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IDENTIFICATION OF (+)- 3-HYDROXY-α-CALACORENE IN COTTON AND KENAF

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Abstract

When attacked by pathogens, cotton responds by synthesizing antimicrobial compounds called phytoalexins. As a means to increasing the resistance of cotton to wilt pathogens, we are investigating biosynthetic pathways that could be utilized to introduce more potent phytoalexins. A phytoalexin produced by kenaf, *Hibiscus cannabinus*, is significantly more potent toward wilt pathogens than is any phytoalexin produced by cotton. We have established that a purported common intermediate in the biosynthetic pathway of phytoalexins in cotton and kenaf, 3-hydroxy-α-calacorene, is the (+)-enantiomer in both plants.

Introduction

Kenaf, *Hibiscus cannabinus,* like cotton is a member of the Malvaceae Family. However, it is significantly more resistant to the wilt pathogens *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum* than cotton is. When either kenaf or cotton is attacked by *V. dahliae*, they produce antimicrobial compounds, i. e., phytoalexins, that kill the pathogen propagules. One of the phytoalexins produced by kenaf, *o*-hibiscanone, is significantly more toxic than any of the phytoalexins produced by cotton (Bell, et al., 1998; Puckhaber, et al., 1998). The phytoalexins produced by cotton and kenaf appear to arise *via* the same biosynthetic pathway (Scheme I). We have previously identified 3-hydroxy-α-calacorene (3HαC) in both cold-shocked kenaf and cotton seedlings (Figures 1 and 2) (Stipanovic, et al., 1998). However, 3HαC can exist as two different optically active enantiomers, and it was not known if the same enantiomer was present in both plants. If they possess the same configuration, then the number of genes that would be required to express the kenaf pathway in cotton would be reduced. Herein, we report that the configuration of 3HαC is the same in cotton and in kenaf. Our long-term goals are to establish the absolute configuration of (+)-3hydroxy-α-calacorene, identify the enzymes and genes involved in the kenaf phytoalexin pathway and express these in cotton. Plants expressing these genes are expected to show enhanced levels of resistance to *V. dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum.*

Results and Discussion

A small amount of epimeric 3HαC was synthesized (McCormick et al., 1984) and then an HPLC method was devised to separate the synthetic material into its two enantiomers. A Pirkle column with a chiral stationary phase was employed resulting in a separation of the synthetic material into two peaks with retention times of 11.5 and 12.1 min (Figure 3A). The amounts of enantiomerically pure samples (Figure 3B and 3C) isolated using the Pirkle column were quite small; nevertheless, there were sufficient amounts to obtain rotations. The compound eluting at 11.5min had $[\alpha]_D^{25}$ = +17.8 (c = 0.058, $CHCl_3$), and the compound eluting at 12.1min had $[\alpha]_D^{25}$ = -25.9 (c = 0.048, $CHCl_3$).

Using an established HPLC technique for the analysis of phytoalexins in cotton stem and seedling extracts, small amounts of 3HαC were isolated from cotton and kenaf extracts. These samples then were subjected to HPLC analysis using the Pirkle column. They were analyzed as pure compounds and as samples spiked with the synthetic enantiomeric material (Figure 4). Analysis proved unequivocally that the absolute configuration of 3HαC is the same in both kenaf and cotton, and that this configuration corresponds to the (+)-enantiomer. However, the absolute configuration of the (+)-enantiomer is unknown.

To establish the absolute configuration of (+)-3HαC, we have turned to a natural source of the compound. Segura, et al. (2000) reported that 3HαC may be isolated in good yield from *Heterotheca inuloides* flowers. They also reported that the 3HαC isolated from *H. inuloides* is the (-)-enantiomer. To obtain sufficient material for additional studies, a bulk extraction of *H. inuloides* was carried out. From 250 gm of dried flowers, we obtained several milligrams of 3HαC whose melting point and rotation was in good agreement with that reported in the literature (Segura et al. 2000). We will use this material to prepare a crystalline derivative of (-)-3HαC using an optically active reactant of known configuration. This derivative will be subjected to X-ray crystallography. Knowing the absolute configuration of the reactant will allow the assignment of (-)-3HαC and, by default, the absolute configuration of (+)-3HαC as found in kenaf and cotton.

Materials and Methods

Seedling Preparation and Extractions

Kenaf (Gregg) or cotton (DP-451) seeds were germinated in paper towels in a 30ºC incubator. After 6 days, they were transferred to a cold chamber and held at 2ºC for 72 hours. The seedlings were then placed in a glass dish (22 cm x 34 cm x 7 cm) containing a ~1.5 cm depth of nutrient medium that had been prepared as follows. A 1.0 L solution of 10 gm sucrose and 1 gm sodium acetate was prepared. To 500 mL of this solution, 1.36 gm KH_2PO_4 was added while 1.41 gm $Na_2H_2PO_4$ was added to the second 500 mL. The KH_2PO_4/sucrose solution (200 mL) was diluted with the $Na_2H_2PO_4$/sucrose solution (~80 mL) until the pH of the combined solution was 6.5. The glass dish with seedlings and medium was covered with plastic wrap and placed in the 30ºC incubator. After 48 hrs, the medium was decanted from the seedlings, the seedlings were washed with ether (2 x 250 mL), and then the ether wash was used to extract the media. This process was repeated a second time. The organic extracts were combined, washed with 50% aqueous brine solution and then dried under vacuum. The samples were reconstituted in 500 µL 90:10 methanol:water.

Extraction of *Heterotheca inuloides* Flowers

Heterotheca inuloides flowers were obtained from Laboratorios Mixim, Naucalpan, Edo. de Mexico. Bulk extraction of *H. inuloides* was carried out by steeping 250g of ground plant material in four consecutive volumes of 1.0L hexane. The four extracts were combined, rotoevaporated and reconstituted in acetone, resulting in the appearance of a white, waxy precipitate. The precipitate was removed *via* filtration and then the extract was subjected to column chromatography procedures to isolate the 3HαC. In the first CC procedure, a column of Baker CC Silica Gel was used with a hexane/acetone gradient and fractions containing cadalene and 3HαC plus 7-hydroxycadalene (7HC) were collected. The 3HαC+7HC fractions were combined and subjected to another CC procedure using Baker CC Silica Gel and a hexane/ethyl acetate gradient. This second procedure yielded fractions with pure 3HαC (>99% based on HPLC area percentages). These fractions were combined into one sample and then the 3HαC was crystallized from the sample using hexane. White needles with a melting point of 103.5-105.0ºC were obtained. The optical rotation of the 3HαC isolated from *H. inuloides* was measured and found to be $[\alpha]_D^{25}$ = -44.53 (c = 0.124, $CHCl_3$). These results agree well with the reported values for the melting point and optical rotation; i.e., Mp = 103.5ºC and $[\alpha]_D^{21}$ = -32.84 (c = 1.0, $CHCl_3$) (Segura, et al., 2000).

HPLC

HPLC analysis for 3HαC in plant extracts was carried out on a Hewlett-Packard 1090LC equipped with a diode array detector (DAD). The method employs a Keystone Hypersil-MOS-1 (250 x 4.6 mm, 5 µm) column maintained at 40ºC and a mobile phase of methanol and water (both containing 0.07% H_3PO_4) run at 1.25 mL/min. The methanol and water percentages change linearly between the following pump timetable points: 20% methanol at 0 min, 70% at 7 min, 80% at 12 min, 90% at 19min, 100% at 19.2min, 100% at 23 min and 20% at 26 min. The chromatogram signal was accumulated at 235 nm (20 nm bandwidth) with reference to 550 nm (100 nm bandwidth) while UV-vis spectra for peaks were collected over 210-600 nm. For isolation of the 3HαC, the relevant peak was collected manually as it eluted.

HPLC analysis of the 3HαC enantiomers was performed on a Hewlett-Packard 1050LC equipped with a 1100DAD module. A Regis Pirkle Type 1-A (250 x 4.6 mm, 5 µm) column was used with a mobile phase of 98.7% hexane and 1.3% isopropanol with a flow rate of 1.00 mL/min and run time of 16 min. The chromatogram signal was collected at 265 nm (20 nm bandwidth) with reference to 550 nm (100 nm bandwidth) while UV-vis spectra for peaks were collected over 210-600 nm. For isolation of the enantiomers, the individual peaks were collected as they eluted using an ISCO Foxy 200 fraction collector set up with a time windows program.

GC/MS Analysis

GC-MS results were obtained on a Hewlett-Packard 5989b mass spectrometer coupled to a 5890 II gas chromatograph. Conditions for analysis were as follows: mass range 50-325 amu at 1.1 scans/s; source 280ºC; quadrupole 100ºC; injector 210ºC; transfer line 280ºC; SGE BP1 (0.2 mm ID, 0.25 µm film, 25 m length) column; oven 60ºC for 7 min to 180ºC at 10ºC/min, hold 1 min then to 280ºC at 15ºC/min, hold 5min; He flow 1.0 mL/min.

Optical Rotations

The optical rotations for the 3HαC samples were taken in $CHCl_3$ on a Perkin-Elmer 241 Polarimeter.

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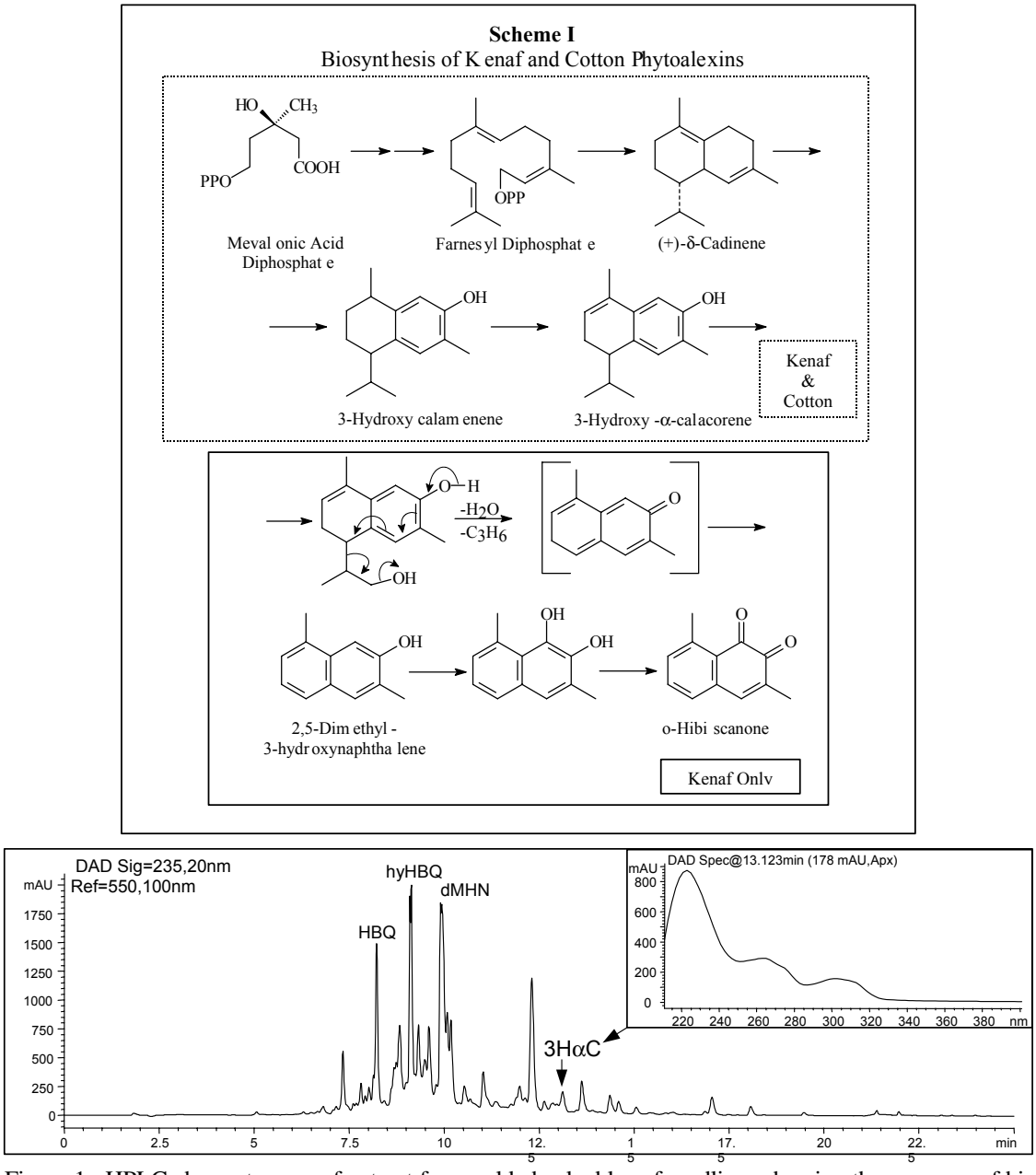

Figure 1. HPLC chromatogram of extract from cold-shocked kenaf seedlings showing the presence of hibiscanone (HBQ), 3-hydroxy-*p*-hibiscanone (hyHBQ), 3-hydroxy-2,5-dimethylnaphthalene (dMHN) and 3-hydroxy-α-calacorene (3HαC) with the UV-vis spectrum for the 3HαC peak displayed in the inset.

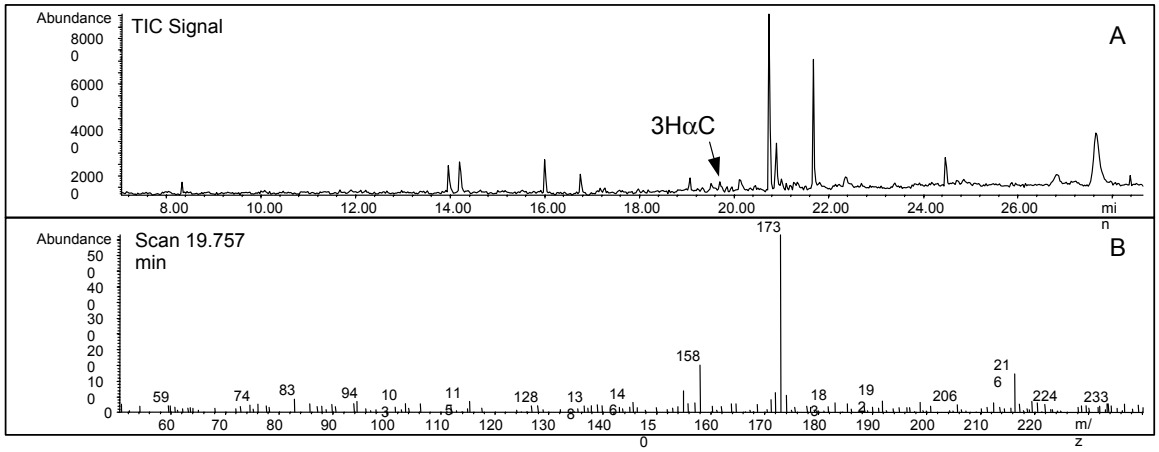

Figure 2. GC-MS results for an extract from cold-shocked cotton seedlings: A) gas chromatogram showing 3-hydroxy-α-calacorene (3HαC) at t_R = 19.75min and B) mass spectrum of the 19.75min peak indicating m/z = 216.

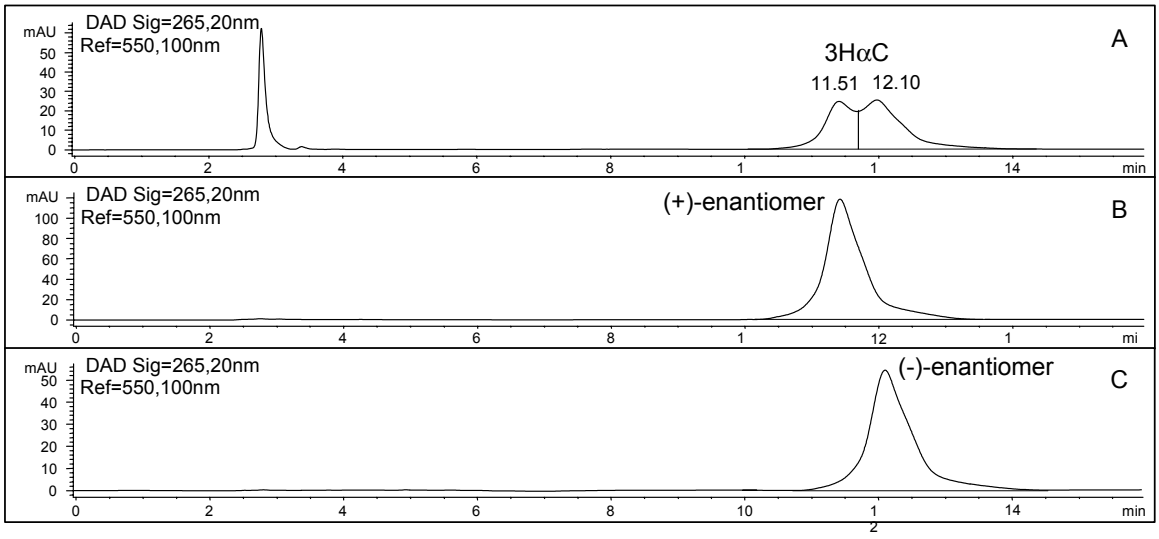

Figure 3. HPLC chromatograms of synthetic 3-hydroxy-α-calacorene (3HαC): A) mixed enantiomer product, B) HPLC isolated (+)- 3-hydroxy-α-calacorene, and C) HPLC isolated (-)- 3-hydroxy-α-calacorene.

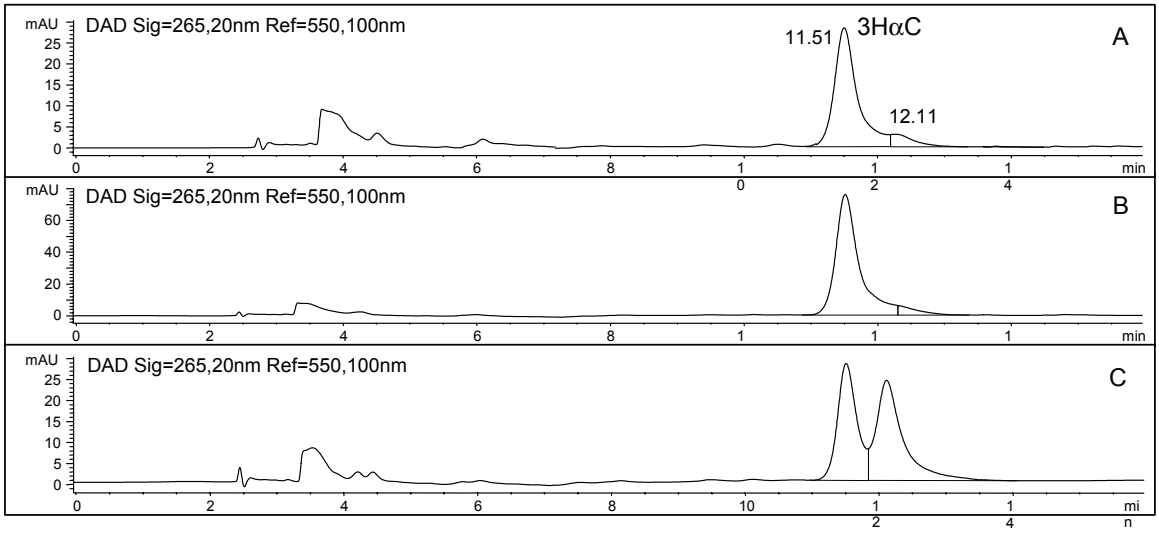

Figure 4. HPLC chromatograms of A) isolated 3-hydroxy-α-calacorene (3HαC) from cold-shocked kenaf seedlings, B) isolated sample spiked with synthetic (+)- 3-hydroxy-α-calacorene (t_R = 11.50min), and C) isolated sample spiked with synthetic (-)- 3-hydroxy-α-calacorene (t_R = 12.11min).