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8-Hydroxy-(+)-δ-cadinene is a precursor to hemigossypol in *Gossypium hirsutum*

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Dedicated to memory of Professor Jeffrey B. Harborne

Abstract

[^{3}H](+)-δ-Cadinene and its 8-hydroxy derivative, prepared from (1*RS*)-[1-^{3}H]FPP by the action of one and two recombinant enzymes, respectively, were infiltrated into cotyledons of bacterial blight-resistant cotton plants as they biosynthesized sesquiterpene phytoalexins in response to infection by *Xanthomonas campestris* pv. *malvacearum*. Following both treatments, tritium appeared in the HPLC fraction that contained hemigossypol. Hemigossypol was isolated from the cotyledons that had been treated with [^{3}H](+)-8-hydroxy-δ-cadinene and was trimethylsilylated and purified. In two experiments, specific radioactivity of the hemigossypol derivative indicated that 5% and 10%, respectively, of the [^{3}H](+)-8-hydroxy-δ-cadinene had been converted to hemigossypol.

Keywords: *Gossypium hirsutum*; Malvaceae, Cotton; *Xanthomonas campestris* pv. *malvacearum*; Biosynthesis; Radioactive tracer; Sesquiterpenes; Cytochrome P450; (+)-δ-Cadinene; 8-Hydroxy-(+)-δ-cadinene; Hemigossypol

1. Introduction

Cotton (*Gossypium* spp.) synthesizes a large group of sesquiterpenes with the cadinane carbon skeleton that function for defense against pathogens and herbivores (Bell, 1986; Bell et al., 1993; Stipanovic et al., 1999). Control of their synthesis is complex, following a developmental program in healthy plants and also responding to stresses of infection, wounding, and chilling. Healthy plants accumulate gossypol **1** and related compounds in sub-epidermal, lysigenous glands of all green tissues and in epidermal cells of young roots (Bell and Stipanovic, 1977). Vascular fungal pathogens elicit production of desoxyhemigossypol (dHG **2**), hemigossypol (HG **3**), and their 3-methyl ethers as phytoalexins (Bell et al., 1993). The bacterial blight pathogen *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) elicits dHG **2**, HG**3**, and also cadalenes (**4**, **5**) and lacinilenes (**6**, **7**) as phytoalexins (Abraham et al., 1999). Wounding and chilling induce production of all of these sesquiterpenes (Bell and Christiansen, 1968; Essenberg et al., 1990).

The biosynthetic pathway to this large group of defensive sesquiterpenes offers possibilities for metabolic engineering to improve defenses of cotton to vascular fungal diseases and to herbivores or to transfer the superb bacterial disease resistance of some lines of cotton (Pierce et al., 1996) to other plant species (Essenberg, 2001). Alternatively, the economic value of cottonseed would be greatly enhanced if the pathway were suppressed in seed. Since cottonseed has a nutritious amino acid composition, prevention of gossypol production in the embryo would increase its usefulness for animal feed and could also provide edible protein products for human consumption (Lusas et al., 1978).

The first step in the pathway committed to biosynthesis of cadinanes is cyclization of *E*,*E*-*trans*-farnesyl diphosphate (FPP **8**) to (+)-δ-cadinene **9** (Fig. 1) (Benedict et al., 1995; Davis and Essenberg, 1995). Administration of (+)-δ-cadinene **9** labeled with tritium

Abbreviations: BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; δC, (+)-δ-cadinene; DHC, 2,7-dihydroxycadalene; dHG, desoxyhemigossypol; FPP, *E*,*E*-*trans*-farnesyl diphosphate; FW, fresh weight; HG, hemigossypol; HMC, 2-hydroxy-7-methoxycadalene; HOδC, 8-hydroxy-(+)-δ-cadinene; TMCS, trimethylchlorosilane; TMSi, trimethylsilyl; *Xcm*, *Xanthomonas campestris* pv. *malvacearum*.

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at C-10 or at C-12 to cotyledons of *Xcm*-inoculated *G. hirsutum* plants led to incorporation of tritium into cadalenes **4**, **5**, lacinilenes **6**, **7**, dHG **2**, HG **3**, and proposed biosynthetic intermediates **10**, **11** and **12** (Davis and Essenberg, 1995), providing evidence that (+)-δ-cadinene **9** is a precursor to all the cadinane-type sesquiterpenes of cotton. Since each of these cadinanes bears a hydroxyl group on either C-7 or C-8, but not on both C-7 and C-8, the pathway appears to have two branches. A cytochrome P450, CYP706B1, that hydroxylates

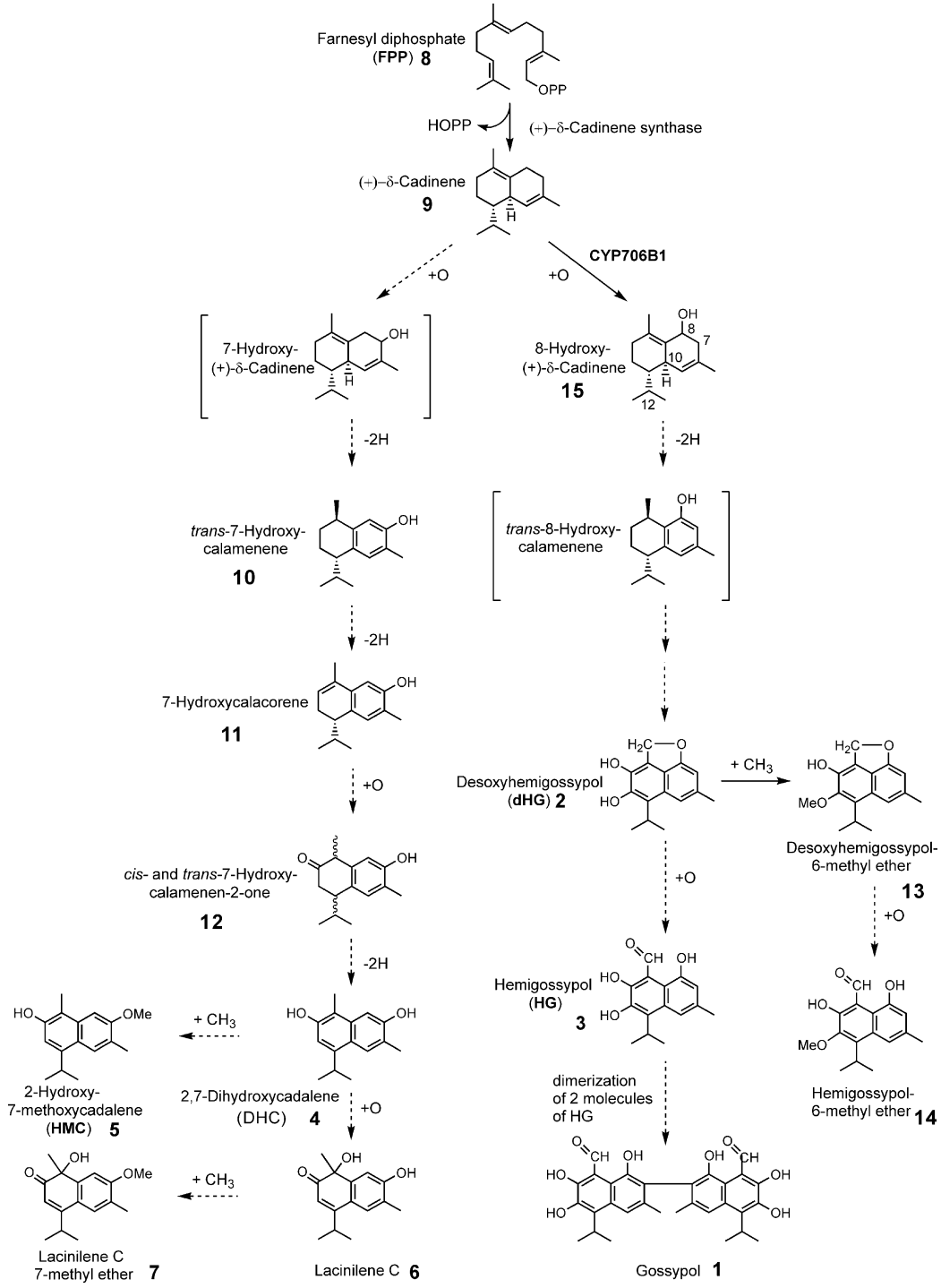
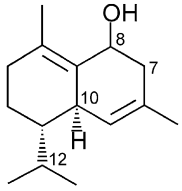


Fig. 1. Proposed biosynthetic pathway for biosynthesis of cadinane sesquiterpenoid phytoalexins and of the bi-sesquiterpene gossypol **1** in cotton cotyledons and leaves. Gossypol **1** is a component of the lysigenous glands of these tissues (Bell and Stipanovic, 1977), but is not a prominent component of the antibacterial hypersensitive response. All other compounds shown, except those in brackets, have been isolated from *Xcm*-inoculated cotton cotyledons (Davila-Huerta et al., 1995; Davis and Essenberg, 1995; and Stipanovic and Essenberg, unpublished). Solid arrows represent steps for which cDNAs have been cloned and functionally expressed.

(+)-δ-cadinene **9** at C-8 was cloned from *G. arboreum* (Luo et al., 2001), suggesting that (+)-δ-cadinene **9** itself is the branch-point intermediate. Fig. 1 presents a working model for the pathway, showing a branch leading from (+)-δ-cadinene **9** to the 7-hydroxylated cadalenes (**4**, **5**) and lacinilenes (**6**, **7**) and another leading to the 8-hydroxylated gossypol compounds (**1**–**3**, **13**–**14**).

Efforts to block production of gossypol **1** in cotton-seed through anti-sense suppression of (+)-δ-cadinene synthase have achieved as much as 70% reduction in gossypol **1** content (Martin et al., 2003). Complete suppression of biosynthetic flux to (+)-δ-cadinene **9** is made difficult by the existence of a family of at least six genes that encode (+)-δ-cadinene synthase in *G. arboreum* (Chen et al., 1995, 1996) and 12 such genes in *G. hirsutum* (Chen, 1998). The 8-hydroxylation step may be easier to block, since a southern blot indicated only one copy of *CYP706B1* in *G. arboreum* (Luo et al., 2001). Does its gene product, (+)-δ-cadinene-8-hydroxylase, catalyze a step in biosynthesis of gossypol **1**? Expression patterns of *CYP706B1* in various tissues of *G. arboreum* and *G. hirsutum*, in developing seeds of *G. arboreum*, and in *G. arboreum* suspension-cultured cells treated with *Verticillium dahliae* elicitors correlated well with levels and kinetics of accumulation of gossypol **1** and its derivatives (Luo et al., 2001). We report here results of a study designed to test whether the enzymic product of CYP706B1 catalysis, 8-hydroxy-(+)-δ-cadinene, is a precursor to hemigossypol **3**. We performed this work with *Xcm*-inoculated *G. hirsutum* cotyledons because of their dramatic induction of phytoalexin biosynthesis. Although the phytoalexin complement produced in this tissue in response to *Xcm* does not include gossypol **1**, hemigossypol **3** has been shown to be converted by peroxidase to gossypol **1** (Veech et al., 1976).

We prepared tritium-labeled 8-hydroxy-(+)-δ-cadinene **15** from (1*RS*)-[1-^{3}H]FPP **8**, using recombinant (+)-δ-cadinene synthase and recombinant (+)-δ-cadinene-8-hydroxylase. Benedict et al. showed that (1-*RS*)-[1-^{2}H]FPP **8** is converted by purified recombinant (+)-δ-cadinene synthase to [10-^{2}H] and [12-^{2}H] δ-cadinene **9** (by the carbon-numbering system shown here) (Alchanati et al., 1998). Their finding confirmed the inference from work of Davis et al. (1991) that cyclization involves a hydrogen transfer from C-1 of FPP **8** to the methine carbon of the isopropyl side chain, perhaps as a 1,3-hydride shift. Thus, our preparation of labeled (+)-δ-cadinene **9** from (1*RS*)-[1-^{3}H]FPP **8** is an equimolar mixture of [10-^{3}H](+)-δ-cadinene **9** and [12-^{3}H](+)-δ-cadinene **9**. We assumed that the enzyme-catalyzed 8-hydroxylation did not alter the positions of tritium labeling. Thus the tritium-labeled product of CYP706B1 that we infiltrated into cotton cotyledons was an equimolar mixture of [10-^{3}H]8-hydroxy-(+)-δ-cadinene **15**, whose tritium atom is expected to be lost during aromatization on the pathway to HG **3** (Fig. 1), and [12-^{3}H]8-hydroxy-(+)-δ-cadinene **15**, whose tritium atom is expected to be retained.



8-Hydroxy-(+)-δ-cadinene **15**

2. Results and discussion

2.1. Incorporation of tritium from [10-^{3}H](+)-δ-cadinene and [12-^{3}H](+)-δ-cadinene *9* into phytoalexins

Tritium from (+)-δ-cadinene **9** labeled on C-10 and C-12 was incorporated into both 7-hydroxylated and 8-hydroxylated cadinane phytoalexins in cotyledons of intact plants 60–68 h after inoculation with *X. campestris* pv. *malvacearum* (Davis and Essenberg, 1995). An incorporation experiment from the same labeled precursors was carried out to test whether cotyledons in the much more convenient experimental system (for radiochemical incorporation) of detached cotyledons in a glove box were also biosynthetically active. The incorporation period used was 30–39 h after inoculation, when dHG **2** and HG **3** production predominates over that of cadalenes (**4**, **5**) and lacinilenes (**6**, **7**) (Abraham et al., 1999). Tritium appeared in peaks migrating with the retention times of HG **3** and dHG **2**, which are incompletely resolved in this chromatographic system (20–21 min), of 2-hydroxy,7-methoxycadalene (HMC **5**, 28 min), of 8-hydroxy-(+)-δ-cadinene **15** (34 min), and of an unidentified metabolite (38 min) (Fig. 2).

2.2. Incorporation of tritium from a mixture of [10-^{3}H]8-hydroxy-(+)-δ-cadinene *15* and [12-^{3}H]8-hydroxy-(+)-δ-cadinene *15* into hemigossypol *3*

The most strongly labeled metabolite of 8-hydroxy-(+)-δ-cadinene **15** labeled on C-10 or C-12 eluted at 38–39 min (Fig. 3). It had also appeared in the product mixture from labeled (+)-δ-cadinene **9** (Fig. 2) and may be one of the yet-unidentified intermediates of the pathway. A small peak of radioactivity eluted with HG **3** at 20 min. Appearance of tritium in other peaks is not surprising, since there are five to six pathway intermediates between 8-hydroxy-(+)-δ-cadinene **15** and dHG **2**, which have not been identified.

The 20-min peaks from five such incorporations were pooled for further purification. To facilitate resolution of HG **3** from dHG **2**, as well as their purification from

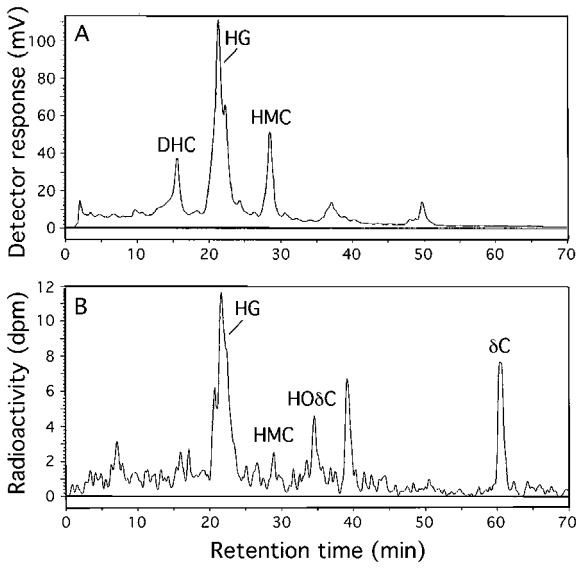


Fig. 2. Elution patterns of A_{230} (A) and radioactivity (B) of metabolites of [^{3}H](+)-δ-cadinene (δC) **9** in HPLC system 3. Extract from 0.15 gFW was injected.

other co-migrating metabolites, the pooled metabolites were trimethylsilylated. Authentic standards of TMSi-dHG and TMSi-HG resolved well in HPLC systems 1 and 2. The TMSi-derivatized, tritium-labeled dHG/HG samples were chromatographically separated using system 1. Very little tritium migrated with TMSi-dHG; however a tritium peak coincided with the TMSi-HG peak. The TMSi-HG fraction was concentrated and also chromatographically separated using system 2. Again a tritium-labeled peak coincided with the UV-absorbing peak of TMSi-HG (Fig. 4). The TMSi-HG fraction was collected. Its UV absorption spectrum was a good match to that of the standard TMSi-HG (described in Experimental Section 3.3). The other peaks shown in Fig. 4 exhibited UV absorption spectra distinct from that of TMSi-HG. Their tritium content indicates that they are other metabolites of 8-hydroxy-(+)-δ-cadinene **15**. Radioactivity in the TMSi-HG fraction was determined (Table 1). Results of two such incorporation experiments are presented in Table 1.

The isotopic dilution that occurred in planta can be computed from the specific radioactivities of the labeled precursor, 8-hydroxy-(+)-δ-cadinene **15**, and of the purified TMSi-HG. Specific radioactivity of the 8-hydroxy-(+)-δ-cadinene **15**, may be assumed to be the same as that of the (1-*RS*)-[1-^{3}H]FPP **8** from which it was prepared for the following reason. When Davis et al. (1991) administered a mixture of [2-^{14}C]mevalonolactone and [5-^{3}H]mevalonolactone to inoculated *G. hirsutum* cotyledons, ^{14}C and ^{3}H were incorporated into 2,7-dihydroxycadalene (DHC **4**) with the isotope ratio predicted from the structure of DHC **4** (retention of 50% of the tritium, since tritium on C-10 is expected to be lost during aromatization), indicating that no kinetic isotope effect discriminates against tritium-labeled molecules in the entire pathway from mevalonolactone to DHC **4**. Degradation analysis accounted for all the tritium on C-12, the methine carbon of the isopropyl group (Davis et al., 1991). Thus, although a carbon-tritium bond is broken during cyclization of [1-^{3}H]FPP **8** to [12-^{3}H](+)-δ-cadinene **9**, the bond-breaking step is evidently not rate-limiting. Therefore, the specific radioactivity of the (+)-δ-cadinene **9** that we prepared

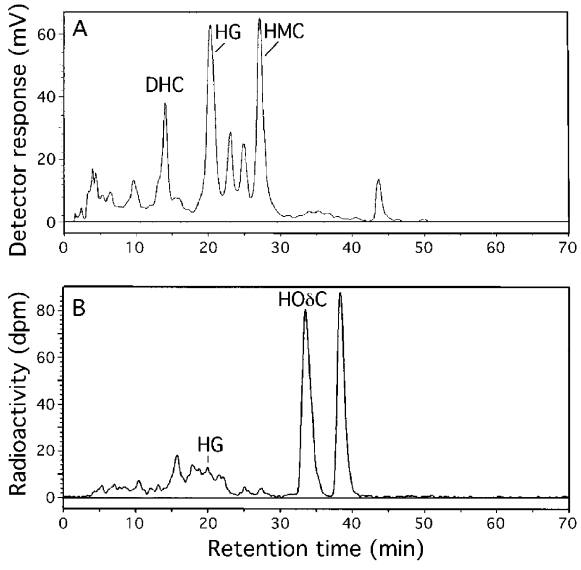


Fig. 3. Elution patterns of A_{214} (A) and radioactivity (B) of metabolites of [^{3}H]8-hydroxy-(+)-δ-cadinene **15** (HOδC) in HPLC system 3. Extract from 0.60 gFW was injected.

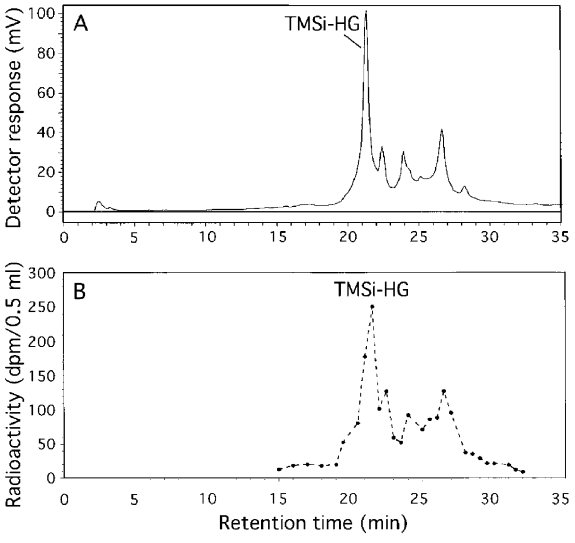


Fig. 4. Elution patterns of A_{230} (A) and radioactivity (B) of the TMSi derivatives of the HG **3**-containing fraction from [^{3}H]8-hydroxy-(+)-δ-cadinene **15** incorporation experiment no. 1 in HPLC system 2. The derivative obtained from 2.30 gFW of tissue was injected. Fractions were collected and subjected to liquid scintillation counting.

Table 1
Incorporation of [^{3}H]8-hydroxy-(+)-δ-cadinene **15** into hemigossypol **3** in cotton cotyledons[a]

Experiment	Tissue (gFW)	8-Hydroxy-(+)-δ-cadinene **15** fed		Hemigossypol **3** isolated		Incorporation (%)[e]
		(μmol)[b]	(dpm)	(μmol)[c]	(dpm)[d]	
1	4.66	0.0359	3.74×10^6	10.6	2.01×10^5	5.4
2	2.30	0.0184	2.45×10^6	0.472	2.28×10^5	9.3

[a] Cotyledons were inoculated with *Xcm* at 0 h, excised and infiltrated with [^{3}H]8-hydroxy-(+)-δ-cadinene **15** at 40 h, incubated under light at 32–35 °C, and harvested at 48 h.

[b] Micromol infiltrated = [8-hydroxy-(+)-δ-cadinene **15** (M)] × 8.9 μl intercellular space cm^{-2} (Miranda, 1993) × 61.8 cm^2 gFW^{-1} × quantity of treated tissue (gFW).

[c] Micromol isolated was computed from HPLC peak areas due to hemigossypol **3** (see Figs. 2 and 3).

[d] Dpm in HG **3** isolated from tissue = specific radioactivity of purified TMSi derivative of hemigossypol **3** (dpm μmol^{-1}, see Fig. 4) × μmol hemigossypol **3** isolated from tissue.

[e] Incorporation (%) = dpm in hemigossypol **3** isolated from tissue at 48 h ÷ dpm in 8-hydroxy-(+)-δ-cadinene **15** infiltrated at 40 h.

from (1-*RS*)-[1-^{3}H]FPP **8** by means of recombinant (+)-δ-cadinene synthase may be inferred to be the same as that of the FPP **8**. It is also reasonable to assume that presence of tritium on C-10 or C-12 of (+)-δ-cadinene **9** exerted no kinetic isotope effect upon its 8-hydroxylation, so that the specific radioactivity of 8-hydroxy-(+)-δ-cadinene **15** may be inferred to be the same as that of the (1-*RS*)-[1-^{3}H]FPP **8** from which it was prepared.

In the two experiments summarized in Table 1, the isotopic dilution that occurred during incorporation of [^{3}H]8-hydroxy-(+)-δ-cadinene **15** into HG **3** in planta was 5500- and 275-fold, respectively. The quantities of [^{3}H]8-hydroxy-(+)-δ-cadinene **15** that were injected in each experiment, listed in Table 1, were computed from the fresh weight of harvested tissue and the known intercellular volume of 14-day-old cotton seedlings' cotyledons, 550 μl gFW^{-1}. The quantities of HG **3** that were extracted from the harvested tissue in each experiment are also shown in Table 1. Had the entire amounts of injected [^{3}H]8-hydroxy-(+)-δ-cadinene **15** been incorporated into these amounts of HG **3**, the isotope dilutions would have been 295- and 26-fold, respectively, in the two experiments. If tritium on C-10 is lost during aromatization, incorporation of the entire amount of [12-^{3}H]8-hydroxy-(+)-δ-cadinene **15** would have resulted in 2-fold greater isotope dilutions, 590- and 52-fold, respectively. Biosynthesis of hemigossypol **3** from 8-hydroxy-(+)-δ-cadinene **15** would require three desaturation steps, three hydroxylations, and cyclic ether formation (Fig. 1). Since isotopic dilution of the tritiated intermediate by endogenous pools of intermediates would occur at any of these steps that do not involve substrate channeling from one enzyme to the next (Chappell, 1995), the observed isotopic dilutions are reasonable for a pathway of this length. The data also indicate conversion of 5.4% and 9.3% of the [^{3}H]8-hydroxy-(+)-δ-cadinene **15** to [^{3}H]HG **3** in the two experiments (Table 1). We conclude from these moderately efficient incorporations of tritium that 8-hydroxy-(+)-δ-cadinene **15** is a biosynthetic precursor to hemigossypol **3** in *G. hirsutum*.

If *G. hirsutum* is found to have only one *CYP706B1* gene like *G. arboreum* or only a small gene family, efforts to silence expression of this P450 hydroxylase in developing cottonseed, perhaps in addition to silencing expression of the gene for (+)-δ-cadinene synthase, may aid in development of cotton varieties with gossypol-free seed.

3. Experimental

3.1. General experimental procedures

Tritium content of HPLC eluates was detected on-line by liquid scintillation counting with a β-RAM radiochemical detector (IN/US Systems, Inc., Tampa, FL, USA). The reversed-phase C_{18} HPLC column was 250 mm × 4.6 mm with 5 μm particle diameter (Alltech Assoc., Deerfield, IL, USA). (1*RS*)-[1^{3}H]FPP **8** was from NEN (Boston, MA, USA). *N*,*O*-*bis*-(Trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA/TMCS reagent) was from Pierce (Rockford, IL, USA).

3.2. Biological materials

Seedlings of upland cotton (*G. hirsutum* L.) of a bacterial blight-resistant line lacking pigment glands, WbM*gl*, were used in all experiments (Davis and Essenberg, 1995). The seedlings were grown in a growth chamber as previously described (Pierce et al., 1993). *Xcm* strain 3631 (Pierce et al., 1993) was cultured in nutrient broth and diluted in sterile H_2O saturated with $CaCO_3$ to an inoculum concentration of ca. 5×10^6 cfu ml^{-1}. Entire cotyledons were inoculated 11–12 days after planting, between 5:15 pm and 6:15 pm, by infiltration from a needleless syringe as previously described (Pierce and Essenberg, 1987).

3.3. Isolation of HG 3 and dHG 2 and preparation of their TMSi-derivatives

HG **3** and dHG **2** were isolated from inoculated cotyledons as previously described (Abraham et al., 1999). Preliminary separation by C_{18} reversed-phase HPLC yielded a fraction containing both HG **3** and HG **2**. Analytical-scale HPLC of this fraction on a C_8 column (Abraham et al., 1999) resolved dHG **2** and HG **3**, whose identities were confirmed by their UV absorption spectra (Bell et al., 1975). The HG **3**/dHG **2** mixture from the first HPLC was trimethylsilylated with BSTFA/TMCS reagent (40 min at 60 °C). The trimethylsilyl (TMSi) derivatives of HG **3** and dHG **2** were subjected to HPLC on a C_{18} column with two different solvent systems. Solvents of System 1 were: A [CH_3CN–H_2O (5:95)] and B [CH_3CN (100%)]. All solvent gradients were linear. The elution program was: 0–20 min, 40–90%B; 20–60 min, 90–100% B; flow rate 1.0 ml min^{-1}. TMSi derivatives of dHG **2** and HG **3** eluted at 37 min and 41.5 min, respectively. Solvents of System 2 were: A [MeOH–H_2O (10:90)] and B [MeOH (100%)]. Elution program was the same as for system 1. TMSi derivatives of HG **3** and dHG **2** eluted at 21.5 min and 35 min, respectively. UV absorption spectra of the TMSi derivatives were essentially identical to those of the free phenols, HG **3** and dHG **2**.

3.4. Preparation of [^{3}H](+)-δ-cadinene 9 and [^{3}H]8-hydroxy-(+)-δ-cadinene 15

[^{3}H](+)-δ-Cadinene **9** was prepared from commercial (1*RS*)-[1^{3}H]FPP **8** which had been diluted with non-radioactive FPP **8** to a specific activity of 47–60 μCi μmol^{-1} employing recombinant (+)-δ-cadinene synthase (Chen et al., 1995) as previously described (Davis and Essenberg, 1995). It was converted to [^{3}H]8-hydroxy-(+)-δ-cadinene **15** with a microsomal preparation containing recombinant (+)-δ-cadinene hydroxylase from a yeast clone as previously described (Luo et al., 2001). To avoid problems with scaling up the reaction, 10 × 1.0-ml reaction mixtures were prepared with 20 μM [^{3}H](+)-δ-cadinene **9** as substrate. The reaction was started by adding the microsomes, incubated at 30 °C for 90 min, and stopped by adding 2 ml 0 °C Et_2O to each vial. Each reaction mixture was extracted 4–5 times with 2 ml of Et_2O. The combined Et_2O extracts plus 2 ml hexane were evapd. to ca. 1.0 ml, applied to a 20-g column of silica gel (14 cm × 2.5 cm diam., 100–200 mesh), and eluted with hexane to remove unreacted [^{3}H](+)-δ-cadinene **9**, followed by Et_2O to elute [^{3}H]8-hydroxy-(+)-δ-cadinene **15**. The Et_2O eluate was conc., again applied to a silica gel column (6 g), and eluted with hexane followed by Et_2O. The evapd. Et_2O eluate was analyzed by HPLC in system 3. Solvents were: A [CH_3CN–H_2O (5:95)] and B [CH_3CN (100%)]. The elution program was a linear gradient: 0–60 min, 40–100% B; flow rate 1.0 ml min^{-1}. [^{3}H]8-Hydroxy-(+)-δ-cadinene **15** eluted at 34 min and was 98% radiochemically pure, with a 2% impurity that eluted at 38 min (in contrast to HG **3**, which elutes at 20 min).

3.5. Incorporation of [^{3}H](+)-δ-cadinene 9 and [^{3}H]8-hydroxy-(+)-δ-cadinene 15 into sesquiterpenes in cotton cotyledons

At 30 h after inoculation, cotyledons were detached from the seedlings, and their petioles were immersed in water, placed in a plexiglass glove box (31 cm × 31 cm × 15 cm) for containment of radiochemicals, and infiltrated with an aqueous solution of [^{3}H](+)-δ-cadinene **9** (20 μM, 60 μCi μmol^{-1}) and 1% Tween 80 from a needleless syringe. The cotyledons were incubated in darkness at 19 °C for 9 h, harvested, quick-frozen in liquid N_2, and stored at −80 °C until extraction.

[^{3}H]8-hydroxy-(+)-δ-cadinene **15** incorporations were conducted similarly, except that the precursor concentrations were 8–52 μM, 47–60 μCi μmol^{-1}, and the incorporation period was 40–48 h after inoculation and was in light at 33–35 °C.

3.6. Isolation, derivatization, and chromatography of tritium-labeled metabolites from cotton cotyledons

Frozen cotyledon tissue was ground to a fine powder in a liquid N_2-chilled mortar and extracted with 80% MeOH/H_2O (12 ml per gFW). The hydro-organic phase was conc. to less than 10% MeOH/H_2O by rotary evaporation. It was applied to a MeOH and H_2O preconditioned C_{18} silica cartridge (for extracts of less than 0.5 gFW) or a 1-g column (3 cm × 1.5 cm diam., for extracts of up to 2.5 g). Sesquiterpenes were eluted with MeOH/H_2O (85:15). The MeOH content was adjusted to less than 30% MeOH in H_2O by addition of H_2O. Sesquiterpenes were extracted three times with 1/3 vol. $CHCl_3$. The combined $CHCl_3$ extract was dried with $MgSO_4$, filtered, and evapd. to dryness.

The resulting crude extracts were subjected to HPLC on a C_{18} column in system 3. The sesquiterpenes labeled from [^{3}H]8-hydroxy-(+)-δ-cadinene **15** were treated further as follows. A fraction containing [^{3}H]HG **3** and [^{3}H]dHG **2** was collected at 19–22 min. [^{3}H]HG **3** and [^{3}H]dHG **2** were recovered by partitioning with $CHCl_3$ and evapd. to dryness. HG **3** and dHG **2** from incorporations conducted on four occasions were combined for experiment no. 1; experiment no. 2 consisted of a single incorporation. For each experiment, the dry, labeled [^{3}H]HG **3** and [^{3}H]dHG **2** mixture was trimethylsilylated with 300 μl of the BSTFA/TMCS reagent at 60 °C for 40 min. CH_3CN (400 μl) was added and the volume was reduced to ca. 200 μl with an argon stream. The derivatives were subjected to HPLC

in system 1 (Section 3.3), and fractions containing [^{3}H]dHG **2** and [^{3}H]HG **3** were collected. The [^{3}H]dHG **2** fractions was not further tested due to its small quantity. The [^{3}H]HG **3** fraction was subjected to HPLC in system 2 (Section 3.3). Fractions (0.5 ml) were collected from 19 min to 39 min. Their UV spectra were recorded, and the fractions were then analyzed for tritium by liquid scintillation counting. Concentrations of TMSi-HG were calculated using the molar extinction coefficient of HG **3** at 374 nm (Bell et al., 1975).

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