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# In vivo Mutational Analysis of the Mupirocin Gene Cluster Reveals Labile Points in the Biosynthetic Pathway: the "Leaky Hosepipe" Mechanism

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*A common feature of the mupirocin and other gene clusters of the AT-less polyketide synthase (PKS) family of metabolites is the introduction of carbon branches by a gene cassette that contains a β-hydroxy-β-methylglutaryl CoA synthase (HMC) homologue and acyl carrier protein (ACP), ketosynthase (KS) and two crotonase superfamily homologues. In vivo studies of* Pseudomonas fluorescens *strains in which any of these components have been mutated reveal a common phenotype in which the two major isolable metabolites are the truncated hexaketide mupirocin H and the tetraketide mupiric acid. The structure of the latter has been confirmed by stereoselective synthesis. Mupiric acid is also the major metabolite arising from inactivation of the ketoreductase (KR) domain of module 4 of the modular PKS. A number of other mutations in the tailoring region of the mupirocin gene cluster also result in production of both mupirocin H and mupiric acid. To explain this common phenotype we propose a mechanistic rationale in which both mupirocin H and mupiric acid represent the products of selective and spontaneous release from labile points in the pathway that occur at significant levels when mutations block the pathway either close to or distant from the labile points.*

## Introduction

Mupirocin, an antibiotic active against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, consists of a mixture of pseudomonic acids produced by *Pseudomonas fluorescens*. It was one of the first members of a group of complex polyketide metabolites produced by the "AT-less" polyketide synthases (PKSs) to have its biosynthetic gene cluster sequenced (Figure 1).[1] These gene clusters are characterised by having modular type I PKSs in which the condensing modules lack the acyl transferase (AT) domains normally found in the paradigm actinomycete modular PKSs.[2] Instead, the AT activity is associated with a separate protein which acts in trans with each condensation module (Scheme 1 A). Metabolites produced by these synthases often feature $C_1$, $C_2$ or $C_3$ branches derived from acetate or propionate, which are introduced by β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) synthase homologues in a manner reminiscent of terpenoid biosynthesis.[3] Apart from pseudomonic acid (mupirocin), the earliest examples to have their gene clusters sequenced were pederin[4] from an unculturable *Pseudomonas* sp. symbiont of *Paederus fuscipes* beetles, the related omnamides[5] from the marine sponge *Theonella swinhoie*, and leinamycin[6] from *Streptomyces astrooliviaceus*. Subsequent examples include jamaicamide A[7] and curacin A,[8] both from the sponge *Lyngbya majuscula*, myxovirescin A[9] from *Myxococcus xanthus*, bacillaene ("PksX") and difficidin from *Bacillus amyloliquifaciens*[10] and *Bacillus subtilis*,[3] bryostatin[11] from an unculturable bacterial symbiont of the marine bryozan *Buglia neritina*, and rhizoxin[12] from a bacterial endosymbiont, *Burkholderia rhizoxina*, of the fungus *Rhizopus microsporus*.

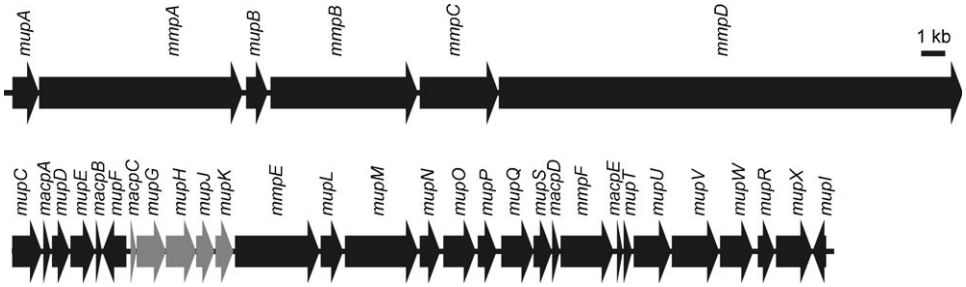


**Figure 1.** Summary of the 74 kb mupirocin biosynthesis gene cluster; the mupH cassette is shown in grey.

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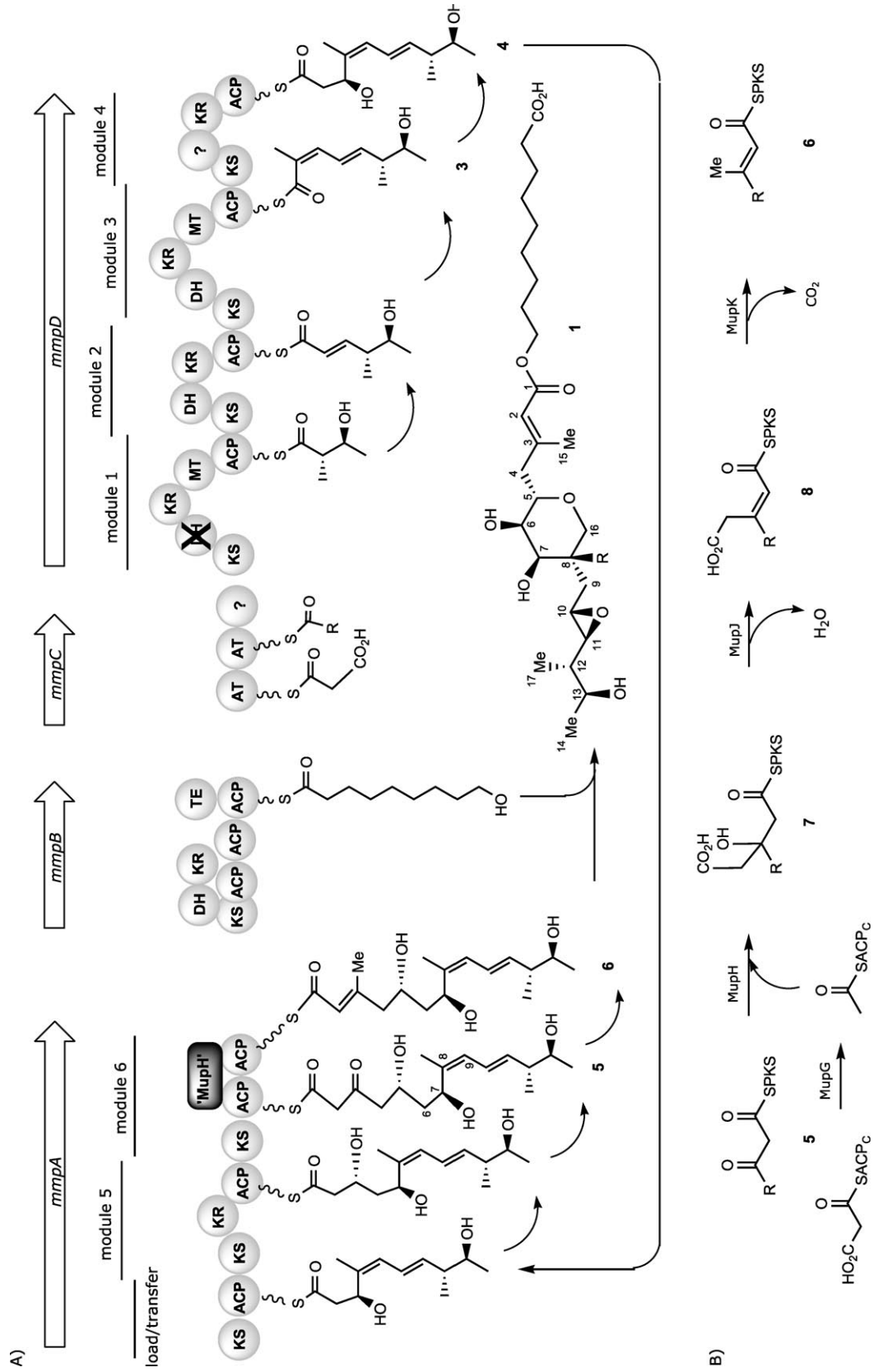



**Scheme 1.** A) Summary of the mupirocin type I PKS and assembly scheme for the biosynthesis of monic acid precursor **6**. B) Proposed mechanism for introduction of the 15-methyl group by the MupH cassette.

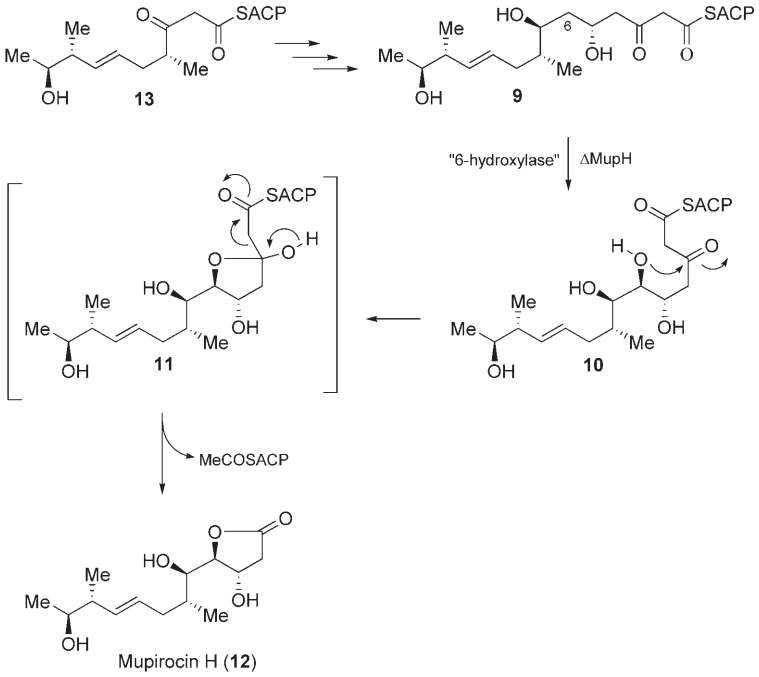
  

Pseudomonic acid A (**1**, R = H),[13] consisting of monic acid (MA) esterified[14] by 9-hydroxynonanoic acid (Scheme 1), is the major component of mupirocin. The thiomarinols isolated from the marine bacterium *Alteromonas rava* have very similar structures and are probably the products of closely related genes.[15] Monic acid is formed from an acetate-derived heptaketide chain containing two carbons (C-16 and C-17) derived from methionine and one from the methyl (C-15) of a cleaved acetate unit.[16] The 74 kb mupirocin gene cluster[1] (Figure 1, Scheme 1 A) can be divided into two distinct parts. The first contains the type 1 modular PKS in which, unusually, the synthetic sequence is not colinear with the genetic sequence. The first four condensation modules are found on MmpD, with *C*-methyl transferase (MT) domains within the first and third condensation modules. The remaining three are on MmpA, which terminates with an acyl carrier protein (ACP) didomain and commences with an apparently silent condensation module (Scheme 1 A). Although this was a feature first noted in the *mup* cluster, other examples including split condensation modules with domains on different proteins are becoming apparent.[9,10]

The mupirocin PKS genes (*mmpA, mmpC* and *mmpD*) would formally generate a PKS-bound intermediate **5** (Scheme 1 A), which requires a number of modifications for conversion into pseudomonic acid A (**1**). These include reduction of the 8,9-alkene, oxidative introduction of the 6-hydroxy and 10,11-epoxide functions, formation of the tetrahydropyran (THP) ring and the 2,3-olefinic double bond with the associated 15-methyl group, and synthesis and addition of 9-hydroxynonanoic acid. These features are proposed to be mainly controlled by *mmpB* and some, or all, of the 26 ORFs found in the "tailoring" region of the cluster (Figure 1), all of which we have shown to be essential for mupirocin production.[17,18] A series of targeted gene knockouts have led to the biosynthesis of several new compounds. Their chemical identification[18–20] has led to the first understanding of mupirocin biosynthesis, showing the involvement of many new features.

The mupirocin tailoring region contains *mupH*, which encodes an HMG-CoA synthase (HCS) analogue, which is part of a cassette that also consists of an ACP (*macpC*), a mutant KS (*mupG*) and a pair of similar genes (*mupJ* and *mupK*) that have been ascribed variously to enoyl reductases and hydratases or the crotonase (CR) superfamily, for which, inter alia, decarboxylase and retro-aldolase activities have been reported.[21] In vitro studies[3] on the expressed proteins of the corresponding HCS cassettes from the bacillaene[10] and myxovirescin pathways[22] (using acetoacetate as a surrogate substrate), following more limited in vitro studies on components of the curacin cassette,[23] have confirmed their likely functions. Normal biosynthesis should thus proceed through MupH-catalysed condensation of PKS-bound β-keto thiolester **5** with malonate-derived acetate (with the KS analogue MupG acting as a malonyl decarboxylase)[24] bound to MacpC to give the glutarate analogue **7** (Scheme 1 B). This would be followed by dehydration (MupJ) to the glutaconate analogue **8** and decarboxylation (MupK) to afford the β-methyl-α,β-unsaturated ester moiety of **6** found in all pseudomonic acid analogues to date.

Mutagenesis of *mupH*, which should block the key acylation step (Scheme 1, **5** to **7**), produced the truncated γ-lactone derivative mupirocin H (**12**; Scheme 2), which is the first novel




**Scheme 2.** Proposed biosynthesis of mupirocin H (**12**).

molecule related to MA biosynthesis identified.[20] Its production can be rationalised[20] as shown in Scheme 2: hydroxylation at C-6 of **9** (see mupC discussion below) gives the tetraol **10**; this allows intramolecular addition of the 6-hydroxyl to the C-3 ketone to create the hemiketal **11**, from which a retro-Claisen process releases the γ-lactone **12** and leaves an acetyl-ACP. This discovery encouraged us to look more carefully for other products that may represent products preceding or following this stage of the biosynthetic pathway. The results presented here, in combination with the in vitro work referred to above, and parallel in vivo mutational studies on myxovirescin discussed later,[25] allowed us to develop a new model that might explain why many of the mutants that should affect quite different steps in the pathway produce the same novel metabolites.

## Results and Discussion

### Mutagenesis of the MupH cluster and discovery of mupiric acid

From consideration of the demonstrated in vitro properties of the HCS cassette components we anticipated that we might isolate products related to mupirocin H from mutation of the

acyl carrier protein and malonyl decarboxylase (*macp*CS38A and *ΔmupG*), and glutarate **7** and glutaconate **8** from mutation of the subsequent dehydratase and decarboxylase (*ΔmupJ* and *ΔmupK*; Scheme 1B). The profile of the *ΔmupG* mutant differed from the others in still containing small but detectable amounts of PA-A. The production of small amounts of PA-A could be explained either by spontaneous decarboxylation of malonyl-mAcpC or by the presence of other unspecific malonyl-decarboxylase activity in vivo. A similar drastic (120-fold), but not complete, reduction in levels of myxovirescin production was observed on mutation of the MupG analogue TaK during in vivo mutational analysis of the myxovirescin cluster in *Myxococcus xanthus*.[25] Apart from the PA-A in the *ΔmupG* mutant, all four mutations gave very similar results (Figure 2),

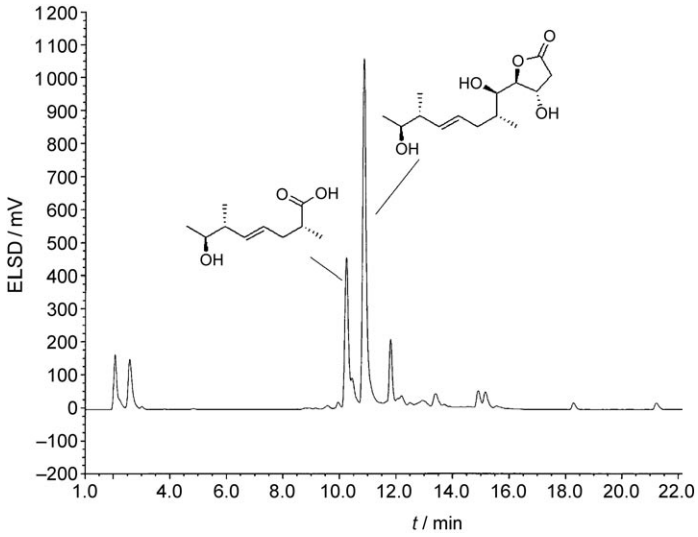
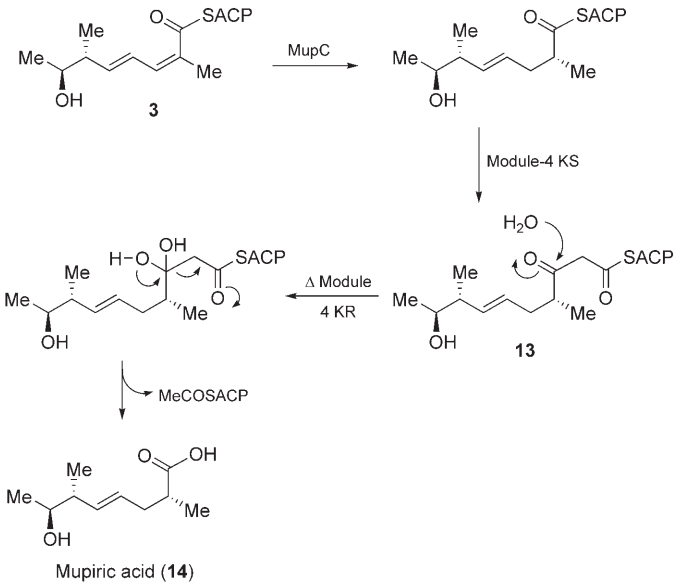



**Figure 2.** Reversed-phase HPLC trace of the extract from the *ΔmupJ* mutant of *P. fluorescens* NCIMB 10586. Column: Phenomenex Prodigy C18 5 μ ODS3 (5 μ, 250×4.6 mm). Eluent: MeCN/$H_2O$. Detector: Alltech ELSD 800.

with the major detectable products being an approximately 2:1 mixture of mupirocin H (**12**) and a novel truncated metabolite, which we named mupiric acid (**14**; Scheme 3). Re-examination of the extracts from the *ΔmupH* mutant by LC-MS showed the presence of a similar proportion of mupiric acid.

### Structure elucidation of mupiric acid

HRMS indicated the empirical formula $C_{10}H_{19}O_3$, and NMR data were in accord with the *C*-methylated tetraketide-derived acid **14**. The *6R,7S* configuration was assumed by analogy with the structures of the pseudomonic acids, but the stereochemistry at C-2 could not be assigned. To confirm the structure of the novel metabolite, synthetic samples of the C-2 epimers **14** and **15** were prepared from a common alkene **19** in a cross-metathesis approach (Scheme 4). The known[26] secondary alcohol **18** was protected as the silyl ether. Reductive cleavage of the sultam auxiliary followed by Wittig olefination gave alkene **19**[27] in good yield. The enantiomeric alkenes **17** and **21** were prepared by allylation of the D- and L-valine-derived propionyl-



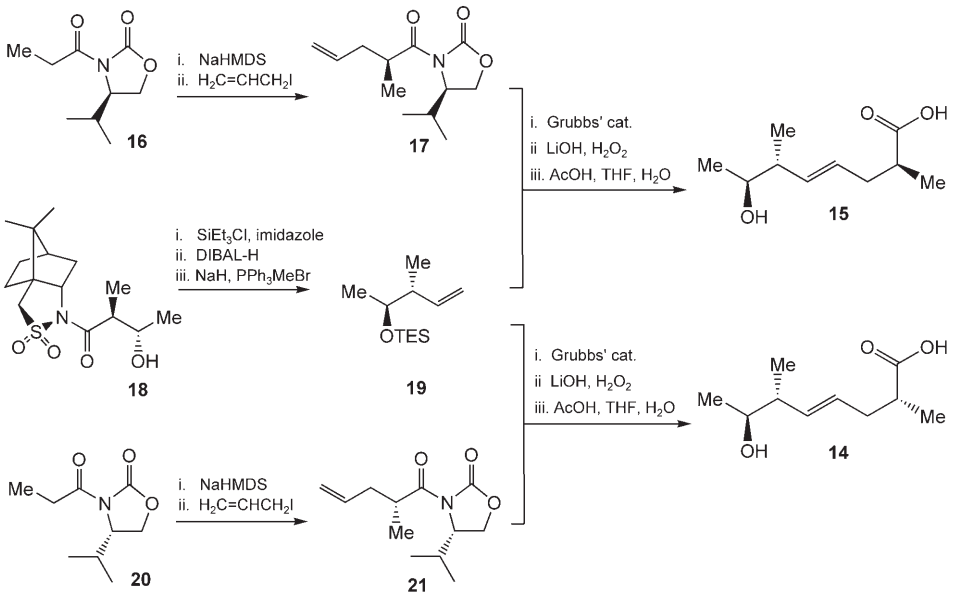

**Scheme 3.** Proposed biosynthesis of mupiric acid (**14**).

oxazolidinones **16** and **20**.[28] Treatment of **17** and **21** separately with alkene **19** in the presence of Grubbs' second-generation catalyst gave the coupled products as 4:1 mixtures of *E*:*Z* olefins. Interestingly, Markó had used alkene **19** in the synthesis of methyl monate C and isolated the analogous *E* alkene as the sole product.[27] Removal of the auxiliary and TES group gave the acids **15** and **14**. The $^1$H NMR spectra of **15** and **14** in $CDCl_3$ were virtually indistinguishable, but comparison of their spectra in $C_6D_6$ with that of the new metabolite clearly demonstrated that it is the same as **14**, and not **15**. Acid **15** has $[\alpha]_D = +35$ ($c = 1.95$, $CHCl_3$), whereas synthetic **14** has $[\alpha]_D = +13$ ($c = 1.05$, $CHCl_3$), which compares well with that of the metabolite ($[\alpha]_D = +16$ ($c = 2.5$, $CHCl_3$)) and confirms its absolute configuration.

### Mutation of the MmpD module 4 ketoreductase (KR) domain (ΔKR6) produces mupiric acid

Further evidence for the origin of mupiric acid came from mutation of the module 4 KR by changing the predicted active-site tyrosine to phenylalanine by site-directed point mutation. The rationale for constructing this mutant was to test hypothetical schemes that might lead to mupirocin W[19] and related shunt products[18] that appear to suggest that keto reduction to form the 7-OH group might be a late event and could imply that the module 4 KR is redundant and might be inactive. On the contrary, the mutation resulted in loss of PA-A (**1**, R = H), PA-B (**1**, R = OH) and any other closely related metabolites, but the KR knockout did produce mupiric acid as the major novel isolable metabolite. This is the first metabolite identified as the result of a mutation in one of the type I PKS modules. Since the point mutation should not result in loss of the ability to create the tetraketide β-keto thiolester **13** it seems likely that release of mupiric acid is the result either of this compound not being processed further at the normal rate, so that KS and ACP-bound intermediates accumulate and are released from




**Scheme 4.** Synthesis of mupiric acid (**14**) and its C-2 epimer **15**.

the module 3 ACP, or that the β-keto group in the tetraketide β-keto thiolester **13** makes the intermediate labile, as indicated in Scheme 3, and results in the release of mupiric acid through a retro-Claisen reaction and leaves an acetyl-ACP in a release mechanism analogous to that proposed for mupirocin H (**12**).

### Possible role of MupC in 8,9-alkene reduction to produce mupirocin H and mupiric acid

A key feature of the mupirocin type I PKS modules is the absence of any predicted ER domains, raising questions about the origin and timing of alkene reduction steps in the biosynthetic pathway. Specifically for the data presented here, the domain structure in module 3 of MmpD lacks an enoyl reductase (ER), yet the 8,9 double bond (pseudomonic acid A numbering) is reduced in both **12** and **14**, as it is in all known pseudomonic acid analogues. We originally proposed[19] that reduction might occur by in trans action of *mupC*, which encodes a putative dienoyl thiolester reductase,[29] on module 3 tetraketide **3** (Scheme 3). However, we have subsequently shown that MupC must normally act after MupO, U, V and other proteins responsible for formation of the THP ring,[18] reducing the 8,9-alkene of an α,β-unsaturated ketone located between C9 and C7. However, isolation of mupirocin H (**12**) and mupiric acid (**14**), which must be formed from pre-THP intermediates, is consistent with the original proposed in trans action of MupC on PKS-bound tetraketide **3**. MupC might thus be able to manifest activity on different substrates. Support for such a dual action of a tailoring enzyme comes from a recent study[30] on the P450 enzymes that control the formation of the biphenyl and diphenyl ether linkages in the biosynthesis of the vancomycin family of glycopeptide antibiotics. These studies have shown that the oxidative enzymes act in a defined sequence on specific substrates in wild-type (WT) organisms. However, analysis of intermediates that accumulate in the culture filtrates of P450 deletion mutants indicate that the enzymes show decreased substrate specificity, possibly because of the accumulation of abnormal substrates only present under the artificial conditions found in the deletion mutants. In the mutants that produce mupirocin H and mupiric acid, it might be (see below) that there is a much higher steady-state concentration of tetraketide **3**; this would increase the chance of it being an alternate substrate for MupC and undergoing reduction as originally proposed. In vitro experiments with over-expressed MupC and synthetic substrates are in progress to address this.

### Other mutations of the *mup* cluster produce the mupirocin H/mupiric acid phenotype

A number of mutations of other *mup* genes were previously reported to result in loss of muprocin production, but no novel metabolites were initially detected by the less sensitive methods used.[17,18] In light of the above observations, however, further analysis of extracts from several of the mutant strains showed the same levels of production of mupirocin H and mupiric acid as the *mupH* cassette mutations. The additional genes so far identified in which mutation results in this phenotype are: *ΔmupB*, *ΔmupL*, *ΔmupQ* and *ΔmupS*, as well as *mmpB* (S1390A) and *mmpF* (C183A). Our hypothesis to rationalise why they all have this effect is that they may all be involved in 9-HN formation and esterification of monic acid or its precursor. It seems likely that the ultimate product of the PKS is removed from MmpA by esterification with 9-HN or a precursor. MmpB is proposed to be part of a fatty acid synthase (FAS) system involved in 9-HN biosynthesis,[1] and point mutation (S1390A) of the first of its unusual triplet of ACP domains known to cause a loss of PA-A production also gives the mupirocin H/mupiric acid phenotype. We predict also that MupS, MupQ and mAcpD are all involved in making a 3-hydroxypropionate precursor for 9-HN. MupS, originally annotated as a 3-oxoacyl-ACP reductase, has homology with the malonyl-CoA reductase responsible for formation of 3-hydroxypropanoic acid in *Chloroflexus aurantiacus*.[31] It is flanked by *mupQ*, which encodes an acyl-CoA synthase, and the *macpD* ACP gene. The difficidin cluster in *B. amyloliquifaciens* also has three adjacent genes *difC, difD* and *difE* that encode the same three activities;[10] this suggests that these genes might have been inherited together through horizontal gene transfer with a common function. It has been suggested that DifC, DifD and DifE are responsible for the formation of the $C_3$ acrylyl thiolester starter unit required for difficidin biosynthesis by reduction and dehy-

dration of pyruvate. By analogy with mupirocin biosynthesis, dehydration of 3-hydroxypropionate is also the likely source of the acrylate starter in difficidin, possibly catalysed by the adjacent *difB* kinase product. In mupirocin biosynthesis, impairment of 9-HN production is likely to result in failure to remove the PKS monic acid product **6** from MmpA. A potential candidate for attachment of 9-HN to monic acid or its precursor is the *mupL*-encoded "hydrolase". A *ΔmupL* mutant again gives the mupirocin H/mupiric acid phenotype, as do two further mutants. The first is an active site cysteine to alanine point mutant of *mupB*—a 3-oxo-acyl-ACP synthase strategically placed between MmpA and MmpB that might facilitate loading of 3HN onto the KS of MmpB or movement of the MA-ester from ACP4 to the KS of MmpB. The second mutation is of *KS10*—part of *mmpF*—which arguably might also be involved in 9-HN synthesis or attachment.

### Formation of mupirocin H and mupiric acid: the hosepipe hypothesis

Two explanations for the formation of mupirocin H (**12**) by all *mupH* cassette mutations appeared possible. One is that, in vivo, the components of the MupH cassette must act as a functioning complex, and mutation of any of the five components renders the whole complex inoperative. While our complementation studies on mupirocin mutants show some evidence that MupJ and MupK must be coexpressed for full functionality,[18] this appears to be inconsistent with in vitro studies of the bacillaene and myxovirescin systems, which show that all the components can display their individual catalytic functions independently.[3,22] A more likely proposal, which would also explain the formation of both **12** and **14**, is that mutation of the individual functionalities impairs the flux of metabolites along the biosynthetic assembly line.

Staunton and co-workers have demonstrated by proteolysis and MS analysis of the DEBS1-TE system, that in modular PKSs all sites that can covalently bind a thiolester intermediate, for example KS, AT and ACP, are simultaneously occupied.[32] The C-terminal thiolesterase (TE) removes the final PKS-bound product and thus allows normal flux along the synthase and normal levels of metabolite production. When the TE is absent, as in DEBS1, product release is drastically reduced, and relies on spontaneous, noncatalysed lactone formation.[33] There is also evidence in modular PKSs for the presence of extra in trans type II TEs that hydrolytically remove incorrectly synthesised intermediates not recognised by the downstream domains. This monitoring function allows blockages in the assembly line to be removed and normal production levels to be maintained.[34]

In the absence of such a function in the mupirocin system, we propose that the *mupH* cassette mutations result in failure of the individual intermediates to be efficiently processed; this increases the dwell time of intermediates at all the covalent sites on the synthase, so that production is blocked and no metabolites are observed. The release mechanisms proposed for the formation of mupirocin H and mupiric acid, however, provide a spontaneous escape route at these two particular points in the assembly. It is interesting that the yields of **12** are consistently two- to three-times higher than those of **14**. The release mechanism for **12** utilising an intramolecular trigger might be expected to be more efficient than that for **14**, which needs an intermolecular source of nucleophile, presumably water. Overall, the levels of metabolite production are drastically reduced relative to the WT. The simple analogy is to that of a hose pipe—it is only when the main flow is blocked that potential leaks elsewhere become apparent.

There are previous examples in which impairment of release of the ultimate product of modular PKSs have resulted in the accumulation of products derived from premature release of assembly intermediates. Inactivation of the *rifF* gene,[35] which encodes an amidase responsible for release of the rifamycin undecaketide as the macrolactam, resulted in the production and structural characterisation of all of the polyketide assembly intermediates, ranging from tetra- to decaketide either as the free acids or the corresponding lactones/pyrones from hydrolysis of the thiolester linkage to the PKS. This was attributed to the presence of a type II editing TE in the rifamycin gene cluster. More recently, Piel and co-workers have shown[36] that deletion of the terminal TE in the bacillaene PKS results in release of virtually all of the possible assembly intermediates. Relocation of the TE to the end of module 6 gave predominantly the product predicted from this module. In contrast to the rifamycin cluster, there has been no report of a type II TE in the bacillaene cluster, so the promiscuous release in the absence of the terminal TE must be due to an inherent, and perhaps unusual, lability of the thiolesters in this system, or to the presence of an editing TE or a non-specific thiolesterase activity in *B. amyloliquifaciens*. These reports, in which wholesale release of intermediates was observed, contrast with the very selective release observed in *P. fluorescens*.

### Implications for in vivo mutagenesis studies

Our observations provide a rationale for several hitherto puzzling results. Despite extensive mutational analysis, we have failed to identify genes for the key oxidative steps responsible for introduction of the 6-hydroxyl group and the 10,11-epoxide. This suggests that these functionalities may be key recognition elements for downstream modifying enzymes so that knock-out of the appropriate genes simply results in complete blockage of the pathway. Consistent with this is the observation that with the exception of the results discussed above, all gene knock-outs that have given isolable products result in metabolites in which both 6-hydroxylation and epoxidation have occurred.[17–20] Isolation of mupirocin H (**12**) requires hydroxylation at C-6 to occur on the PKS module 6-bound heptaketide intermediate **5**, with the 6-hydroxylase acting in trans with the PKS.

These data illustrate the constraints placed by an assembled system on its component parts and highlight the fact that the relevance of in vitro experiments with over-expressed PKS components to the real in vivo situation always need to be kept in mind. While in vitro experiments can provide details of the activities and mechanisms of individual proteins, they

might not accurately reflect the more complex in vivo situation, in which the real mode of action could depend on rate of flux and hence effective concentrations of metabolites. Increased dwell times may assist otherwise kinetically disfavoured reactions, spontaneous or enzyme-catalysed. It is clear that in vivo mutation studies, on the other hand, do depend on release of products to provide an experimental read-out. Awareness of this is important and it may be that design of mutations can be used to create "leaks in the pipeline" to ensure product release.

## Experimental Section

### Mupirocin cluster mutagenesis

*MmpD module 4 ketoreductase (KR6) Y6276F, Y6278F*: Sequence alignments of KR6 to short-chain dehydrogenases/reductases has revealed a consensus catalytic triad that consists of tyrosine, serine and lysine. Thus, to inactivate KR6, two amino acid (aa) replacements Y6276F and Y6278F were constructed through mutagenesis, essentially as previously described.[18] PCR primer pairs KR6YF1F (5′-GGATCCGGCTATTGGTTCAGCGTGAGGCG)/KR6YF1R (5′-CCGCGGATGCCATAGGCTGCCATG) and KR6YF2F (5′-CCGCGGTTCAGCCGATTTTCGGGTTTGCCG)/KR6YF2R (5′-GAATTCGCTCAAGCAGTGCACCCGCGC) were used to amplify two fragments that contained Y6276F and Y6278F. These were ligated into the suicide vector pAKE604 through BamHI/EcoRI sites introduced by the PCR primers and joined by a SacII site created by a silent mutation, nucleotide (nt) t18813c. This vector (pKR6YF) was transferred to *P. fluorescens* NCIMB 10586 by biparental mating. Strains with the plasmid integrated into the chromosome by homologous recombination were selected for by antibiotic resistance to kanamycin. The plasmid was allowed to excise by a second homologous recombination event under unselective conditions. PCR, restriction analysis and DNA sequencing were used to identify kanamycin-sensitive excisants that contained the aa mutations Y6276F and Y6278F. This strain was denoted 10586KR6YF.

*MupB C116A*: MupB shows 25% sequence identity to the β-ketoacyl-ACP synthase FabH of *Pseudomonas aeruginosa*. A putative active site cysteine 116 identified in MupB was mutated to alanine essentially as described above. PCR primer pairs mupB1F (5′-GGATCCGCGGATGAGGTCGATCGC)/mupB1R (5′-CCACGTGACGCCCAGCG) and mupB2F (5′-GTCACGTGGGGCGATGCCACCATGCTG)/mupB2R (5′-GAATTCGCCATGCACCTGCTGCGCAC) amplified fragments that incorporated the aa C116A mutation and two silent mutations nt g333c and a337g to generate an internal PmlI site. The mutation plasmid was denoted pJHBCA and the strain 10586MupBCA.

Other mutations of the mupirocin cluster have been reported previously: point mutagenesis of *macpC* active site Ser38 to alanine, and *mmpF* ketosynthase domain active site Cys183 to alanine, and in-frame deletions of *mupG* aa 27–397, *mupJ* aa 19–228, *mupK* aa 20–230 and *mupL* aa 14–299;[18] and point mutagenesis of *mmpB* acyl carrier protein domain 5 active site Ser1390 to alanine.[37] The *ΔmupS* mutation has been reported previously,[19] but the sequence of the primers used to create the deletion were omitted. These were: pmupS1-F (5′-GAATTCAGTGACTCGCTGTGGATTC)/pmupS1-R (5′-GGATCCGACAATGATGGTGCCCTTTAC) for one arm and pmupS2-F (5′-GGATCCACACCGTTGGGGCGTCTG)/pmupS2-R (5′-TCTAGACAGGCTCGCTCCATTGCTC) for the other arm. These were cloned as above, joined through the BamHI site, transferred into pAKE604 as an EcoRI/XbaI fragment and then used for suicide mutagenesis in the same way. The underlined regions in the primer sequences indicate the restriction sites used for cloning.

**Culture conditions and isolation of mupiric acid (14)**: A *ΔmupK+pJH2* mutant strain of *Pseudomonas fluorescens* NCIMB 10586 was grown at 25 °C on Lennox (L) agar for 24 h. Single colonies were used to inoculate L broth (50 mL in a 250 mL conical flask) supplemented with tetracycline (15 μg mL$^{-1}$), and incubated at 25 °C, overnight, to prepare the seed culture. Secondary stage medium (25 g L$^{-1}$ soya flour, 6.25 g L$^{-1}$ $CaCO_3$, 5.0 g L$^{-1}$ $(NH_4)_2SO_4$, 1.5 g L$^{-1}$ $KH_2PO_4$, 1.0 g L$^{-1}$ $Na_2HPO_4$, 1.0 g L$^{-1}$ KCl, 0.5 g L$^{-1}$ $MgSO_4{\cdot}7H_2O$ and 4% glucose; 1000 mL) in 10×500 mL conical flasks was inoculated with seed culture (5%, *v*/*v*) and grown at 22 °C, 250 rpm for 50 h. Cells were removed by centrifugation at 10000 *g* for 15 min. The supernatant was acidified to pH 4.5 with dilute HCl and extracted with ethyl acetate (0.6 vol) twice. After the ethyl acetate had been removed by rotary evaporation, the residue was subjected to gel filtration chromatography on Sephadex LH-20 with elution with MeOH. Fractions were analysed by $^1$H NMR, which showed the presence of mupirocin H (**12**) and mupiric acid (**14**) in the earlier fractions. Further purification was carried on by gradient flash chromatography on normal-phase silica gel eluted with MeOH in $CHCl_3$ (from 0:100 to 15:85) to give mupirocin H (data as before[20]) and mupiric acid as a colourless viscous oil. $[\alpha]_D = +16$ ($c = 2.5$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta = 1.00$ (d, $J = 6.8$ Hz, 3H; 6-$CH_3$), 1.18 (d, $J = 6.4$ Hz, 3H; 2-$CH_3$), 1.20 (d, $J = 6.5$ Hz, 3H; 8-$H_3$), 2.09 (m, 1H; 6-H), 2.27 (ddd, $J = 13.9$, 7.1, 6.8 Hz, 1H; 3-H*H*), 2.37 (ddd, $J = 13.9$, 7.1, 6.8 Hz, 1H; 3-*H*H), 2.59 (m, 1H; 2-H), 3.53 (appt. br quin, $J = 6.5$ Hz, 1H; 7-H), 5.41 (dd, $J = 15.4$, 8.6 Hz, 1H; 5-H), 5.55 ppm (ddd, $J = 15.4$, 6.8, 6.8 Hz, 1H; 4-H); $^1$H NMR ($C_6D_6$, 400 MHz): $\delta = 0.85$ (d, $J = 6.8$ Hz, 3H; 6-$CH_3$), 1.00 (d, $J = 7.0$ Hz, 3H; 2-$CH_3$), 1.07 (d, $J = 6.2$ Hz, 3H; 8-$H_3$), 1.90 (m, 1H; 6-H), 2.06 (ddd, $J = 13.7$, 6.8, 6.6 Hz, 1H; 3-H*H*), 2.35 (m, 1H; 2-H), 2.17 (ddd, $J = 13.7$, 7.3, 6.7 Hz, 31H; 3-*H*H), 3.53 (dq, $J = 6.3$, 6.2 Hz, 1H; 7-H), 5.41 (dd, $J = 15.6$, 8.2 Hz, 1H; 5-H), 5.37 ppm (ddd, $J = 15.6$, 6.7, 6.7 Hz, 1H; 4-H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta = 16.7$ (6-$CH_3$), 16.7 (2-$CH_3$), 20.3 (C-8), 36.6 (C-3), 41.5 (C-2), 45.1 (C-6), 71.2 (C-7), 128.9 (C-4), 135.1 (C-5), 181.2 ppm (C-1); $^{13}$C NMR ($C_6D_6$, 75 MHz): $\delta = 16.1$ (6-$CH_3$), 16.4 (2-$CH_3$), 20.2 (C-8), 36.4 (C-3), 44.8 (C-2), 44.8 (C-6), 70.9 (C-7),128.4 (C-4), 135.0 (C-5), 180.1 (C-1); IR (neat): $\nu_{max} = 3502$, 2993, 1706, 1635, 1460, 1378, 1098 cm$^{-1}$; ESI: calcd $C_{10}H_{18}O_3Na$: 209.1154 $[M+Na]^+$; found 209.1156.

**(1*S*,2*R*)-*N*-[(2′*S*, 3′*S*)-2-Methyl 3-(triethylsilanyloxy)butanoyl]bornane-10,2-sultam:** Alcohol **18** (4.47 g, 14.17 mmol) was dissolved under nitrogen in dry DMF (17 mL). Imidazole (1.26 g, 18.42 mmol) was added, and the reaction mixture was cooled to 0 °C. Chlorotriethylsilane (2.85 mL, 17.00 mmol) was added, and the mixture was stirred for 19 h at room temperature. The solution was then diluted with $Et_2O$ (50 mL) to produce a white precipitate, and washed with water (3×20 mL). The organic layer was then dried over magnesium sulfate, filtered and concentrated in vacuo. Purification by flash column chromatography ($SiO_2$, 5–10% ethyl acetate/petroleum ether 40–60 °C, dry loaded) afforded the *silyl ether* (5.28 g, 87%) as a white solid; m.p. 148–150 °C (from ethyl acetate and petroleum ether 40–60 °C); $[\alpha]_D^{22} = -13.8$ ($c = 5.35$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta = 0.58$ (q, $J = 8.0$ Hz, 6H; Si($CH_2CH_3$)$_3$), 0.93 (t, $J = 8.0$ Hz, 9H; Si($CH_2CH_3$)$_3$), 0.97 (s, 3H; 8-$H_3$ or 9-$H_3$), 1.11 (d, $J = 7.0$ Hz, 3H; 2′-$CH_3$), 1.14 (d, $J = 6.5$ Hz, 3H; 4′-$H_3$), 1.18 (s, 3H; 8-$H_3$ or 9-$H_3$), 1.26–1.44 (m, 2H; 5-*H*H and 6-*H*H), 1.83–1.96 (m, 3H; 4-H, 5-H*H* and 6-H*H*), 2.03–2.08 (m, 2H; 3-$H_2$), 3.14 (appt. quin, $J = 7.0$ Hz, 1H; 2′-H), 3.42 (d, $J = 13.5$ Hz, 1H; 10-*H*H), 3.50 (d, $J = 13.5$ Hz, 1H; 10-H*H*), 3.88 (t, $J = 6.5$ Hz, 1H; 2-H), 4.16 ppm (appt.

quin, $J=6.5$ Hz, 1H; 3′-H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta=4.9$ (Si-($CH_2CH_3)_3$), 6.8 (Si($CH_2CH_3)_3$), 12.1 (2′-$CH_3$), 19.9 and 20.2 (C-8 and C-9), 21.0 (C-4′), 26.5 and 32.8 (C-5 and C-6), 38.6 (C-3), 44.7 (C-4), 47.7 and 48.1 (C-1 and C-7), 48.2 (C-2′), 53.1 (C-10), 65.3 (C-2), 70.3 (C-3′), 174.6 ppm (C-1′); IR (neat): $\nu_{max}=2955$, 2877, 1685, 1333 and 723 cm$^{-1}$; MS (CI): *m/z* 430 $[M+H]^+$ (19%), 400 (100), 330 (65), 159 (12), 135 (20) and 115 (22); CI: calcd $C_{21}H_{40}NO_4SSi$: 430.2447 $[M+H]^+$; found 430.2439; elemental analysis (%) calcd for $C_{21}H_{39}NO_4SSi$: C 58.70, H 9.15, N 3.26; found: C 58.60, H 9.07, N 3.36.

**(3*R*,4*S*)-3-Methyl-4-(triethylsilanyloxy)pent-1-ene (19):** Diisobutylaluminium hydride (1 M in hexanes, 2.42 mL, 2.42 mmol) was added dropwise under nitrogen at −78 °C over 30 min to the above silyl ether (1.00 g, 2.42 mmol) in dry $CH_2Cl_2$ (12 mL). The reaction mixture was stirred for 5 h and then quenched with saturated aqueous potassium sodium L-tartrate tetrahydrate solution (40 mL). The solution was stirred vigorously overnight and was then extracted with $CH_2Cl_2$ (3×100 mL), dried over magnesium sulfate and concentrated in vacuo. The resulting solid was triturated with petroleum ether 40–60 °C and filtered to remove the precipitated auxiliary. The filtrate was concentrated in vacuo to yield the corresponding aldehyde (0.51 g) as a pale yellow oil. Meanwhile, a solution of methyltriphenylphosphonium bromide (4.37 g, 12.10 mmol) and sodium hydride (2.18 g, 54.6 mmol, 60% dispersion in oil prewashed with dry hexane) in dry THF (50 mL) was gently heated with stirred under nitrogen until a green/grey colour was observed. The solution was stirred overnight at room temperature and the solids were then allowed to settle. The bright yellow solution was transferred by cannula into a solution of the aldehyde in dry THF (3 mL) and stirred at room temperature under nitrogen for 3 h. The solvent was then removed in vacuo, and the resulting yellow residue was purified by flash column chromatography ($SiO_2$, 0–1% ethyl acetate/petroleum ether 40–60 °C, dry loaded) to give alkene **19** (0.24 g, 48%) as a colourless oil. $[\alpha]_D^{21}=+10.5$ ($c=2.48$, $CHCl_3$); lit.[27] $[\alpha]_D=+2.8$ ($c=2.50$, $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta=0.59$ (q, $J=8.0$ Hz, 6H; Si($CH_2CH_3)_3$), 0.96 (t, $J=8.0$ Hz, 9H; Si($CH_2CH_3)_3$), 0.99 (d, $J=7.0$ Hz, 3H; 3-$CH_3$), 1.07 (d, $J=6.5$ Hz, 3H; 5-$H_3$), 2.18 (m, 1H; 3-H), 3.73 (dq, $J=6.5$, 4.5 Hz, 1H; 4-H), 4.98 (m, 1H; 1-*H*H), 5.01 (m, 1H; 1-H*H*), 5.79 ppm (ddd, $J=17.5$, 9.5, 7.5 Hz, 1H; 2-H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta=5.1$ (Si($CH_2CH_3)_3$), 7.0 (Si($CH_2CH_3)_3$), 15.1 (3-$CH_3$), 20.4 (C-5), 45.4 (C-3), 71.6 (C-4), 114.2 (C-2), 141.3 ppm (C-1).

**(4*S*)-*N*-[(2*R*,4*E*,6*R*,7*S*)-2,6-Dimethyl-7-(triethylsilanyloxy)oct-4-enoyl]-4-isopropyloxazolidin-2-one:** A premixed solution of alkene **19** (0.12 g, 0.56 mmol) and the known[28] alkene **21** (0.24 g, 1.12 mmol) in dry toluene (3 mL) was added to a solution of Grubbs' second-generation catalyst (24.3 mg, 0.028 mmol) in dry toluene (2 mL). The reaction mixture was stirred at 55–65 °C, overnight. Upon cooling, the solvent was removed in vacuo, and the mixture was then purified by flash column chromatography ($SiO_2$, 2.5–5% ethyl acetate/petroleum ether 40–60 °C) to give the title compound as a 4:1 mixture of *E*/*Z* isomers, as a colourless oil (0.12 g, 50%). $[\alpha]_D^{25}$ (of 4:1 mixture) $=+14.2$ ($c=3.32$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ (major isomer) $=0.58$ (q, $J=8.0$ Hz, 6H; Si($CH_2CH_3)_3$), 0.87 and 0.91 (each d, $J=7.0$ Hz, each 3H; CH($CH_3)_2$), 0.94 (d, $J=6.0$ Hz, 3H; 6′-$CH_3$), 0.95 (t, $J=8.0$ Hz, 9H; Si($CH_2CH_3)_3$), 1.03 (d, $J=6.0$ Hz, 3H; 8′-$H_3$), 1.13 (d, $J=7.0$ Hz, 3H; 2′-$CH_3$), 2.08–2.19 (appt. sext, $J=7.0$ Hz, 2H; 3′-*H*H and 6′-H), 2.33 (dsept, $J=7.0$, 3.5 Hz, 1H; C*H*($CH_3)_2$), 2.47 (appt. quin, $J=7.0$ Hz, 1H; 3′-H*H*), 3.70 (dq, $J=6.0$, 4.5 Hz, 1H; 7′-H), 3.80 (appt. sext, $J=7.0$ Hz, 1H; 2′-H), 4.20 (dd, $J=9.0$, 3.0 Hz, 1H; 5-*H*H), 4.27 (appt. t, $J=9.0$ Hz, 1H; 5-H*H*), 4.46 (ddd, $J=8.5$, 3.5, 3.0 Hz, 1H; 4-H), 5.35 (ddd, $J=15.5$, 7.5, 6.0 Hz, 1H; 4′-H), 5.45 ppm (dd, $J=15.5$, 7.5 Hz, 1H; 5′-H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ (major isomer) $=5.0$ (Si($CH_2CH_3)_3$), 6.9 (Si-($CH_2CH_3)_3$), 14.7 (CH($CH_3)_2$), 15.3 and 16.0 (2′-$CH_3$ and 6′-$CH_3$), 18.0 (CH($CH_3)_2$), 20.2 (C-8′), 28.4 (*C*H($CH_3)_2$), 37.1 (C-3′), 37.7 (C-2′), 44.2 (C-6′), 58.4 (C-4), 63.1 (C-5), 71.7 (C-7′), 126.4 (C-4′), 135.7 (C-5′), 153.7 (C-2), 176.6 ppm (C-1′); IR (neat): $\nu_{max}=2961$, 2876, 1778, 1700 cm$^{-1}$; MS (CI): *m/z* 412 $[M+H]^+$ (3%), 382 (60), 280 (100), 159 (28), 151 (23); CI: calcd for $C_{22}H_{42}NO_4Si$: 412.2883 $[M+H]^+$; found 412.2885.

**(4*R*)-*N*-[(2*S*,4*E*,6*R*,7*S*)-2,6-Dimethyl-7-(triethylsilanyloxy)oct-4-enoyl]-4-isopropyloxazolidin-2-one:** A premixed solution of alkene **19** (0.60 g, 2.80 mmol) and the known[28] alkene **17** (0.31 g, 1.40 mmol) in dry toluene (7 mL) was added to a solution of Grubbs' second-generation catalyst (60.8 mg, 0.07 mmol) in dry toluene (5 mL). The reaction was stirred at 60–70 °C, overnight. Upon cooling, the solvent was removed in vacuo, and the mixture was then purified by flash column chromatography ($SiO_2$, 2.5–5% ethyl acetate/petroleum ether 40–60 °C) to give the title alkene as a 4:1 mixture of *E*/*Z* isomers as a colourless oil (0.22 g, 38%). $[\alpha]_D^{25}$ (4:1 mixture) $=-22.7$ ($c=2.56$, $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ (major isomer) $=0.58$ (q, $J=8.0$ Hz, 6H; Si($CH_2CH_3)_3$), 0.87 and 0.91 (each d, $J=7.0$ Hz, each 3H; CH($CH_3)_2$), 0.94 (d, $J=6.5$ Hz, 3H; 6′-$CH_3$), 0.95 (t, $J=8.0$ Hz, 9H; Si($CH_2CH_3)_3$), 1.03 (d, $J=6.0$ Hz, 3H; 8′-$H_3$), 1.13 (d, $J=7.0$ Hz, 3H; 2′-$CH_3$), 2.08–2.20 (m, 2H; 3′-*H*H and 6′-H), 2.33 (dsept, $J=7.0$, 3.5 Hz, 1H; C*H*($CH_3)_2$), 2.47 (appt. quin, $J=6.0$ Hz, 1H; 3′-H*H*), 3.70 (dq, $J=6.0$, 4.5 Hz, 1H; 7′-H), 3.80 (appt. sext, $J=7.0$ Hz, 1H; 2′-H), 4.20 (dd, $J=9.0$, 3.0 Hz, 1H; 5-*H*H), 4.27 (appt. t, $J=9.0$ Hz, 1H; 5-H*H*), 4.46 (ddd, $J=8.5$, 3.5, 3.0 Hz, 1H; 4-H), 5.35 (ddd, $J=15.5$, 7.5, 6.0 Hz, 1H; 4′-H), 5.45 ppm (dd, $J=15.5$, 7.5 Hz, 1H; 5′-H); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ (major isomer) $=4.9$ (Si($CH_2CH_3)_3$), 6.8 (Si($CH_2CH_3)_3$), 14.6 (CH($CH_3)_2$), 15.0 and 15.8 (2′-$CH_3$ and 6′-$CH_3$), 17.9 (CH($CH_3)_2$), 20.0 (C-8′), 28.4 (*C*H($CH_3)_2$), 37.0 (C-3′), 37.6 (C-2′), 44.1 (C-6′), 58.3 (C-4), 63.0 (C-5), 71.6 (C-7′), 126.3 (C-4′), 135.6 (C-5′), 153.6 (C-2), 176.4 ppm (C-1′); IR (neat): $\nu_{max}=$ 2960, 2876, 1779, 1700 cm$^{-1}$; MS (CI): *m/z* 412 $[M+H]^+$ (2%), 382 (70), 280 (100), 159 (48), 151 (32); CI: calcd for $C_{22}H_{42}NO_4Si$: 412.2883 $[M+H]^+$; found 412.2866.

**(2*R*,4*E*,6*R*,7*S*)-2,6-Dimethyl-7-hydroxyoct-4-enoic acid (mupiric acid, 14):** Lithium hydroxide monohydrate (0.02 g, 0.50 mmol) and hydrogen peroxide solution (0.1 mL, 0.75 mmol, 30% solution in water) were added successively at 0 °C to a solution of (4*R*)-*N*-[(2*S*,4*E*,6*R*,7*S*)-2,6-dimethyl-7-(triethylsilanyloxy)oct-4-enoyl]-4-isopropyloxazolidin-2-one (0.11 g, 0.28 mmol) in THF (3 mL) and water (1.5 mL). This was then stirred at room temperature, overnight, and quenched with sodium sulfite solution (1.25 mL, 0.75 mmol, 7.56% in water). After 10 min the solvents were removed in vacuo. A mixture of water (3 mL), acetic acid (5 mL) and THF (11 mL) was then added to the residue, and the mixture was stirred overnight. Solvents were then removed in vacuo, and the crude product was purified by flash column chromatography ($SiO_2$, 20% ethyl acetate/1% acetic acid/petroleum ether 40–60 °C) to afford mupiric acid as a 4:1 mixture of *E*/*Z* isomers as a colourless oil (0.20 g, 40%). $[\alpha]_D^{25}$ (of 4:1 mixture) $=+13.4$ ($c=1.05$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ (major isomer) $=0.98$ (d, $J=7.0$ Hz, 3H; 6-$CH_3$), 1.16 (d, $J=6.5$ Hz, 3H; 8-$H_3$), 1.18 (d, $J=7.0$ Hz, 3H; 2-$CH_3$), 2.09 (appt. sept, $J=7.0$ Hz, 1H; 6-H), 2.25 (appt. quin d, $J=7.0$, 1.0 Hz, 1H; 3-*H*H), 2.35 (appt. quin d, $J=7.0$, 1.0 Hz, 1H; 3-H*H*), 2.56 (appt. sept, $J=7.0$ Hz, 1H; 2-H), 3.53 (appt. quin, $J=6.5$ Hz, 1H; 7-H), 5.39 (dd, $J=15.5$, 8.5 Hz, 1H; 5-H), 5.53 (dt, $J=15.5$, 7.0 Hz, 1H; 4-H), 6.50 (br s, 1H; COOH); $^1$H NMR ($C_6D_6$, 400 MHz): $\delta$ (major isomer) $=0.90$ (d, $J=7.0$ Hz, 3H; 6-$CH_3$), 1.04 (d, $J=7.0$ Hz, 3H; 2-$CH_3$), 1.12 (d, $J=6.0$ Hz, 3H; 8-$H_3$), 1.96 (appt. sept, $J=7.0$ Hz, 1H; 6-H), 2.11 (appt.

quin, $J=7.0$ Hz, 1H; 3-*H*H), 2.22 (appt. quin, $J=7.0$ Hz, 1H; 3-H*H*), 2.41 (appt. sept, $J=7.0$ Hz, 1H; 2-H), 3.46 (appt. quin, $J=6.0$ Hz, 1H; 7-H), 5.34 (dd, $J=15.5$, 8.0 Hz, 1H; 5-H), 5.41 (dt, $J=15.5$, 7.0 Hz, 1H; 4-H), 5.94 ppm (brs, 1H; COOH); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ (major isomer) = 16.2 and 16.5 (2-$CH_3$ and 6-$CH_3$), 20.0 (C-8), 36.5 (C-3), 39.8 (C-2), 44.9 (C-6), 71.1 (C-7), 128.7 (C-4), 134.9 (C-5), 181.2 (C-1); IR (neat): $\nu_{max}=3363$, 2971, 2933, 1705 cm$^{-1}$. MS (CI): *m/z* 187 $[M+H]^+$ (5%), 169 (63), 151 (78), 123 (67), 95 (100), 85 (28), 83 (36), 69 (43); CI: calcd for $C_{10}H_{19}O_3$: 187.1334 $[M+H]^+$; found: 187.1332.

**(2*S*,4*E*,6*R*,7*S*)-2,6-Dimethyl-7-hydroxyoct-4-enoic acid (15):** Lithium hydroxide monohydrate (0.02 g, 0.50 mmol) and hydrogen peroxide solution (0.1 mL, 0.75 mmol, 30% solution in water) were added successively at 0 °C to a solution of (4*R*)-*N*-[(2*S*,4*E*,6*R*,7*S*)-2,6-dimethyl-7-(triethylsilanyloxy)oct-4-enoyl]-4-isopropyloxazolidin-2-one (0.13 g, 0.32 mmol) in THF (3 mL) and water (1.5 mL). This was then stirred at room temperature, overnight, and quenched with sodium sulfite solution (1.25 mL, 0.75 mmol, 7.56% in water). After 10 min the solvents were removed in vacuo. A mixture of water (3 mL), acetic acid (5 mL) and THF (11 mL) was then added to the residue, and the mixture was stirred, overnight. Solvents were then removed in vacuo, and the crude product was purified by flash column chromatography ($SiO_2$, 20% ethyl acetate/1% acetic acid/petroleum ether 40–60 °C) to afford *acid* **16** as a 4:1 mixture of *E*/*Z* isomers as a colourless oil (0.20 g, 40%). $[\alpha]_D^{25}$ (4:1 mixture) = +35.0 ($c=1.95$ in $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ = (major isomer) 0.98 (d, $J=7.0$ Hz, 3H; 6-$CH_3$), 1.16 (d, $J=6.0$ Hz, 3H; 8-$H_3$), 1.18 (d, $J=7.0$ Hz, 3H; 2-$CH_3$), 2.07 (appt. sept, $J=7.0$ Hz, 1H; 6-H), 2.21 (appt. quin d, $J=7.0$, 1.0 Hz, 1H; 3-*H*H), 2.34 (appt. quin, $J=7.0$ Hz, 1H; 3-H*H*), 2.52 (appt. sept, $J=7.0$ Hz, 1H; 2-H), 3.52 (appt. quin, $J=6.0$ Hz, 1H; 7-H), 5.35 (dd, $J=15.0$, 8.5 Hz, 1H; 5-H), 5.51 (dt, $J=15.0$, 7.0 Hz, 1H; 4-H), 6.07 (brs, 1H; COOH); $^1$H NMR ($C_6D_6$, 400 MHz): $\delta$ (major isomer) = 0.98 (d, $J=7.0$ Hz, 3H; 6-$CH_3$), 1.05 (d, $J=7.0$ Hz, 3H; 2-$CH_3$), 1.14 (d, $J=6.0$ Hz, 3H; 8-$H_3$), 2.00 (m, 2H; 3-*H*H and 6-H), 2.26 (m, 1H; 3-H*H*), 2.40 (m, 1H; 2-H), 3.47 (appt. quin, $J=6.0$ Hz, 1H; 7-H), 5.31 (dd, $J=15.5$, 8.0 Hz, 1H; 5-H), 5.39 (dt, $J=15.5$, 7.0 Hz, 1H; 4-H), 6.32 ppm (brs, 1H; COOH); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ (major isomer) = 16.6 (2 coincident peaks, 2-$CH_3$ and 6-$CH_3$), 19.9 (C-8), 37.0 (C-3), 39.7 (C-2), 45.1 (C-6), 71.2 (C-7), 129.3 (C-4), 134.9 (C-5), 180.8 ppm (C-1); IR (neat): $\nu_{max}=3333$ (br, OH), 2971, 2933, 1705 cm$^{-1}$. MS (CI): *m/z* 187 $[M+H]^+$ (31%), 169 (100), 151 (93), 123 (82), 95 (98), 85 (10), 83 (16) and 69 (49); CI calcd for $C_{10}H_{19}O_3$: 187.1334 $[M+H]^+$; found: 187.1334.

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