A New Isofuranonaphthalenone and Benzopyrans from the Endophytic Fungus *Nodulisporium* sp. A4 from *Aquilaria sinensis*

by **Zheng-Chao Wu**[a])[b])[c]), **Dong-Li Li**[a]), **Yu-Chan Chen**[a]), and **Wei-Min Zhang***[a])

[a]) Guangdong Provincial Open Laboratory of Applied Microbiology, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou 510070, P. R. China (phone: +86-20-37656321; fax: +86-20-87687286; e-mail: wmzhang58@yahoo.com.cn)

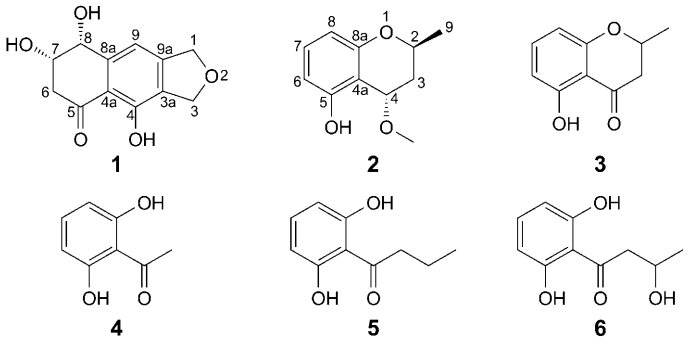
[b]) South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, P. R. China

[c]) Graduate School of the Chinese Academy of Sciences, Beijing 100049, P. R. China

A new isofuranonaphthalenone, (7*R**,8*S**)-3,6,7,8-tetrahydro-4,7,8-trihydroxynaphtho[2,3-*c*]furan-5(1*H*)-one (**1**), and a new benzopyran, (2*R**,4*R**)-3,4-dihydro-4-methoxy-2-methyl-2*H*-1-benzopyran-5-ol (**2**), together with four known related analogs, **3**–**6**, were isolated from the culture of *Nodulisporium* sp. A4, an endophytic fungus from the stem of *Aquilaria sinensis* (LOUR.) GILG. The structures of the isolated compounds were determined by extensive analysis of their spectroscopic data as well as by comparison with literature reports. The isolated compounds **1**–**6** were evaluated for their cytotoxic activities against the NCI-H460 and SF-268 tumor cell lines.

Introduction. – Endophytic fungi are considered as a rich source of bioactive metabolites [1–6], some of which being promising candidates for pharmaceutical application. In our previous research, we had isolated and identified some endophytic fungi from *Aquilaria sinensis* (LOUR.) GILG., a well-known traditional Chinese medicinal plant [7]. Among these fungi, the strain A4 belonging to the genus *Nodulisporium*, a group of common endophytic fungi harbored by many forest trees and known to produce bioactive secondary metabolites [8–10], was selected for further investigation, as its crude extract showed remarkable inhibitory activity against tumor cells SF-268 based on our primary screening. We report here the isolation, structure elucidation, and cytotoxic evaluation of a new isofuranonaphthalenone, **1**, and a new benzopyran, **2**, from this fungus.

Results and Discussion. – 1. *Isolation and Structure Elucidation*. The culture broth of *Nodulisporium* sp. A4 was separated from the mycelia by filtration and then extracted with AcOEt. The concentrated extract was further purified by a combination of silica gel, *Sephadex LH-20*, reversed-phase silica-gel column chromatography, preparative TLC and HPLC to afford one new isofuranonaphthalenone, **1**, and one new benzopyran, **2**, together with four known benzopyran derivatives, **3**–**6**. The known compounds were identified by comparing their NMR data with those reported in the literature: 2,3-dihydro-5-hydroxy-2-methylchromen-4-one (**3**) [11], 1-(2,6-dihydroxyphenyl)ethanone (**4**) [12], 1-(2,6-dihydroxyphenyl)butan-1-one (**5**) [13][14], and 1-(2,6-dihydroxyphenyl)-3-hydroxybutan-1-one (**6**) [8].



Compound **1** was obtained as colorless crystals. Its IR spectrum showed absorption bands for OH (3494 and 3385 cm^{-1}), C=O (1636 cm^{-1}), and aromatic (1578 cm^{-1}) functionalities. The molecular formula was determined as $C_{12}H_{12}O_5$ (seven degrees of unsaturation) by HR-EI-MS (*m/z* 236.0678 (M^+, $C_{12}H_{12}O_5^+$; calc. 236.0679)), which was in agreement with the ^{1}H- and ^{13}C-NMR data (see *Exper. Part*). The ^{1}H-NMR spectrum exhibited signals attributed to one phenolic OH group (δ(H) 12.48 (HO–C(4))), one aromatic H-atom (δ(H) 7.03 (H–C(9))), two aliphatic OH groups (δ(H) 5.80 (HO–C(8)) and 5.40 (HO–C(7))), three aliphatic CH_2 groups (δ(H) 5.04 (CH_2(1)), 4.90 (CH_2(3)), 2.99 (H_{eq}–C(6)) and 2.65 (H_{ax}–C(6))), two aliphatic CH groups (δ(H) 4.50 (H–C(8)), 3.98 (H–C(7))). The ^{13}C-NMR and DEPT spectra indicated the presence of three CH_2 groups including two O-bearing C-atoms (δ(C) 73.5 (C(1)), 70.4 (C(3)), and 43.1 (C(6))), three CH groups including one aromatic (δ(C) 112.2 (C(9))) and two O-bearing (δ(C) 71.2 (C(8)) and 69.8 (C(7))) ones, and six quaternary C-atoms, of which five were aromatic (δ(C) 155.4 (C(4)), 149.1 (C(9a)), 146.0 (C(8a)), 124.7 (C(3a)), 114.5 (C(4a))), and one was a ketone C=O C-atom (δ(C) 203.8 (C(5))). Analysis of the ^{1}H,^{1}H-COSY in combination with the HSQC spectrum led to the identification of the partial structure shown by the bold lines in the *Figure*. In the HMBC spectrum (*Fig.*), correlations observed from HO–C(8) to C(8) and C(8a) indicated that C(8) was attached to the aromatic C-atom C(8a). Other HMBCs from H–C(6) to C(4a) and C(5), and from H–C(8) to C(4a) indicated the connection of C(6) to C(5), and of C(5) to C(4a), forming a six-membered ring (*A*) which was fused to the aromatic ring (*B*) along C(4a) and C(8a). HMBC Cross-peaks observed from HO–C(4) to C(3a), C(4), and C(4a) revealed that the aromatic OH group was attached to C(4). The HMBC spectrum also revealed correlations from CH_2(1) to

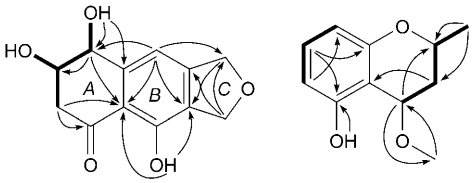


Figure. *^{1}H,^{1}H-COSY* (bold line) *and key HMBCs* (arrow) *data of* **1** *and* **2**

C(3a), and from CH_2(3) to C(9a), suggesting the connections of C(1) to C(9a) and C(3) to C(3a), respectively, which was confirmed by the cross-peak from H–C(9) to C(1). Considering the NMR data of the two O-bearing CH_2 groups, C(1) and C(3), as well as the molecular formula, these two CH_2 groups have to be connected *via* the remaining O-atom to form a five-membered ring (*C*). Thus, the planar structure of **1** was determined. The relative configuration of **1** was assigned by the analysis of H-atom coupling constants and a NOESY spectrum. Small *J* values (3.8 and 7.3 Hz) exhibited by H_{eq}–C(6) and H_{ax}–C(6) with H–C(7) suggested vicinal eq–eq and eq–ax couplings, revealing the equatorial orientation for H–C(7). NOESY Correlation between H_{ax}–C(6) and H–C(8) revealed that H–C(8) should also be in axial position. However, the absolute configuration of **1** remains unknown. Based on the above spectral evidence, the structure of **1** was established as (7*R**,8*S**)-3,6,7,8-tetrahydro-4,7,8-trihydroxynaphtho[2,3-*c*]furan-5(1*H*)-one, which was named as nodulone.

Compound **2** was obtained as a brown gum. The IR spectrum showed absorption bands for OH (3360 cm^{-1}) and aromatic (1616 and 1592 cm^{-1}) functionalities. The molecular formula was determined as $C_{11}H_{14}O_3$ (five degrees of unsaturation) by HR-EI-MS (*m*/*z* 194.0938 (M^+, $C_{11}H_{14}O_3^+$; calc. 194.0937)), which was in agreement with the ^{1}H- and ^{13}C-NMR data (see *Exper. Part*). Interpretation of the ^{1}H-, ^{13}C-, and HSQC NMR data of **2** revealed the presence of one OH group (HO–C(5)), one 1,2,3-trisubstituted (C(4a), C(5), and C(8a)) benzene ring, two CH–O groups (C(2) and C(4)), one CH_2 group (C(3)), one Me group (C(9)), and one MeO group (MeO–C(4)). Comparison of the ^{1}H- and ^{13}C-NMR data of **2** with those of the reported benzopyran derivative 3,4-dihydro-2-methyl-2*H*-1-benzopyran-4,5-diol [15] revealed that the structures of these two compounds are very similar except the presence of a MeO group (δ(H) 3.47; δ(C) 54.6) in **2**. The HMBCs from H–C(4) (δ(H) 4.54) to the MeO C-atom, and from the MeO H-atoms to C(4) (δ(C) 69.8) indicated that the MeO group was attached to C(4) in **2** (*Fig.*), instead of an OH group in 3,4-dihydro-2-methyl-2*H*-1-benzopyran-4,5-diol. The relative configuration of **2** was assigned by the analysis of H-atom coupling constants. Small coupling constants (2.2 and 4.9 Hz) exhibited by H–C(4) revealed that H–C(4) was in an equatorial orientation. The large coupling constant 11.2 Hz observed for H_{ax}–C(3) and H–C(2) suggested the axial orientation for H–C(2), which is in agreement with the literature reports that, in benzopyrans, an equatorial orientation is preferred for the substituent at C(2) [15–17]. However, the absolute configuration of **2** remains unknown. Based on the above spectral evidence, the structure of **2** was established as (2*R**,4*R**)-3,4-dihydro-4-methoxy-2-methyl-2*H*-1-benzopyran-5-ol.

2. *Cytotoxic Activity.* Compounds **1**–**6** were evaluated for their cytotoxic activities against the NCI-H460 and SF-268 cell lines by using the CCK-8 assay [18]. Only **2** exhibited weak cytoxicity (*ca.* 57.9% inhibition rate) against the SF-268 cell line at the concentration of 100 μg/ml, compared with the positive control, cisplatin, which showed significant inhibition of cell proliferation at the concentration of 5 μg/ml (*ca.* 97.1% inhibition rate).

This work was supported by the *National Natural Science Foundation of China* (No. 20902014), by the *Guangdong Provincial Project for Science and Technology* (No. 2008B090500085), by the *Natural Science Foundation of Guangdong Province* (No. 9451007002003074), and by the *Guangzhou Project for Science and Technology* (No. 09B34061446).

Experimental Part

General. Column chromatography (CC): commercial silica gel (SiO_2; 200–300 mesh; *Qingdao Haiyang Chemical Group Co.*), *Chromatorex ODS* (40–75 μm; *Fuji Silysia*), and/or *Sephadex LH-20* (*Amersham Biosciences*). TLC: precoated silica gel plates *GF-254* (*Qingdao Haiyang*). Semiprep. HPLC: *Shimadzu LC-20AB* pump, *Shimadzu SPD-M20A* diode-array detector, and *YMC-pack ODS-A* column (5 μm, 250 × 10 mm). M.p.: *Netzsch DSC 204* apparatus. Optical rotations: *Perkin-Elmer 341* polarimeter. UV Spectra: *Biochrom Ultrospec 6300pro* UV-visible spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Bruker EQUINOX 55* spectrophotometer; in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker Avance-500* spectrometer; at 500 and 125 MHz, resp.; δ in ppm, J in Hz. EI-MS: *Thermo DSQ* mass spectrometer and/or *Thermo MAT95XP* mass spectrometer; in m/z. HR-EI-MS: *Thermo MAT95XP* mass spectrometer; in m/z.

Fungal Material. The endophytic fungal strain *Nodulisporium* sp. A4 was isolated from the stem of *A. sinensis* (LOUR.) GILG. (Thymelaeaceae), which was collected at Xinyi, Guangdong Province, P. R. China, in November, 2007. The strain was indentified by sequence analysis of rDNA ITS (internal transcribed spacer) region. The sequence of ITS region of A4 has been submitted to GenBank (Accession No. EU781661). By using BLAST (nucleotide sequence-comparison program) to search the GenBank database, A4 has almost 100% similarity to *Nodulisporium* sp. JP807 (GenBank Accession No. AF 280629) and less than 95% similarity to the other *Nodulisporium* species [7]. Therefore, A4 and *Nodulisporium* sp. JP807 are of the same species and different from the other *Nodulisporium* species based on ITS sequence analysis. The strain is preserved at the Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology.

Extraction and Isolation. *Nodulisporium* sp. A4 was cultured in liquid medium. The fresh mycelia grown on potato–dextrose agar (PDA) medium at 28° for 3 d was inoculated into three flasks (500 ml) containing potato–dextrose (PD) medium (250 ml). After 5 d of incubation at 28° on a rotary shaker at 150 rpm, a portion of the liquid culture (50 ml) was aseptically transferred into each of a total of 80 flasks (1000 ml), which contain PD medium (500 ml). The liquid cultivation that followed was kept for 7 d at 28° and 150 rpm on a rotary shaker. The culture broth (40 l) was separated from the mycelia by filtration under vacuum and extracted with AcOEt (4 × 15 l). Evaporation of the solvent under reduced pressure at 40° afforded the AcOEt extract as a dark gum (14.9 g). The AcOEt extract was submitted to CC (SiO_2; petroleum ether (PE)/AcOEt 50:1 to 0:1 and $CHCl_3$/MeOH gradient 5:1; 0:1) to yield 20 fractions, *Frs. 1–20*, on the basis of TLC analysis. *Fr. 2* was further fractionated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1) and then purified by CC (reversed-phase (RP) SiO_2 C_{18}; MeOH) to give compound **3** (44.7 mg). *Fr. 7* was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1), followed by CC (RP SiO_2 C_{18}; MeOH) to yield compound **4** (51.7 mg). *Fr. 9* was fractionated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1), and one subfraction was further subjected to CC (RP SiO_2 C_{18}; MeOH), followed by HPLC (70% MeOH/H_2O) to yield compound **5** (7.4 mg). *Fr. 10* was separated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1) and then fractionated by CC (RP SiO_2 C_{18}; MeOH/H_2O 80:20), followed by prep. TLC (PE/AcOEt 8:1) to afford compound **2** (16.1 mg). *Fr. 14* was fractionated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1), and one subfraction was further subjected to CC (SiO_2), followed by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1), and finally purified by recrystallization from $CHCl_3$ to yield compound **6** (31.9 mg). *Fr. 18* was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1) to obtain compound **1** (13.5 mg).

Nodulone (=(*7*R*,*8*S*)*-3,6,7,8-Tetrahydro-4,7,8-trihydroxynaphtho[2,3-c]furan-5(1*H*)-one*; **1**). Colorless crystals. M.p. 226°. $[\alpha]_D^{20} = +17.9$ ($c = 0.61$, DMSO). UV (MeOH): 222 (4.24), 272 (3.98), 329 (3.50). IR (KBr): 3494, 3385, 2925, 2855, 1636, 1578, 1435, 1285, 1071. ^1H-NMR (500 MHz, (D_6)DMSO): 12.48 (br. *s*, HO–C(4)); 7.03 (*s*, H–C(9)); 5.80 (*d*, $J = 5.6$, HO–C(8)); 5.40 (*d*, $J = 3.7$, HO–C(7)); 5.04 (br. *s*, CH_2(1)); 4.90 (br. *s*, CH_2(3)); 4.50 (*t*, $J = 5.6$, H–C(8)); 3.96–3.99 (*m*, H–C(7)); 2.99 (*dd*, $J =$ 17.2, 3.8, H_{eq}–C(6)); 2.65 (*dd*, $J = 17.2$, 7.3, H_{ax}–C(6)). ^{13}C-NMR (125 MHz, (D_6)DMSO): 203.8 (C(5)); 155.4 (C(4)); 149.1 (C(9a)); 146.0 (C(8a)); 124.7 (C(3a)); 114.5 (C(4a)); 112.2 (C(9)); 73.5 (C(1)); 71.2 (C(8)); 70.4 (C(3)); 69.8 (C(7)); 43.1 (C(6)). EI-MS: 236 (36, M^+), 208 (19), 190 (29), 149 (31), 136 (36), 107 (49), 77 (95), 61 (100). HR-EI-MS: 236.0678 (M^+, $C_{12}H_{12}O_5^+$; calc. 236.0679).

*(2*R*,4*R*)-3,4-Dihydro-4-methoxy-2-methyl-2*H*-chromen-5-ol* (**2**). Brown gum. $[\alpha]_D^{20} = -4.8$ ($c =$ 0.92, $CHCl_3$). UV ($CHCl_3$): 281 (3.17). IR (KBr): 3360, 2926, 2855, 1616, 1592, 1466, 1343, 1087, 1022.

^{1}H-NMR (500 MHz, $CDCl_3$): 7.09 (*t*, *J* = 8.1, H–C(7)); 6.46 (*dd*, *J* = 8.1, 2.4, H–C(6), H–C(8)); 6.40 (br. *s*, HO–C(5)); 4.54 (*dd*, *J* = 4.9, 2.2, H–C(4)); 4.09–4.13 (*m*, H–C(2)); 3.47 (*s*, MeO); 2.25 (*dt*, *J* = 14.8, 2.2, H_{eq}–C(3)); 1.73 (*ddd*, *J* = 14.8, 11.2, 4.9, H_{ax}–C(3)); 1.42 (*d*, *J* = 6.3, Me). ^{13}C-NMR (125 MHz, $CDCl_3$): 156.8 (C(5)); 156.1 (C(8a)); 130.0 (C(7)); 109.1 (C(4a), C(6)); 107.9 (C(8)); 69.8 (C(4)); 68.2 (C(2)); 54.6 (MeO); 33.4 (C(3)); 20.8 (Me). EI-MS: 194 (12), 162 (83), 161 (23), 147 (100, M^+). HR-EI-MS: 194.0938 (M^+, $C_{11}H_{14}O_3^+$; calc. 194.0937).

Cytotoxicity Assays. Cytotoxic assays against the NCI-H460 and SF-268 cell lines were carried out as reported in [18].

REFERENCES

[1] R. X. Tan, W. X. Zou, *Nat. Prod. Rep.* **2001**, *18*, 448.
[2] A. H. Aly, R. Edrada-Ebel, V. Wray, W. E. G. Müller, S. Kozytska, U. Hentschel, P. Proksch, R. Ebel, *Phytochemistry* **2008**, *69*, 1716.
[3] B. P. Bashyal, E. M. K. Wijeratne, S. H. Faeth, A. A. L. Gunatilaka, *J. Nat. Prod.* **2005**, *68*, 724.
[4] M. L. Macías-Rubalcava, B. E. Hernández-Bautista, M. Jiménez-Estrada, M. C. González, A. E. Glenn, R. T. Hanlin, S. Hernández-Ortega, A. Saucedo-García, J. M. Muria-González, A. L. Anaya, *Phytochemistry* **2008**, *69*, 1185.
[5] X.-K. Xia, H.-R. Huang, Z.-G. She, J.-W. Cai, L. Lan, J.-Y. Zhang, L.-W. Fu, L. L. P. Vrijmoed, Y.-C. Lin, *Helv. Chim. Acta* **2007**, *90*, 1925.
[6] L. Yuan, P.-J. Zhao, J. Ma, C.-H. Lu, Y.-M. Shen, *Helv. Chim. Acta* **2009**, *92*, 1118.
[7] L. Wang, W. M. Zhang, Q. L. Pan, H. H. Li, M. H. Tao, X. X. Gao, *J. Fungal Res.* (in Chinese) **2009**, *7*, 37.
[8] J. Dai, K. Krohn, U. Flörke, S. Draeger, B. Schulz, A. Kiss-Szikszai, S. Antus, T. Kurtán, T. van Ree, *Eur. J. Org. Chem.* **2006**, 3498.
[9] S. Kamisuki, C. Ishimaru, K. Onoda, I. Kuriyama, N. Ida, F. Sugawara, H. Yoshida, Y. Mizushina, *Bioorg. Med. Chem.* **2007**, *15*, 3109.
[10] J. G. Ondeyka, G. L. Helms, O. D. Hensens, M. A. Goetz, D. L. Zink, A. Tsipouras, W. L. Shoop, L. Slayton, A. W. Dombrowski, J. D. Polishook, D. A. Ostlind, N. N. Tsou, R. G. Ball, S. B. Singh, *J. Am. Chem. Soc.* **1997**, *119*, 8809.
[11] L. E. Gray, H. W. Gardner, D. Weisleder, M. Leib, *Phytochemistry* **1999**, *50*, 1337.
[12] C. K. Lau, P. C. Bélanger, C. Dufresne, J. Scheigetz, *J. Org. Chem.* **1987**, *52*, 1670.
[13] H.-R. Huang, X.-L. Feng, Z.-G. She, Y.-C. Lin, L. L. P. Vrijmoed, E. B. G. Jones, *Acta Crystallogr., Sect. E* **2005**, *o61*, 282.
[14] A. Arnoldi, *Synthesis* **1984**, 856.
[15] H. L. Teles, G. H. Silva, I. Castro-Gamboa, V. da Silva Bolzani, J. O. Pereira, C. M. Costa-Neto, R. Haddad, M. N. Eberlin, M. C. M. Young, A. R. Araújo, *Phytochemistry* **2005**, *66*, 2363.
[16] D.-L. Li, X.-M. Li, T.-G. Li, H.-Y. Dang, P. Proksch, B.-G. Wang, *Chem. Pharm. Bull.* **2008**, *56*, 1282.
[17] V. Joshi, J. R. Merchant, *Indian J. Chem., Sect. B* **1984**, *23*, 266.
[18] G. Hou, L. Xue, Z. Lu, T. Fan, F. Tian, Y. Xue, *Cancer Lett.* **2007**, *253*, 236.

Received August 26, 2009