
Peer reviewed version

Link to published version (if available):
10.1021/ja501731p

Link to publication record in Explore Bristol Research
PDF-document

This is the accepted author manuscript (AAM). The final published version (version of record) is available online via American Chemical Society at http://dx.doi.org/10.1021/ja501731p. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/
The Biosynthesis of Mupirocin by *Pseudomonas fluorescens* NCIMB 10586 Involves Parallel Pathways

Shu-Shan Gao,§,† Joanne Hothersall,§,† Ji’en Wu,§,† Annabel C. Murphy,§,† Zhongshu Song,§ Elton R. Stephens,§ Christopher M. Thomas,§ Matthew P. Crump,§ Russell J. Cox,§ Thomas J. Simpson,*§ and Christine L. Willis*§

# School of Chemistry, University of Birmingham, Birmingham, B15 2TT, UK

§School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

ABSTRACT: Mupirocin, a clinically important antibiotic produced via a *trans-AT* Type I polyketide synthase (PKS) in *Pseudomonas fluorescens*, consists of a mixture of mainly pseudomonic acids A, B and C. Detailed metabolic profiling of mutant strains produced by systematic inactivation of PKS and tailoring genes, along with re-feeding of isolated metabolites to mutant strains, has allowed the isolation of a large number of novel metabolites, identification of the 10,11-epoxidase and the full characterisation of the mupirocin biosynthetic pathway which proceeds via major (10,11-epoxide) and minor (10,11-alkene) parallel pathways.

INTRODUCTION

Mupirocin, a mixture of pseudomonic acids, produced by *Pseudomonas fluorescens* NCIMB 10586, is a clinically important antibiotic against Gram positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). It inhibits bacterial isoleucyl-transfer RNA synthetase and is currently the standard treatment used worldwide for the topical control of MRSA. The major component of mupirocin is pseudomonic acid A (1, PA-A, Fig. 1), which accounts for ca 90% of the mixture. It was identified as one of the first of an extensive family of antibiotics produced by the *trans-AT* class of modular polyketide synthases (PKS). It consists of a C7 polyketide-derived substructure (monic acid) esterified by 9-hydroxynonanoic acid (9-HN). The other main components are pseudomonic acid B (2, PA-B, 8%) which has an additional hydroxyl group at C8, and pseudomonic acid C (3, PA-C, < 2%) which has a double bond in place of the epoxide group at C8-C9. PA-D (1, 4’,5’-alkene) has also been reported as a very minor component. Simple biosynthetic logic would suggest that PA-A is formed by epoxidation of the 10,11-double bond in PA-C 3, and PA-B 2, by a further hydroxylation of PA-A at C8.

Novel pseudomonic acid analogues, the thiomarinols, e.g. thiomarinol A 4, and also active against MRSA, have been isolated from marine organisms, e.g., *Aleromonas* sp. SANK 73390. The thiomarinols are closely related to mupirocin, being produced by a very similar biosynthetic gene cluster with the addition of a non-ribosomal peptide synthase (NRPS)-encoded pyrroline moiety attached via an amide to an 8-hydroxyoctanoic acid moiety. The 74-kb mupirocin biosynthetic gene cluster (Fig. 2A) encodes six modular multifunctional proteins (Mmps) involved in polyketide and fatty acid biosynthesis and 26 single enzymes originally thought to perform largely tailoring functions. The first half of the cluster contains two large Type I multifunctional PKS genes (*mmpA* and *D*) plus associated trans-acyltransferases (*mmpC*), an iterative Type I FAS (*mmpB*), as well as two single ORFs *mupA* and *mupB*, while the second half contains twenty seven single ORFs (*mupC-X, maccA-E*), plus two smaller PKS-like genes *mmpE* and *mmpF*. MmpB and MmpA together contain six modules for condensation and reduction of acetate-derived units, which with two methyl transferase

![Figure 1. Major pseudomonic acids and thiomarinol A.](image-url)
domains could generate the backbone of a C₇-heptaketide monic acid precursor (I). Module 6 of MmpA, the final module involved in monic acid biosynthesis, contains twin ACPs each with a unique recognition motif specific for association with the "β-hydroxymethylglutaryl-CoA synthase" (HCS) cassette (MupG, H, J and K and MacC) responsible for the introduction of the 15-methyl β-branch (see in II). 9-HN is likely formed from a 3-hydroxy-propionate starter unit extended by three malonate condensations by MmpB functioning as an iterative FAS with additional enoyl reductase (ER) activity, perhaps provided by MupE and/or MupD. The resulting backbone would need further modifications to produce the major metabolite PA-A.

In-frame deletions demonstrated that all of the proteins are required for mupirocin production. However, many uncertainties remain, particularly in the timing of 10,11-oxidioperoxidation, tetrahydropyran (TPH) ring formation, 6-hydroxylation, fatty acid chain extension, and the precise roles of, inter alia: the mupW (dioxynogenase), mupC (oxidoreductase), mupF (ketoreductase), mupU (acyl CoA synthase), mupO (PA450), mupV (oxidoreductase), macpE (acyl carrier protein), and mmpE (ketosynthase/oxidoreductase) gene products.

Previous work on mutant strains containing single deletions of these genes led to the isolation of several novel PAs, including mupirocin W1 5, mupirocin C2 6 and mupirocin F1 8 (previously named mupricins W, C and F) from ΔmupW, ΔmupC and ΔmupF strains respectively (Figs. 3 and 4 below). The tetrahydrofuranc-containing metabolites 5 and 6 are likely rearrangement products formed by attack of a β-hydroxyl group onto C-10 of the labile epoxide. Deletions of mupV, mupO, mupU and macpE all resulted in a switch to production of PA-B 2 only 7 and the corresponding double mutants with addition of a ΔmupC mutation gave the same result suggesting that mupV, mupO, mupU and macpE all act before mupC, and by implication mupF. This led to the conclusion that PA-B 2 is produced either as a result of a branch in the pathway 7 or that it could be a precursor leading to PA-A 1 as had been proposed but not proven by Mantle et al. 8 The 10,11-epoxide in PA-A 1 makes it susceptible to intramolecular attack by the 7-OH outside a narrow pH range, which limits its clinical utility. PA-C 3, lacking the epoxide is similarly active to PA-A 1 but is much more stable. Thus a desirable goal in terms of obtaining a clinically more useful antibiotic would be to knock out the epoxidase activity and channel production entirely to PA-C 3. Further correlation of tailoring genes with catalytic function and resulting chemical modification was made difficult due to the so-called "leaky-hosepipe" mechanism 9 in which mutations of the HCS cassette and many others all produce an essentially identical phenotype in which pseudomonic acid biosynthesis is blocked and two truncated metabolites mupiric acid 13 and mupirocin H 14 (Fig 3) are isolated. (Mupirocin H is proposed 9 to result from a retro-aldol cleavage of intermediate 25 in Scheme 1 – see below). This phenotype was attributed to spontaneous release of these two compounds at chemically labile points, as a result of impeding metabolic flux along the assembly pathway by any mutation which interferes with formation of monic acid or its subsequent esterification by the MmpB-derived FAS.

We now report detailed investigations on mupirocin biosynthesis involving single and double gene knockouts leading to new insights into this complex pathway, including association of MmpE with introduction of the 10,11-epoxide. Single mutants as well as the WT strain were grown on a modified L-medium which crucially allowed more facile isolation of minor metabolites not previously observed in these strains. Similarly, a series of double mutants were analysed under the same conditions, and intermediates isolated in these experiments were re-fed to mutant strains. As a result of these studies definitive evidence has been obtained for the biosynthetic relationships among previously reported and novel pseudomonic acid metabolites, showing the presence of two parallel pathways, one major and one minor (see Scheme 1), and that epoxidation, tetrahydropyran ring
Figure 3. (A) Known and novel (*) metabolites isolated from extracts of WT and selected mutant strains of *P. fluorescens*. WT also contains PA-A and PA-B as the major products and a trace amount of PA-C. Metabolites in LH column are epoxide derived, RH column are 10,11-alkenes. Some compounds have been renamed to take account of new metabolites isolated. (B) HPLC traces of extracts of (a) WT, (b) mmpΔOR, (c) ΔmupW, (d) mmpΔOR/ΔmupW strains.

**RESULTS AND DISCUSSION**

**Analysis of WT and mutant strains**

We first investigated metabolite production by the WT strain of *P. fluorescens* NCIMB 10586 cultivated on the modified L-medium to determine if any previously undetected minor metabolites were present (see Supporting Information for details). PA-A (ca. 50 mg L⁻¹), PA-B 2, and PA-C 3 were isolated (Fig. 3B(a) and Table 1). Further detailed analysis of minor components resulted in the isolation of mupirocin W 5 and its analogue mupirocin W2 6 containing a shorter (C₅) fatty acid side chain, as well as four new mupirocin metabolites of which one, a macro lactonic derivative 12 of PA-B contained the 10,11-epoxide. The other three metabolites are: mupirocin W4 9 and mupirocin W5 10 each with a 10,11-alkene and hence lacking the tetrahydrofuran ring formed via the labile 10,11-epoxide, and 10,11-desepoxy-PA-B 11 (Fig. 3B(b)). These were isolated and purified, and their structures determined by full NMR analysis and HR-ESI-MS (see SI for details). These metabolites form two structurally distinct groups. The first group contains the 10,11-epoxide (1, 2 and 12) or rearrangement products (5 and 6) derived from it; while the second group lack the epoxide and contain a 10,11-alkene (3, 9, 10, and 11). This suggests that the first group of PAs (1, 2, 5, 6 and 12) which account for 97% of total PAs in the WT strain (Table 1) are intermediates, shunt or final natural products generated from the same dominant biosynthetic pathway, while a minor pathway involving intermediates lacking the 10,11-epoxide is responsible for the second group of PAs accounting for 1% of the total yield of PAs.

Previous studies failed to identify a monofunctional gene encoding a 10,11-epoxidase. We therefore considered domains of multifunctional proteins. One such candidate is the bifunctional MmpE which has a putative N-terminal KS and a putative C-terminal oxireductase (MmpEOR). Deletion (aa789 to aa1173) of the predicted MmpEOR domain and HPLC analysis (Fig. 3B(b)) indicated that production of PAs with a 10,11-epoxide or derived functionality are completely abolished and only PAs with a 10,11-alkene (PA-C 3, mupirocins W4 and W5 9 and 10, and desepoxy PA-B 11) accumulate in the mmpΔOR mutant, consistent with mmpEOR encoding the 10,11-epoxidase. The titres of these 10,11-alkenes (2.6 - 1.1 mg L⁻¹) isolated from the mmpΔOR mutant, while somewhat higher than in WT (0.4 - 0.2 mg L⁻¹, Table 1), remain very low. As indicated above mutation of *mupW* and also *mupT* (which encode a putative dioxygenase and associated ferredoxin dioxygenase respectively), produce mupirocin W1 5 lacking the THP ring and so these genes appear to be responsible for the oxidative activation required for the formation of the ring. In this study, we first refermented ΔmupW: HPLC (Fig. 3B(c)) showed the presence of 5, and its 7-hydroxyheptanoate analogue mupirocin W2 6 (each ca 1 mg L⁻¹). The ΔmupT mutant gave identical results (data not shown). Interestingly a double ΔmupW/ΔmupO mutant (originally constructed to test the relative timing of action of MupW and MupO) gave slightly higher titres of 5 and 6 and also allowed detection of smaller amounts of mupirocin W3 7, an analogue containing a further truncated 5-hydroxyoctanoate side chain and significantly, mupirocin W6 8, the only monic acid analogue so far isolated lacking a fatty acid side chain. To explore the relationship between epoxidation and other tailoring steps, the double mutant mmpΔOR/ΔmupW was then examined. Production of 5 and 6 was abolished, and only their desoxy
Table 1. Pseudomonic acids isolated from WT and mutant strains.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>mmpEΔOR</th>
<th>ΔmupW</th>
<th>mmpEΔOR/ΔmupW</th>
<th>ΔmupC</th>
<th>mmpEΔOR/ΔmupC</th>
<th>ΔmupF</th>
<th>mmpEΔOR/ΔmupF</th>
<th>ΔmupW/O</th>
<th>ΔKR6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-A 1</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-B 2</td>
<td>3.8</td>
<td></td>
<td></td>
<td></td>
<td>7.6</td>
<td>7.1</td>
<td>7.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-C 3</td>
<td>0.2</td>
<td></td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin W1 5</td>
<td>0.5</td>
<td></td>
<td>0.8</td>
<td></td>
<td>0.8</td>
<td>0.9</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin W2 6</td>
<td>0.3</td>
<td></td>
<td>0.9</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
<td>2.2</td>
<td></td>
<td>&lt;0.5</td>
<td></td>
</tr>
<tr>
<td>mupirocin W3 7</td>
<td>0.2</td>
<td></td>
<td>1.3</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin W6 W</td>
<td>0.4</td>
<td></td>
<td>1.1</td>
<td></td>
<td>1.3</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>mupirocin W5 10</td>
<td>0.4</td>
<td></td>
<td>2.4</td>
<td></td>
<td>1.6</td>
<td>1.1</td>
<td>2.1</td>
<td>1.4</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>10,11-deoxy-PA-B</td>
<td>0.3</td>
<td></td>
<td>2.3</td>
<td></td>
<td>0.7</td>
<td>1.8</td>
<td>1.1</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-B macrolactone</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin C1 15</td>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td>7.8</td>
<td></td>
<td></td>
<td>8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin C2 16</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin C3 17</td>
<td>0.5</td>
<td>8.4</td>
<td></td>
<td></td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin F1 18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mupirocin F2 19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>desepoxy-mupirocin F1</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-keto-mupirocin W4</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-keto-mupirocin W5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*New PAs reported for the first time in this study; †PAs derived from minor pathway. Values are given in mg.L⁻¹.

Figure 4. (A) Known and novel (*) metabolites isolated from extracts of selected mutant strains of *P. fluorescens*. LH column are epoxide derived, RH column are 10,11-alkenes. Some compounds have been renamed to take account of new metabolites isolated. (B) HPLC traces of extracts of (a) ΔmupC, (b) mmpEΔOR/ΔmupC, (c) ΔmupF, (d) mmpEΔOR/ΔmupF.

10,11-alkene analogues, mupirocins W₄ 9 and W₅ 10 (each ca. 1.5 mg.L⁻¹) accumulated (Fig. 3B(d)). Although the actual substrates for MupW/T and MmpEOR remained to be established (see below), it is notable that MmpE (Fig. 2) is positioned immediately after the “HCS cassette” responsible for the introduction of the β-branch 15-methyl. Also in the related thiamarinol gene cluster the tmlK and tmpE genes are fused (Fig. S2), encoding a tri-functional protein, suggesting that these activities act together, or in sequence – i.e. after the action of the final PKS module of MmpA, and immediately before THP formation (see intermediate 25, Scheme 1 below).

The ΔmupC mutant was also analysed and showed the presence of three major, and various minor, metabolites (Fig. 4B(a)). The three major products (80% total PAs, each ca. 8 mg.L⁻¹, Table 1) were identified as the novel mupirocin C1 15 and rearrangement product mupirocin C2 16 (presumably formed from 15), and PA-B 2. Both mupirocin C1 and C2 have C6 and C7 at the keto oxidation level. The minor products (0.7–1.3 mg.L⁻¹) were analogous to those seen in WT: mupirocin W₁ 5, W₂ 6, W₄ 9, W₅ 10, desepoxy-PA-B 11, and macrolactone 12.

The double mutant mmpEΔOR/ΔmupC produced mainly the 10,11-alkene containing PAs 9, 10, 11 (Fig. 4B(b)) and a new product identified as desepoxy-mupirocin 17. MupC was identified as a putative dienoyl-CoA reductase suggesting that it acts here on the enol ketones 15 and 17. A similar enone reductase activity has recently been reported for a reductase from a *Clostridium* sp.
which shows high homology to dienoyl-CoA reductases.\textsuperscript{20} When the ∆mup\textit{F} strain was examined (Fig. 4B(c)), the metabolite profile was almost identical to that from ∆mup\textit{C} with the addition of the previously reported\textsuperscript{16} mupirocin F1 \textit{18a} containing a 6-hydroxy, 7-keto moiety (along with 50\% of its 8-epimer \textit{18b}) and its epoxide-mediated rearrangement (see Scheme Si) product mupirocin F2 \textit{19} as additional major (ca 8 mg/L\textsuperscript{-1}) metabolites. The minor metabolite yields were essentially identical to those in the ∆mup\textit{C} mutant.

As with ∆mup\textit{C}, formation of the double mutant \textit{mmpEDOR}/∆mup\textit{F} simplified the metabolite profile (Fig. 4B(d)) which was very similar to \textit{mmpEDOR}/∆mup\textit{C} apart from a new metabolite which was identified as desepoxy-mupirocin \textit{F1 20}, the 10,11-alkene analogue of mupirocin \textit{F1 18}. This, and the results from the single mutant ∆mup\textit{F}, are consistent with Mup\textit{F} acting as a 7-ketoreductase with both hydroxy-ketones \textit{18} and \textit{20}.

Metabolites from double mutants ∆mup\textit{W}/∆mup\textit{C} and ∆mup\textit{W}/∆mup\textit{U} are identical to those of single mutant ∆mup\textit{W} (Fig. S3) and no other metabolites from the single mutants, ∆mup\textit{C} or ∆mup\textit{U}, are present: this confirms that Mup\textit{W} acts before Mup\textit{O}, Mup\textit{U}, Mup\textit{V} and Mup\textit{C}. The ∆mup\textit{C}/∆mup\textit{F} double mutant is identical to the single mutant ∆mup\textit{C} confirming that Mup\textit{C} acts before Mup\textit{F}. Interestingly, re-examination of the strain in which the KR domain (KR6) responsible for initial reduction at C7 during PKS assembly has been deleted, led to isolation of the previously reported\textsuperscript{19} truncated product mupric acid \textit{13}, along with the 7-keto analogues \textit{21} and \textit{22} of mupirocin W4 and W5. Thus a 7-hydroxy group is clearly required for action of MmpEDOR and MupW/T.

No compounds lacking a 6-OH have been detected. The gene responsible for 6-hydroxylation has not been definitively identified but must function before the HCS cassette as evidenced by the isolation of mupirocin H \textit{14} from mutation of any of the HCS cassette genes,\textsuperscript{19} or either of the MmpA twin acyl carrier proteins.\textsuperscript{17} Mutation of \textit{mupA} which encodes a putative FMNH-dependent oxygenase again gives the shunt metabolite mupric acid \textit{13} as the only detectable product. Thus 6-hydroxylation may be controlled by \textit{mupA}, and seems to be essential for further processing along the main pathway.

Chemical complementation experiments.

To further clarify the later stages of the biosynthetic pathways and to identify the substrates for the later-acting enzymes, a series of feeds to small-scale cultures (25 mL) were performed. Metabolites were fed to the mutant strains immediately after inoculation and the extracts were analysed by HPLC-MS (Fig. 5 and Fig. S4). We first fed PA-C \textit{3} to ∆mup\textit{W} in which the \textit{mmpE} gene is still intact and could potentially catalyse 10,11-epoxidation. After 60 hours fermentation, mupirocin W1 \textit{5} and W2 \textit{6} were produced as normal, but no metabolism of PA-C \textit{3} and no production of PA-A \textit{1} was detected. This is consistent with PA-A \textit{1} and PA-C \textit{3} being formed from a common intermediate by parallel pathways that diverge at 10,11-epoxidation. To further confirm this PA-B \textit{2} was fed to mutants ∆mup\textit{W} and \textit{mmpEDOR}. PA-A \textit{1} was produced in both cases with conversion rates of 35 and 20\% respectively. (Fig. 5 and Fig. S4(a) and (d)), consistent with PA-B \textit{2} being an intermediate on the main pathway to PA-A \textit{1}, (Scheme 1) as originally proposed by Mantle.\textsuperscript{18} On feeding the 10,11-alkene analogue \textit{11} of PA-B to ∆mup\textit{W}, PA-C \textit{3} is produced (7\% conversion, Figs. 5 and S4(b)), but no PA-A \textit{1}. Thus, neither PA-C \textit{3} nor the 10,11-alkene analogue of PA-B \textit{11} or intermediates derived from it can be the actual substrate for MmpEDOR. Thus, it seems that MmpEDOR acts before MupW/T and the parallel pathways must be active at the same time in the WT strain since both epoxidised and non-epoxidised intermediates (see 24 and 25, Scheme 1) can be processed to generate analogous products.

Mupirocin W4 \textit{9} and W5 \textit{10} were also fed to the \textit{mupH} strain, in which biosynthesis is truncated at the β-methylation (15-methyl) stage.\textsuperscript{19} No transformation of mupirocin W4 \textit{9} was observed, but mupirocin W5 \textit{10} (C7 side chain) was elongated to mupirocin W4 \textit{9} (C6 side chain) indicating that these intermediates can be taken up and metabolised by the cells (Fig S4i). This suggests that once the fatty acid side-chain elongation is underway, THP ring formation is not possible, again consistent with both MmpEDOR and MupW/T acting after polyketide assembly and before fatty acid side chain elaboration.

Additions of the enol-ketone mupirocin C1 \textit{15} to deletion strains of ∆mup\textit{U}, ∆mup\textit{V}, ∆mup\textit{O}, and ∆mup\textit{E} (which all produce PA-B \textit{2} but no PA-A \textit{1}) all (e.g. Fig S4e) gave good conversion to PA-A \textit{1} with most of the unincorporated substrate being transformed to its rearrange-ment
product mupirocin C2 16. Similarly PA-A was obtained (Fig S4j) on feeding mupirocin F1 18 to ΔmupV. These experiments confirm that MupC acts as an enone reductase, and MupF as a 7-ketoreductase.

CONCLUSIONS
We have elucidated details of the pathway to PA-B 2 and established its intermediacy in PA-A 1 biosynthesis. The efficient transformation of PA-B 2 to PA-A 1 and the switch to PA-B 2 only production on mutation of any of mupU, V, O, C, F and macpE suggests that modification of the pyran ring occurs after elaboration of the 9-hydroxynonanoic acid moiety on monic acid. This is consistent with the pathway shown in Scheme 1 in which β-keto ester 23, produced by the modular PKS proteins encoded by mmpD, mmpA and mmpC, along with the putative oxidase mupA, is the substrate for the HCS cassette to give the acyclic monic acid thioester 24. Thioester 24 is then epoxidised to give 25 and cyclised to 26 which is the substrate for stepwise fatty acid elongation to PA-B 2. This is then transferred (MupU) to MacpE where it is oxidised (MupO), dehydrated (MupV) and stepwise reduced (MupC and MupF) to give PA-A 1. It appears that release of PA-B from MmpB is dependent on the action of the terminal thioesterase (TE) domain. A mmpBΔTE mutant gives only the mupic acid/mupirocin H phenotype. This is further supported by the isolation of macro lactone 12 from WT and ΔmupC and ΔmupF strains. It is unlikely that macrolactisation could be spontaneous and it suggests that the TE domain can accept either the 13-OH or water to give both intramolecular or intermolecular catalysed release.

Isolation of, e.g. mupirocins 6, 7, 10 and 22, containing shorter fatty acid side chains demonstrates that, while monic acids lacking the THP ring can act as substrates for MmpB-dependent 9HN elongation, it is inefficient, consistent with MupW/T acting before MmpB.

PA-C 3, previously assumed to be a precursor to PA-A 1 is formed by a minor parallel pathway. Several of the intermediates involved in these parallel pathways were isolated from a series of single and double mutants and their involvement demonstrated by their transformation to either PA-C 3 or PA-A 1 on re-feeding to mutant strains. We have identified the gene responsible for epoxidation. This occurs at the end of assembly of the monic acid moiety 24 as indicated by lack of epoxidation on mupiric acid 13 and mupirocin H 14 but epoxidation has taken place in formation of the acyclic monic acid, mupirocin W6 8. Thus epoxidation can occur before MupW and MupT catalysed THP ring formation and ring formation is not essential for epoxidation. Nevertheless, since in all mutant strains and WT, non-epoxy metabolites appear in very low titres, epoxidation, like THP ring formation seems to be important for further processing of intermediates by later stage enzymes. Thus our goal of engineering P. fluorescens to give high titres of only PA-C may be unachievable and other means of achieving this are currently being explored.

ASSOCIATED CONTENT
Supporting Information.
Complete description of materials and methods, Tables 1 and 2, and Figures S1-S3, Scheme S1, including full 1H and 13C NMR data for all new compounds.

AUTHOR INFORMATION
Corresponding Author
tom.simpson@bristol.ac.uk
chris.willis@bristol.ac.uk

Scheme 1. Biosynthetic pathway to mupirocin and related metabolites. Structures of intermediates on the main pathway are shown. For minor pathway and other compounds, see Figs 3 and 4.
Present Addresses

1 Institute of Molecular and Cell Biology, Proteos, Singapore.
1 Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA.

Author Contributions

S-S.G. and J.H. contributed equally.

ACKNOWLEDGMENT

This work was funded by BBSRC/EPSRC grant E02161.

REFERENCES

Pseudomonic acid A

Pseudomonic acid B

Pseudomonic acid C