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Antifungal Activity and Fungal Metabolism of Steroidal Glycosides of Easter Lily (*Lilium longiflorum* Thunb.) by the Plant Pathogenic Fungus, *Botrytis cinerea*

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ABSTRACT: *Botrytis cinerea* Pers. Fr. is a plant pathogenic fungus and the causal organism of blossom blight of Easter lily (*Lilium longiflorum* Thunb.). Easter lily is a rich source of steroidal glycosides, compounds which may play a role in the plant−pathogen interaction of Easter lily. Five steroidal glycosides, including two steroidal glycoalkaloids and three furostanol saponins, were isolated from *L. longiflorum* and evaluated for fungal growth inhibition activity against *B. cinerea*, using an in vitro plate assay. All of the compounds showed fungal growth inhibition activity; however, the natural acetylation of C-6‴ of the terminal glucose in the steroidal glycoalkaloid, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**2**), increased antifungal activity by inhibiting the rate of metabolism of the compound by *B. cinerea*. Acetylation of the glycoalkaloid may be a plant defense response to the evolution of detoxifying mechanisms by the pathogen. The biotransformation of the steroidal glycoalkaloids by *B. cinerea* led to the isolation and characterization of several fungal metabolites. The fungal metabolites that were generated in the model system were also identified in Easter lily tissues infected with the fungus by LC-MS. In addition, a steroidal glycoalkaloid, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**6**), was identified as both a fungal metabolite of the steroidal glycoalkaloids and as a natural product in *L. longiflorum* for the first time.

KEYWORDS: *Lilium longiflorum* Thunb., Liliaceae, Easter lily, steroidal glycoside, steroidal glycoalkaloid, furostanol saponin, *Botrytis cinerea*, antifungal activity, fungal metabolite

INTRODUCTION

Botrytis cinerea Pers. Fr. is a necrotrophic plant pathogenic fungus with a broad host range and is the cause of gray mold disease, one of the most important postharvest diseases of fruits and vegetables worldwide. *B. cinerea* infects over 200 species of economically important plants and is a causal organism of blossom blight of Easter lily (*Lilium longiflorum* Thunb.) and other ornamental lily species.[1−3] In contrast to *Botrytis elliptica*, the cause of fire blight of Easter lily, *B. cinerea* typically does not infect healthy plant tissues and is often found growing on stressed, wounded, or senescing tissues.[4] *B. cinerea* over winters as sclerotia in crop debris and penetrates young plant tissues where it remains latent until the environmental conditions are conducive to infection.[3] The fungus flourishes in cool temperatures and high humidity, often during the end of the growing season in late summer and early fall.[5] In fact, it is recommended to treat Easter lily flower buds with fungicide prior to cold storage as a postharvest management strategy for the control of blossom blight in potted Easter lily plants and cut flowers prior to shipment.[6]

Host defense responses to *B. cinerea* have been investigated in many plants including thale cress, *Arabidopsis thaliana*, and tomato, *Solanum lycopersicum*.[3] In response to infection by *B. cinerea*, activation of host defense pathways including the production of antifungal metabolites and pathogenesis related proteins have been reported.[4] In addition to the activation of inducible defense pathways, steroidal glycosides including steroidal saponins and steroidal glycoalkaloids, constitutively present in plant tissues, have been shown to play a role in host defense in several plant species.[7−13]

Steroidal saponins are widely distributed secondary metabolites and have been found in over 100 plant families.[14] They are characterized by a steroid type skeleton glycosidically linked to sugar moieties. Steroidal glycoalkaloids are similar in structure to steroidal saponins; however, they have nitrogen present in the steroidal aglycone. In contrast to steroidal saponins, the occurrences of steroidal glycoalkaloids are, thus far, limited to the members of the plant families Solanaceae and Liliaceae.[15,16]

Because of the amphipathic nature of the molecules, steroidal glycosides have been shown to disrupt cell membranes both in vitro and in vivo.[17,18] Some studies suggest that membrane disruption may be due either to the interaction of the aglycone with membrane bound sterols, resulting in the formation of membrane pores[19] or the extraction of membrane bound sterols, causing the loss of lipid bilayer integrity and membrane leakage.[20,21] The antifungal mechanisms of steroidal glycosides remain unclear, and the exact mechanism remains to be elucidated.

Fungal plant pathogens such as *Gaeumannomyces graminis* and *Stagonospora avanae* enzymatically detoxify host plant saponins.[22,23] Interestingly, *B. cinerea* has been shown to produce enzymes that can metabolize a variety of plant defense compounds from active forms to inactive forms.[24] In tomato, *B. cinerea* metabolizes the antifungal steroidal glycoalkaloid, α-tomatine, to an inactive

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form by enzymatic cleavage of the entire saccharide moiety or by the cleavage of the terminal xylose by a β-xylosidase enzyme.[25,26] In addition, other plant pathogenic fungi such as *Septoria lycopersici* and *Fusarium oxysporum* f.sp. *lycopersici* detoxify α-tomatine through independent metabolic pathways.[27,28] Investigations have been conducted on the interaction of steroidal glycosides and *B. cinerea*; however, to date there are no studies on the interaction between steroidal glycosides from *L. longiflorum* and the fungus *B. cinerea*.

Easter lily is a rich source of steroidal glycosides.[29−31] Recently, two steroidal glycoalkaloids, (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**1**), (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-[6-*O*-acetyl-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**2**), and three furostanol saponins, (25*R*)-26-*O*-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**3**), (25*R*)-26-*O*-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside (**4**), and (25*R*)-26-*O*-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside (**5**) were identified and quantified in the various plant organs of *L. longiflorum*.[30,31] Steroidal glycosides are known to be inhibitory to fungal growth and may play a role in the plant−pathogen interaction. The goal of this study was to (1) evaluate the biological activity of five steroidal glycosides isolated from the bulbs of *L. longiflorum* on the growth of the plant pathogenic fungus *B. cinerea*, using an in vitro assay and (2) to use a model system to generate fungal metabolites of the steroidal glycosides, isolate and characterize the metabolites, and determine if the fungal metabolites were present in plant tissues infected with *B. cinerea*.

MATERIALS AND METHODS

Plant Material. *L. longiflorum*, cultivar 7−4, plants were provided by the Rutgers University lily breeding program. Young bulbs were treated with Captan (Bayer CropScience AG, Monheim am Rhein, Germany) fungicide prior to planting. Bulbs were planted in raised beds containing Pro-Mix (Premier Horticulture Inc., Quakertown, PA) and were grown to mature plants under greenhouse conditions for 9 months prior to harvest. The greenhouse temperatures were set to provide a minimum day temperature of 24 °C and a minimum night temperature of 18 °C. Plants were fertilized biweekly with a 100 mg L^{-1} solution of NPK 15−15−15 fertilizer (J.R. Peters Inc., Allentown, PA). Each plant produced 3−5 new bulbs, which were used for extraction. For the purification of steroidal glycosides **1**−**5**, each plant was harvested by hand, and the bulbs were manually separated, immediately frozen under liquid nitrogen, lyophilized on a VirTis AdVantage laboratory freeze-dryer (SP Industries inc.,Warminster, PA), and stored at −80 °C until extraction. For the fungal inoculation studies, small sections of aerial stems and adjacent leaves of healthy growing plants were chosen. Small sections were carefully excised from intact plants approximately 5 cm below the apical meristem.

Fungal Cultures. An isolate of *Botrytis cinerea* was obtained from the Plant Diagnostic Laboratory at Rutgers Cooperative Research and Extension (New Jersey Agricultural Experiment Station). Cultures were maintained on potato dextrose agar (PDA, 39 g L^{-1} deionized water) (Thermo Fisher Scientific Inc., Fairlawn, NJ) and incubated in the dark at 25 °C.

Chemicals. The following compounds were obtained commercially: Sephadex LH-20, hydrochloric acid, sodium hydroxide, Tween-80, chloroform-*d* (0.03% v/v TMS), methanol-d_4 (0.03% v/v TMS), and pyridine-d_5 (0.03% v/v TMS) were purchased from Sigma-Aldrich (St. Louis, MO); and (22*R*,25*R*)-spirosol-5-ene-3β-ol was from Glycomix Ltd. (Reading, UK). All solvents (acetonitrile, chloroform, ethanol, ethyl acetate, formic acid, *n*-butanol, and *n*-pentane) were of chromatographic grade and were purchased from Thermo Fisher Scientific Inc. (Fairlawn, NJ). Potato dextrose agar and potato dextrose broth were purchased from Thermo Fisher Scientific Inc. (Fairlawn, NJ). Water was deionized (18 MΩ cm) using a Milli-Q-water purification system (Milli-Q, Bedford, MA).

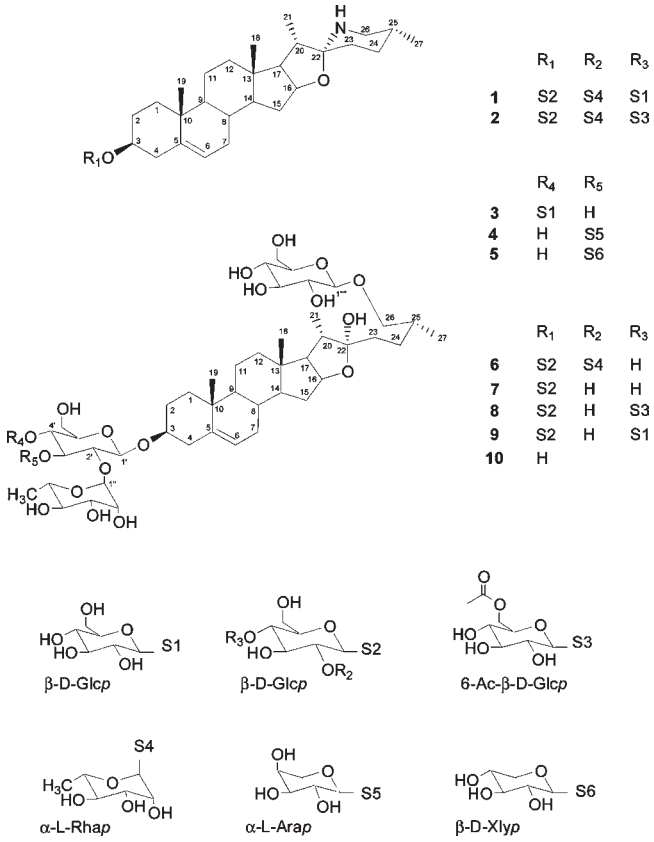

Figure 1. Structures of compounds **1**−**10**.

Isolation and Purification of Steroidal Glycosides 1−5 from *Lilium longiflorum*. Closely following the procedure recently reported in our previous study,[30] the following five steroidal glycosides were isolated from lyophilized *L. longiflorum* bulbs (Figure 1). The compounds were obtained as white amorphous powders in high purity >98%, as determined by LC-MS and NMR.

*Compound **1**, (22R,25R)-Spirosol-5-en-3β-yl O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[32]

*Compound **2**, (22R,25R)-Spirosol-5-en-3β-yl O-α-L-rhamnopyranosyl-(1→2)-[6-O-acetyl-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[30]

*Compound **3**, (25R)-26-O-(β-D-Glucopyranosyl)-furost-5-en-3β,22α, 26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[33]

*Compound **4** ,(25R)-26-O-(β-D-Glucopyranosyl)-furost-5-en-3β,22α, 26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[30]

*Compound **5**, (25R)-26-O-(β-D-Glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-xylopyranosyl-(1→3)-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[30]

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR and ^{13}C NMR spectra were acquired on an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). For NMR analysis, all compounds were dissolved in pyridine-d_5, except for compounds **7** and **10**, which were dissolved methanol-d_4 and chloroform-d, respectively. Chemical shifts were generated as δ values with reference to tetramethylsilane (TMS).

Liquid Chromatography−Mass Spectrometry (LC-MS). LC-MS analysis was performed using a HP 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA) equipped with an autoinjector, quaternary pump, column heater, and diode array detector, interfaced to a Bruker 6300 series ion-trap mass spectrometer equipped with an electrospray ionization chamber. Chromatographic separations were performed using a Prodigy C18 column (250 mm × 4.6 mm i.d.; 5.0 μm particle size) (Phenomenex, Torrance, CA). The flow rate was set to 1.0 mL min^{-1}, and the column temperature was set to 25 °C. The binary mobile phase composition consisted of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. Separations were performed using a linear gradient of 15−43% B over 40 min and then to 95% B over 5 min; thereafter, elution with 95% B was performed for 10 min. The re-equilibration time was 10 min. For instrumentation control and data acquisition, HP ChemStation and BrukerData Analysis software were used. All mass spectra were acquired in positive ion mode over a scan range of m/z 100−2000. Ionization parameters included capillary voltage, 3.5 kV; end plate offset, −500 V; nebulizer pressure, 50 PSI; drying gas flow, 10 mL min^{-1}; and drying gas temperature, 360 °C. Trap parameters included ion current control, 30000; maximum accumulation time, 200 ms; trap drive, 61.2; and averages, 12 spectra.

Partial Acid Hydrolysis of Compound 1. Compound **1** (1 mg) was refluxed in a reaction vial (1 mL) (Reacti-Vial, Thermo Fisher Scientific Inc., Fairlawn, NJ) at 80 °C for 2 h in a solution of 1 N HCl in methanol (0.5 mL). After hydrolysis and titration to pH 7 with NaOH (4 N), the sample was evaporated to dryness under reduced pressure (30 °C; 1.0 × 10^{-3} mbar) using a Labarota 4003 rotary evaporator (Heidolph Brinkman LLC, Elk Grove Village, IL). The residue was dissolved in ethanol and water (7:3, v/v; 2 mL), mixed on a vortex mixer (1 min), and filtered through a 0.45 μm PTFE syringe filter (Thermo Fisher Scientific Inc., Fairlawn, NJ) prior to LC-MS analysis.

***B. cinerea* Growth Inhibition Assay.** Antifungal activity was assessed by an in vitro fungal growth inhibition assay modified from Nicol et al.[34] Fungi were maintained on potato dextrose agar (PDA, 39 g L^{-1} deionized water) and incubated in the dark at 25 °C. The cultures were continuously maintained by transferring a 5 mm plug of mycelium cut with a cork bore from the periphery of actively growing colonies to freshly prepared media. The fungal growth inhibition of compounds **1**−**5** were evaluated at three concentrations (1, 10, and 100 μmol) in the final media. Solutions of compounds **1**−**5** were prepared in ethanol and water (7:3, v/v), filter sterilized with a 0.22 μm sterile syringe filter (Thermo Fisher Scientific Inc., Fairlawn, NJ), and incorporated into autoclaved PDA that was allowed to cool to 50 °C. The media (each plate; 5 mL) were then transferred to polystyrene Petri dishes (50 mm × 12 mm) (VWR International Inc., West Chester, PA) and allowed to solidify. The final concentration of the carrier solvent was 1% of the final volume of media for all treatments and the control. Plates were inoculated with a 5 mm plug taken from the periphery of an actively growing stock culture. Plates were incubated in the dark at 25 °C, and the radial growth of each colony was measured using an ABS Solar Digimatic caliper (Mitutoyo America Corporation, Aurora, IL). Treatment and control colonies were measured when the control colonies reached approximately 80% of the plate diameter. The average control colony diameter minus the average treatment colony diameter was used to calculate the relative growth inhibition. % inhibition = [(average control diameter mm − treatment diameter mm)/average control diameter mm] × 100.

In Vitro Fungal Metabolism of Compounds 1 and 2. Solutions of compound **1** and **2** were separately prepared in ethanol and water (7:3, v/v), filter sterilized with a 0.22 μm syringe filter, and incorporated into autoclaved PDA that was allowed to cool to 50 °C. The media (each plate; 5 mL) were then transferred to polystyrene Petri dishes (50 mm × 12 mm) and allowed to solidify. A total of 4 plates were prepared for each compound. The final concentration for each compound was 100 μM. Plates were inoculated in the center with a 5 mm plug taken from the periphery of an actively growing stock culture. Plates were incubated in the dark at 25 °C and harvested at 48 and 72 h, respectively. At harvest, the total contents of 2 plates (total; 10 mL) were transferred to a centrifuge tube (50 mL) (Thermo Fisher Scientific Inc., Fairlawn, NJ) containing ethanol and water (7:3, v/v; 35 mL). Each sample was then extracted on a wrist-action autoshaker (15 min) (Burrell Scientific, Pittsburgh, PA), sonicated in an ultrasonic water bath (15 min) (B3500A-DTH ultrasonic bath, VWR International Inc., West Chester, PA), and centrifuged (5000 rpm for 10 min) (Sorvall RC-3C Plus, Thermo Fisher Scientific Inc.). The supernatant was then filtered through a 0.45 μm PTFE syringe filter prior to LC-MS analysis.

Scale-Up Fungal Metabolism of Compound 1. Potato dextrose broth (PDB, 39 g L^{-1} deionized water) (each; 100 mL) was prepared and transferred to an Erlenmeyer flask (250 mL) and autoclaved. Once the broth reached room temperature, it was inoculated with *B. cinerea* and incubated on an orbital platform shaker at 200 rpm for 48 h at 25 °C. After 48 h, compound **1** (35 mg) was dissolved in ethanol and water (7:3, v/v; 1 mL), filter sterilized with a 0.22 μm sterile syringe filter, and introduced to the flask. The reaction was monitored by sampling aliquots (each; 0.5 mL) every 24 h for 96 h. Each aliquot was diluted (1:3, v/v) with ethanol and water (7:3, v/v; 1.5 mL), and filtered through a 0.45 μm PTFE syringe filter prior to LC-MS analysis. At the completion of the reaction, the content of the flask was immediately frozen under liquid nitrogen, lyophilized, and stored at −80 °C until extraction.

Semipreparative RP-HPLC Isolation of the Fungal Metabolites of Compound 1. The lyophilized reaction mixture, as described above, was ground into a fine powder with a laboratory mill (IKA Labortechnik, Staufen, Germany) and extracted with ethanol and water (7:3, v/v; 2 × 50 mL) on an autoshaker at room temperature for 15 min. After centrifugation (5000 rpm for 10 min), the supernatant was collected and the residue discarded. The supernatant was then evaporated under reduced pressure, dissolved in a mixture of 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile (75:25, v/v; 5 mL), and filtered through a 0.45 μm PTFE syringe filtered prior to purification. Chromatographic separations were achieved by semipreparative RP-HPLC performed on a Luna C18 column (250 mm × 21.2 mm i.d.; 10 μm particle size) (Phenomenex, Torrance, CA). Chromatography was performed on a Shimadzu LC-6AD liquid chromatograph (Shimadzu Scientific Instruments Inc., Columbia, MD) using a UV/vis detector and a 2 mL injection loop. Mixtures of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile were used as the mobile phase. The flow rate was set to 20 mL min^{-1}, the column temperature was 23 ± 2 °C, and UV detection was recorded at λ = 210 nm. Chromatography was performed using a linear gradient of 5−30% B over 45 min and then to 90% B over 10 min; thereafter, elution with 90% B was performed for 10 min. The re-equilibration time was 10 min. The target compounds were collected, freed from solvent under reduced pressure, and lyophilized, yielding **6** (2 mg), **7** (5 mg), and **10** (1 mg) (Figure 1) as white amorphous powders in high purity >98%, as determined by LC-MS and NMR.

Compound ***6****, (22R,25R)-Spirosol-5-en-3β-yl O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[32]

Compound ***7****, (22R,25R)-Spirosol-5-en-3β-yl O-β-D-glucopyranoside.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[35]

Compound ***10****, (22R,25R)-Spirosol-5-en-3β-ol.* ^{1}H NMR and ^{13}C NMR were consistent with the literature.[36]

Isolation and Purification of Compound 6 from *Lilium longiflorum* Bulbs. *Sequential Solvent Extraction of Lyophilized L. longiflorum Bulbs.* Lyophilized lily bulbs (100 g) were frozen in liquid nitrogen, ground into a fine powder with a laboratory mill, and extracted with *n*-pentane (3 × 100 mL) on an autoshaker at room temperature for 30 min. After centrifugation (5000 rpm for 10 min), the organic layers were discarded, and the pellet was freed from the residual solvent. The residual material was then extracted with a mixture of ethanol and water (7:3, v/v; 2 × 150 mL) on an autoshaker for 45 min at room temperature. After centrifugation (5000 rpm for 10 min) and vacuum filtration through Whatman 114 filter paper (Whatman International Ltd., Maidstone, UK), the supernatant was collected and the residue discarded. The supernatant was then evaporated under reduced pressure and lyophilized, yielding a crude bulb extract (12.9 g). The lyophilized crude bulb extract was then dissolved in deionized water (100 mL) and washed with ethyl acetate (5 × 100 mL), and the organic phase was discarded. The aqueous phase was then extracted with *n*-butanol (5 × 100 mL), and the aqueous phase was discarded. The organic phase was then evaporated under reduced pressure (30 °C; 1.0×10^{-3} bar) and lyophilized, yielding a crude glycoside extract (2.2 g).

Gel Permeation Chromatography (GPC). The crude glycoside extract (1.0 g) was dissolved in a solution of ethanol and water (7:3, v/v; 5.0 mL), filtered with a 0.45 μm PTFE syringe filter, and then applied onto a standard threaded 4.8 cm × 60 cm glass column (Kimble

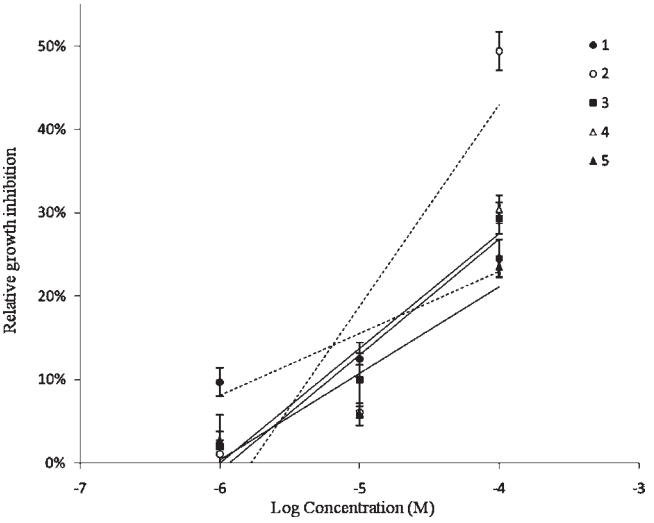

Figure 2. Growth inhibition activity of compounds **1**−**5** on radial mycelia growth of *B. cinerea*. The dashed lines represent steroidal glycoalkaloids, and the solid lines represent furostanol saponins.

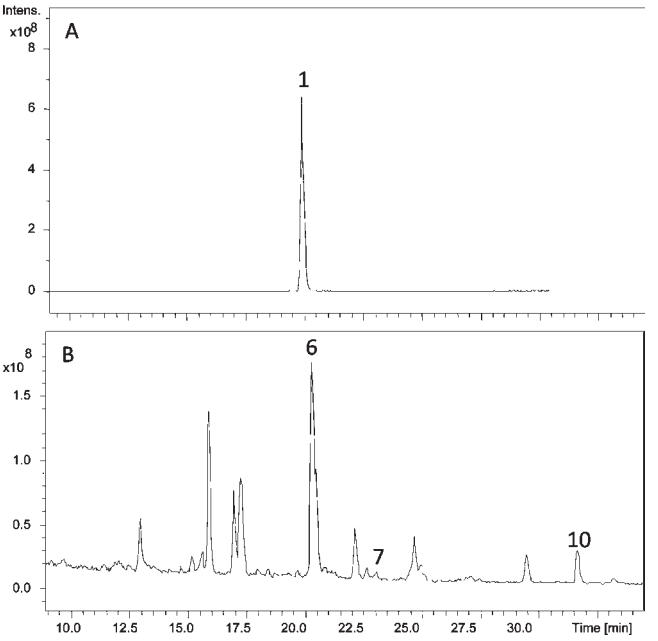

Figure 4. Metabolism of compound **1** by *B. cinerea*: total ion chromatogram (TIC) of fungal media spiked with compound **1** (time = 0) (A) and TIC of metabolites of compound **1** (48 h) (B).

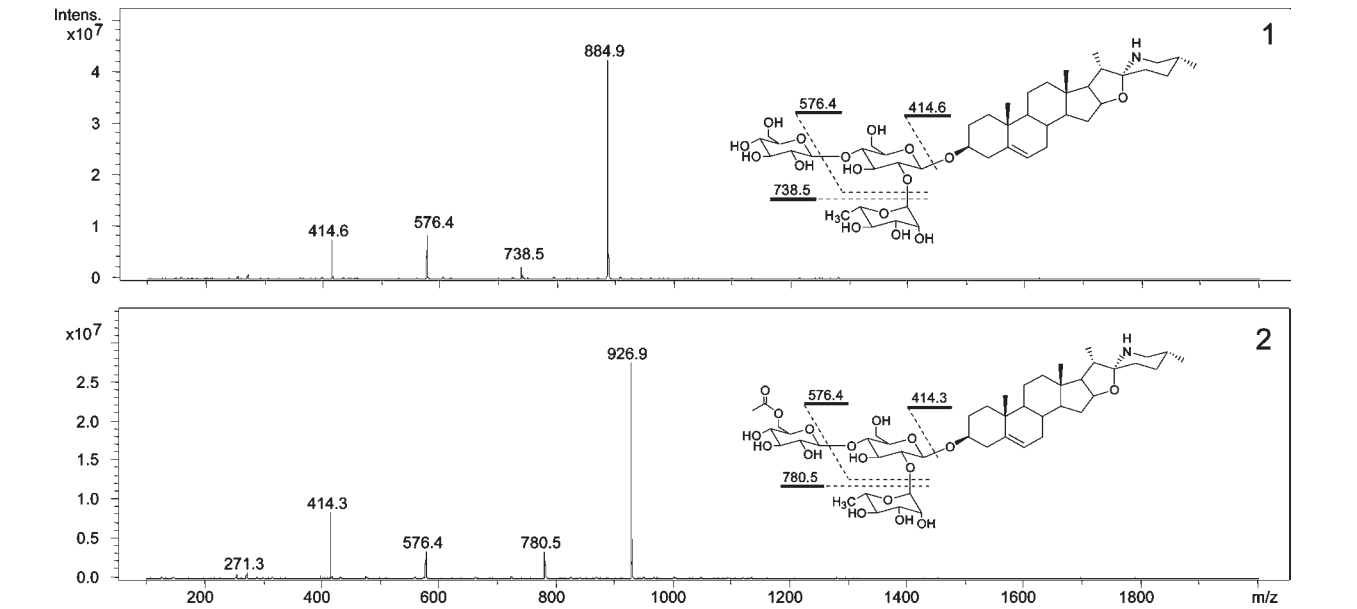

Figure 3. ESI^{+}-MS mass spectra of steroidal glycoalkaloids **1** and **2**.

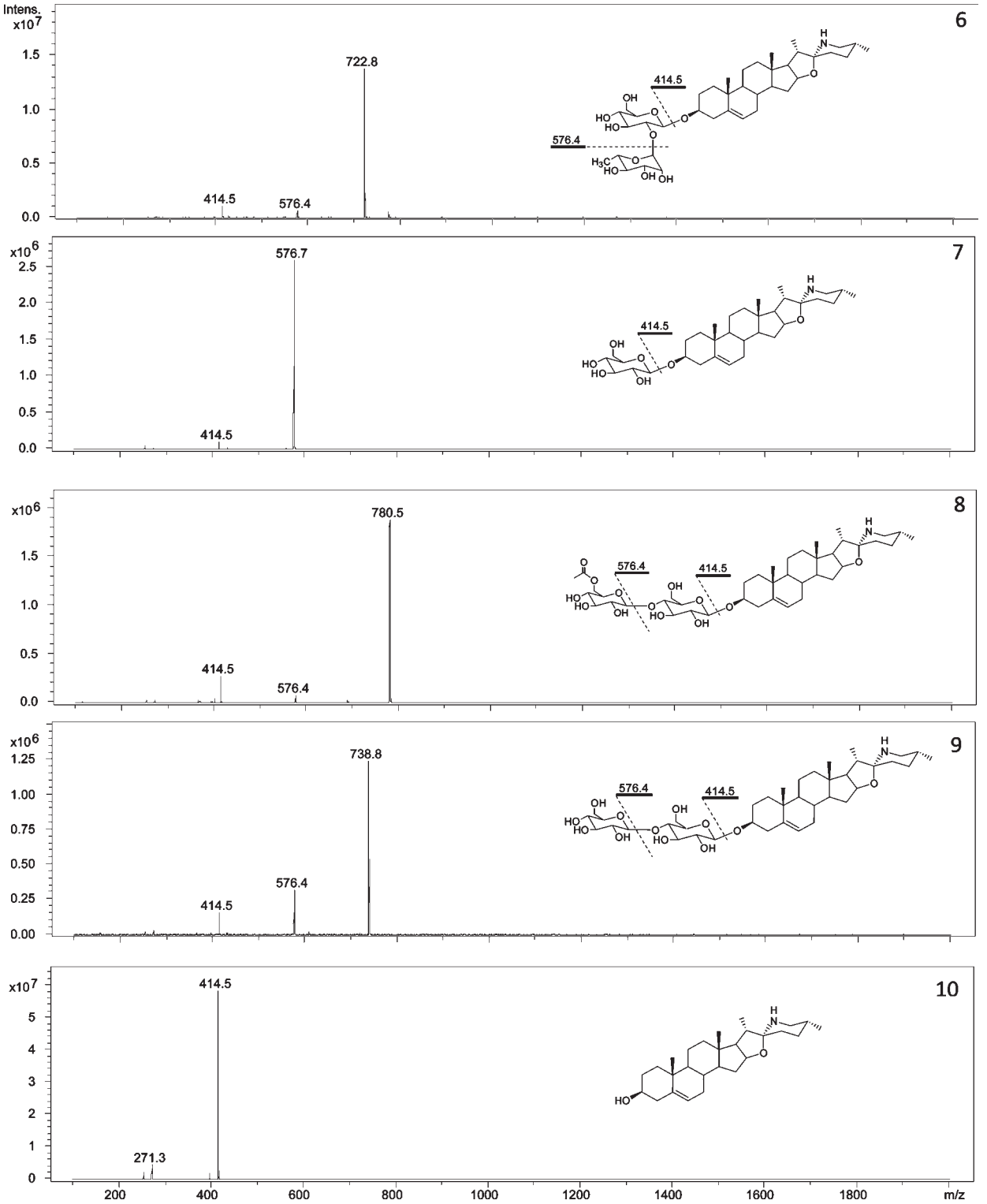

Figure 5. ESI^+-MS mass spectra of fungal metabolites **6**−**10**.

Chase Life Science and Research Products LLC, Vinland, NJ) packed with Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) that was washed and conditioned in the same solvent mixture overnight. Chromatography was performed with isocratic ethanol and water (70:30, v/v) at a flow rate of 3.5 mL min^{-1}. The first 200 mL of effluent was discarded, and 25 fractions (25 mL each) were collected and analyzed by LC-MS as described above. On the basis of the LC-MS analysis, GPC fractions 8−10 contained the highest levels of compound **6** and were combined, evaporated under reduced pressure, and lyophilized, yielding GPC fraction A (25 mg).

Semipreparative Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). Purification of compound **6** from GPC fraction A was achieved by semipreparative RP-HPLC under the same conditions as those described above. Compound **6** was collected, freed from solvent

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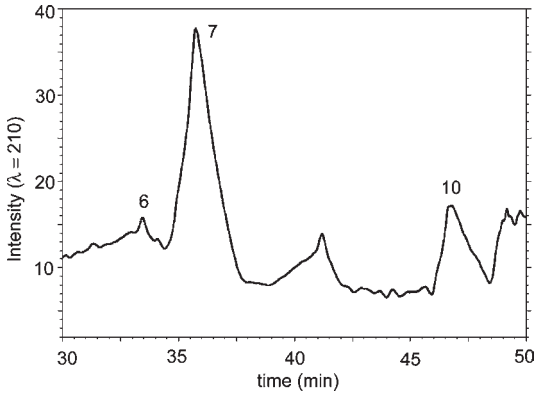

Figure 6. RP-HPLC chromatogram ($\lambda = 210$ nm) of fungal metabolites **6**, 7, and **10** isolated from the biotransformation of compound **1** by *B. cinerea*.

under reduced pressure, and lyophilized yielding **6** (3 mg) as a white amorphous powder in high purity >98%, as determined by LC-MS and NMR. ^{1}H NMR and ^{13}C NMR were consistent with the literature.[32]

Infection of *L. longiflorum* Tissue and Sample Preparation for LC-MS Analysis. Small sections of aerial stems were excised from intact plants approximately 5 cm below the apical meristem, including several small (~3 cm) leaves. The plant tissue was surface sterilized (10% bleach and 0.01% Tween-80, v/v) for 10 min, rinsed with sterilized DI water, and transferred aseptically to a Petri dish (90 mm × 15 mm). Treatment tissues were inoculated with two 5 mm plugs of *B. cinerea* and incubated in the dark at 25 °C. Control samples were treated under the same conditions without fungal inoculation. Once fully colonized with the mycelium (7 days), the samples were frozen under liquid nitrogen and lyophilized. The lyophilized material was ground with a mortar and pestle and passed through a sieve (pore size; 270 mesh) (W.S. Tyler Inc., Mentor, OH). The fine powder (0.5 g) was transferred to a centrifuge tube (15 mL), extracted with ethanol and water (7:3, v/v; 5 mL) on an autoshaker at room temperature for 10 min, and sonicated in an ultrasonic water bath (10 min). After centrifugation (5000 rpm for 10 min), the supernatant was collected and filtered through a 0.45 μm PTFE syringe filter prior to LC-MS analysis. LC-MS analysis was performed as described above.

Statistical Analysis. Data was subjected to analysis of variance (ANOVA) and regression analysis using SAS, version 9.2 for Windows (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Fungal Growth Inhibition Assay. Analysis of variance was performed to determine if there was a significant effect of treatment (compounds **1**−**5**) (Figure 1), rate (1, 10, and 100 μmol), and the interaction between treatment and rate. There was a significant interaction between the treatment and rate ($P > 0.0001$); thus, the main effects were ignored, and a further investigation of the interaction was performed. An equation describing the relationship between the response and the rate was generated for each treatment (Figure 2). All five compounds were weakly inhibitory to *B. cinerea*. The furostanol saponins, compounds **3**−**5**, all had similar activity ranging from approximately 25−30% growth inhibition as compared to that of the control at the highest concentration tested. The steroidal glycoalkaloid, compound **1**, had inhibitory activity similar to that of the furostanol saponins. The steroidal glycoalkaloid, compound **2**, had the highest inhibitory activity of 49% at the highest concentration,

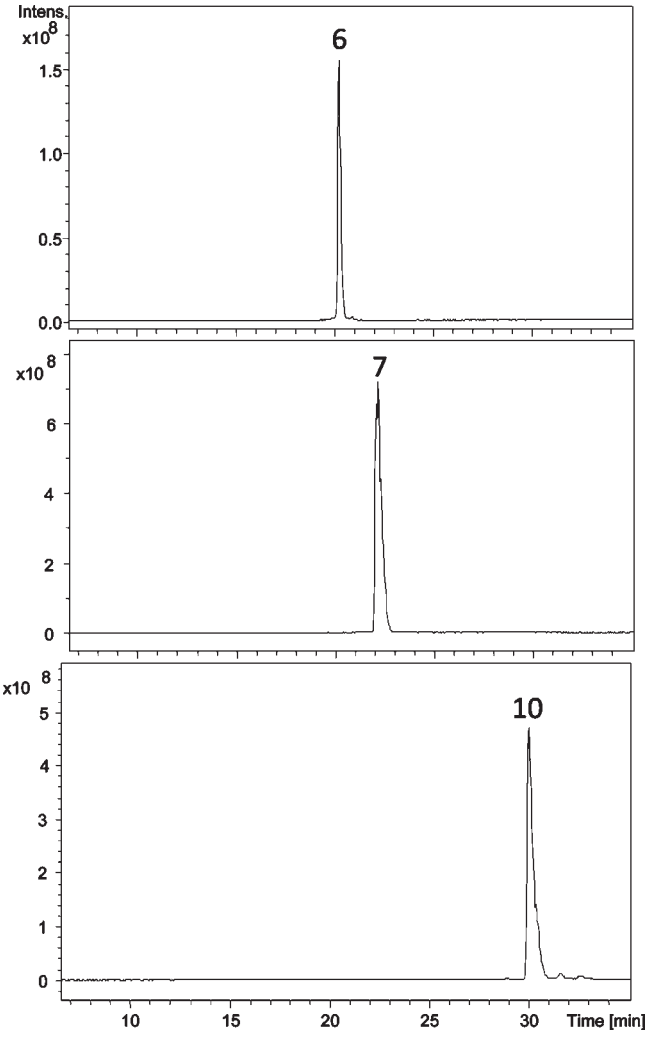

Figure 7. Total ion chromatograms (TIC) of compounds **6**, 7, and **10** isolated by RP-HPLC.

approximately two times the activity of compound **1**. Steroidal glycoalkaloids **1**−**2** are similar in structure and only differ by the presence of an acetyl group linked to the C-6‴ hydroxy position of the terminal glucose of carbohydrate moiety. The acetylation of the terminal glucose unit resulted in an increased rate of fungal growth inhibition, as compared to that of compound **1**. Similar to the solanaceous glycoalkaloids, α-chaconine and α-tomatine, compounds **1** and **2** occur together as a pair, share the same aglycone, only differ in the carbohydrate moiety, and exhibit differential biological activity.[17] Friedman and MacDonald suggested that glycoalkaloid pairs may occur as a plant defense response to the adaptive ability of the pathogen to detoxify the plant's antifungal compounds.[37]

Metabolism of Compounds 1 and 2 by *B. cinerea*. On the basis of the observation that compound **2** had a 2-fold increase in fungal growth inhibition as compared to that of compound **1**, an investigation on the ability of *B. cinerea* to cleave the sugar residues of compounds **1** and **2** was conducted (Figure 3). After 48 h of in vitro metabolism of compound **1** by *B. cinerea*, only trace quantities of compound **1** could be detected by LC-MS (Figure 4). A metabolite of compound **1**, compound **6**, was observed with a base peak at m/z 722.8 (Figure 5). This ion was

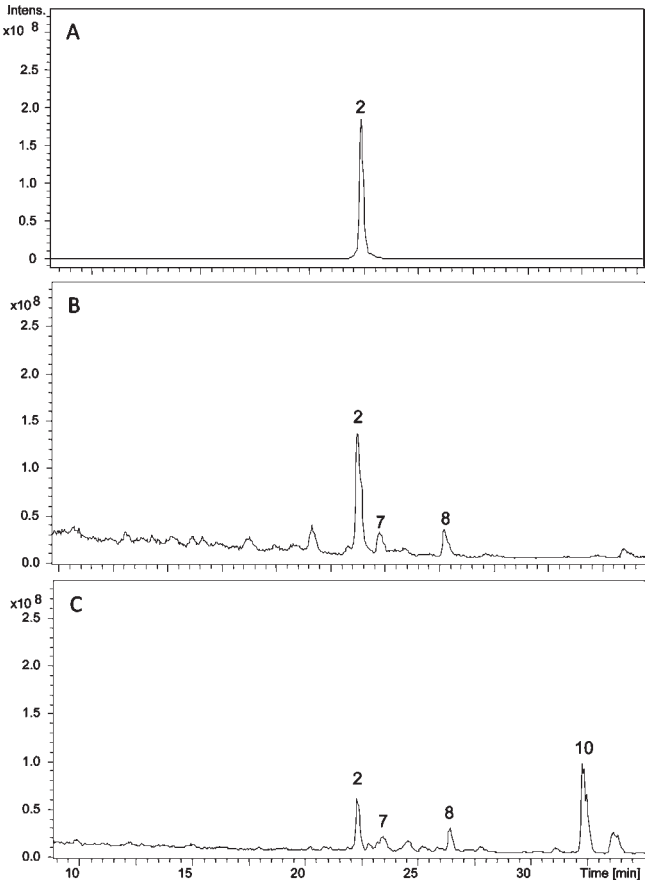

Figure 8. Metabolism of compound **2** by *B. cinerea*: total ion chromatogram (TIC) of fungal media spiked with compound **2** (time = 0) (A), TIC of metabolites of compound **2** (48 h) (B), and TIC of metabolites of compound **2** (72 h) (C).

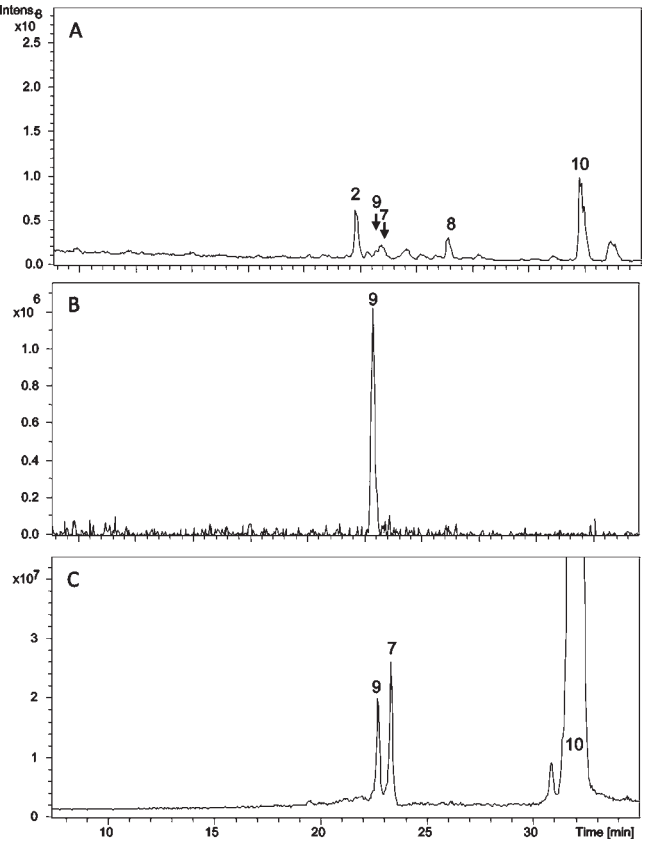

Figure 9. Total ion chromatogram (TIC) of the metabolites of compound **2** (72 h) (A), extracted ion chromatogram (EIC) for compound **9** (m/z 738.8) from the metabolite mixture derived from compound **2** (B), and TIC of the partial acid-catalyzed hydrolysis products of compound **1** (C).

consistent with a molecule containing one less glucose molecule than compound **1**. Additionally, ion fragments at 576.4 $[M-Rha+H]^+$ and 414.5 $[M-Glu-Rha+H]^+$ were observed and were consistent with a disaccharide moiety containing one glucose and one rhamnose molecule. Compound **6** was then isolated by semipreparative RP-HPLC from a scale-up fermentation and subjected to further chemical and spectroscopic analysis (Figures 6 and 7). On the basis of ESI^+-MS and a comparison of ^1H NMR and ^{13}C NMR with the literature, compound **6** was confirmed to be solasodine 3-*O*-α-L-rhamnopyranosyl-(1→2)- β-D-glucopyranoside,[32] previously isolated from the bulbs of *Lilium brownii*. In addition, another metabolite, compound **7**, was observed with a base peak at m/z 576.7 (Figure 5). The mass spectrum of the metabolite was consistent with a molecule containing one less glucose and one less rhamnose from compound **1** or one less rhamnose from compound **6**. Additionally, an ion fragment at 414.5 $[M-Glu+H]^+$ was observed and was consistent with a monosaccharide moiety containing one glucose molecule. Compound **7** was then isolated by semipreparative RP-HPLC from a scale-up fermentation and subjected to further chemical and spectroscopic analysis (Figures 6 and 7). On the basis of a comparison of the retention time of the partial hydrolysis products of compound **1**, ESI^+-MS, and a comparison of ^1H NMR and ^{13}C NMR with the literature, compound **7** was confirmed to be solasodine 3-*O*-β-D-glucopyranoside,[35,38] previously isolated from *Solanum umbelliferum*. A third metabolite, compound **10**, was observed with a based peak at m/z 414.5 (Figure 5). The mass spectrum was consistent with a molecule containing the loss of two glucoses and one rhamnose from compound **1**, one rhamnose and one glucose from compound **6**, or one glucose from compound **7**. Additionally, an ion fragment at m/z 271.3 was observed. Compound **10** was then isolated by semipreparative RP-HPLC from a scale-up fermentation and subjected to further chemical and spectroscopic analysis (Figures 6 and 7). On the basis of a comparison of the retention time with an authentic standard, ESI^+-MS, and comparison of ^1H NMR and ^{13}C NMR with the literature, compound **10** was confirmed to be solasodine,[36] a common aglycone of steroidal glycoalkaloids. Similar to the metabolism of α-tomatine by *Alternaria solani*, sequential cleavage of all of the sugars of the carbohydrate moiety were observed in the model system.[39] In addition to compounds **6**, **7**, and **10**, several other fungal metabolites with differential degrees of glycosylation and regiospecific mono- and polyhydroxylation of the aglycone (data not shown) were observed and will not be discussed this article.

The fungal metabolism of compound **2** by *B. cinerea* was markedly different from that of compound **1**, as a result of the acetylation of the 6‴ hydroxy position of the terminal glucose unit. After 48 h of in vitro metabolism of compound **2**, most of compound **2** was still present in the media (Figure 8), whereas after 48 h of metabolism of compound **1**, only trace amounts

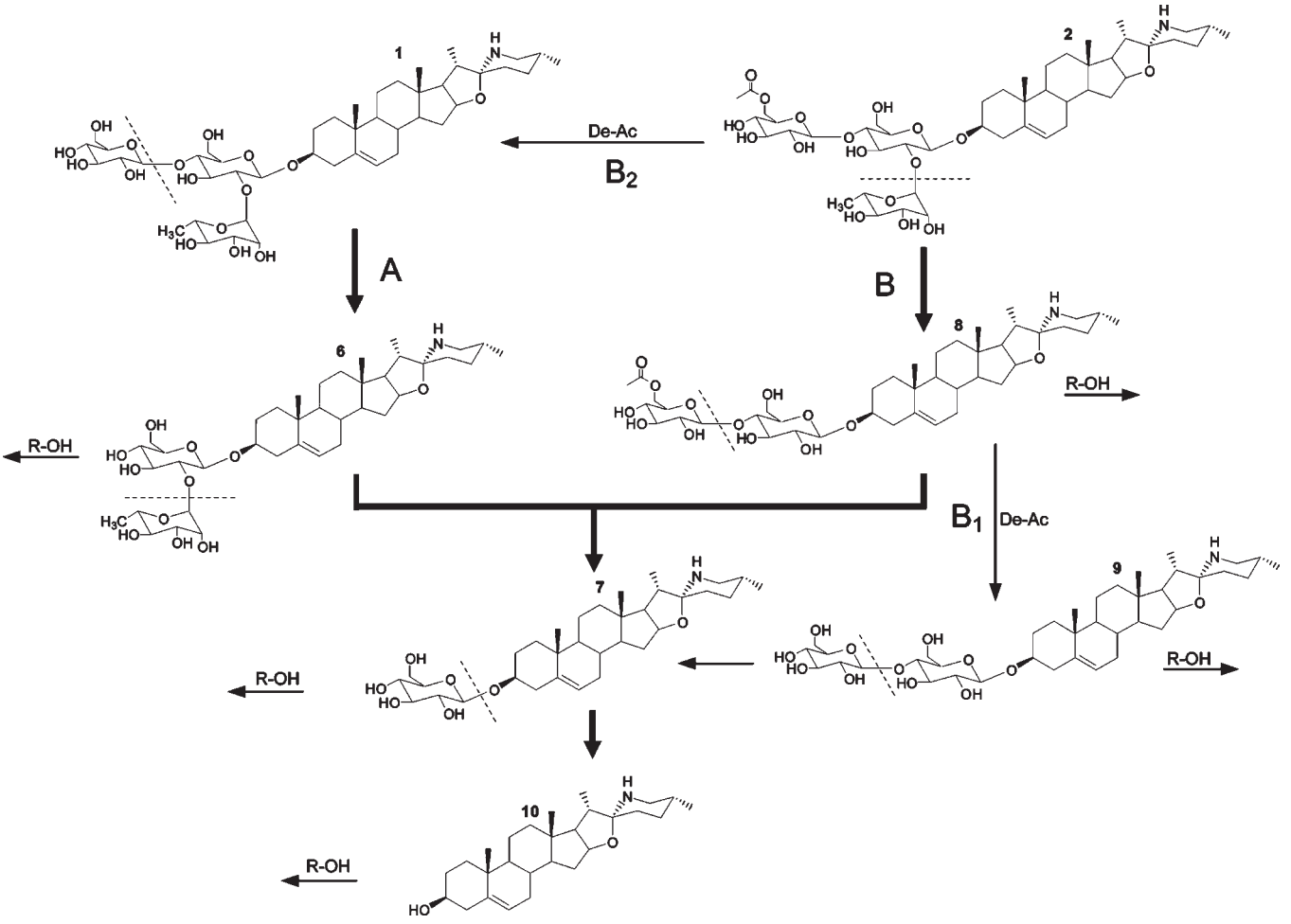

Figure 10. Proposed partial metabolic pathways for compounds **1** and **2** (thick arrows): major metabolic pathway for compound **1** (A). Major metabolic pathway for compound **2** (B). Minor metabolic pathways for compound **2** (B_1 and B_2). De-Ac, deacetylation; R-OH, mono/poly-hydroxylation of aglycone.

were present (Figure 4). In contrast to the metabolism of compound **1**, the metabolite compound **6** was not detected; however, a new metabolite, compound **8**, was observed with a base peak at *m*/*z* 780.5 (Figure 5). The mass spectrum of the metabolite was consistent with a loss of rhamnose from compound **2**. Additionally, ion fragments at 576.4 $[M-Glu-Ac+H]^+$ and 414.5 $[M-2Glu-Ac+H]^+$ were observed and were consistent with a disaccharide moiety containing glucose and an acetylated glucose moiety. On the basis of mass spectral analysis of this metabolite, compound **8** is likely to be (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[6-*O*-acetyl-β-D-glucopyranoside (Figure 1); however, preparative isolation and full characterization are needed for unequivocal confirmation. In addition, compound **7** was detected as a metabolite of compound **2** and is consistent with the loss of one rhamnose and an acetylated glucose moiety of compound **2** or the loss of the acetylated glucose moiety of compound **8**. Compound **10**, was also observed and is consistent with the metabolite of compound **1**. The mass spectrum was consistent with a molecule containing the loss of one rhamnose, one glucose, and an acetylated glucose from compound **2**, the loss of one glucose and an acetylated glucose from compound **8**, or one glucose from compound **7**.

Because of the fact that only a small portion of compound **2** was metabolized after 48 h of incubation, the metabolism experiment was continued for an additional 24 h. After 72 h of metabolism of compound **2** by *B. cinerea*, compound **2** was still present in the media (Figure 8) as compared to the metabolism of compound **1** in which only trace amounts were present after 48 h (Figure 4). The decreased metabolism rate of compound **2** may play a role in the increased fungal growth inhibition of compound **2** as compared to that of compound **1**. Interestingly, after 72 h, compound **10** increased, and small amounts of compound **9** and compound **1** were detected (Figure 8). Compound **9** had a base peak *m*/*z* 738.8 (Figure 5). This ion was consistent with the deacetylation of compound **8**. Ion fragments at 576.4 $[M-Glu+H]^+$ and 414.5 $[M-2Glu+H]^+$ were observed and were consistent with a disaccharide moiety containing two glucose moieties. Additionally, compound **9** had the same retention time and mass spectrum as the product of the partial acid hydrolysis of compound **1** (Figure 9). Accordingly, compound **9** is likely to be (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (Figure 1); however, preparative isolation and full characterization are needed for unequivocal confirmation. The presence of small amounts of compound **1** and compound **9** after 72 h of incubation suggests acetylase activity; however, due to the presence of a greater abundance of compound **8**, the cleavage of the rhamnose moiety at the C-2′ position of the inner glucose is favored over

deacetylation of the acetyl moiety of the C-6‴ position of the terminal glucose under these conditions.

On the basis of these data, the metabolism of compound **1** occurs by the sequential removal of the sugars of the trisaccharide moiety with compounds **6**, **7**, and **10** as intermediates (pathway A; Figure 10). In parallel, hydroxylation of the aglycone occurs (data not shown). Regiospecific microbial hydroxylation of diosgenin and solasodine is well known and is utilized in the production of pharmaceutical steroids.[40,41] In tomato, *B. cinerea* has been shown to metabolize the steroidal glycoalkaloid, α-tomatine, by both cleavage of the entire carbohydrate moiety and by the cleavage of the terminal xylose.[25,26] Sequential sugar cleavage in compound **1** is similar to the metabolism of α-tomatine by *Alternaria solani*, where all four sugars of the tetrasaccharide moiety are sequentially cleaved.[39] In the case of compound **2**, acetylation of the terminal glucose moiety inhibits cleavage from the inner glucose, and metabolism proceeds through the cleavage of rhamnose at the C-2′ position of the inner glucose. The major metabolic pathway proceeds sequentially with compounds **8**, **7**, and **10** as intermediates (pathway B; Figure 10). Alternatively, evidence of acetylase activity was observed, and two minor metabolic pathways of compound **2** are proposed. One minor metabolic pathway may proceed with deacetylation of compound **2** with compounds **8**, **9**, **7**, and **10** as intermediates (pathway B_1; Figure 10), and a second minor metabolic pathway may proceed with compounds **1**, **6**, **7**, and **10** as intermediates (pathway B_2; Figure 10).

In Planta Identification of Compounds 6−10 by LC-MS. On the basis of the in vitro fungal growth inhibition studies and the characterization of the fungal metabolites of compounds **1** and **2**, the objective of this part of the study was to determine if *B. cinerea* has the ability to metabolize compounds **1** and **2**, in planta, into the fungal metabolites that were identified in the model system. In order to investigate this question, plant tissue that was infected with *B. cinerea* was compared to a control tissue by LC-MS analysis. All fungal metabolites that were characterized in the model system were detected in the infected plant tissue. None of the metabolites were detected in the control tissue with the exception of compound **6**. Although the infected tissue had elevated levels of compound **6** as compared to that in the control, interestingly, compound **6** was also present in the control sample that was not infected with *B. cinerea*. This suggests that compound **6** is not only a fungal metabolite of the steroidal glycoalkaloids but also a natural product constitutively present in *L. longiflorum* tissues not infected with the fungus.

Isolation and Identification of Compound 6 from *L. longiflorum* Bulbs. In order to confirm the presence of compound **6** as a natural product in *L. longiflorum*, compound **6** was isolated and purified from *L. longiflorum* bulbs. Briefly, lyophilized lily bulbs were washed with *n*-pentane and extracted with ethanol and water. After the removal of the solvent, the extract was dissolved in deionized water, washed with ethyl acetate, and extracted with *n*-butanol yielding a crude steroidal glycoside extract. The crude glycoside extract was fractionated by gel permeation chromatography and repeated semipreparative RP-HPLC to yield compound **6**. On the basis of ^{1}H NMR and ^{13}C NMR, compound **6** was confirmed as (22*R*,25*R*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, previously isolated from *L. brownii*.[32] This data confirms not only that compound **6** is a fungal metabolite of compounds **1** and **2** but also that it is constitutively present in *L. longiflorum*.

In this present study, two steroidal glycoalkaloids and three furostanol saponins, isolated from *L. longiflorum*, were evaluated for fungal growth inhibition of the plant pathogenic fungus, *B. cinerea*. All compounds showed weak fungal growth inhibition activity. In addition, five fungal metabolites of the glycoalkaloids **1** and **2**, were characterized from a model system and were observed in living plant tissue infected with the fungus. Furthermore, a structure−function relationship for the fungal growth inhibition for compounds **1** and **2** was established on the basis of the acetylation of the terminal glucose moiety. On the basis of these results, *B. cinerea* can metabolize compounds **1** and **2** by the sequential removal of the sugars of the trisaccharide moiety. Additionally, these data suggest that a decreased rate of metabolism of compound **2** may play a role in increased fungal growth inhibition activity. Moreover, compound **6** was determined to be both a fungal metabolite of compounds **1** and **2** and a natural product constitutively present in *L. longiflorum*. This is the first report of compound **6** from *L. longiflorum*. This study can be used as a model for characterizing fungal metabolites of plant derived natural products and a means for the generation of new natural products with novel biological activities.

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