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Ceriporic acid C, a hexadecenylitaconate produced by a lignin-degrading fungus, *Ceriporiopsis subvermispora*

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Abstract

A lignin-degrading basidiomycete, *Ceriporiopsis subvermispora* produces a series of alkyl- and alkenylitaconates (ceriporic acids). Previously, two alkylitaconic acids with tetradecyl and hexadecyl side chains were isolated and identified as 1-heptadecene-2,3-dicarboxylic acid (ceriporic acid A) and 1-nonadecene-2,3-dicarboxylic acid (ceriporic acid B). In the present study, one hexadecenylitaconate (ceriporic acid C) was isolated and its chemical structure was analyzed by glycolation and subsequent (1) trimethylsilation, or (2) acetalation with acetone and acetone-d_6. Analyses of the isolated metabolite demonstrated that the hexadecenylitaconic acid was (*Z*)-1,10-nonadecadiene-2,3-dicarboxylic acid. The structure of the side chain in ceriporic acid C was the same as that of hexadecenylcitraconate, chaetomellic acid B. Thus, it was found that ceriporic acids share close structural similarity with alk(en)yl citraconate derivatives, chaetomellic acids and other lichen lactones, protolichesterinic, lichesterinic, and murolic acids.

Keywords: Itaconic acid; Ceriporic acid; White rot fungi

1. Introduction

Itaconic acid has been found as a metabolite of filamentous fungi such as *Aspergillus itaconicus*, (Kinosita, 1931; Bonnarme et al., 1995), *Helicobasidium mompa* (Araki et al., 1957), *Ustilago zeae* (Haskins et al., 1955), and *U. maydis* (Guevarra and Tabuchi, 1990) and some yeast belonging to the genus *Candida* (Tabuchi et al., 1981). Dicarboxylic acids having an alkyl side chain and itaconate core have been isolated from lichens and other microorganisms. 15-Hydroxyhexadecyl and butylhydroxy itaconic acids are produced by the lichen *Usnea aliphatica* (Keogh and Zurita, 1977) and *Penicillium decumbens* (McCorkindale et al., 1978), respectively. The other analogues of alkylitaconates are alk(en)ylcitraconic acids, called chaetomellic acids (Fig. 1). A series of chaetomellic acids with different chain lengths and degrees of unsaturation have been isolated from lichens. These metabolites are a viable target for the development of anticancer drugs due to their strong inhibitory effects on RAS farnesyl-protein transferase (FPTase;

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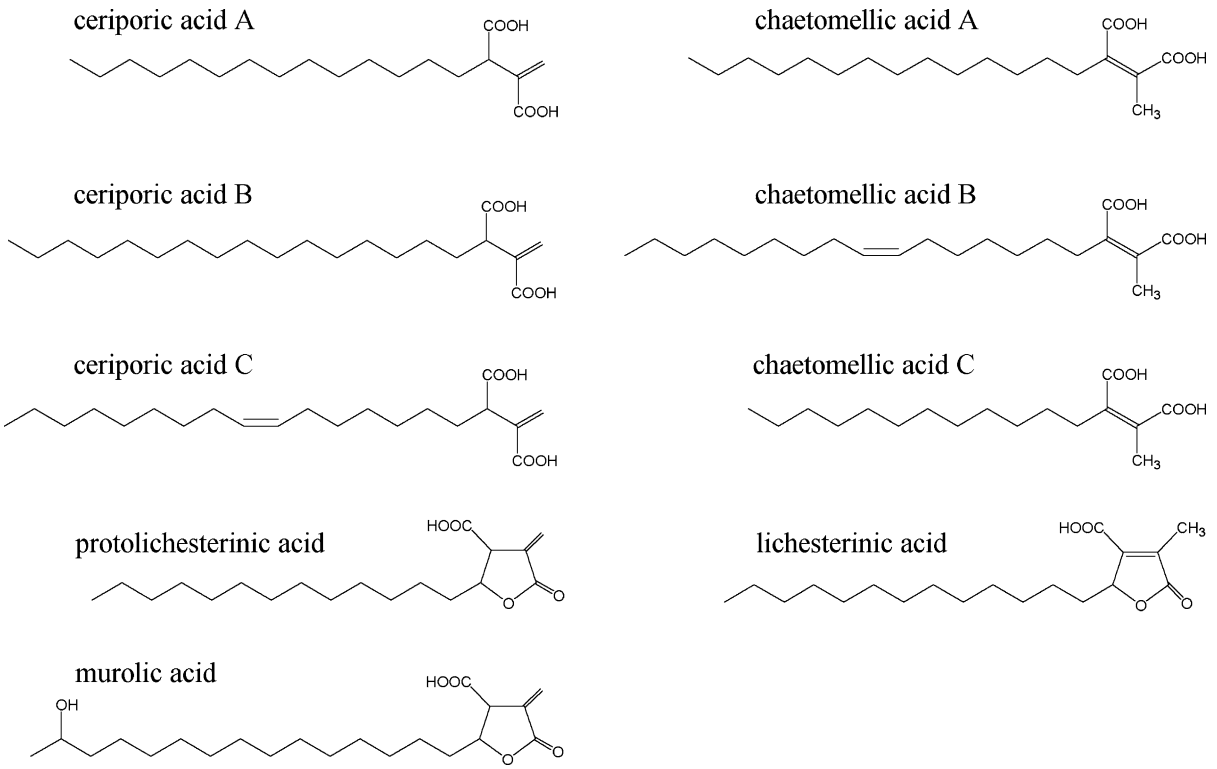

Fig. 1. Chemical structure of ceriporic acids A, B, and C, chaetomellic acids A, B, and C, protolichesterinic acid, lichesterinic acid, and murolic acid.

Singh, 1993; Singh et al., 1993, 2000; Branchaud and Slade, 1994; Gibbs et al., 1993; Gill, 1982; Ratemi et al., 1996; Kates and Schauble, 1996). Chaetomellic acids are lichen aliphatic acids produced by *Chaetomella acutiseta*, *C. raphigera*, *C. circinoseta*, and *C. oblonga* but production of chaetomellic acid B anhydride by a basidiomycete, *Piptoporus australiensis* is also reported (Gill, 1982). The other fungal metabolites related to alk(en)ylitaconate are aliphatic lactones, protolichesterinic, murolic, and lichesterinic acids. Protolichesterinic and murolic acids possess a lactone ring but retain the itaconate core, while lichesterinic acid is an alkyllactone having a citraconate core (Fig. 1) (Huneck et al., 1979).

In 1999, a series of new fungal metabolites, alkylitaconates, were found in cultures of a selective lignin-degrading fungus, *Ceriporiopsis subvermispora* (Enoki et al., 1999). This fungus has potential in biopulping due to its selectivity for lignin-degradation (Akhtar et al., 1998; Urzúa et al., 1998; Daina et al., 2002; Ferraz et al., 2002). *C. subvermispora* produces a series of itaconic acid derivatives having a long alkyl and alkenyl side chain at position C-3 of its core (ceriporic acids) (Fig. 1) (Enoki et al., 1999, 2000). Recently, we synthesized one of the alkylitaconates, 1-nonadecene-2,3-dicarboxylic acid (ceriporic acid B), and found that the new metabolite suppressed iron redox reactions to attenuate $^{\bullet}$OH production by the Fenton reaction in the presence of iron reductants such as hydroquinone and cysteine (Enoki et al., 2002; Watanabe et al., 2002). We proposed that the suppression of the cellulolytic active oxygen species, $^{\bullet}$OH, by this metabolite contributes to the selective lignin-degradation with a minimum loss of cellulose. Among the new itaconate derivatives, a hexadecenyl derivative was reported originally as (*Z*)-1,7-nonadecadiene-2,3-dicarboxylic acid (Enoki et al., 1999, 2000), but del Rio et al. (2002) and Gutiérrez et al. (2002) proposed that the position of the double bond in its side chain be changed to C-10 based on gas chromatograph–mass spectrometory (GC–MS) of a crude extract from wood cultures of *C.*

subvermispora. From the GC–MS of crude extract, it is difficult to identify new compounds, especially those belonging to a large family of metabolites with structural similarities. In the present study, we isolated the hexadecenyl itaconate and analyzed its chemical structure by glycolation and subsequent (1) trimethylsilation, or (2) acetalation with acetone and acetone-d_6, with special emphasis on the position of the double bond in its hexadecenyl side chain. The structural relationship of the new metabolite with other fungal alk(en)yl itaconates and citraconates is discussed.

2. Experimental methods

2.1. General method

The extractive-free wood meals (40–60 mesh) used for fungal cultures were prepared by grinding sapwood of beech (*Fagus crenata*) in a Wiley mill and subsequent refluxing treatment with a mixture of benzene and ethanol (2:1, v/v) for 48 h (Enoki et al., 1999). Milli-Q^{TM} water and peroxide-free organic solvent ($CHCl_3$/MeOH; 2:1, v/v) were used for the extraction of wood meal cultures. All of the chemicals used were of analytical grade. Trimethylsilyldiazomethane and BSTFA–TMCS were obtained from Sigma (Missouri, USA). The structural analysis of ceriporic acid C was conducted using nuclear magnetic resonance (NMR) spectroscopy and gas chromatograph–mass spectrometry (GC–MS). NMR spectra were obtained on a JEOL λ-400 NMR spectrometer (Tokyo, Japan) at 400 MHz and 22 °C in $CDCl_3$. Tetramethylsilane (TMS) was used as an internal standard. GC–MS was performed on a Shimadzu QP-5050 A mass spectrometer with a fused-silica CP-Sil-8-CB capillary column (50 m × 0.25 mm i.d., GL Science, Tokyo, Japan). The electron impact mass spectrum (EIMS) was recorded at an ionization energy of 70 eV. The column oven temperature was raised from 130 to 230 °C at 8 °C min^{-1} and maintained at 230 °C for 10 min and then raised to 280 °C at 8 °C min^{-1} for 20 min.

2.2. Isolation of ceriporic acid C

C. subvermispora ATCC 90467 was cultured on a modified BIII-agar medium at 28 °C for two weeks (Kirk et al., 1986). $(NH_4)_2SO_4$ was used as a nitrogen source instead of ammonium tartrate. Glucose (10 g l^{-1}) was used as a carbon source. Two pellets of inocula from the preculture were added to 4 g of extractive-free beech wood meal containing 7 ml of Milli-Q^{TM} water in a 300-ml Erlenmeyer flask and incubated at 28 °C and 70% relative humidity for 2, 4, and 8 weeks. After the incubation, the cultures were extracted with 50 ml of Milli-Q^{TM} water, washed five times with the water, and then extracted with 50 ml of a peroxide-free $CHCl_3$/MeOH (2:1, v/v) solution per flask. Next, the organic extract from 8 weeks culture obtained was concentrated using a rotary vacuum evaporator, and then dissolved with 4 ml of acetonitrile.

The isolation and purification of ceriporic acid C was performed using a Hitachi L-7100 HPLC system equipped with a Shodex-Asahipak ODP-50 column (250 mm × 6.0 mm i.d. Showa Denko, Japan). Detection was carried out based on UV absorbance at 205 nm. Elution was performed at a flow rate of 8 ml min^{-1} with a mixture of 94% acetonitrile and 6% H_2O. The isolated compound was methylated with 100 μl of trimethylsilyldiazomethane (Sigma, 2.0 M in hexane) in an ice–water bath with continuous stirring for 30 min.

2.3. Structural analysis of ceriporic acid C

Glycolation of the dimethyl ester of ceriporic acid C; dimethyl ester of ceriporic acid C (2 mg), and OsO_4 (1 mg) were mixed in a pyridine/dioxane (1:8, v/v) mixture and allowed to react for 60 min at room temperature. To the reaction mixture, 1.7 ml of 16% Na_2SO_3 and 0.5 ml of methanol were added as described (McCloskey and McClelland, 1965). The reaction mixture was stirred for 90 min and then evaporated to dryness. The concentrate was extracted with diethyl ether and subjected to acetalation or trimethylsilation.

Acetalation of glycol from methylated ceriporic acid C; To the diethyl ether solution of the diol derivative, acetone (300 μl) and $CuSO_4$ (20 mg) were added and allowed to react for 120 min at 50 °C. After the reaction, the diethyl ether was removed with a gentle stream of nitrogen gas to obtain *O*-isopropyridine derivatives.

Trimethylsilation of the diol from methylated ceriporic acid C; trimethylsilation of the diol compounds

was carried out with BSTFA (Sigma) containing 1% trimethylsylilchlorosilan (TMCS). Pyridine (100 μl) and BSTFA–TMCS (10 μl) were added and allowed to react at 60 °C for 40 min. The reaction mixture was directly analyzed by GC–MS.

3. Results and discussion

A lignin-degrading basidiomycete, *C. subvermispora* produces a series of alkyl and alkenylitaconates (ceriporic acids; Enoki et al., 1999). Two alkylitaconic acids with tetradecyl and hexadecyl side chains were isolated and identified as 1-heptadecene-2,3-dicarboxylic acid (ceriporic acid A) and 1-nonadecene-2,3-dicarboxylic acid (ceriporic acid B; Enoki et al., 1999, 2002). Other minor alkylitaconates, dodecanyl-, tridecanyl-, tetradecanyl-, pentadecanyl-, octadecanyl-, and octadecenylitaconic acids were tentatively assigned following GC–MS of the crude extract of wood meal cultures of *C. subvermispora* (Gutiérrez et al., 2002). In addition to the alkylitaconate, one alkenylitaconate was reported (Enoki et al., 1999, 2000). The compound was originally reported as (*Z*)-1,7-nonadecadiene-2,3-dicarboxylic acid. However, del Rio and Gutiérrez proposed that the double bond is actually located at C-10 based on GC–MS of a crude extract from wood meal cultures of this fungus. They proposed the structure by detecting the *m*/*z* 215 fragment after glycolation and trimethylsilation of the crude extract. However, direct structural information is not provided by analyses of crude extract by GC–MS. In addition, detection of a counter fragment for the *m*/*z* 215 fragment was not specified because mass spectra of the diol-TMS derivatives were not shown. Therefore, we isolated the hexadecenyl itaconate (Fig. 2) and determined its chemical structure by glycolation and subsequent trimethylsylation or acetalation with acetone and acetone-d_6.

The mass spectrum of the dimethyl ester of ceriporic acid C was identical to that reported previously (Fig. 2) (Enoki et al., 1999; del Rio et al., 2002). Two fragment peaks produced by the scission of methoxy and carboxylate groups were observed at *m*/*z* 349 and 321, respectively. A fragment peak originating from the itaconate core was found at *m*/*z* 157. The dimethyl ester of ceriporic acid C was glycolated and then trimethylsilylated with BSTFA to obtain a diol-TMS derivative of methylated ceriporic acid C (Fig. 3). In the mass spectra, two mass fragments at *m*/*z* 215 and 343 were observed, in addition to one at *m*/*z* 73 originating from the trimethylsilyl group. The fragments at *m*/*z* 215 and 343 were assigned as products formed by the scission of TMS derivatives between C-10 and C-11. When the same reactions were applied to oleic acid, mass fragments at *m*/*z* 215 and 259 were detected as expected (Fig. 3).

The dimethyl ester of ceriporic acid C was also glycolated and then subjected to acetalation with acetone and acetone-d_6. A mass fragment at *m*/*z* 439 was observed in the mass spectrum of the acetal compound prepared with acetone (Fig. 4). This fragment is produced by demethylation of the 2,2-dimethyl-1,3-dioxorane ring [M − 15]. The corresponding fragment [M − 18] was observed at *m*/*z* 442 in the spectrum of the diol-acetal compound prepared with acetone-d_6 due to the presence of one trideuteriomethyl group (Fig. 4). The demethylated compound [M − 15, *m*/*z* 439] decayed on eliminating CH_3COOH to produce carbocation [*m*/*z* 379, M − 75]. In addition to this route, the demethylated compound decayed on eliminating $CH_2{=}C{=}O$ and methanol to produce a fragment with *m*/*z* 365 [M − 89]. A mass fragment with *m*/*z* 347 [M − 107] was also found as reported by McCloskey and McClelland (1965) (Fig. 5).

In the mass spectrum of the diol-acetal derivative prepared with acetone-d_6, the corresponding mass fragments with *m*/*z* 379 [M − 81], 365 [M − 95], and 347 [M − 113] were observed. In addition to the decay route involving the scission of the 2,2-dimethyl-1,3-dioxorane ring, a mass fragment produced by cleavage of the C_{10}–C_{11} bond adjacent to the 1,3-dioxirane ring was detected at *m*/*z* 213 and 219 from the diol-acetal compounds prepared with acetone and acetone-d_6, respectively (Fig. 5). These results clearly indicate that the double bond in the side chain of ceriporic acid C is located at C-10. Therefore, we reexamined the signal assignment of this compound by NMR and concluded that C-4 and C-5 must be reversed. [^{1}H NMR] 0.88 (t, 3H, CH_2CH_3); 1.27–1.32 (m, 20H, CH_2); 1.66 (m, 1H, H-4); 1.88 (m, 1H, H-4); 2.01 (m, 4H, H-9,12); 3.50 (t, 1H, H-3); 3.68 (s, 3H, $COOCH_3$); 3.77 (s, 3H, $COOCH_3$); 5.34 (m, 2H, H-10,11), 5.75 (s, 1H, CH_2=); 6.36 (s, 1H, CH_2=); [^{13}C NMR] 14.2 (C-19);

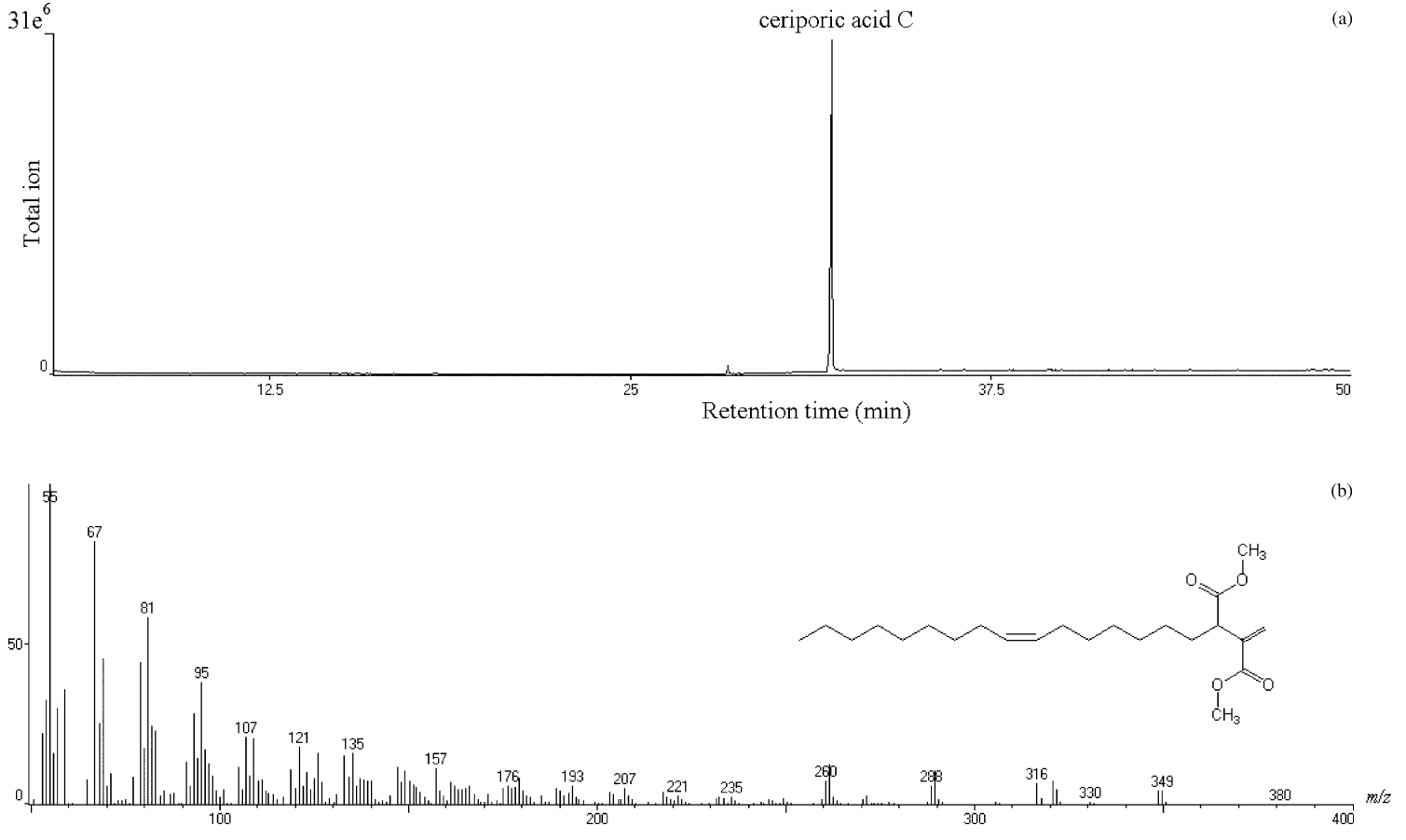

Fig. 2. TIC (a) and mass spectrum (b) of dimethyl ester of ceriporic acid C isolated from cultures of *C. subvermispora*.

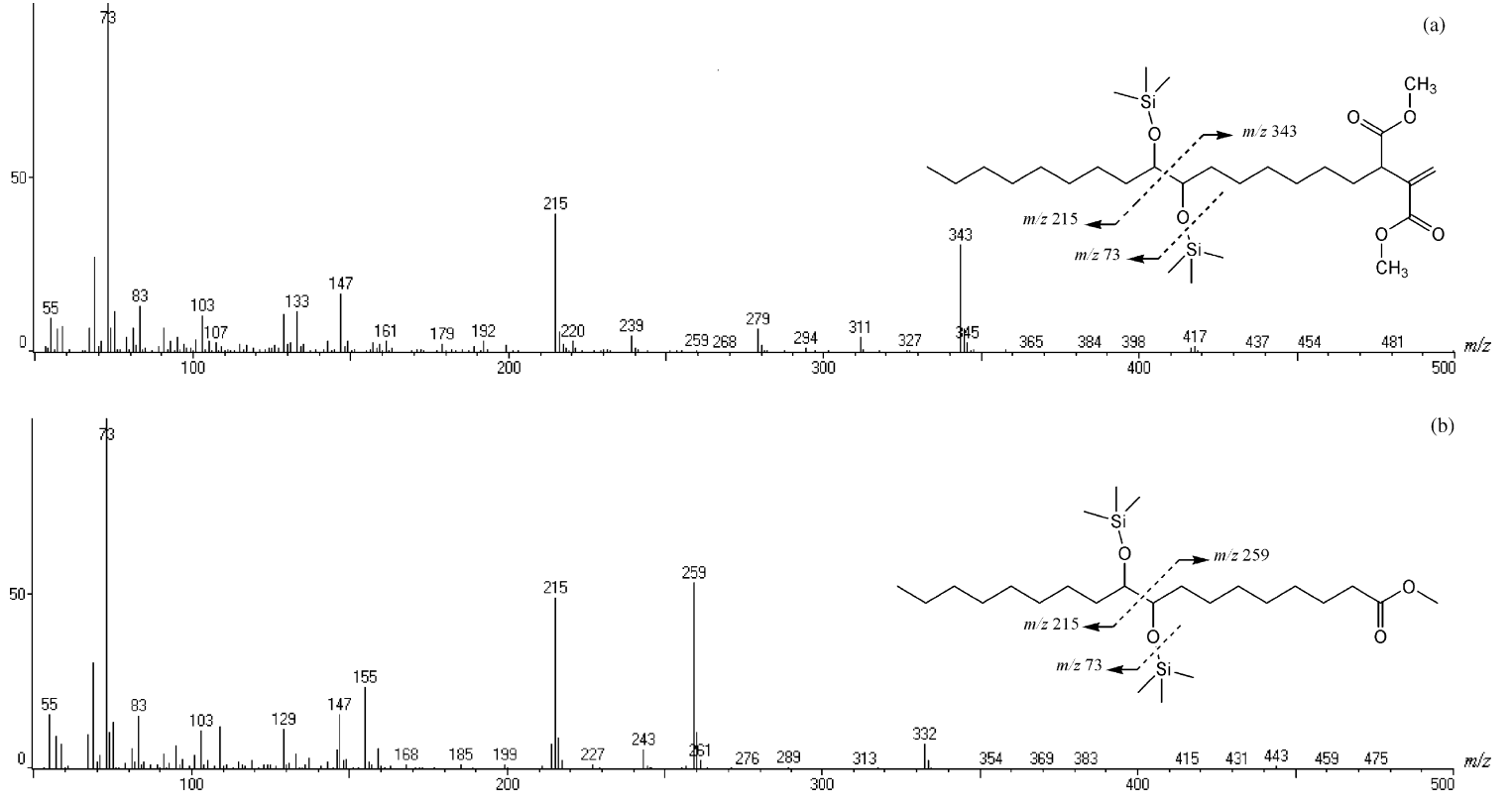

Fig. 3. Mass spectra of the diol-TMS derivative of fatty acids: (a) diol-TMS derivative from the dimethyl ester of ceriporic acid C; (b) diol-TMS derivative from the methyl ester of oleic acid.

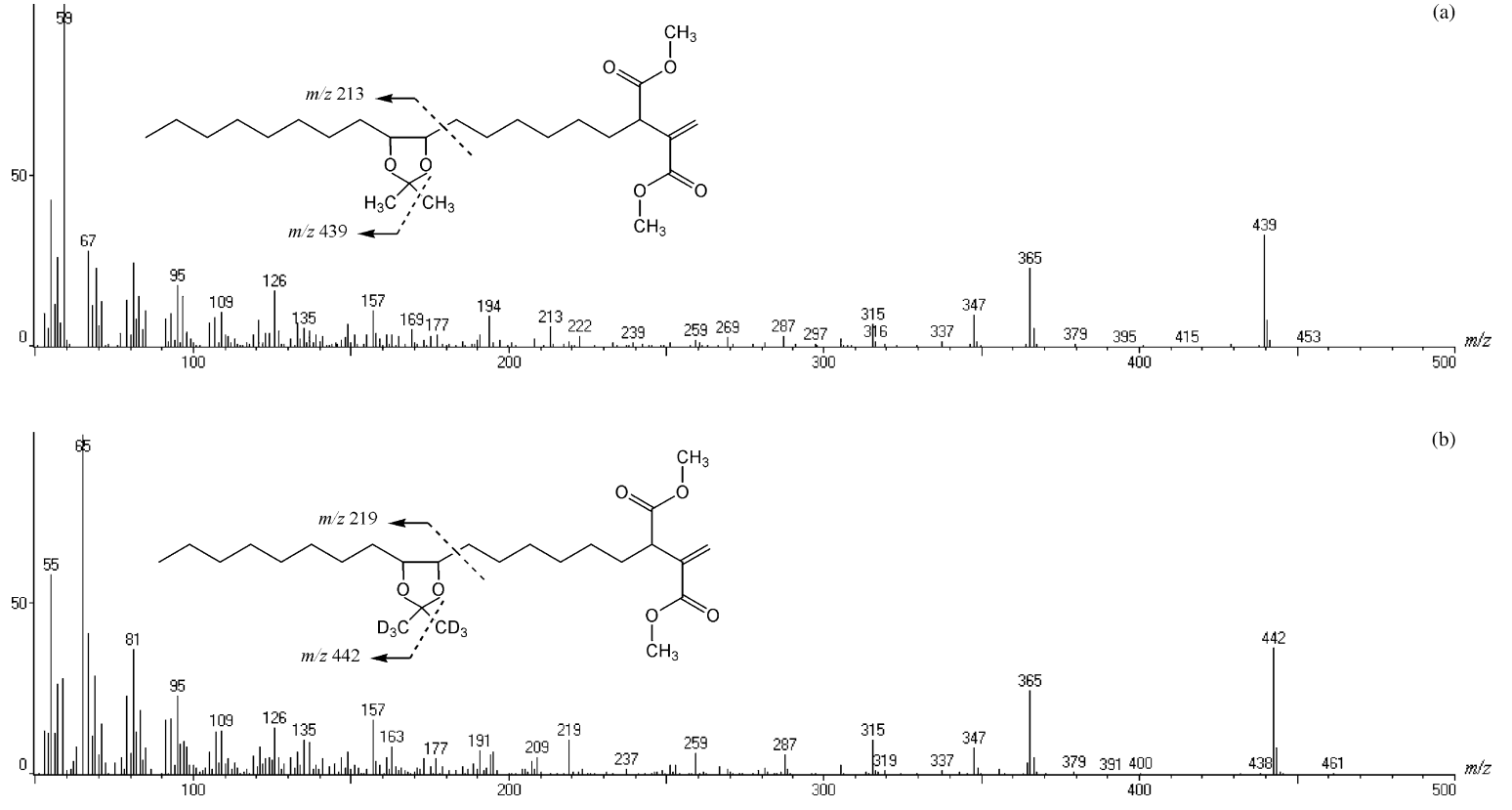

Fig. 4. Mass spectra of the diol-acetal derivative from ceriporic acid C: (a) diol-acetal derivative of methylated ceriporic acid C prepared with acetone; (b) diol-acetal derivative of methylated ceriporic acid C prepared with acetone-d_6.

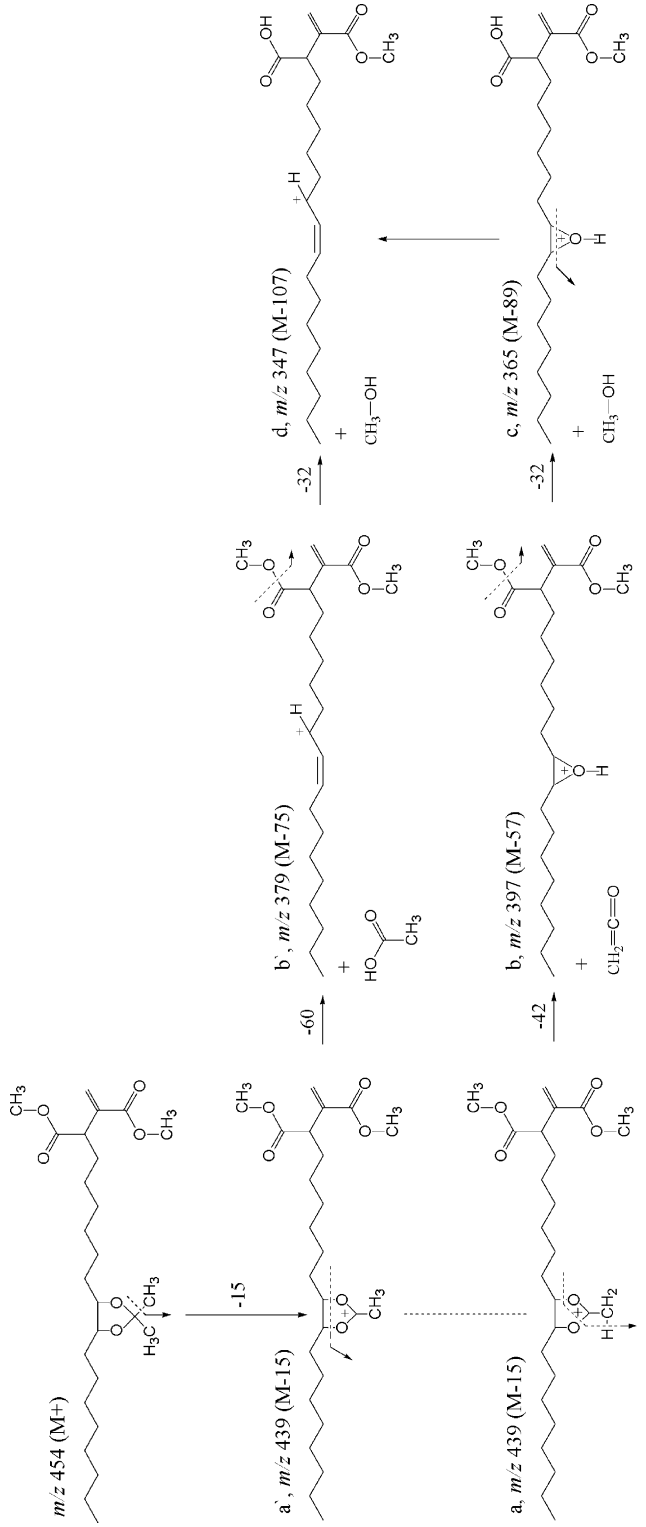

Fig. 5. Fragmentation of the diol-acetal derivative from methylated ceriporic acid C prepared with acetone.

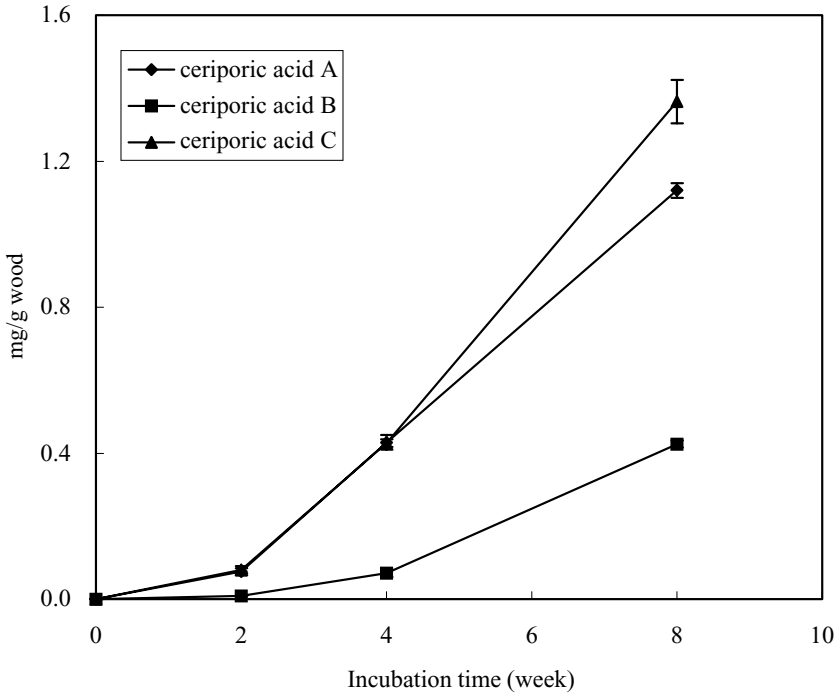

Fig. 6. Production of ceriporic acids by *C. subvermispora* on extractives-free beech wood meal cultures.

22.8 (C-18); 31.3 (C-4); 29.2∼29.9 (C-6∼C-8, C-13∼C-16); 27.3 (C-5,9,12); 32.0 (C-17); 46.7 (C-3); 52.1 ($COOCH_3$); 52.2 ($COOCH_3$); 126.8 (CH_2=); 129.8, 130.1 ($C_{(10)}$=$C_{(11)}$); 138.5 (C-2); 166.8 ($C_{(2)}$-$COOCH_3$); 173.9 ($C_{(3)}$-$COOCH_3$). Recently, Kar and Argade (2002) synthesized (*Z*)-1,7-nonadecadiene-2,3-dicarboxylic acid. They reported that the compound is identical to a fungal metabolite from *C. subvermispora*. However, it should be noted that the chemical shifts of C-10 and C-11 in their compound were 128.8 and 130.6 ppm, respectively. The differences in chemical shift between the two olefinic carbon signals was 1.8 ppm, while that for the ceriporic acid C isolated was 0.3 ppm, as reported for chaetomellic acid B anhydride (Kates and Schauble, 1996). Thus, the ^{13}C NMR chemical shift is consistent with the present structural determination and previous discussion (del Rio et al., 2002). Amount of ceriporic acids produced by *C. subvermispora* on the extractive-free beech wood medium was determined (Fig. 6). The amount of ceriporic acid A, B, and C produced by the fungus reached 1.12, 0.43, and 1.36 mg g^{-1} of wood after 8 weeks. These values were around 9–19 times higher than those estimated by the analysis of lipid-containing eucalypt wood cultures of the fungus (Gutiérrez et al., 2002).

Little is known about the biosynthetic pathways for lichen acids having a citraconate and itaconate core. In 1982, Gill proposed that the chaetomellic acid B anhydride was biosynthesized by condensation between the C-2 of oleic acid and the C-2 of the carbonyl of oxaloacetate, followed by decarboxylation and dehydration (Gill, 1982). Similarly, Gutiérrez et al. (2002) proposed a route for synthesizing alkylitaconate involving condensation of the oxaloacetate with the α-methylene group of acyl-CoA, and the subsequent removal of hydroxyl from the resulting tetradecylcitric acid. In cultures of *C. subvermispora*, condensation products with linoleic acid were not found although linoleic acid is the major unsaturated fatty acid produced by this fungus (Enoki et al., 1999). In the series of alk(en)yl citraconate and its lactone, derivatives having a 1,4-dienyl structure in their side chain are not reported. Thus, a close structural relationship between ceriporic acids and other related lichen acids has been found in the side chain. In chemical synthesis, methyl

alk(en)yl itaconate is converted to the corresponding citraconic acids in alkaline medium at 80 °C (Singh et al., 2000). Refluxing of alkylitaconate with acetic anhydride produced chaetomellic acid anhydride quantitatively (Kar and Argade, 2002). Hydrolysis of the chaetomellic acid anhydride gives the corresponding chaetomellic acid. Therefore, a biosynthetic route from alkylitaconic to chaetomellic acids is possible if the condensation of alkylitaconate and hydrolysis of the alkylcitraconate anhydride is enzymatically catalyzed. However, there has been no report of the co-production of chaetomellic and ceriporic acids by one microorganism. This suggests that, instead of interconversion between itaconate and citraconate derivatives, enzymatic decarbonization and dehydration regulate the biosynthesis of alk(en)yl itaconate and citraconate via the same condensation intermediates from oxaloacetate and acyl-CoA. Research is necessary to elucidate the enzymes responsible for the biosynthesis of unique fungal metabolites, having an itaconic or citraconic core.

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