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Review

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Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants

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Supporting Information

ABSTRACT: Modern analytical techniques can determine a multitude of fungal metabolites contaminating food and feed. In addition to known mycotoxins, for which maximum levels in food are enforced, also currently unregulated, so-called "emerging mycotoxins" were shown to occur frequently in agricultural products. The aim of this review is to critically discuss the relevance of selected emerging mycotoxins to food and feed safety. Acute and chronic toxicity as well as occurrence data are presented for enniatins, beauvericin, moniliformin, fusaproliferin, fusaric acid, culmorin, butenolide, sterigmatocystin, emodin, mycophenolic acid, alternariol, alternariol monomethyl ether, and tenuazonic acid. By far not all of the detected compounds are toxicologically relevant at their naturally occurring levels and are therefore of little or no health concern to consumers. Still, gaps in knowledge have been identified for several compounds. These gaps should be closed by the scientific community in the coming years to allow a proper risk assessment.

KEYWORDS: *fungal metabolites, toxicity, risk assessment, Fusarium, Penicillium, Aspergillus, Alternaria*

INTRODUCTION

Filamentous fungi show a remarkable potential to produce secondary metabolites. Although the exact number of fungal metabolites is unknown, a significant fraction of the ~170,000 currently known natural products[1] are of fungal origin, and it can be assumed that many more compounds are yet to be discovered. Fungal metabolites include important pharmaceuticals such as penicillin or statins, potent poisons such as aflatoxins or trichothecenes, and metabolites that are both toxic and pharmaceutically useful, such as the ergot alkaloids.[2] As fungi can grow on basically any organic material, also food and feed are regularly contaminated with fungal metabolites. Poisonous fungal metabolites—commonly referred to as mycotoxins—are of major importance to food and feed safety. One of the best definitions of mycotoxins states that they "are natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route".[3] In addition to animal toxicity, mycotoxins may exert phytotoxic or antimicrobial effects. A variety of fungi are known to produce mycotoxins, including *Aspergillus*, *Fusarium*, and *Penicillium* species.[4] The most important classes of mycotoxins[5] include the highly carcinogenic aflatoxins (e.g., aflatoxin B_1, AfB1),[6] trichothecenes (e.g., deoxynivalenol, DON),[7] fumonisins (e.g., fumonisin B_1, FB1),[8] ochratoxin A (OTA),[9] and zearalenone (ZEN).[10] These toxins and several others are regulated in many countries of the world[11] after thorough risk assessment, taking into account toxicity, occurrence, and consumption data as well as economic and political considerations.

Sound analytical methods are key to ensuring food safety, and dozens of new methods are developed and published each year.[12,13] Of those, liquid chromatography−mass spectrometry (LC-MS) based methods are becoming more and more popular, as they allow the sensitive simultaneous determination of multiple fungal metabolites in many matrices. These methods also sometimes reveal "surprising" findings, such as known mycotoxins in untypical matrices (e.g., fumonisin B_2 in grapes[14]) or in unusual geographical regions (e.g., aflatoxins in Europe[15]), for which global warming might partly be responsible.[16] LC-MS is of tremendous help in the discovery of new mycotoxins (e.g., NX-toxins[17]), as well as masked[18] or other modified[19] forms of mycotoxins. Moreover, "emerging mycotoxins", which are in the scope of this review, can occur in high frequency and sometimes also in high concentrations in cereals and in other food- and feedstuffs.

The term "emerging mycotoxins", although often used nowadays for certain fungal metabolites, is not clearly defined. One of the first papers to use this term was published in 2008 and deals with the *Fusarium* metabolites fusaproliferin (FP), beauvericin (BEA), enniatins (ENNs), and moniliformin (MON),[20] and the term has mostly been used for these compounds ever since. However, in a more recent paper, emerging mycotoxins were defined as "mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing".[21] According to this definition, many more fungal metabolites with known (or at least suspected) toxicity would fall in the category of emerging mycotoxins.

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The aim of this review is to critically assess the toxicity and occurrence of different fungal metabolites that are currently not regulated in food or feed. We deliberately chose the most "interesting" fungal compounds based on the frequency of occurrence and concentrations in food and feed, as well as current scientific interest. The following sections will deal with the *Fusarium* metabolites ENNs, BEA, MON, FP, fusaric acid (FA), culmorin (CUL), and butenolide (BUT), the *Aspergillus* metabolites sterigmatocystin (STE) and emodin (EMO), the *Penicillium* metabolite mycophenolic acid (MPA), and the *Alternaria* metabolites alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA).

FORMATION, TOXICITY, AND OCCURRENCE OF EMERGING MYCOTOXINS

Enniatins. ENNs are cyclic hexadepsipeptides that are produced by several *Fusarium* species, such as *F. avenaceum*, *F. oxysporum*, *F. poae*, or *F. tricinctum*, which grow mainly on cereals. In total, 29 species of ENNs are known.[22] ENNs A, A1, B (Figure 1), and B1 are most frequently detected in food and feed. The toxicity of ENNs is based on their ionophoric properties. They facilitate the transport of mono- or divalent cations such as K^+ or Ca^{2+} across membranes, thereby disrupting normal physiological concentrations of these ions.[23] Low micromolar concentrations of ENNs were shown to be cytotoxic to different cell lines[24−28] and to reduce the motility of boar spermatozoa.[29] Cell death was shown to be mediated by the induction of apoptosis[28,30,31] via the mitochondrial pathway[24,32] or by the induction of necrosis linked to lysosomal damage.[33,34] There are conflicting data on whether[35] or not[31] ENNs trigger the production of reactive oxygen species (ROS). Several studies found no genotoxic effect of ENNs.[30,31,34] Interestingly, ENN B1 and T-2 toxin showed an antagonistic toxic effect on cultured intestinal epithelial cells and intestinal explants, which indicates that ENN B1 down-modulates the gastrointestinal toxicity of T-2 toxin.[36]

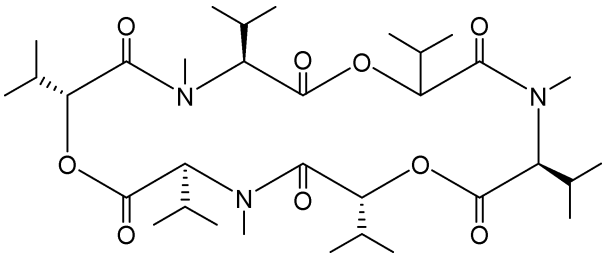

Figure 1. Chemical structure of enniatin B.

ENNs were shown to interact with proteins. In rat liver microsomes ENNs inhibited the activity of acyl-CoA:cholesterol acyltransferase.[37] ENN B was shown to bind calmodulin and to inhibit 3′,5′-cyclo-nucleotide phosphodiesterase.[38] ENNs furthermore interact with multidrug resistance ATP-binding cassette (ABC) transporters. The overexpression of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) counteracted the cytotoxic effect of ENNs on human cancer cell lines.[39] Ivanova and co-workers[40] found that two multidrug resistance ABC transporters facilitate the transport of ENN B1 across a monolayer of the human intestinal cell line CaCo-2 in the apical to basolateral direction. Furthermore, exposure to ENNs counteracted the protective effect of ABC transporters against cytotoxic compounds in yeast[41,42] and human cancer cell lines,[39] and it may consequently alter the bioavailability of pharmaceuticals in vivo.

Several studies investigated the in vivo toxicity of ENNs in rodents. Most of these studies show low toxicity. A single dose of 50 mg ENNs/kg body weight (BW) did not induce signs of toxicity in rats.[43] Oral administration of ENN A to mice at a dosage of 1 mg/kg BW/day for 6 days or subcutaneous (sc) administration of ENN A to mice at a dosage of 0.5 mg/kg BW/day for 6 days did not elicit a toxic effect.[44] A daily intraperitoneal (ip) ENN B dose of 5 mg/kg BW administered for 9 days did not affect BW, food intake, or behavior in mice.[45] In rats fed an ENN A contaminated diet (465 mg/kg feed, corresponding to 20.9 mg/kg BW/day) for 28 days, no effects on body and organ weight, histology of duodenum tissue, biochemical blood parameters,[46] or any visible signs of illness[47] were found. Although altered peripheral blood lymphocyte levels were detected, their consequences are yet unclear. In the sole study that showed toxic effect of ENNs, ip administration of 10−40 mg/kg BW at an interval of 8 h was lethal for immune-deficient mice within 2−5 days, whereas lower levels caused only weight loss.[48]

Two studies investigated the distribution of ENNs in different tissues of rats, and the highest levels were detected in jejunum, liver, and fat tissue.[45,46] These findings led the authors to suggest that the jejunum is a site of ENN absorption[46] and that ENNs primarily accumulate in fat-rich tissues owing to their lipophilic properties.[45] Dietary ENNs were also shown to accumulate in the meat, skin, and liver of broilers,[49] and traces of the toxin were detected in a high fraction of Finish egg samples.[50] ENNs were shown to cross the blood−brain barrier in mice.[51]

Bioavailability and toxicokinetics of ENN B were investigated in pigs.[52] The toxin had a high (∼91%) oral bioavailability and was rapidly absorbed, distributed, and eliminated. Hence, the authors suggested that rapid metabolization rather than low bioavailability may explain the low in vivo toxicity of ENNs. Several metabolites of ENNs have been reported. Monooxygenated, dioxygenated, and N-demethylated metabolites were detected in liver microsomes of rats, dogs, humans, and chickens.[53−55] The same metabolites were detected in liver, plasma, and egg samples of chickens[54] and in liver and colon samples of mice.[45]

A mixture of enniatins termed "fusafungine" is used as a nasal/oromucosal spray with antibiotic and anti-inflammatory properties.[56,57] However, in April 2016, the European Medicines Agency of the European Union recommended to revoke the authorization of fusafungine sprays "due to serious allergic reactions and limited evidence of benefit".[58]

ENNs were detected in 37, 68, and 76% of food (n = 4251), feed (n = 3640), and unprocessed grain (n = 2647) samples collected in Europe between 2000 and 2013.[59] Maximum reported concentrations in grains were 950, 2000, 18,300, and 5720 μg/kg for ENN A, ENN A1, ENN B, and ENN B1, respectively. Maximum reported concentrations in cereal-based food were, 42, 125, 832, and 980 μg/kg for ENN A, ENN A1, ENN B, and ENN B1, respectively. ENNs were detected in 96% of samples (n = 83) of feed and feed raw materials with median and maximum concentrations of 30 and 5441 μg/kg, respectively.[60] In a survey of Chinese medicinal herbs, ENN A, ENN A1, ENN B, and ENN B1 were detected in 8, 7, 12, and 7% of all analyzed samples (n = 60) with maximum concentrations of 355, 253, 291, and 40 μg/kg, respectively.[61]

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In summary, ENNs are toxic in vitro, but most in vivo data indicate no or only low toxicity. ENNs are frequently detected in food, feed, and grains, but feeding studies conducted with rodents indicated a high tolerance to dietary ENN levels 1 order of magnitude higher than the reported maximum levels. In their scientific opinion from 2014, the European Food Safety Authority (EFSA) concluded that acute exposure to ENNs is not a concern to human health, whereas no conclusion could be drawn with respect to chronic exposure due to the lack of relevant in vivo toxicity data.[59] Interestingly, effects of ENNs on the bioavailability of pharmaceuticals have been suggested by in vitro data and should be further clarified before any valid conclusions on that aspect can be drawn.

Beauvericin. BEA is a cyclic hexadepsipeptide (Figure 2), produced by several *Fusarium* species, such as *F. proliferatum*, *F. subglutinans*, *F. verticillioides*, or *F. oxysporum*. BEA is an ionophore; it transports mono- or divalent cations, for example, K^+ or Ca^{2+}, across membranes and thereby disrupts normal physiological concentrations of these ions.[62,63] Accordingly, low micromolar concentrations of BEA were shown to be cytotoxic to different cell lines in vitro.[25,26,31,64] Cell death was shown to be mediated by apoptosis[31,65−67] via the mitochondrial pathway[32,68] or by necrosis.[69] There are conflicting reports on whether[35,70] or not[31] oxidative stress is involved in BEA toxicity. BEA showed no mutagenicity in the Ames test[71] and intercalated into DNA only when applied at high concentrations (>100 μM).[31] However, it showed a mutagenic potential in the alkaline Comet assay.[72] Low micromolar BEA concentrations affected the development of immune cells in vitro. BEA disturbed the maturation process of dentritic cells and decreased the endocytosis ability of macrophages when applied during their differentiation process.[73] The toxin was furthermore shown to act as an enzyme inhibitor in liver microsomes. It inhibited cytochrome P450 enzymes[74] and acyl-CoA:cholesterol acyltransferase, the enzyme that causes the accumulation of cholesteryl ester in atherogenesis.[37] BEA was shown to interact with P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) in vitro.[39] The data suggest that BEA is both a substrate and an inhibitor of the investigated transporters. It was concluded that the presence of homologues of these transporters in the gastrointestinal tract may decrease the bioavailability of BEA. Furthermore, the inhibitory effect of BEA on these transporters may affect the bioavailability of pharmaceuticals.

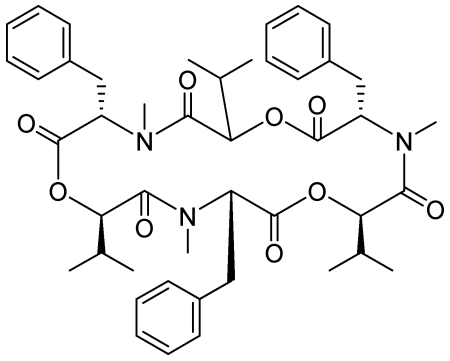

Figure 2. Chemical structure of beauvericin.

In vivo, toxicity and pharmacological behavior of BEA were studied in mice and poultry. A daily ip administration of 5 mg/kg BW for 9 days did not affect BW, food intake, or behavior in mice.[45] The LD_{50} values in mice were ≥100 and ≥10 mg/kg for oral and ip administration, respectively.[75] When the distribution of BEA in different tissues was investigated, the highest concentrations were detected in the liver and in fat tissue, suggesting an accumulation in fat-rich tissue due to the compound's lipophilic properties.[45] BEA was shown to cross the blood−brain barrier in mice.[51] The effect of chronic dietary exposure to BEA was investigated in poultry. In turkey and broiler feeding trials, diets contaminated with ≤2.5 and ≤12 mg/kg BEA, respectively, and cocontaminated with other *Fusarium* mycotoxins, had no effect on performance parameters, biochemical blood parameters, or meat quality.[76−79] However, increased heart weight was detected in one broiler trial.[76] Dietary exposure of broilers and laying hens to 10 and 9 mg/kg BEA, respectively, did not affect growth, feed uptake, or egg production.[49] Surveys of poultry products in Finland found trace amounts of BEA in a high fraction of analyzed egg samples and in a low fraction of analyzed meat and liver samples.[50,80] The authors suggested that BEA accumulates in egg yolk owing to its lipophilicity.

BEA was detected in 20, 21, and 54% of food (n = 732), feed (n = 861), and unprocessed grain (n = 554) samples collected in Europe between 2000 and 2013.[59] Maximum reported concentrations for BEA in grains and in cereal-based food were 6400 and 844 μg/kg, respectively. BEA was detected in 98% of samples (n = 83) of feed and feed raw materials with median and maximum concentrations of 6.7 and 2330 μg/kg, respectively.[60] In a survey of Chinese medicinal herbs, BEA was detected in 20% of all analyzed samples (n = 60) with a maximum concentration of 125 μg/kg.[61]

In summary, BEA is—although toxic in vitro—not toxic to rodents and poultry in vivo. Dietary BEA concentrations applied in poultry feeding trials were comparable to maximum concentrations reported in feed and feed raw materials. In vivo toxicity data for other species are missing. BEA presumably accumulates in fat-rich tissue including eggs of laying hens. However, detected levels should be negligible concerning an acute risk to human consumers. In their scientific opinion from 2014, EFSA concluded that acute exposure to BEA is not a concern to human health, whereas no conclusion could be drawn with respect to chronic exposure due to the lack of relevant in vivo toxicity data.[59] Effects of BEA on components of the immune system and on the bioavailability of pharmaceuticals were suggested by in vitro studies and should be investigated in vivo.

Moniliformin. MON (1-hydroxycyclobut-1-ene-3,4-dione; semisquaric acid; Figure 3) is a small, water-soluble molecule originally isolated from a *Fusarium* strain, initially called *F. moniliforme*, in the 1970s.[81] MON is among the most acidic organic acids and therefore occurs in nature typically as sodium or potassium salt. It is produced by a large variety of *Fusarium* spp. (e.g., *F. proliferatum*,[82] *F. subglutinans*,[83] *F. avenaceum*,[82] *F. tricinctum*,[84] *F. fujikuroi*,[85] *F. nygamai*,[86] *F. pseudonygamai*,[87] *F. temperatum*,[88] or *F. thapsinum*[87]) and only recently has been shown to be a metabolite of *Penicillium melanoconidium*.[89]

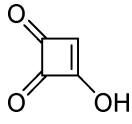

Figure 3. Chemical structure of moniliformin.

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The primary mode of action of MON seems to be the inhibition of thiamin pyrophosphatase dependent enzymes, which compromises the tricarboxylic acid cycle. Soon after the discovery of impaired oxidation of pyruvate and α-ketoglutarate by MON,[90] inhibition of pyruvate dehydrogenase was demonstrated in vitro.[91,92] Later, several enzymes sharing thiamin as common cofactor (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, pyruvate decarboxylase, and acetohydroxy acid synthase) have been shown to be inhibited by MON.[93] The authors concluded that MON meets the criteria for an active site-directed, irreversible affinity label for enzymes utilizing thiamin and that the inhibition of pyruvate dehydrogenase is cofactor-directed and likely caused by the structural similarity of MON and pyruvate.

MON was shown to by phytotoxic to wheat and corn, where it caused necrosis, affected growth regulation, and rendered leaves distorted.[81] Its toxicity on animal cells was tested extensively in vitro, and its effects are highly dependent on the used cell lines. For instance, MON showed no inhibitory effects on the proliferation of human white blood cell progenitors or human platelet progenitors, but was cytotoxic to human red blood cell progenitors with a half-maximal inhibitory concentration (IC_{50}) value of 4.1 μM.[94] After treatment of human hepatocellular carcinoma cells for 2 or 3 days with MON, IC_{50} values of 39.5 μg/mL (403 μM) and 26.8 μg/mL (273 μM) were recorded.[95] With regard to chicken primary cell cultures, MON was not toxic to chondrocytes and macrophages, but was toxic to splenocytes, cardiac (IC_{50} = 95 μM), and skeletal myocytes (IC_{50} = 42 μM).[96] Literature reports on genotoxicity are slightly ambiguous. MON did not show any activity in gene mutation assays with *Salmonella typhimurium* or *Escherichia coli* with or without metabolic activation.[97] However, the same authors elucidated that MON caused pronounced dose-dependent chromosomal aberration in primary rat hepatocytes. Compared to the control, a 9-fold increase was seen after 3 h of exposure to 1 μg/mL (10 μM). Previously, studies with 5−500 μM MON on the same cell type showed no genotoxic effects as measured with the unscheduled DNA synthesis test.[98] The most recent study that investigated genotoxic effects of MON used human peripheral blood lymphocytes.[99] Chromosomal aberrations, sister-chromatid exchanges, and micronucleus frequencies were significantly increased in a dose-dependent manner compared with the negative control after treatment of lymphocytes with MON concentrations between 2.5 and 25 μM.

MON shows even more severe effects in vivo. Incomplete uptake of the polar toxin through the cell membranes in cell culture studies may be a possible explanation for this discrepancy. LD_{50} values of MON have been reported as 4.0 mg/kg BW in 1-day-old cockerels (oral),[81] as 2.2−2.8 mg/kg BW in 9-month-old female mink (ip),[100] as 1.4 mg/kg BW in 7-week-old female broiler chickens intravenous (iv),[101] as 20.9 and 29.1 mg/kg BW in female and male mice (ip),[102] as 3.7 mg/kg BW in 7-day-old ducklings (oral),[103] and as 41.6 and 50.0 mg/kg BW in female and male rats (oral).[103] Besides damage to the heart muscle, including myocardial lesions and increased relative heart weights,[100,103−105] also muscular weakness,[103,104] respiratory distress,[103,104] decreased feed intake and BW gains,[101,105] and impaired immune function[104,106] were noted frequently. In summary, these studies highlight that poultry are the most affected species and that the heart is the main target organ in all investigated animals. Potential synergistic effects of the cardiotoxic MON with the co-occurring BEA and ENNs[107] or FB1[105,106] were disproved for the investigated conditions.

Occurrence of MON in cereals seems to be rather frequent in various regions of the world, albeit at rather low levels on average. For instance, 40% of 151 Finnish cereal samples collected in 2005 were positive for MON, with a mean level of 190 μg/kg and a maximum level of 850 μg/kg.[108] The toxin was also found in maize samples from all agroecological zones of Lesotho from the 2009/2010 and 2010/2011 seasons with levels reaching 1.2 mg/kg.[109] Furthermore, 93% of naturally contaminated maize samples from northwestern Italy (n = 108) collected in 2008, 2009, 2010, and 2011 showed detectable levels of MON with maximum concentrations of 2.6, 0.5, 0.9, and 0.4 mg/kg for the respective years.[110] MON was detected in 26 of 50 samples of poultry feed mixtures of Slovak origin in concentrations up to 1.2 mg/kg.[111]

Two excellent reviews compiled general information on MON[112] or data on the negative health effects caused by MON.[113] The latter paper stated that "MON per se does not pose a clear threat to human health at current levels", if an internal no observed adverse effect level (NOAEL) of 10 mg/kg BW/day was applied. However, a more recently published study indicated a severe impact of MON on the immune system of Sprague−Dawley rats and a lowest observed adverse effect level (LOAEL) value of 3 mg/kg BW.[114] Also, it was assumed a few years ago that MON "is not likely to be a genotoxic carcinogen",[113] which at least has to be questioned given the latest data,[99] and further studies should be conducted to answer this question. The European Commission Directorate-General for Health and Consumers requested a scientific opinion from EFSA about the risks for public health related to MON in feed and food,[115] which is due soon. Disregarding potential genotoxicity, current average contamination levels of cereals imply that the compound is likely not a problem for human health. Still, guidance levels for MON in poultry feed might be warranted to better protect animals from occasional high concentrations and to provide scientific support to poultry farmers.

Fusaproliferin. FP is a bicyclic sesterterpene (Figure 4). It is produced by *Fusarium* species such as *F. proliferatum*,

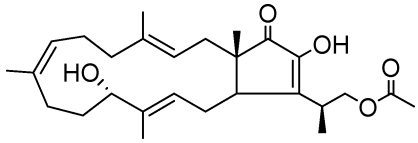

Figure 4. Chemical structure of fusaproliferin.

F. subglutinans, and *F. verticillioides*. FP was moderately cytotoxic to lepidopteran SF-9 cells and to human B lymphocyte IARC/LCL 171 cells. In the case of SF-9 cells, the CC_{50} values (concentration resulting in 50% cell viability) were 100 and 70 μM after 24 and 48 h of exposure, respectively.[116] Fornelli and co-workers found a CC_{50} value of >100 μM after 48 h of exposure.[25] For IARC/LCL 171 cells the CC_{50} values were 60−65 and 55 μM after exposure times of 24 and 48 h, respectively.[116] Moreover, exposure to 30 μM FP inhibited the proliferation of IARC/LCL 171 cells.[116] When the transport of FP across a monolayer of Caco-2 cells was investigated, 80−83% of FP that had been applied to the apical side was detected on the basolateral side after 4 h of incubation, which suggests a high bioavailability in vivo.[117]

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In vivo toxicity data are limited to brine shrimp (*Artemia salina*) larvae and chicken embryos. FP was toxic in the brine shrimp larvae bioassay with an LD_{50} of 53.4 μM or 23.7 μg/mL.[116] FP exerted teratogenic and pathogenic effects on chicken embryos when applied at concentrations of 1 or 5 mM.[118] The toxin was shown to noncovalently interact with oligonucleotides in vitro.[119] Although interactions with DNA may account for the teratogenic effect of FP, they have not yet been confirmed in living organisms or cells.

FP was detected in 17% of samples (n = 83) of feed and feed raw materials with median and maximum concentrations of 2.6 and 14.8 mg/kg, respectively.[60] A review of FP occurrence data published between 1997 and 2011 shows its occasional occurrence in grains and grain-based foodstuffs[120] at concentrations up to 500 mg/kg.[121]

In summary, high levels of FP occasionally occur in grains and grain-based food and feed. However, its toxicity and mode of action have not been comprehensively investigated so far. Toxic effects on chicken embryos and brine shrimp larvae suggest a potential hazard to humans and animals that should be clarified by further experiments.

Fusaric Acid. FA (5-butylpiconilic acid; Figure 5), is a phytotoxin produced by a great variety of *Fusarium* species,

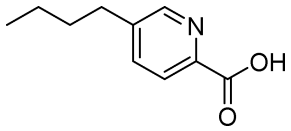

Figure 5. Chemical structure of fusaric acid.

such as *F. oxysporum*, *F. moniliforme*, *F. proliferatum*, *F. subglutinans*, *F. verticillioides*, *F. crookwellense*, *F. napiforme*, and *F. fujikuroi*.[122,123] This off-white to yellowish crystalline compound, a precursor of the beta blocker bupicomide,[124] is understudied for its mode of action and its effects in livestock and humans. FA has been shown to exhibit cytotoxic and growth inhibitory effects in different normal and cancer cell lines. Cell proliferation was suppressed by 500 μM FA in human WI-38 fibroblasts and colorectal adenocarcinoma cell lines.[125] Furthermore, FA was cytotoxic to dog kidney (IC_{50} = 10 μg/mL), McCoy mouse (IC_{50} = 25 μg/mL), and Chinese hamster ovary fibroblast cells (IC_{50} = 10 μg/mL), as well as to rat hepatoma cells (IC_{50} = 50 μg/mL).[126] FA was shown to induce oxidative and mitochondrial stress, as well as to cause apoptosis and necrosis in the human hepatocellular carcinoma cell line HepG2.[127] May and co-workers found that FA inhibits the growth of the rumen microorganisms *Ruminococcus albus* and *Methanobrevibacter ruminantium* at concentrations >84 μM.[128] Another in vitro study demonstrated that FA inactivates several clinical and soil isolates of *Acanthamoeba*, with an IC_{50} value of 0.31 μM.[129] As a phytotoxin, FA is known to play a critical role in accelerating the development of *Fusarium* wilt in numerous plants, such as banana and tomato plants.[130−132]

The LD_{50} values of FA in mice were 100 and 80 mg/kg BW for iv and ip administration, respectively.[133] The average oral bioavailability of FA (25 mg/kg) in Sprague−Dawley rats was 58%.[134] Several studies indicated a neurochemical effect of FA. Researchers demonstrated a hypotensive effect of FA in humans, rabbits, rats, cats, and dogs that was based on the inhibition of dopamine-β-hydroxylase,[133,135,136] the enzyme that converts dopamine to norepinephrine. FA was furthermore shown to alter brain and pineal neurotransmitters in rats.[137] Oral administration of FA (200 mg/kg BW) to swine caused neurochemical changes and vomiting behavior.[138] Co-administration of different doses of FA and DON to swine decreased weight gain and feed consumption, which led the authors to suggest that FA might act synergistically with trichothecenes.[139] Chickens that received FA-contaminated diet (up to 150 mg FA/kg diet), on the other hand, did not show any abnormalities in behavior, feed intake, weight gain, and appearance of visceral organs.[140] FA exerted a teratogenic effect in zebrafish. The compound caused malformations at the notochord due to copper chelating.[141] A synergistic cytotoxic effect was detected for in ovo administration of FA and FB1.[142] Interestingly, oral administration of 1 mg/mL FA inhibits tumor growth of head and neck cancer in an in vivo murine model.[143]

Porter and co-workers showed that FA is a naturally occurring contaminant in several types of cereal grain and mixed livestock and poultry feeds and is synthesized together with other *Fusarium* mycotoxins.[137] Smith and Sousadias reported that in 48 investigated swine feed samples, 85% were positive, with the highest concentration of 136 mg/kg FA.[144]

Because FA is produced by the most common fungal genus, *Fusarium*, due to its neurochemical effects and due to possible synergistic effects with predominant mycotoxins (DON and FB1), this substance might pose a problem to humans and farm animals. FA is not yet regulated in any country or monitored regularly. For an appropriate risk assessment, more studies covering in vivo and in vitro trials as well as occurrence are duly needed.

Culmorin. CUL is a sesquiterpene diol (Figure 6) produced by several *Fusarium* species, such as *F. culmorum*, *F. graminearum*, *F. crookwellense*, and *F. venenatum* (summarized by Pedersen and Miller[145]) and by the marine ascomycete *Leptosphaeria oraemaris*.[146] In addition, the formation of various related compounds, namely, hydroxyculmorins (5-, 12-, 15-hydroxyculmorin), culmorone, and hydroxyculmorones, has been reported for different *Fusarium* strains.[147,148]

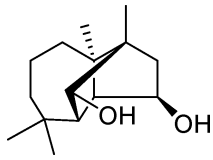

Figure 6. Chemical structure of culmorin.

Information on the toxicological relevance of CUL and its hydroxylated forms is limited. CUL was shown to be phytotoxic[149,150] and to possess antifungal activity.[146] Pedersen and Miller demonstrated that this compound results negative in the Ames test.[145] In baby hamster kidney cells BHK-21, only the highest tested CUL concentration (20 μg/mL; 84 μM) exhibited mild cytotoxicity.[147] The LD_{50} for CUL in the chick embryotoxicity screening test (CHEST) was between 68.0 μg/kg (incubation period of 22 days) and 78.2 μg/egg (incubation period of 7 days).[151] Thus, CUL was approximately 10 times less toxic than DON and approximately 25−30 times less toxic than STE in the same test. In an attempt to extrapolate data from the CHEST, the mouse LD_{50} (ip) for CUL was estimated to range between 250 and 1000 mg/kg BW, indicating low mammalian toxicity.[145]

So far, in vivo studies on the toxicity of CUL are restricted to caterpillars and swine. Administration of a CUL-contaminated

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diet (25 mg/kg) for 7 days did not affect the mortality or weight of corn earworm (*Heliothis zea*) and fall armyworm (*Spodoptera frugiperda*) larvae.[152] Similarly, CUL exposure (2 mg/kg diet, 21 days) had no negative impact on the performance of growing piglets.[153] Interestingly, CUL even seemed to slightly enhance the growth of *H. zea* larvae and to increase the weight gain of pigs. In addition, a potential interaction of CUL and DON was investigated in both studies. The combination of CUL (10 mg/kg diet) with DON (25 mg/kg diet) resulted in a significantly increased mortality and decreased weight of *H. zea* larvae when compared to DON alone. The authors assumed a synergistic effect, which is probably related to an impaired DON detoxification in insects in the presence of CUL.[152] While an interaction of these two mycotoxins could not be confirmed in pigs,[153] CUL was recently proposed to increase DON toxicity in wheat seeds.[150]

The latter is of certain interest, because the levels of CUL as well as hydroxyculmorins strongly correlate with those of DON in cereal grains.[154,155] In a recent study, CUL was detected in 95−100% of Norwegian barley, oat, and wheat samples (n = 76). Highest contamination levels were found in oats (n = 28), where median and maximum CUL concentrations reached 2.0 and 31.5 mg/kg, respectively.[155] Streit and co-workers found 63, 63, 13, and 7% of feed and feed raw materials (n = 83) positive for CUL, 15-hydroxyculmorin, 5-hydroxyculmorin, and 15-hydroxyculmorone.[60] In contrast, hydroxyculmorins were more frequently found than CUL in Italian malting barley samples[156] and groundnuts from Cameroon.[157] Besides factors such as region, season, and sensitivity of the analytical method, variations in the hydroxyculmorin profile between individual *Fusarium* species[148] may account for these differences.

On the basis of available literature data, CUL seems to have limited acute toxicity in mammals. However, due to its frequent occurrence in cereal grains, the mode of action of this mycotoxin should be elucidated and its potential interaction with DON (and other trichothecenes) should be clarified.

Butenolide. BUT (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone; Figure 7) is produced by several *Fusarium* species,

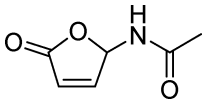

Figure 7. Chemical structure of butenolide.

such as *F. tricinctum*, *F. equiseti*, *F. graminearum*, or *F. sporotrichioides*. BUT was cytotoxic to different established mammalian cell lines[126,158] and to primary cultures of neonatal rat and murine cardiomyocytes.[159,160] Minimal effective concentrations ranged from 1 to 25 μg/mL. In the case of human chondrocytes, exposure to BUT concentrations ≥4 μg/mL had a cytotoxic effect, whereas lower concentrations (1−2 μg/mL) caused an increase in cell viability.[161] ROS production was shown to be a mechanism of BUT cytotoxicity in HepG2 cells.[158] Furthermore, ROS production due to BUT exposure caused lipid peroxidation[159,161] and DNA damage[160] in mammalian cell cultures. BUT was shown to induce mitochondrial dysfunction in rat cardiomyocytes, which may trigger ROS production.[162]

The effect of BUT exposure was investigated in rodents. Intragastric administration of 10 or 20 mg BUT/kg BW to rats for ≥7 weeks caused a loss of BW[163] and injuries to the myocardium[159] and liver.[163] Markers of oxidative stress and oxidative damage were increased in myocardium, liver, and serum.[159,163] Exposure of mice to 0.5 mg BUT/mL drinking water for 3 weeks (corresponds to consumption of ∼2.95 mg BUT/day) had no effect on BW.[164] BUT showed adverse effects on the growth and development of rat embryos in vitro when applied at a concentration of ≥1.25 mg/L, whereas no such effects were observed at 0.625 mg/L.[165] In chicken embryos, oxidative damage was detected in the myocardium,[166] liver, and kidney[167] after in ovo injection of a single dose of BUT (10−100 μg). The toxicity of BUT was investigated in a small number of cows. Application of ≥1.5 mg BUT to the skin of heifers induced local inflammatory encrusted lesions.[168] Intramuscular administration of 1.1 g BUT/day for 3 months, oral administration of 4.5 g BUT/day for 2 months,[168] or intramuscular administration of 3.8 mg BUT/kg BW/day for 90 days[169] caused weight loss and necrosis of the tail tip. Oral administration of 39 and 68 mg/kg BW was lethal to steers within 3 and 2 days, respectively.[170] Oral administration of 31 mg/kg BW/day for 46 days caused weight loss and esophageal and gastric ulcers.[170] BUT was detected in 52% of samples (n = 83) of feed and feed raw materials with median and maximum concentrations of 23 and 1490 μg/kg, respectively.[60]

In summary, the toxicity of BUT was shown in vivo and the compound's potential to trigger oxidative stress and oxidative damage has been reported. However, very high doses of BUT were administered in previous studies, and it is unclear whether BUT levels that can be reached by dietary exposure would also cause adverse effects. Additional experiments with lower concentrations of BUT should be conducted in vivo to study the long-term toxicity. Also, more occurrence data are needed.

Sterigmatocystin. STE (Figure 8) is a toxic precursor of aflatoxins and structurally closely related to AfB1. Its toxic effect

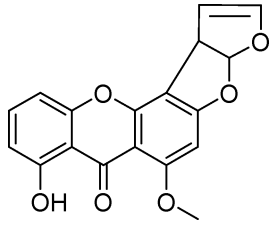

Figure 8. Chemical structure of sterigmatocystin.

is mediated by its furofuran ring structure, which forms DNA adducts after metabolic activation to an epoxide.[171] STE is produced by several fungal species within the genera *Aspergillus*, *Bipolaris*, *Emericella*, *Chaetomium*, *Botryotrichum*, and *Humicola* and the species *Penicillium luteum*. The main producers are *Aspergillus* fungi, such as *Aspergillus flavus*, *A. parasiticus*, *A. nidulans*, and especially *A. versicolor*.[172] The International Agency of Research in Cancer classified STE as a group 2B carcinogen (possibly carcinogenic to humans).[173,174]

Several studies could demonstrate that STE exerts genotoxic and cytotoxic effects on different cell lines, for example, on immortalized ovarian hamster cells (CHO-K1 cells) for which an IC_{50} value of 12.5 ± 2.0 μM was detected after 72 h of incubation.[175] The IC_{50} value of STE was determined to be 7.3 μM by measuring the protein synthesis in liver hepatocellular (HepG2) cells.[176] In addition, Gao and co-workers investigated the STE-induced DNA damage in HepG2 cells. They showed a significant dose-dependent increase of DNA strand breaks and an increased intracellular ROS level if cells were exposed to 3 and 6 μM STE.[177] Also, in the human lung adenocarcinoma cell

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line A549 (IC_{50} = 3.7 μM)[178] and in transformed rat fibroblasts (AWRF)[179] the cytotoxic effect of STE was confirmed. Furthermore, Sun and co-workers reported the induction of apoptosis in human peripheral blood lymphocytes incubated with 2 μg/mL STE.[180] STE induced DNA damage in primary cultured human esophageal epithelial cells and immortalized human esophageal epithelial cells (Het-1A) and caused G1 and G2 phase arrest, respectively.[181]

Only a few recently conducted in vivo studies are available in livestock, fish, and other animals. Whereas the effects of STE are similar to those of AfB1, its acute toxicity is much lower. The LD_{50} values in rat are 120−166 and 60−85 mg/kg BW for oral and ip administration, respectively.[182] In monkeys, the LD_{50} upon ip administration was determined to be 32 mg/kg BW.[183] Studies in ruminants found a negative effect of a STE-contaminated diet in cattle,[184] but not in sheep.[185] Dietary exposure of dairy cows to ≤12 mg STE/kg feed caused bloody diarrhea, reduced milk production, and was lethal in some cases.[184] In one pig trial with STE (30 μg/kg feed), negative effects, such as decreased feed intake, incidental diarrhea, and necrotic alterations of the liver tissue, were observed.[186] An LD_{50} of 5−7 μg was reported for 5-day-old chicken embryos, whereas 10 μg killed 90−100% of the embryos.[187] Newly hatched male chicks suffered from liver cirrhosis and altered biochemical serum parameters after 7 weeks of dietary STE exposure.[188] Also, Sreemannarayana and co-workers described the hepatotoxic and nephrotoxic impact of STE in poultry.[189−192] In carp and catfish, the mycotoxin caused a decrease in body growth and crude protein content.[193] In Nile tilapia fish, STE showed toxic and clastogenic effects indicated by a significantly decreased BW and an increased incidence of micronucleated red blood cells as well as chromosomal aberrations in the kidney.[194] STE down-regulated TNF-α, interleukin (IL) 6, and IL-12 mRNA expression in isolated peripheral blood mononuclear cells and peritoneal macrophage cells of BALB/c mice, as well as TNF-α and IL-6 protein levels in serum, suggesting an immunosuppressive effect.[195] Already a single sc dose of STE (5 μg/g of BW) induced tumors in lung and liver in newborn mice. Males seemed to show an even higher susceptibility to those effects than females.[196] Chromosome aberrations were found in rat bone marrow cells, when a dose of 31.2 mg STE/kg BW was injected ip.[197]

Scarce data about the natural occurrence of STE was available until 2015. An external scientific report, assigned by EFSA, presents data on 1259 samples of cereal grains, cereal products, beer, and nuts collected over a period of 14 months and analyzed for STE by a validated LC-MS/MS method.[198] STE was detected in 10% of all samples, and the highest frequency of contamination was seen in rice (21%) and oat grains (22%) intended for human consumption. In beer, peanut, and hazelnut samples, no STE concentration was found.[198] A review from 2010 reported the occurrence of STE in green coffee beans, spices, nuts, beer, and the outer layer of hard cheese colonized by *A. versicolor*.[199]

The EFSA Panel on Contaminants in the Food Chain (CONTAM) published a scientific opinion in 2013 about the presence of STE in food and feed without characterizing its possible risk due to a lack of occurrence data and the need for more sensitive analytical methods.[172] With new data available now, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is currently working on a safety assessment of STE proposed by the Codex Committee on Contaminants in Foods.[200] Due to the high STE concentrations used in vitro, a clear relevance of the observed results is arguable. Thus, follow-up experiments with low-dose STE treatments should be conducted in vivo to study the long-term toxicity.

Emodin. EMO, an orange-red crystalline compound, is a 1,3,8-trihydroxy-6-methylanthraquinone (Figure 9) and is

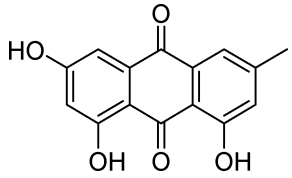

Figure 9. Chemical structure of emodin.

produced by many *Aspergillus* species, but has also been found to be produced by the genera *Penicillium* and *Talaromyces*. Furthermore, it occurs as an active ingredient in many plants,[201,202] mainly in Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex*, and *Polygonum* spp.), and Rhamnaceae (*Rhamnus* and *Ventilago* spp.).[203] In plants, the anthraquinones (also aloe-emodin, chrysophanol, physcion or rhein) are present as aglycones or as glycosides, such as emodin-8-glucoside.[203]

In traditional Chinese medicine, EMO, mainly extracted from the rhizome of *Rheum palmatum* L., is claimed to be a therapeutic alternative for the prophylaxis and treatment of various diseases and physical complaints. Several studies could demonstrate that EMO inhibits the growth and proliferation of cancer cells in vivo and in vitro.[204,205] EMO induced apoptosis in the human lung squamous carcinoma cell line CH27[206] and in murine leukemia WEHI-3 cells.[207] It might also act as a JAK2/STAT3 inhibitor, and it down-regulates the expression of anti-apoptotic Mcl-1, thereby inducing cell death in multiple myeloma cell lines.[208] Antiviral activities were shown in different studies. EMO reduced the entry and replication of Coxsakievirus in HEp-2 cells[209] and inhibited hepatitis B virus and Herpes simplex virus type 1 in vitro.[210,211] EMO exerted antibacterial activity against *Bacillus subtilis* (minimal inhibitory concentration (MIC) = 28.9 μM), *Staphylococcus aureus* (MIC = 14.4 μM)[212] and methicillin-resistant or sensitive *S. aureus* strains (MIC = 64 μg/mL; 237 μM).[213] EMO was suggested to be purgative, as it was shown to induce contraction of rat isolated ileum tissue by releasing endogenous acetylcholine.[214] EMO was also shown to possess antidiabetic activity by activating the PPARγ[215] or the AMPK[216] signaling pathway. Furthermore, EMO is considered to have anti-osteoporotic[217,218] and antiallergic effects.[219] A recently published review describes several positive effects in detail.[220]

In contrast to EMO's manifold beneficial effects, its negative effects have not been thoroughly studied. In human mononuclear cells, EMO (3−30 μM) caused an immunosuppressive reaction by decreasing IL-1 and IL-2 production and IL-2 receptor expression.[221] It induced apoptosis in human T cells[222] and a human proximal tubular cell line (HK-2) via the mitochondrial pathway.[223] Furthermore, it inhibited the stimulation of human peripheral blood mononuclear cells and decreased the plasma IL-2 level.[224] Published studies about the genotoxicity of EMO are controversial. Mueller and co-workers showed a genotoxic effect using the micronucleus test and mutation assay in mouse lymphoma L5178Y cells.[225,226] Morita and co-workers observed an induction of 6-thioguanine-resistant mutation in the mouse mammary carcinoma cell line

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FM3A by 1−10 μg/mL EMO.[227] Other researchers, however, could not demonstrate a mutagenic effect of EMO.[228,229] Furthermore, Westendorf and co-workers discovered only a slight response in the V79-HGPRT mutagenicity assay.[230]

In vivo, therapeutic effects and toxicity were investigated mainly in mice and rats. Two studies with collagen-induced arthritis mice showed that EMO exerted an anti-inflammatory effect by inhibition of the NF-κB-pathway[231] and reduction of TNF-α and IL-6 in plasma, PGE2 production, and COX-2 protein expression.[232] Following successful in vitro experiments, Liu and co-workers confirmed the inhibition of the Coxsakie virus also in mice.[209] Another mouse trial showed the positive effect of EMO on diesel exhaust particle induced lung inflammation.[233] EMO also reduced aldose reductase in the lens of mice, which might play an important role in the development of diabetes cataract.[234] Negative effects of EMO (0.25−2 μg/mL; 0.92−7.4 μM) were demonstrated for the survival rates and hatching success of zebra fish embryos.[235] Furthermore, EMO induced nephrotoxicity in Sprague−Dawley rats.[236]

Long-term dietary exposure to EMO (≤2.5 g/kg) for a period of 2 years showed no carcinogenic effect in rats or mice.[237] Similarly, neither prenatal mortality nor morphologic or genotoxic changes were observed in EMO-fed mice and rats, and NOAELs of 2500 and 850 mg/kg were defined for mice and rats, respectively.[238]

EMO is probably the most ubiquitous anthraquinone found worldwide, especially in higher plant families but also in fungi, trees, shrubs, lianas, and herbs.[203] However, EMO receives little attention as a fungal metabolite, and occurrence data of fungal EMO in food and feed are scarce. One study reports the detection of EMO in 89% of the analyzed 83 feed samples, at median and maximum concentrations of 9.8 and 1570 μg/kg.[60] Because of the high NOAEL in rodents[238] and its use in alternative human medicine for decades, the risk to animal or human health from EMO is very likely marginal.

Mycophenolic Acid. MPA ((4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid; Figure 10) was isolated already 1893 by an Italian physician,

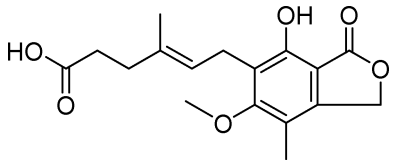

Figure 10. Chemical structure of mycophenolic acid.

Bartolomeo Gosio, from *Penicillium brevicompactum* (reviewed by Bentley[239]). Besides its production by this fungus, MPA can also be produced by *P. roqueforti* and *P. carneum* or by *Byssochlamys nivea*—a known producer of patulin.[4] MPA holds the distinction of being the first ever purified antibiotic,[239] but it is widely used nowadays both as an immunosuppressive drug for prophylaxis and treatment of organ rejection in transplantations and as an antirheumatic drug.[240] The main mode of action of MPA is the specific inhibition of the enzyme inosine monophosphate dehydrogenase, which is highly expressed in proliferating cells such as T- and B-lymphocytes. The inhibition of lymphocyte proliferation leads to immunosuppression.[241]

Although MPA is sometimes referred to as a mycotoxin, its acute toxicity in animals is low, with LD_{50} values of around 450 mg/kg BW in rats and around 1900 mg/kg BW in mice after oral application.[242] The same authors assessed subacute toxicity in rats and detected no adverse effects at 10 mg/kg BW. At higher doses growth retardation and anemia were diagnosed in rats. Dogs died after 12 days when given daily doses of 80 mg/kg BW and at lower doses showed anorexia, diarrhea, and enteritis.[242] MPA was not mutagenic, did not produce chromosome aberrations, and was nontoxic when given to nonhuman primates in higher doses than required for human therapy.[243] Used as an antirheumatic drug in human medicine, daily doses of 1−3 g were generally tolerated, with gastrointestinal intolerance being the major observed side effect.[243] In the following years, a multitude of effects on different cell types, e.g. lymphocytes, monocytes, neutrophils as well as on dendritic, mesangial, mast, vascular smooth muscle and endothelial cells were discovered.[244] The authors concluded in their literature review that while rheumatic patients still largely benefited from MPA treatment, it holds the risk of immune suppression, and therapy has been associated with several, sometimes life-threatening, infections by viruses.

MPA has been shown to occur in various food and feedstuffs in relatively high concentrations compared to other fungal metabolites. Dry-cured ham and liver pâté were shown to be good substrates for *P. brevicompactum*, and after inoculation, levels of 11−14 mg/kg of MPA were found on the food surfaces.[245] All investigated Roquefort cheese samples were found positive for MPA with levels up to 1.2 mg/kg, whereas German blue-white soft cheese contained even 11 mg/kg.[246] In lower concentrations, MPA was also found to occur in red wine[247] and ginger.[248] In silage, MPA was found in 32% of 233 investigated sampled at levels up to 35 mg/kg[249] or even up to 48 mg/kg.[250]

Given the low toxicity of MPA, the only concern is its role in unwanted immunosuppression.[251] The extensive use of MPA in medicine has indicated that these concerns are minimal.[239] Levels in food do not even come close to therapeutic doses in humans, so it is safe to assume that MPA should also not be of any dietary concern. Although in feed the concentrations can be higher, it was verified that 300 mg of MPA per sheep and day from contaminated silage does not show any immunodepressive effects.[252] In conclusion, MPA should not be termed a "mycotoxin" as it is neither toxic nor does it show any other adverse effects on humans or animals at the concentration levels found in food and feed.

Alternariol and Alternariol Monomethyl Ether. AOH and its monomethyl ether AME are dibenzopyrone derivatives (Figure 11), produced by a large variety of *Alternaria* spp., which mainly grow on vegetables, fruits, and cereals.[253] In vitro, AOH and AME were shown to be cytotoxic to HeLa cells, but less toxic to *Bacillus mycoides*.[254] AOH was furthermore shown to be cytotoxic to human colon carcinoma cell lines, and cytotoxicity was shown to be mediated by the activation of the mitochondrial pathway of apoptosis.[255] In Caco-2 cells, AOH

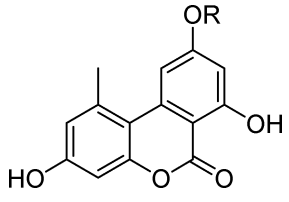

Figure 11. Chemical structure of alternariol (R = H) and alternariol monomethyl ether (R = CH_3).

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induced toxicity via cell cycle disruption, induction of apoptosis/necrosis, and changes in mitochondrial membrane potential.[256] AOH and AME induced DNA strand breaks and chromosomal aberration in different cell lines.[253,257−259] The DNA damaging effect of these compounds was traced back to their binding to the minor groove of DNA and their action as topoisomerase poisons.[258,260] Furthermore, in mammalian cell lines, AOH induced oxidative stress that resulted in oxidative DNA damage in some studies,[261,262] but not in others.[263,264] In line with these observations of DNA-damaging effects, AOH was shown to be mutagenic in mammalian cell lines.[265,266] Mutagenicity assays in bacteria, on the other hand, produced contradictory results. Both compounds were suggested to be strongly mutagenic according to the *B. subtilis* rec-assay[267] and the *E. coli* ND160 reverse mutation assay.[268−270] In the Ames *Salmonella* test, the compounds were not or only weakly mutagenic to strains TA98 and TA100,[271−273] and AOH was mutagenic to strain TA102.[274] Effects of both AOH and AME on reproductive organs and the immune system have been suggested on the basis of in vitro data. An estrogenic potential of AOH was detected in a human endometrial adenocarcinoma cell line; however, this potential was 10,000-fold lower compared to the endogenous hormone 17β-estradiol.[257] Interestingly, AOH showed synergistic estrogenic effects in combination with ZEN or its phase I metabolite α-zearalenol.[275] AOH and AME specifically inhibited progesterone secretion in cultured porcine granulosa cells, which suggests that contaminated food may affect the reproductive performance of pigs and other mammalian species.[276] AOH induced autophagy and senescence in murine macrophages[277] and modified the morphology and cytokine secretion of murine and human macrophages.[278]

With regard to in vivo toxicity, no mortality or teratogenic effects of AOH or AME on chicken embryos were detected at doses up to 1 mg per egg.[279] Chicks exposed to dietary AME at levels up to 100 mg/kg feed for 4 weeks showed no mortality or significant loss in performance.[279] No evidence of toxicity was observed in rats when AME and AOH were fed for 21 days at levels up to 24 and 39 mg/kg feed, respectively.[280] Furthermore, no toxic effects were observed when 3.75 mg of AME was given to rats by oral gavage daily for 30 days.[281] In a study performed by Schuchardt and co-workers, AOH was shown to be nontoxic to mice after single or repeated oral application of doses as high as 2000 mg/kg BW.[282] An in vivo oral toxicokinetic study using 200 or 1000 mg/kg AOH revealed low systemic absorption and rapid metabolization, and thus it was concluded that target organ toxicity would likely be restricted to the gastrointestinal tract.[282] Furthermore, neither the micronucleus assay applied to bone marrow nor the Comet assay applied to liver tissue indicated systemic genotoxicity. Whether AOH exhibits local genotoxicity in the gastrointestinal tract remains to be determined.

The metabolism of AOH and AME was investigated in different animal species and cell lines, and different classes of metabolites have been observed. AOH and AME were shown to be hydroxylated at their aromatic carbon atoms by cytochrome P450, leading to the formation of catechols,[282−284] and they were shown to be conjugated with glucuronic acid or sulfate at their phenolic hydroxyl groups by UDP-glucuronosyltransferases and sulfotransferases, respectively.[285,286] AME was furthermore shown to be demethylated to AOH in vitro.[284]

Two studies investigated the fetotoxicity of AOH and AME. AME was maternally toxic and fetotoxic when administered ip to Syrian golden hamsters at a single dose of 200 mg/kg BW, whereas no such effects were observed at lower doses.[281] In mice, sc administration of AOH (100 mg/kg BW) resulted in an increased percentage of dead and resorbed fetuses/litter and runts/litter, whereas lower doses of AOH or AME did not elicit a fetotoxic effect.[254] Interestingly, a synergistic fetotoxic effect of AOH and AME was observed when both substances were administered at a dose of 25 mg/kg. AOH and AME were suggested as etiological agents of human esophageal cancer,[253,287] but a causal relationship has not been demonstrated so far. Interestingly, precancerous changes were detected in the esophageal mucosa of mice fed 50 or 100 mg AME/kg BW/day for 10 months.[288]

EFSA concluded in 2011 that the estimated chronic dietary exposure of humans to AOH and AME exceeds the threshold of toxicological concern for potentially genotoxic substances, indicating a need to create additional toxicity data.[289] According to a survey of published occurrence data, AOH was detected in 31% and AME was detected in 6% of samples ($n \sim 300$) of European feed and agricultural commodities at maximum concentrations of 1840 and 184 μg/kg for AOH and AME, respectively.[289] Also, in tomato-based foodstuffs collected in Belgium, AOH and AME exceeded the threshold of toxicological concern assigned by EFSA for potentially genotoxic substances.[290]

In conclusion, AOH and AME showed genotoxic effects in vitro. However, administration of high doses that could—on the basis of currently available data on the occurrence in food and feed—hardly be achieved in vivo did not elicit toxic or genotoxic effects in rodents. Nevertheless, given the genotoxic potential, additional toxicity data should be generated. Effects of AOH and AME on reproductive organs and the immune system were suggested by in vitro data and should be investigated in vivo.

Tenuazonic Acid. TeA is a tetramic acid derivative (Figure 12) produced by *Alternaria* species that grow on vegetables,

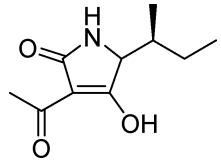

Figure 12. Chemical structure of tenuazonic acid.

fruits, and cereals[253] and by plant pathogenic fungi such as *Phoma sorghina* and *Magnaporthe oryzae*.[291,292] The toxic effect of TeA is presumably based on its interference with protein biosynthesis.[293] The compound was nonmutagenic in the Ames *Salmonella* test with or without metabolic activation or nitrosylation.[272−274]

TeA was toxic to mice and rats, and LD_{50} values were determined.[294,295] Observed symptoms included diarrhea, muscle tremor, and convulsion. For mice, LD_{50} values upon oral administration were 81 mg/kg BW for females and 186 or 225 mg/kg BW for males. Upon iv administration the LD_{50} value was 115 mg/kg BW for females. Upon ip, sc, or iv administration, LD_{50} values were 125−162 mg/kg BW for males. For rats, LD_{50} values were 168 mg/kg BW for females and 180 mg/kg BW for males upon oral administration and 157 mg/kg BW for females and 146 mg/kg BW for males upon iv administration. After dietary exposure of mice to 25 mg TeA/kg BW/day for 10 months precancerous changes were detected

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in the esophageal mucosa.[288] TeA was furthermore toxic to beagle dogs (4 oral doses of 2.5 mg/kg BW per day) and monkeys (daily oral dose of 89.6 mg/kg BW).[294] Symptoms included diarrhea, vomiting, and hemorrhages and were lethal in most cases. In the case of monkeys, no adverse effects were detected for daily doses up to 48.8 mg/kg BW. TeA was toxic to chickens. The compound was toxic in the chicken embryo assay with an LD_{50} of 548 μg/egg, but it showed no teratogenic effect.[279] For day-old chicks, the LD_{50} upon oral administration was 37.5 mg/kg BW.[296] Dietary exposure of broilers to 10 mg/kg feed for 3 weeks or daily oral administration of $\geq$1.25 mg/kg BW to broilers and layers caused macro- and microscopic lesions in different organs and led to decreased weight gain and feed efficiency.[296] In the same study, no adverse effects were detected for doses of 0.63 mg/kg BW. The toxicokinetics of TeA were investigated in pigs and broilers.[297] Oral or iv administration of single doses of 0.05 mg TeA/kg BW had no adverse effects. The orally administered doses were completely absorbed in both species and rapidly eliminated in pigs but not in broilers.

According to a survey of published occurrence data conducted by EFSA, TeA was detected in 15% of samples ($n \sim 300$) of European feed and agricultural commodities at concentrations between 500 and 4310 μg/kg.[289] In a survey of feed and feed raw materials, TeA was detected in 65% of samples ($n = 83$) at median and maximum concentrations of 68 and 1980 μg/kg, respectively.[60] Interestingly, high levels of TeA (up to 1200 μg/kg) were found in *Sorghum*-based infant food.[298]

In summary, administration of TeA was toxic to rodents and chickens. In their scientific opinion from 2011, EFSA concluded, on the basis of exposure estimates and due to a lack of data indicating genotoxicity, that TeA is unlikely to be a human health concern.[289] However, more recent occurrence data suggested that health risks cannot be excluded for infants consuming larger quantities of *Sorghum*-based food and that more data on the subject should be generated. EFSA furthermore concluded that on the basis of in vivo toxicity data[296] and estimated exposure, adverse health effects cannot fully be ruled out for chickens.[289] The recent finding that TeA shows a high bioavailability and slow elimination in chickens reinforces the notion that the toxin may be a cause for concern for this species and that more data on the subject should be collected.

CONCLUSIONS

Fungal spoilage of agricultural goods is accompanied by the formation of mycotoxins, which pose a significant health risk to humans and livestock. As climatic conditions favor the growth of different fungi (and hence the formation of different toxins) in different parts of the world, some populations are more affected than others, but mycotoxins are a global challenge. Without any doubt, aflatoxins, due to their high acute and chronic toxicity, have the most severe impact on human and animal life. In addition, several other mycotoxins including trichothecenes, fumonisins, OTA, or ZEN are of high toxicological significance and have been extensively studied, and regulations are in force in many countries with the aim of ensuring tolerable levels in food and feed. Efficient and reliable analytical methods have been developed and are used to control foodstuffs for their compliance with regulations. During the past decade, advances in—particularly LC-MS based—methods used for food and feed analysis have been enormous with regard to their detection limits, speed, and capability to determine multiple compounds.[299] Nowadays, certain modern analytical methods are able to detect hundreds of different compounds, for instance, fungal metabolites,[300] in a variety of food and feed samples. Many of these metabolites are irrelevant in terms of food and feed safety, but for the first time in history occurrence data can be collected on a large scale before the toxicity of the detected fungal metabolites is (and can be) assessed. Sticking to a basic principle in toxicology, *sola dosis facit venenum* (the dose makes the poison), both toxicity and occurrence data are indispensable for risk assessment. Instead of prioritizing research on natural toxins based on the most bioactive compounds, we might have reached a point where assigning priorities based on actual occurrence data in foodstuffs is at least conceivable.

Given the enormous number of bioactive fungal metabolites, every attempt to provide exhaustive coverage of their potential health impact is doomed to fail. As a limited number of currently nonregulated compounds of fungal origin had to be selected for this paper, it is plausible that equally or even more important ones were missed. Still, our aim was to critically scrutinize the impact of the emerging mycotoxins covered here and to propose a ranking list for the research community to prioritize research efforts. The bottom of this list is clearly reserved for EMO and MPA. Both compounds have already been used in human medicine for decades with negligible side effects even at doses that cannot be reached due to ingestion of contaminated food. CUL would probably rank above these compounds on our imaginary list. Although levels of occurrence can be high, the toxicity of this compound in mammals seems to be marginal. Yet, the mode of action of this substance has not been elucidated and, in addition, its frequent co-occurrence with DON may be a cause for concern. Therefore, it should be clarified whether synergism with trichothecenes, as hinted in a few studies, actually exists. Arguably next on the list are ENNs and BEA. Whereas these compounds are clearly toxic in vitro, most in vivo data indicate limited toxicity. The EFSA risk assessment for these compounds stated no immediate concern for human health, but also highlighted the need for long-term studies to assess potential chronic toxic effects in vivo.[59] Although BUT is toxic in vivo and causes oxidative stress to cells, it is currently unknown if the occurring levels are sufficient to cause adverse effects and toxicity studies with lower doses should be conducted in animals. A similar picture can be drawn for STE, as long-term in vivo studies at lower doses are currently lacking that would be needed to assess chronic toxicity. Whereas the *Alternaria* metabolites AOH and AME showed genotoxic effects in vitro, genotoxicity could so far not be confirmed in vivo, and further studies on this subject would be warranted. Other effects caused by these compounds, for example, on the reproductive or immune system, should also be clarified using in vivo trials. In the case of FP and FA there is clearly a need for more studies. Whereas FP levels in cereals can be high and toxic effects were seen on larvae and chicken embryos, further animal studies are currently missing. Even less is known about FA other than that it shows neurochemical effects and that it is possibly synergistic to co-occurring mycotoxins such as DON or fumonisins. MON would probably end up on the upper end of the ranking. Although its acute toxicity and occurrence levels are unproblematic for humans, the situation is different for poultry. Furthermore, the discrepancies between the outcomes of studies investigating

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genotoxic effects of MON should be clarified as quickly as possible. The situation is similar for TeA. Although toxic to animals, EFSA concluded that this compound is likely of no human health concern mainly due to its low average occurrence.[289] Considering recent studies, risk cannot be excluded, though, both for chickens and for infants consuming *Sorghum*-based food.

With regard to *Alternaria* metabolites, interestingly, AOH, AME, and TeA made only a minor contribution to the genotoxicity of certain *A. alternata* extracts, whereas a high genotoxic activity was attributed to unidentified compounds.[263] Also, altertoxins were shown to be mutagenic in vitro,[273,301,302] and altertoxin I was detected in 42% of analyzed feed samples (n = 83) in a recent survey.[60] Altertoxins are just one example that several fungal metabolites, not explicitly discussed in this paper, show high toxic potential. Because of this, together with the recently available information on frequent occurrence, more data on the exposure of humans and animals as well as in vivo studies clarifying possible toxic effects are warranted.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03413.

ChemDrawings (ZIP)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ABC	ATP-binding cassette
AfB1	aflatoxin B_1
AME	alternariol monomethyl ether
AOH	alternariol
BEA	beauvericin
BUT	butenolide
BW	body weight
CC_{50}	concentration resulting in 50% cell viability
CHEST	chick embryotoxicity screening test
CONTAM	Panel on Contaminants in the Food Chain
CUL	culmorin
DON	deoxynivalenol
EFSA	European Food Safety Authority
EMO	emodin
ENNs	enniatins
FA	fusaric acid
FB1	fumonisin B_1
FP	fusaproliferin
ip	intraperitoneal
iv	intravenous
IC_{50}	half-maximal inhibitory concentration
IL	interleukin
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	liquid chromatography−mass spectrometry
LD_{50}	dose that is lethal to 50% of test subjects
LOAEL	lowest observed adverse effect level
MIC	minimal inhibitory concentration
MON	moniliformin
MPA	mycophenolic acid
NOAEL	no observed adverse effect level
OTA	ochratoxin A
ROS	reactive oxygen species
sc	subcutaneous
STE	sterigmatocystin
TeA	tenuazonic acid
ZEN	zearalenone

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