An allene oxide and 12-oxophytodienoic acid are key intermediates in jasmonic acid biosynthesis by
Fusarium oxysporum

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Abstract Fungi can produce jasmonic acid (JA) and its iso-
leucine conjugate in large quantities, but little is known about the biosynthesis. Plants form JA from 18:3n-3 by 13S- lipoygenase (LOX), allene oxide synthase, and allene oxide cyclase. Shaking cultures of Fusarium oxysporum f. sp. tulipae released over 200 mg of jasmonates per liter. Nitrogen powder of the mycelia expressed 10R-dioxygenase-epoxy alcohol synthase activities, which was confirmed by comparison with the recombinant enzyme. The 13S-LOX of F. oxysporum could not be detected in the cell-free preparations. Incubation of mycelia in phosphate buffer with [17,17,18,18-2H2]18:3n-3 led to biosynthesis of a [17H3]-12-oxo-13-hydroxy-9Z,15Z-octa-
decadienoic acid (α-ketol), [17H3]-12-oxo-10,15Z-phytodiendoic acid (12-OPDA), and [17H3]-13-keto- and [17H3]-13S-hydroxyoctadeca
lstrienoic acids. The α-ketol consisted of 90% of the 13R stereoisomer, suggesting its formation by nonenzymatic hy-
drolysis of an allene oxide with 13S configuration. Labeled and unlabeled 12-OPDA were observed following incubation with 0.1 mM [17H3]18:3n-3 in a ratio from 0.4:1 up to 47:1 by mycelia of liquid cultures of different ages, whereas 10 times higher concentration of [17H3]-13S-hydroperoxyoctadecatrienoic acid was required to detect biosynthesis of [17H3]-12-
OPDA. The allene oxide is likely formed by a cytochrome P450 or catalase-related hydroperoxidase. We conclude that F. oxysporum, like plants, forms jasmonates with an allene oxide and 12-OPDA as intermediates.—Oliw, E. H., and M. Hamberg. An allene oxide and 12-oxophytodienoic acid are key intermediates in jasmonic acid biosynthesis by Fusarium oxysporum. J. Lipid Res. 2017. 58: 1670–1680.

Supplementary key words cytochrome P450 • epoxy alcohol synthase • fatty acid dioxygenase • jasmonates • lipids/oxidation • lipoxygenase • mass spectrometry • methods/high-performance liquid chromatography

Fusarium oxysporum is a plant pathogenic fungus, which infects the root tips and causes wilt disease (1, 2). F. oxysporum can infect virtually all plants except grasses. A special form [forma specialis (f. sp.)] of F. oxysporum is so designated from its capacity to infect a specific host. F. oxysporum f. sp. cubense is one of most infamous strains, as it causes Panama disease of banana, a constant threat to this commodity (3).

The methyl ester of (−)-jasmonic acid [(−)-JA] was iso-
lated and structurally characterized by Demole in 1962 (4). The (+)-7-isojasmonic acid [(+)-JA], which is the bio-
logically relevant form of JA, was conclusively identified more than two decades later in lemon (5) and in media from two fungi, Lasiodiplodia theobromae (6) and Fusarium fujikoroi (7).

The plant biosynthesis of JA has been investigated in detail. Many of the enzymes have been crystallized, and the biological importance of jasmonates as growth hormones and physiological regulators is well-documented (8–14). The 13S-lipoxygenase (LOX) of plants oxidizes 18:3n-3 to 13S-hydroperoxy-octadecatrienoic acid (13S-hydroperoxyoctadecatrienoic acid (HPOTrE)), which is dehydrated by allene oxide synthase (AOS) to an allene oxide, which in turn is converted by allene oxide cyclase (AOC) to 12-oxo-10,15Z-phytodiendoic acid (12-OPDA) in plastids. The 12-OPDA is reduced and subject to β-oxidation with formation of (+)-JA in the peroxisomes and then conjugated with Ile (Fig. 1).

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; α-ketol, 12-oxo-13-hydroxy-9Z,15Z-octa
decadienoic acid; α-ketol-Ile, N-[12-oxo-13-hydroxy-9Z,15Z-octa
decadienoyl]-isoleucine; CDB, Czapec-Dox broth; CP, chiral phase; CYP, cytochrome P450; DOX, dioxygenase; EAS, epoxy alcohol synthase; 12,13-5ET, 12(13S)epoxy-9Z,11,15Z-octa
decatrienoic acid; Fo-MnLOX, lipoxygenase containing catalytic manganese; Fot, F. oxysporum f. sp. tulipae, FoXLOX, lipoxygenase containing catalytic iron; HOTE, hydrogenperoxyoctadecatrienoic acid; HPODe, hydrogenperoxyoctadecatrienoic acid; HPOTE, hydrogenperoxyoctadecatrienoic acid; JA, jasmonic acid; (+)-JA, (+)-7-isomono
yloxy-10(S)-isoleucine; KOTrE, keto-octadecatrienoic acid; LOX, lipoxygenase; MO, O-methyloxime; 18:3n-3-Ile, N-[9Z,12Z,15Z-octa
decatrienoyl]-isoleucine; 12-OPDA, 12-oxo-10,15Z-phytodiendoic acid; PDA, potato dextrose agar; PDB, potato dextrose broth; RP, reversed phase.

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The fungal biosynthesis of (+)-JA is intriguing in many aspects. First, fungi can form remarkably large amounts of (+)-JA in the laboratory, as discussed below. Second, in spite of the large production of (+)-JA, the mechanism of the fungal biosynthesis is not firmly established. Third, (+)-JA produced by fungi can augment plant disease (15, 16); yet many plants form jasmonates as defense reactions to fungal infections (11, 12). Fourth, the initial steps of (+)-JA biosynthesis in plants occur in plastids, which are absent in fungi.

*L. theobromae* and members of the *Fusarium* family remain prototypes for studies of (+)-JA biosynthesis. *L. theobromae* (strain 2334) can routinely produce 300–500 mg (+)-JA per liter of growth medium and occasionally much more (17–19). In the fields, *L. theobromae* causes excessive growth of tropical fruit trees, possibly due to (+)-JA secretion (15, 18). Several subspecies of *F. oxysporum*, which infect garden stock, cabbage, and tulips, are also known to produce (+)-JA, 9,10-dihydro-JA, and their conjugates with isoleucine (15, 20). The most prominent biosynthesis occurs in still cultures of *F. oxysporum* f. sp. *tulipae* (Fot). Fot can produce milligram amounts of jasmonates per liter and induce gum disease in tulips (15, 16).

The mechanism of (+)-JA biosynthesis by *L. theobromae* was investigated by Tsukada, Takahashi, and Nabeta (21), who found that *L. theobromae* produced [2H₄](-)-JA following feeding with [9,10,12,13,14,15-²H₆]18:3n-3 (21). Traces of labeled 12-OPDA were detected (21); however, additional compounds indicative of the plant pathway [13S-HPOTrE and 12-oxo-13-hydroxy-9Z,15Z-octadecadienoic acid (α-ketol)] were not observed. The fungal biosynthesis of jasmonates from 18:3n-3 therefore remains an enigma.

Dioxygenases (DOXs) of C₁₈ fatty acids, which belong to the cyclooxygenase gene family, and LOXs have been investigated by genome analysis of *F. oxysporum* and recombinant protein expression (22–25). The genome codes for two LOXs and three DOX-cytochrome P450 (CYP) fusion proteins (Fig. 2). The LOXs contain either catalytic manganese (Fo-MnLOX) or catalytic iron (FoxLOX). Both enzymes oxidize 18:2n-6 and 18:3n-3 to 13S-hydroperoxy metabolites, and Fo-MnLOX also forms 11-hydroperoxy metabolites (22, 26). These LOXs could be candidates for the first fungal enzyme in a plant pathway to jasmonates (26). The 9S-DOX-AOS oxidizes 18:2n-6 to 9S-HPODE and the 9S-AOS transforms this hydroperoxide to an allene oxide (Fig. 2), but it does not transform 13S-HPOTrE (24, 27). This 9S-AOS is therefore not involved in fungal biosynthesis of (+)-JA. This also applies to 9R-DOX, which does not form 13-hydroperoxy C₁₈ fatty acids (23). The 10R-DOX-epoxy alcohol synthase (EAS) of *F. oxysporum* has, so far, only been characterized as a recombinant homolog to 10R-DOX-EAS of the rice blast fungus, *Magnaporthe oryzae*, but only with respect to oxidation of 18:2n-6 (25). Expression of 10R-DOX-EAS in mycelia of *F. oxysporum* has not been reported, but expressed sequence tags have been found.

The present investigation had three goals. The first goal was to optimize the growth conditions of *F. oxysporum* with respect to biosynthesis of jasmonates, and we chose to
Materials and Methods

Cartridges, silicic acid, and C18 silica (SepPak) were from Waters. Fatty acids were dissolved in ethanol and stored in solid solutions (50–100 mM) at −20°C. The 18:2n-6 (99%) was from Sigma. The 18:1n-9 (99%), 18:5n-3 (99%), [13C18]18:2n-6 (98%), [17,17,18,18,18-2H5]18:3n-3, (+)-JA, 12OPDA, and α-ketol were from Larodan (Stockholm, Sweden). When required, fatty acids were purified by SiO2 chromatography (SepPak) and eluted with 30 ml diethyl ether/hexane/acetic acid, 5/95/0.2, in one step. The [13C18]10S/Ehydroypropoxy-8E,12Zoctadecadienoic acid (HPODE) was prepared by photo oxidation of [13C18]18:2n-6 (24). The [13H1]13S-HPOTrE was prepared with soybean LOX-1 (Sigma) and 13R-HPOTrE with 13R-MnLOX. The (-)-JA, RNAaseA, potato dextrose broth (PDB), and ampicillin were from Sigma. Czapec-Dox broth (CDB) was prepared with reagents from Sigma and VWR (per liter: saccharose (30 g), NaNO3 (3 g), Czapec-Dox broth (CDB) was prepared with reagents from Sigma and VWR (per liter: saccharose (30 g), NaNO3 (3 g), K2HPO4 (1 g), KCl (0.5 g), MgSO4·7H2O (0.5 g), and FeSO4·7H2O (0.01 g)]. JA-Ile was synthesized as described (28). The Ile conjugate of 18:3n-3 was prepared as earlier described (29) and purified by reversed phase (RP)-HPLC using a solvent system of methanol-water-acetic acid (85:15:0.01, v/v/v). Fot (NRRL 26954) was from ARS Culture Collection (Peoria, IL), imported with due permission (Jordbruksverket), and stored on potato dextrose agar (PDA) plates at +4°C. The fungus was recultivated for 7–10 days at room temperature (PDA plates). The 10S/Dox-EAS was prepared as described (28). The Ile conjugate of 18:3n-3 was prepared as earlier described (29) and purified by reversed phase (RP)-HPLC using a solvent system of methanol-water-acetic acid (85:15:0.01, v/v/v). Fot (NRRL 26954) was from ARS Culture Collection (Peoria, IL), imported with due permission (Jordbruksverket), and stored on potato dextrose agar (PDA) plates at +4°C. The fungus was recultivated for 7–10 days at room temperature (PDA plates). The 10S/Dox-EAS was prepared as described (28). The Ile conjugate of 18:3n-3 was prepared as earlier described (29) and purified by reversed phase (RP)-HPLC using a solvent system of methanol-water-acetic acid (85:15:0.01, v/v/v). Fot (NRRL 26954) was from ARS Culture Collection (Peoria, IL), imported with due permission (Jordbruksverket), and stored on potato dextrose agar (PDA) plates at +4°C. The fungus was recultivated for 7–10 days at room temperature (PDA plates). The 10S/Dox-EAS was prepared as described (28).
Enzyme assay

The N2 powder was added to 100 μM [13C3]18:2 n-6 or 100 μM other C18 fatty acids in 0.1 M KH2PO4 (pH 7.4), 2 mM EDTA, and 0.04% Tween-20. After homogenization, the crude homogenate was incubated for 30–40 min on ice. After centrifugation (17,000 g for 10 min, +4°C), we extracted the supernatant with C18 silica, as above.

**GC- and LC-MS/MS analysis**

An Agilent mass selective detector model 5977E connected to an Agilent model 7890A gas chromatograph were used for GC-MS. A capillary column of 5% phenylylmethylsiloxane (12 m, 0.35 μm film thickness) with helium as the carrier gas was used. The temperature was raised from 80°C to 300°C at a rate or 10°C/min. The scan and selected ion monitoring modes were used for data acquisition. For quantitative determination of (-)-JA-Ile and (+)-7-isojasmonoyl(S)-isoleucine [(+)-JA-Ile] in media from cultures of *F. oxysporum*, samples of medium were treated with 30 mM O-methyl hydroxylamine hydrochloride in methanol and 0.5 M sodium acetate (proportions, 1:1:10, v/v/v) at room temperature overnight. After addition of 2.2 μg of [1H3](-)-JA-Ile-MO, the mixture was extracted with ethyl acetate. The product was dissolved in CHCl3/2-propanol, 2:1 (v/v) and applied to an amino propyl cartridge (SupelcoClean LC-NH2). The CHCl3/2-propanol eluate was discarded and the conjugates were obtained by elution with methanol/acetic acid, 98:2 (v/v). The product was treated with diazomethane and subjected to GC-MS using selected monitoring of the ions m/z 335 (unlabeled conjugates) and m/z 338 ([1H3] standard). The peaks of the methyl ester/MO derivatives of (-)-JA-Ile, (+)-JA-Ile, and [1H3](-)-JA-Ile were integrated and the amounts of the two unlabeled conjugates were calculated from the area ratios (following, in the case of (-)-JA-Ile, subtraction of the 0.78% of unlabeled material present in the [1H3] standard). The (+)- and (-)-JA were analyzed in an analogous way using [1H3](-)-JA-MO as an internal standard (M. Hamberg, unpublished observations).

RP-HPLC with MS/MS analysis was performed with a Surveyor MS pump (ThermoFisher) and an analytical or semi preparative octadecyl silica column (5 μm; 2.0 × 150 mm, Phenomenex; 5 μm; 4.6 × 150 mm, Dr. Maisch). The columns were eluted at 0.25–0.3 ml/min or 1 ml/min, respectively, with methanol/water/acetic acid, 600:400:0.05, 650:350:0.05, 700:500:0.05, 750:250:0.05, or 800:200:0.05 (v/v/v). The effluent was subjected to ESI in a linear ion trap mass spectrometer (LTQ; ThermoFisher). The heated transfer capillary was set at 315°C, the ion isolation width at 1.5 amu (5 amu for analysis of hydroperoxides), the collision energy at 35 (arbitrary scale), and the tube lens at −112 V. Samples were injected manually (Rheodyne 7510) or by an autosampler (Surveyor Autosampler Plus; ThermoFisher).

Chiral phase (CP)-HPLC-MS/MS analysis was performed with Reprosil Chiral AM (5 μ; 2 × 300 mm, Dr. Maisch), which was eluted (0.2 ml/min) with hexane/methanol/acetic acid, 95:5:0.02 (v/v/v). The eluate was mixed in-line with 2-propanol/water [3:2 (v/v); 0.15 ml/min] from a second pump (Constametric 3200, LDC/MiltonRoy). The combined effluents were introduced by ESI into the ion trap mass spectrometer above. Normal phase-HPLC-MS/MS was performed in the same way and the silica column (5 μm; 2 × 250 mm; Reprosil 100 SI, Dr. Maisch) was eluted with 2-propanol/hexane/acetic acid, 3:97:0.05 (v/v/v). Hydrogenation of JA-Ile was performed with H2 and catalytic Pd/C, and hydroperoxides were reduced to alcohols with triphenylphosphine.

**RESULTS**

**Analysis of jasmonates in the growth medium**

Cultures were inoculated from agar plates. We first examined the secretion of JA and JA-conjugates by cultures for 3 weeks (22°C) in flasks with CDB with and without shaking (100 rpm) and in the dark or under fluorescent light. Cultures in CDB, which were grown with shaking in the dark, released the largest amounts of (-)-JA-Ile and (+)-JA-Ile, as shown in Fig. 3A. Cultures grown in PDB in the dark (or in subdued light) at 28°C with shaking (100 rpm) released the highest amounts of jasmonates after 3 weeks (Fig. 3A). The amount of (+)- and (-)-JA was only 0.2% of the amount of (+)-JA-Ile in this sample, which is lower than in other strains of *F. oxysporum* (15, 20). Growth in PDB with shaking at 28°C and in the dark yielded the highest levels and these conditions were, therefore, used routinely.

GC-MS analysis showed that the secretion of jasmonates to the PDB was low for the first 4 days, but caught momentum after about a week and reached about 4 mg l⁻¹ (+)-JA-Ile (Fig. 3B). The yield varied between incubations (15).

**Fig. 3.** Formation of JA-Ile conjugates after 3 weeks under different conditions and two time curves. A: Different growth conditions in CDB and a comparison with PDB. Light reduced biosynthesis and it was augmented in shaking flasks (100 rpm). Changing from CDB to PDB and increasing the temperature to 28°C with shaking (100 rpm) yielded the largest amounts of jasmonates and this growth condition was used routinely. B: Partial time curves for biosynthesis of (-)-JA-Ile and (+)-JA-Ile by two different cultures in PDB, which were inoculated from liquid cultures in PDB. Left: The biosynthesis of (+)-JA-Ile and (-)-JA-Ile reached 4.5 and 0.25 mg l⁻¹, respectively, after 8 days. Right: The biosynthesis of (+)-JA-Ile and (-)-JA-Ile reached over 0.22 and 0.01 g l⁻¹, respectively, after 15 days.
Inoculation of PDB with an aliquot of a liquid culture led to accumulation of over 0.23 g l⁻¹ (+)-JA-Ile after 15 days (Fig. 3C). The largest amounts obtained were 0.31 g l⁻¹ (+)-JA-Ile (almost 1 mM).

The elution of jasmonates on RP-HPLC is shown in Fig. 4A. JA-Ile was the main product along with variable relative amounts of 9,10-dihydro-JA-Ile. In addition, small amounts of Ile-Val and 9,10-dihydro-JA-Val were also detected. The MS² spectrum of JA-Ile is shown in Fig. 4B, and dominated by the signal at m/z 130 (Ile carboxylate anion; compare inset in Fig. 4B). The MS² spectra of 9,10-dihydro-JA-Ile, JA-Val, and 9,10-dihydro-JA-Val showed a similar fragmentation pattern (supplemental Fig. S1).

JA was detectable by LC-MS/MS after two rounds of RP-HPLC purification (semi-preparative RP-HPLC, 65% methanol; RP-HPLC-MS/MS, 60% methanol) (Fig. 4C). The MS² spectrum of JA (Fig. 4D) lacked a signal at m/z 59, which is abundant in the mass spectra of JA recorded with triple quadrupole instruments (15, 18).

Oxidation of C₁₈ fatty acids by N₂ powder of mycelia

We first evaluated mycelia grown in CDB. N₂ powder, prepared from nonshaking cultures under light or dark conditions for 3 weeks (22°C) did not oxidize [¹³C₁₈]18:2 n-6, whereas significant oxidation to [¹³C₁₈]10-HPODE was detected in N₂ powder of mycelia from shaking dark cultures at 100 rpm (22°C). This oxidation appeared to be further increased in N₂ powder of mycelia grown with shaking at 28°C (dark). The highest activities were noted of N₂ powder of mycelia, which was grown under these conditions in PDB. The PDB medium also contained the largest amounts of jasmonates, as discussed above (Fig. 3). We conclude that large secretion of jasmonates occurred by mycelia, which also oxidized 18:2 n-6 to 10-HPODE. Mycelia from these cultures in PDB were intensely dark red due to bakaverin expression [confirmed by CHCl₃ extraction and UV analysis, compare (32)].

The N₂ powder oxidized [¹³C₁₈]18:2 n-6 to [¹³C₁₈]10-HPODE and [¹³C₁₈]10-HODE as main products (Fig. 5A). Relatively small amounts of [¹³C₁₈]8-HPODE were also detected. In addition, 10-HODE was formed from endogenous 18:2 n-6 (Fig. 5A). We expected to detect signs of 13S-LOX or 9-DOX activities in the N₂ powder, but neither [¹³C₁₈]9-HPODE nor [¹³C₁₈]13-HPODE could be detected.

Steric analysis of 10-HODE showed that it consisted of the 10R stereoisomer (90%; Fig. 5B). The mass spectrum of [¹³C₁₈]10-HODE is shown in Fig. 5C; the insert compares the mass spectrum with that of unlabeled 10-HODE. A polar metabolite, which was formed from [¹³C₁₈]18:2 n-6, was identified as [¹³C₁₈]12(13)epoxy-10-hydroxy-8E-octadecenoic acid [12(13)Ep-10-HOME] by the mass spectra illustrated in Fig. 5D; the insert compares the spectra of the [¹³C₁₈]12(13) Ep-10-HOME and 12(13) Ep-10-HOME.

The 18:3 n-3 was oxidized in analogy with 18:2 n-6 to 10-HPOTrE and 12(13)epoxy-10-hydroxy-8E,15Z-octadecadienoic acid [12(13)Ep-10-HODE], but 18:1 n-9 was mainly oxidized to 8-HOME (data not shown). The oxidation of 18:2 n-6 was consistent with expression of 10R-DOX-EAS of F. oxysporum (25), but the transformation of 18:1 n-9 and 18:3 n-3 by this enzyme has not been established.

Recombinant 10R-DOX-EAS oxidized 18:1 n-9 to 8-H(P)OME and to small amounts of 10-H(P)OME (supplemental Fig. S2A). The 18:3 n-3 was oxidized in analogy with 18:2 n-6 to 10-HPOTrE and 12(13)Ep-10-HODE (supplemental Fig. S2B, C). In addition, 8- and 16-hydroxyoctadecatrienoic acids (HOTrEs) were also detected. Steric analysis by CP-HPLC-MS/MS showed that the latter was racemic (data not shown). We conclude that the transformations of C₁₈ fatty acids by N₂ powder of mycelia and by recombinant 10R-DOX-EAS are strikingly similar.

Metabolism of 18:3 n-3, [²H₅]18:3 n-3, and [³H₂]13S-HPOTrE by mycelia in buffer

Mycelia, in 2.5 ml of suspended cultures, were harvested by centrifugation and resuspended (2.5 ml) in 0.1 M NaBO₃ (pH 8.2) or 0.1 M KHPO₄ (pH 7.4)/2 mM EDTA/0.02%
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**Tween-20 with or without added 18:3n-3 (2.3 mM) or \([2H5]18:3n-3\) (from 30 μM to 2.3 mM).**

The 2.3 mM 18:3n-3 was extensively metabolized in 1 h to 16-HOTrE, 16-keto-9Z,12Z,13E-octadecatrienoic acid (KOTrE), 16-HOPtE, and 17-KOTrE and to the ω3 epoxide of 18:3n-3 in both buffers within 1 h. The elution of 16- and 17-HOTrE and 16-KOTrE is shown in Fig. 6A. The 16- and 17-KOTrE were separated on ReproSli-Chiral AM, as illustrated by the ion chromatograms for 16-HOTrE (m/z 235; A -58; loss of HCO-CH2-CH3) and 17-HOTrE (m/z 249; A -44; loss of HCO-CH3 or CO2) in Fig. 6B. R stereoisomers of cis-trans conjugated hydroxy fatty acids elute before S stereoisomers on columns with amylose tris-5,5-dimethylphenylcarbamate modified silica (33). The 16R/HOTrE thus appeared to be the main stereoisomer.

**Fig. 6. Oxidation of 18:3n-3 by mycelia of *Fot* during short-time incubation in 0.1 M NaBO3 (pH 8.2).** A: MS/MS analysis of products. Top: MS/MS analysis of HOTrE (m/z 293). Bottom: MS/MS analysis of KOTrE (m/z 291). The insets mark the elution of the identified products, 16- and 17-HOTrE, and 16-KOTrE, respectively. Small amounts of 12-OPDA (marked by arrow) eluted 3 min before 16-KOTrE. B: Chiral HPLC-MS analysis (m/z 293 → full scan) of 16-HOTrE (m/z 235 (A -58); top) and separation of 17-HOTrE (m/z 249 (A -44); bottom).

**Fig. 5. LC-MS/MS analysis of HPODE formed from endogenous and \([^{13}C_{18}]18:2n-6\) by nitrogen powder of *F. oxysporum*.** A: Chromatograms from LC-MS/MS analysis of HODE (top), \([^{13}C_{18}]18:2n-6\) (middle), and \([^{13}C_{18}]10\)HPODE (bottom). The 10-HODE, \([^{13}C_{18}]10\)HODE, and \([^{3}P_{18}]10\)HPODE could be detected along with small amounts of 8-HPODE. B: Steric analysis of 10-HODE formed from 18:2n-6 by the N2 powder. The \([^{13}C_{18}]10R/S\)HODE was added, and the 10-HODE (top) eluted at the same time as the R stereoisomer (bottom). The elution order on this chiral column was established with 10R-HODE formed by recombinant 10R-DOX-EAS (25). C: MS2 spectrum of \([^{13}C_{18}]10\)HODE formed by N2 powder. The insert and the labels describe the corresponding ions in this and the unlabeled spectrum. D: MS2 spectrum of \([^{13}C_{18}]12(13)Ep-10\)HOME. The insert illustrates the ions of the labeled and unlabeled compound.
The transformation of endogenous 18:3\(n\)-3 and 100 \(\mu\)M \([2H5]18:3\(n\)-3 by mycelium of Fot for 1 h appeared to differ. The latter was transformed to \([2H5]12\)-OPDA and also to \([2H5]13\)-KOTriE and \([2H5]\)-labeled 8-, 10-, and 13-HOTrE (Fig. 8A). The \([2H5]11\)-HOTriE could not be detected, which is a major product of Fo-MnLOX (22). Steric analysis by CP-HPLC showed that over 95% of \([2H5]13\)-HOTriE coeluted with 13-SHOTriE (Fig. 8B).

Products formed in a large-scale incubation of mycelia with the KHPO\(_4\) buffer were extracted and purified by semi-preparative RP-HPLC, and 10 \(\mu\)l of 1 ml fractions were analyzed by direct injection into the mass spectrometer. The fraction with 12-OPDA was reduced to dryness and then analyzed by GC-MS, which separated the cis and trans side chain isomers of 12-OPDA (supplemental Fig. S4A). The electron impact mass spectrum is shown in supplemental Fig. S4B. We conclude that 12-OPDA was identified by both LC- and GC-MS analyses.

The \(\alpha\)-ketol was detected when mycelia were incubated with buffer or buffer with \([2H5]18:3\(n\)-3 (Fig. 9A). It is well-known that the allene oxide [12(13)S]epoxy-9Z,11,15Z-octadecatrienoic acid (12,13S-EOT), which is formed from 13S-HOTriE, is hydrolyzed to the \(\alpha\)-ketol by inversion of configuration at C-13 (36). Steric analysis showed that the fungal \(\alpha\)-ketol consisted mainly of the 13R stereoisomer (90%; Fig. 9B), and thus likely originated from 13S-HOTriE via 12,13SEOT.

The MS\(^2\) spectrum of the \(\alpha\)-ketol was noncharacteristic with weak signals except for the trivial signals at m/z 291 (loss of water), 273 (291-18), and 247 (291-44; loss of CO\(_2\)) (supplemental Fig. S5). The MS\(^3\) spectrum of the \(\alpha\)-ketol was more characteristic with strong signals at m/z 165 and 153 (Fig. 9B). These mass spectra were identical with those of the authentic standard. We next purified the [\(^{13}H_3\)]-labeled \(\alpha\)-ketol by normal phase-HPLC. The MS\(^3\) spectrum of [\(^{13}H_3\)]-\(\alpha\)-ketol showed signals, among other things, at m/z 158 (153+5) and 170 (165+5) (Fig. 9C). We also prepared the [\(^{13}H_3\)]-labeled \(\alpha\)-ketol and [\(^{2}H_5\)]12-OPDA from [\(^{13}H_3\)]13S-HOTriE using AOS in acetone powder of flaxseed (37), and their MS\(^2\) and MS\(^3\) spectra were identical with the fungal metabolites.

We expected exogenous 13S-HOTriE to be transformed to 12-OPDA. Incubation of mycelia with 100 \(\mu\)M [\(^{13}H_3\)]13S-HOTriE for 1 h did not lead to significant amounts of

![Fig. 7. LC-MS/MS analysis of the transformation of 300 \(\mu\)M [\(^{13}H_3\)]18:3\(n\)-3 by mycelium of Fot for 1 h. A: The mycelium released endogenous 12-OPDA and newly formed [\(^{13}H_3\)]12-OPDA to the KHPO\(_4\) buffer, as shown by the top and bottom chromatograms, respectively. B: LC-MS/MS analysis of 12-OPDA. C: LC-MS/MS analysis of [\(^{13}H_3\)]12-OPDA. The inset in (C) shows the formation of the fragmentation ion at m/z 165 and 170, respectively, in (A, C) (35).](image)

![Fig. 8. Mycelia transform endogenous and \([^{13}H_3]18:3\(n\)-3 to partly different products. A: RP-HPLC-MS analysis of products. The top chromatogram shows that endogenous 18:3\(n\)-3 is transformed to 12-OPDA, but not to significant amounts of 13-KOTriE. The middle and bottom chromatograms show that \([^{13}H_3]18:3\(n\)-3 is transformed to \([^{13}H_3]12\)-OPDA and also to \([^{13}H_3]13\)-KOTriE and \([^{13}H_3]\)-labeled 8-, 10-, and 13-HOTriE (Fig. 8A). The \([^{13}H_3]11\)-HOTriE could not be detected, which is a major product of Fo-MnLOX (22). Steric analysis by CP-HPLC-MS/MS of \([^{13}H_3]13\)-HOTriE (bottom) showed that over 95% co-chromatographed with 13-SHOTriE (top chromatogram).](image)
to two major products, identified as N-[12-oxo-13-hydroxy-9Z,15Z-octadecadienoyl]-L-isoleucine (α-ketol-Ile; 90%) and 12-OPDA-Ile (10%) (supplemental Fig. S7A). The MS² spectra of 12-OPDA-Ile and the MS³ spectrum of the α-ketol-Ile are shown in supplemental Fig. S7B. C. The spectrum of the former was identical to the reported MS³ spectrum of 12-OPDA-Ile (38). Small amounts of 13HOTrE-Ile were also detected. In contrast, mycelia of Fot oxidized 100 μM 18:3n-3-Ile to three major products, likely formed by the prominent subterminal ω hydroxylase activities. The 12-OPDA-Ile or α-ketol-Ile could not be detected.

Mycelia of Fot did not release detectable amounts of 12-OPDA-Ile or the α-ketol-Ile conjugate. We conclude that conjugation of (+)-JA with Ile likely occurs as a final step.

**DISCUSSION**

We report a prominent biosynthesis of the JA conjugate (+)-JA-Ile by the fungus Fot, about 0.2 g l⁻¹. This is of the same magnitude as that reported for the biosynthesis of (+)-JA by L. theobromae, which commonly produces 0.3–0.5 g l⁻¹ (17, 19). The prominent (+)-JA biosynthesis by Fot enabled our main finding, the detection of two key intermediates in plant biosynthesis of (+)-JA. The [2H₅]₁₈:₃n-3 was converted by mycelia of Fot to [2H₅]₁₂-OPDA and to an allene oxide-derived α-ketol, [2H₅]₁₂-oxo-1₃-hydroxy-9Z,1₅Z-octadecadienoic acid. In addition, 1₃S-HOTrE and 1₃KOTrE could be detected, and [2H₅]₁₅S-HOTrE was also converted to 1₂-OPDA, albeit in low yields. This suggests that the initial steps of the fungal biosynthetic pathway to jasmonates are analogous to those operating in plants (Fig. 10).

The first step is likely oxidation of 1₈:₃n-3 to 1₃S-HOTrE. As far as is known, the fungal DOXs of the cyclo-oxygenase gene family do not oxidize 1₈:₃n-3 at C-1₃, but there are two possible LOX candidates (Fig. 2). These LOXs have only been studied by recombinant expression and expressed sequence tags have not yet been detected in F. oxysporum.² Mo-MnLOX is likely secreted and forms 1₁₁-HOTrE as a major metabolite (22). The latter could not be detected. The 1₃SLOX with catalytic iron is therefore the obvious candidate (26).

We have little information on the other fungal enzymes leading to biosynthesis of (+)-JA-Ile. AOS, AOC, 1₂-OPDA reductase (OPR3), and JAR1 proteins of Arabidopsis thaliana are well-characterized (39–42). The dbEST of NCBI (https://www.ncbi.nlm.nih.gov).

²BLAST analysis at NCBI showed that AOS (GenBank AAF09255) and 1₂-OPDA reductase (OPR3; GenBank OA905653) of A. thaliana could be aligned with putative CYP (27% identity; 24% query cover) and with a putative NADPH-dependent dehydrogenase (45% identity; 97% query cover) of F. oxysporum Fo47. We could not detect homologs of AOC or JAR1 in the genome of F. oxysporum.³ Both

**Metabolism of 1₈:₃n-3-Ile by mycelia of Fot**

Standards were prepared with flaxseed AOS. The 1₃S-HOTrE-Ile was obtained by oxidation with sLOX-1 and further transformed by acetone powder of flaxseed (AOS)
Plant enzymes lack catalytic metals. The active site of JAR1 relies on an adaptable three-dimensional scaffold (8) and the crystal structure of AOC indicates an active site inside a barrel cavity (10). It seems likely that fungal AOC and acyl amino acid synthase could be examples of parallel evolution of active sites.

α-Ketols are formed by nonenzymatic hydrolysis of allene oxides taking place with predominant inversion of configuration (43). The α-ketol of the present work was mainly 13R, suggesting its formation from 12,13S-EOT. The latter is likely formed from 13S-HPOTrE by a 13S-AOS. AOSs belong to two families of heme proteins, CYP of plants and fungi and catalases of corals and cyanobacteria (36, 44, 45). Fungal AOSs have so far only been detected in DOX-CYP fusion proteins, e.g., 8R, 8S, 9R, and 9S-DOX-AOS and not in fungal catalases (24, 46–48). In contrast to plant CYP74, which contains a characteristic insertion sequence of seven to nine amino acids in the Cys pocket, the fungal AOSs of DOX-AOS enzymes do not share common characteristic sequences (9, 47, 49). It will be a challenging task to identify the putative 13S-AOS. The efficient synthesis of jasmonates in fungi suggests a tight coupling between the AOS and cyclase activities and even the possibility that they may reside in the same protein (compare Ref. 50).

The 12-OPDA is formed in plants by AOS and AOC in plastids. The location of the fungal AOS and AOC is unknown. Eukaryotic CYP enzymes are typically membrane-bound and found in the endoplasmic reticulum. β-Oxidation in fungi only occurs in peroxisomes (51). In analogy with plants, 12-OPDA could be transferred to peroxisomes where the ring double bond could be reduced and the side chain shortened by β-oxidation.

We expected N2 powder of mycelia to oxidize 18:3n-3 to 13S-HPOTrE, but this was not detected with certainty. This may explain why the fungal biosynthesis of JA has been enigmatic. Biosynthesis of (+)-JA-Ile in shaking cultures in PDB was associated with prominent expression of 10R-DOX-EAS and bikaverin, which colored the mycelium dark red. Bikaverin biosynthesis in *Fusarium* and JA biosynthesis by *L. theobromae* start when glucose and/or nitrogen have been partly consumed and can be optimized in different ways (17, 18). It is not unlikely that the biosynthesis of jasmonates by Fot also can be optimized.

How can the enzymes in the fungal cascade to (+)-JA-Ile be identified? The classical method to purify enzymes from cell-free preparations does not appear to be feasible due to undetectable enzyme activities. This is in striking similarity to the undetectable prostaglandin biosynthesis by subcellular fractions of the coral, *Plexaura homomalla* (52). This enigma was solved by recombinant expression of the coral cyclooxygenase (52). The 13S-LOX of *F. oxysporum* has also been expressed (26) and we detected biosynthesis of 13S-HOTrE by the mycelium. The enzymes further down the biosynthetic pathway to (+)-JA-Ile are unknown. It is possible that comparison of mRNA expression under growth conditions with little and augmented JA biosynthesis might generate hypotheses for subsequent recombinant enzyme expression and analysis.

Access to strains of *L. theobromae* with a high capacity to form (+)-JA is restricted by environmental and commercial considerations. In contrast, Fot is generally available for future studies from the ARS culture collection. Our study raises many questions, which could be investigated in mycelia of Fot, e.g., the effect of gene deletion of 13SLOX,

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**Fig. 10.** Proposed pathway for the formation in *F. oxysporum* of 12-OPDA and its further conversion to (+)-JA-Ile. Involvement of *F. oxysporum* iron 13SLOX (26) in the initial step was deduced from the parallel formations of 13S-HOTrE, 13KOTrE, and a 13R-configured α-ketol. The nature of the enzyme activities needed for allene oxide formation and cyclization is unknown.
the subcellular location of the biosynthesis of 12-OPDA, the reduction of the ring double bond, and conditions for optimal biosynthesis of (+)-JA-Ile in liquid cultures.

CONCLUSIONS

We have identified 12-OPDA as an intermediate, which was formed from 18:3w-3 and 13S-HPOTrE, in the biosynthesis of jasmonates in F. oxysporum. An α-ketol was also detected, which provides evidence for an allene oxide serving as the immediate precursor of 12-OPDA. It seems likely that further conversions of 12-OPDA take place as established for higher plants, i.e., by reduction of the ring double bond, β-oxidation, and conjugation with Ile.27

REFERENCES

1. Ma, L. J., D. M. Geiser, R. H. Proctor, A. P. Rooney, K. O’Donnell, F. Traill, D. M. Gardiner, J. M. Manners, and K. Kazan. 2013. Fusarium pathogenomics. Annu. Rev. Microbiol. 67: 399–416.
2. Michie, C. B., and M. Rep. 2009. Pathogen profile update: Fusarium oxysporum. Mol. Plant Pathol. 10: 311–324.
3. Ploetz, R. C. 2015. Fusarium wilt of banana. Phytopathology. 105: 1512–1521.
4. Demole, E., L. Deretre, and D. Mercier. 1962. Isolation and determination of the subcellular location of the biosynthesis of 12-OPDA, in the biosynthesis of jasmonates in F. oxysporum. The immediate precursor of 12-OPDA. It seems likely that further conversions of 12-OPDA take place as established for higher plants, i.e., by reduction of the ring double bond, β-oxidation, and conjugation with Ile.27

Production of jasmonic acid by Lasiodiplodia theobromae reveals formation of valuable plant secondary metabolites. PLoS One. 11: e0167627.
19. Eng, F., M. Gutiérrez-Rojas, and E. Favela-Torres. 1998. Culture conditions for jasmonic acid and biomass production by Botryodiplodia theobromae in submerged fermentation. Process Biochem. 33: 715–720.
20. Miersch, O., H. Bohlmann, and C. Wasternack. 1999. Jasmonates and related compounds from Fusarium oxysporum. Phytochemistry. 50: 517–523.
21. Tsukada, K., K. Takahashi, and K. Nabetca. 2010. Biosynthesis of jasmonic acid in a plant pathogenic fungus, Lasiodiplodia theobromae. Phytochemistry. 71: 2019–2023.
22. Wernman, A., A. Magnusson, M. Hamberg, and E. H. Oliiv. 2015. Manganese lipoxygenase of Fusarium oxysporum and the structural basis for biosynthesis of distinct 11-hydroprosteroxyozoners. J. Lipid Res. 56: 1606–1615.
23. Sooman, L., and E. H. Oliiv. 2015. Discovery of a novel linoleate dioxygenase of Fusarium oxysporum and linoleate diole synthase of Colletotrichum graminicola. Lipids. 50: 1243–1252.
24. Hoffmann, I., and E. H. Oliiv. 2013. Discovery of a linoleate 9S-dioxygenase and an allene oxide synthase in a fusion protein of Fusarium oxysporum. J. Lipid Res. 54: 3471–3480.
25. Hoffmann, I., F. Jernner, and E. H. Oliiv. 2014. Epoxy alcohol synthase of the rice blast fungus represents a novel subfamily of jasmonoyl-ester-chymo-xyenzyme P450 fusion enzymes. J. Lipid Res. 55: 2115–2123.
26. Brodthum, F., A. Cristofal-Sarramian, S. Zabel, J. Newie, M. Hamberg, and I. Feussner. 2013. An iron 13S-lipoxygenase with an alpha-linolenic acid specific hydroperoxidase activity from Fusarium oxysporum. PLoS One. 8: e64919.
27. Chen, Y., F. Jernner, and E. H. Oliiv. Purification and site-directed mutagenesis of monogalactose of 9S-dioxygenase-allene oxide synthase of Fusarium oxysporum confirms the oxygenation mechanism. Arch. Biochem. Biophys. Epub ahead of print. May 11, 2017; doi:10.1016/j.abb.2017.05.007.
28. Fonseca, S., A. Chini, M. Hamberg, B. Adie, A. Porzel, R. Kramell, O. Miersch, C. Wasternack, and R. Solano. 2009. (+)-7-isoojasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat. Chem. Biol. 5: 344–350.
29. Koch, T., K. Krumm, V. Jung, J. Engelberth, and W. Boland. 1999. Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. Plant Physiol. 121: 153–162.
30. Monte, I., M. Hamberg, A. Chini, S. Gimenez-Ilizabe, G. Garca-Cardado, A. Porzel, F. Pazos, M. Boter, and R. Solano. 2014. Rational design of a ligand-based antagonist of jasmonate perception. Nat. Chem. Biol. 10: 671–676.
31. Powell, W. S. 1980. Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. J. Lipid Res. 21: 947–952.
32. Balan, J., J. Fuksa, I. Kahr, and V. Kuhrova. 1972. Bikeranin, an antibotic from Gibberella fujikoi, effective against Leishmania brasilensis. Folia Microbiol. (Praha). 15: 470–484.
33. Schneider, C., Z. Yu, W. E. Boeglin, Y. Zheng, and A. R. Brash. 2007. Enantiomeric separation of hydroxy and hydroperoxy eicosanoids by chiral column chromatography. Methods Enzymol. 433: 143–157.
34. Nakayama, N., A. Takemae, and H. Shoun. 1996. Cytochrome P450, a catalytically self-sufficient fatty acid hydroxylase of the fungus Fusarium oxysporum. J. Biochem. 119: 435–440.
35. Bao, J., X. Gao, and A. D. Jones. 2014. Unusual negative charge-directed fragmentation: collision-induced dissociation of cyclopentene oxylipins in negative ion mode. Rapid Commun. Mass Spectrom. 28: 457–464.
36. Brash, A. R. 2009. Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes. Phytochemistry. 70: 1522–1531.
37. Zimmerman, D. C. 1966. A new product of linoleic acid oxidation by a flavoxed enzyme. Biochem. Biophys. Res. Commun. 23: 398–402.
38. Floková, K., F. Feussner, C. Herrfurth, O. Miersch, V. Mik, D. Tarkowsk, M. Strnad, I. Feussner, C. Wasternack, and O. Novák. 2016. A previously undescribed jasmonate compound in flowering Arabidopsis thaliana - the identification of cis- (+)-OPDA-Ile. Phytochemistry. 122: 230–237.
39. Schaller, F., C. Biesgen, C. Musig, T. Altmann, and E. W. Weiler. 2005. 12-Oxophytodienoate reductase 3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta. 210: 979–984.
40. Schaller, F., P. Zerbe, S. Reinbothe, C. Reinbothe, E. Hofmann, and S. Pollmann. 2008. The allene oxide cyclase family of Arabidopsis thaliana: localization and cyclization. *FEBS J.* **275**: 2428–2441.

41. Staswick, P. E., I. Tiryaki, and M. L. Rowe. 2002. Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *Plant Cell.* **14**: 1405–1415.

42. Laudert, D., U. Pfannschmidt, F. Lottspeich, H. Hollander-Czytko, and E. W. Weiler. 1996. Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Mol. Biol.* **31**: 323–335.

43. Hamberg, M. 1987. Mechanism of corn hydroperoxide isomerase: detection of 12(13)-oxide-9(Z),11-octadecadienoic acid. *Biochim. Biophys. Acta.* **920**: 76–84.

44. Oldham, M. L., A. R. Brash, and M. E. Newcomer. 2005. The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide. *Proc. Natl. Acad. Sci. USA.* **102**: 297–302.

45. Gao, B., W. E. Boeglin, Y. Zheng, C. Schneider, and A. R. Brash. 2009. Evidence for an ionic intermediate in the transformation of fatty acid hydroperoxide by a catalase-related allene oxide synthase from the cyanobacterium *Aca-trychloris marina*. *J. Biol. Chem.* **284**: 22087–22098.

46. Hoffmann, I., F. Jernerén, and E. H. Oliw. 2013. Expression of fusion proteins of Aspergillus terreus reveals a novel allene oxide synthase. *J. Biol. Chem.* **288**: 11459–11469.

47. Oliw, E. H., M. Arago, Y. Chen, and F. Jernerén. 2016. A new class of fatty acid allene oxide formed by the DOX-P450 fusion proteins of human and plant pathogenic fungi, C. inimitis and Z. tritici. *J. Lipid Res.* **57**: 1518–1528.

48. Teder, T., W. E. Boeglin, C. Schneider, and A. R. Brash. 2017. A fungal catalase reacts selectively with the 13S fatty acid hydroperoxide products of the adjacent lipoxygenase gene and exhibits 13S-hydroperoxide-dependent peroxidase activity. *Biochem Biophys Acta.* **1862**: 706–715.

49. Stumpe, M., and I. Feussner. 2006. Formation of oxylipins by CYP74 enzymes. *Phytochem. Rev.* **5**: 347–357.

50. Grechkin, A. N., L. S. Mukhtarova, L. R. Latypova, Y. Gogolev, Y. Y. Toporkova, and M. Hamberg. 2008. Tomato CYP74C3 is a multi-functional enzyme not only synthesizing allene oxide but also catalyzing its hydrolysis and cyclization. *ChemBioChem.* **9**: 2498–2505.

51. Martin, J. F., R. V. Ullán, and C. García-Estrada. 2012. Role of peroxisomes in the biosynthesis and secretion of beta-lactams and other secondary metabolites. *J. Ind. Microbiol. Biotechnol.* **39**: 367–382.

52. Valmsen, K., I. Jarving, W. E. Boeglin, K. Varvas, R. Koljak, T. Pehk, A. R. Brash, and N. Samel. 2001. The origin of 15R-prostaglandins in the Caribbean coral *Plexaura homomalla*: molecular cloning and expression of a novel cyclooxygenase. *Proc. Natl. Acad. Sci. USA.* **98**: 7700–7705.