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 causes vomiting upon ingestion (9, 16). Data on the frequency of outbreaks of emetic food poisoning are scarce, since the

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-
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)-t-Val-OH, H-t-Val-OBn (where Bn is a benzylox moiety), Boc-t-Val-OH, and H-t-Val-OBn (Chiralix B.V., Nijmegen, Netherlands). First, Boc-t-Val-OH and H-t-Val-OBn were coupled to give a depsipeptide building block, which was subjected to acidolysis of the Boc protecting group. Analogously, Boc-t-Val-OH and H-t-Val-OBn were coupled, but with inversion of the configuration of t-Val-Leu, affording the corresponding t-Val-Leu-containing depsipeptide building block. This building block was then debenzylated by hydrolysis. Both building blocks were subjected to peptide coupling conditions to form a tetrapeptide having again N-Boc and O-benzyl protection. Selective protection of this tetrapeptide resulted in either the corresponding free amine or the free acid, followed by sequential coupling, resulting in a linear dodecadepsipeptide 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 Hängblom 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 LCO Advantage setup (Thermo Fisher Scientific, Waltham, MA) with a C18 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 C18 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) trifluoroacetic acid anhydride (TFA; Merck KGaA, Darmstadt, Germany) in Milli-Q water with additional NH4OH added as ammonium acetate [Merck, Darmstadt, Germany]) at a concentration of 10 mM and phase B being acetonitrile. The NH4OH 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 well was measured at 450/620 nm to determine the absorbance of the samples.

Extraction and detection of cereulide from food products spiked with cereulide. Cooked rice, a Chinese noodle dish, and french fries were used as model food products to test the extraction of cereulide from foods by using acetonitrile. The cereulide-spiked food products were chopped into small pieces, and 10 g of each food sample was used to prepare each sample. Cereulide concentrations of 10 ng/ml per well were used for calibrating the LC-MS, and the concentration range extended to 100 ng/ml for the high concentration level. The low concentration level (1 ng/ml per well) was used for the calibration curve of cereulide. The LC-MS method was used to determine the concentration of cereulide in the samples.

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

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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%.
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).

Detection of cereulide from a food product spiked with cereulide. The results for recovery, expressed as the SD, 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 and precision data for the quantification of cereulide from cooked rice, a Chinese noodle dish, and french fries are given in Table 1.

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

<table>
<thead>
<tr>
<th>Matrix</th>
<th>% Recovery</th>
<th>SD_{RL} (μg/kg)</th>
<th>SD_{RL} (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low level</td>
<td>High level</td>
<td>Low level</td>
</tr>
<tr>
<td>Cooked rice</td>
<td>96.7</td>
<td>98.2</td>
<td>1.93</td>
</tr>
<tr>
<td>Chinese noodle dish</td>
<td>98.0</td>
<td>102.5</td>
<td>2.74</td>
</tr>
<tr>
<td>French fries</td>
<td>101.1</td>
<td>106.7</td>
<td>2.47</td>
</tr>
</tbody>
</table>

* Low-level samples were spiked with 6.1 to 18.4 μg of cereulide/kg of sample.
* 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 SD₃ and SD₉, on the other hand, 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 Häggblov 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 quantitate 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 Häggblov 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 13C₆ cereulide isotopologue to each sample during extraction, dilution with water, and quantification by liquid chromatography (4). The 13C₆ 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 SD₃ and SD₉ 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. CH₂Cl₂ 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 (Merek 60 F254), and compounds were detected with UV light (254 nm) and/or with potassium permanganate.

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\[ \text{Boc-(d-Ala-L-Val)-OBn (compound 3). DCC (N,N’-dicyclohexylcarbodiimide) (219 mg, 1.06 mmol) was added to the stirred solution of compound 1 (200 mg, 1.06 mmol), compound 2 (208 mg, 1.00 mmol) (6), and DMAP [4-(dimethylamino)pyridine] (24 mg, 0.20 mmol) in CH₂Cl₂ (5 ml) at 0°C. The resulting suspension was allowed to warm to room temperature and stirred for 2 h. The suspension was filtered, and the residue was washed with CH₂Cl₂ (5 ml). The combined filtrates were concentrated in vacuo. Purification by flash col-} \]```

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umn chromatography (ethyl acetate [EtOAc]/heptane, 1:4; Rf 0.28) afforded compound 3 (330 mg, 87%) as a colorless oil.

H-(l-Ala-l-O-Val)-OBn · HCl (compound 4). A freshly prepared solution of HCl (v/v, i.e., added as a gas by continuous injection in the solution) in EtOAc (2.6 M, 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-l-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 NaHCO₃ (three 10-ml volumes). The organic phase was dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:9; Rf 0.23) afforded compound 7 (371 mg, 0.88 mmol) in EtOAc (1 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 7 (330 mg, 87%) as a colorless viscous oil, which was used without further purification.

Na₂SO₄ filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:3; Rf 0.23) afforded compound 9 (250 mg, 62%) as a colorless oil.

Boc-(l-Val-n-O-Leu-n-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-n-O-Leu-n-Ala-l-O-Val)-OBn · HCl (compound 11). A freshly prepared solution of HCl (v/v) 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-n-O-Leu-n-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 CH₂Cl₂ (2 ml) at room temperature, followed by addition to a solution of compound 11 (48 mg, 0.091 mmol) in CH₂Cl₂ (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 NaHCO₃ (twice with 5 ml), water (once with 5 ml), and brine (once with 5 ml), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:2; Rf 0.19) afforded compound 12 (79 mg, 92%) as a white solid.

Boc-(l-Val-n-O-Leu-n-Ala-l-O-Val-l-Val-n-O-Leu-n-Ala-l-O-Val)-OBn (compound 13). Palladium on carbon (10% [wt/wt] Pd, 8 mg, 0.007 mmol) was added to a stirred solution of compound 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-n-O-Leu-n-Ala-l-O-Val-l-Val-n-O-Leu-n-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 CH₂Cl₂ (1.5 ml) at room temperature and stirred for 1 h. 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 NaHCO₃ (twice with 5 ml), water (once with 5 ml), and brine (once with 5 ml), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:2; Rf 0.19) afforded compound 14 (84 mg, 95%) as a white solid.
temperature, followed by a solution of compound 11 (36 mg, 0.052 mmol) in EtOAc (1 ml) at room temperature. The resulting mixture was stirred for 45 min and then concentrated in vacuo. The resulting mixture was stirred for 45 min and then concentrated in vacuo to afford compound 15 (72 mg, 100%) as a white solid, which was used without further purification.

H-Val-Val-Val-Val-Val-Val-Val-Val-Val-Leu-Val-OH (compound 15). A freshly prepared solution of HCl (10% wt/wt) 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 afford compound 15 (61 mg, 98%) as a white solid, which was used without further purification.

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 NaHCO₃ (twice with 50 ml), water (once with 50 ml), and brine (once with 50 ml), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 2:3; Rₚ 0.26) afforded compound 14 (87 mg, 96%) as a white solid.

Boc-(L-Val-d-O-Leu-d-O-Ala-l-O-Val-d-O-Leu-d-O-Ala-l-O-Val-l-O-Val-d-O-Leu-l-O-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 stirred for 16 h. Next, the mixture was filtered over Celite and concentrated in vacuo to afford compound 16 (61 mg, 98%) as a white solid, which was used without further purification.

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