Swinhoeiamide A, a New Highly Active Calyculin Derivative from the Marine Sponge *Theonella swinhoei*

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Analysis of the Papua New Guinean sponge *Theonella swinhoei* afforded a new calyculinamide-related congener for which we propose the name swinhoeiamide A (**1**). The structure of the new compound was unambiguously established on the basis of NMR spectroscopic (^{1}H, ^{13}C, COSY, HMBC) and mass spectrometric (FABMS) data. Swinhoeiamide A exhibited insecticidal activity toward neonate larvae of the polyphagous pest insect *Spodoptera littoralis* when incorporated in an artificial diet offered to the larvae in a chronic feeding bioassay (ED_{50} 2.11 ppm, LD_{50} 2.98 ppm). Furthermore, it was found to be fungicidal against *Candida albicans* and *Aspergillus fumigatus* (MIC 1.2 and 1.0 μg/mL, respectively).

Sponges of the order Lithistida are one of the most prolific sources of novel marine natural products.[1] The genus *Theonella* is among these Lithistid sponges and has been reported to yield unusual secondary metabolites with pronounced biological activity.[1b] Some of these compounds also caught the attention of the pharmaceutical industry as potential lead structures for the development of new drugs. Examples of these secondary metabolites are the macrolides swinholides[2] and bistheonellides,[3] the cyclic peptides theonellamides[4] and cyclotheonamides,[5] and the peptide lactones theonellapeptolides.[6]

Our ongoing search for biologically active metabolites from Indo-Pacific marine organisms led to the isolation of a new calyculinamide-related congener (**1**) from a Papua New Guinean sample of *Theonella swinhoei*, for which we propose the name swinhoeiamide A. In this paper, we report the isolation and structure elucidation of this new compound, together with further biological activity data that complements those in a previous publication on the potent inhibition of tumor cell proliferation.[7]

The calyculins are secondary metabolites of the Japanese sponge *Discodermia calyx*, which belongs to the order Lithistidae.[8] Later, an Epipolasid sponge, *Lamellomorpha strongylata*, was reported to yield calyculins and structurally related compounds called calyculinamides.[9] More recently, calyculinamide congeners were also isolated from the Astrophorid sponge *Myriastra clavosa* and were named clavosines.[10] Similar to the calyculins, both the calyculinamides and clavosines were described as potent inhibitors of type 1 and 2A serine/threonine protein phosphatases.[11−13] In addition to their inhibition of protein phosphatases, the calyculins exhibited pronounced cytotoxicity against several cell lines[14] and have been described as potent natural insecticides.[15] These interesting biological activities eventually led to the commercial marketing of calyculins as molecular tools for studying intracellular signal transduction.[16]

T. swinhoei was collected by diving at a depth of 50 m near the coast of the Karkar Island, Papua New Guinea. The freeze-dried sample was repeatedly extracted with MeOH. The total MeOH extract was subjected to solvent−solvent partitioning between BuOH and H_2O. Swinhoeiamide A (**1**) was separated from other sponge metabolites, most of which were nucleotides, by column chromatography of the resulting BuOH extract over Sephadex LH_{20} using MeOH as eluent. Final purification of **1** was accomplished through a series of normal- and reversed-phase C_{18} Lobar column chromatographic separations.

Swinhoeiamide A (**1**) had the molecular formula $C_{40}H_{65}N_2O_{12}P$, as established by high-resolution FABMS. The ^{1}H and ^{13}C NMR data of **1** were comparable to those of calyculinamide A[9] and clavosine A[10] (Chart 1). Due to limited solubility of **1** in $CDCl_3$, all NMR spectral data were obtained using CD_3OD as a solvent. The spectral data of **1** were initially compared to those of dephosphonocalyculin A,[8f] which had likewise been obtained in CD_3OD. Compound **1** was shown to share many structural features with the calyculinamides[8,9] and clavosines.[10] The ^{1}H NMR spectrum contained signals characteristic for the calyculins such as the oxazole singlet (δ 7.76), the three olefinic methyl singlets (δ 1.91, 1.83, and 1.81), the three methyl doublets (δ 1.07, 0.85, and 0.77, J = 6.9 Hz), and the two aliphatic methyl singlets (δ 1.30 and 1.03). However, the 1D NMR spectra indicated that the side chain located at C-29 of the calyculins was replaced by a methyl substituent. Both the ^{1}H and ^{13}C NMR spectra of swinhoeiamide A (**1**) lacked all respective signals, while the additional aromatic methyl group gave rise to a singlet at δ 2.50 in the ^{1}H NMR spectrum and, correspondingly, an additional quartet at δ 13.5 in the DEPT spectrum.

The different substructures A−F of swinhoeiamide A (**1**) (Chart 2) were elucidated through interpretation of the ^{1}H,^{1}H-COSY spectrum in combination with the HMBC

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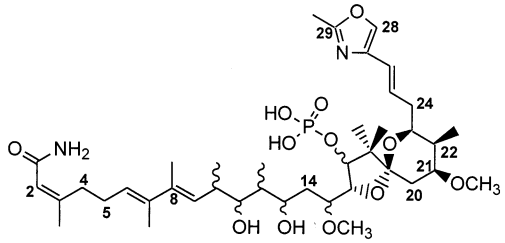
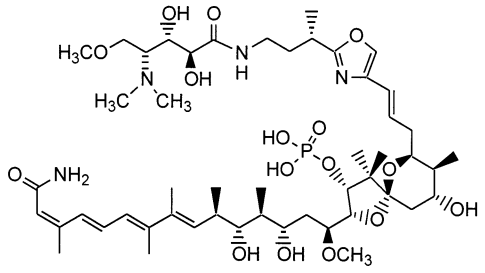
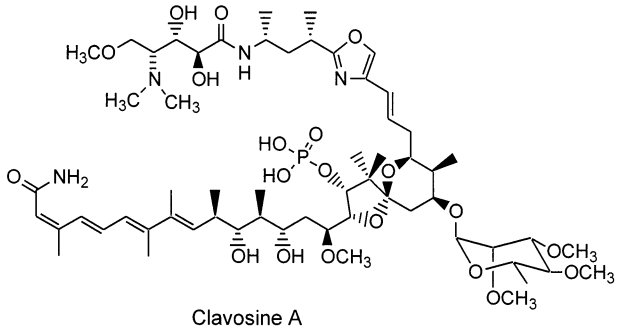
Chart 1



Swinhoeiamide A (**1**)



Calyculinamide A



Clavosine A

spectrum (see Table 1). Starting with substructure A, a long-range allylic coupling was observed from H-2 (δ 5.78) to 3-CH_3 (δ 1.91) in the COSY spectrum, while in the HMBC spectrum, a correlation of H-2 with the amide carbonyl at δ 171.8 (C-1) was detected. The nature of substructure B was evident from the COSY spectrum, which indicated the connectivity of the protons H_2-4 (δ 2.77), H_2-5 (δ 2.36), H-6 (δ 5.56), and 7-CH_3 (δ 1.83). This assignment was confirmed by key HMBC correlations observed from H-4 to C-6 (δ 125.8) and from H-5 to C-7 (δ 138.4).

Substructure C was assembled through analysis of the COSY spectrum, which established the connectivity of the protons at C-9 to C-17, including the allylic coupling from 8-CH_3 (δ 1.81) to H-9 (δ 5.70). The positions of the hydroxyl groups were implied from the HMBC spectrum (see Table 1). The ^{1}H NMR methoxyl singlet at δ 3.53 was positioned at C-15 (δ 80.3), as was evident from the respective ^3J correlation. The attachment of the phosphate ester to C-17 was indicated by an additional coupling ($^3J_{P-H}$ = 10.4 Hz) of H-17 (δ 4.42) which was observed as a doublet of doublets, although the COSY spectrum revealed only one coupling partner. Furthermore, due to long-range couplings to the phosphorus atom, the ^{13}C NMR resonances for C-17 and C-16 were split into doublets ($^2J_{P-C}$ = 6.2 Hz and $^3J_{P-C}$ = 5.5 Hz, respectively). These findings were in full agreement with data reported for other calyculin congeners.[8d]

Substructure D was evident from HMBC correlations of the two *gem*-dimethyl groups 18a-CH_3 (δ 1.30) and 18b-CH_3 (δ 1.03) to C-18 (δ 51.2) as well as the ketal carbon, C-19 (δ 109.9) (see Table 1). Inspection of the COSY spectrum established the connectivity from C-20 to C-26 and, hence, the nature of substructure E. A HMBC correlation from the methoxyl singlet at δ 3.37 to a ^{13}C signal at δ 79.0 revealed that C-21 of swinhoeiamide A (**1**) carried a methoxyl substituent instead of a hydroxyl function as observed in calyculins and calyculinamides. The 2-methyloxazole moiety (substructure F) was evident by comparison with literature data[17] as well as a long-range coupling of the C-29 methyl protons (δ 2.50) to H-28 (δ 7.76) in the COSY spectrum and a ^2J correlation to C-29 (δ 164.3) in the HMBC spectrum.

The connectivities between substructures A, B, and C were established by key HMBC correlations between H_2-4 and 3-CH_3, H-2 and C-4, H-6 and C-8, and H-9 and C-7, respectively (see Table 1). The spiroketal unit comprising substructures C, D, and E was assembled through HMBC correlations which were essentially the same as those reported for the calyculins[8,18] and the clavosines.[10] Important cross-peaks included 18a-CH_3 to C-17, and H_2-20 to C-19. Finally, a correlation between C-27 and H-26 allowed the assignment of the 2-methyloxazole moiety (substructure F) at C-26, and thus the gross structure for swinhoeiamide A (**1**) was assigned as depicted.

The stereochemistries of the double bonds present in **1** were established through a series of NOE experiments. Mutual NOE effects were observed between the proton pairs, H-2/3-CH_3, H-6/8-CH_3, and H-9/7-CH_3 (see Chart 2). Thus, the *Z*-configuration of the double bond between C-2 and C-3 and the all-*E*-configuration for the diene moiety (C-6 to C-9) were established, respectively. Finally, the *E*-configuration of the double bond between C-25 and C-26 was evident from the large vicinal coupling constant ($^3J_{H-25,H-26}$ = 15.9 Hz).

The ^{1}H and ^{13}C NMR data observed for H/C-9 to H/C-17 (acquired in CD_3OD) showed significant differences to the data reported for this identical segment in calyculinamide A (in $CDCl_3$),[9] clavosine A (in CD_2Cl_2),[10] and dephosphonocalyculin A (in CD_3OD).[8f] Thus, the relative stereochemistry of this moiety could not be established by comparison to NMR data previously reported in the literature.[19]

The splitting pattern observed for H-21 ("dt", $J \approx$ 12.0, 4.5 Hz) indicated its axial orientation and, consequently, an equatorially oriented oxygen substituent, whereas the methyl substituent at C-22 was oriented in an axial position. Thus, the relative axial−equatorial orientation of H-21 and H-22 in swinhoeiamide A (**1**) was the same as that of the clavosines,[10] while a diequatorial orientation of both protons as present in calyculins or calyculinamides would result in considerably smaller coupling constants.[8d] This assignment was further supported by a diaxial coupling ($^3J_{H,H} \approx$ 12.0 Hz) between H-21 and H-20B and by the pronounced upfield shift of the C-22 methyl carbon (δ 4.4) due to a γ-gauche effect, which was comparable to data reported for clavosine A (C-42, δ 4.9).[10] In the calyculins or calyculinamides, which feature an axially oriented oxygen substituent, the corresponding methyl group resonated between 10 and 11 ppm.[8d,9]

Swinhoeiamide A (**1**) was identified as a new calyculin congener which differed from the known derivatives, calyculins,[8] calyculinamides,[8e,9] and clavosines,[10] by two novel structural features. The complex side chain attached to C-29 in the oxazole ring system was replaced by a methyl substituent in swinhoeiamide A (**1**). Furthermore, com-

Chart 2

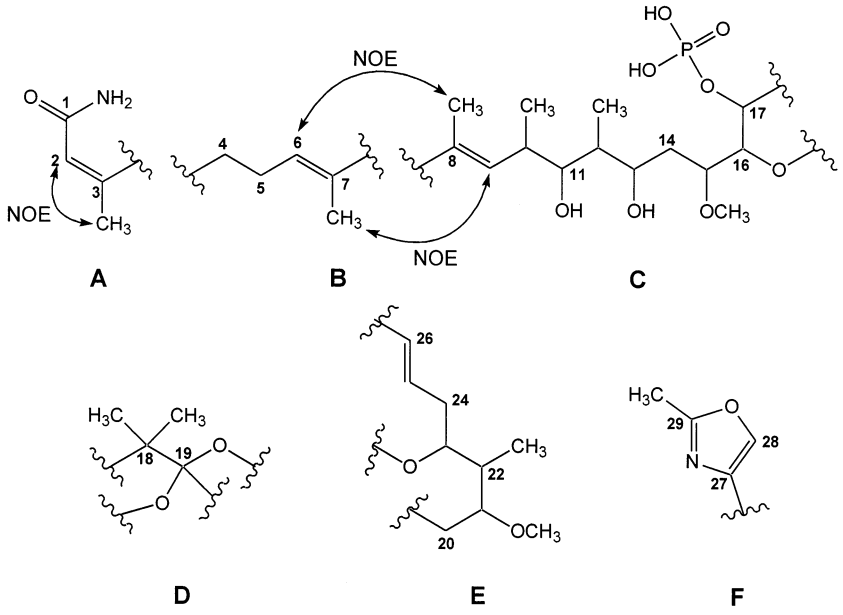


Table 1. NMR Data for Swinhoeiamide A (**1**) in CD_3OD

	δ_C	(mult)	δ_H	(mult, *J* in Hz)	$HMBC_{(\delta H\ to\ \delta C)}$	$COSY_{(\delta H\ to\ \delta H)}$
1	171.8	(s)				
2	119.5	(d)	5.78	(d, 1.5)	1, 4, 3-Me	3-Me
3	156.3	(s)				
3-Me	25.1	(q)	1.91	(d, 1.5)	2, 3, 4	2, 4
4	33.8	(t)	2.77	(m)	3, 5, 6, 3-Me	3-Me, 5
5	28.3	(t)	2.36	(q, 7.2)	4, 6, 7	4, 6
6	125.8	(d)	5.56	(dt, 7.2, 1.2)	8, 7-Me	5, 7-Me
7	138.4	(s)				
7-Me	14.4	(q)	1.83	(d, 1.2)	6, 7	6
8	137.1	(s)				
8-Me	14.4	(q)	1.81	(s)	8, 9	9
9	127.5	(d)	5.70	(d, 9.7)	7, 8-Me	8-Me, 10
10	36.8	(d)	2.71	(m)		9, 10-Me, 11
10-Me	18.9	(q)	1.07	(d, 6.9)	9, 10, 11	10
11	79.8	(d)	3.45	(dd, 9.6, 2.5)		10, 12
12	44.1	(d)	1.63	(m)		11, 12-Me, 13
12-Me	12.1	(q)	0.77	(d, 6.9)	11, 12, 13	12
13	72.3	(d)	3.88	(ddd, 11.0, 6.0, 1.8)	15	12, 14A, 14B
14A	36.1	(t)	1.93	(m)		13, 14B, 15
14B			1.49	(ddd, 14.6, 9.4, 1.8)		13, 14A, 15
15	80.3	(d)	3.82	(brt, 9.6)		14A, 14B, 16
15-OMe	60.9	(q)	3.53	(s)	15	
16	85.7	(d)	4.10	(dd, 9.6, 4.4)		15, 17
17	84.2	(d)	4.42	(dd, 10.4, 4.4)		16
18	51.2	(s)				
18a-Me	18.2	(q)	1.30	(s)	17, 18, 19, 18b-Me	
18b-Me	23.2	(q)	1.03	(s)	18, 19, 18a-Me	
19	109.9	(s)				
20A	31.1	(t)	1.70	(dd, 12.2, 4.6)[a]	19, 21	20B, 21
20B			1.54	("bt", 12.2)[a]	19, 21	20A, 21
21	79.0	(d)	3.75	("dt", 11.8, 4.6)[a]	21-OMe	20A, 20B, 22
21-OMe	55.4	(q)	3.37	(s)	21	
22	35.5	(d)	2.10	(m)	21	21, 22-Me, 23
22-Me	4.4	(q)	0.85	(d, 6.9)	21, 22, 23	22
23	72.8	(d)	3.99	(m)		22, 24A, 24B
24A	37.2	(t)	2.47	(m)		23, 24B, 25, 26
24B			2.16	(m)		23, 24A, 25, 26
25	132.5	(d)	6.75	(ddd, 15.9, 10.1, 5.1)[a]		24A, 24B, 26
26	119.5	(d)	6.27	(bdd, 15.9)	24A, 24B, 27	24A, 24B, 25, 28
27	139.7	(s)				
28	136.1	(d)	7.76	(bs)	29	26, 29-Me
29	164.3	(s)				
29-Me	13.5	(q)	2.50	(s)	29	28

[a] Splittings observed from complex mutiplets.

pound **1** represented the first calyculin congener in which the double bond at C-4 and C-5 was hydrogenated. Additionally, C-21 was methoxylated with this substituent following the equatorial orientation as in the clavosines.[10]

Swinhoeiamide A (**1**) was found to be active in several bioassays other than the previously reported antiproliferative activity against human tumor cells.[7] It demonstrated significant insecticidal activity toward neonate larvae of the polyphagous pest insect *Spodoptera littoralis* (LC_{50} 2.98 ppm) in a chronic feeding assay. In the topical contact assay, swinhoeiamide A (**1**) had a LD_{50} of 10 μg when directly administered to the larvae, while the LD_{50} amounted to 0.98 μg per 1000 mg larval body weight following injection into the hemolymph. In the latter bioassay, calyculin A, in comparison, had a LD_{50} of 1.00 μg per 1000 mg larval body weight. Thus, both compounds were essentially equitoxic to the larvae of *S. littoralis*. Furthermore, swinhoeiamide A (**1**) showed strong fungicidal activity toward *Candida albicans* and *Aspergillus fumigatus* (MIC 1.2 and 1.0 μg/mL, respectively). It was also fungicidal against the plant pathogen *Cladosporium cucumerinum*, causing a 14 mm zone of inhibition at a dose of 25 nmol in the bioautographic assay procedure. Since the pronounced cytotoxicity of swinhoeiamide A (**1**) toward a panel of human cancer cells had already been demonstrated before,[7] it is possible that the broad spectrum of biological activities observed in this study is likewise caused by cytotoxic properties.

Experimental Section

Animal Material. The sponge was collected by diving at a depth of 50 m near the coast of Karkar Island, Papua New Guinea. The sample was directly frozen after collection and stored at −20 °C. The sponge was identified as *Theonella swinhoei* (order Lithistida, family Theonellidae).[20] A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMA POR.10885.

The sponge's surface was uneven, pitted, ridged, and grooved, but smooth in between. Its consistency was hard. The skeleton of the surface was made up of a tangential layer of phyllotriaenes with cladomes up to 400−450 μm and individual cladi of 200−240 × 12−20 μm which formed a characteristic honeycombed-surface network with rounded spaces of 150−240 μm diameter. Scattered over the surface were numerous curved or flexuous 15−18 × 3 μm microrhabds with spined or rugose surface. The skeleton of the interior was almost completely "lithistid"; that is, there were comparatively few bundles of strongyles and all desmas appeared to be zygosed from the surface downward. The strongyles measured 250−400 × 3−6 μm, often with wide axial canals or frequently broken. Desmas were polyrhabdose, with cladome up to 540 μm. Cladi were 35−40 μm in diameter, tuberculated, but smooth between tubercles. In the interior, microrhabds were frequent.

Extraction and Isolation. The sponge sample was freeze-dried and was repeatedly extracted with MeOH. The total extract was evaporated under reduced pressure, taken to dryness, and subjected to solvent−solvent partitioning between BuOH and H_2O. Swinhoeiamide A (**1**) was separated from other sponge metabolites, most of which were nucleotides, by column chromatography of the BuOH extract over Sephadex LH_{20} using MeOH as eluent. Final purification of **1** was accomplished through a series of normal- and reversed-phase C_{18} Lobar column chromatographic steps utilizing CH_2Cl_2−MeOH (70:30) and MeOH−H_2O−TFA (80:20:0.1) as solvents, respectively.

^{1}H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were recorded in CD_3OD on a Bruker Advance DMX 600 NMR spectrometer. Mass spectra (FABMS) were measured on a Finnigan MAT 8430 mass spectrometer. Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. The CD spectrum was recorded on a CD6 ESA Jobin-YVIN/dInstrument S.A. using EtOH as solvent. For HPLC analysis, samples were injected into an HPLC system (Gynkotek) coupled to a photodiode-array detector which recorded the UV spectra online. Routine detection was at 254 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher C-18. UV spectra were recorded in MeOH. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on precoated TLC plates with Si gel 60 F_{254} (Merck, Darmstadt, Germany).

Swinhoeiamide A (1): white powder residue; $[\alpha]^{20}_D$ −21.6° (*c* 0.35, EtOH); UV (MeOH) λ_{max} 223 nm; IR (KBr) ν_{max} 3444, 2931, 1682, 1442, 1383, 1209, 1142 cm^{-1}; CD (EtOH) λ ($\Delta\epsilon$) 200 (0), 207 (−4.0), 210 (−3.0), 230 (−9.0), 250 (0), 253 (+1.0), 259 (+1.0) nm; ^{1}H and ^{13}C NMR data, see Table 1; FABMS pos *m*/*z* 835 $[M + K]^+$, neg *m*/*z* 795 $[M - H]^-$; HRFABMS *m*/*z* 795.4326 $[M - H]^-$ (calcd for $C_{40}H_{64}N_2O_{12}P$, 795.4197).

Bioassays. Experiments with *S. littoralis*. Chronic Feeding Assay. Larvae of *S. littoralis* were reared on an artificial bean-agar diet under controlled conditions as reported previously.[21] Feeding studies were conducted with neonate larvae ($n = 20$) that were kept on an artificial diet that had been treated with various concentrations of the compound under study. After 6 days, survival of the larvae and weight of the surviving larvae were protocolled and compared to controls reared on an untreated diet. LD_{50} and ED_{50} values were calculated from the dose−response curves by probit analysis.[22]

Contact Toxicity by Topical Method. To assess the insecticidal action of a compound following direct contact via the integument, the test compound was dissolved in EtOH and was topically applied on the dorsal region of third instar larvae with the aid of a microliter syringe. After initial contact, the larvae were transferred to boxes containing untreated diet, and the survival rate was assessed after 5 days. Controls were treated with EtOH only.

Hemolymph Injection Method. The assay was performed with last instar larvae by injecting different doses of test compound, dissolved in EtOH, directly into the hemolymph. Survival was recorded after 48 h. Controls were treated with EtOH only.

Evaluation of Fungicidal Activity. Serial Dilution Assay. A 2-fold dilution of 0.5 mL of the test compound (800 μg in 4 mL of solvent) in Shadomy-Bouillon was added to the Bouillon culture containing 10^3 microorganisms per milliliter and incubated at 37 °C for 24 h. The concentration of the test compound in the initial dilution tube was 100 μg/mL. Cultures used in the serial dilution assay included *Aspergillus fumigatus* and *Candida albicans*. Inhibition of growth was assessed by comparing the fungal growth of the test samples with that of the control tubes prepared without the test compound. The concentration of the tube at highest dilution that was still free from growth was assigned the minimum inhibitory concentration (MIC in μg/mL). The fungal strains were from the laboratory cultures of the Institute for Hygiene, University of Würzburg, Germany.

Bioautographic Detection of Fungicidal Activity.[23] Spores of *Cladosporium cucumerinum* were cultivated on carrot-nutrient agar and were inoculated into a liquid yeast culture medium. Silica gel TLC plates were spotted with the isolated compounds at amounts ranging from 10 to 100 nmol, and the plates were sprayed with a spore suspension of *C. cucumerinum* in liquid yeast culture medium. The fungicidal activity of the test compound was observed as a clear white inhibition spot on a dark layer of the mycelia covering the TLC plate after the inoculated plates had been incubated for 2 days at 25 °C. The fungal strain of *C. cucumerinum* used was from the laboratory cultures of the Institute of Plant Biochemistry, University of Halle.

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(19) Due to limited supply of the substance and exhaustion upon biological testing, no further NMR spectra could be measured. Previous attempts to obtain crystals of swinhoeianide A (**1**) suitable for X-ray crystallography were unsuccessful.

(20) The habitus and spicular characteristics conformed to those of the type of *Theonella swinhoei*, but it should be noted that the distinction of *Theonella* species on skeletal characteristics remains problematic.

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