Elucidation of the Biosynthetic Pathway of the Allelochemical

Sorgoleone Using Retrobiosynthetic NMR Analysis

Franck E. Dayan†, Isabelle A. Kagan and Agnes M. Rimando

USDA/ARS, Natural Products Utilization Research Unit, PO Box 8048, University, Mississippi 38677 U.S.A.

† To whom correspondence should be addressed.

Tel: 662-915-1039; Fax: 662-915-1035; E-mail: fdayan@ars.usda.gov

Running Title: Biosynthesis of sorgoleone
Summary

NMR analyses of the labeling pattern obtained using various $^{13}$C-labeled precursors indicated that both the lipid tail and the quinone head of sorgoleone, the main allelopathic component of the oily root exudate of *Sorghum bicolor*, were derived from acetate units, but that the two moieties were synthesized in different subcellular compartments. The 16:3 fatty acid precursor of the tail is synthesized by the combined action of fatty acid synthase and desaturases most likely in the plastids. It is then exported out of the plastids and converted to 5-pentadecatriene resorcinol by a polyketide synthase. This resorcinol intermediate was identified in root hair extracts. The lipid resorcinol intermediate is then methylated by a SAM-dependent $O$-methyltransferase and subsequently dihydroxylated by a P450 monooxygenase to yield the reduced form of sorgoleone.

Key words: Allelochemical, biosynthesis of quinone, fatty acid desaturase, isotopic labeling of natural products, $O$-methyltransferase, polyketide synthase, xenognosin
Introduction

Sorgoleone (2-hydroxy-5-methoxy-3-[(Z,Z)-8',11',14'-pentadecatriene]-p-benzoquinone) (1) is the main allelopathic component of the oily root exudate of sorghum (Sorghum bicolor (L.) Moench) (Figure 1) (1-3). The term sorgoleone is also used to describe a group of lipophilic p-benzoquinones structurally related to 1 that are also produced by sorghum roots, having a hydroxy and a methoxy substitution at positions 2 and 5, respectively; and either a 15- or 17-carbon aliphatic tail with various degrees of unsaturation at position 3 (3).

The primary mechanism of phytotoxic action of 1 is associated with inhibition of photosynthesis in higher plant systems by competing for the binding site of plastoquinone on photosystem II (4-6). This lipophilic p-benzoquinone is also known to hinder electron transfer reactions involved in mitochondrial respiration and to inhibit the enzyme p-hydroxyphenylpyruvate dioxygenase (7,8).

The herbicidal and allelopathic properties of 1 make isolation of the genes responsible for its biosynthesis desirable, as manipulation of those genes in sorghum, or their introduction in other plant species, could provide a better understanding of the role of 1 in plant-plant interaction and natural weed control. To identify the genes and characterize the enzymes they encode, it is necessary to determine the steps involved in the biosynthesis of 1. Little has been reported on this subject.

Sorgoleone is found exclusively as a hydrophobic droplet exuding from the tips of root hairs (2), and a recent investigation of sorghum roots suggested that the production of 1 is compartmentalized in the highly physiologically active root hairs (9). The biosynthesis of secondary metabolites occurring in root hairs has not been studied in
detail, although other specialized cells at the interface between a plant surface and its environment (i.e. trichomes) are known to produce unique secondary metabolites (10-12).

It has been postulated that the biosynthesis of 1 and related resorcinolic lipids is the result of the convergence of two pathways, namely the fatty acid biosynthetic pathway for the synthesis of the aliphatic tail and the activity of polyketide synthase-type enzymes for the formation of the quinone head (13,14). Although this convergence of pathways has been demonstrated for aflatoxin biosynthesis in fungi (15), examples in plants have not been completely elucidated, but derived from incomplete experimental data complemented with biogenetic assumptions that have not necessarily been demonstrated for these compounds (14). In the case of 1, Fate and Lynn demonstrated that exogenous isotopically labeled acetate was incorporated into the quinone head but not into the tail (16). No further isotopic labeling studies of the hydrophobic tail, nor any studies of the biogenesis of the substituents on the quinone head, have been reported. Consequently, many questions regarding the biosynthesis of this important allelochemical have been left unanswered (17). The objective of this study was to determine the entire biosynthetic pathway of 1 via retrobiosynthetic NMR analysis of the root exudate of sorghum seedlings grown in the presence of suitable isotopically labeled substrates, and to identify key metabolic intermediates.

**Experimental Procedures**

**Materials** - Seeds of high sorgoleone-producing *S. bicolor* x *S. sudanense* hybrid (SX17) were purchased from Dekalb Genetics (Dekalb, IL). 1,2-$^{13}$C$_2$-Palmitoyl-CoA was
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synthesized according to a scaled-up protocol of Hajra and Bishop (18). All other chemicals, including 1-\(^{13}\)C-acetate (99% \(^{13}\)C), 2-\(^{13}\)C-acetate (99% \(^{13}\)C), 1-\(^{13}\)C-L-acetyl-
carnitine chloride (99% \(^{13}\)C), 2-\(^{13}\)C-D-glucose (99% \(^{13}\)C), 4-\(^{13}\)C-3-hydroxybutyrate (99%
\(^{13}\)C), 2-\(^{13}\)C-malonate (99% \(^{13}\)C), \(^{13}\)C-methyl-L-methionine (99% \(^{13}\)C), and 1,2-\(^{13}\)C\(_2\)-
palmitate (99% \(^{13}\)C) were purchased from Sigma-Aldrich (Milwaukee, WI).

**Seed sterilization and growth of seedlings** - Sorghum (SX17) seeds were
surface sterilized in 20% Clorox bleach (final concentration of 1% sodium hypochlorite)
for 10 minutes and rinsed with deionized water. Seeds were poured into a gravity
funnel lined with cheesecloth and allowed to dry overnight in a laminar flow hood.
Though not completely aseptic, this method allowed the seed to grow free of observable
microbial contamination during the experiments.

Twenty seeds (ca. 500 mg) were placed in 20 x 100 mm sterile polystyrene Petri
dishes (Falcon) over the surface of sterile Whatman #1 filter paper (90 mm diameter).
Five ml of sterile water was added to the dish, and the seeds were covered with a
second sterile filter paper. The dishes were sealed and incubated in the dark at 25°C.
Sorgoleone was extracted from 6 day-old seedlings in all experiments unless otherwise
indicated.

**Labeling studies** - Stock solutions of the labeled compounds were prepared as
a 1:1 or 1:3 ratio of \(^{13}\)C:\(^{12}\)C isotopes at 10 mM concentration of the combined forms,
except for acetate and pyruvate which were prepared in 1 mM stock solutions. The
labeled precursors were added to the Petri dishes on the fourth day. The Petri dishes
were opened and the upper filters were discarded. Excess water in the dishes was
removed and replaced with 2 ml of either 500 µM 4-\(^{13}\)C-3-hydroxybutyrate, 500 µM 1,2-
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$^{13}$C$_2$-palmitate, 500 µM $^{13}$C-methyl-ß-methionine, 1 mM $^{1}$-$^{13}$C-acetate, 1 mM $^{1}$-$^{13}$C-acetate, or 2 mM $^{2}$-$^{13}$C-D-glucose. The excess water in the Petri dish was discarded since it contained approximately 0.15% of the total amount of 1 produced by the roots. The same procedure was repeated on the fifth and sixth day. At the end of the labeling period, the roots were excised, and 1 was extracted as described below. All labeling procedures were done under low-intensity green light to prevent the formation of anthocyanins by sorghum roots.

**Extraction and purification of sorgoleone** - Sorgoleone was extracted by immersing the roots in chloroform for 1 min. Sorgoleone absorbed to the bottom filter paper (about 20% of the final weight of sorgoleone) was also extracted with chloroform. The extract was filtered through Whatman #1 filter paper and evaporated *in vacuo* (Büchi Rotovapor, Brinkmann Instrument, Westbury, NY) at 30°C. The dried yellow residue was redissolved in chloroform, transferred to vials and dried under nitrogen. The crude extract was applied to 20 x 20 cm aluminum-backed silica thin-layer plates and separated using hexane:2-propanol (9:1 v/v). The sorgoleone band ($R_f$ 0.36) was cut out and eluted from the silica by washing with 200 mL of CHCl$_3$ twice. The pooled material was dried under nitrogen and stored at –20°C until NMR analysis.

**NMR and mass spectrometry analysis** - $^{13}$C-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), with the following variables: 30° pulse (2.17 µs), repetition time. 3.0 s; spectral width, 15.1 kHz, temperature, 27°C. The decoupling pulse was 90 µs at 18 db.
\(^{13}\)C-NMR spectra of labeled and unlabeled (natural abundance) sorgoleone were obtained under the same experimental conditions. Each carbon peak was integrated and \(^{13}\)C enrichments were calculated using the formula: \((a-b)/b\), where \(a\) is the integrated NMR signal of the enriched carbon in the labeled sorgoleone and \(b\) is the integrated NMR signal of the same carbon in the unlabeled sorgoleone (19).

Gas Chromatography-Mass Spectrometry (GC-MS) was 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 and held at this temp for 1 min, then raised to 300°C at a rate of 10°C/min and held at this temp for 4 min. The GC capillary column was ZB-50 (0.25 mm i.d., 0.25 mm film thickness, 30 m length; Phenomenex, Torrance, CA). The carrier gas was ultra high purity helium, 1 mL/min flow rate. The inlet (splitless), GC interface and ion chamber temperatures were 250, 250 and 230°C, respectively. Samples were prepared in CHCl\(_3\) at 1 mg/mL concentrations and the volume of samples injected was 1 \(\mu\)L.

**Isolation and identification of resorcinolic metabolic intermediates in**

**Sorghum bicolor root hairs** – Root hairs were isolated from unlabeled sorghum roots as described by Röhm and Werner (20), with the addition that the liquid nitrogen containing the isolated root hairs was decanted by pouring it through a stainless steel mesh sieve (#60, 250 \(\mu\)m) to remove larger root debris. Isolated root hairs were powdered in liquid nitrogen, extracted with 10 mL of 95% MeOH, and sonicated in a sonication bath (Branson 3510 Ultrasonic Cleaner) for 15 min. The extract was filtered through a Whatman #1 filter. The extraction procedure was repeated with the residue two more times, and the organic supernatants were pooled. The organic phase was
dried with magnesium sulfate and evaporated *in vacuo*. The residue was dissolved in a small amount of ethyl acetate/hexane (80:20 v/v) and applied to aluminum-backed silica plates. The extract was chromatographed in CHCl₃/ethyl acetate/methanol (85:8:7 v/v), and the UV active band with an \( R_f \) identical to that of a 5-pentadecyl resorcinol (Chem Service, Inc. West Chester, PA) was eluted from the plate (21). The semipurified sample (1-2 mg) was diluted in 1 mL of CHCl₃ and injected in a JEOL GCmate mass spectrometer as described above. The injection volume was 1 µL.

**Results and discussion**

Elucidation of the biosynthetic pathway of natural products via isotopic labeling is often a prerequisite to the characterization of the enzymes involved and the identification of the genes encoding them (22). The structural features of 1 and the published pattern of incorporation of acetate in xenognosin/sorgoleone reported by Fate and Lynn suggested that the biosynthesis of 1 involves the convergence of the fatty acid and polyketide pathways (16). Other plant secondary metabolites with some resemblance to 1, such as the resorcinolic lipids, appear to be also synthesized through the convergence of these pathways (13,14).

Labeling with 2-¹³C-acetate and 2-¹³C-malonate yielded similar patterns of incorporation on carbon atoms 2, 4 and 6 (151.9, 182.1, and 102.5 ppm, respectively) (Figures 2B and 3). The percent incorporation was similar for both precursors, ranging from 3.6 to 6.2% (Table 1). Labeling with 1-¹³C-acetate led to enrichment in carbons 1 and 5 (183.2 and 161.5 ppm, respectively) but no incorporation was detected in carbon atom 3 (Figure 2C). These results indicate that at the mechanistic level, the polyketide synthase (PKS) involved in the production of 2 catalyzes an orsellinic acid-type
cyclization of the linear tetraketide intermediate (steps III and IV in Figure 3) such as
catalyzed by stilbene synthase, rather than a phloracetophenone-type cyclization
typically associated with chalcone synthase (23,24). Though stilbene and chalcone
synthase have similar nucleotide sequences, this difference in their reaction mechanism
may provide useful information to help identify the genes encoding for the proper
polyketide synthase participating in the biosynthesis of 1.

The fact that carbon 3 (119.6 ppm) of the quinone head is not labeled in the
presence of 1-13C-acetate (Table 1), whereas carbons 1 and 5 become isotopically
labeled, precludes the possibility that acetate is the starter unit for the PKS involved in
the synthesis of 1. Since 1, with a pentadecatriene lipid tail, accounts for greater than
80% the sorghum root exudate, the starter unit is likely to be a novel Δ9,12,15 C16:3-
CoA. However, the exudate also contains minor components consisting of sorgoleone
derivatives with aliphatic tails of 15 or 17 carbons and various degrees of unsaturation
(3), suggesting that the PKS involved in the synthesis of 1 can utilize other C16 and
C18 fatty acyl-CoAs as starter units.

The product of PKS activity is 2 (2-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]resorcinol), a cardol-like intermediate as shown in Figure 3, which was
isolated and identified by GC-MS in sorghum root hair extracts. The extracted ion
chromatogram showed m/z 313.1 [M+ - H] indicative of 2 (retention time 7 min 55 sec)
with characteristic fragment ions m/z 282 [M+ - O2], 206 (the pentadecatriene fragment),
149 [206+ - C4H7], 135 [149+ - CH2], 123 (methyl resorcinol fragment) supporting the
identity of 2. The existence of 5-pentadecatriene-resorcinol and related analogues has
also been reported in grass species phylogenetically related to sorghum (14,21,25-28).
Attempts to label the lipid tail with acetate, malonate, and pyruvate were unsuccessful. Other substrates tested, such as L-acetyl-carnitine, involved in the transport of fatty acids across the mitochondrial membranes and possibly fatty acid biosynthesis (29,30), and 3-hydroxybutyrate, whose ACP conjugate is an intermediate in the early stages of fatty acid biosynthesis (29), also failed to be incorporated. Further labeling experiments, bypassing fatty acid synthesis by providing labeled palmitate or palmitoyl-CoA, also proved unsuccessful. However, inhibition of root growth caused by palmitate and its low water solubility (log P = 7.15), and the low root uptake of palmitoyl-CoA were limiting factors interfering with incorporation of these substrates.

Labeling of the alkyl carbons, in a pattern consistent with the catalytic mechanism of fatty acid synthase (FAS) (31), was achieved only with 2-\textsuperscript{13}C-D-glucose (Figures 2D and 2F, and step II in Figure 4). Enrichment was observed in carbons 2’, 4’, 6’, 8’, 10’, 12’, and 14’, corresponding to increased signals at 28.4, 29.7, 30.0, 130.8, 25.9, 127.2, and 137.3 ppm, respectively (Figure 4). The level of isotope incorporation in the tail ranged from 1.2 to 3.7% (Table 1).

Glucose is often used in retrobiosynthetic NMR analysis of pathways because it overcomes limitations associated with subcellular compartmentalization (22). In this study, the contrast between the labeling patterns obtained with acetate, malonate and glucose is particularly striking because FAS uses both acetate and malonate as substrate, yet, no incorporation in the tail was observed with these latter substrates.

Incorporation of biosynthetic precursors into fatty acids is known to be problematic because their synthesis is compartmentalized in plastids. In most plant cells, exogenous acetate is not usually incorporated into C18 and shorter fatty acids.
because acetate does not readily cross the plastidic membranes, and plastids have
their own endogenous pool of acetate used for fatty acid synthesis (32-34). Even when
endogenous acetate has been reported to be incorporated in short fatty acids, glucose
has been demonstrated to be a far better substrate because it can either be imported in
nongreen plastids directly as glucose-6-phosphate and used as the source for the
plastidic pool of acetate (35,36), or by being broken down to triose phosphates in the
cytosol, and taken up in plastids via triose phosphate translocators (37).

An important aspect of the synthesis of 1 is the unusual desaturation pattern of
the aliphatic tail. Most of the steps leading to the formation of Δ9 C16:1 fatty acids are
ubiquitous in plants. However, the next two desaturation steps required to yield Δ9,12
C16:2 and especially Δ9,12,15 C16:3 are more unusual and must be catalyzed by as-
et-unknown fatty acid desaturases (FAD) (reaction IV in Figure 4). It is important to
differentiate between this novel Δ9,12,15 hexadecatrienoyl-CoA derivative (3) involved
in the biosynthesis of 1 from the well characterized ω3 and ω6 hexadecatrienoic acids
that are important components of plastidic membranes and (Δ7,10,13 and Δ4,7,10,
respectively) (38-40).

The remaining steps involved in the biosynthesis of 1 are the methylation of the
hydroxyl on carbon 5 and the addition of two hydroxyl groups on carbons 2 and 4.
While the order of these reactions is not certain, the methylation is catalyzed by an O-
methyltransferase (OMT) (41), and the addition of the hydroxyl groups is most likely
catalyzed by the action of a P450 monooxygenase on the resorcinol ring (42-46). As
expected, isotopic labeling of this methyl group was achieved with 13C-methyl –
methionine, suggesting the presence of a S-adenosyl-L-methionine (SAM) dependent
OMT. Methylation with labeled methionine yielded the greatest percent incorporation (nearly 27%), relative to the other substrates used in this study (Table 1).

The presence of 2 in root hair suggests that the methylation most likely occurs on 2 (Figure 3) rather than a putative tetrahydroxy- resorcinolic lipid intermediate. In any case, both intermediates are symmetrical with respect to the position of the hydroxyl groups. Therefore the OMT would not distinguish between the hydroxyl group on carbon 1 from the one on carbon 5. The OMT found in sorghum root hairs appears to be quite specific for this substrate, since a 4,6-dimethylated form of 1 is present only in small amounts in sorghum exudate (16, 47,48). OMTs have been recognized to catalyze highly regiospecific reactions and to be quite substrate specific (43,49-52). In light of the dual hydrophobic/hydrophilic nature of 1, the OMT involved in this pathway is also expected to be substrate specific, with a substrate binding domain that can accommodate the very lipophilic tail of 1. Once a single methylation occurs, subsequent hydroxylation of 4 by a putative P450 monooxygenase (53) leads to the formation of 1 in its reduced hydroquinone form. The enzymatic synthesis apparently ends with the formation of the reduced form of 1 (a hydroquinone known as sorghum xenognosin for *Striga* germination) (54). The oxidation of the hydroquinone into the benzoquinone is most likely the result of autooxidation upon exposure to air (1,16).

In conclusion, the biosynthetic pathway of 1 initiates in the plastids with the synthesis of an unusual Δ9,12,15 hexadecatrienoic acid through the action of FAS and FAD. Outside the plastids, this polyunsaturated fatty acid serves as the starter unit for PKS, yielding a resorcinolic lipid intermediate. The action of a SAM-dependent OMT subsequently methylates the hydroxyl group on carbon 5, and the enzymatic synthesis
ends with the hydroxylation in the aromatic ring by a P450 monooxygenase. Our research laboratory is now focusing on the characterization of the biochemical machinery involved in the biosynthesis of \( 1 \) and identifying the genes encoding these enzymes.

**Acknowledgements**

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**References**


Table 1. Percent incorporation of the various $^{13}$C-labeled substrate used in this study

<table>
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<tr>
<th>Isotopically Labeled Substrates</th>
<th>1-$^{13}$C-acetate</th>
<th>2-$^{13}$C-acetate</th>
<th>2-$^{13}$C-malonate</th>
<th>2-$^{13}$C-d-glucose</th>
<th>$^{13}$C-methyl-L-methionine</th>
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<tr>
<td>Carbon</td>
<td>ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>183.2</td>
<td>6.1</td>
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<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
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<td>6.2</td>
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</table>

* Refer to structure in figure 1 for carbon numbering

b Chemical shifts are in ppm (relative to CDCl$_3$).

* This carbon should be labeled if acetate were the starter unit of the PKS. This carbon was not labeled with 1-$^{13}$C-acetate.
Figure legends

Figure 1. Structure of sorgoleone (1) with atoms numbered according to Chang et al., 1986.1

Figure 2. A. Reference NMR spectrum of unlabeled sorgoleone (90-190 ppm region). B. NMR spectrum of sorgoleone labeled with 2-13C-acetate, showing incorporation in carbon atoms 2, 4 and 6. Similar labeling pattern was obtained with 2-13C-malonate. C. NMR spectrum of sorgoleone labeled with 1-13C-acetate, showing incorporation in carbon atoms 1 and 5. D. NMR spectrum of sorgoleone labeled with 2-13C-D-glucose, showing incorporation in carbon atoms 1, 3 and 5 of the quinone head, as well as carbon atoms 8’, 12’, and 14’ of the tail. E. Reference NMR spectrum of unlabeled sorgoleone (22-30 ppm region). F. NMR spectrum of sorgoleone labeled with 2-13C-D-glucose, showing incorporation in carbon atoms 2’, 4’, 6’, and 10’ of the tail. No isotopic incorporation in the tail was achieved with either 4-13C-3-hydroxybutyric acid, 2-13C-acetate, 2-13C-malonate, 1-13C-L-acetyl-carnitine chloride, 1,2-13C2-palmitate, and 1,2-13C2-palmitoyl-CoA.

Figure 3. Isotopic labeling of the benzoquinone moiety of sorgoleone with 2-13C-acetate. 1-13C-Acetate was incorporated in carbons 1 and 5 but not in 3. Steps I and II are catalyzed by acetyl-CoA ligase and acetyl-CoA carboxylase. Polyketide synthase catalyzes the condensation of 3 malonyl-CoA units to the fatty acyl-CoA starter unit (step III) followed by the cyclization, reduction and decarboxylation steps (step IV), yielding 2. Step V summarizes the remaining transformations leading to the formation of 1 (see text). R = (Z,Z)-8’,11’,14’-pentadecatrienyl.
**Figure 4.** Isotopic labeling of sorgoleone hydrophobic tail of 1 with 2-\(^{13}\)C-D-glucose. In contrast to the labeling pattern obtained with 1-\(^{13}\)C-acetate, labeling with 2-\(^{13}\)C-D-glucose led to incorporation in carbons 3 in addition to 1 and 5. Metabolism of glucose (step I) leads to acetyl-CoA and malonyl-CoA that serve as substrate for the chloroplastic FAS (II) for the synthesis of palmitic acid. Once incorporated in monogalactosyldiacylglycerol (MGD), the trienoic tail is formed by the activity of FAD (IV). The fatty acid is then activated by an acyl-CoA synthetase (V) and serves as a substrate for PKS (VI, see figure 3), a SAM-dependent OMT (VII), and a P450 monooxygenase (VIII), yielding 1.
Figure 1
Figure 2.
Figure 3

[Chemical reaction diagram showing the biosynthesis of sorgoleone with reactions numbered I to V.]

I: Acetyl-CoA + CO₂ → Acetate
II: Acetyl-CoA + CoA → Malonyl-CoA
III: Malonyl-CoA × 3 → S-Enzyme
IV: S-Enzyme + CO₂ → 2
V: 2 → 1

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Figure 4

D-Glucose → Acetyl-CoA + Malonyl-CoA → Palmitoyl-ACP → C16:0 → C16:3 → 3 → 1
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