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VARACIN AND THREE NEW MARINE ANTIMICROBIAL POLYSULFIDES FROM THE FAR-EASTERN ASCIDIAN *POLYCITOR* SP.

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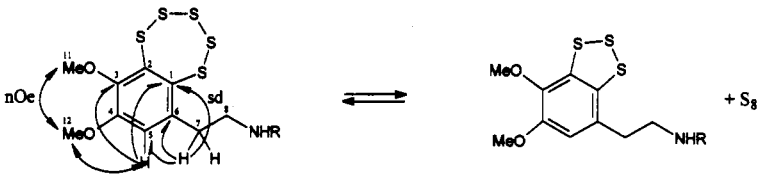
ABSTRACT.—Varacin [**1**] and three new antimicrobial marine polysulfides, varacins A–C [**3–5**], have been isolated from the Far Eastern ascidian *Polycitor* sp. Their structures have been elucidated by spectroscopic methods and by chemical transformations.

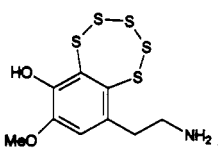
Organic polysulfides have generated significant interest during the past few years due to their chemical properties and physiological activities (1). Recently, the unprecedented natural polysulfides varacin [**1**] (2) and lissoclinotoxin [**2**] (3,4), which exhibit potent antibiotic, cytotoxic, and antiviral activities, have been isolated from ascidians belonging to the genus *Lissoclinum* (Didemnidae), and varacin [**1**] has been synthesized (5,6). In the course of our continuing studies on biologically active marine natural products (7,8) we have isolated varacin [**1**] and three new related polysulfides **3–5** from the ascidian *Polycitor* sp. In addition, we have obtained the corresponding acetates **1a**, **3a–5a**, and have studied the equilibrium between **1a** and **3a**+S_8 as well as other properties of these substances.

The colonial ascidian *Polycitor* sp. (phylum Chordata, subphylum Urochordata, class Ascidiaceae, suborder Aplousobranchia, family Polycitoridae) was collected by dredging from a depth of 90 m in the Sea of Japan (43° 04′N, 134° 30′E) and immediately after collection was extracted with EtOH. The EtOH extract from the ascidian was shown to possess potent inhibitory activities against *Staphylococcus aureus, Bacillus subtilus*, and



1 R=H
1a R=$COCH_3$

3 R=H
3a R=$COCH_3$



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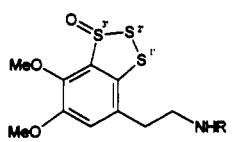
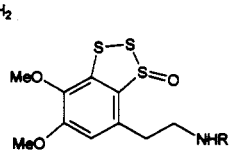


4 R=H
4a R=$COCH_3$



5 R=H
5a R=$COCH_3$

Candida albicans. Solvent partitioning of the concentrated crude extract resulted in the localization of antimicrobial activity in a $CHCl_3$-soluble fraction. Rapid chromatographic separation of these materials over a short column with Si gel resolved inactive nonpolar and polar subfractions from active midpolar ninhydrin-positive components. The latter were further purified by repeated cc on Sephadex LH-20 to give a pale yellow powder, containing **1** with **3–5** as impurities (*Bacillus subtilus*, zone of inhibition: 17 mm/0.1 μg). Structural identification of **1** was carried out by nmr (see Table 1) and ms methods. Positive fabms showed a $[M+H]^+$ ion peak at *m/z* 340 which corresponded, together with nmr data, to a molecular formula of $C_{10}H_{13}NO_2S_5$ for **1**. In order to gain more information on the identification and properties of **1** we prepared a crude acetate **1a** by treatment with Ac_2O/pyridine and subjected it to hplc on a Si gel column to give pure varacin acetate **1a** and the acetates of varacins A–C [**3a–5a**].

The previously undescribed acetate **1a** was isolated as light yellow crystals with a molecular formula of $C_{12}H_{15}NO_3S_5$, as suggested from fabms (see Table 2). Its eims displayed a molecular ion at *m/z* 381 and a predominant peak at *m/z* 317, corresponding to a loss of S_2 from the molecular ion. The ^{13}C-nmr spectrum of **1a** showed 12 signals including six aromatic carbons, two methoxyl carbons, two methylene carbons, and two carbons of one *N*-acetyl group (Table 3). The ^{1}H-nmr spectrum of **1a** had, along with singlets of two methoxyls and an *N*-acetyl group, one multiplet of NH, two 2H multiplets of protons of an ethylamine side-chain at 3.17 and 3.46 ppm, and the singlet of a proton in a pentasubstituted benzene moiety at 6.82 ppm. Data from ^{13}C- and ^{1}H-nmr spectra with nOe difference, noise, gated, and selective decoupling experiments (see Table 3 and structure) confirmed the identification of the isolated compound as **1a**.

A molecular formula of $C_{12}H_{15}NO_3S_3$ for the acetate of varacin A [**3a**] was deduced from the $[M-H]^-$ ion at *m/z* 316 in the fabms (negative) and eims (see Table 2) as well as from other spectral characteristics. The ^{1}H-nmr spectra of **1a** and **3a** were very similar. However, there were some significant differences for chemical shifts of an aromatic proton and signals of methylene groups (see Tables 2 and 3). These data showed that compounds **1a** and **3a** differed from each other in their polysulfide rings, containing S_5 in **1a** and S_3 in **3a**.

It has been reported that some aromatic fused benzopentathiepins are capable of decomposing into sulfur and the corresponding benzotrithioles in solution (9). Examination of **1** or **1a** as well as **3** and **3a** by ^{1}H nmr and ms revealed that

TABLE 1. Chemical Shifts (ppm) and Spin-Spin Coupling Constants (Hz) of Protons of Varacin (**1**, CD_3OD) and Varacin A–C Acetates (**3a–5a**, $CDCl_3$).

Proton(s)	Compound			
	1	**3a**	**4a**	**5a**
H (1H)	7.13, s	6.49, s	6.96, s	6.81, s
NH (1H)		5.56, m	5.60, m	5.90, m
OCH_3 (3H)	3.96, s	3.89, s	4.04, s	3.98, s
OCH_3 (3H)	3.82, s	3.84, s	3.91, s	3.94, s
CH_2 (2H)	3.15, m	2.83, t (J=6.2 Hz)	2.98, m	3.23, m
CH_2 (2H)	3.25, m	3.49, q (J=6.2 Hz)	3.60, m	3.62, m
$COCH_3$ (3H)		1.98, s	1.98, s	1.93, s

TABLE 2. Mass Spectral (Negative and Positive fabms, Eims), and Hplc Data of Acetates **1a**, **3a–5a**.

Compound	Hplc RR_t	Ms fragment (*m/z*; rel. int.)		
		Fabms		Eims
		Negative	Positive	
1a	1.00		382 (11) 317 (21) 87 (100)	$[M+2]^+$ 383 (0.5), $[M]^+$ 381 (2), 317 (100), 258 (48), 245 (24), 230 (15)
3a	0.89	316 (15) 285 (100) 96 (16)	—	$[M+2]^+$ 319 (13.6), $[M]^+$ 317 (100), 258 (50) 245 (30), 230 (15)
4a	1.06		334 (25) 301 (21) 285 (11) 73 (100)	$[M+2]^+$ 335 (4), $[M]^+$ 333 (34), 317 (53), 285 (18), 274 (38), 258 (31), 245 (22), 226 (100)
5a	1.18		334 (7) 301 (15) 285 (6) 73 (100)	$[M+2]^+$ 335 (2), $[M]^+$ 333 (16), 317 (100), 285 (10), 274 (44), 258 (50), 245 (10), 226 (40)

both of these compounds readily equilibrate to a mixture of **1** and **3** or **1a** and **3a**, respectively. In fact, reactions **1**=**3**+S_8 or **1a**=**3a**+S_8 were observed to occur in $CHCl_3$, MeOH, and pyridine. For example, **1** or **1a** generated **3** or **3a** when dissolved in MeOH-d_4. After 100 h, the concentrations of **3** and **3a** in these mixtures amounted to about 25% and 10%, respectively. About 45% of **3a** was detected after standing for 1 year. Approximately 45% of **1a** was converted into **3a** by heating in pyridine for 20 min at 100°. The addition of excess S_8 to the resulting mixture of **1a**+**3a** under the same conditions regenerated some **1a** and the content of **3a** was diminished by up to 10% after 15 min.

The acetates of varacins B [**4a**] and C [**5a**] were studied by fabms and eims (see Table 2). It was shown that both these isomeric polysulfides differed from **3a** by the presence of an additional oxygen atom. The eims spectra of both **4a** and **5a** demonstrated, along with the molecular ion peak at *m/z* 333, a prominent peak at *m/z* 317, corresponding to the loss of an oxygen atom. Such fragmentation was reported to be characteristic of some organic *S*-oxides (10,11). Moreover, the

TABLE 3. ^1H- and ^{13}C-Nmr Spectra of **1a** ($CDCl_3$).

Position	δ ^{13}C, ppm	δ ^1H, ppm	J ^{13}C-^1H (Hz)
1	135.0		8.5, 5.5, 5.5
2	140.5		s
3	149.6		br s
4	154.7		br m
5	115.4	6.82, s, 1H	155, 5.5, 5.5
6	141.0		br s
7	36.1	3.17, m, 2H	
8	40.1	3.46, m, 2H	
9	170.0		
10	23.3	1.99, s, 3H	
11	61.0	3.86, s, 3H	
12	56.3	3.92, s, 3H	

characteristic absorption of a -S-SO-C- group (11) at 1085 cm^{-1} was found in the ir spectra of these compounds. Asymmetrical structures for **4a** and **5a** were confirmed by ^{1}H-nmr studies. The ^{1}H-nmr spectrum of **4a** revealed downfield shifts of the methoxyl group signals, while the spectrum of **5a** revealed similar shifts for signals of the NH- and CH_3CO- groups. These data suggested structures corresponding to the 3′-*S*-oxide for **4a** and the 1′-*S*-oxide for **5a**. It is likely that *S*-oxides **4** and **5** are natural products. Indeed, a mixture of **1a**+**3a** did not give either of these compounds at room temperature in MeOH solution after standing for one year. It is tempting to speculate that varacin A [**3**] is a natural product, although it is also formed by decomposition from **1**. In support of this, crude varacin [**1**], which was obtained on board the research vessel by rapid chromatographic separation of extract, contained **3**. The ^{1}H-nmr spectrum (CD_3OD) of this substance had, along with a characteristic singlet of the aromatic proton of **1** at 7.13 ppm, a similar singlet of **3** at 6.75 ppm.

Varacin [**1**], varacin acetate [**1a**], and varacin A–C acetates [**3a–5a**] all exhibit potent antifungal and antimicrobial activities in vitro against *Candida albicans* (20 mm/0.1 μg for **1** and 20 mm/10 μg for **1a**, **3a–5a**) and *Bacillus subtilus* (20 mm/0.1 μg for **1** and 20 mm/1 μg for **1a**, **3a–5a**). These results show that activities of the series do not depend upon the presence of a free amino group in their side-chains.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H- and ^{13}C-nmr spectra were recorded on a Bruker WM-250 spectrometer with TMS as internal standard. Mps were determined on a Boethius apparatus. Eims were measured on a LKB 9000S spectrometer (ionizing energy 70 eV), and fabms were obtained using a LKB 9091 instrument. Prep. hplc was conducted utilizing a DuPont 8800 instrument, fitted with a RIDK-102 differential refractometer. Polysulfide acetates were fractioned on an Ultrasphere-Si column (10 mm i.d.×25 cm) using EtOAc as mobile phase with a flow rate of 3 ml/min. Tlc and lplc were performed on Si gel L (Chemapol, former Czechoslovakia) 5/40 and 40/100 μm, respectively.

ANIMAL MATERIAL.—The ascidian *Polycitor* sp. was collected during the 13th cruise of the research vessel "Academik Oparin" in July 1991 at the depth of 90 m, near Valentin Bay (northwestern shore of the Sea of Japan, 43°04′ N, 134°30′ E). Numerous colonies of this ascidian were found to be attached to mollusc shells and tubes of Polychaeta. Living colonies have a light gray to pale pink smooth surface with rounded edges. Colonies vary in their form and size. The maximum height of a colony is about 10 mm. Zooids (length 1–1.5 mm) are located in a disorderly fashion. Both siphons are 6-lobed and there are more than 4 rows of stigma. A voucher specimen (013–118) is on deposit in the collection of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia.

EXTRACTION AND ISOLATION.—Animal materials were extracted with twice their volume of EtOH immediately after collection. The crude EtOH extract of the ascidian *Polycitor* sp. (0.20 kg) was evaporated *in vacuo* at 50° to give a brown oil. This oil was dissolved in 100 ml of EtOH-H_2O (9:1). The hexane solubles were extracted three times by partitioning with equal volumes of hexane. The aqueous phase was then diluted with H_2O to EtOH-H_2O (7:1) and partitioned four times against $CHCl_3$. The $CHCl_3$ solubles were concentrated at reduced pressure to give 5.23 g of a brown oil. This residue was chromatographed over a Si gel column, using $CHCl_3$/MeOH mixtures as eluents, to give an active fraction (3.03 g, dry wt), eluted with $CHCl_3$-MeOH (4:1). The latter was chromatographed twice on a column with Sephadex LH-20 (elution with $CHCl_3$-MeOH, 1:1) to obtain 231 mg (0.11% yield) of crude varacin [**1**] as a light yellow powder: mp 258–260° (dec); ir ($CHCl_3$) ν max 3364, 3040, 3008, 2930, 1600, 1578, 1464, 1420, 1240, 1200, 1068, 1008 cm^{-1}; eims (70 eV) *m/z* $[M-2\times S]^+$ 275 (20), $[M-2\times S-NH_3]^+$ 258 (6), $[M-2\times S-CH_2=NH]^+$ 246 (16), $[M-4\times S]^+$ 211 (8), $[2\times S]^+$ 64 (30), $[CH_2=NH_2]^+$ 30 (100); positive fabms, *m/z* $[M+H]^+$ 340. ^{1}H-nmr data, see Table 1.

PREPARATION OF POLYSULFIDE ACETATES.—Acetylation of crude varacin [**1**] (200 mg) with Ac_2O and pyridine (1:1) at room temperature afforded 206 mg of a mixture of acetates. The separation and purification of individual polysulfide acetates were carried out by repeated hplc as described in General Experimental Procedures.

Varacin acetate [**1a**].—Thin yellow crystals (MeOH) (41 mg): mp 136–137°; ir (KBr) ν max 3436, 3280, 3088, 2962, 2928, 2914, 2850,

1644, 1558, 1536, 1468, 1416, 1370, 1256, 1068, 1006, 990, 602 cm^{-1}; uv (MeOH) λ max (ε) 217 (19,000), 254 (12,000) nm; *RR*, and ms data, see Table 2, ^1H- and ^{13}C-nmr data, see Table 3.

Varacin A acetate [**3a**].—Thin yellow crystals (MeOH) (1 mg): mp 140–142°; ir (KBr) ν max 3400, 3255, 2914, 1642, 1528, 1456, 1360, 1241, 1066, 1006 cm^{-1}; *RR*, and ms data, see Table 2, ^1H- nmr data, see Table 1.

Varacin B acetate [**4a**].—Thin yellow crystals (MeOH) (1.0 mg): mp 131–133°; ir (KBr) ν max 3400, 3255, 2914, 1640, 1530, 1463, 1365, 1273, 1085, 1067, 1009 cm^{-1}; *RR*, and ms data, see Table 2, ^1H-nmr data, see Table 1.

Varacin C acetate [**5a**].—Thin yellow crystals (MeOH) (2.5 mg): mp 70–73°; ir (KBr) ν max 3400, 3255, 1640, 1530, 1463, 1366, 1278, 1085, 1065, 1009 cm^{-1}; *RR*, and ms data, see Table 2, ^1H-nmr data, see Table 3.

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