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**Preparation and characterization of the conjugated
Fusarium mycotoxins
zearalenone-4O- β -D-glucopyranoside,
 α -zearalenol-4O- β -D-glucopyranoside and
 β -zearalenol-4O- β -D-glucopyranoside by MS/MS and
2D-NMR**

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**Preparation and characterization of the conjugated
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 α -zearalenol-4O- β -D-glucopyranoside and β -zearalenol-4O-
 β -D-glucopyranoside by MS/MS and 2D-NMR**

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Abstract

Glucosides of several *Fusarium* mycotoxins occur in naturally infected cereals and may contribute to an increased content to the total mycotoxin load of food and feed. Here we present the results of a fermentation procedure to produce zearalenone-4*O*- β -D-glucopyranoside from zearalenone using an engineered *Saccharomyces cerevisiae* strain, expressing the *Arabidopsis thaliana* UDP-glucosyltransferase UGT73C6. About 24 mg of zearalenone-4*O*- β -D-glucopyranoside was obtained from 50 mg of zearalenone and further purified. 10 mg of the glucoside were reduced with sodium borohydride, yielding 4.1 mg α -zearalenol-4*O*- β -D-glucopyranoside and 4.5 mg β -zearalenol-4*O*- β -D-glucopyranoside at purities higher than 99%. To confirm the identities of the three produced glucosides, MS and MS/MS spectra were acquired using negative electrospray ionization. Besides the deprotonated ions at m/z 479 or 481 respectively in full scan mode, fragments, adducts and dimers were recorded and assigned. MS/MS spectra of the glucosylated substances yielded the deprotonated ions of the mycotoxins zearalenone, α -zearalenol, β -zearalenol and their fragments respectively. Unambiguous structural assignment of the three substances was achieved using 2D-NMR methods. This way, the glucose attachment to position C-4, the β -configuration of the sugar unit and the stereo-chemical assignment of the zearalenol hydroxyl group at C-6' were proven.

Keywords: mycotoxins, mass spectrometry, nuclear magnetic resonance, zearalenone

Introduction

Zearalenone (ZON) is an estrogenic mycotoxin produced by several *Fusarium* spp. Both its toxicological relevance (JECFA, 2000) and analytical methods for its determination in cereals (Krska and Josephs) have been reviewed. Living plants have an amazing capability to transform xenobiotics into more polar and usually less toxic conjugates. Since several *Fusarium* spp. excrete mycotoxins like ZON or deoxynivalenol into the tissue of infected host plants, these compounds are also the subject of such biotransformations (Berthiller et al., 2005). About 20 years ago, zearalenone-4*O*- β -D-glucopyranoside (ZON4G) was identified by Engelhardt et al. (1988) as a transformation product of ZON in maize cell suspension cultures. Shortly thereafter, Gareis et al. (1990) proved, that ZON4G can be cleaved during digestion in swine, releasing its estrogenic precursor – ZON. The term “masked mycotoxin” was coined around that time, describing a compound that is usually not detected by routine analytical methods, but still maintains its toxicity for mammals due to reactivation of the mycotoxin after hydrolysis of the glucoside in the digestive tract. Previously, ZON4G was also identified and characterized with ^1H - and ^{13}C -NMR as a transformation product from ZON by *Rhizopus* spp. (Kamimura, 1986). Chemical synthesis (Zill et al., 1990; Grabley et al., 1992) yielded ZON4G from ZON in high purity with yields up to 80% for use as analytical standard. ^1H -NMR spectra were shown in both publications, which were matching with that from the *Rhizopus* biotransformation product. A small survey of the occurrence of ZON4G along with ZON in wheat was conducted, showing that ZON4G was detected in levels of about 10% of the ZON contamination in wheat (Schneeweis et al., 2002).

Engelhardt et al. (1999) also found the phase I metabolites α -zearalenol (αZOL), β -zearalenol (βZOL) and their respective glucosides (αZOL4G , βZOL4G , structures shown in Figure 1) after treatment of maize cell cultures with ZON. Recently, it was demonstrated that *Arabidopsis thaliana* plants were able to metabolize ZON into at least 17 different metabolites, including glucosides, malonylglucosides, dihexosides (presumably diglucosides)

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2
3 and pentosylhexosides (presumably xylosylglucosides) of ZON, α ZOL and β ZOL (Berthiller
4 et al., 2006). Poppenberger et al. (2006) identified the *A. thaliana* UDP-glucosyltransferase
5 (UGT) UGT73C6, that is mainly responsible for the glucosylation of ZON, α ZOL and β ZOL,
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9
10 cloned and expressed the encoding gene into *Saccharomyces cerevisiae*. The resulting yeast
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12 expression system was used to convert ZON into ZON4G. It was briefly shown before by our
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14 group that chemical reduction of ZON4G can yield both α ZOL4G and β ZOL4G in a ratio of
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16 about 1:1 (Krenn et al., 2007).
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18

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20 Here, we report an optimized procedure to obtain ZON4G after fermentation of ZON
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22 treated yeast, expressing UGT73C6. Furthermore, α ZOL4G and β ZOL4G were produced by
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24 chemical reduction of ZON4G with sodium borohydride, purified by preparative HPLC and
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26 crystallized. Both substances were fully characterized for the first time using various MS/MS
27
28 and NMR techniques.
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33

34 **Materials and methods**

35 ***Fermentation of ZON treated yeast expressing UGT73C6***

36
37 *Saccharomyces cerevisiae* strain YGZA515 (Poppenberger et al., 2003) was transformed with
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39 plasmid pBP918 (Poppenberger et al., 2006). The 2 μ -LEU2 plasmid harbors the *Arabidopsis*
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41 *thaliana* glucosyltransferase UGT73C6 with a c-myc epitope tag behind the string constitutive
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43 ADH1 promoter. The transformed strain was streaked out on SC-LEU plates. A single colony
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45 was picked, suspended in 30 mL SC-LEU medium and grown overnight under shaking at
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47 30°C. To this pre-culture, 100 mL SC-LEU medium was added to replenish essential medium
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49 components (OD₆₀₀ = 0.05 after the dilution) and again grown overnight. This was repeated
50
51 two-times by adding 400 mL SC-LEU (OD₆₀₀ = 0.1) at the third and 3000 mL SC-LEU
52
53 (OD₆₀₀ = 0.25) at the fourth day where the liquid culture reached a final OD₆₀₀ of 0.8 and was
54
55 used to inoculate a 42 L bioreactor (Applikon, Schiedam, The Netherlands) equipped with a
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57 Bio Controller ADI 1030 for yeast biomass production. In contrast to a previous fermentation
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3 (Krenn et al., 2007), the yeast for inoculation was collected already at OD₆₀₀ of 0.8 well
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5 before it reached the stationary growth phase. The bioreactor was loaded with 28 L SC-LEU
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7 medium, containing a surplus of the carbon source (+20 g/L glucose) and inoculated with 2 L
8
9 of the yeast culture. The cell suspension was stirred at 600 rpm at a constant pH value of 4.9
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11 at 30°C. Oxygen saturation was kept above 90% using pressurized air. The optical density
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13 values were measured every 2 h to determine the transition from exponential growth to the
14
15 stationary growth phase, which happened about 11 h after the inoculation. At this point, the
16
17 cell suspension was immediately harvested, cooled to 4°C and the biomass separated from the
18
19 medium in five subsequent centrifugation steps (6000×g, 8 min). The cell pellet was re-
20
21 suspended in 300 mL SC-LEU medium and used as biocatalyst for ZON bioconversion in a 7
22
23 L bioreactor (MBR Bioreactor AG, Wetzikon, Switzerland) controlled by an IMCS-2000 unit.
24
25 The reactor was loaded with 5 L SC-LEU medium and the yeast-cell slurry and stirred (600
26
27 rpm) at 30°C. During the conversion, the pH value was kept constant at 5.0 and the oxygen
28
29 saturation was regulated (80%) using compressed air. After 20 min, 25 mg ZON (Biopure,
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31 Tulln, Austria) was dissolved in 1 mL acetone and added to the actively growing biocatalyst.
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33 After 2.5 h, another dose of 25 mg ZON was added and the stirrer speed increased to 1000
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35 rpm thereafter. The bioconversion was stopped after 21 h and the cells were removed by
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37 centrifugation. The obtained 5 L of clear supernatant were mixed with 500 mL methanol (to
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39 assure complete solution of ZON4G) and used for the purification of ZON4G.
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51 ***Purification of ZON4G***

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53 ZON4G was isolated from the yeast supernatant using Bakerbond Octadecyl Polar Plus (1000
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55 mg; J.T. Baker, Phillipsburg, USA) solid phase extraction columns. Columns were
56
57 conditioned successively with 3x10 mL methanol, 3x10 mL methanol/water (10/90, v/v) and
58
59 loaded with 300 mL of the supernatant. Each column was washed with 200 mL
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methanol/water (10/90, v/v) and 50 mL methanol/water (30/70, v/v), before eluting ZON4G

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3 with 300 mL methanol/water (35/65, v/v). The combined eluates containing ZON4G were
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5 evaporated nearly to dryness using a rotavapor and re-dissolved in a total of 5 mL
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7 acetonitrile/water (20/80, v/v). Afterwards ZON4G was purified by an 1100 series preparative
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9 HPLC system (Agilent Technologies, Waldbronn, Germany). Separation was achieved at
10
11 25°C using a Luna RP-C18(2) semi-preparative column (250x10 mm, 10 µm; Phenomenex,
12
13 Aschaffenburg, Germany). An injection volume of 500 µL and a flow rate of 4 mL/min were
14
15 chosen. Mobile phases were MilliQ water (Millipore, Molsheim, France) and LC grade
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17 acetonitrile (J.T. Baker, Phillipsburg, USA). After an initial hold time of 4 min with 20%
18
19 acetonitrile, a linear gradient was applied which reached 85% acetonitrile after 22 min. A 6
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21 min hold time at the same composition was followed by a rapid change to the starting
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23 conditions, which were held for another 5 min to re-equilibrate the column.
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32 ***Reduction of ZON4G to α ZOL4G and β ZOL4G***

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34 A 10-fold molar excess of sodium borohydride was used to reduce ZON4G to both α ZOL4G
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36 and β ZOL4G. Therefore, 10 mg ZON4G and about 3 mg fresh sodium borohydride were
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38 dissolved in 10 mL methanol and stirred for about 30 min (until no more gas bubbles
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40 emerged). The solution was pipetted into 90 mL of a 1% aqueous acetic acid solution to
41
42 quickly stop the reaction and to avoid degradation of the products due to the alkaline
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44 conditions in the reaction mixture. Enrichment was again achieved using SPE columns. 4
45
46 Bakerbond Octadecyl Polar Plus (1000 mg; J.T. Baker, Phillipsburg, USA) columns were
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48 conditioned with 20 mL methanol, equilibrated with 2x20 mL methanol/water (10/90, v/v).
49
50 Aliquots of 25 mL reaction mixture were applied per column and each column was
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52 subsequently washed with 50 mL methanol/water (10/90, v/v), before eluting α ZOL4G and
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54 β ZOL4G with 30 mL acetonitrile. The eluted fractions were pooled, evaporated with a
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56 rotavapor and re-dissolved in 5 mL acetonitrile/water (20/80, v/v). α ZOL4G and β ZOL4G
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58 were then separated using preparative HPLC using the conditions described above.
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MS/MS experiments

A Q TRAP LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) with a TurboIonSpray (ESI) source was used to characterize the purified compounds. 5 mg/L solutions of ZON4G, α ZOL4G and β ZOL4G were prepared in methanol and directly infused into the ion source using a 1 mL syringe with a flow rate of 10 μ L/min. The source was used solely in the negative ionization mode at a temperature of 250°C and 20 scans were summed for a final spectrum. MS parameters were: curtain gas 20 psi (138 kPa), nebulizer gas 20 psi (138 kPa), auxiliary gas 65 psi (448 kPa), ionspray voltage -4200 V, interface heater on, declustering potential -30 V, entrance potential -10 V. For the full scan mode quadrupole 1 was set to scan between 300 and 1000 amu within 1 s. The enhanced product ion scan mode was used to acquire MS/MS spectra between 100 and 500 amu. For this mode, the following further parameters were set: scan rate 250 amu/s, Q0 trapping on, linear ion trap fill time 20 ms, collision activated dissociation gas low, collision energy -5 eV.

NMR spectroscopy

^1H - and ^{13}C -NMR spectra were obtained using an Avance DRX 400 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 300 K using a 5 mm inverse broadband Z-gradient probehead. About 4 mg of crystalline ZON4G, α ZOL4G and β ZOL4G were dissolved in deuterated methanol for analysis. The spectrometer was operated at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C measurements and the spectra were referenced to the respective solvent peaks.

Results and discussion

Production of ZON4G, α ZOL4G and β ZOL4G

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3 A total of 50 mg ZON was used in two doses to produce ZON4G with yeast, expressing
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5 UGT73C6. As fermentation took place in a 5 L scale, the concentration of ZON was always
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7 kept below 10 mg/L. Concentrations above 10 mg/L proved to be toxic for the yeast strain,
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9 resulting in a drastic loss of productivity. The fermentation yielded 24 mg ZON4G. Besides,
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11 also 10 mg β ZOL and minor amounts of α ZOL, β ZOL4G, α ZOL4G and unfermented ZON
12
13 were found in the fermentation supernatant. These side products were presumably formed due
14
15 to the activity of alcohol dehydrogenases of the used yeast strain and further conjugation of
16
17 α ZOL and β ZOL. After purification, 21 mg of pure ZON4G were obtained. Purity was
18
19 determined by LC-UV at 200 nm as shown previously (Krenn et al., 2007) and was estimated
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21 to be 99.7% (data not shown). It has to be pointed out that certain impurities (e.g. salts or
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23 inorganic materials) that do not have a chromophore are not detected by the used method.
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30 Although the used yeast strain can principally be used to convert α ZOL directly into
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32 α ZOL4G and β ZOL into β ZOL4G, we decided to produce the latter compounds by chemical
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34 reduction from ZON4G. This way only ZON was needed as reactant. After purification 4.1
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36 mg α ZOL4G (estimated purity 99.8%) and 4.5 mg β ZOL4G (estimated purity 99.6%) were
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38 obtained from 10 mg ZON4G.
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43 *MS/MS characterization*

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45 The fullscan (LC-)MS scans of ZON4G (Figure 2), α ZOL4G (Figure 3) and β ZOL4G (Figure
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47 4) showed various MS-peaks in negative ionization mode. The negative ionization mode was
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49 used exclusively, since it yielded far higher signal intensities compared to the positive mode.
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51 Besides the expected $[M-H]^-$ ions, also chlorine, formiate and acetate adducts were observed
52
53 in the mass spectra. We suspect that the adduct ions are arising due to unavoidable
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55 contaminations of our mass spectrometer, which is often used with several different solvents
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57 and buffers for other methods. Characteristic $[2M-H]^-$ ions of high intensities were formed for
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59 all three glucosides. In-source-decay yielded the deprotonated ions of the unconjugated
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3 mycotoxins ZON, α ZOL and β ZOL respectively, after neutral loss of the glucose moieties (-
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5 162 amu). Most interestingly, an uncommon $[M+90-H]^-$ adduct could be seen at various
6
7 intensities for ZON4G, α ZOL4G and β ZOL4G. We believe this adduct to be a $C_4H_{10}O_2$
8
9 molecule, formed from methanol, as shown before in negative ESI spectra of glycolipids (Li et
10
11 al., 1993).
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13

14
15 MS/MS spectra were acquired for the three glucosides using the minimum possible
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17 collision energy of 5 eV, as harsher fragmentation conditions resulted in MS/MS spectra that
18
19 no longer showed the selected $[M-H]^-$ precursor ions. The major ions in the MS/MS spectra
20
21 (depicted as inlays in Figures 2-4) clearly were $[ZON-H]^-$ in case of ZON4G, $[\alpha ZOL-H]^-$ in
22
23 case of α ZOL4G and $[\beta ZOL-H]^-$ in case of β ZOL4G. Minor peaks corresponded to losses of
24
25 water or CO_2 from the non-glucosylated compounds. Higher collision energies (data not
26
27 shown) yielded MS/MS spectra that were not distinguishable from those obtained for ZON,
28
29 α ZOL and β ZOL respectively.
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34 35 36 *NMR characterization*

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38 1H and ^{13}C -APT NMR spectra were recorded for all glucosides. The identity of ZON4G was
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40 confirmed by the complete accordance of its NMR data with those published earlier by
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42 Kamimura (1986). Additionally, full structure characterizations and complete signal
43
44 assignments were carried out for the two isomeric zearalenol derivatives by means of 2D
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46 NMR methods: proton-proton (DQF-COSY) and proton-carbon (HSQC) direct correlations as
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48 well as proton-carbon long range correlation (HMBC). Thus, the attachment of the glucoside
49
50 to position 4 of the zearalenols was shown by a long range correlation of the glucosidic proton
51
52 to the C-4 carbon signal, and the β configuration of the sugar unit could be proven by the 7.5
53
54 Hz coupling constant between H-1'' and H-2''. The stereochemical assignment of the -OH
55
56 group within the macrocyclic ring (α - vs. β -isomer) was done by comparing the spectra of our
57
58 products with those of the two isomeric non-conjugated zearalenols, which were produced by
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3 Burckhardt and Ley (2002) *via* total synthesis. Full ^1H and ^{13}C assignments of the zearalenol-
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5
6 4*O*- β -D-glucopyranosides are given in Table 1, the spectra are shown in Figures 5 and 6.
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8
9

10 **Conclusions**

11
12 It is currently unknown to which extent conjugates of mycotoxins occur in food and feed.
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14 Here we biotechnologically and chemically produced three glucosidic conjugates of *Fusarium*
15
16 mycotoxins. All three compounds, ZON4G, α ZOL4G and β ZOL4G were obtained in
17
18 acceptable yields and high purities. MS scans showed the expected nominal molecular mass
19
20 of the produced compounds, while NMR characterization additionally proved the position of
21
22 the glucose group and the stereochemical assignments of the glucose and hydroxyl groups of
23
24 α ZOL4G and β ZOL4G. These substances will be used as standards for a survey of conjugated
25
26 mycotoxins in cereals and cereal-based food using LC-MS/MS.
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34 **Acknowledgements**

35
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37
38 the UK Food Standards Agency for financial support.
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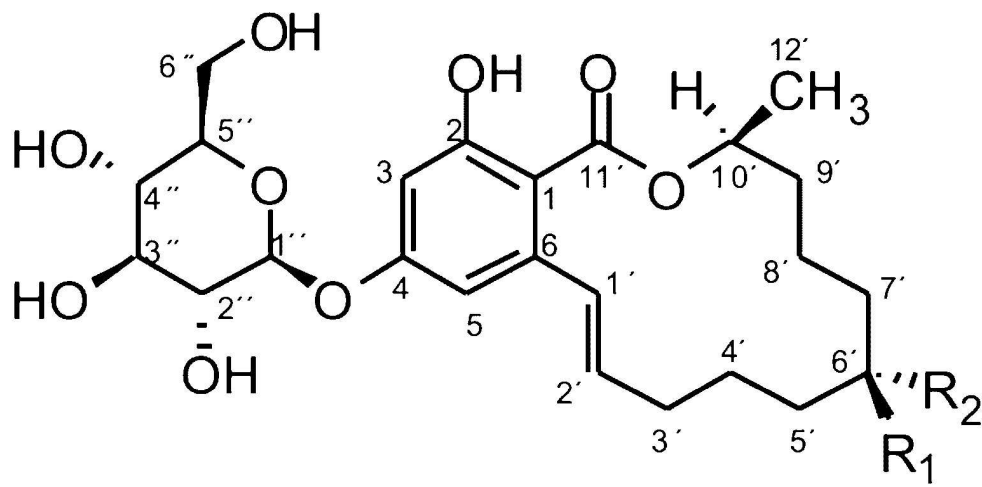
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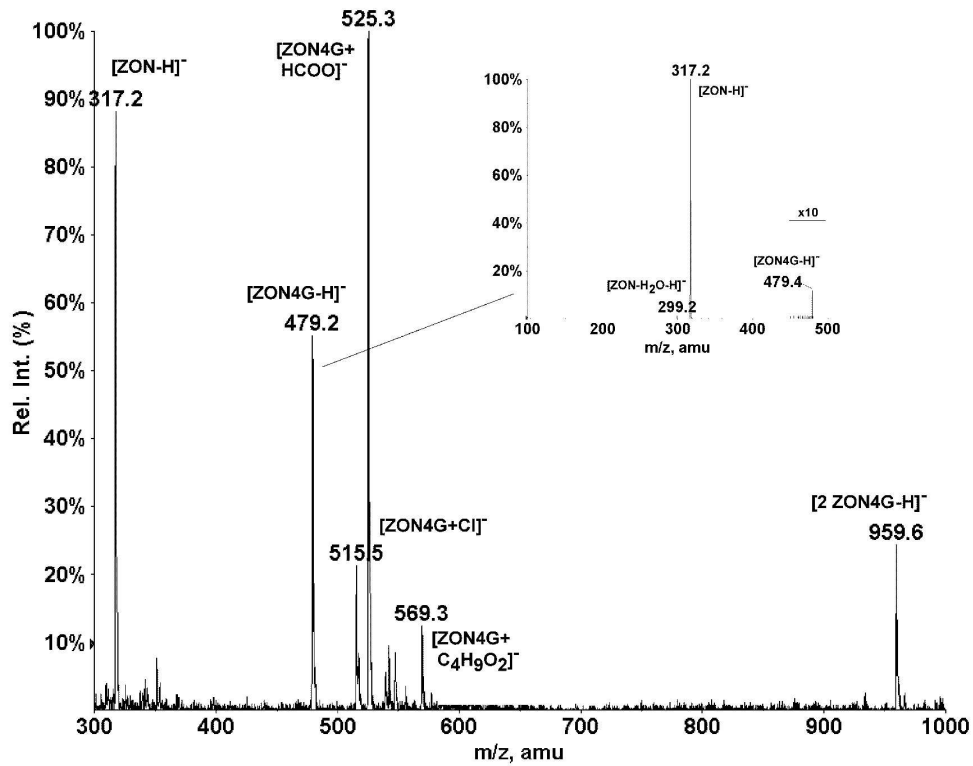
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Table 1. ^1H and ^{13}C NMR shifts of zearalenol-4*O*- β -D-glucopyranosides (ppm)

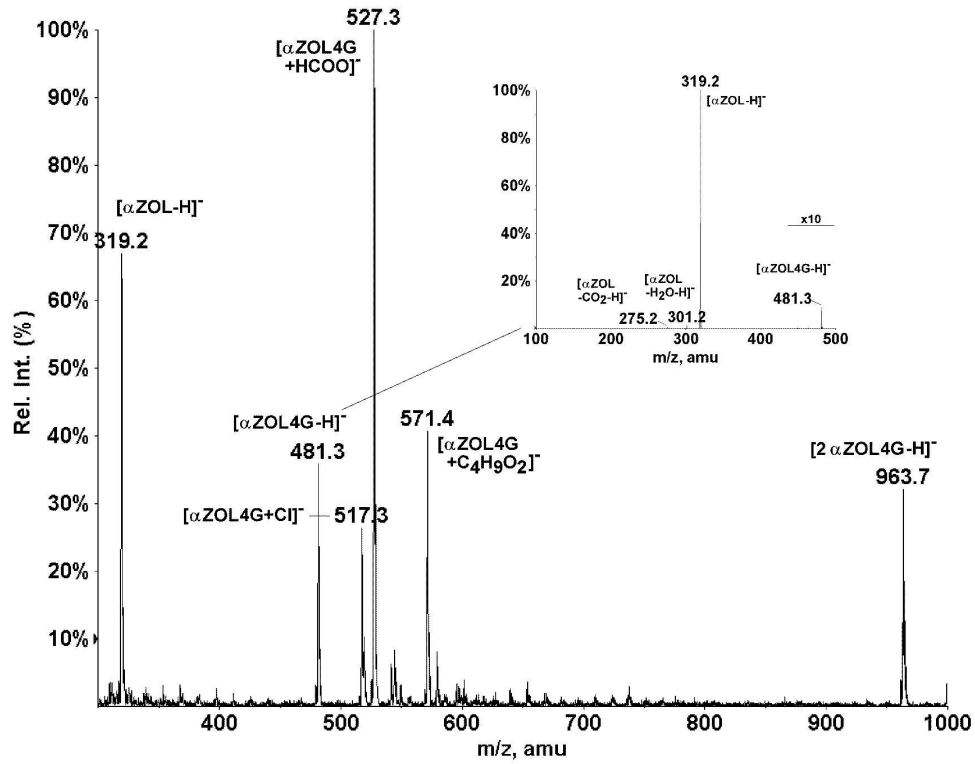
Pos.	^1H		^{13}C	
	α	β	α	β
1	-	-	104.8	110.9
2	-	-	164.6	159.6
3	6.55	6.54	102.6	102.3
4	-	-	161.6	160.4
5	6.69	6.80	108.7	106.2
6	-	-	143.6	139.7
1'	7.14	6.63	132.1	129.6
2'	5.85	6.10	133.9	132.7
3'	2.45-2.25	2.30-2.25	30.1	30.4
4'	1.90; 1.55	1.75; 1.65	22.2	22.2
5'	1.70; 1.18	1.74; 1.29	31.3	31.4
6'	3.78	3.70	65.7	68.0
7'	1.60-1.45	1.66; 1.45	36.1	35.8
8'	1.50-1.40	1.56; 1.42	21.3	18.9
9'	1.90; 1.65	1.88; 1.63	34.7	34.2
10'	5.01	5.22	73.8	72.5
11'	-	-	171.4	170.0
12'	1.43	1.35	19.9	17.8
1''	5.00	4.95	99.8	100.5
2''	3.47	3.47	73.4	73.4
3''	3.48	3.48	76.5	76.5
4''	3.38	3.40	70.0	69.9
5''	3.50	3.48	77.0	76.9
6''	3.93; 3.70	3.92; 3.71	61.1	61.1

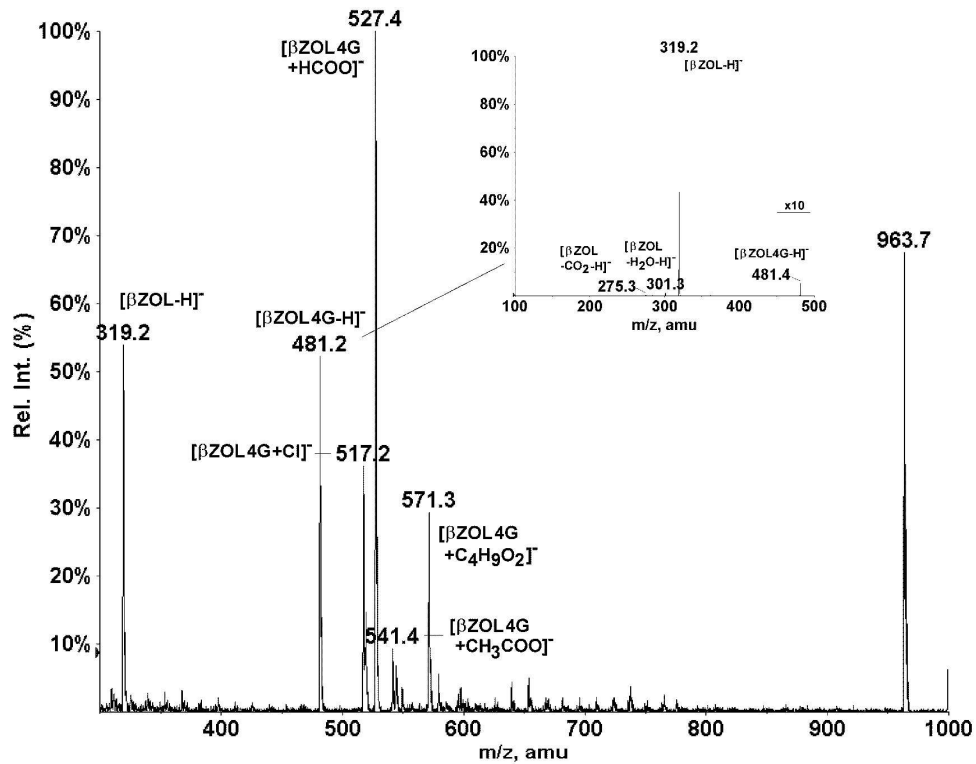


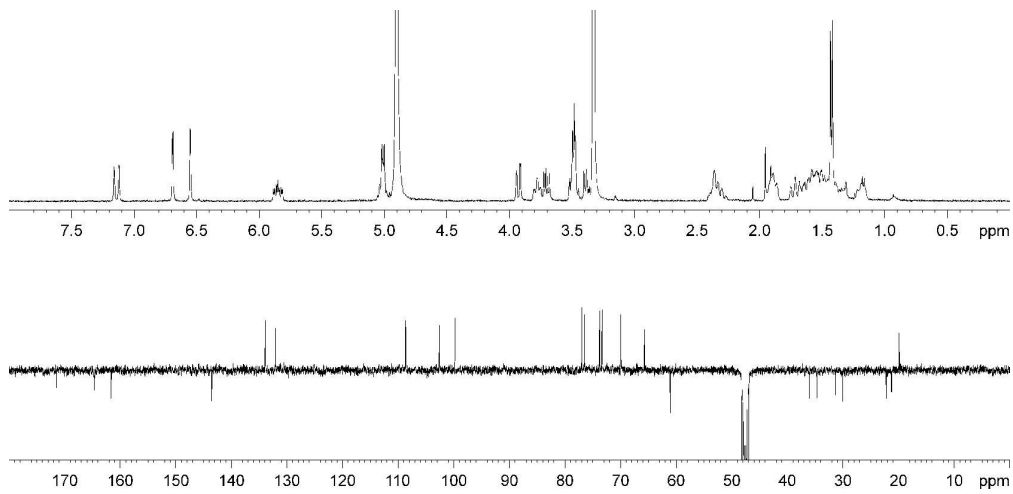
Structures and numbering of α ZOL4G (R₁=H, R₂=OH), and β ZOL4G (R₁=OH, R₂=H)



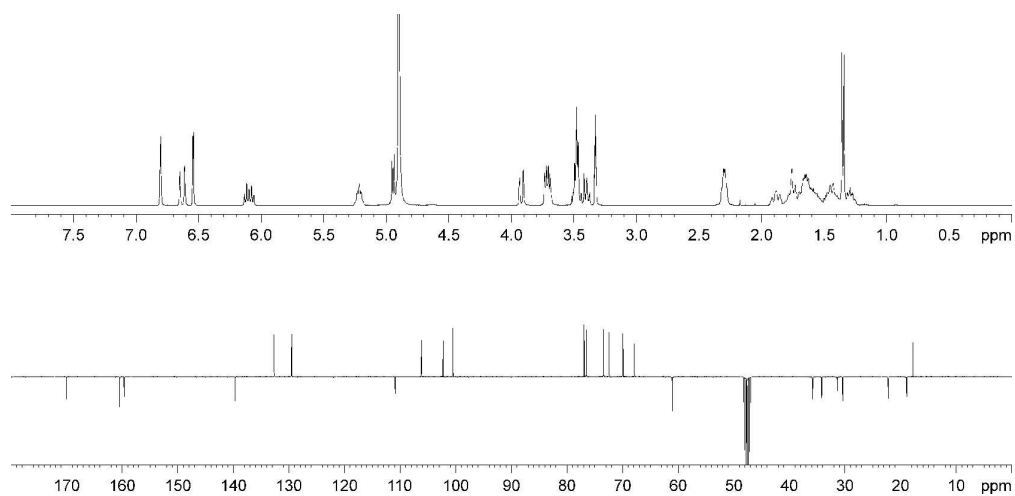
MS and MS/MS (inlay) spectra of ZON4G

MS and MS/MS (inlay) spectra of α ZOL4G

MS and MS/MS (inlay) spectra of β ZOL4G



¹H (top) and ¹³C-APT (bottom) NMR spectra of αZOL4G



^1H (top) and ^{13}C -APT (bottom) NMR spectra of β ZOL4G