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ORIGINAL ARTICLE

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# Screening for condensed tannin-degrading fungi with a synthetic $^{14}C$-labeled compound

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**Abstract** To find fungi that are potent for degradation of condensed tannin, a two-step screening was used. This involved measurement of fungal growth rate on Japanese cedar (*Cryptomeria japonica*) bark, followed by determination of [$^{14}C$]-labeled $CO_2$ generated from fungal degradation of synthetic [$^{14}C$]-labeled condensed tannin model. In the first screening, 75 strains of wood rot fungi were tested, and 19 strains effectively decreased bark weight and/or the weight of the methanol-soluble fraction. For the second screening, [$^{14}C$]-labeled condensed tannin model compound was synthesized in 11.8% yield based on radioactivity measurements. Over the incubation period, *Coriolus hirsutus* K-2671, *Lentinus edodes* Is, and *Lampteromyces japonicus* Nn showed higher cumulative [$^{14}C$]-labeled $CO_2$ emissions than the other strains and mineralized the [$^{14}C$]-labeled condensed tannin model compound by 3.7%, 3.0%, and 3.0%, respectively. Fractionation of the methanol extracts from the medium by gel permeation chromatography after fungal treatment suggested that fungi that can induce the emission of significant levels of [$^{14}C$]-labeled $CO_2$ can extensively depolymerize condensed tannins.

**Key words** Condensed tannins · Biodegradation · Fungi

## Introduction

Condensed tannins, also known as proanthocyanidins, are polyphenols that are widely distributed in the plant kingdom. Condensed tannins are present in the xylem, leaf, root, bark, petal, seed, and fruit.[1] They are particularly abundant in bark and heartwood,[2,3] where they play an important role in protection from insect and animal feeding,[4,5] pathogen infection,[6] rotting induced by fungi,[7] and ultraviolet (UV) radiation.[8,9] Protection by condensed tannins against these attacks is due to a number of mechanisms including the property of astringency[10] and to binding to proteins including enzymes[11] and cytotoxic[12] and antioxidant proteins.[13] Condensed tannins consist of flavonoids such as (+)-catechin and (−)-epicatechin, which are highly polymerized through an interaction between the C4 and C8 of each unit. Moreover, this polymer interacts with cellulose, hemicelluloses, lignins, and proteins in plants,[14] which enhances plant tissue rigidity. Therefore, condensed tannins are extremely resistant to microbial degradation.

Because of the complexity and heterogeneity of tannin structure, there have been few studies on the biodegradation of condensed tannins, even though they are very important products in the natural world. However, it has been shown that some bacteria and fungi are able to degrade these polyphenolic compounds. The first study of the degradation of condensed tannins was published in 1973 and reported that some species of *Aspergillus* and *Penicillium* could degrade wood apple tannin.[15] In 1976 a further study showed that *Penicillium adametzi* could utilize low molecular weight (dimer or trimer) condensed tannins as a carbon source.[16] In a more recent study, using solid-state cross-polarization and magic angle spinning $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy, it was observed that a white rot fungi *Ceriporiopsis subvermispora* could degrade insoluble polymeric condensed tannins.[17] However, it is difficult to precisely evaluate the fungal degradation of condensed tannins described in previous reports because these experiments used plant materials or plant extracts, rather than tannin itself, as the sample for degradation, and they also employed indirect methods for measurement of the extent of degradation.

In this study, we aimed to elucidate mechanisms of tannin biodegradation by microorganisms, which more directly corresponds to degradation occurring in the natural state. As a first step toward this aim, we synthesized a $^{14}C$-labeled condensed tannin model compound to allow a direct

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comparison of the tannin degradation activity by a number of different fungi. Using this potent tool, we undertook a screening of fungal strains to identify strains that would be useful for investigation of the tannin degradation pathway.

## Materials and methods

### Fungi

The 76 strains of fungi, belonging to the class Basidiomycetes, that were analyzed in this study were as follows: *Lentinus edodes* (6 strains), *Grifola frondosa* (19 strains), *Meripilus giganteus* As, *Dendropolyporus umbellatus* Sz, *Hypsizygus marmoreus* (3 strains), *Armillariella mellea* (2 strains), *Flammulina velutipes* (4 strains), *Clitocybe lignatilis*, *Lentinus lepideus* (12 strains), *Hericium erinaceum* Sz, *Pholiota nameko* (2 strains), *Pholiota lubrica*, *Lyophyllum decastes* Iw, *Naematoloma sublateritium* Ic, *Laetiporus sulphureus* (3 strains), *Lampteromyces japonicus* (2 strains), and an unidentified strain of *Polyporaceae* sp. were provided by Iwate Prefectural Forestry Technology Center; *Coriolus versicolor* K-2615, *Coriolus hirsutus* K-2617 and *Pleurotus ostreatus* K-2946 were provided by the Research Institute for Sustainable Humanosphere, Kyoto University; *Merulius tremellosus* IFO 30385, *Grifola frondosa* IFO 30661, *Stereum hirsutum* IFO 6520, *Hymenochaete tabacina* IFO 4969, *Cyclomyces fuscus* IFO 9789, *Inonotus cuticularis* IFO 9788, *Inonotus dryadeus* IFO 9352, *Inonotus mikadoi* IFO 6517, *Onnia orientalis* IFO 30386, *Phellinus linteus* IFO 6989, *Phellinus tuberculosus* IFO 7125, and *Poria aurantiofibrillosus* IFO 9048 were obtained from the Natural Institute of Technology and Evaluation (Tokyo, Japan). All strains were maintained on potato dextrose agar (PDA, Difco) medium and stored at 4°C.

### Synthesis of $^{14}$C-labeled condensed tannin model compound

The $^{14}$C-labeled condensed tannin model compound was synthesized by polymerization of flavan-3,4-carbonate as previously described,[18] followed by the addition of [U-$^{14}$C]-phloroglucinol as a terminal unit. The steps involved in the synthesis are outlined in Fig. 1.

### Synthesis of [U-$^{14}$C]-1,3,5-tribromobenzene (**2**)

[U-$^{14}$C]-1,3,5-Tribromobenzene (**2**) was synthesized according to the procedure of Coleman and Talbot[19] Briefly, [U-$^{14}$C]-aniline (51.3 MBq, Fig. 1) together with nonlabeled aniline (1.05 g, 12.3 mmol) that was used as a carrier, were dissolved in 1 M HCl (11 ml). To the solution, bromine vaporized with heating at 40°–50°C was introduced through connected tubing. The reacting solution was then maintained at 0°C for 4 h. The resulting solid was filtered, washed with water to remove hydrobromic acid, and dried in vacuo. The obtained solid was dissolved in a solution of benzene/ethanol (1:5, v:v, 26 ml) that included concentrated $H_2SO_4$ (1.40 ml) and $NaNO_2$ (1.40 g), and the solution was then refluxed for 2 h. This reaction was then quenched at 0°C. The mixture was extracted with ethyl acetate, and washed successively with water and brine. The organic layer was dried over anhydrous $Na_2SO_4$ and the solvent was evaporated in vacuo. The solid substance obtained was recrystallized from a mixture of ethanol and a small amount of dichloromethane to produce compound **2** in the form of opal white needles (2.73 g, 76.7% yield).

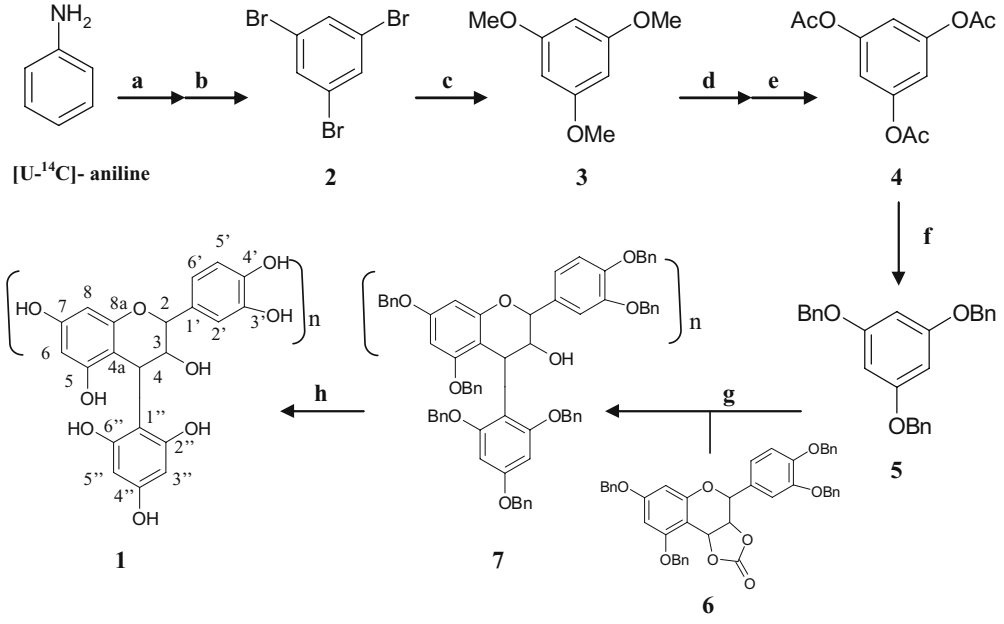


**Fig. 1.** Synthetic procedures for $^{14}$C-labeled condensed tannin model (**1**). Conditions: *a*, $Br_2$, 1 M HCl, 0°C, 4 h; *b*, $NaNO_2$, $H_2NO_4$, ethanol:benzene = 5:1, v:v, reflux, 2 h; *c*, sodium methoxide, CuI, *N*,*N*-dimethylformamide (DMF), reflux, 2 h; *d*, concentrated HCl, reflux, 2.5 h; *e*, acetic anhydride, pyridine, room temperature, overnight; *f*, benzyl chloride, NaH, $H_2O$, DMF, 0°C–room temperature, 12.5 h; *g*, $BF_3{\cdot}Et_2O$, $CH_2Cl_2$, –20°C, 24 h; *h*, 10% Pd-C, tetrahydrofuran:ethanol = 4:1, v:v, $H_2$, 37°C, 24 h

### Synthesis of [ring-$^{14}$C]-phloroglucinol-tri-*O*-methyl ether (**3**)

[Ring-$^{14}$C]-phloroglucinol-tri-*O*-methyl ether (**3**) was synthesized according to the procedure of McKillop et al.[20] In brief, 28% sodium methoxide in methanol (11 ml), *N*,*N*-dimethylformamide (DMF, 11 ml), copper iodide (0.27 g, 1.43 mmol), and compound **2** (2.73 g, 7.97 mmol) were added sequentially to a pear-shaped flask. The reaction was then activated by heating to 107°C for 2 h. After rapid cooling in water, the reaction mixture was diluted with diethyl ether, and then washed successively with water and brine. The organic layer was dried over anhydrous $Na_2SO_4$ and the solvent was evaporated under a $N_2$ stream. The resulting syrup was purified on a silica gel column with ethyl acetate/*n*-hexane (1:10, v:v), and the eluate was concentrated under a $N_2$ stream. The concentrated residue was recrystallized from methanol (1.01 g, 69.3% yield) and was used to produce compound **4**.

### Synthesis of [ring-$^{14}$C]-phloroglucinol-*O*-triacetate (**4**)

[Ring-$^{14}$C]-phloroglucinol-*O*-triacetate (**4**) was synthesized according to the procedure of McKillop et al.[20] In brief, compound **3** (1.01 g, 5.99 mmol) was dissolved in 37% HCl solution (100 ml) and was refluxed for 2.5 h. The reaction was quenched by the addition of sodium hydrogen carbonate and water, and the mixture was thereafter washed with *n*-hexane and extracted with ethyl acetate. The extract was dried over anhydrous $Na_2SO_4$ and the solvent was concentrated in vacuo. The concentrated solution was acetylated by the addition of 1 ml of pyridine/acetic anhydride (1:1, v:v) followed by incubation overnight at 30°C. The precipitate, which formed when the reaction mixture was dropped into cold water, was filtered through filter paper. The residue was collected, recrystallized from ethanol (0.67 g, 44.4% yield), and was used to produce compound **5**.

### Synthesis of [ring-$^{14}$C]-phloroglucinol-*O*-benzyl ether (**5**)

[Ring-$^{14}$C]-phloroglucinol-*O*-benzyl ether (**5**) was synthesized according to the procedure of Kawamoto et al.[21] Briefly, compound **4** (0.67 g, 2.67 mmol), benzyl chloride (1.09 ml), and 60% NaH in mineral oil (0.76 g, 18.91 mmol) were suspended in DMF. After $H_2O$ was added into the suspension at 0°C, the reacted solution was allowed to warm to room temperature and stirred for 12.5 h. The reaction mixture was then diluted with ethyl acetate and washed with water and brine. The organic layer was dried over anhydrous $Na_2SO_4$ and the solvent was evaporated off in vacuo. The evaporated residue was recrystallized from a mixture of methanol and dichloromethane (0.96 g, 90.7% yield), and was used to produce compound **7**.

### Polymerization of flavan-3,4-carbonate with compound **5**

The compound 3-*trans*-3,4-*cis*-5,7,3′,4′-tetrabenzyloxyflavan-3,4-carbonate (**6**) was synthesized according to the procedure of Yoneda et al.,[18] and used as a precursor of the model polymer. Compounds **6** (1.14 g, 1.65 mmol) and **5** (0.093 g, 0.24 mmol, 1.11 MBq) were dissolved in 9.1 ml anhydrous $CH_2Cl_2$. Then $BF_3{\cdot}Et_2O$ (41 µl, 0.32 mmol) was added at –20°C and the solution was stirred for 24 h. The reaction mixture was diluted with ethyl acetate, and was washed successively with water, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over $Na_2SO_4$ and the solvent was evaporated off in vacuo to obtain the polymerized product **7** (1.62 g).

### Debenzylation and purification

These procedures were carried out as described by Yoneda et al.[18] Briefly, the polymerized product **7** (1.16 g) was hydrogenated on 10% Pd–C (8.45 g) in tetrahydrofuran/ethanol (4:1, v:v) at 37°C for 24 h. The reaction mixture was filtered through a Millipore filter (0.45 µm), and after the filtrate was concentrated, the solvent was replaced with methanol (7 ml). In order to remove the low molecular weight fraction, the solution was dropped into benzene (35 ml), and the resultant precipitate was filtered through a Millipore filter (0.45 µm), washed with benzene, and eluted with methanol. After concentration of the solvent to a minimum volume, the eluate was freeze-dried to yield the $^{14}$C-labeled condensed tannin model compound **1** (Fig. 1, 0.26 g, 588 kBq). The structure of compound **1** was confirmed by comparing the$^{13}$C NMR spectrum of **1** with that of the unlabeled condensed tannin model synthesized with the same procedures used for compound **1**. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 38.6 (C-4); 73.9 (C-3); 83.9 (C-2); 96.2 and 97.5 (C-6 and C-8); 106.8, 107.8, 115.9, and 117.7 (C4a, C-1″, C-3″, and C-5″); 120.8 and 121.0 (C-2′, C-5′, and C-6′); 132.3 (C-1′); 145.9 and 150.1 (C-3′ and -4′); 155.2, 156.5, 156.7, 157.0, and 157.6, (C-5, C-7, C-2″, C-4″, and C-6″); 158.4 (C-8a).

### Cultivation of fungi on bark meal medium

Japanese cedar (*Cryptomeria japonica*) bark, milled to 40–100 mesh, was defatted by *n*-hexane extraction. One gram of bark meal was introduced into a test tube (18 × 180 mm), and autoclaved at 121°C for 15 min. Each strain of fungi growing on the PDA medium was inoculated to the bark meal medium. Quadruplicate experimental cultures were prepared for each strain. One milliliter of sterilized water was added to two of these cultures, and 1 ml of 2.4% potato dextrose solution was added to the other two. Four controls were prepared in a similar manner without inoculation. These cultures were grown at 25°C for 28 days in stationary culture. The incubated cultures were freeze-dried and weighed prior to evaluation of their ability to grow on the bark. Thereafter, the freeze-dried samples were extracted by soaking them in methanol for 20 h at room temperature. Each extract was concentrated with a centrifugal evaporator (CVE 3100, Eyela) and weighed prior to estimation of fungal ability to decompose polyphenolic compounds.

### Biodegradation of the [$^{14}$C]-labeled condensed tannin model compound

The 19 strains of fungi tested that showed potential growth on bark meal and/or decomposition of polyphenols were used in the following degradation experiment with the synthesized substrate. The [$^{14}$C]-labeled condensed tannin model compound **1** (147.2 mg) was dissolved in DMF (0.9 ml), and the solution was diluted with 45 ml of sterilized water. One gram of bark meal was introduced into a test tube (18 × 180 mm), and 1 ml of the aqueous suspension of the [$^{14}$C]-labeled condensed tannin model compound (2.78 mg, 2.71 kBq) was added to the bark meal medium. Then each strain of fungi was inoculated to the bark meal medium as described above. Experimental cultures were prepared in duplicate and controls were prepared in quadruplicate. Degradation-test vessels with the design shown in Fig. 2 were constructed using a modification of Kirk's method.[22] Cultures prepared in this way were incubated at 25°C for 26 days. Every second day, or every third day, the gaseous phase in the culture vessel was replaced with fresh air using an electric pump at a flow rate of 50 ml/min for 30 min. When the aeration was carried out, the exhaust tube was connected to a $CO_2$ trap containing 3-methoxypropylamine (10 ml each in two test tubes), and the [$^{14}$C]-labeled $CO_2$ in the effluent gas was collected. The [$^{14}$C]-labeled $CO_2$ was allowed to accumulate until the trap was replaced by a new one after 14 days of cultivation. The radioactivity of the collected solutions was measured with a liquid scintillation counter (Aloka LSC-5100) using Aquasol-2 (PerkinElmer) as a scintillation cocktail.

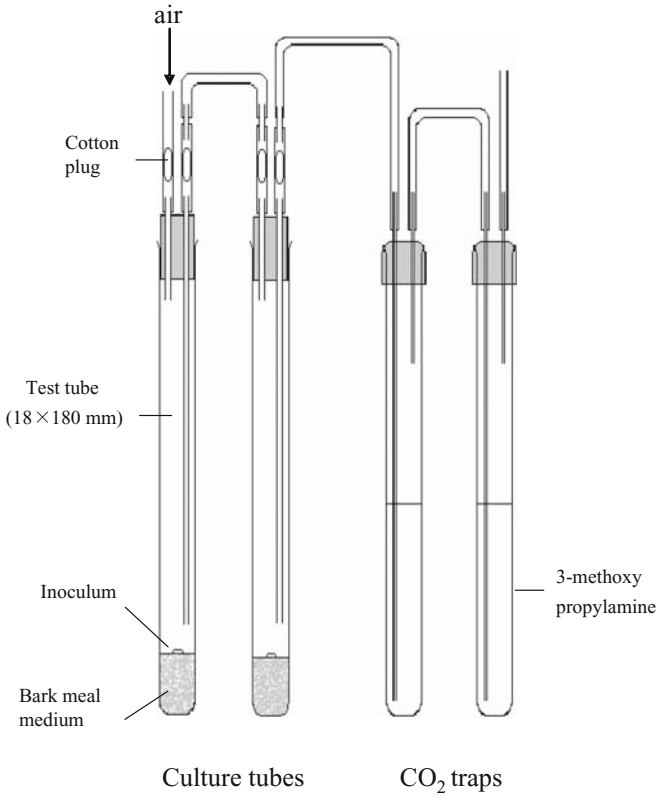


**Fig. 2.** Incubation apparatus for trapping of [$^{14}$C]-labeled $CO_2$

### Gel permeation chromatography of degradation products

Degradation products of the [$^{14}$C]-labeled condensed tannin model compound were extracted from the incubated bark meal with 5 ml of methanol. The extracts were filtered through a cotton plug, and the bark residue was washed with 5 ml of methanol. The solvent was removed by rotary evaporation, and the weight of the concentrated residue was determined. After acetylation with a mixture of acetic anhydride and pyridine (1:1, v:v), the molecular weight distribution of the radioactive degradation products dissolved in tetrahydrofuran was monitored by gel permeation chromatography (GPC). GPC was performed with a Jasco PU-986 pump and a Jasco UV-975 UV-VIS detector. The column used was a TSK Multipore $H_{XL}$-M column (300 mm × 7.8 mm i.d., 5 µm, exclusion limit $2 \times 10^6$, Tosoh), and the chromatography was carried out at room temperature. Tetrahydrofuran was used as the mobile phase and the flow rate was 1.0 ml/min. The analytes were monitored at 280 nm. Two polystyrenes [molecular weight (MW) 4000 and 2000], tri-*O*-benzyl phloroglucinol-4$\alpha$-tetra-*O*-benzyl catechin (MW 1044), and phloroglucinol-*O*-benzyl ether (MW 396) were used as calibration standards. Separation of the degradation products was conducted five times, and the separated products were collected using a fraction collector. The radioactivity of each fraction was measured as described above.

## Results and discussion

### Synthesis and characterization of [$^{14}$C]-labeled condensed tannin model compound

The [$^{14}$C]-labeled condensed tannin model compound was synthesized as outlined in Fig. 1. The intermediate compound [ring-$^{14}$C]-phloroglucinol-*O*-tribenzyl ether (**5**) was synthesized from [U-$^{14}$C]-aniline with a yield of 21.4%. The product weighed 0.96 g and had a specific radioactivity of 11.98 kBq/mg. The radiochemical yield of 22.4% almost corresponded to the synthetic yield. Polymerization of benzylated flavan-3,4-carbonate (**6**) with [ring-$^{14}$C]-phloroglucinol-*O*-tribenzyl ether (**5**), followed by deprotection, gave the [$^{14}$C]-labeled condensed tannin model compound (**1**) with a specific radioactivity of 2.30 kBq/mg. The synthetic yield and radiochemical yield of the [$^{14}$C]-labeled condensed tannin were 53.8% and 52.6%, respectively. Any other radioactivity that might have been included in the small molecular weight oligomers was removed by fractional precipitation. The chemical structure and molecular weight distribution of the condensed tannin compound was determined by $^{13}$C NMR spectroscopy and GPC, respectively, using a nonlabeled condensed tannin model compound prepared by the same procedures used for compound **1**. The chemical shifts of the condensed tannin model com-

pound corresponded to those reported in previous studies,[18] and indicated the addition of a phloroglucinol moiety at the terminal site. The molecular weight distribution of the acetylated condensed tannin model compound was determined by GPC using calibration standards in the range of 396–4000. The molecular weight of the condensed tannin model compound was estimated to range between 786 and 11 280, which corresponded to a degree of polymerization number (DPn) of 1.5–22.5. The average molecular weight was 4308, which corresponded to a DPn of 8.6. In this study, it was assumed that any natural polymeric condensed tannin would be likely to be degraded after extracellular depolymerization by fungi. Therefore, it was considered desirable to use a high molecular weight substrate for this study. The [$^{14}$C]-labeled condensed tannin prepared by the above synthetic process satisfied this requirement, and provided us a potent tool for direct evaluation of the capability of microorganisms to degrade condensed tannin.

## Fungal growth on bark meal medium

Bark contains many substances, including condensed tannins, that affect microbial growth. The first screening was designed to identify fungi that can grow well on Japanese ceder bark meal and actively degrade the bark components. The activities of a range of fungi in the degradation of bark components were estimated as the loss of culture dry weight, and as the loss of methanol extract, resulting from fungal metabolism (Table 1).

**Table 1.** Losses of culture dry weight and methanol extract resulted from fungal metabolism

| Strain | Loss of culture dry weight (%) | | Loss of MeOH extract (%) | |
|---|---|---|---|---|
| | Bark meal | Bark meal + PD | Bark meal | Bark meal + PD |
| *L. edodes* Sz | 5.6 | 5.9 | 48.1 | 38.3 |
| *L. edodes* Mt | 4.0 | 5.9 | 40.5 | 53.3 |
| *L. edodes* Hy | 4.2 | 5.1 | 44.9 | 49.5 |
| *L. edodes* It | 4.0 | 4.7 | 37.3 | 49.5 |
| *L. edodes* Is | 4.9 | 6.1 | 56.8 | 55.1 |
| *L. edodes* Iw | 5.3 | 6.2 | 48.1 | 47.7 |
| *G. frondosa* Iw | 1.0 | 2.3 | 38.4 | 47.7 |
| *G. frondosa* Hm | 0.8 | 1.6 | 30.8 | 27.1 |
| *G. frondosa* Km | 1.1 | 2.1 | 34.1 | 42.1 |
| *G. frondosa* Am | 0.7 | 2.2 | 40.5 | 42.1 |
| *G. frondosa* Tu | 1.0 | 1.9 | 43.8 | 43.9 |
| *G. frondosa* Ap | 1.0 | 2.4 | 35.1 | 53.3 |
| *G. frondosa* Yd | 0.7 | 1.6 | 30.8 | 27.1 |
| *G. frondosa* Km | 0.8 | 2.5 | 52.4 | 53.3 |
| *G. frondosa* Ik-1 | 1.3 | 2.5 | 44.9 | 49.5 |
| *G. frondosa* Ik-2 | 1.6 | 2.7 | 53.5 | 51.4 |
| *G. frondosa* Ik-3 | 1.6 | 2.6 | 49.2 | 58.9 |
| *G. frondosa* Ik-4 | 1.1 | 2.4 | 42.7 | 45.8 |
| *G. frondosa* A | 1.5 | 2.7 | 54.6 | 49.5 |
| *G. frondosa* B | 1.5 | 2.1 | 47.0 | 42.1 |
| *G. frondosa* E | 1.2 | 2.6 | 52.4 | 58.9 |
| *G. frondosa* IV | 1.2 | 2.3 | 48.1 | 47.7 |
| *G. frondosa* Amh | 0.9 | 2.4 | 40.5 | 45.8 |
| *G. frondosa* IV′ | 1.0 | 2.1 | 31.9 | 42.1 |
| *G. frondosa* IV′-2 | 1.5 | 2.5 | 45.9 | 53.3 |
| *M. giganteus* As | –[a] | 0.9 | 3.8 | 6.5 |
| *D. umbellatus* Sz | 0.2 | 0.4 | – | 0.9 |
| *H. marmoreus* Yd | – | 0.1 | – | – |
| *H. marmoreus* Sw | – | – | – | – |
| *H. marmoreus* Sk | – | 0.1 | – | – |
| *A. mellea* Fk | 0.5 | 0.7 | 7.0 | 6.5 |
| *A. mellea* Yh-2 | 0.2 | 0.7 | 4.9 | – |
| *F. velutipes* Kg | – | 0.4 | – | – |
| *F. velutipes* Sg | – | 0.4 | – | – |
| *F. velutipes* Ns | – | 0.4 | – | 0.9 |
| *F. velutipes* Sn | – | 0.4 | – | – |
| *C. lignatilis* Nn | – | 0.2 | – | – |
| *L. lepideus* 84-02 | 2.3 | 4.6 | 26.5 | 36.4 |
| *L. lepideus* 84-03 | 2.3 | 4.7 | 26.5 | 40.2 |
| *L. lepideus* 84-05 | 4.1 | 6.8 | 20.0 | 32.7 |
| *L. lepideus* 84-06 | 4.6 | 7.5 | 27.6 | 36.4 |
| *L. lepideus* 84-07 | 6.9 | 8.8 | 23.2 | 36.4 |
| *L. lepideus* 84-08 | 2.8 | 6.0 | 20.0 | 32.7 |
| *L. lepideus* 84-09 | 1.9 | 3.1 | 33.0 | 42.1 |
| *L. lepideus* 84-10 | 2.1 | 4.1 | 29.7 | 42.1 |
| *L. lepideus* 85-11 | 3.0 | 5.7 | 22.2 | – |

**Table 1.** *Continued*

| Strain | Loss of culture dry weight (%) | | Loss of MeOH extract (%) | |
|---|---|---|---|---|
| | Bark meal | Bark meal + PD | Bark meal | Bark meal + PD |
| *L. lepideus* I-2 | 7.7 | 10.5 | 27.6 | 38.3 |
| *L. lepideus* Sz | 3.2 | 7.5 | 15.7 | 32.7 |
| *L. lepideus* Yh | 3.4 | 6.4 | 17.8 | 29.0 |
| *H. erinaceum* Sz | 1.3 | 3.0 | 30.8 | 40.2 |
| *P. nameko* Yi | 0.4 | 1.6 | 0.5 | – |
| *P. nameko* Gt | 0.4 | 1.3 | 8.1 | 4.7 |
| *L. decastes* Iw | 0.1 | 0.4 | 3.8 | 10.3 |
| *N. sublateritium* Ic | – | 1.0 | 13.5 | 6.5 |
| *P. lubrica* Nn | – | 1.1 | 12.4 | 12.1 |
| *L. sulphureus* Om | 3.4 | 4.7 | 27.6 | 29.0 |
| *L. sulphureus* Mr | 3.5 | 4.0 | 33.0 | 21.5 |
| *L. sulphureus* Ik | 4.2 | 5.9 | 42.7 | 43.9 |
| *L. japonicus* Sg | 4.5 | 5.8 | 56.8 | 64.5 |
| *L. japonicus* Nn | 4.2 | 5.4 | 58.9 | 60.7 |
| *Polyporaceae* sp. | 2.2 | 4.2 | 49.2 | 34.6 |
| *C. versicolor* K-2615 | 5.4 | 8.2 | 61.1 | 66.4 |
| *C. hirsutus* K-2617 | 4.6 | 11.4 | 51.4 | 58.9 |
| *P. ostreatus* K-2946 | – | 0.4 | – | 4.7 |
| *M. tremellosus* IFO 30385 | 1.2 | 2.6 | 27.6 | 43.9 |
| *G. frondosa* IFO 30661 | 0.9 | 1.7 | 40.5 | 42.1 |
| *S. hirsutum* IFO 6520 | 1.6 | 4.4 | 41.1 | 56.6 |
| *H. tabacina* IFO 4969 | 3.7 | 4.7 | 65.4 | 64.5 |
| *C. fuscus* IFO 9789 | 1.7 | 2.9 | 36.2 | 45.8 |
| *I. cuticularis* IFO 9788 | 2.3 | 5.5 | 59.4 | 67.0 |
| *I. dryadeus* IFO 9352 | – | 1.2 | – | 35.6 |
| *I. mikadoi* IFO 6517 | 0.7 | 4.9 | 53.3 | 62.5 |
| *O. orientalis* IFO 30386 | – | 3.4 | 45.2 | 64.0 |
| *P. linteus* IFO 6989 | 1.8 | 5.6 | 49.2 | 70.0 |
| *P. tuberculosus* IFO 7125 | 1.1 | 4.0 | 26.9 | 59.6 |
| *P. aurantiofibrillosus* IFO 9048 | 2.0 | 4.6 | 33.0 | 52.1 |

MeOH, Methanol; PD, potato dextrose
[a] Loss not observed

In cultures where only water was added, many strains of wood rot fungi including *Lentinus edodes* spp., *Lentinus lepideus* spp., *Laetiporus sulphureus* spp., *Lampteromyces japonicus* spp., *Coriolus versicolor*, *Coriolus hirsutus*, and *Hymenochaete tabacina* caused a considerable decrease in bark components compared with other strains. Maximal decomposition was observed with *L. lepideus* I-2, which reduced the bark weight by 7.7%. The followers in terms of efficiency were *L. lepideus* 84–07, *L. edodes* Sz, and *C. versicolor* K-2615, which decreased bark weight by 6.9%, 5.6%, and 5.4%, respectively. In cultures where PD was added, *C. hirsutus* K-2617 showed the highest rate of decrease in bark meal at 11.4%. The second highest was *L. lepideus* I-2, which reduced the bark meal dry weight by 10.5%. This strain was also one of the fungi that showed the highest degradation activity when only water was added to the cultures.

Phenols are a major component of the methanol extract from coniferous bark. Presumably the rate of decrease observed in the methanol extract corresponds to the ability of the fungi to degrade the condensed tannin. Loss of methanol extract from the incubated bark when only water was added to the culture was observed in various strains of many fungal species. In particular, *H. tabacina* IFO 4969, *C. versicolor* K-2615, *Inonotus cuticularis* IFO 9788, and *L. japonicus* Nn strongly decreased the dry weight of the methanol extract by 65.4%, 61.1%, 59.4%, and 58.9% from that of the control, respectively. *Lentinus lepideus* spp., which showed higher rates of decrease in bark weight, degraded less of the components in degradation of the methanol extracts. The addition of PD increased the methanol-extract-degrading activity of *Phellinus linteus* IFO 6989, *Inonotus mikadoi* IFO 6517, *Onnia orientalis* IFO 30386, and *Phellinus tuberculosus* IFO 7125.

The purpose of adding PD to the cultures was to enhance the ability to degrade the bark component and establish the culture condition for the next screening with the [$^{14}$C]-labeled condensed tannin model. However, PD addition did not significantly affect the ability to degrade the bark component and dramatically change the species of fungi that showed considerable degradation of the bark, as compared with adding water to the culture. Therefore, the addition of water alone to the bark meal medium was adopted in the next screening.

The criteria chosen for the selection of fungi for further examination were as follows: (1) the rate of decrease in bark weight should be greater than or equal to 5% on bark meal with addition of water, (2) the rate of decrease in bark weight should be greater than or equal to 7% on bark meal with addition of PD, (3) the rate of decrease in methanol extract should be greater than or equal to 55% on bark meal with addition of water, (4) the rate of decrease in methanol

extract should be greater than or equal to 59% on bark meal with addition of PD. Nineteen strains of fungi that satisfied at least one of these conditions were selected.

## Emission of [$^{14}$C]-labeled $CO_2$ from labeled condensed tannin model compound by fungal degradation

Figure 3 shows the accumulated [$^{14}$C]-labeled $CO_2$ generated by fungal degradation of the $^{14}$C-labeled condensed tannin model compound following incubation from day 0 to day 14, or from day 15 to day 26. The measured amount of [$^{14}$C]-labeled $CO_2$ was small because the bark media contained abundant condensed tannins and the fungi cannot distinguish between natural and synthetic tannins. However, the values were high enough to allow measurement of the degrading capability of the condensed tannin by the fungi. Release of [$^{14}$C]-labeled $CO_2$ from the metabolized $^{14}$C-labeled condensed tannin model compound was detected in 12 of the 19 species of fungi tested. Fungi that did not release [$^{14}$C]-labeled $CO_2$ were *L. lepideus* spp. and *P. tuberculosus* IFO7125 (Fig. 3). The strain *C. hirsutus* K-2617 showed the maximum [$^{14}$C]-labeled $CO_2$ emission at 3.7% over the incubation period, followed by *L. edodes* Is, *L. japonicus* Nn, *L. japonicus* Sg, and *L. edodes* Iw at 3.0%, 3.0%, 2.5%, and 2.5%, respectively. The mineralization rate of the [$^{14}$C]-labeled condensed tannin model correlated with the decrease in weight of the methanol extract by fungal degradation. All fungi that showed higher mineralization of the [$^{14}$C]-labeled condensed tannin model compound belong to the white rot fungi, which are potent ligninolytic microorganisms.[23–25] These results suggest that lignins, as well as condensed tannins with phenylpropane moieties, can be decomposed by oxidoreductases, such as peroxidases or phenol oxidases, from these fungi. In support of this hypothesis, it has been reported that condensed tannins and/or catechins were converted to various oxidative forms by laccase treatment[26] or peroxidase treatment.[27,28]

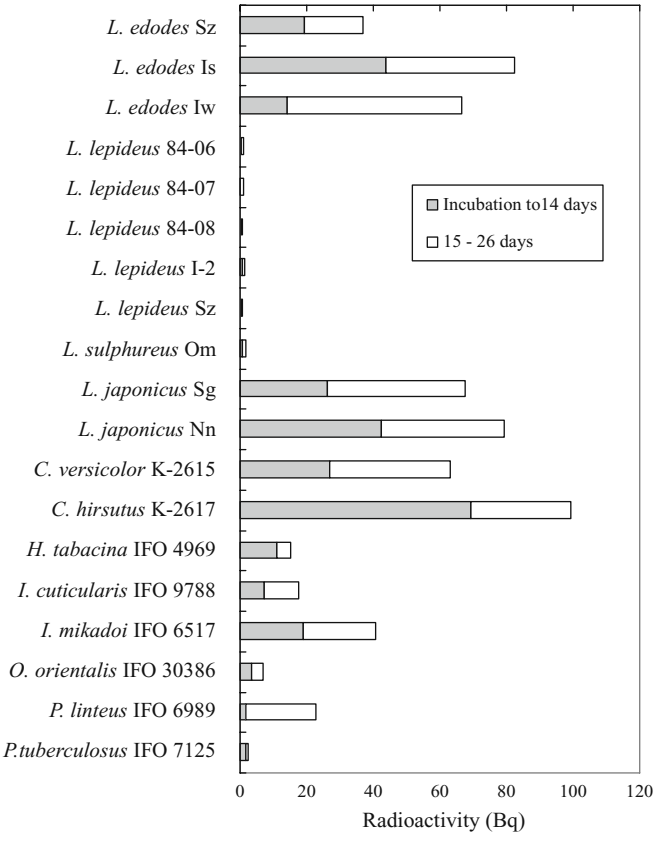


**Fig. 3.** Radioactivity of [$^{14}$C]-labeled $CO_2$ derived from fungal degradation of the $^{14}$C-labeled tannin model compound

During the first 14 days of incubation, *C. hirsutus* K-2617 showed significant mineralization of [$^{14}$C]-labeled condensed tannin model compound, whereas this activity declined by day 26. In contrast, *L. edodes* Iw showed increased emission of [$^{14}$C]-labeled $CO_2$ in the later stage compared with the early stage of incubation. The expression of mineralizing activity of both fungi appeared to closely parallel their growth rates (data not shown). Thus, the ability to metabolize wood condensed tannin may characterize an advantage of those white rot fungi in the competition for existence.

## Depolymerization of [$^{14}$C]-labeled condensed tannin model compound in bark meal cultures

Decayed bark media containing the degraded [$^{14}$C]-labeled condensed tannin model compound after 26 days of incubation were extracted with methanol, and the extracts were acetylated to determine the change in molecular weight of the labeled compounds by GPC. The samples used for this analysis were the cultures of *C. hirsutus* K-2617, which showed the highest emission of [$^{14}$C]-labeled $CO_2$, the cultures of *L. lepideus* I-2 in which no [$^{14}$C]-labeled $CO_2$ emission could be detected, and control culture that had not been inoculated with any fungi. The yields of methanol extracts of these samples were 25.9, 64.3, and 65.9 mg, respectively, and specific radioactivities of the acetylated samples were 2.9, 10.9, and 9.0 Bq/mg, respectively. In the *L. lepideus* culture, the content of methanol extracts and specific radioactivity of the acetylated sample were almost the same as those of the control culture. In contrast, the *C. hirsutus* cultures significantly decreased the content of the methanol extracts by 60.7% and reduced the specific radioactivity of the acetylated sample by 67.8%. It is clear that the decreases in the methanol extract content and specific radioactivity were caused by degradation of condensed tannins in the medium by *C. hirsutus*.

As shown in Fig. 4, GPC analysis clearly indicated that depolymerization of the [$^{14}$C]-labeled condensed tannin model had occurred because the radioactivities of the high DPn fractions of the methanol extracts from the cultures of *C. hirsutus* were decreased compared with the uninoculated control. On the other hand, the radioactivities of fractions with DPn of 1–3 were almost the same after fungal treatment, suggesting that the high DPn fractions were converted into oligomer fractions by fungal degradation. It also was shown that a little part of [$^{14}$C]-labeled condensed tannin polymerized because a small peak was observed at

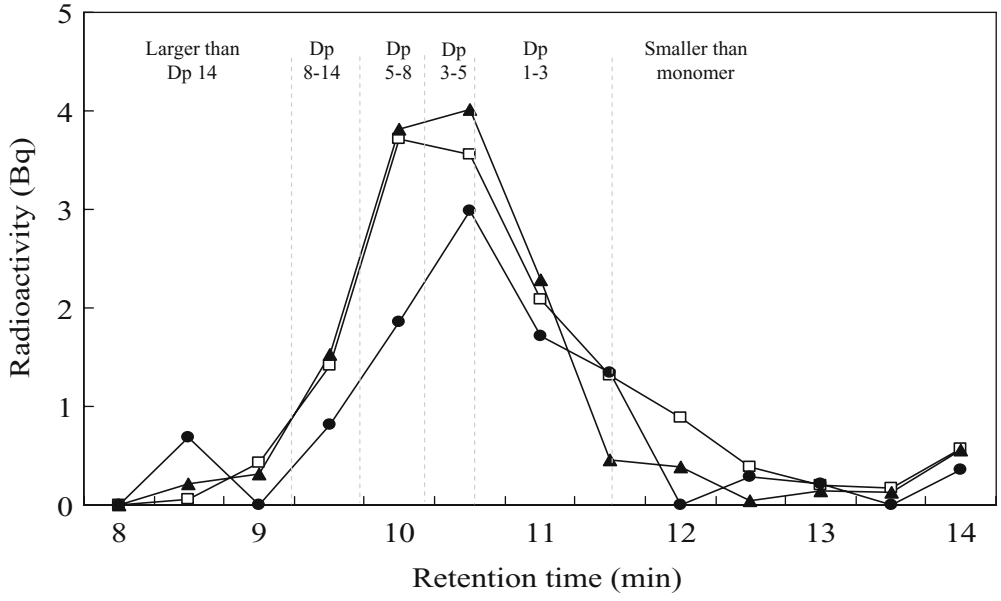


**Fig. 4.** Change in the molecular weight distribution of the [$^{14}$C]-labeled tannin model compound after incubation with *Coriolus hirsutus* (*circles*), *Lentinus lepideus* I-2 (*triangles*), or with the control (*squares*). *Dp*, degree of polymerization

a retention time of 8.5 min in Fig. 4. This is the first report on direct biological degradation of condensed tannins by white rot fungi. In agreement with these data, the cultures of *L. lepideus* were not able to degrade the [$^{14}$C]-labeled condensed tannin model compound to produce [$^{14}$C]-labeled $CO_2$, and as expected no change in the molecular weight distribution was observed over the incubation period. Thus, it is believed that fungi such as *C. hirsutus*, which can significantly generate [$^{14}$C]-labeled $CO_2$ from the labeled tannin model, will actively degrade condensed tannins even in the natural environment.

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## References

1. Haslam E (1989) Plant polyphenols – vegetable tannins revisited. Cambridge University Press, Cambridge
2. Hillis WE (1985) In Higuchi T (ed) Biosynthesis and biodegradation of wood components. Academic, Orlando, p 209
3. Hillis WE (1987) Heartwood and tree exudates. Springer, Berlin Heidelberg New York, p 76
4. Navon A, Hare JD, Federici BA (1993) Interactions among *Heliothis virescens* larvae, cotton condensed tannin and the CryIA(c) δ-endotoxin of *Bacillus thuringiensis*. J Chem Ecol 19: 2485–2499
5. Ayres MP, Clausen TP, MacLean SF, Redman AM, Reichardt PB (1997) Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78:1696–1712
6. Hammerschmidt R (1999) Induced disease resistance: how do induced plants stop pathogens? Physiol Mol Plant Pathol 55: 77–84
7. Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30:3875–3883
8. Shirley BW (1996) Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci 1:377–382
9. Bieza K, Lois R (2001) An *Arabidopsis* mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol 126:1105–1115
10. Haslam E, Lilley TH (1988) Natural astringency in foodstuffs. A molecular interpretation. Crit Rev Food Sci Nutr 27:1–41
11. Hagerman AE, Butler LG (1981) The specificity of proanthocyanidin–protein interactions. J Biol Chem 256:4444–4497
12. Kashiwada Y, Nonaka G, Nishioka I, Chang JJ, Lee KH (1992) Antitumor agents, 129. Tannins and related compounds as selective cytotoxic agents. J Nat Prod 55:1033–1043
13. Tourino S, Selga A, Jimenez A, Julia L, Lozano C, Lizarraga DL, Cascante M, Torres JL (2005) Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. J Agric Food Chem 53:4728–4735
14. Bourvellec CL, Guyot S, Renard CMGC (2004) Non-covalent interaction between procyanidins and apple cell wall material part I. Effect of some environmental parameters. Biochim Biophys Acta 1672:192–202
15. Chandra T, Krishnamurthy V, Madhavakrishna W, Nayyudamma Y (1973) Astringency in fruits: microbial degradation of wood apple tannin. Leather Sci 20:269–273
16. Grand WD (1976) Microbial degradation of condensed tannins. Science 193:1137–1139
17. Gamble GR, Akin DE, Makkar HPS, Becker K (1996) Biological degradation of tannins in sericea lespedeza (*Lespedeza cuneta*) by the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* analyzed by solid-state $^{13}$C-nuclear magnetic resonance spectroscopy. Appl Environ Microbiol 62:3600–3604
18. Yoneda S, Kawamoto H, Nakatsubo J (1997) Synthesis of high molecular mass condensed tannin by cationic polymerization of flavan-3,4-carbonate. J Chem Soc Perkin Trans 1, pp 1025–1030
19. Coleman GH, Talbot WF (1933) *sym*-Tribromobenzene. Org Synth 13:96–98
20. McKillop A, Howarth BD, Kobylecki RJ, Ryszard J (1974) Simple and inexpensive procedure for the preparation of phloroglucinol and phloroglucinol trimethyl ether. Synth Commun 4:35–43
21. Kawamoto H, Nakatsubo F, Murakami K (1996) *O*-Benzylation of phloroglucinol via phloroglucinol triacetate. Synth Commun 26:531–534
22. Kirk TK, Connors WJ, Bleam RD, Hackett WF, Zeikus JG (1975) Preparation and microbial decomposition of synthetic [$^{14}$C]lignins. Proc Nat Acad Sci USA 72:2515–2519
23. Coll PM, Fernández-Abalos JM, Villanueva JR, Santamaría R, Pérez P (1993) Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59:2607–2613
24. Kawai S, Umezawa T, Higuchi T (1988) Degradation mechanisms of phenolic beta-1 lignin substructure model compounds by laccase of *Coriolus versicolor*. Arch Biochem Biophys 262:99–110

25. Kofujita H, Nabeta K, Okuyama H, Miyake M (1989) Biodegradation of milled wood lignin on cellulose particle by *Lentinus edodes*. Mokuzai Gakkaishi 35:268–274
26. Osman AM, Wong KKY (2007) Laccase (EC 1.10.3.2) catalyses the conversion of procyanidin B-2 (epicatechin dimer) to type A-2. Tetrahedron Lett 48:1163–1167
27. Gnanamani A, Sekaran G, Babu M (2001) Removal of tannin from cross-linked and open chain polymeric tannin substrates using heme peroxidases of *Phanerochaete chrysosporium*. Bioprocess Biosyst Eng 24:211–217
28. Hosny M, Rosazza JPN (2002) Novel oxidations of (+)-catechin by horseradish peroxidase and laccase. J Agric Food Chem 50: 5539–5545