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NOVEL CYTOTOXIC METABOLITES FROM THE MARINE-DERIVED FUNGUS *TRICHODERMA CITRINOVIRIDE*

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Abstract – Three new compounds, trichoderiol C (**1**), citrinoviric acid (**4**), and penicillenol D (**5**), together with seven known compounds, trichoderiol A (**2**), lignoren (**3**), penicillenol B_1 (**6**), penicillenol B_2 (**7**), cyclo-(Leu-Pro) (**8**), cyclo-(Ile-Pro) (**9**), and cyclo-(Phe-Pro) (**10**), were isolated from the marine-derived fungus *Trichoderma citrinoviride*. The structures of these compounds were elucidated using spectroscopic methods, including 1D and 2D nuclear magnetic resonance and high-resolution mass spectrometric analyses. Among these compounds, **4** and **5** showed moderate cytotoxic effects on the A-375 cell line, with IC_{50} values of 85.7 and 32.6 μM, respectively.

Numerous natural products with novel structures and distinct biological activities have been discovered as secondary metabolites of marine-derived microbes.[1] Some of these natural products have been used as drugs. For example, echinocandins, ergot alkaloids, cyclosporine, and lovastatin have been used as antifungal, analgesic, immunosuppressive, and cholesterol-lowering drugs,[2] respectively. To search for new anticancer compounds, more than 300 microbial strains isolated from sediment samples that were collected from the Min River estuary in China were screened for cytotoxicity against the A-375 cell line. Among these strains, the fungus *Trichoderma citrinoviride* showed significant cytotoxic activity. The

crude extract of *T. citrinoviride* was separated using chromatography on silica gel and Sephadex LH-20 columns. The crude extract was then purified via reversed-phase high-performance liquid chromatography (HPLC) to yield three new compounds, trichoderiol C (**1**), citrinoviric acid (**4**), and penicillenol D (**5**), together with seven known compounds, trichoderiol A (**2**),[3] lignoren (**3**),[4] penicillenol B_1 (**6**),[5] penicillenol B_2 (**7**),[5] cyclo-(Leu-Pro) (**8**),[6] cyclo-(Ile-Pro) (**9**),[7] and cyclo-(Phe-Pro) (**10**).[8] In the present study, we report the isolation, structural elucidation, and bioactivities of compounds **1**–**10**.

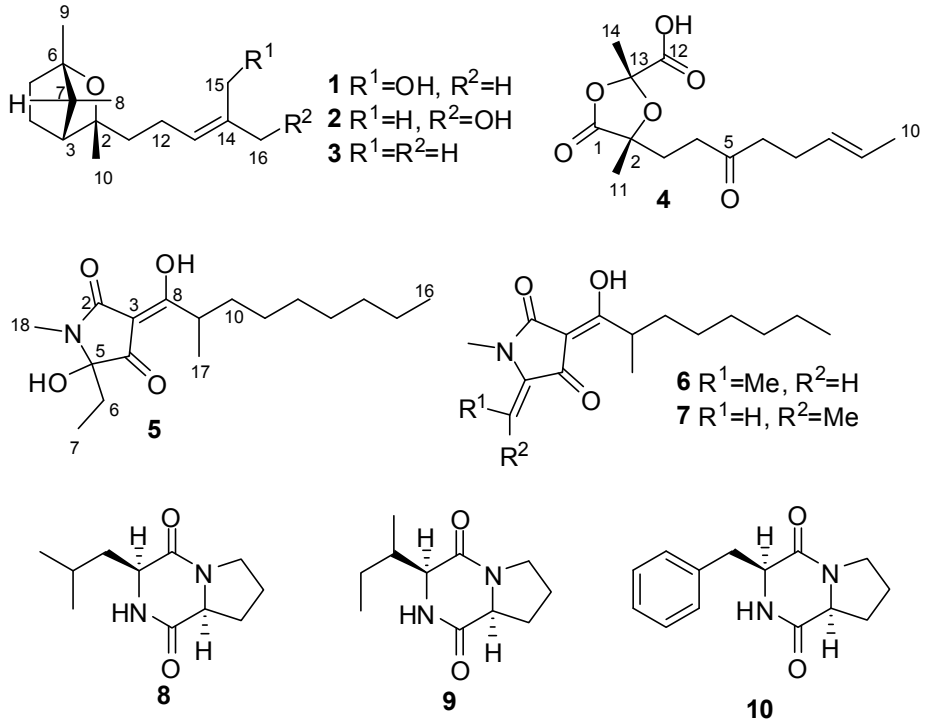

Figure 1. Structures of compounds **1**–**10**

Compound **1**, which is trivially named as trichoderiol C, was obtained as yellow oil and was analyzed to have the molecular formula $C_{15}H_{26}O_2$ through positive high-resolution electrospray ionization mass spectroscopy (HRESIMS) (*m/z*: 239.2003 $[M + H]^+$, Calcd. for $C_{15}H_{27}O_2$, 239.2011). Its nuclear magnetic resonance (NMR) data (Tables 1 and 2), combined with distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum coherence (HMQC) spectrum analyses, revealed 15 carbon signals, including four methyls, five methylenes, three methines, and three quaternary carbons. The plane structure of **1** was revealed via COSY and HMBC spectrum analyses (Figure 2). The COSY correlations of H-4 with H-3 and H-5, H-7 with H-3 and H-8, and H-12 with H-11 and H-13 demonstrated the connections from H-5 to H-8 and from H-11 to H-13. The HMBC correlations of H-8 with C-3, C-6, and C-7, H-9 with C-5, C-6, and C-7, and H-10 with C-2 and C-3 confirmed the presence of the bridged-ring part. The HMBC correlations of H-15 with C-13, C-14, and C-16 and H-16 with C-13,

C-14, and C-15 confirmed the presence of the long-chain part. Finally, the HMBC correlations of H-10 with C-11 and H-11 with C-2 and C-3 linked the two independent parts. The relative stereochemistry of **1**, which contains four asymmetric carbons, was settled based on observable NOE signals between H-8 and H-10 (Figure 3). The *Z* configuration was assigned to the C-13 and C-14 double bond through the NOE correlation of H-13 with H-16. Therefore, the structure of **1** was deduced as shown in Figure 1.

Table 1. ^{1}H NMR data (500 MHz, *J* in Hz and δ in ppm) of compounds **1**, **4**, and **5** in DMSO-d_6

Position	**1**	**4**	**5**
3	1.86 (1H, m)	2.04 (1H, m) 2.10 (1H, m)	
4	1.55 (1H, m) 1.86 (1H, m)	2.31 (1H, m) 2.46 (1H, m)	
5	1.54 (1H, m) 1.68 (1H, m)		
6		2.45 (2H, t, 7.2)	1.84 (1H, dq, 14.6, 7.5) 2.01 (1H, dq, 14.6, 7.3)
7	1.58 (1H, m)	2.20 (2H, dt, 7.2, 7.2)	0.69 (3H, dd, 7.5, 7.3)
8	1.04 (3H, d, 6.8)	5.35 (1H, m)	
9	1.25 (3H, s)	5.40 (1H, m)	3.55 (1H, m)
10	1.16 (3H, s)	1.60 (3H, d, 6.1)	1.45 (1H, m) 1.66 (1H, m)
11	1.51 (2H, t, 8.1)	1.46 (3H, s)	
12	2.15 (2H, m)		
13	5.31 (1H, t, 7.4)		1.19~1.33 (10H, m)
14		1.71 (3H, s)	
15	4.11 (1H, d, 11.8) 4.16 (1H, d, 11.8)		
16	1.79 (3H, s)		0.84 (3H, t, 7.1)
17			1.16 (3H, d, 6.7)
18			2.93 (3H, s)

Compound **4**, which is trivially named as citrinoviric acid, was obtained as white powder and was analyzed to have the molecular formula $C_{14}H_{20}O_6$ using negative HRESIMS (*m/z*: 283.1183 $[M - H]^-$, Calcd. for $C_{14}H_{19}O_6$, 283.1182). The IR absorptions at 3432, 1736, and 1634 cm^{-1}, indicated the presence of a carboxylic acid moiety and an alkenyl moiety.[9] Its NMR data (Tables 1 and 2), combined with DEPT and HMQC spectrum analyses, revealed 14 carbon signals, including three methyls, four methylenes, two methines, and five quaternary carbons. The plane structure of **4** was revealed using COSY and HMBC spectrum analyses (Figure 2). The COSY correlations of H-3 with H-4, H-7 with H-6 and H-8, and H-9 with H-8 and H-10 demonstrated the connections from H-3 to H-4 and from H-6 to H-10. The HMBC

correlations of H-11 with C-1, C-2, and C-3, and H-3, H-4, and H-6 with C-5 confirmed the presence of a long chain with eleven carbons. The residual HMBC correlations of H-14 with C-12 and C-13 suggested the short carbon chain from C-14 to C-12 via C-13. Since the degree of unsaturation of **4** was five, a cycle was necessary considering the three carbonyl groups and an alkenyl group determined. Furthermore, taking into account the molecular formula $C_{14}H_{20}O_6$ and the chemical shifts of C-1 (177.4 s), C-2 (83.1 s), and C-13 (96.4 s), the two carbon chains should be connected via two oxygen atoms, which formed a cyclic acetal structure. The relative stereochemistry of **4** was determined based on the NOE correlation of H-11 with H-14 (Figure 3). The double bond *E* configuration was confirmed through the NOE correlation of H-8 with H-10. Therefore, the structure of **4** was deduced as shown in Figure 1.

Table 2. ^{13}C NMR data (125 MHz, δ in ppm) of compounds **1**, **4**, and **5** in DMSO-d_6

Position	1	4	5
1		177.4 s	
2	75.1 s	83.1 s	173.2 s
3	54.4 d	30.0 t	99.1 s
4	24.3 t	36.5 t	194.2 s
5	40.4 t	210.5 s	90.3 s
6	81.4 s	42.7 t	27.2 t
7	44.3 d	26.7 t	7.6 q
8	14.5 q	129.1 d	192.0 s
9	26.0 q	126.2 d	36.2 d
10	24.8 q	17.8 q	33.7 t
11	40.3 t	23.1 q	29.2 t
12	22.2 t	175.8 s	27.0 t
13	128.7 d	96.4 s	27.1 t
14	134.4 s	5.9 q	22.5 t
15	61.5 t		31.6 t
16	21.5 q		14.0 q
17			17.1 q
18			22.9 q

Compound **5**, which is trivially named as penicillenol D, was obtained as yellow oil and was analyzed to have the molecular formula $C_{17}H_{29}NO_4$ using negative HRESIMS (310.2015 $[M - H]^-$, Calcd. for $C_{17}H_{28}NO_4$, 310.2018). FT-IR microscopy data suggested the presence of the hydroxyl group (3433), the carbonyl group (1712), the amide group (1640), and the alkenyl group (1618). Its NMR data (Tables 1 and 2), combined with DEPT and HMQC spectrum analyses, revealed 17 carbon signals, including four methyls, seven methylenes, one methine, and five quaternary carbons. The COSY correlations from H-16 to H-17 confirmed the presence of a long-chain part, including nine carbons. The COSY correlations of

H-6 with H-7 verified the presence of an ethyl group. Further careful comparison of the molecular formular and the similar NMR spectral data (Tables 1 and 2) of **5** with those of penicillenols A_1,[5] showed **5** differed from the known compound at the chemical shifts of C-5 (90.3 s, 21.6 ppm downfield shifting than A_1, 68.7 d), C-6 (27.2 t, 39.4 ppm highfield shifting than A_1, 66.6 d), C-7 (7.6 q, 10.2 ppm highfield shifting than A_1, 17.8 q), and an additional methylene group (27.0 t) suggesting that **5** owned the similar plane structure as penicillenols A_1 with exception of the position of a hydroxyl group and the longth of a carbon chain. This result was confirmed by the very similar FT-IR data of these two compounds and the HMBC correlations from H-18 to C-2 and C-5, H-6 to C-4 and C-5, H-7 to C-5, and H-17 to C-8, C-9, and C-10. Therefore, the structure of **5** was deduced as shown in Figure 1.

The new compounds (**1**, **4**, and **5**) were tested for their cytotoxic effects on the A-375 cell line using the MTT method.[10] Compounds **4** and **5** showed moderate cytotoxicity against the A-375 cell line, with IC_{50} values of 85.7 and 32.6 μM, respectively.

Trichoderma species are important sources of many bioactive compounds. However, chemical investigations of *T. citrinoviride* focused only on the cellulase[11] and antifeedant[12] activities until now. To the best of our knowledge, this study is the first to reveal the antitumor activity of metabolites from *T. citrinoviride*. Moreover, the metabolite of the carboxylic acid and alkaloid is almost never found in *T. citrinoviride*.

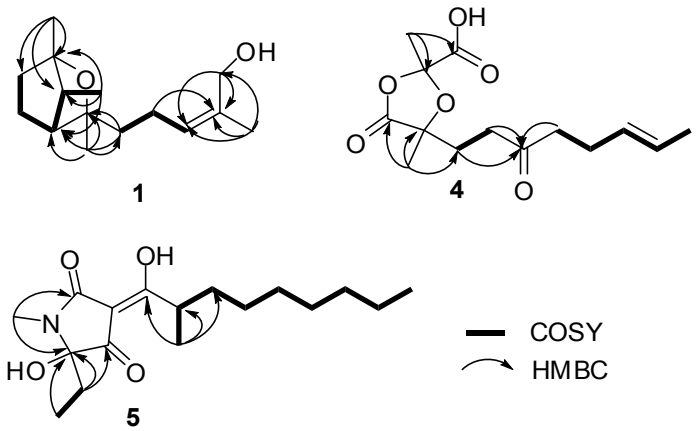

Figure 2. Key COSY and HMBC correlations of compounds **1**, **4**, and **5**

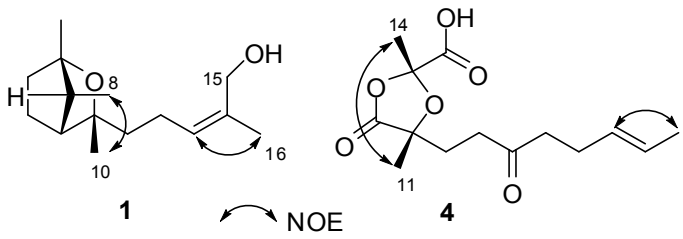

Figure 3. Key NOE correlations of compounds **1** and **4**

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. HRESIMS were obtained by an Agilent Q-TOF 6520 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 *T. citrinoviride* 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 °C. The producing strain was prepared on Martin medium and stored at 4 °C.

Fermentation and Extraction. The fungus *T. citrinoviride* was cultured under static conditions at 28 °C for 30 d in 1000 mL conical flasks containing a 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 the supernatant from the mycelia. The former was extracted two times with EtOAc to obtain an EtOAc solution, whereas the latter was extracted three 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 to give another EtOAc solution. Both EtOAc solutions were combined and concentrated under reduced pressure to obtain a crude extract (46.5 g).

Purification. The crude extract (46.5 g) of the fungus *T. citrinoviride* was separated into six fractions on a silica gel column using a step-gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction A (5.6 g) was further purified on a silica gel column using a step-gradient elution of CH_2Cl_2 and MeOH to obtain four subfractions. Subfraction A-1 (1.8 g) was subjected to Sephadex LH-20 ($CHCl_3$:MeOH, 1:2), followed by semipreparative HPLC (50% MeCN, 0.1% TFA), to yield compounds **1** (12 mg), **2** (6 mg), **3** (23 mg), and **8** (10 mg). Subfraction A-2 (0.9 g) was subjected to Sephadex LH-20 ($CHCl_3$:MeOH, 1:2), followed by semipreparative HPLC (40% MeCN, 0.1% TFA), to yield compounds **4** (9 mg) and **10** (9 mg). Fraction B (5.2 g) was further purified on a silica gel column using a step-gradient elution of CH_2Cl_2 and MeOH to obtain five subfractions. Subfraction B-1 (1.7 g) was subjected to Sephadex LH-20 ($CHCl_3$:MeOH, 1:2), followed by semipreparative HPLC (85% MeCN, 0.1% TFA), to yield compounds **5** (21 mg), **6** (19 mg), and **7** (13 mg). Subfraction B-2 (1.2 g) was subjected to Sephadex LH-20 ($CHCl_3$:MeOH, 1:2), followed by semipreparative HPLC (35% MeCN), to yield compound **9** (15 mg).

Trichoderiol C (**1**): yellow oil ($CHCl_3$); $[\alpha]^{23}_{D}$ -28.8 (*c* 0.10, MeOH); ^{1}H and ^{13}C NMR data (see Tables

1 and 2); HRESIMS (*m/z:* 239.2003 $[M + H]^+$, calcd for $C_{15}H_{27}O_2$, 239.2011); IR (KBr) ν_{max} 3395, 2962, 2925, 2848, 1659, 1454, 1377, 1095, 1021, 955, 915, 882 cm^{-1}.

Citrinoviric acid (**4**): white powder (MeOH); $[\alpha]^{20}_D$ +96.7 (*c* 0.03, $CHCl_3$); ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 283.1183 $[M - H]^-$, calcd for $C_{14}H_{19}O_6$, 283.1182); IR (KBr) ν_{max} 3432, 2958, 2921, 2848, 1736, 1634, 1462, 1381, 1266, 1098, 1025, 800 cm^{-1}.

Penicillenol D (**5**): yellow oil ($CHCl_3$); $[\alpha]^{20}_D$ -60.0 (*c* 0.13, $CHCl_3$); ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS (*m/z*: 310.2015 $[M - H]^-$, calcd for $C_{17}H_{28}NO_4$, 310.2018); IR (KBr) ν_{max} 3433, 2956, 2925, 2856, 1712, 1640, 1618, 1462, 1377, 1344, 1258, 1095 cm^{-1}.

Biological Assays. The cytotoxic activity for the A-375 cancer cell line was evaluated by the MTT method. Doxorubicin was used as the reference drug.

ACKNOWLEDGEMENTS

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