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Engineered Thiomarinol Antibiotics Active against MRSA Are Generated by Mutagenesis and Mutasynthesis of *Pseudoalteromonas* SANK73390**

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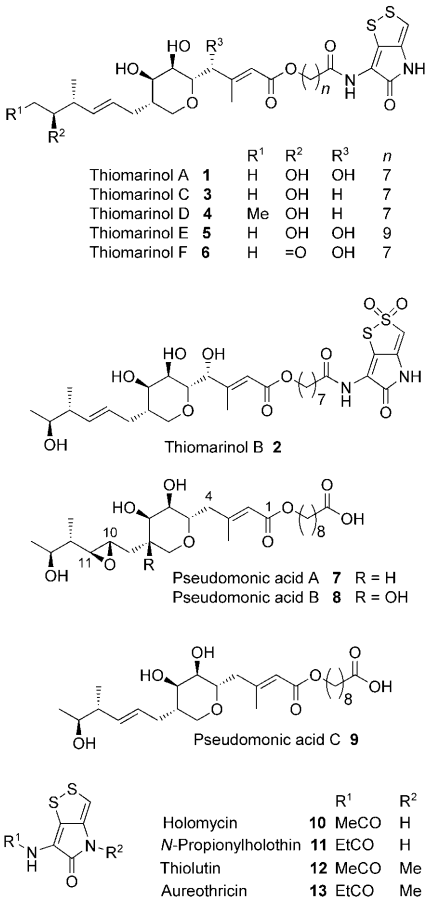
The obligate marine bacterium *Pseudoalteromonas* spp. SANK73390 produces a series of hybrid antibiotics, thiomarinols A–G (Scheme 1),[1–3] in which a pyrrothine moiety is linked through an amide to close analogues of the clinically significant antibiotic[4] mupirocin (pseudomonic acids, for example, **7**–**9**) produced by *Pseudomonas fluorescens*.[5–8] The pyrrothine-containing holomycin (**10**),[9] *N*-propionylholothin (**11**),[10] thiolutin (**12**), and aureothricin (**13**)[11] are also antibiotics but the thiomarinols and mupirocin display particularly potent activity against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) (MIC < 0.01 μg mL^{-1}). Pseudomonic acid A (**7**) was one of the first of an extensive family of antibiotics produced by the "trans-AT" class of modular polyketide synthases (PKSs).[12]

Identification of the thiomarinol (*tml*) biosythetic gene cluster by full genome sequencing of SANK73390 showed that it is contained on a 97 kb plasmid consisting almost entirely of the thiomarinol biosynthetic genes.[13] These consist of trans-AT PKSs and associated tailoring genes with high homology to the mupirocin (*mup*) cluster, along with a nonribosomal peptide synthetase (NRPS) linked to a set of tailoring enzymes similar to that recently shown to control holomycin biosynthesis in *Streptomyces clavuligerus*.[14] In contrast to thiomarinol A (**1**), the major mupirocin component, pseudomonic acid A (**7**) has the 9,10-alkene epoxidized which makes it susceptible to intramolecular rearrangements outside a narrow pH range and limits its clinical utility. Mupirocin inhibits isoleucyl-transfer RNA synthetase.[4] The appended pyrrothine moiety in thiomarinol A improves inhibition of this target,[13] but it is yet to be established whether it also imparts an additional mode of antibacterial action.



Scheme 1. Structures of thiomarinol, pseudomonic acid, and acylpyrrothine natural products.

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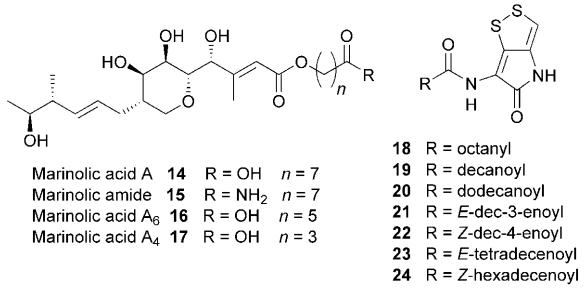
To confirm involvement of the *tml* cluster in thiomarinol A production, two mutant strains ΔPKS and ΔNRPS were generated.[13] For the ΔPKS mutant a ketosynthase segment of the PKS gene (*tmpD* corresponding to mupirocin PKS *mmpD*) was used for suicide mutagenesis using vector pAKE604.[15] An internal segment of the NRPS gene (*holA* cf. *orf3488*)[14] was used similarly to generate the ΔNRPS strain. In each case, thiomarinol production was abolished, but when the two strains were co-fermented thiomarinol production was restored.[13] We now describe determination of the full metabolic profiles and characterization of a number of previously undetected metabolites in wild-type (WT) and mutant strains of *Pseudoalteromonas* SANK73390. An in-frame deletion mutant (Δ*tmlU*) of tailoring enzyme TmlU,

which shows homology to SimL, an amide synthetase in simocyclinone biosynthesis,[16,17] was also generated by suicide mutagenesis.[13] Mutasynthesis[18] experiments with selected biosynthetic substrates in ΔPKS and ΔNRPS mutants gives new analogues which show altered activity against mupirocin-resistant strains of MRSA.

The metabolic profiles of WT *Pseudoalteromonas* SANK73390 and the ΔPKS, ΔNRPS, and Δ*tmlU* mutants were examined. In each case, the strain was fermented in marine media. After 24 h the supernatant and the acetone-lysed cell pellets were separately extracted with ethyl acetate and the extracts were combined and analyzed by reversed-phase HPLC-ESIMS chromatography (Supporting Information, Figure S1). In the case of WT SANK73390, thiomarinol A (**1**) is the main metabolite, but thiomarinol C (**3**) is also observed.[2] A number of less polar metabolites with UV spectra (UV_{max} 387 nm) indicative of pyrrothine derivatives are also present as well as a pyrrothine fragmentation ion ($[M+H]^+$ 173).[9] Two more polar peaks give UV spectra similar to the pseudomonic acids (UV_{max} 220 nm).

The two polar metabolites have molecular formulas of $C_{25}H_{42}O_9$ and $C_{25}H_{43}NO_8$, respectively (HRESIMS). Their ^{1}H NMR spectra show signals corresponding to thiomarinol A but no pyrrothine methine singlet. The molecular formulas, IR signals (1705 cm^{-1}), and ^{13}C NMR spectra ($\delta_C =$ 182.6 ppm) are in accord with the carboxylic acid **14**, which we name marinolic acid A (by analogy with pseudomonic acid A (**7**)), and amide **15** (1659 cm^{-1} and $\delta_C = 180.3$ ppm), which we similarly name marinolic amide A (Scheme 2). HMBC correlations (e.g. from the 2′-methylene ($\delta_H = 2.20$ ppm) to the amide carbonyl ($\delta_C = 180.3$ ppm)) in **15** confirm structures **14** and **15**. Their absolute stereoconfigurations are presumed to be the same as that of thiomarinol A (**1**). Whilst **14** is very similar to pseudomonic acid C (**9**), the origin of amide **15** is less obvious. No equivalent "pseudomonic amide" has been observed in the mupirocin-producing organism, *P. fluorescens*.



Scheme 2. New compounds isolated from WT SANK73390.

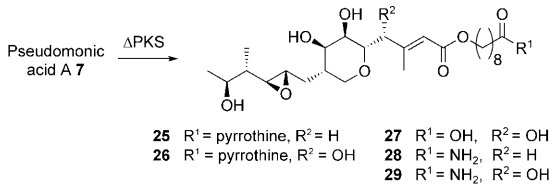
The pyrrothine metabolites **18**–**24** were also isolated. The molecular formula, $C_{13}H_{19}O_2N_2S_2$ (HRESIMS), ^{1}H and 2D NMR data, and comparison with literature values allowed **18** to be assigned as the known *Xenorhabdus* spp. metabolite xenorhabdin 3,[19] while all others are related new compounds. Compounds **19** and **20** (Figure S1) are closely related to **18**, with the principal differences being in the integration in the $\delta_H = 1.4$–1.2 ppm region, suggesting that they have varying fatty acid chain lengths. The molecular formulas, $C_{15}H_{23}O_2N_2S_2$ and $C_{17}H_{27}O_2N_2S_2$ (HRESIMS), are in accord with the fatty acyl chains being decanoyl for **19** and dodecanoyl for **20**. The ^{1}H NMR spectra for compounds **21**–**24** indicate the presence of a disubstituted alkene in each compound. COSY and HMBC spectra confirm that **21** and **22** are *E*-dec-3-enoyl- and *Z*-dodec-5-enoyl-substituted, respectively. Pyrrothines **23** and **24** contain *E*-tetradecenoyl and *Z*-hexadecenoyl ($J_{alkene} = 18$ and 11 Hz, respectively) side chains but the positions of the double bonds could not be unambiguously assigned. Similar compounds have been isolated from *Xenorhabdus* nematode symbionts.[19–21] We name the new homologues (**19**–**24**) as xenorhabdins 8–13, respectively.

LCMS analysis of the ΔPKS mutant extract indicates that, as expected, no thiomarinol A (**1**), marinolic acid A (**14**), or amide **15** are produced but xenorhabdins **18**–**24** remain. All pyrrothine-containing metabolites including thiomarinol A (**1**) are missing from the ΔNRPS mutant extract, marinolic acid A (**14**) is produced but not amide **15** indicating that the former is a biosynthetic product in its own right, and is not formed by hydrolysis of **1** whereas the amide is likely a degradation product of thiomarinol A. In addition to marinolic acid A, two minor metabolites with very similar UV chromophores but which eluted earlier were observed. The molecular formulas of the more abundant metabolite, $C_{23}H_{38}O_9$ (HRESIMS), and the less abundant metabolite, $C_{21}H_{34}O_9$, correspond to C_2H_4 and C_4H_8 less than marinolic acid A. The NMR data confirms that these compounds are closely related to marinolic acid A (**14**), the only difference being in the integration of signals due to the acyl side chain, so they can be assigned as **16** and **17**, named marinolic acids A_6 and A_4, in which the 8-hydroxyoctanoate moiety of **14** is replaced by 6-hydroxyhexanoate and 4-hydroxybutanoate, respectively. Re-examination of the WT extracts revealed trace amounts of **16**. The isolation of these truncated metabolites from both the ΔNRPS and WT strains suggests that incompletely biosynthesized compounds can be released from the producing polyketide synthases. Truncated (C_7 and C_5) side-chain homologues of pseudomonic acid A were also isolated in trace quantities from upregulated[22] WT *Pseudomonas fluorescens*. This suggests that the saturated acyl side chains in mupirocin and thiomarinols are built up by successive elongations from the PKS-derived product and not by ligation of the fully assembled (C_9 or C_8, respectively) hydroxy acids.

Finally, we examined the metabolite profile of the Δ*tmlU* mutant by LCMS and found that marinolic acid A (**14**) and, surprisingly, the same range of xenorhabdins as WT are produced, but no thiomarinol A (**1**) or amide **15**. Thus, while TmlU is responsible for linking the pyrrothine and marinolic acid **14** to generate **1**, the production of xenorhabdins indicates that a second amide ligase activity, other than TmlU, must be responsible for their production.

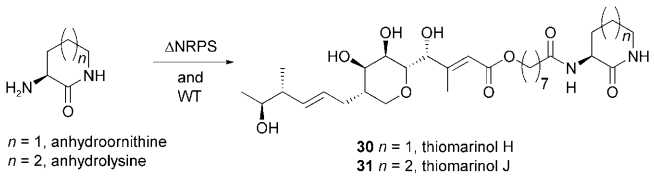
We next examined the potential of the mutants for carrying out mutasynthesis. These experiments were performed by feeding the compound of interest (0.1 $mg\,mL^{-1}$) immediately after inoculation. After 24 h, the extracts were analyzed as before and compared to a control (unfed) mutant extract. When pseudomonic acid A (**7**) was fed to the ΔPKS

mutant, five new compounds along with residual **7** were isolated by reversed-phase HPLC chromatography. Comparison of NMR and MS data with marinolic acid A and pseudomonic acid A allowed the structures of the new metabolites to be assigned as **25**–**29** (Scheme 3). The pyrrothine derivative **25** of pseudomonic acid A and its 4-hydroxylated analogue **26** are produced along with 4-hydroxypseudomonic acid A (**27**), and the pseudomonic acid A and 4-hydroxypseudomonic acid amides **28** and **29**, respectively. Thus the ΔPKS mutant is capable of adding the pyrrothine to pseudomonic acid A (**7**) and also of catalyzing its 4-hydroxylation. The isolation of **27**, in addition to previously observed formation of marinolic acid A by the ΔNRPS and Δ*tmlU* mutants, suggests that 4-hydroxylation does not have to occur prior to or during elongation of the polyketide backbone and also that TmlU and the 4-hydroxylase are able to accept close analogues of thiomarinol.



Scheme 3. Mutasynthetic conversion of pseudomonic acid A by SANK73390 ΔPKS.

The mutasynthetic incorporation of alternative amines in the ΔNRPS mutant was also examined (Scheme 4). Isolation of thiomarinol H (**30**) from a closely related organism,[23] but not previously from SANK73390, prompted us to feed anhydroornithine (Scheme 4). Ornithine itself has no effect on the metabolite profile, but anhydroornithine is efficiently incorporated to give thiomarinol H (**30**). This suggests that SANK73390 may have lost its ability to cyclize ornithine to its anhydro form. When anhydroornithine was fed to WT SANK73390, both **30** and thiomarinol A (**1**) were produced, indicating that anhydroornithine can compete with pyrrothine as a coupling partner. The Δ*tmlU* mutant gave no **30**, confirming that TmlU is responsible for amide ligation in the biosynthesis of both **1** and **30**. α-Aminobutyrolactone was not incorporated, but anhydrolysine gave a small amount of the corresponding 7-membered lactam analogue **31**, for which we propose the name thiomarinol J.



Scheme 4. Mutasynthetic formation of thiomarinols H and J.

Compounds isolated during the course of these studies were tested for antimicrobial activity against *Bacillus subtilis* and MRSA (Table 1). Almost all compounds tested show some activity against *B. subtilis* and MRSA with thiomarinols A (**1**) and B (**3**) and pseudomonic acid A (**7**) being the most active, but **25**–**31** generated by mutasynthesis also show good activity. The xenorhabdins also display activity, though the activity falls with increasing lipophilicity. To the best of our knowledge, the acylpyrrothine class of antibiotics has not been shown to have anti-MRSA activity previously.

Table 1: Results of disk diffusion assays (zone of inhibition, mm).[a]

Compd.	*B. subtilis*	MRSA
1	38.7 ± 0.6	23.3 ± 0.6
3	35 ± 2	20.7 ± 0.6
7	37.7 ± 0.6	21.3 ± 0.6
14	29 ± 2	17.7 ± 0.6
15	16.7 ± 0.6	8.3 ± 0.6
16	15.7 ± 0.6	9.7 ± 0.6
18	25 ± 2	12.7 ± 0.6
19	17 ± 1	9 ± 0
20	11 ± 2	7.3 ± 0.6
22	15 ± 1	*
23	11 ± 2	*
24	9.7 ± 0.6	*
25	26.3 ± 0.6	22.3 ± 0.6
26	23.7 ± 0.6	22.7 ± 0.6
27	18.7 ± 0.6	15.3 ± 0.6
28	21.7 ± 0.6	11 ± 0
29	6.7 ± 0.6	10 ± 0
30	15.3 ± 0.6	7.3 ± 0.3
31	12 ± 1	9.7 ± 0.3
Kan	12.3 ± 0.6	*

[a] Average of three determinations; see the Supporting Information for full experimental details. Kan = kanamycin. * no observable activity.

The results presented here provide evidence for the later stages of thiomarinol biosynthesis. It is proposed that marinolic acid A (**14**) is the first post-PKS intermediate and that this is likely derived by successive two-carbon extensions of the minor metabolites marinolic acids A_4 (**17**) and A_6 (**16**). The marinolic acids differ from the pseudomonic acids in that they are hydroxylated at C-4. This hydroxylation can clearly occur relatively late in the pathway because pseudomonic acid A (**7**) is a substrate for the C-4 hydroxylase. The amide ligase TmlU is responsible for linking marinolic acid to pyrrothine, but the substrate selectivity of TmlU is rather flexible, and different polyketide and amine substrates can be utilized—for example during the synthesis of **25** and **30**. However, TmlU is not responsible for the synthesis of the xenorhabdins which must be synthesized by another amide ligase. Amide **15** is derived by degradation of thiomarinol A (**1**) itself. In combination with our identification of the thiomarinol gene cluster[13] this is the first demonstration that engineered strains of *Pseudoalteromonas* SANK73390 can be used for the rational production of new antibacterial compounds through a combination of mutation and mutasynthesis, and in particular compounds active against MRSA. Thus it holds considerable potential for the development of new anti-MRSA compounds with likely clinical applications and our current and future work focusses on genetic engineering with this aim.

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