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The peroxidative coupling of hemigossypol to (+)- and (−)-gossypol in cottonseed extracts

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Abstract

Peroxidase(s) present in embryo extracts of *Gossypium hirsutum* cv. Texas Marker 1 catalyzed a bimolecular coupling of [4-^{3}H]-hemigossypol to [4,4′-$^3H_2$]-gossypol. The reaction was dependent on the addition of H_2O_2 and was inhibited 71–94% by 1 and 10 mM sodium azide. The phenolic coupling produced 53% (+)-gossypol and 47% (−)-gossypol in close agreement to the 49% (+)-gossypol and 51% (−)-gossypol found in the intact seed. The nearly racemic mixture of (+)-and (−)-gossypol produced in these embryo extracts can be accounted for by non-enzymatic random coupling of the free radicals of hemigossypol produced by the peroxidase. In contrast, peroxidase reaction mixtures containing crude embryo extracts of *G. hirsutum* var. *marie-galante* produced 73% (+)-gossypol and 27% (−)-gossypol. These data from the *marie-galante* extracts and the fact that these intact seed contain 95% (+)-gossypol suggest a regio-stereoselective bimolecular coupling of hemigossypol to gossypol. The development of the peroxidative coupling of hemigossypol to gossypol in maturing seed of *G. hirsutum* cv. Texas Marker 1 was correlated to the formation of gossypol and suggests that peroxidative coupling of hemigossypol contributes to gossypol biosynthesis.

Keywords: *Gossypium hirsutum*; Malvaceae; Cotton; Peroxidative coupling; Gossypol formation; Hemigossypol coupling; Dirigent protein

1. Introduction

Gossypol (**6**) is a polyphenolic *bis*-sesquiterpene found in plants of the *Malvaceae*. A proposed pathway for the biosynthesis of gossypol (**6**) from *E*,*E*-farnesyl diphosphate (**1**) in cotton is shown in Fig. 1. The first step in this pathway is the cyclization of *E*,*E*-farnesyl diphosphate (**1**) to (+)-δ-cadinene (**2**) catalyzed by (+)-δ-cadinene synthase (Benedict et al., 1995; Davis and Essenberg, 1995; Chen et al., 1995; Benedict et al., 2001): The hydroxylation of (+)-δ-cadinene (**2**) to form 8-hydroxy-(+)-δ-cadinene (**3**) is catalyzed by (+)-δ-cadinene-8-hydroxylase, a cytochrome P_{450} monooxygenase (Luo et al., 2001). Intact cotton cotyledons convert 8-hydroxy-(+)-δ-cadinene (**3**) to desoxyhemigossypol (**4**) and hemigossypol (**5**) demonstrating the functioning of the cytochrome P_{450} monooxygenase in the biosynthesis of gossypol (**6**) (Wang et al., 2003). The conversion of 8-hydroxy-(+)-δ-cadinene (**3**) to desoxyhemigossypol (**4**) requires hydroxylations, desaturations and cyclic ether formation. Stipanovic et al. (1992) demonstrated that desoxyhemigossypol (**4**) decomposes to hemigossypol (**5**) in solution by a non-enzymatic free radical oxidation. In studies on the structure of hemigossypol (**5**) it was shown that horseradish peroxidase can dimerize hemigossypol (**5**) to gossypol (**6**) (Veech et al., 1976) but enzymatic studies on the conversion of hemigossypol (**5**) to gossypol (**6**) in cotton tissues are scarce. The purpose of this paper is to present data on the peroxidative coupling of hemigossypol (**5**) to form (+)- and (−)-gossypol (**6**) in cottonseed extracts.

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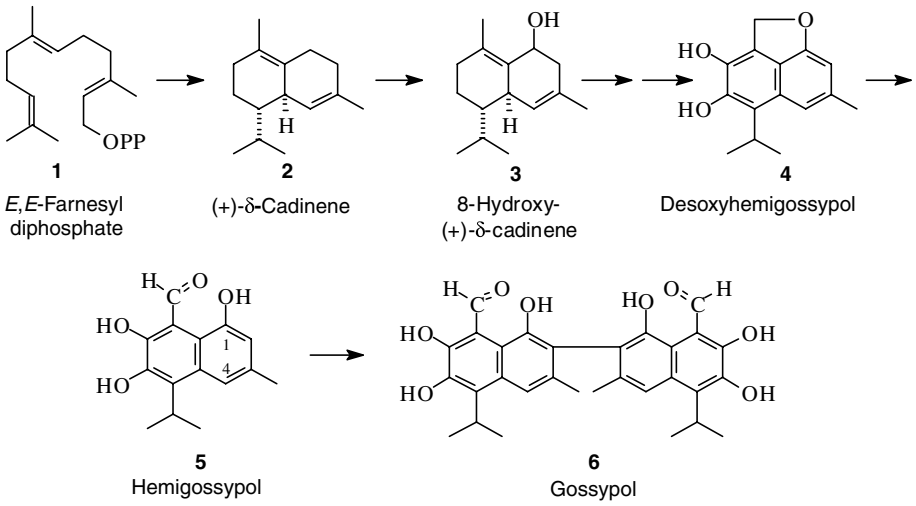

Fig. 1. A proposed pathway for the biosynthesis of gossypol (**6**) in cottonseed from *E,E*-farnesyl diphosphate (**1**) with intermediates (+)-δ-cadinene (**2**), 8-hydroxy-(+)-δ-cadinene (**3**), desoxyhemigossypol (**4**), and hemigossypol (**5**).

2. Results and discussion

*2.1. Identification of gossypol (**6**) formed from hemigossypol (**5**) in the* Gossypium hirsutum *cv. Texas Marker 1 (TM1) embryo extracts*

A compound formed when hemigossypol (**5**) was combined with the TM1 embryo extracts in the presence of H_2O_2 had a HPLC-retention time of 16.6 min; its mass spectrum [EIMS (probe) 70 eV] showed ions at *m/z* (rel. int.): 518 $[M]^+$ (2.2), 500 (17.2), 483 (33.7), 482 (100), 468 (23.6), 467 (73.4), 454 (15.3) and 439 (20.2). This mass spectrum agrees with that reported by Bell et al. (1975). A proposed fragmentation scheme that accounts for the major peaks in the mass spectrum is shown in Fig. 2. A single ion at *m/z* 518 accounts for gossypol (**6**). The loss of H_2O produces a single ion at *m/z* 500 and another loss of H_2O produces an ion at *m/z* 482. The loss of a methyl group gives an ion at *m/z* 467 and the alternative loss of an isopropyl group results in an ion at *m/z* 439. This fragmentation pattern is consistent with the formation of gossypol (**6**) by a bimolecular coupling of hemigossypol (**5**) in the embryo extracts.

*2.2. Formation of [4,4′-$^3H_2$]-gossypol (**6**) from [4-^{3}H]-hemigossypol (**5**) in TM1 embryo extracts*

Gossypol (**6**) is formed by coupling of hemigossypol (**5**) at the 2 position (Fig. 1); Therefore, gossypol (**6**) produced from [4-^{3}H]-hemigossypol (**5**) will retain the tritium label. [4-^{3}H]-Hemigossypol (**5**) was incubated with a cell free extract from TM1 embryo tissue in the presence of H_2O_2. The product was submitted to HPLC analysis. Authentic hemigossypol (**5**) and gossypol (**6**) eluted at 14.83 and 16.62 min, respectively. The product from the reaction contained the same two peaks. Fractions were collected every 15 s and were assayed for radioactivity on a scintillation spectrometer. The radioactivity of the collected fractions peaked at 14.83 and 16.62 min. The UV spectrum and the retention time of the peak eluting at 16.62 min agreed with that of authentic gossypol (**6**). These results are consistent with the peroxidative coupling of [4-^{3}H]-hemigossypol (**5**) at the 2-position to give [4,4′-$^3H_2$]-gossypol (**6**).

In the presence of 1 mM H_2O_2 in the mixture, 86% of [4-^{3}H]-hemigossypol (**5**) was converted to [4,4′-$^3H_2$]-gossypol (**6**) but only 6% of the [4-^{3}H]-hemigossypol (**5**) was converted to [4,4′-$^3H_2$]-gossypol (**6**) in the absence of H_2O_2. Others have shown that azide inhibits peroxidase enzymes (Brill and Weinryb, 1967). We have demonstrated that the enzymatic coupling of hemigossypol (**5**) to gossypol (**6**) in the TM1 embryo extracts was inhibited 71% and 94%, respectively, by the addition of 1 and 10 mM sodium azide to the reaction mixtures. These data support the conclusion that the bimolecular coupling of hemigossypol (**5**) to gossypol (**6**) was catalyzed by peroxidase(s) in the embryo extracts.

*2.3. The enantiomers of gossypol (**6**) formed during the enzymatic coupling of hemigossypol (**5**) to gossypol (**6**) in the TM1 embryo extracts*

Racemic gossypol was first derivatized by reaction with (+)-2-amino-1-propanol (Hron et al., 1999). Since the derivatizing reagent is itself chiral, it was possible to separate the resulting mixture of diastereomers using an achiral column. Two peaks were observed eluting at 3.12 and 4.85 min. The peak eluting at 3.12 min was shown to be the (+)-enantiomer by comparison of the retention time and the UV spectrum (obtained by the diode array detector) to that of a derivatized authentic sample of (+)-gossypol (**6**) obtained from *Thespesia populnea* (King and de Silva, 1968). The reaction product from Section 2.1 above was derivatized with

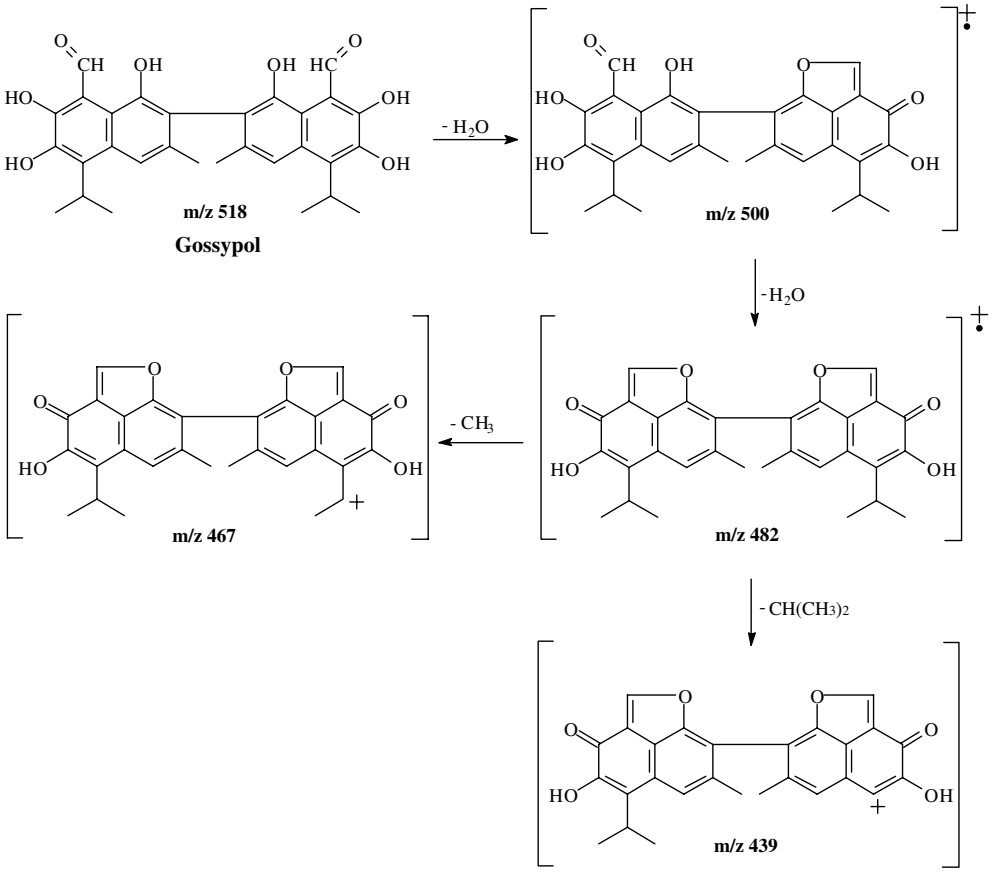

Fig. 2. The MS fragmentation scheme for gossypol (**6**) formed from hemigossypol (**5**) by peroxidative coupling with an enzyme extract from 35 day-post-anthesis embryos of *Gossypium hirsutum* cv. Texas Marker 1.

(+)-2-amino-1-propanol and the resulting derivatives were submitted to HPLC analysis (Fig. 3). The percentages of (+)- and (−)-gossypol (**6**) were 53 and 47, respectively, and indicates the formation of a racemic mixture. These percentages are in close agreement to the percentages of (+)- and (−)-gossypol (**6**) found in the intact TM1 seed [i.e., 49% (+)- and 51% (−)-gossypol (**6**)].

*2.4. The enantiomers of (+)- and (−)-gossypol (**6**) formed in* G. hirsutum *var.* marie-galante *(moco cotton) embryo extracts*

The percentage of (+)- and (−)-gossypol (**6**) in *G. hirsutum* cottonseed ranges from a racemic mixture in *G. hirsutum* cv. TM1 to a preponderance of (+)-gossypol (**6**) in *G.*

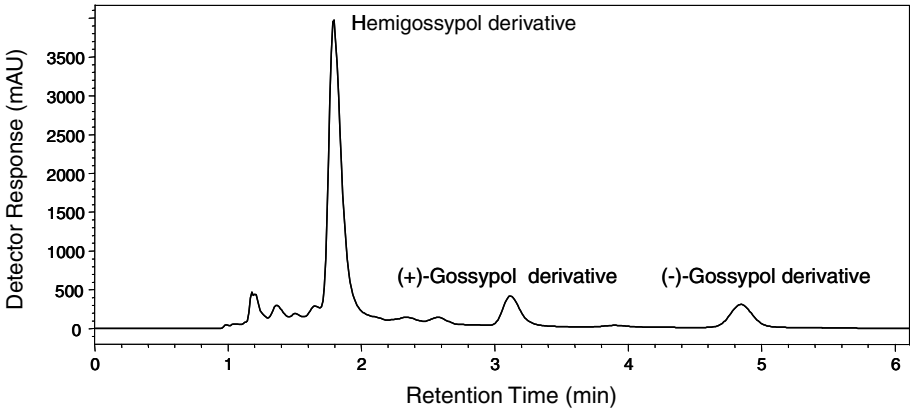

Fig. 3. HPLC showing the (+)-2-amino-L-propanol derivatives of (+)-gossypol (**6**) (retention time 3.12 min, 53%) and (−)-gossypol (retention time 4.85 min, 47%) formed during the enzymatic coupling of hemigossypol (**5**) with an enzyme extract from 35 day-post-anthesis embryos of *Gossypium hirsutum* cv. Texas Marker 1; the derivative of unreacted hemigossypol (**5**) had a retention time of 1.79 min.

hirsutum var. *marie-galante*. In separate experiments we determined that intact seed of moco cotton contained 95% (+)-gossypol (**6**) and 5% (−)-gossypol (**6**) similar to reports by Cass et al. (1991). To assess the role of peroxidase and possible associated protein in the preferential formation of (+)-gossypol (**6**) in these seeds, reaction mixtures were made containing an embryo extract of 35 days-post-anthesis, hemigossypol (**5**), and H_2O_2. The data in Fig. 4 show that 72% (+)-gossypol (**6**) and 28% (−)-gossypol (**6**) were made in these mixtures, these percentages of (+)- and (−)-gossypol (**6**) were different than the racemic mixture of gossypol (**6**) formed in the TM1 embryo extracts.

Several possibilities may account for the formation of predominant amounts of (+)-gossypol (**6**) in intact seed and embryo extracts of the moco cotton. The presence of a peroxidase together with a dirigent protein similar to the dirigent protein isolated from *Forsythia* (Davin et al., 1997; Davin and Lewis, 2000) could direct the coupling of free radicals of hemigossypol formed by the oxidase (peroxidase/H_2O_2) in a stereospecific manner for preferential formation of (+)-gossypol. An incomplete solubilization of the dirigent protein with the peroxidase in the embryo extracts could account for less (+)-gossypol (**6**) formed in the extracts compared to the intact seed. Alternatively, other regio-stereospecific enzymes such as laccase-type phenoloxidase (Niemetz and Gross, 2003) or cytochrome P_{450} (Stadler and Zenk, 1993) that were not measured, may play a role in the regio-stereospecific coupling of hemigossypol (**5**) in moco cotton.

2.5. Correlation of peroxidative coupling activity with gossypol (6) content in maturing TM1 cottonseed

The correlation of the peroxidative coupling activity of hemigossypol (**5**) to gossypol (**6**) with the formation of gossypol (**6**) in maturing cottonseed of TM1 is shown in Fig. 5. There is a high correlation of the coupling activity of hemigossypol (**5**) to gossypol (**6**) in extracts of the maturing seed and the deposition of gossypol (**6**) in the intact maturing seed. The data are consistent with the conclusion that the peroxidative coupling activity of hemigossypol (**5**) contributes to the biosynthesis of gossypol (**6**).

3. Experimental

3.1. Chemicals

The ^3H_2O (1 Ci/g) and ACS grade 30% H_2O_2 in water (v/w) were purchased from Sigma Chemical Company. (+)-2-Amino-1-propanol was purchased from Aldrich Chemical Company.

3.2. Plants

Plants of *G. hirsutum* cv. Texas Marker 1 were planted in Cotton Variety Test Plots on the Texas A&M University Farm. The plants were planted in 1 m wide rows and the stands were thinned early in the season to 1 m between plants. The fertilization, irrigation and pest control procedures were those used throughout the test plots. Plants of *G. hirsutum* var. *marie-galante* (moco cotton) were grown in a greenhouse in 2 gal pots. In the greenhouse, the plants were watered daily and fertilized with 20-20-20 Peter's Soluble Fertilizer containing micronutrients. Pest control spraying was applied weekly. Throughout the growing season flowers of both TM1 and moco cottons were tagged on the day of anthesis (full bloom). Bolls were harvested throughout the boll development period of about 50 days post anthesis.

3.3. Preparation of embryo extracts

Seeds were removed from the developing cotton bolls and the seed coat removed from the individual seed. The embryos were ground to a powder in liquid N_2 in a mortar.

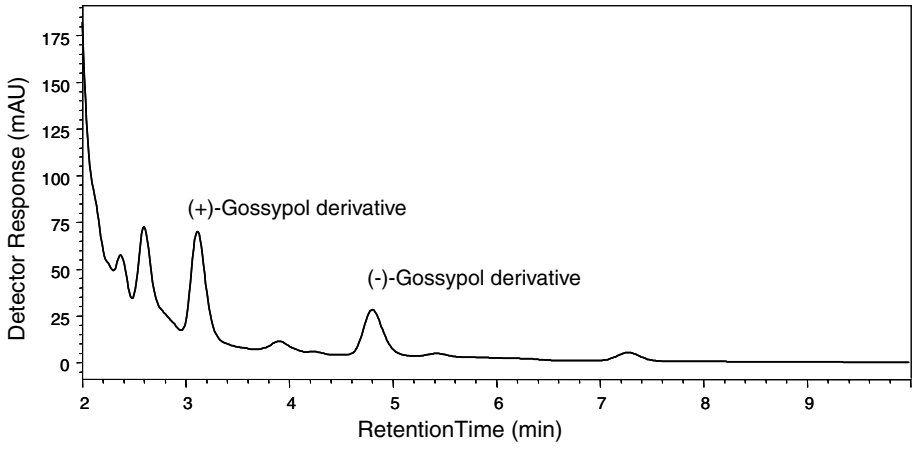

Fig. 4. HPLC showing the (+)-2-amino-L-propanol derivatives of (+)-gossypol (**6**) (retention time 3.11 min, 72%) and (−)-gossypol (retention time 4.80 min, 28%) formed during the enzymatic coupling of hemigossypol (**5**) with an enzyme extracts from 35 days-post-anthesis embryos of *Gossypium hirsutum* var. *marie-galante*.

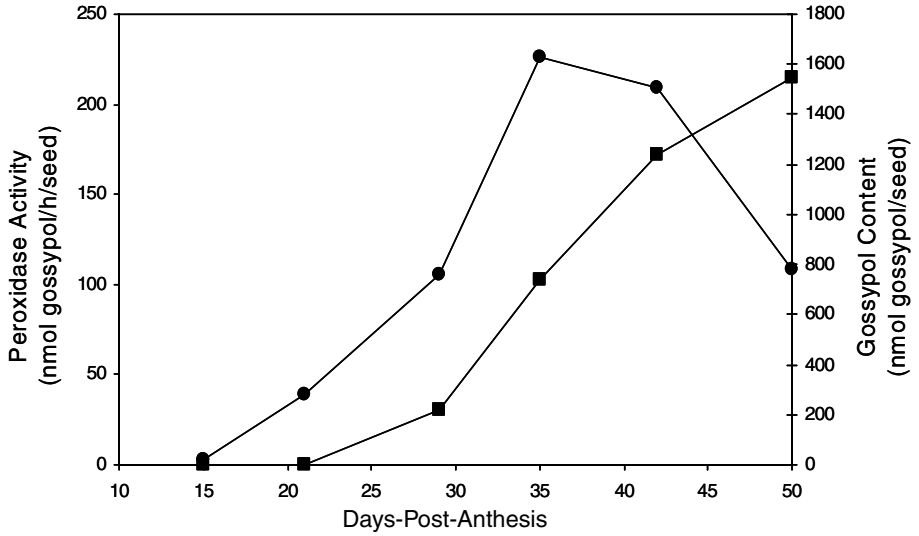

Fig. 5. Correlation between the peroxidative coupling activity and gossypol (**6**) content in maturing embryos of *Gossypium hirsutum* cv. Texas Marker 1 [–●–, peroxidase activity (nmol gossypol/h/seed); –■–, gossypol (**6**) content (nmol gossypol/seed)].

Ten ml/g seed of 0.1 M phosphate buffer pH 5.4 containing 5% insoluble PVP was added to the powder and the mixture stirred to a smooth suspension. The suspension was transferred to epitubes and centrifuged 14,000*g* for 20 min. An aliquot of the supernatant phase was used as the source of the coupling enzyme preparation.

3.4. Preparation of hemigossypol (*5*)

Hemigossypol (**5**) was purified from cotton stele extracts by the procedure of Bell (1967). Hemigossypol (**5**) was identified by ^{1}H NMR spectroscopy on a Bruker ARX-300 instrument and EIMS analysis using a Hewlett–Packard 5989 GC-Electron Impact Quadrupole Mass spectrometer operating at 70 eV using a direct insertion probe method. Source was heated to 260 °C, and the quadrupole to 100 °C.

3.5. Synthesis of [4-^{3}H]-hemigossypol (*5*)

[4-^{3}H]-Hemigossypol (**5**) was synthesized by an exchange reaction of 500 μCi $^{3}H_2O$ with hemigossypol (**5**) in the presence of trifluoroacetic anhydride by the procedure of Stipanovic et al. (1986). The label was almost exclusively located at the 4-position (95%) which would not be expected to be lost during the coupling reaction.

3.6. Assay of the peroxidative coupling of hemigossypol (*5*) to gossypol (*6*) in the embryo extracts

The reaction mixture for the peroxidative coupling of hemigossypol (**5**) to gossypol (**6**) in embryo extracts contained 150 μl of embryo extract, 0.63 mM hemigossypol (**5**) containing 190 nmol and 3.1×10^{5} dpm of [4-^{3}H]-hemigossypol (**5**), and 1.0 mM H_2O_2 in a final volume of 300 μl. The mixture was incubated at 32 °C for 30 min and the reaction stopped by the addition of 200 μl hexane–EtOAc (1:1). The aqueous phase was extracted with 200 μl hexane–EtOAc three times and the combined extracts evaporated to dryness in a rotary evaporator under reduced pressure. The residue was dissolved in 100 μl hexane–EtOAc (1:1 v/v) and 20 μl injected onto a 250 × 4 mm Scientific Glass Engineering Mos-Hypersil-1 C8 column (5 μm) at a column temperature of 40 °C and a flow rate of 1.25 ml/min using a Waters 600 HPLC equipped with a diode array detector. A linear MeOH–H_2O gradient containing 0.07% phosphoric acid was used for column chromatography. The initial MeOH–H_2O ratio was 2:8 progressing to 7:3 over 7 min, to 8:2 over the next 5 min, to 9:1 over the next 7 min, and to 100% MeOH over the last 4 min. Aliquots of the column eluate were collected at 0.25 min intervals and assayed for radioactivity in a Beckman Scintillation Spectrometer. [4-^{3}H]-Hemigossypol (**5**) and [4,4′-$^{3}H_2$]-gossypol (**6**) had elution times of 14.88 and 16.63 min, respectively, identical to the elution times of authentic samples.

3.7. MS analysis of gossypol (*6*) enzymatically synthesized by peroxidative coupling

The identity of the enzymatic reaction product formed by peroxidative coupling with embryo extracts was confirmed by EIMS analysis using non-radioactive hemigossypol (**5**) as substrate. The peak corresponding to gossypol (**6**) with a retention time of 16.63 min was collected. The fraction was diluted with an equal volume of water and extracted in hexane–EtOAc (1:1). The organic layer was evaporated to dryness and analyzed by EIMS using a direct insertion probe 70 eV on a Hewlett–Packard 5989B Mass Spectrometer. Source was heated to 260 °C, and the quadrupole to 100 °C.

3.8. Analysis of the enantiomers of gossypol (**6**) formed during the coupling of hemigossypol (**5**) to gossypol (**6**)

Following the coupling of hemigossypol (**5**) to gossypol (**6**) by embryo extract peroxidase (cf. Section 3.3), the reaction was stopped with hexane–EtOAc (1:1) and the (+)- and (−)-gossypol (**6**) extracted into the organic phase. This phase was evaporated to dryness in a rotary evaporator under reduced pressure. The residue was dissolved in 30 μl of a derivatizing reagent prepared by dissolving 0.2 ml of (+)-2-amino-1-propanol in 8 ml of acetonitrile and 1.0 ml glacial acetic acid as described by Hron et al. (1999). The resulting solution was heated for 30 min at 70 °C. A 10-μl aliquot of the derivatized enantiomers of gossypol (**6**) was injected into a reversed-phase C18 column using a Hewlett–Packard 1090 HPLC equipped with a diode array detector. The mobile phase was CH_3CN–KH_2PO_4 (10 mM) containing 2.5 mM tetrabutylammonium hydrogen sulfate at pH 3.0 at a ratio of 71:29 with a flow rate of 0.6 ml/min. The eluent was monitored at 254 nm.

The enantiomers of gossypol (**6**) present in intact seed were analyzed by grinding 0.14 g of 36 days-post-anthesis cottonseed in a mortar in liquid N_2. The powder was suspended in 1.4 ml of EtOH–H_2O–Et_2O–HOAc (59:24:17:0.2) and shaken vigorously for 30 min. The mixture was filtered through a 0.45-μm filter and the filtrate was analyzed for (+)- and (−)-gossypol (**6**) by HPLC following derivatization with (+)-2-amino-1-propanol as described above.

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