Vibrindole A, a Metabolite of the Marine Bacterium, Vibrio parahaemolyticus, Isolated from the Toxic Mucus of the Boxfish Ostracion cubicus

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VIBRINDOLE A, A METABOLITE OF THE MARINE BACTERIUM, *VIBRIO PARAHAEMOLYTICUS*, ISOLATED FROM THE TOXIC MUCUS OF THE BOXFISH *OSTRACION CUBICUS*

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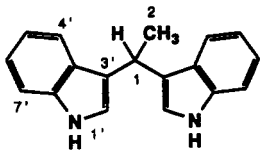
ABSTRACT.—The EtOAc extract of the whole culture medium of *Vibrio parahaemolyticus*, which inhabits the toxic mucus of the box fish *Ostracion cubicus*, afforded a new indole-derived natural product, vibrindole A [**1**], along with some known cyclic dipeptides and indoles. The structure of **1** was determined by analysis of its physicochemical characteristics.

Symbiotic microorganisms are common in all marine organisms, and are believed to be of great importance in the biosynthesis of biologically active natural products within these organisms. Recently, metabolites formally isolated from the sponge *Dysidea herbacea*, and speculated as being cyanobacterial metabolites, were isolated from the symbiotic cyanobacterium, *Oscillatoria spongeliae*, which inhabits the aforementioned sponge (1). The biosynthesis of the latter compounds by the cyanobacterium has yet to be demonstrated. During the past three years we have been involved in the isolation of biologically active natural products from extracts of cultured symbiotic, heterotrophic, and autotrophic microorganisms. The microorganisms were isolated from marine invertebrates (sponges and tunicates), and, as described below, from the toxic skin secretion of *Ostracion cubicus*.

The box fish, *O. cubicus* (Ostraciidae) secretes an ichthyotoxic mucus from its skin when stressed or disturbed. Chemical investigations that have been carried out on the toxic skin secretion of Australian *O. cubicus* (2) resulted in the isolation of pahutoxin, the choline chloride ester of 3-acetoxyhexadecanoic acid, and related compounds (3).

The white foamy mucus that was collected from a stressed fish was diluted in sterile sea water and plated onto Petri dishes containing SLB agar (2). Pure strains were isolated and grown after optimization of growth conditions in a SLB liquid medium. Two of the isolated strains (strains B-2 and B-4), that were identified as the same *Vibrio parahaemolyticus* (Vibrionaceae), showed antimicrobial and hemolytic activities. Strain B-2 was selected for further investigation of its extract constituents.

The EtOAc extract of the whole culture medium was chromatographed on Si gel H and eluted with step gradients from petroleum ether, through EtOAc, to MeOH. The major, and least polar component of the extract was identified as indole according to the red color obtained upon visualization after tlc with vanillin, and by comparing its physicochemical characteristics with those of authentic material. A second, non-polar component of the extract was identified as indole-3-carboxaldehyde. Two other antibacterial compounds of medium polarity were also stained red upon spraying the tlc plate with vanillin, and thus were suspected as being indole derivatives. Purification of these compounds on a reversed-phase (C_{18}) hplc column afforded the pure compounds **1**, designated vibrindole A, and **2** (0.53 and 10.3 mg/liter of culture medium, respectively). Compound **2** was identified as 2,2-di(3-



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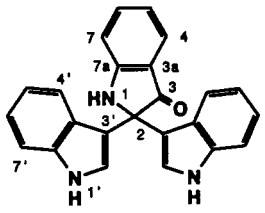

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indolyl)-3-indolone, on the basis of its spectroscopic properties (^{1}H, ^{13}C, COSY, HMQC, HMBC nmr spectra, and hreims data). Compound **2** was previously isolated as the product of indole oxidation by a strain of *Claviceps purpurea* (4). Purification of the polar fraction of the extract (70% EtOAc in petroleum ether through MeOH) by reversed-phase hplc afforded seven diketopiperazine derivatives that are produced by most culturable marine bacteria: cyclo(L-Pro,L-Ile), cyclo(L-Pro,L-Leu), cyclo(L-Pro,L-Val), cyclo(L-Pro,L-Tyr), cyclo[Didehydroaminopropionyl (Dhap),L-Ile], cyclo(Dhap,L-Leu) and cyclo(Dhap,L-Val) (1).

The molecular formula of **1**, $C_{18}H_{16}N_2$, was deduced from its hreims (*m/z* 260.1370). Inspection of the nmr spectra of **1** revealed that the aromatic signals were doubled in their intensity relative to the higher field signals. The two doublets of doublets (δ_H 7.28 and 7.50) and the two doublets of triplets (δ_H 6.96 and 7.09) were interrelated through a COSY-45 experiment to a fragment that fits a 1,2-disubstituted aromatic ring. The two other protons in this region (δ_H 6.86 br d and 7.85 br s) were coupled to each other through three bonds (J=2.2 Hz). The latter two fragments fit an indole system substituted at position 3. Five of these signals (δ_H 6.86, 6.96, 7.09, 7.28, and 7.50) could be correlated in an HMQC experiment to five carbons in the aromatic region (see Table 1). The two identical 3-indolyl fragments could finally be constructed with the assistance of the correlations from an HMBC experiment (see Table 1) and the hreims indole fragment at *m/z* 117.0538 (C_8H_7N). The remaining two signals in the ^{1}H- and ^{13}C-nmr spectra could be assigned to a 1,1-disubstituted ethane (see Table 1). The proton signals of the latter fragment (δ_H 4.61 q, 1H and 1.74 d, 3H) could be correlated in the HMBC map to carbons 2′, 3′, and 3a′ of the two indolyl systems. H-2′ of the two indolyl systems presented correlations to C-1 of the ethane fragment. Based on these data, vibrindole A was identified as 1,1-di(3-indolyl)

TABLE 1. Nmr Data of Vibrindole A [**1**] in $CDCl_3$.[a,b]

Position	δ_C multiplicity	δ_H multiplicity	J (Hz)	No. of Protons	H-H Correlations	Long-range C-H Correlations
1	28.0 d	4.61 q	7.6	1	2	2, 2′
2	21.7 q	1.74 d	7.6	3	1	1
1′		7.85 br s		2	2′	
2′	121.1 d	6.86 d	2.2	2	1′	1
3′	121.8 s					1,2,2′,4′
3a′	127.0 s					1,1′,2′,4′,7′
4′	119.7 d	7.50 d	7.9	2	5′,6′,7′	5′,6′
5′	119.0 d	6.96 dt	0.6, 7.9	2	4′,6′,7′	6′,7′
6′	121.7 d	7.09 t	7.9	2	4′,5′,7′	4′,5′
7′	111.0 d	7.28 dd	0.6, 7.9	2	4′,5′,6′	4′,5′,6′
7a′	136.6 s					2′,4′,6′

[a]Chemical shifts referred to $CHCl_3$ at 7.26 ppm and to $CDCl_3$ at 77.0 ppm.
[b]Assignments assisted by COSY, HMQC, and HMBC experiments.

ethane. Vibrindole A [**1**] is a new natural product, but several synthetic routes have been reported for this compound since 1963 (5–8). A related compound, streptindole (2,2-di(3-indolyl) ethyl acetate), was isolated a few years ago as a genotoxic metabolite of the intestinal bacterium *Streptococcus faecium* (9).

Compound **1** exhibited an 11-mm zone of inhibition against *Staphyloccocus aureus* and *S. albus*, and a 7-mm zone against *Bacilus subtilis* at 100 μg/disk. Compound **2** gave an 11-mm zone of inhibition against *Staphyloccocus aureus* at 100 μg/disk. The standard gentamycin presented 14-, 14-, and 10-mm zones of inhibition against *Staphyloccocus aureus, S. albus,* and *Bacilus subtilis*, respectively, at 1 μg/disk.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ftir spectra were recorded on a Nicolet Ftir in $CHCl_3$ or neat. High-resolution ms were recorded on a Finnegan MAT 711. Uv spectra were recorded on a Kontron 931 plus spectrophotometer. Nmr spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^1H and 125.76 MHz for ^{13}C. ^1H, ^{13}C, DEPT, COSY-45, HMQC, and HMBC spectra were recorded using standard Bruker pulse sequences. Hplc separations were performed with an Applied Biosystem Inc. instrument equipped with two model 150 pumps and a 893 programmable uv detector.

CULTURING CONDITIONS.—Bacteria were isolated from the toxic mucus of *Ostracion cubicus* in the following manner. A fish captured using a hand net was allowed to acclimatize for four to six h in running sea water. The fish was removed from the tank and gently rinsed with sterile sea water. The fish was forced into a graduated cylinder filled with sterile sea water. After five minutes the sea water turned turbid due to the secretion of the toxic mucus. A sample of the mucus was diluted and plated on SLB medium (10). This medium consisted of Bacto tryptone (Difco), 5 g; bacto yeast extract (Difco), 2.5 g; NaCl, 20 g; bacto agar, 30 g; and distilled H_2O to 1000 ml. After incubation at 25° for 4 days, the five most frequent colonies (B-1 to B-5) were isolated. For antibiotic production, *Vibrio parahaemolyticus* strain B-2 was batch cultured. One liter culture of the isolate was grown in liquid SLB medium (SLB without agar) at 27° for 48 h. Growth was followed using a Klett Summerson turbidity meter. After cooling on ice cultures were kept frozen at −70°.

EXTRACTION AND ISOLATION.—The melted whole culture medium (6 liters) was extracted with EtOAc (3×3 liters) and the combined EtOAc fraction dried and evaporated. The crude material (550 mg) was loaded onto a vacuum column (Merck Si gel H, 5 g, packed into a 2 cm i.d.×30 cm high, sintered glass funnel; vacuum by water aspirator) and eluted with petroleum ether with a 10% increase in EtOAc step gradient to yield 12 fractions (50 ml each). The fractions were assayed for antibacterial activity against *Staphyloccocus aureus* and *S. albus* at a concentration of 0.25 mg/disk. Fractions 5, 6, and 7 were active. Fraction 5 contained mainly compound **1**, fraction 6 contained a mixture of compounds **1** and **2**, and fraction 7 contained mainly compound **2**. The latter fractions were combined (112 mg) and separated on a preparative hplc column (Alltech Econosil C_{18}, 10 μm, 22.5 mm×250 mm) using MeOH-H_2O (3:1) as eluent (6 ml/min) and uv detection (238 nm). The major component of this fraction (62.1 mg, R_t 13.3 min) was compound **2**. Compound **1** (3.2 mg) was eluted from the column with a longer retention time (R_t 44.7 min).

Vibrindole A [**1**] was obtained as a colorless oil: Uv ($CHCl_3$) λ max (ε) 241 (12,220), 275 (8,100), 282 (8,160) nm; ir (neat) ν max 3416, 2958, 2936, 1612, 1456, 743 cm^{-1}; ^1H- and ^{13}C-nmr data, see Table 1; hreims (70 eV) *m/z* 260.1370 (M^+, 37, calcd for $C_{18}H_{16}N_2$, mmu error −0.4), 245.1076 (100, calcd for $C_{17}H_{13}N_2$, mmu error −0.3), 117.0538 (18, calcd for C_8H_7N, mmu error −4.1).

2,2-Di(3-indolyl)-3-indolone [**2**] was obtained as a colorless oil: Uv ($CHCl_3$) λ max (ε) 242 (33,600), 268 (31,800) nm; ir (neat) ν max 3407, 1683, 1027, 995, 823, 740 cm^{-1}; ^1H nmr ($CDCl_3$, 500 MHz) δ 7.94 (2H, br s, H-1′), 7.69 (1H, dd, J=0.9 and 7.1 Hz, H-4), 7.52 (1H, dt, J=0.9 and 7.1 Hz, H-6), 7.40 (2H, d, J=7.9 Hz, H-4′), 7.37 (2H, d, J=7.9 Hz, H-7′), 7.13 (2H, d, J=2.0 Hz, H-2′), 7.11 (2H, dt, J=0.8 and 7.9 Hz, H-6′), 6.94 (1H, d, J=7.1 Hz, H-7), 6.93 (2H, dt, J=0.8 and 7.9 Hz, H-5′), 6.87 (1H, dt, J=0.9 and 7.1 Hz, H-5), 6.08 (1H, br s, H-1); ^{13}C nmr ($CDCl_3$, 125 MHz) δ 201.8 (s, C-3), 160.5 (s, C-7a), 137.5 (d, C-6), 136.9 (2×s, C-7a′), 125.3 (2×s, C-3a′), 124.8 (d, C-4), 124.0 (2×d, C-2′), 121.2 (2×d, C-6′), 119.9 (2×d, C-4′), 119.0 (s, C-3a), 118.7 (3×d, C-5 and 5′), 113.7 (2×s, C-3′), 112.3 (d, C-7), 111.2 (2×d, C-7′), 68.2 (s, C-2); hreims (70 eV) *m/z* 363.1404 (M^+, 17, calcd for $C_{24}H_{17}N_3O$, mmu error 3.2).

BIOASSAYS.—Bacterial cultures were obtained from stocks maintained at the Department of Plant Protection and Inspection Clinical Laboratory, Ministry of Agriculture, Bet-Dagan, Israel, as follows: *Bacillus subtilis* (strain Bs1091-1), *Staphylococcus aureus* (strain Sau1091-5) and *Staphylococcus albus* (strain Sal1091-4). Bacteria were main-

tained and tested on Luria's Broth medium (LB) containing 10 g of triptone (Difco), 5 g yeast extract (Difco), 5 g of NaCl, and 16 g of bacto agar (Difco) per liter of distilled H_2O.

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