Purification, Structure Determination and Biological Activities of 20(29)-lupen-3-one from *Daedaleopsis tricolor* (Bull. ex Fr.) Bond. et Sing.

Eun-Mi Kim, Hae-Ryong Jung, and Tae-Jin Min*

Department of Chemistry, College of Science, Dongguk University, Seoul 100-715, Korea
Received July 5, 2000

The dried fruit-body of *Daedaleopsis tricolor* was extracted by the petroleum ether. The extracts were purified by liquid-liquid extraction, column chromatography, and recrystallization. The purified compound was a colorless orthorhombic crystal form. Its melting point, molecular weight and molar extinction coefficient (ε) were estimated 168-170 °C, 424 and 3,935 at 208 nm, respectively. Its structure was elucidated to be 20(29)-lupen-3-one by UV-Vis, FT-IR, NMR and X-ray crystallographic analysis. It showed antifungal activities against *Saccharomyces cerevisiae* and *Microsporum gypseum*, and antibacterial activities against *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas pyocyanea*, *Bacillus subtilis*, and *Staphylococcus aureus*. In addition, this compound showed an antioxidative activity on lipid-peroxidation by 6.4%.

Keywords: Bioactive substance, *Daedaleopsis tricolor*.

Introduction

At present, the mushrooms grown naturally in Korea have been classified and identified[1] on 992 species. Among these, it has been reported that 100 species are edible, and 50 species are toxic. The thirty five families 82 genera 162 species are used for the traditional medicine.[2] The biochemical properties of these bioactive substances and their physiological effects including anticholesterol, antitumor, antibiotics, antihypertensive and antihyperglycemia have been well documented.[3] However, the components of Korean mushrooms have not been well studied. In order to develop the bioactive substances, 144 species of mushrooms have been screened.[4-10] Our previous work showed that *Daedaleopsis tricolor* had uncovered the strong antibiotic activities against fungi, yeasts and bacteria.

Daedaleopsis tricolor belonging to the polyporaceae family has been known as an annual white rotting fungus, and the fruit-body consisted of leathery. This mushroom has been grown in the wilderness throughout the world. However, the study on the bioactive substance of this mushroom have not been reported.

In this work, we have isolated and purified antibioactive substance from the fruit body of *Daedaleopsis tricolor* and determined the structure by UV-vis, FT-IR, NMR spectroscopy and X-ray crystallography. In addition, the antibiotic and antioxidant effect of the compound have been investigated.

Experimental Section

Materials. Fruiting body of *Daedaleopsis tricolor* used was collected from Mt. Kwanak. Silica gel (70-230 and 230-400 mesh) and thin layer chromatography (TLC) plate (silica gel 60 F_{254}) were purchased from Merck Co. Sabouraud agar and mueller-hinton agar were purchased from Difco Co. α-Tocopherol, thiobarbituric acid (TBA), trichloroacetic acid (TCA) and L-ascorbic acid were purchased from Aldrich Co. Bovine serum albumin (BSA), butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) were purchased from Sigma. Co. All the other reagents were used of analytical grade. All solvents were freshly distilled prior to use.

Instruments. Melting points were determined on a Melt-Temp Lab. devices and are uncorrected. UV-Vis spectra were recorded on a HP-8452 spectrophotometer in the chloroform. FT-IR spectra were recorded on a Bomem series 100 spectrophotometer using KBr discs. Mass spectra were determined on a Micromass Trio-2000 spectrometer (ionization voltage 70 eV) using direct inlet system. ^1H and ^{13}C NMR spectra were recorded on a Varian spectrometer (^1H 200 MHz and ^{13}C 50 MHz) in the $CDCl_3$ with TMS as an internal standard. The crystal structure was measured on a MAC sciences MXC3 fourcircle diffractometer.

Extraction and purification of bioactive substances. The air dried and powdered mushroom (12.8 kg) was extracted by petroleum ether (18 L) for one week at room temperature and then the extract was evaporated. This crude extract (32.2 g) was successively extracted with methanol, distilled water : diethyl ether (1 : 3, v/v) and acetonitrile. Methanol fraction was applied to silica gel column (3 × 60 cm) chromatography using *n*-hexane : chloroform : methanol : ammonia water (5 : 6 : 30 : 5, v/v) as eluent. The fraction with antibiotic activity was repeatedly applied to silica gel column (1.5 × 60 cm) chromatography using *n*-hexane : chloroform (10 : 2, v/v) as eluent. The fraction II out of five fractions was purified by recrystallization with ethanol. The purity of this compound was determined by one and two dimensional thin-layer chromatography using mixed solvent of *n*-hexane : chloroform : acetonitrile (30 : 10 : 1 v/v) and petroleum ether : chloroform : methanol (30 : 10 : 1 v/v), re-

*To whom correspondence should be addressed. Tel: +82-2-2260-3211; Fax: +82-2-2268-8204; e-mail: tjmin@dgu.ac.kr

spectively. The purified compound was obtained by 150 mg as needle form of colorless. m.p.: 168-170 °C; UV ($CHCl_3$) λ_{max}: 208 nm (ε 3.935); IR (KBr) cm^{-1}: 870, 1,382, 1,644, 1,705, 2,942, 3,071; EI-MS (70 eV) m/z (relative intensity): 108 (100), 368 (1.55), 409 (3.68), 424 (6.12); ^{13}C-NMR ($CDCl_3$): 39.7 (C-1), 34.1 (C-2), 218.0 (C-3), 47.4 (C-4), 55.1 (C-5), 19.8 (C-6), 33.7 (C-7), 40.9 (C-8), 49.9 (C-9), 37.0 (C-10), 21.6 (C-11), 25.3 (C-12), 38.3 (C-13), 43.0 (C-14), 27.6 (C-15), 35.6 (C-16), 43.1 (C-17), 48.4 (C-18), 48.1 (C-19), 150.9 (C-20), 29.9 (C-21), 40.1 (C-22), 26.8 (C-23), 21.1 (C-24), 15.9 (C-25), 16.0 (C-26), 14.6 (C-27), 18.1 (C-28), 109.5 (C-29), 19.4 (C-30); ^1H-NMR ($CDCl_3$): 2.41 (1H, m, H-19), 1.06 (3H, s, H-23), 0.93 (6H, s, H-24, H-25), 0.95 (3H, s, H-26), 1.02 (3H, s, H-27), 0.79 (3H, s, H-28), 4.57 (1H, d, J = 2 Hz, H_a-29), 4.69 (1H, d, J = 2 Hz, H_b-29), 1.68 (3H, s, H-30); molecular formula : $C_{30}H_{48}O$ and M.W : 424.

Antibiotic assay. The antibiotic activities of this compound were screened *in vitro* by the disk diffusion method.[11] We used microorganism which were strain of *Candida albicans* and three others belong to yeasts, *Aspergillus flavus* and five others belong to fungi, *Escherichia coli* and four others belong to gram-negative bacteria, and *Bacillus cereus* and five others belong to gram-positive bacteria. Filter paper discs, 8 mm in diameter (Advantec, Toyo), were used for this compound. Loading concentration of compound purified was 500 μg/disc. Petri dishs were incubated at 25 °C for yeasts and fungi, and 37 °C for bacteria. Inhibition zone were observed after 24 h of growth.

Antioxidant assay. To measure the antioxidant activity of purified compound, lipid peroxidation of rat liver microsomes was carried out *in vitro* according to the Fe^{2+}/ascorbate method.[12,13] Microsomes of liver cell were prepared by differential centrifugation.[14] The reaction mixture for induction of lipid peroxidation containing 0.5 mL of rat liver microsome (1 mg/mL), 0.5 mL of 10 μM $FeSO_4$, 0.5 mL of 2 mM ascorbate, 0.1 mL of the compound purified (0.706 μmol) in DMSO, and 0.4 mL of 150 mM KCl/50 mM Tris-HCl buffer solution (pH 7.4) was incubated at 37 °C for 60 min in a shaking water bath and the reaction was stopped by addition of 0.75 mL of 2.0 M TCA/1.7 M HCl. After centrifugation (4,000 rpm, 10 min), 0.5 mL of the supernatant was mixed with 1.5 mL of 0.67% TBA and the mixture was heated at 95 °C for 10 min. After cooling, the quantity of malondialdehyde (MDA, $\varepsilon = 1.52 \times 10^5\ M^{-1}cm^{-1}$) was determined by measuring the absorbance at 533 nm. The foregoing, all operation was carried out below 4 °C and protein concentration was determined according to Lowry method[15] using BSA as a standard.

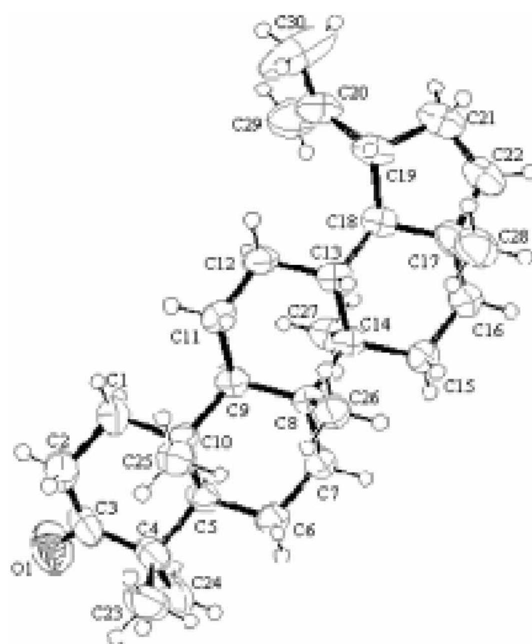

Figure 1. The molecular conformation by X-ray crystallography of 20(29)-lupen-3-one purified from *D. tricolor*.

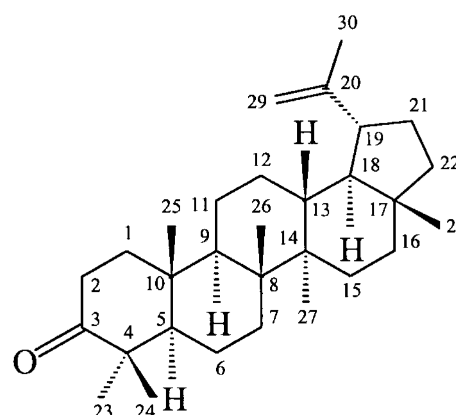

Figure 2. The structure of 20(29)-lupen-3-one purified from *D. tricolor*.

Results and Discussion

The structure of compound purified from *D. tricolor*. The compound purified from *D. tricolor* was colorless single crystal of needle form. In order to classify to this compound, color reactions with various spray reagents[16] on TLC plate were investigated. This compound showed negative response to Dragendroff and ninhydrin test for nitrogen containing compound, while positive response to sulfuric acid, I_2-vapor, anisaldehyde-sulfuric acid, antimony chloride(III)-acetic acid and tungstophosphoric acid test, respectively. The purified compound from the results was classified for steroid or terpene. Melting points of the purified compound were 168-170 °C [lit. (17), 168-170.5 °C]. The UV spectrum of this compound showed strong absorption band at 208 nm[molar extinction coefficient (ε)=3.935]. The IR spectrum indicated the presence of C=O (1705 cm^{-1}), C=C (1644 cm^{-1}), C-H ($CR_1R_2=CH_2$, 3071, 870 cm^{-1}) and C-H (2942, 1382 cm^{-1}) bond [lit. (18), C=O (1709 cm^{-1}), $CH=CH_2$ (1642, 882 cm^{-1})]. In its EI/MS spectrum m/z 424, 409 and 368 correspond to $[M]^+$, $[M-CH_3]^+$ and $[M-CH_3-CH_2=C-CH_3]^+$, respectively. The ^{13}C-NMR spectrum[18,19] showed carbon signal of C=O at δ 218.04 (C-3) and C=C at δ 150.96 (C-20), δ 109.47 (C-29). Down field signals at δ 4.57, δ 4.69 (1H, each d, J = 2 Hz, H_a-29, H_b-29) in the ^1H-NMR

Table 1. The Structural data by X-ray crystallography of 20(29)-lupen-3-one purified from *D. tricolor*

Characters	Data
Empirical formular	$C_{30}H_{48}O$
Formular weight	424.7
Space group	$P2_12_12_1$
Crystal system	Orthorhombic
a (Å)	0.0041
b (Å)	0.0055
c (Å)	0.0034
α (deg)	90
β (deg)	90
γ (deg)	90
V (Å^3)	257.73
Z	8
T (K)	293
λ (Mo Kα) (Å)	0.7107
μ (mm^{-1})	0.06
Scan type	ω - 2θ
wR_2	0.1407
R_1	0.0537

Table 2. Selected bond lengths and angles by X-ray crystallography of 20(29)-lupen-3-one purified from *D. tricolor*

Selected bond lengths (Å)		Selected bond angles (deg)	
C2 - C3	1.4949 (2)	C2 - C3 - C4	118.34 (2)
C3 - O1	1.2223 (2)	C2 - C3 - O1	119.83 (2)
C2 - C4	1.5180 (2)	O1 - C3 - C4	121.83 (2)
C19 - C20	1.4992 (2)	C19 - C20 - C29	124.27 (2)
C20 - C29	1.3639 (2)	C19 - C20 - C30	115.27 (2)
C20 - C30	1.4476 (2)	C29 - C20 - C30	120.21 (2)

spectrum[18] revealed the presence of disubstitued double bond. Other characteristic signals in ^{1}H-NMR spectrum were δ 2.41 (1H, m, H-19), δ 0.79 (3H, s, H-28), δ 0.93 (6H, s, H-24, H-25), δ 0.95 (3H, s, H-26), δ 1.02 (3H, s, H-27), δ 1.06 (3H, s, H-26) and δ 1.68 (3H, s, H-30). The crystal structure of this compound was shown in Figure 1. The structure was found to be orthorhombic which consisted of three different sides and three of 90° angle. The crystallographic data are listed in Table 1. The selected bond lengths and angles are shown in Table 2. The bond length of 1.2223 Å and 1.3639 Å were showed the presence of C=O (C3-O1) and C=C (C20-C29), respectively. X-ray crystallogrphic data agreed with the study[20] of Dampawan *et al.* From the analysis of spectroscopic data, this compound was established to be 20(29)-lupen-3-one with molecular formular of $C_{30}H_{48}O$ (Figure 2).

Table 3. Antibiotic activities of 20(29)-lupen-3-one purified from *Daedaleopsis tricolor*

Microorganism		Activity
Yeasts	*Candida albicans*	−
	Cryptococcus neoformans	−
	Saccharomyces cerevisiae	+
	Trichosporon beigelii	−
Fungi	*Aspergillus flavus*	−*
	Aspergillus niger KCTC 2025	−*
	Aspergillus versicolor	−
	Microsporum canis	−
	Microsporum gypseum	+
	Trichophyton mentagrophytes	−
Gram-negative bacteria	*Escherichia coli*	+
	Proteus vulgaris	+
	Providencia rettigeri	−
	Pseudomonas pyocyanea	+
	Serratia marcescens	−
Gram-positive bacteria	*Bacillus cereus*	−
	Bacillus subtilis	+
	Micrococcus leteus	−
	Mycobacterium fortuitum	−
	Staphylococcus epidermidis	−
	Staphylococcus aureus	+

Disc diameter; 8 mm. Loading concentration: 500 μg/disc. +: Inhibition, −: No inhibition. * The growth of spore in fungus by 20(29)-lupen-3-one has been stimulated.

Antibiotic activity of 20(29)-lupen-3-one in *D. tricolor*. Antibiotic activity of 20(29)-lupen-3-one was shown in Table 3. This compound showed antifungal activities against *Saccharomyces cerevisiae* and *Microsporum gypseum*. However, in the case of *Aspergillus flavus* and *Aspergillus niger*, the growth of spore by this compound have been rather stimulated than inhibited. The reason is planning to research in the future. This compound showed antibacterial activities against *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas pyocyanea*, *Bacillus subtilis* and *Staphylococcus aureus*. This results agreed with the study[21] of Goyal and Rani which antibacterial activities of 20(29)-lupen-3-one. Thus, the present results indicated that the compound from *D. tricolor* is expected to be effective as a natural antibiotics.

Antioxidant activity of 20(29)-lupen-3-one in *D. tricolor*. Antioxidant activity of 20(29)-lupen-3-one was pre-

Table 4. Antioxidant activity of 20(29)-lupen-3-one purified from *Daedaleopsis tricolor* compare with known antioxidants

Compound	Concentration (μmol)	MDA (μmol/mg microsome)	Relative production (%)
Control	−	4.25	100
20(29)-Lupen-3-one	0.706	3.98	93.6
α-Tocopherol	0.706	3.90	91.8
BHT (Buthylated hydroxy toluene)	0.706	1.76	41.4
BHA (Buthylated hydroxy anisole)	0.706	0.74	17.4

sented in Table 4. This compound exhibited 6.4% inhibition of lipid peroxidation at the concentration of 0.706 μmol. Antioxidant activity of 20(29)-lupen-3-one was compared with α-tocopherol, BHT and BHA as well-known antioxidants. This compound showed similar inhibition to α-tocopherol by 8.2%, while less activity than BHT and BHA.

20(29)-lupen-3-one has been previously isolated and identified from whole plants[18] of *Tephrosia villosa*, bark[22] of *Firmiana platanifolia*, and root[23] of *Adenophora axilliflora*. However, this compound was identified in this study from mushroom for the first time. And also, study on bioactive substances of this mushroom have not been reported. This compound exhibited broad spectrum of antibiotic activity against yeasts, fungi and bacteria. Antioxidant activity of this compound show similar effect to α-tocopherol using far and wide as cometics and nutrient. Therefore, 20(29)-lupen-3-one is considered to be as antibiotic, antioxidant and its model compound. However, since the experiment in this study have been carried out *in vitro*, further work is required to determine antibiotic and antioxidant activity of the compound *in vivo* system.

Acknowledgment. The author wish to acknowledge the financial support of the Korea Research Foundation made in the Basic Science Research Program (1998-015-D00202) year of 1998.

References

1. Jung, H. S. *The Kor. Soc. Mycol. News Letter* **1993**, *5*(1), 29.
2. Ahn, D. K. *Kor. J. Mycol.* **1992**, *20*(2), 154.
3. Mizuno, T. *Mushroom Chemistry and Biochemistry*; Hakhoi press center: Tokyo, Japan, 1992; p 13.
4. Min, T. J.; Kim, E. M.; Lee, S. J.; Bae, K. G. *Kor. J. Mycol.* **1995**, *23*(1), 14.
5. Park, S. S.; Lee, K. D.; Min, T. J. *Kor. J. Mycol.* **1995**, *23*(1), 28.
6. Lee, K. D.; Su, Y. C.; Park, S. S.; Min, T. J. *Kor. J. Mycol.* **1995**, *23*(1), 37.
7. Yoon, J. O.; Min, T. J.; Yoon, H. S. *Kor. J. Mycol.* **1995**, *23*(1), 46.
8. Park, S. S.; Lee, K. D.; Min, T. J. *Kor. J. Mycol.* **1995**, *23*(2), 176.
9. Min, T. J.; Kim, E. M.; You, S. H. *Kor. J. Mycol.* **1996**, *24*(1), 25.
10. Min, J. Y.; Kim, E. M.; Min, T. J. *Kor. J. Mycol.* **1996**, *25*(4), 354.
11. Bauer, A. W.; Kirby, W. M. M.; Sherris, J. C.; Turck, M. *Am. J. Clin. Pathol.* **1966**, *45*(4), 493.
12. Placer, Z. A.; Cushman, L. L.; Johxon, B. C. *Anal. Biochem.* **1966**, *16*, 359.
13. Kim, S. W.; Park, S. S.; Min, T. J.; Yu, K. H. *Bull. Korean Chem. Soc.* **1999**, *20*(7), 819.
14. Plummer, D. T. *An Introduction to Practical Biochemistry*, 3rd ed.; McGRAW-HILL Co.: London, UK, 1987; p 268.
15. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
16. Stahl, E. *Thin-Layer Chromatography*, 2nd ed.; Springer-Verlag: Singapore, 1988; p 855.
17. Djerassi, D. *Dictionary of Natural Products*; Buckingham, J., Ed.; Chapman & Hall: London, UK, 1992; Vol. 6, p 3698.
18. Prashant, A.; Krupadanam, G. L. D. *Phytochemistry* **1993**, *32*(2), 484.
19. Dantanarayana, A. P.; Kumar, N. S.; Muthukuda, P. M.; Wazeer, M. I. *Phytochemistry* **1982**, *21*(8), 2065.
20. Dampawan, P.; Huntrakul, C.; Reutrakul, V.; Raston, C. L.; White, A, H. *J. Sci. Soc. Thailand* **1977**, *3*, 14.
21. Goyal, M. M.; Rani, K. K. *Acta Ciencia Indica* **1989**, *15C*(2), 117.
22. Tanabe, Y.; Ogura, K.; Sakai, S.; Takahashi, K. *Yakugaku zasshi* **1964**, *84*(9), 887.
23. Du, S. J.; Gariboldi, P.; Jommi, G. *Planta Medica* **1986**, 317.