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ANTIGERMINATION ACTIVITY OF PHENYLPROPENOIDS FROM THE GENUS *PIMPINELLA*

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ABSTRACT.—Eight phenylpropenyl esters (three new compounds) were identified in extracts of seed from six *Pimpinella* species. Six of these were isolated and tested for antigermination activity. Those with epoxypropenyl groups were active against seed from several different species, while compounds with olefinic groups instead of epoxy groups were either inactive or had minimal activity.

Extracts of the roots and seed of *Pimpinella* species (Umbelliferae) have long been known to contain epoxidized phenylpropenoids (1,2); however, no biological activity has been reported for these compounds. Antifungal (3) and spermicidal (4) activity has been reported for the gross extracts of *Pimpinella diversifolia* DC. root, but the responsible compounds have not been determined. In addition to these phenylpropenoids, this species also contains other compounds with potential antifungal activity such as furanocoumarins (5). We now report strong antigermination activity of the isolated epoxy phenylpropenoid esters contained in the seed of a number of *Pimpinella* species, and we will relate activity to structure.

The original reports of epoxidized phenylpropenoids (1,2) misidentified these compounds as esters of epoxidized isoeugenol, placing the epoxidized propenyl group para to the acylated hydroxy group. Recently, reports by Martin *et al.* (6) and Bottini *et al.* (7) have revised the structures of these compounds to the correct ortho configuration (pseudoisoeugenol). We have identified four epoxy compounds and their non-epoxy analogs as seed constituents in six different species of the genus *Pimpinella*. Three of these epoxides and their previously unreported non-epoxy analogs were isolated for bioassay. One epoxy compound, reported by Stahl and Herting (2), did not contain a methoxy substituent. In this case we have confirmed that the epoxy-containing moiety is in the para position.

RESULT AND DISCUSSION

Epoxyphenylpropenoids have been isolated previously from *Pimpinella* root (1,2,6,7), and in the case of *Pimpinella anisum* L., *Pimpinella major* (L.) Huds., and *Pimpinella saxifraga* L., they were also found in the fruits (2,8). In our program to find growth regulators from wild plant germ plasm, we examined extracts of seed from nine different *Pimpinella* species. Extracts from seed of several of these species showed significant germination inhibition when velvetleaf was used as the test species. Where germination succeeded, obvious growth inhibition was observed. We, therefore, decided to isolate the phenylpropenoids found to be present in six species (Table 1) and to determine if they had antigermination activity.

Isolation by hplc, in general, was straightforward. However, the non-epoxy compounds eluted fairly close to triglycerides (Table 2), which are the major constituents in the hexane extracts. The triglycerides tended to overlap the much less concentrated

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TABLE 1. Source of Phenylpropenoids (Hexane Extract of Seed).

Pimpinella species	Compound	Amount (% of extract)
anisum	**1**	0.1
	2	0.2
diversifolia	**1**	1.6
	5	2.7
gustavobegiana	none detected	
kotschyana	none detected	
major	**7**	10.9
	8	1.5
peregrina	**5**	23.5
	6	tr[a]
saxifraga-1	**5**	19.4
saxifraga-2	**5**	20.4
saxifraga-3	**3**	5.1
	4	tr
stewartii	non detected	
tragium	**1**	9.0
	3	tr

[a]tr = trace amount.

phenylpropenoids, making isolation of them more difficult. Rechromatography of some fractions up to three times was necessary before adequate purity was reached. Isolates were monitored for purity by tlc and gc (Table 2).

The ^{1}H- and ^{13}C-nmr data for compounds **1**, **3**, and **5** are in close agreement with those found by Martin *et al.* (6) (Table 3); therefore, the substitution pattern is as shown. Nmr data confirmed that compound **2** differs from compound **1** in that a propenyl moiety has been substituted for the epoxypropyl group. In the ^{13}C spectrum of **2**, resonances appear at 124.5 and 128.4 ppm instead of the resonances at 58.5 and 55.2 ppm observed for **1**. The ^{1}H spectrum exhibits a change with multiplets at 6.36 and 6.19 ppm observed for **2** replacing multiplets at 3.60 and 2.91 ppm in **1**. An olefinic proton coupling constant of 15 Hz indicates an *E* configuration for the double bond. Similarly, nmr of compound **4** indicates that it is the unsaturated analog of **3**. Thus, compound **4** has nmr signals for two double bonds. The signal representing the propenyl group confirms the *E* configuration just as in **2** (Table 3). Compounds **7** and **8** both lack a methoxy resonance in their nmr spectra. The aromatic ring substitution is

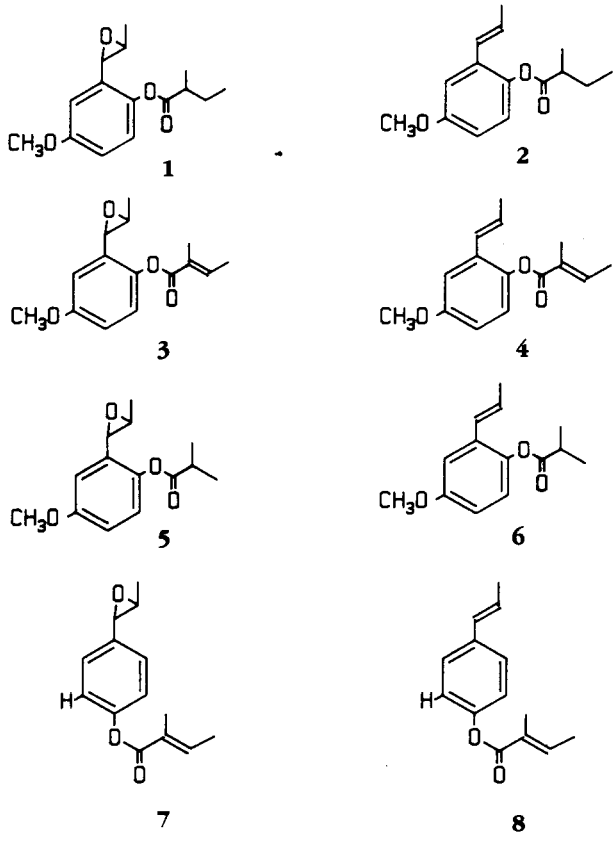
TABLE 2. Relative Chromatographic Data of Phenylpropenoids.

Compound	Gc	Lc	Tlc
1	0.915	1.641	0.64
2	0.846	1.401	0.80
3	1.005	2.040	0.58
4	0.950	1.450	0.71
5	0.769	1.916	0.62
6	0.723	—	—
7	0.862	1.830	0.62
8	0.748	1.182	1.03
16:0[a]	1.000	—	—
Triglyceride	—	1.000	1.00

[a]Methyl palmitate.

TABLE 3. Nmr Data of Compounds from *Pimpinella* Species.

Carbon	Compound						
	1	**2**	**3**	**4**	**5**	**7**	**8**
				^{1}H nmr			
2						7.17 m	7.32 m
3	6.93 d, 8.7	6.88 d, 8.8	6.96 d, 8.7	6.91 d, 8.8	6.93 d, 8.7	6.99 m	7.02 m
4	6.80 dd, 8.7,3	6.74 dd, 8.	6.79 dd, 8.7,3	6.75 dd, 8.8,2.9	6.79 dd, 8.7,3		
5						6.99 m	7.02 m
6	6.76 d, 3	6.98 d, 3	6.75 d, 3	7.00 d, 2.9	6.75 d, 3	7.17 m	7.32 m
1′	3.60 d, 2	6.36 m, 15.	3.56 d, 2	6.35 m, 15.9,1.2	3.58 d, 2	3.47 brs	6.39 m, 15.8,1.6
2′	2.91 m, 2,5.2	6.19 m, 15.	2.90 m, 2,5.1	6.21 m, 15.9,6.4	2.90 m, 2,5.2	2.89 m	6.19 m, 15.8,6.5
3′	1.41 d, 5.2	1.86 dd, 6.	1.38 d, 5.1	1.85 dd, 6.4,1.2	1.41 d, 5.2	1.33 m, 5,1.1	1.87 m, 6.5
2″	2.66 m	2.65 m			2.83 m, 7		
3″	1.64 m, 1.85 m	1.65 m, 1.8	7.11 m, 7.0,1.5	7.12 m, 7.1	1.32 d, 7	7.02 m	7.10 m, 7.0
4″	1.03 t, 7.4	1.03 t	1.87 m, 7.0,1.2	1.89 brd, 7.1	1.33 d, 7	1.76 brd, 7	1.87 m, 7.0,1.2
5″	1.31 d, 7.0	1.31 d	1.95 m, 1.2,1.5	1.96 brs		1.86 m, 1.2	1.95 m, 1.2
OMe	3.77 s	3.79 s	3.79 s	3.80 s	3.77 s		
				^{13}C nmr			
1	131.2 s	131.3 s	131.2 s	131.1 s	131.2 s	134.6 s	135.4 s
2	142.6 s	141.4 s	142.8 s	141.7 s	142.6 s	126.0 d	126.6 d
3	122.7 d	123.1 d	122.7 d	123.3 d	122.6 d	121.3 d	121.6 d
4	114.2 d	113.2 d	114.1 d	113.2 d	114.2 d	150.5 s	149.0 s
5	157.7 s	157.3 s	157.5 s	157.2 s	157.7 s	121.3 d	121.6 d
6	109.8 d	111.0 d	109.9 d	110.9 d	109.8 d	126.0 d	126.6 d
1′	58.5 d	124.5 d	58.2 d	124.5 d	58.4 d	58.4 d	125.6 d
2′	55.2 d	128.4 d	55.3 d	128.1 d	55.3 d	58.4 d	130.1 d
3′	17.8 q	18.8 q	17.7 q	18.8 q	17.8 q	17.3 q	18.4 q
1″	175.0 s	175.3 s	166.3 s	166.7 s	175.0 s	165.7 s	166.5 s
2″	41.1 d	41.2 s	127.8 s	128.1 s	34.1 d	127.6 s	128.1 s
3″	26.8 t	26.8 s	139.3 d	139.1 d	19.0 q	138.9 d	139.1 d
4″	11.7 q	11.7 q	14.6 q	14.6 q	19.1 q	14.1 q	14.6 q
5″	16.8 q	16.8 q	12.1 q	12.2 q		11.6 q	12.1 q
OMe	55.6 q	55.6 q	55.5 q	55.5 q	55.6 q		



supported by calculations for the ^{13}C chemical shifts (9) that are in close agreement with the experimental data (Table 4). As further proof of the substitution pattern, compound **7** was subjected to a 2D INADEQUATE experiment (10). No coupling was observed between the carbons bearing the substituents (Figure 1). Cross sections taken from the 2D INADEQUATE spectrum (Figure 1) show that the carbons bearing the substituents are each connected to two protonated aromatic carbons, confirming the 1,4 substitution. If the substitution were ortho, then coupling would have been observed for only one protonated aromatic carbon. ^{13}C multiplicities were determined with a DEPT experiment (11).

Mass spectral analysis (Table 5) supported the nmr assignments. Molecular ions and ions representing the ester function were compatible with those structures presented in

TABLE 4. Calculated and Experimentally Determined ^{13}C Chemical Shifts for the Two Possible Substitution Patterns for **8**.

Carbon	Calcd 1,4	Calcd 1,2[a]	Experimental
1	135.7	130.6	135.4
2	127.0	148.7	149.8
3	122.2	122.2	121.6
4	151.2	129.5	121.6
5	122.2	126.3	126.6
6	127.0	127.0	126.6

[a]Synthetic compound (synthesis to be reported in subsequent publication).

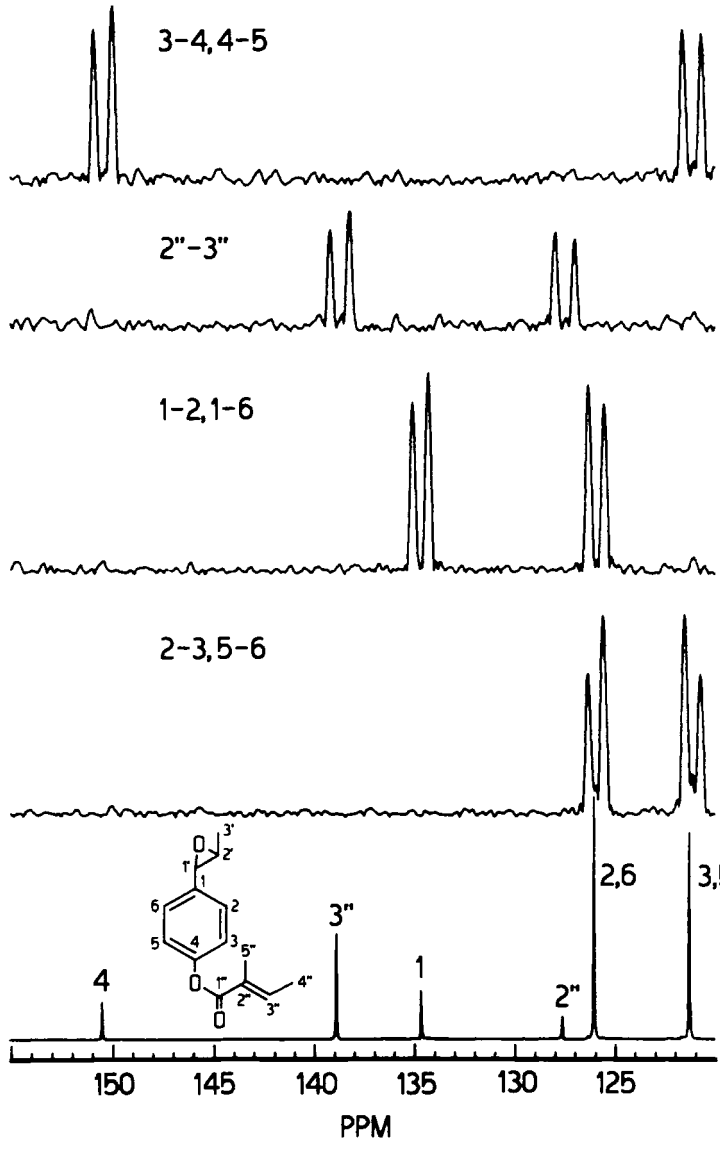


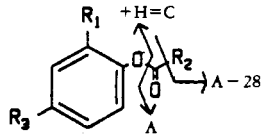
FIGURE 1. Cross section from the 2D INADEQUATE spectrum of **7** that demonstrates ^{13}C-^{13}C connectivity.

the structure table. Our spectra were consistent with those previously reported (6–8) except for minor intensity differences. The location of the functional groups on the aromatic ring in the epoxides was supported by an important diagnostic ion assigned as the benzofuran (ion B, *m/z* 162). This ion is derived only from compounds with a 1,2 epoxypropyl group (**1**, **3**, and **5**), and is not present in spectra from compounds where there is no epoxide or the epoxypropyl is 1,4.

Compound **6** was present in *Pimpinella peregrina* L. seed in such small amounts that it was identified only on the basis of its mass spectrum (Table 5) and chromatographic data (Table 2).

Natural occurrence of compounds **4**, **6**, and **8** has not been previously reported. Compound **2**, in the same series as **4** and **6**, was found in *P. anisum* (8,12,13). The isovaleric isomer of **8** was established in *Coreopsis gigantea* (Kell.) Hall (14).

TABLE 5. Mass Spectral Data.



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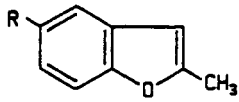

R = −OMe or H

Compound	Ions[a]								
	$[M]^+$	$[M-R_2CO_2]^+$	$[A]^+$	$[A-28]^+$	$[B]^+$	$[C]^+$	$[C-15]^+$	$[C-29]^+$	$[C-43]^+$
1	64 (17)	163 (45)	85 (10)	57 (82)	162 (41)	180 (36)		151 (31)	137 (100)
2	248 (7)	147 (1)	85 (2)	57 (21)	—	164 (100)	149 (27)	135 (3)	121 (3)
3	262 (7)	163 (17)	83 (100)	55 (54)	162 (34)	180 (4)	—	151 (5)	137 (9)
4	246 (12)	147 (1)	83 (100)	55 (47)	—	164 (36)	149 (6)	135 (2)	121 (1)
5	250 (19)	163 (43)	71 (18)	43 (97)	162 (37)	180 (35)	165 (1)	151 (34)	137 (100)
6	234 (14)	147 (1)	71 (3)	43 (11)	—	164 (100)	149 (31)	135 (4)	121 (5)
7	232 (4)	133 (6)	83 (100)	55 (39)	132 (3)	—	—	121 (1)	107 (2)
8	216 (10)	117 (1)	83 (100)	55 (57)	—	134 (34)	119 (1)	105 (3)	91 (1)

[a] *m/z* (rel. int.).

Activity of **1** against velvetleaf (*Abutilon theophrasti* Medic.) seed was significant (28% of control) at 1×10^{-2} M concentration with very little root growth in those seeds that did germinate. At the 5×10^{-3} M level no inhibition of germination was observed, but moderate growth inhibition was present. This compound was far more active with seeds other than velvetleaf, limiting ryegrass (*Lolium multiflorum* Lam.) germination to 0% at 1×10^{-3} M concentration. Significant antigermination activity also was observed with carrot (*Daucus carota* L.), lettuce (*Lactuca sativa* L.), and tomato (*Lycopersicon esculentum* Mill.) seed at this level. Compounds applied to cucumber (*Cucumis sativus* L.) and radish (*Raphanus sativus* L.) showed no significant activity with concentrations as high as 1×10^{-2} M.

Bioassays of compound **3** showed significant activity with all test seeds at the 1×10^{-2} M level except radish. The germination of carrot, ryegrass, and tomato was still significantly inhibited by **3** at the 1×10^{-3} M level.

Compound **5** was the most active of all the compounds in this series. Isolated from the seed of *P. diversifolia*, it inhibited germination of carrot and ryegrass seed completely at the 1×10^{-3} M level and with lettuce seed produced significant inhibition even at the 1×10^{-4} M level, allowing only 56% of the seeds to germinate.

Compound **2**, the unsaturated non-epoxy analog of **1**, isolated from the seed of *P. anisum*, did not inhibit germination of any of the test seeds. Compounds **4** and **6**, the non-epoxy analogs of **3** and **5**, were not tested.

Compounds **7** and **8**, isolated from *P. major* seed, showed an epoxy–non-epoxy activity relationship similar to **1** and **2**. Compound **7** was active with seed of carrot, lettuce, ryegrass, and tomato and not with velvetleaf, cucumber, and radish. Compound **8** had significant activity with carrot, tomato, and ryegrass. However, the activity was in no case as high as with its epoxy analog **7**. For instance, at 1×10^{-3} M only 3% of carrot seed germinated with **7** compared to 55% with **8**. Compounds **7** and **8** differed from the other compounds in this series in that they did not have a methoxy substituent, and the propenyl group was in the para position. With most test seeds, **7** had

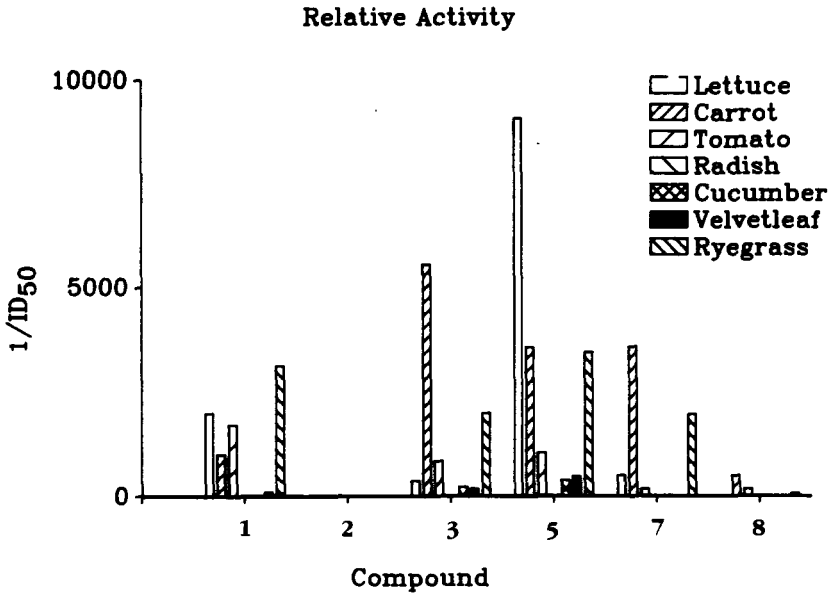

FIGURE 2. Relative bioactivity of *Pimpinella* phenylpropenoids.

less activity than its methoxy 1,2-substituted analog, compound **3**. In fact, it had no activity against velvetleaf at any concentration tested while **3** was significantly active at 5×10^{-3} M. However, with carrot seed, **7** had about the same activity as those compounds having a methoxyl group.

The relative activity of these compounds from *Pimpinella* can be best seen in Figure 2 in which we plot the inverse of the dose that inhibited germination of 50% of the seeds (ID_{50}). Carrot was most affected by compounds **1**, **3**, and **7**, and second to lettuce in its response to compound **5**. Germination of ryegrass seed was also greatly inhibited by **1**, **5**, and **7**, while **2** did not inhibit germination of any of the test seeds. Cucumber and velvetleaf were only marginally affected by some compounds and not at all by others.

As proposed by Bottini *et al.* (7), it is possible that the biosynthesis of this group of compounds may involve *o*-coumarate and compete for the formation of simple coumarins. This must involve decarboxylation of *o*-coumarate and subsequent methoxylation, esterification, and epoxidation. From our study the presence of esterified non-epoxy analogs tends to indicate that esterification occurs before epoxidation.

MATERIALS AND METHODS

Most seeds were collected in the wild—*P. diversifolia* and *Pimpinella stewartii* (Dunn) E. Nasir in Pakistan, *Pimpinella gustavohegiana* in Korea, *Pimpinella kotschyana* in Turkey, *P. peregrina* in Israel, and *P. saxifraga* and *Pimpinella tragium* Vill. in Yugoslavia. *P. anisum* seed was purchased from Harry Saier, Inc., Dimondale, Michigan, and *P. major* seed was purchased from Medigran Seed Co., Amstelveen, The Netherlands. Seeds not purchased were collected in the wild and identified by USDA botanists. Ground seeds were successively extracted in a Soxhlet apparatus with hexane (16 h), Me_2CO (8 h), and H_2O (8 h). Hexane and Me_2CO were removed in a rotary evaporator at 30–50° and H_2O by lyophilization.

BIOASSAY.—The filter paper-petri dish germination bioassays were conducted and evaluated as reported by Spencer *et al.* (15) except that the incubation time was 5 days and menadione (Vitamin K_3) at the 1×10^{-3} M level was used as a positive control. Gross extracts were applied at the rate of 40 mg per dish (two 20-seed dishes) and tested only against velvetleaf. Isolated compounds were tested at a maximum concentration of 1×10^{-2} M. The concentration was decreased until germination was not significantly different from the control. These compounds were applied to lettuce, carrot, tomato, radish, cucumber, velvetleaf, and ryegrass seed. ID_{50}'s were calculated according to Finney (16).

COMPOUND ISOLATION.—Hexane extracts were fractionated using normal phase hplc. For typical isolation we used a Waters model 590 programmable solvent delivery module delivering hexane-EtOAc (80:20) at 8 ml/min to a 21.4 mm i.d. × 25 cm Dynamax (Rainin Instrument Co., Inc.) 60A 8-μm silica column. Injection (150 μl, 50% solution) was made by a Waters WISP model 710A and collection was accomplished with a Gilson model 201 fraction collector, both run in the automatic mode. Isolated components were rechromatographed through this same system to increase purity.

NMR AND MS.—^{1}H- and ^{13}C-nmr spectra were recorded with a Bruker WM 300 WB spectrometer operating at 75.47 MHz for ^{13}C and 300.13 MHz for ^{1}H. Typically 10,000 to 90,000 transients were obtained from $CDCl_3$ solutions and summed in the Aspect 2000 computer to yield, following Fourier transformation, ^{13}C spectra. The solvent served as the internal lock as well as an internal reference standard at 77 ppm for ^{13}C. Sweep widths of 200 ppm 16K data points were used in data collection. A pulse width of 3 μsec (40°) was employed with a 5-mm dual probe. Adequate broadband decoupling power was obtained with 1 watt. Standard software was used for the 2D INADEQUATE experiment. A total of 128 FIDs of 512 scans acquired in 32 step phase cycles with 512 W data points were transformed after exponential line broadening and zero filling. The observed spectral width of 3759.4 Hz was chosen to include only the ^{13}C resonances of interest. A 5-sec recycle delay was used, with the double quantum coherence delay being 4 millisec. The total acquisition time was 24 h for the solution containing 360 mg of compound **7** in 0.35 ml of $CDCl_3$.

Mass spectra were taken in the electron impact mode at 70 eV in a Finnegan model 4600 TSQ from samples introduced through a gas chromatograph.

ANALYTICAL CHROMATOGRAPHY.—Crude extracts and isolated compounds were examined by gc and tlc. Gc was accomplished with a Packard model 428 gas chromatograph equipped with a 15 m × 0.253 mm DB-1 (0.25 μm film thickness) fused silica capillary column (J&S Scientific, Inc.) temperature programmed from 100° to 290° at 4°/min. Estimates of the amount of these materials present in crude hexane extracts were made by first obtaining a response factor by using different proportions of methyl palmitate and compound **5**, then placing 10 mg of each extract in a vial and adding 1 mg of methyl palmitate as internal standard. This mixture was made up to 1 ml, and 2 μl was analyzed by gc as above. The amount of phenylpropenoid was calculated by comparing compounds of interest with the internal standard after correcting for response. All phenylpropenoids were assumed to have the same response relative to methyl palmitate.

Tlc was accomplished by using plates (20 × 20 cm) coated with a 0.25-mm layer of Si gel 60 F_{254} (Merck). The developing solvent was hexane-Et_2O (70:30). Component detection was made under uv light or by charring with H_2SO_4–dichromate solution at 150°.

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