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Antioxidative Catechol Lignans Converted from Sesamin and Sesaminol Triglucoside by Culturing with *Aspergillus*

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Sesamin and sesaminol triglucoside in sesame seeds are major lignans that display an abundance of biological activities. Although their antioxidative activity in vitro is weak, they have been reported to suppress oxidative stress in vivo. We investigated the production of new antioxidative lignans from sesame lignans by culturing with the genus *Aspergillus* to enhance the function of food materials. Media containing sesamin or sesaminol triglucoside increased antioxidative activity for DPPH radical scavenging by culturing with *Aspergillus usamii* mut. *shirousamii* RIB2503. The antioxidative lignans in sesamin medium were identified as sesamin 2,6-dicatechol and episesamin 2,6-dicatechol. Those in sesaminol triglucoside medium were identified as sesaminol 6-catechol and episesaminol 6-catechol, which are novel antioxidative lignans. It is suggested that they may exhibit higher antioxidative activity than sesamin and sesaminol triglucoside because they have the catechol functional moiety.

KEYWORDS: Antioxidant; *Aspergillus*; catechol; lignan; sesame

INTRODUCTION

Sesame has long been categorized as one of the traditional health foods in Japan, China, and other East Asian countries. Sesame seeds contain abundant lignans such as sesamin, sesamolin, and sesaminol glucoside (*1*). Sesamin is a major lignan in sesame oil, and its biological effects have been extensively studied. Previous studies report that sesamin inhibits Δ5 desaturase (*2*), an antihypertensive effect (*3*), and hypocholesterolemic activity through the inhibition of cholesterol absorption and synthesis (*4*). Sesamolin and sesaminol glucoside have their own individual antioxidative activities which are related to their high stability against oxidation and increased storage of sesame oil (*5*). Sesaminol glucoside is contained in the soluble fraction of defatted sesame seed (*6*). Dietary defatted sesame flour containing sesaminol glucoside has been reported to decrease susceptibility to oxidative stress in hypercholesterolemic rabbits (*7*).

Antioxidants such as phenolic phytochemicals in food have demonstrated an ability to scavenge free radicals in vivo and are reportedly linked to anticarcinogenic and antiatherogenic activities (*8*). Sesamin and sesaminol glucoside have no antioxidative properties in vitro (*9*), but they have been reported to be converted to phenolic compounds after oral administration to the rat and to express antioxidative activity in vivo (*10*).

Fungi of the genus *Aspergillus* are used as rice malt in the production process of Japanese distilled spirit. Recently, it has been reported that *Aspergillus* produces hydroxygenistein and hydroxydaizein, which have the catechol functional moiety and are potent antioxidants, from the isoflavone glycosides of genistin and daidzin by hydroxylation during soybean fermentation (*11*). We have also reported production of hydroxyhesperetin and hydroxynaringenin as new potent antioxidants, which have the catechol functional moiety, from the citrus flavonoids of hesperidin or naringin by culturing with *Aspergillus saitoi* that is used to produce "awamori" Japanese distilled spirit (*12*). In this study, we attempted to obtain new antioxidative lignans from sesamin and sesaminol triglucoside by culturing with *Aspergillus* species to produce effective antioxidative lignans for food materials.

MATERIALS AND METHODS

Chemicals and Microorganisms. Reagent grade chemicals were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased for Becton Dickinson and Co., Maryland. Sesamin (a mixture of sesamin and 6-episesamin) and sesaminol triglucoside (sesaminol 2′-*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside) were purified from roasted sesame seed according to reported methods (*6*, *13*). Authentic samples of sesamin, episesamin, and sesaminol for analysis were prepared from refined sesame oil as described previously (*13*, *14*). The microorganisms, *Aspergillus awamorii* RIB2804, *Aspergillus usamii* mut. *shirousamii* RIB2503, *Aspergillus niger* ATCC38857, ATCC10254, ATCC10549, and ATCC9642, and *A. saitoi* IAM2210 were obtained from the National Research Institute of Brewing (RIB), American Type Culture Collection (ATCC), and Institute of Applied Microbiology at Tokyo University (IAM).

Culture Condition of *Aspergillus*. *Aspergillus* species were precultured in a PDA medium at 30 °C for 7 days. The spores were

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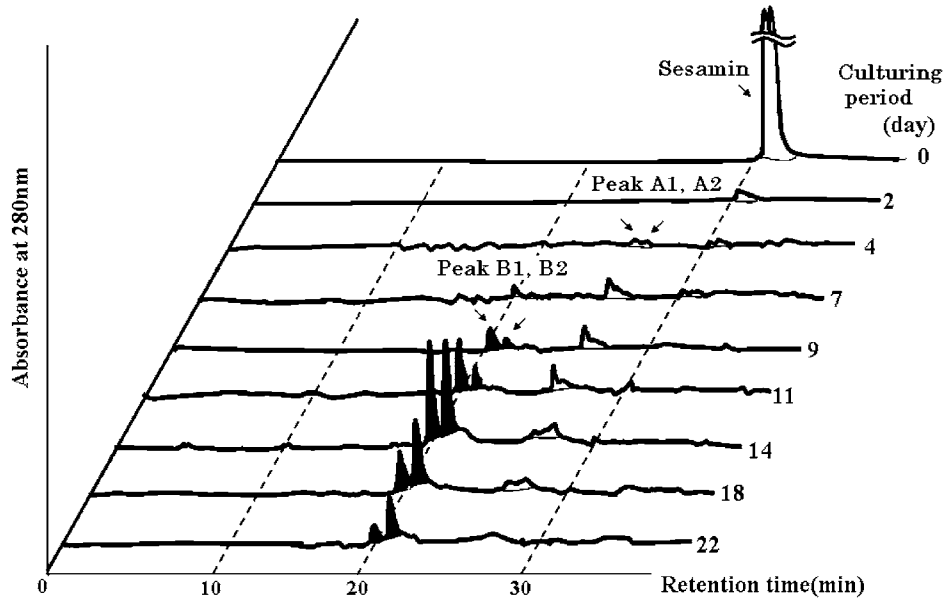

Figure 1. HPLC profile for a medium containing sesamin cultured with *A. usamii* mut. *shirousamii* RIB2503.

suspended in 0.15 M NaCl solution and prepared to 1×10^8 spores/mL. A 100 mL sample of PDB (12 g/L) medium was poured into an Erlenmeyer flask. Sesamin was dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL and added to the medium at a final concentration of 0.2% (w/v). Sesaminol triglucoside was dissolved in water to 500 mg/mL and added to the medium at a final concentration of 0.5% (w/v). After the solution was sterilized at 121 °C for 15 min, the solution was inoculated with 1 mL of each species of *Aspergillus* (1×10^8 spores/mL). It was confirmed in advance that the growth of *Aspergillus* is not influenced at the concentration of DMSO in the medium. The inoculated solution was shake-cultured with a rotary shaker at a speed of 100 rpm and 30 °C. A sample (1 mL) of *Aspergillus* was periodically removed from the medium containing sesamin during a culturing period of 0−12 days. A small sample (1 mL) of the medium containing sesaminol triglucoside was periodically removed during a culturing period of 0−31 days.

Assay of Radical Scavenging Activity. During the culture period, a small sample (1 mL) of the medium solution was periodically removed and centrifuged at 1900*g* for 5 min to remove the residue. The sample was measured for antioxidative activity using the DPPH radical scavenging system by a high-performance liquid chromatographic (HPLC) analysis according to the reported method (*15*). A 100 μL portion of the sample solution was mixed with 2 mL of 20 mg/mL DPPH in ethanol and 1 mL of a 100 mM Tris−HCl buffer (pH 7.4). The mixture was shaken vigorously, left to stand for 40 min at room temperature in the dark, and then subjected to HPLC (LC-10A, Shimadzu Co., Ltd., Kyoto, Japan) analysis. Analyses were performed using a TSKgel Octyl-80Ts column (4.6 × 150 mm, Tosoh, Ltd., Tokyo) at ambient temperature and a spectrophotometric detector (517 nm) with a mobile phase of methanol/water (70:30, v/v) at a flow rate of 1 mL/min. In assaying sesame lignan activity such as sesamin, sesaminol triglucoside, and the isolated lignans, each sample was dissolved in DMSO and assayed at a final concentration of 5 μM. α-Tocopherol was used as the standard antioxidant and assayed at a final concentration of 5 μM. The activity was evaluated from the difference in the decreasing peak area of the DPPH radical detected between the blank and the sample. The values were represented as the mean of the two or three measurements.

Determination of Lignans in the Cultured Medium. The sesame lignans in the medium during the culturing period were analyzed by reversed-phase HPLC on a 4.6 × 250 mm i.d. YMC-pack ODS column (YMC Co. Ltd., Japan) with UV detection at 280 nm. The column was eluted with a linear gradient from solvent A [water/methanol (90:10, v/v)] to solvent B [water/methanol (10:90, v/v)] in 30 min and then eluted with 100% methanol for 5 min at a flow rate of 1 mL/min.

Isolation of the Antioxidative Lignans. *A. usamii* mut. *shirousamii* RIB2503 was cultured by media containing sesamin or sesaminol triglucoside as mentioned above. After confirmation of the increase of DPPH radical scavenging for the cultured media, the media were centrifuged at 1500*g* for 15 min to remove the residue and concentrated in vacuo. Peaks A1, A2, B1, and B2 (**Figure 1**) for HPLC analysis were fractionated from the medium containing sesamin by preparative HPLC using a 50 × 250 mm i.d. YMC-pack ODS column (YMC Co. Ltd.) and a UV detector at 280 nm with a mobile phase of methanol/water (50:50, v/v) at a flow rate of 100 mL/min at room temperature. Compounds B1 and B2 were isolated, and the yields of B1 and B2 were 12.0% and 8.0%, respectively. The peaks sesaminol, C1, and C2 for HPLC analysis (**Figure 2**) were fractionated from the medium containing sesaminol triglucoside by preparative HPLC, and then compounds C1 and C2 were isolated. The yields of C1 and C2 were 4.6% and 3.0%, respectively. The fractionated peaks were examined for DPPH radical-scavenging activity.

Instrumental Analyses. Molecular weight was determined by LC−MS (VG PLATFORM) using a 4.6 × 250 mm i.d. YMC-pack ODS column with an ESP^+ detector. The column was eluted with a linear gradient from solvent A [water/methanol (90:10, v/v)] to solvent B [water/methanol (10:90, v/v)] in 30 min and then eluted with 100% methanol for 5 min at a flow rate of 0.8 mL/min. ^1H NMR and ^{13}C NMR spectra were obtained with a Bruker ARX 400 instrument (400 MHz for ^1H and 100 MHz for ^{13}C) in d_6-dimethyl sulfoxide containing tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

Change of Sesamin and Sesaminol Triglucoside by Culturing with *Aspergillus* Species. The medium containing sesamin was fermented using six species of *Aspergillus* as shown in **Figure 3**. A higher increase in activity was observed in the media cultured with *A. niger* ATCC38857, *A. usamii* mut. *shirousamii* RIB2503, and *A. saitoi* IAM2210 after 4 days compared to the medium before culturing. During continuous observation over 7 days, the media cultured by *A. niger* ATCC38857 and *A. usamii* mut. *shirousamii* RIB2503 exhibited increasing and high activity. The media cultured by other species of *Aspergillus* did not exhibit steady increases.

The medium containing sesaminol triglucoside was also examined for DPPH radical scavenging activity as shown in **Figure 4**. A notable increase in activity was observed in the media cultured by *A. usamii* mut. *shirousamii* RIB2503 and *A.*

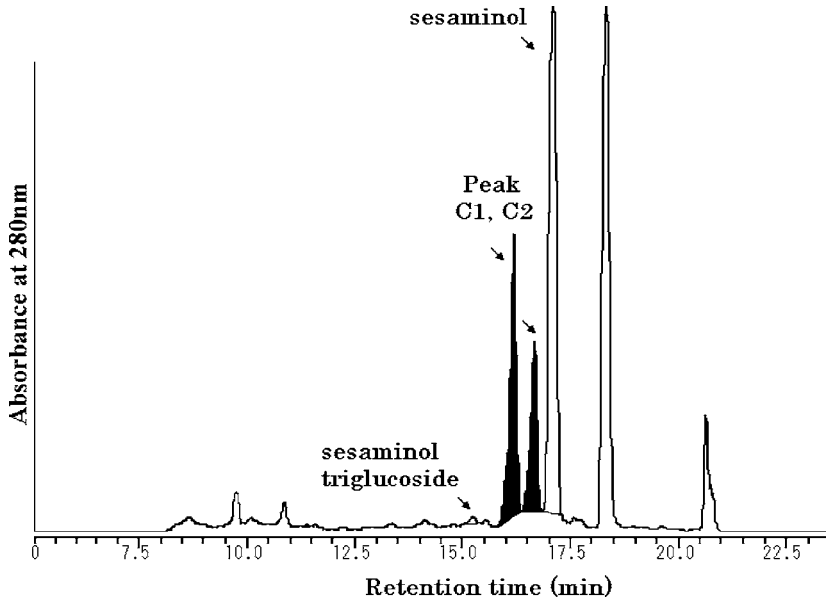

Figure 2. HPLC profile for a medium containing sesaminol triglucoside cultured with *A. usamii* mut. *shirousamii* RIB2503.

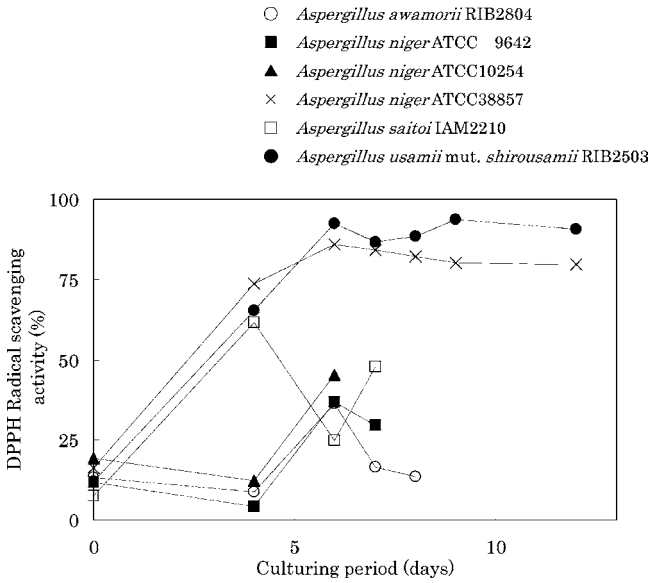

Figure 3. Change in the antioxidative activity of the medium containing sesamin by culturing with *Aspergillus* species.

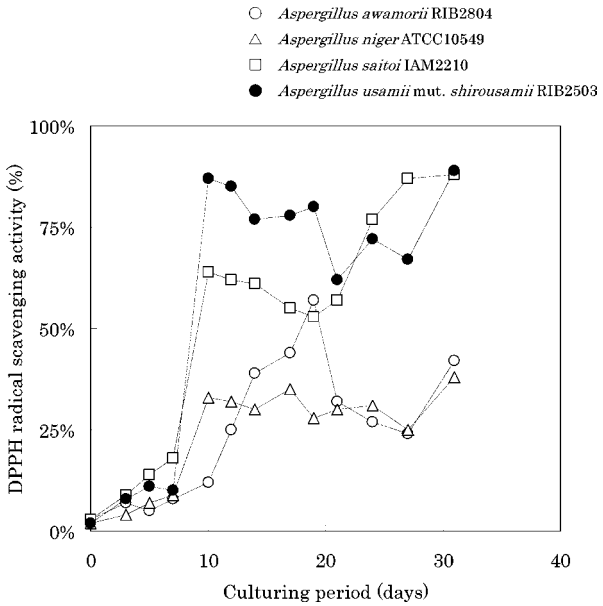

Figure 4. Change in the antioxidative activity of the medium containing sesaminol triglucoside by culturing with *Aspergillus* species.

saitoi IAM2210 after 10 days, although no large variation in activity was observed in the media cultured from all species of *Aspergillus* until 7 days. For *A. awamorii* RIB2804 and *A. niger* ATCC10549, the activity was lower than that of *A. usamii* mut. *shirousamii* RIB2503 and *A. saitoi* IAM2210, although the activity increased slowly when compared to that of the medium before culturing.

Lignans Converted by Culturing with *Aspergillus*. By culturing with *A. usamii* mut. *shirousamii* RIB2503, the medium containing sesamin or sesaminol triglucoside exhibited high antioxidative activity in the DPPH radical scavenging assay (**Figures 3** and **4**). The change of lignans in the medium during the culture period was analyzed by HPLC (**Figure 1**), and the lignans were isolated by preparative HPLC and examined for DPPH radical-scavenging activity. The peak corresponding to sesamin in the HPLC profile decreased after 2 days, and disappeared after 9 days. The two small peaks, A1 and A2, which had DPPH radical scavenging activity, were detected after 4 days, but they decreased after 11 days and disappeared as the culturing progressed. However, the two large peaks, B1 and B2, which had DPPH scavenging activity, were detected after 7 days and maximized at 14 days. HPLC analysis revealed that compounds A1, A2, B1, and B2 were generated from sesamin and were also detected in the media, accounting for the increase of activity, cultured by other species of *Aspergillus*. The compounds corresponding to peaks B1 and B2 were isolated and purified as compounds B1 and B2, respectively.

Compounds B1 and B2 were analyzed by LC−MS, and the molecular weight of both compounds was shown to be 330.34. ^{1}H NMR data for the compounds are shown in **Table 1**. The ^{1}H NMR data for a diastereomer of sesamin and episesamin were obtained by analysis of authentic samples. The chemical shift of the proton of the methylenedioxy moieties (−O−CH_2−O−) in sesamin and episesamin was 5.97 ppm with a signal intensity of four protons, but the proton signal of the methylenedioxy moiety in compounds B1 and B2 was not observed. The data for compounds B1 and B2 were similar to those of sesamin and episesamin, respectively, except that there was no proton signal for the methylenedioxy moiety or the hydroxyl

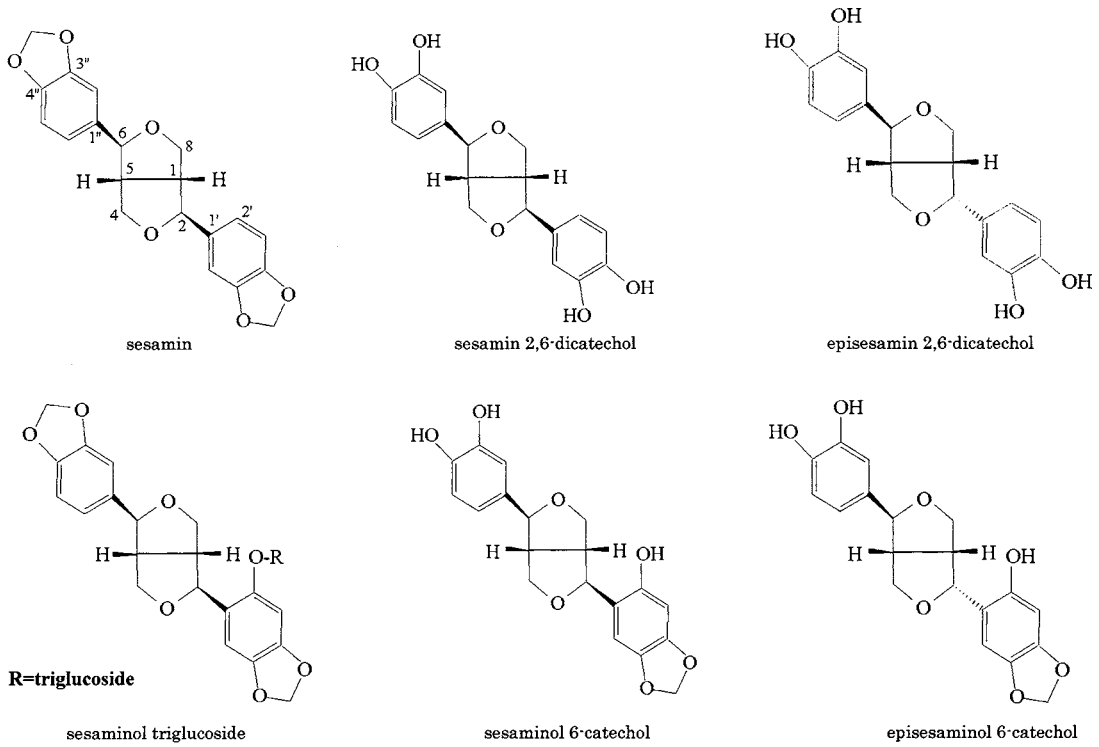

Figure 5. Chemical structures of sesamin, sesaminol triglucoside, and catechol lignans.

Table 1. ^{1}H NMR Spectroscopic Data for Sesamin, Episesamin, and the Compounds Converted from Sesamin by *A. usamii* mut. *shirousamii* RIB2503

position	δ_H[sesamin ($CDCl_3$)]	δ_H[compd B1 (DMSO)]
H-1/5	2.88, 2H, m	2.91, 2H, m
H-2/6	4.75, 2H, d	4.52, 2H, d
H-4a/8a	3.74, 2H, dd	3.68, 2H, dd
H-4e/8e	4.10, 2H, dd	4.07, 2H, dd
$-OCH_2O-$	5.92, 4H, s	
H-2″/5″/6″	6.83, 6H, m	6.67, 6H, m
–OH	8.79	

position	δ_H[episesamin ($CDCl_3$)]	δ_H[compd B2 (DMSO)]
H-1	2.88, 1H, m	2.50, 1H, m
H-5	3.30, 1H, m	3.30, 1H, m
H-2	4.83, 1H, d	4.68, 1H, d
H-6	4.39, 1H, d	4.21, 1H, d
H-4a	3.83, 1H, dd	3.66, 1H, dd
H-4e	4.10, 1H, dd	3.96, 1H, dd
H-8a	3.80, 1H, m	3.30, 1H, m
H-8e	3.30, 1H, m	3.30, 1H, m
$-OCH_2O-$	5.92, 4H, s	
–OH	8.79	

group (8.79 ppm). Furthermore, the B1 data were consistent with those of sesamin 2,6-dicatechol (*10*), which was reported as demethylpiperitol in the literature (*16*). Therefore, the structures of compounds B1 and B2 were identified as sesamin 2,6-dicatechol and episesamin 2,6-dicatechol, respectively (**Figure 5**). Detection of sesamin 6-catechol as a metabolite has been reported in the metabolism of sesamin to sesamin 2,6-dicatechol in rat liver (*10*). Therefore, peaks A1 and A2, having radical scavenging activity, were postulated to be sesamin 6-catechol and episesamin 6-catechol. In this study, sesamin was mainly converted to sesamin 2,6-dicatechol, having radical scavenging activity, by culturing with *Aspergillus*.

For sesaminol triglucoside, the peaks of sesaminol, C1, and C2, which exhibited radical scavenging activity, were detected in the medium cultured with *A. usamii* mut. *shirousamii* RIB2503 (**Figure 2**). The sesaminol peak was identified by comparison with standard sesaminol using HPLC analysis. HPLC analysis revealed that these compounds were generated from sesaminol triglucoside, and they were detected in the medium, having activity, through culturing of other *Aspergillus* species. Peaks C1 and C2 were isolated and purified as compounds C1 and C2. The molecular weight of both compounds was analyzed by LC−MS and shown as 358.0. Compounds C1 and C2 were analyzed by ^{1}H, ^{13}C, HMBC, and HMQC and were assigned by ^{1}H and ^{13}C NMR data to identify the chemical structures as shown in **Table 2**. The structure of these compounds was speculated by comparison with standard sesaminol and episesaminol or with their reported data (*14*, *17*). Compounds C1 and C2 were detected via the signal of the tetrahydrofuran ring by ^{1}H NMR analysis. The proton chemical shifts of the methylenedioxy moiety for sesaminol and episesaminol were detected with a signal intensity of four protons, but those of compounds C1 and C2 were observed at 5.87 and 5.90 ppm, respectively, with a signal intensity of two protons. For compounds C1 and C2, the signals of the hydroxyl moiety were observed at 8.78 and 9.23 ppm, and the chemical shifts of other proton signals were similar to those of sesaminol and episesaminol, respectively. Compound C1 was analyzed by the 2D NMR technique. The proton signal of position 1 was observed to coincide with that of position 5 at 2.87 ppm. The proton signals of positions 4 and 8 were similar to those of sesaminol. As for the 2D NMR analysis of compound C2, the proton signals of positions 1 and 5 were different between 3.37 and 2.75 ppm, respectively. The proton signal of position 1 was detected at a low magnetic field, but that of position 8 was detected at a high magnetic field in comparison with compound C1. The conformation of compound C2 was suggested to be similar to that of 2-episesaminol by the comparison with the reported data (*14*). These results enabled compounds C1 and C2 to be identified as sesaminol 6-catechol and episesaminol 6-catechol, respectively. They are novel compounds, and their chemical structures are shown in **Figure 5**. It is proposed that

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for the Compounds Converted from Sesaminol Triglucoside

	compd C1		compd C2	
position	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C
1	2.87, m	53.6	3.37, m	47.9
2	4.86, d (5)	80.9	4.78, d (6)	77.9
4a	3.83, dd (4, 9)	70.9	3.70, dd (6, 9)	69.8
4e	4.14, dd (6, 9)		3.98, d (9)	
5	2.87, m	53.6	2.75, dd (6, 9)	53.7
6	4.51, d (5)	84.5	4.21, d (7)	86.6
8a	3.71, dd (4, 9)	71.6	3.07, t (8)	68.6
8e	4.07, dd (6, 9)		3.64, t (8)	
1′		120.3		117.0
2′		148.7		147.8
3′	6.43, s	97.5	6.43, s	97.2
4′		146.2		146.0
5′		139.7		139.4
6′	6.75, s	105.4	6.85, s	106.1
1″		132.4		132.5
2″	6.72, s	113.5	6.75, s	113.4
3″		145.1		145.1
4″		144.5		144.6
5″	6.67, d (8)	115.2	6.67, d (8)	115.2
6″	6.58, d (8)	117.0	6.60, d (8)	117.0
$-OCH_2O-$	5.87, d (10)	100.5	5.90, s	100.5
–OH	8.78, br s		8.78, br s	
	9.23, br s		9.24, br s	

they are converted from sesaminol after sesaminol triglucoside is hydrolyzed to sesaminol by *Aspergillus*. In **Figure 4**, the antioxidative activity for media containing sesaminol triglucoside increases slowly compared to that for media containing sesamin. A metabolic process that converts sesaminol triglucoside to an aglycon of sesaminol may be necessary for a certain culturing period with *Aspergillus*. Sesamin may be able to be converted more quickly to catechol lignans than sesaminol glucoside because sesamin does not require hydrolysis of the glucoside bond.

Sesaminol 2,6-dicatechol was not detected from media containing sesaminol triglucoside in this study, although sesamin was converted to sesamin 2,6-dicatechol by *Aspergillus*. The hydroxy group at the 2′-position on sesaminol may inhibit the reaction, which converts the methylenedioxy moiety on carbons 3″ and 4″ to a catechol structural moiety. 8-Hydroxydaidzein and 8-hydroxyhesperetin were reported to be produced by the hydroxylation reaction of daidzein and hesperetin during culturing with *A. saitoi* (*11*, *12*). In this study, sesame lignans were converted to catechol lignans, which have catechol moieties similar to 8-hydroxydaidzein and 8-hydroxyhesperetin, by culturing with *A. usamii* mut. *shirousamii* RIB2503 and other *Aspergillus* species. However, catechol lignan was not generated by an addition reaction of the hydroxyl group on lignans, but it was converted from the methylenedioxy moiety on the lignan to a catechol structural moiety. The production process of catechol lignans by culturing with *Aspergillus* is different from that of hydroxyflavonoids having catechol structural moieties such as 8-hydroxydaidzein and 8-hydroxyhesperetin.

Radical Scavenging Activity of the Converted Lignans. In vivo, antioxidative activities of sesame lignans such as sesamin, sesamolin, and sesaminol glucoside have been reported where the suppressive effect of oxidative stress is observed when administered orally to experimental animals (*18*−*21*). In vitro, sesaminol and sesamolin have antioxidative activity, but sesamin and sesaminol glucoside have no activity (*9*). Antioxidative activity of sesamin 6-catechol and sesamin 2,6-dicatechol, which are also found as sesamin metabolites in the rat liver, has been reported in vitro (*10*). As shown in **Figure 6**, the antioxidative

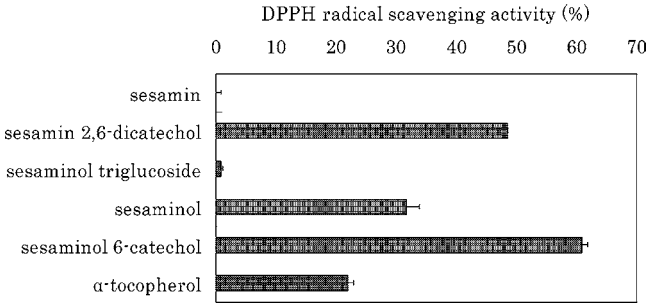

Figure 6. Antioxidative activity of sesamin, sesaminol triglucoside, and catechol lignans. The values are represented as the mean ± SD (N = 3).

activity of the lignans converted by *Aspergillus* was examined using the assay for DPPH radical scavenging activity. In the assay, sesamin and sesaminol triglucoside had little activity, but sesamin 2,6-dicatechol, sesaminol, and sesaminol 6-catechol showed significant activity. Sesamin 2,6-dicatechol and sesaminol 6-catechol exhibited the most potent activity.

Much attention has been focused on antioxidants in food materials, including phenolic acids, flavonoids, and carotenoids, for the prevention and treatment of many diseases (*8*). Flavonoids such as catechin and quercetin show general antioxidative effects, because they have a catechol moiety in their structures (*22*). The *o*-dihydroxyphenyl structure (catechol moiety) at the 3′- and 4′-positions in the B ring of flavonoids is necessary for high antioxidative activity such as radical scavenging and the suppression of lipid peroxidation (*22*, *23*). Sesamin does not have a catechol structural moiety, but it is reported to be metabolized to biologically active compounds having catechol structural moieties after absorption into the body (*10*). Sesamin and sesaminol glucoside have no antioxidative properties in vitro, but they are converted to prominently antioxidative metabolites, and they exert protective actions against oxidative damage in vivo (*7*, *10*). This study showed that catechol lignans such as sesamin 2,6-dicatechol and sesaminol 6-catechol are effective lignans, which have antioxidative activity in vitro because they have a catechol structural moiety. In this study, the catechol lignans which are produced by culture of sesamin and sesaminol glucoside using *Aspergillus* species were found to have high antioxidative activity in vitro, and these compounds were suggested to be expected as effective food antioxidants.

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