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 *slt2*Δ and *bck1*Δ), *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.

The phenolic DFA (**1**, in Figure 1) is a metabolite of several plants[1−3] 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 report[11] 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*.

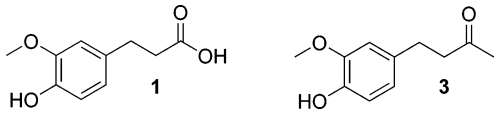
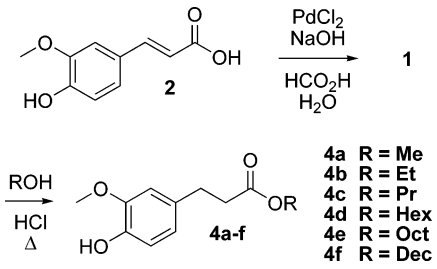


Figure 1. Natural products DFA (**1**) and vanillylacetone (**3**).

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



Results and Discussion

The synthesis of **1** and the corresponding analogues **4a**−**f**, illustrated in Scheme 1, was readily performed making use of traditional chemistry. Reduction of **2** utilized palladium-catalyzed transfer hydrogenation[15] to provide generous quantities of **1**, which was then subjected to standard esterification conditions[16] with the appropriate alcohol to result in the targeted esters.

The synthesized compounds (at 5 mM) were tested against wild-type *S. cerevisiae* and provided the following order of inhibition (% growth inhibition): **4a** (100) > **4c** (99.4) > **4b** (98.7) > **4d** (18.8) > **1** (6.4) > **4e** (6.2) > **4f** (2.8), relative to the control. A notable result was the difference of inhibition between the short-chain and long-chain esters. An additional observation of interest was the point at which the carboxylic acid **1** exhibited enhanced inhibition relative to the longer chain octyl and decyl esters (**4e** and **4f**, respectively).

Due to the observed bioactivity gradient between the short-chain and long-chain esters, the activity for the short-chain esters was 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% (MIC_{50}) and 90% (MIC_{90}) 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 MIC_{50} 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

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Table 1. Determination of MIC_{50} and MIC_{90} of DFA (**1**) and the Effective Ester Analogues (**4a**−**c**) against *Saccharomyces cerevisiae* Wild Type

	1	**4a**	**4b**	**4c**
MIC_{50} (mM)	6.4	2.1	4.0	1.5
MIC_{90} (mM)	nd[a]	5.6	>6.4[b]	4.5

[a] Not determined. [b] 87% growth inhibition at 6.4 mM.

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)**

	DFA propyl ester, **4c**		DFA hexyl ester, **4d**		
conc (mM)	cell number[a]	% growth inhibition[b]	cell number	% growth inhibition	difference in growth inhibition (%)[c]
no treatment	4.44 (0.10)	0.0	4.44 (0.10)	0.0	
0.8	2.99 (0.00)	32.7	3.61 (0.24)	18.7	14.0
1.6	1.89 (0.18)	57.4	2.74 (0.20)	38.3	19.1
3.2	0.03 (0.03)	99.3	1.00 (0.49)	77.5	21.8
6.4	0.0 (0.00)	100.0	0.28 (0.08)	93.7	6.3

[a] Cell number: $\times 10^7$ cells/mL, determined at OD 600 nm. Number in parentheses: std. dev. [b] Percent growth inhibition compared to the "no treatment" control. [c] 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,[20] which allows for use of gene deletion mutants to study functional genomic responses to synthetic and/or natural antifungals (chemogenomics).[21] 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*Δ, which lacks the mitogen-activated protein kinase (MAPK) gene, and *bck1*Δ, 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_{50} 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-STR Pulsar I quadrupole/time-of-flight mass spectrometer. All ^1H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, at 23 °C on a JEOL ECX spectrometer. NMR experiments were performed in $CDCl_3$; proton chemical shifts are reported in ppm and referenced to residual $CHCl_3$ at 7.25 ppm; carbon shifts are referenced to $CDCl_3$ 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 noted eluents. *A. fumigatus* AF293 was kindly provided by Dr. Gregory S. May (The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030). *A. flavus* NRRL3357 was obtained from National Center for Agricultural Utilization and Research, USDA, Peoria, IL.

Microorganisms and Culture Condition. *S. cerevisiae* wild-type BY4741 (*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants, *slt2*Δ and *bck1*Δ, were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL). Yeast strains were grown on YPD (1% Bacto yeast extract, 2% Bacto peptone, 110 μM glucose) or SG (0.67% yeast nitrogen base without amino acids, 110 μM glucose with appropriate supplements: 180 μM uracil, 200 μM amino acids) medium at 30 °C without light. *A. fumigatus* AF293, wild type, was grown at 37 °C on potato dextrose agar (PDA) medium.[22] *A. flavus* NRRL3357 was cultured at 28 °C on PDA medium.

Antifungal Bioassays. Sensitivity of *S. cerevisiae* to test compounds was assessed using modified assays according to the guidelines of National Committee for Clinical Laboratory Standards document M27-A. An initial test was performed where an inoculum of $\sim 5 \times 10^3$ colony forming units (CFU)/mL yeast cells was incubated in flat-bottomed microtiter plates in SG liquid medium (200 μL/well; 30 °C) containing DFA analogues (5 mM). Cell growth was monitored by optical density (OD) at 600 nm after 48 h of incubation. In a second set of assays to confirm the cutoff point (chain length) for the highest antifungal activity, both propyl and hexyl esters of DFA were dissolved in DMSO and serially diluted 2-fold six times to provide test concentrations of 0.1 to 6.4 mM. The corresponding MIC_{50} and MIC_{90} values were calculated by linear regression analysis, and yeast cell growth was monitored as described above. In a third set of assays to differentiate the level of potency, the methyl, ethyl, and propyl esters were serially diluted as above using a larger inoculum (i.e., 5×10^5 CFU/mL) in a larger volume of test solution (300 μL/well). Microtiter plates were incubated at 30 °C, the OD at 600 nm was measured in each well after 24 h of incubation, and MIC_{50} and MIC_{90} values were calculated by linear regression. All treatments were performed in triplicate. Sensitivity of the aspergilli to the test compounds was evaluated on the basis of percent radial growth of treated fungal colonies and compared to control colonies receiving only DMSO: fungi (~200 spores) were diluted in phosphate-buffered saline and spotted on the center of PDA plates with or without antifungal compounds. Growth rate was monitored for 4 days.

Dihydroferulic acid methyl ester (4a): pale brown oil (84%); UV (CH_2Cl_2), λ_{max} 294 nm; IR (CCl_4), ν_{max} 1741 cm^{-1}; ^1H NMR δ 2.59 (2H, t, J = 7.6 Hz), 2.87 (2H, t, J = 7.6 Hz), 3.65 (3H, s, methyl ester), 3.84 (3H, s, aryl methyl ether), 6.66 (1H, dd, J = 2.0 and 8.0 Hz), 6.69 (1H, d, J = 2.0 Hz), 6.81 (1H, d, J = 8.0 Hz); ^{13}C NMR δ 30.6 (CH_2, C-7), 36.2 (CH_2, C-8), 51.7 (CH_3, methyl ester), 55.9 (CH_3, $ArOCH_3$), 111.1 (CH, C-2), 114.5 (CH, C-5), 120.9 (CH, C-6), 132.5 (C, C-1), 144.1 (C, C-4), 146.6 (C, C-3), 173.6 (C, C-9); EIMS m/z 210 $[M]^+$ (100), 179 (8), 150 (65), 137 (100), 122 (20), 91 (17); HRESIMS m/z 211.0929 (calcd for $C_{11}H_{15}O_4$ 211.0964); R_f 0.72 (hexanes/EtOAc, 1:1).

Dihydroferulic acid ethyl ester (4b): pale brown oil (82%); UV (CH_2Cl_2), λ_{max} 290 nm; IR (CCl_4), ν_{max} 1737 cm^{-1}; ^1H NMR δ 1.23 (3H, t, J = 7.2 Hz), 2.58 (2H, t, J = 7.6 Hz), 2.87 (2H, t, J = 7.6 Hz), 3.83 (3H, s), 4.12 (2H, q, J = 7.2 Hz), 6.67 (1H, dd, J = 1.6 and 8.4 Hz), 6.69 (1H, d, J = 1.6 Hz), 6.81 (1H, d, J = 8.4 Hz); ^{13}C NMR δ 14.3 (CH_3, chain C-2′), 30.8 (CH_2, C-7), 36.5 (CH_2, C-8), 55.9 (CH_3, $ArOCH_3$), 60.5 (CH_2, chain C-1′), 111.1 (CH, C-2), 114.5 (CH, C-5), 120.9 (CH, C-6), 132.5 (C, C-1), 144.1 (C, C-4), 146.6 (C, C-3), 173.2 (C, C-9); EIMS m/z 224 $[M]^+$ (67), 179 (5), 150 (80), 137 (100), 122

Table 3. Comparison of Growth Inhibition: *Saccharomyces cerevisiae* Wild Type and Mutant Strains to DFA Propyl Ester (**4c**)

	wild type		*slt2*Δ		*bck1*Δ	
conc	cell number[a]	% growth inhibition[b]	cell number	% growth inhibition	cell number	% growth inhibition
no treatment	4.63 (0.33)		5.05 (0.30)	+9.0	4.88 (0.60)	+5.3
1.6 mM	2.30 (0.13)	50.4	1.13 (0.13)	75.5	1.07 (0.18)	76.8
3.2 mM	1.59 (0.11)	65.7	0.43 (0.03)	90.7	0.32 (0.06)	93.0

[a] Cell number: × 10^7 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.

Table 4. Growth Inhibition of *Aspergillus fumigatus* and *A. flavus* for the DFA Propyl Ester Analogue (**4c**)[a]

	A. fumigatus (AF293)		*A. flavus* 3357	
conc (mM)	radial growth[b]	% growth inhibition[c]	radial growth[b]	% growth inhibition[c]
no treatment	4.7	0.0	3.2	0.0
0.1	4.7	0.0	3.2	0.0
0.2	4.6	2.2	3.1	3.2
0.4	4.2	10.7	3.0	6.3
0.8	3.9	17.1	2.8	12.5
1.6	2.7	42.6	2.3	28.2
3.2	1.0	78.8	1.5	53.2
6.4	0.0	100.0	0.0	100.0

[a] Reported values are means of triplicate testing. Std. dev. for all were <3%. [b] Radial growth of fungi on potato dextrose agar plate (cm). [c] Percent growth inhibition compared to the "no treatment" control.

(10), 91 (15); HRESIMS *m*/*z* 225.1027 (calcd for $C_{12}H_{17}O_4$ 225.1121); R_f 0.25 (hexanes/EtOAc, 4:1).

Dihydroferulic acid propyl ester (4c): pale brown oil (92%); UV (CH_2Cl_2), λ_{max} 292 nm; IR (CCl_4), ν_{max} 1735 cm^{-1}; ^1H NMR δ 0.90 (3H, t, J = 7.2 Hz), 1.61 (2H, sext, J = 7.2 Hz), 2.58 (2H, t, J = 7.6 Hz), 2.86 (2H, t, J = 7.6 Hz), 3.82 (3H, s), 4.01 (2H, t, J = 7.2 Hz), 6.67 (1H, dd, J = 2.0 and 8.4 Hz), 6.69 (1H, d, J = 2.0 Hz), 6.81 (1H, d, J = 8.4 Hz); ^{13}C NMR δ 10.4 (CH_3, chain C-3′), 22.0 (CH_2, chain C-2′), 30.8 (CH_2, C-7), 36.4 (CH_2, C-8), 55.9 (CH_3, $ArOCH_3$), 66.2 (CH_2, chain C-1′), 111.1 (CH, C-2), 114.5 (CH, C-5), 120.9 (CH, C-6), 132.6 (C, C-1), 144.1 (C, C-4), 146.6 (C, C-3), 173.3 (C, C-9); EIMS *m*/*z* 238 $[M]^+$ (42), 195 (5), 179 (6), 150 (45), 137 (100), 122 (5), 91 (7); HRESIMS *m*/*z* 239.1211 (calcd for $C_{13}H_{19}O_4$ 239.1277); R_f 0.33 (hexanes/EtOAc, 4:1).

Dihydroferulic acid hexyl ester (4d): silica gel column (hexanes/EtOAc, 4:1) and Sephadex LH-20 (CH_3OH/CH_2Cl_2, 3:2) to provide a colorless oil (89%); UV (CH_2Cl_2), λ_{max} 292 nm; IR (CCl_4), ν_{max} 1736 cm^{-1}; ^1H NMR δ 0.88 (3H, t, J = 6.8 Hz), 1.27 (6H, m), 1.58 (2H, sext, J = 6.8 Hz), 2.58 (2H, t, J = 7.6 Hz), 2.87 (2H, t, J = 7.6 Hz), 3.84 (3H, s), 4.05 (2H, t, J = 6.8 Hz), 6.67 (1H, dd, J = 2.2 and 8.0 Hz), 6.70 (1H, d, J = 2.0 Hz), 6.81 (1H, d, J = 8.0 Hz); ^{13}C NMR δ 14.1 (CH_3, chain C-6′), 22.6 (CH_2, chain CH_2′), 25.7 (CH_2, chain CH_2′), 28.7 (CH_2, chain CH_2′), 30.8 (CH_2, C-7), 31.5 (CH_2, chain C-2′), 36.4 (CH_2, C-8), 55.9 (CH_3, $ArOCH_3$), 64.8 (CH_2, chain C-1′), 111.0 (CH, C-2), 114.4 (CH, C-5), 120.9 (CH, C-6), 132.6 (C, C-1), 144.1 (C, C-4), 146.5 (C, C-3), 173.3 (C, C-9); EIMS *m*/*z* 280 $[M]^+$ (50), 195 (13), 179 (4), 150 (55), 137 (100), 122 (5), 91 (8); HRESIMS *m*/*z* 281.1644 (calcd for $C_{16}H_{25}O_4$ 281.1747); R_f 0.38 (hexanes/EtOAc, 4:1).

Dihydroferulic acid octyl ester (4e): silica gel column (hexanes/EtOAc, 4:1) to provide a colorless oil (67%); UV (CH_2Cl_2), λ_{max} 290 nm; IR (CCl_4), ν_{max} 1735 cm^{-1}; ^1H NMR δ 0.88 (3H, t, J = 6.8 Hz), 1.27 (10H, m), 1.59 (2H, sext, J = 6.8 Hz), 2.58 (2H, t, J = 7.6 Hz), 2.87 (2H, t, J = 7.6 Hz), 3.86 (3H, s), 4.05 (2H, t, J = 6.8 Hz), 6.68 (1H, dd, J = 2.0 and 7.6 Hz), 6.70 (1H, d, J = 2.0 Hz), 6.82 (1H, d, J = 7.6 Hz); ^{13}C NMR δ 14.2 (CH_3, chain C-8′), 22.7 (CH_2, chain CH_2′), 26.0 (CH_2, chain CH_2′), 28.7 (CH_2, chain CH_2′), 29.26 (CH_2, chain CH_2′), 29.30 (CH_2, chain CH_2′), 30.8 (CH_2, C-7), 31.9 (CH_2, chain C-2′), 36.4 (CH_2, C-8), 55.9 (CH_3, $ArOCH_3$), 64.8 (CH_2, chain C-1′), 111.0 (CH, C-2), 114.4 (CH, C-5), 120.9 (CH, C-6), 132.6 (C, C-1), 144.1 (C, C-4), 146.5 (C, C-3), 173.2 (C, C-9); EIMS *m*/*z* 308 $[M]^+$ (61), 196 (14), 195 (17), 150 (58), 137 (100), 122 (5), 91 (7); HRESIMS *m*/*z* 309.1931 (calcd for $C_{18}H_{29}O_4$ 309.2060); R_f 0.40 (hexanes/EtOAc, 4:1).

Dihydroferulic acid decyl ester (4f): silica gel column (CH_2Cl_2) to provide a colorless oil (97%); UV (CH_2Cl_2), λ_{max} 292 nm; IR (CCl_4), ν_{max} 1734 cm^{-1}; ^1H NMR δ 0.87 (3H, t, J = 6.8 Hz), 1.25 (14H, m), 1.59 (2H, sext, 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); ^{13}C NMR δ 14.2 (CH_3, chain C-10′), 22.8 (CH_2, chain CH_2′), 26.0 (CH_2, chain CH_2′), 28.7 (CH_2, chain CH_2′), 29.3 (CH_2, chain CH_2′), 29.4 (CH_2, chain CH_2′), 29.61 (CH_2, chain CH_2′), 29.62 (CH_2, chain CH_2′), 30.8 (CH_2, C-7), 32.0 (CH_2, chain C-2′), 36.4 (CH_2, C-8), 55.9 (CH_3, $ArOCH_3$), 64.8 (CH_2, chain C-1′), 111.0 (CH, C-2), 114.4 (CH, C-5), 120.9 (CH, C-6), 132.6 (C, C-1), 144.1 (C, C-4), 146.5 (C, C-3), 173.2 (C, C-9); EIMS *m*/*z* 336 $[M]^+$ (76), 196 (17), 195 (17), 179 (3), 150 (57), 137 (100), 122 (5), 91 (7); HRESIMS *m*/*z* 337.2239 (calcd for $C_{20}H_{33}O_4$ 337.2373); R_f 0.47 (hexanes/EtOAc, 4:1).

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Supporting Information Available: ^1H and ^{13}C 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.

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