Oxepine-Containing Diketopiperazine Alkaloids from the Algal-Derived Endophytic Fungus *Paecilomyces variotii* EN-291

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Two new oxepine-containing diketopiperazine-type alkaloids, varioloids A and B (**1** and **2**, resp.), were isolated from the algal-derived fungus *Paecilomyces variotii* EN-291. The structures and absolute configurations were determined by detailed interpretation of 1D- and 2D-NMR spectroscopic data and by analysis of acidic hydrolysates. Compounds **1** and **2** exhibited potent activity against the plant-pathogenic fungus *Fusarium graminearum* with *MIC* values of 8 and 4 μg/ml, respectively.

Introduction. – Marine-derived fungi have been proven to be a prolific source of various novel secondary metabolites in the last two decades [1]. As our continuation of investigations aimed to characterize new antimicrobial secondary metabolites from marine-derived endophytic fungi [2–4], a new 3*H*-oxepine-containing diketopiperazine-type alkaloid, varioxepine A, possessing diverse antimicrobial activities, was isolated and identified from *Paecilomyces variotii* EN-291, an endophytic fungus obtained from a red alga *Grateloupia turuturu* [5]. Further investigation on the same fungal strain has now led to the identification of two new oxepine-containing diketopiperazine-type alkaloids, varioloids A and B (**1** and **2**, resp.; *Fig. 1*). The identification of varioloid A (**1**) as a metabolite of *P. variotii* seemes to confirm its role as a biosynthetic intermediate on the path to varioxepine A, as proposed in our previous report [5]. Herein, we report the isolation, structure elucidation, and antimicrobial activity of the two compounds.

Fig. 1. *Structures of isolated compounds* **1** *and* **2** *and of the reference compound protuboxepin B* (**3**)

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Results and Discussion. – Compound **1** was obtained as colorless oil. Its molecular formula $C_{26}H_{29}N_3O_5$ was established on the basis of the HR-ESI-MS (*m/z* 486.2001 ($[M+Na]^+$; calc. 486.2005)), indicating 14 degrees of unsaturation. The ^{1}H-NMR spectrum (*Table 1*) exhibited signals attributed to nine aromatic and olefinic H-atoms, three aliphatic CH (one N-bearing), two aliphatic CH_2 (one O-bearing), and four Me groups. The coupling patterns and COSY correlations of five H-atom signals at δ(H) 7.13 (*d*, *J* = 6.9, H–C(18,22)), 7.25 (*t*, *J* = 6.9, H–C(19,21)), and 7.20 (*t*, *J* = 6.9, H–C(20)) indicated the presence of a mono-substituted Ph group. Four olefinic signals, comprising H–C(9) (δ(H) 6.60 (*d*, *J* = 7.2)), H–C(10) (5.47 (*t*, *J* = 7.2)), H–C(11) (6.33 (*dd*, *J* = 10.0, 7.2)), and H–C(12) (5.73 (*d*, *J* = 10.0)), were typical for an oxepine ring [6], which was confirmed by the expected COSY correlations (*Fig. 2*). The ^{13}C-NMR chemical shift of C(1) (δ(C) 56.8) indicated that this C-atom was attached to a N-atom, and HMBCs of CH_2(16) and H–C(1) with one of the two amide C=O C-atoms (δ(C) 164.5) positioned this C-atom at C(2). HMBCs of H–C(1) and H–C(23) with C(5), in turn, led to the completion of the diketopiperazine ring in the molecule.

Table 1. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp., $CDCl_3$) *of* **1** *and* **2**. Assignments were corroborated by ^{1}H,^{1}H-COSY, HSQC, and HMBC. Arbitrary atom numbering indicated in *Fig. 1*; δ in ppm, *J* in Hz.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	5.42 (*dd*, *J* = 5.7, 3.6)	56.8 (*d*)	11.64 (*dd*, *J* = 6.8, 3.8)	57.9 (*d*)
2		164.5 (*s*)		167.2 (*s*)
4		123.4 (*s*)		89.0 (*s*)
5		115.9 (*s*)		153.7 (*s*)
7		152.3 (*s*)		162.2 (*s*)
9	6.60 (*d*, *J* = 7.2)	145.2 (*d*)	6.07 (*d*, *J* = 5.4)	143.4 (*d*)
10	5.47 (*t*, *J* = 7.2)	104.6 (*d*)	5.65 (*t*, *J* = 5.4)	117.1 (*d*)
11	6.33 (*dd*, *J* = 10.0, 7.2)	132.4 (*d*)	6.21 (*dd*, *J* = 11.1, 5.4)	129.3 (*d*)
12	5.73 (*d*, *J* = 10.0)	125.7 (*d*)	6.76 (*d*, *J* = 11.1)	125.2 (*d*)
13		75.5 (*s*)		111.6 (*s*)
14		166.2 (*s*)		160.9 (*s*)
16	3.07 (*dd*, *J* = 13.9, 5.7), 3.24 (*dd*, *J* = 13.9, 3.6)	37.4 (*t*)	3.42 (*dd*, *J* = 13.7, 3.8), 3.60 (*dd*, *J* = 13.7, 6.8)	37.2 (*t*)
17		134.8 (*s*)		134.9 (*s*)
18,22	7.13 (*d*, *J* = 6.9)	130.0 (*d*)	7.08 (*d*, *J* = 7.3)	130.0 (*d*)
19,21	7.25 (*t*, *J* = 6.9)	128.8 (*d*)	7.21 (*t*, *J* = 7.3)	128.6 (*d*)
20	7.20 (*t*, *J* = 6.9)	127.7 (*d*)	7.21 (*t*, *J* = 7.3)	128.8 (*d*)
23	3.20–3.22 (*m*)	25.1 (*d*)	2.66–2.69 (*m*)	34.7 (*d*)
24	0.75 (*d*, *J* = 7.0)	18.1 (*q*)	0.71 (*d*, *J* = 6.5)	14.4 (*q*)
25	0.95 (*d*, *J* = 7.1)	20.0 (*q*)	0.96 (*d*, *J* = 6.9)	16.9 (*q*)
26	3.80 (*d*, *J* = 15.8), 4.19 (*d*, *J* = 15.8)	69.7 (*t*)	2.64 (*s*)	50.6 (*q*)
27		210.7 (*s*)		
28	2.81–2.84 (*m*)	36.9 (*d*)		
29	1.05 (*d*, *J* = 7.0)	19.8 (*q*)		
30	1.06 (*d*, *J* = 7.0)	25.1 (*q*)		

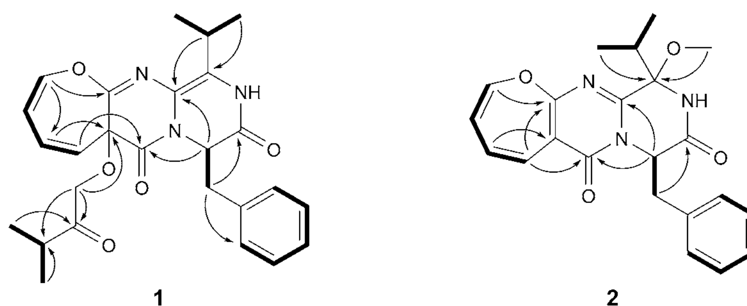

Fig. 2. *Key HMBC* (H → C) *and COSY* (—) *correlations of compounds* **1** *and* **2**

Other HMBCs of H–C(1) and H–C(12) to C(14) positioned another amide C=O C-atom at C(14). Further analysis of the COSY and HMBCs revealed the connection of a side chain consisting of five C-atoms (C(26) – C(30); *Fig. 2*), which was terminated by two Me groups, Me(29) and Me(30), as evidenced by the HMBCs of Me(29) and Me(30) to the keto C(27)=O C-atom and of $CH_2(26)$ to C(28). The key HMBC of the O-bearing $CH_2(26)$ to C(13) indicated the location of the side chain at C(13). The remaining heteroatom N(6) was then connected to C(5) and C(7) to account for the required unsaturation equivalents. The observed NOE correlation between H–C(1) and H–C(12) in the NOESY spectrum indicated the cofacial orientation of the two H-atoms [7]. Thus, the structure and relative configuration of **1** were established as shown in *Fig. 1*.

In our previous report, varioxepine A, a 3*H*-oxepine-containing alkaloid having a condensed 3,6,8-trioxabicyclo[3.2.1]octane unit was isolated, and its plausible biosynthetic pathway was proposed [5]. In this hypothesis, varioloid A (**1**) was proposed as a key intermediate and, thus, a precursor of varioxepine A. The isolation and identification of varioloid A (**1**) as a secondary metabolite of *P. variotii* supported its role in the biosynthetic pathway of related metabolites. Based on the common biosynthetic origin, the absolute configuration of **1** was presumed to be same as that of varioxepine A. Compound **1** was hydrolyzed in a 6N aqueous HCl solution at 110° for 24 h to afford L-phenylalanine, $[\alpha]_D^{20} = -32.8$ ($c = 0.17$, H_2O), which was determined by comparison with a reference sample [8]. Taking into account the desired (*S*)-configuration at C(1) and the above positive NOESY correlations between H–C(12) and H–C(1), the absolute configuration of metabolite **1** has to be (1*S*,13*R*).

Compound **2**, colorless oil, was determined to have the molecular formula $C_{22}H_{23}N_3O_4$ on the basis of HR-ESI-MS, suggesting 13 degrees of unsaturation. The ^{1}H- and ^{13}C-NMR data of **2** (*Table 1*) was almost identical to those of protuboxepin B (**3**) [9] except for the appearance of additional signals of a Me(26)O group (δ(H) 2.64 and δ(C) 50.6) in **2**. The HMBC of Me(26) with C(4) positioned this Me(26)O group at C(4). The assignments of ^{1}H- and ^{13}C-NMR signals were fully established by means of a combination of COSY, HSQC, and HMBC spectral measurements. The relative configuration assigned for **2** was proposed by the observation of NOE correlations of Me(26) with H–C(18,22) [9]. Analysis of the acidic hydrolysate of **2** indicated that the Phe residue in the molecule has L-configuration. Therefore, the absolute configurations

of the stereogenic centers were assigned as (1*S*) and (4*S*). It should be mentioned that the L-configuration of our phenylalanine degradation product in varioloids A and B (**1** and **2**, resp.) as well as in varioxepine A [5] is the same as that reported by *Lu et al.* [7] and *Li et al.* [8] for the related oxepine- or 3*H*-oxepine-containing diketopiperidine metabolites, but opposite to that described by *Lee et al.* [9].

Antimicrobial Activities. Compounds **1** and **2** were evaluated *in vitro* for antibacterial activity against two *Gram*-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*) and five *Gram*-negative bacteria, including *Escherichia coli* and the aquacultural bacteria, *Aeromonas hydrophila*, *Vibrio anguillarum*, *V. harveyi*, and *V. parahaemolyticus.* Both compounds exhibited diverse and promising antibacterial activities with the *MIC* values ranging from 16 to 64 μg/ml (*Table 2*). Compounds **1** and **2** also exhibited activity against the plant-pathogenic fungus *Fusarium graminearum* with *MIC* values of 8 and 4 μg/ml, respectively.

Table 2. *Minimum Inhibitory Concentration* (*MIC* [μg/ml]) *of Compounds* **1** *and* **2** *against Seven Bacterial Strains*

Compound	*A. hydrophila*	*E. coli*	*M. luteus*	*S. aureus*	*V. anguillarum*	*V. harveyi*	*V. parahaemolyticus*
1	64	16	64	16	64	32	64
2	32	32	64	16	32	64	64
Chloramphenicol[a])	4	4	0.5	1	8	4	0.5

[a]) Positive control.

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Experimental Part

General. TLC: Precoated silica gel GF_{254} plates (SiO_2; *Qingdao Haiyang Chemical Group Co.*). Column chromatography (CC): commercial SiO_2 (200–300 mesh; *Qingdao Haiyang Chemical Group Co.*), *Lobar LiChroprep RP-18* (40–63 μm; *Merck*), and *Sephadex LH-20* (*Pharmacia*). Semi-prep. HPLC: *Dionex* HPLC system equipped with a *P680* pump, an *ASI-100* automated sample injector, and a *UVD340U* multiple wavelength detector; ODS column (*Sinochrom ODS-BP*, 10 × 300 mm, 10 μm); flow 3 ml/min; detection by UV (λ_{max} (log ε) in nm). Optical rotations: *AA-55* digital polarimeter (*Optical Activity Ltd.*). UV Spectra: *Lengguang-Gold-Spectrumlab-54* UV/VIS spectrophotometer; λ_{max} (log ε) in nm. NMR Spectra: *Bruker-Avance-500* spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI- and HR-ESI-MS: *VG-Autospec-3000* mass spectrometer; in *m*/*z*.

Fungal Strain and Identification. The endophytic fungus *Paecilomyces variotii* EN-291 was isolated from *Grateloupia turuturu*, a marine red alga collected from the coast of Qingdao, P. R. China, in May 2013. Fungal identification was carried out by a protocol reported previously [10], and the sequence data derived from the fungal strain was submitted to and deposited at *GenBank*, with accession No. KJ577627. A BLAST search result showed that the sequence was the same (100%) as the sequence of *Paecilomyces variotii* (compared with KC311513). The strain is preserved with the Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. For chemical investigations, the fungal strain was statically fermented in a 1000-ml *Erlenmeyer* flask containing 300 ml of the PDB medium (potato dextrose broth:

2% mannitol, 1% glucose, 0.3% peptone, 0.5% yeast extract, and seawater added up to 300 ml, pH 6.5 – 7.0, adjusted with 10% NaOH/flask, 60 flasks) at r.t. for 30 d.

The mycelium and broth were separated by filtration, and then exhaustively extracted with AcOEt to give a crude extract (4.3 g), which was dried and fractionated by vacuum liquid chromatography (VLC; SiO_2) using different solvents of increasing polarity from petroleum ether (PE) to MeOH to yield seven fractions, *Frs. 1 – 7*, based on TLC analysis. *Fr. 3* (0.5 g; $CHCl_3$/MeOH 40 : 1) was further purified by CC (SiO_2; $CHCl_3$/MeOH 80 : 1 to 20 : 1), *Lobar LiChroprep RP-18* (MeOH/H_2O 3 : 7 to 8 : 2), and *Sephadex LH-20* (MeOH)) to afford compounds **1** (8.8 mg) and **2** (3.9 mg), resp.

Varioloid A (=*(5a*R*,8*S*)-5a,10-Dihydro-11-(1-methylethyl)-5a-(3-methyl-2-oxobutoxy)-8-(phenylmethyl)-6*H*-oxepino[2,3-*d*]pyrazino[1,2-*a*]pyrimidine-6,9(8*H*)-dione*; **1**). Colorless oil. $[\alpha]_D^{20} = +71.4$ ($c = 0.14$, MeOH). UV (MeOH): 201 (3.87), 320 (3.21). ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 486 ($[M + Na]^+$). HR-ESI-MS: 486.2001 ($[M + Na]^+$, $C_{26}H_{29}N_3NaO_5^+$; calc. 486.2005).

Varioloid B (=*(8*S*,11*S*)-10,11-Dihydro-11-methoxy-11-(1-methylethyl)-8-(phenylmethyl)-6*H*-oxepino[2,3-*d*]pyrazino[1,2-*a*]pyrimidine-6,9(8*H*)-dione*; **2**). Colorless oil. $[\alpha]_D^{20} = +102.3$ ($c = 0.18$, MeOH). UV (MeOH): 258 (3.70), 328 (3.41). ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 394 ($[M + H]^+$). HR-ESI-MS: 394.1768 ($[M + H]^+$, $C_{22}H_{24}N_3O_4^+$; calc. 394.1761).

Analysis of the Acidic Hydrolysates. Compounds **1** (3.0 mg) and **2** (2.0 mg) were hydrolyzed in a 6N aq. HCl soln. at 110° for 24 h. The solns. were then evaporated to dryness under reduced pressure. The hydrolysates of **1** and **2** were each subjected to CC (SiO_2) to afford L-phenylalanine ($[\alpha]_D^{20} = -32.8$ ($c = 0.17$, H_2O)) which was identified by comparison of its optical rotation with that of authentic sample.

Antimicrobial Assays. The antimicrobial assays against seven bacteria (*A. hydrophila*, *E. coli*, *M. luteus*, *S. aureus*, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus*) and three fungi (*A. brassicae*, *C. gloeosporioides*, and *F. graminearum*) were carried out by using the well diffusion method [11]. Chloramphenicol and amphotericin B were used as antibacterial and antifungal positive controls, resp.

REFERENCES

[1] W. E. Rateb, R. Ebel, *Nat. Prod. Rep.* **2011**, *28*, 290.
[2] D. Liu, X.-M. Li, C.-S. Li, B.-G. Wang, *Helv. Chim. Acta* **2013**, *96*, 437.
[3] F.-Y. Du, X.-M. Li, J.-Y. Song, C.-S. Li, B.-G. Wang, *Helv. Chim. Acta* **2014**, *97*, 973.
[4] P. Zhang, L.-H. Meng, A. Mándi, T. Kurtán, X.-M. Li, Y. Liu, X. Li, C.-S. Li, B.-G. Wang, *Eur. J. Org. Chem.* **2014**, 4029.
[5] P. Zhang, A. Mándi, X.-M. Li, F.-Y. Du, J.-N. Wang, X. Li, T. Kurtán, B.-G. Wang, *Org. Lett.* **2014**, *16*, 4834.
[6] K. Yamada, D. Hashizume, T. Shimizu, S. Ohki, S. Yokohama, *J. Mol. Struct.* **2008**, *888*, 187.
[7] X.-H. Lu, Q.-W. Shi, Z.-H. Zheng, A.-B. Ke, H. Zhang, C.-H. Huo, Y. Ma, X. Ren, Y.-Y. Li, J. Lin, Q. Jiang, Y.-C. Gu, H. Kiyota, *Eur. J. Org. Chem.* **2011**, 802.
[8] G.-Y. Li, T. Yang, Y.-G. Luo, X.-Z. Chen, D.-M. Fang, G.-L. Zhang, *Org. Lett.* **2009**, *11*, 3714.
[9] S. U. Lee, Y. Asami, D. Lee, J. H. Jang, J. S. Ahn, H. Oh, *J. Nat. Prod.* **2011**, *74*, 1284.
[10] S. Wang, X.-M. Li, F. Teuscher, D.-L. Li, A. Diesel, R. Ebel, P. Proksch, B.-G. Wang, *J. Nat. Prod.* **2006**, *69*, 1622.
[11] K. Li, X.-M. Li, J. B. Gloer, B.-G. Wang, *Food Chem*. **2012**, *135*, 868.

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