JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chromatographic Separation and *in Vitro* Activity of Sorgoleone Congeners from the Roots of *Sorghum bicolor*

ISABELLE A. KAGAN,* AGNES M. RIMANDO, AND FRANCK E. DAYAN

Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi 38677

Sorgoleone, 2-hydroxy-5-methoxy-3-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone (**1**), and its corresponding hydroquinone are the major components of the root exudate of *Sorghum bicolor*. The name sorgoleone includes minor analogues differing in the length or degree of unsaturation of the 3-alkyl side chain. These compounds are known to be phytotoxic, probably through inhibition of photosystem II (PSII) driven oxygen evolution, as previously demonstrated for **1**. Isolation of these sorgoleone congeners was achieved by C_8 column chromatography and argentation thin-layer chromatography, and the purified compounds were structurally characterized. The abilities of the minor sorgoleones to inhibit PSII were similar to that of the major compound, suggesting that all of these sorgoleone congeners contribute to the overall allelopathy of sorghum.

KEYWORDS: Sorghum; sorgoleone; allelopathy; argentation chromatography; column chromatography; congeners; phytotoxicity; oxygen evolution; roots

INTRODUCTION

The allelopathic (plant growth inhibiting) properties of sorghum (*Sorghum bicolor*) have been studied for decades, due in part to concern about reduced crop growth in fields previously planted with sorghum (*1*) and the potential use of such inhibitory properties in weed control (*2*). Considerable effort has been devoted to identifying the compounds contributing to this allelopathic effect in sorghum. Although initial studies focused on water-soluble compounds from root exudates or decaying plant tissue (*3*−*6*), more recent work has identified a hydrophobic compound secreted from the root hairs, namely, sorgoleone, 2-hydroxy-5-methoxy-3-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone, **1**, (**Figure 1**) (*7*, *8*). Sorgoleone was studied initially for its ability to stimulate germination of witchweed (*Striga asiatica*) when in its naturally occurring reduced hydroquinone form (*7*−*9*), although later studies have indicated that other compounds may be the key stimulants of witchweed germination (*10*−*13*). Sorgoleone inhibits the growth of many weed species (*8*, *14*−*20*). Studies of the effects of sorgoleone on oxygen evolution in cell cultures (*17*), thylakoid membranes (*15*, *21*, *22*), and intact chloroplasts (*23*), as well as computer modeling studies (*24*), have demonstrated that this natural lipophilic benzoquinone interferes with the binding of plastoquinone at the D1 protein of photosystem II (PSII). Sorgoleone also affects mitochondrial functions (*25*) and inhibits the enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPPD) (*26*).

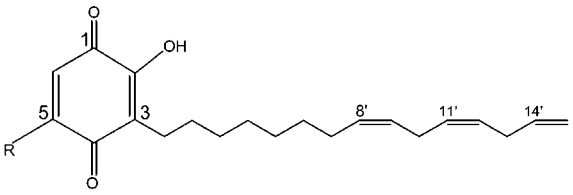
Whereas sorgoleone, **1**, and its 1,4-hydroquinone form, **2**, are the major components of the hydrophobic root hair exudates (*7*, *9*), some minor components have been reported as well. These include 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol, **3**, a resorcinolic lipid (*13*), and 5-ethoxy-sorgoleone, **4** (*15*). All of these compounds have the same aliphatic side chain as **1** and differ in the substituents on the aromatic ring. A quinone compound with different ring substituents and no side chain has also been isolated from the roots (*27*).

In addition to analogues with different substitutions on the quinone moiety of **1**, compounds with differing numbers of carbon atoms and double bonds in the aliphatic side chain have been isolated as well. Netzly et al. (*8*) isolated **1**, **2**, and three other quinone compounds by HPLC. High-resolution mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) confirmed that these other compounds had the same *p*-quinone moiety as sorgoleone but different numbers of double bonds or carbon atoms in the aliphatic side chain (*8*). The following compounds were therefore referred to collectively as sorgoleones: sorgoleone-358 (**1**); sorgoleone-360, with two double bonds in the C_{15} side chain; sorgoleone-362, with one double bond in the C_{15} side chain; and sorgoleone-386, with three double bonds in a C_{17} side chain (*8*). However, the exact location of the double bonds was not determined in that study because the amounts isolated were too small for ^{13}C NMR analysis. More recently, Erickson et al. (*28*) found that GC-MS analysis of sorghum root extract yielded a peak having a mass spectrum that contained molecular ions not only at 358, as expected for **1**, but also at 359, 360, 362, 363, 364, 365, and 366, suggesting the presence of sorgoleone-like compounds with different degrees of unsaturation in the side chain. None of these sorgoleone-like compounds, however, was structurally characterized.

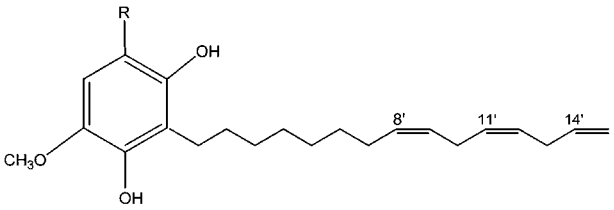
In this study, we performed a scaled-up purification of crude root extract on a C_8 column that enabled the separation and

* Corresponding author [telephone (662) 915-1247; fax (662) 915-1035; e-mail ikagan@olemiss.edu].

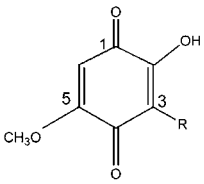
10.1021/jf034789j
Published on Web 11/05/2003



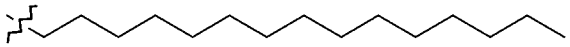
1 Sorgoleone-358; R=OCH_3
4 5-Ethoxy-sorgoleone; R=OCH_2CH_3



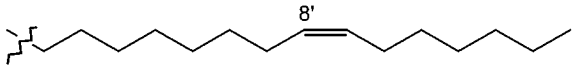
2 Sorghum xenognosin for Striga germination (SXSg); R=OH
3 4,6-dimethoxy-2-[(8'Z, 11'Z)-8', 11', 14'-pentadecatriene]resorcinol, R=OCH_3



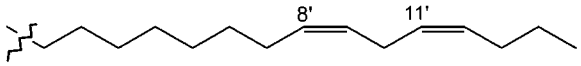
5 Sorgoleone-364, R=



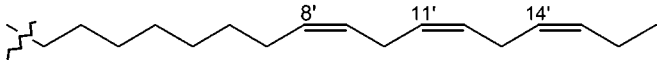
6 Sorgoleone-362, R=

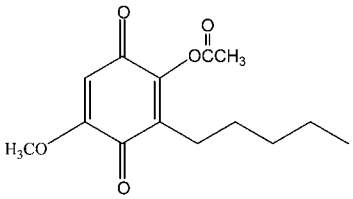


7 Sorgoleone-360, R=



8 Sorgoleone-386, R=





9 2-acetoxy-5-methoxy-3-(pent-1-yl)-1,4-benzoquinone

Figure 1. Previously characterized compounds (**1**–**4**) from *S. bicolor*, analogues isolated in this study (**5**–**8**), and a synthetic analogue (**9**) used by Barbosa et al. (*19*).

isolation of reasonable amounts of the sorgoleone congeners reported by Netzly et al. (*8*), thus enabling their ^{13}C NMR characterization. A congener previously isolated from *Maesa lanceolata* (*26*) and synthesized (*19*) was also isolated by the C_8 column chromatographic method. A rapid small-scale method of separating the congeners by argentation thin-layer chromatography (TLC) was developed as well. The herbicidal activity of several of these congeners was evaluated by comparing the inhibition of PSII-driven oxygen evolution to the inhibitory properties of the major sorgoleone.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Seeds of the sorghum cultivar SX17 (*S. bicolor* × *S. sudanense*), surface-sterilized by soaking for 10 min in 10% bleach and rinsing with tap and deionized water, were grown in the dark on a 63 × 35 cm capillary mat system, as described by Czarnota et al. (*20*), except that the heating element was omitted, and seeds were placed directly on the screen. For small-scale plantings (~300 cm^2, or one-seventh the area of large-scale plantings), used to verify the presence of the sorgoleone congeners in fresh tissue, the same capillary materials were used, but seeds were distributed over a wire screen held in place by a no. 8 plastic embroidery hoop, with the screen side down and raised slightly with Pasteur pipets. The seedlings were grown inside a square plastic 4.9-L closed container (Rubbermaid), and no weight was applied over the seeds. For both large- and small-scale plantings, roots were harvested 5−6 days after planting by excision from below the screen with a razor blade.

Extraction. Roots were immersed in $CHCl_3$ or CH_2Cl_2 for 1−5 min. These solvents had been found in previous studies to extract sorgoleone efficiently (*15*, *16*). Although extraction protocols in previous studies usually consisted of $CHCl_3$ or CH_2Cl_2 plus 0.25% glacial acetic acid (*7*−*9*, *13*−*18*), the acetic acid was omitted, as satisfactory yields of sorgoleone could be obtained with $CHCl_3$ or CH_2Cl_2 alone (*29*). The extract was decanted through a fluted glass funnel lined with Whatman no. 1 filter paper to remove root debris. To obtain higher yields of

sorgoleones, roots were immersed in solvent twice. The crude extract was concentrated under reduced pressure at 30 °C, dried under a stream of nitrogen, and stored at −20 °C.

Separation by Column Chromatography. The crude extract, redissolved in acetonitrile, was filtered through a 0.2 μm nylon filter (Gelman), and 1.0 g was loaded onto a 37 × 4.4 cm i.d., 40−63 μm, Lichroprep RP-8 column (Merck, Darmstadt, Germany) connected to an Altex model 110A pump. Compounds, which were visible on the column as yellow and brown bands, were eluted at a flow rate of 2 mL/min, using a stepwise increase in solvent system polarity as follows: step A, 100 mL of acetonitrile/2.5% aqueous acetic acid (60:40, v/v); step B, 150 mL of acetonitrile/2.5% aqueous acetic acid (65:35, v/v); step C, 225 mL of acetonitrile/2.5% aqueous acetic acid (70:30, v/v); step D, 1725 mL of acetonitrile/2.5% aqueous acetic acid (75:25, v/v); step E, 175 mL of acetonitrile/2.5% aqueous acetic acid (77:23, v/v); step F, 150 mL of acetonitrile/2.5% aqueous acetic acid (79:21, v/v); step G, 150 mL of acetonitrile/2.5% aqueous acetic acid (81:19, v/v); step H, 150 mL of acetonitrile/2.5% aqueous acetic acid (85:15, v/v); step J, 400 mL of acetonitrile/2.5% aqueous acetic acid (90:10, v/v); step K, 200 mL of acetonitrile/2.5% aqueous acetic acid (95:5, v/v); and step L, 400 mL of 100% acetonitrile. Compound **1** was eluted in step D; the compound corresponding to sorgoleone-360 was eluted in step G; the compound corresponding to sorgoleone-362 was eluted in step J; and the compound corresponding to sorgoleone-364 was eluted in step L. The identity of **1**, the most abundant band in the extract, was verified by comparing its R_f value on silica TLC (CH_2Cl_2/MeOH 98:2, v/v) to that of a standard previously isolated from sorghum root extracts and spectroscopically characterized. Fractions from the elution of the same band (corresponding to one congener) were pooled and concentrated under reduced pressure at 25−30 °C to remove the acetonitrile. If crystals formed in the resulting aqueous phase, they were recovered by removing the mother liquor. If no crystals formed upon removal of acetonitrile, the aqueous samples were partitioned twice with CH_2Cl_2 or $CHCl_3$. The pooled organic phases were dried over magnesium sulfate, concentrated under reduced pressure at 25−30 °C, transferred to 4-mL screwcap vials, and dried under nitrogen. All samples were stored at −20 °C.

Preparative TLC. To obtain sorgoleones for argentation TLC, crude extract (30−40 mg) was separated on 10 × 20 cm, 1 mm thick silica F_{254} glass-backed preparative TLC plates (Analtech, Newark, DE), developed twice in hexane/2-propanol (9:1, v/v). The sorgoleones (R_f = 0.35) were eluted with $CHCl_3$/MeOH (19:1, v/v). Eluates were concentrated, dried, and stored, as described above, until separated by argentation TLC.

Crystallization of Sorgoleone. To improve yields of the minor sorgoleone congeners, the band of sorgoleones eluted from preparative TLC plates was sometimes crystallized four times from hexanes. Only **1**, the major component of the band, crystallized. The mother liquor from the fourth crop, consequently enriched in the minor congeners, was concentrated and separated by argentation TLC.

Argentation TLC. Aluminum-backed 10 × 20 cm, 0.2 mm thick silica F_{254} plates (EM Science, Gibbstown, NJ) were washed in EtOAc/MeOH (9:1, v/v) and activated at 80 °C (20−30 min) or 62 °C (30−35 min) to improve adsorption of $AgNO_3$ onto the silica. Plates were impregnated with $AgNO_3$ according to the method of Mahadevan (*30*), by development in a TLC tank containing a saturated (5%) solution of $AgNO_3$ in MeOH/water (95:5, v/v). Plates were developed twice in this solution, after which the $AgNO_3$ had migrated 8−8.5 cm from the bottom edge of the plate, as determined by the presence of a black zone under short-wave UV light. Plates were air-dried for 1−16 h and then oven-dried at 70−80 °C for 2.5−3 h.

Samples (10−25 mg) of sorgoleones, eluted from preparative silica TLC plates and sometimes recovered as the mother liquor from crystallization of **1**, were dissolved in 100−500 μL of CH_2Cl_2 and spotted across the origin of a $AgNO_3$-impregnated plate with a syringe. Plates were developed twice in hexane/acetone/CH_2Cl_2 (7:2:1, v/v/v). This separation, which was improved if the TLC chamber was not saturated with solvent vapors, yielded bands A−F (**Figure 2**), with average R_f values of 0.60, 0.54, 0.44, 0.35, 0.34, and 0.25, respectively. Bands were scraped individually from the plate, eluted with $CHCl_3$, concentrated, and dried as described previously. Bands A−C were then loaded separately to purify them. Bands D and E, which migrated closely together, were scraped together with part of band F. The eluate from those combined bands was then respotted on a 5% $AgNO_3$ plate in order to isolate the bands individually, and the bands were separated using hexane/acetone/CH_2Cl_2 (85:10:5, v/v/v) followed by hexane/acetone/CH_2Cl_2 (7:2:1, v/v/v).

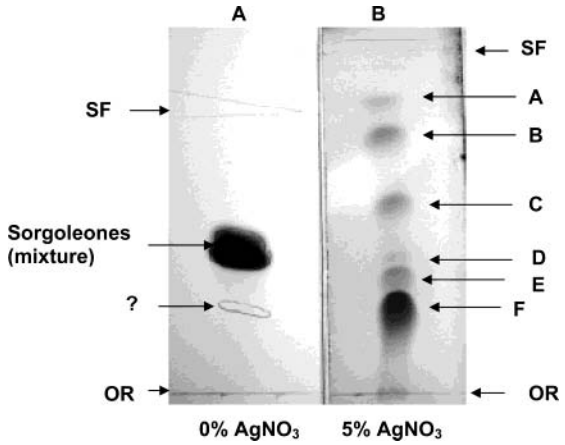


Figure 2. Thin-layer chromatograms of sorgoleones on (**A**) normal silica (0% $AgNO_3$) and (**B**) silica impregnated with 5% silver nitrate, developed in hexane/acetone/methylene chloride (7:2:1, v/v/v). On normal silica, the sorgoleones migrated as a single band, plus a minor unknown compound seen under short-wave UV light. On 5% $AgNO_3$, the sorgoleones separated into bands A (sorgoleone-364, **5**), B (sorgoleone-362, **6**), C (sorgoleone-360, **7**), D (unidentified), E (sorgoleone-386, **8**), and F (sorgoleone-358, **1**). The origin (OR) and solvent front (SF) are shown on each chromatogram.

NMR Analysis. ^1H, ^{13}C, and two-dimensional nuclear magnetic resonance (NMR) experiments were carried out in $CDCl_3$ on a Bruker Avance DPX 300 (Bruker, Billerica, MA) instrument using standard Bruker software (XWINNMR version 1.3).

The molecular weights of **5**−**8** were determined by gas chromatography−mass spectrometry (GC-MS) performed on a JEOL (JEOL USA, Inc., Peabody, MA) GCMate II system. The GC temperature program was as follows: initial, 120 °C; raised to 280 °C at a rate of 20 °C /min; held at this temperature for 1 min; then raised to 300 °C at a rate of 10 °C /min and held at this temperature for 4 min. The GC capillary column used was a 30 × 0.25 mm i.d., 0.25 μm DB-5 (J&W Scientific, Inc., Folsom, CA). The carrier gas was ultrahigh-purity helium (NexAir, Batesville, MS), at a 1 mL/min flow rate. The inlet (splitless), GC interface, and ion chamber temperatures were 250, 250, and 230 °C, respectively. The volume of sample injected was 1 μL.

Ultraviolet (UV) Analysis. UV spectra in EtOH were determined on a Shimadzu UV-3101PC dual-beam spectrophotometer. Twenty-five or 50 μL of a 1 mg/mL stock solution (also in EtOH) was diluted to 3 mL in a quartz cuvette and scanned from 200 to 700 nm.

Preparation of Spinach Thylakoid Membranes. The oxygen evolution assay was performed on spinach thylakoid membranes, prepared as described by Rimando et al. (*15*) except that after the initial homogenization, the filtrate was centrifuged for 10 min instead of 5 min at 4 °C and 1100*g*. The membrane preparation was diluted to a chlorophyll concentration of 4 mg/mL and stored at −80 °C. Because the preparation had high oxygen-evolving activity, it was diluted in resuspension buffer for the assays, to a final chlorophyll concentration of 1.5−1.6 mg/mL.

Oxygen Evolution Assays. Assays were performed at 30 °C with a computer-controlled oxygen electrode (Hansatech Instruments, Norfolk, U.K.). A fiber-optic light source (Fiberoptic Specialties, Inc., Palmetto, FL) provided saturating light (2400 μmol/m^2/s), and a water circulator (Neslab RTE-101, Newington, NH) was connected to the oxygen electrode cell to maintain a temperature of 30 °C throughout the assay. The assay protocol was as described by Rimando et al. (*15*) except that decyl plastoquinone was omitted from the assay buffer, and the rate of oxygen evolution was measured for 25 s over the linear portion

of the curve. Compounds were stored as 33 mM stocks in 100% EtOH at 4 °C. Dilutions, prepared in EtOH, ranged from 0.1 to 10 mM and gave final assay concentrations ranging from 0.01 to 3 μM. Dose−response curves were fitted to a four-parameter logistic function used previously for the analysis of oxygen evolution assays (*31*)

$$f = c + \frac{d - c}{1 + e^{b(\ln(x) - \ln(I_{50}))}}$$

where d = maximum amount of oxygen evolved, in μmol/mL/min, c = minimum amount of oxygen evolved, and b = slope (x-axis, amount of oxygen evolved, versus y-axis, inhibitor concentration). The I_{50} values were calculated from the regressions.

RESULTS

Isolation of Sorgoleone and Analogues by C_8 Column Chromatography. Compound **1** was immediately identified in C_8 column eluates by its abundance and comigration with a sorgoleone standard on normal silica TLC plates developed in CH_2Cl_2/MeOH. Its structure was confirmed by ^1H and ^{13}C NMR. Other bands were eluted from the C_8 column, including some that crystallized, such as **6** and **7**. Despite the chromatographic separation obtained on the C_8 column, all fractions comigrated with a sorgoleone standard on silica TLC in CH_2Cl_2/MeOH (98:2, v/v), $CHCl_3$/MeOH (99:1, v/v), or hexane/iPrOH (9:1, v/v).

Isolation of Sorgoleone and Analogues by Argentation TLC. Although compounds **1**, **5**, **6**, and **7** were eluted separately and isolated by C_8 column chromatography, some C_8 column fractions were shown by NMR analysis to be mixtures of compounds having the same quinone moiety as **1** but different numbers of double bonds in the 3-alkyl side chain. Therefore, these sorgoleone congeners were separated by argentation TLC. This technique of separation, often used to separate lipids, fatty acids, and lipophilic resorcinols differing in numbers of double bonds (*30*, *32*, *33*), is based on the interaction of Ag^+ ions with π-electrons in double bonds (*32*). The mixtures from the C_8 column were separated effectively (not shown). However, due to concern that these compounds, as well as the pure compounds obtained from the column, could be artifacts, the argentation TLC was used primarily to separate the congeners in more recent extracts of sorgoleones and compare their structures to those obtained from the C_8 column. The sorgoleones, eluted as a single band from preparative TLC of crude extract, separated into six bands (A−F) on $AgNO_3$-impregnated plates (**Figure 2**). TLC of a 1-week-old extract from a small-scale sorghum planting yielded the same pattern and relative intensities of bands as TLC of 3−8-month-old extracts, indicating that the bands represented naturally occurring compounds and not degradation products.

Bands A, B, C, E, and F, obtained by argentation TLC, were structurally characterized by NMR as 2-hydroxy-5-methoxy-3-pentadecyl-*p*-benzoquinone (sorgoleone-364, **5**) (**Figure 1**), 2-hydroxy-5-methoxy-3-[8′-pentadecene]-*p*-benzoquinone (sorgoleone-362, **6**), 2-hydroxy-5-methoxy-3-[8′,11′-pentadecadiene]-*p*-benzoquinone (sorgoleone-360, **7**), 2-hydroxy-5-methoxy-3-[8′,11′,14′-heptadecatriene]-*p*-benzoquinone (sorgoleone-386, **8**), and **1**, respectively. The ^1H and ^{13}C NMR chemical shifts of **5**−**8** are given in **Tables 1** and **2**, respectively. The configurations at the double bonds were not determined. Netzly et al. (*8*) reported sorgoleone-360, sorgoleone-362, and sorgoleone-386 as analogues having C15:2, C-15:1, and C17:3 aliphatic side chains, respectively, without specifying the position of the double bonds. In this study, we were able to determine the position of the double bonds, which confirmed that all congeners had a terminal methyl group in the aliphatic chain, as opposed to a terminal methylene group present in the aliphatic chain of **1** (**Tables 1** and **2**). The presence of a methyl group was shown from both ^1H and ^{13}C NMR studies. The assignment of the one double bond at position 8 in the C_{15} aliphatic chain of **6** was determined from a two-dimensional (heteronuclear multiple bond correlation, HMBC) NMR experiment (**Figure 3**). Due to minute sample quantity, not all expected cross-peaks appeared in the spectrum. However, significant correlation peaks to establish the position of the double bond at C-8 were observed, namely, between H-7′ and C-5′, between H-7′ and C-9′, between H-8′ and C-6′, between H-8′ and C-10′, between H-9′ and C-7′, between H-9′ and C-11′, between H-10′ and C-8′, and between H-10′ and C-12′. Similarly, the position of the three double bonds at C-8, C-11, and C-14 in the C-17 aliphatic chain of **8** was also determined from an HMBC experiment. Significant correlation peaks appeared in the spectrum to establish the position of the double bonds, namely, between H-7′ and C-5′, between H-7′ and C-9′,

Table 1. ^1H NMR Data for Compounds **5**–**8**[a]

^1H no.	sorgoleone-364 (**5**)	sorgoleone-362 (**6**)	sorgoleone-360 (**7**)	sorgoleone-386 (**8**)
6	5.83 (s)	5.83 (s)	5.83 (s)	5.83 (s)
1′	2.43 (dd, 7.6, 7.5)	2.43 (dd, 7.6, 7.5)	2.42 (dd, 7.6, 7.5)	2.40 (dd, 7.7, 7.5)
2′	1.42 (m)	1.44 (m)	1.44 (m)	1.44 (m)
3′	1.24 (m)	1.28 (m)	1.29–1.41 (m)	1.24–1.29 (m)
4′	1.24 (m)	1.28 (m)	1.29–1.41 (m)	1.24–1.29 (m)
5′	1.24 (m)	1.28 (m)	1.29–1.41 (m)	1.24–1.29 (m)
6′	1.24 (m)	1.28 (m)	1.29–1.41 (m)	1.24–1.29 (m)
7′	1.24 (m)	1.98–2.0 (m)	2.01–2.04 (m)	2.02–2.11 (m)
8′	1.24 (m)	5.31–5.36 (m)	5.34 (m)	5.30–5.38 (m)
9′	1.24 (m)	5.31–5.36 (m)	5.34 (m)	5.30–5.38 (m)
10′	1.24 (m)	1.98–2.0 (m)	2.76 (m)	2.77–2.81 (m)
11′	1.24 (m)	1.28 (m)	5.34 (m)	5.30–5.38 (m)
12′	1.24 (m)	1.28 (m)	5.34 (m)	5.30–5.38 (m)
13′	1.24 (m)	1.28 (m)	2.01–2.04 (m)	2.77–2.81 (m)
14′	1.24 (m)	1.28 (m)	2.01–2.04 (m)	5.30–5.38 (m)
15′	0.87 (t, 7.6)	0.87 (t, 7.6)	0.89 (t, 7.5)	5.30–5.38 (m)
16′				2.02–2.11 (m)
17′				0.96 (t, 7.5)
OCH_3	3.85 (s)	3.85 (s)	3.85 (s)	3.85 (s)

[a] δ_H (m, J in hertz) relative to $CDCl_3$; recorded at 300 MHz.

Table 2. Chemical Shifts of the Carbons of the Aliphatic Chain of Sorgoleone Congeners[a]

carbon no.	sorgoleone-364 (**5**)	sorgoleone-362 (**6**)	sorgoleone-360 (**7**)	sorgoleone-386 (**8**)
1′	22.6[b]	23.0[d]	23.0	23.0
2′	28.0	28.4	28.4	28.3
3′	29.3	29.3	29.9	29.9
4′	29.6[c]	29.9	30.0	30.0
5′	29.6[c]	29.6	29.60	29.6
6′	29.6[c]	30.1[e]	29.69[h]	29.7
7′	29.6[c]	27.6[f]	27.61	27.6
8′	29.6[c]	130.2[g]	130.5	130.7
9′	29.6[c]	130.2[g]	128.5	128.6[i]
10′	29.6[c]	27.6[f]	26.0	26.0
11′	29.6[c]	30.1[e]	128.3	128.1
12′	29.5	29.7	130.3	128.7[i]
13′	31.9	32.1	29.69[h]	25.9
14′	22.5[b]	23.0[d]	23.2	127.5
15′	14.2	14.4	14.1	132.3
16′				20.9
17′				14.6

[a] Values are in ppm, referenced from $CDCl_3$. Chemical shifts of the carbons of the quinone moiety are similar for all analogues, i.e., 183.1 (C-1), 182.0 (C-4), 161.5 (C-5), 151.9 (C-2), 119.6 (C-3), and 102.5 (C-6). [b] Interchangeable. [c−i] Overlapping signals.

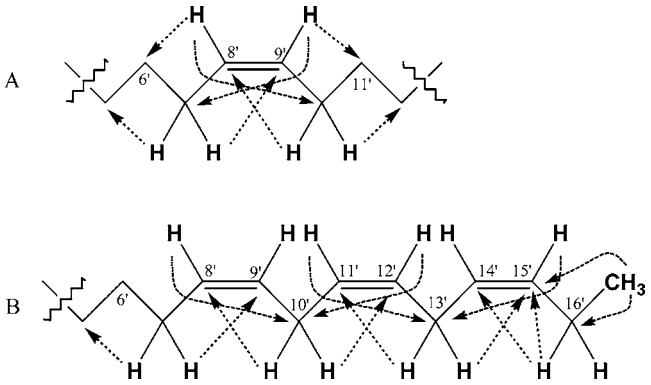


Figure 3. Partial structures of sorgoleone-362 (**A**) and sorgoleone-386 (**B**) showing correlations observed in their HMBC spectra indicating the positions of the double bonds.

between H-8′ and C-10′, between H-10′ and C-8′, between H-10′ and C-12′, between H-11′ and C-13′, between H-12′ and C-10, between H-13′ and C-11′, between H-13′ and C-15′, between H-15′ and C-13′, between H-16′ and C-14′, between H-16′ and C-15′, between H-17′ and C-15′, and between H-17′ and C-16′. The molecular weights of **6**−**8** were previously determined by high-resolution MS (*8*). The molecular weights of **5**−**8** were obtained in this study by low-resolution GC-MS, and the molecular ions of **7** (*m*/*z* 360.7, retention time = 10.48 min), **6** (*m*/*z* 362.7, retention time = 10.38 min), **5** (*m*/*z* 364.5, retention time = 10.68 min), and **8** (*m*/*z* 386.5, retention time = 11.93 min) confirmed their structures. Full structural characterization of band D was not possible because it was present in very small amounts.

The difficulty of isolating **5**−**8** by argentation TLC differed strikingly among the compounds. Four TLC experiments, using a total of 53 mg of sorgoleones, sufficed for obtaining 3−4 mg each of **6** and **7** for NMR analysis. In contrast, obtaining 3.8 mg of compound **5** required ∼380 mg of sorgoleones. The low yield of **5** (∼1%) may explain why this compound was not reported by Netzly et al. (*8*) at the time when other sorgoleones were identified and why it was isolated from the C_8 column, not in crystalline form but as part of a fraction that was subsequently TLC-purified. The yield of compound **8** (3%) was higher than that of **5**, but as **8** was difficult to separate from **1**, isolating enough for ^{13}C NMR required, again, repeated TLC purifications.

UV Spectra. In EtOH, compounds **1**, **5**, **6**, and **7** all had a single absorbance maximum at 287 nm. The extinction coefficients for these four compounds were 16000, 17000, 15000, and 18000, respectively. The UV spectrum of **8** was not obtained, as this compound was used destructively before spectrophotometric testing. The similarities of the extinction coefficients for **1**, **5**, **6**, and **7** indicated that the absorbance at 287 nm was due to the benzoquinone moiety and not to the side chain.

Assay of PSII-Inhibitory Properties. Because **1** is a known inhibitor of PSII-driven oxygen evolution (*15*, *20*), the sorgoleone congeners isolated in this study were tested for PSII-inhibitory activity by measuring their abilities to inhibit oxygen evolution in spinach thylakoid membranes, at concentrations ranging from 0.01 to 3 μM. The I_{50} value for all compounds was ∼0.1 μM (**Table 3**; **Figure 4**), indicating that the differences in length or degree of unsaturation in the 3-alkyl side chain did not affect the PSII-inhibitory properties associated with **1**. These findings are similar to those of Barbosa et al. (*19*), who found that a synthetic version of **5**, as well as **9**, a synthetic analogue of sorgoleone with a five-carbon side chain and an acetoxy group on the benzene ring, did not differ significantly from **1** in phytotoxicity toward five plant species.

Table 3. I_{50} Values of Sorgoleones in Assays of PSII Inhibition in Spinach Thylakoid Membranes[a]

	I_{50} (μmol/L of compound)	
compound	expt 1	expt 2
sorgoleone-358	0.083 ± 0.0049	0.065± 0.011
sorgoleone-360	0.104 ± 0.032	0.110 ± 0.007
sorgoleone-362	0.109 ± 0.022	0.117 ± 0.010
sorgoleone-364	0.103 ± 0.017	0.105 ± 0.013

[a] Each value represents the mean ± standard deviation of three replicates.

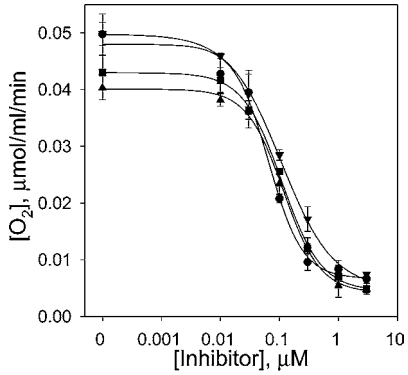


Figure 4. Effect of **1** (●), **5** (▼), **6** (▲), and **7** (■) on oxygen evolution in spinach thylakoid membranes.

DISCUSSION

Compounds **6**−**8**, the general structures of which were determined by high-resolution MS and ^1H NMR of sorghum root exudates (*8*), have now been characterized by both ^1H and ^{13}C NMR, thus establishing the exact locations of the double bonds in the aliphatic side chains of these sorgoleone congeners. In addition, compound **5**, which has been synthesized (*19*), isolated from the plant *Maesa lanceolata* (*26*), and observed in HPLC analysis of sorghum root extracts (*24*), has now been isolated from sorghum in sufficient amounts to spectroscopically characterize and verify that its structure is identical to that of the synthetic compound and the *M. lanceolata* compound.

The presence of these sorgoleone analogues in root exudates raises questions about their biological significance, particularly their potential contribution to the overall allelopathic properties of sorghum. All have similar PSII-inhibitory properties, inhibiting oxygen evolution at concentrations of ∼0.1 μM (**Table 3**), and they probably all contribute collectively to the phytotoxicity of sorghum to other plants.

The low concentrations of **5**−**8**, relative to the abundance of **1**, may reflect the nature of enzymes in the sorgoleone biosynthetic pathway. A key step is formation of the ring. This step is most likely catalyzed by a polyketide synthase (PKS) adding three malonyl-CoA units to a 16-carbon fatty acid starter unit and then cyclizing the linear tetraketide intermediate to form a 5-alkyl resorcinol with a 15-carbon side chain (*29*). The presence of sorgoleone analogues with various degrees of unsaturation in the 3-alkyl side chain suggests that the PKS can accept fatty acid substrates with different numbers of double bonds or carbons. The differing concentrations of these sorgoleone analogues in the root exudates could indicate differences in availability of the different fatty acid precursors, with the C16:3 fatty acid dominating, or differences in the affinity of the PKS for the various fatty acids. Although less likely, it is

also possible that the desaturation of the side chain occurs after the formation of **5**. In that case, the majority of **5** would be converted by desaturases into **1**, with lower desaturase activity, or premature dissociation of substrate from enzyme, yielding **6** and **7**.

The similarity of the side chain of **8** (C17:3 Δ8,11,14) to α-linolenic acid (C18:3 Δ9,12,15) (*34*) strongly suggests that **8** is formed by a PKS adding three malonyl-CoA units to C-1 of α-linolenic acid. Likewise, it is easy to consider palmitic acid (C16:0) as the starter unit for **5** and palmitoleic acid (C16:1 Δ9) (*34*) as the starter unit for **6**, which has a C15:1 Δ8 side chain. The origin of the fatty acid precursors of **1** and **7** is more difficult to hypothesize, as the double bonds are not in the usual position for C_{16} fatty acids. A C16:3 fatty acid usually has a Δ7,10,13 configuration (*34*), which would correspond to a Δ6,9,12 configuration in the side chain instead of the observed Δ8,11,14. Similarly, a C16:2 fatty acid usually has a Δ7,10 configuration (*34*), which would correspond to a Δ6,9 configuration in the side chain instead of the observed Δ8,11. If these sorgoleone congeners are indeed formed by the addition of three malonyl-CoA units to a fatty acid starter unit, sorghum root hairs may contain novel fatty acids with double bonds located at unusual positions. Alternatively, if any of these compounds are formed by desaturation of the side chain after it has bonded to the benzoquinone ring, sorghum roots may contain unusual desaturases.

With the separation of sorgoleone congeners that can serve as standards, HPLC and GC-MS protocols can more readily be developed to quantify the levels of the congeners in different stages of a biosynthetic study. Because **1** is antifungal as well as phytotoxic (*35*), it may eventually be worthwhile to investigate the presence and relative amounts of the sorgoleone congeners in fungal-infected plants. Pure standards are the first step in understanding the role that these congeners may play in nature.

LITERATURE CITED

(1) Breazeale, J. F. The injurious after-effects of sorghum. *J. Am. Soc. Agron.* **1924**, *16*, 689−700.

(2) Einhellig, F. A.; Rasmussen, J. A. Prior cropping with grain sorghum inhibits weeds. *J. Chem. Ecol.* **1989**, *15*, 951−960.

(3) Guenzi, W. D.; McCalla, T. M. Phenolic acids in oats, wheat, sorghum, and corn residues and their phytotoxicity. *Agron. J.* **1966**, *58*, 303−304.

(4) Lehle, F. R.; Putnam, A. R. Allelopathic potential of sorghum (*Sorghum bicolor*): isolation of seed germination inhibitors. *J. Chem. Ecol.* **1983**, *9*, 1223−1234.

(5) Al-Saadawi, I. S.; Al-Uqaili, J. K.; Alrubeaa, A. J.; Al-Hadithy, S. M. Allelopathic suppression of weed and nitrification by selected cultivars of *Sorghum bicolor* (L.) Moench. *J. Chem. Ecol.* **1986**, *12*, 209−219.

(6) Panasiuk, O.; Bills, D. D.; Leather, G. R. Allelopathic influence of *Sorghum bicolor* on weeds during germination and early development of seedlings. *J. Chem. Ecol.* **1986**, *12*, 1533−1543.

(7) Chang, M.; Netzly, D. H.; Butler, L. G.; Lynn, D. G. Chemical regulation of distance: characterization of the first natural host germination stimulant for *Striga asiatica*. *J. Am. Chem. Soc.* **1986**, *108*, 7858−7860.

(8) Netzly, D. H.; Riopel, J. L.; Ejeta, G.; Butler, L. G. Germination stimulants of witchweed (*Striga asiatica*) from hydrophobic root exudates of sorghum (*Sorghum bicolor*). *Weed Sci.* **1988**, *36*, 441−446.

(9) Fate, G.; Chang, M.; Lynn, D. G. Control of germination in *Striga asiatica*: chemistry of spatial definition. *Plant Physiol.* **1990**, *93*, 201−207.

(10) Hauck, C.; Müller, S.; Schildknecht, H. A germination stimulant for parasitic flowering plants from *Sorghum bicolor*, a genuine host plant. *J. Plant Physiol.* **1992**, *139*, 474−478.

(11) Hess, D. E.; Ejeta, G.; Butler, L. G. Selecting sorghum genotypes expressing a quantitative biosynthetic trait that confers resistance to *Striga*. *Phytochemistry* **1992**, *31*, 493−497.

(12) Olivier, A.; Leroux, G. D. Root development and production of a witchweed (*Striga* spp.) germination stimulant in sorghum (*Sorghum bicolor*) cultivars. *Weed Sci.* **1992**, *40*, 542−545.

(13) Fate, G. D.; Lynn, D. G. Xenognosin methylation is critical in defining the chemical potential gradient that regulates the spatial distribution in *Striga* pathogenesis. *J. Am. Chem. Soc.* **1996**, *118*, 11369−11376.

(14) Einhellig, F. A.; Souza, I. F. Phytotoxicity of sorgoleone found in grain sorghum root exudates. *J. Chem. Ecol.* **1992**, *18*, 1−11.

(15) Rimando, A. M.; Dayan, F. E.; Czarnota, M. A.; Weston, L. A.; Duke, S. O. A new photosystem II electron-transfer inhibitor from *Sorghum bicolor*. *J. Nat. Prod.* **1998**, *61*, 972−930.

(16) Netzly, D. H.; Butler, L. G. Roots of sorghum exude hydrophobic droplets containing biologically active components. *Crop Sci.* **1986**, *26*, 775−777.

(17) Nimbal, C. I.; Pedersen, J. F.; Yerkes, C. N.; Weston, L. A.; Weller, S. C. Phytotoxicity and distribution of sorgoleone in grain sorghum germplasm. *J. Agric. Food Chem.* **1996**, *44*, 1343−1347.

(18) De Souza, C. N.; de Souza, I. F.; Pasqual, M. Extração e ação de sorgoleone sobre o crescimento de plantas. *Cienc. Agrotecnol.* **1999**, *23*, 331−338.

(19) Barbosa, L. C. A.; Ferreira, M. L.; Demuner, A. J.; da Silva, A. A.; de Cassia Pereira, R. Preparation and phytotoxicity of sorgoleone analogues. *Quim. Nova* **2001**, *24*, 751−755.

(20) Czarnota, M. A.; Paul, R. N.; Dayan, F. E.; Nimbal, C. I.; Weston, L. A. Mode of action, localization of production, chemical nature, and activity of sorgoleone: a potent PSII inhibitor in *Sorghum* spp. root exudates. *Weed Technol.* **2001**, *15*, 813−825.

(21) Gonzalez, V. M.; Kazimir, J.; Nimbal, C.; Weston, L. A.; Cheniae, G. M. Inhibition of a photosystem II electron-transfer reaction by the natural product sorgoleone. *J. Agric. Food Chem.* **1997**, *45*, 1415−1421.

(22) Nimbal, C. I.; Yerkes, C. N.; Weston, L. A.; Weller, S. C. Herbicidal activity and site of action of the natural product sorgoleone. *Pestic. Biochem. Physiol.* **1996**, *54*, 1−11.

(23) Einhellig, F. A.; Rasmussen, J. A.; Hejl, A. M.; Souza, I. F. Effects of root exudate sorgoleone on photosynthesis. *J. Chem. Ecol.* **1993**, *19*, 369−375.

(24) Czarnota, M. A.; Rimando, A. M.; Weston, L. A. Evaluation of seven sorghum (*Sorghum* sp.) accessions. *J. Chem. Ecol.* **2003**, *29*, 2073−2083.

(25) Rasmussen, J. A.; Hejl, A. M.; Einhellig, F. A.; Thomas, J. A. Sorgoleone from root exudates inhibits mitochondrial functions. *J. Chem. Ecol.* **1992**, *18*, 197−207.

(26) Meazza, G.; Scheffler, B. E.; Tellez, M. R.; Rimando, A. M.; Nanayakkara, N. P. D.; Khan, I. A.; Abourashed, E. A.; Romagni, J. G.; Duke, S. O.; Dayan, F. E. The inhibitory activity of natural products on plant *p*-hydroxyphenylpyruvate dioxygenase. *Phytochemistry* **2002**, *60*, 281−288.

(27) Chang, M.; Lynn, D. G. The haustorium and the chemistry of host recognition in parasitic angiosperms. *J. Chem. Ecol.* **1986**, *12*, 561−579.

(28) Erickson, J.; Schott, D.; Reverri, T.; Muhsin, W.; Ruttledge, T. GC-MS analysis of hydrophobic root exudates of *Sorghum* and implication on the parasitic plant *Striga asiatica*. *J. Agric. Food Chem.* **2001**, *49*, 5537−5542.

(29) Dayan, F. E.; Kagan, I. A.; Rimando, A. M. Elucidation of the biosynthetic pathway of the allelochemical sorgoleone using retrobiosynthetic NMR analysis. *J. Biol. Chem.* **2003**, *278*, 28607−28611.

(30) Mahadevan, V. Thin-layer chromatography of neutral glycerides. In *Lipid Chromatographic Analysis*; Marinetti, G. V., Ed.; Dekker: New York, 1967; pp 191−203.

(31) Rimando, A. M.; Dayan, F. E.; Streibig, J. C. PSII inhibitory activity of resorcinolic lipids from *Sorghum bicolor*. *J. Nat. Prod.* **2003**, *66*, 42−45.
(32) Mangold, H. K. Thin-layer chromatography of lipids. *J. Am. Oil Chem. Soc.* **1964**, *41*, 762−773.
(33) Kozubek, A.; Tyman, J. H. P. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. *Chem. Rev.* **1999**, *99*, 1−25.
(34) Hitchcock, C.; Nichols, B. W. *Plant Lipid Biochemistry*; Academic Press: London, U.K., 1971; pp 2−13.
(35) Suzuki, Y.; Kono, Y.; Inoue, T.; Sakurai, A. A potent antifungal benzoquinone in etiolated sorghum seedlings and its metabolites. *Phytochemistry* **1998**, *47*, 997−1001.

Received for review July 16, 2003. Revised manuscript received September 23, 2003. Accepted October 2, 2003.

JF034789J