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Selected Mutations Reveal New Intermediates in the Biosynthesis of Mupirocin and the Thiomarinol Antibiotics

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Abstract: Mupirocin and the thiomarinols are assembled on similar polyketide/fatty acid backbones and exhibit potent antibiotic activity against MRSA. They both contain a tetra-substituted tetrahydropyran (THP) ring essential for biological activity. Mupirocin is a mixture of pseudomonic acids (PAs). Isolation of the novel mupirocin P containing a 7-hydroxy-6-ketoTHP from a *ΔmupP* strain and chemical complementation experiments confirm that the first step in the conversion of PA-B to the major product PA-A is oxidation at C-6. In addition, nine novel thiomarinol (TM) derivatives with different oxidation patterns decorating the central THP core have been isolated from gene deletion (*tmlF*). These metabolites are in accord with the THP ring formation and elaboration in thiomarinol following a similar order to that found in mupirocin biosynthesis, despite lacking some of the equivalent genes. Novel mupirocin-thiomarinol hybrids have been synthesised using mutasynthesis.

Thiomarinols (**1**-**6**) are an unusual group of hybrid antibiotics produced by the marine bacterium *Pseudoalteromonas* sp. SANK73390 (Figure 1).[1] They combine close analogues of the clinically important agent, mupirocin (a mixture of pseudomonic acids, PA A–C, **7**-**9**)[2] with the pyrrothine subunit of the holomycin class of antibiotics. Both thiomarinols and mupirocin display potent activity against MRSA (methicillin-resistant *Staphylococcus aureus)*,[3] and belong to the *trans*-AT class of modular polyketide-derived antibiotics.[4] The *mup* biosynthetic gene cluster was one of the first of this class to be discovered,[5] but there are now more than 20 at least partially characterised, with many others known from genomic sequences.[4] Thiomarinols can be regarded as comprising three main elements: a highly functionalised polyketide-derived acid, which is esterified by 8-hydroxyoctanoic acid, which itself forms an amide with the bicyclic amino acid-derived pyrrothine. The major components TM-A **1** and PA-A **7** differ in the lack of a 10,11-epoxide, the presence of a 4-hydroxyl, a C_8 rather than a C_9 fatty acid, and the pyrrothine in TM-A.[6]

The 6,7-dihydroxytetrahydropyran (THP) ring is necessary for the biological activity of mupirocin and thiomarinol, so elucidation of the biosynthetic mechanisms for ring formation and further modifications to the 6,7-diol is important. Mutational analysis of mupirocin biosynthesis showed somewhat counter-intuitively: (a) that the 8-hydroxyl of PA-B **8** has to be lost not gained, with feeding experiments confirming that PA-B is an intermediate in the biosynthesis of PA-A **7**[7] and (b) that PA-C **9** is the product of a parallel pathway that branches from the main pathway (to PA-A) following failure to undergo epoxidation of the 10,11-double bond.[8] Analyses of mutant strains[9] indicate that putative biosynthetic precursor **12** (Scheme 1) is cyclised and esterified to give PA-B with the tetrahydropyran ring. Furthermore identification of minor metabolites with C_5 and C_7 fatty acid side-chains are in accord with elaboration of the 9-hydroxynonanoic acid moiety occurring by initial addition of 3-hydroxypropionate followed by 3 successive MmpB-mediated chain elongations.[7b]

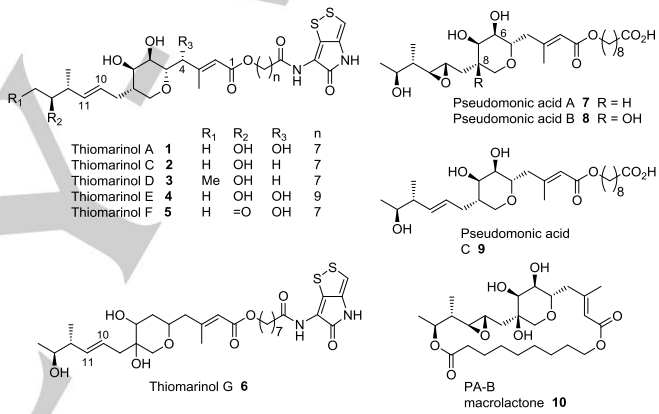

Figure 1. Selected thiomarinols and pseudomonic acids.

New insights into the mechanism of PA-B to PA-A conversion – MupP acts as a dehydratase.

Conversion of PA-B **8** to PA-A **7**, formally removal of the 8-hydroxy, has been shown to require MupU (CoA-ligase) and MacpE (ACP), MupO (P_{450}), MupV (see below), MupC (enoate reductase) and MupF (ketoreductase) *via* the isolated intermediates mupirocin C1 **14** and mupirocin F1 **15**, as shown in Scheme 1. Deletion of any of *mupO, mupU*, mup*V* or *macpE* all gave PA-B **8** as the major product along with the macrolactone **10**.[8] The first step in the conversion of PA-B **8** to PA-A **7** is MupU catalysed ligation of PA-B to MacpE (Scheme 1) followed by oxidation (MupO). However, no metabolite corresponding to the putative α-hydroxyketone product **13** of MupO had been detected. Isolation and identification of these epoxide-containing metabolites is challenging as they are prone to rearrangements but are key to elucidating the remaining biosynthetic steps from PA-B to PA-A. Indeed PA-A itself is limited to mainly topical applications due to its instability in serum.[3]

Our first goal was to establish the proposed 7-hydroxy-6-ketone **13** as a biosynthetic intermediate to PA-A. Analysis of a *ΔmupP* strain revealed a major metabolite with MW 514 in accord with **13**, but it was unstable and gave rearrangement products. Further analysis of this mutant, however, using Sephadex LH-20 fractionation of the crude extract has allowed isolation of the

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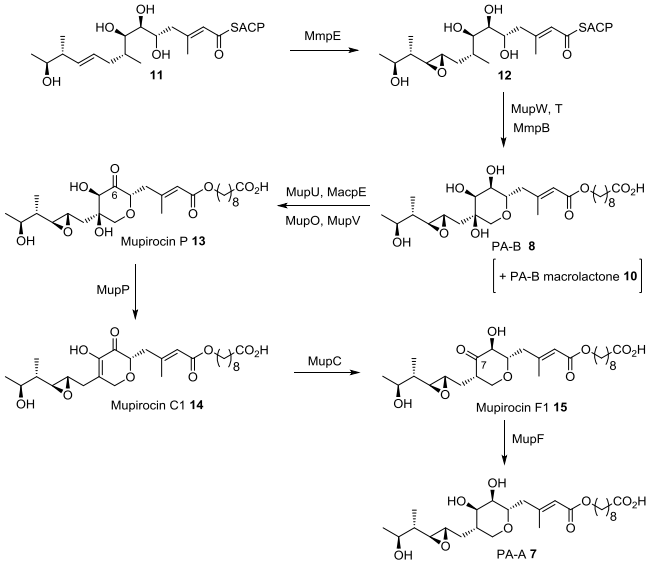
product and detailed 1D and 2D NMR analysis (see Supporting Information) confirms the structure of this key intermediate, which is now named mupirocin P (**13**).



Scheme 1. MupP acts as a dehydratase in mupirocin biosynthesis

We have shown previously that the OR domain of MmpE is responsible for epoxidation.[8] Thus a double mutant, *mmpEΔOR/ΔmupP* strain was constructed. Gratifyingly, this produces the less labile desepoxy-mupirocin P **16** as the main metabolite along with a small amount of desepoxy-PA-B **17** (Figure 2). MupP had not been previously assigned a function. Bioinformatic analysis indicates that it belongs to the glyoxalase/bleomycin resistance protein/dioxygenase superfamily but appears to act here overall as a dehydratase to give enol-ketone **14** (Scheme 1).

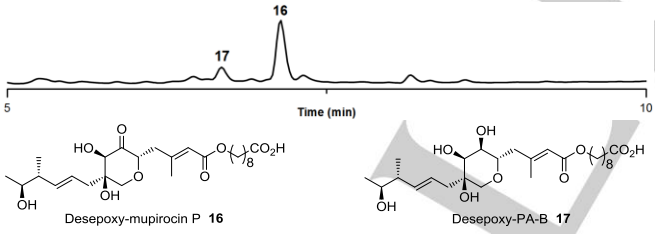
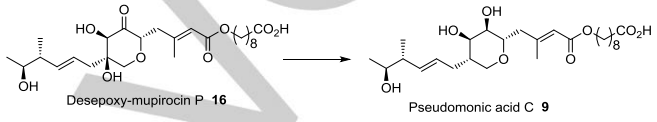


Figure 2. HPLC trace of crude extract of the *mmpEΔOR/ΔmupP* double mutant strain of *P. fluorescens* NCIMB 10586

Previous mutasynthesis studies on mupirocin biosynthesis[8] showed that PA-B **8**, mupirocin C1 **14** and mupirocin F1 **15** were all efficiently (15-50%) converted to PA-A **7** by mutant strains of *P. fluorescens* blocked in earlier parts of the pathway, thus confirming their role as intermediates in mupirocin biosynthesis. To investigate if the 7-hydroxy-6-ketone is also a true intermediate on the pathway, desepoxy-mupirocin P **16**, isolated from the *mmpEΔOR/ΔmupP* strain, was fed to cultures of the *mmpEΔOR/ΔmupW* strain and LC-MS showed a peak with the same retention time as desepoxy-mupirocin P but with MW 484. The metabolite was isolated and NMR revealed that PA-C had been produced confirming that desepoxy-mupirocin P is indeed an intermediate in PA-C **9** biosynthesis (Scheme 2).

Reanalysis of the MupV amino acid sequence, which had previously been identified as a putative oxidoreductase, revealed a second domain with *ca*. 30% sequence identity to many *Pseudomonas* α/β hydrolases. These contain a GXCXG consensus sequence where cysteine replaces the more common nucleophilic serine and includes an amino transferase involved in shuffling intermediates between thiolation domains in the syringomycin biosynthetic pathway in *Pseudomonas syringae*.[10] Thus MupV may act as a thioesterase or perhaps in some related ACP transfer capacity. Previously deletion of *mupV* had provided few clues as this yielded PA-B **8** as the major product.[9c] However, in this study, feeding desepoxy-mupirocin P **16** to the *ΔmupV* strain gave PA-C in accord with a Mup V activity being required before MupP leading to mupirocin P **13** (and desepoxy-mupirocin P **16**) for successive dehydration (MupP) and reductions catalysed by MupC and MupF to PA-A **7** (and PA-C **9**) (Schemes 1 and 2).



Scheme 2. Feeding **16** to either *mmpEΔOR/ΔmupW* or *ΔmupV* strains gives PA-C **9**.

Thiomarinol biosynthesis

The thiomarinol (*tml*) biosynthetic gene cluster was identified *via* complete genome sequencing of SANK73390, which was found to harbour a 97 kb plasmid consisting almost entirely of the thiomarinol biosynthetic genes.[11] These include multimodular *trans*-AT PKSs (*tmpA, C* and *D*), a putative FAS (*tmpB*) and associated tailoring and resistance genes (*tmlA-Z*) most of which exhibit high homology to counterparts in the mupirocin (*mup*) cluster (Figure S1). A non-ribosomal peptide synthetase (NRPS) linked to a set of tailoring enzymes (*holA-H*) similar to that recently shown to control holomycin biosynthesis in *Streptomyces clavuligerus* is also present.[12] We have produced mutant strains of SANK73390 in which the PKS and NRPS parts of the cluster have been insertionally deactivated. Analysis of WT, *NRPS* and *tmlU* mutant strains led to the isolation of marinolic acids A **18**, A_6 **20**, and A_4 **21** lacking the pyrrothine (Figure 3).[13] In the minor components marinolic acids A_6 and A_4 the octanoate moiety of marinolic acid A has been replaced by hexanoate and butanoate respectively. Using a combination of genetic and isotopic labelling studies, we showed that the 8-hydroxyoctanoic acid side-chain is generated *via* successive chain extensions of a C_4 precursor (4-hydroxybutyrate) derived from succinate, to give marinolic acids **18**, **20** and **21** and that pyrrothine is assembled from two molecules of cysteine (HolA-D) prior to intact incorporation into thiomarinol.[13] It has recently been shown that TmlU acts as a substrate selective CoA ligase that activates marinolic acid as a thioester which in turn is the substrate for amide formation catalysed by the acyl transferase HolE.[14] Analysis of the *PKS* mutant revealed a series of acyl-pyrrothines designated as xenorhabdins.[13]

Marinolic acid A **18** has a similar structure to PA-A **7** but lacks the epoxide and has an additional 4-hydroxyl group. The *tml* pathway, however has only some of the homologues of the *mup* genes, viz. *tmlW, T, O, P, C* and *F*, but homologues of the stand alone ACP *macpE, mupU* and *mupV* are missing. The absence of these genes from the *tml* cluster raises the question of how the same overall conversion can be achieved with an apparently simpler gene set. We now report results which indicate that a

similar set of intermediates are indeed involved in THP ring formation and further modification in both mupirocin and thiomarinol biosynthesis.

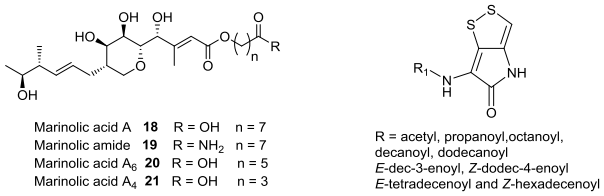


Figure 3. Marinolic acids and acyl-pyrrothine metabolites isolated from WT and mutant strains of *Pseudoalteromonas* sp SANK73390

Chemical investigation of ΔtmlF- late stage biosynthetic steps.

Deletion of *mupF* (7-ketoreductase) in *P. fluorescens* releases a number of intermediates including mupirocin C1 **14** and PA-B **8** and related shunt products in addition to mupirocin F1 **15** (Scheme 1).[9b] TmlF, shows 33% amino acid sequence identity to MupF so it is likely to act as a 7-ketoreductase as does MupF in the final step in mupirocin biosynthesis, which converts mupirocin F1 **15** to the dihydroxytetrahydropyran PA-A **7**. Thus deletion of *tmlF* could release more intermediates and shunt products besides its own substrate analogous to the *mupF* deletion.

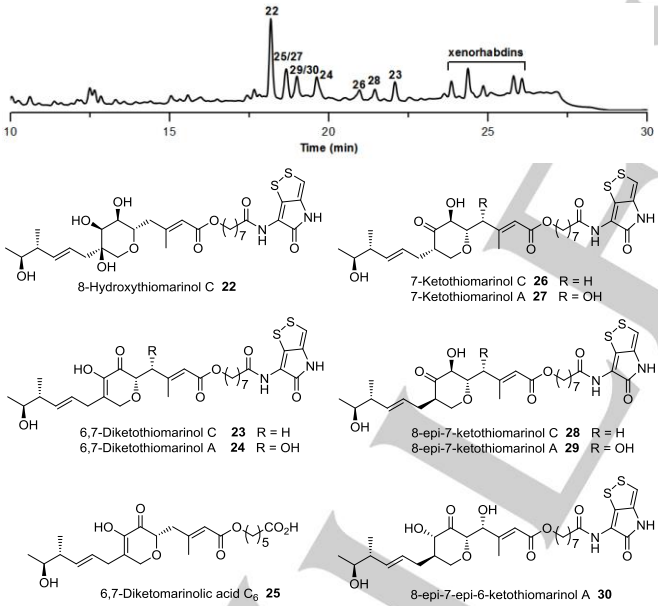


Figure 4. Novel metabolites isolated from extracts of the *ΔtmlF* mutant of *Pseudoalteromonas* sp. SANK73390

WT SANK73390 was cultivated on a modified marine broth medium (see SI for details) which significantly improved the production of the total thiomarinols. Using this medium, a yield of 70–100 mg.L^{-1} TM-A **1** could be obtained, compared to 10-20 mg.L^{-1} in previous studies.[6] Our aim was to carry out a set of gene KOs to parallel those carried out with mupirocin,[8,9] but reverse genetics of SANK73390 proved to be problematic. We were however successful in making the key *ΔtmlF* by the method previously reported[11] using approximately 500bp arms defining an in-frame deletion from aa 10 to aa 327 in TmlF.

Analysis of extracts showed that as anticipated the production of thiomarinols A and C (**1** and **2**, Figure 1) was completely abolished, but the xenorhabdin acyl-pyrrothine metabolites were generated as normal. Further analysis of the HPLC trace indicated that various new thiomarinol-related metabolites were present albeit in minor amounts, 0.1 - 1.2 mg.L^{-1} (Figure 4). A major product among those minor metabolites was identified as 8-hydroxy-thiomarinol C **22**. Its structure, and that of other metabolites reported herein, were determined by full NMR analysis and HR-ESI-MS (see SI for details). The presence of an 8-hydroxy-THP ring as in PA-B **8** suggests that both biosynthetic pathways possess analogous pyran ring closing mechanisms, and will require similar mechanisms for removal of the 8-OH function. The only previous thiomarinol derivative reported to contain an 8-hydroxyl is TM-G **6** (as a very minor metabolite of WT SANK73390),[1c] but it lacks both the 4 and 6-hydroxyls. We have not observed **6** in any of our studies, and its structure remains to be confirmed. Further detailed analysis of minor components resulted in the isolation of metabolites **23**–**30**. These metabolites form two structurally distinct groups. The first consists of 6,7- diketothiomarinol C **23**, analogous to mupirocin C1 **14** isolated from Δ*mupC*, its 4-hydroxy analogue **24**, and **25** a 6,7-diketo analogue of marinolic acid A_6 lacking the 4-hydroxyl and the pyrrothine. These all exist as the enol-ketone tautomers shown. The second group possesses the 6-hydroxy-7-keto THP ring (7-keto-thiomarinol C **26** and its 4-hydroxy analogue **27**) similar to mupirocin F1 **15** isolated from Δ*mupF*, and products (**28**–**30**) derived from **26** and **27** by keto-enol tautomerism and/or epimerisation. This is consistent with a biosynthetic sequence to thiomarinol C **2** being similar to mupirocin after the formation of the 8-hydroxyTHP ring. Interestingly, the 4-hydroxy analogue of 8-hydroxy-thiomarinol C was not detected.

Chemical Complementation Experiments

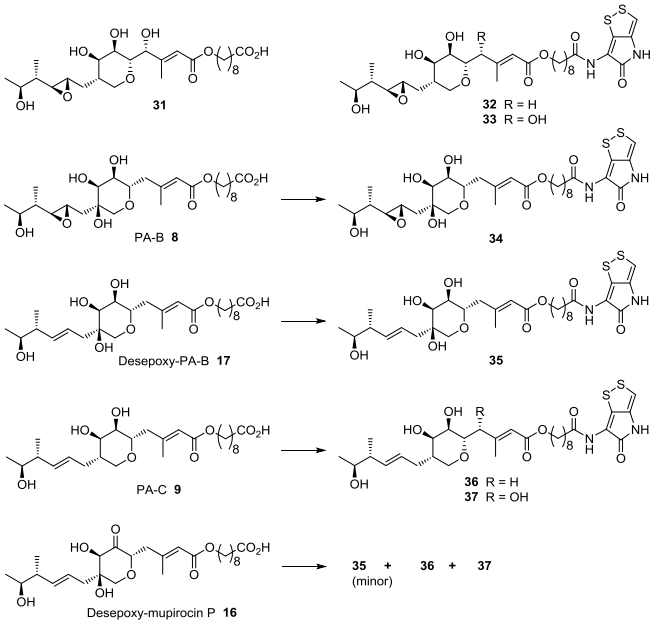
When PA-A **7** was fed to the *PKS* mutant strain of SANK73390 in which all of the tailoring genes are intact, it was metabolised with both 4-hydroxylation and/or pyrrothine addition taking place to give the novel mupirocin/thiomarinol hybrid molecules **31**-**33** (Scheme 3).[6,13] In this study we have investigated biotransformations of further substrates isolated from *P. fluorescens* and the products were isolated and fully characterised by spectroscopic methods.

On feeding PA-C **9**, with the mature 6,7-dihydroxypyran ring, the pyrrothine was added to give the novel metabolite **36** (MW 638) and 4-hydroxylation yielded **37** (MW 654) in similar amounts (Scheme 3). In contrast, feeding PA-B **8** or desepoxy-PA-B **17** simply led to pyrrothine addition (**34** and **35**, respectively) and no C-4 hydroxylated metabolites were detected. Hence it is apparent that the presence of an 8-hydroxyl inhibits 4-hydroxylation.

Desepoxy-mupirocin P **16** was also fed to cultures of the *ΔPKS* mutant giving the 3 metabolites, **35**, **36** and **37** all of which had the pyrrothine added. Importantly it was evident that further processing of the THP ring had occurred leading to loss of the 8-hydroxyl and keto-reduction giving the fully mature 6,7-dihydroxylated product **36**; a minor product detected by LC-MS was the corresponding 4-hydroxylated metabolite **37**. The 6,7,8-trihydroxy metabolite **35** was formed *via* a keto-reduction and pyrrothine addition, but no loss of the 8-hydroxyl was observed.

It is interesting to compare the metabolism of 8-hydroxyTHPs in *P. fluorescens* and *Pseudoalteromonas*. In the former desepoxy PA-B **17** (and PA-B **8**) undergo efficient oxidation, dehydration and keto-reduction to give the bioactive 6,7-diols PA-C **9** (and PA-A **7**). In contrast, in the *PKS* mutant of *Pseudoalteromonas* whilst further processing of the 6,8-dihydroxy-7-ketoTHP ring of desepoxy-mupirocin P **16** to the mature 6,7-dihydroxyTHP as

well as pyrrothine addition readily occurs, feeding the 6,7,8-trihydroxy THP substrates **8** or **17**, only pyrrothine addition was apparent.



Scheme 3. Transformation of mupirocin analogues by the *PKS* mutant strain of *Pseudoalteromona*s SANK73390.

Conclusions

MupP has been identified as a dehydratase involved in mupirocin biosynthesis. The *ΔmupP* strain of *P. fluorescens* was cultured and the novel 6-keto-7-alcohol, mupirocin P **13** was isolated and fully characterised. Results of chemical complementation studies feeding desepoxy-mupirocin P **16** to the *mmpEΔOR/ΔmupW* strain blocked earlier in the biosynthetic pathway, are in accord with **16** being an intermediate in the biosynthesis of the bioactive 6,7-diol PA-C **9** (and the epoxy analogue mupirocin P **13** is deduced to be a biosynthetic precursor of PA-A **7**). Hence the first step in conversion of PA-B **8** to PA-A **7** is oxidation at C-6. Furthermore feeding **16** to the *ΔmupV* strain gave PA-C in accord with MupV acting before MupP.

Despite some divergencies in the gene clusters, the isolation of novel metabolites including **22**, **23** and **26** from cultures of the *ΔtmlF* mutant of SANK73390 point to some similarities in THP ring processing in both thiomarinol and mupirocin. Indeed, feeding desepoxy-mupirocin P **16** to cultures of the *PKS* mutant not only led to addition of the pyrrothine but also confirmed that further processing of the THP ring occurred giving 6,7-dihydroxylated products **36** and **37**. In contrast, feeding desepoxy-PA-B **17** to the *PKS* mutant strain simply led to addition of the pyrrothine and no THP modified products were detected. PA-B **8** is efficiently converted to PA-A **7** by mutant strains of *P. fluorescens* blocked earlier in the biosynthetic pathway indicating an important difference between the two pathways.

Differences are evident in the two gene clusters. Homologues of the mupirocin stand-alone ACP MacpE, along with MupU and MupV are missing in SANK73390. TmpB, however, contains extra KS and ACP domains as compared to MmpB which it is speculated may replace the apparently missing activities.[13] Thus the tetrahydropyran processing reactions occurring on intermediates bound to MacpE, may take place while bound to the additional ACP on TmpB, thus obviating the need for homologues of MacpE and MupU. The lack of ring processing of PA-B **8** and desepoxy-PA-B **17** fed to the *PKS* mutant of *Pseudoalteromonas* thus may be rationalised by an inability of these substrates to be loaded onto TmpB so providing key evidence to support our proposed pathway to the marinolic acids. Studies are in progress to fully understand the differences between the two pathways as well as to identify the genes for 4- and 6-hydroxylation in thiomarinol and 6-hydroxylation in mupirocin biosynthesis.

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Keywords: antibiotic • polyketide • biosynthesis • mutasynthesis • structure determination

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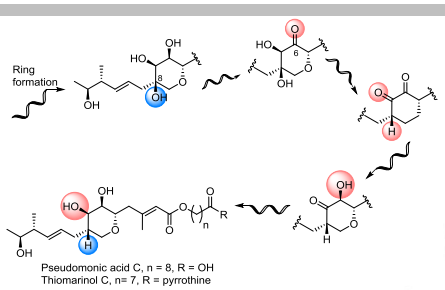
Entry for the Table of Contents (Please choose one layout)

COMMUNICATION

The long and winding road (to replace OH with H): Isolation and structure elucidation of novel metabolites from mutant strains of *Ps. fluorescens* and the marine bacterium *Pseudoalteromonas* combined with complementation experiments reveal that THP ring formation and elaboration of thiomainol follow a similar order to that found in mupirocin despite lacking some of the genes.



*S-S. Gao, L. Wang, Z. Song, J. Hothersall, E.R Stevens, J. Connolly, P.J. Winn, R.J. Cox, M.P. Crump, P.R. Race, C.M. Thomas, T.J. Simpson and C.L. Willis**

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