Three New Cytotoxic Cyclic Acylpeptides from Marine *Bacillus* sp.

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Three new cytotoxic cyclopeptides belonging to iturin class have been isolated from marine bacterium *Bacillus* sp. These three compounds have the cytotoxic activities. Their structures were determined based on chemical analysis and all kinds of spectroscopic techniques.

Key words *Bacillus* sp.; cyclopeptide; cytotoxic activity

Marine microorganisms have proven to be a promising source for the production of novel antibiotic, antitumor and anti-inflammatory agents.[1] In the continuation of previous research work, we have isolated three new cyclic acylpeptides named as mixirins A (**1**), B (**2**) and C (**3**) from marine microorganism. The ethyl acetate extract of the fermentation of marine bacterium *Bacillus* sp.,[2] obtained from sea mud near the Arctic pole, was subjected to silica gel, Sephadex LH-20 column chromatography and reversed-phase HPLC to afford to mixirins A (62 mg), B (16 mg) and C (9 mg). In this paper, we report the isolation and the structure elucidation of these three new cyclic acylpeptides.

Mixirin A (**1**), obtained as a white amorphous powder, has a molecule formula of $C_{48}H_{75}N_{12}O_{14}$ (18 unsaturations) based on the positive-ion high resolution (HR)-FAB-MS, showing a $[M+H]^+$ ion peak at *m/z* 1043.5411 (Calcd for 1043.5426). The ^{13}C-NMR data and molecule formula showed that there were 12 amide-type carbonyls. Additional analysis of the ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR (150 MHz, CD_3OD) revealed resonances for di-substituted aromatic ring. The aromatic residue was identified as Tyr by ^{1}H–^{1}H COSY, HMQC and HMBC. Further analysis showed the presence of one Ser residue, one Pro, one Gln and three Asn residues, all of which were assigned based on ^{1}H–^{1}H COSY, HMQC, HMBC, especially TOCSY spectra and HR-FAB-MS. Derivatization of these residues obtained from the crude hydrolysate with Marfey's reagent[3] and HPLC analysis with co-injection of standards defined the absolute stereochemistry of Tyr as D, Ser as L, Pro as D, three Asn as L and Gln as L.

Extensive analysis of the ^{1}H- and ^{13}C-NMR found that there was a long-chain fatty acid in the molecule. Accounting for all the amino acid residues, a formula of $C_{14}H_{27}NO$ remained for the fatty acid portion of the molecule. The protons signal at δ 0.89 (3H, t, J=7.2 Hz) and at δ 1.30 (nH, br s) in the ^{1}H-NMR, and the carbon signal at δ 14.4 in the ^{13}C-NMR revealed that it was a normal-type[4] long-chain amino fatty acid (β-AA). Analysis of HMBC and ^{1}H–^{1}H COSY spectra showed that the amino group was at β-position. Thus the fatty acid residue should be β-aminotetradecanoic acid residue. The absolute stereochemistry of the residue hasn't been determined.

The full assignment of the ^{13}C- and ^{1}H-NMR data for mixirin A was accomplished by a combination of ^{1}H–^{1}H COSY, TOCSY, DEPT, HMQC and HMBC experiments.[5]

The total sequence of residues in mixirin (A) was obtained by analysis of HMBC, NOESY and by multiple stages of collisionally-activated decompositions (CAD) technique in MS/MS. In HMBC, α-H of every residue (β-H of β-aminotetradecanoic acid) has correlation with carbonyls of neighbored residues (Chart 1). The sequence of the residues of the cyclic peptide was confirmed by multiple stages of collisionally-activated decompositions in MS/MS (Chart 2). Therefore mixirin A was determined to be cyclo (D-Pro–L-Asn-1–L-Ser–L-Gln–L-Asn-2–D-Tyr–L-Asn-3–D-β-AA) (Chart 1).

The ^{13}C- and ^{1}H-NMR of mixirins B and C were essentially identical to that of mixirin A. But the terminal methyl proton signals of mixirin C at δ 0.87 (6H, m) and δ 1.30 (mH, br s) in ^{1}H-NMR were different from those of mixirin A. The terminal methyl carbon signals were at δ 11.5 and 19.4 in ^{13}C-NMR. This showed that the long-chain β-amino acid residue in mixirin C was ante-iso type.[6,7] ^{1}H-NMR of

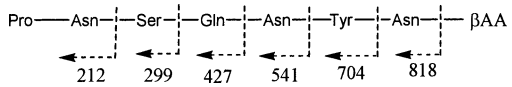

Chart 2. MS^n Fragmentation of Mixirins A, B and C

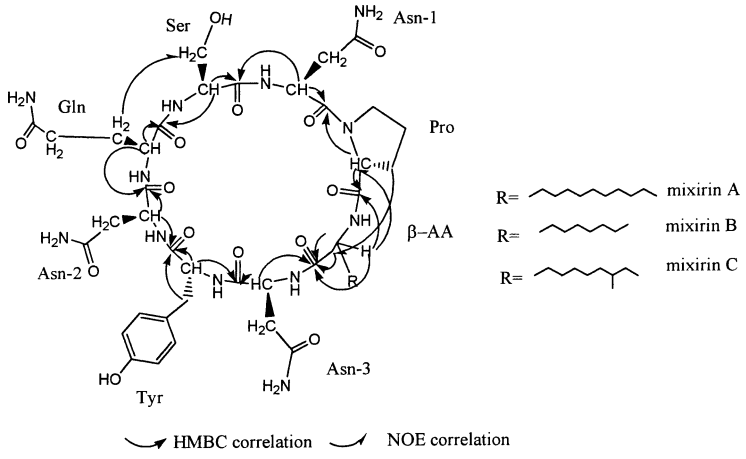

Chart 1. The Structures of Mixirins A, B and C

mixirin B only at δ 1.30 (m′H, br s) was different from that of mixirin A. Due to essentially identical ^{13}C- and ^{1}H-NMR spectra, mixirins B and C were deduced to have the same amino acid residues and sequences as those of mixirin A. The differences were the long-chain β-amino acids. The positive-ion HR-FAB-MS revealed the molecule formula of mixirin B was $C_{45}H_{69}N_{12}O_{14}$, showing a $[M+H]^+$ ion peak at *m*/*z* 1001.4638 (Calcd for 1001.4641), Thus the long-chain β-amino acid residue was β-aminoundecanoic acid residue. The molecule formula of mixirin C was $C_{47}H_{73}N_{12}O_{14}$ based on HR-FAB-MS, showing a $[M+H]^+$ ion peak at *m*/*z* 1029.3245 (Calcd for 1029.3261). Therefore the long-chain β-amino acid residue in mixirin C was β-amino 10-methyl-dodecanoic acid residue (Chart 1).

The activities of antitumor cell of mixirins A, B and C have been evaluated. Mixirins A, B and C inhibited the growth of human colon tumor cells (HCT-116) with IC_{50} of 0.68, 1.6, 1.3 μg/ml. Extensive activities evaluations are in progress.

References and Notes

1) Fenical W., *Chem. Rev.*, **93**, 1673—1683 (1993).
2) The marine microorganism, culture number MIX-62, was isolated from sea mud near the Arctic pole, identified as *Bacillus* sp. by Professor Li Tian, First Institute of Oceanography, State Oceanic Administration, China.
3) Marfey P., *Carlsberg Res. Commun.*, **49**, 591—596 (1984).
4) Higuchi R., Inagaki M., Togawa K., Miyamoto T., Komori T., *Liebigs Ann. Chem.*, **7**, 653—658 (1994).
5) Compound **1**: White amorphous powder, $[\alpha]_D^{22}$ −18.2° (*c*=0.16, CH_3OH), mp 285—286 °C, ^{1}H-NMR (600 MHz, CD_3OD)/^{13}C-NMR (150 MHz, CD_3OD) Pro δ: 4.21 (1H, t, *J*=7.2 Hz, H-α)/63.3, 2.26 (1H, m, H-β), 2.18 (1H, m, H-β)/31.8, 2.12 (1H, m, H-γ), 1.93 (1H, m, H-γ)/26.2, 3.94 (1H, m, H-δ), 3.80 (1H, m, H-δ)/49.0, CO 175.3; Asn-1δ: 4.63 (1H, m, H-α)/52.51, 2.75 (1H, dd, *J*=8.4, 15.6 Hz, H-β), 2.68 (1H, dd, *J*=4.8, 15.6 Hz, H-β)/36.8, CO 173.1, *CO 174.8; Ser δ: 4.40 (1H, dd, *J*=3.6, 4.8 Hz, H-α)/57.4, 4.03 (1H, dd, *J*=4.8, 11.4 Hz, H-β), 3.77 (1H, dd, *J*=3.6, 11.4 Hz, H-β)/62.8, CO 172.9; Gln δ: 4.68 (1H, dd, *J*=4.7, 9.9 Hz, H-α)/51.6, 2.14 (1H, m, H-β), 2.01 (1H, m, H-β)/27.0, 2.31 (2H, m, H-γ)/33.1, CO 172.8, *CO 177.7; Asn-2 δ: 4.50 (1H, dd, *J*=5.2, 7.0 Hz, H-α)/51.8, 2.87 (2H, m, H-β)/36.28, CO 174.2, *CO 174.7; Tyr δ: 4.28 (1H, dd, *J*=4.8, 9.6 Hz, H-α)/57.8, 3.10 (1H, dd, *J*=4.8, 14.4 Hz, H-β), 2.91 (1H, dd, *J*=9.6, 14.4 Hz, H-β)/36.32, 7.05 (1H, d, *J*=6.5 Hz, H-o)/131.2, 6.72 (1H, d, *J*=6.5 Hz, H-m)/116.5, iC 128.9, pC 157.4, CO 174.2; Asn-3 δ: 4.60 (1H, m, H-α)/52.54, 2.47 (1H, dd, *J*=6.0, 15.6 Hz, H-β), 2.51 (1H, dd, *J*=7.8, 15.6 Hz, H-β)/37.8, CO 173.9, *CO 175.3; β-AA δ: 2.39 (1H, dd, *J*=1.8, 15.8 Hz, H-2), 2.56 (1H, dd, *J*=11.0, 15.8 Hz, H-2)/43.7, 4.17 (1H, m, H-3)/48.0, 1.63 (1H, m, H-4), 1.48 (1H, m, H-4)/36.6, 1.28 (2H, m, H-5)/27.7, 1.24—1.27 (nH, br s, H-6—12)/30.7—30.8, 1.30 (2H, m, H-13)/23.7, 0.89 (3H, t, *J*=7.2 Hz, H-14)/14.4. * γCO of Asn residues and δ CO of Gln residue.
6) Normal means a straight chain (–$CH_2CH_2CH_3$); ante-iso means a branched chain possessing a methyl group on the third carbon from the terminal methyl group [–$CH_2CH(CH_3)CH_2CH_3$].
7) Natori T., Morita M., Akimoto K., Koezuka Y., *Tetrahedron*, **50**, 2771—2784 (1994).