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Identification and in Vitro Cytotoxicity of Ochratoxin A Degradation Products Formed during Coffee Roasting

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The mycotoxin ochratoxin A is degraded by up to 90% during coffee roasting. In order to investigate this degradation, model heating experiments with ochratoxin A were carried out, and the reaction products were analyzed by HPLC-DAD and HPLC-MS/MS. Two ochratoxin A degradation products were identified, and their structure and absolute configuration were determined. As degradation reactions, the isomerization to 14-(*R*)-ochratoxin A and the decarboxylation to 14-decarboxy-ochratoxin A were identified. Subsequently, an analytical method for the determination of these compounds in roasted coffee was developed. Quantification was carried out by HPLC-MS/MS and the use of stable isotope dilution analysis. By using this method for the analysis of 15 coffee samples from the German market, it could be shown that, during coffee roasting, the ochratoxin A diastereomer 14-(*R*)-ochratoxin A was formed in amounts of up to 25.6% relative to ochratoxin A. The decarboxylation product was formed only in traces. For toxicity evaluations, first preliminary cell culture assays were performed with the two new substances. Both degradation products exhibited higher IC_{50} values and caused apoptotic effects with higher concentrations than ochratoxin A in cultured human kidney epithelial cells. Thus, these cell culture data suggest that the degradation products are less cytotoxic than ochratoxin A.

KEYWORDS: Ochratoxin A; coffee roasting; degradation; toxicity; cell culture; 14-(*R*)-ochratoxin A; cytotoxicity

INTRODUCTION

Ochratoxin A (14-(*S*)-ochratoxin A, *N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L-phenylalanine) (**Figure 1**) is a toxic fungal secondary metabolite produced by molds of the genera *Penicillium* and *Aspergillus*. It can be found throughout the world in various commodities such as cereal based products, coffee, cocoa, grape juice, beer, and wine (*1*). 14-(*S*)-Ochratoxin A possesses nephrotoxic, teratogenic, and immunotoxic properties (*2*). Furthermore, it was classified as possibly carcinogenic to humans (group 2b) by the International Agency for Research on Cancer (IARC) in 1993 (*3*).

14-(*S*)-Ochratoxin A is generally described as a relatively stable mycotoxin with a reduction of approximately 30% after dry heating at 150 °C for 1 h (*4*). However, an increased loss of 14-(*S*)-ochratoxin A is observed at higher temperatures, as reported for coffee roasting. The reduction of 14-(*S*)-ochratoxin A levels during this process has been investigated in numerous experiments. While some studies report no or little loss of 14-(*S*)-ochratoxin A (*5, 6, 34*), a reduction of the 14-(*S*)-ochratoxin A level ranging from 70 to 90% was observed in most instances (*7–9, 35*). The formation of degradation products has not been investigated in these studies, but isomerization as well as binding to matrix compounds have been suggested as possible degradation reactions (*6, 10, 11*).

The high degradation rates of 14-(*S*)-ochratoxin A observed in most of the publications gives rise to the question whether

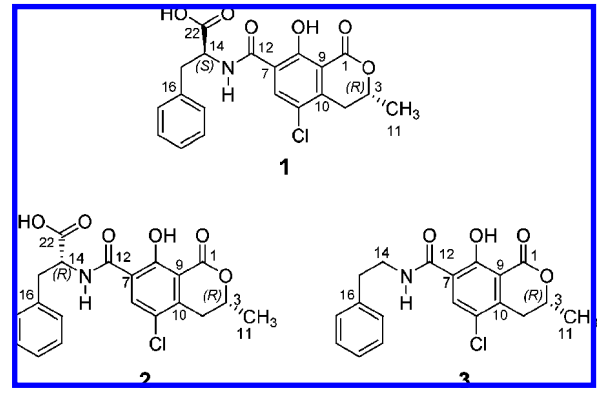

Figure 1. Structures of 14-(*S*)-ochratoxin A (ochratoxin A) (**1**) and the degradation products 14-(*R*)-ochratoxin A (**2**) and 14-decarboxy-ochratoxin A (**3**).

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this loss of 14-(*S*)-ochratoxin A also means a detoxification during coffee roasting or only a masking of 14-(*S*)-ochratoxin A.

The objective of the present work was the identification of thermal degradation products of 14-(*S*)-ochratoxin A in roasted coffee. For this purpose, the isolation and identification of possible thermal degradation products of 14-(*S*)-ochratoxin A derived from model heating experiments were carried out. Subsequently, a method for identification and quantification of these degradation products in roasted coffee using HPLC-MS/MS was developed. Additionally, first data on the cytotoxic and apoptotic effects of the degradation products were determined in a cultured human kidney epithelial cell line.

MATERIALS AND METHODS

General Remarks. All solvents and reagents were purchased from VWR (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) in gradient grade or reagent quality. 14-(*S*)-Ochratoxin A was produced by fungal culture. A modified yeast extract sucrose-medium (YES medium), which contained 200 g/L sucrose, 20 g/L yeast extract, and 500 mg/L potassium sorbate, was inoculated with spores of *Penicillium verrucosum* (DSM 12639), and the fungus was allowed to grow for 28 days at 25 °C. The culture was then autoclaved, acidified with formic acid to pH <3, and extracted with *tert*-butyl methyl ether. Further purification was achieved by centrifugal partition chromatography using a Kromaton-200 fast centrifugal partition chromatograph (Kromaton Technologies, Angers, France) at 1000 rpm in descending mode with a solvent system of water/methanol/acetonitrile/*tert*-butyl methyl ether/ethyl acetate/pentane (5:3:3:3:3:5) and a flow rate of 10 mL/min. The final product was crystallized from toluene. The received 14-(*S*)-ochratoxin A had a purity of ≥98% and contained no detecteable (≤0.2%) 14-(*R*)-ochratoxin A or 14-decarboxy-ochratoxin A.

Water for HPLC separation was purified with a MilliQ Gradient A 10 system (Millipore, Schwalbach, Germany).

NMR Spectroscopy. All NMR experiments were carried out on a Bruker DCX-400 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany). Signals are reported in parts per million referenced to TMS. For structural elucidation, additionally to the 1D ^{1}H- and ^{13}C-experiments, further 2D NMR experiments such as gradient selected correlation spectroscopy (gs-COSY), heteronuclear multiple bond correlation (HMBC), and heteromolecular multiple quantum correlation (HMQC) spectroscopy were performed. Pulse programs for the experiments were taken from the Bruker software library.

Model Experiments. For the 14-(*S*)-ochratoxin A model experiments, 500 μL of a stock solution of 14-(*S*)-ochratoxin A (100 μg/mL in acetonitrile) was evaporated to dryness in a 1.5 mL autosampler vial under a stream of nitrogen at 40 °C. The capped vials were heated at 175, 200, and 225 °C, respectively. Heating periods were 5, 10, and 20 min, and the experiments were carried out in triplicate. The reaction products were dissolved in 500 μL of acetonitrile/water/formic acid (30:70:0.1) and sonicated for 5 min. A portion of the received solutions (10 μL) was injected into an analytical HPLC system equipped with a Jasco PU-2089 pump (Jasco GmbH, Gross-Umstadt, Germany) delivering a binary gradient of acetonitrile (A) and water with 0.1% formic acid (B). Samples were injected by a Jasco AS-2057plus autosampler, and peaks were detected by a Jasco MD-2010plus diode array detector and a Jasco FP-1520 fluorescence detector set to the wavelengths 333 nm for excitation and 460 nm for emission. For separation, a 250 mm × 4 mm i.d., 4 μm, ReproSilPur C18-AQ HPLC column (Dr. Maisch, Ammerbach, Germany) was applied. The following linear gradient was used: 0 min, 20% A, 1 min, 20% A, 30 min 100% A, 40 min 100% A. After each run, the column was equilibrated at starting conditions for 10 min.

Synthesis of 14-(*R*)-Ochratoxin A. 14-(*R*)-Ochratoxin A was produced by thermal isomerization of 14-(*S*)-ochratoxin A. 14-(*S*)-Ochratoxin A (13 mg) was heated in a 4 mL vial at 200 °C for 10 min. The resulting mixture containing the two diastereomers 14-(*R*)-ochratoxin A and 14-(*S*)-ochratoxin A was purified on a semipreparative 250 mm × 16 mm i.d., 4 μm, Synergi Fusion column (Phenomenex, Aschaffenburg, Germany) using an isocratic mixture of 55% water containing 0.1% formic acid and 45% acetonitrile delivered by two Varian ProStar 210 HPLC solvent delivery modules (Varian, Darmstadt, Germany). The flow rate was set at 10 mL/min. 14-(*R*)-Ochratoxin A with a retention time of 26.2 min and 14-(*S*)-ochratoxin A with a retention time of 24.0 min were collected after peak-detection by a Varian ProStar 325 UV/vis detector set at 330 nm. Acetonitrile was removed from each fraction, and the aqueous solution was extracted with *tert*-butyl methyl ether. The organic phases were dried over sodium sulfate, and *tert*-butyl methyl ether was removed under reduced pressure. Yield: 42% 14-(*R*)-ochratoxin A and 45% of 14-(*S*)-ochratoxin A. The 14-(*R*)-ochratoxin A contained ≤0.5% 14-(*S*)-ochratoxin A and no detectable (≤0.2%) 14-decarboxy-ochratoxin A.

14-(R)-Ochratoxin A. ^{1}H NMR (400 MHz, C_6D_6) δ 0.79 (3H, d, J = 6.3 Hz, H-11), 1.79 (1H, dd, J = 12.0 Hz, J = 17.3 Hz, H-4a), 2.38 (1H, dd, J = 3.1 Hz, J = 17.3 Hz, H-4b), 3.16 (1H, dd, J = 7.3 Hz, J = 13.9 Hz, H-15a), 3.34 (1H, dd, J = 4.7 Hz, J = 13.9 Hz, H-15b), 3.67 (1H, m, H-3), 5.20 (1H, br, C-8 OH), 5.25 (1H, m, H-14), 7.00 (1H, t, J = 7.3 Hz, H-19), 7.11 (2H, t, J = 7.5 Hz, H-18, H-20), 7.19 (1H, d, J = 7.4 Hz, H-17, H-21), 8.62 (1H, s, H-6), 8.65 (1H, d, J = 6.6 Hz, N-13-H), 13.00 (1H, s, C-22 OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7 (C-11), 32.3 (C-4), 37.3 (C-15), 54.5 (C-14), 75.9 (C-3), 110.1 (C-9), 120.2 (C-7), 123.2 (C-5), 127.3 (C-19), 128.7 (C-17, C-21), 129.3 (C-18, C-20), 135.8 (C-16), 139.0 (C-6), 141.0 (C-10), 159.1 (C-8), 163.2 (C-12), 169.8 (C-1), 174.8 (C-22). ESI-MS: negative mode: m/z 402 $[M - H]^-$, MS/MS (−20 V): m/z (%) 402 (100), 358 (69), 211 (10), positive mode: m/z 404 $[M + H]^+$, MS/MS (+20 V): m/z (%) 239 (100), 404 (66), 358 (54), 341 (15).

14-(S)-Ochratoxin A. ^{1}H NMR (400 MHz, C_6D_6) δ 0.77 (3H, d, J = 6.3 Hz, H-11), 1.79 (1H, dd, J = 11.8 Hz, J = 17.3 Hz, H-4a), 2.33 (1H, dd, J = 3.4 Hz, J = 17.3 Hz, H-4b), 3.14 (1H, dd, J = 7.3 Hz, J = 14.0 Hz, H-15a), 3.32 (1H, dd, J = 5.2 Hz, J = 14.1 Hz, H-15b), 3.52 (ddd, 1H, J = 3.4 Hz, J = 6.2 Hz, J = 11.8 Hz, H-3), 5.20 (br, 1H, C-8 OH), 5.25 (1H, m, H-14), 7.00 (1H, t, J=7.3 Hz, H-19), 7.09 (2H, t, J = 7.6 Hz, H-18, H-20), 7.17 (2H, d, J = 7.2 Hz, H-17, H-21), 8.64 (1H, s, H-6), 8.65 (1H, d, J = 5.3 Hz, H-13), 13.01 (1H, s, C-22 OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7 (C-11), 32.3 (C-4), 37.3 (C-15), 54.5 (C-14), 75.9 (C-3), 110.1 (C-9), 120.2 (C-7), 123.2 (C-5), 127.3 (C-19), 128.7 (C-17, C-21), 129.3 (C-18, C-20), 135.8 (C-16), 139.0 (C-6), 141.0 (C-10), 159.1 (C-8), 163.2 (C-12), 169.8 (C-1), 174.8 (C-22). ESI-MS: negative mode: m/z 402 $[M - H]^-$, MS/MS (−20 V): m/z (%) 402 (100), 358 (62), 211 (9), positive mode: m/z 404 $[M + H]^+$, MS/MS (+20 V): m/z (%) 239 (100), 404 (94), 358 (65), 341 (15).

Determination of the Absolute Configuration of 14-(*R*)-Ochratoxin A. 14-(*S*)-Ochratoxin A (15 mg) and the diastereomer 14-(*R*)-ochratoxin A were hydrolyzed separately to phenylalanine and ochratoxin α in 8 mL of 6 M hydrochloric acid in a sealed 12 mL screw-capped vessel at 110 °C for 24 h under constant stirring. After cooling down, ochratoxin α was extracted with 5 mL of *tert*-butyl methyl ether, and the organic solvent was removed in vacuum. Ochratoxin α formed from 14-(*S*)-ochratoxin A and 14-(*R*)-ochratoxin A was purified by semipreparative HPLC using the system described above but with 1% formic acid as aqueous solvent. Each of the purified products was dissolved in 3 mL of methanol and directly used for polarimetric measurements. The exact concentration was determined by measuring the absorption at 333 nm (ε (MeOH) = 6640 [mol × cm]$^{-1}$) (*33*). Both compounds showed almost the same optical rotation with α^{25}_{589}(MeOH) = −163.0 for ochratoxin α originated from 14-(*S*)-ochratoxin A and α^{25}_{589}(MeOH) = −167.0 for ochratoxin α from the ochratoxin A diastereomer.

The configuration of phenylalanine derived from 14-(*R*)-ochratoxin A by hydrolysis was determined using chiral HPLC. A portion of the extracted aqueous phase (200 μL) was evaporated to dryness in a stream of nitrogen at 80 °C. Residual hydrochloric acid was removed by addition of 100 μL of water and subsequent evaporation. For analysis, the sample was dissolved in 500 μL of methanol/2 mM copper(II) sulfate in water (30:70) and separated on a 150 mm × 4.6 mm i.d., Chirex 3126 (D)-penicillamine column (Phenomenex, Aschaffenburg, Germany) using the analytical HPLC system described above and an isocratic mixture of methanol and 2 mM copper(II) sulfate in water

(30/70) at a flow rate of 1 mL/min. The injection volume was 50 μL, and the detection wavelength was 254 nm with L-Phenylalanine at a retention time of 17.9 min and D-phenylalanine at a retention time of 24.8 min.

Preparative Hydrolysis of 14-(*S*)-Ochratoxin A to Ochratoxin α. 14-(*S*)-Ochratoxin A was hydrolyzed using a slightly modified method of the hydrolysis previously described by van der Merwe et al. (*19*). In brief, 75 mg (186 μmol) of ochratoxin A was stirred in 50 mL of 6 M hydrochloric acid and heated under reflux for 36 h. After cooling down, the obtained suspension was diluted with 30 mL of water and extracted with 50 mL of *tert*-butyl methyl ether. After drying the combined organic layers over sodium sulfate, the solvent was removed to yield ochratoxin α, which was used for the synthesis of 14-decarboxy-ochratoxin A. Yield: 27 mg (105 μmol, 56%).

Synthesis of 14-Decarboxy-ochratoxin A. A solution of 4.0 mg of ochratoxin α (15.6 μmol) in 2 mL of dry chloroform and 50 μL of 2-phenylethylamine (397 μmol) was stirred at 0 °C under argon atmosphere. After dropwise addition of 100 μL of thionyl chloride (1.37 mmol), the mixture was allowed to warm to room temperature and stirred overnight. Chloroform (15 mL) was added to the reaction mixture, and the solution was washed with 15 mL of 1 M hydrochloric acid. After solvent removal under reduced pressure, the crude 14-decarboxy-ochratoxin A was purified using the semipreparative HPLC system described above and an isocratic gradient of 50% water with 0.1% formic acid and 50% acetonitrile to 100% acetonitrile after 30 min. The flow rate was set to 10 mL/min, and 14-decarboxy-ochratoxin A was collected after peak detection at 330 nm at a retention time of 13.3 min. Yield: 5 mg (13.9 μmol, 89%). The purity was approximately 98% with no detectable 14-(*S*)-ochratoxin A, 14-(*R*)-ochratoxin A, and ochratoxin α (LC/MS/MS).

14-Decarboxy-ochratoxin A. ^{1}H NMR (400 MHz, $CHCl_3$) δ 1.62 (3H, d, $J_{11,3} = 6.3$ Hz, H-11), 2.87 (1H, dd, $J_{AB} = 17.4$, $J_{3,4A} = 11.6$ Hz, H-4A), 2.96 (2H, t, $J_{14,15} = 7.1$ Hz, H-15), 3.31 (1H, dd, $J_{AB} = 17.4$ Hz, $J_{3,4B} = 3.4$ Hz, H-4B), 3.77 (2H, q, $J_{13,14}$ and $J_{14,15} = 7.1$ Hz, H-14), 4.78 (1H, m, H-3), 7.23−7.28 (3H, m, H-17, H-19, H-21), 7.38−7.31 (2H, m, H-18, H-20), 8.10 (1H, s, broad, H-13), 8.50 (1H, s, H-6), 12.70 (1H, s, OH-8). ESI-MS: negative mode: *m/z* 358 $[M - H]^-$, MS/MS (−40 V): *m/z* (%) 167 (100), 211 (63) 358 (27), 314 (13), 123 (11) positive mode: *m/z* 360 $[M + H]^+$, MS/MS (+25 V): *m/z* (%) 239 (100), 360 (18), 221 (11), 105 (10).

Stable Isotope Dilution Analysis. Isotope labeled d_5-14-(*S*)-ochratoxin A was produced by growing *Penicillium verrucosum* (DSM 12639) on d_5-L-phenylalanine enriched minimal growth medium for 28 days at 25 °C (unpublished data). The medium consists of 20 g of sucrose, 1.5 g of agar, 400 mg of urea, 50 mg of potassium hydrogen phosphate, 50 mg of magnesium sulfate heptahydrate, 50 mg of potassium chloride, and 25 mg of zinc sulfate heptahydrate dissolved in 100 mL of distilled water. The medium was autoclaved, and 100 mg of d_5-L-phenylalanine was added to the solution at 70 °C. The final isotopic purity of d_5-14-(*S*)-ochratoxin A was determined using HPLC-MS/MS and was greater than 95%. d_5-14-(*R*)-Ochratoxin A was produced by thermal isomerization of d_5-(*S*)-ochratoxin A, as described above, and d_5-14-decarboxy-ochratoxin A was produced according to the synthesis of the unlabeled compound but with d_5-2-phenylethyl amine as reagent. d_5-2-Phenylethyl amine was synthesized starting from d_5-bromobenzene, as previously described by Morrison and Botting (*13*).

The internal standards (IS) d_5-14-(*S*)-ochratoxin A and d_5-14-(*R*)-ochratoxin A were dissolved in methanol to a concentration of approximately 25 μg/mL. The exact concentration was determined by photometric measurement, as described above. The stock solution of the internal standard d_5-14-decarboxy-ochratoxin A was prepared by dissolving 5 mg of the compound in 10 mL of acetonitrile. This solution was diluted with acetonitrile to a final concentration of 200 ng/mL.

These stock solutions were mixed and diluted with acetonitrile to give a final internal standard solution containing 25 ng/mL d_5-14-(*S*)-ochratoxin A, 12.5 ng/mL d_5-14-(*R*)-ochratoxin A, and 5 ng/mL d_5-14-decarboxy-ochratoxin A. A portion of this internal standard solution (1 mL) was added to 25 g of ground, homogenized roasted coffee, and the sample was extracted for 30 min with 500 mL of methanol/sodium bicarbonate solution (3%) (1:1) on a laboratory shaker at room temperature (*14*). For soluble coffee, 5 g of sample was extracted with 100 mL of methanol/sodium bicarbonate solution (3%) (1:1), and only 200 μL of the internal standard solution was added. After filtration, a 5 mL aliquot was diluted with alkaline phosphate buffered saline (PBS), pH 7.0, to 100 mL, and the whole solution was purified using OchraTest immunoaffinity columns (VICAM, Watertown, MA, USA). 14-(*S*)-ochratoxin A and the degradation products were eluted with 2 mL of methanol. After solvent removal in a stream of nitrogen at 40 °C, the extract was dissolved in 250 μL of methanol/water/formic acid (60:40:0.1) and injected into the HPLC-MS/MS system.

For the determination of the recovery rate, a coffee sample containing 0.1 ± 0.02 μg/kg 14-(*S*)-ochratoxin A, 14-(*R*)-ochratoxin A below the limit of quantification (LOQ), and no detectable 14-decarboxy-ochratoxin A was spiked at three different levels with 14-(*S*)-ochratoxin A (0.1, 1.0, and 10 μg/kg), 14-(*R*)-ochratoxin A (0.05, 0.5, and 5.0 μg/kg), and 14-decarboxy-ochratoxin A (0.02, 0.2, and 2.0 μg/kg). Each spiked sample was worked up independently in duplicate, giving the following recovery rates (±SD): 14-(*S*)-ochratoxin A (99.2 ± 5.3%, 100.9 ± 3.4%, 107.9 ± 7.0%), 14-(*R*)-ochratoxin A (118.1 ± 6.4%, 104.9 ± 6.9%, 106.1 ± 3.7%), and 14-decarboxy-ochratoxin A (78.8 ± 3.9%, 76.3 ± 4.7%, 72.6 ± 6.4%).

Calibration. Calibration solutions were prepared as follows: Aliquots of the standard solutions of 14-(*S*)-ochratoxin A, 14-(*R*)-ochratoxin A, and 14-decarboxy-ochratoxin A were mixed with the respective internal standards d_5-14-(*S*)-ochratoxin A (1.0 ng/mL), d_5-14-(*R*)-ochratoxin A (0.5 ng/mL), and d_5-14-decarboxy-ochratoxin A (0.2 ng/mL) to give various standard/analyte concentration ratios of 1:10 up to 10:1. The mixtures were analyzed by HPLC-MS/MS in the multiple reaction monitoring mode (MRM), as described below. Each calibration point was determined three times. The resulting peak area ratios were plotted against the concentration ratios. The LOQ was 0.1 ng/mL for 14-(*S*)-ochratoxin A, 0.05 ng/mL for 14-(*R*)-ochratoxin A, and 0.02 ng/mL for 14-decarboxy-ochratoxin A. The limit of detection (LOD) for each compound was by a factor of 3 lower.

HPLC-MS/MS. ESI mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. The MS/MS parameters were dependent on the compounds, detecting the fragmentation of the $[M - H]^-$ or $[M + H]^+$ molecular ions into specific product ions after collision with nitrogen (4.2×10^{-5} torr). Both quadrupoles were set at unit resolution.

For HPLC-ESI-MS/MS analysis, an Agilent 1100 series HPLC was linked to the mass spectrometer. Data acquisition was carried out with the Analyst 1.4 software (Applied Biosystems). Chromatographic separation was performed on a 150 mm × 2.0 mm i.d., 5 μm, Gemini C18 column (Phenomenex, Aschaffenburg, Germany) using a binary gradient. Solvent A was methanol with 0.1% formic acid, and solvent B was water with 0.1% formic acid. The following linear gradient was used: 0 min 60% A, 1 min 60% A, 10 min 100% A, 15 min 100% A. After each run, the column was equilibrated for 8 min at starting conditions. The injection volume was 50 μL, and the flow rate was set to 250 μL/ min. For HPLC-MS/MS, the mass spectrometer was operated in the MRM mode, detecting positive ions. Zero grade air was used as the nebulizer gas (30 psi) and heated at 300 °C as turbo gas for solvent drying (50 psi). Nitrogen served as the curtain gas (20 psi) and collision gas (4.2×10^{-5} torr). The following transition reactions were monitored for a duration of 150 ms each (declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are given in parentheses): 14-(*S*)-ochratoxin A and 14-(*R*)-ochratoxin A: *m/z* 404 → 239 (DP 81 V, CE 33 V, CXP 15 V), d_5-14-(*S*)-ochratoxin A and d_5-14-(*R*)-ochratoxin A: *m/z* 409 → 239 (DP 81 V, CE 33 V, CXP 15 V), 14-decarboxy-ochratoxin A: *m/z* 360 → 239 (DP 81 V, CE 29 V, CXP 15 V), d_5-14-decarboxy-ochratoxin A *m/z* 365 → 239 (DP 81 V, CE 29 V, CXP 15 V).

Cell Culture. Immortalized human kidney epithelial cells (IHKE cells) were kindly provided by M. Gekle (Institut für Physiologie, Martin-Luther-Universität Halle Wittenberg, Germany). IHKE cells were cultivated as described by Tveito et al. (*15*) in DMEM/HAM-F-12 medium enriched with 1.1 g/L $NaHCO_3$, 3.57 g/L HEPES, 5 mg/L human apo-transferrin, 5 mg/L bovine insulin, 0.5 mg/L hydrocortisone,

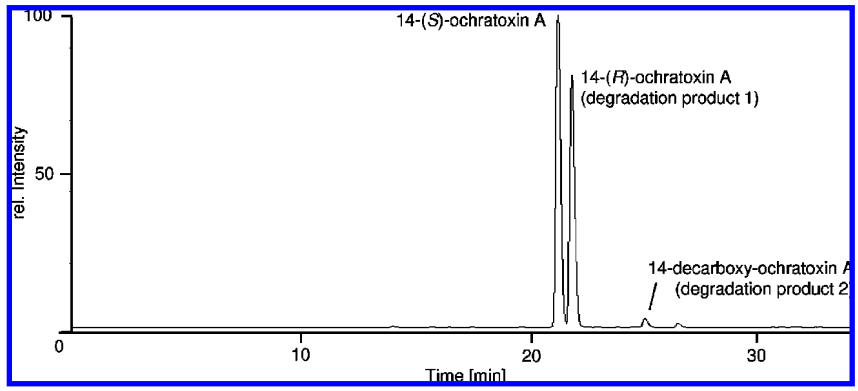

Figure 2. HPLC-FLD chromatogram of 14-(*S*)-ochratoxin A after heating at 225 °C for 5 min.

10 μg/L mouse epidermal growth factor (EGF), 5 μg/L sodium selenite, and 1% fetal calf serum under standard cell culture conditions (37 °C, 5% CO_2). For experiments, cells were seeded in 96-well (CCK-8 assay) or 24-well plates (caspase-3 assay). In plates, cell medium was changed to serum-free medium when cells reached a microscopic confluence of approximately 80%. The cytotoxicity assays were performed under serum-free conditions to exclude any binding of the tested compound to serum proteins. Approximately 24 h after the change into serum-free conditions, 14-(*S*)-ochratoxin A, 14-(*R*)-ochratoxin A, or 14-decarboxy-ochratoxin A (stock solution: 1 mM in methanol) was added in concentrations ranging from 1 nM to 50 μM and incubated for 24 h (14-(*S*)-ochratoxin A/14-(*R*)-ochratoxin A/caspase-3) and 48 h (14-(*S*)-ochratoxin A/14-decarboxy-ochratoxin A/CCK-8 and 14-(*S*)-ochratoxin A/14-decarboxy-ochratoxin A/caspase-3), respectively. Control cells were incubated with an equal solvent concentration. Studies with 14-(*R*)-ochratoxin A were performed between passages 146 and 156, experiments with 14-decarboxy-ochratoxin A between passages 175 and 185. Each experiment was carried out simultaneously with 14-(*S*)-ochratoxin A, 14-(*R*)-ochratoxin A, and 14-decarboxy-ochratoxin A, allowing a better comparability of the effects of 14-(*S*)-ochratoxin A with its degradation products at similar passages.

Cytotoxicity Assay (CCK-8). Cytotoxicity was evaluated colorimetrically with the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan), according to the manufacturer's instructions. Briefly, cells were seeded on 96-well microplates. After toxin exposure, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt solution (WST-8 solution) was added, and the cells were incubated for 1.5 h at 37 °C. The absorbance of each well was measured with a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany) at 450 nm. WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The amount of the formazan generated by the activity of dehydrogenases in cells is directly proportional to the number of viable cells per well. The results of toxin-treated cells were compared with a solvent treated control.

Caspase-3 Assay. Cells were seeded in 24-well plates. After toxin incubation, the medium was discarded, and the cells were washed with cold PBS buffer and incubated with 100 μL of cell lysis buffer (10 mM TRIS, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5) for 15 min on ice. The cell lysates were harvested with a cell scraper and centrifuged at 15 000*g* for 10 min at 4 °C. A portion of the supernatant (50 μL) was incubated with 50 μL of reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT) and 5 μL of caspase-substrate (80 μM DEVD-AFC, dissolved in DMSO) in a black 96-well microplate at 37 °C for up to 15 h. The fluorescence of the cleaved product AFC was measured at 400 nm excitation and 505 nm emission wavelengths using a FLUOstar Optima microplate reader. Cleaved AFC was quantified by a calibration curve using known AFC concentrations (0.32−12.8 μM) and was normalized to the protein content per well. Protein concentrations were determined with a bicinchoninic acid (BCA) assay kit from Sigma-Aldrich (Deisenhof, Germany) using bovine serum albumin (BSA) as standard.

Statistics. All cell culture measurements are given as mean values ± SEM. The significance of difference was determined by the unpaired Student's *t* test. $p \leq 0.05$ was considered to be statistically significant. All experiments were performed at least in three different cell passages using a minimum of four wells per group each time.

RESULTS AND DISCUSSION

In order to investigate the degradation of 14-(*S*)-ochratoxin A during food processing, heating experiments of pure 14-(*S*)-ochratoxin A were performed. As reported previously for other mycotoxins, small amounts of 14-(*S*)-ochratoxin A were heated in glass vials at temperatures between 175 and 225 °C for 5, 10, and 20 min (*16–18*). The analysis of the reaction residues by analytical HPLC with diode array (DAD) and fluorescence (FLD) detection revealed two major degradation products (**Figure 2**).

In the model experiment, heating 14-(*S*)-ochratoxin A at 175 °C and higher temperatures resulted in a degradation product in quantities equal to the parent compound. Higher temperatures and longer heating periods furthermore supported the formation of a second degradation product. However, this compound was formed in much smaller amounts during the model heating. Experiments in larger scale under optimized heating temperature and heating period formed degradation product **1** in large amounts. Thus, the purification of the reaction mixture by semipreparative HPLC provided this compound in high yields and allowed full structure elucidation. Degradation product **1** showed the same molecular ions as 14-(*S*)-ochratoxin A with signals for $[M + H]^+$ at *m*/*z* 404 in the positive and for $[M - H]^-$ at *m*/*z* 402 in the negative mode, respectively. Further signals at *m*/*z* 406 and *m*/*z* 404, characteristic for the ^{37}Cl-isotope of ochratoxin A, could also be observed. The product ion spectra of this degradation product also gave fragmentation patterns similar to those recorded for 14-(*S*)-ochratoxin A with only little differences in the signal intensities. In the positive mode, product ions at *m*/*z* 358 and *m*/*z* 239 could be detected, which are characteristic for the loss of formic acid at C-14 and the cleavage of the phenylalanine moiety, respectively. In the negative mode, the product ion *m*/*z* 358 indicated a decarboxylation of C-22 while *m*/*z* 211 is the product of a fragmentation between C-8 and C-12, the cleavage of phenylalanine, including the carboxylic acid of the dihydroisocoumarin moiety. Summarizing, the MS data indicated that degradation product **1** is an isomer of 14-(*S*)-ochratoxin A. Comparison of the NMR data revealed similar spectra for 14-(*S*)-ochratoxin A and the degradation product **1** with differences only in the chemical shift of four hydrogen signals around the stereocenter of C-3 with benzene as solvent. For H-3 of degradation product **1**, a low field shift of 0.15 ppm is observed. H-4B and H-11 also shifted low field by 0.05 and 0.02 ppm, respectively. Thus, the 1D- and 2D-NMR data proved that degradation product **1** is a 14-(*S*)-ochratoxin A diastereomer. However, the low field shift around

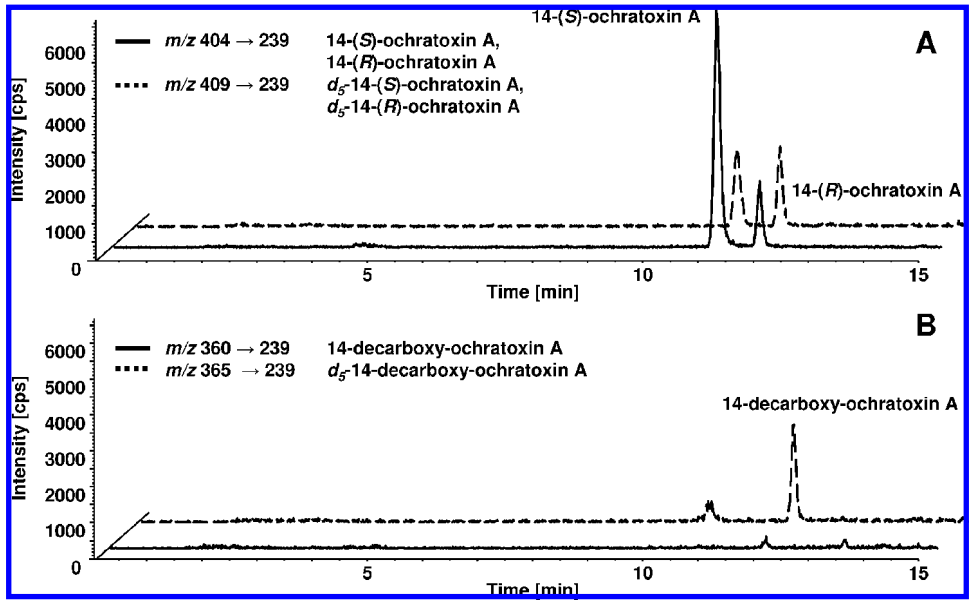

Figure 3. HPLC-MS/MS chromatograms of a soluble coffee sample: (**A**) 14-(*S*)-ochratoxin A (2.04 ± 0.01 μg/kg), 14-(*R*)-ochratoxin A (0.29 ± 0.01 μg/kg); (**B**) 14-decarboxy-ochratoxin A (0.07 ± 0.02 μg/kg).

C-3 observed in the NMR spectrum allowed no conclusion regarding the position of isomerization. The change of the stereochemistry at any of the two stereocenters of 14-(*S*)-ochratoxin A would lead to a diastereomer with this chemical shift as long as no chiral selector is added in the NMR experiments. To determine the position of isomerization, the configuration of the two chiral centers of this diastereomer was investigated. Therefore, this compound was hydrolyzed in 6 M hydrochloric acid, and the obtained products, phenylalanine and ochratoxin α, were analyzed for their respective configuration. Ochratoxin α from both compounds showed nearly identical values in polarimetric measurement; thus, it could be shown that the isomerization did not occur in this part of the ochratoxin A molecule. Phenylalanine derived from hydrolysis of the 14-(*S*)-ochratoxin A diastereomer was analyzed for its configuration by chiral HPLC. It was determined as D-phenylalanine ((*R*)-phenylalanine) and thus as the enantiomer of L-phenylalanine from 14-(*S*)-ochratoxin A (*19*). The 14-(*S*)-ochratoxin A degradation product **1** is therefore the diastereomer 14-(*R*)-ochratoxin A (**Figure 1**). A previously suggested isomerization of 14-(*S*)-ochratoxin A to the 3-(*S*)-ochratoxin A diastereomer was not observed in our heating experiments (*6, 20*). As 3-(*S*)-ochratoxin A would show the same chromatographic behavior as 14-(*R*)-ochratoxin A, the results of the polarimetric measurements show that this diastereomer is generally not formed in significant amounts in the heating experiments and cannot be expected to occur in processed coffee.

The second degradation product of 14-(*S*)-ochratoxin A observed in the model experiment was formed only in small amounts. This compound showed molecular ions of *m/z* 358 $[M - H]^-$ in the negative and *m/z* 360 $[M + H]^+$ in the positive mode. The respective signals for the ^{37}Cl-isotope at *m/z*360 (negative mode) and *m/z* 362 (positive mode) could also be observed. In the positive mode, degradation product **2** showed a fragment at *m/z* 239, known as the characteristic cleavage of the amide bond to the phenylalanine moiety of 14-(S)-ochratoxin A. In the negative mode, degradation product **2** forms a product ion *m/z* 211, which can also be observed in the respective spectrum of 14-(S)-ochratoxin A. Thus, mass spectrometric fragmentation indicated that this compound is the decarboxylation product of 14-(*S*)-ochratoxin A. To confirm this, 14-decarboxy-ochratoxin A was synthesized by amide coupling of ochratoxin α with 2-phenylethylamine, as previously described by Xiao et al. (*21*). Identical retention times and mass spectrometric fragmentation patterns of thermal degradation product **2** and the synthesized reference finally identified this compound as 14-decarboxy-ochratoxin A (**Figure 1**).

Table 1. Concentration of 14-(*S*)-Ochratoxin A, 14-(*R*)-Ochratoxin A, and 14-Decarboxy-ochratoxin A in Representative Coffee Samples

	concentration[a], μg/kg		
coffee sample	14-(*S*)-ochratoxin A	14-(*R*)-ochratoxin A	14-decarboxy-ochratoxin A
1	1.04	0.11	<LOQ
2	1.28 ± 0.42	0.25 ± 0.02	<LOQ
3	0.39	0.10	n.d.[b]
4	0.58	0.13	<LOQ
5	0.83 ± 0.27	0.21 ± 0.04	<LOQ
6	2.94 ± 0.01	0.63 ± 0.01	<LOQ
7	0.56 ± 0.09	0.12 ± 0.01	0.05 ± 0.05
8	1.26	0.22	<LOQ
9	0.06	<LOQ	n.d.[b]
10	0.15	<LOQ	n.d.[b]
11	0.48	0.09	<LOQ
12	0.15	<LOQ	n.d.[b]
13	0.16	<LOQ	n.d.[b]
14 (soluble coffee)	2.04 ± 0.01	0.29 ± 0.01	0.07 ± 0.02
15 (soluble coffee)	2.15 ± 0.04	0.26 ± 0.01	0.07 ± 0.01

[a] Standard deviation given for duplicate analysis. [b] n.d. = not determined.

To study the relevance of these two 14-(*S*)-ochratoxin A degradation products, 13 roasted coffee samples and 2 soluble coffee samples from the German retail market were analyzed for the occurrence of 14-(*R*)-ochratoxin A, 14-decarboxy-ochratoxin A, and 14-(*S*)-ochratoxin A. Because of the different structures of the degradation products compared to the parent compound 14-(*S*)-ochratoxin A, the cleanup by immunoaffinity columns was found to be a crucial step. Comparison of the three different immunoaffinity columns OTAclean (LC-Tech GmbH, Dorfen, Germany), OchraStar (Romer Laboratories, Tulln, Austria), and OchraTest (VICAM, Watertown, MA) showed that only the immunoaffinity columns provided by VICAM were able to bind 14-(*R*)-ochratoxin A and 14-decarboxy-ochratoxin A. However, to overcome any possible experimental uncertainties in the immunoaffinity cleanup of the degradation products

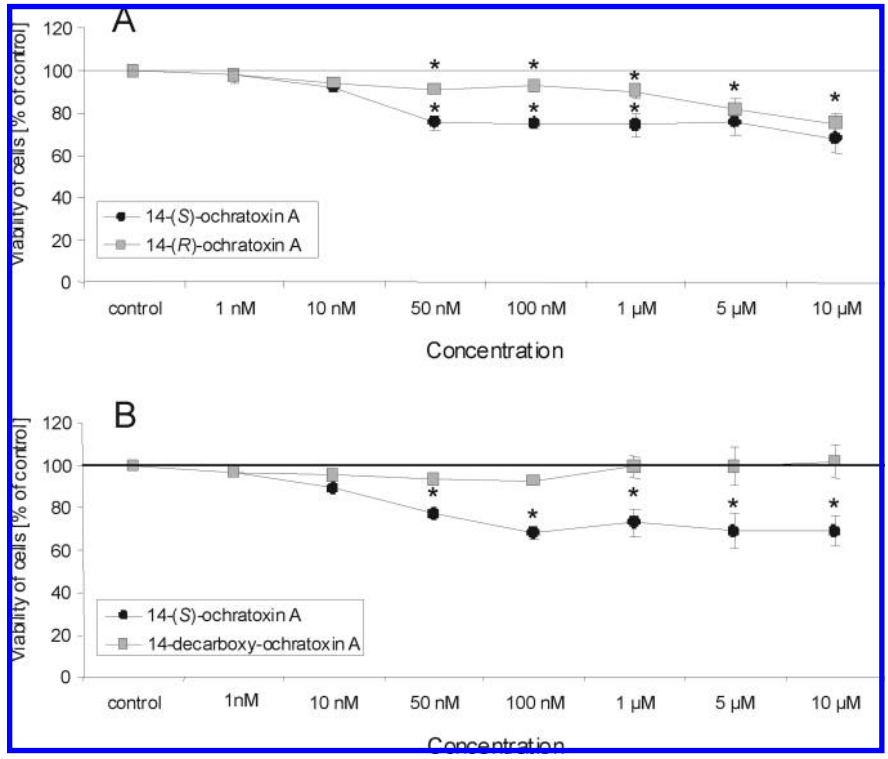

Figure 4. Concentration-dependent viability of IHKE cells after 48 h exposure to the following: (**A**) 14-(*S*)-ochratoxin A (IC_{50} value: 26.2 nM ± 8.4 nM), 14-(*R*)-ochratoxin A (IC_{50} value: 350 nM ± 190 nM); (**B**) 14-(*S*)-ochratoxin A (IC_{50} value: 16.3 nM ± 3.3 nM), 14-decarboxy-ochratoxin A (no cytotoxicity within the concentration range); concentrations as indicated; determined by the CCK-8 assay; number of analyzed wells $n \geq 12$; mean ± SEM; ∗ indicates significant differences from control ($p < 0.01$).

and to allow a low limit of detection, the purified samples were analyzed by HPLC-MS/MS using stable isotope labeled internal standards (*22, 23*). As labeled standard, d_5-14-(*S*)-ochratoxin A with an isotopic purity of greater 95% was available from biosynthetic production by *Penicillium verrucosum* (DSM 12639) using a d_5-L-phenylalanine supplemented growth medium. d_5-14-(*R*)-Ochratoxin A was prepared by thermal isomerization of d_5-14-(*S*)-ochratoxin A. d_5-14-Decarboxy-ochratoxin A was synthesized by amide coupling of ochratoxin α with d_5-2-phenylethylamine, which was synthesized from d_5-bromobenzene.

A chromatographic separation of the ochratoxin A diastereomers and 14-decarboxy-ochratoxin A of a coffee sample is shown in **Figure 3**.

The results of the quantitative analysis of the market samples are given in **Table 1**. The degradation product 14-(*R*)-ochratoxin A could be found in each sample with a maximum concentration of 0.63 µg/kg. Comparison of the 14-(*R*)-ochratoxin A level with the nonisomerized toxin gives values of 10.6−25.6% 14-(*R*)-ochratoxin A relative to 14-(*S*)-ochratoxin A. The second degradation product of 14-(*S*)-ochratoxin A, the 14-decarboxy-ochratoxin A could be detected in seven coffee samples but only three samples showed contents higher than the limit of quantification (LOQ). This compound seems to occur in roasted coffee only in very small amounts.

Cytotoxicity and Apoptotic Potential of the 14-(*S*)-Ochratoxin A Degradation Products. 14-(*S*)-Ochratoxin A is known to be a nephrotoxin (*24, 25*) and to cause changes in the tubule architecture (*26, 27*). In various cells, 14-(*S*)-ochratoxin A is described to induce cell death already at nanomolar concentrations, mainly by apoptosis, whereas necrosis plays a minor role (*28–30*). Thus, to determine the cytotoxic and apoptotic potential of 14-(*S*)-ochratoxin A and its degradation products 14-(*R*)-ochratoxin A and 14-decarboxy-ochratoxin A, two assays were performed using immortalized human kidney epithelial cells. In order to compare the cytotoxic and apoptotic potential of the degradation products to the one of 14-(*S*)-ochratoxin A, we performed each experiment simultaneously with 14-(*S*)-ochratoxin A.

Figure 4 shows the viability of IHKE cells after treatment with 14-(*S*)-ochratoxin A and its degradation products for a period of 48 h as determined by the CCK-8 assay. **Figure 4A** depicts the viability of IHKE cells in passages 146−156 after incubation with 14-(*S*)-ochratoxin A and 14-(*R*)-ochratoxin A concentrations ranging from 1 nM to 10 µM. 14-(*S*)-Ochratoxin A as well as 14-(*R*)-ochratoxin A are cytotoxic for IHKE cells. The treatment with both substances led to a concentration-dependent decrease in viability and a significant reduction of living cells could be observed above concentrations of 50 nM. Although there is a reduction of living cells for both compounds, the treatment of IHKE cells with 50 nM 14-(*S*)-ochratoxin A led to a stronger decrease of viability (75% of untreated control) than the treatment with 14-(*R*)-ochratoxin A (91% of untreated control). The graph showed a steeper slope for 14-(*S*)-ochratoxin A than for 14-(*R*)-ochratoxin A. The highest 14-(*S*)-ochratoxin A concentration of 10 µM led to a viability of 67%, whereas 10 µM of the degradation product 14-(*R*)-ochratoxin A had still a viability of 75% (**Figure 4A**). The calculated IC_{50} value of 14-(*R*)-ochratoxin A (350 nM) is approximately 10-fold higher than the IC_{50} value of 14-(*S*)-ochratoxin A (26 nM). Thus, to obtain the same effects as 14-(*S*)-ochratoxin A, a 10-fold higher amount of 14-(*R*)-ochratoxin A is needed. These preliminary results indicate that 14-(*R*)-ochratoxin A might have a lower cytotoxicity than 14-(*S*)-ochratoxin A. But, for a conclusive verification of differences in cytotoxicity, further work is necessary to establish the relative toxicities of these compounds.

Nevertheless, these first cell culture data demonstrate that 14-(*R*)-ochratoxin A shows a 10-fold lower cytotoxicity in IHKE

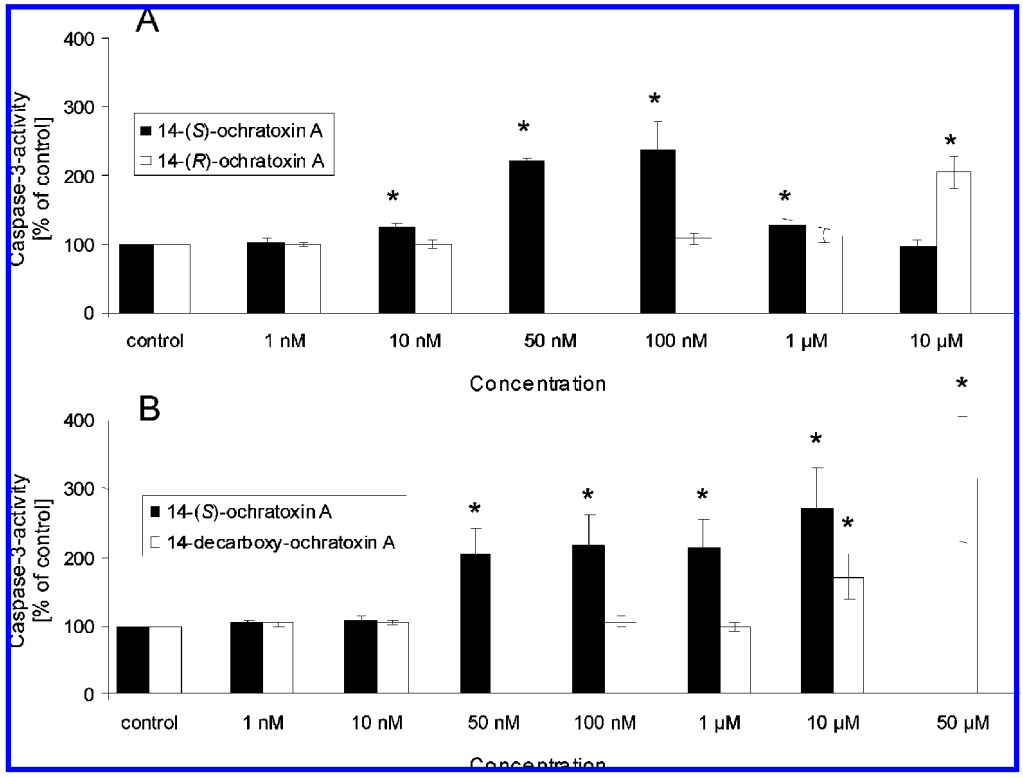

Figure 5. Concentration-dependent caspase-3-activity of IHKE cells: (**A**) after 24 h exposure to 14-(*S*)-ochratoxin A and 14-(*R*)-ochratoxin A, passages 146−156; (**B**) after 48 h exposure to 14-(*S*)-ochratoxin A and 14-decarboxy-ochratoxin A, passages 175−185; no data are measured for 50 nM 14-(*R*)-ochratoxin A (**A**), 50 nM 14-decarboxy-ochratoxin A (**B**), and 50 μM 14-(*S*)-ochratoxin A (**B**); number of analyzed wells $n \geq 12$; mean ± SEM; ∗ indicates significant differences from control ($p < 0.05$).

cells than 14-(*S*)-ochratoxin A. These data are in close agreement with previous literature data. Xiao et al. (*21, 31*) synthesized several ochratoxin A derivatives for cytotoxicity assays in order to investigate the structure−activity relationship of 14-(*S*)-ochratoxin A. In human HeLa cells, the concentration of 14-(*R*)-ochratoxin A necessary to induce comparable effects as 14-(*S*)-ochratoxin A was around 30-fold higher. The IC_{50} value was 5 μM for 14-(*S*)-ochratoxin A and 163 μM for 14-(*R*)-ochratoxin A. Also, the 14-(*R*)-ochratoxin A is less toxic to mice than 14-(*S*)-ochratoxin A, as at a dosage of 200 mg/kg of bodyweight of 14-(*R*)-ochratoxin A caused no death while 20 mg/kg of bodyweight 14-(*S*)-ochratoxin A resulted in a death rate of 3 out of 10. However, an antibacterial activity of 14-(*R*)-ochratoxin A equal to 14-(*S*)-ochratoxin A was reported for studies with *Bacillus brevis*.

Figure 4B demonstrates the viability of IHKE cells in passages 175−185 after incubation with 14-(*S*)-ochratoxin A and 14-decarboxy-ochratoxin A. Only the incubation with 14-(*S*)-ochratoxin A led to a significant reduction of cell viability, whereas 14-decarboxy-ochratoxin A did not affect the cells. Up to a concentration of 10 μM of 14-decarboxy-ochratoxin A, no significant decrease of living cells was observed. Thus, we could show that 14-decarboxy-ochratoxin A, in comparison to 14-(*S*)-ochratoxin A, is not cytotoxic for IHKE cells in the tested concentration range. In studies with *Bacillus brevis*, mice, and HeLa cells, the results were similar. No antibacterial activity was observed at a dose more than 40-fold higher than the toxic dose of 14-(*S*)-ochratoxin A when the carboxyl group was removed. To mice, 14-decarboxy-ochratoxin A was 10-fold less toxic than 14-(*S*)-ochratoxin A. For HeLa cells, 14-decarboxy-ochratoxin A showed an IC_{50} value of 7.5 mM and was therefore even 1500-fold less toxic than 14-(*S*)-ochratoxin A. The determined IC_{50} value of 14-(*S*)-ochratoxin A in HeLa cells was 5 μM (*31*).

As previously described, 14-(*S*)-ochratoxin A in nanomolar concentrations leads to apoptotic cell death. Apoptosis, in contrast to necrosis, is characterized by DNA cleavage, cytosolic as well as nuclear shrinkage, and the occurrence of apoptotic bodies without breakdown of the plasma membrane. These morphological characteristics are attributed to the activation of cystein proteases, the so-called caspases. To study the apoptotic potential of 14-(*S*)-ochratoxin A and its degradation products, we measured the activation of caspase-3 under the influence of these substances (**Figure 5**). **Figure 5A** shows the caspase-3-activation in IHKE cells after a 24 h treatment with 14-(*S*)-ochratoxin A and 14-(*R*)-ochratoxin A. 14-(*S*)-Ochratoxin A causes a significant activation starting at a concentration of 50 nM up to 1 μM. These data are in agreement with the literature. In opossum kidney cells (OK cells), a 24 h incubation with 100 nM 14-(*S*)-ochratoxin A caused a caspase-3 activation of 150% compared to control cells (*30*). Schwerdt et al. (*29*) observed that 10 nM 14-(*S*)-ochratoxin A increased the caspase-3 activation in IHKE cells 2-fold and the highest activation rate was observed at 100 nM.

In comparison, our studies show that the treatment with the 14-(*R*) isomer of ochratoxin A did not lead to a significant activation below 10 μM (**Figure 5A**). Thus, a 200-fold higher concentration of the degradation product is necessary to induce comparable effects in IHKE cells. In **Figure 5B**, the caspase-3 activation after a 48 h treatment with 14-(*S*)-ochratoxin A and 14-decarboxy-ochratoxin A is shown. 14-(*S*)-ochratoxin A caused significant caspase-3 activation from a concentration of 50 nM, whereas 14-decarboxy-ochratoxin A revealed a significant activation not until 10 μM (**Figure 5B**). To induce similar caspase-3 activation as 14-(*S*)-ochratoxin A, a concentration of 50 μM 14-decarboxy-ochratoxin A was necessary. Therefore, to obtain the same apoptotic effects in IHKE cells, a 1000-fold

quantity of 14-decarboxy-ochratoxin A in comparison to 14-(*S*)-ochratoxin A is needed.

In our studies, we could show for the first time that the 14-(*S*)-ochratoxin A degradation products 14-(*R*)-ochratoxin A and 14-decarboxy-ochratoxin A are able to induce a caspase-3 activation in kidney cells. Both substances have an apoptotic potential but at 200- and 1000-fold higher concentrations, respectively, compared to 14-(*S*)-ochratoxin A. The degradation product 14-(*R*)-ochratoxin A showed a lower IC_{50} value than 14-(*S*)-ochratoxin A, as determined using the CCK-8 assay. 14-Decarboxy-ochratoxin A was even not cytotoxic for IHKE cells in the tested concentration range.

In summary, using our model system, we could simulate degradation reactions of the mycotoxin 14-(*S*)-ochratoxin A, and two degradation products of this compound were identified. By using HPLC-MS/MS and stable isotope dilution analysis, it could be confirmed that these compounds were also formed during coffee roasting. 14-(*R*)-Ochratoxin A was found in concentrations up to 0.63 μg/kg or up to 25.6% of the respective 14-(*S*)-ochratoxin A concentration. The second degradation product, 14-decarboxy-ochratoxin A, was found in the coffee samples only in traces. In first preliminary cell culture experiments, both compounds showed lower cytotoxic and apoptotic effects than 14-(*S*)-ochratoxin A. But, for a conclusive verification of differences in cytotoxic and apoptotic potential, further work is necessary to establish the relative toxicities of these compounds. Nevertheless, these data indicate that the formation of the two degradation products might be a possible way for the detoxification of 14-(*S*)-ochratoxin A; the use of Good Agricultural and Manufacturing Practices (GAP/GMP) at the green bean stage to reduce 14-(*S*)-ochratoxin A levels is recommended as a much better approach to control this contaminant in coffee.

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