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METABOLITES OF THE PALAUAN SPONGE *AXINYSSA APLYSINOIDES*

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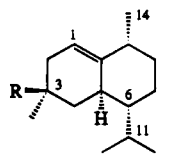
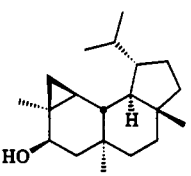
ABSTRACT.—The Palauan sponge *Axinyssa aplysinoides* contained (3*S**,5*R**,6*R**,9*R**)-3-isocyano-1(10)-cadinene [**2**], (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene [**3**], and (*E*)-(4-hydroxystyryl)trimethylammonium chloride [**6**], together with the known diterpenes (−)-neoverrucosan-5β-ol [**4**] and (+)-homoverrucosan-5β-ol [**5**].

Many sponges of the order Halichondrida contain sesquiterpene isonitriles, isothiocyanates, and formamides; in most cases a trio of nitrogenous derivatives has been isolated for each sesquiterpene skeleton (1). Occasionally, sesquiterpene thiocyanates, ureas, or amines are encountered. Of particular relevance to this work is the report by Nakamura *et al.* (2) of the isolation of halipanicine [**1**] from an Okinawan specimen of *Halichondria panicea*. Apparently, only the isothiocyanate was found in this sponge. In this paper we report the isolation of the corresponding isonitrile and formamide from a specimen of *Axinyssa aplysinoides* (Dendy, 1922) from Palau.



1 R=NCS
2 R=NC
3 R=NHCHO



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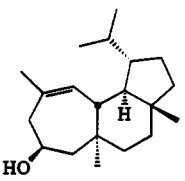

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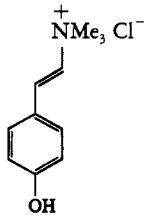

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Crude extracts of the sponge *Axinyssa aplysinoides* showed mild but consistent antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*. Fractionation of the crude extract resulted in the antimicrobial activity being almost equally divided between the H_2O and EtOAc fractions although nmr spectroscopy indicated the presence of very different metabolites in each fraction. Further purification of the EtOAc fraction resulted in the isolation of (3*S**,5*R**,6*R**,9*R**)-3-isocyano-1(10)-cadinene [**2**] (1.1% dry wt), (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene [**3**] (0.39% dry wt), (−)-neoverrucosan-5β-ol [**4**] (0.24% dry wt) and (+)-homoverrucosan-5β-ol [**5**] (0.19% dry wt). After identifying the diterpenes **4** and **5** by interpretation of spectral data, we were surprised, from a chemotaxonomic viewpoint, to read that they had earlier been isolated from the liverworts *Mylvia verrucosa* (3) and *Schistochila rigidula* (4). (*E*)-(4-Hydroxystyryl)trimethylammonium chloride [**6**] was isolated as a major constituent (3.1% dry wt) of the aqueous extract.

(3*S**,5*R**,6*R**,9*R**)-3-Isocyano-1(10)-cadinene [**2**] was isolated as a clear oil of molecular formula $C_{16}H_{25}N$, typical of a sesquiterpene isonitrile. The isonitrile functionality was confirmed by the ir band at 2178 cm^{-1}. In the ^{13}C-nmr spectrum, the expected 16 signals were observed, which included an isonitrile signal at δ 152.5 (s), two olefinic signals at δ 143.9 (s) and 114.9 (d), four methyl

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signals, four methylene signals, four methine signals, and a signal at δ 55.5 that was assigned to a quaternary carbon bearing the isonitrile group. The ^{1}H-nmr spectrum was assigned with the aid of HMQC and COSY experiments and the cadinene carbon skeleton and substitution pattern was deduced from HMBC and COSY experiments. The relative stereochemistry was defined by analysis of coupling constants and NOEDS data; irradiation of both the Me-14 and Me-15 signals caused enhancements of the H-5 signal, which requires that both methyl groups be axial and on the same side of the ring system as the axial bridgehead proton. The presence of two large coupling constants ($J_{5,6}=J_{6,7ax}=11$ Hz) indicated that H-6 is axial. Comparison of the spectral data of (3*S**,5*R**,6*R**,9*R**)-3-isocyano-1(10)-cadinene [**2**] with those of the corresponding isothiocyanate, halipanicine [**1**], revealed the expected similarities and differences associated with a change of functional group.

(3*S**,5*R**,6*R**,9*R**)-3-Formamido-1(10)-cadinene [**3**] was isolated as a clear oil of molecular formula $C_{16}H_{27}NO$ and possessed all the spectral properties expected of a formamide. The ir spectrum contained bands at 3370 and 1665 cm^{-1} due to the formamide group. The ^{1}H-nmr spectrum in $CDCl_3$ solution contained two sets of signals of approximately equal intensity including two formamide proton signals at δ 8.30 (d, J=9 Hz) and 8.03 (br s), due to the (*E*) and (*Z*) geometrical isomers, with the corresponding -NH signals at δ 5.85 (br d) and 5.28 (br s). The ^{13}C-nmr spectrum in $CDCl_3$ solution also showed extensive doubling of the signals. In MeOH-d_4 solution, both nmr spectra were considerably cleaner since the ratio of isomers had changed to 6:1 and in the ^{13}C-nmr spectrum, the doubling of signals was less pronounced. Interpretation of the nmr data, including the COSY, HMQC, and HMBC experiments revealed that the sesquiterpene portion of **3** was identical to that of **2**.

(*E*)-(4-Hydroxystyryl)trimethylammonium chloride [**6**] was obtained as a highly hygroscopic white solid of molecular formula $C_{11}H_{16}NO^+$ Cl^-. The chloride counter-ion was identified by energy dispersive X-ray analysis using a scanning-electron microscope. The ir spectrum contained a very strong and broad band at 3430 cm^{-1}, which was not informative. The uv absorption at 267 nm underwent a bathochromic shift to 294 and 312 nm on addition of base, suggesting the presence of a phenol. The ^{1}H-nmr spectrum consisted of signals at δ 3.41 (9H, s), assigned to the methyl signals of a trimethylammonium salt, at δ 6.92 (1H, d, J=14 Hz) and 7.39 (1H, d, J=14 Hz), due to an (*E*)-disubstituted conjugated olefin, and at δ 6.81 (2H, d, J=9 Hz) and 7.12 (2H, d, J=9 Hz), assigned to a para-substituted phenol. These units can only be assembled in one way to produce the structure of (*E*)-(4-hydroxystyryl)trimethylammonium chloride [**6**]. The ^{13}C-nmr spectrum is completely compatible with this structure.

It is unusual to isolate compounds of different classes from a single sponge. Although sesquiterpenes **2** and **3** are similar to known metabolites from *A. aplysinoides*, the diterpenes **4** and **5** are related to compounds from the sponge *Higginsia* sp. (5,6). However, microscopic examination and spicule analysis of tissue samples from representative pieces of sponge, selected randomly, effectively eliminated the possibility that we had studied a mixed collection of two visually similar sponges. In addition, there was no obvious evidence of the presence of symbionts or other contaminants. The antimicrobial activity of the crude extract was shown to be due to the formamide **3** that inhibits *Staphylococcus aureus* at 25 μg/disk and *Bacillus subtilis* at 10 μg/disk in the disk-diffusion assay.

EXPERIMENTAL

ANIMAL MATERIAL.—The peach colored sponge *Axinyssa aplysinoides* (93-074, SIO Benthic Invertebrate Collection #P1150), which is very similar to specimens of *A. aplysinoides* studied

previously (7,8), was collected by hand using scuba (−30 m) from the fringing reef due west of Malakal Harbor, Palau, and was immediately frozen.

EXTRACTION AND ISOLATION.—After 10 months at −20°, the sponge (155 g dry wt) was extracted with MeOH (3×1 liter) at 2°. The MeOH extract was filtered and evaporated to obtain an oily suspension that was partitioned between H_2O (400 ml) and EtOAc (5×250 ml). A portion (1.8 g) of the EtOAc-soluble material (17.3 g) was subjected to flash chromatography on Si gel using 3% MeOH in CH_2Cl_2 as eluent to obtain three fractions. The first fraction was purified by hplc on Partisil using 5% EtOAc in hexane as eluent to obtain (3*S**,5*R**,6*R**,9*R**)-3-isocyano-1(10)-cadinene [**2**] (179 mg, 1.1% dry wt). The second fraction was separated by cc on Si gel using 15% EtOAc in hexane as eluent to obtain (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene [**3**] (63 mg, 0.39% dry wt) and (−)-neoverrucosan-5β-ol [**4**] (39 mg, 0.24% dry wt). The third fraction was purified by hplc on Partisil using 30% EtOAc in hexane as eluent to obtain (+)-homoverrucosan-5β-ol [**5**] (32 mg, 0.19% dry wt). A portion (812 mg) of the lyophilized H_2O-soluble material (14 g) was chromatographed on Sephadex LH-20 using MeOH as solvent to obtain (*E*)-(4-hydroxystyryl)trimethylammonium chloride [**6**] (287 mg, 3.1% dry wt).

(3S,5R*,6R*,9R*)-3-Isocyano-1(10)-cadinene* [**2**].—Oil: $[\alpha]_D$ −75.1° (*c*=0.6, $CHCl_3$): uv λ max (MeOH) 206 nm (ε 2880); ir ν max (film) 2940, 2910, 2860, 2178, 1455, 1380, 1130 cm^{-1}; ^1H- and ^{13}C-nmr data, see Table 1; hrcims, *m/z* 230.1907 ($C_{16}H_{24}N$ $[M-H]^+$ requires 230.1908).

(3S,5R*,6R*,9R*)-3-Formamido-1(10)-cadinene* [**3**].—Oil: $[\alpha]_D$ −43.6° (*c*=0.55, $CHCl_3$); uv λ max (MeOH) 204 (ε 7790), 238 nm (1375); ir ν max (film) 3700, 3270, 2950, 2875, 1665, 1540, 1455, 1375, 1330, 1055 cm^{-1}; ^1H- and ^{13}C-nmr data (MeOH-d_4), see Table 1; hrcims, *m/z* 250.2175 ($C_{16}H_{28}NO$ $[M+H]^+$ requires 250.2170).

(E)-4-Hydroxystyryl)trimethylammonium chloride [**6**].—Hygroscopic white solid: uv λ max (MeOH) 203 (ε 7545), 267 nm (7050), (MeOH+0.5% NaOH) 294 (ε 10530), 312 nm (10200); ir ν max (KBr) 3430, 1605, 1515, 1270 cm^{-1}; ^1H nmr (MeOH-d_4) δ 3.41 (9H, s), 6.81 (2H, d, *J*=9 Hz), 6.92 (1H, d, *J*=14 Hz), 7.12 (2H, d, *J*=9 Hz), 7.39 (1H, d, *J*=14 Hz); ^{13}C nmr (MeOH-d_4) δ 56.1 (q, 3×C), 116.9 (d, 2×C), 124.1 (s), 128.0 (d), 130.3 (d, 2×C), 134.1 (d), 160.5 (s); hrfabms, *m/z* 178.1226 ($C_{11}H_{16}NO$ requires *m/z* 178.1232).

ACKNOWLEDGMENTS

The sponge was identified by Mary Kay

TABLE 1. ^1H- and ^{13}C-nmr Data for Isonitrile **2** and Formamide **3** (values in parentheses are for the minor isomer).

Position	Compound					
	2			**3**		
	δ_C	δ_H	(mult., *J*)	δ_C	δ_H	(mult., *J*)
1	114.9	5.26	br s	117.9 (117.6)	5.33 (5.36)	br s
2	38.6	2.44	br d, 17	39.6 (41.5)	2.08 (1.90)	m
		2.18	br dd, 17, 6		1.42 (1.32)	m
3	55.5			53.9 (53.3)		
4	40.5	2.04	m	38.0	2.33	br d, 16
		1.56	m		2.16	m
5	33.1	2.07	m	34.6 (34.9)	2.15	m
6	49.6	1.05	br t, 11	51.5	1.01	m
7	18.1	1.44	m	19.3	1.43	m, 2H
		1.32	m			
8	32.1	1.56	m	33.6	1.60	m
		1.50	m		1.41	m
9	37.4	2.42	m	39.9	2.43	m
10	143.9			144.8 (144.9)		
11	26.5	1.90	m	27.7	1.95	m
12	21.4	0.89	d, 3H, 7	21.9	0.92	d, 3H, 7
13	14.7	0.77	d, 3H, 7	15.2	0.82	d, 3H, 7
14	19.6	0.98	d, 3H, 7	20.2	1.04	d, 3H, 7
15	24.7	1.33	s, 3H	22.8 (24.6)	1.29 (1.26)	s, 3H
16	152.5			163.0 (165.8)	7.88 (8.22)	s

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