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Penostatins, Novel Cytotoxic Metabolites from a *Penicillium* Species Separated from a Green Alga

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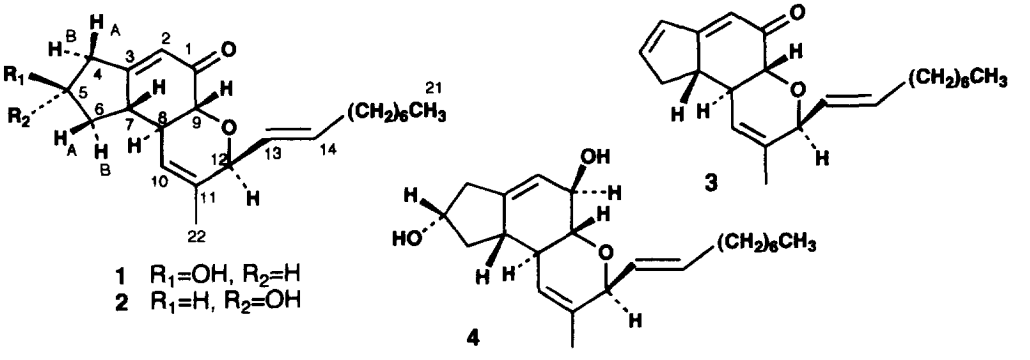
Abstract : Penostatins A (**1**), B (**2**), C (**3**) and D (**4**) have been isolated from a strain of *Penicillium* sp. originally separated from the marine alga *Enteromorpha intestinalis*, and their stereostructures have been established on the basis of spectral analyses. The compounds **1**~**3** exhibited significant cytotoxicity against cultured P388 cells.

Marine microorganisms are potentially prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents. As part of our ongoing search for new antitumour metabolites from microorganisms inhabiting the marine environment, we previously reported that cytotoxic compounds, communesins[1] and penochalasins[2], were produced by *Penicillium* sp. originally isolated from the marine alga *Enteromorpha intestinalis*, and their structures were established. These results prompted us to examine cytotoxic metabolites from this fungal strain cultivated in a different medium from that used for the previous experiment. This investigation has led to the isolation of four new compounds, penostatins A (**1**), B (**2**), C (**3**) and D (**4**). Among them, **1**~**3** exhibited significant cytotoxic activity in the P388 lymphocytic leukemia test system in cell culture.

In the present experiment, the fungal strain was cultured at 27℃ for 3 weeks in a medium (40 l) containing 2% glucose, 1% peptone and 2% malt extract in distilled water. The MeOH extract of the mycelial cake was purified by bioassay-directed fractionation employing a combination of Sephadex LH-20 and



silica gel column chromatographies and HPLC to afford penostatins A(**1**)(45 mg), B(**2**)(9 mg), C(**3**)(37 mg) and D (**4**)(6 mg).

Penostatin A (**1**)[3] had the molecular formula $C_{22}H_{32}O_3$ established by HREIMS. Its IR spectrum contained absorption bands at 3462, 1669 and 1639 cm^{-1}, characteristic of a hydroxyl group, a conjugated ketone and a double bond. A close inspection of the ^1H and ^{13}C NMR spectra of **1** (Table 1) by DEPT and ^1H-^{13}C COSY experiments revealed the presence of a conjugated ketone (C1), one disubstituted (C13 and C14) and two trisubstituted double bonds (C2 and C3, and C10 and C11), an allylic methyl (C22), a primary methyl (C21), eight methylenes (C4, C6 and C15~C20) and five sp^3-hybridized methines (C5, C7, C8, C9 and C12) including three oxygen-bearing methines. Production of the monoacetate derivative by standard acetylation of **1** and its ^1H NMR signals suggested the presence of one secondary alcohol, linked to the C5 methine, and consequently of one ether linkage as deduced from the molecular formula of **1**. The EIMS fragment at *m/z* 245 [M^+ -99] implied that the primary methyl and the six methylenes constitute a heptyl group.

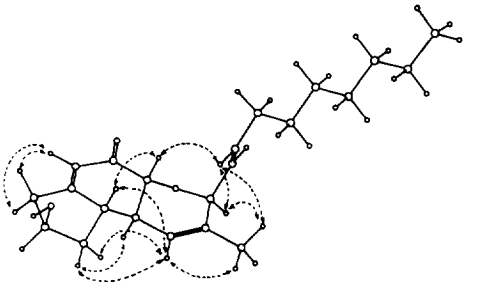


Fig. 1 Energy minimised conformation of **1** and observed NOEs

The ^1H-^1H COSY analysis for the functional groups thus established led to a partial structure representing C2−C22. The connection of C3 to both C4 and C7 was deduced from cross peaks attributed to long-range couplings between H2 and both H4 and H7 in the ^1H-^1H COSY, and from three-bond HMBC correlations from H2 to both C4 and C7. In addition, the connectivity of C11 and C12 was deduced from a cross peak attributed to a long-range coupling between H12 and H22 in the ^1H-^1H COSY, and from an HMBC correlation from H22 to C12. Appearance of the deshielded C3 carbon signal (δ 170.37) indicated that a ketone is located at a β-position of the C3 carbon. HMBC correlations from H9 to C1, C2 and C12 implied that C9 is linked to the ketone (C1) and that the ether linkage is between C9 and C12. The geometrical configuration of the Δ^{13} olefin was deduced as *E* from a large coupling constant ($J_{13,14}$ 14.2 Hz). The above summarised evidence led to planar structure **1** for penostatin A.

The relative stereochemistry for **1** was established by a combination of observed coupling constants (Table 1) and NOESY data. Based on the generalized Karplus relationship,[4] the $J_{7,8}$ and $J_{8,9}$ values (11 Hz) in **1** implied that H8 is arranged *trans*-axial to H7 and H9. This was supported by an NOE between H7 and H9. The observation of an NOE between H8 and H6B and a coupling constant of 12.5 Hz between H7 and H6B suggested a pseudoaxial orientation for H6B. Equivalent coupling constants of 4.5 Hz were observed from H5 to H4B and H6B, whereas no coupling between H5 and neither H4A nor H6A was found. Analysis of the observed coupling constant by the Karplus relationship showed that dihedral angles for H5/H4A, H5/H4B, H5/H6A and H5/H6B were approximately 34°, 80°, 34° and 80°, respectively. The dihedral angles were nearly equivalent to those of the low-energy structure obtained by the CaChe MM2 method (Fig. 1). Assignments for H4A, H4B, H6A and H6B were supported by selected difference NOE values between each of these protons and H5, and a W-type of long-range coupling between H6A and H4A except for the coupling relationship between each of these protons and H5, and the NOE between H6B and H8. The above

Table 1. ^1H and ^{13}C NMR data of penostatin A (**1**) in $CDCl_3$

Position	δ_H	^1H–^1H COSY	δ_C	HMBC[a]
1			196.36 (q)[b]	
2	5.95 q (2.5)	4A, 4B, 7	122.37 (t)	4, 7
3			170.39 (q)	
4 A	2.64 br d (19.5)	2, 4B, 5, 6A	41.67 (s)	2, 3, 5, 6, 7
B	2.84 br dd (19.5, 5.0)	2, 4A, 5		2, 3, 6, 7
5	4.61 t (5.0)	4A, 4B, 6B	70.92 (t)	3, 4, 6, 7
6 A	2.28 ddd (12.5, 7.0, 2.0)	4A, 6B, 7		3, 4, 5, 7, 8
B	1.55 td (12.5, 5.0)	5, 6A, 7	39.15 (s)	3, 5, 7, 8
7	2.86 m	2, 6B, 6A, 8	44.84 (t)	2, 3, 6, 9, 10
8	2.41 tq (11.0, 1.5)	7, 9, 10, 22	44.75 (t)	6, 7, 9, 11
9	4.08 d (11.0)	8	73.78 (t)	1, 2, 7, 8, 10, 12
10	5.55 q (1.0)	8, 22	121.83 (t)	7, 8, 9, 11, 12, 22
11			136.11 (q)	
12	4.60 br d (5.8)	13, 22	77.49 (t)	9, 10, 11, 13, 14, 22
13	5.57 dd (14.2, 5.8)	12, 14	125.91 (t)	11, 12, 15
14	5.68 dt (14.2, 6.5)	13, 15	136.49 (t)	12, 15
15	2.06 q (6.5)	14, 16	32.37 (s)	14, 16
16	1.37 quint (6.5)	15	29.08 (s)	14, 15
17	1.26 br s		29.08 (s)	
18	1.26 br s		29.08 (s)	
19	1.26 br s		31.78 (s)	
20	1.31 br s	21	22.62 (s)	21
21	0.87 t (6.5)	20	14.10 (p)	20
22	1.66 br s	8, 10, 12	20.06 (p)	10, 11, 12
5-OH	1.72 br s			

a Long-range ^1H-^{13}C correlations from H to C . *b* Letters, p, s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT. *a* Long-range ^1H-^{13}C correlation from H to C .

Table 2. ^1H and ^{13}C NMR data of penostatins B (**2**), C (**3**) and D (**4**) in $CDCl_3$

	2		**3**		**4**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		196.07 (q)[a]		196.59 (q)	4.34 m	71.73 (t)
2	5.96 q (2.0)	122.68 (t)	5.91 d (2.5)	117.14 (t)	5.47 quintet (2.0)	120.13 (t)
3		168.82 (q)		170.96 (q)		145.18 (q)
4 A	2.97 ddd (18.5, 6.5, 2.0)	41.47 (s)	6.45 dt (5.5, 2.0)	132.23 (t)	2.71 ddt (16.5, 8.0, 2.0)	40.30 (s)
B	2.46 br dd (18.5, 6.5)				2.19 ddd (16.5, 5.0, 2.0)	
5	4.52 quint (6.5)	71.23 (t)	6.69 dt (5.5, 2.5)	147.82 (t)	4.37 m	71.87 (t)
6 A	2.54 dt (11.0, 6.5)	38.61 (s)	2.86 dddd (17.5, 7.0, 2.5, 2.0)	36.44 (s)	2.40 dt (13.0, 6.5)	39.53 (s)
B	1.54 ddd (11.0, 9.5, 6.5)		2.45 dddd (17.5, 4.0, 2.5, 2.0)		1.33 m	
7	2.51 m	45.12 (t)	2.70 dddd (11.2, 7.0, 4.0, 2.5)	45.71 (t)	2.07 m	43.43 (t)
8	2.49 br t (10.8)	45.27 (t)	2.53 tq (11.2, 2.0)	44.56 (t)	2.03 m	41.50 (t)
9	4.00 d (10.8)	73.93 (t)	4.45 d (11.2)	75.05 (t)	3.45 dd (9.5, 8.0)	75.50 (t)
10	5.54 br s	121.60 (t)	5.58 q (1.5)	121.66 (t)	5.50 q (1.5)	122.00 (t)
11		136.42 (q)		136.64 (q)		135.05 (q)
12	4.59 br d (6.0)	77.24 (t)	4.62 br d (6.0)	77.58 (t)	4.41 d (6.5)	77.27 (t)
13	5.55 dd (5.5, 6.0)	125.95 (t)	5.59 dd (15.5), 6.0)	126.02 (t)	5.57 dd (15.5, 6.5)	126.84 (t)
14	5.67 dt (15.5, 6.5)	136.33 (t)	5.70 dt (15.5, 7.0)	136.22 (t)	5.69 dt (15.5, 6.5)	135.56 (t)
15	2.05 q (6.5)	32.37 (s)	2.06 q (7.0)	32.37 (s)	2.06 q (6.5)	32.39 (s)
16	1.39 quint (6.5)	29.02 (s)	1.38 quint (7.0)	29.01 (s)	1.37 m	29.13 (s)
17	1.25 br s	29.12 (s)	1.26 br s	29.12 (s)	1.27 br s	129.13 (s)
18	1.25 br s	29.12 (s)	1.26 br s	29.12 (s)	1.27 br s	29.13 (s)
19	1.25 br s	31.80 (s)	1.26 br s	31.78 (s)	1.27 br s	31.81 (s)
20	1.31 br s	22.64 (s)	1.31 br s	22.62 (s)	1.30 m	22.65 (s)
21	0.87 t (6.5)	14.12 (p)	0.87 t (7.0)	14.10 (p)	0.88 t (6.5)	14.10 (p)
22	1.66 br s	20.06 (p)	1.68 br s	20.09 (p)	1.63 br s	20.06 (p)
1-OH					2.34 br s	
5-OH	1.83 br s				1.60 br s	

a Letters, p, s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT.

evidence implied that the 5-hydroxyl group is oriented pseudoaxial and consequently *cis* to H7. NOEs from H22 to H12, H13 and H14, and from H9 to H13 and H14 indicated that the dihydropyran ring of **1** exists in a twist chair conformation with H12 and the nonenyl group in equatorial and axial arrangements, respectively, and that the C12-C13 axis rotates in solution.

Penostatin B(**2**)[5] had the same molecular formula as **1**. The general spectral features of **2** closely resembled those of **1** except for the chemical shifts of H4A, H5, H6A, H7 and C3, and a coupling relationship of H5 in the NMR spectra (Table 2). Appearance of H5 as a quintet (7 Hz) and an NOE between H6B and H8 showed the 5-hydroxyl group to be oriented pseudoequatorial and consequently *trans* to H7. This finding led to relative stereostructure **2** for penostatin B.

Penostatin C(**3**)[6] was assigned a molecular formula which contained a molecule of water less than that of **1** and **2**. Comparison of its ^{1}H and ^{13}C NMR signals with those of **1** (Tables 1 and 2) revealed that the 4-methylene and 5-hydroxymethine in **1** are replaced by a double bond (δ_H 6.45 and 6.69; δ_C 132.23 and 147.82) in **3**. Based on close correspondence of the chemical shifts and NOE data of the remaining signals in **3** with those of **1**, the stereostructure for penostatin C was established as **3**.

Penostatin D (**4**)[7] was assigned a molecular formula which contained two proton atoms more than that of **1** and **2**. Comparison of the ^{1}H and ^{13}C NMR data of **4** with those of **2** (Table 2) revealed that the ketone (C1) in **2** is replaced by a hydroxymethine in **4**. The coupling constant (8 Hz) between H1 and H9 indicated that the 1-hydroxyl group is arranged pseudoequatorial. The coupling relationship of H5 to H4 and H6 and the observation of an NOE between H5 and H7 indicated that the 5-hydroxyl group in **4** is arranged *trans* to H7 as in **2**. The configuration of C7, C8, C9 and C12 was shown to be the same as that of **2** by the coupling constants of H8 to H7 and H9, and NOEs from H7 to H9, from H9 to H13 and H14 and from H22 to H13 and H14. This evidence allowed assignment of stereostructure **4** for penostatin D. Work on determination of the absolute configurations of these compounds is in progress.

Penostatins A (**1**), B (**2**), C (**3**) and D (**4**) exhibited cytotoxic activity (ED_{50} 0.8, 1.2, 1.1 and 11.5 μg/ml, respectively) in the P388 lymphocytic leukemia test system in cell culture.

References and Notes

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2. A. Numata, C. Takahashi, Y. Ito, K. Minoura, T. Yamada, C. Matsuda and K. Nomoto, *J. Chem. Soc., Perkin Trans. 1*, **1995**, 2345.
3. **1**: Colourless needles, mp 73−75℃(MeOH), $[\alpha]_D$ +133.3 (*c* 0.18, $CHCl_3$). UV $_{max}$ nm (log ε): 232 (4.22). IR ν_{max} cm^{-1}: 3462, 1669, 1639. HREIMS *m/z* : 344.2376 [M^+] ($C_{22}H_{32}O_3$ requires 344.2351).
4. M. Karplus, *J. Chem. Phys.*, **1959**, *34*, 11.
5. **2**: Colourless powder, mp 63−66℃, $[\alpha]_D$ -103.1 (*c* 0.49, $CHCl_3$). UV λ_{max} nm (log ε): 230 (4.10). IR ν_{max} cm^{-1}: 3465, 1673, 1637. HREIMS *m/z* : 344.2353 [M^+] ($C_{22}H_{32}O_3$ requires 344.2351).
6. **3**: Colourless powder, mp 63−65℃, $[\alpha]_D$ +120 (*c* 1.0, $CHCl_3$). UV λ_{max} nm (log ε): 283(4.30). IR ν_{max} cm^{-1}: 1675, 1610. HREIMS *m/z* : 326.2245 [M^+] ($C_{22}H_{30}O_2$ requires 326.2246).
7. **4**: Colourless powder, mp 106−110℃, $[\alpha]_D$ -26.7 (*c* 0.14, $CHCl_3$). IR ν_{max} cm^{-1}: 3343, 1634. HREIMS *m/z* : 346.2504 [M^+] ($C_{22}H_{34}O_3$ requires 346.2508).

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