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## Glycosylated mycotoxins: a hidden enemy

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Mycotoxins, secondary metabolites produced by filamentous fungi, are highly toxic contaminants of food crops. These contaminated plants pose a significant health risk to livestock and humans. Cereals are the main source of dietary mycotoxin intake in the EU and are often contaminated with *Fusarium* mycotoxins. After fungal infection, mycotoxins are produced and further modified by plant enzymes. The most common mechanism of modification of *Fusarium* mycotoxins and other mycotoxins is conjugation with glucose, but also with oligosaccharides and polysaccharides. Although this reduces their toxicity to the plant, enzymes in the gastrointestinal tract of animals or enzymes used in the food processing can hydrolyze the glycosidic bond, releasing the mycotoxin. While the analysis of free mycotoxins is routine, the quantification of mono-, oligo-, and polyglycosides is difficult or impossible, leading to an underestimation of the actual risk. Most conjugated mycotoxins cannot be routinely quantified as analytical standards are commercially unavailable. This review focuses in particular on the formation and occurrence of glycosylated mycotoxins and their effects on health and their transformations in the food chain. It summarizes and critically compares chemical, enzymatic, plant, and microbial glycosylation. Specific deglycosylation methods (both enzymatic and chemical) required for the evaluation of the content of respective mycotoxins are covered. Indirect quantification of modified mycotoxins using enzymatic hydrolysis methods and subsequent analysis of the free forms, typically performed by HPLC-MS, is discussed. The whole spectrum of mycotoxins, including those that are sometimes neglected in modern literature (typically ergot mycotoxins), is covered in a rather complex way.

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## 1. Introduction

Food contamination by chemicals can arise from various sources, including environmental pollution, excessive or unauthorized use of food additives, technological processing, application of pesticides, and secondary metabolism of plants and co-existing microorganisms.<sup>1</sup> Among the latter, toxic metabolites produced by microscopic filamentous fungi, known as mycotoxins, represent one of the most widespread and challenging groups of natural contaminants.<sup>2,3</sup> Assessing the overall impact of contaminants on public health remains difficult, despite the existence of numerous regulatory and toxicological tools for their monitoring. This is particularly true for natural toxins, including mycotoxins, whose structural diversity and variable production conditions complicate both risk assessment and effective control. In this context, preventive measures such as good agricultural practices, cultivation of resistant crop varieties, soil treatment, and pesticide application remain the primary line of defense. Nevertheless, because the effectiveness of these measures is limited, mycotoxins continue to pose a serious health risk to humans and animals,



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often exceeding the risks associated with pesticide residues used to control their fungal producers.<sup>4</sup>

Among the most insidious and least understood members of this group are the “masked”<sup>5,6</sup> mycotoxins, which are the chemical derivatives of mycotoxins, typically glycosides, produced in various ways. The concept of “*masked mycotoxins*” emerged in the 1990s, when conjugated forms of trichothecenes and zearalenone were first detected in cereals and were shown to escape routine analytical methods. Over subsequent years, the terminology diversified, with descriptors such as bound, conjugated, or modified mycotoxins appearing in the literature.<sup>7,8</sup> To resolve this inconsistency, the German Federal Institute for Risk Assessment (BfR) proposed in 2014 a clear classification,<sup>9</sup> which has since been widely adopted by EFSA and the broader research community. It deals comprehensively with the sorting of these compounds and introduces a classification of these substances into two main groups, namely, “matrix-associated” mycotoxins, including mycotoxins that are covalently or non-covalently bound to biopolymeric food components (proteins, polysaccharides), and “modified” mycotoxins, which comprise a broad spectrum of biological and chemical modifications. This review focuses primarily on mycotoxin glycosides that fall under these two main categories according to the BfR definition. For reasons of clarity and consistency, the term ‘‘glycosylated mycotoxins’’ is therefore preferred throughout the text.

The most important glucosylated derivatives, the phase II plant metabolites, are those of trichothecene mycotoxins such as deoxynivalenol-3- $\beta$ -glucopyranoside (DON-3G), glucosides of T2 (T-2-3- $\beta$ -glucopyranoside/T-2-3- $\alpha$ -glucopyranoside; T-2-G) and HT-2 toxins (HT-2-3- $\beta$ -glucopyranoside/T-2-3- $\alpha$ -glucopyranoside; HT-2-G), zearalenone-14- $\beta$ -glucopyranoside (ZEN-14G),<sup>10-13</sup> and glucosides of *Alternaria* mycotoxins<sup>14</sup> whose presence in food and feed can significantly influence toxicity, bioavailability, and metabolic fate. Relying solely on existing analytical protocols can severely underestimate human and animal exposure. Although the European Food Safety Authority (EFSA), the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the U.S. Department of Agriculture (USDA) have begun to systematically assess the risks associated with masked mycotoxins (or better “modified mycotoxins”), the toxicological data for most glycosylated mycotoxins remains limited and comprehensive risk assessment frameworks are still under development. Based on available data,<sup>15</sup> some conjugated forms, such as DON-3G, have begun to be included in calculations of total dietary exposure. Despite this progress, there is still a lack of a harmonized international methodology for the identification, quantification, and toxicological evaluation of modified mycotoxins. There is also an urgent need for updated regulatory definitions and clear inclusion criteria in food safety legislation.

The challenge posed by modified mycotoxins is exacerbated by the complex interplay among fungal metabolism, plant biochemistry, and food processing technologies.<sup>16</sup> In summary, masked mycotoxins represent a current food safety challenge that requires an interdisciplinary approach that integrates analytical chemistry, toxicology, metabolomics, and regulatory science. This review aims to provide a comprehensive overview of the current

state of knowledge on the occurrence, detection, toxicity, and potential impact of modified mycotoxins on consumer health, while highlighting the research and regulatory gaps that need to be addressed to ensure effective protection of public health.

## 2. Mycotoxins forming glycosides – classes, toxicity, occurrence

### 2.1 *Fusarium* toxins

*Fusarium* species such as *F. graminearum*, *F. culmorum*, *F. oxyphorum*, *F. sporotrichioides*, and *F. langsethiae*, and many others<sup>17</sup> are prominent producers of mycotoxins that contaminate cereals and pose a significant health risk to humans and animals. Among them, trichothecenes and zearalenone are the most studied. Recent research has highlighted the occurrence of glycosylated forms of these toxins, which can alter their toxicity and complicate their detection. Here, we focus on their structural characteristics, biosynthesis, toxicology, occurrence, metabolism, detection, and regulatory aspects.<sup>18</sup>

Trichothecenes (Fig. 1) are sesquiterpenoid compounds characterized by a tricyclic 12,13-epoxytrichothec-9-ene core synthesized by enzymes encoded by the TRI gene cluster.<sup>17</sup> All trichothecenes share the 12,13-epoxy ring crucial for their toxicity. They are classified into four types based on their functional groups: Type A lacking a carbonyl group at C-8, with a free hydroxyl at C-3 (e.g., T-2 toxin (T-2, 1), HT-2 toxin (HT-2, 2)) and diacetoxyscirpenol (DAS)), type B possessing a carbonyl group at C-8 and hydroxyl groups at C-3, C-7, C-15 (e.g., deoxynivalenol (DON, 3), nivalenol (NIV), and fusarenon-X (FUS-X)). These structural differences influence toxicity – type A trichothecenes are generally more acutely toxic than representatives from the type B group. Less common representatives include type C trichothecenes containing an additional epoxide

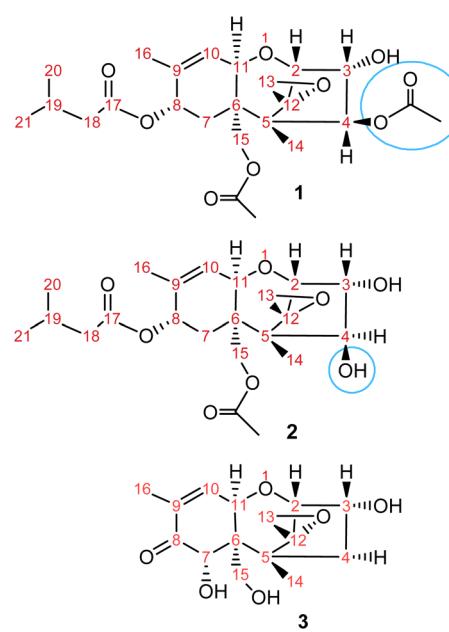


Fig. 1 T-2 toxin (T-2, 1), HT-2 toxin (HT-2, 2), deoxynivalenol (DON, 3).



ring bridging C-7 and C-8 (e.g., crotocin), and type D trichothecenes featuring a macrocyclic ring between C-4 and C-15 (e.g., roridin A and satratoxin H).

### 2.1.1 Type B trichothecenes – nivalenol and deoxynivalenol

2.1.1.1 *Occurrence.* Trichothecenes frequently contaminate cereal grains. *F. graminearum* and *F. culmorum* are widespread pathogens of wheat, barley, and maize, and are the major producers of DON. *F. asiaticum* and *F. cerealis* can produce NIV in Asia and Europe, respectively.<sup>17,19,20</sup>

2.1.1.2 *Toxicity.* Trichothecenes are generally strong inhibitors of eukaryotic protein synthesis. They bind to the center of the ribosomal peptidyltransferase (60S subunit) and thus prevent chain elongation. This mechanism underlies their acute toxicity: rapidly dividing cells (intestinal epithelium, bone marrow, immune cells) are most affected. Recent research also links trichothecenes to oxidative stress and cell cycle disruption, e.g., DON and its metabolites induce reactive oxygen species and DNA damage *in vitro*.<sup>21</sup> DON (vomitoxin) causes nausea, vomiting, and feed refusal in animals – hence its name – and triggers ribotoxic stress at the molecular level by activating mitogen-activated protein kinase (MAPK) signaling pathways and the release of proinflammatory cytokines. The tolerable daily intake (TDI) for DON is defined as a group TDI value for the sum of its glycosylated and acetylated forms and is 1 µg per kg body weight per day.<sup>22</sup> NIV is generally considered more cytotoxic than DON *in vitro* and can cause severe gastrointestinal lesions and hematologic effects in animals at high doses, although it is less abundant in food. The TDI for NIV is 1.2 µg per kg body weight per day.<sup>23</sup>

2.1.1.3 *Metabolism.* Trichothecenes are rapidly metabolized. In animals and humans, phase I metabolism can alter the core of the toxin. For example, DON can be epimerized at C-3 or de-epoxidized by gut microbes, forming less toxic 1-deepoxy-deoxynivalenol (DOM-1).<sup>21</sup> The degree of conversion of DON to DOM-1 is significant, especially in ruminants. Phase II metabolism in humans and animals (conjugation) is also important – DON is extensively glucuronidated in humans and pigs, primarily to DON-15-glucuronide and DON-3-glucuronide, which are excreted in the urine;<sup>24</sup> in humans, glucuronidation is considered the main detoxification pathway for DON. Interestingly, a metabolite coproduced by the same toxicogenic fungus *Fusarium* – culmorin – significantly suppresses the glucuronidation of DON by human liver microsomes.<sup>25</sup>

### 2.1.2 Type A trichothecenes – T-2 and HT-2 toxins

2.1.2.1 *Occurrence.* The primary producers of T-2 and HT-2 toxins in cereals like oats, barley, and wheat are *Fusarium* species, especially *Fusarium langsethiae*, *F. sporotrichioides*, and *F. poae*.<sup>26</sup> Recent review literature confirms that *F. langsethiae* appears to be the most significant contributor, followed by *F. poae* and *F. sporotrichioides*, and that toxin production is strongly influenced by environmental factors such as warm, wet conditions during flowering and high water activity levels.<sup>17,27</sup>

2.1.2.2 *Toxicity.* T-2 and HT-2 belong to the most acutely toxic trichothecenes, implicated in outbreaks of alimentary toxic aleukia. T-2/HT-2 cause diarrhea, hemorrhage, leukopenia, and dermal necrosis; they readily suppress immune function by rapidly killing proliferating cells in the spleen and

thymus. T-2/HT-2 may trigger anorexia *via* neurotransmitters such as substance P and serotonin in the brain.<sup>21</sup> HT-2 is almost as toxic as T-2; toxicity studies often consider the sum T-2 + HT-2. The TDI for the sum of T-2 and HT-2 is 0.02 µg per kg body weight per day. The 12,13-epoxide ring is essential for activity – elimination of the epoxide moiety significantly reduces toxicity.<sup>5</sup>

2.1.2.3 *Metabolism.* T-2 toxin is partially hydrolyzed to HT-2 toxin in the stomach and intestine by esterases that remove the C-4 acetyl group. Both T-2 and HT-2 can be further de-esterified to T-2-triol or T-2-tetraol, and the epoxide may be opened to form trichothecenediols.

### 2.1.3 Zearalenone

2.1.3.1 *Occurrence.* Zearalenone (ZEN, 4) is a resorcylic acid lactone mycotoxin produced by *Fusarium* species (notably *F. graminearum* and *F. culmorum*) and commonly contaminates maize and other cereals in temperate regions.<sup>3</sup> (Fig. 2). It often co-occurs with type B trichothecenes (e.g., deoxynivalenol) since the same fungi can produce both toxins. ZEN contamination levels vary from year to year but can reach several hundred µg kg<sup>-1</sup> in grain during severe *Fusarium* outbreaks.

2.1.3.2 *Toxicity.* ZEN is a nonsteroidal estrogen that binds to estrogen receptors (ER $\alpha$ / $\beta$ ) with lower affinity than 17 $\beta$ -estradiol. Its structure allows metabolic reduction to estrogenic alcohols ( $\alpha$ -zearalenol ( $\alpha$ -ZEL, 5) and  $\beta$ -zearalenol ( $\beta$ -ZEL, 6)) in animals.<sup>28</sup> In livestock (especially pigs, which are highly sensitive), ZEN exposure leads to a hyperestrogenic syndrome – for example, infertility, swelling of the vulva and mammary glands, and disrupted estrous cycles in swine. Recent studies suggest that ZEN can affect epigenetic regulation: *in vitro*, zearalenone exposure altered DNA methylation patterns and histone acetylation in uterine cells.<sup>21</sup> This suggests possible long-term effects on gene expression. ZEN is not classified as carcinogenic to humans, but chronic exposure in animals resulted in an increased incidence of certain tumors (e.g., in the pituitary gland and reproductive tract), likely due to hormonal imbalance. The group TDI for ZEN and its modified forms is 0.25 µg per kg body weight per day.<sup>29</sup>

2.1.3.3 *Metabolism.* ZEN is rapidly absorbed and primarily metabolized in the liver. The main biotransformation is the reduction of its keto-lactone to form  $\alpha$ -ZEL and  $\beta$ -ZEL (mediated by hepatic 3 $\alpha$ /3 $\beta$ -hydroxysteroid dehydrogenases). Pigs predominantly produce the more estrogenic  $\alpha$ -ZEL, whereas cattle and poultry form more  $\beta$ -ZEL<sup>30</sup> – a species difference correlating with sensitivity. ZEN and its metabolites can also disrupt progesterone and androgen receptors to a lesser extent, contributing to reproductive toxicity. ZEN and its phase I metabolites are extensively conjugated (mainly as glucuronides) to facilitate excretion. For example, most of an absorbed ZEN dose is excreted as ZEN-14-O-glucuronide (and similarly as glucuronides of  $\alpha$ -ZEL and  $\beta$ -ZEL) in bile or urine,<sup>31</sup> with minor sulfate conjugates also reported. In many species, hepatic 3 $\alpha$ /3 $\beta$ -hydroxysteroid dehydrogenases carry out this reaction.

## 2.2 *Alternaria* toxins

2.2.1 *Occurrence.* *Alternaria* spp. are filamentous fungi with broad temperature tolerance and a wide host range,

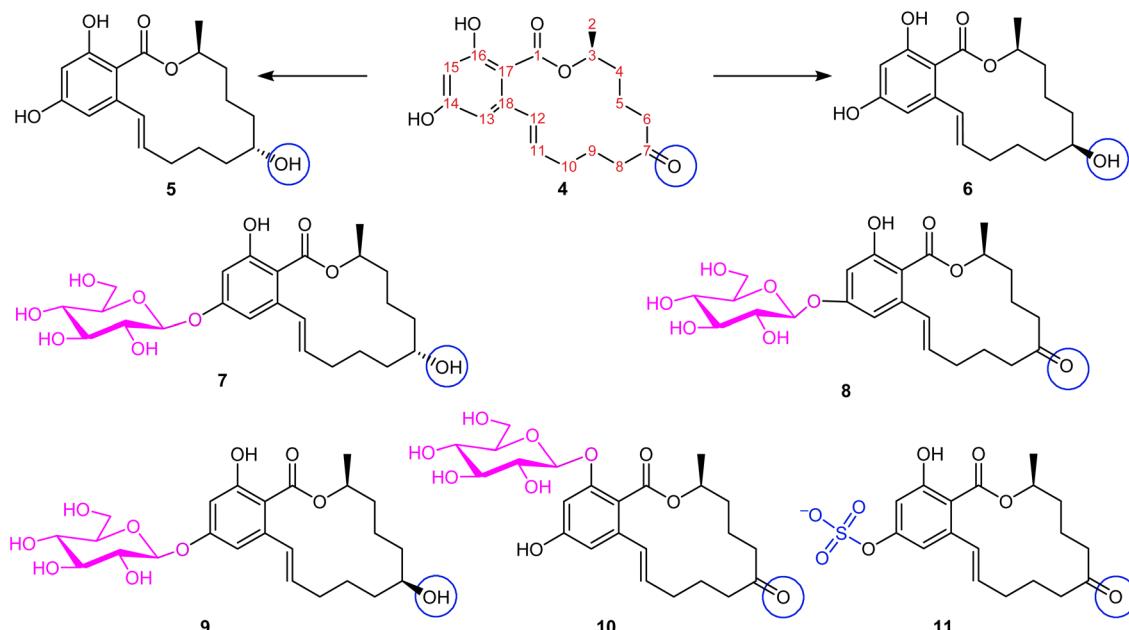


Fig. 2 Zearalenone (ZEN, 4),  $\alpha$ -zearalenol ( $\alpha$ -ZEL, 5),  $\beta$ -zearalenol ( $\beta$ -ZEL, 6),  $\alpha$ -zearalenol-14-O- $\beta$ -glucopyranoside ( $\alpha$ -ZEL-14G, 7), zearalenone-14-O- $\beta$ -glucopyranoside (ZEN-14G, 8),  $\beta$ -zearalenol-14-O- $\beta$ -glucoside (9), zearalenone-16-O- $\beta$ -glucopyranoside (ZEN-16G, 10), zearalenone-14-sulfate (ZEN-14S, 11).

affecting several economically important crops, including vegetables,<sup>32</sup> fruits,<sup>33–35</sup> and wheat,<sup>36</sup> among others.<sup>37</sup> In addition to causing plant diseases, *Alternaria* spp. produce toxic secondary metabolites, collectively known as *Alternaria* toxins. The most important *Alternaria* toxins include alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), tentoxin (TEN), altertoxins (ATX I–III), etc. (Fig. 3). Plants primarily form glucoside conjugates of AOH and

AME, effectively “masking” these mycotoxins.<sup>38</sup> The glycosylation of *Alternaria* toxins has been studied primarily for AOH and AME.

**2.2.2 Toxicity.** Many *Alternaria* toxins are cytotoxic, genotoxic, and potentially carcinogenic. In particular, AOH and AME are of high concern due to their potent DNA-damaging effects, thought to result from intercalating into DNA and inhibiting topoisomerases I/II (leading to strand breaks and mutations).<sup>14</sup> The toxicological concern arises particularly from AOH and AME, which are dibenzopyrone derivatives shown to cause DNA strand breaks and trigger oxidative stress in mammalian cells. *In vitro* assays have demonstrated their ability to intercalate into DNA and inhibit topoisomerases I and II, enzymes essential for

DNA replication and transcription, thereby promoting DNA damage and mutagenesis. TeA, in contrast, has a different mode of action: as a tetramic acid, it inhibits protein synthesis (interfering with translation initiation and elongation). TeA is considered the most acutely toxic *Alternaria* metabolite, yet it lacks the strong genotoxicity of AOH and AME. Consequently, it has a much higher threshold of toxicological concern (TTC  $\sim$ 1500 ng per kg bw per day vs.  $\sim$ 2.5 ng kg $^{-1}$  for AOH/AME).<sup>39–41</sup> Overall, AOH and AME's genotoxicity and TeA's protein synthesis inhibition highlight the need for careful risk assessment of foods contaminated with *Alternaria* toxins.

**2.2.3 Metabolism.** In mammals, *Alternaria* toxins (especially AOH, AME, ALT) undergo phase I oxidation (e.g., hydroxylation at multiple positions by cytochrome P450 enzymes) followed by phase II conjugation (glucuronidation and sulfation) to facilitate elimination.<sup>42</sup> These biotransformations substantially reduce the toxins' bioavailability. However, the presence of phase II metabolites (such as glucuronides or

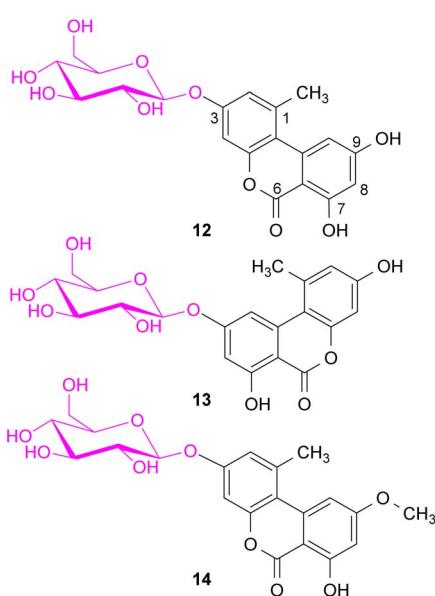


Fig. 3 Alternariol-3-O- $\beta$ -glucopyranoside (AOH-3G, 12), alternariol-9-O- $\beta$ -glucopyranoside (AOH-9G, 13), alternariol methyl ether-3-O- $\beta$ -glucopyranoside (AME-3G, 14).



sulfates) implies that any deconjugation (e.g., by gut microbes) could release the parent toxins, an issue to consider in toxicological evaluations.

### 2.3 Ergot alkaloids

**2.3.1 Occurrence.** Ergot alkaloids (EAs) are a class of mycotoxins with a dual identity: notorious for their historical role in toxic outbreaks (the oldest known mycotoxicosis)<sup>43</sup> and valued for their diverse pharmacological applications. They are classified into major groups, such as clavines, secoclavines, and ergopeptides.<sup>44</sup> These indole-derived compounds are mainly biosynthesized by fungi of the genus *Claviceps*, in particular by *Claviceps purpurea* (ergot), *C. paspali*, and *C. fusiformis*, but are also found in *Aspergillus* and *Penicillium* species as well as endophytic fungi in grasses such as tall fescue (*Epichloë* spp.) and rarely in some plants (e.g., seeds of morning glory, *Ipomoea* sp.). Structurally, EAs are similar to important biogenic amines such as dopamine, serotonin, and noradrenaline, which explains their complex physiological effects as partial agonists or antagonists of various neurotransmitter receptors.<sup>45</sup>

**2.3.2 Toxicity.** The toxicity of ergot alkaloids stems from their ability to mimic biogenic amines like dopamine, serotonin, and norepinephrine, acting as partial agonists or antagonists at neurotransmitter receptors.<sup>46</sup> This pharmacological mimicry causes the classic manifestations of ergotism. Chronically, EAs induce gangrenous ergotism, characterized by severe vasoconstriction leading to tissue necrosis (gangrene, as historically seen in poisoned rye outbreaks), and convulsive ergotism, involving neurologic effects such as hallucinations, convulsions, and odd behavior (paralleling LSD-like symptoms).<sup>46</sup>

Some EAs, such as ergovaline, ergotamine, and their stereoisomers, pose significant risks to human and animal health through ingestion of contaminated grain or forage.<sup>47</sup> In livestock, ergot exposure via endophyte-infected tall fescue causes fescue toxicosis, characterized by hyperthermia, reduced prolactin levels, impaired reproduction, and gangrene ("fescue foot").<sup>48</sup> Recent studies have also revealed that the so-called "inactive" C-8 S-epimers of ergot alkaloids can contribute to toxicity, prompting their inclusion in monitoring alongside the traditionally measured R-epimers.<sup>46,47</sup> Regulatory authorities are responding: bodies like EFSA and the UK COT have updated exposure guidelines for ergot alkaloids due to ongoing cereal contamination (especially in rye) and emerging data on risks to maternal and animal health.<sup>43,49,50</sup>

**2.3.3 Metabolism.** Ergot alkaloids undergo extensive first-pass metabolism, resulting in very low oral bioavailability (<1% of the dose reaches systemic circulation).<sup>48</sup> In mammals, cytochrome P450 enzymes in the liver rapidly transform EAs through reactions such as *N*-dealkylation and hydroxylation, producing a multitude of metabolites (including various *N*-demethylated and oxygenated derivatives).<sup>51</sup> In addition, enterohepatic recirculation prolongs systemic exposure, which, combined with accumulation in tissues and cells, may exacerbate chronic toxicity.<sup>47</sup>

### 2.4 Ochratoxins

**2.4.1 Occurrence.** Ochratoxins A and B are polyketide mycotoxins produced predominantly by *Aspergillus* and *Penicillium* species. They frequently contaminate a variety of commodities – especially cereals, coffee, and grape products (wine) – in both food and feed chains.<sup>52</sup> The most widespread and toxic member of this group is ochratoxin A (OTA, 49) (Fig. 10).

**2.4.2 Toxicity.** OTA is a potent nephrotoxin, teratogen, immunosuppressant, and suspected human carcinogen,<sup>53</sup> with toxicity mainly attributed to the presence of bound chlorine in the OTA molecule.<sup>54</sup> Its toxicity is based on several mechanisms: Potent inhibition of phenylalanyl-tRNA synthetase interferes with protein synthesis; generation of reactive oxygen species leads to oxidative stress, lipid peroxidation, DNA damage, and apoptosis. OTA binds with high affinity to serum albumin (>99%), which prolongs its half-life and facilitates uptake into the kidneys, where it triggers the proximal tubular degeneration seen in Balkan endemic nephropathy.<sup>55</sup> Immunotoxicity is also well documented.<sup>56</sup> Compared with OTA, its dechlorinated form (OTB) is markedly less toxic *in vivo*, although some hydroxylated metabolites (especially 4-R-OH-OTA) may retain significant cytotoxic and immunosuppressive properties.<sup>57</sup>

**2.4.3 Metabolism.** OTA is mainly metabolized by hepatic CYP450 enzymes *via* hydroxylation of both its isocoumarin ring and phenylalanine moiety, yielding metabolites such as 4-OH-OTA and 10-OH-OTA.<sup>56</sup> These reactions, observed in various species, occur mainly in liver microsomes. OTA conjugates, including glucuronides, methyl esters, and cysteine adducts, have been detected in urine.<sup>58</sup> Despite extensive metabolic profiling, the species-specific differences and the precise enzymatic pathways involved remain poorly characterized.<sup>53,59</sup>

## 3. Mycotoxin glycosides

Over the past decade (2015–2025), research has focused heavily on the synthesis and characterization of mycotoxin glycosides, both to understand their occurrence in food and to develop analytical standards.<sup>5,7,60–63</sup> In this section, we provide an overview of the status of glycosylation of the major mycotoxin groups – *Fusarium* toxins, ochratoxins, *Alternaria* toxins, and ergot alkaloids – covering (1) natural biosynthesis by plants and natural occurrence in plants, (2) methods of laboratory synthesis of mycotoxin glucosides for the research purposes comprising chemical, enzymatic, microbial biotransformation, (3) modification of glycosylated mycotoxins during food processing and occurrence of these glycosides in food, and (4) biotransformation of mycotoxin glycosides in the gastrointestinal tract and associated health effects.

### 3.1 Natural biosynthesis by plants and occurrence

In general, plants readily conjugate mycotoxins to glucosides as a defense mechanism by using the UGT enzymes.<sup>64</sup> The biological role of glycosylation in plants is detoxification and compartmentalization. By attaching a glucose moiety to the mycotoxin, the plant renders the toxin more water-soluble and

less able to interact with cellular targets. The glucosides are probably sequestered in vacuoles or incorporated into the matrix of the plant cell wall.<sup>5,6,21</sup>

**3.1.1 Trichothecene glycosides.** As for trichothecenes, DON is converted to deoxynivalenol-3-O- $\beta$ -D-glucoside (DON-3G, 15) *in planta* (Fig. 4).<sup>65,66</sup> The mechanism underlying Fhb1-based resistance of wheat (*Triticum aestivum* L.) to *Fusarium* infection has been traced to either a gene encoding a UDP-glucosyltransferase (UGT) or a regulatory function related to D3G formation. Therefore, these resistant wheat varieties are an excellent tool for the biotransformation of DON into glycosylated metabolites. Nine different biotransformation products of DON formed in wheat during detoxification of the toxin were found by LC-HRMS. The metabolites indicate that DON is conjugated to endogenous metabolites *via* two major metabolic pathways, namely (1) glucosylation (DON-3G (15), DON-di-hexoside (22), 15-acetyl-DON-3-glucoside (20), DON-6'-malonyl-glucoside) and (2) glutathione conjugation (DON-S-glutathione (40), "DON-2H"-S-glutathione, DON-S-cysteinyl-glycine, and DON-S-cysteine) (Figs. 4 and 9).<sup>67</sup>

Similarly, NIV is conjugated to NIV-3G,<sup>6</sup> and Type A trichothecenes T-2 toxin and HT-2 also form glucosides in infected cereals,<sup>68</sup> reducing their ribosomal toxicity.<sup>69</sup> The first report of a natural occurrence of DON-3G in cereals was published by Berthiller *et al.*, and the compound was characterized as deoxynivalenol-3-O- $\beta$ -D-glucopyranoside.<sup>65</sup>

Lattanzio *et al.* (2012) first identified T-2-3-O-glucoside (T-2-3G) and two isomeric HT-2 monoglucosides in naturally contaminated wheat and oats;<sup>68</sup> despite the fact that the original assumption mentioned in that study was that the isomers are positional with glucose bound in C-3 and C-4 positions,<sup>68</sup> the new findings showed that those are more probably the  $\alpha$ -/ $\beta$ -anomers with glucose bound at C-3.<sup>70</sup> DON-3G is frequently detected in *Fusarium*-infected grains, often at a concentration of 5–30% of the molar concentration of DON.<sup>71</sup> In a study of wheat, DON-3G was found in 27% of the samples (mean 42  $\mu$ g kg<sup>-1</sup>) and ranged from 4% to 37% of the co-occurring DON content.<sup>71,72</sup> NIV-3G was also detected in NIV-contaminated wheat, with one study finding that NIV-3G accounted for up to  $\sim$ 30% of the NIV present.<sup>73</sup>

T-2-3G was first identified in naturally contaminated oats and wheat, and was identified as the  $\alpha$ -anomer, suggesting that plants predominantly form T-2- $\alpha$ -D-glucopyranoside.<sup>11</sup> As regards the  $\beta$ -anomer of T-2-G, some authors report that it is not present in crops,<sup>11</sup> but another detected this  $\beta$ -glucoside in toxin-treated barley.<sup>74</sup> To the contrary, HT-2-3G occurs predominantly as the  $\beta$ -anomer. The occurrence of T-2-3G and HT-2-3G was proved in naturally contaminated oats and oat-based foods.<sup>75</sup> Průšová *et al.* reported the presence of HT-2-3G in 92% of oat-based food samples.<sup>76</sup> A recent study by Pierzgalsky *et al.*<sup>77</sup> found that in naturally contaminated oat samples, HT-2- $\beta$ -Glc was the predominant form, detected in up to 91% of samples, while the  $\alpha$ -anomer was present less frequently and at lower concentrations. In contrast, only the  $\alpha$ -anomer of T-2-Glc was detected in these samples (Fig. 5).

In some cases, diglucosides and even tri-glucosides have been experimentally detected in cereals and cereal-based

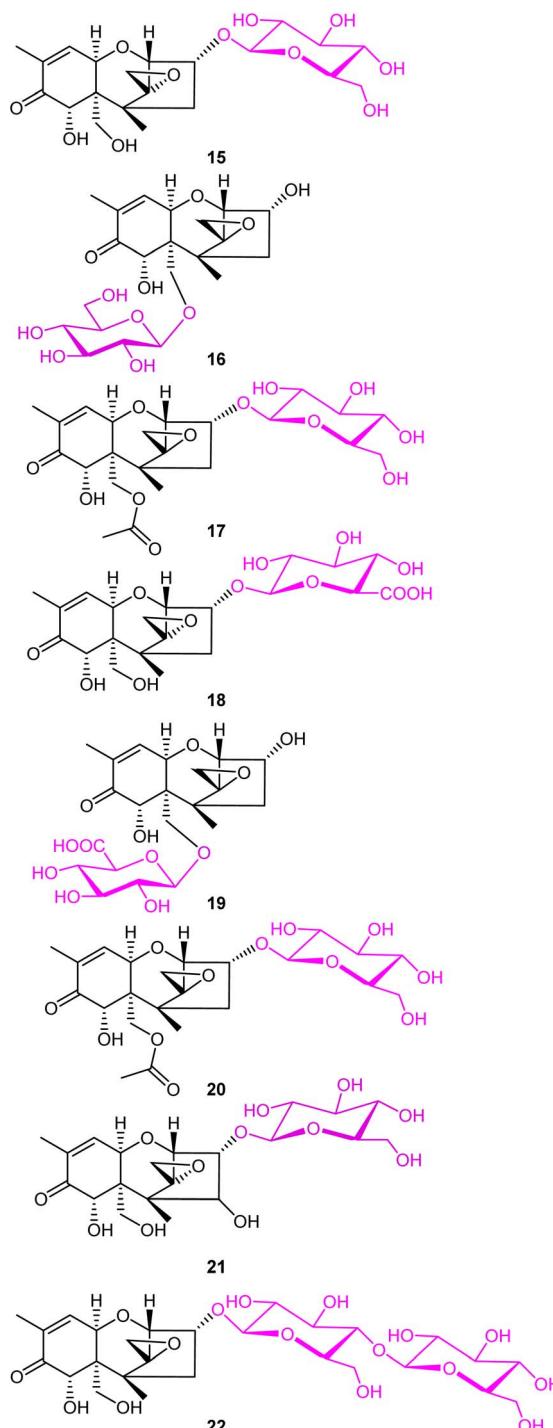


Fig. 4 Deoxynivalenol-3-O- $\beta$ -D-glucoside (DON-3G, 15), deoxynivalenol-15-O- $\beta$ -D-glucopyranoside (DON-15G, 16), 15-acetyl-deoxynivalenol-3-O- $\beta$ -D-glucopyranoside (15Ac-DON-3G, 17), deoxynivalenol-3-O- $\beta$ -D-glucuronide (DON-3GlcA, 18), deoxynivalenol-15-O- $\beta$ -D-glucuronide (DON-15GlcA, 19), 15-acetyl-deoxynivalenol-3-O- $\beta$ -D-glucopyranoside (15Ac-DON-3Glc, 20), fusarenone-X-glucoside (21), deoxynivalenol-3-diglucoside (DON-3-diGlc, 22).

samples. For example, Zachariášová *et al.* found traces of deoxynivalenol diglucoside (DON-di-G, 29) and deoxynivalenol triglucoside (DON-tri-G) in wheat, rye and multigrain baked



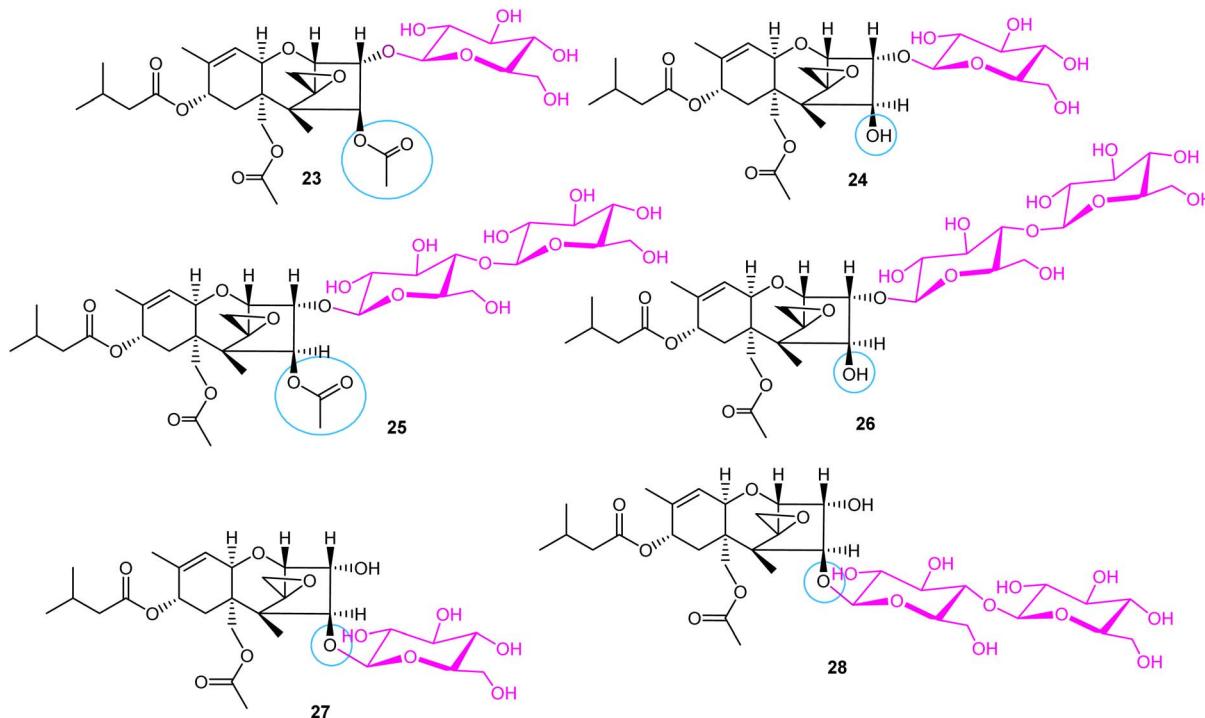


Fig. 5 T-2-toxin-3-O- $\beta$ -glucopyranoside (T-2-3Glc, 23), HT-2-toxin-3-O- $\beta$ -glucopyranoside (HT-2-3Glc, 24), T-2-toxin-3-diglucoside (T-2-3diGlc, 25), HT-2-toxin-3-diglucoside (HT-2-3diGlc, 26), HT-2-toxin-4-O- $\beta$ -glucopyranoside (HT-2-4Glc, 27), HT-2-toxin-4-diglucoside (HT-2-4diGlc, 28).

goods in wheat, rye, and multicerereal bakery products (Fig. 6).<sup>78</sup> Diglucosides (e.g., gentiobiosides) of HT-2 toxin were also observed in some cereal samples;<sup>76,79</sup> however, follow-up studies suggest that these may be mixtures of the two monoglucoside isomers rather than a single molecule with two sugar units.<sup>12</sup> In addition to soluble mycotoxin glycosides, there are also a variety of mycotoxins conjugated to polysaccharides and some other biopolymers, so-called “matrix-associated mycotoxins”, which bind to macromolecular components through covalent or non-covalent interactions. Their “masking” by the polymers leads to an underestimation of the overall exposure risk of mycotoxin.<sup>80</sup>

**3.1.2 Zearalenone glycosides.** Zearalenone is frequently converted into glucosides by plants and microbes and is

therefore also found in plant foods.<sup>72,81</sup> In infected cereals, zearalenone-14-O- $\beta$ -D-glucoside (ZEN-14G, 8) is the predominant masked form.<sup>13,82</sup> Plants like maize or wheat use UGT enzymes to attach glucose to the 14-hydroxyl (phenolic) group of ZEN.<sup>6</sup> ZEN-14G has been detected in infected cereals, though usually in lower amounts than DON-3G. In naturally contaminated maize samples, ZEN-14G was measured at roughly 10–20% of the parent ZEN.<sup>21</sup> The latter identified conjugate is zearalenone-16-glucoside (ZEN-16G, 10).<sup>83</sup> ZEN-16G is a minor form, occurring in barley in about 10% of the total ZEN conjugates.<sup>84</sup> ZEN forms both monoglucosides and, under specific conditions, also diglucosides. Interestingly, diglucosides (one at C-14 and another at C-16) can be formed

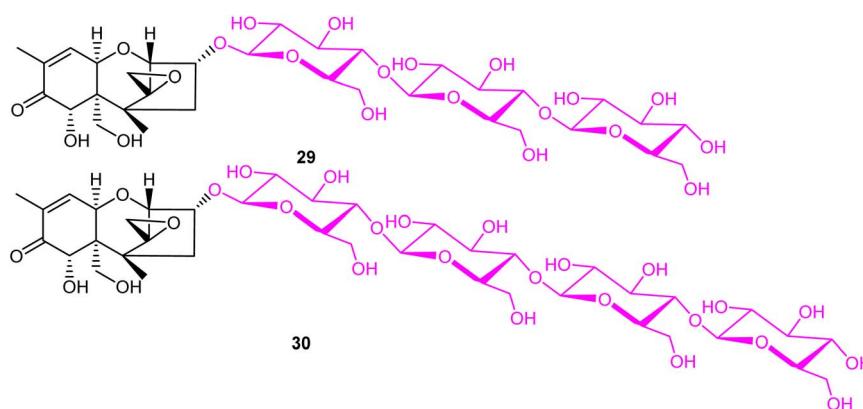


Fig. 6 Deoxynivalenol-3-triglucopyranoside (DON-3-triGlc, 29), deoxynivalenol-3-tetraglucoside (DON-3-tetraGlc, 30).



when ZEN is incubated with an active UGT for prolonged periods of time (Fig. 2).<sup>83</sup>

In addition to glucose conjugates, plants can further decorate ZEN glucosides: malonylation of ZEN-14-Glc, addition of a malonyl group to the 6'-OH of the glucoside, analogous to the malonylation of phytoalexins, has been reported.<sup>85,86</sup> For completeness, it should be mentioned that  $\alpha$ -ZEL (5) and  $\beta$ -ZEL (6) can also be glucosylated at their 14-OH positions (yielding  $\alpha$ -ZEL-14G and  $\beta$ -ZEL-14G)<sup>7</sup> or sulfated ( $\alpha$ -ZEL-14S, 11).<sup>87</sup> These conjugates often co-occur together with ZEN in grains.

**3.1.3 *Alternaria* glycosides.** As for *Alternaria* glycosides, their biological origin can be traced back to the plant rather than the fungus,<sup>14</sup> similarly to other mycotoxins. Studies comparing fungal and plant metabolism of AOH and AME have shown that *Alternaria* fungi themselves do not predominantly produce glucosides, but that fungi modify these toxins *via* other pathways (mainly sulfation). In contrast, plant enzymes convert these toxins into a range of conjugated forms. Early experiments with suspension cultures of tobacco cells have shown that AOH and AME are rapidly modified once inside plant cells.<sup>85</sup> Five different AOH conjugates were identified in tobacco cells: two were simple  $\beta$ -D-glucosides (with glucose attached to either the 3-hydroxyl or the 9-hydroxyl of AOH), two were malonyl-glucosides (malonyl esters of these glucosides at the 6'-position of the sugar) and one was an AOH gentiobioside (a disaccharide conjugate to which a  $\beta$ -D-gentiobiose is attached).<sup>85</sup> In the same study, AME (which has a methoxy at the AOH C-9 position, leaving only the 3-hydroxyl free) was converted to AME-3-glucoside and further to malonylated glucosides.<sup>85</sup> These findings demonstrate that plant UGTs readily form *Alternaria* toxin glucosides. Building on this, recent *in planta* experiments have revealed that the interplay between fungal and plant metabolism can lead to even more complex derivatives (Fig. 3).

Soukup *et al.* (2016) investigated *Alternaria*-infected tomato fruits and found novel "sulfoglucosides" of AOH and AME. This was the first report of mixed sulfate–glucoside conjugates for any mycotoxin.<sup>85</sup> In this scenario, the *Alternaria* fungus first adds a sulfate group to the toxin (*Alternaria* can enzymatically sulfate AOH/AME, which plant cells generally cannot<sup>85</sup>), and then the plant adds a glucopyranosyl moiety to the already sulfated toxin.<sup>85</sup> Three distinct AOH sulfoglucosides have been identified: alternariol-9-sulfate-3-glucoside (each has one sulfate and one glucose at the two different hydroxyls of AOH), and a similar mixed conjugate for AME (in which the only free OH of AME at C-3 was sulfated by the fungus and the plant then attached glucose to the remaining C-7 OH<sup>88</sup>). The structures were confirmed by NMR, which showed, for example, that in AME, the glucose was linked at O-7 in the sulfoglucoside, as the 3-OH carried the sulfate group.<sup>85</sup> In addition to sulfates, acetylated glucosides can also be formed (acetylation is another plant conjugation strategy). There is evidence for alternariol-3-acetylglucoside in some plant systems,<sup>88</sup> but it is less studied than malonyl or sulfated forms. In another *in vitro* study using suspension cultures of tobacco cells, AOH and AME were found to be extensively conjugated.<sup>89</sup> At least five distinct AOH conjugates, including 3-O- (12) and 9-O- $\beta$ -D-glucopyranosides (13) of alternariol and their 6'-O-malonyl-glucoside derivatives,

as well as a gentiobioside ( $\beta$ (1  $\rightarrow$  6)diglucopyranoside) were identified. AME was similarly converted to its 3-O-glucopyranoside and multiple malonyl-glucoside derivatives. The pattern of conjugation can vary – AOH tends to form a greater variety of conjugates (including di-hexose conjugates) than AME.<sup>89</sup>

**3.1.4 Ochratoxin glycosides.** As far as ochratoxin A (OTA) is concerned, plants and some microbes can convert OTA into hydroxylated derivatives, which then undergo glycosylation. Ochratoxin glucosides represent a relatively new but potentially significant category of modified mycotoxins. Their presence in food and feed, combined with the possibility of *in vivo* reactivation and toxic effects, raises important questions for both food safety.<sup>90</sup> Pioneering studies by Ruhland *et al.* in the 1990s demonstrated that OTA is extensively metabolized in cell suspension cultures of wheat and maize to several products, notably OT $\alpha$  (from amide hydrolysis), both (4R)- and (4S)-4-OH-OTA and their corresponding  $O$ - $\beta$ -D-glucosides (Fig. 10).<sup>91</sup> In these plant systems, both stereoisomers of 4-OH-OTA were formed in roughly similar amounts, suggesting that plant oxidative enzymes (perhaps peroxygenases or cytochrome P450s) do not have a strong stereoselectivity or that epimerization may occur post-formation. Any 4-OH-OTA could then be conjugated to glucose by plant UDP-glucosyltransferases. The result is a pair of epimeric glucosides: (4R)-4-OH-OTA- $\beta$ -D-glucoside (44) and (4S)-4-OH-OTA- $\beta$ -D-glucoside (45). The enzymatic pathway likely involves an inducible glycosyltransferase that recognizes the phenolic group of 4-OH-OTA as an acceptor, analogous to how plants detoxify other xenobiotics.<sup>91,92</sup> The two epimers, (4S)-4-OH-OTA and (4R)-4-OH-OTA, are diastereomeric metabolites with likely different properties – *R/S* epimerization significantly affects bioavailability and how they are enzymatically formed or hydrolyzed.<sup>92</sup> Apart from these, no other sugar conjugates (such as OTA glucopyranoside or gentiosides) have been reported, although hydroxyl groups are present in the native OTA molecule.<sup>92</sup>

There is some evidence that the fungus infecting the plant could influence which epimer predominates by affecting the local environment or stereospecifically.<sup>53</sup> In general, however, both glucoside epimers are formed simultaneously *in planta*. Notably, 4-OH-OTA itself has occasionally been isolated as a pair of epimers that slowly isomerize in solution (epimerization at C-4 can occur under certain conditions, possibly *via* keto-enol tautomerism involving the lactone). After conjugation with glucose, epimerization may be hindered so that each glucoside remains in its configuration. In practice, the presence of both 4S and 4R glucosides in samples complicates analytical detection (two peaks may appear) and raises questions about their potentially different toxicokinetic behavior.

In addition to plant-derived conjugation, there is emerging evidence that some fungi may also produce glucosides of ochratoxins, possibly as a self-protection mechanism.<sup>57</sup> Moreover, some biocontrol or detoxification microbes may modify OTA. Certain *Aspergillus* and *Trichoderma* strains can degrade OTA to OT $\alpha$  or to 4-hydroxy-OTA, possibly allowing subsequent glycosylation if a suitable glycosyltransferase is present.<sup>93</sup>



**3.1.5 Ergot alkaloid glycosides.** Although the glycosides of some of the mycotoxins mentioned above are relatively well documented in plant-pathogen interactions, there are few reports of naturally occurring glycosides of ergot alkaloids. One of the earliest discoveries was elymoclavine- $\beta$ -D-fructofuranoside (31) (Fig. 7), isolated from cultures of *Claviceps* sp. SD-58. Glycosylation is mediated by an endogenous invertase enzyme, transferring a  $\beta$ -D-fructofuranosyl unit from sucrose in the medium to the hydroxyl group of elymoclavine.<sup>94</sup> Further work revealed the production of di- (32), tri-, and tetrafructosides of elymoclavine through successive fructosyl transfers, leading to oligosaccharide derivatives that may have altered biological or storage function in fungal metabolism.<sup>95</sup> Later, also fructofuranosides of chanoclavine, *e.g.*, chanoclavine 1- $\beta$ -D-fructofuranoside (35) and chanoclavine 1- $\beta$ -D-fructofuranosyl-(2-1)- $\beta$ -D-fructofuranoside (36) were identified in the culture of *C. fusiformis* strain W1 (Fig. 8).<sup>96</sup> Křen *et al.* investigated the enzymatic fructosylation of chanoclavine, lysergol, elymoclavine, 9,10-dihydrolysergol, and ergometrine maleate using commercial yeast invertase, and they successfully

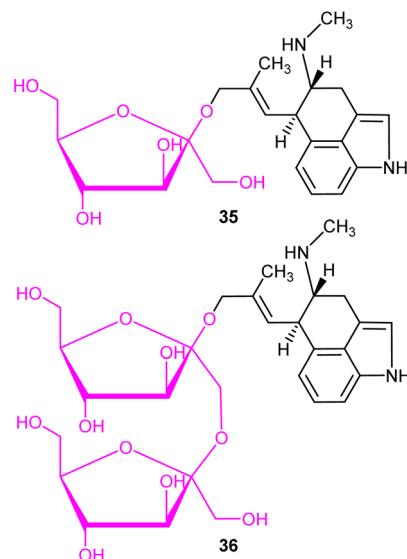


Fig. 8 Chanoclavine- $\beta$ -O-fructofuranoside (35), chanoclavine-di-fructoside (36).

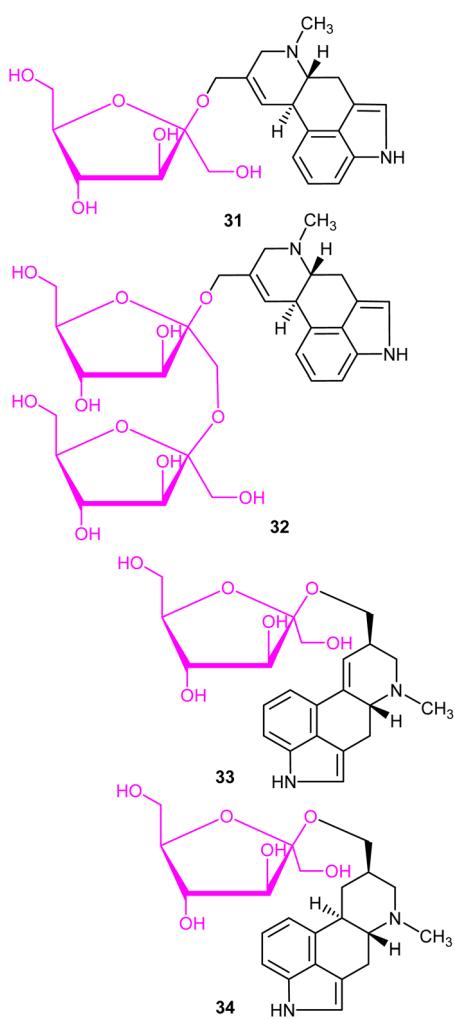


Fig. 7 Elymoclavine- $\beta$ -O-fructofuranoside (31), elymoclavine-di-fructoside (32), lysergol- $\beta$ -O-fructofuranoside (33), 9,10-dihydrolysergol- $\beta$ -O-fructofuranoside (34).

synthesized lysergol- $\beta$ -D-fructofuranoside (33), elymoclavine- $\beta$ -D-fructofuranoside (31), chanoclavine- $\beta$ -D-fructofuranoside (35), and 9,10-dihydrolysergol- $\beta$ -D-fructofuranoside (34) with low yields (*ca.* 8%), while ergometrine maleate gave no detectable fructosides (Fig. 7 and 8).<sup>97</sup>

The filamentous fungus *C. purpurea* possesses an intrinsic enzymatic machinery capable of fructosylating EAs *in vivo*. In the presence of high concentrations of sucrose, endogenous fructosyltransferases catalyze the glycosylation of elymoclavine and related alkaloids.<sup>98</sup> Interestingly, the introduction of 5-fluorotryptophan – a specific inhibitor of 4-dimethylallyltryptophan synthase (the first enzyme in EA biosynthesis) – enabled the selective fructosylation of exogenously added EAs without interference from *de novo* biosynthesis of elymoclavine by the host fungus. This strategy facilitated the glycosylation of otherwise less reactive substrates such as lysergol and dihydrolysergol, albeit in lower yields due to steric and electronic constraints.<sup>99</sup>

It appears that these clavine (oligo)fructosides are among the few naturally occurring EA glycosides known to date. Recently, however,<sup>100</sup> discovered ergometrine (syn. ergonovine)  $\beta$ -galactoside in a plant of the genus *Ipomoea* infected with the endophytic fungus *Periglandula* sp. There exist numerous examples of endophytic fungi producing ergot alkaloids in grasses used as forage. Specific cases indicating the risk of poisoning of livestock by these alkaloids are also reflected in their common names, such as Sleepy grass (*Achnatherum robustum* – endophyte *Neotyphodium* spp.) or Drunken horse grass (*A. inebrians*). They harbor the ergometrine-producing endophytes *Epichloë inebrians* or *E. gansuensis*. Both plant species from the genus *Achnatherum*, which contain ergometrine, also contain ergometrine glycoside. The presence of toxic EA in the form of glycosides in these plants, which are widely used as animal feed, is still an open question and deserves detailed investigation.



### 3.2 Laboratory synthesis of mycotoxin glucosides (chemical, enzymatic, microbial biotransformation)

All mycotoxin glycosides described hereunder were prepared *in vitro* by enzymatic or chemical syntheses to serve as analytical standards and/or for biological testing.

**3.2.1 Trichothecene glycosides.** Although the chemical glycosylation of trichothecenes is a challenge, it has been successfully carried out for analytical and reference purposes. One significant example is the synthesis of T-2 toxin- $\beta$ -D-glucoside, achieved using a classical carbohydrate coupling strategy. In this approach, triisopropylsilyl-protected ethylthioglucoside served as the glycosyl donor, with *N*-iodosuccinimide as the promoter. The final deprotection was accomplished with tetrabutylammonium fluoride, a reagent compatible with the sensitive ester functionalities of the T-2 aglycone.<sup>11</sup> These synthetic glucosides have played an essential role as standards for the characterization of biologically derived T-2-glucosides. Deoxynivalenol-3- $\beta$ -D-O-glucuronide (**19**) was synthesized by the Koenigs-Knorr reaction using acetobromo- $\alpha$ -D-glucuronic acid methyl ester as a glucuronyl donor, catalyzed with  $\text{Ag}_2\text{CO}_3$ .<sup>101</sup> The same method was also later used for the synthesis of isotopically labeled deoxynivalenol-3- $\beta$ -D-[<sup>13</sup>C<sub>6</sub>]-glucopyranoside.<sup>102</sup>

Some studies, such as Schmidt *et al.* (2018), employed a dual approach combining microbial biotransformation and chemical synthesis. Microbial systems (yeasts and bacteria) were used to preferentially form  $\alpha$ -glucosides, while chemical methods yielded  $\beta$ -glucosides.<sup>11,12</sup> The very recent study by Hoogstra *et al.* describes biotransformation of deoxynivalenol to the novel metabolite deoxynivalenol-8,15-hemiketal-7-glucoside by the glycosyltransferase produced by *Bacillus subtilis*.<sup>103</sup>

However, enzymatic glycosylation has become the most prominent, specifically with recombinant UGTs expressed in *E. coli* used for producing glucosides of DON and NIV, particularly their C-3 conjugates.<sup>64</sup> Whole-cell maize culture systems have also been used to generate DON/NIV glucosides for biological studies,<sup>104</sup> whereas wheat suspension culture produced DON-15-G (**16**), 15-acetyl-DON-3-O-glucopyranoside (**20**), and 15-acetyl-DON-3-sulfate (**39**) (Figs. 4 and 9).<sup>105</sup>

In a study by Della Gala *et al.*, a library of 380 recombinant plant UGTs was investigated, and eight novel enzymes glycosylating DON *in vitro* were identified and characterized. Interestingly, four enzymes produced primarily a novel, still uncharacterized glucoside.<sup>64</sup>

Notably, Della Gala *et al.*<sup>64</sup> identified several recombinant plant UGTs capable of glycosylating deoxynivalenol (DON) *in vitro*, four of which produced an as yet uncharacterized DON-glucoside distinct from the known DON-3- and DON-15-glucosides. Based on chromatographic behaviour and MS data, they hypothesized that this conjugate could be a DON-7-O-glucoside, although structural confirmation was not possible due to the low yield of the metabolite. Almost simultaneously, Hoogstra *et al.*<sup>103</sup> reported the full characterization of a DON-8,15-hemiketal-7-O- $\beta$ -D-glucoside generated by a *Bacillus subtilis* glycosyltransferase (YjiC). Given the close correspondence in chromatographic and mass-spectrometric properties and the

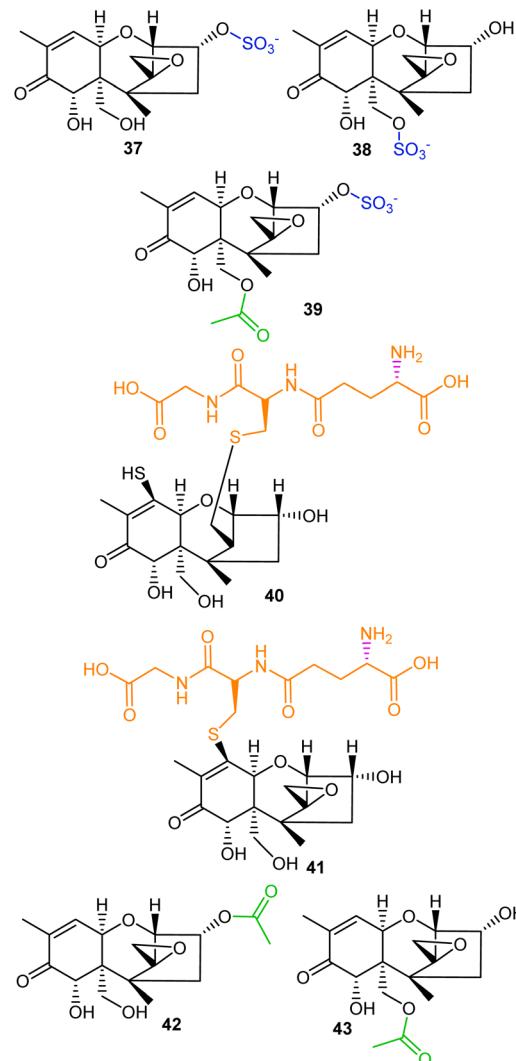


Fig. 9 Deoxynivalenol-3-sulfate (DON-3S, **37**), deoxynivalenol-15-sulfate (DON-15S, **38**), 15-acetyl-deoxynivalenol-3-sulfate (15Ac-DON-3S, **39**), deoxynivalenol-13-glutathione (DON-13GSH, **40**), deoxynivalenol-10-glutathione (DON-10GSH, **41**), 3-acetyl-deoxynivalenol (**42**), 15-acetyl-deoxynivalenol (**15Ac-DON**, **43**).

temporal proximity of both studies, it is plausible that the plant UGT-derived “uncharacterized glucoside” described by Della Gala *et al.* represents the same compound later identified by Hoogstra *et al.* as the DON-7-O-glucoside (existing predominantly in the 8,15-hemiketal form).

Both chemical and enzymatic approaches face key limitations. Regioselectivity is a major concern, as trichothecenes like HT-2 possess multiple hydroxyl groups (*e.g.*, at C-3 and C-4), and unprotected chemical glycosylation can lead to mixtures. While enzymatic reactions with recombinant UGTs offer regio- and stereoselectivity, plant-derived UGT isoenzymes can produce varying products.<sup>68</sup> Chemically controlling the anomeric configuration is a particular challenge:  $\beta$ -anomers typically dominate the synthetic reactions, whereas plants often produce both  $\alpha$ - and  $\beta$ -forms, including some that are likely derived from starch-processing enzymes.<sup>11</sup>



In a study of Svoboda *et al.*, *Fusarium sporotrichioides* was cultivated on sterilized rice and besides the respective aglycon, a mixture of two  $\alpha$ -glucosides, presumably HT-2-3-O- $\alpha$ -glucoside and HT-2-4-O- $\alpha$ -glucoside (unseparable on HPLC) was produced. Authors propose that the two HT-2  $\alpha$ -glucosides are not formed by a glucosyltransferase as they are in plants, but by a *trans*-glycosylating  $\alpha$ -glucosidase expressed by the fungus using starch as a substrate.<sup>70</sup>

In terms of practicality, yields remain a bottleneck. Microbial and plant cell transformations typically produce quantities in the milligram range and may result in multiple side-products that complicate purification. Chemical syntheses, though structurally precise, often require multiple protection and deprotection steps and can suffer from low overall yields.<sup>12</sup> Nevertheless, the combinations of synthetic and biological methods remain essential for the production of analytical standards and the study of masked trichothecenes in food and feed.

**3.2.2 Zearalenone glycosides.** The synthesis of glycosylated zearalenone (ZEN) derivatives is complicated by the presence of two reactive hydroxyl groups at C-14 and C-16 on the resorcylic acid lactone ring. Early efforts to achieve regioselective glycosylation involved protecting-group strategies. Hametner and Fröhlich employed silyl-protected intermediates to direct conjugation to the (less reactive) C-16 position, successfully synthesizing ZEN-16- $\beta$ -D-glucoside and a ZEN-16S.<sup>83,106</sup> However, purely chemical approaches were laborious and required careful protection and deprotection to achieve selectivity.

More recently, enzymatic methods have superseded purely chemical methods, offering greater regio- and stereo-selectivity. A major advance came with the use of recombinant barley UDP-glucosyltransferase HvUGT14077. Expressed in *E. coli*, this enzyme glucosylated ZEN at both the 14- and 16-hydroxyl groups, as well as its phase-I metabolites,  $\alpha$ - and  $\beta$ -zearalenol, yielding their corresponding 14-O-glucosides.<sup>83</sup> Fungal systems also offer effective biotransformations: strains of *Rhizopus* and *Aspergillus oryzae* were shown to convert ZEN into mixtures of glucosides and sulfates, including ZEN-14G, ZEN-16G, and ZEN-14S.<sup>107,108</sup> *Cunninghamella elegans* and *C. echinulata*, which have already been shown to be capable of glycosylating various complex molecules, including flavonoids, were used for the glycosylation of ZEN, yielding ZEN-14G, ZEN-16G, and also ZEN-14S. In the sulfate-depleted medium, *C. elegans* showed a strong preference for the production of ZEN-14G and ZEN-16G.<sup>109</sup>

Preparative-scale enzymatic synthesis using HvUGT14077 has proven especially efficient. *In vitro* reactions using UDP-glucose achieved near-complete conversion of ZEN to its 14-glucoside (~90%), with about 10% going to the 16-isomer.<sup>83</sup> To enhance the yield of the less favored ZEN-16G, an elegant strategy was applied: co-incubating the reaction with a  $\beta$ -glucosidase that selectively hydrolyzed the 14-glucoside, thereby shifting the equilibrium toward the 16-product and boosting its yield to ~85%. Additionally, the inclusion of a sucrose synthase system allowed *in situ* regeneration of UDP-glucose, maintaining high conversion efficiency throughout the reaction.<sup>83</sup>

ZEN glycosylation by the Koenigs-Knorr reaction was used for the preparation of zearalenone-14-O- $\beta$ -D-glucopyranoside and the corresponding  $\beta$ -glucuronide, followed by NaBH<sub>4</sub> reduction of the 7-keto group of the aglycon moiety, yielding respective glycosides of  $\alpha$ -ZEL and  $\beta$ -ZEL.<sup>110</sup> Zearalenone-14- $\beta$ -D-gentiobioside was prepared from peracetylated gentiobiosyl- $\alpha$ -bromide as glycosyl donor using phase-transfer glycosylation (tetrabutylammonium bromide as catalyst in borate buffer pH 10.5/CHCl<sub>3</sub>); after deacetylation with KOH, the yield of ZEN-14- $\beta$ -gentiobioside was 43%.<sup>111</sup>

In conclusion, the preparation and identification of sulfated and multi-conjugated forms (mixed conjugates – sulfates & glucuronides) often require multi-step procedures, either by fungal fermentation<sup>108</sup> or *via* chemical protection followed by sulfation.<sup>106</sup> Until recently, the limited availability of reference standards for conjugates such as ZEN-14-G and ZEN-14-sulfate hindered routine detection, but recent biosynthesis efforts have begun to close this gap.<sup>108</sup>

**3.2.3 *Alternaria* glycosides.** The glycosylation of *Alternaria* toxins has been studied primarily through biological rather than chemical methods, as the range of possible modifications – such as glucosylation, sulfation, and malonylation – would be highly complex to achieve synthetically in a stepwise manner. In practice, plant-based systems, particularly cell suspension cultures like tobacco BY-2 cells, have served as highly effective biocatalytic platforms. When fed with purified AOH or AME, these cultures are capable of converting more than 50% of the parent toxins into glycosides over a few days.<sup>14,85</sup> For example, one study reported the biotransformation of AOH into approximately 58% AOH-3-glucoside and 5% AOH-9-glucoside, which were subsequently isolated using preparative HPLC.<sup>14</sup>

While the chemical glycosylation of AOH – specifically at phenolic hydroxyl groups – is theoretically feasible using methods such as Koenigs-Knorr glycosylation or phase-transfer catalysis, it has not been a major focus in the literature. This is probably due to the high efficiency, regioselectivity, and stereospecificity of the enzymatic systems, which usually produce the desired  $\beta$ -anomers directly. A notable enzymatic alternative to whole-cell systems is the screening of UDP-glucosyltransferase (UGT) enzyme libraries to identify individual UGTs that can catalyze the glucosylation of AOH and AME.<sup>14</sup> This approach provides a more controlled enzymatic strategy and can deliver specific glucoside products without relying on whole plant cells.

A semi-biosynthetic approach was used to obtain more complex conjugates such as sulfoglucosides. For example, Soukup *et al.* synthesized AOH sulfates chemically or enzymatically and then incubated them with plant cells to obtain diconjugated products such as alternariol sulfoglucosides.<sup>85</sup> This two-step method mimics the natural pathway in which a fungal enzyme catalyzes the sulfation and a plant enzyme subsequently adds a glucose moiety. These results also illustrate the contribution of organisms in mixed systems: plant cells alone do not sulfate AOH, and fungi do not glycosylate sulfated AOH, demonstrating the cooperative interplay of fungal and plant enzyme activities.



A wide range of conjugates has now been documented. The most important known glucosides include alternariol-3-*O*- $\beta$ -D-glucoside and alternariol-9-*O*- $\beta$ -D-glucoside, in which the glucose moiety is attached to the 3- and 9-hydroxyl groups, respectively.<sup>85</sup> These can be further transformed in plant systems to produce malonylated derivatives, such as alternariol-3-*O*-(6'-O-malonyl- $\beta$ -D-glucoside) and alternariol-9-*O*-(6'-O-malonyl- $\beta$ -D-glucoside), in which the glucose is esterified with malonic acid. This malonylation step, which adds 86 Da to the mass, is a typical plant detoxification mechanism that increases polarity and facilitates vacuolar sequestration.<sup>85</sup>

In some cases, even more complex derivatives such as disaccharide conjugates have been observed. Alternariol-3-*O*- $\beta$ -D-gentiobioside, for example, contains a  $\beta$ 1 → 6-linked glucose disaccharide (gentiobiose) attached at the 3-position of AOH.<sup>85</sup> Although this type of modification is less common, it suggests that plants can extend detoxification by adding a second sugar moiety to a primary glucoside. In addition, diconjugated molecules containing both sulfate and glucose groups—such as alternariol-3-sulfate-9-glucoside and alternariol-9-sulfate-3-glucoside—have been isolated and structurally confirmed.<sup>85</sup> A similar diconjugate, AME-3-sulfate-7-glucoside (also described as alternariol-9-O-methyl ether-7-O-glucoside-3-O-sulfate), was also identified in the same study. Acetylated glucosides, such as alternariol-3-*O*-acetyl- $\beta$ -D-glucoside, have been postulated but are still poorly characterized.<sup>14</sup> To date, all known *Alternaria* toxin glucosides have glucose as a sugar group; rhamnose-containing conjugates have not yet been reported.

**3.2.4 Ochratoxin A glycosides.** Glycosylation of OTA was achieved by incubation of OTA with plant tissue or microbes. To date, no specific isolated enzyme for OTA glucoside synthesis has been reported, and only trace amounts of OTA-4-Glc have been obtained from plant incubations. Therefore, structural confirmation is mainly based on MS/MS comparison with analogs. In the 2020 EFSA review, the metabolism of OTA to (4*R*)/(4*S*)-4-hydroxy-OTA and their conjugates was detected in spiked plant experiments,<sup>6</sup> suggesting the potential for future *in vitro* expression of plant UGT candidates, although this work has not yet been published.

Chemical glycosylation of OTA has not been the focus of synthetic research to date, likely due to detoxification strategies that emphasize cleavage of phenylalanine to generate non-toxic OT $\alpha$ . Theoretically, glucosyl esters on the carboxyl group of OTA are possible, but have not yet been reported. Currently, the only characterized OTA glucosides are C-4 derivatives formed after enzymatic hydroxylation (Fig. 10). The C-10 (methyl group) position could also be hydroxylated,<sup>112</sup> but this is speculative.

Besides the problems with the low abundance of OTA glycosides, stability is another obstacle. OTA glucosides can decompose during sample preparation, especially under acidic or basic conditions, requiring mild extraction protocols.<sup>58</sup>

**3.2.5 Ergot alkaloid glycosides.** Due to the synthetic complexity, aglycone sensitivity, and low yields of conventional glycosylation techniques, researchers have explored a wide range of chemical and enzymatic methods to prepare EA glycosides. Early synthesis employed Koenigs-Knorr

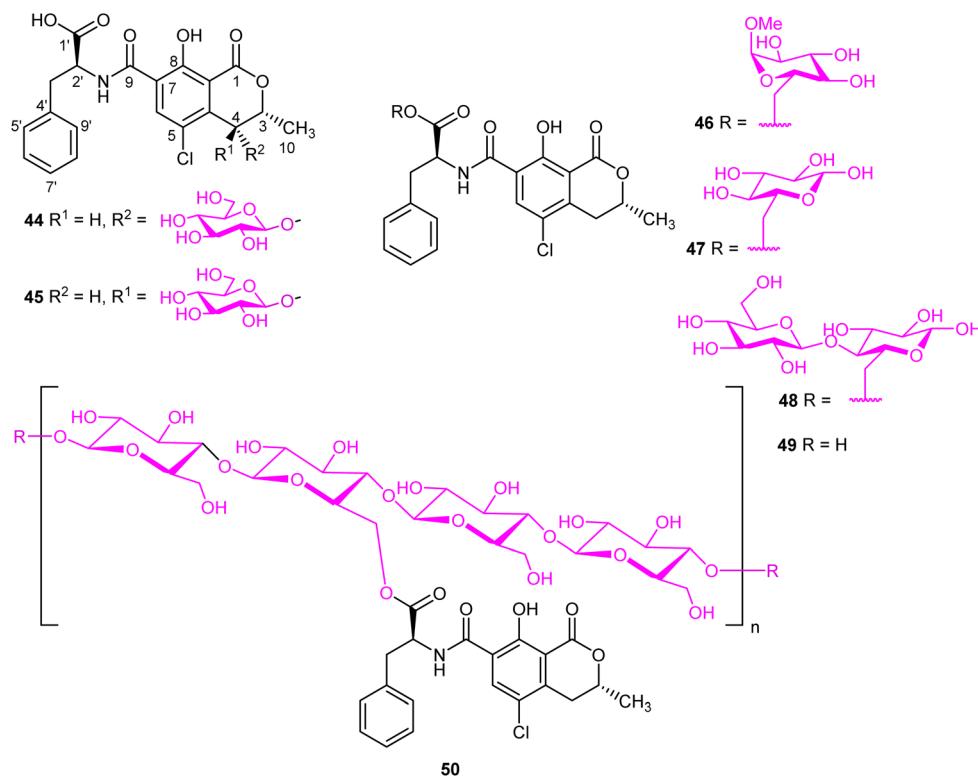


Fig. 10 4(*R*)-Hydroxyochratoxin A  $\beta$ -glucopyranoside (44), 4(*S*)-hydroxyochratoxin A  $\beta$ -glucopyranoside (45), ochratoxin A-methyl- $\alpha$ -D-glucopyranoside ester (46), ochratoxin A glucose ester (47), ochratoxin A cellobiose ester (48), ochratoxin A (OTA, 49), OTA esterified to poly-saccharide (50).

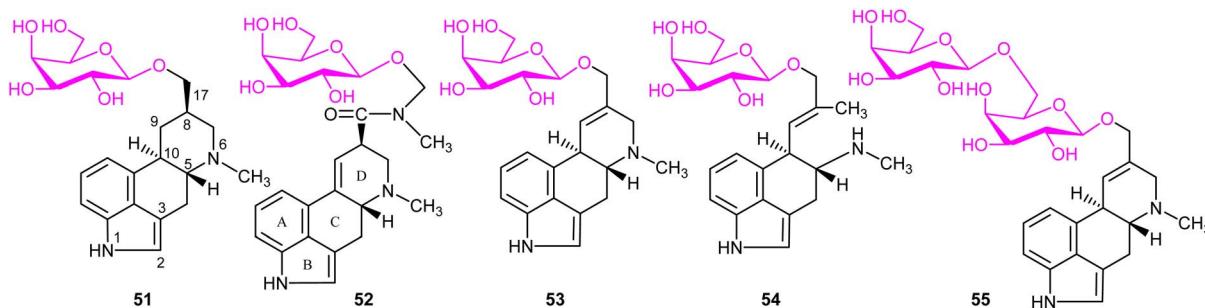


Fig. 11 10-Dihydrolysergol- $\beta$ -O-galactopyranoside (51), ergometrine- $\beta$ -O-galactopyranoside (52), elymoclavine- $\beta$ -O-galactopyranoside (53), chanoclavine- $\beta$ -O-galactopyranoside (54), elymoclavine-digalactoside (55).

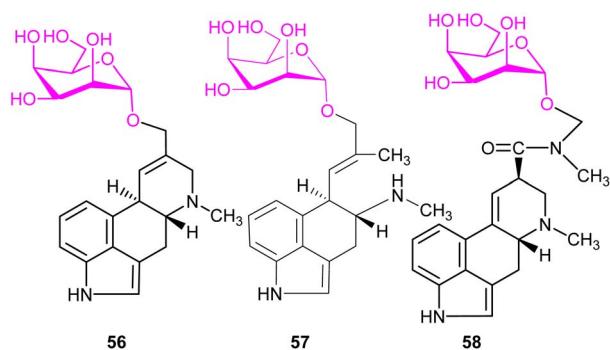


Fig. 12 Elymoclavine- $\alpha$ -O-mannopyranoside (56), chanoclavine- $\alpha$ -O-mannopyranoside (57), ergometrine- $\alpha$ -O-mannopyranoside (58).

glycosylation with peracetylated glycosyl halides and silver salts, but this yielded poor selectivity and unstable orthoesters.<sup>113</sup>

Improved results were obtained using trimethylsilyl triflate as a promoter with peracetylated sugars, affording mono-glycosides of elymoclavine and lysergol (Fig. 11 and 12) in ~40% yield, though side reactions like aglycone acetylation limited scalability.<sup>114</sup>

To address these drawbacks, biocatalytic methods have gained attention. Enzymes such as  $\beta$ -glucosidase,<sup>114</sup>  $\beta$ -galactosidase,<sup>115</sup>  $\alpha$ -mannosidase<sup>116,117</sup> and  $\beta$ -hexosaminidase<sup>118</sup> have been used to transfer sugars from activated donors to various EAs (Fig. 11-13). These enzymatic approaches offer higher regioselectivity and mild conditions, preserving the sensitive EA core. Notably, glycosylation of ergometrine was achieved enzymatically, demonstrating applicability to EAs with labile structures.

Beyond simple mono- and disaccharides, more complex EA glycosides with lactosyl, LacNAc, and sialyl residues have been synthesized using sequential enzymatic steps involving  $\beta$ -1,4-galactosyltransferase, epimerases, and sialyltransferase (Fig. 14).<sup>119</sup> These products are valuable for studying glycan-mediated interactions such as host-pathogen recognition or targeted drug delivery.

EAs have also been converted into  $\beta$ -N-ribosides and  $\beta$ -N-deoxyribosides *via* silylation and Lewis acid-promoted coupling, yielding analogs that mimic antiviral and anticancer nucleosides (Fig. 15).<sup>120,121</sup> Some of these showed reduced cytotoxicity compared to parent compounds, with *N*-ribosylation attenuating toxic effects.

Ergot alkaloid glycosides thus represent promising tools for functional diversification, drug development, and mainly for toxicological evaluation. Their roles as potential masked or modified mycotoxins in food and their synthetic versatility highlight the need for continued research into detection methods and therapeutic applications.

### 3.3 Glycosylated mycotoxins during food processing and occurrence in food

The occurrence and transformation of glycosylated mycotoxins during various stages of food processing, such as milling,

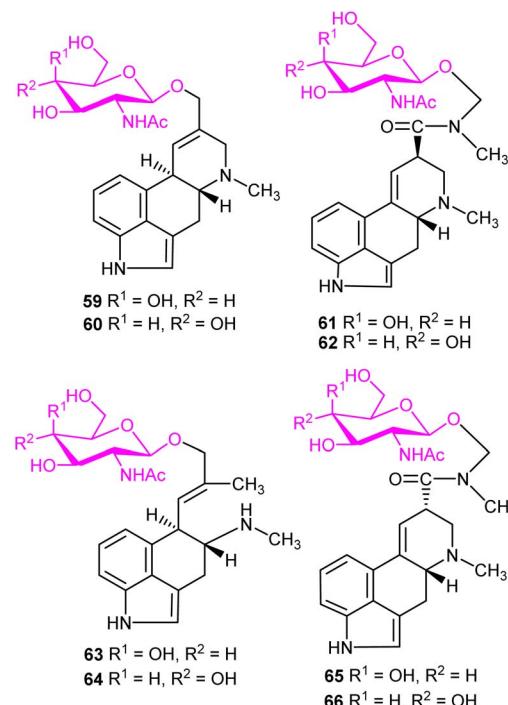


Fig. 13 Elymoclavine 2-acetamido-2-deoxy- $\beta$ -O-galactopyranoside (59), elymoclavine 2-acetamido-2-deoxy- $\beta$ -O-glucopyranoside (60), ergometrine 2-acetamido-2-deoxy- $\beta$ -O-galactopyranoside (61), ergometrine 2-acetamido-2-deoxy- $\beta$ -O-glucopyranoside (62), chanoclavine 2-acetamido-2-deoxy- $\beta$ -O-galactopyranoside (63), chanoclavine 2-acetamido-2-deoxy- $\beta$ -O-glucopyranoside (64), ergometrinine 2-acetamido-2-deoxy- $\beta$ -O-galactopyranoside (65), ergometrinine 2-acetamido-2-deoxy- $\beta$ -O-glucopyranoside (66).



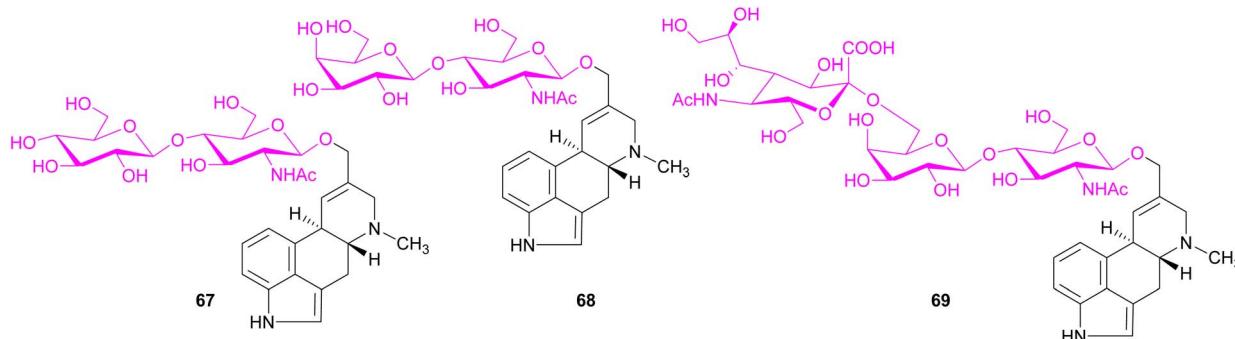


Fig. 14  $\beta$ -D-Glucopyranosyl-(1-4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl-(1-O)-elymoclavine (67),  $\beta$ -D-galactopyranosyl-(1-4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl-(1-O)-elymoclavine (68),  $\alpha$ -5-N-acetylneuraminyl-(2-6)- $\beta$ -D-galactopyranosyl-(1-4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl-(1-O)-elymoclavine (69).

baking, malting, and brewing, represent critical concerns in food safety. Although numerous studies have indicated that glycosylated mycotoxins are highly stable under food processing conditions, suggesting that a significant portion remains unaltered, other research has shown that certain processing techniques can even lead to a substantial increase in these mycotoxin glycosides in food products.

During milling, cereal grains are broken down into flour through various mechanical processes, which can influence the distribution and fate of mycotoxins. Recent studies demonstrate that during dry milling of cereal grains such as wheat and maize, glycosylated mycotoxins, including DON 3G and ZEN 14G, show distinctive distribution patterns among milling fractions. In naturally contaminated wheat, DON and DON 3G are distributed relatively evenly across bran, shorts, and flour fractions, with only slightly higher levels in bran; as a result, dry milling does not effectively remove masked DON forms.<sup>122</sup> By contrast, ZEN 14G and ZEN 14S are significantly enriched in fibre-rich outer fractions, allowing effective reduction in refined flour.<sup>122,123</sup> Thus, dry milling can result in fractionation, where modified mycotoxins may persist or even accumulate in byproducts, highlighting the importance of monitoring these fractions in feed and recycling chains.

Findings by Kostelanská *et al.* showed that white flour contained approximately 60% of the DON and DON-3G levels found in unprocessed cereal grain. In fermented dough, the amount of DON remained relatively unchanged, but DON-3G increased on average by up to 145%. A subsequent decrease in both DON and DON-3-Glc was observed during baking.<sup>124</sup> Conversely, another study reported more unexpected findings – an increase in DON levels during baking, accompanied by a decrease in DON-3G, making a possible release of the parent mycotoxin from the conjugated form.<sup>125</sup> The final concentration of DON-3G may also be significantly influenced by the use of bakery improver enzymes, which have been shown to increase the levels of this conjugated mycotoxin in the final product.<sup>124</sup> Similar outcomes were reported by Vidal *et al.*, who studied the effects of xylanase and  $\alpha$ -amylase addition during breadmaking.<sup>126</sup> Baking involves exposing dough to high temperatures, which might traditionally be expected to partially degrade mycotoxins. This was confirmed by Kostelanská *et al.*, who observed slight thermal degradation of DON and DON-3G, specifically, 10% and 13%, respectively. They also identified several thermal degradation products, including nor-DON A, B, C, D; DON-lactone; nor-DON-3-Glc A, B, C, D; and DON-3-Glc-lactone, primarily detected in the crust of baked bread.<sup>124</sup>

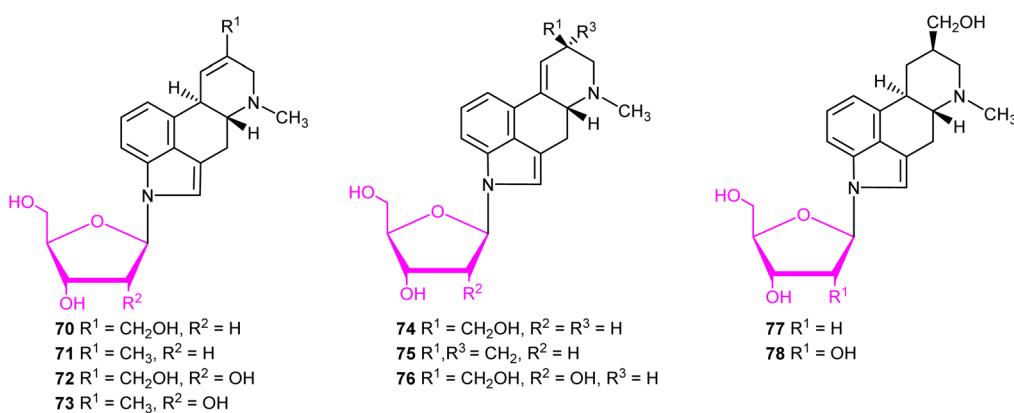


Fig. 15 Elymoclavine- $\beta$ -N-2-deoxyriboside (70), agroclavine- $\beta$ -N-2-deoxyriboside (71), elymoclavine- $\beta$ -N-riboside (72), agroclavine- $\beta$ -N-riboside (73), lysergol- $\beta$ -N-2-deoxyriboside (74), lysergine- $\beta$ -N-2-deoxyriboside (75), lysergol- $\beta$ -N-riboside (76), 9,10-dihydrolysergol- $\beta$ -N-2-deoxyriboside (77), 9,10-dihydrolysergol- $\beta$ -N-riboside (78).



In addition to DON-3G, Bryla *et al.* also investigated the fate of NIV-3G and ZEN-14G during bread production. The concentrations of NIV-3G and DON-3G increased slightly during dough kneading and fermentation, probably due to the enzymatic activity of the malt flour. A significant average decrease of 21% was observed only for ZEN-14G, followed by a further decrease of 19% from ready-to-bake dough to baked loaf crumb. As ZEN concentrations also decreased during bread production, these mycotoxins were likely transformed into degradation products with altered chemical structures.<sup>127</sup>

Data on the behavior of T-2-G and HT-2-G during food processing remains limited, mainly because analytical standards are not commercially available. As a result, many studies only report signal intensities without quantitative values. De Angelis *et al.* found that HT-2-G levels in bread appeared to decrease, while T-2-G levels showed the opposite trend. However, these results may have been influenced by differences in ionization efficiency and matrix effects.<sup>125</sup>

Malting represents a critical phase in the brewing process, during which barley undergoes germination followed by kilning. This stage facilitates enzymatic reactions that can lead to the formation and transformation of mycotoxins into their glycosylated forms. The first study to report an increase in DON-3G levels during malting and brewing was conducted by Lancová *et al.* These findings highlighted that even barley initially deemed safe may release substantial amounts of DON-3G during malting, suggesting that conventional pre-malting screening for *Fusarium* toxins does not fully eliminate the associated risks.<sup>128</sup> Since then, a significant increase in DON-3G during the germination phase of malting has been confirmed by several other studies.<sup>129–131</sup> Ksieniewicz-Woźniak *et al.* observed a similar trend for NIV-G,<sup>130,132</sup> while Průšová *et al.* reported analogous results for T-2-G and HT-2-G.<sup>133</sup> Zachariášová *et al.* also documented a continuous rise in DON-oligoglycoside signals throughout the malting process.<sup>78</sup> This phenomenon may be attributed to the *de novo* growth of *Fusarium* spp. during germination, which leads to the production of additional free mycotoxins, which may subsequently undergo enzymatic “masking” through conjugation with glucose, catalyzed by glycosyltransferases and other related enzymes active during barley germination.<sup>78,134</sup>

The brewing process is highly complex, involving extensive enzymatic activity and fermentation, both of which significantly influence the fate of mycotoxin glycosides. A pilot study by Lancová *et al.* demonstrated a substantial increase in DON-3G levels during brewing – its concentration in sweet wort was approximately ten times higher than in the original malt grist. This increase is attributed to the activity of enzymes released during the mashing stage, which degrade cell walls, membrane-bound proteins, and starch in the grain, thereby liberating DON-3G from insoluble forms.<sup>128</sup> The increase in DON-3G during mashing was also confirmed by Habler *et al.*, who, however, reported a subsequent rapid decline in DON-3G levels during the following stages of lautering and wort boiling. This decrease may be linked to enzymatic activity in the barley, potentially involving the conversion of DON-3G into DON-oligo/polyglucosides, deoxynivalenol-glutathione adducts (**40**, **41**), or

other biologically modified DON derivatives.<sup>135</sup> During fermentation, yeasts and other microorganisms can interact with mycotoxin glycosides, affecting their stability and bioavailability. The metabolic activity during this stage, including the production of ethanol and other fermentation byproducts, may alter the concentration and form of these compounds in the final beer product. A study by Nathanail *et al.* confirmed that the yeast *Saccharomyces pastorianus* reduced DON-3G levels by approximately 17%, likely through adsorption of this glycosylated mycotoxin onto the yeast cell walls. Interestingly, the study also demonstrated that yeast was capable of glucosylating DON to form DON-3G; however, no further metabolism or deglycosylation was observed.<sup>136</sup> The behavior of trichothecene mycotoxins and their glycosylated forms during brewing remains only partially understood. As demonstrated by Kostelanská *et al.*, the overall trend, whether an increase or decrease, can vary significantly throughout the brewing process, depending strongly on the characteristics of the input barley and its initial mycotoxin contamination levels.<sup>137</sup> Nevertheless, in beer, DON-3G is commonly detected at a higher frequency and often at equimolar or even higher levels than DON.<sup>137–139</sup>

In the case of modified *Alternaria* mycotoxins in malt and beer, not glycosylated but predominantly sulfated forms were identified. This indicates that, for *Alternaria* toxins, the malting process favors sulfation over glucosylation.<sup>39,133</sup>

Although *Fusarium* mycotoxin glycosides are predominantly associated with cereals, glycosylated forms of OTA have been identified as a major concern in coffee and wine samples. Humpf *et al.* identified several products of roasted OTA, including an OTA-glucose ester, an OTA-methyl-glucopyranoside ester, and an OTA-cellobiose ester. In these, the sugar is attached *via* an ester linkage to the OTA carboxyl, not *via* the phenolic hydroxyl. Such esterification likely happens when the heat causes OTA's carboxylic acid to react with the hydroxyls on sugars (a condensation reaction, eliminating water).<sup>140,141</sup> In practical terms, the enzyme-formed glycosides (plant-masked OTA) are considered a bioavailable reservoir of OTA because digestive enzymes or gut microflora can cleave them, liberating OTA, whereas the non-enzymatic sugar esters (like those found in coffee) might not readily release OTA in the digestive tract and thus could be less of a toxicological concern. Indeed, Cramer *et al.* noted that the OTA-polysaccharide esters formed in coffee were of significantly reduced cytotoxicity and likely not absorbed. On the other hand, the epimer 2'R-OTA (formed by thermal isomerization at the phenylalanine chiral center during roasting) was detected in the blood of coffee drinkers, showing that some roasting by-products are absorbable.<sup>140</sup>

Winemaking presents a unique case where the glucosylation of OTA in grapes can be further modulated during fermentation, leading to a mixture of parent and conjugated forms in the final product. However, the extent to which these glycosylated forms contribute to the total OTA burden post-consumption is still an open question.<sup>58</sup> Furthermore, thermal processing and interactions with food matrices may influence the formation or stability of OTA conjugates.<sup>57</sup>



### 3.4 Biotransformation in the GI tract and related health effects

An important question is whether glycosylated mycotoxins pose a lower or comparable risk to that of free, non-conjugated toxins. From the plant's perspective, glycosylation markedly reduces toxicity. However, the implications for human and animal health are more complex. The interactions between mycotoxins and the gut microbiota have been comprehensively reviewed by Guerre,<sup>142</sup> while both *in vitro* and *in vivo* models for studying the metabolism of modified mycotoxins are thoroughly discussed in a review by De Boever *et al.*<sup>143</sup>

**3.4.1 Trichothecene glycosides.** The glucosylated forms, such as DON-3G *per se*, are not well absorbed in the upper GI tract.<sup>21</sup> DON-3G has been shown to be relatively stable towards artificial gastric juice.<sup>144</sup> Studies in pigs and poultry showed that DON-3G passes through the stomach and small intestine largely intact and is hydrolyzed to DON by the intestinal microbiota in the large intestine.<sup>145</sup> Administration of DON-3G to rats (p.o.) showed that most of the DON-3G was recovered from the feces in the form of DON and DOM. These results suggest that DON-3G appears to be less bioavailable compared to DON and therefore of less toxicological relevance, at least in rats.<sup>146</sup> Another study showed that the microbiota of the small and large intestine of pigs rapidly hydrolyze DON-3G to DON. The microbiota of the jejunum hydrolyzed DON-3G very slowly, while samples from the ileum, cecum, colon, and feces hydrolyzed it very rapidly and efficiently.<sup>147</sup> The transformation of DON-3G to DOM-1 by fecal microflora was also demonstrated in humans.<sup>144</sup> Another study showed that DON-3G was cleaved by gut microbiota, namely by various *Lactobacilli*.<sup>148,149</sup> This finding was later corroborated in a detailed study with purified  $\beta$ -glucosidases from *Lactobacillus brevis* and *Bifidobacterium adolescentis*.<sup>150</sup> The recombinant enzyme from *B. adolescentis* displayed high flexibility in substrate specificity and showed the highest hydrolytic activity toward 3-O- $\beta$ -D-glucosides of both DON and NIV. Ultimately, the presence of these modified forms can contribute to overall toxin exposure. The phase II metabolism can further detoxify the released aglycones to glucuronides.

Both T-2-Glc and HT-2-Glc are remarkably stable in the upper gastrointestinal tract, resisting acidic hydrolysis and enzymatic digestion. However, they undergo microbial hydrolysis in the colon, where human gut microbiota cleave the glucosidic bond, releasing the parent toxins.<sup>11,149,151,152</sup> For T-2-3G, no structural changes were detected following exposure to saliva or digestive fluids from the small intestine; the study was performed with both T-2- $\alpha$ -G and T-2- $\beta$ -G anomers.<sup>11</sup> Nonetheless, fecal microbiota was able to metabolize both T-2- $\alpha$ -Glc and T-2- $\beta$ -Glc, with different degradation rates depending on the stereochemistry (T-2- $\alpha$ -Glc was converted to T-2 (by 13%) and HT-2 (by 30%), and T-2- $\beta$ -Glc to T-2 (by 58%) and HT-2 (by 12%)).<sup>11</sup> The extent of transformation appears to be strongly influenced by the specific composition of gut microbiota. It has been demonstrated that *Butyrivibrio fibrisolvens*, *Roseburia intestinalis*, and *Eubacterium rectale* are among the bacterial

strains capable of hydrolyzing HT-2-Glc efficiently, with little impact on bacterial growth or diversity.<sup>149</sup>

In some experiments, a full (100%) conversion of HT-2- $\beta$ -Glc to HT-2 was observed after a 24-hour incubation with human fecal samples.<sup>151</sup> A similar effect was confirmed in an *ex vivo* model using porcine jejunum and microbiota, where both T-2-Glc and HT-2-Glc were rapidly and effectively hydrolyzed to their aglycones. Further conversion of these aglycones into T-2 triol and HT-2 was also observed.<sup>153</sup>

**3.4.2 Zearalenone glycosides.** Indeed, modified zearalenone compounds are readily hydrolyzed in the colon, especially in the large intestine, yielding ZEN and  $\alpha$ -ZEL that can then be absorbed.<sup>13</sup> In one *in vitro* study, human fecal microbiota completely converted ZEN-14G to ZEN within 1 hour.<sup>149</sup> Thus, although plants can reduce the estrogenic burden short-term by conjugation, the modified ZEN still contributes to exposure upon consumption. After oral administration of ZEN-14G, metabolism occurs, leading to the formation of  $\alpha$ -ZEL-14G.<sup>154</sup> It has been reported that following intravenous administration of  $\alpha$ -ZEL and  $\alpha$ -ZEL-14G, ZEA-14G,  $\alpha$ -ZEL-14G, and glucuronic acid (GlcA) conjugates were detected in plasma.<sup>87</sup> The calculated elimination rates of  $\alpha$ -ZEL and  $\alpha$ -ZEL-14G indicate low retention of these compounds in the organism.<sup>82</sup> The preferred biochemical pathways of  $\alpha$ -ZEL-14G metabolism include not only conjugation with GlcA but also dehydrogenation and hydrolysis. After apical administration of ZEA-14G and ZEA-16G to the polarized monolayers of Caco-2 cells, both glycosides can be detected in cellular extracts, indicating uptake by intestinal cells. Moreover, the glycosides were cleaved to release ZEN, demonstrating that human cytosolic glucosidase is able to cleave ZEA-14G.<sup>155</sup> *In vitro* experiments using animal and human liver microsomes demonstrated phase II metabolism, resulting in the formation of  $\beta$ -ZEL-14G and ZEN-14G. Additionally,  $\alpha$ -ZEL-14G may undergo hydrolysis, releasing glucose and forming  $\alpha$ -ZEL. This metabolite can be further dehydrogenated to ZEA, which is subsequently conjugated with GlcA to generate various other metabolites.<sup>82</sup> Dehydrogenation followed by GlcA conjugation of  $\alpha$ -ZEL-14G may lead to the formation of the metabolites  $\alpha$ -ZEL-14G-7-GlcA and  $\alpha$ -ZEL-14G-16-GlcA.<sup>154</sup> The preferred site for glucuronidation in liver microsomes of rats, chickens, pigs, and humans was the C-16 carbon. In ruminants (goats and cows), GlcA conjugation occurred primarily at the C-7 position and, to a lesser extent, at C-14.<sup>154</sup> Urine and fecal samples collected after oral administration in pigs did not contain  $\alpha$ -ZEL,  $\alpha$ -ZEL-14G, or their metabolites. However, significant glucuronic acid conjugates derived from  $\alpha$ -ZEL and  $\alpha$ -ZEL-14G were identified in feces after 24 hours, likely associated with enterohepatic recirculation.<sup>156,157</sup>

**3.4.3 Alternaria toxin glucosides.** Unlike other glycosylated mycotoxins, the biotransformation of *Alternaria* toxin glycosides remains largely unexplored. While the metabolism and toxicological relevance of several *Fusarium*-derived glycosides have been well characterized *in vitro* and *in vivo*, only limited data are available for glycosylated *Alternaria* metabolites, and to our best knowledge, their fate in the gastrointestinal tract is practically unknown.



Recent *in vitro* investigations using differentiated Caco-2 human intestinal epithelial cells have shed light on the absorption and metabolism of *Alternaria* monoglucosides, specifically alternariol-3-glucoside (AOH-3G, 12), alternariol-9-glucoside (AOH-9G, 13), and alternariol monomethylether-3-glucoside (AME-3G, 14) (Fig. 3). These studies show that AOH-9G is taken up by Caco-2 cells and efficiently deglucosylated, followed by phase II conjugation into glucuronide and sulfate metabolites, many of which appear on the basolateral side, indicating *trans*-epithelial transport into portal circulation. In contrast, AOH-3G and AME-3G are hydrolyzed to a significantly lesser extent, probably reflecting the positional specificity of  $\beta$ -glucosidase action.<sup>158</sup> Interestingly, AOH diglucoside (AOH-9,9'-DiGlc) shows negligible uptake or hydrolysis, suggesting very low bioavailability of these highly polar forms. The authors nevertheless concluded that modified *Alternaria* toxins can contribute to overall toxicity by efficiently transporting the toxin across the intestinal barrier in a modified form, which is then enzymatically deglucosylated and can exert toxic effects. This result supports earlier assumptions and underlines the need to include glycosylated *Alternaria* toxins in risk assessments.<sup>158</sup>

**3.4.4 Ochratoxin glycosides.** Glycosylated derivatives of OTA, such as OTA-glucosides and 4-hydroxyochratoxin- $\beta$ -D-glucosides (4-OH-OTA-G), are generally considered to be significantly less toxic than respective aglycones. Due to their increased polarity and size, these conjugates do not readily penetrate cell membranes and do not bind effectively to the usual molecular targets of OTA. Therefore, they are widely regarded as pro-toxins, *i.e.*, compounds that can only become toxic by enzymatic hydrolysis, releasing the parent OTA or 4-OH-OTA. *In vitro* studies confirm this lower toxicity: in the absence of glucosidase activity to cleave the glycosidic bond, these forms show minimal cellular effects.<sup>159</sup> After ingestion, partial or complete hydrolysis in the digestive tract may restore toxicity, although bioavailability may be somewhat reduced compared to native OTA. A pig study reviewed by JECFA<sup>53</sup> showed that approximately 50% of an orally administered dose of OTA glucoside was recovered as OTA, with the remainder occurring as metabolites or bound residues. This suggests that glycosylation may mitigate acute toxicity but cannot completely eliminate the risk, especially for highly potent toxins such as OTA. Thus, the overall toxic potential of OTA glucosides depends on factors such as gastrointestinal enzyme activity, microbiota composition, and transit time, which influence how much OTA is ultimately released and absorbed. However, intrinsic toxicity or absorption, as well as bioavailability, cannot be excluded. Animal studies and *in vitro* tests are required to determine whether ochratoxin glucosides are toxic *per se* or only pose a risk through reconversion to OTA.<sup>6</sup>

Mammals primarily metabolize OTA *via* hydrolysis (to OT $\alpha$ ), hydroxylation (to 4-OH-OTA and others), and conjugation with glucuronic acid or sulfate.<sup>160</sup>  $\beta$ -Glucosidation is not a typical mammalian pathway. However, in a notable study in rodents (F344 rats administered OTA), "OTA glucosides" were detected in urine, albeit at low concentrations.<sup>161</sup> This suggests that either the intestinal microflora or low enzyme activity in the

liver produces a glucose conjugate of OTA (or its hydroxylates). It is possible that the intestinal microbiota of rats conjugated a sugar to OTA (some microbes have glycosyltransferases or perform transglycosylation during fermentation in the cecum). However, in pigs or humans, there is no clear evidence of OTA-glucose conjugates *de novo*. Instead, humans and pigs extensively form OTA glucuronides (three positional isomers: OTA conjugated at the carboxyl, at the phenolic OH, or the amide nitrogen).<sup>162</sup>

Many intestinal bacteria produce  $\beta$ -glucosidases; OTA- $\beta$ -D-glucosides would likely be hydrolyzed, releasing 4-OH-OTA (the aglycone) plus glucose. The site of hydrolysis could be the small intestine (*via* brush-border lactase-phlorizin hydrolase or other  $\beta$ -glucosidases with broad specificity) or the large intestine (bacterial enzymes). Once 4-OH-OTA is released, it can be absorbed. 4-OH-OTA is more polar, so its oral bioavailability is somewhat lower than that of OTA, but when absorbed, it circulates and can bind albumin (albeit somewhat less avidly than OTA). When 4-OH-OTA is absorbed, it can be further metabolized: much of it could be glucuronidated and excreted in the urine, resulting in systemic OTA exposure, mainly in the form of the metabolite 4-OH-OTA and subsequent products.<sup>6</sup> One potential difference: the (unchanged) OTA has a very long half-life in humans (days to weeks, due to strong albumin binding).<sup>53</sup> As 4-OH-OTA is more polar, it may not bind as strongly to serum proteins and could be cleared faster. This means that the effective residence time of the toxin could be shorter when OTA is administered in glucosylated form, reducing certain chronic effects but not necessarily acute toxic interactions. However, 4-OH-OTA may still have toxic effects in certain organs (*e.g.*, immunosuppression).

OTA undergoes enterohepatic recirculation in some species (is excreted in the bile as glucuronide, then the intestinal flora can deconjugate and reabsorb free OTA).<sup>163</sup> If OTA was originally ingested as a glucoside, once it is converted to free OTA or 4-OH-OTA and absorbed, it can enter the same cycle. However, if it is converted to OT $\alpha$ , OT $\alpha$  is rapidly excreted (OT $\alpha$  is not recirculated to any significant extent as it is very polar and mostly harmless). The presence of the glucoside could therefore indirectly accelerate excretion if it directs metabolism towards OT $\alpha$  formation by first providing 4-OH-OTA (which can be more easily further hydrolyzed by the enzymes). These mechanistic nuances remain hypothetical, as no *in vivo* study has explicitly compared the toxicokinetics of OTA and OTA glucoside side-by-side.

In humans, exposure to OTA glucosides would occur primarily through plant foods (cereals, perhaps coffee). Human digestive enzymes and microbiota would release OTA. As the half-life of OTA in humans is in the order of weeks,<sup>53</sup> even a slightly lower bioavailability of the glucoside would not eliminate the risk as released OTA accumulates. It is unclear whether humans excrete OTA as a glucoside. Probably not, because extensive biomonitoring studies find OTA and OT $\alpha$  and OTA glucuronides in urine, but no glucose. In a study on biomonitoring in pregnant women, OTA and OT $\alpha$  were mentioned in the urine, but no OTA-Glc.<sup>164</sup>



## 4. Analytical methods

The analysis of glycosylated mycotoxins encompasses several dimensions, each of which reflects the progress of scientific understanding in this area over time. The concept of "masked mycotoxins" emerged when early analytical methods failed to detect glycosylated forms.<sup>13</sup> This was largely due to their altered polarity and distinct chromatographic behavior compared to the free mycotoxins. Furthermore, glycosylation complicates detection, particularly when mass spectrometry (MS) operates in targeted acquisition modes focused on predefined ions, where unexpected glycosylated forms may be overlooked. The attached sugar moiety shifts the molecular mass (and thus the *m/z* ratio) of the parent compound, which affects detection in multiple reaction monitoring (MRM) tandem MS, widely regarded as the "gold standard" in mycotoxin analysis. Such glycosylated toxins may remain unrecognized unless the corresponding mass transitions are predefined, which in practice requires commercial or in-house reference standards.<sup>11,165–171</sup> However, the advent of high-resolution mass spectrometry (HRMS) instruments with full-spectrum acquisition, such as time-of-flight (TOF) or Orbitrap analyzers, has improved the situation considerably.<sup>172</sup> The growing popularity of these instruments for targeted, non-targeted, and screening analysis is related to the wide range of possible combinations of detection modes that operate at high resolution (up to 1 000 000 FWHM) and provide accurate mass measurements (usually <5 ppm), which greatly facilitates subsequent qualitative analysis. Glycosylated mycotoxins and other chemical derivatives can now be more easily identified. Depending on whether the full structural features, properties, or only the summary chemical formulas are known, or whether a dedicated library of HRMS/MS spectra exists, different approaches are possible for their analysis. Among the first HRMS-based methods using Orbitrap MS are those for fusarenone-X-glucoside and nivalenol-glucoside,<sup>173</sup> T-2- and HT-2-glucoside<sup>68</sup> and DON-oligoglycosides,<sup>78</sup> followed by others using different HRMS setups,<sup>174,175</sup> including those combined with ion mobility.<sup>172,176</sup>

It is important to emphasize that the ability of an analytical method to detect glycosylated mycotoxins and confirm their presence in food does not in itself represent complete fulfillment of the analytical objective. From a risk assessment perspective, quantitative information on these compounds is crucial. However, analytical standards for glycosylated mycotoxins are hardly available on the market, with only a few exceptions, such as DON-3G and ZEN-14G. To effectively support research in this area, some research groups are synthesizing these compounds themselves. So far, great efforts have been made to produce various representatives of glycosylated trichothecenes (T-2, HT-2, T-2 triol, neosolaniol, 4,15-diacetoxyscirpenol, and FUS-X), usually by enzymatic synthesis,<sup>177–179</sup> or by chemical synthesis<sup>11,12</sup> and biotransformations.<sup>11,12,180,181</sup> A protocol for in-house chemical synthesis of glycosides of *Alternaria* mycotoxins was published by Mikula *et al.*<sup>182</sup>

Given the different bioavailability of  $\alpha$ - and  $\beta$ -anomers of mycotoxin glycosides (so far specifically  $\alpha$ - and  $\beta$ -anomers of

HT-2 and T-2 toxin in the cereals wheat and oats,<sup>11,12</sup> and DON-oligoglucosides in beer and malt<sup>78</sup>), effective chromatographic separation of these isomers is essential for accurate identification and quantification. In the absence of appropriate analytical standards, it is extremely difficult to determine whether the sample contains only one anomer or both co-eluting anomers. In the study by McCormick *et al.*, T-2  $\alpha$ - and  $\beta$ -glucosides were baseline-separated using a polar end-capped C18 stationary phase.<sup>11</sup> The study by Schmidt *et al.* showed good separation of both HT-2 and T-2- $\alpha$ - and  $\beta$ -glucosides using a hydrophobic base-stable C18 phase.<sup>12</sup> HILIC chromatography with an amide stationary phase was shown to be very suitable for the effective separation of DON oligoglucosides due to the strong polar interactions resulting from the multiple sugar units.<sup>78</sup>

An alternative strategy for the analysis of glycosylated mycotoxins involves an indirect approach, which generally refers to the analysis of the free forms of mycotoxins after hydrolysis pretreatment. Several studies have shown that modified DON in wheat and corn can be quantified by converting it to DON by acid hydrolysis with trifluoromethanesulfonic acid.<sup>183–185</sup> In these studies, acid hydrolysis of cereal samples led to a significant increase in the measured amount of DON. However, the follow-up study by Malachova *et al.* demonstrated that acid hydrolysis with TCA, TFA, or TFMSA does not convert DON-3G (nor acetylated DONs) to DON, casting doubt on the efficacy of previously published indirect methods for DON quantification. The authors explained the apparent increase in DON reported in earlier works as a result of deacetylation of co-occurring acetylated DON derivatives after alkaline neutralization, or extensive matrix artefacts, and recommended enzymatic hydrolysis as a more reliable approach to cleave DON from its bound forms.<sup>186</sup> So far, only a few studies have directly addressed the enzymatic release of mycotoxins from polysaccharides. The study by Bittner *et al.* published a protocol for the enzymatic release of OTA from roasted coffee samples using driselase, a mixture of enzymes with cellulase, xylanase, and  $\beta$ -glucanase activity, and hemicellulase.<sup>141</sup> In another study by Vidal *et al.* that focused on aflatoxins, the authors concluded that these toxins may be linked to carbohydrate structures, as treatment with  $\alpha$ -amylase and cellulase resulted in a 15% and 13% increase in total aflatoxin content, respectively (in contrast, hydrolysis with trifluoromethanesulfonic acid was found to be inefficient).<sup>187</sup> Very recent results obtained by the authors of this review (unpublished so far) suggest that enzymatic hydrolysis of solid cereal matrices using a combination of glucosidase,  $\beta$ -glucanase, glucoamylase, and  $\alpha$ -amylase can increase the total levels of T-2 and HT-2 toxins by several hundred percent. Another study using an indirect quantification approach reported that HT-2 glycosides accounted for up to 129% of the total HT-2 content.<sup>76</sup> However, it is important to note that in the latter case, enzymatic hydrolysis was performed after extraction, meaning that the reported value only reflects the extractable forms. These examples highlight the importance of distinguishing between different indirect approaches and understanding the specific type of information that each method provides. In general, indirect methods for the



determination of glycosylated mycotoxins offer both advantages and limitations. The advantages include their simplicity and the fact that they do not require analytical standards of the native glycoside forms, which are usually not available to routine laboratories. Another advantage is the release of mycotoxins from non-extractable forms (if performed prior to extraction), which allows the determination of the entire mycotoxin pool. The limitation is that this approach inevitably leads to the loss of structural information about the native glycosylated forms and, consequently, denies insights into their specific biological activity.

Despite the high sensitivity of modern MS instruments, the detection of glycosylated mycotoxins remains a challenge, especially in complex matrices such as malt, beer, or cereal hydrolysates. These problems can be partially mitigated by effective sample clean-up and concentration. In this context, immunoaffinity chromatography (IAC) offers remarkable advantages, especially due to the cross-reactivity of antibodies originally developed for the parent mycotoxins, which in some cases can also recognize their glycosylated derivatives. However, it is important to note that this cross-reactivity with glycosylated mycotoxins is essentially random and is usually not declared by the manufacturers. In other words, while such cross-reactivity can be analytically beneficial, it is generally unintentional. The first study investigating commercially available IAC columns for the determination of glycosylated DON was performed by Vendl *et al.* (2009), but the recovery of this analyte was 0% (no cross-reactivity of the columns DONprep (R-Biopharm) and DONtest (Vicam) to DON-3G was detected).<sup>188</sup> In another study by Kostelanská *et al.* 2011, the same DONprep columns were used to analyze DON-3G in beer and brewery intermediates, and method recovery was above 90% in all cases.<sup>189</sup> Remarkably, the follow-up study by Zachariášová *et al.* showed that DONprep columns are even cross-reactive with DON oligoglucosides.<sup>78</sup> Since then, numerous studies have been conducted to analyze glycosylated DON by purification and pre-concentration on IACs,<sup>190,191</sup> including those employing conventional detectors (UV/PDA) instead of MS.<sup>192–194</sup> As for other glycosylated trichothecenes, NIV-G was co-isolated together with DON-3G using IACs developed for NIV and DON (DON-NIV<sup>WB</sup>, Vicam), while HT-2 and T-2 mono- and di-glucosides were effectively co-isolated using T-2/HT-2-specific columns, particularly Easi-Extract (R-Biopharm), T-2/HT-2 HPLC IACs (Vicam), T-2 Test (Vicam) and Aokin Immuno-Clean C T-2/HT-2 (Aokin) IACs.<sup>76,79</sup> As previously indicated, the antibodies used in the IACs are not primarily developed against mycotoxin glycosides, and since their cross-reactivity is not guaranteed,<sup>191,195</sup> it must be thoroughly verified before their use to avoid potentially biased results. Since cross-reactivity can vary not only from manufacturer to manufacturer but also from production batch to production batch, routine performance verification over time is essential to ensure analytical reliability.

However, the phenomenon of cross-reactivity is a double-edged sword and can pose a major challenge, particularly for non-selective methods such as enzyme-linked immunosorbent assay (ELISA), as it affects the accuracy of the measured concentrations. While ELISA is by design a screening method,

and occasional false-positive results are generally not problematic, as all presumptive positive results close to legal limits should be confirmed by instrumental analysis, systematic overestimation due to the presence of glycosylated mycotoxins severely limits its applicability in routine practice. This problem was clearly demonstrated in a case study by Zachariášová *et al.* in which a cross-laboratory comparison of DON and ZEA levels in maize showed a bimodal distribution of results, with ELISA-based measurements yielding statistically significantly higher mean concentrations compared to instrumental methods.<sup>196</sup> Lack of awareness of this phenomenon and lack of appropriate correction strategies may lead to unjustifiably low proficiency test scores for laboratories using selective instrumental techniques.

The analytical aspects of qualitative and quantitative determination of glycosylated mycotoxins are extremely complicated, but important for determining actual exposure, testing bioavailability, and assessing toxicity. In the future, attention must be focused on the establishment of analytical standards for all isomeric/anomeric variants and the development of separation and detection methods that allow their accurate determination.

## 5. Legislative and regulatory aspects

As our understanding of the toxicological relevance of glycosylated mycotoxins and their prevalence in the food chain continues to grow, regulatory bodies such as EFSA and the European Commission have increasingly focused their attention on the associated analytical and legislative challenges. This section provides an update on the status of risk assessment and regulatory developments related to modified mycotoxins, with a particular focus on glycosylated forms, based primarily on official EFSA opinions and applicable EU legislation.

The 2014 EFSA scientific opinion on modified *Fusarium* toxins<sup>197</sup> remains a cornerstone in this field. Surprisingly, despite the increasing scientific attention and growing body of literature on masked or modified mycotoxins, no comprehensive update to this opinion has been issued in over a decade. This gap does not reflect a lack of interest or relevance but rather highlights several persistent challenges. These include the inherent complexity of the chemical structures involved, the limited commercial availability of analytical standards, and a widespread lack of toxicological data for most modified forms. EFSA's 2014 opinion<sup>197</sup> addressed the main modified mycotoxins such as DON, ZEN, NIV, fumonisins, and T-2/HT-2, emphasizing their potential toxicological impact and the significant analytical difficulties they pose. There were also repeated calls for supporting data collection and targeted assessments. A key conclusion of the 2014 opinion was that, in the absence of substance-specific toxicological data, modified forms should be assumed to have equivalent toxicity to their parent compounds unless proven otherwise. In parallel, EFSA estimated the contribution of the modified forms to the overall exposure. For ZEN and its metabolites (including ZEN-14G and ZEN-14S), the contribution was assumed to be 100%. For DON and its main conjugate, DON-3G this value was set at 75%, for



fumonisins at 60% and T-2/HT-2 toxins at 10%. Regarding the exposure assessment based on occurrence data and dietary surveys, EFSA concluded that for most modified mycotoxins, the total exposure (*i.e.*, free plus modified forms) does not exceed the provisional tolerable daily or weekly intakes for the general population, except for zearalenone and fumonisins in certain age groups, in particular infants and children, where the estimated exposure in the upper percentile exceeded the health-based guidance values.<sup>197</sup> These results emphasize the importance of monitoring modified forms in addition to free forms, especially in vulnerable populations. However, it should be noted that some of the recent studies focusing on modified T-2 and HT-2 (ref. 76) suggest that the estimated 10% contribution<sup>15</sup> of their conjugated forms may be underestimated, highlighting the need for more in-depth research on this topic.

Despite increasing scientific concern, modified mycotoxins remain only partially regulated at the EU level. More precisely, Commission Regulation (EU) 2023/915,<sup>198</sup> which consolidates maximum levels for mycotoxins in food, continues to apply only to the free forms. The latest amendment, Regulation (EU) 2024/1022,<sup>199</sup> reduced the maximum levels for DON by approximately 20% in most food categories, creating a safety margin for co-occurring modified forms, in line with the group TDI established for DON and its acetylated and glycosylated derivatives,<sup>22</sup> however, no separate maximum limits have yet been established for DON-3G. According to the attached explanatory memorandum, this decision was based on the observation that many laboratories in the Member States do not yet routinely analyze this compound. Nevertheless, this justification may not fully reflect current analytical capabilities. Numerous academic, commercial, and official laboratories have already implemented and validated LC-MS/MS methods for DON-3G, especially considering its commercial availability as a reference standard. The exclusion of DON-3G thus underlines the tension between scientific readiness and regulatory conservatism and suggests that current monitoring practices should be thoroughly evaluated and objectively assessed prior to any decision on the inclusion of modified mycotoxins in mandatory legislative frameworks.

In summary, although scientific and regulatory progress has been made in the detection and analysis of modified mycotoxins, their risk assessment and legislative control are still evolving. EFSA's precautionary approach, which assumes toxicological equivalence and includes modified forms in the exposure assessment, provides an essential but preliminary framework. Although recent regulatory documents such as Regulations (EU) 2023/915,<sup>198</sup> 2023/2782,<sup>200</sup> and 2023/2783 (ref. 201) reflect a growing awareness of mycotoxin challenges, they do not yet include explicit measures for modified forms. As analytical technologies advance and more data become available, it is plausible that future EU regulations will explicitly address modified mycotoxins, including setting maximum levels. Until then, continued research, method development, and coordinated monitoring remain critical to safeguarding public health from these toxic threats.

## 6. Conclusions, outlook, and outstanding questions

### 6.1 Conclusions

Over the last decade, our knowledge of glycosylated "masked" mycotoxins has increased considerably, and a growing number of publications have documented numerous glycosylated derivatives of the major mycotoxins in crops and foods. Nevertheless, there are still major gaps in translating this knowledge into public health protection, particularly in the areas of digestive behavior, bioavailability, and toxicity. Challenges in the analytical field include, in particular, the limited availability of analytical standards required for accurate quantification, and the primary use of U-HPLC, HRMS/MS, or ion mobility in routine practice to detect "masked" mycotoxins through their advanced separation and mass spectral properties.

Despite this scientific progress, the regulatory framework and risk assessment have been delayed. The crucial 2014 EFSA opinion on modified mycotoxins remains the last comprehensive assessment, and despite intensive research in the intervening period, no major update has been published for over a decade. In addition, current food safety legislation in general still does not set explicit limits for modified mycotoxins. The exclusion of DON-3G – despite its toxicological relevance and the availability of analytical tools – illustrates the tension between scientific progress and regulatory inertia. In summary, glycosylated mycotoxins are a current food safety concern and continued efforts in method development, toxicological research, and policy updates are critical to close the knowledge gaps.

### 6.2 Outlook

Several developments are expected to shape future work on modified mycotoxins:

**6.2.1 Analytical innovations.** Advances in detection technologies remain critical. New generation hybrid mass spectrometers (*e.g.*, Orbitrap-based HRMS) and ion mobility MS provide the resolution and sensitivity required for reliable detection of glycosylated toxins. These platforms support both untargeted screening and targeted quantification.

In addition, AI-assisted expert systems are emerging as valuable tools for processing HRMS data, prioritizing potential conjugates, and aiding structural annotation even in the absence of reference standards. The integration of spectral libraries, *in silico* fragmentation tools, and machine learning algorithms is expected to further improve structural recognition. These improvements, together with the ongoing synthesis of affordable analytical standards, will make modified mycotoxin analysis more reliable and routine.

**6.2.2 Toxicological data gaps.** Current risk assessments are still based on the assumption that modified mycotoxins are as toxic as their parent forms. Future research should rigorously verify this through *in vitro* and *in vivo* studies of key conjugates such as DON-3G, ZEN-14G, and T-2/HT-2 glucosides. Particular attention needs to be paid to bioavailability, metabolic activation, and stereoisomeric differences (*e.g.*,  $\alpha$ - vs.  $\beta$ -anomers). New



methods — such as gut microbiome models and organoids — can help to close toxicological gaps more efficiently and enable evidence-based risk assessment.

**6.2.3 Regulatory framework.** The European Commission, in particular the Directorate-General for Health and Food Safety (DG SANTE), is beginning to address modified mycotoxins, but integration into official food safety standards (the current food safety legislation) remains limited. Progress will depend mainly on the harmonization of analytical protocols and the inclusion of selected conjugates in monitoring programs. Initial steps, such as the inclusion of DON-3G in EFSA's dietary exposure estimates, are promising, but more definitive regulations are needed for effective risk management.

**6.2.4 Interdisciplinary cooperation.** Dealing with masked mycotoxins requires close collaboration between analytical chemists, toxicologists, plant scientists, microbiologists, and the authorities responsible for food safety. Interdisciplinary work can drive innovation in the areas of occurrence, prevention, and risk assessment/management. An active dialogue between researchers and policymakers is key to translating new scientific knowledge into practical food safety strategies.

### 6.3 Outstanding questions

The following outstanding (*i.e.*, unresolved) questions highlight key gaps in our understanding of glycosylated mycotoxins and indicate promising directions for future research. Although some of these questions have been largely addressed for DON-3G, they remain highly relevant for other glycosylated mycotoxins, such as T-2-Glc, HT-2-Glc, ZEN-14-Glc, ergot alkaloid glycosides, and *Alternaria* conjugates, for which knowledge is still limited.

**6.3.1 Toxicity and health impact.** Are glycosylated mycotoxins really as toxic as their parent substances? Although they are currently thought to be equivalent, data on their chronic effects, metabolism, and organ-specific toxicity are lacking, so targeted toxicology studies are needed.

**6.3.2 Bioavailability and metabolism.** How are glycosylated mycotoxins absorbed and metabolized? Their fate—in particular microbial hydrolysis, absorption rates, and anomer-specific behavior—remains poorly understood and crucial for risk assessment.

**6.3.3 Matrix-associated mycotoxins.** How large is the actual “mycotoxin pool”, and are all of these bound toxins bioavailable? Reliable and effective detection methods and appropriate bioavailability models need to be developed.

**6.3.4 Glycosylated ergot alkaloids.** Does the contaminated forage grass pose a hidden risk to livestock? The presence of ergot alkaloids, including their glucosides, in commonly consumed species such as *Achnatherum* remains poorly understood, and the toxicological relevance of these glycosides—including their stability, bioactivation, and effects in animals—should be specifically investigated.

**6.3.5 Risk assessment and regulatory framework.** Which modified mycotoxins should be routinely monitored, and how should modified mycotoxins be regulated? Clearly defining target substances and incorporating new data into the evolving frameworks remain key challenges for international authorities.

## 7. Author contributions

V. K., T. R., and M. S. – conceptualization, methodology, formal analysis, writing original draft, and editing; V. K. and M. S. – funding acquisition; T. R. and V. K. – resources; T. R. and M. S. – data curation.

## 8. Conflicts of interest

There are no conflicts to declare.

## 9. Abbreviations

$\alpha$ -ZEL	$\alpha$ -Zearalenol
$\beta$ -ZEL	$\beta$ -Zearalenol
$\alpha$ -ZEL-14G	$\alpha$ -Zearalenol-14-glucopyranoside
$\beta$ -ZEL-14G	$\beta$ -Zearalenol-14-glucopyranoside
4-OH-OTA-G	4-Hydroxyochratoxin- $\beta$ -D-glucopyranoside
AOH	Alternariol
AOH-3G	Alternariol-3-glucopyranoside
AOH-9G	Alternariol-9-glucopyranoside
AOH-9,9'	Alternariol-9,9'-diglucoside
DiGlc	
AME	Alternariol monomethylether
AME-3G	Alternariol monomethylether-3-glucopyranoside
DAS	Diacetoxyscirpenol
DOM-1	Deepoxy-deoxynivalenol
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3- $\beta$ -glucopyranoside
DON-di-G	Deoxynivalenol-diglucoside
DON-tri-G	Deoxynivalenol-triglucoside
EFSA	European Food Safety Authority
FUS-X	Fusarenon-X
HT-2	HT-2 toxin
HT-2-3G	HT-2-3- $\beta$ -glucopyranoside/T-2-3- $\alpha$ -glucopyranoside
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
NIV	Nivalenol
NIV-3G	Nivalenol-3-glucopyranoside
T-2	T-2 toxin
T-2-3G	T-2-3- $\beta$ -glucopyranoside/T-2-3- $\alpha$ -glucopyranoside
USDA	U.S. Department of Agriculture
ZEN	Zearalenone
ZEN-14G	Zearalenone-14- $\beta$ -glucopyranoside
ZEN-16G	Zearalenone-16- $\beta$ -glucopyranoside
ZEN-14S	Zearalenone-14-sulfate

## 10. Data availability

This review article is based exclusively on publicly available data. All sources of information are fully cited in the reference list and are fully searchable, with data sourced mainly from publicly available databases such as PubMed (<https://pubmed.ncbi.nlm.nih.gov>), Web of Science (<https://www.webofscience.com>), Google Scholar (<https://scholar.google.com>),



[scholar.google.com](https://scholar.google.com)) and other relevant resources. In addition, specific data on mycotoxins were retrieved from public sources such as the Mycotoxin and Food Contaminant Database (<https://mycotoxins.info>) and the Contaminant Candidate List of the European Food Safety Authority (EFSA) (<https://www.efsa.europa.eu>). No new data were generated or analyzed in this study.

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