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

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Abstract: *Thiomarinol and mupirocin are assembled on similar polyketide/fatty acid backbones and exhibit potent antibiotic activity against methicillin-resistant Staphylococcus aureus (MRSA). They both contain a tetrasubstituted tetrahydropyran (THP) ring that is essential for biological activity. Mupirocin is a mixture of pseudomonic acids (PAs). Isolation of the novel compound mupirocin P, which contains a 7-hydroxy-6-keto-substituted THP, from a ΔmupP strain and chemical complementation experiments confirm that the first step in the conversion of PA-B into the major product PA-A is oxidation at the C6 position. In addition, nine novel thiomarinol (TM) derivatives with different oxidation patterns decorating the central THP core were isolated after 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 the lack of some of the equivalent genes. Novel mupirocin–thiomarinol hybrids were also synthesized by 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 (PAs) 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 that have been at least partially characterized, with many others known from genomic sequences.[4] Thiomarinols can be regarded as comprising three main elements: a highly functionalized polyketide-derived acid that 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 the C10/C11 epoxide, the presence of a 4-hydroxy group, a C_8 rather than a C_9 fatty acid, and the pyrrothine in TM-A.[6]

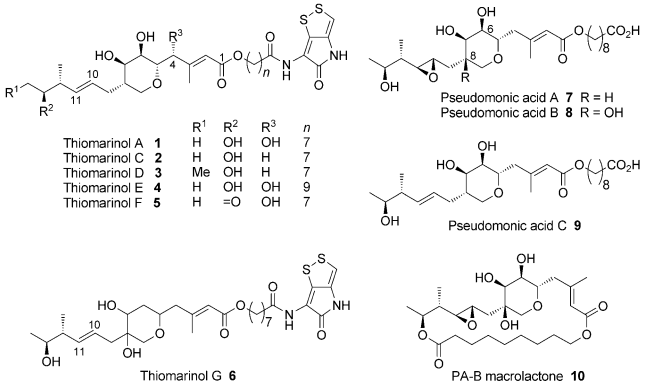

Figure 1. Selected thiomarinols and pseudomonic acids.

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 modification to the 6,7-diol is important. Mutational analysis of mupirocin biosynthesis showed somewhat counterintuitively a) that the 8-hydroxy group 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 C10/C11 double bond.[8] Analyses of mutant strains[9] indicate that the putative biosynthetic precursor **12** (Scheme 1) is cyclized and esterified to give PA-B with the tetrahydropyran ring. In addition, the identification of minor metabolites with C_5 and C_7 fatty acid side chains is in accord with elaboration of the 9-hydroxynonanoic acid moiety occurring by initial addition of 3-hydroxypropionate followed by three successive MmpB-mediated chain elongations.[7b]

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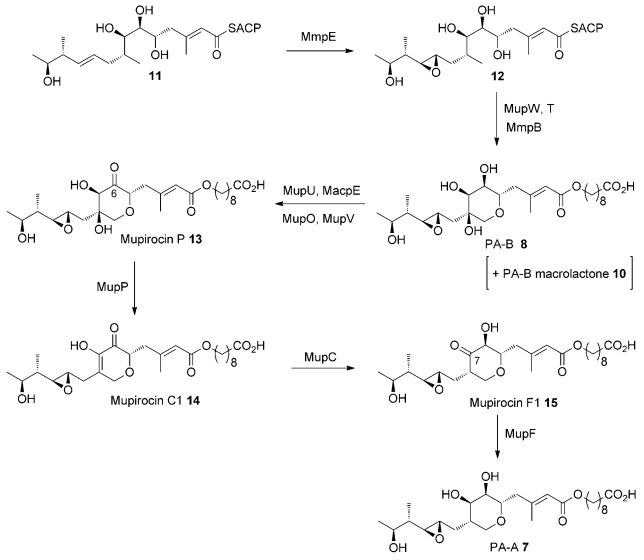


Scheme 1. MupP acts as a dehydratase in mupirocin biosynthesis.

Conversion of PA-B (**8**) into PA-A (**7**; replacing 8-OH with 8-H) 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**; Scheme 1). Deletion of any of the *mupO, mupU*, mup*V*, or *macpE* genes gave PA-B (**8**) as the major product along with macrolactone **10**.[8] The first step in the conversion of PA-B (**8**) into PA-A (**7**) is MupU-catalyzed 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 has been detected. Isolation and identification of these epoxide-containing metabolites is challenging as they are prone to rearrangements but they are key to elucidating the remaining biosynthetic steps from PA-B to PA-A. Indeed, PA-A itself is limited to mainly topical applications owing 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 an unstable metabolite with $M_w = 514$, and detailed 1D and 2D NMR analysis confirmed the structure of this key intermediate, which was named mupirocin P (**13**).

We have shown previously that the OR domain of MmpE is responsible for epoxidation.[8] Thus a double mutant, the *mmpEΔOR/ΔmupP* strain, was constructed. Gratifyingly, this produces the less labile desepoxymupirocin 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 while it here appears to act overall as a dehydratase to give enol ketone **14** (Scheme 1).

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 into PA-A (**7**) by mutant strains of *P. fluorescens* blocked in earlier

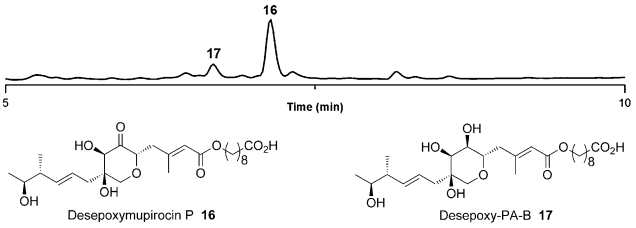
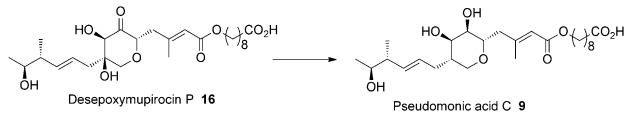


Figure 2. HPLC trace of the crude extract of the *mmpEΔOR/ΔmupP* double-mutant strain of *P. fluorescens* NCIMB 10586.

parts of the pathway, thus confirming their roles as intermediates in mupirocin biosynthesis. To investigate whether the 7-hydroxy-6-ketone is also a true intermediate on the pathway, desepoxymupirocin P (**16**), isolated from the *mmpEΔOR/ΔmupP* strain, was fed to cultures of the *mmpEΔOR/ΔmupW* strain, and LC-MS analysis showed a peak with the same retention time as desepoxymupirocin P but with $M_w = 484$. The metabolite was isolated, and NMR spectroscopy revealed that PA-C had been produced, confirming that desepoxymupirocin P is indeed an intermediate in PA-C (**9**) biosynthesis (Scheme 2).



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

Reanalysis of the MupV amino acid sequence, which had previously been identified as a putative oxidoreductase, revealed a second domain with about 30 % sequence identity to many *Pseudomonas* α/β hydrolases. These contain a GXCXG consensus sequence where cysteine replaces the more common nucleophilic serine and include 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 desepoxymupirocin 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 desepoxymupirocin P (**16**); Schemes 1 and 2).

The thiomarinol (*tml*) biosynthetic gene cluster was identified by complete genome sequencing of SANK73390, which was found to harbor a 97 kb plasmid consisting almost entirely of the thiomarinol biosynthetic genes.[11] These include multimodular *trans*-AT polyketide synthase (PKS) genes (*tmpA, tmpC*, and *tmpD*), putative fatty acid synthase (FAS) genes, *tmpB*, and associated tailoring and resistance genes (*tmlA–tmlZ*), most of which exhibit high homology to counterparts in the mupirocin (*mup*) cluster (see the Supporting Information). A non-ribosomal peptide synthetase (NRPS) linked to a set of tailoring enzymes (*holA–holH*)

similar to that recently shown to control holomycin biosynthesis in *Streptomyces clavuligerus* is also present.[12] We produced mutant strains of SANK73390 in which the PKS and NRPS parts of the cluster had 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] Using a combination of

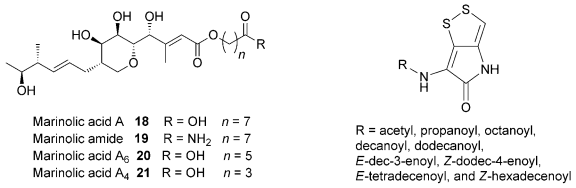


Figure 3. Marinolic acids and acyl pyrrothine metabolites isolated from WT and mutant strains of *Pseudoalteromonas* sp. SANK73390.

genetic and isotopic labeling 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–HolD) 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 catalyzed 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-hydroxy group. The *tml* pathway, however, has only some of the homologues of the *mup* genes, namely *tmlW, T, O, P, C*, and *F*, but homologues of the standalone ACPs *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 that indicate that a similar set of intermediates are indeed involved in THP ring formation and further modification in both mupirocin and thiomarinol biosynthesis.

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**) into the dihydroxytetrahydropyran PA-A (**7**). Thus deletion of *tmlF* could release more intermediates and shunt products aside from its own substrate analogous to the *mupF* deletion.

WT SANK73390 was cultivated on a modified marine broth medium (see the Supporting Information for details), which significantly improved the production of the total thiomarinols. Using this medium, TM-A (**1**) could be obtained in a yield of 70–100 mg L^{-1}, 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 500 bp arms defining an in-frame deletion from amino acid 10 to amino acid 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 of 0.1–1.2 mg L^{-1} (Figure 4). A major product among those minor

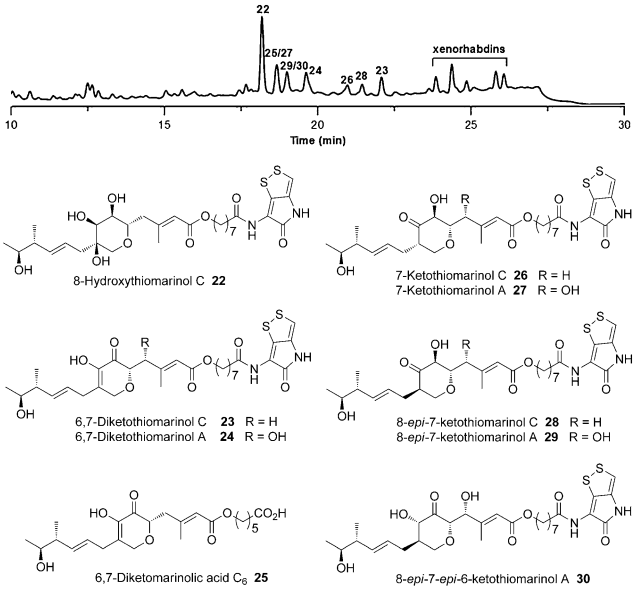
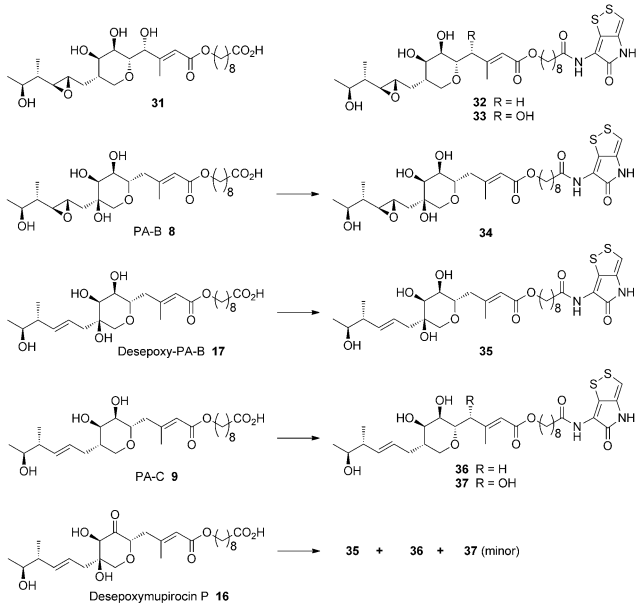


Figure 4. Novel metabolites isolated from extracts of the *ΔtmlF* mutant of *Pseudoalteromonas* sp. SANK73390.

metabolites was 8-hydroxythiomarinol C (**22**). Its structure and that of other metabolites reported herein were determined by full NMR analysis and high-resolution ESI-MS (see the Supporting Information for details). The presence of an 8-hydroxy-substituted THP ring as in PA-B (**8**) suggests that both biosynthetic pathways feature analogous pyran ring-closing mechanisms, and will require similar mechanisms for removal of the 8-hydroxy function. The only previous thiomarinol derivative reported to contain an 8-hydroxy group is TM-G (**6**, as a very minor metabolite of WT SANK73390),[1c] but it lacks both the 4 and 6-hydroxy groups. 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 *ΔmupC*, its 4-hydroxy analogue **24**, and derivative **25**, a 6,7-diketo analogue of marinolic acid A_6 lacking the 4-hydroxy moiety and the pyrrothine. These all exist as the enol ketone tautomers shown. The second group features the 6-hydroxy-7-keto THP ring (7-ketothiomarinol C (**26**) and its 4-hydroxy analogue **27**)

similar to mupirocin F1 (**15**) isolated from *ΔmupF*, and products **28**–**30** derived from **26** and **27** by keto–enol tautomerism and/or epimerization. This is consistent with the biosynthetic sequence to thiomarinol C (**2**) being similar to that towards mupirocin after the formation of the 8-hydroxy-THP ring. Interestingly, the 4-hydroxy analogue of 8-hydroxythiomarinol C was not detected.

When PA-A (**7**) was fed to the *PKS* mutant strain of SANK73390 in which all of the tailoring genes are intact, it was metabolized 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 characterized by spectroscopic methods.



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

On feeding PA-C (**9**), with the mature 6,7-dihydroxypyran ring, the pyrrothine was added to give the novel metabolite **36** ($M_w = 638$), and 4-hydroxylation yielded **37** ($M_w = 654$) 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 C4-hydroxylated metabolites were detected. Hence it is apparent that the presence of an 8-hydroxy group inhibits 4-hydroxylation.

Desepoxymupirocin P (**16**) was also fed to cultures of the *ΔPKS* mutant giving the three 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-hydroxy group 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 by keto reduction and pyrrothine addition, but no loss of the 8-hydroxy group was observed.

It is interesting to compare the metabolism of 8-hydroxy-THPs 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-keto-THP ring of desepoxymupirocin P (**16**) to the mature 6,7-dihydroxy-THP as well as pyrrothine addition readily occur, upon feeding the 6,7,8-trihydroxy-THP substrates **8** or **17**, only pyrrothine addition was apparent.

In conclusion, 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-hydroxy-substituted mupirocin P (**13**) was isolated and fully characterized. Results of chemical complementation studies feeding desepoxymupirocin 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**) was deduced to be a biosynthetic precursor of PA-A (**7**). Hence the first step in the conversion of PA-B (**8**) into PA-A (**7**) is oxidation at the C6 position. Furthermore, feeding **16** to the Δ*mupV* strain gave PA-C in accord with MupV acting before MupP.

Despite some divergences 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 desepoxymupirocin 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 to give the 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 into 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 standalone ACP MacpE, along with MupU and MupV, are missing in SANK73390. TmpB, however, contains extra KS and ACP domains compared to MmpB, which is speculated to replace the apparently missing activities.[13] Thus the tetrahydropyran-processing reactions occurring on intermediates bound to MacpE may take place while the intermediates are 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 rationalized by the inability of these substrates to be loaded onto TmpB, providing key evidence to support our proposed pathway to the marinolic acids. Structure–activity studies as well as investigations to fully understand the differences between the two pathways and to identify the genes for 4- and 6-hydroxylation in thiomarinol and 6-hydroxylation in mupirocin biosynthesis are in progress.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antibiotics · biosynthesis · mutasynthesis · polyketides · structure determination

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Communications

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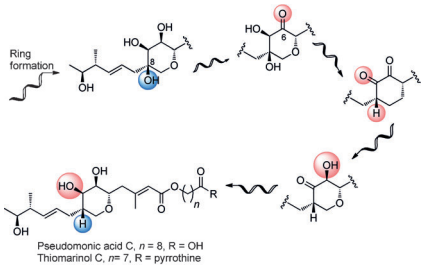
Selected Mutations Reveal New Intermediates in the Biosynthesis of Mupirocin and the Thiomarinol Antibiotics



A long and winding road: Novel metabolites from mutant strains of *P. fluorescens* and the marine bacterium *Pseudoalteromonas* were isolated and structurally characterized. In combination with complementation experiments, it was shown that formation of the tetrahydropyran ring and elaboration of thiomarinol follow a similar order to that in mupirocin biosynthesis despite the lack of some genes.