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SECONDARY METABOLITES FROM THE ENDOPHYTIC FUNGUS *Annulohypoxylon boveri* var. *microspora* BCRC 34012

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A chemical study on the n-*BuOH-soluble fraction of the 95% EtOH extract of long-grain rice (*Oryza sativa*) fermented with the endophytic fungus* Annulohypoxylon boveri *var.* microspora *(BCRC 34012) has resulted in the isolation of one new natural azaphilone derivative, designated as annulohypoxyboverin (**1**) together with 12 known compounds, (3*R,6R,7E*)-3-hydroxy-4,7-megstigma-dien-9-one (**2**),* α*-tocopheryl quinone (**3**), isofraxidin (**4**), coumarin (**5**), cinnamic acid (**6**), a mixture of palmitic acid (**7**) and stearic acid (**8**),* N-cis-*feruloyltyramine (**9**), luteolin (**10**), kaempferol (**11**), kaempferitrin (**12**), and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**13**). Annulohypoxyboverin (**1**) contains a dihydrobenzofuran-2,4-dione backbone, 1-hydroxyhexyl side chain, and one γ-lactone ring. Their structures were elucidated by spectroscopic analyses including 1D and 2D NMR experiments, and by HR-ESI-MS. The relative configuration of **1** was confirmed by NOESY experiment. Other known compounds were identified by comparing their spectral data with those of literature data.*

Keywords: *Annulohypoxylon boveri* var. *microspora*, Xylariaceae, secondary metabolites, dihydrobenzofuran-2,4-dione.

Endophytes, commonly present in almost all plants, are important sources of natural products with pharmaceutical potential [1, 2]. Endophytic fungi live in the intracellular spaces of the tissues of host plants without apparent demonstration of disease [1, 2]. Recently, endophytes have been recognized as a fruitful source of structurally novel and biologically active secondary metabolites to be chemically explored [3–8]. An endophytic fungal strain, named BCRC 34012, was collected from Fu-shan Botanical Garden, I-lan County, Taiwan, during August of 2001. This strain was determined to be *Annulohypoxylon boveri* var. *microspora* (family Xylariaceae) based on their cultural and anamorphic data by Dr. Y.-M. Ju [9]. Ju′s group had placed the taxa assigned to Hypoxylon sect. Annulata in a new genus, for which the name *Annulohypoxylon* is given based on the morphological characteristics and the analyses of β-tubulin and α-actin gene sequences [9].

The Xylariaceae is a large family (Xylariales, Ascomycotina) of 36 or more genera. Secondary metabolites produced by representatives from at least one-third of these genera have now been isolated and identified [10]. A variety of structurally diversified compounds is widely distributed in the *Xylaria* sp [11–19]. Contrary to *Xylaria* sp., the secondary metabolites of the genus *Annulohypoxylon* have received less attention, and only one paper has been reported [20]. To further understand the chemotaxonomy of the genus *Annulohypoxylon* and to continue searching for novel bioactive metabolites from Xylariaceae, *A. boveri* var. *microspora* was chosen for a phytochemical investigation. Carefully examination of the above title fungus has resulted in the isolation of one new dihydrobenzofuran-2,4-dione skeleton combined with a γ-lactone ring, named annulohypoxyboverin (**1**), together with 12 known constituents (**2**–**13**). Compounds **2**–**12** were found for the first time from this species. The structures of these compounds were established by means of spectral experiments. We herein report the isolation and characterization of the new compound.

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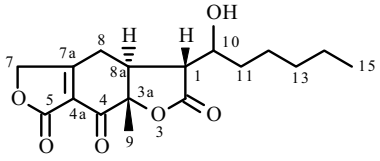
TABLE 1. NMR Data for the New Compound **1** ($CDCl_3$, δ, ppm, J/Hz)[a]

C atom	δ_C[b]	δ_H	C atom	δ_C[b]	δ_H
1	49.2 (d)	2.80 (dd, J = 13.0, 3.0)	8a	43.0 (d)	3.20 (ddd, J = 13.0, 12.0, 4.8)
2	175.2 (s)		9	16.8 (q)	1.50 (s)
3a	84.7 (s)		10	70.0 (d)	4.30 (dt, J = 8.8, 3.0)
4	190.2 (s)		11	35.1 (t)	1.60 (m)
4a	144.0 (s)		12	26.2 (t)	1.31–1.33 (m)
5	172.5 (s)		13	31.4 (t)	1.31–1.33 (m)
7	68.5 (t)	4.97 (dd, J = 17.9, 3.6)	14	22.5 (t)	1.31–1.33 (m)
		5.09 (dd, J = 17.9, 3.0)	15	14.0 (q)	0.89 (t, J = 7.0)
7a	150.4 (s)				
8	25.3 (t)	2.65 (ddd, J = 18.4, 12.0, 3.6) (Hax)			
		3.10 (ddd, J = 18.4, 4.8, 3.0) (Heq)			

[a]All spectra were recorded at 600 MHz (^1H) and 150 MHz (^{13}C); assignment were aided by 2D NMR, COSY, HSQC, and HMBC experiments; [b] ^{13}C NMR multiplicities were determined by a DEPT experiment.



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Compound **1**, obtained as an optically inactive yellowish oil, has the molecular formula $C_{17}H_{22}O_6$, as determined by HR-ESI-MS data (*m/z* 345.1317 ($[M + Na]^+$; calcd 345.1314)) in combination with ^1H NMR, ^{13}C NMR, and DEPT data, requiring 7 degrees of unsaturation. UV absorption bands at 235 (3.81) and 350 (4.10) nm demonstrated that **1** was structurally related to a conjugated ketone skeleton [21]. Its IR spectrum showed absorption bands for a hydroxyl at 3400 cm^{-1}, a conjugated ketone at 1720 cm^{-1}, and an ester (γ-lactone) at 1650 cm^{-1}. Four of the seven degrees of unsaturation inherent in the formula were accounted by ^{13}C NMR as one conjugated carbonyl, two ester carbonyls (γ-lactone rings), and two olefinic carbons. Accordingly, compound **1** contained a dihydrobenzofuran-2,4-dione skeleton combined with a γ-lactone ring. The ^1H and ^{13}C NMR spectra (Table 1) indicated six quaternary C-atoms, three CH, six CH_2, and two Me groups. In the ^1H NMR spectrum, there were typical signals for one aliphatic terminal methyl group at δ 0.89 (3H, t, J = 7.0 Hz, H-15), one methyl signal attached to a quaternary C-atom at δ 1.50 (3H, s, H-9), one methylene moiety at δ 2.65 (ddd, J = 18.4, 12.0, 3.6 Hz, H_{ax}-8)/3.10 (ddd, J = 18.4, 4.8, 3.0 Hz, H_{eq}-8), one oxymethylene moiety at δ 4.97 (dd, J = 17.9, 3.6 Hz, H-7a)/5.09 (dd, J = 17.9, 3.0 Hz, H-7b), two mutually coupling methine signals at δ 2.80 (1H, dd, J = 13.0, 3.0 Hz, H-1)/3.20 (1H, ddd, J = 13.0, 12.0, 4.8 Hz, H-8a), as well as one 1-hydroxyhexyl side chain (δ 0.89 (3H, t, J = 7.0 Hz, H-15), 1.31–1.33 (6H, m, H-12–14), 1.60 (2H, m, H-11), and 4.30 (1H, dt, J = 8.8, 3.0, H-10)), indicating that **1** was probably a dihydrobenzofuran-2,4-dione skeleton possessing another γ-lactone ring [21, 22]. The carbons of the dihydrobenzofuran-2,4-dione derivative were assigned from ^{13}C NMR and DEPT experiments. There were resonances for one C=O function [δ 190.2 (α,β-unsaturated C=O group); 172.5, 175.2 (ester C=O group)], two C=C bonds [δ 144.0, 150.4], two methyl groups [δ 14.0, 16.8], one oxybearing methylene moiety [δ 68.5], one oxymethine moiety [δ 70.0], one methylene moiety [δ 25.3], two methine moieties [δ 43.0, 49.2], and four aliphatic methylene C-atoms [δ 22.5, 26.2, 31.4, 35.1]. The above data of **1** also pointed to a dihydrobenzofuran-2,4-dione derivative as in similar compounds [21, 22].

The two (^2J) and three bond (^3J) ^1H–^{13}C connectivities of the building elements of the molecule were detected by the HMBC. The HMBC spectrum of **1** provided its gross structure; key long-range ^1H, ^{13}C correlations were observed from H-1 to C-2, C-8, C-8a, and C-3a; H-7 to C-5, C-7a, and C-4a; H-8 to C-7a, C-4a, and C-3a; 9-Me protons to C-3a, and C-8a; H-8a to C-4, C-8, and C-2; H-15 to C-13 and C-14; and both H-11 and H-13 to C-12.

The HSQC spectrum of **1** enabled assignment of the directly bonded C-H moieties. The results showed that H-C correlations signals were δ_H 0.89/δ_C 14.0; δ_H 1.31–1.33/δ_C 22.5, 26.2, 31.4; δ_H 1.50/δ_C 16.8; δ_H 1.60/δ_C 35.1; δ_H 2.65, 3.10/δ_C 25.3; δ_H 2.80/δ_C 49.2; δ_H 3.20/δ_C 43.0; δ_H 4.30/δ_C 70.0; and δ_H 4.97, 5.09/δ_C 68.5, respectively.

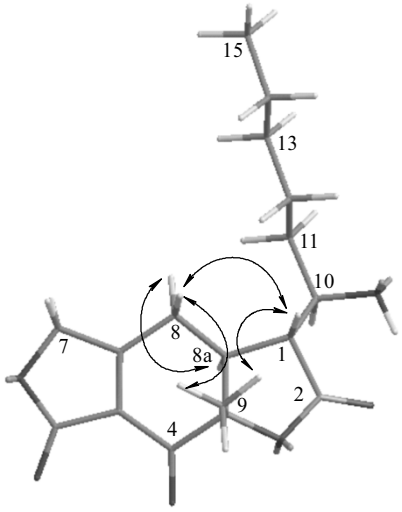

Fig. 1. Most stable conformation for **1** as predicted by molecular mechanics (MM2) calculations and major NOESY (H↔H) correlations.

The δ_H 4.97/δ_C 25.3 cross peak proves that the CH_2-8 methylene is connected to the C-7a atom of the γ-lactone ring, and the δ_H 4.97/δ_C 190.2 correlation shows that the C-4 keto group is attached to C-4a in the same ring. From the HMBC plot between the singlet methyl at δ_H 1.50 and the C-4 signal, we can conclude that these protons are located three bonds distant from C-4. The H_3C-9 protons correlate with one methine carbon atom (δ_C 43.0), and in this way the signal at δ_C 43.0 can be assigned to C-8a. The double doublet at δ_H 2.80 (H-1) correlates with the carbon atoms C-8 and C-8a, and with the lactone carbonyl C-2. This allows the determination of the position of the lactone ring. On the basis of the key cross peaks between H-1/C-14, the position of the $C_5H_{11}CH(OH)$ moiety is also obvious in the HMBC spectrum.

The relative configuration of **1** was derived by a NOESY spectrum in combination with similar compounds [21, 22]. According to the NOESY spectrum, the H-1 was β-oriented, which was confirmed by the NOE CH_3-9/H-1. NOEs for CH_3-9/H_{ax}-8, and H-1/H_{ax}-8 indicated that H-1, H_{ax}-8, and CH_3-9 were on the same side of the molecular plane, tentatively assumed as the β-orientation. In addition, the coupling constant values of ^3J(H-1/H-8a) ≈ ^3J(H-8a/H_{ax}-8) ≈ 12.5 Hz proved the *trans*-antiperiplanar positions of these protons. Because of the optical inactivity ($[\alpha]_D^{22}$ ±0° (*c* 0.05, $CHCl_3$)), **1** was concluded to be racemic.

To further clarify the relative configuration of **1**, a computer-assisted 3D structure was obtained by using the molecular modeling program CS CHEM 3D Ultra 10.0, with MM2 force-field calculations for energy minimization (Fig. 1). The calculated distances between H-1/CH_3-9 (2.328 Å) H-1/H_{ax}-8 (2.739 Å), and CH_3-9/H_{ax}-8 (2.393 Å) are all less than 4 Å. This is consistent with the above-mentioned NOESY interactions between each of these proton pairs.

From the above data, compound **1** was unambiguously characterized as rel-(1*R*,3a*S*,8a*S*)-3-(1-hydroxyhexyl)-3a-methyl-3a,8a-dihydro-3*H*,4*H*,5*H*-benzo[1,2-b;4,5-c′]difuran-2,4,5-trione, named annulohypoxyboverin, and its structure was illustrated as **1**, which was further confirmed by COSY, NOESY, HSQC, and HMBC experiments.

The other known isolates, (3*R*,6*R*,7*E*)-3-hydroxy-4,7-megstigma-dien-9-one (**2**) [23], α-tocopheryl quinone (**3**) [24], isofraxidin (**4**) [25], coumarin (**5**) [26], cinnamic acid (**6**) [26], a mixture of palmitic acid (**7**) and stearic acid (**8**) [27], *N-cis*-feruloyltyramine (**9**) [28], luteolin (**10**) [29], kaempferol (**11**) [30], kaempferitrin (**12**) [30], and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**13**) [31] were readily identified by comparison of their spectral data (UV, IR, ^1H NMR, MS) with the data from the corresponding values in the literature. Among them, all known compounds, except **13**, were isolated from *Annulohypoxylon* species for the first time. Further, the known metabolite that is frequently encountered in *Annulohypoxylon* is 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (also called BNT) (**13**, ubiquitous in all of its species and many other Xylariaceae).

EXPERIMENTAL

General Experimental Procedures. All melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco P-1020 digital polarimeter, UV spectra were obtained on a Jasco UV-240 spectrophotometer in MeOH, and IR spectra (KBr or neat) were taken on a Perkin–Elmer System 2000 FT-IR spectrometer. 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra using $CDCl_3$ as solvent were recorded on Varian Unity Plus 400 (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) and 600 (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR) spectrometers. Chemical shifts were internally referenced to the solvent signals in $CDCl_3$ (^{1}H, δ 7.26; ^{13}C, δ 77.0). Low-resolution ESI-MS spectra were obtained on an API 3000 (Applied Biosystems), and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer. Low-resolution EI-MS spectra were recorded on a Quattro GC/MS spectrometer having a direct inlet system. Silica gel (70–230, 230–400 mesh) (Merck) was used for column chromatography, and silica gel 60 F-254 (Merck) was used for TLC and preparative TLC.

Fungus Material. *Annulohypoxylon boveri* var. *microspora* (BCRC 34012) was used throughout this study, and specimens were deposited at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI). *A. boveri* var. *microspora* BCRC 34012 was maintained on potato dextrose agar (PDA), and the strain was cultured on potato dextrose agar slants at 25°C for 7 days and the spores harvested by sterile water. The spores (5×10^5) were seeded into 300 mL shake flasks containing 50 mL RGY medium (3% rice starch, 7% glycerol, 1.1% polypeptone, 3% soybean powder, 0.1% $MgSO_4$, 0.2% $NaNO_3$) and cultivated with shaking (150 rpm) at 25°C for 3 days. After the mycelium enrichment step, an inoculum mixing 100 mL mycelium broth and 100 mL RGY medium was inoculated into plastic boxes (25 cm × 30 cm) containing 1 kg sterile rice and cultivated at 25°C for producing the rice, and the above-mentioned RGY medium was added to maintain the growth. After 21 days of cultivation, the rice was harvested and used as a sample for further extraction.

Extraction and Separation of Compounds. The rice of the *A. boveri* var. *microspora* BCRC 34012 (2 kg) was extracted three times with 95% EtOH at room temperature. The ethanol syrup extract was partitioned between *n*-BuOH and H_2O (1:1) to afford *n*-BuOH (fraction A, 2.8 g) and H_2O (fraction B, 3.0 g) soluble fractions. The *n*-BuOH-soluble fraction (2.8 g) was chromatographed over silica gel (70–230 mesh), eluting with CH_2Cl_2, and enriched with MeOH to produce 8 fractions (A1–A8). Fraction A-1 (170 mg) was chromatographed over silica gel, eluting with CH_2Cl_2–EtOAc (10:1→5:1→1:1), to obtain 4 fractions (A-1-1–A-1-4). Fraction A-1-4 (45 mg) was purified by preparative TLC to afford (3*R*,6*R*,7*E*)-3-hydroxy-4,7-megstigma-dien-9-one (**2**) (0.8 mg) and a mixture of palmitic acid (**7**) and stearic acid (**8**) (10 mg). Fraction A-2 (345 mg) was chromatographed over silica gel, eluting with CH_2Cl_2–acetone (15:1→1:1), to obtain 6 fractions (A-2-1–A-2-6). Fraction A-2-3 (26.3 mg) was repeatedly purified by preparative TLC to afford annulohypoxyboverin (**1**) (1.8 mg) and α-tocopheryl quinone (**3**) (1.7 mg). Fraction A-2-6 (50 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 1:1) to give isofraxidin (**4**) (2.1 mg) and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**13**) (15.2 mg). Fraction A-4 (140 mg) was resubjected to silica gel column chromatography (CH_2Cl_2–EtOAc, 10:1→1:1) to afford 10 fractions (A-4-1–A-4-10). Fraction A-4-5 (25 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–EtOAc, 8:1) to afford coumarin (**5**) (2.6 mg) and cinnamic acid (**6**) (1.5 mg). Fraction A-5 (220 mg) was resubjected to silica gel column chromatography (CH_2Cl_2–MeOH, 10:1→1:1) to afford 5 fractions (A-5-1–A-5-5). Fraction A-5-2 (20 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–EtOAc, 10:1) to afford *N-cis*-feruloyltyramine (**9**) (1.9 mg). Fraction A-5-5 (48 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–MeOH, 20:1) to afford luteolin (**10**) (1.4 mg). Fraction A-6 (715 mg) was chromatographed over silica gel, eluting with CH_2Cl_2–acetone (10:1), to obtain 10 fractions (A-6-1–A-6-10). Fraction A-6-3 (50 mg) was repeatedly purified by preparative TLC to afford kaempferol (**11**) (4.5 mg) and kaempferitrin (**12**) (1.6 mg).

Annulohypoxyboverin (1). Yellowish oil. $[\alpha]_D^{22}$ ±0° (*c* 0.05, $CHCl_3$). UV spectrum (MeOH, λ_{max}, nm): 235, 350 (log ε 3.81; 4.10). IR spectrum (neat, ν_{max}, cm^{-1}): 3400 (OH), 1720 (conjugated ketone C=O), 1650 (ester C=O). ^{1}H NMR (600 MHz, $CDCl_3$, δ, ppm, J/Hz) and ^{13}C NMR (150 MHz, $CDCl_3$, δ) are shown in Table 1. ESI-MS *m/z* 345 $[M + Na]^+$. HR-ESI-MS *m/z* 345.1317 $[M + Na]^+$ (calcd for $C_{17}H_{22}O_6Na$, 345.1314).

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