Evidence for the Presence of Gossypol in Malvaceous Plants Other than Those in the "Cotton Tribe"

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Acetone extracts from nondefatted and defatted finely ground whole seed of okra, velvetleaf, prickly sida, venice mallow, and a glanded and glandless isoline of cotton were assayed for the presence of gossypol. Reversed-phase TLC and HPLC were used as methods of detection. HPLC served for quantification. Product identification was accomplished with spectral scans of peak apices. Gossypol was identified in all defatted seed examined. Glanded cottonseed contained at least 2500 ppm and velvetleaf seed had as little as ~6 ppm. Gossypol in seed samples from other examined species ranged from ~8 to 70 ppm, with okra containing the largest amount. Gossypol from fat- and/or oil-laden samples will not migrate from the origin on a TLC plate unless the quantity of it is large as in glanded cottonseed. Examination of okra and three other species of the Hibiscadeae confirm the existence of gossypol in plants outside of the Gossypieae.

Gossypol is a major terpenoid secondary product readily detected in commercial cultivars of cotton. It and several structurally related metabolites have antibiotic properties (Bottger et al., 1964; Margolith, 1967; Zaki et al., 1972). The gossypol pathway appears to be nearly ubiquitous in the tribe Gossypieae. Gossypol was first detected and identified in Gossypium 90 years ago (Marchlewski, 1899). Gossypol has been found in other representatives of the tribe Gossypieae, *Ceinfugosia spp.*, *Thespesia spp.*, and one *Kokia sp.* (Lukefahr and Fryxell, 1967). Detection of gossypol in *Montezuma sp.* (Jolad et al., 1975) and hemigossypol in *Hampea sp.* and *Gossipoides sp.* (Bell et al., 1975) points to the broad existence of gossypol synthesis in the Gossypieae.

Pigment glands, like those found to contain gossypol in *Gossypium spp.* (Stanford and Viehoever, 1918), have been observed in each of the aforementioned genera of the Gossypieae (Fryxell, 1968). Glands are probably necessary for the normal accumulation of large quantities of gossypol in the producing plant. Some gossypol is readily detected in glandless cottons (Smith, 1962). However, under certain conditions, high levels of gossypol have been induced in boll cavities and stem tissue of cotton plants in the absence of glands (Bell, 1967).

Chemotaxonomic distinction of the tribe Gossypieae from the tribe Hibiscadeae in the family Malvaceae presently exists on the basis that plants in several genera in the tribe are the only ones known to produce gossypol and related terpenoids (Fryxell, 1968, 1979). One exception, *Bombax melabaricum* in the family Bombacaceae, is the only species outside the cotton tribe reported to produce the gossypol-related sesquiterpenoid hemigossypol (Sankaram et al., 1981). Any expansion of knowledge of the range of gossypol-productive capacity to other genera in the Bombacaceae or proof of productive capacity in the tribe Hibiscadeae will have an effect on present hypotheses of how gossypol metabolism can be incorporated into the evolutionary chemotaxonomy of the order Malvales (Fryxell, 1968).

A possible identification of gossypol was made in the Hibiscadeae when phloroglucinol positive reactions were reported in okra (*Abelmoscus esculentus* L.) seed (Karakoltsidis and Constantinides, 1975). Bell's group (Bell et al., 1975), however, found no trace of three gossypol-related sesquiterpenoids in three hibiscus species including okra. A further positive identification of gossypol in okra was later reported (Al-Wandawi, 1983), but all reported positive information was brought into question by Stipanovic et al. (1984). Stipanovic's group found that fatty acid degradation products could give false-positive reactions with phloroglucinol and may be mistakenly identified as gossypol. The phloroglucinol stained spots had R_f values different from that of a gossypol standard on a TLC plate. The group found phloroglucinol-positive spots, but no spots corresponding with standard gossypol in extracts from seed of nine okra cultivars.

Initial observations of RP-HPLC peaks in okra extracts that mimicked standard gossypol peaks encouraged us to investigate both the literature and the peaks we had repeatedly observed. Since previous reports conflicted regarding the presence or absence of gossypol in the tribe Hibiscadeae, this study was undertaken to determine gossypol content of seeds from selected species of both tribes of the Malvaceae including three malvaceous weed species.

MATERIALS AND METHODS

Seed from okra (*A. esculentus* L.) cultivar Crimson Spineless, velvetleaf (*Abutilon theophrasti* Medick), prickly sida (*Sida spinosa* L.), venice mallow (*Hibiscus trionum* L.), and cotton (*Gossypium hirsutum* L.) cultivar Stoneville 213, glanded and glandless isolines, were used in the investigation. The first four species are members of the tribe Hibiscadeae, and the cotton isolines are in the tribe Gossypieae.

Examination of naked seed from each species was made with use of a dissecting microscope to detect glands.

Whole seeds were ground. Ground seed samples were stirred into 100-mL volumes of hexane/CCl_4 (30%/70%, v/v). This modification of a method (Boatner and Hall, 1946) was used to float pigment glands at their buoyant density.

Seed samples prepared for RP-TLC were ground as above. Ground seed meal was extracted with light petroleum ether (1 g/5 mL), stirred, and centrifuged. The resulting pellet was dried overnight and extracted with acetone. The extract was filtered, and the filtrate was reduced to dryness under vacuum. The residue was redissolved in 1 mL of acetone. Redissolved extracts of from 10 to 25 μL were spotted on a Whatman KC_{18} (20 × 20 cm) reversed-phase TLC plate. Gossypol (Sigma) at 1 mg/mL in acetone served as a standard. The chromatograph

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was developed in 0.1% phosphoric acid in methanol/water (90/10). Phloroglucinol was used to detect terpenoids as described (Bell et al., 1974). Seed samples from each species were also chromatographed with the petroleum ether defatting step excluded. A standard gossypol (Sigma) spiked fat/oil sample (100 μg/25 μL) from okra seed was used as the standard for runs made on samples that had not been defatted.

The RP-HPLC samples were prepared in a manner similar to that described for RP-TLC. Glanded cottonseed was ground as above and extracted with petroleum ether (1 g/5 mL), and the procedure described was followed through acetone evaporation. At this point the residue was dissolved in methanol and filtered through a 0.45-μm syringe filter prior to injection onto an HPLC column. All other ground seed samples required 10 g/50 mL petroleum ether defatting and acetone extraction steps to obtain a sufficient quantity of the terpenoid examined. Residues remaining after acetone evaporation were dissolved in 2.5 mL of methanol and filtered as above. Venice mallow and velvetleaf seed required redissolution in 0.5 mL of methanol to be investigated in the proper concentration range.

A modified form of the method of Nomier and Abou-Donia (1982) was selected for the HPLC determination of gossypol. A 5-μm Alltech Licrosorb RP-18 steel (4.6 × 250 mm) column was used for separation. The TLC mobile phase described above was also used for HPLC separation. The mobile phase was pumped through the system at 1 mL/min isocratically by a Waters 6000A pump. A Waters 710B autosampler provided programmed injection of samples. A Waters 490 variable-wavelength detector was instituted to detect terpenoid peaks at 230 nm. A Digital Professional 350 computer using Waters Expert REV 4.0 software was programmed to process data and integrate terpenoid peaks.

Injection volumes were 25 μL for glanded cottonseed extracts, 50 μL for glandless cotton, okra, and prickly sida, and 100 μL for venice mallow and velvetleaf to obtain gossypol in appropriate quantities.

Peaks from each sample eluting with the same retention time as gossypol were scanned from 190 to 450 nm with use of the scan program in the Waters 490 detector after physical stop-flow intervention to capture the apex of the appropriate peak in the detector cell (Lydon and Duke, 1988).

RESULTS AND DISCUSSION

Pigment glands were detected only in glanded cottonseed by microscopic examination or buoyant density floatation. Our results concur with those of Fryxell (1968) and Stipanovic et al. (1984) who also reported that malvaceous species other than those in the Gossypieae appear to have no pigment glands associated with gossypol production and storage. TLC spots of standard gossypol stained violet with phloroglucinol at R_f 0.54. Defatted/deoiled seed meal samples from all species yielded violet or magenta phloroglucinol-treated spots with R_f 0.52, 0.53, or 0.54. Samples of all but glanded cottonseed extracted without the oil and fat removal step stained violet or magenta only at the origin as has been previously indicated for paper chromatography (Schramm and Benedict, 1958). A Sigma gossypol spiked fat/oil sample from okra retained all phloroglucinol-stainable material at the origin. Glanded cottonseed samples were the only ones in which gossypol could be detected if the meal was not defatted with petroleum ether. These samples probably contained sufficient gossypol to overcome the binding capacity of the fat/oil present in them.

Undefatted okra, prickly sida, and velvetleaf samples developed yellow to orange-yellow streaks after phloroglucinol spraying with R_f 0.41–0.44. Accounting for an expected reversed migration order on RP-TLC from that of normal-phase TLC, these spots or streaks may have been what Stipanovic et al. (1984) observed at R_f 0.75 compared with dianalineogossypol at R_f 0.68. No visible streaks with R_f 0.41–0.44 were present in any of our defatted samples.

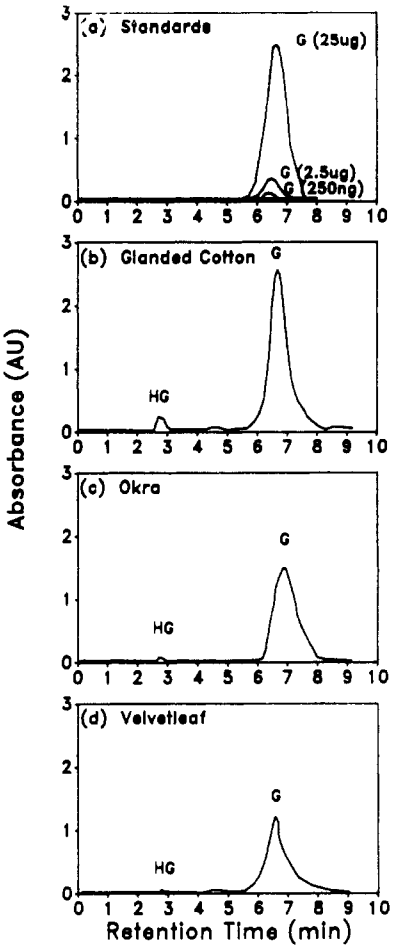

Figure 1. Selected chromatograms showing gossypol and hemigossypol peaks: (a) peaks of three measured quantities of gossypol standard (Sigma); (b) extract from glanded cottonseed showing a hemigossypol peak and a measurable gossypol peak; (c) okra seed extract showing the above-mentioned peaks; (d) velvetleaf seed extract showing a quantifiable gossypol peak.

Table I. Gossypol in Seed Obtained by Comparison with RP-HPLC Gossypol Standards

plant	gossypol, ppm (μg g^{-1} fr wt)
Cotton	
Stoneville 213 (glanded)	2500
Stoneville 213 (glandless)	50
Okra	
Clemson spineless	70
prickly sida	60
venice mallow	~8
velvetleaf	~6

Using RP-HPLC, standard gossypol peaks had an average RT of 6.5 min. Defatted seed meal extract peaks ranged in RT from 6.6 min for velvetleaf to 6.9 min for okra. Representative sample chromatograms show the nearly identical behavior of sample materials (Figure 1). Quantitative information for gossypol in each sample species is shown (Table I). As little as 6 μg/g fresh weight was detected in velvetleaf seed meal and up to 2500 μg/g fresh weight was found in glanded cottonseed meal. Data collected in a presently unpublished study indicate an average of 8500 μg/g fresh weight for gossypol in Stoneville 213 glanded cottonseed if the defatting step is eliminated from the preparation. This would suggest greater than herein reported quantities of gossypol in the other plants in this study; however, the gossypol in them is not readily detected in samples containing oil and/or fat.

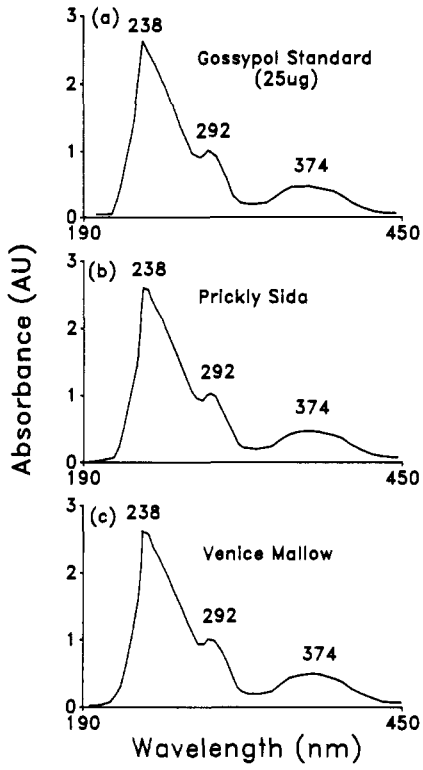

Figure 2. Selected absorbance scans of chromatogram peak apices: (a) peak apex of a 25-μg standard gossypol peak; (b) peak apex of the gossypol RT peak from the prickly sida seed extract; (c) apex scan of the gossypol RT peak from the Venice mallow seed extract.

Procedures useful in increasing extract concentration were required to quantify smaller amounts of gossypol than were found in defatted glandless cottonseed samples.

Spectral scans of peak apices from peaks eluting with the same retention time as gossypol resulted in identical scans for each species examined (Figure 2). Sample curves matched published gossypol absorption curves (Frampton et al., 1948; Stipanovic et al., 1974). TLC migration R_f values were identical, HPLC peak retentions were nearly identical, and spectral scans of peak apices were identical for all species studied. These data provide strong evidence that gossypol is produced by malvaceous plants in the tribe Hibiscadeae as well as in the tribe Gossypieae.

Data from RP-HPLC indicate the presence of as yet unquantifiable hemigossypol in all of the species examined (Figure 1). The presence of both hemigossypol and gossypol in the tribe Hibiscadeae suggests the possibility that the ability to produce gossypol was developed early in the evolution of the Malvaceae and is not specific to the Gossypieae. The chemical relationships between the Gossypieae and other Malvaceae may be closer than earlier implied (Fryxell, 1968).

Gossypol shows antibiotic or toxic effects on insects and many vertebrates including monogastric mammals (Bell et al., 1987). Gossypol in glanded cottons confers a degree of immunity against insect invaders on the host and has not been bred out of commercial cotton, in spite of its harmful effect on vertebrates. A safe workable technique to remove gossypol from foods could provide the opportunity of genetic manipulation to increase okra's natural resistance to insects through increased gossypol production.

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Received for review January 25, 1989. Revised manuscript received June 23, 1989. Accepted September 5, 1989. Mention of a trademark or propietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Registry No. Gossypol, 303-45-7.

Structure–Activity Relationships in (Haloalkyl)pyridazines: A New Class of Systemic Fungicides

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3,6-Dichloro-4-(2-chloro-1,1-dimethylethyl)pyridazine (1) is one of the most active members of a series of new fungicides active against *Oomycetes*. We report on 66 compounds representing modifications of structure 1 and showing that only very limited variation in this structure is possible without loss of activity. Activity is retained only when bromine or iodine is substituted for the chlorine on the *tert*-butyl function. A second chlorine may be added to an adjacent methyl group without large loss of activity. A bromine may be substituted for the 3-chlorine, and to a lesser extent, methyls may be substituted for the ring chlorines. All other changes we explored resulted in partial or complete loss of activity. The parent compound is mobile within plant tissue, moving through the root system to control the pathogen and also toward the tip of the leaf when applied to the middle of the leaf.

We have described the synthesis of 3,6-dichloro-4-(2-chloro-1,1-dimethylethyl)pyridazine (1) (Hackler et al., 1988). This compound is part of a series of compounds (Arnold et al., 1988) with very interesting systemic activity against *Phycomycetes* organisms in plants. We report on a study of the structure–activity relationships in this series of compounds, revealing a narrow set of structural parameters for retention of this activity.

EXPERIMENTAL SECTION

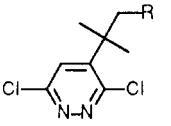
The primary test results reported in Tables I–V result from a foliar application of the test compounds to squash plants inoculated with *Pseudoperonospora cubensis*, the causative organism of downy mildew, 2–4 h after spraying. Formulation consisted of dissolving 48 mg of a selected compound in 1.2 mL of a solvent prepared by mixing 100 mL of Tween 20 (a nonionic surfactant) with 500 mL of acetone and 500 mL of ethanol. The solution of test compound was diluted to 120 mL with deionized water and further diluted to obtain the desired concentration.

The rating system used in the tables is based on a scale of 1–9, as follows: 1, 0–19% control; 2, 20–29%; 3, 30–39%; 4, 40–59%; 5, 60–74%; 6, 75–89%; 7, 90–96%; 8, 97–99%; 9, 100% control, no disease.

Soil and foliar local systemic activity were determined by using the formulation described above. The soil systemic test on squash consisted of using 50 mL of solution at the desired concentration to drench the soil in a 4-in. plastic pot containing 9-day-old Golden Crookneck squash plants. The plants were inoculated 24 h after treatment. The soil systemic test on tobacco was done by diluting the formulated sample to create a solution that would equal the desired concentration in pounds per acre when 20 mL was applied to a 4-in. square pot containing the tobacco plant var. NC2326. Twenty-four hours after application, the tobacco plant was inoculated by placing a 2-cm disk of tobacco black shank agar in a puncture wound in the tobacco stem. The wound was sealed with lanolin. Disease incidence was determined 9 days after inoculation.

The foliar local systemic test consisted of applying the solution to 14-day-old squash plants by spraying the lower leaf surface at right angles to midvein in a 5/8-in. band. Twenty-four hours after treatment, the plants were inoculated on the upper leaf surface with the sporangial suspension and incubated for 24 h in a moist chamber at 70 °F. The plants were then moved to the greenhouse for disease expression.

Table I. Activity of 3,6-Dichloro-4-(substituted *tert*-butyl)-pyridazines against Downy Mildew of Squash



no.	R	mp, °C	400 ppm	100 ppm	25 ppm	6.25 ppm
1	Cl	64–65	9	9	8.5	6
2	Br	86.5–88.5	9	9	8	5.5
3	I	106–108	9	9	8	2
4	F	36–38	9	9	3	
5	H	37–40	4			
6	OH	134–136	1	1	1	
7	CH_3	oil	9	2	1	
8	OAc	32–34	1	1	1	
9	OTos	111–113	1	1	1	
10	CN	80–82	5	2	1	
11	$OCOCH_2Cl$	74–75	1	1	1	
12	CH_2Cl	80–81	6	2	1	
13	CO_2Et	64–65	4	1	1	
14	$OCOC_6H_5$	62–64	3	2	1	
15	OCO_2CH_3	106–107	1	1	1	
16	$OCONHC_6H_5$	146–148	2	1	1	
17	$OCSNHC_6H_5$	135–136	6	4	3	

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0021-8561/90/1438-0508$02.50/0