**Fungicidal Activities of Dihydroferulic Acid Alkyl Ester Analogues**

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The natural product dihydroferulic acid (DFA, 1) and the synthesized DFA methyl (4a), ethyl (4b), propyl (4c), hexyl (4d), octyl (4e), and decyl (4f) esters were examined for antifungal activity. Test fungi included *Saccharomyces cerevisiae* (wild type, and deletion mutants slt2Δ and bek1Δ), *Aspergillus fumigatus*, and *A. flavus*. Growth inhibition of *S. cerevisiae* treated with 5 mM DFA or the corresponding esters was 4a, 4b, and 4c > 98%; 4d 18.8%; 1 6.4%; 4e 6.2%; and 4f 2.8%, relative to the control. The 50% minimum inhibitory concentrations for the more active propyl, methyl, and ethyl esters were 1.5, 2.1, and 4.0 mM, respectively. Compound 4c inhibited 100% growth of both aspergilli at 6.4 mM.

**Results and Discussion**

The phenolic DFA (1, in Figure 1) is a metabolite of several plants1-3 and has been purported to possess antioxidant/radical-scavenging properties.4-5 It has not yet been reported as an antimicrobial, although its biosynthetic precursor ferulic acid (2, in Scheme 1) has been recognized as an antibacterial.6,7 Compound 1 has been reported to be a metabolite of human gut microflora as well as a precursor of vanillic acid.8 Of the ester analogues, only methyl dihydroferulate has been isolated as a plant secondary metabolite and was reported as weakly phytotoxic.9 Bioassay-guided fractionation of the plant material *Gypsophila paniculata* revealed isolated DFA to possess weak inhibitory activity against the fungi *Aspergillus niger* and *Candida kefyr*, in addition to the bacteria *Bacillus subtilis* and *Staphylococcus aureus*.10 This observation, combined with the recent report11 that the structurally similar ketone derivative vanillylacetone (3, in Figure 1) possessed activity against the fungi *Saccharomyces cerevisiae* and *A. flavus*, inspired our investigation of ester analogues of DFA for their antifungal capacities. Control of *A. flavus* is a central goal of several agricultural industries due to its ability to produce the mutagenic and toxic metabolite aflatoxin.12 Given that conventional fungicides are reputed to be hazardous to human health and the environment,13,14 our lab is focusing on development of medically and environmentally benign natural product analogues to aid in the control of targeted fungi, particularly *A. flavus*.

**Scheme 1. Synthesis of DFA (1) and Targeted Esters (4a–f)**

![Scheme 1](image)

confirmed with a 2-fold dilution (0.1 to 6.4 mM) of the compounds, using 1 as the control. The inhibitions of the short-chain esters are provided as the minimum inhibitory concentrations at 50% (MIC50) and 90% (MIC90) of growth of *S. cerevisiae* and are shown in Table 1. The slight difference of inhibition between treatments with short-chain esters became pronounced at lower concentrations, with the propyl ester (4c) exhibiting the highest antifungal activity. Interestingly, the MIC50 value for the ethyl ester (4b) required a considerably higher concentration than its methyl (4a) and propyl (4c) counterparts. The observed activities of these shorter chain esters of DFA are congruent with the bioactivities of the corresponding esters of *p*-hydroxybenzoic acid (parabens), which are commonly used as preservatives in cosmetic, food, and pharmaceutical products.17 No definitive explanations are available for the inhibition trend between the propyl, methyl, and ethyl esters at this time. However, if analogies can be drawn from the similarities between the DFA ester bioactivities to those of the paraben bioactivities, hydrolysis of the ester to corresponding acid and alcohol may be excluded since hydrolysis of parabens results in loss of antimicrobial activity,18 as well as a decrease in preservative efficacy.19 Exploration of a possible mode of action for bioactivity for the DFA esters will be continued.

The chain length at which there was significant decrease in antifungal efficacy, between the propyl (4c) and hexyl (4d) esters, was also examined in greater detail. To confirm this activity cutoff point, an additional bioassay was performed with serial 2-fold dilutions (0.1 to 6.4 mM) of 4c and 4d. The differences observed in antifungal activity are reported in Table 2 as the number of cells for varying concentrations and growth inhibition. The values illustrate substantial differences between the propyl and hexyl DFA esters at the lower concentrations (0.8 to 3.2 mM), before shifting
Table 1. Determination of MIC<sub>50</sub> and MIC<sub>90</sub> of DFA (1) and the Effective Ester Analogues (4a–e) against Saccharomyces cerevisiae Wild Type

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>% Growth inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Growth inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Difference in growth inhibition (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>4.44 (0.10)</td>
<td>0.0</td>
<td>4.44 (0.10)</td>
</tr>
<tr>
<td>0.8</td>
<td>2.99 (0.00)</td>
<td>32.7</td>
<td>3.61 (0.24)</td>
</tr>
<tr>
<td>1.6</td>
<td>1.89 (0.18)</td>
<td>57.4</td>
<td>2.74 (0.20)</td>
</tr>
<tr>
<td>3.2</td>
<td>0.03 (0.03)</td>
<td>99.3</td>
<td>1.00 (0.49)</td>
</tr>
<tr>
<td>6.4</td>
<td>0.00 (0.00)</td>
<td>100.0</td>
<td>0.28 (0.08)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell number: × 10<sup>3</sup> cells/mL, determined at OD 600 nm. Number in parentheses: std. dev. Percent growth inhibition compared to the “no treatment” control. Difference in growth inhibition between DFA propyl ester and hexyl ester.

Table 2. Comparative Cell Growth Inhibition of Saccharomyces cerevisiae Wild Type versus Analogue Chain-Length Cutoff Point for DFA Propyl and Hexyl Ester Analogues (4c and 4d)

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>Cell number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Growth inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cell number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Growth inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Difference in growth inhibition (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>4.44 (0.10)</td>
<td>0.0</td>
<td>4.44 (0.10)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>2.99 (0.00)</td>
<td>32.7</td>
<td>3.61 (0.24)</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>1.89 (0.18)</td>
<td>57.4</td>
<td>2.74 (0.20)</td>
<td>38.3</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.03 (0.03)</td>
<td>99.3</td>
<td>1.00 (0.49)</td>
<td>77.5</td>
<td></td>
</tr>
<tr>
<td>6.4</td>
<td>0.00 (0.00)</td>
<td>100.0</td>
<td>0.28 (0.08)</td>
<td>93.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>b</sup>Cell number: × 10<sup>3</sup> cells/mL, determined at OD 600 nm. Number in parentheses: std. dev. Percent growth inhibition compared to the “no treatment” control. Difference in growth inhibition between DFA propyl ester and hexyl ester.

to similar values at the higher concentration of 6.4 mM. Given the results that the more polar DFA (1) is relatively inactive versus the nonpolar ester analogues 4a–c, the tentative postulation could be made that the receptor site for bioactivity may prefer a hydrophobic compound, but has limited amount of space available for the ester chain length.

With the bioactivity of the propyl ester analogue established, focus was turned to the probing of a possible mode of action using gene deletion mutants of S. cerevisiae. The genome of S. cerevisiae has been fully sequenced and well annotated, which allows for focus to be turned to the probing of a possible mode of action using gene deletion mutants of S. cerevisiae. The genome of S. cerevisiae has been fully sequenced and well annotated, which allows for use of gene deletion mutants to study functional genomic responses to synthetic and/or natural antifungals (chemogenomics). In S. cerevisiae the SLT2 and BCK1 genes are involved in the signal transduction pathway for cell wall construction/integrity. Experimental values listed in Table 3 demonstrate that SLT2, which lacks the mitogen-activated protein kinase (MAPK) gene, and BCK1, lacking the MAPKK kinase gene in cell wall construction, were sensitive to 4c at concentrations of 1.6 and 3.2 mM when compared to the wild-type strain, indicating that the phenolic ester 4c may target these genes.

Finally, and more importantly for application to agricultural issues, the results using the model yeast bioassay to screen for antifungals were used to take the lead compound, DFA propyl ester (4c), forward for testing against target fungi A. fumigatus, the causative agent of invasive aspergillosis in humans, and A. flavus. The results provided in Table 4 demonstrate the efficacy of 4c against the pathogenic Aspergillus. The MIC<sub>50</sub> value of the propyl ester was 2.1 mM against A. fumigatus and 2.9 mM against A. flavus.

Compounds 4a–f were screened for antifungal activity against S. cerevisiae (wild-type and selected signal transduction mutants in the MAPK pathway) and Aspergillus. The short-chain ester analogues 4a–c exhibited moderate growth inhibition against the model fungus S. cerevisiae, and the bioactivity of the propyl ester 4c was conveyed when tested against pathogenic aspergilli. Results of the bioassays of the compound 4c against the signal transduction mutants implied disruption of cell wall construction/ integrity.

Experimental Section

General Experimental Procedures. UV spectra were obtained on an HP-8452A diode array spectrophotometer. IR spectra were obtained on a Mattson Instruments 4020 Galaxy series FTIR spectrophotometer. EIMS spectra were obtained on an Agilent 6890N GC coupled to a 5975B MSD. HRMS spectra were obtained on a Q-STAR Pulsar I quadrupole/time-of-flight mass spectrometer. All 1H and 13C NMR spectra were obtained at 400 and 100 MHz, respectively, at 23 °C on a JEOL ECX spectrometer. NMR experiments were performed in CDC<sub>3</sub>; proton chemical shifts are reported in ppm and referenced to residual CHCl<sub>3</sub> at 7.25 ppm; carbon shifts are referenced to CDCl<sub>3</sub> at 77.1 ppm. Chemicals for the synthetic work were purchased from Aldrich Chemical Co. (St. Louis, MO) and used without further purification. Chromatography was carried out using either 230–400 mesh silica gel or Sephadex LH-20. TLC was performed on precoated silica gel 60 F254 plates using n-butanol:acetic acid:water (4:1:1) and 23 % formic acid:water (8:2) as solvent systems. TLC plates were viewed under long-wave UV light (254 nm) or with 0.5% aqueous KMnO<sub>4</sub>. HRMS spectra were obtained on a Q-STR Pulsar I HRMS spectrometer. Extracted ion chromatography was used to confirm the presence of each peak in the mass spectrum. GC was performed on an Agilent 6890N/5975B MSD spectrometer equipped with a 5975B MSD. HRMS spectra were obtained on a Q-STAR Pulsar I quadrupole/time-of-flight mass spectrometer. All 1H and 13C NMR spectra were obtained at 400 and 100 MHz, respectively, at 23 °C on a JEOL ECX spectrometer. NMR experiments were performed in CDC<sub>3</sub>; proton chemical shifts are reported in ppm and referenced to residual CHCl<sub>3</sub> at 7.25 ppm; carbon shifts are referenced to CDCl<sub>3</sub> at 77.1 ppm. Chemicals for the synthetic work were purchased from Aldrich Chemical Co. (St. Louis, MO) and used without further purification. Chromatography was carried out using either 230–400 mesh silica gel or Sephadex LH-20. TLC was performed on precoated silica gel 60 F254 plates using n-butanol:acetic acid:water (4:1:1) and 23 % formic acid:water (8:2) as solvent systems. TLC plates were viewed under long-wave UV light (254 nm) or with 0.5% aqueous KMnO<sub>4</sub>. HRMS spectra were obtained on a Q-STR Pulsar I HRMS spectrometer.
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Table 3. Comparison of Growth Inhibition: *Saccharomyces cerevisiae* Wild Type and Mutant Strains to DFA Propyl Ester (4e)

<table>
<thead>
<tr>
<th>conc</th>
<th>wild type</th>
<th>% growth inhibition</th>
<th>cell number</th>
<th>sh2Δ</th>
<th>% growth inhibition</th>
<th>cell number</th>
<th>bck1Δ</th>
<th>% growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>4.63 (0.33)</td>
<td>3.0</td>
<td>0.0</td>
<td>5.05 (0.30)</td>
<td>9.0</td>
<td>4.88 (0.60)</td>
<td>90.7</td>
<td>17.6</td>
</tr>
<tr>
<td>1.6 mM</td>
<td>2.30 (0.13)</td>
<td>50.4</td>
<td>1.13 (0.17)</td>
<td>75.5</td>
<td>1.07 (0.18)</td>
<td>76.8</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>3.2 mM</td>
<td>1.59 (0.11)</td>
<td>65.7</td>
<td>0.43 (0.03)</td>
<td>93.0</td>
<td>0.32 (0.06)</td>
<td>93.0</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

"a" Number of cells/mL, determined at OD 600 nm. Number in parentheses: std. dev. "b" Percent growth inhibition compared to the "no treatment" control of the wild type.

Dihydroferulic acid decyl ester (4f): silica gel column (CH2Cl2): to provide a colorless oil (97%); UV (CH2Cl2), 292 nm; IR (CCl4), 1734 cm⁻¹; 1H NMR δ 0.87 (3H, t, J = 6.8 Hz), 1.25 (14H, m), 1.59 (2H, s, t, J = 6.8 Hz), 2.58 (2H, t, J = 7.2 Hz), 2.87 (2H, t, J = 7.2 Hz), 3.86 (3H, s), 4.05 (2H, t, J = 6.8 Hz), 6.68 (1H, dd, J = 1.6 and 7.6 Hz), 6.70 (1H, d, J = 1.6 Hz), 6.82 (1H, d, J = 7.6 Hz); 13C NMR δ 142.0 (CH3, C-10′), 22.8 (CH2, chain CH2(C3)), 26.0 (CH2, chain CH2(C5)), 28.7 (CH3, chain CH2(C4)), 29.3 (CH2, chain CH2(C6)), 29.6 (CH2, chain CH2(C7)), 30.8 (CH2, chain C-8), 31.9 (CH2, chain C-9), 36.4 (CH2, chain C-10), 55.9 (CH2, ArOCH3), 64.8 (CH2, chain C-1′), 111.0 (CH-C-2′), 111.4 (CH-C-5′), 120.9 (CH-C-6′), 143.1 (C-C-4′), 146.5 (C-C-3′), 173.2 (C-C-9′); EIMS m/z 336 [M⁺] (76), 196 (19), 197 (15), 179 (3), 150 (57), 137 (100), 122 (5, 91) (7); HRESIMS m/z 337.2229 (calcd for C22H32O3, 337.2373); Rf 0.47 (hexanes/EtOAc, 4:1).

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Supporting Information Available: 1H and 13C NMR spectra and experimental data for compounds 1 and 4a–f. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(12) Campbell, B. C.; Molyneux, R. J.; Schatzki, T. F. J. Toxicol. 2003, 22, 225–266.
