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Biosynthesis of $^{15}N_3$-Labeled Enniatins and Beauvericin and Their Application to Stable Isotope Dilution Assays

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Supporting Information

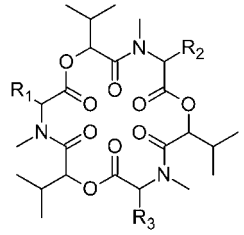
ABSTRACT: The first stable isotope dilution assay for the determination of enniatins A, A1, B, and B1 and beauvericin was developed. The $^{15}N_3$-labeled enniatins and beauvericin were biosynthesized by feeding two *Fusarium* strains $Na^{15}NO_3$ and subsequently isolated from the fungal culture. The chemical structures of the biosynthesized products were characterized by LC-MS/MS and ^1H NMR. Standard solutions of $^{15}N_3$-labeled beauvericin, enniatin A, and enniatin A1 were accurately quantitated by quantitative NMR. On the basis of the use of the labeled products as internal standards, stable isotope dilution assays were developed and applied to various food samples using LC-MS/MS. The sample extracts were directly injected without any tedious cleanup procedures. The limits of detection were 3.9, 2.6, 3.7, 1.9, and 4.4 μg/kg for enniatins A, A1, B, and B1 and beauvericin, respectively. Limits of quantitation were 11.5 (enniatin A), 7.6 (enniatin A1), 10.9 (enniatin B), 5.8 (enniatin B1), and 13.1 μg/kg (beauvericin). Recoveries were within the range between 90 and 120%, and good intraday and interday precisions with coefficients of variation between 1.35 and 8.61% were obtained. Thus, the stable isotope dilution assay presented here is similarly sensitive and precise but more accurate than assays reported before. Analyses of cereals and cereal products revealed frequent contaminations of barley, wheat, rye, and oats with enniatins B and B1, whereas beauvericin was not quantifiable.

KEYWORDS: *beauvericin, enniatins, biosynthesis, Fusarium, LC-MS/MS, stable isotope dilution assay, quantitative NMR*

INTRODUCTION

Enniatins and beauvericin are cyclodepsipeptides consisting of three alternating D-α-hydroxyisovaleryl and *N*-methylamino acid units. They differ in the amino acid units in that beauvericin contains three phenylalanine residues; enniatins A and B each contain three isoleucine or valine residues, whereas enniatins A1 and B1 contain mixtures of these two.[1−3] Their structures are presented in Figure 1.

Enniatins and beauvericin are produced by various *Fusarium* species worldwide[4,5] with *Fusarium tricinctum* and *Fusarium avenaceum* being the most prevalent ones. These toxins are gaining increasing attention due to their diverse biological activities. Enniatins and beauvericin are known to be toxic to brine shrimp[1,6] and insects.[7] Recently, their cytotoxicity on different cell lines of human origin has been reported,[8,9] and they were shown to be phytotoxic[10] and exert antifungal activity.[11]



BEA: $R_1=R_2=R_3=$ -$CH_2C_6H_5$

ENN A: $R_1=R_2=R_3=$ -$CH(CH_3)CH_2CH_3$

ENN A1: $R_1=R_2=$ -$CH(CH_3)CH_2CH_3$, $R_3=$ -$CH(CH_3)_2$

ENN B: $R_1=R_2=R_3=$ -$CH(CH_3)_2$

ENN B1: $R_1=R_2=$ -$CH(CH_3)_2$, $R_3=$ -$CH(CH_3)CH_2CH_3$

Figure 1. Chemical structures of enniatins A, A1, B, B1 and beauvericin.

Different methods for determination of enniatins and beauvericin have been reported, among which HPLC with UV or MS detection is the most often used. As the maximum absorption of enniatins and beauvericin occurs at low wavelengths, UV detection is usually carried out between 192 and 209 nm,[12,13] which makes it easily affected by coeluting compounds. In contrast to this, HPLC coupled with MS or MS/MS detection proved to be more specific and sensitive; thus, a number of methods were developed using different MS interfaces such as ESI and APCI.[14−18] However, for quantitative methods based on LC-MS/MS, one issue that must be addressed is matrix effects. The latter may either decrease (ion suppression) or increase (ion enhancement) the intensity of analyte ions and, therefore, affect the accuracy and reproducibility of the assay. Stable isotope dilution assays offer an ideal solution to overcome matrix effects, because the labeled internal standard and the analyte possess identical chemical and physical properties. Therefore, both are affected identically by matrix effects. In addition, analyte losses during sample preparation also are compensated for by the use of these ideal internal standards.[19] However, no isotope-labeled

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Table 1. ^{1}H NMR Chemical Shift Assignments for Enniatins A, A1, B, and B1

		chemical shift (ppm); J (Hz)			
		enniatin A	enniatin A1	enniatin B	enniatin B1
N-Me-Ile[a]	αH	4.68 (d, J = 6.8, 3H)	4.70 (m, 2H)		4.73 (d, J = 12.8, 1H)
	βH	2.09 (m, 3H)	2.03 (m, 2H)		2.07 (m, 1H)
	$\gamma_1(CH_2)$	1.03 (bs, 3H)	1.04 (s, 2H)		1.03 (1H)
		1.44 (m, 3H)	1.42 (t, J = 7.3, 2H)		1.43 (m, 1H)
	$\gamma_2(CH_3)$	1.02 (d, J = 3.8, 9H)	1.03 (d, J = 6.0, 6H)		1.01 (m, 3H)
	$\delta(CH_3)$	0.89 (m, 9H)	0.90 (m, 6H)		0.87 (m, 3H)
	N−CH_3	3.13 (s, 9H)	3.17 (s, 6H)		3.12 (s, 3H)
N-Me-Val[b]	αH		4.52 (d, J = 9.8, 1H)	4.53 (d, J = 12.0, 3H)	4.51 (d, J = 9.2, 1H); 4.47 (d, J = 8.1, 1H)
	βH		2.22 (m, 1H)	2.29 (m, 3H)	2.30 (m, 2H)
	$\gamma(CH_3)$		1.09 (d, J = 6.6, 3H)	0.93 (d, J = 6.6, 9H)	1.08 (d, J = 6.6, 6H)
			0.92 (s, 3H)	1.09 (d, J = 6.6, 9H)	0.89 (m, 6H)
	N−CH_3		3.19 (s, 3H)	3.17 (s, 9H)	3.14 (s,6H)
Hiv[c]	αH	5.14 (d, J = 8.1, 3H)	5.09 (m, 3H)	5.13 (d, J = 6.2, 3H)	5.16 (m, 3H)
	βH	2.28 (m, 3H)	2.22 (m, 3H)	2.26 (m, 3H)	2.30 (m, 3H)
	$\gamma(CH_3)$	0.91−1.01 (m, 18H)	0.96−1.02 (m, 18H)	0.97 (d, J = 6.8, 9H)	0.90−1.01 (m, 18H)
				1.01 (d, J = 6.6, 9H)	

[a]N-Me-Ile, *N*-methylisoleucine. [b]N-Me-Val, *N*-methylvaline. [c]Hiv, hydroxyisovaleryl.

standards of enniatins and beauvericin are available; therefore, it is the aim of this study to synthesize labeled enniatins and beauvericin and to develop stable isotope dilution assays for these mycotoxins in a series of food samples. Whereas other isotope-labeled *Fusarium* toxins such as ^{13}C-labeled type A trichothecenes have been prepared by chemical syntheses,[20] we here intended to prepare the depsipeptides by fungal biosyntheses.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile (MeCN), methanol, potassium chloride, citric acid, iron(II) sulfate heptahydrate, ammonium sulfate, copper(II) sulfate pentahydrate, and glucose were purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, zinc sulfate monohydrate, manganese(II) sulfate monohydrate, sodium molybdate dihydrate, and sodium nitrate were purchased from Sigma-Aldrich (Steinheim, Germany). The following compounds were obtained from the sources given in parentheses: magnesium sulfate heptahydrate (AppliChem, Darmstadt, Germany), boric acid (Avantor Performance Materials, Deventer, The Netherlands), ^{15}N-sodium nitrate (98 atom % ^{15}N) (Cambridge Isotope Laboratories, Cambridge, MA, USA), beauvericin (AnaSpec, San Jose, CA, USA), enniatin B (Bioaustralis, New South Wales, Australia), and enniatins A, A1, and B1 (Enzo Life Sciences, Lörrach, Germany).

Fungal Culture. Czapek−Dox liquid minimal medium,[21] with the normal $NaNO_3$ replaced by $Na^{15}NO_3$, sucrose replaced by glucose, and $(NH_4)_2SO_4$ eliminated, was used as culture medium. Five 250 mL Erlenmeyer flasks, each containing 100 mL of the modified Czapek−Dox minimal medium, were autoclaved at 121 °C for 25 min. An enniatins producer, *Fusarium sambucinum* strain 4.0979 previously grown on a synthetic agar low in nutrients (Synthetischer Nährstoffarmer Agar, SNA), was transferred to the five flasks and incubated on a shaker (128 rpm) at 25 °C for 7 days. A beauvericin producer, *Fusarium fujikuroi* strain 4.0860, was cultured likewise to produce beauvericin. The *Fusarium* strains were obtained from Prof. Ludwig Niessen, Chair of Technical Microbiology, Technische Universität München.

Extraction of $^{15}N_3$-Labeled Enniatins and Beauvericin. The culture broth was centrifuged at 4000 rpm for 10 min, and the supernatant was discarded as the content of the target compounds was negligible. The residue, that is, the harvested mycelia, was dried in an oven at 50 °C for 18 h, and extracted with 100 mL of MeCN/H_2O (84:16, v/v) in an ultrasonic bath (Bandelin Sonorex Super RX 106, Berlin, Germany) for 3 × 15 min, followed by extraction on a shaker for 2 days. The extract was filtered through 597 $^1/_2$ S&S folded paper filters (Schleicher & Schuell, Dassel, Germany). The filtrate was then processed according to the method of Song et al.[22] with minor modifications. Namely, the filtrate was defatted twice with 200 mL of hexane, the bottom layer was evaporated to dryness, and the residue was dissolved in 200 mL of MeOH/H_2O (55:45, v/v) and extracted twice with 200 mL of CH_2Cl_2. Then the CH_2Cl_2 phase was evaporated, and the residue was dissolved in 5 mL of methanol. This solution was passed through Strata C-18-T (55 μm, 140A, 1000 mg/6 mL, Phenomenex, Torrance, CA, USA) SPE cartridges. The cartridges were eluted with methanol, and then the eluate was collected, concentrated to 2 mL, and filtered through a membrane filter (Spartan 13/0.45 RC, Whatman, Dassel, Germany) prior to HPLC.

Preparation of $^{15}N_3$-Labeled Enniatins and Beauvericin by HPLC. HPLC analyses and preparations were performed using an analytical Merck Hitachi system (Tokyo, Japan) including an L-7455 diode array detector, an L-7200 autosampler, a D-7000 interface, and an L-7100 pump. A 250 mm × 3.0 mm i.d., 4 μm, Synergi Hydro-RP 80A (Phenomenex) column was used. HPLC conditions were set up using a constant flow of 0.6 mL/min and a very shallow gradient elution started with MeCN/H_2O (65:35, v/v), kept for 5 min, linearly increased to 68% MeCN in 10 min, and maintained for 12 min before it was switched back to the starting condition in 3 min. The enniatins and beauvericin were detected at 203 nm. Using these conditions, nine fractions were eluted and collected separately. Each fraction was subjected to LC-MS/MS analysis; fractions 1, 3, and 6 showed fragmentation patterns and retention times similar to those of enniatin B, enniatin B1, and enniatin A1 standards, respectively. Both fractions 8 and 9 showed fragmentation similar to that of enniatin A standard. In subsequent ^{1}H NMR tests, peak 9 was confirmed to be enniatin A, whereas peak 8 remained unknown. The HPLC separation was then repeated, and the five fractions were collected and pooled. Each pooled fraction was evaporated to dryness under reduced pressure and redissolved in 180 μL of methanol.

To further purify the five fractions, a second run of HPLC separation using the same system mentioned above was performed for each of them separately. Only the mobile phase was different; the flow was kept constant at 0.6 mL/min, and MeOH/H_2O (78:22, v/v) was used as starting eluent, maintained for 5 min before rising to 92%

MeOH over 20 min, then kept for 1 min, and taken back to the starting ratio in 4 min.

Each rechromatographed fraction was co-injected with pure standard for confirmation, and their purity was further verified by LC-MS in the full scan mode as described below. According to the results of quantitative NMR described below the yields for $^{15}N_3$-labeled enniatin A, $^{15}N_3$-labeled enniatin A1, and $^{15}N_3$-labeled beauvericin were 430, 450, and 1460 μg, respectively.

^{1}H NMR. The structures of purified compounds were characterized by ^{1}H NMR on a Bruker AV III system (Bruker, Rheinstetten, Germany) operating at a frequency of 500.13 MHz. All five compounds were dissolved in $CDCl_3$.

The ^{1}H NMR chemical shifts for beauvericin, given in δ (ppm) (TMS) are 7.16 (m, 15H, aromatic H, Phe), 5.47 (m, 3H, αH, Phe), 4.80 (d, J = 8.1 Hz, 3H, αH, hydroxyisovaleryl), 3.32 (m, 3H, βH, Phe), 2.95 (s, 9H, N−CH_3), 2.89 (m, 3H, βH, Phe), 1.89 (m, 3H, βH, hydroxyisovaleryl), 0.73 (d, J = 6.6 Hz, 9H, γ(CH_3), hydroxyisovaleryl), and 0.34 (d, J = 6.8 Hz, 9H, γ(CH_3), hydroxyisovaleryl). The ^{1}H NMR chemical shifts for enniatins are listed in Table 1. The data are in good agreement with the literature.[1−3]

Quantitative NMR. The method of quantitative NMR for $^{15}N_3$-labeled beauvericin, enniatin A, and enniatin A1 was similar to that described by Korn et al.[24] Briefly, the purified compounds were dissolved in 600 μL of methanol-d_3 (Euriso-top, Gif sur Yvette Cedex, France) and analyzed in 5 × 178 mm NMR tubes (Norell, ST500-7, Landisville, NJ, USA). A caffeine sample of known concentration was used as external standard. For quantitation, the signals at 7.87 ppm (caffeine), 5.47 ppm ($^{15}N_3$-labeled beauvericin), 5.14 ppm ($^{15}N_3$-labeled enniatin A), and 5.09 ppm ($^{15}N_3$-labeled enniatin A1) were chosen. The intensity of the signal was integrated manually.

LC-MS and LC-MS/MS. Liquid chromatography was carried out on a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a 150 mm × 2.0 mm i.d., 4 μm, Synergi Polar RP 80A column (Phenomenex). The starting mobile phase MeCN/H_2O (65:35, v/v) was kept constant for 5 min and linearly raised to 75% MeCN in 7 min. After 1 min at 75% MeCN, the gradient was increased to 100% MeCN in 2 min and held for 1 min before returning to the starting condition in 3 min. The injection volume was 10 μL, the flow rate was 0.2 mL/min, and the equilibration time between two runs was 5 min. Data acquisition was carried out using Analyst 1.5 software (Applied Biosystems Inc., Foster City, CA, USA).

The LC was interfaced to a hybrid triple-quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc.) operated in the positive ESI mode. The ion source parameters were set as follows: curtain gas, 10 psi; temperature, 450 °C; ion source gas 1, 45 psi; ion source gas 2, 50 psi; ion spray voltage, 5500 V. MS parameters were optimized by direct infusion of each standard solution (40 ng/mL) into the source. (See the Supporting Information for additional parameters.)

Full scan spectra for confirmation of the purified compounds were recorded in a mass range from m/z 200 to 1500 and a scan time of 1.0 s.

For MS/MS measurements, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode; a valve was used to divert the column effluent to the mass spectrometer from 5 to 13.5 min and to waste for the rest of the run.

Preparation of Standard Solutions. All standard solutions were prepared in methanol. The concentrations of labeled enniatin A, enniatin A1, and beauvericin were determined by quantitative NMR described above; stock solutions of 100 μg/mL of each compound were prepared accordingly, from which further dilutions of 10 μg/mL were prepared. The UV absorptions of the 10 μg/mL enniatin A and enniatin A1 were determined on a UV spectrometer Specord 50 (Analytik Jena, Jena, Germany) at the maximum absorption wavelength of 203 nm in triplicates. The ratio between the molar extinction coefficients of enniatin A and enniatin A1 was calculated from the mean of the triplicates, and the result was 1.007, which confirmed the assumption that the molar extinction coefficients of enniatins A, A1, B, and B1 are all the same because they differ only in the side chains, which are devoid of UV chromophores. On this basis, the concentrations of labeled enniatin B and labeled enniatin B1 were determined by comparing their UV absorptions at 203 nm to those of enniatin A and enniatin A1. Stock solutions of 100 μg/mL were prepared for labeled enniatin B and enniatin B1, as well as unlabeled enniatins and beauvericin. Further dilutions of 1 μg/mL, 100 ng/mL, and 10 ng/mL were also prepared. All solutions were stored in the dark at 4 °C.

Sample Preparation. Food samples were purchased from local retail stores except from barley malts, which were obtained from Bavarian malt producers. All samples were ground (Ika Universalmühler M20, Staufen, Germany) into fine powder before extraction. One gram of each dried sample was spiked with 10 ng (100 μL × 100 ng/mL solution in MeCN) of each of the labeled standards; after the solvent was evaporated, the sample was suspended in 10 mL of MeCN/H_2O (84:16, v/v), vortexed (Ika Vortex Genius 3, Staufen, Germany) for 1 min, and extracted for 1.5 h, after which each sample was centrifuged at 4000 rpm for 10 min, and 1 mL of the supernatant was filtered through a membrane filter (Spartan 13/0.45 RC, Whatman, Dassel, Germany) prior to HPLC.

Calibration and Quantitation. Constant amounts (10 ng) of labeled standard (S) were mixed with various amounts of analyte (A) in molar ratios between 0.1 and 10 (1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1). After LC-MS/MS measurement, response curves were obtained from molar ratios [n(A)/n(S)] versus peak area ratios [A(A)/A(S)], and response functions were obtained using linear regression. The response functions were as follows [y = n(A)/n(S), x = A(A)/A(S)]: enniatin A, $y = 1.8692x - 0.0406$ ($R^2 = 0.9975$); enniatin A1, $y = 1.4310x - 0.0821$ ($R^2 = 0.9984$); enniatin B, $y = 1.5138x - 0.0674$ ($R^2 = 0.9958$); enniatin B1, $y = 1.7618x - 0.1002$ ($R^2 = 0.9919$); beauvericin, $y = 0.9042x - 0.1627$ ($R^2 = 0.9971$). According to the Mandel test, all functions were linear within the chosen molar ratios (0.1−10). Residual plots were drawn to examine the appropriateness of using linear regression, and all five plots showed random patterns. The contents of enniatins and beauvericin in samples were calculated using the respective response functions.

Limits of Detection (LODs) and Quantitation (LOQs). LODs and LOQs were calculated according to the procedures suggested by Vogelgesang and Hädrich.[23] A potato starch devoid of enniatins and beauvericin was used as blank for the determination of LODs and LOQs. The blank was spiked with enniatins and beauvericin at four different amounts (5, 20, 35, and 50 μg/kg), each in triplicate. The samples were extracted and analyzed as described before.

Precision. Intraday (n = 5) and interday (n = 3) precisions were determined within 6 weeks. As no single sample that contained all four enniatins as well as beauvericin was found by that time, precision was determined with three samples: a naturally contaminated whole wheat flour sample was used to measure enniatins A1, B, and B1; a naturally contaminated wheat grain sample was used for enniatin A; and a rice sample that contained none of these mycotoxins was spiked with 45 μg/kg of beauvericin for determination because no naturally contaminated beauvericin sample was available.

Recovery. Blank samples (potato starch) were spiked in triplicate with different amounts (20, 35, and 50 μg/kg) of enniatins and beauvericin and analyzed as described before. Recovery was calculated as the mean of the spiking experiments.

RESULTS AND DISCUSSION

Biosynthesis of $^{15}N_3$-Labeled Enniatins and Beauvericin. Synthesis of the $^{15}N_3$-labeled enniatins and beauvericin was achieved by cultivating the enniatin/beauvericin-producing *Fusarium* strains separately in a synthetic medium, Czapek−Dox liquid minimal medium, with modification. To ensure that the $Na^{15}NO_3$ was the only nitrogen source for the fungi, unlabeled $NaNO_3$ was replaced by the labeled one, and $(NH_4)_2SO_4$, which is only a trace element of the medium, was eliminated. The lack of $(NH_4)_2SO_4$ in the medium was proved to have no significant influence on the production of enniatins and beauvericin in a previous experiment (not reported). The

two enniatin/beauvericin-producing strains were screened from 54 *Fusarium* strains (4 species: *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, and *F. sambucinum*) before they were fed with labeled nitrogen. To the best of our knowledge, this is the first literature report on the production of mycotoxins labeled with the nitrogen isotope ^{15}N. Up to now, similar protocols were applied only to produce fungal peptides from $K^{15}NO_3$ or chitin from $(^{15}NH_4)_2SO_4$.[28,29]

Quantitative NMR. Determining the concentration of standard solutions of labeled enniatins and beauvericin is inaccurate by gravimetry due to the small amount of the mycotoxins isolated from fungal culture. The precise amount of commercially bought unlabeled enniatins and beauvericin was also unknown as the purity is not certified. Thus, quantitative NMR was adopted, which revealed the molar concentration of the three mycotoxins as follows: 1.0355 mmol/L ($^{15}N_3$-labeled enniatin A), 1.1203 mmol/L ($^{15}N_3$-labeled enniatin A1), and 3.1019 mmol/L ($^{15}N_3$-labeled beauvericin). As already reported for ochratoxin A,[24] quantitative NMR proved again to be a suitable and accurate tool in mycotoxin quantitation.

LC-MS/MS. Detection of the analytes was carried out by ESI-(+)-MS/MS, and product ion scans of enniatins and beauvericin standards were recorded using the protonated molecules as precursor ions. Generally, the labeled standards gave fragmentation patterns similar to those of the respective unlabeled compounds. As displayed in Figure 2, the three most intense fragments derived from $[M + H]^+$ ion (m/z 668) of unlabeled enniatin A1 were m/z 196, 210, and 228; similar fragments were produced by $[M + H]^+$ ions (m/z 671) of labeled enniatin A1, with m/z 197, 211, and 229 being the three most intense signals. The fragmentation of labeled and unlabeled beauvericin is shown in Figure 3. Whereas the protonated molecules contained a mass increment of three being in accordance with the three ^{15}N incorporated, the fragments contained only a mass increment of one, equivalent to one ^{15}N incorporated. On the basis of this information, a fragmentation pathway of enniatins and beauvericin is proposed, with the protonated molecule in the center of Figure 4 showing an imaginary molecule composed of all side chains incorporated in the different enniatins and beauvericin. In accordance with the observed occurrence of one labeled nitrogen in each fragment, the fragments obviously contained one amino acid moiety. Hypothetically, the ring of the molecule had an even chance to break in either of the three marked C−O bonds. Due to the different substituents on the amino acid residues, enniatins and beauvericin resulted in different fragments. For beauvericin, most plausible were the fragments containing phenylalanine residues (m/z 262), which then lost H_2O to give m/z 244. For enniatin A, fragments of m/z 228 containing *sec*-butyl moieties were formed after breaking of the ring, and subsequent loss of H_2O resulted in m/z 210. Similarly, fragments of m/z 214 and 196 were obtained from enniatin B. For enniatins A1 and B1 containing both isopropyl and *sec*-butyl side chains, a mixture of m/z 196, 214, 210, and 228 fragments was observed.

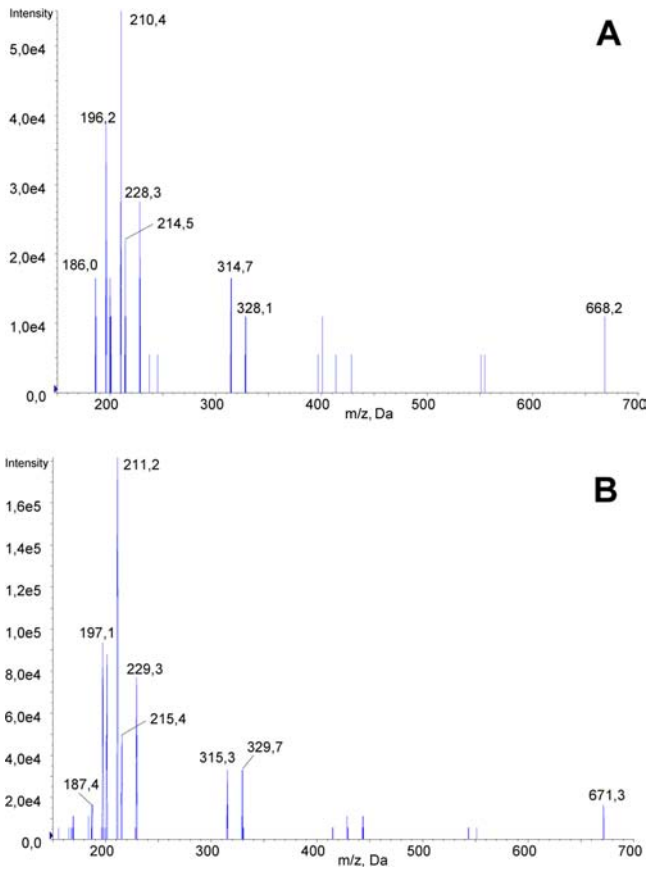

Figure 2. ESI-(+)-LC-MS/MS spectra of (A) enniatin A1 (precursor m/z 668, $[M + H]^+$) and (B) $^{15}N_3$-labeled enniatin A1 (precursor m/z 671, $[M + H]^+$).

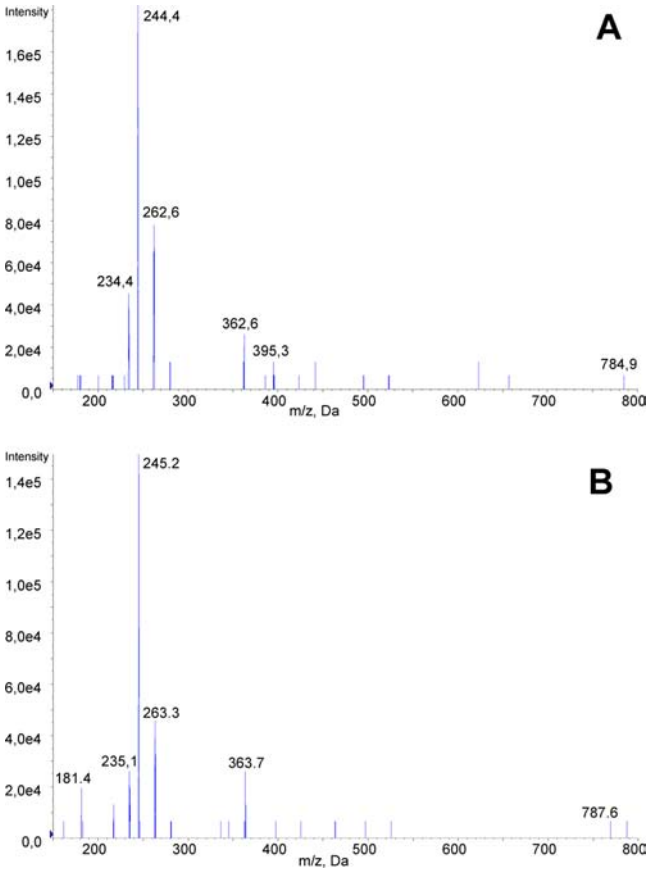

Figure 3. ESI-(+)-LC-MS/MS spectra of (A) beauvericin (precursor m/z 784, $[M + H]^+$) and (B) $^{15}N_3$-labeled beauvericin (precursor m/z 787, $[M + H]^+$).

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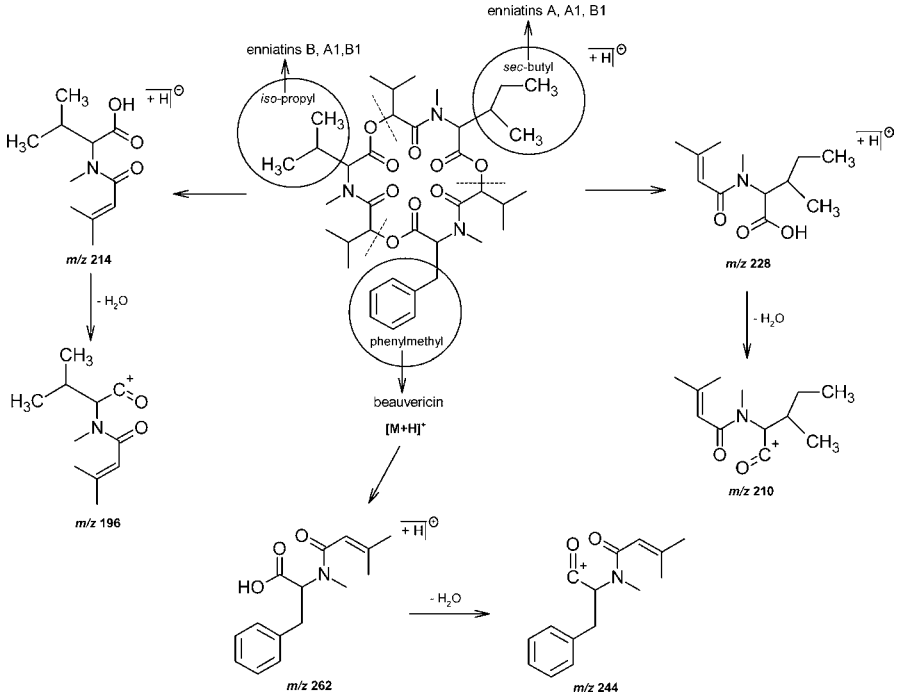

Figure 4. Proposed MS/MS fragmentation routes of enniatins and beauvericin. The depicted structure refers to a hypothetical molecule composed of the amino acids included in enniatins and beauvericin.

Table 2. Validation Data of the Stable Isotope Dilution Assay for Enniatins and Beauvericin

			coefficients of variation (%)		recovery (three spiking levels; %)		
	LOD (μg/kg)	LOQ (μg/kg)	interday (n = 3)	intraday (n = 5)	20 μg/kg	35 μg/kg	50 μg/kg
enniatin A	3.9	11.5	1.36	1.35	98 ± 7.6	105 ± 3.4	107 ± 7.2
enniatin A1	2.6	7.6	8.61	6.31	96 ± 2.6	102 ± 4.8	98 ± 2.2
enniatin B	3.7	10.9	5.58	7.21	99 ± 3.9	100 ± 3.1	106 ± 6.9
enniatin B1	1.9	5.8	4.09	4.89	105 ± 5.1	100 ± 1.8	104 ± 1.9
beauvericin	4.4	13.1	1.48	1.41	110 ± 3.4	109 ± 1.0	103 ± 8.6

In previous studies, MS/MS fragmentations of enniatins and beauvericin were reported but neither explained in detail nor substantiated.

In their LC-MS/MS method, Sørensen et al.[14] used the fragments at m/z 555 as well as 210, at m/z 541 as well as 210, at m/z 527 as well as 196, at m/z 228 as well as 196, and at m/z 362 as well as 244 for enniatin A, enniatin A1, enniatin B, enniatin B1, and beauvericin, respectively. The masses of the quantifier ions (m/z 210, 196, 244) were assigned to protonated "monomers" with phenylmethyl, *sec*-butyl, or isopropyl residues after loss of water without giving any detailed structural suggestions for the fragments. The same fragments were reported by Jestoi et al.,[17] who did not comment on their structure or on the route of formation. In another report, Sewram et al.[15] explained that the fragments of beauvericin resulted from the cleavage of the amide bond, which we could not confirm in our studies.

Calibration and Quantitation. Calibration curves were obtained by linear regression, showing good linearity within the chosen molar ratios (0.1−10) confirmed by the Mandel test. The response factors for enniatins were all above 1.4 and exceeded the usual response factors around 1.0 for stable isotope dilution assays. This can be partly explained by the different isotope abundances between labeled and natural enniatins, as approximately 95.8−96.5% of the isotopologues in the biosynthesized labeled enniatins were M + 3 ones, and the abundance of M isotopologues in unlabeled enniatins standards varied between 51.1 and 62.5% due to natural isotopologues. According to this isotopologic distribution, large response factors between 1.53 and 1.88 would be expected, but as the signals of natural isotopologues in LC-MS/MS are reduced due to higher specificity, the found values between 1.43 and 1.87 are plausible. The isotope abundances for labeled and unlabeled enniatins and beauvericin were estimated by LC-MS full scan, in which the respective fragmentations of M, M + 1, M + 2, and M + 3 of each compound were recorded and calculated. However, this cannot explain the normal response factor (0.9) but abnormally high y intercept (0.16) of beauvericin, because the M isotopologue abundance was 58.1% in unlabeled standard and the M + 3 isotopologue abundance was 93.2% in labeled beauvericin. On the basis of the considerations detailed before, a response factor up to 1.60 would be expected. However, multiple and regular tests of the calibration curve confirmed these unusual values. Therefore, additional isotope effects have to be assumed.

Limits of Detection and Quantitation. LODs and LOQs were calculated according to the method of Vogelgesang and Hädrich,[23] which is based on a calibration curve obtained from spiking experiments in a matrix free from the respective analyte. As shown in Table 2, the LODs ranged from 1.9 to 4.4 μg/kg, and LOQs ranged from 5.8 to 13.1 μg/kg. Thus, the stable isotope dilution assay presented here is 2 orders of magnitude

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Table 3. Presence of Enniatins and Beauvericin in Analyzed Food Samples (Micrograms per Kilogram)

		enniatin A			enniatin A1			enniatin B			enniatin B1			beauvericin	
sample[a]	no.	positive samples	min−max[b]	mean[c]	positive samples	min−max[b]	mean[c]	positive samples	min−max[b]	mean[c]	positive samples	min−max[b]	mean[c]	positive samples	min−max[b]
barley malts	6	5	52−448	220	6	24−2721	1225	6	196−6998	3668	6	138−6762	3624	2	nq
wheat grains	6	5	nq−38	17	6	33−232	111	6	508−2125	1306	6	210−1066	658	2	nq
oat grains (organic)	2	0	−	−	0	−	−	2	nq	5	2	nq	3	1	nq
rice grains	6	0	−	−	0	−	−	0	−	−	0	−	−	0	−
maize grains	2	0	−	−	0	−	−	2	nq	5	2	3−4	4	2	nq
maize grits (organic)	2	0	−	−	0	−	−	1	11	6	−	−	−	0	−
maize flour	1	0	−	−	0	−	−	1	nq	5	1	nq	3	1	nq
wheat bread	5	0	−	−	3	nq	3	5	17−90	47	5	7−35	21	0	−
rye bread	5	0	−	−	3	nq−23	8	5	25−735	263	5	9−256	88	0	−
wheat flour	5	1	7	3	4	7−45	15	5	41−332	125	5	13−217	75	0	−
wheat flour (organic)	5	0	−	−	4	nq−21	7	4	48−114	65	5	nq−74	33	1	nq
oat flakes	5	0	−	−	2	9−13	5	5	nq−94	42	4	nq−50	21	3	nq
oat flakes (organic)	5	0	−	−	0	−	−	5	nq−62	24	3	nq−21	9	4	nq
spaghetti	5	0	−	−	4	nq−12	5	5	22−642	234	5	6−134	54	0	−
spaghetti (organic)	5	0	−	−	2	nq	2	5	nq−68	25	5	nq−19	11	0	−

[a]The samples were conventional unless indicated as organic. [b]Min = minimum detected value; max = maximum detected value. [c]Mean = mean value of all samples in the category, with not detectable (−) and not quantifiable (nq; detected, but below limit of quantitation) results considered as nq = $^1/_2$(LOQ + LOD) and − = $^1/_2$(LOD).

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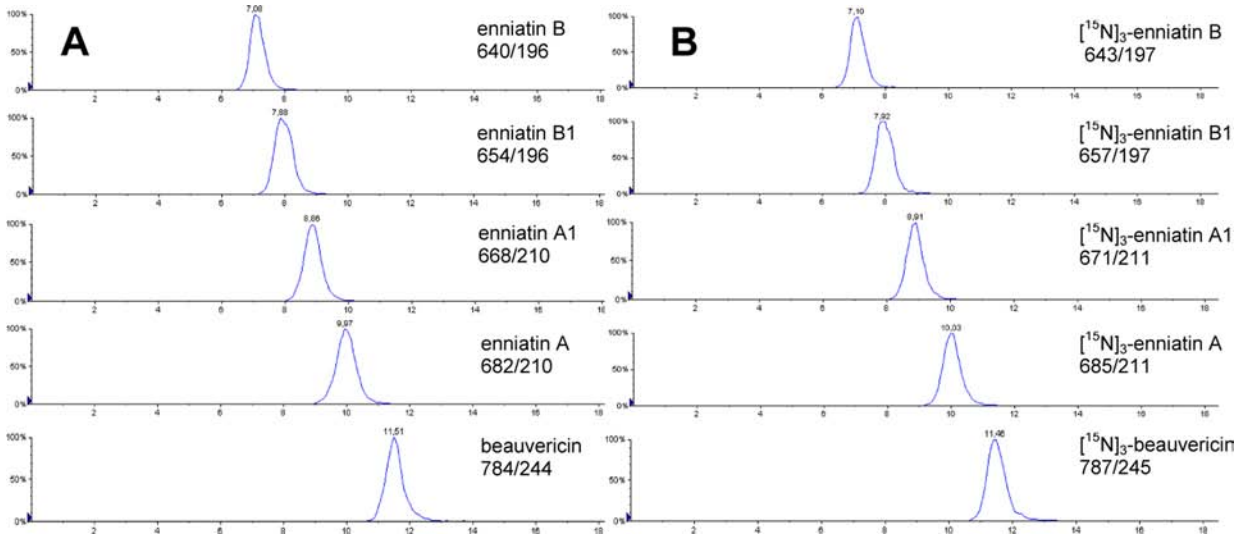

Figure 5. LC-MS/MS chromatograms of a barley malt sample: (A) analytes; (B) $^{15}N_3$-labeled standards.

more sensitive than methods previously reported[13,26] and 5 times more sensitive than that presented by Pamel et al.[18] The methods recently reported[14,15] are similarly sensitive as our assay. In contrast to this, two further LC-MS/MS assays were reported to be approximately 10 times more sensitive. For the first one, Jestoi et al.[17] applied 5 times more sample weight and did not report how LOD was determined, and for the second one, Sewram et al.[15] used a 20 times higher sample weight than we did. Similarly to Jestoi et al.,[17] the deduction of LOD in food samples remains unclear in the latter study.

Precision. The interday (n = 3) and intraday (n = 5) coefficients of variation are given in Table 2; they varied between 1.35 and 8.61%. With these results the stable isotope dilution assay presented here was similarly precise as those methods reported by Mahnine et al.[13] In contrast to this, the method of Pamel et al.[18] revealed relative standard deviations ranging between 8 and 49% and, therefore, was less precise.

Recovery. Table 2 shows the recoveries determined with different spiking levels (20, 35, and 50 μg/kg) of each mycotoxin. All of the recoveries fell in the range between 90 and 120%, with low standard deviations. These recoveries confirmed the expected superiority of stable isotope dilution assays over other assays as the other methods all showed recoveries for at least one depsipeptide as low as or far below 85%.[14−18,26]

Analysis of Cereals and Relating Food Samples. A series of cereals and relating food samples were analyzed for enniatin and beauvericin contamination using the stable isotope dilution assays developed. The results are summarized in Table 3. Figure 5 presents the LC-MS/MS chromatograms of a barley sample.

Overall, our findings show high incidence of, particularly, enniatins B and B1. Except for rice, all of the samples analyzed contained at least one of the five mycotoxins. The percentages of samples contaminated with enniatins A, A1, B, and B1 and beauvericin were 16.9, 52.3, 87.7, 83.1, and 24.6%, respectively.

The occurrence and concentrations of enniatins were in a distinct ratio (enniatin B > enniatin B1 > enniatin A1 > enniatin A), which was in accordance with previous investigations on Norwegian grains and Danish maize.[14,25] Wheat grains and barley malts were the most severely contaminated with enniatins; indeed, the highest levels of all four enniatins were detected in barley malts, with the amounts of enniatins B and B1 reaching 6998 and 6762 μg/kg, respectively. The contents of enniatins in other food samples were significantly lower, ranging from not detectable to 735 μg/kg. Earlier studies have reported the presence of enniatins in a variety of food samples, including wheat-, barley-, oat-, maize-, and cereal-based products with the levels of enniatins covering a wide range between <3.0 μg/kg and 814 mg/kg.[13,14,25,26] Therefore, our results of enniatin levels (from <5.8 to 6998 μg/kg) fell within the range of previous studies, but the maximum level was considerably lower.

Interestingly, none of our samples contained beauvericin above the LOQ (13.1 μg/kg), whereas other groups have reported cereals from Spain[26] and Italy[27] having beauvericin levels up to 11.8 and 520 mg/kg, respectively.

In addition, three sets of organic and conventional cereal products (wheat flour, oat flakes, and spaghetti) were compared for their contamination of enniatins and beauvericin. The organic products were found to be less contaminated with enniatins on average; their maximum levels were also lower. This result is similar to that for the trichothecene deoxynivalenol, which was found more abundantly in conventionally grown cereals.[30] The frequency of beauvericin in organic products was slightly higher than that in conventional ones; however, due to the negligibly low amount of beauvericin in all samples, this would not mean the organic products were a hazard to the consumer.

■ ASSOCIATED CONTENT

Supporting Information

Additional table of LC-MS/MS parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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