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CYTOTOXIC METABOLITES FROM A FUNGAL ADHERENT OF A MARINE ALGA

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Key Word Index— *Leptosphaeria* sp.; fungus; *Sargassum tortile*; marine alga; epipolythiodioxopiperazine; leptosin; cytotoxicity.

Abstract—Leptosins G, G_1, G_2 and H, belonging to a series of dimeric epipolythiodioxopiperazines, were isolated from the mycelium of a strain of *Leptosphaeria* sp. stuck on the marine alga *Sargassum tortile*. Their stereostructures were elucidated by chemical and spectral evidence.

INTRODUCTION

As part of our ongoing search for new antineoplastic materials from marine microorganisms [1, 2], we found that cytotoxic compounds, leptosins A (**1**), B (**2**), C (**3**) and D–F, belonging to a series of epipolythiodioxopiperazines, were produced by a strain of *Leptosphaeria* sp. isolated from the marine alga *Sargassum tortile*. Compounds **1** and **3** exhibited significant antitumour activity against Sarcoma 180 ascites [3]. Further investigation of the secondary metabolites of this fungus has led to the isolation of four additional cytotoxic compounds, named leptosins G (**4**), G_1 (**5**), G_2 (**6**) and H (**7**).

RESULTS AND DISCUSSION

The molecular formulae of leptosins G (**4**)–H (**7**) and the general features of their UV, IR and NMR (Tables 1 and 2) spectra suggested that they were analogues with different numbers of sulphur atoms in the polysulphide bridges from those of leptosins A (**1**)–C (**3**) belonging to a series of dimeric epipolythiodioxopiperazines [3]. Leptosin G (**4**) was assigned the molecular formula $C_{32}H_{32}N_6O_7S_7$ (HRFABMS). The ^{13}C NMR signals (C-1′ to C-14′) for the hydroxymethyl-bearing half of the molecule of **4** (Table 2) showed close correspondence with those of the corresponding half of **1** with the tetrasulphide bridge [3]. In addition, the FABMS of **4** revealed a fragment peak at *m/z* 428 $[a]^+$, corresponding to the hydroxymethyl-bearing half of the molecule of **4** together with the peaks at *m/z* 709 $[MH-4S]^+$ and 691 $[MH-4S-H_2O]^+$ arising from the loss of sulphur atoms from the polysulphide bridge in the hydroxymethyl-bearing dioxopiperazine ring and successive dehydration, respectively. Assignment of the $[MH-4S]^+$ peak was based on the report that sulphur atoms in a hydroxymethyl-bearing dioxopiperazine ring were eliminated more easily than those of an isopropyl-bearing ring [3]. This evidence indicated that the hydroxymethyl-bearing dioxopiperazine ring of **4** has a tetrasulphide bridge. The FABMS of **4** also exhibited fragment peaks at *m/z* 408 $[b]^+$, corresponding to the isopropyl-bearing half of the molecule of **4**, 312 $[b-3S]^+$, 525 $[cH]^+$, 429 $[cH-3S]^+$ and 411 $[cH-3S-H_2O]^+$, indicating that the isopropyl-bearing ring in **4** has a trisulphide bridge (see structures **1**–**7** for the fragment ions). The above-mentioned evidence allowed assignment of planar structure **4** to leptosin G.

Leptosin G_1 (**5**) was assigned a molecular formula which contained one sulphur atom less than that of **4**. The two sets of ^{13}C NMR signals (C-1 to C-16 and C-1′ to C-14′) for the isopropyl- and hydroxymethyl-bearing halves of the molecule of **5** (Table 2) revealed similarities with those of the isopropyl-bearing half of **4** and the hydroxymethyl-bearing half of **2** with the trisulphide bridge [3], respectively. The FABMS of **5** exhibited fragment peaks at *m/z* 709 $[MH-3S]^+$, arising from the loss of sulphur atoms from the polysulphide bridge in the hydroxymethyl-bearing dioxopiperazine ring, and *m/z* 397 $[d+H]^+$ together with the fragment ions *m/z* 408 $[b]^+$, 312 $[b-3S]^+$, 524 $[c]^+$, 428 $[c-3S]^+$ and 410 $[c-3S-H_2O]^+$ which indicated that the isopropyl-bearing ring in **5** has a trisulphide bridge. This evidence led to planar structure **5** for leptosin G_1.

Leptosin G_2 (**6**) was assigned a molecular formula which contained one sulphur atom less than that of **5**. Most of the ^{13}C NMR signals of **6** were not observed due to peak broadening. The FABMS of **6** exhibited a fragment

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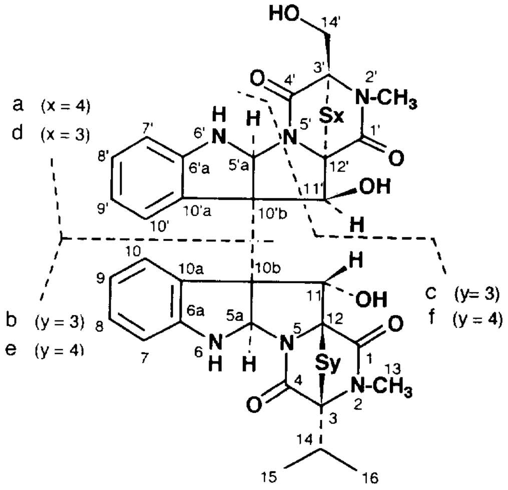
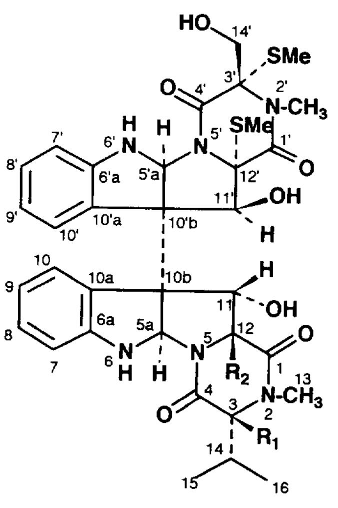


1 x = 4, y = 2 **5** x = 3, y = 3
2 x = 3, y = 2 **6** x = 2, y = 3
3 x = 2, y = 2 **7** x = 2, y = 4
4 x = 4, y = 3



R₁ R₂
8 -S₃-
9 SMe SMe

Table 1. ^{1}H NMR spectral data of leptosins **4**–**7** and derivative **8** (300 MHz, $CHCl_3$, TMS as int. standard)*

H	**4**	**5**	**6**	**7**	**8**
5a	6.28 *s*	6.31 *s*	6.50 *br s*	6.29 *br s*	6.64 *s*
6	5.28 *s*	5.31 *br s*	5.36 *br s*†	5.47 *br s*	‡
7	6.70 *d* (8.0)	6.72 *d* (7.8)	6.74 *br s*	6.23 *br d* (7.8)	6.69 *d* (7.8)
8	7.14 *t* (8.0)	7.15 *t* (7.8)	7.17 *t* (7.8)	7.07 *br t* (7.8)	7.12 *td* (7.8, 1.0)
9	6.41 *t* (8.0)	6.43 *t* (7.8)	6.50 *br s*	6.81 *br t* (7.8)	6.38 *t* (7.8)
10	5.49 *d* (8.0)	5.55 *d* (7.8)	5.36 *br s*	7.65 *br d* (7.8)	5.53 *dd* (7.8, 1.0)
11	4.90 *s*	4.94 *s*	4.80 *br s*	5.28 *br s*	4.91 *d* (1.5)
13	3.20 *s*	3.20 *s*	3.21 *s*	3.06 *s*	3.21 *s*
14	2.47 *heptet* (6.8)	2.46 *heptet* (6.8)	2.48 *heptet* (6.8)	2.70 *heptet* (6.8)	2.50 *heptet* (6.8)
15	1.23 *d* (6.8)	1.23 *d* (6.8)	1.21 *d* (6.8)	1.15 *d* (6.8)	1.19 *d* (6.8)
16	1.44 *d* (6.8)	1.43 *d* (6.8)	1.43 *d* (6.8)	1.43 *d* (6.8)	1.45 *d* (6.8)
11-OH	4.18 *br s*	4.22 *br s*	4.35 *br d* (2.0)	4.40 *br s*	4.19 *d* (1.5)
5′a	5.41 *br s*	5.60 *br s*	5.80 *br s*	5.96 *br d* (6.8)	5.60 *s*
6′	5.28 *br s*	5.14 *br s*	†‡	6.97 *br d* (6.8)	‡
7′	6.53 *d* (8.0)	6.52 *d* (7.8)	6.54 *d* (7.8)	6.59 *br d* (7.8)	6.48 *d* (7.8)
8′	7.22 *t* (8.0)	7.22 *t* (7.8)	7.08 *br t* (7.8)	7.05 *br t* (7.8)	7.17 *td* (7.8, 1.0)
9′	6.92 *t* (8.0)	6.92 *t* (7.8)	6.74 *br s*	6.40 *br t* (7.8)	6.88 *t* (7.8)
10′	7.74 *d* (8.0)	7.76 *d* (7.8)	7.62 *br s*	6.27 *br d* (7.8)	7.72 *dd* (7.8, 1.0)
11′	5.33 *s*	5.33 *s*	6.12 *br s*	6.21 *br s*	5.26 *s*
13′	3.00 *s*	2.99 *s*	2.93 *br s*	2.91 *br s*	3.02 *s*
14′	3.94 *d* (12.8) 4.12 *d* (12.8)	3.74 *d* (12.6) 4.52 *d* (12.6)	4.17 *dd* (12.5, 9.2) 4.33 *dd* (12.5, 5.2)	4.17 *br d* (12.5) 4.32 *br d* (12.5)	3.70 *d* (12.0) 4.03 *d* (12.0)
11′-OH	3.82 *br s*	4.42 *br s*	3.3 *br s*	3.40 *br s*	‡
14′-OH	‡	‡	†‡	‡	‡
2′-SMe					2.22 *s*
12′-SMe					2.39 *s*

*^{1}H chemical shift values (δppm) followed by multiplicity and then the coupling constant (*J*/Hz) in parentheses.
†Interchangeable within each column.
‡Not detected.

peak at 709 $[MH - 2S]^+$ arising from the loss of sulphur atoms from the hydroxymethyl-bearing dioxopiperazine ring together with the fragment ions $[b]^+$, $[c]^+$, $[c - 3S]^+$ and $[c - 3S - H_2O]^+$. Leptosin G_2 (**6**) was transformed into bis(methylthio) and tetrakis(methylthio) derivatives **8** and **9** by treatment with $NaBH_4$ and MeI. The presence of two methylthio groups on the hydroxymethyl-bearing dioxopiperazine ring of **8** was established from

Table 2. ^{13}C NMR spectral data of leptosins **4**, **5** and **7** (75.4 MHz, $CHCl_3$, TMS as int. standard)*

C	**4**	**5**	**7**
1	170.1 (*q*)†	170.1 (*q*)	168.8 (*q*)‡
3	77.6 (*q*)	77.4 (*q*)	81.6 (*q*)
4	165.1 (*q*)	165.1 (*q*)	167.7 (*q*)‡
5a	79.3 (*t*)	79.7 (*t*)	79.2 (*t*)
6a	150.9 (*q*)	151.0 (*q*)	148.1 (*q*)
7	110.7 (*t*)	110.8 (*t*)	108.6 (*t*)
8	131.0 (*t*)	131.0 (*t*)	130.2 (*t*)
9	119.3 (*t*)	119.4 (*t*)	118.9 (*t*)
10	126.7 (*t*)	126.7 (*t*)	127.4 (*t*)
10a	123.5 (*q*)	123.8 (*q*)‡	127.1 (*q*)
10b	60.7 (*q*)	60.6 (*q*)	61.6 (*q*)
11	83.2 (*t*)	83.2 (*t*)	83.0 (*t*)
12	83.3 (*q*)	83.4 (*q*)	80.0 (*q*)
13	28.0 (*p*)	27.9 (*p*)	30.2 (*p*)
14	35.5 (*t*)	35.5 (*t*)	36.0 (*t*)
15	19.2 (*p*)	19.2 (*p*)	18.3 (*p*)
16	18.0 (*p*)	18.1 (*p*)	18.4 (*p*)
1′	168.4 (*q*)	167.7 (*q*)	167.7 (*q*)
3′	79.0 (*q*)	80.6 (*q*)	79.2 (*q*)
4′	169.3 (*q*)	165.8 (*q*)	164.7 (*q*)
5′a	80.5 (*t*)	79.7 (*t*)	83.2 (*t*)
6′a	149.8 (*q*)	149.9 (*q*)	149.6 (*q*)
7′	109.8 (*t*)	109.7 (*t*)	109.6 (*t*)
8′	130.0 (*t*)	130.0 (*t*)	129.7 (*t*)
9′	119.0 (*t*)	118.8 (*t*)	119.3 (*t*)
10′	130.2 (*t*)	130.3 (*t*)	129.8 (*t*)
10′a	122.9 (*q*)	123.2 (*q*)‡	121.7 (*q*)
10′b	64.2 (*q*)	65.2 (*q*)	63.3 (*q*)
11′	79.0 (*t*)	79.7 (*t*)	78.8 (*t*)
12′	80.5 (*q*)	76.3 (*q*)	74.5 (*q*)
13′	28.9 (*p*)	28.5 (*p*)	26.4 (*p*)
14′	63.6 (*s*)	62.0 (*s*)	60.6 (*s*)

* Signal assignments were based on long range ^{1}H–^{13}C HETCOR and/or ^{1}H–^{13}C HETCOR spectra.

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

‡ Assignments may be interchangeable in each column.

Table 3. Cytotoxicity of **4–7** against tumour cells

Compound	Cell line P-388 (ED_{50} ng ml^{-1})
Leptosin G (**4**)	4.6
Leptosin G_1 (**5**)	4.3
Leptosin G_2 (**6**)	4.4
Leptosin H (**7**)	3.0
Mitomycin C (standard)	21.5

the fact that the chemical shifts of the ^{1}H NMR signals for the isopropyl-bearing half of **8** were similar to those of the corresponding half of **4–6** (Table 1), and a polysulphide bridge in a hydroxymethyl-bearing dioxopiperazine ring could react more easily than that of an isopropyl-bearing ring on treatment with $NaBH_4$ and MeI as reported previously [3]. The derivative **9** was identical to that derived from **1** [3] in all respects examined, including the CD spectrum. The above-mentioned evidence led to stereostructure **6** for leptosin G_2.

For the purpose of desulphurization, **4** was treated with triphenylphosphine to afford **5** and **6**. This proved **4** and **5** to have the same absolute configuration as that of **6**.

Leptosin H (**7**) had the same molecular formula as **5**. The ^{13}C NMR signals (C-1′ to C-14′) for the hydroxymethyl-bearing half of the molecule of **7** (Table 2) revealed similarities with those of the same half of **3** with a disulphide bridge [3]. The FABMS exhibited fragment peaks at *m/z* 740 $[M-2S]^+$, arising from the loss of sulphur atoms from the polysulphide bridge in the hydroxymethyl-bearing dioxopiperazine ring, 440 $[e]^+$, 556 $[f]^+$, 428 $[f-4S]^+$ and 410 $[f-4S-H_2O]^+$, the last four indicating that the isopropyl-bearing ring has a tetrasulphide bridge. Treatment of **7** with $NaBH_4$ and MeI afforded bis(methylthio) and tetrakis(methylthio) derivatives **8** and **9**. Formation of **8** suggested that the reductive elimination of one sulphur atom from the tetrasulphide bridge in the isopropyl-bearing dioxopiperazine ring of **7** coincided with reductive cleavage of the disulphide bridge in the hydroxymethyl-bearing ring on $NaBH_4$ reduction. Based on the above evidence, the stereostructure for leptosin H is represented as **7**.

The cytotoxic activities of **4–7** were examined in the P388 lymphocytic leukaemia test system in cell culture according to the method reported previously [4]. All the compounds tested exhibited cytotoxic activity greater than that of mitomycin C, used as a standard sample (Table 3). The cytotoxicities were equivalent to those of the previously reported compounds **1–3** and suggested that a difference in the numbers of sulphur atoms of the polysulphide bridges had no influence on the cytotoxicities of such compounds against P388 cells.

EXPERIMENTAL

Isolation of metabolites. As reported previously [3], a strain of *Leptosphaeria* sp. isolated from the marine alga *Sargassum tortile* C. Agaroh (Sargassaceae) was cultured in a medium (20 l) containing 2% glucose, 1% peptone and 0.5% yeast extract in artificial seawater at 27°. The CH_2Cl_2–MeOH (1:1)-soluble fr. (21.5 g) of the mycelium was successively subjected to CC on Sephadex LH-20 (CH_2Cl_2–MeOH, 1:1) and silica gel (hexane–CH_2Cl_2 gradient). One fr. each of the hexane–CH_2Cl_2 (6:4) and (4:6), and CH_2Cl_2 eluates [fr. 4 (117 mg), fr. 7 (141 mg) and fr. 10 (45 mg)] was purified by HPLC (SIL) using CH_2Cl_2, Me_2CO–CH_2Cl_2 and MeOH–CH_2Cl_2 (1:49) as eluents, respectively, to afford **6** (7.2 mg), **5** (5.4 mg) and **7** (6.2 mg), and **4** (7.6 mg).

Leptosin G (**4**). Pale yellow powder, mp 205–210°, $[\alpha]_D^{24}$ +481° ($CHCl_3$; *c*0.40). UV λ_{max}^{EtOH} nm (log ε): 206 (4.91), 240 (4.42), 296 (3.91); IR ν_{max}^{KBr} cm^{-1}: 3423 (OH, NH), 1686, 1666 (CON), 1607, 1597 (Ar. C-C); FABMS *m/z* (rel. int): 837 $[M+H]^+$ (1), 709 $[MH-4S]^+$ (9), 691 $[MH-4S-H_2O]^+$ (2), 525 $[cH]^+$ (4), 429 $[cH-3S]^+$ (4), 428

[a]$^+$ (2), 411 [cH$-$3S$-$H$_2$O]$^+$ (3), 408 [b]$^+$ (4), 312 [b $-$ 3S]$^+$ (14), 232 [bi-indolyl]$^+$ (59), 197 [cH $-$ 3S $-$ 232]$^+$ (33), 155 [cH $-$ 3S $-$ 232 $-$ *i*-Pr + H]$^+$ (63), 119 (100); HRFABMS *m/z* 837.0459 [M + H]$^+$ ($C_{32}H_{33}N_6O_7S_7$ requires: 837.0456); CD λ_{ext}^{EtOH} nm [*c*2.77 × 10^{-5} M] ($\Delta\varepsilon$): 233 (+32.9), 249 (+39.6), 316 (+4.8); ^{1}H and ^{13}C NMR: Tables 1 and 2.

Leptosin G_1 (**5**). Powder, mp 210–212°, $[\alpha]_D^{24}$ +558° (CHCl$_3$; *c*0.45). UV λ_{max}^{EtOH} nm (log ε): 210 (4.72), 240 (4.14), 296 (3.79); IR ν_{max}^{KBr} cm^{-1}: 3503 (OH, NH), 1691, 1665 (CON), 1606, 1594 (Ar. C-C); FABMS *m/z* (rel. int): 805 [M+H]$^+$ (4), 709 [MH$-$3S]$^+$ (2), 691 [MH$-$3S $-$H$_2$O]$^+$ (0.6), 613 [MH$-$6S]$^+$ (0.5), 524 [c]$^+$ (1), 428 [c $-$3S]$^+$ (1), 410 [c$-$3S$-$H$_2$O]$^+$ (1), 408 [b]$^+$ (2), 397 [d +H]$^+$ (2), 312 [b$-$3S]$^+$ (5), 307 (100), 289 (21), 232 [bi-indolyl]$^+$ (100), 197 [cH$-$3S$-$232]$^+$ (11), 155 [cH$-$3S $-$ 232 $-$ *i*-Pr + H]$^+$ (63), 136 (100); HRFABMS *m/z* 805.0738 [M + H]$^+$ ($C_{32}H_{33}N_6O_7S_6$ requires: 805.0735); CD λ_{ext}^{EtOH} nm [*c*5.53 × 10^{-5} M] ($\Delta\varepsilon$): 230 (+41.1), 238 (+39.4), 251 (+43.5), 304 (+4.9), 318 (+5.3); ^{1}H and ^{13}C NMR: Tables 1 and 2.

Leptosin G_2 (**6**). Pale yellow powder, mp 210–215°, $[\alpha]_D^{24}$ +303° (CHCl$_3$; *c*0.58). UV λ_{max}^{EtOH} nm (log ε): 206 (4.79), 240 (4.31), 296 (3.80); IR ν_{max}^{KBr} cm^{-1}: 3398 (OH, NH), 1690, 1666 (CON), 1608, 1594 (Ar. C-C); FABMS *m/z* (rel. int): 773 [M+H]$^+$ (3), 709 [MH$-$2S]$^+$ (0.2), 613 [MH $-$5S]$^+$ (0.2), 524 [c]$^+$ (0.2), 428 [c$-$3S]$^+$ (0.2), 410 [c $-$3S$-$H$_2$O]$^+$ (0.2), 408 [b]$^+$ (0.1), 307 (28), 289 (14), 232 [bi-indolyl]$^+$ (11), 197 [cH$-$3S$-$232]$^+$ (3), 154 [cH$-$3S $-$232$-$*i*-Pr]$^+$ (100), 136 (61); HRFABMS *m/z*: 773.1012 [M+H]$^+$ ($C_{32}H_{33}N_6O_7S_5$ requires: 773.1014). CD λ_{ext}^{EtOH} nm (*c*2.63 × 10^{-5} M] ($\Delta\varepsilon$): 229 (+43.1), 256 (+15.0), 292 (+0.3), 320 (+3.9); ^{1}H NMR: Table 1.

Leptosin H (**7**). Pale yellow powder, mp 214–215°, $[\alpha]_D^{24}$ +298° (CHCl$_3$; *c*0.47). UV λ_{max}^{EtOH} nm (log ε): 207 (4.81), 240 (4.37), 302 (3.85); IR ν_{max}^{KBr} cm^{-1}: 3397 (OH, NH), 1672 (CON), 1609, 1594 (Ar. C-C); FABMS *m/z* (rel. int): 805 [M+H]$^+$ (0.8), 740 [M$-$2S]$^+$ (0.4), 613 [MH $-$6S]$^+$ (0.2), 556 [f]$^+$ (0.3), 440 [e]$^+$ (0.1), 428 [f$-$4S]$^+$ (1), 410 [f$-$4S$-$H$_2$O]$^+$ (0.3), 307 (29), 289 (14), 232 (bi-indolyl]$^+$ (16), 197 [fH$-$4S$-$232]$^+$ (8), 154 [fH$-$4S $-$232$-$*i*-Pr]$^+$ (100), 136 (62); HRFABMS *m/z*: 805.0732 [M+H]$^+$ ($C_{32}H_{33}N_6O_7S_6$ requires: 805.0735); CD λ_{ext}^{EtOH} nm (*c*2.24 × 10^{-5} M] ($\Delta\varepsilon$): 232 (+40.6), 250 (+23.7), 257 (+24.4), 346 ($-$1.7); ^{1}H and ^{13}C NMR: Tables 1 and 2.

Formation of the bis(methylthio) and tetrakis(methylthio) derivatives **8** *and* **9** *from leptosins G_2* (**6**) *and H* (**7**). Leptosin G$_2$ (**6**, 5 mg) was dissolved in a soln (0.13 ml) of pyridine and MeOH (5:8). MeI (0.5 ml) and NaBH$_4$ (2 mg) were added, and the mixt. was stirred for 20 min at room temp. The reaction mixt. was then diluted with H$_2$O and extracted with Et$_2$O. The solvent was evapd *in vacuo*, and the residue was chromatographed on a silica gel column with a CH$_2$Cl$_2$–MeOH gradient as the eluent. The MeOH–CH$_2$Cl$_2$ (1:99) eluate afforded **8** (1.3 mg) and **9** (1.0 mg). Compound **8** was obtained as a powder; IR λ_{max}^{KBr} cm^{-1}: 3490 (OH, NH), 1660, 1640 (CON), 1609, 1594 (Ar. C-C); FABMS *m/z* (rel. int.): 803 [MH]$^+$ (7); ^{1}H NMR: Table 1. Compound **9** was obtained as a pale yellow oil. It was identical with the sample derived from **1** in all respects [3]. The same reaction with **7** (6.5 mg) gave **8** (3.5 mg) and **9** (1.3 mg).

Formation of leptosins G_1 (**5**) *and G_2* (**6**) *from leptosin G* (**4**). Triphenylphosphine (2 mg) was added to a CHCl$_3$ soln (5 ml) of **4** (5.5 mg), and the reaction mixt. was left standing at room temp. for 1 hr. The solvent was evapd *in vacuo*, and the residue was purified by silica gel CC using MeOH–CH$_2$Cl$_2$ (1:99) as the eluent to afford **5** (1.0 mg) and **6** (1.2 mg), which were identified by IR, ^{1}H NMR, CD and TLC.

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