New Cytotoxic Metabolites from the Marine-Derived Fungus *Penicillium* sp. ZLN29

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One new penicillide derivative, prenpenicillide (**1**), and one new xanthone derivative, prenxanthone (**2**), were isolated from the culture extract of the marine-derived fungus *Penicillium* sp. ZLN29, together with six known polyketide compounds, **3**–**8**. Their structures were elucidated on the basis of spectroscopic and spectrometric analyses. The cytotoxicities of these eight compounds were evaluated on four tumor-cell lines by SRB (=sulforhodamine B) or MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) methods. Compounds **1** and **3** exhibited weak cytotoxicities against HepG2 cell line with IC_{50} values of 9.9 and 9.7 μM, respectively.

Introduction. – Marine-derived microorganisms are widely recognized as emerging sources of active secondary metabolites, and increasing attention has been paid to them in recent years [1][2]. In the course of our ongoing investigations of structurally new and bioactive compounds from microorganisms [3–5], a fungus strain ZLN29, identified as *Penicillium* sp., was obtained from the sediments collected in the Jiaozhou Bay of China. Investigation of the extract of this fungus led to the isolation of one new penicillide derivative, named prenpenicillide (**1**), and one new xanthone derivative, named prenxanthone (**2**), along with six known metabolites, penicillide (**3**) [6], NG-011 (**4**), NG-012 (**5**), 15G256 β (**6**), 15G256α-2 (**7**) [7–10], and bioxanthracene 2 (**8**) [11] (*Fig. 1*). Herein, we report the isolation, structure elucidation, and cytotoxic activities of these compounds.

Results and Discussion. – The AcOEt extract of the fungus *Penicillium* sp. was concentrated *in vacuo* to give a crude extract, which showed significant antitumor activity *in vitro* with the inhibitory rate 59% at 10 μg/ml. Bioassay-guided fractionation of the residue by a combination of column chromatography (silica gel and *Sephadex LH-20*) and semi-preparative HPLC yielded compounds **1**–**8**.

Prenpenicillide (**1**) was obtained as a shallow yellow oil, with molecular formula $C_{21}H_{22}O_5$, established on the basis of HR-ESI-MS data (*m*/*z* 353.1388 ($[M-H]^-$, calc. 353.1389)). This formula was consistent with the ^{1}H- and ^{13}C-NMR data (*Table 1*), indicating eleven degrees of unsaturation. ^{1}H- and ^{13}C-NMR data of compound **1** displayed resonances that were assigned to three Me, one MeO, two CH_2, four aromatic CH, one olefinic CH group, six O-bearing C-atoms (among them, five ester or aromatic C-atoms), and five additional quaternary C-atoms (*Table 1*). These data were very similar to those of penicillide (**3**) [6], except for the difference of the isoprenoid moiety.

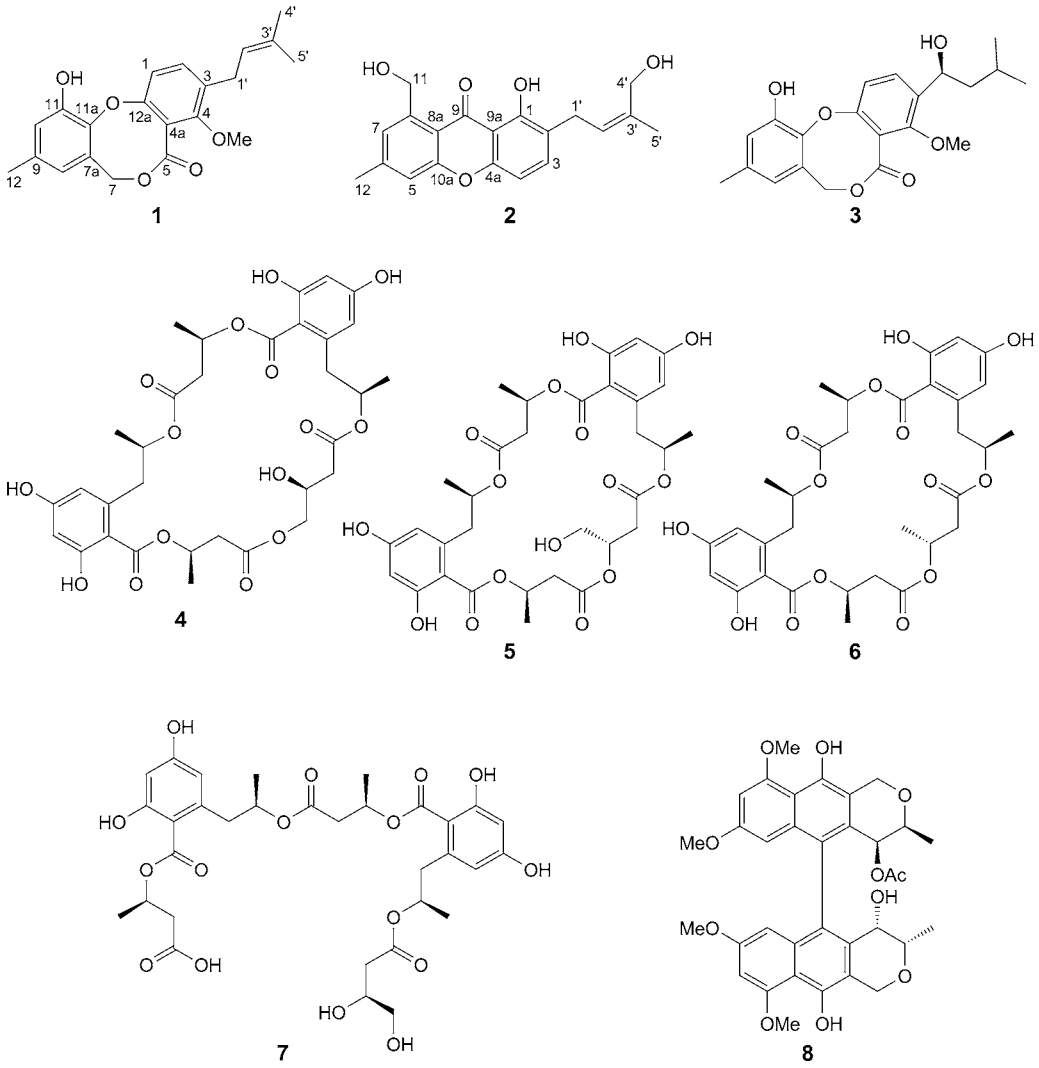


Fig. 1. *Compounds* **1**–**8**, *isolated from* Penicillium *sp. ZLN29*

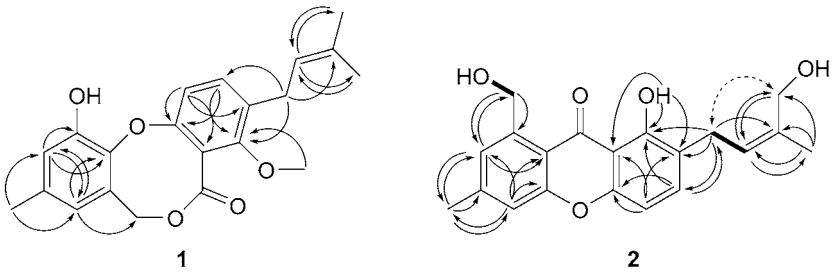
In the HMBC experiment (*Fig. 2*), long-range correlations were observed between $CH_2(1')$ and C(3′), between H–C(2′) and C(1′), C(4′), C(5′), between Me(4′) and C(2′), between Me(5′) and C(2′), and which revealed the presence of an isopentenyl (= 3-methylbut-2-enyl) group, and between $CH_2(1')$ and C(2), C(4), indicating the position of the isopentenyl group (*Fig. 2*). Finally, the structure of compound **1** was deduced as 11-hydroxy-4-methoxy-9-methyl-3-(3-methylbut-2-en-1-yl)-5*H*,7*H*-dibenzo[*b*,*g*][1,5]-dioxocin-5-one.

Prenxanthone **2** was separated as a yellowish amorphous powder, and its molecular formula was determined as $C_{20}H_{20}O_5$ on the basis of HR-ESI-MS data (*m*/*z* 339.1239

Table 1. *^{1}H- and ^{13}C-NMR* (at 600 and 150 MHz, resp.) *Data of* **1** (in $CDCl_3$). δ in ppm, J in Hz. Atom numbering as indicated in *Fig. 1*.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
1	6.81 (d, J = 8.2)	117.5	10	6.84 (s)	117.5
2	7.26 (d, J = 8.2)	133.9[a])	11		147.4
3		134.0	12	2.23 (s)	20.9
4		155.6	OH	6.20 (br. s)	
MeO	3.93 (s)	62.7	11a		141.5
4a		120.4	12a		150.3
5		167.6	1′	3.35 (d, J = 7.2)	27.9
7	5.06 (br. s)	69.1	2′	5.21 (t, J = 7.2)	121.7
7a		125.9	3′		133.9[a])
8	6.36 (s)	121.0	4′	1.74 (s)	25.8
9		134.9	5′	1.71 (s)	18.0

[a]) Signals may be interchanged.



1 **2**

Fig. 2. *Selected ^{1}H,^{1}H-COSY* (—), *HMB* (H → C), *and NOESY* (H ←- - → H) *correlations of compounds* **1** *and* **2**

([M − H]$^-$, calc. 339.1232)), indicating eleven degrees of unsaturation. Its UV spectrum (λ_{max} 236, 260, 298, and 373 nm) indicated that **2** was a xanthone derivative [12][13]. The ^{1}H- and ^{13}C-NMR spectra featured signals of two Me, three CH_2, four aromatic CH, one olefinic CH groups, three O-bearing aromatic C-atoms, one C=O C-atom, and six other quaternary C-atoms, also indicating that **2** was a xanthone derivative (*Table 2*). On the basis of a series of 2D-NMR experiments including ^{1}H,^{1}H-COSY and HMBC (*Fig. 2*), structure **2** was proposed. Particularly, in the HMBC experiment, long-range correlations were observed between CH_2(1′) and C(3′), between H–C(2′) and C(1′), C(4′), C(5′), revealing the presence of an isoprenoid moiety. The correlations between O*H*–C(1), and C(1), C(2), and C(9a); between CH_2(1′), and C(1), C(2), and C(3); between CH_2(11), and C(7) and C(8); and between Me(12), and C(5), C(6), and C(7), determined the positions of the OH, isoprenoid, $HOCH_2$, and Me groups in the xanthone skeleton, respectively. In addition, the relative configuration of **2** was elucidated on the basis of NOESY experiments. The cross-peaks CH_2(1′)/CH_2(4′) confirm the (*Z*)-configuration at C(2′) (*Fig. 2*). Thus, **2** was identified as 1-hydroxy-8-(hydroxymethyl)-2-[(2*Z*)-4-hydroxy-3-methylbut-2-en-1-yl]-6-methyl-9*H*-xanthen-9-one.

Table 2. *^1H- and ^{13}C-NMR* (at 600 and 150 MHz, resp.) *Data of* **2** (in (D_6)DMSO). δ in ppm, J in Hz. Atom numbering as indicated in *Fig. 1*.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
1		158.7	9a		108.8
OH–C(1)	13.10 (br. *s*)		10a		157.5
2		122.4	11	5.11 (br. *s*)	62.5
3	7.51 (*d*, *J* = 8.5)	137.3	OH–C(11)	5.38 (br. *s*)	
4	6.91 (*d*, *J* = 8.5)	106.5	12	2.46 (*s*)	22.3
4a		153.8	1′	3.31 (*d*, *J* = 7.7)	26.7
5	7.26 (br. *s*)	116.3	2′	5.51 (*t*, *J* = 7.7)	121.3
6		147.3	3′		137.3
7	7.53 (br. *s*)	122.9	4′	3.82 (br. *s*)	66.8
8		146.5	OH–C(4′)	4.71 (br. *s*)	
8a		114.8	5′	1.67 (*s*)	14.1
9		184.2			

All compounds were evaluated for their cytotoxicities against HepG2, HL-60, BEL-7402, and K562 cell lines using the SRB (= sulforhodamine B) [14] or MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) [15] methods with adriamycin (ADM) as positive control. Interestingly, only compounds **1** and **3** exhibited weak cytotoxicities against HepG2 cell line with IC_{50} values of 9.9 and 9.7 μM, respectively, indicating that these compounds can selectively inhibit the growth of HepG2 cell line. Compounds **5**–**7** exhibited only weak cytotoxicities against HL-60 cell line with IC_{50} values of 40.7, 10.8, and 38.8 μM, respectively. All these compounds did not exhibit any cytotoxicity against BEL-7402 and K562 cell lines (*Table 3*).

Table 3. *Cytotoxicities of Compounds* **1**–**8** (IC_{50} [μM])[a]) *against Tumor Cell Lines*

Cell line	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
HepG2	9.9	> 50	9.7	> 50	> 50	> 50	> 50	> 50
HL-60	> 50	> 50	> 50	> 50	40.7	10.8	38.8	> 50
BEL-7402	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
K562	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50

[a]) Data represent mean values of five independent experiments and were determined by the SRB method with the HepG2 and BEL-7404 cell line, and the MTTmethod with HL-60 and K562 cell lines.

Penicillides are characterized by 5*H*,7*H*-dibenzo[*c*,*f*][1,5]dioxocin-5-one skeleton, which were found only in fungi. A few simple derivatives of these penicillides were isolated, and these compounds exhibited diverse biological activities: cytotoxic [16] and antifungal [17] activities, and inhibition of oxytocin [18], acyl-CoA:cholesterol acyltransferase (ACAT) [19], cholesterol ester transfer protein (CETP) [20], and calpain [21]. The biological evaluation of this kind of compounds, reported here, using other bioassays is still in progress.

Experimental Part

General. TLC: SiO_2 *GF_{254}* (10–40 mm; *Qingdao Marine Chemical Inc.*, P. R. China). Column chromatography (CC): Silica gel (SiO_2; 200–300 mesh, 10–40 mm; *Qingdao Marine Chemical Inc.*, P. R. China), *Sephadex LH-20* (*GE Healthcare*, Sweden). Semi-prep. HPLC: *ODS* column (*YMC-Pack ODS-A*, 10 × 250mm, 5 μM, 4 ml/min). UV Data: HPLC, mobile phase; λ_{max} in nm. IR Spectra: *NICOLET NEXUS 470* spectrophotometer, in KBr discs; $\tilde{\nu}_{max}$ in cm^{-1}. ^1H-, ^{13}C-, DEPT, and 2D-NMR: *JEOL JNM-ECP 600* spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS: *Micromass Q-TOF ULTIMA GLOBAL GAA076 LC Mass* spectrometer; in *m/z* (rel. %).

Fungal Material. The fungal strain *Penicillium* sp. ZLN29 was isolated from the sediments collected in the Jiaozhou Bay of China. The voucher specimen is deposited with our laboratory at −20°. Working stocks were prepared on potato dextrose agar slants stored at 4°.

Fermentation and Extraction. Spores were directly inoculated into 1000-ml *Erlenmeyer* flasks containing 300-ml fermentation media (mannitol (20 g), maltose (20 g), glucose (10 g), monosodium glutamate (10 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7\,H_2O$ (0.3 g), yeast extract (3 g), and corn steep liquor (1 g), dissolved in 1 l of sea water; pH 6.5). The fungus was grown under static conditions at 28°. After 30 d of cultivation, 30 l of whole broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The former was extracted with AcOEt, while the latter was extracted with acetone. The acetone extract was evaporated under reduced pressure to afford an aq. soln., and then extracted with AcOEt. The two AcOEt extracts were combined and concentrated *in vacuo* to give a crude extract (22.5 g).

Purification. The crude extract (22.5 g) was applied to a SiO_2 (300–400 mesh) column and was separated into seven fractions, *Frs. 1–7*, using a step gradient elution with petroleum ether/$CHCl_3$ and $CHCl_3$/MeOH. The active fraction, *Fr. 3*, eluted with $CHCl_3$/MeOH 100:1, was fractionated on a SiO_2 column using a step gradient elution of petroleum ether/acetone and was separated into ten fractions, *Frs. 3.1–3.10*. *Fr. 3.2* was further separated on *Sephadex LH-20* column with $CHCl_3$/MeOH 1:1 and by semi-prep. HPLC (MeOH/H_2O 60:40, 4 ml/min) to yield compound **1** (t_R 13.5 min, 5.3 mg) and **3** (t_R 9.5 min, 15.0 mg), resp. *Fr. 3.5* was further separated on *Sephadex LH-20* column with $CHCl_3$/MeOH 1:1 and by semi-prep. HPLC (MeOH/H_2O 65:35, 4 ml/min) to yield compound **2** (t_R 11.0 min, 8.7 mg). *Fr. 3.7* was further separated by CC (*Sephadex LH-20*; $CHCl_3$) and semi-prep. HPLC (MeOH/H_2O 65:35, 4 ml/min) to yield compound **8** (t_R 13.5 min, 4.0 mg). *Fr. 4* was subjected to CC (*Sephadex LH-20*; $CHCl_3$; and SiO_2; petroleum ether/acetone 4:1) to afford five fractions, *Frs. 4.3.1–4.3.5*. *Fr. 4.3.2* was further purified by semi-prep. HPLC (MeOH/H_2O 75:25, 4 ml/min) to yield compound **7** (t_R 11.4 min, 13 mg). *Fr. 4.3.3* was further submitted to by semi-prep. HPLC (MeOH/H_2O 80:20, 4 ml/min) to yield compound **4** (t_R 9.4 min, 29.0 mg), **5** (t_R 11.7 min, 20.2 mg), and **6** (t_R 15.7 min. 35.0 mg), resp.

Prenpenicillide (=*11-Hydroxy-4-methoxy-9-methyl-3-(3-methylbut-2-en-1-yl)-5*H,*7*H-*dibenzo[*b,g*][1,5]dioxocin-5-one*; **1**). Shallow yellow oil. UV (HPLC, mobile phase): 208, 261. IR (KBr): 3400, 2900, 1720, 1412, 1253, 1145. ^1H- and ^{13}C-NMR: *Table 1*. HR-ESI-MS: 353.1388 ($[M-H]^-$, $C_{21}H_{21}O_5^-$; calc. 353.1389).

Prenxanthone (=*1-Hydroxy-8-(hydroxymethyl)-2-[(2Z)-4-hydroxy-3-methylbut-2-en-1-yl]-6-methyl-9*H-*xanthen-9-one*; **2**). Yellowish amorphous powder. UV (HPLC, mobile phase): 236, 260, 298, 373. IR (KBr): 3430, 2903, 1700, 1413, 1258, 1140. ^1H- and ^{13}C-NMR: *Table 2*. HR-ESI-MS: 339.1239 ($[M-H]^-$, $C_{20}H_{19}O_5^-$; calc. 339.1232).

Biological Assay. In the MTT assay, cell lines were grown in *RPMI-1640* medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37°. Cell suspensions (200 μl), at a density of 5×10^4 cell/ml were plated in 96-well microtiter plates and incubated for 24 h. Then, 2 μl of the test solns. (in MeOH) were added to each well and further incubated for 72 h. Then, 20 μl of the MTT soln. (5 mg/ml in IPMI-1640 medium) was added to each well and incubated for 4 h. Old medium containing MTT (150 μl) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a *Spectra Max Plus* plate reader at 540 nm.

In the SRB assay, 200 μl of the cell suspensions were plated in 96-cell plates at a density of 2×10^5 cell/ml. Then, 2 μl of the test soln. (in MeOH) was added to each well, and the culture was further incubated for 24 h. The cells were fixed with 12% CCl_3COOH, and the cell layer was strained with 0.4% SRB. The absorbance of SRB soln. was measured at 515 nm. Dose–response curves were generated, and

the IC_{50} values (the concentration of a compound required to inhibit cell proliferation by 50%), were calculated from the linear portion of log dose–response curves.

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