Palmarumycins from the Endophytic Fungus *Lasiodiplodia pseudotheobromae* XSZ-3

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One new and three known spiro-dioxynaphthalene compounds, related to palmarumycins, were isolated from the extracts of fungus *Lasiodiplodia pseudotheobromae* XSZ-3. Their structures were established by spectroscopic methods (1D- and 2D-NMR, HR-ESI-MS, *etc.*). The absolute configuration of **1** was determined by comparison of quantum-chemical TDDFT (time-dependent density-functional theory) calculated and experimental ECD (electronic circular dichroism) spectra. The cytotoxic activities of **1**–**4** against HL-60 cell line were evaluated by trypan blue-staining assay.

Introduction. – Palmarumycins belong to a structurally remarkable class of natural products mainly isolated from various fungal cultures. Their common structural feature is the 1,8-dioxy-naphthalene moiety linked to a decalin unit *via* a spiroketal C-atom [1]. Because of its interesting structural patterns and important biological properties, a large number of investigations on this family have been carried out in the last few years [2]. These metabolites display a wide range of biological features, including antibacterial [3][4], antifungal [4][5], and antitumor activities [6][7].

Lasiodiplodia pseudotheobromae is a common pathogenic fungus found on a great variety of host plants. This fungus causes numerous diseases, in particular causing rotting of fruits and root crops during storage. As a part of our research on the discovery of biologically active metabolites from endophytic fungus *L. pseudotheobromae* XSZ-3, four palmarumycins have been isolated, one of which was characterized as a new compound, **1**, named palmarumycin LP1, while the other three compounds were the known palmarumycins, identified as cladospirone B (**2**) [8], ascochytatin (**3**) [9], and Sch 50676 (**4**) [10] (*Fig. 1*). To the best of our knowledge, all of these compounds are isolated from this species of fungus for the first time. Herein, we report the isolation and structure elucidation of the new compound **1**, and the cytotoxic activities of **1**–**4**.

Results and Discussion. – Compound **1** was isolated as a gray powder. Its molecular formula, $C_{20}H_{14}O_7$ (14 degrees of unsaturation), was deduced from its HR-ESI-MS (*m*/*z* 367.0808 ($[M+H]^+$, $C_{20}H_{15}O_7^+$; calc. 367.0818)). Its IR absorptions implied the presence of OH (3399 cm^{-1}) and ketone C=O (1642 cm^{-1}) functionalities.

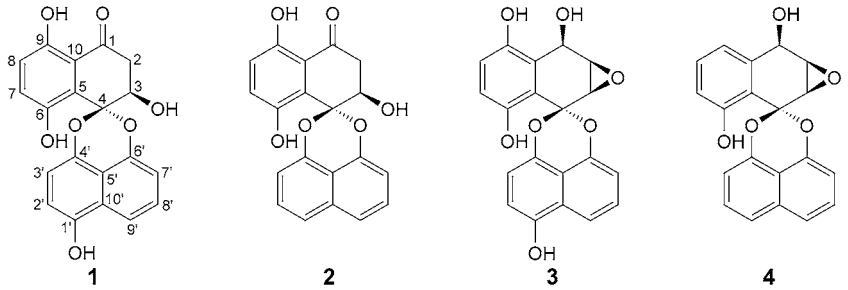

Fig. 1. *Structures of* **1**–**4**

The ^{1}H-NMR spectrum of **1** (*Table*) exhibited seven aromatic H-atom signals, of which three are assigned to a 1,2,3-trisubstituted phenyl ring (δ(H) 7.69 (*d*, $J = 8.5$, 1 H), 7.39 (*dd*, $J = 8.5$, 7.4, 1 H), 6.95 (*d*, $J = 7.4$, 1 H)) and others to 1,2,3,4-tetrasubstituted phenyl rings (7.25 (*d*, $J = 9.1$, 1 H), 7.01 (*d*, $J = 9.1$, 1 H), and 6.89 (*d*, $J = 8.1$, 1 H), 6.86 (*d*, $J = 8.2$, 1 H)), three aliphatic H-atom signals (4.22–4.24 (*m*, 1 H), 3.16 (*dd*, $J = 16.2$, 2.3, 1 H), 2.64 (*dd*, $J = 16.4$, 4.1, 1 H)), along with four OH H-atom signals (12.00 (*s*), 9.90 (*s*), 9.00 (*s*), 5.66 (*d*, $J = 3.0$)), which were induced by HSQC spectrum. The ^{13}C-NMR spectrum displayed signals for 20 C-atoms, including

Table. *^{1}H- and ^{13}C-NMR Data of* **1** (600 and 150 MHz, resp., (D_6)DMSO), *and its HMBCs.* δ in ppm, J in Hz. Atom numbering as indicated in *Fig. 1.*

Position	δ(H)	δ(C)	HMBC (H → C)
1		203.5	
2	3.16 (*dd*, $J = 16.2$, 2.3), 2.64 (*dd*, $J = 16.4$, 4.1)	43.4	1, 3
3	4.22–4.24 (*m*)	65.6	
4		100.4	
5		119.9	
6		149.8	
7	7.25 (*d*, $J = 9.1$)	128.6	4, 5, 6, 9
8	7.01 (*d*, $J = 9.1$)	120.7	6, 9, 10
9		154.5	
10		115.6	
1′		148.1	
2′	6.86 (*d*, $J = 8.2$)	109.7	1′, 4′, 10′
3′	6.89 (*d*, $J = 8.1$)	110.7	1′, 4′, 5′
4′		138.5	
5′		113.9	
6′		147.9	
7′	6.95 (*d*, $J = 7.4$)	109.3	5′, 6′, 9′
8′	7.39 (*dd*, $J = 8.5$, 7.4)	126.4	10′
9′	7.69 (*d*, $J = 8.5$)	115.8	1′, 5′, 7′
10′		124.9	
3-OH	5.66 (*d*, $J = 3.0$)		4
6-OH	9.00 (*s*)		5, 6, 7
9-OH	12.00 (*s*)		8, 9, 10
1′-OH	9.90 (*s*)		

one C=O C-atom (δ(C) 203.5), 16 aromatic C-atoms (δ(C) 154.5, 149.8, 148.1, 147.9, 138.5, 128.6, 126.4, 124.9, 120.7, 119.9, 115.8, 115.6, 113.9, 110.7, 109.7, 109.3), one ketal C-atom (δ(C) 100.4), and two aliphatic C-atoms (δ(C) 65.6, 43.4).

The assignments of all H-bearing C-atoms were achieved by HSQC experiment (*Table*). The HMBC spectrum showed signal correlations of δ(H) 6.86 (H–C(2′)) to δ(C) 148.1 (C(1′)), 138.5 (C(4′)), and 124.9 (C(10′)), of δ(H) 6.89 (H–C(3′)) to δ(C) 148.1 (C(1′)), 138.5 (C(4′)), and 113.9 (C(5′)), of δ(H) 6.95 (H–C(7′)) to δ(C) 113.9 (C(5′)), 147.9 (C(6′)), and 115.8 (C(9′)), of δ(H) 7.39 (H–C(8′)) to δ(C) 124.9 (C(10′)), and of δ(H) 7.69 (H–C(9′)) to δ(C) 148.1 (C(1′)), 113.9 (C(5′)), and 109.3 (C(7′)), confirming the presence of the trioxygenated naphthalene unit 1a (*Fig. 2*). The correlations δ(H) 12.00 (HO–C(9))/δ(C) 120.7 (C(8)), 154.5 (C(9)), and 115.6 (C(10)); δ(H) 9.00 (HO–C(6))/δ(C) 149.8 (C(6)), 128.6 (C(7)), and 119.9 (C(5)); δ(H) 3.16, 2.64 (H–C(2))/δ(C) 203.5 (C(1)) and 65.6 (C(3)); δ(H) 5.66 (HO–C(3))/δ(C) 100.4 (C(4)); δ(H) 7.01 (H–C(8))/δ(C) 149.8 (C(6)), 154.5 (C(9)), and 115.6 (C(10)); and δ(H) 7.25 (H–C(7))/δ(C) 100.4 (C(4)), 119.9 (C(5)), 149.8 (C(6)), and 154.5 (C(9)) established the moiety 1b (*Fig. 2*). The trioxynaphthalene unit should be linked to C(4) (δ(C) 100.4) *via* two O-atoms of the ketal moiety, thereby completing the planar structure of **1** as shown in *Fig. 1*.

The small coupling constants ($^3J(2,3) = 4.1$ and 2.4) indicated that the OH group at C(3) is axially oriented with a half-chair conformation of the six-membered ring. The absolute configurations of C(3) and C(4) were determined by comparison of the quantum-chemical TDDFT-calculated with the experimental electronic circular dichroism (ECD) spectra. There are four possible isomers of **1** (*Fig. 3*), which were optimized using DFT at the B3LYP/6-31G (d) level in the GAUSSIAN 09 program. Each optimized conformation was calculated using DFT at the B3LYP/6-311G (d, p) in the GAUSSIAN 09 program to generate its ECD property (*Fig. 4*). The calculated

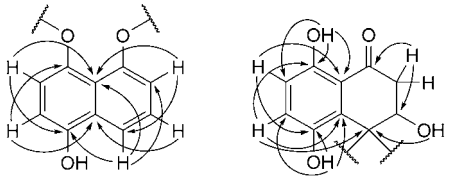

Fig. 2. *Key HMBCs* (H → C) *of the moieties of* **1**

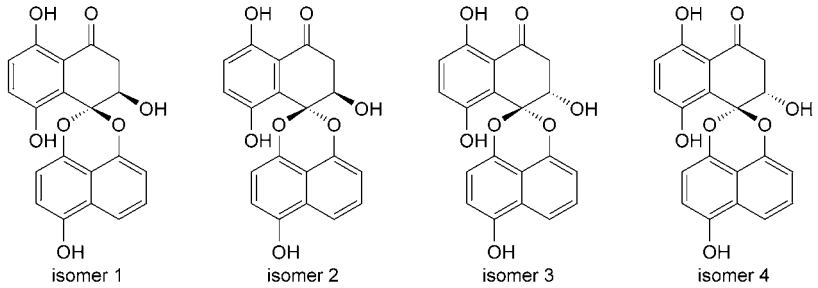

Fig. 3. *Four stereoisomers of* **1**

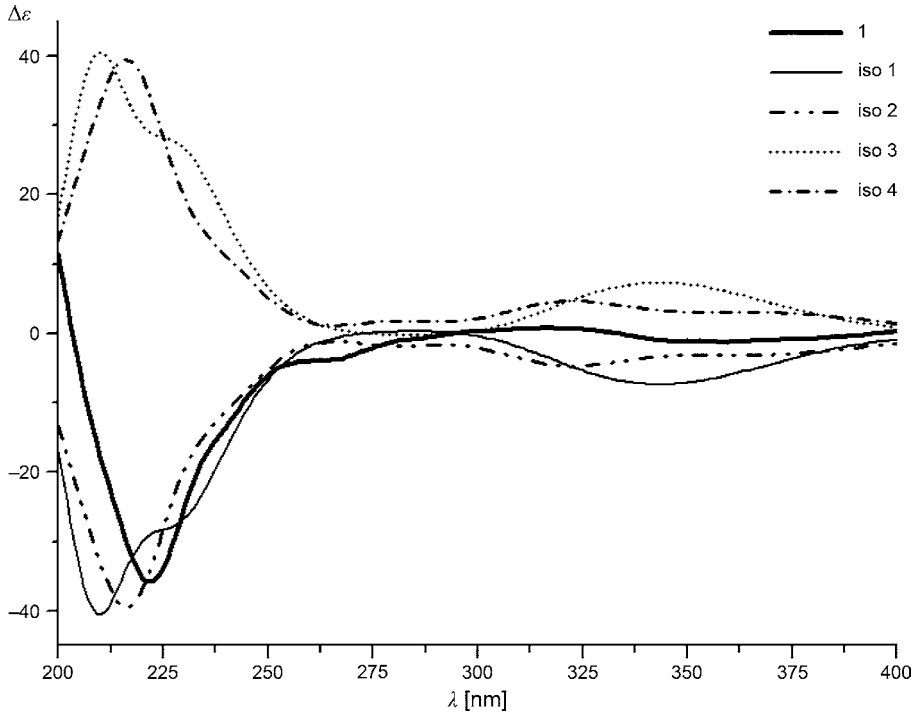

Fig. 4. *Quantum-chemical TDDFT-calculated ECD spectra of the four stereoisomers and experimental ECD spectrum of* **1**

ECD spectrum of isomer 2 was the most similar compared with the experimental CD spectrum of **1**. So the absolute configurations at C(3) and C(4) were determined to be (*R*) and (*S*), respectively.

The cytotoxic activities of **1**–**4** against HL-60 (human promyelocytic leukemia cells) cell line were evaluated. Compounds **1** and **4** exhibited significant inhibitory effects with IC_{50} values of 2.39 and 1.41 μM, respectively. Compound **2** exhibited a moderate inhibitory effect with an IC_{50} value of 10.91 μM, and **3** exhibited no cytotoxicity against HL-60 with an IC_{50} value > 100 μM (IC_{50} value of 5-fluorouracil (5-FU), 1.87 μM).

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 100–200 and 200–300 mesh; *Qingdao Haiyang Chemical Co., Ltd.*, China), *Sephadex LH-20* (*Pharmacia*, USA). HPLC: *Hitachi L-6000* pump, *Hitachi L-7400* UV detector, and *YMC-pack ODS-A* (10 × 250 mm, 5 μm) column. Optical rotations: *241MC PerkinElmer* polarimeter. UV Spectra: *Shimadzu-UV-1700* spectrophotometer; λ_{max} in nm. ECD: *JASCO CD-2095* Chiral detector, in MeOH; λ_{max} ($\Delta\varepsilon$) in nm. IR Spectra: *PerkinElmer IFS-55*

spectrophotometer; KBr discs; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker ARX-300* and *AV-600* NMR spectrometers; δ in ppm rel. to Me_4Si as internal standard, J in Hz. HR-ESI-MS: *Varian QFT-ESI* mass spectrometer, in m/z.

Microorganism Material and Fermentation. The fungal strain XSZ-3 was isolated from the branch of plant *Camptotheca acuminata* collected from Panzhihua, Sichuan Province, P. R. China, and identified as *Lasiodiplodia pseudotheobromae* (GenBank accession No. KF 938553) by the morphological evaluation and sequence ITS1-5.8S-ITS2 of rRNA. The voucher specimen is deposited at $-80°$ with the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

The fungus was cultivated in a flask at r.t. The liquid medium was composed of mannitol, 2%; D-glucose, 2%; yeast extract, 0.5%; peptone, 1%; KH_2PO_4, 0.05%; $MgSO_4 \cdot 7\,H_2O$, 0.03%; corn syrup, 0.1%, which were dissolved in dist. H_2O.

Extraction and Isolation. After 30 d of cultivation, the fermented broth (75 l) was filtered through cheesecloth to be separated into supernatant and mycelia. The supernatant was concentrated and extracted four times with AcOEt to yield the AcOEt extract. The mycelia mass was ultrasonically extracted with 80% acetone for three times (1 h each), and concentrated by rotary evaporation, then extracted four times with AcOEt to yield the AcOEt extract. The two AcOEt extracts were combined to give the crude extract (60.0 g).

The crude extract was subjected to CC (SiO_2; CH_2Cl_2/MeOH 100:0 to 0:100) to yield twelve fractions. *Fr. 4* (4.0 g) was subjected to CC (*Sephadex LH-20*; MeOH) to give four subfractions. *Fr. 4.4* was submitted to HPLC (MeOH/H_2O 5.8:4.2; flow rate, 4 ml min^{-1}) to give **1** (t_R 38 min; 6.0 mg), **2** (t_R 11 min; 2.4 mg), and **3** (t_R 18 min; 3.0 mg). *Fr. 3* (4.5 g) was purified by CC (SiO_2; petroleum ether/acetone 100:0 to 0:100) to yield ten subfractions. *Fr. 3.8* was subjected to CC (*Sephadex LH-20*; MeOH) to give **4** (3.3 mg).

Palmarumycin LP1 (=*(1*S,*2*R*)-2,3-Dihydro-2,5,6′,8-tetrahydroxy-4*H-*spiro[naphthalene-1,2′-naphtho[1,8-*de*][1,3]dioxin]-4-one*; **1**). Gray powder (MeOH). $[\alpha]_D^{25} = -35$ ($c = 0.4$, MeOH). UV (MeOH): 219, 309, 325, 340. ECD (MeOH): 221 (−38.5), 311 (1.49), 363 (−2.14). IR: 3399, 2927, 2833, 1642, 1450, 1384, 1118, 1030. ^1H- and ^{13}C-NMR, HSQC, and HMBC: see the *Table*. HR-ESI-MS: 367.0808 ($[M+H]^+$, $C_{20}H_{15}O_7^+$; calc. 367.0818).

Cytotoxic Activities of the Compounds. RPMI-1640 medium (*Gibco*, New York, NY, USA) contained 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 1 mmol glutamine, and 10% heat-inactivated fetal bovine serum (*Gibco*). Human leukemia HL-60 cells (*American Type Culture Collection*, Rockville, MD, USA) were cultured in the above medium at a density of $5 \cdot 10^4$ cells/ml at 37° under an atmosphere of 5% CO_2. The compounds were dissolved in DMSO, and the amount of DMSO was maintained lower than 0.1% in the final concentration. Cells were incubated with drug concentrations of 1, 2, 4, 8, and 16 μM for 3 d. The number of cells was determined by hemocytometer, and its viability was determined using trypan blue staining. The growth-inhibitory abilities of the compounds were calculated and expressed using the IC_{50} value (half-inhibitory concentration). 5-FU and 0.1% DMSO were used as positive and negative control, resp.

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