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Synthesis of the reported structure of homocereulide and its vacuolation assay

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ABSTRACT

Homocereulide, isolated from marine bacterium *Bacillus cereus*, is an analog of emetic toxin cereulide. There is no report on its structure determination and involvement in *B. cereus*-associated food poisoning. Homocereulide is a cyclic dodecadepsipeptide composed of L-*O*-Val-L-Val-D-*O*-Leu-D-Ala and L-*O*-*allo*-Ile-D-Val-D-*O*-Leu-D-Ala. Here, we synthesized homocereulide using liquid phase fragment condensation. The NMR spectrum of synthesized homocereulide confirmed the intended structure and LC-MS results were consistent with natural products. Morphological evaluation using HEp-2 cells showed higher toxicity with homocereulide (1.39 nM) than cereulide (3.95 nM). Though cereulide is the main component in broth culture, homocereulide is also likely involved in *B. cereus*-associated food poisoning.

Bacillus cereus is a Gram-positive bacterium found in various environments and often causes food poisoning, particularly the emetic or diarrheal type. There are many cases of the diarrheal type in North America and Northern Europe, while the emetic type mainly occurs in Japan and the United Kingdom.[1] In such cases, vomiting is usually mild, but rare cases of death have occurred.[2] Cereulide, an emetic toxin in *B. cereus*-associated food poisoning, was isolated from culture broth and its chemical structure was determined by Isobe and co-workers.[3,4] Cereulide shows heat resistance even at 126 °C and does not decompose during food processing.[5–7]

Cereulide is a 36-membered cyclic depsipeptide containing 12 stereogenic centers and a *cyclo* [L-*O*-Val-L-Val-D-*O*-Leu-D-Ala]$_3$ depsipeptide sequence (Fig. 1). Cereulide shows ionophore activity with specific affinity for K^+ and inhibits respiration in mitochondria.[4,8,9] Its food toxicity is assessed by vacuolation activity in HEp-2 cells, a human laryngeal carcinoma cell line.[6,8] Homocereulide, one of the analogs of cereulide, was isolated from marine bacterium *B. cereus*.[10] The chemical structure of homocereulide is proposed to be *cyclo* (L-*O*-*allo*-Ile-D-Val-D-*O*-Leu-D-Ala [L-*O*-Val-L-Val-D-*O*-Leu-D-Ala]$_2$) depsipeptide, as one *O*-Val of cereulide is replaced by *O*-*allo*-Ile.[10] Although strong cytotoxic activity is reported, chemical and biological properties, such as food poisoning, of homocereulide have not yet been reported. Herein, we described the total synthesis of the proposed structure of homocereulide and the measurement of vacuole activity in HEp-2 cells for its toxicity evaluation.

Homocereulide was synthesized by the liquid phase fragment condensation method using commercially available amino acids. The starting hydroxy acids were prepared through sterically retaining hydroxylation of the corresponding amino acids.[11] Amino acids and hydroxy acids were coupled to afford the corresponding three tetradepsipeptides, which were sequentially condensed to lead to a precursor dodecadepsipeptide. Finally, homocereulide was synthesized by a head-to-tail intramolecular coupling reaction.

Dipeptide **5** was obtained in good yield by coupling D-*O*-Leu **3** and D-alanine *p*-toluene sulfonate **4** using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDCI) and *N*-hydroxybenzotriazole (HOBt) in the presence of *N,N*-diisopropylethylamine (DIPEA). Dipeptide **8** was prepared using the same method from L-*O*-*allo*-Ile **6** and D-valine benzyl ester hydrochloride **7**. The hydroxy group in **8** was protected as *tert*-butyldimethylsilyl ether to provide dipeptide **9**. Subsequently, the benzyl group in **9** was cleaved to obtain acid **10**. Dipeptides **5** and **10** were coupled with *p*-toluoyl chloride in the presence of triethylamine

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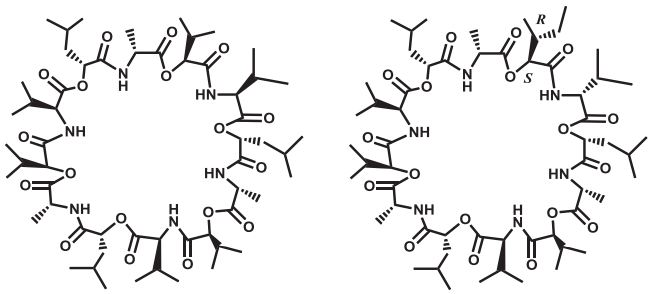


Fig. 1. Chemical structure of cereulide and homocereulide.[10]

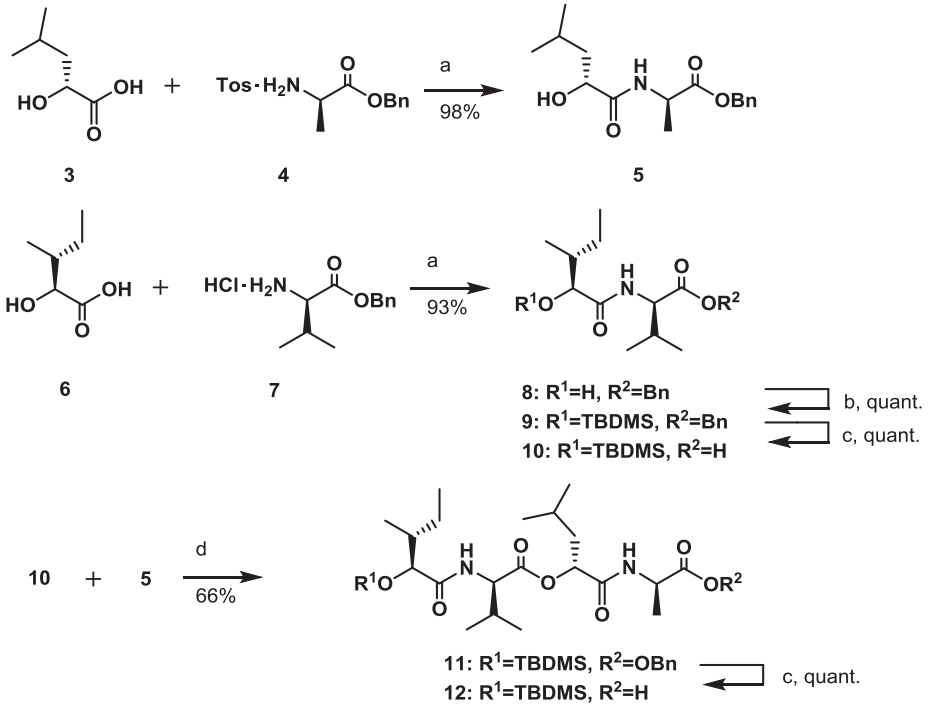
and 4-(*N,N*-dimethylamino)pyridine (DMAP) to afford tetradepsipeptide **11**. In the course of this reaction, a small amount of partially epimerized by-product was observed by TLC. The minor by-product was easily removed by silica gel chromatography. The compound **11** was then hydrogenated to afford acid **12** (Scheme 1).

Dipeptide **15** was prepared from L-*O*-Val **13** and L-valine benzyl ester hydrochloride **14** using the same method for the preparation of dipeptide **5**. The hydroxy group in **15** was protected as *tert*-butyldimethylsilyl ether to afford dipeptide **16**. Subsequently, the benzyl group in **16** was cleaved to obtain acid **17**. Dipeptides **5** and **17** were coupled using the same method used for the preparation of **11** to obtain tetradepsipeptide **18**. Hydrogenation of **18** gave tetradepsipeptidic acid **19** in quantitative yield. Removal of the *tert*-butyldimethylsilyl group in **18** with hydrofluoric acid/pyridine gave tetradepsipeptidic alcohol **20** in 85% yield. The coupling reaction between **19** and **20** with *p*-toluoyl chloride afforded octadepsipeptide **21** in 61% yield. Subsequent cleavage of the *tert*-butyldimethylsilyl group gave alcohol **22** (Scheme 2).

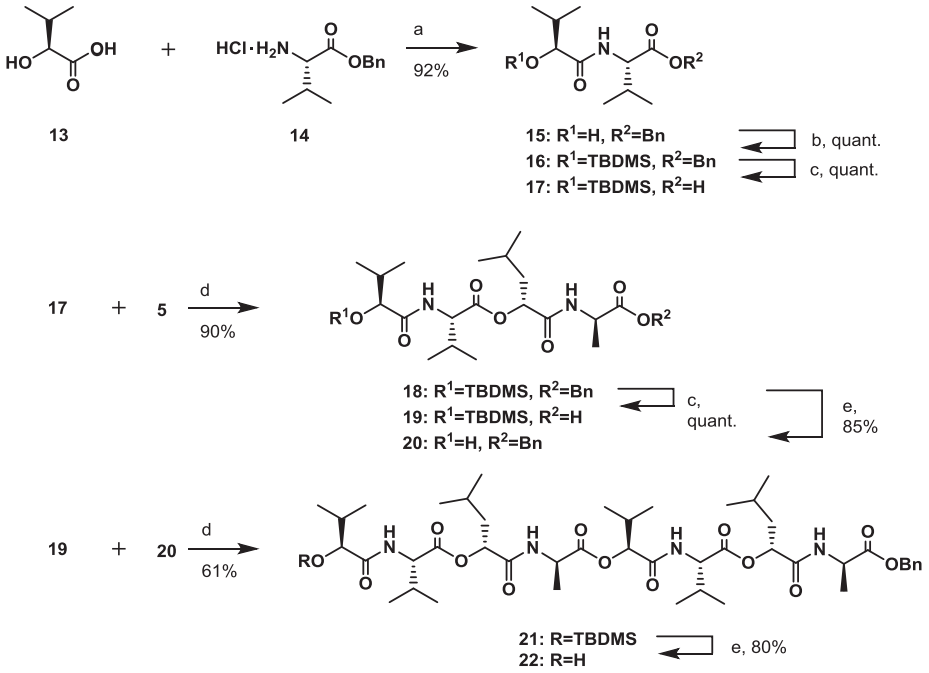
Tetradepsipeptidic acid **12** and octadepsipeptidic alcohol **22** were coupled using the same method used for the preparation of **11** to obtain **23** in 74% yield. Removal of the *tert*-butyldimethylsilyl group in **23** with hydrofluoric acid/pyridine gave dodecadepsipeptidic alcohol **24**. Subsequent cleavage of the benzyl group gave dodecadepsipeptide precursor **25**. Macrolactonization of dodecadepsipeptide **25** was carried out under high-dilution conditions (1.5 mM) using *m*-nitrobenzoic anhydride (MNBAN)[12] to give the reported structure of homocereulide (**2**) in 74% yield, $[\alpha]_D$ +10.8° (*c* 0.40, CH_3OH, lit. +10.5°, *c* 0.12, CH_3OH) (Scheme 3). The synthesis of homocereulide was achieved in 11% overall yield in 18 steps.

The methanol solution of synthetic homocereulide was analyzed by LC-MS using C18 reversed-phase chromatography. The retention time of synthetic homocereulide and natural product in trypticase soy broth (TSB) were both 16.1 min (Fig. 2). In the analytical chromatogram of these mixed solutions, one peak formed without separation. The mass spectrum of synthetic homocereulide was $[M+H]^+$ *m/z* 1167.6961 and that of natural homocereulide was $[M+H]^+$ *m/z* 1167.6926, which were consistent (Fig. 3). Therefore, synthetic homocereulide is considered to be the same as the natural product.

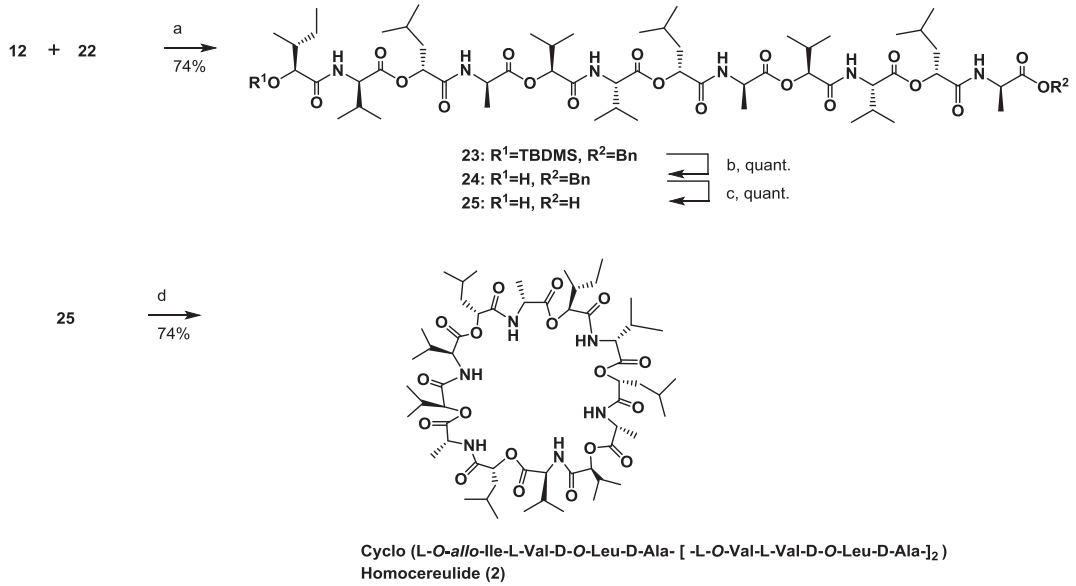
Subsequently, ^1H, ^{13}C NMR, and HSQC spectra in $CDCl_3$ of synthetic homocereulide were obtained. These results supported the synthesized structure. In comparison with the literature,[10] our obrain spectra were highly coincident with the reported ones. However, the multiplet signal of *O-allo*-Ile (measured δ = 1.42 ppm, Lit.[10] 1.55 ppm) was confirmed to be lower than multiplet signals of Ala (measured δ = 1.43–1.46 ppm, Lit. 1.45 ppm) in ^1H NMR, which was opposite to that in the literature (Table 1). In ^{13}C NMR, the side chain [2 × CH_3] of *O-allo*-Ile had a different measured value (11.8, 14.1 ppm) with respect to the literature



Scheme 1. Synthesis of tetradepsipeptidic acid **12**. Reagents and conditions: (a) EDCI, HOBt, DIPEA, CH_3CN, r.t.; (b) *tert*-butyldimethyl-chlorosilane (TBDMSCl), imidazole, *N,N*-dimethylformamide (DMF), 50 °C; (c) H_2, Pd/C, CH_3OH, r.t.; (d) *p*-toluoyl chloride, Et_3N, DMAP, toluene, r.t.



Scheme 2. Synthesis of octadepsipeptidic alcohol 22. Reagents and conditions: (a) EDCI, HOBt, DIPEA, CH_3CN, 0 °C to r.t.; (b) TBDMSCl, imidazole, DMF, 50 °C; (c) H_2, Pd/C, CH_3OH, r.t. (d) *p*-toluoyl chloride, Et_3N, DMAP, toluene, r.t.; (e) 70% HF/pyridine, tetrahydrofuran (THF), −10 °C to r.t.



Scheme 3. Synthesis of homocereulide (2). 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, Pd/C, CH_3OH, r.t.; (d) MNBAN, DMAP, CH_2Cl_2 (*c* 1.5 mM), r.t.

value (15.1, 23.3 ppm). The reason for these disagreements is currently under investigation.

Morphological changes in HEp-2 cells were examined using the method described by Sakurai et al.[8] An aliquot of 2-fold serially diluted sample solution was mixed with a suspension of HEp-2 cells in each well of a 96-well tissue culture plate, incubated at 37 °C for 24 h, and observed for vacuole responses in HEp-2 cells (Fig. 4). The concentration of the highest dilution of the test sample producing vacuole formation in more than 30% of HEp-2 cells at 10 vacuoles/cell was determined (Table 2). Cereulide showed vacuolation activity at 3.95 nM, whereas homocereulide showed activity at 1.39 nM, indicating that homocereulide has stronger toxicity than cereulide. The LC-MS

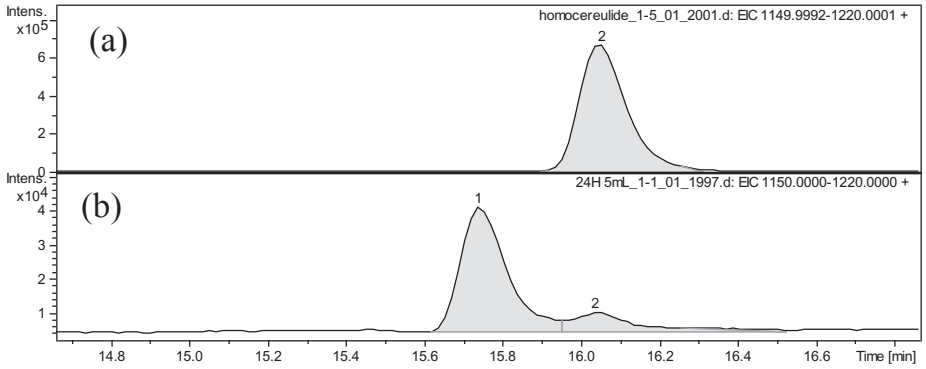


Fig. 2. LC-MS chromatogram of homocereulide. (a) Synthetic homocereulide, (b) Culture broth of *B. cereus* cultivated in 5 mL TSB for 24 h at 35 °C and 180 rpm. Peak No. 1 indicates cereulide and peak No. 2 indicates homocereulide.

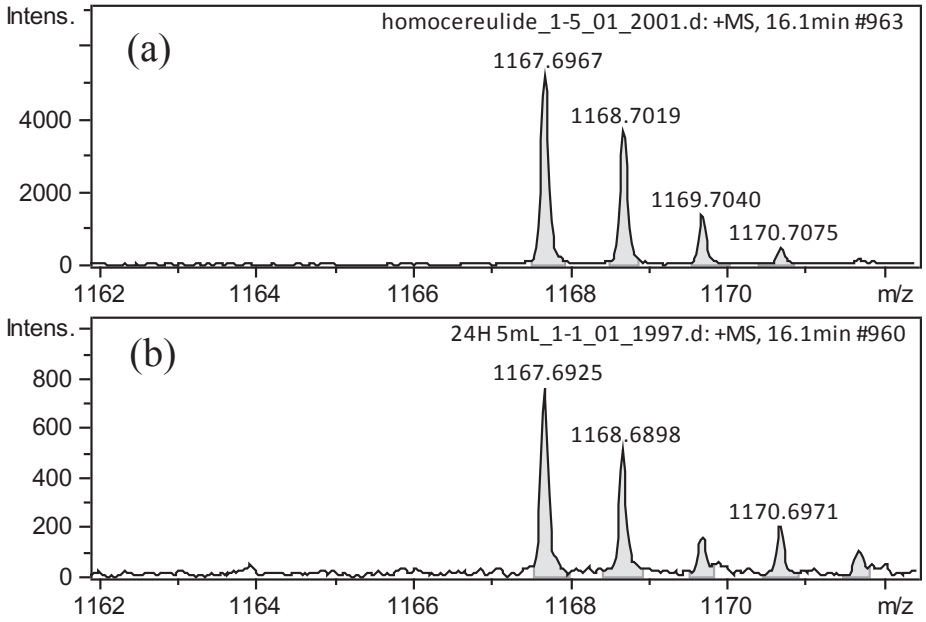


Fig. 3. Mass spectra of homocereulide. (a) Synthetic homocereulide. (b) Culture broth of *B. cereus* cultivated in 5 mL TSB for 24 h at 35 °C and 180 rpm.

quantitative results of the culture broth cultivated for 24 h (Fig. 2, TSB medium) showed that the concentration of cereulide was 65.4 nM and that of homocereulide was 9.3 nM. Obviously, homocereulide is a minor component in culture but has strong toxicity. Thus, it is considered to be involved in *B. cereus*-associated food poisoning. The three synthetic intermediates (**23–25**) exhibited vacuole change at 1000 nM or more. These results suggest that the cyclic structure is important for food poisoning induced by depsipeptide compounds.

In conclusion, homocereulide was prepared efficiently and conveniently using liquid phase condensation from readily available chemicals. The structure of synthesized homocereulide was confirmed by NMR and LC-MS. In this study, we demonstrated the emetic toxicity of homocereulide by vacuolating assay using HEp-2 cells. Homocereulide showed higher toxicity than cereulide. These data suggest the need for examination of minor components in emetic type food poisoning caused by *B. cereus*.

Table 1
^{1}H and ^{13}C NMR spectroscopic data for homocereulide (**2**).

	Synthetic homocereulide ($CDCl_3$, 500 MHz)		Lit. ($CDCl_3$, 400 MHz)[10]	
	[a]δ_H	δ_C	δ_H	δ_C
NH	7.80 (1H, d, [a]J = 7.0) 7.71–7.74 (4H, m) 7.68 (1H, d, J = 6.5)		7.76 (6H, m)	 171.5 (s) 48.9 (d) 15.7 (q)
Ala	4.28–4.40 (3H, m) 1.43–1.46 (9H, m)	171.2, 171.3 (2C) 48.7, 48.8, 48.9 15.7, 15.8 (2C)	4.35 (3H, m) 1.451 (3H, d, J = 7.0) 1.442 (3H, d, J = 7.0) 1.438 (3H, d, J = 7.0)	
O-Leu	5.26 (2H, dd, J = 4.0, 8.5) 5.28 (1H, dd, J = 4.0, 11.0) 1.63–1.83 (6H, m) 1.63–1.83 (3H, m) 0.89–0.93 (18H, m)	171.7 (2C), 171.8 72.8 (2C), 72.9 40.5 (3C) 24.4 (3C) 23.3 (3C) 21.2 (3C)	5.30 (3H, dd, J = 8.1, 4.7) 1.76 (6H, m) 1.68 (3H, m) 0.92 (9H, d, J = 6.2) 0.89 (9H, d, J = 6.2)	171.9 72.8 (d) 40.6 (t) 24.4 (d) 23.3 (q) 21.3 (q)
Val	4.09–4.19 (3H, m) 2.25–2.35 (3H, m) 1.05 (9H, d, J = 6.0) 0.96–0.97 (9H, m)	170.3, 170.4 (2C) 59.3 (3C) 28.7 (3C) 19.3 (3C) 19.2 (3C)	4.10 (3H, m) 2.31 (3H, m) 1.05 (9H, d, J = 6.6) 0.95 (9H, d, J = 6.6)	170.4 (s) 59.3 (d) 28.7 (d) 19.3 (q) 19.3 (q)
O-Val	4.99 (2H, d, J = 3.5) 2.25–2.35 (2H, m) 0.97–0.99 (12H, m)	170.9 (2C) 78.8 (2C) 30.6 (2C) 18.5 (2C) 16.9 (2C)	4.99 (1H, d, J = 3.3) 4.98 (1H, d, J = 3.3) 2.31 (2H, m) 0.97 (12H, d, J = 7.3)	171.0 (s) 78.8 (d) 30.5 (d) 18.6 (q) 16.9 (q)
O-allo-Ile	5.10 (1H, d, J = 3.0) 2.05–2.10 (1H, m) 1.42–1.46 (1H, m) 1.21–1.29 (1H, m) 0.93–0.95 (6H, m)	171.6 77.4 37.1 25.8 14.1 11.8	5.05 (1H, d, J = 4.6) 2.01 (1H, m) 1.55 (1H, ddq) 1.30 (1H, ddq) 0.94 (3H, d, J = 7.0) 0.91 (3H, d, J = 7.0)	170.9 (s) 78.3 (d) 37.2 (d) 24.5 (t) 23.3 (q) 15.1 (q)

[a] Chemical shifts (δ) are expressed in ppm, and J values are presented in Hz.

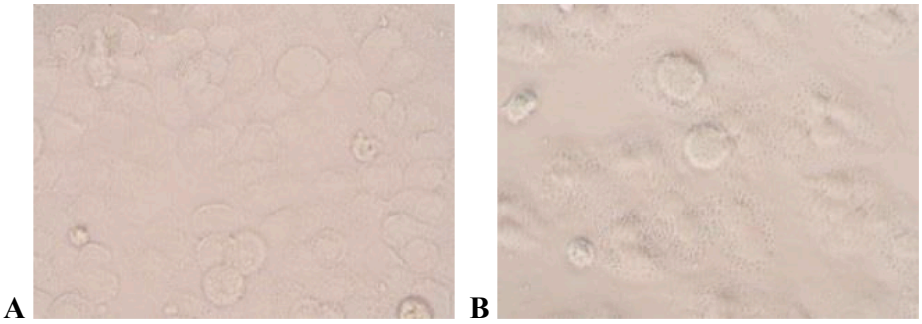


Fig. 4. Micrographs of HEp-2 cells. (A) Control cells with no treatment. (B) Vacuolar cells incubated with 2.0 nM homocereulide.

Table 2
Concentration of indicated compounds in vacuole formation and *B. cereus* culture broth.

Compound No.	Vacuole formation (nM)[a]	Concentration in *B. cereus* culture broth (nM)[b]
Cereulide (1)[c]	3.95 ± 1.22	65.4 ± 15.0
Homocereulide (2)	1.39 ± 0.43	9.3 ± 1.2
25	1845 ± 1,081	–
24	1592 ± 723	–
23	1124 ± 348	–

[a] Data are presented as mean concentration ± standard deviation (N = 6).
[b] Quantitative results of LC-MS analysis of *B. cereus* culture cultivated in 5 mL TSB for 24 h at 35 °C and 180 rpm. Data are presented as mean concentration ± standard deviation (N = 3).
[c] A previously reported synthesized cereulide was used.[13]

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.2019.01.007. These data include MOL files and InChiKeys of the most important compounds described in this article.

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