New Pyridinium Alkaloids from a Marine Sponge of the Genus *Spongia* with a Human Phospholipase A_2 Inhibitor Profile

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Four new bioactive pyridinium alkaloids, named spongidines A−D (**5**−**8**), have been isolated from a Vanuatu sponge of the genus *Spongia*, together with known petrosaspongiolides D (**1**) and G (**2**). Compounds **3** and **4** are 21-hydroxy derivatives of petrosaspongiolides K and P. Structure elucidation was accomplished through extensive 2D NMR experiments (COSY, ROESY, HMBC, HMQC) and IR, UV, and FABMS data. All compounds significantly inhibited human synovial phospholipase A_2 (PLA_2) at 10 μM, with an IC_{50} value of 5.8 μM for compound **4**, which is the most potent inhibitor, with a higher selectivity toward this enzyme than the reference inhibitor manoalide. Pyridinium alkaloids (**5**−**8**) mainly inhibited human synovial PLA_2. Compound **8**, which contains a sulfonic acid group, is the most interesting inhibitor.

Marine sponges of the genus *Spongia* have been extensively investigated and have gained importance for their high content of malonate-derived metabolites[1] with a variety of biological activities. A broad array of sesquiterpenoids, diterpenoids, and sesterterpenoids[2−4] have also been isolated from other Dictyoceratida and Dendroceratida sponges and soft corals. Potent antiinflammatory agents[5−7] are sesterterpenes with some structural features, like a γ-hydroxy-butenolide moiety, exemplified by the monocarbocyclic manoalide[8] and by the tricarbocyclic luffolide,[9] which are inhibitors of various secreted forms of phospholipase A_2 (PLA_2).[10]

In our ongoing research program on new bioactive metabolites from Vanuatu Islands marine invertebrates, we found significant activity in the chloroform extracts of a sponge of the genus *Spongia* (family Spongiidae; order Dictyoceratida). Along with the major metabolites petrosaspongiolides D (**1**) and G (**2**) recently reported in the sponge *Petrosaspongia nigra* (Dictyoceratida),[11,12] we isolated six new metabolites. Two of them are closely related to petrosaspongiolides K[12] and P[13] (**3** and **4**), and four are new pyridinium alkaloids (**5**−**8**) named spongidines A−D.

Herein we report their isolation, structure elucidation, and the data of their in vitro tests on the inhibition of five different secretory PLA_2 ($sPLA_2$), belonging to the groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant enzyme), and III (bee venom enzyme), as well as on cytosolic PLA_2 ($cPLA_2$), from macrophage line RAW 264.7 (group IV), using manoalide as reference inhibitor. None of these compounds exerted cytotoxic effects on human neutrophils at the concentrations tested (see Table 3).

Table 1. ^1H and ^{13}C NMR Assignments for Spongidine A (**5**) and HMBC Correlations (CD_3OD)

position	δ_H[a]	δ_C	HMBC[b]
1	0.99, 1.88	40.6	
2	1.54, 1.77	19.6	
3	1.23, 1.45	43.2	
4		34.3	
5	0.99	57.3	C-4, C-10, C-18, C-19
6	1.70, 1.83	19.4	
7	1.58, 2.53 dd (12.4, 3.0)	40.4	C-5, C-9
8		40.5	
9	1.36	54.7	C-8, C-12, C-19, C-20
10		40.0	
11	1.80, 2.05 m	18.0	C-8, C-12, C-13
12	2.97 m, 3.15 dd (18.0, 6.2)	28.5	C-9, C-11, C-13, C-14, C-21
13		137.7	
14		170.2	
15	7.90 d (6.3)	124.7	C-8, C-13, C-16
16	8.49 d (6.3)	143.3	C-14, C-15,C-21, $-CH_2$COOH
17	0.93 s	33.8	C-3, C-4, C-5, C-18
18	0.93 s	21.9	
19	1.04 s	16.8	C-1, C-5, C-9, C-10
20	1.32 s	25.6	C-7, C-8, C-9, C-14
21	8.53 s	146.3	C-12, C-13, C-14, C-16, $-CH_2$COOH
$-CH_2COOH$		170.5	
$-CH_2$COOH	5.07 s	63.9	C-16, C-21, $-CH_2COOH$

[a] Coupling constants are in parentheses and given in Hz. ^1H assignments aided by COSY experiments. [b] HMBC optimized for $^{2,3}J_{CH} = 10$ Hz.

Results and Discussion

Freeze-dried specimens (1.0 kg), collected near Tongoa (Vanuatu Islands) were extracted with MeOH. The methanol extract was subjected to Kupchan's partitioning methodology.[14] The chloroform-soluble material was then fractionated by DCCC using $CHCl_3$−MeOH−H_2O (7:13:8) followed by reversed-phase HPLC (MeOH−H_2O, 7:3) to yield compounds **3**−**8** and a large amount of the previously

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Table 2. ^{1}H and ^{13}C NMR Assignments for Spongidine D (**8**) and HMBC Correlations (CD_3OD)

position	δ_H[a]	δ_C	HMBC[b]
1	0.97, 1.88	40.8	
2		19.9	
3	1.22, 1.46	43.2	
4		34.6	
5	0.95	57.3	C-4, C-10, C-18, C-19
6	1.70, 1.88	19.6	
7	1.58, 2.50 dd (12.4, 3.0)	39.7	C-5, C-9
8		40.2	
9	1.35	54.6	C-10, C-12, C-19, C-20
10		39.2	
11	1.79, 2.06 m	16.9	C-8, C-9, C-12, C-13
12	2.98 m, 3.15 dd (18.0, 6.2)	28.5	C-9, C-11, C-13, C-14, C-21
13		137.8	
14		171.0	
15	7.93 d (6.3)	124.9	C-8, C-13, C-16
16	8.65 d (6.3)	142.8	C-14, C-15, C-21, $-CH_2CH_2SO_3H$
17	0.93 s	33.8	C-3, C-4, C-5, C-18
18	0.93 s	21.3	C-17
19	1.04 s	16.9	C-1, C-5, C-9, C-10
20	1.32 s	25.6	C-7, C-8, C-9, C-14
21	8.68 s	146.1	C-12, C-13, C-14, C-16, $-CH_2CH_2SO_3H$
$-CH_2CH_2SO_3H$	4.88 t (6.2)	57.5	C-16, C-21, $-CH_2CH_2SO_3H$
$-CH_2CH_2SO_3H$	3.43 t (6.2)	51.1	$-CH_2CH_2SO_3H$

[a] Coupling constants are in parentheses and given in Hz. ^{1}H assignments aided by COSY experiments. [b] HMBC optimized for $^{2,3}J_{CH} = 10$ Hz.

isolated petrosaspongiolides D (**1**) and G (**2**). The identity of the known compounds was established by comparison of their spectral properties with authentic samples.[12]

21-Hydroxy petrosaspongiolide K (**3**), showed in the FABMS spectrum (positive ion), a molecular ion peak at *m/z* 389 $(M + H)^+$, 16 mass units more than petrosaspongiolide K isolated from *Petrosaspongia nigra*.[12] The presence of an α,β-unsaturated ketone was supported by UV (λ_{max} 235 nm; $\epsilon = 5450$). NMR data were very similar to those of petrosaspongiolide K except for one tertiary methyl proton signal replaced by an AB system at δ_H 3.73 and 3.38 (2H, AB system, $J = 12.0$ Hz, H_2-21), indicating that **3** is a hydroxy-derivative of petrosaspongiolide K.[12] The protonated carbon signals were first assigned from HMQC data, and then an HMBC experiment revealed long-range coupling between the H-5 proton signal at δ_H 1.02 and carbon signals at δ_C 65.0 (C-21) and 17.6 (C-22). The signal of H_2 protons at C-21 (δ_H 3.73 and 3.38) showed correlations to the C-20 (δ_C 27.8), C-3 (δ_C 36.8), C-4 (δ_C 40.0), and C-5 (δ_C 58.8). These correlations confirm that the signal at δ_C 65.0 is due to C-21 and that the oxymethylene is joined to C-4. (^{1}H and ^{13}C NMR are given in the Experimental Section.)

21-Hydroxy petrosaspongiolide P (**4**) had a molecular formula $C_{25}H_{38}O_6$ as established by HRFABMS. Analysis of the ^{1}H and ^{13}C NMR spectra revealed the presence of two interconverting species formed by a tricyclic carbon framework in its structure, as observed in terpenoids, and a γ-hydroxybutenolide unit. NMR data are consistent with those reported for petrosaspongiolide P,[13] which revealed the typical hemiacetal functionality of the γ-hydroxybutenolide ring with an additional hemiacetal function in the molecule. The only significant differences in the NMR data were for signals corresponding to ring A. The upfield shift of C-3 (δ_C 36.7 ppm) and the presence of an oxygenated methylene carbon at δ_C 65.1 are consistent with a hydroxyl group at C-21. (^{1}H and ^{13}C NMR are given in the Experimental Section.)

Spongidine A (**5**) had a molecular formula of $C_{23}H_{34}NO_2$ as established by HRFABMS (*m/z* 356.2499; calcd 356.2590) and NMR data. The FAB (positive ion) showed a fragment peak at *m/z* 312 (loss CO_2), thereby indicating the presence of a carboxyl group. The ^{1}H NMR spectrum exhibited signals for four methyl singlets (δ_H 0.93 × 2, 1.04, and 1.32), three aromatic protons (δ_H 7.90, d, $J = 6.3$ Hz; δ_H 8.49 d, $J = 6.3$ Hz, and δ_H 8.53 s), and a methylene proton signal at δ_H 5.07 (s) (Table 1).

The ^{13}C NMR (Table 1) spectrum revealed 23 carbons, including signals for one carboxyl group (170.5 ppm), four quaternary methyls (33.8, 25.6, 21.9, and 16.8 ppm), and three quaternary carbons (40.5, 40.0, and 34.3 ppm), consistent with a tricarbocyclic skeleton with a *gem*-dimethyl group at C-4 and two methyl groups at the ring junction C-8 and C-10. In the ^{13}C NMR spectrum are also detectable five aromatic carbons, two quaternary (170.2 and 137.7 ppm) and three CH (146.3, 143.3 and 124.7 ppm), implying the presence of a disubstituted pyridinium salt. The UV [λ_{max} 267 nm, 273 (sh) and 212] and the IR (1637 cm^{-1}) spectra exhibited absorption characteristic of an alkylpyridinium salt.[15] The large, broad absorption at 1637 cm^{-1} was also due to the presence of a carboxylate group as shown by ^{13}C NMR data. Inspection of the COSY spectrum allowed us to assemble three partial structure units: C-1 to C-3, C-5 to C-7, and C-9 to C-12. The H_2-12 protons, downfield shifted at δ_H 2.97 and δ_H 3.15, are typical for a benzylic methylene, indicating that the pyridinium moiety was fused to tricarbocyclic skeleton at C-13 and C-14. In the HMBC experiment this was supported by cross-peaks: H-15/C-8,C-13,C-16; H-21/C-14,C-16,C-13,C-12; H-16/C-14,C-15,C-21. The remaining methylene protons at δ_H 5.07 were long-range coupled to the C-16,C-21 and to the carboxylic group (Table 1).

Spongidine B (**6**) had a molecular formula of $C_{25}H_{36}NO_4$, $C_2H_2O_2$ larger than **5** as shown in the FAB (positive ion) at *m/z* 414 $[M]^+$. ^{1}H and ^{13}C NMR data were quite similar to those of **5**, except for the lack of a methyl group replaced by an acetoxymethyl group in **6**. A significant downfield shift of C-4 (δ_C 38.2 vs. 34.3 ppm in **5**) and an upfield shift

Table 3. Effect of Compounds **3**−**8** on Different $sPLA_2$ Activities[a]

compound	*N. naja* venom % I (10 μM)	pancreas % I (10 μM)	human synovial %I (10 μM) IC_{50}(μM)	RAP[b]+zymosan % I (10 μM)	bee venom %I(10 μM) IC_{50}(μM)
3	1.3 ± 0.8	14.3 ± 6.8	34.4 ± 6.5[d]	18.8 ± 3.2	37.1 ± 6.3[d]
4	8.7 ± 3.9	19.5 ± 3.6[d]	87.2 ± 2.1[d] 5.8	25.6 ± 1.9[d]	5.4 ± 2.1
5	0.5 ± 0.5	18.0 ± 8.1	40.1 ± 7.7[d]	17.1 ± 4.6	33.1 ± 6.0[c]
6	0.4 ± 0.4	14.2 ± 5.1	34.6 ± 5.8[d]	17.9 ± 4.2	32.2 ± 6.0[c]
7	3.1 ± 2.2	9.1 ± 3.5	40.4 ± 5.7[d]	30.9 ± 5.3[c]	36.2 ± 5.4[d]
8	0.0 ± 0.0	7.6 ± 4.0	48.2 ± 3.8[d]	19.6 ± 5.4	37.6 ± 6.5[c]
manoalide	17.0 ± 1.7[c]	32.3 ± 2.7[d]	93.2 ± 0.2[d] 3.9	38.4 ± 0.5[d]	62.5 ± 3.8[d] 7.5

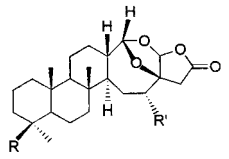
[a] Results show percentages of inhibition at 10 μM and IC_{50} (μM) values determined only for those compounds that reach 50% of inhibition. Mean ± S.E.M. ($n = 6$). [b] RAP: Rat air-pouch PLA_2. [c] $p<0.05$; [d] $p<0.01$.

of C-3 (δ_C 37.4 ppm vs. 43.2 in **5**) and C-18 (δ_C 27.8 ppm vs. 21.9 in **5**) implied the presence of an acetoxymethyl group at C-4 (δ_H 4.36−3.98, AB system, $J = 11.0$ Hz, CH_2) resonating in the ^{13}C NMR at 68.2 ppm. The stereochemistry at C-4 was established by a ROESY experiment. The acetoxymethyl group was assigned as axial because of intense cross-peaks between the H_2-17 protons and the angular methyl protons at C-19 (δ_H 1.04). (For NMR data, see Experimental Section.)

Spongidine C (**7**) showed a molecular ion peak at *m/z* 428 in the FAB (positive ion), in agreement with the composition $C_{26}H_{38}NO_4$ (72 mass units more than **5**). The UV and IR spectra were suggestive of an alkylpyridinium salt, as observed in spongidines A (**5**) and B (**6**). Analysis of ^1H and ^{13}C NMR spectra and interpretation of $^1H-^1H$ COSY data established the relationship between **7** and spongidines A (**5**) and B (**6**). All signals assigned to rings A, B, and C, and the glycine residue in the ^1H NMR spectrum were present, whereas in the aromatic region a signal of the pyridinium system was missing. Two aromatic protons resonating as singlets (δ_H 8.42 and 7.81) are detectable, implying a trisubstituted pyridinium salt. Inspection of the COSY spectrum showed two methylene proton signals at δ_H 3.20 (t, $J = 6.8$) and 2.68 (t, $J = 6.8$) mutually coupled. This latter exhibited, in the HMBC experiment, a cross-peak with a carbonyl signal at 179.4 ppm, which allowed constructing a propionic side chain. The observation that the C-24 aromatic proton and C-18 methylene proton exhibited HMBC interaction with C-16 (δ_C 157.4) helped to locate the side chain at C-16 of a pyridinium moiety. This compound is apparently a 25-norsesterterpene derived by condensation with glycine of a 16-keto-24-al precursor.[12]

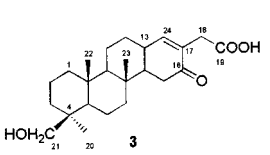
The HRFABMS of **8** showed a molecular ion peak at *m/z* 406.2366, in agreement with the molecular formula $C_{23}H_{36}NO_3S$ (calcd 406.2416). ^1H and ^{13}C NMR data of **8** are superimposable on those of **5** for the tricyclic carbon system, but significantly different NMR shifts are seen for the signals of the pyridinium salt. Inspection of the COSY spectrum showed two methylene triplets at δ_H 4.88 ($J = 6.2$ Hz) and δ_H 3.43 ($J = 6.2$ Hz) mutually coupled, showing correlation by HMQC with carbon signals at δ_C 57.5 and 51.1, respectively. The IR spectrum contained an absorbance at 1637 cm^{-1}, typical for an alkylpyridinium salt, and two strong bands at 1216 and 1048 cm^{-1}, suggestive of a sulfonic acid salt.[16] All these data suggested the presence of a taurine residue. The HMBC data (Table 2) showed cross-peaks $-CH_2-CH_2-SO_3H$/C-21,C-16 and $-CH_2-CH_2-SO_3H$, which imply that the taurine moiety is bonded to the pyridinium ring through the nitrogen atom.

Sesterterpenes with a γ-hydroxybutenolide moiety have received special attention after the discovery of manoalide,[8] a potent antiinflammatory agent that binds and blocks the enzyme PLA_2 through the formation of a covalent inhibitor-enzyme adduct.[17] Two main groups of PLA_2 enzymes have been reported,[18] the secretory PLA_2 (sPLA_2 groups I, II, III, V, IX, and X with a relatively small molecular weight) and the cytosolic PLA_2 (cPLA_2 groups IV, VI, VII, and VIII with a higher molecular weight). They also differ in Ca^{2+} requirements and molecular characteristics. It is well-known that inhibition of specific PLA_2 constitutes a potentially useful approach for treating a great variety of inflammatory disorders such as septic shock, adult respiratory distress syndrome, arthritis, and acute pancreatitis. Unfortunately, no potent and absolutely type-specific PLA_2 inhibitors are widely available. Recently, petrosaspongiolides M−R, isolated from *Petrosaspongia nigra*,[13] have been tested in vitro on PLA_2 from different sources, where they showed a high degree of specificity toward human synovial PLA_2. Compounds **3**−**8** have been tested under the same experimental conditions on five different sPLA_2[18] belonging to the groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant and rat air-pouch secretory enzymes[19]), and III (bee venom enzyme). Among petrosaspongiolide derivatives, compound **4** inhibited preferentially human synovial PLA_2 in the μM range (Table 3), showing a slightly lower potency toward this enzyme than that of the reference inhibitor, manoalide. In contrast, compounds **3**−**8** had no inhibitory effects on cPLA_2, although this enzyme was partially inhibited by manoalide at 10 μM (44.6 ± 5.6 of inhibition, mean ± SEM, $n = 6$).



1 R=CH_2OH R'=OAc Petrosaspongiolide D

2 R=CH_2OH R'=OH Petrosaspongiolide G



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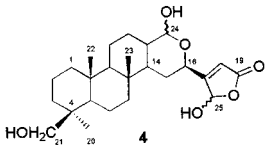
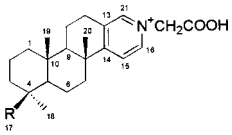
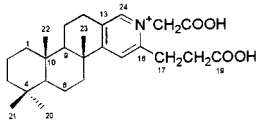


4

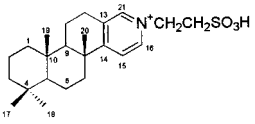


5 R=CH_3 Spongidine A

6 R=CH_2OAc Spongidine B



7 Spongidine C



8 Spongidine D

It is interesting to note that pyridinium alkaloids **5**−**8** inhibited mainly human synovial PLA_2 at 10 μM. Among them, compound **8**, containing a sulfonic acid group, can be considered the most interesting inhibitor. In this regard, these pyridinium alkaloids can offer new structural requirements for further studies about mechanistic interactions between PLA_2 enzymes and inhibitors as it has been described for manoalide-like sesterterpene molecules.[20] None of the pyridinium alkaloids (**5**−**8**) inhibited cPLA_2 (data not shown).

On the other hand, compounds **3**−**8** were devoid of significant cytotoxic effects on human neutrophils at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (data not shown).

Experimental Section

General Experimental Procedures. NMR spectra were obtained on a Bruker AMX-500 (^1H at 500 MHz, ^{13}C at 125

MHz) instrument, with δ (ppm), J in Hz, and spectra referred to CD_3OD as internal standard. MS were recorded with a VG PROSPEC instrument equipped with a FAB source (Cs^+ ion bombardment), using a glycerol or glycerol−thioglycerol (3:1) matrix. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. Reversed-phase HPLC were performed on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.; flow rate 2 mL min^{-1}); Waters model 6000 A pump equipped with U6K injector and a differential refractometer, model 401; DCCC, on a DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes.

Chemicals and Enzymes. Human synovial recombinant PLA_2 were kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K. Both [9,10-^{3}H]oleic acid] and l-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] were purchased from Du Pont (Itisa, Madrid, Spain). The rest of the reagents were from Sigma Chemicals (St. Louis, MO). *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain.

Assay of sPLA$_2$. $sPLA_2$ was assayed by using a modification of the method of Franson et al.[21] *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% NaCl, and 0.6% sodium dihydrogen orthophosphate, pH 5.0, and grown for 6−8 h at 37 °C in the presence of 5 μCi/mL [^{3}H]oleic acid (sp. act. 10 Ci/mmol). After centrifugation at 2500 × g for 10 min, the cells were washed in buffer [0.7 M Tris−HCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin (BSA) pH 8.0], resuspended in saline, and autoclaved for 30 to 45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom enzyme, porcine pancreatic enzyme, human recombinant synovial enzyme, and bee venom enzyme were diluted in 10 μL of 100 mM Tris−HCl and 1 mM $CaCl_2$ buffer, pH 7.5. Supernatants (10 μL) of exudates from zymosan-injected rat air-pouch[18] were also used as a source of $sPLA_2$. Enzymes were preincubated at 37 °C for 5 min with 2.5 μL of test compound solution or its vehicle in a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 10 μL of autoclaved oleate-labeled membranes and was terminated by addition of 100 μL ice-cold solution of 0.25% BSA in 100 mM Tris−HCl and 1 mM $CaCl_2$ buffer, pH 7.5, to a final concentration of 0.07% w/v. After centrifugation at 2500 × g for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting.

Assay of cPLA$_2$. $cPLA_2$ activity was measured as the release of radiolabeled arachidonic acid according to the method of Clark et al.[22] using cytosolic fractions of murine monocyte cell line RAW 264.7 as the source of enzyme and 1-palmitoyl-2-[^{14}C] arachidonyl-*sn*-glycero-3-phosphocholine (57.0 mCi/mmol, 2 × 10^6 cpm) as substrate. Test compounds were dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1%, which showed no effect on the enzyme activity. The reaction was stopped after a 60-min incubation period at 37 °C by mixing with 0.5 mL of isopropyl alcohol−heptane−0.5 M H_2SO_4 (10:5:1). Heptane (0.7 mL) and water (0.2 mL) were then added, and the solution was vigorously mixed for 15 s. The heptane phase was mixed with 100 mg of Si gel 60 (Merck, 70−230 mesh) and centrifuged, and the radioactivity in each supernatant was measured.

Cytotoxicity Assays. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[23] was used to assess the possible cytotoxic effects of test compounds on human neutrophils.

Statistical Analysis. The results are presented as mean ± SEM Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations (n = 6). The level of statistical significance was determined by analysis of variance (ANOVA), followed by Dunnett's t-test for multiple comparisons.

Animal Material. The sponge was collected near Tongoa (Vanuatu Islands) in 1996, and identified only to the genus *Spongia* by John Hooper of Queensland Museum, South Brisbane, Australia (QM species no. 2128).

Voucher specimen R1644 was deposited at ORSTOM center in Noumea. The sponge was found growing on sides of coral heads, in gullies, and on walls of coral reefs, at 10−35 m depth. It has a thickly encrusting lobate ridge-like growth form, spreading over the coralline substrate. Alive, the sponge is light gray with a mauve tinge, but gray in EtOH. There are numerous, discrete oscules up to 5 mm in diameter, regularly distributed over the surface, with slightly raised membranous lip and slightly raised on papillae, about 3−8 mm high. The sponge is firm and springy, but only slightly compressible. The surface ornamentation is described as opaque, membranous, optically smooth; it has uneven, microconulose goose-flesh. The ectosomal skeleton is slightly arenaceous with a thin scattering of fine sand-grains and spicule detritus; surface membrane was pushed up into microconules by ascending choanosomal fibres. The choanosomal skeleton is composed mainly of a regular reticulation of secondary spongin fibers infrequently interconnected by finer teriary fibers. Primary fibers are reduced in number and occur throughout the choanosome, partially cored by sand detritus, whereas secondary and tertiary fibers are completely clear. Fibers are moderately well developed and lack any sign of lamination. Mesohyl collagen is moderately light and homogeneous, containing scattered sand grains and spicule detritus only in peripheral regions.

This species is clearly a member of the widely distributed genus *Spongia*, in having a reduced primary fiber system (cored by foreign debris) and a highly developed secondary network of fine, intertwined fibers making up the bulk of the skeleton (clear of any coring material) (Bergquist, 1980). Unfortunately, there are many, possibly hundreds of, species of *Spongia*, some of which are described in the literature and certainly many still undescribed. Furthermore, many of those known to science were described nearly a century ago, with descriptions poor by today's standards and type material scattered all over the world. This particularly applies to the many species of *Spongia* (and its synonym *Euspongia*) described by von Lendenfeld at the end of the 1800s, many of which were collected from Australasian waters. Until these ancient species are reexamined and reconciled with those collected more recently, with underwater observations pertaining to their living characteristics, it is not possible to name the present species. Nevertheless, this species shows some similarities to *Spongia reticulata* (Lendenfeld, 1885) from New South Wales (and allegedly also from New Zealand), but differs in that the latter has a much more prominently conulose surface, fewer larger oscules grouped around a central pore area, "shaggy" fiber endings around oscules, and a massive cushion shape. The fiber network and fiber diameter are otherwise similar between the two species, with the present one possibly new to science.

Extraction and Isolation. Preliminary assays for cytotoxic activity showed marked activity for the ethanolic extract (KB cells, 96% inhibition at 10 μg/mL). The organisms were freeze-dried, and the lyophilized material (1.0 kg) was extracted with MeOH (3 × 2 L) at room temperature. The extracts were combined and partitioned according to the modified Kupchan[14] procedure as follows.

The methanol extract (80 g) was dissolved in a mixture of MeOH−H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 20% and 40%, and partitioned against CCl_4 and $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butanol. The bioactive chloroform extract (3.2 g) was chromatographed by DCCC using $CHCl_3$−MeOH−H_2O (7:13:8) in the ascending mode (the upper phase was the stationary phase).

Fractions 19−30 (380 mg) were purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.) with MeOH−H_2O 70% to yield pure petrosaspongiolide D (**1**) (10 mg), petrosaspongiolide G (**2**) (7.0 mg), compound **3** (5.0 mg), compound **4** (8.0 mg), spongidine A (**5**) (3.0 mg), spongidine B (**6**) (2.8 mg), spongidine C (**7**) (1.2 mg), and spongidine D (**8**) (3.0 mg).

Compound 3: $[\alpha]_D$ −37.1° (*c* 0.015, MeOH); ^{1}H NMR (CD_3OD) δ_H 6.58 (1H, br s, H-24), 3.73−3.38 (2H, d, J = 12.0, H_2-21), 3.09−2.97 (2H, d, J = 15.6, H_2-18), 2.43 (1H, br t, J =

10.4, H-13), 2.37 (1H, dd, J = 16.0, 3.7, H-15), 2.23 (1H, t, J = 16.0, H-15), 2.04−1.22 (2H, H_2-12), 1.83−0.93 (2H, H_2-3), 1.76−0.88 (2H, H_2-1), 1.75−1.02 (2H, H_2-7), 1.72−1.49 (2H, H_2-11), 1.69−1.47 (2H, H_2-6), 1.62−1.42 (2H, H_2-2), 1.54 (1H, H-14), 1.02 (1H, H-5), 0.95 (3H, s, Me-20), 0.94 (3H, s, Me-23), 0.93 (1H, H-9), 0.89 (3H, s, Me-22); ^{13}C NMR (CD_3OD) δ_C 202.8 (C-16), 178.5 (C-19), 154.4 (C-24), 135.4 (C-17), 65.0 (C-21), 61.0 (C-9), 58.8 (C-5), 55.7 (C-14), 41.6 (C-7), 41.3 (C-1), 40.0 (C-4), 38.7 (C-18), 38.6 (C-15), 38.6 (C-10), 38.2 (C-13), 37.8 (C-8), 36.8 (C-3), 34.1 (C-12), 27.8 (C-20), 21.8 (C-11), 19.6 (C-6), 19.3 (C-2), 17.6 (C-22), 15.7 (C-23), HMBC H-5/C21, C22; H-15/C13, C14, C16, C17; H_2-18/C17, C24, C19, C16; H_3-20/C3, C4, C5, C21; H_2-21/C20, C3, C4, C5; H_3-22/C10, C1, C5, C9; H_3-23/C8, C7, C14, C9; H-24/C12, C14, C18, C16.

Compound 4: $[\alpha]_D$ −14.3° (*c* 0.015, MeOH); ^{13}C NMR (CD_3OD) δ_C 173.4 (C-19), 164.7 (C-17), 118.2 (C-18), 101.8 (C-24), 95.5 (C-25), 73.0 (C-16), 65.1 (C-21), 61.1 (C-9), 58.5 (C-5), 53.4 (C-14), 42.4 (C-13), 41.2 (C-1), 40.2 (C-10), 38.5 (C-4), 39.7 (C-7), 37.6 (C-8), 36.7 (C-3), 30.7 (C-15), 29.5 (C-12), 27.6 (C-20), 20.7 (C-11), 19.6 (C-6), 19.2 (C-2), 17.3 (C-22), 15.3 (C-23); ^{1}H NMR (CD_3OD) δ_H 6.09 (1H, br s, H-25), 5.02 (1H, d, J = 3.1 Hz, H-18), 4.37 (1H, d, J = 8.0 Hz, H-24), 4.36 (1H, ddd, J = 11.0, 2.2, 2.2, H-16), 3.74−3.39 (2H, d, J = 11.7 Hz, H_2-21), 1.00 (3H, s, Me-20), 0.94 (3H, s, Me-22), 0.93 (3H, s, Me-23).

Spongidine A (5): $[\alpha]_D$ −16.2° (*c* 0.010, MeOH); ^{1}H and ^{13}C NMR, see Table 1.

Spongidine B (6): $[\alpha]_D$ +7.8° (*c* 0.012, MeOH); ^{13}C NMR (CD_3OD) δ_C 173.3 (CH_3*CO*), 170.4 (C-14), 169.9 (−CH_2*COOH*), 146.4 (C-21), 143.3 (C-16), 137.7 (C-13), 124.8 (C-15), 68.2 (C-17), 63.9 (−*CH_2*COOH), 57.7 (C-5), 54.8 (C-9), 40.8 (C-1), 40.4 (C-8), 39.6 (C-10), 38.9 (C-7), 38.2 (C-4), 37.4 (C-3), 28.6 (C-12), 27.8 (C-18), 25.6 (C-20), 20.9 (CO*CH_3*), 20.1 (C-6), 19.2 (C-2), 18.0 (C-11), 17.4 (C-19); ^{1}H NMR (CD_3OD) δ_H 4.36−3.98 (2H, d, J = 11.0 Hz, CH_2-17), 2.08 (3H, s, CO*CH_3*), 1.06 (3H, s, Me-18), other signals are superimposable on those reported for **5**.

Spongidine C (7): $[\alpha]_D$ −10° (*c* 0.012, MeOH), ^{13}C NMR (CD_3OD) δ_C 179.4 (C-19), 170.4 (−CH_2*COOH*), 168.4 (C-14), 157.4 (C-16), 147.3 (C-24), 137.5 (C-13), 123.5 (C-15), 64.2 (−*CH_2*COOH), 57.5 (C-5), 55.9 (C-9), 43.2 (C-3), 40.6 (C-1), 40.2 (C-7), 39.6 (C-10), 39.5 (C-8), 36.2 (C-18), 34.3 (C-4), 33.8 (C-21), 33.2 (C-17), 28.4 (C-12), 25.9 (C-23), 21.9 (C-20), 20.0 (C-2), 19.7 (C-6) 18.3 (C-11), 17.0 (C-22); ^{1}H NMR (CD_3OD) δ_H 5.15 (2H, s, −*CH_2*COOH), 3.09 (1H, dd, J = 18.0, 6.2 Hz, H-12), 2.93 (1H, m, H-12), 1.32 (3H, s, Me-23), 1.03 (3H, s, Me-22), 0.94 (3H, s, Me-20), 0.93 (3H, s, Me-21).

Spongidine D (8): $[\alpha]_D$ −6° (*c* 0.016, MeOH); ^{1}H and ^{13}C NMR, see Table 2.

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