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PAPER

Alkadienyl and alkenyl itaconic acids (ceriporic acids G and H) from the selective white-rot fungus *Ceriporiopsis subvermispora*: a new class of metabolites initiating ligninolytic lipid peroxidation†

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New ceriporic acids—alkadienyl and alkenyl itaconic acids having a bis-allyl (3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconic acid; ceriporic acid G) and a monoene (3-[(*Z*)-octadec-9-enyl]-itaconic acid; ceriporic acid H) structure in their side chains—were isolated from the cultures of the selective lignin-degrading fungus *Ceriporiopsis subvermispora*. The new metabolites ceriporic acid G and H were synthesized by a cross-aldol condensation and a Grignard substitution reaction, respectively. Ceriporic acid G triggered the manganese peroxidase (MnP)-catalyzed lipid peroxidation and decomposed a recalcitrant non-phenolic lignin substructure model compound. Except for simple fatty acids, this is the first report of a fungal metabolite that induced ligninolytic lipid peroxidation.

Introduction

White-rot fungi play a key role in the carbon cycle in earth's ecosystems because they efficiently degrade plant cell walls that are impregnated with lignin. Most white-rot fungi simultaneously degrade lignin and cellulose, while selective white-rot fungi such as *Ceriporiopsis subvermispora* degrade lignin without intensive damage to cellulose. In lignin biodegradation, the cleavage of recalcitrant non-phenolic substructures has been regarded as an essential prerequisite for the efficient degradability of lignin by enzymatic reactions. For instance, lignin peroxidase has been proposed to drive lignin degradation because of its high oxidation potential, which is sufficiently high to degrade the non-phenolic lignin substructure. However, the selective white-rot fungus *C. subvermispora* degrades the recalcitrant non-phenolic lignin substructure without expressing detectable LiP.[1,2] Lipid peroxidation catalyzed by manganese peroxidase (MnP) was proposed as a mechanism for the lignin biodegradation, because the reaction decomposed the non-phenolic lignin dimer model compound.[3] Accumulation profiles of fatty acids, lipid hydroperoxides, aldehydes, and titers of MnP in wood cultures of *C. subvermispora* supported the fact that lipid peroxidation by MnP is involved in the incipient stage of wood decay by the fungus.[4]

In lignin degradation by *C. subvermispora*, enzymes secreted by the fungus remain inside the wood lumens and do not penetrate into the degradation sites in the wood cell walls, even after extensive decay.[5,6] This phenomenon implies that diffusible low-molecular-weight metabolites are principally responsible for lignin degradation. Manganic ion, an oxidation product of MnP, is stabilized by organic acids like oxalate so it can diffuse into wood cell walls. Although the oxidation potential of the Mn^{3+} chelate is very low to decompose the non-phenolic lignin substructure, the diffusible oxidant initiates lipid peroxidation by abstracting hydrogen from unsaturated fatty acids like linoleic acid to decompose the lignin structure. Analysis of the oxidation pathway of linoleic acid revealed that the diffusible Mn^{3+} chelate generates free radicals from unsaturated fatty acids and hydroperoxides. This accounts for the lignin degradation at a site far from enzymes.[7,8] Recently, we demonstrated that alkoxy- and carbon-centered radicals are primary agents in degrading non-phenolic lignin substructure model compounds.[9]

In addition to the fatty acids involved in lipid peroxidation, new lipid-related secondary metabolites suppressing the ion redox cycle have been found from the culture of *C. subvermispora*. Thus far, six novel alk(en)ylitaconic 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), (*R*)-3-[(*Z*)-tetradec-7-enyl]-itaconic acid (ceriporic acid E), and (*R*)-3-[(*Z*)-tetradec-5-enyl]-itaconic acid (ceriporic acid F)—and epoxy ceriporic acid have been isolated and identified from the cultures of *C. subvermispora*.[4,10–16] Minor ceriporic acids having odd-numbered side chain lengths were also identified.[16] It was demonstrated that alkyl itaconic acids suppressed the

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reduction of ferric ions, thereby suppressing the production of celluloytic hydroxyl radicals (˙OH) *via* the Fenton reaction system.[17–19]

Ceriporic acids are the major class of extracellular metabolites of this fungus and may play versatile roles in wood decay in addition to suppressing the Fenton reaction system. Here we report new alkenyl and alkadienyl itaconic acids from the family of ceriporic acids. The alkadienyl itaconic acid is a readily oxidizable metabolite and plays a role in initiating ligninolytic lipid peroxidation. Possible roles of the new ceriporic acids in lignin biodegradation are discussed.

Experimental methods

Materials

Dimethyl itaconate was obtained from Wako Pure Chemical Industries (Osaka, Japan). Magnesium turnings were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Oleyl chloride was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). ^{13}C-glucose (U-^{13}C6, 99%) was obtained from Cambridge Isotope Laboratories (Massachusetts, USA). All reagents used were of analytical grade.

Nylon mesh filter (30 μm, NY-30, Tokyo Rikagaku Kikai co., Ltd, Tokyo, Japan) was washed with ethanol, acetone, and hot water in turn before use. Non-phenolic syringyl and guaiacyl types of β-aryl ether lignin model compounds were synthesized as previously described.[20]

C. subvermispora culture conditions and extraction of metabolites

C. subvermispora ATCC90467 was grown on a potato dextrose agar medium at 25 °C for 5 days. In a 500 mL Erlenmeyer flask, 200 mL of the preculture was inoculated into an SDW medium and incubated statically at 28 °C for 4 weeks. The SDW medium contained glucose (10.0 g L^{-1}), Difco Yeast Nitrogen base without amino acid (3.35 g L^{-1}), and Japanese beech wood blocks (2.0 × 2.0 × 1.0 cm, four pieces per flask). After incubation, the mycelia and culture fluid were filtered by a nylon mesh filter (30 μm, NY-30, Tokyo Rikagaku Kikai Co., Ltd, Tokyo, Japan) using a vacuum pump. The extracellular fraction was freeze-dried and extracted with acetonitrile–water (v/v, 90 : 10).

Administration of ^{13}C-labeled glucose to *C. subvermispora*

C. subvermispora was precultured in a modified BIII medium[16] at 28 °C for 12 days. In the modified medium, $(NH_4)_2SO_4$ was used as the nitrogen source instead of NH_4NO_3, and the amount of glucose was reduced from 1.0% to 0.5%. The blended mycelium from the precultures was inoculated into 50 mL of the modified BIII medium in a 300 mL Erlenmeyer flask and incubated statically at 28 °C for 19 days. In the main cultures, ^{13}C-[U, 99%]-glucose was used as the sole carbon source. After cultivation, the fungal mycelium was filtered. The filtrate containing extracellular metabolites was freeze-dried and extracted with acetonitrile–water (v/v, 90 : 10). The extract was subjected to LC/IT-TOF-MS analysis.

Chemical synthesis of ceriporic acid H

Synthesis of dimethyl-α-(bromomethyl) fumarate. Dimethyl itaconate (7.91 g) was dissolved in tetrachloromethane (40 mL) and refluxed at 90 °C in a two-necked flask. To the flask, bromine (2.61 mL) was added dropwise with constant stirring. The reaction mixture was cooled on ice, stirred for 16 h, and extracted with CCl_4–water. After drying over Na_2SO_4, the lower layer was separated and concentrated. Dimethyl-α-(bromomethyl) fumarate was purified by silica-gel chromatography (eluent: hexane–Et_2O, 85 : 15, yield: 4.71 g, 40%).

Lithium copper bromide ($LiCuBr_2$). Lithium copper bromide was synthesized by mixing CuBr (286.2 mg) and LiBr (176.8 mg) in 2 mL of THF for 30 min on ice in an argon atmosphere.

Grignard reagent (octadec-9-en-1-magnesium chloride). To a vigorously stirred suspension of magnesium turnings in THF (30 mL) was added oleyl chloride (15.1 g) dropwise. The progress of the reaction was monitored by GC. For the reaction to proceed to the completion, a total of 60 μL of 1,2-dibromoethane (0.045 mol%, 2.11 mmol) was added and the reaction mixture was occasionally heated by a heat gun to keep the gentle refluxing. After the GC analysis showed the consumption of oleyl chloride, the concentration of the Grignard reagent was determined to be 1.01 M by titration.

Grignard substitution reaction

Dimethyl-α-(bromomethyl) fumarate (2.63 g) in THF (55 mL) was cooled at −78 °C. $LiCuBr_2$ (551 μL) was added to the solution under an argon atmosphere. To this mixture at −78 °C was added octadec-9-en-1-magnesium chloride (13.1 mL) dropwise over 1 h with constant stirring. Saturated aqueous ammonium chloride (150 mL) was added to the mixture, and the mixture was extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated. The product was purified by silica-gel chromatography (eluent: hexane–EtOAc, 95 : 5) to give dimethyl 3-(octadec-9-enyl)-itaconate (yield: 3.2 g, 71%).

Hydrolysis of the methyl esters

A solution of dimethyl 3-(octadec-9-enyl)-itaconate (2.0 g) in THF (75 mL) was added to a 1.95 N aqueous solution of LiOH (25 mL). The mixture was stirred for 15 h at room temperature. The reaction mixture was cooled on ice, acidified with 2 N HCl to pH 2.0, and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated. 3-(Octadec-9-enyl)-itaconic acid was purified by silica-gel chromatography (eluent: hexane–EtOAc–AcOH, 3 : 1 : 0.05) (yield: 2.0 g, 92.0%).

Dimethyl 3-[(*Z*)octadec-9-enyl]-itaconate (ceriporic acid H dimethyl ester). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (3H, *t*, *J* = 6.8, H21), 1.27–1.31 (20H, *br*, H5–10, H15–20), 1.64 (1H, *m*, H4), 1.87 (1H, *m*, H4), 2.00, 2.02 (4H, *d*, *J* = 6.0, H14, H11), 3.50 (1H, *t*, *J* = 7.4, H3), 3.68 (3H, *s*, 3-COOCH_3), 3.77 (3H, *s*, 2-COOCH_3), 5.34 (2H, *m*, H12, H13), 5.75 (1H, *s*, $H1_a$), 6.35 (1H, *s*, $H1_b$).

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3-[(*Z*)octadec-9-enyl]-itaconic acid (ceriporic acid H). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.89 (3H, *t*, *J* = 7.0, H21), 1.27–1.31 (20H, *br*, H5–10, H15–20), 1.73 (1H, *m*, H4), 1.94 (1H, *m*, H4), 2.00, 2.01 (4H, *d*, *J* = 6.2, H14, H11), 3.43 (1H, *t*, *J* = 7.4, H3), 5.34 (2H, *m*, H12, H13), 5.86 (1H, *s*, $H1_a$), 6.55 (1H, *s*, $H1_b$). ^{13}C NMR (150 MHz, $CDCl_3$): δ 14.1 (C21), 22.7 (C20), 27.2–27.4 (C14, C11), 29.2–30.0 (C4–10, C15–17), 31.9 (C19), 46.9 (C3), 129.8 (C1, C12), 129.9 (C13), 137.3 (C2), 171.8 (C2–*C*OOH), 179.7 (C–*C*OOH).

Chemical synthesis of ceriporic acid G

Cross-aldol condensation with methyl linoleate and methyl pyruvate. To a cooled solution of diisopropylamine (2.2 mL, 15 mmol) in THF (10 mL) at −78 °C was added dropwise *n*-butyllithium (1.6 M/hexane, 8.5 mL, 13.6 mmol). After the mixture was warmed to 0 °C for 20 min, the mixture was cooled to −78 °C. To this mixture at −78 °C was added a solution of methyl linoleate (22.5 mL, 67.9 mmol) in THF (135.8 mL) dropwise over 30 min and stirred for an additional 10 min to give the lithium enolate anion. Then, the solution of the lithium enolate anion solution was transferred *via* cannula to a solution of methyl pyruvate (7.36 mL, 81.5 mmol) in THF (68 mL) at −78 °C, and the resulting mixture was stirred for 30 min. To this mixture was added an aqueous citric acid solution (10%), followed by EtOAc, and the resulting solution was stirred vigorously. The separated organic layer was washed with brine and dried over Na_2SO_4. A diastereomeric mixture (1 : 1) of dimethyl 3-[(7*Z*,10*Z*)-hexadeca-7,10-dien-1-yl]-2-hydroxy-2-methylsuccinate (yield: 7.44 g, 28%) was purified by silica-gel chromatography (eluent: hexane–EtOAc = 4 : 1). Each isomer was separable by silica-gel chromatography (eluent: hexane–EtOAc = 4 : 1). After partial separation of each isomer, their structures were characterized by NMR analysis.

Tosylation

A solution of the aldol product (500 mg, 0.43 mmol) in CH_2Cl_2 (3.0 mL) was added to pyridine (2.0 mL). Then, to this mixture was added 2,6-di-*tert*-butyl-4-methylpyridine (625 mg, 32.3 mmol), and followed by *p*-toluenesulfonic anhydride (1.78 g, 5.45 mmol), and the resulting mixture was stirred at 50 °C for 12 h. After the reaction, CH_2Cl_2 and pyridine were removed *in vacuo*.

β-Elimination reaction

One milliliter of preheated 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) was added to the crude tosylated aldol product, and the mixture was heated and stirred at 130 °C for 10 min. Then, the mixture was cooled on ice, acidified with 2 N HCl to pH 2.0, and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated. Dimethyl 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconate (yield: 439.6 mg, 16%) was separated from the byproducts, such as citraconate diester (*Z*-isomer) and mesaconate diester (*E*-isomer), by silica-gel chromatography (eluent: hexane–EtOAc, 95 : 5).

Hydrolysis of the dimethyl ester and purification

A solution of 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconate (377.2 mg) in THF (19 mL) was added to a 1.73 N aqueous solution of LiOH (5.7 mL). The mixture was stirred for 16 h at room temperature. The reaction mixture was cooled on ice, acidified with 2 N HCl to pH 2.0, and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated. 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconic acid was purified by silica-gel chromatography (hexane–EtOAc–AcOH, 3 : 1 : 0.05) (yield: 337 mg, 96%).

Dimethyl 3-[(7*Z*,10*Z*)-hexadeca-7,10-dien-1-yl]-2-hydroxy-2-methylsuccinate. *erythro* isomer (*S**,*R**) ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (*t*, 3H, *J* = 6.9), 1.30 (*m*, 18H), 1.42 (*s*, 3H), 1.85 (*m*, 2H), 2.05 (*m*, 4H), 2.75–2.78 (*br s*, 3H), 3.51 (*br s*, 1H), 3.69 (*s*, 3H), 3.76 (*s*, 3H), and 5.34 (*m*, 4H).

***threo* isomer** (*S**,*S**) ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (*t*, 3H, *J* = 6.9), 1.27 (*m*, 18H), 1.43 (*s*, 3H), 1.85 (*m*, 2H), 2.05 (*m*, 4H), 2.75–2.78 (*br s*, 3H), 3.52 (*br s*, 1H), 3.73 (*s*, 3H), 3.81 (*s*, 3H), 5.36 (*m*, 4H).

Dimethyl 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconate (ceriporic acid G dimethyl ester). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (3H, *t*, *J* = 6.8, H19), 1.30 (14H, *br*, H5–8, H16–18), 1.63 (1H, *m*, H4), 1.86 (1H, *m*, H4), 2.04, 2.05 (4H, *d*, *J* = 6.4, H9, H15), 2.77 (2H, *t*, *J* = 6.2, H12), 3.50 (1H, *t*, *J* = 7.2, H3), 5.34 (4H, *m*, H10–11, H13–14), 3.68 (3H, *s*, 3-COOCH_3), 3.74 (3H, *s*, 2-COOCH_3), 5.75 (1H, *s*, $H1_a$), 6.36 (1H, *s*, $H1_b$).

3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconate (ceriporic acid G). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.89 (3H, *t*, *J* = 6.9, H19), 1.32 (14H, *br*, H5–8, H16–18), 1.72 (1H, *m*, H4), 1.93 (1H, *m*, H4), 2.04, 2.05 (4H, *d*, *J* = 6.7, H9, H15), 2.77 (2H, *t*, *J* = 6.3, H12), 3.37 (1H, *t*, *J* = 7.4, H3), 5.34 (4H, *m*, H10–11, H13–14), 5.81 (1H, *s*, $H1_a$), 6.52 (1H, *s*, $H1_b$). ^{13}C NMR (150 MHz, $CDCl_3$): δ 14.2 (C19), 22.7 (C18), 25.7 (C12), 27.3–27.4 (C9, C15), 29.2–29.7 (C4–8, C16), 31.6 (C17), 47.5 (C3), 128.0–128.2 (C11, C13), 129.7 (C1), 130.1–130.3 (C10, C14), 137.5 (C2), 171.9 (C2–*C*OOH), 179.9 (C–*C*OOH).

Purification of ceriporic acids by HPLC

Ceriporic acid G and byproducts were separated on a reversed-phase Inertsil ODS-SP column (250 mm × 8 mm, 5 μm, GL Sciences Inc., Japan) using a Shimadzu HPLC system equipped with an SPD10A UV detector. The column was eluted in the linear gradient mode using two different solvent mixtures—CH_3CN/0.1% aq. HCOOH = 20 : 80 (A) and CH_3CN (B)—at a flow rate of 3.5 mL min^{-1}. Initially, 67% of solvent A was used. The percentage of solvent B was constant for the initial 25 min, increased to 95% during the 25–35 min, and maintained at 95%. The elution was monitored at 210 nm.

LC/IT-TOF-MS measurements

The extract was pretreated by solid phase extraction (SPE) using an Oasis HLB cartridge (Waters, 500 mg, 6 mL). After conditioning with methanol and then ultrapure water, 0.5–1.0 mL of the extracted fraction was loaded onto the cartridge. First, the

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cartridge was washed with 1.0 mL of ultrapure water and 6.0 mL of 80% methanol, and then eluted with 5.0 mL of methanol and acetone, respectively. The eluent was dried by blowing nitrogen gas and dissolved in a mixture of acetonitrile and water at the initial composition of the eluent for LC/IT-TOF-MS analyses.

The sample solutions containing metabolites were analyzed with a Prominence HPLC system coupled to an LC-IT-TOF MS (LC-20AD pump, SIL-20AC auto-injector, CTO-20A column oven, SPD-20A UV/Vis detector, CBM-20A system controller, and ESI-IT-TOF MS; Shimadzu, Kyoto, Japan). A reversed-phase column (Inertsil ODS-SP, 250 × 2.1 mm i.d., 5 μm, GL Sciences Inc., Japan) was eluted at a flow rate of 0.24 mL min^{-1} with 44% aq. acetonitrile containing 0.1% formic acid for the initial 2 min, changed to the linear gradient mode from 44% to 96% aq. acetonitrile for 2–50 min, and maintained at 96% for 50–62 min. The column temperature was maintained at 40 °C. The MS was operated at a probe voltage of 4.50 kV, CDL temperature of 200 °C, block heater temperature of 200 °C, nebulizer N_2 gas flow of 1.5 L min^{-1}, ion accumulation time of 30–40 ms, repeat time of 3 s, MS range from *m*/*z* 100 to 700, and CID energy of 70%. The multiple-stage MS was operated under the following conditions: MS2, ion accumulation time of 30–40 ms, repeat time of 2 s, MS range from *m*/*z* 100 to 500, CID energy of 60%; MS3, ion accumulation time of 50 ms, repeat time 1, MS range from *m*/*z* 50 to 500, and CID energy of 50–60%.

GC-MS measurements

GC-MS analysis was performed with a Shimadzu GCMS-QP5050A gas chromatograph–mass spectrometer (Kyoto, Japan) on a DB-5MS column (30 m × 0.25 mm, 0.25 μm, J & W Scientific Inc., California, USA). The electron impact mass spectrum (EI-MS) was recorded at an ionization energy of 70 eV. About 1.0 μL of the sample was injected in a split (ratio 14 : 1) and splitless mode and helium was used as a carrier gas at a constant flow rate of 1.20 mL min^{-1} and 1.81 mL min^{-1}, respectively. In the split mode, the column oven temperature was subsequently increased from 80 to 170 °C at the rate of 15 °C min^{-1}, from 170 to 250 °C at the rate of 10 °C min^{-1}, from 250 to 280 °C at the rate of 2 °C min^{-1}, and maintained at 280 °C for 2 min. In the splitless mode, oven temperature was subsequently increased from 100 to 196 °C at the rate of 8 °C min^{-1}, from 196 to 280 °C at the rate of 2.5 °C min^{-1}, and maintained at 280 °C for 6.4 min.

NMR measurement

NMR spectra were recorded at room temperature (295 K) in deuterated chloroform (CD_3OD). Chemical shifts are expressed in δ (ppm) scale downfield from tetramethylsilane (TMS) (internal standard; δ_{TMS}, 0 ppm) for ^{1}H-NMR and ^{13}C-NMR. One-dimensional ^{1}H-NMR spectroscopy and two-dimensional ^{1}H–^{1}H correlation spectroscopy (COSY) were recorded on JEOL λ-400 and JEOL JNM-ECA 600 NMR spectrometers operating at 400 and 600 MHz Larmor frequencies, respectively. ^{1}H–^{13}C heteronuclear multiple-quantum coherence (HMQC) and heteronuclear multiple-bond correlation (HMBC) spectroscopy were also recorded to assign signals. ^{13}C-NMR and DEPT spectroscopy were recorded at 100 and 150 MHz Larmor frequencies, respectively.

Degradation of a non-phenolic β-*O*-4 lignin model dimer by ceriporic acid G and H in reactions with MnP

Degradation of the lignin model compound. The degradation of the lignin model compound GOS-Et was examined *via* MnP-induced lipid peroxidation. Manganese peroxidase (MnP) was prepared from the wood meal culture fluid of *C. subvermispora* as previously described[8] and purified on DEAE-Sepharose (DEAE Sepharose Fast Flow, Amersham Bioscience) before use. Reaction conditions were as follows: Sodium acetate buffer adjusted to pH 4.5, 0.5 mM $MnSO_4$, 0.1 mM H_2O_2, 140 mU MnP, 0.2 mM GOS-Et (*threo*/*erythro* = 1.13), 3 mM lipid, 0.3% (wt/vol) Tween 20 were mixed in a 1.5 mL vial and reacted for 24 h at 37 °C with stirring. Then, 250 μL of chloroform and 50 μL of *n*-tetracosane (100 μg mL^{-1}) were added to the reaction mixture, dried under N_2 gas, and acetylated with acetic anhydride and pyridine (1 : 1, v/v). As a lipid, ceriporic acid H (20 : 1), ceriporic acid G (18 : 2), oleic acid (18 : 1), or linoleic acid (18 : 2) was used. Control experiments were carried out by omitting MnP, lipid, H_2O_2, or surfactant (Tween 20).

Results

New extracellular metabolites, ceriporic acid G and H were isolated from wood cultures of the selective white-rot fungus *C. subvermispora* (Fig. 1). The metabolites were chemically synthesized and characterized as having capability to trigger the ligninolytic lipid peroxidation.

LC/IT-TOF-MS analyses of extracellular metabolites

We incubated *C. subvermispora* in a liquid medium containing wood blocks. The culture fluid of the fungus was separated by filtration and immediately freeze-dried. The metabolites were extracted with aq. acetonitrile, partially purified by SPE, and then analyzed by LC/IT-TOF-MS with negative-ion electrospray ionization (ESI). From the precursor ion spectra of the cultural

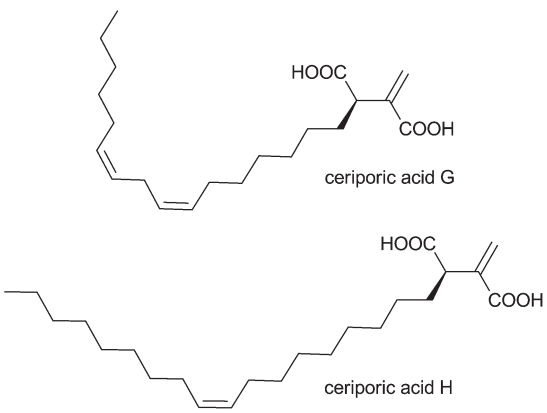

Fig. 1 Chemical structures of ceriporic acid G and H.

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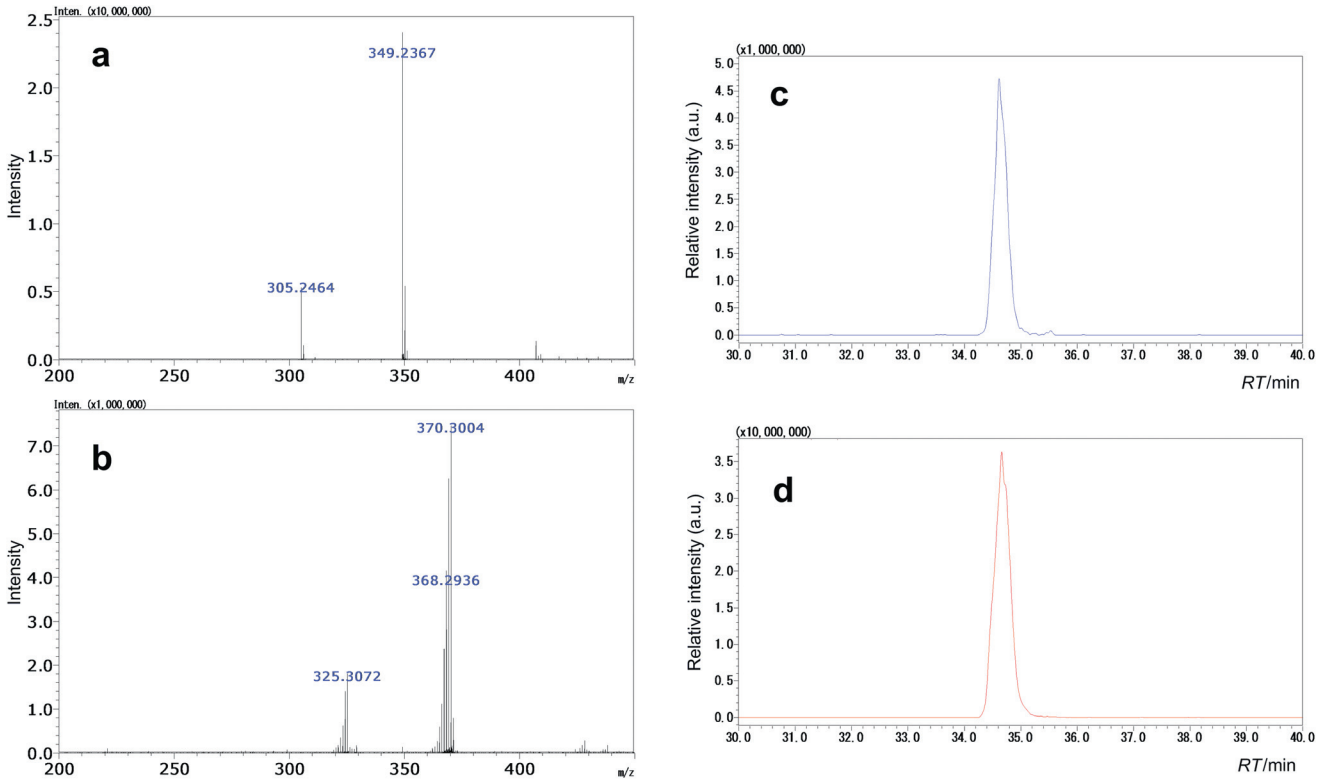
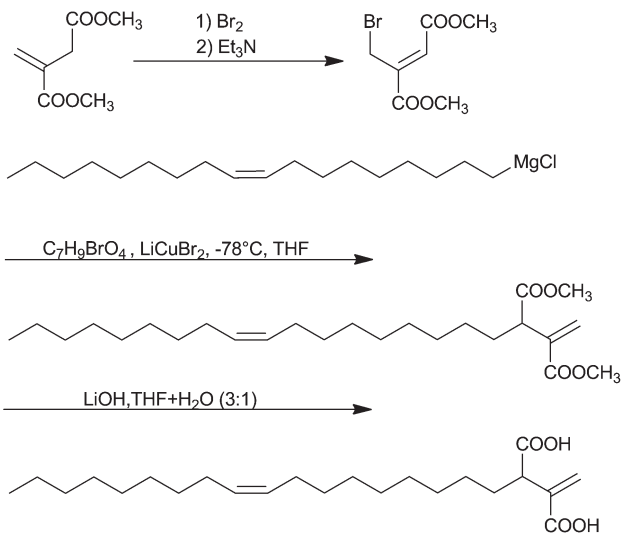


Fig. 2 Identification of ceriporic acid G by LC/IT-TOF-MS analyses. ESI-Mass spectra of natural metabolite (a) and that of administration experiments of [^{13}C–U, 99%] glucose (b). Extracted ion chromatograms (*m/z* 349.24) from LC/IT-TOF-MS of natural metabolite (c) authentic ceriporic acid G (d).

extract, we found an unidentified ion at *m/z* 349.237, as shown in Fig. 2a. The fragment ions appeared at *m/z* 305.246. The high-resolution MS^n data, including the isotope pattern, suggested that this compound was a polyunsaturated ceriporic acid with the compositional formula $C_{21}H_{34}O_4$. We confirmed the chemical formula of CAG by feeding experiments with [^{13}C–U] glucose as reported.[16] The compositional formula $^{13}C_{21}H_{34}O_4$ was determined by detecting precursor and fragment ions at *m/z* 370.300 and *m/z* 325.307, respectively as shown in Fig. 2b.

Synthesis of ceriporic acid H

We estimated the chemical structure of the metabolite as octadecenyl itaconic acid having the fatty acid moiety of eicosenoic acid in the molecule and designated the metabolite tentatively as ceriporic acid H. Ceriporic acid H was synthesized by a Grignard substitution reaction. Previously, the Grignard substitution reaction of dimethyl α-(bromomethyl) fumarate with appropriate Grignard reagents was carried out using $LiCuBr_2$ as a catalyst under N_2.[11,19] We modified these methods using THF as a reaction solvent to synthesize the Grignard reagent and react at −78 °C to improve the yield. Our synthetic route of ceriporic acid H is shown in Scheme 1. GC/EI-MS spectra of ceriporic acid H and its dimethyl ester are shown in Fig. 3.



Scheme 1 Synthetic routes of 3-(octadec-9-enyl)-itaconic acid (ceriporic acid H).

Synthesis of ceriporic acid G

LC/IT-TOF-MS analysis of the culture fluid from *C. subvermispora* suggests an existence of alkadienyl itaconic acid. We estimated the chemical structure of the metabolite as hexadecadienyl itaconic acid having a linoleic acid fatty-acid moiety in the molecule and designated the metabolite tentatively as ceriporic acid G.

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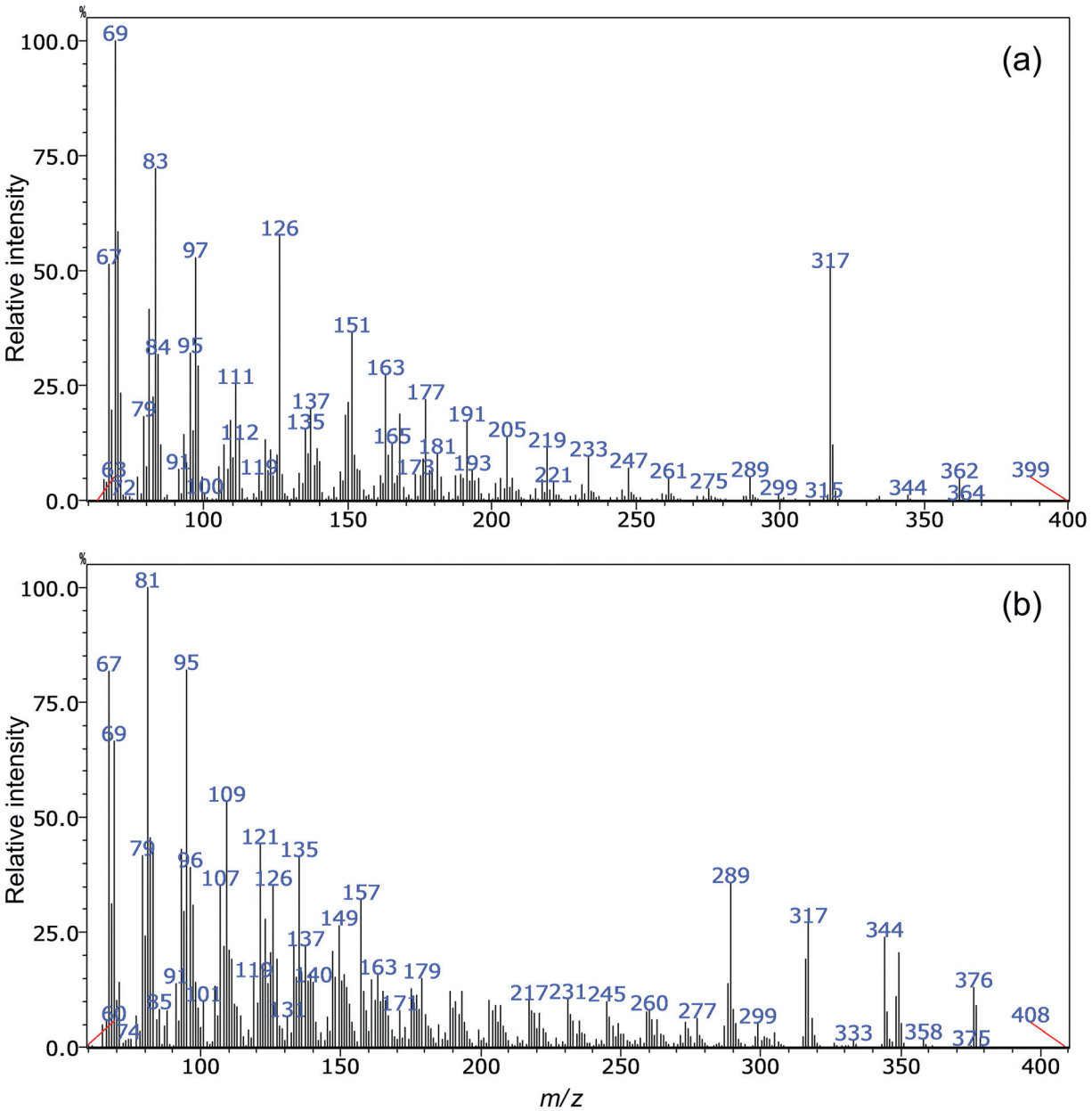


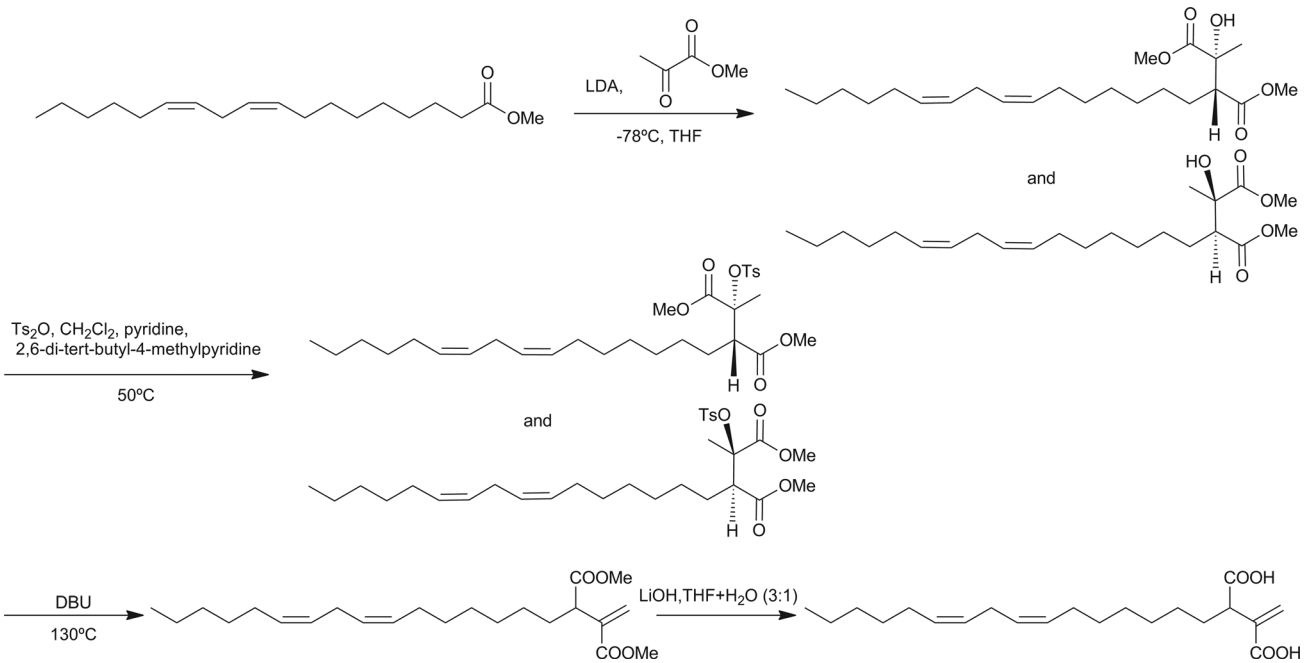
Fig. 3 EI-MS spectra of 3-(octadec-9-enyl)-itaconic acid (ceriporic acid H) (a) and dimethyl 3-(octadec-9-enyl)-itaconate (ceriporic acid H dimethyl ester) (b). Mass fragment ions—*m/z* 362 ($M^+ - H_2O$), *m/z* 344 (362-H_2O), *m/z* 317 (362-$CHO_2^{\cdot}$), *m/z* 289 (317-$CO_2^{\cdot}$)—were observed in spectrum (a) and *m/z* 408 (M^+), *m/z* 376 ($M^+ - CH_3OH$), *m/z* 344 (376-CH_3OH), *m/z* 349 ($M^+ - C_2H_3O_2^{\cdot}$), *m/z* 317 (349-CH_3OH), *m/z* 289 (317-$CO_2^{\cdot}$), *m/z* 157 ($C_7H_9O_4^{\cdot}$) were observed in spectrum (b).

Since ceriporic acid G has a non-conjugated diene unsaturated chain, we synthesized the compound by a biomimetic-type aldol condensation of linoleic acid ester with methyl pyruvate, which would give an aldol product with the carbon skeleton of ceriporic acid G.[21] Our synthetic route to ceriporic acid G is shown in Scheme 2.

The GC/EI-MS spectrum of ceriporic acid G is shown in Fig. 4.

Identification of ceriporic acid G and H

Chemically synthesized authentic compounds were used to identify the new metabolites from *C. subvermispora*. These new metabolites were purified from the culture fluid of *C. subvermispora* by reversed-phase HPLC. The two natural metabolites had retention times and mass spectra similar to those of authentic ceriporic acids G and H by LC/IT-TOF-MS. The retention time of authentic ceriporic acid G was identical with that of a natural metabolite at 34.81 min as shown in Figs. 2c, d; and gave $[M - H]^-$ at *m/z* 349.2391. The retention time of authentic ceriporic acids H was identical with that of a natural metabolite at 44.54 min and gave $[M - H]^-$ at *m/z* 379.2864 similar with that of a natural metabolite *m/z* 379.2839. GC/EI-MS spectra of natural metabolites were also identical with those of authentic ceriporic acid G and H (Figs. 3 and 4, respectively).



Scheme 2 Synthetic routes of 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconic acid (ceriporic acid G).

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Degradation of a non-phenolic β-*O*-4 lignin model dimer by ceriporic acid G and ceriporic acid H in reactions with MnP

New natural metabolites ceriporic acids G and H were produced by *C. subvermispora*. To evaluate functions of the unsaturated ceriporic acids as a lignin decomposer, we examined degradation of a non-phenolic β-*O*-4 lignin model dimer GOS-Et by reactions of MnP with ceriporic acid G and H. Lignin contains both phenolic and non-phenolic substructures. Lignin subunits with phenolic hydroxyl groups are much more labile than those with non-phenolic subunits due to its low oxidation potential. Phenolic hydroxyl content is less than 13% per monolignol unit.[22] Therefore, it is important to demonstrate the degradation of non-phenolic structure of lignin to assess the capability of lignin degradation by the radical chain reactions. GOS-Et used in this study was a mixture of *threo* and *erythro* diastereomers. In reactions of ceriporic acid G with Mn^{2+}/MnP, approximately 77% of GOS-Et was degraded to smaller fragments by oxidation, C_α–C_β cleavage, and aryl ether cleavage. The MnP-catalyzed lipid peroxidation with ceriporic acid G and H were compared with the similar lipid peroxidation system using oleic and linoleic acids instead of the ceriporic acids. In the absence of MnP, no degradations were observed, regardless of the presence of lipids throughout the experiments. In reactions of MnP with ceriporic acid G and linoleic acid, significant degradation was observed, even in the absence of H_2O_2. GC/MS chromatograms of the degradation products from GOS-Et by MnP and cerioric acid G are shown in Fig. 5, together with chemical structure of the identified degradation products. The percentage of GOS-Et decomposition under different reaction conditions are summarized in Table 1.

Discussion

Selective white-rot fungi and their ligninolytic systems have significant potentials in production of biofuels, chemicals, pulp, feed for ruminant animals, and other applications when the main objective is to separate cell wall polysaccharides from lignin for subsequent use. As a possible mechanism for selective ligninolysis, *in situ* lipid peroxidation, a free radical generation system at a distance far from enzymes, has been proposed, and the production and oxidation of saturated and unsaturated fatty acids by the catalysis of MnP have been demonstrated in selective white-rot fungus *C. subvermispora*. In the present study, we identified new alkadienyl and alkenyl itaconic acids belonging to the family of ceriporic acids and chemically synthesized the metabolites to characterize their functions in lignin degradation. We found that the new metabolites degraded the recalcitrant non-phenolic β-*O*-4 lignin model compound in the lipid peroxidation system by MnP and that the alkadienyl itaconic acid, designated as ceriporic acid G, decomposed the lignin model compound more than linoleic acid. Except for simple fatty acids, this is the first report of fungal metabolites degrading the non-phenolic lignin substructure in the MnP-catalyzed lipid peroxidation system. This finding also expands the role of ceriporic acid from the suppression of cellulose depolymerization to lignin degradation.

Lipid peroxidation proceeds by initiation, propagation, and termination phases. To propagate the radical chain reactions, chain-carrying radicals have to be produced. Unlike lipoxygenase, which directly abstracts hydrogen from a bis-allylic position, manganic ion abstracts hydrogen from the enolic form of carboxylic acids at the position vicinal to a carboxyl group. In the reaction of linoleic acid with MnP, a carboxylalkyl radical is initially formed. It abstracts hydrogen from a bis-allylic position to generate chain-carrying radicals. Therefore, distinct differences in the initial oxidation rate by MnP or by its reaction product, the Mn^{3+} chelate were observed between linoleic acid and methyl linoleate.[7] The oxidation of ceriporic acids by MnP or the Mn^{3+} chelate should proceed by the same pathway but

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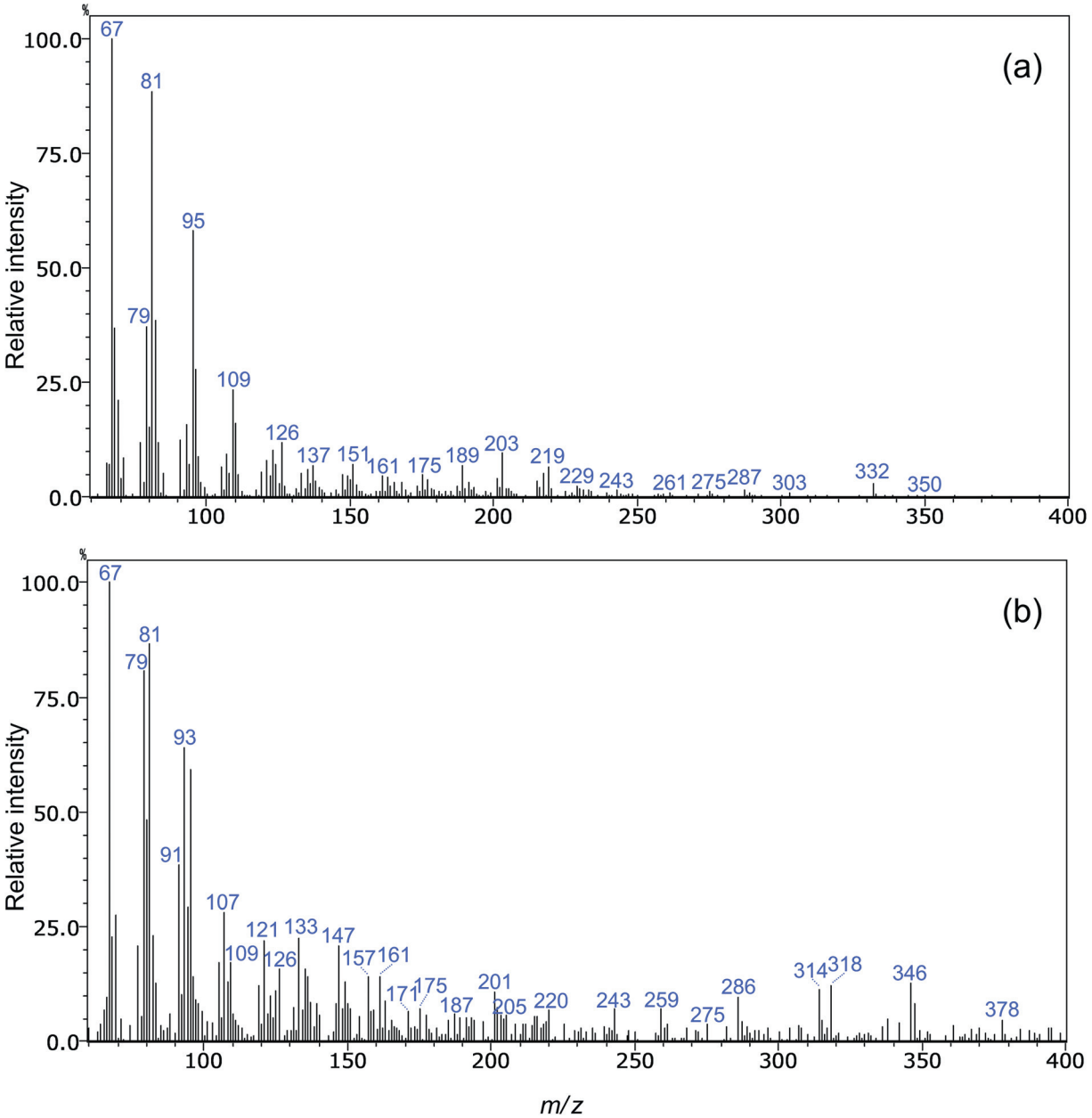

Fig. 4 EI-MS spectrum of 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconic acid (ceriporic acid G) (a) and dimethyl 3-[(*Z*,*Z*)-hexadec-7,10-dienyl]-itaconic acid (ceriporic acid G dimethyl ester) (b). Mass fragment ions—*m*/*z* 350 (M^+), *m*/*z* 332 ($M^+ - H_2O$), *m*/*z* 315 (332-$HO^{\cdot}$), *m*/*z* 287 (315-$CHO_2^{\cdot}$), *m*/*z* 126 ($C_6H_6O_3$)—were observed in spectrum (a) and *m*/*z* 378 (M^+), *m*/*z* 346 ($M^+ - CH_3OH$), *m*/*z* 314 (346-CH_3OH), *m*/*z* 157 ($C_7H_9O_4^{\cdot}$) were observed in spectrum (b).

differences can be found in the structure of the initial radicals between linoleic acid and ceriporic acid G. The initial carboxyalkyl radical from ceriporic acid G is tertiary and stabilized with neighboring terminal vinyl group, while linoleic acid produces a secondary carbon radical by the reaction with MnP or the Mn^{3+} chelate (Fig. 6a and b). The initiation of ceriporic acid G *via* the formation of carboxylalkyl radicals and subsequent hydrogen abstraction from the bis-allylic position may proceed more efficient in ceriporic acid G than linoleic acid because lifespan of the initiation radical is important factor in the hydrogen abstraction from the labile bis-allylic C–H bond.[7,9]

Significant differences in the degradation of lignin model compounds were found among the four metabolites with and without the bis-allylic moiety. The degradation of the model compound was significantly less for oleic acid and ceriporic acid H, which have a monoene structure but lack a bis-allylic structure, than for linoleic acid and ceriporic acid G. This suggests that the bis-allylic moiety is crucial for generating the chain-carrying radicals that propagate the reactions, sufficiently crucial to the extent of intensively degrading the model compound, although differences in fragment radicals after the initial phase may cause differences in degradation.

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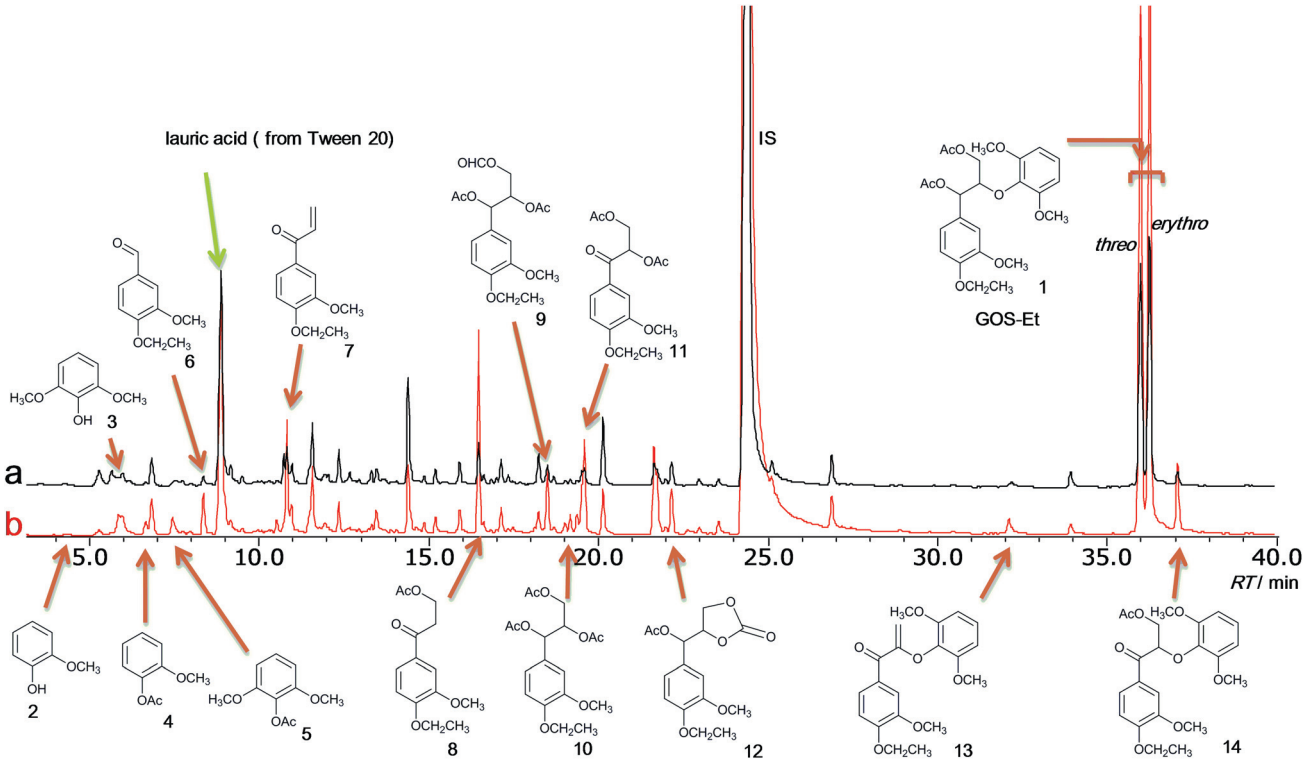

Fig. 5 Total ion current chromatogram **a** and extracted ion chromatogram **b** of the non-phenolic β-*O*-4 lignin model dimer, GOS-Et after reacting with ceriporic acid G and MnP for 24 h in the absence of H_2O_2. Reaction mixture contained 50 mM AcONa buffer (pH 4.5), 0.5 mM $MnSO_4$, 140 mU MnP, 3 mM ceriporic acid G, 0.3% (w/v) Tween20, 0.2 mM GOS-Et (*thre*/*erythro* = 1.13). Decomposed compounds are marked in the chromatograms and write in those chemical structures.

Table 1 Percentage of the non-phenolic β-*O*-4 lignin dimmer model compound, GOS-Et decomposed by the lipid peroxidation system with MnP. Reactions were conducted using different lipid-related compounds (LA: linoleic acid, CAG: ceriporic acid G, OA: oleic acid, CAH: ceriporic acid H) in the presence and absence of H_2O_2

Reaction condition	LA	LA + H_2O_2	CAG	CAG + H_2O_2	OA + H_2O_2	CAH + H_2O_2
% decomposed	59	70	74	77	28	32

In GC/MS chromatograms after the degradation experiments of GOS-Et, we identified degradation products (compounds No. **2–14**). Possible formation mechanisms of those compounds are summarized in Fig. 6. The initial carboxyalkyl radicals from ceriporic acid G (CAG) or linoleic acid (LA) caused radical chain reactions, resulting in production of carbon-centred radical (CR) and alkoxy radical (AR). We proposed a degradation pathway of GOS-Et by radicals initiated by hydrogen abstraction at the benzyl position. The benzyl radical, an initial product from lignin can be transformed to the aryl cation radical intermediate by protonation. The initial benzyl radical and the aryl cation radical decay into smaller fragments by the different routes. Compound **9′** and **12′** are formed by aryl ether and aromatic ring cleavage from the aryl cation radical, while benzyl ketone **14′** was produced by reaction of the benzyl radical intermediate with molecular oxygen and subsequent release of hydroperoxy radical.[9,23,24]

In the MnP-catalyzed lipid peroxidation of ceriporic acid G and linoleic acid, the decomposition of the lignin model compound GOS-Et was observed even in the absence of H_2O_2. Electron acceptors to drive MnP are H_2O_2 or organic hydroperoxide. H_2O_2 can be formed by the reduction of molecular oxygen to superoxide by radicals and subsequent disproportionation. Once the catalytic cycle starts using a small amount of H_2O_2 formed by autoxidation, the enzymatic reaction continuously produces H_2O_2, accompanied by the degradation of lipids. Another candidate for the electron acceptor, organic hydroperoxides, can be formed by the addition of molecular oxygen to carbon-centered radicals during the course of lipid peroxidation. However, we demonstrated that a lipid hydroperoxide from linoleic acid—13(S)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(S)-HPODE)—cannot serve as the electron acceptor for MnP.[7] The pathway for H_2O_2 formation from molecular oxygen should play a major role in the reaction. The degradation of non-phenolic lignin models by the MnP-catalyzed lipid peroxidation without H_2O_2 was reported previously.[25,26]

We synthesized ceriporic acid G by a cross-aldol condensation. With this reaction pathway, a simple fatty acid can be

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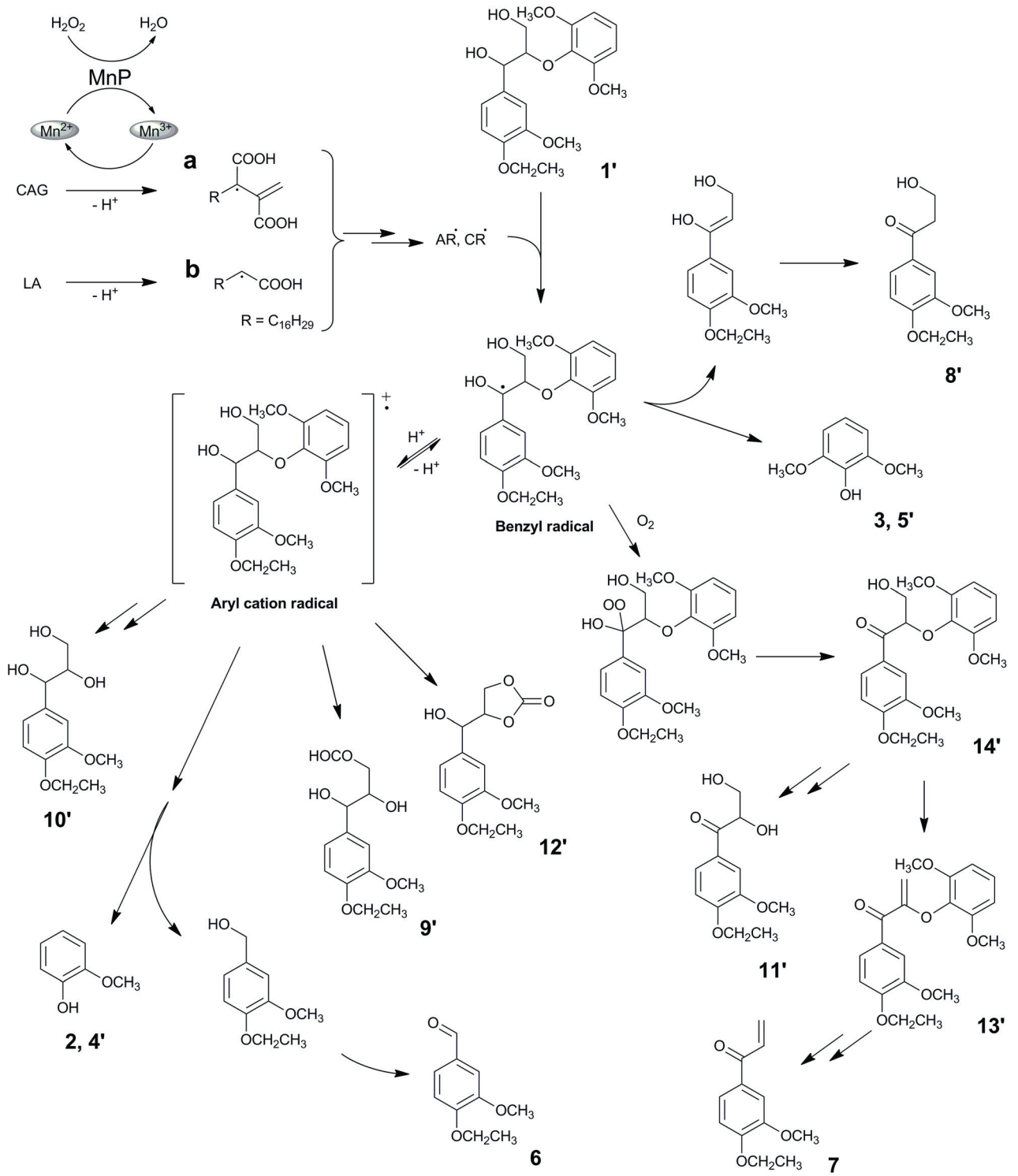

Fig. 6 Proposed pathway for the degradation of non-phenolic β-*O*-4 lignin model dimer, GOS-Et in the lipid peroxidation system by MnP and ceriporic acid. Carboxyalkyl radical by hydrogen abstraction at the position vicinal to the carboxylic group of (a) ceriporic acid and (b) fatty acid. Additional dash character in numbers shows that those compounds were detected as acetylated derivatives in GC/EI-MS spectra as shown in Fig. 5.

used as a starting molecule for the synthesis of alkyl, alkenyl, and alkadienyl itaconic acids. The synthesis by a cross aldol condensation promotes functional studies on itaconic acid derivatives related to selective white rot, plant growth regulation and antitumor activity.[27,28]

Conclusions

In this study, new alkenyl (ceriporic acid H) and alkadienyl itaconic acids (ceriporic acid G) were obtained as extracellular metabolites of *Ceriporiopsis subvermispora*. The unsaturated

metabolites—ceriporic acids H and G—were synthesized by a Grignard substitution reaction and a cross-aldol condensation reaction, respectively. Ceriporic acid G initiated lipid peroxidation in combination with MnP and degraded a recalcitrant non-phenolic β-*O*-4 lignin model compound. The new metabolite serves as a trigger to initiate lignin-degrading lipid peroxidation.

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