Filipins: the first antifungal “weed killers” identified from bacteria isolated from the trap-ant

Hong Gao\textsuperscript{a,b}, Sabine Grüschow\textsuperscript{a,b}, Jörg Barke\textsuperscript{c}, Ryan F. Seipke\textsuperscript{c}, Lionel M. Hill\textsuperscript{d}, Jérôme Orivel\textsuperscript{e}, Douglas W. Yu\textsuperscript{c,f}, Matthew Hutchings\textsuperscript{c} and Rebecca J.M. Goss\textsuperscript{a,b,*}

\textsuperscript{a} School of Chemistry, University of St. Andrews, St. Andrews, Fife, KY16 9ST, UK
\textsuperscript{b} School of Chemistry, University of East Anglia, Norwich Research Park, Norwich, Norfolk, NR4 7TJ, UK
\textsuperscript{c} School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, Norfolk, NR4 7TJ, UK
\textsuperscript{d} John Innes Centre, Norwich Research Park, Norwich, Norfolk, NR4 7UH, UK
\textsuperscript{e} CNRS, UMR Ecologie des Forêts de Guyane, Campus Agronomique, BP 316, 97379 Kourou Cedex, France
\textsuperscript{f} State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Kunming, Yunnan, 650223, China

* Email: rimg@st-andrews.ac.uk; Tel: +44 (0) 1334 463856.

Materials and Methods

Bacterial strains and culture conditions
The Streptomyces strain FG26 was isolated by Seipke \textit{et al.}\textsuperscript{1}. The strain was streaked on SFM agar plates (2.0% soy flour, 2.0% mannitol, and 2.0% agar) and incubated at 28 °C for 7 d. Then a single colony was picked up to inoculate ISP2 media (0.4% yeast extract, 1.0% malt extract and 0.4% glucose; pH 7.2) as the starter culture. Following 3 d of growth at 28 °C and 180 rpm shaking, the starter culture was inoculated into the fermentation media (Table S1) at 1% culture volume to produce antifungal compounds and was grown for 8 d at 28 °C and 180 rpm shaking (incubator throw 19 mm).

Isolation and detection of filipins complex
After fermentation, the whole cell culture was extracted with an equal volume of ethyl acetate, then the organic solvent phase was evaporated. The solid residue from 50 mL of culture was dissolved in 1 mL of methanol.

The extract was fractionated by column chromatography (Sephadex, 3 cm * 40 cm column, mobile phase: methanol), 60 fractions were collected, and the volume for each fraction was 10 mL. The resulting fractions were analyzed for antifungal activity.

Samples were analyzed by HPLC with a Phenomenex Luna 5a C18(2) column. For HPLC analysis, the column was developed using a gradient of 10-95% acetonitrile in water (0.01% trifluoroacetic acid added) for 6 min at a flow rate of 0.6 mL min\textsuperscript{-1}.

For LC-MS analysis samples were re-dissolved in water: methanol 50:50 and separated using a Surveyor LC
(Thermo Finnigan) on a Phenomenex Luna C18(2) column (100×2mm, 3μm) with a linear gradient of 20 – 95 % methanol against 0.1 % formic acid in water over 25 min at a flow rate of 0.24 ml min\(^{-1}\).

A LCQ DecaPlus\(^{XP}\) ion trap (Thermo Finnigan) was used for nominal mass LC-MS/MS, and a LTQ Orbitrap mass spectrometer (Thermo Scientific) was used at 60,000 resolution for HR mass analysis. The assignment of individual filipins (II, III, IV) is based on comparison to the published relative retention times \(^2\).

**Antifungal Assay**

The antibacterial activity of the crude extracts and pure compounds was determined using the paper disc diffusion method\(^3\). Briefly, crude extracts and nystatin (as positive control) was reconstituted in an appropriate volume of MeOH, after which they were dispensed into paper bioassay discs. An equal volume of MeOH solvent, which was used as a negative control, was also applied onto a disc. *Candida albicans* was grown in LB medium at 37 °C overnight, then 100 μl of culture was dispensed into LB agar medium. The dried discs were placed onto agar plates and incubated at 37 °C overnight. The diameter of the zone of inhibition was used to evaluate the antibacterial activity. All assays were performed in triplicate.

**Identification and characterization of the filipins' biosynthetic cluster cluster**

Following genome sequencing, genes coding for type I polyketide synthases (PKS) were identified and analyzed by software Open Reading Frame Finder protocol of the NCBI (ORF Finder, NCBI)\(^4\). The corresponding deduced proteins were compared with the known proteins for filipin biosynthesis in *Streptomyces avermitilis*\(^5\) using available BLAST methods (http://www.ncbi.nlm.nih.gov/blast).
Table S1. Fermentation media used in this study

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Medium composition per litre</th>
<th>Reference</th>
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<tbody>
<tr>
<td>M1</td>
<td>10 g soluble starch, 4 g yeast extract, 2 g peptone</td>
<td>6</td>
</tr>
<tr>
<td>M2</td>
<td>1 g L-arginine, 1 g K$_2$HPO$_4$, 0.5 MgSO$_4$·7H$_2$O, 6 ml of 100% glycerol</td>
<td>6</td>
</tr>
<tr>
<td>M3</td>
<td>ISP-2: 4 g yeast extract, 10 g malt extract, 4 g glucose</td>
<td>7</td>
</tr>
<tr>
<td>M4</td>
<td>0.1 g asparagine, 0.5 g K$_2$HPO$_4$, 0.001 g FeSO$_4$·7H$_2$O, 0.1 g MgSO$_4$·7H$_2$O, 2 g peptone, 4 g sodium propionate</td>
<td>8</td>
</tr>
<tr>
<td>M5</td>
<td>10 g beef extract, 4 g peptone, 10 g brain heart infusion, 5 g yeast extract, 5 g glucose, 15 g K$_3$PO$_4$, 1 g starch, 1 g (NH$_4$)$_2$SO$_4$, 1 g cysteine, 0.2 g MgSO$_4$·7H$_2$O, 0.01 g CaCl$_2$</td>
<td>9</td>
</tr>
<tr>
<td>M6</td>
<td>4 g beef extract, 4 g peptone, 1 g yeast extract, 10 g glucose</td>
<td>9</td>
</tr>
<tr>
<td>M7</td>
<td>2 g peptone, 0.1 g asparagine, 4 g sodium propionate, 4 g K$_2$HPO$_4$, 0.1 g MgSO$_4$·7H$_2$O, 0.001 g FeSO$_4$·7H$_2$O, 5 g glycerol</td>
<td>9</td>
</tr>
<tr>
<td>M8</td>
<td>4 g yeast extract, 15 g soluble starch, 1 g K$_2$HPO$_4$, 0.5 g MgSO$_4$·7H$_2$O</td>
<td>9</td>
</tr>
<tr>
<td>M9</td>
<td>20 g soluble starch, 1 g KNO$_3$, 0.5 g K$_2$HPO$_4$, 0.5 g MgSO$_4$·7H$_2$O, 0.01 g FeSO$_4$·7H$_2$O</td>
<td>9</td>
</tr>
<tr>
<td>M10</td>
<td>Terrific broth (Sigma)</td>
<td>-</td>
</tr>
<tr>
<td>M11</td>
<td>TSB medium (Difco)</td>
<td>-</td>
</tr>
<tr>
<td>M12</td>
<td>SFM medium</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure S1: LC-MS of filipins complex produced in FG26
Figure S2: HR-MS of filipin III produced in FG26

For M = C_{35}H_{58}O_{11}, [M+Na]^+ found 677.3872; expected 677.3871; error 0.127ppm
For \( M = C_{35}H_{58}O_{11} \), \([M+Na]^+\) found 677.3871; expected 677.3871; error -0.005 ppm
Figure S4: HR-MS of filipin II produced in FG26

For $M = C_{35}H_{58}O_{10}$, $[M+Na]^+$

found 661.3922; expected 661.3922; error -0.014 ppm
Figure S5: LC-MS of a co-injection of FG26 extract and filipin complex standard. The intensity of the peak of filipin III was increased after co-injected with filipin complex standard.
Figure S6: MS² spectra for filipin III (A) and II (B)

FG26 extract

FG26 extract

NL: 8.44E6
21b06#1081-1186 RT: 20.58-22.02 AV: 3 F: + c d Full
ms2 677.36@cid35.00 [175.00-1365.00]

NL: 8.04E6
21b04#1126-1172 RT: 20.60-20.93 AV: 2 F: + c d Full
ms2 677.36@cid35.00 [175.00-1365.00]

NL: 7.25E6
21b06#1219-1260 RT: 23.24-23.30 AV: 2 F: + c d Full
ms2 661.41@cid35.00 [170.00-1335.00]

NL: 5.31E6
21b04#1239-1294 RT: 23.22-23.27 AV: 2 F: + c d Full
ms2 661.38@cid35.00 [170.00-1335.00]
Figure S7. UV absorption of filipin III

Filipin complex
standard

NL: 6.12E5  
21b08#1229-1242  
RT: 20.47-20.68  
AV: 14 F: + c ESI Full ms  
[150.00-2000.00]

FG26 extract

NL: 4.50E5  
21b04#1231-1245  
RT: 20.50-20.73  
AV: 15
Figure S9. Structure-based sequence alignments of PteC/FlpC (A) and PteD/FlpD (B) generated using SwissModel. Substrate binding residues are highlighted.

References: