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Molecular diversity of cereulide detected by means of nano-HPLC-ESI-Q-TOF-MS

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Abstract

Cereulide is a cyclic dodecadepsipeptide from a pathogenic bacteria *Bacillus cereus*, which shows the emetic toxicity. Molecular diversity, or variety in homologation was found as a minor constituent of this cyclic peptide. Its molecular weight is 1152 but its homologs were observed as 1138 and 1166, which had 14 mass lower and higher differences from cereulide. This homologation was observed in about 10% of cereulide. It seemed to be difficult to determine the heterogeneous amino acids directly by MS/MS analysis on the intact molecules of cereulide. And hydrolysis of this cyclic peptide gave dipeptides, which were analyzed to determine their heterogeneous components by means of nano-HPLC-ESI-Q-TOF-MS and MS/MS. Among all amino- and oxy-acids, we found that *O*-Val and *O*-Leu were the keys of variation in cereulide. These findings will be significant to establish an identification method for pathogenic bacteria on the basis of biosynthetic pathways.

Keywords: Cereulide; Valinomycin; Homolog; HPLC-ESI-Q-TOF-MS

1. Introduction

Cereulide and valinomycin are similar, 36-membered cyclic depsipeptides having component sequences of *cyclo* (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ and *cyclo* (L-*O*-Ala-L-Val-D-*O*-Val-D-Val)$_3$, respectively. These two peptides are constructed by six peptide bonds and six ester bonds one after another and are composed of three repeating tetrapeptides. Cereulide has been known as an emetic toxin produced by *Bacillus cereus* causing an emetic-syndrome in some people for many years. The isolation of cereulide was first reported by Agata et al. [1] from this bacteria in 1994 and, subsequently, cereulide was found to cause swelling of mitochondria in HEp-2 cells [2]. Its structure was elucidated by Suwan et al. [3] in 1995 by means of NMR analysis on various metal complexes of cereulide. Valinomycin, on the other hand, had been known as an antibiotic drug produced by *Streptomyces fulvissimus* [4]. In Fig. 1, the structure of cereulide is shown in comparison to that of valinomycin. Valinomycin has been studied for its ionophoric properties with the alkali metal ion by Ivanov and coworkers [5,6], and had been shown to be a K^+ ion-selective ionophore without toxicity. Cereulide has also been found to act selectively as an ionophore with the K^+ ion [3]. The stereochemistry of cereulide was reported through alkali hydrolysis into two kinds of dipeptides, which were further determined by Marfey's method. The acid hydrolysates of the dipeptide mixture from cereulide were coupled with (5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDDA) comparison with the authentic L/D-Ala-dinitrophenyl (DNP)-L-Ala-NH_2 and L/D-Val-DNP-L-Ala-NH_2. The K^+ complex structure was determined from combination of NMR and molecular mechanic calculation to be a caged structure [3]. Its ionophoric nature was reported with alkali metal ions such as Li^+, Na^+, K^+, Rb^+, and Cs^+, among them K^+ being a highly selective guest in the complexation [3]. Cereulide was chemically synthesized in 1995

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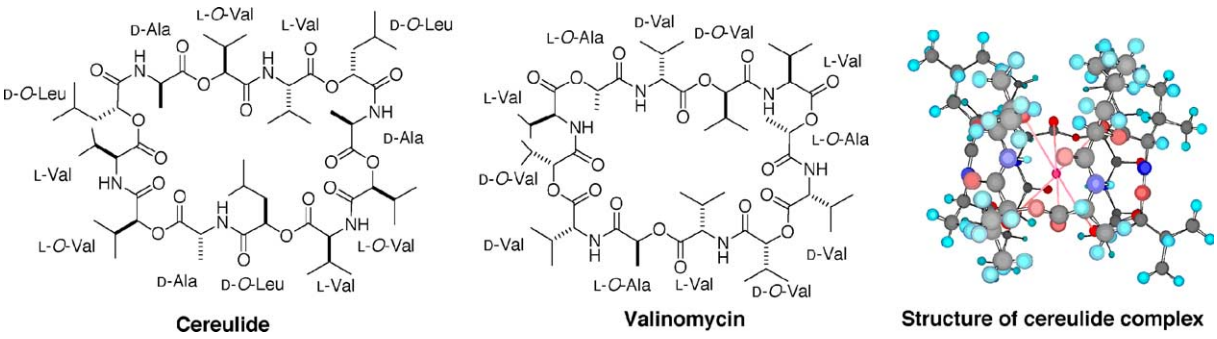


Fig. 1. Structure of cereulide, valinomycin, and main chain of their complexes.

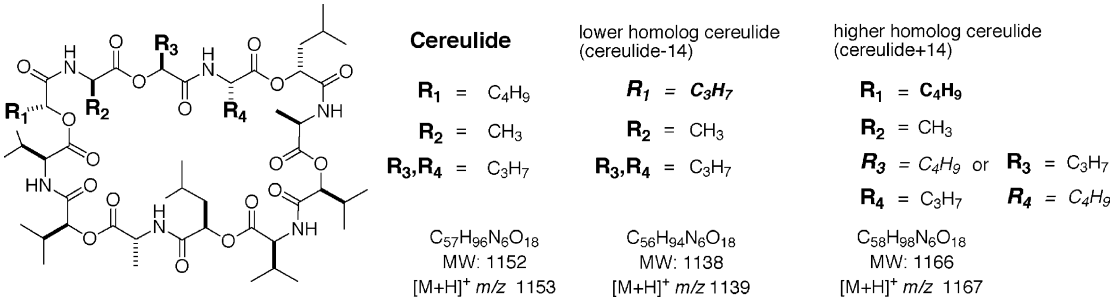


Fig. 2. Structure of cereulide and variable structures of cereulide homologs (cereulide lower homolog (MW 1138), cereulide (MW 1152), cereulide higher homolog (MW 1166).

by Isobe and coworkers [7,8], which in fact showed the same emetic, ionophoric and pathogenic activities. In 2000, a biosynthetic route of cereulide was proposed for this unusual cyclic depsipeptide toxin through NMR and electrospray ionization mass spectrometry (ESI/MS) studies in which ^{13}C-labeled (at carboxylic carbon) L-amino acid precursors (L-Val, L-Leu, L-Ala) were shown to have 95% incorporation into *O*-Val, *O*-Leu, and L-Val, while only 40% incorporation into D-Ala [9]. We have been studying the cereulide complex with various ammonium salts, both inorganic and organic, through spectroscopic methods, and the results have been reported by Pitchayawasin et al. [10].

From all previous information, including fast atom bombardment mass spectrometry analysis (FAB-MS), the molecular mass of cereulide was determined to have a mass-to-charge (*m*/*z*) value of 1152 [3]. We also reported about the solvent effect for ESI-Q-TOF-MS analysis of cereulide in the previous paper [10]. Cereulide can form the most stable complex with K^+, the same as valinomycin, but we always observe the peak cereulide–NH_4^+ at *m*/*z* 1170 and the valinomycin–NH_4^+ complex at *m*/*z* 1128 when acetonitrile is used as the solvent. Under this HPLC condition with acetonitrile as eluent, we found that the ammonium adduct is predominant instead of potassium adducts but in the case of using methanol as the solvent, potassium is still the dominant guest ion in both cereulide and valinomycin [10].

From HPLC-MS analysis of the extracted sample from *B. cereus*, wild strain NC7401 (natural strain from Agata et al.), we observed two new homologs of cereulide which have a ±14 mass units difference and have the same ionophoric properties as cereulide (Fig. 2). These results informed us that cereulide has heterogeneities in their biosynthetic pathways. This compound, thus, became more interesting for us to analyze the structure of their homologs. In addition, structural information on the cereulide homologs will provide us with an understanding about diversity in the biosynthetic pathways of bacteria. We should consider more about the identification and toxicity of cereulide and its homologs. The homologs of cereulide were studied by means of HPLC-ESI-MS and MS/MS. Determination of which amino acid has the heterogeneity proved to be very difficult even through the variable MS/MS fragmentation experiments, when we directly analyzed the intact molecules of cereulide. This was simply because random fragmentation occurred at different bonds of these cyclic peptides. In this report, we further describe the KOH hydrolysis method to give dipeptides and a combined method with nano-(micro-)-HPLC-ESI-Q-TOF-MS and MS/MS for analyses of these dipeptides, and we also state our conclusion on the component heterogeneity of cereulide.

2. Experimental

2.1. Materials and sample preparation

An isolated fraction of cereulide from *B. cereus*, strain NC7401 was obtained from Norio Agata et al. and synthetic

cereulide (was prepared by the method in reference [8]). Alkaline hydrolysis of cereulide was achieved with 1.2 M KOH (in a methanol solution) in water by heating at 50 °C for 2 h to give dipeptide components such as *O*-Leu-Ala and *O*-Val-Val together with each homolog. After neutralization, the hydrolysates were used for LC-MS analyses.

2.2. Mass spectrometry

LC-MS/MS analyses were performed on ESI-Q-TOF mass spectrometer (Micromass, Manchester, UK) connected to a nano-HPLC system via a capillary UV detector (210 nm) (JASCO, Tokyo, Japan). Chromatographic separation for

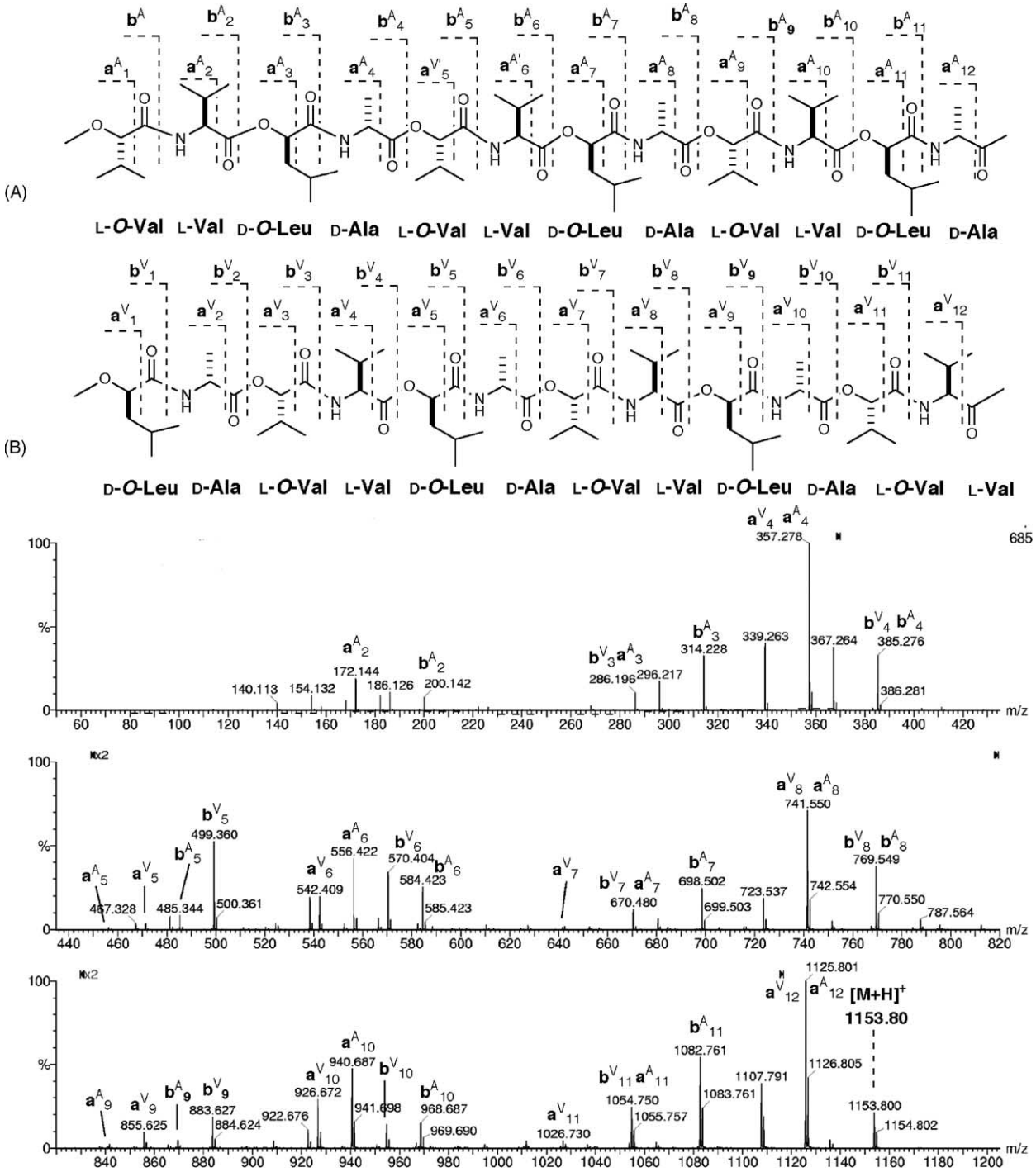


Fig. 3. MS/MS spectrum of cereulide (precursor ion at *m*/*z* 1153) in 50% MeOH–0.2% TFA by syringe injection (cone voltage 40 V, collision energy 35 eV). The calculated monoisotopic masses (*m*/*z*) are 99.07 (Val), 100.05 (*O*-Val), 71.04 (Ala), 114.07 (*O*-Leu) and 27.99 (CO). The fragmentation starts by cleavage at two ester bonds between *O*-Leu and Val (a^V, b^V series); and *O*-Val and Ala (a^A, b^A series).

cereulide was performed on Develosil ODS-HG-5 (Nomura Co. Ltd., Aichi, Japan, 0.3 mm ∅ × 50 mm which had 95% acetonitrile containing 0.1% trifluoroacetic acid as the mobile phase) and separation of hydrolyzed cereulide was performed on Develosil ODS-HG-5 (Nomura Co. Ltd., 0.3 mm ∅ × 150 mm which was equilibrated with 260 μL of water containing 0.025% trifluoroacetic acid at a flow rate 10 μL/min and then developed using a linear gradient from 0 to 30% of acetonitrile containing 0.025% trifluoroacetic acid for 40 min at a flow rate of 5 μL/min before on-line ESI-MS and MS/MS analysis).

All MS experiments were performed in the positive-ion mode. Full-scan mass spectra were recorded between 200 and 2500 mass units with a scan rate of 1 s per scan in both MS and MS/MS modes. The source temperature was 80 °C and the desolvation temperature was 100 °C. The ESI probe (needle) voltage was 3.0 kV. The ESI drying and nebulizing gases were nitrogen and the collision gas for MS/MS measurement was argon. The sample cone voltage was 40 V for the MS mode, and the collision energy was varied between 10 and 75 eV for optimal fragmentation. Data were acquired and processed by MassLynx version 3.4 (Micromass, Manchester, UK). The LC-MS and MS/MS experiments were conducted utilizing the appropriately adjusted nano-HPLC system (JASCO, Tokyo, Japan).

3. Results and discussion

As shown in Fig. 3, the precursor ion of cereulide at *m*/*z* 1153 (collision energy 35 eV), the fragmentation starts to open the ring from cleavage of the ester bond and the straight chain shows preferential fragmentation from the C-terminal; thus, Ala or Leu being the first fragmentation. The ester bond seems to be easier to break than the peptide bond. Cereulide subsequently fragments following its sequence from Ala → *O*-Leu → Val → *O*-Val or Val → *O*-Val → Ala → *O*-Leu.

Cereulide consist of three repeating tetrapeptide units forming cyclic dodecadepsipeptide structures as well as cereulide. The mixture of cereulide and valinomycin is separable by the LC-MS system with 95% acetonitrile–0.1% TFA, column ODS-HG-5 0.3 mm ∅ × 50 mm, Develosil, Nomura Co. Ltd., as shown in Fig. 4. We can observe the high intensity peak of ammonium complex ions $[M+NH_4]^+$ of cereulide and valinomycin under this condition and we also observe potassium complex ions $[M + K]^+$ of both compounds. The ammonium and potassium complex ions of cereulide are observed at *m*/*z* 1170.89 and *m*/*z* 1191.83, respectively, at retention time 8.69 min. In the case of valinomycin, we observe the ammonium complex ions at *m*/*z* 1128.83, and potassium complex ions at *m*/*z* 1149.78, at retention time 8.19 min.

From another HPLC-ESI-Q-TOF-MS analysis of cereulide and its homologs (Fig. 5), we observe the ammonium complex ion of cereulide (*m*/*z* 1170.84) at retention time 8.67 min. The ammonium complex ions of the higher homolog (*m*/*z* 1184.89) and the lower homolog (*m*/*z* 1156.86) are also observed at retention time 9.78 and 8.06 min, respectively. In Fig. 6, the MS/MS fragmentation pattern from $[M + NH_4]^+$, precursor ion at *m*/*z* 1170, after loss of NH_4^+, is as the same as the protonated ion of cereulide $[Mc + H]^+$. The MS/MS fragmentation pattern from the higher homolog of cereulide, the precursor at *m*/*z* 1184, also shows the same fragmentation pattern from Ala → *O*-Leu → Val → *O*-Val or Val → *O*-Val → Ala → *O*-Leu (Fig. 7). The MS/MS pattern of the cereulide homologs seems to be complicated. From the MS/MS

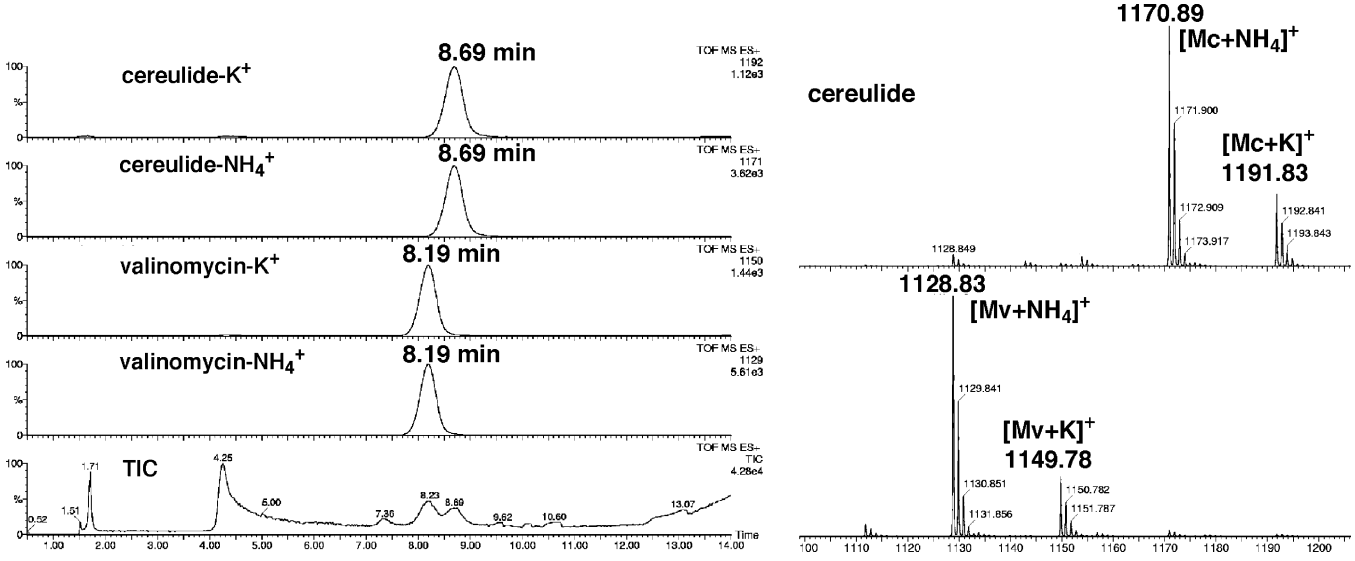


Fig. 4. Chromatogram and corresponding MS spectra of cereulide–NH_4^+ (*m*/*z* 1170.89), cereulide–K^+ (*m*/*z* 1191.83) at retention time 8.69 min valinomycin–NH_4^+ (*m*/*z* 1128.83), valinomycin–K^+ (*m*/*z* 1149.78), at retention time 8.19 min and with solvent 95% acetonitrile–0.1% TFA, a column ODS-HG-5 0.3 mm ∅ × 50 mm, Develosil, Nomura Co. Ltd. (Mv: valinomycin, Mc: cereulide).

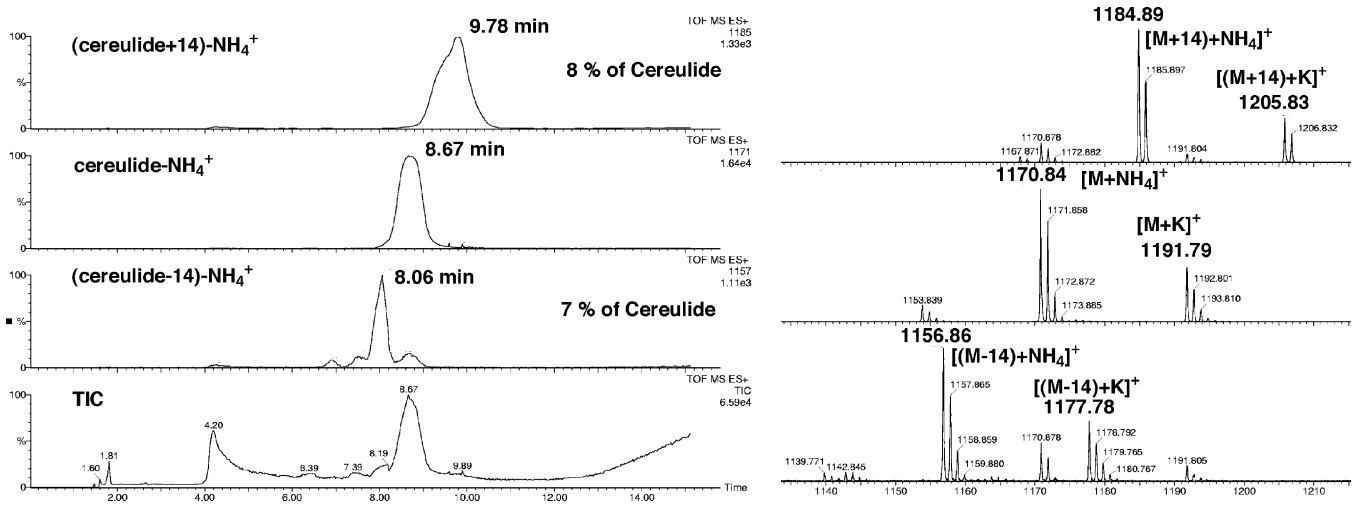


Fig. 5. Chromatogram and corresponding MS spectra of ammonium complex ions of cereulide (at *m/z* 1170.84), the higher homolog (at *m/z* 1184.89), and the lower homolog (at *m/z* 1156.86).

fragmentation pattern of the cereulide homolog, it is difficult to determine which amino acids have been substituted and are responsible for the increase (or decrease) for 14 molecular weight. We have to use another method, which is KOH hydrolysis, to hydrolyze the respective cyclic peptides at six ester bonds to give dipeptides.

Fig. 8 shows the dipeptide structures obtainable from hydrolysis of cereulide. Cereulide was hydrolyzed with KOH and the hydrolysates were acidified and analyzed by nano-HPLC-MS and MS/MS. Fig. 9 shows an ion chromatogram of the hydrolyzed cereulide following at *m/z* 204 (at 10.33 min), *m/z* 218 (at 12.28 min), and *m/z* 232 (at

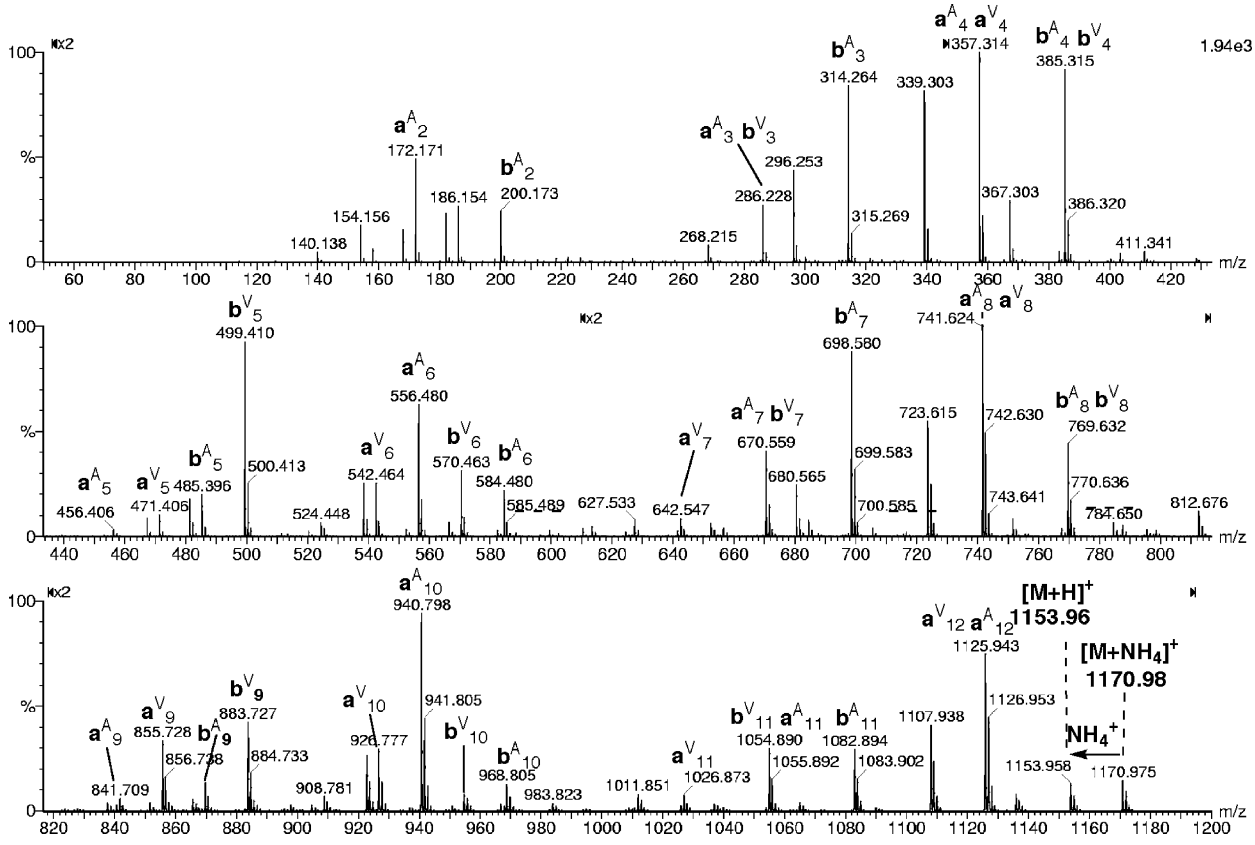


Fig. 6. MS/MS spectrum of cereulide from the precursor ion *m/z* at 1170 as NH_4^+ complex.

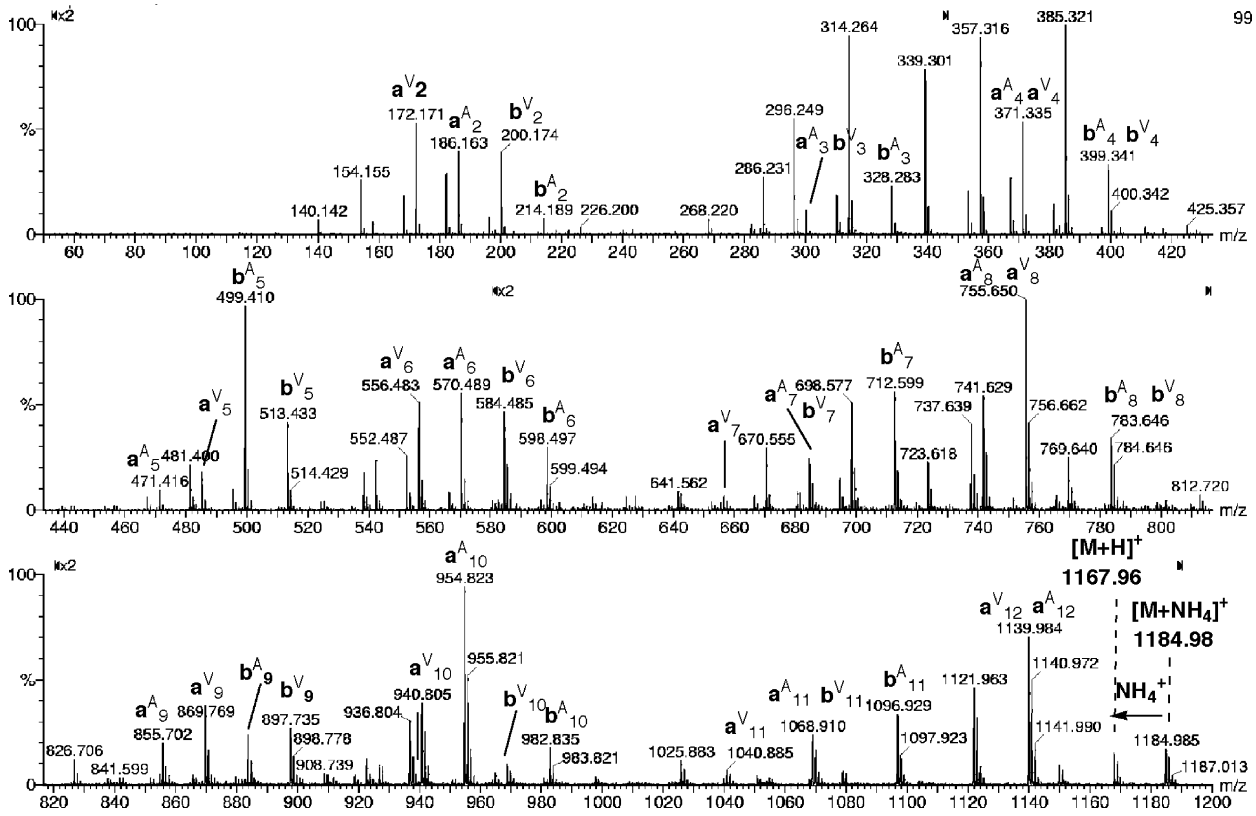
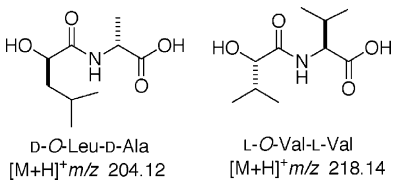


Fig. 7. MS/MS spectrum of a higher homolog (+14 mass) of cereulide from the precursor ion at *m*/*z* 1184 as NH_4^+ complex.

14.17 and 14.80 min) but not *m*/*z* 190. In the precursor ion at *m*/*z* 232 of cereulide two peaks, which have different retention times (14.17 and 14.80 min) are shown. This incident means that we have two different dipeptides, which have the same molecular ions. The structures **a**, **b**, **c**, and **d** are confirmed by MS/MS spectra of the dipeptides from hydrolyzed cereulide (Fig. 10). The precursor ion at *m*/*z* 204 is **10a** (*O*-Leu-Ala) and the precursor ion at *m*/*z* 218 is **10b** (*O*-Val-Val) from the authentic samples. The MS/MS spectrum of the precursor ion at *m*/*z* 232, the major peak (retention time 14.17 min) confirms the structure as **10c** (*O*-Leu-Val, higher homolog of **10b**) and the minor peak (retention time 14.80 min) confirms the structure as **10d** (*O*-Val-Val-OCH_3), which has a similar MS/MS fragmentation pattern to **10b** (*O*-Val-Val). **10d** might overlap with **10c** (*O*-Leu-Val) because there is the weak fragment peaks of **10c** (at *m*/*z* 214, 186, 158) in the MS/MS spectrum of **10d**. The structure of **10d** is identified as ester to have



D-*O*-Leu-D-Ala
$[M+H]^+$*m*/*z* 204.12

L-*O*-Val-L-Val
$[M+H]^+$*m*/*z* 218.14

Fig. 8. Dipeptide structures from hydrolyzed cereulide and valinomycin.

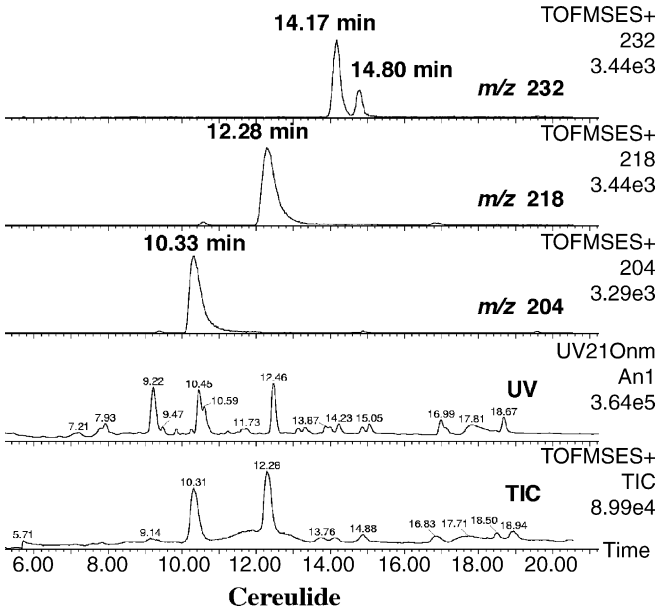


Fig. 9. HPLC-MS chromatogram and ion chromatogram of the dipeptides (obtainable from KOH hydrolysis of cereulide) using condition acetonitrile–0.025% TFA and H_2O–0.025% TFA (with a column ODS-HG-5 0.3 mm ∅ × 150 mm Develosil, Nomura Co. Ltd).

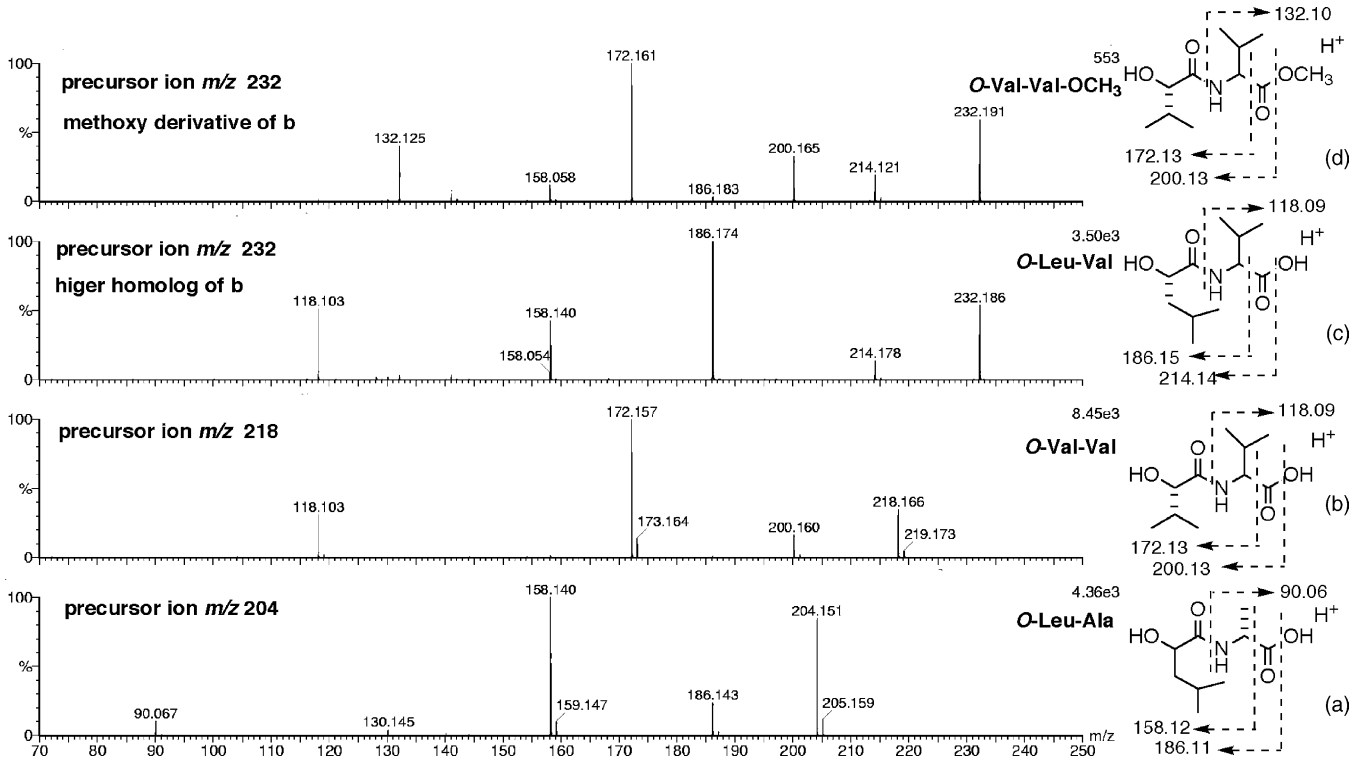


Fig. 10. MS/MS spectra and fragmentation of the dipeptides from hydrolyzed cereulide.

the methoxy group (–OCH_3) instead of the hydroxy group (–OH) as an artifact during alkali hydrolysis in methanol. From these MS/MS results of hydrolyzed cereulide, we conclude that homologation occurs at *O*-Val (to result in *O*-Leu) and *O*-Leu (to result in *O*-Val).

4. Conclusions

Cereulide from *B. cereus*, is often prepared via the ammonium sulfate precipitation process, so that the guest ions partially exchange from K^+ to NH_4^+. This is why it is always observed as an ammonium complex at *m*/*z* 1170 when the HPLC condition employs acetonitrile solvent [10]. In regard to MS/MS spectra of cereulide, this compound are preferentially cleaved at an ester bond to open the ring. Then the straight chain form is fragmented following their sequence. The homologs of cereulide, which have ±14 different mass units from cereulide, were observed from natural. To determine which amino acids are the keys of variation, we employed the KOH hydrolysis method. These dipeptides were subjected to HPLC-MS and MS/MS, and the heterogeneity was clearly shown. In the case of cereulide, variation was found to occur on *O*-Val (resulting *O*-Leu). The above heterogeneity analysis might be related to other mutations in the nature, which would be installed in the genetic codes for the production of such unusual cyclic depsipeptides.

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