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Spongidepsin, a new cytotoxic macrolide from *Spongia* sp.

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Abstract—A novel macrolide, spongidepsin (**1**) has been isolated from the Vanuatu marine sponge *Spongia* sp. The structure of **1**, which contains 9-hydroxy-2,4,7-trimethyltetradeca-14-ynoic acid and *N*-methylphenylalanine residues joined in a 13-membered ring, was elucidated by spectroscopic analysis. Spongidepsin (**1**) showed cytotoxic activity against J774.A1, WEHI-164 and HEK-293 cancer cell lines with an IC_{50} in the sub-micromolar range.

1. Introduction

Marine porifera of the genus *Spongia* (family Spongidae, class Demospongiae) have proved to be particularly rich sources of polycyclic terpenes.[1–7] The discovery of spongistatin,[8] a macrocyclic lactone with potent antitumor activity, from *Spongia* sp. is surprising when compared to the normal secondary metabolites of these animals, and suggests that symbiotic microorganisms can sometimes overthrow the biosynthetic pathways of the host.

In our continuing search for bioactive metabolites from marine invertebrates[9] we have investigated the active crude extract of the sponge *Spongia* sp. collected off the Vanuatu islands. Bioassay guided fractionations of the CCl_4 extract led us to the isolation of the new cytotoxic metabolite spongidepsin **1** (Fig. 1) which co-occurred with the known metabolites petrosaspongiolides A, B and I.[10,11]

We now report the isolation and the structure determination of the new macrolide **1**, containing an amino acid residue of *N*-methylphenylalanine joined to a polyketide unit in a 13-membered macrocycle.

2. Results and discussion

Freeze dried specimens of *Spongia* sp. were subjected to extraction with MeOH, at room temperature, followed by a Kupchan partition[12] affording four extracts of different polarity: *n*-hexane, CCl_4, $CHCl_3$, *n*-butanol. The CCl_4 soluble material was chromatographed by medium pressure liquid chromatography (MPLC) on a silica gel column using a gradient elution system *n*-hexane/EtOAc from 100% *n*-hexane to 100% EtOAc. The first eluting fractions were further purified by HPLC (Whatman ODS-2, MeOH/H_2O 75:25), affording the minor spongidepsin **1** (1.4 mg of colorless material) along with greater amounts of the previously described petrosaspongiolides A, B and I.[10,11]

The ESIMS of **1** gave pseudomolecular ions at *m/z* 426 $[M+H]^+$ and at *m/z* 448 $[M+Na]^+$. The peak at *m/z* 426.2985, observed at high resolution (HRESIMS), established the molecular formula $C_{27}H_{39}NO_3$ for **1** (calculated for $C_{27}H_{40}NO_3$: 426.3008). The collective ^1H and ^{13}C NMR data indicated the presence of 27 carbon and 39 hydrogen atoms: eight sp^3 methylenes, five sp^3 methines, four methyls, eight sp^2 carbons (five CH and three C) and two sp carbons (one CH and one C). A careful analysis of COSY, TOCSY and HSQC spectra allowed us to recognize a long chain aliphatic spin system assigned to a 9-hydroxy-2,4,7-trimethyltetradeca-14-ynoic acid. In fact, a COSY

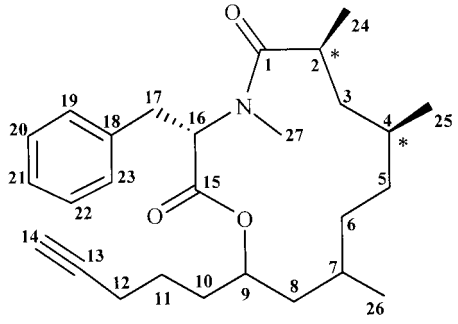

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Figure 1. Chemical structure of spongidepsin (**1**). Stereocenters marked with stars indicate relative stereochemistry.

Keywords: alkynes; cytotoxic; macrocyclic lactone; marine metabolites.
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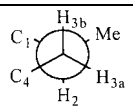
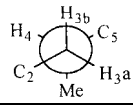
Table 1. NMR data for spongidepsin (600 MHz, CD_3OD)

	^{1}H δ, m, J (Hz)	^{13}C (ppm)	COSY	HMBC
1		179.3		
2	2.96 m	34.3	H3b; H24	
3a	0.81 m	40.2	H3b; H4	
3b	1.94 m		H3a; H2	C2; C25
4	1.43 m	28.2	H3a; H5a; H25	
5a	1.09 m	33.5	H4; H5b; H6b	C7
5b	1.52 m		H5a; H6a	
6a	1.08 m	32.5	H5b; H6b; H7	C4; C7
6b	1.59 m		H5a; H6a	
7	1.65 m	25.1	H6a; H8a; H26	
8a	0.92 m	38.0	H7; H8b	
8b	1.70 m		H8a; H9	C9; C26
9	5.18 q (5.9)	73.9	H8b; H10	C15
10	1.68 m	35.8	H9; H11	C9
11	1.56 m	25.3	H10; H12	C12; C13
12	2.25 m	18.7	H11	C10; C11; C13; C14
13		84.6		
14	2.26 brs	69.9		C13
15		172.0		
16	4.00 dd (4.4; 11.0)	67.6	H17a; H17b	C1; C15; C27
17a	3.27 dd (4.4; 13.2)	35.1	H16; H17b	C16; C18; C19; C23
17b	3.42 dd (11.0; 13.2)		H16; H17a	C16; C18; C19; C23
18		139.5		
19	7.21 d (7.3)	130.5	H20	C17; C21; C23
20	7.32 t (7.3)	129.4	H19; H21	C18; C22
21	7.25 t (7.3)	127.6	H20; H22	C19; C23
22	7.32 t (7.3)	129.4	H21; H23	C18; C20
23	7.21 d (7.3)	130.5	H22	C17; C19; C21
24	1.10 d (6.6)	18.9	H2	C1; C2; C3
25	0.95 d (6.6)	22.3	H4	C3; C4; C5
26	0.92 d (6.6)	21.5	H7	C6; C7; C8
27	2.75 s	40.3		C1; C16

spectrum of **1** clearly showed the proton connectivities for the spin system H2–H12. Furthermore, NMR connectivities between signals at δ 1.1 (3H, d, J=6.6 Hz), 0.95 (3H, d, J=6.6 Hz), 0.92 (3H, d, J=6.6 Hz) and the methine signals at 2.96 (m, H-2), 1.43 (m, H-4), 1.65 (m, H-7), respectively, placed the secondary methyl groups at C2, C4 and C7 of the polyketide chain. The resonance at δ 5.18 (1H, m), correlated to the carbon signal at 73.9 ppm, suggested an oxymethine function, that on the basis of COSY data was assigned to C-9. The low field resonance of H2 (δ 2.96) and a correlation peak observed in HMBC spectrum between H24 and the carbonyl centered at 179.3 ppm allowed us to identify the carboxylic head (C1) of the polyketide chain which, as an additional feature, includes a terminal alkynyl moiety. The presence of the alkyne function was suggested primarily on the basis of ^{13}C NMR resonances at 84.6 and 69.9 ppm, which are in good agreement with those expected for a monosubstituted alkynyl group;[13] moreover key long-range ^{1}H–^{13}C NMR correlations peaks (HMBC) from H_2-11 and H_2-12 to C-13 (84.6 ppm) established the location of the two sp carbon system. An HSQC spectrum optimized for a $^1J_{C-H}$ of 250 Hz allowed us to unambiguously assign the proton at δ_H 2.26 as attached to the terminal sp carbon (δ_C 69.9). The remaining resonances in ^{1}H and ^{13}C spectra (see Table 1) indicated that the molecule still contained seven sp^2 carbons, one of which could be assigned to an ester function (δ 172.0) and the other six to a monosubstituted aromatic unit, one deshielded methyl (δ_C 40.3, δ_H 2.75) and one methylene (δ_C 35.1; δ_H 3.42, 3.27) coupled to a low field resonating methine (δ_C 67.6, δ_H 4.0). These data corroborated by HMBC correlations suggested us the presence of a *N*-methylphenylalanine residue. Comparison of the above chemical shifts to those assigned to the same residue found in other metabolites[14,15] gave conclusive support to this assignment. Finally, the diagnostic HMBC correlations between the C9 oxymethine proton (δ 5.18) and the ester carbonyl (δ 172.0, C-15), and between the Hα of the aminoacid residue (H-16) and the carbonyl of the hydroxyacid moiety (δ 179.3, C-1) allowed us to close a 13-membered macrocyclic ring and establish the complete carbon framework of **1**. The L-series was assigned to the *N*-methylphenylalanine residue through the application of Marfey HPLC method[16] on the acidic hydrolysate of **1** (HCl 6N, 110°C, 15 h). The relative configuration at C2 and C4 stereocenters of spongidepsin (**1**) was investigated using the NMR approach recently described by Murata,[17] that relies on proton–proton ($^3J_{H-H}$) and carbon–proton ($^{2,3}J_{C-H}$) coupling constant measurements in combination with ROESY responses. The latter method allows the

Table 2. Dominant rotamers of each of the two C_2-fragments along with the pattern of homo and heteronuclear J couplings used for their determination

Fragment	Segment	$^3J_{H-H}$ (Hz)	$^3J_{C-H}$ (Hz)
C2–C3 *anti*		$^3J_{H2-H3b}$ 12.2 $^3J_{H2-H3a}$ 3.0	$^3J_{H3b-Me}$ 2.1 (1–3)[a] $^3J_{H3b-C1}$<2.1 (1–3)[a] $^3J_{H3a-Me}$ 1.6 (1–3)[a]
C3–C4 *anti*		$^3J_{H4-H3a}$ 11.1 $^3J_{H4-H3b}$ 1.7	$^3J_{H3b-Me}$ 6.5 (5–7)[a] $^3J_{H3b-C5}$<2.1 (1–3)[a]

[a] Values in parentheses refer to the range of heteronuclear J couplings expected for this stereochemistry on the basis of Murata method.[17]

Table 3. In vitro antiproliferative activity of spongidepsin

Cell lines	Spongidepsin IC_{50} (μM)	6-mercaptopurine IC_{50} (μM)
J774.A1	0.56	0.003
HEK-293	0.66	0.007
WEHI-164	0.42	0.017

identification of the predominant conformers among the six staggered rotamers with *threo* and *erythro* relative configurations. Heteronuclear $^{2,3}J$ values are usually measured by using HETLOC[18] and phase-sensitive HMBC experiments while accurate values of homonuclear coupling constants can be extracted from PE-COSY cross-peaks. In our case, the two stereocenters were connected through a methylene requiring the analysis of both C2–C3 and C3–C4 fragments, in order to determine the relative spatial orientations of the two methyl groups. The dominant rotamers with correct relative stereochemistry of each of the two C_2 fragments investigated, are reported in Table 2 along with the pattern of homo and heteronuclear *J* couplings used for their determination. Unfortunately, we could not assign the relative stereochemistry of the two other stereogenic centers (C7 and C9) because the ^{1}H NMR resonances under investigation closely overlapped and therefore prevented the measurement of the *J* couplings.

Table 3 shows the results obtained in the antiproliferative assay for spongidepsin (**1**) expressed as IC_{50} values (μM), the concentration that inhibited cell growth by 50% as compared to the control (6-mercaptopurine).

3. Experimental

3.1. General experimental procedures

Spectral data were acquired on the following instruments: Bruker DRX 600 (NMR spectra); LCQ Finnigan mass spectometer (ESIMS); $[\alpha]_D$-Perkin Elmer 161 polarimeter; UV-Beckman DU 640 spectrophotometer. HPLC was performed with a Waters model 510 pump with refractive index detection.

3.2. Biological material

The sponge *Spongia* sp. was collected off the Vanuatu Islands (Australia) in May 1997. The taxonomic identification was made by Dr John Hooper (Museum of Queenland, Brisbane, Australia) and voucher specimens (reference no. R1739) are available at the IRD (ex ORSTOM) Center of Nouméa, New Caledonia.

3.3. Isolation

The organism (lyophilized material, 500 g) was extracted exhaustively with MeOH (3×1.5 L) at room temperature. The methanolic extract, filtered through paper and concentrated under reduced pressure gave a red brown oil. The oily residue was successively extracted using a modified Kupchan partition procedure: the extract was dissolved in 1 L of a mixture of MeOH/H_2O containing 10% of H_2O and partitioned against 1.5 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 1.5 L of CCl_4 and 1.5 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butanol (1 L). The CCl_4 soluble material (5.08 g) was chromatographed by medium pressure liquid chromatography (MPLC) on a silica gel column (230–400 mesh) using a gradient elution system *n*-hexane/EtOAc from 100% *n*-hexane to 100% EtOAc (30 mL/fraction). The collected fractions were controlled by TLC on silica gel (Merck, kieselgel F_{254}, 0.25 mm) and revealed by spraying with $Ce(SO_4)_2$ in sulfuric acid solution. Homogeneous fractions were pooled into 24 groups. MPLC fractions 8–38 (15.7 mg) eluted with 75% *n*-hexane were further purified by HPLC on analytical Whatman ODS-2 column, eluted with MeOH/H_2O (75:25) to afford spongidepsin (1.7 mg) as pure metabolite.

Petrosaspongiolides, cytotoxic polycyclic sesterterpens already described as secondary metabolites of *Petrosaspongia nigra*, were also found in the CCl_4 extract. MPLC fractions 51–53 (5.6 mg) were purified by HPLC on analytical Whatman ODS-2 column and eluted with MeOH/H_2O (85:15) to afford petrosaspongiolide A (2.8 mg), while MPLC fraction 26 (9.1 mg) eluted in the same conditions afforded petrosaspongiolide B (6.1 mg). Moreover MPLC fractions 31–72 (41.3 mg) eluted with MeOH/H_2O (8:2) yielded petrosaspongiolide I (6.6 mg). The compounds were identified by comparison of spectra with those of authentic samples.[10,11]

3.4. Spongidepsin

White amorphous solid. $[\alpha]_D$=−61.8 (MeOH, *c*=0.014 g/100 mL); UV λ_{max} (ϵ): 210 nm (2308); 258 nm (750); ESIMS (MeOH): $[M+H]^+$ *m/z* 426 (42); $[M+Na]^+$ *m/z* 448 (100). HRESIMS: 426.2985 (calculated for $C_{27}H_{40}NO_3$: 426.3008). NMR data: see Table 1.

3.5. Antiproliferative assay

J774.A1, WEHI-164 and HEK-293 (3.4×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5% CO_2 and 95% air for 2 h.

Thereafter, the medium was replaced with 50 μL of fresh medium and a 75 μL aliquot of 1:4 serial dilution of each test compound was added and then the cells incubated for 72 h. In some experiments, serial dilutions of 6-mercaptopurine (6-MP) were added. The cells' viability was assessed through an MTT conversion assay.[19] Briefly, 25 μL of MTT (5 mg/mL) were added and the cells were incubated for an additional 3 h. Thereafter, the cells were lysed and the dark blue crystals solubilized with 100 μL of an aqueous solution containing 50% (v/v) *N*,*N*-dimethylformamide, 20% (w/v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP was calculated as: % dead cells=100−(OD treated/OD control)×100.

The results obtained in the antiproliferative assay for spongidepsin, expressed as an IC_{50} value (μM), are: J774.A1=

0.56; HEK-293=0.66; WEHI-164=0.42. The IC_{50} value is the concentration of compound that affords 50% reduction in cell growth (after 3 day incubation). J774.A1 is a murine monocyte/macrophage cell line; HEK-293 is a human epithelial kidney cell line; WEHI-164 is a murine fibrosarcoma cell line. In the same experimental conditions, the reference drug 6-MP displayed an IC_{50} of 0.003 μM on J774.A1 cell line, an IC_{50} of 0.007 μM on HEK-293 cell line, and an IC_{50} of 0.017 μM on WEHI-164 cell line.

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