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Structural elucidation and analysis of thermal degradation products of the *Fusarium* mycotoxin nivalenol

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The major class of mycotoxins produced by *Fusarium* moulds are trichothecenes, a large group of sesquiterpenes sharing the same basic chemical structure, a 12,13-epoxytrichothec-9-ene ring system. Their toxic effects range from causing diarrhoea, vomiting and gastro-intestinal inflammation to non-competitive inhibition of the biosynthesis of proteins in eukaryotic cells. Trichothecenes in general are relatively stable compounds, their degradation is observed only at high temperatures and prolonged heating time. In order to investigate the stability of the trichothecene nivalenol (NIV) under food processing conditions such as cooking or baking, we performed model heating experiments and screened the residue for degradation products using gas chromatography-mass spectrometry (GC-MS). Heating of nivalenol, especially under mild alkaline conditions, gave a mixture of four compounds (norNIV A, norNIV B, norNIV C, and NIV lactone), which where isolated and identified by nuclear magnetic resonance (NMR) and MS experiments. Although their formation was also demonstrated in heating experiments with spiked flour samples, only norNIV B was detectable in a screening of several commercially available samples, possibly due to the very low contamination with nivalenol. Furthermore, cell culture experiments using immortalized human kidney epithelial (IHKE) cells showed that the four compounds are less cytotoxic (formazan dye cytotoxicity assay) compared to nivalenol. Whereas nivalenol revealed an EC_{50} at 0.9 µmol, all other compounds did not show any significant effect up to 100 µmol.

Keywords: Gas chromatography-mass spectrometry / High-performance liquid chromatography-tandem mass spectrometry / Mycotoxin / Nivalenol / Thermal degradation

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1 Introduction

Fusarium moulds are a large group of phytopathogenic fungi, which are able to infect cereal crops in the field and contaminate them with toxic secondary metabolites, the so-called mycotoxins. The *Fusarium* genus consists of more than 100 different species, such as *F. culmorum* or *F. graminearum*. These particular species are common on soil and the most important in causing head blight and producing trichothecenes in various cereal crops and food and feed. The major class of mycotoxins produced are trichothecenes, a large group of sesquiterpenes sharing the same basic chemical structure, a 12,13-epoxytrichothec-9-ene ring system [1]. The group is subdivided in type A trichothecenes, such as diacetoxyscirpenol or T2-toxin, and type B trichothecenes, such as deoxynivalenol (DON **1**, Fig. 1) or nivalenol (NIV **2,** Fig. 1), the difference being a keto substitution at C-8 in the latter case. Our research has focused on nivalenol, being one of the most potent toxins produced by several species, such as *F. cerealis, F. poae, F. culmorum* and *F. graminearum* [2].

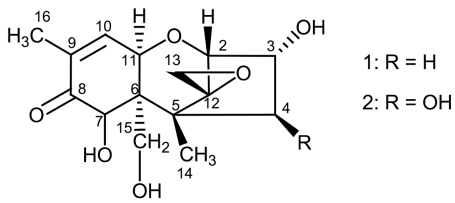

Figure 1. Structure of type B trichothecenes DON **1**, NIV **2**.

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Abbreviations: **BOC**, *t*-butyloxycarbonyl; **DON**, deoxynivalenol; **HMBC**, heteronuclear multiple bond correlation; **HMQC**, heteronuclear multiple quantum correlation; **IHKE cells**, immortalized human kidney epithelial cells; **NIV**, nivalenol; **NOESY**, nuclear Overhauser effect spectroscopy

The toxicity of NIV is attributed to its general ability to noncompetitively inhibit the biosynthesis of proteins in eukaryotic cells likely by affecting the ribosomal function. Inhibition of the synthesis of nucleic acid was observed *in vitro*, although only at very high concentrations. Cell culture studies showed that NIV induces apoptosis in HL60 cells. Oral LD_{50} of NIV was determined to be 19.5 mg/kg body weight (b.w.) in Fischer 344 rats, while it was 38.9 mg/kg b.w. in mice. Although chromosomal aberrations and DNA damage were observed both in cell culture assays and in several organs of mice fed 20 mg/kg b.w., evidence on genotoxicity and carcinogenicity of NIV is still inadequate. Mice fed with 3.5 mg/kg b.w. for 24 days showed signs of immuno- and hematotoxicity, namely erythropenia and leukopenia, a decrease in red and white blood cells. So far no data are available on the effects of NIV on humans, but summarizing the results of the animal experiments, the critical factors seem to be its immunotoxicity/hematotoxicity. A summary of the toxicity of nivalenol can be found in the SCF report [2].

Trichothecenes in general are relatively stable compounds. Most of the studies on their stability are focused on model experiments, observing their degradation depending on time, temperature, and pH in aqueous solutions. Using such a system, Wolf and Bullerman [3] studied the amount of decomposition of DON. Lauren *et al.* [4] included NIV in their research. These model experiments proved the trichothecenes to be especially unstable under alkaline conditions and at high temperatures. Further studies showed, that DON is even stabilized by heating in food matrix compared to pH-adjusted solutions [4, 5]. However, there are only a few studies concerning the stability of trichothecenes in thermal-treated food. Abbas *et al.* [6] studied the degradation of DON during the production of tortillas from contaminated corn, observing a reduction of up to 82%. This is possibly due to the nixtamalization process, the traditional alkaline treatment of corn kernels in Latin America. Only recently, Wolff [7] studied the reduction of DON in the process of bread baking using contaminated flour. These results showed a decrease of 25% of DON. In summary, the available data clearly show, that the concentration of trichothecenes is reduced during thermal food processing, such as cooking or baking. However, little is known about specific degradation products and their toxicological properties.

In an attempt to investigate the stability of NIV under food processing conditions, we used a model heating system, which we have recently developed to investigate the stability of fumonisins [8, 9]. Briefly, fumonisins were heated with several model compounds simulating important food constituents [8, 9]. NIV was heated with α-D-glucose (sugar model), with methyl α-D-glucopyranoside (starch model), and with the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and *t*-butyloxycarbonyl (BOC)-L-cysteine methyl ester (protein models) (for details of the used model compounds see [8]). The degradation products formed were analyzed by gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). After optimization of the heating conditions for maximum yield, we identified four substances, one of which was NIV lactone already described by Grove [10]. We completely elucidated the structure of the three other compounds and provided additional spectroscopic data for NIV lactone.

2 Materials and methods

2.1 Reagents

NIV was purchased from Sigma-Aldrich (Deisenhofen, Germany). Water for HPLC separation was purified with a Milli-Q Gradient A10 (Millipore, Schwalbach, Germany) system, the other solvents for HPLC and HPLC-MS as well as all other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich in gradient- or reagent-grade quality.

2.2 Model experiments

The following compounds were used as food models: α-D-glucose as sugar model, methyl-α-D-glucopyranoside as starch model, and the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester as protein models. All experiments were performed by heating 25 µg of NIV with 250 µg model substance without solvent. To get a homogeneous mixture, aliquots of stock solutions of the reactants (1–20 mg/mL in methanol) were thoroughly vortexed in a 1.5 mL glass vial and the solvent was removed under a stream of nitrogen. The mixture was then heated in a heating block for various periods (10–60 min) at different temperatures (150–200°C). The residue was further analyzed by GC-MS.

2.3 GC-MS

Electron impact (EI) GC-MS data were acquired on an HP6890 series gas chromatograph and HP5973 mass spectrometer (Hewlett Packard/Agilent, Böblingen, Germany) after derivatization of the compounds with 200 µL trimethylsilylimidazol and addition of 300 µL toluene. Data acquisition was carried out with the ChemStation software (Agilent). Chromatographic separation was performed on a 60 m × 0.25 mm ID fused silica, 0.25 µm methyl silicone coating J&W Scientific DB-1 column (Agilent) using

1 mL/min helium as carrier gas. The injector temperature was set at 250°C, the injection volume was 1 µL with split injection (1 : 9). The column temperature was held initially at 120°C for 1 min, then programmed at 4°C/min to 260°C, then with 15°C/min to 320°C, which was held isothermally for 10 min. The transfer line was heated at 320°C. The mass spectrometer was operated in the electron impact mode (EI; 70 eV electron energy) with a source temperature of 230°C and the quadrupole heated at 150°C. Mass spectra were acquired in the full scan mode ranging from *m/z* 40 – 800 with a scan rate of 2.0 scans/s.

2.4 Preparation and isolation of NIV lactone, norNIV A, norNIV B, and norNIV C

The compounds were obtained by alkaline degradation of NIV. Ten mg NIV was heated in 1 mL 0.1 M aqueous NaOH at 75°C for 60 min. The mixture was neutralized with 1 mL 0.1 M aqueous HCl and 400 µL MeOH was added to adjust the solution to the HPLC starting conditions. The compounds were separated on an analytical Knauer Eurospher 100 column (250 × 4.6 mm ID, 5 µm; Knauer, Berlin, Germany) using a linear binary gradient delivered by a Knauer Wellchrom Maxi-Star K-1000 HPLC pump with water as solvent A and methanol as solvent B. The following gradient was used: 0 min, 20% solvent B; 1 min, 20% solvent B; 24 min, 40% solvent B. The flow rate was 700 µL/min. For injection, a Knauer A-0258 six-port valve with a 250 µL sample loop was used. Three fractions were collected after peak detection on a Knauer A-0293 single-wavelength detector set at 220 nm. The new compounds were named norNIV A, norNIV B, and norNIV C according to their elution order and using the nomenclature of Young *et al.* [11]. Fraction 1 contained a mixture of norNIV A and NIV lactone. All compounds (approx. 0.5 – 1 mg each) were further analyzed after solvent evaporation under nitrogen stream.

2.5 Exact mass measurements

Mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer with flow injection and on a Quattro LCZ (Waters-Micromass, Manchester, UK) mass spectrometer with NanoSpray interface and referenced on sodium formiate and polyethylene glycol (PEG) 600, respectively. The compounds were dissolved in 1 mL MeOH and 10 µL of a saturated solution of $NaBF_4$ in MeOH was added to measure the exact mass of the sodium adducts.

2.6 ESI-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. Nitrogen served as curtain gas (20 psi), the declustering potential, being the accelerating current from atmospheric pressure into high vacuum, was set at −30 to −40 V in the negative mode and 50 V in the positive mode. The mass spectrometer was operated in the full-scan mode detecting positive or negative ions. The MS/MS parameters were dependent on the substances, detecting the fragmentation of the $[M-H]^-$ or $[M+H]^+$ molecular ions into specific product ions after collision with nitrogen as collision gas (4×10^{-5} torr). The collision energies are given below.

2.7 MS data

norNIV A: exact mass: *m/z* 305.0996 (calculated for $C_{14}H_{18}O_6$ + Na^+ 305.1001); ESI-MS, negative mode: *m/z* 281 (100) $[M-H]^-$, MS/MS (−17 V): *m/z* 281 (100), 251 (57), 221 (46), 233 (39), 203 (27), 205 (26), 191 (24), positive mode: *m/z* 283 (100) $[M+H]^+$, MS/MS (16 V): *m/z* 247 (100), 283 (75), 177 (59), 229 (43). norNIV B: exact mass: *m/z* 305.0978 (calculated for $C_{14}H_{18}O_6$ + Na^+ 305.1001); ESI-MS, negative mode: *m/z* 281 (100) $[M-H]^-$, MS/MS (−17 V): *m/z* 281 (100), 203 (94), 191 (29), 221 (27), positive mode: *m/z* 283 (100) $[M+H]^+$, MS/MS (17 V): *m/z* 179 (100), 247 (84), 283 (57), 177 (45). norNIV C: exact mass: *m/z* 305.1004 (calculated for $C_{14}H_{18}O_6$ + Na^+ 305.1001); ESI-MS, negative mode: *m/z* 281 (100) $[M-H]^-$, MS/MS (−17 V): *m/z* 281 (100), 203 (64), 191 (31), 221 (29), positive mode: *m/z* 283 (100) $[M+H]^+$, MS/MS (17 V): *m/z* 205 (100), 283 (42), 247 (38), 175 (24). NIV lactone: exact mass: *m/z* 335.1101 (calculated for $C_{15}H_{20}O_7$ + Na^+ 335.1107); ESI-MS, negative mode: *m/z* 311 (100) $[M-H]^-$, MS/MS (−28 V): *m/z* 311 (100), 125 (70), 309 (53), 251 (39); positive mode: *m/z* 313 (100) $[M+H]^+$, MS/MS (28 V): *m/z* 313 (100), 159 (45), 219 (38), 161 (36).

2.8 HPLC-ESI-MS/MS analysis

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 × 2.1 mm ID, 4 µm Phenomenex SynergiFusion column (Phenomenex, Aschaffenburg, Germany) using a linear binary gradient. The injection volume was 5 µL, the flow rate was 200 µL/min. Solvent A was methanol and solvent B was water. The following gradient was used: 0 min, 10% solvent A; 1 min, 10% solvent A; 21 min, 80% solvent A. After each HPLC run, the column was washed with 100% solvent A and equilibrated for 10 min at the starting conditions. For HPLC-MS/MS the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode, detecting negative ions. Zero-grade air served as nebulizer

Table 1. ^{1}H-NMR spectra of the NIV degradation products in MeOH-d_4

C	norNIV A			norNIV B			norNIV C			NIV lactone		
2	4.54	d	$J_{2,3}$ = 4.0	4.25	d	$J_{2,3}$ = 8.8	4.14	d	$J_{2,3}$ = 4.4	3.89	d	$J_{2,3}$ = 4.8
3	3.79	dd	$J_{3,2}$ = 4.0	3.98	dd	$J_{3,2}$ = 8.8	3.89	dd	$J_{3,2}$ = 4.4	4.50	dd	$J_{3,2}$ = 4.8
			$J_{3,4}$ = 7.2			$J_{3,4}$ = 6.4			$J_{3,4}$ = 4.4			$J_{3,4}$ = 3.6
4	3.97	d	$J_{4,3}$ = 7.2	3.80	d	$J_{4,3}$ = 6.4	3.96	d	$J_{4,3}$ = 4.4	3.93	d	$J_{4,3}$ = 3.6
9	–			–			–			2.65	m	$J_{9,10}$ = 3.0/10.6
												$J_{9,16}$ = 7.2
10A	2.64	m	$J_{10,11}$ = 8.0/7.5	6.65	d	$J_{10,11}$ = 8.0	6.52	d	$J_{10,11}$ = 8.0	1.75	dd	$J_{10,9}$ = 3.0/10.6
			$J_{10A,16}$ = 1.5									$J_{10B,11}$ = 4.8
10B	2.83	dd	J_{AB} = 16.5	–			–			2.60	m	J_{AB} = 15.0
11	4.58	dd	$J_{11,10}$ = 8.0/7.5	6.78	d	$J_{11,10}$ = 8.0	6.59	d	$J_{11,10}$ = 8.0	4.28	d	$J_{11,10B}$ = 4.4
13A	4.05	d	J_{AB} = 12.8	3.88	d	J_{AB} = 12.4	3.78	d	J_{AB} = 12.0	4.12	d	J_{AB} = 12.4
13B	4.13	d		4.29	d		3.82	d		4.57	d	
14	1.45	s		1.22	s		1.36	s		1.17	s	
15A	–			–			–			4.00	d	J_{AB} = 12.4
15B	–			–			–			4.48	d	
16	1.96	d	$J_{16,10A}$ = 1.5	2.16	s		2.16	s		1.29	d	$J_{16,9}$ = 7.2

Chemical shifts are reported in ppm and J in Hz.

gas (35 psi), and, heated at 400°C, as turbo gas for solvent drying (45 psi). The following transition reactions were monitored for a duration of 150 ms each (collision energy in brackets): NIV: *m/z* 311/281 (–14 V), NIV lactone: *m/z* 311/125 (–28 V), all norNIV compounds: *m/z* 281/203 (–20 V) (for other MS parameters see ESI-MS/MS). Due to the limited amount of isolated NIV degradation products we were not able to validate the used HPLC-MS/MS method and therefore only semiquantitative data are given.

2.9 NMR spectroscopy

^{1}H- and 2-D NMR data were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany), ^{13}C-NMR on a Unity plus (Varian, Palo Alto, CA, USA) NMR spectrometer. Signals are reported in parts per million relative to MeOH-d_4, respectively D_2O. For structural elucidation and NMR signal assignment 2-D NMR experiments, such as double-quantum filtered correlated spectroscopy (DQF-COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and 2-D nuclear Overhauser effect spectroscopy (NOESY) were carried out. Pulse programs for the experiments were taken from the Bruker software library. For NMR-data see Tables 1 and 2.

Table 2. ^{13}C-NMR spectra of NIV degradation products in MeOH-d_4

C	norNIV A	norNIV B	norNIV C	NIV lactone
2	83.4	76.2	74.7	80.4
3	82.3	74.9	78.5	79.7
4	74.8	87.4	83.4	80.4
5	46.0	45.7	56.9	59.6
6	47.2	128.7	135.9	47.2
7	180.2	141.4	139.7	189.4
8	144.5	144.3	147.2	84.5
9	126.6	123.1	125.8	42.3
10	34.7	123.6	114.4	38.3
11	74.7	118.8	123.8	77.5
12	54.1	77.3	97.6	77.1
13	54.4	70.3	64.3	69.4
14	6.5	16.7	17.3	8.5
15	–	–	–	70.4
16	16.3	15.6	15.7	15.7

Chemical shifts are reported in ppm.

2.10 Model baking experiment

Dry samples were prepared by weighing 0.6 g NIV-spiked wheat flour (concentration 0.5, 1, and 2 mg/kg flour) in a 1.5 mL glass vial. For wet samples, 400 µL water was added and the "dough" was thoroughly mixed. The samples were then heated in a heating block at 200°C for 60 min, reflecting the conditions during the process of baking bread. The resulting crust was extracted three times with 1 mL methanol and filtered through a Spartan 13/0.45 RC filter unit (Schleicher & Schuell, Dassel, Germany). The solvent was evaporated to dryness, the residue dissolved in 300 µL methanol/water (1 : 1 v/v) and analyzed by HPLC-MS/MS.

2.11 Sample preparation

Commercially available food samples (n = 25, bread, crisp bread, cookies, corn flakes, tortilla chips, pretzels, and pretzel sticks) were manually desalted when possible and finely ground in a laboratory blender. 25 g of the samples was extracted with 100 mL methanol/water (75 : 25 v/v) by

shaking it for 60 min on a laboratory shaker. The supernatant was filtered and 25 mL of the filtrate were evaporated to dryness. The residue was redissolved in 2 mL methanol, filtered through a Spartan 13/0.45 RC filter unit, and further analyzed by HPLC-MS/MS. Recovery rates were not determined due to the limited amount of available degradation products.

2.12 Cell culture

Human immortalized proximal tubule cells immobilized human kidney epithelial ((IHKE) cells, passage 150 – 156) were kindly provided by M. Gekle (Würzburg, Germany) (IHKE cells are originally from S. Mollerup, National Institute of Occupational Health, Norway). They were cultured as described by Tveito [12] in DMEM/Ham's-F12 medium (100 μL/cm^2) enriched with 13 mmol/L $NaHCO_3$, 15 mmol/L HEPES, 36 μg/L hydrocortisone, 5 mg/L human apotransferrin, 5 mg/L bovine insulin, 10 μg/L mouse epidermal growth factor, 5 μg/L Na-selenite, 10% fetal calf serum, and in addition 1% penicillin/streptomycin under standard cell culture conditions (37°C, 5% CO_2).

2.13 Cytotoxicity assay

Cytotoxicity was evaluated colorimetrically with the Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan) similar to the manufacturer's instruction. Briefly, cells were grown on a 96-well microplate. One-hundred microliters of a cell suspension, containing 3×10^3 cells, was added to each well. After 48 h growth, culture medium was replaced by serum-free medium for 24 h. Test compounds were dissolved in methanol and added to serum-free medium (the final MeOH concentration was <1%). After 48 h incubation, the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) solution was added and the cells were incubated for 1 h. WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The absorbance of each well was measured with a microplate reader (FLUOstar Optima; BMG Labtechnologies, Jena, Germany) at 450 nm. The amount of the produced formazan generated by the activity of dehydrogenases in cells is directly proportional to the number of viable cells per well. The absorbance of the treated wells was compared with the untreated control.

3 Results and discussion

In order to study the formation of degradation products of NIV during food processing such as baking, we heated NIV in model experiments with α-D-glucose (sugar model), methyl-α-D-glucopyranoside (starch model), and the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein models) at various temperatures (150 – 200°C) for different time periods (10 – 60 min). The resulting residues were screened using GC-MS after silylation.

Degradation of NIV was observed under all conditions, generally accelerating with increasing temperatures. Heating with *N*-α-acetyl-L-lysine methyl ester resulted in the fastest degradation of NIV: after 10 min at 175°C, only 40% of NIV was left, while it was approximately 70% with all the other model compounds. After 60 min, only trace amounts of NIV were detectable compared to 20 – 45% with the other model compounds. Although the heating experiments with α-D-glucose, methyl-α-D-glucopyranoside and BOC-L-cysteine methyl ester showed a significant loss of NIV depending on the heating time (see above), we were only able to detect traces of degradation products. This can probably be explained by pyrolysis and/or polymerization reactions.

In the heating experiments with the lysine-derivative we observed appreciable amounts of degradation products. Therefore, we supposed an alkaline catalyzed mechanism for the degradation of NIV, which was already reported for DON [11]. Our assumption was confirmed by treatment of NIV with 0.1 M NaOH, observing the same degradation products with even higher yield. After optimization of the heating conditions for maximum product yield, four compounds were isolated by semipreparative HPLC and used for further structure elucidation. One of these compounds appeared to be NIV lactone, already described by Grove [10]. HPLC-MS and HPLC-MS/MS measurements showed its protonated and deprotonated molecular ion $[M+H]^+$ at *m/z* 313 and $[M-H]^-$ at *m/z* 311. The exact mass of this compound corresponded with the molecular formula $C_{15}H_{20}O_7$ showing the substance to be an isomer of NIV. The three other compounds were new and were named norNIV A, norNIV B, and norNIV C according to their elution order and using the nomenclature of Young *et al.* [11]. HPLC-MS and HPLC-MS/MS measurements showed their protonated molecular ion $[M+H]^+$ at *m/z* 283 and their deprotonated one $[M-H]^-$ at *m/z* 281. The exact mass of the three compounds corresponded to the molecular formula $C_{14}H_{18}O_6$, showing that they are structural isomers. For structure elucidation and NMR signal assignment ^1H, ^{13}C, H,H-COSY, H,C-HMQC, H,C-HMBC, and H,H-NOESY spectra were recorded (see Tables 1 and 2). The structures of the degradation products are shown in Fig. 2. The absolute configuration shown was deduced from coupling constants and H,H-NOESY-experiments. The responses obtained in the NOESY spectra are shown in Fig. 3 (due to the complexity of the molecules, the figures are limited to 2-D display; 3-D models of the molecules are helpful to

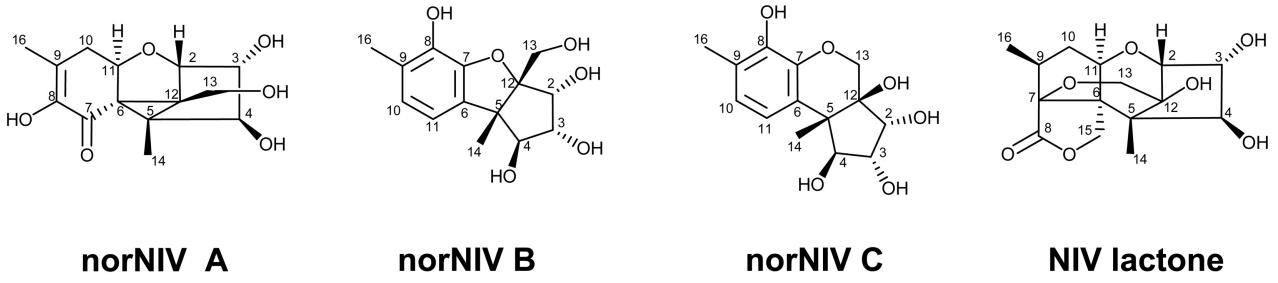

Figure 2. Structures of the NIV degradation products.

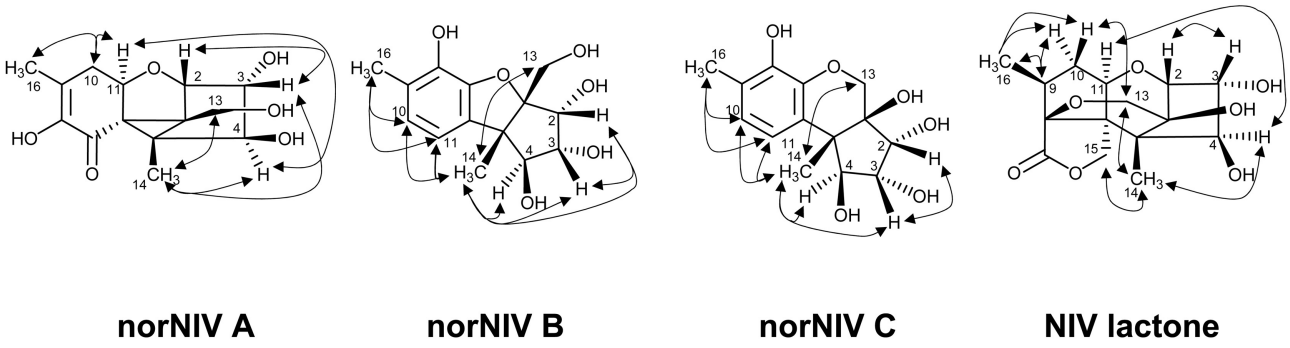

Figure 3. 2-D NOESY correlations of the NIV degradation products.

fully comprehend the NOESY data). For norNIV A, norNIV B, and norNIV C the protons on the same side of the five-membered ring show correlation signals. Additionally, H_3-14 shows a weak correlation to H-4 in all three molecules, although they are not on the same side of the ring. However, NOESY signals are detected for H-2/H-3 but not for H-3/H-4 in the molecules. This can be explained by the steric properties of five-membered rings, in which the distance between *trans*-substituents can be small enough to get correlation signals, when one of substituents shows a strong response, such as the H_3-14 methyl group. Furthermore, we confirmed the absolute configuration of NIV lactone: The coupling constants and the low field shift of H-9, indicating its proximity to the carbonyl oxygen of C-8, show that only the 9-S-enantiomer is formed. This is consistent with the mechanism of formation of NIV lactone proposed by Grove [10].

In order to study the formation of NIV degradation products in real samples, we spiked flour with NIV and heated the samples both with and without added water. The samples were extracted with methanol, filtered, and further analyzed by HPLC-MS/MS. The results showed qualitatively the formation of the norNIV products in the heat-treated flour. As a representative example, Fig. 4 shows the HPLC-MS/MS chromatogram of a heated flour sample (without water) spiked with 1 mg/kg NIV. After a heating time of 60 min approximately 95% of the NIV was degraded and under these conditions norNIV B was formed in the highest concentration compared to all other compounds (due to the limited amounts of isolated NIV degradation products we were not able to quantify the exact concentration). A significant difference between wet and dry heating could not be detected. We further analyzed samples from the German market to study the significance of the NIV degradation products in commercially available food samples. Only one out of 25 samples contained NIV and norNIV B. This is most likely due to the low NIV-contamination of the available samples. According to the SCOOP report on the occurrence of *Fusarium* toxins in food, only 14% of wheat and up to 35% of corn samples are contaminated with NIV. The mean contamination of these samples is between 76 to 98 μg/kg [13]. Considering the fact, that NIV decomposes into four different products, the limit of detection of the compounds is easily reached.

In order to determine the cytotoxicity of the NIV degradation products, cell culture experiments were performed using IHKE cells. The results clearly showed that the four compounds are less cytotoxic compared to NIV. Whereas NIV revealed an EC_{50} at approximately 0.89 ± 0.09 μmol, all other compounds did not show any significant effect up to 100 μmol. As representative example Table 3 shows the results for norNIV C. These findings are in agreement with the fact that the epoxy-group plays an important role in the toxicity of trichothecenes [14, 15]. Cell culture experiments

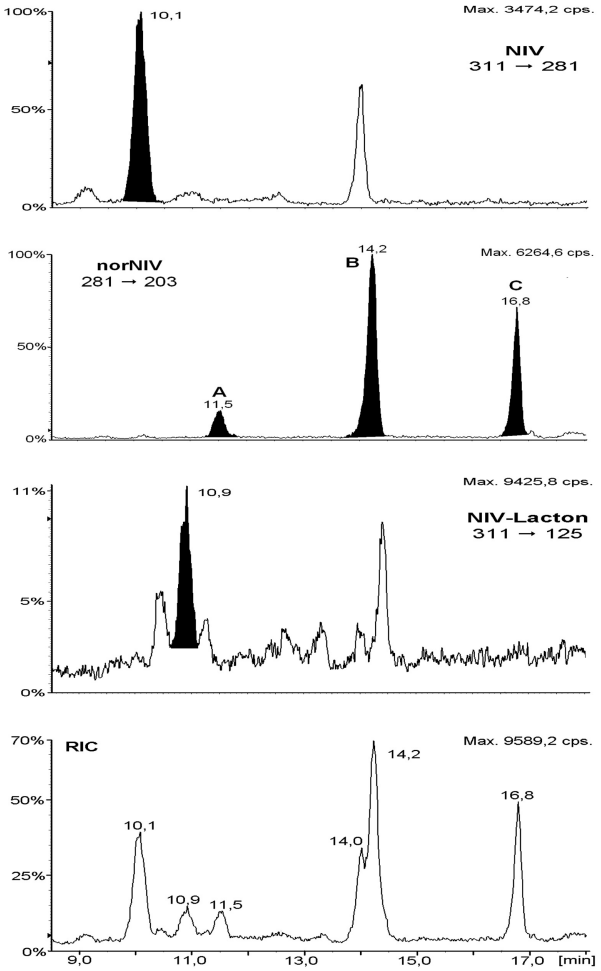

Figure 4. HPLC-ESI-MS/MS analysis of a baked flour sample spiked with 1 mg/kg NIV.

Table 3. Cytotoxicity of norNIV C in IHKE cells

Concentration norNIV C	Viable cells (% of control)
5 µM	100.1 ± 6.6
10 µM	100.5 ± 9.7
50 µM	96.5 ± 5.6
100 µM	92.8 ± 6.5

The cells were incubated with the shown concentrations for 48 h, then the viable cells were determined colorimetrically (for details see Section 2).

using the 5-bromo-2′-deoxyuridine incorporation assay accessing DNA synthesis showed that the de-epoxides of DON and NIV were 24 and 51 times less toxic compared to the toxins with an intact epoxide ring [15]. It was also shown in the brine shrimp bioassay that the de-epoxides are much less acutely toxic than the corresponding trichothecenes [16]. Furthermore, in the case of the T2 trichothecene i.p. injection of 60 mg de-epoxy T2-toxin/kg b.w. did not show any significant effect in rats, whereas seven out of nine rats died after injection of 12 mg T2 toxin/kg b.w. [17]. De-epoxy T2 was also 400 times less toxic that T2-toxin in the rat skin irritation assay [17]. Therefore, the degradation products of NIV found in our study are less cytotoxic due to the missing epoxy group. It is known from literature data that feeding of 0.05 mg NIV/kg b.w. to swine did not result in the formation of any metabolites in plasma, urine, or feces [18]. Furthermore, the gastrointestinal microflora of unexposed swine did not form de-epoxidated metabolites of NIV. However, after one week of exposure to NIV, the microflora became capable of NIV de-epoxidation [19]. Since *in vitro* and *in vivo* experiments clearly showed that de-epoxy metabolites are less toxic [14 – 17], this might explain the decreased toxicity during a longer exposure to NIV [20]. On the other hand, a detoxification via a de-epoxidation of trichothecenes by the gut microflora of pigs seems critical, since it was shown for DON that the formation of de-epoxy-DON occurred in the lower gastro-intestinal tract, namely in the colon and not in the duodenum or jejunum [21].

4 Concluding remarks

Model compounds mimicking major food components were heated with NIV to study the degradation process of trichothecenes during thermal treatment of food. After optimization of the heating condition for maximum yield, four substances (norNIV A, norNIV B, norNIV C, and NIV lactone) were isolated and their structures completely elucidated, including their absolute configuration. We then demonstrated the formation of these compounds in a heating experiment of NIV with flour, simulating baking conditions. We further analyzed samples from the German market to study the significance of the NIV degradation products in commercially available food stuff. Only norNIV B was detectable in one of these samples, this is most likely due to the low mean contamination of food stuff with NIV. Since the degradation products are less cytotoxic compared to NIV, we conclude that the formation of the degradation products during heating processes reduces the toxicity of NIV-contaminated samples.

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