SHORT REPORT

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Peniciloxatone A, a New Polyoxygenated Ergostane Steroid Isolated from the Marine Alga-Sourced Fungus *Penicillium oxalicum* 2021CDF-3

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Abstract: Chemical investigations on the culture of *Penicillium oxalicum* 2021CDF-3, a marine red alga-sourced endophytic fungus, revealed a new steroid, namely peniciloxatone A (**1**). With the aid of high-resolution electrospray ionization mass spectrometry (HRESIMS) and nuclear magnetic resonance (NMR) analyses, the structure of peniciloxatone A (**1**) was determined to be a polyoxygenated ergostane steroid. Cytotoxic activities of **1** were tested against A549, FADU, and HepG2 cells, and **1** was active against the FADU and HepG2 cells, with IC_{50} values of 9.5 ± 0.1 and 18.1 ± 0.3 μM, respectively.

Keywords: *Penicillium oxalicum*; secondary metabolites; natural product; steroid; cytotoxicity. © 2024 ACG Publications. All rights reserved.

1. Fungal Source

The fungal strain *Penicillium oxalicum* 2021CDF-3 investigated in the present study was separated from the marine red alga *Rhodomela confervoides*, which was obtained from Lianyungang, China, in October 2021. By amplification of the internal transcribed space (ITS) rDNA sequence (GenBank accession number OP349593), the species was identified as *P. oxalicum* (99.82% identity). This strain was preserved at the School of Food and Pharmacy, Zhejiang Ocean University.

2. Previous Studies

Filamentous fungi pertaining to the *Penicillium* genus have long been extensively studied because of their high biosynthetic potential to generate abundant secondary metabolites [1−3]. These secondary metabolites are appealing sources of beneficial agrochemicals and pharmaceuticals [4]. *P. oxalicum*, a patented industrial fungus, was found to be rich in bioactive substances, including catabolic enzymes and small metabolites [5]. For example, *P. oxalicum* is a famous producer of bioactive secondary metabolites,

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including chromones [6], *N*-containing alkaloids [7], butyrolactones [8], and monoterpenoids [9]. In our previous studies, *P. oxalicum* obtained from the red alga *R. confervoides* was chosen for scale-up fermentation. As a result, ten structurally diverse polyketides were isolated when cultured on solid rice media [10]. By employing the one strain many compounds (OSMAC) strategy, this fungus was cultured in liquid PDB media and produced six indole derivatives [11]. Although many metabolites have been isolated, the discovery of additional bioactive metabolites from the fungus *P. oxalicum* is still highly important.

3. Present Study

As previously described, the fungus was cultivated in potato dextrose agar (PDA) media at 28 °C for four days to produce seeds. The agar blocks were subsequently divided into smaller blocks (1.5 cm × 1.5 cm) and transferred into 1 L Erlenmeyer flasks accommodating 300 mL of liquid potato dextrose broth (PDB) media. One hundred flasks in total were prepared and incubated at 28°C for 30 days. Afterwards, the whole cultures were combined, filtered with three layers of gauze, and extracted with EtOAc three times. A crude extract (approximately 28.0 g) was obtained after evaporation of the organic solvent in vacuo. The same crude extract was subsequently sectionalized by silica gel column chromatography with petroleum ether−EtOAc (from 20:1 to 1:1, v/v) and CH_2Cl_2−MeOH (from 20:1 to 5:1, v/v) elution mixtures. Eight fractions (A1−A8) were ultimately obtained. Among them, fraction A7 (1.5 g) eluting with CH_2Cl_2−MeOH 10:1 was selected for further purification. First, this fraction was subjected to silica gel column chromatography eluted with CH_2Cl_2−MeOH (from 20:1 to 10:1, v/v) to give three subfractions A7.1−A7.3. Then, subfraction A7.1 was chromatographed on a Sephadex LH-20 column (MeOH). Finally, peniciloxatone A (**1**) was obtained with a yield of 8.5 mg.

Peniciloxatone A (1): Colorless oil; $[\alpha]_D^{25}$ = +66.7° (*c* = 0.098, MeOH); UV (MeOH): λ_{max} (log ε): 205 (4.10), 256 (3.75) nm; for ^{1}H and ^{13}C NMR (measured in DMSO-d_6) data, see Table 1; HRESIMS: *m/z* 529.2773 $[M + Na]^+$ (calcd for $C_{28}H_{42}O_8Na^+$, 529.2777).

Cytotoxic Assay: Standard CCK-8 assays using A549 (human non-small cell lung cancer cell line), FADU (human pharyngeal squamous cancer cell line), and HepG2 (human hepatocellular carcinoma cell line) cell lines, which were described previously [12], were carried out to assess the cytotoxicity of the isolated compound **1**. Commercial doxorubicin was used as the positive control. The concentrations of **1** and doxorubicin used in this assay were 0, 3.125, 6.25, 12.5, 25, 50, and 100 μM.

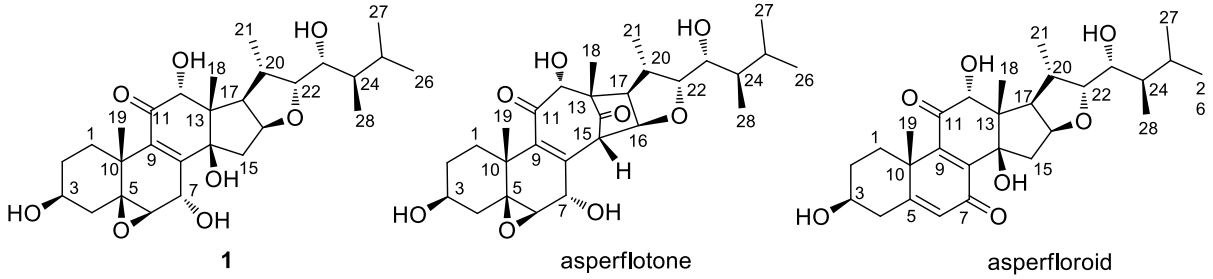

Figure 1. Structures of peniciloxatone A (**1**) and reference compounds asperflotone and asperfloroid

Compound **1** was purified as a colorless oil. The molecular formula of **1** was determined to be $C_{28}H_{42}O_8$ on the basis of the Na^+-ligand ion peak at *m/z* 529.2773 ($[M + Na]^+$, calcd for $C_{28}H_{42}O_8Na^+$, 529.2777) in its HRESIMS data. The ^{1}H NMR data combined with the HSQC spectra of **1** (Table 1) displayed signals of seven oxygenated methine protons at δ = 4.63 (dd, *J* = 6.4, 2.7 Hz, H-7), 4.25 (td, *J* = 9.1, 6.8 Hz, H-16), 4.06 (d, *J* = 3.2 Hz, H-12), 3.94 (dd, *J* = 7.3, 2.2 Hz, H-22), 3.56 (m, H-3), 3.09 (ddd, *J* = 8.8, 6.4, 2.2 Hz, H-23), 3.06 (d, *J* = 2.7 Hz, H-6), four methylenes (δ 2.58, 1.65; 2.33, 1.44; 2.03, 1.26; 1.68, 1.37), four methines (δ 2.18, 2.00, 1.91, 1.44), six methyls including two methyl singlets at δ = 1.15 (s, H_3-19), 1.08 (s, H_3-18), and four methyl doublets at δ = 0.94 (d, *J* = 7.2 Hz, H_3-21), 0.83 (d, *J* = 6.8 Hz,

H_3-26), 0.66 (d, J = 6.8 Hz, H_3-27), 0.62 (d, J = 7.0 Hz, H_3-28). Besides, five additional exchangeable protons at δ = 5.49 (d, J = 6.4 Hz, 7-OH), 5.24 (s, 14-OH), 4.82 (d, J = 4.7 Hz, 3-OH), 4.60 (d, J = 3.2 Hz, 12-OH), and 4.18 (d, J = 6.4 Hz, 23-OH) were observed. The ^{13}C NMR data of **1** indicated a total of 28 carbons, including one ketone group, one double bond, six methyls, four methylenes, eleven methines, and four sp^3 quaternary carbons. Detailed analysis of the spectroscopic features indicated that **1** closely resembled asperflotone and asperfloroid, two new ergostane steroids that were isolated from *Aspergillus flocculosus* 16D-1 [13]. The chemical shifts of compound **1**, asperflotone, and asperfloroid were almost identical (measured in DMSO-d_6), especially for the side chain from C-20 to C-28 (Table 1). The main differences between **1** and asperflotone were the presence of an oxygenated sp^3 quaternary carbon in **1** (δ 80.6, C-14) rather than a ketone (δ 209.4, C-14 in asperflotone) (Table 1). Moreover, the key differences between **1** and asperfloroid were as follows: (1) there were two oxygenated methines in **1** (δ 62.1, C-6; 62.3, C-7) rather than the double bond (δ 168.4, C-5; 125.4, C-6) present in asperfloroid; (2) there was an additional OH group in **1** (δ 5.49, d, J = 6.4 Hz, 7-OH) rather than a ketone (δ 187.8, C-7 in asperfloroid) (Table 1). The key COSY correlations of H-20/H_3-21/H-22/H-23/H-24/H-25/H_3-26/H_3-27/H_3-28 indicated a C-22 alkanol side chain (Figure 2). HMBC correlations from the angular methyl group H_3-18 to C-12/C-14/C-17 and from H_3-19 to C-1/C-5/C-9, together with the COSY correlations between H_2-1 and H_2-4, revealed the presence of a steroidal skeleton [13]. The HMBC correlations from H-6 (δ_H 3.06) to C-4/C-5/C-7/C-8/C-19, from H-7 (δ_H 4.63) to C-5/C-6/C-8/C-9/C-14, and from OH-7 (δ_H 5.49) to C-6/C-7/C-8, by the important COSY correlations of H-6/H-7/OH-7 confirmed that the two oxygenated methines were located at C-6 and C-7 (Figure 2). In addition, the remaining four exchangeable protons (δ_H 5.24, 4.82, 4.60, and 4.18), together with the upfield chemical shifts of C-5 (δ_C 60.4) and C-6 (δ_C 62.1), suggested the formation of an epoxy ring between C-5 and C-6 [13], which was in accordance with its molecular formula and related degrees of unsaturation. Other COSY and HMBC correlations allowed the complete assignment of the chemical structure of **1**. As shown in Figure 1, compared with other classical ergostane steroids, compound **1** possesses a polyoxygenated steroidal skeleton.

The relative configuration of **1** was solved by analysing its NOESY data and J coupling constants (Figure 2). NOE correlations between H-3/H-4b (δ_H 1.26) and H-6/H-4b suggested that H-3 and H-6 were located in the same orientation (tentatively assigned as α). The correlations of H_3-19/H-12, H-12/OH-14, H-12/H_3-18, and OH-14/H-7 with H-3/H-1b (δ_H 1.44) and H_3-19/H-1a (δ_H 2.33) indicated that H-7/H-12/OH-14/H_3-18/H_3-19 was located in the opposite orientation (assigned as β). Moreover, the correlations among H-16/H-17/H_3-21 and the absence of correlations between H_3-18/H-17 suggested that H-16, H-17 and H_3-21 were α-oriented. NOE correlations of H-20/H-22 indicated that they were located in the β orientation. The relative configurations of 23*R** and 24*R** on the alkanol side chain were solved on the basis of the coupling constants of $^3J_{H22-H23}$ (δ 3.94, dd, J = 7.3, 2.2 Hz in **1** vs δ 4.01, dd, J = 7.7, 1.6 Hz in asperfloroid) and $^3J_{H23-H24}$ (δ 3.09, ddd, J = 8.8, 6.4, 2.2 Hz in **1** vs δ 3.12, ddd, J = 9.0, 7.2, 1.6 Hz in asperfloroid) as previously depicted [9]. In terms of chemical stability, the relative configuration of C-5 was temporarily assigned to be 5*S** [9].

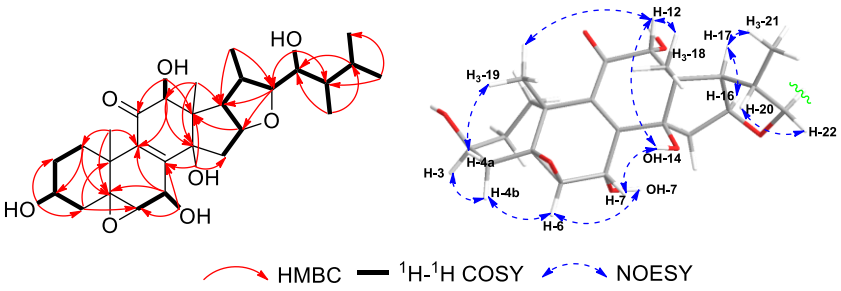

Figure 2. Key ^{1}H-^{1}H COSY, HMBC, and NOE (on the ring skeleton) correlations for **1**

Table 1. 1D NMR data of compound **1**, asperflotone, and asperfloroid (δ in ppm, measured in DMSO-d_6)

No	**Compound 1**		**Asperflotone** [13]		**asperfloroid** [13]	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1	2.33 dt (12.7, 3.2)	33.7 CH_2	2.36 br d (13.8)	35.1 CH_2	2.43 dt (13.9, 3.4)	31.9 CH_2
	1.44 m		1.41 br t (13.8)		1.06 td (13.9, 4.0)	
2	1.68 m	30.7 CH_2	1.69 m	30.5 CH_2	1.76 m	29.8 CH_2
	1.37 m		1.34 m		1.60 tdd (13.9, 11.0, 3.4)	
3	3.56 m	67.9 CH	3.50 m	67.7 CH	3.46 tt (11.0, 4.7)	70.3 CH
4	2.03 m	40.1 CH_2	1.95 t (12.0)	39.9 CH_2	2.60 m	41.5 CH_2
	1.26 m		1.26 br d (12.0)		2.47 ddd (13.1, 11.0, 1.4)	
5		60.4 C		61.3 C		168.4 C
6	3.06 d (2.7)	62.1 CH	3.01 br s	61.0 CH	6.24 d (1.4)	125.4 CH
7	4.63 dd (6.4, 2.7)	62.3 CH	4.07 br d (7.6)	67.9 CH		187.8 C
8		147.2 C		133.7 C		144.1 C
9		135.3 C		139.9 C		147.2 C
10		36.9 C		38.1 C		40.6 C
11		201.8 C		207.2 C		200.7 C
12	4.06 d (3.2)	76.9 CH	3.77 d (4.3)	82.0 CH	3.60 d (3.6)	80.2 CH
13		51.1 C		64.6, C		52.2 C
14		80.6 C		209.4 C		81.0 C
15	2.58 dd (12.0, 6.8)	41.4 CH_2	2.81 s	59.4 CH	2.34 dd (14.3, 7.9)	47.9 CH_2
	1.65 m				2.07 dd (14.3, 3.2)	
16	4.25 td (9.1, 6.8)	79.2 CH	4.23 d (8.1)	84.3 CH	4.60 td (8.2, 3.2)	83.9 CH
17	1.91 dd (9.1, 7.6)	53.9 CH	2.76 t (9.3)	50.2 CH	2.30 t (8.5)	57.7 CH
18	1.08 s	17.6 CH_3	1.15 s	14.9 CH_3	1.00 s	15.2 CH_3
19	1.15 s	17.7 CH_3	0.90 s	16.8 CH_3	1.46 s	22.3 CH_3
20	2.18 q (7.2)	37.3 CH	1.79 m	38.8 CH	2.60 m	38.4 CH
21	0.94 d (7.2)	14.1 CH_3	1.02 d (6.8)	13.6 CH_3	1.04 d (7.0)	14.7 CH_3
22	3.94 dd (7.3, 2.2)	83.6 CH	3.90 br d (7.9)	82.2 CH	4.01 dd (7.7, 1.6)	82.4 CH
23	3.09 ddd (8.8, 6.4, 2.2)	72.6 CH	3.08 br t (8.5)	71.9 CH	3.12 ddd (9.0, 7.2, 1.6)	72.7 CH
24	1.44 m	40.0 CH	1.51 m	39.8 CH	1.54 m	40.2 CH
25	2.00 m	25.5 CH	2.06 m	25.2 CH	2.07 m	25.4 CH
26	0.83 d (6.8)	21.9 CH_3	0.83 d (6.8)	21.6 CH_3	0.85 d (7.0)	21.7 CH_3
27	0.66 d (6.8)	15.6 CH_3	0.66 d (6.8)	15.2 CH_3	0.69 d (6.8)	15.5 CH_3
28	0.62 d (7.0)	10.1 CH_3	0.59 d (6.9)	9.7 CH_3	0.65 d (6.9)	10.0 CH_3
3-OH	4.82 d (4.7)		4.85 br s		5.09 br s	
7-OH	5.49 d (6.4)		5.90 d (7.6)		-	
12-OH	4.60 d (3.2)		5.54 d (4.3)		4.71 s	
14-OH	5.24 s		-		5.99 s	
23-OH	4.18 d (6.4)		4.43 d (7.1)		4.04 d (7.2)	

Ergostane steroids possess a wide range of biological activities and have long been used as privileged leads in drug discovery [14]. Steroids have been reported to display anti-inflammatory, antimicrobial, anticancer, and enzyme inhibitory activities [15−17]. In this study, cytotoxic activities of compound **1** against the A549, FADU, and HepG2 cell lines were tested using the standard CCK-8 assay, with commercial doxorubicin as a positive control (Table 1). Compound **1** showed promising cytotoxicity against the FADU cells, with an IC_{50} value of 9.5 ± 0.1 μM (compared with that of doxorubicin, IC_{50} = 5.6 ± 0.7 μM). Moreover, compound **1** also exhibited mild activity against the HepG2 cells (IC_{50} = 18.1 ± 0.3 μM), whereas it was inactive against A549 cells (IC_{50} > 100 μM) (Table 2).

Table 2. Cytotoxicity data of peniciloxatone A (**1**) (IC_{50}, mean ± SD, μM)

Compound	**A549**	**FADU**	**HepG2**
1	> 100 μM	9.5 ± 0.1	18.1 ± 0.3
doxorubicin	1.8 ± 0.1	5.6 ± 0.7	4.5 ± 0.2

In conclusion, chemical studies on the marine red alga-sourced endophytic fungus *P. oxalicum* 2021CDF-3 revealed a new polyoxygenated ergostane steroid, peniciloxatone A (**1**). Cytotoxic results indicated that **1** possessed considerable inhibitory effects on FADU and HepG2 cells. This study added the chemical diversity of classical ergostane steroids and revealed the new compound **1** as a potential lead compound. Further research will particularly focus on its mechanism of action in selected FADU and HepG2 cells to determine its potential use in the development of anti-tumour drugs.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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