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Chemical structure of hydrolysates of cereulide and their time course profile

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ABSTRACT

Hydrolysates of an emetic toxin cereulide were found in the broth of *Bacillus cereus*. The ester cleaved depsipeptides of cereulide were synthesized using liquid phase fragment condensation method starting from commercially available amino acids. The chemical structure of hydrolysates was verified tetradepsipeptide L-*O*-Val-L-Val-D-*O*-Leu-D-Ala and dodecadepsipeptide $(D\text{-}O\text{-Leu-D-Ala-L-}O\text{-Val-L-Val})_3$ using LC-TOFMS. Quantitative analysis of cereulide in the broth revealed production of cereulide in the stationary phase and decomposition in the death phase. The increase in tetradepsipeptide continued after the stationary phase until decomposition occurred.

Bacillus cereus is a bacterium that lives in soil. It forms heat-resistant spores, so it can grow after being cooked in contaminated food, causing food poisoning. *Bacillus cereus* can cause two types of food poisoning: an emetic type and a diarrhea type.[1] *B. cereus* strains that cause the emetic

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type of food poisoning produce cereulide.[2] Cereulide is resistant to heat even at 126 °C and does not decompose during food processing.[3–5] In addition, cereulide has been reported to be resistant to enzymes such as peptidase and pepsin.[3–10]

Cereulide is a K^+ ionophore that damages the cell membrane potential,[11] resulting in expansion of mitochondria in the cell and formation of vacuoles in the protoplasm of sensitive cells, causing apoptosis.[12–14] Cereulide causes vacuolation in HEp-2 cells, which are from a human laryngeal cancer cell line,[10,15] and this effect is used to determine toxicity. Cereulide has homologues and these compounds also cause HEp-2 vacuolation.[16–19]

Incubation at room temperature (21 ± 1 °C, Helsinki) is suitable for producing cereulide, and the production at 8 °C or 40 °C is only a few percent that at room temperature.[20] Cereulide is produced in the stationary phase that occurs after the logarithm phase, and the concentration forms a plateau.[20–22] Therefore, a few reports exist on cereulide production, but no study has reported on decompose of cereulide after the stationary phase.

The spontaneous degradation of toxins in bacterial colony is important for food hygiene analysis. Only small amounts of food poisoning toxins produced by bacteria can be obtained from nature. Therefore, development of a general method for synthesizing standards is needed. The present report describes the synthesis and structural determination of the cereulide hydrolysates found in culture broth. The chemical structure was determined using HPLC-TOFMS. Quantitative analysis using synthetic compounds as standards revealed the time course profile of cereulide production and degradation.

B. cereus strain No. JCM 17690 (derived from R. Gilbert F4810/72) was used in this study. Five milliliters of trypticase soy broth (TSB) was inoculated and incubated at 35 °C with shaking at 180 rpm for 24 h. The culture broth was extracted with methanol and analyzed using an LC-TOFMS instrument equipped with a C18 column without solid phase treatment. Elution involved a gradient program with 0.1% aqueous formic acid solution and 0.1% methanolic formic acid solution as the mobile phase.

Cereulide was confirmed by comparison with its previously synthesized reference standard[23] *via* retention time of 36.0 min (Fig. 1) and mass spectrum (Fig. 2). Analysis of NH_4^+ adduct ions indicated elution of linear dodecadepsipeptide, which is the primary hydrolysate of cereulide at 30.3 min. In addition, the H^+ adduct ion of tetradepsipeptide, a secondary hydrolysate, was confirmed at a retention time of 20.4 min. Side chains of the amino acids and hydroxy acids in the cereulide structure contain only hydrocarbons. Hydrolysis increases the polarity and so hydrolysates elute in short times in reversed-phase chromatography. The octadepsipeptide should elute between 20.4 and 30.3 min but was not found in the culture broth.

Cereulide (**1**) is a cyclic depsipeptide, *cyclo* (L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)$_3$, which contains three tetradepsipeptide repeat units (Fig. 3). In general, the ester bonds are easier to cleave than the amide bonds. Therefore, the hydrolysates found in the culture broth were presumed to be dodecadepsipeptides (L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)$_3$ (**2**) or (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (**3**), and tetradepsipeptides L-*O*-Val-L-Val-D-*O*-Leu-D-Ala (**4**) or D-*O*-Leu-D-Ala-L-*O*-Val-L-Val (**5**). The compounds were synthesized to confirm their chemical structure.

Four depsipeptides were synthesized from commercially available amino acids, condensing agents, and protective reagents using a previously reported liquid-phase fragment condensation method. The synthetic intermediates tetradepsipeptide (**15** and **16**) and octadepsipeptidic alcohol (**18**) were used as prepared (Scheme 1).[19]

Further extension of the depsipeptide chain gave dodecadepsipeptide (**2**). Cleavage of the benzyl group in compound **16** gave tetradepsipeptide **4** in 51% overall yield in 7 steps (Scheme 2). Moreover, tetradepsipeptidic acid **15** and octadepsipeptidic alcohol **18** were coupled using *p*-toluoyl chloride as an acylation reagent to produce dodecadepsipeptide **19** in 98% yield. Removing the *tert*-butyldimethylsilyl (TBDMS) group with 70% hydrofluoric acid/pyridine and

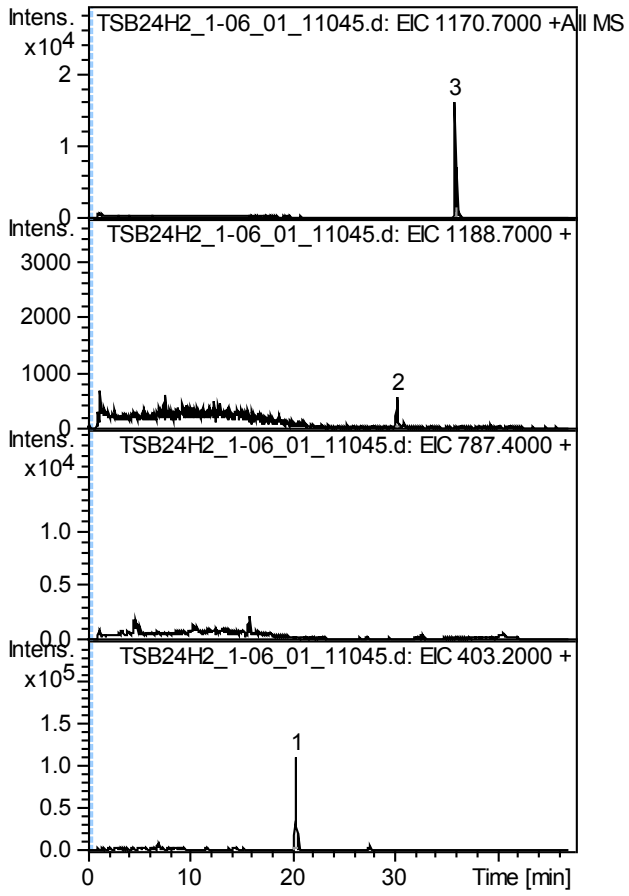


Fig. 1. The LC-MS chromatogram of broth methanol extracts. The broth was cultured in 5 mL TSB for 24 h at 35 °C and 180 rpm. Chromatograms: 1st line: *m/z* 1170.7 (peak 3 represents cereulide); 2nd line: *m/z* 1188.7 (peak 2 represents dodecadepsipeptide); 3rd line: *m/z* 787.4 (no peak identified); 4th line: *m/z* 403.2 (peak 1 represents tetradepsipeptides).

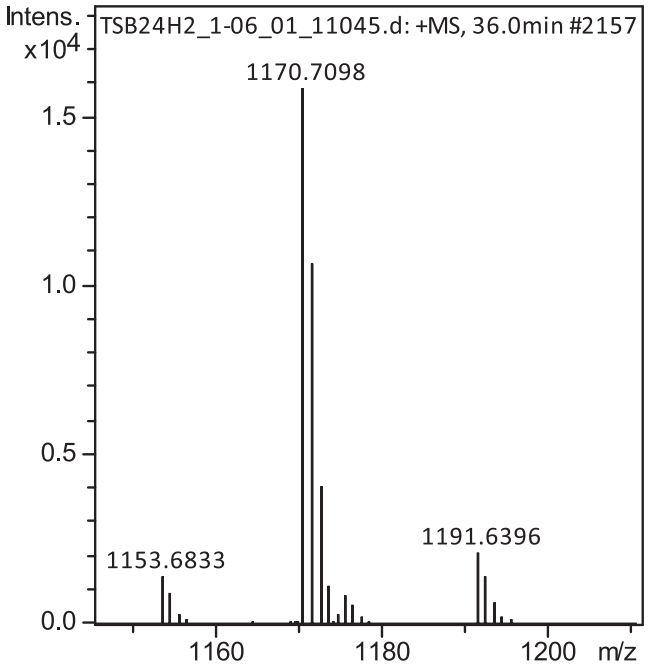


Fig. 2. Mass spectrum of peak 3 {Cereulide, $[M + H]^+$ 1153.6833 (Exact mass 1153.6859), $[M + NH_4]^+$ 1170.7098, $[M + K]^+$ 1191.6396} from Fig. 1.

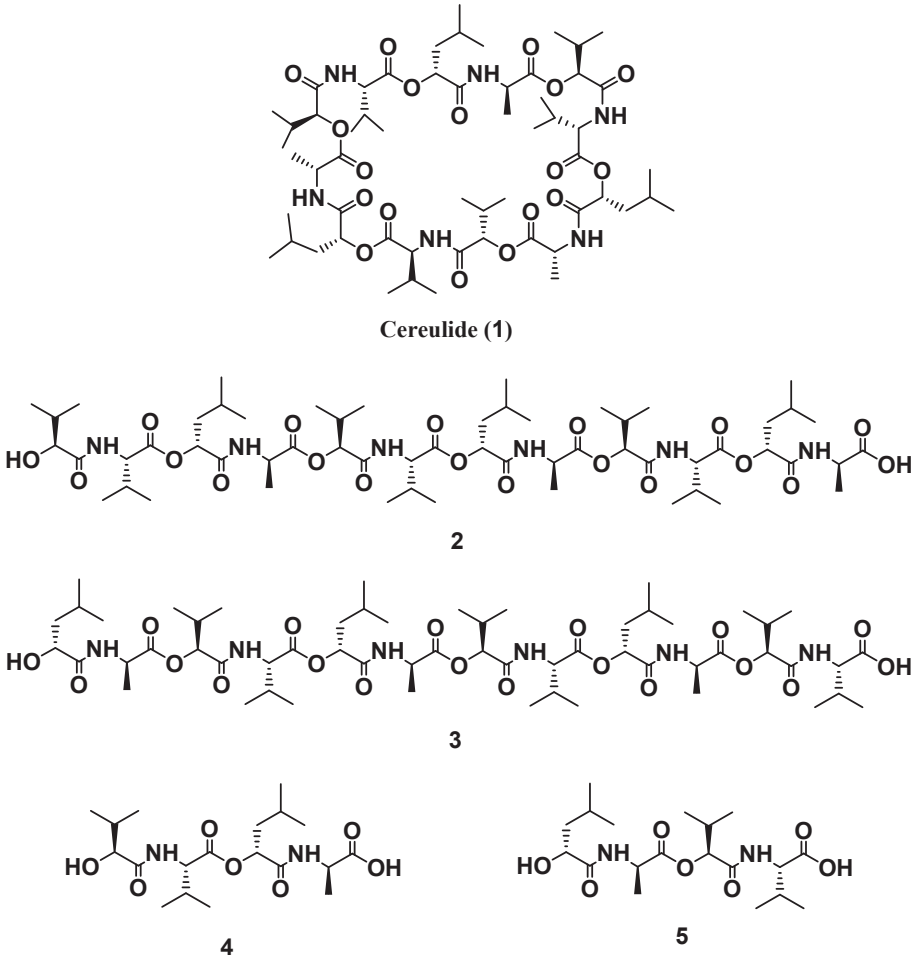


Fig. 3. The chemical structure of cereulide and its hydrolysates.

cleavage of the benzyl group in compound **19** gave dodecadepsipeptide **2** in 12 steps with 19% overall yield.

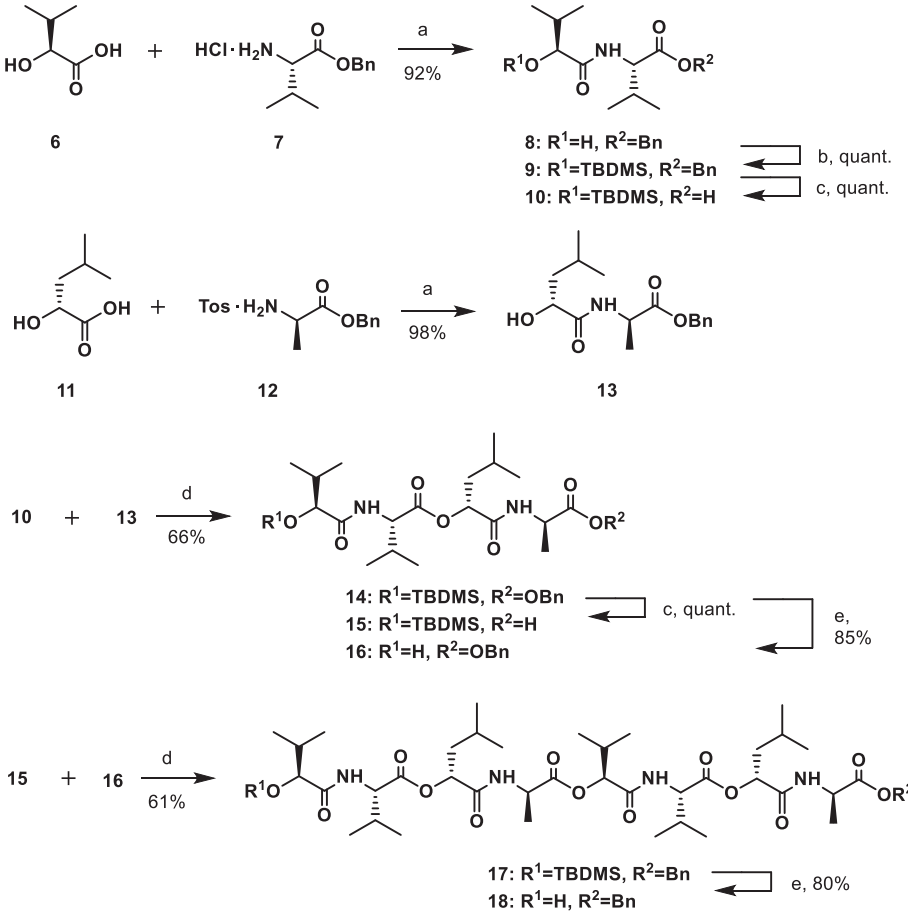
Subsequently, dodecadepsipeptide (**3**) and tetradepsipeptide (**5**) were synthesized *via* a similar reaction scheme. The hydroxy group in dipeptide **11** was protected with the TBDMS group using *tert*-butyldimethylsilyl chloride to afford compound **21**, followed by cleavage of the benzyl ester to obtain dipeptidic acid **22** (Scheme 3). Then, dipeptidic acid **22** and dipeptidic alcohol **8** were condensed using *p*-toluoyl chloride to afford tetradepsipeptide **23**. Hydrogenation of compound **23** over 10% Pd/C eliminated the TBDMS and benzyl groups simultaneously, giving tetradepsipeptide (**5**) in 78% overall yield in 6 steps. A small amount of HCl remaining in the 10% Pd/C catalyst removed the TBDMS group; only the benzyl group was removed by hydrogen reduction using 20% $Pd(OH)_2$ catalyst to obtain tetradepsipeptidic acid **24** in 80% yield. Removing the TBDMS group of compound **23** using 70% HF/py gave tetradepsipeptidic alcohol **25** in good yield.

Coupling of tetradepsipeptidic acid **24** and tetradepsipeptidic alcohol **25** using *p*-toluoyl chloride gave octadepsipeptide **26** and removing the TBDMS group in compound **26** gave octadepsipeptidic alcohol **27** (Scheme 4). Coupling of tetradepsipeptidic acid **24** with octadepsipeptidic alcohol **27** under conditions identical to those used for synthesis of compound **26** provided dodecadepsipeptide **28** in 66% yield. Removal of the TBDMS and benzyl protecting groups gave dodecadepsipeptide **3** in 12% overall yield in 12 steps.

The chemical structures of the natural depsipeptides were confirmed with reversed-phase HPLC-TOFMS using synthetic standards. A comparison of retention times showed that natural component peak occurred at 30.3 min in the chromatogram with *m/z* 1188.7 corresponding to the mass of the NH_4^+ adduct ion of dodecadepsipeptide (Fig. 4, 1st line). No broth compound was found at the retention time for (L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)$_3$ (**2**), which was 31.3 min (Fig. 4, 2nd line). Retention time of dodecadepsipeptide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (**3**) was similar to that of the natural constituent (Fig. 4, 3rd line). The spectrum of the mixed solution of broth and compound **3** contained only one peak that did not separate. The composition of dodecadepsipeptide is $C_{64}H_{104}N_6O_{19}$, and the exact mass of its H^+ adduct ion is 1171.6965. The measured mass of the natural product was 1171.7003, with a measurement error of 3.2 ppm, and the error of the synthetic compound **3** was 0.1 ppm, which showed high agreement. Therefore, peak 2, as Fig. 2 shows, was determined to be compound **3**.

The composition of tetradepsipeptide is $C_{19}H_{34}N_2O_7$, and the exact mass of its H^+ adduct ion is 403.2444. The natural depsipeptide peak eluted with a retention time of 20.5 min with *m/z* 403.2 (Fig. 5a, 1st line). The tetradepsipeptide L-*O*-Val-L-Val-D-*O*-Leu-D-Ala (**4**) eluted at the same retention time of 20.5 min (Fig. 5a, 2nd line). The measured mass of the natural product was 403.2441, and that of synthetic compound **4** was 403.2440, both of which were within 0.5 ppm of the theoretical value. In the culture medium, a small peak eluted at 18.5 min (Fig. 5b, 1st line). The tetradepsipeptide D-*O*-Leu-D-Ala-L-*O*-Val-L-Val (**5**) eluted at the same time of 18.5 min (Fig. 5b, 3rd line). The peak in the culture broth was below the quantification limits. The mass of the synthetic compound was 403.2435 and the mass of the natural compound was 403.2414, both of which were within 3 ppm of the theoretical value.

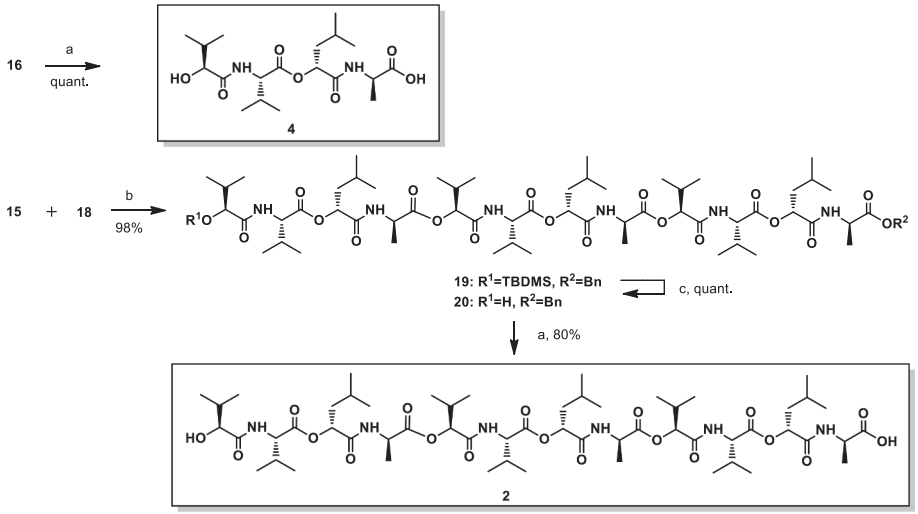
Recovery rate was examined by adding cereulide to the culture



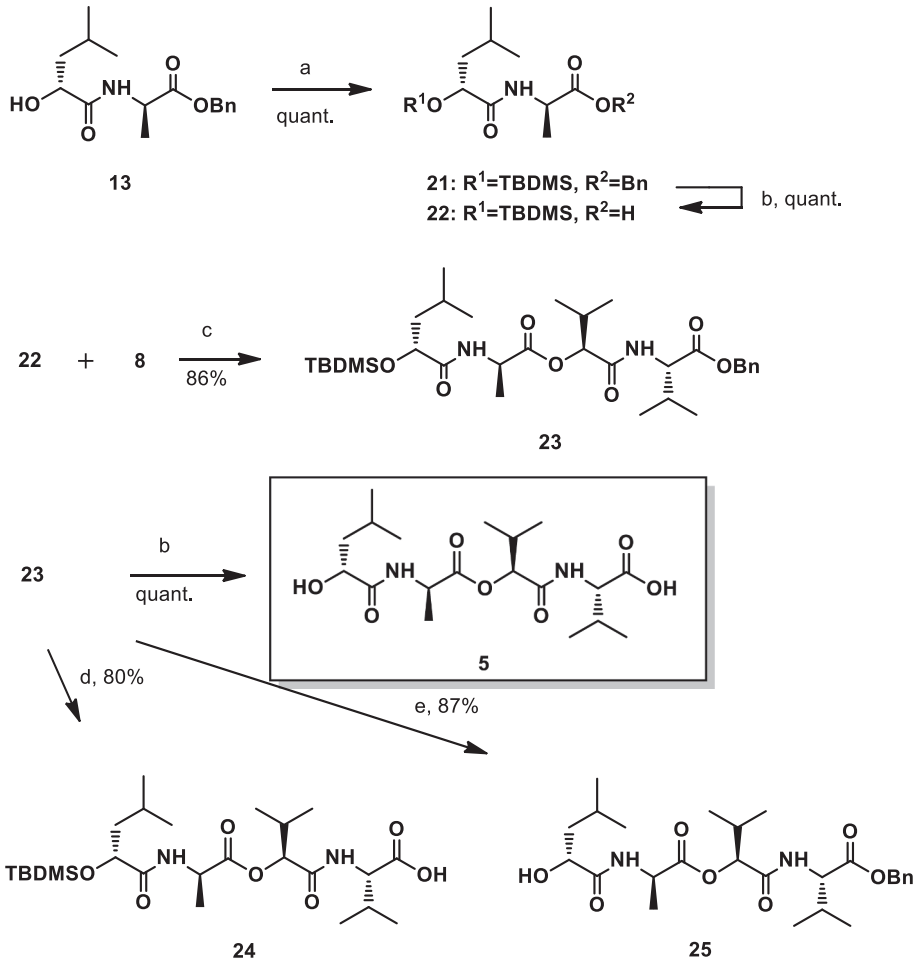
Scheme 1. Synthesis of octadepsipeptidic alcohol (**18**)[19] Reagents and conditions: (a) EDCI (*N*-ethyl-*N*'-(3-dimethylaminopropylcarbodiimide), HOBt (*N*-hydroxybenzotriazole), DIPEA (*N,N*-diisopropylethylamine), CH_3CN, r.t.; (b) *tert*-butyldimethylchlorosilane (TBDMSCl), imidazole, *N,N*-dimethylformamide (DMF), 50 °C; (c) H_2, 10% Pd/C, CH_3OH, r.t.; (d) *p*-toluoyl chloride, Et_3N, DMAP (4-(*N,N*-dimethylamino)pyridine), toluene, r.t. ; (e) 70% HF/pyridine, tetrahydrofuran (THF), −10 °C to r.t.

broth. The TSB broth cultured at 35 °C for 2 weeks in a shaking incubator (180 rpm) was sterilized through a 0.22-μm membrane filter. Methanol (200 μL) containing 36 μg cereulide was added to 10 mL of culture broth, and shaken at 40 °C for 20 min. The sample solutions were ice-cooled, and quantitative analysis was performed using LC-MS. The unused TSB, acting as a negative control, showed a good recovery rate of 93.3% (Table 1). The recovery rate of the untreated broth cultured for 2 weeks was 1.6%, while recovery of the boiled broth was 92.4%. Since cereulide hydrolysis activity in the culture broth was inactivated by boiling, the cereulide was thought to be hydrolyzed by esterase secreted outside of the cells.

The hydrolysates found in the culture broth were dodecadepsipeptide (**3**) ester cleaved between Val-*O*-Leu, and tetradepsipeptide (**4**) cleaved between Ala-*O*-Val. The substrate specificity of the esterase was not high enough to recognize two points. It was expected that the subsequent ester degradation between Val-*O*-Leu was slow, leaving a large amount of tetradepsipeptide (**31**) in the culture broth and only trace of tetradepsipeptide (**32**) were observed due to the rapid



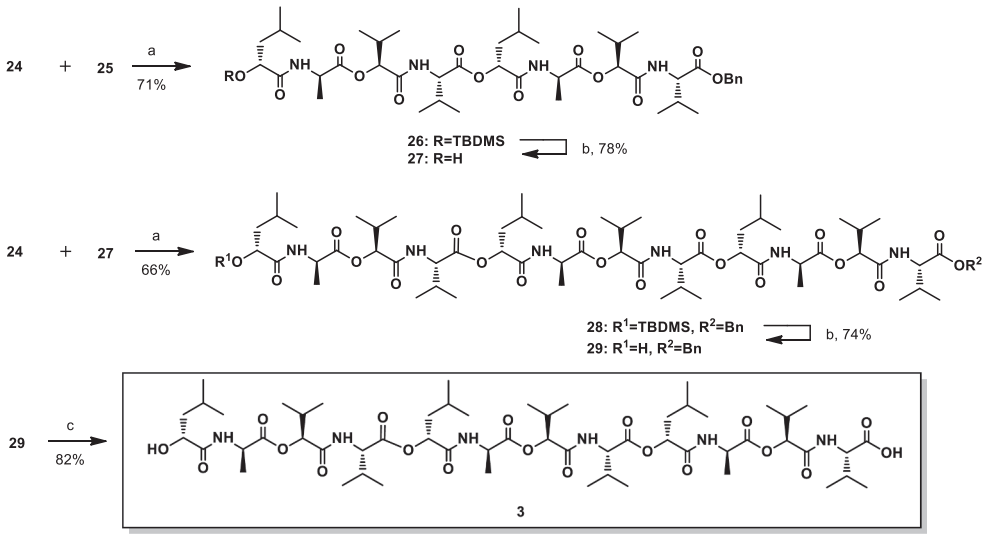
Scheme 2. Synthesis of tetradepsipeptide (**4**) and dodecadepsipeptide (**2**). Reagents and conditions: (a) H_2, 10% Pd/C, CH_3OH, r.t.; (b) *p*-toluoyl chloride, Et_3N, DMAP, toluene, r.t.; (c) 70% HF/pyridine, THF, −10 °C to r.t.



Scheme 3. Synthesis of tetradepsipeptide (**5**). Reagents and conditions: (a) TBDMSCl, imidazole, DMF, 50 °C; (b) H_2, 10% Pd/C, CH_3OH, r.t.; (c) *p*-toluoyl chloride, Et_3N, DMAP, toluene, r.t.; (d) H_2, 20% $Pd(OH)_2$/C, ethyl acetate, r.t.; (e) 70% HF/pyridine, THF, −10 °C to r.t.

degradation between Ala-*O*-Val. In addition, it was not found in octadepsipeptide and other depsipeptide, they were speculated to degrade rapidly to tetradepsipeptide. The dipeptide that is a hydrolysate of tetradepsipeptide was not found.

Using the synthetic compounds as standards for LC-MS analysis, a concentration profile of cereulide and hydrolysates with culture time was produced (Fig. 6). *Bacillus cereus* was cultured in TSB in a shaking incubator at 35 °C. The viable count after the lag phase indicated that the logarithm phase was up to 12 h and then gradually decreased to the death phase *via* the stationary phase. Few viable bacteria were found at



Scheme 4. Synthesis of dodecadepsipeptide (**3**). Reagents and conditions: (a) *p*-toluoyl chloride, Et_3N, DMAP, toluene, r.t.; (b) 70% HF/pyridine, THF, −10 °C to r.t.; (c) H_2, 20% $Pd(OH)_2$/C, ethyl acetate, r.t.

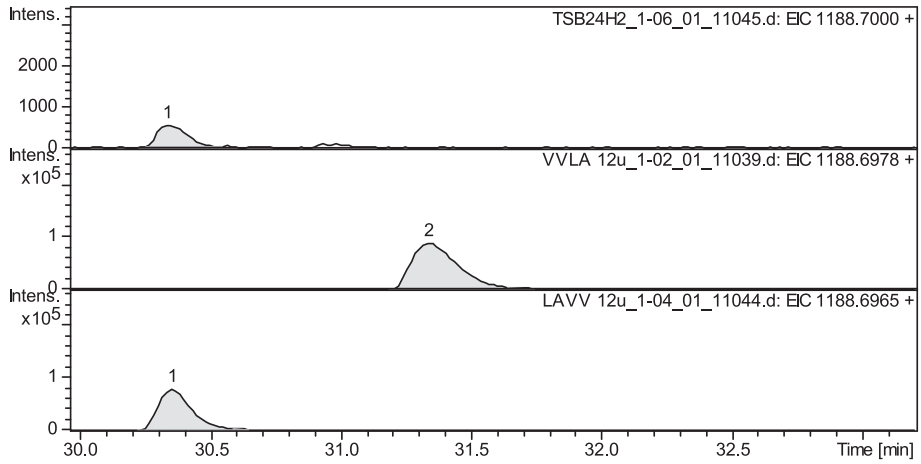


Fig. 4. The LC-MS chromatograms of dodecadepsipeptides with *m/z* 1188.7. 1st line: an extract of 5 mL TSB cultured for 24 h at 35 °C and 180 rpm; 2nd line: dodecadepsipeptide (L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)$_3$ (**2**) 10 μg/mL methanol; 3rd line: dodecadepsipeptide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (**3**) 10 μg/mL methanol.

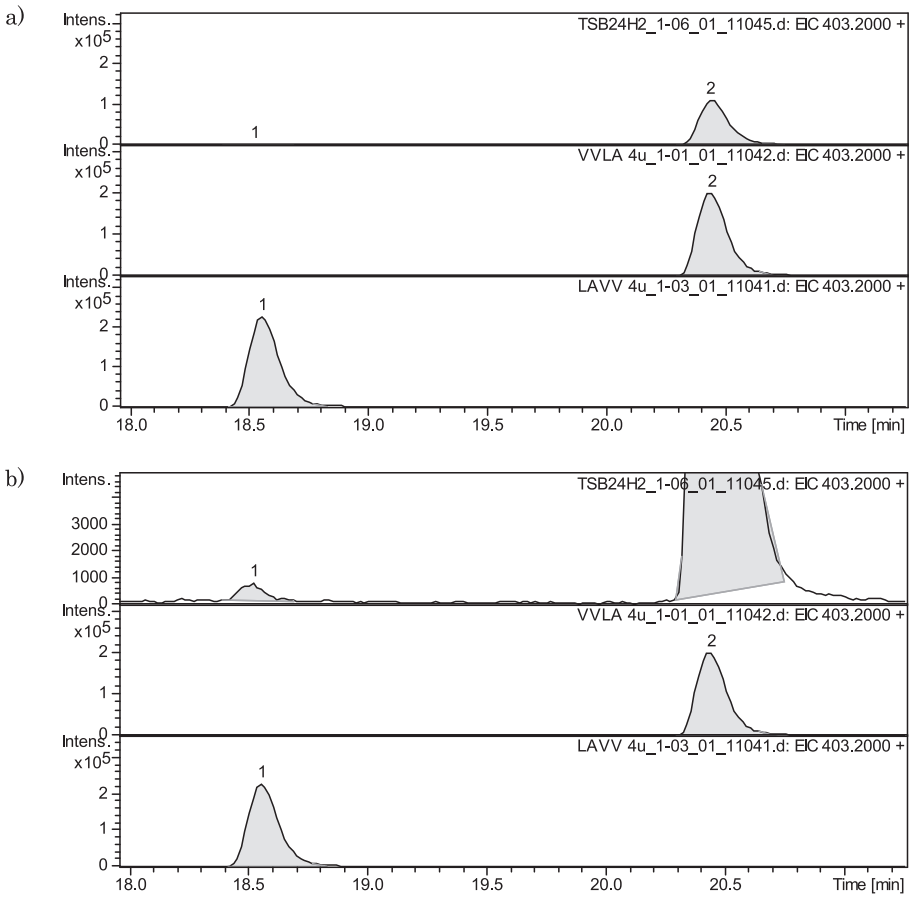


Fig. 5. The LC-MS chromatogram of tetradepsipeptides: (a) mass chromatogram *m/z* 403.2, (b) enlarged y-axis view of the natural product in (a). 1st line: an extract of 5 mL TSB cultured for 24 h at 35 °C and 180 rpm; 2nd line: tetradepsipeptide L-*O*-Val-L-Val-D-*O*-Leu-D-Ala (**4**) 10 μg/mL methanol; 3rd line: tetradepsipeptide D-*O*-Leu-D-Ala-L-*O*-Val-L-Val (**5**) 10 μg/mL methanol.

Table 1
Recovery of cereulide.

Matrix	Recovery [a]	
	μg	%
Negative control [b]	33.6 ± 0.4	93.3 ± 1.2
Culture broth for 2 weeks	0.6 ± 0.0	1.6 ± 0.1
Boiled culture broth for 2 weeks	33.3 ± 0.2	92.4 ± 0.6

[a] A previously reported synthesized cereulide was used.[23] Added 36 μg per 10 mL broth; values represent mean ± standard deviation of 3 measurements.
[b] Unused fresh TSB.

70 h.

Cereulide was produced in the stationary phase, which is consistent with previous reports.[20–22] Cereulide decreased after a maximum concentration of 0.23 μM was achieved at 39 h, and traces were found after 70 h. Dodecadepsipeptide **30** was found at 14 h, with the maximum concentration of 0.02 μM occurring at 32 h, and the compound disappeared in about 60 h. This trend was similar to that of cereulide. Tetradepsipeptide **31** increased (up to 2.31 μM at 57 h) as the cereulide amount decreased. Although the concentration in the broth was monitored, no molar equivalents between cereulide and hydrolysates were found. Future studies will monitor the concentrations of hydroxy acids and amino acids in the broth over time.

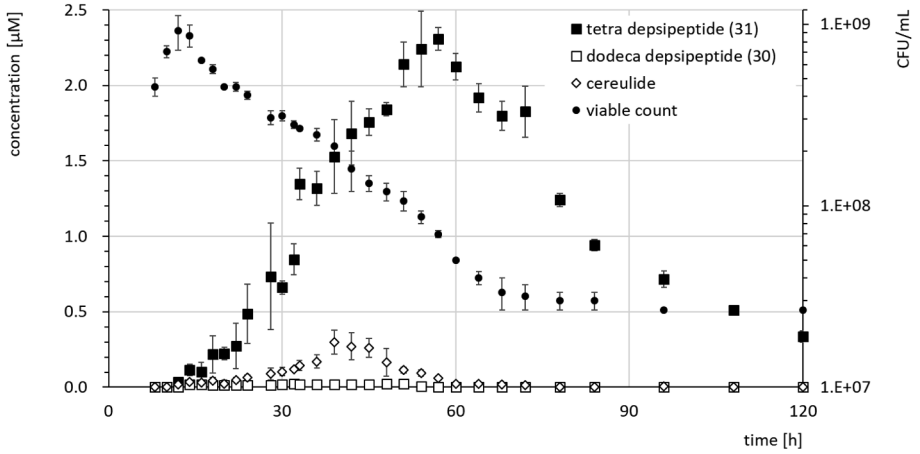


Fig. 6. The concentration variation of cereulide and its hydrolysate depsipeptides in culture broth. A previously reported synthesized cereulide was used.[23]

Table 2
Vacuole response on HEp-2 cells.

Compound	Vacuole formation (nM)[a]
Cereulide (**1**)[b]	3.24 ± 0.66
(L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)$_3$ (**2**)	1,630 ± 331
(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (**3**)	1,482 ± 419
L-*O*-Val-L-Val-D-*O*-Leu-D-Ala (**4**)	> 10,000
D-*O*-Leu-D-Ala-L-*O*-Val-L-Val (**5**)	> 10,000

[a] Data are presented as mean concentration ± standard deviation (n = 6).
[b] A previously reported synthesized cereulide was used.[23]

The vacuole response of the hydrolysates was examined using the HEp-2 cells. The two dodecadepsipeptides **2** and **3** were weaker than cereulide but vacuole positive. The two negative tetradepsipeptides **4** and **5** appeared to lose toxicity due to their small molecular size (Table 2).

In conclusion, hydrolysate depsipeptides in the culture supernatant of a *Bacillus cereus* strain were found and their chemical structures elucidated by synthesis of authentic samples. The depsipeptides included the tetradepsipeptide L-*O*-Val-L-Val-D-*O*-Leu-D-Ala and dodecadepsipeptide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$. The recovery rate in the broth with added cereulide suggested enzymatic degradation of cereulide. The two depsipeptides appeared to remain in the culture broth because of their slow ester cleavage between L-Val-D-*O*-Leu.

Quantitative analysis of cereulide and the depsipeptides using synthetic standards revealed production of cereulide in the stationary phase and decomposition in the death phase. The increase in tetradepsipeptide continued after the stationary phase. These results indicate that cereulide is not enzymatically stable. Therefore, elucidating the chemical structure of the hydrolysates and clarifying their characteristics is important in food hygiene. The present study should stimulate further investigation into the biological activities and the results are expected to lead to the development of treatment for severely ill patients or to be used as a marker in simple analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127050. These data include MOL files and InChiKeys of the most important compounds described in this article.

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