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CHEMICAL CONSTITUENTS FROM THE ENDOPHYTIC FUNGUS *Annulohypoxylon squamulosum*

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Chemical investigation of the EtOAc-soluble fraction of the 95% EtOH extract of rice fermented with the endophytic fungus Annulohypoxylon squamulosum *(BCRC 34022) led to the isolation of a new γ-lactone, 4-hentriacontyl-dihydrofuran-2-one (**1**), together with nine known compounds, taraxasterol (**2**), oleanolic acid (**3**), β-sitostenone (**4**), ergosta-4,6,8(14),22-tetraen-3-one (**5**), cinnamic acid (**6**), a mixture of palmitic acid (7) and stearic acid (**8**), octadecyl ferulate (**9**), and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**10**). Their structures were elucidated by spectroscopic analyses, including 1D- and 2D NMR experiments, and by ESI-MS. Other known compounds were identified by comparing their spectral data with literature data.*

Keywords: *Annulohypoxylon squamulosum*, Xylariaceae, secondary metabolites, γ-lactone.

Endophytes, commonly present in almost all plants, are important sources of natural products with pharmaceutical potential [1, 2]. An endophytic fungal strain, named BCRC 34022, was collected from Fu-shan Botanical Garden, I-lan County, Taiwan, during August of 2004. This strain was determined to be *Annulohypoxylon squamulosum* (family Xylariaceae), based on their cultural and anamorphic data, by Ju [3]. 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 [4]. The secondary metabolites of the genus *Annulohypoxylon* have received less attention, and only one paper has been reported [4]. To further understand the chemotaxonomy of the genus *Annulohypoxylon* and to continue searching for novel bioactive metabolites from Xylariaceae, *A. squamulosum* was chosen for chemical investigation. Careful examination on the above title fungus has resulted in the isolation of one new γ-lactone, named 4-hentriacontyl-dihydrofuran-2-one (**1**), together with nine known constituents (**2**–**10**). Compounds **2**–**9** were obtained 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.

(*S*)-4-Hentriacontyl-dihydrofuran-2-one (**1**) was obtained as a colorless powder. The absence of UV absorption showed that the structure has no conjugated chromophore. IR spectrum revealed the γ-lactone absorption at 1780 cm^{-1}. The ^1H NMR spectra of **1** showed one *n*-hentriacontyl group at δ 0.89 (3H, t, J = 7.2 Hz), 1.24–1.50 (58H, br.s), 1.62 (1H, m), and 1.89 (1H, m), which was assigned to H-35, H-6–34, and H-5b/H-5a, respectively. The presence of a γ-butyrolactone moiety was suggested by the signals of two CH_2 groups at 1.91 (1H, m, H-3b), 2.35 (1H, m, H-3a), and 2.56 (2H, J = 9.4, 8.9 Hz, H-2) and an oxymethine signal at 4.55 (1H, m, H-4). The *n*-hentriacontyl group is occupied at C-4 of the γ-butyrolactone ring according to the contacts of the signals at 4.55 (H-4) and 1.62, and 1.89 (H-5) in the COSY spectrum. The molecular formula of **1** was established as $C_{35}H_{68}O_2$ by ESI- and HR-ESI-MS data, and the existence of a butyrolactone ring was supported by prominent mass fragments at *m/z* 85 $[C_4H_4O_2]^+$. The configuration at C-4 was determined to be 4*S* based on the correlation between the $[\alpha]_D$ –20.7° and the known *S*-configuration for the levorotatory (5*S*)-3,4-dihydro-5-nonyl-2(5*H*)-furanone ($[\alpha]_D^{25}$–32.2°) [5].

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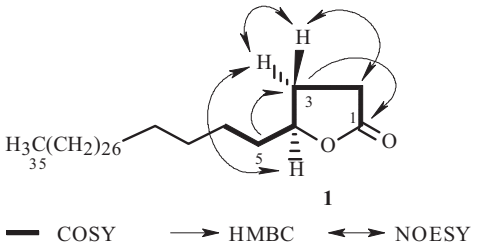

Fig. 1. Key COSY, HMBC, and NOESY correlations of **1**.

The correlations of H-5/C-3, H-2, 3/C-1, and H-4/C-2 were also observed in the HMBC experiment and further supported the position of the *n*-hentriacontyl group substitution. Accordingly, the structure of **1** was elucidated as (*S*)-4-hentriacontyl-dihydrofuran-2-one, which was further confirmed by ^{13}C NMR, COSY, NOESY, HMQC, and HMBC (Fig. 1) experiments.

The other known isolates, taraxasterol (**2**) [6], oleanolic acid (**3**) [7], β-sitostenone (**4**) [8], ergosta-4,6,8(14),22-tetraen-3-one (**5**) [9], cinnamic acid (**6**) [10], a mixture of palmitic acid (**7**) [11] and stearic acid (**8**) [12], octadecyl ferulate (**9**) [13], and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**10**) [14] were readily identified by comparison of their spectral data (UV, IR, ^{1}H NMR, MS) with data from the literature. Among them, all known compounds except **10** were isolated from *Annulohypoxylon* species for the first time.

EXPERIMENTAL

General Experimental Procedures. 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 a 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) with TMS as the internal standard. 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.

Extraction and Separation of Compounds. Rice with the fungi *A. squamulosum* BCRC 34022 (1 kg) was extracted three times with 95% EtOH at room temperature. The ethanol extract was partitioned with EtOAc to afford an EtOAc- soluble fraction (fraction A, 1.5 g). The EtOAc-soluble fraction (1.5 g) was chromatographed over silica gel (70–230 mesh), eluting with CH_2Cl_2 and enriched with acetone to produce five fractions (A1–A5). Fraction A-1 (85 mg) was chromatographed over silica gel, eluting with hexane–EtOAc (5:1→1:1) to obtain three fractions (A-1-1–A-1-3). Fraction A-1-3 (48 mg) was purified by preparative TLC to afford 4-hentriacontyl-dihydrofuran-2-one (**1**) (5.2 mg) and ergosta-4,6,8(14),22-tetraen-3-one (**5**) (1.2 mg). Fraction A-2 (155 mg) was chromatographed over silica gel, eluting with CH_2Cl_2–EtOAc (8:1→1:1) to obtain 10 fractions (A-2-1–A-2-10). Fraction A-2-1 (11.7 mg) was repeatedly purified by preparative TLC to afford a mixture of palmitic acid (**7**) and stearic acid (**8**) (8 mg). Fraction A-2-5 (16.7 mg) was repeatedly purified by preparative TLC to afford taraxasterol (**2**) (2.2 mg) and oleanolic acid (**3**) (1.5 mg). Fraction A-2-8 (45 mg) was purified by preparative TLC (*n*-hexane–EtOAc, 1:1) to give β-sitostenone (**4**) (1.7 mg) and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (**10**) (4.3 mg). Fraction A-4 (140 mg) was subjected to silica gel column chromatography (CH_2Cl_2–EtOAc, 5:1) to afford eight fractions (A-4-1–A-4-8). Fraction A-4-4 (42 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–EtOAc, 2:1) to afford octadecyl ferulate (**9**) (7.6 mg). Fraction A-5 (80 mg) was resubjected to silica gel column chromatography (CH_2Cl_2–MeOH, 5:1→1:1) to afford five fractions (A-5-1–A-5-5). Fraction A-5-3 (16 mg) was repeatedly purified by preparative TLC (CH_2Cl_2–EtOAc, 10:1) to afford cinnamic acid (**6**) (7.1 mg).

(*S*)-4-Hentriacontyl-dihydrofuran-2-one (1). Colorless powder; $[\alpha]_D^{22}$ –20.7° (*c* 0.074, $CHCl_3$). IR spectrum (KBr, ν_{max}, cm^{-1}) 1780 (lactone). ^{1}H NMR (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.89 (3H, t, J = 7.2, CH_3-35), 1.24–1.50 (58H, br.s, H-6–34), 1.62 (H, m, H-5b), 1.89 (H, m, H-5a), 1.91 (1H, m, H-3b), 2.35 (1H, m, H-3a), 2.56 (2H, dd, J = 9.4, 8.9, H-2), 4.55 (1H, m, H-4). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 14.2 (C-35), 22.7 (C-34), 25.2 (C-6), 27.9 (C-3), 28.9 (C-2), 29.4–30.0 (C-7-32),

32.1 (C-33), 35.9 (C-5), 81.5 (C-4), 178.2 (C-1). ESI-MS: *m/z* 543 $[M + Na]^+$. HR-ESI-MS *m/z* 543.5117 $[M + Na]^+$ (calcd for $C_{35}H_{68}O_2Na$, 543.5118).

Fungus Material. *Annulohypoxylon squamulosum* (BCRC 34022) 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. squamulosum* BCRC 34022 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 using 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 containing 100 mL mycelium broth and 100 mL RGY medium was introduced into plastic boxes (25 cm × 30 cm) containing 1 kg sterile rice and cultivated at 25°C, and the above mentioned RGY medium was added to maintain growth. After 21 days of cultivation, the rice was harvested and used as a sample for further extraction.

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