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Novel Antitumour Metabolites Produced by a Fungal Strain from a Sea Hare

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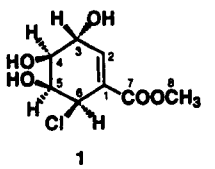
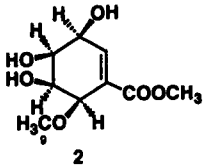
Abstract: Pericosines A (**1**) and B (**2**), and macrosphelides E – H (**3** – **6**) have been isolated, along with known macrosphelide C (**7**), from a strain of *Periconia byssoides* originally separated from the sea hare *Aplysia kurodai*, and their structures have been established on the basis of spectral analyses. Compounds **1** and **2** exhibited significant inhibitory activity *in vitro* against tumour cells, and the former also showed significant *in vivo* tumour-inhibitory activity. © 1997 Elsevier Science Ltd.

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 marine microorganisms,[1] we have found that a strain of *Periconia byssoides* separated from the gastrointestinal tract of the sea hare *Aplysia kurodai* produces two novel cytotoxic compounds, pericosines A (**1**), and B (**2**), along with four new macrolides, macrosphelides E (**3**) – H (**6**). The producing microorganism was cultured at 27°C for 4 weeks in a medium (50 l) containing 1% malt extract, 1% glucose and 0.05% peptone in artificial seawater adjusted to pH 7.5. The AcOEt extract of the culture filtrate was purified by bioassay-directed fractionation employing a combination of Sephadex LH-20 and silica gel column chromatographies and reversed phase HPLC to afford pericosines A (**1**) (1.03 g) and B (**2**) (8.2 mg),



1



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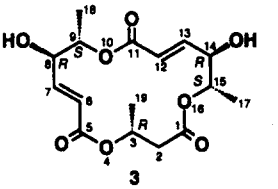

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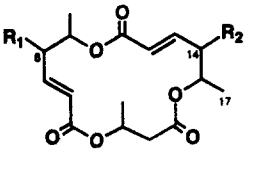
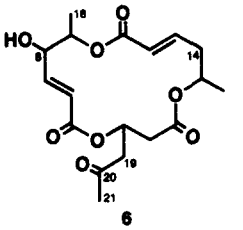


4 R_1=H R_2=OH
5 R_1=OH R_2=H



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and macrosphelides E (**3**)(12.4 mg), F (**4**)(1 mg), G (**5**)(2.3 mg), H (**6**)(2.1 mg) and C (**7**)(5.4 mg). The last compound **7** was in all respects identical to macrosphelide C, isolated from the fermentation broth of *Microsphaeropsis* sp. by Omura and co-workers,[2] who had established its planar structure.

Pericosine A (**1**)[3] was assigned a molecular formula of $C_8H_{11}O_5Cl$ as deduced from MH^+ peak in HREIMS and the ratio of intensity of isotope peaks (MH^+/$[MH+2]^+$). A close inspection of the ^1H and ^{13}C NMR spectral data of **1** by DEPT and ^1H-^{13}C COSY experiments revealed the presence of three hydroxy-methines, one sp^3-hybridized methine linked to a chlorine atom, one trisubstituted double bond and a methoxycarbonyl group. Analysis of the ^1H-^1H COSY and long-range ^1H-^{13}C COSY correlations (C7 to H2 and H6) led to the planar structure of **1** for pericosine A. The relative stereochemistry for **1** was established by a combination of observed coupling constants and NOE data in the 3, 4-*trans*-acetonide **1a** derived from **1**. The observation of an NOE between H4 and H6 in **1a** implied that the cyclohexene ring of **1a** exists in a twist chair conformation, with the two protons in a copseudoaxial arrangement. The $J_{3,4}$ and $J_{4,5}$ values (7.4 and 3.9 Hz, respectively) in **1a** suggested pseudoaxial and pseudoequatorial orientations for H3 and H5, respectively. Based on this evidence, the relative configuration of **1** was established.

Pericosine B (**2**)[4] was assigned a molecular formula of $C_9H_{13}O_6$ as deduced from MH^+ peak in HREIMS. The general spectral features of **2** closely resembled those of **1** except that proton and carbon signals for an additional methoxyl group appeared in the NMR spectra of **2**. ^1H-^1H COSY correlations between hydroxy-methine protons and hydroxyl protons in **2** suggested that the additional methoxyl group is at C6. An NOE between H3 and H5 in the 3, 4-*cis*-acetonide (**2a**) of **2** implied that the cyclohexene ring of **2a** exists in a twist chair conformation, with the two protons in a copseudoaxial arrangement. The $J_{3,4}$, $J_{4,5}$ and $J_{5,6}$ values (5.5, 3.0 and 5.0 Hz, respectively) of **2a** suggested pseudoequatorial orientations for both H4 and H6. Thus, the relative stereostructure of **2** was established.

Macrosphelide E (**3**)[5] had the molecular formula $C_{16}H_{22}O_8$ established by HREIMS. Its UV and IR spectra contained absorption bands, characteristic of a hydroxyl group and a conjugated ester. A close inspection of the ^1H and ^{13}C NMR spectral data (Table 1) of **3** by DEPT and ^1H-^{13}C COSY experiments revealed the presence of three secondary methyl, one sp^3-hybridized methylenes, five oxygen-bearing sp^3-methines, two 1, 2-disubstituted double bonds and three ester carbonyl groups. ^1H-^1H COSY and HMBC (H2/C1, H7/C5 and H14/C11) correlations and coupling constants ($J_{6,7}$ = 15.7 Hz and $J_{12,13}$ = 15.6 Hz) showed that **3** consists of one molecule of 3-hydroxybutyric acid (**8**) and two molecules of 4, 5-dihydroxy-2 *E*-hexenoic acid (**9**), which are connected by an ester linkage to form a 16-membered macrolide as deduced from the chemical shifts of H3, H9 and H15 corresponding to H3 of **8** and H5 of **9**. Connection of three carboxylic acid moieties, supported by HMBC correlations (H3/C5, H9/C11 and H15/C1), led to the planar structure of **3**.

Alkaline hydrolysis of **3** gave two products, 3*R*-hydroxybutyric acid (**8**) and 4*R*, 5*S*-dihydroxy-2*E*-hexenoic acid (**9**). They were isolated as esters obtained by the reaction of the hydrolysis product with diazomethane followed by *p*-bromobenzoyl chloride, and **9** also was isolated as an acetonide of the methyl ester. The resulting ester of **8** was identified by comparison of the spectral data, including CD spectrum, with an authentic sample as methyl 3*R*-(*p*-bromobenzoyloxy)-butyrate. The relative stereochemistry at C4 and C-5 in **9** was defined as *anti* by the following fact; in the acetonide of methyl ester derived from **9**, the proton signals of the two isopropylidene methyl groups showed the different chemical shifts (δ_H 1.38 and 1.52),[6] and

Table 1. ^1H and ^{13}C NMR data of **3-6** in $CDCl_3$[a]

	3			**4**			**5**			**6**		
No.	δ_H	*J* Hz	δ_C	δ_H	*J* Hz	δ_C	δ_H	*J* Hz	δ_C	δ_H	*J* Hz	δ_C
1			170.8			170.8			169.2			169.7
2	2.58 dd	15.8, 7.0	40.4	2.58 dd	15.8, 7.5	40.7	2.51 dd	15.7, 6.1	40.0	2.58 dd	15.6, 3.4	36.8
	2.75 dd	15.8, 3.3		2.66 dd	15.8, 3.3		2.70 dd	15.7, 3.9		2.90 dd	15.6, 4.7	
3	5.31 dqd	7.0, 6.5, 3.3	66.8	5.30 dqd	7.5, 6.5, 3.3	66.5	5.21 qdd	6.6, 6.1, 3.9	67.2	5.50 dddd	7.2, 6.6, 4.7, 3.4	66.6
5			166.5			165.1			165.3			165.1
6	6.12 dd	15.7, 1.5	122.4	5.79 dd	15.7, 1.8	124.6	6.11 dd	15.7, 1.6	121.8	6.07 dd	15.6, 1.9	121.4
7	7.03 dd	15.7, 4.2	145.5	6.88 dt	15.7, 7.5	143.4	7.00 dd	15.7, 3.9	145.3	7.05 dd	15.6, 3.6	146.0
8	4.37 br s		74.9	2.41 dt	14.6, 7.5	37.7	4.33 br s		75.3	4.35 br s		75.3
				2.70 dddd	14.6, 7.5, 5.0, 1.8							
9	5.11 qd	6.8, 2.0	75.7	5.14 dqd	7.5, 6.4, 5.0	68.9	5.05 m		76.4	5.09 q	6.8	77.2
11			165.4			164.9			166.9			167.3
12	6.04 dd	15.6, 1.5	123.0	6.09 dd	15.6, 1.9	123.0	5.80 d	15.6	123.3	5.81 d	15.6	123.3
13	6.81 dd	15.6, 5.3	145.2	6.85 dd	15.6, 4.2	144.1	6.78 dt	15.6, 7.6	145.3	6.79 ddd	15.6, 8.7, 6.3	145.6
14	4.16 br dt	7.1, 5.3	73.6	4.21 dddd	7.7, 4.2, 3.7, 1.9	73.6	2.38 m		38.3	2.39 m		38.6
							2.47 m			2.45 m		
15	4.97 qd	6.5, 5.3	75.0	4.96 qd	6.6, 3.7	76.0	5.03 m		70.0	5.03 m		70.0
17	1.30 d	6.5	17.3	1.31 d	6.6	17.5	1.22 d	6.4	20.3	1.26 d	6.0	20.4
18	1.41 d	6.8	17.6	1.39 d	6.4	19.9	1.37 d	6.8	17.9	1.41 d	6.8	17.8
19	1.40 d	6.5	19.5	1.35 d	6.5	19.8	1.42 d	6.6	19.3	3.14 dd	18.2, 6.6	45.3
										3.18 dd	18.2, 7.2	
20												205.6
21										2.19 s		30.5
8-OH	3.59 d	7.3					3.38 br s			3.56 d	8.3	
14-OH	3.36 d	7.1		2.91 d	7.7							

[a]Measured at 300 and 75.4 MHz for ^1H and ^{13}C, respectively.

NOEs were observed from one side of the isopropylidene methyl protons (δ_H 1.38) to both H4 and H5 and from the other side of methyl protons to H2. Use of the modified Mosher's method[7] for **3** led to the absolute stereostructure for **3** with 3*R*, 8*R*, 9*S*, 14*R* and 15*S*-configurations. As the result, the absolute configuration of **9** was clarified. Based on the above evidence, **3** was found to be the stereoisomer, in which the configuration of C3 was different from that of macrosphelide A (**10**)[8] isolated from the culture broth of *Microsphaeropsis* sp. by Omura *et al.* as anti-adhesion compounds.

Macrosphelide F (**4**)[9] was assigned a molecular formula of $C_{16}H_{22}O_7$ as deduced from HREIMS. The general features of its UV, IR and NMR spectra closely resembled those of **3** except that the proton and carbon signals of the hydroxymethine (C8) in **3** were replaced by those of a methylene in **4** (Table 1). Analysis of ^1H-^1H COSY and HMBC (H8/C9, H9/C11 and H15/C1) correlations led to the planar structure of **4**. This compound is expected to be a stereoisomer of macrosphelide C (**7**).

Macrosphelide G (**5**)[10] had the same molecular formula as **4** as deduced from HREIMS. Its ^1H and ^{13}C NMR signals showed close correspondence with **4** (Table 1), suggesting that **5** is comprised of the same carboxylic acid moieties as **4** except for stereochemistry. Analysis of ^1H-^1H COSY and HMBC (H9/C11 and H3/C5) data led to the planar structure of **5**. Thus, compound **5** was expected to be a positional isomer of the hydroxyl group for **4** and **7**.

Macrosphelide H (**6**)[11] had the molecular formula $C_{18}H_{24}O_8$ established by HREIMS. A close inspection of its ^1H and ^{13}C NMR spectra (Table 1) revealed that the methyl group of the 3-hydroxybutyric acid moiety in **5** was replaced by an acetonyl group (C19 – C21) in **6**. Connection of the three carboxylic acid moieties

confirmed by ^{1}H-^{1}H COSY correlations was deduced from HMBC analysis (H9/C11, H3/C5, H19/C20, H19/C3 and H21/C20) to led to the planar structure of **6**. Study of the stereochemistry of macrosphelides F (**4**)-H (**6**) is in progress.

Among the compounds mentioned above, pericosines A (**1**) and B (**2**) exhibited significant cytotoxicity (ED_{50} 0.12 and 4.0 μg/ml, respectively) in the P388 lymphocytic leukemia test system in cell culture,[12] though ED_{50} values of macrosphelides C (**7**) and E (**3**)-H (**6**) were more than 100 μg/ml. Furthermore, compound **1** showed significant *in vivo* tumour-inhibitory activity.[13]

References and Notes

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2. T. Sunatsuka, S. Takamatsu, Y-P. Kim, T. Hirose, Y. Haritani, M. Hayashi, K. Komiyama, S. Omura, H. Hiraoka, M. Natori, Abstr. 117th Annu. Meet. Pharm. Soc. Japan, Tokyo, **2**, p. 182 (1997).
3. Data for **1** : Colorless plates, mp 95-97°C (MeOH); $[\alpha]_D$ +57° (*c* 3.16, EtOH) ; UV λ max (EtOH) nm (log ε) 217 (3.90); IR υ max (KBr) cm^{-1} 3353, 1720, 1651; HREIMS *m/z* 223.0363 $[MH]^+$, Δ - 1.0 mmu; ^{1}H NMR (300 MHz, Acetone-d_6) δ 3.79 (s, H8), 4.07 (ddd, *J* 6.7, 4.4, 1.8 Hz, H4), 4.13 (ddd, *J* 5.8, 4.5, 1.8 Hz, H5), 4.15 (d, *J* 8.6 Hz, 3-OH), 4.20 (d, *J* 6.7 Hz, 4-OH), 4.38 (ddd, *J* 8.6, 4.4, 3.9 Hz, H3), 4.89 (d, *J* 4.5 Hz, H6), 4.91 (d, *J* 5.8 Hz, 5-OH), 6.92 (d, *J* 3.9 Hz, H2); ^{13}C NMR (75.4 MHz, Acetone-d_6) δ 52.4 (C8), 57.6 (C6), 67.0 (C3), 68.6 (C4), 75.4 (C5), 130.3 (C1), 141.8 (C2), 168.2 (C7).
4. Data for **2**: Colorless oil, $[\alpha]_D$ +22.3° (*c* 0.82, EtOH) ; UV λ max (EtOH) nm (log ε) 218 (3.85); IR υ max (liquid) cm^{-1} 3327, 1720, 1635; HREIMS *m/z* 219.0853 $[MH]^+$, Δ -1.4 mmu; ^{1}H NMR (300 MHz, Acetone-d_6) δ 3.59 (s, H9), 3.77 (s, H8), 3.80 (m, 3, 4-OH), 3.86 (br d, *J* 4.0 Hz, H5), 3.93 (br s, H4), 4.16 (br d, *J* 1.5 Hz, H3), 4.26 (d, *J* 4.0 Hz, H6), 4.43 (br s, 5-OH), 6.72 (d, *J* 1.5 Hz, H2); ^{13}C NMR (75.4 MHz, Acetone-d_6) δ 51.9 (C8), 61.2 (C9), 69.3 (C3), 69.7 (C4), 72.5 (C5), 76.7 (C6), 130.2 (C1), 141.7 (C2), 166.6 (C7).
5. Data for **3** : Colorless oil, $[\alpha]_D$ + 56.8° (*c* 0.46, EtOH) ; UV λ max (EtOH) nm (log ε) 213 (4.18); IR υ max (liquid) cm^{-1} 3452, 1717, 1665, 1648; HREIMS *m/z* 343.1390 $[MH]^+$, Δ - 0.2 mmu.
6. H. Oikawa, I. Matsuda, T. Kagawa, A. Ichihara and K. Kohmoto, *Tetrahedron*, **50**, 13347 (1994).
7. The proton chemical shift difference between the (+) and (-)-MTPA esters of **3**: Δδ (Hz) +41.3 (C6), +18.1(C7), - 13.3 (C9), - 8.1 (C18), +11.4 (C12), +11.3 (C13), - 27.4 (C15), - 16.8 (C17).
8. S. Takamatsu, Y-P. Kim, M. Hayashi, H. Hiraoka, M. Natori, K. Komiyama and S. Omura, *J. Antibiotics*, **49**, 95 (1996).
9. Data for **4** : Colorless oil, $[\alpha]_D$ + 23.3° (*c* 0.09, EtOH) ; UV λ max (EtOH) nm (log ε) 216 (4.15); IR υmax (liquid) cm^{-1} 3445, 1720, 1658, 1651; HREIMS *m/z* 327.1461 $[MH]^+$, Δ 1.9 mmu.
10. Data for **5** : Colorless oil, $[\alpha]_D$ + 66.7° (*c* 0.48, EtOH) ; UV λmax (EtOH) nm (log ε) 217 (4.17); IR υmax (liquid) cm^{-1} 3443, 1718, 1658, 1648; HREIMS *m/z* 326.1362 $[M]^+$, Δ - 0.2 mmu.
11. Data for **6** : Colorless oil, $[\alpha]_D$ + 41.7° (*c* 0.22, EtOH) ; UV λmax (EtOH) nm (log ε) 214 (4.19); IR υmax (liquid) cm^{-1} 3452, 1724, 1661, 1648; HREIMS *m/z* 369.1540 $[MH]^+$, Δ - 0.7 mmu.
12. A. Numata, P. Yang, C. Takahashi, R. Fujiki, M. Nabae and E. Fujita, *Chem. Pharm. Bull.*, **37**, 648, (1989).
13. Mice were inoculated intraperitoneally (i.p.) with P388 leukemia cells on day 0, and administered i.p. with 25 mg/kg of **1** on days 1 and 5. The median survival days of non-treated mice and **1**-treated mice were 10.7 and 13.0, respectively, suggesting antitumour activity of this compound.

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