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Antifungal cyclopeptides from *Halobacillus litoralis* YS3106 of marine origin

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Abstract—Three new cyclopeptides, including halolitoralin A (a cyclic hexapeptide), halolitoralin B and C (two cyclic tetrapeptides), together with three known cyclic dipeptides, cyclo(Pro-Val), cyclo(Pro-Leu) and cyclo(Ile-Val) were isolated from the ferment broth of a marine sediment-derived *Halobacillus litoralis* YS3106. The cyclopeptides show surprisingly simple architectures with highly repeated residue units, which showed moderate antifungal and weak antitumor activities in vitro.

Antimicrobial peptides have played a crucial role in the pharmaceutical research as biomedically useful agents or as lead compounds for drug development.[1] Whereas many natural peptides show therapeutic potential in in vitro biological screening, small cyclopeptides are generally among the more promising lead structures owing to their reduced conformation flexibility and increased in vivo stability compared with their linear counterparts.[2] As the attention of many chemists was concentrated on increasing the size and diversity of the peptide libraries by means of chemical synthesis or biosynthesis,[3,4] continuing efforts are still put on the isolation of novel cyclopeptides from natural resources.[5] Marine microorganisms are the biggest reservoir providing a wide variety of structurally unique, biologically significant nonribosomal peptides, especially cyclopeptides derivatives.[6] As part of our ongoing efforts toward finding novel antifungal and antitumor chemicals from natural resources,[7] we started to investigate the secondary metabolites of microorganisms under the high salt circumstance.

Our interest in a bacterium strain YS3106 was stimulated by the fact that the culture of this strain showed broad antifungal and antibacterial activities in vitro. YS3106 was separated from the high salt sediment of Huanghai Sea and identified as *Halobacillus litoralis*.[8] The strain was fermented in a mechanical agitation reactor with rotary agitation at 150 rpm and aerating pressure at 0.05 MPa for 4 days at 29°C using artificial seawater medium (water solution of yeast extract 1.5%, peptone 0.5%, NaCl 0.5%, sea salt 1.5%, pH 6.8–7.0). Then the ferment broth was extracted with EtOAc and concentrated to afford 50 g of a dark-brownish residue. The crude extract was soaked in MeOH to eliminate wax by decreasing temperature from the room temperature to −8°C gradually and standing at this temperature for 24 h. Resulted alcohol-soluble portion was fractionated over silica gel eluting with gradient of $CHCl_3$/MeOH to give the mixture of cyclic dipeptides, which showed specific signals in ^1H NMR spectrum. This fraction was then chromatographed on Sephadex LH-20 columns eluted with $CHCl_3$/MeOH (1:1), followed by preparative HPLC (Hitachi semi-preparative column, MeOH/H_2O) to yield three known products: cyclo(L-Pro-L-Val) **1**,[9] cyclo(L-Pro-L-Leu) **2**[10] and cyclo(L-Ile-L-Val) **3**.[11] The structures were assigned by comparing the optical rotations and the NMR spectra with reported data.[12]

To confirm the stereochemistry of the amino acid residues, Marfey's analysis was performed.[13] Two milli-

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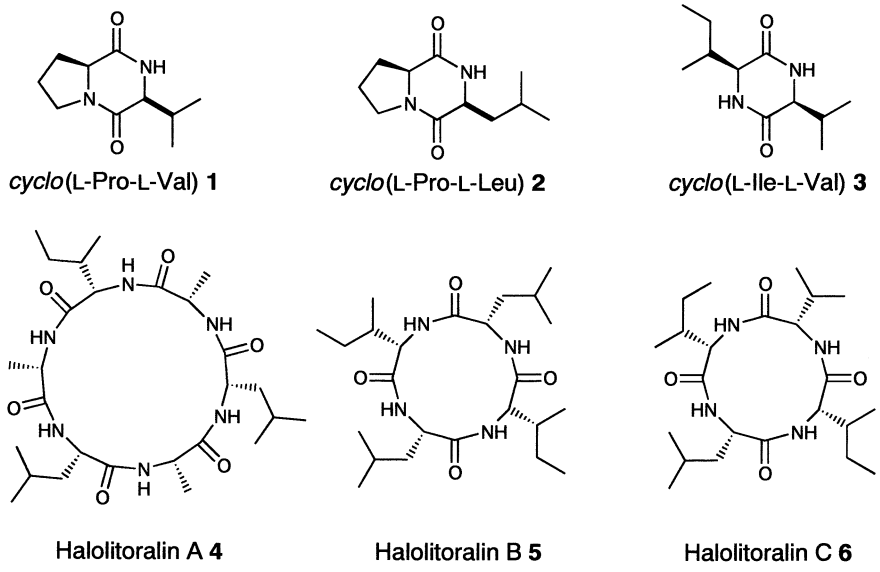
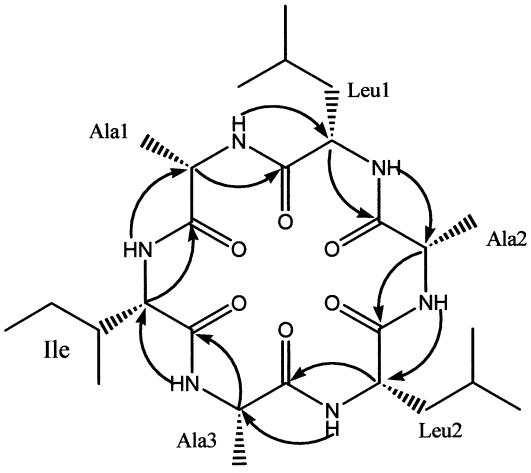
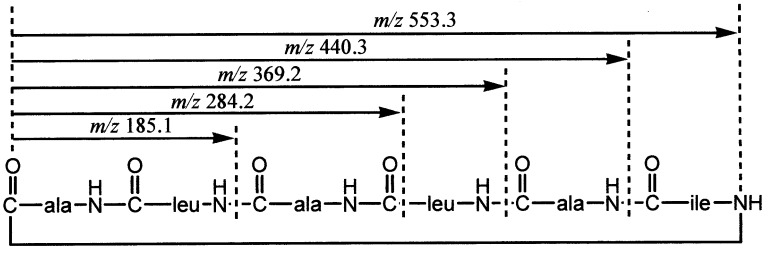


grams of the cyclopeptides were dissolved in 6N HCl (2 mL) and heated at 110°C for 24 h in a sealed glass tube. After cooling to room temperature and being neutralized to pH 6.0 with 2N NaOH, the solution was concentrated in vacuum and redissolved in distilled water (2 mL), then reacted with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA), which was prepared by the reaction of 1,5-difluoro-2,4-dinitrobenzene and L-alaninamide hydrochloride. The resulted solution in acetone was analyzed by HPLC and compared with derivatized authentic amino acids. As a result, all the amino acid residues showed L-configurations.

Directed by TLC monitoring and antifungal bioassays, the fractions with inhibiting ability were purified by combination of silica gel chromatography and



(a)



(b)

Figure 1. (a) Arrows show key HMBC correlations and (b) ESI MS fragments of halolittoralin A **4**.

Saphadex LH-20 chromatography to afford halolitoralin A **4**, halolitoralin B **5** and halolitoralin C **6** as colorless amorphous solids.[14] Compound **4** showed a molecular ion peak in high resolution ESI MS at m/z 575.3521 $[M+Na]^+$ corresponding to molecular formula $C_{27}H_{48}O_6N_6$ (calcd 575.3528), while **5** and **6** appealed as isomers with formula $C_{23}H_{42}O_4N_4$.[15] The ^1H and ^{13}C NMR spectra of **4**–**6** showed typical signals of cyclopeptides.[16] The ^1H NMR spectrum of halolittoralin A **4** in deuterated DMSO revealed six NH proton signals between δ 7.9 and 8.2, pointing to six amino acid residues. The ^{13}C and DEPT NMR spectra revealed the presence of nine methyl groups, three methylene carbons, and six carboxyl carbonyls (δ 168 to 172 ppm). Combining above information with HMQC and ^1H–^1H COSY data, we could easily interpret that **4** is a cyclic hexapeptide composed of Ala (×3), Leu (×2) and Ile. Similarly, **5** was found as a cyclic tetrapeptide with Leu (×2), Ile and Val, and **6** was also a cyclic tetrapeptide composed of Ile (×2), Leu and Val.

The sequences of the amino acid residues in **4**–**6** were established by the analysis of the HMBC data, which showed correlations from the α-methine protons of each amino acid residues to the carbonyl carbons of the neighboring residues as well as the correlations from the α-amide protons to the α-carbons of the adjacent residues (Fig. 1a). The appearance of fragments of Ala-Leu or Ala-Ile, Ala-Leu-Ala or Ala-Ile-Ala, but lack of the fragment of Ala-Ala in ESI-MS analyses also confirmed this proposed linkage pattern (Fig. 1b). Upon hydrolysis and Marfey's analysis, the stereochemistries of all the amino acid residues in **4**–**6** were determined as L-configuration.

Antifungal activity of halolittoralin A **4**, B **5** and C **6** were tested in vitro by agar dilution method,[17] against two human fungi (*Candida albicans* and *Tricophyton rubrum*) and four crop-threatening fungi (*Gaeumannomyces graminis*, *Rhzioctonia cerealis*, *Helminthosporium sativum* and *Fusarium graminearum*). All of them showed moderated antifungal activities, while **4** was the most active one in all the cases (Table 1). In addition, these three cyclopeptides showed moderate anti human gastric tumor activities in vitro (with a cell line of BGC).

Most cyclopeptides or cyclic depsipeptides from marine resources presented D-amino acids or unusual amino acids.[6] However, all six cyclic peptides we report herein show surprisingly simple architectures with highly repeated residue units, composed of only usual L-amino acids residues with hydrophobic sides chains. We also found that compound **4** underwent conformational charge upon binding with potassium but not with sodium. Furthermore, with the appearance of potassium, the antifungal activity remarkably decreased.[18] Thus, it will be possible to generate potential ionophorestic cyclopeptides based on similar structures by combinatorial synthesis.

Table 1. Minimal inhibition concentrations (MIC, μg/mL) of the halolittoralins

Fungi	**4**	**5**	**6**	Positive control
Candida albicans	20	30	30	2[a]
Tricophyton rubrum	25	35	40	5[a]
Gaeumannomyces graminis	300	400	350	150[b]
Rhzioctonia cerealis	200	350	350	100[b]
Helminthosporium sativum	300	400	400	120[b]
Fusarium graminearum	350	600	800	150[b]

[a] Ketoconazole used as positive control.
[b] Triadimefon used as positive control.

It is a very promising research area to screen the microorganisms in extreme environments for the generation of new natural molecules with diversified structures and bio-properties. To our knowledge, no precedent investigation of the secondary metabolites of *H. litoralis* (or other *Halobacillus* sp.) has been reported so far. Our discovery will be very important to understand how the high salt environment affects the metabolites of microorganism compared with the ordinary system.

Supplementary material

NMR data of halolittoralin A–C were listed with all two dimensional NMR spectra.

Acknowledgements

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14. Halolitoralin A **4**: mp 170–171°C, $[\alpha]_D^{20}$ −175.2 (*c* 0.24 in MeOH); λ_{max} (MeOH)/nm (ε) 204 (3.6×10^4); ν_{max} (KBr)/cm^{-1} 3432, 3197, 3082, 2962, 2876, 1676, 1449, 1323. Halolitoralin B **5**: mp 198–199°C, $[\alpha]_D^{20}$ −115° (*c* 0.28 in MeOH); λ_{max} (MeOH)/nm (ε) 204 (3.2×10^4); ν_{max} (KBr)/cm^{-1} 3319, 3191, 3056, 2963, 2937, 1661, 1447. Halolitoralin C **6**: mp 201–202°C, $[\alpha]_D^{20}$ −105 (*c* 0.25 in MeOH); λ_{max} (MeOH)/nm (ε) 204 (3.7×10^4); ν_{max} (KBr)/cm^{-1} 3316, 3192, 3057, 2964, 2938, 1660, 1448.
15. HR ESI MS: halolitoralin B **5** was *m*/*z* 439.3278 [*M*+Na]$^+$ and halolitoralin C **6** was *m*/*z* 439.3274 [*M*+Na]$^+$, calcd for a molecular formula of $C_{23}H_{42}O_4N_4$ gives 439.3279.
16. For all the NMR data and spectra see supplemental materials.
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18. The study of solution conformational changes of halolitoralin A will be reported elsewhere.