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Article

Sterin C, a New Antioxidant from the Mycelial Culture of the Mushroom *Stereum hirsutum*

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Structurally new antioxidative metabolite was isolated from mycelial culture of mushroom *Stereum hirsutum*. Culture broth was subjected to Diaion HP-20 column chromatography, and 70% aqueous MeOH eluent was extracted with EtOAc. EtOAc extract was purified through silica gel and Sephadex LH-20 column chromatographies, and reversed phase C_{18} HPLC. Compound was revealed to be new dihydroxylated derivative of sterin B with molecular formula of $C_{12}H_{16}O_5$ (MW 240) by MS and various NMR spectral data analyses, and designated as sterin C. Sterin C showed superoxide radical-scavenging activity with EC_{50} value of 0.31 mM.

Key words: Stereum hirsutum, *sterin C, superoxide radical, antioxidant*

Free radicals are involved in the pathogenesis of various diseases such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, cancer-initiation, and aging process,[1-5] thus, many antioxidants of microbial origin have been studied.[6-8] Because free radical scavengers have the potential as protective agents against diseases related with oxidative stress, we have searched for natural free radical scavengers from chemical constituents of mushrooms.[9-13]

Mushrooms, used in the Orient as traditional foods and medicines, produce various classes of primary and secondary metabolites, which exhibit significant biological activities such as immuno-stimulating, antimicrobial, antitumor, and antiviral effects. However, in spite of their potential as resources for drug development, few bioactive metabolites have been reported from mushrooms, compared with higher plants and other microbes.

As part of our continuing search for antioxidative substances from mushrooms, we isolated novel antioxidative compounds, hirsutenols A-C[14] and sterins A and B,[15] from the fermentation broth of *Stereum hirsutum* (Willd.: Fr.) S. F. Gray (Stereaceae). Further investigation of a fraction of this mushroom has resulted in the discovery of a new antioxidative compound designated sterin C (**1**), which is a derivative of sterin B (**2**) (Fig. 1). *Stereum* species produce sesquiterpenes such as hirsutane,[16] sterepolide,[17] sterpurene,[18] benzaldehydes,[19] and benzofurans.[20] This paper describes the isolation, structure determination, and superoxide radical-scavenging activity of sterin C (**1**).

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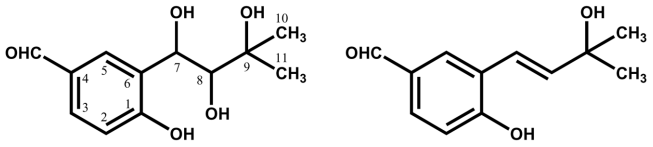
Materials and Methods

Mushroom strain. The sterin C-producing strain was generated from the tissue culture of the fruiting body of *S. hirsutum* collected at Duckyu Mountain, Korea in September 2001, and identified by the staff of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea, according to the taxonomic key of Imazeki and Hongo.[21] A voucher specimen (MVS39) was deposited in the Laboratory of Antioxidant, KRIBB.

Reagents. Diaion HP-20 was purchased from Nippon Rensui Company (Japan). Silica gel (Merck Kiesel gel 60, 70-230 mesh) for column chromatography and silica TLC plates (Silica gel 60 F_{254}) were purchased from Merck company (USA), Sephadex LH-20 (bead size 25~100 μm) from Pharmacia (USA), and nitrobluetetrazolium (NBT), vitamin E, and trolox from Sigma (USA). Organic solvents for HPLC were purchased from Baxter (Burdick & Jackson, USA) and CD_3OD for NMR from Aldrich (USA). All other chemicals were reagent grade and obtained from commercial sources.

Fermentation. The strain grown on potato sucrose agar medium was used to inoculate two 500-m*l* baffled Erlenmeyer flasks containing 100 m*l* YM medium consisting of yeast extract 0.4%, malt extract 1.0%, and glucose 0.4% (pH 6.0 before sterilization). The flasks were shaken on a rotary shaker for 4 days at 27°C. The seed culture was transferred into a 5-L jar fermenter containing 3.5 L YM medium to produce sterin C, and cultivation was carried out at 28°C for 10 days with aeration of 2 L/min and agitation of 200 rpm.

General experiments. ^1H, ^{13}C, and 2D NMR spectra were measured with a Bruker DMX600 spectrometer (USA) in CD_3OD with TMS as an internal standard. UV-Vis, IR, and EI



Sterin C (**1**) Sterin B (**2**)

Fig. 1. Chemical structures of sterin C (1) and sterin B (2).

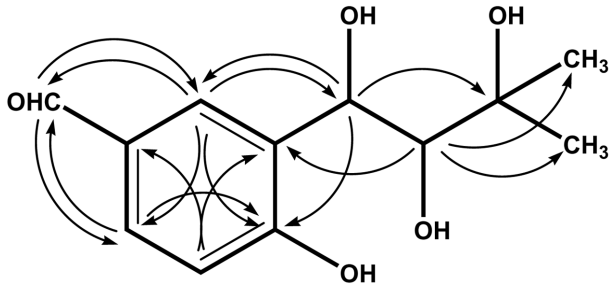


Fig. 2. Key long-range correlations in the HMBC spectrum of sterin C.

mass spectra were obtained using a Mecasys Optizen 2120 spectrophotometer (Korea), a Research series FT-IR spectrometer (England), and a JEOL JMS-SX 102A spectrometer (Japan), respectively. Optical rotation was obtained on a Perkin-Elmer 343 polarimeter (USA) with MeOH as solvent. HPLC was run on a Waters 510 instrument equipped with a 991 photodiode array detector using YMC (ODS, 250 × 20 mm, Japan) for preparative column and YMC (ODS, 150 × 4.6 mm) for analytical column. $Versa_{max}$ microplate reader (Molecular Devices, USA) was used to read 96 well-plate for the measurement of superoxide radical-scavenging activity.

Isolation and purification. Mycelial culture of *S. hirsutum* was separated into supernatant and mycelia by centrifugation at 3000 g for 10 min. The supernatant was applied to a Diaion HP-20 column, and the column was washed with 30% aqueous MeOH and eluted with 70% aqueous MeOH. After evaporation of the 70% aqueous MeOH eluent *in vacuo*, the resultant residue was partitioned between EtOAc and H_2O. The EtOAc extract was then concentrated and subjected to a silica gel column with $CHCl_3$: MeOH (30 : 1-1 : 1, stepwise) as the eluent to give active fractions. The active fractions were concentrated and rechromatographed on a Sephadex LH-20 column (MeOH), followed by a reversed phase preparative HPLC eluted with 25% aqueous ACN at a flow rate of 7.0 m*l*/min to give 3.3 mg of compound **1**.

Physico-chemical properties of sterin C(1). Yellow powder; $[\alpha]^{20}_{D} = -46.1^{\circ}$ (*c*0.18, MeOH); IR (KBr) ν_{max}: 3433, 2925, 1607, 1117 cm^{-1}; EIMS (*m/z*): 240 (M^+); UV (MeOH) λ_{max} = 287 nm; ^{1}H-NMR (600 MHz, CD_3OD, δ): 9.82 (1H, s, CHO), 8.04 (1H, br. s, H-5), 7.73 (1H, dd, *J* = 8.4, 2.4 Hz, H-3), 6.89 (1H, d, *J* = 8.4 Hz, H-2), 4.55 (1H, d, *J* = 8.4 Hz, H-7), 3.53 (1H, d, *J* = 8.4 Hz, H-8), 1.48 (3H, s, H-10), 1.23 (3H, s, H-11); ^{13}C-NMR (150 MHz, CD_3OD, δ): 192.8 (4-CHO), 159.5 (C-1), 132.6 (C-5), 131.4 (C-3), 131.2 (C-4), 126.8 (C-6), 118.5 (C-2), 81.3 (C-9), 76.3 (C-8), 69.4 (C-7), 27.0 (C-10), 19.8 (C-11).

Superoxide radical-scavenging activity. Irradiated riboflavin/EDTA/NBT system[22] was employed to measure the superoxide radical-scavenging activity with minor modifications. The mixture consisted of 140 μ*l* of 0.030 mM riboflavin, 1 mM EDTA, 0.60 mM methionine and 0.030 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 μ*l* of a sample solution, which includes the test compound and the reference compounds at various concentrations in MeOH, as well as MeOH as a control. The photoinduced reactions to generate superoxide anion were carried out in an aluminum foil-lined box with two 20-W fluorescent lamps. The distance between the reactant and the lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated for 8 min at 25°C. The photochemically reduced riboflavin generated superoxide anion, which reduced NBT to form the blue formazan. The un-illuminated reaction mixture was used as a blank. Reduction of NBT was measured by the absorbance change at 560 nm before and after irradiation using a microplate. Scavenging activity was calculated based on the changes in the absorbances of the control and test samples:

$$\text{Scavenging activity (\%)} = (1 - \Delta A_{sample}/\Delta A_{control}) \times 100,$$

where ΔA_{sample} and $\Delta A_{control}$ are the changes in the absorbances of the wells containing the test compounds and MeOH, respectively.

The EC_{50} value is defined as the concentration of substrate that causes 50% loss of the reduced NBT. The assays were performed in triplicates, and the changes in the absorbance were averaged before calculation.

Results and Discussion

Structure determination. The molecular formula of **1** was determined to be $C_{12}H_{16}O_5$ on the basis of EIMS (*m/z* 240, M^+) and ^{13}C NMR spectral data. The ^{1}H and ^{13}C NMR spectral data of **1** were in accordance with those of sterin B, except for the presence of two hydroxylated methines instead of the two olefinic methines found in sterin B. In the ^{1}H NMR spectrum, three aromatic proton signals were assigned to H-2 (δ_H6.89, d, *J* = 8.4 Hz), H-3 (δ_H7.73, dd, *J* = 8.4, 2.4 Hz), and H-5 (δ_H8.04, br. s), which suggested the existence of 1,2,4-tri substituted benzene ring. In addition, two coupled hydroxylated methine protons of H-7 (δ_H4.55, d, *J* = 8.4 Hz) and H-8 (δ_H3.53, d, *J* = 8.4 Hz), and two singlet methyl protons of H-10 (δ_H1.48, s) and H-11 (δ_H1.23, s) were assigned. The singlet proton signal at δ_H9.82 was assigned to aldehyde proton.

The ^{13}C NMR data depicted 12 carbon signals, distributed into 1,2,4-tri substituted benzene ring, a carbonyl carbon, an sp^3 quaternary carbon, two hydroxylated methine carbons, and two isolated methyl carbons. The ^{1}H and ^{13}C NMR spectral data suggested the existence of a formyl group (δ_H9.82, 1H, s; δ_C 192.8). From its upfield-shifted ^{13}C chemical shift, the

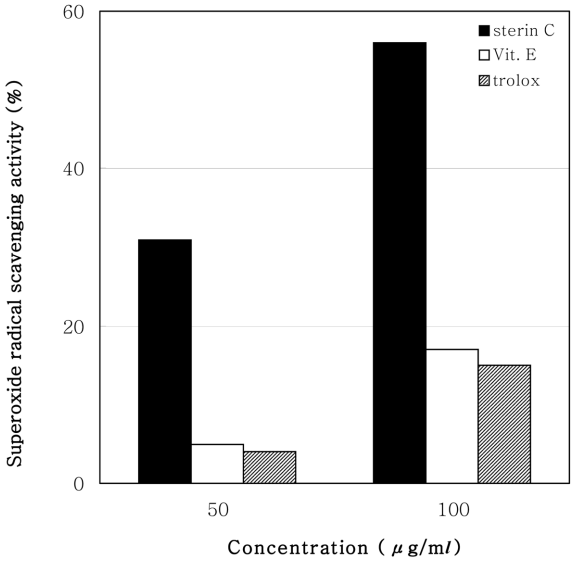


Fig. 3. Superoxide radical-scavenging activity of sterin C.

formyl group was considered to be attached to an aromatic ring.

Additional information permitting the complete structure assignment of sterin C was provided through the analysis of HMBC experiment (Fig. 2). The HMBC long-range correlations observed between the carbonyl carbon at 192.8 (4-CHO) and the aromatic protons at δ_H8.04 (H-5) and 7.73 (H-3) showed that the formyl group is directly attached to C-4 (δ_C131.2) of the benzene ring. The 3-methyl-1,2,3-tri-hydroxybutyl unit (C7-C11) was assigned based on the long-range correlations between the hydroxylated methine proton at δ_H4.55 (H-7) and the quaternary carbon at δ_C81.3 (C-9), and between the proton at δ_H3.53 (H-8) and methyl carbons at δ_C27.0 (C-10) and δ_C19.8 (C-11). This unit was connected to C-6 of the benzene ring as revealed through the long-range correlations of H-7 to the carbons at C-5 (δ_C132.6) and C-1 (δ_C159.5), and H-8 to C-6 (δ_C126.8).

Based on these results, **1** was identified as a new dihydroxylated derivative of sterin B and designated as sterin C. Sterin B has been isolated from the fermentation broth of *S. hirsutum*.[15]

Superoxide radical-scavenging activity. Superoxide radical-scavenging activity of sterin C was compared with those of well-known antioxidants such as vitamin E and trolox (Fig. 3). Sterin C scavenged superoxide radical with EC_{50} value of 0.31 mM, which is higher than those of vitamin E (0.85 mM) and trolox (1.56 mM).

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References

1. Wulf, D. (2001) Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47-95.
2. Coyle, J. T. and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689-695.
3. Halliwell, B. and Gutteridge, J. M. C. (1989) Oxygen is a toxic gas-an introduction to oxygen toxicity and reactive oxygen species. In *Free radicals in biology and medicine* (3rd ed) pp. 1-35, Oxford University Press, NY, USA.
4. Hammond, B., Kontos, H. A. and Hess, M. L. (1985) Oxygen radicals in the adult respiratory distress syndrome, in myocardial ischemia and reperfusion injury, and in cerebral vascular damage. *Can. J. Physiol. Pharmacol*. **63**, 173-187.
5. Blois, M. S. (1958) Antioxidant determinations by the use of a stable free radical. *Nature* **181**, 1199-1200.
6. Tanaka, M., Shin-Ya, K., Furihata, K. and Seto, H. (1995) Isolation and structural elucidation of antioxidative substances, carbazoquinocins A to F. *J. Antibiot.* **48**, 326-328.
7. Shin-Ya, K., Hayakawa, Y. and Seto, H. (1993) Structure of benthophoenin, a new free radical scavenger produced by *Streptomyces prunicolor. J. Nat. Prod*. **56**, 1255-1258.
8. Shin-Ya, K., Furihata, K., Kato, Y., Hayakawa, Y., Clardy, J. and Seto, H. (1991) The structure of benthocyanin A, a new free radical scavenger of microbial origin. *Tetrahedron Lett*. **32**, 943-946.
9. Kim, W. G., Kim, J. W., Ryoo, I. J., Kim, J. P., Kim, Y. H. and Yoo, I. D. (2004) Boletunones A and B, highly functionalized novel sesquiterpenes from *Boletus calopus. Org. Lett*. **6**, 823-826.
10. Lee, I. K., Yun, B. S., Kim, J. P., Ryoo, I. J., Kim, Y. H. and Yoo, I. D. (2003) Neuroprotective activity of *p*-terphenyl leucomentins from the mushroom *Paxillus panuoides*. *Biosci. Biotechnol. Biochem*. **67**, 1813-1816.
11. Lee, I. K., Yun, B. S., Han, G. H., Cho, D. H., Kim, Y. H. and Yoo, I. D. (2002) Dictyoquinazols A, B, and C, new neuroprotective compounds from the mushroom *Dictyophora indusiata*. *J. Nat. Prod*. **65**, 1769-1772.
12. Yun, B. S., Lee, I. K., Kim, J. P. and Yoo, I. D. (2000) Curtisians A-D, new free radical scavengers from the mushroom *Paxillus curtisii. J. Antibiot*. **53**, 114-122.
13. Kim, J. P., Yun, B. S., Shim, Y. K. and Yoo, I. D. (1999) Inoscavin A, a new free radical scavenger from the mushroom *Inonotus xeranticus. Tetrahedron Lett.* **40**, 6643-6644.
14. Yun, B. S., Lee, I. K., Cho, Y. R., Cho, S. M. and Yoo, I. D. (2002) New tricyclic sesquiterpenes from the fermentation broth of *Stereum hirsutum. J. Nat. Prod.* **65**, 786-788.
15. Yun, B. S., Cho, Y. R., Lee, I. K., Cho, S. M., Lee, T. H. and Yoo, I. D. (2002) Sterins A and B, new antioxidative compounds from *Stereum hirsutum*. *J. Antibiot*. **55**, 208-210.
16. Amouzou, E., Ayer, W. A. and Browne, L. M. (1989) Antifungal sesquiterpenoids from an arthroconidial fungus. *J. Nat. Prod*. **52**, 1042-1054.
17. Ayer, W. A. and Saeedi-Ghomi, M. H. (1981) The sterepolides: new isolactaranes from *Stereum purpureum*. *Tetrahedron Lett*. **22**, 2071-2074
18. Xie, J. L., Li, L. P. and Dai, Z. Q. (1992) Isolation and

identification of two new metabolites from silver leaf fungus *Stereum hirsutum*. *J. Org. Chem*. **57**, 2313-2316.
19. Nair, M. S. R. and Anchel, M. (1977) Frustulosinol, an antibiotic metabolite of *Stereum frustulosum*: Revised structure of frustulosin. *Phytochem*. **16**, 390-392.
20. Bu'Lock, J. D., Kaye, B. and Hudson, A. T. (1971) New benzofurans from *Stereum subpileatum*, their biosynthesis, and related processes of aromatic aminoacid metabolism in a basidiomycete. *Phytochem*. **10**, 1037-1046.
21. Imazeki, R. and Hongo, T. (1989) In *Colored illustrations of mushrooms of Japan*, Vol. 2, Hoikusha Press, Osaka, Japan.
22. Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem*. **44**, 276-287.