Vergosin and Hemigossypol, Antifungal Compounds Produced in Cotton Plants Inoculated with Verticillium albo-atrum

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ABSTRACT

The chemical structures of two antifungal compounds formed in the stele of cotton stems in response to inoculation with *Verticillium albo-atrum* have been determined as 8-carboxaldehyde, 1-hydroxy, 5-isopropyl, 7-methoxy, 3-methyl naphthalene, and 8-carboxaldehyde, 1,6,7-trihydroxy, 5-isopropyl, 3-methyl naphthalene. The compounds have been assigned the trivial names "vergosin" and "hemigossypol", respectively, and are structurally related to the cottonseed naphthaldehyde, gossypol.

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Additional key words: phytoalexins, NMR, mass spectra, sesquiterpenes.

Bell (3, 4) reported that cotton (*Gossypium hirsutum* L. and *G. barbadense* L.) plants inoculated with *Verticillium albo-atrum* Reinke & Berth. accumulate antifungal substances (phytoalexins) in the xylem. Schnathorst et al. (16) also observed inhibition of fungus development in the presence of xylem fluids from inoculated tolerant plants. Bell presented considerable evidence that this biologic activity is due to de novo production of gossypol and/or gossypol-related compounds in the cotton stems after fungus inoculation, and that a direct relationship exists between rates of production of these compounds by different cotton cultivars and their resistance to Verticillium wilt. Because the antifungal substances have not been previously characterized, we undertook the isolation and structural elucidation of these compounds. We report here the isolation of two of them for which the trivial names "vergosin" and "hemigossypol" are proposed (Fig. 1, I and II, respectively).

MATERIALS AND METHODS.–Cotton (*G. barbadense* L. 'Seabrook Sea Island 12B') plants were grown in the greenhouse as described (8), and inoculated when 30-40 days old with conidial suspensions (10^7 cells/ml) of isolate V3H of *V. albo-atrum* by the stem puncture method. Stems from inoculated plants were harvested after 12-20 days.

Cortical tissues were stripped from stems, and the stelar tissues were cut into small pieces and extracted with 95% ethanol in a Sorvall Omni-Mixer. Following concentration in vacuo, the solution was extracted with ethyl acetate which was dried with $MgSO_4$ and concentrated. These crude extracts were chromatographed on 0.5 mm Silica Gel GF_{254} (E. Merck, Darmstadt, Germany) or polyamide thin-layer chromatography (TLC) plates with various solvent systems (Table 1). A TLC antifungal bioassay with *Cladosporium cucumerinum* (12, 13) revealed that two major and several minor compounds were present in the crude extracts. Compounds 1 and 2, comprising the major antifungal spots in the TLC bioassay, were isolated from the crude extracts by preparative TLC and found to be antifungal in the TLC bioassay and active against *V. albo-atrum*. Their biologic properties will be considered in more detail in the accompanying paper (17).

Gossypol was not detected in the crude plant extracts by TLC, but was used as a reference compound in the structural elucidation of compounds 1 and 2. Gossypol acetate was converted to the free compound before use. Acetates of vergosin and hemigossypol were formed conventionally with acetic anhydride and pyridine.

TLC was performed using various solvent systems with silica gel GF_{254} or polyamide plates (Table 1). Although unknown compounds 1 and 2 chromatographed as well-defined spots on all the solvent systems, authentic gossypol streaked in all solvents except 4, 5, 8, and 9 (Table 1).

Ultraviolet (UV) spectra were taken in ethanol or methanol; infrared (IR) spectra were taken in chloroform. Nuclear magnetic resonance (NMR) spectra were taken in deuterated chloroform in microtubes for vergosin and in macrotubes for gossypol using a Varian T-60 NMR spectrometer. Aromatic hydroxyl protons were assigned following D_2O exchange of the compounds. Mass spectra were obtained by direct probe insertion of samples into a Finnigan Model 1015C quadrupole mass spectrometer which was operated at ionizer settings of 70 ev and 300 μamp.

RESULTS AND DISCUSSION.–*Gossypol.*–The structure of gossypol (Fig. 1, III) was originally deduced by the classic work of Adams et al. (2), and subsequently fully confirmed by synthesis (7). Our UV and IR data for gossypol generally agreed with literature values (10, 15). Although mass spectra have been taken for derivatives of gossypol, Abou-Donia et al. (1) were unable to obtain spectra for the underivatized compound using a high resolution magnetic mass spectrometer. We obtained excellent

spectra on the underivatized compound with the quadrupole mass spectrometer (Table 2). Gossypol exhibited a strong molecular ion, and had strong peaks for loss of water and methyl. The NMR spectrum of gossypol (Table 3) was readily interpreted on the basis of its known structure.

Compound 1—vergosin.—Compound 1 appeared colorless to faintly yellow on TLC plates, and absorbed short wavelength (254 nm) light. It reacted slowly with phloroglucinol (3), forming a pink color. Upon elution from the chromatograms, faintly yellow crystals were formed. Compound 1 was very soluble in ethanol, methanol, acetone, ethyl acetate, and ethyl ether, but only slightly soluble in benzene and water. The UV spectrum of compound 1 showed λ_{max} (ethanol): 225, 248, 258(s) (shoulder or inflection), 293, 306, 323, and 338 nm; (ethanolic NaOH): 213, 258, 298, 309, 343(s), and 354 nm. The infrared spectrum showed hydroxyl absorbance at 2.9 μ which was weak, relative to that exhibited by gossypol or compound 2. A band at 3.4 μ was attributed to aromatic CH-stretching, and a prominent conjugated carbonyl band was observed at 5.95 μ. Compound 1 showed aromatic stretching bands at 6.25 and 6.77 μ, and exhibited an ether absorbance band at 9.0 μ that was not present in gossypol or compound 2. The mass spectrum of compound 1 (Table 2) gave a strong parent ion at m/e (mass to charge ratio) 258. The compound appeared to contain at least four methyl groups (loss of four 15 atomic mass units [AMU] fragments), a free aldehyde group (M-29 [CHO]), one aromatic hydroxyl group, based on the loss of 28 amu (CO), a methoxyl (M-CH_3, followed by M-31 [OCH_3], and underwent ring fissure leading to loss of 43 [C_2H_3O]). The loss of 45 and 46 mass units observed in the spectra was probably due to carbonyl-C_1-hydroxyl

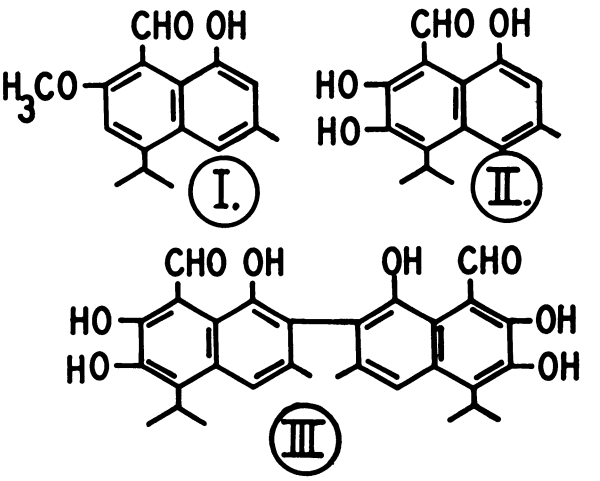

Fig. 1. Proposed structures of vergosin (I) and hemigossypol (II), and structure of gossypol (III). The numbering of carbon atoms follows that proposed by Adams et al. (2).

tautomerization in the molecule, with elimination of CHO_2 or CH_2O_2, respectively. The NMR spectrum of compound 1 (Table 3) clearly showed the presence of methoxyl, methyl, and isopropyl groups.

The acetate derivative of compound 1 showed a parent ion in the mass spectrum at m/e 300 and a base peak at m/e 258, further indicating that compound 1 contained but one hydroxyl group.

The IR and NMR spectral data indicated that compound 1 contained a naphthalene nucleus (Fig. 1). The positioning of the free methyl and isopropyl groups was made by similarity of the NMR data to

TABLE 1. R_F values of gossypol, hemigossypol, and vergosin on Silica Gel GF_{254} or polyamide thin-layer plates developed with various solvent systems

Adsorbent	Solvent system	$R_F \times 100$		
		Gossypol	Compound 1 (vergosin)	Compound 2 (hemigossypol)
Silica gel[a]	Chloroform:acetone:formic acid:conc. HCl 95/5/2/0.3[b]	51[c]	52	40
Silica gel	Petroleum ether (30-60°):ethyl ether:conc. HCl 70/30/0.3[b]	19[c]	24	14
Silica gel	Hexane:ethyl acetate:methanol 60/40/1	28[c]	56	28
Silica gel	Toluene:ethyl formate:formic acid 88% 50/40/10	73	67	60
Silica gel	Benzene:ethyl formate:formic acid 88% 75/24/1	66	55	20
Silica gel	Benzene:methanol 97/3	19[c]	50	13
Silica gel	Benzene:methanol 95/5	23[c]	56	21
Polyamide[d]	Chloroform:acetone:formic acid 95/5/2	86	79	45
Polyamide	Methanol:formic acid 98/2	51	47	42

[a] 0.375-mm layers were applied to glass plates and heated at 110 C for 30 min before use; all solvents are (v/v).
[b] Suggested by A. A. Bell, USDA, Cotton Disease Laboratory, College Station, Texas.
[c] Compound streaked.
[d] 0.250-mm layers were activated by an air drying before use.

TABLE 2. Mass spectra of gossypol, compound 1 (vergosin), and compound 2 (hemigossypol) [a,b]

Compound	m/e	Relative intensity[c]	Interpretation
Gossypol	519	7	M+1
	518	17	M^+
	500	100	$M-H_2O$
	485	33	$500-CH_3$
	482	20	$500-H_2O$
	472	9	$M-CH_2O_2$
	467	23	$482-CH_3$
	457	40	$472-CH_3$
	439	3	$485-CH_2O_2$
Compound 1, vergosin	259	19	M+1
	258	94	M^+
	243	100	$M-CH_3$
	229	17	M–CHO
	228	42	$243-CH_3$
	227	13	$M-OCH_3$
	215	6	243–CO and $M-CH_2O_2$
	214	11	$229-CH_3$
	213	14	$M-CHO_2$ or $228-CH_3$
	212	9	$M-CH_2O_2$ or $243-OCH_3$
	211	8	
	201	14	229–CO
	200	5	228–CO
	199	14	
	198	5	$213-CH_3$ or $229-OCH_3$
	197	8	$228-OCH_3$ and $215-CH_3$
	196	2	$214-CH_3$
	195	5	
	187	6	
	186	6	$229-C_2H_3O$
	185	13	$228-C_2H_3O$ and 213–C
	184	5	$229-CHO_2$
	183	9	$197-CH_3$ and $229-CH_2O_2$
	182	5	$213-OCH_3$ and $197-CH_3$
	181	6	
	171	13	$214-C_2H_3O$
	169	13	197–CO and $184-CH_3$
Compound 2, hemigossypol	261	17	M+1
	260	90	M^+
	245	35	$M-CH_3$
	244	42	M– (O) ?
	243	16	M– (OH) ?
	242	45	$M-H_2O$
	230	52	$245-CH_3$
	229	57	$244-CH_3$
	228	22	$243-CH_3$
	227	100	$242-CH_3$
	215	62	$M-CHO_2$
	214	45	$M-CH_2O_2$
	201	30	228–CO
	199	52	227–CO
	197	13	215–CO
	185	13	
	171	27	199–CO

[a] Direct probe sample insertion; gossypol was run at ca. 200 C; vergosin and hemigossypol were run at 100-120 C.

[b] Only higher mass peaks were tabulated.

[c] Intensities are expressed as a percentage of the base peak intensity.

that for gossypol (Table 3). The single hydroxyl group was not placed at C_6 because the rather high wavelength (5.85 μ) carbonyl band in the IR spectrum suggested tautomerization in the molecule. Of the two remaining possible positions for the hydroxyl, C_7 is not allowed because of the failure to observe loss of water in the mass spectrum, which would occur from the tautomer of such a structure, analogous to the formation of anhydrogossypol from gossypol (2, Table 2). The occurrence of this predicted fragmentation was confirmed by the mass spectrum of a model compound, salisaldehyde (M^+ = 122; major fragments at m/e 121, 104, 93, and 76). Furthermore, the observed loss of m/e 45 and 46 fragments from compound 1 would be expected from tautomerization between the C_1 hydroxyl and the aldehyde grouping. It was impossible to assign unambiguously the single methoxyl group in compound 1. The tentative placement at C_7 (Fig. 1) appears to best fit the spectral data, but synthesis will be required to definitively establish this point.

Compound 2—hemigossypol.—Compound 2 was a yellow substance when separated from the crude extracts by TLC, and absorbed light at 254 nm. The color of TLC spots changed to greenish brown upon standing in the air for 15 min, indicating that compound 2 was unstable in the presence of oxygen. Compound 2 reacted rapidly with phloroglucinol, forming a deep pink color. The purified compound was a yellow-brown viscous oil, readily soluble in acetone, methanol, and ethanol. It showed UV absorption at λ_{max} (methanol); 228, 255, 288, 294, and 370 nm. The infrared spectrum was very similar to that of gossypol, showing hydroxyl absorbance at 2.8 μ and a carbonyl band at 5.85 μ. The mass spectrum of compound 2 showed a parent ion at m/e 260 (Table 2). The mass spectral fragmentation pattern (Table 2) was also very similar to gossypol, with both compounds losing methyl groups, water, and CO. Neither compound lost CHO, indicating the occurrence of considerable tautomerization. Both compound 2 and gossypol also appeared to lose fragments at m/e 45 and 46, similar to compound 1. Compound 2 formed a tri-acetate (M^+ = 386), indicating that it contained three hydroxyl groups. Because of the instability of compound 2 when concentrated, it was not possible to obtain suitable NMR spectra. However, based on the similarity of the UV and IR data and the extreme similarity of the mass spectral fragmentation pattern to that of gossypol, we suggest that compound 2 is hemigossypol (Fig. 1). Hemigossypol has also been described by Bell based on chemical properties of the free and derivatized compound (5).

The antifungal properties of vergosin and hemigossypol are not surprising in view of their chemical similarity to the antifungal 1-naphthaldehyde, gossypol. Douros et al. (6) showed that a wide range of synthetic disubstituted naphthalenes are active antifungal agents. Although a number of naturally occurring antifungal 2-naphthaldehydes have been described (9, 11, 14), gossypol and the related compounds reported here

TABLE 3. Nuclear magnetic resonance data for gossypol and compound 1 [a]

Compound	Peak(s)	No. protons	Interpretation
Gossypol	1.55 Doublet	12	Methyl groups of isopropyls
	2.2 Singlet	6	Methyl groups at C_3
	3.75 Septet	2	Single protons of isopropyl carbons
	5.9 Singlet	2	Aromatic hydroxyl protons
	6.4 Singlet	2	Aromatic hydroxyl protons
	7.8 Singlet	2	Aromatic protons
	11.3 Singlet	2	Aldehyde protons
	15.2 Singlet	2	Aromatic hydroxyl protons
Compound 1	1.55 Doublet	6	Methyl groups of isopropyl
	2.4 Singlet	3	Methyl group at C_3
	3.5 Broad singlet	1	Aromatic hydroxyl proton
	3.7 Septet	1	Single proton of isopropyl carbon
	3.9 Singlet	3	Methoxyl protons
	5.8 Singlet	2	Aromatic protons
	6.5 Singlet	1	Aromatic proton
	7.3 Singlet	1	Aldehyde proton

[a] Spectra were taken in deutero-chloroform; aromatic hydroxyl groups were located by D_2O exchange.

appear to be the only known examples of naturally occurring antifungal 1-naphthaldehydes.

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