Leptosins, Antitumour Metabolites of a Fungus Isolated from a Marine Alga

Chika Takahashi,[a] Atsushi Numata,*[,a] Yoshinori Ito,[a] Eiko Matsumura,[a] Hiromasa Araki,[b] Hideo Iwaki[b] and Katsuhiko Kushida[c]

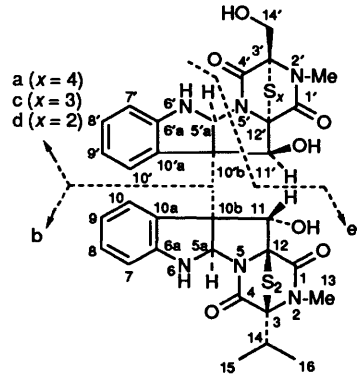
[a] *Osaka University of Pharmaceutical Sciences, Matsubara, Osaka 580, Japan*
[b] *Fuso Pharmaceutical Industries, Ltd, Joto-ku, Osaka 532, Japan*
[c] *Varian Instruments Ltd, Shinjuku-ku, Tokyo 169, Japan*

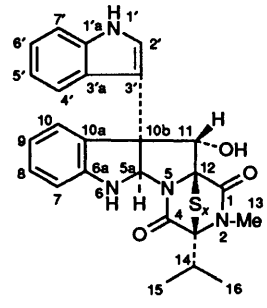
Leptosins A B, C, D, E and F, chaetocin derivatives, have been isolated from the mycelium of a strain of *Leptosphaeria* sp. attached to the marine alga *Sargassum tortile*. Their stereostructures, with a different configuration from that of related compounds, have been elucidated by spectroscopic analyses using various 1D and 2D NMR techniques and some chemical transformations. All the compounds showed potent cytotoxicity against cultured P388 cells, and leptosins A and C exhibited significant antitumour activity against Sarcoma 180 ascites.

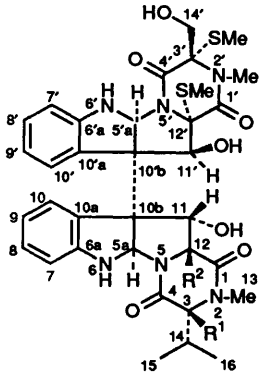
Studies on the natural-product chemistry of marine animals have illustrated that they are prolific sources for structurally unique, highly bioactive and biomedically utilitarian secondary metabolites. Of the many bioactive compounds found in marine animals, toxic principles of several animals (tetrodotoxin, neosurugatoxin, saxitoxin and palytoxin) have proven to be produced by bacteria.[1–4] This has evoked wide interest in marine microorganisms because of the potential for the development of new pharmaceutical agents and also in the search for the origin of marine animal metabolites. We have focussed our attention on antineoplastic and/or cytotoxic metabolites from microorganisms which inhabit the marine environment. As part of this programme, we previously reported that cytotoxic substances, three fumiquinazolines and two communesins, were produced by a strain of *Aspergillus fumigatus*, isolated from the gastrointestinal tract of the saltwater fish *Pseudolabrus japonicus*,[5] and by a strain of *Penicillium* sp. isolated from the marine alga *Enteromorpha intestinalis*,[6] respectively. In the present study, we examined secondary metabolites from a strain of *Leptosphaaeria* sp. isolated from the marine alga *Sargassum tortile*, and isolated six novel antitumour and cytotoxic metabolites, designated leptosins A–F **1**–**6**, which belong to a series of dimeric epipolysulfanyldioxopiperazines such as chaetocin and chetoracin A.[7–9] We describe herein the structure elucidation and cytotoxic activity of these metabolites.

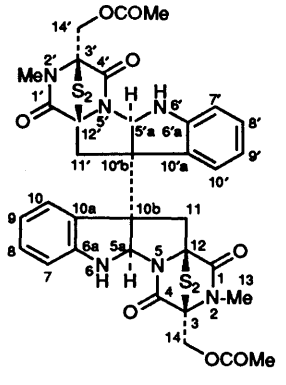
The fungal strain was cultured at 27 °C for 3 weeks in a medium containing 2% glucose, 1% peptone and 0.5% yeast extract in artificial seawater. The MeOH extract of the mycelium was purified by bioassay-directed fractionation employing a combination of Sephadex LH-20 and silica gel column chromatographies and high-performance liquid chromatography (HPLC) to afford leptosins A **1**, B **2**, C **3**, D **4**, E **5** and F **6**.

Leptosin A **1** had the molecular formula $C_{32}H_{32}N_6O_7S_6$ established by high-resolution fast atom bombardment mass spectrometry (HRFABMS) [*m*/*z* 805.0740 (MH^+), Δ +0.5 mmu]. Its IR spectrum exhibited bands at 3412, 1686, 1664, 1608 and 1593 cm^{-1}, characteristic of an alcohol, an amine, an amide and an aromatic ring. A close inspection of the ^1H and ^{13}C NMR spectra of **1** (Table 1) by distortionless enhancement by polarization transfer (DEPT) and ^1H–^1H and ^1H–^{13}C correlation spectroscopy (COSY) experiments and comparison with spectral data for related compounds revealed signals for two hydroxy methine groups (C-11 and C-11′) linked to two quaternary sp^3-hybridized carbons, two methines (C-5a and C-5′a) bearing two nitrogens and a quaternary sp^3-carbon, four quaternary sp^3-carbons (C-3, C-12, C-3′ and C-12′) each









bearing a nitrogen and a sulfur, four amides (C-1, C-4, C-1′ and C-4′), two *N*-methyl groups (C-13 and C-13′), isopropyl (C-14, C-15 and C-16) and hydroxymethyl C-14′) groups each linked to a quaternary sp^3-carbon, and two 1,2-disubstituted benzenes (C-6a to 10a and C-6′a to C-10′a). The signals for one quaternary sp^2-carbon (C-6a and C-6′a) of each of the two aromatic rings appeared lowfield (δ 148.3 and 149.9) in the ^{13}C NMR spectrum, indicating that one substituent on each benzene is an amino group.

The connection of the functional groups was demonstrated

Table 1 ^1H (300 MHz) and ^{13}C (75.4 MHz) NMR data of leptosin A **1** in $CDCl_3$

Position	δ ^1H[a]	NOEs[b]	δ ^{13}C	HMBC[d]
1			167.1 (q)[c]	13-H
3			80.3 (q)	13-H, 14-H, 15-H, 16-H
4			160.9 (q)	14-H
5a	6.43 s	11-OH, 5'a-H, 11'-H	80.3 (t)	11-H
6	5.25 br s			
6a			148.3 (q)	5a-H, 8-H, 10-H
7	6.64 d (8.0)		110.4 (t)	9-H
8	7.10 t (8.0)		130.0 (t)	10-H
9	6.46 t (8.0)		119.7 (t)	7-H
10	5.59 d (8.0)	11-H	125.7 (t)	8-H
10a			126.0 (q)	5a-H, 6-H, 7-H, 9-H, 11-H
10b			62.7 (q)	
11	4.95 s	10-H, 10'-H	80.9 (t)	6-H, 11-OH, 5'a-H, 11'-H
12			75.5 (q)	5a-H
13	3.04 s	15-H, 16-H	27.8 (p)	
14	2.64 heptet (6.8)		32.3 (t)	15-H, 16-H
15	1.42 d (6.8)	13-H	17.9 (p)	16-H
16	1.42 d (6.8)	13-H	18.6 (p)	15-H
11-OH	5.69 s	5a-H		
1'			168.2 (q)	13'-H, 11'-H
3'			79.0 (q)	13'-H, 14'-H
4'			169.3 (q)	14'-H, 15'-H
5'a	5.42 s	5a-H	80.2 (t)	11'-H
6'	3.90 br s[e]			
6'a			149.9 (q)	5'a-H, 8'-H, 10'-H
7'	6.55 d (7.8)		109.8 (t)	9'-H
8'	7.24 t (7.8)		130.0 (t)	10'-H
9'	6.95 t (7.8)		119.0 (t)	7'-H
10'	7.87 d (7.8)	11-H	130.3 (t)	8'-H
10'a			123.1 (q)	5'a-H, 7'-H, 9'-H, 11'-H
10'b			64.5 (q)	5a-H, 11'-OH
11'	5.43 s	5a-H	79.3 (t)	
12'			80.2 (q)	5'a-H
13'	3.00 s		28.9 (p)	
14'A	3.93 d (12.0)		63.6 (s)	
14'B	4.10 m			
11'-OH	5.30 br s[e]			
14'-OH	3.27 br s[e]			

[a] ^1H Chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constant (J/Hz) in parentheses. [b] Observed in the NOESY experiment. [c] Letters, p. s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT. [d] Long range ^1H–^{13}C correlation from H to C observed in the HMBC experiment. [e] Interchangeable.

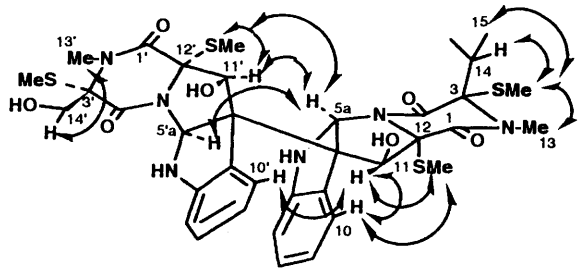


Fig. 1 NOESY data summary for **8**

on the basis of heteronuclear multiple-bond connectivity (HMBC) correlations (Table 1). The principal correlations are as follows: 13-H to C-1 and C-3, 14-H to C-4, 11-H to C-5a and C-10a, 5a-H to C-6a, C-12 and C-10'b, 13'-H to C-1' and C-3', 14'-H to C-4', 11'-H to C-1', C-5'a, C-10'a and C-10b, and 5'a-H to C-10b, C-4', C-6'a and C-10'a. This evidence led to planar structure of **1** for leptosin A. The number of sulfur atoms in the polysulfide bridges of **1** were determined by chemical and mass spectral evidence as follows. Leptosin A **1** was transformed into bis(methylsulfanyl) and tetrakis(methylsulfanyl) derivatives **7** and **8** by treatment with $NaBH_4$ and MeI in pyridine. The position of the two methylsulfanyl groups in **7** was established from the fact that the NMR signals of all the carbons (C-1–C-16) on the isopropyl-bearing half of the molecule of **7** (Table 3) showed close correspondence with those of **1** (Table 1). Formation of **7** from **1** and the molecular formula of **1** indicated that the tetrasulfide and disulfide bridges existed in the hydroxymethyl- and isopropyl-bearing dioxopiperazine rings of **1**, respectively. This was supported by the FABMS fragments at m/z 428 (a^+) and 377 ($[b + H]^+$), corresponding to the hydroxymethyl- and isopropyl-bearing halves of the molecule of **1**, respectively, as well as five other ions at m/z 282 ($[a - 4S - H_2O]^+$), 312 ($[b - 2S]^+$), 493 ($[e + H]^+$), 429 ($[eH - 2S]^+$) and 411 ($[eH - 2S - H_2O]^+$) (see structure **1** for a, b and e). The FABMS of **1** also exhibited the fragment peaks at m/z 232 ($[bis\text{-}indol\text{-}3\text{-}yl]^+$), 197 ($[eH - 2S - 232]^+$), 677 ($[MH - 42]^+$) and 659 ($[MH - 4S - H_2O]^+$), the last two fragments showing that the sulfur atoms of the hydroxymethyl-bearing dioxopiperazine ring are eliminated more easily than those of the isopropyl-bearing ring.

The relative configuration of **1** was deduced from detailed nuclear Overhauser enhancement (NOE) spectral analysis of **1** (Table 1) and **8** (Fig. 1). NOE observed between 11-H and 12-SMe in **8** was indicative of their *cis* configuration while NOEs between 10-H and 12-SMe, and 10-H and 11-H indicated that 11-H and the C-10b–C-10'b bond, and 5a-H and 11-H have *trans* configurations. If 5a-H and 11-H have a *cis* configuration, then no NOE between 10-H and 12-SMe should be observed. The *trans* configuration of 11-H and 5a-H was supported by an NOE between 5a-H and 5'a-H. On the other hand, the NOE between 11'-H and 12-SMe was indicative of their *cis*

Table 2 ^{1}H (300 MHz) NMR data of leptosins B–F **2–6** and derivatives **7** and **8** in $CDCl_3$ [a]

Position	2	3	4	5	6	7	8
5a	6.48 s	6.47 br s	6.34 br s	6.20 s	6.51 s	6.71 s	6.96 s
6	5.30 s	*c*	5.40 s	5.62 s	5.15 s	5.35 s	5.30 s [b]
7	6.66 d (8.0)	6.56 d (8.0)	6.72 dd (7.8, 1.0)	6.76 d (7.8)	6.52 d (7.8)	6.65 d (7.8)	6.54 d (7.8)
8	7.11 t (8.0)	7.14 t (8.0)	7.16 td (7.8, 1.0)	7.15 t (7.8)	7.04 t (7.8)	7.09 t (7.8)	7.06 t (7.8)
9	6.50 t (8.0)	6.47 br s	6.84 td (7.8, 1.0)	6.74 t (7.8)	6.72 t (7.8)	6.45 t (7.8)	6.33 t (7.8)
10	5.66 d (8.0)	5.68 br s	7.45 dd (7.8, 1.0)	7.33 d (7.8)	7.32 d (7.8)	5.60 d (7.8)	5.58 d (7.8)
11	4.99 s	4.80 s	5.37 s	5.41 d (2.0)	5.21 d (2.3)	4.97 s	4.84 d (2.8)
13	3.03 s	3.05 s	3.08 s	3.23 s	3.05 s	3.05 s	3.07 s
14	2.65 heptet (6.8)	2.67 heptet (7.0)	2.72 heptet (7.0)	2.52 heptet (6.8)	2.73 heptet (6.8)	2.67 heptet (7.0)	2.63 heptet (7.0)
15	1.416 d (6.8)	1.41 d (7.0)	1.47 d (7.0)	1.22 d (6.8)	1.19 d (6.8)	1.44 d (7.0)	1.12 d (7.0)
16	1.424 d (6.8)	1.42 d (7.0)	1.49 d (7.0)	1.48 d (6.8)	1.53 d (6.8)	1.45 d (7.0)	1.20 d (7.0)
11-OH	5.65 br s	5.73 br s	5.22 br s	3.71 d (2.0)	3.43 br s	5.62 br s	3.63 d (2.8)
3-SMe							2.17 s
12-SMe							1.84 s
1′			8.01 br s	8.05 br s	8.12 br s		
2′			7.02 d (2.7)	7.12 d (3.0)	7.09 d (3.0)		
4′			7.96 dd (7.5, 1.0)	7.87 dd (7.5, 1.0)	7.87 dd (7.5, 1.0)		
5′			7.18 td (7.5, 1.0)	7.18 td (7.5, 1.0)	7.12 td (7.5, 1.0)		
5′a	5.62 s	5.97 br s				5.57 s	5.49 s
6′	3.08 br s	*c*	7.19 td (7.5, 1.0)	7.20 td (7.5, 1.0)	7.20 td (7.5, 1.0)	4.97 br s [b]	5.02 br s [b]
7′	6.56 d (7.8)	6.26 br s	7.30 dd (7.5, 1.0)	7.34 dd (7.5, 1.0)	7.33 dd (7.5, 1.0)	6.50 d (7.8)	6.50 d (7.8)
8′	7.25 t (7.8)	7.14 br s				7.18 t (7.8)	7.19 t (7.8)
9′	6.95 t (7.8)	6.88 br s				6.91 t (7.8)	6.89 t (7.8)
10′	7.91 d (7.8)	7.80 br s				7.85 d (7.8)	7.72 d (7.8)
11′	5.44 s	5.36 br s				5.38 s	5.13 d (2.6)
13′	2.99 s	2.94 br s				3.02 s	3.02 s
14′A	3.73 d (12.8)	4.18 m				3.71 d (12.0)	3.70 br s (12.0)
14′B	4.53 br d (12.8)	4.34 br d				4.03 d (12.0)	4.02 br s (12.0)
11′-OH	4.51 s	*c*				3.73 br s [b]	3.75 d (2.6)
14′-OH	2.67 br s	3.35 br s [b]				2.69 br s [b]	1.84 br s [b]
3′-SMe						2.22 s	2.22 s
12′-SMe						2.43 s	2.37 s

[a] ^{1}H Chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constant (*H*/Hz) in parentheses. [b] Assignments interchangeable. [c] Not detected.

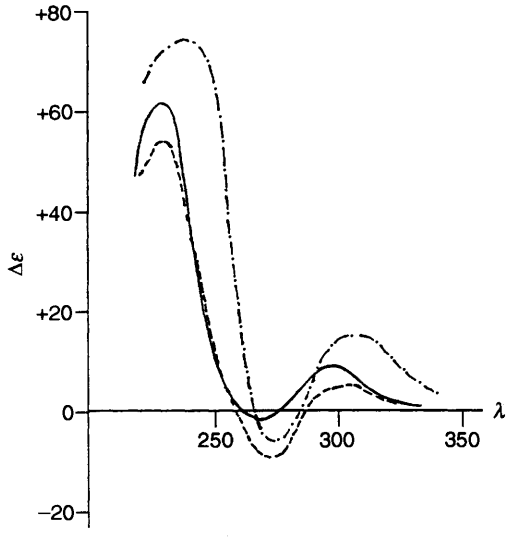


Fig. 2 CD spectra of leptosins C **3** (– – –), D **4** (——) and di-*O*-acetylchaetocin **9** (— · —) in EtOH

configuration while NOEs between 5′a-H and 5a-H and 11′-H and 5a-H showed that 11′-H and 5′a-H, and 11′-H and the C-10′b–C-10b bond have *cis* configurations. Because of the presence of sulfide bridges between C-3 and C-12, and C-3′ and C-12′ in **1**, the two S-Me groups at C-3 and C-12, and the two S-Me groups at C-3′ and C-12′ in **8** should have *cis* orientations. The above summarized evidence, supported by an NOE experiment of **1**, allowed assignment of the relative configuration of **1**, and also suggested that **1** and **8** exist in the conformation shown in Fig. 1 in $CHCl_3$ solution. The relative configuration of C-5′a and C-10′b in **1** was different from that of related compounds, chaetocin and chetracin A,[7] and this is the first isolation of a dimeric epipolysulfanyldioxopiperazine with such a configuration.

Leptosins B **2** and C **3** were assigned the molecular formulae $C_{32}H_{32}N_6O_7S_5$ and $C_{32}H_{32}N_6O_7S_4$, respectively, as deduced from MH^+ peaks in HRFABMS (*m*/*z* 773.1024, Δ + 0.9 mmu and 741.1316, Δ + 2.2 mmu, respectively). The general features of their UV, IR and NMR spectra (Tables 2 and 3) closely resembled those of **1** except that some ^{13}C NMR signals (C-4′, C-14′, C-10′b, *etc.*) for their hydroxymethyl-bearing dioxopiperazine rings exhibited a chemical shift difference relative to those of **1**. Both **2** and **3** afforded **7** and **8** on treatment with $NaBH_4$ and MeI. In the FABMS, **2** exhibited two fragments [*m*/*z* 396 (c^+) and 377 (bH^+)], corresponding to the two halves of the molecule, together with two fragments [*m*/*z* 282 ([c − 3S − H_2O]$^+$) and 312 ([b − 2S]$^+$), while **3** showed two fragments [*m*/*z* 364 (d^+) and 377 (bH^+), corresponding to the two halves of the molecule, together with two other fragments [*m*/*z* 282 ([d − 2S − H_2O]$^+$) and 312 ([b − 2S]$^+$). In addition, both the compounds showed other fragment ions at *m*/*z* 493 (eH^+), 429 ([eH − 2S]$^+$), 411 ([eH − 2S − H_2O]$^+$), 232 ([bis-indol-3-yl]$^+$), 197 ([eH − 2S − 232]$^+$), 677 ([MH − 3S or 2S]$^+$) and 659 ([677 − H_2O]$^+$), the last two fragments illustrating that the hydroxymethyl-bearing dioxopiperazine rings in **2** and **3** have three and two sulfide bridges, respectively. This evidence led to relative stereostructures **2** and **3** for leptosins B and C, respectively.

In the circular dichroism (CD) spectrum of leptosin C **3**, there was a negative band at 271 nm due to an S→CO charge-transfer transition, comparable in strength to that of the related compound, di-*O*-acetylchaetocin **9** (Fig. 2),[8,10] showing the asymmetric centres of the dioxopiperazine ring in **3** to have the

Table 3 ^{13}C (75.4 MHz) NMR data of leptosins B–F **2–6** and derivatives **7** and **8** in $CDCl_3$

Position	2	3	4	5	6	7	8
1	167.1 (q)[a]	167.3 (q)	167.6 (q)	170.2 (q)	168.2 (q)[b]	167.2 (q)	165.9 (q)
3	80.2 (q)	80.4 (q)	80.5 (q)	78.0 (q)	81.6 (q)[c]	80.3 (q)	77.8 (q)
4	160.9 (q)	160.7 (q)	161.3 (q)	165.4 (q)	167.8 (q)[b]	161.0 (q)	165.3 (q)
5a	80.7 (t)	79.8 (t)	82.6 (t)	81.7 (t)	83.0 (t)	80.8 (t)	80.7 (t)
6a	148.5 (q)	149.0 (q)	147.2 (q)	149.7 (q)	147.4 (q)	148.7 (q)	149.6 (q)
7	110.6 (t)	109.4 (t)	110.6 (t)	110.9 (t)	109.0 (t)	110.1 (t)	108.8 (t)
8	130.0 (t)	130.1 (t)	129.2 (t)	130.1 (t)	129.3 (t)	129.9 (t)	129.5 (t)
9	119.8 (t)	119.0 (t)	119.7 (t)	119.6 (t)	119.4 (t)	119.4 (t)	118.2 (t)
10	125.7 (t)	126.6 (t)	124.4 (t)	125.2 (t)	124.5 (t)	125.7 (t)	124.2 (t)
10a	126.3 (q)	126.8 (q)	130.9 (q)	128.6 (q)	131.2 (q)	126.5 (q)	127.0 (q)
10b	62.6 (q)	63.2 (q)	60.7 (q)	58.6 (q)	59.0 (q)	62.8 (q)	61.2 (q)
11	80.8 (t)	81.4 (t)	81.3 (t)	83.5 (t)	83.1 (t)	81.3 (t)	79.7 (t)
12	75.5 (q)	75.6 (q)	76.2 (q)	83.9 (q)	80.2 (q)[c]	75.8 (q)	72.9 (q)
13	27.8 (p)	27.8 (p)	27.8 (p)	28.1 (p)	30.1 (p)	27.8 (p)	30.2 (p)
14	32.3 (t)	32.3 (t)	32.4 (t)	35.5 (t)	36.0 (t)	32.3 (t)	37.0 (t)
15	18.0 (p)	18.0 (p)	18.7 (p)	19.0 (p)	18.4 (p)	18.7 (p)	18.3 (p)
16	18.6 (p)	18.6 (p)	18.1 (p)	18.2 (p)	18.3 (p)	18.0 (p)	18.3 (p)
3-SMe							13.9 (p)
12-SMe							16.4 (p)
1′	167.6 (q)	167.3 (q)				165.2 (q)	165.0 (q)
1′a			136.9 (q)	136.9 (q)	137.0 (q)		
2′			123.4 (t)	122.9 (t)	123.5 (t)		
3′	80.6 (q)	79.2 (q)	113.2 (q)	113.5 (q)	114.0 (q)	73.3 (q)	73.8 (q)
3′a			126.2 (q)	125.7 (q)	125.8 (q)		
4′	165.7 (q)	164.7 (q)	121.5 (t)	120.9 (t)	121.0 (t)	165.2 (q)	165.1 (q)
5′			119.8 (t)	120.0 (t)	120.0 (t)		
5′a	79.4 (t)	81.4 (t)				80.2 (t)	80.5 (t)
6′			122.3 (t)	122.4 (t)	122.5 (t)		
6′a	150.0 (q)	149.2 (q)				150.1 (q)	150.3 (q)
7′	109.7 (t)	109.9 (t)	111.5 (t)	111.6 (t)	111.7 (t)	109.5 (t)	109.6 (t)
8′	130.0 (t)	129.7 (t)[b]				129.8 (t)	129.7 (t)
9′	118.8 (t)	119.2 (t)				118.9 (t)	118.9 (t)
10′	130.4 (t)	130.0 (t)[b]				130.2 (t)	130.0 (t)
10′a	123.4 (q)	122.3				123.7 (q)	123.9 (q)
10′b	65.5 (q)	63.2 (q)				64.9 (q)	66.1 (q)
11′	79.9 (t)	79.2 (t)				79.1 (t)	78.8 (t)
12′	75.8 (q)	75.6 (q)				70.0 (q)	69.6 (q)
13′	28.4 (p)	26.5 (p)				28.4 (p)	28.4 (p)
14′	62.0 (s)	60.5 (s)				64.9 (s)	65.0 (s)
3′-SMe						13.5 (p)	13.4 (p)
12′-SMe						15.4 (p)	15.2 (p)

[a] Letters, p, s, t and q, in parentheses indicate respectively primary, secondary, tertiary and quaternary carbons, assigned by DEPT. [b,c] Assignments interchangeable.

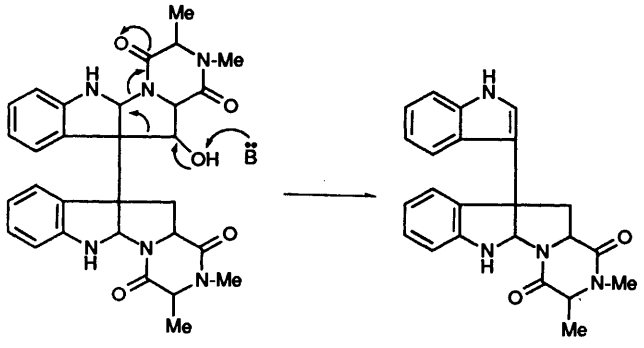
same configuration (*S*) as those of **9**.[10] A relatively weak negative band ($\Delta\varepsilon$ − 17.3) at 233 nm due to the indolinyl chromophore was observed in the tetrakis(methylsulfanyl) derivative **8**, which has no 231 nm band due to disulfide $n\sigma^*$ transitions and exist in the same conformation as that of **3**. This evidence suggests that the contribution of the disulfide $n\sigma^*$ transitions to the 231 nm band ($\Delta\varepsilon$ + 62.6) in the CD spectrum of **3** is more important than that of the indolinyl chromophore and the weak band due to the latter is hidden by overlapping with the strong band attributable to the former in the CD spectrum of **3**. Therefore, it is assumed that the stereochemical difference between **3** and **9** at C-5′a does not appear in their CD spectra. Based on the above evidence, the absolute stereostructures of leptosin C and, consequently, leptosins A and B are represented as **3**, **1** and **2**, respectively.

Leptosin D **4** was assigned the molecular formula $C_{25}H_{24}N_4O_3S_2$ deduced from HRFABMS [*m/z* 493.1366 (MH^+), Δ − 0.2 mmu]. A close inspection of its ^1H and ^{13}C NMR spectra (Tables 2 and 3) revealed that the hydroxymethyl-bearing half of the molecule of **1** was replaced by a 3-substituted indole moiety in **4**. A chemical shift difference of the ^{13}C NMR signals for C-5a, C-10a and C-10b of **4** relative to those of **1** revealed that C-10b was linked at C-3 (namely C-3′ of **4**) of the indole moiety in **4**. In addition, the FABMS of **4** exhibited a fragment ion at *m/z* 429 ($[MH - 2S]^+$), arising from desulfurization of MH^+, together with other fragments at *m/z* 411 ($[MH - 2S - H_2O]^+$), 232 ([bis-indol-3-yl]$^+$), 197 ($[MH - 2S - 232]^+$), 154 ($[197 - isopropyl]^+$) and 136 ($[154 - H_2O]^+$). For the purpose of desulfurization, **2** was treated with triphenylphosphine[7] to afford **4** together with **3**. It has been reported that the tetradesulfanyl-derivative **10** of verticillin A was treated with methanolic potassium hydroxide to give compound **11**.[9] It is considered that a similar reaction took place on treatment of **2** with triphenylphosphine as a nucleophile to give **4**. Formation of **4** from **2** showed the absolute configuration of **4** to be the same as that of **1**–**3**. This was supported by CD spectral comparison of **4** with **1** (Fig. 2). The above-mentioned evidence allowed assignment of stereostructure **4** to leptosin D.

Leptosins E **5** and F **6** were shown to have molecular formulae of $C_{25}H_{24}N_4O_3S_3$ and $C_{25}H_{24}N_4O_3S_4$, respectively, by HRFABMS (MH^+, *m/z* 525.1061, Δ − 2.8 mmu; 557.0828, Δ + 1.8 mmu). The general features of their UV, IR and NMR spectra (Tables 2 and 3) closely resembled those of **4** except that some ^{13}C NMR signals for their dioxopiperazine rings exhibited a chemical shift difference relative to those of **4**. Desulfurization of **6** and **5** with triphenylphosphine afforded **4** and **5**, and **4**, respectively. In addition, the FABMS of both **5** and **6** showed a fragment peak at *m/z* 429 ($[MH - 3S]^+$ or $[MH - 4S]^+$), arising from desulfurization of MH^+, together

Table 4 Cytotoxicity of compounds **1–6** against tumour cells

Compound	Cell line P-388(ED_{50} μg cm^{-3})
Leptosin A **1**	1.85×10^{-3}
Leptosin B **2**	2.40×10^{-3}
Leptosin C **3**	1.75×10^{-3}
Leptosin D **4**	8.60×10^{-2}
Leptosin E **5**	4.60×10^{-2}
Leptosin F **6**	5.60×10^{-2}
Mitomycin C (standard)	4.40×10^{-2}



10 **11**

with the same fragments (*m/z* 411, 232, 197, 154 and 136) as those of **4**. This evidence led stereostructures **5** and **6** for leptosins E and F, respectively.

The cytotoxic activities of compounds **1–6** were examined in the P-388 lymphocytic leukemia test system in cell culture, according to the method reported previously.[11] As shown in Table 4, all the compounds tested exhibited potent cytotoxic activity. Among them, dimeric epipolysulfanyldioxopiperazines **1–3** showed more potent activity than the monomeric epipolysulfanyldioxopiperazines **4–6** with the indole moiety, and the number of sulfur atoms in dioxopiperazine rings were found not to influence the activity.

The antitumour activity of compounds **1** and **3** was also examined against Sarcoma-180 ascites tumour. The ascites cells (about 10^6 cells per mouse) were inoculated intraperitoneally into ICR mice. The test sample was injected intraperitoneally once 24 h after the inoculation of ascitic cells. Prolongation of survival of mice bearing the Sarcoma-180 ascites was evaluated by the ratio of the mean survival time of the treated animal (*T*) to that of control animals (*C*) (*T/C* %). As the result, both compounds **1** and **3** were found to show significant antitumour activity (*T/C* 260 and 293, respectively) at doses of 0.5 mg kg^{-1} and 0.25 mg kg^{-1}, respectively.

The cytotoxicity of **1** and **3** to other tumour cells and the detailed results on their *in vivo* screening will be reported elsewhere.

Experimental

General Procedures.—M.p.s were obtained on a Yanagimoto micromelting point apparatus and are uncorrected. UV spectra were recorded on a Shimadzu spectrophotometer and IR spectra on a Perkin-Elmer FT-IR spectrometer 1720X. Optical rotations were obtained on a JASCO ORD/UV-5 spectropolarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. CD spectra were recorded on a JASCO J-500A spectrometer. NMR spectra were recorded at 27 °C on a Varian XL-300 spectrometer, operating at 300 and 75.4 MHz for ^1H and ^{13}C, respectively, in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. The ^1H–^1H and ^1H–^{13}C COSY spectra were recorded on a Varian XL-300 spectrometer, and the HMBC and NOESY spectra on a Varian UNITY-400 spectrometer with the usual parameters.

FABMS was determined using a VG ZAB-SE mass spectrometer (low resolution) and a JEOL JMS-HX 100/110A mass spectrometer (high resolution) in 3-nitrobenzyl alcohol matrix. Liquid chromatography over silica gel (mesh 230–400) was performed at medium pressure. HPLC was run on a Waters ALC-200 instrument equipped with a differential refractometer (R 401) and Shim-pack PREP-SIL (25 cm × 20 mm i.d.). Analytical TLC was performed on precoated Merck aluminium sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) with CH_2Cl_2–MeOH (97:3), and compounds were viewed under a UV lamp and sprayed with 10% H_2SO_4 followed by heating.

Culturing and Isolation of Metabolites.—A strain of *Leptosphaeria* sp. was initially isolated from the marine alga *Sargassum tortile* C. Agaroh (Sargassaceae), collected in the Tanabe Bay of Japan. The marine alga was homogenized with sterile artificial seawater and applied onto the surface of nutrient agar layered in a Petri dish. Serial transfers of one of the resulting colonies provided a pure strain of *Leptosphaeria* sp. The fungal strain was grown in a liquid medium (20 dm^3) containing 2% glucose, 1% peptone and 0.5% yeast extract in artificial seawater adjusted to pH 7.5 for three weeks at 27 °C. The culture was filtered under suction and the mycelium collected was extracted thrice with MeOH. The combined extracts were evaporated under reduced pressure to give a mixture of crude metabolites, the CH_2Cl_2–MeOH (1:1) soluble fraction (21.5 g) of which exhibited cytotoxicity (ED_{50} < 1 μg cm^{-3}). This fraction was passed through Sephadex LH-20, using CH_2Cl_2–MeOH (1:1) as the eluent. The second fraction (8.4 g) was chromatographed on a silica gel column with a hexane–CH_2Cl_2 gradient as the eluent. The hexane–CH_2Cl_2 (6:4) and (4:6) and CH_2Cl_2 eluates were collected as 4 fractions [Fr. 1 (469 mg), Fr. 2 (78 mg), Fr. 3 (139 mg) and Fr. 4 (117 mg)], 4 fractions [Fr. 5 (965 mg), Fr. 6 (106 mg), Fr. 7 (141 mg) and Fr. 8 (210 mg)] and 2 fractions [Fr. 9 (142 mg) and Fr. 10 (45 mg)], respectively. Fr. 2 and Fr. 9 were purified by HPLC (SIL) using CH_2Cl_2 and MeOH–CH_2Cl_2 (1:99) as the eluents, respectively, to afford **4** (11 mg) and **1** (31 mg), respectively. Fr. 4, Fr. 3 and Fr. 7 afforded **3** (47 mg) and **6** (6 mg), **5** (5 mg) and **2** (80 mg), respectively, after purification by HPLC using acetone–CH_2Cl_2 (2:98) as the eluent.

Leptosin A **1**. This was obtained as a pale yellow powder, m.p. 216–218 °C, $[\alpha]_D$ +237 (*c* 0.49 in $CHCl_3$); λ_{max}(EtOH)/nm 209 (log ε 4.61), 242 (4.19) and 298 (3.83); ν_{max}(KBr)/cm^{-1} 3412 (OH, NH), 1686, 1664 (CON), 1608 and 1593 (Ar C–C); *m/z* (FAB) 805 (2%, MH^+), 677 (18, $MH^+ - 4S$), 659 (3, $MH^+ - 4S - H_2O$), 493 (7, eH^+), 429 (13, $eH^+ - 2S$), 428 (3, a^+), 411 (9, $eH^+ - 2S - H_2O$), 377 (3, bH^+), 312 (15, $b^+ - 2S$), 296 (16), 282 (6, $a^+ - 4S - H_2O$), 232 (100, [bis-indol-3-yl]$^+$), 197 (64, $eH^+ - 2S - 232$) and 185 (29) (Found: MH^+, 805.0740. $C_{32}H_{33}N_6O_7S_6$ requires MH^+, 805.0735); CD λ(*c* 0.93 × 10^{-5} mol dm^{-3} in EtOH)/nm 240 (Δε +46.0), 287 (+13.2) and 321 (−1.62). ^1H and ^{13}C NMR data are listed in Table 1.

Leptosin B **2**. This was obtained as a pale yellow powder, m.p. 210–213 °C, $[\alpha]_D$ +392 (*c* 0.50 in $CHCl_3$); λ_{max}(EtOH)/nm 208 (log ε 4.45), 244 (4.00) and 299 (3.68); ν_{max}(KBr)/cm^{-1} 3394 (OH, NH), 1687, 1666 (CON), 1608 and 1593 (Ar C–C); *m/z* (FAB) 773 (8, MH^+), 677 (8, $MH^+ - 3S$), 659 (2, $MH^+ - 3S - H_2O$), 512 (4), 493 (5, eH^+), 429 (11, $eH^+ - 2S$), 411 (9, $eH^+ - 2S - H_2O$), 396 (3, c^+), 377 (3, bH^+), 312 (13, $bH^+ - 2S$), 296 (19), 282 (6, $c^+ - 3S - H_2O$), 232 (100, [bis-indol-3-yl]$^+$), 197 (63, $eH^+ - 2S - 232$) and 185 (29) (Found: MH^+, 773.1024. $C_{32}H_{33}N_6O_7S_5$ requires MH^+, 773.1014); CD λ(*c* 2.07 × 10^{-5} mol dm^{-3} in EtOH)/nm 234 (Δε +45.3), 286

(+12.1) and 322 (−1.32). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

Leptosin C **3**. This was obtained as a pale yellow powder, m.p. 208–210 °C, $[\alpha]_D$ +237 (*c* 0.36 in $CHCl_3$); λ_{max}(EtOH)/nm 206 (log ε 4.78), 2.40 (4.23) and 301 (3.71); ν_{max}(KBr)/cm^{-1} 3406 (OH, NH), 1685, 1665 (CON), 1610 and 1593 (Ar C–C); *m/z* (FAB) 741 (3%, MH^+), 677 (6, $MH^+ - 2S$), 659 (2, $MH^+ - 2S - H_2O$), 493 (5, eH^+), 429 (11, $eH^+ - 2S$), 411 (8, $eH^+ - 2S - H_2O$), 397 (28), 395 (35), 377 (57, bH^+), 364 (2, d^+), 312 (12, $b^+ - 2S$), 296 (8), 282 (3, $d^+ - 2S - H_2O$), 232 (100, [bis-indol-3-yl]$^+$), 197 (51, $eH^+ - 2S - 232$) and 185 (17) (Found: MH^+, 741.1316. $C_{32}H_{33}N_6O_7S_4$ requires MH^+, 741.1294); CD λ(*c* 2.57 × 10^{-5} mol dm^{-3} in EtOH)/nm 231 (Δε +62.6), 271 (−9.3), 301 (+3.1) and 357 (−0.71). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

Leptosin D **4**. This was obtained as a pale yellow powder, m.p. 190–192 °C, $[\alpha]_D$ +436 (*c* 0.51 in $CHCl_3$); λ_{max}(EtOH)/nm 206 (log ε 4.60), 219 (4.62), 240 (4.06), 272 (3.78), 282 (3.83) and 290 (3.83); ν_{max}(KBr)/cm^{-1} 3404 (OH, NH), 1688, 1665 (CON), 1607 and 1595 (Ar C–C); *m/z* (FAB) 493 (10%, MH^+), 429 (8, $MH^+ - 2S$), 411 (3, $MH^+ - 2S - H_2O$), 307 (19), 289 (10), 232 (100, [bis-indol-3-yl]$^+$), 197 (23, $MH^+ - 2S - 232$), 154 (77, [197-isopropyl]$^+$) and 136 (49, $[154 - H_2O]^+$) (Found: MH^+, 493.1366. $C_{25}H_{25}N_4O_3S_2$ requires MH^+, 493.1368); CD λ(*c* 3.92 × 10^{-5} mol dm^{-3} in EtOH/nm 229 (Δε +41.7), 264 (−1.66), 294 (+8.50) and 367 (−0.70). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

Leptosin E **5**. This was obtained as a pale yellow powder, m.p. 229–231 °C, $[\alpha]_D$ +563 (*c* 0.32 in $CHCl_3$); λ_{max}(EtOH)/nm 206 (log ε 4.63), 218 (4.65), 240 (4.12), 273 (3.83), 282 (3.84) and 291 (3.81); ν_{max}(KBr)/cm^{-1} 3406 (OH, NH), 1676, 1655 (CON) 1608 and 1595 (Ar C–C); *m/z* (FAB) 525 (40%, MH^+), 460 (51, $MH^+ - 2S$), 429 (11, $MH^+ - 3S$), 411 (10, $MH^+ - 3S - H_2O$), 307 (50), 289 (25), 232 (30, [bis-indol-3-yl]$^+$), 197 (8, $MH^+ - 3S - 232$), 154 (99, [197 − isopropyl]$^+$) and 136 (100, $[154 - H_2O]^+$) (Found: MH^+, 525.1061. $C_{25}H_{25}N_4O_3S_3$ requires MH^+, 525.1089); CD λ(*c* 3.85 × 10^{-5} mol dm^{-3} in EtOH)/nm 225 (Δε +29.9), 255 (+17.0) and 305 (+9.0). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

Leptosin F **6**. This was obtained as a pale yellow powder, m.p. 219–221 °C, $[\alpha]_D$ +452 (*c* 0.39 in $CHCl_3$); λ_{max}(EtOH)/nm 206 (log ε 4.66), 216 (4.69), 240 (4.19), 272 (3.88), 281 (3.90) and 290 (3.90); ν_{max}(KBr)/cm^{-1} 3408 (OH, NH), 1677, 1655 (CON), 1609 and 1595 (Ar C–C); *m/z* (FAB) 557 (21%, MH^+), 460 (13, $MH^+ - 3S$), 429 (11, $MH^+ - 4S$), 411 (4, $MH^+ - 4S - H_2O$), 307 (35), 289 (21), 232 (93, [bis-indol-3-yl]$^+$), 197 (31, $MH^+ - 4S - 232$), 154 (100, [197 − isopropyl]$^+$) and 136 (100, $[154 - H_2O]^+$) (Found: MH^+, 557.0828. $C_{25}H_{25}N_4O_3S_4$ requires MH^+, 557.0810); CD λ(*c* 3.24 × 10^{-5} mol dm^{-3} in EtOH)/nm 232 (Δε +24.2), 253 (+13.6), 290 (+11.8) and 344 (−2.11). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

Formation of the Bis(methylsulfanyl) and Tetrakis(methylsulfanyl) Derivatives **7** *and* **8** *from Leptosins A* **1**, *B* **2** *and C* **3**.—Leptosin C **3** (12 mg) was dissolved in a solution (0.26 cm^3) of pyridine and MeOH (5:8). MeI (1 cm^3) and $NaBH_4$ (4.8 mg) were added, and the mixture was stirred for 20 min at room temperature. The reaction mixture was then diluted with water and extracted with diethyl ether. The solvent was evaporated off under reduced pressure, and the residue was chromatographed on a silica gel column with a CH_2Cl_2–MeOH gradient as the eluent. The MeOH–CH_2Cl_2 (1:99) eluate afforded **7** (5.0 mg) and **8** (4.6 mg). **7** was obtained as a pale yellow oil; ν_{max}(KBr)/cm^{-1} 3529 (OH, NH), 1680, 1658 (CON), 1608 and 1593 (Ar C–C); *m/z* (FAB) 771 (20%, MH^+). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3. **8** was obtained as a pale yellow oil; λ_{max}(EtOH)/nm 213 (log ε 4.70), 238 (4.23) and 303 (3.76); ν_{max}(KBr)/cm^{-1} 3527 (OH, NH), 1658, 1641 (CON), 1610 and 1593 (Ar C–C); *m/z* (FAB) 801 (6%, MH^+); CD λ(*c* 1.31 × 10^{-5} mol dm^{-3} in EtOH)/nm 233 (Δε −17.3), 252 (+12.0), 270sh (+3.5) and 296 (−4.6). ^{1}H and ^{13}C NMR data are listed in Tables 2 and 3.

The same reaction with leptosin A **1** (3 mg) and B **2** (3 mg) gave **7** (0.2 mg and 0.8 mg, respectively) and **8** (1.9 mg and 1.1 mg, respectively).

Formation of Leptosins C **3** *and D* **4** *from Leptosin B* **2**.—Triphenylphosphine (10 mg) was added to a $CHCl_3$ solution (5 cm^3) of leptosin B **2** (28 mg), and the reaction mixture was left at room temperature for 2 h. The solvent was evaporated off under reduced pressure, and the residue was purified by silica gel column chromatography using MeOH–CH_2Cl_2 (1:99) to afford leptosins C **3** (2.6 mg) and D **4** (2.2 mg), which were identified by IR, ^{1}H NMR, CD and TLC.

Formation of Leptosins D **4** *and E* **5** *from Leptosin F* **6**.—Using the same procedure as above with leptosin B **2**, leptosin F **6** (14 mg) was treated with triphenylphosphine (5 mg) to yield leptosins D **4** (1.2 mg) and E **5** (1.4 mg), which were identified by ^{1}H NMR, CD and TLC.

Formation of Leptosins D **4** *from Leptosin E* **5**.—Using the same procedure as above, leptosin E **5** (7 mg) was treated with triphenylphosphine (2.5 mg) to yield leptosin D **4** (1.1 mg), which was identified by ^{1}H NMR, CD and TLC.

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