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Leptosins M–N_1, cytotoxic metabolites from a *Leptosphaeria* species separated from a marine alga. Structure determination and biological activities

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Abstract—Leptosins M (**4**), M_1 (**5**) N (**6**) and N_1 (**7**) have been isolated from a strain of *Leptosphaeria* sp. originally separated from the marine alga *Sargassum tortile*. Their absolute stereostructures have been elucidated by spectral analyses and some chemical transformations. Their NMR and NOE spectral analyses revealed that **4**–**7** exist in a single conformer of B type in acetone-d_6. All the compounds exhibited significant cytotoxicity against cultured P388 cells. In addition, leptosin M (**4**) proved to exhibit significant cytotoxicity against human cancer cell lines, and to inhibit specifically two protein kinases, PTK and CaMKIII, and human topoisomerase II.

1. Introduction

Based on the fact that some of the bioactive materials isolated from marine animals have been produced by bacteria, we have chosen to seek new antitumour metabolites from microorganisms inhabiting the marine environment.[1–4] As part of this program, we reported that antitumour and cytotoxic compounds, leptosins A–J and K (**1**)–K_2 (**3**), belonging to a series of epipolysulfanyldioxopiperazines, were produced by a strain of *Leptosphaeria* sp. OUPS-4 isolated from the marine alga *Sargassum tortile* C. AGAROH (Sargassaceae).[5–8] Our continuing search for cytotoxic metabolites from this fungal strain led to the isolation of four new epipolysulfanyldioxopiperazine designated leptosins M (**4**), M_1 (**5**), N (**6**) and N_1 (**7**). All these metabolites exhibited significant cytotoxic activity against the murine P388 lymphocytic leukemia cell line. In addition, leptosin M (**4**) proved to show appreciable cytotoxicity against a disease-oriented panel of 39 human cancer cell lines, and to inhibit specifically two protein kinases and topoisomerase II. We report herein their absolute stereostructures, conformation and biological activities.

2. Results and discussion

The fungal strain was cultured at 27°C for 3 weeks in a liquid medium (90 l) containing 2% glucose, 1% peptone and 0.5% yeast extract in artificial seawater adjusted to pH 7.5 as reported previously.[5] The MeOH extract of the mycelium was purified by bioassay-directed fractionation (cytotoxicities against P388 cell line) employing a combination of Sephadex LH-20 and silica gel column chromatography and reversed phase HPLC to afford leptosins M–N_1 (**4**–**7**).

Leptosin M (**4**) is a pale yellow powder with the molecular formula $C_{33}H_{36}N_6O_8S_4$ as established by HRSIMS. Its IR spectrum contained absorption bands at 3503, 1687, 1658, 1610 and 1599 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 **4** (Table 1) by DEPT and ^1H–^{13}C COSY experiments and comparison with spectral data for the other leptosins revealed the presence of the following functionalities: two hydroxymethines (C-11 and C-11′), two methines (C-5a and C-5′a) each bearing two nitrogen atoms, one methine (C-3′) bearing a nitrogen atom and a carbonyl

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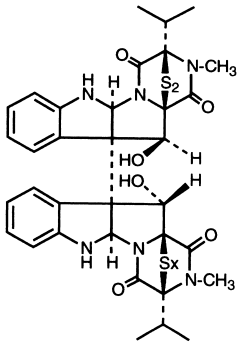

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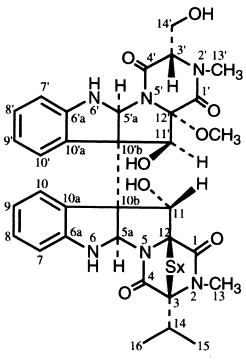

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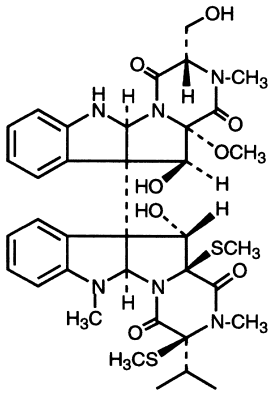

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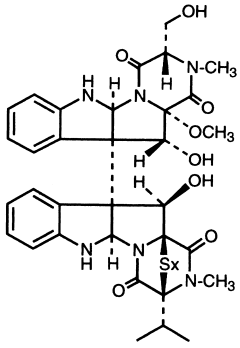

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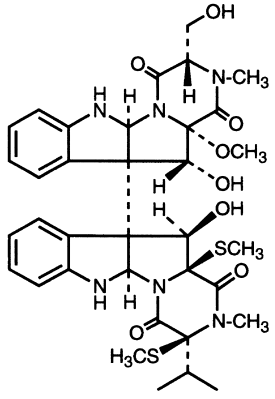

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group, two quaternary sp^3-carbons (C-3 and C-12) each bearing both nitrogen and sulfur atoms, one quaternary sp^3-carbon (C-12′) bearing both nitrogen and oxygen atoms, four amide carbonyls (C-1, C-4, C-1′ and C-4′), two *N*-methyls (C-13 and C-13′), one isopropyl (C-14, C-15 and C-16), one hydroxymethyl (C-14′), two 1,2-disubstituted benzenes (C-6a–10a and C-6′a–C-10′a), each bonding to an amino group as one substituent, two benzylic quaternary sp^3-carbons (10b and 10′b) and one methoxy group. The connection of these functional groups was determined on the basis of ^1H–^1H COSY and HMBC correlations summarized in Fig. 1, and the planar structure of **4** was elucidated. The presence of a tetrasulfide bridge in the isopropyl-bearing dioxopiperazine ring of **4** was supported by the following experiment. Treatment of **4** with $NaBH_4$ and CH_3I gave bis(methylsulfanyl) derivatives **8**, which had the molecular formulae $C_{36}H_{44}N_6O_8S_2$ established by the $[M]^+$ peak in HRSIMS. The ^1H and ^{13}C NMR signals of **8** (Table 2) were similar to those of **4** except for additional signals for one *N*-methyl and two *S*-methyl groups. The positions of the *S*-methyl groups were shown to be C-3 and C-12 by HMBC correlations from H-13, H-15 and 3-SMe to C-3, and from H-5a, H-11 and 12-SMe to C-12. In addition, HMBC correlations from N-Me to C-5a and C-6a implied that the *N*-methyl group is located at N-6.

The relative configuration of **4** was deduced from detailed analysis of NOE data of **4** and **8** in acetone-d_6 (Tables 1 and 2). NOE correlations from H-5′a to H-5a, H-15 and H-14′ observed in the NOESY of **4** implied that H-5a, H-5′a, 3-isopropyl and 3′-hydroxymethyl groups are oriented on the same side, and H-5a and H-5′a are both *cis* to the C-10b–C-10′b bond (Fig. 2). This observation also demonstrated that the hydroxymethyl-bearing dioxopiperazine ring exists in a boat conformation. NOEs from H-10 to H-11 and H-11′ in **4** and **8**, and from H-11 to 12-SMe in **8** implied that H-11, 12-SMe and the C-10a–C-10b bond are oriented *cis* to one another, and H-11′ and the C-10b–C-10′b bond are also arranged *cis*. In other words, H-11 is oriented *trans* to H-5a while H-11′ *cis* to H-5′a. In addition, NOEs from H-11′ to 12′-OMe in **4** and from 12′-OMe to H-11′ and H-14′ in **8** were indicative of the *cis* configuration of 12′-OMe and 3′-hydroxymethyl group. The relative configuration of **4** was thus elucidated.

Previously we found that leptosins K_1 (**2**) and K_2 (**3**) exist in a mixture of two conformers of A and B types, which are formed by rotation of two monomeric subunits about the C-10b–C-10′b bond, in $CDCl_3$, while leptosin K (**1**) exists in a single conformer of an A type (Fig. 3).[8] These conformers can be deduced from a combination of chemical shifts of H-10 and H-10′, and NOE data between H-5a, H-10, H-11, H-5′, H-10′ and H-11′. In compound **4**, NOEs were observed between H-10 and H-11′, and H-5a and H-5′a in acetone-d_6 as mentioned earlier, and the signals for H-10 and H-10′ appeared at lower (δ 7.72) and higher (δ 5.86) fields, respectively. This evidence demonstrated that **4** exists in a B type of conformer in acetone-d_6 (Fig. 3).

In order to determine the absolute configuration of **4**,

Table 1. NMR spectral data of leptosin M (**4**) in acetone-d_6

Position	δ_H[a]	J (Hz)	^1H–^1H COSY	NOESY	δ_C	HMBC (C)[b]
1					167.81 (q)[c]	
3					82.95 (q)	
4					166.9 (q)	
5a	5.94 d	1.8 (6)	6	6, 15, 16, 5′a	80.74 (t)	4, 6a, 10a, 10b, 11, 12, 10′b
6	5.60 s		5a	5a		5a, 6a, 10a, 10b
6a					152.37 (q)	
7	6.28 d	7.8 (8)	8	8	108.42 (t)	6a, 9, 10a
8	7.02 t	7.8 (7, 9)	7, 9	7, 9	130.24 (t)	6a, 10
9	6.68 t	7.8 (8, 10)	8, 10	8, 10	118.07 (t)	7, 8, 10, 10a
10	7.72 d	7.8 (9)	9	9, 11, 10′, 11′	127.73 (t)	6a, 8, 10b
10a					129.11 (q)	
10b					64.25 (q)	
11	5.41 d	4.4 (11-OH)	11-OH	10, 12′-OCH_3	79.72 (t)	1, 5a, 10a
12					81.39 (q)	
13	3.00 s			14, 15	30.41 (p)	1, 3
14	2.78 heptet	6.6 (15, 16)	15, 16	13, 15, 16	36.41 (t)	3, 4, 15, 16
15	1.21 d	6.6 (14)	14	5a, 13, 14, 5′a	18.79 (p)	3, 14, 16
16	1.42 d	6.6 (14)	14	5a, 14, 5′a	18.73 (p)	3, 14, 15
11-OH	5.66 br d	4.4 (11)	11			11, 10b
1′					165.08 (q)	
3′	4.05 t	2.4 (14′)	14′	13′	66.05 (t)	
4′					166.59 (q)	
5′a	6.24 d	1.4 (6′)	6′	5a, 15, 16, 6′, 14′	78.44 (t)	10b, 4′, 6′a, 10′a, 10′b, 11′, 12′
6′	6.03 d	1.4 (5′a)	5′a	5′a		5′a, 6′a, 10′a, 10′b
6′a					151.96 (q)	
7′	6.54 d	7.6 (8′)	8′	8′	108.59 (t)	9′, 10′a
8′	6.9 t	7.6 (7′, 9′)	7′, 9′	7′, 9′	129.0 (t)	6′a, 7′, 10′
9′	6.15 t	7.6 (8′, 10′)	8′, 10′	8′, 10′	117.03 (t)	7′, 8′, 10′a
10′	5.86 d	7.6 (9′)	9′	10, 9′	128.85 (t)	6′a, 8′, 10′a, 10′b
10′a					125.85 (q)	
10′b					61.37 (q)	
11′	5.36 s			10	84.41 (t)	10b, 1′, 5′a, 10′a, 10′b
12′					89.82 (q)	
13′	2.91 s			3′, 14′	32.60 (p)	1′, 3′
14′	4.03 d	2.4 (3′)	3′	5′a, 13′	62.03 (s)	3′, 4′
11′-OH	4.48					
12′-OCH_3	2.98 s			11	51.80 (p)	12′
14′-OH	3.32 br s					

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

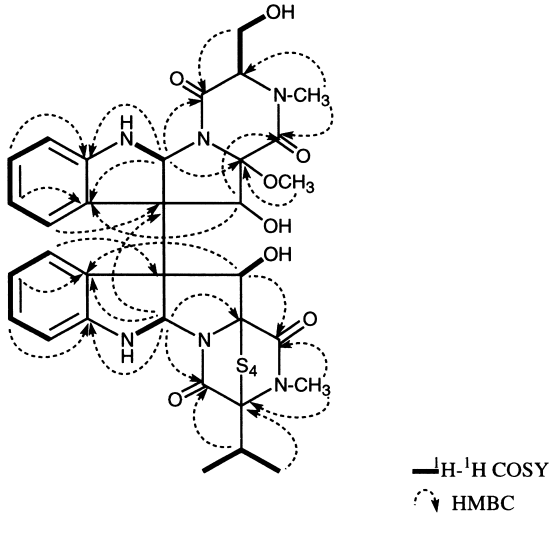

Figure 1. Selected ^1H–^1H COSY and HMBC correlations in leptosin M (**4**).

Marfey's analysis[9] was applied to *N*-methylserine produced by the acid hydrolysis of **4**. The result revealed that the *N*-methylserine moiety of **4** has the L-(*S*)-configuration. The above-summarized evidence led to absolute stereostructure **4** for leptosin M.

Leptosin M_1 (**5**) had a molecular formula two sulfurs less than that of **4** as deduced from HRSIMS. The general features of its UV, IR and NMR spectra (Table 3) closely resembled those of **4** except that some ^{13}C NMR signals (C-4, C-12, C-10b, C-14 and C-10′b) for the isopropyl-bearing dioxopiperazine ring exhibited a chemical shift difference relative to those of **4**. Treatment of **5** with $NaBH_4$ and CH_3I afforded a bis(methylsulfanyl) derivative which was identical with **8** as judged by comparison of spectral data and specific optical rotations. The absolute stereostructures of **5** was elucidated on the basis of this evidence and the molecular formula of **5**. The same conformational analysis as above-mentioned with **4** demonstrated that **5** exists in a B type of conformer in acetone-d_6 as in **4**.

Leptosin N (**6**) had the same molecular formula as **4** as deduced from HRSIMS. The general features of its spectral

Table 2. NMR spectral data of bis(methylsulfanyl) derivative **8** in $CDCl_3$

Position	δ_H[a]	J (Hz)	^1H–^1H COSY	NOESY	δ_C	HMBC (C)[b]
1					166.06 (q)[c]	
3					79.12 (q)	
4					164.47 (q)	
5a	5.56 s			15, 16, 5′a, 6-CH_3	86.15 (t)	4, 6a, 10a, 10b, 11, 12, 10′b
6a					153.34 (q)	
7	6.21 d	8.0 (8)	8	8	106.94 (t)	8, 9
8	7.20 t	8.0 (7, 9)	7, 9	7, 9	129.79 (t)	6a, 10
9	6.75 t	8.0 (8, 10)	8, 10	8, 10	117.24 (t)	7, 10a
10	7.68 d	8.0 (9)	9	9, 11, 11′	125.10 (t)	6a, 10a
10a					128.87 (q)	
10b					60.73 (q)	
11	5.16 d	4.4 (11-OH)	11-OH	10, 12-SCH_3, 12′-OCH_3	80.81 (t)	5a, 10a
12					72.79 (q)	
13	3.06 s			14, 15	29.99 (p)	1, 3
14	2.49 heptet	6.8 (15, 16)	15, 16	13, 15, 16	37.20 (t)	3, 4, 15, 16
15	1.09 d	6.8 (14)	14	5a, 13, 14, 5′a	18.22 (p)	3, 14, 16
16	1.29 d	6.8 (14)	14	5a, 14, 5′a	18.29 (p)	3, 14, 15
11-OH	3.34 br s		11			
6-CH_3	2.50			5a	33.28 (p)	6a, 5a
3-SCH_3	2.12				13.88 (p)	3
12-SCH_3	1.95			11	16.24 (p)	12
1′					164.70 (q)	
3′	3.98 t	1.8 (14′A, 14′B)	14′A, 14′B	13′, 14′B	65.27 (t)	1′, 4′, 14′
4′					166.30 (q)	
5′a	6.06 s			5a, 15, 16, 6′	77.22 (t)	10b, 4′, 6′a, 10′a, 10′b, 11′, 12′
6′	5.50 br s			5′a		
6′a					149.12 (q)	
7′	6.54 d	7.3 (8′)	8′	8′	108.29 (t)	9′, 10′a
8′	6.97 t	7.3 (7′, 9′)	7′, 9′	7′, 9′	129.11 (t)	6′a, 10′
9′	6.29 t	7.3 (8′, 10′)	8′ 10′	8′, 10′	117.69 (t)	7′, 10′a
10′	5.69 d	7.3 (9′)	9′	9′	128.12 (t)	6′a, 8′
10′a					123.91 (q)	
10′b					62.21 (q)	
11′	5.24 d	8.7 (11-OH′)	11′-OH	10	78.83 (t)	10′b
12′					88.74 (q)	
13′	3.09 s			3′, 14′A	32.97 (p)	1′, 3′
14′A	3.99 dd	11.7 (14′B), 1.8 (3′)	3′, 14′-OH	13′, 14′B, 12′-OCH_3	61.96 (s)	1′
14′B	4.08 dd	11.7 (14′A), 1.8 (3′)	3′	3′, 14′A		
11′-OH	3.02 br d	8.7 (11′)	11′			
12′-OCH_3	2.97 s			11, 14′	52.1 (p)	12′
14′-OH	2.38 br s		14A			

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

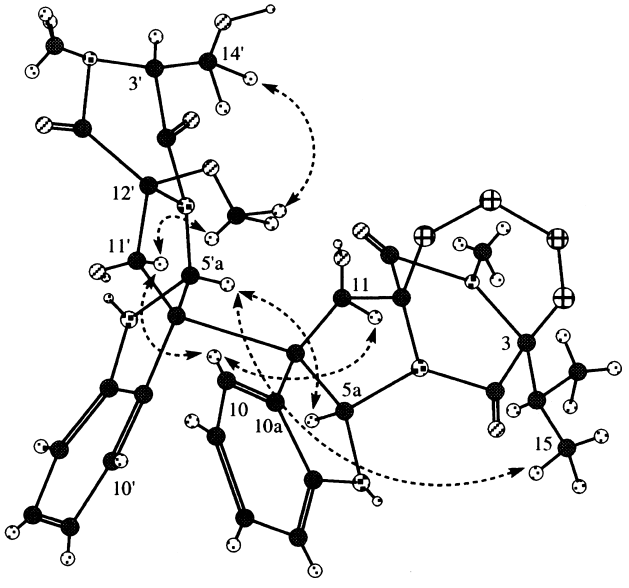

Figure 2. Observed NOEs for leptosin M (**4**).

data (Table 4) closely resembled those of **4** except that the NMR signals for H-5a, H-11, H-5′a, H-6′, H-11′, C-10, C-10a, C-10b, C-4′, C-10′, C-10′a and C-14′ in **6** revealed a chemical shift difference relative to those of **4**, implying that **6** is a stereoisomer of **4**. Treatment of **6** with $NaBH_4$ and CH_3I gave bis(methylsulfanyl) derivatives **9**, which had two *S*-methyl groups but no additional *N*-methyl group. HMBC correlations (from H-13, H-15 and 3-SMe to C-3, and from H-5a, H-11 and 12-SMe to C-12) in **9** demonstrated that a tetrasulfide bridge in **6** exists between C-3 and C-12 of the isopropyl-bearing dioxopiperazine ring as in **4**. In detailed NOESY analysis of **6**, NOE correlations regarding H-11 and H-11′ were found to be different from those of **4**, indicating that they are useful for the elucidation of the stereochemistry of **6**. NOEs from H-10′ to H-11′, and from H-10 to 11-OH and 11′-OH were observed in **6** though not in **4**, suggesting that both C-11 and C-11′ in **6** have opposite configurations of those of **4**. This was supported by the observation of NOEs from H-11 to H-5a, H-5′a and 12′-OMe in **6**. NOEs from 12′-OMe to H-5′a and H-14′

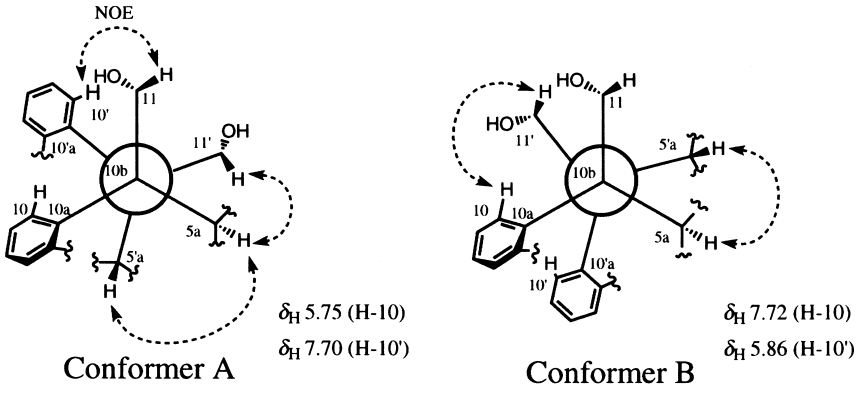

Figure 3. Respective conformers A and B in leptosins K (**1**) and M (**4**).

implied that H-5′a, 12′-OMe and 3′-hydroxymethyl group are oriented *cis* to one another, and the hydroxymethyl-bearing dioxopiperazine ring exists in a boat conformation. In addition, NOE correlations from H-5′a to H-5a and H-15 implied that H-5a, H-5′a and 3-isopropyl group are oriented on the same side, and H-5a and H-5′a are both *cis* to the C-10b–C-10′b bond. This stereochemistry for **6** was supported by NOEs observed in **9**. The above-summarized evidence indicated that **6** is a stereoisomer of **4** at C-11 and C-11′. Application of Marfey's analysis for **6** revealed that the *N*-methylserine moiety of **6** has the L-(*S*)-configuration as in **4**. Thus the absolute stereostructure **6** for leptosin N was elucidated. The signals for H-10 and H-10′ in **6** appeared at lower (δ 7.87) and higher (δ 5.52) fields,

Table 3. NMR spectral data of leptosin M_1 (**5**) in acetone-d_6

Position	δ_H[a]	*J* (Hz)	^1H–^1H COSY	NOESY	δ_C	HMBC (C)[b]
1					166.90 (q)[c]	
3					81.84 (q)	
4					161.21 (q)	
5a	5.79 s		6	6, 15, 16, 5′a	81.34 (t)	6a, 10a, 10b, 12
6	5.56 s		5a	5a		10a
6a					151.79 (q)	
7	6.40 d	7.8 (8)	8	8	109.74 (t)	9, 10a
8	7.10 t	7.8 (7, 9)	7, 8	7, 9	130.31 (t)	6a, 10
9	6.78 t	7.8 (8, 10)	8, 10	8, 10	118.47 (t)	7, 10a
10	7.74 d	7.8 (9)	9	9, 11, 11′	127.59 (t)	8
10 a					128.95 (q)	
10 b					60.46 (q)	
11	5.39 d	3.2 (11-OH)	11-OH	10, 11-OH, 11′, 12′-OCH_3	79.81 (t)	5a, 10a
12					77.19 (q)	
13	3.03 s			14	29.21 (p)	1, 3
14	2.78 heptet	6.6 (15, 16)	15, 16	13, 15, 16	32.98 (t)	3, 4, 15, 16
15	1.37 d	6.6 (14)	14	5a, 14	18.23 (p)	3, 14, 16
16	1.39 d	6.6 (14)	14	5a, 14	18.70 (p)	3, 14, 15
11-OH	5.59 br s		11	11		
1′					164.92 (q)	
3′	4.08 t	2.4 (14′)	14′	13′, 14′	66.30 (t)	
4′					167.78 (q)	
5′a	6.32 s		6′	5a, 6′, 11′	78.60 (t)	10′b, 12′
6′	5.91 s		5′a	5′a		6′a
6′a					151.77 (q)	
7′	6.52 d	7.7 (8′)	8′	8′	108.59 (t)	9′, 10′a
8′	6.88 t	7.7 (7′, 9′)	7′, 9′	7′, 9′	128.97 (t)	6′a
9′	6.13 t	7.7 (8′, 10′)	8′ 10′	8′, 10′	117.33 (t)	7′, 10′a
10′	5.87 d	7.7 (9′)	9′	10, 9′	128.68 (t)	6′a, 8′, 10′a
10′a					126.06 (q)	
10′b					65.55 (q)	
11′	5.27 d	5.5 (11′-OH)	11′-OH	10, 11, 5′a, 11′-OH	83.30 (t)	10b, 1′, 10′a, 10′b
12′					89.74 (q)	
13′	3.05 s			3′, 14′	32.60 (p)	1′, 3′
14′	4.03 d	2.4 (3′)	3′	3′, 13′, 12′-OCH_3, 14′-OH	62.57 (s)	
11′-OH	3.31 d	5.5 (11′)	11′	11′		
12′-OCH_3	2.95 s			14′	51.84 (p)	12′
14′-OH	4.34 br s		14	14′		

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

Table 4. NMR spectral data of leptosin N (**6**) in acetone-d_6

Position	δ_H[a]	J (Hz)	^1H–^1H COSY	NOESY	δ_C	HMBC (C)[b]
1					167.61 (q)[c]	
3					82.50 (q)	
4					167.59 (q)	
5a	5.25 d	2.3 (6)	6	11, 14	79.07 (t)	6a, 10a, 10b, 10′b
6	4.21 br s		5a			
6a					152.40 (q)	
7	6.46 d	7.8 (8)	8	8	109.51 (t)	9, 10a
8	7.09 t	7.8 (7, 9)	7, 8	7, 9	129.47 (t)	6a, 10
9	6.75 t	7.8 (8, 10)	8, 10	8	118.13 (t)	7, 10a
10	7.87 d	7.8 (9)	9	11-OH, 11′, 11′-OH	131.00 (t)	6a, 8, 10b
10 a					125.94 (q)	
10 b					66.77 (q)	
11	6.58 s		11-OH	5a, 11-OH, 5′a, 12′-OCH_3	80.42 (t)	10b, 12
12					81.65 (q)	
13	3.01 s			14, 15	30.32 (p)	1, 3
14	2.82 heptet	6.8 (15, 16)	15, 16	5a, 13, 15, 16	36.48 (t)	3, 4, 15, 16
15	1.16 d	6.8 (14)	14	13, 14, 16, 5′a, 12′-OCH_3	18.80 (p)	3, 14, 16
16	1.42 d	6.8 (14)	14	14, 15	18.80 (p)	3, 14, 15
11-OH	6.14 br s		11	11		
1′					164.40 (q)	
3′	4.04 t	2.5 (14′)	14′	13′	67.05 (t)	1′, 4′
4′					169.05 (q)	
5′a	5.24 d	2.3 (6′)	6′	11, 15, 6′, 11′-OH, 12′-OCH_3	77.66 (t)	10′b, 12′
6′	5.39 d	2.3 (5′a)	5′a	5′a		6′a, 10′a, 10′b
6′a					151.45 (q)	
7′	6.54 d	7.6 (8′)	8′	8′	108.84 (t)	6′a, 9′, 10′a
8′	6.90 t	7.6 (7′, 9′)	7′, 9′	7′, 9′	130.22 (t)	6′a, 10′
9′	6.20 t	7.6 (8′, 10′)	8′ 10′	8′, 10′	118.92 (t)	7′, 10′a
10′	5.52 d	7.6 (9′)	9′	9′, 11′, 11′-OH	126.31 (t)	6′a, 8
10′a					129.47 (q)	
10′b					62.73 (q)	
11′	4.80 d	4.5 (11′-OH)	11′-OH	10, 10′, 11-OH	83.90 (t)	
12′					91.84 (q)	
13′	3.05 s			3′, 14′	33.20 (p)	1′, 3′
14′	4.01 d	2.5 (3′)	3′	5′a, 13′, 12′-OCH_3	64.21 (s)	3′, 4′
11′-OH	5.80 br s		11′	10, 5′a		10′b
12′-OCH_3	3.45 s			11, 15, 5′a, 14′	52.99 (p)	12′
14′-OH	4.33 br s					

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

respectively. In addition, a further NOE from H-10 to H-11′ as well as an NOE between H-11 and H-5′a showed that **6** exists in a B type of conformer in acetone-d_6.

Leptosin N_1 (**7**) was assigned a molecular formula which contained one sulfur atom less than that of **6** as deduced from HRSIMS. Its UV, IR and NMR spectra (Table 5) revealed similarities with those of **6** except that some ^{13}C NMR signals (C-1, C-3, C-4, C-10a, C-13 and C-10′a) mainly for the isopropyl-bearing dioxopiperazine ring exhibited a chemical shift difference relative to those of **6**. Treatment of **7** with $NaBH_4$ and CH_3I afforded a bis(methylsulfanyl) derivative which was identical with **9** as judged by comparison of spectral data and specific optical rotations. This evidence and the molecular formula of **7** led to absolute stereostructure **7** for leptosin N_1.

The cancer cell growth inhibitory properties of leptosins M–N_1 (**4**–**7**) were examined using the murine P388 lymphocytic leukemia cell line and a disease-oriented panel of 39 human cancer cell lines (HCC panel) in the Japanese Foundation for Cancer Research.[10] All of these metabolites (**4**–**7**) exhibited significant cytotoxic activity against the murine P388 cell line (Table 6), and the activities of **6** and **7** were almost 10-fold potent than those of **4** and **5**. In addition, leptosin M (**4**) showed appreciable cytotoxic activities against the 39 human cancer cell lines (Table 7). As shown in Table 7, the delta and range values of **4** were 0.54 and 1.18, respectively (effective value: delta$\geq$0.5 as well as range$\geq$1.0), disclosing that this compound showed selective cytotoxic activity. Furthermore, evaluation of the pattern of differential cytotoxicity using the COMPARE program[10] suggested the possibility that the mode of action for **4** might be different from that shown by any other anticancer drug developed to date.

Moreover, inhibitory activities of **4** against five protein kinases[11–13] and human topoisomerases were examined. Compound **4** inhibited two protein kinases PTK and CaMKIII at a concentration of 10 $\mu g\ ml^{-1}$ by 40–70% whereas showed no inhibition against other protein kinases PKA, PKC and EGFR at extreme doses such as 100 $\mu g\ ml^{-1}$. On the other hand, compound **4** was demonstrated to inhibit specifically topoisomerase II with IC_{50} value of 59.1 μM without affecting topoisomerase I ($IC_{50}>300\ \mu M$).

Table 5. NMR spectral data of leptosin N_1 (**7**) in acetone-d_6

Position	δ_H[a]	J (Hz)	^1H–^1H COSY	NOESY	δ_C	HMBC (C)[b]
1					171.47 (q)[c]	
3					85.20 (q)	
4					163.29 (q)	
5a	5.18 d	2.8 (6)	6	6, 11, 15, 5′a	79.07 (t)	4, 6a, 10
6	4.18 d	2.8 (5a)	5a	5a		6a, 10a, 10b
6a					153.50 (q)	
7	6.50 d	7.8 (8)	8	8	109.79 (t)	6a, 9, 10a
8	7.17 t	7.8 (7, 9)	7, 8	7, 9	130.02 (t)	6a, 10
9	6.89 t	7.8 (8, 10)	8, 10	8, 10	118.76 (t)	7, 10a
10	7.72 d	7.8 (9)	9	10′, 11′	129.92 (t)	6a, 8, 10a, 10b
10 a					123.76 (q)	
10 b					65.88 (q)	
11	6.77 d	1.8 (11-OH)	11-OH	5a, 15, 12′-OCH_3	79.38 (t)	10b, 12, 10′b
12					80.25 (q)	
13	3.21 s			14, 15	27.28 (p)	1, 3
14	2.62 heptet	6.8 (15, 16)	15, 16	13, 15, 16	36.09 (t)	3, 4, 15, 16
15	1.21 d	6.8 (14)	14	5a, 11, 13, 14, 16, 12′-OCH_3	18.72 (p)	3, 14, 16
16	1.45 d	6.8 (14)	14	14, 15	18.52 (p)	3, 14, 15
11-OH	5.80 d	1.8 (11)	11			
1′					164.27 (q)	
3′	4.03 t	2.5 (14′)	14′	13′	66.79 (t)	
4′					169.86 (q)	
5′a	5.27 d	2.5 (6′)	6′	5a, 14′, 12′-OCH_3	77.46 (t)	6′a, 10′a, 12′
6′	5.41 br s		5′a			6′a
6′a					152.18 (q)	
7′	6.52 d	7.6 (8′)	8′	8′	110.02 (t)	9′, 10′a
8′	6.89 t	7.6 (7′, 9′)	7′, 9′	7′, 9′	129.56 (t)	6′a, 7′, 10′
9′	6.22 t	7.6 (8′, 10′)	8′ 10′	8′, 10′	118.12 (t)	7′, 10′a
10′	5.54 d	7.6 (9′)	9′	10, 11′	125.48 (t)	6′a, 8′, 10′b
10′a					124.52 (q)	
10′b					61.23 (q)	
11′	4.80 d	4.8 (11′-OH)	11′-OH	10, 10′	82.71 (t)	10b, 10′b, 12′
12′					92.21 (q)	
13′	3.05 s			3′, 14′	33.18 (p)	1′, 3′
14′	4.01 d	2.5 (3′)	3′, 14′-OH	5′a, 13′, 12′-OCH_3	63.86 (s)	3′, 4′
11′-OH	5.89 br s		11′			
12′-OCH_3	3.43 s			11, 15, 5′a, 14′	52.47 (p)	12′
14′-OH	4.32 br s		14′			

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

Table 6. Cytotoxity of leptosins M–N_1 (**4**–**7**) against P388 cells (DMSO was used for vehicle)

Compound	ED_{50} ($\mu g\ ml^{-1}$)
Leptosin M (**4**)	1.05
Leptosin M_1 (**5**)	1.40
Leptosin N (**6**)	0.18
Leptosin N_1 (**7**)	0.19
5-FU (standard)	5.80×10^{-2}

3. Experimental

3.1. General procedures

Melting points were determined on a Yanagimoto micro melting 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. NMR spectra were recorded at 27°C on a Varian UNITY INOVA-500 spectrometer, operating at 500 and 125 MHz for ^1H and ^{13}C, respectively, with tetramethylsilane (TMS) as an internal reference. EIMS was determined using a Hitachi M-4000H mass spectrometer. Liquid chromatography over silica gel (230–400 mesh) was performed in a medium pressure. Preparative 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 HPLC was run on a Waters 484 instrument equipped with a photodiode array detector (900J) and Cosmosil 5C_{18}-MS (15 cm×4.6 mm i.d.). Analytical TLC was performed on precoated Merck aluminum sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) with the solvent system CH_2Cl_2–MeOH (19:1), and compounds were viewed under UV lamp and sprayed with 10% H_2SO_4 followed by heating.

3.2. Culturing and isolation of metabolites

As reported previously,[5] a strain of *Leptosphaeria* sp. OUPS-N80 was isolated from the marine alga *S. tortile*. The fungal strain was grown in a liquid medium (90 ml) containing 2% glucose, 1% peptone and 0.5% yeast extract in artificial seawater adjusted to pH 7.5 for 4 weeks at 27°C. The MeOH extract (52.3 g) of the mycelium was dissolved

Table 7. Cytotoxicity of leptosin M (**4**) against a panel of 39 human cancer cell lines

Origin of cancer	Cell line	Log GI_{50} (M)[a]
Breast	HBC-4	−5.55
	BSY-1	−5.76
	HBC-5	−5.62
	MCF-7	−5.29
	MDA-MB-231	−5.17
Central nervous system	U-251	−5.62
	SF-268	−4.96
	SF-295	−4.79
	SF-539	−5.59
	SNB-75	−5.64
	SNB-78	−5.59
Colon	HCC2998	−5.36
	KM-12	−5.21
	HT-29	−5.14
	HCT-15	−4.75
	HCT-116	−5.57
Lung	NCI-H23	−5.11
	NCI-H226	−5.61
	NCI-H522	−5.79
	NCI-H460	−5.09
	A549	−4.67
	DMS273	−5.55
	DMS114	−5.59
Melanoma	LOX-IMVI	−5.37
Ovary	OVCAR-3	−5.44
	OVCAR-4	−4.77
	OVCAR-5	−5.03
	OVCAR-8	−5.44
	SK-OV-3	−4.61
Kidney	RXF-631L	−4.80
	ACHN	−4.73
Stomach	St-4	−4.72
	MKN1	−5.65
	MKN7	−5.45
	MKN28	−5.41
	MKN45	−4.82
	MKN74	−5.76
Prostate	DU-145	−4.76
	PC-3	−4.87
MG-MID[b]		−5.25
Delta[c]		0.54
Range[d]		1.18

[a] Log concentration of compound (**4**) 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 MG-MID value.
[d] The difference in log GI_{50} value of the most sensitive cell and the least sensitive cell.

in CH_2Cl_2–MeOH (1:1), and the soluble fraction (32.7 g) was passed through Sephadex LH-20, using CH_2Cl_2–MeOH (1:1) as the eluent. The second fraction (12.8 g), in which the activity was concentrated, was chromatographed on a silica gel column with a hexane–CH_2Cl_2–MeOH gradient as the eluent. The MeOH–CH_2Cl_2 (2:98) eluate (211.7 mg) was further purified by HPLC using MeOH–H_2O (8:2) as the eluent to afford **6** (11.7 mg, 0.02%) and **7** (12.1 mg, 0.02%). The MeOH–CH_2Cl_2 (5:95) eluate (426.6 mg) afforded **4** (50.8 mg, 0.1%) and **5** (25.9 mg, 0.05%) after purification by HPLC using MeOH–H_2O (7:3) as the eluent.

3.2.1. Leptosin M (4). Obtained as a pale yellow powder, mp 223–226°C, $[\alpha]_D$=+478° (*c* 0.10, $CHCl_3$); λ_{max} (EtOH)/nm: 304 (log ε 3.62), 243 (4.08) and 206 (4.64); ν_{max} (KBr)/cm^{-1}: 3503 (NH, OH), 1687, 1658 (amide), 1610 and 1599 (Ar); *m/z* (SIMS): 773 ($[M+H]^+$, 0.3%), 645 (MH^+-4S, 0.5), 627 (MH^+-4S-H_2O, 1.8), 557 (0.8), 429 (4.5), 312 (17.3) and 232 (bis-indol-3-yl, 100); *m/z* (HRSIMS): 773.1553 $[M+H]^+$ (Calcd for $C_{33}H_{37}N_6O_8S_4$: 773.1550); CD λ (*c* 1.63×10^{-5} M in EtOH)/nm: 390 ($\Delta\varepsilon$ 0), 345 (−5.95), 323 (0), 270 (+20.45), 258 (0), 250 (−19.52), 244 (0), 234 (+54.84) and 222 (0). ^1H and ^{13}C NMR data are listed in Table 1.

3.2.2. Leptosin M_1 (5). Obtained as a pale yellow powder, mp 219–222°C, $[\alpha]_D$=+140° (*c* 0.18, $CHCl_3$); λ_{max} (EtOH)/nm: 300 (log ε 3.83), 238 (4.30) and 212 (4.73); ν_{max} (KBr)/cm^{-1}: 3528 (NH, OH), 1674, 1658 (amide), 1609 and 1594 (Ar); *m/z* (SIMS): 731 ($[M+Na]^+$, 2.0%), 667 ($M^++Na-2S$, 0.5), 627 ($M^++Na-2S-H_2O$, 1.2), 493 (4.6), 429 (6.4), 312 (19.5) and 232 (bis-indol-3-yl, 100); *m/z* (HRSIMS): 731.1936 $[M+Na]^+$ (Calcd for $C_{33}H_{36}N_6O_8S_2Na$: 731.1931); CD λ (*c* 2.51×10^{-5} M in EtOH)/nm: 380 ($\Delta\varepsilon$ 0), 358 (−0.36), 337 (0), 304 (+3.92), 281 (0), 252 (−5.07), 246 (0) and 229 (+51.31). ^1H and ^{13}C NMR data are listed in Table 3.

3.2.3. Leptosin N (6). Obtained as a pale yellow powder, mp 226–228°C, $[\alpha]_D$=+276° (*c* 0.16, $CHCl_3$); λ_{max} (EtOH)/nm: 302 (log ε 3.86), 240 (4.36) and 212 (4.75); ν_{max} (KBr)/cm^{-1}: 3524 (NH, OH), 1688, 1658 (amide), 1610 and 1598 (Ar.); *m/z* (SIMS): 773 ($[M+H]^+$, 0.9%), 645 (MH^+-4S, 2.4), 627 (MH^+-4S-H_2O, 6.5), 557 (0.6), 429 (6.1), 312 (16.8) and 232 (bis-indol-3-yl, 100); *m/z* (HRSIMS): 773.1550 $[M+H]^+$ (Calcd for $C_{33}H_{37}N_6O_8S_4$: 773.1553); CD λ (*c* 2.11×10^{-5} M in EtOH)/nm: 387 ($\Delta\varepsilon$ 0), 343 (−2.44), 318 (0), 304 (−0.72), 299 (0), 252 (−5.07) and 229 (+31.60). ^1H and ^{13}C NMR data are listed in Table 4.

3.2.4. Leptosin N_1 (7). Obtained as a pale yellow powder, mp 227–229°C, $[\alpha]_D$=+347° (*c* 0.14, $CHCl_3$); λ_{max} (EtOH)/nm: 302 (log ε 3.82), 240 (4.34) and 212 (4.74); ν_{max} (KBr)/cm^{-1}: 3528 (NH, OH), 1688, 1658 (amide), 1609 and 1597 (Ar); *m/z* (SIMS): 741 ($[M+H]^+$, 3.2%), 645 (MH^+-3S, 7.2), 627 (MH^+-3S-H_2O, 15.6), 525 (0.8), 429 (5.8), 312 (18.7) and 232 (bis-indol-3-yl, 100); *m/z* (HRSIMS): 741.1832 $[M+H]^+$ (Calcd for $C_{33}H_{37}N_6O_8S_3$: 741.1834]; CD λ (*c* 1.95×10^{-5} M in EtOH)/nm: 371 ($\Delta\varepsilon$ 0), 321 (+7.77), 308 (0), 294 (−6.85), 283 (0) and 223 (+40.25). ^1H and ^{13}C NMR data are listed in Table 5.

3.3. Formation of bis(methylsulfanyl) derivatives

3.3.1. The bis(methylsulfanyl) derivative 8 from leptosins M (4) and M_1 (5). Leptosin M (**4**)(10.3 mg) was dissolved in a solution (0.5 ml) of pyridine and MeOH (5:8). To the solution were added MeI (1.5 ml) and $NaBH_4$ (5.0 mg), 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 ether layer was evaporated off under reduced pressure, and the residue was purified by HPLC using MeOH–H_2O (8:2) as the eluent to afford **8** (6.5 mg) as a pale yellow powder; mp 203–206°C, $[\alpha]_D$=+15° (*c* 0.16, $CHCl_3$); λ_{max} (EtOH)/nm: 305

(log ε 3.56), 248 (4.12) and 211 (4.78); ν_{max} (KBr)/cm^{-1}: 3510 (NH, OH), 1682, 1659 (amide), 1610 and 1598 (Ar); *m/z* (SIMS): 752 $[M]^+$; *m/z* (HRSIMS): 752.2259 $[M]^+$ (Calcd for $C_{36}H_{44}N_6O_8S_2$: 752.2260); CD λ (*c* 2.12×10^{-5} M in EtOH)/nm: 338 ($\Delta\varepsilon$ 0), 320 (+7.11), 272 (0), 255 (−50.37), 242 (0) and 225 (+21.73). ^{1}H and ^{13}C NMR data are listed in Table 2.

The same reaction with leptosin M_1 (**5**) (6.6 mg) gave **8** (3.9 mg).

3.3.2. The bis(methylsulfanyl) derivative 9 from leptosins N (6) and N_1 (7). Using the same procedure as above with compound **4**, leptosins N (**6**) (8.6 mg) was treated with MeI (1.2 ml) and $NaBH_4$ (4.0 mg) in pyridine and MeOH (5:8) (0.4 ml) and the product was purified by HPLC [MeOH–H_2O (8:2)] to afford **9** (4.7 mg) as a pale yellow powder; mp 196–198°C, $[\alpha]_D$=+46° (*c* 0.18, $CHCl_3$); λ_{max} (EtOH)/nm: 306 (log ε 3.78), 245 (4.51) and 210 (4.76); ν_{max} (KBr)/cm^{-1}: 3515 (NH, OH), 1682, 1659 (amide), 1609 and 1595 (Ar); *m/z* (SIMS): 738 $[M]^+$; *m/z* (HRSIMS): 738.2103 $[M]^+$ (Calcd for $C_{35}H_{42}N_6O_8S_2$: 738.2101); ^{1}H NMR δ ppm ($CDCl_3$): 1.11 (3H, d, *J*=6.8 Hz, H_3-15), 1.20 (3H, d, *J*=6.8 Hz, H_3-16), 1.82 (3H, s, 12-SCH_3), 2.19 (3H, s, 3-SCH_3), 2.23 (1H, br s, 14′-OH), 2.60 (1H, heptet, *J*=6.8 Hz, H-14), 2.91 (3H, s, H_3-13′), 3.08 (3H, s, H_3-13), 3.41 (3H, s, 12′-OCH_3), 3.53 (1H, br s, 11′-OH), 3.65 (1H, br s, 11-OH), 3.90 (1H, t, *J*=1.8 Hz, H-3′), 4.14 (2H, d, *J*=2.2 Hz, H-14′), 4.80 (1H, br s, H-6), 4.92 (1H, d, *J*=3.7 Hz, H-11′), 4.97 (1H, br s, H-11), 5.09 (1H, s, H-5a), 5.31 (1H, br s, H-6′), 5.63 (1H, d, *J*=7.3 Hz, H-10′), 6.21 (1H, d, *J*=8.0 Hz, H-7), 6.35 (1H, s, H-5′a), 6.47 (1H, d, *J*=7.3 Hz, H-9′), 6.48 (1H, d, *J*=7.3 Hz, H-7′), 6.89 (1H, t, *J*=8.0 Hz, H-9), 7.04 (1H, t, *J*=7.3 Hz, H-8′), 7.19 (1H, t, *J*=8.0 Hz, H-8) and 7.75 (1H, d, *J*=8.0 Hz, H-10); ^{13}C NMR δ ppm ($CDCl_3$): 14.17 (3-SCH_3), 15.92 (12-SCH_3), 18.26 (C-15), 18.28 (C-16), 30.07 (C-13), 33.21 (C-13′), 37.24 (C-14), 52.37 (12′-OCH_3), 62.37 (C-10b), 63.12 (C-10′b), 63.39 (C-14′), 66.67 (C-3′), 72.65 (C-12), 76.53 (C-5′a), 78.23 (C-11′), 78.88 (C-3), 81.08 (C-11), 84.79 (C-5a), 90.56 (C-12′), 107.52 (C-7), 108.69 (C-7′), 117.38 (C-9), 118.65 (C-9′), 124.84 (C-10a), 126.12 (C-10′), 127.97 (C-10′a), 128.86 (C-8), 130.25 (C-8′), 130.48 (C-10), 148.95 (C-6′a), 154.12 (C-6a), 163.56 (C-4), 164.26 (C-1′), 166.14 (C-4′) and 166.21 (C-1′); CD λ (*c* 1.26×10^{-5} M in EtOH)/nm: 372 ($\Delta\varepsilon$ 0), 345 (−0.29), 317 (0), 255 (+10.70) and 225 (+18.40).

The same reaction with leptosin N_1 (**7**) (5.4 mg) gave **9** (2.9 mg).

3.4. Marfey analyses of *N*-methylserine produced from leptosins M (4) and N (6)

Leptosins M (**4**) (1.5 mg) and N (**6**) (1.2 mg) were dissolved in MeOH (0.2 ml) and 6N HCl (2 ml) and sealed in separate vials. The vials were heated at 110°C for 36 h, and the solution was evaporated in vacuo. To the acid hydrolyzates of **4** and **6** were added a solution of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA) in acetone (100 μl) and 1 M $NaHCO_3$ (100 μl). The mixtures were heated at 80°C for 30 min followed by neutralization with 2N HCl (50 μl) and evaporated off under reduced pressure. The residues were dissolved in 50% aqueous MeOH and subjected to reversed phase HPLC: Cosmosil 5C_{18}-MS (15 cm×4.6 mm i.d.), MeOH–H_2O–TFA (30:70:0.1) as the eluent, flow rate 1 ml min^{-1}, UV detection 340 nm. *N*-Me-L-Ser-L-FDAA derivative was detected from both the acid hydrolyzates of **4** and **6**; retention time (min): *N*-Me-D-Ser-L-FDAA (55.2), *N*-Me-L-Ser-L-FDAA (56.8). The identity of the peak was confirmed by co-injection with a solution of a standard derivatized in the same manner.

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