Mupirocin H, a novel metabolite resulting from mutation of the HMG-CoA synthase analogue, *mupH* in *Pseudomonas fluorescens*†

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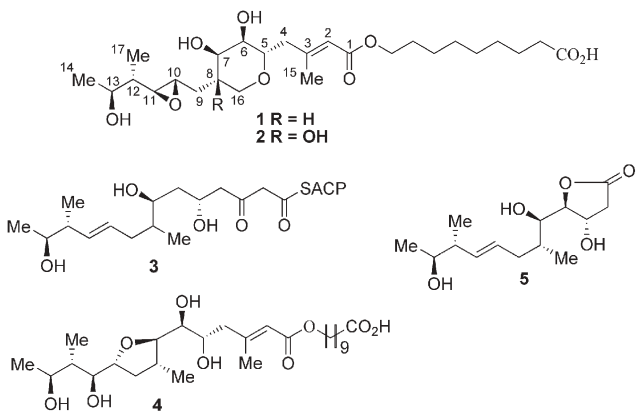
Mutation of the HMG-CoA synthase encoding *mupH* gene in *Pseudomonas fluorescens* gives rise to a new metabolite formed from a truncated polyketide intermediate, providing *in vivo* evidence for the roles of *mupH* and cognate genes found in several "AT-less" and other bacterial PKS gene clusters responsible for the biosynthesis of diverse metabolites containing acetate/propionate derived side chains.

Mupirocin is a polyketide antibiotic active against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and is used clinically for the treatment of bacterial skin infections.[1] It consists of a mixture of pseudomonic acids produced by *Pseudomonas fluorescens* NCIMB 10586. The major component, pseudomonic acid A (PA-A, **1**), which accounts for 90% of the mixture, consists of a C_{17} unit (monic acid [MA]) esterified by 9-hydroxynonanoic acid (9-HN).[2] Other components include pseudomonic acid B (PA-B, **2**), with an extra tertiary hydroxyl group at C-8.[3,4] MA is formed from an acetate-derived heptaketide chain containing two branching carbons derived from methionine (C-16 and C-17) and one (C-15) from the methyl of a cleaved acetate unit.[5]



The 74 kb mupirocin gene cluster has been sequenced and many of the open reading frames have been assigned putative functions.[6] The gene cluster (Fig. 1) can be divided into two approximately equal regions.

The first region contains a Type 1 modular polyketide synthase (PKS) with a number of unusual features, some, but not all, of which are shared with a number of other (mainly non-actinomycete) bacterial polyketides. These include *C*-methyl transferase (*C*-MeT) domains within PKS condensation modules which themselves lack distinct acyl transferase (AT) domains. The former feature has been found in several bacterial PKSs, *e.g.* pederin **7**,[7] myxovirescin A **6**[8] and bryostatin,[9] and the latter feature is found in several gene clusters, including those of leinamycin,[10] pederin,[7] TA antibiotic[11] and the *pksX* of *Bacillus subtilis*.[12,13] In the *mup* cluster, the acyl transferase function has been assigned to *mmpC* which contains two AT domains believed to be responsible for the *in trans* transfer of acetyl and malonyl units.[6]

The PKS genes (*mmpA, mmpC* and *mmpD*), along with *mupC* which encodes a putative dienoyl CoA reductase,[14] would generate a PKS-bound heptaketide intermediate **3**.[15] This heptaketide requires a number of modifications for conversion into PA-A **1** including: introduction of the 6-hydroxyl, and 10,11-epoxide functions; formation of the tetrahydropyran (THP) ring; and introduction of the 2,3-olefin and associated 15-methyl group. In addition, the synthesis of 9-HN and its esterification[16] to monic acid need to be achieved. These features are believed to be controlled mainly by *mmpB* and some or all of the 26 ORFs found in the latter 'tailoring' region of the *mup* cluster, all of which have been shown to be essential for mupirocin production.[15,17]

In order to determine the role and sequence of these ORFs we have conducted directed knockout experiments and isolated new analogues of the pseudomonic acids. Thus, we recently isolated the shunt product mupirocin W **4** from the corresponding *ΔmupW* mutant. This demonstrated that *mupW* encodes a dioxygenase and is responsible for the oxidative activation of the 16-methyl group necessary for THP formation.[18]

An important feature of the tailoring region is the presence of a putative HMG-CoA synthase (HCS) analogue, *mupH*. *MupH* homologues have been reported from the myxovirescin **6**[8] (*taC*), pederin **7**[19] (*pedP*), jamaicamide A **8**[20] (*jamH*) and curacin A **9**[21] (*curD*) gene clusters where they are proposed to be responsible,

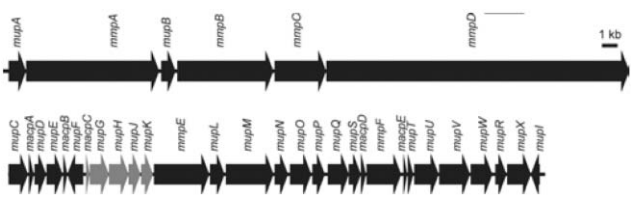


Fig. 1 The 74 kb mupirocin (*mup*) gene cluster. The putative *mupH* cassette is shown in grey.

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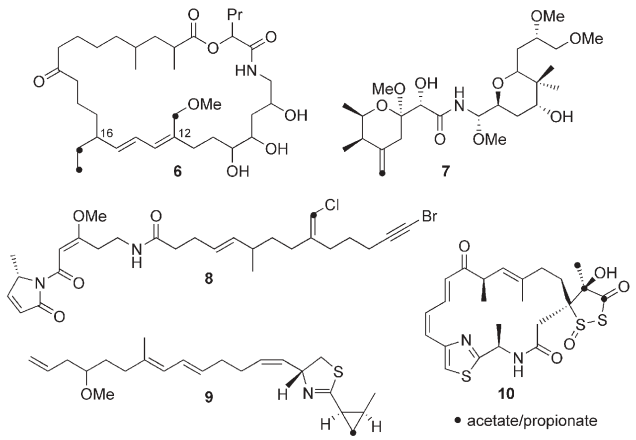


Fig. 2 Bacterial metabolites containing HCS catalysed acetate/propionate derived side branches.

inter alia, for the introduction of olefinic methyl (as in PA-A), exomethylene, vinyl chloride and cyclopropyl moieties respectively (Fig. 2). In each case the single carbon derives from the methyl carbon of a cleaved acetate unit.

MupH analogues are usually found as part of a cassette (Fig. 1) consisting of an ACP (*macpC*), KS (*mupG*), HCS (*mupH*) and two similar genes (*mupJ* and *mupK*) which have been ascribed variously to enoyl reductases, enoyl CoA hydratases or the crotonase (CR) superfamily for which decarboxylase and retro-aldol activities have been reported.[22] In addition, it has been proposed that another HCS analogue, *LnmM*, is responsible for the introduction of the branched propionyl moiety found in the unusual 1,3-dioxo-1,2-dithiolane moiety of leinamycin **10**.[23] The bacillaene (PksX) pathway from *Bacillus subtilis* also contains similar genes.[13] We now report mutagenesis studies of *mupH* in *P. fluorescens* which provide support for its essential role in the introduction of the 15-methyl group during PA-A biosynthesis. A mechanism is proposed which accounts for the role of *mupH* and its associated genes *macpC, mupG, mupJ* and *mupK*.

An in frame deletion of *mupH* was created.[15,24] Bioassay[6] of three *ΔmupH* mutants revealed a consistent reduction in antibacterial activity against *Bacillus subtilis* 1064 to *ca.* 14% of wild-type levels. The *mupH* ORF itself was amplified by PCR and cloned into the IncQ *tac* promoter expression vector pJH10, giving pSCCH. The resulting plasmid was introduced into the *ΔmupH* strain and bioassay showed activity being fully restored to wild-type levels, confirming that the defect in PA production must be due to loss of *mupH* function and not due to a polar effect on downstream genes.

HPLC analysis of extracts of wild-type *P. fluorescens* NCIMB 10586 showed a major component at 27.4 min corresponding to PA-A on UV detection. The mutated strain (*ΔmupH*) failed to produce any detectable PA-A and no new UV active peaks were observed. However, the extract from 10 l of *ΔmupH* culture was purified by LH-20 Sephadex chromatography followed by flash chromatography on normal phase silica gel to give 5.7 mg of a colourless oil. The structure of this new compound, was elucidated by HRMS and detailed analysis of 1D and 2D NMR spectra.[24] HRMS revealed a molecular ion peak at [M + Na]/e, 295.1525, which indicated a molecular formula, $C_{14}H_{24}O_5Na$ (calc. 295.1516). Correlations in the ^{1}H-^{1}H COSY spectrum established

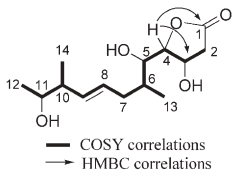


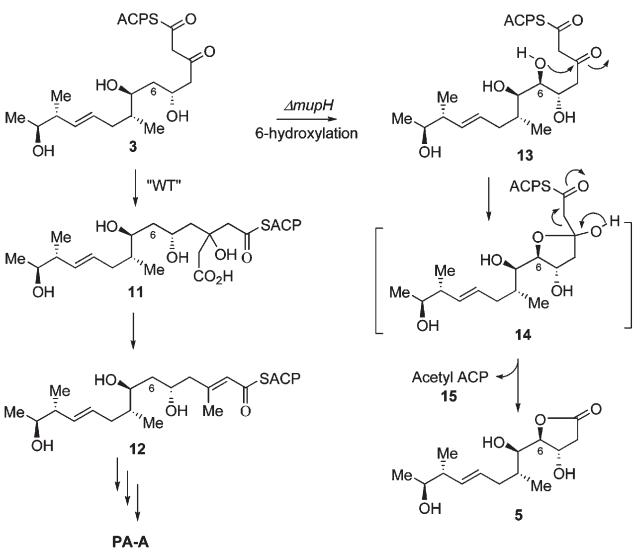
Fig. 3 COSY and selected HMBC correlations for mupirocin H **5**.

the skeleton shown in Fig. 3. HMBC correlations from H-4 (δ_H 4.44) to both C-1 and C-3 at 179.2 and 68.4 ppm respectively indicated the presence of a 3-hydroxy-γ-lactone ring. Consistent with this, the FTIR spectrum showed an absorbance at 1793 cm^{-1}.

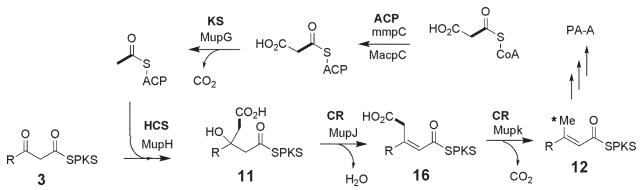
The relative stereochemistry in the γ-lactone ring was suggested from the NOESY spectrum which showed NOE enhancements between H-3 at δ_H 4.61 and both H-2a and H-2b and H-5 but not with H-4. H-4 showed enhancements to H-5 and the 13-methyl hydrogens only, data consistent with an *anti* arrangement of the C-3 and C-4 oxygen functionalities. This relative stereochemistry was confirmed by comparison with literature data for a series of 3-hydroxy-γ-lactones with both the *syn* and *anti* relationship between C-3 and C-4.[25] In the ^{1}H NMR spectra of the *anti* compounds, H-3 typically appears at *ca.* 4.5 ppm with three doublet splittings of *ca.* 7, 4 and 3 Hz to the vicinal 2 and 4 hydrogens, whereas the corresponding splittings in the *syn* series are *ca.* 4, 3 and 1 Hz. The observed H-3 couplings of 7.6, 4.3 and 3.2 Hz are again clearly consistent with the *anti* relative stereochemistry.

The full relative and absolute configuration shown for **5** are based on comparison, and are fully consistent, with the established stereochemistry of PA-A **1** and mupirocin W **4**. Following the precedent set for mupirocin W, we propose the name mupirocin H for **5** which is the first member of the pseudomonic acid family to be derived from a truncated monic acid analogue.

Thus it appears that mutagenesis of *mupH* has resulted in derailment of the normal biosynthetic pathway. We propose the mechanism shown in Scheme 1 for formation of mupirocin H **5** which accounts for the truncation of the polyketide chain and also rationalizes formation of the γ-lactone. Normal biosynthesis would



Scheme 1 Proposed biosynthesis of mupirocin H (**5**) *via* PKS-bound intermediates.



Scheme 2 Putative operation of the MupH cassette.

proceed *via* MupH-catalysed condensation of **3** with a malonyl CoA or acetyl CoA derived acetate unit (see below) to give the β-hydroxy-β-methyl-glutaryl CoA analogue **11**. Dehydration and decarboxylation of **11** would generate the β-methyl-α,β-unsaturated thiolester moiety of **12** found in all pseudomonic acid analogues to date. On mutagenesis of *mupH*, however, the key carbon–carbon bond forming step catalyzed by MupH does not take place and so this sequence is blocked. We propose that the next programmed modification would be hydroxylation at C-6 of **3** to give the tetraol **13**. The new hydroxyl would then act as a "trigger" for release and truncation of the polyketide assembly intermediate. Thus as shown in Scheme 1, the intramolecular addition of the 6-hydroxyl to the C-3 ketone of **13** forms the hemiketal intermediate **14**. A subsequent retro-Claisen reaction allows formation of the observed γ-lactone **5** with concomitant facile release of acetyl-ACP **15**. This process is not possible once MupH-catalysed condensation occurs, and the alternative of intramolecular displacement by attack of the 5-hydroxyl on the C-1 thiolester of **3** to give uncatalysed release of a six-membered δ-lactone ring is presumably less favoured.

As described above, *mupH* appears as part of a cassette of five genes: *macpC, mupG, H, J* and *K* which respectively encode an isolated ACP, a KS in which the normal active site cysteine has been mutated to serine (and hence a putative decarboxylase), putative β-hydroxy-β-methyl-glutaryl CoA synthase (HCS) and two crotonase (CR) super family activities. Mutagenesis studies have shown that all of the genes are essential for normal PA production.[15] In recent work on the curacin[26] and PksX clusters,[27] the products of the analogues of these genes have been overexpressed and their *in vitro* activities studied with acetoacetate in the form of CoA or ACP thiolesters. These have shown that the MacpC analogue is malonylated and then decarboxylated by KS to form acetyl-ACP which is then the substrate for the HCS catalysed formation of β-hydroxy-β-methylglutaryl–SACP. This then undergoes successive CR-mediated dehydration to the β-methylglutaconic thiolester and facile decarboxylation to 3-methylbutenyl-SACP.

We therefore propose by analogy (Scheme 2) that malonyl CoA is transferred by MmpC to MacpC to give malonyl-MacpC, which is decarboxylated by MupG to give acetyl-MacpC. MupH would then catalyse a condensation between the β-ketothiolester moiety of **3**, with acetyl-MacpC, to give the glutaryl thiolester **11**. MupJ catalysed dehydration would generate the glutaconate intermediate **16**, which on MupK mediated decarboxylation gives the 3-methylbut-2-enoyl thiolester moiety in monic acid precursor **12**. This therefore provides a rationale for the role of all 5 genes in these cassettes fully consistent with their previously reported activities.

Interestingly, work in progress on analysis of further *mupG*, *mupJ* and *mupK* mutant strains, indicates that mupirocin H **5** is the major product of all of them.[28] Thus a divergence between *in vivo* and *in vitro* studies is apparent which perhaps points to a role for functioning protein complexes *in vivo*. This is consistent with our previous work[15] which indicates that *inter alia*, *mupJ* and *mupK* need to be expressed together for *in vivo* activity to be observed.

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