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1 **Metabolism of Versicolorin A, a genotoxic precursor of Aflatoxin B1: Characterization of**
2 **metabolites using *in vitro* production of standards**

3

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26 **Abstract**

27 The toxicity of mycotoxins containing bisfuranoid structures such as aflatoxin B1 (AFB1) depends
28 largely on biotransformation processes. While the genotoxicity and mutagenicity of several bisfuranoid
29 mycotoxins including AFB1 and sterigmatocystin have been linked to *in vivo* bioactivation of these
30 molecules into reactive epoxide forms, the metabolites of genotoxic and mutagenic AFB1 precursor
31 versicolorin A (VerA) have not yet been characterized. Because this molecule is not available
32 commercially, our strategy was to produce a library of metabolites derived from the biotransformation
33 of in-house purified VerA, following incubation with human liver S9 fractions, in presence of
34 appropriate cofactors. The resulting chromatographic and mass-spectrometric data were used to identify
35 VerA metabolites produced by intestinal cell lines as well as intestinal and liver tissues exposed *ex vivo*.
36 In this way, we obtained a panel of metabolites suggesting the involvement of phase I (M + O) and phase
37 II (glucuronide and sulfate metabolites) enzymes, the latter of which is implicated in the detoxification
38 process. This first qualitative description of the metabolization products of VerA suggests bioactivation
39 of the molecule into an epoxide form and provides qualitative analytic data to further conduct a precise
40 metabolism study of VerA required for the risk assessment of this emerging mycotoxin.

41

42

43 **Keywords:** Versicolorin A, Aflatoxin B1, S9 fractions, Biomonitoring, Metabolites,

44

45 1. Introduction

46 Mycotoxins are widespread toxic secondary fungal metabolites that contaminate food and feed (Payros
47 et al., 2021). Aflatoxins (AFs) are considered to be the most dangerous family of mycotoxins, while
48 aflatoxin B1 (AFB1) is the most frequent and most toxic member of this family, and is the strongest
49 known natural carcinogen (classified as Group 1 by IARC) (IARC, 1993; Schrenk et al., 2020). The
50 presence of AFs in food and animal feed, in particular of AFB1, is regulated in most parts of the world
51 (European Commission, 2006; US Food and Drugs Administration, 2021, 2019). In Europe, the
52 maximum authorized levels of aflatoxins range from 0.1 to 15 µg/kg (European Union, 2006).

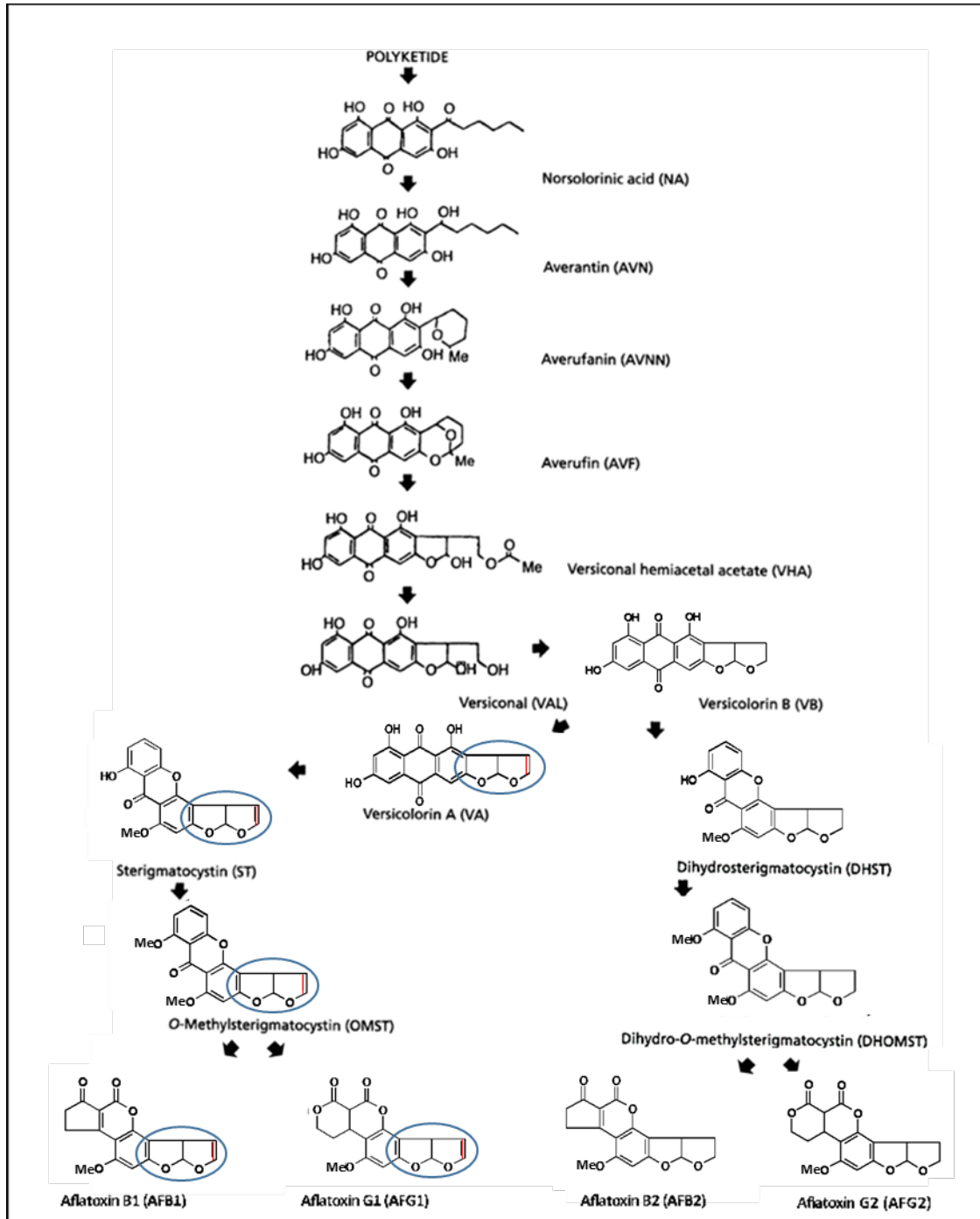
53 AF synthesis involves several enzymatic reactions and at least 15 compounds have been reported as
54 intermediates of the aflatoxin biosynthetic pathway (Fig. 1; Caceres et al., 2020). Notably during the
55 final steps of the synthesis of AFB1 and Aflatoxin G1 (AFG1) a characteristic dihydrobisfuran ring
56 structure is formed, which is also present in three intermediate precursors: sterigmatocystin (STC), O-
57 methyl sterigmatocystin (OMST) and versicolorin A (VerA; Fig.1). The mycotoxins containing this
58 structure are known as bisfuranoids. This structure drives the toxicity of these molecules, since the
59 compounds containing an unsaturated vinyl ether double bond of the terminal furan ring have been
60 described as mutagens and liver carcinogens (Hendricks et al., 1980; Mori et al., 1986; Wong et al.,
61 1977). The involvement of this specific structure in the toxicity of AFB1 is well characterized, and is
62 determined by epoxidation of the vinyl ether double bond mainly by the action of several cytochrome
63 P450 (CYP450) enzymes in exposed tissues which metabolise AFB1 into the highly reactive aflatoxin-
64 8,9-*exo*-epoxide known as AFBO (Eaton et al., 2010; Smela et al., 2001). AFBO reacts with critical
65 biological nucleophiles, such as proteins and DNA, and causes point mutations, chromosomal
66 aberrations, and genetic damage (Eaton et al., 2010). The detoxification of AFBO is achieved by
67 glutathione-S-transferases (GST). Depending on the relative importance of these biotransformation
68 routes and the GST-dependent detoxification of AFBO, differences can be found in the susceptibility of
69 tissues or species to developing cancer, liver being the most sensitive organ, and humans and pigs being
70 more sensitive than rats or mice, respectively (Chu, 2003; Eaton et al., 2010).

71

72

73 **Fig. 1. Aflatoxin biosynthetic pathway**

74 Schematization of the main biosynthetic reactions leading to aflatoxins synthesis produced by
75 *Aspergillus* species: *A.flavus* (B1, B2) and *A.parasiticus* (B1, B2, G1, G2) (Adapted from Trail et al.
76 (1995)). The structure of dihydrobisfuran ring is circled and the double bonds are highlighted in red.



77

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79

80 The biotransformation of other bisfuranoid AFB1 precursors has been less extensively investigated. The
81 formation of reactive *exo*-epoxide forms of AFG1 and STC is described in the literature, along with their
82 relationship with genotoxic effects (Baertschi et al., 1989; EFSA, 2013; Walkow et al., 1985). In the
83 case of OMST, to our knowledge, no production of an epoxide form has been reported so far, and in
84 fact, the genotoxicity of this compound is not established, as the results are contradictory. The distinctive
85 methyl group present only in OMST may explain its apparently lower toxicity compared to other
86 bisfuranoid mycotoxins (Mori et al., 1986; Theumer et al., 2018; Wehner et al., 1978). Hence,
87 methylation of STC may be linked to a reduction in toxicity (Kawai et al., 1986). No data is available
88 in the literature on the biotransformation of VerA or on its circulating concentrations in blood, even
89 though this mycotoxin is highly genotoxic and mutagenic (Gauthier et al., 2020; Jakšić et al., 2012; Mori
90 et al., 1986; Theumer et al., 2018).

91 VerA is considered to be an emerging threat, as it shows high *in vitro* cytotoxic, genotoxic and
92 clastogenic potency in different cell lines, in some cases, even higher than AFB1 (Gauthier et al., 2020;
93 Jakšić et al., 2012; Mori et al., 1986; Theumer et al., 2018). The characterization of the biotransformation
94 of VerA is thus indispensable for a complete assessment of the risk, as indicated in a report published
95 in 2017 by the joint FAO/WHO Expert Committee on Food Additives (JECFA; JECFA, 2017), which
96 underlined the importance of producing sound data on the emerging threats represented by AF
97 precursors. Moreover, metabolites derived from VerA would also need to be characterized to monitor
98 the toxicokinetics of the molecule *in vivo* and to estimate actual exposure to VerA.

99 Biotransformation studies are usually conducted using radiolabeled compounds to allow their specific
100 detection and quantification, followed by mass spectrometry to enable their identification (Jacques et
101 al., 2010). However, this approach depends on the commercial availability of the radiolabeled standard,
102 and is not easy to apply due to the complexity involved in handling radiolabeled compounds (Staack
103 and Hopfgartner, 2007). Another possible solution is to analyze only using liquid chromatography
104 combined with high-resolution mass spectrometry (HPLC-HRMS) (Fæste et al., 2011), but this
105 approach requires validation of all each identification using metabolite standards, which are rarely
106 commercially available. To get round the lack of standards, an approach aimed at *in vitro* production of

107 metabolites using human liver S9 fraction incubations was recently proposed for human biomonitoring
108 (Huber et al., 2021). This method provides relevant data, such as MS spectra, MS/MS spectra and
109 chromatographic retention times of metabolites that can confirm their identification in more diluted and
110 complex samples. We previously applied this kind of structural validation of phase I and II metabolites
111 in human urine samples during an exposome study, but using *in vivo* experiments (Jamin et al., 2014).
112 Because VerA is not commercially available, human liver S9 fractions containing both microsomal and
113 cytosolic enzymes were incubated with appropriate cofactors to explore the metabolic fate of VerA, to
114 provide a comprehensive list of metabolites produced *in vitro*, and to elucidate major metabolite
115 structures. In a second step, we obtained a broader overview of the metabolites possibly present in more
116 complex samples using intestinal porcine epithelial cell line (IPEC1), porcine jejunum explants, and
117 precision-cut liver slices exposed to VerA. Based on this workflow, we provide a first qualitative
118 characterization of the metabolites derived from the metabolization of VerA. The list comprises
119 degradation products, as well as phase I and phase II metabolites, including a metabolite compatible
120 with possible bioactivation of VerA.

121

122 **2. Material and Methods**

123 2.1. Chemicals and reagents

124 HPLC grade solvents (methanol, acetonitrile), acetic acid, dichloromethane and chloroform were
125 purchased from Fisher Scientific (Illkirch, France). Dulbecco's Modified Eagle Medium/Nutrient
126 Mixture F-12 Ham (DMEM/HAMs F12 medium), William's E Medium, phosphate buffered saline
127 (PBS), insulin transferrin-selenium (ITS), alanine, dexamethasone, ethanol, DMSO, monobasic sodium
128 phosphate, dibasic heptahydrate sodium phosphate, NADP, glucose, D-glucose 6-phosphate sodium
129 salt, glucose-6-phosphate dehydrogenase, magnesium chloride (MgCl₂), reduced L-glutathione (GSH),
130 sodium chloride (NaCl), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), uridine 5'-
131 diphosphoglucuronic acid (UDPGA), Krebs-Henseleit buffer (KHB), calcium chloride dihydrate
132 (CaCl₂, 2H₂O) and sodium bicarbonate (NaHCO₃) were purchased from Sigma-Aldrich Merck (Saint
133 Quentin Fallavier, France). Streptomycin/penicillin, L-glutamine, fetal bovine serum (FBS) and
134 gentamycin were purchased from Eurobio (Courtaboeuf, France). Epidermal growth factor (EGF) was

135 purchased from Becton-Dickinson (Le Pont de Claix, France). Alamethicin was purchased from
136 Cayman Chemical (Ann Arbor, MI, USA). Ultrapure water was generated by a Milli-Q system
137 (Millipore, Saint Quentin en Yvelines, France) with a specific resistance of 18.2 M Ω at 25 °C and total
138 organic carbon (TOC) value < 3 ppb.

139 2.2. Purification of VerA

140 As VerA is not available commercially, it was purified using a previously described in-house protocol
141 (Gauthier et al., 2020) from wheat grains colonized by a pathway-blocked strain of *Aspergillus*
142 *parasiticus* that specifically accumulates VerA (*A. parasiticus* strain SRRC 0164). Briefly, VerA was
143 extracted from wheat and mycelia in chloroform and then purified by high-performance liquid
144 chromatography (HPLC) using an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Courtaboeuf,
145 France). An authentic VerA standard previously produced in our lab and checked by high resolution
146 mass spectrometry (HRMS) and nuclear magnetic resonance analysis (NMR) was used as reference for
147 both purification and to check purity (Theumer et al., 2018). The identity and purity of the purified VerA
148 were confirmed by HPLC with a diode-array detector (DAD), following the protocol already used by
149 our team and detailed in Theumer et al. (2018). The concentration of VerA produced was determined
150 by reading absorbance at 290 nm ($\epsilon_{25^\circ\text{C}}^{\text{EtOH}} = 25,825$) and 450 nm ($\epsilon_{25^\circ\text{C}}^{\text{EtOH}} = 7,585$) (Cole and Cox,
151 1981). Stock solution of VerA (10 mM) was prepared in DMSO and stored at -20 °C until use.

152

153 2.3. Incubation of VerA with human liver S9

154 Reference metabolites of VerA were produced *in vitro* using the S9 liver fraction, containing both phase
155 I and phase II xenobiotic metabolizing enzymes (XME). Mixed-gender human liver S9 (pool comprising
156 50 donors) was purchased from Tebu-bio (Le Perray-en-Yvelines, France). The incubation method was
157 adapted from previous studies (Cabaton et al., 2008; Jaeg et al., 2004). S9 incubations were prepared in
158 a final volume of 0.5 mL of 0.1 M sodium/phosphate buffer pH 7.4 with 5 mM MgCl₂, with a protein
159 content fixed at 6 mg/mL and with appropriate cofactors. A 15-min pre-incubation was performed with
160 alamethicin (50 $\mu\text{g}/\text{mg}$ protein, 0.15 mM in DMSO, 0.5% in the final volume) (pore-forming agent).
161 Incubations were started with the addition of 50 μM VerA (0.6% ethanol in the final volume), GSH 12.5

162 mM, UDPGA 2 mM, PAPS 0.2 mM and a NADPH generating system consisting of NADP 1.3 mM,
163 glucose-6-phosphate 5 mM and glucose-6-phosphate dehydrogenase 2 IU/mL. S9, VerA and cofactors
164 were incubated for 3 h at 37 °C under shaking. Incubations were quenched with 1.5 mL acetonitrile,
165 kept for 30 min on ice and centrifuged for 10 min at 6,500 g at 4 °C. Supernatants were collected, placed
166 in HPLC vials and stored at -20 °C until HPLC-HRMS analyses. All the experiments were performed
167 in triplicate. Control incubations were performed without VerA (proteins, cofactors and 0.6% ethanol)
168 and with the addition of cofactors after the quenching step (proteins, VerA and cofactors), in order to
169 distinguish non-cofactor-mediated VerA transformation and cofactor-dependant metabolites.

170

171 2.4 Exposure of cultured intestinal IPEC-1 cells to VerA

172 Cells were cultured in Petri dishes (100 x 20 mm; Cellstar, Greiner Bio-One, Germany) in
173 DMEM/HAMs F12 medium, completed with 1% ITS, 1% streptomycin/penicillin, 1% L-glutamine, 5%
174 FBS, and 5 µg/mL EGF. Cultures were kept under standardized conditions at 39 °C in a humidified
175 atmosphere with 5% CO₂.

176 To mimic real conditions, prior to exposure, IPEC-1 cells were differentiated into monolayers. We chose
177 to work at the same dose of VerA (50 µM) and time of exposure (3 h) as for *in vitro* S9 fraction
178 experiments and to complete the study using an additional exposure condition of a less cytotoxic dose
179 (10 µM) and longer exposure times (24 and 48 hours). IPEC-1 cells were differentiated as previously
180 described (Pinton et al., 2009). Briefly, cells were plated at a density of $2 \cdot 10^5$ cells/mL in the
181 aforementioned medium in permeable supports suitable for a 6-well plate with 0.4 µm polyethylene
182 terephthalate membranes (Coring Falcon, Corning, NY, USA) until confluence was reached. The cells
183 were then differentiated into monolayers in the same medium containing 20 µg/mL dexamethasone but
184 without FBS until transepithelial electrical resistance stability was reached, which takes between 8 to
185 10 days. After washing with PBS, VerA was added on the apical side and incubated in the conditions
186 described above. Control samples were treated with the vehicle (DMSO) in all cases.

187 At the end of the treatments, cells were scraped off using 0.9% cold isotonic NaCl solution, recovered
188 and centrifuged at 300 g for 10 minutes at 4 °C. After removal of the supernatant, the dry cell pellets
189 were stored at - 80 °C until further analysis.

190

191 2.5 Exposure of jejunum explants and precision-cut liver slices to VerA

192 Porcine tissues were used to evaluate VerA biotransformation in the intestine and liver. Jejunum
193 explants and precision-cut liver slices were prepared from four 35-day-old castrated male piglets.
194 Animal care and use for this study were carried out in accordance with the French Ministry of
195 Agriculture guidelines. All animal tissue procedures were performed in accordance with the ethics
196 Committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées (APAFIS
197 #N2016080314392462). The dose of VerA (50 µM) and time of exposure (3 h) used in this study was
198 equivalent to that used for S9 fraction experiments.

199 The method of obtaining jejunum explants is described in detail in Lahjouji et al. (2020). Briefly, the
200 jejunum was rapidly extracted, flushed with phenol red-free William's E Medium completed with 1%
201 penicillin/streptomycin and 0.5% gentamycin, and opened longitudinally. A 6 mm diameter biopsy
202 punch was used to obtain jejunum explants that were placed with the mucosa facing upwards on sponges
203 in 6-well plates containing 3 mL medium (3 explants per well). Jejunum explants were then exposed for
204 3 h to 50 µM VerA or DMSO in William's E Medium supplemented with 25 g/L of glucose, 1% ITS,
205 1% alanine-glutamine, 1% penicillin/streptomycin and 0.5% gentamycin at 39 °C, and 5% CO₂-
206 controlled atmosphere with orbital shaking.

207 To prepare the liver slices (Hasuda et al., 2022), the liver was rapidly resected and the right lateral
208 hepatic lobe flushed with a 0.9% ice-cold isotonic NaCl solution to limit ischemia and remove
209 hemoglobin. To maintain explant viability, KHB supplemented with NaHCO₃ (2.1 g/L) and CaCl₂,
210 2H₂O (0.373 g/L), previously bubbled with carbogen for 1 h, was used in the coring, slicing, and storage
211 procedures. After placing the perfused liver under a cylinder-shaped tissue coring tool (diameter 8 mm),
212 the tissue was drilled to make liver cores, and immediately placed in ice-cold buffer. Next, the cylindrical
213 cores were transferred to the holder of the Krumdieck tissue slicer (Alabama Research and
214 Development, AL, USA). Slices (250 µm thick) were prepared to optimize oxygen and nutrient intake

215 (De Graaf et al., 2010). Damaged slices were discarded. Before the slices were treated, a regeneration
216 step was required, consisting of incubating the slices in William's E Medium supplemented with 1%
217 glutamine and 0.5% gentamycin (2 mL per well) bubbled in carbogen for 1 h to enable homeostasis
218 recovery. Liver slices were distributed in 12-well tissue culture plates (1 slice per well) using a spatula
219 and exposed for 3 h to 50 μ M VerA or DMSO in William's E Medium supplemented with 1% glutamine
220 and 0.5% gentamycin (2 mL per well), at 37 °C under a 90% O₂ and 5% CO₂-controlled atmosphere.
221 After the incubation period, the jejunum explants, liver slices, and their culture media were stored
222 separately at -80 °C.

223

224 2.5 Extraction of metabolites from IPEC-1 cells, jejunum explants, and liver slices

225 Metabolites were extracted from IPEC-1 intestinal cell pellets by adding 1 mL of acetonitrile/ultrapure
226 water (90:10 v/v) and vortexing for 1 min. This operation was repeated twice. After centrifugation at
227 5,340 g for 10 min, the supernatants were evaporated with a SpeedVac® (Thermo Scientific, Les Ulis,
228 France) at ambient temperature. The extracts were finally dissolved in 250 μ L of water/methanol/acetic
229 acid (95:5:0.1 v/v/v).

230 Approximately 50 mg of tissue was weighed and placed in a 2 mL lysing matrix S tube (Fisher Scientific,
231 Illkirch, France) for jejunum explants, or a 2 mL lysing matrix M tube (Fisher Scientific, Illkirch,
232 France) for liver slices. Cold methanol was added to each tissue at a concentration of 4 mL/g, and cold
233 water at 0.85 mL/g of tissue. Tissues were homogenized using a FastPrep® System (MP Biomedicals,
234 Illkirch, France) to extract the metabolites. Jejunum explants were homogenized twice for 30 sec. at a
235 speed of 6 a.u. (arbitrary units). Liver slices were homogenized for 40 sec. at a speed of 6 a.u. A volume
236 of 2 mL/g of tissue of dichloromethane was added and the extracts were vortexed, followed by a second
237 addition of 2 mL/g of tissue of dichloromethane and 2 mL/g of tissue of water. Samples were centrifuged
238 at 2,870 g for 15 min at 4 °C. The lipid supernatant was removed and the aqueous phase was evaporated
239 in a SpeedVac® (Thermo Scientific, Les Ulis, France). Finally, the extracts were suspended with 500
240 μ L of water/methanol/acetic acid (95:5:0.1 v/v/v).

241

242 2.6 HPLC-HRMS analyses

243 Samples were analyzed with an RSLC 3000 HPLC system (Thermo Scientific, Les Ulis, France) coupled
244 with an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Les Ulis, France) equipped with a
245 heated electrospray ionization source (HESI). A volume of 10 μ L was injected onto a Hypersil Gold
246 C18 column (100 x 2.1 mm, 1.9 μ m; Thermo Fisher Scientific, Les Ulis, France) maintained at 40 $^{\circ}$ C.
247 Analytical mobile phases were composed of water/methanol/acetic acid (95:5:0.1 v/v/v) for phase A and
248 methanol/acetic acid (100:0.1 v/v) for phase B. Chromatographic separation was performed at a flow
249 rate of 0.3 mL/min with the following gradient: 0% to 100% of B from 0 to 30 min, 100% of B from 30
250 min to 34 min. HRMS detection was performed between m/z 80 and 1,500 at a resolution of 30,000 (at
251 m/z 400). For the positive ionization mode, the following HESI settings were applied: capillary
252 temperature 300 $^{\circ}$ C, vaporizer temperature 400 $^{\circ}$ C, source voltage 4 kV, sheath gas (N_2) flow rate 30
253 a.u., auxiliary gas (N_2) flow rate 10 a.u. and tube lens offset 80 V. For the negative ionization mode, the
254 following parameters were used: capillary temperature 300 $^{\circ}$ C, vaporizer temperature 400 $^{\circ}$ C, source
255 voltage 2 kV, sheath gas (N_2) flow rate 40 a.u., auxiliary gas (N_2) flow rate 5 a.u. and tube lens offset -
256 70 V. The high-resolution mass analyzer was calibrated in each ionization mode with calibration
257 mixtures (Thermo Scientific, Les Ulis, France) based on the supplier's protocols, which allowed m/z
258 measurements at ± 5 ppm.

259 A list of potential metabolites including hydroxylated, hydrolyzed, demethylated, conjugation with
260 glucuronic acid or sulfate, was produced. Suspected metabolites were monitored using Xcalibur[®]
261 (Thermo Scientific, Les Ulis, France) according to their exact mass with a window of ± 5 ppm. A
262 complementary screening step was performed using MetaSense software[®] (ACD/Labs, Strasbourg,
263 France) to identify unexpected metabolites. When a potential metabolite was detected according to its
264 m/z in extracts, and not in blank samples, targeted MS/MS and MS³ experiments with the collision
265 induced dissociation (CID) mode at a low resolution, or at a resolution of 7500 were triggered with a
266 normalized collision energy of 25%. Identification nomenclature was based on the nomenclature of the
267 metabolomics standards initiative (Sumner et al., 2007). The results were interpreted by looking at
268 characteristic fragmentation patterns, assisted when necessary using CFM-ID (Allen et al., 2014).

269

270 3 Results

271

272 3.1 Construction of a library of VerA metabolites using the *in vitro* S9 fraction

273 VerA was first incubated with human liver S9 fractions combined with different cofactors to
274 biosynthesize phase I and II metabolites. Six compounds were detected in negative mode ionization
275 along with intact VerA (Table 1). Their m/z ratio was measured by HRMS with an error < 5 ppm,
276 confirming their molecular formulae according to the technical specificities of the mass analyzer. Their
277 product ions observed by MS/MS enabled us to propose an identification level for each compound
278 (Sumner et al., 2007). Their structures are displayed in Figure 2. Positive ionization mode data are
279 available in supplementary table 1. No metabolites were detected in control incubations. Unmodified
280 VerA was detected in S9 fraction incubations and identification confirmed by a control. Phase I
281 metabolites displayed a similar fragmentation pattern to that of VerA (i.e. mainly losses of CO, CO₂ as
282 indicated in Table 1), but with m/z shifts in fragment ions in agreement with modifications in their
283 chemical structure and with additional loss of water. Phase II metabolites showed characteristic fragment
284 ions (Table 1) of glucuronic acid and sulfate groups with a loss of 176 u and 80 u, respectively (Jamin
285 et al., 2014). Conjugated metabolites were confirmed by MS³ experiments, displaying a similar
286 fragmentation pattern as the non-conjugated molecule (see supplementary table 2).

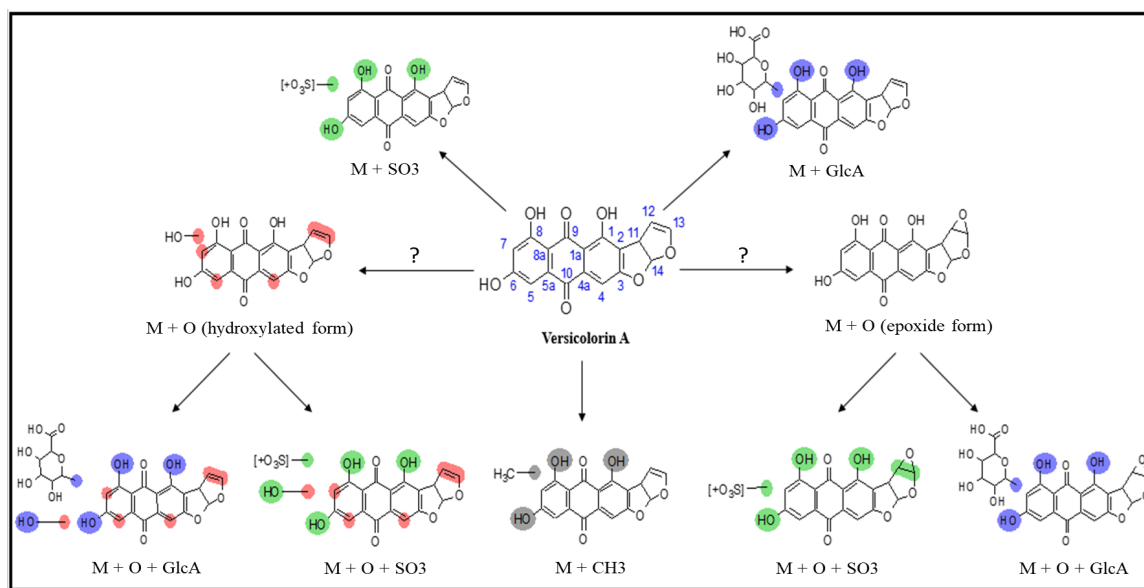
287 The case of the M + O metabolite is particular since its chemical formula could correspond to different
288 kind of structures. As illustrated in the Figure 3 with the well-studied AFB1, the 8,9-epoxide metabolite
289 or hydroxylated metabolite (AFM1) displayed the same chemical formula, which correspond to the
290 addition of one oxygen atom to the structure of the parent molecule. Therefore, it is not possible to
291 differentiate the hydroxylated metabolite from the epoxide one only by the mass measurement. These
292 isomer structures could potentially displayed different chromatographic retention times, but in our
293 analytical conditions only one peak was detected, which can correspond to one or several co-eluted M
294 + O metabolites (i.e. hydroxylated or epoxide). Furthermore, these similar structures are expected to
295 produced similar fragment ions by low energy CID in MS/MS experiments according to *in silico*

296 fragmentation tools as CFM-ID. Thus, none of the detected fragment ions of M + O were specific of
 297 one hydroxylated or epoxide structure. Since VerA has asymmetric carbon atoms, diastereoisomers of
 298 epoxide metabolites are possible (known as endo or exo epoxide for AFB1) and could potentially be
 299 differentiated by MS/MS or chromatography. However, it was not more possible to differentiate them
 300 in our experimental conditions, than it was for hydroxylated metabolites.

301 **Fig. 2. Identified structures of VerA metabolites**

302 Highlighted atoms correspond to possible bonding positions. Red positions correspond to hydroxylation
 303 (-OH), green to conjugation with sulfate (-SO₃), blue to conjugation with glucuronic acid, grey to
 304 methylation (-CH₃), and pink to double bond reduction. Question marks indicate the uncertain
 305 biotransformation of VerA into an epoxide and/or hydroxylated form.

306



307

308

309 **Table 1: Compounds detected as [M-H]⁻ after VerA incubations with human liver S9**, according to
 310 their measured *m/z* corresponding to the expected molecular formula with an *m/z* error below 5ppm.
 311 Observed chromatographic retention times are expressed in minutes, and detected MS/MS fragment ions
 312 are reported with their relative abundance.

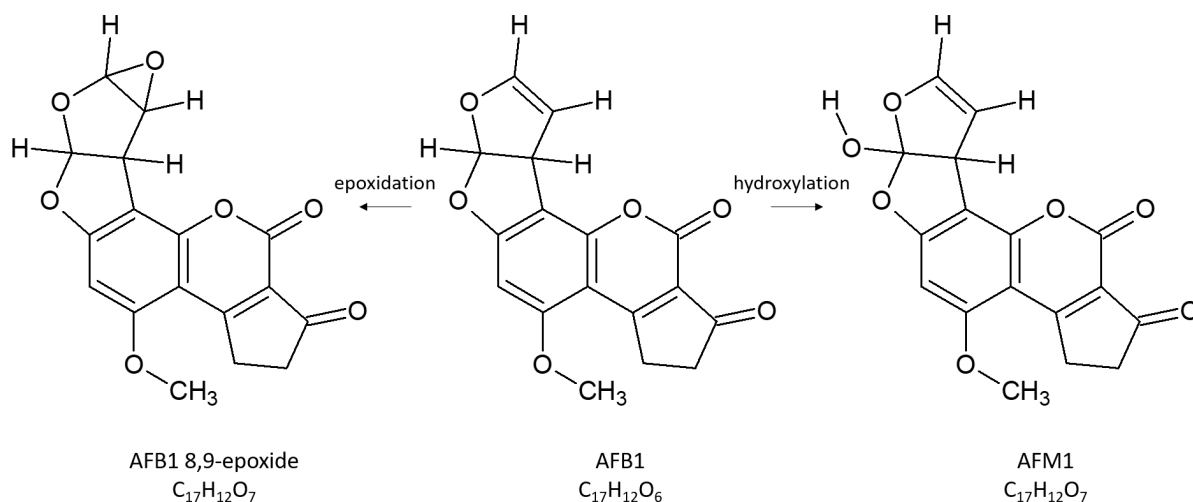
Compound	Measure d <i>m/z</i>	Error (ppm)	Molecular formula	Retention time (min)	Identification level ^a	MS/MS spectrum Observed product ions <i>m/z</i> (relative
----------	-------------------------	----------------	----------------------	----------------------------	--------------------------------------	---

						abundance expressed in %)
VerA	337.0359	-2.1	C ₁₈ H ₁₀ O ₇	23.2	1	309.1 (100), 308.1 (55), 293.1 (14), 337.1 (12), 320.0 (5), 265 (5), 252 (4), 280 (3)
M + O epoxide and/or hydroxylated	353.0304	-2.8	C ₁₈ H ₁₀ O ₈	18.9	3	309.1 (100), 335.2 (97), 325.1 (93), 281.1 (23), 310.1 (17), 265.1 (11), 297.1 (10), 307.1 (5), 308.0 (5), 311.1 (3), 324,2 (3), 353.0 (2)
M + H₂O	355.0465	1.7	C ₁₈ H ₁₂ O ₈	18.4	3	337.1 (100), 309.1 (60), 310.1 (24), 327.1 (21), 355.2 (14), 299.1 (13), 325.1 (9), 311.1 (6), 293.2 (5), 284.0 (5), 297.2 (4), 270.1 (3)
M + GlcAc	513.0677	2.7	C ₂₄ H ₁₈ O ₁₃	19.1	3	337.1 (100), 175.0 (2), 513.1 (2)
M + O + GlcAc epoxide and/or hydroxylated	529.0619	-2.1	C ₂₄ H ₁₈ O ₁₄	14.2	3	353.1 (100), 529.1 (2)
M + SO₃	416.9910	-2.9	C ₁₈ H ₁₀ O ₁₀ S	22.0	3	337.1 (100), 417.0 (2)
M + O + SO₃ epoxide and/or hydroxylated	432.9859	-2.8	C ₁₈ H ₁₀ O ₁₁ S	14.9	3	353.1 (100), 433.0 (2)

313 ^a Identification level as defined by Sumner et al., 2007

314 **Figure 3: structures of the known epoxide and hydroxylated (AFM1) metabolites of AFB1.**

315



316

317 3.2 VerA metabolites identified in IPEC-1 intestinal epithelial cells

318 To obtain a first overview of VerA metabolites that could be produced in the intestine, cultured intestinal
 319 IPEC-1 cells were exposed to VerA. Although the amount of material obtained using cultured cells is
 320 limited, the use of a biological sample of intermediate complexity compared to tissues helped interpret
 321 the results as a whole. Metabolites were characterized according to the chromatographic, MS and
 322 MS/MS results obtained with S9 fractions.

323 Reduced sensitivity was observed for this type of sample, which was reflected in the relatively short list
 324 of metabolites identified in cells, and none in culture medium. Two additional metabolites (M + O and
 325 M + SO₃) were identified in cells exposed for 24 h and 48 h (Table 2). Non-metabolized VerA was
 326 detected at all exposure times.

327

328 **Table 2: Compounds detected as [M-H]⁻ in extracts of IPEC-1 intestinal epithelial cells exposed to**
 329 **VerA for 24 and 48 hours.**

Compound	Measured <i>m/z</i>	Error (ppm)	Molecular formula	Retention time (min)	Identification level ^a	MS/MS spectrum Observed product ions <i>m/z</i> (relative abundance expressed in %)
VerA	337.0348	-1.5	C ₁₈ H ₁₀ O ₇	23.2	1	309.1 (100), 308.1 (55), 293.1 (13), 337.1 (13), 320.1 (5), 265.2 (5), 252.1 (4), 280.1 (3)

M + O epoxide and/or hydroxylated	353.0295	-2.3	C ₁₈ H ₁₀ O ₈	18.9	3	309.1 (100), 325.1 (90), 335.2 (69), 281.1 (23), 310.1 (14), 353.4 (10) 265.1 (8), 311.2 (6), 297.2 (6)
M + SO₃	416.9911	-2.4	C ₁₈ H ₁₀ O ₁₀ S	22.4	3	337.2 (100), 417.0 (2)

330 ^a Identification level as defined by Sumner et al., 2007

331 3.3 VerA metabolites identified in porcine jejunum explants and liver slices

332 Extracts of liver and jejunum *ex vivo* incubations were analyzed to characterize metabolites of VerA.

333 These models display features close to the *in vivo* situation, including complex tissue architecture and

334 cell diversity. Although still relatively weak, higher sensitivity of the tissues was observed, and a more

335 complete list of metabolites was obtained. Four compounds were identified from tissue samples, all

336 characterized metabolites based on data acquired from human liver S9 incubations, except for one

337 metabolite (M + CH₂) (Table 3). Concerning the sample liver slices, all metabolites were detected in

338 tissue extracts and culture media except the metabolite M + CH₂, which was only detected in liver slices.

339 Only one compound, (M + GlcAc), were detected in the jejunum explant culture medium. Only one

340 epoxide and/or hydroxylated metabolite, (M + O), was found in all tissues from liver origin and cultured

341 cells, whereas the sulfate conjugate (M + SO₃) identified in intestinal cells was not detected in tissues.

342 MS³ analyses were performed for conjugated metabolites, (M + GlcAc and M + O + GlcAc), to confirm

343 their identification.

344

345 Table 3: Compounds detected as [M-H]⁻ in extracts of porcine jejunum explants and precision-cut

346 liver slices exposed to VerA

Compound	Liver slices samples	Jejunum samples	Liver slice: culture media	Jejunum culture media	Measured <i>m/z</i>	Error (ppm)	Molecular formula	Retention time (min)	Observed product ions <i>m/z</i> (relative abundance)
VerA	Yes	Yes	Yes	Yes	337.0348	-1.5	C ₁₈ H ₁₀ O ₇	23.2	309.1 (100), 308.1 (64), 293.1 (34),337.1 (11)
M + O epoxide and/or hydroxylated	Yes	No	Yes	No	353.0303	0	C ₁₈ H ₁₀ O ₈	18.9	325.1 (100), 309.1 (98), 335.2 (94), 281.1 (25),

									310.1 (20), 265.1 (11), 297.1 (9), 353.2 (6), 324.3 (5), 308.2 (5), 307.1 (4)
M + GlcAc	Yes	Yes	Yes	Yes	513.0674	0	C ₂₄ H ₁₈ O ₁₃	19.1	337.1 (100), 513.1 (2), 175.0 (2)
M + O + GlcAc epoxide and/or hydroxylated	Yes	No	Yes	No	529.0624	0	C ₂₄ H ₁₈ O ₁₄	14.2	353.1 (100), 529.1 (2)
M + CH₂	Yes	No	No	No	351.0503	-2.0	C ₁₉ H ₁₃ O ₇	24.9	323.2 (100), 322.2 (54), 351.2 (12), 307.2 (11), 336.2 (6), 266.2 (6), 279.2 (6),

347

348 4 Discussion

349 The toxicity of the bisfuranoid mycotoxins such as AFB1 and its precursor STC largely depends on their
350 biotransformation, which can either lead to the production of less toxic metabolites (detoxification) or
351 of metabolites (or reactive intermediates) that trigger adverse effects following XME-driven
352 bioactivation pathways. One of the pathways involved in the toxicity of mycotoxins is the production of
353 reactive intermediates, which are mutagenic compounds. The balance between bioactivation and
354 detoxification pathways may differ according to the target tissue, depending on the expressed XME.
355 The capability of a given tissue to metabolize mycotoxins into reactive species may define its sensitivity
356 to the adverse effects of these substances. It is therefore essential to characterize the molecules that
357 derive from the metabolization of these mycotoxins. Despite being highly mutagenic and genotoxic, the
358 metabolites of VerA have not been described to date.

359 The presence of a bisfuran moiety in the structure of VerA and previous literature suggest that one of
360 the biotransformation pathways of this substance could lead to the production of a reactive *exo*-epoxide,
361 as already demonstrated for STC and AFB1 (Díaz Nieto et al., 2018; Rushing and Selim, 2019). In the
362 present study, we provide novel qualitative data concerning VerA, as this is the first study to investigate
363 the metabolization of this molecule.

364 Since VerA metabolites are not commercially available, our strategy was based on the generation of
365 high quality chromatographic and MS data produced following incubations of large amounts of in-house
366 purified VerA *in vitro* S9 fractions. This strategy was selected so as to produce sufficient amounts of
367 each metabolite in a matrix relatively less complex than biofluids or cell/tissue extracts.

368 Human S9 fractions are an appropriate well-recognized *in vitro* model to study both phase I and II
369 biotransformation pathways of xenobiotics. Human S9 incubations potentially enable the formation of
370 different metabolites catalyzed by CYP450, flavin-containing monooxygenase (FMOs), UDP-
371 glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and glutathione *S*-transferases
372 (GSTs). With the addition of diverse cofactors, a wider panel of XME activities was investigated than in
373 a previous study (Huber et al., 2021). This approach was then applied to study the VerA metabolism in
374 pigs. Porcine S9 fractions would have been more appropriate to conduct a detailed study of porcine
375 metabolism. However, this protocol was developed to be applied to any xenobiotics in human or any
376 animals after this proof of concept on pigs.

377 In this study, analytical data were acquired on seven VerA-derived products, including phase I and phase
378 II biotransformation metabolites. Along with the intact toxin, the phase I metabolite M + O compatible
379 with an hydroxylated, but also with an epoxide form of VerA was detected in liver. According to
380 previous data, VerA induces AhR transactivation and the subsequent induction of the expression of the
381 CYP450, enzymes involved in the bioactivation of AFB1 (Budin et al., 2021; Gauthier et al., 2020).
382 VerA up-regulates the expression of multiple CYP450 enzymes in intestinal cells including, CYP1A1,
383 CYP1A2 and CYP3A4 (Gauthier et al., 2020). These three isoforms were previously shown to be
384 involved in the oxidation of AFB1 into the highly mutagenic *exo*-8-9 epoxide (Rendic and Guengerich,
385 2021) and CYP3A isoforms have been shown to be important in the metabolization of AFB1 in pig
386 (Jiang et al., 2018; Wu et al., 2016). The presence of an epoxide metabolite of VerA is also supported
387 by the high genotoxicity and mutagenicity (Mori et al., 1984; Theumer et al., 2018) that are partially
388 explained by the intense oxidative and replication stress induced by this toxin, leading to double strand
389 DNA breaks (Gauthier et al., 2020; Smela et al., 2002). Although, hydroxylated, endo or exo epoxide
390 structures could not be differentiated from our experimental data, the observed genotoxicity of VerA
391 suggested the production of an exo epoxide metabolite. Further investigations are needed to confirm this

392 hypothesis, notably the structure of this metabolite by NMR as well as the presence of VerA-DNA or
393 VerA-protein adducts. The epoxide form is usually detoxified into glutathione-conjugated forms
394 mediated by glutathione S-transferases (GSTs), other phase II enzymes (Behrens et al., 2019) and could
395 be consecutively metabolized into other adducts (Hinchman and Ballatori, 1994). The *exo*-AFB1-8-9-
396 epoxyde can be detoxified to a glutathione conjugate by rat and human GSTs (Raney et al., 1992).
397 Regarding the STC, previous experiments using human recombinant CYP1A1 and CYP3A4 enabled
398 the detection of a glutathione conjugate, suggesting the formation of STC-epoxide (Cabaret et al., 2010).
399 Such conjugates were not detected in the present experiment. However, in our experiments, the
400 incubation times of exposed tissues were relatively short. Indeed 24/48 h incubation with VerA enabled
401 the detection of more metabolites than incubation for 3 h in IPEC1 cells. Additional research is
402 warranted to enrich GST-transformed VerA metabolites to overcome possible sensitivity problems. In
403 parallel with epoxide, the M + O metabolite could also represent hydroxylated metabolites. Among four
404 C-positions likely to be hydroxylated, the hydroxylation at C-7 of VerA, which is equivalent to C-9 in
405 STC, could lead to the formation of a catechol. As other possibility, the hydroxylation at C-11 could
406 lead to a hydroxylated metabolite homologous to aflatoxin M1 (Marchese et al., 2018) and 12c-hydroxy-
407 STC (Pfeiffer et al., 2014).

408 Conjugation pathways (phase II metabolism) are predominantly detoxification processes, leading to
409 more readily excreted metabolites (Kedderis, 2010). Glucuronide metabolites (M + GlcAc and M + O
410 + GlcAc) of VerA produced by UGTs were observed in human liver S9 incubations and in liver. Only
411 the metabolite M + GlcAc was detected in intestinal porcine tissues exposed to the toxin. These results
412 suggest that this probable detoxification pathway are crucial in these organs. This is in line with the
413 known induction of the expression of UGTs by VerA (Gauthier et al., 2020). It has been already shown
414 that glucuronidation, which acts directly on the parent molecule or after hydroxylation, is an important
415 pathway for metabolism of STC and 5-methoxysterigmatocystin (Cabaret et al., 2013, 2011, 2010) and
416 is involved in its urinary and biliary elimination in the vervet monkey (Steyn and Thiel, 1976; Thiel and
417 Steyn, 1973).

418 Additional sulfate groups were found in human liver S9 incubations (M + SO₃ and M + O + SO₃) and
419 intestinal porcine epithelial cells (M + SO₃). In the porcine tracheal epithelial cells, STC metabolism

420 resulted in a sulfo-conjugate of hydroxysterigmatocystin (Cabaret et al., 2011), suggesting that sulfonate
421 conjugation may also correspond to a detoxification pathway for VerA. Further studies are needed to
422 investigate the non-toxicity of these conjugated VerA.

423 **5 Conclusion**

424 In the present study, we established the first list of reference metabolites of VerA using human liver S9
425 fraction incubations coupled with UPLC-HRMS, which we subsequently used to identify VerA
426 metabolites produced in intestinal porcine epithelial cells as well as intestinal and hepatic porcine tissues
427 exposed *ex vivo* to the toxin. Reference metabolites produced *in vitro* were used to produce
428 chromatographic and MS and MS/MS data, thereby improving the identification of VerA metabolites
429 in complex biological matrices. This approach was successfully applied to identify emerging
430 mycotoxins in biological samples from animals, but the same strategy could be used to characterize the
431 exposure of humans to other emerging contaminants. As metabolites corresponding to emerging
432 contaminants of food or of human environments are not currently commercially available, their
433 identification is complex (Bonvallot et al., 2021) and the *in vitro* synthesis of reference metabolites using
434 S9 fractions is a promising approach to improve non-targeted human biomonitoring of xenobiotics.

435 By this way, a large set of VerA metabolites was characterized that will be useful for further metabolic
436 studies of VerA. A relatively high concentration of VerA was studied to allow the production of this
437 first list of metabolites, which might not be representative of real circulating concentrations. However,
438 no data are now available on these internal concentrations of VerA in exposed animals. The metabolites
439 identified include phase I and phase II metabolites of VerA, thereby revealing potential detoxification
440 pathways (i.e. glucuronidation and sulfation pathways). In addition, we identified a metabolite
441 consistent with the bioactivation of VerA into an epoxide form. If this pathway is confirmed, it could be
442 involved in the genotoxic and mutagenic effects described in the literature for this emerging toxin. The
443 present results warrant further investigation of the metabolism of this dangerous compound, including
444 quantitative measurements of metabolization rates and kinetics of transformations, as well as the
445 detection of VerA-DNA adducts to help explain its high genotoxicity. Taken together, the results of the
446 present study provide analytical data and biological information, which would be valuable to detect

447 biomarkers of exposure, and biomarkers of effect of VerA useful for the risk assessment of this emerging
448 mycotoxin.

449

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458

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