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The polyketide backbone of thiolactomycin is assembled by an unusual iterative polyketide synthase†

Marie E. Yurkovich,^a Robert Jenkins,^b Yuhui Sun,^c Manuela Tosin*^b and Peter F. Leadlay*^a

Following the *in vivo* investigation of thiotetronate assembly in *Lentzea* sp. and in *S. thiolactonus* NRRL 15439 (Havemann *et al.*, *Chem. Commun.*, 2017, DOI: 10.1039/c6cc09933e), the minimal set of genes required for thiolactomycin production was determined through heterologous expression and the mechanism for polyketide assembly was established *in vitro* through incubation of recombinant TlmB with its substrates in the presence of either nonhydrolysable or hydrolysable chemical probes. The results presented here constitute unequivocal evidence of enzymatic processing by an unusual iterative polyketide synthase.

In 2014, the World Health Organisation (WHO) declared antimicrobial resistance a global health crisis, with a ‘post-antibiotic era’ looming unless critical measures are taken.² While rigorous changes in policy are required, there is also an urgent need for effective new antibiotics, and in particular, for compounds that act on biological targets that are under-exploited, highly conserved, essential for survival, and unique to bacteria.³ Thiolactomycin (TLM, **1**, Fig. 1C), a thiotetronate antibiotic first discovered in 1982 from a soil *Nocardia* strain (ATCC 31319,⁴ since re-named *Lentzea* sp.) reversibly inhibits the β -ketoacyl-acyl carrier protein synthase (KAS) enzymes of type II (dissociated) fatty acid synthase (FAS), an essential metabolic pathway for bacterial cell viability.⁵ By binding preferentially to the acyl-enzyme intermediate of the KAS enzymes,⁶ TLM and other thiotetronates, such as Tü 3010,⁷ confer broad antimicrobial activity against both Gram-positive and Gram-negative bacteria and in murine models of infection.⁸

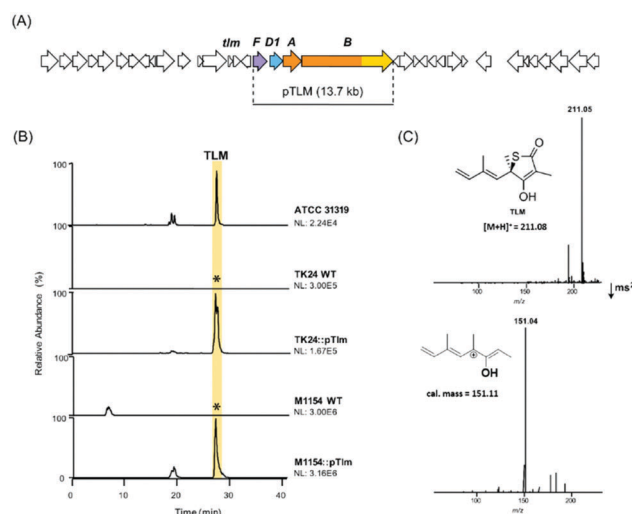


Fig. 1 (A) Organisation of the proposed *tlm* cluster in *Lentzea* sp. and the limits of the fragment cloned into pTLM. (B) LC-MS analysis of the selective ion trace of thiolactomycin ($[M + H]^+ = 211.1$ *m/z*) within WT and recombinant heterologous strains. The asterisk means not detected. (C) Confirmation of TLM production by MS-MS analysis.¹⁴

Additionally, thiotetronates show promise as a *Mycobacterium tuberculosis* therapeutic⁹ as well as anti-malarial and anti-trypansomal activity through the inhibition of apicomplast type II FAS.¹⁰ The broad spectrum antimicrobial activity displayed by TLM, its effectiveness in murine models of infection, and its favourable physical properties, have all established a rationale for the development of TLM derivatives. To date, the more ramified thiotetronate, Tü 3010, has been reported to be 15-fold more effective as an antibacterial *in vivo*, and various recent synthetic modifications to the TLM C3- and C5-methyl groups have led to increased activity against *Plasmodium falciparum*, *Francisella tularensis*, methicillin-sensitive *Staphylococcus aureus* (MSSA), and methicillin-resistant *Staphylococcus aureus* (MRSA).^{8b,11} The C5-chiral centre of thiotetronates highly complicates analogue synthesis,¹² making this class of natural products an attractive target for genetic engineering. Early experiments with

^a Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK. E-mail: pfl10@cam.ac.uk; Tel: +441223336710

^b Department of Chemistry, University of Warwick, Library Road, CV4 7AL, UK. E-mail: M.Tosin@warwick.ac.uk; Tel: +442476572878

^c Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education, Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, People's Republic of China

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isotopically-labelled precursors indicated that this class of molecules were likely of polyketide origin.¹³ While the genetic cluster was not initially identified, it was predicted that **1** would be possibly assembled by a multimodular type I polyketide synthase (PKS) as various levels of β -keto reduction would be expected within the TLM polyketide scaffold. In 2015 the biosynthetic gene clusters for TLM (*tlm*) and Tü 3010 (*tue*, *stu*, *ssu*) were identified by comparative genetic analysis by our group¹⁴ and others.¹⁵ *In silico* analysis of the predicted *tlm* biosynthetic gene cluster within the TLM producing strain *Lentzea* sp. revealed only four open reading frames (ORFs): a gene encoding a copy of the known TLM intracellular target KASI/II enzyme, *tlmF*; a gene encoding an unexpected cytochrome P450, *tlmD1*, which we have shown to be essential for TLM biosynthesis through in-frame genetic mutation;¹⁴ *tlmA*, encoding a PKS-protein housing a KSQ domain,¹⁶ and an acyl carrier protein (ACP) domain, therefore likely acting as the initiation module; and *tlmB*, encoding a novel multienzyme containing a single PKS extension module juxtaposed at its C-terminus with several NRPS domains. Bioinformatic analysis of this peculiar biosynthetic cluster led to the prediction that the TLM tetraketide backbone would be assembled by the ORFs *tlmA* and *tlmB*. TlmA was proposed to catalyse the attachment of a malonyl group to the ACP and its subsequent decarboxylation providing the acetate starter unit for TLM, likely borrowing its required acyltransferase (AT) domain from the malonyl-CoA:ACP acyl-transferase of fatty acid biosynthesis (MCAT), as presented earlier in actinorhodin¹⁷ and FK228 biosyntheses.¹⁸ The predicted domain order within TlmB from the N-terminus is ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), acyl-carrier protein (ACP) domains, followed by the NRPS domains: cyclisation (Cy), adenylation (A) and peptidyl carrier protein (PCP). Therefore TlmB was envisaged to act as an iterative type I PKS by recruiting three successive propionate units and catalyzing three cycles of chain elongation. In-frame gene mutation and complementation studies on *tlmA* within the ATCC 31319 *tlm* cluster have revealed that *tlmA* is essential for thiotetronate biosynthesis, strengthening our proposed mechanism for thiotetronate polyketide assembly unexpectedly involving an iterative, rather than modular, system.¹⁴ An encouraging precedent for such an iterative assembly-line is the recently-uncovered PKS for the 14-macrocyclic polyketide galbonolide.¹⁹ Validation of this mechanism requires isolation and identification of the intermediate species. While this has historically been a significant obstacle due to the covalent attachment of all intermediates to polyketide synthase multi-enzymes throughout chain assembly, a recently developed chemical strategy has provided a useful way to sample and identify PKS intermediates from both modular²⁰ and iterative systems.²¹ This strategy employs nonhydrolysable synthetic mimics of the natural PKS extender units recruited for polyketide formation. These small molecules act as competitive substrates for the natural ACP-bound extender units, carrying out decarboxylative condensation with the KS-tethered polyketide: by decoying and removing the growing chain from the PKS, the product from each round of chain extension can be isolated and analysed. As reported by Havemann *et al.* in the

accompanying paper,¹ *in vivo* feeding of nonhydrolysable synthetic mimics to the TLM-producing strain *Lentzea* sp. resulted in the capture and identification of putative thiolactomycin polyketide intermediates¹ including di-, tri- and tetraketide species with the expected states of β -keto-reduction.¹ However, this work alone cannot conclusively demonstrate that only TlmA and TlmB are sufficient for polyketide assembly. To do this, the minimal set of genes required for TLM production was first determined through heterologous expression of the gene cluster in several heterologous *Streptomyces* hosts, and then the mechanism for TLM polyketide assembly was enzymatically reconstituted *in vitro*. The *tlm* cluster was cloned and transplanted into two genetically well-defined *Streptomyces* heterologous host strains. The 13.7 kbp *tlm* cluster was PCR amplified as a fragment containing terminal regions identical to the *NdeI*- and *EcoRI*-digested ends of pIB139. This fragment was then inserted into digested pIB139 through Gibson assembly, transformed into *Escherichia coli* DH10B, and apramycin-resistant pTLM containing colonies were confirmed through PCR analysis and Sanger sequencing. pTLM (Fig. 1S, ESI[†]) was introduced into the *Streptomyces coelicolor* M1154 and *Streptomyces lividans* TK24 genomes by conjugation through the triply methylation-deficient *E. coli* strain ET12567/pUZ8002. The resulting transformed strains M1154::pTlm and TK24::pTlm were cultured and the extracts analysed by LCMS. As shown in Fig. 1, the recombinant strains gained the ability to produce TLM at comparable/higher levels than those found in *Lentzea* sp. Having independently identified a *tlm* cluster within *Salinispora pacifica* CNS863, Tang *et al.* have also been successful in demonstrating heterologous production of TLM in the recombinant host M1152/pMXT13.¹⁵ TLM production in heterologous *Streptomyces* hosts has confirmed that only TlmA and TlmB are required for assembly of the thiolactomycin polyketide chain, and that the sulfur insertion machinery is borrowed from primary metabolism.

To attempt the reconstitution of polyketide chain assembly *in vitro*, we first undertook recombinant expression of the giant PKS-NRPS multienzyme, TlmB. The N-terminally His₆-tagged 316.8 kDa recombinant protein was expressed in *E. coli* BL21(DE3) and purified by selective ammonium sulfate precipitation (Fig. 2A). Matrix-assisted laser desorption/ionisation (MALDI) mass fingerprinting of recombinant TlmB, following tryptic digest, was used to confirm the identity of the expressed protein (Fig. 2S, ESI[†]). To ensure that the ACP domain of TlmB was in the active *holo*-form through addition of a 4'-phosphopantetheine (4'PP) arm, the broad-specificity 4'PP transferase Sfp from *B. subtilis*²² was co-expressed with recombinant TlmB. MALDI-TOF analysis to determine whether co-expression had been successful was inconclusive (Fig. 3S, ESI[†]), so CoASH and heterologously purified Sfp were included in TlmB enzymatic assays to ensure complete *in vitro* conversion of *apo*-ACP to *holo*. Based on the assumption that TlmA is the initiating enzyme providing an acetate starter unit for TlmB *via* malonate decarboxylation, we replaced TlmA in these assays with acetyl-CoA (2). To reconstitute thiolactomycin polyketide assembly and probe the proposed iterative mechanism of TlmB, the same chain termination



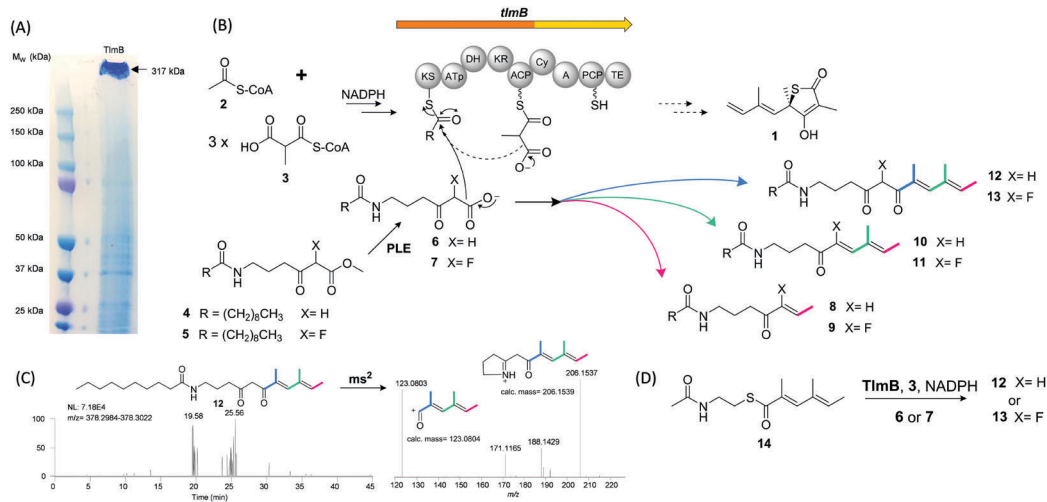


Fig. 2 (A) SDS-PAGE analysis of recombinant TlmB. (B) Chain termination probes **6** and **7**, generated from PLE-catalysed hydrolysis of **4** and **5**, compete with ACP-bound methylmalonate *in vitro* to off-load recombinant TlmB-bound intermediates (representative structures shown, full overview of captured species is given in Table 4S, ESI[†]). (C) HR-MSⁿ analysis of the putative dienoyl tetraketide **12** captured by probe **6** from recombinant TlmB. (D) Incubation of recombinant TlmB with the synthetic SNAc triketide **14** in the presence of probe **6** or **7** results in the formation of species **12** or **13** (ESI[†]).

probes utilised by Havemann and co-workers¹ *in vivo* were employed *in vitro*. Prior to incubation with recombinant TlmB, the malonate and fluoromalonate esters **4–5** were hydrolysed to the corresponding carboxylates **6–7** through incubation with pig liver esterase (PLE)^{20a} (Fig. 2B). Purified TlmB, co-expressed with Sfp, was incubated with each probe in the presence of assay substrates acetyl-CoA (**2**) and (2*RS*)-methylmalonyl-CoA (**3**), as well as DTT and NADPH. The enzyme assay mixtures were quenched and extracted with ethyl acetate. High-resolution mass-spectrometry (HRMS) analyses of the organic extracts showed the presence of putative enoyl-diketide intermediates from both malonyl- and fluoromalonyl terminator assays (**8–9**, Fig. 2B and Fig. 4S, 10S, ESI[†]), indicating that acetyl-CoA could indeed replace TlmA to initiate chain extension through loading of acetyl groups onto recombinant TlmB. Additionally, for each terminator species, β -keto and β -hydroxy diketide intermediates were also detected (Table 4S and Fig. 4S, 10S, ESI[†]), revealing all the reductive enzyme-catalysed steps employed during the first round of chain extension. Furthermore, dienoyl triketide intermediates from the second round of TlmB chain extension (**10–11**) were also detected, albeit in minor amounts, from both malonyl- and fluoromalonyl terminator assays, as well as triketide β -keto and β -hydroxy species (Fig. 6S and 12S, ESI[†]). Finally, from the third and final round of TlmB chain extension, we were able to detect off-loaded dienoyl tetraketides (*e.g.* **12**) in organic extracts (Fig. 1B, C and Fig. 8S, ESI[†]). None of these putative intermediates, each characterised by HR-MSⁿ analyses, were found in controls containing boiled TlmB; and the intermediates were virtually identical to those identified from *in vivo* feeding studies,¹ supporting substrate processing by TlmB as an iterative synthase. To further confirm substrate processing by TlmB to dienoyl tetraketides, the hydrolysable *N*-acetylcysteamine triketide **14** was synthesised according to Scheme 1S (ESI[†]) and utilised to prime TlmB. Addition of chain termination probes **6–7** to the newly primed TlmB in the presence of the natural

extender unit **3** led to the formation of dienoyl tetraketides **12–13** (Fig. 2D and Fig. 9S, 13S, ESI[†]). No further elaborated polyketide species (*e.g.* reduced tetraketides) were detected. These results taken together constitute unequivocal evidence of polyketide chain building and processing by an iterative enzyme in thiotetronate bio-assembly. The discovery that the thiotetronate polyketide backbone is assembled by an iterative PKS is particularly interesting given that a different level of β -keto reduction is required during the final cycle. Further, in the case of the more elaborate Tü 3010 and thiotetronomycin structures, both methylmalonyl-CoA and ethylmalonyl-CoA extension units are selected and activated by a single AT domain. The successful reconstitution of this system *in vitro* makes this recombinant enzyme an attractive system for further structural studies. High resolution structural investigation of TlmB could provide insights into the poorly-understood²³ factors that control reduction within iterative PKSs, and pave the way to future engineering of unnatural thiotetronates.

Complete *in vitro* reconstitution of the PKS enzymology within TLM biosynthesis has confirmed an unexpectedly iterative method of assembly. This work has now paved the way for the investigation of post-PKS mechanisms leading to sulfur-insertion and thiolactone formation.

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