Mupirocin W, a novel pseudomonic acid produced by targeted mutation of the mupirocin biosynthetic gene cluster†

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Mutation of the *mupW* gene in the mupirocin biosynthetic gene cluster in *Pseudomonas fluorescens* results in efficient production of a novel pseudomonic acid metabolite, mupirocin W, which lacks the characteristic tetrahydropyran ring, and reveals the role of the *mupW* gene in pseudomonic acid biosynthesis.

Mupirocin is a potent inhibitor of Gram positive bacterial and mycoplasmal pathogens. It is used clinically for the treatment of bacterial skin infections and as a means of controlling *Staphylococcus aureus*, particularly MRSA when other antibiotics are ineffective.[1] It consists of a mixture of highly unusual polyketide metabolites (pseudomonic acids – PAs) produced by *Pseudomonas fluorescens*.[2] The major component, pseudomonic acid A **1** which accounts for 95% of the mixture, consists of a C_{17} unit (monic acid) which is derived from an unsaturated polyketide containing a tetrahydropyran ring and a C_9 saturated fatty acid (9-hydroxynonanoic acid, 9-HN). PA-B has an extra hydroxyl group at C-8, in PA-C the 10-11 epoxide is replaced by a double bond, and PA-D has further unsaturation in the 4′-5′ position of 9-HN. Other PA derivatives such as thiomarinol have been isolated from marine bacteria, *e.g. Altermonas* sp.[3] These contain an extra hydroxyl group at C-4 and various complex amide derivatives at C-1′. Due to their structural complexity and important biological activity, the PAs have been the subject of many total syntheses,[4] of which our recently reported route to PA-C is one of the most versatile.[5]

Mupirocin biosynthesis has been studied by classical methods which have revealed that the monic acid moiety of PA-A is derived from a linear combination of acetate units with introduction of one-carbon branches at C-16 and C-17 from methionine, whereas the 15-methyl is derived from the methyl of a cleaved acetate unit (Fig. 1).[6] 9-HN would appear to be formed (formally) by a fatty acid synthase (FAS) catalysed chain extension of a 3-hydroxypropionate unit whose origins remain obscure. Oxygen labelling studies[7] indicate that the oxygens attached to carbons 1, 5, 7, 13 and 9′ are acetate derived whereas the 6-hydroxyl and the 10,11-epoxide oxygens probably derive from the atmosphere by oxidative processes. Thus, the C-1 ester linkage should be formed from condensation of monic acid and 9-HN rather than through insertion of oxygen into a single linear precursor. The latter is proposed to occur in the biosynthesis

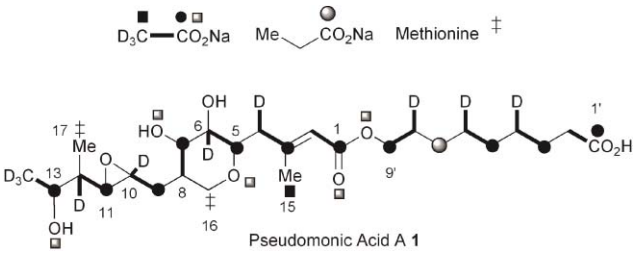

Fig. 1 Origin of labelled precursors in pseudomonic acid A **1**.

of the truncated polyketide metabolite pederin, believed to be formed in beetles of the *Paederus* genus by a bacterial (pseudomonad) symbiont.[8] The results are also in accord with the proposal[7] that the tetrahydropyran ring is formed from an intramolecular S_N2 type attack of the 5-OH onto an activated 16-hydroxy group, resulting from oxidative activation of the 16-methyl. The timing of the cyclisation relative to other steps such as 6-hydroxylation, epoxidation or esterification at C-1 is not known.

More recently, we have isolated[9] and sequenced[10] the 74 kb mupirocin biosynthesis gene cluster and assigned putative enzymatic functions to many of the open reading frames (ORFs). The mupirocin cluster is a combination of 6 larger ORFs (*mmp1–6*) each containing several domains resembling the multifunctional proteins of bacterial Type I polyketide synthase (PKS) and FAS Type I systems, and a multitude of individual genes (*mupA–mupZ*) some of which show similarity to Type II systems (*e.g. mupB*, *mupG* and *mupD*, *mupS* – ketosynthases (KSs) and ketoreductases (KRs) respectively),[11] which could be involved in the production of the monic and 9-HN backbones and others which could be involved in downstream "tailoring". *MupO*, *mupW*, *mupT* and *mupC* encode, for example, respectively a putative cytochrome P450, dioxygenase, ferrodoxin dioxygenase and NADH/NADPH dependent oxidoreductase which could be involved in 10,11 epoxidation, 6, 8 and 16-hydroxylations. In many cases the function of the gene is not obvious and in order to determine the role of all the genes we have developed a system for carrying out selective deletions and site directed mutations in the mupirocin gene cluster and have initiated a programme of in-frame deletions to assay the effect of these on metabolite production.[12] In this paper we report the efficient production of a new metabolite through mutation of the *mupW* gene. The *mupW* mutant showed reduced activity (37%) compared to the WT *P. fluorescens* in a standard plate assay against *Bacillus subtilis*.[10]

† Electronic supplementary information (ESI) available: details of purification and characterisation of compounds **2–4**. See http://www.rsc.org/suppdata/cc/b4/b414781b/

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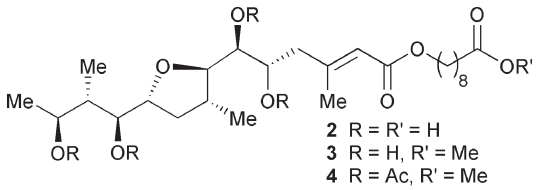


HPLC analysis of extracts of shake flask cultures of wild type *P. fluorescens* NCIMB 10586 shows a peak at 21.5 minutes corresponding to the major metabolite PA-A. Similar analysis of extracts from mutated strains revealed a loss of PA-A production and in several cases the appearance of other products. In the case of the *mupW* mutant, a new peak appeared at 22.0 minutes and preliminary LC-MS analysis revealed that it had a molecular ion at *m/e* 503 in contrast to PA-A (*m/e* 501), suggesting that the new metabolite, which we designate mupirocin W **2**, is a formal dihydro-derivative of PA-A. Initial 400 MHz NMR analysis in d_6-acetone indicated a number of features expected for a pseudomonic acid metabolite but with several important differences: the 10,11-epoxide methines which normally lie between 2.6 and 2.8 ppm were absent; the 3–4 ppm region, where the remaining hydrogens attached to oxygen-bearing carbons normally appear, showed a much altered pattern and appeared to have two extra hydrogens; and crucially an extra methyl doublet was apparent in the 0.9–1.1 ppm region.

Mupirocin W **2** was fully characterised by conversion to the corresponding methyl ether by treatment with diazomethane. HRMS analysis of the resulting methyl mupirocin W **3** gave the molecular formula $C_{27}H_{48}O_9$ consistent with mupirocin W being a dihydro-analogue of PA-A. Subsequent 600 MHz NMR analysis in $CDCl_3$ confirmed the conclusions from the lower field NMR spectrum and a deuterium exchange experiment indicated the presence of 4 hydroxyl protons at 2.14, 2.43, 2.85 and 3.90 ppm respectively. The presence of 4 secondary hydroxyl groups was confirmed on the formation of a tetra-(trimethylsilyl) ether ($C_{39}H_{80}O_9Si_4$), and the tetra-acetate **4** on treatment of methyl mupirocin W with acetic anhydride in pyridine.

The appearance of an extra methyl doublet in the ^{1}H NMR spectrum of mupirocin W immediately suggested a structure lacking the tetrahydropyran ring and this was confirmed by analysis of the COSY spectrum of **3** which provided the connectivities summarised in Fig. 2(a). The remaining problems were the positions of the four secondary hydroxyl groups and thus the exact location of the ether linkage required to satisfy the molecular formula. This was resolved by further examination of the COSY spectrum and the changes observed in the 1D spectrum following D_2O exchange.

Direct correlations were seen between H-5 and the hydroxyl proton at 3.95 and 2.43 ppm respectively, H-6 and OH at 3.62 and 2.14 ppm, and H-11 and OH at 3.76 and 2.85 ppm. On addition of D_2O, no change was observed on either H-7 or H-10 at 4.12 or 3.76 ppm respectively, whereas, H-13 at 3.84 ppm sharpened from an apparent broad quintet with coupling of 7 Hz to a sharp doublet of quartets with couplings of 8 and 6 Hz respectively. These observations are entirely consistent with the tetrahydrofuran ring of **2**, with C-7 and C-10 being linked by the ether oxygen. The relative configuration around the tetrahydrofuran ring was established by observation of the NOEs summarised in Fig. 2(b). These indicated that H-8, H-9β and H-10 showed mutual relaxations, whereas irradiation of H-9α gave enhancement of the 16-methyl and H-9β only. The absolute configuration is assumed from that observed in the pseudomonic acids and the biosynthetic rationale below. This established the structure of mupirocin W **2**.

It is known that PA-A **1** is sensitive to pH and on either mild acid or mild base treatment, it is converted to structures containing either of the 5- or 6-membered cyclic ethers arising from attack of the 7-hydroxyl group at C-10 or C-11 of the epoxide ring. Thus one can propose that mupirocin W **2** is formed as shown in Scheme 1 from an acyclic biosynthetic intermediate, *e.g.* **5** by analogous opening of a 10-11 epoxide at C-10 by 7-OH. It is possible that greater conformational mobility of **5** compared to **1** results in spontaneous cyclisation to form the tetrahydrofuran ring.

It is now clear that *mupW* is the gene responsible for 16-hydroxylation. Mutation of mupW blocks hydroxylation, but it appears that the other proposed late stage modifications, 5-hydroxylation, 10,11 epoxidation and esterification with 9-hydroxynonanoic acid still take place. This may imply that 16-hydroxylation and subsequent tetrahydropyran ring formation occurs as the last step in the biosynthesis of pseudomonic acid A, which would be consistent with a genetic location close to the end of the cluster, on the basis that there is often an approximately co-linear relationship between gene position and the biosynthetic

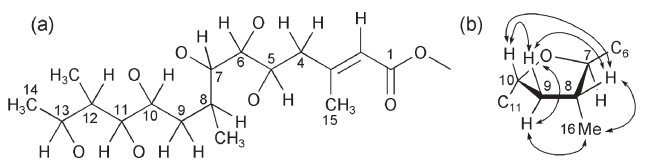
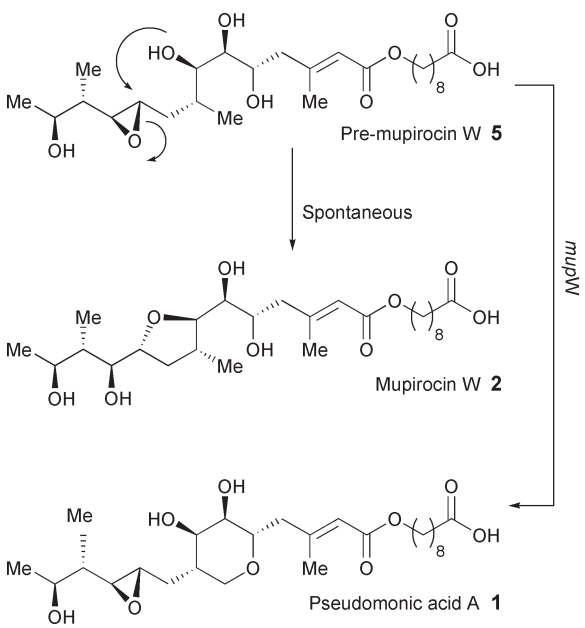


Fig. 2 (a) Proton connectivities and (b) NOEs observed for methyl mupirocin W **3**.



Scheme 1 Mechanism of formation of mupirocin W **2** *via* inhibition of *mup*W catalysed 16-methyl hydroxylation.

stage in a metabolic pathway. Alternatively, the enzymes responsible for the other late modifications may have sufficiently relaxed substrate specificity to accept non-THP containing intermediates. This explanation would be favoured by other results[12] which show that the balance between production of PA-A and PA-B is shifted in favour of the latter in certain other knock-out mutants implying that the THP ring formation is not the last step. Studies to elucidate the role of the remaining modifying genes are in progress.

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