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Three New Potent Cytotoxic Macrolides Closely Related to Sphinxolide from the New Caledonian Sponge *Neosiphonia superstes*°

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Abstract: Three new macrolides **2-4** have been isolated with sphinxolide **1** from the marine sponge *N. superstes* collected off New Caledonia . The structures of the new compounds were determined by interpretation of NMR spectral data as well as by comparison of spectral data with those of **1**. These compounds were highly cytotoxic against various human carcinoma cells.

INTRODUCTION

Macrolides of marine origin are of considerable interest, because of their structural novelty and strong biological activity. They include polymethylated polyhydroxy(methoxy)lated macrolides, such as the dimeric lactones swinholide A, B, C, isoswinholide A and the related misakinolide A, from the sponges of the genus *Theonella* [1,2] , the 1,3-oxazole-bearing macrolides, ulapualides[3], kabiramides[4,5] and halichondramides[5,6] , from nudibranch eggmasses, and also from sponges of genus *Halichondria* [6], mycalolides, and pateamine, a thiazole-containing dilactone, both from sponges of the genus *Mycale* [7,8].

Recently it has been reported on a novel 26-membered macrolide of the first type, isolated from an unidentified Pacific Nudibranch, which, because of the difficulties in defining the source was named sphinxolide (**1**) from the misterious Egyptian Sphinx[9]. During our continuing search for bioactive metabolites from New Caledonian marine invertebrates, we found that the lipophilic extract of the marine living fossil sponge *Neosiphonia superstes* Sollas [10] collected from deep waters (515-505 m) in the region

°This paper is dedicated to the memory of Dr. Pierpaolo Grimaldi.

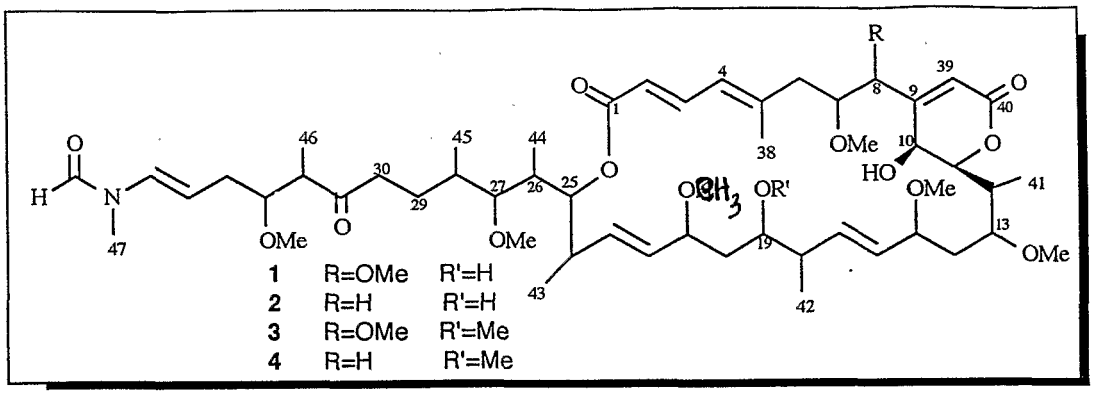

Table 1. *In vitro* cytotoxic activity (IC_{50} in μg/ml) of sphinxolides (**1**), (**2**), (**3**) and (**4**)[a].

Tumor cells	1	2	3	4	6-mercaptopurine
NSCLC-N6	$27x10^{-3}$	$16x10^{-3}$	$30x10^{-3}$	$60x10^{-3}$	0.76
P388/Dox	$0.33x10^{-3}$	$0.02x10^{-3}$	$30x10^{-3}$	$8x10^{-3}$	0.25
P388	$4.1x10^{-3}$	$3.1x10^{-3}$	$40x10^{-3}$	$3x10^{-3}$	0.70
KB	$7x10^{-3}$	$0.03x10^{-3}$	$40x10^{-3}$	$3x10^{-3}$	0.54
HT29	$115x10^{-3}$	$2.4x10^{-3}$	$30x10^{-3}$	$22x10^{-3}$	0.87

a) NSCLC-N6: human bronchopulmunary non-small-cell-lung-carcinoma. P388: murine leukemia. P388/Dox : murine leukemia expressing the multi-drug resistance gene mdr, expecially towards doxorubicine. KB : human nasopharyngeal carcinoma. HT29 : human colon carcinoma. Results corresponding to the averages of three to five experiments.

Banc Eponge showed marked activity in antifungal and cytotoxic assays. We now wish to report the discovery of sphinxolide (**1**) and of three new congeneric macrolides sphinxolide B (**2**), C (**3**) and D (**4**) from the extracts of *Neosiphonia superstes*. The sphinxolides proved to be potent cytotoxins against various human carcinoma cells *in vitro* (Table**1**) and the activity of **2** exceed those of the previous marine macrolides. The discovery of a defined source of sphinxolide and its congeners is of the most importance and allow detailed biological and farmacological studies, which are now underway in our laboratory.. In this paper we describe evidences consistent with structures **2**, **3** and **4** of sphinxolide B , C and D, respectively, and report their cytotoxic activities *in vitro* (Table **1**).

RESULTS AND DISCUSSION

The lyophilized sponge (1 Kg) was extracted with *n* -hexane and then CH_2Cl_2/MeOH, 8:2. The latter

extract was chromatographed on silica gel (flash column MeOH/$CHCl_3$, 0-20%) followed by reverse-phase C18 μ-Bondapak HPLC, 73% MeOH aq., to give the major compound, later identified with sphinxolide (**1**)[9] and the new congeneric macrolides, named sphinxolide B (**2**), C (**3**) and D (**4**).

Structure determination was carried out mostly with **1**, $C_{54}H_{87}O_{15}N$ from FABMS, *m/z*=1012 $(M+Na)^+$ and ^{13}C NMR data (Table3). Restricted rotation about the N-methyl formyl terminus gives rise to doubled signals for H-37, N-Me, H-36, H-35, and 33-OMe and ^{13}C signals for C-37, N-Me, C-36, C-35, and C-32, all in a 2:1 ratio, a well known phenomenon previously encountered in stylocheilamide[11], scytophycins[12], ulapualides[3], kabiramides[4,5] and halichondramides[5,6]. Interpretation of 2D (^1H-^1H)-COSY and 2D-HOHAHA[13] indicated that the segments C_{37}-C_{30} C_1-C_5 and C_{12}-C_{24}, were present, and inspection of literature indicated that the same segments were present in the structure of sphinxolide (**1**). Identification of the major compound from the sponge *Neosiphonia superstes* was confirmed by comparison of ^1H, ^{13}C NMR (Table **2** and **3**), and UV spectral data of **1** with those reported for sphinxolide[9].

Sphinxolide B (**2**). The 30 mass units (FABMS, *m/z* =982) difference between **1** and **2** is reflected by the presence of only six methoxyl singlets in the ^1H NMR spectrum of **2**; thus sphinxolide B (**2**) was presumed to be a demethoxy analog of sphinxolide (**1**). The H-8 signals at δ 3.78 d in **1** was replaced in **2** by two allylic methylene protons, one at δ 2.23 dd (J=13.7, 6.1Hz), which does not overlap with anything else, and the other in a crowded region at δ 2.48. The lack of 8-methoxyl group in **2** also influences the chemical shift of H-39 observed shifted upfield to δ 5.93 s (δ 6.08 in **1**). Interpretation of partial structure C_6-C_8 in **2** was supported by a (^1H-^1H)-COSY experiment and confirmed by a ^{13}C NMR spectral analysis (Table **2** and **3**). The C-8 methine at 84.7 ppm in **1** was replaced by a methylene carbon at 40.7 ppm whereas the methylene carbon-6 was shifted downfield (δ_c 44.6 *vs.* 40.7 ppm in **1**) because of the removal of a γ-substituent. Thus, the novel sphinxolide B can be defined as the 8-demethoxysphinxolide (**2**).

Sphinxolide C (**3**) was larger than sphinxolide (**1**) by a CH_2 unit, FABMS (positive ion mode), *m/z* 1026 $(M+Na)^+$. The ^1H NMR spectrum indicated the presence of eight methoxyl singlets, thus suggesting **3** to be an O-methyl derivative of **1**. The ^1H and ^{13}C NMR spectra (Table **2** and **3**) were almost superimposable on those of sphinxolide, except for extra signals for an O-methyl group (δ_H 3.16; δ_C 57.3 ppm). In addition the C-19 in **3** was shifted downfield (δ_C 81.5 *vs.* 72.9 ppm in **1**), while H-19 signals was shifted upfield (δ_H 3.01 *vs.* 3.52 ppm in **1**) This indicated that C19-OH in sphinxolide was replaced by a O-methyl group in **3** . Interpretation of the COSY spectrum confirmed the sequence C_{13}-C_{25}, and according the location of extra methyl group at C19-OH.

Sphinxolide D (**4**) is the minor component of the mixture. The molecolar formula was deduced from FAB mass spectrometry, *m/z* 996 $(M+Na)^+$ and ^{13}C NMR data, indicating that one oxygen in sphinxolide was missing in **4**. This deduction was supported by the ^1H and ^{13}C spectra (Table **2** and **3**) which revealed that the methoxy group at C-8 was missing in **4**. In addition signals for an O-methyl group at δ_H 3.18 and δ_C 57.3 ppm and signals for C-19 observed shifted downfield (δ_C 81.2 *vs.* 72.9 ppm in **1**) and for H-19 shifted upfield (δ_H 3.12 *vs.* 3.52 ppm in **1**) were consistent with the 8-demethoxy-19-O-methyl sphinxolide structure (**4**).

As in the case of other macrolides existing as a mixture of two slowly interconverting isomers, the stereochemistry of the sphinxolides remain to be determined.

Table 2. ^{1}H NMR data for sphinxolide (1), B (2), C (3) and D (4)[a] ($CDCl_3$,500 MHz)

Position	1	2	3	4
1	-	-	-	-
2	5.79 d (15.3)	5.82 d (15.3)	5.79 d (15.3)	5.80 d (15.3)
3	7.52 dd (15.3,11.8)	7.52 (15.3,11.8)	7.52 (15.3,11.8)	7.50 (15.3,11.8)
4	6.07 d (11.8)	6.07 d (11.8)	6.07 d (11.8)	6.04 d (11.8)
5	-	-	-	-
6	2.32,2.46 dd	2.35,2.46 dd	2.32,2.42 dd	2.30,2.48
7	3.46 m	3.46 m	3.62 m	3.52 m
8	3.76 d (6.4)	2.23 dd,2.48 m	3.80 d (6.4)	2.41 dd,2.47 m
9	-	-	-	-
10	4.10 d (1.8)	4.08 d (1.8)	4.01 d (1.8)	3.98 d (1.8)
11	4.08 dd (9.5,1.8)	4.06 dd (9.5,1.8)	4.12 dd (9.5,1.8)	4.08 dd (9.5,1.8)
12	2.35 dd	2.35 dd	2.28 dd	2.31 dd
13	3.45 m	3.38 m	3.42 m	3.46 m
14	1.52,1.84 m	1.52,1.88 m	1.68,1.88 m	1.61,1.89 m
15	3.52 m	3.54 m	3.56 m	3.58 m
16	5.23 dd (15.4,8.5)	5.26 dd (15.4,8.5)	5.26 dd (15.4,8.5)	5.25 dd (15.4,8.5)
17	5.43 dd (15.4,7.8)	5.55 dd (15.4,7.8)	5.58 dd (15.4,7.8)	5.68 dd (15.4,7.8)
18	2.27 dd	2.31 dd	2.30 dd	2.42 dd
19	3.52 m	3.62 m	3.01 m	3.12 m
20	1.42,1.49 m	1.48,1.52 m	1.40,1.68 m	1.36,1.75 m
21	3.63 m	3.67 m	3.45 m	3.47 m
22	5.15 dd	5.26 dd	5.16 dd	5.18 dd
23	5.52 dd (15.1,10.0)	5.45 dd	5.50 dd	5.50 dd
24	2.44 dd	2.42 dd	2.50 dd	2.42 dd
25	5.12 dd	5.12 dd	5.18 dd	5.12 dd
26	1.93 dd	1.93 dd	1.94 dd	1.92 dd
27	2.69 dd	2.69 dd	2.70 dd	2.69 dd
28	1.68 m	1.68 m	1.68 m	1.70 m
29	1.68,1.73 m	1.68,1.38 m	1.72,1.40 m	1.72,1.40 m
30	2.45,2.53 m	2.45,2.55 m	2.45,2.57 m	2.45,2.50 m
31	-	-	-	-
32	2.72 d	2.72 d	2.72 d	2.72 d
33	3.43 m	3.46 m	3.43 m	3.44 m
34	2.11,2.44 m	2.15,2.44 m	2.11,2.41 m	2.11,2.44 m
35	5.11-5.08[b] m	5.11-5.08[b] m	5.11-5.08[b] m	5.11-5.08b m
36	6.5-7.18b d (15.0)	6.5-7.18[b] d (15.0)	6.5-7.18[b] d (15.0)	6.5-7.18b d (15.0)
37	8.28-8.02[b] s	8.28-8.02[b] s	8.28-8.02[b] s	8.28-8.02b s
38	1.93 s	1.93 s	1.93 s	1.93 s
39	6.08 s	5.93 s	6.11 s	5.93 s
40	-	-	-	-
41	1.14 d (6.9)	1.14 d (6.9)	1.13 d (6.9)	1.14 d (6.9)
42	0.84 d (6.9)	0.91 d (6.9)	0.84 d (6.9)	0.84 d (6.9)
43	1.03 d (6.9)	1.03 d (6.9)	1.03 d (6.9)	1.03 d (6.9)
44	0.91 d (6.9)	0.91 d (6.9)	0.92 d (6.9)	0.91 d (6.9)
45	0.97 d (6.9)	0.97 d (6.9)	0.98 d (6.9)	0.97 d (6.9)
46	0.97 d (6.9)	0.97 d (6.9)	0.98 d (6.9)	0.97 d (6.9)
47	3.03-3.08[b] s	3.03-3.08[b] s	3.03-3.08e s	3.03-3.08b s
OMe-7	3.35 s	3.29 s	3.31 s	3.30 s
OMe-8	3.39 s	-	3.40 s	-
OMe-13	3.22 s	3.23 s	3.23 s	3.25 s
OMe-15	3.22 s	3.24 s	3.23 s	3.26 s
OMe-19	-	-	3.16 s	3.18 s
OMe-21	3.28 s	3.29 s	3.28 s	3.32 s
OMe-27	3.38 s	3.37 s	3.37 s	3.37 s
OMe-33	3.35-3.27[b] s	3.37-3.28[b] s	3.33-3.26[b] s	3.37-3.29b s

[a]from 2D-COSY and 2D-HOHAHA experiments. The coupling constants are enclosed in parentheses and given in Hz. [b]signals for minor conformer.

Table 3. ^{13}C NMR data for sphinxolide (**1**), B (**2**) C (**3**) and D (**4**) ($CDCl_3$, 125 MHz).

Position	1	2	3	4
1	167.2	167.1	166.7	166.7
2	120.1	120.1	120.1	120.5
3	140.7	140.3	140.4	140.3
4	125.5	126.0	125.3	125.9
5	147.2	145.5	146.2	145.4
6	40.7	44.6	39.7	44.0
7	78.6	78.8	82.1	78.0
8	84.7	40.7	84.2	39.2
9	155.4	156.2	156.0	156.4
10	61.1	62.3	62.2	63.0
11	83.2	82.2	83.2	82.3
12	35.6	35.6	35.5	35.7
13	78.5	78.3	79.0	78.4
14	35.1	35.0	35.1	34.8
15	78.9	79.0	79.6	79.0
16	131.2	131.0	131.0	130.2
17	137.6	137.3	138.1	138.4
18	41.9	41.2	39.3	38.1
19	72.9	73.5	81.5	81.2
20	39.1	38.9	37.6	36.9
21	82.4	82.2	78.3	79.3
22	129.6	129.6	130.1	130.6
23	138.7	137.8	138.1	138.0
24	40.6	40.5	40.9	40.5
25	75.5	75.6	75.6	75.5
26	36.5	36.4	76.1	36.7
27	87.2	87.2	87.5	87.2
28	34.4	34.3	34.6	34.4
29	23.4	23.3	23.9	23.3
30	41.0	40.9	41.0	40.8
31	213.5	213.5	213.5	213.5
32	49.0(49.1)[a]	48.9(49.0)[a]	49.2(49.3)[a]	48.9(49.1)[a]
33	82.3	82.3	82.4	82.3
34	30.4(30.2)[a]	30.4(30.3)[a]	30.8(30.2)[a]	30.4(30.2)[a]
35	105.4(107.1)[a]	105.4(107.1)[a]	105.7(107.0)[a]	105.4(107.0)[a]
36	130.3(126.3)[a]	130.3(126.3)[a]	130.4(126.5)[a]	130.4(126.3)[a]
37	162.1(160.8)[a]	162.0(160.0)[a]	162.0(160.7)[a]	162.1(160.7)[a]
38	18.6	17.4	18.8	18.1
39	121.4	120.7	120.3	119.9
40	164.0	164.1	163.9	164.3
41	11.5	11.6	11.2	11.1
42	15.7	14.3	15.1	14.6
43	17.6	17.8	18.4	17.9
44	9.9	9.9	10.3	10.0
45	17.4	17.2	17.4	17.4
46	12.7(12.8)[a]	12.7(12.8)[a]	12.7(12.8)[a]	12.7(12.8)[a]
47	27.5(32.9)[a]	27.6(33.0)[a]	27.6(32.9)[a]	27.6(33.1)[a]
OMe-7	57.6	57.6	57.6	57.7
OMe-8	58.2	-	58.1	-
OMe-13	55.6	55.8	55.6	55.7
OMe-15	55.9	55.9	55.8	55.8
OMe-19	-	-	57.3	57.3
OMe-21	57.6	57.7	57.2	57.2
OMe-27	61.5	61.4	61.3	61.4
OMe-33	57.6	57.5	57.6	57.5

[a]Signals for minor conformer. The assigments were aided by HETCOR correlation experiments and DEPT measurements

EXPERIMENTAL

General Methods.

All NMR measurements were performed on a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer, using the UXNMR software package.

Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional pulse sequence. The COSY spectra were obtained using a data set (t_1 X t_2) of 1024 X 1024 points for a spectral width of 4201.68 Hz (relaxation delay 1 s). The data matrix was processed using a unshifted sine bell window function, following transformation to give a magnitude spectrum with symmetrization (digital resolution in both F2 and F1 dimensions 1.13 Hz/pt).

The 2D-HOHAHA experiment was performed in the phase-sensitive mode (TPPI) using a MLEV-17 sequence for mixing. The spectral width (t_2) was 4201.68 Hz; 512 experiments of 40 scans each (relaxation delay 1.5 s, mixing time 100 ms) were acquired in 2K data points. For processing, a unshifted sine bell window function was applied in both dimension before transformation. The resulting digital resolution in F2 was 0.48 Hz/pt.

^{13}C, ^{1}H NMR shift correlation experiments (HETCOR): the spectral width in ^{13}C dimension was 23801.52 Hz (1024 points) and 4337.29 Hz (256 time uncrements) along the ^{1}H domain; for each feed 200 scans were recorded. A sine square window function was applied in both dimension prior to Fourier trasformation.

Optical rotation were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. Fast atom bombardment mass spectra (FAB MS) were recorded in a glycerol-tioglicerol matrix (with 10 μl of a solution of NaCl 0.1 N) in the positive ion mode on a VG ZAB instrument (Argon atoms of energy of 2-6 kV).

Animal Collection and Preliminary Experiments.

Neosiphonia superstes Sollas (Demospongiae, Lithistida, Phymatellidae) was collected during the dredging campaigns (1987, 1989) of the ORSTOM-CNRS, Programme "Substances Marines d'Interest Biologique (SMIB)" in the South of New Caladonia (Banc Eponge region) at depth of 500-515 m. Taxonomic identification was performed by Lèvi and Lévi[10] at the ORSTOM Centre de Nouméa where reference specimens are on file (reference 1408). Preliminary assays for cytotoxic (KB cells and P388 leukemia cells) and antifungal activities (*Fusarium oxysporum*, *Phythophthora hevea* and *Penicillium digitatum*) showed a marked activity of chloroformic extract.

Extraction.

The organisms were freeze dried and the lyophilized material (1 Kg) was extracted with *n*-hexane (3x1L), then with CH_2Cl_2:MeOH 8:2 (3x1L) and finally with MeOH (3L). The dichloromethane-methanol extract was filtered and concentrated under reduced pressure to give 12 g of a yellow cytotoxic oil (*Artemia salina*, IC_{50} 10μg/mL).

Isolation.

The crude dichloromethane-methanol extract was chromatographed by MPLC on a SiO_2 column (1 Kg) using a solvent gradient system from $CHCl_3$ to $CHCl_3$:MeOH 8:2. Fractions eluted with $CHCl_3$:MeOH 98:2

(605 mg) were further purified by HPLC on a Waters C-18 μ-Bondapak column (7.8 mm i.d. x 30 cm) with MeOH:H_2O (73:27) as eluent (flow rate 5 mL/min.) to give 85.4 mg of sphinxolide (**1**) (t_r=12.0 min.), 30.9 mg of sphinxolide B (**2**) (t_r=15.6 min.), 27.1 mg of sphinxolide C (**3**) (t_r=19.8 min.) and 15.7 mg of sphinxolide D (**4**) (t_r=21.6 min.).

*Compound **1**. m/z* 1012 $(M+Na)^+$; $[\alpha]_D$(MeOH) =-3.1°.

*Compound **2** m/z* 982 $(M+Na)^+$; $[\alpha]_D$(MeOH) =+2.8°.

*Compound **3** m/z* 1026 $(M+Na)^+$; $[\alpha]_D$(MeOH) =-11.8°.

*Compound **4** m/z* 996 $(M+Na)^+$; $[\alpha]_D$(MeOH) =-3.2°.

Determination of biological activity.

KB cells were maintained in a.suspension culture of BME medium supplemented with 5% calf serum containing 1% of glutamine at 200 mM and 1% of antibiotic solution (streptomycin : 10000 UI/mL). NSCLC-N6-L16, P388 and HT29 cells were maintained in a suspension culture of RPMI supplemented with 5% calf serum containing 1% of glutamine solution at 200 mM and 1% of a penicillin-streptomycin mixture (10000 UI/mL). P388/Dox cells were maintained in a suspension culture of RPMI supplemented with 5% calf serum containing 1% of glutamine solution at 200 mM, 10 ng doxorubicine and 1% of a penicillin, streptomycin mixture (10000 UI/mL). A 50 μL aliquot of each cell culture [NSCLC cells ($1x10^4$), HT29 cells ($1x10^4$), KB cells ($1x10^3$), P388 cells ($5x10^3$) or P388/Dox cells ($5x10^3$)] was mixed with a 50 μL aliquot of serial dilution of compounds and the mixture was incubated in a microtiter well plate (96-well Falcon 3072) for 72 h at 37°C in a humified incubator containing 5% CO_2 in air. Cell proliferation was estimated by a colorimetric test : 10 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (SIGMA) was added. After 4 h the dark blue cristals, formed in mitochondria of living cells during the reduction of MTT, were solubilized with 100 μL of isopropanoic acid. Microplates were read by ELISA using a multiskar Titertek multiscan MK2 with a 570 nm filter. The optical dentity of the wells then enable a dose/effect curve to be plotted and the IC_{50} (growth cell inhibition for each product relative to the controls), to be determined.

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