Phytochemistry 122 (2016) 165–171

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Gossypolhemiquinone, a dimeric sesquiterpenoid identified in cotton (*Gossypium*)

Robert Stipanovic [a,*], Lorraine Puckhaber [a], James Frelichowski Jr. [a], Jesus Esquivel [a], John Westbrook [a], Mike O'Neil [a], Alois Bell [a], Michael Dowd [b], Kater Hake [c], Sara Duke [a]

[a] USDA, Agricultural Research Service, Southern Plains Agricultural Research Center, College Station, TX 77845, United States
[b] USDA, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70124, United States
[c] Cotton Incorporated, 6399 Weston Parkway, Cary, NC 27513, United States

ARTICLE INFO

Article history:
Received 14 September 2015
Received in revised form 4 December 2015
Accepted 14 December 2015
Available online 22 December 2015

Keywords:
Gossypium thurberi
Malvaceae
Insect herbivory resistance
Di-sesquiterpenoid
Helicoverpa zea
Gossypol
Gossypolhemiquinone

ABSTRACT

The report that the cotton leaf perforator, *Bucculatrix thurberiella*, is one of the few insect herbivores to attack *Gossypium thurberi* prompted an investigation of the terpenoids present in the leaves of this wild species of cotton. Members of *Gossypium* produce subepidermal pigment glands in their leaves that contain the dimeric sesquiterpenoid gossypol as well as other biosynthetically related terpenoids. In addition to gossypol, a previously unknown dimeric sesquiterpenoid, gossypolhemiquinone (GHQ), was identified in trace amounts in *G. thurberi*, a member of the D genome. Other members of the D genome of *Gossypium* were subsequently found to contain this compound, but GHQ was not detected in commercial cotton cultivars. When fed to *Helicoverpa zea* in an artificial diet, GHQ delayed days-to-pupation, reduced pupal weights, and survival to adulthood to a lesser or equal extent than gossypol in comparison to the control diet. However, GHQ had a synergistic effect on survival and days-to-pupation when combined with gossypol at the highest dosage tested (0.18%; 15.5:84.5 GHQ:gossypol). Because gossypol exhibits anti-cancer activity, GHQ was also evaluated for its anti-cancer activity against the National Cancer Institute's 60-Human Tumor Cell Line Screen. Significant inhibitory activity against most of these cell lines was not observed, but the results may offer some promise against leukemia cancer cell lines.

Published by Elsevier Ltd.

1. Introduction

Members of the genus *Gossypium* produce pigment glands in the foliage that contain a mixture of terpenoid aldehydes such as gossypol (**1**) (Fig. 1). Gossypol (**1**) and biosynthetically related compounds such as heliocides H_1–H_4 (**3**–**6**) and hemigossypolone (**7**) (Fig. 1) suppress the growth of some herbivorous insect pests (Lukefahr and Martin, 1966; Stipanovic et al., 1977). Based on taxonomy, as well as cytological and cross pollination studies, cotton breeders have conveniently given alphanumeric designations to *Gossypium* species where those sharing the first letter have some degree of interfertility (Percival et al., 1999). An early TLC study of foliar extracts from *Gossypium* species found that most members of the D genome produce only gossypol (**1**) in the foliage pigment glands (Stipanovic et al., 1977). Most members of the D genome inhabit mainly dry regions of the western portions of Mexico, with one species found in the Galapagos (*Gossypium klotzschianum* Andersson) and one in Peru (*Gossypium raimondii* Ulbrich); the latter is exceptional in that it is the only species that produces the terpenoid raimondal (**8**) (Fig. 1). One species, *Gossypium thurberi*, extends north as far as central Arizona. It is in Arizona that Karban studied *G. thurberi* Todaro and found that the cotton leaf perforator (*Bucculatrix thurberiella* Busek) is one of the few insect herbivores that feed on this plant (Karban, 1993). This report prompted the current investigation of the terpenoid aldehydes present in the leaves of this plant which resulted in the identification of a previously unknown minor component in addition to gossypol (**1**). The structure of this compound, which we call gossypolhemiquinone (**9**, GHQ) (Fig. 1), was established based on synthesis and subsequent detailed 1-D and 2-D NMR analyses, and high resolution MS. Phillips and Hedin (1990) had previously identified the related compound gossypolone (**2**, GQ) (Fig. 1) as a component of the pigment glands in flower buds in some cotton cultivars.

In the current study, *Helicoverpa zea* (Boddie) was selected as a test-insect to determine if GHQ (**9**) might augment resistance to insect herbivores. Its effect on the growth and development of this insect was determined by adding 0.06%, 0.12% and 0.18% GHQ (**9**) to artificial diets. Mortality, days to pupation, and pupal weight were determined for larvae fed the GHQ (**9**) diets and compared

* Corresponding author.
E-mail address: bob.stipanovic@ars.usda.gov (R. Stipanovic).

http://dx.doi.org/10.1016/j.phytochem.2015.12.009
0031-9422/Published by Elsevier Ltd.

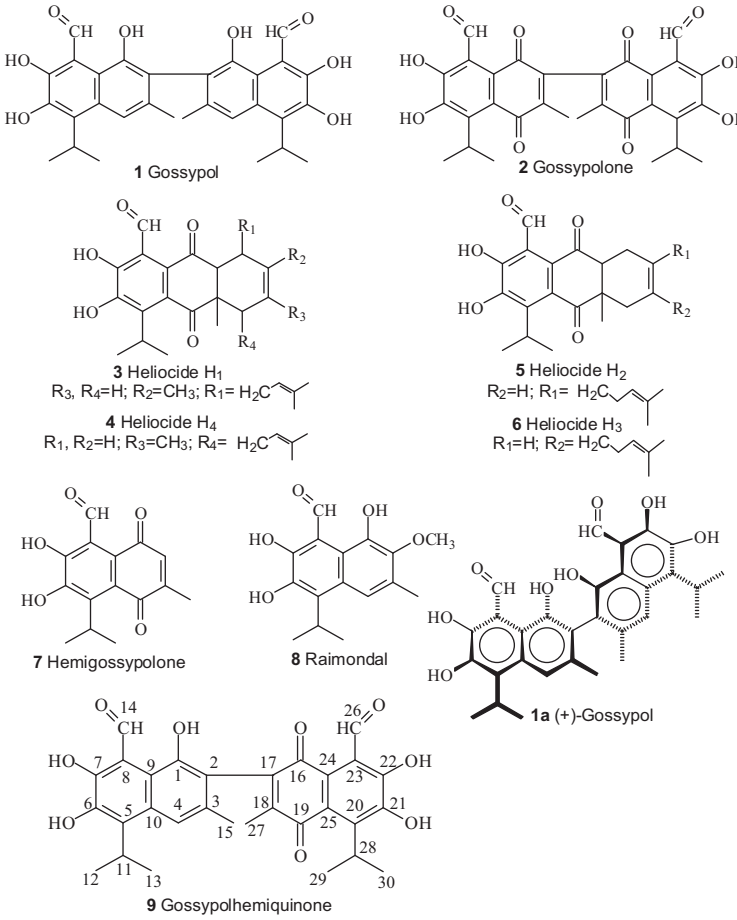

Fig. 1. Terpenoids identified in *Gossypium* foliage.

to that from larvae raised on a diet containing gossypol (**1**) at the same concentrations or a control diet containing no terpenoids. Diets with a mixture of GHQ (**9**) and gossypol (**1**) (15.5:84.5) at the same concentrations as GHQ (**9**) were also studied.

Gossypol (**1**) shows a wide range of biological activity including anticancer (Shelly et al., 2000; Oliver et al., 2005), spermicidal (Kim et al., 1984), antiamoebic (Gonzalez-Garza and Said-Fernandez, 1988), antibacterial (Yildirim-Aksoy et al., 2004) and antifungal (Mellon et al., 2011). Since both gossypol (**1**) and GQ (**2**) show anticancer activity, GHQ (**9**) was tested for its anticancer activity in the National Cancer Institute's 60-Human Tumor Cell Line Screen.

Gossypol (**1**) exists as stable enantiomers at room temp. due to restricted rotation around the central binaphthyl bond (Jaroszewski et al., 1992). The anti-cancer activity of (+)- and (−)-gossypol (**1**) differ, with (−)-gossypol being more active (Loberg et al., 2007). Since the enantiomers of gossypol (**1**) are stable at room temperature, but those of GQ (**2**) racemize at room temp. (Dao et al., 2004), the optical stability of (+)-GHQ was investigated.

2. Results and discussion

2.1. Identification of gossypolhemiquinone (GHQ) (9)

Extracts from the young leaves of *G. thurberi* showed the presence of gossypol (**1**) and an unidentified compound. The extract was subjected to LC/MS analysis and the unidentified compound provided a base peak of *m*/*z* 515. Gossypol-type compounds with a hydroxyl group *peri* to the aldehyde group and a second hydroxyl group *ortho* to the aldehyde have a propensity to lose a molecule of water during mass spectrometry. A small peak (0.4%) was observed at *m*/*z* 533, but a substantially larger peak was observed at *m*/*z* 555 (10%). The latter peak was suspected to be due to [M] (532 amu) + Na (23 amu), while the *m*/*z* 533 peak was tagged as the parent [M]+1 peak; this corresponds to the [M]+1 molecular weight of several gossypol-like terpenoids including gossypolhemiquinone (GHQ) (**9**). To further characterize this metabolite, the compound was purified by semi-prep HPLC. The purified metabolite was subject to EI/MS analysis using a direct exposure probe. When only masses above 400 amu were considered, the compound provided a base peak at *m*/*z* 514, a fragment ion at *m*/*z* 499 (62.9%) and an apparent molecular ion at *m*/*z* 532 (2.2%). This molecular weight agreed with the ready loss of H_2O to provide the base peak and a subsequent loss of a CH_3 group. High resolution ESI/MS provided an ion at *m*/*z* 533.1814 with a molecular formula of $C_{30}H_{29}O_9$ (Calcd. $C_{30}H_{29}O_9$: 533.181158, accuracy 0.45 ppm).

2.2. Synthesis of (±)-gossypolhemiquinone (GHQ) (9) and (+)-gossypolhemiquinone (2)

To establish the identity of the unknown, GHQ (**9**) was synthesized [along with GQ (**2**)] using a modification of the $FeCl_3$ oxidation of gossypol (**1**) developed by Hass and Shirley (1965). The final synthetic product was >97% pure (HPLC) [m.p. 158–160 °C; UV: (95% EtOH) λ_{max} (log ε) 234, (3.81), 274 (3.57), 372 (3.08)] and the structure was established to be GHQ (**9**) based on 1-D and 2-D NMR analyses [chemical shifts are reported in Tables 1 and 2 (HMBC proton–carbon couplings are illustrated in the online

Table 1
^{13}C chemical shifts for gossypolhemiquinone (GHQ) (**9**) compared to the dimeric sesquiterpenoids gossypol (Goss) (**1**) and gossypolone (GQ) (**2**) (for specific carbon assignments refer to Fig. 1).

C#	Goss (**1**) δ	GHQ (**9**) δ	C#	GQ (**2**) δ	GHQ (**9**) δ
1	150.4	150.2	16	186.5	186.7[a]
2	115.8	115.6	17	147.2[b]	149.0
3	133.7	131.6	18	138.1[b]	140.3
4	118.1	117.9	19	184.4	186.8[a]
5	134.1	133.8	20	141.5	141.4
6	143.4	143.4	21	149.4	149.4
7	156.0	156.0	22	152.4	152.3
8	111.7	111.8	23	115.9	115.6
9	114.6	117.3	24	126.7	127.2
10	129.6	129.4	25	127.6	127.5
11	27.8	27.8	26	198.5	197.9
12	20.2	20.1	27	14.8	14.6
13	20.2	20.3	28	28.7	28.8
14	199.3	199.3	29	19.7	20.0
15	20.3	20.8	30	19.6	19.7

[a] Assignments may be interchanged.
[b] Assignments are based on that in GHQ; in the HMBC experiment, no coupling was shown between the protons on C-27 with either C-17 or C-18.

Table 2
^{1}H chemical shifts for gossypolhemiquinone (GHQ) (**9**) compared to the dimeric sesquiterpenoids gossypol (Goss) (**1**) and gossypolone (GQ) (**2**) (for carbon assignments refer to Fig. 1).

C#	Type	Goss (**1**) δ	GHQ (**9**) δ	C#	Type	GQ (**2**) δ	GHQ (**9**) δ
1	C1-OH	5.89	6.60[a]	21	C21-OH	6.59	6.39[a]
4	Ar-H	7.77	7.65	22	C22-OH	13.01	12.97
6	C6-OH	6.39	6.37[a]	26	HC=O	10.58	10.50
7	C7-OH	15.10	15.05	27	Ar-CH_3	2.04	1.97
11	$CHMe_2$	3.88	3.82	28	$CHMe_2$	4.12	4.05
12	CH_3	1.53	1.48	29	CH_3	1.42	1.39
13	CH_3	1.53	1.53	30	CH_3	1.44	1.42
14	HC=O	11.1	10.90				
15	Ar-CH_3	2.13	2.19				

[a] Assignments for GHQ (**9**) C1-OH, C6-OH and C21-OH may be interchanged.

Supplementary material section)] and direct probe EI/MS. The identity of the unknown from *G. thurberi* was then confirmed to be GHQ (**9**) based on HPLC retention times [10.83 min for GHQ (**9**) compared to 9.54 min for GQ (**2**) and 15.66 min for gossypol (**1**)] and UV spectra (shown in Supplementary material section) that matched the synthesized compound.

(+)-GHQ (**9**) was prepared from (+)-gossypol (**1a**) (99% ee). The synthesized (+)-GHQ (**9**) had a specific rotation of $[\alpha]_D^{20}$ +36.4° (*c* 0.278, EtOH) and was established to be 82% enantiomeric excess *via* HPLC analysis using a Phenomenex Lux Cellulose column and an iPrOH:hexane mobile phase. HPLC analysis showed that in a solution of iPrOH:hexane (~1:1), the GHQ (**9**) slowly racemized at room temperature. Over 5 days, solutions that were 65% ee were reduced to 12% ee. The ee of an aliquot of this solution held at −20 °C did not significantly racemize after 5 days. The solid samples also could be stored at −20 °C without detectable racemization. Others have shown that GQ (**2**) readily undergoes racemization at room temperature (Dao et al., 2004). Thus, GHQ (**9**) appears to be intermediate between gossypol (**1**) and GQ (**2**) in its propensity to racemize.

2.3. GHQ (*9*) and gossypol (*1*) in the D genome of Gossypium and in commercial cottons

G. thurberi is a member of the D genome of *Gossypium*. In a previous investigation of terpenoids in the young leaves of *G. thurberi* using TLC plates, only gossypol (**1**) was identified in this tissue (Stipanovic et al., 1977). Thus, terpenoids such as the sesquiterpenoid hemigossypolone (**7**) and the sesterterpenoids heliocides H_1, H_2, H_3 and H_4 (**3**–**6**) (Fig. 1) that are found in commercial Upland cottons (*Gossypium hirsutum*), as well as other members of *Gossypium*, were not found. To extend this investigation, several *Gossypium* accessions of the D genome available in the USDA Cotton Germplasm collection were grown in the greenhouse; leaves were collected, freeze dried, and ground. The ground tissue was extracted and immediately subjected to HPLC analysis; the results are shown in Table 3. As found previously, other sesquiterpenoids and sesterterpenoids normally in *G. hirsutum* were not detected in *G. thurberi* nor in most other members of the D genome. However, GHQ (**9**) was present in small amounts in these cottons. As previously reported (Stipanovic et al., 1977), within the D genome only the leaves of *Gossypium gossypioides* (Ulbrich) contained hemigossypolone (**7**) and heliocides (**3**–**6**); the leaves of other members of the D genome did not contain these terpenoids. In addition, the leaves of *G. raimondii* contained only gossypol (**1**) and the sesquiterpenoid, raimondal (**8**) (Fig. 1). Four commercial cultivars of *G. hirsutum* cotton also were analyzed (Table 4). GHQ (**9**) was not detected in these plants, but, as expected, gossypol (**1**), hemigossypolone (**7**) and heliocides H_1–H_4 (**3**–**6**) were detected.

2.4. Effect of GHQ (*9*) on growth and development of H. zea larvae

Since gossypol (**1**) and related terpenoids are important components in the plant's arsenal of terpenoids that protect it from herbivorous insects, it seemed appropriate to test the toxicity of GHQ (**9**) to insects. Thus, racemic gossypol (**1**) and racemic GHQ (**9**) were individually incorporated into a soybean artificial diet using the non-nutritive additive, Alphacel, at concentrations of 0.00% (control), 0.06%, 0.12% and 0.18%. To determine if GHQ (**9**) has a synergistic effect with gossypol (**1**), diets were also prepared with a total concentration of terpenoids of 0.06%, 0.12% and 0.18%, and a 15.5:84.5 ratio of GHQ (**9**):gossypol (**1**). One-day-old *H. zea* larvae were placed in plastic cups containing the various diets described above. After pupation, the pupae were allowed to harden for one day and then weighed. Days-to-pupation, pupal weights and percent survival to adulthood were recorded.

The mean number days-to-pupation increased as the concentration of gossypol (**1**) and GHQ (**9**) increased compared to larvae fed the control diet (Fig. 2). However, the difference in days-to-pupation between the larvae fed the gossypol (**1**) and GHQ (**9**) were not significantly different. When the larvae were fed the 15.5:84.5 mixtures of GHQ (**9**) and gossypol (**1**), an extension in days-to-pupation was not observed at the 0.06% and 0.12% concentrations. However, at the 0.18% concentration, the days-to-pupation were significantly longer compared to all other diets.

The mean pupal weights (Fig. 3) were significantly less for diets containing 0.12% and 0.18% gossypol (**1**), GHQ (**9**), or the 15.5:84.5 mixture of the two terpenoids compared to those fed the control diet. However, pupal weights for larvae fed gossypol (**1**) were significantly less than those fed an equivalent concentration of GHQ (**9**). No synergistic effect in reducing pupal weight was observed with the mixture.

The percent survival to adulthood of larvae fed 0.06% gossypol (**1**) or the 0.06% 15.5:84.5 GHQ (**9**):gossypol (**1**) mixture was greater than the control (Fig. 4). This may be due to a hormetic effect (i.e., a biphasic dose–response exhibited by some toxic substances when fed at low concentrations) that has been demonstrated in other gossypol (**1**) feeding studies (Stipanovic et al., 1986; Celorio-Mancera et al., 2011). Survival of larvae fed 0.06% or 0.12% GHQ (**9**) or the 15.5:84.5 mixtures were not affected. At 0.18%, gossypol (**1**) and GHQ (**9**) reduced survival with gossypol

Table 3
Concentration of gossypol (Goss) (**1**) and gossypolhemiquinone (GHQ) (**9**) in freeze dried leaves of select D genome *Gossypium* greenhouse grown plants.

Gossypium species (Accession #)	Genome #	GHQ (**9**) μg/mg	Goss (**1**) μg/mg	Gossypium species	Genome #	GHQ (**9**) μg/mg	Goss (**1**) μg/mg
G. thurberi (#6)	D1	0.17	10.12	*G. raimondii* (#4)[a]	D5	nd[b]	0.76
G. thurberi (#21)		0.12	13.46	*G. raimondii* (#20) [c]		nd	0.73
G. thurberi (#22)		0.13	19.94	*G. raimondii* (#26)[d]		nd	0.71
G. armourianum (#1–6)	D2	0.18	9.34	*G. gossypioides* (#1)[e]	D6	nd	0.52
G. armourianum (#1–7)		0.12	5.72	*G. gossypioides* (#3)[f]		nd	0.24
G. harknessii (#2–2)	D2–2	0.15	12.87	*G. gossypioides* (#7)[g]		nd	0.28
G. davidsonii (#d-2)	D3	0.10	1.97	*G. gossypioides* (#8)[h]		nd	0.24
G. davidsonii (#d-3)		0.15	6.34	*G. lobatum* (#4)	D7	0.11	6.59
G. davidsonii (#d-4)		0.10	3.61	*G. trilobum* (#1)	D8	0.25	22.39
G. davidsonii (#d-21)		0.10	2.16	*G. trilobum* (#4)		0.14	15.33
G. davidsonii (#d-23)		0.11	4.14	*G. trilobum* (#5)		0.33	23.52
G. davidsonii (#d-26)		0.12	2.86	*G. trilobum* (#8)		0.18	14.73
G. davidsonii (#d-30)		0.13	9.56	*G. trilobum* (#9)		0.18	23.81
G. davidsonii (#d-32)		0.10	1.87	*G. laxum* (#4)	D9	0.15	7.72
G. klotzschianum (#k-57)	D3	0.13	8.50	*G. turneri* (#2)	D10	0.10	9.35
G. aridum (#12)	D4	0.17	12.02	*G. turneri* (#7)		0.12	6.10
G. aridum (#13)		0.15	7.19	*G. turneri* (#8)		0.13	5.24
				G. schwendimanii (#1)	D11	0.13	23.92

[a] Raimondal (**8**) 8.72 μg/mg.
[b] nd = not detected (limit of detection 0.05 μg/mg).
[c] Raimondal (**8**) 5.20 μg/mg.
[d] Raimondal (**8**) 5.82 μg/mg.
[e] Hemigossypolone (HGQ) (**7**) 6.46 μg/mg, $H_1 + H_3$ (**3**, **6**) 10.50 μg/mg, H_2 (**5**) 5.43 μg/mg, H_4 (**4**) 5.76 μg/mg; HPLC method did not separate heliocide H_1 (**3**) from H_3 (**6**).
[f] HGQ (**7**) 0.34 μg/mg, $H_1 + H_3$ (**3**, **6**) 5.52 μg/mg, H_2 (**5**) 4.10 μg/mg, H_4 (**4**) 2.69 μg/mg.
[g] HGQ (**7**) 2.28 μg/mg, $H_1 + H_3$ (**3**, **6**) 7.24 μg/mg, H_2 (**5**) 4.27 μg/mg, H_4 (**4**) 3.90 μg/mg.
[h] HGQ (**7**) 0.41 μg/mg, $H_1 + H_3$ (**3**, **6**) 7.06 μg/mg, H_2 (**5**) 3.19 μg/mg, H_4 (**4**) 3.93 μg/mg.

Table 4
Concentration (μg/mg) of gossypol (Goss) (**1**), hemigossypolone (HGQ) (**7**), heliocides H_{1-4} (3–6) and gossypolhemiquinone (GHQ) (**9**) in freeze dried leaves of commercial cultivars (the HPLC method did not separate heliocide H_1 (**3**) from heliocide H_3 (**6**); gossypolone (**2**) was not detected.

Cultivar	HGQ (**7**)	GHQ (**9**)	Goss (**1**)	$H_1 + H_3$ (**3**, **6**)	H_2 (**5**)	H_4 (**4**)
Coker-312	1.18	nd[a]	0.36	0.44	1.10	0.06
FiberMax 958	0.49	nd	0.13	0.25	0.40	0.07
FiberMax 966	1.27	nd	0.27	0.43	0.44	0.16
Stoneville 474	0.56	nd	1.92	0.60	0.76	0.17

[a] nd = not detected (limit of detection 0.05 μg/mg).

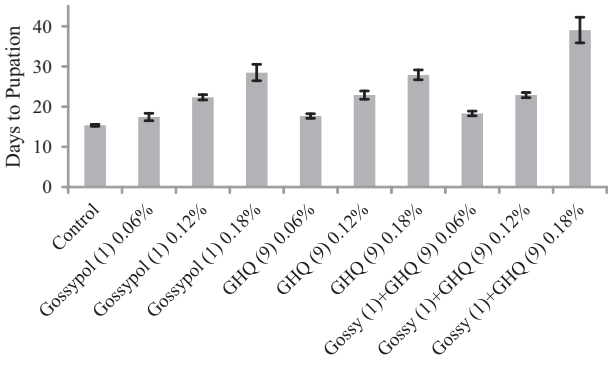

Fig. 2. Mean number (with standard error bars) of days-to-pupation for *Helicoverpa zea* one-day-old larvae fed various concentrations of gossypol (**1**) or gossypolhemiquinone (GHQ) (**9**) or a 15.5:84.5 mixture of GHQ (**9**):gossypol (**1**) in an artificial diet.

(**1**) showing mortality >50% compared to larvae fed the control diet. However, the survival to adulthood of larvae fed the 0.18% 15.5:84.5 GHQ (**9**):gossypol (**1**) diet was less than one-third those fed the control diet indicating a potential synergistic effect.

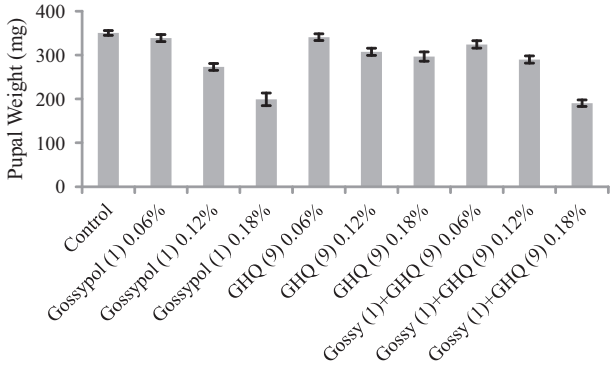

Fig. 3. Mean (with standard error bars) pupal weights for *Helicoverpa zea* one-day-old larvae fed various concentrations of gossypol (**1**) or gossypolhemiquinone (GHQ) (**9**) or a 15.5:84.5 mixture of GHQ (**9**):gossypol (**1**) in an artificial diet.

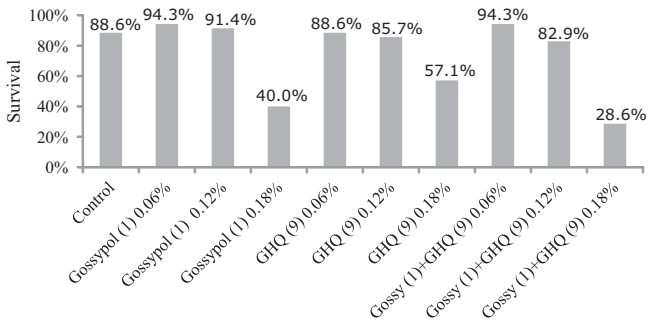

Fig. 4. Percent survival for *Helicoverpa zea* larvae fed various concentrations of gossypol (**1**), gossypolhemiquinone (GHQ) (**9**) or a 15.5:84.5 mixture of GHQ (**9**):gossypol (**1**) in an artificial diet.

2.5. Effect of GHQ (**9**) on cancer cell lines

Both gossypol (**1**) and GQ (**2**) have been extensively tested for anti-cancer activity. (−)-Gossypol (termed AT-101) is currently in

clinical trials as an anticancer agent (Oliver et al., 2005; Ready et al., 2011). GQ (**2**) shows some anticancer activity but appears to be less active than gossypol (**1**) (Gilbert et al., 1995). Since GHQ (**9**) possesses structural features of both molecules, it seemed reasonable to investigate its anticancer activity. The compound was submitted to the National Cancer Institute 60-Human Tumor Cell Line Screen. Experimental details for the Tumor Cell Line Screen can be found online (http://dtp.nci.nih.gov/branches/btb/ivclsp.html). Results are shown in Supplementary section. No potent activity was noted against slow-growing tumors; however, the results offer some promise against leukemia cancer cell lines. Additional screening for apoptosis, angiogenesis, cell invasion and migration may be indicated.

3. Conclusion

In these artificial insect feeding studies, GHQ (**9**) was no more effective or was less effective than gossypol (**1**) at comparable concentrations. However, at the highest concentration tested (0.18%), the 15.5:84.5 GHQ (**9**):gossypol (**1**) mixture demonstrated a significant increase in days-to-pupation, as well as a reduced survival rate compared to gossypol (**1**) or the control. Since GHQ (**9**) is present at very low concentrations in *G. thurberi*, it is probably not responsible for the plant's resistance to herbivorous insects. However, if the concentration of GHQ (**9**) could be increased, then a synergistic interaction as observed in this diet study could provide cotton plants with enhanced resistance to herbivorous insects. This may be an achievable goal, since the conversion of gossypol (**1**) to GHQ (**9**) may involve a single enzyme, possibly an oxidation *via* a P_{450} enzyme (see Supplementary material section for proposed mechanism). A molecular marker assisted breeding program directed at a P_{450} gene might facilitate incorporation of GHQ (**9**) at levels high enough to increase resistance to herbivorous insects in cotton.

Since GHQ (**9**) is not responsible for the apparent resistance reported by Karban, then the high concentration of gossypol (**1**) in the leaves may be a major contributor to this resistance. However, increasing the gossypol (**1**) content in leaves usually leads to an increase in levels of gossypol (**1**) in the seed. For example, in a study of 28 accessions of *G. thurberi*, the mean concentration of gossypol (**1**) in seed ranged from 17.02 μg/mg to 3.10 μg/mg with a mean of 13.77 μg/mg (Stipanovic et al., 2005). In the current study, the concentrations of gossypol (**1**) in the seed of *G. thurberi* D1–6, D1–21 and D1–22 (greenhouse grown plants) were 21.86 μg/mg, 21.08 μg/mg, and 25.02 μg/mg, respectively. In contrast, a study of gossypol (**1**) concentrations in the seed of 14 commercial and experimental *G. hirsutum* cottons grown at five different locations from the Lower Rio Grande Valley to the high plains of Texas varied from 1.03 μg/mg to 0.41 μg/mg (Stipanovic et al., 1988). Unfortunately, cottonseed with high levels of gossypol (**1**) is unsuitable as a feed for cattle and dairy cows, which is the major market for this important byproduct of cotton production. Therefore, a phytochemical such as GHQ (**9**) that augments the activity of gossypol (**1**) may offer an alternative strategy to increase resistance to herbivorous insects.

4. Experimental

4.1. General

HPLC isolations, quantitative analyses and enantiomeric ratio determinations were performed on an Agilent Technologies HPLC instrument (Waldbronn, Germany) equipped with a 1200 solvent degasser, 1200 quaternary pump, 1100 autosampler, and 1100 diode array detector (DAD). The LC/MS chromatography was carried out with the Agilent HPLC instrument in conjunction with a Varian 500-MS Ion Trap Mass Spectrometer (Walnut Creek, CA). That is, samples were injected on the Agilent instrument then a splitter sent 81% of the column eluent to the DAD detector and 19% to the Varian MS. The MS was operated in the positive polarity full scan electrospray ionization (ESI) mode with spray chamber 50 °C, nebulizer gas N_2, pressure 35 psi, drying gas 350 °C, drying gas pressure 10 psi, needle voltage 4200 V, spray shield 600 V, capillary 80 V, and RF loading 100%. The product ion start mass was 100 m/z and end mass was 600 m/z.

Direct exposure probe MS were acquired on a Thermo Electron DSQ in positive ion EI mode (70 EV, source 200 °C, scan rate 300 amu/s, scan 50–600 amu).

^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMBC and HSCQ spectra were acquired on a Bruker Avance III 500 instrument (Billerica, MA, USA) equipped with a cryoprobe operating at 500 MHz for ^{1}H and 125 MHZ for ^{13}C. Spectra were determined in $CDCl_3$, which was used as an internal standard (^{1}H: 7.24 δ; ^{13}C: 72.0 δ). One and two dimensional ^{1}H and ^{13}C NMR spectra (COSY, HMBC, and HSQC) were used to assign specific proton and carbon assignments. The optical rotation for GHQ (**9**) synthesized from (+)-gossypol (**1a**) was determined using a Perkin Elmer Model 241 polarimeter utilizing a 1 dm temperature controlled microsample holder. UV spectra were recorded on a Hewlett Packard ultraviolet–visible spectrometer Model 8456.

4.2. Plant material

Leaves of accessions listed in Table 3 were collected from greenhouse grown plants; these accessions are part of the U.S. National Cotton Germplasm Collection (accessible through http://www.ars-grin.gov). Leaves from commercial cultivars (Table 4) also were taken from greenhouse grown plants. After collection, the leaves were frozen and then freeze dried; the freeze dried leaves were ground to a fine powder in an agate mortar and pestle. Ground samples were stored at 2 °C until used. Leaves from *G. thurberi* plants grown in the field also contained GHQ (**9**).

4.3. HPLC isolation of GHQ (*9*) from G. thurberi

Isolation of GHQ (**9**) was initially achieved from a *G. thurberi* extract [10 g tissue in CH_3CN (10 mL)] on the Agilent HPLC instrument using a Scientific Glass Engineering ProteCol-GP-C18-125 (4.6 × 250 mm) column at 23–24 °C and an isocratic mobile phase of 3:1 CH_3CN:H_2O with 0.1% HCO_2H. The flow rate was 1.25 mL/min and the run time was 20 min. The eluent was monitored at 272 ± 10 nm (referenced to 550 ± 50 nm) and spectra of detected peaks were stored over 210–600 nm. Multiple injections of 50 μL were carried out and the peak of interest was manually collected from the eluent from the DAD. The collected fractions were extracted three times with Et_2O after the addition of an equal volume of H_2O. Back washing with H_2O (2×), drying over Na_2SO_4 and final evaporation provided the compound of interest (>95% pure).

4.4. Preparation of GHQ (*9*) and (+)-GHQ

A modification of the method described by Hass and Shirley (1965) was used to prepare GHQ (**9**). Specifically, gossypol acetic acid (400 mg) was dissolved in acetone (48 mL) and glacial AcOH (32 mL) in a round bottom flask equipped with a stirring bar. While the solution was stirred at room temperature, a freshly prepared aqueous solution of 10% $FeCl_3{\cdot}6H_2O$ (25 mL) was added over four minutes. The solution was stirred for a total of 35 min. Dilute H_2SO_4 (20%, 20 mL) then was added followed by H_2O (25 mL). The solution (dark black) was extracted with Et_2O (1 × 100 mL and 3 × 50 mL). The combined organic layer was washed 1× with saturated brine, dried over Na_2SO_4 (anhydrous) and evaporated to

dryness. The dried material was stored overnight at 2 °C. The product was purified by low pressure chromatography on a dry-packed silica gel (Mallinckrodt CC-4; 48 g) column as follows. The crude material was dried onto silica gel (2.5 g) and placed on the top of the column. The column was developed with a gradient of acetone:cyclohexane beginning with 1% acetone and increasing in 1% increments to 7% acetone (210 mL solvent per increment), and finally to 10% acetone (200 mL). Beginning at 6% acetone, fractions were collected and checked for GHQ (**9**) purity via HPLC as described above in Section 4.3 except 1 μL injections were used. Individual fractions with GHQ (**9**) purity >97% were allowed to evaporate slowly in a fume hood. Crystals began to form after about two days [m.p. 158–160 °C; UV: (95% EtOH) λ_{max} (log ε) 234 (3.81). 274 (3.57), 372 (3.08)] (spectra shown in Supplementary material section). After about 5 days, the mother liquor was removed from tubes in which crystals formed and the crystals were washed with cyclohexane and allowed to dry at room temperature.

(+)-GHQ was prepared from (+)-gossypol (**1a**) (>98% ee) as detailed above. It was found that (+)-GHQ slowly racemizes in solution at room temperature over a period of days, such that after 5 days the enantiomeric excess of (+)-GHQ was <1%. Thus, the synthetic (+)-GHQ was allowed to crystalize over a period of only two days.

4.5. HPLC determination of the GHQ (*9*) enantiomeric ratio

The enantiomeric ratio of the isolated and the synthesized GHQ (**9**) was determined *via* HPLC using the Agilent Technologies instrument with a Phenomenex Lux-Cellulose-4–5 μm (4.6 × 150 mm) column at 23–24 °C and an isocratic mobile phase of 4:1 hexane:iPrOH with 0.1% CF_3CO_2H. The flowrate was 1.5 mL/min and the run time was 10 min. The eluent was monitored at 254 ± 10 nm (referenced to 550 ± 50 nm) and spectra were stored over 240–600 nm. Under these conditions, (+)-GHQ (**9**) elutes at 2.2 min and (−)-GHQ elutes at 3.8 min.

4.6. Extraction and HPLC quantitation of terpenoids in D genome leaf tissue

Freeze dried leaf powder was accurately weighed (100 mg ± 2 mg) into a test tube and CH_3CN:H_2O with 0.1% H_3PO_4 was added (10 ml, 4:1, v/v). The tube was sonicated for 5 min, vortexed momentarily and then centrifuged for 5 min at 3000 rpm. A portion of the resulting clear supernatant was transferred to a vial for quantitative HPLC analysis. For each sample, the total time from the beginning of the extraction until HPLC injection was <15 min. The HPLC analysis was performed using the same method as that for the isolation of GHQ (**9**) (Section 4.2) except that a 20 μL injection was used; when the H heliocides (**3**–**6**) were present, the run time was 30 min instead of 20 min. Standard μg versus peak area curves obtained from pure terpenoids were employed to calculate the concentration of the compounds in the leaf tissue from the peak areas in the chromatogram. The limit of detection of GHQ (**9**) was 0.05 μg/mg. Each cotton line was analyzed once.

4.7. Insect feeding studies

Insect feeding studies were conducted as previously described (Stipanovic et al., 2014). Thus, Alphacel (ICN Nutritional Biochemicals, Cleveland, OH) a non-nutritive polysaccharide was used as a carrier in all diets. The control diet contained Alphacel alone, while terpenoid diets contained three different concentrations of either gossypol (**1**), GHQ (**9**) or a 15.5:84.5 ratio of GHQ (**9**):gossypol (**1**) mixed with Alphacel. The diets were prepared as follows. Weighed amounts of terpenoids were dissolved in acetone (12 mL) and quantitatively added to Alphacel (5.04 g). Hexane (10 mL) was added, and the suspensions were dried under vacuum at room temperature on a rotoevaporator. Acetone (10 mL) was added, followed by hexane (25 mL). The suspension was evaporated to dryness as before. Gossypol (**1**) was added as the acetic acid complex, but the final concentrations of gossypol (**1**) and GHQ (**9**) were 0.06%, 0.12% and 0.18% of the final product. To account for the AcOH in the gossypol (**1**) samples, an equivalent amount of AcOH was added with the GHQ (**9**). The Alphacel samples were stored at −20 °C and then placed under vacuum for 20 h. The control diet was prepared as described above including all solvents and Alphacel but without any added terpenoids. The Alphacel samples (5.04 g) were mixed with a diet-premix of Instant Soybean-Wheat Germ Insect Diet, Stonefly Industries, Inc., Bryan, TX (42 g). Next, a dilute vinegar solution [H_2O and 5% vinegar (Albertson's, Boise, ID; white vinegar 0.2% volume) without 0.1% formalin] was prepared and added to the Alphacel and diet-premix mixtures to yield a total wet weight of 168 g per diet.

H. zea larvae were obtained from laboratory reared eggs (Mississippi State University). Insects were maintained at a photoperiod of 14:10 light:dark hours at 27 °C (relative humidity 50–70%) in an insect-rearing room at the College Station, TX laboratory. A one-day-old bollworm larva was placed in a 22-mL plastic cup with 4–5 g of diet. Thirty larvae were used per treatment. Ten days after initiation of the experiment, the cups were inspected daily. After pupation, pupae were allowed to harden for 1 day, weighed, and returned to their respective cups; pupae were inspected daily until adult emergence. Days-to-pupation, pupal weight, and number of survivors reaching adulthood were recorded.

4.8. Anti-cancer activity

GHQ (**9**) was submitted to the National Cancer Institute's Developmental Therapeutics Program (DPI) in the small molecule In Vitro Cell Line Screening Project. This assay utilizes 60 different human tumor cell lines representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. The object is to identify compounds that exhibit selective growth inhibition or cell killing of selected tumor cell lines.

Acknowledgements

We thank Howard Williams of Texas A&M University for providing NMR spectra, and Stephanie Sullivan and Christine Soriaga for excellent technical assistance. We thank Cotton Inc. for partial support for this work. Kathy Knighten of Mississippi State University provided *H. zea* eggs from which larvae were reared for the insect feeding study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.12.009.

References

Celorio-Mancera, M.P., Ahn, S.J., Vogel, H., Heckel, D.G., 2011. Transcriptional responses underlying the hormetic and detrimental effects of the plant secondary metabolite gossypol on the generalist herbivore *Helicoverpa armigera*. BMC Genomics 12, 575–590.

Dao, V.-T., Dowd, M.K., Martin, M.-T., Gaspard, C., Mayer, M., Michelot, R.J., 2004. Cytotoxicity of enantiomers of gossypol Schiff's bases and optical stability of gossypolone. Eur. J. Med. Chem. 39, 619–624.

Gilbert, N.E., O'Reilly, J.E., Chang, C.J.G., Lin, Y.C., Brueggemeier, R.W., 1995. Antiproliferative activity of gossypol and gossypolone on human breast cancer cells. Life Sci. 57, 61–67.

Gonzalez-Garza, M.T., Said-Fernandez, S., 1988. *Entamoeba histolytica*: potent *in vitro* antiamoebic effect of gossypol. Exp. Parasitol. 66, 253–255.

Hass, R.A., Shirley, D.A., 1965. The oxidation of gossypol. II. Formation of gossypolone with ferric chloride. J. Org. Chem. 30, 411–413.

Jaroszewski, J.W., Strom-Hansen, T., Hansen, S.H., Thastrupo, O., Kofod, H., 1992. On the botanical distribution of chiral forms of gossypol. Planta Med. 58, 454–458.

Karban, R., 1993. Costs and benefits of induced resistance and plant density for a native shrub, *Gossypium thurberi*. Ecology 74, 9–19.

Kim, I.C., Waller, D.P., Marcelle, G.B., 1984. Comparative *in vitro* spermicidal effects of (±)-gossypol, (+)-gossypol, (−)-gossypol, and gossypolone. Contraception 30, 253–259.

Loberg, R.D., McGregor, N., Ying, C., Sargent, E., Pienta, K.J., 2007. *In vivo* evaluation of AT-101 (R-(−)-gossypol acetic acid) in androgen-independent growth of VCaP prostate cancer cells in combination with surgical castration. Neoplasia 9, 1030–1037.

Lukefahr, M.J., Martin, D.F., 1966. Cotton-plant pigments as a source of resistance to the bollworm and tobacco budworm. J. Econ. Entomol. 59, 176–179.

Mellon, J.E., Zelaya, C.A., Dowd, M.K., 2011. Inhibitory effects of gossypol-related compounds on growth of *Aspergillus flavus*. Lett. Appl. Microbiol. 52, 406–412.

Oliver, C.L., Miranda, M.B., Shangary, S., Land, S., Wang, S., Johnson, D.E., 2005. (−)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-XL-mediated apoptosis resistance. Mol. Cancer Ther. 4, 23–31.

Phillips, V.A., Hedin, P.A., 1990. Spectral techniques for structural analysis of cotton terpenoid aldehydes gossypol and gossypolone. J. Agric. Food Chem. 38, 525–528.

Percival, A.E., Wendel, J.E., McStewart, J.M., 1999. Taxonomy and germplasm resources. In: Smith, C., Cothren, J.T. (Eds.), Cotton: Origin, History, Technology, and Production. John Wiley and Sons Inc, New York, pp. 33–63.

Ready, N., Karaseva, H.A., Orlov, S.V., Luft, A.V., Popovych, O., Holmlund, J.T., Wood, B.A., Leopold, L., 2011. Double blind, placebo-controlled, randomized phase 2 study of the proapoptotic agent AT-101 plus docetaxel, in second-line non-small cell lung cancer. J. Thorac. Oncol. 6, 781–785.

Shelly, M.D., Hartley, L., Groundwater, P.W., Fish, R.G., 2000. Structure-activity studies on gossypol in tumor cell lines. Anticancer Drugs 11, 209–216.

Stipanovic, R.D., Altman, D.W., Begin, D.L., Greenblatt, G.A., Benedict, J.H., 1988. Terpenoid aldehydes in upland cotton: analysis by aniline and HPLC methods. J. Agric. Food Chem. 88, 509–515.

Stipanovic, R.D., Bell, A.A., Lukefahr, M.J., 1977. Natural insecticides from cotton (*Gossypium*). In: Hedin, P.A. (Ed.), Host Plant Resistance to Pests, Symposium Series #62. American Chemical Society, Washington, DC, pp. 197–214.

Stipanovic, R., Esquivel, J., Westbrook, J., Puckhaber, L., O'Neil, M., Bell, A., Duke, S., Dowd, M., Lopez Jr., J.D., Hake, K., 2014. The effect of gossypolone on the growth and development of *Helicoverpa zea* (Boddie). Southwest Entomol. 39, 1–7.

Stipanovic, R.D., Puckhaber, L.S., Bell, A.A., Percival, A.E., Jacobs, J., 2005. Occurrence of (+)- and (−)-gossypol in seed of wild species of cotton and in *Gossypium hirsutum* var. *marie-galante* (Watt) Hutchinson. J. Agric. Food Chem. 53, 6266–6271.

Stipanovic, R.D., Williams, H.J., Smith, L., 1986. Cotton terpenoid inhibition of *Heliothis virescens* development. In: Green, M.A., Hedin, P.A. (Eds.), Natural Resistance of Plants to Pests, Symposium Series #296. American Chemical Society, Washington, DC, pp. 79–94.

Yildirim-Aksoy, P., Lim, C., Dowd, M.K., Wan, P.J., Klesius, P.H., Shoemaker, C., 2004. *In vitro* inhibitory effect of gossypol from gossypol-acetic acid, and (+)- and (−)-isomers of gossypol on the growth of *Edwardsiella ictaluri*. J. Appl. Microbiol. 97, 87–92.