Isolation of Microbial Antibiotics from a Marine Ascidian of the Genus *Didemnum*

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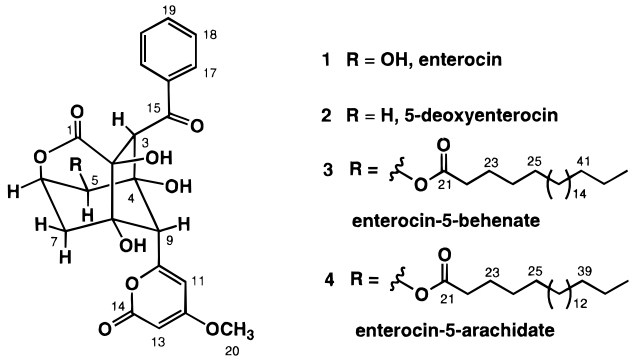
A major debate in marine natural products chemistry is the true origin of metabolites isolated from invertebrates which frequently possess symbiotic microorganisms. Numerous examples exist in which identical compounds have been isolated from taxonomically distinct invertebrates. The isolation of the cyclic peptides cycloxazoline[1] or westiellamide,[2] a tetrapyrrolic blue pigment,[3] bipyrroles of the tambjamine class,[4] and the alkaloids dercitamide or kuanoniamine D,[5] is difficult to explain considering the secondary metabolite specificity of most invertebrate groups. In many cases, closely related compounds of identical structural classes have been observed from marine invertebrates and terrestrial microorganisms. Examples are lissoclinolide,[6] tetrenolin,[7] saframycin B,[8] renieramycin E,[9] ecteinascidins,[10] and most recently staurosporine derivatives.[11] The isolation of similar compounds from two unrelated invertebrates, or the isolation of compounds reminiscent of microbial metabolites, supports the potential microbial origins of these invertebrate-derived compounds. The symbiotic relationship between sponges and cyanobacteria has served as the model for such potential symbioses, and in one case the location of several secondary metabolites has been shown to be within the associated cyanobacteria.[12] Symbioses between ascidians and microorganisms are also well known, suggesting that some ascidian metabolites may also have microbial origins.[13] In this paper, we report the isolation of four metabolites, all derivatives of the well-known microbial antibiotic enterocin (or vulgamycin), from an unidentified Western Australian ascidian of the genus *Didemnum*. Isolated in this study were enterocin (**1**) itself, 5-deoxyenterocin (**2**), enterocin-5-behenate (**3**), and enterocin-5-arachidate (**4**), specific derivatives of this rare class of microbially-produced antibiotics. This study adds further support for the concept that bacteria may produce some of the interesting molecules isolated from marine invertebrates.

Chart 1



The brown, encrusting ascidian, *Didemnum* sp. (specimen #s WA-90-122 and WA-90-129) was collected by hand using SCUBA (-8 m) near Exmouth, Western Australia, in December 1990 and 1992. The samples were frozen immediately after collection and then lyophilized, and the residue was extracted twice with 70% $MeOH/CH_2Cl_2$. Solvent partition and silica flash chromatography ($MeOH/CHCl_3$), followed by reversed-phase HPLC (25% MeCN/2% $MeOH/H_2O$), yielded the known *Streptomyces* metabolite enterocin[14] (**1**, 30 mg, 0.11% dry wt), 5-deoxyenterocin (**2**, 935 mg, 3.5% dry wt), and impure enterocin esters. The mixtures were further purified using successive reversed-phase HPLC (100% MeOH and 2% H_2O/MeOH) to give the related compounds, enterocin-5-behenate (**3**, 6.8 mg, 0.026% dry wt) and enterocin-5-arachidate (**4**, 3 mg, 0.011% dry wt).

The known compounds, enterocin (**1**) and 5-deoxyenterocin (**2**), originally isolated from three strains of soil-derived *Streptomyces* species, were identified by comprehensive spectral analyses and by comparison of limited literature spectral data. 5-Deoxyenterocin, mentioned only in a Japanese patent,[15] is not fully characterized in the literature. Hence a comprehensive structural assignment was undertaken, applying 2D NMR analyses, to assure that NMR assignments were correct (see Table 1). 5-Deoxyenterocin was analyzed for $C_{22}H_{20}O_9$ by high resolution FAB mass spectrometry and by ^{13}C NMR. The spectral similarities between enterocin (**1**) and 5-deoxyenterocin (**2**) were striking. Spectral features for the 3-methoxy-α-pyrone and phenyl ketone functionalities were easily assigned based upon the spectral data published for enterocin. The major difference in the spectral data was that compound **2** contained one less

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Table 1. NMR Data for 5-Deoxyenterocin (2)[c]

no.	^{13}C	^{1}H[a]	COSY[a]	HMBC[a] (8 Hz)	NOESY[a]
1	174.1				
2	79.7	5.80(s, OH)		C3, C2, C1	
3	61.0	4.00 (s, 1H)		C5, C9, C4, C8, C2, C1, C15	$H5_{eq}$, H17, H17′
4	75.9	5.49 (s, OH)		C5, C9, C4, C3	
5	39.5	2.06 (bd, $1H_{eq}$, $J = 15$)[b]	H6, H9, $H5_{ax}$	C7, C9, C3, C6, C4	H3, $H5_{ax}$, H6
		2.65 (dd, $1H_{ax}$, $J = 15, 4$)[b]	H6, $H5_{eq}$	C3, C4, C9	$H5_{eq}$, H6, H11
6	72.6	4.83 (m, 1H)	H5, H7	C7, C5, C4, C8, C1	H5, H7
7	36.8	1.62 (bd, $1H_{eq}$, $J = 14$)[b]	H6, H9, $H7_{ax}$	C5, C9, C6, C8	H6, $H7_{ax}$
		2.22 (dd, $1H_{ax}$, $J = 14, 3$)[b]	H6, $H7_{eq}$	C9, C8, C2	H6, $H7_{eq}$
8	77.1	5.91 (s, OH)		C7, C9, C8	
9	54.3	4.61 (bs, 1H)	$H5_{eq}$, $H7_{eq}$	C7, C5, C4, C8, C11, C10	H11
10	162.0				
11	105.6	6.26 (d, 1H, $J = 2.5$)	H13	C12, C10, C13, C9	$H5_{ax}$, H9, H20
12	170.7				
13	87.8	5.61 (d, 1H, $J = 2.5$)	H11	C12, C14, C11	H20
14	163.5				
15	195.1				
16	139.4				
17, 17′	128.0	7.78(d, 2H, $J = 8$)	H18, H18′	C15, C19, C17′	H3, H18, H18′
18, 18′	128.5	7.49 (t, 2H, $J = 8$)	H17, H17′	C16, C18′	H17, H17′, H19
19	132.7	7.59 (t, 1H, $J = 8$)	H18, H18′	C17, C17′	H18, H18′
20	56.3	3.81 (s, 3H)		C12	H11, H13

[a] Spectra were obtained at 500 MHz. [b] In MeOH-d_4 the C-5 protons were observed at δ 2.17 (dd, 1H, $J = 15$, 1.5 Hz), 2.82 (dd, 1H, $J = 15$, 4.5 Hz), while the C-7 protons were observed at δ 1.82 (dd, 1H, $J = 15$, 2 Hz), and 2.55 (dd, 1H, $J = 15$, 3 Hz). [c] All spectra, except for NOESY (MeOH-d_4), were recorded in DMSO-d_6. Assignments were aided by DEPT and HMQC experiments. Chemical shifts are reported in δ units (down field of TMS). Coupling constants are presented in Hertz units. ^{13}C NMR experiments were performed at 125 MHz.

oxygen atom. NMR experiments involving 2D HMQC and HMBC heterocorrelation measurements allowed all carbons and their respective protons to be assigned (Table 1). The relative stereochemistries of the chiral centers at C-2, -3, -4, -6, -8, and -9 were defined by NOESY experiments that placed protons within spatial proximities on the rigid cyclohexane ring. The proton at C-3 showed correlations with the β (eq) proton at C-5 and with the C-17 aromatic protons. The C-11 pyrone proton showed correlations with the C-5 α proton, thus placing the pyrone in the axial and down position at C-9.

Enterocin-5-behenate (**3**) was obtained as a white amorphous solid. The molecular formula $C_{44}H_{62}O_{11}$, established by HRFABMS [$(M + H)^+$ obsd m/z 767.4354 dev −2.14 ppm], showed 14 degrees of unsaturation, one more than enterocin itself. A strong IR absorption at 3385 cm^{-1}, three quaternary carbon signals in the ^{13}C NMR spectrum at δ 80.9, 78.8 and 77.5, all of which correlated with D_2O-exchangeable proton signals at δ 5.87 (s, 1H), 6.06 (s, 1H), and 6.08 (s, 1H), suggested the presence of three tertiary alcohols. From ^{13}C NMR, COSY, and HMBC spectral analysis, the same 3-methoxy-α-pyrone and phenyl ketone functionalities were readily assigned (Table 2). The 3-methoxy-α-pyrone unit was supported by strong IR absorptions at 1643 and 1564 cm^{-1}. The presence of the phenyl ketone functionality was also supported by the downfield shift of the proton signal at δ 7.89 (d, 2H, $J = 8$ Hz) and a strong IR absorption at 1694 cm^{-1}. In the ^{1}H NMR and COSY spectra of **3**, three proton signals at δ 1.90 (ddd, 1H, $J =$ 15, 3, 2 Hz), 2.70 (dd, 1H, $J = 15$, 3 Hz), and 5.84 (d, 1H, $J = 5$ Hz), coupled to a proton signal at δ 4.94 (ddd, 1H, $J = 5$, 3, 3 Hz), while the proton signal at δ 1.90 only coupled to a proton signal at δ 4.81 (d, 1H, $J = 2$ Hz). These results suggested that the protons at δ 1.90 and 4.81 couple through a 1,3-diequatorial interaction (W-coupling), thus leading to their assignment at C-7 (eq) and C-9, respectively. The proton at δ 5.84 (corresponding to a methine carbon at δ 73.2) was significantly downfield shifted compared with the same proton (δ 4.64) in enterocin (**1**). Given the additional degree of unsaturation in this metabolite, the presence of an IR carbonyl absorption at 1740 cm^{-1}, and the presence of an additional ester carbonyl at δ 174.2, it was concluded that esterification had taken place at C-5. This assignment was also supported by a long range correlation between H-5 (δ 5.84) and C-21 (δ 174.2), thus linking the ester carbonyl to this position. The ^{1}H NMR signals for **3** showed several characteristic signals for a saturated fatty acid chain [δ 2.42 (dt, 1H, $J = 14$, 7 Hz), 2.46 (dt, 1H, $J = 14$, 7 Hz), 1.63 (q, 2H, $J = 7$ Hz), 1.33 (m, 2H), 1.30 (m, 34H), 0.89 (t, 3H, $J = 7$ Hz)]. Since accurate proton numbers for the fatty acid chain were difficult to obtain by integration, the identity of the fatty acid was assigned by evaluation of fragments observed in the LREI mass spectrum of **3**. The major fragment observed (base peak at m/z 340 amu) was ascribed to the expected "McLafferty + 1" rearrangement. The saturated fatty acid was, therefore, identified as behenic acid (22:0).

Enterocin-5-arachidate (**4**) was obtained as an amorphous solid. The ^{1}H NMR and UV spectra of **4** were almost identical to those of **3** (Table 2). Thus, this enterocin derivative was recognized as a structurally similar fatty acid ester of **1**. Through HRFAB mass spectral analysis, the molecular formula $C_{42}H_{58}O_{11}$ was confidently established. Low resolution EI mass spectral analysis, in a similar fashion with **3**, showed the expected ester fragmentation (base peak at m/z 312), but showed that the ester in this derivative was derived from arachidic acid (20:0), the two carbon lower homolog.

The absolute stereochemistries of **1**-**4** appear identical to those found in the *Streptomyces*-produced antibiotics. Enterocin (**1**) showed $[\alpha]_D = -18.7°$, in close accord with the reported literature values.[14] Similarly, 5-deoxyenterocin (**2**) showed $[\alpha]_D = -31.3°$, and the ester derivatives **3** and **4** showed $[\alpha]_D = -75°$ and $[\alpha]_D = -81°$, respectively. Since the new enterocin derivatives all show optical rotations of the same sign, and the absolute stereochemistry of **1** is well known, the absolute stereochemistries for all metabolites were assigned as C-3 = *S*, identical with that reported for enterocin.

Table 2. NMR Data for Enterocin-5-behenate (3) and Enterocin-5-arachidate (4)[d]

	compound **3**					
no.	^{13}C	^{1}H[a]	COSY[a]	HMBC[a] (8 Hz)	NOESY[a]	compound **4**: ^{1}H[a,c]
1	175.1					
2	80.9	6.08 (s, OH)[b]		C1, C3	H9	
3	55.9	4.59 (s, 1H)		C1, C2, C4, C5, C8, C9, C15	4-OH, H17, H17′	4.59 (S, 1H)
4	78.8	5.87 (s, OH)[b]		C4, C9	H3, H9	
5	73.2	5.84 (d, 1H, $J = 5$)	H6	C3, C4, C21	H6, $H7_{ax}$	5.84 (d, 1H, $J = 5$)
6	74.4	4.94 (ddd, 1H, $J = 5, 3, 3$)	H5, H7	C1, C4, C5, C8	H5, H7	4.94 (m, 1H)
7	36.5	1.90 (ddd, 1Heq, $J = 15, 3, 2$)	H6, $H7_{ax}$, H9		H6, $H7_{ax}$	1.90 (ddd, $1H_{eq}$, $J = 15, 3, 2$)
		2.70 (dd, $1H_{ax}$, $J = 15, 3$)	H6, $H7_{eq}$	C2, C8, C9	H5, H6, $H7_{eq}$	2.70 (dd, $1H_{ax}$, $J = 15, 3$)
8	77.5	6.06 (s, OH)[b]		C7, C8	H9	
9	56.8	4.81 (d, 1H, $J = 2$)	$H7_{eq}$	C4, C5, C7, C8, C10, C11	4-OH, 8-OH, H11	4.81 (d, 1H, $J = 2$)
10	161.6					
11	107.3	6.40 (d, 1H, $J = 2$)	H13	C10, C12, C13	H9, H20	6.40 (d, 1H, $J = 2$)
12	173.3					
13	89.2	5.65 (d, 1H, $J = 2$)	H11	C11, C12	H20	5.65 (d, 1H, $J = 2$)
14	166.7					
15	196.7					
16	140.8					
17, 17′	129.5	7.89 (d, 2H, $J = 8$)	H18, H18′	C15, C19, C17′, C17	H3, H18, H18′	7.89 (d, 2H, $J = 8$)
18, 18′	129.6	7.48 (t, 2H, $J = 8$)	C15, H18′, H18	C15, C18′, C18	H17, H17′, H19	7.48 (t, 2H, $J = 8$)
19	134.2	7.59 (t, 1H, $J = 8$)	H18, H18′	C17, C17′	H18, H18′	7.59 (t, 1H, $J = 8$)
20	57.0	3.89 (s, 3H)		C12	H11, H13	3.89 (s, 3H)
21	174.2					
22	35.0	2.42 (dt, 1H, $J = 14, 7$)	H23	C21, C23, C24	H23	2.42 (dt, 1H, $J = 14, 7$)
		2.46 (dt, 1H, $J = 14, 7$)				2.46 (dt, 1H, $J = 14, 7$)
23	26.1	1.63 (q, 2H, $J = 7$)	H22, H24	C21, C22, C25	H22	1.63 (q, 2H, $J = 7$)
24	30.1	1.33 (m, 2H)	H23, H25			1.33 (m, 2H)
25	30.5	1.30 (m, 2H)	H24, H26			1.30 (m, 2H)
26	30.6	1.30 (m, 2H)		C27		1.30 (m, 2H)
27−40	30.8	1.30 (m, 26H)		C27-C39		1.30 (m, 26H)
41	33.1	1.30 (m, 2H)				0.89 (t, 3H, $J = 7$)
42	23.7	1.30 (m, 2H)			H43	
43	14.4	0.89 (t, 3H, $J = 7$)	H42	C41, C42	H42	

[a] Spectra were obtained at 500 MHz. [b] Signals were observed in DMSO-d_6. [c] Assignments were based on a comparison of the ^{1}H NMR assignments for enterocin-5-behenate (**3**). [d] All spectra were recorded in MeOH-d_4, except for HMBC and NOESY spectra which were recorded in DMSO-d_6. Assignments were provided by HMQC experiments. Chemical shifts are reported in δ units (down field of TMS). Coupling constants are presented in Hertz. ^{13}C NMR experiments were performed at 50 MHz.

This is apparently the first observation of enterocin being isolated from nonmicrobial sources. The limited production of enterocin (vulgamycin) antibiotics by various *Streptomyces*, and the uniqueness of this structural class, creates significant questions about their true origins in biologically-complex marine invertebrates. Microscopic examination of a thin section of the alcohol-fixed ascidian tunic of this *Didemnum* sp. showed the presence of large amounts of V-shaped bacteria similar in size and shape to *Arthrobacter* species. Although considerable effort was expended to recollect fresh samples and cultivate these endobiotic microorganisms, none of the 20 strains obtained could be confirmed, in culture, to produce the enterocin-based metabolites isolated from the whole animal. NMR examination of the corresponding chromatography fractions of extracts of these cultures failed to show the presence of signals attributable to the enterocins. Negative evidence such as this is discouraging; however, we are still struggling to gain an understanding of the nutrient requirements and culture conditions for the cultivation of symbiotic bacteria. Even though we could not demonstrate the microbial production of these natural products, this study provides a direct correlation between the presence of large numbers of morphologically-distinct bacteria and the isolation of what are most likely microbial metabolites.

Experimental Section

General. NMR spectra were recorded at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C). Infrared spectra were recorded as thin films on NaCl plates, while ultraviolet spectra were obtained in methanol solutions. Low resolution electron ionization mass spectral analyses were performed at 70 eV. High and low resolution FAB mass measurements were provided by the Mass Spectrometry Facility at the University of California, Riverside. Optical rotations were measured in methanol using a 10-cm cell. The melting point reported was uncorrected.

Collection, Extraction, and Isolation Procedures. Two brown, encrusting ascidian collections, (WA-90-122 and WA-90-129), both apparently colormorphs of one *Didemnum* species, were collected by hand using SCUBA (−8 m) near Exmouth, Western Australia, in December 1990. Since both species possessed the identical metabolites, the collections and extracts were ultimately combined. For chemical investigation, the specimens were immediately frozen after collection. The lyophilized animals (26.6 g dry wt) were extracted twice with 70% MeOH/CH_2Cl_2. The combined extract was concentrated and partitioned between hexane and methanol, and the methanol fraction was then further partitioned between ethyl acetate and water. Silica vacuum flash chromatography of the ethyl acetate fraction using MeOH/$CHCl_3$ mixtures, followed by reversed-phase HPLC (25% MeCN/2% MeOH/H_2O), yielded enterocin (**1**, 30 mg, 0.11% dry wt), 5-deoxyenterocin (**2**, 935 mg, 3.5% dry wt), and a mixture of two enterocin esters. The mixture was further purified using successive reversed-phase HPLC (100% MeOH and 2% H_2O/MeOH) to give enterocin-5-behenate (**3**, 6.8 mg, 0.026% dry wt), and enterocin-5-arachidate (**4**, 3 mg, 0.011% dry wt).

Enterocin (1). Spectral data for enterocin were identical to those reported in the literature.[14] The rotation recorded for enterocin isolated in this study was $[\alpha]_D = -18.7°$.

5-Deoxyenterocin (2): white solid, mp 218−220 °C; $[\alpha]_D$ (*c* 1.0, MeOH) −31.5°; HRFABMS $(M + H)^+$ obsd *m*/*z* 429.1205, $C_{22}H_{21}O_9$, dev 4.53 ppm; IR (NaCl) 3404, 1751, 1716, 1691, 1640, 1564, 1458, 1410, 1375, 1336, 1295, 1226, 1224, 1153, 1134,

1025, 1005 cm^{-1}; UV (MeOH) 284 nm (ϵ 5650), 248 (8110), 203 (24900); UV (MeOH + NaOH) 385 nm (ϵ 1710), 284 (5600), 244 (8800), 205 (70000); LREIMS: *m/z* 428, 348, 323, 269, 243, 235, 185, 167, 140, 125, 105, 77, 69, 44. See Table 1 for NMR spectral data.

Enterocin-5-behenate (3): noncrystalline solid; $[\alpha]_D$ (*c* 0.19, MeOH) −75.5°; HRFABMS $(M + H)^+$ obsd *m/z* 767.4354, $C_{44}H_{63}O_{11}$, dev −2.14 ppm; IR (NaCl) 3385, 2915, 2848, 1740, 1694, 1643, 1564, 1465, 1378, 1254, 1223, 1125, 1019 cm^{-1}; UV (MeOH) 284 nm (ϵ 12700), 248 (19100), 203 (58000); UV (MeOH + NaOH) 385 nm (ϵ 3010), 282 (11600), 246 (19600), 205 (57000); LREIMS *m/z* 341, 340, 297, 288, 259, 241, 211, 185, 167, 140, 129, 125, 115, 111, 105, 97, 85, 83, 77, 73, 71, 69, 60, 57, 43, 41. See Table 2 for NMR spectral data.

Enterocin-5-arachidate (4): noncrystalline solid; $[\alpha]_D$ (*c* 0.15, MeOH) −81°; HRFABMS $(M + H)^+$ obsd *m/z* 739.4052, $C_{42}H_{59}O_{11}$, dev −0.73 ppm; IR (NaCl) 3376, 2922, 2852, 1741, 1694, 1638, 1453, 1253, 1224, 1119 cm^{-1}; UV (MeOH) 284 nm (ϵ 12900), 247 (20400), 203 (57200); UV (MeOH + NaOH) 382 nm (ϵ 4000), 282 (12000), 244 (19800), 205 (78000); LREIMS: *m/z* 313, 312, 269, 221, 185, 149, 147, 129, 125, 105, 97, 85, 83, 77, 73, 69, 60, 57, 55, 43. See Table 2 for NMR spectral data.

Observation and Chemical Investigation of Bacterial Associates from *Didemnum* sp. For microbial evaluation, a second collection was made in December of 1992. Specimens (WA-92-04) were carefully compared with a previous voucher specimen. The presence of 5-deoxyenterocin as the major compound in the crude extract was determined by TLC comparison with previously purified standards. Once the ascidian was confirmed as the same collections made earlier (WA-90-122 and WA-90-129), a small piece of the ascidian was macerated in sterile seawater, and the resultant suspension was further diluted with sterile seawater (to ca. 1/1000 and 1/10000). Dilute suspensions were plated onto solid agar prepared with B1 media and onto B1 media enriched with a sterile, filtered seawater extract of the ascidian. Colony-forming bacteria were obtained and transferred into pure cultures. In addition, small pieces of the animal were frozen in 10% glycerol seawater solution, transported to La Jolla, and after defrosting sampled using the identical procedure above. In total, 20 bacterial strains were obtained. All cultures grew well in B1 media in shake flask cultures. After dense cultures were obtained (3−7 days), the whole cultures were extracted with ethyl acetate, and the reduced extracts were chromatographed on silica gel to obtain the midpolarity chromatography fractions (observed earlier to contain the enterocin antibiotics). Analysis of the fractions showed that none contained any of the corresponding signals from enterocin, 5-deoxyenterocin, nor the ester derivatives. B-1 Media consisted of the following: peptone (2.5 g), yeast extract (1.5 g), 50% glycerol (3.0 mL), seawater (750 mL), and deionized water (250 mL).

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Supporting Information Available: Copies of relevant spectral data for metabolites **1**−**4** (13 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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