Halicylindramides A-C, Antifungal and Cytotoxic Depsipeptides from the Marine Sponge *Halichondria cylindrata*[1]

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Halicylindramides A-C (**1**-**3**) have been isolated from the Japanese marine sponge *Halichondria cylindrata*. They are tetradecapeptides with the N-terminus blocked by a formyl group and the C-terminus lactonized with a threonine residue. Their total structures including absolute stereochemistry were determined by a combination of spectral and chemical methods. Halicylindramides A-C were antifungal against *Mortierella ramanniana* and cytotoxic against P388 murine leukemia cells.

Bioactive peptides are an increasing class of sponge metabolites,[2] some of which showed medicinally interesting activities: PLA_2 inhibitory (discodermins[3]), serine protease inhibitory (cyclotheonamides[4]), protein phosphatase inhibitory (motuporin[5]), and cytotoxic activities (polytheonamides[6] and many others[2]). In the course of our studies of bioactive metabolites in Japanese invertebrates, we have isolated a cytotoxic macrolactam cylindramide[7] from *Halichondria cylindrata* collected off the Sata Peninsula, 900 km southwest of Tokyo. Further investigation of the same sponge collected off Atami,[8] 90 km southwest of Tokyo led to the isolation of cytotoxic and antifungal peptides related to the discodermins[3] along with cylindramide. This paper deals with the isolation and structure elucidation of these new peptides.

Results and Discussion

The concentrated EtOH extract of the frozen specimens (1 kg) was successively extracted with $CHCl_3$, EtOAc, and *n*-BuOH. The EtOAc and *n*-BuOH extracts were combined and fractionated by reversed phase and normal phase column chromatographies, followed by reversed phase HPLC to yield halicylindramide A (**1**, 8.1×10^{-4}%; based on wet weight), B (**2**, 5.4×10^{-3}%), and C (**3**, 1.1×10^{-3}%) along with the known compound cylindramide (**5**, 7.2×10^{-3}%) (Chart 1). Cylindramide was identified by comparison of ^1H NMR data and HPLC behavior with those of an authentic sample.

Halicylindramide B (**2**), the major component, exhibited an $(M + H)^+$ ion cluster at *m/z* 1789:1790:1791:1792 with an intensity ratio of 7:9:10:6 in the FAB mass spectrum, suggesting the presence of one bromine atom. The molecular formula of $C_{78}H_{111}BrN_{20}O_{22}S$ was established on the basis of positive ion high resolution FABMS [MH^+ *m/z* 1790.7053, Δ −3.3 mmu]. The IR spectrum showed an intense amide carbonyl band at 1660 cm^{-1} and an ester carbonyl band at 1745 cm^{-1}. The ^{13}C NMR spectrum (DMSO-d_6)[9] revealed a total of 16 carbonyl signals between δ 173.5 and 168.2, while 15 amide protons were observed between δ 8.37 and 6.67 in the ^1H NMR spectrum, thereby implying its peptide nature. In fact, standard amino acid analysis revealed the presence of Ala, Pro, Val, Trp, Arg, Asp, Phe, Thr, Gly, $Cys(O_3H)$, and three unusual amino acids. A negative ninhydrin reaction suggested a blocked *N*-terminus. Interpretation of the COSY, HOHAHA,[10] HMQC,[11] HMBC,[12] and NOESY[13] spectra allowed the structural assignment of 14 amino acid residues, which were eventually connected by tracing inter-residual cross peaks obtained by NOESY and HMBC experiments.

The assignments of ^1H NMR spin systems for the Ala, Pro, Arg, Val, Thr, and Gly residues were straightforward from interpretation of the COSY and HOHAHA spectra (Table 1).

Three aromatic amino acids were identifed by NMR spectra together with amino acid analysis. Four contiguous aromatic protons (δ 7.58, 6.94, 7.02, and 7.31) and an exchangable proton (δ 10.75) coupled to an aromatic proton (δ 7.13) were consistent with a 3-substituted indole, which was supported by the UV spectrum [λ_{max} 290, 283, 273 (sh) nm]. The presence of a monosubstituted and a 1,4-disubstituted benzene ring was evident from ^1H NMR data. ^{13}C NMR signals of the latter were reminiscent of a *p*-bromobenzene. HMBC cross peaks indicated that each aromatic ring was linked to a β-carbon, thus confirming Trp, Phe, and *p*-bromophenylalanine (BrPhe) residues.

The assignments of the MeGln and Asn residues were established by the HOHAHA and NOESY spectra. The spin system of the MeGln residue, which lacked an α-amide proton, was assigned by the HOHAHA spectrum. NOESY cross peaks were observed between *N*-methyl (δ 2.94) and Hα and between a pair of primary amide protons (δ 6.67 and 7.17) and Hδ, which indicated that the α-nitrogen was *N*-methylated and the γ-carbonyl was linked to an NH_2 group. The Asn residue was assigned in the same way by HOHAHA and NOESY data. The presence of *t*-Leu was inferred from an HMQC cross peak between a 9H-singlet proton (δ 0.70) and an exceptionally strong carbon signal (δ 26.4) and HMBC cross peaks between Hγ (δ 0.70) and Cβ (δ 33.7) and Cα (δ 60.6). The last amino acid residue exhibiting characteristic NMR signals [δ_H 4.45 (m, Hα), 2.91 (2H, m, $H_2\beta$); δ_C 50.9d, 52.3t] was identified as cysteic acid [$Cys(O_3H)$]. Additionally, a formamide group was evident from NMR data ($\delta_{H/C}$ 7.89/160.5).

Sequencing of these residues was carried out by interpretation of the NOESY and HMBC spectra (Scheme

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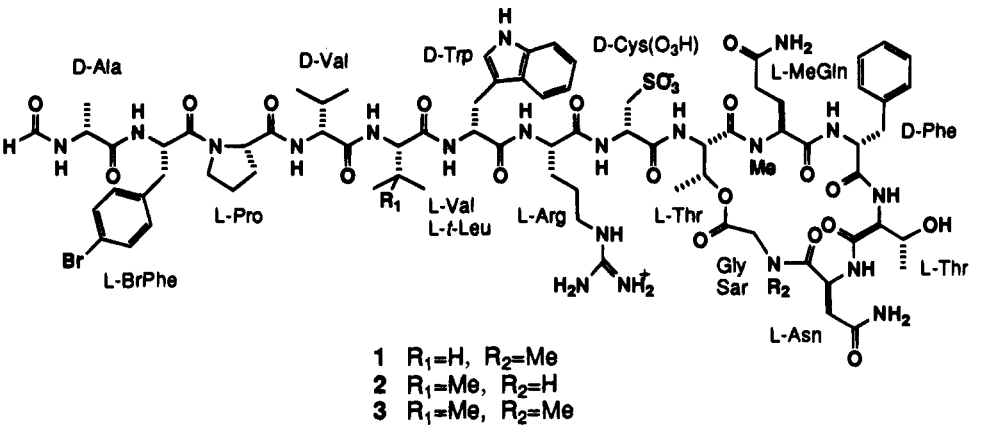
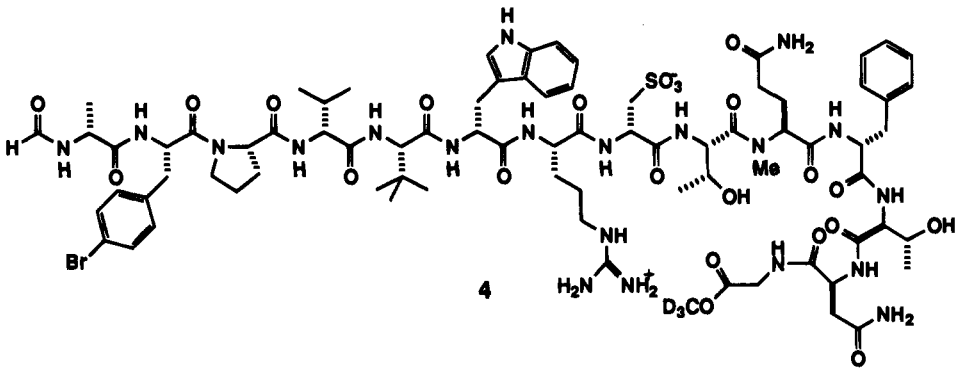
Chart 1



1 R_1=H, R_2=Me
2 R_1=Me, R_2=H
3 R_1=Me, R_2=Me



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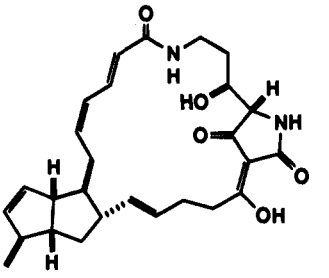

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1 and Table 1). Since Hβ of Thr-1 resonated at δ 5.16, its hydroxyl group must be esterified. An HMBC cross peak observed between Hβ of Thr-1 residue and the carbonyl carbon of the Gly residue allowed the presence of a macrocylic lactone ring.

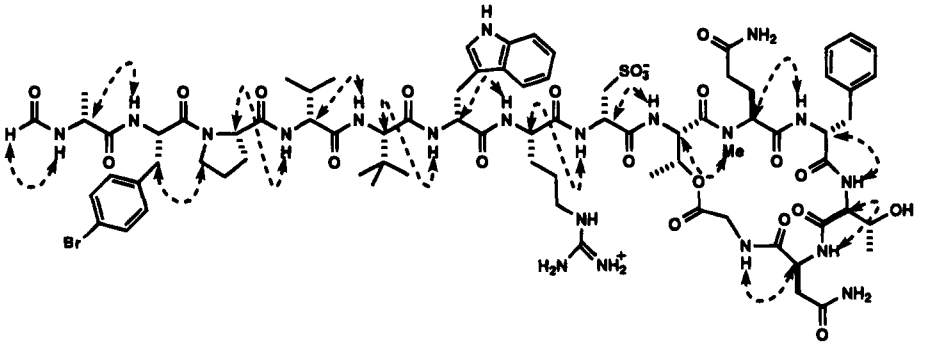
Halicylindramide A (**1**), which displayed the shortest HPLC retention time, had a molecular formula of $C_{78}H_{111}BrN_{20}O_{22}S$, the same as that of **2**. The UV spectrum (λ_{max} 214, 272, 280, 288 nm) indicated the presence of a Trp residue. ^1H and ^{13}C NMR data indicated that halicylindramide A contained Sar (*N*-methylglycine) and Val residues in place of Gly and *t*-Leu residue in halicylindramide B. The amino acid sequence of **1** was determined as in the case of halicylindramide B. Formation of a lactone ring between the hydroxyl group of Thr-1 and the carbonyl group of Sar was deduced from NOESY cross peaks. The ^1H NMR spectrum exhibited two sets of signals in a ratio of 6:1 for Thr-1, Sar, MeGln, and Asn residues, indicating the presence of two conformers probably due to *cis–trans* isomerism of the amide bond between Asn and Sar residues. Conspicuous exchange cross peaks were observed between the major and minor signals in the NOESY spectrum. Incidentally, halicylindramide B, which had a Gly residue instead of the Sar residue, adopted only one conformation.

The high-resolution FAB mass spectrum and NMR data of halicylindramide C (**3**) led to a molecular formula of $C_{79}H_{113}BrN_{20}O_{22}S$, one CH_2 unit larger than that of **2**. The ^1H and ^{13}C NMR spectra indicated that Gly was replaced by a Sar residue. An additional *N*-methyl signal (δ 2.95), which gave intense NOESY cross peaks with the methylene protons in Sar, was observed. Sequence analysis by interpretation of NOESY data allowed assignment of structure **3**. In order to further corroborate the amino acid sequence, halicylindramide C was deformylated and saponified to produce the deformyl *seco* acid, which was subjected to automatic protein sequencing. The resulting sequence was Ala-X-Pro-Val-*t*-Lut-Trp-Arg-X-Thr-X-Phe-Thr-Asn-X, where X denotes an unusual amino acid residue, thereby confirming the sequence obtianed from NMR data. Incidentally, the ^1H NMR spectrum exhibited a pair of signals for Sar, Thr-1, MeGLn, Asn, and Sar residues, reflecting conformational isomerism at the Asn-Sar bond.

Table 1. NMR Data for Halicylindramide B (**2**) in DMSO-d_6

position	^{1}H	^{13}C	HMBC	position	^{1}H	^{13}C	HMBC
NCHO	7.89 (s)	160.5, d	Alaα, Ala NH	Arg α	4.34 (dd, 12.5, 6.6)	51.9, d	
Ala α	4.30 (q, 7.0)	46.5, d	β, CHO	β	1.59 (2H, m)	29.6, t	α
β	0.93 (3H, d, 7.0)	19.4, q	α	γ	1.23 (m), 1.30 (m)	23.6, t	α
NH	8.13 (d, 6.8)			δ	2.94 (2H, m)	39.7, t	
CO		171.5, s	α, β	guanidine		156.8, s	
BrPhe α	4.72 (m)	51.5, d		NH	7.93 (brd, 7.5)		
β	2.71 (dd, 13.5, 10.9)	36.2, t	2, 6	CO		170.8, s	CysNH
	2.99 (m)						
C1		137.1, s	2, 6, β	Cys (SO_3) α	4.56 (m)	50.9, d	
C2/C6	7.21 (2H, d, 8.2)	131.7, d	3, 5	β	2.91 (2H, m)	52.3, t	α
C3/C5	7.39 (2H, d, 8.2)	130.8, d	2, 6	NH	8.32 (brd, 6.4)		
C4		119.5, s		CO		170.6	α, I-ThrNH
NH	8.37 (brd, 8.1)			Thr-1 α	4.90 (dd, 2.3, 7.9)	51.0	γ
CO		169.5, s	α, β	β	5.16 (dq, 2.3, 6.1)	69.5, d	γ
Pro α	4.47 (m)	60.2, d		γ	1.18 (3H, d, 6.3)	17.3, q	
β	1.82 (m), 2.08 (m)	29.0, t		NH	7.98 (brd, 7.6)		
γ	1.84 (2H, m)	24.1, t		CO		168.6, s	α, β, NMe
δ	3.63 (m)	47.0, t		NMeGln α	4.95 (m)	55.0, d	β, γ, NMe
CO		171.7, s	ValNH	β	1.74 (m), 1.82 (m)	24.1, t	γ
Val α	4.42 (dd, 7.7, 5.5)	57.2, d	β, γ	γ	1.89 (2H, m)	31.3, t	NH_2
β	2.10 (m)	30.7, d	α, γ	NMe	2.94 (3H, s)	29.7, q	
γ	0.77 (3H, d, 6.7)	15.9, q	α, β, γ'	$CONH_2$	6.67 (brs), 7.17 (brs)	173.5, s	β, r, NH_2
γ''	0.82 (3H, d, 6.6)	18.5, q	α, β, γ				
NH	7.82 (brd, 9.8)			CO		169.3, s	PheNH
CO		171.0, s		Phe α	4.63 (m)	54.9, d	β
t-Leu α	4.14 (d, 8.3)	60.6, d	γ	β	2.94 (2H, m)	37.9, t	α, 2, 6
β		33.7, s	α, γ	C1		136.8, s	2, 6, 3, 5, α, β
γ	0.70 (9H, s)	26.4, q	α	C2/C6	7.22 (2H, m)	129.6, d	3, 5, 4, β
NH	7.76 (brt, 7.4)			C3/C5	7.14 (2H, m)	128.2, d	2, 6, 4
CO		170.1, s	α, TrpNH	C4	7.12 (m)	126.7, s	2, 6, 3, 5
Trp α	4.55 (m)	54.9, d		NH	7.80 (brs)		
β	3.13 (m), 2.92 (m)	27.5, t	α	CO		172.5, s	α, β
C3		109.8, s	α, β, 2, 4	Thr-2 α	3.67 (m)	62.7, d	γ, β
C2	7.13 (s)	124.1, d	β	β	3.93 (brd, 5.3)	65.2, d	γ
C3a		136.1, s	2, 4, 6	γ	0.93 (3H, d, 7.0)	20.1, q	
C7a		127.2, s	2, 6, 7	OH	4.85 (brs)		
C4	7.58 (d, 7.7)	118.5, d		NH	8.39 (brs)		
C5	6.94 (dd, 7.5, 7.4)	118.1, d	5, 6	CO		170.4, s	AsnNH, α
C6	7.02 (dd, 7.3, 7.6)	120.8, d	7a, 5	Asn α	4.49 (m)	50.1, d	β
C7	7.31 (d, 8.0)	111.2, d	5	β	2.57 (dd, 15.6, 8.8)	35.8, t	α, NH_2
					2.63 (dd, 15.6, 5.8)		
1-NH	10.7 (brs)			NH	8.17 (brd)		
NH	8.24 (brd, 6.4)			$CONH_2$	6.81 (brs), 7.30 (brs)	171.7, s	α, β, NH_2
CO		171.1, s	ArgNH	CO		171.5, s	II-ThrNH
				Gly α	3.47 (brd, 16.5)	41.2, t	
					4.02 (dd, 16.6, 6.9)		
				NH	7.47 (brt)		
				CO		168.2, s	α, I-Thrβ

Scheme 1. Critical NOESY Cross Peaks for Amino Acid Sequence of Halicylindramide B



The configuration of component amino acids in halicylindramides was determined by Marfey analysis of the acid hydrolysis products.[14] Assignment of D- and L-Val residues in **1** was accomplished as in the case of discodermin A. Deformylhalicylindramide A was subjected to four Edman degradation cycles, and the resultant decapeptide, which had lost four residues from the *N*-terminus, was subjected to acid hydrolysis followed by Marfey analysis, revealing the L-configuration of the fifth Val residue. Therefore, the fourth residue from the *N*-terminus was D-Val.

Halicylindramides are closely related to discodermins[15] and polydiscamide A.[16] Halicylindramide A differed from discodermin D in two amino acid residues; L-Phe and D-*t*-Leu residues of the latter were replaced by L-BrPhe and D-Phe. It should be noted that *H. cylindrata* collected off the Sata Peninsula, 800 km apart from Atami, did not contain the cytotoxic peptides,

although cylindramide was common to both collections. Moreover, the occurrence of closely related peptides in taxonomically remote sponges, *Halichondria* and *Discodermia*, may indicate a microbial origin of these peptides.

Halicylindramide A-C were antifungal against *Mortierella ramanniana* at 7.5 μg/disk as well as cytotoxic against P388 murine leukemia cells with IC_{50}'s of 0.54, 0.2, and 0.2 μg/mL, respectively. Interestingly, the methyl ester **4**, which was generated from halicylindramide B during NMR measurement in CD_3OD, was not cytotoxic at 10 μg/mL and was antifungal only at 120 μg/disk, indicating that the macrocyclic structure of halicylindramides is essential for their cytotoxic and antifungal activities.

Experimental Section

General Procedures. UV spectra were recorded on a Hitachi 330 spectrophotometer. Infrared spectra were taken on a JASCO-IR-G infrared spectrometer. ^1H and ^{13}C NMR spectra were recorded either on Bruker AM-600, JEOL GMX-500, or Bruker AC-300 instruments. ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks: $\delta_{H/C}$ 2.49/39.5 for DMSO-d_6. Optical rotations were determined by a JASCO DIP-371 digital polarimeter. Mass spectra were measured on a JEOL JMX-SX102 mass spectrometer. High-resolution FAB mass spectra were measured using a dual target inlet probe. Amino acid analysis was performed with a Hitachi L-8500A amino acid autoanalyzer. Automatic protein sequence analysis was carried out with an Applied Biosystems 476A protein sequencer and 120A PTH analyzer.

Cytotoxicity Assay. P388 murine leukemia cells (JCRB17) were cultured in RPMI 1640 medium (Nissui Pharm. Co., Tokyo) supplemented with 100 μg/mL of kanamycin (Nacalai Tesque Inc., Kyoto), 10% fetal bovine serum (Lot 42H0342, Sigma Chemical Co., St. Louis, MO), and 10 μM/mL of 2-hydroxyethyl disulfide (Nacalai Tesque Inc., Kyoto) at 37 °C under an atmosphere of 5% CO_2. To each well of 96-well microplates which contained 100 μL of a tumor cell suspension of 1×10^4 cells/mL was added 100 μL of test solution (sample dissolved in RPMI 1640 medium), and the plates were incubated for 96 h. After addition of 50 μL of a 3-(4,4-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and saline solution (1 μg/mL) to each well the plates were incubated for 3 h under the same conditions. The mixture was centrifuged, and the supernatants were removed. The precipitates obtained were dissolved in DMSO, and absorbance at 525 nm was measured with a microplate reader.

Antifungal Assay. See reference 17 for the assay procedure.

Isolation. The frozen sponge (1 kg wet weight) was homogenized and extracted with EtOH (3 × 3 L). The combined extract was concentrated and partitioned between chloroform and water; the aqueous layer was successively extracted with ethyl acetate and *n*-butanol. The ethyl acetate and *n*-butanol soluble materials were combined and subjected to flash column chromatography on ODS with 30%, 50%, 70%, 85% MeOH, MeOH, $CHCl_3$/MeOH/H_2O (6:4:1 and 7:3:0.5). The fractions eluted with MeOH and $CHCl_3$/MeOH/H_2O (7:3:0.5 and 6:4:1), which were antifungal against *Mortierella ramanniana*, were combined and evaporated to yield a yellowish solid (560 mg). This was purified by reversed-phase HPLC [ODS, 10 × 250 mm, flow rate 2 mL/min; UV (254 nm) detection] with 80% MeOH containing 0.05% TFA to afford cylindramide (**5**, 72 mg, t_R 58 min). The other antifungal fraction eluted with 70% MeOH from the ODS flash column was chromatographed on silica gel with $CHCl_3$/MeOH systems to afford a peptide fraction, which was finally separated by ODS HPLC with 62% MeOH to yield halicylindramide A (1, 5.4 mg, t_R 37 min), halicylindramide B (**2**, 20.1 mg, 47 min), and halicylindramide C (**3**, 3.0 mg, 55 min). The fraction eluted from the ODS flash column with 85% MeOH was separated on a silica gel open column with MeOH/$CHCl_3$ systems. The active fraction thus obtained was purified by MPLC on ODS with 64% MeOH [4 × 100 cm, flow rate 5 mL/min; UV (254 nm) detection], followed by ODS HPLC with 62% MeOH to furnish **1** (2.2 mg), **2** (34.0 mg), and **3** (7.9 mg).

Halicylindramide A (1): colorless solid; $[\alpha]^{23}_D$ −1.4° (c 0.61, MeOH); UV λ max (MeOH) 214 (46700), 272 sh (5200), 283 (5400), 290 nm (ε 4600); IR (film) 3300 (br), 1745, 1660, 1530, 1225 cm^{-1}; FABMS (positive ion, glycerol matrix) *m/z* 1831, 1830, 1829, 1828, 1827, 1815, 1814 $[M(Br^{81}) + Na]^+$, 1813, 1812 $[M(Br^{79}) + Na]^+$, 1811, 1792 $[M(Br^{81}) + H]^+$, 1791, 1790 $[M(Br^{79}) + H]^+$, 1789, 1713, 1712, 1711, 1710, 1709, 1708, 1707, 1706, 1465, 1466, 1467, 1021, 1019, 995, 993, 424, 422; HRFABMS *m/z* 1792.6932 (Δ −13.3 mmu) for $C_{78}H_{111}Br^{81}N_{20}O_{22}S$, 1790.7134 (Δ +4.8 mmu) for $C_{78}H_{111}Br^{79}N_{20}O_{22}S$; for ^1H NMR see Table 2; ^{13}C NMR (75 MHz in DMSO-d_6) δ 17.2, 17.5, 18.5, 19.0, 19.3, 20.1, 24.3, 24.6, 27.8, 28.7, 28.8, 29.0, 29.6, 30.3, 30.5, 30.6, 31.5, 35.5, 36.1, 36.9, 38.0, 46.0, 46.4, 46.9, 48.3, 50.8, 51.3, 51.5, 51.8, 52.3, 53.6, 54.1, 54.4, 57.5, 58.0, 59.8, 60.7, 62.8, 65.5, 68.8, 69.7, 109.8, 111.2, 118.1, 118.4, 119.4, 120.7, 124.0, 126.2, 127.1, 127.9, 129.3, 130.7, 131.7, 136.1, 137.0, 137.3, 156.8, 160.5, 167.6, 169.2, 169.3, 169.5, 169.8, 170.0, 170.4, 170.6, 170.7, 170.9, 171.0, 171.1, 171.4, 171.5, 172.1, 172.9, 173.5.

Halicylindramide B (2): colorless solid, $[\alpha]^{23}_D$ −4.5° (c 4.07, MeOH); UV λ max (MeOH) 215 (45000), 273 sh (5000), 283 (5300), 290 nm (ε 4500); IR (film) 3300 (br), 2950, 1745, 1660, 1540, 1040 cm^{-1}; FABMS (positive ion, glycerol as a matrix) *m/z* 1831, 1830, 1829, 1828, 1827, 1815, 1814 $[M(Br^{81}) + Na]^+$, 1813, 1812, $[M(Br^{79}) + Na]^+$, 1811, 1792 $[M(Br^{81}) + H]^+$, 1791, 1790 $[M(Br^{79}) + H]^+$, 1789, 1713, 1712, 1711, 1710, 1709, 1708, 1707, 1706, 1465, 1466, 1467, 1007, 1005, 981, 979, 424, 422; HRFABMS *m/z* 1792.7111 (Δ +4.5 mmu) for $C_{78}H_{111}Br^{81}N_{20}O_{22}S$, 1790.7053 (Δ −3.3 mmu) for $C_{78}H_{111}Br^{79}N_{20}O_{22}S$; for ^1H and ^{13}C NMR see Table 1.

Halicylindramide C (3): colorless solid, $[\alpha]^{23}_D$ −6.1° (c 0.52, MeOH); UV λ max (MeOH) 214 (43000), 273 sh (5100), 283 (5500), 289 nm (ε 4500); IR (film) 3300 (br), 1745, 1650, 1540, 1460, 1230, 1040 cm^{-1}; FABMS (positive ion, glycerol as a matrix) *m/z* 1845, 1844, 1843, 1842, 1841, 1829, 1828 $[M(Br^{81}) + Na]^+$, 1827, 1826 $[M(Br^{79}) + Na]^+$, 1825, 1806 $[M(Br^{81}) + H]^+$, 1805, 1804 $[M(Br^{79}) + H]^+$, 1803, 1728, 1726, 1725, 1724, 1723, 1722, 1721, 1720, 1479, 1477, 1021, 1019, 995, 993, 424, 422; HRFABMS *m/z* 1806.7207 (Δ −1.5 mmu) for $C_{79}H_{113}Br^{81}N_{20}O_{22}S$, 1804.7351 (Δ +10.9 mmu) for $C_{79}H_{113}Br^{79}N_{20}O_{22}S$; for ^1H NMR see Table 2; ^{13}C NMR (75 MHz in DMSO-d_6) δ 17.3, 17.4, 18.6, 19.4, 20.1, 23.6, 24.3, 24.6, 26.5, 27.5, 28.7, 28.8, 28.9, 29.7, 30.6, 31.0, 31.5, 33.8, 24.7, 35.5, 36.2, 36.9, 38.0, 46.1, 26.4, 46.9, 49.4, 50.8, 51.4, 51.6, 51.8, 52.4, 53.8, 53.9, 54.1, 54.4, 57.3, 59.9, 60.4, 61.0, 5.6, 68.9, 69.8, 109.7, 111.3, 118.1, 118.3, 119.4, 120.8, 123.5, 125.9, 126.3, 127.1, 129.3, 130.8, 131.7, 136.1, 137.1, 137.3, 155.9, 160.5, 167.6, 169.2, 169.4, 169.5, 169.8, 170.0, 170.7, 170.8, 170.9, 171.0, 171.4, 171.5, 172.0, 173.6.

***seco*-Methyl Ester of Halicylindramide B.** After overnight measurement of the ^{13}C NMR spectrum in CD_3OD, halicylindramide B (**2**) gave an additinal HPLC peak, which was collected to afford the *seco*-methyl ester **4**: colorless solid, $[\alpha]^{23}_D$ −5.1° (c = 1.01, MeOH); UV λ max (MeOH) 214 (43500), 273 sh (5100), 283 (5300), 290 nm (ε 4500); IR (film) 3300 (br), 2950, 1745, 1660, 1530, 1230 cm^{-1}; FABMS (positive ion, glycerol as a matrix) *m/z* 1849, 1847, 1846, 1829, 1828, 1827, 1826, 1749, 1748, 1747, 1746, 1745, 1743, 1262, 1260, 1021, 1019, 995, 993, 424, 422; HRFABMS *m/z* 1828.7526 (Δ −5.3 mmu) for $C_{79}H_{111}D_4Br^{81}N_{20}O_{23}S$, 1827.7679 (Δ +16.4 mmu) for $C_{79}H_{112}D_3Br^{81}N_{20}O_{23}S$, 1825.7704 (Δ +16.8 mmu) for $C_{79}H_{112}D_3Br^{79}N_{20}O_{23}S$; for ^1H NMR see Table 2; ^{13}C NMR (75 MHz in DMSO-d_6) δ 17.4, 18.6, 18.9, 19.0, 19.3, 23.4, 23.6, 24.3, 26.4, 27.6, 28.5, 29.7, 30.9, 31.2, 31.4, 33.5, 36.2, 36.8, 37.4, 40.7, 46.4, 46.9, 51.0, 51.4, 51.9, 52.3, 53.7, 54.0, 54.3, 55.0, 55.7, 57.1, 59.8, 60.6, 62.6, 63.1, 64.4, 65.2, 66.5, 66.8, 67.5, 109.8, 111.2, 118.1, 118.4, 119.4, 120.8, 124.0, 126.2, 127.1, 128.0, 129.2, 130.8, 131.7, 136.1, 137.0, 137.5, 156.6, 160.5, 169.4, 169.8, 169.9, 170.0, 170.1, 170.8, 170.9, 171.1, 171.3, 171.4, 173.8.

Amino Acid Analysis of Acid Hydrolysate. Halicylindramide A-C (each 70 μg) were heated at 110 °C for 16 h with

Table 2. ^{1}H NMR Data for Halicylindramides A (1) and C (**3**) and the *seco*-Methyl Ester **4** in DMSO-d_6

position	1	3	4
NCHO	7.89 (s)	7.89 (s)	7.89 (s)
Ala α	4.29 (q, 7.0)	4.30 (dq, 7.0)	4.31 (dq, 7.3, 7.0)
β	0.92 (3H, d, 7.0)	0.93 (3H, d, 7.0)	0.93 (3H, d, 7.0)
NH	8.13 (brs)	8.12 (d, 8.0)	8.14 (d, 8.0)
BrPhe α	4.73 (m)	4.72 (m)	4.71 (m)
β	2.70 (m), 2.97 (m)	2.70 (dd, 14.2, 11.0), 2.99 (m)	2.71 (dd, 13.5, 10.4), 3.02 (m)
C2/C6	7.20 (2H, d, 8.3)	7.21 (2H, d, 8.2)	7.22 (2H, d, 8.8)
C3/C5	7.39 (2H, d, 8.3)	7.39 (2H, d, 8.2)	7.40 (2H, d, 8.8)
NH	8.36 (brs)	8.37 (brd, 8.7)	8.37 (d, 8.6)
Pro α	4.45 (dd, 4.0, 8.2)	4.46 (dd, 3.9, 8.2)	4.62 (m)
β	1.82 (m), 2.07 (m)	1.82 (m), 2.08 (m)	1.84 (m), 2.10 (m)
γ	1.84 (2H, m)	1.84 (2H, m)	2.06 (2H, m)
δ	3.64 (m)	3.64 (m)	3.65 (m)
Val-1 α	4.31 (m)	4.41 (m)	4.43 (dd, 8.9, 5.6)
β	2.07 (m)	2.07 (m)	2.02 (m)
γ	0.78 (3H, d, 6.7), 0.82 (3H, d, 6.6)	0.77 (3H, d, 6.7), 0.82 (3H, d, 6.7)	0.77 (3H, d, 6.7), 0.82 (3H, d, 6.7)
NH	7.85 (brd, 8.5)	7.81 (brd, 9.5)	7.84 (brd, 9.0)
t-Leu α		4.15 (d, 8.4)	4.12 (d, 8.3)
γ		0.70 (9H, s)	0.73 (9H, s)
NH		7.76 (brd, 9.3)	7.77 (brd, 8.0)
Val-2 α	4.12 (dd, 7.0, 5.5)		
β	1.82 (m)		
γ	0.59 (3H, d, 6.7), 0.62 (3H, d, 6.6)		
NH	7.89 (brs)		
Trp α	4.59 (m)	4.56 (m)	4.59 (m)
β	3.13 (dd, 5.5, 14.1), 2.90 (m)	3.12 (m), 2.92 (m)	3.13 (dd, 5.2, 10.8), 2.92 (m)
C2	7.11 (s)	7.13 (s)	7.13 (s)
C4	7.58 (d, 7.9)	7.58 (d, 7.8)	7.58 (d, 7.9)
C5	6.94 (dd, 7.5, 7.3)	6.94 (dd, 7.6, 7.4)	6.94 (dd, 7.5, 7.6)
C6	7.02 (dd, 7.3, 7.7)	7.02 (dd, 7.4, 7.7)	7.02 (dd, 7.3, 7.7)
C7	7.31 (d, 7.9)	7.31 (d, 8.2)	7.31 (d, 8.2)
1-NH	10.7 (brs)	8.22 (brd, 6.4)	8.15 (brd, 7.5)
NH	8.15 (brd, 6.7)	10.7 (brs)	10.5 (brs)
Arg α	4.34 (m)	4.35 (m)	4.36 (m)
β	1.61 (2H, m)	1.60 (2H, m)	1.59 (2H, m)
γ	1.24 (m), 1.31 (m)	1.24 (m), 1.30 (m)	1.31 (2H, m)
δ	2.95 (2H, m)	2.94 (2H, m)	2.95 (2H, m)
NH	7.97 (brs)	7.89 (m)	8.09 (brd, 8.1)
Cys (SO_3) α	4.57 (m)	4.58 (m)	4.56 (m)
β	2.99 (2H, m)	2.91 (2H, m)	2.94 (2H, m)
NH	8.32 (brd, 6.7)	8.31 (brd, 6.1)	8.25 (brd, 7.1)
Thr 1 α	4.82 (d, 8.5)	4.82 (d, 8.7)	4.73 (brd, 8.1)
β	5.14 (q, 5.9)	5.16 (q, 5.9)	4.02 (m)
γ	1.14 (3H, d, 6.6)	1.14 (3H, d, 6.6)	1.01 (3H, d, 6.3)
NH	7.90 (m)	7.92 (m)	7.80 (brd, 8.7)
OH			5.01 (d, 4.8)
MeGln α	4.94 (m)	4.94 (m)	4.87 (dd, 5.0, 10.7)
β	1.68 (m), 1.91 (m)	1.68 (m), 1.91 (m)	1.62 (2H, m)
γ	1.89 (2H, m)	1.89 (2H, m)	1.90 (2H, m)
NMe	2.92 (3H, s)	2.93 (3H, s)	2.86 (3H, s)
$CONH_2$	6.62 (brs), 7.13 (brs)	6.62 (brs), 7.13 (brs)	6.69 (brs), 7.13 (brs)
Phe α	4.67 (q, 6.6)	4.67 (brd, 6.6)	4.61 (m)
β	3.02 (dd, 7.2, 13.5), 2.95 (m)	3.00 (2H, m)	2.99 (m), 2.80 (dd, 10.4, 13.6)
C2/C6	7.22 (2H, m)	7.22 (2H, m)	7.22 (2H, m)
C3/C5	7.14 (2H, m)	7.14 (2H, m)	7.13 (2H, m)
C4	7.12 (m)	7.12 (m)	7.12 (m)
NH	7.56 (m)	7.47 (m)	7.74 (brd, 7.8)
Thr 2 α	3.85 (dd, 3.7, 7.3)	3.85 (dd, 3.7, 7.3)	4.25 (dd, 8.3, 3.9)
β	3.95 (m)	3.94 (m)	3.93 (m)
γ	0.89 (3H, d, 6.3)	0.89 (3H, d, 6.4)	0.91 (3H, d, 6.4)
OH	4.82 (brs)	4.82 (d, 8.7)	5.05 (d, 4.9)
NH	7.90 (m)	7.89 (m)	8.00 (d, 8.2)
Asn α	4.98 (m)	4.98 (m)	4.64 (m)
β	2.09 (m)	2.10 (m)	2.45 (dd, 15.6, 7.8)
	2.64 (dd, 15.1, 6.2)	2.64 (dd, 15.3, 6.8)	2.57 (dd, 15.3, 5.3)
NH	7.49 (d, 9.8)	7.48 (m)	8.07 (brd, 7.6)
$CONH_2$	6.72 (brs), 7.28 (brs)	6.72 (brs), 7.28 (brs)	6.90 (brs), 7.33 (brs)
Gly α	3.47 (d, 17.3), 4.42 (d, 17.3)	3.46 (d, 17.3), 4.02 (d, 17.3)	3.78 (dd, 16.3, 5.8), 3.84 (dd, 16.3, 5.8)
NH			8.07 (brt, 5.4)
NMe	2.72 (3H, s)	2.73 (3H, s)	

6 N HCl (200 μL) in an evacuated tube. After the removal of HCl, the residues were dissolved in 1% HCl (500 μL) and subjected to an automatic amino acid analyzer. Trp, Pro, Phe, Ala, Val, Thr, Asp, Cys (O_3H), Arg, and Gly were found in the hydrolysate of **2**, while Trp, Pro, Phe, Ala, Val, Thr, Asp, Cys (O_3H), and Arg were detected in the hydrolysates of 1 and **3**.

Sequence Analysis by an Automatic Protein Sequencer. Halicylindramide C (100 μg) was deformylated with 10% HCl/MeOH (0.3 mL) at room temperature for 5 h. After removal of HCl, the reaction mixture was treated with 0.1 N NaOH at room temperature for 5 h, desalted with an ODS column, and subjected to an automatic protein sequencer.

Determination of the Stereochemistry of Amino Acids. Halicylindramide B (**2**, 200 μg) was treated with 6 N HCl (200 μL) at 108 °C for 16 h. The reaction products obtained by lyophilization were reacted with a 10% acetone solution (50 μL) of FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and 1 M $NaHCO_3$ (100 μL) at 80–90 °C for 3 min. After cooling to room temperature, the reaction mixture was neutralized with 2 N HCl (50 μL). The solution was diluted with 50% MeCN (1 mL) and subjected to HPLC analysis (10 μL/injection). Halicylindramides A and C (100 μg each) were processed in the same manner.

The FDAA derivatives of standard amino acids were prepared by the procedure described above. The FDAA derivatives of hydrolyzates of **1**–**3** and standard amino acids were subjected to HPLC analysis using an ODS column (4.6 × 250 mm, 5 μm, 1.3 mL/min) and the gradient elution as follows: solvent A for 20 min, then programmed to solvent B in 80 min and solvent B for 20 min, where solvent A was 10% MeCN containing 5 mM triethylamine phosphate buffer (pH 3.0) and solvent B was 40% MeCN containing 5 mM triethylamine phosphate buffer (pH 3.0). The assignment of peaks was made by coinjection with the standard sample. The retention times (min) of FDAA derivatives of the hydrolysates of **1**–**3** were as follows: D-Cys (O_3H) (36.1), L-Arg (36.8), L-Thr (45.7), L-Asp (47.8), L-MeGln (52.0), L-Pro (57.1), D-Ala (63.5), L-Val (70.6), L-*t*-Leu (77.7), D-Val (79.6), D-Trp (84.7), L-BrPhe (91.2), D-Phe (99.1).

Determination of the Configuration for the Two Valine Residues of Halicylindramide A (1). Halicylindramide A (1, 100 μg) was deformylated with 4 N HCl (0.2 ml) and MeOH (0.2 mL) at room temperature for 5 h. To desformylcylindramide A was added a mixture (20 μL) of PITC (phenyl isothiocyanate)/H_2O/EtOH/$N(Et)_3$ (1:1:7:1), and the mixture was allowed to stand at 50 °C for 8 min. The reaction mixture was partitioned between H_2O and *n*-heptane/EtOAC (15:1) (300 μL), and the aqueous layer was further washed twice with *n*-heptane/EtOAC (7:1) (300 μL). The H_2O layer was lyophilized; the residue was treated with TFA (20 μL) at 50 °C for 7 min. The residue obtained by lyophilization was taken up in H_2O (20 μL), washed twice with MeCN/C_6H_6 (1:1), and evaporated to dryness to yield the dealanyl peptide. Three more Edman degradation cycles were performed to obtain a decapeptide containing only one Val residue. The Marfey analysis of the hydrolysate resulted in L-Val and D-Val in a ratio of 8:2.

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Supplementary Material Available: ^1H NMR spectra of **1**–**4**, COSY spectrum of **2**, DQFCOSY spectrum[18] of **3**, HOHAHA spectra of **1**–**4**, HMQC spectra of **1**–**4**, HMBC spectrum of **2**, ^{13}C NMR spectra of **1**–**4**, and NOESY spectra of **1**–**4** (23 pages). This material is contained in many 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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