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TUMONOIC ACIDS K AND L, NOVEL METABOLITES FROM THE MARINE-DERIVED FUNGUS *PENICILLIUM CITRINUM*

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Abstract – Three new compounds, namely tumonoic acids K (**1**), L (**2**), and methyl 2-(2-acetyl-3,5-dihydroxy-4,6-dimethylphenyl)acetate (**3**), together with nine known compounds, were isolated from the marine-derived fungus *Penicillium citrinum*. The structures of these compounds were elucidated by spectroscopic methods and chemical derivatization. This study is the first to report on tumonoic acid analogues derived from a fungus.

It has been demonstrated that marine fungal species have evolved unique metabolic mechanisms where the metabolites have potential applications in drug discovery.[1] During the continuing research of antitumor natural products produced by *Penicillium citrinum* collected from the Min River estuary of China,[2] we found that the extract of fermentation broth and mycelia showed potent cytotoxic activity against the A-375 cell. Therefore, we investigated the secondary metabolites of this fungus, and obtained two new tumonoic acid analogs, tumonoic acids K (**1**) and L (**2**), a new benzene derivative methyl 2-(2-acetyl-3,5-dihydroxy-4,6-dimethylphenyl)acetate (**3**), as well as nine known compounds including penicillenols A_1, A_2, B_1, and B_2 (**4**–**7**),[3] dihydrocitrinone (**8**),[4] phenol A acid (**9**),[4] 7-carboxyl-6,8-dihydroxy-1,1,3,4,5-pentamenthylisochroman (**10**),[5] 1-acetonyl-7-carboxyl-6,8-dihydroxy-3,4,5-trimethylisochroman (**11**),[5] and 2,4,5-trimethylbenzene-1,3-diol (**12**).[6] It is exciting to note that our group is the first to report the isolation of tumonoic acids from a fungus, whereas all of the previous reports of tumonic acids were derived from marine cyanobacterium.[7,8]

The bioactive extract of P. *citrinum* was subjected to column chromatography (CC) on Si gel and Sephadex LH-20, and further purified by semipreparative HPLC, which resulted in the separation of 12 pure compounds, **1**–**12** (Figure 1).

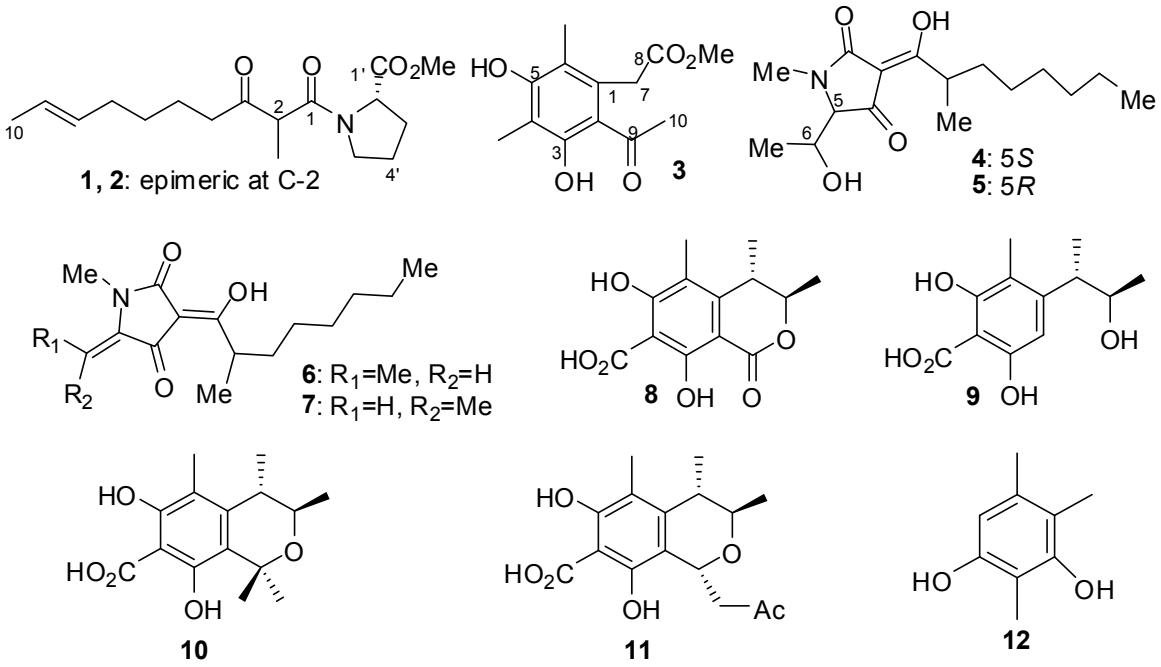

Figure 1. Structures of compounds **1**–**12**

Compound **1** was found to have the molecular formula $C_{17}H_{27}NO_4$ by its HRESIMS (*m/z* 310.2020 $[M + H]^+$; Calcd for $C_{17}H_{28}NO_4$: 310.2018). The ^{1}H NMR spectrum of **1** indicated the presence of nine methyl protons (δ 1.38, 1.62, and 3.73) and four methine protons, two of which (δ 3.58 and 4.51) were attached to oxygenated carbons, and the others (δ 5.39 and 5.40) were double-bond protons. The ^{13}C NMR and DEPT data indicated the presence of the following: three methyl carbons (δ 52.3, 17.9, and 13.2); seven methylene carbons (δ 23.0, 24.8, 29.0, 29.2, 32.3, 39.5, and 47.3); four methine carbons (δ 53.2, 59.0, 125.2, and 131.0); and three quaternary carbons (δ 169.1, 172.6, and 207.3). Two-dimensional NMR analysis (Figure 2) facilitated assignment of all protons and carbons in the proline residue and the fatty acid moiety, respectively. However, no HMBC correlations were observed linking the proline and fatty acid portions of **1**, but on the basis of the downfield shifts of H-2′ and H-5′, it was clear that the proline nitrogen was part of an amide group, which was consistent with its MS data leading to definition of the planar structure.[8] The *E* configuration was assigned to the C-8 to C-9 double bond based on comparison of the chemical shifts of C-7 (δ_C 32.3) and C-10 (δ_C 17.9) with the reported calculated values [δ_C (*E*) 33.0, (*Z*) 27.0 for C-7 and δ_C (*E*) 17.0, (*Z*) 11.0 for C-10].[3,9] The L-configuration of proline was determined by acid hydrolysis and derivatization of **1** using Marfey's reagent, followed by HPLC analysis.[10] In order to determine the stereochemistry of C-2 methyl, several unsuccessful reactions were attempted, resulting in

decomposition of compound **1**. Thus, the stereochemistry of C-2 methyl of **1** remains undermined at this time.

Table 1. ^{1}H and ^{13}C NMR data for compounds **1**–**2**[a]

Position	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
fatty acid				
1	169.1 s		169.0 s	
2	53.2 d	3.58 (H, q, 7.0)	53.2 d	3.51 (H, q, 7.0)
3	207.3 s		206.9 s	
4	39.5 t	2.51 (H, t, 7.2) 2.49 (H, t, 7.2)	39.0 t	2.59 (H, dt, 18.0, 7.4) 2.48 (H, dt, 18.0, 7.4)
5	23.0 t	1.53 (2H, m)	22.9 t	1.55 (2H, m)
6	29.0 t	1.32 (2H, m)	29.0 t	1.31 (2H, m)
7	32.3 t	1.95 (2H, m)	32.4 t	1.95 (2H, m)
8	131.0 d	5.39 (H, m)	131.1 d	5.39 (H, m)
9	125.2 d	5.40 (H, m)	125.0 d	5.40 (H, m)
10	17.9 q	1.62 (3H, d, 4.4)	17.9 q	1.62 (3H, d, 3.5)
2-Me	13.2 q	1.38 (3H, d, 7.0)	13.2 q	1.33 (3H, d, 7.0)
proline				
1'	172.6 s		172.3 s	
2'	59.0 d	4.51 (H, dd, 8.7, 3.9)	59.0 d	4.52 (H, dd, 8.7, 4.0)
3'	29.2 t	2.21 (H, m) 1.98 (H, m)	29.1 t	2.21 (H, m) 1.98 (H, m)
4'	24.8 t	2.08 (H, m) 1.97 (H, m)	24.9 t	2.08 (H, m) 2.00 (H, m)
5'	47.3 t	3.64 (H, m) 3.52 (H, m)	47.4 t	3.61 (H, m) 3.55 (H, m)
1'-OMe	52.3 q	3.73 (3H, s)	52.2 q	3.72 (3H, s)

[a] Spectra were recorded in $CDCl_3$ at 400 and 100 MHz for ^{1}H and ^{13}C, respectively.

Compound **2** was found to have the same molecular formula ($C_{17}H_{27}NO_4$) as **1**, based on HRESIMS data (*m/z*: 332.1834 $[M + Na]^+$; Calcd for $C_{17}H_{27}NO_4Na$: 332.1838). The NMR (1D NMR, COSY, HMQC, and HMBC) data revealed that **2** and **1** possessed the same planar structure. Analysis of the amino acid revealed its proline was also L-configuration. However, there was a notable high-field shifting of chemical shifts of H-2 (δ_H 3.51), CH_3-2 (δ_H 1.33), C-3 (δc 206.9), and C-4 (δc 39.0) in **2**, indicating that **2** was an isomer of **1** at C-2.

Compound **3** was obtained as a yellow gum. Its molecular formula, $C_{13}H_{16}O_5$, was established using HRESIMS (*m/z* 251.0910 $[M - H]^-$). The ^{1}H NMR spectrum of **3** indicated the presence of 12 methyl

protons (δ 2.01, 2.06, 2.40, and 3.57) and two methylene protons (δ 3.58). The ^{13}C NMR and DEPT data indicated the presence of the following: four methyl carbons (δ 10.0, 12.5, 32.7, and 52.0); one methylene carbon (δ 35.2), and eight quaternary carbons (δ 112.3, 117.3, 124.1, 128.6, 152.1, 155.4, 171.6, and 205.4). The connectivities of these groups and carbons were deduced from the HMBC spectra (Figure 2), and the structure of **3** was elucidated as shown.

Compounds **1**–**12** were evaluated for cytotoxicity against the A-375 cell line by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[3] Compounds **4**, **6**, and **7** displayed moderate activity, with IC_{50} values of 3.2, 2.8, and 0.97 μg/mL, compounds **1**, **2**, **5**, **8**, **10**, **11**, and **12** showed weak activity with IC_{50} values of 20.3, 38.5, 13.8, 71.7, 27.7, 53.3, and 62.1 μg/mL, whereas compounds **3** and **9** were inactive (IC_{50} >100 μg/mL).

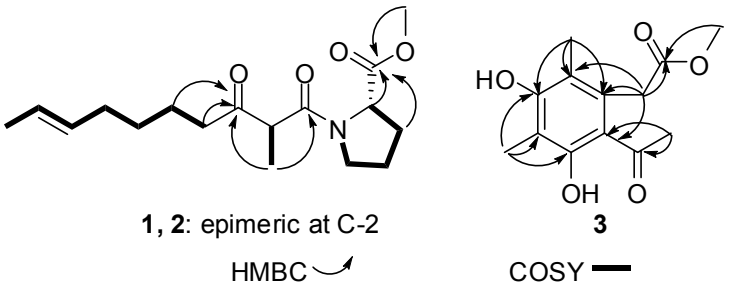

Figure 2. Key COSY and HMBC correlations of compounds **1**–**3**

EXPERIMENTAL

General Experimental Procedures. Optical rotations were obtained from a Shenguang SGW-1 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. ^{1}H NMR, ^{13}C NMR, DEPT spectra and 2D NMR were recorded on a BRUKER BIOSPIN AVANCE III spectrometer using TMS as the internal standard. ESI-MS were obtained by an AGILENT 1200/Q-TOF 6510 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (ODS-A, 10×250 mm, 5 μm) at 5 mL/min.

Fungal Material. The fungus P. *citrinum* was isolated from marine sediments collected from Langqi Island, Fujian, China. It was identified according to its morphological characteristics and ITS by Beijing Sunbiotech Co. Ltd, and preserved in our laboratory at −80 ºC. The producing strain was prepared on Martin medium and stored at 4 ºC.

Fermentation and Extraction. The fungus was cultured under static conditions at 28 ºC for 30 days in 1000-mL conical flasks containing the liquid medium (400 mL/flask) composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), yeast extract (3 g/L), and seawater. The fermented whole broth (60 L) was filtered through

cheese cloth to separate supernatant from mycelia. The former was extracted two times with EtOAc to yield an EtOAc solution that was concentrated under reduced pressure to give a broth extract (32.0 g), while the latter was extracted two times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted two times with EtOAc and concentrated in vacuo to give a mycelia extract (20.7 g).

Purification. The broth extract (32.0 g) was separated into 11 fractions on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction 7 (4.2 g) eluted with CH_2Cl_2/MeOH (100:1) was further purified on a Si gel column using a step gradient elution. Subfraction 7−3 (1.5 g) was purified by a reversed-phase column (MeOH/H_2O, 3:2) and semipreparative HPLC (80% MeOH containing 0.1% TFA), yielding compounds **4** (81.6 mg), **5** (228.2 mg), **6** (111.7 mg), and **7** (125.8 mg). Fraction 10 (1.9 g) eluted with CH_2Cl_2/MeOH (20:1) was further purified on a Sephadex LH-20 (MeOH). Subfraction 10−4 (326 mg) was purified by a reversed-phase column (MeOH/H_2O, 3:2) and semipreparative HPLC (55% MeCN), yielding compounds **1** (8.7 mg) and **2** (15.4 mg). Subfraction 10−7 (409 mg) was purified by a reversed-phase column (MeOH/H_2O, 2:3) and semipreparative HPLC (35% MeCN), yielding compounds **3** (13.9 mg) and **12** (8.1 mg). The mycelia extract (20.7 g) was separated into 7 fractions on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction 5 (4.9 g) eluted with CH_2Cl_2/MeOH (20:1) was further purified on a Sephadex LH-20 ($CHCl_3$/MeOH, 1:2). Subfraction 5−4 (885 mg) was purified by a reversed-phase column (MeOH/H_2O, 3:2) and semipreparative HPLC (45% MeCN), yielding compounds **8** (28.2 mg), **9** (41.5 mg), **10** (23.7 mg), and **11** (55.5 mg).

Tumonoic acid K (**1**): yellow oil; $[\alpha]^{31}_D$ −47.1 (*c* 0.26, MeOH); ^{1}H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 310.2020 $[M + H]^+$ (Calcd for $C_{17}H_{28}NO_4$: 310.2018).

Tumonoic acid L (**2**): yellow oil; $[\alpha]^{31}_D$ −82.6 (*c* 0.45, MeOH); ^{1}H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 332.1834 $[M + Na]^+$ (Calcd for $C_{17}H_{27}NO_4Na$: 332.1838).

Methyl 2-(2-acetyl-3,5-dihydroxy-4,6-dimethylphenyl)acetate (**3**): yellow gum; UV λ_{max} (MeCN) nm (log ε): 277 (3.86); ^{1}H NMR (500MHz) δ 3.58 (2H, s, CH_2-7), 3.57 (3H, s, OCH_3-8), 2.40 (3H, s, CH_3-10), 2.06 (3H, s, CH_3-4), 2.01 (3H, s, CH_3-6); ^{13}C NMR (125MHz) δ 205.4 (s, C-9), 171.6 (s, C-8), 155.4 (s, C-5), 152.1 (s, C-3), 128.6 (s, C-1), 124.1 (s, C-2), 117.3 (s, C-6), 112.3 (s, C-4), 52.0 (q, OCH_3-8), 35.2 (t, C-7), 32.7 (q, CH_3-10), 12.5 (q, CH_3-6), 10.0 (q, CH_3-4); HRESIMS *m/z* 251.0910 $[M - H]^-$ (Calcd for $C_{13}H_{15}O_5$, 251.0919).

Hydrolysis and L-FDAA Derivatization of Tumonoic Acids K and L (1 and 2). Pure tumonoic acids K and L (1 mg each) were hydrolyzed with 4 mL of 6 N HCl for 4 h in a sealed, thick-walled vial at 110 ºC, respectively. The hydrolysates were evaporated under nitrogen and then derivatized by treatment first with 400 μL of 1 M $NaHCO_3$ and second with 200 μL of *N*-α-(2,4-dinitro-5-flourophenyl)-L-alaninamide

(L-FDAA) solution (10 mg/mL in acetone). Each reaction mixture was heated to 80 ºC for 30 min, cooled to room temperature, and finally quenched with 200 μL of 2 N HCl. MeCN (1.2 mL) was then added for subsequent HPLC analysis. Each standard proline (2 mg) was derivatized under the same procedure with L-FDAA. For each reaction product, a linear gradient of 5% to 50% MeCN (contain 0.1% TFA) over 60 min was used to separate the derivatized products by HPLC (ODS column, 4.6 mm × 250 mm, UV 340 nm, flow rate 1 mL/min). Retention times of the derivatized hydrolysate were monitored by UV (340 nm) and compared to that of the derivatized commercially available standard prolines. Retention times for standard proline-FDAA derivatives: L-Pro 44.0 min, D-Pro 45.4 min. Retention times for the FDAA hydrolysate derivatives of tumonoic acids **K** and **L**: **K** 44.0 min, **L** 44.0 min.

Biological Assay. Cytotoxic activity was evaluated by the MTT method using A-375 cell line.[3] The cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37 ºC. Those cell suspensions (200 μL) at a density of 5×10^4 cell mL^{-1} were plated in 96-well microtiter plates and incubated for 24 h at the above condition. The test compound solution (2 μL in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same condition. Then 20 μL of the MTT solution (5 mg/mL in RPMI-1640 medium) was added to each well and incubated for 4 h. The 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.

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