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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 IC50 values of 9.5 ± 0.1 and 18.1 ± 0.3 μ M, 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 CH₂Cl₂–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 CH₂Cl₂–MeOH 10:1 was selected for further purification. First, this fraction was subjected to silica gel column chromatography eluted with CH₂Cl₂–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; $[\alpha]_D^{25} = +66.7^\circ$ (c = 0.098, MeOH); UV (MeOH): λ_{max} (log ε): 205 (4.10), 256 (3.75) nm; for ¹H and ¹³C 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 ¹H NMR data combined with the HSQC spectra of **1** (Table 1) displayed signals of seven oxygenated methine protons at $\delta = 4.63$ (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 $\delta = 1.15$ (s, H₃-19), 1.08 (s, H₃-18), and four methyl doublets at $\delta = 0.94$ (d, J = 7.2 Hz, H₃-21), 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 $\delta = 5.49$ (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 ¹³C 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 sp³ 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-d₆), 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 sp³ 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/H₃-21/H-22/H-23/H-24/H-25/H₃-26/H₃-27/H₃-28 indicated a C-22 alkanol side chain (Figure 2). HMBC correlations from the angular methyl group H₃-18 to C-12/C-14/C-17 and from H₃-19 to C-1/C-5/C-9, together with the COSY correlations between H₂-1 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 ($\delta_{\rm H}$ 4.63) to C-5/C-6/C-8/C-9/C-14, and from OH-7 ($\delta_{\rm H}$ 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 $(\delta_{\rm H}$ 5.24, 4.82, 4.60, and 4.18), together with the upfield chemical shifts of C-5 ($\delta_{\rm C}$ 60.4) and C-6 ($\delta_{\rm C}$ 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 ($\delta_{\rm H}$ 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 H₃-19/H-12, H-12/OH-14, H-12/H₃-18, and OH-14/H-7 with H-3/H-1b ($\delta_{\rm H}$ 1.44) and H₃-19/H-1a ($\delta_{\rm H}$ 2.33) indicated that H-7/H-12/OH-14/H₃-18/H₃-19 was located in the opposite orientation (assigned as β). Moreover, the correlations among H-16/H-17/H₃-21 and the absence of correlations between H₃-18/H-17 suggested that H-16, H-17 and H₃-21 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 ${}^3J_{\rm H22-H23}$ (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 ${}^3J_{\rm H23-H24}$ (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].

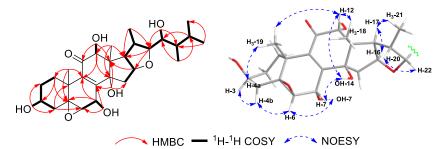


Figure 2. Key ¹H-¹H COSY, HMBC, and NOE (on the ring skeleton) correlations for 1

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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]	
	$\delta_{\rm H} \left(J \text{ in Hz} \right)$	$\delta_{\rm C}$, type	$\delta_{\rm H}$ (J in Hz)	$\delta_{\rm C}$, type	$\delta_{\mathrm{H}}\left(J\ \mathrm{in}\ \mathrm{Hz}\right)$	$\delta_{\rm C}$, 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,	72.6 CH	3.08 br t (8.5)	71.9 CH	3.12 ddd (9.0, 7.2, 1.6)	72.7 CH
	2.2)					
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 IC₅₀ value of $9.5 \pm 0.1 \, \mu M$ (compared with that of doxorubicin, IC₅₀ = $5.6 \pm 0.7 \, \mu M$). Moreover, compound 1 also exhibited mild activity against the HepG2 cells (IC₅₀ = $18.1 \pm 0.3 \, \mu M$), whereas it was inactive against A549 cells (IC₅₀ > $100 \, \mu M$) (Table 2).

Table 2. Cytotoxicity data of peniciloxatone A (1) (IC₅₀, mean \pm SD, μ M)

	<i>-</i>	\ / \	
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

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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 $\underline{\text{http://www.acgpubs.org/journal/records-of-natural-products}}$



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