DELTA-CADINENE SYNTHASE FROM HYPER-SENSITIVELY RESPONDING COTTON COTYLEDONS: IDENTIFICATION OF SUBSTRATE AND PRODUCT AND PARTIAL PURIFICATION OF THE ENZYME

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PREFACE

The general subject of this thesis was an investigation into the early portion of the metabolic pathway responsible for the biosynthesis of sesquiterpenoid phytoalexins in cotton tissues inoculated with *Xanthomonas campestris* pv. *malvacearum*, the causative agent of bacterial blight of cotton. The purpose of the work was to specifically elucidate the enzymology of the first step of the metabolic pathway responsible for the production of the sesquiterpenoid phytoalexins. Characterization of this part of the pathway might be very important as it could be the first committed step in the biosynthesis of the phytoalexins. Study of this enzymatic step could yield basic scientific information concerning regulation of biosynthesis of the phytoalexins; this knowledge may have practical importance if the pathway (or portions of it) are used to create transformed plants. The major accomplishments described in this thesis include: 1) determination that tritium-labelled farnesyl pyrophosphate is an appropriate substrate for assay of cyclase enzyme activity, 2) identification of δ-cadinene as the most prominent product of induced cyclase activity in bacterially-inoculated cotton tissues and in cell-free reactions catalyzed by homogenates of these cotton tissues, and 3) initial purification from bacterially-inoculated tissues of the induced cyclase activity responsible for the conversion of farnesyl pyrophosphate to δ-cadinene.

A number of special difficulties were overcome during the course of this work. An unexpected increase in ^{3}H to ^{14}C ratio in degradation products was eventually explained when we uncovered what is possibly the only other description of the phenomenon in the literature. The abnormal ratio could have resulted from a relatively slower degradation rate for tritium labeled fragments as compared to ^{14}C labeled fragments due to an isotope effect involved in the abstraction of a strategically place tritium.

Special "problems" followed us in the characterization of δ-cadinene as the product of the inducible cyclase activity. After isolation of δ-cadinene from very large amounts of bacterially inoculated tissues and in preparative amounts from cade oil, we were initially confused by what at first appeared to be odd ^{1}H NMR chemical shifts and extra correlations in 2D NMR. However, extensive review of the literature revealed that δ-cadinene, along with a number of other terpenes, violates a number of the venerable rules that have been used to predict NMR spectra. We were further assured of the identity of the δ-cadinene when it matched other literature characteristics ascribed to δ-cadinene.

A few final challenges were presented in the isolation of the inducible cyclase activity. Initial attempts to isolate the enzyme were thwarted by catastrophic losses of activity during attempted concentration with centrifugally-driven ultrafiltration concentrators. We found that concentration with acceptable activity losses could be obtained by using bulk anion-exchange media and hydrophilic immersible vacuum-drawn concentrators. HPLC characterization of the enzyme product from two active protein fractions showed that we had two separate fractions which both converted farnesyl pyrophosphate to δ-cadinene. Although we were initially disappointed by the poor trailing peak shape of our active protein fractions, we found later that this is often characteristic of hydrophobic proteins; most characterized cyclases have been shown to have substantial hydrophobic character.

I thank my advisor, Margaret Essenberg, for allowing me to "follow my nose" and being patient while I found experimental and literature support for the "strange" results which somehow turned out to be correct after all. My thanks also go to the other members of my committee: Dr. Ulrich K. Melcher, Dr. Earl D. Mitchell Jr., Dr. Robert K. Gholson, Dr. E. J. Eisenbraun, and Dr. Carol L. Bender.

Many portions of this work were made possible by the assistance I received in specific portions of the work as shown by the following acknowledgements. I thank Dr. E. J. Eisenbraun for assistance in the aspects of the work which required application of organic chemistry to our biochemical problems. I thank Dr. Paul Geno and Mr. Jeff

Archer for the initial GC/MS work and Mr. Thayne Vickstrom for the final, intensive GC/MS characterization of my samples. Additional thanks go to Dr. Paul Devlin for infrared analysis and Dr. Neil Purdie and Mr. Allen Engles for circular dichroism analysis of cade oil δ-cadinene. Mr. Stan O. Sigle rendered extensive assistance in performing NMR experiments on δ-cadinene and Dr. K. D. Berlin greatly assisted in interpretation of the NMR experimental results. I must also thank the laboratories of Dr. Andrew Mort and Dr. John Sauer for the loan of HPLC equipment and the gift of advice in HPLC methods. I am endebted to Dr. George Waller for loan of the historic gas chromatograph which was formerly attached to the mass spectrometer now residing in the Smithsonian Institution. I also thank Robin Hurst, Steve Hartson, and Zuoyu Xu for help with protein electrophoresis. Within the local group, I thank Marlee Pierce (especially for getting the native gel electrophoresis going when all I could think of was sleep!!), Joy Steidl for keeping the grins going, Judy Hall for loans of trivial but essential equipment, Lupe Davila for commiserating with me when the HPLC pumps misbehaved (which seemed like every day!), Ken McNally for those notorious puns, Roushan Samad for helping with the plants and those massive inoculations, and Dr. Charles Gardner, Sharlene Matten, Janet Rogers-Best and SueAnn Hudiburg for being good neighbors. And for sage advice, I thank Craig Roddy, Kenny Ferreira, and Dr. Aaron Guenzi. And my thanks to everyone who watered plants, patiently washed my glassware, and otherwise looked out for me (surely sometimes without my noticing).

To the new crew members, Jun Tsuji and Ed Davis, I give hearty encouragement. Just remember, terpenes are tricky, but with lots of effort you can wrestle one of them to the mat.

I wish to thank the Heads of the Department during my stay at OSU, Dr. Roger Koeppe, Emeritus, and Dr. James Blair. I acknowledge support from the National Science Foundation, the United States Department of Agriculture, the Oklahoma Agricultural

Experimental Station. I also acknowledge the Phillips Petroleum for providing a pre-doctoral fellowship which included a generous travel allowance.

To some other friends who played significant roles, my hearty thanks: Rick McMillan, Mike and Cynthia Daggett, Peter and Tammy Bowers, Larry Hawkins, and everyone at Medical Arts Lab, including Steve, Mike, Gordon, Vicki and Mark.

In this season of "family values", I could not forget my parents Marilyn and Gordon Sr. who provided (too much!!) funding and endless patience in helping me secure my education. And I also thank my brother Doug, my sister-in-law Georgia, my Grandmother Della, my cousin Eric, and my aunt Maureen for their encouragement.

I heartily acknowledge my debt to the examples of A. J. Cronin, James B. Sumner, Ignaz Philipp Semmelweis, Nikola Tesla and others who "kept the faith" even in the face of contrary "expert" authority. Even though I would not be worthy to tie the laces of any of these legends, they provided a sense of direction even during detours through the woods of confusion. Additionally, I learned from the lives of these people to never be suprised when a good deed is punished, but to persevere even if it appears foolish to the surrounding crowd.

And if I left anyone out-I'm sorry, but you know my intentions are always good even if my memory is not!

I especially dedicate this work to the memory of departed friends: Les Muchmore, Martha White, Jerry Bond, Gershon Tucker and one of the "real people" of my high school days, Gary Boyer. Gary, I hope your last climb was a good climb and I'm sorry we did not have a chance to chat again on this plane of existence. "Montani semper liberi!"

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LIST OF ABBREVIATIONS

A_{215},A_{254},or A_{280}	Absorbance at 215, 254, or 280 nm
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
°C	Degrees Centigrade
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
C-18	Octadecyl bonded silica
δ-Cadinene	Delta-Cadinene
Carbowax 20M	Polar gas chromatographic phase
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Methylene chloride (dichloromethane)
COSY	Correlated Spectroscopy (two-dimensional ^1H-^1H NMR technique)
COSY LR	Correlated Spectroscopy (two-dimensional ^1H-^1H NMR technique) optomized for detection of long range correlations of protons
DB1	Proprietary version of SE-30 gas chromatographic phase
DB5	Proprietary version of SE-54 gas chromatographic phase
DB-17	Proprietary gas chromatographic phase
DBWAX	Proprietary version of Carbowax 20M gas chromatographic phase
1D-NMR	One-Dimensional Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer (A ^{13}C NMR technique)

Et_2O	Diethyl ether
FPLC	Fast Protein Liquid Chromatography
dG	Desoxygossypol
[1-^{3}H]δ-cadinene	δ-Cadinene (created by enzymatic transformation of [1-^{3}H]FPP)
[1-^{3}H]FPP	Farnesyl pyrophosphate labeled at carbon number one
dHG	Desoxyhemigossypol
dMHG	Desoxymethoxyhemigossypol
DHC	2, 7-Dihydroxycadalene
EIMS	Electron Impact Mass Spectrometry
FID	Free Induction Decay (NMR term for the exponentially decaying sine wave with a frequency equal to the difference between the applied frequency and the resonance frequency for the excited nucleus)
FID	Flame Ionization Detection (Gas Chromatography term for detection of eluted compounds by a flame ionization detector)
FPP	Farnesyl pyrophosphate
FSOT	Fused Silica Open Tubular (as in capillary gas chromatography columns)
FT-IR	Fourier Transform-Infrared spectrometry
G	Gossypol
GC-EIMS	Gas Chromatography-Electron Impact Mass Spectrometry
GC-FID	Gas Chromatography-Flame Ionization Detection
GPC-300	Proprietary name for gel permeation chromatography media (glyceryl-propyl bonded to silica gel; 300 mm pore size)
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{1}H-^{1}H COSY	Homonuclear Correlation nuclear magnetic resonance

HETCOR LR	Long Range Heteronuclear Correlation nuclear magnetic resonance
^{1}H-^{13}C HETCOR	Heteronuclear Correlation nuclear magnetic resonance
HEPES (or Hepes)	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HMC	2-Hydroxy-7-methoxycadalene
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive response
INADEQUATE	Incredible Natural Abundance Double Quantum Transfer Experiment (two-dimensional NMR technique to find ^{13}C-^{13}C correlations
Instagel XF	Proprietary name for Instagel Xylene-Free, a scintillation fluid
KCl	Potassium chloride
kDa	Kilodalton (unit denoting one thousand molecular weight increment)
L	Liter
LC	Lacinilene C
LCME	Lacinilene C 7-methyl ether
Liquid N_2	Liquid nitrogen
µg	Microgram
MHG	Methoxyhemigossypol
MHz	Megahertz
µl	Microliter
ml	Milliliter
µM	Micromolar (concentration)
mM	Millimolar (concentration)
µPorasil	Proprietary name for HPLC silica gel

M_r	Molecular weight, as estimated by chromatographic retention
MVA	Mevalonic acid (or mevalonolactone)
N_2 gas	Nitrogen gas
Native PAGE	Native (non-denaturing) Polyacrylamide Gel Electrophoresis
NMR	Nuclear Magnetic Resonance
PA	Phytoalexin
PAGE	Polyacrylamide Gel Electrophoresis
Polyclar AT	Proprietary trademark of insoluble polyvinylpyrrolidone
R_f	Retention
$Ru(bpy)_2Cl_2$	Rubidium bipyridyl chloride
SDS-PAGE	Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis
SE-30	Non-polar gas chromatographic phase equivalent to proprietary DB-1
SE-54	Low-polar gas chromatographic phase equivalent to proprietary DB-5
Sorvall SS-34	Sorvall centrifuge rotor, type SS-34
Tris	Tris (hydroxymethyl) aminoethane
UV	Ultraviolet absorbance spectroscopy
V_o	Void volume (as in liquid chromatography)
V_t	Total volume (as in liquid chromatography)
WbM	Westburn M, a line of glanded cotton
WbM*gl*	Westburn M, a line of glandless cotton
XAD-4	Rohm and Haas Company beaded non-polar absorbent
Xcm	*Xanthomonas campestris* pv. *malvacearum*

CHAPTER I

INTRODUCTION

Bacterial blight of cotton occurs in the interaction between the bacterium *Xanthomonas campestris* pv. *malvacearum* and susceptible *Gossypium hirsutum* (L.) (upland cotton). The cotton plant undergoes several changes while responding to the bacterial infection: production of colored pigments, accumulation of terpenoid compounds (in sufficient levels to assure bacteriostasis in resistant plant), small lesions which grow into large "window-pane" wounds and, eventually, the death of susceptible plants.

In resistant plants, the hypersensitive response (HR) occurs. A prominent portion of the HR is the accumulation of bacteriocidal terpenoid compounds not found in healthy cotton. Three of these compounds have been thoroughly characterized as phytoalexins. However, additional knowledge of the biosynthetic mechanism responsible for these compounds was desired in order to lay the groundwork for future manipulations of the system by the tools of molecular biology. Increased understanding of the possible first committed step of the pathway and identification of the genetic passages responsible for the biosynthetic enzymes will increase the possibility of transferring the pathway to another type of plant. Additionally, new modes for regulating the pathway may be found, such as induction of biosynthesis by events other than bacterial infection or even constitutive biosynthesis. To pave the way to future benefits the possible first step in the pathway was studied: conversion of the terpenoid substrate farnesyl pyrophosphate by a sesquiterpene cyclase to a precursor sesquiterpene molecule which may be subsequently converted to the sesquiterpenoid phytoalexins.

Glanded cotton produces many terpenoid compounds, many of which are created from farnesyl pyrophosphate. We employed a glandless line of cotton lacking much of the constitutive sesquiterpenoid production of glanded cotton in order to simplify our biochemical investigations.

The first step of the investigation was the identification of an appropriate and convenient enzyme substrate. By feeding mixtures of [2-^{14}C]mevalonolactone and [5-^{3}H]mevalonolactone to cotton cotyledons which were accumulating phytoalexins during the hypersensitive response to *Xanthomonas campestris* pv. *malvacearum*, labeled phytoalexins were obtained. Analysis of the labeling pattern in the chemical degradation products of these labeled phytoalexins revealed that [1-^{3}H]farnesyl pyrophosphate would be an appropriate substrate to assay the cyclase enzyme involved in phytoalexin production and to label intermediates which give rise to the phytoalexins.

The second step of the investigation was the characterization of the enzyme product. Chemical investigation of cotton tissues undergoing the hypersensitive response and analysis of the radioactive enzyme products created when [1-^{3}H]farnesyl pyrophosphate was added to cell-free reactions catalyzed by homogenates of hypersensitively-responding cotton tissues revealed the presence of a sesquiterpene olefin not present in untreated cotton tissues. Rigorous analysis and comparison with known standards allowed identification of the sesquiterpene olefin as δ-cadinene and resolved some confusion in the literature concerning the physical properties of δ-cadinene.

The third step of this investigation was the partial purification of the cyclase enzyme responsible for the conversion of the substrate farnesyl pyrophosphate to the product δ-cadinene. By monitoring the production of δ-cadinene in the cell-free assays used to quantify enzyme purification, two enzyme activities of 39.2 and 27 kDa were identified which could convert farnesyl pyrophosphate to δ-cadinene. The 39.2 kDa enzyme was purified 320-fold.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Plant Defenses to Pathogens

The struggle between plants and the organisms that attack them has been continuous for millions of years (1). Plants have been under unrelenting pressure from fungi, bacteria, viruses, viroids, mycoplasmas, parasitic higher plants, nematodes, protozoa, insects and larger herbivores, including mankind (2). Natural selection and the intervention of plant breeding to improve plant health are now augmented by biochemical studies and molecular biological manipulations. The current scientific investigations are performed with anticipation of improved plant health which can result in increased yields of food crops and even improved aesthetic qualities in horticultural plants. An added benefit of improved plant health will be higher yields with lower inputs of labor, chemical pesticides and, ultimately, financial resources.

The defenses of plants to invading pathogens generally fall into the two categories of structural defenses and diffusible chemical defenses. One form of structural defense is that of a pre-existing defense. An example of this type of plant protection is found in alfalfa. Varieties of alfalfa resistant to the pathogen *Corynebacterium insidiosum* have shorter vessels and fewer vascular bundles; this structural arrangement severely restricts the movement of invading bacteria (3).

Plants erect many structural defenses in response to attacks by micro-organisms. These defenses include abscission layers, accumulation of tyloses, gum deposition, cork layers and changes in the morphology of the invaded cell. The necrosis seen in hypersensitively responding plant tissues can functionally present a structural barrier to

obligate parasites by isolating them from healthy plant cells containing nutrition necessary for survival of the parasite (2).

The other major category of plant protection is diffusible biochemical defense. Some of the biochemical defenses are pre-formed, i. e., synthesized and stored prior to invasion by the pathogens. These pre-formed defenses include inhibitors that are released to the environment or inhibitors that are stored within the plant tissue. This strategy of defense has the temporal advantage of immediate contact of the defense compound with the invading pathogen but also has the disadvantage of requiring energy inputs to create a defense against a pathogen that may never attack the plant tissue.

In contrast to the pre-formed biochemical defenses, other biochemical defense compounds are synthesized or accumulated at a greater rate in response to attack by pathogens. These compounds may be present at modest concentrations in healthy plant tissues, but the concentration of the defense compound is rapidly elevated to a level needed to inhibit the invading pathogen. Phenolic compounds such as chlorogenic acid, caffeic acid, and scopoletin (2) are examples of this type of defense. Some plants also secrete proteins or enzymes which can neutralize the offensive weaponry of the invading pathogen or even directly attack the pathogen itself.

A class of induced chemical defenses that has come under increased study is the phytoalexins. By definition, "Phytoalexins are low molecular weight antimicrobial compounds that are both synthesized by and accumulate in plants after their exposure to microorganisms" (4). In practice, this definition includes compounds which, by modern chemical analysis, are undetectable or present in healthy plant tissues at concentrations insignificant in comparison to concentrations in infected tissues. Phytoalexins arise in the incompatible interaction between resistant plants and pathogens such as fungi, bacteria, viruses, and nematodes. In these incompatible interactions, the pathogen may be able to invade a small area of plant tissue, but is successfully prevented from spreading to plant tissue beyond the vicinity of the small local lesions. It should also be noted that elicitation

can occur in compatible interactions between susceptible plants and their pathogens, but the phytoalexins are not produced in sufficient amounts or quickly enough to prevent the spread of the pathogen in the plant tissues.

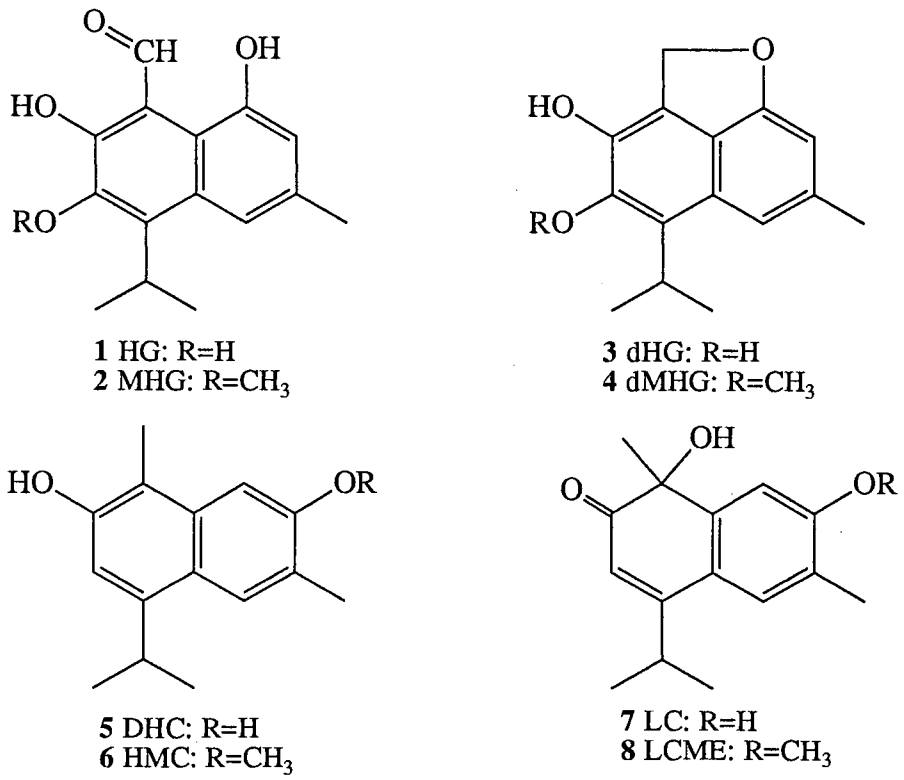
Currently, the regulation of phytoalexin biosynthesis is an area of interest, as phytoalexin defense systems may eventually be transferred into susceptible plants by conventional plant breeding or genetic engineering techniques (5). The events surrounding the induction of biosynthesis of the phytoalexins are interesting because elicitation represents a mechanism by which the plant recognizes the attack of the pathogen and then "turns on" the biosynthetic machinery that produces the phytoalexins quickly and in amounts which could contribute to containment of the pathogen (6). While elicitation occurs upon the attack by fungal or bacterial pathogens on a resistant plant, biosynthesis of the phytoalexin can be provoked by other means (7-9). Elicitation can occur upon treatment of the tissues with fragments of plant cell wall pectin, viruses, fractions from pathogens or non-pathogens, denatured RNAase or degradative enzymes; these agents are termed "biotic elicitors". Additionally, "abiotic elicitors", which include wounding (by abrasion), heavy metal ions and ultraviolet light treatments, can induce biosynthesis of phytoalexins.

Phytoalexins have been found in many plant sources, with certain chemical classes of phytoalexins occurring predominantly in certain families of plants. The plants in the Leguminosae (Fabaceae) have been found to possess phytoalexins that are isoflavonoids, ketonic furanoacetylenes, ethylated chromones, benzofurans, and stilbenes (10). The Solanaceae have prominent phytoalexins that are phenylpropanoids from the shikimic acid pathway, acetylenes and polyacetylenes from the acetate-malonate pathway, and terpenoid phytoalexins from the acetate-mevalonate pathway (11). Other chemical classes of phytoalexins include polyketides and anthranilate-derived compounds (12,13).

In cotton, the subject of this work, the phytoalexins characterized to this point are of the cadalene class of sesquiterpenoids (C-15 compounds). Some dimers of

sesquiterpenoids (*bis*-sesquiterpenes [C-30 compounds]) (14) such as gossypol are prominent constitutive compounds in cotton. The sesquiterpene phytoalexins identified from *Verticillium*-infected glanded cotton include hemigossypol (HG, **1**), hemigossypol-6-methyl ether (MHG, **2**), desoxyhemigossypol (dHG, **3**), and desoxyhemigossyol-6-methyl ether (dMHG, **4**) (15-18) (Figure 1). The sesquiterpenoid compounds accumulated during the incompatible interaction of cotton with *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) include 2,7-dihydroxycadalene (DHC, **5**), 2-hydroxy-7-methoxycadalene (HMC, **6**), lacinilene C (LC, **7**), and lacinilene C methyl ether (LCME, **8**) (19) (Figure 1). While DHC, LC, and LCME are phytoalexins, HMC (**6**) does not possess sufficient anti-bacterial activity to merit classification as a phytoalexin.

The terpenoid origin of these cadalene class phytoalexins has been either inferred from study of the structurally related compound gossypol or determined directly for one of the phytoalexins (DHC). The folding pattern of the putative farnesyl precursor, which gives rise to the constitutive *bis*-sesquiterpenoid gossypol (a dimer of hemigossypol [HG] **1**), has been established by radiochemical analysis of derivatives of degradation products of radioactive gossypol created from feeding of [2-^{14}C]mevalonate (MVA), [4-^{14}C]MVA or a mixture of [5-^{3}H]MVA and [2-^{14}C]MVA to cotton roots (20) and by analysis of ^{13}C NMR of gossypol (**9** in Figure 2) created with [1,2-$^{13}C_2$]acetate fed to cotton roots (21) (Figure 2). The terpenoid origin of DHC and the folding pattern of the farnesyl precursor have been shown by analysis of ^{13}C NMR of DHC (**5** in Figure 3) created by injection of cotton seedlings with [1,2-$^{13}C_2$]acetate (22). It should be noted that the folding patterns of the putative *cis, trans*-farnesyl precursor (or equivalent) inferred for DHC (22) and for gossypol (21) are in disagreement with the earlier experimental findings of Heinstein *et al.* that indicated gossypol was created through a putative *cis, cis*-farnesyl intermediate (23). As will be discussed later (page 13), cyclase enzymes can convert *trans,trans*-farnesyl pyrophosphate to cyclized product(s); this may be accomplished by conversion of the *trans,trans*-FPP into *cis, trans*-FPP (or equivalent) which is utilized for cyclization.



1 HG: R=H
2 MHG: R=CH_3

3 dHG: R=H
4 dMHG: R=CH_3

5 DHC: R=H
6 HMC: R=CH_3

7 LC: R=H
8 LCME: R=CH_3

Figure 1. Sesquiterpenoid Phytoalexins of Cotton (**1-5**, **7**, and **8**) and HMC (**6**).

While these initial investigations have given valuable information concerning the role of putative farnesyl precursors in the creation of sesquiterpenoid phytoalexins, important biosynthetic information can be gained by studies of the conversion of farnesyl pyrophosphate to an initial enzyme product. These studies in fungal, bacterial, and plant systems have shown that this conversion of FPP to an olefin (and in at least one case, a sesquiterpene alcohol) is catalyzed by enzymes termed cyclases (or synthases) (24). The study of these enzymes in an early step of biosynthesis of sesquiterpenoid compounds has a heightened importance in comparison to some of the following biosynthetic transformations leading to the end-product sesquiterpenoids. This is because this initial conversion of FPP to an enzyme product may represent a first committed step involved in diverting metabolic flux into the specific pathways for creation of sesquiterpenoids and from pathways for creation of other terpenoid compounds (25). Additionally, a fuller understanding of this enzymatic step is important if these biosynthetic pathways are to be manipulated by means of molecular biology techniques.

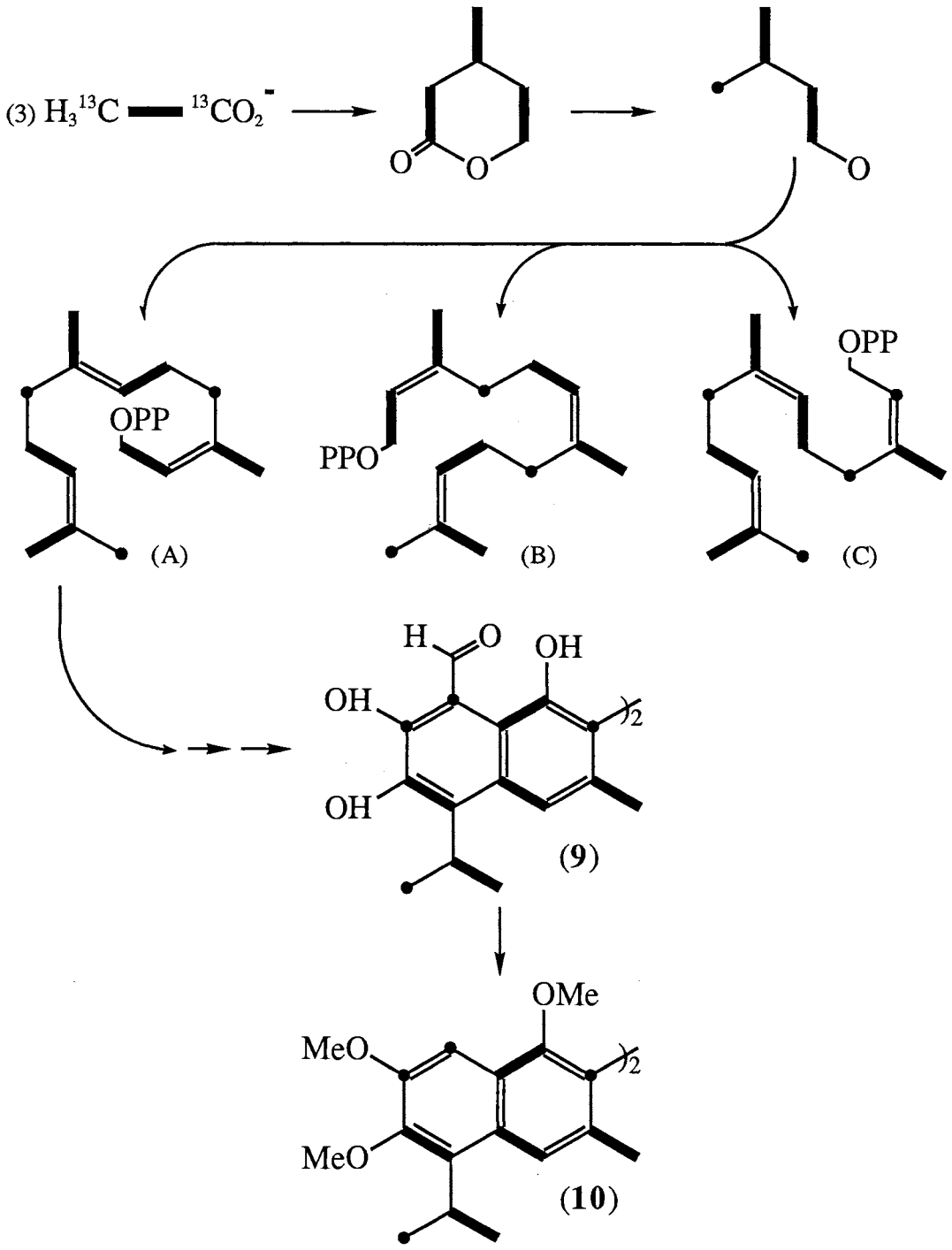


Figure 2. Folding pattern of putative *cis, trans*-farnesyl precursor (A) (or equivalent) to gossypol (**9**) as revealed by analysis of ^{13}C NMR of ^{13}C-labelled derivative apogossypol hexamethyl ether (**10**). (Thick lines signify intact ^{13}C-labeled acetates, dots represent isolated, ^{13}C-enriched sites.This figure is modeled after Scheme 1 in Reference 21).

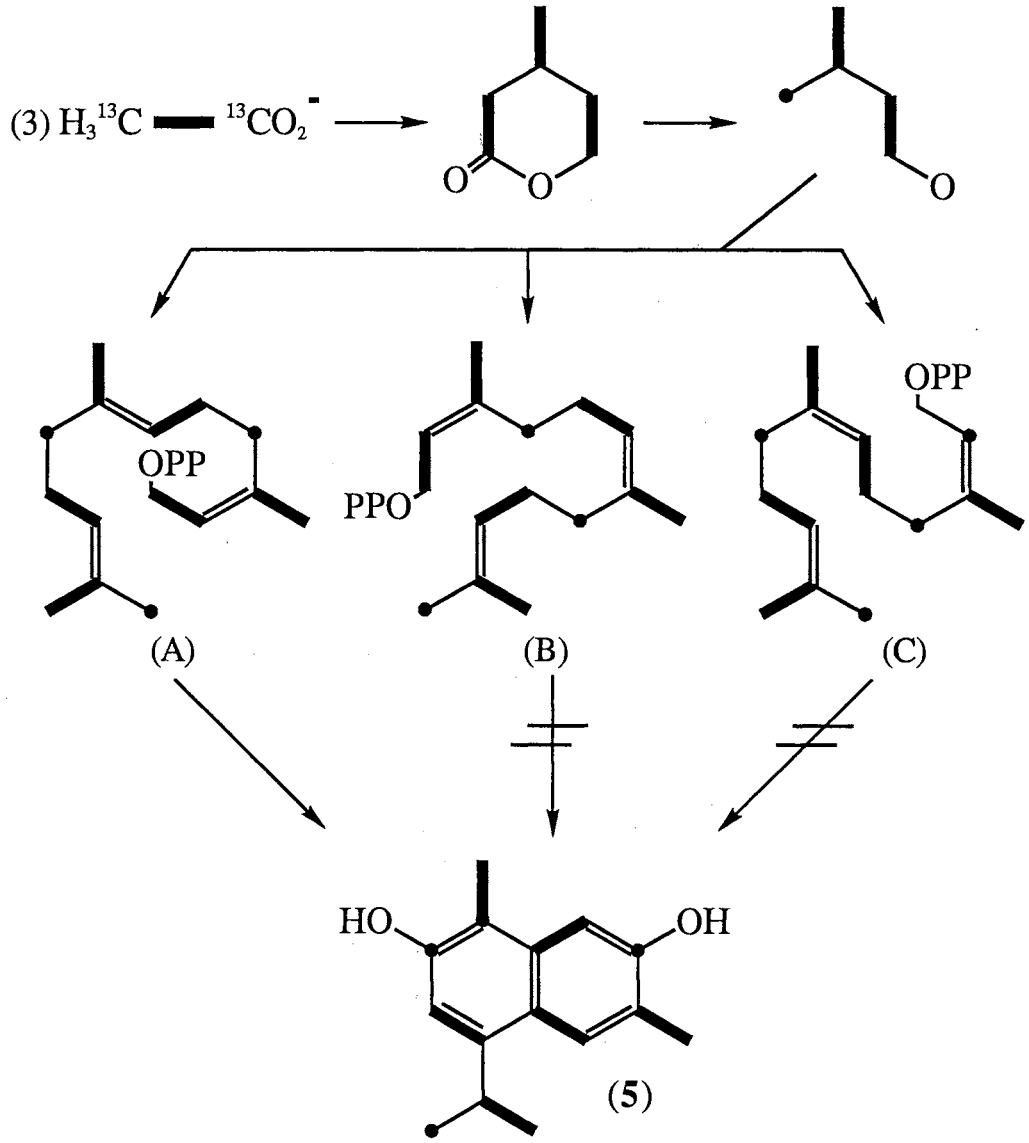


Figure 3. Folding pattern of putative *cis, trans*-farnesyl precursor (A) (or equivalent) to produce DHC (2,7-dihydroxycadalene)(**5**) as determined by ^{13}C NMR analysis of the ^{13}C-labelled DHC. (Thick lines signify intact ^{13}C-labeled acetate; dots represent isolated, ^{13}C-enriched sites. This figure is modeled after Scheme 1 in Reference 22).

Review of Sesquiterpene Cyclase

(Synthase) Enzyme Systems

A simple review of the current state of knowledge surrounding cyclase enzymes can be performed by discussion of the 1) substrate(s), 2) initial enzyme product(s), and 3) the purification and subsequent characterization of the cyclase enzymes. In the review of literature that follows, the cyclase enzymes are often called "synthase" enzymes. As shown in Figure 4, the cyclase enzymes represent a small, but crucial part of a terpenoid biosynthetic system.

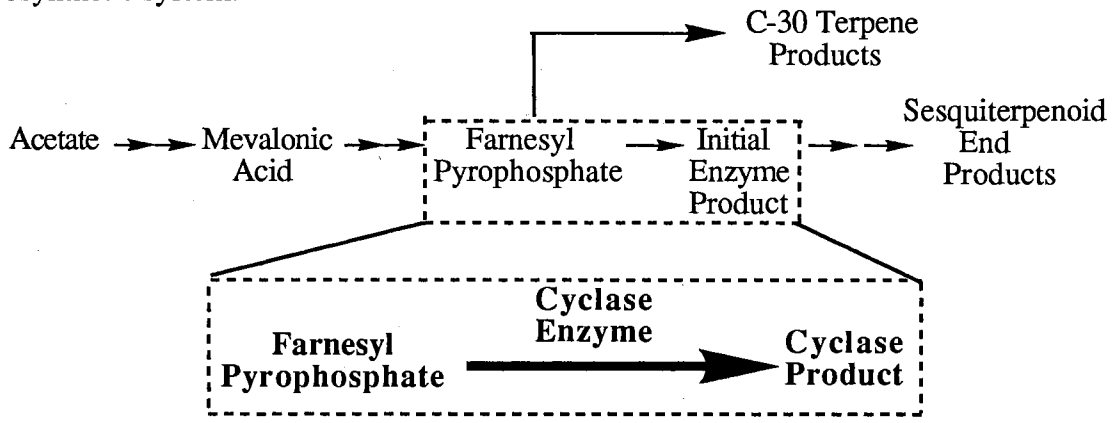


Figure 4. Sesquiterpenoid Biosynthetic Pathway

These enzymes are difficult to name by conventional International Union of Biochemistry (IUB) nomenclature because these enzymes appear to catalyze multiple enzymatic steps (26). However, the IUB has apparently accepted the convention of naming these cyclase enzymes as an "enzyme product" synthase, *e.g.*, trichodiene synthase for the enzyme that converts farnesyl pyrophosphate to trichodiene. Much of the initial work on terpenoid cyclase enzymes was performed by Croteau and colleagues in the study of monoterpene cyclases (27). The work performed on sesquiterpene cyclases has been a natural extension of the research on monoterpene cyclases.

The Substrates

The substrates utilized by sesquiterpene cyclase enzymes are isomers of farnesyl pyrophosphate or nerolidyl pyrophosphate (24). Preliminary evidence of the involvement of a sesquiterpene cyclase enzyme in biosynthesis of a specific compound can be obtained by incorporation of labelled acetate or mevalonolactone into a specific sesquiterpene (28). If the locations of label found in the enzyme product are consistent with a series of metabolic conversions from acetate to mevalonate to farnesyl pyrophosphate to the labelled enzyme product, then there is a strong possibility that the enzyme product is a sesquiterpene compound. Such evidence is often obtained prior to committing resources to the purchase or synthesis of farnesyl- or nerolidyl-type compounds for use as substrate in the assay of cyclase enzymes.

Although labelled farnesyl pyrophosphate is often used as the substrate in cell-free assay of cyclase activity involved in sesquiterpenoid phytoalexin biosynthesis, other sesquiterpene substrates are, to varying degrees of biosynthetic efficiency, able to function as substrates in crude enzyme preparations. An early example of this suitability of various substrates (with varying efficiencies) for conversion to terpenoid products is found in the work on biosynthesis of gossypol by Heinstein and colleagues (29, 30). This research showed that the order of efficiency of incorporation of isomers of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) into gossypol by a 105 000 *g* supernatant from cotton roots was: *cis*-GPP>*trans*-GPP; *cis,cis*-FPP>*cis,trans*-FPP>*trans,cis*-FPP>*trans,trans*-FPP (**10** in Figure 5)); [2-^{14}C]mevalonate (MVA) was also able to form labelled gossypol. This ability to utilize all isomers of FPP was apparently aided by a soluble protein fraction isolated from cotton root homogenates which catalyzed formation of all four isomers of FPP from isopentenyl pyrophosphate (30).

As previously shown in Figures 2 and 3, the use of ^{13}C NMR analysis of gossypol and DHC created from ^{13}C-labelled acetate has led to the conclusion that putative farnesyl

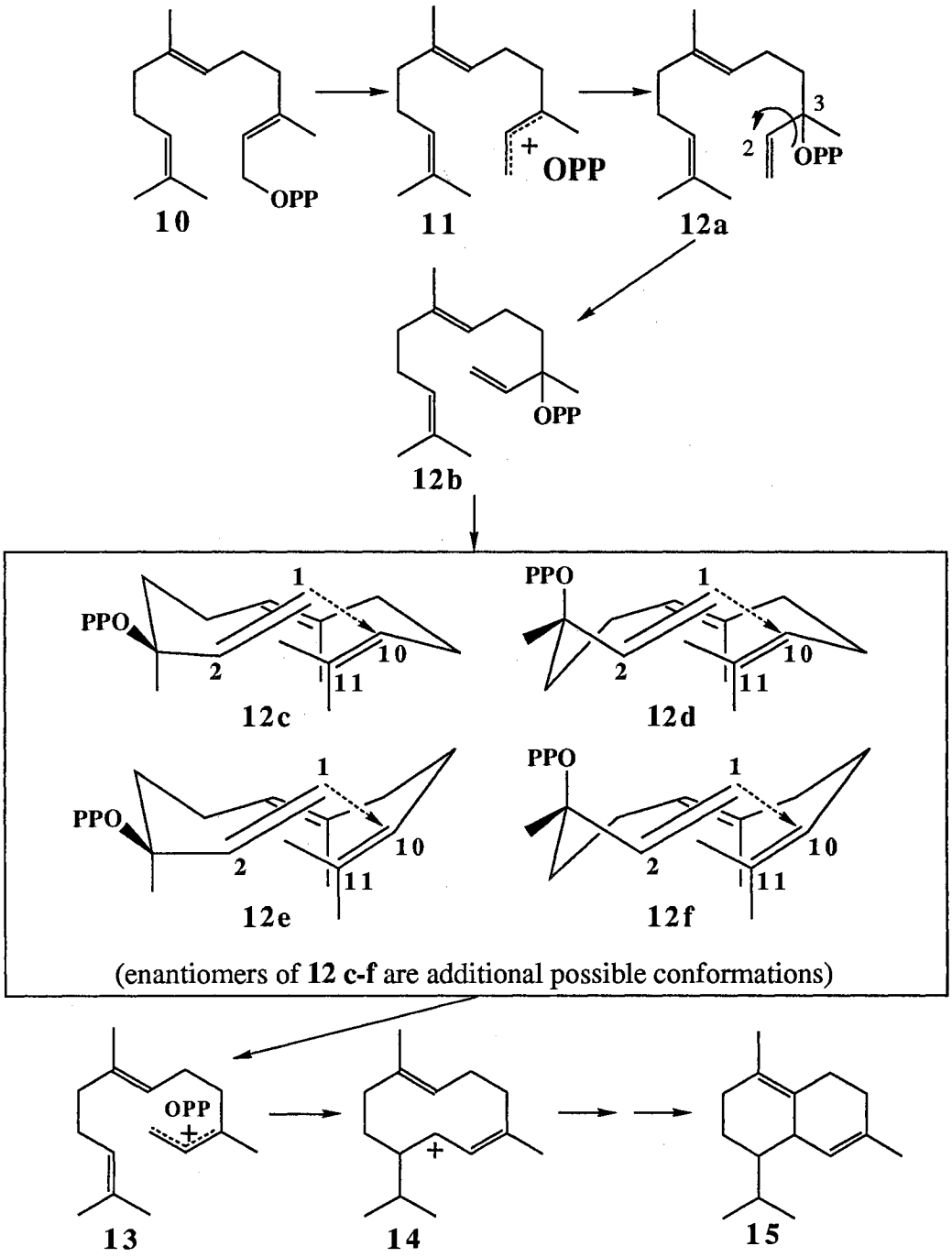


Figure 5. Proposed conversion of *trans, trans*-farnesyl pyrophosphate (**10**) to an example sesquiterpene olefin (**15**) via nerolidyl pyrophosphate (**12 a-f**). Structures **12 c-f** within the enclosed box (and their enantiomers) represent possible conformations of nerolidyl pyrophosphate involved in the biosynthetic pathway; no specific conformer has yet been identified as a biosynthetic intermediate.

precursors must assume a *cis, trans* (or sterically equivalent) conformation in order that cyclization may be accomplished. However, it is expected that *trans, trans*-FPP is a precursor of all sesquiterpenes, because *trans, trans*-FPP is a product of prenyltransfersase [E.C. 2.5.1.1] (31) and of farnesyl pyrophosphate synthase (or synthetase) [E. C. 2.5.1.10] (32). These two enzymes may be responsible for providing the *trans, trans*-FPP utilized by sesquiterpene cyclases *in vivo*. The ability of the purified sesquiterpene cyclase trichodiene synthase to utilize nerolidyl pyrophosphate as well as *trans, trans*-FPP as a substrate is another example of the ability of sesquiterpene cyclases to utilize substrates that are the steric equivalent of *cis, trans*-FPP (33,34). This suggests that the *trans, trans*-FPP may be transformed into nerolidyl pyrophosphate by cyclases before production of the cyclized end-product. (The enzyme names "prenyltransferase" and "farnesyl pyrophosphate synthase" (synthetase) are sometimes used interchangeably as shown by a reading of references listed for E.C. 2.5.1.1 and E. C. 2.5.1.10 in Reference 26; additionally, the name "farnesyl pyrophosphate synthetase" is identified with E. C. 2.5.1.1 in Reference 24).

A proposed pathway from *trans, trans*-FPP to a putative sesquiterpenoid product is shown in Figure 5. This figure shows compounds likely to be involved in such a pathway and reflects current thought on the nature of putative intermediates. As reviewed by Cane (24), isomers of FPP [*e.g., trans, trans* FPP (**10** in Figure 5) are often suitable substrates for cyclase enzymes which create sesquiterpenoid products. The proposed formation of the enzyme products requires two major transformations. First, the farnesyl isomer is proposed to isomerize through an ionic species (**11**) to nerolidyl pyrophosphate (**12 a, b**). This allows for rotation about the new single bond between carbons 2 and 3, thus permitting a more favorable disposition of the reactive portions of the molecule. The second important step is the shaping of the NPP intermediate into a proper conformation. Forms of NPP which could be cyclized are shown as structures **12c-f**. The two double bonds (C1-C2 and C10-C11) need to be mutually co-planar with what is termed "the plane

of cyclization". Thus, for the example in Figure 5, the two double bonds are mutually perpendicular to the plane containing the new bond that will connect C1 to C10.

Once the proper conformer is created, the next ion **13** undergoes cyclization to another putative intermediate ion **14**. Further transformations eventually complete the creation of the sesquiterpenoid product, *e.g.*, δ-cadinene **15,** which is sufficiently stable for isolation. While this biosynthetic scheme is hypothetical, it is interesting to note that nerolidyl pyrophosphate is perhaps the preferred substrate for trichodiene synthase as the enzyme converts nerolidyl pyrophosphate to trichodiene at greater efficiencies than it does *trans, trans*-FPP (33, 34).

In addition to possibly required isomerizations and conformational reshaping of substrates, it has been proposed that other intermediate compounds may be created prior to the release of the isolated cyclic sesquiterpenoid enzyme product. Germacrene D (**16** in Figure 6) has been proposed as an intermediate in many sesquiterpene cyclization schemes (24) and α-humulene has been proposed as an intermediate in the formation of the sesquiterpenoid pentalenene (35, 36). Other researchers (7) have proposed that farnesyl pyrophosphate is converted to an initial cyclized product such as a germacrene by a first enzyme and then the germacrene is converted to the isolated sesquiterpenoid product by a second enzyme. However, each reported purification of a cyclase enzyme has led to purification of an apparently homogeneous single enzyme which can perform the total transformation of substrate to cyclized product (24). It has been proposed that cyclized compounds such as germacrene D (24) or α-humulene (37) may be examples of initial products created by individual cyclase enzymes. Then the initial cyclase product can in theory be transformed by enzymatic or non-enzymatic means into stable product(s). However, germacrene D and α-humulene have not yet been found among the products created by the cell-free reactions catalyzed by crude homogenates or purified cyclase enzymes (24). It is still possible these compounds may be synthesized as initial enzyme-bound products which are converted to the final cyclase product which is released

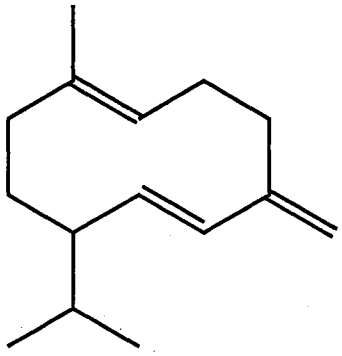


Figure 6. Germacrene-D (**16**)

from the enzyme and recovered from the cell-free reaction.

Although [1-^{3}H]farnesyl pyrophosphate is probably a suitable substrate for assay of sesquiterpene cyclase activity, it is wise to find the final position of the label in the sesquiterpene olefin or derived sesquiterpenoid phytoalexin (28). The information that can be rationalized from the radioactive analysis includes: identification of the final site of the label, detection of hydride or methyl shifts, estimation of the stoichiometric retention or loss of label, and a determination of the suitability of any particular compound for use as a substrate to assay cyclase activity.

Examples of rigorous analysis of labelling patterns are the investigations of the labelling patterns of two sesquiterpenoids: the cadalene-class compound gossypol (**9** in Figure 7) from cotton (20) and avocettin (**17** in Figure 7) from the fungus imperfectus *Anthostoma avocetta* (38). The investigation of labelling patterns in gossypol revealed a 1, 3-hydride shift in the putative farnesyl intermediate (Panel a in Figure 7). Assuming a similar transfer occurs if [1-^{3}H]farnesyl pyrophosphate were used as substrate in creation of a cadalene-class compound (e.g. DHC), the tritium label might be localized at the methine carbon of the isopropyl side chain in the intermediates and end-product phytoalexins. A similar 1,3 shift was detected during the analysis of labelled fragments recovered from labelled avocettin (Panel b in Figure 7). The analysis of labelling in avocettin is of additional interest as it gives a rationalization for the observation

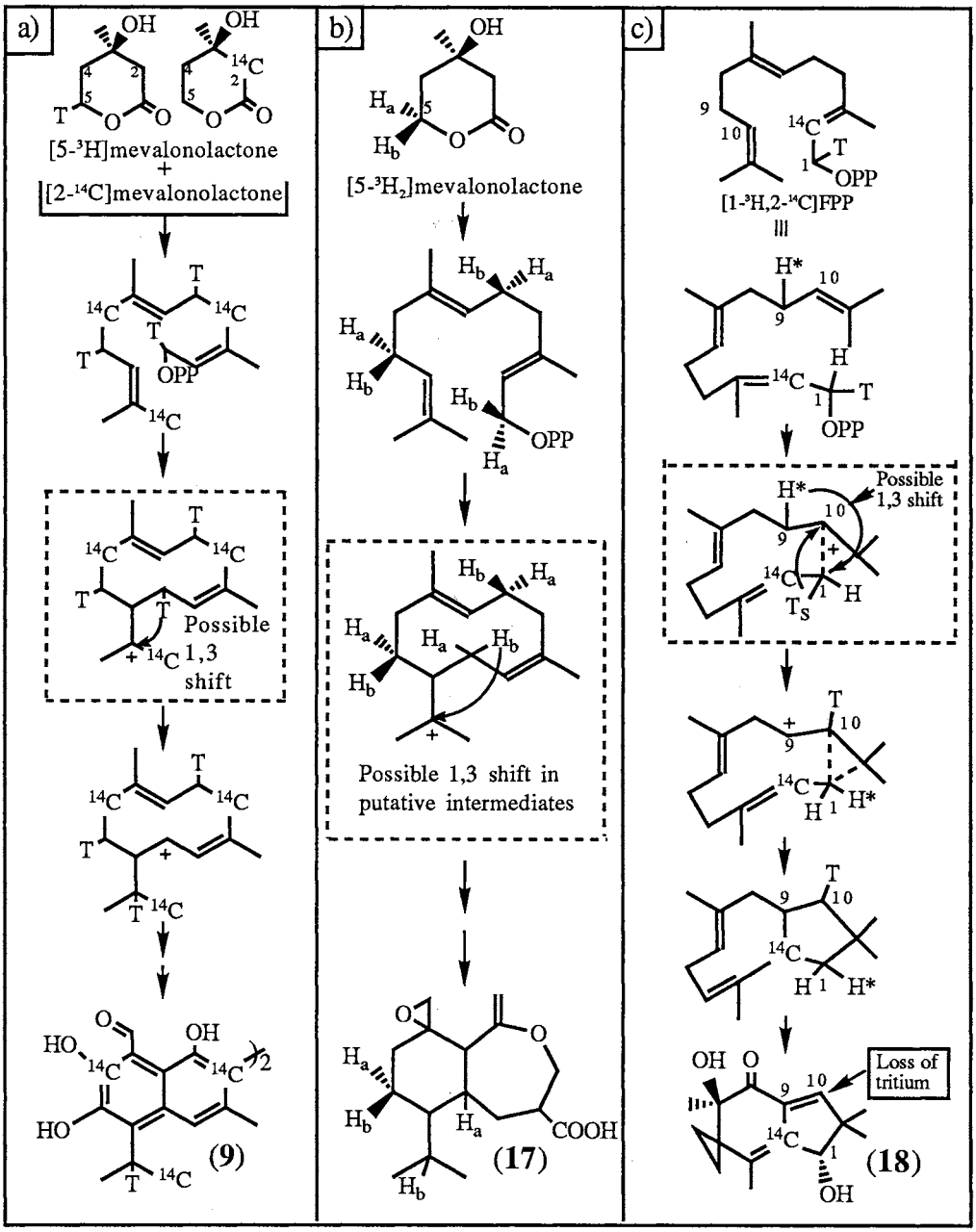


Figure 7. Examples of probable 1,3 hydride shifts in the biosynthesis of gossypol (**9**), avocettin (**17**) and illudin M (**18**) shown in panels a), b) and c), respectively.
Note loss of tritium in biosynthesis of illudin M (**18**).
(This figure illustrates information from references 20, 38, and 39).

of unexpectedly high ^{3}H:^{14}C ratios in the degradation products of labelled avocettin. The proposed explanation for this suprising observation in the degradation of avocettin and similarly high ^{3}H:^{14}C ratios seen in this study is discussed in Chapter III.

Investigation of the biosynthesis of illudins by Hanson and colleagues (39) shows that the site at which label is localized in the enzyme product can be suprising. In the biosynthesis of illudin M (**18** in Figure 7), it was shown that a hydrogen (or tritium) label in the FPP substrate is transferred from C-9 of the farnesyl precursor to C-1 of the cyclized end-product (Panel c in Figure 7). There is an apparently simultaneous movement of a hydrogen (or tritium) to C-1 of the FPP (or C-3' of illudin M, following the numbering system of Hanson [39]) which causes inversion of configuration at C-1. Another important finding of the analysis of the labelling pattern of illudin M is that the tritium label in the substrate [1-^{3}H]farnesyl pyrophosphate is lost during biosynthesis of illudin M, thus [1-^{3}H]FPP is not an appropriate substrate for assay of the cyclization of FPP to illudin M. This illustrates that empirical work is often necessary before an appropriate substrate for assay of sesquiterpene cyclase can be found. A similar shifting of a hydrogen is seen in gossypol (20); however, the shifted hydrogen (or tritium) is retained throughout biosynthesis in the isopropyl side chain. The movement of the hydrogen atoms in both illudin M and gossypol could be the result of 1,3 hydride shift. This demonstration of the mobile nature of tritium labels in illudin M (39) and in gossypol (20), along with the loss of tritium from specific sites during biosynthesis of some sesquiterpene compounds (38-40), shows why investigators should be cautious in assigning the final site of a label and in assessing the final number of labelled atoms in an enzyme product.

In the current work, the labelling study yielded much of the information discussed above. The incorporation of a mixture of [5-^{3}H]mevalonolactone and [2-^{14}C]mevalonolactone generated labelled DHC (and HMC) for analysis by scintillation counting to determine if the expected stoichiometric loss of tritium during aromatization of the cadalene nucleus occurs. Radiochemical analysis of labelled fragments liberated from

the labelled DHC (or HMC) provided information about the final site of the label. Label found at sites that should not have been labelled in the putative farnesyl pyrophosphate precursor raised the possibility of a hydride or methyl transfer. Determination of the final site of label can provide information in support of a proposed folding pattern; in our particular case we speculate that a 1,3-hydride shift (from C-1 of farnesyl pyrophosphate to the methine carbon of the isopropyl group in DHC or HMC) occurs during biosynthesis; a similar pattern was seen in gossypol biosynthesis. This stoichiometric transfer is of special interest, because it indicates that [1-^{3}H]farnesyl pyrophosphate should be an appropriate substrate for cyclase activity and should label all biosynthetic compounds in the pathway to the sesquiterpenoid phytoalexins and HMC. Definitive information about the stereochemistry of the transformations will not be obtained in this study. To obtain such information would require that several versions of farnesyl pyrophosphate labelled with deuterium or tritium in specific positions be obtained or synthesized, converted into labelled products by cell-free reactions, and then chemically degraded to labelled fragments to be separated and analyzed. It is possible that David Cane of Brown University may perform such studies with the cyclase activity that is found during this study.

The Products of Sesquiterpene Cyclase Enzymes

The chemical analysis of plant tissues has successfully revealed the presence of many sesquiterpenoid phytoalexins which are often cyclized and substituted derivatives of acylic precursors (e.g. farnesyl precursors). However, when labelled farnesyl precursors are added to the cell-free homogenates of these tissues, the predominant labelled compounds recovered are often labelled sesquiterpene olefins (24), with the substituted (e.g., hydroxylated) compounds present only at low levels. This finding suggests that the recovered sesquiterpene olefin may be an intermediate in the pathway leading to the more modified sesquiterpenoid products. One notable exception to the usual recovery of sesquiterpene olefins from these systems is the production of the sesquiterpene alcohol,

patchoulol (41), in cell-free reactions catalyzed by homogenates of *Pogestemon cablin.* The patchoulol synthase is also interesting for its ability to produce mixtures of other sesquiterpenoids in small amounts.

Identification of a sesquiterpene olefin requires careful processing of plant tissues or cell-free reactions to avoid sample loss due to volatility, because these compounds have sufficient vapor pressure to be used as aroma compounds in foodstuffs, medicinal preparations or perfumes (42, 43). Additionally, sesquiterpene olefins, and terpene hydrocarbons in general, can undergo isomerization (44) or be converted to other compounds during distillation (45) or gas chromatography with certain stationary phases (46). These characteristics of sesquiterpene olefins can lead to loss of the actual enzyme product or an incorrect identification based on isolation and characterization of a derivative of the actual enzyme product.

Labelled sesquiterpene olefins from cell-free reactions (using labelled farnesyl pyrophosphate) can be conveniently identified by co-chromatography with known standards in gas chromatographic systems using simultaneous thermal conductivity/radioactivity detection (47, 48). If sufficient enzyme and unlabelled substrate are available, the product of the cyclase enzyme activity can be identified by conventional GC/MS, with further confirmation by comparison of Kovat's Indices on multiple gas chromatographic columns, *e.g.*, a non-polar SE-54 column and a polar Carbowax column, with indices of known compounds (49). Another non-radioactive method employed to identify an isolated compound is HPLC co-chromatography with a standard compound; this technique requires purification from more plant tissue and available standards. If more extensive characterization of a putative sesquiterpene olefin enzyme product is desired, even larger amounts of extract can be fractionated to obtain purified compound for characterization by ultraviolet absorption, circular dichroism, infrared, and nuclear magnetic resonance spectrometry. If the product is a new compound which has not been previously described, such characterization is necessary. Unfortunately, the amount of

tissue, extraction solvents, and time investment needed for recovery of sufficient sample for these analyses may be prohibitively large.

Sesquiterpene Cyclase (Synthase) Enzymes

The creation of sesquiterpenoid phytoalexins appears to depend on the action of cyclases, the enzymes responsible for the conversion of an acyclic precursor (e.g. farnesyl pyrophosphate) to a cyclic compound. The fungal and plant cyclase enzymes rigorously studied to this point have these general characteristics (24, 47): molecular weight of 40 000-100 000, operationally soluble proteins, monomers or homodimers, moderately lipophilic (hydrophobic), and requiring only a divalent metal ion (Mg^{2+} usually preferred). The apparent K_m values for farnesyl pyrophosphate are, with only one documented exception, in the 0.5-5 μM range. The turnover numbers are modest, falling in the range 0.02-0.3 s^{-1}. The cyclase enzymes also are usually present at relatively low concentrations *in planta*. In general, purification on the order of 120-2900 fold is required to obtain a homogeneous, pure cyclase enzyme, but it should be noted that specific activity determinations may not accurately reflect the level of purification because crude enzyme preparations can contain inhibitors (50) which may falsely elevate the calculated level of purification (over the actual enzyme purification). In addition, substantial amounts of purified enzyme may denature in the later steps of purification (41), thus a highly purified, yet lower specific activity protein may be recovered. Cyclase (synthase) enzymes are often very unstable in crude homogenates, cannot tolerate ammonium sulfate precipitation, and may not have long-term stability upon purification to homogeneity, even if stored at -70°C (41). Activity may also be lost upon exposure to ultrafiltration membranes (8, 51). These characteristics make purification of terpene cyclase enzymes quite challenging.

Sesquiterpene cyclases identified in non-plant sources include: bisabolene synthase from *Andrographis paniculata* (52), trichodiene synthase from the apple mold fungus *Trichothecium roseum* (24), *Gibberella pulicaris (Fusarium sambucinum)* (24), and

Fusarium sporotrichioides (53), bergamotene synthase from *Pseudeurotium ovalis* (54), pentalenene synthase from *Streptomyces* UC5319 (51), and aristolochene synthase from *Aspergillus terreus* and *Penicillium roquefortii* (55).

Purification of sesquiterpene cyclases from plant sources include: β-caryophyllene synthase and α-humulene synthase from *Salvia officinalis* by Dehal and Croteau (50), *epi*-aristolochene synthase from tobacco cell cultures treated with fungal elicitor prepared from *Phytophthora megasperma* or related species, by Vögeli, Freeman, and Chappell (8), the β-selinene cyclase from *Citrofortunella mitis* fruits (56), and patchoulol synthase purified from *Pogestomon cablin* by Munck and Croteau (41). The patchoulol synthase is notable for its production of a combination of the sesquiterpene alcohol patchoulol as a major product and a low abundance mixture of sesquiterpene olefin products. This formation of product mixtures by a single enzyme is unusual, but has also been seen in the action of other terpene cyclases such as pinene synthases of sage (57-59) and of longifolene-sativene cyclase of plant and fungal origin (60-62).

A general scheme for the isolation of a cyclase enzyme is given in Figure 8. The procedures employed to purify these enzymes generally include the use of low resolution anion-exchange media (*e.g.*, Whatman DE-52), higher resolution anion exchange and gel filtration FPLC or HPLC columns, and hydroxyapatite HPLC. To monitor enzyme purification, the protein fractions obtained by chromatography are used to catalyzed cell-free reactions which convert labelled substrate to labelled enzymatic product(s). The enzymatic products created during the cell-free reaction are often separated by gas chromatography and detected by simultaneous thermal conductivity detection (TCD) and scintillation counting. Alternatively, unreacted substrate may be removed by adsorption to silica and then analyzed by liquid scintillation counting.

Use of these labor-intensive, multiple-step purifications has produced homogeneous cyclase enzymes. A possible improvement in the methodology of cyclase purification may result from use of a new commercially available preparative-scale electrophoresis unit, the

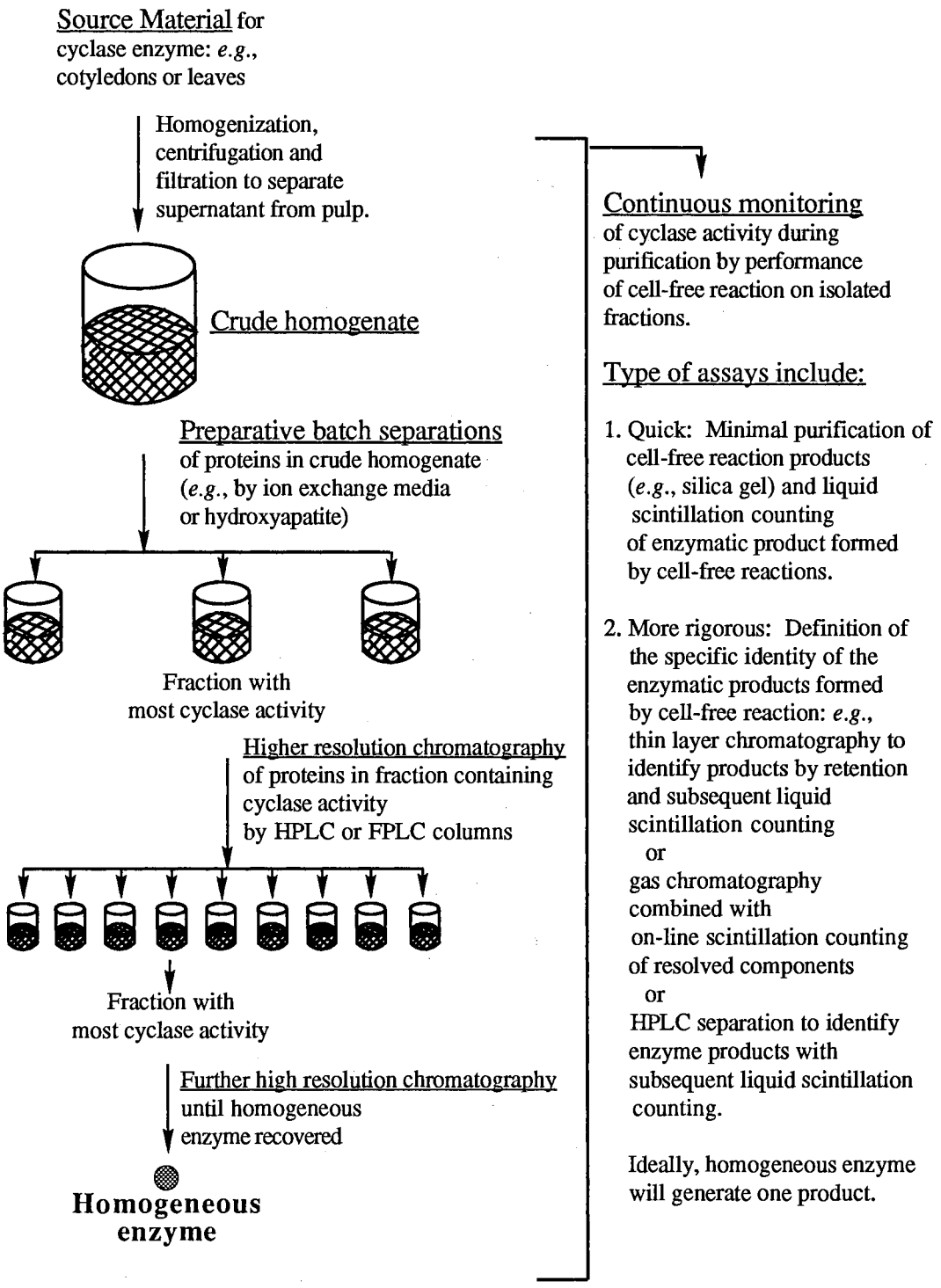


Figure 8. General scheme for purification to obtain homogeneous cyclase which generates a single enzyme product. (Some purified cyclases can yield multiple enzyme forms and, occasionally, multiple products).

Prep-Cell (63). An efficient two-step purification of a monoterpene cyclase included an 80x purification step accomplished by the Prep-Cell (63); this type of purification method may be applicable to sesquiterpene cyclases.

Two characteristics of terpene cyclases are apparently contradictory. With one exception (64,65), cyclases are characterized as operationally soluble enzymes, *i.e.*, they appear to solubilize in buffer solutions without detergents. However, most of these enzymes are relatively hydrophobic (24) which is consistent with the broad peaks seen during chromatography of these enzymes on various media, including capillary electrophoresis (66). High losses of activity sometimes occur upon contact of the cyclase with hydrophobic membranes used for ultrafiltration (8,51). And finally, cyclase enzymes are often extremely unstable, even when stored at low temperatures (41). These characteristics are reminiscent of some enzymes which are peripheral (associated, but not anchored) membrane proteins (67).

The possibility that cyclases are functionally soluble, but may be membrane-associated, could be supported by two reports of membrane-associated sesquiterpene cyclases in the tissues of plants. Belingheri and colleagues (56) found that the β-selinene cyclase isolated from the fruits of *Citrofortunella mitis* was located in the endoplasmic reticulum of the exocarpium. Bernard-Dagan and colleagues (64, 65) have reported that the sesquiterpene cyclase activity found in homogenates of maritime pine leaves precipitates with membrane fragments during centrifugation. However, this is not definitive proof of specific interaction between the membrane and the cyclase activity *in planta*. The *in vitro* association, even if non-specific, of cylase activity and membranes may explain the low yield of cyclase activity during most purifications. It is possible that a large part of the total cyclase activity is lost during centrifugation of the crude homogenate and that only a small fraction of the total activity is recovered as "soluble" enzyme in the supernatant. Another possibility is that a lipid required for maximal activity of a cyclase is only present in the pellet, thus the apparent yields of cyclase purifications are very low.

The characterization of purified cyclase enzyme can be complicated by the occasional recovery of multiple proteins, each capable of converting substrate to the same cyclized product (24). The most prominent mechanism proposed to account for the multiple forms is the action of proteases on the original, single cyclase (24). A second possible explanation for the appearance of the multiple enzyme forms is differing post-translational processing of the enzyme. A third plausible explanation for the multiple cyclase forms may be found in the duplication of genes for rate-determining enzymes for phytoalexin biosynthesis (68). In some plants, multiple genes apparently responsible for multiple isozymes provide for proper temporal-, spatial-, or stress-specific biosynthesis of phytoalexins. While the presence of possible genetic sequences may be responsible for the multiple active enzymes , the relationship of these multiple active protein systems awaits clarification at the protein level as researchers have only purified to homogeneity a single active enzyme in each system.

Another complex attribute of these enzymes is the occassional biosynthesis of multiple enzyme products by one sesquiterpene cyclase. The sesquiterpene cyclase patchoulol synthase supports biosynthesis of the sesquiterpene alcohol patchoulol and smaller amounts of a mixture of five cyclic sesquiterpene olefins (41). The phenomenon of multiple product formation by a single homogeneous monoterpene cyclase has been well documented in the action of pinene synthases of sage and appears to operate in the longifolene-sativene biosynthetic system of plants and fungi (24) The biological machinery involved in this delicately controlled production of phytoalexins should support many interesting biochemical and genetic investigations.

The existence of multiple enzyme forms catalyzing the same transformation and of cyclases capable of producing mixtures of products might be expected to create difficulties in assignment of nomenclature. The Enzyme Commission has generally overcome these potential problems by assigning the same Enzyme Commission (E.C.) number (and name) to different enzymes (or isozymes) which catalyze the same enzymatic reaction, regardless

of the tissue source of the enzymes. However, as these different enzymes are more intensively studied, the nomenclature may become more complicated. This is because separate E.C. numbers (and names) are issued "...when the specificity of two enzymes catalyzing the same reactions is sufficiently different (the degree of difference being a matter of arbitrary choice)..." or "...for enzymes having similar catalytic functions, but known to differ basically with regard to reaction mechanism or to the nature of the catalytic groups..." [quotations from page 13 of Reference 26]. Additionally, the production of mixtures of sesquiterpene products by a single homogeneous cyclase can complicate the nomenclature because "When an enzyme catalyses more than one type of reaction, the name should normally refer to one reaction only. Each case must be considered on its merits, and the choice must be, to some extent, arbitrary." [quotations from page 13 of Reference 26]. While the rules used by the Enzyme Commission do a good job of organizing the large mass of characterized enzymes, it is important for the novice to realize that the single name assigned to a particular cyclase enzyme may not fully describe the relation of that enzyme to similar proteins that catalyze the same reaction or may not fully describe the biosynthetic capability of the enzyme to synthesize a mixture of products.

The Interaction of *Gossypium hirsutum* L. (Upland Cotton) with *Xanthomonas campestris* pv. *malvacearum* : Bacterial Blight of Cotton

Cotton [*Gossypium* spp. (L.)] is a plant genus which has been important to mankind throughout antiquity and up to the present day (69). These hardy semi-tropical plants have provided fiber which clothed primitive man, then fueled the explosive growth of the Industrial Revolution by creating the demand for advances in mechanical knowledge to separate the fiber from the seed (the cotton gin) and to convert crude cotton fiber to value-added cloth and clothing (cotton mills). The success of cotton as a commodity, in a past era, enriched plantation owners (the "King Cotton" era) and, due to the labor investment

required for cultivation, provided a criminal rationale for the physical, then economic, enslavement of a large number of field laborers.

With the increased importance of other clothing fiber sources, mechanized cultivation and increased social enlightenment, the effect of cotton on mankind has changed. While cotton is no longer a totally dominant crop in much of the world, it is still very popular as a clothing fiber, having survived attempts to replace it with synthetic fibers. The endurance of cotton as a fiber source is a fortunate development, as cotton represents a renewable resource, while polyester and other synthetic fibers are derived from non-renewable petroleum. The people who grow cotton today are still economically dependent on the success of their cotton crops. This requires that today's cotton be easily cultivated and able to withstand attack by pathogens, including insects such as the boll weevil, fungi such as *Verticillium*, and the bacterium *Xanthomonas campestris* pv. *malvacearum*. Conventional plant breeding has endowed most commercial cotton lines with resistance or tolerance to these and other pests and pathogens (70). This protection against many cotton pathogens is known as "multiadversity resistance system of genetic improvement" (71). Despite this powerful protection against pathogens delivered by plant breeding, vigilance is needed as a new strain of pathogen may, without warning, arise to threaten the health of the cotton crop. An example of this sudden threat was the appearance in Africa in the early 1980's of strains of *Xanthomonas campestris* pv. *malvacearum* which were able to defeat the defenses of commercially available cotton plants (72). Once again, conventional plant breeding was able to provide cotton plants with sufficient defense to deter this potent new strain of pathogen (73).

In the system studied in this work, bacterial blight-resistant cotton infected by *Xanthomonas campestris* pv. *malvacearum* undergoes a hypersensitive response. The hypersensitive response includes production of the three sesquiterpenoid phytoalexins (19). As mentioned earlier, these are 2,7-dihydroxycadalene, lacinilene C and lacinilene C-7-methyl ether (compounds **5**, **7**, and **8**, respectively, in Figure 1). The same three

phytoalexins are produced during hypersensitive responses of seven genetically distinct cotton lines which are resistant to infection by *Xcm* (19,74). Evidently the biosynthetic pathway to these phytoalexins is common to the seven *Xcm*-resistant cotton lines, but its induction is regulated by different race-specific genes for bacterial blight resistance. Previous work has shown that the phytoalexins are accumulated at infection sites (74) in sufficient local concentrations *in planta* to account for the bacterial inhibition that occurs after inoculation of the cotton plants (75). Chemical analysis of the hypersensitively responding cotton tissues has revealed the presence of a number of compounds of molecular weight 204, 216, 218, and 232 that may be intermediates leading to the three phytoalexins [and 2-hydroxy-7-methoxycadalene (HMC, **6** in Figure 1)] (75). These findings lead to the subject of this work, the identification of the substrate and product and the purification of the cyclase enzyme of this pathway.

CHAPTER III

TRITIUM TRANSFER DURING BIOSYNTHESIS OF CADALENE STRESS COMPOUNDS IN COTTON

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Key Word Index - *Gossypium hirsutum* L.; Malvaceae; upland cotton; biosynthesis; sesquiterpenes; phytoalexins; 2,7-dihydroxycadalene; 2-hydroxy-7-methoxycadalene.

ABSTRACT

Chemical degradation of 2,7-dihydroxycadalene and 2-hydroxy-7-methoxy-cadalene biosynthesized in cotton cotyledons from a mixture of [2-^{14}C]mevalonolactone and [5-^{3}H]mevalonolactone has revealed that formation of each of these cadalene sesquiterpenoids involves a hydrogen transfer to the methine carbon of the isopropyl side chain. The transfer may occur as a 1,3-hydride shift following cyclization of the farnesyl precursor to a ten-membered ring cation.

INTRODUCTION

2,7-Dihydroxycadalene (DHC) (**1**) and 2-hydroxy-7-methoxycadalene (HMC) (**2**) are stress 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* and to pathovars of *X. campestris* that are not pathogenic to cotton [1,2]. They are concentrated in the mesophyll cells closest to the intercellular colonies of the pathogen [3]. DHC has antibacterial activity and thus is considered a phytoalexin [1]. The terpenoid origin of DHC has been established [4], and the folding pattern of its farnesyl precursor was shown to be the same as that for other sesquiterpenes of the cadalane type [5,6]. Biosynthesis in cotton of gossypol, another cadalene sesquiterpene, involves a hydrogen transfer from C-1 of the farnesyl precursor to the methine carbon of the isopropyl group, probably via a 1,3-hydride shift during cyclization [6]. Our experiments were undertaken to ascertain if a similar hydrogen transfer occurs during formation of DHC and HMC. This question was of practical interest, because if the 1,3-hydride shift occurs, [1-^{3}H] farnesyl pyrophosphate (FPP) would be an appropriate substrate for assay of the cyclase involved in biosynthesis of DHC and HMC.

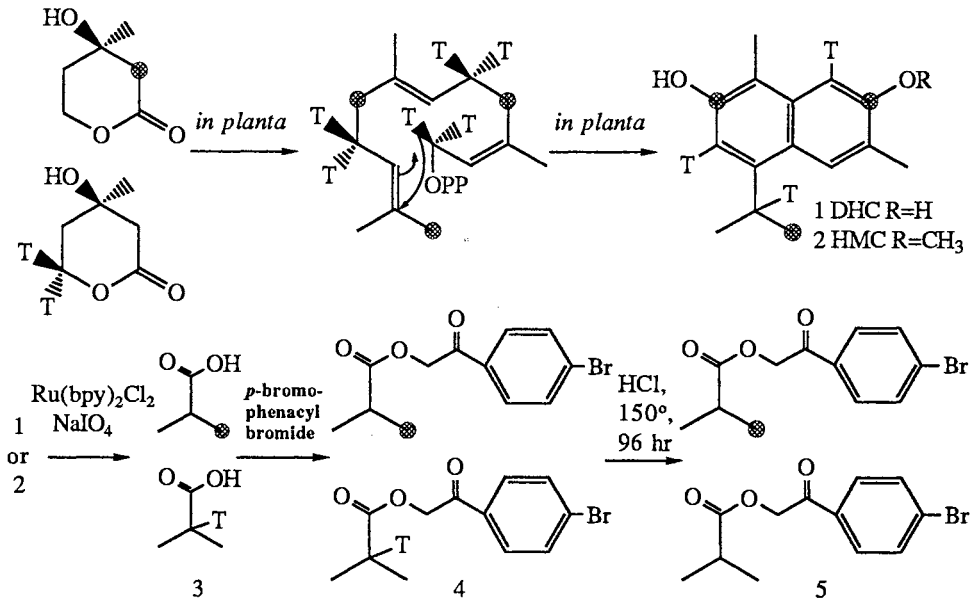


Figure 1. Predicted labelling patterns from [2-^{14}C] MVA and [5-^{3}H] MVA into DHC, HMC, and their degradation products.

RESULTS AND DISCUSSION

The chemical manipulations applied to labelled DHC and HMC are summarized in Figure 1. Cotton cotyledons inoculated with an incompatible race of *Xanthomonas campestris* pv. *malvacearum*, then fed a mixture of *RS*-[5-^{3}H] MVA and *RS*-[2-^{14}C] MVA, were extracted and chromatographed (Figure 2) to yield the doubly-labelled phytoalexins DHC (**1**) and HMC (**2**). As shown in Table 1, the ^{3}H:^{14}C ratio for the isolated labelled DHC was 2.82:3 (=0.94:1.00) and for HMC was 2.56:3 (=0.85:1.00). While the ^{3}H:^{14}C ratios for the labelled DHC and HMC were lower than the "ideal" value of 1.00:1 (*i.e.*, one-half of the 2.00:1 ratio in the mevalonolactone precursors), the apparently low ^{3}H:^{14}C ratios can be explained by invoking the labile nature of tritium label (as compared to ^{14}C label). An example of the magnitude of an acceptable ^{3}H:^{14}C ratio in an similar radiochemical experiment can found in the creation of the labelled sesquiterpene γ-patchoulene from [4*R*-^{3}H, 2-^{14}C]mevalonolactone [7]. In that experiment, an ideal value for the ^{3}H:^{14}C ratio of the recovered γ-patchoulene was 1.00:1, based on the ^{3}H:^{14}C ratio of the mevalonolactone precursors; the ^{3}H:^{14}C ratio found in the isolated γ-patchoulene

TABLE 1

INCORPORATION OF A MIXTURE OF [2-^{14}C]MVA AND [5-^{3}H]MVA INTO DHC, HMC AND THEIR DEGRADATION PRODUCTS

Compound	^{14}C Relative Specific Activity (pCi/nmol)		Relative Atomic Ratio (^{3}H:^{14}C)	
Mevalonolactone	5.01 X 10^4		2.00:1	
	Normal* Phase HPLC	Reversed* Phase HPLC	Normal Phase HPLC	Reversed* Phase HPLC
DHC(**1**)		518		2.82:3
Ester (**4**) from DHC				
First half+	5.45 5.51 5.45	4.95 4.85	1.30:1 1.27:1 1.33:1	1.33:1 1.36:1
Second half+	6.97 6.96 7.27	7.27 7.09	1.31:1 1.30:1 1.28:1	1.25:1 1.29:1
HMC (**2**)		831		2.56:3
Ester (**4**) from HMC‡	14.2 13.1 13.2	13.4 13.1	1.30:1 1.26:1 1.27:1	1.25:1 1.28:1
Ester (**5**) from HMC after exchange (no carrier)	12.1		0.075:1	
Ester (**5**) from HMC after exchange (with carrier)	2.09 2.10 2.14		0.076:1 0.059:1 0.042:1	

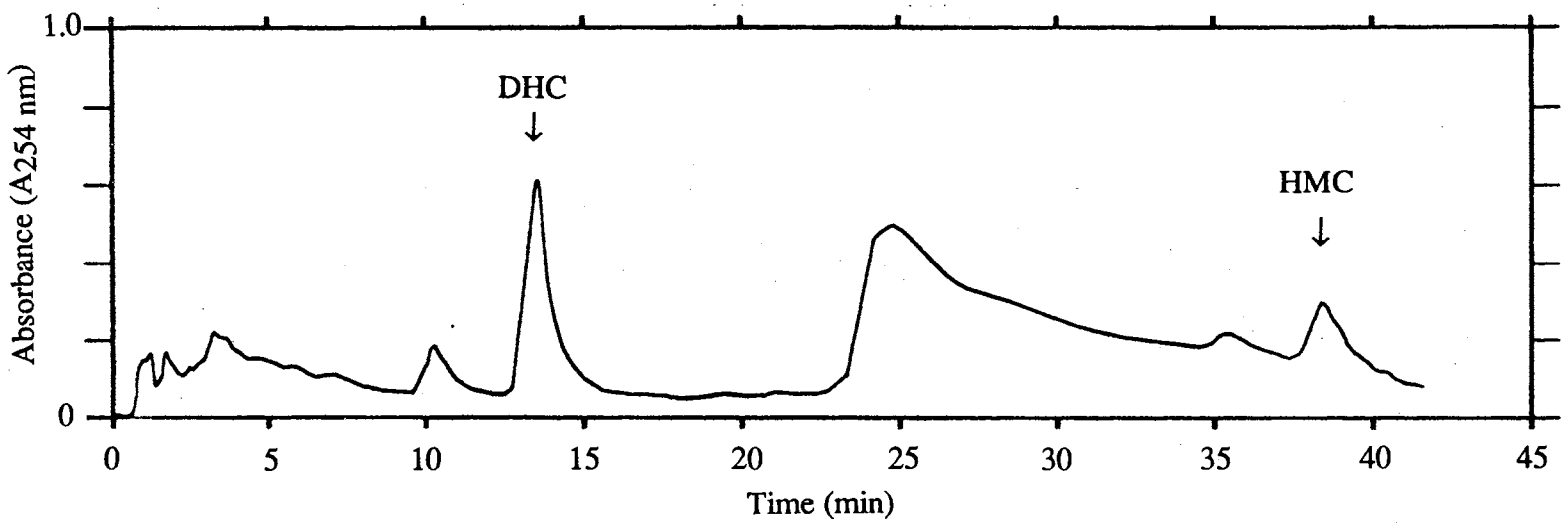
* Values from sequential chromatographies. The ester (**4**) or (**5**) was first chromatographed by normal phase HPLC, then reversed phase HPLC.

+ The labelled sample of DHC was divided into two halves, which were degraded separately. Carrier ester was generated *in situ* by addition of unlabelled isobutyric acid prior to esterification.

‡ One-half of this purified ester was used for the exchange experiment, results shown below.

Figure 2. Reversed phase isolation of labelled DHC and HMC from cotton cotyledon tissue.

Instrument: Spectra/Physics SP8700 Solvent Delivery System.
Separation performed with Hibar HPLC column using octadecylsilane media (C_{18}, 5μm particle diameter)
(Elution regime described in Experimental section)


1.0
0
Absorbance (A254 nm)
DHC
HMC
0
5
10
15
20
25
30
35
40
45
Time (min)

was 0.92:1. Although it is obvious that approximately 8% of the tritium label was lost, relative to the ^{14}C label, it was concluded that this ^{3}H:^{14}C ratio "... suggests that no tritium was lost..." during cyclization [7].

The identity of the isolated labelled compounds was confirmed by obtaining the UV spectra of the DHC (Figure 3) and HMC. Subsequent degradation by *cis*-Ru(bipyridine)$_2Cl_2 \cdot 2H_2O$ and sodium metaperiodate [8] of each of these compounds yielded doubly-labelled isobutyric acid (**3**) from the isopropyl side chain and unlabelled acetic acid from the methyl substituents due to the formation of carboxylic acid functions at the points of attachment to the naphthalene nucleus of DHC and HMC. These acids were esterified to yield *p*-bromophenacyl esters [9], which were then isolated by HPLC [10] on silica. Isolation of the esterification reaction mixture is shown in Figure 4 and chromatography of standard compounds is shown in Figure 5.

The doubly-labelled *p*-bromophenacyl isobutyrate (**4**) from DHC or HMC was chromatographed (Figure 6) to constant specific radioactivity in the same system, then re-chromatographed by reversed phase HPLC on octadecylsilane (Figure 7), maintaining constant specific radioactivity. The chromatography of the doubly-labelled *p*-bromophenacyl isobutyrate (**4**) from DHC or HMC was accomplished by adjusting to an interesting chromatographic phenomenon. It was found that during chromatography of the labelled isobutyrate ester (actually a mixture of ^{3}H-labelled isobutyrate ester, ^{14}C-labelled isobutyrate ester and unlabelled isobutyrate ester) that the ^{3}H:^{14}C ratio was not constant "across the peak": the tritium-labelled ester was more retained than the unlabelled and ^{14}C-labelled ester on the polar silica gel media. Conversely the tritium-labelled ester eluted slightly before the unlabelled and ^{14}C-labelled ester during chromatography on the octadecylsilane (C-18) media. Thus the ^{3}H-labelled isobutyrate ester (at least in liquid chromatography) behaves as a more polar or hydrophilic entity than the ^{14}C-labelled isobutyrate ester or unlabelled isobutyrate ester.

Figure 3. UV absorbance spectrum of labelled DHC (2,7-dihydroxycadalene) in 60% methanol [60:40; methanol:H_2O (v/v)], prior to ruthenium degradation.

Instrument: Hitachi 100-80 UV Recording Spectrophotometer

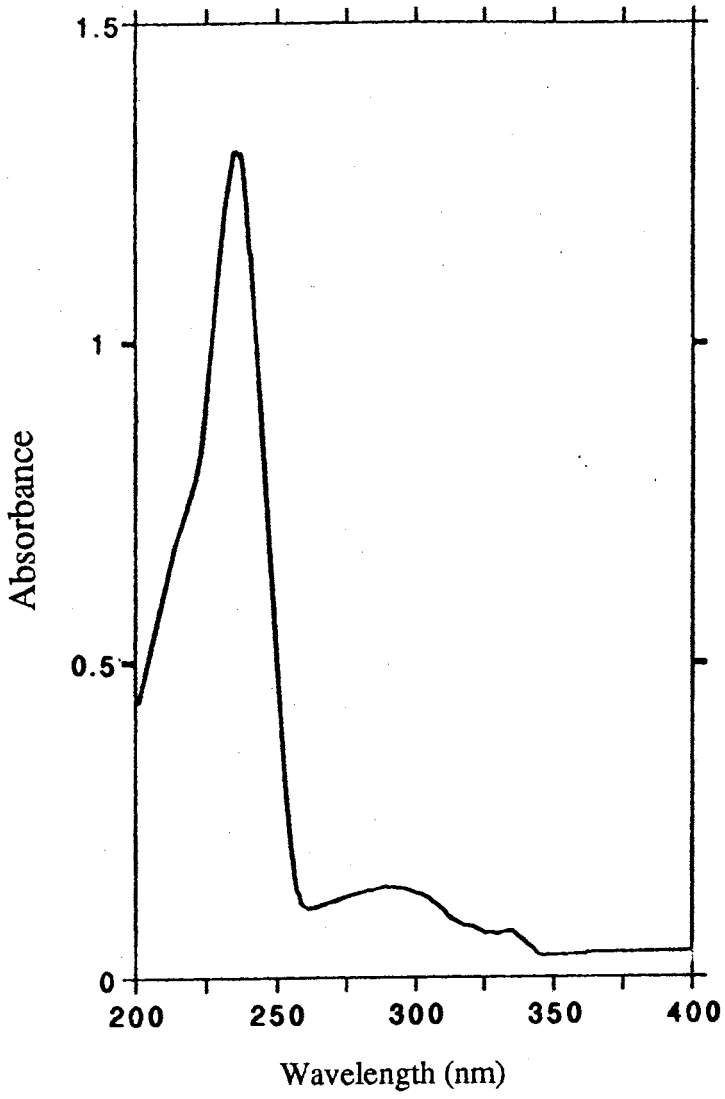

1.5
1
0.5
0
Absorbance
200
250
300
350
400
Wavelength (nm)

Figure 4. Normal phase HPLC isolation of products from degradation of labelled DHC and esterification of resulting degradation products.

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with an Alltech HPLC column with Lichrosorb Si-60 packing (silica gel, 5μm particle diameter). (Elution regime described in Experimental section)

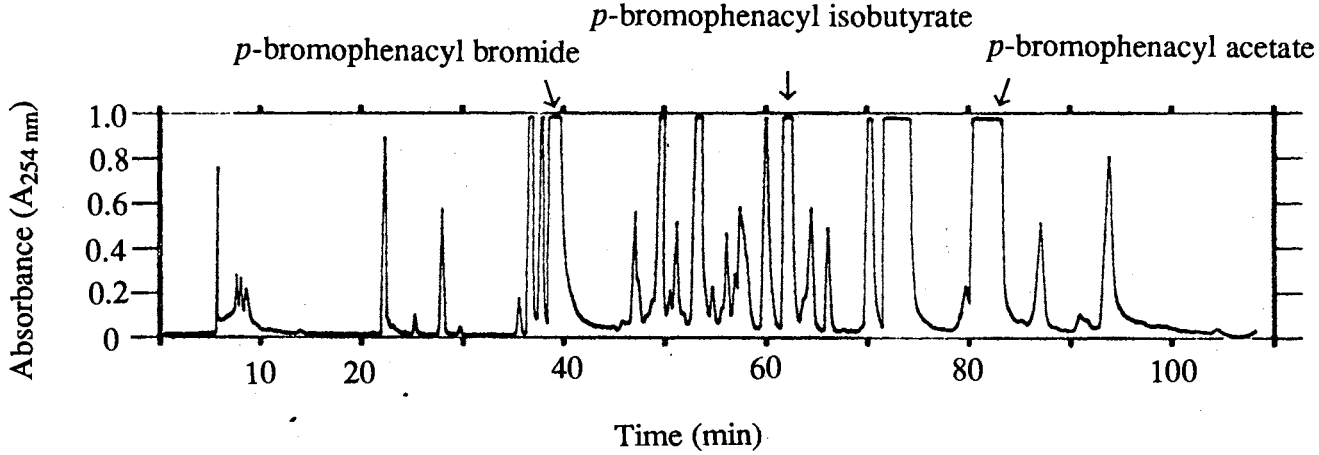

p-bromophenacyl bromide
p-bromophenacyl isobutyrate
p-bromophenacyl acetate
Absorbance (A254 nm)
1.0
0.8
0.6
0.4
0.2
0
10
20
40
60
80
100
Time (min)

Figure 5. Normal phase HPLC separation of standard compounds (in order of elution): *p*-bromophenacyl bromide, *p*-bromophenacyl isobutyrate, and *p*-bromophenacyl acetate.

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with an Alltech HPLC column containing Lichrosorb Si-60 packing (silica gel, 5μm particle diameter). (Elution regime described in Experimental section)

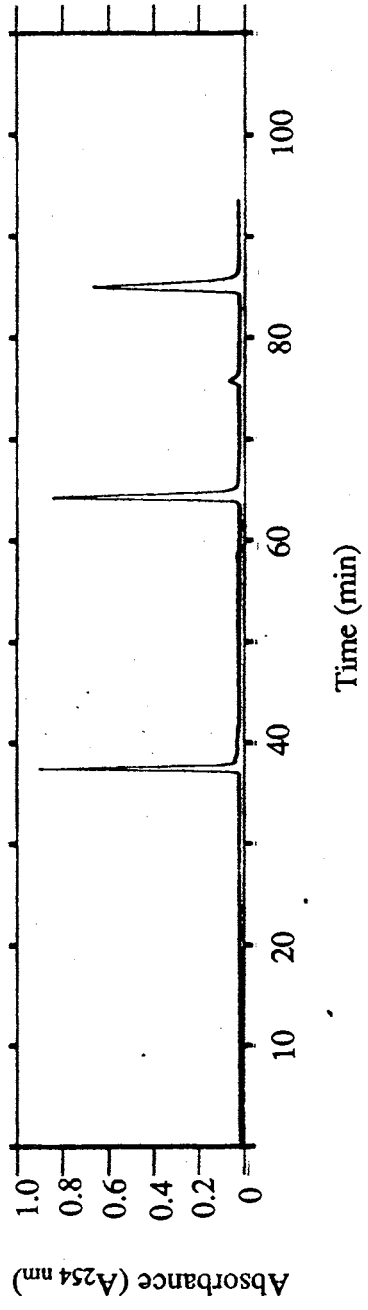

Absorbance ($A_{254\ nm}$)
1.0
0.8
0.6
0.4
0.2
0
10
20
40
60
80
100
Time (min)

Figure 6. Example of normal phase HPLC re-chromatography of labelled *p*-bromophenacyl isobutyrate to constant specific activity:

a) Initial re-chromatography of labelled *p*-bromophenacyl isobutyrate obtained by degradation and esterification of labelled DHC created by cotton tissue inoculated with labelled MVA.

b) Re-chromatography of labelled *p*-bromophenacyl isobutyrate chromatographed in a).

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with an Alltech HPLC column containing Lichrosorb Si-60 packing (silica gel, 5μm particle diameter). (Elution regime described in Experimental section)

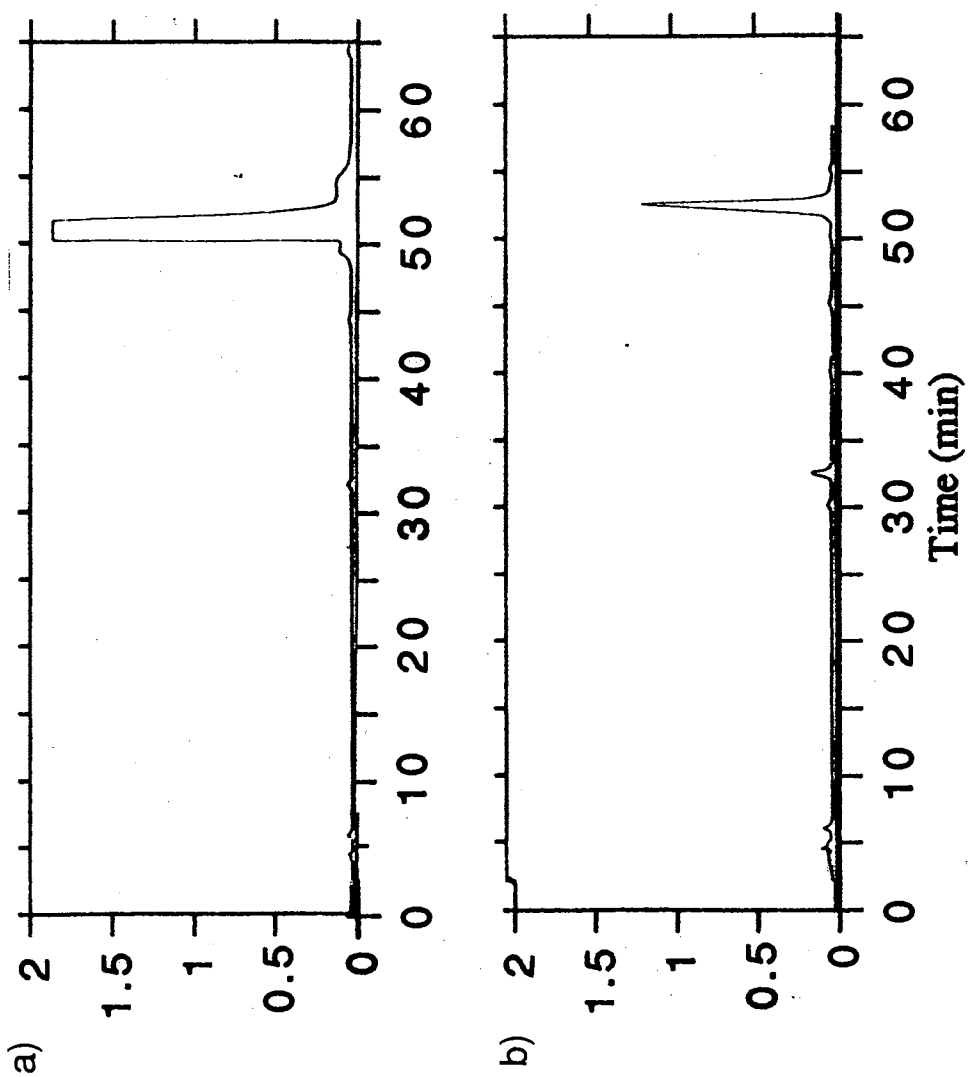
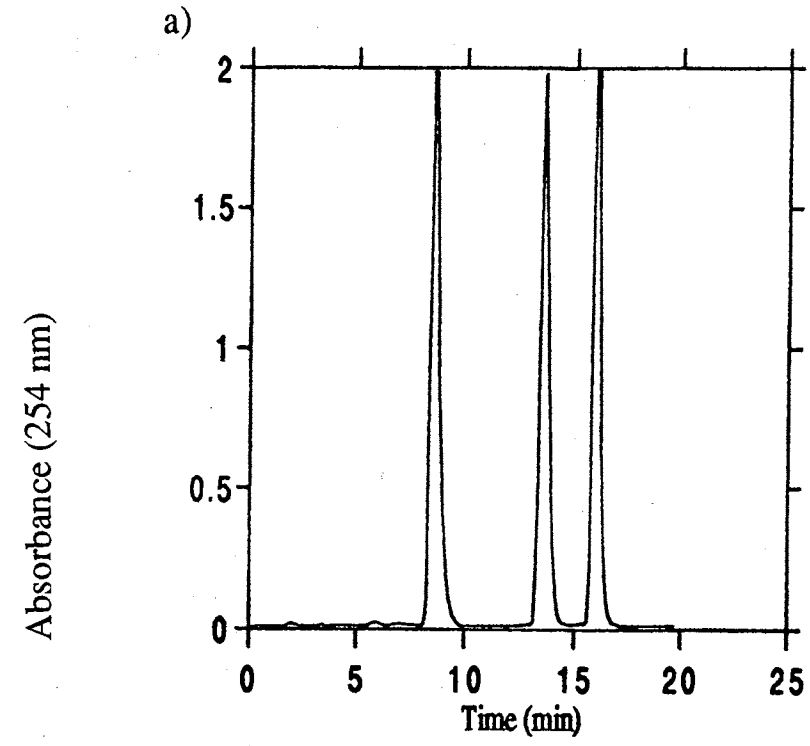

a)
b)
Absorbance (254 nm)
Time (min)
0
0.5
1
1.5
2
0
10
20
30
40
50
60

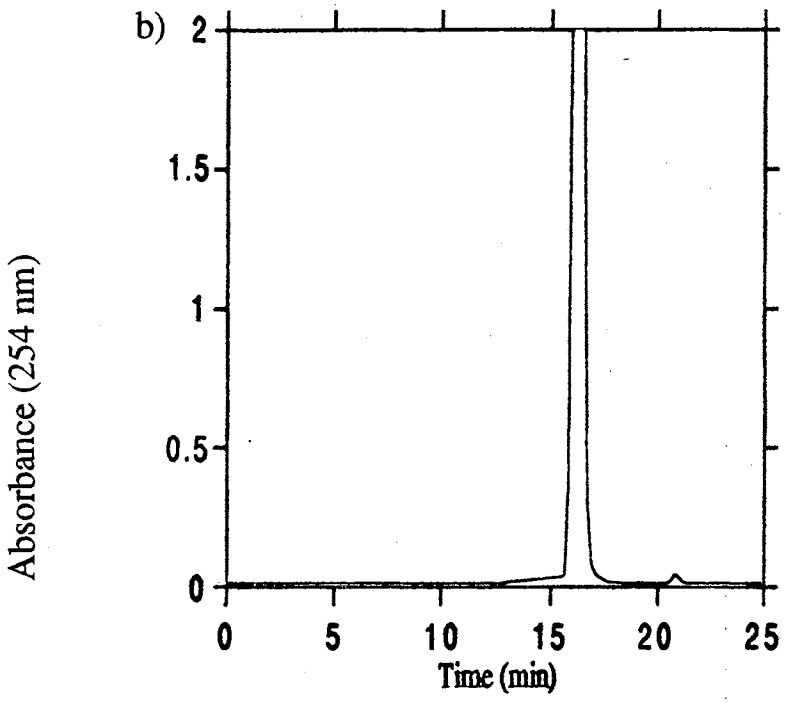
Figure 7. Reversed phase separations:

a) HPLC separation of standard compounds (in order of elution): *p*-bromophenacyl bromide, *p*-bromophenacyl acetate, and *p*-bromophenacyl isobutyrate.

b) HPLC rechromatography of *p*-bromophenacyl isobutyrate previously chromatographed to constant specific activity by normal phase HPLC.

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with Hibar HPLC column containing octadecylsilane media (C_{18}, 5μm particle diameter) (Elution regime described in Experimental section)


a)
Absorbance (254 nm)
2
1.5
1
0.5
0
0
5
10
15
20
25
Time (min)


b)
Absorbance (254 nm)
2
1.5
1
0.5
0
0
5
10
15
20
25
Time (min)

While this chromatographic behaviour, known as "isotopic fractionation", is suprising and even upsetting to some researchers (see footnote on p. 7 of Reference [11]), many examples have been compiled in a review [12]. Researchers may ignore this effect to their peril, because if they simply collect a peak fraction based on the UV absorbance of a mixture of the ^{3}H-labelled, ^{14}C-labelled and the unlabelled versions of a compound, some of the ^{3}H-labelled component will be likely be accidentally discarded because the "time window" of the collection based on the UV absorbance will be too narrow. (Repetition of this type of fraction collection will lead to a constantly declining ^{3}H:^{14}C ratio during subsequent chromatographies.) This type of potential problem has been described for the chromatography of mixtures of deuterium-labelled and unlabelled plant hormones such as indole acetic acid (IAA), abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) [13]. Isotopic fractionation has been documented in low resolution chromatographic systems such as thin layer chromatography [10,11], gas chromatography with packed columns [14] and even during simple concentration of nitrogen compounds prior to isotope-ration analysis [15]. Baseline resolution of mixtures of deuterium-labelled compounds from the unlabelled analog can be attained with ease in high resolution capillary gas chromatographic systems as shown by the separation of deuterated BHT (butylated hydroxytoluene) from unlabelled BHT [16].

The ^{3}H:^{14}C relative atomic ratio of the doubly-labelled *p*-bromophenacyl isobutyrate (Table 1) was significantly and reproducibly higher than the expected ratio of 1.00:1. A similar observation was made by Arigoni after HNO_3 degradation of doubly-labelled avocettin [5]. He explained the abnormally high ^{3}H:^{14}C ratio by proposing that enolization of the product isobutyric acid is the rate-determining step in its further degradation by HNO_3. Due to the kinetic isotope effect expected for enolization of isobutyric acid bearing tritium at the α-position, tritium-labelled isobutyric acid molecules would be relatively protected from further degradation. Since the doubly-labelled sesquiterpene preparations in Arigoni's study and in ours were obtained from separate preparations of ^{3}H- and ^{14}C-

labelled MVA, no isobutyric acid molecules would be expected to bear both ^{3}H and ^{14}C; ^{14}C-labelled molecules would not be protected from further degradation, and the ^{3}H:^{14}C ratio would increase during the degradation. Application of this proposed relative protection of tritium-labelled fragments from labelled DHC and HMC (and the resulting observed rise in ^{3}H:^{14}C ratio) is detailed in Figure 8.

Relevant to this proposal are kinetic and product studies of the oxidation of cyclohexanone catalyzed by the tris-2,2'-bipyridyl ruthenium cation [17], which provided evidence that it is the enol form of the substrate that is oxidized. Those studies also suggest that at the relatively high concentration of ruthenium catalyst that we employed, enolization is the rate-limiting step in further oxidation of isobutyric acid, as proposed above to account for our abnormally high ^{3}H:^{14}C ratio in *p*-bromophenacyl isobutyrate.

To test whether all of the tritium in the isobutyrate ester **4** was in the α-position, as shown in Figure 1, exchangeability of the tritium was tested. Purified ester **4** obtained by degradation of HMC was heated at 150° for 96 hr in 12N HCl. The isobutyric acid liberated by hydrolysis under these conditions was re-esterified and chromatographed to constant specific activity. The specific activities revealed that approximately 96% of the tritium had been lost by exchange whereas approximately 90% of the ^{14}C label was retained (Table 1 and **5**, Figure 1). This exchangeability confirmed the localization of tritium on the methine carbon atom of the isopropyl side chain of HMC, as shown in Figure 1. The process of exchange with water and the lowering of the ^{3}H:^{14}C ratio is illustrated in Figure 9.

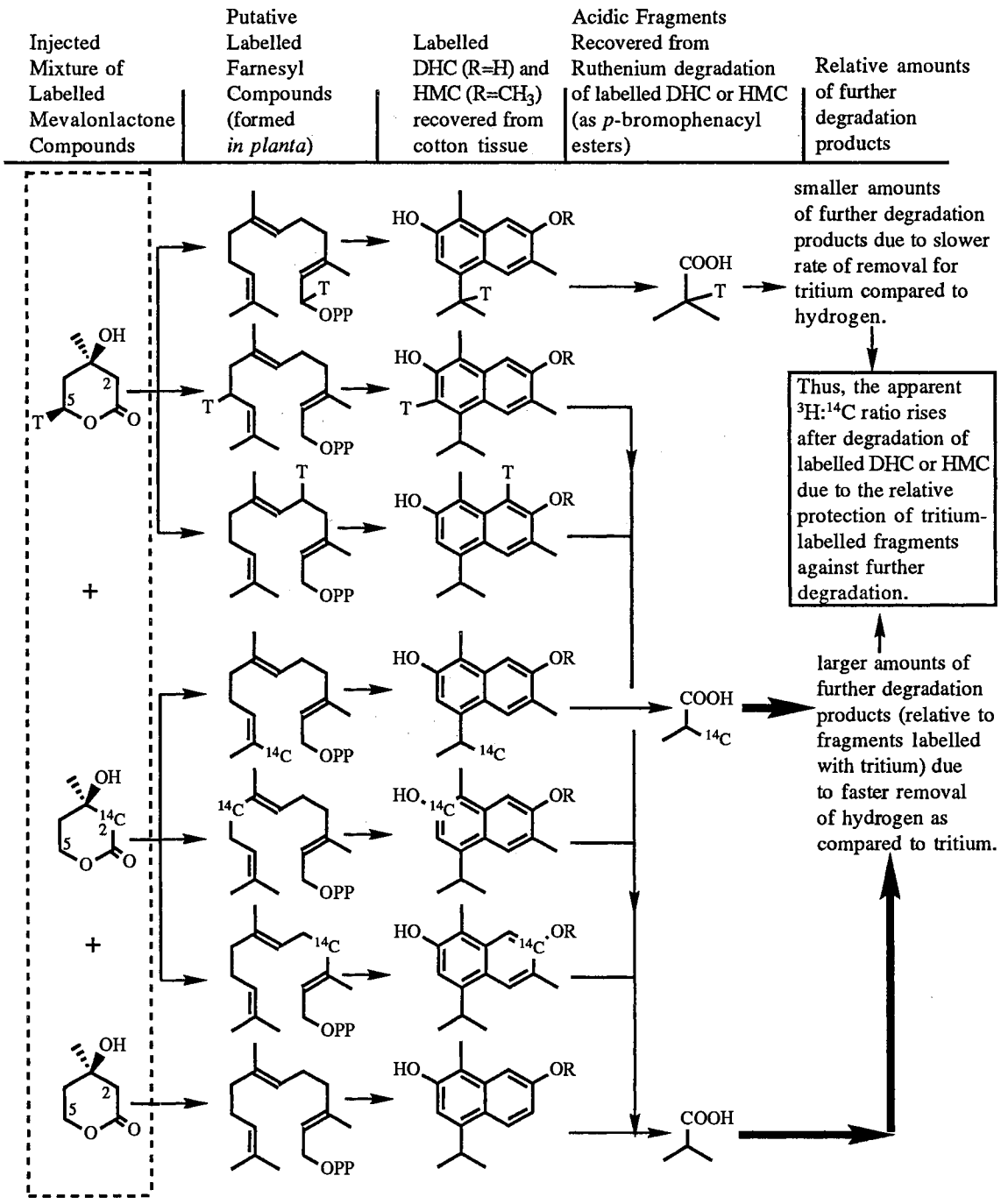


Figure 8. Detailed scheme to show species created during creation of labelled DHC and HMC.

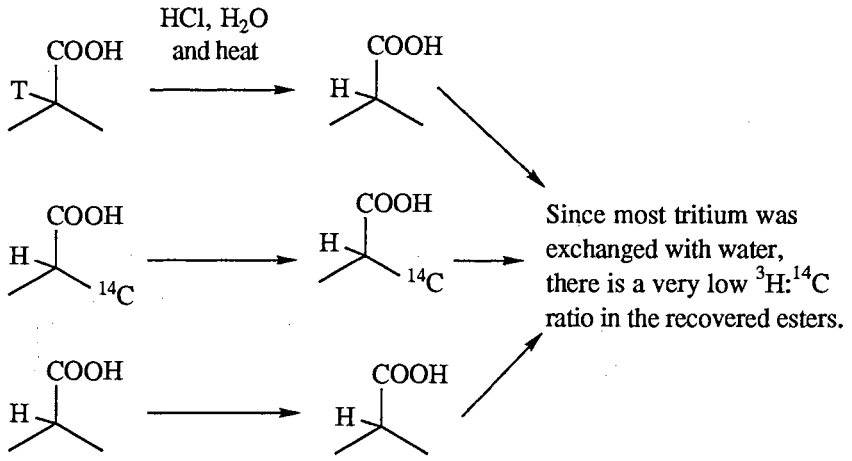


Figure 9. Scheme to show effect of exchange with water resulting in lowering of ^{3}H:^{14}C ratio.

Our evidence strongly suggests that DHC and HMC are, like gossypol, formed by cyclization of FPP or the biosynthetic equivalent of FPP via a ten-membered ring cation [6] that undergoes a 1,3-hydride shift from C-1 of FPP to the methine carbon of the isopropyl group. The observed retention of tritium in this position through the subsequent biosynthetic steps to DHC and HMC suggests that [1-^{3}H]FPP will be an appropriate substrate for assay in cell-free extracts of the first enzyme of this pathway.

EXPERIMENTAL

Isotopic labelling of stress compounds in planta

RS-[5-^{3}H] MVA lactone (sp. act. 30 Ci/mmol) and *RS*-[2-^{14}C] MVA lactone (sp. act. 50.1 mCi/mmol) were purchased from New England Nuclear. The two central quarters of fourteen leafy cotyledons of seven 12-day-old seedlings of cotton line OK1.2 (*Gossypium hirsutum* L.) were infiltrated with a suspension of 5.4 x 10^{6} colony-forming-units/ml of *Xanthomonas campestris* pv. *malvacearum* race 3 as previously described [3]. At 26 hr post-inoculation, the cotyledons were infiltrated with the radioactive precursors (126 μCi ^{3}H and 88 μCi ^{14}C) in 4 ml H_2O solution (pH=2.2). At 43 hr post-inoculation,

the cotyledons were harvested, quick-frozen in liquid N_2 and stored at -70°. DHC and HMC were extracted from frozen cotyledons and purified by reversed phase HPLC as previously described [3]. Yields were 388 nmol of DHC and 286 nmol of HMC.

Degradation of stress compounds

The doubly-labelled DHC and HMC were separately degraded by an adaptation of the method of Chakraborti and Ghatak [8]. The oxidizing reagent (*cis*-Ru(bipyridine)$_2$Cl$_2$·2H$_2$O) used to degrade the labelled DHC and HMC was synthesized by a published method [18]. Commercial $RuCl_3$-3H_20 (7.8 g, 29.8 mmol), bipyridine (9.36 g, 60.0 mmol), and LiCl (8.4 g, 2 mmol) were heated at reflux with stirring in dimethylformamide (50 mL) for 8 h. The reaction was cooled to room temperature, 250 mL of acetone was added and the solution was cooled at 0°C overnight. Filtering yielded a red-violet solution and finely divided green-black crystals. The solid was washed three times with 25-ml portions of water followed by three 25-mL portions of diethyl ether, and then dried by suction prior to use in degradation of DHC or HMC.

To begin the oxidation 15 ml of a 1:1 mixture of H_2O:CH_3CN containing DHC or HMC was added 10 mg of *cis*-Ru(bipyridine)$_2$Cl$_2$·2H$_2$O and 1g of sodium metaperiodate. The mixture was refluxed with stirring for 1 hr. Unlabelled carrier isobutyric acid (1.7 μmol) and acetic acid (3.4 μmol) were added. After cooling, the mixture was filtered to remove sodium periodate. Water (40 ml) was added to improve phase separation, and after acidification with 0.1 ml of 12N HCl, the solution was extracted with 3 x 15 ml of Et_2O. The combined Et_2O extracts were chilled at -20° for 3 hr to freeze contaminating H_2O and then the Et_2O extracts were transferred to another flask. Et_2O was evaporated with a low velocity stream of N_2 gas.

Esterification

The *p*-bromophenacyl esters were prepared by modification of the method of Miller *et al.* [9]. The preparation of acids was dissolved in 15 ml of CH_3CN and acidified with 0.1ml of 12N HCl. KF (1 g) and *p*-bromophenacyl bromide (7 mg) were added, and the resulting mixture was stirred for 1 hr at room temperature in a 50-ml round-bottom flask fitted with a water-cooled condenser. Water (40 ml) was added, and the esters were extracted with 3 x 15ml of Et_2O. Solvent was evaporated from the combined Et_2O extracts at reduced pressure with an Evapomix (Haake Buchler Instruments, Inc.). A series of esters were synthesized. Melting points were taken to assure identity of the synthesized ester (Table 2). [In Table 2, note the series of reference melting points for *p*-bromophenacyl formate. It is possible that the higher reference values (in the range of 135°C to 140°C) represent original incorrect values that were appropriated for subsequent publication in melting point tables without experimental validation. The existence of the one reference value that matches our experimental melting point value for *p*-bromophenacyl formate, combined with the good match between our experimental melting point values for the remaining esters, does increase our confidence that the reference melting point value of 99°C for *p*-bromophenacyl formate is correct.]

HPLC of esters

The *p*-bromophenacyl esters of isobutyric and acetic acids were isolated, and the isobutyrate ester was re-chromatographed on an Alltech/Lichrosorb Si-60 (5μm particle diameter), 4.6 X 250 mm silica column, using a Spectra/Physics model 8700 solvent delivery system and a Waters model 440 254-nm absorbance detector or dual Waters 510 pumps with automated gradient controller as well as Waters Lambda Max variable wavelength detector set at 254 nm. Injection volumes ranged from 50 to 400 μl. The normal phase system was capable of resolving esters synthesized from the acids that were structural isomers *e.g.*, isobutyric and butyric acids as shown in Figure 10. The amount

TABLE 2

COMPARISON OF EXPERIMENTALLY DETERMINED MELTING POINTS OF *p*-BROMOPHENACYL ESTERS WITH REFERENCE VALUES

p-bromophenacyl ester:	Experimental melting point of *p*-bromophenacyl ester (°C)	Reference values for melting point of *p*-bromophenacyl ester (°C)
Formate	98-99	140 or 135[a] 140[b] 140 or 135.2[c] 99[e]
Acetate	83-84	86.0[a] 86[b] 86.0 or 85.0[c]
Propionate	61	63.4[a] 63[b] 63.4 or 59.0[c]
Butyric	62	63[a] 63[b] 63.0 or 63.2[c]
Isobutyric	75-76	76.8[a] 76[b] 76.8[c] 76.8[d]

a. Reference 19. *Handbook of Tables for Organic Compound Identification.* Third Edition. (1967) Compiled by Z. Rappoport. p.190, The Chemical Rubber Co. Cleveland, Ohio.

b. Reference 20. Vogel, A. I. (1989) *Vogel's Textbook of Practical Organic Chemistry.* Fifth Edition. Longman Scientific & Technical. Furniss, B. S., Hannaford, A. J., Smith, P. W. G., and Tatchell, A. R. (eds.) pp. 1342-1343, Essex, England .

c. Reference 21. Huntress, E. H. and Mulliken, S. P. (1941) *Identification of Pure Organic Compounds.* p. 651, John Wiley & Sons, Inc. London.

d. Reference 22. Utermark, W. and Schicke, W. (1963) *Melting Point Tables of Organic Compounds.* Interscience Publishers, New York. (See compound number 47, 0 for *p*-bromophenacyl isobutyrate).

e. Reference 23. Buckingham, J. (ed.). (1982). *Dictionary of Organic Compounds.* Fifth Edition. p. 2677, Chapman and Hall, London.

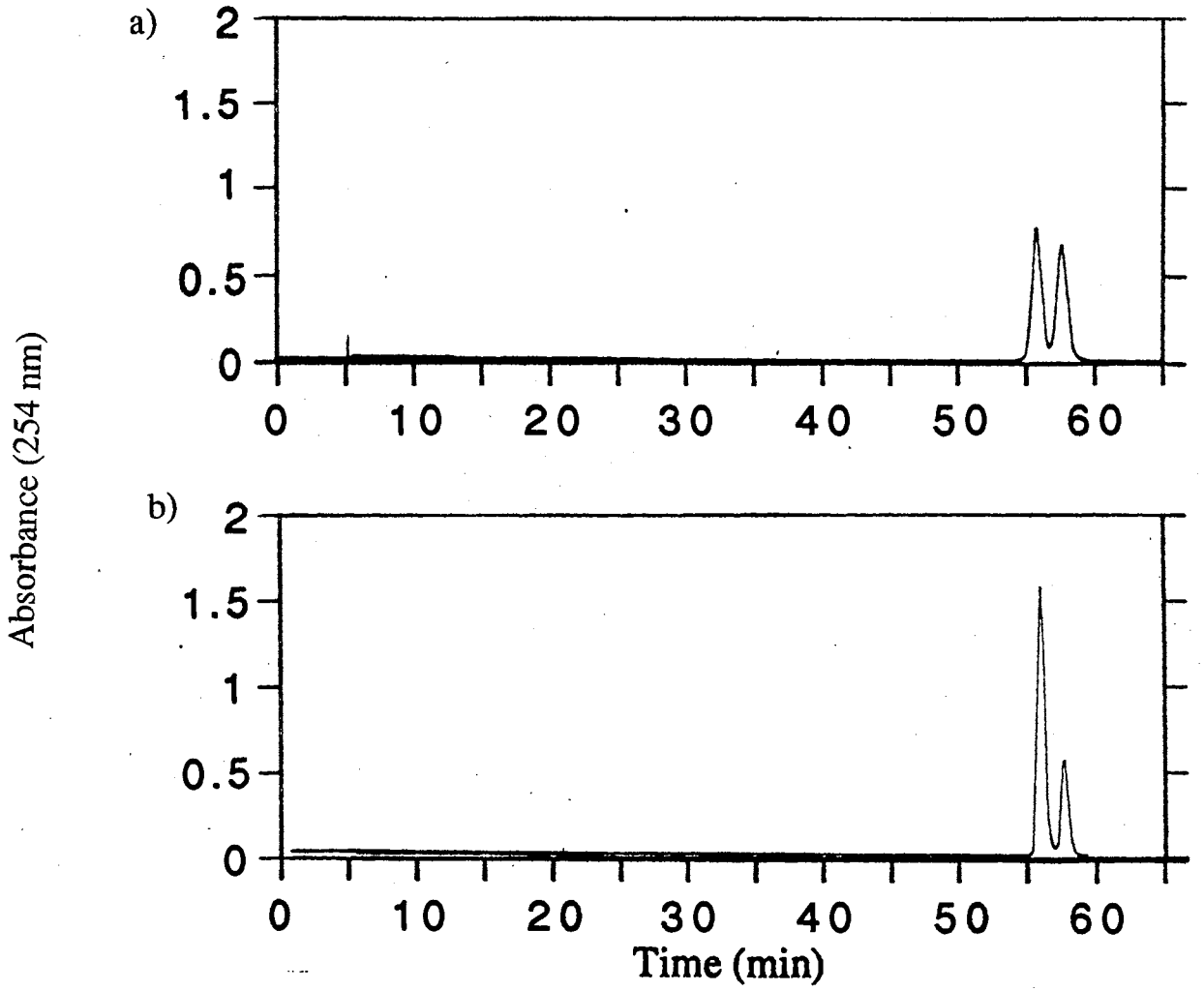
Figure 10. Normal phase HPLC to test ability of the normal phase system to separate structurally similar *p*-bromophenacyl esters:

a) Separation of mixture of *p*-bromophenacyl isobutyrate and *p*-bromophenacyl butyrate

and

b) Separation of sample chromatographed in a), spiked with additional *p*-bromophenacyl isobutyrate

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with an Alltech HPLC column containing Lichrosorb Si-60 packing (silica gel, 5μm particle diameter). (Elution regime described in Experimental section)


a)
b)
2
1.5
1
0.5
0
0
10
20
30
40
50
60
Absorbance (254 nm)
Time (min)

and activity of the isobutyrate ester samples initially were approximately 900 nmol containing approximately 12 μCi decreasing to approximately 150 nmol containing approximately 2 μCi during the process of re-chromatography. Elution, based on the method of Weatherston *et al.* [10], was with 5% CH_2Cl_2 in hexane (v/v) for 5 min, followed by a gradient of 1%/min increase in CH_2Cl_2 until 70 min. Flow rate was 1 ml min^{-1} throughout. Void volume (V_o) for the HPLC column was determined by injection of pentane and was found to be 3.8 mls [24]. Capacity factors (k') were 12 and 16 for the isobutyrate and acetate esters, respectively. (Chromatography for V_o and k' is shown in Figure 11).

For subsequent reversed phase HPLC of the *p*-bromophenacyl isobutyrate, an E. Merck Hibar RP C18 (5 μm particle diameter), 4.6 x 250 mm column was employed with a 30-min linear gradient program of 60-87% MeOH in H_2O (v/v) at 1 ml min^{-1}. Void volume (V_o) was determined to be 2.1 ml by injection of $1x10^{-5}$ M sodium nitrate as described by Wells and Clark [25]. Capacity factors (k') were 6.0 and 2.5 for the isobutyrate and acetate esters, respectively. (Chromatography for V_o and k' is shown in Figure 12).

^3H exchange with water

One-half of the doubly-labelled *p*-bromophenacyl isobutyrate, which had been isolated from HMC and chromatographed three times on the silica column was dissolved in 3 ml of hexane and divided among three vacuum hydrolysis tubes. These tubes were manufactured in-house by sealing tubes to Ace stopcocks equipped with sidearm vent tubes. The hexane was removed with a gentle stream of N_2 gas, and 0.5 ml of 12 N HCl was added to each tube. The tubes were cautiously and slowly heated to 150° in a Tecam DB-3H heating block with 8-mesh aluminum beads filling extra space in the heating block wells. The tubes were maintained at 150° for 96 hr, then slowly cooled. The contents were removed, diluted with 50 ml of H_2O, and extracted with 3 x 15 ml of Et_2O. Isobutyric acid in the

Figure 11. Normal phase HPLC to obtain void volume and capacity factors (k') for (in order of elution): *p*-bromophenacyl bromide, *p*-bromophenacyl isobutyrate and *p*-bromophenacyl acetate.

Inset tracing to show determination of V_o by injection of pentane (V_o =3.8 ml)

Instrument: Spectra/Physics SP8700 Solvent Delivery System. Separation performed with an Alltech HPLC column containing Lichrosorb Si-60 packing (silica gel, 5μm particle diameter). (Elution regime described in Experimental section)

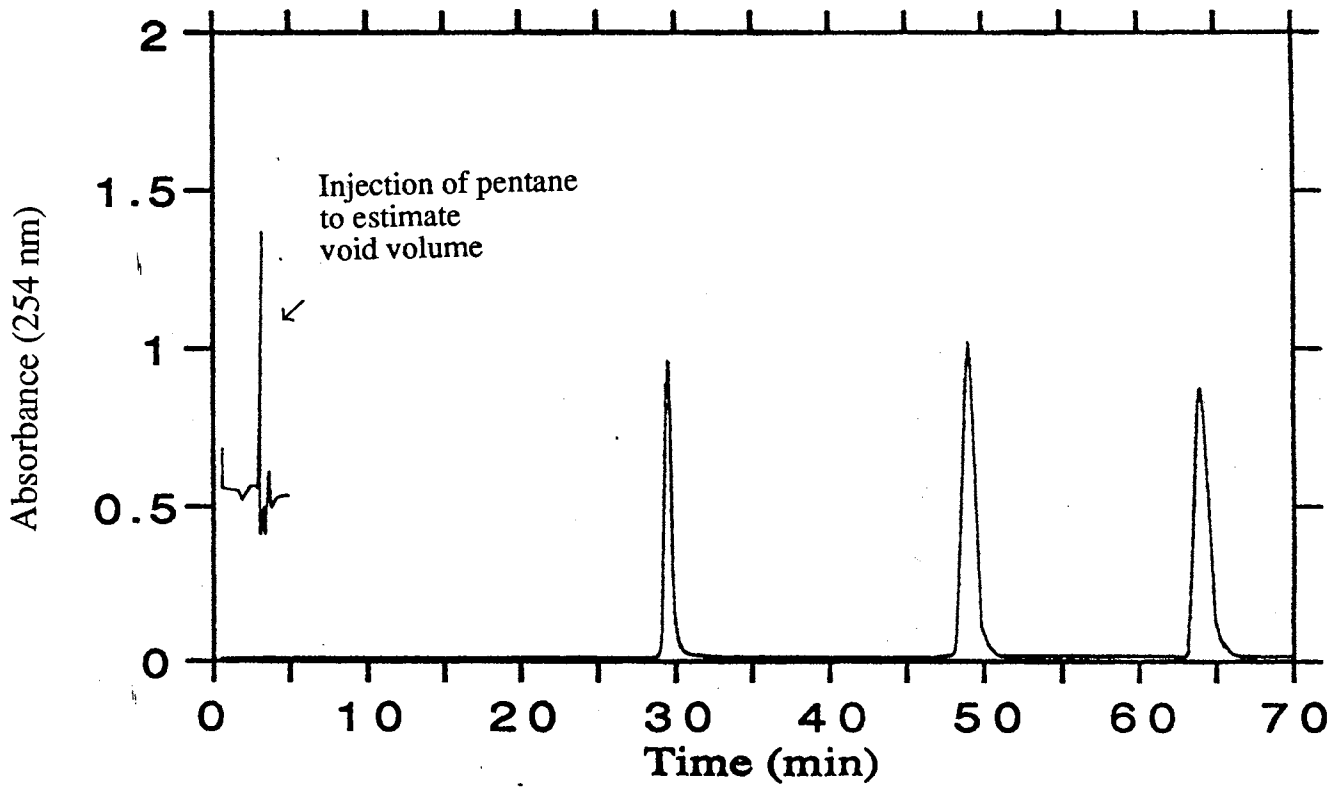
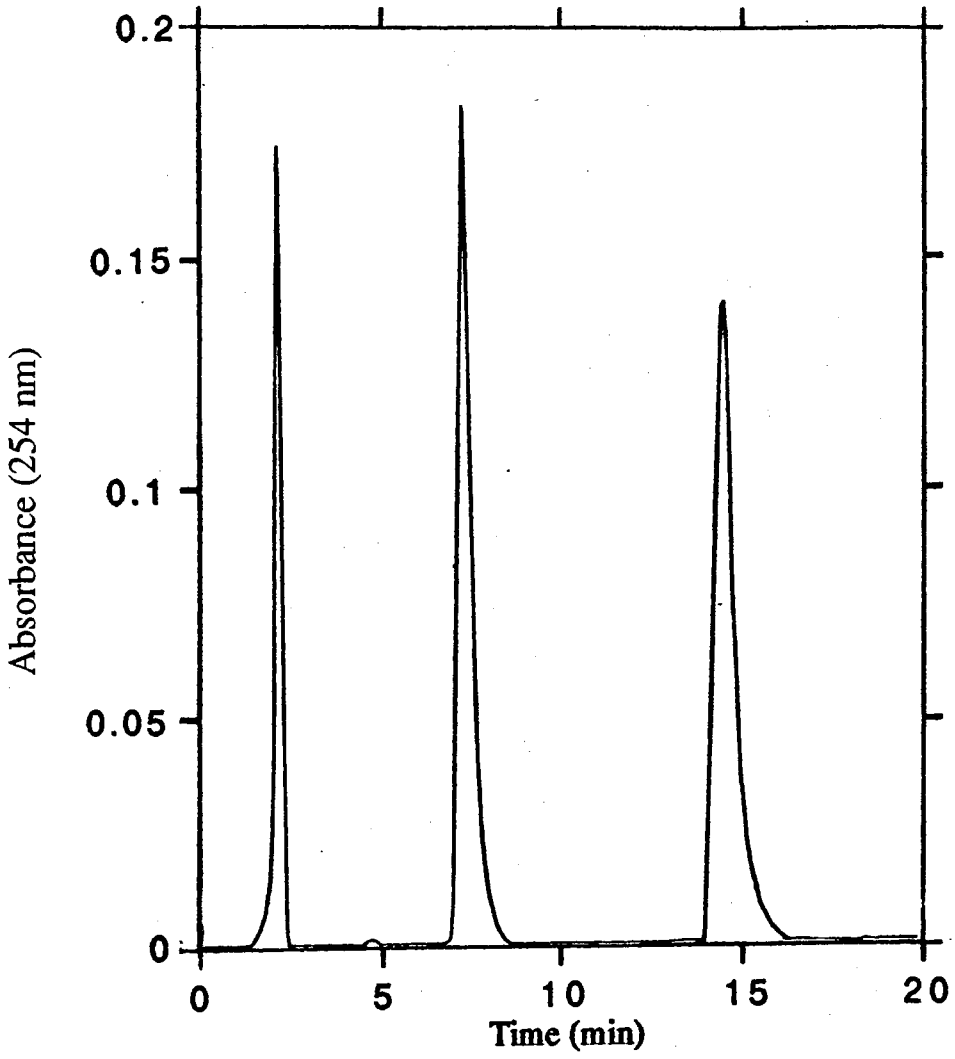

Absorbance (254 nm)
2
1.5
1
0.5
0
Injection of pentane
to estimate
void volume
0
10
20
30
40
50
60
70
Time (min)

Figure 12. Reversed phase HPLC to obtain void volume and capacity factors for (in order of elution): $NaNO_3$, *p*-bromophenacyl acetate, and *p*-bromophenacyl isobutyrate.

Void volume (V_o) by $NaNO_3$ retention = 2.1 ml

Instrument: Spectra/Physics SP8700 Solvent Delivery System.
Separation performed with Hibar HPLC column containing octadecylsilane media (C_{18}, 5μm particle diameter)
(Elution regime described in Experimental section)


0.2
0.15
0.1
0.05
0
Absorbance (254 nm)
0
5
10
15
20
Time (min)

extract was re-esterified with 7 mg of *p*-bromophenacyl bromide and 1 g of KF and purified by HPLC on the silica column. After initial isolation of the *p*-bromophenacyl isobutyrate ester, carrier nonradioactive ester (350 nmol) was added to facilitate re-chromatography by normal phase HPLC to constant specific activity.

Analytical methods

Concentrations of DHC, HMC, and *p*-bromophenacyl isobutyrate were determined by UV absorption; DHC, λ_{max}^{MeOH} nm (log ε): 237 (4.86); HMC, λ_{max}^{MeOH} nm (log ε): 238 (4.77); *p*-bromophenacyl isobutyrate, $\lambda_{max}^{n-hexane}$ nm (log ε): 255 (4.28) (Figure 13) or λ_{max}^{MeOH} nm (log ε): 256 (4.25) (Figure 14). Comparison of the experimental UV spectrum *p*-bromophenacyl isobutyrate (in methanol) to *p*-bromophenacyl propionate (in methanol or chloroform) is shown in Figure 15. Radioactivity was determined by a Packard model 1900 CA Tricarb liquid scintillation analyzer. Samples in 10-250 μl hexane were dissolved in 5 ml of Instagel (Packard) and were analyzed to accumulate at least 4 x 10^4 scintillations. Quench corrections were made by comparison of the samples' transformed spectral indices of the external standard (tSIE) with curves of tSIE versus scintillation efficiency for chemically quenched ^3H and ^{14}C standard samples. Sample scintillation efficiencies were 58% for ^3H and 85% for ^{14}C.

ACKNOWLEDGEMENTS

We wish to thank the National Science Foundation for support of this work (grant number DMB86-16650) and the Phillips Petroleum Company for a predoctoral fellowship to G.D.D.

Figure 13. UV absorbance spectrum of 2.5 x 10^{-5} M *p*-bromophenacyl isobutyrate in hexane to obtain extinction coefficient.

Instrument: Hitachi 100-80 UV Recording Spectrophotometer

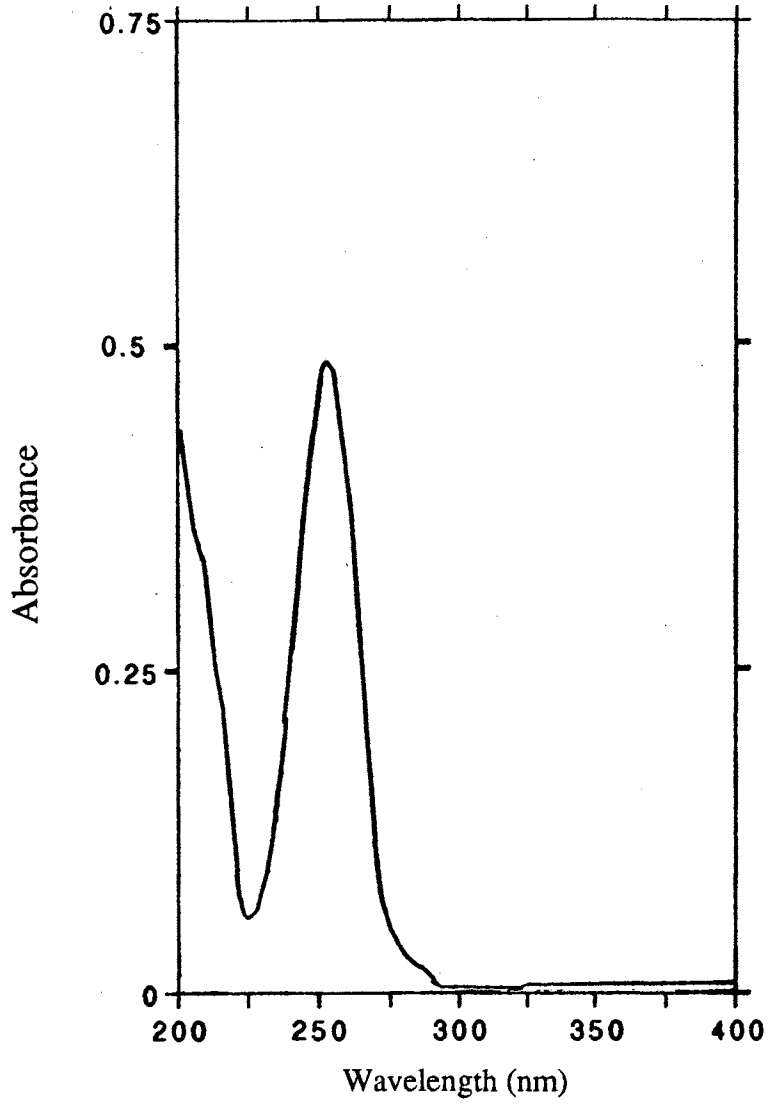
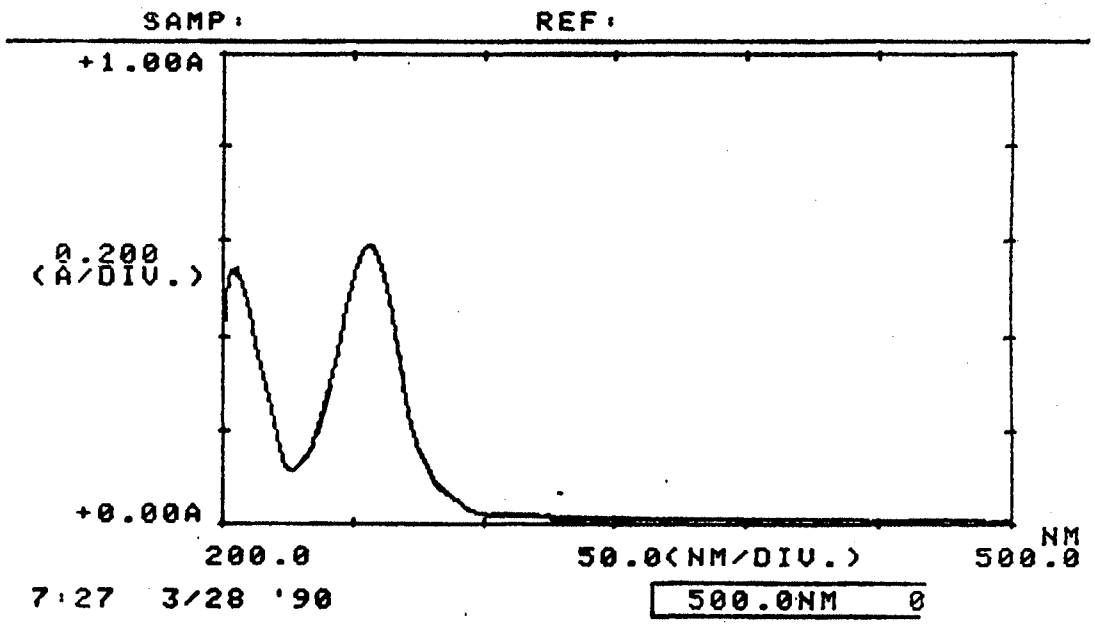

0.75
0.5
0.25
0
Absorbance
200
250
300
350
400
Wavelength (nm)

Figure 14. UV absorbance spectrum of 3.3×10^{-5} M *p*-bromophenacyl isobutyrate in methanol to obtain extinction coefficient.

Instrument: Shimadzu UV-160 UV-Visible Recording Spectrophotometer

x3 dil of 1.43 mg of ~~p-bromo~~phenacyl isobutyrate in MeOH



Stock solution:

$\frac{0.00143\ g\ ester / 285\ g/mole}{0.050\ L\ hexane} = 1.00 \times 10^{-4}$

Solution scanned:

x3 dil $\Rightarrow 3.33 \times 10^{-5}$ mole/L

$A = \frac{\epsilon}{cl} \Rightarrow \epsilon = \frac{A}{cl}$

$\epsilon = \frac{0.589}{(3.33 \times 10^{-5}\ mole/L)\ cm^{-1}}$

$= 17{,}700\ M^{-1}\ cm^{-1}$

$\log = 4.25$

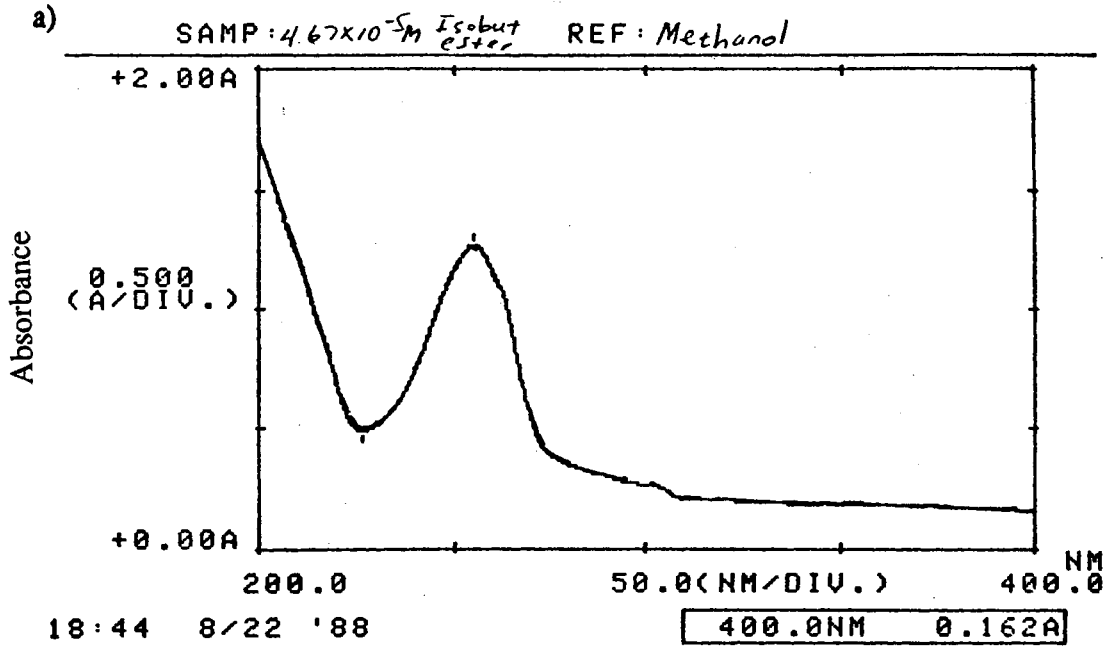
Figure 15. UV absorbance spectra of *p*-bromophenacyl esters:

a) experimental determination of UV absorbance spectrum of *p*-bromophenacyl isobutyrate in methanol.

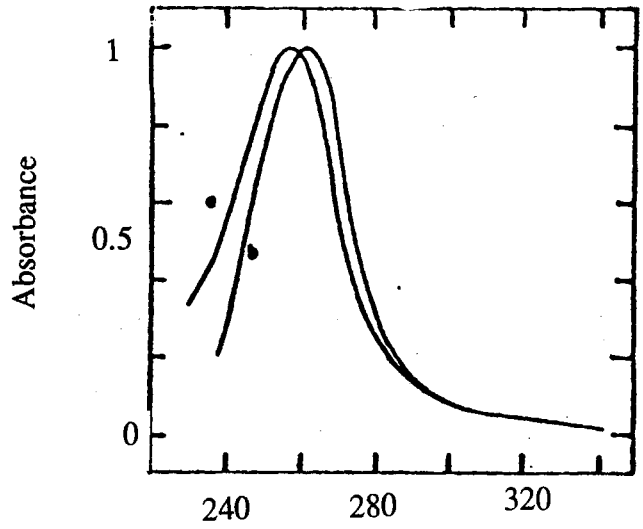
Instrument: Shimadzu UV-160 UV-Visible Recording Spectrophotometer

b) reference spectra of *p*-bromophenacyl propionate in methanol and in chloroform. [From reference 19; enlarged for comparison to spectrum a)]

a)


SAMP : 4.67x10⁻⁵M Isobut ester
REF : Methanol
+2.00A
0.500
(A/DIV.)
+0.00A
Absorbance
200.0
50.0(NM/DIV.)
400.0
NM
18:44 8/22 '88
400.0NM 0.162A

b)


1
0.5
0
Absorbance
240
280
320
Wavelength (nm)

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CHAPTER IV

COMPLETE ^{1}H AND ^{13}C NMR SPECTRAL ASSIGNMENT OF δ-CADINENE, A BICYCLIC SESQUITERPENE HYDROCARBON

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Key words δ-Cadinene ω-cadinene Sesquiterpene hydrocarbon ^{1}H NMR ^{13}CNMR Gossypium hirsutum L. bacterial blight of cotton upland cotton *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye

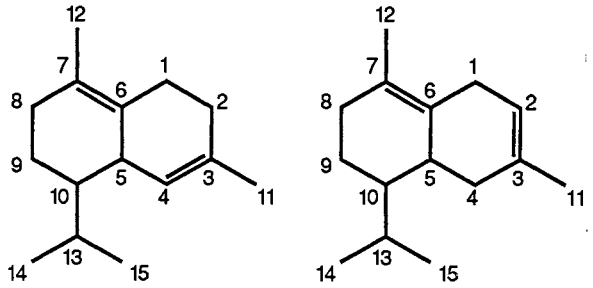
ABSTRACT

A bicyclic sesquiterpene hydrocarbon, δ-cadinene, was isolated from cade oil to assist identification of low abundance δ-cadinene from cotton. Detailed 2D NMR spectroscopy confirmed the previously reported structure of δ-cadinene and permitted the complete assignment of ^{1}H and ^{13}C spectra.

INTRODUCTION

δ-Cadinene (**1**)[1] (Figure 1) is a sesquiterpene hydrocarbon found in many biological sources[2-14] and essential oils such as ylang-ylang oil[15] and cade oil.[5,16] After isolating δ-cadinene from cade oil for use as a standard, NMR characterization of the δ-cadinene was necessary because ^{1}H NMR data for δ-cadinene published by Buttery and colleagues[17], Ohta and Hirose[18] and Formacek and Kubeczka[19] were incomplete. Additionally, no published ^{1}H NMR spectrum for this ubiquitous compound could be found during an extensive search of the literature. We found inconsistencies in some of the reported ^{13}C NMR chemical shift designations for δ-cadinene.[15,16,20,21]. The assignment of ^{13}C chemical shifts of δ-cadinene by Oyarzún and Garbarino[20] included six carbon shift assignments which disagreed greatly with the other three reports The ^{13}C chemical shift assignments for δ-cadinene by Formacek and Kubeczka[21] included an incorrect multiplicity assignment.

Another reason for performing the NMR identification was prior confusion concerning a proposal by Vlahov and coworkers[22] that δ-cadinene was represented by structure **2** (Figure 1). Our rigorous NMR analysis by numerous modern routine pulse sequences established unambiguous ^{1}H and ^{13}C NMR chemical shift designations. The shift designations confirmed chemical analysis of cadinene derivatives by Connell and colleagues.[23] Furthermore, our complete NMR profile of δ-cadinene is consistent with partial ^{1}H NMR evidence produced by Nagasampagi and coworkers[24] that supported structure **1**, not structure **2** (now known as ω-cadinene[23,25]) as the correct representation of δ-cadinene.



1 **2**

Figure 1. Structural formulae of δ-cadinene (**1**) and ω-cadinene (**2**).

Our need for a fully characterized δ-cadinene standard occurred when we found that δ-cadinene appeared to be biosynthesized during attempted infection of bacterial blight-resistant varieties of upland cotton (glanded or glandless) by the bacterium *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye.[26] δ-Cadinene is a possible biological precursor for numerous cadinene and cadalene compounds including the antibacterial compound 2,7-dihydroxycadalene that is generated in bacterially infected cotton tissues.[27] δ-Cadinene and other sesquiterpenes found in cotton are secondary metabolites which may function as constitutive defenses against herbivory, inducible defenses against microbial infection, or attractants to pollinating insects.[28] δ-Cadinene has also been found in the essential oil from buds of glanded upland cotton plants.[29] Our report expands the 2D NMR record of sesquiterpenoid compounds[30] which have been isolated from cotton.[31]

Our 2D NMR analysis of δ-cadinene may serve as a model for investigators in the expanding field of sesquiterpene biosynthesis.[32] Successful isolation of a single biosynthetic enzyme from the complex metabolic apparatus commonly found in an organism often requires quantitative monitoring of a precisely identified specific sesquiterpene.[33] Such identification of sesquiterpenoid substrates or products is assisted by 2D NMR analysis, which has revealed incorrect chemical shift assignments for some sesquiterpenes[30] and triterpenes[34] previously based on 1D NMR analysis.

RESULTS AND DISCUSSION

The experimental mass spectrum (Figure 2) and infrared spectrum (Figure 3) of the δ-cadinene isolated from cade oil matched the literature mass spectrum[35-38] and infrared spectrum[39,40] of δ-cadinene. The ^{13}C NMR spectrum (attached to the ^{1}H-^{13}C HETCOR plot in Figure 4) of the cade oil δ-cadinene displayed the expected 15 carbon shift signals. Integration of the ^{1}H NMR spectrum (Figure 5) was in agreement with the partial ^{1}H NMR data[17-19] and consistent with a total of 24 protons, thus the one-dimensional ^{1}H and ^{13}C NMR data (for ^{13}C NMR spectrum, see Figure 6) were consistent with the molecular formula of δ-cadinene ($C_{15}H_{24}$). Further ^{13}C DEPT (Figures 7, 8. and 9), ^{13}C off-resonance (Figures 10, 11, and 12), decoupled ^{1}H (example expansions in Figure 13), ^{1}H-^{13}C HETCOR (Figure 4), ^{1}H-^{1}H COSY (Figure 14), and ^{1}H-^{13}C long-range HETCOR experiments (Figures 15, 16, and 17) were conducted. The ^{1}H and ^{13}C NMR spectral data, correlations from long-range ^{1}H-^{13}C HETCOR spectra, and long-range connectivities from the ^{1}H-^{1}H COSY spectrum are summarized in Tables 1-3, respectively.

Analysis of the NMR spectra showed that the ^{13}C NMR spectrum (Figure 6) of the cade oil δ-cadinene was in good agreement with all literature sources,[15,16,20,21] except for discrepancies with two reports.[20,21] One report[20] assigned 1D ^{13}C NMR shifts of 130.03 ppm, 124.29 ppm, 32.09 ppm, 26.90 ppm, 23.86 ppm, and 15.79 ppm to C-7, C-6, C-1, C-2, C-15, and C-11, respectively; our detailed 2D NMR analysis assigns corresponding ppm shifts to C-6, C-7, C-2, C-1, C-11, and C-15, respectively (See Table 1). Multiplicities of $C(H)_n$ signals determined by ^{13}C DEPT subspectra and off-resonance ^{13}C NMR spectra agreed with the literature,[15,16,20,21] with the exception of an apparent typographical error in one report[21] which assigned singlet multiplicity to four carbon shifts, including a 45.75 ppm shift [corresponding to our 45.23 ppm shift (CH, C-10)]; this multiplicity assignment should be a doublet in order to be reconciled with the structure of δ-cadinene which must have only 3 carbons with singlet multiplicity. Our ^{13}C

Figure 2. GC/EIMS spectrum of:

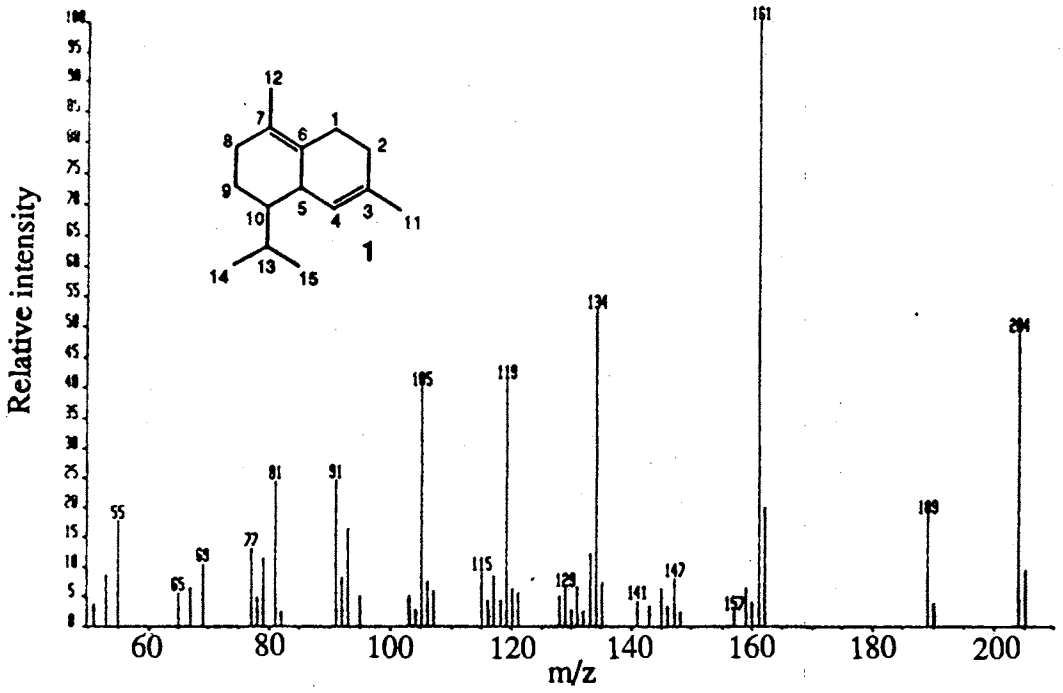
a) δ-cadinene (**1**) isolated from cade oil

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 100% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-1)
and
VG TS-250 mass spectrometer.

and

b) reference GC/EIMS spectrum of δ-cadinene from the National Bureau of Standards compilation stored in data system of VG TS-250 mass spectrometer (also available in book form; see Reference 51)

a)


Relative intensity
m/z
60
80
100
120
140
160
180
200
161
134
204
105
119
81
91
55
189
77
63
65
115
129
141
147
157
1

b)

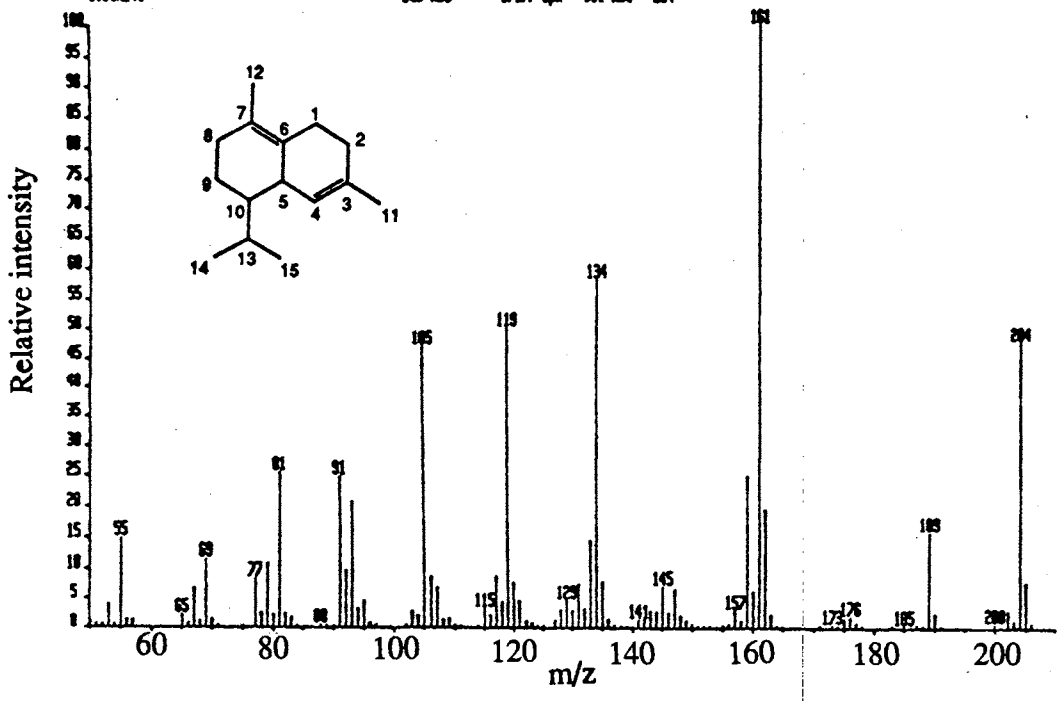

Relative intensity
m/z
60
80
100
120
140
160
180
200
161
134
204
105
119
81
91
55
189
77
63
65
115
129
141
145
157
173
176
185
1

Figure 3. a) Full scale FT/IR (Fourier Transform/Infrared) spectrum of δ-cadinene (**1**) isolated from cade oil.

Instrument: Bio-Rad Digilabs FTS-40A FT/IR (Fourier Transform/Infrared) Spectrometer.;
192 scans accumulated on a sample of 50-100 ug δ-cadinene (**1**) isolated from cade oil.

b) Full scale Reference Infrared spectrum of δ-cadinene isolated from copaiba balsam oil (reproduced from Reference 55).

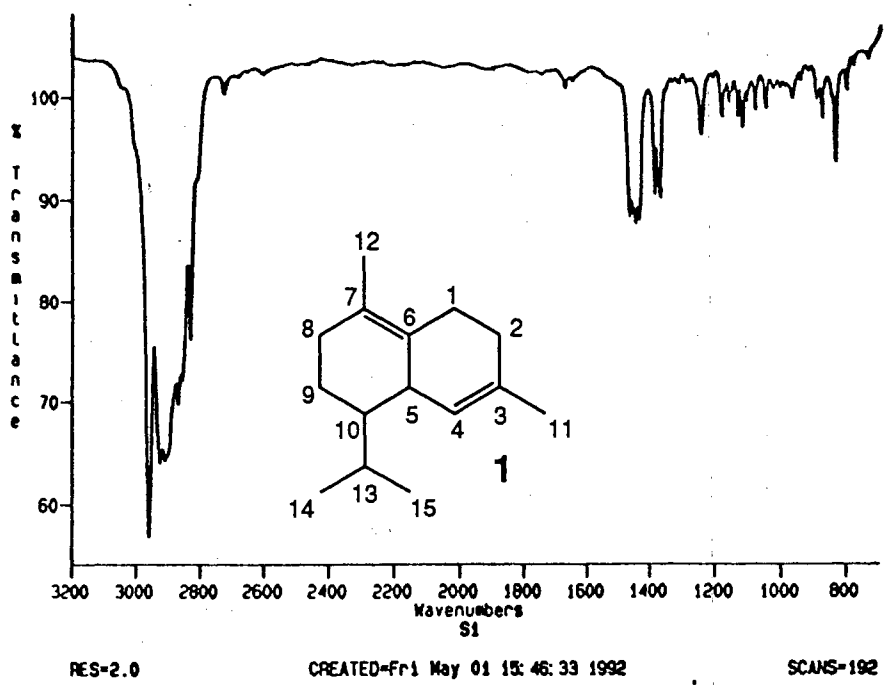
c) Expansion from FT/IR (Fourier Transform/Infrared) spectrum of the δ-cadinene (**1**) isolated from cade oil.

Instrument: Bio-Rad Digilabs FTS-40A FT/IR (Fourier Transform/Infrared) Spectrometer.;
192 scans accumulated on a sample of 50-100 ug δ-cadinene (**1**) isolated from cade oil.

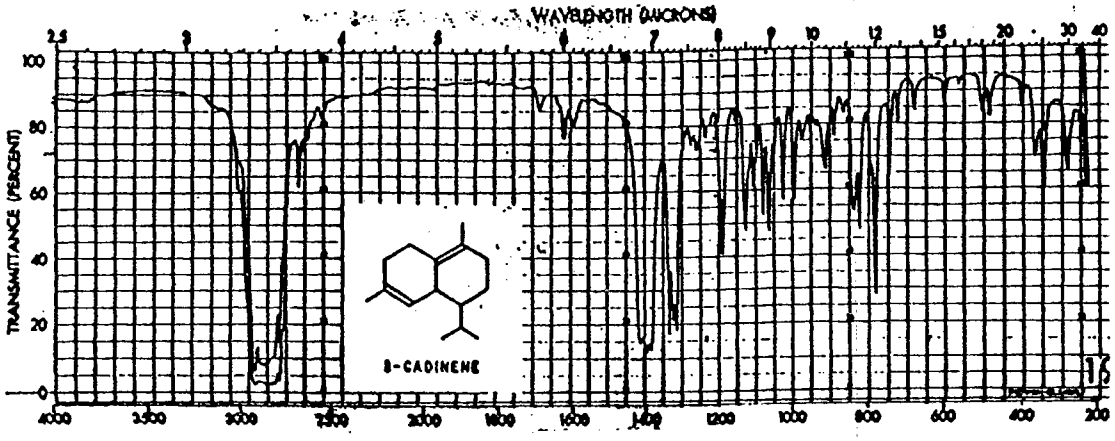
and

d) Full scale Reference Infrared spectrum of δ-cadinene isolated from copaiba balsam oil (reproduced from Reference 55).

a)

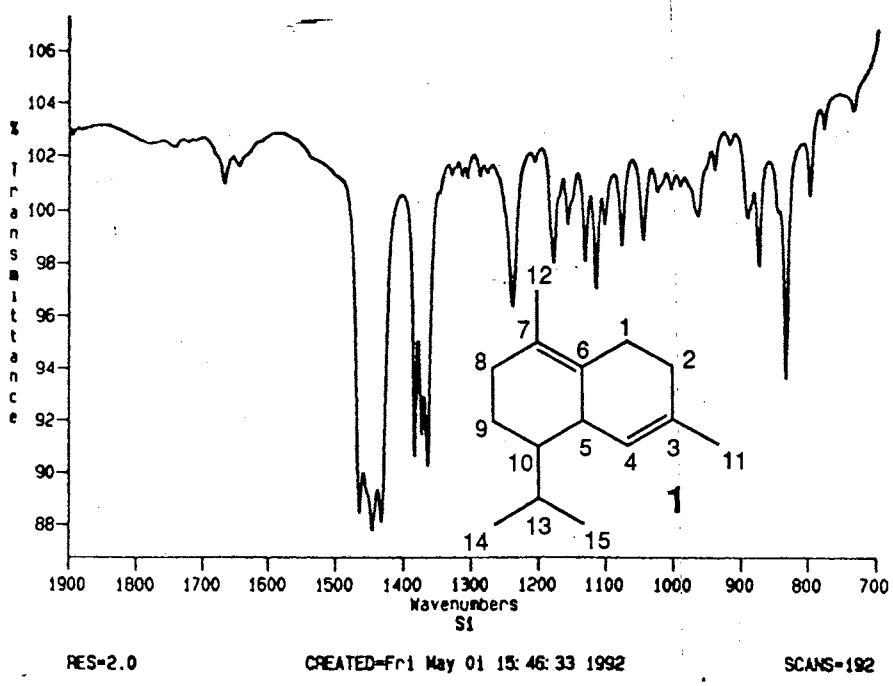


b)



Freqency (cm^{-1})

c)


% Transmittance
106
104
102
100
98
96
94
92
90
88
1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700
Wavenumbers
S1
RES=2.0
CREATED=Fri May 01 15:46:33 1992
SCANS=192
12
7
1
6
2
8
9
5
4
3
11
10
13
14
15
1

d)

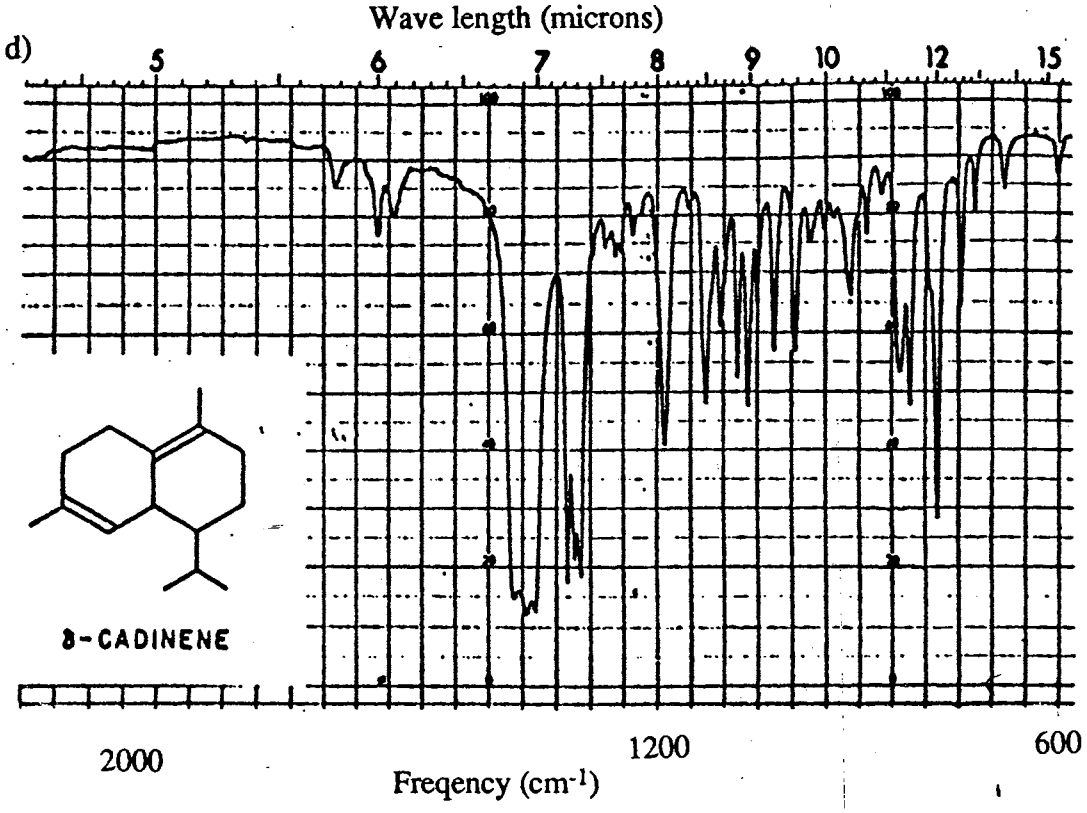

Wave length (microns)
5
6
7
8
9
10
12
15
β-CADINENE
2000
1200
600
Freqency (cm-1)

Figure 4. Contour plot of a ^{1}H-^{13}C heteronuclear shift correlation (HETCOR) of δ-cadinene (**1**) at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer. 400 MHz ^{1}H and 100 MHz ^{13}C

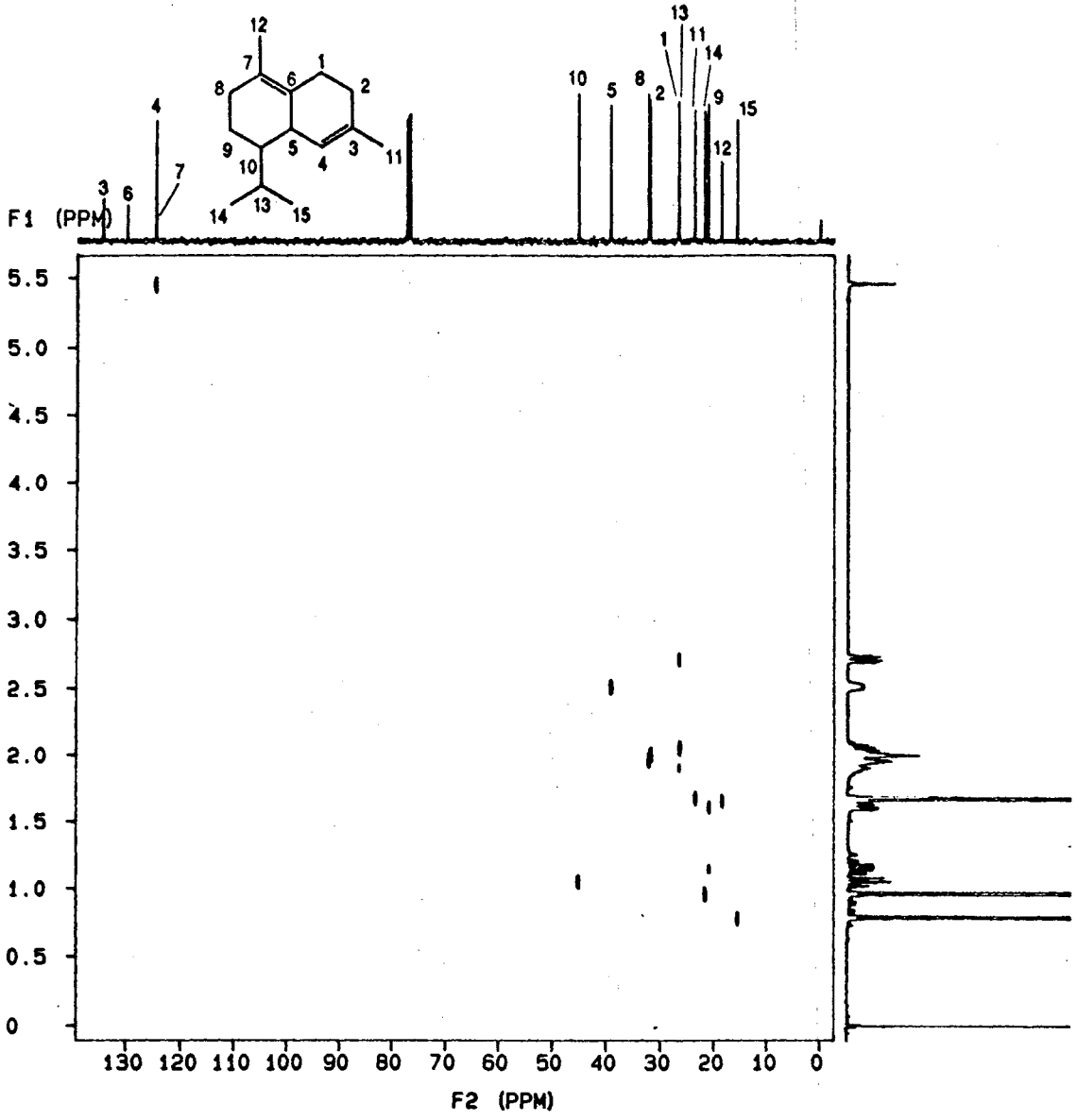

F1 (PPM)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
130 120 110 100 90 80 70 60 50 40 30 20 10 0
F2 (PPM)

Figure 5. Integrated ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

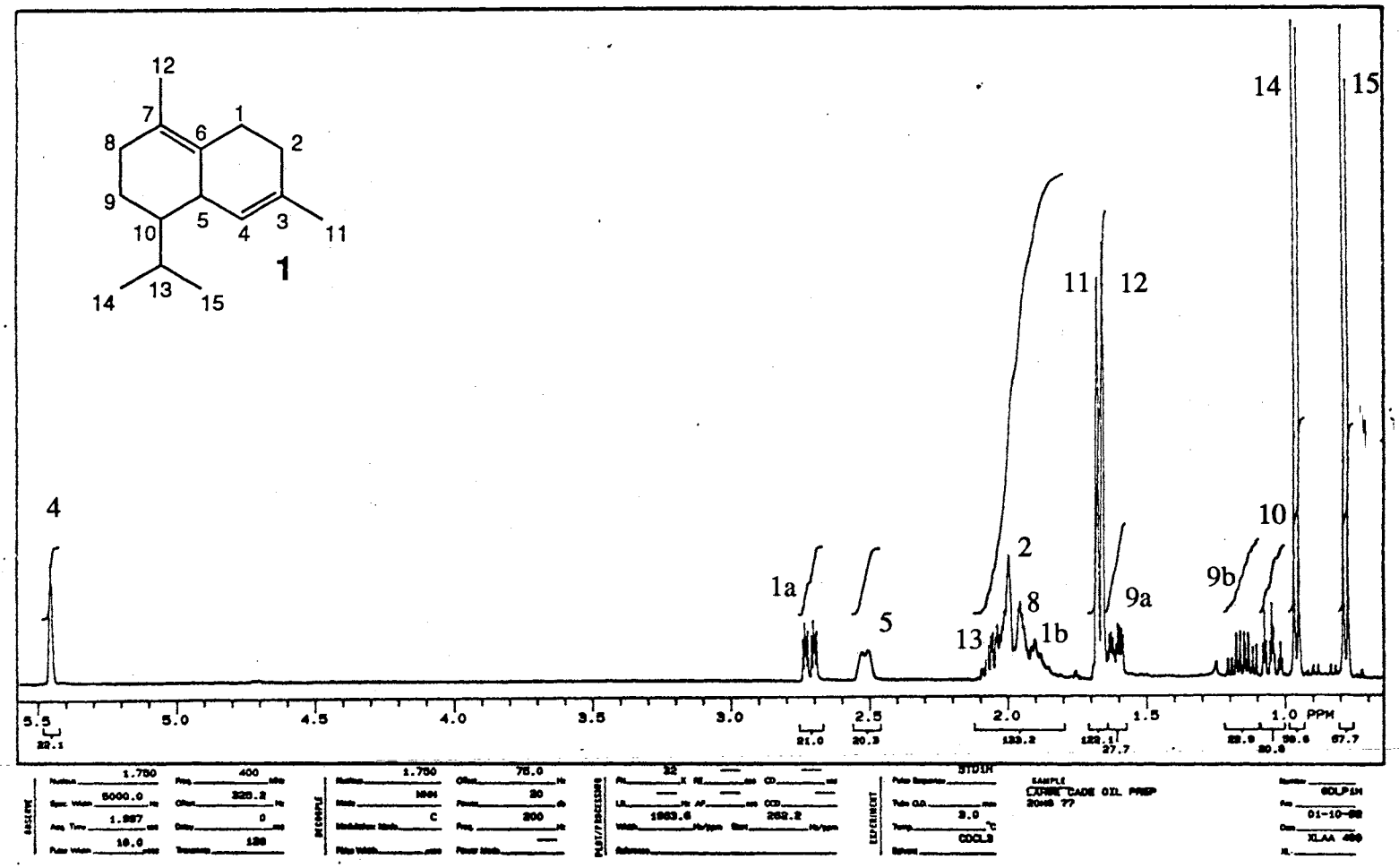

12
7
1
8
6
2
9
5
4
3
11
10
1
14
13
15
4
1a
5
13
2
8
1b
11
12
9a
9b
10
14
15
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0 PPM

Figure 6. ^{13}C NMR spectrum of δ-cadinene (**1**) at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.
100 MHz ^{13}C

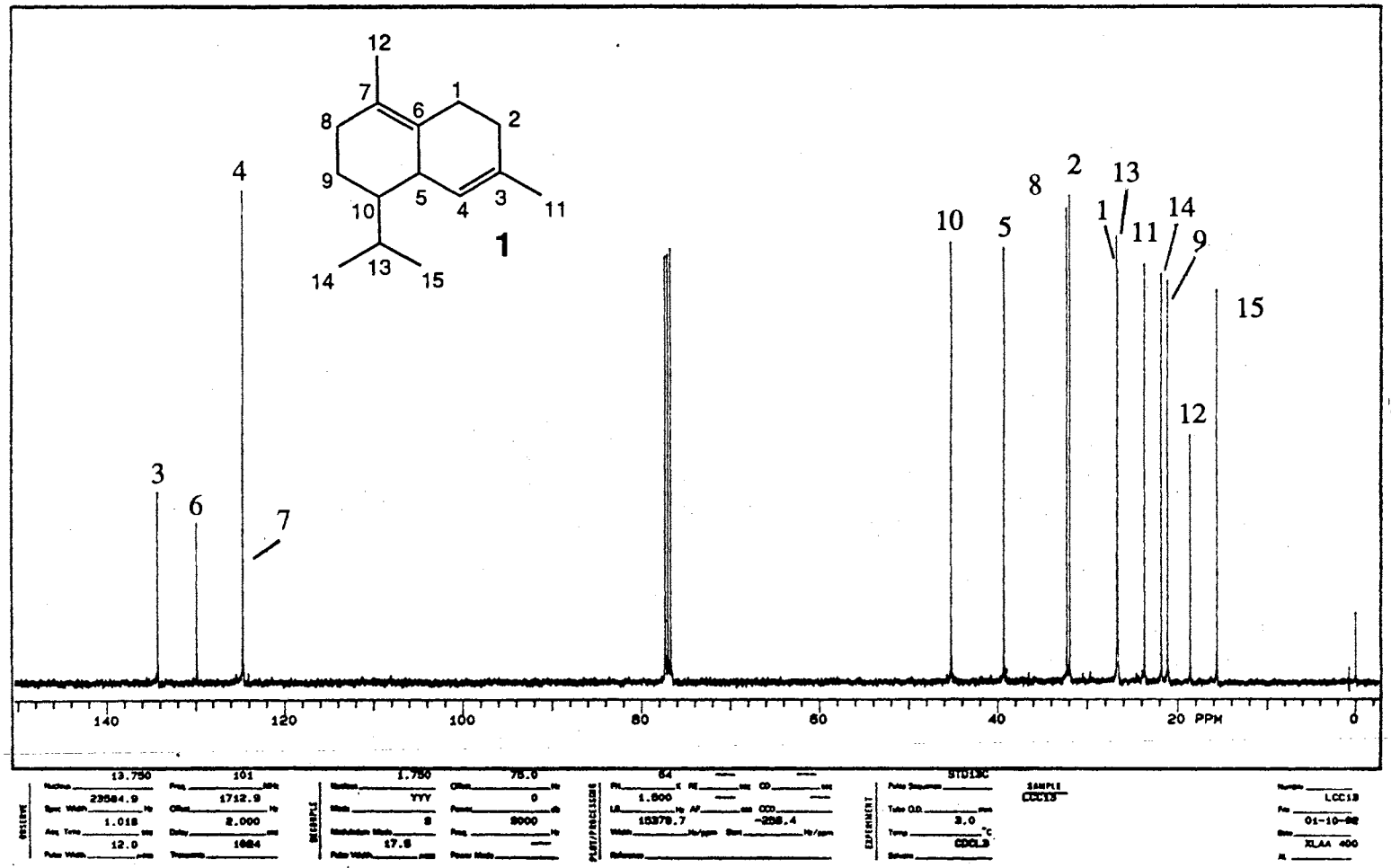

12
7
1
6
8
2
9
5
4
3
11
10
13
14
15
1
4
3
6
7
10
5
8
2
13
1
11
14
9
15
12
140
120
100
80
60
40
20 PPM
0

Figure 7. Full ppm scale ^{13}C DEPT (Distortionless Enhancement by Polarization Transfer) NMR spectrum of cade oil δ-cadinene (**1**) at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

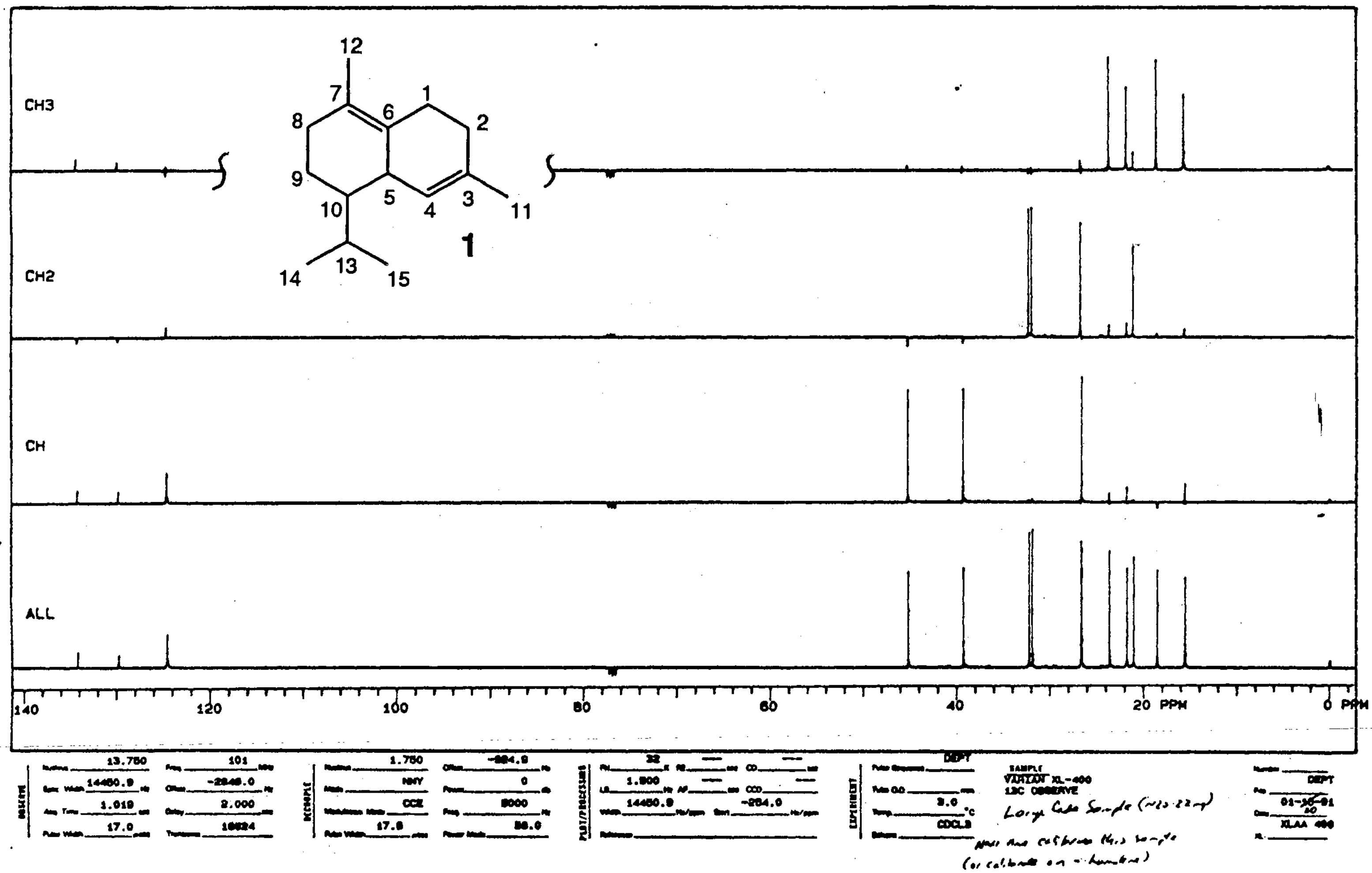

CH3
CH2
CH
ALL
140
120
100
80
60
40
20 PPM
0 PPM
DEPT
VARIAN XL-400
13C OBSERVE
CDCL3
XLAA 400

Figure 8. Expansion from full ppm scale ^{13}C DEPT NMR spectrum (Figure 6) of cade oil δ-cadinene (**1**) at 7°C for detail on lower ppm signals.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

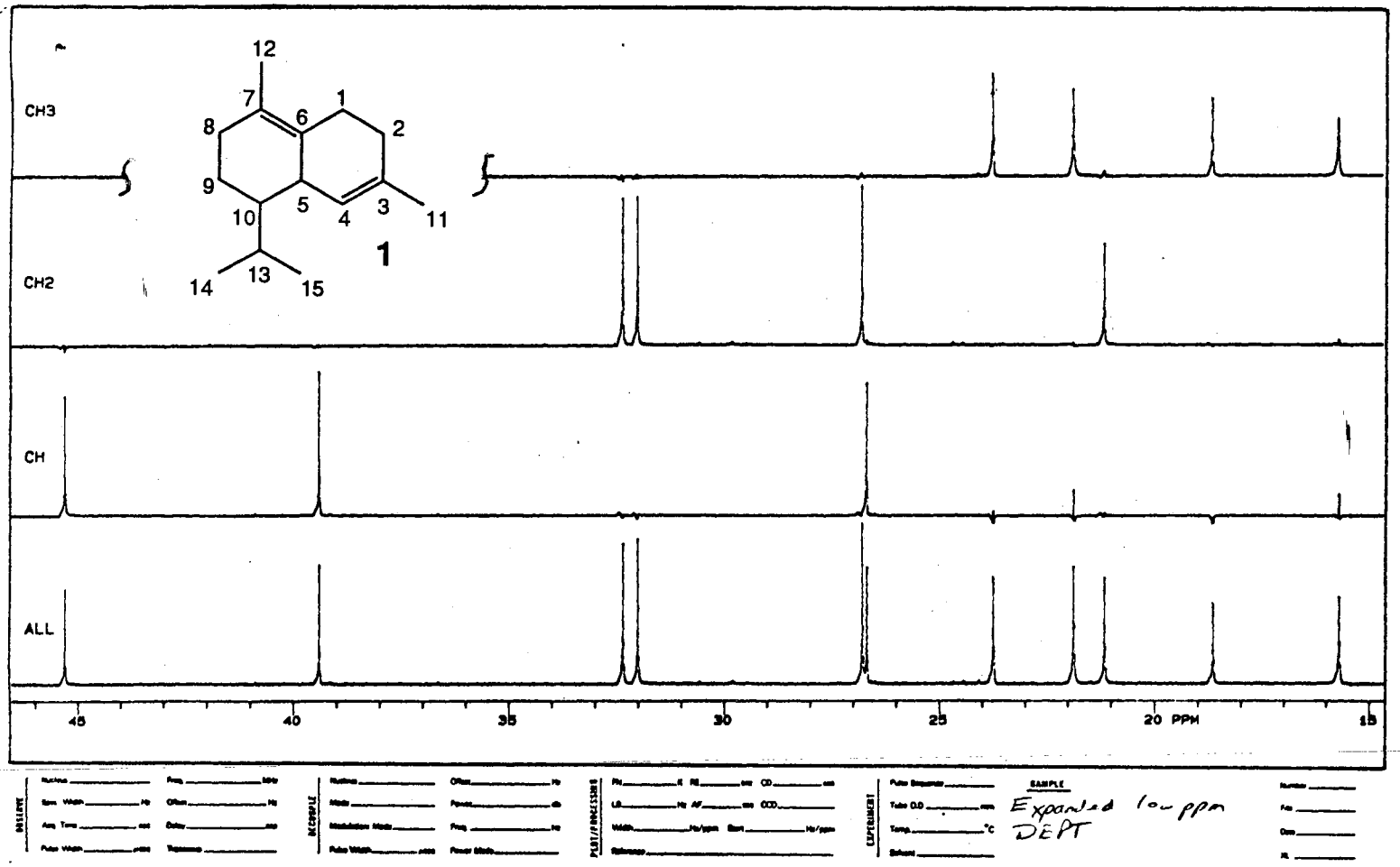
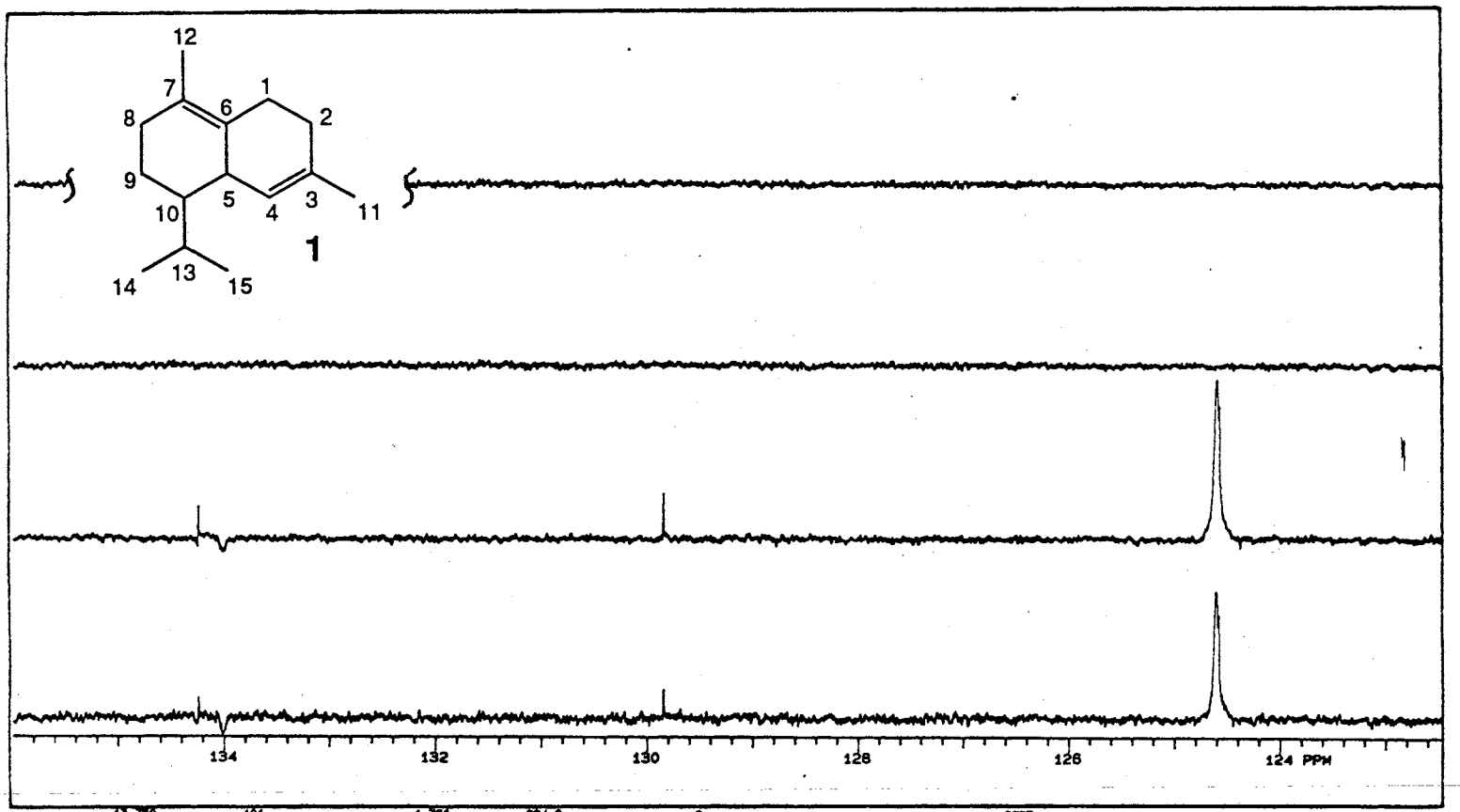

12
7
1
8
6
2
9
5
4
3
11
10
14
13
15
1
CH3
CH2
CH
ALL
45
40
35
30
25
20 PPM
15
SAMPLE
Expanded low ppm
DEPT

Figure 9. Expansion from full ppm scale ^{13}C DEPT NMR spectrum (Figure 6) of cade oil δ-cadinene (**1**) at 7°C for detail on higher ppm signals.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.


12
7
6
1
8
2
9
5
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3
11
10
1
14
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15
134
132
130
128
126
124 PPM
13.750
101
1375.0
3184.2
1.024
2.000
17.0
4760
1.750
-824.8
NNY
0
CCE
9000
17.0
66.0
8
0.500
1368.4
12318.1
DEPT
3.0
CDCL3
SAMPLE
VARIAN XL-400
13C OBSERVE
Large Code 8.1
Narrow DEPT
Overnight 1/30-31/92
~16hr.
Standard
DEPT NDPT-01
6/21/92
XLAA 400

Figure 10. Off-resonance ^{13}C NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. (Decoupler offset = "Array")

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

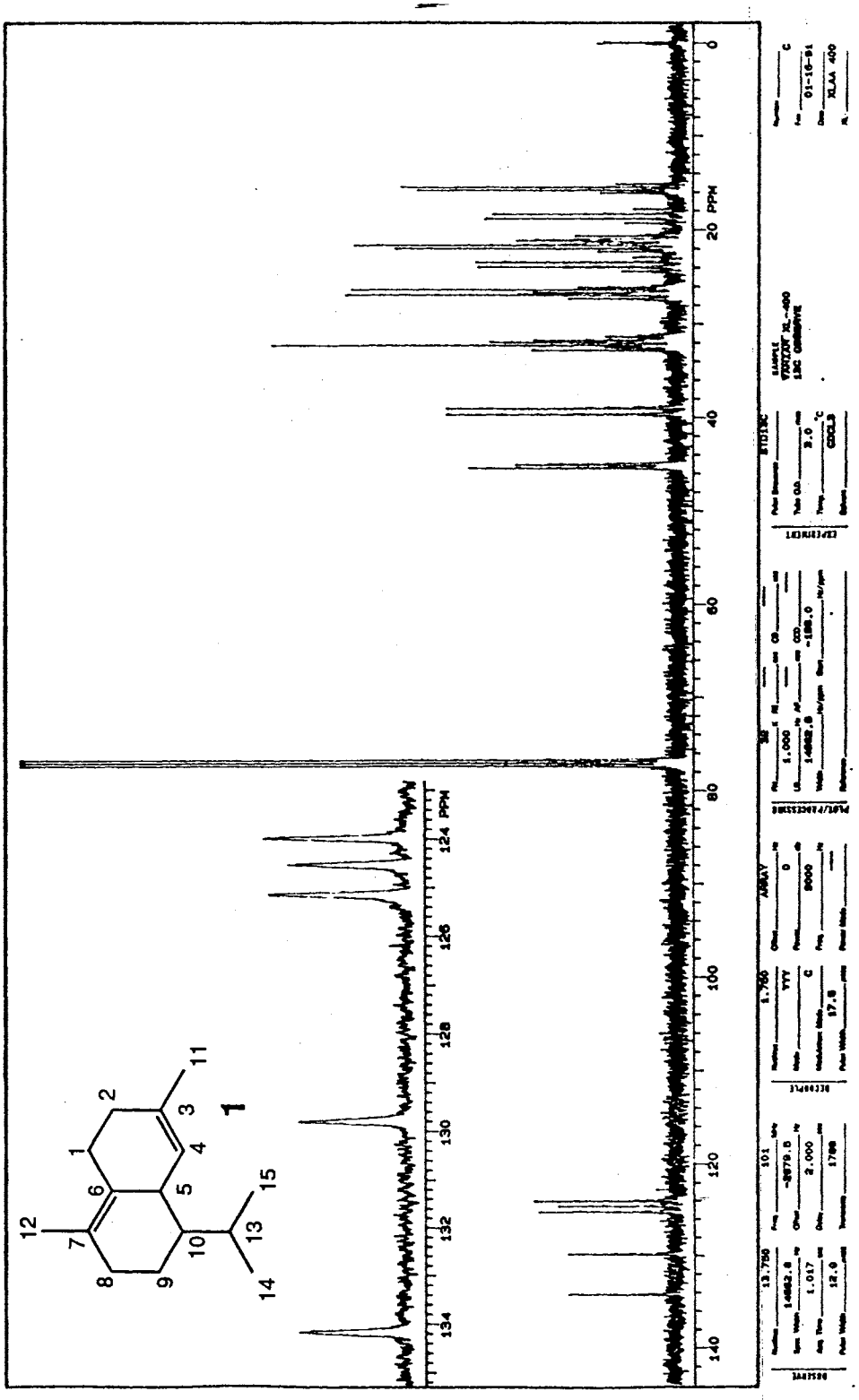
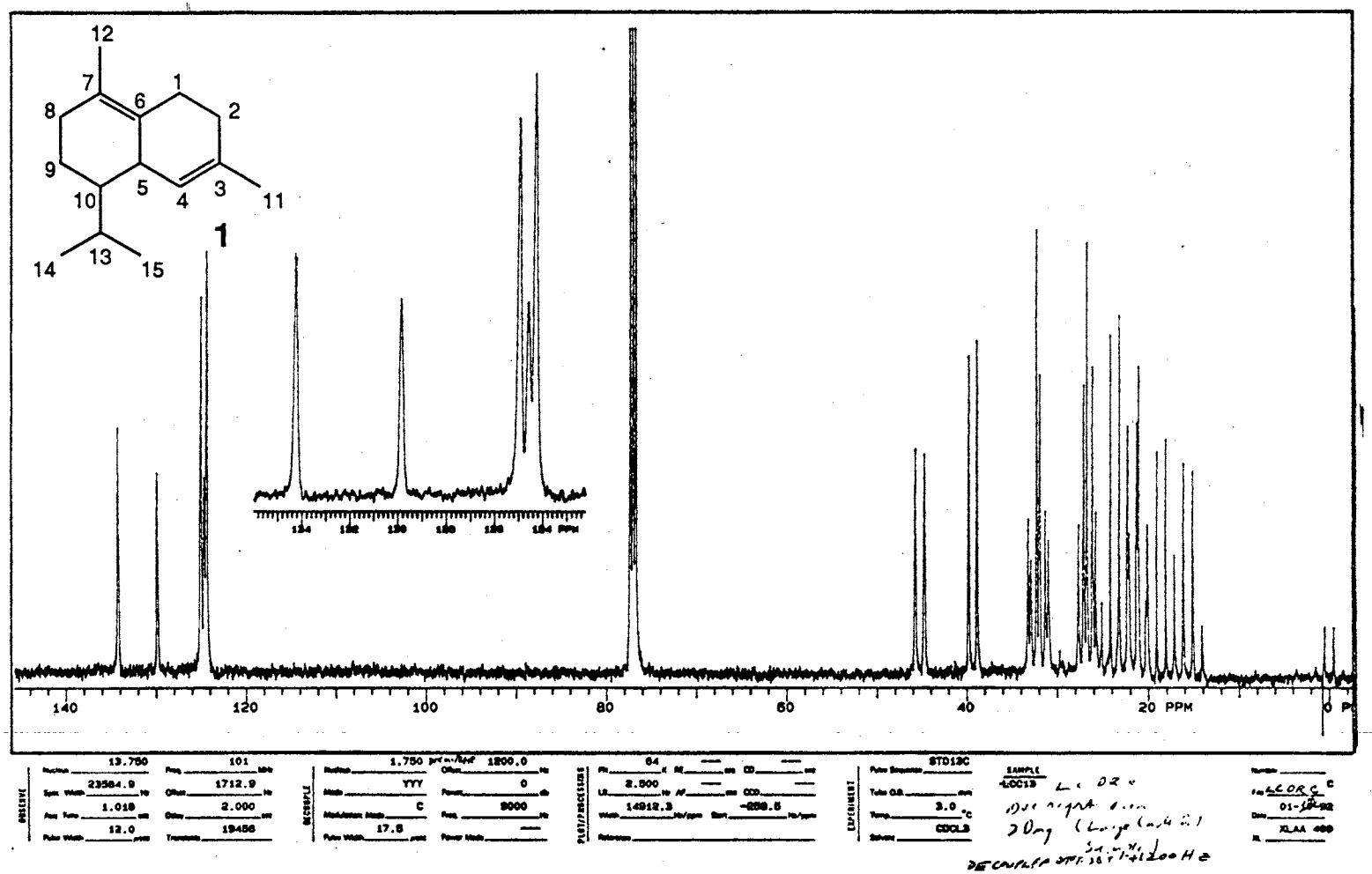

1
PPM
0
20
40
60
80
100
120
140
124
126
128
130
132
134

Figure 11. Off-resonance ^{13}C NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. (Decoupler = 1200 Hz)

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.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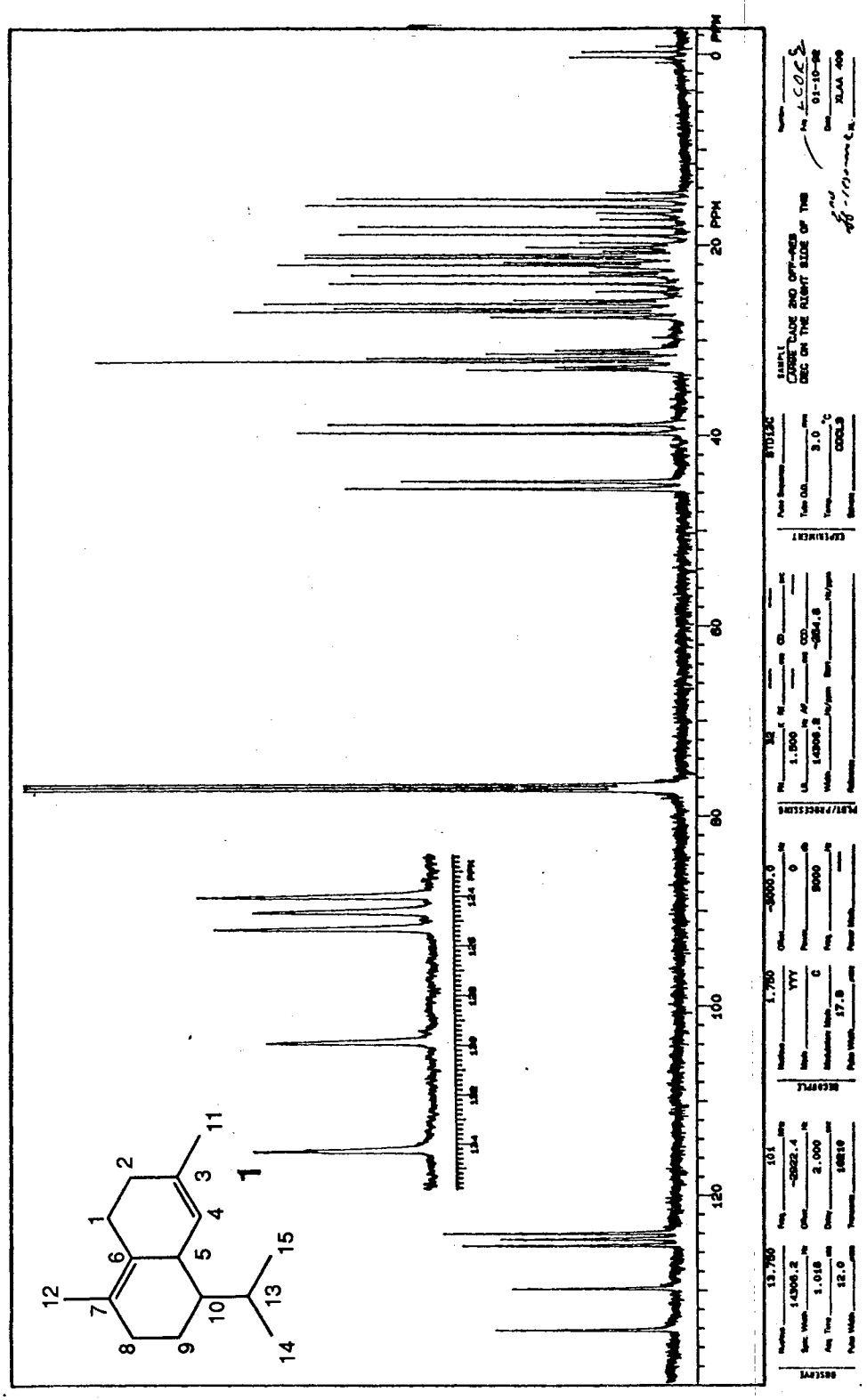
2
9
5
4
3
11
10
14
13
15
1
140
120
100
80
60
40
20 PPM

Figure 12. Off-resonance ^{13}C NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. (Decoupler offset = -3000 Hz)

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.


1
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11
0 PPM
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60
80
100
120

Figure 13. Expansions from:

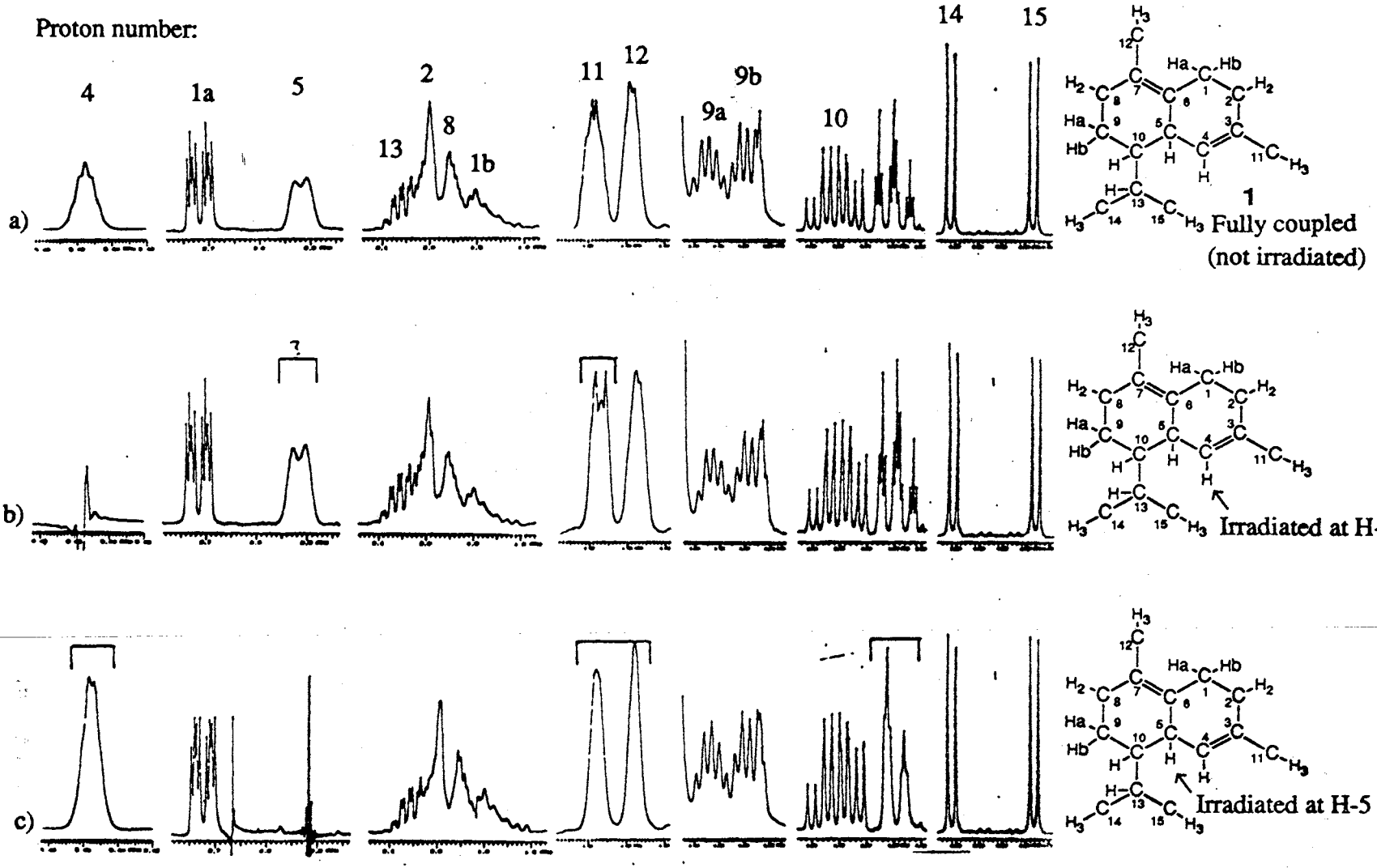
a) Fully coupled ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C.

b) Decoupled ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. Sample was irradiated at H-4 (olefinic proton) signal.

c) Uncoupled ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. Sample was irradiated at H-5 (bridgehead proton) signal.

Decoupled spectra in b) and c) provided as additional evidence of possible long-range connectivities found in COSY plot (Figure 13) and compiled in Table 3.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.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4
1a
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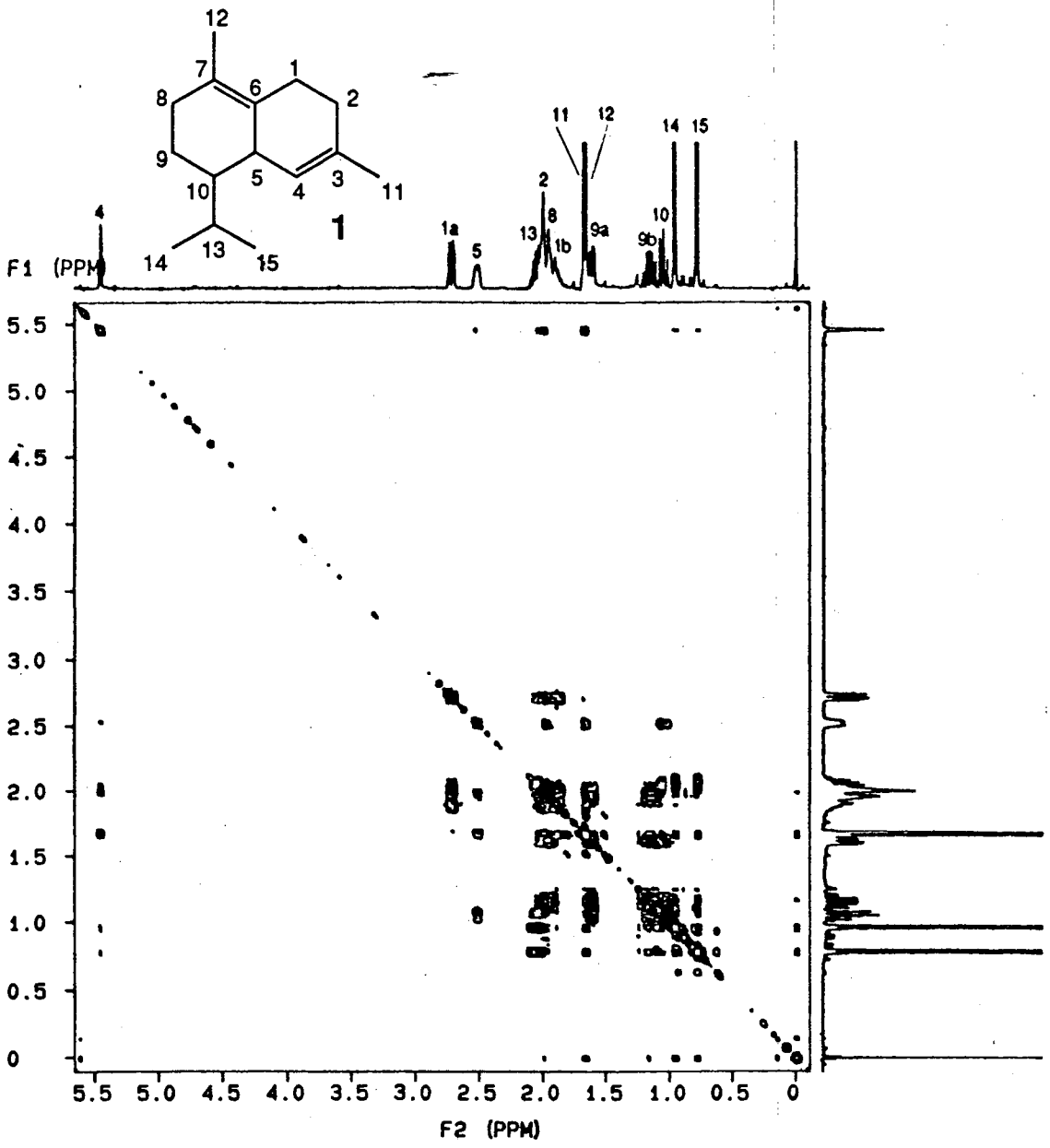
2
13
8
1b
11
12
9a
9b
10
14
15
a)
b)
c)
1
Fully coupled
(not irradiated)
Irradiated at H-4
Irradiated at H-5

Figure 14. Contour plot of a homonuclear ^{1}H-^{1}H chemical shift correlation (COSY) of δ-cadinene (**1**) at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.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F1 (PPM)
4
1a
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13
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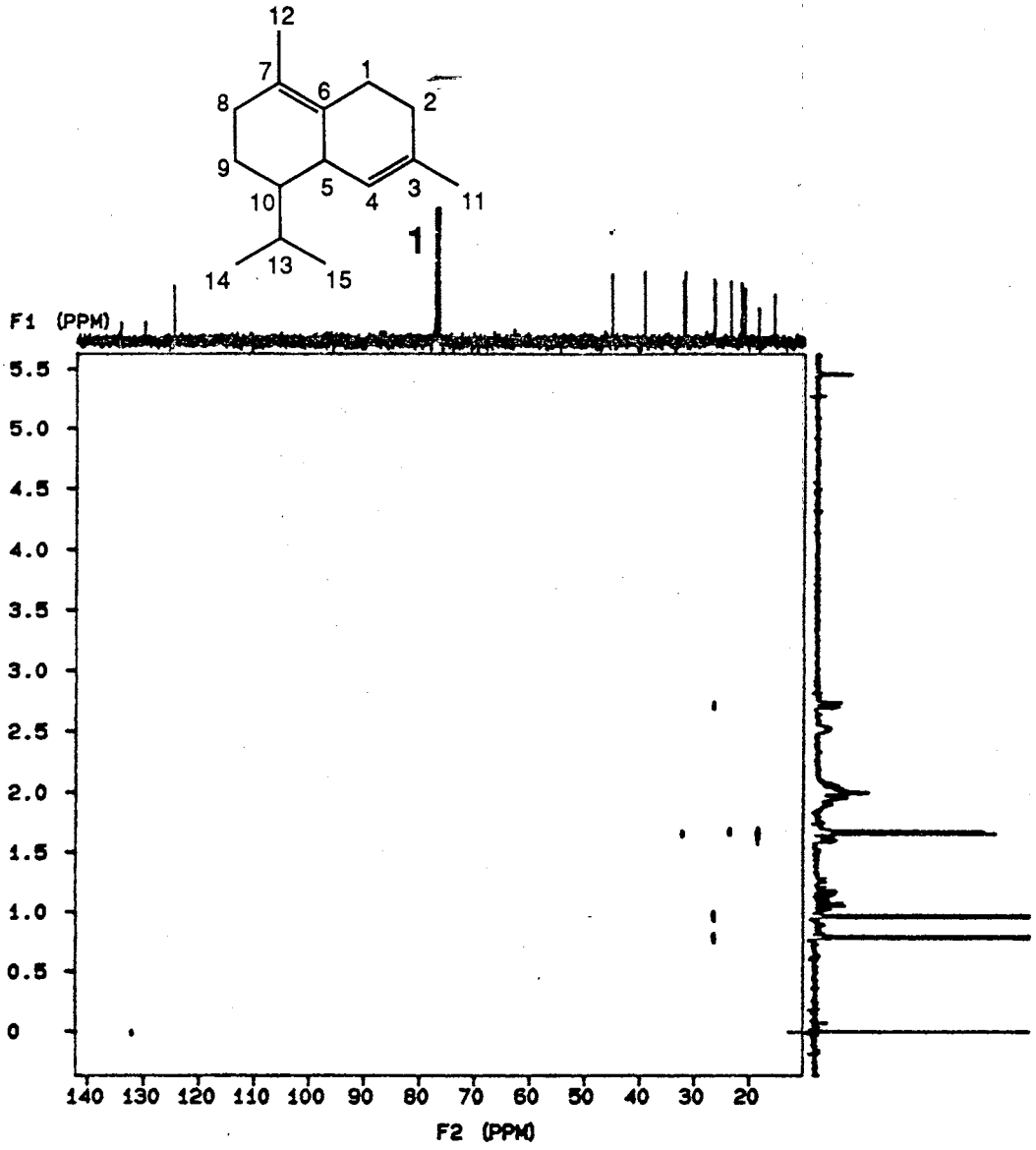
8
1b
11
12
9a
9b
10
14
15
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
F2 (PPM)

Figure 15. Contour plot of a long-range ^{1}H-^{13}C heteronuclear shift correlation (HETCOR) of δ-cadinene (**1**) at 7°C. (JNXH = 5 Hz)

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.
400 MHz ^{1}H and 100 MHz ^{13}C.
JNXH value= 5 Hz.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F1 (PPM)
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4.0
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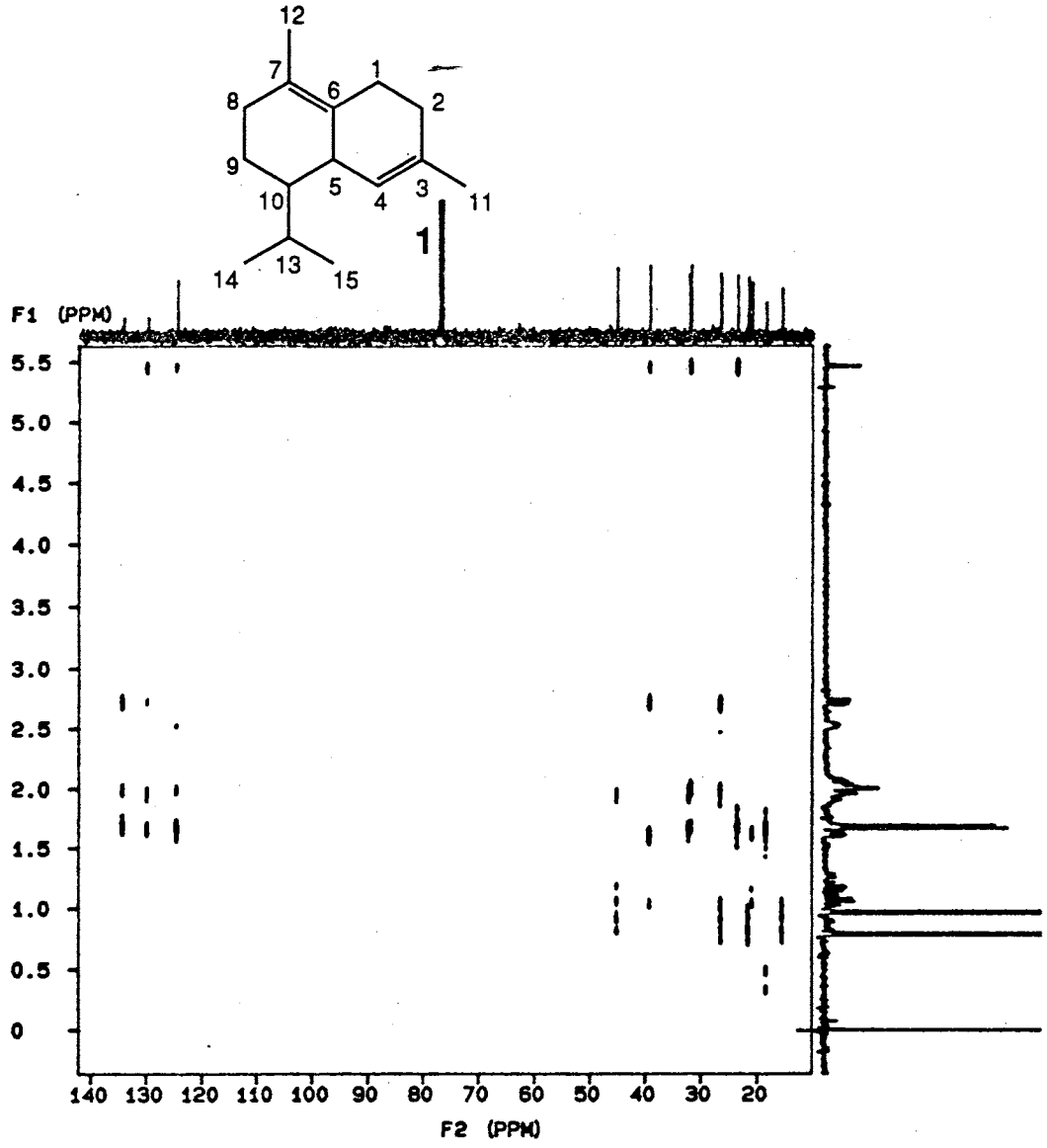
3.0
2.5
2.0
1.5
1.0
0.5
0
140 130 120 110 100 90 80 70 60 50 40 30 20
F2 (PPM)

Figure 16. Contour plot of a long-range ^{1}H-^{13}C heteronuclear shift correlation (HETCOR) of δ-cadinene (**1**) at 7°C. (JNXH value =10 Hz)

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.
400 MHz ^{1}H and 100 MHz ^{13}C.
JNXH value = 10 Hz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F1 (PPM)
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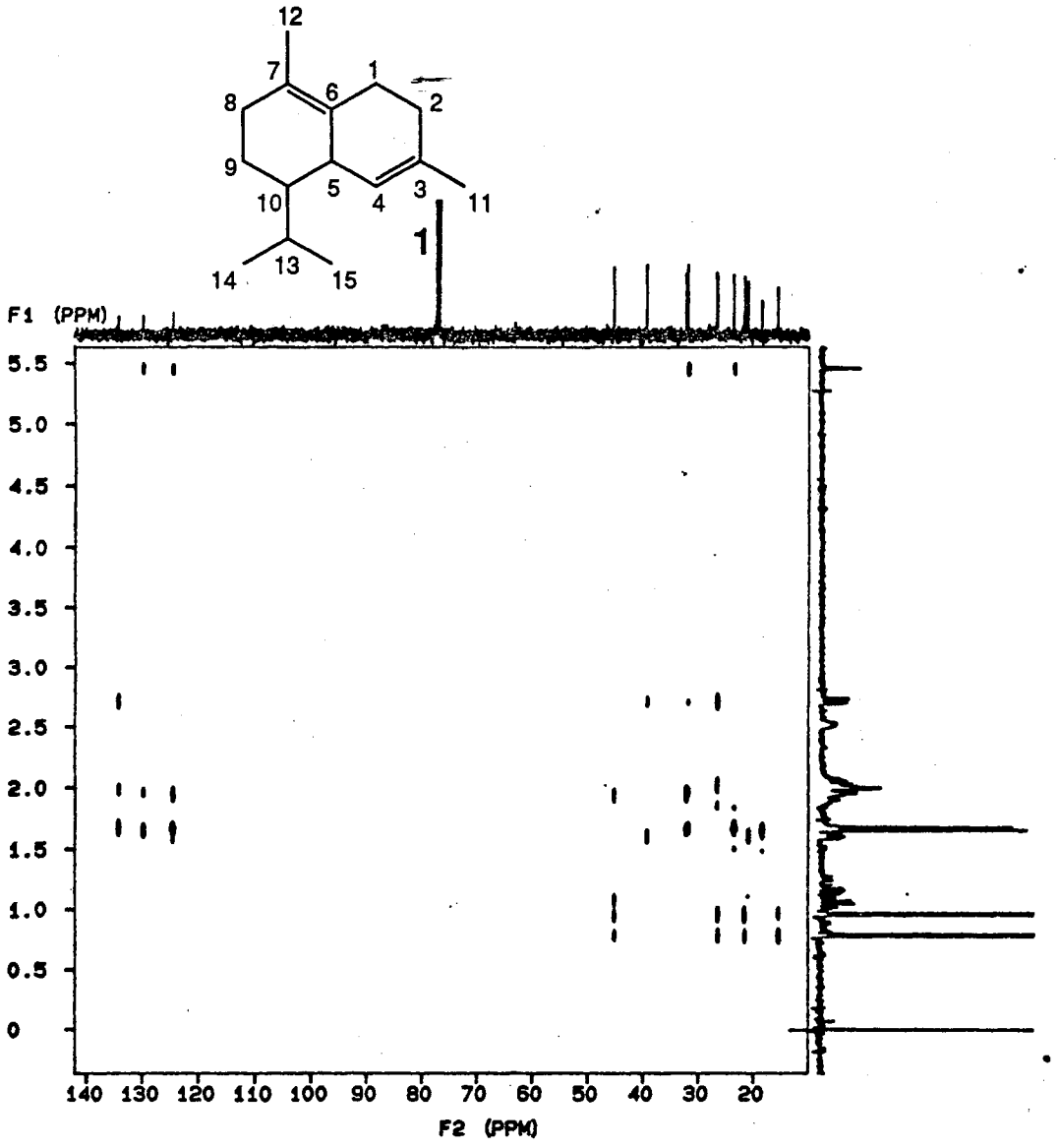
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
140 130 120 110 100 90 80 70 60 50 40 30 20
F2 (PPM)

Figure 17. Contour plot of a long-range ^{1}H-^{13}C heteronuclear shift correlation (HETCOR) of δ-cadinene (**1**) at 7°C. (JNXH value = 15 Hz)

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.
400 MHz ^{1}H and 100 MHz ^{13}C.
JNXH value = 15 Hz.


12
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15
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F1 (PPM)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
140 130 120 110 100 90 80 70 60 50 40 30 20
F2 (PPM)

TABLE 1

^{1}H AND ^{13}C NMR CHEMICAL SHIFTS OF δ-CADINENE (**1**)

^{13}C			^{1}H
δ[a]	Group[b]	Carbon	δ[a]
134.23	C	3	—
129.84	C	6	—
124.61	CH	4	5.45
124.58	C	7	—
45.23	CH	10	1.05
39.32	CH	5	2.52
32.27	CH_2	8	1.95
31.93	CH_2	2	2.00
26.70	CH_2	1	2.72
			and 1.90
26.60	CH	13	2.06
23.65	CH_3	11	1.68
21.77	CH_3	14	0.96
21.06	CH_2	9	1.61
			and 1.16
18.56	CH_3	12	1.66
15.61	CH_3	15	0.78

J [13(14)-CH3, H-12] = 6.9 Hz

[a] In ppm with respect to TMS.
[b] Information obtained from off-resonance ^{13}C NMR and DEPT subspectra.

TABLE 2

LONG-RANGE ^{1}H-^{13}C CORRELATIONS (LONG-RANGE HETCOR) OF δ-CADINENE (**1**) (400 MHz, DEUTERATED CHLOROFORM).

C	Observed long-range correlations
1	H-2
2	H-1a[a], H-4, H-11
3	H-1a, H-2, H-11
4	H-2, H-11[a]
5	H-1a, H-4, H-9a, H-10
6	H-1a, H-2, H-4, H-8, H-12
7	H-5, H-8[a], H-9a, H-12[a]
8	H-12
9	H-10
10	H-8, H-9b, H-14, H-15
11	H-4
12	H-9a[a]
13	H-10[a], H-14, H-15
14	H-15
15	H-10[a], H-14

[a] Tentative correlation due to weak or poorly resolved signal.

TABLE 3

LONG-RANGE CONNECTIVITIES IN COSY CONTOUR PLOT OF δ-CADINENE (**1**)

^4J	^5J
H-2, H-4[b]	H-2, H-5[b]
H-2, H-11	H-4, H-13
H-4, H-11[a,b]	H-5, H-8[b]
H-8, H-12	H-5, H-11[a,b]
H-9a, H-13[c]	H-5, H-12[a,b]
H-9b, H-13[c]	H-9b, H-12
H-14, H-15	

[a] Confirmed by decoupled ^1H NMR spectrum (Figure 13).

[b] Connectivity possibly involving intervening double bonds.

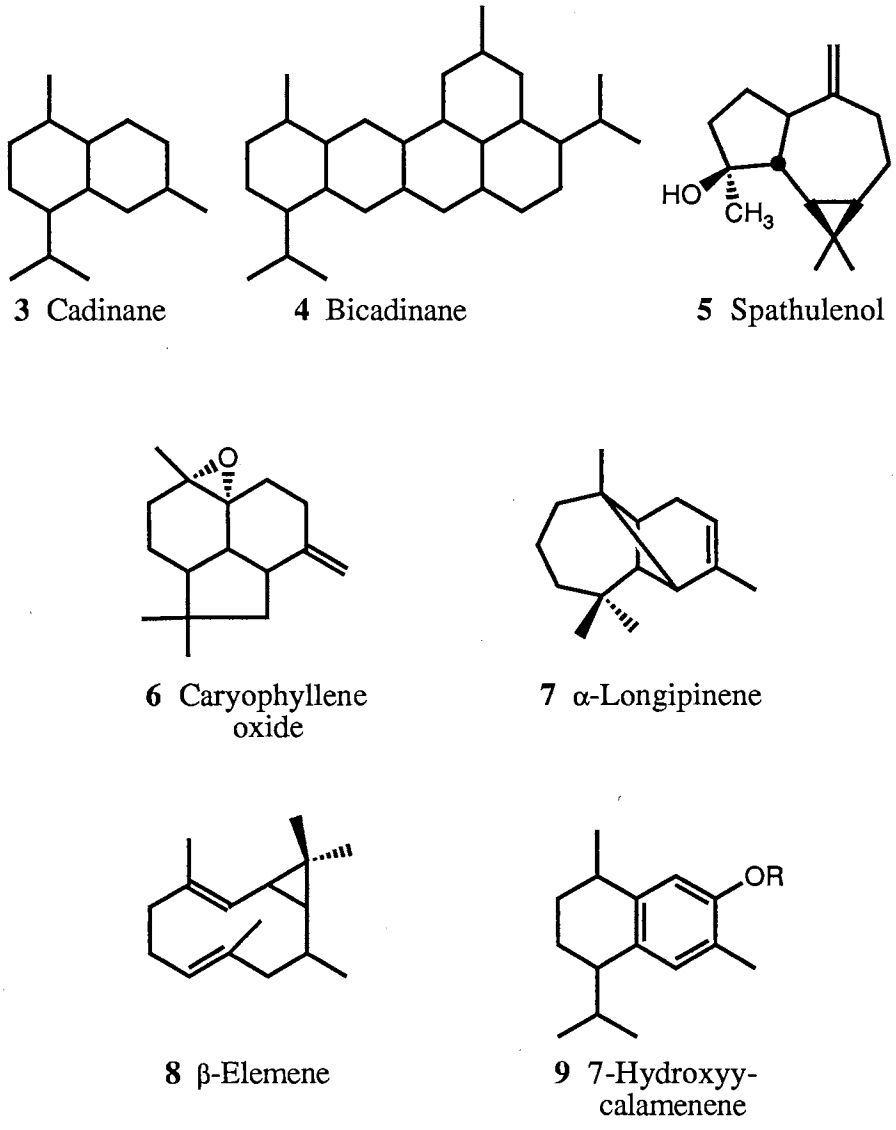
[c] Tentative correlation due to weak or poorly resolved signal.

DEPT subspectra (Figures 7, 8, and 9) failed to reveal the multiplicity of the carbon signals at higher ppm readings, but the off-resonance ^{13}C NMR spectra (Figures 10, 11, and 12) allowed final determination of all carbon signal multiplicities.

The ^{1}H-^{1}H COSY spectrum of δ-cadinene (Fig. 14) displayed all expected proton connectivities. The exhibition of numerous long-range connectivities in the ^{1}H-^{1}H COSY spectrum was similar to the behavior of other sesquiterpenoid compounds: cadinane,[35] bicadinane,[35] spatulenol,[30] caryophyllene oxide,[30] α-longipinene,[36] β-elemene,[37] and 7-hydroxy-calamenene[38] (chemical formulae **3-9** in Figure 18). Some of the long-range connectivities are possibly enhanced by double bonds between the connected nuclei.[39] The ^{1}H-^{1}H COSY spectrum confirmed the assignments for each of the geminal diastereotopic protons attached to C-1 and to C-9.

The normal ^{1}H-^{13}C HETCOR spectrum (Fig. 4) revealed all expected one-bond correlations for δ-cadinene. This allowed assignment of proton shifts in the complex multiplet located from 2.10 to 1.90 ppm in the ^{1}H spectrum; these ^{1}H shift assignments would have been difficult to determine by 1D NMR. The long-range ^{1}H-^{13}C HETCOR correlations (performed at J_{CH} values of 5, 10, and 15 Hz) (Table 2) supported the long-range connectivities in the ^{1}H-^{1}H COSY.

A cumulative analysis of the normal and long-range connectivities in the ^{1}H-^{1}H COSY spectrum and the correlations from normal and long-range ^{1}H-^{13}C HETCOR spectra permitted construction and joining of the fragments **A** and **B** (Figure 19) to give the structure of δ-cadinene (**1**). Use of this strategy[40] to "construct and join" fragments into a molecular structure was necessary because the 22 mg sample of δ-cadinene was insufficient for the performance of the INADEQUATE NMR method. INADEQUATE can provide the specific ^{13}C-^{13}C connections between the carbon nuclei of a molecule, thus revealing the carbon skeleton of the compound. Although it can deliver very useful structural information, the INADEQUATE technique has the drawback of low sensitivity, as the miniumun sample size is in excess of 200 mg.[40]



3 Cadinane

4 Bicadinane

5 Spathulenol

6 Caryophyllene oxide

7 α-Longipinene

8 β-Elemene

9 7-Hydroxyy-calamenene

Figure 18. Chemical formulae of terpenoid compounds which provided 2D NMR information useful in analysis of NMR spectra of δ-cadinene (**1**).

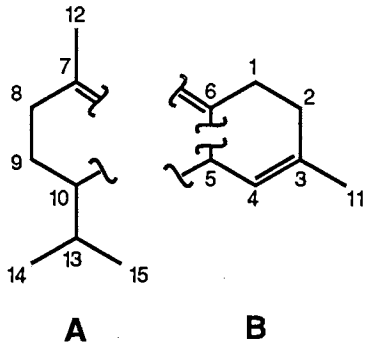


Figure 19. Structural fragments **A** and **B** constructed from NMR spectra.

The H-12 protons exhibited long-range connectivity to the methylene H-8 protons (2H, m, H-8) in the ^{1}H-^{1}H COSY spectrum, and C-8 (32.27 ppm) was coupled to the H-12 protons in the long-range ^{1}H-^{13}C HETCOR. A long-range correlation of the quaternary carbon absorbing at 124.58 ppm (C-7) to the adjacent H-8 methylene protons at 1.95 ppm (2H, m, H-8) was tentatively identified in long-range ^{1}H-^{13}C HETCOR spectra; attempts to increase resolution to clarify this correlation were unsuccessful. The ^{1}H-^{1}H COSY spectrum revealed connectivities of the H-8 protons to the diastereotopic protons absorbing at 1.61 ppm (1H, m, H-9a) and 1.16 ppm (1H, m, H-9b). These couplings supported the order of C-12→C-7→C-8→C-9 in fragment **A**.

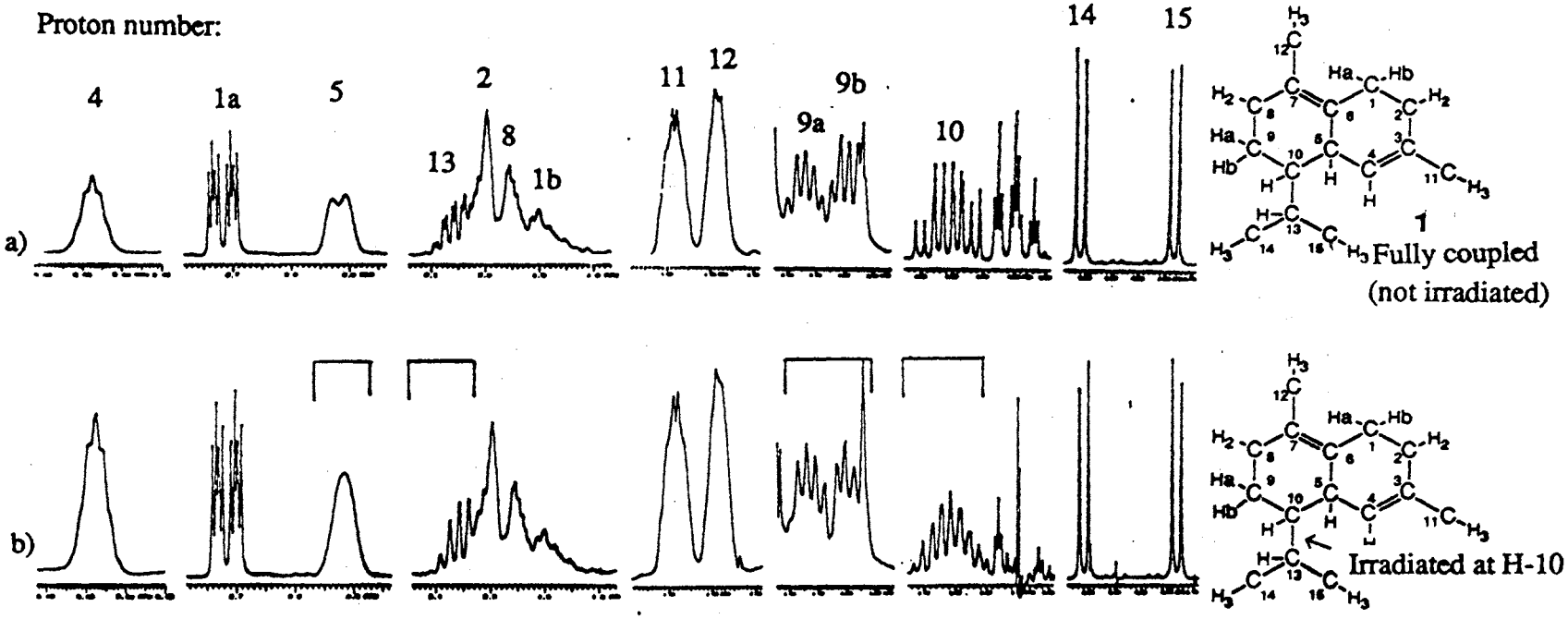
The H-9 protons in turn coupled with the methine proton absorbing at 1.05 ppm (1H, m, H-10). The H-10 proton coupled with the methine proton located on the isopropyl side chain which absorbs at 2.06 ppm (1H, m, H-13); the H-13 methine proton also coupled with the protons of the methyl groups of the isopropyl side chain which absorb at 0.96 ppm (3H, d, H-14) and 0.78 ppm (3H, d, H-15). Identification of H-10 was supported by decoupled ^{1}H NMR (Figure 20) because decoupling of H-10 simplified the H-13 signal (multiplet of doublet peaks simplified to multiplet of single peaks) and the H-5 signal (broad doublet simplified to a broad singlet), and altered the signals of H-9a and H-9b. Long-range connectivities in the ^{1}H-^{1}H COSY spectrum showed coupling between the H-9 protons and the H-13 proton and also coupling between the H-14 and H-15 protons. This

Figure 20. Expansions from:

a) Fully coupled ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C.

b) Decoupled ^{1}H NMR spectrum of cade oil δ-cadinene (**1**) at 7°C. Sample was irradiated at H-10 signal.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

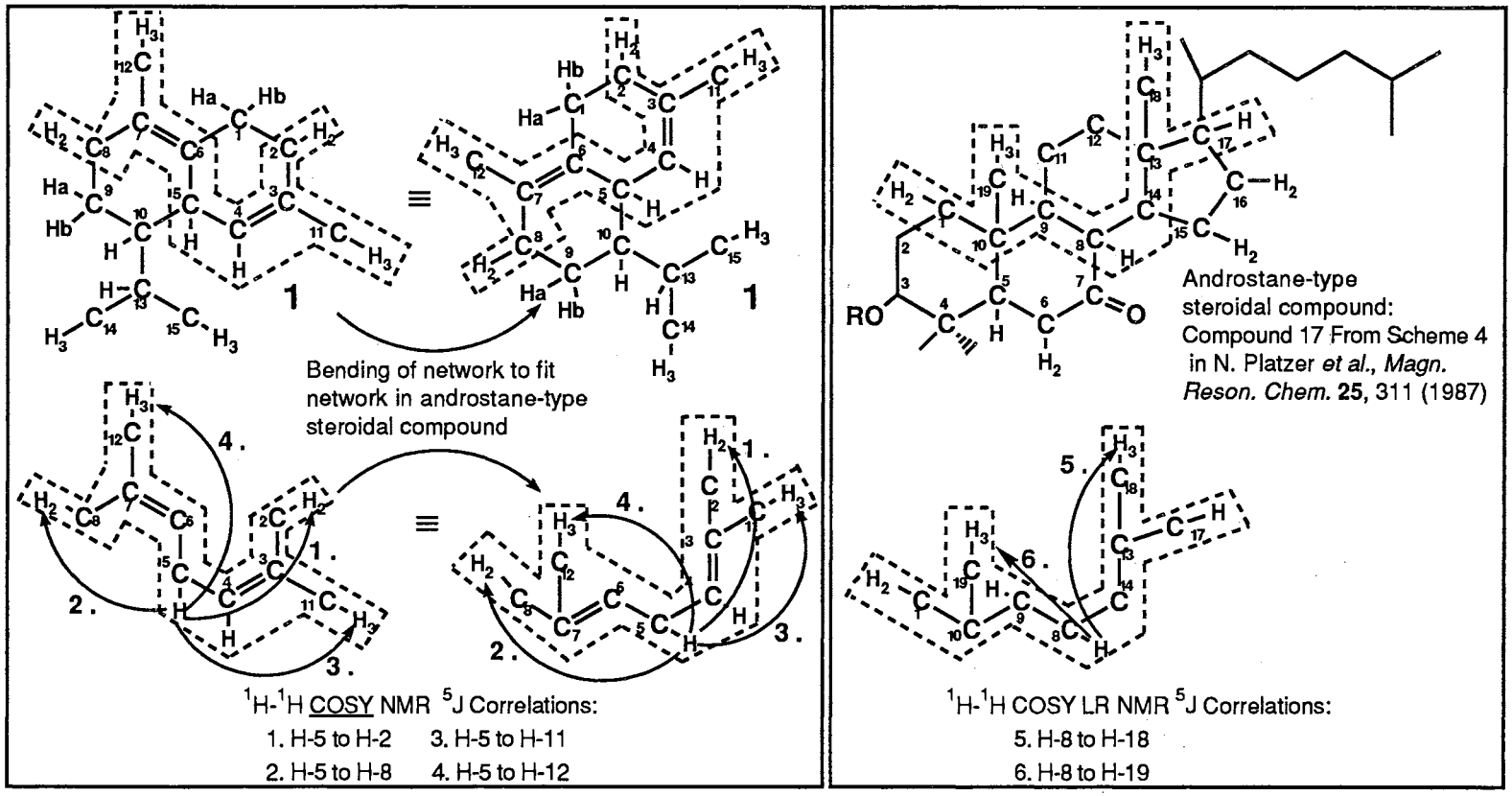

Proton number:
4
1a
5
2
13
8
1b
11
12
9b
9a
10
14
15
a)
b)
1
Fully coupled
(not irradiated)
Irradiated at H-10

evidence of C-12→C-7→C-8→C-9→C10→C-13→(C14 and C-15) order supported the structure of fragment **A**. Long-range ^{1}H-^{13}C HETCOR correlations (Table 2) were consistent with and supported the structure of fragment **A**.

Construction of fragment **B** followed in similar fashion. The methine proton absorbing at 2.52 ppm (1H, br d, H-5) showed coupling to the olefinic proton absorbing at 5.45 ppm (1H, br s, H-4) in the ^{1}H-^{1}H COSY spectrum (See Figure 13; irradiation of H-4 changes H-5 signal and irradiation of H-5 alters H-4 signal). The ^{1}H-^{1}H COSY spectrum also displayed long-range connectivities from methyl protons absorbing at 1.68 ppm (3H, s, H-11) to both the olefinic H-4 proton and the methylene protons absorbing at 2.00 ppm (2H, m, H-2); the long range ^{1}H-^{13}C HETCOR spectrum showed the quaternary carbon absorbing at 134.23 ppm (C-3) correlated with the methyl H-11 protons (1.68 ppm) and the methylene H-2 protons (2.00 ppm). The ^{1}H-^{1}H COSY spectrum exhibited connectivities of the methylene H-2 protons to the diastereotopic protons absorbing at 2.72 ppm (1H, m, H-1a) and 1.90 ppm (1H, m, H-1b). Finally, the long-range ^{1}H-^{13}C HETCOR spectrum revealed correlation of the quaternary carbon absorbing at 129.84 ppm (C-6) to H-1a, H-2 protons, and H-4; this analysis supported existence of fragment **B**.

The connection between fragments **A** and **B** was deduced by consideration of the ^{1}H-^{13}C long-range HETCOR spectrum which showed C-6 correlated protons absorbing at 1.66 ppm (H-12) and 1.95 ppm (H-8, thus supporting the bond between C-6 and C-7. The long-range ^{1}H-^{13}C HETCOR revealed C-5 coupled with protons absorbing at 1.05 ppm (H-10) and 1.61 ppm (H-9a); the ^{1}H-^{1}H COSY spectrum revealed H-5 coupled to H-10, thus the bond between C-5 and C-10 was strongly supported. Finally, the bond between C-5 and C-6 fulfilled the need to satisfy the multiplicity pattern. The extended network of C- 11, 3, 4, 5, 6, 7 and 12 in **1** is supported by a report of the COSY LR pattern for an androstane-type steroidal compound which contains a similar extended network (Figure 21; also, see Fig. 17 in Ref. 41). The COSY LR of the androstane-type compound exhibited two ^{5}J connectivities which were analogous to the long-range ^{5}J

Figure 21. Comparison of similar long-range connectivities found in ^{1}H-^{1}H COSY plot of δ-cadinene (**1**) and ^{1}H-^{1}H COSY LR plot of androstane-type steroidal compound from Reference 41.



[NOTE: NMR technique used for δ-cadinene was normal COSY which yielded numerous long-range connectivities; the NMR technique for the androstane-type steroidal compound was COSY LR (COSY Long-Range)]

connectivities of H-5 to H-2, H-8, H-11, and H-12 seen in the ^1H-^1H COSY of **1**; apparently the structurally similar networks give rise to comparable long-range ^1H-^1H COSY connectivities; put simply, the sets of protons located in very similar environments in the two molecules displayed very similar connectivities. Our analysis of long-range connectivities in the ^1H-^1H COSY spectrum and the correlations in the long-range ^1H-^{13}C HETCOR spectra has allowed unambiguous assignment of all ^{13}C and ^1H chemical shifts for δ-cadinene. The chemical shift assignments C-2/H-2 and C-8/H-8 were marked as possibly interchangeable in previously published work;[15] i.e., there were two sets of protons which could reasonably be attached to C-2 or to C-8. This present work has now established that the set of two protons at 2.00 ppm (^1H NMR scale) are attached to C-2 and that the set of two protons at 1.95 ppm (^1H NMR scale) are attached to C-8.

The ^1H-^1H COSY connectivity between the olefinic proton and one set of methylene protons (H-4 proton and H-2 protons, respectively, in structure **1**) and the long-range ^1H-^{13}C HETCOR correlations C2-H4 (in **1**) and C4-H2 (in **1**) could be used in an attempt to fit the NMR data to **2**. If the connectivities and correlations were considered to be evidence of adjacent carbons, one bearing the olefinic proton and the other bearing methylene protons, then the signals assigned to carbons (and attached protons) numbered 1, 2 and 4 in structure **1** could be reassigned to carbons (and attached protons) numbered 4, 1 and 2, respectively, in structure **2**. However, the COSY spectrum (with the reassignment of signals to **2**) lacks the predicted correlation of the H-5 proton to either of the H-4 protons. Additionally, the reassignment of chemical shifts to **2** would lead to unlikely ^4J correlations in the long-range ^1H-^{13}C HETCOR for these carbon-proton pairs: C5 to H2 (olefinic proton), C4 to H1 and C1 to H-11.

The 2D NMR results are consistent with **1**, not **2**. The fragment of C1-C2 of **1** is supported by ^1H-^1H COSY, and a long-range ^1H-^{13}C HETCOR correlations of C1-H2 and C2-H1a (tentative) in **1**. The adjacent disposition of carbons 5 and 4 is shown by a C5 to H4 correlation in the long-range ^1H-^{13}C HETCOR spectrum. Evidence for two pairs of

adjacent methylene groups (C1-C2 and C8-C9) and the C5-C4 connection eliminated structure **2** as the representation of δ-cadinene (**1**).

Consideration of the ^{1}H-^{1}H COSY spectrum, ^{1}H-^{13}C HETCOR spectrum, long-range ^{1}H-^{13}C HETCOR spectrum, off-resonance ^{13}C spectra and confirmatory decoupled ^{1}H NMR spectra allowed unambiguous assignment of all proton and carbon signals (Table 1) for this prevalent natural product. All long range ^{1}H-^{13}C HETCOR correlations were consistent with assignments made on the basis of ^{1}H-^{1}H COSY and normal ^{1}H-^{13}C HETCOR. The abundance of positive evidence and lack of negative evidence strongly supports structure **1** as the correct representation of δ-cadinene. Our assignments compared well with those of structurally related compounds in the literature.[16,42-47]

EXPERIMENTAL

For the isolation of **1** 50 ml of cade oil (product of destructive distillation of *Juniperus oxycedrus*; obtained from Penta Manufacturing, East Hanover, New Jersey, U.S.A.) was diluted with 200 ml of hexane; dark polar components were removed by extraction with H_2O and subsequent mixing with silica gel. The hexane decolorized by the silica gel was mixed with an Et_2O:hexane (1:50) rinse of the silica gel (to enhance recovery of δ-cadinene). After concentration by rotary evaporation (0°C bath temperature), the hexane solution was purified by passage through silica Sep-Paks (Waters). An Et_2O:hexane solution (1:19; 30 ml volume) was used to elute any retained δ-cadinene from the silica Sep-Paks; this solution was mixed with the flow-through fluid. The combined solution was concentrated to small volume (approximately 3 ml) by rotary evaporation (0°C bath temperature). The solution was dissolved in 50 ml of HPLC-grade acetonitrile:H_2O (35:65) which was then slowly passed through four octadecylsilane Environmental Sep-Paks (Waters) connected in tandem, and flow-through fluid was discarded. δ-Cadinene was eluted from the octadecylsilane Sep-Paks with 50 ml of HPLC-grade acetonitrile which

had been used to rinse any remaining δ-cadinene from the previous sample container. Water was added to the acetonitrile and the δ-cadinene was back-extracted into hexane. After concentration of the hexane by rotary evaporation (0°C bath temperature), the δ-cadinene was purified by HPLC with a semi-preparative 10 x 250 mm octadecylsilane column (5 μm media, Maxsil, Phenomenex), eluting with acetonitrile:H_2O (85:15). The extract was further purified by sub-ambient temperature HPLC[48] at -30°C through four 3.9 x 300 mm silica HPLC columns (10 μm particle diameter, μPorasil, Waters) attached in tandem;[49] the δ-cadinene was eluted with hexane with detection by UV absorption at 215 nm. The sample was applied to a tandem[49] arrangement of a 4.0 x 125 mm octadecylsilane (5 μm particle diameter, Partisphere, Whatman) and a 4.0 x 250 mm octadecylsilane (5 μm particle diameter, Hibar, E. Merck) and eluted with acetonitrile:H_2O (85:15) at ambient temperature. Twenty-two milligrams of δ-cadinene (**1**) were recovered at >95% purity (by gas chromatographic analysis with flame ionization detection (Figure 22).

The isolated δ-cadinene had an enantiomeric excess of the (+)-isomer, as the circular dichroism reading taken from the recorded spectrum (Figure 23) was $\Delta\varepsilon_{212} = -1.9$; $\Delta\varepsilon_{193} = +2.8$ (reference circular dichroism values for (+)-δ-cadinene isolated from cade oil:[5] $\Delta\varepsilon_{212} = -3.7$; $\Delta\varepsilon_{193} = +10.6$). The difference between our experimental and the literature circular dichroism values is not disturbing because specific rotation values for isolated (+)- or (-)-isomers of δ-cadinene vary.[10] The differences in these values may reflect variations in the source material. Examples of such variability include the terpenoid compounds lacinilene C and LCME[27] and gossypol[50] which have been isolated from cotton in varying degrees of enantiomeric excess. The experimental mass spectrum and infrared spectrum for the δ-cadinene from cade oil matched the literature mass spectrum[51-54] and infrared spectrum,[55,56] respectively, of δ-cadinene. (These results are produced here after the style of *Magnetic Resonance in Chemistry* . This information is referred to in the experimental section of Chapter V of this thesis to avoid duplication.)

Figure 22. GC/FID chromatography of:

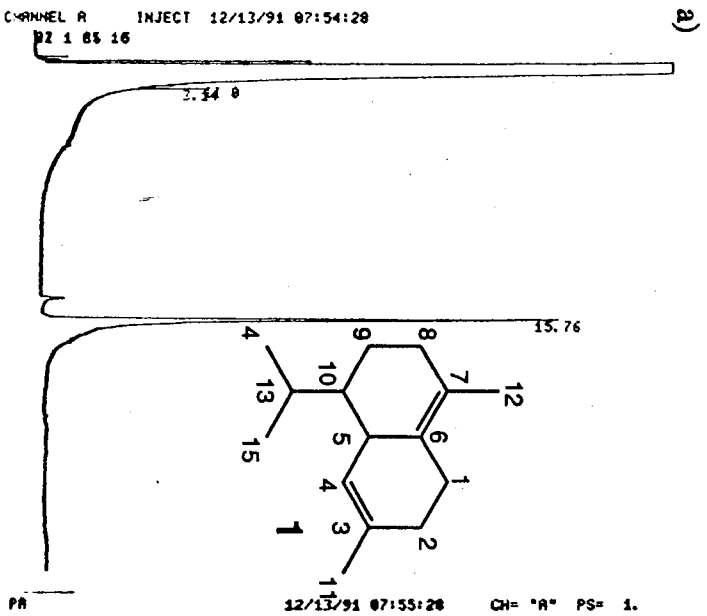
a) cade oil δ-cadinene (**1**); injection of approximately 5 nanograms of cade oil δ-cadinene (**1**). The cade oil δ-cadinene (**1**) was contained in an on-column injection volume of 0.5 μl hexane.

and

b) cade oil δ-cadinene (**1**); injection of approximately 25 nanograms of cade oil δ-cadinene (**1**) to visualize contaminant peaks. The cade oil δ-cadinene (**1**) was contained in an on-column injection volume of 0.5 μl hexane.

Instrument: United Technologies Packard Model 438A gas chromatograph employing a 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-5). Detection made by flame ionization detection (FID).
Oven gradient: 1 min. hold at 85°C, then 3 min. at 21.7°C min^{-1}, then 1 min. hold at 150°C, then 50 min. at 2°C min^{-1} (final temperature of 250°C).

a)



PA 12/13/91 07:55:28 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 309 INDEX 309

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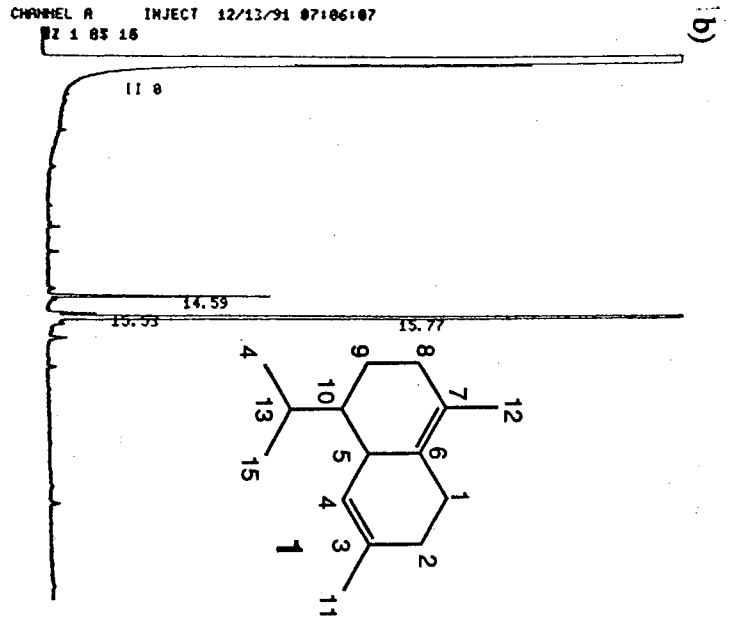
1	100.	15.76	195832 01
TOTAL	100.		195832

b)



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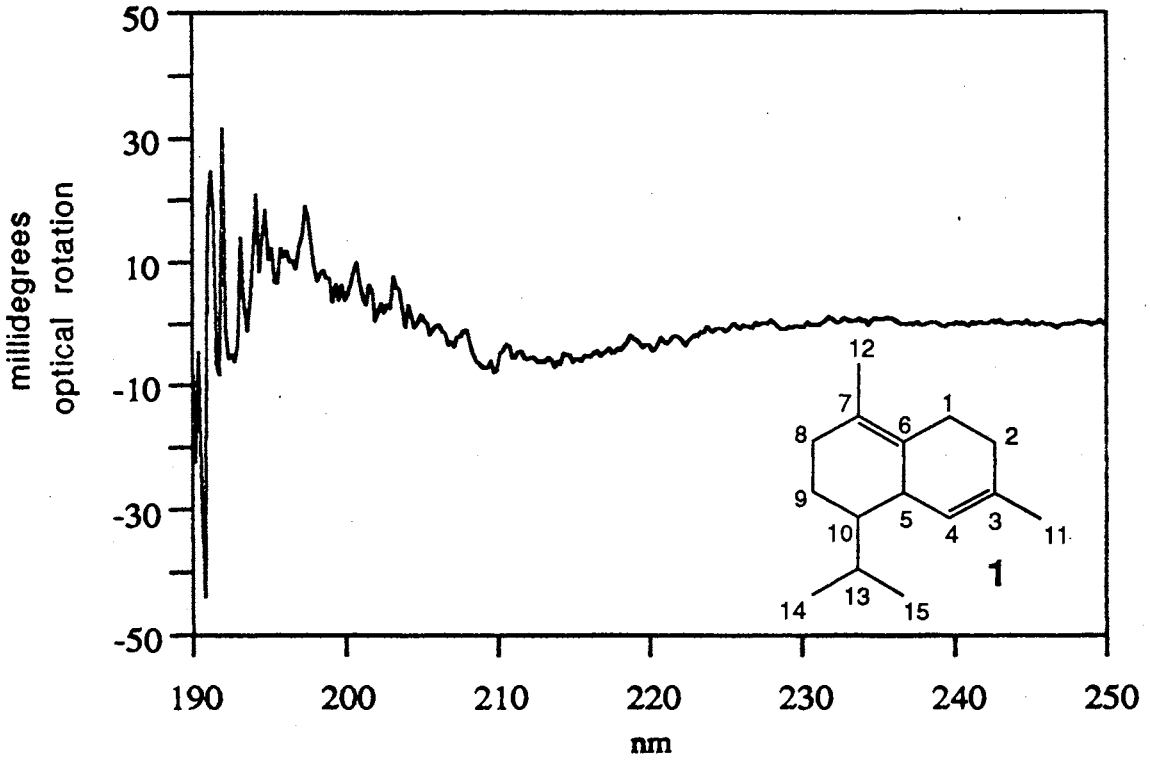
FILE 1. METHOD 0. RUN 308 INDEX 308

PEAK#	AREA%	RT	AREA BC
1	4.063	14.59	47039 01
2	95.937	15.77	1110799 01
TOTAL	100.		1157838

Figure 23. The circular dichroism spectrum of the δ-cadinene (**1**) isolated from cade oil.

Instrument: JASCO J-600 recording spectropolarimeter; 16 scans accumulated on a sample of 1 x 10^{-5} M solution of δ-cadinene isolated from cade oil in hexane.


50
30
10
-10
-30
-50
millidegrees
optical rotation
190
200
210
220
230
240
250
nm
12
7
1
8
6
2
9
5
4
3
11
10
14
13
15
1

NMR spectra were obtained in the Fourier transform mode from the sample of **1** contained in a 5 mm o.d. tube on a Varian XL-400A NMR spectrometer with data acquisition at 399.9 MHz (^1H) and 100.6 MHz (^{13}C). Spectra were recorded for solution of **1** [2% (w/v) in $CDCl_3$ with TMS as the reference] at 7°C. All downfield shifts are on the δ scale from the reference and are labelled as positive. Signal-to-noise enhancement and baseline linearization were achieved by suitable exponential weighting and apodization of the free induction decay (FID). (Apodization[57] is a treatment of the free induction decay [FID] in order to obtain the best resolution and signal-to-noise ratio in the final NMR spectrum. Two popular apodization procedures are exponential multiplication and Gaussian multiplication.) Proton decoupled spectra were obtained by appropriate adjustment of decoupler frequency. DEPT subspectra were obtained by use of standard Varian software. ^{13}C NMR off-resonance spectra were acquired by appropriate adjustment of decoupler frequency offset.

The 2D NMR spectra were obtained by using standard sequences. For the ^1H-^1H COSY 90 spectra, 256 experiments were performed, with 592 scans. Block size was 64. The relaxation delay was 1 s for the spectra. The spectral width was 2304.7 Hz. The data size was 2K. Proton pulse width was 30.0 ms. Quadrature detection was used. The final data matrix was symmetrized about the diagonal.

For the heteronuclear two-dimensional ^1H-^{13}C chemical shift correlation experiments, the spectra were acquired with 2 K x 2 K data points and a data acquisition of 128 experiments with 576 scans. The relaxation delay was 1 s. Spectral width was 2304.7 Hz and 14224.8 Hz in the (^1H) and F_2 (^{13}C) domains, respectively. Digital resolution was 1.13 for ^1H and 6.95 for ^{13}C. Pulse width was 17.5 ms for ^1H and 17.0 ms for ^{13}C. Long range ^1H-^{13}C HETCOR spectra were obtained by setting the JNXH variable of the Varian software to 5, 10, or 15 Hz.

ACKNOWLEDGEMENTS

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CHAPTER V

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; farnesyl pyrophosphate; 2,7-dihydroxycadalene; lacinilene C; bacterial blight of cotton.

ABSTRACT

Glandless cotton tissues 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 noninoculated tissues. This hydrocarbon, labelled with tritium, was formed from [1-^{3}H]farnesyl pyrophosphate in a cell-free reaction catalyzed by homogenates of cotton tissues which had been inoculated with *Xanthomonas campestris* pv. *malvacearum* or treated with elicitor. A larger amount of the hydrocarbon product was isolated from cell-free reactions of unlabelled farnesyl pyrophosphate catalyzed by homogenates of inoculated cotton tissues. In all analyses, the isolated hydrocarbon was identical to δ-cadinene isolated from cade oil. The biosynthesis of this cadinene in bacteria-inoculated or elicitor-treated cotton tissues suggests that δ-cadinene may be an early enzymatic intermediate in the biosynthesis of the sesquiterpenoid phytoalexins 2,7-dihydroxycadalene, lacinilene C and lacinilene C 7-methyl ether by upland cotton.

INTRODUCTION

2,7-Dihydroxycadalene (DHC) (**1**), 2-hydroxy-7-methoxycadalene (**2**), lacinilene C (**3**) and lacinilene C 7-methyl ether (**4**) are sesquiterpenoid compounds produced in leaves and cotyledons of upland cotton (*Gossypium hirsutum* L.) during the hypersensitive response to incompatible races of *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (*Xcm*) and to pathovars of *X. campestris* that are not pathogenic to cotton [1, 2] (Compounds designated by bold numeral are listed in Figure 1). *Xcm* is the causal agent of bacterial blight of cotton. The hypersensitive response enables resistant lines of upland cotton to withstand infection with *Xcm*, 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].

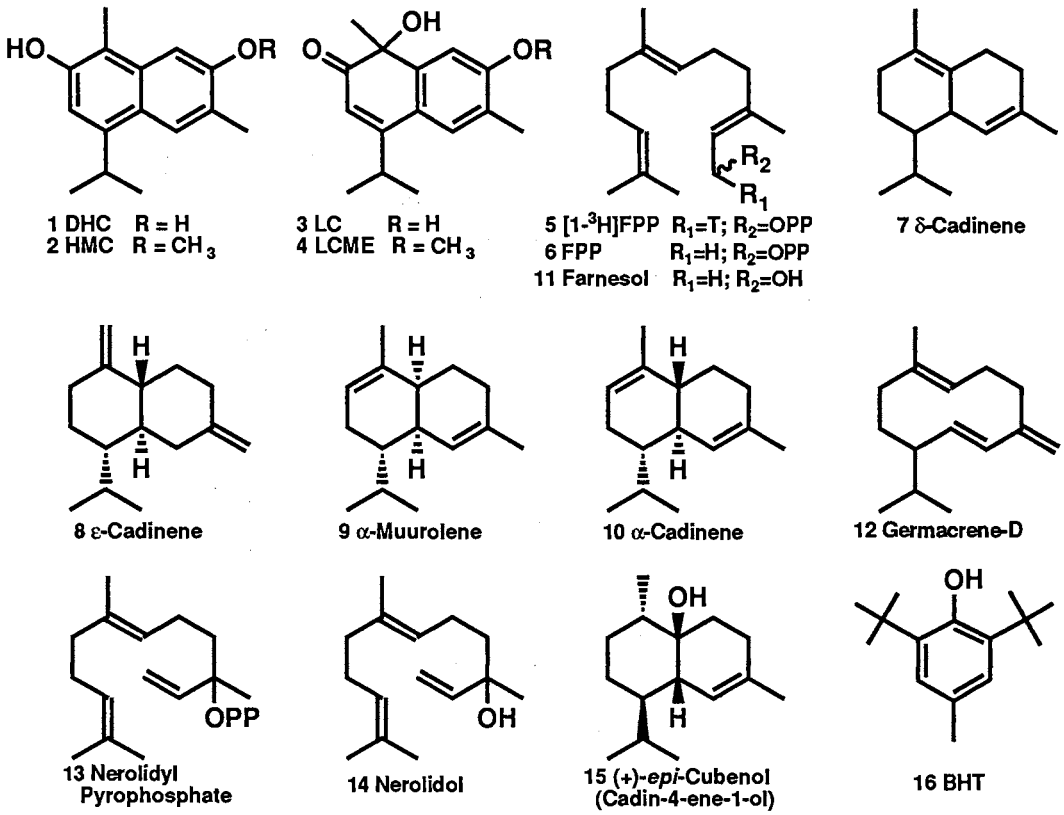


Figure 1. Sesquiterpenoid compounds and BHT.

In previous work [4], we showed that [1-^{3}H]farnesyl pyrophosphate ([1-^{3}H]FPP) (**5**) 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 [5-7], we identified a glandless cotton line (WbM*gl*) whose healthy tissues lack the constitutive terpene production of glanded cotton, but which still accumulates DHC upon inoculation with a strain of *Xcm* to which it is resistant. A prominent infection-induced hydrocarbon not present in mock-inoculated or noninoculated cotton cotyledons was recovered from *Xcm*-inoculated glandless cotton cotyledons and from cell-free extracts of such cotyledons upon incubation with either [1-^{3}H]FPP or unlabelled FPP (**6**). During this work, the same hydrocarbon was found in elicitor-infiltrated tissues and in cell-free extracts of these tissues incubated with [1-^{3}H]farnesyl pyrophosphate. We now report our identification of this hydrocarbon product of the cyclase enzyme as δ-cadinene (**7**).

RESULTS AND DISCUSSION

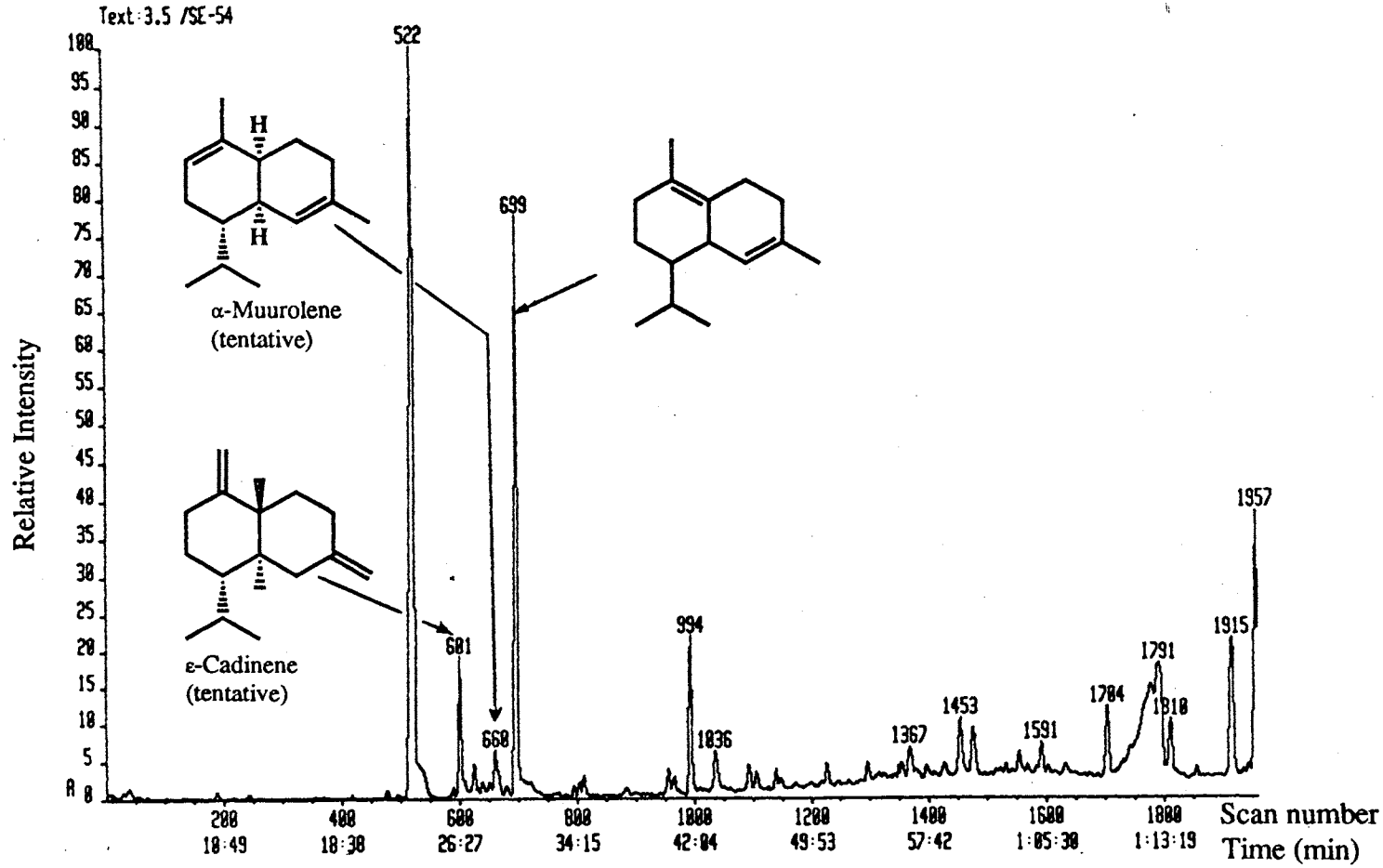
Identification of an infection-induced hydrocarbon from cotyledons

Hexane extracts of noninoculated and mock-inoculated glandless cotton (WbM*gl*) tissues which had been eluted from silica gel with hexane were analyzed by gas chromatography-electron impact mass spectrometry (GC-EIMS). Virtually no volatile sesquiterpenes were detected in the hexane extracts. GC-EIMS analysis (Figure 2) of similar hexane extracts of *Xcm*-inoculated WbM*gl* tissues revealed three infection-induced compounds, each possessing 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 present in smaller amounts. The identification of each compound was tentative because we did not have known standards for identification by co-chromatography. The tentative identification of ε-cadinene was based upon a close match with the published Kovat's Index value for ε-cadinene chromatographed on a Carbowax 20M GC column [8] the experimental mass spectrum was similar, but not identical, to the published mass spectrum of racemic ε-cadinene [9]. The tentative identification of α-muurolene was based upon a close match with the Kovat's Index value calculated from the published retention time for chromatography of α-muurolene on an SE-54 GC column [10] and reference mass spectra for α-muurolene from numerous sources [10,11,12].

To obtain more of the major sesquiterpene for further characterization, 1300 g of *Xcm*-inoculated WbM*gl* tissues were extracted. The yield was approximately 500 μg of a compound exhibiting the chromatographic characteristics of a hydrocarbon: it was weakly retained by silica gel and strongly retained by octadecylsilane. Its mass spectrum (Figure 3; Figure 4 is an blank run to check for detectable compounds in the extracting solvent) exhibited an *m/z* 204 $[M]^+$ and most closely matched the reference mass spectrum of δ-cadinene from different literature sources [11,13-15]. However, the mass spectrum was

Figure 2. The total ion current chromatogram of the hexane extract of *Xcm*-inoculated glandless cotton (WbM*gl*). Infection-induced compounds include ε-cadinene (tentative), α-muurolene (tentative), and δ-cadinene.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; Alltech equivalent to J&W Scientific DB-5)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 2°C min^{-1} until run terminated.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Relative Intensity
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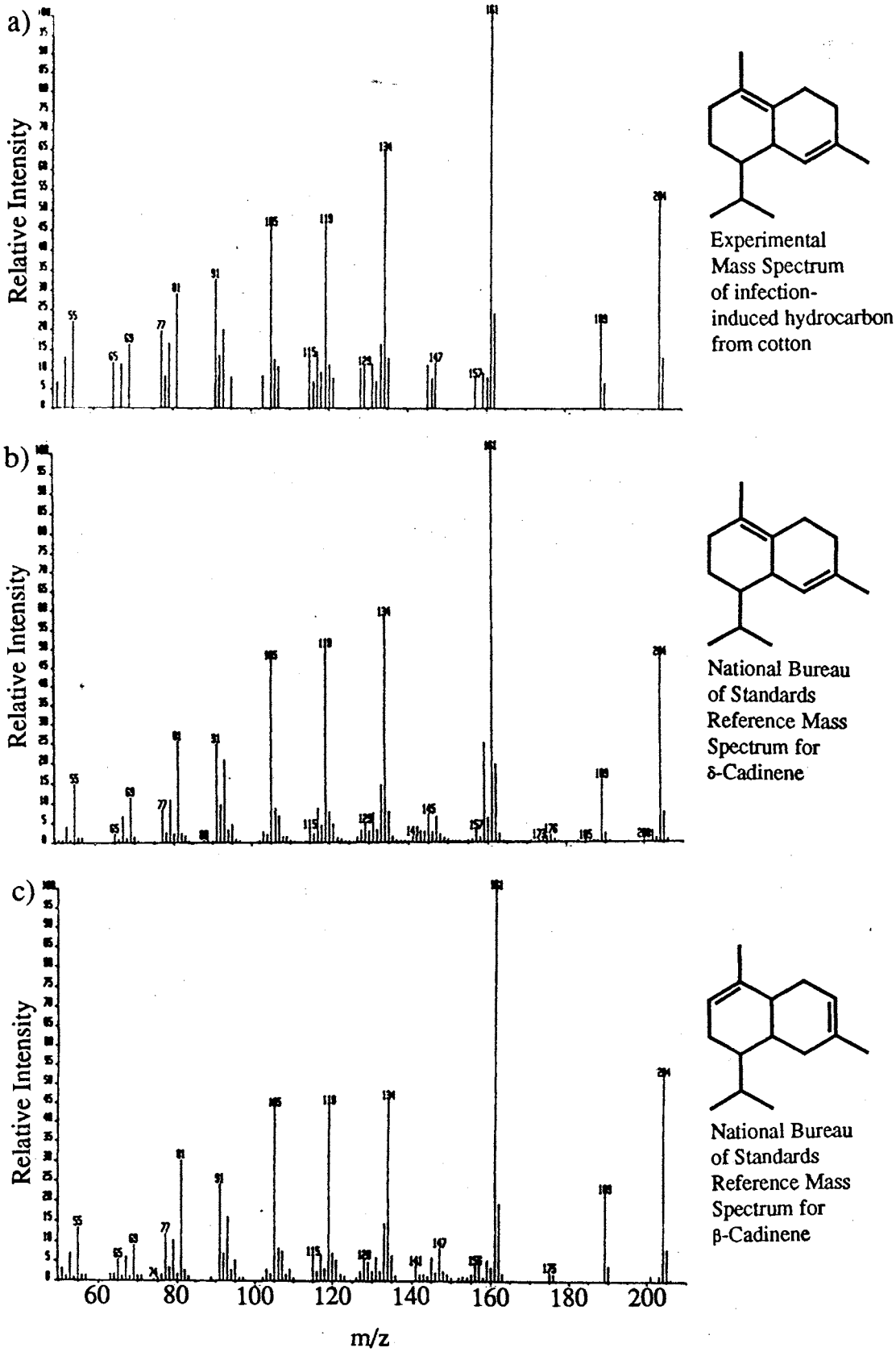
A 0
522
699
681
668
994
1836
1367
1453
1591
1784
1791
1810
1915
1957
α-Muurolene
(tentative)
ε-Cadinene
(tentative)
H
H
200
18:49
400
18:38
600
26:27
800
34:15
1000
42:04
1200
49:53
1400
57:42
1600
1:05:30
1800
1:13:19
Scan number
Time (min)

Figure 3. EI mass spectrum of

a) the experimentally obtained infection-induced hydrocarbon in *Xcm*-inoculated glandless cotton (WbM*gl*) cotyledons.

b) δ-Cadinene from National Bureau of Standards data base

c) β-Cadinene from National Bureau of Standards data base.



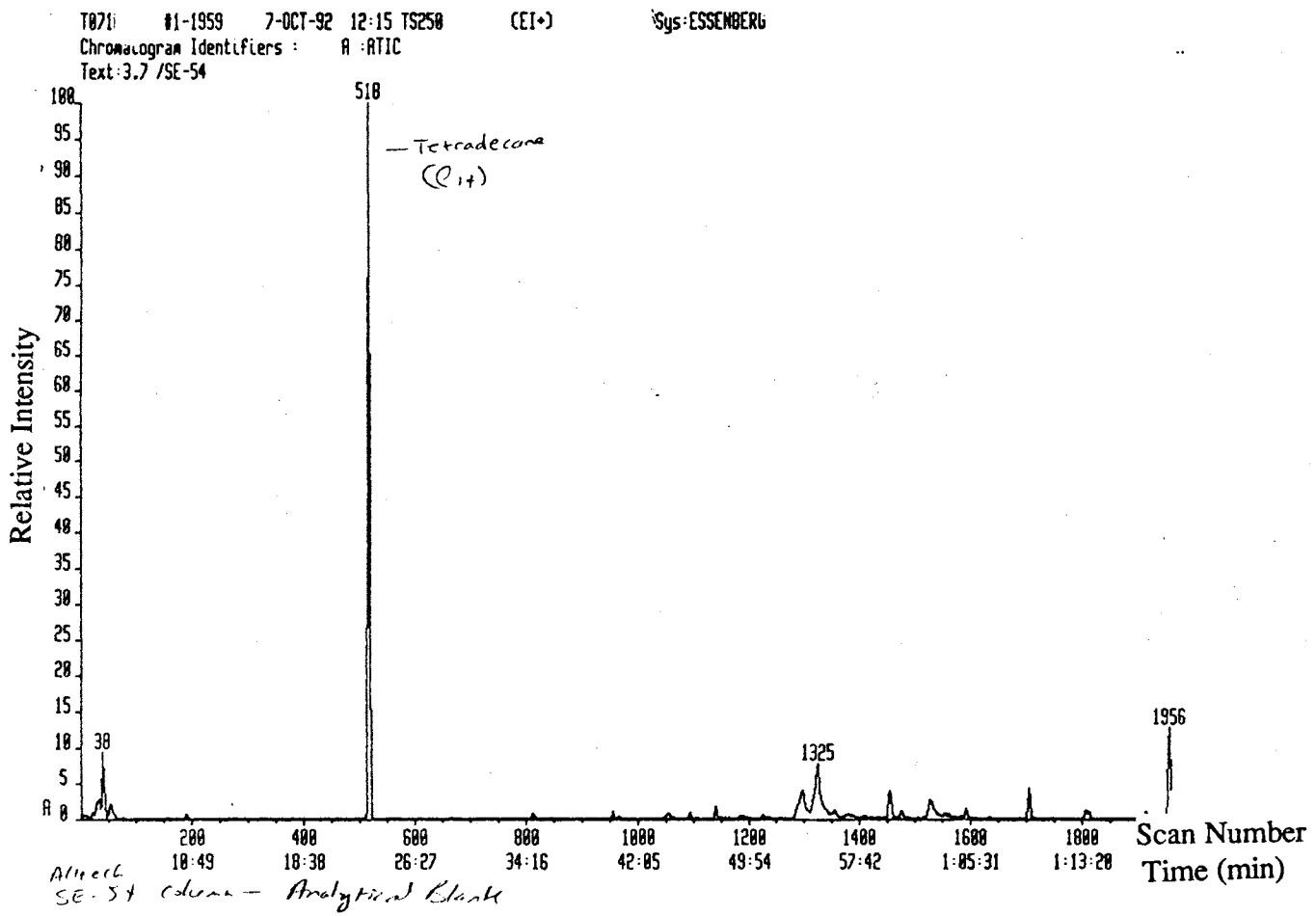
Experimental Mass Spectrum of infection-induced hydrocarbon from cotton

National Bureau of Standards Reference Mass Spectrum for δ-Cadinene

National Bureau of Standards Reference Mass Spectrum for β-Cadinene

Figure 4. The total ion current chromatogram of the analytical blank; test for detectable compounds in extracting solvent. Spiked with tetradecane.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; Alltech equivalent to J&W Scientific DB-1) and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 2°C min^{-1} until run terminated.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Relative Intensity
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30
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20
15
10
5
A 0
518
Tetradecane
(C14)
38
1325
1956
200 400 600 800 1000 1200 1400 1600 1800
10:49 18:38 26:27 34:16 42:05 49:54 57:42 1:05:31 1:13:20
Scan Number
Time (min)
Allech
SE-54 column — Analytical Blank

not diagnostic for δ-cadinene because a number of sesquiterpene hydrocarbons of molecular weight 204 (e.g., β-cadinene [13]) exhibit very similar mass spectra. ^{1}H NMR of the compound (Figures 5 and 6) corresponded well with the incomplete ^{1}H NMR data found in the literature for δ-cadinene [16-18] (and ^{1}H NMR of cade oil δ-cadinene in Chapter IV of this thesis; see Figure 5 on page 81).

To obtain a δ-cadinene standard for comparison to the infection-induced compound, δ-cadinene was isolated from cade oil and identified as described in Chapter IV (see p. 118 of this thesis). The rigorously characterized cade oil δ-cadinene co-chromatographed with the infection-induced hydrocarbon compound isolated from *Xcm*-inoculated cotton in four GC phases of varying polarities and yielded Kovat's index values (R_I) [19] which were very close to literature values for δ-cadinene (Table 1) (see Figures 7 and 8). Co-chromatography was performed on a 5%phenyl-95%methyl (SE-54) column of 120 m length formed by the union of two 60 m columns; calculation of Kovat's index values for the cade oil δ-cadinene and the infection-induced compound by the routine method employing *n*-paraffin standards yielded a value in disagreement with the literature value (Table 1). However, the normal *n*-paraffin standards could be replaced by the sesquiterpene hydrocarbons β-caryophyllene and α-humulene to generate a "self-consistent" Kovat's index value [17,20] matching the literature value [10] for δ-cadinene retention on a 5%phenyl-95%methyl (SE-54) GC phase (Table 1).

The co-chromatography studies and similarities of the ^{1}H NMR and mass spectra between the cotton infection-induced hydrocarbon from *Xcm*-inoculated glandless cotton and the rigorously identified cade oil δ-cadinene demonstrated that δ-cadinene was accumulated during the course of the hypersensitive response of cotton to its pathogen *Xcm* to a level of approximately 300 ng per g of inoculated tissue [by gas chromatography-flame ionization detection (GC-FID)]. In contrast, δ-cadinene was not detected in mock-inoculated and noninoculated cotton tissues, even though as little as 5 ng δ-cadinene per g

TABLE 1

COMPARISON OF REFERENCE KOVAT'S INDEX[19] VALUES FOR δ-CADINENE (7) WITH EXPERIMENTALLY DETERMINED VALUES

(from co-injection of the induced hydrocarbon component from *Xcm*-inoculated cotton tissues and cade oil δ-cadinene onto gas chromatographic phases of varying polarity.) [Marker compounds (β-caryophyllene and α-humulene) included to assure confidence in calculated values.]

	100% Methyl (SE-30) 20M)		5% Phenyl 95% Methyl (SE-54)*		50% Phenyl 50% Methyl		Polyethylene Glycol (Carbowax	
Compound(s)	Ref.[21]	Exp.	Ref.[10]	Exp+	Ref.[22]	Exp.	Ref.[21]	Exp.
Co-injected Cotton Hydrocarbon and Cade Oil δ-Cadinene (7)	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 Literature Value		1667	1665

* 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 Kovat's Indices standard curve [19] which could produce a "self-consistent" Kovat's index value [17,20] for δ-cadinene.

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

Figure 5. ^{1}H NMR spectrum of the infection-induced compound from cotton at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

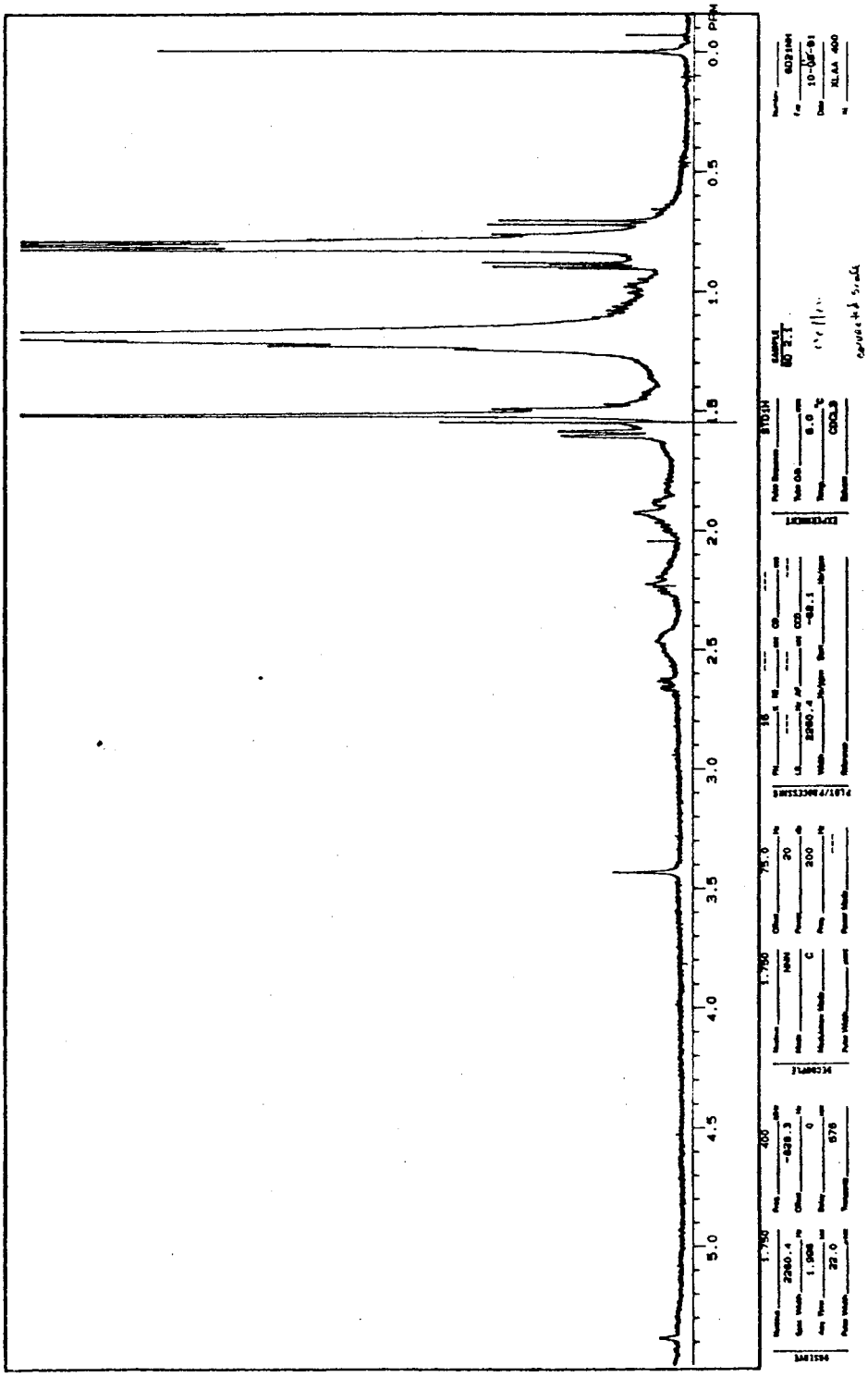

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0 PPM

Figure 6. Expansion to visualize detail of the ^{1}H NMR spectrum of infection-induced compound from cotton at 7°C.

Instrument: Varian Associates XL-400A 400 MHz NMR spectrometer.

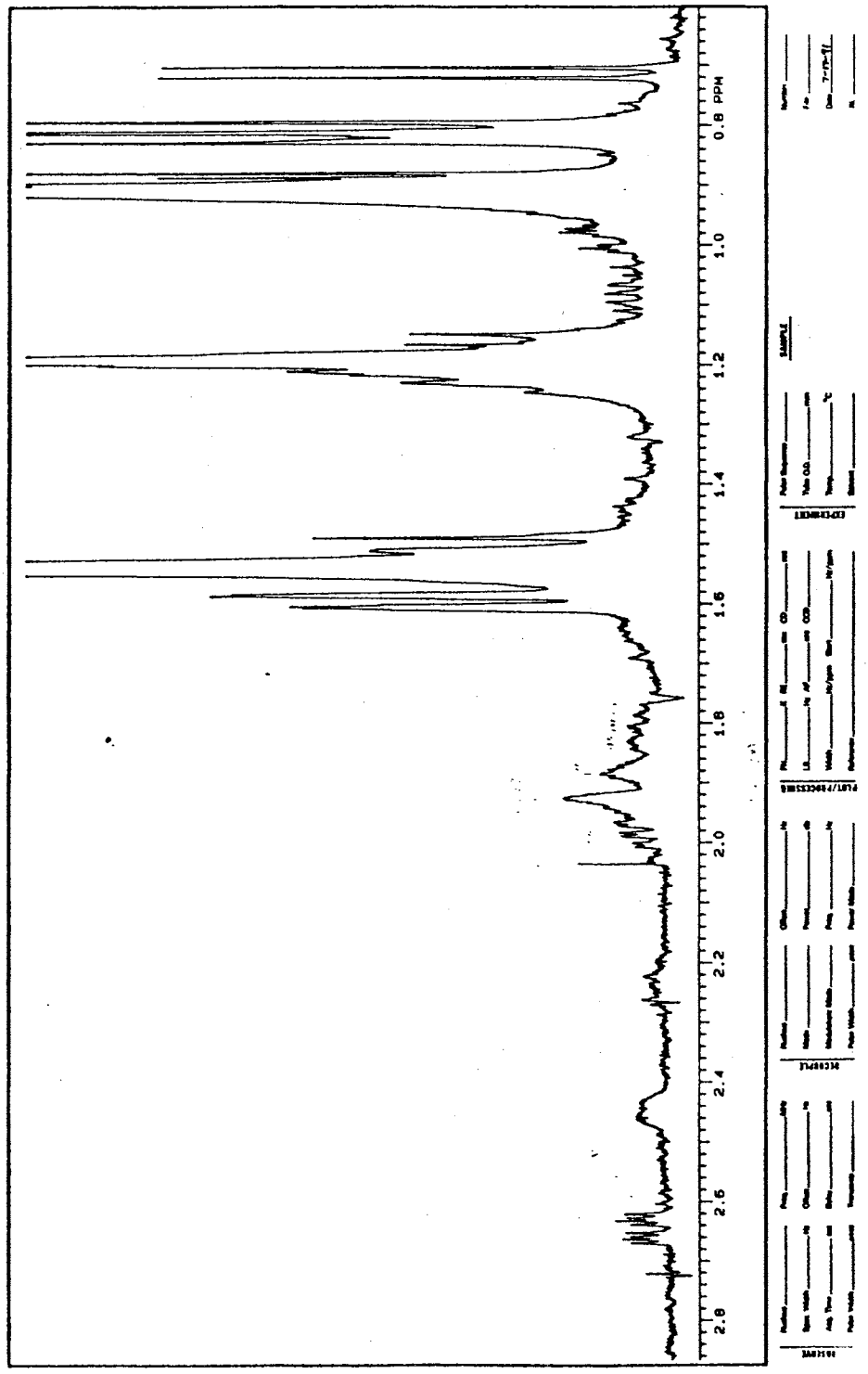

2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8 PPM

Figure 7. The total ion current chromatogram of

a) the hexane extract of *Xcm*-inoculated glandless cotton (WbM*gl*).

b) the hexane extract of cade oil

and

c) 1:1 (v/v) mixture of samples injected to obtain a) and b) above

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-5)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.

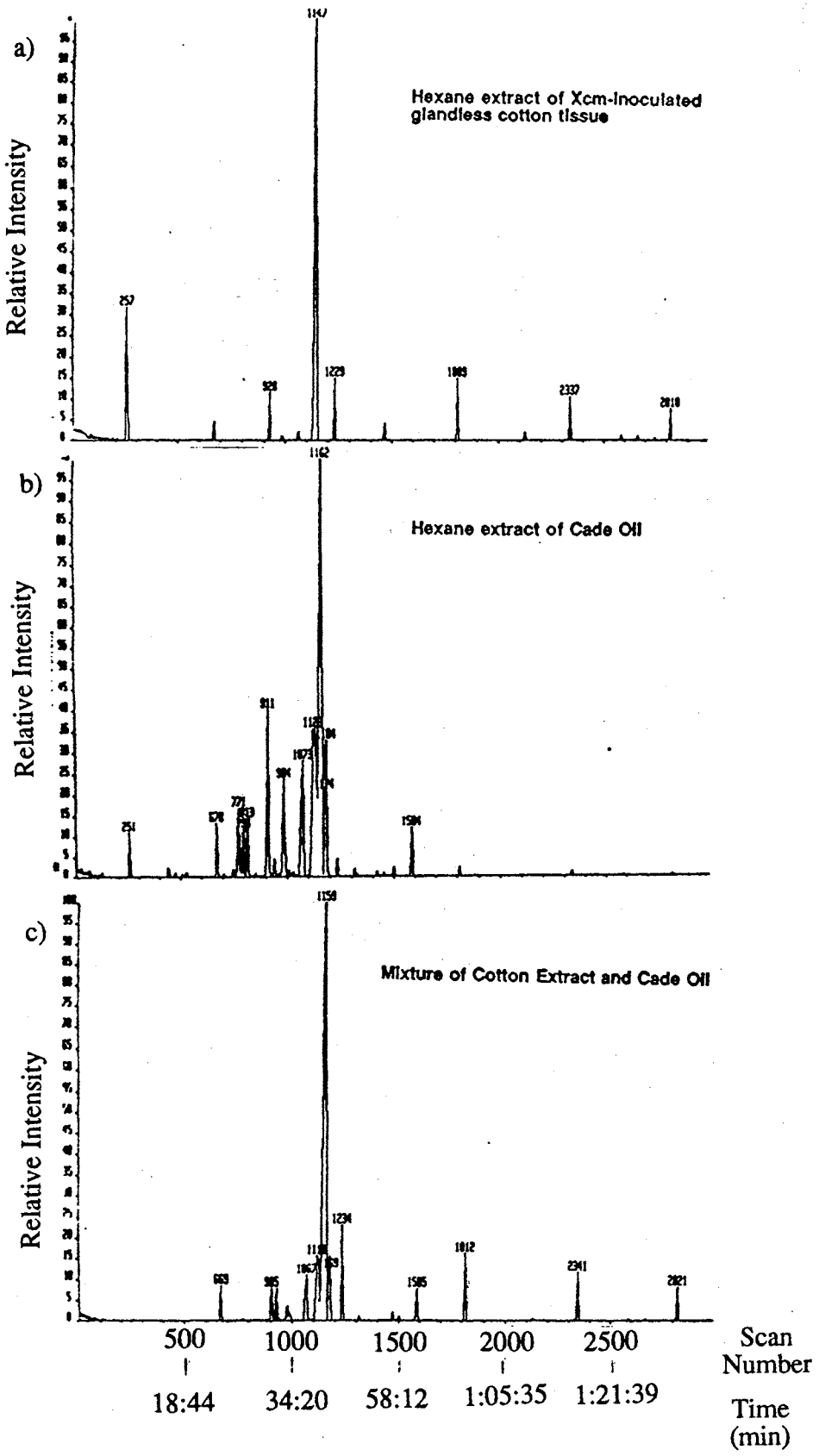

a)
Hexane extract of Xcm-Inoculated glandless cotton tissue
Relative Intensity
b)
Hexane extract of Cade Oil
Relative Intensity
c)
Mixture of Cotton Extract and Cade Oil
Relative Intensity
500
1000
1500
2000
2500
Scan Number
18:44
34:20
58:12
1:05:35
1:21:39
Time (min)

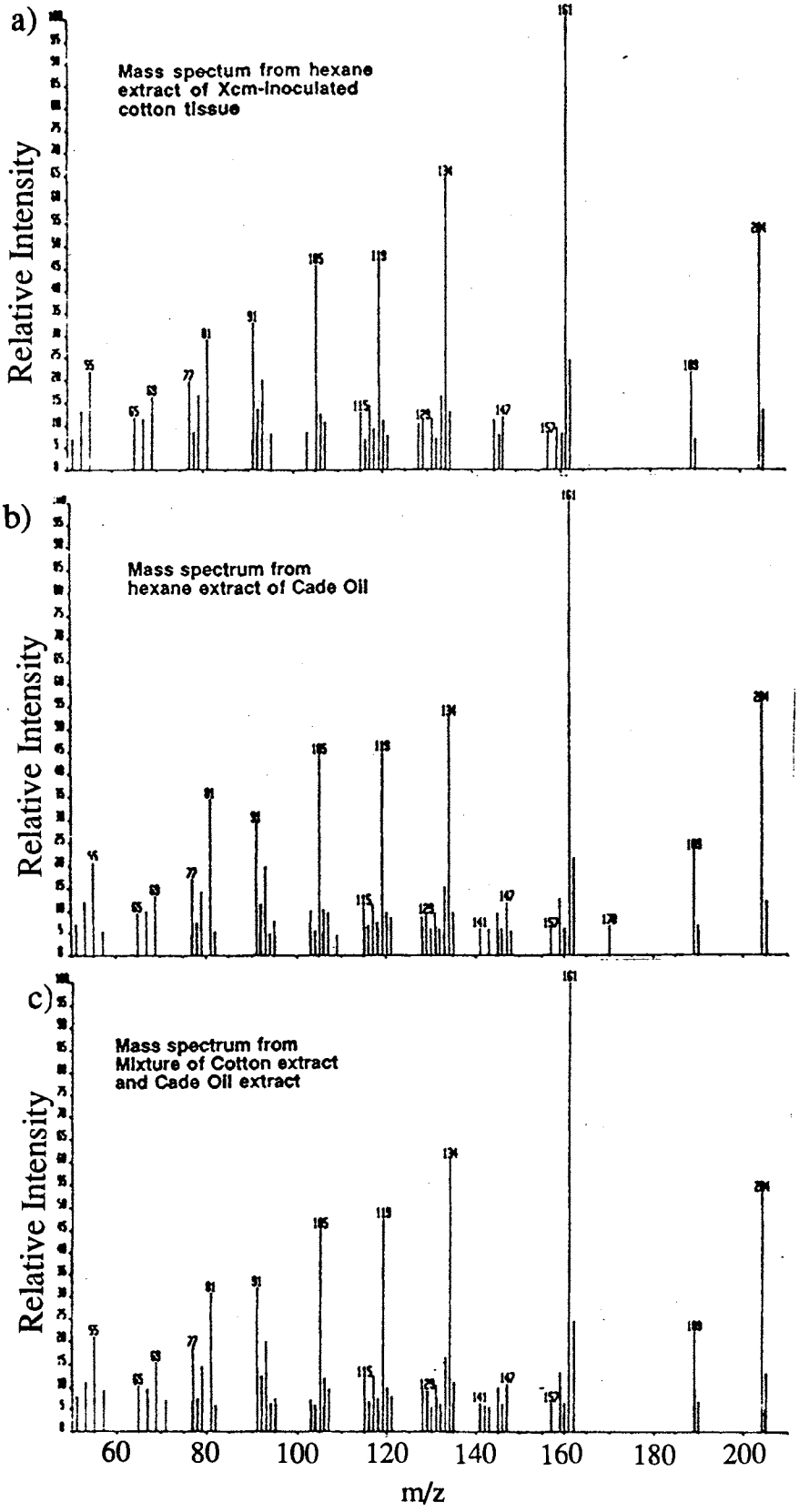
Figure 8. EI mass spectrum of:

a) the experimentally obtained infection-induced hydrocarbon in *Xcm*-inoculated glandless cotton (WbM*gl*) cotyledons.

b) the hexane extract of cade oil (suspected δ-cadinene)

and

c) 1:1 (v/v) mixture of a) and b) above.


a)
Mass spectum from hexane extract of Xcm-inoculated cotton tissue
Relative Intensity
b)
Mass spectrum from hexane extract of Cade Oil
Relative Intensity
c)
Mass spectrum from Mixture of Cotton extract and Cade Oil extract
Relative Intensity
60
80
100
120
140
160
180
200
m/z

of cotton tissue (<2% of *Xcm*-inoculated tissue content) could be detected by GC-FID analysis.

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

Homogenates of the *Xcm*-inoculated WbM*gl* cotton tissues which accumulated δ-cadinene were able to catalyze conversion of [1-^{3}H]FPP to a predominant radioactive product displaying chromatographic characteristics of a 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 tissues co-chromatographed in both analytical-scale sub-ambient temperature normal phase [23] and ambient temperature tandem column [24] reversed phase HPLC systems (Figures 9-13). Additionally, the radioactive product co-chromatographed with the cade oil δ-cadinene in both analytical-scale HPLC systems. These findings support the conclusion that the radioactive product generated by the cell-free reaction is [^{3}H]δ-cadinene. Catalytic rates by the homogenates were in the range of 1-10 nmoles hr^{-1} mg $protein^{-1}$

Further support for identification of the cell-free reaction product as δ-cadinene was given by results from reactions utilizing non-radioactive FPP as substrate. The cell-free reactions were conducted with controls [25] which assured that all measured biosynthesis was derived only from the action of enzyme(s) on added FPP. Endogenous terpenoid compounds presumably were removed from the enzyme preparation by routine treatment with XAD-4 resin [25]. As a result of the XAD-4 resin treatment, the GC-EIMS analysis of a hexane extract of a nonincubated aliquot of the enzyme preparation revealed the absence of endogenous δ-cadinene or other sesquiterpenes. Analysis of boiled enzyme preparation incubated with FPP showed that there was no conversion of FPP to δ-cadinene by a heat-denatured enzyme solution. Analysis of a hexane extract of the enzyme

Figure 9. Normal phase chromatography of non-radioactive, infection-induced component and product of conversion of [1-^{3}H]-FPP by cell-free reaction catalyzed by homogenate of *Xcm*-inoculated glandless cotton cotyledons.

System: Sub-ambient temperature; four μPorasil HPLC silica gel columns in tandem eluted at 1 ml min^{-1} with 100% hexane.

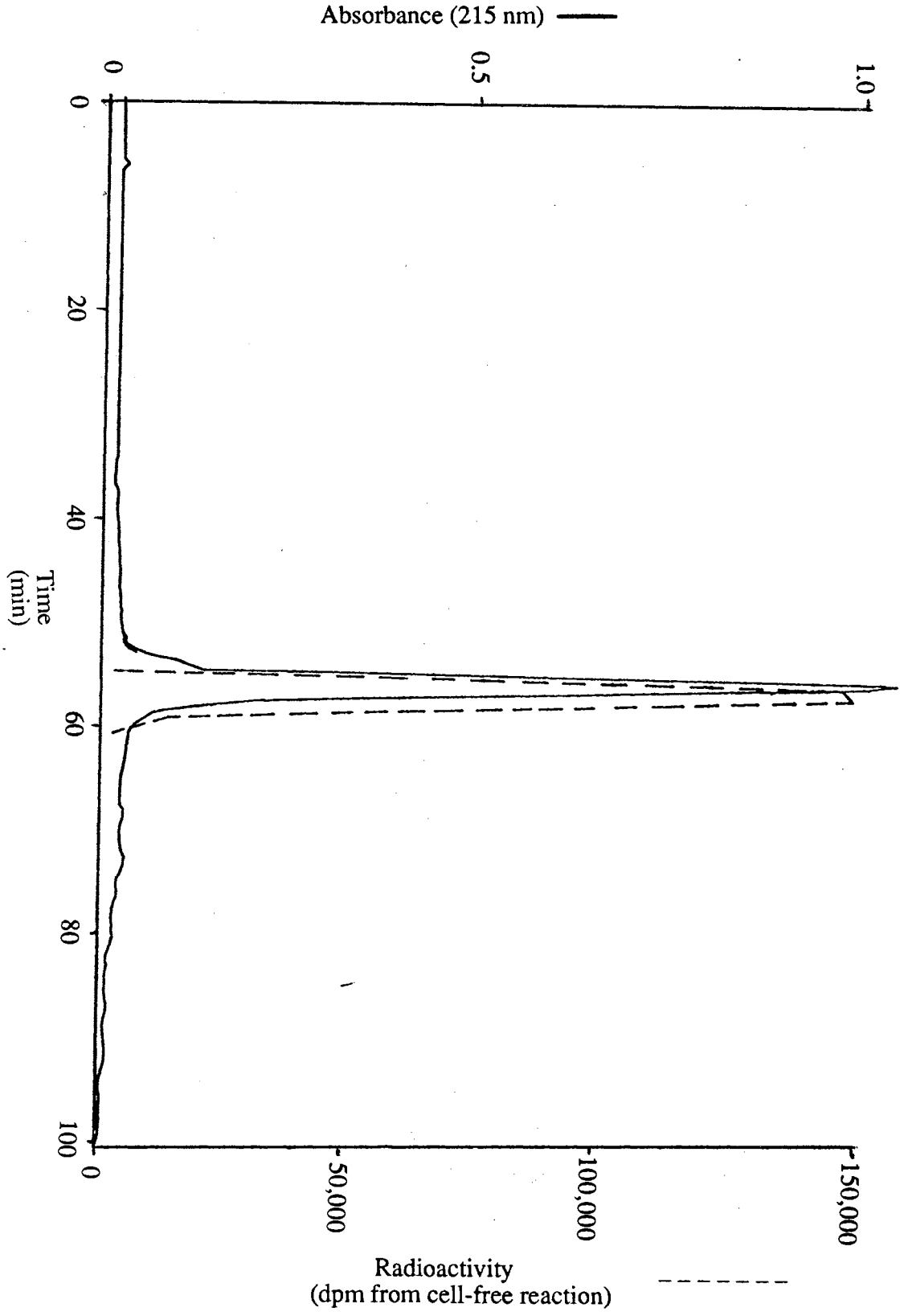

Absorbance (215 nm)
0
0.5
1.0
0
20
40
60
80
100
Time
(min)
0
50,000
100,000
150,000
Radioactivity
(dpm from cell-free reaction)

Figure 10. First normal phase HPLC co-chromatography: validation of co-chromatography in normal phase HPLC system of infection-induced compound accumulated in *Xcm*-inoculated WbM*gl* cotton tissues with major radioactive product in hexane extract from cell-free reaction.

Purified infection-induced compound (δ-cadinene) was co-injectedwith hexane extract from a cell-free reaction which converted[1-^{3}H]FPP to a predominant olefinic product.

Radioactivity of the eluted samples was checked by scintillation counting. To estimate the concentration of olefins in the separated fractions, a Beers Law plot was generated on the Shimadzu UV-Vis spectrophotometer by obtaining absorbance readings from solutions of varying concentrations of β-caryophyllene (Fluka) at 220 nm. The experimental log ε of β-caryophyllene at 220 nm was 3.66 (A_{220nm} = 0.222 for a solution of 10 μg β-caryophyllene (Fluka) per ml hexane. The UV absobance (A_{220nm}) of the HPLC-separated fractions was then obtained, and the approximate concentration of the total olefin (including putative δ-cadinene) was generated by reference to the Beers Law plot of β-caryophyllene.

Instrumentation:

Sub-ambient temperature (approximately -30°C); Waters 6000 pump connected to four μPorasil HPLC silica gel columns in tandem. Sample was eluted at 1ml min^{-1} with 100% hexane.

Shimadzu UV-160 UV-Vis recording spectrophotometer

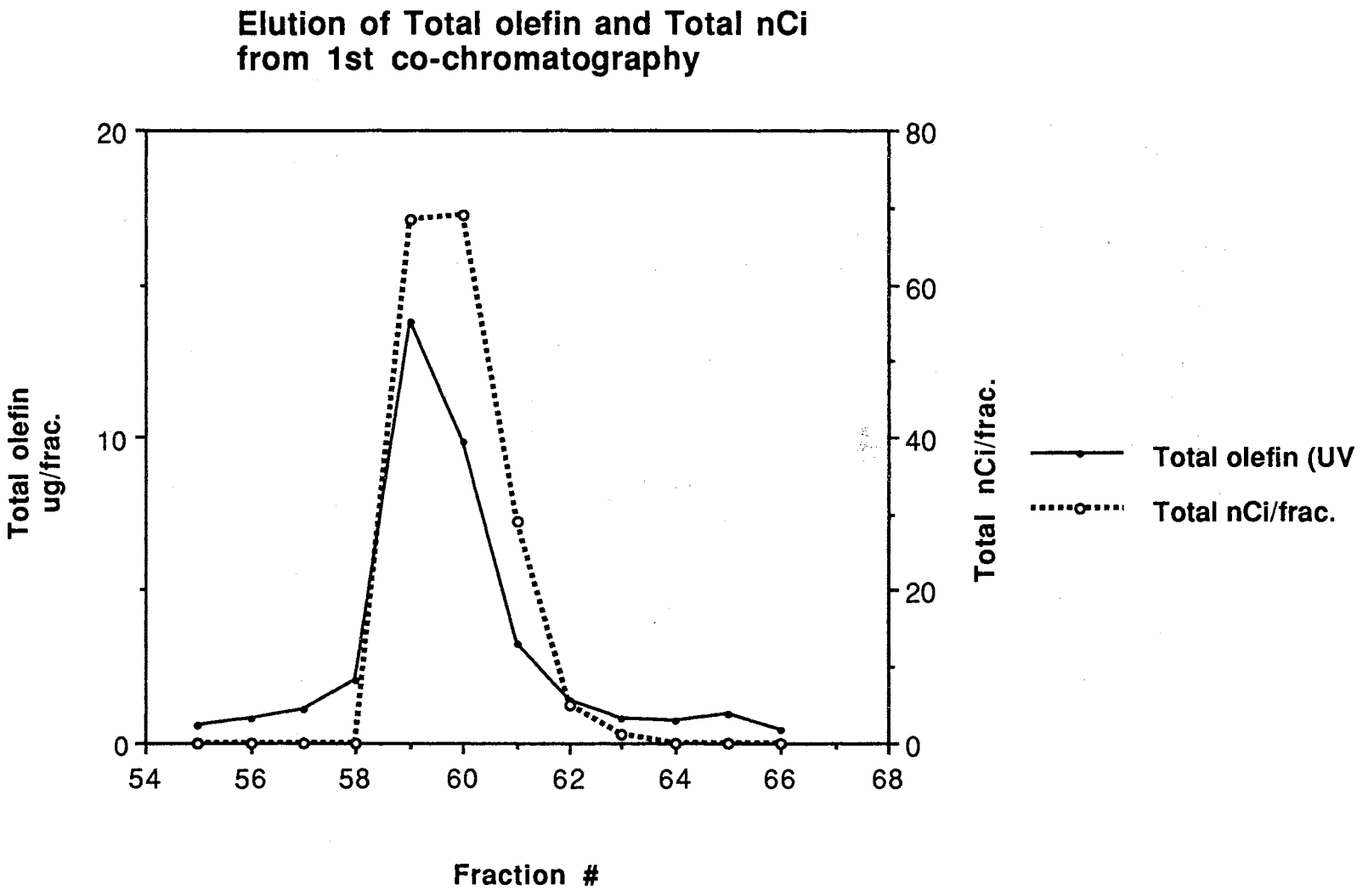

Elution of Total olefin and Total nCi
from 1st co-chromatography
Total olefin
ug/frac.
20
10
0
80
60
40
20
0
Total nCi/frac.
54
56
58
60
62
64
66
68
Fraction #
Total olefin (UV
Total nCi/frac.

Figure 11. Validation of co-chromatography in reversed phase HPLC system of infection-induced compound accumulated in *Xcm*-inoculated WbM*gl* cotton tissues with major radioactive product in hexane extract from cell-free reaction .

Fractions collected from normal phase co-chromatography in Figure 10 were pooled and concentrated, then injected into HPLC system.

Radioactivity of the eluted samples was checked by scintillation counting. To estimate the concentration of olefins in the separated fractions, a Beers Law plot was generated on the Shimadzu UV-Vis spectrophotometer by obtaining absorbance readings from solutions of varying concentrations of β-caryophyllene (Fluka) at 220 nm. The experimental log ε of b-caryophyllene at 220 nm was 3.66 (A_{220nm} = 0.222 for a 10 μg β-caryophyllene (Fluka) per ml hexane. The UV absobance (A_{220nm}) of the HPLC-separated fractions was then obtained, and the approximate concentration of the total olefin (including putative δ-cadinene) was generated by reference to the Beers Law plot of β-caryophyllene.

Instrumentation:

Waters 6000 HPLC pump, Valco injector, tandem Whatman Partisphere and E. Merck octadecylsilane columns eluted at 1ml min^{-1} with 85:15 (v:v) acetonitrile:H_2O.

Shimadzu UV-160 UV-Vis recording spectrophotometer

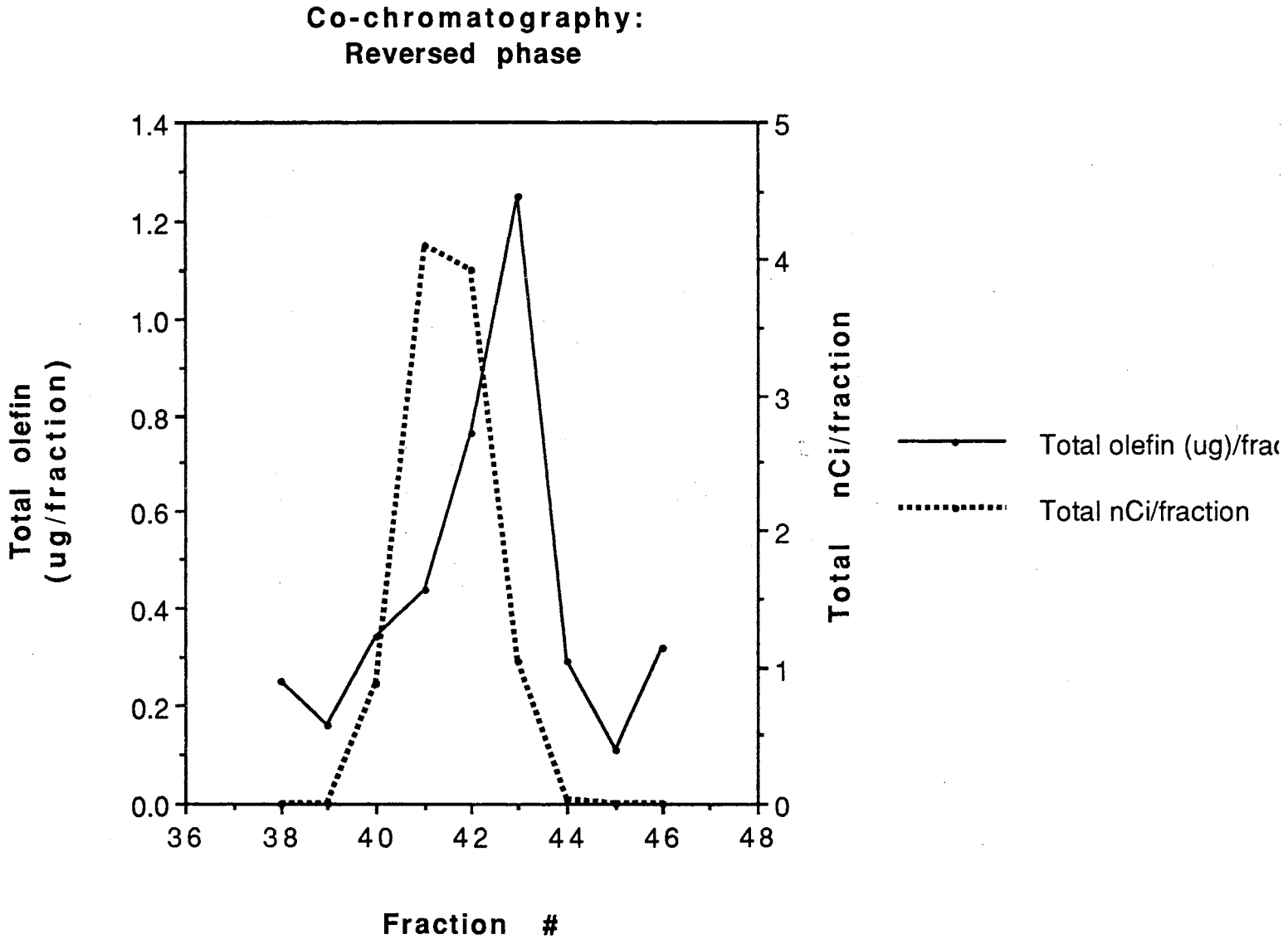

Co-chromatography:
Reversed phase
Total olefin (ug/fraction)
Total nCi/fraction
Fraction #
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5
4
3
2
1
0
36
38
40
42
44
46
48
Total olefin (ug)/frac
Total nCi/fraction

Figure 12. Second normal phase HPLC co-chromatography: validation of co-chromatography in normal phase HPLC system of infection-induced compound accumulated in *Xcm*-inoculated WbM*gl* cotton tissues with major radioactive product in hexane extract from cell-free reaction .

Fractions collected from normal phase co-chromatography in Figure 10 were pooled and concentrated, then injected into HPLC system.

Radioactivity of the eluted samples was checked by scintillation counting. To estimate the concentration of olefins in the separated fractions, a Beers Law plot was generated on the Shimadzu UV-Vis spectrophotometer by obtaining absorbance readings from solutions of varying concentrations of β-caryophyllene (Fluka) at 220 nm. The experimental log ε of b-caryophyllene at 220 nm was 3.66 (A_{220nm} = 0.222 for a 10 μg β-caryophyllene (Fluka) per ml hexane. The UV absobance (A_{220nm}) of the HPLC-separated fractions was then obtained, and the approximate concentration of the total olefin (including putative δ-cadinene) was generated by reference to the Beers Law plot of β-caryophyllene.

Instrumentation:

Sub-ambient temperature (approximately -30°C); Waters 6000 pump connected to four μPorasil HPLC silica gel columns in tandem. Sample was eluted at 1ml min^{-1} with 100% hexane.

Shimadzu UV-160 UV-Vis recording spectrophotometer

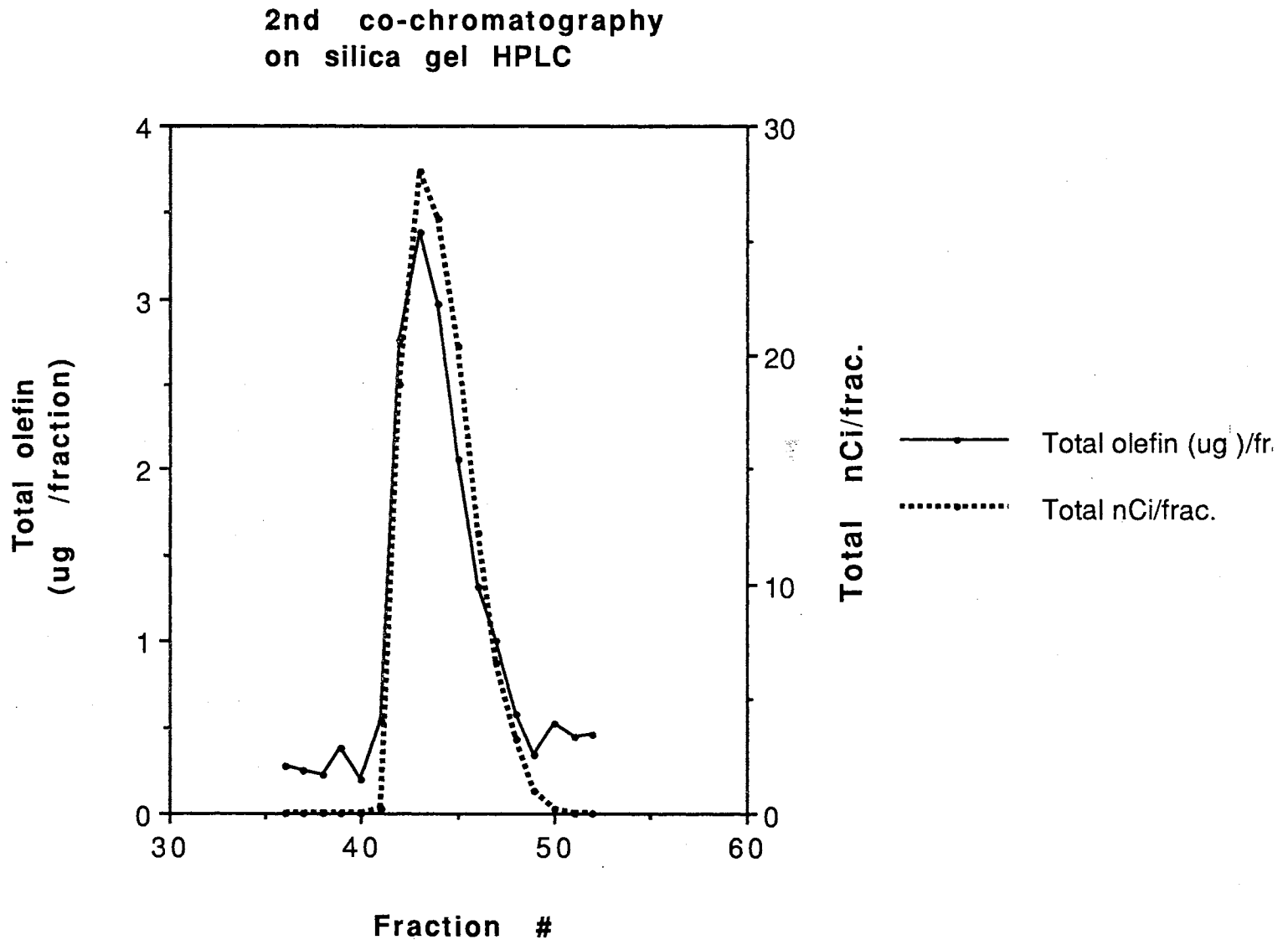

2nd co-chromatography
on silica gel HPLC
Total olefin
(ug /fraction)
Total nCi/frac.
Fraction #
0
1
2
3
4
0
10
20
30
30
40
50
60
Total olefin (ug)/fr
Total nCi/frac.

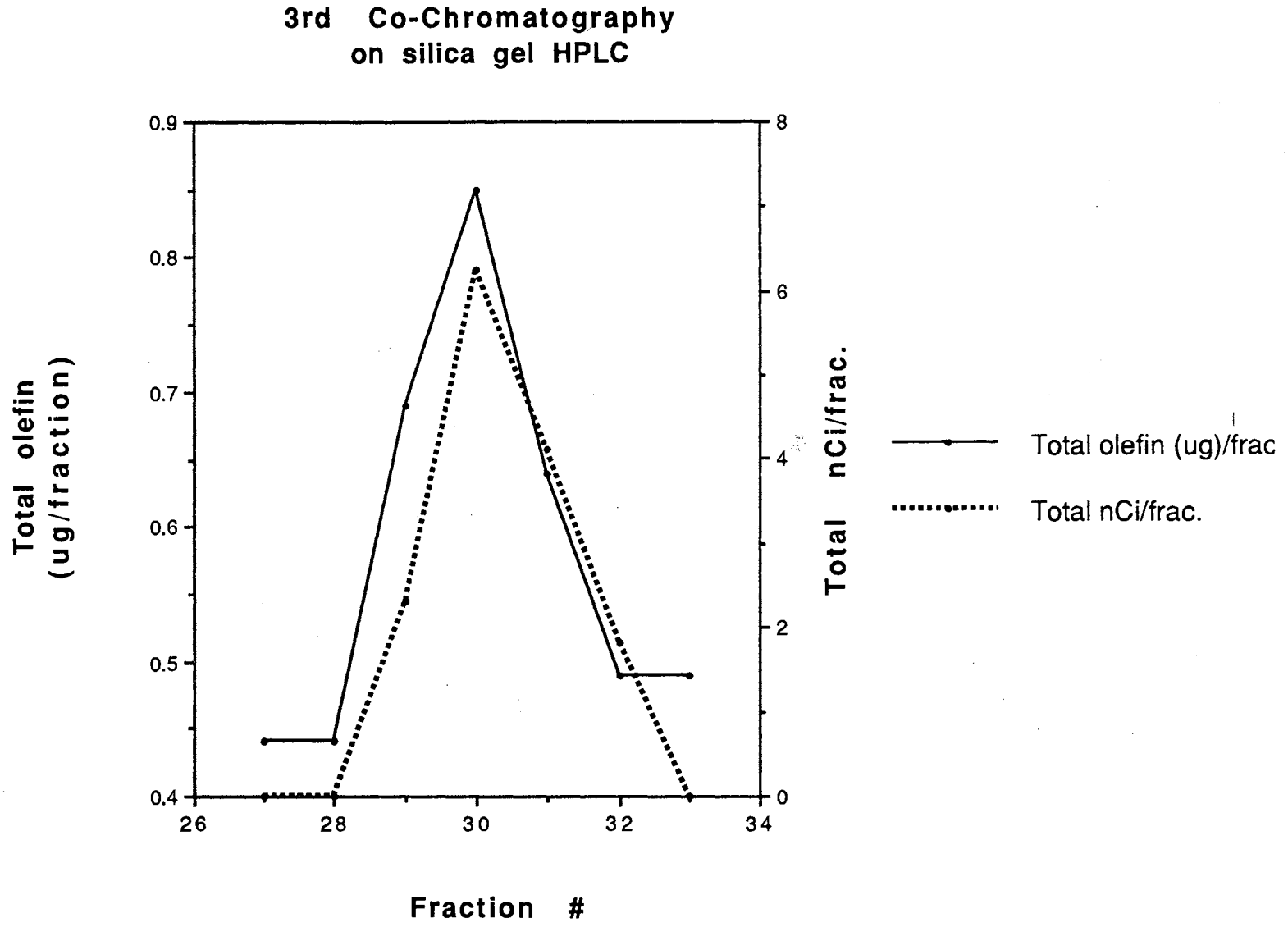
Figure 13. Third normal phase HPLC co-chromatography: validation of co-chromatography in normal phase HPLC system of infection-induced compound accumulated in *Xcm*-inoculated WbM*gl* cotton tissues with major radioactive product in hexane extract from cell-free reaction .

Fractions collected from normal phase co-chromatography in Figure 12 were pooled and concentrated, then injected into HPLC system.

Radioactivity of the eluted samples was checked by scintillation counting. To estimate the concentration of olefins in the separated fractions, a Beers Law plot was generated on the Shimadzu UV-Vis spectrophotometer by obtaining absorbance readings from solutions of varying concentrations of β-caryophyllene (Fluka) at 220 nm. The experimental log ε of b-caryophyllene at 220 nm was 3.66 (A_{220nm} = 0.222 for a 10 μg β-caryophyllene (Fluka) per ml hexane. The UV absobance (A_{220nm}) of the HPLC-separated fractions was then obtained, and the approximate concentration of the total olefin (including putative δ-cadinene) was generated by reference to the Beers Law plot of β-caryophyllene.

Instrumentation:
Sub-ambient temperature (approximately -30°C); Waters 6000 pump connected to four μPorasil HPLC silica gel columns in tandem. Sample was eluted at 1ml min^{-1} with 100% hexane.

Shimadzu UV-160 UV-Vis recording spectrophotometer


3rd Co-Chromatography
on silica gel HPLC
Total olefin (ug/fraction)
Total nCi/frac.
Fraction #
0.9
0.8
0.7
0.6
0.5
0.4
8
6
4
2
0
26
28
30
32
34
Total olefin (ug)/frac
Total nCi/frac.

preparation incubated without substrate revealed that no δ-cadinene was produced in the absence of added FPP. The cell-free preparations converted non-radioactive farnesyl pyrophosphate into a component which matched the GC-EIMS characteristics of the cade oil δ-cadinene; co-injection of *ca* equal amounts of the enzymatic product and the δ-cadinene from cade oil resulted in co-chromatography as revealed by a single homogeneous peak exhibiting a mass spectrum that matched that of δ-cadinene (Figures 14-17). GC-EIMS analysis also revealed minor amounts of a component tentatively identified as α-cadinene (**10**). The identification of α-cadinene was based upon a published mass spectrum [10]. Although no published retention value for α-cadinene was found for the intermediate polarity GC phase we employed, the tentatively identified α-cadinene eluted after δ-cadinene; similar elution order was seen for chromatography on polar Carbowax 20M GC columns [26]. Farnesol (**11**) was also present, presumably arising from action of phosphohydrolases [27] on the added FPP substrate. It was found that the farnesol found in the cell-free reaction exhibited a mass spectrum and retention on gas chromatographic capillary column comparable to that of commercially available *trans, trans*-farnesol (Aldrich); *trans*-nerolidol (Aldrich) exhibited significantly shorter retention time on the capillary column.

It was found that the cade oil δ-cadinene (chromatographed with standards in Figure 18) co-chromatographed with the product of cell-free reactions utilizing non-radioactive FPP with cade oil δ-cadinene in a reversed phase HPLC system (Figure 19). When the product of cell-free reactions generated from [1-^{3}H]-FPP was added to the the mixture chromatographed in Figure 19, the cade oil δ-cadinene, the non-radioactive cell-free reaction product and the radioactive cell-free product co-chromatographed (Figure 20).

The evidence supports the conclusion that the non-radioactive hydrocarbon accumulated in the inoculated cotton tissues, the radioactive product generated by the radioactive cell-free reaction and the non-radioactive product generated by the non-radioactive cell-free reaction are all identical to δ-cadinene. Because δ-cadinene is the most

Figure 14. The total ion current chromatogram of the hexane extract from cell-free reaction catalyzing conversion of non-radioactive farnesyl pyrophosphate.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with
DB-17 methyl capillary gas chromatography column
(0.25 mm i. d. x 30 m; J&W Scientific DB-17)
and
VG TS-250 mass spectrometer.
Oven gradient: 2 min hold at 85° then a gradient of 85° to 170° at 3° min^{-1}, followed by a second gradient from 170° to 270° at 10° min^{-1}.

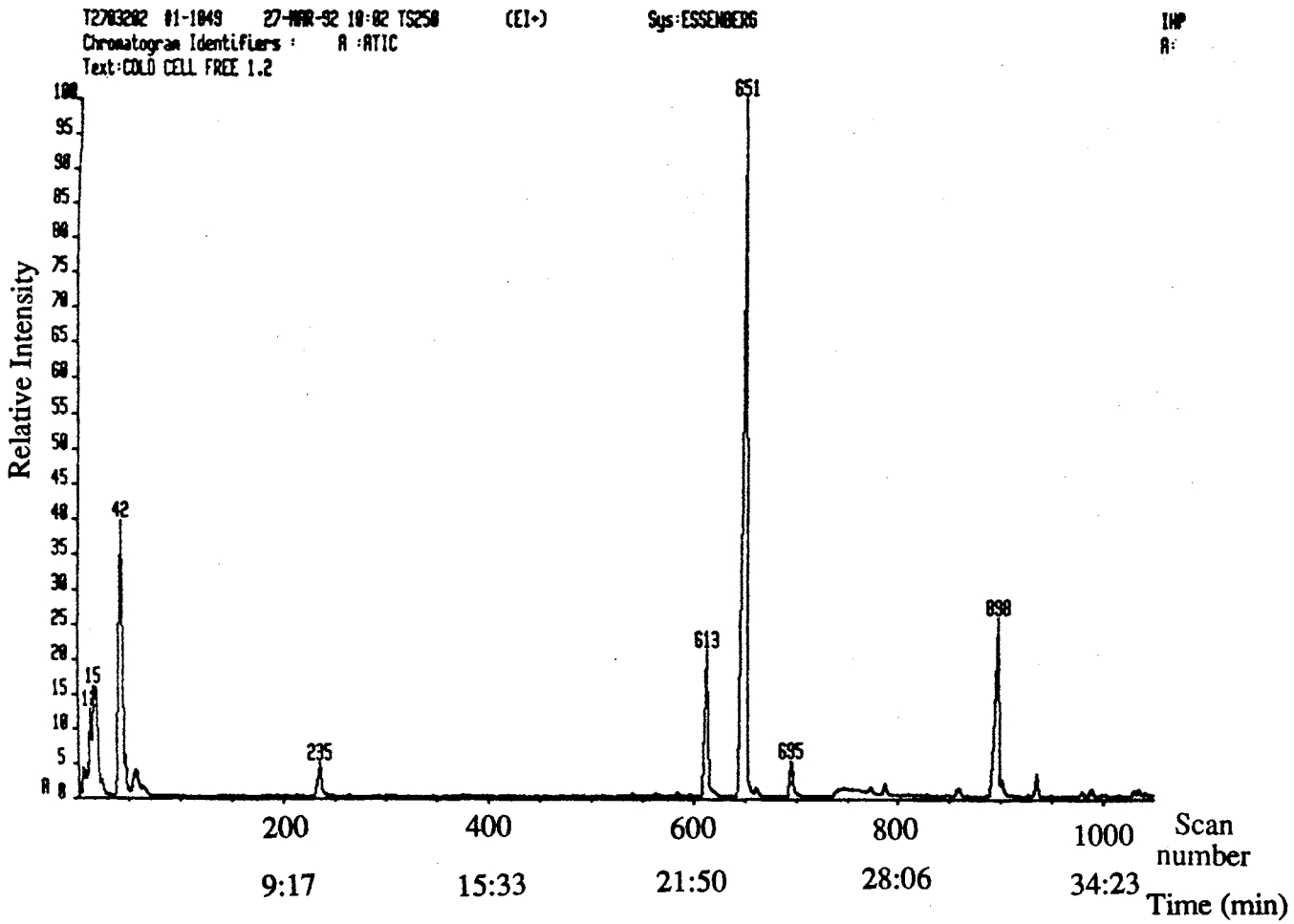

T2703202 #1-1049 27-MAR-92 10:02 TS250 (EI+) Sys:ESSENBERG IHP
Chromatogram Identifiers : A :ATIC
Text:COLD CELL FREE 1.2
Relative Intensity
42
15
235
613
651
695
898
200
400
600
800
1000
Scan number
9:17
15:33
21:50
28:06
34:23
Time (min)

Figure 15. EI mass spectrum of apparent δ-cadinene (Scan number 613 in Figure 14) generated by cell-free reaction utilizing non-radioactive FPP.

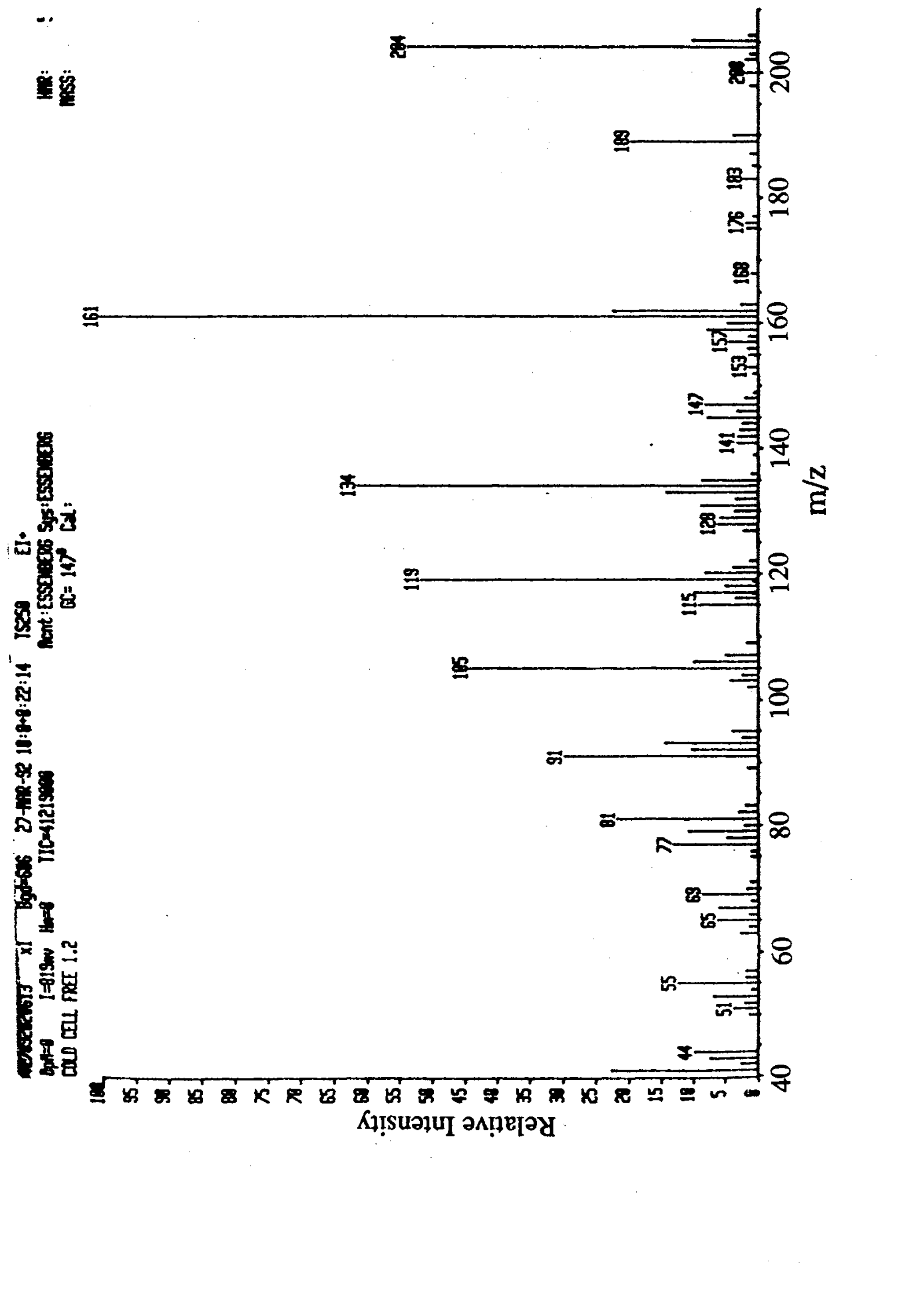

Relative Intensity
m/z

Figure 16. The total ion current chromatogram of the hexane extract from cell-free reaction catalyzing conversion of non-radioactive farnesyl pyrophosphate mixed with a spike of cade oil δ-cadinene.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with
DB-17 methyl capillary gas chromatography column
(0.25 mm i. d. x 30 m; J&W Scientific DB-1)
and
VG TS-250 mass spectrometer.
Oven gradient: 2 min hold at 85° then a gradient of 85° to 170° at 3° min^{-1}, followed by a second gradient from 170° to 270° at 10° min^{-1}.

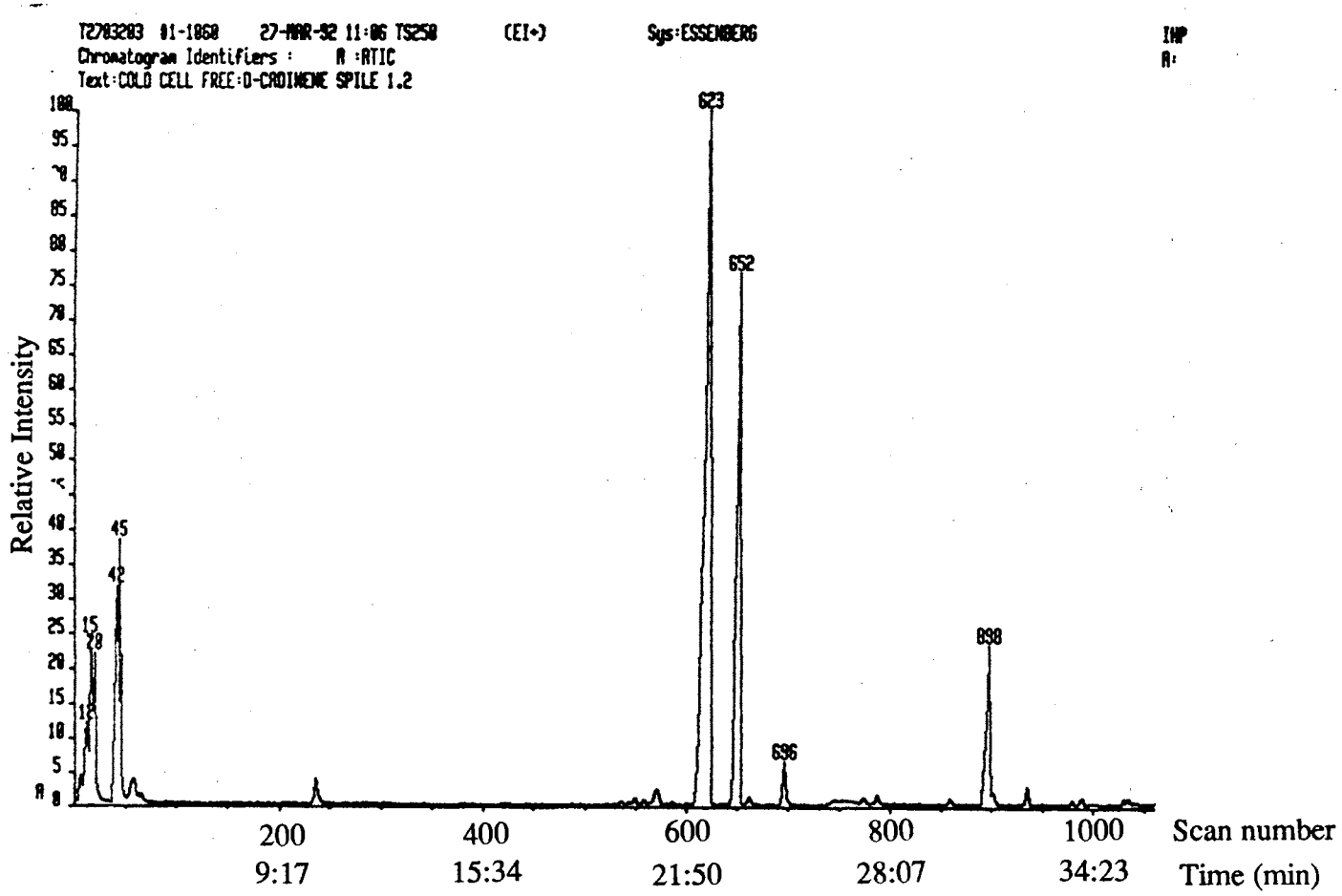

T2703203 01-1868 27-MAR-92 11:06 TS250 (EI+) Sys:ESSENBERG IHP
Chromatogram Identifiers : A :RTIC A:
Text:COLD CELL FREE:D-CADINENE SPILE 1.2
Relative Intensity
623
652
45
42
15
28
698
696
200
400
600
800
1000
Scan number
9:17
15:34
21:50
28:07
34:23
Time (min)

Figure 17. EI mass spectrum of apparent δ-cadinene generated by cell-free reaction utilizing non-radioactive FPP mixed with cade oil δ-cadinene (Scan number 623 in Figure 16).

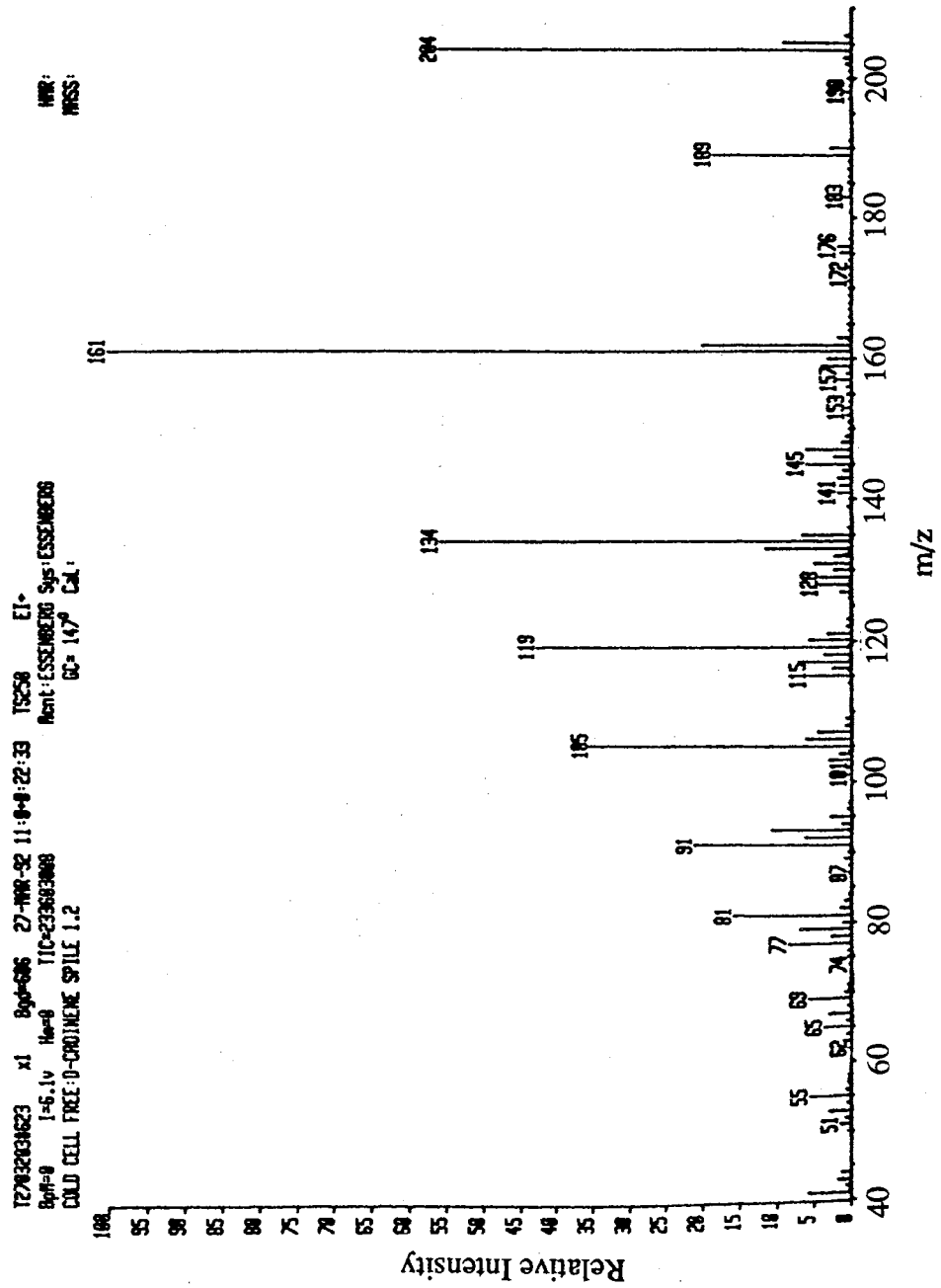

Relative Intensity
m/z

Figure 18. Reversed phase chromatogram of sesquiterpene standards; UV detection at 215 nm. δ-Cadinene is last eluting compound in this system.

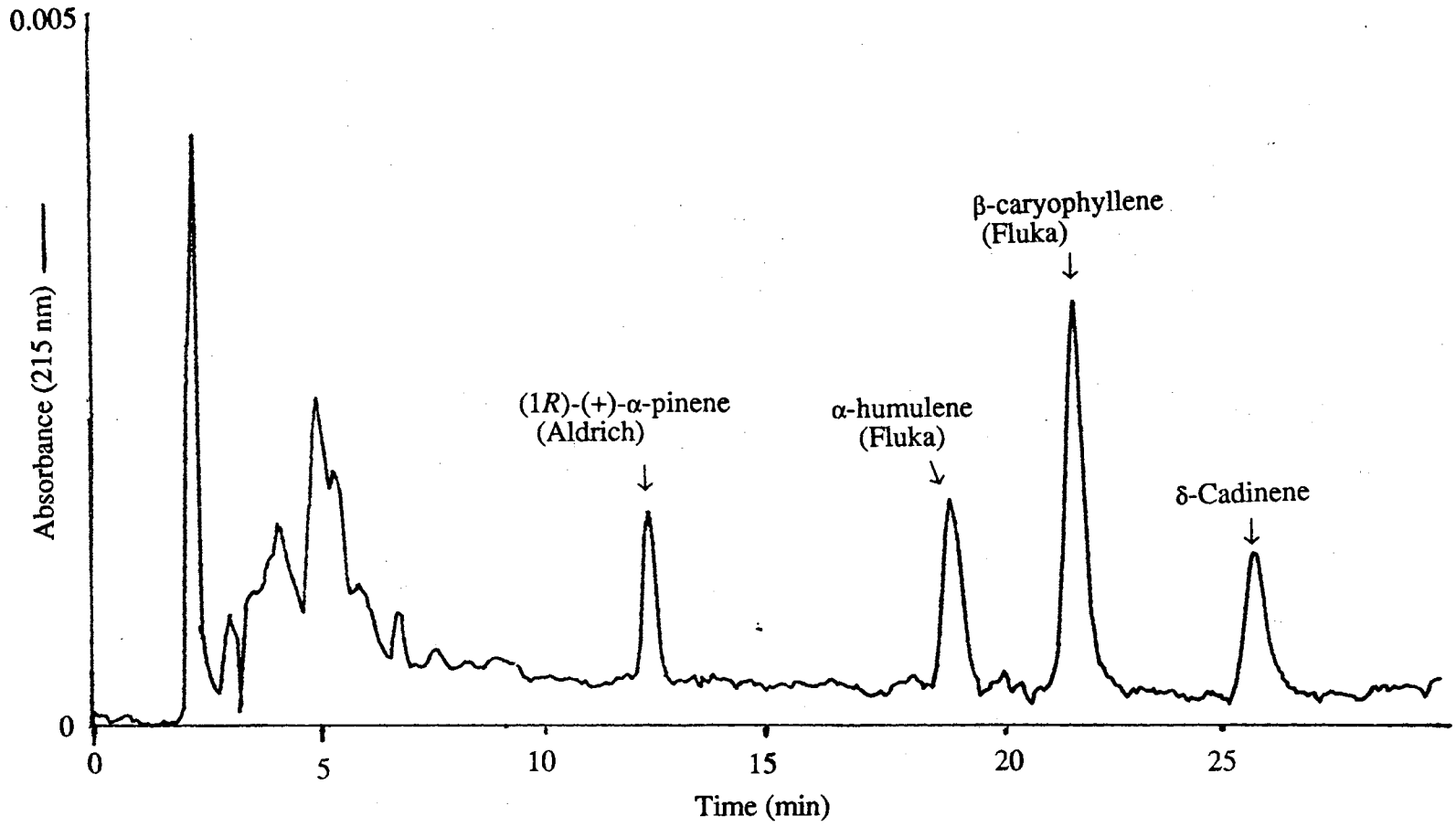

0.005
0
Absorbance (215 nm)
0
5
10
15
20
25
Time (min)
(1R)-(+)-α-pinene
(Aldrich)
α-humulene
(Fluka)
β-caryophyllene
(Fluka)
δ-Cadinene

Figure 19. Reversed phase chromatogram of sesquiterpene standards spiked with δ-cadinene; UV detection at 215 nm.

Product of cell-free reaction utilizing non-radioactive farnesyl pyrophosphate substrate added; peak enhancement seen for δ-cadinene peak (Last eluting peak)

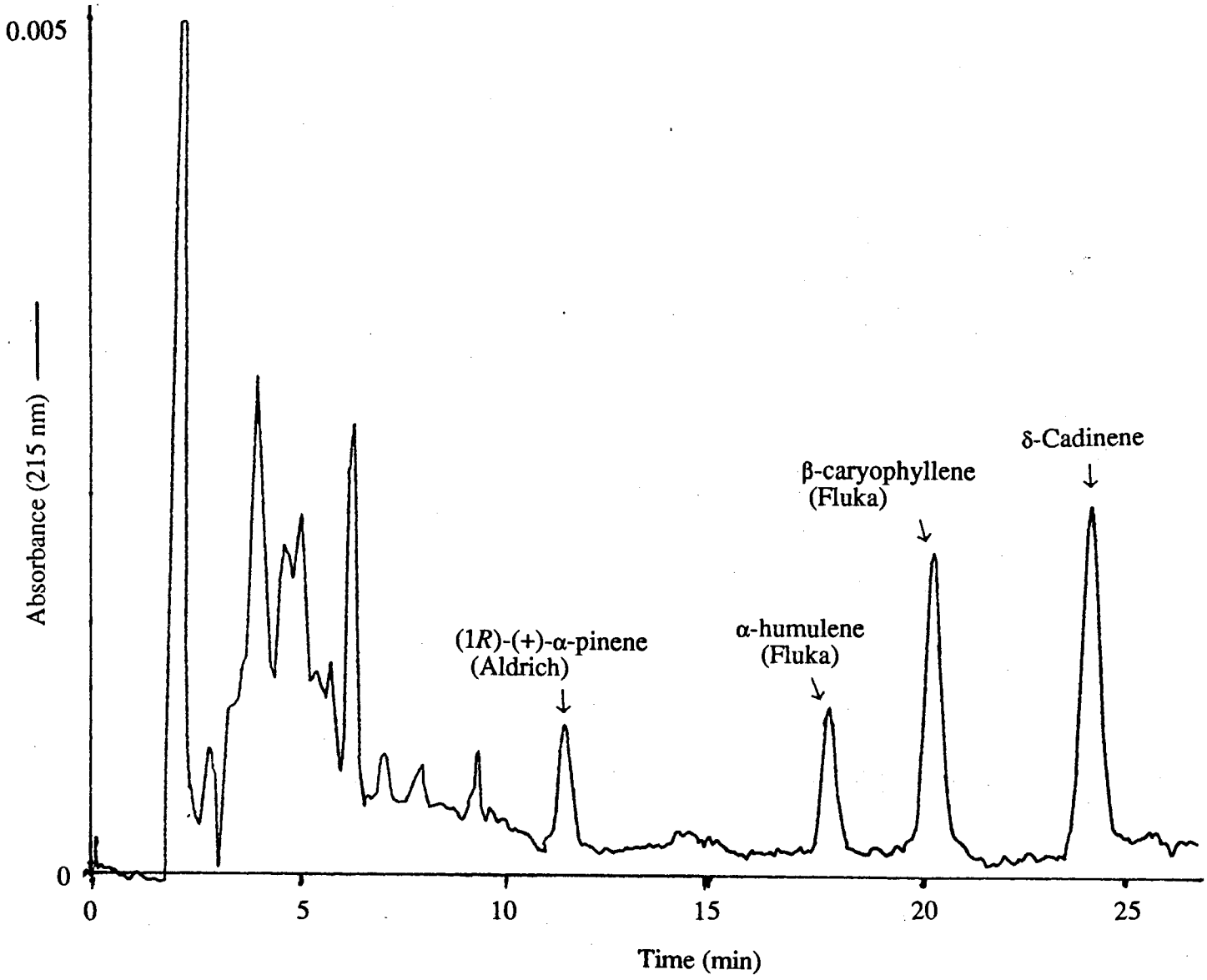
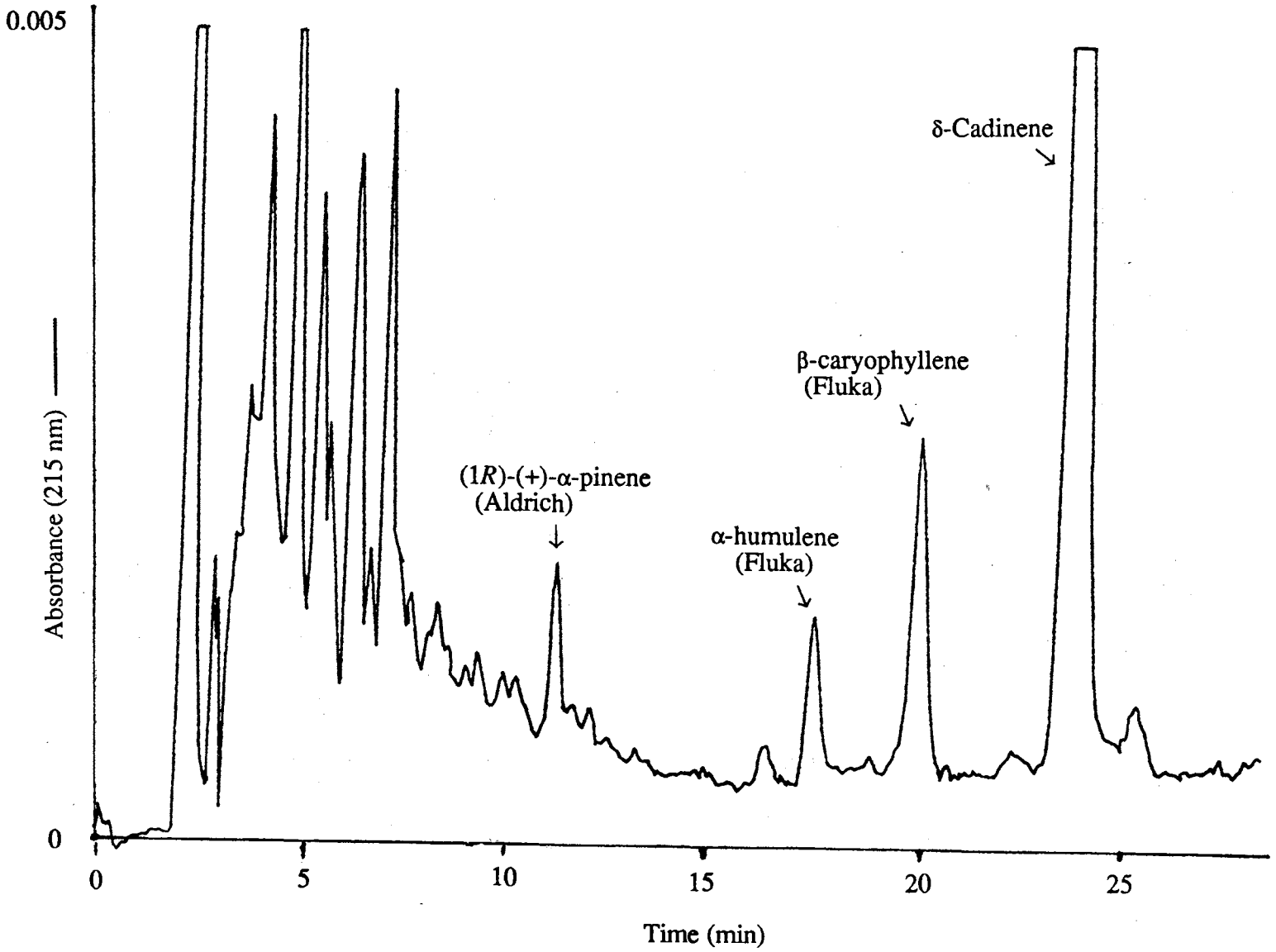

0.005
Absorbance (215 nm)
0
0
5
10
15
20
25
Time (min)
(1R)-(+)-α-pinene
(Aldrich)
α-humulene
(Fluka)
β-caryophyllene
(Fluka)
δ-Cadinene

Figure 20. Reversed phase chromatogram of non-radioactive cell-free reaction product, cade oil δ-cadinene, and radioactive cell-free product. All three compounds co-elute in last peak. UV detection at 215 nm.

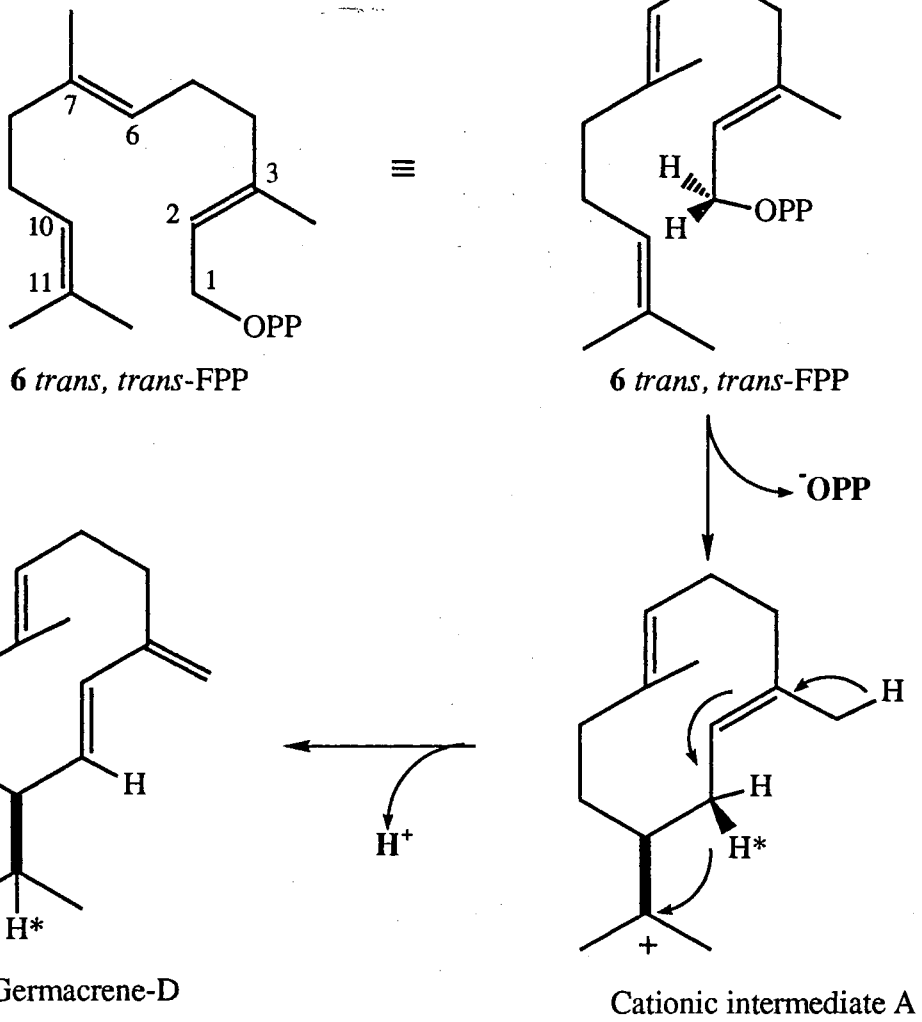

0.005
0
Absorbance (215 nm)
0
5
10
15
20
25
Time (min)
δ-Cadinene
β-caryophyllene
(Fluka)
(1R)-(+)-α-pinene
(Aldrich)
α-humulene
(Fluka)

commonly documented sesquiterpene hydrocarbon produced in healthy glanded cotton tissues that has the cadinane carbon skeleton [5,28,29] of our sesquiterpene phytoalexins [*e.g.*, DHC (**1**)], it is a plausible precursor to the cadinane-type phytoalexins which are prominent in the hypersensitive response of *Xcm*-inoculated cotton plants. This hypothesis is supported by our finding that δ-cadinene is the predominant sesquiterpene hydrocarbon generated in *Xcm*-inoculated glandless plants and in our cell-free reactions, and by the coincidence in time between δ-cadinene and DHC production (Davis, G. D. *et al.*, unpublished results).

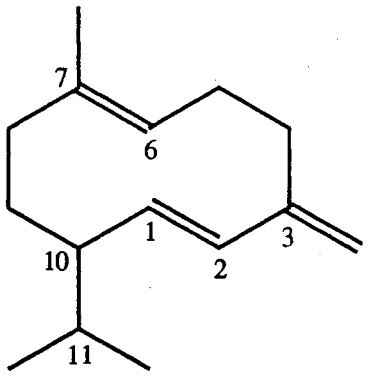
Although it is geometrically possible for *trans, trans*-FPP to cyclize to a ten-membered ring, its 2,3 *trans* double bond would prevent formation of the C-1 to C-6 bond. This problem and two types of solution to it were described by Arigoni [30]. One possible solution involves cyclization of *trans, trans*-FPP to form a ten-membered ring, followed by deprotonation to yield an intermediate, germacrene-D (**12**), which can undergo a conformational change to cisoid configuration at carbons 2 and 3 and, upon reprotonation, cyclize to δ-cadinene (see Figure 21). Germacrene-D and δ-cadinene often occur together in plants [31] and nonenzymic, acid-catalyzed isomerization of germacrene-D to δ-cadinene in high yield has in fact been observed [32]. 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 generating δ-cadinene, nor was it detected by GC-FID in inoculated cotton tissue extracts. If it is an intermediate, it does not accumulate above *ca* 5 ng/g tissue.

The second possible solution to the geometric problem posed by the 2,3 *trans* double bond involves initial isomerization of *trans, trans*-FPP to nerolidyl pyrophosphate (NPP, **13**), followed by rotation of the 2,3 bond to bring C-1 within bonding distance of C-6 [30]. NPP could ionize and cyclize to a pyrophosphate-germacrene cation pair, and progress through a series of cationic intermediates [8] involving a 1,3-hydride shift from C-1 to C-11 and the second cyclization, ending with deprotonation to δ-cadinene (see

Figure 21. Scheme for cyclization via Germacrene-D intermediate.

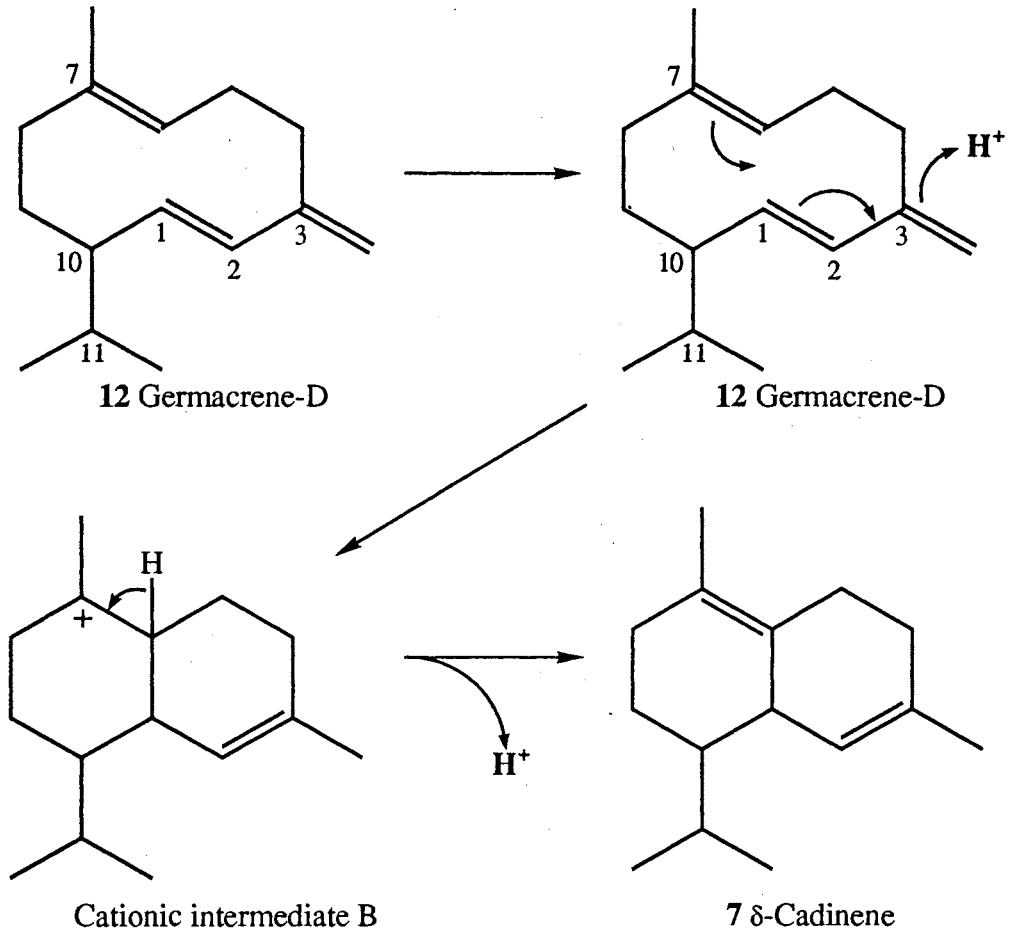


III



12 Germacrene-D

Continue Scheme
on next page.



12 Germacrene-D

12 Germacrene-D

Cationic intermediate B

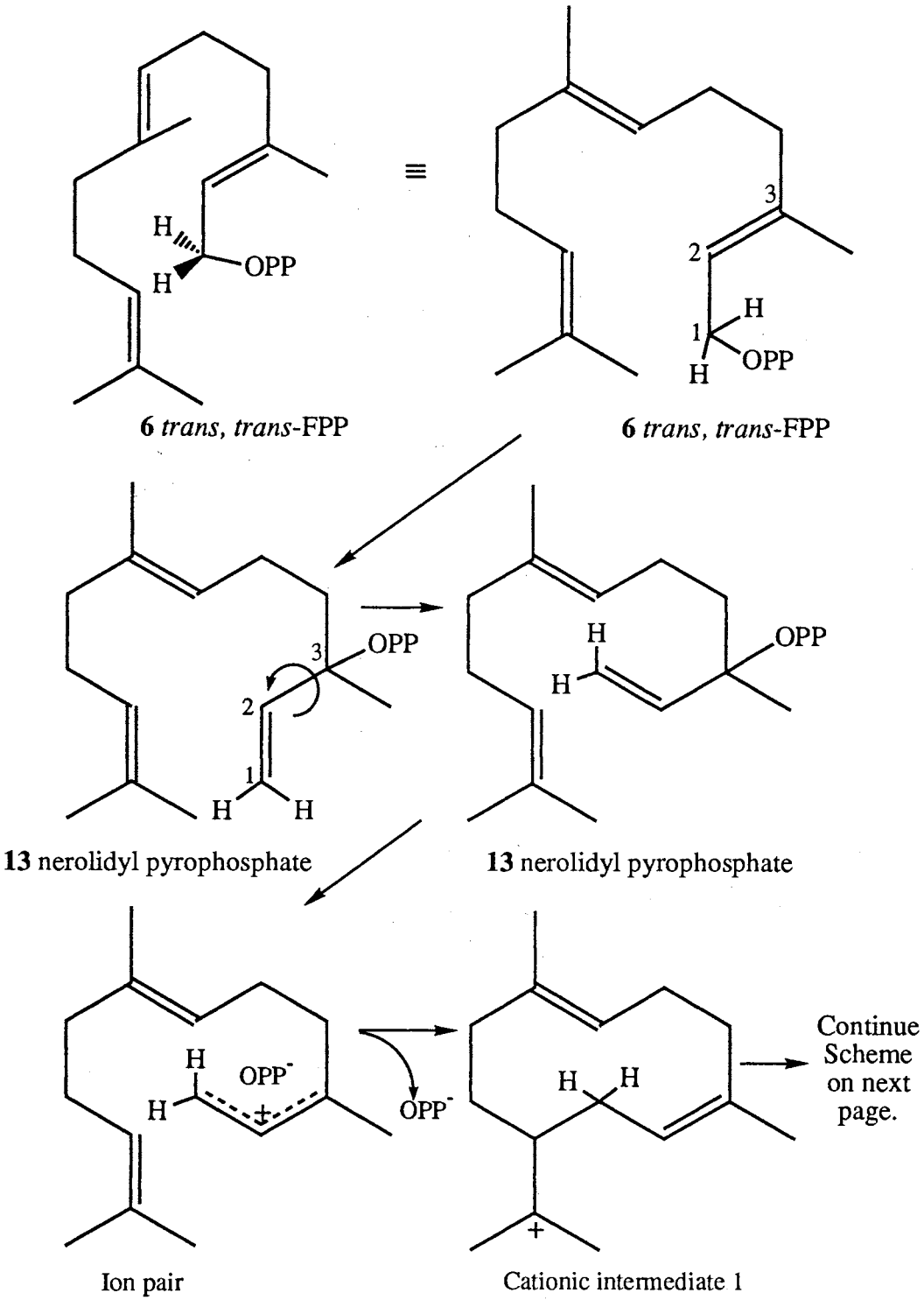
7 δ-Cadinene

Figure 22). There is strong evidence for the intermediacy of NPP in the enzymatic cyclization of *trans, trans*-FPP to trichodiene [33] and for a corresponding ten-carbon tertiary allylic intermediate in monoterpene cyclizations [34]. Therefore, biochemical precedents make NPP a more likely intermediate than germacrene-D. No nerolidol (**14**) was detected in our non-radioactive cell-free reactions, while farnesol was found, presumably generated from FPP by the phosphohydrolases which are common in crude enzyme preparations [27]. However, nerolidol was hardly expected, since Cane *et al.* were unable to detect any release of NPP from trichodiene synthase [33]. The 1,3-tritium transfer that we demonstrated during biosynthesis of DHC and HMC [4] is predicted by both mechanisms.

Identification of the hydrocarbon product from elicitor-treated cotyledon tissues

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. Several species of the bacterial genus *Streptomyces* produce (+)-*epi*-cubenol (cadin-4-ene-1-ol) (**15**) [35], a sesquiterpene structurally similar to δ-cadinene. (Chemical formula **15** represents the correct stereochemistry of (+)-*epi*-cubenol [Cane, D. E., personal communication].) This prompted an experiment to determine if the enzymes of the cotton tissues could generate δ-cadinene in the absence of the bacterial enzymes of *Xcm*. The treatment we chose was injection of a mixture of oligogalacturonides obtained by hydrolysis of the plant cell wall component polygalacturonic acid, because it contains no bacterial products, yet elicits phytoalexin accumulation in cotton tissues [36]. GC-FID and GC-EIMS analysis of hexane extracts of elicitor-treated WbM*gl* cotyledons showed that δ-cadinene was indeed present in these cotyledons at *ca* 60 ng per g of elicitor-treated tissue (*ca* 20% of the level in *Xcm*-inoculated tissue). The gas chromatography of hexane extracts from the elicitor treated tissues is shown in Figure 23; note the peak enhancement

Figure 22. Scheme for cyclization via nerolidyl pyrophosphate.


H
H
OPP
≡
3
2
1
H
H
OPP
6 trans, trans-FPP
6 trans, trans-FPP
3
OPP
2
1
H
H
H
H
OPP
13 nerolidyl pyrophosphate
13 nerolidyl pyrophosphate
H
H
OPP⁻
OPP⁻
H
H
+
Continue Scheme on next page.
Ion pair
Cationic intermediate 1

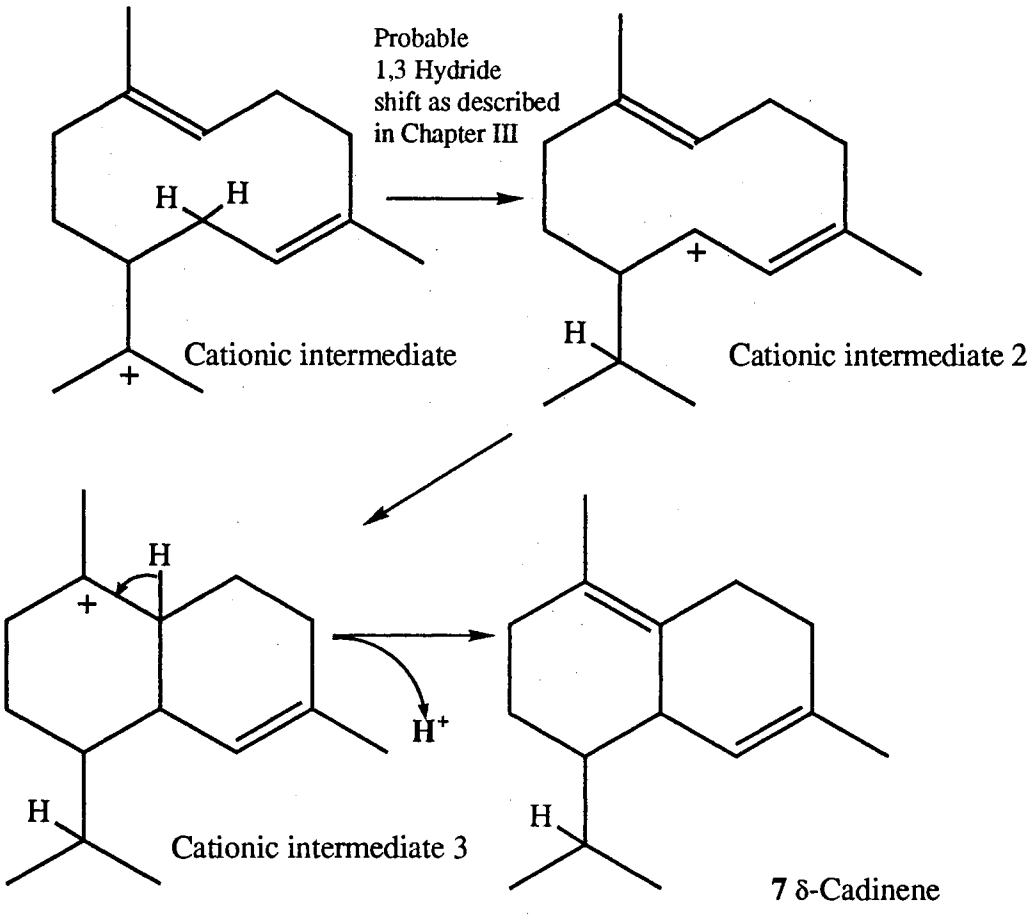
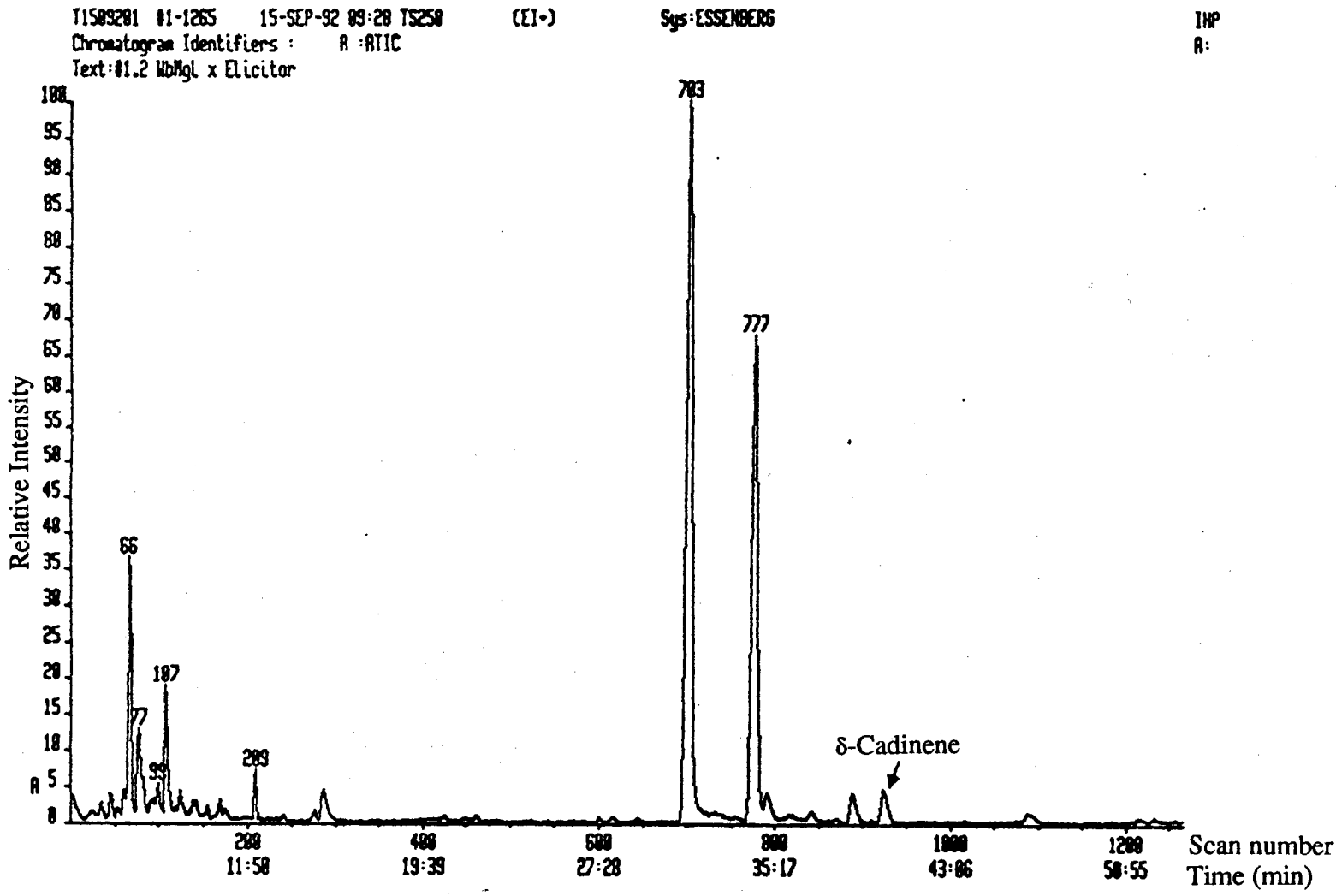

Probable
1,3 Hydride
shift as described
in Chapter III
H
H
+
Cationic intermediate
H
+
Cationic intermediate 2
H
+
H
H+
H
Cationic intermediate 3
H
7 δ-Cadinene

Figure 23. The total ion current chromatogram of the hexane extract of elicitor-treated glandless cotton (WbM*gl*). δ-Cadinene mass spectrum was obtained on peak indicated by arrow.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; Alltech equivalent to J&W Scientific DB-5) and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.


T1509201 01-1265 15-SEP-92 09:20 TS250 (EI+) Sys:ESSENBERG INP
Chromatogram Identifiers : A :RTIC A:
Text:01.2 MbMgL x Elicitor
Relative Intensity
66
77
99
107
203
703
777
δ-Cadinene
200 400 600 800 1000 1200
11:50 19:39 27:28 35:17 43:06 50:55
Scan number
Time (min)

of the δ-cadinene produced in the elicitor-treated tissues by the addition of cade oil δ-cadinene as shown in Figure 24. Gas chromatography of extracts from *Xcm*-inoculated tissues are shown in Figure 25 for comparison purposes (although there is a minor variation in the retention time of the putative δ-cadinene in Figures 23-25, the peaks marked by an arrow in each figure exhibited a mass spectrum that matched the reference mass spectrum for δ-cadinene.) There was no detectable accumulation of δ-cadinene in noninoculated and mock-inoculated WbM*gl* cotyledons) (Figures 26 and 27). The elicitor-treated tissues were also used as a source of homogenate for cell-free reactions utilizing 1-[^{3}H]FPP 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 performed with *Xcm*-inoculated tissue homogenate. The catalytic rate was *ca* 4 nmole hr^{-1} mg $protein^{-1}$. The combined chromatographic evidence supports the conclusion that the cotton tissues are able to biosynthesize δ-cadinene without catalysis by bacterial enzymes. Additionally, GC-FID analysis showed that δ-cadinene does not accumulate in *Xcm* cells and culture medium prior to inoculation of the cotton cotyledon tissue with the injected mixture of Xcm cells, culture medium, and $CaCO_3$.

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] and to degrade during distillation [8] 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 [37]. Thus, the possibility remains that δ-cadinene may be the stable, isolatable product of a very unstable intermediate

Figure 24. The total ion current chromatogram of the hexane extract of elicitor treated glandless cotton (WbM*gl*) spiked with δ-cadinene. δ-Cadinene mass spectrum was obtained on peak indicated by arrow.

This sample was "spiked" with cade oil δ-cadinene and the suspected δ-cadinene peak was "enhanced" and still yielded a mass spectrum matching the NBS standard.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-1)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.

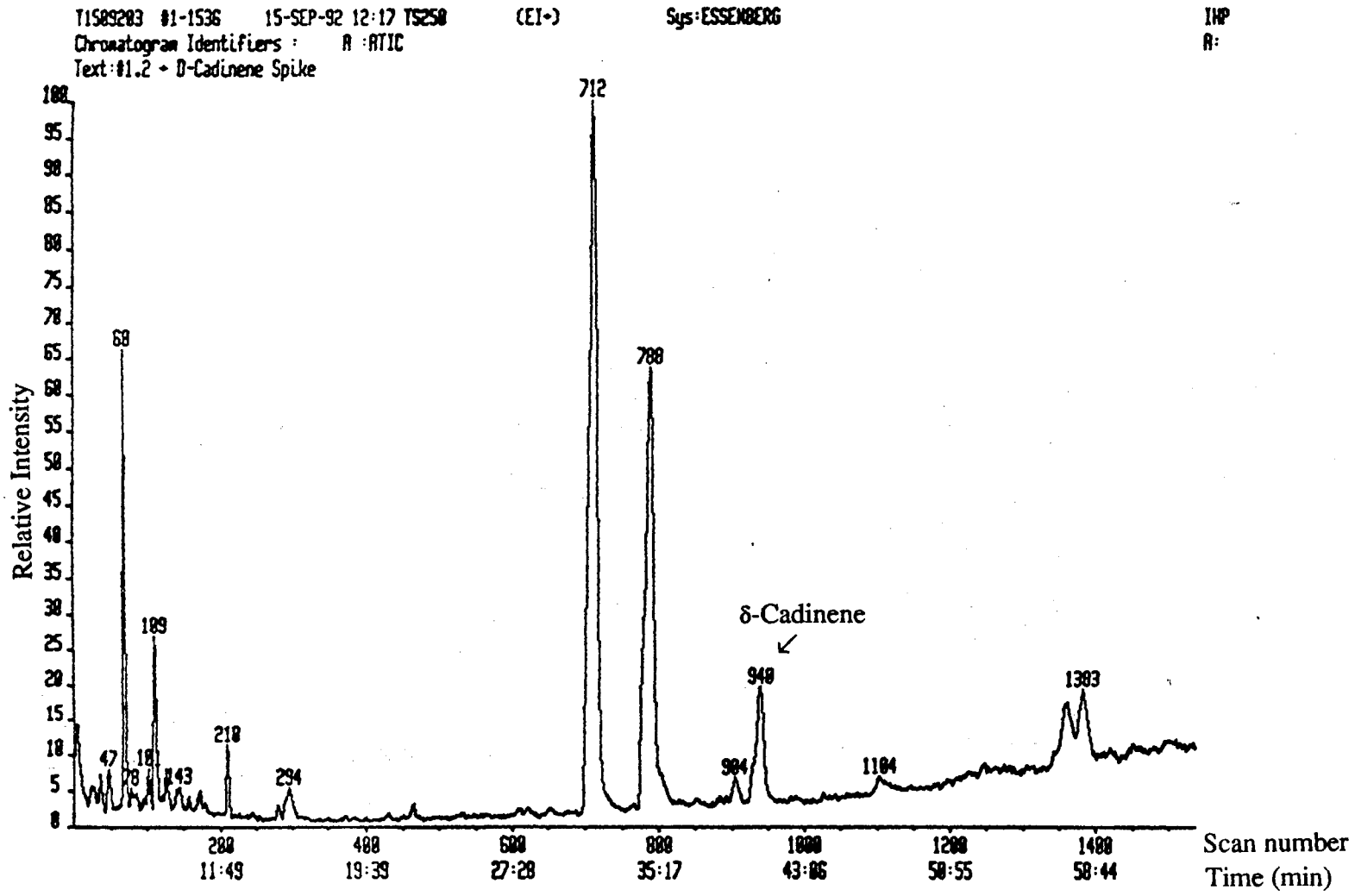

T1589283 #1-1536 15-SEP-92 12:17 TS250 (EI+) Sys:ESSENBERG IHP
Chromatogram Identifiers : A :ATIC A:
Text:#1.2 + D-Cadinene Spike
Relative Intensity
712
68
788
δ-Cadinene
949
1383
189
218
294
984
1184
47
Scan number
Time (min)

Figure 25. The total ion current chromatogram of the hexane extract of *Xcm*-inoculated glandless cotton (WbM*gl*) cotyledons. δ-cadinene mass spectrum was obtained on peak indicated by arrow.

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-1)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.

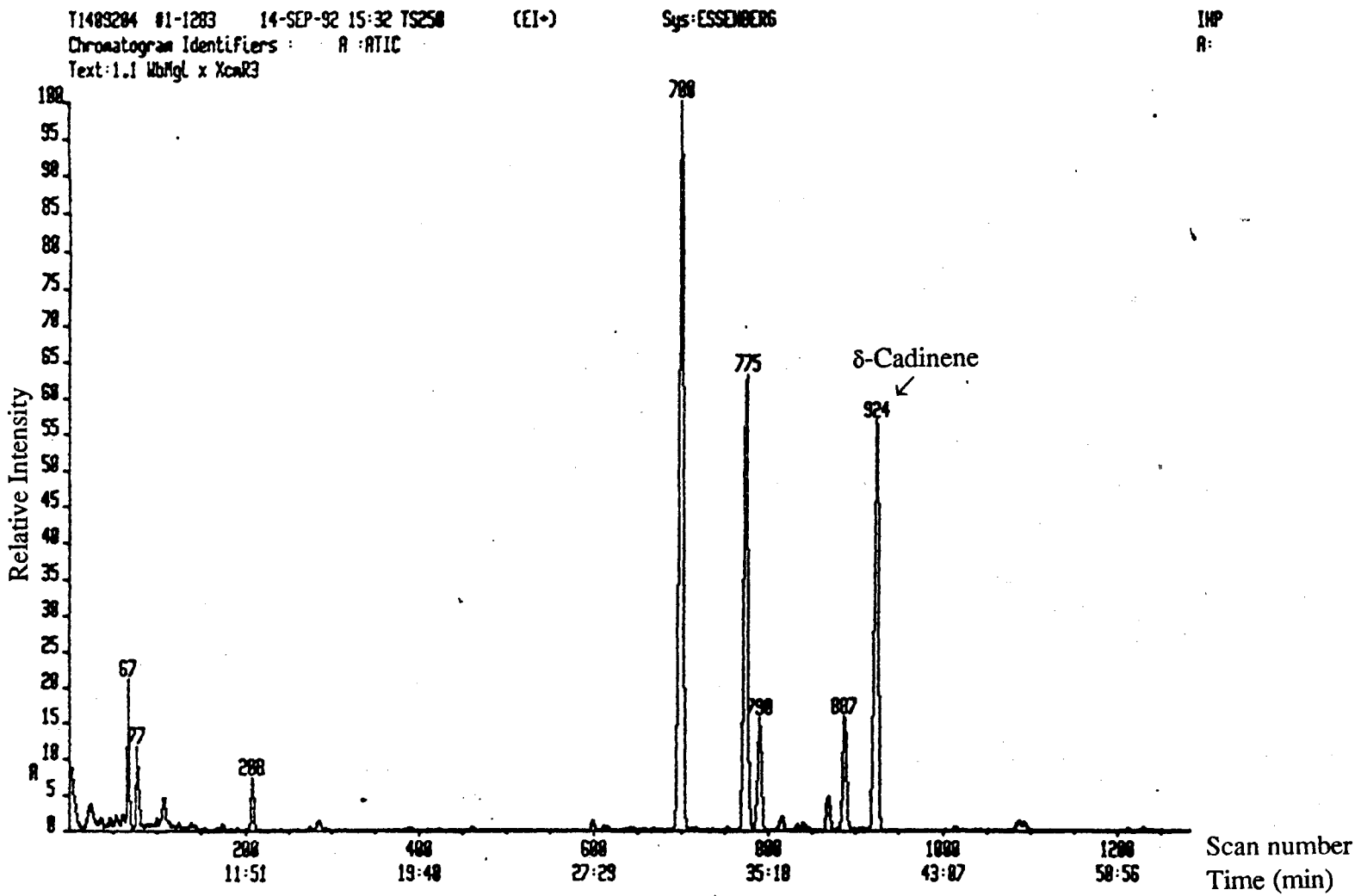

T1489204 #1-1283 14-SEP-92 15:32 TS250 (EI+) Sys:ESSENBERG IHP
Chromatogram Identifiers : A :ATIC A:
Text:1.1 WbMgL x XcmR3
Relative Intensity
δ-Cadinene
67
77
205
700
775
798
887
924
11:51
19:48
27:29
35:18
43:07
50:56
Scan number
Time (min)

Figure 26. The total ion current chromatogram of the hexane extract of noninoculated glandless cotton

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; Alltech equivalent to J&W Scientific DB-5)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.

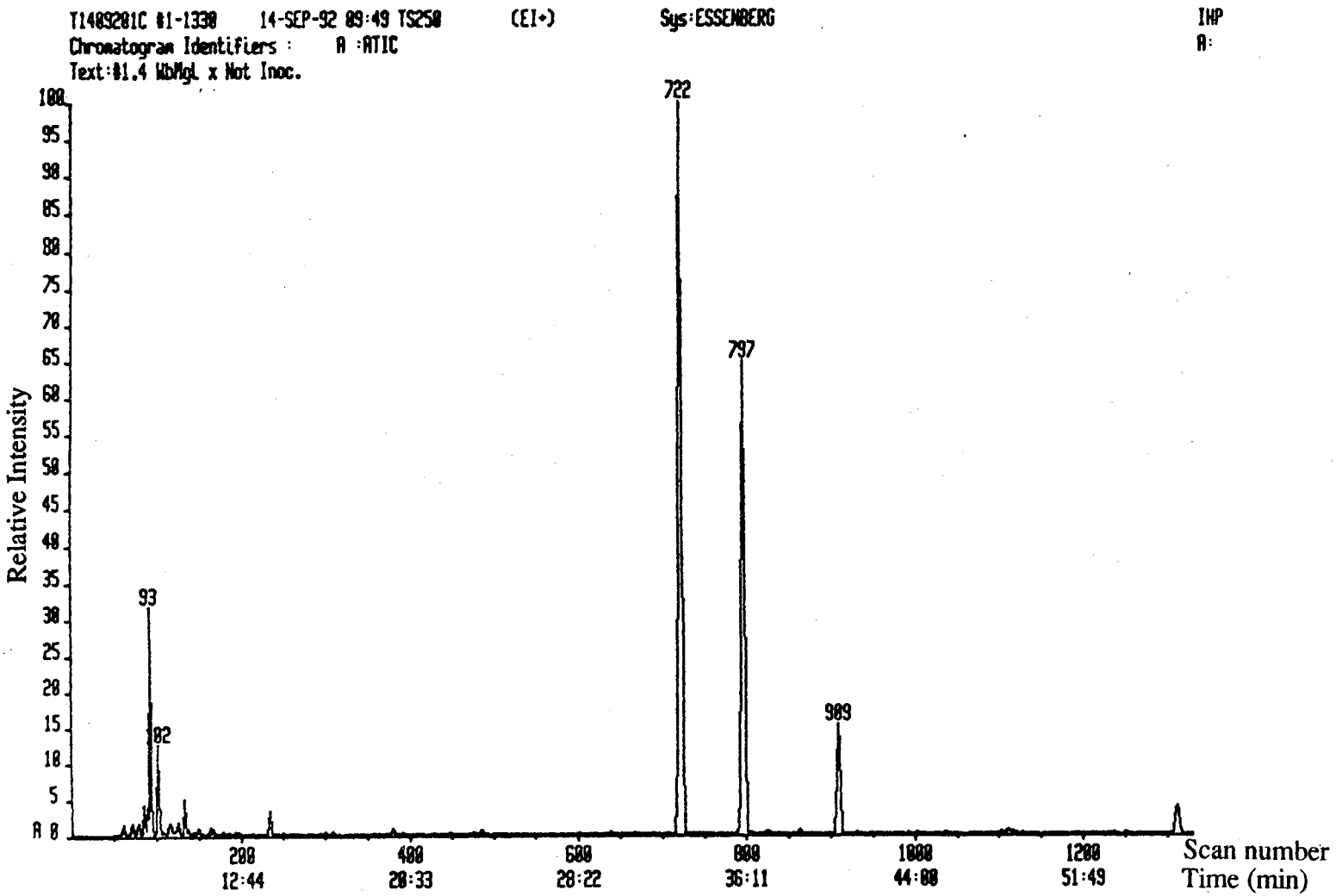
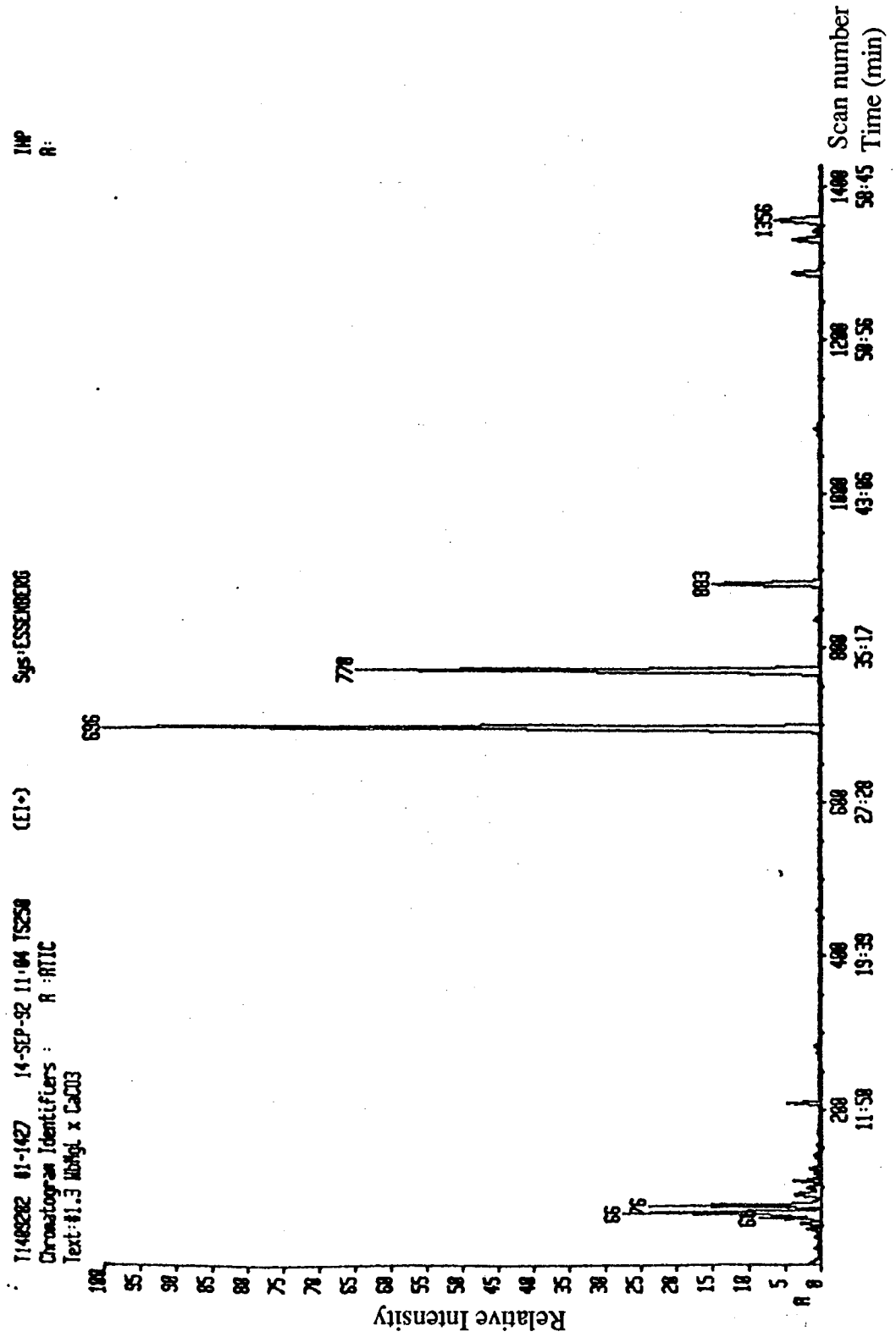

T1489201C #1-1338 14-SEP-92 09:49 TS250 (EI+) Sys:ESSENBERG IHP
Chromatogram Identifiers : A :ATIC A:
Text:#1.4 WbMgL x Not Inoc.
Relative Intensity
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
A 0
93
82
722
797
909
200
12:44
400
20:33
600
28:22
800
36:11
1000
44:00
1200
51:49
Scan number
Time (min)

Figure 27. The total ion current chromatogram of the hexane extract of calcium carbonate-inoculated cotton cotyledons

Instrument: Hewlett-Packard (HP) 5890 Gas Chromatograph with 5% phenyl/95% methyl capillary gas chromatography column (0.25 mm i. d. x 30 m; J&W Scientific DB-1)
and
VG TS-250 mass spectrometer.
Temperature program: 85°C hold for 1 min.; then 1°C min^{-1} until run terminated.


Relative Intensity
Scan number
Time (min)
836
778
883
1356
66
76
68
11:58
19:39
27:20
35:17
43:06
50:56
58:45

involved in the biosynthesis of the sesquiterpenoid phytoalexins of cotton. The role of δ-cadinene in sesquiterpenoid phytoalexin biosynthesis will be evaluated in future experiments.

EXPERIMENTAL

Radiochemicals, chemicals, and seed

[1-^{3}H]farnesyl pyrophosphate (sp. act. 32.14 μCi/μmol) was provided as a gift by Joseph Chappell, University of Kentucky. Non-radioactive farnesyl pyrophosphate was synthesized [38, 39]. 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 [Verhalen, L. M., personal communication] with the glandless line "21D111-112", which is homozygous in each of the recessive alleles gl_2 and gl_3 [40]. 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, B. E. and Verhalen, L. M., unpublished work). The elicitor was prepared from polygalacturonic acid by autoclaving according to the method of [41].

Cautionary note concerning GC trace analysis

In the following experimental section, we often employed an Et_2O:hexane (5:95) extraction solution and silica gel for the isolation of sesquiterpenoids. If a solution of CH_2Cl_2:hexane (5:95) is used in place of the Et_2O:hexane solution, the analyst can avoid the 2, 6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) (**16**) commonly added by commercial vendors to Et_2O as a preservative. We found that the BHT may obscure recovered compounds, since it displays retention on the 100% methyl (SE-30) and 5% phenyl-95% methyl (SE-54) gas chromatographic phases characteristic of a sesquiterpene hydrocarbon. It is important to use a high quality silica gel which has been

washed with acid, base, and then with water to neutrality. Drying, extraction with CH_2CL_2, and a final drying of the silica gel will remove many low-abundance contaminants that can complicate GC analyses of concentrated extracts.

Isolation and characterization of δ-cadinene from
Xcm-*inoculated glandless cotton tissue.*

Entire cotyledons of 11- or 12-day-old WbM*gl* seedlings were infiltrated with a suspension of *ca* 5 x 10^6 colony-forming units ml^{-1} of *Xanthomonas campestris* pv. *malvacearum* strain 3631 as previously described [42]. At 42 hr post-inoculation, the cotyledons were harvested, quick-frozen in liquid N_2 and stored at -70°.

Thirteen hundred g of inoculated tissue were processed by repetition of the following procedure to recover hexane-extractable compounds: 100 g of tissues 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; repeated 4 times). The combined supernatants were conc by rotary evaporation (0° bath). The extract was also 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. Fractions containing substances with GC retention times characteristic of sesquiterpene hydrocarbons were combined and conc by rotary evaporation (0° bath). Fats and waxes precipitated by overnight storage of the concentrate at -20° were removed by centrifugation. The extract was further conc by rotary evaporation (0° bath) to minimal volume, then suspended in acetonitrile:H_2O (35:65) and loaded onto a crude column constructed of four octadecylsilane cartridges attached in tandem (total wt of packing 4 g). Flow-through was discarded. Retained polar compounds were eluted with acetonitrile:H_2O (35:65) and discarded; hydrocarbons were eluted with HPLC-grade acetonitrile (which had been used to rinse the sample container to

enhance recovery). After addition of water, the hydrocarbon eluate was back-extracted into hexane, conc by rotary evaporation (0° bath) and diluted with hexane. The extract was subjected to sub-ambient temperature HPLC [23] using four 3.9 mm i.d. x 300 mm silica columns (10 μm particle diameter) attached in tandem and submerged in an acetone bath cooled to *ca* -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 separations was UV absorption at 215 nm.

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

Isolation of δ-cadinene (7) from cade oil

The isolation of the cade oil δ-cadinene (**7**) is describe in Chapter IV on p.118.

Enzyme preparations

Xcm-inoculated cotton tissues were homogenized in 50 mM HEPES (pH 7.2), 5 mM dithiothreitol, 10 mM sodium metabisulfite, 30 mM $MgCl_2$, and 10% glycerol (5 ml per g of tissue) plus 0.3 g of insoluble polyvinylpyrrolidone and 0.3 g of XAD-4 resin per g of tissue. The tissues were homogenized with a motorized homogenizer at full speed (30 sec on/ 30 sec off for 4 cycles) 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 [43] with bovine serum albumin as standard.

Radioactive cell-free reactions

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 preparation (27 000 *g* supernatant) containing 10-60 μg of protein was diluted into this buffer to a total volume of *ca* 490 μl in an 8-ml screw cap tube and pre-incubated at 30° for 5 min. [1-^{3}H]FPP (**5**) (*ca* 0.3 μCi in 10 μl) was added to give a final concentration of *ca* 20 μM FPP and a final volume of 500 μl. The capped tube was then incubated at 30° for 30 minutes (conditions were determined to give linear kinetics), and the mixture was extracted twice with 1.5 ml of hexane. The hexane extract was transferred to a tube containing 200 mg of silica gel which absorbed more than 99.9% of the total dpm attributable to unconverted [1-^{3}H]FPP, farnesol generated by cotton phosphohydrolases [27] and non-enzymatic hydrolysis, and other polar compounds. One ml of the hexane extract was added to 5 ml of scintillation fluid and subjected to liquid scintillation counting. One ml of the hexane extract was conc by argon gas at 0° for identification of radioactive product(s) by analytical-scale normal phase or reversed phase HPLC.

Non-radioactive cell-free reactions

These reactions were performed in the same manner as the radioactive cell-free reactions, except for an increase in tissue amount, the use of non-radioactive FPP as substrate and the addition of the controls [25] described under Results and Discussions. *Xcm*-inoculated glandless tissue was homogenized; by use of smaller 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 minutes in screw-cap tubes. One-half ml of an FPP solution (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 radioactive cell-free solution, but larger amounts of hexane were used to enhance recovery of non-radioactive enzyme product; thus, the reaction mixture was extracted four times in a separatory funnel with 30 ml of hexane. The hexane extract was then conc by rotary evaporation (0° bath). Silica gel was not employed; this assured availability of intermediate polarity compounds for GC analyses. The conc extract was analyzed by GC-FID and by GC-EIMS (70 eV) to obtain a mass spectrum for comparison to δ-cadinene reference spectra from EPA/NIH [13] and other literature sources [11,14,15].

HPLC co-chromatography of radioactive cell-free reaction product with cotton tissue hydrocarbon or with cade oil δ-cadinene (7)

The hexane extract of the radioactive cell-free reaction was mixed with purified sesquiterpene hydrocarbon isolated from *Xcm*-inoculated glandless cotton tissues; the mixture was then co-chromatographed by analytical-scale normal phase and reversed phase HPLC. A mixture of the radioactive cell-free reaction product with cade oil δ-cadinene 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 fractions. The void volume (V_o) of the analytical-scale normal phase column system was found to be 13.1 ml by injection of pentane [44]. Variability in capacity factors (k') observed in this sub-ambient temperature normal phase HPLC system was probably due to fluctuations in the temperature (*ca* -30°) of the acetone bath used to cool the HPLC columns; changes in the operating temperature influence the capacity factors (k') in sub-ambient temperature HPLC systems [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_o)of the analytical-scale reversed phase column system was found to be 2.4 ml by injection of 1 x 10^{-5} M $NaNO_3$ [45]. The capacity factors (k') determined from co-injections of commercial α-pinene, α-humulene and β-caryophyllene with δ-cadinene (from cade oil or cotton) were 4.2, 7.1, 8.3 and 9.9, respectively.

GC co-chromatography of cade oil δ-cadinene (7) with cotton tissue hydrocarbon or with non-radioactive cell-free reaction product.

Co-chromatography of cade oil δ-cadinene with the infection-induced hydrocarbon from *Xcm*-inoculated cotton tissue was conducted on four different gas chromatographic phases in FSOT capillary columns [0.25 mm i.d. x 30 m 100% methyl (SE-30); 0.25 mm i.d. x 60 m 5% phenyl-95% methyl (SE-54) x 2 (two of the 60 m columns were joined by glass union to make a 120 m column); 0.25 mm i.d. x 30 m 50% phenyl-50% methyl; and 0.25 mm i.d. x 30 m polyethylene glycol (Carbowax 20M equivalent)]. The FID signal was plotted and integrated. Detector temperature was 300°; on-column injection of 0.2 to 1.0 μl was made at injector temperature of 55° and oven temperature of 85°; carrier gas was He, and linear flow rate was 28 cm s^{-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 Kovat's Index values were obtained by use of a linear regression line calculated from retention times of co-injected *n*-paraffin hydrocarbon standards [20]. β-caryophyllene and α-humulene were co-injected with samples as time marker compounds on each column and as standards in place of *n*-paraffins to construct the Kovat's Indices plot with "self-consistent" Kovat's indices [17,20] for the 5%phenyl-95%methyl (SE-54) column.

Tentative identification of low abundance compounds in crude hexane extracts of Xcm-*inoculated glandless cotton tissue and non-radioactive cell-free reactions*

GC-EIMS analysis of the crude hexane extract of *Xcm*-inoculated glandless cotton tissues was conducted on two different gas chromatographic phases in FSOT capillary columns [0.25 mm i.d. x 30 m 5% phenyl-95% methyl (SE-54) and 0.25 mm x 30 m polyethylene glycol (Carbowax 20M equivalent)]. 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 Kovat's Index value for ε-cadinene chromatographed on a Carbowax 20M GC column [8]; the experimental mass spectrum was similar, but not identical, to the published mass spectrum of racemic ε-cadinene [9]. The tentative identification of α-muurolene was based upon a close match with the Kovat's Index value calculated from the published retention time for chromatography of α-muurolene on an SE-54 GC column [10] and reference mass spectra for α-muurolene from numerous sources [10-12]. GC/FID and GC/EIMS (VG TS-250 system; 70eV) analyses of the extract from the non-radioactive cell-free reaction were conducted with a 0.25 mm i.d. x 30 m 50% phenyl-50% methyl FSOT capillary GC column. The oven gradient was 2 min hold at 85° followed by a gradient of 85° to 170° at 3° min^{-1} and followed by a second gradient of 170° to 270° at 10° min^{-1}. The identification of α-cadinene was based upon a published mass spectrum [10]. Although no published retention value for α-cadinene was found for the intermediate polarity GC phase we employed, the tentatively identified α-cadinene eluted after δ-cadinene; similar elution order was seen for chromatography on polar Carbowax 20M GC columns [26].

Identification of sesquiterpene hydrocarbon from elicitor-infiltrated cotton; lack of hydrocarbon in mock-inoculated and noninoculated control tissues

The elicitor preparation was the hydrolysate of polygalacturonic acid prepared by the method of Robertsen [41]. The elicitor solution was filter-sterilized by 0.22 μm filter prior to use. WbM*gl* cotyledons were infiltrated with elicitor (5 mg ml^{-1}) in our inoculation medium, which is sterile, saturated $CaCO_3$ solution. At the same time, separate WbM*gl* cotyledons were mock-inoculated by infiltration with the sterile, saturated $CaCO_3$ solution or inoculated with an *Xcm* suspension (*ca* 5 x 10^6 colony-forming units ml^{-1}) in the same solution. The *Xcm*-, elicitor- and $CaCO_3$-infiltrated tissues and noninoculated tissues were cultivated in the same growth chamber and harvested at 42 hr post-infiltration. The tissues were immediately frozen in liquid N_2 and stored at -70° until analysis. Five hundred mg of each type of tissue was separately extracted by hand homogenizer (Duall) with 10 ml, then 5 ml of Et_2O:hexane (5:95). Each solution was centrifuged, and the supernatant was conc to small volume (*ca* 0.5 ml) by evaporation (0° bath). Each sample was chromatographed on a small homemade silica gel column (70-230 mesh silica gel in a pasteur pipet with glass wool plug) with hexane until the first colored (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 conc to *ca* 2 ml by rotary evaporation (0° bath) and then to dryness with argon gas stream with sample container at 0°. Samples were suspended in hexane (10-20 μl) and 0.2-1.0 μl injections were made for GC-FID analysis on the 0.25 mm i.d. x 30 m polyethylene glycol column (Carbowax 20M equivalent). The oven gradient was 2 min hold at 85° followed by a gradient of 85° to 220° at 2° min^{-1}. Identity of any possible δ-cadinene peak was confirmed by co-chromatography of co-injected cade oil δ-cadinene and GC-EIMS identification of the sesquiterpene hydrocarbon using the 0.25 mm i.d. x 30 m

polyethylene glycol column (Carbowax 20M equivalent) with a VG TS-250 mass spectrometer.

Cell-free reaction using elicitor-infiltrated tissue homogenate

A separate aliquot of the tissues used for the preceeding GC-EIMS identification of δ-cadinene in elicitor-infiltrated tissues was utilized for this experiment. Two grams of tissue from each treatment were homogenized as described for the enzyme preparations above, and cell-free reactions using [1-^{3}H]FPP as substrate were also prepared, incubated and extracted as described above for *Xcm*-inoculated tissues. One-sixth of the extract from each cell-free reaction (0.5 ml) was subjected to scintillation counting; the remaining extract (*ca* 2.5 ml) was conc to near dryness by argon gas at 0°. The extracts were each suspended in hexane containing α-pinene, β-caryophyllene, and cade oil δ-cadinene as time retention markers detected by UV absorption at 215 nm during fractionation by the sub-ambient temperature analytical-scale normal phase HPLC system. Fractions were collected and subjected to scintillation counting to locate radioactive fractions. A second series of homogenizations, cell-free reactions, and hexane extracts were made, but the dried extracts were resuspended in acetonitrile:H_20 (85:15) containing α-pinene, α-humulene, β-caryophyllene, and cade oil δ-cadinene as time retention markers detected by UV absorption at 215 nm during fractionation by the analytical-scale reversed phase HPLC system at room temperature. Fractions were subjected to scintillation to locate radioactive fractions.

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 concentration of *ca* 5 X 10^6 colony-forming units ml^{-1}. The bacteria and nutrient broth were separated by centrifugation (27 000 *g* for 20 min). The separated bacteria and broth were each extracted with CH_2Cl_2:hexane (5:95). Each extract was conc

by rotary evaporation (0° bath), chromatographed on 70-230 mesh silica gel with hexane to remove polar components and the hexane eluant was then conc by rotary evaporation (0° bath) and argon at 0° to *ca* 10-20 µl. Injection of 0.5 µl of each extract allowed for GC-FID analysis employing 0.25 mm i.d. x 30 m 5% phenyl-95% methyl (SE-54) or 0.25 mm i.d. x 30 m polyethylene glycol (Carbowax 20M equivalent) FSOT capillary GC columns. The oven gradient was 2 min hold at 85° followed by a gradient of 85° to 220° at 2° min^{-1}.

Other analytical methods employed to identify cade oil δ-cadinene

Circular dichroism (CD) was performed at ambient temperature (23°-26°); sixteen scans accumulated on a 1 x 10^{-5} M solution of cade oil δ-cadinene 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 cade oil δ-cadinene spread on a compressed zinc sulfide plate. UV absorption was recorded in hexane.

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CHAPTER VI

Partial Purification of the Inducible Sesquiterpene Cyclase δ-Cadinene Synthase from Glandless *Gossypium hirsutum*[1]

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[3] Abbreviations used: FPP, pyrophosphate ester of 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienol; DHC, 2,7-dihydroxycadalene; *Xcm*, *Xanthomonas campestris* pv. *malvacearum*; WbM *gl*, Westburn M glandless cotton; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; FPLC, fast protein liquid chromatography; A_{280}, absorbance at a wavelength of 280 nm; Native PAGE, non-denaturing polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminoethane.

ABSTRACT

The sesquiterpene cyclase δ-cadinene synthase from glandless *Gossypium hirsutum* L. (cotton) inoculated with the bacterium *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) was purified 320-fold by batch processing with anion-exchange and hydroxyapatite media followed by anion-exchange and gel permeation chromatography. The enzyme exhibited a maximum specific activity of 15,800 nmol/min/mg protein and a native molecular weight of M_r 39,200 as determined by gel permeation chromatography. A second protein of M_r 27,800, also purified from the cotton tissues, could function at lower efficiency as δ-cadinene synthase. In general properties, both enzymatically active proteins resemble fungal and plant sesquiterpene olefin cyclases. Failure to detect Germacrene D during the enzyme purification or in extracts of *Xcm*-inoculated cotton tissues suggests Germacrene D does not function as a free intermediate in the biosynthesis of δ-cadinene.

INTRODUCTION

Plant, insects, and microbes synthesize several thousand sesquiterpenoid compounds of nearly 200 skeletal types (1). The synthesis of many of these terpenes includes cyclization of the ubiquitous isoprenoid precursor *trans, trans*-farnesyl pyrophosphate (FPP)[3] (**1**). Much information concerning this type of cyclization is available from studies of the biosynthesis of monoterpenes (2) and diterpenes (3, 4), and progress has been made in the study of sesquiterpene cyclases (5) from a number of fungal (6, 7), bacterial (8) and a few plant sources (9, 10,11).

Glanded *Gossypium hirsutum* (cotton) is a versatile biosynthetic factory which can constitutively produce many sesquiterpene and derived dimeric compounds (*bis*-sesquiterpenoids) (12). Glanded cotton has been selectively bred to yield resistant lines that can withstand infection by *Xanthomonas campestris* pv. *malvacearum* (*Xcm*), the

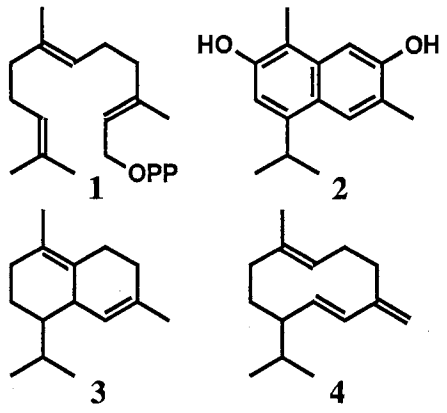


Figure 1. Sesquiterpenes of interest in isolation of δ-cadinene synthase.

causative agent of bacterial blight of cotton. This resistance response includes the inducible production of sesquiterpenoid phytoalexins, e.g., 2,7 dihydroxycadalene (DHC) (**2**) (13). Previous work has shown that these induced phytoalexins are derived from a *cis, trans*-farnesyl precursor or an equivalent substrate (14) and that [1-^{3}H]-FPP (**1**) should be a suitable substrate for the assay of any cyclase activity involved in biosynthesis of the induced phytoalexins (15). To simplify purification of the cyclase activity, a line of glandless cotton (WbM *gl*) was used because it could be induced to produce the sesquiterpenoid phytoalexins, but did not exhibit the complex constitutive production of terpene compounds seen in glanded cotton. Initial studies showed that cell-free extracts of *Xcm*-inoculated glandless cotton tissues converted [1-^{3}H]-FPP (**1**) to δ-cadinene (**3**) (Davis, G. D. et al., unpublished work). (In contrast, cell-free extracts of uninoculated glandless cotton tissues did not convert [1-^{3}H]-FPP (**1**) to δ-cadinene (**3**); the major labelled product recovered from the cell-free reaction catalyzed by uninoculated tissue was tritium-labelled farnesol, presumably produced by hydrolysis of the [1-^{3}H]-FPP.) By monitoring production of δ-cadinene (**3**), the δ-cadinene synthase activity was purified 315-fold. Although germacrene D (**4**) (16) has been proposed to be a biosynthetic precursor of δ-cadinene (17-19) or cadinene compounds (20), often occurs in the same plants with δ-cadinene (21), and is converted to δ-cadinene by acid catalysis *in vitro* (22,23), we have

not found any evidence for involvement of Germacrene D (**4**) as a free intermediate in the biosynthesis of δ-cadinene (**3**).

EXPERIMENTAL PROCEDURES

Plant materials, substrates, and reagents

Glandless Westburn M (WbM *gl*) cotton seeds were obtained from Laval Verhalen of Oklahoma State University. The cotton seedling were grown and inoculated with *Xanthomonas campestris* pv. *malvacearum* strain 3631 (24) when the seedlings were 11 or 12 days old, as previously described. The inoculated cotton cotyledons were harvested at 42 h post-inoculation, quick-frozen with liquid N_2, and stored at -70°C until homogenized.

[1-^{3}H]farnesyl pyrophosphate (50.1 Ci/mol) was purchased from Dr. C. Dale Poulter of the University of Utah, received as a gift from Dr. Joseph Chappell of the University of Kentucky (32.14 Ci/mol), or prepared by mixing of labelled farnesyl pyrophosphate purchased from New England Nuclear (26,900 Ci/mol) with unlabelled farnesyl pyrophosphate prepared by the methods described in references (25,26) to yield a specific activity of approximately 35 Ci/mol.

δ-Cadinene used as an HPLC retention marker was isolated and purified (>95% by gas chromatography) from cade oil (Penta Manufacturing) and thoroughly characterized (Davis, G. D. et al., unpublished work). Other retention markers for HPLC analysis of enzyme products were (1*R*)-(+)-α-pinene (Aldrich), β-caryophyllene (Fluka) and α-humulene (Fluka). Hexane (E. Merck) and acetonitrile (Baker) were of HPLC quality. Water used for enzyme assays and enzyme purification was distilled and polished by a dual Barnstead column system (organic removal and mixed bed) to attain a conductivity of 2.0 μmho or less.

δ-Cadinene synthase isolation

The initial isolation by DE-52 and hydroxyapatite batch processes was conducted under conditions of yellow light or very low light, as *Xcm*-inoculated cotton tissues contain photoactivatible compounds that form free radical species (27,28) that might inactivate enzyme activities. For initial homogenization, 100 g (fresh wt.) of cotyledons were ground to fine powder with mortar and pestle in liquid N_2 and the powder was stirred into a cold slurry consisting of 0.3 g insoluble polyvinylpyrrolidone ("Polyclar AT", GAF Chemicals) and 0.3 g of XAD-4 resin (Rohm and Haas Corp.; obtained from Sigma) in 5 ml/g fresh tissue weight of 50 mM Hepes buffer, pH 7.2, containing 5 mM dithiothreitol, 10 mM $Na_2S_2O_5$, 30 mM $MgCl_2$, and 10% (v/v) glycerol. The insoluble polyvinylpyrrolidone was cleaned as previously described (29) and the XAD-4 resin was cleaned by extraction with a series of methanol, acetone, methanol, and deionized water rinses. Both the polyvinylpyrrolidone and the XAD-4 resin were washed with aliquots of homogenization buffer before being mixed with homogenization buffer to constitute the homogenization buffer slurry. The tissues were homogenized using a Sorvall homogenizer at 4°C (30s on/ 30s off for four cycles). The homogenate was strained through 6 layers of cheesecloth and filtrate was centrifuged at 27,000*g* for 20 min at 0-4°C in 30-ml Corex tubes to provide a supernatant to be used as enzyme source.

The combined supernatant from homogenization of 500g of tissue (2000 ml) was added to 400 g of DE-52 cellulose (Whatman) pre-equilibrated with homogenization buffer. After mixing and settling of the DE-52 cellulose, the supernatant was removed and discarded. Enzyme was eluted by an aliquot of buffer containing 150 or 600 mM KCl; since both eluted fractions had substantial activity, the fractions were combined to give an enzyme solution of 3000 ml. The enzyme still was able to bind to 65g of hydroxyapatite, as KCl has neglible power to elute many acidic proteins from hydroxyapatite (30). The enzyme was eluted from the hydroxyapatite with solutions containing 80, 160, and 400

mM potassium phosphate (pH 7.2). Because each fraction (including the 0 mM KCl wash) had substantial activity, the solutions were mixed to give a total volume of 820 ml.

δ-Cadinene purification

All further purification was made with either FPLC columns or conventional steel HPLC columns. The chromatography was accomplished by two Waters 6000 pumps and Waters Model 660 solvent controller with a Valco injector utilizing 0.2, 0.4, or 1.0 ml sample loops. The enzyme fraction eluted from the hydroxyapatite was concentrated by immersible concentrators (Waters, regenerated cellulose membranes, 10,000 nominal molecular weight cutoff) connected to a water vacuum aspirator. The buffer composition at this point was 0 mM KCl, 50 mM potassium phosphate, 10 mM $MgCl_2$, and 10% (v/v) glycerol. The concentrate was applied to a Q-Sepharose HR16/50 column (16 x 500 mm, anion exchange, Pharmacia) equilibrated with 50 mM Hepes, pH 7.2, containing 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% (v/v) glycerol. The column was washed with 4 ml of starting buffer followed by elution by a linear gradient of 0-600 mM KCl (140 mls, 2.0 ml fractions, 1.0 ml/min). Activity was found in two distinct portions of the elution pattern at approximately 230 mM KCl and 340 mM KCl. All active fractions were combined and concentrated by four immersible concentrators to 6 ml.

The concentrated enzyme was divided into three 2 ml aliquots each of which was chromatographed on a Superose 6 HR 16/50 gel permeation column (16 x 500 mm, Pharmacia). The column was pre-equilibrated and the protein was eluted with 50 mM Hepes buffer, pH 7.2, containing 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% (v/v) glycerol (240 mls, 1.0 ml fractions, 1.0 ml/min). Activity was found in a broad peak in fractions containing proteins of approximate molecular weight range of 20 to 50 kDa. The column had been calibrated with blue dextran as a void volume marker, thyroglobulin (670 kDa), ovalbumin (44 kDa), and myoglobin (17.2 kDa); a smaller molecular weight marker could not be used due to excessive retention in the chromatographic media. Fractions

containing activity were combined and concentrated by immersible ultrafiltration devices to a volume of 4 ml.

The concentrated solution was applied to a MonoQ HR 5/5 column (5 x 50 mm, anion exchange, Pharmacia) previously equilibrated in 50 mM Hepes buffer, pH 7.2, containing 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% (v/v) glycerol. The column was washed to remove any unbound proteins. The column was then washed with a 0-600 mM KCl gradient in the buffer (100 ml, 0.4 ml fractions, 0.8 ml/min). As with the previous elution on the Q-Superose column, the activity eluted in two distinct portions, one at 220 mM KCl (designated Activity I) and the other at 340 mM KCl (designated Activity II). Activity I and Activity II were separately concentrated by immersible concentrators.

The concentrated activities were separately chromatographed by repetitive injections on a conventional steel HPLC column (GPC 300, glycerylpropyl phase bonded to 5 μm silica, 7.8 x 300 mm, Synchrom). The column was pre-equilibrated and the activities were eluted with 50 mM Hepes buffer, pH 7.2, containing 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% (v/v) glycerol (10 ml, 0.05 ml fractions, 0.5 ml/min). The column was calibrated with thyroglobulin (670 kDa), bovine serum albumin (69 kDa), ovalbumin (44 kDa), myoglobin (17.2 kDa), with dextran blue as V_0 marker and glyceryl-tyrosine as V_t marker. Activity I eluted with an apparent molecular weight of 27.8 kDa and Activity II eluted with an apparent molecular weight of 39.2 kDa. Each of the activities was separately concentrated by immersible ultrafiltration device.

An attempt was made to elute Activity I by elution with tetrasodium pyrophosphate and KCl from MonoQ as described by Hohn for trichodiene synthetase (6) and for aristolochene synthase (7). Unfortunately this provided no further purification.

Activities I and II were separately eluted from MonoQ again with a gradient with a low rate of increase in KCl concentration (0-500 mM KCl in 180 min). Activity I and II eluted near 220 and 310 mM KCl, respectively. Activity I and II fractions were very dilute; protein concentration of Activity II was determined by performing Bio-Rad (Bradford

Coomassie blue dye-binding) determination on a low enzyme activity fraction which eluted near Activity II and exhibited an A_{280nm} similar to that of the Activity II fractions. The total protein cited in the purification table is elevated over the value of the Bio-Rad determination; this should provide a conservative estimation of specific activity in the final Activity II fraction. Activity I eluted from the MonoQ column had very low protein concentration so that estimation of protein and activity were unreliable. All fractions containing Activity I or Activity II from the final MonoQ elution were stored at -70°C.

Gel electrophoresis

For native gel electrophoresis samples containing KCl were exchanged into electrophoresis buffer by concentration in spin concentrators (Millipore Ultrafree; 400 µl volume; 10,000 nominal molecular weight cut-off regenerated cellulose membrane) and dilution in electrophoresis buffer. A discontinuous electrophoresis system employing a stacking gel of 150 mm x 20 mm x 1.5 mm and a separating gel of 150 mm x 90 mm x 1.5 mm was used. It was polymerized by 0.0005% riboflavin which was caused to polymerize by 2 hr exposure to white fluorescent light. Duplicate aliquots of 1.5 or 15 µg of Activity II from the first MonoQ elution were loaded in wells of 150 µl total volume (80 µl volume per sample). One of the duplicate lanes was subjected to staining by Coomassie brilliant blue, an unstained lane of the 15 µg loading of Activity II was analyzed by standard assay of 2 mm slices (9); Activity II eluted at an R_f 0.61. A control lane loaded with sample buffer and tracking dye was also analyzed by standard assay of 2mm slices. A mixture of standard proteins eluted to give thyroglobulin (Sigma) an R_f of 0.36 and ovalbumin (Sigma) an R_f of 0.58.

δ-Cadinene synthase assay

Assays were run in 250 µl of assay buffer, 50 mM Hepes, pH 7.2, 30 mM $MgCl_2$, 10% (v/v) glycerol. Small aliquots of the protein samples (1-25 µg in less than 10 µl of

homogenization or elution buffer) were diluted into the assay buffer in small screw-cap tubes. The samples were then pre-incubated at 30°C for 5 min. The assay reactions were initiated by addition of 20 μM [1-^{3}H]FPP, and the reaction was allowed to proceed at 30°C for 0.5 hr. The reaction was stopped by chilling of the assay mixture, followed by extraction with hexane (1.5 ml x 2). The hexane extracts were pooled in a 13 x 100 mm tube containing 200 mg of 70-230 mesh high purity silica (Aldrich). One milliliter of the hexane extract was then analyzed by aliquot counting; aliquots of hexane were transferred to 7 ml vials containing 5 ml of Packard Insta-gel XF scintillation fluid for direct determination of radioactivity by a Packard model 1900 CA Tricarb liquid scintillation analyser and were counted to accumulate at least 4 x 10^4 scintillations (^{3}H counting efficiency = 58%). Quench corrections were made by comparison of the samples transformed spectral indices of the external standard (tSIE) with curves of tSIE versus scintillation efficiency for chemically quenched ^{3}H samples. Boiled enzyme controls were included in each experiment (nonenzymatic formation of sesquiterpene olefins was neglible).

When needed, the product(s) of the cell-free assay were separated and analyzed by sub-ambient temperature (approximately -30°C) normal phase HPLC system (31) with tandem column arrangement (32). The two milliliters (approximate volume) of the hexane extract of the cell-free reaction not analyzed by scintillation counting was concentrated by gentle N_2 gas stream at 4°C prior to injection into the HPLC system. The HPLC consisted of a Waters 6000 pump eluting 100% HPLC-grade hexane through four Waters μPorasil columns (5 μm media; 3.9 x 300mm) connected in tandem with detection of eluted components by UV absorption at 215 nm with a Dionex detector. The flow rate was 1ml/min or less because back-pressures (>4000 psi) in the system cooled to approximately -30°C. Adequate resolution of the components could be achieved by cooling of the columns to 0°C with an ice bath. In this system, a concentrated aliquot of the hexane extracts from a cell-free assay was co-injected with retention markers (1R)-(+)-α-pinene, β-

caryophyllene, and α-humulene; the trailing edge of the δ-cadinene cell-free product merged with the leading edge of the UV-detectable β-caryophyllene marker peak. Higher resolution to completely separate δ-cadinene and the β-caryophyllene standard was achieved when the columns were cooled to approximately -30°C by immersion of the columns in an acetone bath cooled by a Cryocool CC-60 (Neslab).

Protein determination was made by the method of Bradford (33) with the Bio-Rad protein assay kit using bovine albumin, fraction V (Pierce Chemical) as a standard.

RESULTS AND DISCUSSION

Enzyme extraction, stability and assay

Glanded cotton lines produce many sesquiterpenoid products, both on a constitutive and inducible basis (12). Use of a glandless cotton line that does not produce a complex constitutive mixture of sesquiterpenes but can produce sesquiterpene phytoalexins after inoculation with *Xcm* provided us with a biosynthetic background virtually devoid of terpenoid compounds. The predominant product of cell-free reactions utilizing either radioactive or non-radioactive farnesyl pyrophosphate (**1**) and the predominant sesquiterpene olefin generated *in planta* upon bacterial infection have all been shown to be δ-cadinene (**3**) (Davis, G. D. et al., unpublished results; see p.161 of Chapter V of this thesis). This characterization of the enzymatic product proved useful as we isolated two farnesyl pyrophosphate converting enzymes. We show below that both enzymes produce δ-cadinene from [1-^{3}H]FPP (**1**), and that one enzyme was biosynthetically more efficient than the other protein.

The cyclase activity was found to be inducible . The first indirect evidence of this induction was found during the chemical analysis of uninoculated and *Xcm*-inoculated glanded (OK 1.2) cotton tissue; both tissues were harvested at 60 hours after the inoculation with *Xcm*- (Figure 2). Figure 2a shows the population of volatile compounds

Figure 2. Gas chromatograms of volatiles (hydrocarbons) extracted from

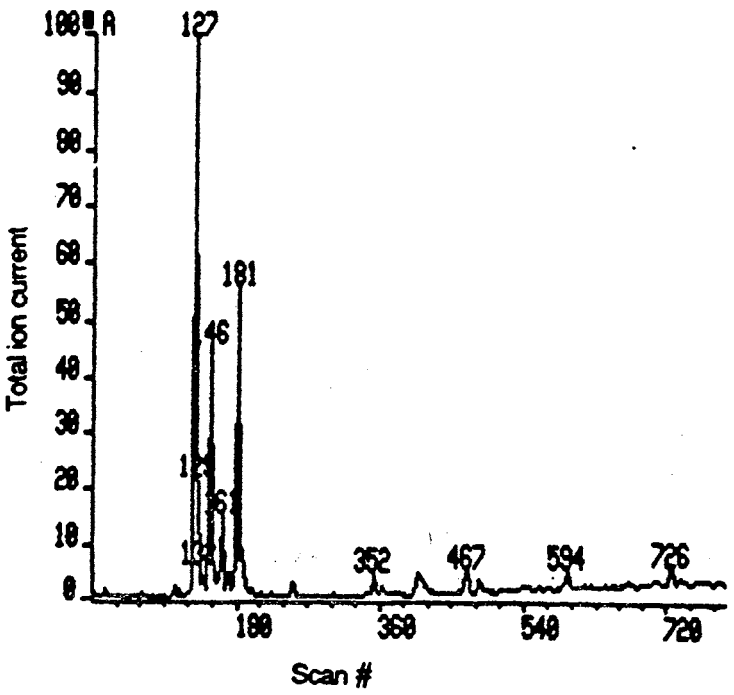
a) mock-inoculated cotyledons of glanded (OK 1.2) cotton seedlings

and

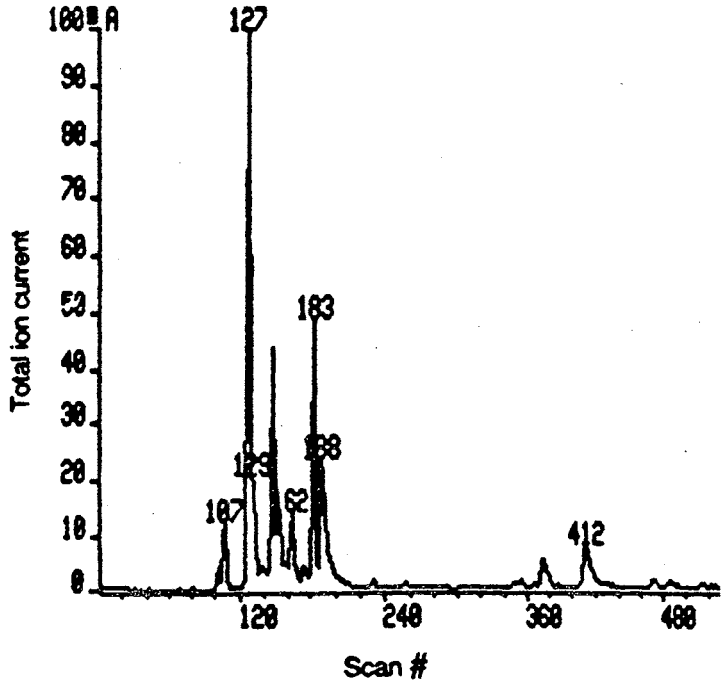
b) *Xcm*-inoculated cotyledons of glanded (OK 1.2) cotton seedling.

Cotyledons were harvested at 60 hr post-inoculation and frozen in liquid nitrogen. Tissues (0.5 g) were extracted twice with 10 ml of ethyl acetate/hexane (25:75, v/v) in a glass homogenizer. After low-speed centrifugation, the extract was evaporated to dryness, redissolved in hexane, and applied to a 1 cm x 25 cm silica column. Hydrocarbons were eluted with 25 ml hexane and evaporated to dryness. Small aliquots in ethyl acetate were sujected to GC/M on a methylsilicone capillary column (J&W Scientific DB-1, 0.25 mm x 30 m, 0.25 mm thick stationary phase) linked to a VG TS-250 mass spectrometer. Kovats indices and mass spectra, compared with published values for hydrocarbons of cotton buds (34), suggested the following identities, (scan #, compound name): 127, β-caryophyllene; 146, α-humulene; 188, δ-cadinene.

a)


100 A
90
80
70
60
50
40
30
20
10
0
Total ion current
127
181
46
352
467
594
726
180
360
540
720
Scan #

b)


100 A
90
80
70
60
50
40
30
20
10
0
Total ion current
127
183
188
129
62
107
412
120
240
360
480
Scan #

recovered in the hexane extract of uninoculated glanded cotton. The gas chromatographic profile in Figure 2a is similar to the gas chromatographic profiles performed on from glanded DeltaPine cotton tissues (34) and supports the tentative identification of the compounds designated by scan number 127 as β-caryophyllene and scan number 146 as α-humulene. Inoculation of the glanded tissues with Xcm provoked the appearance of the compound designated as scan number 188 (Figure 2b) which had very similar retention to that of δ-cadinene. Additionally, the constitutive accumulation of δ-cadinene in glanded cotton tissue has been documented (34).

A cell-free reaction catalyzed by extracts of the *Xcm*-inoculated glanded cotton (OK 1.2) tissue were also found to convert [1-^{3}H]FPP into a labelled compound which was very weakly retained during normal phase HPLC on silica gel (Figure 3a). The other labelled compounds in Figure 3a may have arisen from conversion of [1-^{3}H]FPP by constitutive enzymes of the cotton tissue, including phospohydrolases (35) that can convert [1-^{3}H]FPP to farnesol and by other enzymes induced by inoculation with *Xcm*.. By comparison, a similar cell-free reaction catalyzed by a boiled extract of the *Xcm*-inoculated glanded cotton (OK 1.2) converted the [1-^{3}H]FPP into minor amounts of a weakly-retained compound (Figure 3b). Very little of any other labelled products were recovered in the boiled control, presumably due to effective inactivation of the enzymes in the boiled extract, including the common phophohydrolases (35) which can produce tritium-labelled farnesol by hydrolyzing the [1-^{3}H]FPP substrate. Any [1-^{3}H]FPP not utilized during the cell free reaction may have been adsorbed by the glass of the cell-free reaction vessel; alternatively, the [1-^{3}H]FPP may have been so strongly retained by the silica gel of the HPLC column that it did not present a recognizable peak and would only be removed from the HPLC column upon washing with a solvent of very high eluting strength such as methanol or water.

Seeking a less complicated metabolic environment for our work, we decided to employ a glandless cultivar of cotton, Westburn M *gl* (WbM*gl*), that could was capable of

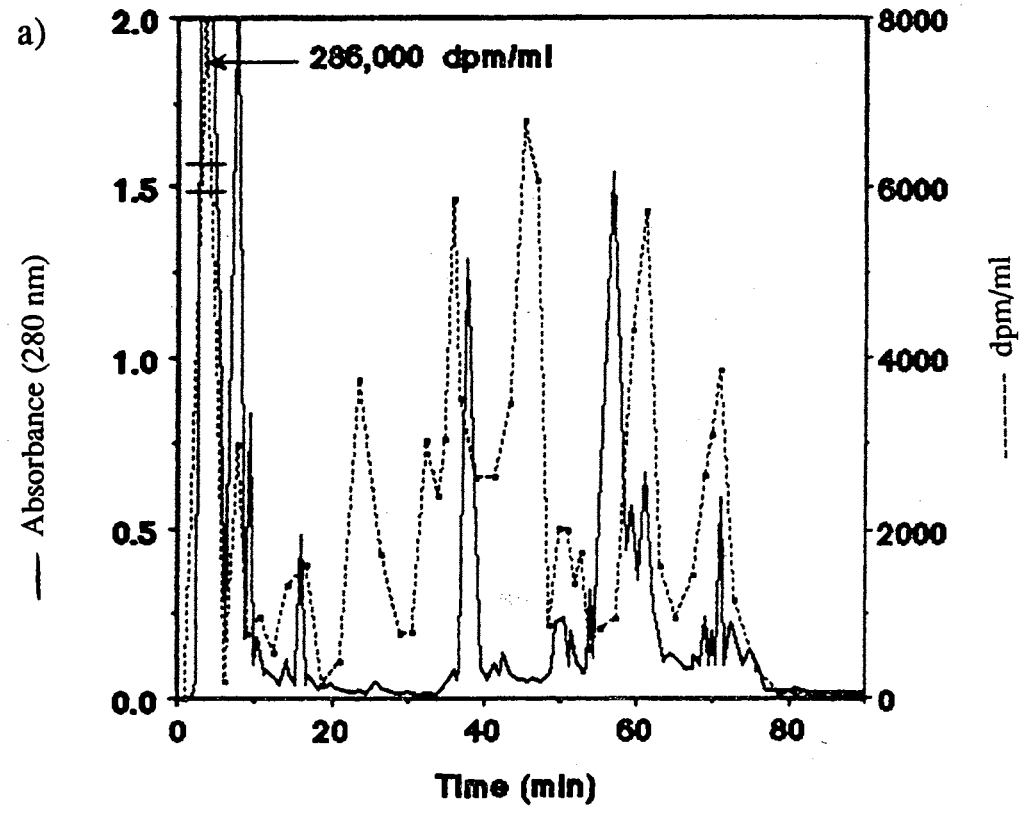
Figure 3. Normal phase HPLC separation of hexane-extractable products from cell-free reactions catalyzed by:

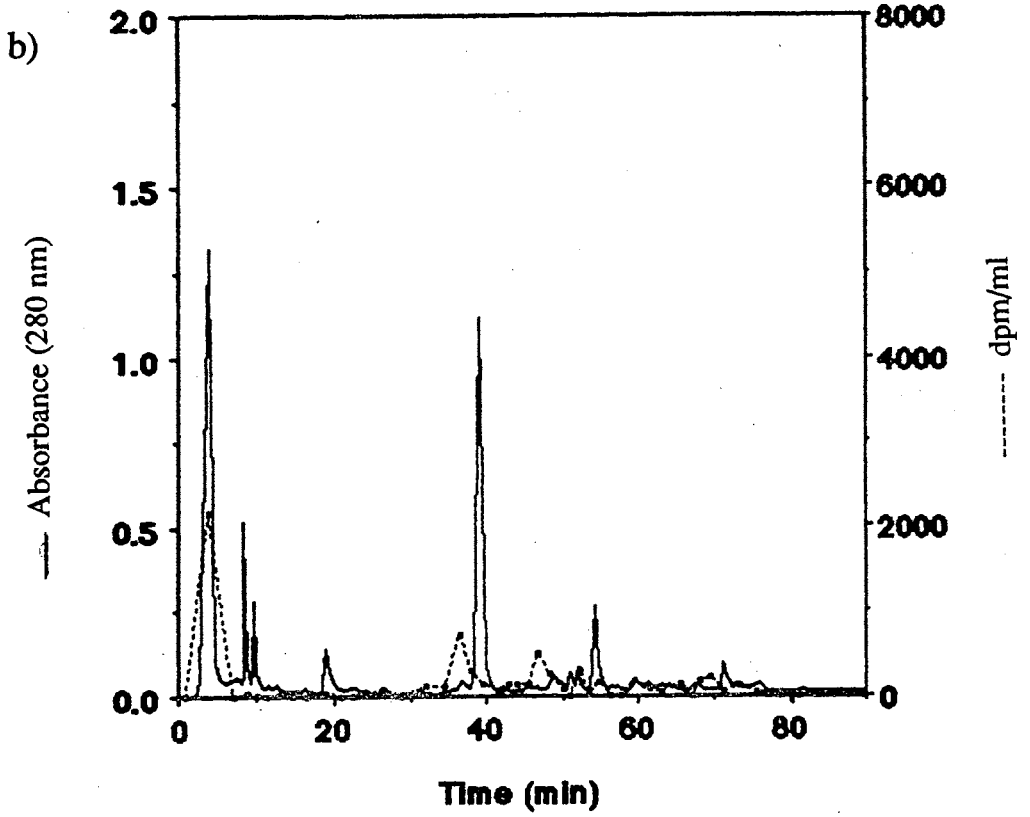
a) extracts of *Xcm*-inoculated glanded cotton (OK 1.2) cotyledons

and

b) boiled extract of *Xcm*-inoculated glanded cotton (OK 1.2) cotyledons.

Cotyledons of glanded cotton (OK 1.2) seedlings were inoculated with Xcm. The cotyledons were harvested at 60 hours post-inoculation and quick-frozen in liquid N_2. The tissues were extracted by homogenization with HEPES-based homogenization buffer, which was clarified by passage through cheesecloth and centrifugation. The supernatant was then divided; one-half of the supernatant was then subjected to boiling and allowed to cool to 30°C. Then two cell-free reactions then performed with catalysis by : a) with the untreated supernatant or b) the boiled supernatant solution. After addition of [1-^{3}H]FPP to the two cell-free reactions and 30 minutes incubation at 30°C, the cell-free reactions were ceased (and extracted) by addition of hexane. The hexane extract was concentrated and applied to a 4.6 x 250 mm silica HPLC column. Elution was by a gradient of hexane and ethyl acetate using Waters HPLC pump at 1 ml min^{-1} and UV detection at 280 nm. Fractions were also analyzed by scintillation counting.


a)
286,000 dpm/ml
— Absorbance (280 nm)
------ dpm/ml
2.0
1.5
1.0
0.5
0.0
8000
6000
4000
2000
0
0
20
40
60
80
Time (min)


b)
— Absorbance (280 nm)
------ dpm/ml
2.0
1.5
1.0
0.5
0.0
8000
6000
4000
2000
0
0
20
40
60
80
Time (min)

induced production of sesquiterpenoid phytoalexins following inoculation with *Xcm*. The cell-free extracts from uninoculated glandless cotton tissue converted [1-^{3}H]-FPP (**1**) into only minor amount of compound(s) with the retention characteristics of δ-cadinene (3) (Figure 4a). The major product of the incubation of the [1-^{3}H]-FPP (**1**) with the cell-free extracts of the uninoculated glandless cotton was a labelled compound which exhibited retention in our normal phase HPLC system similar to that of unlabelled *trans, trans*-farnesol obtained from a commercial source (Aldrich). Thus the predominant labelled compound seen in the normal phase HPLC analysis (R_t≈48–49 minutes in Figure 4a) of the products recovered from the cell-free reaction catalyzed by uninoculated WbM*gl* tissues is very likely tritium-labelled farnesol which can be arise from the hydrolysis of [1-^{3}H]-FPP by phophohydrolases (34). In great contrast, cell-free extracts of *Xcm*-inoculated glandless cotton tissue could convert [1-^{3}H]-FPP (**1**) into a labelled compound (R_t≈3–4 minutes in Figure 4b) which displayed the chromatographic characteristics consistent with those of δ-cadinene (**3**). The radiochromatography of the cell-free reaction products has also been performed by revered phase HPLC; the cell-free reaction catalyzed by uninoculated tissue extracts predominantly converts [1-^{3}H]-FPP into (presumably) tritium-labelled farnesol (R_t≈35 minutes in Figure 5a) and the cell-free reaction catalyzed by the *Xcm*-inoculated tissue extracts converts [1-^{3}H]-FPP into tritium-labelled δ-cadinene (R_t≈58–60 minutes in Figure 5b). The [1-^{3}H]-FPP was also converted into a number of other more polar compounds, some of which appear to arise after inoculation with *Xcm*. (Figures 4b and 5b). Some of these labelled polar compounds have also been found to have retention times that match possible intermediates in the pathway leading to the sesquiterpene phytoalexins (G. DaVila-Huerta; unpublished results). This inducibility of enzyme activity by *Xcm*-inoculation is consistent with the chemical analysis of the tissues: δ-cadinene begins accumulating in the glandless cotton at approximately 40 hours after the inoculation with *Xcm*; whereas the δ-cadinene is not detected in the uninoculated glandless cotton tissues. Inducibility of the cyclase activity can also be obtained by injection of the

Figure 4. Normal phase HPLC separation of hexane-extractable products from cell-free reactions catalyzed by:

a) extract of noninoculated glanded cotton (WbM*gl*) cotyledons

and

b) extract of *Xcm*-inoculated glandless cotton (WbM*gl*) cotyledons.

Inoculation was with 5 x 10^6 cfu/ml *Xanthomonas campestris* pv. *malvacearum* in sterile, saturated $CaCO_3$ solution. Control cotyledons were uninoculated. Cotyledons were harvested 40 hr post-inoculation and frozen in liquid nitrogen. Frozen tissues (5g fresh wt. of each) were homogenized with an Omnimixer (3 x 30 sec) in cold 50 mM HEPES, pH 7.0, containing 150 mM sucrose, 10 mM ascorbic acid, 10 mM sodim metabisulfite, 30 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM β-mercaptoethanol, and insoluble polyvinylpyrollidone (Polyclar AT, 1g/g fresh weight cotton tissue). The homogenates were centrifuged at 27 000 g for 20 min. The supernatant fluids were concentrated to 3 ml by ultrafiltration in a Centriprep 10 concentrator, and half of each was incubated for 1 hr at 30°C with 1 μCi of *RS*-[1-^{3}H]FPP (51 μci/μmol). Products were extracted twice with 3 ml hexane then twice with 3 ml chloroform. The combined extracts were evaporated to dryness and subjected to reversed phase chromatography on μPorasil silica (an 8 mm x 10 cm Waters Z-module cartridge). Elution was with hexane/ethyl acetate (99:1, v/v) for 25 min, then with a 35 min gradient to 18% ethyl acetate and held at 18% ethyl acetate for 30 minutes. Flow rate was 1.5 ml/min, and fractions were 1.5 ml. UV absorbance was performed by Waters Lambda-Max detector set at 280 nm. Fractions were assayed for radioactivity by scintillation counting.

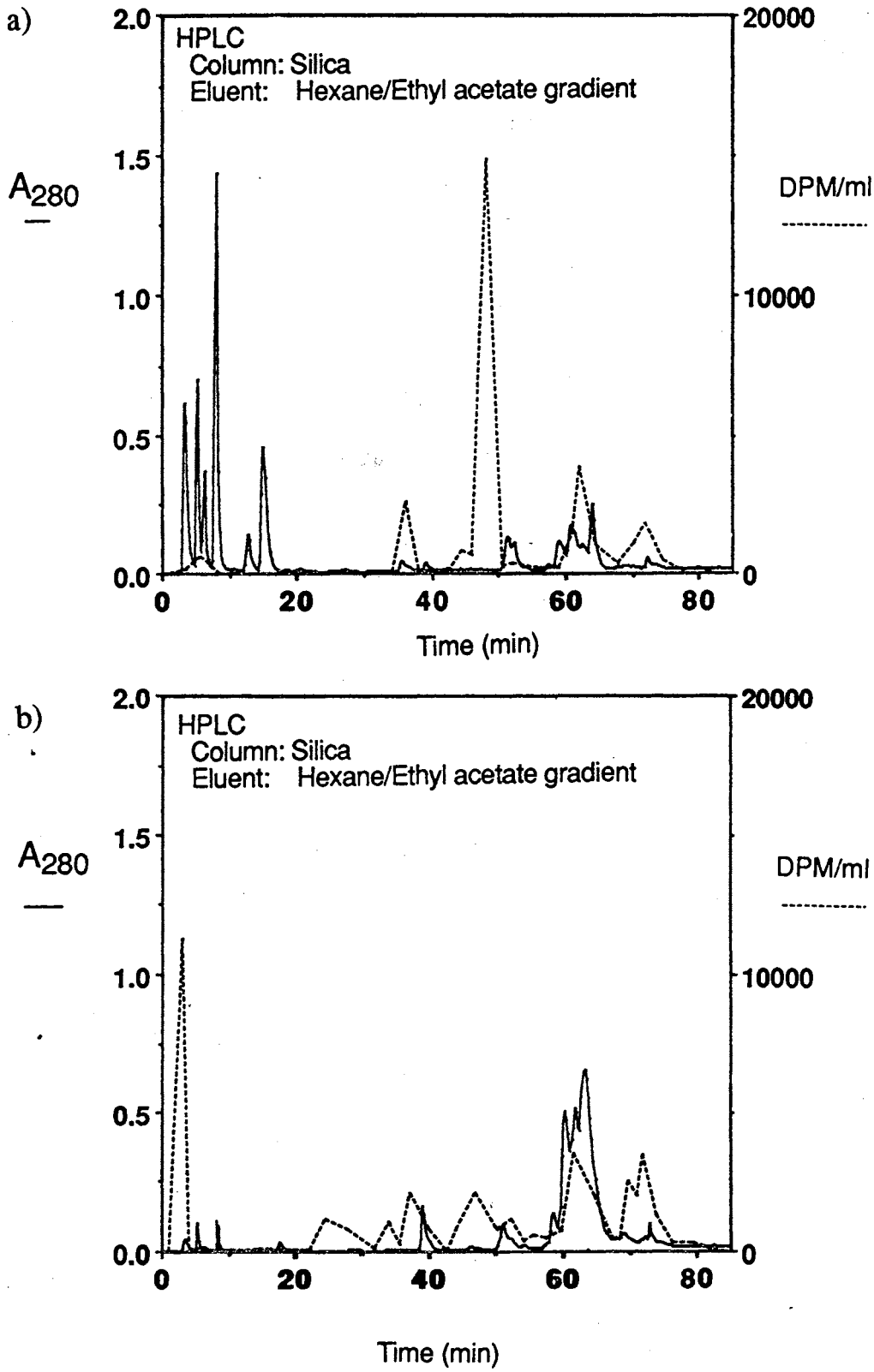

a)
HPLC
Column: Silica
Eluent: Hexane/Ethyl acetate gradient
A280
DPM/ml
2.0
1.5
1.0
0.5
0.0
20000
10000
0
0
20
40
60
80
Time (min)
b)
HPLC
Column: Silica
Eluent: Hexane/Ethyl acetate gradient
A280
DPM/ml
2.0
1.5
1.0
0.5
0.0
20000
10000
0
0
20
40
60
80
Time (min)

Figure 5. Reversed phase HPLC separation of hexane-extractable products from cell-free reaction catalyzed by:

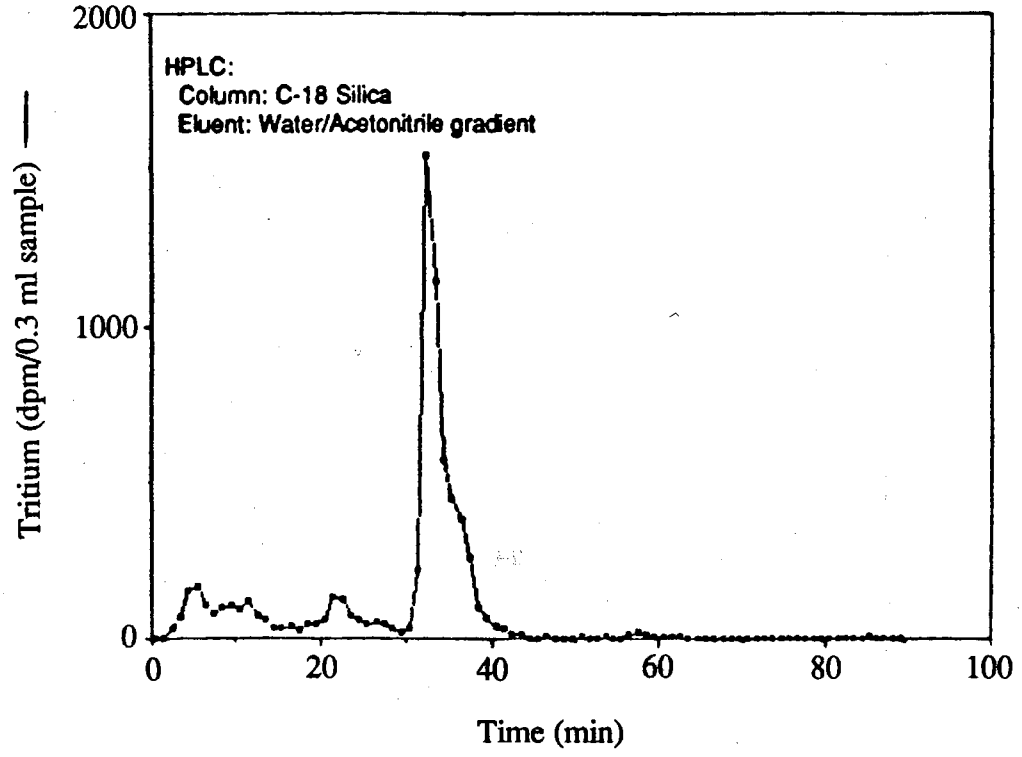
a) extract of noninoculated glandless cotton (WbM*gl*) cotyledons

and

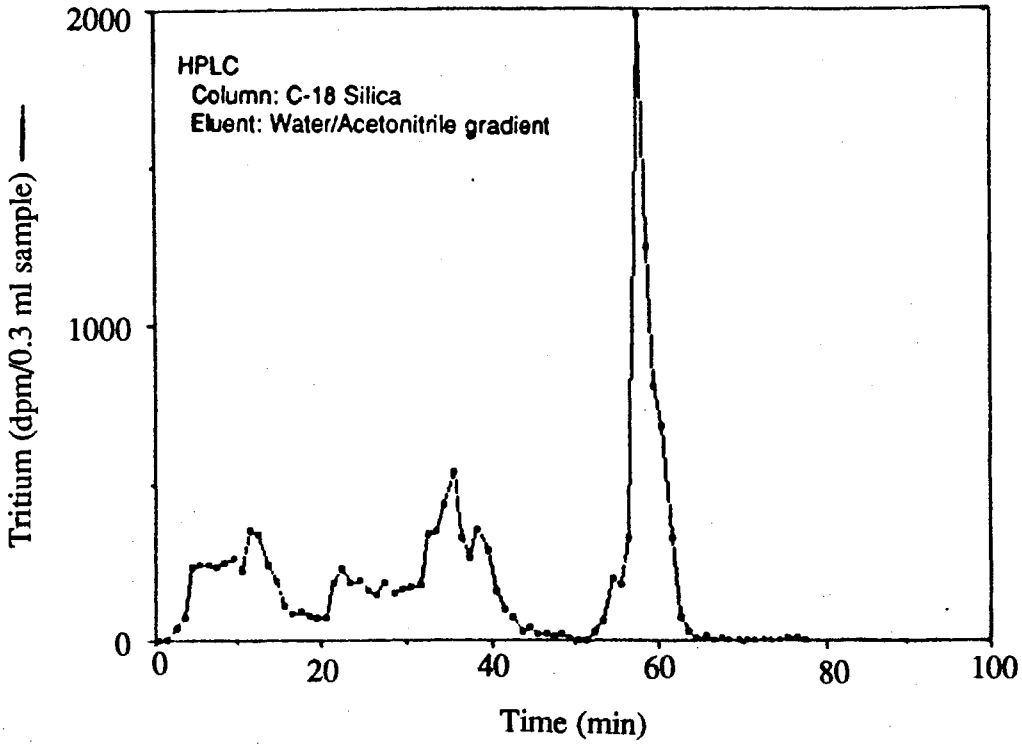
b) extract of *Xcm*-inoculated glandless cotton (WbM*gl*) cotyledons.

Inoculation was with 5 x 10^6 cfu/ml *Xanthomonas campestris* pv. *malvacearum* in sterile, saturated $CaCO_3$ solution. Control cotyledons were infiltrated with sterile, saturated $CaCO_3$ solution. Cotyledons were harvested 40 hr post-inoculation and frozen in liquid nitrogen. Frozen tissues (5g fresh wt. of each) were homogenized with an Omnimixer (3 x 30 sec) in cold 50 mM HEPES, pH 7.0, containing 150 mM sucrose, 10 mM ascorbic acid, 10 mM sodim metabisulfite, 30 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM β-mercaptoethanol, and insoluble polyvinylpyrollidone (Polyclar AT, 1g/g fresh weight cotton tissue). The homogenates were centrifuged at 27 000 g for 20 min. The supernatant fluids were concentrated to 3 ml by ultrafiltration in a Centriprep 10 concentrator, and half of each was incubated for 1 hr at 30°C with 1 μCi of *RS*-[1-^{3}H]FPP (51 μCi/μmol). Products were extracted twice with 3 ml hexane then twice with 3 ml chloroform. The combined extracts were evaporated to dryness and subjected to reversed phase chromatography on C_{18} silica (an 8 mm x 10 cm Waters Z-module cartridge). Elution was with acetonitrile/H_2O (60:40, v/v) for 10 min, then with a 60 min gradient to 100% acetonitrile. Flow rate was 1.5 ml/min, and fractions were 1.5 ml. Total recovered radioactivity and radioactivity in the peak centered at 58 minutes (suspected δ-cadinene) was a) 8.3%, 0.006% and b) 17.7%, 6.5%.

a)


HPLC:
Column: C-18 Silica
Eluent: Water/Acetonitrile gradient
Tritium (dpm/0.3 ml sample)
2000
1000
0
0
20
40
60
80
100
Time (min)

b)


HPLC
Column: C-18 Silica
Eluent: Water/Acetonitrile gradient
Tritium (dpm/0.3 ml sample)
2000
1000
0
0
20
40
60
80
100
Time (min)

glandless cotton tissues with an abiotic elicitor (hydrolysate of polygalacturonic acid), thus induction of the activity is not specifically dependent on the presence of *Xcm* (See Chapter V of this thesis, p.180).

δ-Cadinene synthase was unstable in crude extracts (27,000*g* supernatants in Hepes homogenization buffer system). Attempts to increase activity by use of detergent or changes in buffer salt were unsuccessful. We found that 50 mM Hepes (pH 7.2) with 5mM dithiothreitol, 10% (v/v) glycerol, and 10 to 30 mM $MgCl_2$ provided a crude extract that adequately stabilized activity until subsequent purification could be performed. Dithiothreitol, glycerol, and $MgCl_2$ were included in all purification steps unless the components would degrade chromatographic separations.

The assay for δ-cadinene activity was standardized with the crude homogenate. The requirement for divalent metal ion and saturating concentrations of Mg^{2+} and substrate (20 μm) were established. Linear assay conditions were determined (60 min at up to 100 μg protein/ml crude homogenate; <5% conversion of substrate); all subsequent assays were performed within these limits. The product of the cell-free assays showing significant activity was confirmed by normal phase HPLC separation of the extracts of cell-free assays and scintillation of HPLC-separated fractions. Boiled controls displayed neglible nonenzymatic conversion of *trans,trans*-farnesyl pyrophosphate to δ-cadinene or any other product extractable by 2% diethyl ether in hexane.

Purification

δ-Cadinene cyclase activity in crude extracts could not be effectively concentrated by $(NH_4)_2SO_4$ precipitation or by ultrafiltration without great loss of activity. This instability of cyclase enzyme activity is similar to that of patchoulol synthase (10) and of cyclase activity in crude homogenate of elicitor-induced tobacco suspension cultures (11). Cane and Pargellis also noted severe loss of pentalenene synthase activity upon contact with ultrafiltration membranes (8). By using batch treatments (DE-52 and hydroxyapatite),

small ions and competing and potentially degrading activities were rapidly removed, providing a relatively stable enzyme preparation for further purification. Additionally, the use of ultrafiltration devices employing hydrophilic membranes (regenerated cellulose) allowed for sample concentration with reasonable recovery. Early attempts at concentration using ultrafiltration devices possessing relatively hydrophobic surfaces and membranes caused significant loss of activity. This apparent affinity for hydrophobic membranes suggests that δ-cadinene synthase is, like many sesquiterpene cyclases, modestly hydrophobic (5). The results of our purification scheme through the second (and final) MonoQ chromatography are summarized in Table I.

The final step of MonoQ purification of Activity II and capillary electrophoretic analysis of some of the final MonoQ fractions deserve comment. The elution of Activity II during the final MonoQ purification step is shown in Figure 6. While the shape of the eluted components fails to meet the ideal Gaussian shape for a single eluted component, some of the fractions collected from the final MonoQ elution may contain homogeneous enzyme. To assess the possible homogeneity of the protein in one of the fractions of Activity II recovered from the final MonoQ purification step, a fraction was chromatographed on a Bio-Rad capillary electrophoresis system (BioFocus 2000). The tracing of this chromatography is shown in Figure 7. Although the peak of the eluted Activity II is somewhat broad for a single protein eluted by a capillary electrophoretic method, the operator of the Bio-Rad system (Jeff Verdier) mentioned that hydrophobic proteins often exhibit broad peaks (by comparison to more polar proteins). Given this information, the broad peak exhibited by Activity II may simply be an indication that δ-cadinene synthase is, like many cyclases, relatively hydrophobic. The Activity II chromatographed by the capillary electrophoresis system was not collected (and Activity II had been already been degraded by storage for approximately 2 months before the separation), so no attempt was made to determine the activity of the eluted protein.

TABLE 1

PARTIAL PURIFICATION OF δ-CADINENE SYNTHASE FROM *G. hirsutum* INOCULATED WITH *Xanthomonas campestris* pv. *malvacearum*

Fractionation step		Total Protein (mg)[a]	Total Activity nmol/h)[b]	Specific Activity (nmol) mgxh(%)[c]	Recovery	Purification
27,000g supernatant		3320	64,000	49.5	100	1.0
Batch DE-52		1430	90,430	76.9	55	1.6
Batch Hydroxyapatite		1320	104,000	79.1	64	1.6
Q-Sepharose		208	15,000	72.3	9.2	1.5
Superose 6		19.2	6,510	339	4.0	6.8
MonoQ						
MonoQ	Activity I	2.92	1,540	529	0.94	10.7
	Activity II	5.66	5,470	966	3.34	19.5
GPC 300	Activity I	0.529	268	507	0.16	10.2
	Activity II	0.403	748	1,850	0.46	37.4
Mono Q	Activity I	(Very Low Protein Concentration and Activity)				
	Activity II (estimate)	approx. 8ug[d]	126	15,750	0.077	318.2

[a] Data based on 500 g cotyledon tissue.

[b] A unit is defined as nmol δ-cadinene per hour under the assay conditions described under Experimental Procedures.

[c] Recovery figures include any losses in protein concentration and buffer change for the indicated step.

[d] 8 μg value is over-estimate of total protein value used to calculate a conservative value for specific activity.

Figure 6. Expansion of portion of the chromatogram from final MonoQ chromatography to purify Activity II (δ-cadinene synthase).

Concentrated protein fraction enriched in Activity II (δ-cadinene synthase) was chromatographed on MonoQ (5 x 50 mm; Pharmacia FPLC) column (0.8 ml min^{-1}; 0.4 ml fractions)with a 0-500 mM KCl gradient over 180 min. Protein tracing was obtained by Waters 440 detector at 280 nm. Fractions containing δ-cadinene synthase were located by cell-free assay of fractions.

Fraction 112 was subsequently analyzed by Bio-Rad capillary electrophoresis (Figure 6).

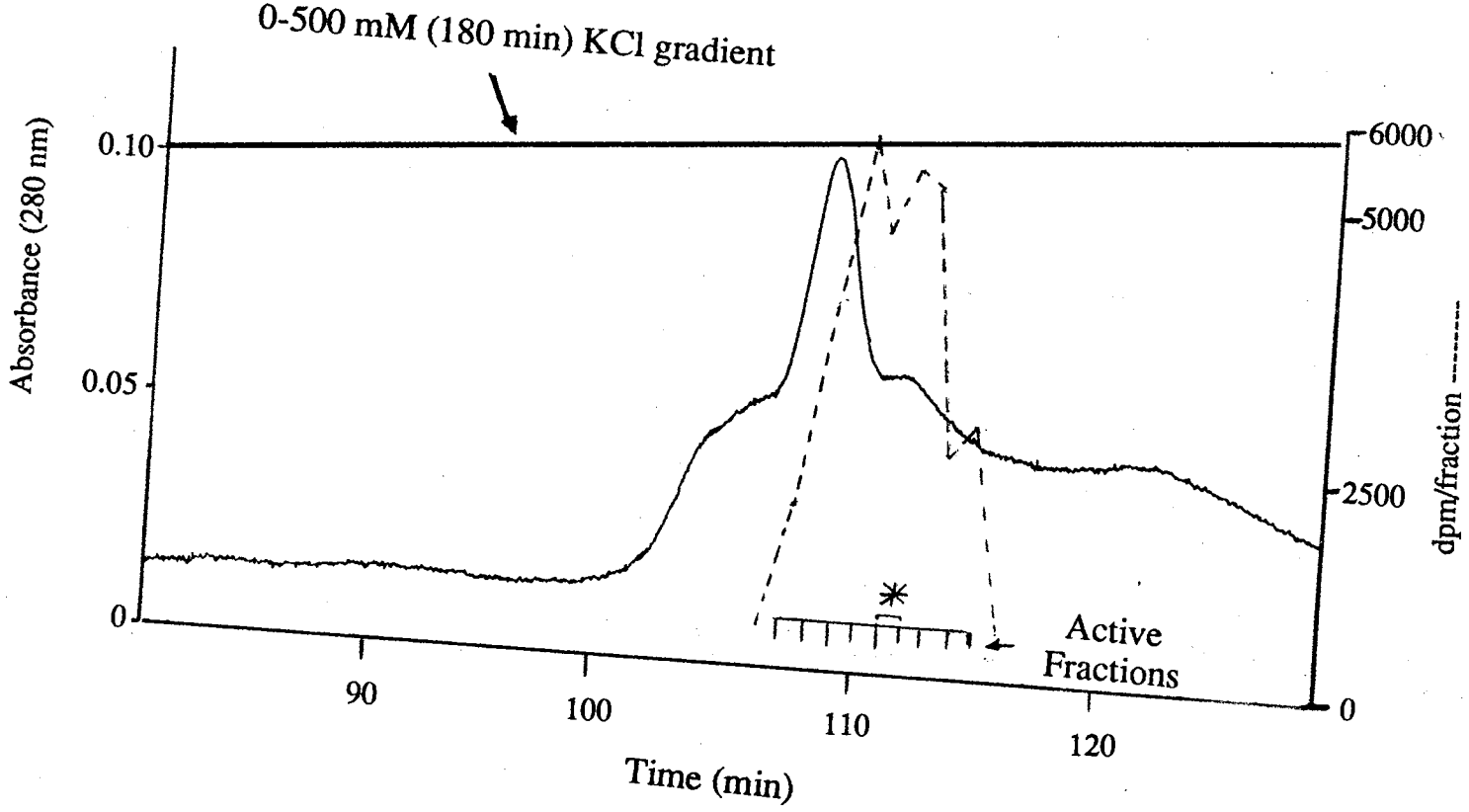
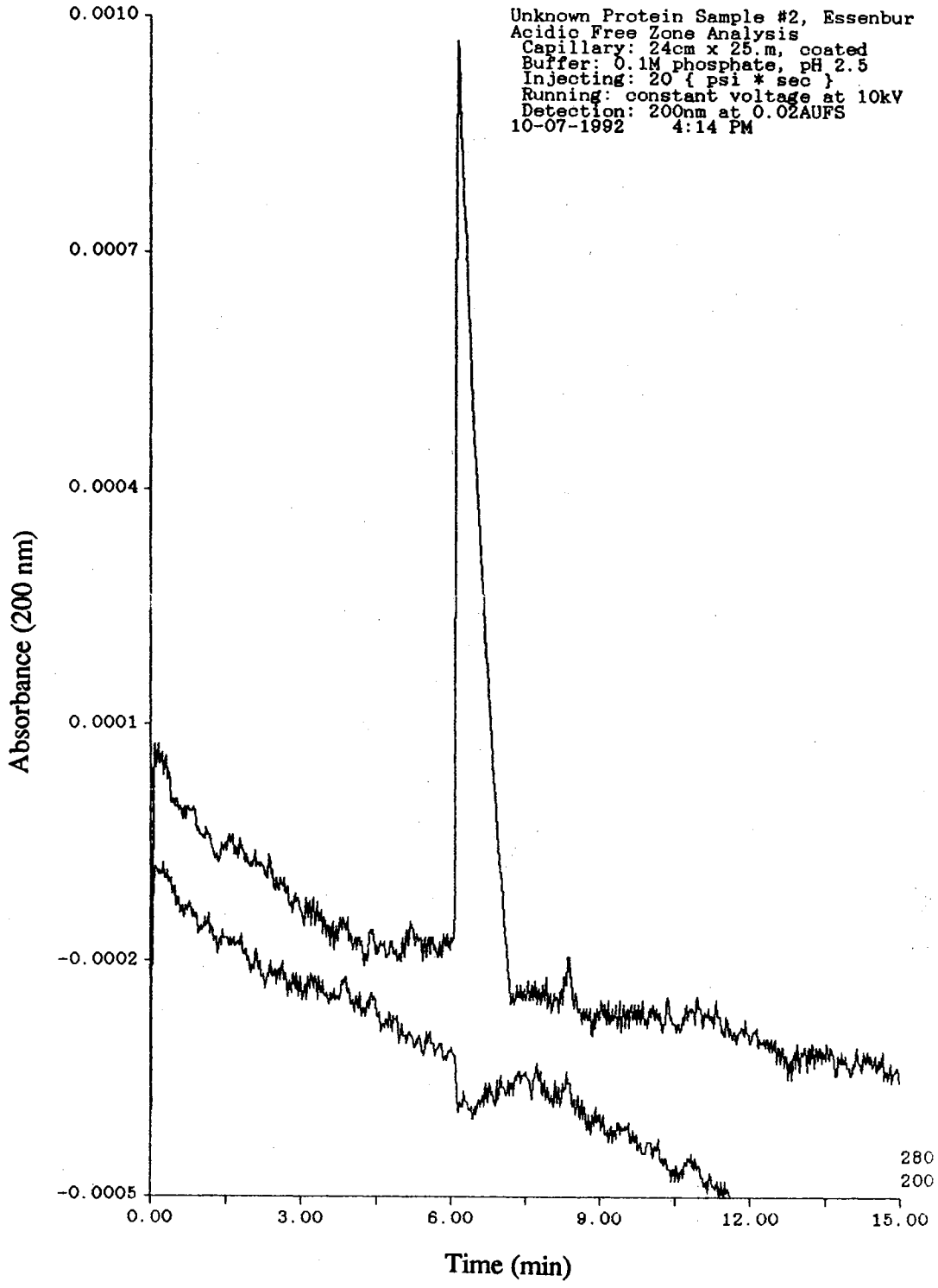

0-500 mM (180 min) KCl gradient
Absorbance (280 nm)
0.10
0.05
0
6000
5000
2500
0
dpm/fraction
90
100
110
120
Time (min)
Active
Fractions

Figure 7. Capillary electropherogram of Fraction 112 from final MonoQ chromatography of Activity II (δ-cadinene synthase).

Fraction 112 from the final MonoQ chromatography of Activity II (δ-cadinene synthase) was desalted into Hepes buffer (50 mM; pH 7.2) with 1mM dithiothreitol, 10 mM $MgCl_2$, and 10% glycerol by repeated concentration/dilution with Waters Ultrafree spin concentrators. The sample was then chromatographed by acidic free zone analysis in a Bio-Rad coated capillary, 24 x 25 μm. The upper tracing is A_{200}.

Instrument: Bio-Rad BioFocus Capillary Electrophoresis System
Chromatography performed by Jeff Verdier of Bio-Rad.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Acidic Free Zone Analysis
Capillary: 24cm x 25.m, coated
Buffer: 0.1M phosphate, pH 2.5
Injecting: 20 { psi * sec }
Running: constant voltage at 10kV
Detection: 200nm at 0.02AUFS
10-07-1992 4:14 PM
0.0010
0.0007
0.0004
0.0001
-0.0002
-0.0005
Absorbance (200 nm)
0.00
3.00
6.00
9.00
12.00
15.00
Time (min)
280
200

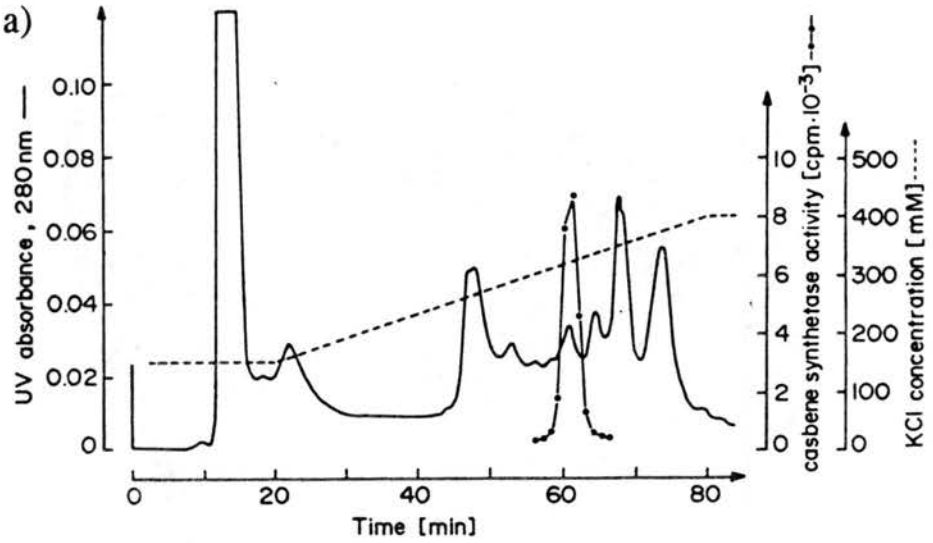
The somewhat confusing co-existence between a chromatography step that apparently should not deliver a homogeneous protein and a subsequent high resolution analysis which reveals that some of the eluted protein is (at least effectively) homogeneous is similar to that seen in a published purification. In the purification of the diterpene cyclase casbene synthetase, Moesta and West (4) performed a final chromatography of their protein mixture on anion-exchange media (DEAE) with a very shallow salt gradient (chromatogram reproduced in Figure 8a). As shown in the chromatogram of the separation, the fractions containing casbene synthetase activity (denoted by dotted peak) were "crowded" on either side by fractions that (apparently) contained undesired proteins. It was found that by making a "...cut containing 60% of the enzyme..." apparently homogeneous protein could be recovered. After the appropriate fractions were pooled, SDS-polyacrylamide gel electrophoresis (reproduced in Figure 8b) of the "cut" revealed a single band of apparently homogeneous protein. Although our purification of a sesquiterpene cyclase (δ-cadinene synthase) may not be directly comparable to that of the diterpene synthase (casbene synthetase), it is interesting to note that, in both purifications, judicious collection of fractions from a complex chromatography resulted in recovery of apparently homogeneous enzyme, at the expense of a reduction in recovery of total activity. Regardless of the apparent achievement of homogeneity, it may still be that the "homogeneous" protein (in either purification) could be contaminated with a modest concentration of multiple undesired proteins, each of which is present at low concentration. If this were the case, the numerous low-abundance contaminant proteins conceivably could "fade into the baseline" during high resolution electrophoretic analysis and the analyst would be deceived in thinking that the "homogeneous" enzyme is 100% pure. By rigorous monitoring of total protein contents and slight overloading of the electrophoretic method (to assure visualization of low-abundance contaminants), the analyst may avoid the error of describing an impure enzyme preparation as "homogeneous".

Figure 8. Example of isolation of homogeneous enzyme (the diterpene cyclase casbene synthetase) from a complex mixture as performed by Moesta and West (4):

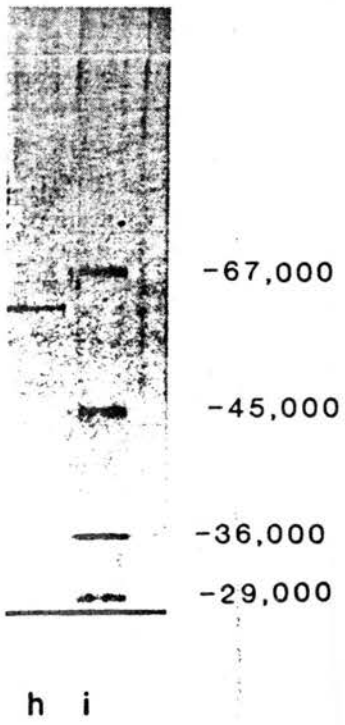
a) Final anion-exchange HPLC separation to isolate fractions active in casbene synthetase (inset at 60 minutes).

b) SDS-polyacrylamide gel electrophoresis of 250 ng of the isolated casbene synthestase (lane h)
and protein standards. Protein standards include: bovine serum albumin (Mr 67 000), eff albumin (Mr 45 000); glyceraldehyde-3-phosphate dehydrogenase (Mr 36 000) and carbonic anhydrase (Mr 29 000) (lane i).

a)


UV absorbance, 280nm —
0.10
0.08
0.06
0.04
0.02
0
0
20
40
60
80
Time [min]
casbene synthetase activity [cpm·10⁻³]----
10
8
6
4
2
0
KCl concentration [mM]-----
500
400
300
200
100
0

b)


-67,000
-45,000
-36,000
-29,000
h i

Viewed in a favorable light, the chromatogram of the final MonoQ elution of Activity II (Figure 6) may reflect a mixture of Activity II with some non-protein contaminant which has substantial absorbance at 280 nm. However, the possibility of a worst-case scenario cannot be ignored. The chromatogram may reflect chromatography of Activity II which is substantially contaminated with undesired protein, thus the capillary electrophoretic chromatography may simply be revealing that the predominant protein is a contaminant, while the δ-cadinene cyclase is a very small part of the total recovered protein. However, this is unlikely as the δ-cadinene synthase activity would be exhibiting a very high turnover number; purified sesquiterpene cyclases have exhibited low turnover numbers (5). A substantial scale up of the enzyme purification and incorporation of a high capacity and high resolution step (e.g., preparative scale electrophoresis by a Bio-Rad Prep Cell) may allow recovery of a homogeneous cyclase in amounts that can be measured with more confidence.

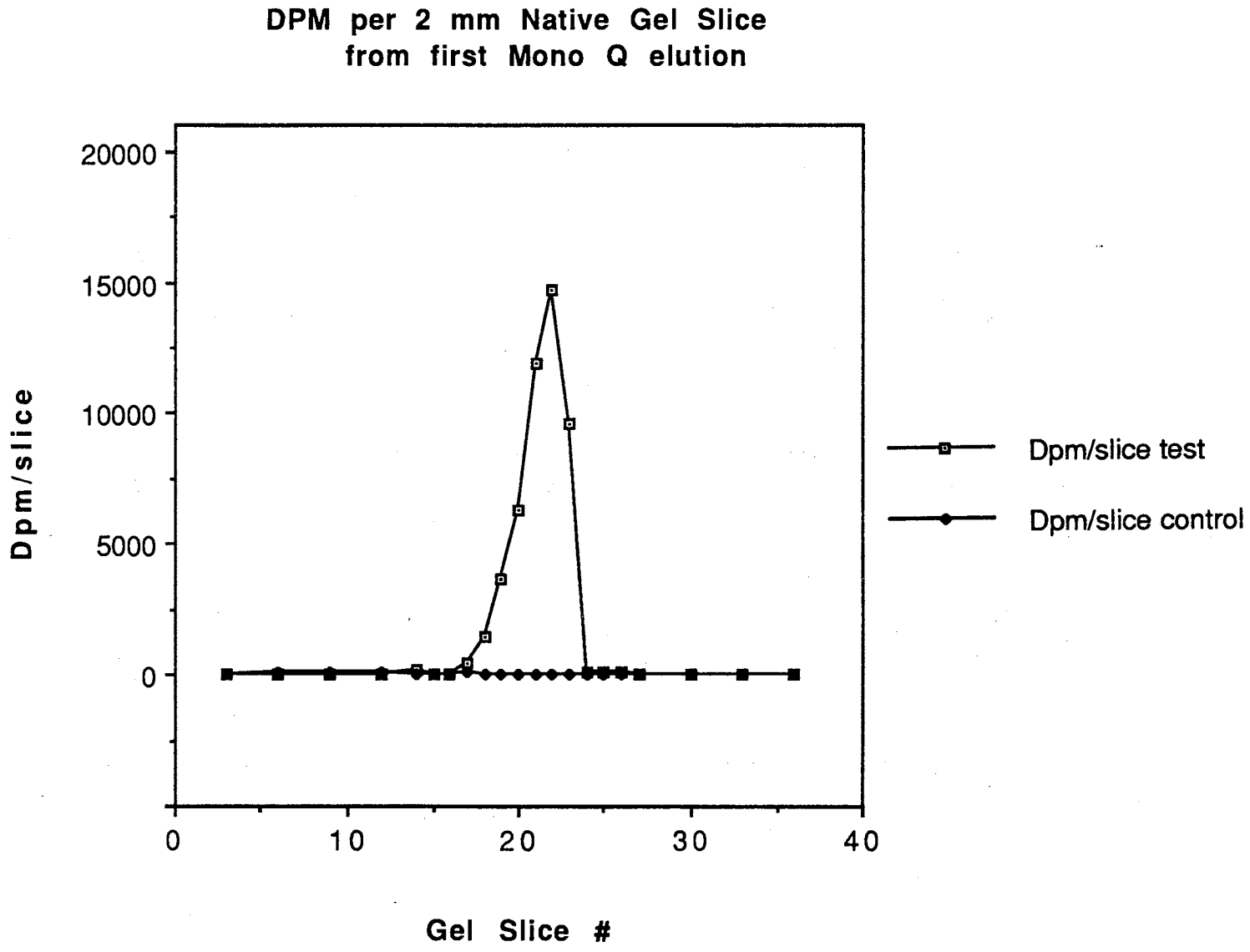
Molecular weight

The molecular weight of the enzyme was determined by gel permeation chromatography (GPC 300, Synchrom). Calibration of the column with a series of standards (670 to 17.2 kDa) indicated a molecular weight of M_r 39.2 kDa for Activity II and a molecular weight of M_r 27.8 kDa for Activity I. Activity II and the ovalbumin standard (44 kDa) exhibited similar elution in the native PAGE system. The pattern of elution in the native PAGE system is shown in Figure 9. Although the native PAGE system was not calibrated with a standard of lower molecular weight than ovalbumin, it did display retention consistent with an M_r of slightly lower molecular weight than ovalbumin, which is consistent with the calculated molecular weight for Activity II by the calibrated gel permeation column.

Figure 9. Native Gel Electrophoresis of Activity II (δ-cadinene synthase). Activity II from the first MonoQ elution migrated in a native gel system.

At cessation of electrophoresis, the gel lane containing δ-cadinene synthase was cut into 2-mm slices. Each slice was individually homogenized in a separate micro-mortar and pestle in 1 ml assay buffer. After centrifugation, the supernatant was removed and incubated with [1-^{3}H]FPP at 30°C in glass tube, then extracted with 2 x 1.5 ml hexane. Polar compounds were removed from the hexane extract by silica gel, then one ml of the hexane extract was assayed by scintillation counting.

A lane that was loaded with buffer was treated in the same way as a control.


DPM per 2 mm Native Gel Slice
from first Mono Q elution
Dpm/slice
20000
15000
10000
5000
0
0
10
20
30
40
Gel Slice #
Dpm/slice test
Dpm/slice control

Olefin synthesis by δ-cadinene synthase

δ-Cadinene is constitutively produced in glanded cotton tissues (34). In contrast, δ-cadinene is not produced constitutively in glandless cotton tissues (36, Davis, G. D. *et al.*, unpublished results), but is accumulated in Westburn M *gl* (glandless) tissues inoculated with *Xanthomonas campestris* pv. *malvacearum* or by infiltration with sterile biotic elicitor. δ-Cadinene is the predominant sesquiterpenoid product of crude homogenate cell-free reactions catalyzed by homogenates of *Xcm*-inoculated or elicitor-injected WbM*gl* tissues which utilize either radioactive or non-radioactive FPP (**1**) as substrate (Davis, G. D. *et al.*, unpublished results).

By separating the product(s) generated by the cell-free assays during purification, a number of observations were made. It was found that both Activity I and Activity II biosynthesize δ-cadinene, with Activity II having higher specific activity and being more stable. Given the lower apparent molecular weight, instability, and lower biosynthetic efficiency of Activity I (relative to Activity II), it is possible that Activity I is an enzymatically degraded and/or structurally rearranged form of Activity II. The appearance of multiple enzyme forms varying in molecular weight that synthesize the same product has also been found during cyclase activity purification in a number of other systems (5, 11) Purification of larger amounts of both forms of δ-cadinene synthase and sequencing of these two proteins should clarify their relationship.

A number of researchers have proposed that δ-cadinene arises though the cyclization of *trans, trans*-FPP (**1**) to germacrene D (**4**) (or other intermediate such as nerolidyl pyrophosphate) and then the intermediate rearranges or is enzymatically transformed into δ-cadinene (17-19). Monitoring of cell-free reaction products by HPLC and gas chromatography of *Xcm*-inoculated WbM *gl* tissues (Davis, G. D. et al., unpublished work) failed to reveal any quantifiable accumulation of germacrene D during the biosynthesis of δ-cadinene, despite extraction methods that should have permitted recovery of the modestly unstable germacrene D. Also, throughout the purification, at least for

Activity II, specific activity increased, supporting the hypothesis that a single enzyme activity was being purified and thus δ-cadinene biosynthesis was not dependent on a second enzyme that would generate free germacrene D. Given the radiochemical, chemical, and enzymatic evidence, it appears the inducible biosynthesis of δ-cadinene in cotton can be accomplished by a single enzyme activity that converts FPP (**1**) to δ-cadinene (**3**) without release of detectable intermediates. This proposed biosynthesis by one enzyme is illustrated as Scheme 1 or Scheme 2 in Figure 10. The evidence does not rule out alternate mechanisms that could allow germacrene D (or other equivalent compound) to play a role in δ-cadinene biosynthesis. Since some monoterpene cyclases (2) and at least one sesquiterpene cyclase (10) have been found to be multifunctional, it is possible that the purified enzyme could first convert FPP to germacrene D, then convert germacrene D to δ-cadinene so rapidly that germacrene D never accumulates in solution to detectable levels. This possibility is illustrated by Scheme 3 in Figure 10. This biosynthetic pathway is unlikely, as the δ-cadinene synthase does not produce a multiplicity of products as seen in the known examples of multifunctional monoterpene (2, 5) and sesquiterpene cyclases (10).

Whitehead and colleagues have proposed a double cyclase system for the biosynthesis of 5-*epi*-aristolochene (37): a first cyclase to convert FPP to germacrene A and a second cyclase to convert the germacrene A to 5-epi-aristolochene. A similar biosynthetic pathway for δ-cadinene is shown in Scheme 4 in Figure 10. This arrangement is very unlikely for our system as it would require the two enzymes to effectively co-chromatograph and would require the second enzyme to rapidly convert the germacrene D to δ-cadinene to account for our failure to detect the germacrene D. The sesquiterpene cyclases that have been rigorously characterized appear to be single enzymes which fail to release detectable amounts of free intermediates (3, 5, 6, 38, 39).

As another alternative, germacrene D (or equivalent) could be very unstable and be rapidly converted to δ-cadinene by a non-enzymatic mechanism(s); this possible route is

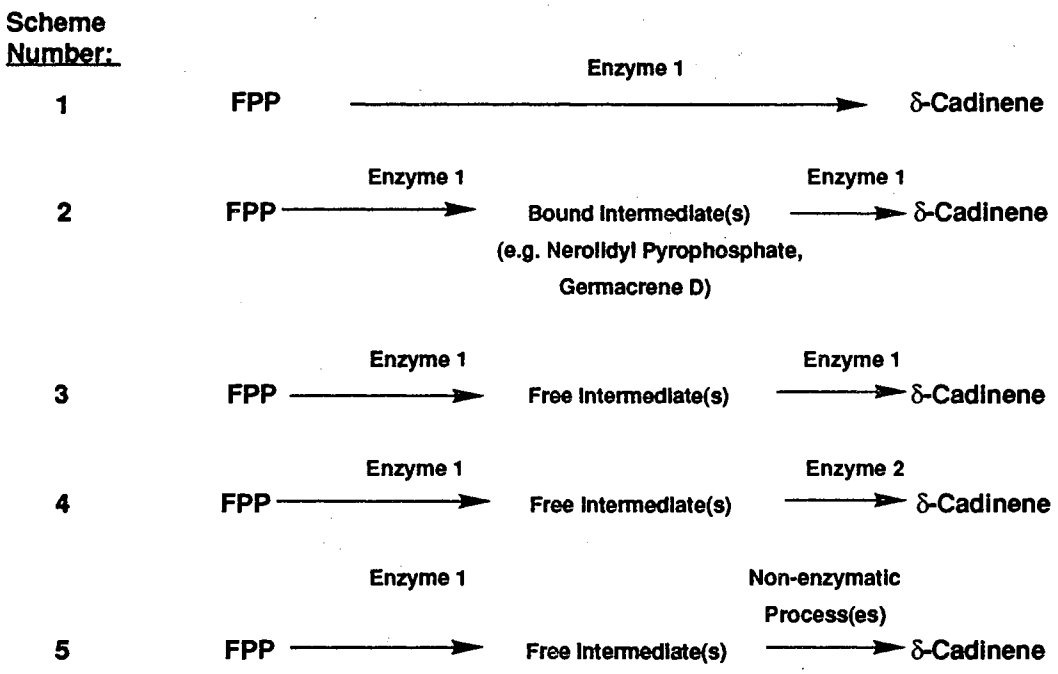


Figure 10. Proposed schemes for the directing of metabolic flux involved in biosynthesis involving cyclase enzymes.

shown by Scheme 5 in Figure 10. Germacrene D would not likely be a participant in this mechanism, as it is sufficiently chemically stable to be isolated routinely in a number of essential oils (40). Our cell-free assays were conducted under conditions of mild temperatures (30°C) under low light conditions, and in one instance without contact with glass, yet no germacrene D was ever detected. If germacrene D were a free intermediate, these conditions should have increased the accumulation of any free germacrene D by diminishing any non-enzymatic conversion (22,23) of germacrene D to δ-cadinene.

Our purification will be scaled up and modified to obtain sufficient enzyme for partial sequencing, antibody generation, classical kinetic studies and generation of sufficient labelled δ-cadinene for production of labelled biosynthetic intermediates on the pathway to the sesquiterpene phytoalexins such as DHC (**2**).

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CHAPTER VII

CONCLUSIONS AND PROJECTIONS

This work has revealed important findings concerning the cyclase enzyme which may catalyze the first reaction in the biosynthetic pathway from FPP to sesquiterpenoid phytoalexins of cotton. The findings from each chapter are summarized in Figure 1.

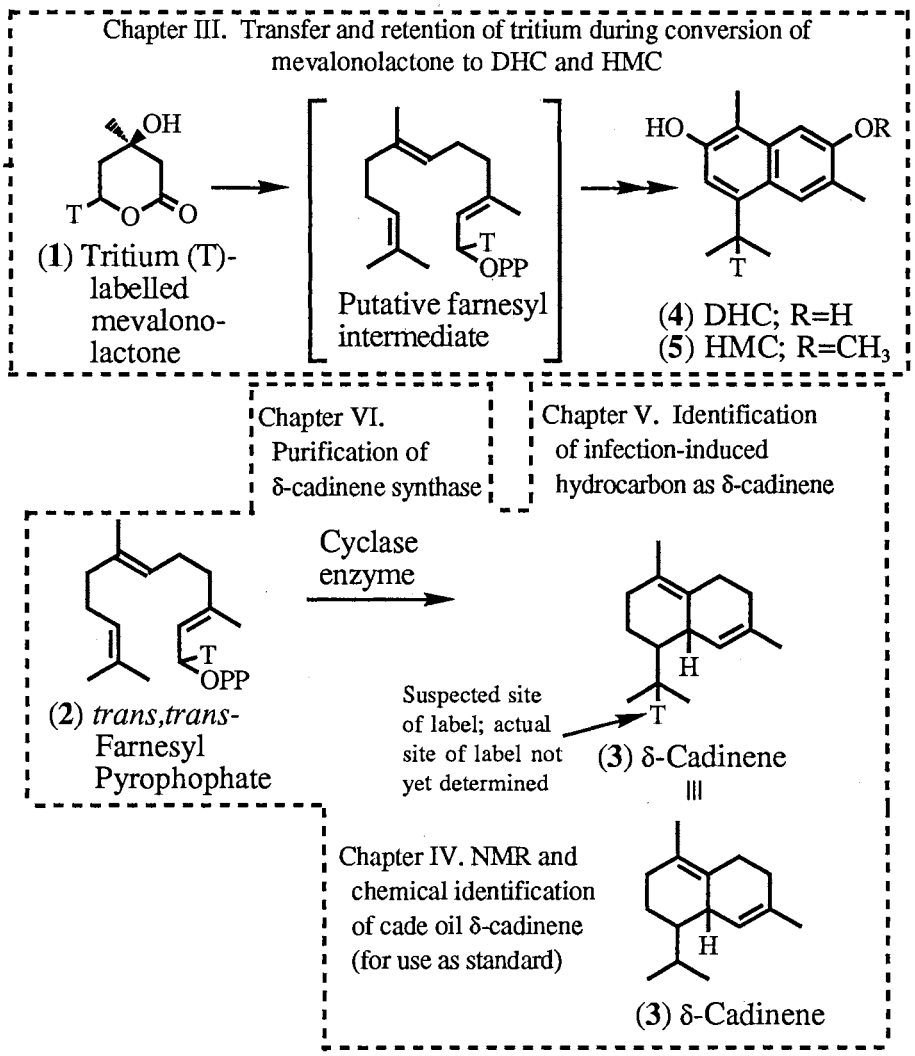

Figure 1. Summary of experimental findings detailed in each chapter.

Conclusions

Tritium Transfer during Biosynthesis

The incorporation of label from mevalonolactone into the sesquiterpenoid phytoalexin DHC and into HMC at reasonable efficiency was strong proof of the terpenoid origin of these compounds (1/96 and 1/60 fold isotopic dilution of ^{14}C for DHC and HMC, respectively). The determination that a tritium transfer occurred from C-1 to C-11 (methine carbon of isopropyl side chain) of the putative farnesyl precursor during HMC biosynthesis showed that these cadalene type compounds undergo a cyclization process similar to that found for the prominent cotton terpenoid compound, gossypol. This rigorous determination of labeling pattern during the biosynthesis of the sesquiterpenoid phytoalexins increased confidence that [1-^{3}H]FPP could be used to assay cyclase enzyme activity and to label biosynthetic intermediates from [1-^{3}H]FPP to the end-product sesquiterpenoid phytoalexins (and HMC).

NMR characterization of δ-cadinene from cade oil

This work was important because it firmly supported the generally accepted structure of δ-cadinene and showed that the alternative proposed structure (ω-cadinene) could not be the correct molecular representation of δ-cadinene. This information was very important in the proper identification of the product of the cyclase enzyme. With this firm identification of the product formed from *trans,trans*-FPP, along with future purification and characterization of the enzyme, there is a high possibility that an Enzyme Commission number can be obtained for this enzyme (26).

A prominent finding of this work was the exhibition by δ-cadinene of ^{1}H-^{1}H COSY long-range connectivities and of ^{1}H-^{13}C HETCOR long-range correlations. Even though the NMR characteristics of δ-cadinene are not totally consistent with the traditional textbook rules of NMR behavior (77), it was found that the 2D NMR plots of other terpenoid

compounds (78) displayed patterns of long-range couplings and long-range correlations similar to those seen in 2D NMR plots of δ-cadinene. These types of couplings are being more widely recognized in the literature (78-81).

Another outcome of this work is that the NMR characterization of δ-cadinene has supported assignments of the unconventional NMR characteristics of other potential biosynthetic intermediates of molecular weights 216, 218, and 232 that are likely intermediates in the biosynthesis of the cotton sesquiterpenoid phytoalexins (DHC, LC, LCME) and HMC. An additional benefit of this work is that the standard δ-cadinene which was isolated from cade oil will have further usefulness in isotopic dilution experiments to test whether δ-cadinene is an intermediate in the conversion of [1-^{3}H]FPP to the sesquiterpenoid phytoalexins.

Identification of δ-cadinene as the enzyme product of the cyclase enzyme (δ-cadinene synthase)

This portion of the research proved identity of the compound produced by cyclase action on FPP. It was found that δ-cadinene accumulated in *Xcm*-inoculated and elicitor-injected glandless cotton tissues and was generated from non-radioactive FPP in cell-free reactions. Furthermore, cell-free reactions utilizing [1-^{3}H]FPP generated ^{3}H-labeled δ-cadinene.

Supporting the prominence of δ-cadinene as a cyclase product was the discovery that only minor amounts of other sesquiterpenoid compounds were found to accompany accumulation or biosynthesis of δ-cadinene. Minor amounts of α-muurolene and ε-cadinene were the only other sesquiterpene hydrocarbons that accumulated in *Xcm*-inoculated tissues, and a minor amount of α-cadinene was generated in the cell-free reaction utilizing non-radioactive FPP. No likely products of sesquiterpene cyclase action were found in $CaC0_3$-injected cotton tissues, in uninoculated tissues, or in *Xcm* cells and bacterial broth prior to inoculation.

Purification of Cyclase (δ-cadinene synthase)

The isolation of the cyclase enzyme (Activity II) was accomplished with a purification of approximately 320-fold (perhaps an underestimate of purification due to the overestimation of the protein concentration in the final fraction). The lower catalytic efficiency conversion of *trans, trans*-FPP to δ-cadinene by the lower molecular weight Activity I and its lower stability compared to Activity II was consistent with the speculation that Activity I (27.8 kDa) may be a proteolytically clipped form of the more efficient Activity II (39.2 kDa).

The purification also revealed that δ-cadinene synthase possesses characteristics common to many sesquiterpene cyclases. Activity II (39.2 kDa) is within the molecular weight range of sesquiterpene cyclases (40-100 kDa), has hydrophobic characteristics, and is unstable (especially in crude homogenates).

No obvious intermediate compound (*e. g.*, germacrene D or nerolidol) was detected in the conversion of FPP to δ-cadinene. This strongly suggests that, like other reported sesquiterpene cyclases, δ-cadinene synthase is a single enzyme and converts substrate to product without detectable accumulation of free intermediates.

Projections Concerning the Major Areas of Research

Tritium transfer

The results of this experimentation have already paid dividends. Having identified [1-^{3}H]FPP as a suitable substrate for assay of cyclase activity, the substrate is also being used to help identify other compounds that are in the biosynthetic pathway. Any intermediate should incorporate label from [1-^{3}H]FPP at a specific radioactivity higher than that of the end-product phytoalexins.

NMR characterization of cade oil δ-cadinene

The 1D and 2D NMR characterization of cade oil δ-cadinene will help to assist NMR identification of any other sesquiterpenoid intermediates found during investigation of the biosynthetic pathway leading to the sesquiterpenoid cotton phytoalexins. The documentation of the unexpected characteristics of δ-cadinene NMR spectra (^{1}H-^{1}H COSY long-range connectivities, long-range ^{1}H-^{13}C HETCOR correlations) may help explain any surprising NMR spectra generated by putative intermediates.

Identification of δ-cadinene as enzyme product of δ-cadinene synthase

The identification of δ-cadinene as the product of purified δ-cadinene synthase activity has shown that it is unnecessary to investigate any other potential intermediates such as Germacrene D or nerolidol pyrophosphate. Having found δ-cadinene to be the cyclase enzyme product, we can build a "bridge" to sesquiterpene metabolic intermediates. It may be possible to use cell-free reactions to convert larger amounts of [1-^{3}H]FPP to ^{3}H-labelled-δ-cadinene. If the [^{3}H]δ-cadinene is then used as substrate in a cell-free reaction, labelled intermediates that lead to the sesquiterpenoid phytoalexins (DHC, LC, and LCME) and HMC may be identified. Repetition of this technique could lead to the identification of all compounds in the pathway leading to the sesquiterpenoid phytoalexins and HMC. Alternatively, it may be found that δ-cadinene is not involved in the synthesis of the sesquiterpenoid phytoalexins and HMC. It could be that δ-cadinene is involved in the biosynthesis of other terpenoid compounds or is simply a minor end-product of secondary metabolism.

Purification of the δ-cadinene synthase

The initial purification of δ-cadinene synthase which yielded very small amounts of very pure enzyme, will lead to a scaled-up enzyme isolation to allow recovery of increased

amounts of the enzyme. The use of a preparative scale native PAGE electrophoresis (Bio-Rad Prep Cell) and possibly a preparative isoelectric focusing device (Bio-Rad Rotofor) should assist scale-up of the purification (63). Purification will be validated by the use of higher resolution methods, *e.g.*, analytical-scale native, SDS PAGE and capillary electrophoresis. The increased amount of enzyme will allow for performance of classical kinetics studies, generation of antibodies, and partial sequencing of the enzyme.

The increased availability of synthase will allow for full characterization of the enzyme, including intensive investigation of the active site of the enzyme. Investigations may also be made concerning the hydrophobic nature of the enzyme, i.e., whether the enzyme requires association with membrane fractions for maximal activity and/or stability. With full purification and characterization, the δ-cadinene synthase should be qualified for registration with the International Union of Biochemistry (26).

Additional General Projections

Investigation of other phytoalexin intermediates and of a possible connection to gossypol biosynthesis

In addition to the proposed investigation of the biosynthesis of the sesquiterpenoid phytoalexins and HMC mentioned above, δ-cadinene may provide a starting point in the study of the metabolism of other sesquiterpenoid compounds. Because δ-cadinene is a normal constituent of healthy glanded cotton, there is a possibility that δ-cadinene may be a biosynthetic precursor of gossypol and other cadalene-type compounds produced in glanded cotton and secreted into the glands. If further investigations show that gossypol is generated during the incompatible reaction between resistant WbM*gl* cotton tissue and *Xcm*, then the role of δ-cadinene in gossypol biosynthesis could be investigated in our biochemically "cleaner" system of glandless cotton tissue. If our system does not produce measurable gossypol, the investigation could be carried on by infection with *Verticillium*, which has been shown to cause accumulation of gossypol in glandless cotton bolls (82).

[Gossypol and related terpenoids are not found in the tissues of healthy glandless cotton (83)].

If intermediates leading to gossypol can be found in our system, new possibilities for the genetic engineering of glanded cotton plants will be available. Identification of the metabolites will make possible identification and partial sequencing of the enzymes responsible for their conversion. Full characterization of the enzymes could lead to identification of genetic and/or biochemical factors controlling the activity of the enzymes. Creation of slightly modified enzymes based on those found *in planta* might lead to production of a more desirable mixture of defense compounds or may be active in a favorable developmental fashion (active in leaves and stems of the plant, but not in the cotton seed). More powerful will be experiments to find what genetic sites control the expression of the DNA responsible for production of these enzymes. Once the controlling genes are found, transformation of these sequences (and comparison to any homologous sequences in glandless cotton) may yield insight into the mechanisms by which production of gossypol is controlled and can be modified. These studies could be very beneficial, as proper alteration of gossypol production could lead to cotton plants that can be more fully utilized. One example of the possible benefit would be the production of cotton plants that could produce glandless cotton seed, yet produce sufficient gossypol and related compounds in the remainder of the plant to discourage herbivory. The glandless seed produced by this type of cotton plant could be used as meal for humans and ruminants, whereas glanded cotton seed can not be used as a food source for mammals. (Researchers are currently trying to obtain the combination of a glanded cotton plant that produces glandless seed by means of cytogenetic techniques such as wide crosses and embryo rescue (84).

Testing the importance of the sesquiterpene phytoalexins in resistance to Xcm

Identification of δ-cadinene as a possible precursor of the sesquiterpenoid phytoalexins identified in cotton (DHC, LC, and LCME) may allow a test of the hypothesis that phytoalexins are responsible for the inhibition of plant pathogens *in planta*. Once a partial sequence of the δ-cadinene synthase is determined, it may be possible to transform resistant cotton plants (*e.g.*, the line OK1.2) to produce anti-sense RNA for that sequence. The production of phytoalexins in these plants should in consequence be genetically blocked. The contribution of the phytoalexins to the resistance response could then be assessed by inoculation of the plants with *Xcm*. If the phytoalexins are crucial to resistance, these re-engineered plants should be susceptible to bacterial infection. However, if the phytoalexins are not crucial to resistance to the *Xcm*, the inoculated plants may successfully resist the bacterial infection. The current system under study may be well suited to this sort of study because the genetic block of the conversion of FPP to δ-cadinene will probably not deprive the transformed plant of needed metabolites. This speculation is supported by the observation that glandless cotton lines, which do not have δ-cadinene synthase activity under normal conditions, are in the absence of herbivorous pests as robust as their glanded counterparts. Thus, the developmental abnormalities seen during genetic blocking of an early step in isoflavanoid phytoalexin biosynthesis (85) may be avoided. Additionally, the cotton plants (especially glanded ones) have many alternate routes for utilization of any additional farnesyl pyrophosphate that might accumulate during bacterial infection of plants which are genetically blocked at δ-cadinene synthase. Thus there is unlikely to be significant pooling of farnesyl pyrophosphate and resulting toxic effects as are likely to occur in plants blocked in late steps of isoflavanoid phytoalexin biosynthesis (86).

Utilization of secondary metabolism of synomones

It has been found that some volatile sesquiterpene compounds released by wounded cotton tissues can attract beneficial insects which are predatory to herbivorous insects. These beneficial compounds are called "synomones". Given the rich mixture of volatile sesquiterpenes accumulated constitutively in glanded cotton tissues (87,88), much genetic diversity exists to allow customizing of the possible synomones to lure whatever insects are most beneficial to the cotton plants in any particular farming location. Although current investigations concern the biosynthesis and release of these attractant compounds upon herbivory (89), it may be beneficial to engineer plants that have constitutive production of beneficial synomones. Production of volatile sesquiterpenes that lure harmful organisms could be abolished. Genetic techniques could also be used to produce transformed plants capable of biosynthesizing synomones not normally found in cotton which attract beneficial insects.

Investigation of allelochemical potential of δ-cadinene

It may also be useful to modify production of δ-cadinene may also be modified for allelochemical purposes. It has been found (90) that δ-cadinene volatilized from the Devil's Claw plant (*Proboscidea louisicnica*) can function as an allelochemical that inhibits cotton and wheat. This could lead to the utilization of δ-cadinene by different routes. In the allelochemical study, it was found that some cultivars of cotton do not suffer negative effects from the δ-cadinene which comes from the Devil's Claw plant (90). Since other cotton cultivars produce δ-cadinene constitutively, it may be possible to find cotton cultivars that produce δ-cadinene, are not themselves inhibited by it, and release it as an allelochemical to create a barren zone around the cotton plant. Planting of such cotton cultivars could result in decreased need for herbicides to inhibit growth of weeds in cotton fields. Alternatively, if the production of δ-cadinene actually causes a self-inhibition of a cotton plant or is harmful to neighboring cotton plants, it may be advantageous to create

cotton plants by genetic methods or conventional breeding that do not produce δ-cadinene. This may permit closer planting of cotton plants (due to diminuation of allelochemical effects) and lead to higher yields per acre.

Possible use of δ-cadinene in biocides.

It has been shown that the δ-cadinene and other sequiterpenes from green tea leaves exhibit anti-bacterial qualities (91). δ-Cadinene was found to inhibit the growth of *Propionbacterium acnes* in *in vitro* assays. Given that the δ-cadinene is potentially available from natural sources such as tea leaves or cotton tissue, it is possible that δ-cadinene or a crude mixture of δ-cadinene with other terpenes could be obtained for use as a natural, non-toxic (to humans) medicinal or cleansing biocidal solution. If recovery from natural sources is not practical, δ-cadinene can be chemically synthesized (92,93) (though only in low percentage yield).

Compounds that are structurally related to δ-cadinene, such as gossypol derivatives, have been found to inhibit replication of the human immunodeficiency virus type I (AIDS virus) (94). Other potential anti-bacterial cadinene compounds have been identified (95). Further pharmaceutical and toxicological screening of δ-cadinene and similar compounds may reveal new, safe medicinal compounds that may be obtained from natural sources.

Final Summation

The work presented here is only an elucidation of a very small part of an important metabolic pathway in cotton. The combination of the intricate biological machinery and agronomic importance of the cotton plant provides the best of both worlds for the scientific investigator: a playground in which experimentation can reveal the elegant inner working of the organism and a chance to apply the results of the experiments to the positive goal of increased yields from cotton plants. Investigations may yet produce the "dream" plant: a cotton plant that is glandless or produces glandless seeds, produces its own biochemical

protection in the proper quantities and at the proper time, and requires less application of expensive or environmentally harmful chemicals. A cotton plant which produces cotton fiber and cottonseed meal suitable for human consumption could make a substantial contribution in the struggle to reduce the pervasive poverty and hunger in many parts of the world (96).

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: DELTA-CADINENE SYNTHASE FROM HYPERSENSITIVELY RESPONDING COTTON COTYLEDONS: IDENTIFICATION OF SUBSTRATE AND PRODUCT AND PARTIAL PURIFICATION OF THE ENZYME

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