

Chemodiversity of cereulide, the emetic toxin of *Bacillus cereus*

Sandra Marxen · Timo D. Stark · Elrike Frenzel ·
Andrea Rüttschle · Genia Lücking · Gabriel Pürstinger ·
Elena E. Pohl · Siegfried Scherer ·
Monika Ehling-Schulz · Thomas Hofmann

Received: 9 October 2014 / Revised: 17 January 2015 / Accepted: 22 January 2015 / Published online: 10 February 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract Food-borne intoxications are increasingly caused by the dodecadepsipeptide cereulide, the emetic toxin produced by *Bacillus cereus*. As such intoxications pose a health risk to humans, a more detailed understanding on the chemodiversity of this toxin is mandatory for the reliable risk assessment of *B. cereus* toxins in foods. Mass spectrometric screening now shows a series of at least 18 cereulide variants, among which the previously unknown isocereulides A–G were determined for the first time by means of UPLC-TOF MS and ion-trap MSⁿ sequencing, ¹³C-labeling experiments, and post-hydrolytic dipeptide and enantioselective amino acid analysis. The data demonstrate a high microheterogeneity in cereulide and show evidence for a relaxed proof reading function of the non-

ribosomal cereulide peptide synthetase complex giving rise to an enhanced cereulide chemodiversity. Most intriguingly, the isocereulides were found to differ widely in their cell toxicity correlating with their ionophoric properties (e.g., purified isocereulide A showed about 8-fold higher cytotoxicity than purified cereulide in the HEp-2 assay and induced an immediate breakdown of bilayer membranes). These findings provide a substantial contribution to the knowledge-based risk assessment of *B. cereus* toxins in foods, representing a still unsolved challenge in the field of food intoxications.

Keywords Cereulide · Isocereulide · *Bacillus cereus* · Depsipeptide · Ionophore · UPLC-TOF MS

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8511-y) contains supplementary material, which is available to authorized users.

S. Marxen · T. D. Stark · T. Hofmann (✉)
Chair of Food Chemistry and Molecular Sensory Science,
Technische Universität München, Lise-Meitner-Straße 34,
85354 Freising, Germany
e-mail: thomas.hofmann@tum.de

E. Frenzel · M. Ehling-Schulz
Functional Microbiology, IBMH, Department of Pathobiology,
University of Veterinary Medicine Vienna, 1210 Vienna, Austria

A. Rüttschle · G. Lücking · S. Scherer
Department of Microbiology, Central Institute for Food and Nutrition
Research, Technische Universität München,
85350 Freising, Germany

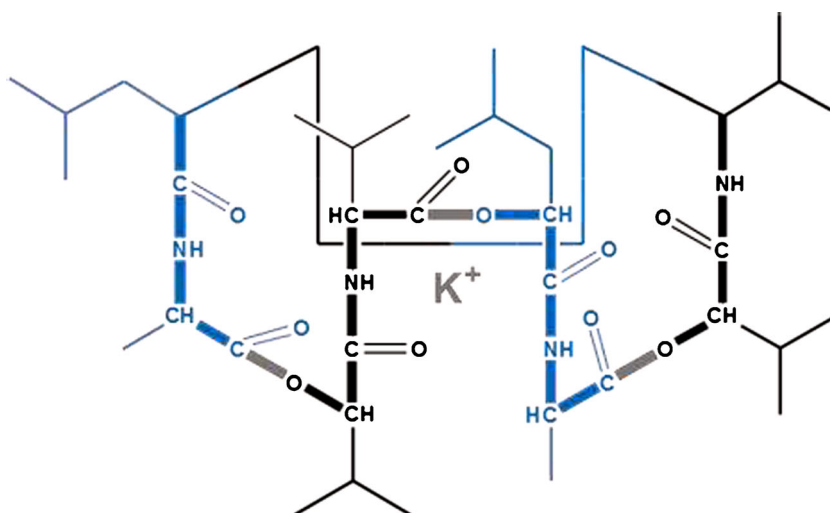
G. Pürstinger · E. E. Pohl
Unit of Physiology and Biophysics, Department of Biomedical
Sciences, University of Veterinary Medicine Vienna,
1210 Vienna, Austria

S. Scherer
Chair of Microbial Ecology, Department of Biosciences, Technische
Universität München, 85350 Freising, Germany

Introduction

The endospore-forming bacterium *Bacillus cereus* is increasingly recognized as a food-borne pathogen causing gastrointestinal diseases such as diarrhea or emesis. Whereas diarrheal symptoms are induced by hemolytic and nonhaemolytic enterotoxins, emesis is triggered by the heat-stable dodecadepsipeptide cereulide (Fig. 1), which is preformed in contaminated food [1, 2]. The enzymatic machinery, the so called cereulide synthetase Ces, required for the biosynthesis of cereulide is located on a virulence megaplasmid that shares its backbone with the *B. anthracis* toxin plasmid pX01 [3, 4]. Ces belongs to the family of non-ribosomal peptide synthetases (NRPS), which are responsible for the production of a broad variety of nonribosomal peptides in bacteria and fungi. The *ces* gene cluster comprises, beside the structural genes *cesA* and *cesB*, a putative hydrolase (*cesH*), a phosphopantetheinyl transferase (*cesP*), a type II thioesterase (*cesT*), as well as a putative ABC transporter (*cesC/D*) [3]. 4-Phosphopantetheinyl transferases are essential for activation of the NRPSs while type II

Fig. 1 (Marxen et al.) Chemical structure of the cyclic dodecadepsipeptide cereulide, [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]₃; black: L-*O*-Val-L-Val, blue: D-*O*-Leu-D-Ala



thioesterase, frequently associated with NRPS structural genes, are thought to have a proof reading function by offloading of incorrectly processed peptide intermediates [5]. The dodecadepsipeptide toxin, composed of six α -amino acid and six α -hydroxy acid moieties arranged in three repeating tetrapeptide units, [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]₃, forms a rectangular cylindrical shape [6–8], giving the toxin its ability for high-affinity complex formation with alkali metal (Li⁺, Na⁺, K⁺) and ammonium ions and its biological function as a strong K⁺ ionophore [9, 10].

Recently, mass spectrometric profiling of 78 *B. cereus* strains showed a high variability in cereulide production and indicated the occurrence of a series of unknown cereulide variants [11]. Although two structural homologues have been reported earlier to differ in molecular weight by 14 Da compared with cereulide [12], neither the exact chemical structure nor their membrane toxicity had been determined.

The objectives of the present investigation were, therefore, to locate structure homologues of cereulide in *B. cereus* culture extracts and to decipher their chemical structures by means of UPLC-TOF MS and ion-trap MSⁿ sequencing, ¹³C-labeling experiments, as well as post-hydrolytic dipeptide and enantioselective amino acid analysis. Furthermore, the biological ionophore activity of the *B. cereus* fractions, containing cereulide and its variants, were compared by measuring their cytotoxic activity using the well-known HEp-2 cell assay [13, 14] and studying their impact on membrane conductance using a lipid bilayer assay [15].

Materials and methods

Chemicals

The following compounds were obtained commercially: KOH, potassium tetraborate tetrahydrate (B₄K₂O₇·4H₂O), trifluoroacetic acid (reagent plus, 99 %), *N,N,N'*,

N'-diisopropylethylamine (DIPEA), piperidine, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluoro phosphate (HBTU), *N*-isobutyryl-L-cysteine (IBLC), *ortho*-phthalaldehyde (OPA), D-(+)-glucose monohydrate, and (*S*)-(-)-2-hydroxyisocaproic acid (L-*O*-Leu) from Sigma-Aldrich (Seelze, Germany), Fmoc-D- and L-Ala-Wang resin, Fmoc-D- and L-Val-Wang resin (100–200 mesh, each), Fmoc-D-Ser(*t*Bu)-Wang resin, D- and L-alanine, D- and L-valine, D- and L-serine, HCl (37 %), *N,N*-dimethylformamide, and HCOOH from Merck (Darmstadt, Germany), NaCl, CH₂Cl₂, and buffer compounds for biophysics experiments using planar lipid bilayers from Carl Roth (Karlsruhe, Germany), Fmoc-L-Ser(*t*Bu)-Wang resin (200–400 mesh), D-lactic-acid (D-*O*-Ala), L- α -hydroxyisovaleric acid (L-*O*-Val), D- α -hydroxyisovaleric acid (D-*O*-Val), D- α -hydroxyisocaproic acid (D-*O*-Leu) from Bachem (Bubendorf, Switzerland), (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid (L-*O*-Ile) from Interchim (Montiucan Cadex, France), pepton tryptone and yeast extract from Oxoid (Hampshire, England), and EtOH from Australco (Spillern, Austria). H₂O for chromatography was purified with an integral 5 system (Millipore, Schwabach, Germany); solvents used were of HPLC or LC-MS grade (J.T. Baker, Deventer, Holland).

Following the protocol reported previously, ¹³C₆-labeled cereulide was biosynthetically prepared using the emetic *B. cereus* reference strain F4810/72 in a defined mineral medium (MOD-medium; 500 mL batch) containing glucose (20 mM) and ¹³C₁-L-valine instead of L-valine, followed by chromatographic purification [16]. Dipeptide synthesis was performed by following a literature procedure (see Supporting Information).

Bacterial culture and growth conditions

Three *B. cereus* strains were employed for this study: the emetic reference strain F4810/72 [17], the foodborne intoxication strain WSBC 10925, which has recently been identified

as a high producer of cereulide [11], and an isogenic mutant of F4810/72 (F4810/72_mut) producing enhanced levels of cereulide variants as shown by a mass spectrometric prescreening. As previously reported [16], the reference strain F4810/72 was cultivated as follows: LB broth (100 mL) supplemented with D-glucose (0.2 %) was inoculated with an overnight pre-culture (10^3 cfu/mL) and then incubated for 24 h at 24 °C in baffled flasks (500 mL) whilst rotary shaking (150 rpm). *B. cereus* strains WSBC 10925 and F4801/72_mut were similarly inoculated with 10^3 cfu/mL in MOD-medium [18, 19], which was modified by adding sucrose (20 mM) and adjusting the final L-valine concentration to 20 mM while omitting L-isoleucine. In total, 16, 8, and 4 L of the cultures containing strain F4801/72, WSBC 10925, and F48010/72_mut, respectively, were produced. Thereafter, the cultures were autoclaved (15 min, 121 °C) and centrifuged ($7800\times g$, 20 °C, 12 min, Sigma 3-18 K), pellets from portions of 100 mL cultures were frozen in liquid nitrogen and then stored at -20 °C until further use.

Preparation of *B. cereus* culture extracts

Liquid cultures (100 portions) of *B. cereus* strains (F4801/72, WSBC 10925, and F48010/72_mut) were harvested by centrifugation, and bacteria were extracted with EtOH (10 mL) by shaking overnight (15 h) at room temperature, the solvent extracts were centrifuged twice ($7800\times g$; 12 min each), and the supernatants were membrane-filtered (0.2 μm ; PTFE; Phenomenex, Aschaffenburg, Germany) to remove remaining cells and debris. The EtOH extracts were stored at 4 °C until further use for isolation of cereulide and its structural variants, named isocereulides.

Isolation and structure determination of cereulide variants

The combined EtOH extracts obtained from bacteria cultures (100 mL) were concentrated to about 25 mL by means of a rotor evaporator, then diluted with H₂O (1:10), and aliquots (15 mL) were applied on top of a C18 SPE cartridge (60 mL, 10 g, Strata, Phenomenex), conditioned with MeOH (30 mL), followed with H₂O (2×60 mL). After sample application, the cartridge was rinsed with MeOH/H₂O (70/30, v/v; 30 mL), dried by sucking air into the cartridge by means of a vacuum pump (30 min), and, finally, rinsed with EtOH (30 mL) to elute the target compounds. The effluent collected from 20 SPE separations for each bacterial culture was combined, concentrated in vacuum to about 50 mL, followed by compound isolation by means of preparative RP-HPLC on a PrepStar system (Varian, Darmstadt, Germany) consisting of two HPLC-pumps (model SD-1), a two-wavelength UV detector (Prostar 325), a fraction collector (model 701), and equipped with a 250×21.2 mm, 4 μm , 90A, Jupiter Proteo column (Phenomenex). Monitoring the effluent at 220 nm, chromatography was performed at a flow rate of

18.0 mL/min starting with a MeOH/H₂O mixture (85/15, v/v) for 1 min, increasing the MeOH content to 100 % within 10 min, holding for 10 min, followed by a decrease of the MeOH content to 85 % within 1 min and re-equilibration for 2 min at 85 % MeOH. Separation of the isolates obtained from strains F4810/72 and WSBC 10925 afforded a total of 14 fractions collected between 6 and 18 min (Fig. 2). Chromatography of the isolates from strain F4810/72_mut yielded 22 fractions collected between 1 and 19 min. After separation of the solvent in vacuum, the residue of each fraction was suspended in H₂O (20 mL), freeze-dried twice, and kept at -20 °C until further use.

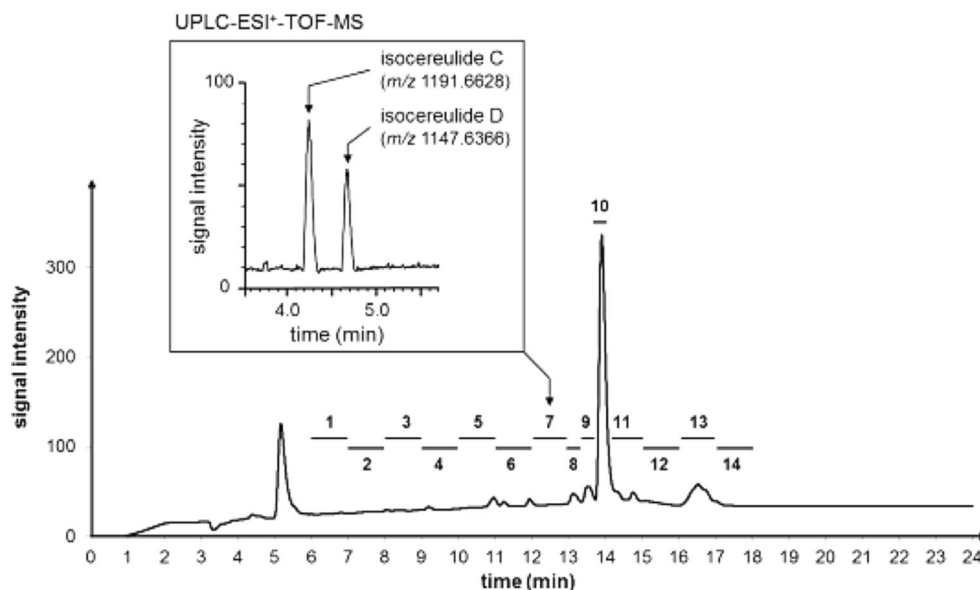
The individual HPLC fractions collected from the strain extracts were then screened for cereulide variants by means of the UPLC-TOF MS system G2-S (Waters, Manchester, UK) equipped with a 2.1×150 mm, 1.8 μm , HSS T3 C18 column (Waters) using the instrument settings and chromatographic conditions reported recently [11].

For structure elucidation of cereulide variants, the HPLC fractions obtained above were re-chromatographed using a 250×4.6 mm, 4 μm , 90 A, Jupiter Proteo column (Phenomenex) and a HPLC system consisting of a HPLC pump system PU-2080, a DAD/UV Detector MD-2010 Plus, and an autosampler AS-2055 (Jasco, Groß-Umstadt, Germany). A total of 18 cereulide variants were detected (Table 1), among which seven variants, coined isocereulides A–G, were purified and unequivocally identified (Table 2).

Alkaline hydrolysis of cereulide and isocereulides and analysis of cleavage dipeptides

Aliquots (~ 500 μg) of purified cereulide and isocereulides, respectively, were dissolved in methanolic KOH solution (1.2 M, 80 % MeOH), incubated at 50 °C for 2 h, then adjusted to pH 5 and analyzed by means of UPLC-TOF MS in the negative electrospray mode. Chromatography was performed on a 2.1×150 mm, 1.7 μm , BEH C18 column (Waters) at 45 °C using aqueous HCOOH (0.1 % in H₂O) as solvent A and MeCN containing 0.1 % HCOOH as solvent B and the following gradient (0.4 mL/min): for dipeptide analysis in hydrolysates obtained from isocereulide A (m/z 1189.6835), B (m/z 1161.6522), E (m/z 1161.6522), F (m/z 1189.6835), and G (m/z 1175.6679), chromatography started with 1 % solvent B for 2.5 min, which was increased to 95 % within 5.5 min, held for 1.5 min, and then decreased again to 1 % within 0.5 min. Analysis of the hydrolysate of isocereulide D (m/z 1147.6366) started with 1 % solvent B for 2.5 min, solvent B was then increased to 50 % within 5.5 min, held for 1.5 min, and was, finally, decreased again to 1 % within 0.5 min. Analysis of hydrolyzed cereulide (m/z 1175.6679) and isocereulide C (m/z 1191.6628), respectively, started with 10 % solvent B for 2.5 min, followed by an increase to 50 % within 5.5 min, and another increase to 100 % within 0.5 min, was kept for 1.0 min, and was then decreased

Fig. 2 (Marxen et al.) Preparative RP-HPLC separation of an ethanolic cell extract obtained from strain F4810/72 and UPLC-TOF MS (ESI⁺) detection of isocereulide C and D in HPLC fraction 7



to 10 % within 0.5 min. For TOF MS analysis, scan time for the MS^E method (centroid) was set to 0.1 s, high-resolution negative ionization mode, and the following ion source parameters were selected: capillary voltage (-2.5 kV), sampling cone (50 V), source temperature (150 °C), desolvation temperature (450 °C), cone gas (10 L/h), and desolvation gas (850 L/h). For the MS^E method, the second MS scan function used a transfer collision energy ramp from 20 to 40 eV.

Analysis of L-O-Ile and L-O-Leu in hydrolysates of isocereulide E

To prove that the structure of isocereulide E (*m/z* 1161.6522) contains besides the α -hydroxy acid 2-hydroxy-4-methylpentanoic acid (*O*-Leu) also 2-hydroxy-3-methylpentanoic acid (*O*-Ile), the purified material was treated by alkaline hydrolysis as described above and an aliquot (200 μ L) of the hydrolysate was subjected to acidic hydrolysis (110 °C, 24 h) with HCl (3 mL, 6 M). The hydrolysate was concentrated using solid phase extraction on a C18ec chromabond cartridge (6 mL, 1 g; Machery-Nagel, Düren, Germany) conditioned with MeOH (6 mL), followed with H₂O (6 mL). After application of the sample adjusted to pH 7, the cartridge was rinsed with H₂O (2 mL), dried by sucking air into the cartridge by means of a vacuum pump (10 min), and, finally, rinsed with MeOH (1 mL) to elute the target compounds. MeOH was evaporated using a stream of N₂, the sample was redissolved in H₂O (100 μ L) and analyzed by means of UPLC-TOF MS. Chromatography was performed on a 2.1 \times 150 mm, 1.7 μ m, BEH C18 column (Waters) at 45 °C using aqueous HCOOH (0.1 % in H₂O; solvent A) and MeCN (containing 0.1 % HCOOH; solvent B) and the following gradient (0.4 mL/min): starting with 10 % solvent B for 1.0 min, solvent B was increased to 15 % within

7.0 min and then to 100 % within 0.5 min, held for 0.5 min, and, finally, decreased to 10 % within 0.5 min. Comparison of chromatographic retention times with those of the reference compounds L-*O*-Ile and L-*O*-Leu was performed using UPLC-TOF MS operating in the ESI negative mode.

Acidic hydrolysis and amino acid analysis

For the determination of the amino acid configuration in dipeptides, showing another stereochemistry of the amino acid or the α -hydroxy acid compared with the cereulide components, the cereulide variants were first treated by alkaline hydrolysis as detailed above and then, aliquots (200 μ L) were treated with HCl (3 mL, 6 M) for 24 h at 110 °C, followed by enantioselective amino acid derivatization using OPA and IBLC [20]. To achieve this, the acidic hydrolysate was adjusted to pH 9 with aqueous KOH (6 M), followed by a saturated aqueous solution (600 μ L) of B₄K₂O₇ \times 4H₂O. Thereafter, the derivatization reagent (800 μ L, 85 mM OPA and 130 mM IBLC in saturated B₄K₂O₇ \times 4H₂O) and 1:2 diluted saturated B₄K₂O₇ \times 4H₂O (500 μ L) were added and kept at r.t. for 15 min. The sample was then adjusted to pH 7 with aqueous HCl (20 % in H₂O) and was then loaded on a C18ec, Chromabond C18-SPE cartridge (6 mL, 1 g, Machery-Nagel) conditioned with MeOH (6 mL), followed by H₂O (12 mL). After rinsing the cartridge with H₂O (6 mL), the analytes were eluted with MeOH (1 mL) and analyzed by means of UPLC-TOF MS (ESI⁻). For comparison, reference amino acids (L-/D-Val, L-/D-Ala, L-/D-Ser; 55 μ M, 600 μ L) were treated as described above with derivatization reagent (400 μ L), saturated B₄K₂O₇ \times 4H₂O solution (600 μ L), and 1:2 diluted saturated B₄K₂O₇ \times 4H₂O (1000 μ L), and were analyzed by means of UPLC-TOF MS (ESI⁻). Chromatography (0.3 mL/min; 45 °C) was done on a 2.1 \times 150 mm, 1.7 μ m BEH

Table 1 Chromatographic, mass spectrometric data, and cereulide variants **1-19** isolated from *B. cereus* cultures

No. ^{aa}	Variant ^b	F no. ^c	UPLC-TOF MS data				Structural modification ⁱ	
			RT ^d (min)	EM/AM ^e (var., ppm)	AM ^f (¹³ C ₁ -Val)	EC ^g Δmass ^h (Da)	Cereulide	Variant
1	Isocereulide C	I-7	4.25	1191.6628/1191.6615 (-1.1)	1197.6793 (¹³ C ₆)	C ₅₇ H ₉₆ N ₆ O ₁₉ Na (+16)	D-Ala	D-Ser
2	n.i.		4.45	1191.6628/1191.6648 (+1.7)	1197.6802 (¹³ C ₆)	C ₅₇ H ₉₆ N ₆ O ₁₉ Na (+16)	Ala	Ser
3	Isocereulide D	I-7	4.68	1147.6366/1147.6379 (+1.1)	1152.6511 (¹³ C ₅)	C ₅₅ H ₉₂ N ₆ O ₁₈ Na (-28)	L-Val	L-Ala
4			4.96	1147.6366/1147.6392 (+2.3)	1152.6520 (¹³ C ₅)	C ₅₅ H ₉₂ N ₆ O ₁₈ Na (-28)	O-Val/Val	O-Ala/Ala
5	n.i.		4.96	1205.6784/1205.6785 (+0.1)	1210.6947 (¹³ C ₅)	C ₅₈ H ₉₈ N ₆ O ₁₉ Na (+30)	O-Val/Val, Ala	O-Leu/Leu, Ser
6	n.i.		5.11	1174.6839/1174.6877 (+3.2)	1180.7015 (¹³ C ₆)	C ₅₇ H ₉₇ N ₇ O ₁₇ Na (+1)	peptide bond	ester bond
7	n.i.		5.27	1161.6522/1161.6564 (+3.6)	1167.6716 (¹³ C ₆)	C ₅₆ H ₉₄ N ₆ O ₁₈ Na (-14)	Ala	Gly
8	n.i.		5.27	1147.6366/1147.6360 (-0.5)	1153.6537 (¹³ C ₆)	C ₅₅ H ₉₂ N ₆ O ₁₈ Na (-28)	2x Ala	2x Gly
9	n.i.		5.41	1161.6522/1161.6538 (+1.4)	1167.6711 (¹³ C ₆)	C ₅₆ H ₉₄ N ₆ O ₁₈ Na (-14)	Ala	Gly
10	n.i.		5.41	1147.6366/1147.6416 (+4.4)	1155.6616 (¹³ C ₈)	C ₅₅ H ₉₂ N ₆ O ₁₈ Na (-28)	2x Leu	2x Val
11	n.i.		5.79	1147.6366/1147.6398 (+2.8)	1152.6517 (¹³ C ₅)	C ₅₅ H ₉₂ N ₆ O ₁₈ Na (-28)	O-Val/Val	O-Ala/Ala
12	Isocereulide B	II-9	5.97	1161.6522/1161.6528 (+0.5)	1168.6761 (¹³ C ₇)	C ₅₆ H ₉₄ N ₆ O ₁₈ Na (-14)	D-O-Leu	D-O-Val
13	Isocereulide E	II-9	5.97	1161.6522/1161.6543 (+1.8)	1165.6656 (¹³ C ₄)	C ₅₆ H ₉₄ N ₆ O ₁₈ Na (-14)	D-O-Leu	O-Ile
14	Cereulide	I-10	6.52	1175.6679/1175.6697 (+1.5)	1181.6880 (¹³ C ₆)	C ₅₇ H ₉₆ N ₆ O ₁₈ Na (-)	-	-
15	n.i.		6.84	1189.6835/1189.6891 (+4.7)	1194.6965 (¹³ C ₅)	C ₅₈ H ₉₈ N ₆ O ₁₈ Na (+14)	O-Val/Val	O-Leu/Leu
16	Isocereulide G	II-11	6.91	1175.6679/1175.6678 (-0.1)	1181.6849 (¹³ C ₆)	C ₅₇ H ₉₆ N ₆ O ₁₈ Na (-)		CI
17	Isocereulide A	I-11	7.11	1189.6835/1189.6837 (+0.2)	1194.6992 (¹³ C ₅)	C ₅₈ H ₉₈ N ₆ O ₁₈ Na (+14)	L-O-Val	L-O-Leu
18	Isocereulide F	II-11	7.27	1189.6835/1189.6852 (+1.4)	1197.6873 (¹³ C ₈)	C ₅₈ H ₉₈ N ₆ O ₁₈ Na (+14)		CI
19	n.i.		8.07	1203.6992/1203.7017 (+2.1)	1207.7112 (¹³ C ₄)	C ₅₉ H ₁₀₀ N ₆ O ₁₈ Na (+28)	2x O-Val/Val	2x O-Leu/Leu

n.i. not identified, CI constitutional isomer

^a Compound number of cereulide variant detected

^b The structures of identified cereulide variants are given in Fig. 4

^c HPLC fraction used for isolation of target compounds from *B. cereus* strain culture F4810/72 (I) and WSBC 10925 (II), respectively

^d Retention time on RP-18 UPLC

^e Exact mass (EM, calcd) and accurate mass (AM) of analytes [M+Na]⁺ ion determined by means of TOF MS

^f Accurate mass of the [M+Na]⁺ ion of the ¹³C-enriched analyte detected in the ¹³C₁-valine supplemented culture

^g Elemental composition of analyte

^h Mass difference between cereulide and the target variant

ⁱ Amino acids and/or α-hydroxy acids in cereulide are replaced by the ones given under variants

C18 column (Waters) starting with a mixture (1/99, v/v) of MeOH and 0.1 % HCOOH; after maintaining for 1 min, increasing to 60 % MeOH within 39 min and then to 100 % within another 10 min, held for 4 min, and then, decreased again to 1 % within 1 min, followed by re-equilibration (5 min).

Analysis of the isoindole derivative generated was performed using the same MS parameters as described above for alkaline hydrolysis. The analytical data obtained for the individual amino acid derivatives are the following: L-Ser derivative: accurate mass: *m/z* 393.1117, Δ (ppm): -0.8; calcd: *m/z* 393.1120 (C₁₈H₂₁N₂O₆S); D-Ser derivative: accurate mass: *m/z* 393.1121, Δ (ppm): +0.3; calcd: *m/z* 393.1120 (C₁₈H₂₁N₂O₆S); L-Ala derivative: accurate mass: *m/z* 377.1171, Δ (ppm): -0.0; calcd: *m/z* 377.1171 (C₁₈H₂₁N₂O₅S); D-Ala derivative: accurate mass: *m/z* 377.1172, Δ (ppm): +0.3; calcd: *m/z* 377.1171

(C₁₈H₂₁N₂O₅S); L-Val derivative: accurate mass: *m/z* 405.1486, Δ (ppm): +0.5; calcd: *m/z* 405.1484 (C₂₀H₂₅N₂O₅S); D-Val derivative: accurate mass: *m/z* 405.1483, Δ (ppm): -0.2; calcd: *m/z* 405.1484 (C₂₀H₂₅N₂O₅S).

Planar bilayer formation and measurements of the membrane conductance

Planar lipid bilayers (~200 μm i.d.) were formed from a polar lipid extract of *Escherichia coli* (Avanti Polar Lipids, Inc., Alabaster, AL, USA) on the tip of plastic pipettes as previously described [15] in a solution containing 100 mM KCl, 10 mM MES, 10 mM TRIS, and 0.6 mM EGTA. The membrane conductance and capacity, which were measured by a patch-clamp amplifier (EPC 10; HEKA Elektronik Dr. Schulze GmbH, Lambrecht, Germany), were performed to

Table 2 UPLC-TOF MS (ESI)-data of dipeptides and ratio of L/D-amino acids after hydrolytic release from cyclic depsipeptides

Variant ^a	Copy number ^b of		Peptide ratio ^c		L/D-amino acid ratio ^d (%)			Additional dipeptide			
	D- <i>O</i> -Leu-D-Ala	L- <i>O</i> -Val-L-Val	A	T	L/D-Ser	L/D-Ala	L/D-Val	AM (<i>m/z</i>) ^e (var., ppm)	EM (<i>m/z</i>) ^f	EC ^g	Sequence
Cereulide	3	3	0.87	1.05	—/—	1.1/98.9	99.0/1.0				
Isocereulide F	2	4	0.51	0.53	—/—	1.6/98.4	98.3/1.7				
Isocereulide G	3	3	0.84	1.05	—/—	1.1/98.9	96.8/1.2				
Isocereulide A	3	2	1.00	1.60	—/—	1.4/98.6	97.6/2.4	230.1393 (+0.4)	230.1392	C ₁₁ H ₂₀ NO ₄	L- <i>O</i> -Leu-L-Val
Isocereulide B	2	3	0.54	0.70	—/—	3.5/96.5	99.2/0.8	188.0920 (−1.6)	188.0923	C ₈ H ₁₄ NO ₄	D- <i>O</i> -Val-D-Ala
Isocereulide C	2	3	0.49	0.70	11.7/88.3	1.9/98.1	99.1/0.9	218.1025 (−1.4)	218.1028	C ₉ H ₁₆ NO ₅	D- <i>O</i> -Leu-D-Ser
Isocereulide D	3	2	0.82	1.60	—/—	19.0/81.0	99.2/0.8	188.0919 (−2.1)	188.0923	C ₈ H ₁₄ NO ₄	L- <i>O</i> -Val-L-Ala
Isocereulide E	3	2	0.91	1.60	—/—	3.0/97.0	98.7/1.3	202.1077 (−1.0)	202.1079	C ₉ H ₁₄ NO ₄	<i>O</i> -Ile-D-Ala

^a The structures of identified cereulide variants are given in Fig. 4

^b Copy number of the dipeptides D-*O*-Leu-D-Ala (AM: *m/z* 202.1081 (+1.0 ppm), EM: *m/z* 202.1079; C₉H₁₆NO₄) and L-*O*-Val-L-Val (AM: *m/z* 216.1249 (+1.9 ppm), EM: *m/z* 216.1236; C₁₀H₁₈NO₄) in the dodecadepsipeptide

^c The analyzed (A) molar ratio was determined by multiplying peak area ratios of both target dipeptides obtained by UPLC-TOF MS after alkaline hydrolysis considering a determined MS response factor of 1.05; the theoretical (T) molar ratio was calculated by multiplying the ratio of the theoretical amounts of the dipeptide D-*O*-Leu-D-Ala to L-*O*-Val-L-Val with the determined MS response factor of 1.05 (see Supporting Information)

^d Ratio of L- and D-amino acids released after acidic hydrolysis of the dipeptide mixture formed from dodecadepsipeptide under alkaline conditions

^e Accurate mass of analytes [M – H][−] ion determined by means of UPLC-TOF MS

^f Exact mass calculated for the [M – H][−] ion of the analyte

^g Elemental composition of the analyte

ensure the correct lipid bilayer formation and integrity. A linear fit of current data at applied voltages (−50 to 50 mV) was used for determining the total membrane conductance as reported earlier [21]. Aliquots of ethanolic solutions of the 22 HPLC fractions isolated from the F4810/72_mut culture were added to the lipid bilayer membranes at a concentration of 1 ng/mL. Solutions of HPLC fractions lacking cereulide homologues (negative control) as well as 1 % aqueous EtOH were used for control measurements.

HEp-2 cell cytotoxicity bioassay

The cytotoxicity of the 22 HPLC fractions isolated from the F4810/72_mut was measured with a cell culture assay employing HEp-2 cells. The HPLC fractions were dissolved in 99 % HPLC-grade EtOH to a final concentration of 2 mg/mL and the cytotoxic activity of the fractions was tested with a maximum concentration of 500 ng in the HEp-2 cell-based cell culture assay as described previously [13, 14]. The structurally related ionophore valinomycin served as the internal control standard.

Mass spectrometry

High-resolution mass spectrometric analyses (UPLC-ESI-TOF MS) were performed on a Waters Synapt G2-S HDMS

spectrometer coupled to an Acquity UPLC core system (Waters, Milford, MA, USA) consisting of a binary solvent manager, sample manager, and column. Data processing was performed by using MassLynx 4.1 SCN 851 (Waters, Manchester, UK) and the elemental composition tool for determining the accurate mass. All data were lock mass corrected on the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, *m/z* 556.2771, [M+H]⁺ and *m/z* 554.2615, [M–H][−]) in a solution (2 ng/μL) of MeCN/0.1 % HCOOH (1/1, v/v). Scan time for the lock mass was set to 0.3 s, an interval of 10 s, and 3 scans to average with a mass window of ±0.5 Da. Calibration of the Synapt G2-S in the range from *m/z* 50 to 1300 was performed using a solution of sodium formate (5 mmol/L) in 2-propanol/H₂O (9/1, v/v). The UPLC and Synapt G2-S systems were operated with MassLynx software (Waters, Manchester, UK).

MSⁿ-experiments were performed on a Bruker Daltonics HCTultra PTM Discovery System (Bruker Daltonics Inc., Billerica, MA, USA) using direct sample infusion via a syringe pump (4 μL/min; KD Scientific, Holliston, MA, USA) and manual MSⁿ (*m/z* 50–1300) using the following instrument parameters: ultrascan mode (26,000 *m/z*/s), max. accumulation time (300 ms), isolation width (*m/z* 4), collision amplitude (1 V; ramp: 30 %–200 %). The following source parameters were applied: capillary voltage (+4 kV), end plate offset (−500 V), nebulizer (30 psi), dry gas (8 L/min), dry temperature (300 °C), skimmer (40 V), capillary exit (166 V). Data

acquisition and processing were done by using Bruker Daltonics Data Analysis ver. 3.4 (Bruker Daltonics Inc.).

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy was performed on an Avance III 400 MHz spectrometer with a Broadband Observe BBFOplus and on an Avance III 500 MHz spectrometer with a CTCI probe (Bruker, Rheinstetten, Germany). The solvent signal (d_4 -MeOH) was used for referencing chemical shifts. Data processing was performed by using Topspin ver. 3.2/3.0 (Bruker, Rheinstetten) and MestReNova ver. 7.0.2-8636 software (Mestrelab Research, Santiago de Compostela, Spain).

Results and discussion

The mass spectrometric strain profiling of a total of 78 *B. cereus* strains recently indicated a series of cereulide variants in strains producing high levels of the emetic toxin [11]. As the yields of these variants were too low for an unequivocal structure determination, first the cultivation conditions were optimized to allow the production of increased levels of the cereulide variants. TOF MS analysis of a series of *B. cereus* culture incubations varying in the carbon source and amino acids revealed increased yields of cereulide variants by cultivation of *B. cereus* in a mineral medium, which was supplemented with sucrose (20 mM) and L-valine (20 mM), while L-isoleucine was lacking.

Mass spectrometric detection and isolation of cereulide variants

Targeting the isolation of cereulide variants, the emetic reference strain F4810/72 [17], the foodborne intoxication strain WSBC 10925, which has recently been identified as a high producer of cereulide [11], and F4810/72_mut, an isogenic mutant of F4810/72 producing enhanced levels of cereulide variants, were cultivated under tailored conditions, autoclaved, centrifuged, and the pellets were extracted with EtOH. The extracts prepared from strain F4810/72 and WSBC 10925, respectively, were separated by HPLC to give 14 fractions. As exemplified in Fig. 2 for strain F4810/72, a series of minor peaks were detectable in a rather small retention time window of 4 min before and after the major cereulide peak, thus suggesting the occurrence of cereulide variants showing minor differences in hydrophobicity. To gain a more detailed insight into the individual cereulide variants, the effluent of each of the individual HPLC fractions was collected and analyzed by means of UPLC-TOF MS (Table 1).

Cereulide and 18 additional cereulide variants, 1–19 (Table 1), were detected with accurate masses between 1191.6615 and 1205.6785 Da and showed a constant ratio

of ~100:40:25 for the pseudomolecular ions $[M+Na]^+$, $[M+K]^+$, and $[M+NH_4]^+$ (see [Electronic Supplementary Material \(ESM\) Figure S1](#)), thus indicating the ability of 1–19 to form high-affinity complexes with alkali metal and ammonium ions as reported for cereulide [7, 9]. This characteristic cation cluster helped to locate cereulide-type molecules in the culture extracts. A total of seven variants, namely isocereulide A (m/z 1189.6835), B (m/z 1161.6522), C (m/z 1191.6628), D (m/z 1147.6366), E (m/z 1161.6522), F (m/z 1189.6835), and G (m/z 1175.6679), respectively, were isolated in suitable amounts to enable structure determination (Table 1). As each isocereulide was found to be produced by both strains, F4810/72 and WSBC 10925, but in somewhat different ratios (data not shown), cereulide (fraction 10) and isocereulide A (fraction 11), C (fraction 7), and D (fraction 7) were isolated from the F4810/72 culture, isocereulide B (fraction 9), E (fraction 9), F (fraction 11), and G (fraction 11) were purified from the WSBC 10925 culture. As an example, UPLC-TOF MS analysis revealed the pseudomolecular ions of isocereulides C and D in HPLC fraction 7 of strain F4810/72 (Fig. 2), from which they were purified by means of analytical HPLC.

Structure determination of isocereulides

In order to gain a first insight into the substituted amino acids and/or α -hydroxy acids in the isocereulides A–G, the accurate mass of their $[M+Na]^+$ ions were compared with that of purified cereulide (m/z 1175.6697). For example, isocereulide D (m/z 1147.6379) showed a mass shift of –28 Da, thus indicating the lack of two methylene groups and matching with the elemental composition of $C_{55}H_{92}N_6O_{18}Na$ (Table 1). Compared with cereulide (3x Ala, 3x Val, 3x *O*-Val, 3x *O*-Leu), the MS data showed evidence for the replacement of either two *O*-Leu moieties by *O*-Val, two Ala moieties by Gly, or *O*-Val by *O*-Ala and Val by Ala (Table 1). To narrow down the number of candidate structures for the isocereulides A–G, a stable isotope labeling experiment was performed by cultivating strain F4810/72 in the presence of $^{13}C_1$ -L-Val instead of L-Val. Comparative UPLC-TOF MS analysis of the natural abundant isocereulides and their corresponding isotopologue obtained upon $^{13}C_1$ -L-Val supplementation revealed the presence of a $^{13}C_4$ -labeled isocereulide E, $^{13}C_5$ -labeled isocereulides A and D, $^{13}C_6$ -labeled isocereulides C and G, $^{13}C_7$ -labeled isocereulide B, and $^{13}C_8$ -labeled isocereulide F (Table 1). In addition, $^{13}C_6$ -cereulide was generated, which has recently been reported to show 95 % ^{13}C enrichment in the carbonyl atoms of the three L-Val and the three L-*O*-Val moieties [16] (Table 1). For example, the increase of the accurate mass of isocereulide D by five units upon $^{13}C_1$ -L-Val supplementation clearly demonstrated that either one Val or *O*-Val is replaced by Ala or *O*-Ala compared with cereulide.

In order to investigate the replacement of single amino or hydroxy acids in isocereulides, the dipeptides released from

cereulide and the individual isocereulides upon alkaline ester hydrolysis were analyzed. Well in line with the [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]₃ units in cereulide, UPLC-TOF MS analysis revealed two peaks in the hydrolysate corresponding to the dipeptides D-*O*-Leu-D-Ala (m/z 202.1079, [M – H][–]) and L-*O*-Val-L-Val (m/z 216.1236, [M – H][–]). Considering slightly different mass responses of the individual dipeptides (MS response factor: 1.05), UPLC-TOF MS analysis revealed the target dipeptides D-*O*-Leu-D-Ala and L-*O*-Val-L-Val in a ratio of 0.87, which is close to the equimolar ratio in cereulide (Table 2).

Comparative MS analysis of the alkaline hydrolysate of isocereulide D revealed D-*O*-Leu-D-Ala, L-*O*-Val-L-Val, and another dipeptide (m/z 188.0923, [M – H][–]; C₈H₁₄NO₄) in a ratio of 3:2:1 (Fig. 3a), thus indicating the exchange of

one *O*-Val or one Val moiety in one of cereulide's three L-*O*-Val-L-Val motifs by *O*-Ala or Ala (Table 2). The discrimination between the exchanged amino acid and the hydroxy acid within the candidate dipeptide was possible by the specific MS fragmentation pattern recorded by means of UPLC-ESI-TOF MS^E. MS analysis of the dipeptide L-*O*-Val-L-Val (m/z 202.1079, [M – H][–]) released from a cereulide reference sample showed the expected fragment ion m/z 172.1335 upon decarboxylation and m/z 116.0708 upon fragmentation of the amino bond (ESM Figure S2a). In comparison, the candidate dipeptide (m/z 188.0923, [M – H][–]) released from isocereulide D showed other fragment ions with m/z 144.1021 and 88.0396, respectively, fitting well to those expected for L-*O*-Val-D/L-Ala (ESM Figure S2b) and indicating the exchange of one Val moiety in cereulide by Ala (Table 2).

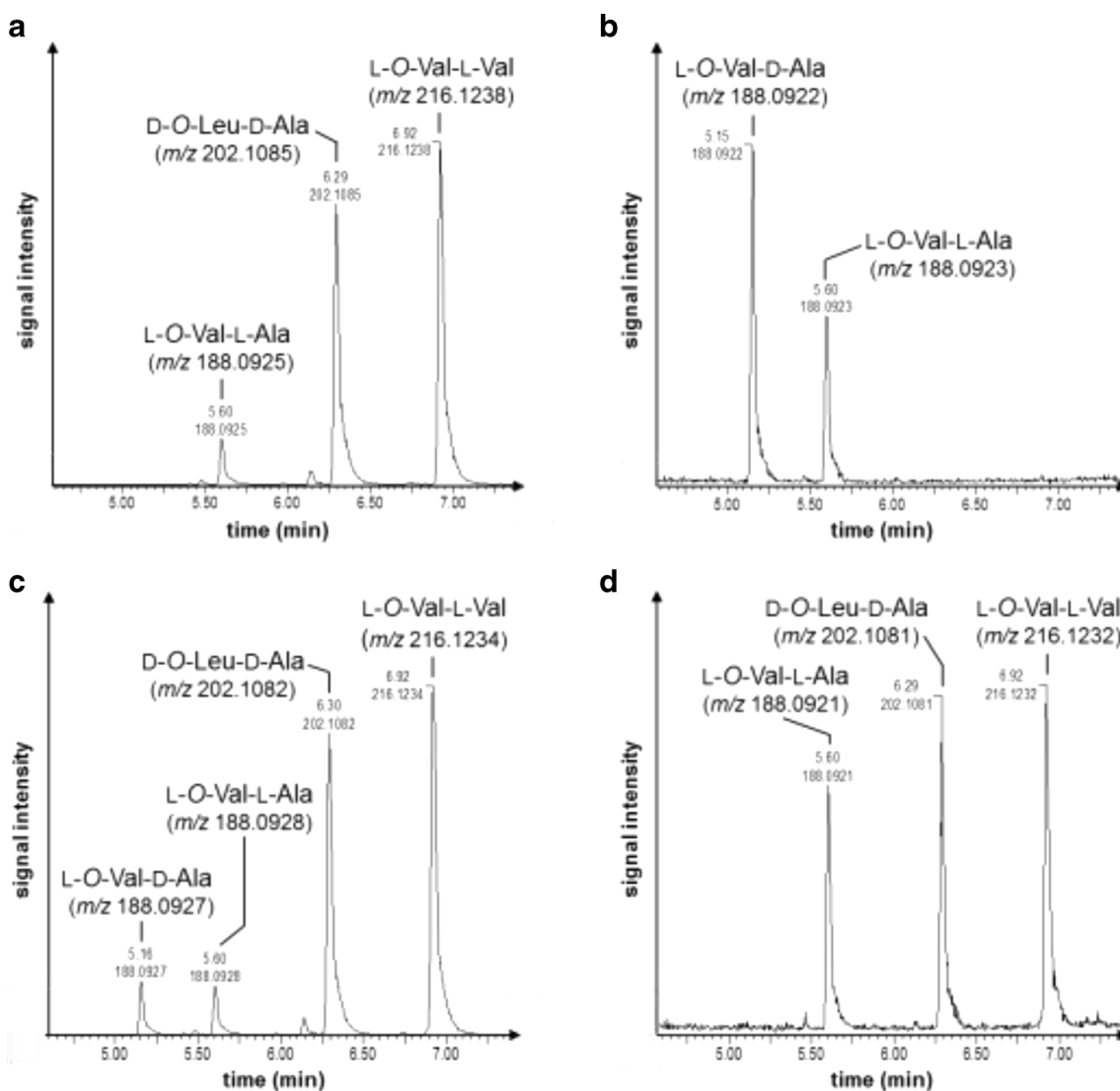


Fig. 3 (Marxen et al.) UPLC-TOF MS (ESI[–]) analysis of (a) the alkaline hydrolysate of isocereulide D, (b) a mixture of synthetic L-*O*-Val-D-Ala and L-*O*-Val-L-Ala, (c) the alkaline isocereulide D hydrolysate spiked

with synthetic L-*O*-Val-D-Ala, and (d) alkaline isocereulide D hydrolysate spiked with synthetic L-*O*-Val-L-Ala

To elucidate the stereochemistry of the amino acid and α -hydroxy acid moieties in the cleavage peptides, candidate dipeptides were prepared by means of solid-phase peptide synthesis (e.g., L-*O*-Val-L-Ala and L-*O*-Val-D-Ala were synthesized for the structure elucidation of isocereulide D.) Comparative UPLC-TOF MS analysis of the alkaline hydrolysate of isocereulide D (Fig. 3a) and a mixture of the synthesized diastereomers L-*O*-Val-D-Ala and L-*O*-Val-L-Ala (Fig. 3b) showed the same retention time (5.60 min) for the candidate peptide released from cereulide and the L-*O*-Val-L-Ala reference. Co-chromatography of the isocereulide D hydrolysate and the reference material of L-*O*-Val-D-Ala (Fig. 3c) or L-*O*-Val-L-Ala (Fig. 3d), respectively, gave evidence for L-*O*-Val-L-Ala as the peptide in isocereulide D.

However, as L-*O*-Val-L-Ala would co-elute with its enantiomeric D-*O*-Val-D-Ala, the latter dipeptide could not be ruled out as a potential partial structure in isocereulide D. To exclude this possibility, the dipeptides released from cereulide and isocereulides upon alkaline hydrolysis were cleaved into free amino acids and α -hydroxy acids by means of acidic hydrolysis. In a control experiment, a cereulide reference was treated alike. After OPA/IBLC-derivatization, the diastereomeric isoindoles of the amino acids were analyzed by UPLC-ESI-TOF MS, followed by comparison of exact mass and retention time with those of enantiopure amino acid references each derivatized using the same procedure (ESM Figure S3a, b). Control experiments with enantiopure amino acids did not show more than 0.2–0.9 % of racemization (ESM Figure S3c), thus demonstrating only a marginal degree of racemization artificially induced during the acidic/alkaline sample treatment. For example, analysis of the isocereulide D hydrolysate revealed a L/D-Ala ratio of 19.0/81.0 and L/D-Val ratio of 99.2/0.8 (Table 2, ESM Figure S3c), which is in agreement with the theoretical composition of 25 % L-Ala, 75 % D-Ala, 100 % L-Val, and the absence of D-Val. In consequence, the structure of isocereulide D comprises one L-*O*-Val-L-Ala, two L-*O*-Val-L-Val, and three D-*O*-Leu-D-Ala dipeptides (Fig. 4a).

The dipeptides identified still did not enable an unequivocal structure determination of the isocereulides as the theoretical recombination of the dipeptides allows for various constitutional arrangements in the cyclic dodecadepsipeptides, e.g., the three dipeptides identified in isocereulide D can be arranged to give six theoretical combinations (Fig. 4b). Therefore, the constitutional arrangement of the dipeptides in the target molecules were determined by means of MSⁿ experiments. Utilizing the favored ester cleavage during MS analysis, cereulide and each isocereulide were expected to deliver a maximum of six open-chain pseudomolecular ions, among which one was selected as identifier ion for MSⁿ sequencing (Fig. 5, ESM Figure S4a–h). Using cereulide as a reference and the candidate isocereulide D as an example, MS, MS², and MS³ experiments revealed a similar fragmentation

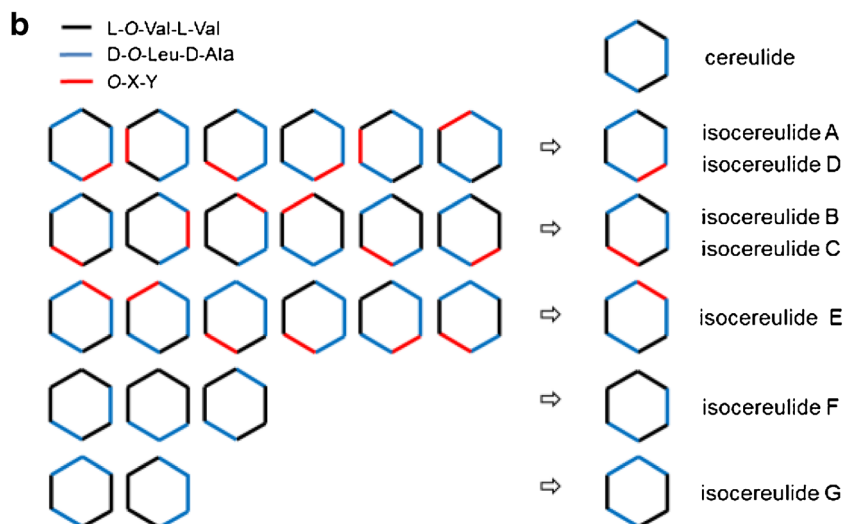
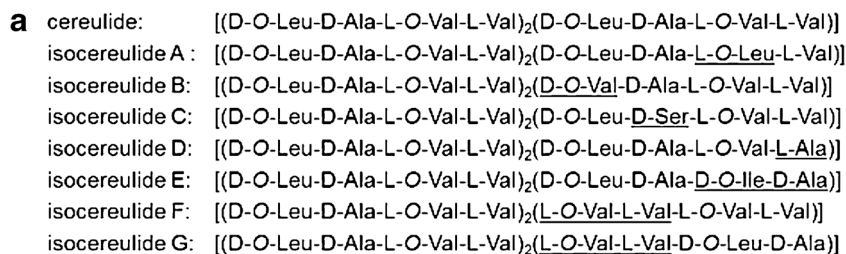
pattern for both molecules just with a mass shift of 28 Da between cereulide (m/z 1191.7 \rightarrow 992.6 \rightarrow 807.5) and isocereulide D (m/z 1163.7 \rightarrow 964.5 \rightarrow 779.4) (ESM Figure S4a, e). The detection of both fragment ions m/z 608.3 and 708.4 in the MS⁴ spectrum of cereulide (m/z 807.5 \rightarrow 708.4/608.3) and isocereulide D (m/z 779.4 \rightarrow 708.4/608.3) clearly indicated the exchange of a Val by an Ala moiety in the latter compound. Subsequent MS⁵ and MS⁶ experiments revealed again the same fragmentation pattern (m/z 608.3 \rightarrow 423.1 \rightarrow 324.2) for both molecules, thus demonstrating that the basic structure of cereulide is conserved in isocereulide D with the exception of the substitution of one Val by an Ala moiety. The sequence of the previously not reported isocereulide D was therefore identified as [(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)₂(D-*O*-Leu-D-Ala-L-*O*-Val-L-Ala)] (Fig. 4a) with the constitution shown in Fig. 4b.

Using the same analytical strategy, the structure of each isocereulide was unequivocally identified (Fig. 4a, b, Table 1). The structures of isocereulide A and B were well in line with previously reported MS data indicating the occurrence of two cereulide variants showing a substitution of *O*-Val by *O*-Leu and *O*-Leu by *O*-Val, respectively [12]. Interestingly, for none of the isocereulides was the replacement of an amino or an α -hydroxy acid accompanied by a change in stereochemistry.

In contrast to isocereulides A–D, alkaline hydrolysis of isocereulide F and G did not reveal any additional dipeptide next to cereulide's dipeptides D-*O*-Leu-D-Ala and L-*O*-Val-L-Val (Fig. 4, Table 2). Differing from cereulide by +14 Da, hydrolysis of isocereulide F (m/z 1189.6835) showed D-*O*-Leu-D-Ala and L-*O*-Val-L-Val in a molar ratio of 0.51 fitting with a theoretic ratio of 1:2 (Table 2). MSⁿ experiments revealed the structure of isocereulide F to be [(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)₂(L-*O*-Val-L-Val-L-*O*-Val-L-Val)] (Figs. 4a, b and 5). Hydrolysis of isocereulide G (m/z 1175.6679) delivered the same dipeptides D-*O*-Leu-D-Ala and L-*O*-Val-L-Val (1:1) as found for the isobaric cereulide (Table 2). MSⁿ analysis clearly identified isocereulide G as the constitutional cereulide isomer [(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)₂(L-*O*-Val-L-Val-D-*O*-Leu-D-Ala)] (Figs. 4a, b and 5).

Interestingly, alkaline hydrolysis of isocereulide E (m/z 1161.6522) released D-*O*-Leu-D-Ala (m/z 202.1079), L-*O*-Val-L-Val (m/z 216.1236), and another dipeptide isobaric to D-*O*-Leu-D-Ala (m/z 202.1079), in a molar ratio of 3:2:1 (Table 2). Synthesis of reference peptides and enantioselective analysis of hydrolytically released amino acids excluded the stereoisomers L-*O*-Leu-D-Ala, D-*O*-Leu-L-Ala, and L-*O*-Leu-L-Ala, respectively, as potential candidates of the latter dipeptide. Acidic hydrolysis of the alkaline hydrolysate of isocereulide E, followed by LC-MS analysis and co-chromatography with and without a L-*O*-Ile reference

Fig. 4 (a) Chemical structures for cereulide and isocereulides A–G with compositional changes underlined, (b) theoretical constitutional isomers (left) and constitution of isocereulides elucidated by MSⁿ sequencing (right)



confirmed the exchange of *O*-Leu in cereulide by the isobaric *D*-*O*-Ile or *L*-*O*-Ile, respectively, in isocereulide E. The synthesized *L*-*O*-Ile-*D*-Ala was chromatographically well separated from the isobaric candidate dipeptide released from isocereulide E, thus ruling out the presence of *L*-*O*-Ile-*D*-Ala and its enantiomer *D*-*O*-Ile-*L*-Ala. Amino acid analysis of isocereulide E showed a composition of 3.0 % *L*-Ala, 97.0 % *D*-Ala, 98.7 % *L*-Val, and 1.3 % *D*-Val (Table 2). Any *L*-Ala containing dipeptide could be excluded as they would result in a theoretical *D*/*L*-Ala ratio of 1:2, which is not in line with the experimental findings. Taking all these data into account, the structure of isocereulide E comprises three *D*-*O*-Leu-*D*-Ala, two *L*-*O*-Val-*L*-Val, and one *O*-Ile-*D*-Ala peptide and is proposed as [(*D*-*O*-Leu-*D*-Ala-*L*-*O*-Val-*L*-Val)₂(*D*-*O*-Leu-*D*-Ala-*D*-*O*-Ile-*D*-Ala)] on the basis of MSⁿ sequencing (Fig. 4a, b).

Although variants of non-ribosomal produced peptide toxins are known from a broad variety of cyanobacteria and are commonly based on the molecular diversity in the structural genes [22], the isocereulides reported here are produced by one single non-ribosomal peptide synthetase, known as Ces-NRPS, which is encoded on a mega plasmid [3]. Since the genome of the reference strains F4810/72 has been fully sequenced, it can be excluded that potential paralogous *ces* genes confer the observed structural diversity. It can therefore be assumed that all isocereulides are synthesized by the Ces-NRPS [3, 23]. Although ATP exchange assays demonstrated a

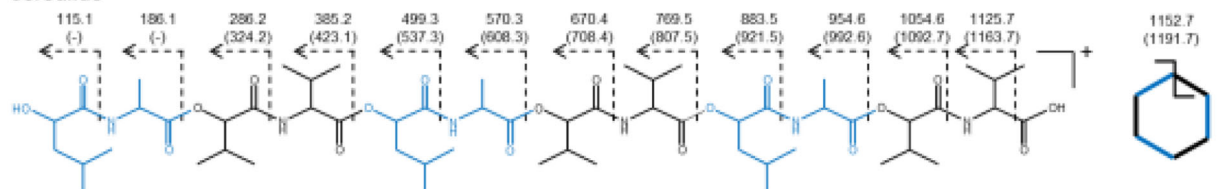
strict monomer preference for Ces and a thioesterase II (*cesT*), which is co-transcribed with the structural *ces* genes as a polycistronic transcript embedded in the *ces* gene locus [3, 24], a high microheterogeneity in cereulide was reported for the first time. Type II thioesterases embedded in NRPS gene loci are known for their proof-reading function and contribute to the NRPS substrate specificity by removing misprimed monomers [5]. It is therefore tempting to speculate that the relaxed proof-reading function of CesT is giving rise to an enhanced chemodiversity of cereulide. Interestingly, all three strains analyzed produced the same variants although at different amounts.

Cytotoxic and membrane activity of cereulide and isocereulides

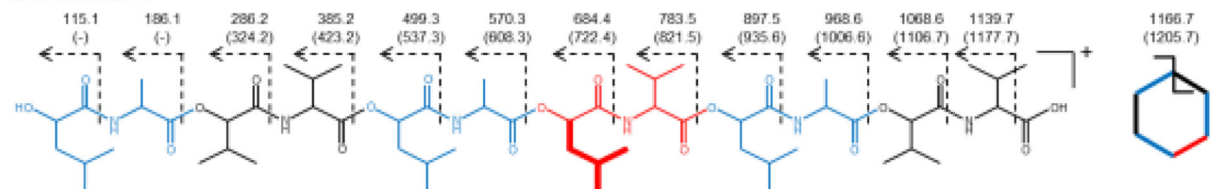
To reveal whether the isocereulides are differently toxic to living cells, we evaluated the effect of 22 HPLC fractions (F1–F22, Fig. 6a) in the HEP2-cell culture assay designed to respond specifically to ionophores causing the uncoupling of

Fig. 5 MSⁿ sequencing of selected pseudomolecular ions of cereulide and isocereulides (upper masses: calculated, lower masses given in parenthesis: measured [M+K]⁺ ions) and position of cleaved ester bond in the ring structures (black: *L*-*O*-Val-*L*-Val; blue: *D*-*O*-Leu-*D*-Ala; red: *L*-*O*-Leu-*L*-Val, *D*-*O*-Val-*D*-Ala, *D*-*O*-Leu-*D*-Ser, *L*-*O*-Val-*L*-Ala, or *D*-*O*-Ile-*D*-Ala)

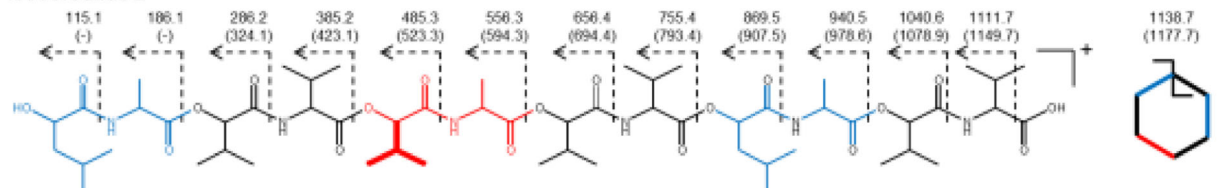
cereulide



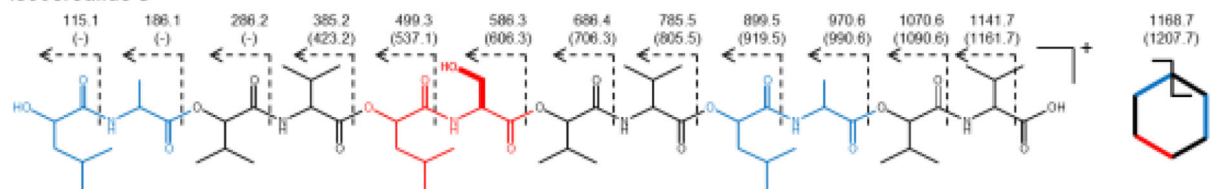
isocereulide A



isocereulide B



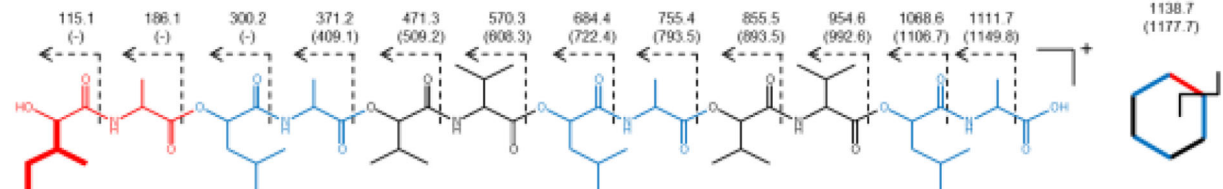
isocereulide C



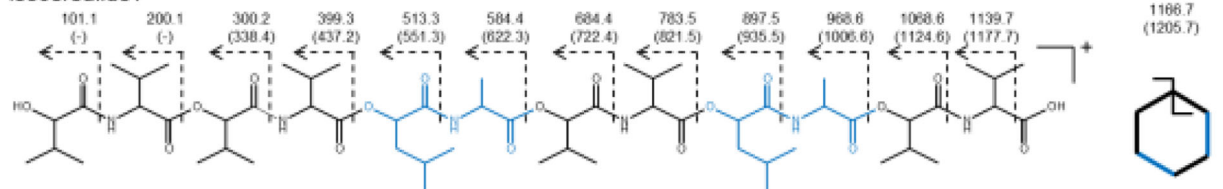
isocereulide D



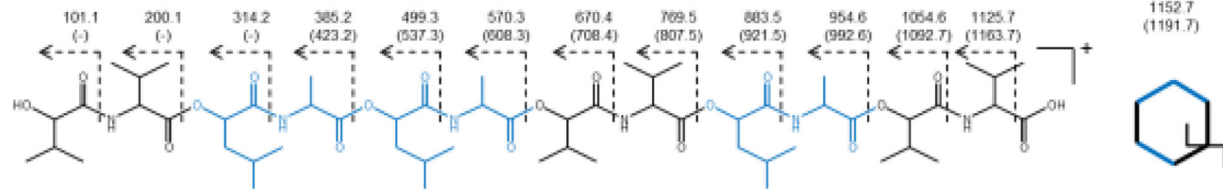
isocereulide E



isocereulide F



isocereulide G



ATP synthesis by facilitating an ion influx into mitochondria and the disruption of the membrane potential [13, 14, 25]. By far the highest bioactivities were found for HPLC fraction F16–18. Whereas cereulide was the major component in fraction F16/F17, isocereulide A and F were predominant in fraction F18 and showed the highest cytotoxicity in the test (Fig. 6a, c). The purified isocereulide A sample was found to show an approximately eight times increased cytotoxic activity in the HEp-2 bioassay compared with the well-known cereulide [e.g., 10, 26].

Previously, toxicity of cereulide was reported to be due to its function as a K^+ ionophore [9, 10]. We now hypothesized that the different activity of HPLC fractions in the HEp2 assay can be explained by the different K^+ transport properties of cereulide variants and their different concentration in each fraction. To test this hypothesis, we evaluated the effect of the 22 fractions (Fig. 6b) on the conductance of bilayer

membranes prepared from *E. coli* polar lipids. Neither fractions lacking isocereulides (F1–11) nor 1 % aqueous EtOH (vehicle) changed the basic membrane conductance ($G_0=13.4$ nS/cm²). In contrast, the addition of F12–19 induced an increase in membrane conductance to different extent (e.g., a 30, 34, 50, and 11 times increase in membrane conductance was measured in the presence of F16, F17, F18, and F19, respectively). This matched the results of the cytotoxicity assay (Fig. 6a) and indicated that the fractions' toxic effect on cells is associated with the ionophoric properties of (iso)cereulides.

UPLC-TOF MS analysis identified cereulide to be present predominantly in the membrane-active fractions F16 and F17 (Fig. 6c). However, most interestingly, isocereulide A and F, accompanied by only trace amounts of cereulide, were the major components in fraction F18 inducing by far the highest increase in membrane conductance (Fig. 6b, c). To reveal whether the more hydrophobic homologues would cause a

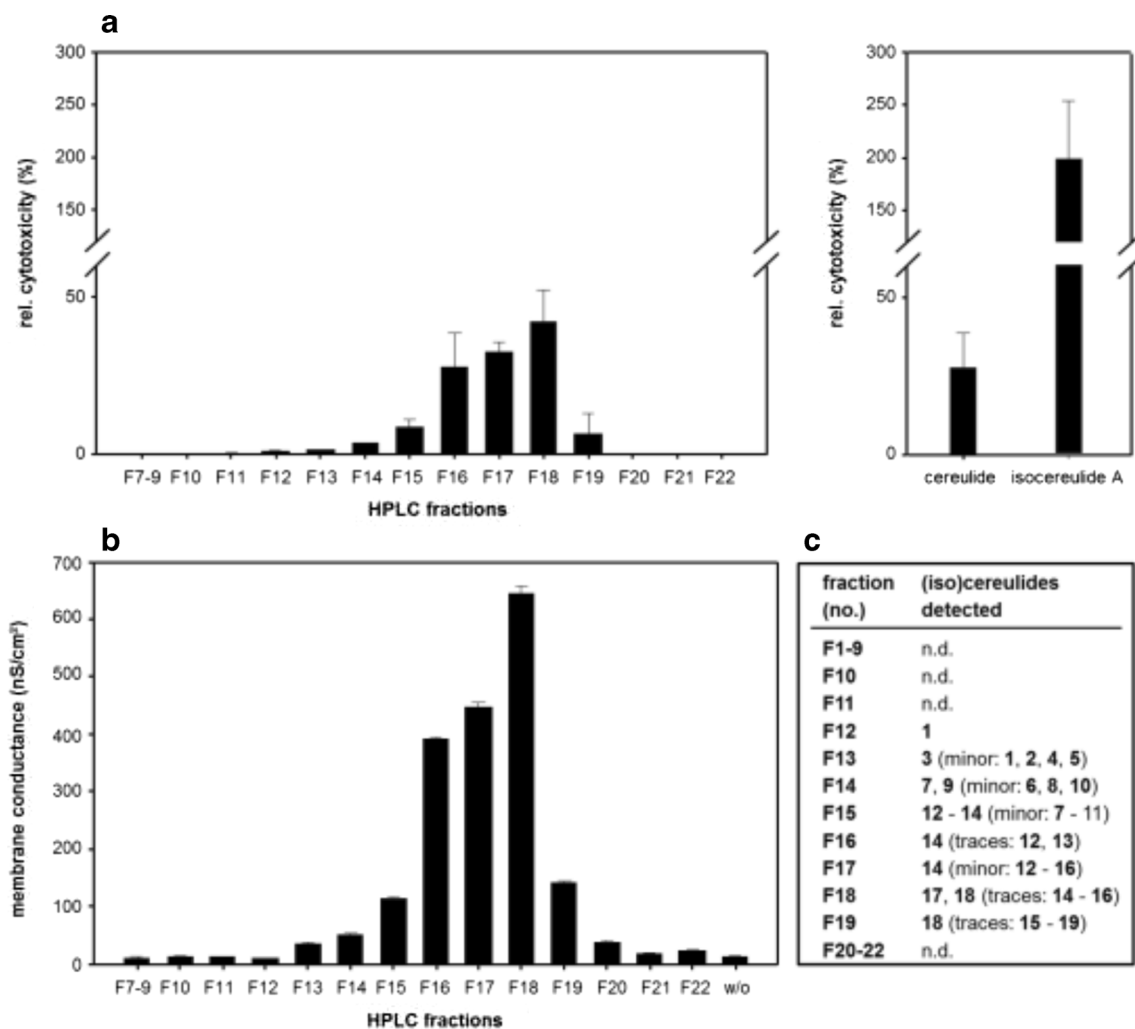


Fig. 6 Membrane and cytotoxic activity of fractions of a F4810/72_mut culture extract; (a) 1 % ethanolic solutions of the fractions (1 ng/mL each) were used for measuring lipid bilayer membrane conductance; (b) 1 % ethanolic solutions of the fractions (500 ng/10⁴ cells) were tested for

cytotoxicity using HEp-2 cells; (c) (iso)cereulides detected in F1-F22 by UPLC-TOF MS; n.d.: no isocereulides detectable. Bold numbers of compounds refer to Table 1

higher effect because of their increased insertion to the membrane, a highly purified isocereulide A sample (>98 %) was tested. Intriguingly, already the lowest concentration of 0.1 ng/mL isocereulide A caused an immediate breakdown of the membrane (data not shown). In comparison, a much lower effect was found for fractions F13–15, containing isocereulide D (F13), isocereulide B and E (F15), as well as cereulide (F15) besides minor unknown isomers (Fig. 6c). Fraction F12 was found to contain primarily isocereulide C exhibiting D-Ser instead of D-Ala as present in cereulide. The lower hydrophobicity of this structure homologue induced by the hydroxy group in D-Ser goes well in line with its lower ability to permeate into cell membranes and function as a less active membrane ionophore. In comparison, the membrane ion current was not increased by the early eluting fractions (<no. F11), lacking cereulide and any of its variants (Fig. 6c).

Conclusions

Next to the well-known emetic toxin cereulide, *B. cereus* was found to produce a series of at least 18 structure homologues, amongst which seven isomers, coined isocereulides A–G, were unequivocally determined for the first time in their chemical composition. Based on the measured cytotoxic and membrane activity of fractions of increasing hydrophobicity, it might be speculated that the structural modification of cereulide leads to the amplification of its ionophoric properties attributable to increase in its lipophilicity and/or the channel transport rate. The hydrophobic isocereulide A seems to be most effective regarding the toxicity against living cells. Based on the differences in the bioactivity of the dodecadepsipeptides, ranging from non/less toxic (e.g., isocereulide C) to highly toxic for eukaryotic cells (e.g., isocereulide A), it might be speculated that the severity of gastrointestinal diseases ranging from acute and limited phases of vomiting to deadly intoxications might be somehow related to varying concentrations of isocereulides. Further studies are needed to explore the ecological function and decipher the health risk of the various isocereulides described here. Moreover, new quantitative tools are urgently required to measure the diversity of cereulide variants in food and feed products. These are expected to provide a substantial contribution to the knowledge-based risk assessment of *B. cereus* toxins in foods, representing a still unsolved challenge in the field of food intoxications.

Acknowledgments This research project (project AiF 16845N) was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). The authors are grateful to Sofie Lösch, Ines Otte, and Comelia Berger for technical assistance.

References

- Ehling-Schulz M, Fricker M, Scherer S (2004) *Bacillus cereus*, the causative agent of an emetic type of food borne illness. *Mol Nutr Food Res* 48:479–487
- Stenfors ALP, Fagerlund A, Einar GP (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol Rev* 32:579–606
- Ehling-Schulz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: Structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXOI. *BMC Microbiol* 6:20
- Rasko DA, Rosovitz MJ, Økstad OA, Fouts DE, Jiang L, Cer RZ, Kolstø AB, Gill SR, Ravel J (2007) Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXOI. *J Bacteriol* 189(1):52–64
- Schwarzer D, Mootz HD, Linne U, Marahiel MA (2002) Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases. *Proc Natl Acad Sci U S A* 99(22):14083–14088
- Agata N, Mori M, Ohta M, Suwan S, Ohtani I, Isobe M (1994) A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiol Lett* 121:31–34
- Suwan S, Isobe M, Ohtani I, Agata N, Mori M, Ohta M (1995) Structure of cereulide, a cyclic dodecadepsipeptide toxin from *Bacillus cereus* and studies on NMR characteristics of its alkali metal complexes including a conformational structure of the K⁺ complex. *J Chem Soc Perkin Trans 1*(7):765–775
- Makarasin A, Yoza K, Isobe M (2009) Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus*. *Chem Asian J* 4(5):688–698
- Pitchayawasin S, Kuse M, Koga K, Isobe M, Agata N, Ohta M (2003) Complexation of cyclic dodecadepsipeptide, cereulide with ammonium salts. *Bioorg Med Chem Lett* 13:3507–3512
- Mikkola R, Saris NEL, Grigoriev PA, Andersson MA, Salkinoja-Salonen MS (1999) Ionophoretic properties and mitochondrial effects of cereulide: the emetic toxin of *B. cereus*. *Eur J Biochem* 263:112–117
- Stark T, Marxen S, Rüttschle A, Lücking G, Scherer S, Ehling-Schulz M, Hofmann T (2013) Mass spectrometric profiling of *Bacillus cereus* strains and quantitation of the emetic toxin cereulide by means of stable isotope analysis and HEp-2 bioassay. *Anal Bioanal Chem* 405:191–201
- Pitchayawasin S, Isobe M, Kuse M, Franz T, Agata N, Ohta M (2004) Molecular diversity of cereulide detected by means of nano-HPLC-ESI-Q-TOF-MS. *Int J Mass Spectrom* 235:123–129
- Lücking G, Dommel MK, Scherer S, Fouet A, Ehling-Schulz M (2009) Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator AbrB, but not by the virulence regulator PlcR. *Microbiology* 155:922–931
- Frenzel E, Letzel T, Scherer S, Ehling-Schulz M (2011) Inhibition of cereulide toxin synthesis by emetic *Bacillus cereus* via long-chain polyphosphates. *Appl Environ Microbiol* 77:1475–1482
- Beck V, Jaburek M, Breen EP, Porter RK, Jezek P, Pohl EE (2006) A new automated technique for the reconstitution of hydrophobic proteins into planar bilayer membranes. Studies of human recombinant uncoupling protein 1. *Biochim Biophys Acta* 1757:474–479
- Bauer T, Stark T, Hofmann T, Ehling-Schulz M (2010) Development of a stable isotope dilution analysis for the quantification of the *Bacillus cereus* toxin cereulide in foods. *J Agric Food Chem* 58: 1420–1428

17. Ehling-Schulz M, Svensson B, Guinebretiere M-H, Lindbäck T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Märtlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S (2005) Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology* 151:183–197
18. Rosenfeld E, Duport C, Zigha A, Schmitt P (2005) Characterization of aerobic and anaerobic vegetative growth of the food-borne pathogen *Bacillus cereus* F4430/73 strain. *Can J Microbiol* 51:149–158
19. Frenzel E, Doll V, Pauthner M, Lücking G, Scherer S, Ehling-Schulz M (2012) CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits. *Mol Microbiol* 85(1):67–88
20. Brückner H, Jaek P, Langer M, Godel H (1992) Liquid chromatographic determination of D-amino acids in cheese and cow milk. Implication of starter cultures, amino acid racemases, and rumen microorganisms on formation, and nutritional considerations. *Amino Acids* 2:271–284
21. Rupprecht A, Sokolenko EA, Beck V, Ninnemann O, Jaburek M, Trimbuch T, Klisshin SS, Jezek P, Skulachev VP, Pohl EE (2010) Role of the transmembrane potential in the membrane proton leak. *Biophys J* 98:1503–1511
22. Dittmann E, Fewer DP, Neilan BA (2013) Cyanobacterial toxins: biosynthetic routes and evolutionary roots. *FEMS Microbiol Rev* 37(1):23–43
23. Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E, Scherer S (2005) Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl Environ Microbiol* 71(1):105–113
24. Dommel MK, Frenzel E, Strasser B, Blöching C, Scherer S, Ehling-Schulz M (2010) Identification of the main promoter directing cereulide biosynthesis in emetic *Bacillus cereus* and its application for real-time monitoring of *ces* gene expression in foods. *Appl Environ Microbiol* 76(4):1232–1240
25. Jääskeläinen EL, Teplova V, Andersson MA, Andersson LC, Tammela P, Andersson MC, Pirhonen TI, Saris NE, Vuorela P, Salkinoja-Salonen MS (2003) In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus*. *Toxicol In Vitro* 17:737–744
26. Teplova VV, Mikkola R, Tonshin AA, Saris NEL, Salkinoja-Salonen MS (2006) The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. *Toxicol Appl Pharmacol* 210(1/2):39–46



Sandra Marxen studied food chemistry at the University of Hamburg, Germany, and worked as a PhD student from 2011 to 2014 at the Chair of Food Chemistry and Molecular Sensory Science of the TU München (supervisor: Professor Dr. T. Hofmann).



structure elucidation, and quantitation of bioactive compounds from food, botanicals, and microorganisms.

Timo D. Stark studied food chemistry at the Technical University of Munich (TUM) and received his PhD (2006) *summa cum laude* at the Chemistry Department of the TUM (supervisor: Professor Dr. T. Hofmann). After a post-doc at the Chair of Food Chemistry of the University of Münster (2005 – 2007), he became a permanent senior scientist at the Chair of Food Chemistry and Molecular Sensory Science at TUM. His research activities



related research and, furthermore, responsible for food diagnostics from 2011 to 2014. Since 2014, she is post-doctoral Fellow at the University of Groningen, The Netherlands, focusing on phenotypic heterogeneity in bacteria and on the optimization of reporter proteins for *Bacillus* species.

Elrike Frenzel studied biology supported by a grant of the German National Academic Foundation at the University of Hamburg (Germany) and completed her diploma with honors in 2008. In 2011, she received her PhD with *summa cum laude* at the Technical University of Munich, Germany, Department of Microbiology. During her PhD and her subsequent Post-doc position at the University of Veterinary Medicine Vienna, Austria, she was a project leader in *Bacillus cereus*-



Andrea Rüttschle studied Molecular Biotechnology at the Technical University of Munich (TUM) and worked from 2011 to 2014 as PhD student (supervisor: Professor Dr. S. Scherer) at the Chair of Microbial Ecology at TUM.



Genia Lücking studied biology at the Julius-Maximilian University of Würzburg and received her PhD (2009) at the Microbiology Department of Central Institute for Nutrition and Food Research (ZIEL) of the Technical University of Munich (TUM). Since 2009, she continues her research work as a post-doctoral scientist.



Siegfried Scherer studied Biology at the University of Konstanz (PhD in 1983, Plant Physiology). After postdoctoral research in China (Beijing) and USA (VirginiaTech, Blacksburg), he was professor and director of the Institute of Bacteriology at the Technical University of Munich (TUM, 1991–2003). In 2003, he took over the Chair of Microbial Ecology at TUM. From 2003–2014 he served as the managing director of the TUM Central Institute for Nutrition and Food Research.

His chair has a research focus on the molecular ecology of foodborne pathogens.



Gabriel Pürstinger studied biochemistry and molecular biomedical sciences at Graz University of Technology, Austria. Since 2013, he is a PhD student at the Unit of Physiology and Biophysics of the University of Veterinary Medicine, Vienna.



Monika Ehling-Schulz has been trained at the Technical University of Munich (TUM) in microbial ecology and food microbiology. In 2008 she joined the University of Veterinary Medicine, Vienna, Austria, as visiting Professor to establish innovative research fields at the interface between animal health and food safety. In 2011 she was appointed full Professor for functional microbiology, and since 2013 she is chairing the Institute for Microbiology at the Vetmeduni Vienna. Her current research interests focus on the pathometabolism of toxigenic bacteria and on microbial community dynamics.

Her current research interests focus on the pathometabolism of toxigenic bacteria and on microbial community dynamics.



Elena E. Pohl studied Biophysics at the Medical University in Moscow, Russia and Human Medicine at the Martin Luther University in Halle/Saale, Germany. She received her MD (1995) at the Martin Luther University and her *Venia docendi* in Biophysics (2008) at the Charité University Hospital in Berlin, Germany. Since 2009, Elena Pohl is full professor and head of the Unit of Physiology and Biophysics at the University of Veterinary Medicine, Vienna, Austria. Her main

research focus is the investigation of membrane proteins, protein–ligand and protein–lipid interactions.



Thomas Hofmann studied food chemistry at the University of Erlangen-Nürnberg and received his PhD (1995) and habilitation (1998) at the Chemistry Department of the Technical University of Munich (TUM). From 1999 to 2002, he was deputy director of the German Research Center for Food Chemistry of the Leibniz Society. In 2002, he took over the Chair of Food Chemistry of the University of Münster, and since 2007 he holds the Chair of Food Chemistry and Molecular

Sensory Science at TUM. Since 2009, he is senior vice president for research and innovation of TUM.