A New Photosystem II Electron Transfer Inhibitor from *Sorghum bicolor*

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Our study of the mechanism(s) by which sorgoleone (**1**) acts as a photosystem II (PS II) inhibitor led to the isolation of a new benzoquinone derivative, 2-hydroxy-5-ethoxy-3-[(*Z*,*Z*)-8′,11′,14′-pentadecatriene]-*ρ*-benzoquinone (**2**), from the root exudate of sorghum. The structure of **2**, which is being given the name 5-ethoxy-sorgoleone, was determined by spectroscopic means. A methoxy derivative (**3**) of **1** was also prepared. Both **2** and **3** caused a reduction in oxygen evolution by thylakoid membranes and induced variable chlorophyll fluorescence. These compounds, however, were less active inhibitors of PS II than **1**.

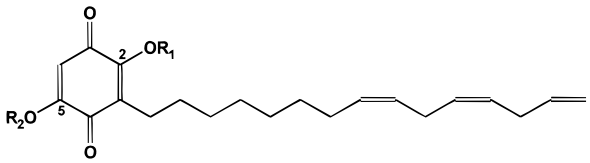
Sorgoleone refers to a group of structurally related benzoquinones having hydroxy and methoxy substitutions at positions 2 and 5, respectively, and either a 15- or 17-carbon chain with one, two, or three double bonds at position 3. These compounds were first isolated from hydrophobic root exudates of *Sorghum bicolor* (L.) Moench (Poaceae) and were distinguished from one another on the basis of their mass spectral data.[1] The name sorgoleone was first given to 5-methoxy-3-(8′*Z*,-11′*Z*)-pentadeca-8′11′14′-trienylbenzene-1,2,4-triol; however, this dihydroquinone form is unstable and is easily oxidized to the quinone, sorgoleone-358 (**1**), which is found as the major constituent of the root exudate (greater than 90%). Thus, **1** has been invariably referred to as sorgoleone. Compound **1** is highly phytotoxic, reducing growth of broadleaf and grass weeds in hydroponic assays at a concentration as low as 10 μM.[2,3]

Studies on the possible site of action of **1** have shown it to be a strong inhibitor of mitochondrial respiration in soybean and corn, blocking electron flow between the cytochrome *b* and c_1 complex.[4] Compound **1** was also found to inhibit photosynthetic electron transport by competing for the Q_B binding site of plastoquinone on photosystem II (PS II).[5−7] This mechanism of action can be investigated by monitoring the rate of photosynthetic oxygen evolution and light-induced variable chlorophyll fluorescence.

Our efforts in studying further the mechanism(s) by which **1** inhibits photosynthesis led to the identification of a new sorgoleone derivative (**2**) and the synthesis of a methylated derivative (**3**). The structural elucidation of **2** is discussed, and the in vivo and in vitro biological activities of these sorgoleone analogues are presented.

Results and Discussion

Separation of **1** and **2** was achieved by preparative layer chromatography of the crude extract of sorghum roots (see Experimental Section). Based on UV and IR spectra, the two compounds were very similar in structure. The ^{1}H NMR spectrum of **2**, however, showed a distinct difference from that of **1**; that is, the disappearance of the methoxy singlet at δ 3.85 found in **1** and the appearance of two additional resonances, a quartet centered at δ 4.03 integrating for two protons and a triplet at δ 1.49 (integrating for three protons). Coupling between these two signals (J = 7 Hz) was also observed in COSY. All other resonances in **2** were similar to those found in **1**. The ^{13}C NMR spectrum of **2** also revealed the absence of the peak corresponding to the methoxy carbon at δ 56.59 in **1**; and instead, two additional peaks were observed—one at δ 65.69 (t) and another at δ 13.69 (q), which showed correlation with the signals at δ 4.03 and 1.49, respectively, from an HMQC experiment. The presence of an ethoxy substituent at C-5 in **2** instead of a methoxy substituent became evident. This was confirmed from an HMBC experiment in which a correlation was observed between the methylene protons of the ethoxy group and C-5 of the benzoquinone ring and also between the methyl protons and the methylene carbon of the ethoxy substituent. Compound **2** is a new sorgoleone derivative, and to distinguish it from **1** it is being given the name 5-ethoxy-sorgoleone. A methoxy derivative of **1**, 2,5-dimethoxy-sorgoleone (**3**), was prepared to investigate the effect of modifying the substituent at position 2 in **1** on its activity.



1 Sorgoleone-358; R_1=OH, R_2=OCH_3
2 5-Ethoxy-sorgoleone; R_1=OH, R_2=OCH_2CH_3
3 2,5-Dimethoxy-sorgoleone; R_1=R_2=OCH_3

The phytotoxic activity of the new sorgoleone derivatives **2** and **3** was tested against lettuce seed germination and radicle elongation in a 24-well plate assay, and compared to **1** and the synthetic PS II inhibitor herbicide atrazine [6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine]. Compound **2** was not as active as **1**. Neither **1** nor any of its analogues was as active

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Table 1. Effects of **1**, **2**, and **3** on Development of Lettuce Seedlings[a]

concentration (mM)	1	2	3	atrazine
0	0	0	0	0
0.03	0	0	0	0
0.1	1	1	1	4
0.3	3	2	3	4
1	4	4	4	4

[a] Activity based on visual ratings of lettuce seedlings growth 4 d after treatment: 0 = no effect, 5 = death.

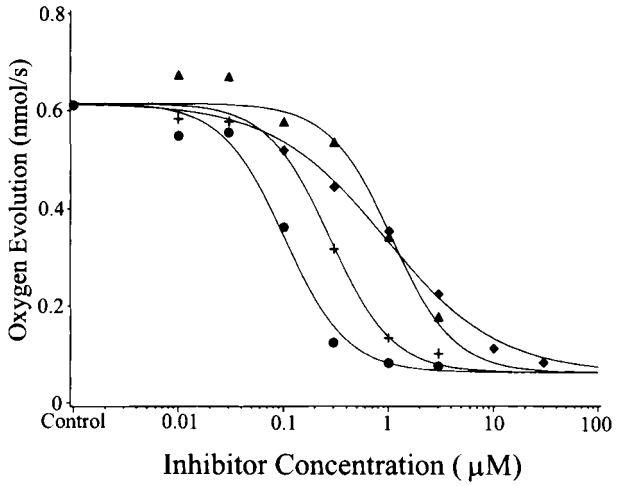

Figure 1. Effect of **1** (●), **2** (▲), **3** (+), and atrazine (◆) on photosynthetic oxygen evolution from isolated thylakoid membranes.

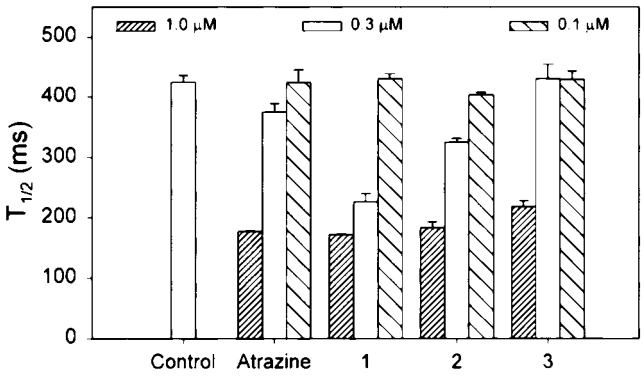

Figure 2. Light-induced $t_{1/2}$ chlorophyll fluorescence of isolated thylakoid membranes incubated with the test compounds.

as atrazine. None of the compounds was active at concentrations lower than 0.1 mM. At concentrations greater than 0.3 mM, the development of lettuce seedlings was strongly inhibited by all the sorgoleone analogues and atrazine (Table 1). The root exudates of sorghum have been previously demonstrated to be highly inhibitory to whole-plant growth[5] and to be more active on some plant species than others.[3] Compound **2** has not been previously isolated from the root exudates, and, although it is a relatively minor component of the sorghum root exudates, this compound actually may have contributed to the overall allelopathic effects observed in the field and laboratory.

The inhibitory activity of **2** and **3** on photosynthetic electron transport was evaluated to determine the effect of the changes in the functional groups of **1** (Figures 1 and 2). Both **2** and **3** were potent PS II inhibitors and efficiently reduced photosynthetic oxygen evolution, with I_{50} values of 1.07 ± 0.09 and 0.28 ± 0.02 μM, respectively. The inhibitory activity of **2** is similar to that of the synthetic PS II inhibitor atrazine (I_{50} values at 0.93 ± 0.12 μM). Both **2** and **3**, however, were less active than **1** (I_{50} values at 0.10 ± 0.01 μM) (Figure 1). The inhibition of oxygen evolution from isolated thylakoid membranes by **1** is due to its ability to competitively bind to the Q_B binding site on PS II.[6] Under such inhibitory circumstances, one would expect faster light-induced $t_{1/2}$ chlorophyll fluorescence in dark-adapted thylakoid membranes.[7] Compounds **2** and **3** had reduced $t_{1/2}$ relative to the control, but these compounds remained less active than **1** in this assay (Figure 2). It appears that **2** is more active than **3** or atrazine when potency is determined by change in $t_{1/2}$.

The structural characteristics of **1**−**3**, and the inhibitory activities associated with them, suggest that they share the same mechanism of action as other PS II inhibiting quinones.[8] The slopes of the dose−response curves for **1**−**3** (Figure 1) were also similar, suggesting similarity in the dynamics of their inhibition; however, the slope for atrazine was shallower compared with those of **1**−**3**. This difference in inhibition dynamics may be a reflection of the different mechanism of binding on PS II. Sorgoleone and atrazine compete for the same binding site on PS II,[6] but they probably interact with different amino acid residues in the binding pocket.[8] Supporting evidence for this can be found in the fact that resistance to quinone-type inhibitors is achieved by point mutation of His-215, whereas resistance to synthetic triazine inhibitors, such as atrazine, is obtained with substitutions at Ser-264.[8]

Data obtained from this study indicate that although dynamics in PS II inhibition are similar for **1**−**3**, modification of the 2-hydroxyl and 5-methoxy substituents of **1** has a negative effect on its activity. Research is currently being conducted in order to determine whether this negative effect is due to unfavorable molecular interaction at the binding site or to changes in lipophilic properties of the molecules.

Experimental Section

General Experimental Procedures. Melting point (uncorrected) of **1** was obtained using a Mel-Temp (Laboratory Devices Inc.) apparatus. UV spectra were obtained on a Shimadzu UV-3101 PC spectrophotometer. IR spectra were recorded with an ATI Mattson Genesis FT-IR instrument. Mass spectra were acquired on a Hewlett−Packard 5989A mass spectrometer using direct inlet probe. HRMS was obtained from a Bruker FT-MS, BioApex 3.0T spectrometer. NMR experiments were carried out using a Bruker Avance DPX 300 (300 MHz) instrument. HMBC experiments were performed on a Bruker Avance DRX (500 MHz) instrument.

Plant Material. *Sorghum bicolor* (L.) Moench (Poaceae), DeKalb Hybrid SX 17 (DeKalb, IL) was grown in Petri dishes as previously described.[7]

Isolation and Purification. Crude sample of **1** was obtained by dipping sorghum roots in acidified (0.25% HOAc) $CHCl_3$. Compounds **1** and **2** were isolated by direct application of the crude sample onto a preparative layer Si gel plate using hexane−*i*PrOH (90:10) as developing solvent.

Sorgoleone (1): mp 50−51 °C (lit. 37−39 °C[9]); UV,

IR, MS, and NMR data are the same as those reported in the literature.[9,10]

5-Ethoxy-sorgoleone (2): yellow amorphous powder; UV (CH_2Cl_2) λ_{max} (log ϵ) 285 (4.33), 289 (4.19), 413 (2.89) nm; IR (film) ν_{max} 3345, 3010, 2926, 2853, 1664, 1642, 1601, 1463, 1441, 1386, 1368, 1217, 1158 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.25−1.37 (8H, m, H-3′,4′,5′,6′), 1.45 (2H, m, H-2′), 1.49 (3H, t, J = 7.0 Hz, OCH_2CH_3), 2.03 (2H, m, H-7′), 2.43 (2H, t, J = 7.5 Hz, H-1′), 2.78 (2H, m, H-10′), 2.82 (2H, m, H-13′), 4.03 (2H, q, J = 7.0 Hz, OCH_2CH_3), 4.97 (1H, ddt, J = 10.1, 1.7, 1.7 Hz, H-15′a), 5.04 (1H, ddt, J = 17.1, 1.7, 1.7 Hz, H-15′b), 5.30−5.47 (4H, m, H-8′, 9′, 11′, 12′), 5.77−5.86 (1H, m, H-14′), 5.80 (1H, s, H-6), 7.26 (1H, br s, OH); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 182.88, 181.51 (each s, C-1, C-4), 160.27 (s, C-5), 151.34 (s, C-2), 136.73 (d, C-14′), 130.31 (d, C-8′), 129.21 (d, C-11′), 127.45 (d, C-9′), 126.71 (d, C-12′), 119.06 (s, C-3), 114.57 (t, C-15′), 102.30 (d, C-6), 65.69 (t, $-OCH_2CH_3$), 31.42 (t, C-13′), 29.01, 29.17, 29.39, 29.51 (each t, C-3′, C-4′, C-5′, C-6′), 27.89 (t, C-2′), 27.11 (t, C-7′), 25.47 (t, C-10′), 22.54 (t, C-1′), 13.69 (q, $-OCH_2CH_3$); HRMS MH^+ 373.2368 (calcd for $C_{23}H_{32}O_4 + H^+$ 373.2374); EIMS (70 eV) m/z 372 $[M]^+$ (14), 249 (16), 189 (41), 182 (95), 153 (100), 125 (56).

2,5-Dimethoxy-sorgoleone (3): UV (CH_2Cl_2) λ_{max} (log ϵ) 283 (3.98), 289 (3.97), 400 (2.44) nm; IR (film) ν_{max} 2863, 2848, 1644, 1601, 1454, 1309, 1287, 1198, 1141 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 1.20−1.39 (8H, m, H-3′,4′,5′,6′), 1.44 (2H, m, H-2′), 2.04 (2H, m, H-7′), 2.44 (2H, t, J = 7.5 Hz, H-1′), 2.76−2.84 (4H, m, H-10′, 13′), 3.79 (3H, s, 2-OCH_3), 3.84 (3H, s, 5-OCH_3), 4.97 (1H, ddt, J = 10.1, 1.7, 1.7 Hz, H-15′a), 5.04 (1H, ddt, J = 17.1, 1.7, 1.7 Hz, H-15′b), 5.32−5.43 (4H, m, H-8′, 9′, 11′, 12′), 5.73−5.85 (1H, m, H-14′), 5.83 (1H, s, H-6); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 182.70, 181.60 (each s, C-1, C-4), 161.08 (s, C-5), 158.70 (s, C-2), 136.75 (d, C-14′), 130.33 (d, C-8′), 129.24 (d, C-11′), 127.50 (d, C-9′), 126.73 (d, C-12′), 119.15 (s, C-3), 114.62 (t, C-15′), 102.10 (d, C-6), 61.26 (q, C2−OCH_3), 56.63 (q, C5−OCH_3), 31.44 (t, C-13′), 29.63 × 2, 29.21, 29.12 (each t, C-3′, C-4′, C-5′, C-6′), 27.95 (t, C-2′), 27.15 (t, C-7′), 25.50 (t, C-10′), 22.55 (t, C-1′); EIMS (70 eV) m/z 372 $[M]^+$ (1), 362 (10), 168 (100), 139 (30).

Methylation of 1. Compound **1** (15 mg) was dissolved in 1 mL of CH_2Cl_2 and treated with an excess (0.5 mL) of freshly prepared ethereal CH_2N_2 solution generated using a Wheaton apparatus.[11] The treated solution was allowed to stand overnight at room temperature. Compound **3** (50% yield) was purified by Si gel preparative layer chromatography using hexane−*i*PrOH (90:10) as developing solvent.

Phytotoxicity Assay. Phytotoxic activity of **2** and **3** on radicle elongation was compared to that of **1** in a 24-well plate assay, using atrazine as positive control. Lettuce seeds were placed on filter paper in the wells and treated with the test compounds. Seeds were grown in growth chamber at 25 °C with a 16-h photoperiod at 400 μmol/m^2/sec photosynthetically active radiation (PAR) for 4 days. Each treatment consisted of two replicates. All compounds were dissolved in Me_2CO and tested at final concentrations of 0.1−3 mM in a final volume of 200 μL (10% Me_2CO in H_2O) in the wells. Seedlings were rated on a scale of 0 (no symptoms) to 5 (death).

Preparation of Photosynthetic Thylakoid Membranes. Spinach leaves (*Spinacea oleracea* L.) were homogenized in ice-cold extraction buffer (330 mM sorbitol, 50 mM tricine−NaOH, 10 mM $MgCl_2$, 5 mM Na-ascorbate, 2 mM EDTA, and 0.05% bovine serum albumin, pH 7.8) and filtered through a layer of miracloth and four layers of cheesecloth. The filtrate was centrifuged at 1100 × g for 5 min at 4 °C. The chloroplast pellet was resuspended in ice-cold lysing buffer (25 mM tricine−NaOH, 10 mM $MgCl_2$, pH 7.8) and centrifuged at 3000 × g for 5 min. The pelleted thylakoid membranes were resuspended in ice-cold resuspension buffer (100 mM sorbitol, 25 mM tricine−NaOH, 10 mM $MgCl_2$, 10 mM NaCl, pH 7.8). For the oxygen evolution experiments, the thylakoid membranes were diluted to 4 mg of chlorophyll/mL according to Arnon[12] as modified by Hiscox and Israelstam.[13] For the chlorophyll fluorescence experiments, two batches of thylakoid membrane extracts were collected as described above but were used undiluted. The chlorophyll content was found to be 3.88 and 5.23 mg/mL, respectively. All thylakoid membrane extracts were used immediately or stored at −80 °C for fewer than 7 days.

Oxygen Evolution Assays. O_2 evolution was initiated under saturating light conditions (2400 μmol/m^2/sec PAR), using a fiber-optic light source and measured polarographically using a computer-controlled Hansatech DW1 oxygen probe. The reaction assay buffer consisted of 800 mM sucrose, 50 mM Mes−NaOH (pH 6.2), 15 mM $CaCl_2$, 1 mM FeCN, and 300 μM decyl plastoquinone. All assays were performed at 30 °C. The inhibitory activity of **2** and **3** was compared to that of **1** and of atrazine. Test compounds were diluted in EtOH, and control treatments received the same concentration of EtOH (less than 1% v/v). Membrane preparations were incubated with test compounds (0 to 10 μM) on ice for 20 min prior to the assay. The assay was initiated by adding the thylakoid membranes to the reaction assay buffer, and the rate of oxygen evolution was measured for 100 s over the linear portion of the curve. Nonlinear log−logistic regression model was fitted simultaneously to the response curves for the three sorgoleone species and atrazine. The slopes of the sorgoleone species were the same, whereas that of atrazine was significantly shallower. The statistical analysis has been described elsewhere.[14]

Chlorophyll Fluorescence. Thylakoid membranes (200 μg of chlorophyll/mL) were added to cold assay buffer (0.4 M sucrose, 50 mM Mes−NaOH, and 15 mM NaCl) in 1.5-mL round-bottom tubes. Test compounds were mixed into the assay and incubated in darkness for 30 min at 3−5 °C in an OptiScience suspension cuvette. Compounds **1**−**3** and atrazine were diluted in EtOH and tested at 1, 0.1, and 0.3 μM with at least three replicates at each concentration. Control treatments contained the same concentration of EtOH as treated samples (<0.01%). An OptiScience pulse-modulated fluorometer (model OS5-FL) was used to measure chlorophyll fluorescence and its decay of the dark-adapted thylakoid membranes. Flash-induced fluorescence and subsequent decay were digitally recorded. The entire experiment was duplicated using a freshly collected batch of spinach thylakoid membranes.

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