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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Sulfoglucosides as Novel Modified Forms of the Mycotoxins Alternariol and Alternariol Monomethyl Ether

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Supporting Information

ABSTRACT: The mycotoxins alternariol and alternariol-9-*O*-methyl ether have recently been reported to be extensively conjugated with glucose and malonyl glucose in tobacco suspension cells. However, only trace amounts of glucosylated conjugates were detected in tomatoes inoculated with *Alternaria alternata* in the present study. Instead, mostly sulfate conjugates were observed. In studies using cultures of *A. alternata* and incubations of alternariol and alternariol-9-*O*-methyl ether with tomato tissue in the absence of the fungus, it was clarified that sulfate conjugates were produced by the fungus, whereas tomato tissues converted alternariol and alternariol-9-*O*-methyl ether to glucosylated metabolites. Alternariol-3-sulfate, alternariol-9-sulfate, and alternariol-9-*O*-methyl ether-3-sulfate were unambiguously identified as fungal metabolites using MS and ^{1}H and ^{13}C NMR spectroscopy. When these sulfate conjugates were incubated with tobacco suspension cells or ex planta tomato tissues, three sulfoglucosides of alternariol and one sulfoglucoside of alternariol-9-*O*-methyl ether were formed. Using NMR spectroscopy, the chemical structures of alternariol-3-sulfate-9-glucoside, alternariol-9-sulfate-3-glucoside, and alternariol-9-*O*-methyl ether-3-sulfate-7-glucoside were established. These conjugates were also detected in the *A. alternata*-inoculated tomato. This is the first report on a mixed sulfate/glucoside diconjugate of a mycotoxin. Diconjugates of this novel type may be formed by all mycotoxins and their phase I metabolites with two or more hydroxyl groups and should be taken into account in the future analysis of modified mycotoxins.

KEYWORDS: *masked mycotoxins, modified mycotoxins, Alternaria toxins, sulfates, sulfoglucosides*

INTRODUCTION

Numerous species of the genus *Alternaria* are important plant pathogens, causing early blight diseases of vegetables, brown spot of tangerines, or postharvest black rot of fruit.[1] In addition, *Alternaria* species produce several groups of toxins, which contaminate food and feed and may pose a health problem.[2] For example, *Alternaria* toxins have been associated with an increased incidence of esophageal cancer in certain areas of China.[3] In 2011, the European Food Safety Authority (EFSA) published a Scientific Opinion on the risks for animal and public health related to the presence of the *Alternaria* toxins alternariol, **1**, alternariol-9-*O*-methyl ether, **2**, tenuazonic acid, iso-tenuazonic acid, altertoxins, tentoxin, altenuene, and AAL-toxins in feed and food.[4] The most prevalent *Alternaria* toxins found in many food items, for example, tomatoes, carrots, cereals, sunflower seeds, and olives, were alternariol, **1**, and alternariol-9-*O*-methyl ether, **2** (Figure 1).[4] Alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, exhibit genotoxicity in vitro by inducing gene mutations, DNA strand breaks, and inhibition of topoisomerases I and IIα.[5−8]

It is increasingly realized by health organizations that the exposure of consumers to mycotoxins or other harmful substances, for example, pesticides, must take into account both their parental and modified forms, that is, derivatives formed by thermal reactions or by mammalian or plant metabolism of the parental toxin.[9,10] Of particular concern for mycotoxins are their conjugated metabolites formed in the infested plant, because such conjugates, after oral ingestion by humans or animals, may be hydrolyzed in the digestive tract, thereby releasing the parental form and increasing the total exposure to the toxin. As conjugated mycotoxins escape routine methods of analysis if the method aims exclusively at the detection of the parental compounds, the conjugates are also termed "masked" mycotoxins.[10,11]

Recently, it was reported that alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, both of which carry free hydroxyl groups, readily form a variety of conjugated metabolites in tobacco suspension cells.[12] Five conjugates of alternariol were identified by NMR and mass spectrometry as β-D-glucopyranosides (with the glucose attached in the 3- or 9-position) as well as their 6′-malonyl derivatives and as a gentiobiose conjugate. Conjugation of alternariol-9-*O*-methyl ether gave rise to the D-glucopyranoside at position 3 and its 6′- and 4′-malonyl derivatives.

Received: July 19, 2016
Revised: October 23, 2016
Accepted: October 24, 2016
Published: October 24, 2016

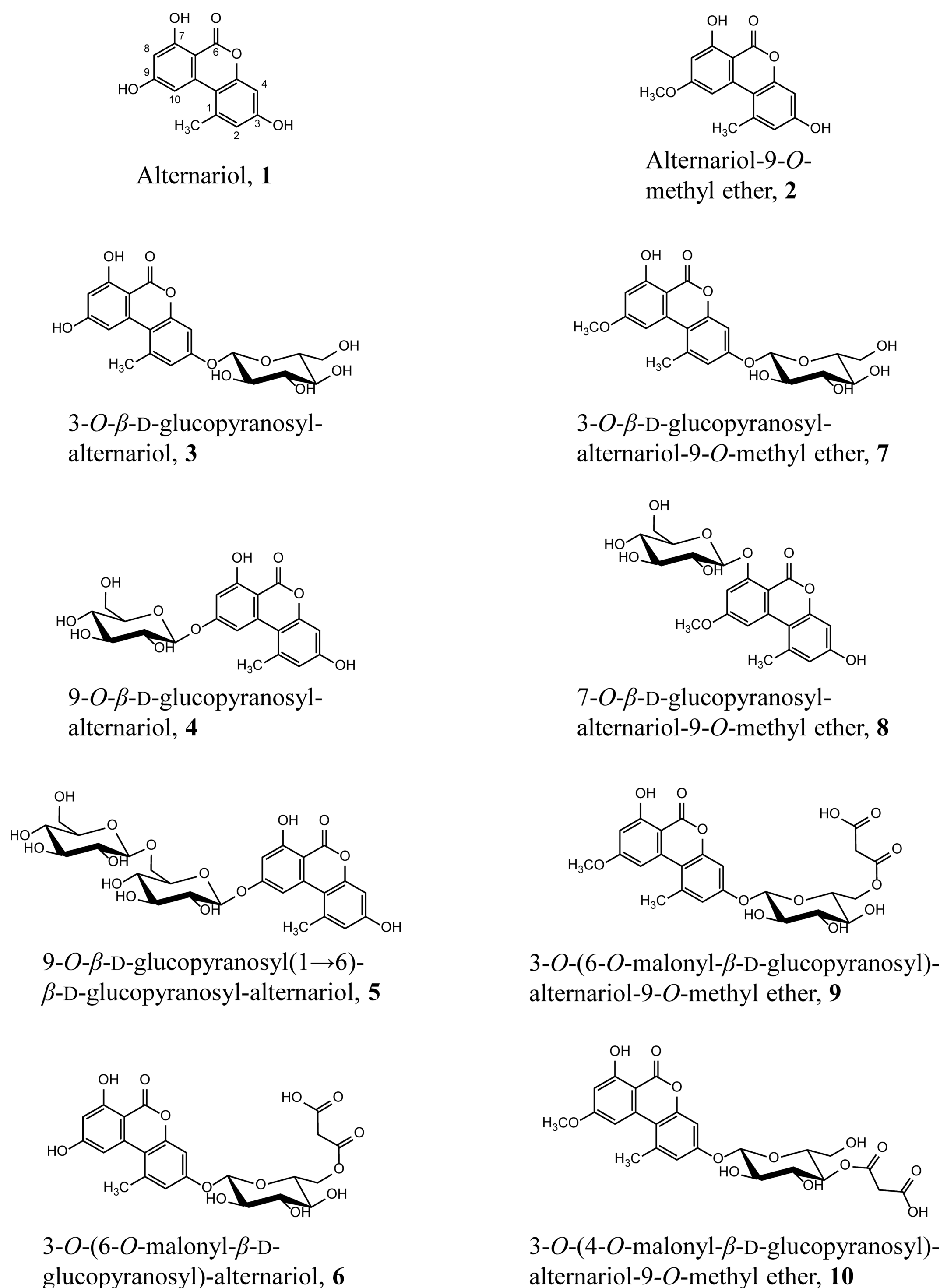

Figure 1. Chemical structures of alternariol, **1**, alternariol-9-*O*-methyl ether, **2**, and their corresponding glycosides.

Although the study with cultured tobacco cells clearly demonstrates that conjugates of alternariol and alternariol-9-*O*-methyl ether can be formed in plant cells, it is necessary to clarify whether food plants commonly infested by *Alternaria* are also capable of "masking" these toxins. To this end, we here report on the conjugation of alternariol and alternariol-9-*O*-methyl ether in tomato fruits inoculated with *Alternaria alternata*, as well as in ex planta cultures of tomato fruits in the absence of the fungus. In addition to glucosides, appreciable amounts of sulfate conjugates and novel sulfoglucosides of alternariol and alternariol-9-*O*-methyl ether, that is, diconjugates in which two different hydroxyl groups of the parent toxin are substituted with a sulfate and a glucoside group, were detected in tomato fruits carrying *A. alternata*, and evidence is provided that the sulfate conjugates were generated by the fungus and not the tomato, whereas the sulfoglucosides are formed in the tomato tissue from the fungal sulfate conjugates.

DOI: 10.1021/acs.jafc.6b03120
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■ MATERIALS AND METHODS

Chemicals and Reagents. For the ex planta culture experiments alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, were isolated from a culture of *A. alternata* strain TA7 grown on rice flour containing media for 24 days at 25 °C as described earlier.[13] The purity was >98% according to HPLC analysis with UV detection at 254 nm.

The following conjugates of alternariol and alternariol-9-*O*-methyl ether were obtained as reference compounds using tobacco suspension cells:[12] 3-*O*-*β*-D-glucopyranosyl-alternariol, **3**; 9-*O*-*β*-D-glucopyranosyl-alternariol, **4**; 9-*O*-*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranosyl-alternariol, **5**; 3-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)-alternariol, **6**; 3-*O*-*β*-D-glucopyranosyl-alternariol-9-*O*-methyl ether, **7**; 7-*O*-*β*-D-glucopyranosyl-alternariol-9-*O*-methyl ether, **8**; 3-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)-alternariol-9-*O*-methyl ether, **9**; and 3-*O*-(4-*O*-malonyl-*β*-D-glucopyranosyl)-alternariol-9-*O*-methyl ether, **10** (Figure 1).

Unless otherwise stated, chemicals and reagents were obtained from Sigma-Aldrich/Fluka (Taufenkirchen, Germany) and Duchefa (Haarlem, The Netherlands) and were of the highest quality available (96−100% depending on the chemical). Agar Kobe I was from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). HPLC-MS grade acetonitrile and methanol were purchased from VWR International (Bruchsal, Germany). NMR solvents were from Deutero GmbH (Kastellaun, Germany) and VWR International (Bruchsal, Germany).

***A. alternata* Strains and Inoculation of a Tomato Fruit with *A. alternata*.** The *A. alternata* strains BFE1346 and BFE1348, which are able to produce alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, were used throughout the experiments. Both strains were isolated from moldy tomatoes. For maintenance the strains were grown on potato dextrose agar (PDA) medium, which was purchased from BioKar Diagnostics (Beauvais, France) and composed of 4 g of potato extract/L, 20 g of glucose/L, and 15 g of agar/L, which was fortified with 10 g of agar Kobe I/L. For inoculum preparation, spores of 7-day-old cultures of *A. alternata* strain BFE1346 grown on PDA medium were suspended at a concentration of 10^5 cfu/mL in Tween salt solution (TWS), which was composed of 1 g of Tween 80/L, 9 g of NaCl/L, and 1 g of agar Kobe I/L. For inoculation, tomatoes from a local market were incised with a sterile scalpel (length of the cut = 50 mm) at three positions, and 10 *μ*L of the spore suspension was applied onto the cut. The tomatoes were incubated at 25 °C in glass beakers covered with aluminum foil. After 7 days, cubes of about 1 cm^3 were cut out of the infected areas and stored at −20 °C. For analysis, the excised cubes were homogenized in liquid nitrogen using a mortar and pestle. An aliquot of 1.0 g of the homogenized samples was mixed with 4 mL of methanol and extracted gently for 60 min at room temperature using an overhead rotation shaker (75 rpm). The suspensions were centrifuged at 17000*g* for 5 min and filtered using 0.2 *μ*m PTFE syringe filters. The filtrate was diluted by 2 volumes of water and analyzed by LC-MS (method A).

Explant Incubation of Tomato Fruits with Alternariol and Alternariol-9-*O*-methyl Ether and Their Sulfates. Ripe red tomatoes from a local supermarket were free of visible fungal or yeast infections. They were thoroughly washed with water, peeled, and cut into cubes of about 2.4 g. Three of these freshly prepared cubes were incubated with 15 mL of complete MS medium containing 600 nmol of alternariol, **1**, alternariol-9-*O*-methyl ether, **2**, or the monosulfates of alternariol and alternariol-9-*O*-methyl ether for 48 h at 25 °C with gentle shaking. Subsequently, the medium was decanted, and the cubes were rinsed with water and freeze-dried. The unconjugated toxins and their conjugates were then extracted from the dried cubes as described before,[12] and the extracts were analyzed by LC-MS (method B).

Cultures of *A. alternata* Using Different Media. Spore suspensions of 10^5 cfu/mL were prepared from strains BFE1346 and BFE1348 as described above. Ten microliters of each suspension was three-point-inoculated on different solid media, those being PDA, MG (composed of 5 g of malt extract/L, 20 g of glucose/L, and 16 g of agar Kobe I/L), and YES medium (composed of 20 g of yeast extract/L, 150 g of sucrose/L, and 16 g of agar Kobe I/L), and incubated at 25 °C in the dark. After incubation for 7 days, samples were taken for toxin analysis. For this purpose, two agar plugs of each colony were taken by a sterile corer near the center and near the margin of the colony. Both agar plugs (229−512 mg total weight) were extracted together by gentle shaking with 1 mL of methanol for 60 min at room temperature using an overhead rotation shaker (75 rpm). The samples were centrifuged at 16000*g* for 5 min and filtered using 0.2 *μ*m PTFE syringe filters. The filtrate was diluted by 2 volumes of water and analyzed by LC-MS (method A).

LC-DAD-MS Analysis. Two different LC-MS devices were used for this study. A UPLC separation module coupled to a high-resolution TOF MS and a DAD was used for the detection and structure elucidation of the conjugates (LC-DAD-MS method A). An HPLC separation module coupled to a DAD and a linear ion trap MS system served for the analysis of most of the incubations (LC-DAD-MS method B). All chromatographic profiles depicted in this paper refer to the DAD response at 254 nm, a wavelength at which all compounds with an intact alternariol or alternariol-9-*O*-methyl ether moiety exhibit absorbance.

LC-DAD-MS method A used a TripleTOF 5600 mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a 1290 Infinity LC system (Agilent, Waldbronn, Germany). The LC-MS system was controlled by the software Analyst TF 1.6.0. LC separation was carried out on a Waters Acquity HSS T3 small-bore UPLC column (100 mm × 2.1 mm i.d., 1.8 *μ*m) equipped with a Waters VanGuard HSS C18 precolumn (5 mm × 2.1 mm i.d., 1.8 *μ*m) and a Phenomenex KrudCatcher ultra. Eluent A was an aqueous 5 mM ammonium acetate buffer, pH 7.0, and eluent B was an acetonitrile/methanol mixture (1:2.5, v/v). A linear gradient was used with a flow rate of 0.5 mL/min and the following elution profile: 0.0−2.0 min, isocratic with 5% B; 2.0−20.0 min, from 5 to 95% B; 20.0−22.6 min, isocratic with 95% B; 22.6−23.0 min, from 95 to 5% B; and 23.0−27.0 min, isocratic with initial conditions. The column oven was set to 40 °C, and the injection volume was 10 *μ*L. The DAD recorded data from 220 to 500 nm with a sampling rate of 10 Hz. The DuoSpray source was operated in negative ESI mode using the following source parameters: curtain gas, 45 psi; ion spray voltage, −4500 V; ion source gas 1, 80 psi; ion source gas 2, 70 psi; ion source gas 2 temperature, 650 °C. The declustering potential was adjusted to −100 V. The MS full scans were recorded from *m*/*z* 100 to 1000 with an accumulation time of 100 ms and a collision energy voltage of −10 V. The MS/MS spectra (product ion) were recorded from *m*/*z* 50 to 1000 in the high-sensitivity mode with an accumulation time of 40 ms, a collision energy voltage of −45 V, and a collision energy spread of 25 V. Nitrogen was used as collision gas. All used organic solvents and reagents were of LC-MS grade quality, and the water was taken from an in-house ultrapure water system at 0.055 *μ*S/cm.

LC-DAD-MS method B used an LXQ Linear Ion Trap MS^n system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Finnigan Surveyor HPLC-DAD system. Separation was carried out on a reversed phase column (Phenomenex Luna C8(2), 250 mm × 4.6 mm i.d.). Solvent A was an aqueous 5 mM ammonium acetate solution, solvent B was acetonitrile with 0.1% formic acid, and the flow rate was 0.5 mL/min. The linear gradient started with 20% B and was held for 5 min, ramped to 50% B in 10 min, ramped to 70% B in 9 min, ramped to 100% B in 5 min, held for 2 min, decreased to 20% B in 1 min, and equilibrated for 2 min before the next injection. The metabolites were detected at 254 nm and characterized with MS operating in ESI negative or positive mode.

Isolation and Purification of Sulfates of Alternariol and Alternariol-9-*O*-methyl Ether. *A. alternata* strain BFE 1346 was grown on six agar plates with PDA medium for 7 days as described above, and the plates were stored at −20 °C. For the extraction of unconjugated toxins, the combined agar and mycelium of each plate was minced with a scalpel and magnetically stirred with 200 mL of ethyl acetate at 20 °C for 2 h, followed by filtration through a paper filter. The residue was then extracted with 200 mL of methanol for 2 h at 20 °C with magnetic stirring, and the extract containing the conjugated metabolites was separated by filtration. After partial evaporation using a rotary evaporator and freeze-drying, the residue was dissolved in methanol and fractionated on a reversed phase column (Phenomenex, Luna C18(2), 5 *μ*m, 250 mm × 25.0 mm i.d.)

DOI: 10.1021/acs.jafc.6b03120
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using a preparative HPLC system consisting of LC-8A pumps and a SPD-20A UV detector (Shimadzu, Kyoto, Japan). Solvent A was an aqueous 5 mM ammonium acetate solution, solvent B was acetonitrile with 0.1% formic acid, and the flow rate was 8 mL/min. The linear gradient started with 20% B and was held for 5 min, then ramped to 65% B in 20 min, ramped to 90% B in 1 min, and held for 4 min. Fractions of the separated metabolites were collected manually according to the chromatogram. After vacuum concentration to about half of their volumes, the fractions were freeze-dried and used for LC-MS and NMR analyses. LC-MS (method B) showed that the purity of the two alternariol sulfates and the alternariol-9-*O*-methyl ether sulfate used for NMR analysis was >97% (based on UV absorbance at 254 nm).

Isolation and Purification of Sulfoglucosides of Alternariol and Alternariol-9-*O*-methyl Ether. To generate sulfoglucosides, pure sulfates of alternariol and alternariol-9-*O*-methyl ether were incubated with tobacco BY-2 (*Nicotiana tabacum* L. cv. 'Bright Yellow 2') suspension cells. Culturing these cells and incubations with sulfate conjugates were conducted as described before for the glycosylation of alternariol and alternariol-9-*O*-methyl ether.[12]

NMR Spectroscopy. NMR spectra were recorded on an Ascend[M] 500 spectrometer (Bruker, Rheinstetten, Germany) equipped with a Prodigy cryoprobe. Freeze-dried samples were dissolved in 500 μL of DMSO-d_6. The structures of the isolated alternariol and alternariol-9-*O*-methyl ether sulfoglucosides were identified by using ^{1}H NMR, heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) experiments; the structures of the alternariol and alternariol-9-*O*-methyl ether sulfates were confirmed by using ^{1}H NMR and HSQC experiments. Standard Bruker pulse sequences were used; spectra were acquired at 298 K. Chemical shifts (δ) were referenced to the central solvent signals (δ_H 2.50 and δ_C 39.5).

RESULTS

Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether Detected in Tomato Fruits Inoculated with *A. alternata*. In a pilot study, a tomato fruit from a local supermarket was inoculated with spores of *A. alternata* strain 1346. After 7 days at ambient temperature, the inoculation site and surrounding tissue together with the fungal mycelium were methanol extracted and analyzed using LC-DAD-MS (method A).

The LC-DAD profile of the extract is depicted in Figure 2. In addition to unconjugated alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, four peaks (a–d) were detected, but none of them was identical with the glucosylated alternariol or alternariol-9-*O*-methyl ether conjugates (compounds **3**–**5** and **7**–**10**) identified earlier.[12] However, traces of alternariol-3-glucoside, **3**, and alternariol-9-glucoside, **4**, were detected at the corresponding retention times by MS in negative ESI mode. The mass spectra of metabolites b and c revealed the same quasimolecular ion $[M - H]^-$ for these compounds. On the basis of the measured accurate masses and the isotopic patterns, the calculated elemental composition of metabolites b and c was $C_{14}H_{10}O_8S$. The MS^2 spectra of the $[M - H]^-$ ions demonstrated a loss of 79.96 Da. This is consistent with the structure of sulfate conjugates, and metabolites b and c were termed alternariol-sulfate-1, **11**, and alternariol-sulfate-2, **12**, respectively. Also, peak d was tentatively identified as the sulfate of alternariol-9-*O*-methyl ether, **13**. The $[M - H]^-$ ion of peak a was represented by m/z 499.0546 and a loss of 79.96 Da, followed by a loss of 162.05 Da in its MS^2 spectrum. The mass spectra and the accurate mass as well as the isotopic pattern were consistent with the structure of an alternariol conjugate carrying both a sulfate and a glucose moiety, and metabolite a was therefore tentatively designated alternariol-sulfoglucoside.

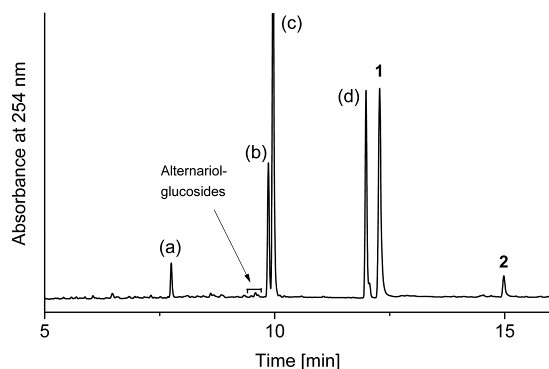

Figure 2. LC-DAD profile of metabolites extracted from tomato fruits 7 days after inoculation with *A. alternata*. LC-DAD-MS method A was used.

Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether Formed in Tomato Fruits. The detection of putative sulfate conjugates in tomato fruits after inoculation with *A. alternata* raised the question of whether these conjugates are generated by the tomato or by the fungus. To study the metabolism of alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, in the absence of the fungus, specimens of noninfected tomato fruit tissue without skin were incubated with alternariol, **1**, or alternariol-9-*O*-methyl ether, **2**, in culture medium for 48 h. After extraction of the tissues, the extracts were analyzed by LC-DAD-MS (method B). In the case of alternariol, two metabolites were unambiguously identified as alternariol-3-glucoside, **3**, and alternariol-9-glucoside, **4**, by comparison of their HPLC retention times, UV spectra, and mass spectra to those of authentic reference compounds (Figure 3A). Also, alternariol-9-*O*-methyl ether-3-glucoside, **7**, and alternariol-9-*O*-methyl ether-7-glucoside, **8**, were identified in the extracts of the tissues, which were incubated with alternariol-9-*O*-methyl ether, **2** (Figure 3B). Alternariol, **1**, appeared to be more readily glucosylated than alternariol-9-*O*-methyl ether, **2**. At the end of the incubation, neither unconjugated alternariol, **1**, nor alternariol-9-*O*-methyl ether, **2**, nor their conjugates were detected in the incubation medium. These data show that alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, were completely taken up and efficiently glucosylated by the tomato cells. A similar behavior of alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, has previously been observed with tobacco suspension cells.[12] However, tobacco cells also formed malonyl derivatives of the glucosides, which were not detected in tomatoes. Importantly, none of the presumed sulfate conjugates formed as major metabolites in tomatoes inoculated with *A. alternata* (Figure 2) were observed by UV detection in the extracts of noninfected tomato fruits after exposure to alternariol, **1**, and alternariol-9-*O*-methyl ether, **2** (Figure 3).

Although it was not the aim of this study to quantitate the metabolites, a semiquantitative analysis is possible because the metabolic profiles were determined by measuring absorbance, and it can be assumed that the molar absorbances at 254 nm of the aglycones and conjugates are very similar. Thus, it is concluded from Figure 3 that 90% of the alternariol, **1**, but only 48% of the alternariol-9-*O*-methyl ether, **2**, were converted to glucosides. The total recovery of glucosides and aglycones from the tomato tissue was about 50% of the incubated toxins, and the losses are probably due to incomplete liquid/liquid extraction and possibly to nonextractable binding to cell wall

DOI: 10.1021/acs.jafc.6b03120
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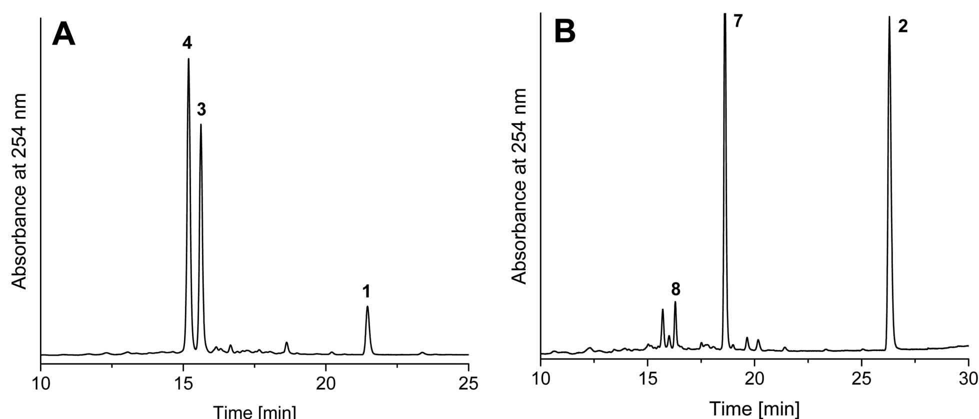

Figure 3. LC-DAD profile of the extracts of tomato fruits after incubation with (A) alternariol, **1**, or (B) alternariol-9-*O*-methyl ether, **2**. LC-DAD-MS method B was used.

constituents, as has also been observed in earlier studies with tobacco suspension cells.[12]

Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether Detected in Cultures of *A. alternata*. The striking difference in the pattern of metabolites observed after incubation of noninfected tomato fruits with the pure toxins, that is, monoglucosides (Figure 3), as compared to tomatoes inoculated with the fungus, that is, sulfate conjugates (Figure 2), suggests that the sulfates are generated by the fungus. To study the fungal metabolites, *A. alternata* strain 1346 was cultured for 7 days on agar plates using three different media (malt glucose (MG), potato dextrose (PDA), and yeast extract sucrose (YES) agar), and samples of the mycelium were subsequently extracted with methanol and analyzed using LC-MS method A. The results are shown in Figure 4. In addition to

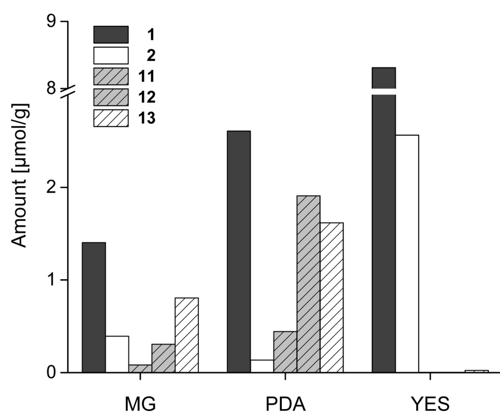

Figure 4. Pattern of fungal metabolites of *A. alternata* strain 1346 in three different media.

unconjugated alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, three products were observed that had identical retention times and MS and MS^2 spectra as the metabolites b (alternariol-sulfate-1, **11**), c (alternariol-sulfate-2, **12**), and d (alternariol-9-*O*-methyl ether-sulfate, **13**) found before in the fungus-inoculated tomato fruit (Figure 2). Whereas these sulfates were formed in appreciable amounts in MG and PDA media, only trace amounts were detected in YES medium. These results were confirmed in a second experiment. Moreover, the same pattern of conjugates was obtained with *A. alternata* strain 1348 (data not shown). None of the strains generated detectable quantities of a glucoside or sulfoglucoside of alternariol and alternariol-9-*O*-methyl ether.

Structure Elucidation of the Sulfate Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether. To clarify the chemical structures of the sulfate-conjugated fungal metabolites, that is, which hydroxyl group carries the sulfate moiety, strain 1346 was grown on several agar plates with PDA medium for 7 days. The complete mycelium and agar of all plates was then extracted with ethyl acetate to remove the unconjugated metabolites, followed by methanol extraction to obtain the conjugates. Analysis of the methanol extract using LC-DAD-MS (method B) revealed the presence of three major products (Figure 5). According to their mass spectra, peak g

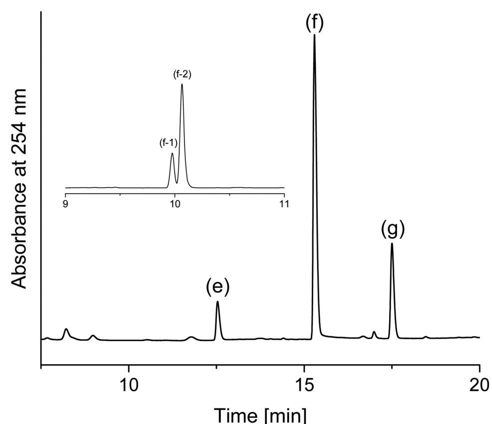

Figure 5. LC-DAD profile of fungal metabolites obtained when *A. alternata* strain 1346 was grown on agar plates with PDA medium and analyzed using LC-DAD-MS method B. Inset: Section of the profile obtained from the analysis using LC-DAD-MS method A.

was alternariol-9-*O*-methyl ether-sulfate, **13**, and peak f was alternariol sulfate. Peak e contains a compound with a molecular weight of 418 and two subsequent losses of 80 Da in the MS^2 mode, suggesting this structure to be an alternariol disulfate, **14**. As the pilot study had shown the formation of two monosulfates of alternariol (alternariol-sulfate-1, **11**, and alternariol-sulfate-2, **12**; Figure 2 peaks b and c, respectively), the methanol extract was also analyzed using LC-DAD-MS (method A), which separated peak f into peaks f-1 (ca. 30%, alternariol-sulfate-1, **11**) and f-2 (ca. 70%, alternariol-sulfate-2, **12**), as shown in the inset of Figure 5. Additionally, in this

DOI: 10.1021/acs.jafc.6b03120
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measurement (negative mode) the suggested structure of peak e (alternariol disulfate, **14**) was confirmed by the calculated elemental composition ($C_{14}H_{10}O_{11}S_2$) based on the measured accurate mass and the isotopic pattern as well as by the accurate MS^2 (two subsequent losses of 79.96 Da (SO_3)).

Peaks e, f, and g were isolated using preparative HPLC and analyzed by NMR (^{1}H, HSQC). The NMR data are listed in Table 1. The electron-withdrawing sulfate group causes a downfield shift of the protons and carbons in ortho-position to the substitution site in comparison to the nonconjugated toxin. The compound representing peak e in Figure 5 exhibited a downfield shift of the H and C atoms at positions 2, 4, 8, and 10, indicating the attachment of sulfate groups at positions 3 and 9. Thus, this metabolite was tentatively identified as alternariol-3,9-disulfate, **14**, which represents a new fungal metabolite. The dominant peak of the methanol extract (peak f) contained a mixture of two regioisomers of alternariol sulfate (peaks f-1 and f-2) (Figure 5). For one of them the protons and carbons shifted downfield by about 0.4 ppm (H-8, H-10) and about 3 ppm (C-8, C-10), suggesting conjugation with sulfate at C-9. The other isomer is conjugated at C-3, as demonstrated by the downfield shifts of the H-2, H-4, C-2, and C-4 signals (Table 1). Integration of the NMR signals suggested that the smaller peak (f-1) (alternariol-sulfate-1, **11**) represents alternariol-3-sulfate, **11**, and the larger peak (f-2) (alternariol-sulfate-2, **12**) alternariol-9-sulfate, **12**. Finally, peak g of the methanol extract (Figure 5) was identified as alternariol-9-*O*-methyl ether-3-sulfate, **13** (downfield shifts of the H-2, H-4, C-2, and C-4 signals).

Table 1. ^{1}H and ^{13}C NMR Data of Sulfate Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether[a]

		δ_H	J (Hz)	δ_C
alternariol-3,9-disulfate, **14**	2	7.06	d (2.6)	120.59
	4	7.17	d (2.6)	105.80
	8	6.89	d (2.1)	104.78
	10	7.74	d (2.1)	107.48
	11	2.75	s	24.66
alternariol-9-sulfate, **12**	2	6.73	d (2.6)	117.37
	4	6.66	d (2.6)	101.33
	8	6.82	d (2.0)	104.13
	10	7.69	d (2.0)	106.84
	11	2.71	s	24.70
alternariol-3-sulfate, **11**	2	7.06	d (2.5)	120.68
	4	7.14	d (2.5)	105.96
	8	6.43	d (1.9)	101.29
	10	7.33	d (1.9)	104.85
	11	2.76	s	24.87
alternariol-9-*O*-methyl ether-3-sulfate, **13**	2	7.08	d (2.6)	120.63
	4	7.17	d (2.6)	105.87
	8	6.70	d (2.2)	99.63
	10	7.33	d (2.2)	103.89
	11	2.80	s	24.66
	12	3.93	s	55.57

[a]For ^{1}H and ^{13}C NMR data of unconjugated alternariol and alternariol-9-*O*-methyl ether, refer to Table 2.

Glucosylation of the Sulfates of Alternariol and Alternariol-9-*O*-methyl Ether in Tobacco Suspension Cells. Our studies described so far have shown that noninfested tomato fruits were able to generate glucosides but not sulfates of alternariol and alternariol-9-*O*-methyl ether, whereas *A. alternata* gave rise to sulfates but not glucosides. This raised the question of how the sulfoglucoside observed in the pilot study (Figure 2, peak a) was formed. One possibility would be that sulfate conjugates formed by the fungus were taken up by the tomato cells and further metabolized to sulfoglucosides. To test this hypothesis, the sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether were incubated with tobacco suspension cells as a model for plant cells known to efficiently glucosylate alternariol and alternariol-9-*O*-methyl ether.[12] Therefore, the mixture of sulfates as shown in Figure 5 was incubated with tobacco suspension cells during their growth phase for 2 days, and the freeze-dried cells were extracted according to the method of Hildebrand et al.[12] Analysis of the extract using LC-DAD-MS (method B) resulted in the metabolite profile depicted in Figure 6.

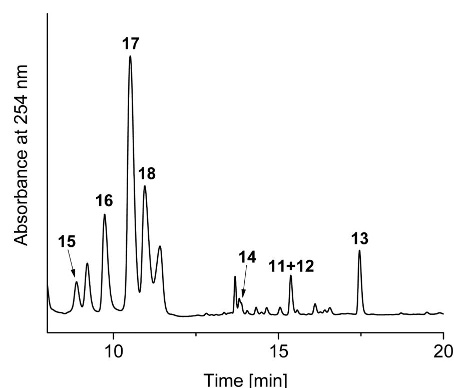

Figure 6. LC-DAD profile of metabolites extracted from tobacco suspension cells after incubation with a mixture of alternariol sulfates and alternariol-9-*O*-methyl ether sulfate. LC-DAD-MS method B was used.

In addition to small amounts of alternariol sulfates (**11** and **12**) and alternariol-9-*O*-methyl ether-3-sulfate, **13**, several prominent peaks were present that eluted earlier than the sulfates from the reverse-phase column. Three of them had the molecular weight of sulfoglucosides of alternariol, that is, 500 (peaks alternariol-sulfoglucoside-1, **15**, alternariol-sulfoglucoside-2, **16**, and alternariol-sulfoglucoside-3, **17**) and one of a sulfoglucoside of alternariol-9-*O*-methyl ether, that is, 514 (peak alternariol-9-*O*-methyl ether-sulfoglucoside, **18**). These data demonstrate that the sulfates of alternariol and alternariol-9-*O*-methyl ether are taken up by the tobacco cells and are efficiently converted to sulfoglucosides.

Structure Elucidation of the Sulfoglucoside Conjugates of Alternariol and Alternariol-9-*O*-methyl Ether. To clarify the exact chemical structures of the alternariol and alternariol-9-*O*-methyl ether sulfoglucoside conjugates, a mixture of the sulfate conjugates as obtained from fungal cultures was prepared on a larger scale and subsequently incubated with tobacco suspension cells, followed by isolation of alternariol-sulfoglucoside-1, **15**, alternariol-sulfoglucoside-2, **16**, alternariol-sulfoglucoside-3, **17**, and alternariol-9-*O*-methyl ether-sulfoglucoside, **18**, using preparative HPLC. The isolated fractions alternariol-sulfoglucoside-2, **16**, alternariol-sulfoglucoside-3, **17**, and alternariol-9-*O*-methyl ether-sulfoglucoside, **18**, were fully characterized by NMR spectroscopy, whereas

DOI: 10.1021/acs.jafc.6b03120
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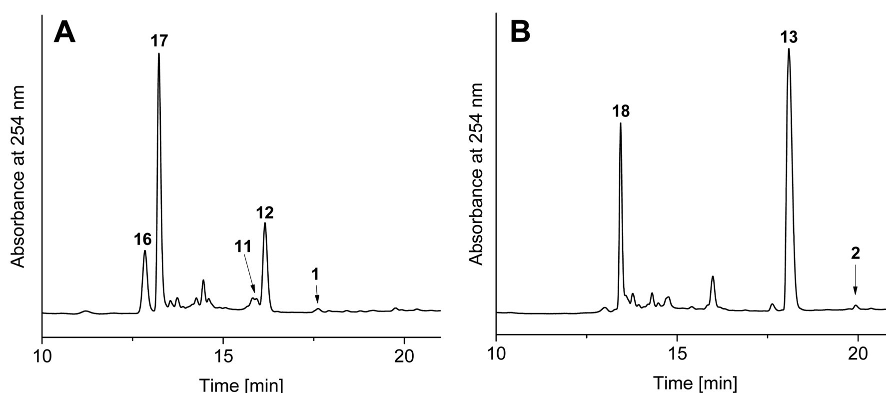

Figure 7. LC-DAD profiles of metabolites extracted from tomato fruit tissues after incubation with a mixture of (A) alternariol-3-sulfate and alternariol-9-sulfate and with (B) alternariol-9-*O*-methyl ether-3-sulfate, using LC-DAD-MS method B.

impurities of fraction alternariol-sulfoglucoside-1, **15**, did not allow for an unambiguous structural characterization. The ^{1}H NMR spectrum of alternariol-sulfoglucoside-2, **16**, showed an anomeric proton signal of the glucose moiety at 5.15 ppm with a coupling constant of 7.3 Hz, indicating the β-configuration of the glucopyranose unit. An H1′(glucose)/C9 (alternariol) HMBC cross peak demonstrates the linkage between the β-anomer of the glucose unit and the phenolic hydroxyl group in position 9 of alternariol via a glycosidic bond, identifying this compound as alternariol-3-sulfate-9-glucoside, **16**. Interpretation of the proton and carbon NMR data of alternariol-sulfoglucoside-3, **17**, suggests this compound to be alternariol-9-sulfate-3-glucoside, **17**, with the coupling constant of the anomeric proton of the glucopyranose unit suggesting a β-configuration. The linkage position between alternariol and the glucose unit was unambiguously confirmed using the HMBC experiment, showing an H1′(glucose)/C3 (alternariol) cross peak. Different from the alternariol sulfoglucosides, NMR experiments of the alternariol-9-*O*-methyl ether-sulfoglucoside, **18**, suggest the linkage of β-glucopyranose to position O-7 of alternariol-9-*O*-methyl ether, the only remaining conjugation site after O-3-sulfation. Again, the anomeric configuration of the glucopyranose unit and its attachment to alternariol-9-*O*-methyl ether was identified via the proton coupling constant (7.8 Hz) and an HMBC cross peak of H1′(glucose)/C7 (alternariol-9-*O*-methyl ether).

The structures of the sulfoglucosides of alternariol and alternariol-9-*O*-methyl ether were further confirmed by high-resolution MS and MS^2 in negative ESI mode. The calculated elemental compositions based on the measured accurate masses and the isotopic patterns of the regioisomeric alternariol-3-sulfate-9-glucoside, **16**, and alternariol-9-sulfate-3-glucoside, **17**, were in both cases $C_{20}H_{20}O_{13}S$. For alternariol-9-*O*-methyl ether-3-sulfate-7-glucoside, **18**, an elemental composition of $C_{21}H_{22}O_{13}S$ was calculated on the basis of the measured accurate mass and the isotopic pattern. The MS^2 of the $[M - H]^-$ ions of all three sulfoglucosides exhibited fragment ions resulting from the initial loss of 79.96 Da (sulfate moiety) and the subsequent loss of 162.05 Da (glucose moiety) (data not shown).

Glucosylation of the Sulfates of Alternariol and Alternariol-9-*O*-methyl Ether in Tomato Fruits. When a mixture of alternariol-3-sulfate, **11**, and alternariol-9-sulfate, **12**, (peak f) (Figure 5) was incubated with noninfested tomato fruit tissues and analyzed under the same conditions as described above for the incubation of alternariol sulfates with tobacco suspension cells, the metabolic profile depicted in Figure 7A was obtained. In addition to small amounts of the applied alternariol sulfates, which were separated on the HPLC column used in this experiment, alternariol-3-sulfate-9-glucoside, **16**, and alternariol-9-sulfate-3-glucoside, **17**, were present in a ratio of about 30−70%, the same ratio as determined for the sulfate conjugates prior to glucosylation (Figure 5). When alternariol-9-*O*-methyl ether-3-sulfate, **13**, was incubated with tomato fruit tissues, it was metabolized to alternariol-9-*O*-methyl ether-3-sulfate-7-glucoside, **18** (Figure 7B), although the extent of glycosylation was apparently lower for the alternariol-9-*O*-methyl ether sulfate as compared to the alternariol sulfates. Neither sulfates nor sulfoglucosides could be detected in the medium at the end of the incubation. These findings demonstrate that the sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether are taken up by the tomato cells and further metabolized to sulfoglucosides. The semi-quantitative analysis of Figure 7 showed that the sulfates of alternariol and alternariol-9-*O*-methyl ether were glucosylated to extents of 72 and 25%, respectively.

Eventually, the two major sulfoglucosides of alternariol and the sulfoglucoside of alternariol-9-*O*-methyl ether were also detected in the tomato inoculated with *A. alternata* in the pilot study (Figure 2). By using LC-DAD-MS (method A), the corresponding mass spectra of alternariol-9-sulfate-3-glucoside, **17**, (peak a, Figure 2) as well as of alternariol-3-sulfate-9-glucoside, **16**, and alternariol-9-*O*-methyl ether-3-sulfate-7-glucoside, **18** (detected only in the MS trace), were obtained at the respective retention times (data not shown). The reason for the low amounts of sulfoglucosides and the trace amounts of glucosides observed under these conditions is probably due to the fact that the analyzed specimens contained mostly fungal material and very little intact tomato tissue.

■ DISCUSSION

There is increasing awareness in the field of mycotoxins that the formation of conjugated metabolites of these harmful compounds in the fungus and/or plant poses a problem in assessing the exposure of humans and animals from food and feed, because such conjugates may escape analysis but release the parental toxin after ingestion.[9−11] Conjugation must especially be expected, and has been shown, for mycotoxins carrying free hydroxyl groups, such as zearalenone and several trichothecenes.[9,11] Another class of ubiquitous mycotoxins are *Alternaria* toxins, with alternariol, **1**, and alternariol-9-*O*-methyl ether, **2**, being prevalent members. Alternariol, **1**, has three and

DOI: 10.1021/acs.jafc.6b03120
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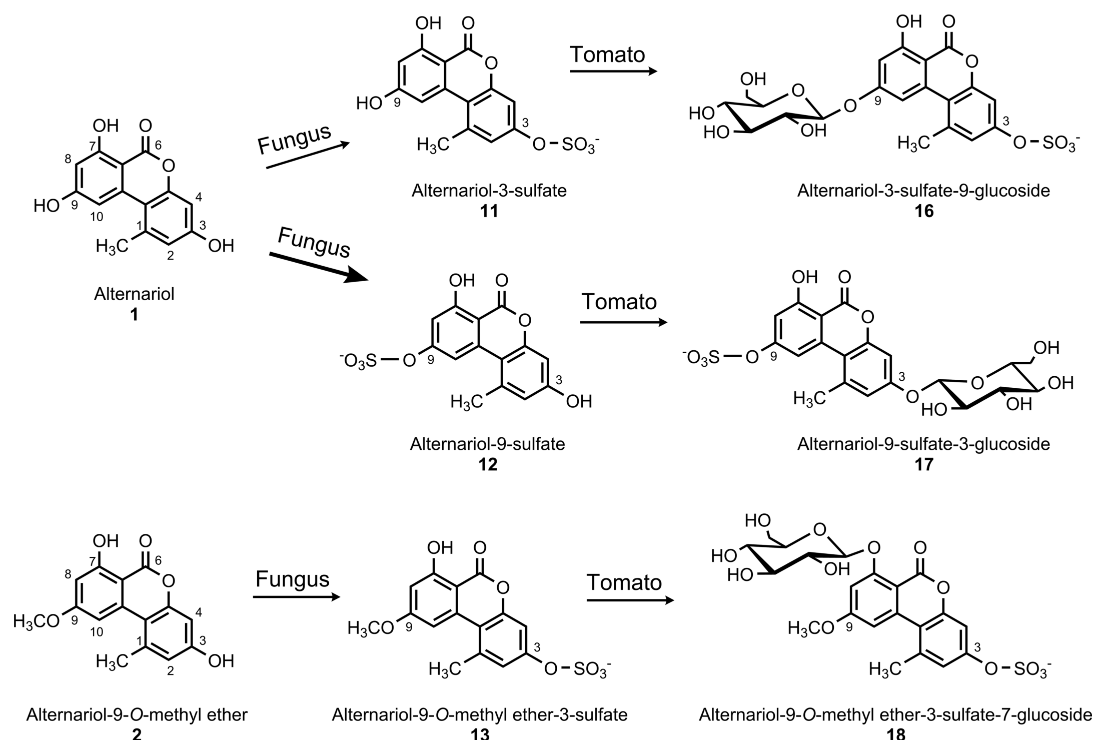

Figure 8. Formation of conjugated forms of alternariol and alternariol-9-*O*-methyl ether. The parent toxins were metabolized to sulfate conjugates in cultures of *A. alternata*, and the sulfates were subsequently conjugated to sulfoglucosides in tomato tissues and cultured tobacco cells. The bold arrow indicates the predominant sulfate conjugation of alternariol.

Table 2. ^{1}H and ^{13}C NMR Data of Alternariol and Alternariol-9-*O*-methyl Ether and Their Sulfoglucosides

	alternariol, **1**			alternariol sulfoglucoside-2, **16**			alternariol sulfoglucoside-3, **17**			alternariol-9-*O*-methyl ether, **2**			alternariol-9-*O*-methyl ether sulfoglucoside, **18**		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1			138.10			137.64						138.30			136.92
2	6.72	d (2.3)	117.32	7.08	d (2.6)	120.70	6.98	d (2.7)	117.86	6.73	d (2.3)	117.42	7.01	d (2.6)	120.00
3			158.25			154.23			157.31			158.33			153.28
4	6.64	d (2.3)	101.36	7.17	d (2.6)	105.85	7.00	d (2.7)	102.20	6.65	d (2.3)	101.29	7.06	d (2.6)	105.30
4a			152.51			151.12			151.90			152.52			nd
6			163.99			nd			nd			nd			nd
6a			97.21			99.96			99.94			98.47			103.81
7			164.44			163.60			162.95			164.00			161.54
8	6.37	d (1.6)	100.63	6.71	d (2.1)	102.11	6.88	d (2.0)	104.71	6.63	d (1.9)	98.94	7.03	d (2.2)	101.93
9			165.29			163.85			160.70			165.89			164.26
10	7.25	d (1.6)	104.07	7.48	d (2.1)	104.61	7.75	d (2.0)	107.44	7.23	d (1.9)	103.23	7.44	d (2.2)	104.78
10a			138.10			138.95			137.89			138.30			n.d.
10b			108.75			111.83			111.03			108.60			111.49
11	2.71	s	24.97	2.82	s	24.69	2.77	s	24.70	2.74	s	24.81	2.80	s	24.49
12										3.91	s	55.60	3.95	s	55.37
1′				5.15	d (7.3)	99.56	5.03	d (7.4)	99.68				5.05	d (7.8)	101.49
2′				3.31		72.87	3.27		72.90				3.42		73.11
3′				3.33		76.04	3.29		76.16				3.32		76.01
4′				3.18		69.33	3.18		69.35				3.18		69.62
5′				3.44		77.16	3.44		76.85				3.45		77.34
6′				3.72		60.37	3.72		60.31				3.73		60.64
6′				3.47		60.37	3.47		60.31				3.47		60.61

alternariol-9-*O*-methyl ether, **2**, has two free phenolic hydroxyl groups (Figure 1), and a recent study demonstrated their facile glycosylation in tobacco suspension cells as a plant cell model system.[12] Therefore, it was somewhat surprising to find only traces of the glucosides of alternariol in a tomato inoculated with *A. alternata*; instead, sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether were detected as major metabolites (Figure 2). However, when tomato tissues were incubated with alternariol, **1**, or alternariol-9-*O*-methyl ether, **2**, in the absence of the fungus, the respective glucosides were formed without detectable amounts of the sulfate conjugates (Figure 3). Further experiments demonstrated that the sulfate

DOI: 10.1021/acs.jafc.6b03120
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conjugates are fungal metabolites (Figures 4 and 5), and the chemical structures of alternariol-3-sulfate, **11**, alternariol-9-sulfate, **12**, and alternariol-9-*O*-methyl ether-3-sulfate, **13** (Figure 8), were unambiguously elucidated by ^{1}H and ^{13}C NMR spectroscopy of the purified metabolites. These three sulfate conjugates are not novel compounds, because alternariol-9-sulfate, **12**, and alternariol-9-*O*-methyl ether-3-sulfate, **13**, have previously been isolated from cultures of an endophytic *Alternaria* strain growing on the leaves of the traditional Egyptian medicinal plant *Polygonum senegalense*, and the chemical structures of both sulfate conjugates were established by ^{1}H and ^{13}C NMR as well as mass spectrometry.[14] Also, monosulfates of alternariol and alternariol-9-*O*-methyl ether have recently been chemically synthesized and characterized by NMR spectroscopy.[15]

The formation of monosulfates of alternariol and alternariol-9-*O*-methyl ether by *Alternaria* fungi raises the question about the fate of such conjugates in the plant. A first hint was obtained by the detection of a putative sulfoglucoside of alternariol in the inoculated tomato (Figure 2), possibly arising through glycosylation of alternariol sulfate. Again, using tobacco suspension cells, it was demonstrated that the sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether were readily metabolized to sulfoglucosides (Figure 6). Two of the three alternariol sulfoglucosides formed in tobacco cells were subsequently identified by ^{1}H and ^{13}C NMR spectrometry as alternariol-3-sulfate-9-glucoside, **16**, and alternariol-9-sulfate-3-glucoside, **17** (Table 2 and Figure 8). The third alternariol sulfoglucoside could not yet be isolated in sufficient amounts and purity for NMR spectroscopy, but is assumed to arise from the predominant sulfate conjugate alternariol-9-sulfate through glucosylation at C-7. The alternariol-9-*O*-methyl ether sulfoglucoside, **18**, was clearly identified by ^{1}H and ^{13}C NMR spectrometry as alternariol-9-*O*-methyl ether-3-sulfate-7-glucoside, **18** (Table 2 and Figure 8). Finally, glucosylation of sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether was also demonstrated to occur in ex planta cultures of tomatoes (Figure 7).

To the best of our knowledge, sulfoglucosides, that is, diconjugates carrying both a sulfate and a glucose moiety at different hydroxyl groups of the same aglycone molecule, have not been reported for any mycotoxin before. The potential formation of such conjugates under field conditions in infested plants makes the analysis of masked mycotoxins more complex, because sulfoglucosides must now be added to the list of modified forms. For example, when 24 samples of rice and oat flakes from Belgian supermarkets were analyzed for alternariol-3-glucoside, **3**, alternariol-3-sulfate, **11**, alternariol-9-*O*-methyl ether-3-glucoside, **7**, and alternariol-9-*O*-methyl ether-3-sulfate, **13**, none of the conjugates were detected in these cereal-based products.[16] In contrast, alternariol-3-sulfate, **11**, and alternariol-9-*O*-methyl ether-3-sulfate, **13**, but not alternariol-3-glucoside, **3**, and alternariol-9-*O*-methyl ether-3-glucoside, **7**, were detected in samples of tomato products (juice, sauce, and concentrate).[17] In a recent survey on *Alternaria* toxins in dried figs, sunflower products, and tomato products carried out in The Netherlands, none of seven conjugates (monoglucosides, malonylglucosides, and monosulfates) of alternariol and alternariol-9-*O*-methyl ether were detected.[18] The failure to find conjugated forms of alternariol and alternariol-9-*O*-methyl ether in two of the studies may, in part, be due to the possible circumstance that sulfoglucosides were the predominant conjugates.

In the present study, sulfate conjugates of alternariol and alternariol-9-*O*-methyl ether were formed by the fungus and further metabolized in the plant tissue. This implies that the formation of sulfoglucosides is more favorable if appreciable amounts of sulfate conjugates are generated. For zearalenone, which has two hydroxyl groups, it has recently been reported that the amount of zearalenone-14-sulfate exceeds the amount of free zearalenone in certain fractions of naturally contaminated wheat grains, whereas zearalenone-glucosides were present only in low amounts.[19] Thus, it is conceivable that a sulfoglucoside occurs as a modified form of zearalenone. In general, the possible formation of sulfoglucosides should be considered in the future for all mycotoxins and their phase I metabolites with two or more hydroxyl groups. The analysis of this novel type of conjugate requires optimized methods of extraction and quantitation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03120.

Measured accurate masses of sulfates and sulfoglucosides of alternariol and alternariol-9-*O*-methyl ether as well as HMBC spectra of alternariol sulfoglucosides and alternariol-9-*O*-methyl ether sulfoglucoside identified in this study (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Nicole Mischke, Bettina Schindler, and Heidi Schön for excellent technical assistance.

■ REFERENCES

(1) Logrieco, A.; Moretti, A.; Solfrizzo, M. *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin J.* **2009**, *2*, 129−140.

(2) Ostry, V. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175−188.

(3) Liu, G. T.; Qian, Y. Z.; Zhang, P.; Dong, W. H.; Qi, Y. M.; Guo, H. T. Etiological role of *Alternaria alternata* in human esophageal cancer. *Chin. Med. J. (Beijing, China, Engl. Ed.)* **1992**, *105*, 394−400.

(4) EFSA Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA J.* **2011**, *9*, 2407.10.2903/j.efsa.2011.2407

(5) Lehmann, L.; Wagner, J.; Metzler, M. Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food Chem. Toxicol.* **2006**, *44*, 398−408.

(6) Brugger, E. M.; Wagner, J.; Schumacher, D. M.; Koch, K.; Podlech, J.; Metzler, M.; Lehmann, L. Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. *Toxicol. Lett.* **2006**, *164*, 221−230.

(7) Pfeiffer, E.; Eschbach, S.; Metzler, M. *Alternaria* toxins: DNA strand-breaking activity in mammalian cells in vitro. *Mycotoxin Res.* **2007**, *23*, 152−157.

(8) Fehr, M.; Pahlke, G.; Fritz, J.; Christensen, M. O.; Boege, F.; Altemoller, M.; Podlech, J.; Marko, D. Alternariol acts as a

topoisomerase poison, preferentially affecting the IIα isoform. *Mol. Nutr. Food Res.* **2009**, *53*, 441−451.

(9) EFSA Scientific opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA J.* **2014**, *12*, 3916.10.2903/j.efsa.2014.3916

(10) Rychlik, M.; Humpf, H. U.; Marko, D.; Danicke, S.; Mally, A.; Berthiller, F.; Klaffke, H.; Lorenz, N. Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. *Mycotoxin Res.* **2014**, *30*, 197−205.

(11) Berthiller, F.; Crews, C.; Dall'Asta, C.; De Saeger, S.; Haesaert, G.; Karlovsky, P.; Oswald, I. P.; Seefelder, W.; Speijers, G.; Stroka, J. Masked mycotoxins: a review. *Mol. Nutr. Food Res.* **2013**, *57*, 165−186.

(12) Hildebrand, A. A.; Kohn, B. N.; Pfeiffer, E.; Wefers, D.; Metzler, M.; Bunzel, M. Conjugation of the mycotoxins alternariol and alternariol monomethly ether in tobacco suspension cells. *J. Agric. Food Chem.* **2015**, *63*, 4728−4736.

(13) Fleck, S. C.; Burkhardt, B.; Pfeiffer, E.; Metzler, M. *Alternaria* toxins: altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol. Lett.* **2012**, *214*, 27−32.

(14) Aly, A. H.; Edrada-Ebel, R. A.; Indriani, I. D.; Wray, V.; Müller, W. E. G.; Totzke, F.; Zirrgiebel, U.; Schächtele, C.; Kubbutat, M. H. G.; Lin, W. H.; Proksch, P.; Ebel, R. J. Cytotoxic metabolites from the fungal endophyte *Alternaria* sp. and their subsequent detection in its host plant *Polygonum senegalense*. *J. Nat. Prod.* **2008**, *71*, 972−980.

(15) Mikula, H.; Skrinjar, P.; Sohr, B.; Ellmer, D.; Hametner, C.; Fröhlich, J. Total synthesis of masked *Alternaria* mycotoxins − sulfates and glucosides of alternariol (AOH) and alternariol-9-methyl ether (AME). *Tetrahedron* **2013**, *69*, 10322−10330.

(16) Walravens, J.; Mikula, H.; Rychlik, M.; Asam, S.; Ediage, E. N.; Di Mavungu, J. D.; Van Landschoot, A.; Vanhaecke, L.; De Saeger, S. Development and validation of an ultra-high-performance liquid chromatography tandem mass spectrometric method for the simultaneous determination of free and conjugated *Alternaria* toxins in cereal-based foodstuffs. *J. Chromatogr. A* **2014**, *1372*, 91−101.

(17) Walravens, J.; Mikula, H.; Rychlik, M.; Asam, S.; Devos, T.; Ediage, E. N.; Di Mavungu, J. D.; Jacxsens, L.; Van Landschoot, A.; Vanhaecke, L.; De Saeger, S. Validated UPLC-MS/MS methods to quantitate free and conjugated *Alternaria* toxins in commercially available tomato products and fruits and vegetable juices in Belgium. *J. Agric. Food Chem.* **2016**, *64*, 5101−5109.

(18) López, P.; Venema, D.; Mol, H.; Spanjer, M.; De Stoppelaar, J.; Pfeiffer, E.; De Nijs, M. *Alternaria* toxins and conjugates in selected foods in the Netherlands. *Food Control* **2016**, *69*, 153−159.

(19) Schwake-Anduschus, C.; Proske, M.; Sciurba, E.; Muenzing, K.; Koch, M.; Maul, R. Distribution of deoxynivalenol, zearalenone, and their respective modified analogues in milling fractions of naturally contaminated wheat grains. *World Mycotoxin J.* **2015**, *8*, 433−443.

DOI: 10.1021/acs.jafc.6b03120
J. Agric. Food Chem. XXXX, XXX, XXX−XXX