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Trichoderlates A and B, Two Bioactive Metal Complexes of Hydroxyaspergillic Acid from the Marine-derived Fungus *Trichoderma erinaceum* F1-1

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Abstract

Two new metal complexes of hydroxyaspergillic acid, trichoderlates A (1) and B (2), and five previously reported compounds (3-7) were obtained from *Trichoderma erinaceum* F1-1. The structures of new compounds were determined by their NMR, HRMS data, optical rotation calculations and chemical methods. This is the first report about metal complexes of hydroxyaspergillic acid from the genus *Trichoderma*. Trichoderlates A (1) and B (2) exhibited significant cytotoxicities against MDA-MB-435 human melanocyte cancer cells with IC_{50} values of 3.3 and 9.5 μM, respectively. Trichoderlate A (1) also showed significant cytotoxic activity against A549 adenocarcinomic human alveolar basal epithelial cells with IC_{50} value of 6.1 μM.

Keywords: Metal complexes; Cytotoxic activities; Marine-derived fungus; *Trichoderma erinaceum*

Introduction

Secondary metabolites (SMs), as chemical entities of defense against biological warfare, were produced by organisms in answer to environmental stimuli [1]. Those SMs isolated from plants, bacteria and fungi provide a diverse and unique source of candidates for drugs discovery. SMs derived from the marine fungi are well known for showing extraordinary potential biological activity due to the marine-derived fungi adapting to the specific habitat environment relative to terrestrial ones. For instance, pyrazin-2(1H)-one derivatives derived from amino acids were well known for showing significant cytotoxicities against virous cancer cell lines and strong toxicity towards brine shrimp [2-7]. By thoroughly literature survey, less than 40 natural pyrazin-2(1H)-one derivative in the literatures have been reported, and only five metal complexes of pyrazin-2(1H)-one derivative have been isolated from *A. ochraceus* Wilh, *A. flavus*, *Aspergillus sp.* SCSGAF0093, *Aspergillus* sp. 16-02-1, the co-culture of *A. sclerotiorum* and *P. citrinum*, *A. ochraceus* LCJ11-102 [2-7]. Despite these findings, the potential of marine fungi as source of pyrazin-2(1H)-one derivative have not been fully exploited. Whole genome sequencing of several marine fungi has revealed that considerable biosynthetic gene clusters of SMs appear to be silent under laboratory culture conditions [8]. Consequently, potential marine fungal secondary metabolites, including pyrazin-2(1H)-one derivative, have been under explored [9].

The genus *Trichoderma*, which is widely spread in nature, is commonly considered a biocontrol agent in agriculture. By 2008, more than 180 metabolites have been isolated from species belonging to this genus [10]. And a wide arrange of bioactivities of these compounds, including antibacterial, antifungal, antiviral activities, have been reported. In our previous work, two harziane-related diterpenoids were discovered from the glucose-peptone-yeast (GPY) medium of the marine-derived fungus *T. erinaceum* F1-1 associated with the Sea Star *A. planci* [11].

In our continued research project, the *Trichoderma erinaceum* F1-1 was cultured in potato dextrose broth (PDB) medium. Two new metal complexes of hydroxyaspergillic acid, trichoderlate A (1) and trichoderlate B (2), and five previously reported compounds (3-7) were obtained from this culture. This is the first report about metal complexes of hydroxyaspergillic acid from *Trichoderma* genus. Herein, we describe the isolation, structure characterization, and bioactivities of compounds 1-7.

Materials and Methods

General experimental procedures

The NMR data were obtained from Bruker Avance IIIT 500HD and 400 spectrometer made in Bruker Bio Spin AG. Referencing the signals of $CDCl_3$ (δ_H7.26 and δ_C77.2) and Methanol-d_4 (δ_H 3.31 and δ_C 49.0) to obtain the relative NMR chemical shifts. HRESIMS data were measured on Thermo MAT95XP EI high-resolution mass spectrometers made in Thermo Fisher Scientific In Preparative HPLC and column chromatography was performed adopting a Shim-pack PRC-ODS HPLC column (250 × 20 mm) made in Shimadzu Corporation, Silica

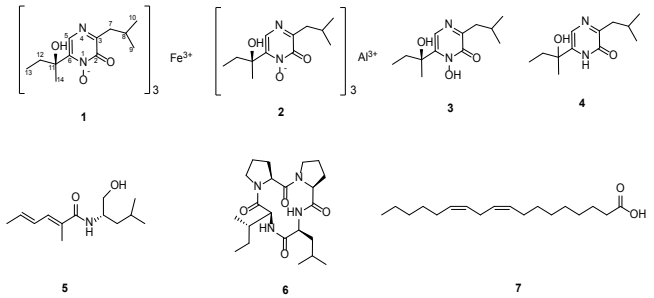

Figure 1: Chemical structures of compounds (1-7).

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gel (SiO_2, 200-300 mesh) made in Qingdao Marine Chemical Inc., and Sephadex LH-20 (green herbs) made in Beijing. UV, IR, and Optical rotations data were recorded on a UV-Vis-NIR spectrophotometer (Shimadzu Corporation, Nakagyo-Ku, Kyoto, Japan), a Frontier FT-IR spectrophotometer (PerkinElmer Inc., Waltham, MA, USA), and an Anton paar MCP500 polarimeter, respectively.

Fungal strain and culture method

T. erinaceum F1-1 was purified from the inner tissue of the sea star *A. planci*, which habited in Hainan Sanya National Coral Reef Reserve, China. Based on morphology and the results of the ITS regions of rDNA, the genus was identified. And its sequence data have been deposited in GenBank with the accession number KF999808. The PDB fermentation medium contains potatoes 200 g, dextrose 20 g, seawater 1 L and pH 7.5. In a sterile condition, transferring the mycelia to sterilized 200 mL of the PDB liquid medium in 500 mL Erlenmeyer flasks. After incubating 40 d at 28°C, 100 L culture broth were filtered and extracted four times with EtOAc, and then were concentrated to afford a crude extract 20 g.

Extraction and isolation

The extract (20 g) was fractionated on the Si gel column with a petroleum-EtOAc gradient (100:0-0:100) and then EtOAc-MeOH gradient (100:0-0:100) to obtain 12 fractions (Fr.1-Fr.12). Fr.4 was separated into six fractions (Fr.4-1-Fr.4-6) by the Sephadex LH-20 (MeOH). Fr.4-2 was further fractionated using the preparative HPLC with MeOH-H_2O (30:70-100:0) to obtain 1 (30.0 mg), 2 (16.0 mg) and 3 (5.2 mg). 5 (23.0 mg) and 7 (4.3 mg) were yielded from Fr.5 by the column chromatography on the Si gel with petroleum-EtOAc (100:0-0:100). Compound 4 (8.7 mg) was purified from Fr. 7 by repeated CC on the Si gel eluting with petroleum-EtOAc (30:70). 6 (2.0 mg) was purified from Fr.9 by the Sephadex LH-20 (MeOH).

Trichoderlate A (1): A red solid; UV (MeCN) λ_{max} (log ε): 312 (2.36) nm, 255 (4.50) nm; $[\alpha]^{20}_D$ -40.0 (*c* 0.5, MeOH); IR υ_{max} 3435, 2961, 1524, 1492, 1411, 1167 cm^{-1}; ^1H and ^{13}C NMR data (Table 1) HRESIMS $[M+H]^+$ ion at *m/z* 774.3618 (calculated (calcd) for $C_{36}H_{58}FeN_6O_9$, 774.3615).

Trichoderlate B (2): a yellow solid; UV (MeCN) λ_{max} (log ε): 316 (2.98) nm, 223 (3.88) nm; $[\alpha]^{20}_D$ -24.0 (*c* 0.5, MeOH); IR υ_{max} 3408, 2979, 1590, 1531, 1497, 1418, 1169 cm^{-1}; ^{1}HNMR data, (Table 1) HRESIMS $[M + H]^+$ ion at *m/z* 745.4079 (calcd for $C_{36}H_{58}AlN_6O_9$, 745.4081). Hydroxyaspergillic acid (3): red solid; UV (MeCN) λ_{max} (log ε): 348 (2.00) nm, 239 (3.10) nm; $[\alpha]^{20}_D$ -8.0 (*c* 0.5, MeOH) (calcd for 11*S*-1, $[\alpha]^{20}_D$ -10.1; 11*R*-1, $[\alpha]^{20}_D$ +15.6) (Figure 1).

Optical rotation calculations

MOE software in the MMFF94 force field was used to the conformational analysis of compounds [12]. The optical rotations were calculated using time-dependent density functional theory (TDDFT) [13]. We employed the B3LYP/6-311+G (d) level for optimizing the conformers and calculating optical rotations at λ=589.3 nm in methanol (Figures 2 and 3).

Bioassay

Cytotoxic assay: Compounds 1-7 were evaluated against human tumor cell line MDA-MB-435 and A549 by MTT method described in the literature [14]. Cisplatin was used as positive control (IC_{50} of cisplatin is 35.1 μM for A549 cell line and 8.5 μM for MDA-MB-435 cell line).

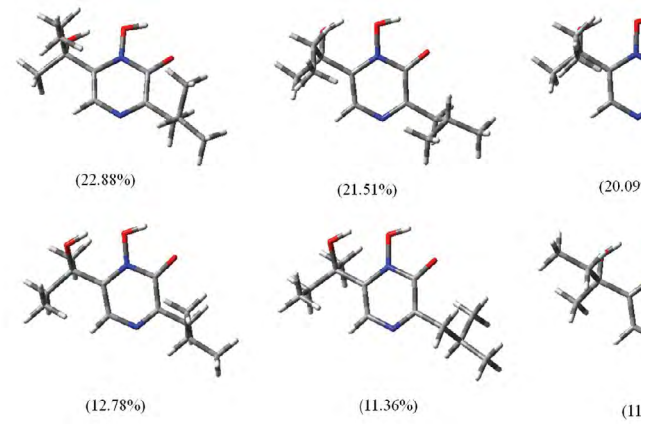


Figure 2: The optimized conformers and equilibrium populations of 11R-3.

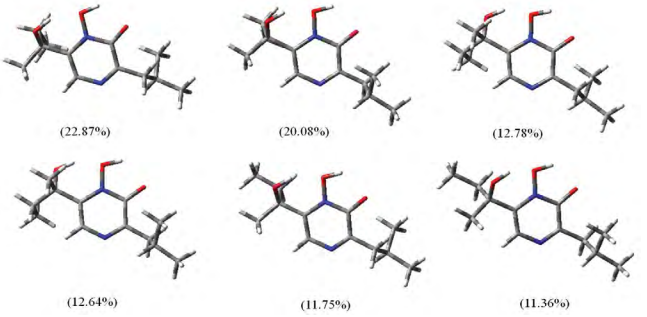


Figure 3: The optimized conformers and equilibrium populations of 11S-3.

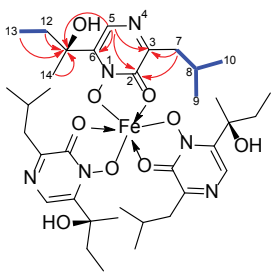
Results and Discussion

Structural elucidation

Trichoderlate A (1) was obtained as a red solid and gave a molecular formula of $C_{36}H_{57}FeN_6O_9$ by HRESIMS ion at *m/z* $[M+H]^+$ 774.3618 (calcd for $C_{36}H_{58}FeN_6O_9$, 774.3615). A dozen of carbons (Table 1) were classified as four methyl, two methylenes, two methines, and four non-protonated carbons. COSY correlations between H_2-7 (δ_H 2.64, m), H-8 (δ_H 2.01, m) and H_3-9 (δ_H 0.83, m), as well as between H-8 (δ_H 2.01, m) and H_3-10 (δ_H 0.83, m) indicated the existence of the segment -CH_2-CH-$(CH_3)_2$. According to the HMBC correlation between H_2-7 (δ_H 2.64, m) and C-3 (δ_C 150.7), the segment -CH_2-CH-$(CH_3)_2$ was placed in the C-3. In addition, the COSY correlation between H_2-12 (δ_H 1.93, m) and H_3-13 (δ_H 0.80, m) and HMBC correlations between H_2-12 (δ_H 1.93, m), H_3-13 (δ_H 0.80, m), H_3-14 (δ_H 1.58, s) and C-11 (δ_C 73.5) revealed the existence of the attachment -C (OH) (CH_3)-CH_2-CH_3-. Furthermore, HMBC correlations between H-5 (δ_H 7.73, s) and C-11 (δ_C 73.5), C-6 (δ_C 141.7) indicated that the substructure -C (OH) (CH_3)-CH_2-CH_3- was placed in C-6 (δ_C 141.7) (Figure 4). The comparison of spectroscopic data and the molecular formula showed that 1 was structurally similar to hydroxyaspergillic acid (3) [15]. Futhermore, alkali hydrolysis of 1 gave 3 and Fe $(OH)_3$ sediment [4], indicating 1 was an iron complex of 3. Based on the molecular formula, it was assured that trichoderlate A (1) was a complex consisting of three molecules of hydroxyaspergillic acid (3) and one iron atom. For the known metal complexes of pyrazin-2(1H)-one derivative, aluminium neohydroxy aspergillinand

Position	1 δH (J in Hz)	δC	2 δH (J in Hz)
1	-	-	-
2	-	155.4, C	-
3	-	150.7, C	-
4	-	-	-
5	7.73, s	122.8, CH	7.82, s
6	-	141.7, C	-
7	2.64, m	41.6, CH_2	2.74, m
8	2.01, m	28.0, CH	2.13, m
9	0.83, m	22.5, CH_3	0.88, m
10	0.83, m	22. 5, CH_3	0.88, m
11	-	73.5, C	-
12	1.93, m	32.6, CH_2	2.02, m
13	0.80, m	8.6, CH_3	0.88, m
14	1.58, m	24.3, CH_3	-

Table 1: ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for 1-2 (δ in ppm) in $CDCl_3$.



1

COSY HMBC

Figure 4: ^1H–^1H COSY and key HMBC correlations of trichoderlate A (1).

ferri-neo hydroxy aspergillin were racemic mixture [6], ochralate A showed negative optical activity (-3.2) [7]. Both trichoderlate A (1) and hydroxyaspergillic acid (3) obtained by alkali hydrolysis of 1 showed negative optical activity. Therefore, trichoderlate A (1) was not racemic mixture. Furthermore, the calculated optical rotation of 11*S*-3 (-10.1) was similar to the experimentally measured rotation (-8.0). Accordingly, the chemical structure of trichoderlate A (1) was established.

Trichoderlate B (2) was obtained as a yellow solid and gave a molecular formula of $C_{36}H_{57}AlN_6O_9$ by HRESIMS ion at *m/z* 745.4079 $[M+H]^+$ (calcd for $C_{36}H_{58}AlN_6O_9$, 745.4081). The ^1H NMR data revealed the presence of δ_H 7.82 (H-5), δ_H 2.74 (H_2-7), δ_H 2.02-2.12 (H-8, H_2-12), δ_H 0.87-0.89 (H_3-9, H_3-10, H_3-13) and δ_H 1.58 (H_3-14). All of these data are slightly different from trichoderlate A (1) (Table 1 and Figure 5). However, ^{13}C NMR and 2D NMR data couldn't be measured. Alkali hydrolysis of 2 gave Al $(OH)_3$ sediment and 3 [4], which means that 2 is an aluminum complex of 3. Based on the molecular formula, 2 were identified as a complex consisting of one aluminum atom and three molecules of 3. Similar to trichoderlate A (1), the absolute configuration of trichoderlate B (2) also was established by optical rotation calculations as shown in Figure 1.

The chemical structures of the known compounds hydroxyaspergillic acid (3) [15], 4-(1-hydroxy-1-methylpropyl)-2-isobutyl-pyrazin-2(1H)-one (4) [16], dichotomocej A (5) [17] and fenestin A (6) [18], linoleic acid (7) [19] were confirmed respectively by comparing their spectroscopic data of relevant references.

Biological activity

Compounds 1-7 were evaluated against human tumor cell line MDA-MB-435 and A549 by MTT method described in the literature [14]. Trichoderlate A (1), trichoderlate B (2) and hydroxyaspergillic acid (3) exhibited cytotoxic activities against MDA-MB-435 human melanocyte cancer cells with IC_{50} values of 3.3, 9.5, 49.9 μM, respectively. Trichoderlates A (1) and B (2) also showed cytotoxic activities against A549 adenocarcinomic human alveolar basal epithelial cells with IC_{50} values of 6.1, 15.6 μM.

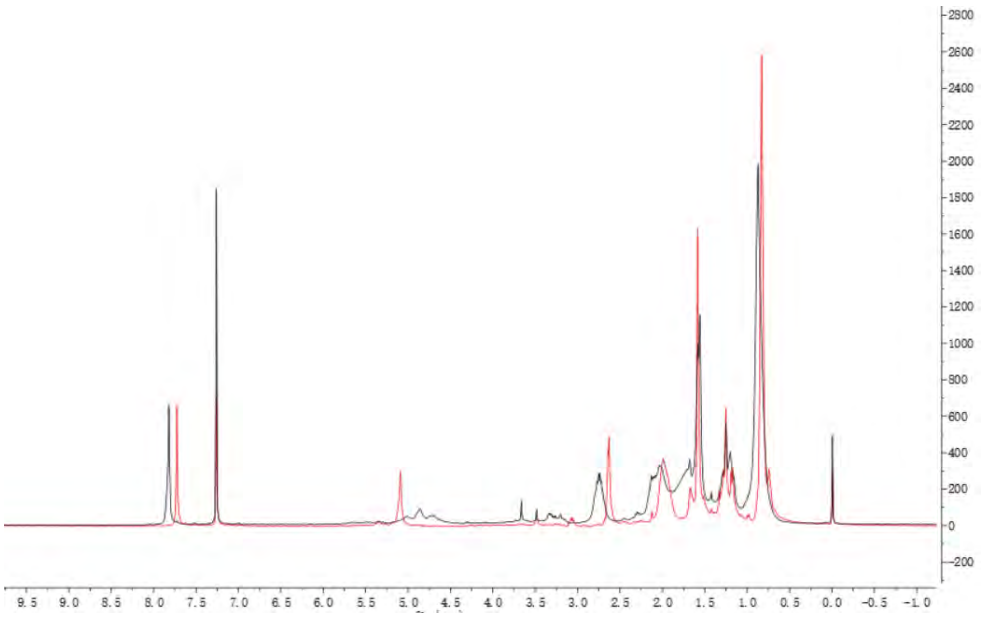


Figure 5: The comparison of 1H NMR spectra of 1 (red line) and 2 (black line) in CDCl3 (400 MHz).

Conclusion

Overall, two new metal complexes of hydroxyaspergillic acid, trichoderlates A (1) and B (2) were afforded from the PDB medium culture of *Trichoderma erinaceum* F1-1. And five known compounds (3-7) were also identified. Furthermore, this is the first report about metal complexes of hydroxyaspergillic acid from the genus *Trichoderma*. How the metals iron and aluminium are involved in the biosynthesis of the fungus, and how the enzymes catalyze the biosynthesis of complexes remain unknown.

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Conflicts of Interest

The authors declare no conflict of interest.

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