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Article

Rapid Detection of ACTG- and AK-Toxins in *Alternaria alternata* by LC-ESI-MS/MS Analysis and Antifungal Properties of *Citrus* Compounds

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A methanol-soluble fraction of the dichloromethane extract from the culture broth of *A. alternata* AT4303 strain afforded the following five known mycotoxins: alternariol, alternariol monomethyl ether, altenusin, altenuene and altertoxin I. A hexane-soluble fraction of the dichloromethane extract yielded the steroids ergosterol and ergosterol peroxide, and alternariol monomethyl ether. The concentrated ethyl acetate extract afforded the nucleoside uridine, uracil and inosine. The micro-extracts obtained from this strain in solid media were analyzed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), and the following five known toxins were identified: ACTG-C, D, E and F and AK-toxin II. The major toxins produced by the tangerine pathotype ACT-toxins appear to be absent in strain AT4303. However, the low concentration of ACT-toxins appears to be responsible for their lack of detection in this study. Some compounds isolated from citrus plants were tested for *in vitro* activity against this strain. The results indicated that 17.3 μM apigenin-7-*O*-rutinoside inhibited by 80% conidial germination and appressorium development.

Keywords: *Alternaria alternata*, mycotoxins, host-selective toxins, *Citrus*

Introduction

The fungi of the *Alternaria* genus Nees ex Wallr. are well known to be a source of many bioactive substances with vastly differing structures, many of which are phytotoxic, acting as either host-specific or non-specific toxins. Most of these are produced by *Alternaria alternata* (Fries) Keissler. For this species there are at least six known host-parasite combinations including apple, citrus, Japanese pear, strawberry, tobacco, and tomato pathotypes, in which toxins produced in culture display the same host-specificity as that of the pathogen itself.[1] In citrus, species of *Alternaria* cause tangerine diseases known as Alternaria brown spot (ABS) and their hybrids, namely Alternaria leaf spot of rough lemon (*C. jambhiri* Lush.), black rot of citrus (a post-harvest disease) and citrus leaf spot of Mexican lime [*C. aurantifolia* (Christm.) Swingle], which occurs only in Western Mexico.[2-4]

Over time, the phylogenetic classification of *Alternaria* citrus-associated species has been unclear and confusing. Isolates causing ABS were originally classified as *A. citri* because of their morphological similarity to isolates causing black rot.[5,6] Further analysis, based on descriptions of the morphology and size of the conidia, revealed their similarity to *A. alternata*.[1] They have also been referred to as *A. alternata* f.sp. *citri*, in order to differentiate them from saprophytic isolates of *A. altenata*, and as *A. alternata* f.sp. *citri* tangerine (*C. reticulata* Blanco), in order to differentiate them from isolates that infect rough lemon.[7,8] To differentiate the isolates that cause ABS in tangerines from those that cause Alternaria leaf spot on rough lemons, the infrasubspecific classification of pathotypes is usually adopted. For example, isolates causing ABS are referred to as *A. alternata* "tangerine pathotype", while those causing Alternaria leaf spot are referred to as *A. alternata* "rough lemon pathotype". Although they are morphologically similar, they produce distinct host-selective toxins.

Little is known about the chemistry of *A. alternata* f.sp. *citri* tangerine from Brazilian citrus. Souza *et al.*[9] used liquid chromatography-solid phase extraction-nuclear magnetic resonance spectroscopy to analyze *A. alternata* crude extracts and obtained alternariol (**1**), alternariol

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monomethyl ether (**2**), 3'-hydroxyalternariol monomethyl ether (**3**), and altenusin (**4**), all of which are *Alternaria* mycotoxins (Figure 1).

An intensive selection program performed by Centro APTA Citrus Sylvio Moreira has evaluated the field resistance of several varieties of tangerines and tangors. These varieties are part of the Citrus Active Germplasm Bank of this institution. Murcott tangor is one of the most susceptible to *A. alternata* f.sp. *citri* tangerine. Souza *et al.*[9] did not cite which *A. alternata* strain was used in their studies. Because metabolites production may vary from strain to strain, it is important that toxin production is studied in different strains of *A. alternata*. Thus, to determine the specific toxins of the tangerine pathotype of *A. alternata*, this study has further investigated the AT4303 strain previously obtained from severely infected Murcott tangor leaves. Because the tangerine pathotype is characterized by the presence of various host-selective toxins, our analysis required a rapid and sensitive method for determination of these toxins in the AT4303 strain. Therefore, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was the predominant method used to identify these host-selective toxins during secondary metabolites screening from the organic extract of *A. alternata* AT4303.

We first endeavored to compile the observations recorded in the literature concerning the chemistry and distribution of several *A. alternata* toxins that appear to be the most suitable candidates for our study. Isolates causing ABS were originally classified as *A. citri*, and the toxins were called AC-toxins. Later, to distinguish the toxins produced by the two different pathotypes, the tangerine-pathotype toxin was named ACT- or ACTG-toxin, and the rough lemon-pathotype toxin was named ACR- or ACRL-toxin.[10]

The ACTG-toxins are terpenoid compounds referred to as bicyclo- and tricycloalternarenes (Figure 2). Three isoprenoid units are recognizable in each of the ACTG-toxins, in which a six-membered ring is bonded to the last methylene C-15 unit. The low concentrations of host-selective toxins in *A. alternata* f.sp. *citri* tangerine from Brazilian citrus make it easy to comprehend how these substances have not been identified to date. Identifying these toxins in crude extracts using LC-MS is relatively difficult because commercial standards are not available. However, it may be possible to determine such trace toxins via LC-MS/MS and comparing the results to published data regarding the above host-selective toxins.

Moreover, Murcott tangor with and without symptoms of ABS were examined to determine whether the secondary metabolites in this plant are associated with a chemical defense response. The preliminary results indicate that flavonoids are present at higher concentrations in leaves exhibiting symptoms compared to leaves without symptoms, suggesting that these compounds play a role in plant-pathogen interactions, probably as phytoanticipins. Thus, another objective of the present study was to evaluate the effects of *Citrus* compounds on the mycelial growth and germination of *A. alternate in vitro*.

Experimental

Biological materials

Experiments were conducted by grafting susceptible Murcott tangor (*Citrus sinensis* Osb. × *C. reticulata* Blanco) onto Rangpur lime (*C. limonia* Osb.). Citrus budwood was obtained from the mother blocks of the Centro APTA Citrus Sylvio Moreira - IAC, Cordeirópolis, SP, Brazil. Inoculations were performed using the AT4303 strain of the tangerine pathotype of *A. alternata*, which was previously obtained from severely infected Murcott tangor fruit collected in Campo Alegre Farm, Aguaí, SP, Brazil.

Preparation of plant samples for *A. alternate* inoculation

Murcott tangor plants were produced in the greenhouse of the Centro APTA Citrus Sylvio Moreira. When they reached approximately 30 cm in height, the plants were pruned to stimulate the emergence of new shoots and

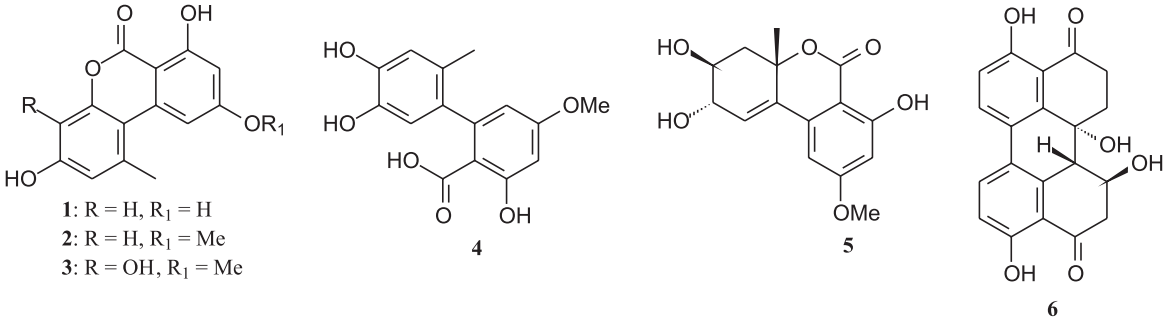


Figure 1. *Alternaria* mycotoxins.

young leaves. To prepare fresh isolated fungi, the abaxial surfaces of young leaves (2-3 cm long) from the tip of the shoots were inoculated with a conidial suspension (10^5 conidia mL^{-1}) of *A. alternata*, approximately 35 days after pruning.

To produce conidial suspensions for inoculation of the leaves, 50 mycelial plugs (5 mm^2), taken from five-day-old cultures on potato dextrose agar (PDA) supplemented with carbendazim fungicide (640 mg L^{-1}), were placed on a $CaCO_3$ sporulation media (30 g $CaCO_3$; 20 g sucrose; 20 g agar L^{-1}), covered with 2 mL of sterile distilled water and incubated for a 12-hour photoperiod for four days at 27 °C. Subsequently, 5 mL of autoclaved distilled water were added to the plates, and the conidia were scraped from the surface of the medium using a sterile spatula. The conidial suspension was filtered through two layers of sterile gauze, quantified in a Neubauer counting chamber, and adjusted to 10^5 conidia mL^{-1}. Inoculation was performed using a sprayer, applying approximately 2 mL of the conidial suspension *per* leaf.[11,12] After inoculation, the shoot tips were covered with plastic bags to create a microenvironment of high humidity. Plastic bags were removed 48 h post inoculation, and symptoms were evaluated.

Fungal cultivation and PCR analyses

Symptomatic leaves were surface sterilized by placing them sequentially in 70% ethanol, followed by 1 min in sodium hypochlorite (3% watery solution), and rinsing with sterile water. Approximately 5-mm-diameter disks were cut from tissue-bearing lesions of Alternaria brown spot. Leaf disks bearing a single lesion were placed onto half-strength potato dextrose agar (PDA) in Petri dishes and incubated on biochemical oxygen demand (B.O.D) at 25 °C. All growing mycelium cultures were sub-cultured onto PDA and incubated on B.O.D. at 25 °C. DNA and PCR analyses were performed by Stuart, R. M., and DNA was purified from fungal cultures according to Stuart, R. M. and Sasseron, G. R.[13,14] Amplification of ITS1-5.8S-ITS2 rDNA was performed using the universal primer pair ITS5F (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4R (5'TCCTCCGCTTATTGATATGC3'). The ACTT1 gene, from the ACT-toxin biosynthesis gene cluster, was amplified using the primer pair ACTT1F2 (5'CACAGGCTATCTTCACATGC3') and ACTT1R2 (5'CGTAGCTCCATAGCATTGCT3').[15] PCR consisted of 35 cycles as follows: denaturing for 30 s at 94 ºC, primer annealing for 30 s at 55 ºC, and extension for 45 s at 72 ºC for both primers pairs. PCR products were visualized under UV light in 1.0% (m/v) agarose gels stained with ethidium bromide.

Rice culture of *A. alternate* and isolation of the compounds

The fungus was statically cultured in 45 Erlenmeyer flasks (500 mL) containing 110 g of rice ("Uncle Ben's", parboiled) and 77 mL of distilled water *per* flask, each of which was autoclaved twice at 120 °C for 45 min. Five small discs (0.5 cm) of the PDA medium from the Petri dish containing mycelium of *A. alternata* (obtained as described above) were transferred under sterile conditions to Erlenmeyer flasks. Three flasks were used for control purposes. After 26 days of growth at 28 °C, ethanol (450 mL) was added to each flask, after which extraction was performed using an Ultra Turrax homogenizer at 20000 rpm. Subsequently, the flasks were allowed to stand for 24 h before being filtered by gravity. Ethanol (450 mL) was added to each precipitate in the flasks, after which each mixture was allowed to stand for 24 h before being filtered by gravity into the same flask. The ethanol was then evaporated under reduced pressure to provide 80 g of a dark residue. This residue was suspended in 500 mL MeOH:H_2O (1:3) solution. The suspension was partitioned into dichloromethane (3 × 1000 mL), EtOAc (3 × 1000 mL), MeOH (3 × 1000 mL), and *n*-BuOH (3 × 1000 mL), after which it was further concentrated under vacuum. After the extracts were analyzed by NMR and compared to the control extracts (flasks without fungus), the concentrated CH_2Cl_2 (45 g) and EtOAc (9.9 g), that showed features that were absent in the control samples, were selected for compounds isolation. The concentrated CH_2Cl_2 was partitioned into hexane (3 × 1000 mL; 17.9 g), and MeOH (3 × 1000 mL; 22.4 g).

The concentrated MeOH extract was chromatographed over gel permeation Sephadex LH 20 (MeOH) to give 5 fractions. Fraction 1 was twice re-chromatographed as above, after which HPLC separation using a semipreparative HPLC column [Gemini C-18, 25.0 × 10.0 cm column, 5 μm; H_2O-MeOH (8:2); flow rate 3.8 mL min^{-1}; UV = 217 and 234 nm] yielded altenuene (**5**, 3 mg). Fraction 2 was also re-chromatographed as above, after which HPLC separation using a preparative column [Gemini C-18, 25.0 × 2.1 cm column, 10 μm; H_2O-acetonitrile (gradient 5-100% B), flow rate 20 mL min^{-1}, UV = 217, 234 and 365 nm] yielded altenusin (**4**, 5 mg). Likewise, fraction 3 was re-chromatographed as above, followed by column chromatography over silica gel (230-400 mesh). Elution with CH_2Cl_2, EtOAc and MeOH yielded alternariol (**1**, 12 mg). Fraction 4 was re-chromatographed as above, after which HPLC separation using a semi-preparative column [Gemini C-18, 25.0 × 10.0 cm column, 5 μm; H_2O-MeOH (8:2), flow rate 3.8 mL min^{-1}, UV = 217 and 234 nm] yielded alternariol monomethyl ether (**2**, 3.4 mg). Fraction 5 was separated using a preparative HPLC column [Gemini C-18,

25.0 × 2.1 cm column, 10 μm; H_2O-acetonitrile (gradient 5-100% B), flow rate 20 mL min^{-1}, UV = 217, 234 and 365 nm], yielding altertoxin I (**6**, 3 mg).

The concentrated EtOAc extract was chromatographed over gel permeation Sephadex LH 20 (MeOH) to give 2 fractions. Both fractions were repeatedly subjected to gel permeation column chromatography (Sephadex LH 20, MeOH) affording a mixture of (6 mg) uridine and uracil, and a new fraction containing inosine (2.5 mg), which was purified by HPLC separation using a semipreparative column [Gemini C-18, 25.0 × 10.0 cm column, 5 μm; H_2O-MeOH (8:2), flow rate 3.8 mL min^{-1}, UV = 217 nm].

The concentrated hexane extract was subjected to column chromatography over silica gel (230-400 mesh). Elution with hexane, CH_2Cl_2, EtOAc and MeOH yielded ergosterol (5 mg), ergosterol peroxide (4.5 mg), alternariol monomethyl ether (**2**, 2 mg) and fatty acids. Methyl esters of the fatty acids (20.7 mg) were prepared adding *p*-toluene sulfonic acid (6.0 mg) as a catalyst and methanol (1.5 mL) and heating until the reflux temperature (50 °C). After 12 h, stirring was stopped and, after cooling, the sample was partitioned into saturated aqueous sodium bicarbonate solution to remove excess acid and further concentrated under vacuum. Methyl esters were identified by low-resolution GC-MS as a mixture of methyl palmitate, methyl octadecadienoate and methyl octadecanoate.

Solid-media cultures in Petri dishes and extraction procedure

For solid-media cultures, a 5-mm agar plug of a fungal pre-culture ($CaCO_3$ sporulation media, see above) was inoculated in the center of a 9-cm Petri dish containing 30 mL of potato dextrose agar media (PDA). The Petri dishes were incubated on B.O.D. for 13 days at 25 °C. Five plugs (6-mm diameter) were cut out along the diameter of the fungal colony, after which they were extracted and then transferred into an extraction vessel with solvent (CH_2Cl_2 33%, EtOAc 17% and MeOH 50%). The solvent mixtures were freshly prepared, and extractions were performed three times in an ultrasound bath for 15 minutes. The extract was filtered through a small Pasteur pipette plugged with pre-cleaned cotton wool into a clean vial. The solvent was evaporated under nitrogen, re-suspended in MeOH (1.5 mL) and filtrated through a polytetrafluoroethylene (PTFE) membrane Millipore filter (0.45 μm) for LC-MS/MS analysis.

High-performance liquid chromatography (HPLC) and MS parameters

LC-ESI-MS/MS data were acquired using an Alliance 2695 HPLC (Waters, Manchester, United Kingdom) coupled with a triple quadrupole Waters Micromass Quattro LC mass spectrometer equipped with an ESI-"Z" spray ion source and operated in positive mode. All LC-MS analyses were carried out using Luna ODS analytical column (150 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) and employing a linear gradient of H_2O/CH_3CN (85:15 → 0:100, v/v, H_2O buffered with 0.1% TFA) over the course of 40 min. Samples (20 μL) were injected into the analytical column under a flow rate of 0.4 mL min^{-1}, and the column was maintained at 20 °C. Desolvation and ion-source block temperatures were set at 250 and 80 °C, respectively; nitrogen was used for nebulization (50 L h^{-1}) and desolvation (475 L h^{-1}); and the optimal voltage of 3.90 kV was applied to the capillary, 20 V for the sample cone and 5 V for the extractor cone. During MS/MS analysis, argon was added to the collision cell for collision induced dissociation (CID) utilizing 15 or 20 eV of collisional energy. The molecular $[M + H]^+$ ions associated with the ACTG- and AK-toxins [*m/z* 347 for **7** and **8** (ACTG-D and E); *m/z* 363 for **9** (ACTG-F); *m/z* 365 for **10** (ACTG-C); and *m/z* 400 for **11** (AK-toxin II; Figure 2)] as well as their typical product ions were analyzed. All MS data were acquired and processed using MassLynx V4.1 software (Waters, Cheshire, United Kingdom).

Evaluation of the effects of citrus natural products on *A. alternata* mycelial growth

Alternaria alternata was sub-cultured on PDA and incubated on B.D.O. at 25 °C as described in the above section. The compound (10 mg) was solubilized in DMSO (300 μL) and then mixed with PDA at 50 °C. The resulting media was poured into 9-cm plates (20 mL *per* plate) to obtain a final concentration of 0.1 mg mL^{-1} *per* plate. PDA lacking the selected compounds and containing DMSO (300 μL) served as a control, with cercobin {ethyl thiophanate, ethyl *N*-[2-(ethoxycarbonylcarbamothioylamino)phenyl] carbamothioyl]carbamate, 0.1 mg mL^{-1}} added as a positive control. After solidification, one 5-mm mycelial disc cut from a 20-day-old colony was inoculated in the center of each plate, after which each plate was incubated on B.O.D. at 25 °C with a light phase of 12:12 h L:D. Each treatment was replicated three times. The colony diameter was measured daily until the third day of incubation, when mycelial growth covered the surface of all control cultures. Inhibition of growth was calculated in relation to the growth of the control samples, according to the equation proposed by Sztejnberg *et al.*[16]

$$\%\text{Inhibition} = (1 - \text{diameter of treated colony} / \text{diameter of control colony}) \times 100 \quad (1)$$

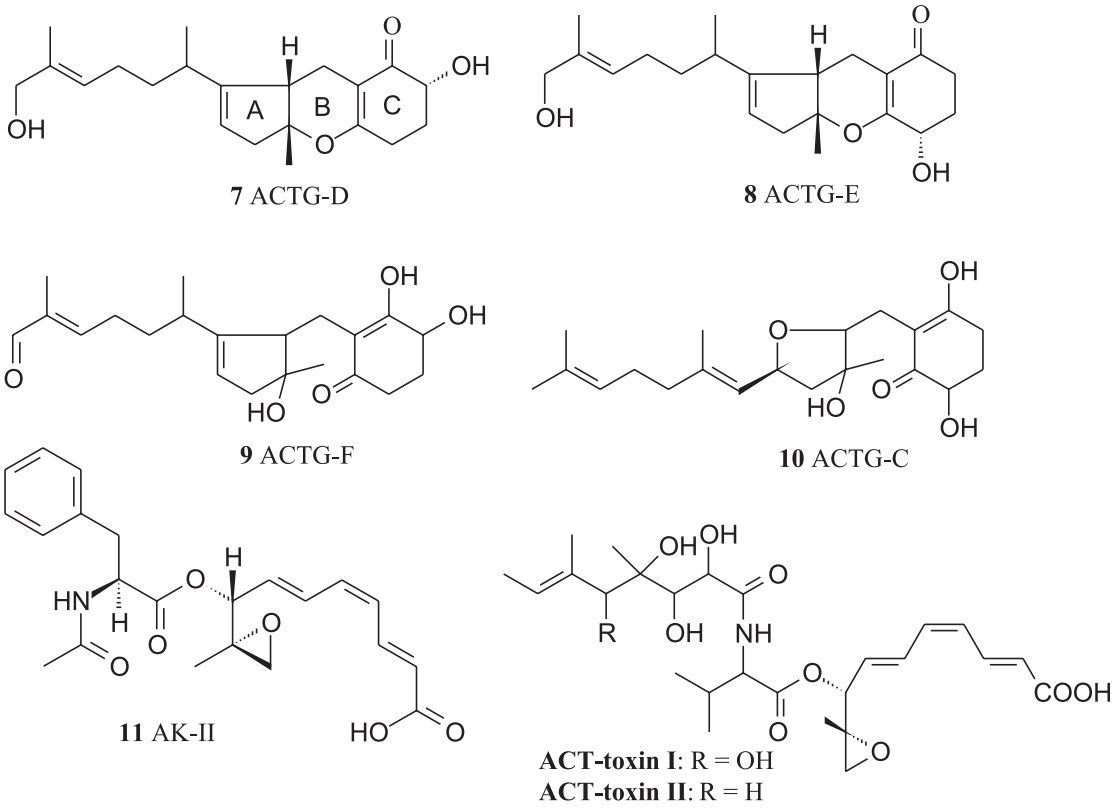


Figure 2. *Alternaria* toxins.

Evaluation of the effects of citrus natural products on *A. alternata* conidial germination

A spore-suspension was obtained from 7-day-old cultures by flooding the cultures with sterile distilled water containing 0.05% (v/v) Tween 80, followed by filtering through two layers of sterile cheesecloth to remove hyphal fragments. The spore concentrations of these suspensions were adjusted to 10^5 conidia mL^{-1} with the aid of a hemocytometer. Four 40-µL aliquots of each spore suspension were aseptically arranged independent from one another, in triplicate, onto polystyrene plates. The inhibitory effects of the compounds on spore germination were evaluated by adding the compounds (10 mg), solubilized in DMSO (0.3 mL) and 57 mL of H_2O (0.1 mg mL^{-1}), to each aliquot. Forty-microliter aliquots of the compound solution (0.1 mg mL^{-1}) were poured onto each spore suspension. After incubation on B.O.D. at 25 °C with a light phase of 12 h L, germination was determined for each concentration by observing at least 100 conidia under a light microscope (magnification ×200). A spore was determined to be germinated if the germ tube length was at least equal to that of the conidium. An appressorium was considered mature when melanization was evident. Inhibition of growth was calculated as described above. DMSO (3 µL) and 37 µL of H_2O served as a control, and cercobin (0.1 mg mL^{-1}) was added as a positive control.

Results and Discussion

The inoculation of *A. alternata* AT4303 strain in plants of Murcott tangor was efficient, causing symptoms typical of ABS on leaves. The fungal colonies recovered from symptomatic leaves were identified as *A. alternata* based on morphology. To confirm the identity of the isolated fungi as the tangerine pathotype *A. alternata*, the rDNA and ACT-toxin gene (ACTT1) were amplified by Stuart R. M.[13] The isolates demonstrated positive amplification of the ACT-toxin biosynthesis gene, confirming their identities.[15]

After successive chromatographic separations, a methanol-soluble fraction of the dichloromethane extract from the culture broth afforded the following five known *Alternaria* mycotoxins: alternariol (**1**), alternariol monomethyl ether (**2**), altenusin (**4**), altenuene (**5**) and altertoxin I (**6**).[17] A hexane-soluble fraction of the dichloromethane extract was subjected to column chromatography on silica gel, yielding the steroids ergosterol and ergosterol peroxide,[18] alternariol monomethyl ether (**2**) and fatty acids. Methyl esters of the fatty acids were identified using GC-MS as a mixture of methyl palmitate, methyl octadecadienoate and methyl octadecanoate. The concentrated EtOAc extract was purified by repeated column chromatography, yielding the nucleoside uridine, uracil and inosine.[19] These compounds were identified

by spectroscopic analysis (Supplementary Information, Figures S1-S21).

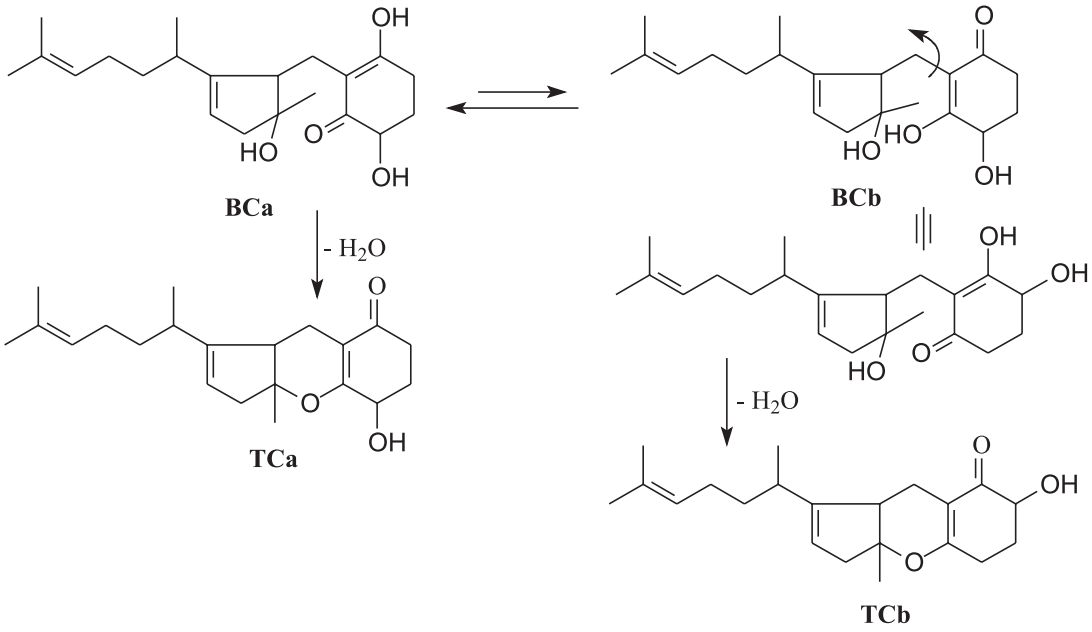
The absence of the selected toxins in the *A. alternata* AT4303 strain stimulated reinvestigation of this strain's growth in solid media (PDA) using micro-scale extraction. In this regard, LC-MS screening was first performed to determine whether compounds other than the above mycotoxins were present. The investigation initially focused on analyzing the molecular ions of the host-specific toxins with the aim of identifying one ion that would distinguish the target compound from any others having similar retention times. Using LC-ESI-MS/MS, five known host-selective toxins, namely ACTG-C (**10**), D (**7**), E (**8**) and F (**9**), and AK-toxin II (**11**), were identified (Figure 2).

Due to the keto-enol tautomerism of the 1,3-diketone structure in the six-membered ring of the bicycloalternarene and because of the free rotation two isomeric tricycloalternarenes can form (Scheme 1).[20-22]

Regarding LC-MS data analysis, the extracted ion chromatogram (EIC) for the ACTG- and AK-toxins revealed more than one peak corresponding to their molecular ions (Figure 3). Thus, production of the *A. alternate*-produced host-specific toxins of interest was confirmed via identification of the product ions detected in the MS/MS spectra by comparison with published data and the chromatographic behavior reported for the four ACTG- and AK-toxins.

The molecular masses of the bicyclo-ACTG-toxins each increased by one H_2O unit compared to its related tricycloalternarene. However, because of rapid ring closure via the loss of water, each bicycloalternarene yielded to a great extent the same ESI-MS spectrum as the corresponding tricycle.[23] The structures of the two isomeric tricycloalternarenes can be elucidated by differences in their chromatographic behaviors, which can be explained by the diminished polarity of TCb (Scheme 1) caused by the presence of the acyloin group.[24] A characteristic cleavage results from the *retro*-Diels-Alder reaction in ring B.[25]

The peaks detected at 25.36 and 30.53 minutes (Figure 3) in the ECI chromatogram for *m/z* 347 $[M + H]^+$ indicate the presence of isomeric ACTG-D (**7**) and E (**8**), $C_{21}H_{30}O_4$ (Figure 2). For both compounds, a prominent peak was found at *m/z* 329, representing one H_2O loss (Figure 4, Scheme 2). Three out of four oxygens are neighboring, as seen in fragment *m/z* 247 in the (+)-ESI-MS/MS spectrum. The fragment ions *m/z* 207, associated with *retro*-Diels-Alder cleavage of ring B, and *m/z* 189 (*m/z* 207 – H_2O) confirmed the presence of a hydroxyl group and a double bond in the side-chain (Scheme 2). The lower polarity of TCb (Scheme 1), caused by the presence of the acyloin group, indicates that the retention times at 30.53 and 25.36 min correspond to ACTG-D (**7**) and ACTG-E (**8**), respectively, because the analysis was performed by reversed-phase liquid chromatography.[24] The *E* geometry of the double bond in the side chain has been verified by NOE correlations.[21] ACTG-D and E (**7**, **8**) were isolated from the fungus *Ulocladium* sp.[26] Recently, the two isomers have been differentiated by HMBC experiments, and NOE experiments have revealed a *cis*-fused A/B cyclic system.[26] Circular dichroism (CD) has been used to determine the configuration of the allylic hydroxyl and acyloin group at ring C, as shown in Figure 2.[26]



Scheme 1. Formation of two isomeric tricycloalternarenes from a parent bicycloalternarene.

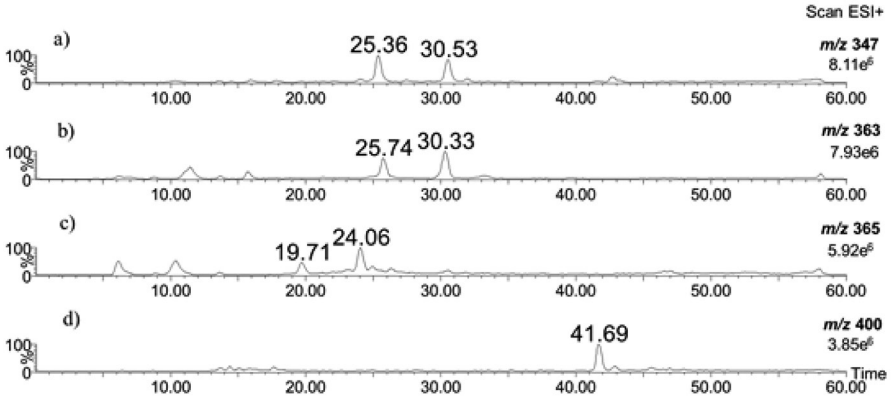
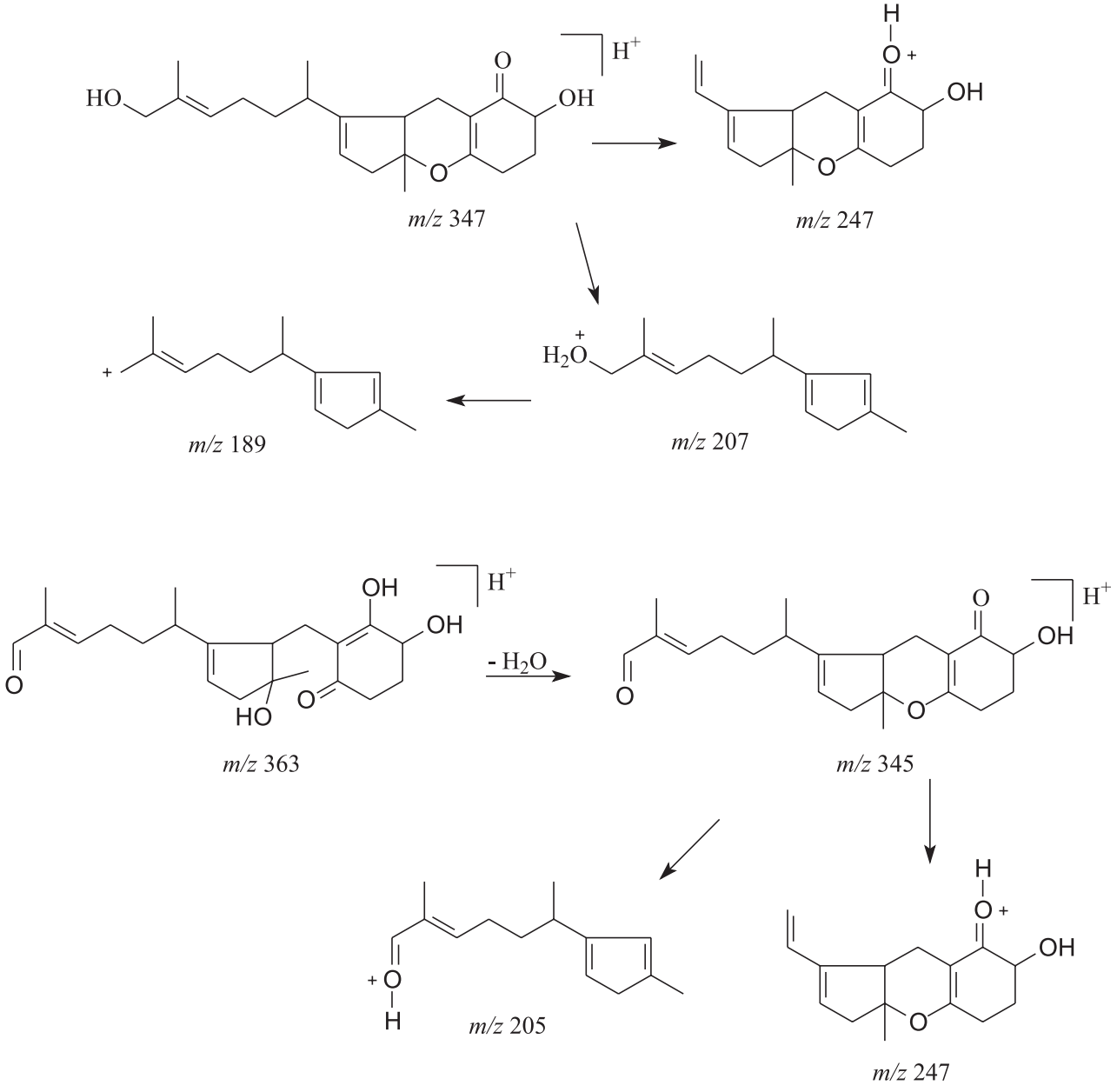


Figure 3. Extracted ions chromatograms (EICs) for the selected molecular ions $[M + H]^+$ of (a) ACTG *m/z* 347; (b) ACTG *m/z* 363; (c) ACTG *m/z* 365 and (d) AK-toxin *m/z* 400 from the crude extract of *A. alternata* AT4303 strain growth in solid media (PDA).



Scheme 2. Fragmentation pathways of ACTG-D (*m/z* 347, **7**) and ACTG-F (*m/z* 363, **9**) obtained via (+)-ESI-MS/MS.

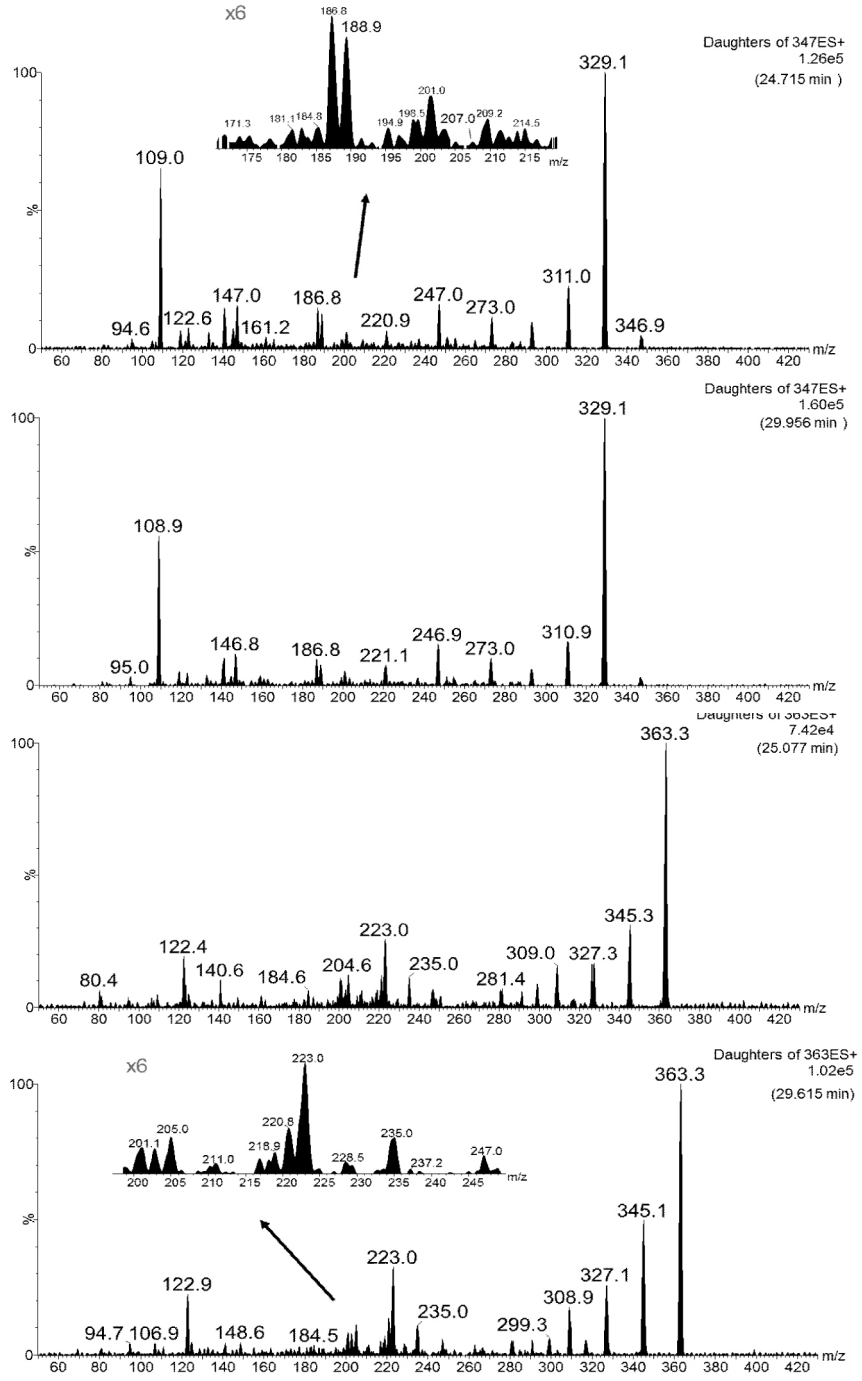


Figure 4. (+)-ESI-MS/MS spectra of ACTG-toxins (*m/z* 347, 363) from the crude extract of *A. alternata* AT4303 strain growth in solid media (PDA).

The latest molecular revision of *Alternaria* delineated phylogenetic lineages within *Alternaria* and allied genera based on nucleotide sequence data of parts of the 18S nrDNA, 28S nrDNA, ITS, GAPDH, RPB2 and TEF1-alpha gene regions.[27] The data indicates that the genus *Ulocladium* is synonymous with *Alternaria*.[27]

Inspection of the chromatogram of ion *m/z* 363 $[M + H]^+$, with retention times at 25.74 and 30.33 min (Figure 3), indicates the presence of two tautomers (BCa/BCb, Scheme 1) of ACTG-F (**9**) (Figure 2). Fragment ion *m/z* 345, which is associated with rapid ring closure via the loss of H_2O and conversion to a tricyclo, confirms the presence of a bicycle type compound (EIS-MS/MS spectrum, Figure 4, Scheme 2). Fragment ion *m/z* 247 clearly indicates the presence of four oxygens in rings A, B and C, postulating the formation of a tricycle. In addition,

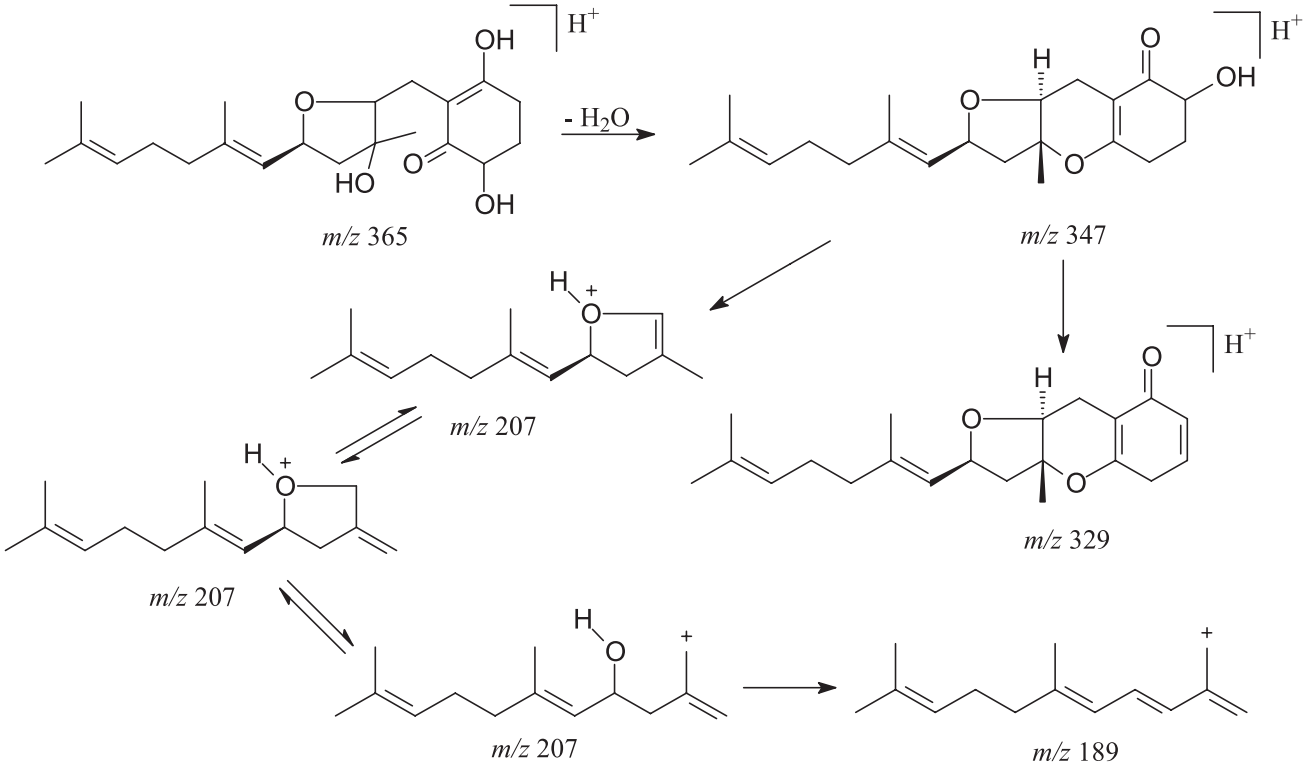
fragment ion *m/z* 205 indicates the aldehyde positioned at the end of the side chain. These data confirm the presence of ACTG-F (**9**) toxins tautomers and indicate the assignment of ACTG-Fb and ACTG-Fa, respectively, to the peaks at 30.33 and 25.74 min, because the acyloin group decreases the compounds polarity, similar to BCa/BCb in Scheme 1.[23] The *E* geometry of the double bond in the side chain was estimated by Kono *et al*.[21] via comparison of its NMR spectrum with that of ACTG-D (**7**).

At first, the ACTG-toxins were regarded as host-specific. However, the existence of **7** and **8** in *Ulocladium* sp. (*Alternaria* sp.) isolated from the lichen *Everniastrum* sp.[26] and in *A. alternata* from *Brassica sinensis*;[28] of **7** in *A. alternata* from *Maytenus hookeri*[29] and in *Alternaria* sp. from a sponge *Callyspongia* sp.;[30] of **8** in *Alternaria* sp. from marine sediment;[31] and of **7** in the allied genus *Paradendryphiella arenariae* (a marine fungus classified with *Alternaria* in the same family, Pleosporaceae),[32,33] supports the opinion that these compounds are non-specific toxins.

Two tautomers (BCa/BCb,) of ACTG-C (**10**) are indicated by the peaks at 19.71 and 24.06 min (Figure 3) in the EIC chromatograms of *m/z* 365 $[M + H]^+$. This evidence is supported by the MS/MS spectra (Figure 5, Scheme 3) which show fragments at *m/z* 347, 207 and 189 due to rapid ring closure through the loss of H_2O and converting in tricyclo, *retro*-Diels-Alder cleavage of ring B followed by allylic isomerization and loss of H_2O, respectively. The base peak *m/z* 329 may arise from the likely tricycle formed (*m/z* 347) followed by the loss of H_2O. ACTG-C (**10**) was isolated from *A. alternata* pathotype citri as a mixture of two tautomers, in which BCa (Scheme 1) is dominant.[20,22]

AK-toxins have likely remained undiscovered in tangerine pathotype because of their low concentrations, stimulating an investigation of the *A. alternata* AT4303 strain to identify these toxins. Observation of the product ions detected from the precursor *m/z* 400 $[M + H]^+$, eluted at 41.69 min (Figures 3 and 5), suggests that the strain produces AK-toxin II (**11**). Fragment ion *m/z* 382 indicates opening of the epoxide ring, which takes place through one of the carbon-oxygen bond cleavages (Scheme 4). Opening begins via epoxide protonation, after which the allyl proton is lost, giving rise to a hydroxyl group and afterwards to a double bond (Scheme 4). In the second step, the rearranged ion loses H_2O to form $C_{22}H_{24}NO_5$ (*m/z* 382).

The small fragment ion *m/z* 211 indicates 9,10-epoxy-8-hydroxy-9-methyl-2*E*,4*Z*,6-*E*-decatrienoic acid in AK-toxin, but this fragment can also lose one methyl group to produce a moderately intense *m/z* 196 ion. In addition, the small fragment ion *m/z* 341 indicates that an *N*-acetyl-phenylalanyl residue is a structural component of AK-toxin [*m/z* 400 – H_2NCOMe]. Hydrogen migration and elimination of a neutral heptatrienoic acid attached to the enol ether of the fragment ion *m/z* 341 can occur, as indicated by an extremely weak *m/z* 219 ion, which then loses H_2O to yield the base



Scheme 3. Fragmentation pathways of ACTG-C (**10**) determined by (+)-ESI-MS/MS.

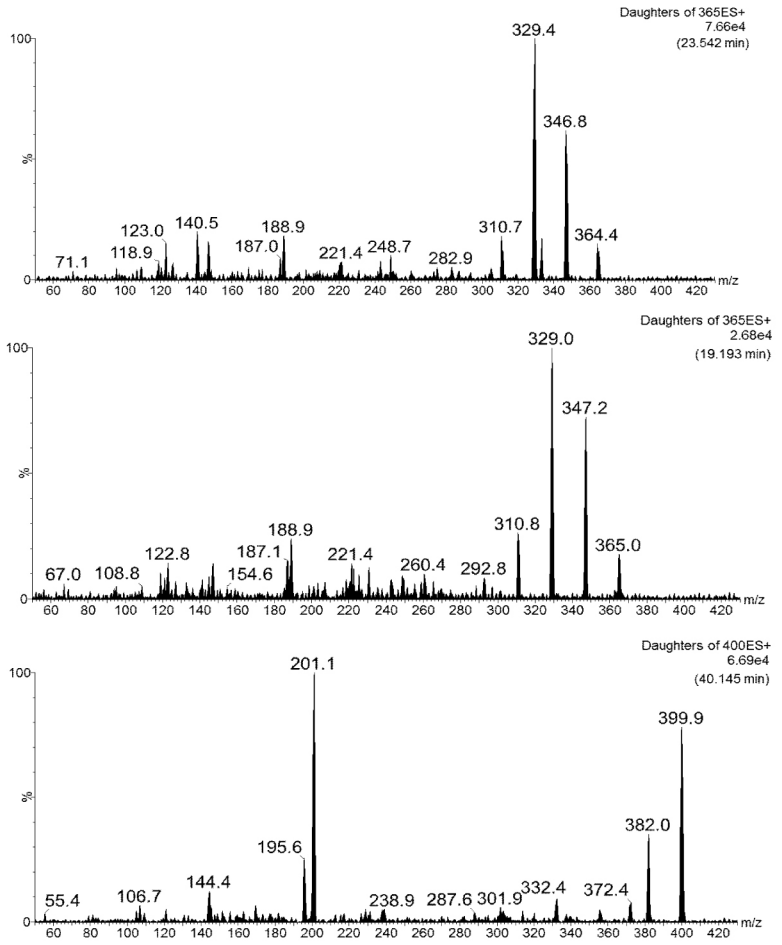


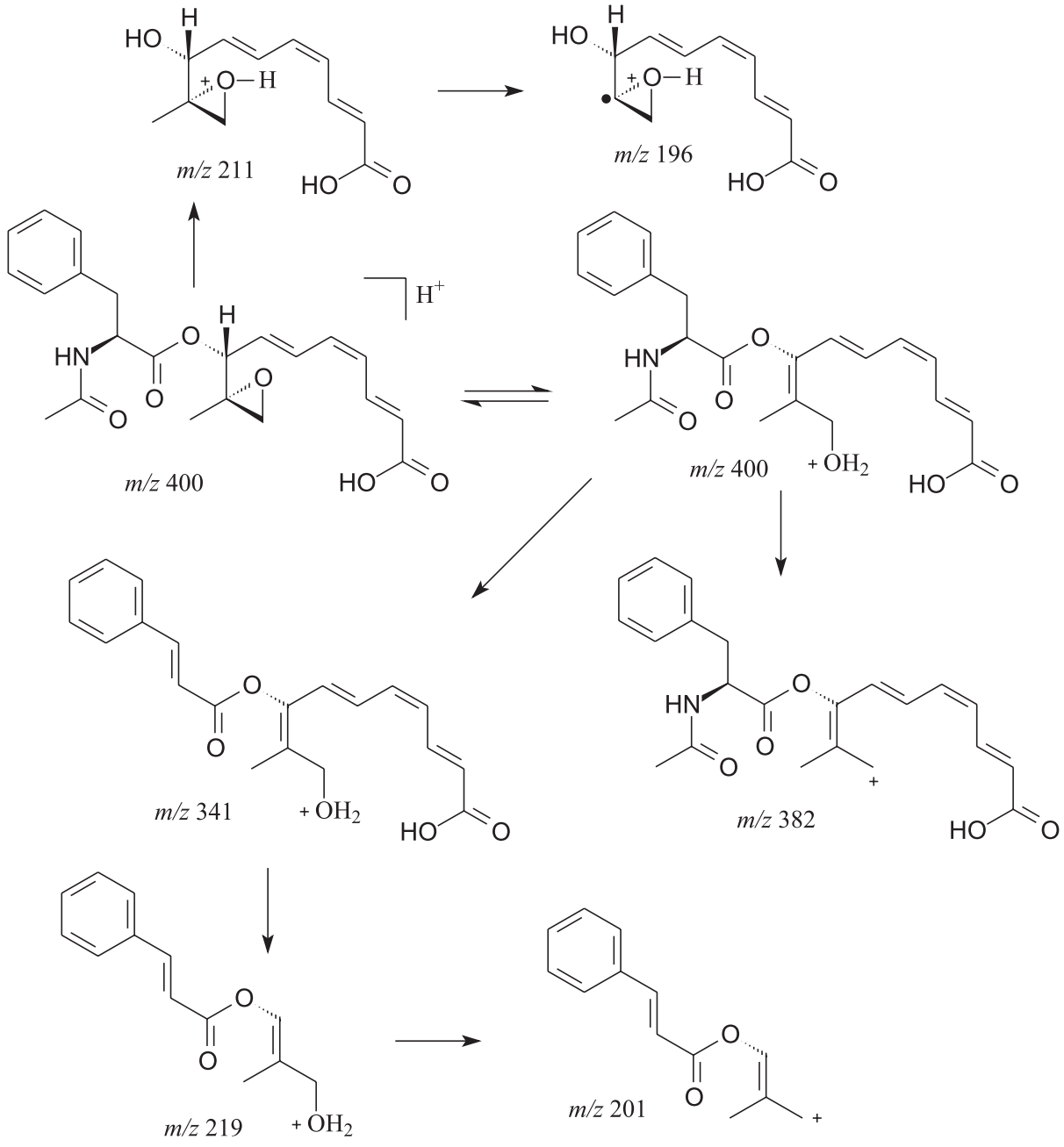
Figure 5. (+)-ESI-MS/MS spectra of ACTG-toxin (*m/z* 365) and AK-toxin (*m/z* 400) from a crude extract of *A. alternata* AT4303 strain growth in solid media (PDA).

ion *m/z* 201. These fragment ions confirm, for the first time, the presence of AK-toxin II (**11**) in a tangerine pathotype.

The host-selective toxins produced by this tangerine pathotype appear to be predominantly ACT-toxins (Figure 2), which are closely related to AK-toxins. These two toxins share a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety. In ACT-toxins, 9,10-epoxy-8-hydroxy-9-methyl-2*E*,4*Z*,6*E*-decatrienoic acid is bonded to a carboxyl group of valine amino acid, while in AK-toxins this moiety bonds to a carboxyl group of phenylalanine amino acid.[10,15,34] ACT-toxins are at least 10 times more toxic to citrus than AK-toxins.[22] ACTG- and AK-toxins were identified in *A. alternata* AT4303 strain growth in solid media (PDA) after micro-scale extraction. This extraction was acceptably reproducible, but the concentrations of the extracts were insufficient to determine the identities of the ACT-toxins reportedly produced by this tangerine pathotype. Conditions known to promote the production of ACT-toxins were used,[13] and the isolates demonstrated positive amplification of the ACT-toxin biosynthesis gene. Thus, the low concentrations of ACT-toxins are likely the reason why these compounds have remained undetected until now. Because of this lack of previous detection, we do not have enough experimental evidence to confirm the absence of ACT-toxins in the AT4303 strain. New methodology that utilizes linear trap quadropole (LTQ) Orbitrap Velos mass spectrometry, which has high sensitivity, is currently being developed so that high-quality data can be acquired using nano-liquid elution.

Some compounds isolated in large amount from Murcott tangor (leaves: nobiletin, tangeretin, apigenin-7-*O*-rutinoside, lupeol and limonin; roots: xanthyletin and seselin) and *C. sinensis* grafted on *C. limonia* cv. Pêra (leaves: umbelliferone, hesperidin, sinensetin and limonin) were tested for *in vitro* activity against *A. alternata*. Among the compounds tested (**12**-**22**, Figure 6) for *in vitro* activity against the *A. alternata* AT4303 strain, the highly oxygenated flavones nobiletin (**19**), tangeretin (**17**) and sinensetin (**18**), the flavanone hesperidin (**20**), and the triterpenes lupeol (**12**) and limonin (**13**) showed weak activities (Figures 6-8).

The flavone apigenin-7-*O*-rutinoside (**21**) and the coumarins xanthyletin (**16**) and seselin (**15**) inhibited mycelial growth (Figure 7) and conidial germination and



Scheme 4. Fragmentation pathways of AK-toxin II (**11**) determined by (+)-ESI-MS/MS.

appressorium development (Figure 8), suggesting that this flavonoid and the coumarins [excepting umbelliferone (**14**)] can behave as good barriers to *A. alternate* colonization.

The results reported herein indicate that apigenin-7-*O*-rutinoside inhibited conidial germination (82%) and appressorium development (88%) at 17.3 μM, while xanthyletin (89, 92%), seselin (84, 89%), and cercobin (**22**) (91, 94%) were most active, but required high concentrations (43.8, 43.8 and 27.0 μM, respectively) (Figure 8). Thus, the antifungal properties of apigenin-7-*O*-rutinoside appear to be superior to those of cercobin, a systemic fungicide belonging to the benzimidazole group, whose wide and indiscriminate agricultural use has caused concern with regard to environmental contamination.[35]

Conclusions

In light of the results reported herein, analysis by tandem MS appears to be an attractive strategy for detecting ACTG- and AK-toxins and confirming the known production of host-selective toxins by the pathogen *A. alternata* AT4303 strain. Furthermore, assuming it is possible to modify the

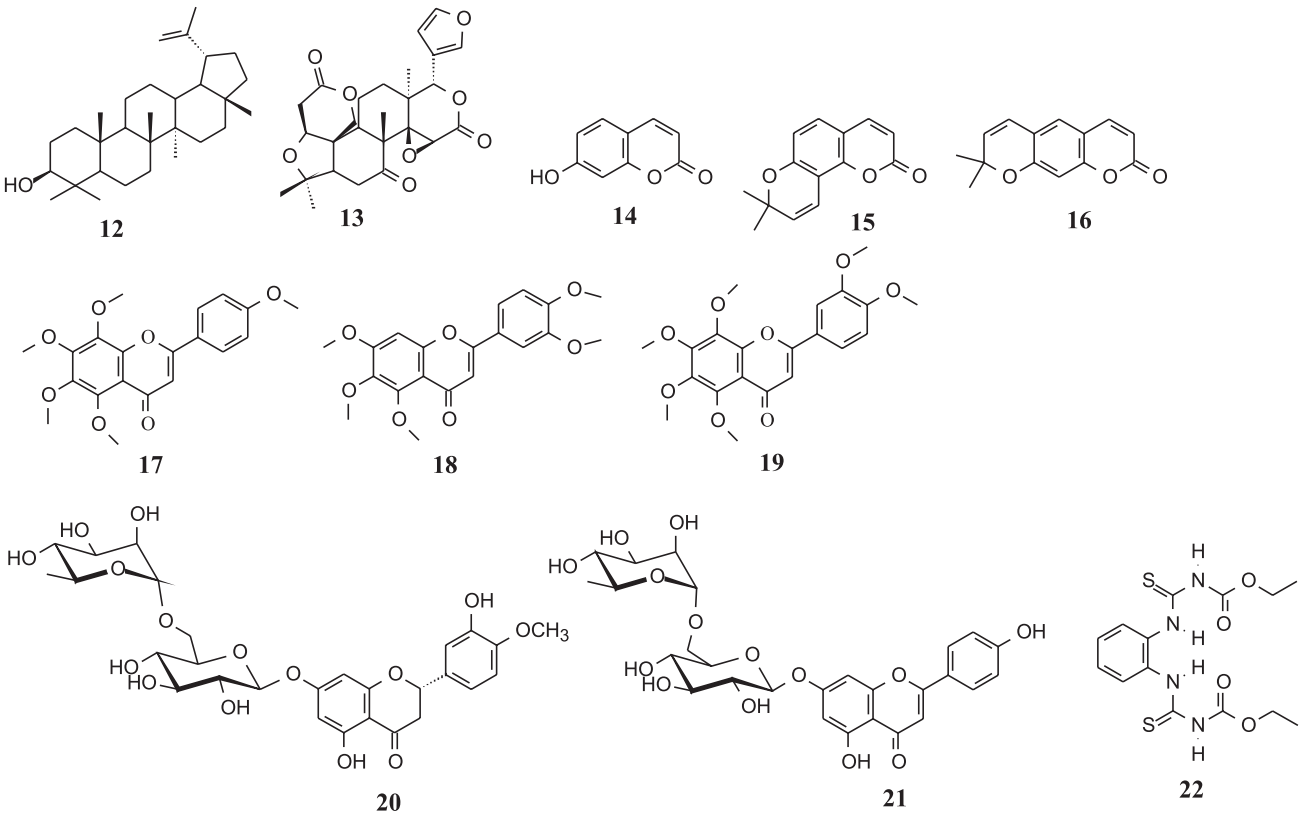


Figure 6. Compounds assayed in *A. alternata* AT4303 strain growth.

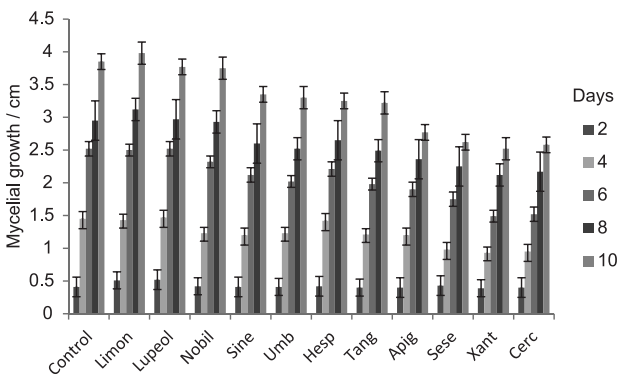


Figure 7. Effects of *Citrus* compounds on mycelial growth of the *A. alternata* AT4303 strain. Limon: limonin; Nobil: nobiletin; Sine: sinensetin; Umb: umbelliferone; Hesp: hesperidin; Tang: tangeretin; Apig: apigenin-7-*O*-rutinoside; Sese: seselin; Xant: xanthyletin; Cerc: cercobin.

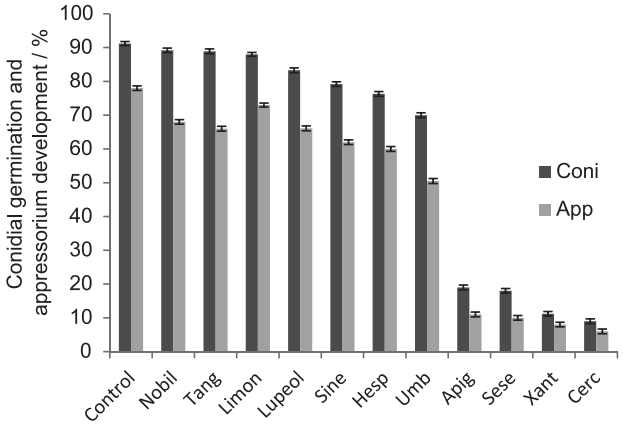


Figure 8. Effects of *Citrus* compounds on conidial germination and appressorium development of the *A. alternata* AT4303 strain. Limon: limonin; Nobil: nobiletin; Sine: sinensetin; Umb: umbelliferone; Hesp: hesperidin; Tang: tangeretin; Apig: apigenin-7-*O*-rutinoside; Sese: seselin; Xant: xanthyletin; Cerc: cercobin.

chemical structures of compounds to improve activity and selectivity, the results of the present study could help in directing the rational design of flavonoid derivatives for use as potent and effective fungicidal agents.

Supplementary Information

Supplementary Information (^{1}H, ^{13}C NMR) is available free of charge at http://jbcs.sbq.org.br.

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