Two New Cyclic Pentapeptides from the Marine-Derived Fungus *Aspergillus versicolor*

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Two new cyclic pentapeptides, named versicotides A (**1**) and B (**2**), were obtained from a marine-derived fungus strain ZLN-60, identified as *Aspergillus versicolor*. Their structures were established on the basis of chemical and spectroscopic evidence. Versicotides are new cyclic pentapeptides which contain an L-alanine residue, two anthranilic acid (=2-aminobenzoic acid) residues, and two *N*-methyl-L-alanine residues. Antitumor activities were evaluated by the SRB and MTT methods.

Introduction. – Fungi are widely recognized as emerging sources of active secondary metabolites, and more and more attention has been paid to those obtained from marine environments in recent years [1][2]. In our continuous search for new bioactive natural products from marine-derived fungi, a fungus strain, ZLN-60, identified as *Aspergillus versicolor*, was isolated from the sediment of the Yellow Sea. The AcOEt extract showed weak cytoxicity *in vitro* against the P388 cell line. Investigation of the AcOEt extract of this fungus led to the isolation of two new cyclic pentapeptides, versicotides A (**1**) and B (**2**) (*Fig. 1*). In this article, we describe the isolation, structural elucidation, and cytotoxic activities of the new compounds.

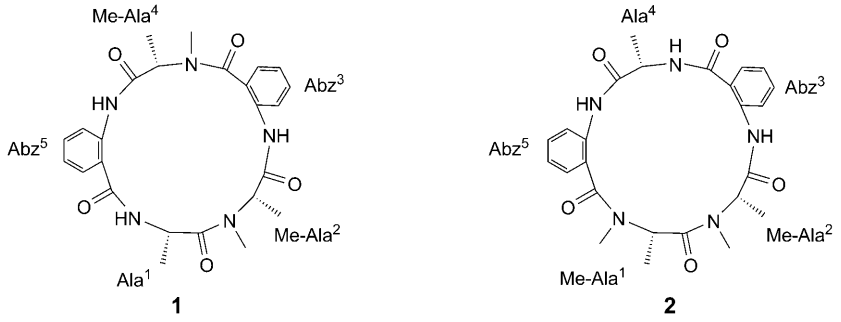

Fig. 1. *Compounds* **1** *and* **2** *isolated from* Aspergillus versicolor *ZLN-60*

Results and Discussion. – The AcOEt extract was concentrated and the residue subjected to repeated column chromatography (SiO_2, *Sephadex LH-20*) followed by recrystallization to yield a mixture of the two new cyclic pentapeptides **1** and **2**.

[1]) These authors made equal contributions to this article.

The isomeric versicotides A (**1**) and B (**2**) were obtained as a 2:1 mixture. They had the same molecular formula $C_{25}H_{29}N_5O_5$ as deduced by the $[M+H]^+$ ion peak at *m/z* 480.2223 in the HR-ESI-MS which indicated 19 degrees of unsaturation. The ^{1}H- and ^{13}C-NMR spectra of versicotide A (**1**) (*Table 1*) showed five amide C=O groups (δ(C) 168.0, 168.5, 169.6, 169.7, and 173.1), two MeN groups (δ(H) and δ(C) 2.70 and 29.4 and 2.83 and 29.9) and three characteristic CH(α), signals of amino acid residues (δ(H) and δ(C) 5.19 and 56.3, 4.85 and 46.8, and 4.75 and 59.3), indicating the peptide nature of **1**. The ^{1}H,^{1}H-COSY and HMBC data (*Table 1*, *Fig. 2*) indicated the presences of the following amino acid residues: one alanine, two *N*-methylalanine, and two anthranilic acid (=2-aminobenzoic acid; Abz) residues which accounted for 19 degrees of unsaturation. The connections of these residues were established by an HMBC

Table 1. *NMR Data* ((D$_6$)DMSO) *of Versicotide A* (**1**). δ in ppm, *J* in Hz.

	Position[2])	δ(H)	δ(C)	^{1}H,^{1}H-COSY	HMBC
Ala[1]	C(1)		173.1 (*s*)		
	H–C(2)	4.85 (br. *q*, *J* = 6.9)	46.8 (*d*)	Ala[1] Me(3), Ala[1] NH	
	Me(3)	1.30 (*d*, *J* = 6.9)	15.1 (*q*)	Ala[1] H–C(2)	Ala[1] C(1)
	NH	9.40 (*s*)			Ala[1] C(2), Abz[5] C(1)
Me-Ala[2]	C(1)		169.6 (*s*)		
	H–C(2)	5.19 (br. *q*, *J* = 6.9)	56.3 (*d*)	Me-Ala[2] Me(3)	
	Me(3)	1.40 (*d*, *J* = 6.9)	15.4 (*q*)	Me-Ala[2] H–C(2)	Me-Ala[2] C(1)
	MeN	2.83 (*s*)	29.9 (*q*)		Me-Ala[2] C(2), Ala[1] C(1)
Abz[3]	C(1)		168.0 (*s*)		
	C(2)		125.4 (*s*)		
	H–C(3)	7.42 (*d*, *J* = 7.7)	127.5 (*d*)	Abz[3] H–C(4)	Abz[3] C(1), Abz[3] C(5), Abz[3] C(7)
	H–C(4)	7.23–7.26 (*m*)	124.4 (*d*)	Abz[3] H–C(3), Abz[3] H–C(5)	
	H–C(5)	7.47–7.55 (*m*)	130.7 (*d*)	Abz[3] H–C(6)	
	H–C(6)	8.05 (*d*, *J* = 7.7)	125.5 (*d*)	Abz[3] H–C(5)	Abz[3] C(2)
	C(7)		136.8 (*s*)		
	NH	9.51 (*s*)			Me-Ala[2] C(1), Abz[3] C(6)
Me-Ala[4]	C(1)		168.5 (*s*)		
	H–C(2)	4.75 (br. *q*, *J* = 6.9)	59.3 (*d*)	Me-Ala[1] Me(3)	
	Me(3)	1.51 (*d*, *J* = 6.9)	16.7 (*q*)	Me-Ala[4] H–C(2)	Me-Ala[4] C(1)
	MeN	2.70 (*s*)	29.4 (*q*)		Me-Ala[4] C(2), Abz[3] C(1)
Abz[5]	C(1)		169.7 (*s*)		
	H–C(2)		116.9 (*s*)		
	H–C(3)	8.00 (*d*, *J* = 7.7)	129.7 (*d*)	Abz[5] H–C(4)	Abz[5] C(1), Abz[5] C(7)
	H–C(4)	7.15–7.18 (*m*)	122.9 (*d*)	Abz[5] H–C(3), Abz[5] C(5)	Abz[5] C(2)
	H–C(5)	7.47–7.55 (*m*)	133.8 (*d*)	Abz[5] H–C(4), Abz[5] H–C(6)	Abz[5] C(3), Abz[5] C(7)
	H–C(6)	8.58 (*d*, *J* = 7.7)	119.5 (*d*)	Abz[5] H–C(5)	Abz[5] C(2), Abz[5] C(4)
	C(7)		140.4 (*s*)		
	NH	12.22 (*s*)			Me-Ala[4] C(1), Abz[5] C(6)

[2]) Arbitrary atom numbering of the 2-aminobenzoic acid residues (Abz).

spectrum. The correlation Abz^3 NH/Me-Ala^2 C(1) showed the neighborhood of Me-Ala^2 and Abz^3. The connections of Ala^1 to Me-Ala^2 and Abz^5 were shown by the correlations Me-Ala^2 MeN/Ala^1 C(1) and Ala^1 NH/Abz^5 C(1)^2). The vicinity of Me-Ala^4 to Abz^5 and Abz^3 were also evident from the HMBC data, *i.e.*, from the correlations Abz^5 NH/Me-Ala^4 C(1) and from Me-Ala^4 MeN/Abz^3 C(1) (*Fig. 2*). Thus, the structure of versicotide A (**1**) was elucidated as cyclo-[Ala^1-(Me-Ala^2)-Abz^3-(Me-Ala^4)-Abz^5].

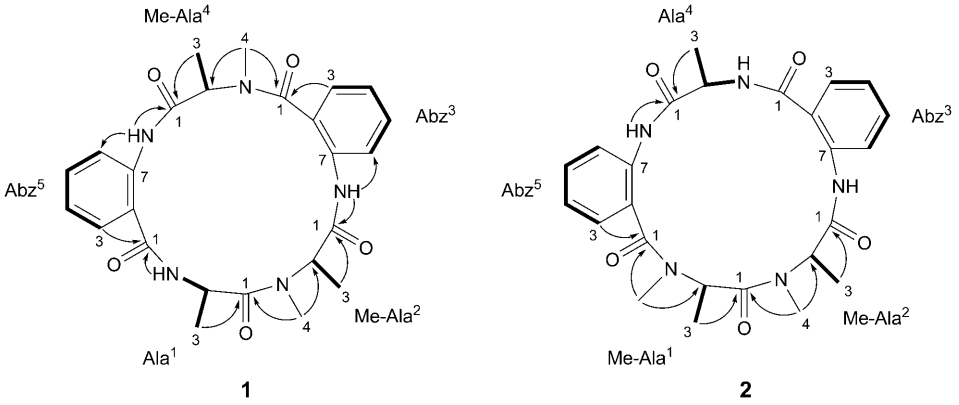

Fig. 2. *^{1}H,^{1}H-COSY* (—) *and key HMBC* (H → C) *features of compounds* **1** *and* **2**

Careful analysis of the NMR spectra and HR-ESI-MS of the mixture **1**/**2** indicated that **2** was structurally related to **1**, and they had the same amino acid residues (for NMR data of **2**, see *Table 2*). The difference between them was the residue sequence which was evidenced by the HMBC experiment. Thus, the correlation Me-Ala^2 MeN/Me-Ala^1 C(1) showed the neighborhood of Me-Ala^1 and Me-Ala^2. The connections of Abz^5 to Me-Ala^1 and Ala^4 were shown by the correlations Me-Ala^1 MeN/Abz^5 C(1) and Abz^5 NH/Ala^4 C(1) (*Fig. 2*). Thus, Abz^3 could be located only between Ala^4 and Me-Ala^2. Versicotide B (**2**) was thus elucidated as *cyclo*-[(Me-Ala^1)-(Me-Ala^2)-Abz^3-Ala^4-Abz^5].

The absolute configurations of the amino acid residues of **1** and **2** were determined by the advanced *Marfey* method [3]. HPLC Analysis of hydrolyzated derivatives indicated that only two types of amino acid were present in the mixture, which were identified as L-alanine and *N*-methyl-L-alanine by comparation with the derivatives of authentic samples. Thus, the absolute configurations of versicotides A (**1**) and B (**2**) were elucidated.

The cytotoxicities of the mixture **1**/**2** were evaluated *in vitro* on the P388, BEL-7402, HL-60, and A-549 cells by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method [4] and the SRB (= sulforhodamine B) method [5], and no cytotoxicities were observed on these cell lines.

The versicotide class of cyclic peptides is characterized by five amino acid residues connected through amide bonds; compounds **1** and **2** are the first two examples of cyclic pentapeptides that contain two 2-aminobenzoic acid residues. A variety of cyclic

Table 2. *NMR Data* ((D$_6$)DMSO) *of Versicotide B* (**2**). δ in ppm, J in Hz.

	Position[2])	δ(H)	δ(C)	^{1}H,^{1}H-COSY	HMBC
Me-Ala[1]	C(1)		174.0 (*s*)		
	H–C(2)	5.37 (br. *q*, *J* = 6.9)	45.8 (*d*)	Me-Ala[1] Me(3)	
	Me(3)	1.33 (*d*, *J* = 6.9)	15.7 (*q*)	Me-Ala[1] H–C(2)	Me-Ala[1] C(1)
	MeN	2.74 (*s*)	28.9 (*q*)		Me-Ala[1] C(2), Abz[5] C(1)
Me-Ala[2]	C(1)		167.7 (*s*)		
	H–C(2)	5.37 (br. *q*, *J* = 6.9)	55.7 (*d*)	Me-Ala[2] Me(3)	
	Me(3)	1.40 (*d*, *J* = 6.9)	17.6 (*q*)	Me-Ala[2] H–C(2)	Me-Ala[2] C(1)
	MeN	2.77 (*s*)	29.4 (*q*)		Me-Ala[2] C(2), Me-Ala[1] C(1)
Abz[3]	C(1)		168.6 (*s*)		
	C(2)		119.4 (*s*)		
	H–C(3)	8.53 (*d*, *J* = 7.7)	130.7 (*d*)	Abz[3] H–C(4)	Abz[3] C(2), Abz[3] C(5)
	H–C(4)	7.47 – 7.55 (*m*)	124.8 (*d*)	Abz[3] H–C(3), Abz[3] H–C(5)	Abz[3] C(2), Abz[3] C(6)
	H–C(5)	7.23 – 7.26 (*m*)	129.4 (*d*)	Abz[3] H–C(4), Abz[3] H–C(6)	
	H–C(6)	7.16 (*d*, *J* = 7.7)	123.0 (*d*)	Abz[3] H–C(5)	
	C(7)		136.2 (*s*)		
	NH	9.04 (*s*)			
Ala[4]	C(1)		171.1 (*s*)		
	H–C(2)	5.03 (br. *q*, *J* = 6.9)	59.3 (*d*)	Ala[1] Me(3)	
	Me(3)	1.30 (*d*, *J* = 6.9)	13.5 (*q*)	Ala[4] H–C(2), Ala[4] NH	Ala[4] C(1)
	NH	9.27 (*s*)			
Abz[5]	C(1)		169.4 (*s*)		
	C(2)		126.7 (*s*)		
	H–C(3)	7.85 (*d*, *J* = 7.7)	129.1 (*d*)	Abz[5] H–C(4)	Abz[5] C(1), Abz[5] C(5), Abz[5] C(7)
	H–C(4)	7.15 – 7.18 (*m*)	126.7 (*d*)	Abz[5] H–C(3), Abz[5] H–C(5)	
	H–C(5)	7.47 – 7.55 (*m*)	133.1 (*d*)	Abz[5] H–C(4), Abz[5] H–C(6)	
	H–C(6)	7.77 (*d*, *J* = 7.7)	119.8 (*d*)	Abz[5] H–C(5)	Abz[5] C(4)
	C(7)		139.3 (*d*)		
	NH	11.43 (*s*)			Ala[4] C(1)

pentapeptides have cytotoxic [6], fibrinolytic [7], and thrombolytic activity [8], or are LH-RH receptor antagonists [9] and endothelin A receptor antagonists [10]. The versicotides **1** and **2** show no cytotoxicity but might have other bioactivities which need to be investigated in the future.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh, 10–40 mm; *Qingdao Marine Chemical Inc.*, P. R. China), *Sephadex LH-20* (*GE Healthcare*, Sweden), *RP-18* (40–63 mm; *YMC Co.*, Japan). TLC: SiO_2 GF_{254} (10–40 mm, *Qingdao Marine Chemical Inc.*, P. R. China). HPLC: *RP-18* (40–63 mm; *YMC Co.*, Japan); UV data (λ in nm) and amino acids analysis determined with a *Waters 996* photodiode array (PDA) detector; t_R in min. IR Spectra: *Nicolet-Nexus-470* spectrophotometer; in KBr discs; Gñ in cm^{-1}. ^{1}H- and ^{13}C-NMR, DEPT, and 2D-NMR Spectra: *Jeol-Eclips-600* spectrometer; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: Micromass *Q-TOF-Ultima-Global GAAo76* LC mass spectrometer; in m/z (rel. %).

Fermentation and Extraction of the Fungal Strain. The fungus strain ZLN-60 was isolated from the mud (depth, 20 m) of the Yellow Sea (36°02.694′N, 120°18.538′E). The fungus was grown under static conditions at 28° for 30 d in 130 1-liter *Erlenmeyer* flasks containing the liquid medium (300 ml/flask) composed of mannitol (20 g/l), maltose (20 g/l), glucose (10 g/l), monosodium glutamate (10 g/l), KH_2PO_4 (0.5 g/l), $MgSO_4 \cdot 7\,H_2O$ (0.3 g/l), yeast extract (3 g/l), corn steep liquor (1 g/l), and seawater after adjusting its pH to 6.5. The fermented whole broth (40 l) was filtered through a cheesecloth to separate it into supernatant and mycelia. The former was concentrated to about a quarter of the original volume and then extracted three times with AcOEt to give an AcOEt soln., while the latter was extracted three times with acetone. The acetone soln. was concentrated to afford an aq. soln. The aq. soln. was extracted three times with AcOEt to give another AcOEt soln. Both AcOEt solns. were combined and concentrated to give a crude extract (13.0 g).

Purification. The crude extract (13.0 g) was applied to CC (SiO_2 (300–400 mesh), step gradient of petroleum ether/$CHCl_3$ and $CHCl_3$/MeOH): *Fractions 1–6*. *Fr. 3* (350 mg) was separated by CC (SiO_2, $CHCl_3$). *Frs. 3.3–3.7* were further separated into *Fr. (3.3–3.7).1–(3.3–3.7).4* by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1). *Fr. (3.3–3.7).2* was purified by recrystallization in MeOH: **1/2** as a 2:1 mixture (13.0 mg).

Acid Hydrolysis of Versicotides A/B (**1/2**). A mixture **1/2** (1 mg) was hydrolyzed in 1 ml of 6N HCl at 108° for 14 h in a 3 ml reaction vial. The cooled mixture was concentrated, and traces of HCl were removed from the residual hydrolyzate by repeated concentration from H_2O (3×1 ml) with N_2 gas.

Absolute Configuration of Amino Acids. To a 1.5 ml vial containing pure amino acid standards (0.25 μmol) in H_2O (50 μl) was added N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA; 0.25 μmol) in acetone (100 μl) followed by 1N $NaHCO_3$ (25 μl). The mixture was heated for 1 h at 43°. After cooling to r.t., 2N HCl (25 μl) was added, and the resulting soln. was filtered through a small 4.5 mm filter and stored in the freezer until HPLC analysis. Half of the peptide hydrolyzate mixture (see above) was dissolved in H_2O (50 μl), and to this was added L-FDAA (0.25 μmol) in acetone (100 μl) followed by 1N $NaHCO_3$ (25 μl). The derivatization reaction was carried out and the product mixture worked up as described above for the amino acid standards. An aliquot (8 μl) of the resulting mixture of L-FDAA derivatives was analyzed by reversed-phase HPLC (5×250 mm *YMC* C_{18} column (5 μm), linear gradient of MeCN (*A*) and 0.05% aq. CF_3COOH (*B*) from 10% to 50% *A* over 60 min, flow rate 1 ml/min, detection at 320 nm). Each chromatographic peak was identified by comparing its t_R with the L-FDAA derivative of the pure L- and D-amino acid standard. The standards had t_R 40.20 (L-FDAA), 38.61 (Ala), 43.92 (D-Ala), 41.15 (Me-Ala), and 39.63 (Me-D-Ala).

Versicotides A (=*Cyclo(*L-*alanyl*-N-*methyl*-L-*alanyl*-2-*aminobenzoyl*-N-*methyl*-L-*alanyl*-2-*aminobenzoyl)*; **1**) *and B* (=*Cyclo(*N-*methyl*-L-*alanyl*-N-*methyl*-L-*alanyl*-2-*aminobenzoyl*-L-*alanyl*-2-*aminobenzoyl)*; **2**): White amorphous powder. UV (HPLC, mobile phase): 210, 282. IR (KBr): 3356, 3191, 2906, 1666, 1613, 1427, 756. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 480.2223 ($[M+H]^+$, $C_{25}H_{30}N_5O_5^+$; calc. 480.2247).

Biological Assay. In the MTT assay, cell lines were grown in RPMI-1640 supplemented with 10% FBS (fetal bovine serum) under a humidified atmosphere of 5% CO_2 and 95% air at 37°. Cell suspensions (200 μl) at a density of $5 \cdot 10^4$ cell/ml were plated in 96-well microtiter plates and incubated for 24 h. Then, 2 μl of the test solns. (in MeOH) were added to each well and further incubated for 72 h. Then the MTT soln. (20 μl; 5 mg/ml in IPMI-1640 medium) was added to each well and incubated for 4 h. Old medium containing MTT (150 μl) was then gently replaced by DMSO and pipetted to dissolve any

formazan crystals formed. Absorbance was then determined on a *Spectra-Max-Plus* plate reader at 540 nm.

In the SRB assay, the cell suspensions (200 µl) were plated in 96 cell plates at a density of $2 \cdot 10^5$ cell/ml. Then, 2 µl of the test solns. (in MeOH) were added to each well, and the culture was further incubated for 24 h. The cells were fixed with 12% CCl_3COOH, and the cell layer was stained with 0.4% SRB. The absorbance of SRB soln. was measured at 515 nm. Dose/response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of the log dose/response curves.

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