Xenognosin Methylation Is Critical in Defining the Chemical Potential Gradient That Regulates the Spatial Distribution in *Striga* Pathogenesis

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Received April 29, 1996[⊗]

Abstract: *Striga asiatica* (Scrophulariaceae) is a parasitic plant requiring a host-derived signal, xenognosin, to initiate a cascade of events necessary for the establishment of host contact. By attempting to model the distribution of the xenognosin around the host, the activity of the signal is shown to be strongly dependent on the presence of another component in the host exudate. Surprisingly this component, characterized as 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,-14′-pentadecatriene]resorcinol, is structurally related and shares the same biosynthetic pathway as the xenognosin. This compound is shown to function as an antioxidant and its ability to enhance the activity of the xenognosin is consistent with its ability to extend its lifetime in the exudate. This endogenous antioxidant activity is required to explain the spatial sensing in the establishment of the host−parasite interface and its characterization provides insight into how chemical potential may be regulated within and around plant tissues.

Introduction

Of the many biological processes that are dependent on spatial relationships, including cellular organization and trafficking, formation of differentiated tissues, and communication between distinct organisms, it is the developing interface between a parasite and its host that may be the most readily analyzed and defined chemically.[1] The successful establishment of such an interface between two distinct organisms requires precise spatial and temporal organization. For example, the seeds of the parasitic angiosperm, *Striga asiatica*, are viable in the soil for up to 20 years, but food reserves available to the embryo limits the time for host attachment to within just a few days of germination.[1−4] Without attachment, the seedling dies. Given this time limitation and the rate of seedling growth, germination must be initiated <5 mm from the host root surface.[5]

Remarkably precise control of this spatial relationship between host and parasite germination has been repeatedly documented in *Striga*.[5−7] As in other seed plants, it is germination that irreversibly commits embryonic development to a particular environment and, in this case, defines the parasite host range. Therefore, the molecular processes which define the geography of the commitment zone are of particular interest both because they establish the conceptual field of view for the parasite and represent the critical weak point in the establishment of the host-parasite interface.

Striga seeds have been shown to require, in addition to H_2O and appropriate temperature preconditioning, the exposure to a specific host-derived stimulant, xenognosin, to germinate.[2,8] The sorghum xenognosin for *Striga* germination (SXSg) was previously characterized as the only component exuded from sorghum roots capable of inducing parasite seed germination.[7] SXSg has been shown to be unstable and to decompose on standing; it was suggested that this facile decomposition could be essential to the spatial dependence of the germination of *Striga* seeds about sorghum roots.[5,7] Here we show that direct mathematical models of this distance relationship fail. This failure has now led to the characterization of a separate component, biosynthetically related to SXSg, which plays a critical role in the chemical events that govern the spatially restricted commitment of this parasite to its host.

Materials and Methods

General Methods. ^{13}C-NMR data were acquired with a 3 μs (22°) pulse width, 2 s repetition delay, and >17 000 transients. For the nOe experiment, a decoupler power of 1 db was used with a 3 s presaturation. UV spectra were acquired on a lambda V Perkin Elmer spectrophotometer. IR spectra were acquired as a film on a Nicolet spectrometer. CI^+ and EI^+ mass spectra were obtained on a VG 7070 spectrometer. Secondary ion mass spectra (SIMS) were obtained in a *m*-nitrobenzyl alcohol matrix with Ar bombardment. MS/MS analyses of ions selected from the SIMS spectra, accelerated at 8 eV and fragmentation was induced with He collision with a Finnegan TSQ 7000.

Seeds of *Sorghum bicolor* (L) Moench cv IS8768 or sudan grass hybrid 855f (∼10 g) were surface-sterilized with a 1.6% solution of sodium hypochlorite (100 mL) for 20 min and then washed three times with sterile, twice-distilled H_2O (100 mL). The seeds were transferred aseptically onto moist filter paper in 10 disposable petri dishes (100 × 15 mm), wrapped with parafilm to prevent evaporation, and grown in the dark. These seedlings were used to collect exudate[5] or for the feeding experiments. *Striga* seed pretreatment and assay were as previously described.[5]

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

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S0002-7863(96)01395-9 CCC: $12.00

Bioassay for Stabilizing Activity. Sorghum exudate (two aliquots of 50 μg) was subjected to reverse phase HPLC. Each of the minor components of the exudate was collected and added to SXSg (100 μg, also purified by HPLC). The resultant mixtures of each minor component with SXSg were evaporated to dryness *in vacuo*. Twice distilled water (1 mL) was added to each sample for assay and the contents were sonicated for 30 s to dissolve the material. An aliquot of each sample (100 μL) was added to pretreated seeds of *Striga asiatica* in twice distilled sterile water (900 μL), to give a total assay volume of 1 mL. The germination percentage was determined 24 h later.

A 1% aqueous agar solution (10 g difco bacto/1L) was prepared and autoclaved for 20 min. After cooling for 20 min, the agar solution was either poured directly into disposable petri dishes (50 mL × 20 dishes) or mixed with the required additives (e.g., methylene blue (40 mg/L, 1×10^{-4} M) and then poured and allowed to cool until gelling started. Methylene blue functioned as an effective fungicide for long-term experiments and was added to the agar. Four-day-old etiolated seedlings (cv. IS8768) were transferred to the gelling agar. Striga seeds were pipetted into the still gelling agar as a 1 cm wide swath perpendicular to the sorghum root. Distances from the root were measured with a ruler and lines parallel to the root were drawn on the petri dish at 0, 0.3, 0.5, 0.8, 1.0, and 1.5 cm. The percentages were determined by counting the number of seeds which germinated within each zone and dividing by the total number of seeds. Six replicates were typically run simultaneously and four plates are included in the averaged germination data.

Isolation of Component 1. Sorghum exudate was collected from 7-day-old germinated seedlings by dipping the entire root in methylene chloride containing 1% acetic acid for 3 s.[5,7] The solvent was removed *in vacuo* and this dried exudate (0.52 mg/g seeds) was subjected to Zorbax ODS reverse phase HPLC (HOAc/H_2O/MeOH; 0.025:4.975:95.0)[7] in aliquots of ~50 μg. **1** was collected over multiple injections, dried *in vacuo* to remove methanol, frozen, and lyophilized to dryness. Alternatively, the dried exudate was applied to a Brinkman silplate F-12 (200 μm gel thickness) as a solution in chloroform. The plate was eluted twice with pentane:diethyl ether (50:50) and **1**, R_f 0.71, was collected. Both methods gave **1** in pure form as determined by HPLC. ^{1}H-NMR ($CDCl_3$) δ 2.03, dt, 2H, J = 6.6, 6.9 Hz; δ 2.68, t, 1H, J = 7.59; δ 2.78, dd, 2H, J = 5.5, 5.9; δ 2.82, dd, 2H, J = 5.1, 5.5; δ 3.78, s, 6H; δ 4.97, dd, 1H, J = 1.7, 10.1; δ 5.03, dd, 1H, J = 1.7, 17.1; δ 5.3−5.4, m, 4H; δ 6.39, m, 1H, J = 5.5, 10.1, 17.1; δ 6.41, s, 1H). ^{13}C NMR ($CDCl_3$): δ 22.6 (C-1′), δ 25.6 (C-10′), δ 27.2 (C-7′), δ 28.0−29.6 (C-2′ to C-6′), δ 31.5 (C-13′), δ 57.0 (−OCH_3), δ 95.1 (C-5), δ 114.7 (C-15′), δ 116.4 (C-2), δ 126.8−130.4 (C-8′,9′,11′,12′), δ 136.4 (C-1, C-3), δ 136.8 (C-14′), δ 138.8 (C-4, C-6). EI$^+$ MS (70 eV) *m*/*z* (relative intensity) 374 (M^+, 57), *m*/*z* 183 ($M^+ - C_{14}H_{22}$, 57). Trimethylsilylation of **1** (bis(trimethylsilyl)trifluoroacetamide)[9] gave a molecular ion at *m*/*z* 518 (CI$^+$ (CH_4), 180 °C) consistent with the bis-silylated resorcinol.

Biosynthetic Experiments. Sorghum dabar seeds were sterilized and planted as described above but it was generally observed that the addition of compounds prior to the emergence of the radicle inhibited the germination process. In addition, low yields of exudate were isolated from seedlings <5 days old. For these reasons, excess bathing media was removed and a solution of labeled acetate (≈25 mL, 1 mM) was added on the fourth day after planting and removed and replaced daily with fresh material for 5 days. For the feeding of [$^{13}C_2$]-acetate (sodium salt) the labeled material was diluted, 1:3, to a 1 mM overall concentration. Exudate for spectral analyses was collected as previously described[7] and the percent enrichment was calculated according to the equations below.[10] Several different carbons were evaluated as standards to ensure accurate relative enrichment determinations for the monoacetate precursors. Here R is the integrated area of the resonance of interest, and St is the integrated area of another carbon not labeled in the experiment.

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$$\text{relative enrichment of resonance } R = \frac{R_{\text{labeled}}/\text{St}_{\text{labeled}}}{R_{\text{control}}/\text{St}_{\text{control}}}$$

Percent incorporation in the [$^{13}C_2$]-acetate experiment was evaluated using the following relationship.

$$\% \text{ incorporation} = \frac{\sum \text{doublet intensity}}{110(\text{singlet intensity}) + \sum \text{doublet intensity}} \times 100\%$$

(a) Haloxyfop. Haloxyfop, a gift from Dow Chemical Company, was prepared as a stock solution (1.81 mg/L, 50 μM) with twice distilled water, sterilized by membrane filtration, and stored at −4 °C. Sorghum seedlings were planted in four petri dishes on sterile filter paper and on the fourth day excess water was withdrawn and haloxyfop solutions (50, 5, and 0.5 μM, ≈10 mL) were add to three of the four plates. The plates were resealed and allowed to incubate in the dark for an additional 3 days.

(b) 2-Fluoroacetate. An aqueous stock solution of 2-fluoroacetate (21.1 mg/ 13.7 mL, 10 mM) was prepared with twice distilled water, sterilized by membrane filtration, and stored at −4 °C. Sorghum was planted in four petri dishes on sterile filter paper and on the fourth day excess water was withdrawn and replaced with fluoroacetate solutions. The plates were resealed and allowed to incubate in the dark for an additional 3 days. Initial experiments were watered with a solution of sodium acetate and 2-fluoroacetate sodium salt (10:1 and 4:1, 1 mM overall concentration) in order to avoid deleterious effects to the plants. In later experiments, sodium acetate was omitted and the concentration of 2-fluoroacetate was increased to 1 mM for quantification. This treatment could be replaced at least every 2 days with no detectable damage for over 2 weeks.

Antioxidant Activity. Polyvinyl chloride tubing (1/2 in. × 1/4 in.) was soaked overnight in saturated sodium dichromate solution (Na_2CrO_7, 60 g/500 mL of twice-distilled water, concentrated H_2SO_4, 800 mL) and finally washed thoroughly with twice-distilled water to remove organic antioxidants. Benzaldehyde and acetophenone were distilled under N_2. Granular, pale yellow sodium dithionite (100 g sodium hydrosulfite, Fischer Scientific) was purified on a fritted glass funnel by washing with distilled water (200 mL). The compound formed a very pale pink slurry and the filtrate was yellow. The slurry was quickly transferred to a round bottom flask and lyophilized to dryness *in vacuo*. Decomposition is evident by loss of the pink tint. *tert*-Amyl alcohol and azabisisobutryonitrile (AIBN) were purchased from Aldrich Chemical Company and used without further purification.

(a) Autoxidation of Benzaldehyde. Reactions initiated with AIBN (50 μL, 1.2 M, 0.606 mmoles) contained benzaldehyde (1.5 mL, 14.9 mmol), acetophenone (500 μL, 4.3 mmol), and *tert*-amyl alcohol (3.0 mL). These mixtures were maintained at 60 °C in a Thermolyne dry bath fitted with heating blocks to accommodate 16 mm diameter test tubes. Reactions were run in the test tubes with slow perfusion of air through thin hand pulled disposable glass pipetts connected with treated polyvinyl chloride tubing to house air. For inhibited reactions, the antioxidant was added as a solution in 50 μL of acetonitrile. For each data point, an aliquot (25 μL) of the reaction mixture was withdrawn and placed in a 1.5 mL Eppendorf microfuge tube and a 0.5% solution of dimethyl sulfide in acetonitrile (1 mL) was added to quench hydroperoxides present in the reaction mixture. The percent reaction was determined by HPLC on a Dupont Zorbax ODS column, eluting with acetonitrile /tetrahydrofuran/water (29/2/69, 1.0 mL/min) and calculated according to the equation below where A_b and A_a are peak areas of benzaldehyde and acetophenone, respectively. The retention times were 6.3 and 7.0 min for benzaldehyde and acetophenone, respectively.

$$\left[1 - \frac{A_b A_{ao}}{A_a A_{bo}}\right] \times 100 = \% \text{ reaction}$$

(b) Autoxidation of SXSg. The SXSg quinone was isolated from sorghum seedlings as previously described.[7] Generally 1 mg (3 μmol) was dissolved in methanol (0.1 mL) and reduced with aqueous sodium dithionite (10 mM, 600 μL, 2 equiv). The reduction occurred

immediately with a concomitant disappearance of the yellow color of the quinone. The resulting solution was acidified with acetic acid and extracted into dichloromethane. The extract was blown dry under a stream of nitrogen and dissolved in acetonitrile for addition to the reaction mixture. Resorcinol **1** was purified from sorghum root exudate as described above.

The exudate, or purified SXSg, was dissolved in twice-distilled water (100 μg in 10 mL, 30 μM, pH 6.9) containing 100 μM O_2. The concentration of molecular oxygen in solution was determined using an oxygen-sensitive electrode and represents water-saturated oxygen from the atmosphere. The reactions were conducted in a 25 mL Erlenmeyer to allow for a large liquid/air interface and the concentration of oxygen was constant during the initial rate measurements. For each time point an aliquot of the reaction mixture (250 μL) was analyzed by HPLC, Zorbax ODS reverse phase, at 420 nm as described above. At this wavelength only the quinone form absorbs and the hydroquinone was not detected. The amount of quinone formed was quantified by integrated peak areas.

Results

Reaction Model. Methylene blue was developed as a redox indicator to image the production of SXSg around sorghum roots.[5] This experiment established that SXSg was produced homogeneously along the entire root axis of the host. In addition, SXSg did not accumulate along the axis but rather the [SXSg] was found to reach a steady state and remain at that level for many days.[5] Given the instability of SXSg, such a result could be derived from two competing rates, the rate of diffusion and the rate of decay, which are equal at steady state. When the seedlings are placed in a thin (< 1 cm) agar plate this field can be approximated as a one dimensional source-sink gradient[11] and, at steady state, expressed as

$$D\frac{\partial^2[\mathrm{SXSg}]}{\partial x^2} - k[\mathrm{SXSg}] = 0 \qquad (1)$$

where D is the diffusion coefficient and k is the pseudo-first-order rate constant for the disappearance of SXSg. Rearrangement of this equation and setting

$$\alpha^2 = k/D \qquad (2)$$

gives a common differential equation

$$\frac{\mathrm{d}^2[\mathrm{SXSg}]}{\mathrm{d}x^2} - \alpha^2[\mathrm{SXSg}] = 0 \qquad (3)$$

which has the solution

$$[\mathrm{SXSg}] = A\mathrm{e}^{-\alpha x} + B\mathrm{e}^{\alpha x} \qquad (4)$$

Under boundary conditions where

$$\text{at} \quad x = 0, \quad [\mathrm{SXSg}] = [\mathrm{SXSg_0}] \qquad (5)$$

and

$$\text{as} \quad x \rightarrow \infty, \quad [\mathrm{SXSg}] \rightarrow 0 \qquad (6)$$

B must equal 0 and A can be assigned as equal to $[\mathrm{SXSg_0}]$ giving

$$[\mathrm{SXSg}]/[\mathrm{SXSg_0}] = \mathrm{e}^{-\alpha x} \qquad (7)$$

where the critical piece of information needed for the evaluation of the gradient is α.

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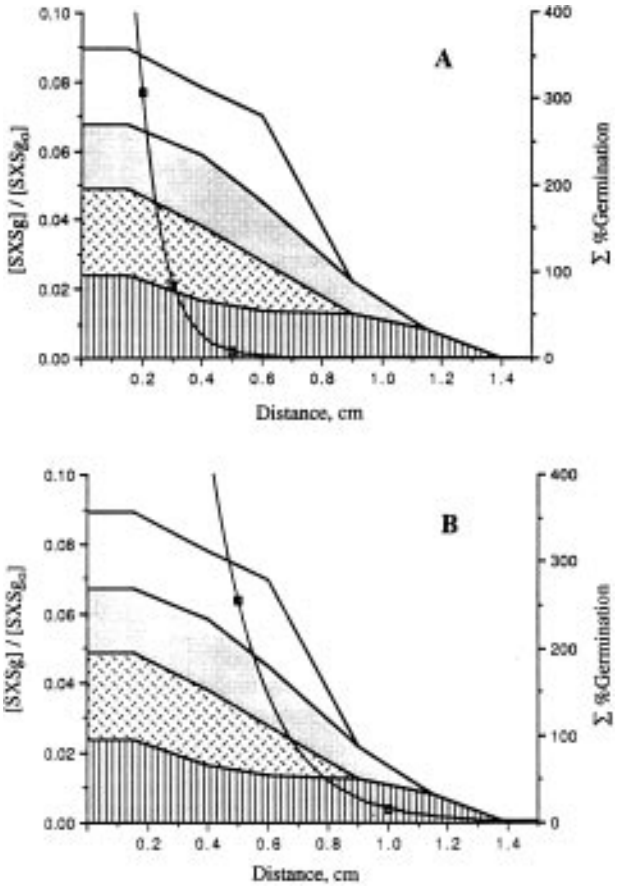

Figure 1. Superposition of four separate bar graphs generated by measuring the germination as a function of distance around a sorghum root surface. The theoretical curve is a plot of the ratio of [SXSg] to the initial concentration [$SXSg_0$] plotted as a function of distance. Panel A represents purified SXSg and panel B is SXSg within the exudate.

The diffusion coefficient, D, of SXSg in agar was determined to be 1.02×10^{-6} cm^2/s.[12] The initial rates for the decomposition of SXSg appeared first order in SXSg and the observed rate constant over three runs was $2.67 \pm 0.60 \times 10^{-5}$ s^{-1}. This value of α, 12.8, predicts the [SXSg] would disappear far faster than was indicated by the response of the *Striga* seeds distributed around the host root.[5] Four separate experiments, where seed germination was monitored as a function of distance from the host, are represented in Figure 1A. While the resolution in these experiments is limited by the density and distribution of the *Striga* seeds around the host, the predicted [SXSg] was clearly not consistent with the observed response.

When the same decay rate determinations were made on the initially collected exudate, the rate of decomposition of SXSg, $1.69 \pm 0.61 \times 10^{-6}$ s^{-1}, was significantly slower. An extended exposure time to SXSg was previously shown to be required to induce germination.[5] This reduced rate of decomposition in the exudate appears to explain two previous observations: (i) that purified SXSg showed a higher ED_{50} than the [SXSg] estimated to be in the exudate and (ii) that multiple additions of SXSg made over the assay period reduced the ED_{50} to that estimated for the exudate.[5]

The theoretical concentration curve for the exudate (Figure 1B), using the much lower value of α, 5.2, and a saturating concentration of SXSg in H_2O of 3×10^{-4} M, predicts that the [SXSg] would be 28 μM at 0.45 cm from the root surface. This concentration of SXSg typically gives 90% germination *in vitro* and a high germination rate is observed in the agar plates. At

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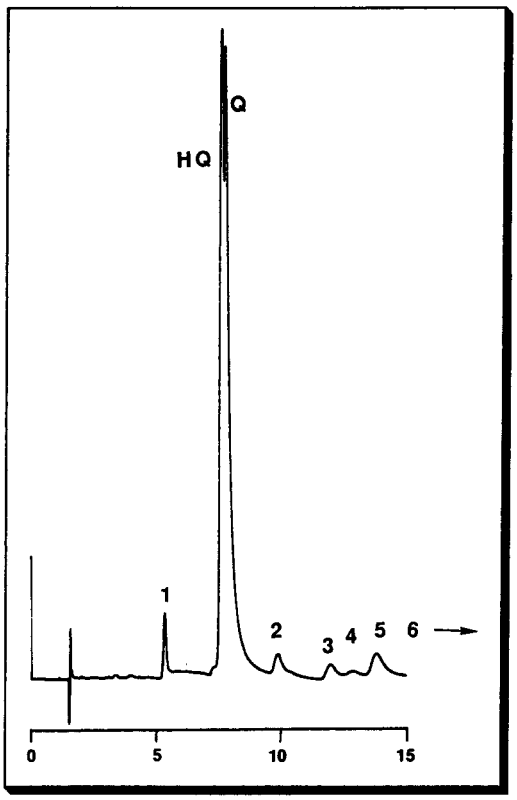


Figure 2. Preparative HPLC of sorghum exudate. HQ and Q here represent SXSg and its quinone form, respectively.

0.9 cm, the concentration would be 2.7 μM, approximatly the ED_{50} of SXSg. Further support for this predicted concentration range came from direct measurements of the [SXSg] in the agar; $\sim 10^{-7}$ M was found in the zone between 1 and 1.5 cm from the host root.[5] Therefore, both the biological response of the seeds and the measured concentrations of SXSg around the host root are consistent with the proposed model. The model predicts that another component of the exudate plays a critical role in the distribution of SXSg around the host.

Analysis of Sorghum Exudate. The root exudate consists primarily of a 1:1 mixture of SXSg and its quinone, together amounting to ~90% of the mixture. Further analysis by HPLC revealed the presence of at least five minor components (Figure 2). Each of these was collected and assayed with a sub-optimal [SXSg] for the induction of germination. Only the early eluting component, **1**, when added with purified SXSg, induced germination (Table 1). Passage through the HPLC had no

Table 1. Bioassay of the Minor Components of Sorghum Root Exudate for the Ability To Enhance the Germination Activity of SXSg

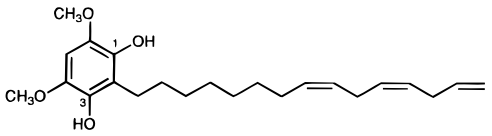
component added to SXSg	% germination[a,b]	component added to SXSg	% germination[a,b]
1	92 ± 5	5	0
2	0	6	0
3	0	1, 2, 3, 4, 5, 6[c]	89 ± 6
4	0		

[a] Each assay represents the average of five replicates. A seed is said to have germinated when a viable radicle protrudes from the seed coat. [b] Errors represent the standard deviation of values obtained from replicates. [c] Assay represents root exudate subjected to HPLC and collected in total.

significant effect on the activity as shown by the combined samples in the last table entry. Under typical assay conditions, the minor components were <1 μg/mL. None of these components assayed in that range induced germination and purified **1** (see below) showed no activity at concentrations of 10, 1, and 0.1 μg/mL. Therefore, component **1** does not induce germination but rather functions to enhance the activity of SXSg.

Purification and Characterization of Component 1. Component **1** was purified preparatively from Sudan Grass Hybrid using the developed analytical HPLC conditions. The material was found to be unstable under these conditions and subsequent isolations greatly benefited by collection under a N_2 atmosphere. The accumulated material gave ^{1}H-NMR and ^{13}C-NMR spectra that were remarkably similar to SXSg. The alkene side chains were identical. The assignment of the aromatic nucleus of **1** was supported by an intense electronic absorption with a maximum at 283 nm (log ϵ 4.0). The ring system was heavily substituted showing a single proton resonance (δ 6.41) and four oxygen bearing ring carbons present as two sets of equivalent signals. Two equivalent aromatic methoxy groups (δ 57.0) were assigned to one set and the IR spectrum, acquired as a film, suggested intramolecularly bonded hydroxyl groups, ν(O−H) at 3500 cm^{-1}, be assigned to the other. The regiochemistry of the methoxy groups (δ 3.78, 6H) was established by a strong 36% nuclear Overhauser enhancement of the single aromatic ring proton. This sets the position of the equivalent methoxyl groups adjacent to the one unsubstituted aromatic position.

Secondary ion mass spectra (SIMS) analyses gave the final confirmation of the structure for component **1**. In a *m*-nitrobenzyl alcohol matrix with argon as the bombardment gas, component **1** gives a base peak at *m*/*z* 375 $[M + H]^+$ and a weak adduct ion (12%) at *m*/*z* 396 $[M + H + Na]^+$. SXSg, under these conditions, gives a similar spectrum, *m*/*z* 361 (28%, $[M + H]^+$) and a base peak at *m*/*z* 382 $[M + H + Na]^+$, each mass 14 amu less than component **1**. Both compounds undergo a characteristic retro-ene fragmentation to give ions at *m*/*z* 183 (15%) and *m*/*z* 169 (15%), respectively, consistent with the phenolic OH being adjacent to the side chain in **1**. Interestingly, these initial retro-ene fragments, when collisionally decomposed (He) to yield daughter ion spectra (Figure 3), show somewhat different fragmentation patterns. The major difference is the loss of 29 and 28 mass units from **1** and SXSg, respectively. A mechanism has been proposed which attributes this difference to different retro-ene fragments and the corresponding loss of CO or CHO (Figure 4). These data establish the structure of **1** as 4,6-dimethoxy-2-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]resorcinol. The similarity of this structure to SXSg suggest that it is likely to originate biosynthetically via methylation of the xenognosin.



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Biosynthetic Studies. The aromatic nucleus of SXSg could in principle be synthesized via the shikimate or polyketide pathways and biosynthetic experiments were designed to distinguish between them. [1-^{13}C]-Acetate was clearly incorporated into positions C_1 and C_5 of the aromatic moiety (Figure 5). [2-^{13}C]-Acetate showed comparative enrichments, $C_6 \approx C_4 > C_2$, supporting the polyketide biosynthesis pathway. Surprisingly, very little enrichment of C_3 was detected in the [1-^{13}C]-acetate feeding and no detectable label was found in the other carbons of the side chain.

The polyketide pathway requires both cyclization of an acyclic precursor and decarboxylation to give the odd-numbered C_{21}-carbon chain of SXSg. An assignment of the position of decarboxylation, C_2 vs C_4, would define the sense of the

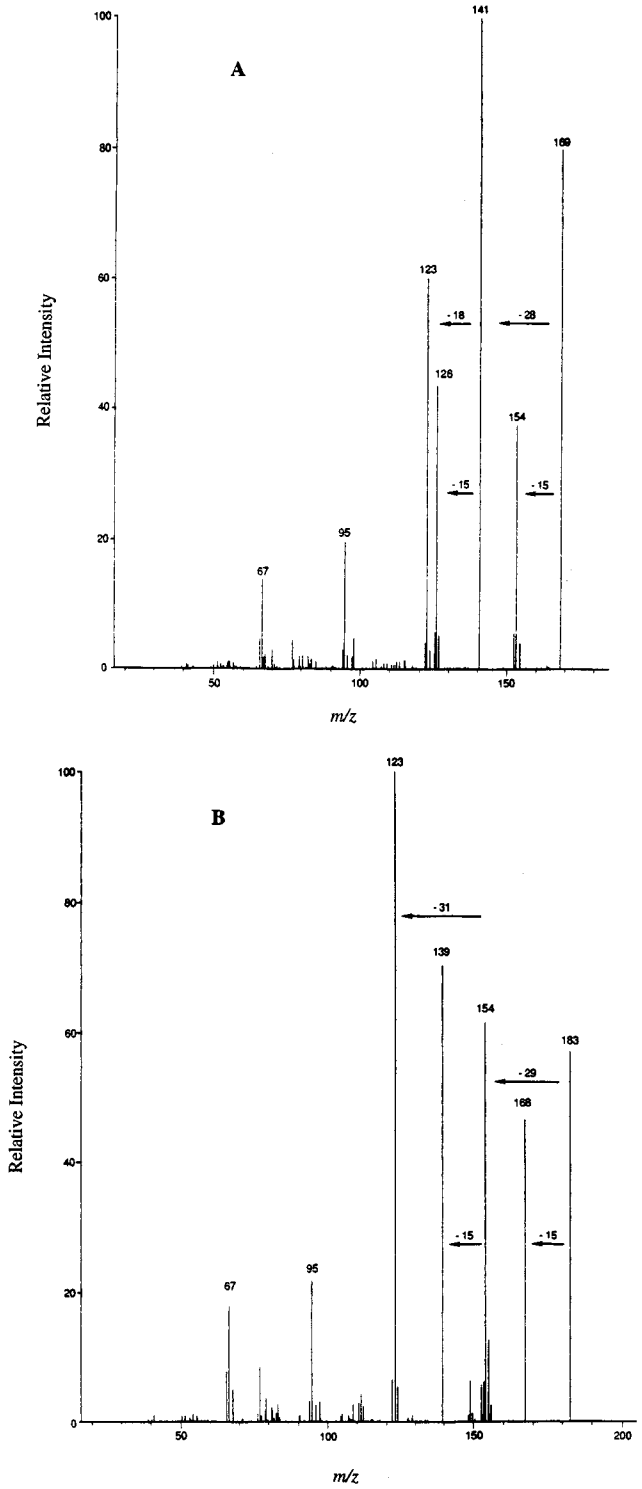

Figure 3. Daughter ion analyses (Ar collision) of the retro-ene fragments from the SIMS spectra of SXSg, panel A, and **1**, panel B.

cyclization (Figure 6). Bond incorporation from [$^{13}C_2$]-acetate was significant under the feeding conditions, 0.60% (C_1), 0.73% (C_6), 0.88% (C_4), 0.90% (C_5), with a 2.34 relative enrichment of the singlet at C_2 in the oxidized quinone. No coupling satellites were detected for C_2 and the one-bond constants, $^1J_{C1-6} = 63$ Hz and $^1J_{C4-5} = 55$ Hz, clearly established the counterclockwise nature of the cyclization. Again in this spectrum, there was no coupling detected in the carbons of the side chain, indicative of different rates of incorporation, arguing that the quinone might be biosynthetically added onto a pre-existing (16-carbon) fatty acid.

The resorcinol, **1**, was also purified from these experiments and found to have labeling patterns and enrichments identical to that seen for SXSg. In addition, two inhibitors, haloxyfop, an inhibitor of acetyl-CoA carboxylase (ACC),[13] and fluoroacetate, were investigated for their ability to modulate the production of the exudate in two varieties, sorghum sudan grass hybrid and Sorghum bicolor (L.) moench cv. IS8768. When exposed to 0.05 μM haloxyfop, exudate production was reduced 70 and 50% (to 59 and 15 nmol/seedling), respectively. More significantly, no differences were observed in the **1**/SXSg ratio, 6.4% and 10.3%, respectively, after inhibition. With fluoroacetate, a 1 mM solution was replaced every 2 days in the sudan hybrid and exudate production was reduced to 27% of control with no detectable effect on the growth of the seedlings. Again, no differences in the relative composition of the resulting exudate were observed. While these inhibitors may either reduce fatty acid availability or directly inhibit the synthase involved in ring construction, their inability to alter the exudate composition is most consistent with a model where **1** is produced by partitioning SXSg through an additional methylation step (Figure 6).

Mechanism of Enhancing SXSg Activity. The oxidative lability of SXSg suggested that autoxidative events were important to its activity. For that reason it was important to evaluate the activity of **1** in a well-defined autoxidation system. A system for the controlled oxidation of benzaldehyde was developed for that purpose. The chromophore of the starting material and product benzoic acid were easily detected and analyzed by HPLC. The autoxidation was initiated with AIBN at 60 °C in *tert*-amyl alcohol. Though any benzoyl hydroperoxide product could participate in the reaction, its thermally induced rate of decomposition is more than an order of magnitude slower than that of AIBN.[14] Also, under the reaction conditions of excess benzaldehyde (3 M), the concentration of oxygen was not in excess but, with continuous perfusion, the [O_2] remained constant. Acetophenone, which was unreactive under the conditions, was used as an internal standard.

In the absence of an inhibitor, a constant rate of consumption of benzaldehyde was observed over the first 35% of reaction. SXSg, up to 63 μM, did not alter the rate of oxidation and there was no delay before the autoxidation of benzaldehyde began. In contrast, the addition of both 2,6-di-*tert*-butyl-4-methylphenol (BHT, 2 nM) and resorcinol **1** (500 nM) to the autoxidation reaction resulted in a biphasic autoxidation rate (Figure 7). Virtually no oxidation of benzaldehyde was observed prior to the breakpoint with BHT, and afterwards the oxidation rate was comparable to that without stabilizer. No significant oxidation was observed by 14 h when BHT was added at 20 μM. In the presence of 500 nM resorcinol **1**, there was a slow basal rate of oxidation and a clear breakpoint at 9.3 h. The rate of oxidation after the stabilizer was exhausted was slower than that observed with the BHT experiment. These data establish the resorcinol as a respectable antioxidant, though not as effective as BHT, and SXSg, consistent with its inherent oxidative lability, as ineffective.

Repeated addition of SXSg over the course of the *Striga* germination assay significantly reduces the [SXSg] necessary for induction.[5] The addition of BHT (4.5 μM) decreases the [SXSg] necessary for activity by the same amount.[5] Therefore it is not a unique function of the structure of **1** that enhances the activity of SXSg but rather its ability to function as a general

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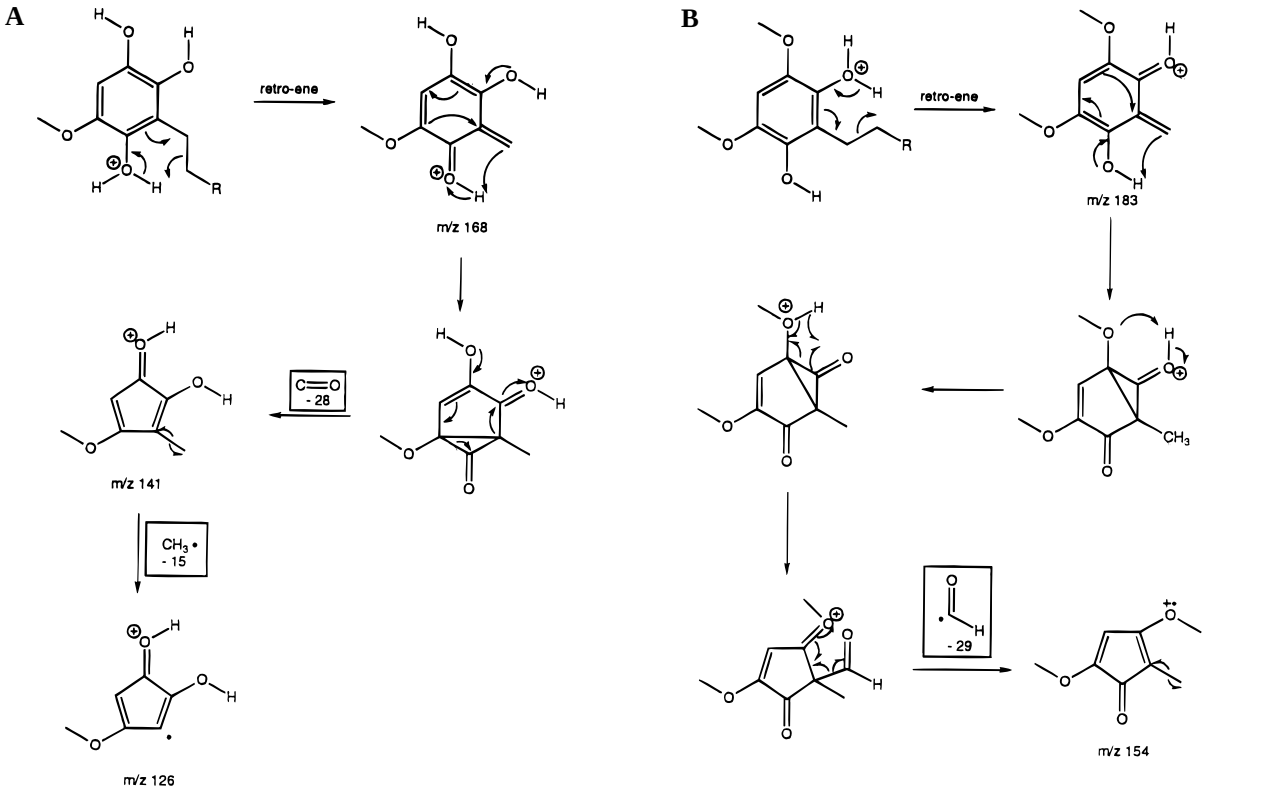

Figure 4. Proposed mechanism for the collisionally induced decomposition of the retro-ene fragments from SXSg, panel A, *m*/*z* 169 and **1**, panel B, *m*/*z* 183.

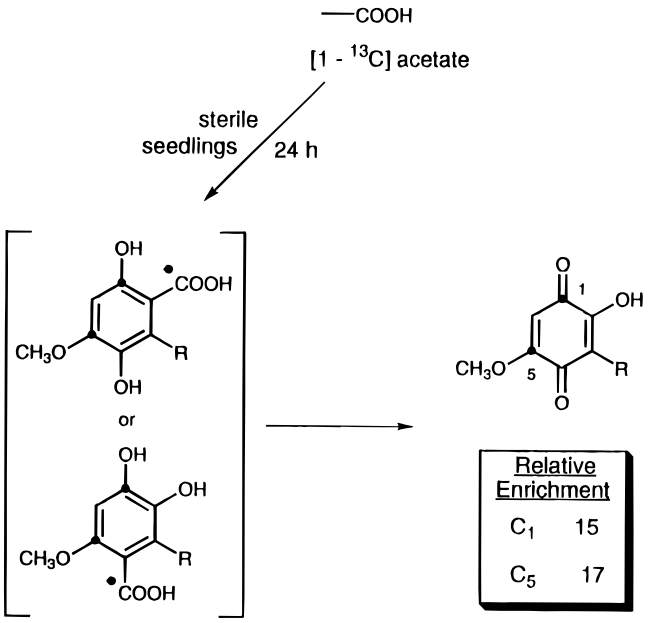

Figure 5. Relative enrichments from [1-^{13}C]-acetate incorporation into the quinone form of SXSg.

antioxidant. Therefore **1** serves as an endogenous antioxidant within the exudate.

Discussion

Striga spp are generally debilitating to their host plants and responsible for significant loss of grain production throughout Asia and Africa.[15] Clearly with *Striga asiatica*, geography dictates destiny. Without a xenognostic signal, the new generation of seeds do not germinate. If the seeds germinate at a distance that precludes host attachment, the seedlings perish. A single component of the sorghum root exudate, SXSg, is necessary and sufficient to induce *Striga* seed germination.[5,7] However, the activity of the exudate from sorghum varieties is not explained simply by the amount of SXSg produced[16] nor is the biological activity of purified SXSg consistent with the activity of SXSg in the sorghum exudate.[5]

While such observations have led others to search for additional active components in host plants,[16,17] two critical observations about the germination event in this organism are important to remember. First, the freshly collected exudate looses all activity when exposed to air over a 24-h period at room temperature. This loss of activity corresponds directly with the loss of [SXSg]; moreover, the activity can be completely recovered by the addition of synthetic SXSg.[7] Therefore the sole component that is physiologically relevant for the induction of germination is SXSg.[1]

Second, there is a definite and rather protracted exposure time to the inducing signal (10−12 h) necessary for the seed to commit to germination. If the concentration of the xenognosin drops significantly below the micromolar threshold, dormancy is not broken. There appears to be some latitude for fluctuations in the [SXSg]. As with haustorial induction,[18] where the concentration can drop below the threshold for a short time (<2 h), it appears to be the integral of time above the threshold that is important. The stability of SXSg around the host is therefore critical to an understanding of the spatial and temporal relationship between host and parasite.

The effectiveness of **1** in the exudate is most clearly seen in a survey of three different sorghum varieties, Piper Sudan Grass, Sorghum cv. dabar, and Sudan Grass Hybrid.[3] While each

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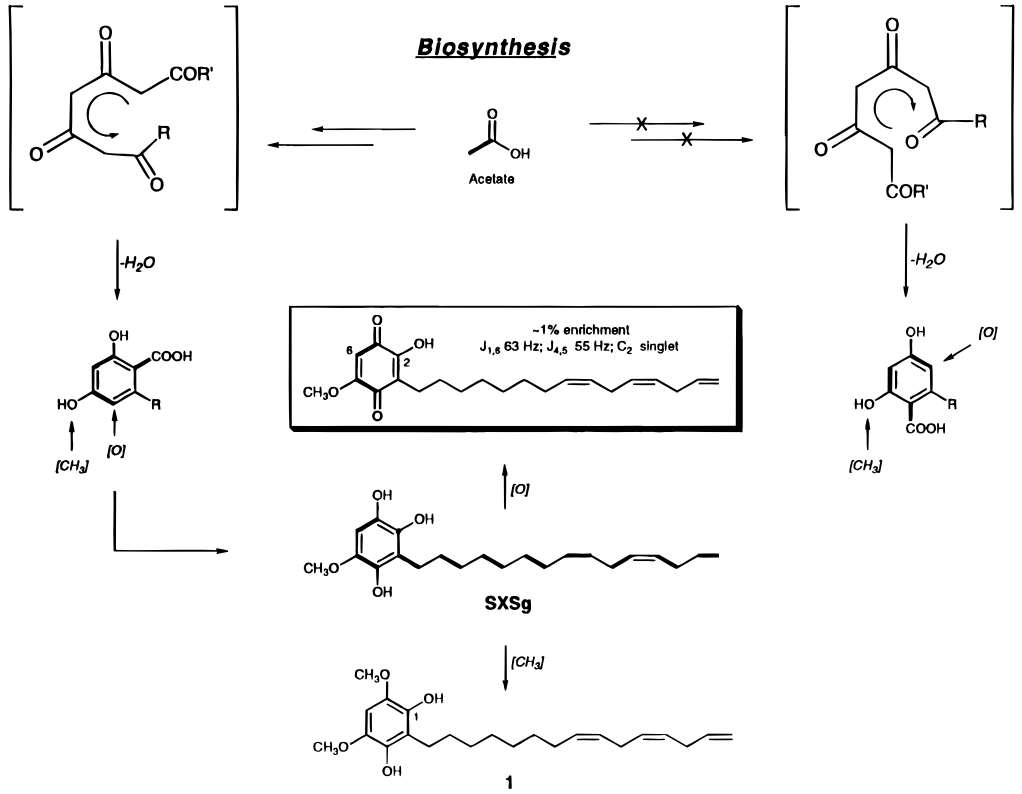

Figure 6. Biosynthetic pathway for the production of SXSg and **1** showing the incorporation of [$^{13}C_2$]-acetate.

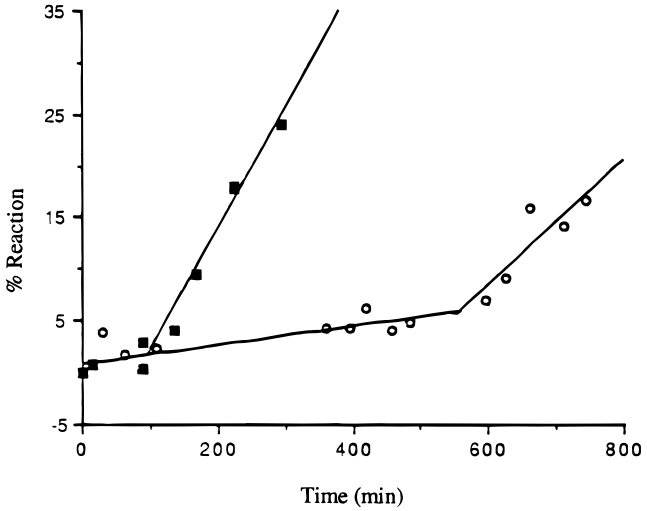

Figure 7. Autoxidation of benzaldehyde in the presence of BHT (■) (2 nM, break point at 95 min) and resorcinol **1** (●) (500 nM, break point at 560 min).

showed small differences in the ratio of resorcinol to SXSg, 6.4%, 7.6%, and 9.8%, respectively, in their exudates, these varieties showed significant differences in ED_{50} for *Striga* seed germination: 14, 5.6, and 1.0 μM, respectively. The addition of stabilizer to each of these exudates brings the ED_{50}'s to 10^{-6} M. Therefore roughly 10% stabilizer is required to maintain a 1.0 μM [SXSg] throughout the required exposure period for germination in these *in vitro* assays.

The mechanism by which **1** stabilizes the autoxidation of SXSg in the exudate can take several forms. Initiating steps involving hydrogen atom abstraction from SXSg could be chain terminated by hydrogen atom exchange between the resorcinol and the hydroquinone radical, a reaction well documented in synergistic mixtures of antioxidants.[19] For example, 2,6-di-*tert*-butylphenols exhibit a synergistic effect when added to the autoxidation of 9,10-dihydroanthracene inhibited with 4-methoxyphenol.[20] The radical of the resorcinol should have a longer lifetime than that of SXSg, and as shown for benzaldehyde oxidation, effectively participate in chain termination steps.

The effect of the triene side chain in both compounds must also be considered. The homolytic bond dissociation energies for the bis-allylic methylene C−H bonds are of comparable energy to a phenolic O−H. The energies for a bis-allylic methylene C−H bond and, for example, the phenolic O−H of 4-methoxyphenol are $D_{298} = 80$, and $D_{333} = 86$ kcal/mol, respectively.[21] Furthermore, reaction of molecular oxygen with these alkenes would provide an initiation pathway and the breakdown of the resulting hydroperoxides may be accelerated by the intramolecular phenolic group.[22] Therefore, autoxidation of the side chain is expected to play a central role in both initiation and chain propagation rates. In addition, polymerization may occur via reactions of the phenol and side-chain moieties.[23] Such bimolecular reactions would be particularly important on the surface of the sorghum root where the concentration of SXSg is the highest. The inability to identify accumulating concentrations of the quinone around the sorghum roots is consistent with the involvement of such reactions in the further decomposition of the SXSg quinone.[5]

The presence of **1** in the exudate means that the decomposition of SXSg with time would be at least biphasic, its rate of decomposition accelerating in the absence of **1**. The fact that the observed germination around the host fits well with the

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stabilized rate of oxidation argues that the concentration gradient around the host root may be more related to [**1**] than that of SXSg. The observed breakpoint distance, where [**1**] becomes ineffective as a stabilizer, will be highly dependent on the rates of initiation which could change radically depending on the soil environment. Nevertheless, the methods developed here should apply equally well and be of practical value in the *in situ* analyses.

The biosynthesis experiments provided initial evidence for a methyl transferase capable of methylating the xenognosin and thereby regulating the spatial distribution of the stimulant around the host. This remarkably simple strategy for spatial and temporal control certainly did not evolve for signaling the parasite and must have been selected for some positive advantage; the nature of this advantage has been the subject of speculation.[1,5] This knowledge opens new molecular genetic strategies for manipulating this critical first step in pathogenesis and the development of pathogen resistant host varieties are now being actively pursued.

Acknowledgment. We thank the Department of Energy (DE-FG02−91ER20024.A002) for financial support and Jeffrey Shabanowitz, University of Virginia, for assiatance with the MS/MS experiments.

JA961395I