New C-4″-Hydroxy Derivatives of Maysin and 3′-Methoxymaysin Isolated from Corn Silks (*Zea mays*)

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Reduced derivatives of maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin] and 3′-methoxymaysin have been isolated and identified from several corn (*Zea mays* L.) inbreds. These include 2″-*O*-α-L-rhamnosyl-6-*C*-quinovosylluteolin (equatorial 4″-OH-maysin, eq-4″-OH-maysin), 2″-*O*-α-L-rhamnosyl-6-*C*-fucosylluteolin (axial 4″-OH-maysin, ax-4″-OH-maysin), and 2″-*O*-α-L-rhamnosyl-6-*C*-fucosyl-3′-methoxyluteolin (ax-4″-OH-3′-methoxymaysin). In addition to maysin and 3′-methoxymaysin, inbred Tx501 contains minor amounts of ax-4″-OH-maysin and ax-4″-OH-3′-methoxymaysin. Corn lines A103, GE275, ESDJ1, and CML131 contained relatively large levels of both ax-4″-OH-maysin and eq-4″-OH-maysin. Synthetic 4″-OH-maysin (obtained by $NaBH_4$ reduction of maysin) was tested for growth inhibition of corn earworm (*Helicoverpa zea* Boddie) larvae in a laboratory bioassay and found to be almost as active as maysin, suggesting that incorporation by breeders of these compounds into silks could enhance the resistance of corn to corn earworm larvae.

Keywords: *Corn; Zea mays; corn earworm; Helicoverpa zea; maysin analogues; insect resistance*

INTRODUCTION

Natural resistance in crops is an important aspect of any integrated pest management program. The development of natural resistance in corn (*Zea mays* L.) to the corn earworm (*Helicoverpa zea* Boddie) has been a major part of our research goals. Maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin], a *C*-glycosylflavone, has been identified as one of the most important natural resistance factors in corn silks (Waiss et al., 1979, 1981; Elliger et al., 1980a,b). Our recent work has shown that corn silk with maysin levels >0.2% fresh weight reduced larval weights by 50% of controls, while silk maysin >0.4% reduced weights by about 70% (Wiseman et al., 1992). Elliger et al. (1980b) showed that the presence of *o*-dihydroxy substituents on the B ring of the aglycon (a structure maysin possesses, Figure 1) is important for antibiosis activity. We have shown that rutin, an *o*-dihydroxyflavonol glycoside, is active against the corn earworm (Snook et al., 1994). The nature of the sugar residue is not important in these compounds because the aglycon, luteolin, was found to be just as active as maysin or rutin in reducing worm weights (Snook et al., 1994). Furthermore, chlorogenic acid (a constituent of corn silks), which is an ester of caffeic acid (*o*-dihydroxycinnamic acid) and quinic acid, is also active in the corn earworm bioassay. Consequently, the discovery of other *o*-dihydroxyflavonoids (i.e., luteolin derivatives) in corn silks would be advantageous in breeding resistance with a broad and diverse chemical basis. We have recently surveyed over 1000 corn inbreds, populations, plant introductions, and various unassigned lines for flavonoid content. In addition to discovering many new corn lines rich in maysin, apimaysin (the apigenin analogue of maysin), and 3′-methoxymaysin levels (Snook et al., 1993, 1994), we found several lines with interesting maysin analogues. We report here the discovery of four inbred corn lines with relatively high levels of 4″-hydroxymaysin (4″-OH-maysin) analogues. Synthetic 4″-hydroxymaysin was tested in a laboratory bioassay for its activity against the corn earworm. We also report the isolation and identification of 4″-hydroxy-3′-methoxymaysin.

MATERIALS AND METHODS

Plant Material. Plants were grown at the Coastal Plain Experiment Station, Tifton, GA, and the University of Missouri, Columbia, MO, under standard cultural practices of fertilizer and weed control for their respective areas. Irrigation was provided as needed. Ears were covered before silk emergence to prevent pollination, and 3–5-day-old silks were sampled by excising at the ear tip.

HPLC Analysis. Sufficient numbers of plants were sampled to give approximately 30 g of fresh silk/sample. The silks were weighed and immediately placed in 8 oz jars (Teflon-lined cap), and the jars were filled with 100% MeOH (approximately 180 mL). Samples were stored at 0 °C until analyzed. Samples were warmed to room temperature, and 6 mL of chrysin, dissolved in methanol (1.6 mg/mL, recrystallized from amyl alcohol) was added as an internal standard. After sonication for 20 min, aliquots of the solution were analyzed by reversed-phase HPLC, as described before (Snook et al., 1989), on an Ultrasphere C_{18}, 5 μm (4.6 × 250 mm) (Beckman Instruments, Norcross, GA), column, using a H_2O/MeOH linear gradient from 20% to 90% MeOH in 35 min, a flow rate of 1 mL/min, and detection at 340 nm (Hewlett-Packard, HP 1050 liquid

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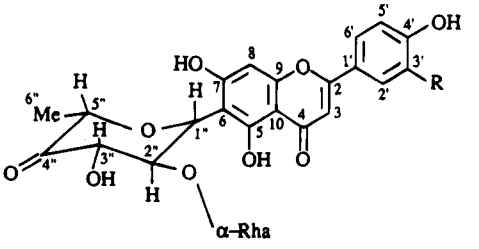
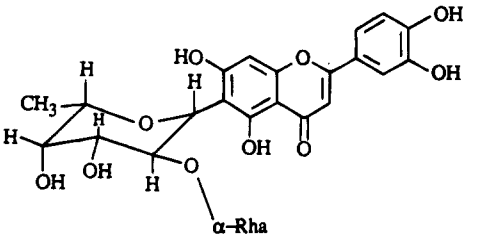
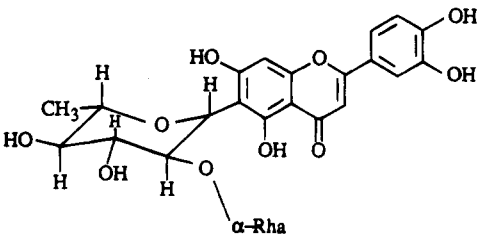


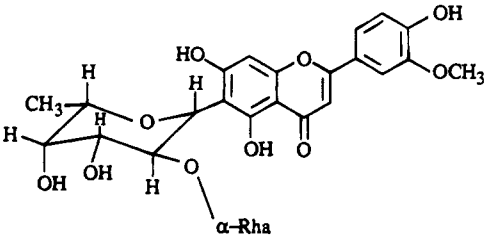
R = OH Maysin
R = H Apimaysin
R = OCH_3 3'-Methoxymaysin



2"-*O*-α-L-Rhamnosyl-6-*C*-fucosyl-luteolin
(ax-4"-OH-Maysin)



2"-*O*-α-L-Rhamnosyl-6-*C*-quinovosyl-luteolin
(eq-4"-OH-Maysin)



2"-*O*-α-L-Rhamnosyl-6-*C*-fucosyl-3'-methoxyluteolin
(ax-4"-OH-3'-Methoxymaysin)

Figure 1. Structures of maysin and related analogues.

chromatograph). Each solvent contained 0.1% H_3PO_4. Additional analyses were made with a Hypersil Phenyl, 5 μm (4.6 × 250 mm, Alltech Associates, Deerfield, IL), column. This column will separate apimaysin from 3'-methoxymaysin.

Isolation of Flavone Glycosides. All solvents were of analytical reagent grade. Fast atom bombardment mass spectral data (FAB-MS) were obtained in a glycerol matrix, as described before (Snook et al., 1991). ^1H and ^{13}C NMR data were obtained in either CD_3OD or DMSO-d_6 at room temperature with either a Bruker AM250 or AC300 spectrometer.

Evaporation of MeOH/H_2O solvents from maysin or the other flavones produced an orange-yellow, glasslike residue, possibly due to tightly bound water and/or methanol. Residual water/methanol was conveniently removed by dissolving the residue in MeOH and adding an equal amount of acetonitrile. Upon evaporation of this solution, an amorphous yellow powder was obtained. All isolated compounds were treated this way.

Maysin. Maysin was obtained as described previously (Snook et al., 1989).

Sodium Borohydride Reduction of Maysin. Maysin (0.5 g) was dissolved in 50 mL of MeOH, and $NaBH_4$ was added (with stirring) until gas evolution ceased. The solution was acidified to pH 2–3 with concentrated HCl and the MeOH evaporated. The residue was dissolved in water and submitted to preparative reversed-phase column chromatography. Approximately 100 g of the packing material from a Waters PrepPAK 500 C_{18} cartridge (Millipore Corp., Milford, MA) was repacked into a smaller glass chromatography column (LabCrest chromatography column, Andrews Glass Co., Vineland, NJ; 54 × 2.54 cm, 15 psi of nitrogen pressure used to aid flow), and the packing was washed with MeOH and recycled to H_2O. Salts from the reaction were eluted with water, and the reduced maysin (a mixture of 4"-OH-maysin epimers, see Figure 1) was eluted with 50% MeOH/H_2O. After evaporation of the solvent, the residue was dissolved in 75 mL of MeOH and 75 mL of acetonitrile. Evaporation of the solvent, as described above, yielded 0.48 g of 4"-OH-maysin (light yellow powder); HPLC analysis indicated an almost equal amount of equatorial 4"-OH-maysin (eq-4"-OH-maysin) and axial 4"-OH-maysin (ax-4"-OH-maysin) (Figure 1). The mixture of epimers was used in the bioassay experiments described below. Apimaysin and 3'-methoxymaysin were reduced in the same way.

2"-*O*-α-L-Rhamnosyl-6-*C*-quinovosylluteolin (eq-4"-OH-maysin). Silks of a corn inbred, ESDJ1, were used to isolate this compound. Approximately 200 silks (total silk of one corn ear, 1500 g of fresh weight) were slurried with 6.4 L of 100% MeOH in a Waring blender and filtered, and the MeOH/H_2O solution was concentrated with a rotary evaporator to an aqueous solution of approximately 500 mL. The resulting water/sample mixture was extracted with CH_2Cl_2 (3 × 200 mL), followed by extraction with 1-butanol (3 × 300 mL). The

Table 1. ^{13}C NMR Chemical Shift Assignments of Maysin, 3′-Methoxymaysin, and Their 4″-Hydroxy Derivatives

carbon assignment[a]	maysin[b]	eq-4″-OH-maysin[b]	ax-4″-OH-maysin[b]	3′-methoxymaysin[c]	ax-4″-OH-3′-methoxymaysin[c]
C-4″	205.6			204.4	
C-4	184.1	184.0	184.0	181.6	181.7
C-2	166.3	166.2	166.2	163.1 (C-2′+7′)	163.3
C-7	164.7	164.6	164.5		162.4
C-5	162.3	161.5	160.6	160.5	160.0
C-9	158.9	158.7	158.8	156.4	156.4
C-4′	151.0	150.9	150.9	147.9	147.9
C-3′	147.0	147.0	146.9	150.7	150.7
C-1′	123.6	123.5	123.6	121.3	121.4
C-6′	120.3	120.3	120.3	120.1	120.2
C-5′	116.8	116.8	116.7	115.7	115.8
C-2′	114.2	114.1	114.1	110.4	110.4
C-6	108.9	109.7	110.0	107.6	107.5
C-10	105.3	105.3	105.4	102.9	103.0
C-3	104.0	103.9	104.0	103.2	103.6
C-1‴	102.1	102.4	102.3	100.1	100.3
C-8	94.9	95.1	96.1	93.5	93.5
C-3″	82.3	81.2[d]	77.6[d,e]	80.2	75.6[d,f]
C-2″	81.1	78.1	76.2	78.9	73.3
C-5″	78.0	77.2	75.7	75.6	72.0
C-4‴	73.5	73.5 + C-4″	73.6 + C-4″	71.5	71.5 +C-4″
C-1″	73.3	72.3	73.3	71.2	71.3
C-2‴	72.3	72.0	72.2	70.4	70.4
C-3‴	72.0		72.0	70.3	70.3
C-5‴	70.1	69.8	70.0	68.2	67.9
C-6‴	18.0	18.2	17.9	17.3	17.3
C-6″	14.2	18.0	17.1	13.7	16.6
OCH_3				55.9	56.0

[a] See Figure 1. [b] Spectra obtained in CD_3OD. [c] Spectra obtained in [$^2H_6$]dimethyl sulfoxide. [d] Chemical shifts unassigned. [e] Additional unassigned chemical shift: 73.9. [f] Additional unassigned chemical shift: 73.6.

1-butanol was evaporated to dryness (a small amount of water, added at the end of the evaporation, facilitated the removal of the last traces of 1-butanol). The residue was dissolved in 50 mL of H_2O and submitted to preparative low-pressure reversed-phase column chromatography as described above. The column was eluted with the following solvents: H_2O, 500 mL; 50% MeOH/H_2O (v/v), 325 mL (fraction A) followed by 250 mL (fraction B). Fraction A contained chlorogenic acid, maysin, and eq- and ax-4″-OH-maysin. Fraction B contained maysin and ax-4″-OH-maysin. Fraction A was evaporated to dryness and submitted to silicic acid column chromatography (SA-50 g, Mallinckrodt, 100 mesh, washed with methanol and activated at 155 °C for 1 h). The column was packed in ethyl acetate, and the sample was applied to the top of the column as a SA/sample deposited mixture. The column was then eluted with 500 mL of ethyl acetate. After evaporation to dryness, the SA separated flavones were dissolved in 40% MeOH/H_2O and chromatographed on a Cheminert LC column (54 × 2.54 cm, Valco Instruments Co., Inc., Houston, TX, packed with the Waters PrepPAK 500 C_{18} cartridge material). The linear solvent gradient was from 40% MeOH/H_2O to 60% MeOH/H_2O in 400 min, and the column flow rate was 2 mL/min. Fractions of 8 mL were collected, and the column eluant was monitored at 340 nm. Individual column fractions were also monitored by HPLC and combinations made that contained chlorogenic acid, eq-4″-OH-maysin (fraction AI), and a mixture of maysin and ax-4″-OH-maysin (fraction AII). Fraction AI containing eq-4″-OH-maysin was chromatographed again on a reversed-phase column (column size, 108 × 1.25 cm; solvent gradient as before). Fractions from this run containing >97% pure eq-4″-OH-maysin (by HPLC) were combined, evaporated, and treated a second time to SA column chromatography and eluted with 10% acetone/ethyl acetate. Approximately 100 mg of 2″-*O*-α-L-rhamnosyl-6-*C*-quinovosyl-luteolin was obtained: mp 212 °C; ^{13}C NMR given in Table 1; fast atom bombardment MS (FAB-MS) *m/z* 579 M + H, 433 M + H − rhamnose.

2″-*O*-α-L-Rhamnosyl-6-*C*-fucosylluteolin (ax-4″-OH-maysin). Fractions B and AII from above were combined and chromatographed on reversed-phase columns (two 108 × 1.25 cm Cheminert LC columns connected in series); linear solvent gradient was as described above. Fractions containing ax-4″-OH-maysin in greater than 95% purity were combined and evaporated to dryness. The residue was dissolved in MeOH and an equal quantity of acetonitrile added. On evaporation, a dark yellow powder (75 mg) was obtained.

Axial 4″-OH-maysin was also isolated from silks of Tx501 as described below, and ^{13}C NMR (Table 1) and FAB-MS data obtained: *m/z* 579 M + H, 433 M + H − rhamnose.

2″-*O*-α-L-Rhamnosyl-6-*C*-fucosyl-3′-methoxyluteolin (ax-4″-OH-3′-methoxymaysin). Silks of Tx501 were the source of this compound. Approximately 110 silks (640 g) were homogenized with 4 L of MeOH, filtered, concentrated to approximately 200 mL, and extracted with CH_2Cl_2 and 1-butanol as described above for ESDJ1 silks. The residue from the 1-butanol extraction was chromatographed on the nitrogen pressure column described above. The compound of interest eluted with 20% and 30% MeOH/H_2O. Repeated chromatographic passes of the material through the 108 × 1.25 cm Cheminert LC columns yielded 32 mg of 2″-*O*-α-L-rhamnosyl-6-*C*-fucosyl-3′-methoxyluteolin (ax-4″-OH-3′-methoxymaysin): mp 192 °C; ^{13}C NMR (Table 1); FAB-MS *m/z* 593 M + H, 447 M + H − rhamnose. During the above chromatographic separations, fractions were combined that contained relatively pure levels of maysin, ax-4″-OH-maysin (used for FAB-MS and NMR studies), and 3′-methoxymaysin, respectively.

Bioassay Procedures. Two larval feeding assays were performed, one with maysin and the other with the mixture of 4″-OH-maysin epimers produced by $NaBH_4$ reduction described above. Maysin (480 mg) was dissolved in MeOH in a 200 mL volumetric flask. One hundred milliliters of the resulting solution was removed and placed in a 500 mL round-bottom flask, containing 2 g of Celufil (U.S. Biochemical, Cleveland, OH). The solvent was evaporated to deposit the compound onto the Celufil. Serial dilutions were prepared to produce four dosages (240, 120, 60, and 30 mg) per test. The 4″-OH-maysin epimeric mixture was treated in the same way. The compound/Celufil mixtures were bioassayed according to the method of Wiseman (1993). The samples were added to 25 g of diluted pinto bean diet (3 mL of diet/2 mL of water). Diet/sample mix (2 mL) was dispensed into plastic diet cups and one neonate corn earworm added to each sample. After 8 days, the weight of the larvae was recorded. Appropriate MeOH/Celufil blanks were used. The experiment was arranged in a randomized complete block design with 10 replications. Results are expressed as percent of controls.

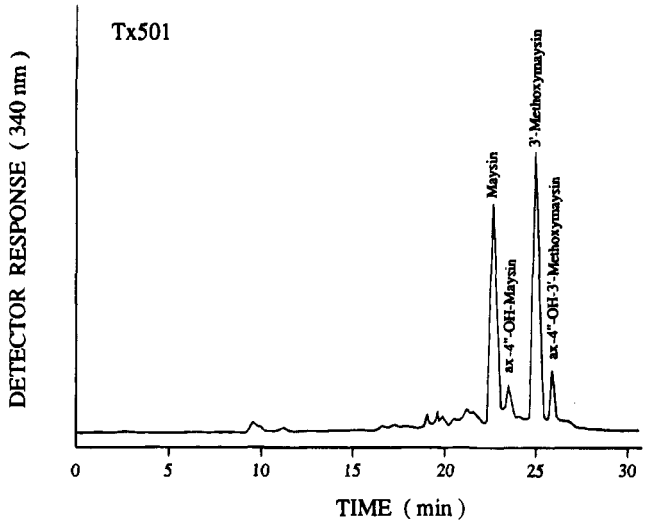


Figure 2. HPLC profile of corn inbred Tx501.

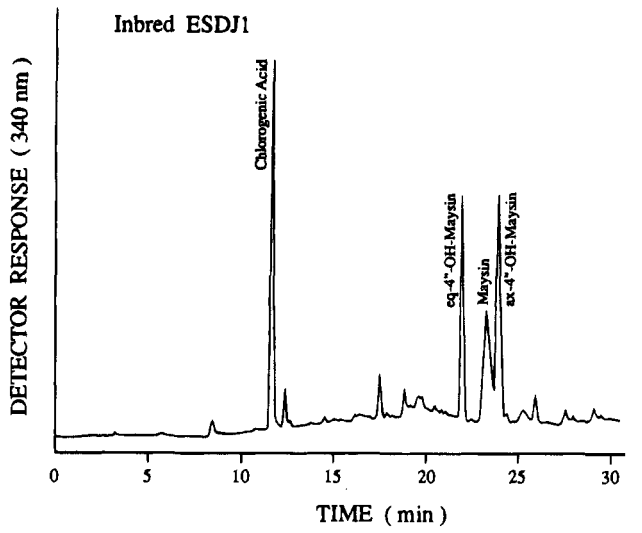


Figure 3. HPLC profile of corn line ESDJ1.

RESULTS AND DISCUSSION

Identification of Flavonoids in Tx501. During our survey of corn inbreds, populations, and plant introductions for maysin content, we discovered several lines with relatively high levels of maysin analogues. Lines with high levels of apimaysin (the apigenin analogue of maysin) and 3′-methoxymaysin (Figure 1) previously have been reported (Snook et al., 1993). One of these lines (Tx501), containing high levels of 3′-methoxymaysin also had two other compounds in its HPLC chromatogram, one eluting just after maysin (post-maysin, Figure 2) and one eluting just after 3′-methoxymaysin (post-3′-methoxymaysin). In the present study, these compounds were isolated and FAB-MS and ^{13}C NMR spectral data were obtained. Post-maysin was found (by MS) to have a molecular weight of 2 more than maysin (578 vs 576), while post-3′-methoxymaysin had a molecular weight of 2 more than 3′-methoxymaysin (592 vs 590). Ultraviolet spectra indicated the two unknowns contained the luteolin chromophor. The ^{13}C NMR data of the two compounds are given in Table 1 and compared to those of maysin and 3′-methoxymaysin. Important features in the spectra of both of the unknown compounds are as follows: (1) the absence of the C-4″ carbonyl signal at 204−205 ppm; (2) the two methyl signals of maysin (C-6″ 14.2 ppm and C-6‴ 18.0 ppm) that have become almost equivalent (C-6″ 17.1 ppm and C-6‴ 17.9 ppm for post-maysin; C-6″ 16.6 ppm and C-6‴ 17.3 ppm for post-3′-methoxymaysin); and (3) the chemical shifts of the aromatic carbons were similar for maysin and post-maysin, while those for 3′-methoxymaysin and post-3′-methoxymaysin were similar. The data indicated that the structures of the compounds were derivatives of maysin and 3′-methoxymaysin in which the carbonyl at C-4″ has been reduced.

Reduction of the C-4″-carbonyl can result in the formation of two epimeric compounds: one with the hydroxyl equatorial, forming a quinovose, and the other with an axial hydroxyl, forming a fucose. Elliger et al. (1980a) reported that the $NaBH_4$ reduction of enzymatically rhamnose-cleaved maysin (derhamnosylmaysin) produced an equal mixture of two products, chromatographically isolated and identified as 6-*C*-quinovosylluteolin (4″-hydroxyl equatorial) and 6-*C*-fucosylluteolin (4″-hydroxy axial). Samples of these two compounds (kindly supplied by Dr. Elliger), when separated by HPLC as above, showed that the equatorial hydroxyl

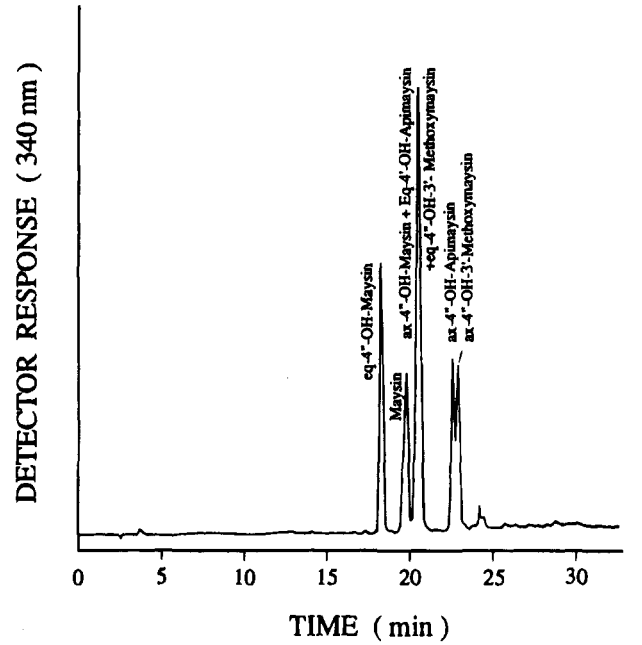


Figure 4. HPLC separation of eq- and ax-4″-hydroxy derivatives of maysin, apimaysin, and 3′-methoxymaysin.

epimer eluted from the column several minutes earlier than the axial epimer. $NaBH_4$-reduced maysin also produced equal amounts of two compounds which were separated by several minutes upon HPLC. In light of the discussion above for $NaBH_4$-reduced derhamnosylmaysin, the earliest HPLC eluting isomer of $NaBH_4$-reduced maysin must be the equatorial hydroxy epimer (eq-4″-OH-maysin or 2″-*O*-α-L-rhamnosyl-6-*C*-quinovosylluteolin) and the latest eluting isomer the axial epimer (ax-4″-OH-maysin or 2″-*O*-α-L-rhamnosyl-6-*C*-fucosylluteolin). Synthesized ax-4″-OH-maysin coeluted with post-maysin from Tx501 and, consequently, post-maysin is 2″-*O*-α-L-rhamnosyl-6-*C*-fucosylluteolin. Similarly, $NaBH_4$-reduced 3′-methoxymaysin produced two epimers that were separated by HPLC into eq-4″-OH-3′-methoxymaysin and ax-4″-OH-3′-methoxymaysin. Synthesized ax-4″-OH-3′-methoxymaysin coeluted with post-3′-methoxymaysin of Tx501 and, thus, post-3′-methoxymaysin was identified as 2″-*O*-α-L-rhamnosyl-6-*C*-fucosyl-3′-methoxyluteolin.

Identification of Flavonoids in Inbred ESDJ1. Inbred ESDJ1, an experimental line from the Insect

Table 2. Corn Lines with Significant Levels of C-4″-Hydroxy Derivatives of Maysin

	% fresh weight						
corn line	eq-4″-OH-maysin	ax-4″-OH-maysin	maysin	ax-4″-OH-3′-methoxymaysin	3′-methyoxymaysin	total flavones listed	chlorogenic acid
A103	0.056	0.176	0.185		0.019	0.436	0.082
CML131	0.059	0.213	0.173		0.030	0.475	0.013
ESDJ1	0.085	0.114	0.111		0.015	0.325	0.118
GE275	0.053	0.038	0.052		0.011	0.154	0.030
Tx501			0.215	0.021	0.252	0.522	0.013
2–043	0.041	0.051	0.249	0.010	0.037	0.388	0.016

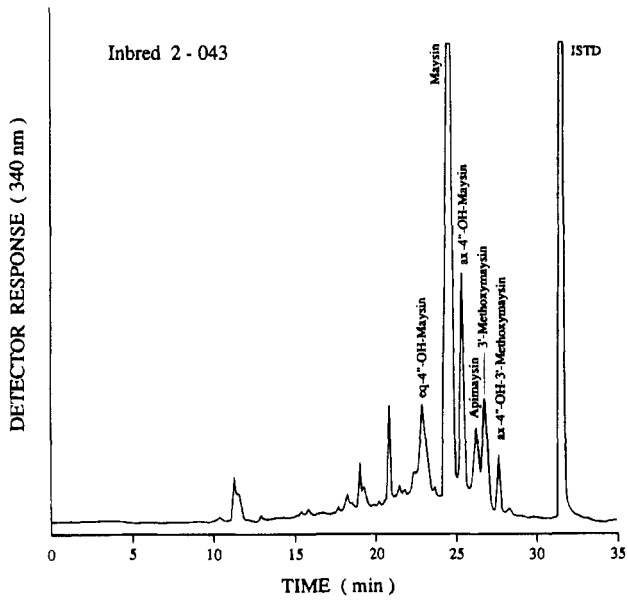


Figure 5. HPLC profile of inbred 2-043.

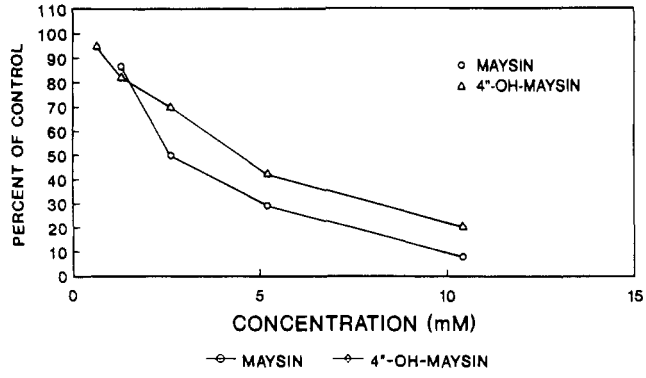


Figure 6. Growth of corn earworm larvae versus concentration of maysin and 4″-hydroxymaysin.

Biology and Population Management Research Laboratory, Tifton, GA, was found to contain high levels of maysin, ax-4″-OH-maysin, and another compound eluting from the HPLC before maysin (Figure 3). This compound coeluted with the eq-4″-OH-maysin obtained by $NaBH_4$ reduction of maysin. The FAB-MS and ^{13}C NMR (Table 1) spectral data are in agreement that this compound is 2″-*O*-α-L-rhamnosyl-6-*C*-quinovosylluteolin.

Occurrence of C-4″-Hydroxymaysin, -Apimaysin, and -3′-Methoxymaysin in Silks. The separation of all six isomers of C-4″-reduced maysin, apimaysin, and 3′-methoxymaysin by HPLC is given in Figure 4. Ax-4″-OH-maysin, eq-4″-OH-apimaysin, and eq-4″-OH-3′-methoxymaysin coeluted in the HPLC systems tested. Many corn lines with high levels of maysin (>0.2% fresh weight) contain a minor compound with the same retention time as that of ax-4″-OH-maysin. Levels are typically $^1/_{10}$ or less the level of maysin, however. Corn lines with relatively high levels of 4″-OH-maysin and 4″-OH-3′-methoxymaysin are given in Table 2. Ax-4″-OH-3′-methoxymaysin was also found in inbred 2-043 (Figure 5), which is unique in that it also contains the reduced maysin analogues. We have found that levels of these compounds in inbred 2-043 were extremely variable from year to year.

The HPLC profiles of lines A103, GE275, and CML131 are very similar to those of inbred ESDJ1 (Figure 3), suggesting either a common ancestry or a rare enhancement of a specific flavonoid synthetic pathway. It is not known whether these lines have any common ancestry, but that is unlikely since each was developed at widely separated geographic locations and selected for differing agronomic purposes. Elliger et al. (1980a) proposed a possible pathway to the 4-ketofucosyl structure of maysin based on previous work on the conversion of UDP-D-glucose to UDP-L-rhamnose (Barber 1963). This involved oxidation of the hydroxy group at C-4 of the glucose to a ketomoiety and later reduction back to a hydroxyl group. Thus, retention of the hydroxyl group at the fucosyl C-4 in the maysin analogues could occur at two different stages during the biosynthesis. Although several corn lines have high levels of apimaysin, only trace levels of components were observed at the HPLC retention times of reduced apimaysin.

Biological Activity of C-4″-Hydroxymaysin toward the Corn Earworm. Sodium borohydride reduced maysin (mixture of epimers) was tested for corn earworm larval growth inhibition in a laboratory bioassay. Results are given in Figure 6 and show that 4″-OH-maysin is almost as active as maysin in reducing larval growth. This suggests that incorporation of these types of flavones into corn silks by plant breeders could increase the resistance of the silks to this very important agricultural pest.

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