Pericosines, antitumour metabolites from the sea hare-derived fungus *Periconia byssoides*. Structures and biological activities†

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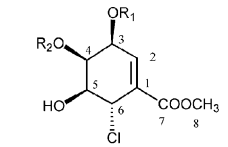
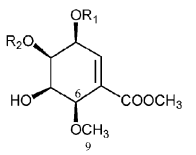
Pericosines A–E **1–5** have been isolated from a strain of *Periconia byssoides* originally separated from the sea hare *Aplysia kurodai*. Among them, pericosines C **3** and E **5** were separated as enantiomeric mixtures. Their stereostructures, except for compound **1**, have been elucidated or identified on the basis of spectroscopic analyses, including 1D and 2D NMR techniques, and X-ray analysis. In addition, conformation for all the compounds has been discussed. Compounds **1–3** exhibited significant growth inhibition against tumour cell lines. Pericosine A **1** also showed significant *in vivo* tumour inhibitory activity. In addition, compound **1** inhibited the protein kinase EGFR and topoisomerase II.

Introduction

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,[1–3] we previously reported that cell-adhesion inhibitors, macrosphelides[4,5] and peribysins,[6–8] were produced by a strain of *Periconia byssoides* OUPS-N133 originally separated from the sea hare *Aplysia kurodai*. Further investigation for metabolites of this fungal strain has now led to the isolation of designated pericosines A–E **1–5**. Among them, pericosines A **1**, B **2** and D **4** exhibited significant growth inhibition against the murine P388 cell line. Moreover, pericosine A **1** showed selective growth inhibition against human cancer cell lines, and significant *in vivo* tumour inhibitory activity against mice inoculated intraperitoneally with P388 leukemia cells. In addition, pericosine A **1** was demonstrated to inhibit the protein kinase EGFR and topoisomerase II. We report herein detailed study of their isolation, relative stereostructures, conformation and biological activities. The relative stereostructures of pericosines A **1** and B **2** have been briefly reported by us in a preliminary form,[9] and it has been recently reported by Usami *et al.*,[10] part of our research group, that the proposed structure **6** for pericosine A[9] is incorrect and should be revised as **1** on the basis of the synthesis of the enantiomer of **1**.



1 $R_1 = R_2 = H$
1a R_1 - $R_2 = C(CH_3)_2$



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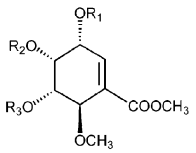
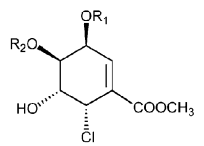
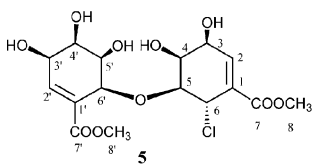
2a R_1 - $R_2 = C(CH_3)_2$



3 $R_1 = R_2 = R_3 = H$
3a R_1 - $R_2 = C(CH_3)_2$, $R_3 = H$
3b $R_1 = H$, R_2 - $R_3 = C(CH_3)_2$



4 $R_1 = R_2 = H$
4a R_1 - $R_2 = C(CH_3)_2$



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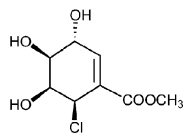

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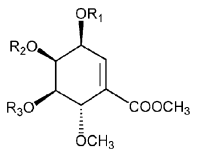

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Results and discussion

The fungal strain was cultured at 27 °C for 4 weeks in a medium 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 (P388 cell line)-guided fractionation employing a combination of Sephadex LH-20 and silica gel column chromatography procedures as well as reversed-phase HPLC to afford pericosines A–E **1–5**.

Pericosine D (**4**) has the molecular formula $C_8H_{11}ClO_5$ established by the $[M + H]^+$ peak of **4** in HREIMS and the ratio of relative intensity of isotope peaks (MH^+ : $[MH + 2]^+$ = *ca.* 3 : 1)

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Table 1 ^{1}H and ^{13}C NMR spectral data of pericosine D **4** in acetone-d_6

Position	δ ^{1}H[a]		J/Hz	^{1}H–^{1}H COSY	NOE	δ ^{13}C		HMBC[c]
1	—	—	—	—	—	129.06	(q)[b]	—
2	6.90	d	2.5 (3)	3	3	144.78	(t)	1, 3, 4, 6, 7
3	4.46	ddd	8.5 (4), 6.2 (3-OH), 2.5 (2)	2, 4, 3-OH	2, 4	69.79	(t)	1, 2, 4, 5
4	3.99	ddd	8.5 (3), 6.0 (4-OH), 2.6 (5)	3, 5, 4-OH	3, 5	71.10	(t)	2, 5, 6
5	4.21	dt	3.1 (5-OH), 2.6 (4, 6)	4, 6, 5-OH	4, 6	75.19	(t)	1, 4, 6
6	4.83	dd	2.6 (5), 1.1 (3)	5	5	56.02	(t)	1, 2, 4, 7
7	—	—	—	—	—	165.86	(q)	—
8	3.77	s	—	—	—	52.38	(p)	7
3-OH	4.57	d	6.2 (3)	3	—	—	—	—
4-OH	4.33	d	6.0 (4)	4	—	—	—	—
5-OH	4.83	d	3.1 (5)	5	—	—	—	—

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (J/Hz). Figures in parentheses indicate the proton coupling with that position. [b] Letters, p, s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT. [c] Long range ^{1}H–^{13}C correlations from H to C.

in EIMS. Its IR spectrum exhibited bands at 3332, 1720 and 1635 cm^{-1}, characteristic of a hydroxy group, an ester and a double bond. A close inspection of the ^{1}H and ^{13}C NMR spectra of **4** (Table 1) by DEPT and ^{1}H–^{13}C COSY experiments revealed the presence of three hydroxymethines (C-3, C-4 and C-5), one sp^3-methine linked to a chlorine atom (C-6), one trisubstituted double bond (C-1 and C-2) and one methoxycarbonyl group (C-7 and C-8). The ^{1}H–^{1}H COSY analysis of **4** led to a partial structural unit as shown by bold-faced lines in Fig. 1, which was supported by HMBC correlations (Table 1). The connection of this unit and the remaining functional groups was determined on the basis of the key HMBC correlations summarized in Fig. 1, and the planar structure of **4** was thus elucidated.

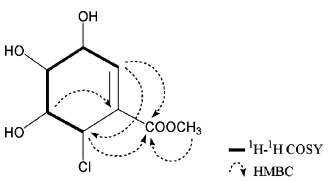

Fig. 1 Selected ^{1}H–^{1}H COSY and HMBC correlations in pericosine D **4**.

The relative stereochemistry and conformation for **4** was established by a combination of observed coupling constants and NOESY experiments in the 3,4-*cis*-acetonide **4a** derived from **4**. Acetonide **4a** showed NOE correlations from the protons of one side of the isopropylidene methyl group (δ_H 1.42 ppm) to both H-3 and H-4 and from the other side of the methyl protons (δ_H 1.50 ppm) to H-5, and coupling constants of $J_{3,4}$ 7.1 Hz and $J_{4,5}$ 8.5 Hz (Table 2). This evidence implied that pseudoaxial H-4 is arranged *cis* to pseudoequatorial H-3, and *trans* to pseudoaxial H-5. Furthermore, an NOE correlation between H-5 and H-6 and a small value of the coupling constant ($J_{5,6}$ 3.7 Hz) between H-5 and H-6 suggested that H-5 is oriented *cis* to pseudoequatorial H-6. This consideration implied that acetonide **4a** in $CDCl_3$ exists in a half-chair conformation (Fig. 2), in which C-5 is above the plane of the olefinic system (C_6–C_1–C_2–C_3) and C-4 is below it. In addition to NOEs for H-3/H-4 and H-5/H-6, pericosine D **4** showed NOE for H-4/H-5 (Table 1), which was not observed in acetonide **4a**. Furthermore, a small value of the coupling constant ($J_{4,5}$ 2.6 Hz) between H-4 and H-5 was observed in compound **4**. This evidence implied that pericosine D **4** in acetone-d_6 exists in a

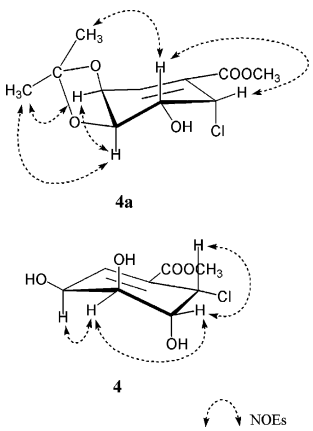

Fig. 2 Observed NOEs and conformation for pericosine D **4** and its acetonide **4a**.

half-chair conformation (Fig. 2), in which C-5 is below the plane of the olefinic system and C-4 is above it. This above-summarized evidence led to relative stereostructure **4** for pericosine D.

Pericosine B **2** was assigned the molecular formula $C_9H_{14}O_6$ as deduced from the $[M + H]^+$ peak of **2** in HREIMS. The general spectral features of compound **2** closely resembled those of **4** except that the chlorine atom in **4** was replaced by a methoxyl group [δ_H 3.59 (H-9), δ_C 61.24 (C-9); δ_H 4.26 (H-6), δ_C 76.72 (C-6) ppm] (Table 3) in **2**. The planar structure of **2** was confirmed by analysis of ^{1}H–^{1}H COSY and HMBC correlations (H-2/H-3, H-3/H-4, H-4/H-5, H-5/H-6, H-9/C-6, H-8/C-7, H-2/C-7, H-6/C-7, and H-5/C-1; Table 3). The 3,4-*cis*-acetonide **2a** derived from **2** showed NOEs from the protons of one side of the isopropylidene methyl group (δ_H 1.38 ppm) to both H-3 and H-4 and from H-5 to H-3, H-4 and H-6, and coupling constants of $J_{3,4}$ 5.7 Hz, $J_{4,5}$ 3.2 Hz and $J_{5,6}$ 4.1 Hz (Table 2). In addition to an NOE correlation between H-3 and H-5, the observation of a *W*-type long-range coupling (0.9 Hz) between H-4 and H-6 implied that acetonide **2a** in $CDCl_3$ exists in a half-chair conformation (Fig. 3) with H-3 and H-5 in a co-pseudoaxial arrangement and with H-4 and H-6 in a co-pseudoequatorial arrangement, in which C-5 is below the plane of the olefinic system (C_6–C_1–C_2–C_3) and C-4 is above it. Pericosine B **2** showed similar NOEs and coupling constants to those of **2a**, including an NOE between H-3 and

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Table 2 ^{1}H NMR spectral data of pericosines A, B and D acetonides (**1a**, **2a** and **4a**) in $CDCl_3$

Position	**1a**				**2a**			**4a**		
	δ ^{1}H[a]		*J*/Hz	NOE	δ ^{1}H[a]	*J*/Hz	NOE	δ ^{1}H[a]	*J*/Hz	NOE
1	—	—	—	—	—	—	—	—	—	—
2	7.15	d	3.2 (3)	3, 8	6.83	dd	3	7.09	d	3
3	4.77	dd	7.6 (4), 3.2 (2)	2, 4, CH_3[b]	4.66	ddd	2, 4, CH_3[b]	4.90	dd	2, 4, CH_3[b]
4	4.70	dd	7.6 (3), 3.9 (5)	3, 5, CH_3[b]	4.50	ddt	5.7 (3), 3.2 (5), 0.9 (2, 6)	4.40	dd	3, CH_3[b]
5	4.30	t	3.9 (4, 6)	4, 6	3.84	br m	—	3.86	br m	6, CH_3[c]
6	5.04	d	3.9 (5)	5	4.30	dt	4.1 (5), 0.9 (3, 4)	5.04	d	5
7	—	—	—	—	—	—	—	—	—	—
8	3.83	s	—	2	3.82	s	—	3.85	s	—
9	—	—	—	—	3.59	s	—	—	—	—
3-OH	—	—	—	—	—	—	—	—	—	—
4-OH	—	—	—	—	—	—	—	—	—	—
5-OH	2.62	br s	—	—	3.12	br s	—	2.39	br d	7.1 (5)
CH_3[b]	1.46	s	—	3, 4	1.38	s	3, 4	1.42	s	—
CH_3[c]	1.52	s	—	—	1.41	s	—	1.50	s	—

[a] As in Table 1. [b] Methyl group of acetonide. [c] Methyl group of acetonide.

H-5, and a *W*-type long-range coupling (1.1 Hz) between H-4 and H-6 (Table 3). The coupling constants of H-3/H-4, H-4/H-5 and H-5/H-6 in **2** were respectively observed as 2.8, 2.0 and 4.1 Hz in acetone-d_6 with a drop of D_2O. This finding indicated that compound **2** in acetone-d_6 exists in the same conformation as that of acetonide **2a** (Fig. 3). The above-summarized evidence led to relative stereostructure **2** with an all *cis*-configuration for pericosine B. The total synthesis of **2** was accomplished by Donohoe *et al.*[11] after our preliminary report,[9] and proved that the absolute configuration of **2** is 3*S*,4*S*,5*S*,6*R*.

Fig. 3 Conformation for pericosines A **1**, C **3** and their acetonides **1a**–**3b**.

Pericosine C **3** has the same molecular formula as pericosine B **2** as deduced from HREIMS. The general features of its ^{1}H and ^{13}C NMR spectra closely resembled those of **2** except that the signals for H-5, H-9, C-3, C-6 and C-9 in **3** revealed a chemical shift difference relative to those of **2** (Tables 3 and 4). ^{1}H–^{1}H COSY and HMBC correlations (H-9/C-6, H-6/C-7, H-2/C-7, H-6/C-2 and H-5/C-1) suggested that compound **3** has the same planar structure as **2** and is the stereoisomer of **2**. Treatment of compound **3** with 2,2-dimethoxypropane (0.5 ml) and pyridinium *p*-toluenesulfonate (PPTS) gave two kinds of acetonides, 3,4-*cis*-acetonide **3a** and 4,5-*cis*-acetonide **3b**, of which the *cis*-configuration was deduced from observation of NOE correlations from the protons of one side of the isopropylidene methyl group [δ_H 1.43 (**3a**) or δ_H 1.35 (**3b**) ppm] to both H-3 and H-4 or to both H-4 and H-5 (Table 5). This result implied that all the protons at the positions 3–5 in **3**, **3a** and **3b** are oriented *cis* and consequently compound **3** is the stereoisomer of **2** at position 6. NOE correlations and a small value of the coupling constant ($J_{5,6}$ 2.3–4.8 Hz) between H-5 and H-6 in **3**, **3a** and **3b** (Tables 4 and 5) showed that these compounds in solution ($CDCl_3$ or acetone-d_6) exist in a half-chair conformation (Fig. 3), in which C-5 is above the plane of the olefinic system (C_6–C_1–C_2–C_3) and C-4 is below it. NMR spectral data of compound **3** and its acetonides **3a** and **3b** were in agreement with those of the epimer of pericosine B **2** and the derivatives which have already been synthesized by us,[12] whereas the specific optical rotation of the natural product **3** and the synthetic compound **7** was different and was found to be −4.8 and +35.1 deg cm^3 g^{-1} dm^{-1}, respectively. This result suggested that the natural pericosine C is a mixture of two enantiomers as described below for pericosine E **5** and contains more enantiomer **3** than synthetic compound **7**.

Pericosine A **1** was shown to have the same molecular formula as pericosine D **4** by HREIMS. The general features of its ^{1}H and

Table 3 ^{1}H and ^{13}C NMR spectral data of pericosine B **2** in acetone-*d*

Position	δ ^{1}H[a]		*J*/Hz	^{1}H–^{1}H COSY	NOE	δ ^{13}C		HMBC[a]
1	—	—	—	—	—	130.23	(q)[a]	—
2	6.72	dd	2.8 (3), 1.1 (6)	3	3	141.72	(t)	1, 6, 7
3	4.16	br s	—	2, 4	2, 4, 5	69.27	(t)	2, 4, 5
4	3.93	br s	—	3, 5	3, 5	69.73	(t)	2, 6
5	3.86	br s	—	4, 6	3, 4, 6	72.48	(t)	1, 3, 4, 6
6	4.26	dt	4.5 (5), 1.1 (2, 4)	5	5, 9	76.72	(t)	1, 2, 5, 7
7	—	—	—	—	—	166.61	(q)	—
8	3.77	s	—	—	—	51.91	(p)	7
9	3.59	s	—	—	6	61.24	(p)	—
3-OH	2.62	br s	—	—	—	—	—	—
4-OH	1.46	br s	—	—	—	—	—	—
5-OH	1.52	br s	—	—	—	—	—	—

[a] As in Table 1.

Table 4 ^{1}H and ^{13}C NMR spectral data of pericosines A **1** and C **3** in acetone-d_6

	1						**3**					
Position	δ ^{1}H[a]		*J*/Hz	NOE	δ ^{13}C		δ ^{1}H[a]		*J*/Hz	NOE	δ ^{13}C	
1	—	—	—	—	130.28	(q)[a]	—	—	—	—	131.15	(q)[a]
2	6.93	d	4.0 (3)	3	141.89	(t)	6.76	d	3.9 (3)	3	140.08	(t)
3	4.41	br t	4.0 (2, 4)	2, 4	66.96	(t)	4.25	t	3.9 (2, 4)	2, 4	67.13	(t)
4	4.10	dd	4.0 (3), 2.0 (5)	3, 5	68.64	(t)	3.89	dd	3.9 (3), 2.1 (5)	3, 5	69.83	(t)
5	4.13	dd	4.5 (6), 2.0 (4)	4	75.42	(t)	3.98	dd	4.8 (6), 2.1 (4)	4, 6, 9	72.85	(t)
6	4.90	dd	4.5 (5), 0.9 (3)	—	57.69	(t)	4.19	d	4.8 (5)	5, 9	78.81	(t)
7	—	—	—	—	168.18	(q)	—	—	—	—	167.22	(q)
8	3.79	s	—	—	52.88	(p)	3.75	s	—	—	51.64	(p)
9	—	—	—	—	—	—	3.48	s	—	5, 6	59.01	(p)
3-OH	n.d.	—	—	—	—	—	4.20	br s	—	—	—	—
4-OH	n.d.	—	—	—	—	—	4.22	br s	—	—	—	—
5-OH	n.d.	—	—	—	—	—	4.57	br s	—	—	—	—

[a] As in Table 1.

Table 5 ^{1}H NMR spectral data of pericosine C acetonides **3a** and **3b** in $CDCl_3$

	3a				**3b**			
Position	δ ^{1}H[a]		*J*/Hz	NOE	δ ^{1}H[a]		*J*/Hz	NOE
1	—	—	—	—	—	—	—	—
2	7.06	d	3.0 (3)	3	7.13	q	1.8 (3, 4, 6)	3
3	4.66	dd	7.0 (4), 3.0 (2)	2, 4, CH_3[b]	4.50	ddd	10.8 (OH), 5.0 (4), 1.8 (2)	2, 4
4	4.53	dd	7.0 (3), 4.2 (5)	3, 5, CH_3[b]	4.65	dd	7.1 (5), 5.0 (3), 1.8 (2)	3, 5, CH_3[b]
5	4.19	td	4.2 (4, 6), 2.7 (OH)	4, 6, 9, 5-OH	4.61	dd	7.1 (4), 2.3 (6)	4, 6, CH_3[b]
6	4.47	d	4.2 (5)	5, 9	4.47	dd	2.3 (5), 1.8 (2)	5, 9
7	—	—	—	—	—	—	—	—
8	3.80	s	—	—	3.80	s	—	—
9	3.46	s	—	5, 6	3.29	s	—	6
3-OH	—	—	—	2.68	br d	10.8 (3)	—	—
4-OH	—	—	—	—	—	—	—	—
5-OH	2.35	d	2.7 (5)	5	—	—	—	—
CH_3[b]	1.43	s	—	3, 4	1.35	s	—	4, 5
CH_3[c]	1.50	s	—	—	1.29	s	—	—

[a] As in Table 1. [b] Methyl group of acetonide. [c] Methyl group of acetonide.

^{13}C NMR spectra closely resembled those of **4** except that the signals for H-4, C-2–C-4 and C-7 revealed a chemical shift difference relative to those of **4** (Tables 1 and 4). Analysis of ^{1}H–^{1}H COSY and HMBC correlations (H-2/C-7, H-2/C-6, H-6/C-7, H-4/C-2 and H-5/C-1) for **1** suggested that compound **1** is the stereoisomer of **4**. The stereochemistry of **1** was previously reported as **6** on the basis of NMR spectral analysis (NOEs and coupling constants) in the 3,4-acetonide of **1** in the preliminary form.[9] After the report we found that the 3,4-acetonide of **1** was not *trans* as previously reported, but *cis*. Furthermore, it was considered that reinvestigation by spectral analysis was difficult and synthesis was effective for determination of the stereochemistry of **1**. Recently, Usami and Ueda, part of our research group, has accomplished the synthesis of compound

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Table 6 ^{1}H and ^{13}C NMR spectral data of pericosine E **5** in acetone-d_6

Position	δ ^{1}H[a]		J/Hz	^{1}H–^{1}H COSY	NOE	δ ^{13}C		HMBC[a]
1	—	—	—	—	—	129.23	(q)[a]	—
2	7.01	d	3.9 (3)	3	3	143.17	(t)	1, 3, 4, 6, 7
3	4.25	br s	—	2, 4	2	65.57	(t)	1, 2, 5
4	4.22	br s	—	3, 5, 4-OH	5	66.75	(t)	2, 3, 5, 6
5	4.36	m	—	4, 6	4, 6, 6′	85.52	(t)	1, 4, 6, 6′
6	5.24	d	3.0 (5)	5	5, 6′	53.06	(t)	1, 2, 4, 7
7	—	—	—	—	—	166.09	(q)	—
8	3.79	s	—	—	—	52.44	(p)	7
3-OH	n.d.	—	—	—	—	—	—	—
4-OH	5.37	br s	—	4	—	—	—	4
1′	—	—	—	—	—	129.91	(q)	—
2′	6.74	t	1.8 (3′, 4′)	3′	3′	143.50	(t)	1′, 3′, 7′
3′	4.26	br s	—	2′, 4′	2′, 4′, 5′	69.22	(t)	2′, 4′, 5′
4′	4.07	br s	—	3′, 5′, 4′-OH	3′, 5′	72.42	(t)	2′, 5′, 6′
5′	3.76	br s	—	4′, 6′, 5′-OH	3′, 4′, 6′	70.43	(t)	1′, 3′, 4′
6′	4.53	d	4.1 (5′)	5′	5, 6, 5′	77.07	(t)	5, 1′, 4′, 7′
7′	—	—	—	—	—	166.87	(q)	—
8′	3.79	s	—	—	—	52.48	(p)	7′
3′-OH	n.d.	—	—	—	—	—	—	—
4′-OH	4.23	br s	—	4′	—	—	—	—
5′-OH	5.64	br s	—	5′	—	—	—	5′, 6′

[a] As in Table 1.

6 with the proposed structure[13] followed by the enantiomer of natural pericosine A **1**,[10] and the report showed that the proposed structure **6** for pericosine A was incorrect and should be revised as **1** with a 3*S*,4*S*,5*S*,6*S* configuration. The revised stereochemistry of **1** was consistent with the observed coupling constant and NOE data of natural pericosine A and its acetonide **1a** (Tables 2 and 4), and the conformation of **1a** and **1** was deduced from those data as follows. The observation of NOEs from H-4 to H-3 and H-5, and from H-5 to H-6, and the coupling constants ($J_{3,4}$ 7.6 Hz, $J_{4,5}$ 3.9 Hz and $J_{5,6}$ 3.9 Hz) in acetonide **1a** (Table 2) implied that acetonide **1a** in $CDCl_3$ exists in a half-chair conformation (Fig. 3) with pseudoequatorial H-3, H-5 and H-6, and pseudoaxial H-4, in which C-5 is above the plane of the olefinic system (C_6–C_1–C_2–C_3) and C-4 is below it. NOEs and coupling constants observed in **1** (Table 4) showed that pericosine A **1** in acetone-d_6 exists in the same conformation as that of acetonide **1a** (Fig. 3).

Pericosine E **5** was assigned the molecular formula $C_{16}H_{21}ClO_{10}$ as deduced from the $[M + H]^+$ peak of **4** in HREIMS. Its UV and IR spectra exhibited the absorption bands similar to those of the above-mentioned pericosines. A close inspection of the ^{1}H and ^{13}C NMR spectra of **5** (Table 6) by DEPT and ^{1}H–^{13}C COSY experiments revealed the presence of five hydroxymethines (C-3, C-4, C-3′, C-4′ and C-5′), two oxygen-bearing sp^3-methines (C-5 and C-6′), one sp^3-methine (C-6) linked to a chlorine atom, two trisubstituted double bonds (C-1, C-2, C-1′ and C-2′) and two methoxycarbonyl groups (C-7 and C-8, and C7′ and C8′), implying that **5** is a dimer of molecules like the pericosines mentioned above. The ^{1}H–^{1}H COSY analysis of **5** led to a partial structural unit as shown by bold-faced lines in Fig. 4, which was supported by HMBC correlations (Table 6). The connection of this unit and the remaining functional groups was determined on the basis of the key HMBC correlations (Fig. 4), and the HMBC correlation (H-5/C-6) revealed that C-5 and C-6′ were connected by an ether linkage. Thus the planar structure of **5** was elucidated. The observation of an NOE between H-5′ and H-3′ in **5** (Table 6) suggested that the cyclohexene ring (C-1′–C-6′) with

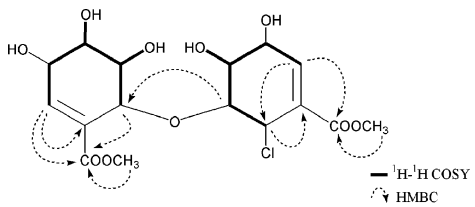

Fig. 4 Selected ^{1}H–^{1}H COSY and HMBC correlations in pericosine E **5**.

three hydroxyl groups in **5** exists in a half-chair conformation with H-3′ and H-5′ in a co-pseudoaxial arrangement, in which C-5 is below the plane of the olefinic system (C_6–C_1–C_2–C_3) and C-4 is above it. NOEs from H-5′ to H-4′ and H-6′, and a small value of the coupling constant ($J_{5',6'}$ 4.1 Hz) between H-5′ and H-6′ implied that H-5′ is oriented *cis* to H-4′ and H-6′ in a co-pseudoequatorial arrangement. In addition, an NOE between H-6′ and H-5 showed H-6′ to be oriented on the same side as H-5. On the other hand, a small value of the coupling constant ($J_{5,6}$ 3.0 Hz) between H-5 and H-6, and NOEs from H-6 to H-5 and H-6′ were observed in the cyclohexene ring (C-1–C-6) with chlorine, implying that H-6 is oriented *cis* to H-5. However, no appropriate data were found for determination of a conformation of the cyclohexene ring and the configurations of C-3 and C-4.

In order to determine the unsolved configuration and conformation of **5**, an X-ray crystal structure analysis was carried out for a single crystal of **5** (obtained by recrystallization from MeOH). The result allowed assignment of the relative configuration of all the asymmetric centers and the conformation of **5** (Fig. 5). The crystal data in the X-ray analysis showed the crystal of **5** to be a racemate. In fact, a specific optical rotation was not observed in the crystal of **5**. The first purification employing silica gel column chromatography as well as reversed-phase HPLC afforded compound **5** as an oil, which was pure in spectral characteristics and HPLC analysis, and showed a specific optical rotation ($[a]_D$ −31.5). When this oil was left at room temperature in MeOH, compound **5** was obtained as plates, showing NMR

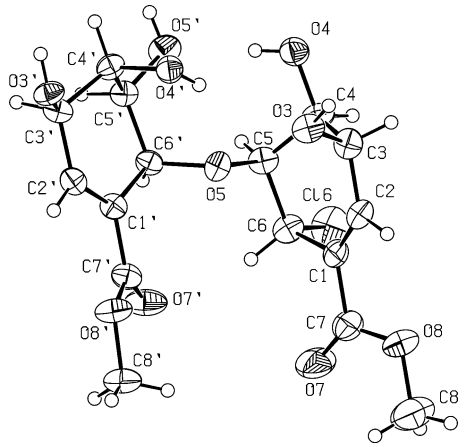

Fig. 5 X-Ray crystal structure for pericosine E **5** with displacement ellipsoids at the 40% probability level.

spectra identical to the oily material. This result suggested that compound **5** was obtained as an enantiomeric mixture. Since a racemate generally crystallizes more readily compared to an optical active compound, it was considered that a racemate of compound **5** was separated as plates in this experiment. Kitagawa and co-workers[14] have reported similar results on natural products that are found as enantiomeric mixtures. Furthermore, we have reported that trichodenone A, with cancer cell growth inhibition, was separated as an enantiomeric mixture from a strain of *Trichoderma harzianum* OUPS-N115 which was isolated from the sponge *Halichondria okadai*.[15] Pericosine E **5** and above-mentioned pericosine C **3** in this experiment are such examples.

The cancer cell growth inhibitory properties of compounds **1**–**5** were examined using the murine P388 lymphocytic leukemia cell line and a disease-oriented panel of human cancer cell lines (HCC panel) in the Japanese Foundation for Cancer Research.[16] Pericosines A **1**, B **2** and D **4** exhibited significant growth inhibition (ED_{50} 0.1, 4.0 and 3.0 μg cm^{-3}, respectively) against the murine P388 cell line, whereas pericosines C **3** and E **5** were inactive (ED_{50} 10.5 and 15.5 μg cm^{-3}, respectively). Among them, pericosines A **1** and E **5** were evaluated for growth inhibition against 38 and 39 human cancer cell lines, respectively. Their mean values (MG-MID) of log GI_{50} over all cell lines tested were moderate (−4.82 and −4.01, respectively, Table 7). However, the delta and range values of **1** were 2.45 and 2.66, respectively (effective value: delta ≥0.5 as well as range ≥1.0), disclosing that this compound showed selective growth inhibition. Namely, pericosine A **1** showed remarkable growth inhibition against HBC-5 (log GI_{50} −5.22) and SNB-75 (log GI_{50} −7.27) cell lines. Furthermore, pericosine A **1** showed significant *in vivo* tumour inhibitory activity. Mice were inoculated intraperitoneally (i.p.) with P388 leukemia cells on day 0, and administered i.p. with 25 mg kg^{-1} 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.

Moreover, inhibitory activities of pericosine A **1** against five protein kinases[17–19] and human topoisomerases were examined. Compound **1** was demonstrated to inhibit the protein kinase EGFR at a concentration of 100 μg ml^{-1} by 40–70%, and to inhibit topoisomerase II with IC_{50} value of 100–300 mM.

Table 7 Growth inhibition of pericosines A **1** and E **5** against a panel of human cancer cell lines

		Log GI_{50}/M[a]	
Origin of cancer	Cell line	**1**	**5**
Breast	HBC-4	−4.76	−4.00
	BSY-1	−4.75	−4.00
	HBC-5	−5.22	−4.00
	MCF-7	−4.66	−4.06
	MDA-MB-231	−4.74	−4.00
Central nervous system	U-251	−4.76	−4.00
	SF-268	−4.72	−4.00
	SF-295	−4.62	−4.00
	SF-539	−4.71	−4.00
	SNB-75	−7.27	−4.17
	SNB-78	−4.71	−4.00
Colon	HCC2998	−4.75	−4.00
	KM-12	−4.73	−4.00
	HT-29	−4.70	−4.00
	WiDr	−4.64	—
	HCT-15	−4.77	−4.00
	HCT-116	−4.75	−4.00
Lung	NCI-H23	−4.78	−4.00
	NCI-H226	−4.80	−4.00
	NCI-H522	−4.95	−4.00
	NCI-H460	−4.72	−4.00
	A549	−4.61	−4.00
	DMS273	−4.68	−4.00
	DMS114	−4.82	−4.00
Melanoma	LOX-IMVI	−4.72	−4.00
Ovary	OVCAR-3	−4.85	−4.00
	OVCAR-4	−4.68	−4.00
	OVCAR-5	−4.79	−4.00
	OVCAR-8	−4.78	−4.00
	SK-OV-3	−4.76	−4.00
Kidney	RXF-631L	−4.73	−4.00
	ACHN	−4.72	−4.00
Stomach	St-4	−4.65	−4.00
	MKN1	−4.78	−4.00
	MKN7	−4.70	−4.00
	MKN28	−4.72	−4.00
	MKN45	−4.75	−4.00
	MKN74	−4.69	−4.00
Prostate	DU-145	—	−4.00
	PC-3	—	−4.00
MG-MID[b]		−4.82	−4.01
Delta[c]		2.45	0.16
Range[d]		2.66	0.17

[a] Log concentration of compound for inhibition of cell growth at 50% compared to control. [b] Mean value of log GI_{50} over all cell lines tested. [c] The difference in log GI_{50} value of the most sensitive cell and the MG-MID value. [d] The difference in log GI_{50} value of the most sensitive cell and the least sensitive cell.

Experimental

General procedures

UV spectra were recorded on a Shimadzu spectrophotometer and IR spectra on a Perkin Elmer FT-IR spectrometer 1720X. NMR spectra were recorded at 27 °C on Varian UNITY INOVA-500 and MERCURY spectrometers with tetramethylsilane (TMS) as an internal reference. EIMS was determined using a Hitachi M-4000H mass spectrometer. ORD and CD spectra were recorded on a JASCO J-820 polarimeter. Liquid chromatography over silica gel (mesh 230–400) was performed under medium pressure.

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HPLC was run on a Waters ALC-200 instrument equipped with a differential refractometer (R 401) and Shim-pack PREP-ODS (25 cm × 20 mm i. d.). Analytical TLC was performed on precoated Merck aluminium sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) with the solvent system CH_2Cl_2–MeOH (9 : 1), and compounds were viewed under UV lamp and sprayed with 10% H_2SO_4 followed by heating.

Biological materials. The fungal strain (OUPS-N133) was separated from the sea hare *Aplysia kurodai* and identified as *Periconia byssoides* as reported previously.[5] A voucher specimen of *P. byssoides* has been deposited at the National Institute of Technology and Evaluation.

Culturing and isolation of metabolites. The fungal strain was grown in a liquid medium (90 dm^3) containing 1% malt extract, 1% glucose and 0.05% peptone in artificial seawater adjusted to pH 7.5 for four weeks at 27 °C. The culture was filtered under suction and the mycelia collected were extracted thrice with MeOH. The combined extracts were evaporated *in vacuo* to give a mixture of crude metabolites (21.5 g), the CH_2Cl_2–MeOH (1 : 1) soluble fraction of which was inactive against the P388 cell line. The culture filtrate was extracted thrice with AcOEt. The combined extracts were evaporated *in vacuo* to afford a mixture of crude metabolites (5.7 g, ED_{50} 4.3 μg cm^{-3}). The AcOEt extract was passed through Sephadex LH-20 using CH_2Cl_2–MeOH (1 : 1) as eluent. The second fraction (3.5 g), in which the activity was concentrated, was chromatographed on a silica gel column with a CH_2Cl_2–MeOH gradient as eluent. The MeOH–CH_2Cl_2 (1 : 9) eluates were collected as 2 active fractions [Fr. 1 (532.8 mg) and Fr. 2 (253.7 mg)] against the P388 cell line. Fr. 1 was purified by HPLC using MeOH–H_2O (4 : 6) as eluent to afford **1** (105.7 mg), **2** (10.7 mg), **3** (6.6 mg), and **4** (5.2 mg). Fr. 2 was purified by HPLC using MeOH–H_2O (35 : 65) as eluent to afford **1** (72.6 mg) and **5** (17.6 mg).

Pericosine A 1. Obtained as plates (MeOH), mp 95–97 °C, $[\alpha]_D$ +57.0 (*c* 3.16 in EtOH); λ_{max} (EtOH)/nm 217 ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1} 3.90); ν_{max} (KBr)/cm^{-1} 3353 (OH), 1720 (ester) and 1651 (C=C); *m/z* (EI) 223 ([M + H]$^+$, 1.8%), 187 ($MH^+ - HCl$, 3.6) and 126 ([$C_7H_9O_4$]$^+$, 100) [*m/z* (HREI) found: [M + H]$^+$, 223.0364. $C_8H_{12}ClO_5$ requires 223.0363]; CD λ (*c* 1.40 × 10^{-3} mol dm^{-3} in EtOH)/nm 289 ($\Delta\varepsilon$ 0), 248 (−0.59), 238 (0) and 227 (+1.75). ^{1}H and ^{13}C NMR data are listed in Table 4.

Pericosine B 2. Obtained as an oil, $[\alpha]_D$ +22.3 (*c* 0.82 in EtOH); λ_{max} (EtOH)/nm 218 ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1} 3.85); ν_{max} (liquid)/cm^{-1} 3327 (OH), 1720 (ester) and 1635 (C=C); *m/z* (EI) 219 ([M + H]$^+$, 0.3%), 187 ($MH^+ - MeOH$, 1.7) and 126 ([$C_7H_9O_4$]$^+$, 100) [*m/z* (HREI) found: [M + H]$^+$, 219.0867. $C_9H_{15}O_6$ requires 219.0853]; CD λ (*c* 3.75 × 10^{-3} mol dm^{-3} in EtOH)/nm 345 ($\Delta\varepsilon$ 0), 266 (−0.1), 258 (0) and 240 (+1.07). ^{1}H and ^{13}C NMR data are listed in Table 3.

Pericosine C 3. Obtained as an oil, $[\alpha]_D$ −4.8 (*c* 0.17 in EtOH); λ_{max} (EtOH)/nm 217 ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1} 3.53); ν_{max} (liquid)/cm^{-1} 3330 (OH), 1721 (ester) and 1635 (C=C); *m/z* (EI) 219 ([M + H]$^+$, 1.2%), 187 ($MH^+ - MeOH$, 3.7) and 126 ([$C_7H_9O_4$]$^+$, 100) [*m/z* (HREI) found: [M + H]$^+$, 219.0867. $C_9H_{15}O_6$ requires 219.0854]; CD λ (*c* 7.5 × 10^{-4} mol dm^{-3} in EtOH)/nm 274 ($\Delta\varepsilon$ 0), 248 (+0.21), 237 (0) and 240 (−0.89). ^{1}H and ^{13}C NMR data are listed in Table 4.

Pericosine D 4. Obtained as oil, $[\alpha]_D$ +1.9 (*c* 1.05 in EtOH); λ_{max} (EtOH)/nm 216 ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1} 4.11); ν_{max} (liquid)/cm^{-1} 3332 (OH), 1720 (ester) and 1635 (C=C); *m/z* (EI) 223 ([M + H]$^+$, 0.6%), 187 ($MH^+ - HCl$, 2.4) and 126 ([$C_7H_9O_4$]$^+$, 100) [*m/z* (HREI) found: [M + H]$^+$, 223.0365. $C_8H_{12}ClO_5$ requires 223.0363]; CD λ (*c* 9.00 × 10^{-4} mol dm^{-3} in EtOH)/nm 283 ($\Delta\varepsilon$ 0), 258 (+0.74), 250 (0) and 232 (−5.06). ^{1}H and ^{13}C NMR data are listed in Table 1.

Pericosine E 5. Obtained as oil, $[\alpha]_D$ −31.5 (*c* 0.43 in EtOH), by chromatography procedures, and as plates, mp 213–215 °C, $[\alpha]_D$ 0, by crystallization of oily material (**5**) in MeOH. Oily and crystalline materials showed the same spectral data. λ_{max} (EtOH)/nm 214 ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1} 4.03); ν_{max} (liquid)/cm^{-1} 3326 (OH), 1721 (ester) and 1638 (C=C); *m/z* (EI) 409 ([M + H]$^+$, 1.1%), 390 ($M^+ - H_2O$, 1.0), 354 ($M^+ - H_2O - HCl$, 6.3) and 139 ([$C_8H_{10}O_4$]$^+$, 100) [*m/z* (HREI) found: [M + H]$^+$, 409.0904. $C_{16}H_{22}ClO_{10}$ requires 409.0900]; CD λ (*c* 3.56 × 10^{-4} mol dm^{-3} in EtOH)/nm 283 ($\Delta\varepsilon$ 0) and 244 (+5.36). ^{1}H and ^{13}C NMR data are listed in Table 6.

Acetonide 1a of pericosine A 1. To a solution of pericosine A **1** (5.5 mg) in CH_2Cl_2 (1.2 ml) was added 2,2-dimethoxypropane (0.5 ml) and pyridinium *p*-toluenesulfonate (PPTS) (1.9 mg), and the reaction mixture was stirred at room temperature for 1 h. The mixture was concentrated to dryness under reduced pressure, and the residue was purified by HPLC using MeOH–H_2O (7 : 3) as eluent to afford acetonide **1a** (3.8 mg) as a pale yellow oil. EIMS: *m/z* 263 ([M + H]$^+$, 2.3%), HREIMS: *m/z* 263.0688 [M + H]$^+$ (calcd for $C_{11}H_{16}ClO_5$: 263.0682). ^{1}H NMR data are listed in Table 2.

Acetonide 2a of pericosine B 2. Using the same procedure as above with compound **1**, a solution of pericosine B **2** (2.1 mg) in CH_2Cl_2 (1.0 ml) was treated with 2,2-dimethoxypropane (0.5 ml) and PPTS (1.1 mg), and purified by HPLC [MeOH–H_2O (7 : 3)] to afford acetonide **2a** (1.7 mg) as a pale yellow oil. EIMS: *m/z* 259 ([M + H]$^+$, 2.8%), HREIMS: *m/z* 259.1176 [M + H]$^+$ (calcd for $C_{12}H_{19}O_6$: 259.1179). ^{1}H NMR data are listed in Table 2.

Acetonides 3a and 3b of pericosine C 3. Using the same procedure as above with compound **1**, a solution of **3** (5.7 mg) in CH_2Cl_2 (1.5 ml) was treated with 2,2-dimethoxypropane (1.0 ml) and PPTS (1.9 mg), and purified by HPLC [MeOH–H_2O (6 : 4)] to afford acetonides **3a** (1.8 mg) and **3b** (2.1 mg) as pale yellow oils.

3a: EIMS: *m/z* 258 ([M]$^+$, 1.7%). HREIMS: *m/z* 258.1105 [M]$^+$ (calcd for $C_{12}H_{18}O_6$: 258.1098). **3b**: EIMS: *m/z* 258 ([M]$^+$, 1.5%). HREIMS: *m/z* 258.1104 [M]$^+$ (calcd for $C_{12}H_{18}O_6$: 258.1098). ^{1}H NMR data of **3a** and **3b** are listed in Table 5.

Acetonide 4a of pericosine D 4. Using the same procedure as above with compound **1**, a solution of pericosine D **4** (3.3 mg) in CH_2Cl_2 (0.5 ml) was treated with 2, 2-dimethoxypropane (0.4 ml) and PPTS (1.6 mg), and purified by HPLC [MeOH–H_2O (7 : 3)] to afford acetonide **4a** (2.7 mg) as a pale yellow oil. EIMS: *m/z* 263 ([M + H]$^+$, 1.5%), HREIMS: *m/z* 263.0685 [M + H]$^+$ (calcd for $C_{11}H_{16}ClO_5$: 263.0682). ^{1}H NMR data are listed in Table 2.

X-Ray crystallography of pericosine E 5. Pericosine E **5** was crystallized from a MeOH solution by the vapor diffusion

method. Crystal data: $C_{16}H_{21}ClO_{10}$, $M = 408.78$, triclinic, $P\bar{1}$, $a = 9.516(10)$ Å, $b = 16.66(2)$ Å, $c = 6.226(4)$ Å, $\alpha = 98.72(9)°$, $\beta = 105.35(7)°$, $\gamma = 98.7(1)°$, $V = 922(2)$ Å^3, $Z = 2$, $D_x = 1.473$ g cm^{-3}, $F(000) = 428$, μ (Cu-Kα) $= 2.328$ mm^{-1}. Data collection was performed by Rigaku AFC5R using graphite-monochromated radiation Cu-Kα ($\lambda = 1.5418$ Å). A total of 2907 reflections were collected until $2\theta = 120.4°$ ($R_{int} = 0.1335$). The crystal structure was solved by direct methods using SHELXS-97.[20] The structure was refined by the full matrix least-squares method on F^2 using SHELXL-97.[21] For the structure refinements, non-hydrogen atoms were refined with anisotropic temperature factors. Hydrogen atoms were calculated on the geometrically ideal positions and fitted the electron density map by the riding method, and were included in the calculation of structure factors with isotropic temperature factors. At the final stage, $R1 = 0.1030$, w$R2 = 0.2516$ [$I > 2\sigma(I)$] and $S = 1.101$ were obtained.

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