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Complexation of Cyclic Dodecadepsipeptide, Cereulide with Ammonium Salts☆

Suthasinee Pitchayawasin,[a] Masaki Kuse,[a] Kazushi Koga,[a] Minoru Isobe,[a,*] Norio Agata[b] and Michio Ohta[c]

[a]*Laboratory of Organic Chemistry, School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan*
[b]*Nagoya City Public Health Research Institute, 1-11 Hagiyamacho, Mizuho, Nagoya 467-0011, Japan*
[c]*Department of Bacteriology, School of Medicine, Nagoya University, 65 Tsurumaicho, Showa, Nagoya 466-8550, Japan*

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Abstract—Cereulide is a principal toxin causing emetic syndrome which is produced by *Bacillus cereus* and has been known as potassium selective ionophore. This paper deals with its complexation with inorganic and organic ammonium ions to assign the higher structures similar to the complex with potassium ion by means of NMR and ESI-MS spectroscopy. Of particular interest, the detectable ions are not only at m/z 1191.8 as K^+ complex but also (or sometimes exclusively) at m/z 1170.8 as NH_4^+ complex in its LC–MS analyses depending upon the conditions. This difference is due to the sample preparation and measurement condition.

Cereulide is an emetic toxin produced by *Bacillus cereus* to cause emetic-syndrome to people for many years. The isolation of cereulide was first reported by Agata et al. in 1994.[1] Cereulide was found that it caused swelling of mitochondria of Hep-2 cell.[2] Its structure was elucidated by Suwan et al. in 1995[3] to be a 36-membered cyclic dodecadepsipeptide having the sequence of *cyclo* (L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)$_3$ (Fig. 1). Valinomycin had been known as K^+ ion selective ionophore but it has no emetic toxicity. Structure of valinomycin is similar to cereulide except one different amino acid and different stereochemistry from cereulide. The stereochemistry of cereulide has been established through chemical degradation and the higher structure was assigned from the NMR and molecular mechanic calculation to be a caged structure as shown in Figure 2. The ionophoric nature was reported with alkali metal ions such as Li^+, Na^+, K^+, Rb^+, Cs^+ and H^+, among them potassium ion being a highly selective guest in the complexation.[3] Cereulide was chemically synthesized in 1995 by Isobe et al., which in fact showed the same emetic, ionophoric and pathogenic activities.[4] In 2000, biosynthetic route of cereulide was proposed on this unusual cyclic depsipeptide toxin through NMR and ESI/MS (electrospray ionization mass spectrometry) studies on ^{13}C-labeled L-amino acid precursors (Val, Leu, Ala) showing high (94, 95 and 40%) incorporation when cultivated in the synthetic media.[5]

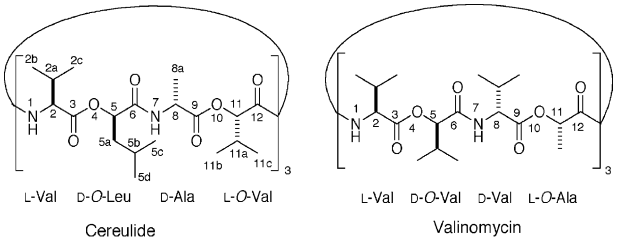

Figure 1. Structure of cereulide and valinomycin.

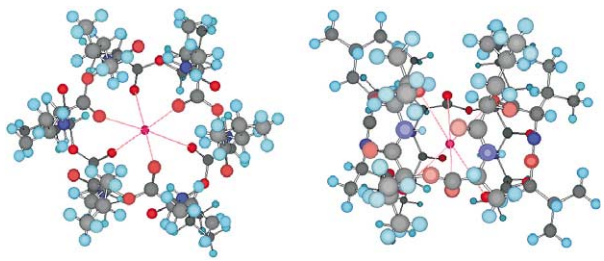

Figure 2. 3D model of cereulide–K^+ complex.

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*Corresponding author. Tel.: +81-52-789-4109; fax: +81-52-789-4111; e-mail: isobem@agr.nagoya-u.ac.jp

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We have been studying cereulide complex with various ammonium salts including inorganic and organic through spectroscopic methods, and the results are described herein.

ESI Mass Spectra of Cereulide and Valinomycin Complexes

The mass spectrum (FAB) of cereulide (MF = 1152.68) gives peaks at *m*/*z* at 1191.64 $[M+K\ (1152.7+39)]^+$, which is dominant and most important signal from LC–MS trace detection method for the emetic toxin.[3] On the other hand, when the sample was treated with ammonium sulfate solution, the peak is often observed differently at *m*/*z* 1170.71 $[M+NH_4\ (1152.7+18)]^+$. Andersson et al., in fact, reported the identification of cereulide as the active principle of spermatozoa toxin through mass spectroscopic methods.[6] They re-studied complexation of cereulide with its K^+ selective ionophoric nature[7] as we have already reported in 1995.[3] In order to evaluate the complexation between K^+ and NH_4^+, we re-examined cereulide with ESI/MS in three solutions containing different ratio of the inorganic ions.[8] Figure 3a–c shows the comparison of cereulide complex masses at *m*/*z* 1170.8:1191.8) in three different molar ratios of NH_4^+/K^+ (1:10, 1:1 and 10:1) in CH_3CN solvent. When cereulide was sprayed in the 1:10 solution, we found the peaks 10 times higher intensity of K^+ complex. With the molar ratio of NH_4^+ to K^+; 1:1 the predominant mass ion peaks were 6:10; and with the molar ratio of 10:1, the MS peak ratio changed to 10:7. This suggests that the MS signal appears at *m*/*z* 1170.8 predominantly under the K^+ lacking conditions. These results may be rationalized due to the fact that the ion radius of K^+ and NH_4^+ are similar to each other. Fig. 3d shows the different result in case of spraying solvent in MeOH for MS measurement with the molar ratios of NH_4^+/K^+ at 10:1, we found the peaks 10 times higher intensity of K^+ complex than NH_4^+ complex. These intensities confirm that cereulide is still the most K^+ selective ionophore, and stronger binding (complexation) than with ammonium ion, which forms fairly stable complex next with K^+.

Similar tendency was observed with the other famous K^+ ionophore, valinomycin (MF = 1110.63). In CH_3CN as ESI solvent having the different molar ratios of NH_4^+/K^+ (1:10, 1:1 and 10:1) in Fig. 3e–g, the observed ratios of the corresponding complex peaks at *m*/*z* 1128.7:1149.7 were 1:10, 2:10 and 10:7, respectively. In Fig. 3h, the spray in MeOH containing the molar ratio of NH_4^+/K^+ (10:1) resulted in observation of the predominant mass ion peaks of K^+ complex with 10 times higher intensity. Such a solvent effect might be due to the different equilibrium of the complexation.

NMR Spectra of Cereulide Complex

We have reported that the complex formation of cereulide with alkali metal ions is particularly detectable from the chemical shift difference of the NH and alpha protons, as well as the coupling constants. The coupling value $J\alpha/\beta$ of *O*-Leu with no guest ion was observed as triplet at 6.4 Hz, while it sprited into two different values (*dd*, 10 and 3.5 Hz) after complexation with K^+.[3] In the current studies, we found that similar complexation with inorganic ammonium was also proved from these data. In Figure 4, are summarized the selected signals of ^1H NMR (600 MHz)[8] for comparison in the presence of different salt; thus, H^+ shows those signals in acidic media (having no metal ion, guest free), K^+

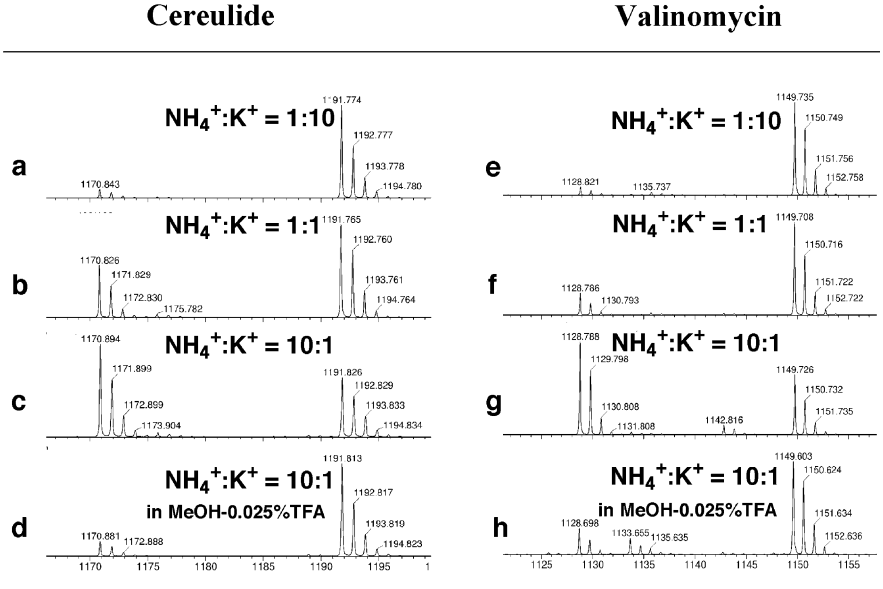

Figure 3. ESI/MS spectra of cereulide (a–d) and valinomycin (e–h) complexes with different NH_4^+/K^+ ratios (measured in acetonitrile solvent (a,b,c,e,f,g) containing 0.025% CF_3COOH, and in methanol solvent (d,h) containing 0.025% CF_3COOH.

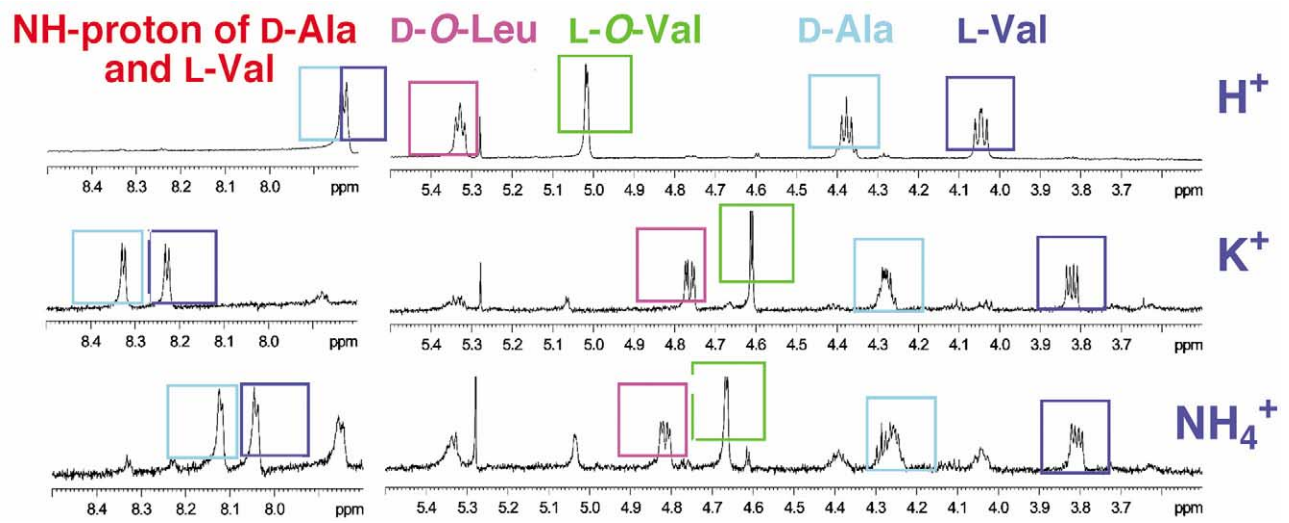

Figure 4. ^{1}H NMR spectra showing NH and α-protons of cereulide in H^+, K^+ and NH_4^+ in $CDCl_3$.

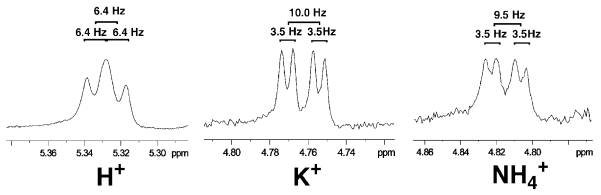

Figure 5. α-Protons coupling constant of on D-*O*-Leu of free cereulide and cereulide complexes: K^+ and NH_4^+.

indicates NH protons shifting to down field, while alpha H's shift to up field. These are consistent with our previous observation in K^+ case.[3] The spectrum of NH_4^+ was measured in $CDCl_3$ after exchanging the guest ion by treatment with NH_4SCN.[9] The chemical shifts of NH_4^+ complex were very similar to those of K^+ complex rather than 'H^+' (or other alkali metal treatments; data not shown here). For better comparison of the α-protons of *O*-Leu, these peaks are expanded in Figure 5, in which the coupling constants of NH_4^+ (dd, $J = 9.5$ and 3.5 Hz) are similar to K^+ (dd, $J = 10.0$ and 3.5 Hz) complexes, but not to the guest free form (t, $J = 6.4$ Hz). All these facts suggest that NH_4^+ bind with cereulide to form a similar higher structure as the case in K^+. The detailed data of the complexes are tabulated in Table 1, where the chemical shifts and coupling constants are quite similar to those values already reported previously.

Complexation of Cereulide with Alkyl Ammonium Ions

During the course of attempted derivatization of cereulide for application to enzyme-immunoassay (not described herein), we became interested in the possibility of complexation with organic ammonium ions such as butyl mono-ammonium ion $CH_3(CH_2)_3NH_3^+$. The sample was prepared in two steps; the first procedure was the same to make guest free form,[9] but the second was slightly changed to make the complexes due to the different solubility of the organic ammonium salts in chloroform.[10] The complexation was monitored by NMR as the case as above.

We have also tried the di-ammonium alkyls such as butyl and hexyl di-ammonium salts, $NH_3^+(CH_2)_4NH_3^+$ and $NH_3^+(CH_2)_6NH_3^+$. The complexes were prepared similarly[11] and the complexation was monitored through NMR and MS spectra. The chemical shifts of NH's and alpha-H's of these three complexes of cereulide with organic ammonium ions appeared at the similar position as inorganic ammonium complex cases as shown in Figure 6. The coupling constants of *O*-Leu were not recordable due to overlap of the signals with ammonium ions.

The Q-TOF-MS spectra of these three complexes of cereulide are shown in Figure 7. The molecular peak of

Table 1. Chemical shifts and coupling constants of NH and α-protons of cereulide complexes

Residue	Assignment (multiplicity)	Chemical shift in δ and *J* (Hz)		
		H^+	K^+	NH_4^+
L-Val	1NH (d)	7.85 (7.5)	8.23 (5.0)	8.04 (5.0)
	$2_{\alpha\beta}$ (dd)	4.04 (10.0, 7.5)	3.82 (10.5, 5.0)	3.80 (11.0, 5.0)
D-*O*-Leu	$5_{\alpha\beta}$ (dd)	5.33 (6.4, 6.4)	4.76 (10.0, 3.5)	4.82 (9.5, 3.5)
D-Ala	7NH (d)	7.83 (7.5)	8.32 (4.6)	8.12 (3.9)
	$8_{\alpha\beta}$ (qd)	4.38 (6.8, 6.8)	4.28 (7.4, 4.5)	4.26 (7.3, 3.9)
L-*O*-Val	$11_{\alpha\beta}$ (d)	5.02 (2.9)	4.61 (3.3)	4.66 (3.3)

the complex was observed at m/z 1226.79 $[M+CH_3(CH_2)_3NH_3^+$ (1152.7 + 74)]. In case of the diammonium salts such as $NH_3^+(CH_2)_4NH_3^+$ and $NH_3^+(CH_2)_6NH_3^+$), the ions appeared as divalent ions at m/z 621.41 $[M+NH_3^+(CH_2)_4NH_3^+$ (1152.7 + 90)/2 = 1242/2] and at m/z 635.47 $[M+NH_3^+(CH_2)_6NH_3^+$ (1152.7 + 118)/2 = 1270/2], respectively. In all cases, the inorganic ammonium complexes appeared at m/z 1170.79 $[M+NH_4]^+$ together at m/z 1191.72 $[M+K]^+$ existing as complexes with contaminants in the reagent.

The Q-TOF-MS spectra of the three complexes of valinomycin are also depicted in Figure 8. The complexs of three organic ammonium ions showed the monovalent peak at m/z 1184.73 $[M+CH_3(CH_2)_3NH_3^+$ (1110.6 + 74)], and the divalent ions appeared at 600.41 $[M+NH_3^+(CH_2)_4NH_3^+$ (1110.6 + 90)/2 = 1200.6/2] and at 614.38 $[M+NH_3^+(CH_2)_6NH_3^+$ (1110.6 + 118)/2 = 1228.6/2]. In addition to the above assigned peaks of the divalent ions, monovalent peaks were confirmed with valinomycin at m/z 1199.75 and 1227.75. These results of ESI-MS implied that both cereulide and valinomycin have made rather stable complexes with the alkyl ammonium ions and alkyl diammonium ions. From butyl diammonium ion of valinomycin in Figure 8. we also observed the divalent peak of valinomycin:butyl diammonium ion (molar ratio = 2:1) at m/z 1155.72 [2 $M+NH_3^+(CH_2)_4NH_3^+$ $((2\times1110.6)+90)/2 = 2311.2/2$] which showed that both side of ammonium ions in alkyl diammonium ions formed the good complexes under an

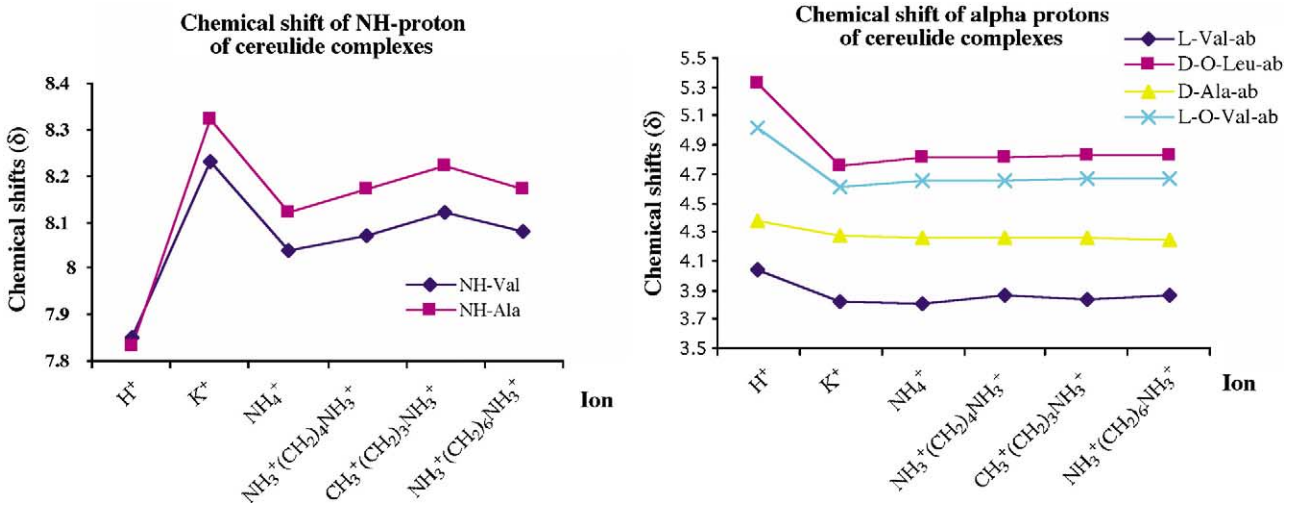

Figure 6. Chemical shift changes of NH-protons (left) and α-protons (right) in cereulide.

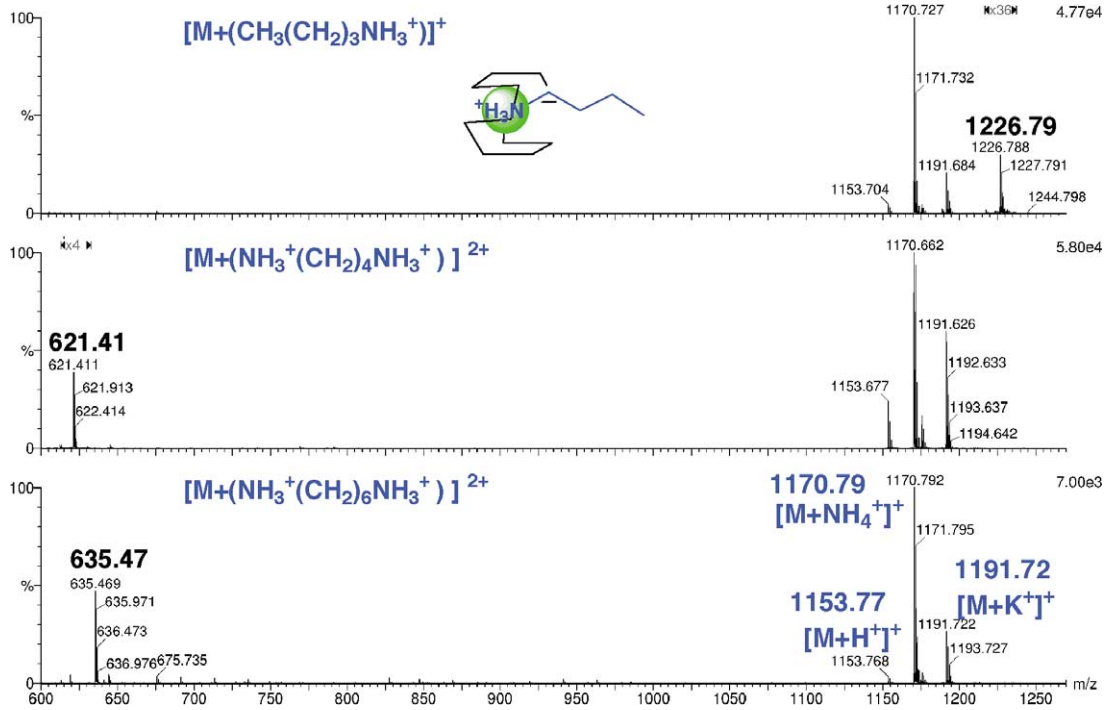

Figure 7. ESI-MS spectra of cereulide complexes (1–10 pmol/μL): butyl ammonium (monovalent peak at m/z 1226.79), butyl 1,4-diammonium (divalent peak at m/z 621.41), hexyl 1,6-diammonium (shown divalent peak at 635.47), NH_4^+ (monovalent peak at m/z 1170.79) and K^+ (monovalent peak at m/z 1191.72). All samples were measured in acetonitrile solvent containing 0.025% CF_3COOH.

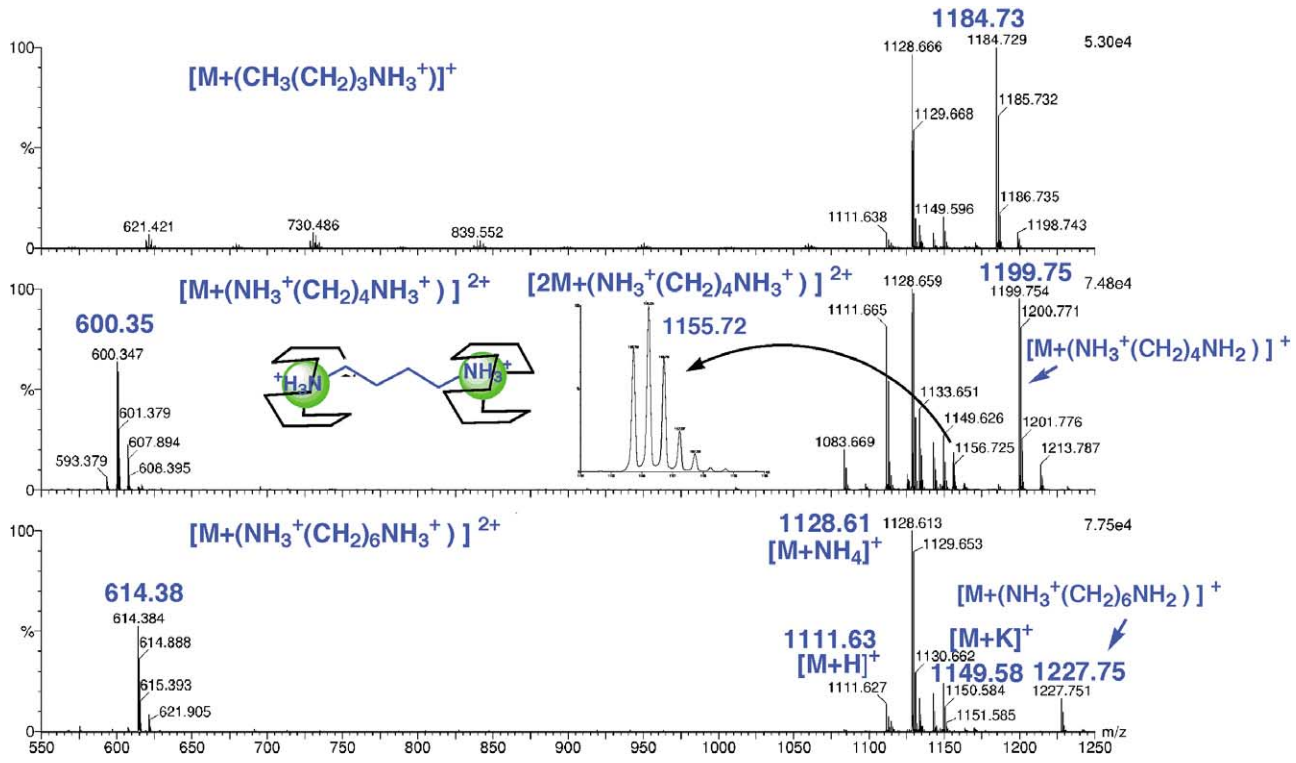

Figure 8. MS spectra of valinomycin complexes (1–10 pmol/μL): butyl ammonium (monovalent peak at *m*/*z* 1184.73), butyl 1,4-diammonium (shown divalent peak at *m*/*z* 600.35), divalent peak of valinomycin/butyl 1,4-diammonium (2:1) at *m*/*z* 1156.22, hexyl 1,6-diammonium (shown divalent peak at *m*/*z* 614.38), NH_4^+ (shown monovalent peak at *m*/*z* 1128.61) and K^+ (shown monovalent peak at *m*/*z* 1149.58). All samples were measured in acetonitrile solvent containing 0.025% CF_3COOH.

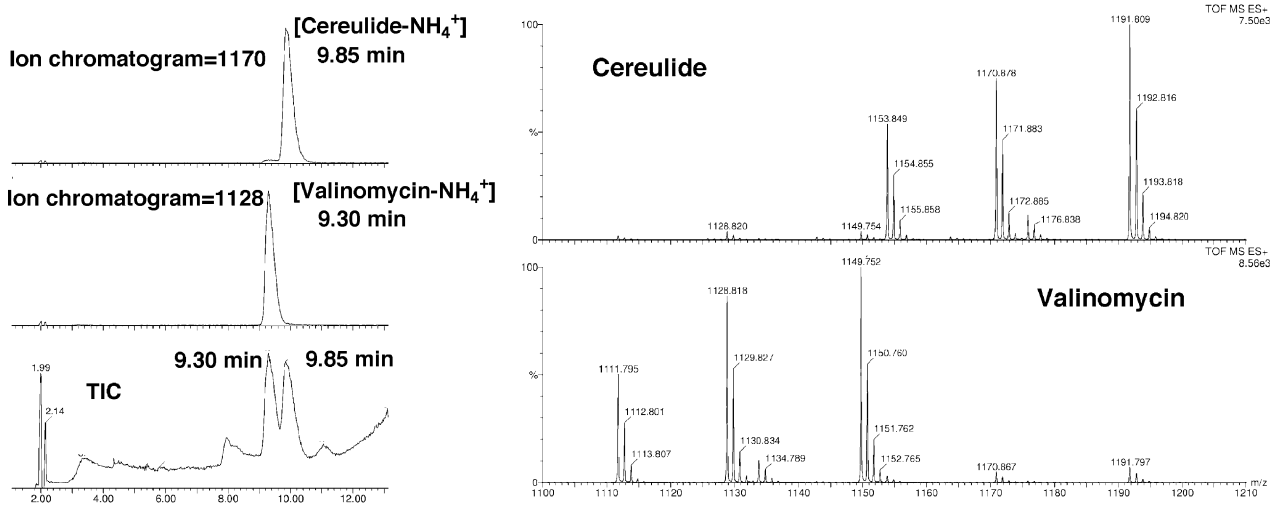

Figure 9. Micro-HPLC-MS analysis of cereulide and valinomycin: (a) HPLC chromatogram and ion chromatogram (column, ODS-HG-5, Develosil, Nomura Co. Ltd.; size, 0.3 mmΦ×5 cm; eluent, 95% acetonitrile containing 0.1% CF_3COOH; flow rate, 10 μL/min); (b) MS spectra of cereulide and valinomycin.

excess valinomycin condition. The alkyl diammonium ions is interest in utilize this complexation for further derivatization of these ionophores. Further progress of derivatization of cereulide will be reported elsewhere in future.

Conclusion

We have confirmed by spectroscopic methods that cereulide is K^+ selective ionophore and it forms fairly stable complex with inorganic ammonium ion as well. NMR studies provided evidence of the higher structure of cereulide ammonium complex being very similar to that of potassium complex. Since cereulide has been identified as emetic toxin of food born illness as well as spermatozoa toxin, the detection should be as rapid and as sensitive as possible. Micro(nano)-LC-ESI-Q-TOF/MS and MS/MS method is one of the best methods to allow detection of cereulide in femt mol level as examplified in Figure 9. The ammonium complex of this cyclic peptide potentially provide problems of its apparent molecular weights different from its own molecular ion

at m/z 1152, but also m/z 1170 and 1191 for NH_4^+ and K^+ complexes, respectively. The best method should be detecting not only one of these values, but also to detect them all ion chromatograms in femt mol quantity to make sure appearing at the same retention time.

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8. **Instrumentation**: MS spectra were measured with a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray type ESI ion source. All experiments were performed in the positive ion mode. NMR spectra were recorded with a Bruker AMX-600 spectrometer in $CDCl_3$.
9. Ammonium ion complex of cereulide: A solution of 254 μg of cereulide (0.22 μmol) in 0.6 μL of $CDCl_3$ (0.37 mM) was treated by trifluoroacetic acid (TFA) to make uncomplexed (guest free) form. To chloroform layer was added a solution of 500 μL ammonium thiocyanate (1 M) and vortexed for 2 min and kept for 30 min. Then the chloroform layer was transfered to an NMR tubing.
10. ***n*-Butyl ammonium complex**: To a solution of TFA (130 μL, 0.80 M) in $CDCl_3$ was added *n*-butylamine (200 μL, 0.75 M) in $CDCl_3$. The pH of the mixture was adjusted to 7. Then 300 μL of methanol and 300 μL of $CDCl_3$ were added to give clear solution of *n*-butyl ammonium ion (0.16 M). Then 4 μL of 0.16 M *n*-butyl ammonium ion was added to 500 μL of 0.58 mM uncomplexed cereulide in $CDCl_3$. Addition of MeOH (100 μL) made a clear solution, which was kept at 5 °C overnight. It was dried in vacuum and dissolved in $CDCl_3$ (500 μL). (cereulide/*n*-butyl ammonium ion = 1:2).
11. **1,4-Diammoniumbutane complex**: To a solution of TFA (57 μL of 0.54 M) in $CDCl_3$ was added 1,4-diaminobutane (23 μL of 0.50 M in $CDCl_3$). The pH of the mixture was adjusted to 7. Then 50 μL of methanol was added to give clear solution of 1,4-diamimoniumbutane ion (0.090 M). Then 6 μL of 0.090 M 1,4-diamimoniumbutane ion was added to 500 μL of 1.0 mM uncomplexed cereulide in $CDCl_3$ and the mixture was kept at 5 °C for 3 days. The mixture was treated as *n*-butyl ammonium complex: (cereulide/1,4-diamimoniumbutane ion = 1:1).