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The absolute configuration of (−)-3-hydroxy-α-calacorene

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Abstract

3-Hydroxy-α-calacorene was identified in extracts from cold-shocked seedlings of cotton (*Gossypium hirsutum* L.) and kenaf (*Hibiscus cannabinus* L.), both of which are members of the Malvaceae family. (−)-3-Hydroxy-α-calacorene was isolated from *Heterotheca inuloides* Cass. (Asteraceae). HPLC on a chiral stationary phase column showed that the 3-hydroxy-α- calacorene from cotton and kenaf had the same relative configuration, while that from *H. inuloides* was of the opposite configuration. X-ray crystallographic analysis established the absolute configuration of the compound in *H. inuloides* as (8*R*)-(−)-3-hydroxy-α-calacorene.

Keywords: *Heterotheca inuloides*; *Gossypium hirsutum*; *Hibiscus cannabinus*; Malvaceae; Cotton; Kenaf; Biosynthesis; Sesquiterpene; (−)-3-Hydroxy-α-calacorene

1. Introduction

Cotton (*Gossypium hirsutum* L.) and kenaf (*Hibiscus cannabinus* L.) are both in the Malvaceae Family. Both are adversely affected by the plant pathogen *Verticillium dahliae*, but some cultivars of kenaf are significantly more resistant than cotton. When infected by pathogens, plants produce secondary metabolites termed phytoalexins that are toxic to or inhibit the growth of the pathogen. We have identified a phytoalexin in kenaf, *o*-hibiscanone (**1**) (Fig. 1) that is ten times as toxic to *V. dahliae* as compared to the most potent phytoalexin produced by cotton (Bell et al., 1998). We have proposed that the phytoalexins in cotton and kenaf are derived from common intermediates. One of these intermediates is 3-hydroxy-α-calacorene, which we have identified in cold-shocked cotton and kenaf seedlings (Stipanovic et al., 1998). The absolute configuration of the 3-hydroxy-α-calacorene in cotton and kenaf was unknown.

In 1976, Bohlmann and Zdero identified and characterized 3-hydroxy-α-calacorene in the flowers of *Heterotheca inuloides* Cass. However, the absolute configuration of the compound was not determined.

As part of our goal to elucidate the biosynthetic pathway for terpenoids in cotton and ultimately to increase cotton's resistance to fungal pathogens, it was essential to ascertain whether the relative configuration of 3-hydroxy-α-calacorene was the same in cotton and kenaf. Determination of the absolute configuration of this compound was an outgrowth of that goal.

2. Results and discussion

Racemic 3-hydroxy-α-calacorene was synthesized (McCormick et al., 1984). The racemic mixture was chromatographed on a Pirkle Type-A chiral column (Pirkle et al., 1981). Although this column did not provide baseline

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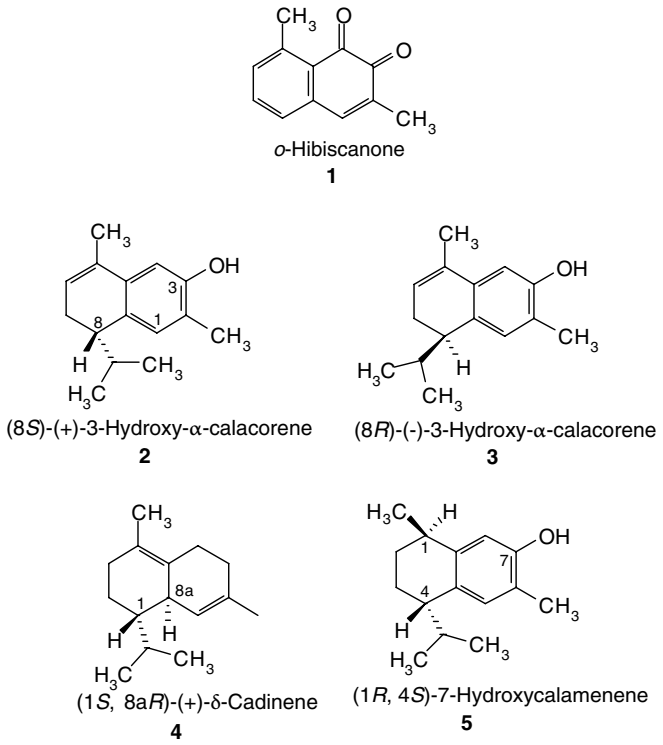

Fig. 1. Some terpenoids found in Malvaceae.

resolution of the two peaks, it was possible to collect sufficient amounts of ~92% enantiomerically pure material to determine the rotations of each enantiomer. The enantiomer eluting at 11.2 min provided a rotation of $[\alpha]_D^{25} = +17.8(CHCl_3; c0.058)$, while the enantiomer eluting at 11.8 min had a rotation of $[\alpha]_D^{25} = -25.9(CHCl_3; c0.048)$. These smaller optical rotation values, as compared to that reported by Segura et al. (2000) for the (−)-3-hydroxy-α-calacorene isolated from *H. inuloides* $\{[\alpha]_D^{21} = -32.84\text{-}(CHCl_3; c1.0)\}$, reflect the lack of complete resolution of the peaks on the Pirkle column. We also isolated 3-hydroxy-α-calacorene from *H. inuloides*. Careful chromatographic separation and multiple crystallizations provided crystals of 3-hydroxy-α-calacorene that had a rotation of $[\alpha]_D^{25} = -44.53(CHCl_3; c0.124)$. The 3-hydroxy-α-calacorene isolated from *H. inuloides* that is spiked with the optically pure (+)-enantiomer isolated from the racemic mixture are shown in Fig. 2.

Using an established achiral reverse phase HPLC technique for the analysis of phytoalexins in cotton stems (Bianchini et al., 1999), 3-hydroxy-α-calacorene was identified as a minor component in extracts of cold-shocked cotton and kenaf seedlings. From these extracts, small amounts of the compound were isolated. The identity of 3-hydroxy-α-calacorene from cotton, kenaf and *H. inuloides* was established by comparing their GC retention times, mass spectra, achiral reverse phase HPLC retention times and unique UV spectra to that of the synthetic racemic compound (McCormick et al., 1984). Subsequent chiral HPLC analyses using the Pirkle column established that: (1) the purified 3-hydroxy-α-calacorene samples from cotton and kenaf were at least 99% optically pure; (2) they had identical retention times; and (3) their retention times were the same as the (+)-enantiomer isolated from the synthetic racemate. Thus, the enantiomer of 3-hydroxy-α-calacorene is the same in both kenaf and cotton, but it is the mirror image of that in *H. inuloides*.

To establish the absolute configuration of the 3-hydroxy-α-calacorene from *H. inuloides*, we subjected the crystals to crystallographic analysis by the anomalous X-ray scattering from the oxygen atom (Flack and Bernardinelli, 2000). By this method, the absolute configuration at C-8 for (−)-3-hydroxy-α-calacorene from *H. inuloides* was determined to be *R*. The FLACK parameter was $-0.2 \pm .09$ where a value of zero represents the correct

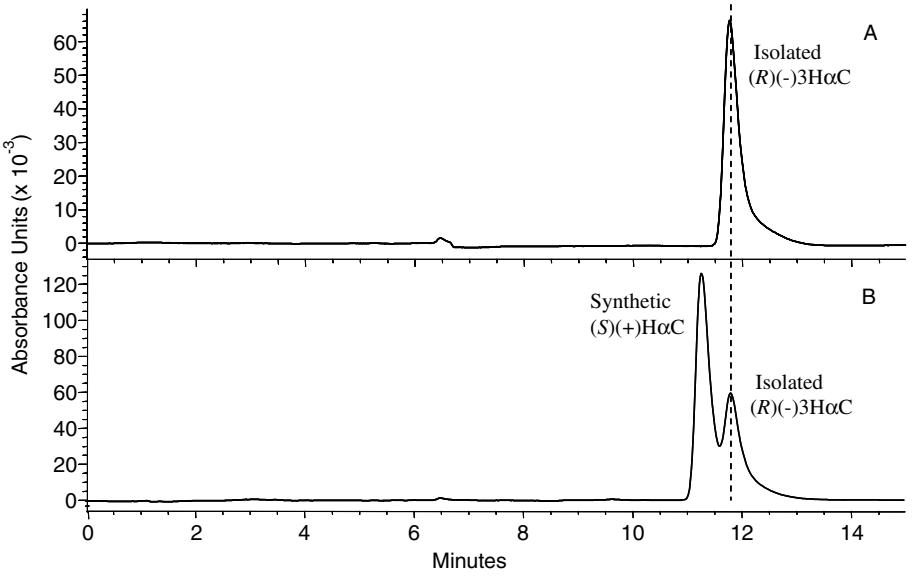

Fig. 2. HPLC chromatograms on a Pirkle Type-A column of: (A) (*R*)-(−)-3-hydroxy-α-calacorene [(*R*)(−)3HaC] isolated from *Heterotheca inuloides* flowers, and (B) isolated (*R*)-(−)-3-hydroxy-α-calacorene spiked with pure synthetic (*S*)-(+)-3-hydroxy-α-calacorene [(*S*)(+)3HaC].

structure and a value of one represents the inverted structure. The parameter was determined from the refinement of over 32,000 Bjvioet pairs (TWIN/BASF, SHELX97). The crystals from *H. inuloides* are considered to be enantiomerically pure based on chromatography of a concentrated solution (UV response of >20,500 mAU) on the Pirkle column where no absorption could be detected in the area where the (+)-enantiomer would have eluted.

Since the absolute configuration for (−)-3-hydroxy-α-calacorene (**3**) found in *H. inuloides* is *R* at C-8 as shown in **3**, the (+)-3-hydroxy-α-calacorene from cotton and kenaf must have the *S* configuration at C-8 as shown in **2**. In addition to (+)-3-hydroxy-α-calacorene, we also identified 7-hydroxycalamenene in the cold-shocked cotton and kenaf extracts (Stipanovic et al., 1998). Davila-Huerta et al. (1995) also identified 7-hydroxycalamenene in cotton cotyledons. Furthermore, they established that the 7-hydroxycalamenene in *G. hirsutum* is the *trans*-isomer based on comparisons of the ^{1}H NMR and ^{13}C NMR spectra of **5** found in diseased cotton tissues with that of *cis*- and *trans*-isomers reported in the literature and the isomer synthesized in their laboratories. However, the absolute configuration of 7-hydroxycalamenene was not established. Subsequently, Davis and Essenberg (1995) found that 7-hydroxycalamenene in *G. hirsutum* cotyledons is derived from (+)-δ-cadinene (**4**). In (1*S*, 8a*R*)-(+)-δ-cadinene (**4**), the carbon to which the isopropyl group is attached has the *S* configuration. Davis, Davila-Huerta, and Essenberg also found that 3-hydroxy-α-calacorene is derived from (+)-δ-cadinene (M. Essenberg, personal communication).

3. Conclusion

Based on Essenberg's work and the present study, we now know that the carbon to which the isopropyl group is attached has the *S* configuration in both (1*S*, 8a*R*)-(+)-δ-cadinene (**4**) and in (8*S*)-(+)-3-hydroxy-α-calacorene (**2**) in *G. hirsutum*. Therefore, it seems likely that 7-hydroxycalamenene in *G. hirsutum* has the 1*R*, 4*S* configuration as shown in **5**, although this remains to be confirmed. Furthermore, we conclude that because the absolute configuration of the phytoalexin biosynthetic precursors in both cotton and kenaf have the same absolute configuration, expression of appropriate kenaf genes in cotton can be used to enhance the potency of the phytoalexin cocktail in the latter.

4. Experimental

4.1. General

Mps are uncorr. EI–MS were obtained with a Thermo Electron DSQ [ion source 205 °C, scan rate 500 amu/s] coupled to a Trace GC 2000 [carrier gas: He at 1 ml min^{-1}; injector 250 °C; transfer line 200 °C; column: Scientific Glass Engineering, BP1 0.25 μ (25 m × 0.22 mm); initial temperature 60 °C (hold 4 min), ramp to 180 °C at 10 °C min^{-1} (hold 1 min), ramp to 280 °C at 15 °C min^{-1} (hold 5 min). The high resolution mass measurement was run in negative ion mode on a QStar MDS Sciex spectrometer using electrospray ionization in the presence of LiCl. Rotations were measured at 25 °C in $CHCl_3$ using a Perkin Elmer Model 241 polarimeter using a 1 dm temperature controlled microsample holder. Air dried flower petals of *H. inuloides* were provided by a commercial source (Laboratorios Mixim, Mexico).

4.2. Isolation of phytoalexins from cold-shocked kenaf and cotton seedlings

Approximately 20 cotton (*G. hirsutum*) or kenaf (*Hibiscus cannabinus*) seed were spaced evenly across a piece of damp paper-towel (28.5 × 46 cm). The towels (20 to 40) were rolled into cylinders and wrapped with a piece of wax paper. The bundles were set into beakers (500 ml) that were one-third full of deionized H_2O. The beakers were covered with a plastic bag and placed in an incubator where the seeds were allowed to germinate and grow at 28 °C without light for 72 h. The beakers of seedlings were transferred to a 2 °C cold-room and kept under no light conditions for 96 h. After this cold-shock treatment, the seedlings were removed from the paper rolls and laid into glass pans containing a nutrient medium of 0.02 M KH_2PO_4/Na_2HPO_4 buffer at pH6.5 with 1.0% sucrose and 0.1% NaOAc. Just enough nutrient medium (~175 ml) was used to cover the seedling roots. The pans were covered with plastic wrap and returned to the incubator to allow the seedlings to grow for another 48 h at 28 °C without light. The nutrient medium was decanted from the cold-shocked seedlings; the seedlings were reserved and the nutrient media extracted three times with Et_2O (20 ml). The Et_2O extracts were evaporated under vacuum and the residue was mixed first with MeOH (20 ml) then with H_2O (200 ml). The MeOH–H_2O sample was extracted with cyclohexane (4 × 10 ml). The reserved seedlings were rinsed with cyclohexane (50 ml) and the rinsate combined with the organic extracts obtained from the MeOH–H_2O sample. The organic extracts were dried under vacuum. The residue was reconstituted in 0.5 ml of 99:1 MeOH:H_2O. The extracts were analyzed on an achiral reverse phase HPLC column (Bianchini et al., 1999), and the 3-hydroxy-α-calacorene was identified by its retention time and unique UV spectrum. Next, multiple injections were performed on the achiral column and the 3-hydroxy-α-calacorene was collected. The collected fraction was extracted with an equal volume of cyclohexane and the organic layer was separated and evaporated to dryness. Reanalysis of the 3-hydroxy-α-calacorene indicated the compound was 90% pure. Its identity was further confirmed by CG/MS analysis. The compound by itself and spiked individually with each enantiomer of 3-hydroxy-α-

calacorene obtained from the synthetic racemate was analyzed using the chiral HPLC method described below.

4.3. HPLC Analysis of the Enantiomers of 3-Hydroxy-α-calacorene

Analysis and separation of the synthetic racemate of 3-hydroxy-α-calacorene was achieved using a computer-operated HP1050 liquid chromatograph equipped with a diode array detector, a Regis Pirkle Type-A (4.6 × 250 mm, 5 μm) column and a Foxy 200 fraction collector. The Regis column has a spherical aminopropyl packing modified with (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine (Pirkle et al., 1981). The column was maintained at room temperature and the isomers were eluted using an isocratic mix of 98.7:1.3 hexane:isopropyl alcohol run at 1.00 ml/min for 16.0 min. Detection was at 265 ± 10 nm (referenced to 550 ± 50 nm) and spectra were stored over 190–600 nm. The (+)- and (−)-3-hydroxy-α-calacorene had retention times of 11.2 and 11.8 min, respectively. For isolation of the pure enantiomers from the synthetic 3-hydroxy-α-calacorene racemate, the Foxy module was set to collect time windows corresponding to the first 2/3 of the (+)-enantiomer peak and to the last 2/3 of the (−)-enantiomer peak. The collected fractions were evaporated to dryness, reconstituted in 100% hexane and analyzed *via* HPLC on the Pirkle column. Both samples were >99.9% enantiomerically pure.

4.4. Isolation of (*R*)-(−)-3-Hydroxy-α-calacorene (*3*) from *H. inuloides*

Ground flowers from *H. inuloides* (250 g) were stirred in hexane (1000 ml) overnight. The suspension was filtered through Whatman #4 filter paper, and both the filtrate and flower material were reserved. The filtrate was evaporated to a minimum volume under vacuum at 25 °C. The extraction procedure was repeated four times on the reserved flower material using the re-condensed hexane from the evaporation step. The concentrated samples were combined and reduced to dryness under vacuum. The dry sample was reconstituted in acetone (100 ml) and chilled (2 °C), resulting in the precipitation of a white waxy material. The acetone solution was filtered through Whatman #4 filter paper to remove the precipitate.

The acetone was evaporated until only an oily sample (5 g) remained. Baker CC silica gel (5.5 g) was added to the oily sample and the mixture dried under vacuum at 40 °C. The gel sample was loaded onto a column of 80 g of the Baker CC silica gel. The sample was eluted using 300 ml volumes of a series of hexane:EtOAc mixtures (98:2, 96:4, 94:6, 92:8 and 90:10). Fractions (∼40 ml) from the column were collected and subjected to achiral HPLC analysis (Bianchini et al., 1999). 3-Hydroxy-α-calacorene was found to elute from the Baker CC column along with increasing amounts of 7-hydroxycadalene during the 94:6 hexane:EtOAc portion of the run. Fractions with more than 80% 3-hydroxy-α-calacorene were reserved separately from those with 30–80% of the compound. The procedure for extracting the *H. inuloides* flowers and partially isolating the 3-hydroxy-α-calacorene was repeated twice. Fractions from the second and third column chromatography runs were combined into the appropriate reserved samples from the first isolation. Separation of the 3-hydroxy-α-calacorene from the 7-hydroxycadalene was achieved via preparative HPLC using a computer-operated Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector, a Phenomenex Hypersil-C18 (10.0 × 250 mm, 5 μm) column and a Foxy 200 fraction collector. The solvent system was a gradient of H_2O and MeOH (both with 0.07% H_3PO_4) run at 5.0 ml/min with linear changes between the following set points: 20% MeOH (0.0 min), 70% (7.0 min), 80% (16.0 min), 80% (20.0 min), 100% (20.2 min), 100% (24.0 min) and 20% (25.0 min). The chromatogram was monitored at 235 ± 10 nm (reference 550 ± 50 nm) and spectra were stored over 190–600 nm. The Foxy unit collected fractions over two time windows: 18.25–18.75 min for 7-hydroxycadalene **5** and 19.0–21.0 min for 3-hydroxy-α-calacorene **3**. The fractions collected over the course of a day were stored at 2 °C overnight, and the next morning they were evaporated under vacuum at 25 °C to remove the methanol. The 7-hydroxycadalene **5** and 3-hydroxy-α-calacorene **3** were extracted from their respective aqueous samples into 1:1 hexane:EtOAc (∼100 ml) and evaporated to dryness. The isolation provided 837 mg of 97.7% pure 3-hydroxy-α-calacorene. Further purification of the 3-hydroxy-α-calacorene was accomplished via crystallization, first from hexane:EtOAc, and then from pentane:Et_2O, yielding 143 mg of 99.9% pure compound (**3**).

4.5. (*R*)-(−)-3-Hydroxy-α-calacorene (*3*)

White needles [m.p. 103.5–105 °C (pentane:ether); Lit. m.p. 103.5 °C (Bohlmann and Zdero, 1976)]; $[\alpha]_D^{25} = -44.53(CHCl_3; c0.124)$; GC–MS 70 eV, *m/z* (rel. int.): 216 [M^+] (14), 174 (13), 173 [M–$CH(CH_3)_2$] (100), 172 (12), 158 (32), 157 (10), 155 (10), 145 (19), 129 (10), 128 (12). The UV [Bohlmann and Zdero, 1976] and ^{13}C NMR and ^1H NMR (Kubo et al., 1994) spectra have been published. HRMS [electrospray ionization (LiCl)/negative ion mode] m/z 215.1441 [Calc. for $C_{15}H_{19}O$: 215.1436 (diff. 2.3 ppm)].

4.6. X-ray analysis of (*R*)-(−)-3-Hydroxy-α-calacorene (*3*)

Crystal data: $C_{15}H_{20}O$, $M = 216.31$, colorless needle, $0.20 \times 0.05 \times 0.05$ mm, monoclinic, space group $P2_1$ (No. 4), $a = 5.1062(2)$, $b = 25.0019(7)$, $c = 15.2519(5)$ Å, $\beta = 93.276(2)°$, $V = 1943.95(11)$ Å^3, $Z = 6$, $Z' = 3$, $D_c = 1.109$ g/cm^3, $F_{000} = 708$, MWPC area detector, CuKα radiation, $\lambda = 1.54178$ Å, $T = 110(2)$ K, $2\theta_{max} = 126.4°$, 35,995 reflections collected, 35,995 unique (reflections were not merged). A BRUKER D8 GADDS general purpose

three-circle X-ray diffractometer was employed for sample screening and data collection. The structure was solved and refined using the programs SHELXS-97 (Sheldrick, 1997 b) and SHELXL-97 (Sheldrick, 1997a), respectively. The program XSHEL was used as an interface to the SHELX programs, and to prepare the figures. Final $GooF = 1.003$, $R_1 = 0.0389$, $wR2 = 0.1061$, R indices based on 34,156 reflections with $I > 2$sigma(I) (refinement on F^2), 433 parameters, 1 restraint. Lp and absorption corrections applied, $\mu = 0.514\ \mathrm{mm}^{-1}$. Absolute structure parameter $= -0.20(9)$ (Flack, 1983). Three molecules were found in the asymmetric volume of the unit cell. The absolute configuration (stereochemistry) at position C8 (*a*, *b* and *c*) was assigned as ***R***. The absolute configuration was determined from the anomalous X-ray scattering from the oxygen atoms. The FLACK absolute configuration parameter was $-0.2 \pm .09$ where a value of zero represents the correct structure and a value of one represents the inverted structure. The parameter was determined from the refinement of over 32,000 Bjvioet pairs. The absolute value of the Flack parameter $|x|$ is 0.20 which is 2.2 times u where $u = 0.09$. This value is slightly larger than the ideal value of $2u$ but is less than $3u$ which is suggested limit for enantiopure compounds (Flack and Bernardinelli, 2000). Crystallographic data have been deposited at the Cambridge Crystallographic Data Center (CCDC) (deposition number CCDC 603411). These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail, deposit@ccdc.cam.ac.uk).

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