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Research Article

Oxidative *in vitro* metabolism of the *Alternaria* toxins altenuene and isoaltenuene

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The mycotoxins altenuene (ALT) and isoaltenuene (iALT) frequently occur in food and feed items infested by fungi of the genus *Alternaria*, but nothing is known about their oxidative metabolism in mammals. We have therefore incubated ALT and iALT with microsomes from rat liver in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system and analyzed the extracted metabolites with HPLC and GC-MS after trimethylsilylation. Both toxins formed a major metabolite, which was tentatively identified as the 8-hydroxylation product by GC-MS analysis and by its methylation by the enzyme catechol-*O*-methyltransferase. Three minor metabolites were tentatively identified as 10-hydroxy- and two stereoisomers of 4-hydroxy-ALT and –iALT. The same metabolic pattern was observed in microsomes from different rat strains and from pigs and humans. Moreover, incubation of ALT with rat liver slices provided evidence that the same oxidative metabolites were formed under *in vivo*-like conditions. Thus, ALT and iALT exhibit a considerable propensity for undergoing metabolic hydroxylation reactions, and the toxicological properties of the oxidative metabolites should now be studied.

Keywords: Altenuene / Alternaria toxins / Isoaltenuene / Microsomes / Mycotoxin

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1 Introduction

Fungi of the genus *Alternaria* grow at moderate temperatures on numerous food items and other materials, *e.g.*, soil, wallpapers, and textiles [1–3]. Their toxins are frequently detected in moldy wheat and other grains, and in various fruits and processed fruit products such as apple, tomato, and grape juice and also in red and white wine [1, 4, 5]. The major *Alternaria* toxins are depicted in Fig. 1.

Despite their wide-spread occurrence in food and feed items, little is known about the toxicology and fate of these toxins in the mammalian organism. Consumption of food contaminated with *Alternaria* toxins has been associated with an increased incidence of esophageal cancer [6–8]. Research conducted in our laboratory has recently disclosed that alternariol (AOH) is mutagenic and clastogenic in cultured mammalian cells [9, 10], and AOH and alternariol methyl ether (AME) have been reported to inhibit topoisomerase I and IIa under cell-free conditions [11]. Moreover, an extensive oxidative metabolism of AOH and AME was demonstrated with rat, pig and human liver microsomes *in vitro* [12]. In contrast, nothing is known about the oxidative metabolism of the *Alternaria* toxins altenuene (ALT) and isoaltenuene (iALT) to date. We report here that four monohydroxylated metabolites are formed from ALT and iALT in rat hepatic microsomes. The chemical structures of these novel metabolites were tentatively elucidated by GC-MS analysis and enzymatic derivatization. The same metabolites were detected in microsomes from pig and human liver. Moreover, when ALT was incubated with precision-cut rat liver slices, which are much closer to the *in vivo* situation than microsomes, the formation of the same oxidative metabolites could be demonstrated.

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Abbreviations: ALT, altenuene, (*2R,3R,4aR*)-2,3,7-trihydroxy-9-methoxy-4a-methyl-2,3,4,4a-tetrahydrobenzo[*c*]chromen-6-one; **AME,** alternariol methyl ether; **AOH,** alternariol; **COMT,** catechol-*O*-methyltransferase; **CYP,** cytochrome P450; **EI,** electron impact; **iALT,** isoaltenuene, (*2R,3R,4aS*)-2,3,7-trihydroxy-9-methoxy-4a-methyl-2,3,4,4a-tetrahydrobenzo[*c*]chromen-6-one; **NADP(H),** nicotinamide adenine dinucleotide phosphate (reduced form); **SAM,** *S*-adenosyl-L-methionine; **TMS,** trimethylsilyl

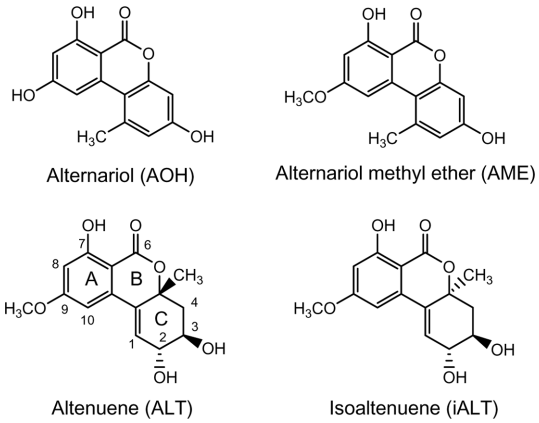

Figure 1. Chemical structures of major *Alternaria* toxins.

2 Materials and methods

2.1 Chemicals, animals, and cell fractions

ALT and iALT were synthesized in the laboratory of J. Podlech as previously reported [13] and had a purity of >96% according to HPLC analysis. *S*-Adenosyl-L-methionine (SAM), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and other chemicals and reagents were of the highest quality available and were purchased from Sigma/Aldrich/Fluka (Taufkirchen, Germany). HPLC grade ACN was from Carl Roth (Karlsruhe, Germany).

Male Wistar rats and male and female Sprague–Dawley 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 male Wistar rats 5 days prior to their sacrifice. Microsomes were prepared from the livers of male and female rats and of adult female pigs immediately after slaughter, as well as from the liver of a 63-year-old male Caucasian (kindly provided by Dr. J. Weymann, former Knoll AG, Ludwigshafen, Germany) as described by Lake [14]. Protein concentrations were measured according to Bradford [15] with BSA as standard. The concentration of active cytochrome P450 (CYP) was determined according to Omura and Sato [16]. Rat liver cytosol, obtained as the 100 000 × *g* supernatant of the microsomal preparation, was used as source of catechol-*O*-methyltransferase (COMT).

2.2 Microsomal incubations

Microsomal incubations contained 50 μM ALT or iALT dissolved in DMSO (final DMSO concentration 0.5%), 1 mg of microsomal protein and a NADPH-generating 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 toxins with the microsomes for 5 min at 37°C, the NADPH-generating system was added and the mixture incubated for 40 min at 37°C. The incubation mixture was then 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-generating system or the toxin was omitted.

2.3 Incubations with COMT

Oxidative metabolites of ALT and iALT, extracted from the microsomal incubations and fractionated by HPLC (see below), were dissolved in DMSO and added to 250 μL of 0.1 M phosphate buffer pH 7.4 containing 0.1 M magnesium chloride and 10 μL rat liver cytosol from male Sprague–Dawley rats. The concentration of DMSO in the final incubation did not exceed 1%. After 5 min incubation at 37°C, 7 μL of a 20 mM solution of SAM in phosphate buffer was added and the incubation continued for another 30 min. Control incubations were run without SAM. The aqueous incubation mixtures were then extracted with ethyl acetate and processed as described for the microsomal incubations.

2.4 Incubations with liver slices

Precision-cut slices were prepared from the liver of untreated female Sprague–Dawley rats as previously described [17]. Four liver slices were separately incubated with 200 μM ALT for 24 h at 37°C in 1.7 mL of Waymouth's medium according to the method of Fisher *et al*. [18]. The incubation media were then separated from the slices, mixed with 17 μL of a 5 mM solution of 4,4′-isopropylidenebis(2,6-dimethylphenol) in DMSO as standard, and stored at −80°C. For analysis, 200 μL aliquots were extracted with 2 × 500 μL ethyl acetate and the extracts analyzed for unconjugated metabolites by HPLC. Other 200 μL aliquots were used to determine glucuronides and sulfates: after adding 200 μL of 0.15 M acetate puffer pH 5.0 and 1000 U of β-glucuronidase type B-1 from bovine liver and/or 0.1 U of sulfatase type IV from *Acetobacter aerogenes*, the mixtures were kept at 37°C for 2 h, and the aglycones were subsequently extracted with 2 × 500 μL ethyl acetate. The yield of the extraction procedure was determined with ALT, iALT and the standard, and found to exceed 95% for all three compounds. The relative amounts of parent and hydroxylated compounds present as unconjugated material or as glucuronides and sulfates in the medium were estimated after HPLC analysis from the peak areas, assuming the same extraction yield and absorbance for the oxidative metabolites as determined for the parent compounds. The standard 4,4′-isopropylidene-

bis(2,6-dimethylphenol) was used as a marker compound to ensure error-free extraction procedures.

2.5 HPLC analysis

A Beckman system equipped with a binary pump, a photodiode array detector and 32 Karat 7.0 software for data collection and analysis was used. Separation was carried out on a 250×4.6 mm^2 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 2 min after injection, changing from 17% B to 45% B in 10 min, then to 50% B in 10 min, then to 100% B in 5 min. After 5 min of eluting the column with 100% B, the initial 17% B were reached in 3 min. The flow rate was 1 mL/min and the detector was set to 254 nm. HPLC fractions of the metabolites were collected, extracted with ethyl acetate, and used for GC-MS analysis or for incubations with COMT.

2.6 GC-MS analysis

A Finnigan GCQ capillary gas chromatograph equipped with a $30\ m \times 0.25$ 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, and 1 µL was injected, using the splitless mode for 90 s. The injection port temperature was 50°C at the time of injection and, after 30 s, raised to 275°C at 8°C/min. The oven temperature was programmed from 60°C (1 min hold) to 290°C (15 min hold) at a rate of 15°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 650 at a rate of 0.5 s/scan.

3 Results and discussion

3.1 Oxidative metabolites and their tentative chemical structures

Hepatic microsomes from aroclor-induced male Wistar rats were incubated with ALT or iALT in the presence of a NADPH-regenerating system and the incubations subsequently extracted with ethyl acetate. Control incubations without NADPH had shown that more than 95% of the parent mycotoxins were recovered under these conditions. The extracts from the complete incubations were then analyzed by HPLC, using conditions very similar to those yielding a good separation of the hydroxylation products of AOH and AME in an earlier study [12]. The HPLC profiles of the extracts are depicted in Fig. 2.

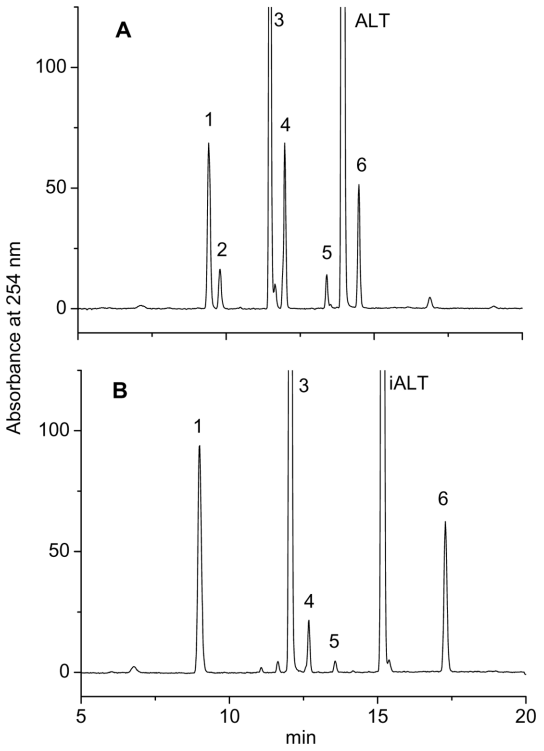

Figure 2. HPLC profiles of the extracts from the incubations of ALT (A) and iALT (B) with NADPH-fortified rat liver microsomes. In addition to the parent compounds, six oxidative metabolites (peaks 1 – 6) are present in each extract.

With ALT, the formation of six new products (peaks 1 – 6 in Fig. 2A) was observed which were not generated in the control incubations without NADPH. About 41% of ALT was converted to metabolites. With iALT, 53% was metabolized in the microsomal incubation and five new peaks were formed (Fig. 2B). Because of the similarities in relative HPLC retention times and amounts of metabolites formed, it was suspected that ALT and iALT followed the same metabolic routes, and the corresponding HPLC peaks were allocated the same numbers. Thus, peak 3 was the major metabolite with both mycotoxins, followed by peak 1. The only difference between the HPLC profiles of ALT and iALT was the lack of peak 2 for iALT.

For tentative structure elucidation of the metabolites of ALT and iALT, HPLC peaks 1 – 6 were collected separately. An aliquot of each fraction was rechromatographed by HPLC to ensure that the compounds were unchanged. The other aliquots were analyzed by GC-MS after trimethylsilylation. The EI mass spectra of ALT and of the ALT metabolites 1 and 3 are depicted in Fig. 3.

The mass spectrum of ALT containing three trimethylsilyl (TMS) groups exhibits a very small molecular ion at *m/z* 508 and a base peak at *m/z* 377 (Fig. 3). Other characteristic ions are at *m/z* 392, 403, and 493. The proposed fragmentations leading to these ions are depicted in Fig. 4.

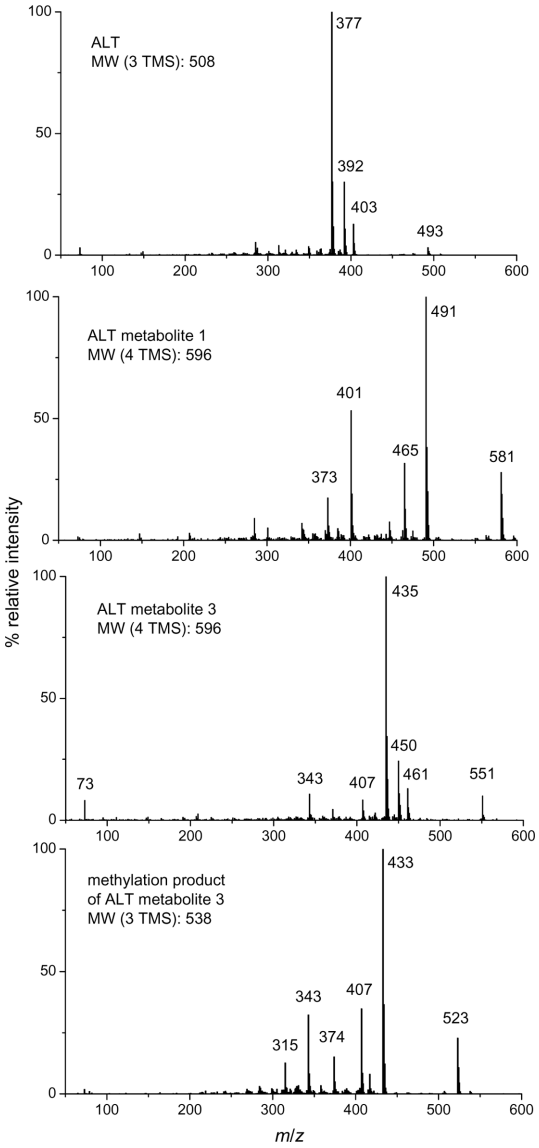

Figure 3. EI mass spectra of ALT and major ALT metabolites after trimethylsilylation.

The ion at *m/z* 493 can best 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 group in *ortho*-position to a phenolic group, such as AOH and AME [12] and gingerol [19, 20]. The peak at *m/z* 392 is probably generated by a retro Diels–Alder reaction of ring C with the concomitant loss of 116 amu (Fig. 4). The same reaction of the ion at *m/z* 493, or the loss of methyl from *m/z* 392 give rise to the base ion at *m/z* 377. Finally, the ion at *m/z* 403 is proposed to arise from *m/z* 493 through the elimination of trimethylsilanol (Fig. 4), which is a common fragmentation of aliphatic alcohols after trimethylsilylation [19, 20].

The mass spectrum of ALT metabolite 1 after trimethylsilylation exhibits its highest ion at *m/z* 581 (Fig. 3).

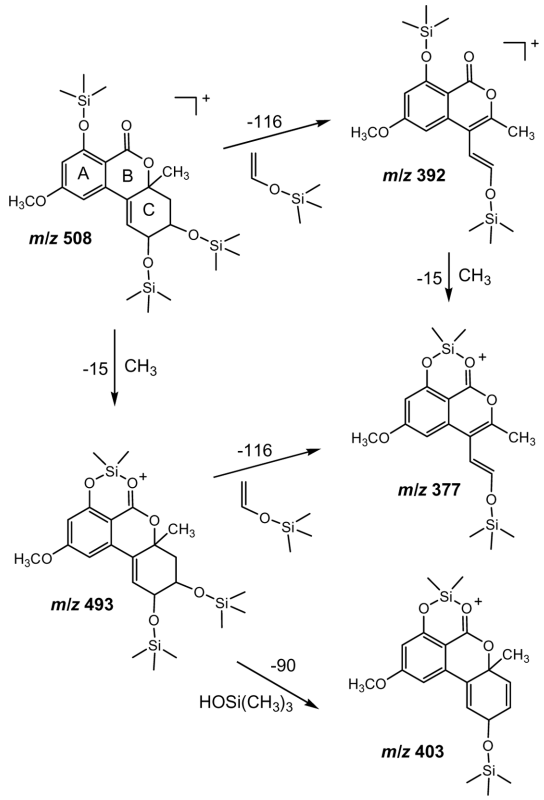

Figure 4. Proposed fragmentation of trimethylsilylated ALT after EI ionization.

Assuming that this is a fragment ion arising through the loss of one methyl group, the molecular ion should be *m/z* 596. In contrast to the fragmentation of ALT, which is dominated by the retro Diels–Alder reaction, the mass spectrum of metabolite 1 exhibits two subsequent eliminations of trimethylsilanol (90 amu) from the ion at *m/z* 581, leading to the base ion at *m/z* 491 and another prominent ion at *m/z* 401 (Fig. 3). This suggests that the newly introduced hydroxyl group is located at an aliphatic position. Because the mass spectrum of ALT metabolite 2 is virtually identical with that of metabolite 1 (data not shown), we propose that metabolites 1 and 2 are the two stereoisomers of 4-hydroxy-ALT.

The mass spectra of the ALT metabolites 3 contains characteristic ions at *m/z* 435, 450, 461, and 551 (Fig. 3). The pattern of these ions resembles that observed in the mass spectrum of ALT, with a shift of 58 amu. Monohydroxylation of ALT at C-8 or C-10 could explain these mass spectra. The presence of a trimethylsilyloxyl group in *ortho*-position to the methoxyl group at C-9 allows a new fragmentation, *i.e.*, the elimination of ethane and formation of a six-membered dimethylsiloxane ring, as exemplified in Fig. 5 for 8- and 10-hydroxy-ALT. This type of fragmentation has been observed before in the mass spectra of analogous metabolites of AME and gingerol [12, 20]. The elimi-

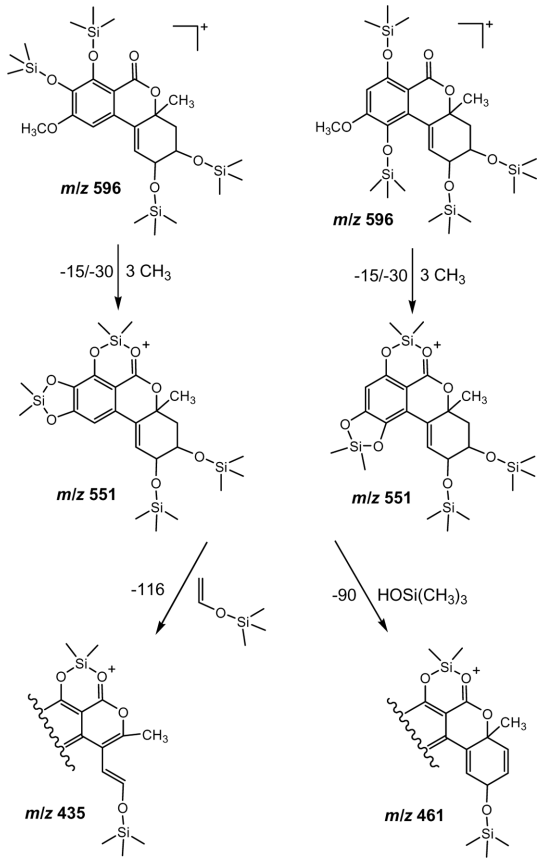

Figure 5. Proposed fragmentation of trimethylsilylated 8-hydroxy-ALT and 10-hydroxy-ALT after EI ionization.

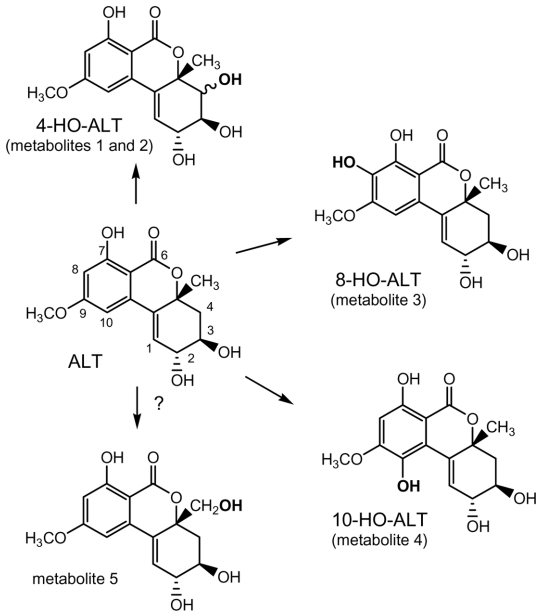

Figure 6. Proposed pathways in the oxidative metabolism of ALT.

nation of ethane, together with the loss of methyl from the 7-trimethylsiloxyl group, would account for the ion at *m/z* 551. This ion still has the same ring C as ALT and can either undergo retro Diels – Alder reaction to yield the base ion at *m/z* 435, or eliminate trimethylsilanol to yield *m/z* 461. The ion at *m/z* 450 could arise by retro Diels – Alder reaction from the ion at *m/z* 566, as shown in Fig. 5. The same fragmentations must be expected in the mass spectrum of 10-hydroxy-ALT (Fig. 5). Indeed, the mass spectrum of the ALT metabolite 4 was found to have a mass spectrum virtually identical with that of metabolite 3 (data not shown).

Because MS could not distinguish the two monohydroxylation products of ALT at the aromatic ring, metabolites 3 and 4 were subjected to methylation by COMT. This enzyme converts catechols to their monomethyl ethers. When ALT metabolites 3 and 4 were incubated with COMT in the presence of SAM and the extract of the incubations analyzed by HPLC, a methylation product was only obtained with metabolite 3, whereas metabolite 4 did not react with COMT. Therefore, it must be tentatively concluded that metabolite 3 is the catechol 8-hydroxy-ALT whereas metabolite 4 is the hydroquinone 10-hydroxy-ALT.

The mass spectrum of the methylation product of metabolite 3 after trimethylsilylation (Fig. 3) strongly suggested the structure of 8-methoxy-ALT because of the loss of 15 amu (from the TMSO group at C-7, leading to *m/z* 523) and the lack of elimination of 30 amu, which would have been expected for the isomeric methyl ether 7-*O*-methyl-8-hydroxy-ALT with a TMSO group between two methoxyl groups (see discussion of mass spectra above). Other ions in the spectrum of 8-methoxy-ALT (Fig. 3) arise from *m/z* 523 through elimination of trimethylsilanol (base ion at *m/z* 433) or retro Diels – Alder reaction (*m/z* 407). The formation of only one methyl ether from 8-hydroxy-ALT by COMT is probably due to the hydrogen bonding of the hydroxyl group at C-7 with the neighboring carbonyl group, as discussed earlier.

GC-MS analysis of the collected HPLC fractions of metabolites 5 and 6 did not lead to convincing proposals for their chemical structures so far. In analogy with the metabolism of AOH and AME [12], it is suspected that metabolite 5 is monohydroxylated at the methyl group and metabolite 6 may be a dimer of ALT generated as an artifact in the microsomal incubations, because this compound was not formed in liver slices (see Section 3.3).

The tentative structures of the ALT metabolites 1 – 5 are arranged in a metabolic scheme in Fig. 6. Thus, the major microsomal metabolite is proposed to be the catechol 8-hydroxy-ALT, whereas the hydroquinone 10-hydroxy-ALT and the stereoisomers of 4-hydroxy-ALT are formed in much smaller amounts (see also Fig. 2A).

The same GC-MS analysis and reaction with COMT as described above for the ALT metabolites were conducted

Table 1. EI mass spectra of iALT and its oxidative metabolites after trimethylsilylation

HPLC peak[a]	Derived structure	*m/z* (relative intensity)
iALT	iALT	493 (24), 403 (49), 392 (20), 377 (100)
1[b]	4-Hydroxy-iALT	581 (30), 491 (96), 465 (10), 401 (100), 373 (23)
3	8-Hydroxy-iALT	551 (24), 461 (67), 450 (17), 435 (100), 407 (5)
4	10-Hydroxy-iALT	551 (22), 461 (70), 450 (18), 435 (100), 407 (3)

a) see Fig. 2b.
b) Two stereoisomers with identical mass spectra were found in HPLC fraction 1 upon GC-MS analysis.

with the microsomal metabolites of iALT (Fig. 2B). The mass spectra of the iALT metabolites, which are listed in Table 1, closely resembled those of the corresponding ALT metabolites, indicating the same metabolic routes. The major microsomal metabolite of iALT was the catechol 8-hydroxy-iALT, but hydroxylation at C-10 and C-4 also took place. Two isomers of 4-hydroxy-iALT were detected upon GC-MS analysis in HPLC fraction 1, suggesting that both stereoisomers of this metabolite were formed in microsomes and could be separated in GC, but not HPLC, due to their diastereomeric nature.

3.2 Comparison of various rat strains and other species

The pattern of oxidative ALT and iALT metabolites discussed above has been obtained with hepatic microsomes from aroclor-treated male Wistar rats. Aroclor is a mixture of numerous polychlorinated biphenyls and often used to induce a broad spectrum of CYPs in experimental animals. When ALT and iALT were incubated with liver microsomes from untreated male and female Sprague–Dawley rats under identical conditions of time and concentrations of microsomal protein and substrate, a lower extent of metabolic conversion was observed, which is probably due, at least in part, to the lower P450 content of the noninduced livers (Table 2). The pattern of oxidative metabolites of ALT and iALT was quite similar among the rat liver microsomes, with major amounts of the 8-hydroxylated product and small amounts of the 4- and 10-hydroxy metabolites (Fig. 7). No significant difference concerning the extent of metabolic oxidation or the metabolic pattern was found between male and female Sprague–Dawley rats. In all rat strains, iALT appeared to be a better substrate for oxidative metabolism than ALT (Table 2).

The preferential hydroxylation of iALT was also observed with hepatic microsomes from pig and human liver; in particular, microsomes from human liver exhibited a high activity for the oxidative biotransformation of iALT despite their low CYP content, whereas hydroxylation of ALT was more than fivefold lower (Table 2). 8-Hydroxy-iALT constituted nearly 100% of the oxidative metabolites, together with traces of 4-hydroxy-iALT (Fig. 7). The reasons for the high propensity of iALT for hydroxylation in human liver are presently under investigation in our laboratory.

Table 2. Extent of hydroxylation of ALT and iALT by hepatic microsomes from various species and rat strains under identical conditions[a]

Origin of microsomes	CYP content[b]	Metabolic conversion (%)	
		ALT	iALT
Aroclor-induced male Wistar rat	1.94	41.4 ± 0.8	53.0 ± 0.6
Male Sprague–Dawley rat	0.76	24.6 ± 6.3	34.6 ± 4.9
Female Sprague–Dawley rat	0.42	23.5 ± 0.1	29.3 ± 1.3
Female pig	0.39	5.8 ± 0.1	10.0 ± 0.1
Male human	0.20	8.0 ± 1.5	42.2 ± 2.4

Data represent percent of parent compound converted to oxidative metabolites and are from two independent experiments.
a) All incubations contained 1 mg of microsomal protein and were conducted with 50 μM substrate for 40 min at 37°C (see Section 2).
b) nmol CYP *per* mg microsomal protein.

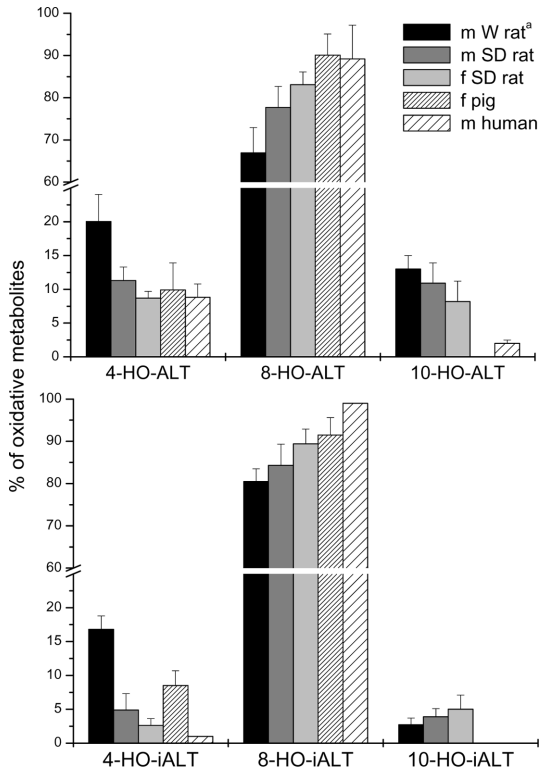

Figure 7. Patterns of oxidative ALT metabolites in hepatic microsomes of different rat strains and other species. Data represent mean ± SD of two independent experiments. [a]Aroclor-induced. The SDs for 4-HO-iALT and 8-HO-iALT formed in human microsomes were too small to be visible in the chart.

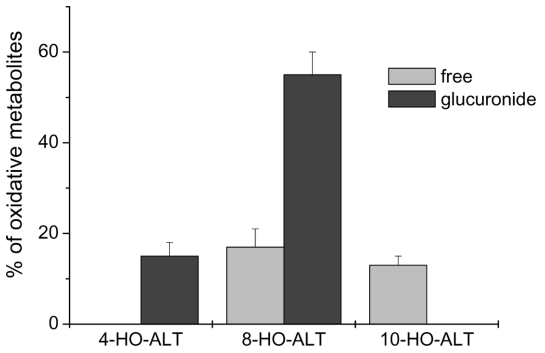

Figure 8. Free and conjugated oxidative metabolites of ALT formed in liver slices from female Sprague – Dawley rat. Data represent mean ± SD of four slices.

3.3 Oxidative metabolites of ALT in rat liver slices

Although hepatic microsomes from all rat strains and other species studied so far exhibit the capability to hydroxylate ALT and iALT, it remains to be shown whether these oxidative metabolites are formed under *in vivo* conditions because of the competition of phase II metabolism leading to glucuronides and sulfates. Therefore, preliminary experiments were conducted with ALT in precision-cut liver slices, which are much closer to the *in vivo* situation than microsomes because they represent intact liver cells in the natural tissue architecture and exhibit metabolic activities and excretory functions for prolonged periods of time [21]. When ALT was incubated with liver slices from a female Sprague – Dawley rat for 24 h incubation and the incubation medium extracted prior to and after treatment with β-glucuronidase and sulfatase, the extract contained a total of 88.4% ALT (38.6% unconjugated and 49.8% as glucuronide) and 11.6% oxidative metabolites. The lower conversion of ALT to oxidative metabolites in rat liver slices (11.6%) as compared with liver microsomes of the same rat strain and gender (23.5%, see Table 2) may reflect the competition by glucuronidation in slices but not in microsomes, in addition to differences in the substrate to CYP ratio between the two metabolic systems.

The major oxidative metabolite of ALT in rat liver slices was 8-hydroxy-ALT, about 75% of which was glucuronidated, whereas 4-hydroxy-ALT and 10-hydroxy-ALT were found as minor metabolites (Fig. 8). The observation that 10-hydroxy-ALT does not form a glucuronide remains to be confirmed, because control experiments showed that this metabolite is not stable enough to survive the enzymatic hydrolysis of the glucuronides. Formation of sulfates was not observed, nor was a methylation product of 8-hydroxy-ALT detected; the latter suggests that 8-hydroxy-ALT is a poor substrate of COMT. These results were confirmed with liver slices from another female Sprague – Dawley rat. Despite the preliminary nature of the liver slice study, the results strongly suggest that oxidative metabolism of ALT may also occur *in vivo*.

4 Concluding remarks

Our study on the *in vitro* metabolism of ALT and iALT has clearly shown that these mycotoxins are prone to the formation of several oxidative metabolites. The major product of both toxins, formed by hepatic microsomes from rats, pigs, and humans, could be tentatively identified as 8-hydroxy-derivative from its mass spectrum and its methylation by the enzyme COMT. Because of their catechol structure, the 8-hydroxy-metabolites of ALT and iALT may be of toxicological relevance, and further investigations should clarify their genotoxic potential and metabolic fate. The other product of aromatic hydroxylation, *i.e.*, the 10-hydroxy-metabolite of ALT and iALT, may also be of toxicological importance, because it has a hydroquinone structure and appears to be chemically unstable in aqueous solution.

Although the chemical structures proposed for the oxidative metabolites of ALT and iALT in this study are most likely correct due to the specificity of the mass spectrometric fragmentation and of the methylation reaction with COMT, final structural confirmation is needed either by NMR spectroscopy or synthetic reference compounds. Whereas the isolation of the metabolites from the biological matrices in sufficient amounts and purity for NMR spectroscopy is very difficult and time-consuming, the synthesis of standards has the additional advantage of providing sufficient quantities of the metabolites for toxicological testing. Therefore, efforts to chemically synthesize the major oxidative metabolites of ALT and iALT are under way in our laboratory.

Taken together, we have shown that ALT and iALT share the propensity for oxidative metabolism recently reported for the other *Alternaria* toxins AOH and AME [12]. Future studies are warranted to clarify the role of oxidative metabolites in the assessment of the health risk posed by these mycotoxins.

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The authors have declared no conflict of interest.

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