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Large-Scale Synthesis of Isotopically Labeled $^{13}C_2$-Tenuazonic Acid and Development of a Rapid HPLC-MS/MS Method for the Analysis of Tenuazonic Acid in Tomato and Pepper Products

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ABSTRACT: Tenuazonic acid is a fungal secondary metabolite that is produced by a number of *Alternaria* species and is therefore a natural contaminant of food and feed samples. This paper describes a new strategy for the efficient and economical large-scale synthesis of the isotopically labeled internal standard $^{13}C_2$-tenuazonic acid via a three-step procedure. Furthermore, a new reliable and quick method based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) cleanup is presented for the determination of tenuazonic acid in food and feed samples utilizing high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) by application of the stable isotope dilution analysis. This new method has a limit of detection (LOD) of 0.86 μg/kg and a limit of quantitation (LOQ) of 2.89 μg/kg. In total 26 tomato samples and 4 bell pepper samples from the German market were analyzed. Tenuazonic acid was found in each sample with levels from 3 to 2330 μg/kg.

KEYWORDS: *Alternaria, tenuazonic acid, synthesis, HPLC-MS/MS, QuEChERS, stable isotope dilution analysis*

INTRODUCTION

Tenuazonic acid (**1**) ((*S*)-3-acetyl-5-(*S*)-*sec*-butyltetramic acid) (Figure 1), a tetramic acid derivative, is a toxic secondary fungal metabolite, which belongs to the group of *Alternaria* toxins and was first isolated by Rosett and co-workers in 1957.[1] It is produced by a number of *Alternaria* species, including the common plant pathogen *Alternaria alternata*, as well as other species of fungi, including *Phoma sorghina* and *Pyricularia oryzae*.[2−4]

Among the *Alternaria* toxins, **1** is regarded as the one with the highest acute toxicity, displaying LD_{50} values (mice, applied orally) of 81−225 mg/kg body weight.[5−7] Yekeler et al.[8] revealed that precancerous changes occurred in the esophageal mucosa of mice when fed 25 mg/kg body weight per day of **1** for 10 months. The molecular mechanism for the toxic action of **1** involves inhibition of the protein biosynthesis at the ribosomal level in mammalian cells by suppression of the release of newly formed proteins from the ribosomes.[9,10] In several papers **1** showed biological activity, including cytotoxic, phytotoxic, antibacterial, and antiviral effects.[11−14]

Because *Alternaria* molds are frequently found in spoiled fruits, vegetables, and crops, processed food and feed samples infested by this fungus might be contaminated with **1**.[15,16] Due to the potential health risk for humans and animals posed by **1**, monitoring of different foods and feeds as well as physiological samples is of importance. Therefore, fast and reliable methods for the detection and quantitation of this mycotoxin in complex matrices are mandatory.

So far, several analytical methods for the quantitation of **1** are described in the literature, including thin layer chromatography (TLC) and visualization with ethanolic $FeCl_3$ solution (red-brown color),[3] gas chromatography coupled with mass spectrometric detection (GC-MS) of its trimethylsilyl ether,[17,18] and high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV).[19] However, the major problem of separation with HPLC is the poor chromatographic performance of **1** on reversed-phase columns because this mycotoxin has strong metal chelating properties and is a relatively strong acid (pK_a = 3.5).[20,21] To overcome this limitation, ion-pairing techniques by addition of alkylammonium phosphate as well as ligand exchange or metal complexation by addition of dodecyldiethylenetriamine and $Zn(II)SO_4$ to the mobile phase are adopted, when using reversed-phase.[19,21] However, the application of such additives to improve chromatography is often not compatible when HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is used. This is one reason why there was for a long time no HPLC-MS/MS method for the determination of **1** described in the literature. Recently, Siegel et al.[22] established a HPLC-MS/MS method that overcomes the above-described chromatographic difficulties by derivatization of **1** with 2,4-dinitrophenylhydrazine (DNPH) using electrospray ionization (ESI) in the negative mode (LOQ of 50 μg/kg). However, this derivatization step is laborious and not compatible with multimycotoxin methods. Furthermore, Shephard et al.[20] had already shown in 1991 that it is possible to analyze **1** by HPLC-UV without derivatization or the use of the above-described additives when a specific end-capped high carbon load HPLC column is used. On the basis of these results as well as the data provided by Kocher et al.,[23] the aim of the current work was to develop a method for trace analysis of **1** utilizing HPLC-MS/MS without prior derivatization.

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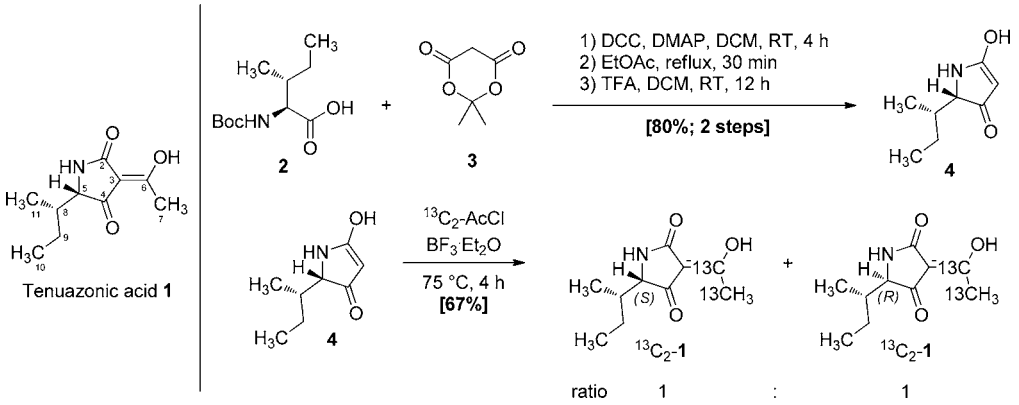

Figure 1. Structure of tenuazonic acid (**1**) ((*S*)-3-acetyl-5-(*S*)-*sec*-butyltetramic acid) and synthesis of $^{13}C_2$-tenuazonic acid ($^{13}C_2$-**1**). Isolated yields are given.

In contrast to all of the advantages featured by HPLC-MS/MS analysis, for instance, the high selectivity and sensitivity, this technique is susceptible to matrix effects. The ionization of the target analyte can be affected by coeluting matrix compounds, which can result in ion suppression or enhancement.[24−26] To compensate for matrix effects, stable isotope dilution analysis (SIDA) using isotopically labeled standards is often applied.[27,28] Recently, Asam et al.[29] developed a SIDA for **1** by applying [^{13}C,^{15}N]-**1** as isotopically labeled standard following the sample preparation published by Siegel et al.[22] (LOQ of 0.3 μg/kg). [^{13}C,^{15}N]-**1** was synthesized from commercially available [^{13}C,^{15}N]-L-isoleucine via Lacey−Dieckmann condensation in three steps (5.6 mg, overall yield = 27%). However, the disadvantages of this synthetic approach are the early introduction of the isotope labeling and the use of a relatively expensive labeled amino acid. This prompted us to develop a new strategy for the preparation of isotopically labeled **1**, which allows the economical and efficient synthesis of $^{13}C_2$-tenuazonic acid ($^{13}C_2$-**1**) on a large scale.

Finally, the objective of the present work was the development of a rapid HPLC-MS/MS method for the routine analysis of **1** in tomato and pepper products without any derivatization step using $^{13}C_2$-**1** as internal standard.

MATERIALS AND METHODS

Chemicals and Reagents. All solvents were provided from Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) in gradient grade. Dichloromethane was distilled and dried over molecular sieves 4 Å. Formic acid for mass spectrometry and anhydrous magnesium sulfate were obtained from Fluka (Steinheim, Germany). For all experiments, ultrapure water provided by a Millipore Milli-Q-System (Billerica, MA, USA) was used. Sodium chloride was purchased from Merck. The reference substance of **1**, as copper salt, and a certified analytical standard of **1**, for determination of exact concentration, were purchased from Sigma-Aldrich (Steinheim, Germany). Acetyl chloride-1,2-$^{13}C_2$, 99 atom % ^{13}C and ≥98% chemical purity, was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Other reagents were obtained from Carl Roth, Merck, and Sigma-Aldrich and used as received. The reusable, stackable plastic cups for the storage of the preweighed salt mixture were purchased from GML Alfaplast (Munich, Germany). The disposable 14 mL PP centrifuge tubes with screw caps were obtained from Simport (Beloine, Canada), and the disposable 50 mL polypropylene-centrifuge tubes with screw caps were obtained from Sarstedt (Nürmbrecht, Germany). Reactions, unless stated otherwise, were performed under an inert atmosphere of argon in flame-dried glassware. The LiChroprep RP_{18} material, 40−63 μm, for the purification of the synthesized products was purchased from Merck.

Nuclear Magnetic Resonance (NMR) Spectroscopy. All nuclear magnetic resonance (NMR) spectra were performed on a Bruker DPX-400 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). Chemical shifts are reported in parts per million relative to TMS using chloroform (δ 7.26), methanol (δ 3.31) or acetonitrile (δ 1.94) for ^{1}H NMR and chloroform (δ 77.0), methanol (δ 49.0), or acetonitrile (δ 118.26) for ^{13}C NMR as reference.

High-Resolution Mass Spectrometry. High-resolution mass spectrometry (HRMS) was performed on an LTQ Orbitrap XL using heated electrospray ionization (HESI) (Thermo Fisher Scientific, Bremen, Germany). Positive ionization mode was used for the detection of tetramic acid (**4**) and **1**. Source conditions were as follows: spray voltage, 3.5 kV; vaporizer temperature, 275 °C; capillary temperature, 275 °C; sheath gas, 30 arbitrary units; auxiliary gas, 10 arbitrary units; capillary voltage, 17 V; tube lens voltage, 85 V. The high-resolution mass spectrometer was operated at a resolution setting of 100,000. Data were processed using Xcalibur software (Thermo Fisher Scientific), version 2.0.7.

Synthetic Procedure. *N-Boc-L-Isoleucine (2).* L-Isoleucine (3.64 g, 27.6 mmol, 1.00 equiv) was suspended in 1 M sodium hydroxide solution (50 mL) and dioxane (25 mL). After the mixture had been cooled to 0 °C, di-*tert*-butyl dicarbonate (Boc anhydride) (6.64 g, 30.4 mmol, 1.10 equiv) in dioxane (20 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred overnight. After dilution with water (100 mL), the solution was washed with pentane (3 × 100 mL). The aqueous phase was acidified with solid citric acid to pH 4 and extracted with ethyl acetate (EtOAc) (3 × 100 mL). After the organic phase had been dried over dried Na_2SO_4, the solvent was removed under reduced pressure and *N*-Boc-L-isoleucine (**2**) was obtained as a colorless oil (6.10 g, 26.4 mmol, 96% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.19 (s, 1H, OH), 5.08 (d, J = 8.8 Hz, NH), 4.29 (dd, J = 8.8, 4.5 Hz, 1H, NHCHCOOH), 1.90 (m, 1H, $CH_3CHCH_2CH_3$), 1.43 (s, 9H, $COOC(CH_3)_3$), 1.29−1.12 (m, 2H, $CH_3CHCH_2CH_3$), 0.98−0.89 (m, 6H, $CH_3CHCH_2CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.00 (COOH), 155.83 ($COOC(CH_3)_3$), 67.14 ($COOC(CH_3)_3$), 57.93 (CHCOOH), 37.89 ($CHCH_3$), 28.43 ($C(CH_3)_3$), 25.01 (CH_2), 15.62 ($CHCH_3$), 11.76 (CH_2CH_3). HESI-HRMS: positive mode, m/z 232.1543 $[M + H]^+$, calculated for $[C_{11}H_{21}O_4N + H]^+$ 232.1543.

Tetramic Acid (4). *N*-Boc-L-isoleucine (**2**) (559 mg, 2.42 mmol, 1.00 equiv), Meldrum's acid (**3**) (2,2-dimethyl-1,3-dioxane-4,6-dione) (523 mg, 3.63 mmol, 1.50 equiv), and 4-dimethylaminopyridine (DMAP) (354 mg, 2.90 mmol, 1.20 equiv) were dissolved in dichloromethane (CH_2Cl_2) (12 mL). The reaction mixture was cooled to 0 °C, and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (598 mg, 2.90 mmol, 1.20 equiv) in CH_2Cl_2 (2 mL) was added slowly with stirring over a period of 10 min. The yellow reaction mixture was allowed to warm to room temperature, and stirring was continued for an additional 4 h. To remove formed *N*,*N*′-dicylcohexylurea (DCU), the reaction mixture was filtered. After the addition of EtOAc (50 mL),

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the organic phase was washed with a saturated solution of sodium chloride (3 × 50 mL), 5% aqueous citric acid (3 × 50 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The remaining residue was dissolved in EtOAc (30 mL) and refluxed for 30 min. Evaporation of the solvent afforded the *N*-Boc protected tetramic acid, which was used without further purification. The residue was dissolved in a two-neck round-bottom flask in CH_2Cl_2 (12 mL), and trifluoroacetic acid (5 mL) was added. The reaction mixture was stirred at room temperature for 12 h, and the conversion was monitored by TLC. After quenching with saturated sodium bicarbonate ($NaHCO_3$) solution, the suspension was transferred to a separatory funnel and the phases were separated. The aqueous phase was extracted with chloroform ($CHCl_3$) (3 × 50 mL), and the combined organic phases were dried over Na_2SO_4. After removal of solvent under reduced pressure, the raw product was purified by column chromatography (30 × 300 mm) over LiChroprep RP_{18} material (40−63 μm) (CH_3CN/H_2O, 2/8, v/v), and tetramic acid (**4**) was obtained as a yellow solid (468 mg, 3.02 mmol, 80% yield, over two steps).

^{1}H NMR (400 MHz, CD_3CN) δ 6.88 (s, 1H, NH), 3.89−3.85 (m, 1H, NHCHCO), 2.86 (s, 2H, $COCH_2CO$), 1.86−1.74 (m, 1H, $CH_3CHCH_2CH_3$), 1.37−1.17 (m, 2H, $CH_3CHCH_2CH_3$), 0.93 (d, J = 7.0 Hz, 3H, CH_3CH), 0.88 (t, J = 7.4 Hz, 3H, CH_2CH_3). ^{13}C NMR (101 MHz, CD_3CN) δ 209.91($CHCOCH_2$), 171.95 ($NHCOCH_2$), 69.36 (NHCHCO), 42.22 ($COCH_2CO$), 38.48 ($CH_3CHCH_2CH_3$), 25.14 ($CHCH_2CH_3$), 15.51 ($CHCH_3$), 11.92 (CH_2CH_3). HESI-HRMS: positive mode, m/z 156.1016 $[M + H]^+$, calculated for $[C_8H_{13}O_2N + H]^+$ 156.1019. HESI-MS^2 (CID = 25%) m/z (% rel int): 156 ($[M + H]^+$, 100), 128 (42).

*$^{13}C_2$-Tenuazonic acid ($^{13}C_2$-**1**).* In a two-neck round-bottom flask tetramic acid (**4**) (463 mg, 2.97 mmol, 1.00 equiv) was dissolved in boron trifluoride diethyl ether complex ($BF_3{\cdot}Et_2O$) (10 mL), and acetyl chloride-1,2-$^{13}C_2$ (0.80 mL, 0.88 g, 10.9 mmol, 4.00 equiv) was added. The reaction mixture was stirred at 75 °C for 5 h, and the conversion was monitored by TLC. After the mixture had cooled to room temperature, water (40 mL) was added, and the reaction mixture was extracted with EtOAc (3 × 100 mL). The organic phase was extracted with aqueous sodium hydroxide (5% w/v; 3 × 100 mL), and the combined aqueous phases were washed with $CHCl_3$ (2 × 100 mL). The aqueous phase was acidified with hydrochloric acid (concentrated) to pH 4 and extracted with $CHCl_3$ (3 × 100 mL). The combined organic phases were dried over Na_2SO_4, and the solvent was removed under reduced pressure. Purification by column chromatography (30 × 300 mm) over LiChroprep RP_{18} material (40−63 μm) (CH_3CN/H_2O + 1% formic acid, 4/6, v/v) afforded $^{13}C_2$-**1** as a diastereomeric mixture (1:1) (401 mg, 2.00 mmol, 67% yield).

^{1}H NMR (400 MHz, MeOD) δ 3.92 (s, 0.5H, H-5, 5*R*,8*S*), 3.84 (s, 0.5H, H-5, 5*S*,8*S*), 2.58 (dd, J = 6.3, 2.5 Hz, 1.5H, H-7), 2.26 (dd, J = 6.3, 2.5 Hz,, 1.5H, H-7), 1.91 (ttt, J = 10.6, 8.8, 4.3 Hz, 1H, H-8), 1.53−1.16 (m, 2H, H-9), 0.99 (d, J = 7.0 Hz, 1.5H, H-11, 5*S*,8*S*), 0.97 (t, J = 7.4 Hz, 1.5H, H-10, 5*R*,8*S*), 0.90 (t, J = 7.4 Hz, 1.5H, H-10, 5*S*,8*S*), 0.77 (d, J = 6.8 Hz, 1.5H, H-11, 5*R*,8*S*). ^{13}C NMR (101 MHz, MeOD) δ 187.33 (bs, ^{13}C-6), 38.43 (C-8, 5*S*,8*S*), 38.01 (C-8, 5*R*,8*S*), 27.62 (C-9, 5*R*,8*S*), 24.89 (C-9, 5*S*,8*S*), 20.38 (d, J = 44.8 Hz, 13C-7, 5*S*,8*S*), 20.20 (d, J = 44.9 Hz, 5*R*,8*S*), 15.90 (C-11, 5*S*,8*S*), 13.34 (C-11, 5*R*,8*S*), 12.20 (C-10, 5*S*,8*S*), 12.15 (C-10, 5*R*,8*S*). UV_{max} = 277 nm. HESI-HRMS: positive mode, m/z 200.1190 $[M + H]^+$, calculated for $[C_8{}^{13}C_2H_{15}O_3N + H]^+$ 200.1192. HESI-MS^2 (CID = 25%) m/z (% rel int): 200 ($[M + H]^+$, 100), 155 (35), 183 (12), 127 (9), 182 (7).

Stock Solution and Working Solution. A purchased certified analytical standard of the free acid of **1** (100 μg, evaporated to dryness) was dissolved in 1 mL of methanol, which resulted in a certified stock solution (100 μg/mL). From this stock solution a working solution (5 μg/mL) was prepared by dilution with methanol. Additionally, 10 mg of **1** copper salt was dissolved in 20 mL of methanol (stock solution of **1** copper salt) and further diluted with methanol to a concentration of approximately 5 μg/mL (working solution of **1** copper salt). The exact concentration of this working solution was determined by the HPLC-MS/MS method described below using the working solution prepared from the respective certified analytical standard of **1** as reference. The solutions of the **1** copper salt were necessary due to the limited availability of the certified analytical standard of **1**.

The internal standard (IS) (2 mg), $^{13}C_2$-**1**, was dissolved with methanol, and the solution was transferred to a 50 mL volumetric flask. The flask was repeatedly flushed with methanol, which was completely transferred into the 50 mL volumetric flask. The volume was filled to 50 mL to obtain a $^{13}C_2$-**1** stock solution (40 μg/mL). The stock solution was diluted with methanol to a concentration of 10 μg/mL (theoretically), and the correct concentration was determined by UV spectroscopy (DU 800 Beckman Coulter GmbH, Krefeld, Germany) using the molar extinction coefficient of **1** given by Scott and Kanhere ($\varepsilon_{277\ nm}$ = 12980 L/mol/cm)[17] in methanol, which is mostly used in current publications (11.04 μg/mL).[20,22,29] However, in the literature, also slightly different molar extinction coefficients have been reported in the past.[30−32] A working solution of $^{13}C_2$-**1** with a concentration of 5.52 μg/mL was prepared in methanol. All solutions were stored at −20 °C under exclusion of light to ensure stability.

Sample Preparation. In preparation for the sample extraction, the mixtures of solids ($MgSO_4$/NaCl and $MgSO_4$) were portioned using two different sample dividers: Laborette 27 (Fritsch, Idar-Oberstein, Germany) and Repro A (Bürkle, Bad Bellingen, Germany). For the phase separation step, 4 g of $MgSO_4$ and 1 g of NaCl were used per 20 mL of extract. For the drying step of the CH_3CN extract, 150 mg of $MgSO_4$ was used per milliliter of CH_3CN phase. Samples of 2.5 g (concentrated tomato products) or 5 g (liquid tomato products), respectively, of the homogenized samples were weighed into a 50 mL centrifuge tube and spiked with 50 μL of the $^{13}C_2$-**1** working solution (5.52 μg/mL) as internal standard. Then 10 mL of water for the initial weight of 2.5 g or 5 mL of water for the initial weight of 5 g, respectively, was added. After slurrying the samples with water, 10 mL of CH_3CN and 110 μL of formic acid were added, and the toxins were extracted within 25 min by shaking on a horizontal shaker (first extraction). For the phase separation the preweighed mixture of salts (4 g $MgSO_4$/1 g NaCl) was added and the samples were shaken manually for 1 min (second extraction with phase separation). After centrifugation at 1029*g* for 10 min, the samples were cooled to room temperature. An aliquot of the upper layer (6 mL) was transferred to a 14 mL centrifuge tube, which already contained 1.2 g of $MgSO_4$. The extracts were manually shaken for 1 min and then centrifuged at 1029*g* for 5 min (drying step of the CH_3CN phase). One hundred microliters of the supernatant were diluted with 400 μL of mobile phase, and the vortexed sample solution was directly used for HPLC-MS/MS analysis.

Calibration Curves in Solvent and LOD and LOQ. For external quantitation, six standard solutions containing 5−25 ng/mL **1**, with 5 ng/mL of $^{13}C_2$-**1** in each calibration point, were prepared in mobile phase. The peak area ratios of the analyte and the IS were plotted against the concentration ratios, and calibration curves were calculated by linear regression. The signal-to-noise (S/N) ratio of the lowest calibration level was calculated via Analyst 1.6 software, and LOD and LOQ were determined based on S/N = 3 and S/N = 10 in solvent-assisted standard as well as in matrix-assisted standard. For the matrix-assisted standards a blank sample of tomato paste was prepared according to the sample preparation, and 200 μL of the CH_3CN extract was evaporated in a heated aluminum block at 40 °C using a gentle stream of nitrogen. The residue was redissolved in the mobile phase and used for the preparation of matrix standard solutions with different concentration levels, which were used for the determination of LOD and LOQ.

Recovery Experiments. For recovery experiments the samples were spiked with a spiking solution of 1.25 μg/mL **1** in methanol prior to extraction. The spiking level was 99.3 μg/kg. For each group of tomato and pepper products one matrix was tested for recovery.

HPLC-MS/MS. HPLC analysis was performed using a Waters Acquity system consisting of a binary solvent manager, column manager, and sample manager (Waters GmbH, Eschborn, Germany). Chromatographic separation of the analyte and IS was performed on a 100 mm × 2.1 mm i.d., 1.7 μm, BEH C18 column with a 5 mm × 2.1 mm i.d. VanGuard precolumn (Waters GmbH). The column

temperature was set to 40 °C, and the injection volume was 5 μL. Solvent A was 1 mM ammonium bicarbonate in methanol/water (5:95, v/v), and solvent B was methanol. A binary gradient at a flow rate of 0.2 mL/min was performed as follows: within 10 min, initial condition of 100% solvent A was held for 2 min, then changed to 75% solvent B within 1 min and changed to 95% solvent B within another minute, which was held for 3 min. Then, the content of solvent B was lowered within 1 min to 0%, which was held for an additional 2 min.

The MS/MS measurements were performed on an AB Sciex QTRAP 5500 mass spectrometer equipped with a Turbo V ion electrospray ionization (ESI) source (ABSciex, Darmstadt, Germany) in the negative ionization mode. The ion spray voltage was set at −3500 V. Zero grade air was used for nebulizer and heated at 500 °C as turbo gas. Nitrogen served as curtain gas and as collision gas in quadrupole 2 (1.6×10^{-5} Torr). Quantitation was achieved using scheduled multiple reaction monitoring (SMRM) with a target scan time of 0.5 s and a MRM detection window of 70 s. The following transition reactions of **1** and the internal standard $^{13}C_2$-**1** with the respective declustering potential (DP), collision energy (CE), and cell exit potential (CXP) in parentheses were recorded using the first mass transition for quantitation: **1** *m/z* 196.0 → 139 (DP −70 V, CE −26 V, CXP −15 V), *m/z* 196.0 → 112.0 (DP −70 V, CE −32 V, CXP −13 V); $^{13}C_2$-**1** *m/z* 198.0 → 141.0 (DP −45 V, CE −26 V, CXP −15 V), *m/z* 198.0 → 114 (DP −40 V, CE −32 V, CXP −13 V).

RESULTS AND DISCUSSION

Synthesis of Isotopically Labeled Tenuazonic Acid. The most widely used method for the synthesis of tetramic acid, the core structure of tenuazonic acid (**1**), is the Lacey-Dieckmann cyclization. After *N*-acylation of L-isoleucine methyl ester with diketene, the *N*-acetoacetyl amino acid is cyclized under basic conditions to afford **1**.[29] As already mentioned, one disadvantage of this route is that the isotope labeling via [^{13}C,^{15}N]-L-isoleucine is introduced in the first step of the synthesis.[28] Thus, we have developed a new strategy for the synthesis of isotopically labeled **1**, which allows the introduction of the isotope labeling in the last step (Figure 1). On the basis of a protocol published by Jouin et al.[33] as well as Hosseini et al.,[34] an unlabeled tetramic acid (**4**) precursor is synthesized in two steps from *N*-Boc-L-isoleucine (**2**) and Meldrum's acid (**3**) via a condensation and a subsequent thermal cyclization and decarboxylation reaction. The introduction of isotope labeling is performed in the last step via a 3-*C*-acylation of tetramic acid (**4**) with labeled acetyl chloride.

The synthesis started with the preparation of tetramic acid (**4**), which was synthesized efficiently in two steps using a modified approach published by Hosseini et al.[34] In their protocol 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was employed as activating reagent for the condensation reaction between a *N*-Boc-*α*-amino acid and Meldrum's acid (**3**). In our study EDC as well as DCC were tested. As both reagents gave comparable yields of tetramic acid (**4**) (86% for EDC and 80% for DCC), the cheaper DCC was chosen for large-scale synthesis.

In detail, *N*-Boc-L-isoleucine (**2**), which was derived from L-isoleucine in 99% yield, was reacted with Meldrum's acid (**3**) in a condensation reaction, activated by DCC. Becuase the condensation intermediate is unstable, it was used directly in the next step without any purification. Subsequent cyclization and decarboxylation provided *N*-Boc-tetramic acid by refluxing in EtOAc. After deprotection with trifluoroacetic acid/CH_2Cl_2, we obtained the key unit **4**.

Having tetramic acid (**4**) in hand, the key step of this reaction pathway, the 3-*C*-acylation, was performed by reacting tetramic acid (**4**) with acetyl chloride. The isotope labeling was introduced at this stage by application of acetyl chloride-1,2-$^{13}C_2$. We decided to introduce ^{13}C instead of deuterium labeling, because d_3-tenuazonic acid would undergo rapid D/H exchange due to the keto−enol tautomerism given in this molecule. Under basic conditions the 4-*O*-acylation of tetramic acid (**4**) with acid chloride is generally favored.[35] There are some reports of the in situ conversion of the 4-*O*-acylated product to the 3-*C*-acylated product by acyl migration.[36] However, with regard to the application of expensive labeled starting material, a direct selective 3-*C*-acylation of tetramic acid (**4**) is of great interest. Jones et al.[37] developed a Lewis acid mediated procedure using $BF_3{\cdot}Et_2O$, which allows the otherwise difficult selective acylation in C-3 position. After a slight modification of this approach, tetramic acid (**4**) was acylated with acetyl chloride-1,2-$^{13}C_2$ in $BF_3{\cdot}Et_2O$, and the desired isotopically labeled $^{13}C_2$-**1** was obtained in good yield (401 mg, 67%) with an isotope purity of >99% (HPLC-HRMS) and a purity of 97% (HPLC-UV-ELSD). During this reaction step epimerization of $^{13}C_2$-**1** was observed. The obtained product exists as a mixture of the natural occurring (5*S*,8*S*)-diastereoisomer and of the unnatural (5*R*,8*S*)-diastereoisomer in a ratio of 1:1 (determined by ^{1}H NMR and ^{13}C NMR). The NMR data of $^{13}C_2$-**1** were in accordance with those of (5*S*,8*S*)-**1** and (5*R*,8*S*)-**1** (D-allo-**1**) published by Gallardo and co-workers.[38] With the employed reversed-phase column we did not observe any separation of both diastereomers with common analytical methods. Therefore, the diastereomeric mixture of $^{13}C_2$-**1** can be used as internal standard. However, separation of both diastereomers is possible under certain conditions, and methods dealing with this problem are described in the literature.[20,21]

The incorporation of two ^{13}C atoms was proven by the accurate masses and the characteristic fragmentation patterns of **1** and $^{13}C_2$-**1** as well as by NMR experiments. Figure 2 displays the accurate mass and the mass spectrometric fragmentation

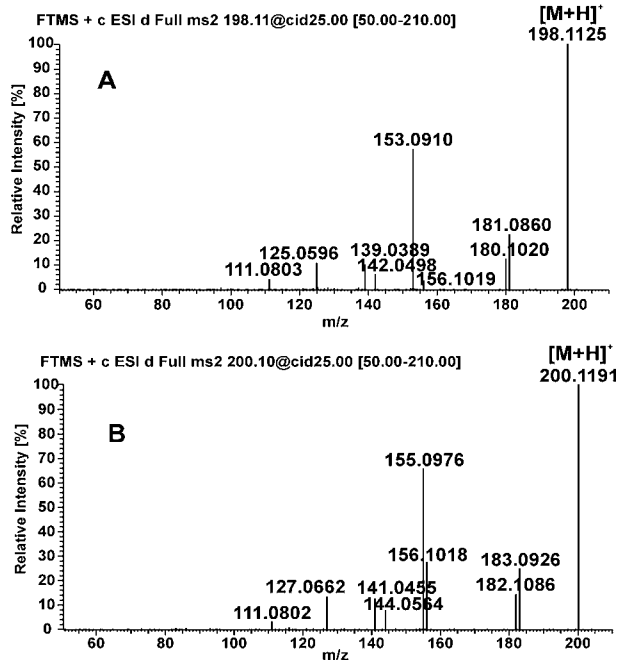

Figure 2. HESI-MS^2 product ion spectra obtained by CID (25%) of the pseudomolecular ion $[M + H]^+$ *m/z* 198.1125 (**1**, A) and *m/z* 200.1191 ($^{13}C_2$-**1**, B).

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patterns of **1** (Figure 2A) and $^{13}C_2$-**1** (Figure 2B), recorded in the positive HESI mode. The pseudomolecular mass $[M + H]^+$ 200.1191 of $^{13}C_2$-**1** is 2 Da higher than that of **1** $[M + H]^+$ 198.1125. The main fragment ions in both spectra are *m/z* 183.0926 ($^{13}C_2$-**1**) and 181.0860 (**1**), which are derived from the loss of ammonia, and *m/z* 155.0976 ($^{13}C_2$-**1**) and 153.0910 (**1**), which result from further fragmentation (loss of carbon monoxide). The proposed fragmentation of $^{13}C_2$-**1** is displayed

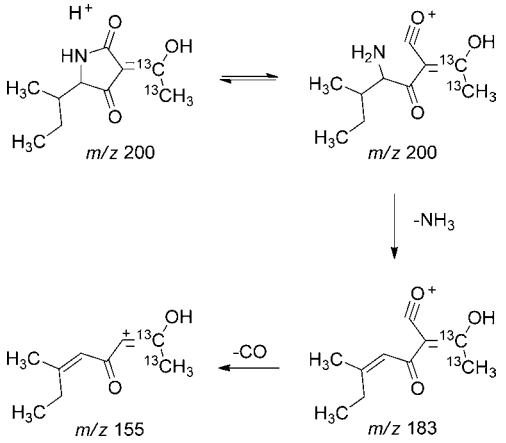

Figure 3. Proposed fragmentation pattern of $^{13}C_2$-tenuazonic acid ($^{13}C_2$-**1**) *m/z* 200.

in Figure 3. In addition, ^{1}H NMR and ^{13}C NMR experiments of $^{13}C_2$-**1** and **1** confirmed the structure of the synthesized standard $^{13}C_2$-**1**. The ^{1}H NMR spectrum of $^{13}C_2$-**1** displays a signal splitting of the H-7 due to the $^1J(^{13}C\text{-}7, ^1H\text{-}7)$ and $^2J(^{13}C\text{-}6, ^1H\text{-}7)$ coupling with the neighboring ^{13}C atoms C-7 and C-6. The ^{13}C spectrum shows two significant signals of the two incorporated ^{13}C atoms at δ 187.56 (C-6) and δ 20.29 (C-7). Moreover, the UV spectrum of $^{13}C_2$-**1** is similar to the spectrum of **1** with an absorbance maximum of 277 nm.

Stable Isotope Dilution Analysis. The determination of **1** was performed using an in-house validated method, which was combined with the SIDA by addition of $^{13}C_2$-**1** as internal standard. The sample preparation based on the QuEChERS protocol, which is widely used for pesticide analysis, has recently been also applied for mycotoxin analysis, including the mycotoxins deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, and fusarin C.[39−42]

This method is well established and has several advantages because it is quick, easy, cheap, effective, rugged, and safe. Slight modifications of the protocol during the extraction step were performed. Furthermore, the dispersive solid phase material, primary-secondary amine, which is mainly used for the analysis with GC, was omitted, because it is known to have the ability to bind acidic compounds.[43] Due to the strong acidity of **1** the usage of primary-secondary amine material was avoided, which is also timesaving regarding the application of this method in routine analytics. Possible matrix interferences, which can be caused by omitting the purification step, are reduced by dilution of the CH_3CN extract before analysis. To obtain reliable quantitative results even with complex matrices, for instance, tomato sauces and pastes, no additional cleanup step was necessary, due to the application of $^{13}C_2$-**1** as IS. The IS was added at the beginning of the sample preparation, which results in a compensation of matrix effects during the ionization process and simultaneous correction of losses during sample preparation. The biggest disadvantage of previously described methods using the HPLC-MS/MS technique for the determination of **1** is the derivatization step, which is necessary due to the poor chromatographic performance of **1**.[29] Recently, Kocher et al.[23] published in a poster presentation a method that allows the reliable chromatographic separation of **1** without any derivatization step before HPLC-MS/MS analysis by adding ammonium bicarbonate to the mobile phase (pH 7.5). Therefore, this method is more time efficient and avoids the sometimes troublesome derivatization step. Additionally, the use of the end-capped BEH C18 column prevents binding of **1** on free silanol groups, which results in an improved chromatographic performance and sharper peaks of **1**. This observation is consistent with the study reported by Shephard et al.[20] For the mass spectrometric detection a QTRAP 5500 mass spectrometer was used. Figure 4 displays an example of a

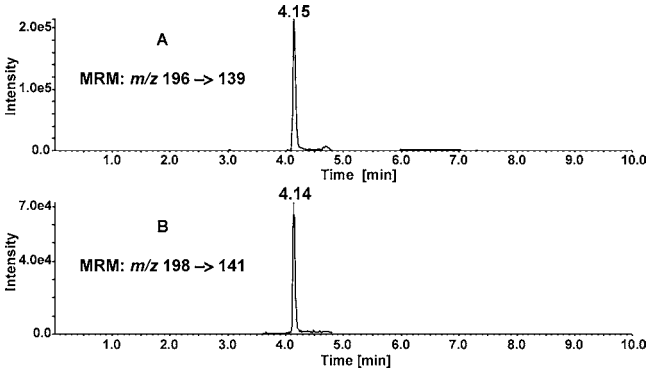

Figure 4. Negative ESI-HPLC-MS/MS chromatograms of an extract of a tomato sauce (A) tenuazonic acid and (B) $^{13}C_2$-tenuazonic acid, presenting the quantifier MRM transitions.

negative ESI-HPLC-MS/MS chromatogram of an extract of a tomato sauce. The LOD and the LOQ of this method were determined by the S/N without as well as with matrix. With this method a LOD (S/N of 3) of 0.59 μg/kg and a LOQ (S/N of 10) of 1.97 μg/kg were obtained for the solvent-assisted calibration. In a matrix-assisted calibration solution a LOD (S/N of 3) of 0.86 μg/kg and a LOQ (S/N of 10) of 2.89 μg/kg were obtained.

Recovery. The recovery rates were determined in every major group of tomato and pepper matrix, spiked at the level of 99.3 μg/kg **1**. The samples were worked up in triplicate for each matrix, and the results were calculated taking the natural levels of **1** of each matrix into account. The recovery rates for **1** were 102 ± 4.4% for minced tomato products and juices, 98.9 ± 4.3% for tomato pastes, 101 ± 2.2% for tomato sauces (ready to use), 98.6 ± 1.7% for tomato ketchups, 91.0 ± 1.2% for pepper pastes, and 96.7 ± 3.6% for tomato powder.

Tenuazonic Acid in Tomato and Pepper Products. With the herein described method 26 different tomato and four different pepper products purchased from the German market were analyzed for their **1** content in triplicate (Table 1).

The analysis revealed that all tested products contained **1**. The highest content of **1**, at a concentration of 2330 μg/kg, was found in a sample of tomato powder, which is used for the production of instant soups. In ready-to-use powder products that contain approximately 50% tomato powder, values from 118 to 508 μg/kg were found. With regard to the concentration factor in the powder of around 10 times, the contents are

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Table 1. Tenuazonic Acid Levels of Analyzed Samples from the German Market

sample	concentration[a] (μg/kg ± SD)	sample	concentration[a] (μg/kg ± SD)
tomato paste triple-concentrated	38 ± 1.2	curry tomato ketchup	81 ± 1.2
tomato paste triple-concentrated	145 ± 5.7	salsa	86 ± 1.2
tomato paste double-concentrated	541 ± 6.4	tomato sauce mozarella (53% tomato powder)	119 ± 0.2
minced tomatos	98 ± 0.6	soup of vine tomatos (42% tomato powder)	508 ± 4.0
minced tomatos 2	23 ± 0.1	tomato powder (pure)	2330 ± 30.6
tomato purree	77 ± 1.7	tomato sauce with herbs	17 ± 0.3
tomato purree 2	29 ± 1.0	tomato sauce with herbs	7 ± 0.1
tomato juice	48 ± 0.7	tomato sauce basilikum	24 ± 0.3
tomato ketchup	80 ± 1.9	tomato sauce pasta basilikum	43 ± 0.7
tomato ketchup 2	19 ± 1.0	tomato sauce vegetables	52 ± 1.2
tomato ketchup 3	96 ± 1.0	tomato sauce for kids	145 ± 6.4
tomato ketchup 4	11 ± 0.2	pepper sauce	712 ± 19.9
tomato ketchup 5	105 ± 5.8	Ajvar pepper sauce	116 ± 1.7
tomato ketchup 6	85 ± 2.6	Ajvar pepper sauce 2	41 ± 1.6
tomato ketchup "fruity"	58 ± 3.0	Calabrian pepper sauce	3 ± 0.3

[a]Each sample was analyzed in triplicate.

comparable to those found in tomato concentrates with a median of 144 μg/kg and in minced tomatoes or tomato sauces with a median of 38 or 42 μg/kg, respectively.

The concentration values of the analyzed tomato ketchups ranged from 11 to 104 μg/kg with a median level of 81 μg/kg. One can assume from the results for the content of **1** in tomato products that the levels are comparable to previously published concentrations in tomato products.[29,44]

In addition, we analyzed four samples of pepper sauces and pastes for their content of **1**. Surprisingly, we found relatively high levels ranging from 3 to 712 μg/kg with a median level of 78 μg/kg. To date, high levels of **1** are described only in pepper powder.[45] Therefore, the analysis of further pepper products should be performed in the future.

In summary, we have developed a new efficient and economical strategy for the large-scale synthesis of isotopically labeled tenuazonic acid, $^{13}C_2$-**1**. This pathway allows access to large amounts of $^{13}C_2$-**1** in only three steps and an overall yield of 54%. With the newly synthesized internal standard $^{13}C_2$-**1** a reliable quantitation of **1** in tomato and pepper products using a SIDA with HPLC-MS/MS techniques was developed. Time-consuming derivatization of **1** was not necessary due to the improved chromatographic conditions. The analysis was performed according to an in-house validated method, which is also suitable for the determination of other *Alternaria* toxins, including alternariol, alternariol monomethyl ether, tentoxin, and altenuene.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CXP, collision cell exit potential; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMAP, 4-dimethylaminopyridine; DP, declustering potential; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HESI, heated electrospray ionization; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum correlation

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