Redox silencing of the Fenton reaction system by an alkylitaconic acid, ceriporic acid B produced by a selective lignin-degrading fungus, *Ceriporiopsis subvermispora*

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Received 3rd October 2006, Accepted 9th January 2007
First published as an Advance Article on the web 26th January 2007
DOI: 10.1039/b614379b

The selective lignin-degrading fungus, *Ceriporiopsis subvermispora* secretes alkylitaconic acids (ceriporic acids) during wood decay. We reported that ceriporic acid B (hexadecylitaconic acid) was protective against the depolymerization of cellulose by the Fenton reaction. To understand the redox silencing effects, we analyzed the physicochemical and redox properties of itaconic, octylitaconic and hexadecylitaconic acids. The initial rate of $HO^{\bullet}$ production by the Fenton system with Fe^{3+}, H_2O_2 and L-cysteine was suppressed by hexadecylitaconic and octylitaconic acids by 0.04 and 0.16 of the reaction rate without chelators. ESR, O_2 uptake and the assay of Fe^{2+} with BPS demonstrated that Fe^{3+} reduction by L-cysteine was suppressed by hexadecylitaconic and octylitaconic acids while the reaction of Fe^{2+} with H_2O_2 was not suppressed by the two alkylitaconic acids. Ligand exchange experiments with NTA demonstrated that Fe^{3+} chelation by two carboxyl groups of alkylitaconic acids is a critical step in iron redox modulation. In stark contrast, the production of $HO^{\bullet}$ and reduction of Fe^{3+} were not suppressed by itaconic acid due to $HO^{\bullet}$-initiated degradation of the chelator. The strong redox silencing effects by a series of alkylitaconic acids have attracted interest in controlling microbial plant cell wall degradation and chemoprotection against cellular oxidative injury.

Introduction

Iron ions play a key role in fungal metabolism and growth. Since the bioavailability of iron is limited in nature, a number of microorganisms produce iron-chelating agents, such as siderophores, to aid in the assimilation of ferric iron.[1] On the other hand, some microorganisms produce iron-chelating metabolites to sequester Fe ions to facilitate metal tolerance.[2] Wood rot fungi secrete Fe^{3+}-reducing extracellular metabolites to promote the production of hydroxyl radicals by the Fenton reaction to attack wood cell walls.[3–10] The Fenton-based $^{\bullet}OH$-producing system plays a central role in wood decay by brown rot fungi.[3–13] It was shown that natural Fe^{3+}-reductants secreted by the brown rot basidiomycete *Gloeophyllum trabeum* depolymerized cellulose in combination with iron ions and H_2O_2.[14] Structural analysis of cellulose after brown rot decay supports that the Fenton reaction is the major pathway in the breakdown of cellulose by these microorganisms.[15] The production of hydroxyl radicals is also proposed as a possible low molecular mass agent that increases the porosity of wood cell walls in non-selective white rot.[8,16–19] In contrast to wood rot fungi with high cellulolytic activity, a selective white rot fungus, *Ceriporiopsis subvermispora* is able to decompose lignin in wood cell walls without excessive cellulose damage.[4] Lignin biodegradation proceeds by a free radical process in the presence of molecular oxygen and transition metals. Reductive radicals such as the semiquinone radical reduce molecular oxygen to produce superoxide, which in turn reduces Fe^{3+} or disproportionate into H_2O_2. Fe^{3+} is directly reduced by semiquinone radicals and lignin-derived phenols such as guaiacol and catechol. Thus, if some inhibition systems for the iron redox reactions were not involved in the wood decaying systems, production of the cellulolytic oxidant, hydroxyl radical is inevitable. Therefore, we hypothesized that selective white rot fungi secrete low-molecular-mass chelators which inhibit the depolymerization of cellulose by inhibiting the redox cycling of iron ions in the Fenton system during free-radical-mediated lignin biodegradation.[20] We then demonstrated that ceriporic acid B, an alkylitaconic acid produced by *C. subvermispora*, inhibited the depolymerization of cellulose by the Fenton reaction even in the presence of Fe^{3+}-reductants such as hydroquinone.[21] The suppressive effects of ceriporic acids in the Fenton system are in contrast to the effects of itaconic acid, a metabolite of fungi such as *Aspergillus itaconicus*,[22] *Helicobasidium mompa*,[23] *Ustilago zeae*,[24] *U. maydis*[25] and some yeasts belonging to the genus *Candida*.[26] To assess redox modulation by (alkyl)itaconic acids, we synthesized octyl (1-undecene-2,3-dicarboxylic acid) and hexadecyl (ceriporic acid B; 1-nonadecene-2,3-dicarboxylic acid) itaconic acids, and comparatively analyzed the physicochemical and redox properties of itaconic acid derivatives with a long, medium and no alkyl side chain.

Materials and methods

Materials

Itaconic acid, 1-bromohexadecane, 1-bromooctane, $FeCl_3$, $FeSO_4$, bathophenanthrolinedisulfonic acid disodium salt (BPS), H_2O_2,

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L-cysteine and L(+)-ascorbic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Magnesium turnings, nitrilotriacetic acid (NTA) and hydroquinone were obtained from Nacalai Tesque (Kyoto, Japan). A spin trapping reagent, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was obtained from Labotec (Tokyo, Japan). All other chemicals used were of analytical reagent grade. Water was purified using an EASYpure© compact ultrapure water system (Barnstead, IA) throughout this study.

Instrumental analysis

GC-MS analysis was carried out with a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer (Kyoto, Japan) equipped with a DB-5HT column (length, 30 m; i.d., 0.25 mm; thickness, 1 μm; J & W Scientific Inc., CA). The electron impact mass spectrum (EI-MS) was recorded at an ionization energy of 70 eV. The column oven temperature was maintained at 50 °C for 3 min and subsequently raised from 50 °C to 100 °C at 40 °C min^{-1}, from 100 °C to 210 °C at 3 °C min^{-1}, and from 210 °C to 300 °C at 12 °C min^{-1} and maintained for 15 min. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL λ-400 NMR spectrometer (Tokyo, Japan) at 400 MHz, 20 °C in $CDCl_3$. Surface tension was measured using a KRÜSS Digital Tensiometer K9 (Hamburg, Germany). pH titration was carried out using a HORIBA pH meter D21 (Kyoto, Japan). UV–vis absorption spectra were measured with a HITACHI U-3310 Spectrophotometer (Tokyo, Japan) at 25 °C. ESR spectra were measured with a JEOL JES-FR30 Free Radical Monitor (Tokyo, Japan) at room temperature under the following conditions: frequency, 9.425 GHz; center field, 335.6 mT; sweep width, 5.0 mT; modulation width, 0.10 mT; receiver gain, 320; data points, 4096; time constant, 0.10 s; sweep time, 1.0 min; power, 4.0 mW.

Synthesis of ceriporic acid B and its analogues

Hexadecylitaconic acid (ceriporic acid B; 1-nonadecene-2,3-dicarboxylic acid, **5**) and dimethyl hexadecylitaconate (dimethyl 1-nonadecene-2,3-dicarboxylate, **4**) were synthesized according to Fig. 1 as described.[27]

Dimethyl octylitaconate (dimethyl 1-undecene-2,3-dicarboxylate, **6**) was synthesized using the Grignard reaction from dimethyl (α-bromomethyl)fumarate, **3**. Procedures for the reaction and purification were as described for hexadecylitaconic acid[27] except for the preparation of the Grignard reagent. The temperature for the Grignard reaction was lowered from 50–55 °C to 30–35 °C due to the higher reactivity of 1-bromooctane with magnesium compared to that of 1-bromohexadecane. Distilled tetrahydrofuran (74.8 mL), compound **3** (3.94 g, 16.6 mM) and 1-bromooctane (4.24 g, 22.0 mM) were prepared to give compound **6** (1.54 g, 5.70 mM: 34.3% from **3**). Compound **6** was then demethylated by acidolysis. A mixture of **6** (0.88 g, 3.3 mM), formic acid (22.9 mL), hydroquinone (4.6 mg) as a polymerization inhibitor, and sulfuric acid (0.323 g) was heated at 100 °C for 3 hours with stirring to give octylitaconic acid (1-undecene-2,3-dicarboxylic acid, **7**, 0.40 g, 1.7 mM: 51% from **6**): δ_H (400 MHz, $CDCl_3$) 0.85 (3H, s, CH_2CH_3); 1.23 (12H, m, CH_2); 1.64 (1H, m, H-4); 1.86 (1H, m, H-4); 3.37 (1H, t, H-3); 5.78 (1H, s, CH_2=, *trans*); 6.44 (1H, s, CH_2=, *cis*), δ_C (400 MHz, $CDCl_3$) 14.1 (C-11); 22.6 (C-10); 27.4 (C-5); 29.3–29.6 (C-6–C-9); 31.8 (C-4); 47.8 (C-3); 127.4 (C-2); 137.6 (C-1); 171.1 ($C_{(2)}$–*C*OOH); 179.8 ($C_{(3)}$–*C*OOH), *m/z* 224(1%), 196 (3), 179 (1), 163 (1), 150 (4), 139 (7), 126 (100), 111 (8), 98(35), 81 (8), 70 (10), 57 (28), 43 (60).

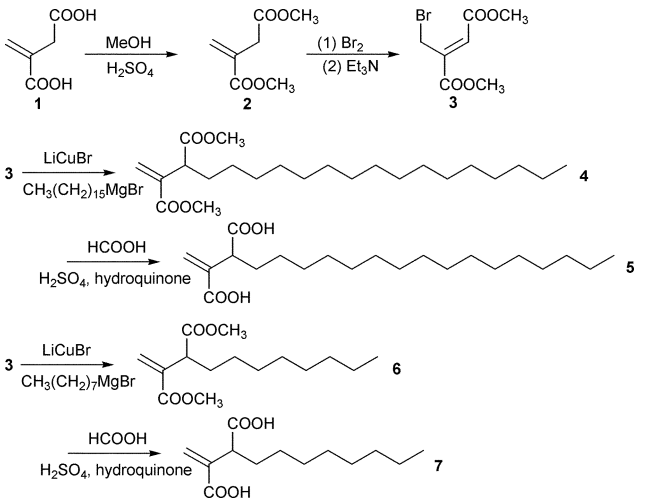

Fig. 1 Synthetic route for 1-nonadecene-2,3-dicarboxylic acid and 1-undecene-2,3-dicarboxylic acid. **1**, itaconic acid; **2**, dimethyl itaconate; **3**, dimethyl (α-bromomethyl)fumarate; **4**, dimethyl hexadecylitaconate (dimethyl 1-nonadecene-2,3-dicarboxylate); **5**, hexadecylitaconic acid (ceriporic acid B; 1-nonadecene-2,3-dicarboxylic acid); **6**, dimethyl octylitaconate (dimethyl 1-undecene-2,3-dicarboxylate); **7**, octylitaconic acid (1-undecene-2,3-dicarboxylic acid).

Determination of surface tension activity of octylitaconic acid

The surface tension of octylitaconic acid was compared with that of Tween 20 and Tween 80. Each compound was dissolved in water at a concentration range of 0.1 mg L^{-1} to 100 mg L^{-1}. Each sample (5 mL) was placed in a vessel and surface tension was measured in triplicate.

Acid dissociation constants of ceriporic acid B and its analogues

To determine the acid dissociation constant of hexadecylitaconic acid, octylitaconic acid, and itaconic acid, pH titration was carried out at room temperature. The electrode was calibrated using standard aqueous buffers (pH 4.0 and 7.0). Solutions were prepared with 50% (v/v) EtOH(aq) due to the low solubility of hexadecylitaconic acid in water, and ionic strength was adjusted to 100 mM by adding $NaNO_3$. Ten millilitres of hexadecylitaconic acid, octylitaconic acid, and itaconic acid solution (1 mM each) containing HNO_3 (4 mM) were titrated with 10 mM NaOH in 50% (v/v) EtOH(aq). The pH was measured after every incremental addition of NaOH solution (20 μL) with stirring until the pH value reached 12.0.

Effects of ceriporic acid B and its analogues on HO• production

To investigate the effects of itaconic acid derivatives on HO• production in the Fenton system, the spin trapping method was carried out as described.[28] With an excess of ethanol *versus* 4-POBN, HO• produced by the Fenton reaction reacts with ethanol to yield the α-hydroxyethyl radical according to (eqn (1)). The

α-hydroxyethyl radical is trapped with 4-POBN (eqn (2)), and the spin adduct produced is quantified by ESR:

$$CH_3CH_2OH + HO^{\bullet} \rightarrow CH_3C^{\bullet}HOH + H_2O \quad (1)$$

$$CH_3C^{\bullet}HOH + \text{4-POBN} \rightarrow \text{4-POBN-}CH_2(CH_3)OH \quad (2)$$

Concentrated stock solutions of hexadecylitaconic acid, octylitaconic acid, dimethyl hexadecylitaconate, dimethyl octylitaconate and itaconic acid (30 mM each) were prepared in ethanol. Those of $FeSO_4$ and $FeCl_3$ (1 mM each), 4-POBN (1 M), reductants (0.5 mM each) and H_2O_2 (2 mM) were prepared in succinate buffer (25 mM, pH 4.0).

The required amounts of succinate buffer and $FeCl_3$ stock solution were poured into a 10 mL vial immediately after preparation, and then the stock solution of itaconic acid derivatives and ethanol were added to the solution. After pre-incubation for 30 min, stock solutions of 4-POBN, reductants, and H_2O_2 were added sequentially. The total volume of the reaction solution was 200 μL. The amount of spin adduct was measured at 1, 5, 10, and 15 minutes after the addition of H_2O_2. These experiments were carried out in triplicate. The final concentrations of each reagent at the starting point of the reaction are described in each legend of the figures and tables.

The experiments using $FeSO_4$ instead of $FeCl_3$–reductant were carried out in the same way.

Effects of ceriporic acid B and its analogues on dissolved oxygen consumption in the process of iron redox reaction

The production of Fe^{2+} was determined by oxygen uptake experiments. Stock solutions of hexadecylitaconic acid, octylitaconic acid and itaconic acid (30 mM each) were prepared in ethanol, whereas those of $FeSO_4$ and $FeCl_3$ (10 mM each), L-cysteine (5 mM) and H_2O_2 (20 mM) were prepared in water.

Stock solutions of H_2O_2, L-cysteine, and itaconic acid derivative in ethanol were mixed in a 10 mL test tube equipped with an oxygen electrode. Aqueous $FeCl_3$ was added to start the reaction. Experiments using $FeSO_4$ instead of $FeCl_3$–L-cysteine were carried out in the same way.

Effects of ceriporic acid B and its analogues on the reduction of Fe^{3+}

BPS, a strong chelator of Fe^{2+}, was used to determine Fe^{2+} produced by the reduction of Fe^{3+}. The production of $[Fe(BPS)_3]^{2+}$ was determined by the absorbance at 534 nm (eqn (3)) (ε_{534} is 22 140 L eq^{-1} cm^{-1}).[29,30]

$$Fe^{2+} + 3BPS \rightarrow [Fe(BPS)_3]^{2+} \quad (3)$$

Stock solutions of hexadecylitaconic acid, octylitaconic acid and itaconic acid (4 mM each) were prepared using ethanol. Those of NTA (4 mM), BPS (10 mM) and the reductant for Fe^{3+} (100 mM) were prepared using succinate buffer (25 mM, pH 4.0). $FeCl_3$ (2 mM) was dissolved in succinate buffer (25 mM, pH 4.0) and immediately used for the assay of Fe^{3+} reduction. A stock solution of the itaconic acid derivative was added to $FeCl_3$ solution in a 10 mL vial. Stock solutions of BPS and the reductant were finally added after incubating Fe^{3+} with NTA for 30 min. Absorption was measured at 534 nm. The total volume of the reaction solution was 1.0 mL and the experiments were carried out in triplicate. The final concentrations of each reagent at the starting point of the reaction are described in the legends of Fig. 8 and Table 3.

Theoretical calculation of HOMO energy of ceriporic acid B and its analogues

Differences in HOMO (highest occupied molecular orbital) energies of geometrically optimized itaconic acid derivatives including itaconic acid, octylitaconic acid, and hexadecylitaconic acid were estimated by the MOS-F module in FUJITSU WinMOPAC Version 3.5 (Tokyo, Japan, 2002), semi-empirical molecular orbital calculation software examining the effect of alkyl chain lengths of itaconic acid derivatives on the inhibitory effect on Fe^{3+} reduction and $HO^{\bullet}$ production.

Results

Surface tension activity of octylitaconic acid

Surface tensions of Tween 20, Tween 80, and octylitaconic acid are shown in Fig. 2. Surface activities of octylitaconic acid were lower than those of Tween 20 and Tween 80 between the concentrations of 0.1 mg L^{-1} and 10 mg L^{-1}. At concentrations higher than 50 mg L^{-1}, surface activities increased linearly and exceeded those of Tween 20 and Tween 80. It was difficult to measure the surface activities of octylitaconic acid at concentrations higher than 100 mg L^{-1} due to low solubility.

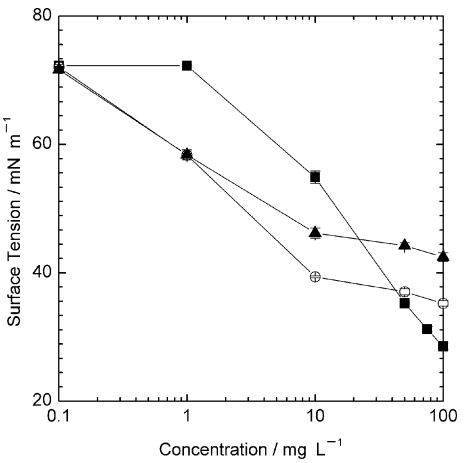

Fig. 2 Surface tension activities of octylitaconic acid (■), Tween 20 (○) and Tween 80 (▲).

Acid dissociation constants of ceriporic acid B and its analogues

The pH titration curves for (alkyl)itaconic acid are shown in Fig. 3. The pK_{a_1} and pK_{a_2} values of itaconic acid in aqueous ethanol solution were 4.5 and 6.5, respectively. These values were slightly higher than those in a previous report (pK_{a_1}, 3.8 and pK_{a_2}, 5.7) measured in water.[31] The pK_{a_1} and pK_{a_2} of hexadecylitaconic acid were 4.5 and 7.8, respectively. For octylitaconic acid, pK_{a_1} and pK_{a_2} were 4.6 and 7.6, respectively. Differences between the pK_{a_1} values of these three itaconic acid derivatives were within 0.1; however, the pK_{a_2} of itaconic acid was lower than those of hexadecylitaconic acid and octylitaconic acid by 1.3 and 1.1,

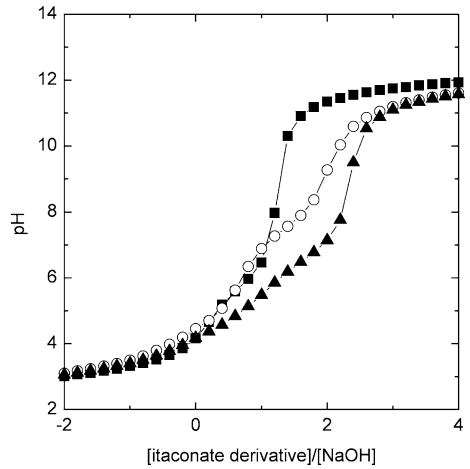

Fig. 3 pH titration curves of 1 mM itaconic acid (■), octylitaconic acid (○) and hexadecylitaconic acid (▲) in the presence of 100 mM $NaNO_3$ and 4 mM HNO_3 in aqueous EtOH (50%, v/v) at room temperature.

respectively. The chain lengths of the two alkylitaconic acids, hexadecylitaconic acid and octylitaconic acid, did not affect the pK_{a1} or pK_{a2} values significantly.

pH dependence on the molar fraction of each acid was calculated using the pK_a values, as shown in Fig. 4(a)–(c). It was found that the pH dependence of $[HA^-]$ of itaconic acid is different from that of octylitaconic acid and hexadecylitaconic acid; the maximum $[HA^-]$ of itaconic acid was lower than that of octylitaconic acid and hexadecylitaconic acid. The highest $[HA^-]$ molar fraction of itaconic acid was 81.6% at pH 5.5 although those of hexadecylitaconic acid and octylitaconic acid were 94.1% and 95.7% at pH 6.1, respectively. The $[HA^-]$ of itaconic acid was the primary fraction at pH 4.6–6.3 although with hexadecylitaconic acid and octylitaconic acid the primary fraction was at pH 4.7–7.5 and pH 4.6–7.7, respectively. Around 80% of the three itaconic acid derivatives exist as "H_2A" at pH 4, used in the experiments for the determination of their inhibitory effects on Fe^{3+} reduction and $HO^{\bullet}$ production.

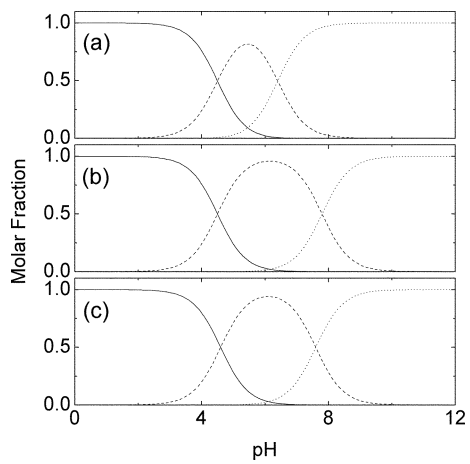

Fig. 4 The pH dependencies of molar fraction diagrams of itaconic acid derivatives (1 mM) in aqueous EtOH (50%, v/v) at 100 mM ionic strength and room temperature. (a) Itaconic acid; (b) octylitaconic acid; (c) hexadecylitaconic acid. Each line shows the molar fraction of $[H_2A]$ (——), $[HA^-]$ (---) and $[A^{2-}]$ (· · ·).

Effects of ceriporic acid B and its analogues on $HO^{\bullet}$ production

The production of $HO^{\bullet}$ in the Fenton system containing (alkyl)itaconic acid (octylitaconic acid, dimethyl octylitaconate and itaconic acid) and a reductant for Fe^{3+}, L-cysteine, was determined (Fig. 5). No suppressive effects were found for dimethyl octylitaconate (▲). Itaconic acid (◇) slightly accelerated $HO^{\bullet}$ production, whereas $HO^{\bullet}$ production was suppressed by octylitaconic acid (○).

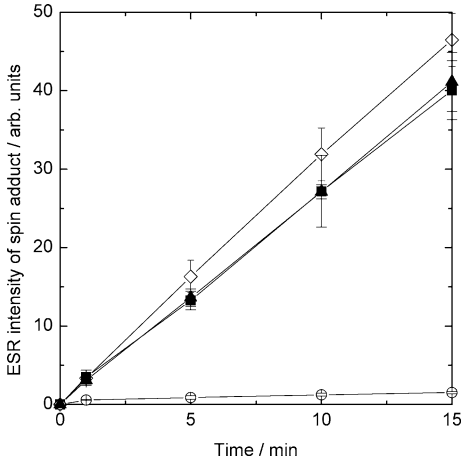

Fig. 5 Effects of octylitaconic acid (○), dimethyl octylitaconate (▲), and itaconic acid (◇) on $HO^{\bullet}$ production by Fe^{3+}–L-cysteine and H_2O_2 in succinate buffer (pH = 4.0) containing 20% (v/v) EtOH at room temperature. $[Fe^{3+}]$ = 0.1 mM, [itaconate derivative] = 3 mM, $[H_2O_2]$ = 0.5 mM, [L-cysteine] = 0.05 mM, [POBN] = 100 mM. In a control experiment (■), no itaconate derivatives were added.

Fig. 6 shows the results of the direct reactions of Fe^{2+} with octylitaconic acid, dimethyl octylitaconate or itaconic acid. In the Fe^{2+} system, the amount of spin adduct rapidly increased within a minute and reached a plateau. Among the three (alkyl)itaconic

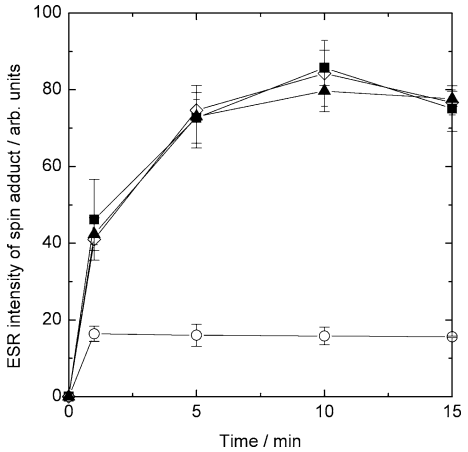

Fig. 6 Effects of octylitaconic acid (○), dimethyl octylitaconate (▲), and itaconic acid (◇) on $HO^{\bullet}$ production by Fe^{2+} and H_2O_2 in succinate buffer (pH = 4.0) containing 20% (v/v) EtOH at room temperature. $[Fe^{2+}]$ = 0.1 mM, [itaconate derivative] = 3 mM, $[H_2O_2]$ = 0.5 mM, [POBN] = 100 mM. In control experiment (■), no itaconic acid derivatives were added.

Table 1 Initial rate and cumulative amount of HO• production by the reaction of Fe^{3+}–reductant–itaconate derivative

Reductant	Itaconate derivative	Initial rate[a]	Cumulative amount[a,b]
L-Cysteine	—	1.00	1.00
	Hexadecylitaconic acid	0.04	0.04
	Dimethyl hexadecylitaconate	0.94	0.96
	Octylitaconic acid	0.16	0.05
	Dimethyl octylitaconate	0.89	1.03
	Itaconic acid	0.96	1.16
L(+)-Ascorbic acid	—	1.00	1.00
	Hexadecylitaconic acid	0.11	0.13
	Dimethyl hexadecylitaconate	0.84	1.03
	Octylitaconic acid	0.38	0.11
	Dimethyl octylitaconate	0.98	1.02
	Itaconic acid	1.04	1.21
Hydroquinone	—	1.00	1.00
	Hexadecylitaconic acid	0.12	0.13
	Dimethyl hexadecylitaconate	0.97	1.03
	Octylitaconic acid	0.16	0.04
	Dimethyl octylitaconate	0.81	1.02
	Itaconic acid	0.80	1.06

Experiments were carried out in succinate buffer (pH = 4.0) containing 20% (v/v) EtOH at room temperature. [Fe^{3+}] = 0.1 mM, [H_2O_2] = 0.5 mM, [itaconate derivative] = 3 mM, [reductant] = 0.05 mM, [4-POBN] = 100 mM. Experimental procedure is described in Materials and Methods.[a] Values are expressed as a ratio of the two reaction systems with and without itaconic acid derivatives. [b] Cumulative amount of HO• produced during the first 15 min.

acid derivatives, suppression of HO• production was found only for octylitaconic acid as in the Fe^{3+}–reductant system. The production of HO• in the presence of octylitaconic acid was less than 3% of the control.

All results in the reactions for the Fe^{3+}–reductant system and those for Fe^{2+} are shown in Table 1 and 2, respectively. When hexadecylitaconic acid or octylitaconic acid was added to the Fe^{3+}–reduction and Fe^{2+} systems, HO• production was inhibited 15 minutes after starting the reaction. In the comparison between the system of Fe^{3+}–reductant and Fe^{2+}, the inhibition effect of HO• production by hexadecylitaconic acid and octylitaconic acid in the Fe^{3+}–reductant system was larger than that in the Fe^{2+} system. Furthermore, the initial rates of HO• production in the Fe^{2+} system containing hexadecylitaconic acid and octylitaconic acid were bigger than in the Fe^{3+}–reductant system.

Table 2 Initial rates of HO• production and cumulative amount of it at 15 min after reaction started in the Fe^{2+}–itaconate derivative system

Itaconate derivative	Initial rate[a]	Cumulative amount[a,b]
—	1.00	1.00
Hexadecylitaconic acid	0.45	0.26
Dimethyl hexadecylitaconate	0.91	1.05
Octylitaconic acid	0.36	0.21
Dimethyl octylitaconate	0.92	1.03
Itaconic acid	0.89	1.02

Experiments were carried out in succinate buffer (pH = 4.0) containing 20% (v/v) EtOH at room temperature. [Fe^{2+}] = 0.1 mM, [H_2O_2] = 0.5 mM, [itaconate derivative] = 3 mM, [4-POBN] = 100 mM. Experimental procedure is described in Materials and Methods.[a] Values are expressed as a ratio of the two reaction systems with and without itaconic acid derivatives. [b] Cumulative amount of HO• produced during the first 15 min.

Effects of ceriporic acid B and its analogues on dissolved oxygen consumption in the process of iron redox reaction

The effects of hexadecylitaconic acid and its analogues on dissolved oxygen consumption in the iron redox reaction are shown in Fig. 7. Dissolved oxygen was consumed by Fe^{2+} in the presence of hexadecylitaconic acid, octylitaconic acid and itaconic acid according to eqn (1). Mixing Fe^{3+}, itaconic acid and L-cysteine decreased dissolved oxygen; however, dissolved oxygen was not consumed when Fe^{3+} was premixed with hexadecylitaconic acid or octylitaconic acid followed by addition of L-cysteine as a reductant for Fe^{3+}.

Effects of ceriporic acid B and its analogues on the reduction of Fe^{3+}

Effects of hexadecylitaconic acid, octylitaconic acid and itaconic acid on Fe^{3+} reduction by a reductant, L-cysteine, were investigated in the presence and absence of a chelating reagent, NTA. When NTA was not added (hexadecylitaconic acid +, NTA −; ○), the production rate of $[Fe(BPS)_3]^{2+}$ decreased compared with a control experiment (hexadecylitaconic acid −, NTA −; ■) as shown in Fig. 8. On the other hand, when hexadecylitaconic acid and NTA were added (hexadecylitaconic acid +, NTA +; ▲), the initial rate of $[Fe(BPS)_3]^{2+}$ production increased about five times compared with when only hexadecylitaconic acid was added (○).

All results are shown in Table 3. When hexadecylitaconic acid or octylitaconic acid was added to the Fe^{3+}–reduction and the Fe^{2+} systems, HO• production was inhibited 15 minutes after starting the reaction. In a comparison of the system of Fe^{3+}–reductant with Fe^{2+}, the inhibition effect of HO• production by hexadecylitaconic acid and octylitaconic acid in the Fe^{3+}–reductant system was greater than that with Fe^{2+}. Furthermore, the initial rate of HO• production in the Fe^{2+} system containing hexadecylitaconic acid or octylitaconic acid was greater than in the Fe^{3+}–reductant system.

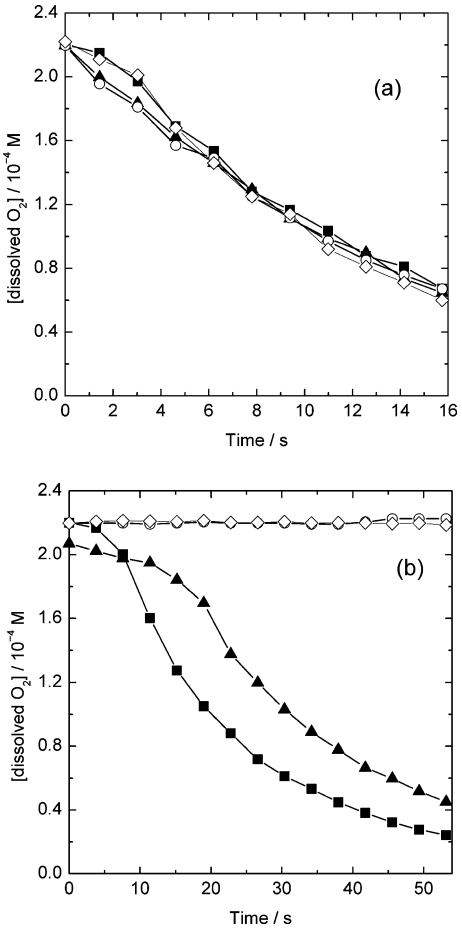

Fig. 7 Time course of dissolved oxygen consumption in Fe^{2+}–H_2O_2 (a) and Fe^{3+}–L-cysteine–H_2O_2 (b) systems containing 20% (v/v) EtOH in the presence of hexadecylitaconic acid (○), itaconic acid (▲) and octylitaconic acid (◇). [itaconate derivatives] = 3 mM, [H_2O_2] = 5 mM. (a): [Fe^{2+}] = 1 mM. (b): [Fe^{3+}] = 1 mM, [L-cysteine] = 0.5 mM. In the control experiment (■), itaconaic acid derivatives were not added.

Theoretical calculation of HOMO energy of ceriporic acid B and its analogues

The HOMO energy of itaconic acid derivatives with different chain length is shown in Fig. 9. Alkylitaconic acids with a long alkyl side chain have higher HOMO energies. Differences in HOMO energy were marked between itaconic acid and the two alkyl itaconic acids.

Discussion

The biodegradation of lignin by wood rot fungi is an extracellular free radical event that proceeds in concert with the activation of molecular oxygen and redox cycling of transition metals. Hydroxyl radicals (HO•), a radical species highly destructive to cellulose and lignin,[8,9] are proposed as principal low molecular mass oxidants that erode wood cell walls to enhance the accessibility of extracellular enzymes to wood cell wall components. Wood rot fungi have versatile enzymatic and non-enzymatic systems to accelerate the reductive half cycle from Fe^{3+} to Fe^{2+}.[9,20,21,27,32–36] The reduction of Fe^{3+} promotes the overall production rate of hydroxyl radicals in the Fenton system. Therefore, the development of inhibitors for Fe^{3+} reduction is a viable target for antifungal agents in wood preservation.[37]

In contrast to the wood decay accompanied by the erosion of wood cell walls and cellulose depolymerization, a selective white rot fungus, *C. subvermispora*, decomposes lignin in wood

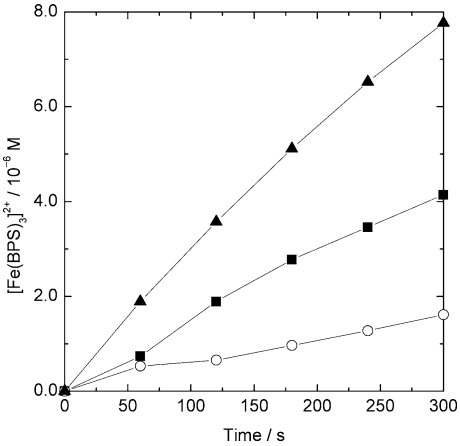

Fig. 8 Effects of hexadecylitaconic acid and NTA on $[Fe(BPS)_3]^{2+}$ production by Fe^{3+}–L-cysteine–BPS in succinate buffer (pH = 4.0) containing 10% (v/v) EtOH at room temperature. [Fe^{3+}] = 0.02 mM, [hexadecylitaconic acid] = 0.04 mM, [L-cysteine] = 10 mM, [NTA] = 0.04 mM, [BPS] = 1.0 mM. The complete system (hexadecylitaconic acid +, NTA+; ▲) contained all reagents. The reaction system of (hexadecylitaconic acid +, NTA −, ○) did not contain NTA. In the control experiment (hexadecylitaconic acid −, NTA −, ■), hexadecylitaconic acid and NTA were omitted.

Table 3 Initial rate of $[Fe(BPS)_3]^{2+}$ production in reactions of Fe^{3+}–reductant–itaconic acid derivatives

Reductant	Itaconate derivative	Chelator	Initial rate[a]
L-Cysteine	—	—	1.00
	Hexadecylitaconic acid	—	0.41
	Hexadecylitaconic acid	NTA	2.06
	Octylitaconic acid	—	0.69
	Octylitaconic acid	NTA	1.72
	Itaconic acid	—	0.53
	Itaconic acid	NTA	1.16
L(+)-Ascorbic acid	—	—	1.00
	Hexadecylitaconic acid	—	0.33
	Hexadecylitaconic acid	NTA	>10
	Octylitaconic acid	—	0.56
	Octylitaconic acid	NTA	2.00
	Itaconic acid	—	1.11
	Itaconic acid	NTA	1.06
Hydroquinone	—	—	1.00
	Hexadecylitaconic acid	—	0.34
	Hexadecylitaconic acid	NTA	1.47
	Octylitaconic acid	—	0.62
	Octylitaconic acid	NTA	2.20
	Itaconic acid	—	0.44
	Itaconic acid	NTA	1.66

Experiments were carried out in succinate buffer (pH = 4.0) containing 10% (v/v) EtOH at room temperature. [Fe^{3+}] = 0.02 mM, [BPS] = 1.0 mM, [itaconate derivative] = 0.04 mM, [reductant] = 10 mM, [NTA] = 0.1 mM. Experimental procedure is described in Materials and Methods.[a] Values are expressed as a ratio of two reactions systems with and without itaconic acid derivatives. The rate of $[Fe(BPS)_3]^{2+}$ production in the reduction of Fe^{3+} with L-cysteine, L(+)-ascorbic acid and hydroquinone was 1.2×10^{-8} M s^{-1}, 7.2×10^{-8} M s^{-1} and 4.6×10^{-9} M s^{-1}, respectively.

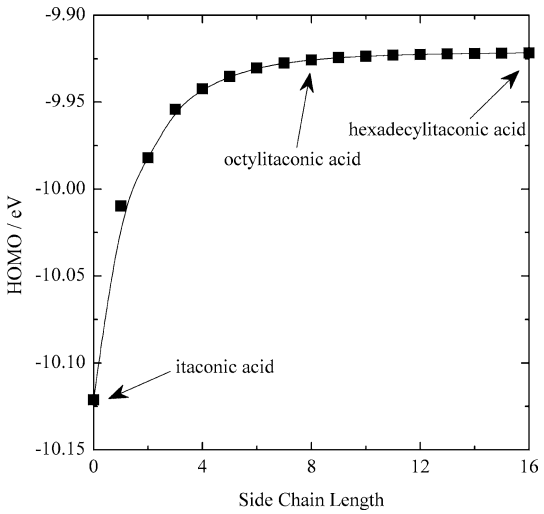

Fig. 9 The highest occupied molecular orbital (HOMO) energies of itaconate derivatives with various lengths of side chain.

without erosion of the wood cell walls at a site far from enzymes. Wood decay proceeds without extensive damage to cellulose in an environment where free radicals, lignin-derived phenols, are produced in the presence of O_2 and Fe ions. Therefore we hypothesized that this fungus produces inhibitors for Fenton-based HO$^{\bullet}$ production, and isolated three novel alkylitaconic acids: 1-heptadecene-2,3-dicarboxylic acid (ceriporic acid A), 1-nonadecene-2,3-dicarboxylic acid (ceriporic acid B) and (*Z*)-1,10-nonadecadiene-2,3-dicarboxylic acid (ceriporic acid C) from extract-free wood cultures of *C. subvermispora*.[20,27,35,36] *In vitro* experiments showed that ceriporic acid B (hexadecylitaconic acid) inhibited the depolymerization of cellulose by the Fenton reaction at the physiological pH of wood decay.[21] In this study, we comparatively analyzed the physicochemical and redox properties of itaconic acid derivatives with long, medium and no alkyl side chains to discuss the structure–function relationship of this series of metabolites.

Production of HO$^{\bullet}$ was strongly suppressed by octylitaconic acid and hexadecylitaconic acid in the Fenton reaction starting from Fe^{3+} in the presence of a reductant, cysteine, ascorbic acid or hydroquinone. Octylitaconic acid has strong surfactant activity comparable to that of Tween 20 and Tween 80 (Fig. 2). Hexadecylitaconic acid decreased the initial rate of HO$^{\bullet}$ production more intensively than octylitaconic acid did, but differences between the two alkylitaconic acids in the total amount of HO$^{\bullet}$ produced during 15 min were negligible. When the carboxyl groups of hexadecylitaconic acid and octylitaconic acid were esterified, inhibitory effects on HO$^{\bullet}$ production disappeared, indicating that free carboxyl groups and the alkyl side chain are essential components for the suppression of HO$^{\bullet}$ production.

The reactions of Fe^{2+} with H_2O_2 in the presence of (alkyl)itaconic acids exhibited an HO$^{\bullet}$ production profile distinctively different from the reactions initiated by mixing Fe^{3+}, H_2O_2 and a reductant. In reactions starting from Fe^{2+}, the production of HO$^{\bullet}$ increased rapidly in one minute, regardless of the presence of alkylitaconic acids; however, the rate of HO$^{\bullet}$ production decreased to zero within one minute after the reaction. This is explained by the mechanism by which alkylitaconic acids inhibited a reductive half cycle from Fe^{3+} to Fe^{2+} by coordinating Fe^{3+}. As alkylitaconic acids have oxygen atoms as the sole coordination site, coordination to Fe^{2+} is much weaker than that to Fe^{3+} as explained by the HSAB theory.[38] For example, stability constants (log*K*) of iron-dicarboxylic acid complexes are as follows: Fe(II)-malonic acid, 2.8 (k_1); Fe(III)-malonic acid, 15.7($k_1k_2k_3$); Fe(II)-oxalic acid, 5.2 ($k_1k_2k_3$); Fe(III)-oxalic acid, 18 ($k_1k_2k_3$).[39] Therefore stability constants of Fe(II)-alkylitaconic acid must be smaller than those of Fe(III)-alkylitaconic acid.

Suppression of the reductive half cycle from Fe^{3+} to Fe^{2+} by alkylitaconic acids was clearly demonstrated by oxygen uptake experiments. In general, iron chelators affected iron-mediated Haber–Weiss reactions involving the oxidation of Fe^{2+} by O_2 ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$).[40] In the experiments of Fe^{2+} with H_2O_2 in the presence of three itaconic acid derivatives, no differences were found in the initial reaction rate of O_2 consumption. In contrast, O_2 uptake was strongly inhibited by hexadecylitaconic acid and octylitaconic acid when reactions were started by mixing Fe^{3+}, H_2O_2 and a reductant, cysteine. No inhibitory effects were found for itaconic acid in the reaction starting from Fe^{3+} as well as in the reaction from Fe^{2+}.

The effects of (alkyl)itaconic acids on Fe^{3+} reduction were analyzed using a ligand for Fe^{2+}, BPS. At a ligand to Fe^{3+} molar ratio of 30 : 1, Fe^{3+} reduction was completely inhibited by hexadecylitaconic acid (data not shown). No ligand exchange reactions with NTA and EGTA occurred under this condition due to steric crowding. When the molar ratio of the ligand to Fe^{3+} was 2 : 1, hexadecylitaconic acid and octylitaconic acid suppressed Fe^{3+} reduction, but the addition of a strong iron chelator, NTA, abolished these suppressive effects due to a ligand exchange reaction and the high oxidation potential of the resultant Fe^{3+}-NTA complex.[41] These observations illustrate that alkylitaconic acids suppressed the reductive half cycle in the Fenton system by coordinating Fe^{3+} as discussed in the ESR and oxygen uptake experiments.

Itaconic acid is oxidized by HO$^{\bullet}$ to form the β-hydroxy itaconate radical, which in turn polymerizes (Fig. 10).[42] Under aerobic conditions, part of the radical intermediate reacts with molecular oxygen to form the β-hydroxy peroxyl radical. The differential profiles between itaconic and alkylitaconic acids in oxygen consumption are ascribed to the susceptibility to oxidation by HO$^{\bullet}$ as expected from the differences in steric effects of the side chain rather than HOMO energy. GCMS analyses after the reactions showed that itaconic acid was decomposed due to the radical reaction but hexadecylitaconic acid and octylitaconic acid remained intact after the Fenton reaction in the presence and absence of NTA (data not shown).

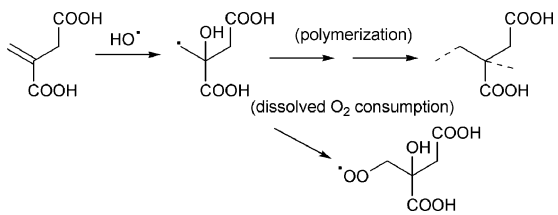

Fig. 10 Proposed pathway for the oxidation of itaconic acid by HO$^{\bullet}$.

In conclusion, the strong redox silencing ability of alkylitaconic acids—hexadecylitaconic acid and its analogue octylitaconic acid—driven by the suppression of Fe^{3+} reduction has been

established. Iron is an essential element for living organisms but iron can damage tissues by catalyzing the production of reactive oxygen species (ROS). A number of iron chelators like desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) have been studied to suppress iron-mediated oxidative injury. In view of anitioxidative therapy, it is of considerable interest to control ROS *in vivo* using a series of lipophilic and amphipathic alkylitaconic acids with different chain lengths.

Conclusion

The reactivity of iron varies greatly depending on its ligand environment. A selective white rot fungus, *C. subvermispora* secretes alkylitaconic acids to suppress the production of hydroxyl radicals by the Fenton reaction even in the presence of reductants for Fe^{3+}. The alkyl side chain and coordination by the two carboxylic groups in the metabolite are essential for redox silencing and high stability against oxidative degradation by $HO^{\bullet}$. These findings indicate that the alkyl side chain in metabolites functions as a switching "device" to control antioxidant activities in addition to the hydrophobicity and amphipathic properties of the metabolites.

Acknowledgements

The authors gratefully acknowledge support from the Advanced Research Program of the Research Institute of Innovative Technology for the Earth (RITE) and a Grant-in-Aid for JSPS Fellows (to Y. O.).

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