Mol. Nutr. Food Res. 2007, *51*, 867 – 871 DOI 10.1002/mnfr.200600295

Research Article

Novel oxidative metabolites of the mycoestrogen zearalenone *in vitro*

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The estrogenic mycotoxin zearalenone (ZEN) is known to get metabolized to the α-and β-isomers of zearalenol, but no hydroxylation products of ZEN have yet been reported as metabolites in animals or humans. We have therefore incubated ZEN with microsomes from rat liver in the presence of a nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-regenerating system and analyzed the extracted metabolites with HPLC and GC-MS after trimethylsilylation. A total of 17 *in vitro* metabolites were observed. The two major metabolites were tentatively identified as monohydroxylated ZEN with the newly introduced hydroxyl group localized in the aliphatic macrocyclic ring. According to the GC-MS analysis, other six monohydroxylation products of ZEN were formed as minor metabolites, together with α-and β-zearalenol and monohydroxylated zearalenols. Thus, ZEN has a considerable propensity for undergoing metabolic hydroxylation reactions *in vitro*, and the *in vivo* formation and biological properties of such oxidative metabolites should now be studied.

Keywords: Microsomes / Mycoestrogen / Mycotoxin / Oxidative metabolites / Zearalenone

Received: December 22, 2006; revised: February 21, 2007; accepted: February 22, 2007

1 Introduction

Zearalenone (ZEN, Fig. 1) is a resorcylic acid lactone produced by *Fusarium* species infesting not only corn but also other grains [1, 2]. It has a rather low acute toxicity, and the biological activity of this mycotoxin is dominated by its estrogenicity, resulting in severe effects on the reproductive system of farm animals, particularly pigs [1, 2]. Little is known on the biotransformation of ZEN in mammals, and the only metabolites identified to date in several species including humans are the α- and β-isomers of zearalenol (Fig. 1), arising through reduction of the carbonyl group at C-7 [1–5]. These reductive metabolites of ZEN have been identified in rat liver homogenate and microsomes as early as 1978 [6, 7] and were found in 1983 [8] to be formed in liver cell fractions from several other species such as pig, goat, cow, hen, and sheep. More recent studies have demonstrated notable inter-species differences in the formation of α- and β-zearalenol [9]. Moreover, it has been reported that a further reduction may occur in cattle, sheep, and pig, leading to α- and β-zearalanol with a saturated bond between C-11 and C-12 [10–12]. *In vivo*, all these reductive phase I metabolites of ZEN are conjugated with glucuronic acid [5, 10–12]. Surprisingly, no oxidative phase I metabolites of ZEN have yet been reported despite the numerous potential sites for aliphatic and aromatic hydroxylation of the ZEN molecule. The aim of the present study was therefore to explore the propensity of ZEN for metabolic hydroxylation *in vitro* by rat hepatic microsomes.

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Abbreviations: d9-BSA, *N,O*-bis(d9-trimethylsilyl)acetamide; **BSTFA,** *N,O*-bis(trimethylsilyl)trifluoroacetamide; **EI,** electron impact; **NADP(H),** nicotinamide adenine dinucleotide phosphate (reduced form); **TMS,** trimethylsilyl; **ZEN,** zearalenone

2 Materials and methods

2.1 Chemicals, animals, and cell fractions

ZEN and the α- and β-isomers of zearalenol and zearalanol were purchased from Sigma/Aldrich/Fluka (Taufkirchen, Germany) and had a purity of >96% according to HPLC analysis. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and other chemicals and reagents were of the highest quality available and were purchased from Sigma/Aldrich/Fluka. *N,O*-bis(d9-trimethylsilyl)acetamide (d9-BSA) was from Campro Scientific (Berlin, Germany) and HPLC-grade ACN from Carl Roth (Karlsruhe, Germany).

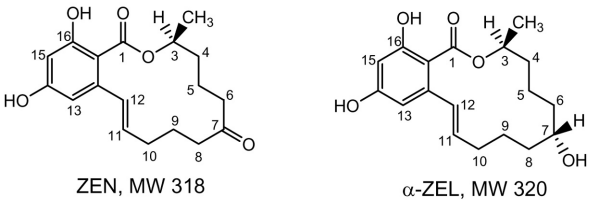

Figure 1. Chemical structures and molecular weights (MW) of zearalenone (ZEN) and α-zearalenol (α-ZEL).

Male Wistar rats were purchased from Harlan Winkelmann (Borchen, Germany). Animals were kept under a 12-h light/dark cycle and received water and commercial lab chow *ad libitum*. For the induction of monooxygenase activity, one intraperitoneal injection of aroclor 1254 with a dose of 500 mg/kg body weight dissolved in sesame oil at 100 mg/mL was administered to the rats 5 days prior to their sacrifice.

2.2 Microsomal incubations

Microsomes, which contain the ER and thus the cytochrome P450-dependent monooxygenases catalyzing hydroxylation reactions, were prepared by differential centrifugation from the homogenized livers of untreated and aroclor-treated male rats with 200–300 g body weight as described by Lake [13]. Protein concentrations were measured according to Bradford [14] with BSA as a standard. Incubations contained 1 mg microsomal protein, 50 μM ZEN dissolved in DMSO (final DMSO concentration 0.5%) and a NADPH-regenerating system (0.9 U isocitrate dehydrogenase, 9.4 mM isocitrate, 1.21 mM $NADP^+$, and 4.3 mM magnesium chloride) in a final volume of 1 mL of 0.1 M phosphate buffer pH 7.4. After preincubation of the microsomes and ZEN for 5 min at 37°C, the NADPH-regenerating system was added and the mixture incubated for 40 min at 37°C. Subsequently, the incubation mixture was extracted with 3 × 0.5 mL ethyl acetate and the pooled extract evaporated to dryness. The residue was dissolved in 50 μL methanol for HPLC analysis. In control incubations, either the NADPH-regenerating system or ZEN was omitted. In order to obtain larger amounts of the metabolites for mass spectrometric analysis, the microsomal incubation was scaled up by a factor of ten.

2.3 HPLC analysis

An HP 1100 system equipped with a binary pump, photodiode array detector, and HP ChemStation Version RevA.07.01 software for data collection and analysis were used. Separation was carried out on a 250 × 4.6 mm id, 5 μm, RP Luna C8 column (Phenomenex, Torrance, CA, USA). Solvent A was deionized water adjusted to pH 3.0 with formic acid and solvent B was ACN. A linear solvent gradient was started directly after injection, changing from 30 to 70% B in 30 min, then to 100% B in 3 min, staying at 100% B for 6 min, and returning to initial conditions within 1 min. The flow rate was 1 mL/min and the detector was set to 280 nm. HPLC fractions of the metabolites were collected, extracted with ethyl acetate, and used for GC-MS analysis.

2.4 GC-MS analysis

The reference compounds and microsomal metabolites were analyzed using a Finnigan GCQ capillary gas chromatograph equipped with a 30 × 0.25 mm id, 0.25 μm, 5% phenylmethyl MDN-5S fused-silica column (Supelco, Bellefonte, PA, USA) and coupled to an IT detector was operated with electron impact (EI) ionization at 70 eV (Thermo Finnigan, Austin, TX, USA). Samples dissolved in methanol or ethyl acetate were evaporated to dryness, dissolved in 30 μL of BSTFA or d9-BSA, and 1 μL was injected, using the splitless mode for 90 s. The injection port temperature was 60°C at the time of injection and raised to 275°C at a rate of 8°C/s. The oven temperature was programmed from 60°C (1 min hold) to 150°C at 30°C/min and then to 295°C at 10°C/min. The transfer line and ion source were kept at 275 and 250°C, respectively. Helium was used as carrier gas with a flow rate of 40 cm/s. Mass spectra were scanned from *m/z* 50 to 800 at a rate of 0.5 s/scan.

3 Results and discussion

Hepatic microsomes from aroclor-induced male Wistar rats were incubated with ZEN in the presence of a NADPH-regenerating system and the incubations subsequently extracted with ethyl acetate. Control incubations without NADPH had shown that ZEN was virtually quantitatively recovered under these conditions. The extracts from the complete incubations were then analyzed by HPLC, using conditions which had been shown before to yield a good separation of ZEN and the four commercially available standards α- and β-zearalenol and -zearalanol. The formation of 17 new products was observed which were not generated in the control incubations and which eluted prior to ZEN from the RP column (Fig. 2A). About 75% of the ZEN was converted to metabolites. When liver microsomes from untreated rats were used, 30% of ZEN was metabolized, and the proportion of the more polar metabolites, M1 to M6, was lower than with induced microsomes (Fig. 2B). This decrease in metabolic conversion and shift in the pattern of metabolites is probably due to the lower content of total cytochrome P450 and differences in the amount of the various isoforms in noninduced liver as compared to aroclor-induced liver.

In order to obtain sufficient quantities of the metabolites M7 to M17 for structure elucidation, larger incubations

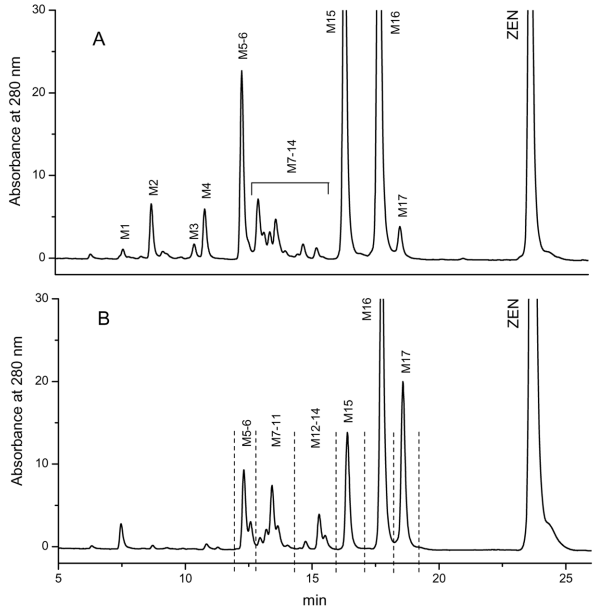

Figure 2. HPLC profiles of the extract of an incubation of ZEN with hepatic microsomes from aroclor-induced (A) and noninduced (B) male Wistar rats in the presence of a NADPH-regenerating system. Peaks M1 to M17 represent ZEN metabolites. M14 and M17 were identified as β- and α-zearalenol, respectively, by cochromatography with authentic standards in HPLC and by GC-MS of the collected HPLC fractions.

with uninduced microsomes were conducted, which gave a less complex pattern of metabolites than aroclor-induced microsomes. The metabolites M5 to M17 were collected in six HPLC fractions as indicated in Fig. 2B. The metabolites were then extracted from each fraction and an aliquot of the extract rechromatographed by HPLC to ensure that they were unchanged. The other aliquots were analyzed by GC-MS after trimethylsilylation. To facilitate structure elucidation, trimethylsilylation was conducted with BSTFA as well as perdeuterated BSA. The EI mass spectra of ZEN and of the major ZEN metabolites M15–M17 are depicted in Fig. 3.

The mass spectrum of ZEN containing unlabeled trimethylsilyl (TMS) groups exhibits a molecular ion at *m/z* 462 and a base peak at *m/z* 333 (Fig. 3). The corresponding ions of ZEN with perdeuterated TMS groups are shifted by 18 amu to *m/z* 480 and 351, respectively, indicating the presence of two intact TMS moieties. Further characteristic ions of the unlabeled ZEN derivative are *m/z* 447, 444, and 429. In the deuterated derivative, only *m/z* 444, which arises by the loss of water from the molecular ion, is shifted by 18 amu to *m/z* 462, whereas the two others are only shifted by 15 amu to *m/z* 462 and 444, indicated the loss of one perdeuterated methyl group. As depicted in Fig. 4, this can be explained by the formation of a six-membered dimethylsiloxane ring, which is a common fragmentation reaction in the TMS derivatives of compounds containing a carbonyl

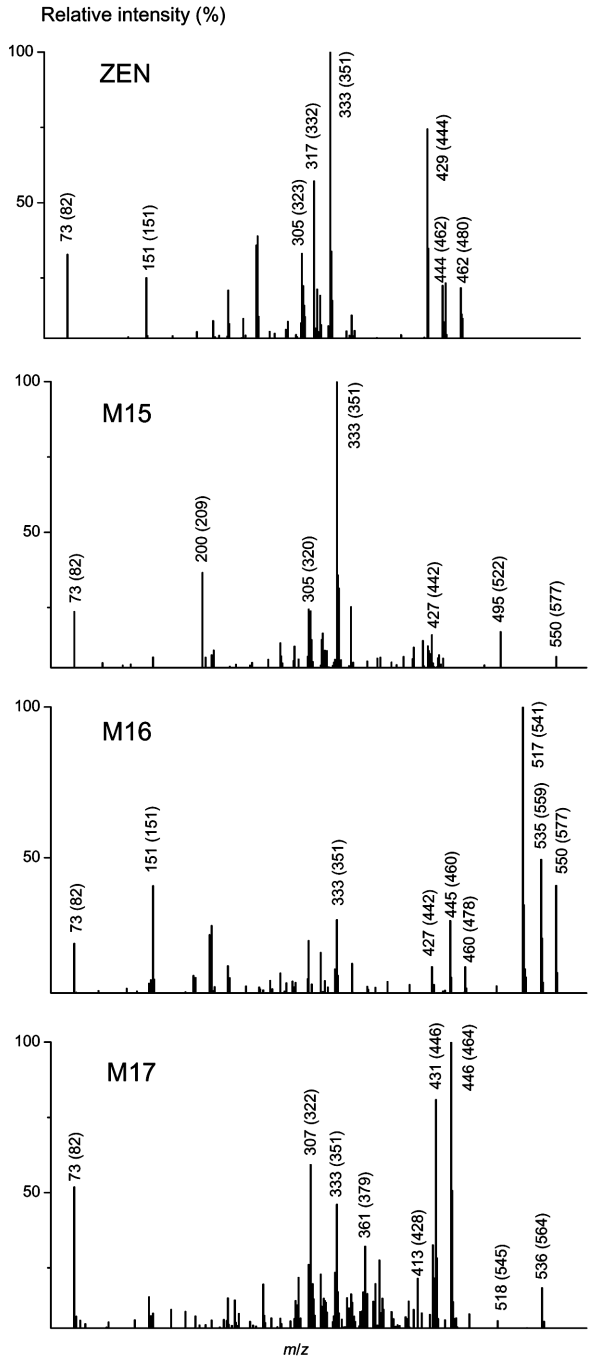

Figure 3. EI mass spectra of the TMS derivatives of ZEN and its major metabolites. Numbers in parentheses indicate the m/z values observed in the corresponding mass spectra of the perdeutero-trimethylsilylated compounds.

group in *ortho*-position to a phenolic group, such as gingerol [15, 16] and alternariol [17]. The base peak at *m/z* 333, which still contains both intact TMS groups, is probably generated by a McLafferty rearrangement involving the carbonyl at C-7 and the hydrogen at C-10, as has previously been proposed [18]. The loss of carbon monox-

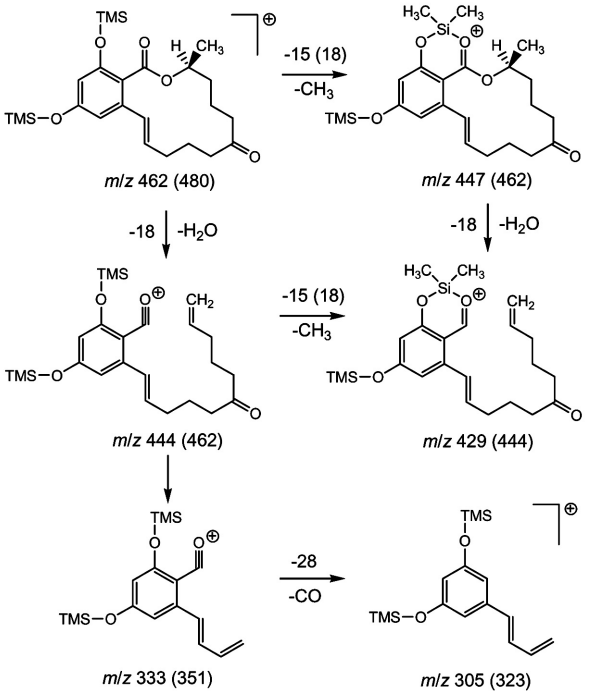

Figure 4. Proposed mass spectrometric fragmentation of trimethylsilylated ZEN. Numbers in parentheses indicate the *m/z* values observed in the corresponding mass spectrum of the perdeutero-trimethylsilylated compound.

ide from *m/z* 333 accounts for the fragment ion at *m/z* 305 (Fig. 4).

The mass spectra of the major ZEN metabolites M15 and M16 both exhibit molecular ions at *m/z* 550, which are shifted by 27 amu in the perdeuterated derivatives (Fig. 3). This clearly suggests the presence of three hydroxyl groups, indicating that M15 and M16 are monohydroxylated metabolites of ZEN. In the mass spectrum of M15, the base peak is at *m/z* 333 and contains two hydroxyl groups like in the mass spectrum of ZEN. This is strong evidence that the newly introduced hydroxyl group must be localized in the aliphatic part of the molecule. However, its exact position in the macrocyclic ring cannot be unambiguously derived from the mass spectrum, and product ion experiments by using the MS/MS technique did not help to clarify the structure so far.

The mass spectrum of M16, which is also a monohydroxylated ZEN metabolite, exhibits a fragmentation markedly different from that of M15 and ZEN (Fig. 3). The ion at *m/z* 333 represents only a minor fragment, and the base ion has a *m/z* of 517, together with a prominent ion at *m/z* 535. The latter must be due to the loss of a methyl from a TMS group, according to *m/z* 559 in the mass spectrum of the perdeuterated derivative of M16. The fragment ion at *m/z* 535 loses water to yield *m/z* 517, but further fragmentation is not favorable. One possible explanation is the location of the newly introduced hydroxyl group at C-5, which would enable the interaction of the respective TMS group with the C-7 keto group to generate another six-membered ring system. This would block the C-7 keto function for the McLafferty rearrangement and explain the low intensity of *m/z* 333. A trimethylsilyloxy group at C-5 could also form a six-membered ring with the ester group, explaining the fact that an ion reflecting the loss of water directly from the molecular ion is absent in the mass spectrum of M16 (Fig. 3). This is in distinct contrast to the mass spectrum of ZEN itself (Figs. 3 and 4). However, the conclusive assignment of the chemical structure of 5-hydroxy-ZEN to M16 must await comparison with an authentic reference compound.

The mass spectrum of M17 exhibited a low intensity molecular ion at *m/z* 536 and a base ion at *m/z* 446, followed by major ions at *m/z* 431 and 333 (Fig. 3). The molecular ion, its loss of 90 amu, and the corresponding ions in the mass spectrum of the perdeuterated derivative are consistent with the structure of zearalenol (Fig. 1), which can easily eliminate trimethylsilanol (90 amu) from the aliphatic ring. Comparison of the mass spectra and GC retention times of the trimethylsilylated reference compounds α- and β-zearalenol clearly showed that M17 was α-zearalenol. Likewise, the mass spectrum and GC retention time of the ZEN metabolite M14 were identical with that of β-zearalenol (data not shown).

GC-MS analyses were also conducted with the minor and more polar ZEN metabolites M5–M14. None of them had identical retention times in HPLC and GC nor identical mass spectra with authentic α- and β-zearalanol, which were also available as standards. The mass spectra of M8, M9, M10, M11, M12 and M13 exhibited molecular ions at *m/z* 550, suggesting monohydroxylated metabolites. Moreover, fragments similar to those of M15 and M16 were observed in the mass spectra. However, due to the small amounts and partly incomplete separation in HPLC and GC, the mass spectra are not yet suitable for publication. The mass spectra of M5, M6, and M7 exhibited a molecular ion at *m/z* 624 and two subsequent eliminations of 90 amu, suggesting monohydroxylated metabolites of zearalenol. More work is needed to purify and characterize these minor metabolites of ZEN.

4 Concluding remarks

Our study on the *in vitro* metabolism of ZEN has clearly shown that this mycoestrogen is prone to the formation of several oxidative metabolites. The major product could be tentatively identified as 5-hydroxy-ZEN from its mass spectrum, whereas the chemical structures of seven other monohydroxylated ZEN metabolites remain unknown. Isolation of sufficient amounts of these products for NMR spectroscopy, together with the chemical synthesis of authentic reference compounds, will be needed for structure elucidation. To date, only α- and β-zearalenol have

been reported in the literature as metabolites of ZEN in several animal species *in vitro* and *in vivo*, together with small amounts of their further reduced metabolites α- and β-zearalanol [2–12]. Hydroxylated ZEN, *i. e.*, two isomers each of 5- and 10-hydroxy-ZEN, have thus far been solely demonstrated as minor products in cultures of *Fusarium* and other fungi, but not as mammalian metabolites [18–21]. It is now important to clarify whether oxidative ZEN metabolites are also formed *in vivo*, and to elucidate their chemical structures and biological activities. The reductive metabolites α-zearalenol and -zearalanol have long been known to be stronger estrogens than ZEN, and their pronounced formation in pigs may explain the high susceptibility of this animal species to the hormonal effects of ZEN-contaminated feed [22, 23]. Likewise, it is conceivable that compounds with increased estrogenicity are among the monohydroxylated ZEN metabolites reported here.

This study was supported by the State of Baden-Württemberg (Research Program "Mycotoxins" as part of the State Research Initiative "Food and Health"). We thank Doris Honig for conducting the GC-MS analyses.

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