Phytochemistry, 1975, Vol. 14, pp. 1077–1081. Pergamon Press. Printed in England.

ANTIMICROBIAL TERPENOIDS OF *GOSSYPIUM*: 6-METHOXYGOSSYPOL AND 6,6′-DIMETHOXYGOSSYPOL*

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(*Revised Received* 16 *October* 1974)

Key Word Index—*Gossypium hirsutum*; *Gossypium barbadense*; Malvaceae; cotton; disease resistance; antibiotics; triterpenoid aldehydes; gossypol; 6-methoxygossypol; 6,6′-dimethoxygossypol; hemigossypol; 6-methoxyhemigossypol.

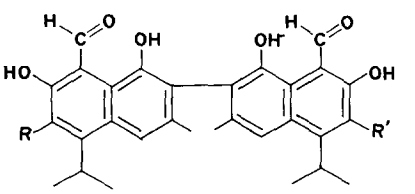
Abstract—The triterpenoid aldehydes, gossypol (**1**), 6-methoxygossypol (**2**) and 6,6′-dimethoxygossypol (**3**); and the sesquiterpenoid aldehydes, hemigossypol (**4**) and methoxyhemigossypol (**5**), were isolated from 1-week-old roots of *Gossypium hirsutum* and *G. barbadense* and identified. This is the first report of **2** and **3** in nature and of **4** and **5** from healthy roots. Compounds **2** and **3** also constituted 30% of the total terpenoid aldehydes in the seeds of 1 cultivar of *G. barbadense*, but occurred only in trace quantities in those of *G. hirsutum*. Spectral data (UV, IR, NMR, MS) and proof of structure for **2** and **3** are presented.

INTRODUCTION

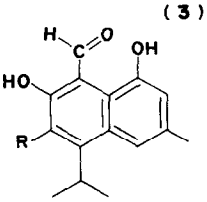
Gossypol (**1**) is a triterpenoid aldehyde that occurs extensively in seed, foliar parts and root bark of *Gossypium* and related genera. Its chemistry and biochemistry have been reviewed [1–3]. Because the compound is toxic to nonruminant animals, it is very important in the processing of cottonseed for food [4]. Gossypol is also important in the resistance of the cotton plant to insects [5], and its induced synthesis [6] is probably important in resistance to fungal pathogens [7].

Recent work has shown that gossypol may exist as either of two optical isomers [8, 9, 11]. In *Thespesia populnea* (L.) Sol. ex Corr. (+)-gossypol predominates [8, 9, 11], whereas both (±)- and (+)-gossypol occur in *Gossypium* seeds [10]. Other variations in the structure of triterpenoid aldehydes from cotton have not been reported.

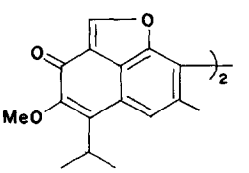
During histochemical investigations of gossypol in seedling roots of *Gossypium*, Mace *et al* [12] found four terpenoid aldehydes besides gossypol. Two of these were the sesquiterpenoid aldehydes, hemigossypol (**4**) and methoxyhemigossypol (**5**), which we identified previously from diseased stele tissue [13]. Isolation and identification of the other two compounds as the 6-methoxy (**2**) and 6,6′-dimethoxy (**3**) ethers of gossypol (**1**) are reported herein.



(**1**) R, R′ = OH
(**2**) R = OMe, R′ = OH
(**3**) R, R′ = OMe



(**4**) R = OH
(**5**) R = OMe



(**6**)

* Presented in part at the 1974 Beltwide Cotton Production Research Conference Proceedings, Dallas, Texas, R. D. Stipanovic, A. A. Bell, M. E. Mace and C. R. Howell, pp. 41–45, National Cotton Council, Memphis, Tennessee.

RESULTS

Identification of 6,6′-*dimethoxygossypol* (**3**) *from roots*

Compound **3** isolated from cotton roots was obtained as bright yellow crystals with mp 181–184° from C_6H_6-hexane. On prolonged exposure to air, it developed an orange brown color. **3** Formed a salmon–rose derivative with phloroglucinol [λ_{max}^{EtOH} 545 nm (ϵ 26800)], a yellow–orange chelate with antimony trichloride, and a burnt orange colored 2,4-dinitrophenylhydrazone.

The MS of **3** showed a M^+ at *m/e* 546 (12%). Diagnostically significant ions occurred at *m/e* 528 (M^+-H_2O, 82%), 513 (M^+-H_2O, Me; 29%), 510 (M^+-2H_2O, 100%), and 495 (M^+-2H_2O, Me; 85%). Only a small ion peak appeared at *m/e* 480 (M^+-2H_2O, 2Me; 4%), but a significant peak occurred at *m/e* 240 (13%); this was probably caused by a doubly charged ion *m/e* 480 since a significant peak is also observed at *m/e* 240·5 (5%).

The NMR spectrum of **3** is compared to that of gossypol (**1**) in Table 1. The methoxyl groups in **3** are at equivalent positions on the dimeric molecule because they appear as a single sharp singlet at δ3·94 (6H). The C_{-1}, C_{-6}, and C_{-7} methoxyls of the hexamethyl ether of gossypol each give peaks at different locations [14]. The methoxyl groups of **3** are not at the C_{-7} and $C_{-7'}$ positions, because these OH groups are H-bonded with the O of the aldehydes as evidenced by a large downfield shift of the aldehydic protons (δ11·10; 2H, *s*) and two hydroxyl protons (δ14·51; 2H, *s*) in the NMR spectrum. The C_{-1} and $C_{-1'}$ OH are free, because **3** readily loses two H_2O molecules on electron impact in the mass spectrometer. This fragmentation shows that the anhydroderivative (**6**) is readily formed from **3** by reactions involving free hydroxyl groups at the C_{-1}, $C_{-1'}$, C_{-7} and $C_{-7'}$ positions. Compound **3** is thus 6,6′-dimethoxygossypol.

Table 1. NMR spectra* of gossypol (G), 6,6′-dimethoxygossypol (DMG) and 6-methoxygossypol (MG)

	G	DMG	MG
$(Me)_2C$†	1·54	1·53	1·53
$Me-C_{-3}$	2·13	2·12	2·12
$(Me)_2C\underline{H}$‡	3·85	3·90	3·90
$MeO-C_{-6}$		3·94	3·95
$MeO-C_{-6'}$		3·94	
$HO-C_{-1'}$	5·81	5·78	5·68
$HO-C_{-1}$	5·81	5·78	5·78
$HO-C_{-6}$	6·31		
$HO-C_{-6'}$	6·31		6·37
$H-C_{-4,4'}$	7·70	7·75	7·75
CHO	10·96	11·10	11·15
CHO	10·96	11·10	11·17
$HO-C_{-7}$	14·84	14·51	14·57
$HO-C_{-7'}$	14·84	14·51	15·16

* All spectra were recorded at 23° in $CDCl_3$ using TMS as an internal standard and are reported in δ units. Phenolic protons were identified by D_2O exchange. Unless indicated in the footnotes the peaks appear as singlets.

† Doublet.

‡ Septet.

The proposed structure of **3** isolated from roots was confirmed by synthesis. Adams and Geissman [15] reported synthesis of 6,6′-dimethoxygossypol (mp 191–193°, corr.) in 1938. Their procedures and those of Datta *et al.* [14] were adapted to obtain 6,6′-dimethoxygossypol (**3**) as a yellow crystalline material (mp 184–186° C_6H_6–hexane). The MS, NMR and IR spectra of the synthetic compound agreed with those of **3** obtained from *Gossypium*.

Identification of 6-*methoxygossypol* (**2**) *from roots*

Compound **2** isolated from cotton roots was obtained as yellow crystals mp 146–149° from C_6H_6-hexane. It formed a deep rose derivative with phloroglucinol [λ_{max}^{EtOH} 551 nm (ϵ 31800)], an orange–red chelate with antimony trichloride, and a burnt orange colored 2,4-dinitrophenylhydrazone. The MS showed a M^+ at *m/e* 532 (3%). Fragment ions included *m/e* 514 (M^+-H_2O, 20%), 499 (M^+-H_2O, Me; 9%), 496 (M^+-2H_2O, 89%), and 481 (M^+-2H_2O, Me; 100%).

Several similarities in the NMR spectra of **2** and that of 6,6′-dimethoxygossypol (**3**) were noted (Table 1), except a 3 proton singlet was observed at δ3·95. Hydroxyl protons occurred at δ5·68 (1H, br. *s*), 5·78 (1H, br. *s*), and 6·37 (1H, br. *s*). The aldehyde protons appeared as 2 distinct signals at δ11·15 (1H, *s*) and 11·17 (1H, *s*). They were shifted downfield because of H-bonding with the C_{-7} and $C_{-7'}$ OH groups, which fall at δ14·57 (1H, *s*) and 15·16 (1H, *s*).

Compound **2** appeared to be 6-methoxygossypol. The NMR data show that **2** is an asymmetrical molecule of methoxylated gossypol. The chemical shift of the methoxy groups in the NMR spectra are the same for both **2** and **3**. The ready loss of 2 H_2O molecules from **2** in the mass spectrometer requires hydroxyls at C_{-1}, $C_{-1'}$, C_{-7}

Table 2. Content of terpenoid aldehydes in cottonseed and seedling roots

Cotton cultivar	Terpenoid content* HG	MHG	G	MG	DMG
	Mature seed (μmol/100 g fresh embryo)				
G. hirsutum					
Acala 4-42	T‡		729	16	5
Deltapine 16	T		1233	26	6
G. barbadense					
SBSI†	T	T	995	413	35
Pima S-2	T	T	1925	62	8
	7-day-old roots (nmol/g fresh root)				
Acala 4-42	100	83	232	123	82
SBSI	34	24	72	42	62

* HG = hemigossypol (**4**), MHG = 6-methoxyhemigossypol (**5**), G = gossypol (**1**), MG = 6-methoxygossypol (**2**), and DMG = 6,6′-dimethoxygossypol (**3**).
† SBSI = Seabrook Sea Island 12B2.
‡ Only trace quantities of sesquiterpenoids found in seed.

and C_{-7} and further confirms that methoxylation is at C_{-6}.

Occurrence of 6-*methoxygossypol* (**2**) *and* 6,6′-*dimethoxygossypol* (**3**)

Seed, seedling roots, and root and stem bark of flowering plants of *G. arboreum*, *G. hirsutum*, and *G. barbadense* were examined for the presence of compounds **2** and **3**. The distribution of the terpenoid aldehydes in seedling roots and seed embryos of some *G. hirsutum* and *G. barbadense* cultivars is shown in Table 2. Quantitative data for **2** and **3** were not obtained for other tissues. However, chromatographic separations were followed by spraying with antimony trichloride or phloroglucinol reagents. They revealed that **2** and **3** were present in root bark of flowering plants of each of five cultivars of *G. hirsutum* and *G. barbadense*. Also, **2** and **3** were present in the lower stem bark of the cultivars of *G. barbadense*. Appreciable quantities of 6-methoxyhemigossypol (**5**) have been shown in diseased cambial tissues of numerous *Gossypium* species and related genera [13]. These species also contain **2** and **3** in the diseased cambium. The methoxylated terpenoids **2** and **3**, like **5** [13], were not found in tissues of *G. arboreum*.

DISCUSSION

Compounds **2** and **3** showed fungitoxicity to *Penicillium* and *Cladosporium* spp. when assayed directly on TLC plates by methods described previously [16]. The ED_{50} values to these or other fungi have not been determined. The presence of a methoxyl group at the C_{-6} position in **2**, **3**, and **5** prevents their oxidation to C_{-6}, C_{-7} *o*-quinones. As a result, their stability to auto-oxidation and their biological specificity might differ considerably from that of the C_{-6}, C_{-7} *o*-dihydroxy terpenoids (**1** and **4**).

Some quantitative estimates of gossypol in cotton tissues and in related genera are in error. All 6 of the terpenoid aldehydes reported here and elsewhere [12, 13] in *Gossypium* and related genera have similar structures at the C_{-1}, C_{-4}, C_{-5}, C_{-7} and C_{-8} positions. Consequently, they undergo similar chemical reactions. Most quantitative estimates of gossypol are based upon its reaction with aniline to form a yellow color, but all six terpenoid aldehydes undergo this reaction to form similar yellow derivatives [17]. Likewise, the six terpenoid aldehydes give similar colored derivatives with phloroglucinol, antimony trichloride and 2,4-dinitrophenylhydrazine (other reagents used to estimate gossypol) [17]. Thus, previous data reflect total terpenoid aldehyde content rather than gossypol alone.

Our observations here and elsewhere [13] show that the methoxylated terpenoid aldehydes frequently form a large percentage of the total aldehydes in seedling roots and the root bark from various *Gossypium* spp. and related genera. In diseased stele tissues the sesquiterpenoid aldehydes were in higher concentrations than triterpenoid aldehydes [13]. However, in healthy or diseased seeds, roots and barks, the aldehydes were mostly triterpenoids.

EXPERIMENTAL

Gossypium barbadense. "Seabrook Sea Island 12B2" and "Pima S-2" and *G. hirsutum*. "Acala 4-42" and "Deltapine 16" cotton plants were grown in the field at College Station, Texas. Seed and various plant parts were harvested, rinsed with dist. H_2O and freeze-dried for 1 week. Whole embryos were removed from seed coats and embryos and other dried tissues were ground to 60-mesh in a Wiley mill. Fresh roots were obtained from seedlings grown in germination towels for 1 week at 28° with a 14:10 hr, light (200 lx): dark regime as described previously [12].

Preparation of crude terpenoid fraction. A soln of 0·25% $NaHSO_3$ in 95% EtOH was used as extracting solvent. Dried, ground tissues were stirred in the solvent (1 g/9 ml) for 30 min

and filtered; this procedure was repeated with the residue and the filtrates were combined. Fresh roots were excised, weighed and immediately ground with 4 vol. of the solvent in a blendor. After filtration, the residue was resuspended and stirred in the solvent (2 vol./g fr. root) for 1 hr. The soln was then filtered and the filtrates were combined and mixed with 2 vol. of 50%-satd aq NaCl and 1 vol. of EtOAc. The H_2O phase was discarded and the EtOAc phase was washed twice with equal vol. of 50% satd NaCl, once with satd NaCl and dried. The EtOAc extract was reduced to *ca* 50 ml in a rotary evaporator at 30°, mixed with 3 vol. of hexane and filtered through a Si-gel column (3 × 5 cm). The column was eluted with 200 ml of EtOAc–hexane (1:3) and the combined eluates were dried *in vacuo* at 30°. The resulting residue is referred to as "crude terpenoids".

Chromatography and bioassay. All procedures were conducted with minimum light or in the dark. Air-dried Si-gel G_{254} layers (0·5 mm) were used and terpenoids in C_6H_6 or EtOAc were applied to the layers and residual solvent was removed with N_2. Terpenoid aldehydes were detected on developed TLC plates by spraying with (a) a fresh mixture of 5% phloroglucinol in EtOH and conc HCl (1:1), (b) 2,4-dinitrophenylhydrazine (saturated) in 2 N HCl, or (c) 2% $SbCl_3$ in $CHCl_3$. During preparation, compounds were located by their yellow color and UV quenching. Fungitoxic activity of the terpenoids to *Cladosporium* and *Penicillium* species was shown by direct bioassays on TLC plates [16].

Spectral data. UV spectra were determined in 95% EtOH or 95% EtOH containing 0·03 M NaOH (EtONa) and are reported as λ_{max}. IR spectra were determined in KBr. NMR spectra (100 MHz) were determined in $CDCl_3$ soln at 23°. Chemical shifts are reported as δ units. Hydroxyl protons were detected by D_2O exchange. MS were determined at 70 eV with a probe temp. of 180° and a source temp. of 200°.

Isolation of 6,6′-dimethoxygossypol (**3**). Crude terpenoids from 2 kg of 7-day-old seedling roots were dissolved in 200 ml EtOAc–hexane (1:3) and this soln was extracted × 4 with 100 ml of an aq. soln containing 9% NaCl and 1% $Na_2B_4O_7$. The aq. extracts were combined and retained for later isolation of **1** and **2**. The EtOAc phase, which contained **3** and related materials, was washed twice with 0·1 N HCl and then washed repeatedly with 50%-satd NaCl soln to remove traces of acid. The EtOAc extract was finally dried and the EtOAc was removed *in vacuo* at 30°. The residue was dissolved in C_6H_6, and chromatographed by TLC, EtO_2–naphtha solvent (1:3). The chromatograms showed 4 bands that quenched UV light (254 nm). The band (R_f = 0·17–0·25) contained compound **3**, which was eluted with Et_2O. The Et_2O was removed *in vacuo* at 20° and the residue was dissolved in a minimum vol. of hot C_6H_6. When an equal vol. of hexane was added to the C_6H_6 soln. **3** was obtained as yellow crystals. Recrystallization from C_6H_6–hexane (1:1) gave pure **3** as golden yellow crystals. Mp 181–184°. UV: λ_{max}^{EtOH} 231 (ε 69900), 253 (48200), 287 (32200), 360 (11200), 390 (sh) nm; λ_{max}^{EtONa} 239 (ε 61200), 271 (32000), 343 (10700) nm. IR: ν_{max}^{KBr} 1612 cm^{-1}. $[\alpha]_D$ −7·7° ($CHCl_3$).

Isolation of 6-methoxygossypol (**2**). The aq. phase obtained during purification of **3** was extracted × 4 with 0·5 vol. of EtOAc–hexane (1:1) and then was retained for purification of gossypol (**1**). The organic phase was washed × 3 with equal vols. of 0·1 N HCl and finally with 50%-satd NaCl soln, until all traces of acid were removed. The EtOAc–hexane extract was dried and the solvent removed *in vacuo* at 30°. The residue was dissolved in C_6H_6 and applied to TLC plates. After development with $CHCl_3$, the yellow band (R_f 0·45–0·55), which contained **2**, was eluted with Et_2O. **2** was then crystallized by the techniques used for **3**. Recrystallization from C_6H_6–hexane (1:1) gave pure **2** as yellow crystals. Mp 146–149°. UV: λ_{max}^{EtOH} 235 (ε 77100), 288 (31200), 369 (13900) nm; λ_{max}^{EtONa} 240 (ε 63500), 269 (sh), 296 (sh), 340 (9000), 380 (14600) nm. IR: ν_{max}^{KBr} 1615 cm^{-1}. $[\alpha]_D$ +38·5° (EtOH).

Isolation of gossypol (**1**) *from roots.* The aq. phase remaining from the purification of **2** was adjusted to pH 3 with HCl and extracted twice with ½ vol. of Et_2O. The organic phase was thoroughly washed with H_2O, dried and reduced to dryness *in vacuo* at 30°. The residue was dissolved in EtOAc and gossypol was purified using the same TLC system as used for **3**. The residual material, obtained from elution of the only major band present after TLC, was dissolved in a minimum vol. of warm $CHCl_3$; the soln was chilled to give crude crystals. Recrystallization ($CHCl_3$) gave pure **1** as yellow crystals. Mp 184–187°. UV: λ_{max}^{EtOH} 236 (ε 76100), 283 (sh), 289 (28800), 376 (15500) nm; λ_{max}^{EtONa} 240 (ε 65400), 273 (sh), 340 (7000), 386 (15000) nm. IR: ν_{max}^{KBr} 1625 cm^{-1}. MS (*m/e*): 518 (M^+, 12%), 510 (4%), 502 (6%), 501 (24%), 500 (M^+-H_2O, 85%), 486 (4%), 485 (M^+-H_2O, Me; 17%), 484 (11%), 483 (38%), 482 (M^+-2H_2O, 100%), 468 (16%), 467 (M^+-2H_2O, Me; 51%), and 438 (10%).

Synthesis of 6,6′-dimethoxygossypol (**3**). Gossypol acetate (2·3 g), MeOH (10 ml), and Me_2SO_4 (6 ml) were mixed in a 500-ml flask under N_2 in the dark. A soln of 10% KOH in MeOH (10 ml) was added with constant stirring in small portions over a period of 1·5 hr, so that the color of the reaction mixture remained yellow-brown and did not turn green. K_2CO_3 (10 g) was then added, followed by Me_2CO (375 ml). The soln was refluxed for 48 hr; Me_2SO_4 (2 ml) was added and refluxing was continued another 48 hr. Most of the solvent was evaporated *in vacuo*, H_2O (500 ml) was added and the mixture was extracted twice with Et_2O–naphtha solvent (1:1; 500 ml total). The combined organic extracts were washed successively with 0·5 M NaOH, H_2O (until neutral) and satd NaCl soln and dried. The solvent was evaporated to give crude gossypol hexamethyl ether. This material was heated in HOAc (100 ml) until refluxing began and then conc H_2SO_4 (1 ml) in HOAc (20 ml) was added rapidly. Refluxing was continued for 30 min and the soln was cooled and poured into H_2O (400 ml). The aq. soln was extracted × 2 with Et_2O (400 ml total). Naphtha solvent (400 ml) was added to the Et_2O phase, which was then extracted × 4 with 2% $Na_2B_4O_7$ (adjusted to pH 10 with NaOH; 500 ml total). The combined aq. extracts were filtered, adjusted to pH 5 with HCl and extracted × 4 with 100 ml portions of Et_2O–hexane (1:3; 400 ml total). The organic phase was washed × 4 with an aq. soln containing equal vol. of 2% $Na_2B_4O_7$ and satd NaCl (adjusted to pH 10; 800 ml total) and then successively with 0·5 N HCl, H_2O and satd NaCl soln. Finally, the soln was dried and the solvent was removed *in vacuo*. Recrystallization of the residual material from C_6H_6–hexane (1:1) provided 161 mg of **3** (mp 184–186°). Physical and spectral properties of synthetic **3** were identical to those of **3** isolated from cotton roots.

Quantitative measurements of terpenoid aldehydes. Details for the TLC separation and quantitation of terpenoid aldehydes are published elsewhere [17]. Crude terpenoids from 0·2 g embryo or 10 g root were streaked along one edge of a 20 × 20-cm TLC plate layered with 0·5 mm of polyamide. The chromatogram was developed with C_6H_6–$CHCl_3$–MeOH–HOAc (150:50:3:2), dried for about 5 min, and sprayed with the phloroglucinol reagent. After the burgundy and rose colors had fully developed (*ca* 2 hr), the bands containing individual terpenoids were removed from the plates and placed in 5 ml of EtOH. R_f (× 100) were: **4**, 11; **5**, 23; **1**, 35; **2**, 51; and **3**, 69. Polyamide fractions containing each phloroglucinol derivative were extracted overnight in EtOH and centrifuged, and an aliquot of the extract was removed from each to determine A at λ_{max}^{EtOH}. These data were compared with known ε values to estimate the amounts of terpenoids. The λ_{max}^{EtOH} (ε) of the phloroglucinol derivatives were: **4**, 553 (ε 21500); **5**, 548 (20300); **1**, 550 (46700); **2**, 551 (31800); **3**, 545 (26800) nm.

Acknowledgements—The authors are grateful to Mrs. Mildred Hummel and Mr. Gilbert Tribble for technical assistance.

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