Halicylindramides D and E, Antifungal Peptides from the Marine Sponge *Halichondria cylindrata*[1]

Hong-yu Li, Shigeki Matsunaga, and Nobuhiro Fusetani*

Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agriculture and Agricultural Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Halicylindramides D (**1**) and E (**2**) have been isolated from the marine sponge *Halichondria cylindrata*. Halicylindramide D is a tridecapeptide with the N-terminus blocked by a formyl group and the C-terminus lactonized with a threonine residue, while halicylindramide E is a truncated linear peptide with a C-terminal amide. Their structures, including absolute stereochemistry, were determined by a combination of spectral and chemical methods. Halicylindramide D was antifungal against *Mortierella ramanniana* and cytotoxic against P-388 murine leukemia cells.

Marine sponges of the genus *Halichondria* contain a diverse array of secondary metabolites, for example, nitrogenous terpenoids,[2−5] trisulfated sterols,[6] polyethers,[7−10] and macrolides.[11] In the course of our studies on bioactive metabolites from Japanese marine invertebrates, we isolated a variety of cytotoxic metabolites including a macrolactam (cylindramide),[12] *N*-acetylglucosaminyl cerebrosides (halicylindrosides),[13] and tetradecapeptides (halicylindramides A−C)[14] from *Halichondria cylindrata* (Axinellidae) collected off Atami in the Gulf of Sagami in 1992. Further investigation of the 1993 collection of the same sponge led to the isolation of smaller antifungal peptides, halicylindramides D and E. In this paper, we describe the isolation and structure elucidation of these peptides.

The combined EtOH and 70% EtOH extracts of the frozen sponge (1.67 kg wet weight) were partitioned between H_2O and $CHCl_3$; the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH-soluble material was subjected to ODS flash column chromatography with MeOH/H_2O systems. The active fraction eluted with 70% MeOH was chromatographed on a Si gel column, followed by ODS HPLC (MeCN/H_2O/TFA, 31:69:0.05) to yield halicylindramides D (**1**, 3.5 mg, 2.1 × 10^{-4}% yield, based on wet weight) and E (**2**, 6.5 mg, 3.9 × 10^{-4}%) along with the known halicylindramides A-C (Chart 1).

Halicylindramide D (**1**) had a molecular formula of $C_{73}H_{99}BrN_{19}O_{20}SNa$, which was established by positive ion high resolution FABMS. The ^{1}H-NMR, ^{13}C-NMR (Table 1), and IR spectra were consistent with a peptide. A negative ninhydrin reaction suggested a blocked N-terminus. Standard amino acid analysis proved the presence of Ala, Pro, Val (2 mols), Trp, Arg, Asp, Phe, Thr, Gly, and Cys(O_3H) residues. An additional peak had the same retention time as *p*-bromophenylalanine (BrPhe).[15]

Interpretation of the COSY and HOHAHA spectra led to assignment of Ala, Pro, Arg, Val, Thr, and Gly residues, while a formamide group, MeGln, and three aromatic amino acids were identified by the HOHAHA,[16] NOESY,[17] HMQC,[18] and HMBC[19] spectra. Sequencing of these amino acid residues was carried out by interpreting NOESY cross peaks between NH_{i+1} (or NMe_{i+1}) and α-Hi, except for the linkage between BrPhe and Pro, which was inferred from a strong NOE between BrPhe-αH and Pro-δH. The chemical shift of Thr-βH (δ_H 5.12) together with HMBC cross peaks between Thr-βH and two carbonyl carbons (δ_C 168.8 and 169.4) indicated that the β-hydroxyl group of this residue was esterified. However, the formation of a lactone with the C-terminal of an Asn residue was not implied by NMR data. This difficulty was solved by treatment of halicylindramide D with [bis(trifluoroacetoxy)iodo]benzene, followed by acid hydrolysis, which yielded 2,3-diaminopropionic acid,[20] thus revealing that the Asn residue was α-linked and its α-carboxyl group participated in the lactone formation.[15]

Stereochemistry of each amino acid residue in halicylindramide D was determined by Marfey analysis of the acid hydrolysate.[21] Because two Val residues appeared to be a mixture of D/L forms, halicylindramide D was subjected to four cycles of the Edman degradation,[22] followed by Marfey analysis,[14] disclosing that the fifth residue was L-Val. Therefore, the fourth residue was D-Val.

The high resolution FAB mass spectrum and NMR data established that halicylindramide E (**2**) had a molecular formula of $C_{68}H_{95}BrN_{17}O_{17}SNa$. Amino acid analysis revealed the presence of Ala, Pro, Val, *t*-Leu, Trp, Arg, Phe, Thr, Cys(O_3H), and BrPhe residues in **2**. Interpretation of COSY and HOHAHA spectra allowed the assignment of ^{1}H NMR signals for the abovementioned amino acid residues together with that of MeGln. Though a good quality HMBC spectrum was not obtained because of the paucity of material, the NOESY data gave cross peaks necessary to sequence the peptide, revealing that halicylindramide E had a structure identical with a partial structure of halicylindramide B (residue 1-11).[14] Similarly, the stereochemistry of the component amino acids was determined by Marfey's method.

Halicylindramides D and E were antifungal against *Mortierella ramanniana* at 5 and 160 μg/disk, respectively. Interestingly, halicylindramide D was cytotoxic against P-388 murine leukemia cells with an IC_{50} value of 2.1 μg/mL, while halicylindramide E was inactive at

* To whom correspondence should be addressed. Tel: +81-3-3812-2111 ext. 5299. Fax: +81-3-5684-0622. E-mail: anobu@hongo.ecc.u-tokyo.ac.jp.

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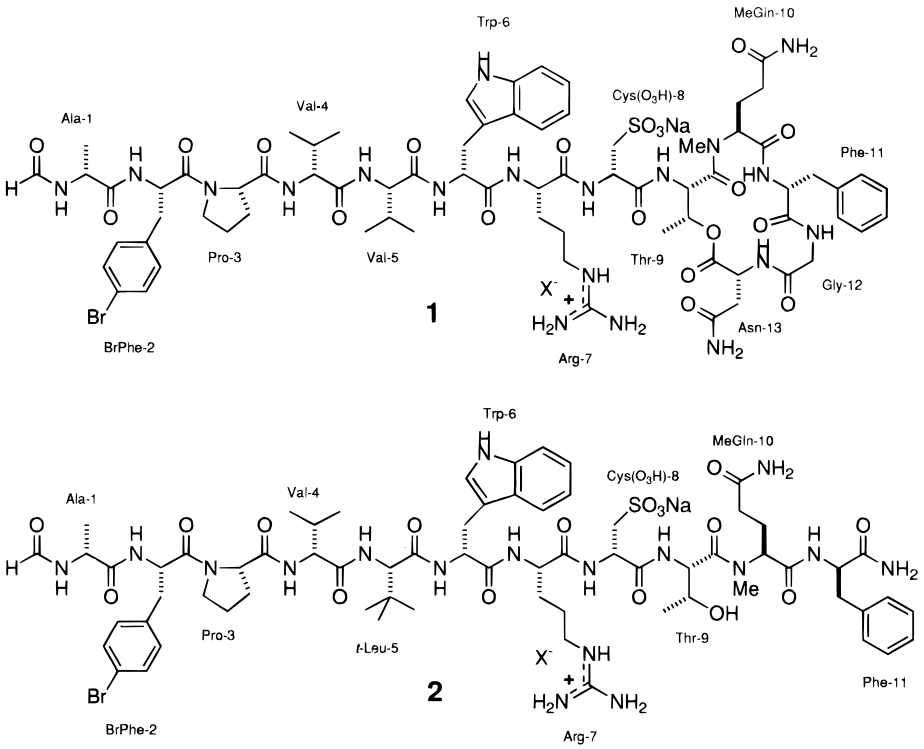
Chart 1



5 μg/mL. In fact, we already knew that secohalicylindramide B was not cytotoxic.[14] The amino acid sequence of halicylindramides D and E is identical or similar to those of halicylindramides A-C or of the discodermins[23] up to Phe-11, but D has a smaller ring[24] and E is linear.

Experimental Section

General Experimental Procedures. For general procedures, see Li *et al.*[14]

Isolation of halicylindramides D and E. The yellowish cylindrical sponge was collected by scuba in 1993 off Atami at depths of 5−15 m. The sponge was identified as *Halichondria cylindrata* Tanita and Hoshino by Dr. Rob van Soest. A voucher specimen was deposited at the Institute of Zoological Taxonomy of the University of Amsterdam (ZMA POR. 10599). The frozen sponge (1.7 kg) was homogenized and extracted with EtOH (3 × 3 L) and then with EtOH/H_2O (7:3, 2 × 3 L). The combined extracts were concentrated and partitioned between H_2O and $CHCl_3$; the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH-soluble material was fractionated by flash column chromatography on ODS with aqueous MeOH. The fraction eluted with MeOH/H_2O (7:3) was chromatographed on a Si gel column with $CHCl_3$/MeOH/H_2O (7:3:0.5) to afford a peptide fraction (20 mg), which was purified by ODS HPLC (MeCN/H_2O/TFA, 31:69:0.05) to afford halicylindramide D (**1**; yield, 3.5 mg) and halicylindramide E (**2**; yield, 6.5 mg).

Halicylindramide D (1): colorless powder, $[\alpha]_D$ +5.1° (*c* 0.16, MeCN/H_2O (3:2)); UV λ max (MeOH) 213 (ϵ 42 000), 279 (5600), 288 nm (4500); IR (film) 3300 (br), 1760, 1660, 1540, 1200 cm^{-1}; FABMS (glycerol)[25] *m/z* 1699, 1697 $[M + 2H]^+$ 1677, 1675, 1007, 1005, 424, 422; HRFABMS *m/z* found 1699.6393, calcd for $C_{73}H_{101}{}^{81}BrN_{19}O_{20}SNa$, 1699.6259; ^1H and ^{13}C NMR data see Table 1.

Halicylindramide E (2): Colorless powder, $[\alpha]_D$ +9.0° (*c* 0.30, MeOH); UV λ max (MeOH) 214 (43 500), 280 (br, 5400), 288 nm (ϵ 4300); IR (film) 3300 (br), 1660, 1530, 1200 cm^{-1}; FABMS *m/z* 1559, 1557 $[M + 2H]^+$, 1022, 1020, 424, 422; HRFABMS (glycerol) *m/z* found 1559.6013, calcd for $C_{68}H_{97}{}^{81}BrN_{17}O_{17}SNa$, 1559.6037; ^1H NMR (DMSO-d_6) Ala δ 8.79 (s, NCHO), 4.30 (q, J = 7.3 Hz, α), 0.95 (d, J = 7.0 Hz, β), 8.17 (d, J = 8.1 Hz, NH); BrPhe 4.76 (m, α), 2.97 (m, β), 2.70 (dd, J = 13.5, 10.6 Hz, β), 7.38 (2H, d, J = 8.5 Hz, H-3, 5), 7.20 (2H, d, J = 8.5 Hz, H-2, 6), 8.41 (d, J = 8.8 Hz, NH); Pro 4.47 (dd, J = 8.4, 3.5 Hz, α), 1.82 (m, β), 2.10 (m, β), 1.84 (2H, m, γ), 3.64 (2H, m, δ); Val 4.43 (m, α), 2.00 (m, β), 0.81 (3H, d, J = 6.7 Hz, γ), 0.67 (3H, d, J = 7.0 Hz, γ), 7.85 (m, NH); *t*-Leu 4.12 (d, J = 8.1 Hz, α), 0.71 (9H, s, γ), 7.78 (d, J = 9.4 Hz, NH); Trp 4.40 (m, α), 3.06 (dd, J = 13.9, 4.4 Hz, β), 2.87 (dd, J = 14.0, 3.7 Hz, β), 7.10 (s, H-2), 7.59 (d, J = 7.7 Hz, H-4), 7.31 (d, J = 8.0 Hz, H-7), 7.03 (d, J = 7.7, 7.3 Hz, H-6), 6.94 (dd, J = 7.7, 7.0 Hz, H-5), 7.84 (d, J = 7.7 Hz, NH), 10.63 (br s, 1-NH); Arg 4.25 (m, α), 1.60 (2H, m, β), 1.30 (2H, m, γ), 2.95 (2H, m, δ), 8.11 (d, J = 6.6 Hz, NH), 7.41 (br t, guanidine NH); Cys(O_3H): 4.56 (m, α), 3.00 (m, β), 2.94 (m, β), 8.27 (d, J = 6.2 Hz, NH); Thr 4.69 (m, α), 3.97 (dq, J = 6.2, 6.1 Hz, β), 1.01 (d, J = 5.9 Hz, γ), 7.83 (d, J = 7.7 Hz, NH); MeGln 4.92 (dd, J = 10.2, 4.8 Hz, α), 1.61 (2H, m, β), 2.00 (m, γ), 1.88 (m, γ), 2.86 (3H, s, NMe), 6.72 (s, $CONH_2$), 7.14 (s, $CONH_2$); Phe 4.59 (m, α), 3.12 (dd, J = 13.6, 5.2 Hz, β), 2.91 (m, β), 7.24 (2H, m, H-2, 6), 7.19 (2H, m, H-3, 5), 7.17 (m, H-4), 8.23 (d, J = 7.3 Hz, NH). ^{13}C NMR (DMSO-d_6) δ 17.4, 18.5, 19.0, 19.3, 23.4, 24.3, 24.6, 26.4, 27.6, 28.5, 29.6, 30.5, 31.1, 31.3, 33.5, 36.2, 36.4, 46.4, 46.9, 50.9, 51.4, 52.0, 52.2, 53.3, 53.7, 53.8, 54.2, 55.3, 56.2, 57.2, 59.8, 60.5, 66.9, 109.8, 111.2, 118.1, 118.3, 119.4, 120.7, 124.0, 126.3, 127.1, 128.1, 129.0, 130.8, 131.7, 136.1, 137.0,

Table 1. ^{1}H- and ^{13}C-NMR Data for Halicylindramide D (**1**) in DMSO-d_6

position	^{1}H	^{13}C	position	^{1}H	^{13}C
CHO	7.88 (s)	160.5d	NH	8.20 (brd, 7.9)	
Ala α	4.28 (q, 7.3)	46.1d	CO		171.2s[a]
β	0.90 (3H, d, 7.0)	18.5q	Arg α	4.37 (m)	52.1d
NH	8.16 (d, 7.9)		*β*	1.63 (m), 1.67 (m)	29.5t
CO		171.4s	*γ*	1.30 (2H, m)	23.9t
BrPhe α	4.70 (ddd, 3.7, 9.2, 9.8)	51.4d	*δ*	2.93 (2H, m)	40.2t
β	2.78 (dd, 13.4,10.8)	36.2t	guanidine	7.43 (brt)	156.7s
	2.93 (m)		NH	8.01 (brd, 7.4)	
C1		137.6s	CO		170.7s
C2/C6	7.21 (2H, d, 8.5)	131.7d	Cys (O_{3H}) α	4.59 (m)	50.9d
C3/C5	7.39 (2H, d, 8.5)	130.8d	*β*	2.90 (2H, m)	51.9t
C4		119.4s	NH	8.40 (brd, 8.3)	
NH	8.39 (brd, 8.3)		CO		170.7s[a]
CO		169.6s	Thr-1 α	4.88 (brd, 7.9)	51.9d
Pro α	4.45 (dd, 8.2, 4.0)	59.8d	*β*	5.12 (dq, 1.5, 6.4)	69.2d
β	1.81 (m), 2.07 (m)	28.7t	*γ*	1.10 (3H, d, 6.1)	16.4q
γ	1.83 (2H, m)	23.5t	NH	7.93 (brd, 7.6)	
δ	3.63 (2H, m)	46.9t	CO		168.8s
CO		171.5s	*N*MeGln α	4.80 (m)	56.3d
Val-I α	4.33 (dd, 8.5, 5.5)	58.1d	*β*	1.70 (2H, m)	23.9t
β	2.04 (m)	30.7d	*γ*	1.88 (m), 1.92 (m)	31.7t
γ	0.74 (3H, d, 6.7)	17.4q	*N*Me	2.93 (3H, s)	31.3q
γ′	0.81 (3H, d, 6.7)	19.3q	$CONH_2$	6.73 (brs), 7.19 (brs)	173.4s
NH	7.83 (brd, 8.2)		CO		169.9s
CO		171.7s	Phe α	4.39 (m)	54.5d
Val-II α	4.09 (t, 7.3)	57.5d	*β*	2.83 (dd, 13.7, 9.8)	37.0t
β	1.78 (m)	30.1d		2.87 (m)	
γ	0.54 (3H, d, 6.7)	18.9q	C1		136.9s
γ′	0.57 (3H, d, 6.7)	17.9q	C2/C6	7.23 (2H, m)	129.6d
NH	7.76 (brd, 7.9)		C3/C5	7.18 (2H, m)	128.1d
CO		170.7s	C4	7.17 (m)	126.3s
Trp α	4.61 (m)	53.5d	NH	8.10 (brd, 6.7)	
β	3.15 (m), 2.87 (m)	27.7t	CO		171.6s
C3		109.9s	Gly α	3.41 (m)	42.5t
C2	7.11 (s)	124.0d		3.79 (dd, 17.0, 6.7)	
C3a		136.1s	NH	8.60 (brt)	
C7a		127.1s	CO		168.6s
C4	7.58 (d, 7.6)	118.4d	Asn α	4.59 (m)	47.7d
C5	6.94 (dd, 7.6, 7.3)	118.1d	*β*	2.44 (m), 2.86 (m)	35.5t
C6	7.02 (dd, 7.3, 7.6)	120.7d	NH	7.27[b]	
C7	7.30 (d, 7.9)	111.2d	CO		169.4s
1-NH	10.66 (brs)		$CONH_2$	*c*	171.5s

[a] May be interchanged. [b] Overlapped. [c] Not observed.

137.5, 156.7, 160.5, 169.4, 169.6, 170.0, 170.1, 170.8, 170.9, 171.1, 171.4, 171.7, 173.7.

Amino Acid Analysis of the Acid Hydrolysate. Each peptide (100 μg) was dissolved in 0.2 mL of 6 N HCl and heated in an evacuated sealed tube at 110 °C for 16 h. After removal of the aqueous HCl by lyophilization, the residue was dissolved in 1% HCl and subjected to automatic amino acid analyzer.

Marfey Analysis of the Acid Hydrolysate. To a half portion of the above acid hydrolysate dissolved in 1 M $NaHCO_3$ (100 μL) was added 10% solution of FDAA (10 μL) and the resulting mixture kept at 80 °C for 3 min. The reaction mixture was neutralized with 2 N HCl (50 μL), diluted with MeCN/H_2O (1:1, 0.3 mL), and subjected to reversed-phase HPLC to assign the chirality of amino acids as described.[14] Retention times of Marfey derivatives of constituent amino acids in halicylindramide D (min): D-Cys(O_3H) (36.1), L-Arg (36.8), L-Thr (45.7), L-Asp (47.8), L-MeGlu (52.0), L-Pro (57.1), D-Ala (63.5), L-Val (70.6), D-Val (79.6), D-Trp (84.7), L-BrPhe (91.2), D-Phe (99.1).

Edman Degradation of Halicylindramide D. Halicylindramide D (100 μg) was deformylated and subjected to four cycles of Edman degradation as described[14] to give a nonapeptide as the major product. The resulting peptide was hydrolyzed (6 N HCl, 110 °C, 16 h) and subjected to Marfey analysis, revealing the ratio of L-Val and D-Val to be 8:3.

Treatment of Halicylindramide D with [Bis-(trifluoroacetoxy)iodo]benzene. To a solution of halicylindramide D (100 μg) in MeCN/H_2O (1:1, 0.1 mL) was added [bis(trifluoroacetoxy)iodo]benzene (1 mg) and pyridine (0.1 mL). The solution was stirred at room temperature for 3 days. The reaction mixture was extracted with EtOAc: the aqueous phase was hydrolyzed (6 N HCl, 110 °C, 16 h) and subjected to amino acid analysis.

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References and Notes

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(25) Halicylindramides gave $[M + 2H]^+$ ion peaks in the FABMS spectra as in the case of a related peptide polydiscamide A.[24]

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