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Diverse rare lipid-related metabolites including ω-7 and ω-9 alkenylitaconic acids (ceriporic acids) secreted by a selective white rot fungus, *Ceriporiopsis subvermispora*

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ABSTRACT

Ceriporiopsis subvermispora is a selective white-rot fungus that secretes alk(en)ylitaconic acids named ceriporic acids, known as ion redox silencers. In this study, we analysed a series of extracellular lipid-related metabolites produced by the fungus and found that a wide variety of ceriporic acids and fatty acids, including those with odd-numbered and very long-chains, were produced in wood meal cultures. Two new ceriporic acids, (*R*)-3-[(*Z*)-tetradec-7-enyl]-itaconic acid (ceriporic acid E) and (*R*)-3-[(*Z*)-tetradec-5-enyl]-itaconic acid (ceriporic acid F), were for the first time identified by dimethyl disulfide derivatisation, followed by GC/EI-MS, ^{1}H and homonuclear J-resolved 2D NMR and feeding experiments with [^{13}C-U] glucose coupled with multiple-stage mass spectrometry. In separation by GC and LC, a reversed correlation of elution sequences between a nonpolar GC column and an ODS-LC column for *cis* and *trans* isomers of ω7 and ω9 lipids was found, and the elution of new metabolites was in accordance with the prevailing theory. The biosynthetic precursors of ceriporic acid F can be proposed as oxaloacetate and 16:1Δ7-CoA. Because fatty acids biosynthesised from 16:1Δ7-CoA have been reported for only a limited number of organisms, the highly individual structure of ceriporic acid F is highlighted.

1. Introduction

In nature, the degradation of lignin in wood occurs primarily through the action of lignin-degrading basidiomycetes known as white-rot fungi; hence, this ecological group has received considerable research attention. Most white-rot fungi simultaneously decompose lignin and cellulose, accompanied by the erosion of wood cell walls, while some fungi – called selective white-rot fungi such as *Ceriporiopsis subvermispora* – are able to degrade lignin without causing extensive damage to the cellulose (Messner and Srebotnik, 1994; Blanchette et al., 1997). In the biological conversion of woody biomass, selective lignin degradation is a key process because cell wall polysaccharides in wood are surrounded by lignin. Ceriporic acid is a major extracellular metabolite of a selective white-rot fungus *C. subvermispor*a. The chemical structure of ceriporic acid is alkyl- and alkenylitaconate. An important function of ceriporic acid is the suppression of Fe(III) reduction to inhibit hydroxyl radical (•OH) production by the Fenton reaction system in the presence of iron reductants such as hydroquinone and cysteine. The metabolite inhibits depolymerisation of cellulose by the Fenton reaction system (Watanabe et al., 2002; Rahmawati et al., 2005; Ohashi et al., 2007). Previously, we reported that five ceriporic acids – (*R*)-3-tetradecylitaconic acid (ceriporic acid A), (*R*)-3-hexadecylitaconic acid (ceriporic acid B), (*R*)-3-[(*Z*)-hexadec-7-enyl]-itaconic acid (ceriporic acid C), (*R*)-3-[(*E*)-hexadec-7-enyl]-itaconic acid (ceriporic acid D) and (*R*)-3-(7,8-epoxy-hexadecyl)-itaconic acid (epoxy ceriporic acid) – have been isolated and identified from the cultures of *C. subvermispora* (Enoki et al., 1999, 2000, 2002; Amirta et al., 2003; Nishimura et al., 2008, 2009, 2011). It has been proposed that ceriporic acids should be biosynthesised from oxaloacetate and acyl-CoA (Gutiérrez et al., 2002). A stereoselective biosynthetic pathway was proposed on the basis of the identification of the (*R*)-configuration of the asymmetric centre at the C-3 position of ceriporic acid (Nishimura et al., 2009). The biosynthetic route from acyl-CoA for ceriporic and fatty acids attracts interest because of the relationship between the production of ceriporic acids and fatty acids by the fungus. To further understand the lipid metabolism and its relationship with selective wood decay, we analysed ceriporic acids and fatty acids produced by *C. subvermispora* in wood meal cultures. In this study, a wide variety of ceriporic acids and fatty acids, including those with odd-numbered and very long-chains, were produced in wood meal cultures. New ceriporic acids with ω7 and ω9 alkenyl structures were identified (Fig. 1). In addition, the biosynthesis of lipid-related metabolites is discussed, with special emphasis on the position of a double bond in a side chain.

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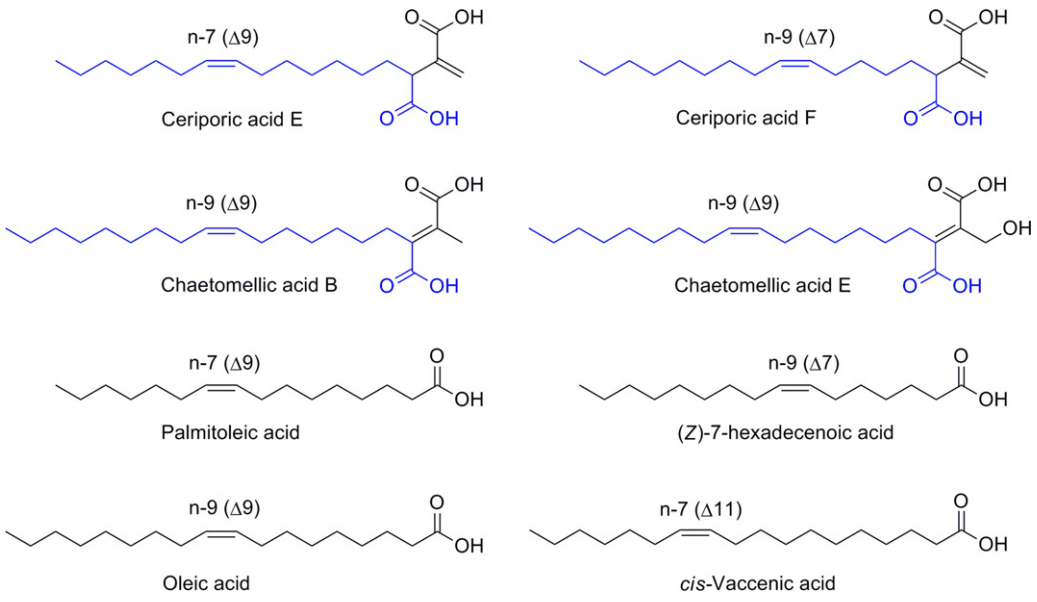

Fig. 1. Chemical structures of ceriporic acids E and F. Structurally related lichen acids and fatty acids having unsaturated side chains (*n*-7 and *n*-9).

2. Experimental methods

2.1. Materials

Acetonitrile and formic acid (LCMS analytical grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), as were oleic acid (C18:1 *n*-9 *cis*) and elaidic acid (C18:1 *n*-9 *trans*). *cis*-vaccenic acid (C18:1 *n*-7 *cis*) and vaccenic acid (C18:1 *n*-7 *trans*) were obtained from GL Sciences Inc. (Tokyo, Japan). Other standard fatty acid methyl esters, methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1 *n*-7 *cis*), methyl heptadecanoate (C17:0), methyl stearate (C18:0), methyl oleate (C18:1 *n*-9 *cis*), methyl nonadecanoate (C19:0), methyl arachidate (C20:0), methyl *cis*-11-eicosenoate (C20:1 *n*-9) and methyl behenate (C22:0) were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Methyl linoleate (C18:2 *n*-6 *cis*) and dimethyl disulfide (DMDS) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). DMDS was distilled before use. [U-^{13}C] glucose (99% ^{13}C enriched) was obtained from Cambridge Isotope Laboratories (Mass, USA). *n*-Tetracosane was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Trimethylsilyldiazomethane (2 M solution in hexanes) was obtained from Sigma–Aldrich Japan (Tokyo, Japan). The other reagents used were of analytical grade.

2.2. Culture conditions of *C. subvermispora* and extraction of metabolites

C. subvermispora ATCC90467 was grown onto a potato dextrose agar medium at 28 °C for five days. Next, *C. subvermispora* was statically pre-cultured in a modified BIII medium (Nishimura et al., 2008) at 28 °C for two weeks. After the pre-cultivation, fungal mycelia (1 g, wet weight) were collected and blended with 20 mL of the modified BIII medium in a Waring blender. In a 300-mL Erlenmeyer flask, 500 μL of the mycelium suspension was inoculated onto a wood meal medium and incubated statically at 28 °C for one to four weeks. The wood meal medium contained 5.0 g of Willey milled wood (dry weight per flask, sapwood of Japanese cedar: *Cryptomeria japonica* D. Don) and 14.5 mL of modified BIII medium without glucose (per flask). After incubation, the mycelia and culture fluid were filtrated through a nylon mesh filter (30 μm, NY-30, Tokyo Rikagaku Kikai Co., Ltd., Tokyo, Japan) and washed with 50 mL of water using a vacuum pump. The extracellular fraction (filtrate) was concentrated and analysed by GC/EI-MS and LC/IT-TOF-MS.

2.3. Purification of ceriporic acids E and F by HPLC

Ceriporic acids E and F were separated on a reversed-phase Inertsil ODS-SP column (250 mm × 4.6 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) using a Shimadzu HPLC system. The column was eluted in the linear gradient mode using two solvent mixtures: CH_3CN/0.1% aq. HCOOH = 20:80 (A) and CH_3CN (B) at a flow rate of 1.2 mL min^{-1}. The initial percentage of solvent A was 70%. The percentage of solvent B was constant for the initial 5 min, increased to 55% during the 5–55 min range and then further increased to 95% over the 55–80 min range. In the 80–90 min range, the percentage of solvent A was maintained at 95%. The elution was monitored at 210 nm.

2.4. GC/EI-MS measurements

GC/EI-MS analyses of the extracted metabolites were performed using a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer (Kyoto, Japan) on a DB-5HT column (30 m × 0.25 mm, 0.1 μm; J & W Scientific Inc., CA, USA). The electron impact-mass spectrum (EI-MS) was recorded at an ionization energy of 70 eV. Helium was used as a carrier gas at a constant flow rate of 1.2 mL min^{-1}. A splitless injection of 1 μL was made at 280 °C. The column oven temperature was maintained at 50 °C for 3 min and subsequently raised from 50 to 160 °C at the rate of 30 °C min^{-1}, from 160 to 250 °C at 3 °C min^{-1}, from 250 to 300 °C at 15 °C min^{-1} and maintained at 300 °C for 5 min.

For dimethyl ceriporic acid, the following GC oven temperature program was used: initial temperature 50 °C held for 3 min and subsequently raised from 50 to 155 °C at the rate of 30 °C min^{-1}, from 155 to 190 °C at 8 °C min^{-1}, from 190 to 250 °C at 3 °C min^{-1} from 250 to 300 °C at 12 °C min^{-1} and maintained at 300 °C for 5 min.

Elution sequence analyses of *n*-7 and *n*-9 fatty acids and ceriporic acids E and F were performed using a Shimadzu GCMS-QP2010A gas chromatograph mass spectrometer (Kyoto, Japan) on a DB-5MS column (30 m × 0.25 mm, 0.25 μm; J & W Scientific