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Penicisteroids A and B, antifungal and cytotoxic polyoxygenated steroids from the marine alga-derived endophytic fungus *Penicillium chrysogenum* QEN-24S

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ABSTRACT

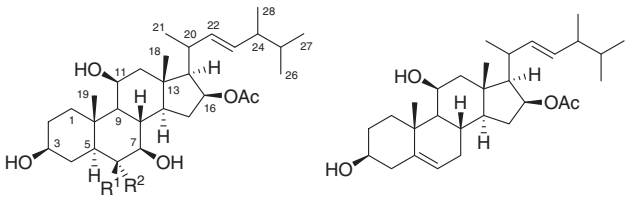
Two new polyoxygenated steroids, namely, penicisteroids A and B (**1** and **2**), were obtained from the culture extract of *Penicillium chrysogenum* QEN-24S, an endophytic fungus isolated from an unidentified marine red algal species of the genus *Laurencia*. In addition, seven known steroids (**3**–**9**) were also isolated and identified. The structures of these compounds were established on the basis of extensive spectroscopic analysis. The absolute configuration for compound **1** was determined by application of the modified Mosher's method. Penicisteroid A (**1**), which is a structurally unique steroid having tetrahydroxy and C-16-acetoxy groups, displayed potent antifungal and cytotoxic activity in the preliminary bioassays. Preliminary structure–activity relationships are discussed.

Polyoxygenated steroids are well known secondary metabolites from marine macroorganisms such as corals, sponges, and starfish.[1–4] These steroids are also occasionally isolated from terrestrial fungi.[5] However, polyoxygenated steroids, especially those containing five or more oxygenated carbons were rarely isolated from marine-derived fungi. Here we wish to describe the isolation, structure elucidation, and biological activity of two new polyoxygenated steroids, penicisteroids A and B (**1** and **2**, Fig. 1), together with seven known steroids (**3**–**9**) (see Scheme S1 in the Supplementary data) from *Penicillium chrysogenum* QEN-24S, an endophytic fungus obtained from an unidentified marine red algal species of the genus *Laurencia*. Penicisteroid A (**1**) displayed potent antifungal and cytotoxic activity in the preliminary bioassay.

P. chrysogenum QEN-24S was grown on rice solid medium at room temperature under static conditions for 30 days. The rice culture (10 flasks) of the fungal strain was exhaustively extracted with EtOAc to give a crude extract, which was dried and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH-soluble fraction (2 g) was purified by a combination of silica gel, Sephadex LH-20, and Lobar LiChroprep RP-18 column chromatography to yield compounds **1**–**9**.

Penicisteroids A and B (**1** and **2**)[6] were obtained as amorphous powders. Detailed analyses of their NMR (Table 1) and MS data as well as by comparison with literature reported data revealed that both compounds belong to steroid derivatives related to anicequol (**3**), a known compound previously isolated from a soil-derived fungus *Penicillum aurantiogriseum* Dierckx[7] as well as isolated from *P. chrysogenum* QEN-24S in our current study.

The IR spectrum of **1** showed absorption bands for OH (3386 cm^{-1}) and C=O (1712 cm^{-1}) functionalities. Low-resolution ESIMS displayed a *quasi*-molecular ion peak at *m/z* 529 $[M+Na]^+$. The molecular formula was determined as $C_{30}H_{50}O_6$ on the basis of positive HRESIMS. The ^1H NMR spectrum (Table 1) exhibited resonances for seven methyl groups, two olefinic protons, and five oxymethine protons. The ^{13}C NMR along with the DEPT spectra (Table 1) revealed the presence of 30 carbon atoms including



1 R^1=OH, R^2=H
3 R^1,R^2= =O

2

Figure 1. The structures of isolated compounds **1**–**3**.

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Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2**[a]

No.	**1**[b]		**2**[c]		No.	**1**[b]		**2**[c]	
	^{1}H	^{13}C	^{1}H	^{13}C		^{1}H	^{13}C	^{1}H	^{13}C
1	1.81 m	39.2	2.00 m	36.8	16	4.99 dt (7.9, 4.6)	76.1	5.01 dt (11.2, 4.6)	75.1
	1.02 m	CH_2	1.21 m	CH_2			CH		CH
2	1.74 m	32.3	1.55 m	32.0	17	1.20 m	60.8	1.20 m	60.5
	1.47 m	CH_2	1.44 m	CH_2			CH		CH
3	3.52 m	71.7	3.51 m	71.3	18	1.19 s	15.8	1.14 s	15.3
		CH		CH			CH_3		CH_3
4	1.86 m	36.2	2.28 m	41.5	19	1.26 s	19.4	1.27 s	22.5
	1.52 m	CH_2		CH_2			CH		CH_3
5	1.16 m	47.9		141.6 C	20	2.54 m	35.3	2.52 m	34.4
		CH					CH		CH
6	3.60 dd (3.8, 2.4)	75.2	5.23 br s	120.4	21	1.09 d (6.8)	21.6	1.07 d (6.8)	21.5
		CH		CH			CH_3		CH_3
7	3.20 dd (9.5, 3.8)	77.6	1.56 m	31.5	22	5.21 m	136.8	5.15 m	135.1
		CH	1.46 m	CH_2			CH		CH
8	2.02 m	36.1	1.93 m	28.0	23	5.22 m	133.1	5.17 m	132.9
		CH		CH			CH		CH
9	0.76 dd (11.3, 3.5)	57.4	1.04 m	54.0	24	1.78 m	44.3	1.76 m	43.2
		CH		CH			CH		CH
10		36.2 C		36.7 C	25	1.44 m	33.9	1.40 m	33.1
							CH		CH
11	4.31 br s	68.2	4.37 m	68.6	26	0.82 d (7.1)	20.5	0.81 d (6.9)	20.0
		CH		CH			CH_3		CH_3
12	2.26 dd (13.7, 2.6)	49.8	2.18 m	49.3	27	0.83 d (7.1)	20.1	0.79 d (6.9)	19.7
	1.36 dd (13.7, 3.2)	CH_2	1.43 m	CH_2			CH_3		CH_3
13		43.7 C		41.6 C	28	0.88 d (6.8)	18.5	0.85 d (6.9)	18.0
							CH_3		CH_3
14	1.13 ddd (14.3, 5.5, 2.7)	56.5	0.94 m	56.5	OAc		170.1 C		170.3 C
		CH		CH					
15	2.68 m	38.8	2.41 m	35.0		1.96 s	21.5	1.99 s	21.0
	1.48 m	CH_2	1.17 m	CH_2			CH_3		CH_3

[a] Measured at 500 MHz for ^{1}H and 125 MHz for ^{13}C with reference to the solvent signals, δ in ppm and J in Hz.
[b] Measured in acetone-d_6.
[c] Measured in $CDCl_3$.

three quaternary carbons (with one carbonyl carbon), fifteen methines (with two olefinic and five oxygenated), five methylenes, and seven methyls. Detailed comparison of the ^{1}H and ^{13}C NMR data of **1** with those of anicequol (**3**)[7] revealed that the structures of these two compounds were similar, except the carbonyl carbon signal of C-6 in **3** (δ_C 210.3)[7] was replaced by the oxygenated methine carbon signal at δ_C 75.2, in the ^{13}C NMR spectrum of **1** (Table 1). Accordingly, an additional oxygenated double doublet signal at δ_H 3.60 (1H, dd, J = 3.8, 2.4 Hz, H-6) was observed in the ^{1}H NMR spectrum of **1**. This observation was supported by the correlations from H-6 to H-5 and H-7 in the COSY spectrum (Fig. 2). In addition, the ^3J correlations from H-6 to C-4, C-8 and C-10 in the HMBC spectrum also supported this deduction (Fig. 2).

The relative configuration of **1** was determined by analysis of the proton coupling constants and by NOESY experiments as well as by comparison with literature data. The large coupling constants for H-7/H-8 (J = 9.5 Hz) indicated the *trans*-axial relationship for the proton pair, while the small coupling constants for H-5/H-6 (J = 2.4 Hz) and for H-6/H-7 (J = 3.8 Hz) suggested that the hydroxyl groups in C-6 and C-7 should be in the *cis*-relationship. In addition,

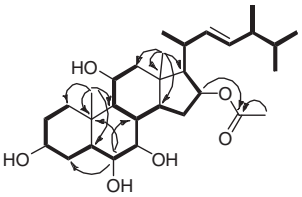

Figure 2. Key COSY (bold lines) and HMBC (arrows) correlations of compound **1**.

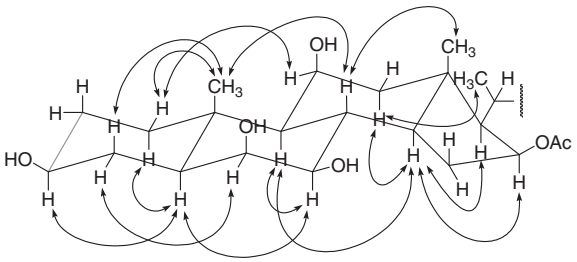

Figure 3. Key NOESY correlations of compound **1**.

the small coupling constant (J = 3.5 Hz) for H-9/H-11 suggested that these two protons should be *cis* arranged. Furthermore, the NOE correlations from H-3 to H-5, from H-5 to H-7, from H-7 to H-9, from H-9 to H-14, and from H-14 to H-16 and H-17 clearly indicated the co-facial orientation of these hydrogens (Fig. 3). Sim-

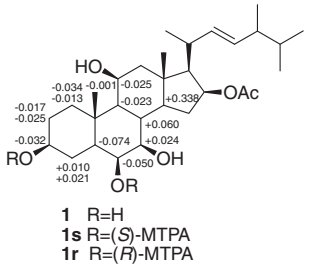

Figure 4. Values of $\Delta\delta_{H(S-R)}$ (measured in $CDCl_3$) of the MTPA esters of compound **1**.

ilarly, NOE correlations from H_3-18 to H-8 and from H-8 to H_3-19 confirmed these groups on the opposite face. These data enabled the assignment of the relative configurations of compound **1**. It should be noted that to date all of the reported naturally occurring steroid derivatives of this class possess the same relative configurations as that assigned for **1**.[8–10] However, the relative configuration on the side chain remains unknown.[9] The structure for compound **1** was established as 16β-acetoxy-3β,6β,7β,11β-tetrahydroxyergost-22*E*-ene, which was named as penicisteroid A.

The absolute configuration of **1** was determined by application of the modified Mosher's method.[11] Acylation of **1** with *R*-(−) and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenyl acetyl chloride (MTPA-Cl) furnished 3,6-bis-MTPA esters **1s** and **1r**, respectively (Fig. 4). The ^{1}H NMR signals of the two MTPA esters were assigned on the basis of their COSY spectra, and the $\Delta_{H(S-R)}$ values were then calculated (Fig. 4). Although the absolute configuration at C-6 cannot be established directly by Mosher method due to axial orientation of 6-OMTPA group that may spatially repel by axial 19-Me group and thus take an orientation not suitable to Mosher method, analysis of the calculated data around C-3 allowed the absolute configuration assignment of C-3 to be *S* (Fig. 4). Therefore, the absolute configurations of C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-13, C-14, C-16, and C-17 were assigned as *S*, *S*, *R*, *S*, *S*, *S*, *S*, *S*, *S*, and *R*, respectively.

The ESIMS of **2** exhibited a molecular ion peak at *m/z* 495 $[M+Na]^+$. Its molecular formula was determined as $C_{30}H_{48}O_4$ on the basis of positive HRESIMS. The general features of its ^{1}H and ^{13}C NMR data (Table 1) closely resembled those of **1**. However, the signals for three methines at C-5, C-6, and C-7 in **1** disappeared in **2**. Instead carbon signals resonated at δ_C 141.6 (s, C-5) and 120.4 (d, C-6) for the double bond at C-5 and at δ_C 31.5 (t, C-7) for the methylene carbon C-7 were observed in the ^{13}C NMR spectrum of **2**. Moreover, an olefinic proton signal at δ_H 5.23 (H-6) and methylene proton signals at δ_H 1.56 and 1.46 (H_2-7) were detected in the ^{1}H NMR spectrum of **2**. The observed COSY correlation from H-6 to H-7 as well as the HMBC correlations from H-4 to C-5 and C-6 and from H-7 and H_3-19 to C-5 confirmed the above deduction.

The relative configuration for the chiral centers of **2** was determined to be the same as that of **1** by NOESY experiments as well as by detailed comparison of the NMR data with that of **1**. The structure for compound **2** was therefore established as 16β-acetoxy-3β,11β-dihydroxyergost-5, 22*E*-diene, which was named as penicisteroid B.

Although steroids are one of the most abundant natural products reported so far, there are only two structurally similar steroids containing a 11-OH and 16-OAc groups, including anicequol (**3**) and its 3-*O*-acetyl derivative, byssochlamysol, have been reported from *P. aurantiogriseum* Dierckx TP-F0213[7] and *Byssochlamys nivea* M#5187,[8,9] respectively. Anicequol (**3**) was independently isolated from *Acremonium* sp. TF-0356 and was assigned the trivial name NGA0187.[10]

In addition to the isolation of three polyoxygenated steroids **1**–**3**, six other known steroids including (22*E*, 24*R*)-ergosta-4,6,8(14),22-tetraen-3-one (**4**),[12] (22*E*, 24*R*)-ergosta-7,22-dien-3,6-dione (**5**),[13] (22*E*, 24*R*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol (**6**),[14] (22*E*, 24*R*)-ergosta-5α,6α-epoxide-8, 22-dien-3β,7α-diol (**7**),[14] (22*E*, 24*R*)-ergosta-7,22-dien-3β,5α,6β-triol (**8**),[15] and (22*E*, 24*R*)-ergosta-7,22-dien-3β,6β-diol (**9**)[16] were also isolated and identified.

The biological activity of purified compounds **1**–**3** was examined in antifungal[17] and cytotoxicity[18] bioassays. In the initial antifungal screening, penicisteroid A (**1**) displayed potent inhibitory activity against the pathogenic fungus *Aspergillus niger* with a clear inhibition zone of 18 mm diameter at the concentration of 20 μg/disk (Table 2). Penicisteroide A (**1**) and anicequol (**3**) also displayed moderate acitivity against the pathogenic fungus *Alternaria brassicae*, giving inhibition zones of 8 and 6 mm diameters at the concentration of 20 μg/disk, respectively. The cytotoxicity against seven tumor cell lines was also determined and penicisteroide A (**1**) displayed selective activity against the tumor cell lines HeLa, SW1990, and NCI-H460 with the IC_{50} of 15, 31, and 40 μg/mL, respectively, while the other compounds displayed weak or no appreciable activity.

The inactivity of penicisteroid B (**2**) and anicequol (**3**) against *A. niger* demonstrated that the hydroxyl group at C-6 in B ring is likely a structure feature important for the antifungal activity against *A. niger*, whereas the moderate activity of penicisteroid A (**1**) and anicequol (**3**) against *A. brassicae* showed that one or more substitutions of hydroxy group at B ring could contribute to the antifungal activity against *A. brassicae*. The hydroxyl group at C-6 in B ring also seems essential for their cytotoxicity, which is likely the reason for that penicisteroid A (**1**) showed cytotoxic activity against the cell lines HeLa, SW1990, and NCI-H460, while penicisteroid B (**2**) and anicequol (**3**) showed no activity.

In summary, we described two new polyoxygenated steroids penicisteroids A and B (**1** and **2**) from *P. chrysogenum* QEN-24S, an endophytic fungus isolated from an unidentified marine red algal species of the genus *Laurencia*. Penicisteroid A (**1**), which is a tetrahydroxy steroid with five oxygenated carbons, displayed better activity than its two analogues (**2** and **3**) in antifungal and cytotoxic activity evaluation. Though anicequol (**3**) is reported to be an active inhibitor of anchorage-independent growth tumor cells,[7] it showed weak or no appreciable activity against all of seven cell lines evaluated in our cytotoxicity assays.

Table 2
Inhibitory activity of compounds **1**–**3** against pathogenic fungus *A. niger* and *A. brassicae*[a]

	AMPB[b]	1	2	3
Aspergillus niger	24	18	–	+
Alternaria brassicae	16	8	–	6

[a] The diameter of the zone of inhibition is indicated in mm. Each compound and the positive control were added 20 μg to each disk. The plus (+) means slight inhibition, and the minus (–) means no inhibition.
[b] AMPB: amphotericin B, positive control.

Acknowledgments

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Supplementary data

Supplementary data (selected 1D and 2D NMR spectra of new compounds **1** and **2** and the chemical structures of compounds **1**–**9**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.076.

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6. *Physical and spectroscopic data of new compounds:* (a) Penicisteroid A (**1**): White amorphous powder; $[\alpha]_D^{20}$: +72.3 (*c* 0.47, MeOH); IR (KBr) υ_{max} 3386, 2954,

2923, 2869, 1712, 1457, 1380, 1265, 1168, 1103, 1037, 871, 733, 675, 548, 506 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 529 $[M+Na]^+$; HRESIMS *m*/*z* 529.3500 $[M+Na]^+$ (cald $C_{30}H_{50}O_6Na^+$, 529.3505). (b) Penicisteroid B (**2**): White amorphous powder; $[\alpha]_D^{20}$: +19.4 (*c* 0.31, MeOH); IR (KBr) υ_{max} 3444, 2958, 2931, 2870, 1716, 1457, 1373, 1264, 1184, 1030, 968, 872, 802, 756 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 495 $[M+Na]^+$; HRESIMS: *m*/*z* 495.3441 $[M+Na]^+$ (cald $C_{30}H_{48}O_4Na^+$, 495.3450).

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11. *Preparation of the (R)- and (S)-MTPA ester derivatives of compound* **1**[19]: (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL) and 4-(dimethylamino)pyridine (2 mg) were added to penicisteroid A (**1**, 2.0 mg) which was dissolved in dried pyridine (400 μL). The mixture was kept at room temperature for 12 h. The acylation product was purified by preparative TLC on silica gel [eluent: petroleum ether/EtOAC (2:1, v/v)] to yield corresponding (*R*)-Mosher ester **1r**. Treatment of **1** (2.0 mg) with (*R*)-MTPA-Cl (10 μL) as described above yielded the corresponding (*S*)-Mosher ester **1s**.
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17. *Antifungal assay:* Antifungal assay against pathogenic fungi *Aspergillus niger* and *Alternaria brassicae* was carried out using the well diffusion method.[20] Amphotericin B (AMPB) was used as positive control.
18. *Cytotoxicity assay:* The cytotoxic activities against NCI-H460 (human nonsmall cell lung cancer), SMMC-7721 (human hepatoma), SW1990 (human pancreatic cancer), DU145 (human prostate carcinoma), HepG2 (human hepatocellular liver carcinoma), Hela (human epithelial carcinoma), and MCF-7 (human breast adenocarcinoma) cell lines were determined according to previously reported methods.[21]
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