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Total synthesis and cytotoxicity evaluation of all ochratoxin A stereoisomers

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ABSTRACT

The mycotoxin ochratoxin A is a potent inhibitor of the protein biosynthesis and known to be cytotoxic in nanomolar concentrations. In order to investigate the relationship between stereochemistry and cytotoxicity of this compound, all four ochratoxin A stereoisomers have been synthesized. Using the liver cell line Hep G2, the compounds were tested for cytotoxic and apoptotic potential. It could be shown, that the L-configuration of the phenylalanine moiety of the molecule is mostly responsible for the high cytotoxicity of ochratoxin A while the stereocenter at the dihydroisocoumarine structure is of less importance.

1. Introduction

The mycotoxin ochratoxin A (3*R*14*S*-ochratoxin A, (*R*)-*N*-((5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl)-L-phenylalanine) (Fig. 1) is a known toxic fungal secondary metabolite first identified by van der Merwe et al. in 1965.[1] It is produced by moulds of the genera *Penicillium* and *Aspergillus* and its occurrence is reported for various commodities, especially for cereal based food products, coffee, cocoa, grape juice, beer, and wine.[2–6] Ochratoxin A is known to possess nephrotoxic, teratogenic and immunotoxic properties and was classified as a possible carcinogen to humans (group 2b) by the International Agency for Research on Cancer (IARC).[7] Gekle et al. recently reviewed the broad spectrum of effects caused by ochratoxin A in nanomolar concentrations in vitro.[8] Beside general effects on cell viability, the main observations were reduced protein and DNA synthesis. The genotoxicity of this compound is still a matter of dispute. Pfohl-Leszkowicz and Manderville in a recent review on the genotoxic effects of ochratoxin A as observed in various studies of different groups, proposed DNA adduct formation as a critical event in ochratoxin A toxicity.[9] However, using highly sensitive isotope dilution mass spectrometry, Delatour et al. could not detect any DNA adduct formation in vivo.[10]

Beside the discussion about genotoxicity, also the mode of action of ochratoxin A that is responsible for the high acute toxicity is still unclear. Early studies with *Bacillus brevis* suggested an inhibition of the phenylalanyl-tRNA-synthetase by ochratoxin A.[11] However, subsequent experiments with recombinant expressed and purified phenylalanyl-tRNA-synthetase could not confirm these findings.[12] Furthermore computational approaches based on crystal structures of the phenylalanyl-tRNA-synthetase gave only very low binding affinities of ochratoxin A to the reactive center of enzyme.[13] This debate on the mode of action of ochratoxin A,

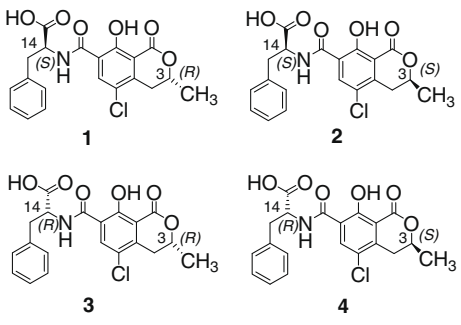

Figure 1. Chemical structures of the ochratoxin A stereoisomers (3*R*14*S*-ochratoxin A, natural compound, **1**), 3*S*14*S*-ochratoxin A (**2**), 3*R*14*R*-ochratoxin A (thermal isomerization product, **3**) and 3*S*14*R*-ochratoxin A (**4**).

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as well as the recently detected 3*R*14*R*-ochratoxin A as a thermal degradation product in food gives rise to the question how the stereochemistry of this compound affects its toxicity.[14] Thus the objective of the present work was the total synthesis of all four stereoisomers of ochratoxin A shown in Figure 1, to compare cytotoxicity and apoptotic effects observed in a cultured human liver cell line (Hep G2).

2. Experimental

Reagents were purchased from Merck, Sigma–Aldrich, Enzo Life Sciences or Nacalai Tesque and used as received. Nuclear magnetic resonance (NMR) spectra were taken either on a JNM-A400 (JEOL, Tokyo, Japan) or on a Bruker DCX-400 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). Mass spectra were recorded on a API 4000QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) or a LTQOrbitrap XL (Thermo-Fisher, Bremen, Germany). Exact mass was measured by a LTQOrbitrap XL. The measurements of absorbance and fluorescence based microplate assays were performed with a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany).

2.1. Synthesis of 2-butynal (6)

2-Butynol (**5**) (11.0 mL, 147 mmol) was added dropwise under cooling to a suspension of manganese dioxide (125.0 g, 1.4 mol) and powdered molecular sieve (0.4 nm, 20 g) in CH_2Cl_2 (150 mL) and stirred for 22 h at room temperature. The reaction mixture was filtered through Celite and most of the CH_2Cl_2 removed by distillation at 50 °C. Subsequent distillation over a 15 cm Vigreux column (bp: 98–102 °C) obtained the pure aldehyde **6** as a colorless product (3.19 g, 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.06 (s, 3H), 9.14 (s, 1H).

2.2. Synthesis of dimethyl-2-hydroxy-4-methylbenzene-1,3-dicarboxylate (7)

NaH (60% in paraffin, 660 mg, 16.5 mmol) was suspended in 25 mL THF and cooled to −10 °C before dimethyl-1,3-acetonedicarboxylate (2.88 g, 16.5 mmol) was added dropwise. 2-Butynal (**6**) (1.0 g, 14.7 mmol) was added carefully and the solution stirred for 1 h at −10 °C before allowing to warm to room temperature whilst stirring overnight. The reaction was stopped by addition of 2 N HCl (20 mL) and the mixture extracted with diethyl ether (2 × 30 mL). The combined organic phases were washed with brine (2 × 30 mL), dried ($MgSO_4$), and the solvent removed. The oily residue was purified by column chromatography (hexane/diethyl ether 8:2) followed by crystallization (hexane/diethylether 9:1) to yield **7** as colorless crystals (1.56 g, 48.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.35 (s, 3H), 3.94 (s, 3H), 3.95 (s, 3H), 6.74 (d, *J* = 8.2 Hz, 1H), 7.77 (d, *J* = 8.2 Hz, 1H), 11.15 (s, 1H).

2.3. Synthesis of ochratoxin β-methyl ester (8)

n-Butyllithium (1.6 M) in hexane (4.70 mL, 7.38 mmol) were added to a solution of diisopropylamine (1.25 mL, freshly distilled over CaH_2) in THF (8.25 mL) at 0 °C. The solution was stirred for 30 min before cooling to −78 °C and addition of a solution of **7** (670 mg, 2.99 mmol) in THF (1.5 mL). After 10 min of stirring at −78 °C, acetaldehyde (3.6 mmol) in THF (1.5 mL) was added and the mixture stirred for further 10 min before the temperature was increased to 0 °C. After 1 h the reaction was quenched by addition of acetic acid (1 mL), diluted with Et_2O (20 mL) and washed with water (20 mL). The aqueous phase was re-extracted with Et_2O (20 mL), the combined organic phases dried and the solvent removed to give **8**. Further purification was achieved by crystallization (hot toluene/hexane) to yield nearly colorless crystals (534.7 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.50 (d, *J* = 6.3 Hz, 3H), 2.93 (d, *J* = 7.2 Hz, 2H), 3.90 (s, 3H); 4.59–4.80 (m, 1H), 6.72 (d, *J* = 7.9 Hz, 1H), 8.00 (d, *J* = 7.9 Hz, 1H), 12,11 (s, 1H).

2.4. Synthesis of ochratoxin α (9)

Ochratoxin β-methyl ester (**8**) (62 mg, 0.26 mmol) was dissolved in 5 mL CH_2Cl_2 at room temperature and sulfuryl chloride (350 μL, 78 mmol) added dropwise. After stirring overnight the reaction residue was evaporated to dryness and hydrolyzed by the addition of lithium hydroxide monohydrate (280 mg, 6.7 mmol) in 2 mL methanol and heating under reflux for 2 h followed by stirring at room temperature for further 10 h. 10 mL water were added to the reaction mixture and the received solution acidified with 2 M hydrochloric acid to pH 3. The aqueous phase was extracted with Et_2O (2 × 10 mL), the combined organic phases dried and the solvent removed to give **9** as a racemate. The product was further purified by crystallization (acetone/hexane) to yield nearly colorless crystals (38.2 mg, 56.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.52 (s, br, 1H), 1.63 (d, *J* = 6.3 Hz, 3H), 2.90 (dd, *J* = 11.7 Hz, 17.6 Hz, 1H), 3.33 (dd, *J* = 3.4 Hz, 17.6 Hz, 1H); 4.79–4.85 (m, 1H), 8.41 (s, 1H), 13.3 (s, 1H).

2.5. Synthesis of 3*R*14*S*-ochratoxin A (1) and 3*S*14*S*-ochratoxin A (2)

L-Phenylalanine methyl ester hydrochloride (**10**) (18.5 mg, 86 μmol) and the racemic mixture of **9** (19.8 mg, 18 μmol) were dissolved in 2 mL *N*,*N*-dimethylformamide. *N*,*N*-Diisopropylethylamine (10 μL, 100 μmol) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uranium hexafluorophosphate (HATU, 38.6 mg, 102 μmol) were added and the solution stirred for 2 h. Subsequently, the reaction mixture was diluted with 10 mL sodium bicarbonate solution (5%) and extracted with Et_2O (2 × 15 mL). The combined organic phases were washed with brine (10 mL) and dried ($MgSO_4$). After solvent removal, a mixture of the methyl ester of **1** and **2** (31 mg, 74 μmol 86%) was received. The pale yellow oil was hydrolyzed by stirring overnight in 0.5 M sodium hydroxide solution (8 mL). The reaction mixture was acidified with 6 M hydrochloric acid to pH <1, stirred for 2 h and extracted with $CHCl_3$ (3 × 5 mL). The combined organic phases were dried ($MgSO_4$), the solvent removed, and the products **1** and **2** separated by semi-preparative HPLC using a 250 × 16 mm id, 4 μm, Synergi Fusion column (Phenomenex, Aschaffenburg, Germany) with an isocratic mixture of 40% water containing 0.1% formic acid and 60% methanol delivered by two Varian ProStar 210 HPLC solvent delivery modules (Varian, Darmstadt, Germany). The flow rate was set at 9 mL/min and peak-detection was done by a Varian ProStar 325 UV/vis detector set at 330 nm. 3*R*14*S*-Ochratoxin A (**1**) eluted at a retention time 24.0 min, 3*S*14*S*-ochratoxin A (**2**) eluted at 26.3 min. Methanol was removed from each fraction and the aqueous solution extracted with *tert*-butyl methyl ether. The organic phases were dried over sodium sulfate and *tert*-butyl methyl ether was removed under reduced pressure to yield **1** (10 mg, 25 μmol) and **2** (8.5 mg, 21 μmol) (**1** + **2**: 62%).

The purity of each fraction was checked by analytical HPLC using the same solvent mixture with a flow rate of 1 mL/min and an Agilent Eclipse XDB-C18 4.6 × 150 mm column (Agilent Technologies, Böblingen, Germany).

2.5.1. 3*R*14*S*-Ochratoxin A (1)

^{1}H NMR (400 MHz, C_6D_6) δ 0.77 (d, *J* = 6.3 Hz, 3H, H-11), 1.79 (dd, *J* = 11.8 Hz, *J* = 17.3 Hz, 1H, H-4a), 2.31 (dd, *J* = 3.4 Hz, *J* = 17.3 Hz, 1H, H-4b), 3.11 (dd, *J* = 7.3 Hz, *J* = 14.0 Hz, 1H, H-15a),

3.29 (dd, J = 5.2 Hz, J = 14.1 Hz, 1H, H-15b), 3.52 (ddd, 1H, J = 3.4 Hz, J = 6.2 Hz, J = 11.8 Hz, H-3), 5.12 (m, 1H, H-14), 6.95–7.18 (m, H-17–H-21), 8.57 (d, J = 5.3 Hz, 1H, H-13), 8.63 (1H, s, H-6), 13.02 (s, 1H). ^{13}C NMR (101 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). $[\alpha]_D^{23}$ = 68 (c 0.41 mg/mL, methanol), ESI-MS (negative mode): HRMS m/z 402.07422 (calculated mass for $[C_{20}H_{17}O_6NCl]^-$: m/z 402.07499), MS/MS (HCD@90.00): m/z (%) 211.02 (100), 358.08 (74), 166.99 (51), 314.06 (28), 254.02 (14), 239.01 (7).

2.5.2. 3*S*14*S*-Ochratoxin A (2)

^{1}H NMR (400 MHz,) δ 0.78 (d, J = 6.3 Hz, 3H, H-11), 1.75 (dd, J = 17.3 Hz, 11.9 Hz, 1H, H-4a), 2.34 (dd, J = 17.3 Hz, 3.2 Hz, 1H, H-4b), 3.10 (dd, J = 13.8 Hz, 7.3 Hz, 1H, H-15a), 3.28 (dd, J = 13.9 Hz, 4.6 Hz, 1H, H-15b), 3.64 (ddd, J = 11.7 Hz, 6.0 Hz, 3.3 Hz, 1H, H-3), 5.16 (m, 1H), 6.95–7.18 (m, H-17–H-21), 8.57 (d, J = 6.4 Hz, 1H, H-13), 8.62 (s, 1H, H-6), 13.01 (s, 1H), ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.9 (C-11), 32.5 (C-4), 37.5 (C-15), 54.6 (C-14), 76.2 (C-3), 110.3 (C-9), 120.4 (C-7), 123.4 (C-5), 127.5 (C-19), 128.9 (C-17, C-21), 129.5 (C-18, C-20), 135.9 (C-16), 139.2 (C-6), 141.3 (C-10), 159.3 (C-8), 163.5 (C-12), 170.0 (C-1), 174.8 (C-22). $[\alpha]_D^{23}$ = 101 (c 0.33 mg/mL, methanol). ESI-MS (negative mode): HRMS m/z 402.07420 (calculated mass for $[C_{20}H_{17}O_6NCl]^-$: m/z 402.07499), MS/MS (HCD@90.00): m/z (%) 211.02 (100), 358.08 (76), 166.99 (51), 314.06 (28), 254.02 (14), 239.01 (7).

2.6. Synthesis of 3*R*14*R*-ochratoxin A (3) and 3*S*14*R*-ochratoxin A (4)

Synthesis and purification were carried out as described under 4.4 but **9** was coupled with D-phenylalanine methyl ester hydrochloride (**11**). The reaction product 3*R*14*R*-ochratoxin A (**3**) eluted at a retention time of 24.0 min, 3*S*14*R*-ochratoxin A (**4**) at a retention time of 26.2 min.

2.6.1. 3*R*14*R*-Ochratoxin A (3)

^{1}H NMR (400 MHz, C_6D_6) δ 0.77 (d, J = 6.3 Hz, 3H, H-11), 1.75 (dd, J = 12.0 Hz, J = 17.3 Hz, 1H, H-4a), 2.33 (dd, J = 3.3 Hz, J = 17.3 Hz, 1H, H-4b), 3.09 (dd, J = 7.3 Hz, J = 13.9 Hz, 1H, H-15a), 3.27 (dd, J = 4.9 Hz, J = 13.9 Hz, 1H, H-15b), 3.58 (m, 1H, H-3), 5.15 (m, 1H, H-14), 6.95–7.18 (m, H-17–H-21), 8.56 (d, J = 5.7 Hz, 1H, H-13) 8.62 (s, 1H, H-6), 13.01 (1H, s). ^{13}C NMR (101 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). $[\alpha]_D^{23}$ = −104 (c 0.44 mg/mL, methanol). ESI-MS (negative mode): HRMS m/z 402.07428 (calculated mass for $[C_{20}H_{17}O_6NCl]^-$: m/z 402.07499), MS/MS (HCD@90.00): m/z (%) 211.02 (100), 358.08 (76), 166.99 (51), 314.06 (28), 254.02 (13), 239.01 (6).

2.6.2. 3*S*14*R*-Ochratoxin A (4)

^{1}H NMR (400 MHz, C_6D_6) δ 0.77 (d, J = 6.3 Hz, 3H, H-11), 1.76 (dd, J = 17.3 Hz, 11.5 Hz, 1H, H-4a), 2.32 (dd, J = 17.3 Hz, 3.4 Hz, 1H, H-4b), 3.08 (dd, J = 14.0 Hz, 7.3 Hz, 1H, H-15a), 3.28 (dd, J = 14.2 Hz, 5.0 Hz, 1H, H-15b), 3.50 (m, 1H, H-3), 5.21 (m, 1H, H-14), 6.96–7.20 (m, H-17–H-21), 8.56 (d, J = 6.6 Hz, 1H, H-13), 8.63 (s, 1H, H-6), 12.99 (s, br, 1H, C-22 OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.9 (C-11), 32.5 (C-4), 37.5 (C-15), 54.6 (C-14), 76.2 (C-3), 110.3 (C-9), 120.4 (C-7), 123.5 (C-5), 127.5 (C-19), 128.9 (C-17, C-21), 129.5 (C-18, C-20), 135.9 (C-16), 139.2 (C-6), 141.2 (C-10), 159.3 (C-8), 163.5 (C-12), 169.9 (C-1), 174.8 (C-22). $[\alpha]_D^{23}$ = −66 (c 0.44 mg/mL, methanol). ESI-MS (negative mode): HRMS m/z 402.07382 (calculated mass for $[C_{20}H_{17}O_6NCl]^-$: m/z 402.07499), MS/MS (HCD@90.00): m/z (%) 211.02 (100), 358.08 (74), 166.99 (51), 314.06 (28), 254.02 (14), 239.01 (6).

2.7. Cultivation of the Hep G2 cell line

Human hepatocellular carcinoma cells (Hep G2, ACC 180, DSMZ, Braunschweig, Germany) were cultivated in DMEM medium enriched with 10% fetal calf serum using standardized culture conditions (37 °C, 8.5% CO_2). After the cultivation of cells for 48 h in 96-well (cytotoxicity assay) or 24-well plates (caspase-3 assay, lactate dehydrogenase release assay) the 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. After 24 h of serum-free cultivation **1**, **2**, **3** or **4** (stock solution: 1 mM in methanol) were added in concentrations ranging from 10 nM to 50 μM and incubated for 24 h (caspase-3) and 48 h (CCK-8 and LDH), respectively. The cells of the control group were incubated with according solvent concentrations. Studies with all stereoisomers were performed in triplicate with cells from three independent passages (n = 9). Each experiment was carried out simultaneously with all four compounds allowing a better comparability of the effects.

2.8. Cytotoxicity assay

The cytotoxicity of ochratoxin A derivatives was evaluated colorimetrically using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) according to the literature and the manufacturer's instructions.[15,16] Briefly, cells were seeded on 96-well microplates (4×10^3 cells/well). After toxin exposure the dye solution (WST-8) was added to the cells, followed by the incubation for 1 h at 37 °C. The reduction of WST-8 dye by cellular dehydrogenases of viable cells increases the absorbance at λ = 450 nm and was measured with a microplate reader. The results for toxin-treated cells were normalized to the values of the untreated negative control.

2.9. Caspase-3 activity assay

The assay was carried out according to the literature.[17,16] Cells were seeded in 24-well plates (2×10^4 cells/well). After toxin incubation, 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, 0.1% Triton X-100, pH 7.5) for 15 min on ice. The cell lysates were centrifuged at 7000g for 10 min at 4 °C. Fifty microliters of the supernatant were incubated with 50 μL reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT) containing 8 μM fluorogenic caspase-3 substrate (Ac-Asp-Glu-Val-Asp-7-Amino-4-trifluoromethylcoumarin, DEVD-AFC) at 37 °C for 1 h. The fluorescence of 7-amino-4-trifluoromethylcoumarin (AFC), released by proteolytic cleavage, was measured with a microplate reader (excitation: λ = 400 nm; emission: λ = 505 nm). Released AFC concentrations were quantified using an AFC standard for the calibration and were normalized to the cellular protein content in each sample. Protein concentrations were determined with the bicinchoninic acid assay using bovine serum albumin (BSA) as standard for the calibration.

2.10. Lactate dehydrogenase release assay

The assay was carried out according to the literature.[18] Cells were seeded in 24-well plates (2×10^4 cells/well). After toxin incubation, 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 centrifuged at 7000*g* for 10 min at 4 °C. Concordant samples of cell lysates and cell media were incubated with reaction buffer (2 μM NADH, 10 mM pyruvate, 100 mM HEPES, pH 7.0). The decrease of NADH related absorption at $\lambda = 340$ nm was measured every 2 min to monitor the kinetic parameters of the enzymatic reaction for 60 min at 37 °C using a microplate reader with temperature control. Thereof the amounts of total cellular lactate dehydrogenase (LDH) enzyme and released LDH were calculated for every sample and were normalized to positive and negative controls.

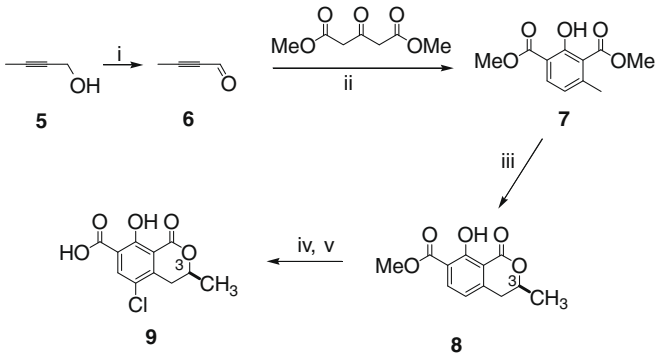
2.11. Statistics

All cell culture measurements are given as mean values ± SD. The levels of significance were determined by the unpaired student's *t*-test and are indicated in the according data sets (Figs. 2–4). EC_{50} and IC_{50} values were calculated with a statistical evaluation software (Sigmaplot 8.01).

3. Results and discussion

3.1. Synthesis of ochratoxin A stereoisomers

In order to investigate the impact of the stereochemistry on the cytotoxicity and apoptotic potential of ochratoxin A in a human liver cancer cell line, a synthesis of the four stereoisomers 3*R*14*S*-ochratoxin A (**1**, natural toxin), 3*S*14*S*-ochratoxin A (**2**), 3*R*14*R*-ochratoxin A (**3**, thermal degradation product of **1**) and 3*S*14*R*-ochratoxin A (**4**), shown in Figure 1 was carried out.



Scheme 1. Reagents and conditions: (i) MnO_2,molecular sieve, CH_2Cl_2, rt, 24 h, 32%; (ii) NaH, THF, -10 °C to rt, 20 h, 48%; (iii) LDA, CH_3CHO, THF, −78 °C, 1 h, 80%; (iv) SO_2Cl_2, CH_2Cl_2, rt, 12 h; (v) LiOH, MeOH, reflux, 2 h, 68% from **8**.

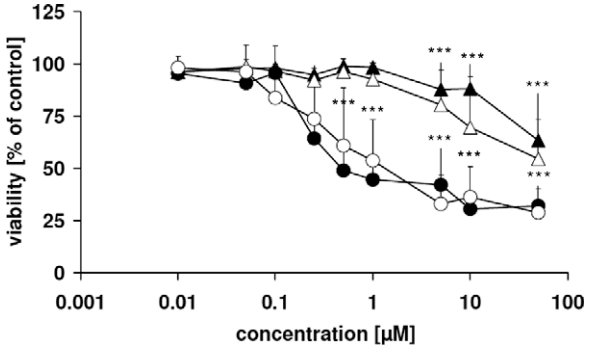


Figure 2. Concentration dependent decrease of viability of Hep G2 cells due to incubation with 3*R*14*S*-ochratoxin A (**1**, Δ), 3*S*14*S*-ochratoxin A (**2**, ●), 3*R*14*R*-ochratoxin A (**3**, ▲), and 3*S*14*R*-ochratoxin A (**4**, ○) ($n = 9$; level of significance: *** = p <0.01).

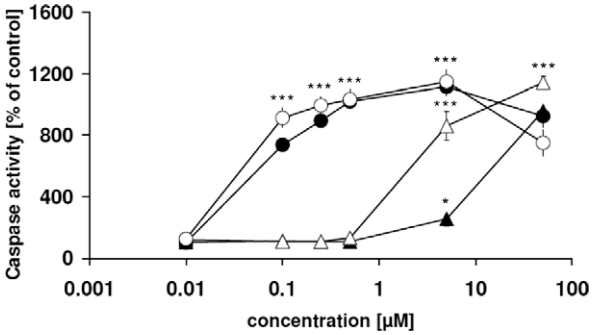


Figure 3. Concentration dependent activation of caspase-3 due to incubation with 3*R*14*S*-ochratoxin A (**1**, Δ), 3*S*14*S*-ochratoxin A (**2**, ●), 3*R*14*R*-ochratoxin A (**3**, ▲), and 3*S*14*R*-ochratoxin A (**4**, ○) ($n = 9$; levels of significance: * = p <0.05, *** = p <0.01).

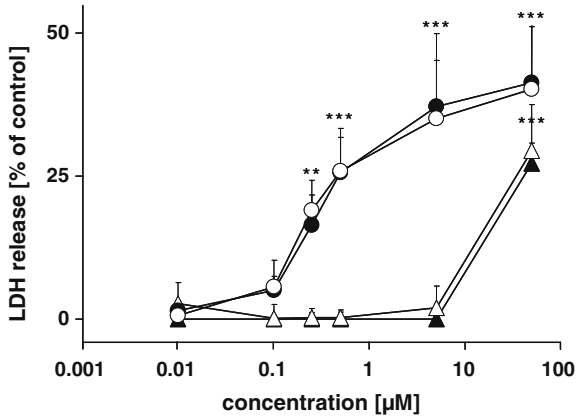
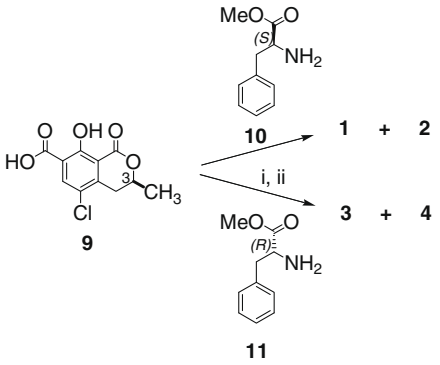


Figure 4. Concentration dependent release of lactate dehydrogenase (LDH) due to incubation with, 3*R*14*S*-ochratoxin A (**1**, Δ), 3*S*14*S*-ochratoxin A (**2**, ●), 3*R*14*R*-ochratoxin A (**3**, ▲), and 3*S*14*R*-ochratoxin A (**4**, ○) ($n = 9$; levels of significance: ** = p <0.025, *** = p <0.01).

Despite a reported stereoselective synthesis of 3*R*-ochratoxin α (**9**), a facile synthesis of a racemic mixture of **9** was found to be more suitable to provide all stereoisomers of ochratoxin A.[19–21] Using this approach, subsequent coupling of **9** with L- or D-phenylalanine methyl ester yielded two diastereomers, which could easily be separated by HPLC (Scheme 2).[14]

The synthesis of racemic **9** shown in Scheme 1 is based on the synthesis of a tetrasubstituted aromatic ring **7**, followed by the setup of the second ring system via aldol condensation with acetaldehyde to **8**. Chlorination with sulfuryl chloride and deprotection of the carboxylic acid were the last steps to a racemic mixture of **9**. Compared to Kraus et al. the formation of **7** was found to be easier achievable in a one pot reaction of dimethyl-acetone-1,3-dicarboxylate with butynal using NaH.[22] The synthesis of the four stereoisomers was finalized by coupling of the activated **9** with either L-phenylalanine methyl ester (**10**) or D-phenylalanine methyl ester (**11**) and deprotection as shown in Scheme 2. For the activation, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uranium hexafluorophosphate (HATU) was found to give the best yield (86%) compared to an activation with thionyl chloride or *N*,*N*′-dicyclohexylcarbodiimide. Reaction of **9** with **11** yielded 3*R*14*S*-ochratoxin A (**1**) and 3*S*14*S*-ochratoxin A (**2**). Both could be separated from each other by preparative HPLC. The configuration of **1** was confirmed by comparison of retention time and specific rotation with the natural reference. The configuration of **2** was confirmed by comparison of the retention time and specific rotation with 3*S*14*R*-ochratoxin A (**3**), an enantiomer of **2**.[14] For both compounds,



Scheme 2. Reagents and conditions: (i) DIPEA, HATU, DMF, rt, 2 h, 88%; (ii) NaOH, rt, 12 h, 62%.

the same retention time on an achiral HPLC-column and a specific rotation with the same absolute value but with opposite directions was found. The reaction of **9** with **12** yielded 3*R*14*R*-ochratoxin A (**3**) and 3*S*14*R*-ochratoxin A (**4**). The configuration of both compounds was also confirmed using the retention time and specific rotation. **3** was compared with the available reference, **4** was compared with its enantiomer **1**.

Using the method described, **9** could be synthesized in an overall yield of 7% and the ochratoxin A stereoisomers (sum of the diastereomers **1** + **2** and **3** + **4**, respectively) in a yield of 53% from **9**.

3.2. Cytotoxicity and apoptotic potential of the four ochratoxin A stereoisomers (1–4)

3*R*14*S*-Ochratoxin A (**1**) is known to be nephrotoxic and to cause changes in the tubule architecture.[23–26] In various cells **1** is described to induce cell death already at nanomolar concentrations, mainly by apoptosis, whereas necrosis plays a minor role.[27–29] Thus for assessment of the structure–activity relationship of ochratoxin A, the parameter cytotoxicity and apoptotic potential were measured. Due to the known high cytotoxic response of Hep G2 cells to **1**, this cell line was chosen to elucidate differences between the cytotoxicity of the ochratoxin A stereoisomers.

The basic assay applied was the CCK-8 assay to determine the acute cytotoxicity of the compounds **1**–**4**. As shown in Figure 2, concentration dependent decrease of cell viability was observed for all compounds. However, among the four compounds, two groups could be identified. IC_{50} values of 0.3 μM and 0.4 μM were obtained for **1** and **2**, respectively, while the IC_{50} values of **3** and **4** were 3.2 μM and 5.0 μM. Thus the change of the configuration of the stereogenic center C-14 from *S* (L-phenylalanine) to *R* (D-phenylalanine) resulted in a decrease of cytotoxicity by a factor of 10. The configuration at C-3 has only a minor effect on the cytotoxicity.

The results of the apoptotic potential of the ochratoxin A stereoisomers are shown in Figure 3. In agreement with the data on the cytotoxicity also two groups could be identified regarding caspase-3 activation. However, the EC_{50} values for all compounds were lower compared to the cytotoxicity. The EC_{50} values of the first group are nearly identical with 0.06 μM for **1** and 0.08 μM for **2**. In contrast the EC_{50} values differ for the group of the 14*R*-isomers according to the configuration of C-3 with EC_{50} values of >10 μM for **3** and 3.8 μM for **4**.

In order to verify apoptosis as the initiator of cell death, LDH release assays were performed 24 h after the measurement of the endpoint for apoptosis. Thus, an observed increase of LDH release can directly be assigned as a secondary effect of apoptosis. The determined EC_{50} values resulting from LDH release were 0.32 μM (**1**), 0.26 μM (**2**), >10 μM (**3**), and >10 μM (**4**) and confirmed the results of the previous assays. Again compounds can be classified in two groups depending on the configuration of C-14. No significant difference was observed between the cytotoxicity of **3** and **4** in this assay.

Based on these data, it could be clearly shown that the configuration of the phenylalanine moiety of ochratoxin A is responsible for the high cytotoxicity and apoptotic activity of ochratoxin A. The importance of the stereochemistry indicates that the specificity of enzymes could be the critical basis for the ochratoxin A induced cytotoxicity. Therefore only **1** and **2,** possessing the L-configuration of the phenylalanine moiety, show a very high cytotoxic potential in all assays applied to determine the toxicity in the in vitro model of Hep G2 cells.

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