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To cite this article: Xiao-Rui Liang, Feng-Ping Miao, Yin-Ping Song, Zhan-Yong Guo & Nai-Yun Ji (2016) Trichocitrin, a new fusicoccane diterpene from the marine brown alga-endophytic fungus Trichoderma citrinoviride cf-27, Natural Product Research, 30:14, 1605-1610, DOI: 10.1080/14786419.2015.1126264

To link to this article: http://dx.doi.org/10.1080/14786419.2015.1126264
Trichocitrin, a new fusicoccane diterpene from the marine brown alga-endophytic fungus *Trichoderma citrinoviride* cf-27

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\section*{ABSTRACT}
One new diterpene, trichocitrin (1), and four known secondary metabolites, nafuredin (2), 5-hydroxy-2,3-dimethyl-7-methoxy-chromone (3), 24-methylenecycloartanol (4) and citrostadienol (5), were isolated from the culture of marine brown alga-endophytic *Trichoderma citrinoviride* cf-27. Trichocitrin (1) represents the first *Trichoderma*-derived and furan-bearing fusicoccane diterpene, and its structure and relative configuration were identified by analysis of 1D/2D NMR and mass spectroscopic data. Compounds 1 and 2 exhibited 8.0- and 9.5-mm inhibition zones, respectively, against *Escherichia coli* at 20 μg/disc and 54.1 and 36.7% growth inhibition, respectively, of *Prorocentrum donghaiense* at 80 μg/mL.

\section*{1. Introduction}
Secondary metabolites of marine-derived fungi have attracted a great attention for natural product research since the end of last century, which afforded more than 1000 new compounds so far (Rateb & Ebel 2011). Among them, over 20% were obtained from marine algicolous fungi, including endophytic and epiphytic strains (Rateb & Ebel 2011). The marine alga-derived endophytic fungi were the focus of our researches, and a series of new
terpenes, steroids, polyketides and alkaloids have been isolated and identified (Miao, Li, et al. 2012; Miao, Liang, et al. 2012; Ji et al. 2013; Liu et al. 2013; Sun et al. 2013; Miao et al. 2014). Trichoderma species were known for the production of functional products (Ahluwalia et al. 2015; Iqtedar et al. 2015). In our continuing chemical investigation towards the marine-derived Trichoderma species, Trichoderma citrinoviride cf-27 isolated from the marine brown alga Dictyopteris prolifera has been studied. As a result, one new furan-containing diterpene, trichocitrin (1), and four known compounds, nafuredin (2) (Ui et al. 2001), 5-hydroxy-2,3-dimethyl-7-methoxychromone (3) (Takenaka et al. 2000), 24-methylenecycloartanol (4) (Yan et al. 2015), and citrostadienol (5) (Yan et al. 2015), were characterised (Figure 1). The isolation, structure elucidation and bioactivity of these compounds are the main subjects of this article.

2. Results and discussion

Compound 1 was obtained as colourless oil. A molecular formula of $C_{20}H_{28}O$ was assigned by HREIMS (m/z 284.2149 [M]$^+$, Calcld for $C_{20}H_{28}O$, 284.2140), requiring seven degrees of unsaturation. The $^1H$ NMR spectrum (Table S1) showed two methyl singlets at $\delta_H 0.84$ (3H, s, H-15) and 1.68 (3H, brs, H-20), one methyl doublet at $\delta_H 1.04$ (3H, d, $J = 6.8$ Hz, H-16), a pair of broad singlets at $\delta_H 4.70$ (1H, brs, H-19a) and 4.75 (1H, brs, H-19b) assignable to two terminal olefinic protons, and one singlet at $\delta_H 7.03$ (1H, s, H-17) attributable to an aromatic proton. The $^{13}C$ NMR spectrum (Table S1) exhibited 20 resonances, sorted into three methyl, seven methylene, five methine and five quaternary carbon atoms by DEPT and HSQC experiments. The $^1H$-$^1H$ COSY correlations (Figure S1) indicated the presence of two spin systems, $-\text{CH}_2-\text{CH}-\text{CH}(-\text{CH}_3)-\text{CH}_2-$ (C-1 to C-4 and C-16) and $-\text{CH}_3-\text{CH}_2-\text{CH}-\text{CH}-\text{CH}_2-\text{CH}_2-$ (C-8 to C-13). The connectivity of them was established by the HMBC correlations (Figure S1) from H-15 to C-1, C-10, C-13 and C-14, and the attachment of an isopropenyl to C-11 was verified by the HMBC correlations from H-19 to C-11 and C-20 and from H-20 to C-11, C-18 and C-19. Moreover, the HMBC correlations from H-1 and H-2 to C-6, from H-4 to C-5, from H-8 to C-6 and C-17, from H-9 to C-7 and from H-17 to C-5 and C-6 established the presence of a trisubstituted furan unit. The above data evidenced the gross structure of 1, and its relative configuration was confirmed by NOESY correlations. In the NOESY spectrum, the correlations of Me-15 with H-11 and H-1a located them on the same face of the molecule, while the correlations of H-2
with Me-16, H-10 and H-1b placed them on the opposite face (Figure S2). Thus, the structure and relative configuration of compound 1 were established, trivially named trichocitrin.

Although a large number of fusicocane diterpenes with various bioactivities have been characterised from plants and fungi (Muromtsev et al. 1994; de Boer & de Vries-van Leeuwen 2012), trichocitrin (1) represents the first Trichoderma-derived and furan-bearing fusicocane diterpene. As shown in Figure 2, a plausible biogenetic pathway is proposed for 1. On the other hand, four known compounds, including nafuredin (2) (Ui et al. 2001), 5-hydroxy-2,3-dimethyl-7-methoxychromone (3) (Takenaka et al. 2000), 24-methylenecycloartanol (4) (Yan et al. 2015) and citrostadienol (5) (Yan et al. 2015), were identified by NMR data comparison with literature values. Among them, nafuredin (2) with a strong anthelmintic activity was originally isolated from Aspergillus niger associated with a marine sponge (Omura et al. 2001; Ui et al. 2001), which was also obtained from the plant-endophytic Trichoderma sp. recently (Damour et al. 2015). To the best of our knowledge, T. citrinoviride cf-27 is the third fungal strain to produce nafuredin (2).

Compounds 1 and 2 were evaluated for antibacterial activity against Escherichia coli and Staphylococcus aureus, antimicroalgal activity against Prorocentrum donghaiense, and brine shrimp toxicity against Artemia salina (Schrader et al. 1997; Miao, Liang, et al. 2012). The results showed that 1 and 2 could inhibit E. coli with inhibitory diameters of 8.0 and 9.5 mm, respectively, at 20 μg/disc, but they were inactive to S. aureus. Additionally, 1 and 2 exhibited 54.1 and 36.7% growth inhibition of P. donghaiense, respectively, at 80 μg/mL. However, 1 and 2 were almost incapable to kill A. salina, with inhibitory rates of 9.3 and 0%, respectively, at 100 μg/mL.

3. Experimental

3.1. General

The optical rotation was measured on a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). The IR spectrum was obtained on a JASCO FT/IR-4100 spectrometer (JASCO, Tokyo, Japan).
The NMR spectra were recorded at 500 and 125 MHz for $^1$H and $^{13}$C, respectively, on a Bruker Avance III 500 NMR spectrometer (Bruker Corp., Billerica, MA, USA) using TMS as an internal standard. The low- and high-resolution mass spectra were determined on an LCQ Fleet mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Autospec Premier P776 mass spectrometer (Waters Corp., Milford, MA, USA), respectively. HPLC separation was carried out on an Agilent 1260 infinity HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) using an Eclipse SB-C18 (5 μm, 9.4 x 250 mm) column. Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Thin-layer chromatography (TLC) was carried out with precoated silica gel plates (GF-254, Qingdao Haiyang Chemical Co., Qingdao, China). The solvents were of analytical grade except for the spectral-grade MeOH for HPLC.

3.2. Fungal material, fermentation, extraction and isolation

The fungal strain *T. citrinoviride* cf-27 was isolated from the fresh tissue of the surface-sterilised marine brown alga *D. prolifera* collected from Zhoushan, China, in August, 2010. The fungus was identified by morphological observation and analysis of the ITS regions of its rDNA, whose sequence data have been deposited at GenBank with the accession number KT259441. The strain is preserved at the Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences. The fresh mycelia were grown on the potato dextrose agar plates. Then, pieces of mycelia were cut into small segments and aseptically inoculated into 200 Erlenmeyer flasks (1 L), each containing 100 g mice, 0.6 g peptone and 100 mL natural sea water. Static fermentations were performed at room temperature for 30 days.

The whole culture was extracted with EtOAc to yield 388.5 g gum after the removal of the solvent by evaporation (40 °C) at reduced pressure. The extract was subjected to silica gel CC with a step-gradient solvent system consisting of 0–100% petroleum ether (PE)/(EtOAc gradient) to afford 21 fractions (Fr. 1–21), monitored by TLC. Fr. 2 (eluted with PE/EtOAc, 20:1) was further purified by HPLC (EtOH/H$_2$O, 4:1) to give 1 (3.0 mg). Fr. 3 (eluted with PE/EtOAc, 10:1) was further purified by silica gel CC (PE/EtOAc, 10:1) to yield three subfractions, 3–1, 3–2 and 3–3. Subfraction 3–1 was further purified by CC on Sephadex LH-20 (CHCl$_3$/MeOH, 1:1) to afford 3 (56.0 mg). Subfraction 3–2 was further purified by HPLC (MeOH) to produce 4 (4.8 mg). Subfraction 3–3 was further purified by HPLC (MeOH/H$_2$O, 4:1–100% MeOH) to yield 5 (3.0 mg). Fr. 5 (eluted with PE/EtOAc, 5:1) was further purified by HPLC (MeOH/H$_2$O, 4:1) to give 2 (100.8 mg).

**Trichocitrin (1):** Colourless oil; [α]$_D^{25}$ = −8.5 (c 0.07, MeOH); IR (KBr) $\nu_{\text{max}}$ 2924, 2858, 1631, 1431, 1072 cm$^{-1}$; $^1$H NMR (500 MHz,CDCl$_3$) δ$_H$ 1.21 (1H, dd, $J = 13.8, 11.8$ Hz, H-1a), 1.68 (1H, dd, $J = 13.7, 1.4$ Hz, H-1b), 2.85 (1H, m, H-2), 2.90 (1H, m, H-3), 2.20 (1H, dd, $J = 15.0, 4.1$ Hz, H-4a), 2.87 (1H, dd, $J = 15.3, 8.0$ Hz, H-4b), 2.22 (1H, dddd, $J = 15.2, 11.1, 3.8, 1.2$ Hz, H-8a), 2.53 (1H, dddd, $J = 15.2, 6.0, 3.3$ Hz, H-8b), 1.41 (1H, m, H-9a), 1.47 (1H, m, H-9b), 1.74 (1H, dddd, $J = 11.4, 5.4, 3.8$ Hz, H-10), 2.42 (1H, td, $J = 10.5, 7.5$ Hz, H-11), 1.49 (1H, ddd, $J = 13.2, 7.5, 2.8$ Hz, H-12a), 1.80 (1H, ddd, $J = 13.2, 9.9, 8.6$ Hz, H-12b), 1.30 (1H, ddd, $J = 12.4, 8.6, 2.8$ Hz, H-13a), 1.68 (1H, m, H-13b), 0.84 (3H, s, H-15), 1.04 (3H, d, $J = 6.8$ Hz, H-16), 7.03 (1H, s, H-17), 4.70 (1H, brs, H-19a), 4.75 (1H, brs, H-19b), 1.68 (3H, brs, H-20); $^{13}$C NMR (125 MHz,CDCl$_3$) δ$_C$ 38.8 (CH$_2$, C-1), 36.7 (CH, C-2), 41.7 (CH, C-3), 33.7 (CH$_2$, C-4), 158.6 (C, C-5), 129.3 (C, C-6), 124.3 (C, C-7), 24.3 (CH$_2$, C-8), 26.2 (CH$_2$, C-9), 46.8 (CH, C-10), 53.9 (CH, C-11), 26.4 (CH$_2$, C-12),
38.3 \((\text{CH}_2\text{, C-13})\), 43.2 (\text{C, C-14}), 23.2 \((\text{CH}_3\text{, C-15})\), 17.1 \((\text{CH}_2\text{, C-16})\), 141.1 (\text{CH, C-17}), 147.7 (\text{C, C-18}), 110.9 \((\text{CH}_2\text{, C-19})\), 18.4 \((\text{CH}_3\text{, C-20})\); APCl+MS and ESI+MS \(m/z\) 285 \([\text{M} + \text{H}]^+\); HREIMS \(m/z\) 284.2149 \([\text{M}]^+\), Calcd for \text{C}_{20}\text{H}_{28}\text{O}, 284.2140.

### 3.3. Bioassays

Antibacterial activity against \textit{E. coli} and \textit{S. aureus} (chloramphenicol as positive control, 22-mm zone at 20 \(\mu\text{g/disc}\)) , antimicroalgal activity against \textit{P. donghaiense} (\text{K}_2\text{Cr}_2\text{O}_7 as positive control, 68.3% inhibition at 80 \(\mu\text{g/mL}\)) and brine shrimp lethality against \textit{A. salina} (\text{K}_2\text{Cr}_2\text{O}_7 as positive control, 100% inhibition at 100 \(\mu\text{g/mL}\)) were assayed as described previously (Schrader et al. 1997; Miao, Liang, et al. 2012).

### 4. Conclusion

Chemical investigation towards the marine brown alga-endophytic \textit{T. citrinoviride} cf-27 resulted in the isolation and identification of one new diterpene (1) and four known compounds (2–5). Trichocitrin (1) represents the first \textit{Trichoderma}-derived and furan-bearing fusicoccane diterpene, and it features antibacterial and antimicroalgal activities.

### Supplementary material

Supplementary details relating to this paper are available online, alongside Table S1, Figures S1 and S2, NMR and mass spectra.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Funding

This work was supported by Strategic Priority Research Program [grant number XDA11020403]; National Natural Science Foundation of China [grant number 41106136], [grant number 41106137].

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