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Quantification of the Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Mass Spectrometry Using Synthetic Cereulide as a Standard▽

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Bacillus cereus **produces the emetic toxin cereulide, a cyclic dodecadepsipeptide that can act as a K^+ ionophore, dissipating the transmembrane potential in mitochondria of eukaryotic cells. Because pure cereulide has not been commercially available, cereulide content in food samples has been expressed in valinomycin equivalents, a highly similar cyclic potassium ionophore that is commercially available. This research tested the biological activity of synthetic cereulide and validated its use as a standard in the quantification of cereulide contents in food samples. The synthesis route consists of 10 steps that result in a high yield of synthetic cereulide that showed biological activity in the HEp-2 cell assay and the boar sperm motility assay. The activity is different in both methods, which may be attributed to differences in K^+ content of the test media used. Using cereulide or valinomycin as a standard to quantify cereulide based on liquid chromatography-mass spectrometry (LC-MS), the concentration determined with cereulide as a standard was on average 89.9% of the concentration determined using valinomycin as a standard. The recovery experiments using cereulide-spiked food products and acetonitrile as extraction solute showed that the LC-MS method with cereulide as a standard is a reliable and accurate method to quantify cereulide in food, because the recovery rate was close to 100% over a wide concentration range.**

The emetic type toxin cereulide is produced in food products, such as rice, pasta, and noodles, by cells of *Bacillus cereus*. This happens during temperature abuse, and the cereulide causes vomiting upon ingestion (9, 16). Data on the frequency of outbreaks of emetic food poisoning are scarce, since the symptoms are often mild and therefore not reported. One fatal outbreak was reported in Belgium in 2003, where a child died due to the presence of emetic toxin in pasta salad eaten during a picnic (5). In the Netherlands an outbreak occurred in 2000, where 116 students got ill after consumption of a rice dish containing emetic toxin (7).

The emetic toxin, cereulide, is a cyclic dodecadepsipeptide (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (1). The toxic effects of cereulide are caused by the ionophoric uptake of K^+, resulting in dissipation of the transmembrane potential, stimulating swelling and respiration in mitochondria, and finally resulting in their inactivation (18). Cereulide structurally resembles valinomycin, another cyclic potassium ionophore (1). Both toxins form complexes with alkali metal ions (22), but cereulide has been suggested to have a higher toxicity than valinomycin due to its higher affinity for potassium (23). The described effect of cereulide on mitochondria is used in several assays to detect the presence of cereulide in food products, like the sperm motility inhibition assay (2, 11, 19) and the HEp-2 (human carcinoma of the larynx) cell vacuolation assay (6, 8, 14).

Chemical assays for cereulide quantification using liquid chromatography-mass spectrometry (LC-MS) have been described in the literature by Häggblom et al., Bauer et al., and Hormazábal et al. (4, 10, 12). This quantitative method is based on high-performance LC (HPLC) connected to ion trap MS, which enables very accurate detection of low cereulide concentrations in food and with small between-experiment variations.

For all three methods, a solution with a known concentration of cereulide is needed as an external standard in order to quantify the cereulide level in a sample. To date, such an external standard of cereulide could not be prepared due to the lack of commercially available pure cereulide. As an alternative, the cereulide-like ionophore valinomycin has been used as a standard, since it is commercially available in known concentrations and purity. The use of valinomycin as a standard results in quantification of cereulide in terms of valinomycin equivalents, which is an elegant method but also scientifically debatable, since the compounds are different and might there-

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fore show different behaviors in biological and chemical assays. In 1995 Isobe et al. (15) chemically synthesized cereulide which was identical to cereulide produced by emetic *B. cereus* and also showed biological activity.

This study describes an improved synthesis route for cereulide, resulting in a higher yield and a more pure final product. Additionally, the cereulide MS pattern was compared to that of valinomycin by using an improved LC-MS analysis method. The synthetic cereulide was tested for its biological activity in both the HEp-2 cell culture assay and the boar sperm motility assay. Finally, cereulide recovery from three cereulide-spiked food products was evaluated using acetonitrile as the extraction medium and the LC-MS method for detection and quantification, with an external standard of cereulide prepared according to the improved protocol.

MATERIALS AND METHODS

Cereulide synthesis. The cyclic dodecadepsipeptide cereulide was synthesized from readily available *tert*-butyl carbonate (Boc)-D-Ala-OH, H-L-*O*-Val-OBn (where Bn is a benzyl moiety), Boc-L-Val-OH, and H-L-*O*-Leu-OBn (Chiralix B.V., Nijmegen, Netherlands). First, Boc-D-Ala-OH and H-L-*O*-Val-OBn were coupled to give a depsipeptide building block, which was subjected to acidolysis of the Boc protecting group. Analogously, Boc-L-Val-OH and H-L-*O*-Leu-OBn were coupled, but with inversion of the configuration of L-*O*-Leu, affording the corresponding D-*O*-Leu-containing depsipeptide building block. This building block was then debenzylated by hydrogenolysis. Both building blocks were subjected to peptide coupling conditions to form a tetrapeptide having again *N*-Boc and *O*-benzyl protection. Selective deprotection of this tetrapeptide resulted in either the corresponding free amine or the free acid, followed by sequential coupling, resulting in a linear dodecapeptide which was finally cyclized to obtain cereulide. The complete synthesis route, including all intermediate steps, is described in the appendix.

LC-MS analysis of synthetic cereulide and valinomycin. Ten milligrams of the synthetic cereulide (Chiralix B.V., Nijmegen, Netherlands) was dissolved in 1 ml methanol (Merck KGaA, Darmstadt, Germany). Solution A was obtained by diluting this stock solution in methanol to a concentration of 50 μg/ml. Solution A was diluted in acetonitrile (Merck KGaA, Darmstadt, Germany) to a concentration of 1,000 ng/ml (solution B). The working stock solution C was obtained by diluting solution B in acetonitrile to a final concentration of 10 ng/ml. The same procedure as described for dilution of synthetic cereulide was used for valinomycin (Sigma-Aldrich GmbH, Steinheim, Germany) to obtain a working stock solution of 10 ng/ml.

For both components, samples for LC-MS analysis were prepared by adding 200, 160, 120, 80, or 40 μl of solution C to an LC-MS vial. The vials were filled with additional acetonitrile to reach a total volume of 240 μl to obtain a concentration range of solution C. Subsequently, 30 μl Milli-Q water was added to the vials, resulting in final concentrations of cereulide or valinomycin of 1.5, 3, 4.5, 6, and 7.5 ng/ml. The samples were analyzed using an LC-MS method derived from the method described by Häggblom et al. (10). The samples were analyzed on an ion trap LC-MS apparatus by injecting 10-μl aliquots of the samples and subsequent elution and analysis using the positive electrospray ionization mode using either a Thermofinnigan LCQ Advantage setup (Thermo Fisher Scientific, Waltham, MA) with a C_{18} column (100 mm by 2.1 mm by 5 μm; Discovery, Supelco, Bellefonte, PA) or a Thermo Scientific LTQ XL setup (Thermo Fisher Scientific, Waltham, MA) with a C_{18} column (100 mm by 2.1 mm by 5 μm; Acquity UPLC, Waters Ltd., Hertfordshire, United Kingdom). The column was eluted at a flow rate of 0.2 ml/min with the following gradient: from 0 to 18 min, 13% solution A and 87% solution B; from 18 to 35 min, 100% B; from 35 to 50 min, 13% A and 87% B, with phase A being 2% (vol/vol) trifluoracetic acid anhydride (TFA; Merck KGaA, Darmstadt, Germany) in Milli-Q water with additional NH_4^+ (added as ammonium acetate [Merck, Darmstadt, Germany]) at a concentration of 10 mM and phase B being acetonitrile. The NH_4^+ adducts of both compounds were used for quantification (*m/z* for valinomycin, 1,128.5; *m/z* for cereulide, 1,170.7). The peak surface of every sample was plotted as a function of the concentration of the sample, resulting in two calibration curves for both synthetic cereulide and valinomycin.

Natural cereulide was produced by culturing *Bacillus cereus* (NCTC 11143) on tryptone soya agar (Oxoid CM 131) plates for 24 h at 28°C. After incubation the cells were harvested by using a 10-μl loop. The biomass was transferred to a screw-cap bottle and suspended in methanol (HPLC grade; Merck 1.06007) for extraction (10 ml methanol per g of biomass). The methanol extract was dried by evaporation over nitrogen, and the residue was suspended in 50 ml pentane (Merck 1.07177) and filtered over a paper filter (Schleicher & Schuell 589/3) to trap the undissolved particles. The pentane solution was subsequently dried by evaporation over nitrogen, and the residue was dissolved in 100 ml methanol. This solution was used to make a calibration curve consisting of various dilutions of natural cereulide of unknown purity (containing 1.5, 3, 4.5, 6, and 7.5 ng/ml valinomycin equivalents). A sample of synthetic cereulide with a final concentration of 4.95 ng/ml was quantified using this natural cereulide calibration curve, with the cereulide content expressed in valinomycin equivalents.

Testing biological activity of synthetic cereulide in a HEp-2 cell assay. The biological activity of the synthetic cereulide was determined using the HEp-2 cell assay as described by Ehling-Schulz et al. (6). In short, Earle's minimal essential cell culture medium (MEM) with supplements was mixed with 2% ethanol as a diluent, and 50 μl was added to every well of a 96-well plate. Valinomycin or cereulide was added to the first well of a row at an initial concentration of 500 ng/ml per well, and the content was serially diluted up to the 10th (valinomycin) or the 20th (cereulide) well, respectively. The HEp-2 cells were prepared and counted, and 150 μl of the HEp-2 cell suspension was added to all wells by using a multichannel pipette. The 96-well plates were incubated at 37°C for 48 h under a 5% CO_2 atmosphere. The cells were investigated microscopically for toxicity, since intoxicated cells will show malformations. Subsequently, 100 μl of the liquid was removed from each well. Ten microliters of the cell proliferation reagent WST-1 was added to every well, and the plate was incubated for 20 min at 37°C. The absorption of every well was measured at 450/620 nm to determine the live/dead cell ratio.

Testing biological activity of synthetic cereulide in the boar sperm motility assay. The effect of the synthetic cereulide on boar sperm was compared to valinomycin of known concentrations according to the modified protocol of Rajkovic et al. (20, 21). The aliquot of the stock solution of 50 ng cereulide per ml of methanol was evaporated under N_2 and diluted in 2-fold serial dilutions to 0.78 ng/ml using dimethyl sulfoxide (DMSO; Sigma-Aldrich, Steinheim, Germany) as the diluent. Volumes of 5 μl of each dilution were mixed with 195 μl of sperm (from the Belgian Piétrain extramuscled boar breed; standardized to a concentration of approximately 30 million cells per milliliter) in wells of a microtiter plate. The mixture was immediately transferred into a 37°C prewarmed counting slide (standard count two-chamber 20-μm slide; Leja, Nieuw-Vennep, Netherlands). The motility behavior was observed for 10 min, and the time to complete cessation of motility was recorded. The lowest concentration at which motility ceased within 10 min was taken as the limit of detection. Exposure was limited to 10 min, as any longer exposure of semen to DMSO had toxic effects that resulted in impaired motility. Semen alone and semen mixed with 5 μl of methanol served as a negative control and blank, respectively.

Extraction and detection of cereulide from food products spiked with cereulide. Cooked rice, a Chinese noodle dish, and french fries were used as model systems to test the extraction of cereulide from foods by using acetonitrile. The samples were spiked with natural cereulide of known purity. The chosen concentrations of the spiked cereulide in the food product were based on the estimated limits of quantification (1.5, 3, and 4.5 times the limit of quantification [LOQ] for the low concentration level and 7.5, 15, and 22.5 times the limit of quantification for the high concentration level). The low concentration level corresponded to 6.1, 12.3, and 18.4 μg cereulide/kg of sample, and the high concentration level corresponded to 30.7, 46.1, and 92.2 μg cereulide/kg of sample. The precision of the method was determined as the standard deviation of replicate measurements of the same sample (SD_r) and standard deviation of the replicate experiment within the same laboratory (SD_{RL}).

Five grams of every food sample was extracted with 50 ml acetonitrile (HPLC grade; Labscan C02C11X) by shaking it for 1 h at 100 rpm on an orbital shaker. The sample was left to settle after shaking, and 1,000 μl of the clear upper layer was transferred to a vial. To each vial 200 μl internal standard (valinomycin at a concentration of 25 ng/ml) and 150 μl Milli-Q water (HPLC grade) were added, mixed, and sealed. If the solution turned turbid, it was centrifuged for 10 min at $\geq$10,000 $\times$ *g*, and the clear supernatant was transferred to a new vial. The concentration of cereulide was determined by LC-MS with the settings described above.

RESULTS

Cereulide synthesis and LC-MS analysis. The synthesis route as described in this study (see the appendix) consists of 10 steps, compared to 11 steps in the synthesis route reported by Isobe et al. (15). The overall yield of this synthesis was 28.5%, a 3-fold

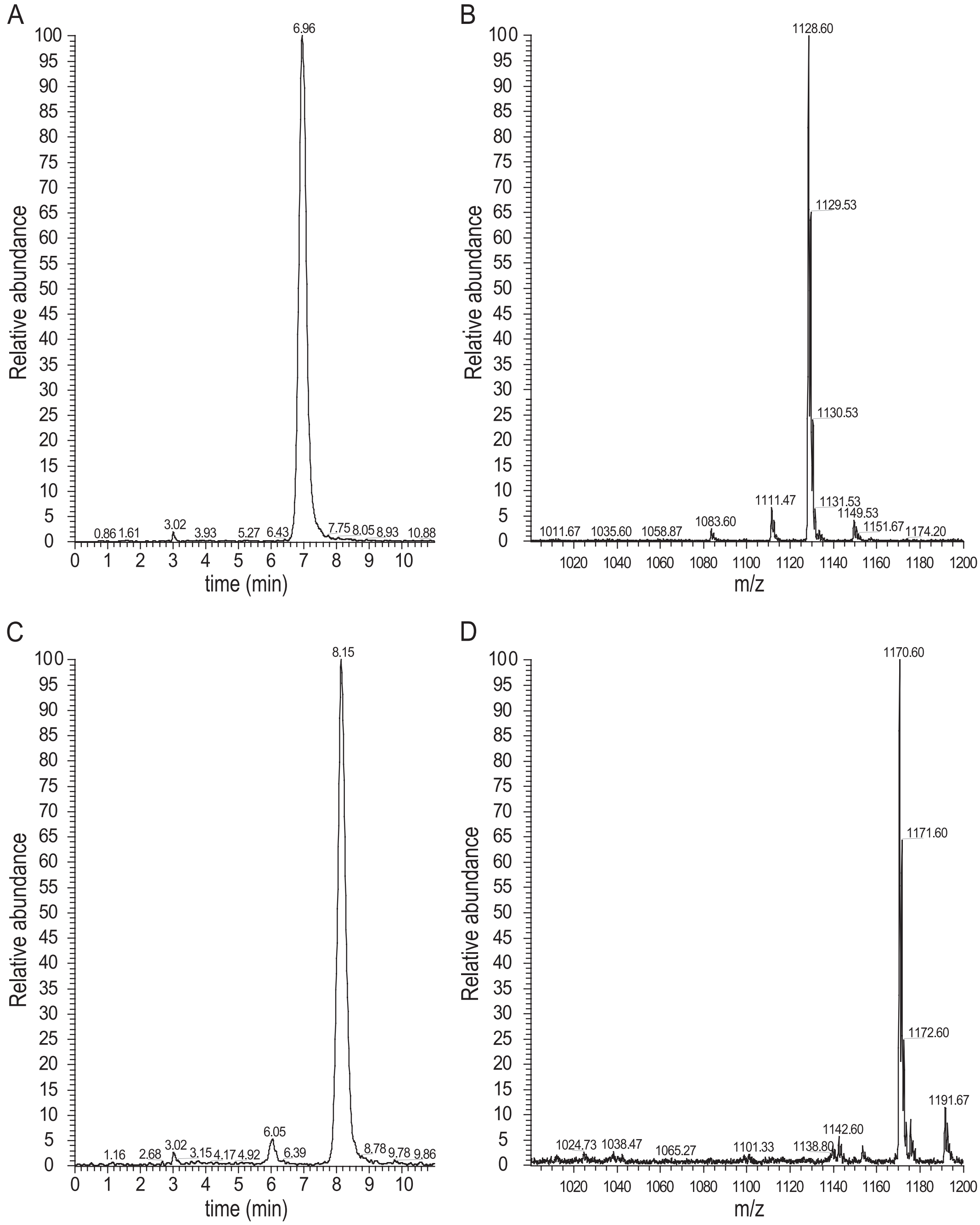


FIG. 1. (A) LC chromatogram of valinomycin with elution of the compound after 7 min. (B) Full ion spectrum of valinomycin with *m/z* 1,128.6. (C) LC chromatogram of cereulide with elution of the compound after 8 min. (D) Full ion spectrum of cereulide with *m/z* 1,170.60.

improvement over the yield of the synthesis route proposed by Isobe et al. The purity of the solution was >95%. Figures 1A to D present the chromatogram and MS scans for, respectively, valinomycin and synthetic cereulide. Figure 1A represents the chromatogram (time versus relative abundance) of the valinomycin NH_4^+ adduct, and Fig. 1C shows the cereulide NH_4^+ adducts. Figure 1B shows the typical mass spectrum of the valinomycin NH_4^+ adduct, and Fig. 1D shows that of the cereulide NH_4^+ adduct. The NH_4^+ adducts of valinomycin and cereulide had molecular weights of 1,128.6 and 1,170.6, respectively. The MS scans for synthetic and natural cereulide proved to be identical (results not shown).

Figure 2 presents the calibration curves for synthetic cereulide and valinomycin in the concentration range of 1.5 to 7.5 ng/ml. The results prove that the LC-MS response was linear over this range. The difference in peak areas for equal concentrations of the two components was 10.3%, indicating that the cereulide content of a sample that was estimated by using valinomycin to establish the calibration curve resulted in an overestimation of the cereulide content by 10.3%.

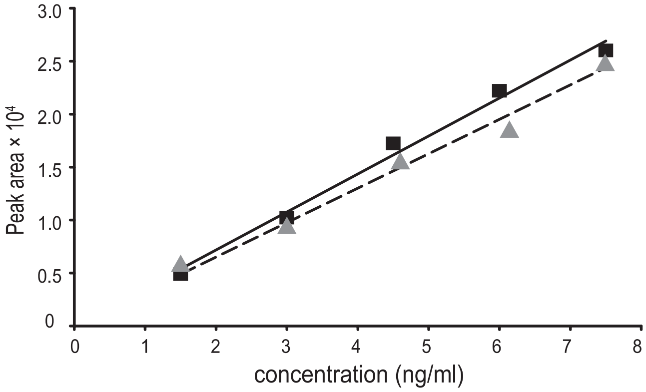


FIG. 2. Calibration curve of synthetic cereulide (black squares) and valinomycin (gray triangles), with the peak area of the compound measured based on LC-MS results as a function of known initial concentrations.

The reverse approach, in which natural cereulide of unknown purity was used to establish a calibration curve (cereulide concentration expressed as valinomycin equivalents) and the peak area of synthetic cereulide with a known concentration of 4.95 ng/ml was compared to the calibration curve, gave similar results. The synthetic cereulide concentration determined was on average 5.520 ng/ml with a standard deviation of 0.205 ng/ml ($n = 11$), 89.9% of the valinomycin concentration.

Testing the biological activity of synthetic cereulide. The effects of various concentrations of valinomycin and cereulide on HEp-2 cells as a function of the toxin concentration are displayed in Fig. 3A. When the toxin concentration was above 7.81 ng/ml (cereulide) or 62.5 ng/ml (valinomycin), less than 10% of the HEp-2 cells survived the 48 h of incubation. An increase in surviving cells was observed with a serial decrease in toxin concentration. The toxic effect of valinomycin decreased faster along dilution series of equal initial concentrations compared to cereulide. The 50% effective concentrations for valinomycin and cereulide, the amounts of toxin required to inactivate half of the cells, showed a 15-fold difference, indicating that cereulide was 15 times more toxic to the cells than valinomycin at an equal incubation concentration. Concentrations of cereulide of 1 ng/ml did not result in a measurable toxic effect to the cells. The effects of equal concentrations of synthetic and natural cereulide on the HEp-2 cells were similar (data not shown).

The effects of various concentrations of valinomycin and cereulide on the motility of the boar sperm are represented as a function of the toxin concentration in Fig. 3B. When the toxin concentration was higher than 6.25 ng/ml, the boar semen lost their motility. A serial decrease in toxin concentration resulted in an increase in time required to reduce semen motility. The results indicated that synthetic cereulide in the range of 6.25 to 25 ng/ml resulted in a shorter time to complete cessation of semen motility than with equal concentrations of valinomycin. Although statistically significant (t test, $P < 0.05$), this difference was not as extensive as the difference observed with the HEp-2 cell assay. For both compounds, concentrations below 6.25 ng/ml did not result in complete cessation of motility within 10 min. At 50 ng/ml both synthetic cereulide and valinomycin were too toxic (complete cessation of semen motility within a minute of exposure).

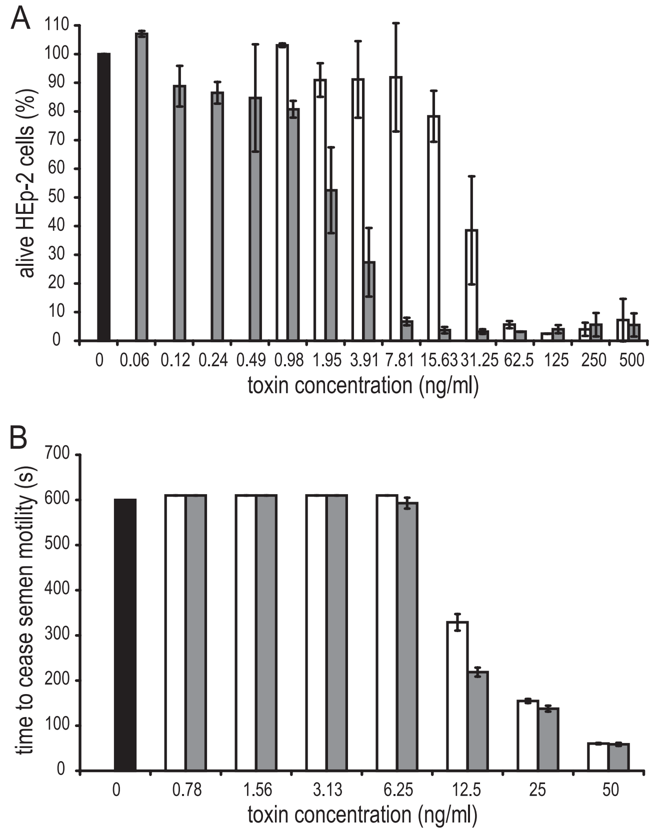


FIG. 3. (A) Live HEp-2 cells, incubated with 2-fold-diluted concentrations of cereulide and valinomycin, as a function of nonincubated HEp-2 cells. (B) Time needed for boar semen to lose motility in the presence of 2-fold-diluted concentrations of cereulide and valinomycin. For both panels, white bars represent valinomycin, gray bars represent cereulide, and black bars represent nonincubated HEp-2 cells or semen.

Detection of cereulide from a food product spiked with cereulide. The results for recovery, expressed as the SD_r and the SD_{RL} for both high and low concentrations of cereulide as determined in the matrices cooked rice, a Chinese noodle dish, and french fries, are presented in Table 1. The concentrations used to determine the recovery were based on the LOQ. The LOQ (defined as six times the background noise) was determined to be 4.1 μg/kg of sample. The values for recovery

TABLE 1. Recovery and precision data for the quantification of cereulide from cooked rice, a Chinese noodle dish, and french fries

Matrix	% Recovery		SD_r		SD_{RL}	
	Low level[a]	High level[b]	Low level	High level	Low level	High level
Cooked rice	96.7	98.2	1.93	3.09	2.85	4.56
Chinese noodle dish	98.0	102.5	2.74	4.38	2.88	4.60
French fries	101.1	106.7	2.47	3.96	3.42	5.47

[a] Low-level samples were spiked with 6.1 to 18.4 μg of cereulide/kg of sample.
[b] High-level samples were spiked with 30.7 to 92.2 μg of cereulide/kg of sample.

ranged between 96.7% and 107.6%. The values for SD_r and SD_{RL} were the lowest in cooked rice and the highest in french fries, although the differences between the results were not significant.

DISCUSSION

The key differences of the new synthesis route, compared to the synthesis route described by Isobe et al., were that in the new strategy the dipeptide building blocks were constructed by ester bond formation and subsequent couplings of the building blocks and that the final cyclization was achieved by amide bond formation. In contrast, in the Isobe et al. synthesis method the dipeptides were prepared by amide bond formation, and the subsequent couplings of the building blocks and the final cyclization were done by ester bond formation. Since amide bond formation is generally more facile than ester bond formation, the new strategy resulted in a more efficient synthesis, with significantly higher overall yield (10 steps with a 28.5% overall yield, compared to 11 steps with a 9.3% overall yield with the Isobe et al. synthesis method).

The mass spectra of natural and synthetic cereulide were identical (data not shown). Using synthesized cereulide allows the direct determination of the concentration of cereulide in (food) samples, instead of expressing the cereulide concentration in terms of valinomycin equivalents. Quantification of cereulide in samples by using valinomycin as the standard overestimated the concentration compared to that obtained using synthetic cereulide as the standard. Using valinomycin, the levels were approximately 10% higher. This is in correspondence with observations of Häggblom et al. (10), who found that MS results for valinomycin and cereulide are within 10%.

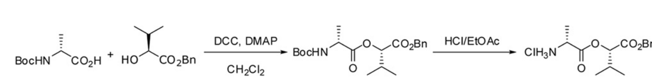
Both the HEp-2 cell assay and the boar sperm motility assay proved that the synthetic cereulide is biologically active. In the HEp-2 cell assay synthetic cereulide had a 15-fold-higher toxic effect than valinomycin at the same concentration (Fig. 3A). In the boar sperm motility assay this difference was of a lesser extent (Fig. 3B). These results are in agreement with the findings of Teplova et al. and Makarasen et al. (17, 23), who suggested that the differences are caused by the different potassium levels in the two assay mixtures, i.e., the boar semen assay mixture contains 10 to 19 mM and the HEp-2 cell assay mixture contains around 1 mM. The lower concentration of potassium in the HEp-2 cell proliferation assay favors the activity of cereulide, based on its higher affinity for potassium. The current study also demonstrates that it is possible to quantify cereulide in a variety of starch-rich food products at low concentration levels (5 μg cereulide/kg of sample) with good reproducibility. The low concentration could be detected by increasing the volume injected in the LC-MS apparatus to 20 μl instead of the 1 μl used by Häggblom et al. (10). In addition, methanol was replaced by acetonitrile for extraction in order to optimize peak shape in the LC chromatogram (data not shown). The acetonitrile itself might also enhance the extraction of cereulide from the food, since the solvent is more apolar than methanol (polarity indices, 5.8 versus 5.1). An alternative approach to increase the accuracy of cereulide quantification was recently published and is based on the addition of a $^{13}C_6$ cereulide isotopologue to each sample during extraction, dilution with water, and quantification by liquid chromatography (4). The $^{13}C_6$ cereulide isotopologue can be considered the perfect internal standard for cereulide extraction and therefore has promising implications for further research concerning cereulide extraction. On the other hand, our study proposes extraction of cereulide with acetonitrile, without dilution in water, and to increase the injection volume into the LC-MS apparatus. This relatively simple protocol can be applied both for research purposes and during routine analysis of (food) samples. Commercially available cereulide may be used as a standard of known concentration, and it can also be considered the perfect standard for recovery and detection experiments.

The values for recovery with the method used were good (ranging between 96.7% and 107.6%) and were well within the laboratory's internal limits (acceptability range, 60 to 115% at a level of 10 μg/kg), which are based upon the AOAC peer-verified methods program (3). The values found for SD_r and SD_{RL} were compared to the Horwitz ratio, the index of method performance with respect to precision, or HorRat values (13), and all values were at least three times lower than the Horwitz values, indicating the good precision of the method.

In conclusion, this research provides a novel route for the synthesis of biologically active cereulide, with a high yield and purity. Recovery rates of cereulide from three tested food matrices, using acetonitrile as extraction solvent, were close to 100%, with low SD values by LC-MS analysis. The commercial availability of cereulide should encourage method development for cereulide detection and quantification, since results no longer need to be extrapolated due to the use of nonidentical standards.

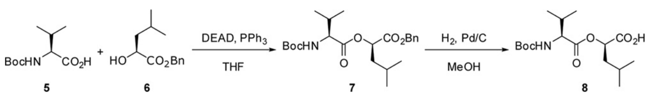
APPENDIX

Unless noted otherwise, materials were purchased from commercial suppliers (Sigma-Aldrich GmbH, Steinheim, Germany, or Acros, Thermo Fisher Scientific, Geel, Belgium) and used without purification. CH_2Cl_2 was freshly distilled from calcium hydride. Celite was obtained from Sigma-Aldrich. All air- and moisture-sensitive reactions were carried out under an inert atmosphere of dry argon. Column chromatography was performed using Acros silica gel (0.035 to 0.070 mm, 6 nm). Thin-layer chromatography was performed using silica gel-coated glass plates (Merck 60 F254), and compounds were detected with UV light (254 nm) and/or with potassium permanganate.



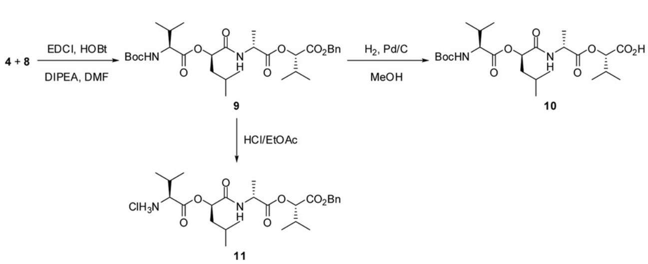
Boc-(D-Ala-L-*O*-Val)-OBn (compound 3). DCC (*N,N′*-dicyclohexylcarbodiimide) (219 mg, 1.06 mmol) was added to a stirred solution of compound 1 (200 mg, 1.06 mmol), compound 2 (208 mg, 1.00 mmol) (6), and DMAP [4-(dimethylamino)pyridine] (24 mg, 0.20 mmol) in CH_2Cl_2 (5 ml) at 0°C. The resulting suspension was allowed to warm to room temperature and stirred for 2 h. The suspension was filtered, and the residue was washed with CH_2Cl_2 (5 ml). The combined filtrates were concentrated *in vacuo*. Purification by flash column chromatography (ethyl acetate [EtOAc]/heptane, 1:4; R_f, 0.28) afforded compound 3 (330 mg, 87%) as a colorless oil.

H-(D-Ala-L-*O*-Val)-OBn · HCl (compound 4). A freshly prepared solution of $HCl_{(g)}$ (i.e., added as a gas by continuous injection in the solution) in EtOAc (2.6M, 10 ml) was added to a stirred solution of compound 3 (317 mg, 0.83 mmol) in EtOAc (3 ml) at room temperature. The resulting mixture was stirred for 45 min and then concentrated *in vacuo*, to afford compound 4 (261 mg, 99%) as a colorless viscous oil, which was used without further purification.



Boc-(L-Val-D-*O*-Leu)-OBn (compound 7). At 0°C, triphenylphosphine (976 mg, 3.72 mmol) was added to a stirred solution of compound 5 (270 mg, 1.24 mmol) and compound 6 (276 mg, 1.24 mmol) (10) in dry tetrahydrofuran (THF) (10 ml). Next, DEAD (diethyl azodicarboxylate) (40 wt% in toluene, 1.70 ml, 3.72 mmol) was added dropwise. The resulting yellow solution was allowed to warm to room temperature and stirred for 1 h. Subsequently, the mixture was concentrated *in vacuo*, redissolved in EtOAc (25 ml), and washed with saturated aqueous $NaHSO_4$ (three 10-ml volumes). The organic phase was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1:9; R_f, 0.25) afforded compound 7 (398 mg, 76%) as a colorless oil.

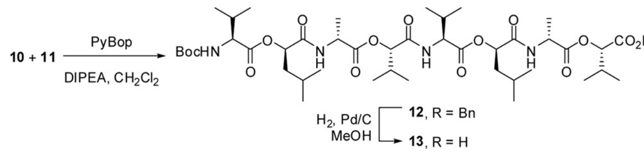
Boc-(L-Val-D-*O*-Leu)-OH (compound 8). Palladium on carbon (10% [wt/wt] Pd, 94 mg, 0.088 mmol) was added to a stirred solution of compound 7 (371 mg, 0.88 mmol) in methanol (MeOH; 10 ml) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated *in vacuo* to afford compound 8 (288 mg, 99%) as a colorless oil, which was used without further purification.



Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OBn (compound 9). DIPEA (diisopropylethylamine) (0.30 ml, 1.7 mmol) was added dropwise to a stirred solution of compound 8 (225 mg, 0.68 mmol) in DMF (dimethylformamide) (4 ml) at 0°C. Next, HOBt (1-hydroxybenzotriazole) (101 mg, 0.75 mmol) and EDCI [*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide] (144 mg, 0.75 mmol) were added successively. Finally, a solution of compound 4 (225 mg, 0.71 mmol) in DMF (2 ml) was added dropwise. The resulting mixture was allowed to warm to room temperature and stirred for 16 h. Next, the mixture was diluted with EtOAc (30 ml). The organic phase was washed with aqueous citric acid (10% [wt/wt], two times with 5 ml), water (once with 5 ml), saturated aqueous $NaHCO_3$ (two times with 5 ml), water (once with 5 ml), and brine (once with 5 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1:3; R_f, 0.23) afforded compound 9 (250 mg, 62%) as a colorless oil.

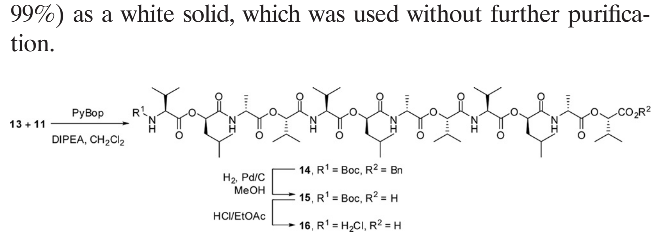
Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OH (compound 10). Palladium on carbon (10% [wt/wt] Pd, 13 mg, 0.01 mmol) was added to a stirred solution of compound 9 (72 mg, 0.12 mmol) in MeOH (3 ml) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated *in vacuo* to afford compound 10 (61 mg, 99%) as a white solid, which was used without further purification.

H-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OBn · HCl (compound 11). A freshly prepared solution of $HCl_{(g)}$ in EtOAc (2.6 M, 4 ml) was added to a stirred solution of compound 9 (125 mg, 0.21 mmol) in EtOAc (1 ml) at room temperature. The resulting mixture was stirred for 45 min and then concentrated *in vacuo* to obtain compound 11 (106 mg, 95%) as a colorless viscous oil, which was used without further purification.



Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OBn (compound 12). DIPEA (34 μl, 0.19 mmol) was added to a stirred solution of compound 10 (44 mg, 0.088 mmol) in CH_2Cl_2 (2 ml) at room temperature, followed by addition to a solution of compound 11 (48 mg, 0.091 mmol) in CH_2Cl_2 (1 ml). Finally, PyBop [(benzotriazol-1-yloxy)tripyrrolidinophosphonium] (48 mg, 0.092 mmol) was added, and the mixture was stirred for 40 min. Next, the mixture was diluted with EtOAc (15 ml). The organic phase was washed with aqueous citric acid (10% [wt/wt], twice with 5 ml), water (once with 5 ml), saturated aqueous $NaHCO_3$ (twice with 5 ml), water (once with 5 ml), and brine (once with 5 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1:2; R_f, 0.23) afforded compound 12 (79 mg, 92%) as a white solid.

Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OH (compound 13). Palladium on carbon (10% [wt/wt] Pd, 8 mg, 0.007 mmol) was added to a stirred solution of 12 (72 mg, 0.074 mmol) in MeOH (2 ml) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated *in vacuo* to obtain compound 13 (65 mg, 99%) as a white solid, which was used without further purification.



Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OBn (compound 14). DIPEA (26 μl, 0.15 mmol) was added to a stirred solution of compound 13 (59 mg, 0.067 mmol) in CH_2Cl_2 (1.5 ml) at room temperature, followed by a solution of compound 11 (36 mg, 0.068 mmol) in CH_2Cl_2 (0.75 ml). Finally, PyBop (37 mg, 0.70 mmol) was added, and the mixture was stirred for 1 h. Next, the mixture was diluted with EtOAc (15 ml). The organic phase was washed with aqueous citric acid (10% [wt/wt], twice with 5 ml), water (once with 5 ml), saturated aqueous $NaHCO_3$ (twice with 5 ml), water (once with 5 ml), and brine (once with 5 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 2:3; R_f, 0.26) afforded compound 14 (87 mg, 96%) as a white solid.

Boc-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OH (compound 15). Palladium on carbon (10% [wt/wt] Pd, 6 mg, 0.005 mmol) was added to a stirred solution of compound 14 (78 mg, 0.057 mmol) in MeOH (2.5 ml) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated *in vacuo* to afford compound 15 (72 mg, 100%) as a white solid, which was used without further purification.

H-(L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-D-*O*-Leu-D-Ala-L-*O*-Val)-OH · HCl (compound 16). A freshly prepared solution of $HCl_{(g)}$ in EtOAc (2.6 M, 4 ml) was added to a stirred solution of compound 15 (66 mg, 0.052 mmol) in EtOAc (1 ml) at room temperature. The resulting mixture was stirred for 45 min and then concentrated *in vacuo*, to obtain compound 16 (61 mg, 98%) as a white solid, which was used without further purification.

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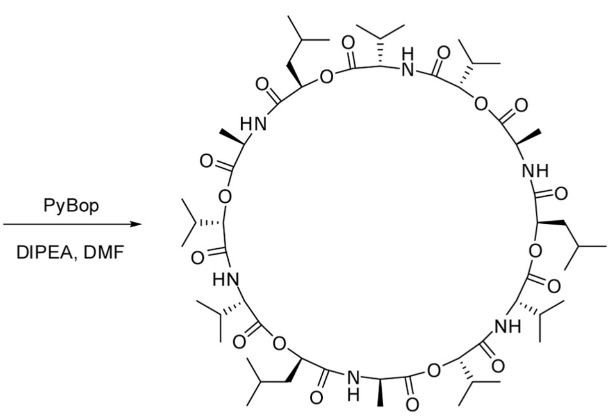

cereulide

Cereulide. DIPEA (22 μl, 0.12 mmol) was added to a stirred solution of compound 16 (50 mg, 0.041 mmol) in DMF (40 ml) at room temperature, followed by PyBop (23 mg, 0.043 mmol), and the mixture was stirred for 16 h. Next, the mixture was diluted with EtOAc (200 ml). The organic phase was washed with aqueous citric acid (10% [wt/wt]; twice with 50 ml), water (once with 50 ml), saturated aqueous $NaHCO_3$ (twice with 50 ml), water (once with 50 ml), and brine (once with 50 ml), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (EtOAc/heptane, 1:3; R_f, 0.28) afforded cereulide (34 mg, 72%) as a white solid.

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