Synthetic molecular mimics of naturally occurring cyclopentenones exhibit antifungal activity towards pathogenic fungi

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INTRODUCTION

Plant oxylipins are a diverse class of lipid metabolites that are derived from the enzyme or non-enzyme oxidation of polyunsaturated fatty acids (Mosblech et al., 2009). They play an important role in plant defence systems, such as detoxification of reactive oxidative species (Mueller et al., 2005; Weber, 2002), induction of phytoalexins (Harborne, 1999), and direct antimicrobial activities (Hamberg, 1999; Hamberg et al., 2003; Prost et al., 2005).

The major oxylipin studied to date is jasmonic acid (JA) (Wasternack, 2007), a plant hormone which is involved in signal transduction pathways in many plant–microbe interactions (Adie et al., 2007). JA is a cyclopentenone derivative of x-linolenic acid elaborated by 13-lipoxygenase (Andreou & Feussner, 2009; Leon et al., 2002), allene oxide synthase (Laudert & Weiler, 1998), allene oxide cyclase (Stenzel et al., 2003), 12-oxo-phytodienoic acid (12-oxo-PDA) reductase (Schaller & Stintzi, 2009) and ß-oxidation (Li et al., 2005). Although JA plays an important role in plant defence systems (Wasternack, 2007), the compound itself exerts little direct antimicrobial activity. By contrast, its biosynthetic precursor, 12-oxo-PDA, not only acts as a modulator of gene expression (Taki et al., 2005) but also shows strong antifungal activity in vitro (Prost et al., 2005). Structurally, 12-oxo-PDA has a double bond in the cyclopentenone ring and an eight-carbon arm at the C-4 position of the ring, whereas JA has a cyclopentanone ring and a two-carbon arm. Both molecules have a pentenyl arm at the 5-position of the carbon ring (Fig. 1). The presence of the double bond adjacent to the carbonyl function (x,ß-unsaturated carbonyl) endows it with electrophilic properties that mean it can react with electron donor (nucleophilic) groups common to many biological molecules (e.g. -SH groups) (Farmer & Davoine, 2007). The presence of such an x,ß-unsaturated carbonyl is a common feature of many enzymically and non-enzymically derived fatty acid products that are termed reactive electrophilic species (RES). These species include many lipid oxidation products such as 2-(E)-hexenal, 12-oxo-PDA and malondialdehyde (Almeras et al., 2003). Plant RES could act as Michael acceptors, reacting not only with reduced glutathione (GSH) but also directly with other enzymes and transcription factors, thereby modulating cellular function (Mueller & Berger, 2009). In addition to the electrophilicity of the x,ß-carbonyl group, another important parameter of oxylipin RES is the hydrophobicity...
NH₄Cl (5 ml) and an aqueous solution of NH₄OH (28 %, 5 ml) with 2 dropwise. The reaction was then stirred at 1,3-diol 1-acetate (1 : 3, w/w). A solution of RMgCl (octylmagnesium chloride in THF; 1.8 ml), and the resultant slurry was stirred magnetically and cooled to 0 °C. Freshly prepared Jones reagent was then added dropwise until the colour of the reagent persisted and the resultant orange solution was stirred for a further 30 min at 0 °C. After this, the reaction was quenched by the addition of 2-propanol, and the resultant mixture was filtered through a pad of silica gel using diethyl ether as the eluent. The filtrate was washed with brine (3 × 25 ml) before being dried by standing over anhydrous MgSO₄ collected by filtration and concentrated under reduced pressure to furnish the crude products 3–5 as yellow oils. The crude products were purified by column chromatography (hexane/ethyl acetate, 50 : 50, v/v) to give the pure compounds 3–5.

4-Octyl cyclopentenone (3) was obtained as a yellow oil (47.3 mg, 0.24 mmol, yield 36 %) (TLC hexane/ethyl acetate, 50 : 50, v/v, Rₑ=0.75). IR (neat): v_max (cm⁻¹) 3052, 1716, 1669, 1178. ¹H NMR (300 MHz, CDCl₃): δ 0.97 (t, 3, ₃J=6.9 Hz, CH₃), 1.28–1.80 (m, 14, CH₂), 2.07 (dd, 1, ₂J₂a,₃b=18.9 Hz, ₃J₂a=2.1 Hz, 5-H₃), 2.61 (dd, 1, ₂J₂b,₃a=18.9 Hz, ₂J₂b=6.3 Hz, 5-H₃), 2.96–3.30 (m, 1, 4-H), 6.22 (dd, 1, ₂J₂a,₃b=5.7 Hz, ₃J₂a=2.1 Hz, CH=, 2.71 (dd, 1, ₂J₂b,₃a=5.7 Hz, ₂J₂b=2.4 Hz, CH=), 5.71 (CH₃). ¹³C NMR (75.58 MHz, CDCl₃, PENDANT): δ 14.01(CH₃), 22.70(CH₂), 27.67(CH₂), 29.28(CH₃), 29.50(CH₃), 29.65(CH₃), 31.89(CH₂), 34.80(CH₂), 41.11(C-3), 42.01(C-4), 133.30(C-3), 168.90(C-2), 210.20(C-1). LRMS (APCI +): m/z 195 [(M+H)⁺]; calculated for C₁₃H₂₂O: 194.32.

4-Hexyl cyclopentenone (4) was obtained as a yellow oil (11.0 mg, 0.066 mmol, yield 26 %) (TLC hexane/ethyl acetate, 50 : 50, v/v, Rₑ=0.75). IR (neat): v_max (cm⁻¹) 3046, 1716, 1669, 1181. ¹H NMR (300 MHz, CDCl₃): δ 0.95 (t, 3, ₃J=6.9 Hz, CH₃), 1.25–1.72 (m, 10, CH₂), 2.05 (dd, 1, ₂J₂a,₃b=18.8 Hz, ₂J₂a=4.0 Hz, 5-H₃), 2.59 (dd, 1, ₂J₂a,₃b=19.0 Hz, ₂J₂b=6.4 Hz, 5-H₃), 2.93–3.06 (m, 1, 4-H), 6.22 (dd, 1, ₂J₂b,₃a=5.6 Hz, ₂J₂b=2.0 Hz, CH=, 2.71 (dd, 1, ₂J₂a,₃b=5.7 Hz, ₂J₂a=2.4 Hz, CH=), 6.22 (CH₃). ¹³C NMR (75.58 MHz, CDCl₃, PENDANT): δ 14.10(CH₃), 22.61(CH₂), 27.72(CH₂), 29.30(CH₃), 31.89(CH₂), 34.79(CH₂), 41.13(C-5), 41.56(C-4), 133.65(C-3), 168.87(C-2), 210.13(C-1). LRMS (APCI +): m/z 167 [(M+H)⁺]; calculated for C₁₃H₂₄O: 166.26.

4-Butyl cyclopentenone (5) was obtained as a yellow oil (9.2 mg, 0.067 mmol, yield 26 %) (TLC hexane/ethyl acetate, 50 : 50, v/v, Rₑ=0.75). IR (neat): v_max (cm⁻¹) 3049, 1716, 1669, 1183. ¹H NMR (300 MHz, CDCl₃): δ 0.99 (t, 3, ₃J=6.9 Hz, CH₃), 1.26–1.74 (m, 6, CH₂), 2.14 (dd, 1, ₂J₂a,₃b=18.8 Hz, ₃J₂a=2.1 Hz, 5-H₃), 2.61 (dd, 1, ₂J₂b,₃a=18.9 Hz, ₂J₂b=6.3 Hz, 5-H₃), 2.90–3.08 (m, 1, 4-H), 6.22 (dd, 1, ₂J₂a,₃b=5.6 Hz, ₂J₂b=2.0 Hz, CH=, 2.72 (dd, 1, ₂J₂a,₃b=5.6 Hz, ₂J₂b=2.4 Hz, CH=), 6.22 (CH₃). ¹³C NMR (75.58 MHz, CDCl₃, PENDANT): δ 13.92(CH₃), 22.83(CH₂), 29.90(CH₃), 34.64(CH₂), 41.00(C-5), 41.63(C-4), 133.61(C-3), 168.82(C-2), 210.42(C-1). LRMS (APCI +): m/z 139 [(M+H)⁺]; calculated for C₁₃H₂₃O: 138.21.

Synthesis of cyclopentenone compounds (8). The simplified synthetic pathway of 8-(4-oxocyclopentenyl)octanoic acid (8) was modified from that of 12-oxo-PDA given by Kobayashi & Matsuumi (2002) (Fig. 2b).

Preparation of CI⁺Mg(Ch₃)₂OTBDPS (8-(4-tet-butylphenylsilyl)oxy)octyl)magnesium chloride). A one-necked flask (25 ml) was charged with 8-chloro-1-octanol (1.0 g, 6.0 mmol), tert-butylphenylsilyl (TBDDS) chloride (2.0 g, 7.2 mmol) and dimethylformamide (10 ml) in an ice bath with stirring. Imidazole (1.0 g, 15.0 mmol) was added slowly and stirred until complete dissolution in DMF was achieved. The reaction was carried out at room temperature overnight. The reaction was quenched by the addition of water and extracted with dichloromethane (DCM; 3 × 25 ml) and the combined extracts were then washed with 1 M HCl (3 × 25 ml). After being dried with MgSO₄, the mixture was concentrated to a transparent oil. The crude oil was purified by column chromatography (hexane/ethyl acetate, 90 : 10) to give the pure oil (CI⁺Mg(Ch₃)₂OTBDPS, 2.27 g, 5.64 mmol, yield: 94 %) (TLC hexane/ethyl acetate, 90 : 10, v/v, Rₑ=0.86). IR (neat): v_max (cm⁻¹) 2952, 2854, 1469, 1450, 1383, 1264, 1232, 1160, 1099, 1046, 802.
A two-necked round-bottomed flask (25 ml) was charged with fresh magnesium turnings (54.1 mg) and dry THF (0.5 ml) which just covered the turnings. Several drops of dichloroethane (0.06 ml) were added, and the mixture was then heated to 50 °C for a few minutes without stirring. When the heat was removed, effervescence was observed from the surface of the turnings and the solution turned grey and turbid. TBDPSO(CH2)8Cl (0.45 g) in THF (1.5 ml) was added dropwise slowly with vigorous stirring. The reaction was then heated to refluxing temperature and left overnight. Grignard reagent [CIMg(CH2)8OTBDPS] was used in the next step without any further purification.

4-(8-Hydroxyoctyl)cyclopentenol (7). The synthetic pathway was simplified from Kobayashi’s method (Kobayashi & Matsuumi, 2002). Grignard reagent, CIMg(CH2)8OTBDPS (2.0 ml, 0.53 M in THF, 1.06 mmol), was added slowly to a slurry of CuI (10.5 mg, 0.054 mmol) in THF (1.0 ml) at −18 °C. The reaction mixture was then stirred for a further 20 min at −18 °C, after which a solution of (1R,3S)-(−)-cis-4-cyclopentene-1,3-diol-1-acetate (1) (50 mg, 0.35 mmol) in THF (0.2 ml) was added dropwise. The resultant reaction mixture was then stirred at −18 °C for 5 h before being quenched by the addition, with vigorous stirring, of a saturated aqueous solution of NH4Cl (5 ml) and an aqueous solution of NH4OH (28 %, 5 ml). The crude product was extracted with ethyl acetate (3 × 10 ml). The combined organic extracts were dried by standing over anhydrous MgSO4, collected by filtration and concentrated under reduced pressure to furnish the crude product as an oil. The crude product was then purified by column
chromatography (hexane/ethyl acetate, 75:25, v/v) to afford the major 1,4-isomer 6 (142.5 mg, 0.316 mmol, yield 90 %) (TLC hexane/ethyl acetate, 75:25, v/v, R<sub>f</sub> = 0.35). Finally, the purified product was used directly in a subsequent deprotection step without being subjected to any analysis.

A mixture of 6 (107.9 mg, 0.24 mmol), tetrabutylammonium fluoride (TBAF) (1.20 ml, 1.0 M in THF, 1.20 mmol), and 4 Å (0.4 nm) molecular sieves (89 mg) and THF (2.50 ml) was stirred and heated to 55 °C for 1 h before being cooled to 0 °C and diluted by the addition of ethyl acetate (2.5 ml) and an aqueous solution of saturated NH<sub>4</sub>Cl (2.5 ml). The resultant mixture was filtered through a pad of silica gel using ethyl acetate as the eluent. After filtration, the phases were separated and the aqueous phase was extracted with ethyl acetate (3 × 25 ml). The combined organic extracts were dried by standing over anhydrous MgSO<sub>4</sub>, collected by filtration and concentrated under reduced pressure to furnish the crude product as a yellow oil. The crude product was purified by column chromatography (DCM:acetone: 75:25, v/v) to give 4-(8-hydroxyxycyclopentenyl)cyclopentenone (7) (34.7 mg, 0.158 mmol, yield 68 %). (TLC DCM:acetone, 75:25, v/v, R<sub>f</sub> = 0.40).

IR ( neat): ν<sub>max</sub> (cm<sup>-1</sup>) 3418, 3034, 1465, 1022. ¹H NMR (300 MHz, CDCl<sub>3</sub>): δ 0.10–1.51 (m, 14, CH₃), 1.59–1.61 (m, 2, OH), 1.80 (dd, 1, J<sub>5α,5β</sub> = 14.0 Hz, J<sub>5α,5β</sub> = 7.1 Hz, J<sub>5α,5β</sub> = 5.2 Hz, 5-H), 1.92 (dd, 1, J<sub>5α,5β</sub> = 14.1 Hz, J<sub>5α,5β</sub> = 7.5 Hz, J<sub>5α,5β</sub> = 2.9 Hz, 5-H), 2.82–2.90 (m, 1, 4-H), 3.71 (dd, 2, 1, J<sub>7,6</sub> = 12.0 Hz, 1, J<sub>7,6</sub> = 6.6 Hz, CH<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub>OH), 4.83–4.91 (m, 1, 1-H), 5.83 (dd, 1, J<sub>1,2</sub> = 5.4 Hz, 1, J<sub>2,3</sub> = 2.2 Hz, 3-H), 5.96 (dd, 1, J<sub>1,2</sub> = 5.6 Hz, 1, J<sub>2,3</sub> = 2.0 Hz, 1, J<sub>2,3</sub> = 0.8 Hz, 2-H). ¹³C NMR (75.58 MHz, CDCl₃, PENDANT): δ 14.91 (CH<sub>3</sub>), 20.26 (CH₂), 26.52 (CH₂), 32.86 (CH₂), 35.22 (CH₂), 39.44 (CH₂), 44.09 (C-4), 63.13 (CH₂-OH), 77.20 (C-1), 132.30 (C-3), 140.40 (C-2).

8-(4-Oxocyclopentenyl)octanoic acid (8). 4-(8-Hydroxyxycyclopentenyl)cyclopentenone (7) (34.7 mg, 0.158 mmol) was dissolved in freshly prepared Jones reagent (34.7 mg, 0.158 mmol) was dissolved in freshly prepared Jones reagent (34.7 mg, 0.158 mmol) was dissolved in freshly prepared Jones reagent (34.7 mg, 0.158 mmol) was dissolved in freshly prepared Jones reagent (34.7 mg, 0.158 mmol). After 1 min pre-incubation at room temperature, 4-octyl cyclopentenone or 8-(4-oxocyclopentenyl)cyclopentenone (100 µM and 200 µM) were mixed with the fungal spores (10⁶ spores ml⁻¹) at room temperature for 1 min (Kubo et al., 2003). The suspension was vortexed for 5 s, and the absorbance of the supernatants obtained by centrifugation at 4000 g for 5 min was measured at 227 nm [JENWAY 6505 UV/Vis spectrophotometer]. Test compounds in the absence of spores were used as controls.

Uptake of compounds by spores. The method used for measuring the uptake of 4-octyl cyclopentenone and 8-(4-oxocyclopentenyl)octanoic acid by spores was modified that described by Fujita & Kubo (2002). Briefly, spores of C. herbarum, B. cinerea (GLUK-1), F. oxysporum and A. brassicicola were harvested and washed three times with HPLC water. After 10 min pre-incubation at room temperature, 4-octyl cyclopentenone or 8-(4-oxocyclopentenyl)cyclopentenone (100 µM and 200 µM) were mixed with the fungal spores (10⁶ spores ml⁻¹) at room temperature for 1 min (Kubo et al., 2003). The suspension was vortexed for 5 s, and the absorbance of the supernatants obtained by centrifugation at 4000 g for 5 min was measured at 227 nm (JENWAY 6505 UV/Vis spectrophotometer).

Test compounds in the absence of spores were used as controls.

Lipid extraction and analysis. Lipids were extracted from freshly harvested spores obtained from cultures grown on PDA according to Griffiths et al. (2003). Dried spores were transferred to test tubes and extracted with hot 2-propanol (70 °C, 3 ml). After vortexing, the mixture was left to extract for 15 min in a heating block at 70 °C before centrifuging at 1000 g for 10 min. The supernatant was decanted and the pellet was washed twice with hot 2-propanol (2 × 3 ml) and then with 2-propanol/chloroform (3 ml, 1:1, v/v), dried under N₂ and resuspended in chloroform/methanol (1:1, v/v).

Lipids were separated and purified by TLC on pre-coated silica gel plates (Merck, silica gel 60) using a non-polar solvent system of isoamyl ether/acetatic acid (70:30:1, by vol). Iodine-stained lipids were scraped from TLC plates and methylated according to Jackson et al. (1998). Fatty acid methyl esters (FAMEs) were extracted with isoamyl ether, dried under N₂ and analysed by GC (Auto System XL; Perkin Elmer) coupled to a flame-ionization detector (FID), using a DB-23 capillary column (Agilent 30 m, 0.25 mm; J&W Scientific); peaks were identified by co-chromatography with authentic standards. The oven temperature programme was as follows: 100 °C, 20 °C min⁻¹ to 200 °C, hold for 5 min, 10 °C min⁻¹ to 240 °C, hold for 5 min. H₂ injector and FID temperature were 230 °C. FAMEs were identified by comparing retention times with those of standard by low-speed centrifugation. Spores were then suspended in the appropriate culture medium to give a concentration of 2000 spores per well (C. herbarum) or 5000 spores per well (B. cinerea, F. oxysporum and A. brassicicola) in sterile, 96-well flat-bottomed microplates (NUNC; Sigma-Aldrich) and sealed with Parafilm, in a final volume of 100 µl. F. oxysporum and A. brassicicola were grown in potato dextrose broth (48 g l⁻¹; Sigma-Aldrich) in an incubator at 250 r.p.m. at 25 °C. C. herbarum and B. cinerea were grown in clarified 5% V8 juice broth prepared as described previously (Prost et al., 2005). Test compounds were dissolved in ethanol to a maximum final concentration of 1% in each well. Controls were treated with 1% ethanol. Spore germination in the presence of compounds was observed by optical microscopy (MAX Mono Monocular Biology Microscope; Fisher, magnification ×100) and 50–100 spores were assessed for germination status.

Spores with visible germ tubes were counted as being germinated and results were expressed as a percentage of germination.

Test for leakage of potassium ions from spores. Leakage of potassium ions was monitored by measuring their concentration in the medium with a potassium-selective electrode (Corning Electrode; Corning Ltd) recorded on a mV/pH meter (Corning 240 pH meter).

A range of increasing concentrations of KCl solutions was measured to give a standard curve of K⁺ before each test. The pre-cultured fungal spores were harvested and washed three times with HPLC water and then resuspended in HPLC water to give 2 × 10⁶ spores ml⁻¹. After 1 min pre-incubation at room temperature, 4-octyl cyclopentenone and nystatin were added and the conductivity of suspension was measured every 5–10 min with continuous stirring. Spore suspensions without compounds were used as controls.

Fungal cultures. Cladosporium herbarum (Pers.: Fr.), Botrytis cinerea MUCL30158, Fusarium oxysporum f. sp. radicis-lycopersici and Alternaria brassicicola MUCL20297 were cultivated from stocks as previously described (Prost et al., 2005). Botrytis cinerea GLUK-1, obtained from an isolate from infected peppers in Scotland, was kindly supplied by Dr Katherine J. Denby (Crop Science, Warwick University, UK).

Sporos germination and mycelial growth assays. Fungal cultures were grown on agar plates [39 g potato dextrose agar (PDA) l⁻¹; Sigma-Aldrich] and the spores were harvested in water and recovered

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FAMEs and were quantified using an internal standard, methyl heptadecanoate (17:0).

RESULTS

Effect of cyclopentenones and 2(E)-nonenal on fungal spore germination and growth

To evaluate the effect of \(\alpha,\beta\)-carbonyl-containing compounds on spore germination and mycelial growth we synthesized cyclopentenone derivatives varying in the chain length of the arm at the C-4 position of the cyclopentenone ring (Fig. 2c). Two eight-carbon chain length compounds were synthesized, one terminating in a methyl group (4-octyl cyclopentenone) and the other in a carboxyl terminus (8-(4-oxocyclopentenyl)octanoic acid), the latter reflecting the group present in fatty acid derivatives. A six-carbon (4-hexyl cyclopentenone) and a four-carbon (4-butyl cyclopentenone) compound were also synthesized and tested against the parent compound, cyclopentenone lacking any

Fig. 3. Effect of test compounds (100 \(\mu\)M) on growth of (a) \textit{F. oxysporum}, (b) \textit{A. brassicicola}, (c) \textit{C. herbarum}, (d) \textit{B. cinerea} MUCL30158 and (e) \textit{B. cinerea} GLUK-1. The fungal strains were exposed to a series of test compounds in appropriate liquid culture medium, starting from the time of spore addition to wells (\textit{F. oxysporum} and \textit{A. brassicicola}) or 16 h later (\textit{C. herbarum} and \textit{B. cinerea}). Results are means ± SD (\(n=6\)). An asterisk indicates a significant difference at \(P<0.01\) (Dunnett’s test) versus the control.
side arm additions. For comparative purposes, 2(E)-nonenal was also examined for effects on spores as it contains an α,β-carbonyl function and is an aliphatic compound, unlike the cyclic derivatives described above (Fig. 2c).

Four fungal pathogens, namely F. oxysporum, A. brassicicola, C. herbarum and B. cinerea, were exposed to each of the compounds at an initial concentration of 100 μM (Fig. 3). For F. oxysporum 4-octyl cyclopentenone suppressed growth at 24 h, whereas for A. brassicicola, despite initial growth suppression with many compounds over the first 48 h, none was effective at 72 h. By contrast, both B. cinerea and C. herbarum were sensitive to 4-octyl cyclopentenone, with the latter species also showing some suppression with 2(E)-nonenal. The cyclopentenone ring itself had very little effect on fungal growth. Interestingly, 8-(4-oxocyclopentenyl)octanoic acid, with the carboxyl group on the end of the alkyl chain, was less effective than 4-octyl cyclopentenone. To evaluate whether the compounds suppressed spore germination or mycelial growth we evaluated the percentage spore germination following short exposure times to the test compounds (max. 12 h, Fig. 4). The percentage of germinated spores for each treatment was compared with the values for 1 % ethanol controls. 4-Octyl cyclopentenone was the most effective compound tested against all three fungi, with 80–90 % inhibition of spore germination observed for C. herbarum and B. cinerea (GLUK-1) and 60 % for F. oxysporum. Data for A. brassicicola were not included due to clustering of the spores, which made it difficult to assess germination quantitatively. The results suggest that, of the compounds examined, 4-octyl cyclopentenone was the most effective at suppression of spore germination and growth for three of the four pathogens tested. Further tests were therefore performed using this compound to determine the dose-dependent relationship with spore growth suppression. In addition, given the sensitivity of B. cinerea MUCL30158 to the compound, we also included in the next screen another strain obtained from the UK, B. cinerea GLUK-1, to evaluate its potential broader application. Concentrations of 4-octyl cyclopentenone up to 400 μM were examined (Fig. 5). The results clearly indicate that increasing the dose of the compound suppressed growth of all species, and at 200 μM it completely inhibited the growth of both C. herbarum and B. cinerea MUCL30158 and caused significant suppression in B. cinerea GLUK-1. The minimum fungicidal concentration (MFC) is the concentration at which there is no growth recovery and the cells are perceived as dead. The MFC of 4-octyl cyclopentenone against C. herbarum and B. cinerea MUCL30158 was between 100 and 200 μM, whereas for B. cinerea GLUK-1 it was between 200 and 400 μM; for both F. oxysporum and A. brassicicola, MFC values were above 400 μM.

**Spore uptake of 4-octyl cyclopentenone**

The observation that 4-octyl cyclopentenone was more effective than the carboxyl derivative 8-(4-oxocyclopentenyl)octanoic acid in suppressing spore germination and growth prompted the determination of the uptake of these compounds by the fungal pathogens. Both compounds were analysed by UV spectroscopy from 200 to 300 nm and the λ_max was determined to be 227 nm. Fungal spores were exposed to test compounds for 1 min and then removed

![Fig. 4. Effect of test compounds (100 μM) on spore germination. Freshly harvested spores of F. oxysporum, C. herbarum and B. cinerea (GLUK-1) were assessed for germination status after 8 h (F. oxysporum and B. cinerea) and 12 h (C. herbarum) incubation as described previously (Prost et al., 2005). Each bar represents the mean of three independent experiments ± SD. An asterisk indicates a significant difference at P<0.01 (Dunnett's test) versus the control.](image-url)
from solution by centrifugation. The resulting supernatant was then assessed spectrophotometrically to determine the level of each compound remaining in solution. The results revealed that for *B. cinerea*, *C. herbarum* and *A. brassicicola*, approximately 55% of 4-octyl cyclopentenone was removed from the solution (adsorbed and/or absorbed) by the spores and 30% was removed by *F. oxysporum*. By contrast, only 2% of 8-(4-oxocyclopentenyl)-octanoic acid was taken up by all four fungi examined (results not shown).

These results show that 4-octyl cyclopentenone is much more readily taken up than the carboxyl derivative; this uptake may account, at least in part, for the observed difference in spore suppression.

**Effect of 4-octyl cyclopentenone on electrolyte leakage**

Perturbation of cell membrane function often results in electrolyte leakage from cells (Leverenz et al., 2002), which can be monitored by determining the conductivity of the medium. 4-Octyl cyclopentenone was readily taken up by all four fungal pathogens, but their sensitivity to the compound varied significantly. To establish whether uptake of the compound disrupts membrane function, we determined electrolyte leakage profiles for the four species and compared them with that for nystatin, a commercial antifungal agent which induces K⁺ efflux in cells, as a positive control. The K⁺ leakage caused by
4-octyl cyclopentenone in B. cinerea and C. herbarum was 35% and 25%, respectively, of that caused by nystatin, whereas for A. brassicicola and F. oxysporum, electrolyte leakage was almost undetectable (Fig. 6). These results suggest that membrane disruption is more acute in those species that are the most sensitive to the compound, namely C. herbarum and B. cinerea.

**Lipid composition of fungal spores**

Lipids constitute one of the major components of cell membranes, and the phospholipids are largely responsible for the bilayer structure. This in turn influences the fluidity properties of the membrane and associated enzymic functions, including ion transport and signalling processes.

**Table 1. Total fatty acid composition of fungal spores**

Total lipid extracts were separated via TLC by the non-polar solvent system and the separated classes were quantified by GC of their derived fatty acids. Results (wt %) are means ± SD of three separate experiments.

<table>
<thead>
<tr>
<th>Lipid composition (wt %)*</th>
<th>A. brassicicola</th>
<th>F. oxysporum</th>
<th>C. herbarum</th>
<th>B. cinerea GLUK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate (16:0)</td>
<td>21.6 ± 1.3</td>
<td>27.6 ± 3.0</td>
<td>12.8 ± 0.5</td>
<td>27.0 ± 0.8</td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>9.9 ± 1.7</td>
<td>11.3 ± 2.5</td>
<td>5.2 ± 0.4</td>
<td>12.5 ± 1.0</td>
</tr>
<tr>
<td>Oleate (18:1 n-9)</td>
<td>15.2 ± 2.1</td>
<td>29.1 ± 2.7</td>
<td>32.1 ± 0.5</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Linoleate (18:2 n-6)</td>
<td>48.3 ± 0.6</td>
<td>29.6 ± 3.2</td>
<td>49.5 ± 0.5</td>
<td>48.1 ± 1.0</td>
</tr>
<tr>
<td>α-Linolenate (18:3 n-3)</td>
<td>5.0 ± 0.4</td>
<td>2.6 ± 0.5</td>
<td>0.3 ± 0.1</td>
<td>8.1 ± 1.2</td>
</tr>
<tr>
<td>Unsaturated/saturated (mol mol⁻¹)</td>
<td>4.8 ± 0.4</td>
<td>2.4 ± 0.6</td>
<td>8.2 ± 0.3</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Δ mol⁻¹</td>
<td>1.7 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>34.6 ± 3.1</td>
<td>11.9 ± 0.8</td>
<td>62.2 ± 2.6</td>
<td>60.7 ± 0.8</td>
</tr>
<tr>
<td>Non-polar lipids</td>
<td>65.4 ± 3.1</td>
<td>88.1 ± 0.8</td>
<td>37.8 ± 2.6</td>
<td>39.3 ± 0.8</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>1.0 ± 0.7</td>
<td>0.2 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>4.7 ± 0.6</td>
<td>1.1 ± 0.3</td>
<td>5.5 ± 0.1</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>5.2 ± 0.8</td>
<td>2.9 ± 1.5</td>
<td>4.0 ± 0.1</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>51.9 ± 4.6</td>
<td>82.9 ± 1.2</td>
<td>25.8 ± 2.3</td>
<td>21.4 ± 1.2</td>
</tr>
<tr>
<td>Sterol esters</td>
<td>2.6 ± 0.6</td>
<td>1.1 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>
The differences observed in electrolyte leakage patterns between species, and hence their sensitivity to 4-octyl cyclopentenone, could be accounted for by differences in their lipid composition. In order to evaluate this we extracted the lipids from the fungal spores and determined their composition.

Hot 2-propanol was used to extract the lipids as it inactivates lipid-degrading enzymes which can yield artefactual results (Griffiths et al., 2003). Palmitate (16:0), stearate (18:0), oleate (18:1 n-9), linoleate (18:2 n-6) and α-linolenate (18:3 n-3) were detected in varying proportions in all fungal spores, with 18:2 (n-6) being the major component in all species (Table 1). Lipid extracts were purified by TLC into lipid classes, yielding polar lipids, monoacylglycerols, diacylglycerols, non-esterified (free) fatty acids, triacylglycerols and sterol esters (Table 1). In C. herbarum and B. cinerea, polar lipids were the major components (>60%) with significantly lower levels of storage triacylglycerols. However, in A. brassicicola and F. oxysporum, the converse was true, with storage triacylglycerols being the major components, constituting 52 and 83%, respectively, of the total lipid mass. The triacylglycerols in all species contained significant levels of all of the major fatty acids detected. However, the acyl composition of the phospholipids was more conservative and was dominated in all species by 18:2 (n-6) (Fig. 7). Triacylglycerols are absent from cell plasma membranes (Harwood & Russell, 1984), which are rich in polar lipids and whose fluidity is largely influenced by the degree of unsaturation of the constituent fatty acids. The ratio of unsaturated to saturated fatty acids and the degree of unsaturation, calculated as Δ mol⁻¹ = [1 × (%18:1) + 2 × (%18:2) + 3 × (%18:3)]/100, are given in Table 1. The value of Δ mol⁻¹ was similar for all four species, reflecting the high content of 18:2 (n-6) in the polar lipids. However, the ratio of unsaturated fatty acids to saturated fatty acids varied between the species, with the highest ratio observed for C. herbarum and the lowest for F. oxysporum. These data reveal no obvious correlation between membrane polar lipid composition and antifungal activity of the test compounds.

![Fig. 7. Fatty acid composition (μg fatty acid per 100 μg lipid extract) of various lipid classes for (a) A. brassicicola, (b) F. oxysporum, (c) C. herbarum and (d) B. cinerea GLUK-1. Fatty acid abbreviations: 16:0, palmitate; 18:0, stearate; 18:1 (n-9), oleate; 18:2 (n-6), linoleate; 18:3 (n-3), α-linolenate. Lipid abbreviations: DAG, diacylglycerol; MAG, monoacylglycerol; NEFA, non-esterified fatty acids; TAG, triacylglycerol. Results are means ± SD of three separate experiments.](http://mic.sgmjournals.org)
DISCUSSION

The synthetic compound 4-octyl cyclopentenone, having an eight-carbon alkyl side arm, showed the highest and most consistent suppression of spore germination and mycelial growth of fungal pathogens. Decreasing the side arm chain length to six or four carbons greatly reduced efficacy, as did introducing a carboxyl terminus to the eight-carbon chain. Complete suppression of spore germination and mycelial growth was observed for C. herbarum and B. cinerea, while both F. oxysporum and A. brassicicola showed recovery after prolonged exposure. Previously, we have shown that 12-oxo-PDA, a naturally occurring cyclopentenone and a precursor of JA, was highly effective at suppressing spore germination and growth in a number of fungal pathogens, including C. herbarum and B. cinerea (Prost et al., 2005). 4-Octyl cyclopentenone shows similar structural features to 12-oxo-PDA in that it consists of an alkyl side arm attached at the C-4 position of the cyclopentenone ring. 12-Oxo-PDA, however, also contains an additional side arm at the C-5 position consisting of a pentenyl group; however, this group appears to play little direct role in antifungal activity since JA and 12-oxo-PDA share this structural feature and JA is ineffective at suppressing spore germination and mycelial growth. The observations here indicate that 4-octyl cyclopentenone can mimic the spore suppression activity of 12-oxo-PDA. Calculations of the lipophilicity of the two molecules indicate that they have similar log P values (partition coefficients) of 4.5 and 4.6, respectively. This is in contrast to the other derivatives, including one with an eight-carbon alkyl side arm with a carboxyl terminus whose log P is considerably lower (2.8) (Fig. 2c). The lack of activity of the carboxyl group can be accounted for by its reduced uptake by spores. Such low affinity could result from the lack of a lipophilic pentenyl chain on C-5 of the cyclopentenone ring. Another possibility could be that the anionic carboxyl group interacts either inter- or intramolecularly with the keto carbon of the ring (C\( ^{\dagger} \)) to form dimers or induce alkyl chain bending due to the close proximity of these moieties. Either arrangement would be likely to hinder uptake across the plasma membrane due to increased steric hindrance. These results are in overall agreement with work on antifungal activity of non-ionic surfactants, such as 2(E)-alkenals (Kubo et al., 2003), and confirm that lipophilicity is an important factor in determining the antifungal activity of reactive electrophilic agents by balancing the hydrophobic and hydrophilic moieties (Kubo et al., 2003). Interestingly, eight-carbon oxylipins have recently been shown to inhibit spore germination and growth in Aspergillus nidulans (Herrero-Garcia et al., 2011).

The most sensitive species, C. herbarum and B. cinerea, showed increased electrolyte leakage following exposure to 4-octyl cyclopentenone, indicating membrane perturbation, which was not evident for the species that recovered from the challenge. However, this increased sensitivity was not related to the degree of unsaturation or to the unsaturated/saturated fatty acid ratio of the membrane polar lipids. Interestingly, however, the two species which recovered from the 4-octyl cyclopentenone challenge, F. oxysporum and A. brassicicola, also have a high triacylglycerol (oil) content. As triacylglycerols are not present in the plasma membrane, direct interaction with the compound is unlikely. Membranes damaged by exposure to the compound such as by increased oxidation and or hydrolysis of lipid constituents would be subject to membrane repair mechanisms. This would involve de-acylation and re-acylation reactions (Shah, 2005) and it is possible that the energy requirements for this process could be better supported by a large store of triacylglycerols acting as both a potential energy source, through \( \beta \)-oxidation (Gurr et al., 2002), and as a direct source of pre-synthesized fatty acids. Besides these differences in acyl lipid composition, other important factors are the concentration of ergosterol (Avis & Belanger, 2001) and the modification of proteins (Myung et al., 2007) following exposure to the test compounds; these are currently being investigated.

The results of this study reveal that 4-octyl cyclopentenone is highly effective as an antifungal agent against C. herbarum and B. cinerea. The compound is relatively simple to synthesize, involving only a two-step process that is considerably easier than the multistep synthesis of 12-oxo-PDA (Ainai et al., 2003), a compound that has previously been demonstrated to have spore-suppressing activity (Prost et al., 2005). The two compounds share structural similarities and lipophilicity, and hence 4-octyl cyclopentenone has potential as a commercial fungicidal agent.

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REFERENCES


8-Carbon oxylipins inhibit germination and growth, and activation in plants: biosynthesis of antifungal trihydroxy oxylipins in leaves of Aspergillus nidulans stimulate aerial conidiation in Saccharomyces cerevisiae. Characterization of a novel lipoxygenase-independent senescence mechanism in Arabidopsis thaliana reveals a specific role in supplying fatty acid hydroperoxides for aliphatic aldehyde production.


