Phellifuropyranone A: A New Furopyranone Compound Isolated from Fruit Bodies of Wild *Phellinus linteus*

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A new furopyranone, phellifuropyranone A, was isolated from fruit bodies of wild *Phellinus linteus* as well as phelligridin G, and their chemical structures were determined by various spectroscopic methods including measurement of NMR spectra. Phellifuropyranone A together with meshimakobnol A and meshimakobnol B showed antiproliferative activity against mouse melanoma cells and human lung cancer cells *in vitro*.

Key words *Phellinus linteus*; fruit body; meshimakobnol A; meshimakobnol B; phellifuropyranone A; antiproliferative activity

Phellinus linteus (BERK. *et* CURT) Aoshima ("meshimakobu" in Japanese) is a Basidomycota fungus which has been reported to have anticancer activity derived from its polysaccharide constituents.[1,2] In Japan, many kinds of dietary supplements containing *P. linteus* are available in the markets. These commercial products are prepared from either fruit bodies of the wild or cultivated mushrooms, the cultured mycelia or their mixtures and have been used for the same purpose irrespective of their origins. It may be possible that chemical constituents are different among *P. linteus* preparations produced from the different origins, which may lead to difference in pharmacological activities.

No report has described small molecule organic compounds in *P. linteus* and none has focused on the chemical difference among fruit bodies of the wild and cultivated mushrooms and the cultured mycelia. In a previous paper[3] we identified meshimakobnol A (**1**) and meshimakobnol B (**2**) for the first time from the fruit body of wild *P. linteus* (Fig. 1) and suggested that these compounds may be used as indicative constituents for *P. linteus* product produced from the wild fruit body. In continuation of our investigation on low molecular weight components in *P. linteus*, we further isolated one new furanopyranone compound, phellifuropyranone A (**3**), together with one known compound phelligridin G (**4**) from the fruit body of wild *P. linteus* in addition to **1** and **2**, and determined their chemical structures by mesurement of IR, UV, FT-MS, and 1D- and 2D-NMR spectra. Furthermore, the antiproliferative activities of phellifuropyranone A (**3**), meshimakobnol A (**1**) and meshimakobnol B (**2**) against mouse melanoma cells and human lung cancer cells were evaluated.

Results and Discussion

The compound **3** was obtained as dark yellow amorphous powder. The molecular formula of **3** was given as $C_{21}H_{14}O_7$ from the HR-ESI-FT-ICR MS spectrum (*m*/*z* 401.0632 $[M+Na]^+$, Calcd for $C_{21}H_{14}O_7Na$ 401.0632). In the UV spectrum of **3**, the absorption maxima were observed at λ_{max} 400 ($\log \varepsilon = 3.8$) and 314 nm (3.6), which indicated long conjugation system. The IR spectrum of **3** showed the absorption bands at 1700 and 3418 cm^{-1} ascribable to carbonyl and hydroxyl, respectively. In the ^{1}H-NMR spectra of **3**, the signals of two ABX systems of tri-substituted benzene rings [δ_H 7.13 (H-8′), δ_H 7.23 (H-7′) and δ_H 7.55 (H-4′), and δ_H 7.30 (H-5″), δ_H 7.40 (H-6″) and δ_H 7.77 (H-2″)], a *trans*-olefine doublet signals [δ_H 6.81 (H-1′) and δ_H 7.59 (H-2′), $J = 15.9$ Hz], and two singlet aromatic protons [δ_H 6.66 (H-7) and δ_H 7.25 (H-3)] were observed (Table 1). All signals in the ^{13}C-NMR spectrum were sp^2 carbons, and 10 of them were methine and the other 11 were quarternary carbons. The lowest carbon signal was that at δ_C 160.9 (C-7a), which suggested the presence of carbonyl group of the α-pyrone. For the next step, measurements of FG-HMQC and FG-HMBC were carried out. It was assumed that the two trisubstituted benzene rings were 3,4-dihydroxyphenyl groups from the correlation from H-4′ to C-8′, H-7′ to C-3′ and C-5′ (δ_C 147.8), from H-8′ to C-4′ and C-6′ (δ_C 149.5, overlapped with pyridine), from H-2″ to C-3″ (δ_C 147.9), C-4″ (δ_C 148.9) and C-6″, from H-5″ to C-1″ and C-3″, from H-6″ to C-4″, and from the chemical values of C-5′, C-6′, C-3″ and C-4″. As shown in Fig. 2, the correlation from H-1′ to C-3′ (δ_C 128.4), from H-2′ to C-4′ (δ_C 115.2) and C-8′ (δ_C 121.0), from H-4′ to C-2′ (δ_C 134.6) and from H-8′ to C-2′, indicated that the one of dihydroxyphenyl groups was combined

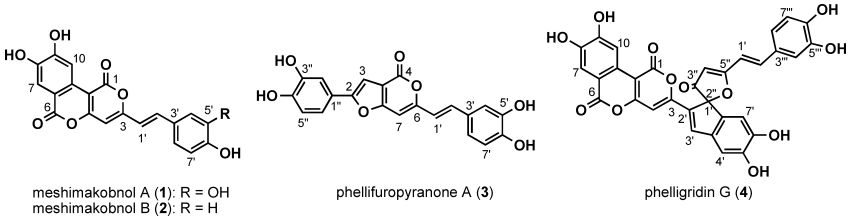

Fig. 1. Chemical Structures of Meshimakobnol A (**1**), Meshimakobnol B (**2**), Phellifuropyranone A (**3**) and Phelligridin G (**4**)

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to *trans*-olefine. The other 3,4-dihydroxyphenyl group was suggested to bind to olefine carbon combined with oxygen by HMBC correlations from the protons of H-2″ and H-6″ to C-2 (δ_C 157.0). The conjugation of the *trans*-olefine with pyrone ring was showed from the observations of HMBC correlations from one singlet proton of H-7 to the carbons of C-3a (δ_C 111.9), C-1′ (δ_C 116.9), C-6 (δ_C 157.3) and C-7a, and from the olefine protons at H-1′ and H-2′ to C-6. The furo[3,2-*c*]pyran-4-one moiety combined with 3,4-dihydroxy phenyl group at C-2 was estimated by the observation of HMBC correlation from the other singlet proton of H-3 to C-2, C-3a and C-7a, and from the phenyl protons of H-6″ and H-2″ to C-2, and by the spectroscopic results mentioned above. The chemical shift value of H-3 was relatively lower field than that of usual furan, which suggested the effect of long conjugation and the influence from the carbonyl carbon of C-4 (δ_C 158.8). Although no HMBC correlations was observed from any protons to δ_C 158.8, the furopyranone ring structure of **3** was also supported by the chemical shifts of C-2, C-3a, C-4, C-6, C-7 and C-7a, which were in good agreement with the corresponding ones of other furopyranone compounds such as inoscavin C or methylinoscavin C.[4] Thus, we determined the structure of the compound **3** as new compound, 2-(3,4-dihydroxyphenyl)-6-(2′-(3,4-dihydroxyphenyl)-*E*-ethenyl)-furo[3,2-*c*]pyran-4-one and named phellifuropyranone A. Phellifuropyranone A (**3**) has 6-(3,4-dihydroxystyryl)pyrone part, which is supposed to be derived from 6-(3,4-dihydroxystyryl)-4-hydroxypyrone (hispidin). As hypholomine B,[5] inoscavin A,[6] and inoscavin B[7] isolated from various mushrooms also have the same partial structure, phellifuropyranone A (**3**) and these compounds might give some information to consider biosynthetic pathway of furopyranone compounds from hispidin in several mushrooms.

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of **3** (in Pyridine-d_5, ^{1}H: 500 MHz, ^{13}C: 125 MHz)

Position	Phellifuropyranone A (**3**)		
	δ_C (ppm)	δ_H (ppm), J (Hz) (each 1H)	HMBC correlations ($^{2,3}J_{CH}$=8 Hz)
2	157.0		
3	100.5	7.25, s	2, 3a, 7a
3a	111.9		
4	158.8		
6	157.3		
7	95.7	6.66, s	3a, 6, 7a, 1′
7a	160.9		
1′	116.9	6.81, d, 15.9	6, 3′
2′	134.6	7.59, d, 15.9	6, 4′, 8′
3′	128.4		
4′	115.2	7.55[a]	2′, 8′
5′	147.8		
6′	149.5[a]		
7′	116.8	7.23, d, 8.2	3′, 5′
8′	121.0	7.13, dd, 8.2, 1.8	2′, 4′, 6′
1″	121.6		
2″	113.1	7.77, d, 1.6	2, 3″, 4″, 6″
3″	147.9		
4″	148.9		
5″	117.1	7.30, d, 8.2	1″, 3″
6″	117.2	7.40, dd, 8.2, 1.6	2, 4″

a) Overlapped with pyridine.

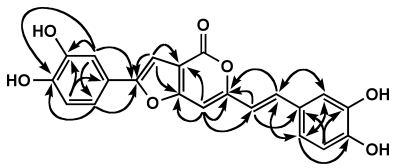

Fig. 2. The HMBC Correlations of Phellifuropyranone A (**3**)
The arrows indicate the selected HMBC correlations. (H→C, $^{2,3}J$=8 Hz).

Phelligridin G (**4**) was identified by measurements of ^{1}H-NMR spectrum and other spectra including ^{13}C-NMR and comparison of the spectral data (data not shown) with those reported previously.[8] The absolute configuration of **4** has not been determined yet and now under investigation.

The antiproliferative activities of phellifuropyranone A (**3**), in addition to meshimakobnol A (**1**) and meshimakobnol B (**2**) against five kinds of B16 cell lines and three kinds of human lung cancer cell lines A549, EBC-1 and SBC-3 were evaluated.[9,10] As shown in Table 2, phellifuropyranone A (**3**) suppressed the proliferation of mouse melanoma and human lung cancer cell lines with a 50% growth-inhibitory concentration (GI_{50}) of 5.6—31.3 μM. Meshimakobnol A (**1**) suppressed them with GI_{50} of 7.1—22.6 μM, and meshimakobnol B (**2**) also did with GI_{50} of 6.1—15.0 μM. These results showed that the antiproliferative activities of phellifuropyranone A (**3**), meshimakobnol A (**1**) and meshimakobnol B (**2**) were almost the same.

Conclusion

We isolated a new furopyranone compound named phellifuropyranone A (**3**) together with phelligridin G (**4**), meshimakobnol A (**1**) and meshimakobnol B (**2**) from fruit bodies of wild grown *P. linteus*. Though phellifuropyranone A (**3**) was obtained in much lower yield than meshimakobnol A (**1**), the phellifuropyranone A (**3**) may be used as an indicator compound to discriminate the wild plant-derived preparations of *P. linteus* from the cultivated plant-derived and cultured mycelia-derived preparations. HPLC analysis of the various *P. linteus* plants and commercially available prepara-

Table 2. GI_{50} Values of **1**—**3** against Five B16 Cell Lines and Three Human Lung Cancer Cell Lines

	B16 mouse melanoma cells					Human lung cancer cells		
	/wild	/BL6	/F1	/F10	/C2M	A549	EBC-1	SBC-3
Meshimakobnol A (**1**)	8.3	9.4	14.8	15.2	9.3	22.6	7.9	7.1
Meshimakobnol B (**2**)	7.3	10.7	8.2	6.1	8.2	15.0	7.7	7.0
Phellifuropyranone A (**3**)	9.4	7.7	13.6	5.6	8.7	31.3	15.6	14.4
Paclitaxel	0.011	0.020	0.0022	0.0087	0.011	0.015	0.0019	0.0044

Antiproliferative activity of **1**—**3** and paclitaxel (positive control) were determined as described in Materials and Methods. GI_{50} values are represented as μM.

tions is now under investigation.

Phellifuropyranone A (**3**) together with meshimakobnol A (**1**) and meshimakobnol B (**2**) showed low antiproliferative activity compared with paclitaxel against mouse melanoma cells and human lung cancer cells *in vitro*. It was not observed the structure–activity relationship and the specificity of antiproliferative activity against the cancer cell lines among compounds **1**—**3**. More investigations about biological activities of **1**—**3** and other small organic molecules in *P. linteus* such as **4** are currently in progress.

Experimental

General Methods Optical rotation was measured with a JASCO P-1020 spectrometer. IR and UV spectra were measured with a JASCO FT-IR 4100 spectrometer and JASCO V-530 spectrometer, respectively. ESI-FT-IR-MS spectra were obtained using a Bruker Apex III, and ^{1}H- and ^{13}C-NMR spectra were acquired with a JEOL ECA-500 spectrometer. NMR spectra were recorded in pyridine-d_5. The absorbance in MTT assay were measured with a Beckman-Coulter DTX-880 multimode plate reader.

Fungal Material Dried and powdered fruit bodies of wild *P. linteus* collected in China was supplied from Nihon Shoyaku Co., Ltd., Tokyo, Japan, in 2003. The voucher specimen is deposited in Laboratory of Pharmacognosy, College of Pharmacy, Kinjo Gakuin University.

Extraction and Isolaion Two 70 g portion of dried and powdered *P. linteus* natural fruit body (total 140 g) was extracted with hot MeOH (500 ml, 3×3 h), and the solvent was removed to give the MeOH extract (5.5 g). The extract was applied to ODS column [MeOH–H_2O (60 : 40→80 : 20, v/v)] and then separated by HPLC [Nomura Chemical RP AQUEOUS, C30, MeOH–H_2O (90 : 10, v/v)] to give meshimakobnols A (76 mg) and B (3 mg). The residual fraction except for meshimakobnols A and B was separated by HPLC [Nomura Chemical RP AQUEOUS, C30, MeOH–H_2O (70 : 30→80 : 20, v/v)] to yield phellifuropyranone A (2 mg) and phelligridin G (6 mg).

Phellifuropyranone A (**3**): A dark yellow amorphous powder, UV λ_{max}^{MeOH} nm (log ε): 400 (3.8), 314 (3.6). ESI-FT-ICR-MS *m/z*: 401.0632 (Calcd for $C_{21}H_{14}O_7Na$, 401.0632). IR (KBr) cm^{-1}: 3418 (–OH), 1700 (>C=O). ^{1}H-NMR data (500 MHz, pyridine-d_5, each 1H) δ: 6.66 (s, H-7), 6.81 (d, J=15.9 Hz, H-1′), 7.13 (dd, J=8.2, 1.8 Hz, H-8′), 7.23 (d, J=8.2 Hz, H-7′), 7.25 (s, H-3), 7.30 (d, J=8.2 Hz, H-5″), 7.40 (dd, J=8.2, 1.6 Hz, H-6″), 7.55 (overlapped with C_5D_5N, H-4′), 7.59 (d, J=15.9 Hz, H-2′), 7.77 (d, J=1.6 Hz, H-2″). ^{13}C-NMR data (125 MHz, pyridine-d_5) δ_C: 95.7 (C-7), 100.5 (C-3), 111.9 (C-3a), 113.1 (C-2″), 115.2 (C-4′), 116.8 (C-7′), 116.9 (C-1′), 117.1 (C-5″), 117.2 (C-6″), 121.0 (C-8′), 121.6 (C-1″), 128.4 (C-3′), 134.6 (C-2′), 147.8 (C-5′), 147.9 (C-3″), 148.9 (C-4″), 149.5 (overlapped with C_5D_5N, C-6′), 157.0 (C-2), 157.3 (C-6), 158.8 (C-4), 160.9 (C-7a).

Phelligridin G (**4**): A brown amorphous powder, $[\alpha]_D^{25}$ −26.5° (MeOH, c=0.05). UV λ_{max}^{MeOH} nm (log ε): 397 (4.4), 265 (4.3). ESI-FT-ICR-MS *m/z*: 617.0682 (Calcd for $C_{52}H_{18}O_{12}Na$, 617.0696). IR (KBr) cm^{-1}: 3423 (–OH), 1693 (>C=O). ^{1}H-NMR data (500 MHz, pyridine-d_5, each 1H) δ: 6.16 (s, H-4″), 6.18 (s, H-4), 7.09 (d, J=16.1 Hz, H-1‴), 7.24 (d, J=8.4 Hz, H-7‴), 7.28 (dd, J=8.4, 1.8 Hz, H-8‴), 7.33 (s, H-7′), 7.34 (s, H-4′), 7.67 (d, J=1.8 Hz, H-4‴), 7.77 (s, H-3′), 7.93 (d, J=16.1 Hz, H-2‴), 8.15 (s, H-7), 9.08 (s, H-10). ^{13}C-NMR data (125 MHz, pyridine-d_5) δ_C: 95.4 (C-1′(2″)), 96.8 (C-4), 100.6 (C-10b), 104.0 (C-4″), 111.3 (C-7′), 111.9 (C-10), 112.2 (C-4′), 112.4 (C-1‴), 112.7 (C-6a), 115.7 (C-7), 116.2 (C-4‴), 116.9 (C-7‴), 123 (overlapped with C_5D_5N, C-8‴), 127.1 (C-3‴), 128.0 (C-10a), 133.3 (C-3′a), 133.6 (C-2′), 136.3 (C-7′a), 141.4 (C-3′), 141.8 (C-2‴), 147.9 (C-5‴), 149.2 (C-6′, exchangeable for C-5′), 149.4 (overlapped with C_5D_5N, C-9, exchangeable for C-8), 149.6 (overlapped with C_5D_5N, C-5′, exchangeable for C-6′), 151.3 (C-6‴), 153.9 (C-3), 155.8 (C-8, exchangeable for C-9), 155.9 (C-1), 159.5 (C-6), 160.6 (C-4a), 185.3 (C-5″), 198.1 (C-3″).

Cell Culture B16 mouse melanoma cell lines: /wild (TKG 0144), /BL6 (TKG 0598), /F1 (TKG 0347), /F10 (TKG 0348) and /C2M (TKG 0346) were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Miyagi, Japan). Human lung cancer cell lines: A549 (adenocarcinoma, JCRB0076), EBC-1 (squamous cell, JCRB0820), SBC-3 (small cell, JCRB0818) were obtained from Health Science Research Resources Bank (Sennan, Osaka, Japan). All kinds of cells were maintained in Eagle's minimum essencial medium (MEM, Sigma, St. Louis, MO, U.S.A.), supplemented with 10% heat-inactivated fetal calf serum (FCS, Nippon Bio-Supp. Center, Tokyo, Japan), 50 U/ml penicillin, and 50 mg/ml streptomycin (Sigma, St. Louis, MO, U.S.A.). Each cell was maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation Assay B16/wild, /BL6, /F1, /F10 and /C2M cells were plated at the density of 2.0×10^3 cells/well, and A549, EBC-1 and SBC-3 were plated at the density of 5.0×10^3 cells/well into 96-well plates and cultured for 24 h, respectively. Then, the medium were exchanged with the ones including the isolated compounds and paclitaxel (Sigma, St. Louis, MO, U.S.A.), and incubated for 48 h. After exchanging with fresh medium, 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 5 mg/ml in MEM) was added to each well and incubated for another 4 h. Following the absorbance at 595 nm subtracted the one at 620 nm were measured and also the cell growth percentage (GP); GP was calculated according to the following formula: GP (>0%)=[(absorbance of cells treated with samples)−(absorbance at 0 times)]/[(absorbance of control cells)−(absorbance at 0 time)], GP (−100—0%)=[(absorbance of cells treated with samples)−(absorbance at 0 times)]/(absorbance at 0 time).

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