J. Antibiot. 61(9): 573–576, 2008

NOTE

THE JOURNAL OF ANTIBIOTICS

A New Antioxidant, Clitocybin A, from the Culture Broth of *Clitocybe aurantiaca*

Young-Hee Kim, Sung-Min Cho, Jin-Won Hyun, In-Ja Ryoo, Soo-Jin Choo, Sangku Lee, Soon-Ja Seok, Jae Sung Hwang, Eui Dong Sohn, Bong-Sik Yun, Ki Hwan Bae, Ick-Dong Yoo

Received: June 20, 2008 / Accepted: August 28, 2008

Abstract Clitocybin A (**1**), a new antioxidant, was isolated from the culture broth of *Clitocybe aurantiaca*. This compound was purified by solvent extraction, silica gel column chromatography, Sephadex LH-20 column chromatography and preparative HPLC. Its structure was determined as 4,6-dihydroxy-2-*ρ*-hydroxyphenyl-isoindol-1-one on the basis of the UV, NMR, and MS spectroscopic analysis. The compound **1** showed potent free radical scavenging activity against superoxide, ABTS, and DPPH radicals, and protective effect against cellular DNA damage induced by oxidative stress.

Keywords clitocybin A, *Clitocybe aurantiaca*, antioxidant activity, comet assay

Free radicals are considered to induce oxidative damage in biomolecules and to play an important role in aging, cardiovascular diseases, cancer, and inflammatory diseases [1]. In addition, they are also well known to be major causes of material degradation and food deterioration. Consequently, antioxidants are now known to be protective or therapeutic agents [2]. In the past few years, the addition of synthetic antioxidants has begun to be restricted, because of their health risks and toxicity. The importance of exploiting natural antioxidants from various sources and replacing synthetic antioxidants with natural ingredients has attracted increasing attention. Most natural antioxidants such as traditional nutrients, polyphenols, and flavonoids are obtained from plants. Recently, mushrooms have been reported to harbor a host of bioactive substances [3, 4]. The study of known and new natural derivatives in higher fungi might also support the development of new drugs, as well as health-promoting substances.

In the course of our screening program for free radical scavengers, we isolated a novel compound, clitocybin A (**1**, Fig. 1), from the culture broth of *Clitocybe aurantiaca* [5, 6]. In this paper, we report upon the isolation, structure elucidation and biological activities of **1**. We were provided this strain from the staff of mushroom taxonomy laboratory at the National Institute of Agricultural Science and Technology.

The producing strain, *C. aurantiaca*, was cultivated in a producing medium consisting of 2.0% glucose, 0.5% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 0.6∼5.8) for 7 days at 28°C on a reciprocal shaker. The whole cultured broth (3.0 liters) was

I.-D. Yoo (Corresponding author), **Y.-H. Kim, S.-M. Cho, I.-J. Ryoo, S.-J. Choo, S. Lee, B.-S. Yun:** Functional Metabolomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305–806, Korea,
E-mail: idyoo@kribb.re.kr
J.-W. Hyun: College of Medicine, Cheju National University, Jeju 690–756, Korea
S.-J. Seok: National Institute of Agricultural Science and Technology, RDA, Suwan 441–707, Korea
J.-S. Hwang, E.-D. Sohn: Amorepacific Corporation R&D Center, Giheung, Gyeonggi, 442–729, Korea
K.-H. Bae: Department of Pharmacy, Chungnam National University, Daejeon 305–764, Korea

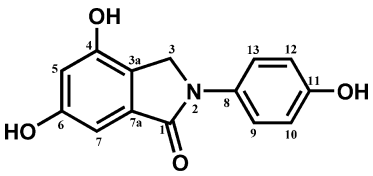

Fig. 1 Structure of clitocybin A.

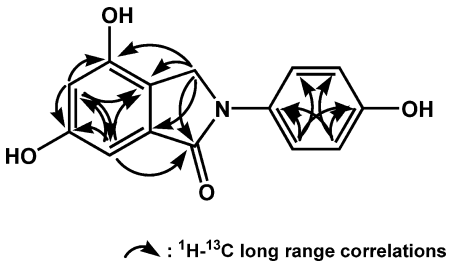

Fig. 2 HMBC correlations of clitocybin A.

sequentially extracted with *n*-hexane and EtOAc. The EtOAc layer was concentrated and applied to a column of silica gel eluted with MeOH - $CHCl_3$ (1 : 50～1 : 1, v/v). The active eluate was then chromatographed on a Sephadex LH-20 column eluted with MeOH. Finally, **1** was obtained by HPLC using a YMC-pack ODS-A column (4.6 mm i.d.×150 mm) eluted with 30% MeOH.

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as $C_{14}H_{11}NO_4$ by high-resolution ESI-mass measurement. IR absorptions at 3399 and 1616 cm^{-1} implied the presence of hydroxyl groups and amide moieties, respectively. A heteronuclear multiple-quantum coherency (HMQC) experiment established all one-bonded ^{1}H-^{13}C connectivity, as shown in Table 2. The ^{1}H-NMR spectrum of **1** displayed signals for one methylene proton and six aromatic protons. The ^{13}C-NMR spectrum of **1** revealed the presence of 14 carbons comprised of one sp^3 methylene, six sp^2 methines and seven quaternary carbons including an amide carbonyl and three oxygenated sp^2 carbons. The HMBC spectrum of **1** revealed two partial structures, isoindolone moiety and 1,4-disubstituted benzene group, as shown in Fig. 2. In the isoindolone moiety, a methylene protons at δ 4.71 showed long range correlations to C-1 (δ_C 172.4), C-3a (δ_C 120.6), C-4 (δ_C 155.4) and C-7a (δ_C 136.4). In addition, the long-range correlations from H-5 to C-3a (δ_C 120.6), C-4 (δ_C 155.4), C-6 (δ_C 162.2), and C-7 (δ_C 102.0), and from H-7 to C-1 (δ_C 172.4), C-3a (δ_C 120.6), C-5 (δ_C 108.0) and C-6 (δ_C 162.2) were observed. The long-range correlations from methine proton at δ 7.54 to C-8 (δ_C 133.9), C-11 (δ_C 157.9) and C-13 (δ_C 124.7), and from methine proton at δ 6.84 to C-8 (δ_C 133.9), C-11 (δ_C 157.9) and C-12 (δ_C 117.6) revealed the presence of a 1,4-disubstituted benzene moiety. Based on the above HMBC experiment, **1** was assigned as a new compound of isoindolone class.

The antioxidant activity of **1** was evaluated by measuring its free radical scavenging effects using three different assays, the superoxide radical anion scavenging activity assay, ABTS (2,2′-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid]) radical cation decolorization assay, and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (Table 3). The superoxide anion scavenging activity

Table 1 Physico-chemical properties of clitocybin A

	Clitocybin A
Appearance	Pale brown powder
$[\alpha]_D^{20}$	−1.40° (*c* 0.3, MeOH)
Molecular formular	$C_{14}H_{11}NO_4$
Molecular weight	257
HR-ESI-MS *m*/*z*	
Found	258.07571 ($M+H^+$)
Calcd.	258.07608
UV λ_{max}^{MeOH} nm (log ε)	211 (1.6), 286 (0.4)
IR ν_{max} (KBr) cm^{-1}	3399, 2925, 1616, 1516, 1453, 1256, 1144, 1097
Soluble	MeOH, DMSO
Insoluble	*n*-hexane, H_2O

Table 2 ^{1}H- and ^{13}C-NMR data of clitocybin A in methanol-d_4

Position	δ_C	δ_H
1	172.4	
3	50.3	4.71 (s)
3a	120.6	
4	155.4	
5	108.0	6.50 (d, *J*=1.8 Hz)
6	162.2	
7	102.0	6.71 (d, *J*=1.8 Hz)
7a	136.4	
8	133.9	
9, 13	124.7	7.54 (d, *J*=9.0 Hz)
10, 12	117.6	6.84 (d, *J*=9.0 Hz)
11	157.9	

Chemical shift in ppm from TMS as internal standard.
^{1}H- and ^{13}C-NMR were measured at 600 and 150 MHz, respectively.

Table 3 Free radical scavenging activities of clitocybin A (IC_{50} μM)

Compound	Superoxide radical[a]	ABTS radical[b]	DPPH radical[c]
Clitocybin A	10.3±1.8	6.4±0.2	>100
Catechin	16.0±0.9	5.6±0.2	57.5±2.5
Ferulic acid	48.4±2.6	7.1±0.1	>100

[a] Xanthine/xanthine oxidase.
[b] 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid).
[c] 2,2-Diphenyl-1-picrylhydrazyl.
In all three assays, results percentaged as the mean (n=3)±SD.

Table 4 Protective effect of clitocybin A on DNA damage induced by H_2O_2

Group	DNA tail (%)*
Control	5.7±0.7
H_2O_2	61.1±2.4
Clitocybin A[a]	7.0±0.8
Clitocybin A[a]+H_2O_2	35.4±2.6
N-Acetylcysteine[b]+H_2O_2	16.2±1.3

[a] Concentration of clitocybin A was at 10 μg/ml.
[b] Concentration of *N*-acetylcysteine was at 2.0 mM.
* Results percentaged as the mean (n=3)±SE.

was evaluated by the xanthine/xanthine oxidase method [7]. In brief, each well of a 96-well plate containing 100 μl of the following reagents: 50 mM potassium phosphate buffer (pH 7.8), 1.0 mM EDTA, 0.04 mM NBT (nitroblue tetrazolium), 0.18 mM xanthine, 250 mU/ml xanthine oxidase, and the sample at various concentrations was incubated for 30 minutes at 37°C in the dark. The xanthine oxidase catalyzes the oxidation of xanthine to uric acid and superoxide, and the superoxide reduces NBT to blue formazan. The reduction of NBT to blue formazan was measured at 560 nm in a microplate reader. As a result, **1** exhibited potent superoxide radical scavenging activity with an IC_{50} value of 10.3 μM, which was comparable to those of well-known antioxidants, catechin and ferulic acid, used as a control.

The ABTS radical scavenging activity was measured using the ABTS radical cation decolorization assay with minor modifications [8]. ABTS was dissolved in water to a concentration of 7.0 mM. The $ABTS^{\cdot+}$ cation radical was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate and by allowing the mixture to stand in the dark for 12 hours. After adding 0.1 ml of the ABTS radical cation solution to 5.0 μl of the antioxidant compounds in EtOH, the absorbance was measured by an ELISA reader at 734 nm after mixing for up to 6 minutes. The compound **1** exhibited a significant ABTS radical cation scavenging effect with an IC_{50} value of 6.4 μM.

To investigate the scavenging effect on the DPPH radicals, **1** at various concentrations was added to 95 μl of 150 μM DPPH EtOH soln, the mixture was incubated for 20 minutes at room temperature, and the absorbance was measured at 517 nm using an ELISA reader [9]. The compound **1** showed no activity against the DPPH radical.

The ability of **1** to inhibit cellular DNA damage in H_2O_2 treated cells was investigated by the comet assay, which assesses oxidative DNA damage [10, 11]. The cells used in this experiment was Chinese hamster lung fibroblast cells (V79-4). Concentration of H_2O_2 was 1.0 mM. The cell pellets were applied to electrophoresis, stained with ethidium bromide and observed using a fluorescence microscope and image analysis. The percentage of total fluorescence in the tail and the tail length of 50 cells per slide were recorded. The comet assay showed that the exposure of the cells to H_2O_2 increased their tail length to 61.1, whereas their treatment with **1** resulted in a decrease of their tail length to 35.4 as shown in Table 4. This data suggested that **1** had a protective effect against cellular DNA damage induced by oxidative stress.

Acknowledgment This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A-050432).

References

1. Palinski W, Rosenfeld ME, Yla HS, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Careow TE, Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification *in vivo*. Proc Natl Acad Sci USA 86: 1372–1376 (1989)
2. Solak MH, Kalmis E, Saglam H, Kalyoncu F. Antimicrobial activity of two wild mushrooms *Clitocybe alexandri* (Gill.) Konr. and *Rhizopogon roseolus* (Corda) T.M. Fries collected from Turkey. Phytother Res 20: 1085–1087 (2006)
3. Yoo NH, Kim JP, Yun BS, Ryoo IJ, Lee IK, Yoon ES, Koshino H, Yoo ID. Hirsutenols D, E and F, new sesquiterpenes from the culture broth of *Stereum hirsutum*. J Antibiot 59: 110–113 (2006)
4. Kim YH, Yun BS, Ryoo IJ, Kim JP, Koshino H, Yoo ID. Methoxylaricinolic acid, a new sesquiterpene from the fruiting bodies of *Stereum ostrea*. J Antibiot 59: 432–434 (2006)
5. Arnone A, Colombo A, Nasini G, Meille SV. Secondary mold metabolites. Part 42. Eleganthol, a sesquiterpene from

Clitocybe elegans. Phytochemistry 32: 1493–1497 (1993)
6. Bradshaw AP, Hanson JR, Hitchcock PB. 4α-Hydroxydihydroilludin M. A new sesquiterpenoid metabolite of *Clitocybe illudens*. Phytochemistry 21: 942–943 (1982)
7. Matsushige K, Basnet P, Kadota S, Namba T. Potent free radical scavenging activity of dicaffeoyl quinic acid derivatives from propolis. J Trad Med 13: 217–228 (1996)
8. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med 26: 1231–1237 (1999)
9. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 181: 1199–1200 (1958)
10. Singh NP. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. Mutat Res 455: 111–127 (2000)
11. Rajagopalan R, Ranjan SK, Nair CK. Effect of vinblastine sulfate on gamma-radiation-induced DNA single-strand breaks in murine tissues. Mutat Res 536: 15–25 (2003)