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High Incorporation of L-Amino Acids to Cereulide, an Emetic Toxin from *Bacillus cereus*†

Masaki Kuse,[a] Thomas Franz,[a] Kazushi Koga,[a] Sathorn Suwan,[a,b] Minoru Isobe,[a,*] Norio Agata[c] and Michio Ohta[d]

[a]*Laboratory of Organic Chemistry, School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan*
[b]*Sci. & Techno. Res. Equip. Center, Chulalongkorn University, Chulalongkorn Soi 62, Phaya-Thai Rd., Bangkok 10330, Thailand*
[c]*Nagoya City Public Health Research Institute, 1-11 Hagiyamacho, Mizuho, Nagoya 467-0011, Japan*
[d]*Department of Bacteriology, Nagoya University School of Medicine, 65 Tsurumaicho, Showa, Nagoya 466-8550, Japan*

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Abstract—Cereulide is a principal toxin causing emetic syndrome produced by *Bacillus cereus*. This paper deals with biosynthetic studies on this unusual cyclic depsipeptide toxin from ^{13}C labeled L-amino acid precursors (Val, Leu, Ala) upon cultivation in synthetic media. The analyses were made at atomic level of the constituent amino- or oxy-acids through NMR and ESI-MS/MS spectroscopic methods on cereulide and its hydrolysate dipeptides. The incorporation of the ^{13}C atom was 95% in each *O*-Val, *O*-Leu and L-Val, while 40% in D-Ala of cereulide.

Bacillus cereus is known to cause two different types of food poisoning, a diarrheal-type syndrome and an emetic-type syndrome.[1] Most of the food poisonings caused by *B. cereus* are of the latter type. Cereulide has been elucidated to have the structure as **1**, a cyclic dodecadepsipeptide, with the 12 stereogenic centers.[2] Stereochemistry was shown from alkaline hydrolysate dipeptides; D-*O*-Leu, D-Ala, L-*O*-Val and L-Val. It is a strong K^+ ionophore similar to valinomycin. It weakly binds Li^+, Na^+, Cs^+ as well, but Rb^+ bounds strongest than any other alkali metal ions.[2] Cereulide takes a higher structure such as **2** from NMR and molecular mechanic calculations (Fig. 1).[2] Chemical synthesis of cereulide has been achieved with stereoselectivity.[3] This toxin causes problems to mitochondria in various tissues.[1,4] We became interested in the biosynthetic pathways of this toxin for a future program to avoid food poisoning. Biosynthetic studies of similar dodecadepsipeptide, valinomycin, were performed using radio active precursor.[5] One of the authors, Agata, examined the growth and toxin production by *B. cereus* in a fully synthetic medium comprising from CADM (mixture of amino acids) and sucrose. They found that (i) it allowed the production of cereulide **1** and (ii) three amino acids, Val, Leu and Thr were essential.[6] More studies on the biosynthesis of cereulide **1** may be necessary to prompt searching a method for prevention from food born illness. Judging from the structure of **1**, the precursors of D-Ala, L-*O*-Val and D-*O*-Leu would relate to the amino acids, L-Ala, L-Val and L-Leu.

In this experiment we employed the synthetic medium having three labeled amino acids, L-Val, L-Leu and L-Ala (0.1 g/L having each 99% enriched ^{13}C at the carboxylic carbon atom, Isotec Inc., USA), all other 15 amino acids (0.1 g/L), K_2HPO_4 (5 g/L) and $MgSO_4{\cdot}7H_2O$ (0.05 g/L). It was cultured at 30 °C for 24 h with constant shaking at 200 rpm.[6] We obtained ca. 2 mg of labeled cereulide having the same toxicity under this condition and used for the following analytical studies. ^1H NMR of this sample was identical with authentic spectrum except the effects of ^{13}C isotopes. Its ESI mass spectrum appeared in 7 peaks centered at *m*/*z* 1201.48 rather than the usual M + K at *m*/*z* 1191.55 (Fig. 2); thus, average 10 mass units higher due to high incorporation of ^{13}Cs. High incorporation of the ^{13}Cs into cereulide molecule **1** prompted us of measuring its ^{13}C NMR spectrum of the K^+ complex in $CDCl_3$ to obtain the spectrum as in Figure 3. The signals at 171.4 (L-*O*-Val), 172.2 (D-*O*-Leu), 175.7 (L-Val) and 176.2 (D-Ala) were assigned through its C–H correlation. Relative intensity of the three carbons (C3, C9 and C12) of L-*O*-Val, D-*O*-Leu and L-Val was more than twice as much as that of C6 D-Ala.

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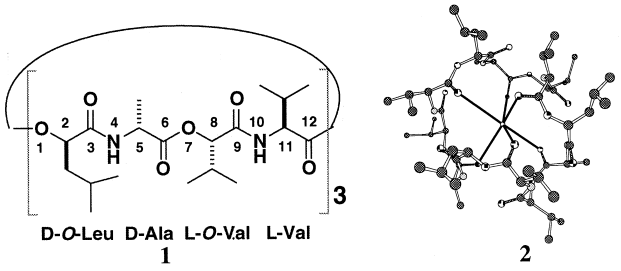


Figure 1. Structure of Cereulide and its 3D model incorporating potassium ion.

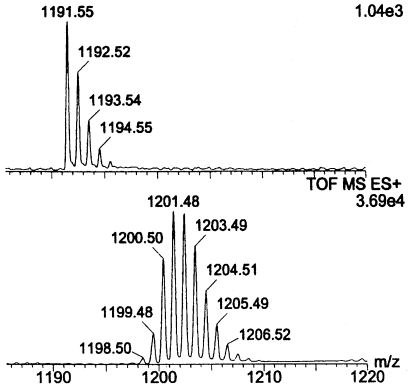


Figure 2. ESI-mass spectrum of cereulide-K^+ complex upper trace. Natural isotope abundance lower trace, ^{13}C enriched cereulide.

Two decoupling experiments showed the N–H signals of L-Val (8.22 ppm) and D-Ala (8.32 ppm) as in Figure 4; namely, both of the α protons at 3.82 and 4.27 ppm were respectively irradiated to result in changing from each triplet into each doublet. Figure 4 also illustrates that both of the coupling constants are 3.9 Hz with the ^{13}C of L-*O*-Val and D-*O*-Leu, respectively. These facts suggested that the uptake of ^{13}C carboxylic carbon into D-*O*-Leu and L-*O*-Val was nearly 100%.

Similar decoupling experiments were achieved with the β proton irradiation of the four constituted amino- or oxy-acids. Clear results were obtained with the α protons of *O*-Val at 4.61 ppm by irradiating its β-H at 2.30 ppm to change from triplet into doublet ($J=$ 3.8 Hz coupling with ^{13}C of *O*-Val) as shown in Figure 5. Irradiation of the β-H of L-Val at 2.24 ppm changed the α proton of Val at 3.82 ppm from quintet into br-triplet ($J=5$ Hz). Both of these results suggested the high incorporation of the carbonyl carbon of the ^{13}C precursor amino acids to L-*O*-Val and L-Val. Indefinite results of ^{13}C incorporation, on the other hands, were the cases with Ala and *O*-Leu (right side of Fig. 5). Irradiation of the methyl signal of Ala at 1.47 ppm caused its α-H (4.27 ppm) as a mixture of apparent doublet and triplet with $J=4$ and 5 Hz, respectively, meant that the incorporation ratio to the carbonyl carbon was not very high (far from 100%). Irradiation of the β-Hs of *O*-Leu around 1.84 ppm was unsuccessful due to their broad band nature. It was, however, ^{13}C NMR spectrum (Fig. 3) and decoupling experiments (Figs. 4 and 5) are the evidence of the nearly 100% incorporation of ^{13}C amino acid precursors into the three components (*O*-Leu, Val and *O*-Val) of cereulide.

Percent incorporation of ^{13}C was quantified by mass spectrometry from cereulide hydrolysate; thus, the two dipeptides were obtained by an alkaline hydrolysis of cereulide (**1**) with 0.1 N KOH (or 1 N NH_4OH) at rt for 30 min.[2] The hydrolysate dipeptides, D-*O*-Leu-D-Ala and L-*O*-Val-L-Val, were analyzed by means of ESI (electrospray ionization)-MS/MS measurement on a Q-TOF mass spectrometer (Micro Mass Co. Ltd, Manchester, UK). To do this, stock of the above digested sample solution (natural isotope abundance and ^{13}C incorporated samples) were diluted to each working solution of 10∼100 pmol/μL in 99.8% methanol:0.2% formic acid before being electrosprayed at 5 μL/min. On the other hand, another digested sample for proton/deuterium (H/D) exchange experiments (vide supra) was

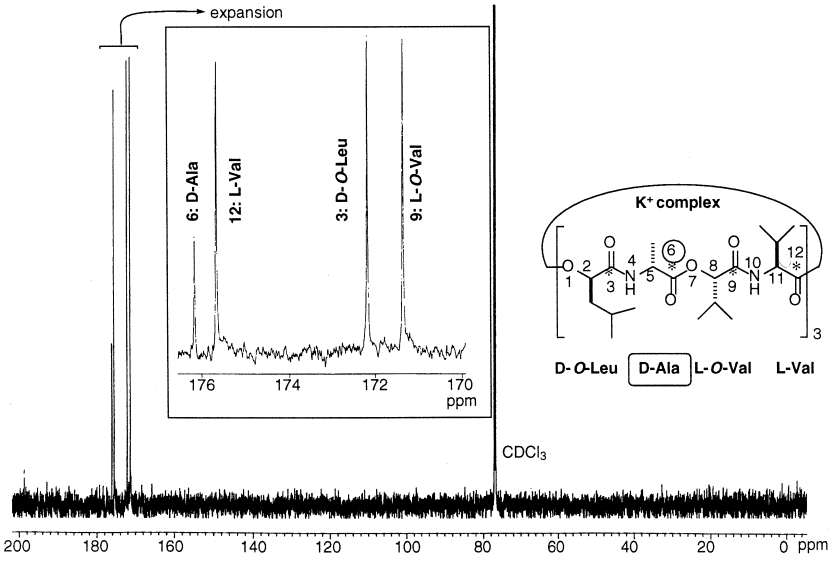


Figure 3. ^{13}C NMR spectrum of cereulide-K^+ complex enriched at the 4 carboxyl or carboamide atoms (*). Brucker AMX-600, 150 MHz for ^{13}C at 300 K.

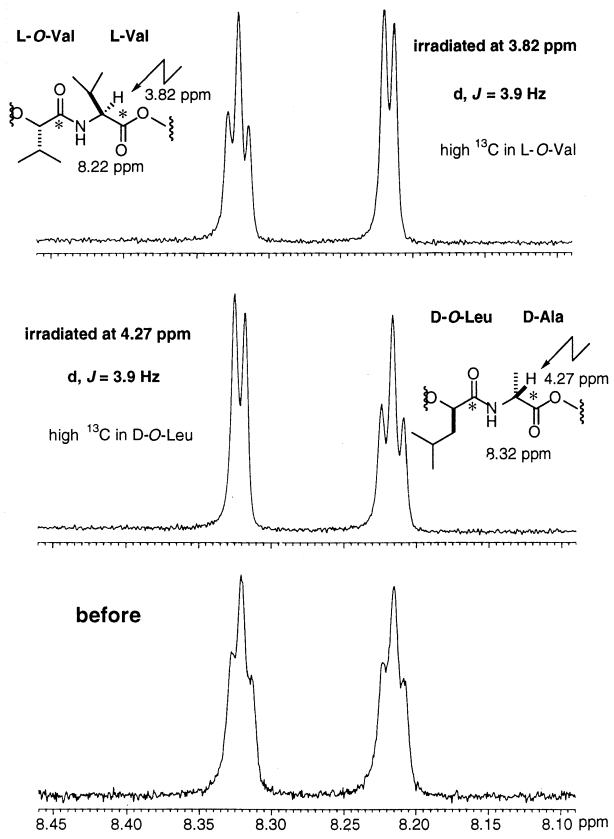


Figure 4. Decoupled NHs by irradiating α-protons.

prepared as such that the hydrolysate solution was completely dried in vacuo then re-dissolved with 99% CH_3OD:1% CH_3COOD.

Protonated dipeptide ions (**2**, **3**) can be seen at *m*/*z* 204 and 218; each of which was employed as the precursor ion for MS/MS measurements (sample cone 20 V, collision 12 V), and the results are shown as in Figure 6(A) for D-*O*-Leu-D-Ala (**2**) and in Figure 7(A) for L-*O*-Val-L-Val (**3**), respectively. The product ions of these natural abundance isotopes are seen at *m*/*z* 186, 158 and 90 from the *m*/*z* 204 precursor, and at *m*/*z* 200, 172, 118 and 72 from the *m*/*z* 218 one. Assignment of these fragments are shown in Table 1. The interesting steps are the loss of C=O (28 unit) corresponding to the carboxylic carbon, which can tell the incorporation ratios of the ^{13}Cs. In Figures 6(B) and 7(B) are found the similar MS/MS spectra of the dipeptides from ^{13}C incorporated cereulide from the precursor ions at *m*/*z* 205 + 206 and *m*/*z* 220. In Figure 6(B) the fragment sets of *m*/*z* 187 + 188 as well as 90 + 91 appear as doublet, while *m*/*z* 159 being almost singlet. The intensity ratio of *m*/*z* 159 to 158 (3480:123) represents the incorporation ratio of D-*O*-Leu being 95%. Similarly the ratio of *m*/*z* 91 to 90 (2040:3459) represents the value of D-Ala being 40%. Figure 7(B) also shows the results of 95% incorporation of L-*O*-Val and 95% of L-Val through the dipeptide L-*O*-Val-L-Val (**3**).

For validation of the above product ions generated under MS/MS collision process, deuteration of all the

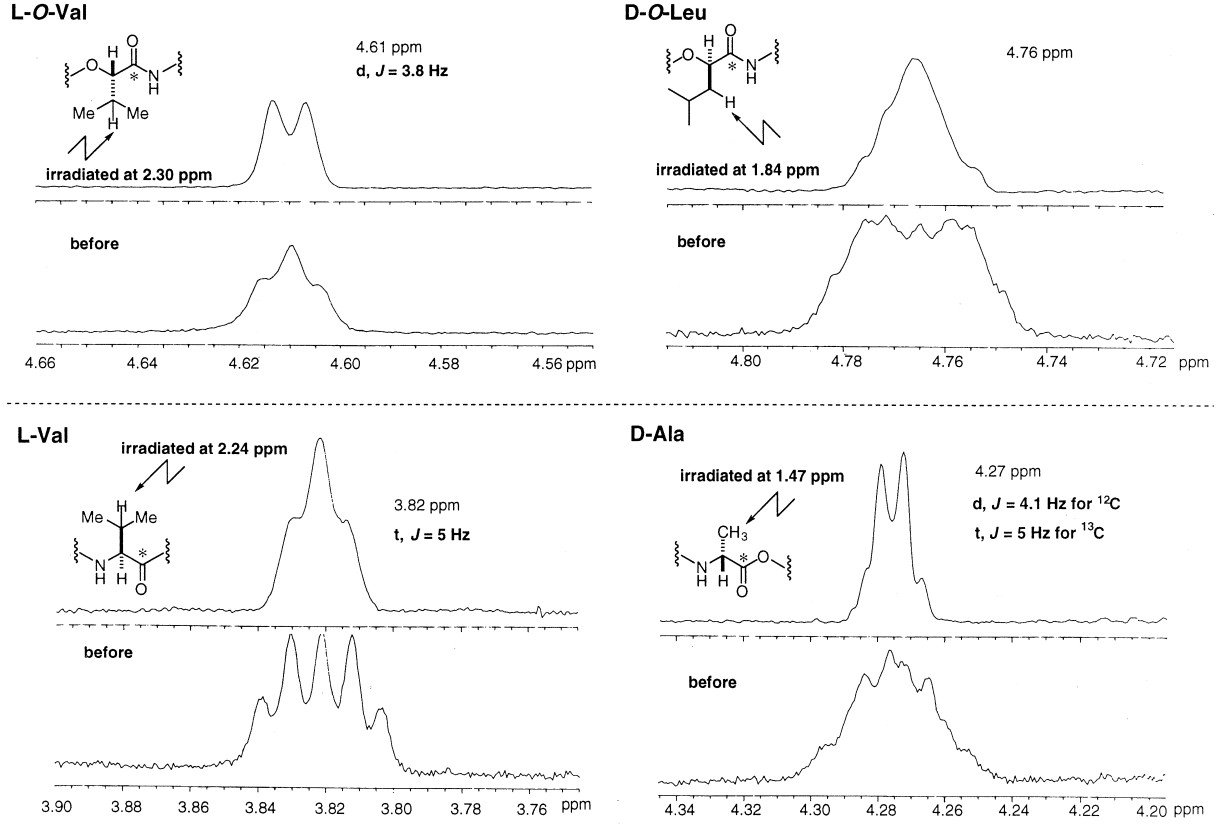
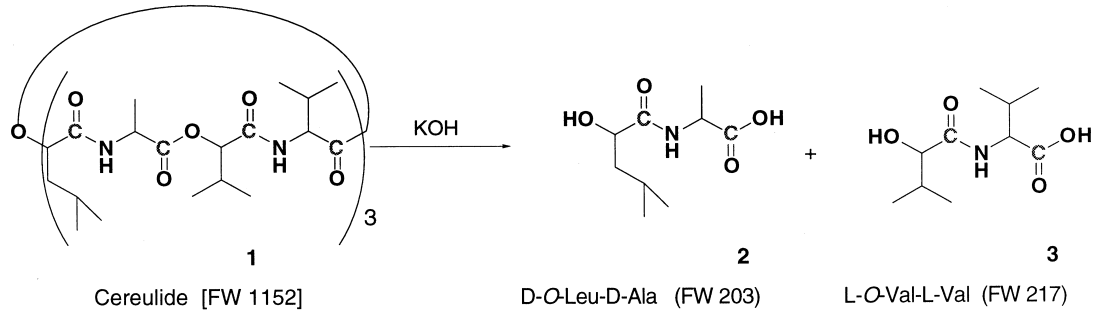


Figure 5. Decoupling experiments of α protons of the four components by irradiating β protons.



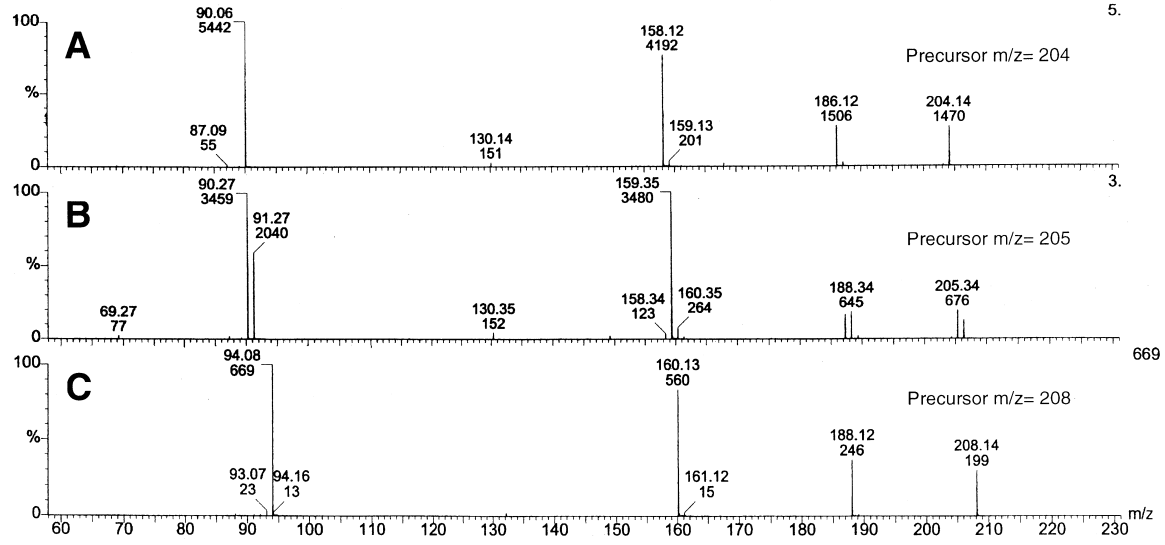


Figure 6. MS/MS spectra of D-*O*-Leu-D-Ala which have a formula weight 203: (A) natural isotope abundance precursor *m*/*z* 204; (B) ^{13}C incorporated sample precursor *m*/*z* 205–206; (**C**) deuteriated sample of the exchangeable protons of the natural isotope sample, precursor *m*/*z* 208.

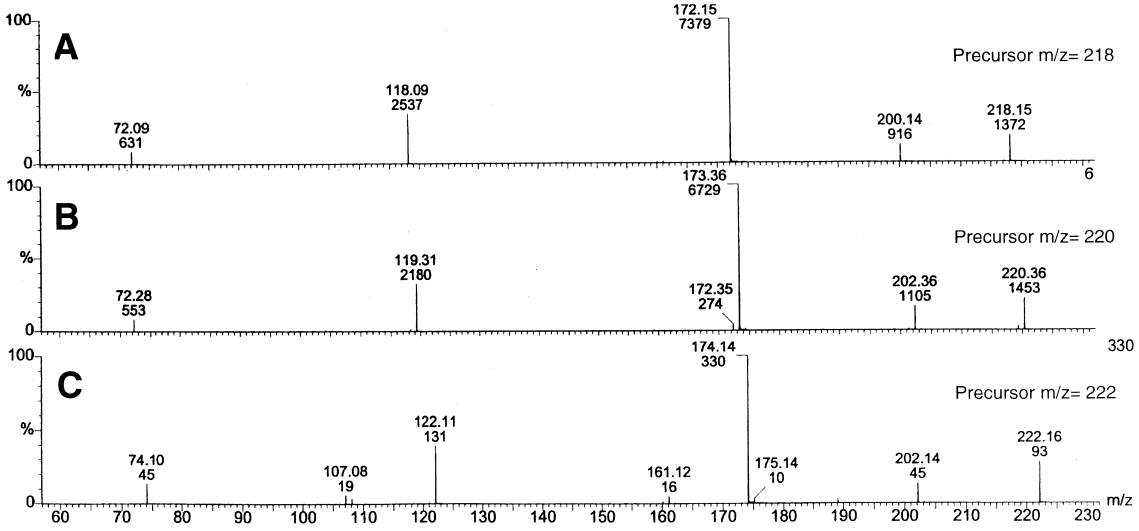
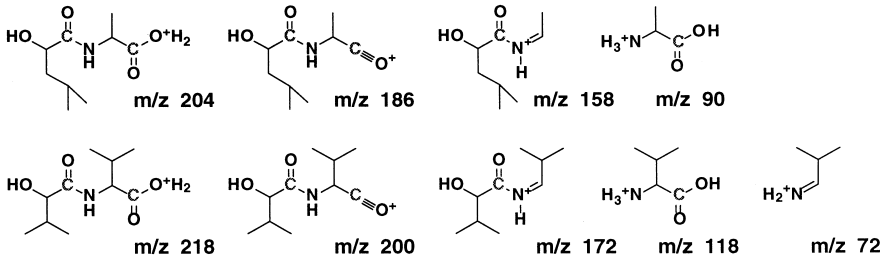


Figure 7. MS/MS spectra of L-*O*-Val-L-Val which have a formula weight 217: (A) natural isotope abundance precursor *m*/*z* 218 (M + H); (B) ^{13}C incorporated sample precursor *m*/*z* 220; (C) deuteriated sample of the exchangeable protons of the natural isotope sample, precursor *m*/*z* 222.

exchangeable protons, as shown in Figures 6(C) and 7(C), made mass increase of the natural isotope abundance precursor ions, *m*/*z* 204 and 218, to *m*/*z* 208 and 222, respectively. The added 4 or 2 mass units to the product ions also provided evidence of their structures as in Table 1. A set of four substructures from *O*-Leu-Ala (**2**) as well that of five substructures from *O*-Val-Val (**3**) well explain the numbers of protonation by deuteriation.

These mass spectrometric experiments as well as the above NMR studies gave conclusion that the enriched

Table 1. MS/MS product ions from the dipeptides: L-*O*-Leu-L-Ala and L-*O*-Val-L-Val[a]



Dipeptide precursor ions	D-*O*-Leu-D-Ala product ions				L-*O*-Val-L-Val product ions				
A (*m*/*z*) M + H	204	186	158	90	218	200	172	118	72
B (*m*/*z*) M + H	205	187	159	90	220	202	173	119	72
	206	188		91					
% of ^{13}C uptake			95	40			95	95	0
C (*m*/*z*) M + D_n	208	188	160	94	222	202	174	122	74
n (exchanged Ds)	4	2	2	4	4	2	2	4	2

[a](A) Natural isotope abundance precursors (204 and 218); (B) ^{13}C incorporated sample precursor (205, 206 and 220); (**C**) deuteriated sample of the exchangeable protons *n* of natural isotope abundance.

precursors, L-Leu, L-Ala and L-Val, have been incorporated into the carboxylic carbon atoms of the four constituents of cereulide, D-*O*-Leu-D-Ala-L-*O*-Val-L-Val in 95, 40, 95 and 95%, respectively.

L-Leu and L-Val are now proven to be definite precursor for the biosynthesis of cereulide, since these two amino acids are essential for *B. cereus*. As one of the possibilities, it may be speculated that all three L-amino acids might be once convert into α-keto acids, and then reduced into D-*O*-Leu and L-*O*-Val, or transaminated to D-Ala. In this case only the pyruvic acid might be diluted due to high amount stock, since L-Ala is not essential to *B. cereus*.[6] These speculation can explain the high (95%) incorporation to D-*O*-Leu, L-*O*-Val and L-Val, and fair (40%) incorporation to D-Ala, which should be further supported in the future experiments.

Biosynthetic studies might help to establish preventing the production of the toxin in the near future, or such analytical methods could help an identification of the toxin cereulide in nature even now. Further studies are necessary along this line.

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