Insights into Lasalocid A Ring Formation by Chemical Chain Termination In Vivo**

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Synthesis and purification of 5 a-c

The methyl esters 5 a-c were prepared as previously reported.\(^1,2\) 5 a-c were purified by preparative HPLC on a Phenomenex Luna C18(2) 100A AX1A Pa column (250.0 x 21.2 mm, 10 μm) using an Agilent HP 1200 HPLC. Mixtures of water and methanol (HPLC grade, both with 0.1% formic acid) were used with a flow rate of 20.0 mL/min and an elution gradient starting from 100 % water and linearly increasing to 100 % methanol over 30 min, with UV detection set at 254, 280 and 210 nm. 5 a-b eluted at 11.0 min, whereas 5 c eluted at 13.6 min. The NMR data for 5 a-c corresponded to those previously reported.\(^1,2\)

Construction of mutant strains of *Streptomyces lasaliensis*

The cultivation of the lasalocid-producing strain *S. lasaliensis* NRRL3382 was as previously described.\(^3\) The construction of *S. lasaliensis* ΔlasB and ΔlasC has also been previously reported.\(^4\) The construction of *S. lasaliensis* ACP12 (S970A) and ACP7 (S987A) was accomplished by site-directed mutagenesis as follows:

- **S. lasaliensis** ACP12 (S970A): a pair of complementary primers was designed to replace the ACP active site serine codon of module 12 (lasAVII) with an alanine codon. These primers were labelled as o12_SDMF, oP12R, o12_SDMR and oP12L (Table 1S). The template for PCR was cosmid DNA encoding the appropriate portion of the cloned lasalocid gene cluster.\(^4\) The forward primer o12_SDMF was paired with oP12R, which primes 1.5 kbp downstream of the active site and incorporates a HindIII site, to amplify a 1.5 kbp DNA fragment containing a HindIII restriction site. The reverse primer o12_SDMR was paired with oP12L, which incorporates an NdeI site to amplify the 1.5kbp upstream fragment which overlaps with the downstream fragment over the length of the mutagenesis primers. The two flanking regions were combined and overlap extension PCR was carried out using the primer pair oP12R and oP12L to give a 3 kbp DNA fragment containing flanking regions as well as the mutated active site and incorporating NdeI and HindIII restriction sites. This DNA

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fragment was gel purified and sequentially digested with *Nde* I and *Hind*III before being gel purified and ligated into *Nde* I- and *Hind*III- digested pHY7 to give the plasmid pA12M.

- *S. lasaliensis* ACP7 (S987A): similarly a mutant in which the ACP domain of module 7 was inactivated was prepared utilising primer pairs o7_SDMF, oP7R, o7_SDMR and oP7L (Table 1S). Overlap extension PCR and cloning into pYH7 was carried out as described above to give plasmid pA7M.

The ligated plasmids were transformed into *E. coli* strain DH10B and positive colonies were tested by restriction mapping and sequencing before a correct clone was transferred to *E. coli* ET12567/pUZ8002. Conjugation was carried out with the *S. lasaliensis* wild type as well as mutants lasΔB and lasΔC as previously described. Candidate mutants were confirmed by sequencing of the mutant region utilising oligonucleotides oA12SF and oA12SR, and oA7SF and oA7SR (Table 1S). Individual verified mutants for each targeted ACP were fermented and LC-MS analysis of the ethyl acetate extracts of each culture showed that lasalocid production was completely abolished in all cases.

**Table 1S.** Primers utilised for the construction and the sequencing of *S. lasaliensis* ACP12 (S970A) and ACP7 (S987A).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>o12_SDMF</td>
<td>CATCGACGCACGACGGCGGTGGAACCTGCGC</td>
</tr>
<tr>
<td>o12_SDMR</td>
<td>GCGCAGTTCCACCACGCGTGTGCATCGCATG</td>
</tr>
<tr>
<td>oP12L</td>
<td>GTGCCGTGCTCATTGCGCCA</td>
</tr>
<tr>
<td>oP12R</td>
<td>GGAATCGCAAGCTTGACACGC</td>
</tr>
<tr>
<td>oA12SF</td>
<td>GCATCGTGCGAGATCTTCATGG</td>
</tr>
<tr>
<td>oA12SR</td>
<td>CATCGCTGCTCCTTGTCTGTG</td>
</tr>
<tr>
<td>o7_SDMF</td>
<td>GACCTGGGCTTCGACGCGCCCTACCGCGACG</td>
</tr>
<tr>
<td>o7_SDMR</td>
<td>GCGTCGCGGTGAGCAGCGTCAAGCCAGGTC</td>
</tr>
<tr>
<td>oP7L</td>
<td>GGCAGCCATATGAGCCCAGG</td>
</tr>
<tr>
<td>oP7R</td>
<td>GCGGTGAGACAAAGCTTTGTCCA</td>
</tr>
<tr>
<td>oA7SF</td>
<td>GTCGGCGTACTGGCTCGA</td>
</tr>
<tr>
<td>oA7SR</td>
<td>CGGTGTTCACCACGTCTGC</td>
</tr>
</tbody>
</table>

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Growth of *S. lasaliensis* strains and mass spectrometry analysis

All the strains were grown in M79 medium (10 mL) for 3 days at 30 °C. The seed cultures (100 µl aliquots) were plated onto MYM-agar (5 mL, in duplicate/triplicate copy) containing 5 a-c (10 mM), 2,6-O-dimethyl-β-cyclodextrin (0.6 mM) and 4-pentynoic acid (as oxidation inhibitor, 2 mM). Control plates in absence of 5 a-c or the strains were also prepared. After incubation at 30 °C for 5 days the agar plates were extracted twice with ethyl acetate (10 mL x 2). The extracts were concentrated and the residues were redissolved in HPLC-grade methanol (1 mL) for mass spectrometry analysis.

The seed cultures were also used to inoculate MYM liquid cultures (10mL, in duplicate/triplicate copy), for which an established feeding protocol was followed. After incubation of the production cultures for 2 days at 30 °C, 2,6-O-dimethyl-β-cyclodextrin (5 mg) was added, followed within two hours by more 2,6-O-dimethyl-β-cyclodextrin (3 mg), 4-pentynoic acid (2 mg) and 5 a-c (10 mM). After further shaking at 30 °C for 3 days, the cultures were extracted twice with ethyl acetate (10 mL x 2). The extracts were concentrated and the residues were redissolved in HPLC-grade methanol (1 mL) for mass spectrometry analysis.

HPLC-HR-ESI-MS analyses of *S. lasaliensis* extracts were performed on a Thermo Electron LTQ-Orbitrap. Samples (1- 10 µl) were injected onto a Dionex Acclaim C18 PepMap 100 column (150 mm x 1.0 mm, 3 µm), eluting with a linear gradient of 0 % to 100 % B in 28 min with a flow rate of 50 µl/min (A: 98 % H₂O, 2 % MeCN, 0.1 % formic acid, B: 90 % MeCN, 10 % H₂O, 0.1 % formic acid). The mass spectrometer was run in positive ionization mode, scanning from m/z 100 to 1800, with the FTMS analyser resolution set at 60K. Selected ion search within 5 ppm was performed, as well as high resolution fragmentation (collision energy set to 30-35%) for the putative biosynthetic intermediates.

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Off-loading of intermediates from wild type *S. lasaliensis* via 5 a

**Figure 1S:** LC-ESI-HRMS analysis of the organic extracts of wild type *S. lasaliensis* grown in the presence of methyl 6-acetamido-3-oxohexanoate 5 a (10 mM). The Total Ion Current and the \([M+Na]^+\) extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 7-9 a are shown. These species have been also detected as ammonium adducts (data not shown) and were absent in all the control samples (e.g. in the absence of 5 a, Figure 2S). Their stereochemistry is yet to be separately determined; further work is in progress to establish this, as well as the origin of multiple peaks for 7 a (possibly arising from the presence of isomers).
Figure 2S: LC-ESI-HRMS analysis of the organic extracts of wild type *S. lasaliensis* grown in the absence of methyl 6-acetamido-3-oxohexanoate 5a (10 mM). The Total Ion Current and the $[\text{M+Na}]^+$ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 7-9 a show no evidence of the presence of these compounds.
Off-loading of intermediates from wild type *S. lasaliensis* via 5 c

**Figure 3S:** LC-ESI-HRMS analysis of the organic extracts of wild type *S. lasaliensis* grown in the presence of methyl 6-acetamido-2-methyl-3-oxohexanoate 5 c (10 mM). The Total Ion Current and the [M+Na]^+^ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 8-9 c are shown. These species have been also detected as ammonium adducts (data not shown) and were absent in all the control samples (e.g. in the absence of 5 c, Figure 4S); their stereochemistry is yet to be separately established.
Figure 4S: LC-ESI-HRMS analysis of the organic extracts of wild type *S. lasaliensis* grown in the absence of methyl 6-acetamido-2-methyl-3-oxohexanoate 5 c (10 mM). The Total Ion Current and the [M+Na]$^+$ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 8-9 c shown no evidence of the presence of these compounds.
Off-loading of intermediates from *S. lasaliensis* ACP12 (S970A) via 5 a

**Figure 5S:** LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ACP12 (S970A) grown in the presence of methyl-6-acetamido-3-oxohexanoate 5 a (10 mM). The Total Ion Current and the [M+Na]⁺ extracted ion traces (5 ppm mass accuracy) for the off-loaded intermediates 7-9 a are shown. These species are identical to those off-loaded from wild type *S. lasaliensis* with the aid of 5 a (Figure 1S) and were absent in control samples (Figure 6S).
Figure 6S: Above: LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ACP12 (S970A) grown in the absence of methyl-6-acetamido-3-oxohexanoate 5a (10 mM). Below: LC-ESI-HRMS analysis of the organic extracts of MYM plates containing methyl 6-acetamido-3-oxohexanoate 5a (10 mM). The Total Ion Current and the [M+Na]+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 7-9a show no evidence of the presence of these compounds.
Off-loading of intermediates from *S. lasaliensis* ACP12 (S970A) via 5 b

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**Figure 7S:** LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ACP12 (S970A) grown in the presence of methyl 6-(acetamido-\(\text{d}_3\))-3-oxohexanoate 5 b (10 mM). The Total Ion Current and the [M+Na]+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 7-9 b (identical in R\(_T\) and fragmentation pattern to their non-deuterated counterparts 7-9 a, Figures 5S, 10S and 12 S) are shown.
Figure 8S: Above: LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ACP12 (S970A) grown in the absence of methyl 6-(acetamido-d₃)-3-oxohexanoate 5 b (10 mM). Below: LC-ESI-HRMS analysis of the organic extracts of MYM plates containing methyl 6-(acetamido-d₃)-3-oxohexanoate 5 b (10 mM). The Total Ion Current and the [M+Na]⁺ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 7-9 b show no evidence of the presence of these compounds.
HR-MS$^n$ characterization of 7 a-b

C$_{39}$H$_{63}$NNaO$_8$

Calc.: 696.4446
Found: 696.4463 (±2.3 pm)
Figure 9S (previous page): HR-ESI-MS (top) and -MS$^2$ (bottom) analyses of the off-loaded dodecaketide 7 a. The right-hand fragment (m/z 377) is characteristic of lasalocid A. Further fragmentation of m/z 377 afforded exclusively m/z 359 (corresponding to loss of water, data not shown), whereas further fragmentation of m/z 342 did not lead to significant detectable fragments.

Figure 10S. Top: HR-ESI-MS$^2$ analysis of the off-loaded dodecaketide 7 b. Fragmentation of the [M+Na]$^+$ adduct still generates a right-hand fragment of m/z 377, as well as a left-hand fragment of increased mass (m/z 345, + 3Da) compared to that of 7 a (m/z 342, Figure 9S).

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HR-MS<sup>n</sup> characterization of 8a

The fragmentation pattern (the mechanisms of which are currently under detailed investigation) is identical to that of the analogous compounds 8b and c (Figures 12S and 13S).
HR-MS$^n$ characterization of 8 b and 8 c

Figure 12S: HR-ESI-MS$^2$ analyses of the putative undecaketide dienes 8 b (top) and 8 c (bottom). Their fragmentation pattern is identical to that of 8 a (Figures 11S and 13S).
Figure 13S: HR-ESI-MS$^3$ analyses of the putative undecaketide diene 8 c. The fragmentation pattern is identical to that of 8 a (Figure 11S) and is currently under investigation.
HR-MS$^2$ characterization of 9 a and 9 c

Figure 14S: HR-ESI-MS$^2$ analyses of the putative oxidized undecaketides 9 a (top) and 9 c (bottom): the right–hand m/z 377 fragment characteristic of lasalocid A$^9$ is generated, together with a left-hand fragment consistent with the proposed β-hydroxy structure.
Off-loading of intermediates from *S. lasaliensis* ΔlasB via 5 a

Figure 15S: LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ΔlasB grown in the presence of methyl 6-acetamido-3-oxohexanoate 5 a (10 mM). The Total Ion Current and the [M+Na]^+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 8 a and 10-11 a are shown. These species have been also detected as ammonium adducts (data not shown). The increase in retention time of 11 a and 10 a compared to 9 a-b and 7 a-b (off-loaded from wild-type and ACP12 (S970A) *S. lasaliensis* strains, cfr. Figures 1S, 5S and 7S) is consistent with their proposed iso-lasalocid structure (Note: *S. lasaliensis* ΔlasB produces iso-lasalocid exclusively). Their stereochemistry is yet to be determined; further work is in progress to establish this, as well as the origin of multiple and/or broad peaks.
Figure 16S: LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ΔlasB grown in the absence of methyl 6-acetamido-3-oxohexanoate 5a (10 mM). The Total Ion Current and the [M+Na]^+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 8a and 10-11a show no evidence of the presence of these compounds.
Off-loading intermediates from *S. lasaliensis* ΔlasB ACP12 (S970A) via 5a

**Figure 17S:** LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ΔlasB ACP12 (S970A) grown in the presence of methyl 6-acetamido-3-oxohexanoate 5a (10 mM). The Total Ion Current and the [M+Na]^+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded intermediates 8a and 10-11a are shown. These species have been also detected as ammonium adducts (data not shown) and are identical to those of Figure 11S. In the absence of 5a no 8a, 10-11a were detected (data not shown).
HR-MS$^2$ characterization of 11 a

Figure 18S: HR-ESI-MS$^2$ analysis of the putative oxidized undecaketide 11 a (based on iso-lasalocid). A right-hand fragment of m/z 377 is generated, together with a left-hand m/z 322 fragment consistent with the proposed β-hydroxy structure, likewise to 9 a (Figure 14S). In the same way HR-ESI-MS$^2$ analysis of the dodecaketide 10a (isolasalocid-like) resulted in an identical fragmentation pattern to that of 7 a (Figure 9S). Further fragmentation of m/z 377 and of the left-hand fragments of 7 a-b, 9 a-b and 10-11 a did not lead to smaller significant fragments. Currently lasalocid and iso-lasalocid species (e.g. 9 a in Figure 14S, and 11 a herein) are distinguishable only on the basis of their different retention time. Further analyses are in progress in order to discriminate the different structural feature of 9 a and 11 a related to their polyether portion.
Off-loading of intermediates from *S. lasaliensis* ΔlasC via 5 a-b

**Figure 19S:** LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ΔlasC grown in the presence of methyl 6-acetamido-3-oxohexanoate 5 a-b (both 10 mM, top and bottom trace respectively). The Total Ion Current, the [M+Na]+ extracted ion traces (5 ppm mass accuracy) for the putative off-loaded octaketides 12 a-b and the [M+H]+ extracted ion trace for the putative δ-lactone 13 are shown. These species were absent in control samples (e.g. cfr *S. lasaliensis* ΔlasC ACP7
(S987A), Figure 20S). Besides no further advanced intermediates (such as the undecaketide dienes 8a/c) nor prelasalocid 3 (or prelasalocid-based species) were identified.

Figure 20S: LC-ESI-HRMS analysis of the organic extracts of *S. lasaliensis* ΔlasC ACP7 (S987A) grown in the presence of methyl 6-acetamido-3-oxohexanoate 5 a. The extracted ion traces (5 ppm mass accuracy) for the putative octaketide 12 a ([M+Na]+) and the putative δ-lactone 13 ([M+H]+) show no evidence of the presence of these compounds.
HR-MS$^n$ characterization of 12 a

Figure 21S: HR-ESI-MS$^2$ (top) and MS$^3$ (bottom) analyses of the putative octaketide diene 12a. The fragmentation pattern (the mechanisms of which are currently under detailed investigation) is identical to that of the analogous deuterated compounds 12 b (Figure 22S).
HR-MS$^n$ characterization of 12 b

Figure 22S: HR-ESI-MS$^2$ (top) and MS$^3$ (bottom) analyses of the putative octaketide diene 12 b. The fragmentation pattern is identical to that of 12 a (Figure 21S). Both 12 a and 12 b major fragments (m/z 308 and 311 respectively) derive from the formal loss of 207 Da. m/z 207 was found to be a significant fragment of the δ-lactone 13 (Figure 23S). Further investigation of the mechanisms of fragmentation of 12 a-b and 13 are currently ongoing in our laboratories and the results will be reported in due course.
**HR-MS^n characterization of the putative δ-lactone 13**

![HR-MS^n characterization of the putative δ-lactone 13](image)

**Figure 23S:** HR-ESI-MS² (top) and MS³ (bottom) analyses of the putative δ-lactone 13.