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Two new sesquiterpenoids from the marine-sediment-derived fungus *Trichoderma harzianum* P1-4

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**ABSTRACT**
Three cyclonerane sesquiterpenoids, including the known cyclonerodiol (1), together with its new derivatives, (10E)-12-acetoxy-10-cyclonen-3,7-diol (2) and 12-acetoxycycloneran-3,7-diol (3) were isolated from the cultures of marine-sediment-derived fungus *Trichoderma harzianum* P1-4. The structures of the new compounds (2 and 3) were elucidated based on extensive spectroscopic methods (1D/2D NMR and HR-MS) and optical rotation analysis.

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**KEYWORDS**
Trichoderma harzianum; sesquiterpenoid; marine fungus; cyclonerodiol

**1. Introduction**
In agriculture, *Trichoderma* species have received considerable attention as biocontrol agents of plant pathogens, for their production of a wide range of bioactive metabolites (Reino et al. 2007; Vinale et al. 2012). *T. harzianum* is perhaps the most studied of the *Trichoderma* species for novel antibiotic substances (Reino et al. 2007; Ahluwalia et al. 2015; Han et al. 2018). Harzianic acid is a novel secondary metabolite isolated from *T. harzianum*, showing antifungal and plant growth promotion activities (Vinale et al. 2009). Harziandione represents a new class of diterpenes and first isolated from the biological control agent *T. harzianum* (Ghisalberti et al. 1992). Another compound harzianolide is a butenolide metabolite which has been isolated from three different strains of *T. harzianum* (Almassi et al. 1991; Claydon et al. 1991; Ordentlich et al. 1992), and recent study showed that it can be acted as a plant growth regulator and...
systemic resistance elicitor, and may play a novel role in both plant growth regulation
and plant defense responses (Cai et al. 2013). A novel trichothecone harzianum A, a
sesquiterpene-based mycotoxin with antifungal activity, also had been reported from
T. harzianum (Corley et al. 1994). Trichosetin, a novel tetramic acid with remarkable
antimicrobial activity, had been isolated from the dual culture of T. harzianum and
Catharanthus roseus Callus (Marfori et al. 2002). In addition, a novel 6-pentyl-α-pyrone
from T. harzianum, displaying plant growth inhibitory and antimicrobial properities
(Cutler et al. 1986).

Although Trichoderma is commonly considered as a terrestrial genus, halotolerant
strains have been continuously reported from mangrove plants, marine sediments,
invertebrates and macroalgae (Song et al. 2018). As for their special living environ-
ment, marine-derived Trichoderma strains can produce more structural novelty and
biological diversity natural products (Zhu et al. 2015). In recent years, the secondary
metabolites of many marine-derived Trichoderma species such as T. asperellum
(Song et al. 2018), T. longibrachiatum (Miao et al. 2012), T. virens (Shi et al. 2018a),
T. harzianum (Suzue et al. 2016) and T. citrinoviride (Liang et al. 2016), had been inves-
tigated. Some of these metabolites have been found to exhibit antimicrobial, cyto-
toxic and anti-inflammatory activities (Song et al. 2018; Zhang et al. 2016a, 2017). As
part of our ongoing study on new and bioactive secondary metabolites from marine-
derived Trichoderma species, the culture of the marine-sediment-derived strain T. harzia-
num P1-4 had been examined, which resulted in the isolation and identification of two
new cyclonerane sesquiterpenoids, (10E)-12-acetoxy-10-cycloneren-3,7-diol (2) and 12-
acetoxycycloneran-3,7-diol (3), along with the known cyclonerodiol (1) (Figure 1). Herein,
we describe the isolation and structure elucidation of the two new compounds.

2. Results and discussion

Compound 1 was isolated as colorless oil, and identified to be cyclonerodiol by the
identical NMR and specific optical rotation data in the literature reported (Langhanki
et al. 2014; Song et al. 2018). Cyclonerodiol (1) is a well-known fungi-derived sesquiter-
penoid, and also a characteristic metabolite of Trichoderma species strains. Previously,
the biosynthetic pathway of cyclonerodiol has been specifically established with cell-
free extracts of G. fujikuroi (Can et al. 1981). Meanwhile, a similar pathway probably
exists in the genus Trichoderma species.

Compound 2 was also obtained as a colorless oil. Its molecular formula was deter-
mined to be C_{17}H_{30}O_{4} by analysis of HREIMS (m/z 298.2150 [M]^+, calcd for C_{17}H_{30}O_{4},
298.2144), implying three degrees of unsaturation. The IR spectrum indicated the

Figure 1. Structures of compounds 1–3.
presence of hydroxyl (3455 cm\(^{-1}\)) and carbonyl (1720 cm\(^{-1}\)) groups. The \(^1\)H NMR spectrum (Table S1) in combination with HSQC data showed four methyl singlets at \(\delta_H\) 2.10 (3 H, s, H-2'), 1.70 (3 H, s, H-15), 1.29 (3 H, s, H-13), and 1.20 (3 H, s, H-14), one methyl doublet at \(\delta_H\) 1.07 (3 H, d, \(J = 6.9\) Hz, H-1), one oxymethylene singlet at \(\delta_H\) 4.48 (2 H, s, H-12), and one double triplet at \(\delta_H\) 5.49 (1 H, td, \(J = 7.2, 1.0\) Hz, H-10) for an olefinic proton. The \(^1\)C NMR and DEPT spectra (Table S1) showed 17 resonances for five methyls, five methylene, three methine, and four quaternary carbons (including an ester carbonyl carbon). The above NMR data closely resembled those of cyclonerodiol (1) (Langhanki et al. 2014), except for the absence of signals for a methyl group. However, HMBC correlations (Figure S1) from H-2' and H-12 to C-1' indicated the presence of an acetoxymethylene group, which was attached to C-11 by HMBC correlations from H-12 to C-11 and C-15 and from H-15 to C-11 and C-12. Thus, 2 was deduced to be an acetoxylated derivative of 1 at C-12, supported by the other HMBC and COSY correlations (Figure S1). A comparison of NMR data with those of (2E)-TCA 12a revealed that the double bond between C-10 and C-11 features an E geometry (Shi et al. 2018b), validated by the NOE correlations between H-12 and H-10, and between H-9 and H-15 (Figure S2). The relative configurations around C-2, C-3, and C-6 in the five-membered ring of 2 were proposed to be the same as those of 1 by the identical NMR data. This was also confirmed by the NOE correlation of H-1 with H-6, and of H-2 with H-13 (Figure S2). However, the stereochemistry of C-7 in the side chain was also in accordance with that of 1 based on the NMR data and the biosynthetic pathway (Evans et al. 1976). The absolute configuration of 2 was assigned as 2S, 3R, 6R, and 7R based on the similar specific optical rotation value ([\(\alpha\])\(^{22}\)_D \(-9.7\)) to that of 10(E)-cyclonerotriol (Li et al. 2007), which it structure was determined by X-ray method (Evans et al. 1976). Thus, the structure of 2 was elucidated as (10E)-12-acetoxy-10-cycloneren-3,7-diol.

Compound 3 gave the molecular formula C\(_{17}\)H\(_{32}\)O\(_4\) as established by HREIMS (m/z 300.2293 [M\(^+\)], calcd for C\(_{17}\)H\(_{32}\)O\(_4\), 300.2301), two more hydrogen atoms than 2. A careful comparison of the \(^1\)H and \(^1\)C NMR data (Table S1) revealed that 3 differed from 2 mainly at the side chain terminus. The splitting pattern of H-15 suggested it was bonded to a methine group (C-11), which was attached to a methylene group and an acetoxymethylene group by COSY correlations of H-11 with H-10 and H-15 and HMBC correlations from H-15 to C-10, C-11, and C-12, from H-12 to C-10, C-15, and C-1', and from H-2' to C-1'. These evidences established 3 to be a hydrogenated derivative of 2, corroborated by the other COSY and HMBC correlations (Figure S1). The relative and absolute configurations at C-2, C-3, C-6, and C-7 of 3 were in accordance with those of 1 and 2 based on the identical NMR data and biogenic consideration, but the stereochemistry of C-11 remained unresolved. So, the structure of 3 was established to be 12-acetoxycycloneren-3,7-diol.

By comparison the structures of cyclonerane sesquiterpenoids 2 and 3 with those of all isolated analogues, there are no 12-acetoxy derivatives reported. So, we doubted the compounds 2 and 3 are artifacts resulting from the treated with EtOAc during the extraction and/or isolation procedure, which would be confirmed in our further studies.

Previously, the antibacterial activities of the known compound 1 had been reported, but no significant inhibition was observed toward to some Gram-positive
and Gram-negative strains (Cutler et al. 1991). In this our study, the new compounds 2 and 3 were also tested no active against Bacillus subtilis and Staphylococcus aureus with concentration up to 100 μg/disk, through the disk diffusion method (Miao et al. 2012).

3. Experimental

3.1. General experimental procedures

Mass spectra were determined on an Autospec Premier P776 mass spectrometer (Waters Corp., Milford, MA, USA). IR spectra were obtained on a JASCO FT/IR-4100 Fourier Transform InfraRed spectrometer (JASCO, Tokyo, Japan). Optical rotations were measured on a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). HPLC separation was operated on an Agilent HPLC system (1260 infinity quaternary pump, 1260 infinity diode-array detector) using an Eclipse SB-C18 (5 μm, 9.4 × 250 mm) column (Agilent Technologies Inc., Santa Clara, CA, USA). Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China), RP-18 reversed-phase silica gel (AAG12S50, YMC Co. Ltd., Kyoto, Japan), and Sephadex LH-20 (GE, Uppsala, Sweden). Thin-layer chromatography (TLC) was carried out with pre-coated silica gel plates (GF-254, Qingdao Haiyang Chemical Co., Qingdao, China). The solvents were of analytical grade except for the spectral-grade CH3CN for HPLC.

3.2. Fungal materials and culture conditions

The fungal strain Trichoderma harzianum P1-4 was isolated from a marine sediment sample collected at E 119°18′ 26′′ and N 38°5′ 05′′ of the Bohai Sea, China, in April 2015 (Ma et al. 2018). The fungus was identified by analysis of the morphology and ITS regions of its rDNA. The sequence data have been deposited at GenBank with the accession number MH290488, and the strain has been preserved at the Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences. The fungal strain was grown on potato dextrose agar (PDA) plates at 28°C for several days, which was then cultured statically at room temperature for 30 days in 100 × 1 L Erlenmeyer flasks. Each flask contained 300 mL of the modified potato dextrose broth (PDB) medium (50% seawater), which was prepared by adding 20.0 g glucose, 6.6 g sucrose, 6.6 g mannitol, 3.0 g sodium glutamate, 0.3 g CaBr2, 5.0 g peptone, and 5.0 g yeast extract powder into 1 L potato (200 g) broth.

3.3. Extraction and isolation

The whole cultures (300 mL ×100 flasks) were filtered through cheesecloth to separate it into filtrate and mycelia. The filtrate was extracted three times with EtOAc to yield an EtOAc solution, whereas the mycelia were extracted three times with a mixture of CH2Cl2/MeOH (1:1, v/v) to get the CH2Cl2-MeOH solution. The EtOAc and CH2Cl2-MeOH solutions were evaporated under reduced pressure and combined to afford a crude residue. The crude residue (82.9 g) was subjected to CC on silica gel with a gradient of petroleum (PE)-EtOAc system (50:1, 20:1, 10:1, 5:1, 2:1, 1:1, and 1:5, v/v) to afford seven
fractions (A-G) based on TLC analysis. Fraction D (756.6 mg) eluted with PE/EtOAc (5:1) was further purified by RP-18 CC (MeOH/H2O, 70:30 to 80:20, v/v) and repeated silica gel CC (CH2Cl2/MeOH, 100:1 to 30:1 and CH2Cl2/EtOAc, 8:1, v/v) to afford compound 1 (10.5 mg). Fraction E (3.50 g) eluted with PE/EtOAc (2:1) and was further purified by RP-18 CC (MeOH/H2O, 70:30 to 100:0, v/v) to afford nine subfractions (F1-F9). Fraction F4 (52.8 mg) was further purified by semi-preparative HPLC (CH3CN/H2O, 43:57, v/v, 15 min, flow rate = 1.8 mL/min) to obtain compound 2 (tR = 14.0 min, 2.9 mg). Compound 3 (3.2 mg) was obtained from subfraction F5 (83.4 mg) after purification by silica gel CC (CH2Cl2/EtOAc, 1:1, v/v).

3.3.1. (10E)-12-Acetoxyl-cyclonerodiol (2)

Colorless oil; [α]22D = -9.7 (c 0.29, CHCl3); IR (KBr) νmax 3455, 2966, 1720, 1637, 1384, 1246 cm⁻¹; EIMS m/z (%) 298 [M]+ (< 1%), 263 (7), 238 (12), 221 (16), 203 (15), 139 (38), 125 (100), 107 (40); HREIMS m/z 298.2150 [M]+, calcd for C17H30O4, 298.2144; ¹H NMR (500 MHz, CDCl3): δ 5.49 (1 H, td, J = 7.1, 1.0 Hz, H-10), 4.48 (2 H, s, H-12), 2.15 (2 H, m, H-9), 2.10 (3 H, s, H-2'), 1.88 (2 H, m, H-5a and H-6, overlap), 1.71 (1 H, m, H-4a), 1.70 (3 H, s, H-15), 1.63 (1 H, m, H-2), 1.58 (2 H, m, H-4b and H-5b, overlap), 1.55 (2 H, t, J = 8.4 Hz, H-8), 1.29 (3 H, s, H-13), 1.20 (3 H, s, H-14), 1.07 (3 H, d, J = 6.9 Hz, H-1); ¹³C NMR (125 MHz, CDCl3): δ 171.0 (s, C-1'), 130.2 (s, C-3), 129.6 (d, C-10), 81.3 (s, C-7), 74.7 (s, C-7), 70.2 (t, C-12), 54.3 (d, C-6), 44.3 (d, C-2), 40.4 (t, C-4), 39.9 (t, C-8), 26.1 (q, C-13), 25.1 (q, C-14), 24.3 (t, C-5), 22.4 (t, C-9), 21.0 (q, C-2'), 14.5 (q, C-1), 14.0 (q, C-15). (Table S1)

3.3.2. 12-Acetoxyl-10,11-dihydro-cyclonerodiol (3)

Colorless oil; [α]22D = -10.1 (c 0.22, CHCl3); IR (KBr) νmax 3455, 2961, 2927, 1723, 1637, 1384, 1260, 750 cm⁻¹; EIMS m/z (%) 300 [M]+ (< 1%), 265 (28), 187 (36), 157 (27), 139 (100), 127 (53), 109 (58); HREIMS m/z 300.2293 [M]+, calcd for C17H32O4, 300.2301; ¹H NMR (500 MHz, CDCl3): δ 3.98 (1 H, dd, J = 10.7, 6.0 Hz, H-12a), 3.89 (1 H, dd, J = 10.7, 6.8 Hz, H-12b), 2.08 (3 H, s, H-2'), 1.87 (1 H, m, H-5a), 1.86 (1 H, m, H-6), 1.81 (1 H, m, H-11), 1.70 (1 H, m, H-4a), 1.63 (1 H, m, H-2), 1.58 (1 H, m, H-4b), 1.57 (1 H, m, H-5b), 1.46 (2 H, m, H-8), 1.45 (1 H, m, H-9a), 1.40 (1 H, m, H-10a), 1.33 (1 H, m, H-9b), 1.29 (3 H, s, H-13), 1.18 (3 H, s, H-14), 1.18 (1 H, m, H-10b), 1.07 (3 H, d, J = 6.9 Hz, H-1), 0.96 (3 H, d, J = 6.8 Hz, H-15); ¹³C NMR (125 MHz, CDCl3): δ 171.3 (s, C-1'), 81.3 (s, C-3), 74.8 (s, C-7), 69.4 (t, C-12), 54.2 (d, C-6), 44.2 (d, C-2), 40.8 (t, C-8), 40.4 (t, C-4), 34.0 (t, C-10), 32.6 (t, C-11), 26.1 (q, C-13), 25.2 (q, C-14), 24.3 (t, C-5), 21.1 (t, C-9), 21.0 (q, C-2'), 16.8 (q, C-15), 14.5 (q, C-1). (Table S1)

4. Conclusion

Trichoderma harzianum strains are noted for their rich novel bioactive metabolites as biocontrol agent of soil-borne plant pathogens in agriculture. But, in this study, the secondary metabolites of a marine-sediment-derived strain T. harzianum P1-4 had been investigated. Two new cyclonerane sesquiterpenoids, (10E)-12-acetoxyl-10-cycloneren-3,7-diol (2) and 12-acetoxycycloneran-3,7-diol (3), together with the known...
cyclonerodiol (1) were isolated, and their structures were established based on extensive spectroscopic methods and optical rotation analysis. In addition, the antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* of two new compounds had been tested, but no activity was observed.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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