Three New 3,6-Dioxygenated Diketopiperazines from the Basidiomycete *Lepista sordida*

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Three new 3,6-dioxygenated diketopiperazines, lepistamides A – C (**1** – **3**), along with a known compound, diatretol (**4**), were isolated from the mycelial solid cultures of the basidiomycete *Lepista sordida*. Their structures were elucidated by spectroscopic means. The isolated diketopiperazines were evaluated for the cytotoxic activity against Astc-a-1, Bel 7402, and HeLa cell lines, and their biogenetic pathway was discussed.

Introduction. – Recently, much attention has been paid to higher basidiomycetes because of their potential as a source of biologically active substances [1]. *Lepista sordida* (SCHUMACH.) SINGER, a basidiomycetous fungus of the family Tricholomataceae, is an edible agaric species [2]. Two diterpenes (lepistal and lepistol) with the activity to induce differentiation in human leukaemia cells were previously isolated from the culture filtrate of this fungus [3]. During a screening for bioactive metabolites produced by macrofungi collected from South China, an EtOH extract from the mycelial solid cultures of a strain (GIM 5.315) of this basidiomycete showed cytotoxic activity against HeLa cells and antibacterial activity against *Staphylococcus aureus*. The metabolites of this fungus were therefore investigated. Three new diketopiperazines, trivially named lepistamides A – C[1]) (**1** – **3**), and a known one, diatretol (**4**) [4], were isolated from the mycelial cultures of the basidiomycete. The isolation, structure elucidation, and possible biogenetic pathway of these new compounds are described as follows.

Results and Discussion. – The EtOH extract of the mycelial solid cultures of *L. sordida* GIM 5.315 was fractionated with petroleum ether, AcOEt, and BuOH. The fractions soluble in petroleum ether and AcOEt were combined and subjected to repeated column chromatography over silica gel and *ODS*, followed by prep. HPLC, to yield the three new compounds **1** – **3** along with diatretol (**4**) [4]. The structure of **4** was

[1]) Arbitrary atom numbering; for systematic names, see *Exper. Part.*

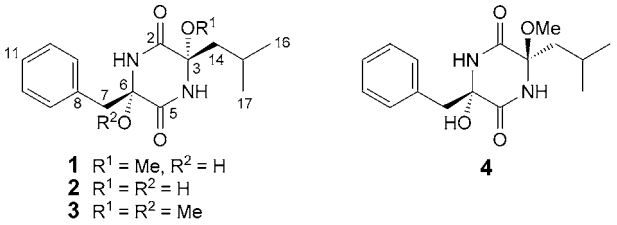

Fig. 1. *Compounds* **1**–**4**, *isolated from* Lepista sordida

determined by interpretation of its spectroscopic data as well as by comparison with reported data.

Lepistamide A (**1**), a white amorphous powder, was determined to have the molecular formula $C_{16}H_{22}N_2O_4$ from HR-ESI-MS and NMR data. Its ^{1}H- and ^{13}C-NMR spectra (*Table*) exhibited signals for two secondary Me groups (δ(H) 0.41 and 0.29; δ(C) 24.1 and 23.3), a MeO group (δ(H) 3.06; δ(C) 50.3), two CH_2 groups (δ(H) 3.41 and 2.79, δ(C) 44.4; δ(H) 1.35 and 1.12, δ(C) 46.1), one CH group (δ(H) 0.56; δ(C) 22.4), two O-bearing quaternary C-atoms (δ(C) 86.8 and 82.5), a monosubstituted benzene ring (δ(H) 7.16–7.24; δ(C) 126.6–135.1), two amide C=O groups (δ(C) 165.2 and 167.5), and two amide NH groups (δ(H) 8.40 and 9.01). Analysis of the ^{1}H,^{1}H-COSY, HSQC, and HMBC data readily derived a planar structure identical to that of diatretol (**4**) [4], a known diketopiperazine also obtained in the present study. However, comparison of the ^{1}H-NMR data with those of **4** showed that the signal of MeO–C(3) was dramatically downfield shifted (δ(H) 3.06 in **1** *vs.* δ(H) 1.97 in **4**), while resonances for the i-Bu group at C(3) were all upfield shifted by 0.31–1.11 ppm relative to those of **4**, indicating that in **1**, the i-Bu group was within the shielding region of the benzene ring of the Bn group at C(6), instead of the MeO group in **4** [4]. This, in combination with the absence of correlations between the aromatic H-atoms and the MeO group in the NOESY spectrum [4], showed that the MeO group at C(3) and the Bn group at C(6) were *trans*-positioned at the diketopiperazine ring. Thus, the structure of **1** was determined as 3-epidiatretol[1]).

Lepistamide B (**2**) was isolated as a yellowish amorphous powder. It had the molecular formula $C_{15}H_{20}N_2O_4$ as determined from the combined analysis of its ESI-MS, NMR, and HR-ESI-MS data. Its ^{1}H- and ^{13}C-NMR spectra (*Table*) were closely similar to those of compound **1**, except that the resonances for the MeO group were absent, and the signal of C(3) was shifted upfield to δ(C) 81.1 (δ(C) 86.8 in **1**). These data demonstrated that the MeO group at C(3) of **1** was replaced by an OH group in **2**. The relative configuration of **2** was deduced to be identical to that of **1** from the ^{1}H-NMR chemical shifts of the i-Bu group at C(3). Therefore, the structure of **2** was determined as 3-*O*-demethyl-3-epidiatretol[1]).

Lepistamide C (**3**), also obtained as a yellowish amorphous powder, was determined to have the molecular formula $C_{17}H_{24}N_2O_4$ from HR-ESI-MS and NMR data. Analysis of its ^{1}H- and ^{13}C-NMR spectra (*Table*) in combination with ^{1}H,^{1}H-COSY, HSQC, and HMBC data indicated that this compound was also a diketopiperazine alkaloid with a structure closely similar to that of **1**, except for the presence of an additional MeO group (δ(H) 3.19; δ(C) 50.5). Comparison of the ^{13}C-NMR spectrum

Table. *^{1}H- and ^{13}C-NMR Data* (600 and 150 MHz, resp.; (D_6)DMSO) *of* **1**–**3**[1]). δ in ppm, J in Hz.

	1		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
H–N(1)	8.40 (*s*)		8.44 (*s*)		8.74 (*s*)	
C(2)		165.2		167.2		165.8
C(3)		86.8		81.1		86.3
H–N(4)	9.01 (*s*)		8.74 (*s*)		9.00 (*s*)	
C(5)		167.5		166.7		164.9
C(6)		82.5		82.4		87.6
H_a–C(7)	3.41 (*d*, *J* = 12.7)	44.4	3.34 (*d*, *J* = 12.6)	43.8	3.27 (*d*, *J* = 12.7)	43.2
H_b–C(7)	2.79 (*d*, *J* = 12.7)		2.74 (*d*, *J* = 12.6)		2.83 (*d*, *J* = 12.7)	
C(8)		135.1		135.2		134.2
H–C(9,13)	7.16–7.24 (*m*)	130.6	7.15–7.25 (*m*)	130.6	7.17–7.25 (*m*)	130.8
H–C(10,12)	7.16–7.24 (*m*)	127.9	7.15–7.25 (*m*)	127.7	7.17–7.25 (*m*)	128.1
H–C(11)	7.16–7.24 (*m*)	126.6	7.15–7.25 (*m*)	126.5	7.17–7.25 (*m*)	127
H_a–C(14)	1.35 (*dd*, *J* = 13.5, 6.0)	46.1	1.55 (*dd*, *J* = 13.5, 6.5)	46.0	0.89 (*dd*, *J* = 13.8, 5.9)	45.7
H_b–C(14)	1.12 (*dd*, *J* = 13.5, 5.3)		1.15 (*dd*, *J* = 13.5, 6.5)		0.76 (*dd*, *J* = 13.8, 5.8)	
H–C(15)	0.56 (*m*)	22.4	0.58 (*m*)	23.0	1.03 (*m*)	22.2
Me(16)	0.41 (*d*, *J* = 6.6)	24.1	0.40 (*d*, *J* = 6.7)	23.8	0.49 (*d*, *J* = 6.7)	24.1
Me(17)	0.29 (*d*, *J* = 6.6)	23.3	0.22 (*d*, *J* = 6.7)	22.8	0.40 (*d*, *J* = 6.7)	23.7
*Me*O–C(3)	3.06 (*s*)	50.3			3.05 (*s*)	50.4
*Me*O–C(6)					3.19 (*s*)	50.5
OH–C(3)			5.89 (*s*)			
OH–C(6)	6.80 (*s*)		6.36 (*s*)			

with that of **1** revealed that the signal of C(6) of **3** was shifted downfield to δ(C) 87.6 (δ(C) 82.5 in **1**). This, in combination with the presence of a long-range correlation between the MeO group (δ(H) 3.19) and C(6) (δ(C) 87.6) in the HMBC spectrum, demonstrated that the additional MeO group was bound to C(6). The relative configuration of **3** was also identical to that of **1** as deduced from the ^{1}H-NMR chemical shifts and NOESY correlations. Thus, the structure of **3** was determined as 6-*O*-methyl-3-epidiatretol[1]).

Lepistamides A – C (**1**–**3**) are members of the 3,6-dioxygenated diketopiperazine family, and this type of compounds are rare in nature and mainly obtained from fungi and actinomycetes [5–13]. Picroroccellin is the first natural substance displaying this substitution pattern [5]. Other natural compounds with this molecular skeleton were reported from *Aspergillus* [6][7], *Fusarium* [8], *Metarhizium* [9], *Penicillium* [10][11], *Phoma* [12], and *Streptomyces* [13]. Interestingly, these compounds as derivatives of cyclodipeptides are all having an aromatic ring, at least at one of the β-positions of the two amino acid residues, except for bicyclomycin [14]. Considering this finding, it can be reasonably assumed that the aromatic ring at the β-position is necessary for the biosynthesis of these substances. Then, it could be hypothetically proposed that compounds **1**–**4** were biosynthetically transformed from cyclo(leucyl-phenylalanyl) by oxidative dehydrogenation [15], in which the cationic intermediates might be stabilized by a large conjugated system formed by participation of the Bn–C(6) group.

The 3,6-dioxygenated diketopiperazines were reported to possess antibacterial, immunomodulating, phytotoxic, and cytotoxic activities [5–13]. However, compounds

1–**4** were all found to be inactive ($IC_{50} > 100$ μg/ml) in the evaluation for the cytotoxic activity against Astc-a-1 (lung cancer), Bel-7402 (liver cancer), and HeLa (cervical carcinoma) cell lines by means of the MTT assay method [16].

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Experimental Part

General. Column chromatography (CC): silica gel *60* (SiO_2; 100–200 mesh; *Qingdao Haiyang Chemical Co., Ltd.*, Qingdao, P. R. China) and *Develosil ODS* (10 μm; *Nomura Chemical Co., Ltd.*, Seto, Japan). TLC: SiO_2 *HSGF*$_{254}$ plates (*Yantai Jiangyou Silica Gel Development Co., Ltd.*, Yantai, P. R. China). Prep. HPLC: *Shimazu-LC-6A* pump and *Shimazu-RID-10A* refractive index detector; *XTerra* prep. MS C_{18} column (10 μm, 300 × 19 mm); t_R in min. Optical rotations: *Perkin-Elmer-341* polarimeter. UV Spectra: *Perkin-Elmer-Lambda-35* UV/VIS spectrophotometer; λ_{max} (log ε) nm. 1D- and 2D-NMR Spectra: *Bruker-AV-600* instrument; in (D_6)DMSO; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *MDS-SCIEX-API-2000* LC/MS/MS apparatus; in *m*/*z*. HR-ESI-MS: *Waters UPLC*/*Q-TOF* micromass spectrometer; in *m*/*z*.

Producing Fungus and Identification. The fungus strain GIM 5.315 was isolated from a basidiomata collected from Baiyun Mountain in Guangzhou, P. R. China, in March 2005. The fungus was authenticated with morphological and molecular methods. In morphological analysis, the basidiomata showed the characteristics of *Lepista sordida* [2] in shape, color, hymenial surface, hyphal system, and presence/absence of sterile structures and basidiospores [17]. In molecular analysis, the genomic DNA of the cultured mycelium was extracted and the ITS1-5.8S-ITS2 segment from the ribosomal DNA (rDNA) was amplified with primer sets ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) by polymerase chain reaction (PCR) techniques [18]. Amplified products were examined with agarose gel electrophoresis and a 2 kb DNA marker. The amplified PCR products were directly sequenced and deposited in the GenBank with the accession number JF 420892. The ITS sequence was compared with the sequences submitted to the GenBank by using the BLAST program that is available from the *National Center for Biotechnology Information* (*NCBI*, www.ncbi.nlm.nih.gov/BLAST/), and showed 100% similarity with other *L. sordida* strains sequences (accession No. FJ428582, and FJ501575).

A voucher specimen of the mushroom, numbered GDGM 21933, is deposited with the Fungal Herbarium of the Guangdong Institute of Microbiology (GDGM). The mycelial strain (GIM 5.315) is deposited with the Microbial Culture Collection of the Guangdong Institute of Microbiology, Guangzhou, P. R. China. For maintenance on agar slants and submerged cultures, the fungus was grown on PDA medium.

Fermentation. Mycelia of *L. sordida* GIM 5.315 grown on PDA (potato-dextrose agar) plates were inoculated into 20 *Erlenmeyer* flasks (250 ml), each flask containing 100 ml of YMG (yeast/malt/glucose) medium (yeast-extract paste (3 g), maltose (20 g), glucose (10 g), KH_2PO_4 (3 g), and $MgSO_4$ (1.5 g), dissolved in 1000 ml of H_2O, pH 6.5). The flasks were incubated on a rotary shaker for 7 d in the dark at 25° with shaking at 120 rpm. Seven days later, the cultures were transferred into 50 *Erlenmeyer* flasks (500 ml), each flask containing 200 g of YMG solid medium (yeast extract paste (3 g), maltose (20 g), glucose (10 g), KH_2PO_4 (3 g), and $MgSO_4$ (1.5 g), mixed with 1000 g of boiled wheat grains, pH 6.5), and cultivated in the stationary phase in the dark at 25° for 4 weeks.

Extraction and Isolation. Mycelial solid cultures (10 kg) were extracted 3 × with 95% EtOH (24 h each) at r.t. The resulting EtOH soln., after concentration *in vacuo*, was suspended in H_2O, and this aq. suspension was sequentially extracted with petroleum ether (60–90°, 3 × 500 ml), AcOEt (3 × 500 ml), and BuOH (3 × 500 ml) to yield the petroleum ether (56.5 g), AcOEt (32.5 g), and BuOH extracts

(40 g). The petroleum ether and AcOEt extracts were combined and subjected to CC (SiO_2, $CHCl_3$/MeOH 100:0 → 60:40): *Fractions E1 – E10. Fr. E7* (3.5 g), obtained on elution with $CHCl_3$/MeOH 9:1, was further applied to CC (*ODS*, MeOH/H_2O 1:9 → 8:2): *Frs. E7.1 – E7.19. Fr. E7.11* (224 mg) was separated by HPLC (MeOH/H_2O 52:48, flow rate of 5 ml/min): **4** (t_R 16.9; 25 mg), **2** (t_R 25.1; 16 mg), **1** (t_R 28.4; 34 mg), and **3** (t_R 34.5; 6 mg).

Lepistamide A (= rel-*(3*R*,6*R*)-3-Hydroxy-6-methoxy-6-(2-methylpropyl)-3-(phenylmethyl)-piperazine-2,5-dione*; **1**): White amorphous powder. $[\alpha]_D^{20} = -8.9$ ($c = 0.37$, MeOH). UV (MeOH): 206 (3.18). ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 329 ($[M+Na]^+$), 635 ($[2M+Na]^+$), 341 ($[M+Cl]^-$). HR-ESI-MS: 329.1469 ($[M+Na]^+$, $C_{16}H_{22}N_2NaO_4^+$; calc. 329.1477).

Lepeistamide B (= rel-*(3*R*,6*R*)-3,6-Dihydroxy-3-(2-methylpropyl)-6-(phenylmethyl)piperazine-2,5-dione*; **2**): Yellowish amorphous powder. $[\alpha]_D^{20} = 0$ ($c = 0.27$, MeOH). UV (MeOH): 204 (3.36). ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 315 ($[M+Na]^+$), 607 ($[2M+Na]^+$), 291 ($[M-H]^-$), 327 ($[M+Cl]^-$). HR-ESI-MS: 315.1326 ($[M+Na]^+$, $C_{15}H_{20}N_2NaO_4^+$, calc. 315.1321).

Lepistamide C (= rel-*(3*R*,6*R*)-3,6-Dimethoxy-3-(2-methylpropyl)-6-(phenylmethyl)piperazine-2,5-dione*; **3**): Yellowish amorphous powder. $[\alpha]_D^{20} = 0$ ($c = 0.17$, MeOH). UV (MeOH): 205 (3.48). ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS (pos.): 343 ($[M+Na]^+$), 359 ($[M+K]^+$). HR-ESI-MS: 343.1641 ($[M+Na]^+$, $C_{17}H_{24}N_2NaO_4^+$; calc. 343.1634).

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