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Polychlorinated compounds with PPAR-γ agonistic effect from the medicinal fungus *Phellinus ribis*

In-Kyoung Lee [a], Jeong-Hyung Lee [b], Bong-Sik Yun [a,*]

[a] *Functional Metabolites Research Center, KRIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea*
[b] *Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea*

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ABSTRACT

During the search for natural substances with PPAR-γ agonistic effect, unique polychlorinated compounds named chlorophellins A–C have been isolated together with the known compound, drosophilin A, from the methanolic extract of the fruiting body of the fungus *Phellinus ribis*. Their structures were assigned on the basis of NMR and mass spectrometric analyses. Chlorophellin C of compounds tested exhibited the most potent PPAR-γ agonistic effect and was comparable to rosiglitazone, a well-known PPAR-γ agonist that has been used for the therapy of type 2 diabetes.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors, a family that includes the receptors for steroid hormones, retinoids, thyroid hormone, and vitamin D. Three mammalian PPARs have been identified to date, termed PPAR-α, PPAR-γ, and PPAR-δ. PPARs function as regulators of lipid and lipoprotein metabolism, glucose homeostasis, and cellular differentiation, and also appear to control the inflammatory response.[1,2] PPAR-γ agonists have therapeutic potential in the treatment of type 2 diabetes, inflammatory disease, and certain cancers.[3,4]

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial medicines. They produce diverse classes of primary and secondary metabolites, many that exhibit potent biological activity including antiviral, anticancer, and antidiabetes activities.[5,6] *Phellinus ribis* (Schumach.) Quél. (Hymenochaetaceae), distributed in East Asia including Korea, Japan, and China, is a white-rotting fungus preferably living on a stump of *Rosa polyantha* and *Weigela subsessilis*. The fruiting body of *P. ribis* has been used as a traditional medicine for the treatment of gastrointestinal cancer and diabetes in Korea, and its biological activity was thought to be attributed to polysaccharides.[7,8] In the course of screening for PPAR-γ agonists from the fruiting bodies of medicinal fungi, we found that the methanolic extract of *P. ribis* exhibited potent PPAR-γ agonistic activity. The bioassay-guided fractionation revealed that the hexane-soluble portion was responsible to the activity. Our investigation on active ingredients in the hexane-soluble portion led to the isolation of new polychlorinated compounds, chlorophellins A (**1**), B (**2**), and C (**3**), together with known compound, drosophilin A (**4**). In this letter, we describe the isolation and structure determination of these compounds, and their PPAR-γ agonistic effect (Fig. 1).

The ground fruiting bodies of *P. ribis* (2 kg) were extracted twice with methanol at room temperature for 2 days. After removal of methanol under reduced pressure, the aqueous concentrate was partitioned between hexane, chloroform, ethyl acetate, and BuOH and H_2O, consecutively. The bioassay revealed that the hexane-soluble portion was responsible to PPAR-γ agonistic activity. The hexane-soluble fraction was subjected to silica gel flash column chromatography and eluted with hexane/ethyl acetate (30:1–2:1, v/v, stepwise). An active fraction eluted with the hexane/ethyl acetate (10:1, v/v) was concentrated and then rechromatographed on a column of silica gel with same solvent as that of flash column. An active fraction of the hexane/ethyl acetate (20:1) was separated by Sephadex LH-20 column chromatography with $CHCl_3$/MeOH (1:2) to provide two active fractions. The first fraction was purified by the ODS column chromatography eluting with 90% aqueous MeOH, followed by preparative HPLC using reversed-phase column with 90% aqueous acetonitrile to provide **1** (15 mg), **2** (10 mg), and **3** (12 mg). The second fraction was purified by the ODS column chromatography eluting with 90% aqueous MeOH to afford **4** (150 mg).

Compound **4** was identified as drosophilin A on the basis of its mass spectrum and NMR data, which were in good agreement with previously published literatures.[9] Drosophilin A has been known as the first halogenated metabolite identified in a basidiomycete.[10]

Chlorophellin A (**1**) was obtained as a white crystalline and its molecular formula was established as $C_{21}H_{10}Cl_{10}O_6$ by high resolution FAB-mass.[11] The molecular formula required 12 degrees of

* Corresponding author. Tel.: +82 42 860 4339; fax: +82 42 860 4595.
E-mail address: ybs@kribb.re.kr (B.-S. Yun).

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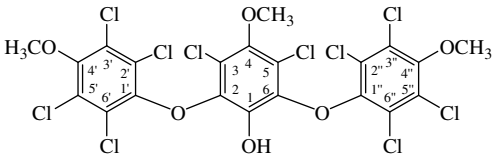

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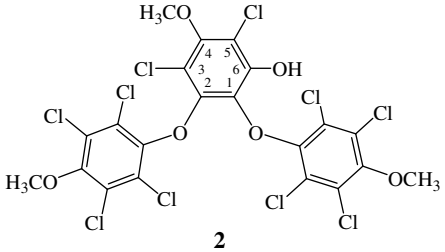

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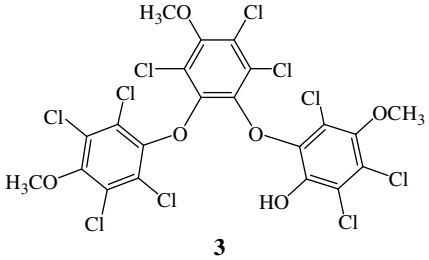

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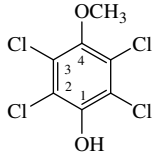

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Figure. 1. Structures of compounds **1**–**4**.

unsaturation. Ten chlorines were proposed by unique isotopic abundance ratio of *m/z* 707.7 (21%), 709.7 (66%), 711.7 (100%), 713.7 (85%), 715.7 (52%), and 717.7 (23%). The ^{1}H NMR spectrum of **1** in $CDCl_3$ exhibited two peaks at δ 3.82 (6H) and 3.93 (3H) assignable to three aromatic methoxyl groups. In the ^{13}C NMR spectrum, 10 carbon peaks with quite different intensity were evident. In intensity, the methoxyl carbon peak at δ 61.0 was two times higher than the methoxyl peak at δ 61.2, suggesting that the carbon peak at δ 61.0 was an overlapped signal of two methoxyl carbons. Eight peaks, except for two methoxyl peaks, were observed in the aromatic/olefinic region. In their intensities, the peaks at δ 125.4 and 127.8 were approximately four times higher (peaks for four overlapped carbons) and the peaks at δ 116.1, 139.1, 146.6, and 150.7 were two times higher (peaks for two overlapped carbons) than the peaks at δ 136.5 and 146.4. The carbon numbers and their intensities suggested that the structure of compound **1** should be a symmetrical dimer. The carbon chemical shifts at δ 125.4, 127.8, 146.6, and 150.7, and their intensities suggest the presence of two 2,3,5,6-tetrachloro-4-methoxyphenol (drosophilin A, **4**) moieties in **1**. This was also supported by the long-range correlations of methoxyl protons at δ 3.82 to the carbons at δ 150.7. The remaining six carbons at δ 116.1 (2×), 136.5, 139.1 (2×), and 146.4 suggested the presence of a symmetrical benzene ring with an axis by the carbons at δ 136.5 and 146.4. Their chemical shift values and the long-range correlation of the methyl protons at δ 3.93 to the carbon at δ 146.4 established the presence of 3,5-dichloro-4-methoxyphenol. Therefore, the structure of **1** was determined as a new polychlorinated compound, 2,6-bis(2,3,5,6-tetrachloro-4-methoxyphenoxy)-3,5-dichloro-4-methoxyphenol.

Chlorophellin B (**2**) was also obtained as a white crystalline, and its molecular formula, $C_{21}H_{10}Cl_{10}O_6$, was determined to be the same as that of **1** by high resolution FAB-mass with a unique isotopic abundance ratio for 10 chlorines.[11] The structure of **2** was determined by comparison of ^{1}H and ^{13}C NMR spectral data with those of compound **1**. The ^{1}H NMR spectrum of **2** in $CDCl_3$ exhibited the signals attributable to three methoxyl groups at δ 3.89 (2×) and 3.90. In the ^{13}C NMR spectrum, 17 carbons were evident. Fourteen carbons except three methoxyl carbons were observed in the aromatic/olefinic region. The carbons at δ 124.6, 125.2, 127.5, and 127.7 were approximately two times higher than others in intensity, and their chemical shift values were in good agreement with C-2/C-6 and C-3/C-5 of 2,3,5,6-tetrachloro-4-methoxyphenol, suggesting the presence of two 2,3,5,6-tetrachloro-4-methoxyphenol moieties. In addition, this was supported by the long-range correlations of the methoxyl protons at δ 3.89 to the carbons at δ 150.6 and 150.4. To satisfy the 12 degrees of unsaturation, the remaining six carbons should form an asymmetrical benzene ring. Their chemical shifts very similar to the corresponding carbons of compound **1** and the long-range correlation of the methyl protons at δ 3.90 to the carbon at δ 149.1 established the structure of chlorophellin B to be 2,3-bis(2,3,5,6-tetrachloro-4-methoxyphenoxy)-4,6-dichloro-5-methoxyphenol.

Chlorophellin C (**3**) was obtained as a white crystalline, and its molecular formula, $C_{21}H_{10}Cl_{10}O_6$, was determined to be the same as that of **1** by high resolution FAB-mass measurement.[11] The ^{1}H NMR spectrum of **3** in $CDCl_3$ exhibited signals attributable to three methoxyl groups at δ 3.82 (2×) and 3.94, and was very similar to those of chlorophellins A and B. However, the ^{13}C NMR spectrum of **3** was too different to be capable of deducing the structure of **3** from chlorophellins A and B. Consequently, the structure of **3** was determined by X-ray crystallography as 2,3,5-trichloro-4-methoxy-6-[2,3,5-trichloro-4-methoxy-6-(2,3,5,6-tetrachloro-4-methoxy-phenoxy)phenoxy]phenol and was previously reported.[12]

The effect on the activation of PPAR-γ by compounds **1**–**4** was evaluated using a reporter gene assay described by Forman et al.[13] with some minor modifications. Briefly, NIH-3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. NIH-3T3 cells, grown in 24-well culture plates, were transiently transfected with expression vectors (500 ng each) for pFA-GAL4-PPAR, pFR-Luc, and pSV-β-galactosidase using Lipofectamine plus reagent for 3 h. Various concentrations of **1**–**4** were added 24 h after transfection, and whole cell lysates were prepared 16 h after treat-

Table 1
^{1}H and ^{13}C NMR spectral data for chlorophellins A (**1**) and B (**2**) in $CDCl_3$[a]

No.	1		2	
	δ_C	δ_H	δ_C	δ_H
1	136.5		132.1	
2,6	139.1		141.8, 142.3	
3,5	116.1		111.9, 112.7	
4	146.4		149.1	
4-OMe	61.2	3.93 (3H, s)[b]	61.1	3.90 (3H, s)
1′,1″	146.6		146.4, 146.6	
2′,6′,2″,6″	125.4		124.6, 125.2	
3′,5′,3″,5″	127.8		127.5, 127.7	
4′,4″	150.7		150.4, 150.6	
4′-, 4″-OMe	61.0	3.82 (6H, s)	60.9, 61.0	3.89 (6H, s)

[a] NMR data were measured at 400 MHz for proton and at 100 MHz for carbon.
[b] Proton resonance integral and multiplicity are in parentheses.

ment and assayed for luciferase and β-galactosidase activity using a luminometer and spectrophotometer, respectively. Transfections were performed in triplicate, and activation was normalized to galactosidase activity. Compounds **1**–**4** activated the PPAR-γ dose-dependently, and the potency of compound **3** was comparable to that of rosiglitazone.[14] The maximum activation by chlorophellin C (10 μM) was 8.5-fold compared to the negative control, while that by rosiglitazone was 9.7-fold. Drosophilin A also showed significant PPAR-γ agonistic activity with 8.4-fold activation at 100 μM although it was less active than chlorophellin C and rosiglitazone. However, chlorophellins A (**1**) and B (**2**) displayed weak activity and their maximum fold activation was about 2-fold at 10 μM, whereas rosiglitazone was 9.7-fold. The activation of PPAR-γ by polychlorinated compounds is reported for the first time in this study.

Many chlorinated compounds were reported to have antimicrobial activity.[15,16] However, compounds **1**–**3** did not display antimicrobial activity, whereas compound **4** showed marginal activity against *Bacillus subtilis*, *Staphylococus aureus*, and *Escherichia coli*.

Acknowledgments

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11. *Chlorophellin A:* White crystalline; UV λ_{max} (MeOH)(log ε) 222 (4.50), 298 (3.11) nm; IR ν_{max} 3400, 1460, 1420, 1200 cm^{-1}; for ^{1}H NMR and ^{13}C NMR, see Table 1; FAB-mass (positive mode) *m/z* 707.7 (M^+, 21%), 709.7 (66%), 711.7 (100%), 713.7 (85%), 715.7 (52%), and 717.7 (23%) (theoretical isotopic abundance ratio for 10 chlorines 707.7 (21%), 709.7 (68%), 711.7 (100%), 713.7 (87%), 715.7 (50%), and 717.7 (20%)); high resolution FAB-mass *m/z* 707.7363 [M^+] (Calcd for $C_{21}H_{10}O_6Cl_{10}$, 707.7363). *Chlorophellin B:* White crystalline; UV λ_{max} (MeOH)(log ε) 226 (4.61), 297 (3.50) nm; for ^{1}H NMR and ^{13}C NMR, see Table 1; FAB-mass (positive mode) *m/z* 707.7 (M^+, 22%), 709.7 (63%), 711.7 (100%), 713.7 (87%), 715.7 (58%), and 717.7 (29%) (theoretical isotopic abundance ratio for 10 chlorines 707 (21%), 709 (68%), 711 (100%), 713 (87%), 715 (50%), and 717 (20%)); high resolution FAB-MS *m/z* 707.7363 [M^+] (Calcd for $C_{21}H_{10}O_6Cl_{10}$, 707.7363). *Chlorophellin C:* White crystalline; UV λ_{max} (MeOH)(log ε) 222 (4.59), 297 (3.58) nm; FAB-mass (positive mode) *m/z* 707.7 (M^+, 27%), 709.7 (74%), 711.7 (100%), 713.7 (84%), 715.7 (48%), and 717.7 (25%) (theoretical isotopic abundance ratio for 10 chlorines 707 (21%), 709 (68%), 711 (100%), 713 (87%), 715 (50%), and 717 (20%)); high resolution FAB-MS *m/z* 707.7363 [M^+] (Calcd for $C_{21}H_{10}O_6Cl_{10}$, 707.7363).
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