
Peer reviewed version

Link to published version (if available):
10.1002/cbic.201800036

Link to publication record in Explore Bristol Research
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Wiley-VCH at http://onlinelibrary.wiley.com/doi/10.1002/cbic.201800036/abstract. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
http://www.bristol.ac.uk/pure/about/ebr-terms
Accepted Article

Title: Fine Tuning of Antibiotic Activity by a Tailoring Hydroxylase in a Trans-AT Polyketide Synthase Pathway

Authors: Hadi H Mohammad, Jack A Connolly, Zhongshu Song, Joanne Hothersall, Paul R Race, Christine L Willis, Thomas J Simpson, and Christopher Morton Thomas

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: ChemBioChem 10.1002/cbic.201800036

Link to VoR: http://dx.doi.org/10.1002/cbic.201800036
Fine Tuning of Antibiotic Activity by a Tailoring Hydroxylase in a Trans-AT Polyketide Synthase Pathway

Hadi H. Mohammad[a,b,d], Jack A Connolly[a], Zhongshu Song[b], Joanne Hothersall[a], Paul R Race[c], Christine L Willis[b], Thomas J Simpson[b], Peter J Winn[a] and Christopher M Thomas[a]

Abstract: Addition or removal of hydroxyl groups modulates the activity of many pharmacologically active biomolecules. It can be integral to the basic biosynthetic factory or result from associated tailoring steps. For the anti-MRSA antibiotic mupirocin, removal of a C8-hydroxy group late in the biosynthetic pathway gives the active pseudomonic acid A. An extra hydroxylolation, at C4, occurs in the related but more potent antibiotic thiomarional A. We report here in vivo and in vitro studies that show putative non-heme-iron(II)/L-2-ketoglutarate-dependent dioxygenase TmuB, from the thiomarional cluster, 4-hydroxylates various pseudomonic acids while C8-OH, and other substituents around the pyran ring, block enzyme action but not substrate binding. Molecular modelling suggested a basis for selectivity but mutational studies showed limited ability to rationally modify TmuB substrate specificity. 4-hydroxylation had opposite effects on the potency of mupirocin and thiomarional. Thus TmuB can be added to the toolbox of polyketide tailoring technologies for in vivo generation of new antibiotics in the future.

Polyketide synthases (PKSs) build carbon backbones of complex molecules from simple building blocks, such as acetate and malonate, using enzymes similar to those found in fatty acid biosynthesis.[1] Further “tailoring” of these backbones (e.g. via hydroxylation) to produce the final product can be critical and understanding the specificity of such enzymes is essential for assembly of new pathways to novel compounds. For example, cyclosporin loses its immunosuppressive activity after regio-specific hydroxylation at the 4th N-methyl leucine but retains its side effects on hair growth.[2]

Hydroxyl groups in polyketides generally occur either by ketoreduction of β-thio ketol esters, or on α-carbons of thiol esters by a tailoring step. β-hydroxy groups can be predicted from the pks gene sequences while α-hydroxylation is less predictable. PKS pathways use a number of enzyme families for hydroxylation of α-carbons: cytochrome P450s,[3] FAD-binding monooxygenases[4] and non-hemeFe(II)/α-ketoglutarate dependent dioxygenases.[5]

To provide new tools for engineering biosynthetic pathways we have investigated tailoring reactions in pathways of two closely related antibiotics from the trans-AT family of modular polyketides,[6] namely the thiomarinals and mupirocin. Thiomarinals (e.g. 1 and 2) are produced by marine bacteria of the genus Pseudoalteromonas and are closely related to the commercially important mupirocin[7] (a mixture of pseudomonic acids including A-C 3-5, Fig. 1) produced by Pseudomonas fluorescens which is used topically against Methicillin Resistant Staphylococcus aureus (MRSA).[8] The structures of the major metabolites, thiomarinal A 1 and pseudomonic acid A (PA-A, 3), differ by the presence of a 10, 11-alkene in 1 but an epoxide in 3, an 8-hydroxyoctanoyl acid rather than 9-hydroxyoctanoyl acid side-chain and an additional pyrothine moiety. A further important difference for the current study, is that the more bioactive thiomarinal A 1 possesses a 4-hydroxyl group[7]. Most genes in the mupirocin gene cluster have been allocated a role[9] and sequence comparisons of the mup and tml gene clusters confirms the similarities but important differences in their biosynthetic pathways.[10]

Figure 1. Structures of key compounds in this study. (A) Selected thiomarinals and pseudomonic acids; (B) Synthetic conversion of PA-A 3; (C) Further 4-hydroxylated metabolites and mupirocin F; (D) Mupirocins not 4-hydroxylated by TmuB.
Understanding these differences might allow modification of mupirocin and other molecules to give new antibiotic leads. Feeding experiments with the PKS mutant of the thiomarinol producer Pseudoalteromonas spp SANK73390 showed that PA-A 1, could be 4-hydroxylated and addition of the pyrrothine occurred giving 6, 7 and 8 (Figure 1B). In contrast, PA-B 2, with the 8-OH, gained the pyrrothine but no 4-hydroxylated occurred. Here we identify the 4-hydroxylase as TmuB and report its characterisation.

Results and Discussion

TmuB can 4-hydroxylate metabolites in P. fluorescens

Comparison of the mupirocin and thiomarinol gene clusters identified genes that lack paralogues in the mupirocin cluster and might encode the 4-hydroxylase, BLAST searches[12] identified TmuB as related to phytanoyl-CoA dioxygenase, an enzyme of the non-heme-iron(II)/αKG-dependent dioxygenase superfamily. We inserted the tmuB coding region into broad host range vector pJH10 under control of the tac promoter (pJH10-tmuB) and transferred it to WT P. fluorescens NCIMB 10586. HPLC analysis of culture supernatant after tmuB expression showed a new peak more polar than PA-A (Fig. 2). The new metabolite was isolated and confirmed by NMR spectroscopy to be 4-hydroxy PAA 6.

Expression in NCIMB10586 ΔmupC producing mupirocin C 13, and ΔmupT or ΔmupW producing mupirocin W 14 (structures shown in Fig. 1) gave no significant differences in metabolite profiles (data not shown). However, the mmpEDQR strain producing PA-C 5, gave a new metabolite (Fig. 2) confirmed as 4-hydroxy PA-C 9 following full characterisation by MS and NMR (Table 1S). Likewise ΔmupF, producing mupirocin F 10 gave the novel metabolite (Fig. 2) confirmed to be 4-hydroxy mupirocin F by LC-ESI-MS (MW=514) and NMR (Table 1S). Interestingly the mupirocin F peak at 22.3 min increased two-fold (Fig. 2), an example of increased overall production observed when tmuB is expressed. Thus the tetrahydropryan ring and its state of oxidation are critical for substrate modification by TmuB and it is not only an 8-OH group that can block the action of TmuB.

PA-B 4 is a competitive inhibitor of purified TmuB

WT tmuB was inserted into pET28a and expressed in E. coli BL21(DE3) with N-terminus His tag. As a control, tmuB from inactive mutant H109N, described below, was expressed in parallel. Purified WT and H109N TmuB protein were tested on purified PA-A 3. Co-substrate (α-ketoglutaric acid) and co-factor (FeSO4) were essential and WT TmuB generated a new peak at 18.2 min (Fig. 3A) confirmed to be 4-hydroxy PA-A 6 but TmuB H109N did not. Reactions at 23 °C, 25 °C, 30 °C and 37 °C for 180 min, showed maximum activity at 23 °C, the optimum growth temperature for the thiomarinol producer.

TmuB activity on PA-B was only observed after >6h incubation, generating a new metabolite (Figure 3A), confirmed as 4-hydroxy PA-B 12 (Fig. 1) by LCMS and NMR. Initial rates were determined at substrate concentrations from 3.75 to 240 µM to determine Vₘₐₓ and Kₘₐₚ for PA-A is three orders of magnitude higher than for PA-B, the major difference between turnover number rather than substrate binding (Fig. 3D).

Since the Kₘₐₚ for PA-B 4 is only 3-fold higher than for PA-A, while turnover number decreases 1000-fold, it may be a competitive inhibitor of TmuB. Reactions on PA-A 3 carried out with 30, 60 and 120 µM PA-B showed an increased Kₘₚ for PA-A while Kₘₚ remained unchanged (Figure 1S). The apparent absence of hydroxylation of PA-B and other derivatives by TmuB in vivo may be due to this slow reaction rate: the product may be released from the cell before hydroxylation occurs or remain below the detection limit.

Mutational analysis of the TmuB substrate binding pocket

In the absence of TmuB crystallographic data, structural predictions were made from the coordinates of EasH[13] (PDB 4NAO, 25% identity), plus AsqJ (PDB 5DAW, 21% identity) and FtmOx1 (PDB 4YST, 23% identity)[15] because 4NAO lacks coordinates for the α-helix2/β3 loop which is resolved as a flap covering the active site entrance in the others (Fig. 2S). The TmuB homology model contains a double stranded β-helix (DSBH), or Jelly-roll fold, characteristic of the nonheme-Fe(II)/α-ketoglutarate-dependent dioxygenase super-family (Fig. 3S) [16].

PA-A 3 was docked with the best scoring models based on the criteria listed in Methods, with and without the α-helix2/β3 loop folded properly, to find dominant conformations/orientations consistent with the known biochemistry and identify residues interacting with the substrate. Without the α-helix2/β3 loop, the results show monic acid fitting the active site pocket and 9HN in the external groove (Fig. 4A). When the α-helix2/β3 loop is included it is located above the active site and interacts with the fatty acid chain (Fig. 4S). The tetrahydropryan ring was similarly
COMMUNICATION

located in all models. Evolutionary Trace Analysis (ETA)
identified about half the residues lining the pocket as likely to be
functionally important (Fig.5S) and this is supported by
mutagenesis experiments in other proteins[17]. For PA-A the
target C-4 is 4.4 Å from the Fe cofactor which is close to the
norm (4.5 Å) for these enzymes[18]. The pyran ring sits near the
pocket entrance which consists of residues R69, K105, I109 and
M208 and interacts with R69, K105 and I109 plus L141 and
A206 (Fig. 4B and C).

To explore specificity, PA-B 4 was docked with the TmuB
model using the same parameters as for PA-A 3. Similar
orientations were obtained and although binding energy and
inhibitory constants for both PA-A 3 and PA-B 4 were similar, the

Figure 4. A. Homology model of TmuB showing PA-A 3 docked in the active
site. SHN fits into the external groove while monic acid inserts into the pocket,
B. PA-A (Pink stick) and monic acid in the active site. The target site C-4 is
4.38 Å from the Fe II (Orange sphere), C. The active site residues surrounding
the pyran ring and were selected for mutagenesis. D. The effect of
mutations on TmuB activity against PA-A.

C4 to active site Fe²⁺ distance was 6.3 Å compared to 4.4 Å for
PA-A 3. Thus the C-8 OH must alter the active site fit and
detailed analysis showed that R69, D104, K105, I109, Q126,
L141 and M208 may be involved. All these residues were
mutated to smaller, hydrophilic amino acids to accommodate
the extra groups. Substitutions least likely to cause TmuB
inactivation were identified by alignments with other superfamily
members and in silico mutation. Single and double tmuB point
mutants were created in pJH10-tmuB and tested for PA-B
hydroxylation in P. fluorescens: HPLC analysis showed that
none gave observable hydroxylation but some reduced activity
on PA-A (Fig. 4D). That 4-OH PA-B 12 might be lethal is unlikely
given the MIC data presented below (Fig. 5) and the possibility
that it is trapped in the cell seems unlikely since hydroxylated
metabolites were easily isolated from culture supernatants. Thus
it may not be possible to manipulate TmuB to allow in vivo
hydroxylation of PA-B.

Effect of hydroxylation on antibacterial activity

Plate bioassay (plus 0.5 mM IPTG) showed that
antibacterial activity of 4-hydroxylated mupirocin produced in vivo
by NCIMB10586 (pJH10-tmuB) (mainly 4-hydroxy PA-A 6) was
reduced against B. subtilis 1064 (Fig. 5A) and MIC tests
confirmed this (Fig. 5C). Lowered activity of 4-OH PA-B makes it
unlikely that failure to convert in vivo is due to lethality. To
explore explanations for the reduction, PA-A was docked to the
crystal structure of the isoleucyl-tRNA synthetase (IleRS) in
silico, yielding a conformation similar to the crystal complex
of IleRS-PA-A (1FFY PDB)[19]. The hydroxy group in 4-OH PA-A 6
disrupted this conformation apparently because the 4-OH
causes steric repulsion by H64 and D557 (Fig. 6S). To test the
effect of thiomarinol lacking the 4-hydroxy group, a sample of
thiomarinol C 2 (Fig. 1) was prepared after the tmuB 1109N
mutation was recombined into the thiomarinol producer. Bioassay and MIC tests with *B. subtilis*, *E. coli* and *S. aureus* showed that thiomarinol C (TMC) 2 was less potent than thiomarinol A 1 (Fig. 5). The different consequences of 4-hydroxylation on potency of PAs versus TMs may indicate that the length of hydroxy-acid moiety (9NA v BHO) alters how these antibiotics bind their targets and emphasises the need for structures of more IleRS-antibiotic complexes.

*In vitro* tests with purified TmUB showed thiomarinol C 2 is a good substrate for TmUB giving thiomarinol A 1 with similar catalytic activity to PA-A 3 (*Km* = 29.5 µM and *Kcat* = 2212 s⁻¹) (Fig. 3B).

![Figure 5](image)

**Figure 5.** Antibacterial activity of the new derivatives. (A) Plate bioassays against *B. subtilis* from *P. fluorescens* strains expressing TmUB in-trans: *P. fluorescens* WT (producing 4-OH PA-A 6), *P. fluorescens* mmpEΔO (producing 4-OH PA-C 9) and *P. fluorescens* Δmupf (producing 4-OH mupirocin F 11). (B) Plate bioassays on Thiomarinol A and G (1 and 2) extracted from WT and PM 11029 SANK respectively. The discs were saturated with 100 µg of purified thiomarinol A and C and tested against *B. subtilis* 1064, *E. coli* DH5α and *S. aureus* MRSA NCTC 12493. (C) Minimal inhibitory concentrations (MIC).

**Conclusions**

We have shown that TmUB 4-hydroxylates thiomarinol and its analogues including PA-A 3, PA-C 5 and mupirocin F 10 in *P. fluorescens*. However, its activity is severely inhibited, not just by the 8-OH of PA-B 4, but also by the further structural changes to the tetrahydropyran core (as in mupirocins C and W, 13 and 14). It therefore seems likely that 4-hydroxylation occurs late in biosynthesis especially since an ACP-tethered substrate is not required, as TMC 2 and PA-C 5 are good substrates. We also conclude that the 9HN/8HO moiety must remain outside the active site pocket since its internal dimensions of 6.8 Å by 4.4 Å would be too small to accommodate the C1-3 of monic acid, 8HO and pyrroline in the pocket. 4-hydroxylation could therefore be one of the final steps in biosynthesis and TmUB could have been acquired relatively recently in thiomarinol cluster evolution, consistent with it being one of the genes (tmY, tmU and tmUB) not present in the mup cluster[10]. The fact that 4-hydroxylation increases thiomarinol potency could explain this acquisition but since tmUB expression also increased product levels in culture supernatants, enhancing passage across the membrane could be another explanation.

The detectable but low activity of TmUB on PA-B 5 suggested that TmUB might be modified to hydroxylate a broader range of substrates. Active site mutagenesis of the nonheme-Fe(II)/αKG-dependent dioxygenase superfamily has changed specificity and diverted catalytic function as reported recently with fumitremorgin B endoperoxidase from *Aspergillus fumigatus*[15]. However, structure-guided site directed mutagenesis failed to improve the activity of our enzyme. This may be due to TmUB having flexible finger-like loops like those found in enzymes such as phytanoyl-CoA dioxygenase[20] and prolyl hydroxylase[21], which undergo conformational changes on substrate binding, making in silico substrate docking difficult. Therefore, determining accurate TmUB crystal structures with and without different substrates will be an important goal to increase its uses in polyketide tailoring technologies and facilitate the in vivo generation of new antibiotics in the future.

**Acknowledgements**

This work was funded by BBSRC grants BB/1014373/1 and BB/1014039/1 and through BисSynBio, the Bristol Centre for Synthetic Biology (BB/L01386X/1). HHM was funded by an Iraqi Government Scholarship (HCED). JAC was funded by a BBSRC MBTP (BB/1014532/1) studentship.

**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** antibiotics • hydroxylation • polyketides • mupirocin • thiomarinol


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

Layout 1:

COMMUNICATION

Text for Table of Contents

Author(s), Corresponding Author(s)*
Page No. – Page No.
Title

((Insert TOC Graphic here))

Layout 2:

COMMUNICATION

Text for Table of Contents

Author(s), Corresponding Author(s)*
Page No. – Page No.
Title

((Insert TOC Graphic here))