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De novo synthesis of (*Z*)- and (*E*)-7-hexadecenylitaconic acids by a selective lignin-degrading fungus, *Ceriporiopsis subvermispora*

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ABSTRACT

Ceriporic acids are a class of alk(en)ylitaconic acids produced by a selective lignin-degrading fungus, *Ceriporiopsis subvermispora*. Their structural units have similarity with biologically important lichen acids, such as chaetomellic and protolichesterinic acids. The unique function of alkylitaconic acid is the redox silencing of the Fenton reaction system by inhibiting reduction of Fe^{3+}. As estimated by the catalytic function of Δ9-desaturases, 7-hexadecenyl derivatives bearing a *trans* configuration have not been reported in the family of alk(en)ylitaconic acids, i.e. the structurally similar lichen acids—alk(en)ylcitraconic and paraconic acids. In this paper, we discuss the isolation of an itaconic acid derivative with an (*E*)-7-hexadecenyl chain from cultures of *C. subvermispora*. To identify the natural metabolite, (*E*)- and (*Z*)-7-hexadecenylitaconic acids were chemically synthesised. The isolated metabolite was identical to the synthetic (*E*)-hexadecenylitaconic acid and was designated as ceriporic acid D. Administration of ^{13}C-[U]-glucose demonstrated that ceriporic acid C and *trans*-7-hexadecenylitaconic acid (ceriporic acid D) were biosynthesised *de novo* by *C. subvermispora*.

1. Introduction

White rot fungi are primarily responsible for initiating depolymerisation of lignin in lignified plants. The basidiomycetous fungi decompose lignin in wood by free radical reactions involving a combination of extra- and intra-cellular enzymes with secondary metabolites such as enzyme mediators, metal chelators and peroxidisable precursors. Most white rot fungi simultaneously decompose lignin and cellulose, accompanied by erosion of wood cell walls, whereas selective white rot fungi, such as *Ceriporiopsis subvermispora*, are able to degrade lignin without extensive damage to cellulose (Messner and Srebotnik, 1994; Blanchette et al., 1997). In selective white rot, the lignin is degraded at a site far from the enzymes. The unique wood decay pattern indicates that extracellular low-molecular-mass metabolites possess the ability to catalyze lignin degradation. To explain lignin biodegradation by metabolites at a site far from enzymes, the possible involvement of lipid peroxidation by chelated Mn^{3+} produced by manganese peroxidase (MnP) has been proposed (Enoki et al., 1999b; Watanabe et al., 2000, 2001; Jensen et al., 1996). Another possibility of wood decay is hypothesized as "the migrating electron hole theory" that enzymes and diffusing agents generate chain radical reaction among the lignin aromatic structures (Henriksson et al., 2005).

From extractive-free wood cultures of *C. subvermispora*, we have isolated three novel alkylitaconic acids—tetradecylitaconic (ceriporic acid A, **1**), hexadecylitaconic (ceriporic acid B, **2**) and (*Z*)-7-hexadecenylitaconic acid (ceriporic acid C, **3**) as shown in Fig. 1 (Enoki et al., 1999a,b, 2000; Amirta et al., 2003). Production of a trace amount of the other alkenylitaconic acids with different chain lengths has been suggested by GC–MS of crude extracts from eucalyptus wood cultures of *C. subvermispora* (del Rio et al., 2002) but only the three metabolites, ceriporic acids A, B and C (**1**–**3**) were isolated.

Previously, we chemically synthesised ceriporic acid B (hexadecylitaconic acid, **2**) and found that this metabolite suppressed the iron redox reactions to attenuate $HO^{\bullet}$ production by the Fenton reaction, even in the presence of iron reductants such as hydroquinone and cysteine (Enoki et al., 2002; Watanabe et al., 2002). Later, we demonstrated that ceriporic acid B (**2**) actually inhibited depolymerisation of cellulose by the Fenton reaction system at the physiological pH of wood decay (Rahmawati et al., 2005). Chemical synthesis of a series of alkylitaconic acids with different chain lengths indicated that the alkyl side-chain in ceriporic acids is a key factor for controlling suppression of reactive oxygen species and stability against oxidative degradation by $HO^{\bullet}$ (Ohashi et al., 2007).

In this paper, we focus on ceriporic acids based on the alkenylitaconic acids and report a new alkenylitaconic acid, (*E*)-7-hexadecenylitaconic acid, which we designate ceriporic acid D (**4**). We

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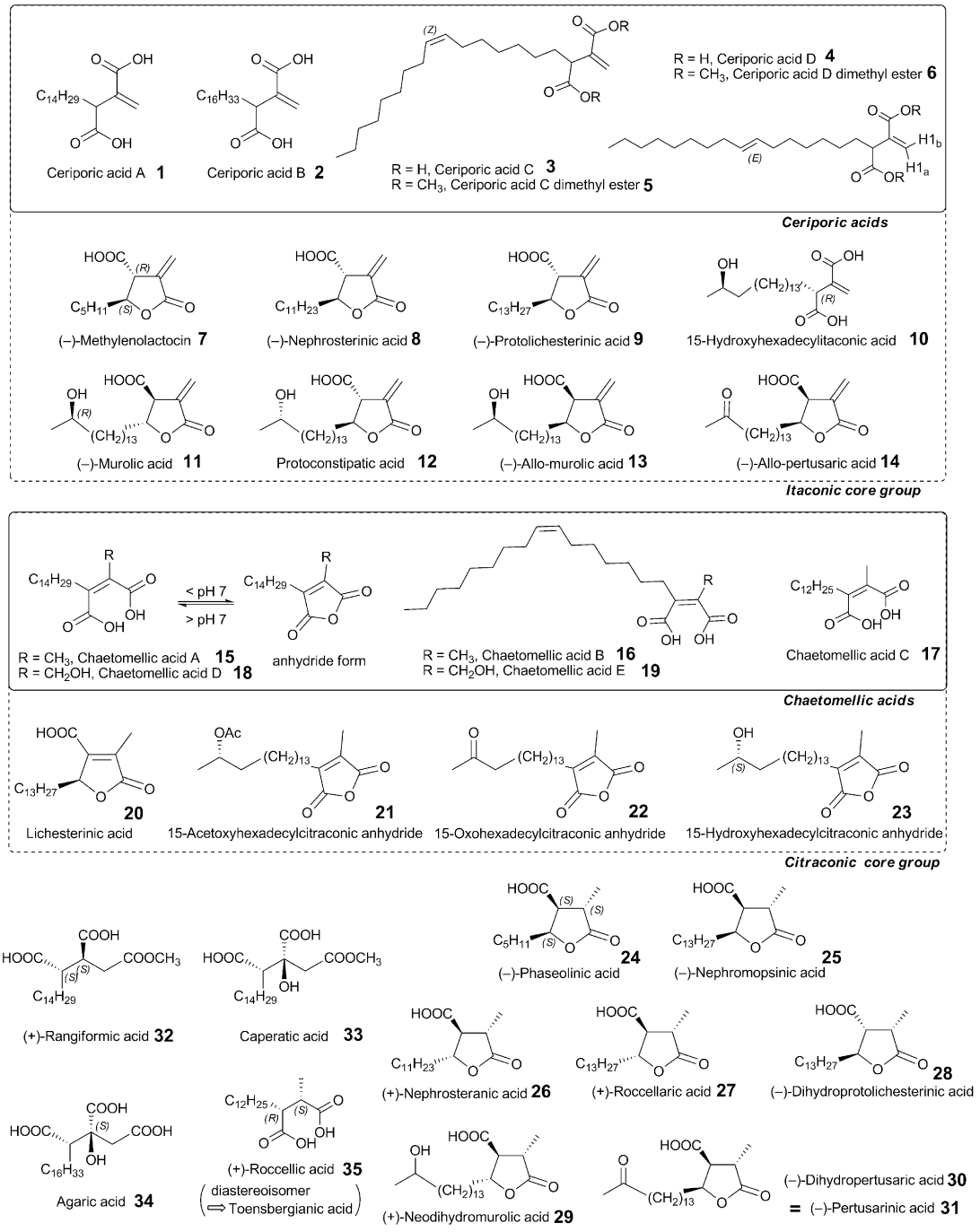

Fig. 1. Chemical structures of ceriporic acids (A–D, **1**–**4**) and similar fungal metabolites.

present unequivocal evidence for the *de novo* synthesis of ceriporic acid C (**3**) and D (**4**) by *C. subvermispora* and discuss the biosynthesis of the *trans*-hexadecenyl side-chain in the new metabolite.

2. Results

2.1. Production of ceriporic acids by C. subvermispora

C. subvermispora was cultured in an SMYW (sucrose–molt extract–yeast extract–wood piece) medium, and extracellular lipid-related metabolites were extracted from the culture fluid. GC–MS analysis showed that, as found in the wood medium previously reported (Amirta et al., 2003), ceriporic acids A, B and C (**1**–**3**) were secreted in the SMYW medium. In addition to these metabolites, we found that an unknown compound appeared just after the peak of ceriporic acid C (**3**) on GC–MS analysis. The mass spectrum of the unknown compound was identical to that of ceriporic acid C (**3**), suggesting that the new compound is a stereoisomer of ceriporic acid C (**3**).

2.2. Chemical synthesis of (Z)- and (E)-7-hexadecenylitaconic acids (3 and 4)

To obtain unambiguous evidence for the identification of the new metabolite, (*Z*)- and (*E*)-7-hexadecenylitaconic acids (**3** and **4**) were chemically synthesised by the Grignard reaction. Retention

times of (*Z*)- and (*E*)-7-hexadecenylitaconic acids (**3** and **4**) on HPLC and GC–MS were 24.4/27.0 min and 21.0/21.1 min, respectively. Mass spectra of (*Z*)-7-hexadecenylitaconic acid (**3**) and its dimethyl ester, dimethyl (*Z*)-7-hexadecenylitaconate (**5**), were very similar to those of (*E*)-7-hexadecenylitaconic acid (**4**) and dimethyl (*E*)-7-hexadecenylitaconate (**6**), respectively. Although the mass spectra were not distinguishable, differences were found in chemical shifts and vicinal ^{1}H–^{1}H coupling constants of the double bond at C10 and C11 in the ^{1}H NMR spectra. Chemical shift and coupling constants of (*Z*)- and (*E*)-7-hexadecenylitaconic acids (**3** and **4**) are summarised in Table 1. Allylic protons of the (*Z*) isomer at 2.02 and 2.03 ppm were shifted downfield by 0.06 ppm from those of the (*E*) isomer, while alkenyl protons of the (*Z*) isomer at 5.34 ppm were shifted upfield by 0.04 ppm from those of the (*E*) isomer (Fig. 2). The (*Z*) and (*E*) isomers were clearly distinguished by vicinal ^{1}H–^{1}H coupling constants of the double bond. Using Schaumburg and Bernstein's parameters (Schaumbu and Berstein, 1968) and the NMR spectra, vicinal coupling constants $^3J_{10,11}$ for the (*Z*) and (*E*) isomers were calculated to be 10.75 and 15.23 Hz, respectively (Fig. 2). The coupling constants observed are in accordance with the theory that the coupling constant of the (*Z*) isomer in alkenyl chains (10–12 Hz) is smaller than that for the (*E*) isomer (15–16 Hz) (Frost and Gunstone, 1975). Further evidence for the stereoisomers was obtained by ^{13}C NMR spectroscopic analysis as shown (Table 2). In ^{13}C NMR spectra of dimethyl (*Z*)- and (*E*)-7-hexadecenylitaconates (**5** and **6**), the alkenyl carbons of the (*Z*) isomer were shifted upfield from those of the (*E*) isomer by 0.5 ppm, while the allylic carbons of the (*Z*) isomer were shifted downfield by 5.2–5.4 ppm from the (*E*) isomer.

2.3. Identification of alkenylitaconic acids produced by *C. subvermispora*

The authentic compounds chemically synthesised were used for identification of the new metabolite from *C. subvermispora*. These new metabolite was purified from the culture fluid of *C. subvermispora* by reversed-phase HPLC. The natural metabolite had the same retention time and mass spectrum as that of the authentic (*E*)-7-hexadecenylitaconic acid by GC–MS and HPLC. In addition, the dimethyl ester of the target metabolite from *C. subvermispora* had the same retention time (22.0 min) and mass spectrum as those of the authentic compound as shown in Fig. 3.

The position of the double bond in the isolated metabolite was analyzed as a DMDS derivative by GC–MS (Fig. 4). In the mass spectrum, molecular ion (M^+) was found at *m/z* 474. Cleavage of the carbon–carbon bond between the two CH_3S groups introduced in the double bond gave two major fragment ions, *m/z* 173 and 301. The fragment ions at *m/z* 173 and 301, and the other secondary fragment ions, *m/z* 427 ($M^+ - CH_3S^\bullet$), 395 (427 – CH_3OH), 269 (301 – CH_3OH) and 237 (269 – CH_3OH), clearly showed that the double bond in the metabolite is located between C10 and C11 of the hexadecenyl side-chain, similar to the synthetic compound. The DMDS derivative of ceriporic acid C showed the same mass spectrum, but its retention time (33.9 min) on GC–MS was different from that of ceriporic acid D (34.0 min).

From these results we conclude that *C. subvermispora* produced (*E*)-7-hexadecenylitaconic acid (**4**). (*E*)-7-Hexadecenylitaconic acid

Table 1
^{1}H NMR (CD_3OD, 400 MHz) data of ceriporic acid C and D

Position	Ceriporic acid C (**3**) δ^1H[a]	Ceriporic acid D (**4**) δ^1H[a]
H19	0.90, *t*, 3H, *J* = 6.79	
H5–8, H13–18	1.29–1.32, *br*, 20H	
H4	1.69, *m*, 1H	
H4	1.84, *m*, 1H	
H9, H12	2.02, 2.03, *d*, 4H, *J* = 5.33	1.96, 1.97, *d*, 4H, *J* = 5.37
H3[b]	3.35, *t*, 1H, *J* = 7.39	
H10, H11	5.34, *m*, 2H, *J* = 10.75	5.38, *m*, 2H, *J* = 15.23
$H1_a$[c]	5.63, *s*, 1H	5.58, *s*, 1H
$H1_b$[c]	6.18, *s*, 1H	6.11, *s*, 1H

[a] ^{1}H NMR data are given in the form: δ/ppm, multiplicity (*s*, singlet, *d*, doublet, *t*, triplet, *m*, multiplet, *br*, broad signal), integration, coupling constants *J*/Hz.
[b] Overlapping signals.
[c] See Fig. 1.

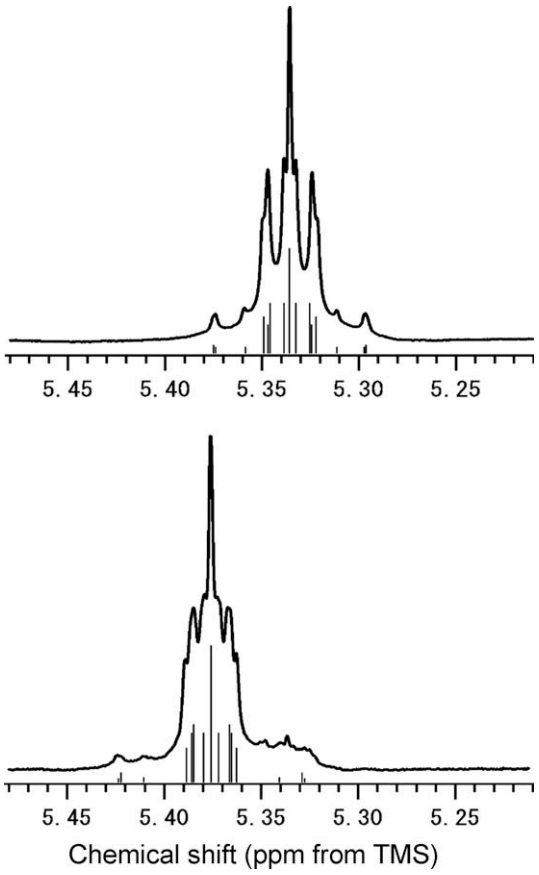

Fig. 2. ^{1}H NMR spectra of the double-bond protons in (a) ceriporic acid C and (b) D. Chemical shifts of alkenyl protons of (*Z*) isomer and (*E*) isomers were 5.34 and 5.38 ppm, respectively. Vicinal coupling constants $^3J_{10,11}$ for (*Z*) and (*E*) isomers were calculated to be 10.75 and 15.23 Hz by Schaumburg and Bernstein's parameters.

Table 2
^{13}C NMR ($CDCl_3$, 100 MHz) data of ceriporic acid C and D dimethyl esters

Position	Ceriporic acid C-Me (**5**) δ^{13}C[a]	Ceriporic acid D-Me (**6**) δ^{13}C[a]
C19	14.1	
C18	22.7	
C9, C12[b]	27.1–27.4	32.5, 32.6
C6–8, C13–16	28.8–29.7	
C5, C17	31.8–31.9	
C4[b]	31.2	
C3	46.5	
$COOCH_3$	52.0, 52.1	
C1	126.7	
C10[b]	129.7	130.2
C11[b]	130.0	130.5
C2	138.3	
2-*C*OOMe	166.7	
3-*C*OOMe	173.8	

[a] ^{13}C NMR data are given in δ/ppm.
[b] Assignments were also based on 2D-HMQC spectra.

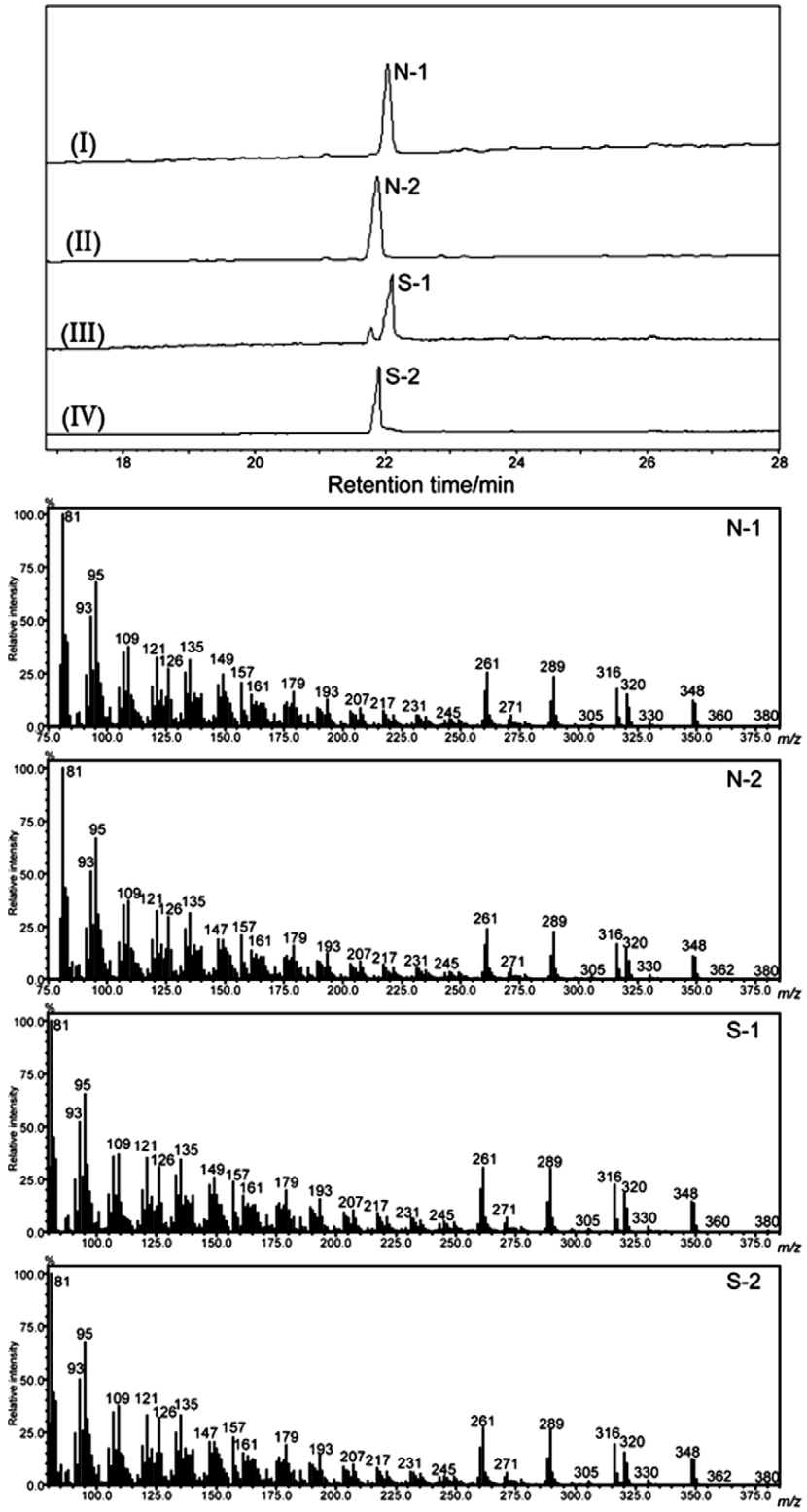

Fig. 3. Total ion chromatograms (I) –(IV) and mass spectra (N-1, N-2, S-1 and S-2) for natural (II, N-2) and synthetic dimethyl-(*Z*)-7-hexadecenylitaconate (IV, S-2) and those for natural dimethyl-(*E*)-7-hexadecenylitaconate (I, N-1) and synthetic dimethyl-(*E*)-7-hexadecenylitaconate (III, S-1) by GC–MS.

is the fourth alk(en)ylitaconic acid isolated from the fungus; we thus designated the metabolite ceriporic acid D (**4**).

On a wood meal medium, *C. subvermispora* secreted ceriporic acid C (**3**) most abundantly among the series of ceriporic acids (**1**–**3**) (Amirta et al., 2003). In this study, the amounts of ceriporic acids A, B, C and D (**1**–**4**) produced on the SMYW medium after 1 week were 136, 69, 296 and 13 μg per liter, respectively.

*2.4. Analysis of biosynthesis of ceriporic acids C and D (**3** and **4**)*

7-Hexadecenyl derivatives with the *trans* configuration have not been reported in the family of alk(en)ylitaconic acids and structurally similar lichen acids—alkenylcitraconic and paraconic acids. This attracts interest in the formation mechanism of the *trans* double bond in the side-chain. Because SMYW is not a

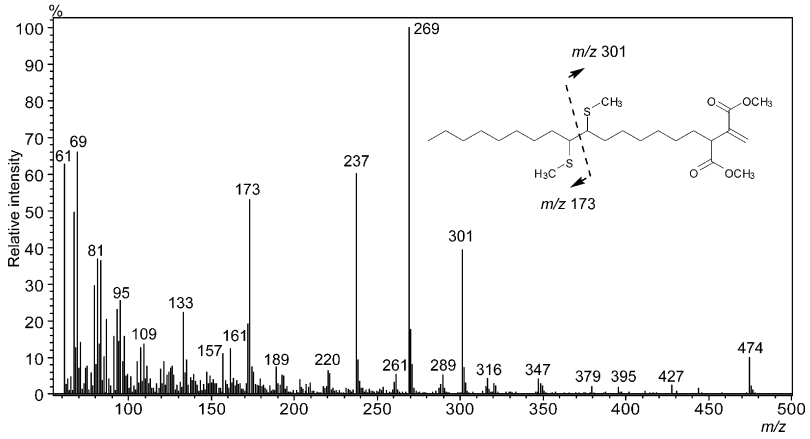

Fig. 4. EI Mass spectrum of a DMDS derivative of ceriporic acid D dimethyl ester (dimethyl (*E*)-7-hexadecenylitaconate, **6**). Key fragment ions produced by the cleavage at Δ9 were found at *m/z* 173 and 301. Molecular ion (M^+) was observed at *m/z* 474.

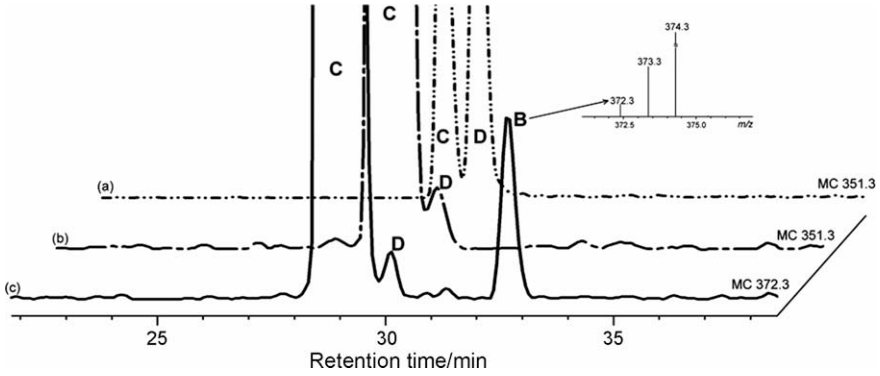

Fig. 5. LC/ESI-MS mass chromatograms of synthetic (*Z*)- and (*E*)-7-hexadecenylitaconic acids (**3** and **4**) (a) and ceriporic acids extracted from the culture fluid of *C. subvermispora* incubated in the modified BIII medium using natural abundance (b) and ^{13}C-[U, 99%]-glucose (c) as the sole carbon source for 19 days. Peaks C and D on the chromatograms are ceriporic acids C (**3**) and D (**4**), respectively. In (a) and (b), mass chromatograms at *m/z* 351.3 corresponding to the deprotonated molecules of ceriporic acids C and D were observed. In (c), *m/z* 372.3 corresponding to the ^{13}C-labelled deprotonated molecules of ceriporic acids C and D were found. Peaks B is the isotope of the ^{13}C-labelled deprotonated molecule of ceriporic acids B (**2**) $[^{13}C_{19}{}^{12}C_2H_{38}O_4{-}H]^-$.

chemically defined medium, it is not clear whether the double bond is biosynthesised *de novo* or incorporated from *trans* fatty acids originally present in the medium. To clarify this, we analyzed production of (*E*)- and (*Z*)-7-hexadecenylitaconic acids (**3** and **4**) in a chemically defined medium using natural abundance and ^{13}C-[U, 99%]-glucose as the sole carbon source. Extracellular metabolites were recovered from 19-day-old culture fluid of the synthetic medium and analyzed by LC/ESI-MS on a negative ion mode (Figs. 5 and 6). The synthetic (*E*)- and (*Z*)-7-hexadecenylitaconic acids (**3** and **4**) were used as authentic compounds. In LC/ESI-MS of the extract from the cultures administered with the natural abundance glucose, ceriporic acids A and B (**1** and **2**) were detected at 21.2 and 27.8 min, respectively. Deprotonated molecules from these metabolites were observed at *m/z* 325.3 and 353.3, respectively. In addition to the alkylitaconic acids, ceriporic acids C and D (**3** and **4**) were detected at 24.5 and 25.1 min, respectively. The two metabolites produced the same deprotonated molecule, $[^{12}C_{21}H_{36}O_4{-}H]^-$, at *m/z* 351.3. Synthetic ceriporic acids C and D (**3** and **4**) also produced the deprotonated ion, $[^{12}C_{21}H_{36}O_4{-}H]^-$, at *m/z* 351.3. In administration experiments using the stable isotope, ^{13}C-[U, 99%]-glucose, ceriporic acids C and D (**3** and **4**) from the cultures had the same retention time as those from the natural abundance medium, but those M_r were observed at *m/z* 372.3, which is 21 amu larger than that from the natural abundance medium. This indicates that ^{13}C was incorporated into ceriporic acids C and D (**3** and **4**) to produce a deprotonated molecule, $[^{13}C_{21}H_{36}O_4{-}H]^-$, at *m/z* 372.3. These results clearly demonstrate that ceriporic acids C and D (**3** and **4**) were biosynthesised *de novo*.

3. Discussion

The important function of ceriporic acid (**4**) is inhibition of cellulose degradation by hydroxyl radicals ($HO^•$) produced by the Fenton reaction system. This occurs because phenols are good reductants of Fe^{3+}, and reductive radicals such as formate anion and semiquinone radicals from oxalate and benzoquinones reduce molecular oxygen to yield superoxide, which in turn disproportionates into H_2O_2 or reduces Fe^{3+} to Fe^{2+}. The combination of reduction of Fe^{3+} and formation of H_2O_2 leads to the production of $HO^•$, a cellulolytically active oxygen species. To explain the selective ligninolysis in an environment where free radicals and lignin-derived phenols are produced in the presence of O_2 and Fe ions, suppression of the Fenton reaction system is essential.

In this study, we first isolated a new alkenylitaconic acid, ceriporic acid D (**4**). Ceriporic acid D (**4**) is predicted to have similar function to ceriporic acid B (**2**) because of its long alkenyl side-chain and itaconic core like ceriporic acid B (**2**). To identify the new metabolite, authentic ceriporic acids C and D (**3** and **4**) were chemically synthesised. The *cis* and *trans* stereoisomers, ceriporic acids C and D (**3** and **4**), were distinguished by vicinal coupling constants of alkenyl protons and chemical shifts at alkenyl and allylic positions in ^1H and ^{13}C NMR spectra. The two stereoisomers

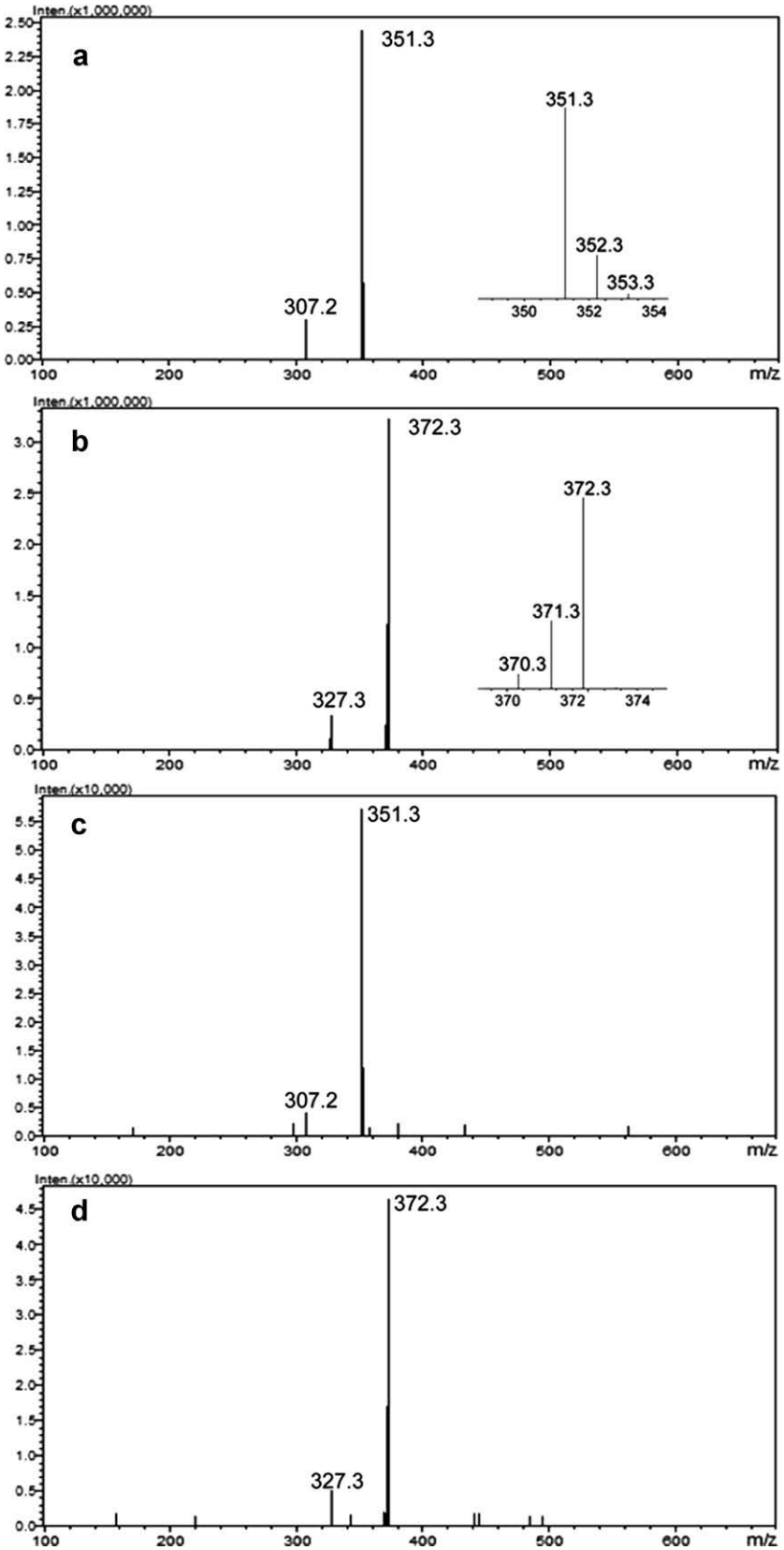

Fig. 6. Negative ESI mass spectra of ceriporic acids extracted from the culture fluid of *C. subvermispora* incubated in the modified BIII medium using natural abundance and ^{13}C-[U, 99%]-glucose as the sole carbon source for 19 days. (a) Natural abundance ceriporic acid C (**3**). (b) ^{13}C-labelled ceriporic acid C (**3**). (c) Natural abundance ceriporic acid D (4) and (d) ^{13}C-labelled ceriporic acid D (**4**). $[^{12}C_{21}H_{36}O_4-H]^-$, *m/z* 351.3; $[^{12}C_{21}H_{36}O_4-COOH]^-$, *m/z* 307.2; $[^{13}C_{21}H_{36}O_4-H]^-$, *m/z* 372.3 and $[^{13}C_{21}H_{36}O_4-COOH]^-$, *m/z* 327.3 were observed in mass spectra.

were separated by HPLC and GC. The *trans* configuration permits a flexible linear structure, whereas the alkyl side-chain in the *cis* isomer is bent at the double bond position, giving in higher affinity to ODS columns during HPLC. The position of the double bond in the hexadecenyl side-chain in the natural metabolite ceriporic acid D (**4**) was determined by GC–MS after derivatisation with dimethyl disulfide.

Alkylitaconic acid derivatives having structural similarities with ceriporic acids have been reported for lichens and other microorganisms (Chen et al., 2007). 15-Hydroxyhexadecyl (**10**) and butylhydroxy itaconic acids are produced by the lichens *Usnea aliphatica* (Keogh and Zurita, 1977) and *Penicillium decumbens* (McCorkindale et al., 1978), respectively. The absolute configuration of 15-hydroxyhexadecyl itaconic acid has been established as 3*R*, 18*R* (Huneck and Snatzke, 1980). Chaetomellic acids (**15**–**19**), produced by *Chaetomella acutiseta*, *C. raphigera*, *C. circinoseta* and *C. oblonga* (Desai and Argade, 1997; Kar and Argade, 2002; Lingham et al., 1993; Ratemi et al., 1996; Gibbs et al., 1993; Singh et al., 2000; Branchaud and Slade, 1994; Kates and Schauble, 1996), are alk(en)ylcitraconic acids and are known anti-cancer agents that inhibit Ras farnesyl-protein transferase (FPTase). In addition to the lichens, a basidiomycete, *Piptoporus australiensis*, produced 3-[(7*Z*)-hexadecenyl]-4-methylfuran-2,5-diene, a chaetomellic acid B anhydride (Gill, 1982). It was reported that a *cis*-citraconic core linking to an alkyl side-chain is required for the inhibition of FTPase activity. Analogues having *trans*-citraconic, itaconic (vinyl acid) and *cis*-citraconic anhydride cores are much less active or almost inactive (Singh et al., 2000). Garneau reported that chaetomellic acid A (**15**) analogues are potential inhibitors against transglycosylase of penicillin-binding protein 1b, a key bacterial enzyme responsible for the formation of the polysaccharide backbone of peptidoglycan (Garneau et al., 2004).

Lichesterinic acid (**20**) (Cavallito et al., 1948; Boll, 1968) from *Cetraria* sp. and 15-acetoxyhexadecyl-, 15-oxohexadecyl- and 15-hydroxyhexadecylcitraconic anhydrides (**21**–**23**) (Selva et al., 1980) from *Aspergillus Wentii* are also reported as citraconic acid derivatives like chaetomellic acid. The other lichen metabolites related to ceriporic acids are (−)-methylenolactocin (**7**) (Park et al., 1987, 1988; Vantamelen and Bach, 1958), nephrosterinic acid (**8**) (Zopf, 1904; Forster et al., 1998), protolichesterinic acid (**9**) (Boll, 1968; Murta et al., 1993), murolic acid (**11**) (Huneck et al., 1979), protoconstipatic acid (**12**), allo-murolic acid (**13**) and allo-pertusaric acid (**14**) (Rezanka and Guschina, 2000; Huneck et al., 1986; Pohmakotr et al., 2002). Those acids (**7**–**9**, **11**–**14**) possess an α-alkyl substituted γ-lactone ring but retain the itaconate core as shown in Fig. 1 (Rezanka and Guschina, 2000; Takahata et al., 1995; Sibi et al., 1996). Phaseolinic acid (**24**) (Mahato et al., 1987; Marta et al., 2004), nephromopsinic acid (**25**), nephrosteranic acid (**26**), roccellaric acid (**27**), dihydroprotolichesterinic acid (**28**), neodihydromurolic acid (**29**), dihydropertusaric acid (**30**) and pertusarinic acid (**31**) (Shimada et al., 1993; Maier et al., 1999; Mulzer et al., 1993; Huneck and Follmann, 1967; Jacobi and Herradura, 1996) are similar α-alkyl substituted γ-lactones. A dicarboxylic acid with a dodecyl alkyl side-chain, called roccellic acid (35), has been isolated (Devlin et al., 1975; Huneck et al., 1994), and its diastereomer, toensbergianic acid was also reported (Bayerova and Haas, 2005). Absolute configuration of dihydropertusaric acid (**30**) was originally determined as 2*S*3*R*4*S* (Huneck et al., 1986), but revised to 2*S*3*S*4*S* (Shimada et al., 1993; Maier et al., 1999), indicating that dihydropertusaric acid (**30**) is identical to pertusarinic acid (**31**). Rocellic acid (**35**) formed a water-soluble metal complex (Iskandar and Syers, 1972). The other dicarboxylic acids with tetradecyl and hexadecyl alkyl side-chains, rangiformic acid (**32**) (Benn et al., 1998), caperatic acid (**33**) (Benn et al., 1998; Brandange et al., 1977) and agaric acid (**34**) (Thoms and Vogelsang, 1907; Brandange et al., 1977) are also isolated. These metabolites are potential precursors for the biosynthesis of ceriporic acids A and B (**1** and **2**). Those lichen metabolites are noted for their biological activities, being antibacterial, antifungal and antitumor substances (Cavallito et al., 1948; Benn et al., 1998; Turk et al., 2003).

Despite numerous reports on lichen acids, there have been no reports of metabolites having a *trans*-hexadecenyl side-chain linking to itaconate, citraconate and maleic anhydride cores. It has been proposed that lichen acids having such cores are biosynthesised *via* condensation of oxaloacetic acids with fatty acids or their CoA esters. We proved that ceriporic acids C and D (**3** and **4**) were biosynthesised *de novo* using ^{13}C-[U, 99%]-glucose administration experiments in chemically defined media. This attracts interest in the biosynthesis of the *trans*-monounsaturated bond in ceriporic acid D (**4**) and in the existence of potential *trans*-analogues of the other lichen acids that are structurally similar to ceriporic acids.

In lipids of microorganisms, monounsaturated bonds are mostly in a *cis* configuration, although *trans*-monounsaturated bonds can be found in free unsaturated fatty acids and membrane phospholipids in some microorganisms. In membrane phospholipids, incorporation of *trans* fatty acids decreases membrane fluidity and increases tolerance against environmental stresses such as toxic compounds, organic solvents and desiccation (Keweloh and Heipieper, 1996).

trans-Monounsaturated bonds can form via three different synthetic routes. *trans* fatty acids are biosynthesised by direct isomerisation of the *cis*-double bond by *cis/trans* isomerase without positional shift (Keweloh and Heipieper, 1996; Heipieper et al., 2003). Another route is formation by an intra-cellular free radical reaction. Free radicals, such as biologically important thiyl radicals (RS$^{\bullet}$), isomerise the *cis*-double bond to its corresponding *trans* form (Chatgilialoglu and Ferreri, 2005; Chatgllialoglu et al., 2006). The third route for the *trans* monounsaturated bond is direct formation by (*E*)-desaturase, or (*Z*), (*E*)-desaturase that had been reported for two kinds of moths, *Epiphyas postvittana*, and *Argyrotaenia velutinana* (Bolton et al., 1994; Jurenka, 1997; Liu et al., 2002). Studies on desaturase and isomerase of *C. subvermispora* are important to elucidate the biosynthetic pathway.

4. Conclusions

In summary, we determined that a white rot fungus, *C. subvermispora*, produced an (*E*)-7-hexadecenylitaconic acid, which we named ceriporic acid D (**4**), and that ceriporic acids C and D (**3** and **4**) were biosynthesised *de novo* using glucose as a sole carbon source.

5. Experimental

5.1. General methods

Itaconic acid, 1-bromohexadecane, 1-bromooctane and phosphorous tribromide were obtained from Wako Pure Chemical Industries (Osaka, Japan), magnesium turnings, dimethyl disulfide (DMDS) and diethyl sulfide were from Nacalai Tesque Inc. (Kyoto, Japan). DMDS was distilled before use. (*Z*)-7-Hexadecen-1-ol was provided by Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). ^{13}C-glucose (U-13C6, 99%) was obtained from Cambridge Isotope Laboratries (Massachusetts). The other reagents used were of analytical grade.

GC–MS analysis was performed with a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer (Kyoto, Japan) on a DB-5HT column (30 m × 0.25 mm, 1 μm, J & W Scientific Inc., California). Electron impact mass spectrum (EI-MS) was recorded at an ionisation energy of 70 eV. Flow rate of carrier gas

(He) were 50 ml min^{-1} (sprit 38:1). The column oven temperature was maintained at 50 °C for 3 min and subsequently raised from 50 to 100 °C at 40 °C min^{-1}, from 100 to 210 °C at 3 °C min^{-1}, and from 210 to 300 °C at 12 °C min^{-1}, and maintained at 300 °C for 15 min. The ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD with a JEOL λ-400 NMR spectrometer (Tokyo, Japan) using tetramethylsilane (TMS) as an internal standard. LC/ESI-MS analysis was performed with a Shimadzu LCMS-2010A liquid chromatograph mass spectrometer with electrospray ionisation (Kyoto, Japan) using a GeminiC18 column (150 mm × 2.0 mm, 5 μm, Phenomenex, California). CH_3CN and 0.01 M NH_4OAc buffer (pH 4.5) were used as mobile phase. Gradient elution was performed by increasing the percentage of CH_3CN as follows: 40% at 0–3 min, 40–97% at 3–40 min, and 97% at 40–60 min.

Product analyses and purification by high performance liquid chromatography (HPLC) were performed on a HITACHI HPLC system with a L6200 gradient pump and a L4200 UV detector monitored at 220 nm using a reversed-phase Cosmosil Cholester column (250 mm × 4.6 mm, Nacalai Tesque Inc., Japan). Gradient elution was performed by a linear gradient mode using two different solvent mixtures, CH_3CN/0.05 M acetate buffer, pH 3.80, 0.05 M NaCl = 50/50 (A) and CH_3CN/0.05 M acetate buffer, pH 5.40, 0.05 M NaCl = 90/10 (B). The initial percentage of solvent A was 100%. The percentage of solvent B was increased to 40% for the initial 3 min, and then to 55% for 3–10 min, and kept at 55% for 10–40 min.

5.2. Chemical synthesis of ceriporic acids C and D (**3** and **4**)

(*Z*)- and (*E*)-7-Hexadecene 1-bromide were prepared from an isomeric mixture of (*Z*)-7-hexadecen-1-ol containing 5% (*E*)-7-hexadecen-1-ol (1.0 g). PBr_3 (1.3 g) was added to the alcohol, and the mixture was stirred at room temperature for 16 h. After the reaction, the products were partitioned between Et_2O and H_2O. The organic phase was separated and washed subsequently with 5% $NaHCO_3$ and satd NaCl, and dried Na_2SO_4. The 7-hexadecene 1-bromide obtained was purified by silica gel column chromatography (1.7 × 20 cm) using *n*-hexane as an eluent (yield, 89.4%).

Grignard reaction of dimethyl α-(bromomethyl) fumarate (2.6 g) with (*Z*)- and (*E*)-7-hexadecene 1-magnesium bromide was carried out using $LiCuBr_2$ as a catalyst under N_2, as previously described (0.132 g, yield, 10.6%) (Enoki et al., 2002; Ohashi et al., 2007). The ratio of (*Z*)- and (*E*)-isomer was 22:3 which was estimated by using integral area of the allylic protons. A mixture of the dimethyl (*Z*)- and (*E*)-7-hexadecenylitaconates (**4** and **5**) were separated on a Discovery Ag-ion SPE cartridge (750 mg, 3 ml, Supelco, Pennsylvania). The dimethyl esters were dissolved in *n*-hexane and loaded onto the cartridge, which had been equilibrated with *n*-hexane. The column was washed with *n*-hexane/acetone (96:4, v/v, 6 ml). Dimethyl (*E*)- and (*Z*)-7-hexadecenylitaconates were recovered separately by stepwise elution with 4 ml of *n*-hexane/acetone (90:10) followed by *n*-hexane/acetone (80:20, v/v).

Demethylation of dimethyl 7-hexadecenylitaconates (1.0 g) was performed with LiOH. The dimethyl ester dissolved in THF was poured into 2.6 M LiOH aq. and stirred for 15 h at room temperature. The reaction products were extracted with $CHCl_3$–MeOH (2:1, v/v) under acidic conditions to give the corresponding free fatty acids. (0.87 g, yield, 94.0%)

5.2.1. (Z)- and (E)-7-hexadecene 1-bromide

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (3H, *t*, H-16), 1.29 (16H, H-3–5 and H-10–14), 1.36 (2H, *m*, H-15), 1.86 (2H, *m*, H-2), 2.05 and 2.08 (4H, H-6 and H-9), 3.49 (2H, *m*, H-1), 5.36 and 5.41 (2H, *Z*-isomer and *E*-isomer, *m*, H-7 and H-8). The ratio of (*Z*)- and (*E*)-isomer was 8:1 which was estimated by using integral area of the alkenyl protons. EIMS, 70 eV, *m/z* (rel. int.): 304 $[M(^{81}Br)]^+$ (4), 302 $[M]^+$ (4), 164 $[M(^{81}Br) - C_{10}H_{20}]^+$ (8), 162 $[M-C_{10}H_{20}]^+$ (8), 150 (16), 148 (18), 111 (28), 97 (83).

5.3. Culture conditions of C. subvermispora and extraction of ceriporic acids (3 and **4**)

C. subvermispora ATCC90467 was grown on a potato dextrose agar medium at 25 °C for 5 days. The preculture was inoculated onto SMYW medium 100 ml in an Erlenmeyer flask 500-ml and incubated statically at 28 °C for 1 week. The SMYW medium used contained sucrose (10.0 g/l), Bacto™ malt extract (10.0 g/l), Bacto™ yeast extract (4.0 g/l) and 10 pieces/l of Japanese beech wood block (2.0 × 2.0 × 1.0 cm). Extracellular fluid of the fungus was filtered and extracted with $CHCl_3$–MeOH (2:1, v/v). The extracts were purified using HPLC and analyzed by GC–MS as previously described.

5.4. Administration of ^{13}C-labelled glucose to C. subvermispora

C. subvermispora was pre-incubated in a modified BIII medium (Kirk et al., 1986) at 28 °C for 12 days. $(NH_4)_2SO_4$ was used as a nitrogen source instead of NH_4NO_3 and reduce the glucose amount to 0.5%. Blended mycelium was inoculated onto the modified BIII medium 50 ml in an Erlenmeyer flask 300-ml and incubated statically at 28 °C for 19 days. ^{13}C-[U, 99%]-labelled and natural abundance glucose were used as the sole carbon source. After cultivation, the fungal mycelium was filtered and washed with distilled H_2O (3 × 10 ml). Then the extracellular fluid was extracted with $CHCl_3$–MeOH (2:1, v/v) and analyzed using LC/ESI-MS as previously described.

5.5. Analysis of position of double bond by DMDS derivatisation

Natural dimethyl (*E*)- and (*Z*)-7-hexadecenylitaconates (**5** and **6**, 1–10 μg) were derivatised in *n*-hexane 50 μl by addition of DMDS 50 μl and I_2 soln. (5 μl, 60 mg/ml in diethyl ether) (Francis and Veland, 1981; George and Knut, 1981; Hans Rudolf et al., 1983). The reaction was carried out at 40 °C for 20 h. The reaction products were diluted with *n*-hexane 200 μl, and I_2 was removed by treating with 5% aqueous $Na_2S_2O_3$ solution 100 μl. The organic phase was separated and concentrated to 20–50 μl and analyzed by GC–MS.

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