JOURNAL OF NATURAL PRODUCTS

Article

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Cochliotoxin, a Dihydropyranopyran-4,5-dione, and Its Analogues Produced by *Cochliobolus australiensis* Display Phytotoxic Activity against Buffelgrass (*Cenchrus ciliaris*)

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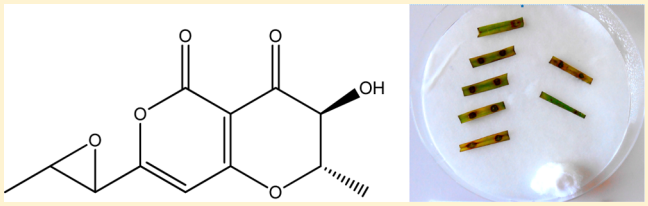
Supporting Information

ABSTRACT: Buffelgrass (*Pennisetum ciliare* or *Cenchrus ciliaris*) is a perennial grass that has become highly invasive in the Sonoran Desert of southern Arizona. In the search for novel control strategies against this weed, strains of the foliar fungal pathogen *Cochliobolus australiensis* from buffelgrass have been screened for their ability to produce phytotoxic metabolites that could potentially be used as natural herbicides in an integrated pest management strategy. A new phytotoxin, named cochliotoxin, was isolated from liquid culture of this fungus together with radicinin, radicinol, and their 3-epimers. Cochliotoxin was characterized, essentially by spectroscopic methods, as 3-hydroxy-2-methyl-7-(3-methyloxiranyl)-2,3-dihydropyrano[4,3-*b*]pyran-4,5-dione. Its relative stereochemistry was assigned by ^{1}H NMR techniques, while the absolute configuration (2*S*,3*S*) was determined applying the advanced Mosher's method by esterification of its hydroxy group at C-3. When bioassayed in a buffelgrass coleoptile elongation test and by leaf puncture bioassay against the host weed and two nontarget grasses, cochliotoxin showed strong phytotoxicity. In the same tests, radicinin and 3-*epi*-radicinin also showed phytotoxic activity, while radicinol and 3-*epi*-radicinol were largely inactive. All five compounds were more active in leaf puncture bioassays on buffelgrass than on the nontarget grass tanglehead (*Heteropogon contortus*), while the nontarget grass Arizona cottontop (*Digitaria californica*) was more sensitive to radicinin and 3-*epi*-radicinin. Cochliotoxin at low concentration was significantly more active on buffelgrass than on either native grass, but the difference was small.



Buffelgrass (*Pennisetum ciliare* or *Cenchrus ciliaris*) is a perennial bunchgrass native to Africa and southern Asia that is an important pasture grass in many semiarid regions of the world.[1,2] However, during the last three decades, buffelgrass has spread into undisturbed natural areas, causing significant ecological damage.[3,4] It has become highly invasive in some parts of its introduced range, particularly in the Sonoran Desert of southern Arizona.[5−7] In fact, it has infested thousands of acres of public and private lands, including Saguaro National Park and the Coronado and Tonto National Forests, competing with the native vegetation for water, nutrients, and space.[3,8] At present the only approaches available to deal with buffelgrass invasion into natural ecosystems are broad-spectrum herbicides and physical removal with hand tools.

Biological control has become an effective approach to combat many weeds that invade natural systems,[9] but direct application of living biocontrol agents for buffelgrass in southern Arizona is problematic for both ecological and sociological reasons. The research reported here is the latest in a series of studies carried out to determine whether fungal pathogens can produce novel bioactive metabolites with potential herbicidal activity.[10] The goal of this study is to select a fungal metabolite to be used as bioherbicide showing more effective phytotoxic effects against the target weed than against nontarget species, thus reducing the collateral damage to native vegetation that often complicates buffelgrass control with broad-spectrum herbicides.

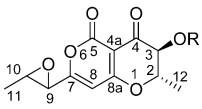
This paper reports on the chemical characterization of a new phytotoxin, named cochliotoxin, as well as on the identification of radicinin, 3-*epi*-radicinin, radicinol, and 3-*epi*-radicinol produced by *Cochliobolus australiensis* in liquid culture. The phytotoxic activity of these compounds was evaluated against buffelgrass as well as co-occurring nontarget native grasses of the Sonoran Desert.

Received: July 27, 2016
Published: April 19, 2017

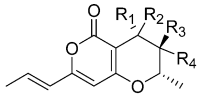
DOI: 10.1021/acs.jnatprod.6b00696
J. Nat. Prod. 2017, 80, 1241−1247

RESULTS AND DISCUSSION

The organic extract of *C. australiensis* liquid culture filtrates was fractionated by a combination of column and preparative TLC chromatography as detailed in the Experimental Section. A new phytotoxic metabolite, named cochliotoxin (**1**), was isolated together with four closely related metabolites. These were identified as radicinin,[11,12] 3-*epi*-radicinin,[11] radicinol,[13] and 3-*epi*-radicinol[11,14] (**2**−**5**) by comparing their physical (OR) and spectroscopic properties (^{1}H NMR and ESIMS) with the data reported in the literature.



1 R=H
6 R=*S*-MTPA
7 R=*R*-MTPA



2 R_1+R_2=O, R_3=OH, R_4=H
3 R_1+R_2=O, R_3=H, R_4=OH
4 R_1=R_3=OH, R_2=R_4=H
5 R_1=R_4=OH, R_2=R_3=H

The new metabolite showed a molecular formula of $C_{12}H_{12}O_6$ as deduced from its HRESIMS, consistent with seven hydrogen deficiencies. This result combined with a preliminary spectroscopic investigation of its ^{1}H and ^{13}C NMR (Table 1) showed that it is closely related to radicinin (**2**, Figure 1). In fact, the ^{1}H NMR spectrum showed a singlet typical of a proton (H-8) of a trisubstituted olefinic group and a double quartet (J = 12.4 and 6.2 Hz) and a doublet (J = 12.4 Hz) at δ 6.07 and 4.37 and 4.01, typical of protons (H-2 and H-3) of secondary oxygenated carbons. H-8 in the COSY spectrum[15] coupled with a doublet (J = 1.3) resonating at δ 3.38 and the latter, in turn, with a double quartet (J = 5.3 and 1.3) at δ 3.17, typical of protons (H-9 and H-10) of a 1,2-*trans*-disubstituted epoxy ring. H-10 and H-3 appeared to be coupled in the same spectrum with the respective geminal methyl groups (Me-11, Me-12), which both resonated as doublets (J = 5.3 and 6.2 Hz) at δ 1.45 and 1.66. A broad singlet due to a hydroxy group also appeared at δ 3.80.[16] These results are in agreement with the bands of hydroxy and olefinic groups observed in the IR spectrum, which also showed absorptions typical of α,β-unsaturated ketone and ester carbonyl groups.[17] These results were consistent with the absorption maxima observed in the UV spectrum and due to extended conjugated chromophores.[18]

Table 1. ^{1}H and ^{13}C NMR Data of Cochliotoxin (1) Recorded in $CDCl_3$[a,b]

position	δ_C[c]	δ_H (J in Hz)	HMBC
2	80.4 CH	4.37, dq (12.4, 6.2)	H-3, Me-12
3	72.0 CH	4.01, d (12.4)	H-2, Me-12
4	188.6 C		H-2, H-3
4a	98.2 C		H-8
5	156.3 C		
7	168.1 C		H-8, H-9
8	97.4 CH	6.07, s	H-9
8a	176.2 C		H-8
9	54.9 CH	3.38, d (1.3)	H-8, Me-11
10	58.4 CH	3.17, dq (5.3, 1.3)	H-9, Me-11
11	17.3 CH_3	1.45, d (5.3)	H-10
12	18.0 CH_3	1.66, d (6.2)	H-3
OH		3.80, brs	

[a]The chemical shifts are in δ values (ppm) from TMS. [b]2D ^{1}H,^{1}H (COSY) and ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectrum.

The relative configuration of **1** was deduced from investigation of the coupling constants observed in its ^{1}H NMR spectrum. In particular, the large value (J = 12.4 Hz) observed for the coupling between H-2 and H-3 allowed location of these two protons both as pseudoaxial and consequently their geminal methyl and hydroxy groups as pseudoequatorial.[20] Furthermore, the value (J = 1.3 Hz) observed for couplings between the two oxyran protons (H-9 and H-10) allowed assignment of a *trans* stereochemistry to this ring.[21]

The absolute configuration of **1** was determined by applying a modified Mosher's method.[22] Cochliotoxin was treated with *R*-(−)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) and *S*-(+)-MTPA chlorides, to convert **1** into the corresponding diastereomeric esters at C-3 (**6**, **7**). The spectroscopic data for the *S*-MPTA and *R*-MTPA esters (**6** and **7**, respectively) were

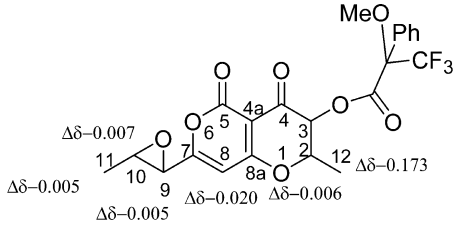


Figure 1. Structures of 3-*O*-*S*- and 3-*O*-*R*-MTPA of cochliotoxin (**6** and **7**, respectively), reporting the $\Delta\delta$ value obtained by comparison (**6** and **7**) of each proton system.

DOI: 10.1021/acs.jnatprod.6b00696
J. Nat. Prod. 2017, 80, 1241−1247

consistent with the structure assigned to **1**. Subtracting the chemical shift of the protons (Table 2) of the 3-*O*-*R*-MTPA (**7**)

Table 2. ^{1}H NMR Data of Cochliotoxin Derivatives (6 and 7) Recorded in $CDCl_3$[a]

	6	7
position	δ_H (J in Hz)	δ_H (J in Hz)
2	4.678 (1H) dq (12.4, 6.2)	4.851 (1H) dq (12.5, 6.2)
3	5.458 (1H) d (12.4)	5.384 (1H) d (12.5)
8	6.085 (1H) s	6.105 (1H) s
9	3.395 (1H) d (1.3)	3.400 (1H) d (1.3)
10	3.184 (1H) dq (1.3, 5.3)	3.191 (1H) dq (1.3, 5.2)
11	1.457 (3H) d (5.3)	1.462 (3H) d (5.2)
12	1.669 (3H) d (6.2)	1.675 (3H) d (6.2)
OMe	3.582 (3H) s	3.545 (3H) s
Ph	7.540−7.438 (5H)	7.541−7.432 (5H)

[a]The chemical shifts are in δ values (ppm) from TMS.

from that of 3-*O*-*S*-MTPA (**6**) esters, the $\Delta\delta$ (**6** - **7**) values for all of the protons were determined as reported in Figure 1. The positive $\Delta\delta$ values were located on the right side, and those with negative values on the left side of model A as reported in Othani et al. (1989).[22] This model allowed the assignment of the *S* configuration at C-3. Consequently, the *S* configuration was assigned to C-2. **1** was therefore formulated as (2*S*,3*S*)-3-hydroxy-2-methyl-7-(3-methyloxiranyl)-2,3-dihydropyrano[4,3-*b*]pyran-4,5-dione.

Cochliotoxin is closely related to the two epimeric radicinins (**2** and **3**) and the two epimeric radicinols (**4** and **5**). These metabolites are produced by different fungi such as *Alternaria longipes*,[23] *Bipolaris coicis*,[11] *Alternaria chrysanthemi*,[14] *Alternaria radicina*,[24] *Curvularia* sp. FH01,[25] and *Cochliobolus* sp.[12] A compound containing a 1,2-epoxypropyl moiety instead of the 1-propenyl moiety of 3-*epi*-radicinol has been previously reported,[11,26] but this is the first report of an epoxide derived from radicinin. α- and γ-Pyrones as well as epoxides are common naturally occurring compounds.[27,28]

Compounds **1**−**5** were bioassayed in a buffelgrass coleoptile elongation test and by leaf puncture bioassay against the host weed and two nontarget grasses, as reported in the Experimental Section.

In the coleoptile elongation bioassay on buffelgrass, cochliotoxin (**1**) was significantly more phytotoxic than the other four compounds, with decreased and delayed germination and severe effects on early seedling growth (Figure 2). Most seeds treated with **1** produced coleoptiles but no radicles, which resulted in a very short mean radicle length and effective seedling death. Radicinin and 3-*epi*-radicinin (**2** and **3**) were also strongly phytotoxic to seedlings, with large impacts to the radicle, whereas radicinol and 3-*epi*-radicinol (**4** and **5**) had little or no negative effect relative to the control.

In the leaf puncture bioassay on buffelgrass at the higher concentration (5×10^{-3} M), cochliotoxin and 3-*epi*-radicinin (**1** and **3**) were strongly and equally phytotoxic (Figure 3). Radicinin (**2**) was also strongly phytotoxic, but significantly less so than the other two compounds. Radicinol and 3-*epi*-radicinol (**4** and **5**) showed much lower phytotoxicity, although both were significantly different from the control. On tanglehead, all three highly phytotoxic compounds (**1**−**3**) were significantly less phytotoxic than on buffelgrass, indicating that tanglehead is generally less sensitive to these compounds. On Arizona cottontop, both radicinin and 3-*epi*-radicinin (**2** and **3**) were significantly more phytotoxic than on buffelgrass and tanglehead. The effect of cochliotoxin on Arizona cottontop at high concentration was not determined. Radicinol and 3-*epi*-radicinol (**4** and **5**) were completely inactive and not

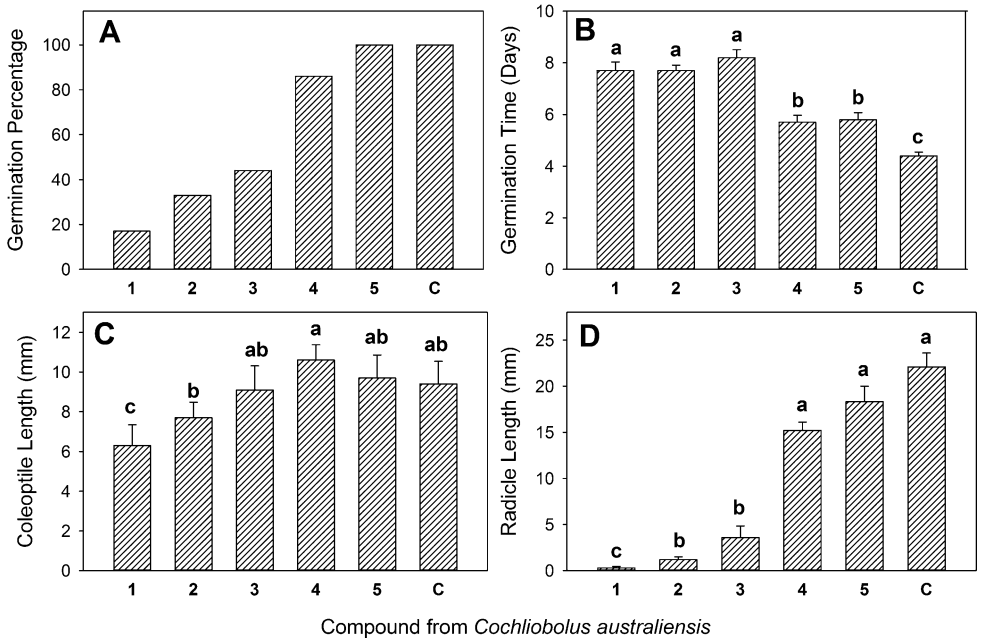


Figure 2. Effect of compounds **1**−**5** tested at 5×10^{-3} M on buffelgrass (*Cenchrus ciliaris*) in a seedling coleoptile elongation test: (A) seed germination percentage after 10 days at 25 °C, (B) mean germination time at 25 °C, (C) mean coleoptile length 3 days after germination, and (D) mean radicle length 3 days after germination. Error bars represent standard error of the mean. For each response variable, columns headed by the same letter are not significantly different at $P < 0.05$ based on mean separation from analysis of variance on log-transformed data. Compounds are cochliotoxin (**1**), radicinin (**2**), 3-*epi*-radicinin (**3**), radicinol (**4**), 3-*epi*-radicinol (**5**), and control (C).

DOI: 10.1021/acs.jnatprod.6b00696
J. Nat. Prod. 2017, 80, 1241−1247

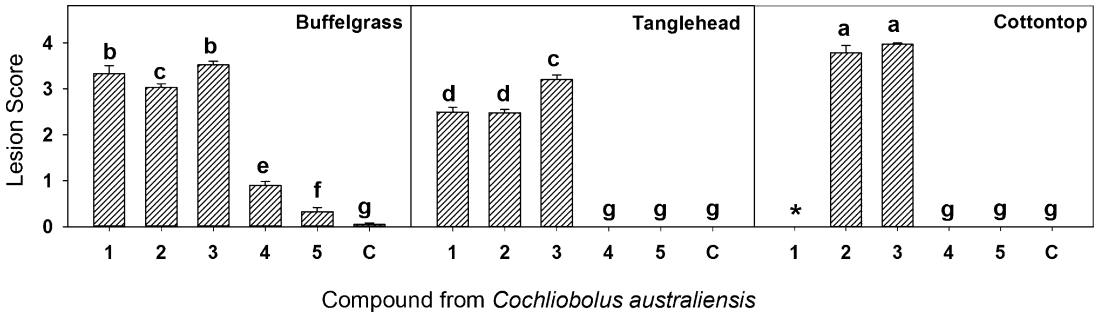


Figure 3. Effect of compounds **1**–**5** tested at 5×10^{-3} M in leaf puncture bioassays on buffelgrass (*Cenchrus ciliaris*) and two nontarget native grasses, tanglehead (*Heteropogon contortus*) and Arizona cottontop (*Digitaria californica*). Lesion severity was scored on an approximately linear scale of 0–4 (n = 40). Error bars represent standard error of the mean. C represents the solvent-only negative control. Positive controls (not shown) were consistent across all tests. Bars headed by the same letter across all three test species are not significantly different at $P < 0.05$ based on mean separation from analysis of variance. The asterisk denotes missing data. Compounds are cochliotoxin (**1**), radicinin (**2**), 3-*epi*-radicinin (**3**), radicinol (**4**), 3-*epi*-radicinol (**5**), and control (C).

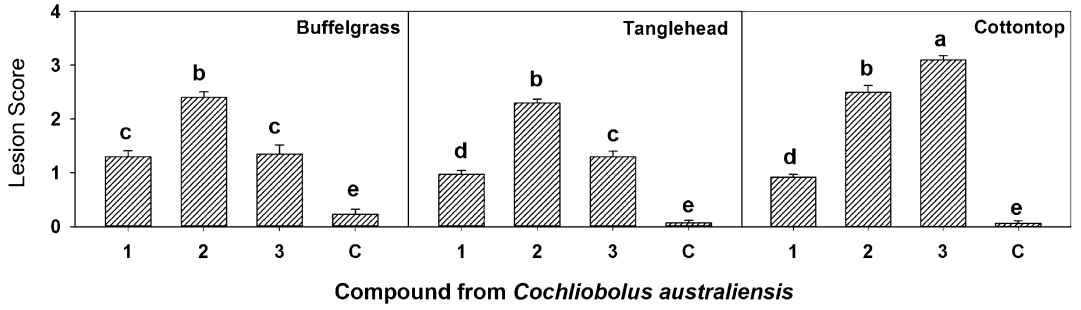


Figure 4. Effect of compounds **1**–**3** tested at 2.5×10^{-3} M in leaf puncture bioassays on buffelgrass (*Cenchrus ciliaris*) and two nontarget native grasses, tanglehead (*Heteropogon contortus*) and Arizona cottontop (*Digitaria californica*). Lesion severity was scored on an approximately linear scale of 0–4 (n = 40). Error bars represent standard error of the mean. C represents the solvent-only negative control. Bars headed by the same letter across all three test species are not significantly different at $P < 0.05$ based on mean separation from analysis of variance. Compounds are cochliotoxin (**1**), radicinin (**2**), 3-*epi*-radicinin (**3**), and control (C).

significantly different from the control on both tanglehead and Arizona cottontop.

Only the three most phytotoxic compounds were tested at the lower concentration (2.5×10^{-3} M). In this test, cochliotoxin was less toxic than either radicinin or 3-*epi*-radicinin on all three grass species (Figure 4). Radicinin was similarly toxic on all three species, while 3-*epi*-radicinin was significantly more toxic on Arizona cottontop, as in the test at higher concentration. Cochliotoxin at this lower concentration was significantly less toxic on both native grasses than on buffelgrass, but the biological significance of this small difference is unclear.

Among the three strong phytotoxins tested here, only **1** exhibited any selective phytotoxicity against buffelgrass relative to native grasses. This may make it a possible candidate as a natural herbicide against buffelgrass, but further evaluation at a range of concentrations and across a wider array of nontarget native species is needed.

Radicinin is well known as a phytotoxin,[11,24,29] but it has also shown strong antifungal activity against *Magnaporthe grisea* (IC_{50} = 16.3 μg/mL) and *Valsa mali* (IC_{50} = 18.2 μg/mL),[25] as well as strong antibacterial activity against *Xylella fastidiosa*.[12] The evidence for toxicity of radicinol and its derivatives is mixed. Nakajima et al. (1997)[11] reported that both radicinol and its epoxide from *Cochliobolus* on the grass Job's tears (*Coix lachrya-jobi*) were nontoxic in leaf puncture bioassays on the host, whereas Solfrizzo et al. (2004)[24] reported that *epi*-radicinol was toxic to carrot seedlings.

These results allow us to consider structure–activity relationships between the new cochliotoxin (**1**) and its analogues (**2**–**5**). The presence at C-4 of the α,β-unsaturated ketone in **1**, **2**, and **3** seems to play a central role in the strong phytotoxic activities of these compounds. In fact, the absence of this moiety in **4** and **5** causes a noticeable activity reduction at 5×10^{-3} M on buffelgrass and the complete inactivity in the leaf puncture assay at 2.5×10^{-3} M on the native grasses. Furthermore, the stereochemistry of the chiral C-3 in **1**, **2**, and **3**, as well as the presence of the epoxy group in **1**, seems also to be important features involved in modulating activity of these compounds.

Other α- and γ-pyrones are also recognized as fungal metabolites with phytotoxic activity against weeds of both agriculture and forestry.[10,30–33] They may also show antifungal activity[34–36] and even anticancer activity.[37,38] In general the presence of an α,β-unsaturated ketone group is already recognized as a factor important for the activity due to the Michael addition of a nucleophilic residue.[39,40] The importance of the epoxy groups for the biological activity of some natural products is also well demonstrated.[41–43] These findings suggest that there may be additional applications for cochliotoxin beyond its use as a natural herbicide for buffelgrass control.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a Jasco P-1010 digital polarimeter; IR spectra were recorded as glassy film on a Thermo Nicolet 5700 FT-IR

DOI: 10.1021/acs.jnatprod.6b00696
J. Nat. Prod. 2017, 80, 1241–1247

spectrometer; UV spectra were recorded in MeCN solution on a Jasco V-530 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 500/125 MHz on a Varian or at 400/100 MHz on a Bruker spectrometer. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra;[15] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[15] were performed using Bruker or Varian microprograms. HRESI and ESI mass spectra were recorded on Thermo LTQ Velos and Agilent Technologies 1100 LC/MS TOF instruments. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 mm) and on reversed-phase (Merck, Kieselgel 60 RP-18, F_{254}, 0.20 mm) plates; the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063−0.200 mm).

Fungal Strain. The *Cochliobolus* strain used in this study (LJ-4B) was obtained from leaf spot lesions on a buffelgrass leaf tissue collection made near La Joya, Hidalgo County, in south Texas, USA, in September 2014. It was identified by Sanger sequencing of the ITS region, which is the "barcode" region for routine fungal identification.[44] It belongs to a group of closely related species that includes *C. australiensis*, to which it is provisionally assigned here. Its ITS sequence was identical to that of GenBank Accession JX960591, documented as an unidentified species of *Cochliobolus* isolated from grain sorghum (*Sorghum bicolor*) tissue collected in Corpus Christi, south Texas, USA.

Production, Extraction, and Purification of *Cochliobolus australiensis* Secondary Metabolites. The strain of *Cochliobolus* was grown in PDB (potato dextrose broth) culture at room temperature (22 °C) by inoculating 500 mL of sterile broth in 1 L Erlenmeyer flasks with fragments of mycelial mat produced on PDA (potato dextrose agar) and incubating in shaker culture for 14 days. The mycelium was then removed from the medium by centrifugation and filtering, and the resulting filtrates were lyophilized and frozen at −20 °C until extraction and analysis. The lyophilized culture filtrate (1 L) was dissolved in distilled H_2O (1/10 of its original volume), acidified to pH 2 with formic acid, and then extracted with EtOAc (3 × 100 mL). The organic extracts were combined and dried on Na_2SO_4, and the solvent was evaporated under reduced pressure, yielding a colored solid (449.8 mg). The organic extract was crystallized from MeOH, yielding radicinin (**2**, 90.3 mg) as white needles. The mother liquors (359.5 mg) were fractionated by column chromatography on silica gel, eluted with $CHCl_3$−*i*-PrOH (93:7). Ten homogeneous fraction groups were collected. The residue (55.1 mg) of the second fraction group was further purified by preparative TLC on silica gel, eluent $CHCl_3$−*i*-PrOH (97:3), yielding a further amount of radicinin as a white solid (**2**, R_f 0.68, 20.1 mg, for a total of 110.4 mg) and cochliotoxin (**1**, R_f 0.64, 7.3 mg) as an amorphous solid. The residues of the third and fourth fractions were combined (for a total of 100.4 mg) and fractionated by column chromatography on silica gel, eluted with CH_2Cl_2−MeOH (96:4), yielding six homogeneous fractions. The residue of the fourth fraction of this column (52.0 mg) was further purified by TLC eluted with CH_2Cl_2−MeOH (96:4), yielding 3-*epi*-radicinin (**3**, R_f 0.33, 10.5 mg) and radicinol (**4**, R_f 0.23, 5.5 mg) as amorphous solids.

The residue (44.2 mg) of the fifth fraction of the first column was purified by two further steps of TLC on silica gel, eluent CH_2Cl_2−MeOH (9:1), and reverse phase, eluent (EtOH−H_2O), yielding 3-*epi*-radicinol (**5**, R_f 0.52, 14.5 mg) as a homogeneous amorphous solid.

*Cochliotoxin (**1**):* $[\alpha]^{25}_D$ −104.0 (*c* 0.2 $CHCl_3$); IR ν_{max} 3463, 1763, 1724, 1637, 1538, 1410, 1268 cm^{-1}; UV λ_{max} nm (log ε) 317 (4.12), 225 (4.08); ^{1}H and ^{13}C NMR spectra, see Table 1; HRESIMS (+) *m*/*z* 291.0276 $[M + K]^+$ (calcd for $C_{12}H_{12}KO_6$ 291.0271), 275.0537 $[M + Na]^+$ (calcd for $C_{12}H_{12}NaO_6$ 275.0532), 253.0715 $[M + H]^+$ (calcd for $C_{12}H_{13}O_6$ 253.0712).

*Radicinin (**2**):* $[\alpha]^{25}_D$ −125.1 (*c* 0.2 $CHCl_3$) [lit.:[12] $[\alpha]^{23}_D$ −11 (*c* 0.317 $CHCl_3$); lit.[11] $[\alpha]^{20}_D$ −236 (*c* 0.1 $CHCl_3$)]; ^{1}H and ^{13}C NMR spectra were very similar to those reported in the literature;[12] HRESIMS (+) *m*/*z* 237.0754 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$ 237.0757).

*3-epi-Radicinin (**3**):* $[\alpha]^{25}_D$ −65.0 (*c* 0.2 $CHCl_3$) [lit.[11] $[\alpha]^{20}_D$ −105 (*c* 0.25 EtOH)]; ^{1}H and ^{13}C NMR spectra were very similar to those reported in the literature;[11] HRESIMS (+) *m*/*z* 237.0760 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$ 237.0757).

*Radicinol (**4**):* $[\alpha]^{25}_D$ −148.2 (*c* 0.2 $CHCl_3$) [lit.[14] $[\alpha]_D$ −168 (*c* 1.0 $CHCl_3$)]; ^{1}H and ^{13}C NMR spectra were very similar to those reported in the literature;[45] HRESIMS (+) *m*/*z* 239.0918 $[M + H]^+$ (calcd for $C_{12}H_{15}O_5$ 239.0920).

*3-epi-Radicinol (**5**):* $[\alpha]^{25}_D$ −100.1 (*c* 0.2 $CHCl_3$) [lit.[23] $[\alpha]_D$ −84 (*c* 1.0 $CHCl_3$); lit.[13] $[\alpha]_D$ −92 (*c* 0.48 $CHCl_3$)]; ^{1}H and ^{13}C NMR spectra were very similar to those reported in the literature;[14,23] HRESIMS (+) *m*/*z* 239.0924 $[M + H]^+$ (calcd for $C_{12}H_{15}O_5$ 239.0920).

*3-O-(S)-α-Methoxy-α-trifluoromethyl-α-phenylacetate Ester of Cochliotoxin (**6**).* (*R*)-(−)-MPTA-Cl (10 μL) was added to **1** (1.0 mg) dissolved in dry pyridine (20 μL). The mixture was kept at room temperature for 1 h, and then the reaction was stopped by adding MeOH. Pyridine was removed under a stream of nitrogen. The residue (1.5 mg) was purified by preparative TLC, eluted with $CHCl_3$−*i*-PrOH (98:2), yielding **6** as a homogeneous oil (R_f 0.41, 1.1 mg): IR ν_{max} 1774, 1727, 1629, 1542, 1446, 1271 cm^{-1}; UV λ_{max} nm (log ε) 319 (4.22), 225 (4.03); ^{1}H NMR spectrum, see Table 2; HRESIMS (+) *m*/*z* 469.3841 $[M + H]^+$ (calcd for $C_{22}H_{20}F_3O_8$ 469.3846).

*3-O-(R)-α-Methoxy-α-trifluoromethyl-α-phenylacetate Ester of Cochliotoxin (**7**).* (*S*)-(+)-MPTA-Cl (10 μL) was added to **1** (1.0 mg) dissolved in dry pyridine (20 μL). The reaction was carried out under the same conditions used for preparing **6**. The purification of the crude residue (1.4 mg) by preparative TLC eluted with $CHCl_3$−*i*-PrOH (98:2) gave **7** as homogeneous oil (R_f 0.41, 1.2 mg): IR ν_{max} 1768, 1718, 1636, 1543, 1445, 1271 cm^{-1}; UV λ_{max} nm (log ε) 318 (4.20), 226 (4.05); ^{1}H NMR spectrum, see Table 2; HRESIMS (+) *m*/*z* 469.3839 $[M + H]^+$ (calcd for $C_{22}H_{20}F_3O_8$ 469.3846).

Biological Assays. *Coleoptile Elongation Bioassay.* Seeds of buffelgrass (*Cenchrus ciliaris*) were used for this assay. Each compound (**1**−**5**) was first dissolved in DMSO and then brought to a concentration of 5 × 10^{-3} M with distilled water (final DMSO concentration 2%). For each compound, 250 μL of the solution was pipetted into each of three 3.5 cm Petri dishes onto the surface of one filter paper. Seeds were incubated in 2% DMSO in the control treatment. Six buffelgrass seeds were arranged onto the surface of each filter paper in a pattern that made it possible to track individual seeds. Petri dishes were sealed with parafilm to retard moisture loss and incubated at 25 °C with a 12:12 h photoperiod. Germination, defined as radicle emergence to >1 mm, was scored each day for 10 days, and germination day was tracked individually for each seed. Three days after germination, the coleoptile and radicle length for each seedling was recorded using electronic calipers. Seeds that produced a coleoptile but no radicle were scored as ungerminated but with a measured coleoptile length and a radicle length of zero, whereas seeds that produced neither a radicle nor a coleoptile were scored as ungerminated. Differential effects of the compounds on germination time and coleoptile and radicle length were evaluated using mean separations from analysis of variance on log-transformed data. Germination percentage was not replicated and was therefore not subjected to statistical analysis. This experiment was not performed with the nontarget grasses species because of problems with seed dormancy.

Leaf Puncture Bioassay. Leaves of buffelgrass (*C. ciliaris*) and two nontarget native grasses, tanglehead (*Heteropogon contortus*) and Arizona cottontop (*Digitaria californica*), were used for this assay. Compounds **1**−**5** were assayed at 5 × 10^{-3} M, and compounds **1**−**3** were also assayed at 2.5 × 10^{-3} M. Compounds were first dissolved in MeOH (final concentration 4%), and then a stock solution with sterile distilled water was made for each compound. For each species × compound combination, four replicates of five 3 cm leaf sections each were placed in a Petri dish on the surface of a water-saturated filter paper, along with leaf sections for negative controls (4% MeOH only). A droplet (10 μL) of test solution was applied on the axial side of the

DOI: 10.1021/acs.jnatprod.6b00696
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leaf over each needle puncture. Each leaf section was needle-punctured twice for a total of 40 leaf punctures for each species × compound combination. Data are missing for cochliotoxin on Arizona cottontop at high concentration due to insufficient quantities of **1** to realize all the bioassays. The dishes were sealed with parafilm and incubated at 20 °C for 3 days under 24 h of fluorescent light. Leaves were observed daily and scored for symptoms after 3 days. Lesions were scored using an approximately linear semiquantitative scale: 0, no symptoms; 1, small slight necrosis; 2, slight necrosis; 3, large necrotic areas; 4, extensive necrotic areas. Leaf puncture bioassay data at each concentration were examined statistically using analysis of variance with lesion score as the interval response variable and compound and grass species as independent variables. Mean separations were obtained from this analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00696.

Spectra of **1** (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by grants from the U.S. Forest Service, State and Private Forestry, BCIP (Biological Control of Invasive Plants) Program, and through supplemental funding from this same agency. We thank R. Reardon and A. White of the U.S. Forest Service and B. Geary of Brigham Young University for logistical support. We also gratefully acknowledge F. Freda of BBCA Onlus, who helped with the leaf puncture assay. Prof. A. Evidente is associated to Istituto di Chimica Biomolecolare, CNR, Pozzuoli, Italy.

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