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

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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-dihydropyran[4,3-b]pyran-4,5-dione. Its relative stereochemistry was assigned by 1H NMR techniques, while the absolute configuration (2S,3S) 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 non-target 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 non-target grass tanglehead (Heteropogon contortus), while the non-target 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.

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RESULTS AND DISCUSSION

The organic extract of C. australis liquid culture filtrates was fractionated by a combination of column and preparative TLC chromatography as detailed in the Experimental Section. A new phytophagic 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-radicinol11,14 (2–5) by comparing their physical (OR) and spectroscopic properties (1H NMR and ESIMS) with the data reported in the literature.

The new metabolite showed a molecular formula of C12H12O6 as deduced from its HRESIMS, consistent with its UV chromophores.18 The 13C NMR spectrum showed the presence of 12 carbons, six of which appeared to be quaternary sp2; among these, those resonating at δ 188.6 (C-4) and 156.3 (C-5) are typical of an α,β-unsaturated ketone and ester, respectively.19 Thus, considering the total of seven unsaturations, the presence of three rings could be hypothesized. The couplings observed in the HSQC spectrum15 allowed assignment of the signals resonating at δ 97.4, 80.4, 72.0, 58.4, 54.9, 18.0, and 17.3 to the protonated carbons and in particular to C-8, C-2, C-3, C-10, C-9, Me-12, and Me-11. The long-range couplings observed in the HMBC spectrum allowed assignment of the chemical shifts of the other three olefinic carbons, including the bridgehead carbons (C-4a and C-8a) of the junction between two rings, one of which appeared to be oxygenated. In particular, the couplings observed between the carbon at δ 176.2 with H-8, the carbon at δ 168.1 with H-8 and H-9, and that at δ 98.2 with H-8 allowed assignment of these signals at C-8a, C-7, and C-4a, respectively. The same couplings of C-9 with H-8 also allowed location of the 2-methyloxiranyl residue at C-7. These couplings together with those observed between C-4 and H-2 and H-3 allowed identification of the two rings as an α-pyrone and a dihydro-γ-pyryne, with the latter bearing a hydroxy and a methyl group in α and β positions with respect to the carbonyl. Thus, these results allowed us to formulate cochliotoxin as 3-hydroxy-2-methyl-7-(3-methyloxiranyl)-2,3-dihydropyrano[4,3-β]pyran-4,5-dione.

This structure was confirmed from all the other couplings observed in the HMBC spectrum (Table 1) and from the data of its HRESIMS. Indeed, the latter spectrum recorded in CDCl3 showed that it is closely related to radicinin (1), was determined by applying a modified Mosher’s method.22 Cochliotoxin was treated with R(-)-α-methoxy-α-trifluoromethylphenacyl (MTPA) and S(+)-MTPA chlorides, to convert 1 into the corresponding diastereomeric esters at C-3 (6 and 7). The spectroscopic data for the S-MPTA and R-MTPA esters (6 and 7, respectively) were

<table>
<thead>
<tr>
<th>position</th>
<th>δC (ppm)</th>
<th>δH (in Hz)</th>
<th>HMBC</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>80.4 CH</td>
<td>4.37, dq (12.4, 6.2)</td>
<td>H-3, Me-12</td>
</tr>
<tr>
<td>3</td>
<td>72.0 CH</td>
<td>4.01, d (12.4)</td>
<td>H-2, Me-12</td>
</tr>
<tr>
<td>4</td>
<td>188.6 C</td>
<td></td>
<td>H-2, H-3</td>
</tr>
<tr>
<td>4a</td>
<td>98.2 C</td>
<td></td>
<td>H-8</td>
</tr>
<tr>
<td>5</td>
<td>156.3 C</td>
<td></td>
<td>H-8, H-9</td>
</tr>
<tr>
<td>7</td>
<td>168.1 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>97.4 CH</td>
<td>6.07, s</td>
<td>H-9</td>
</tr>
<tr>
<td>8a</td>
<td>176.2 C</td>
<td></td>
<td>H-8</td>
</tr>
<tr>
<td>9</td>
<td>54.9 CH</td>
<td>3.38, d (1.3)</td>
<td>H-8, Me-11</td>
</tr>
<tr>
<td>10</td>
<td>58.4 CH</td>
<td>3.17, dq (5.3, 1.3)</td>
<td>H-9, Me-11</td>
</tr>
<tr>
<td>11</td>
<td>173.3 CH3</td>
<td>1.45, d (5.3)</td>
<td>H-10</td>
</tr>
<tr>
<td>12</td>
<td>18.0 CH3</td>
<td>1.66, d (6.2)</td>
<td>H-3</td>
</tr>
<tr>
<td>OH</td>
<td></td>
<td>3.80, brs</td>
<td></td>
</tr>
</tbody>
</table>

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

In fact, the 1H 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 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 carboxyl groups.17 These results were consistent with the absorption maxima observed in the UV spectrum and due to extended conjugated chromophores.18

The 13C NMR spectrum showed the presence of 12 carbons, five of which appeared to be quaternary sp2; among these, those resonating at δ 188.6 (C-4) and 156.3 (C-5) are typical of an α,β-unsaturated ketone and ester, respectively.19 Thus, considering the total of seven unsaturations, the presence of three rings could be hypothesized. The couplings observed in the HSQC spectrum15 allowed assignment of the signals resonating at δ 97.4, 80.4, 72.0, 58.4, 54.9, 18.0, and 17.3 to the protonated carbons and in particular to C-8, C-2, C-3, C-10, C-9, Me-12, and Me-11. The long-range couplings observed in the HMBC spectrum allowed assignment of the chemical shifts of the other three olefinic carbons, including the bridgehead carbons (C-4a and C-8a) of the junction between two rings, one of which appeared to be oxygenated. In particular, the couplings observed between the carbon at δ 176.2 with H-8, the carbon at δ 168.1 with H-8 and H-9, and that at δ 98.2 with H-8 allowed assignment of these signals at C-8a, C-7, and C-4a, respectively. The same couplings of C-9 with H-8 also allowed location of the 2-methyloxiranyl residue at C-7. These couplings together with those observed between C-4 and H-2 and H-3 allowed identification of the two rings as an α-pyrone and a dihydro-γ-pyryne, with the latter bearing a hydroxy and a methyl group in α and β positions with respect to the carbonyl. Thus, these results allowed us to formulate cochliotoxin as 3-hydroxy-2-methyl-7-(3-methyloxiranyl)-2,3-dihydropyranopyran[4,3-β]pyran-4,5-dione.

This structure was confirmed from all the other couplings observed in the HMBC spectrum (Table 1) and from the data of its HRESIMS. Indeed, the latter spectrum recorded in CDCl3 showed that it is closely related to radicinin (1), was determined by applying a modified Mosher’s method.22 Cochliotoxin was treated with R(-)-α-methoxy-α-trifluoromethylphenacyl (MTPA) and S(+)-MTPA chlorides, to convert 1 into the corresponding diastereomeric esters at C-3 (6 and 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 Δδ value obtained by comparison (6 and 7) of each proton system.

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consistent with the structure assigned to 1. Subtracting the chemical shift of the protons (Table 2) of the 3-O-R-MTPA (7) 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). 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 (2S,3S)-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, Bipolaris coicis, Alternaria chrysanthemi, Alternaria radicina, Curvularia sp. FH01, and Cochliobolus sp. A compound containing a 1,2-epoxypropyl moiety instead of the 1-propenyl moiety of 3-epi-radicinol has been previously reported, but this is the first report of an epoxide derived from radicinin. $\alpha$- and $\gamma$-Pyrones as well as epoxides are common naturally occurring compounds.

Table 2. $^1$H NMR Data of Cochliotoxin Derivatives (6 and 7) Recorded in CDCl$_3$.

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_H$ (ppm)</th>
<th>$\Delta \delta$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
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</tbody>
</table>

The chemical shifts are in $\delta$ values (ppm) from TMS.

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 radicle length and effective seedling death. Radicinin and 3-epi-radicin (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 $\times$ 10$^{-5}$ M), cochliotoxin and 3-epi-radicin (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. Radicinin 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-radicin (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 $\times$ 10$^{-5}$ 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-radicin (3), radicinol (4), 3-epi-radicinol (5), and control (C).
significant differences 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 radicinol or 3-epi-radicinol on all three grass species (Figure 4). Radicinin was similarly toxic on all three species, while 3-epi-radicinol 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, 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), as well as strong antibacterial activity against Xylella fastidiosa. The presence at C-4 of the epoxy group for the biological activity of some natural products is also well demonstrated. In general the presence of an α,β-unsaturated ketone, γ, and even anticancer activity. 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. The importance of the epoxy groups for the biological activity of some natural products is also well demonstrated. These findings suggest that there may be additional applications for cochliotoxin beyond its use as a natural herbicide for buffelgrass control.

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. They may also show antifungal activity and even anticancer activity. 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. The importance of the epoxy groups for the biological activity of some natural products is also well demonstrated. 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.

**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).
spectrometer; UV spectra were recorded in MeCN solution on a Jasco V-530 spectrophotometer. 

4 It belongs to a group of closely related species that includes

region, which is the

September 2014. It was identi-

fied by preparative TLC were very similar to those reported in the literature; HRESIMS (+) m/z 237.0760 [M + H]+ (calc for C12H12O3 237.0754). 

3-epi-Radixinol (3): [α]25 D = −65.0 ([0.2 CHCl3] [lit.15 [α]25 D = −105 ([0.25 EtOH]); H and 13C NMR spectra were very similar to those reported in the literature; HRESIMS (+) m/z 237.0754 [M + H]+ (calc for C12H12O3 237.0757).

Radixinol (4): [α]25 D = −148.2 ([0.2 CHCl3] [lit.15 [α]25 D = −168 ([1.0 CHCl3]); H and 13C NMR spectra were very similar to those reported in the literature; HRESIMS (+) m/z 239.0918 [M + H]+ (calc for C12H12O3 239.0920).

3-epi-Radixinol (5): [α]25 D = −100.1 ([0.2 CHCl3] [lit.15 [α]25 D = −84 ([1.0 CHCl3]); H and 13C NMR spectra were very similar to those reported in the literature; HRESIMS (+) m/z 469.3841 [M + H]+ (calc for C22H20F3O8 469.3846).

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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 X 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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The authors declare no competing financial interest.

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