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Chemical ‘chain termination’ probes were utilised for the investigation of thiotetronate antibiotic biosynthesis in the filamentous bacteria Lentzea sp. and Streptomyces thialactonus NRRL 15439. The use of these tools led to the capture of biosynthetic intermediates involved in the thiotetronate polyketide backbone assembly, providing first insights into substrate specificity and in vivo intermediate processing by unusual iterative synthases.

Polyketide natural products constitute a prominent class of secondary metabolites that interact with a wide range of intracellular targets associated with diseases. The presence of heterocyclic moieties in polyketides, such as pyran and furan rings in ionophore antibiotics, is common and often decisive for the physical properties and biological activity of the compound. Although sulphur-ring based structures are relatively common in peptides of both ribosomal and non-ribosomal origin, polyketides bearing sulphur-containing rings are rare. They include potent antitumor agents such as leinamycin and the family of thiotetronate-bearing sulphur-containing rings are rare. They include potent antitumor agents such as leinamycin and the family of thiotetronate antibiotics (Fig. 1). The best-known thiotetronate is thiolactomycin (1, TLM): originally isolated from a soil Nocardia strain (ATCC 31319, recently reclassified as Lentzea sp.), 1 is a reversible inhibitor of the β-ketoacyl-acyl carrier protein synthase (KAS) enzymes of the bacterial type II (dissociated) fatty acid synthase. TLM constitutes a promising lead structure for the development of novel anti-tuberculosis, anti-malarial and antitrypanosomal agents. The closely structurally related Tü 3010 (2) has been reported to be the most potent antibacterial tetronate to date, capable of targeting FabH/FabF in Staphylococcus aureus at lower doses in comparison to TLM and other known FabH/FabF inhibitors. Only very recently these medicinally promising molecules were unequivocally established to be of polyketide origin. Comparative genomics-based approaches to orphan cluster identification led to the uncovering of putative polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) enzymes (A–C, Fig. 1B) responsible for thiotetronate production in both soil and marine bacteria, in association with tailoring enzymes (e.g. P450 oxidases, D), cysteine desulfurases (J) and (thiouridylase-like) sulfur transferases (S) proposed to provide the sulfur atom for thiotetronate ring formation. Indeed, genes A, B, D1, D2, J and S are essential for thiotetronate formation, as proved by in vivo knock-out and complementation studies. Genes A and B encode for an acetyl-loading PKS module and a hybrid PKS-NRPS, respectively. For the thialactomycin biosynthetic gene cluster tlm, the B protein comprises ketosynthase (KS), acyltransferase (AT),...
Polyketide biosynthesis proceeds via decarboxylative Claisen condensation of malonate units anchored to acyl carrier proteins (ACPs) with acyl moieties bound to ketosynthase (KS) domains (Fig. 3, path A): the resulting polyketide chain is subjected to variable ketoreduction, dehydration and enol reduction after chain extension, before being released from PKSs (e.g. via thioester hydrolysis) and/or post-PKS tailoring (e.g. via methyltransferases, glycosyltransferases, ...). A detailed elucidation of the polyketide assembly is crucial for knowledge-based enzyme and cell engineering aiming at novel polyketide production. Advances in molecular biology, synthetic chemistry and analytical techniques have greatly aided the probing of PKS pathways in vitro and in vivo. Nonetheless, several challenges remain concerning the elucidation of timing and mechanisms related to the polyketide assembly, mostly related to the inaccessibility of biosynthetic intermediates covalently linked to PKSs throughout the product assembly.

We have developed a general strategy for probing polyketide biocatalysis based on the use of synthetic ‘chain termination’ probes: these are nonhydrolysable small-molecule mimics of malonate extender units recruited in polyketide formation that competitively interfere in the decarboxylative Claisen condensation to capture and off-load prematurely truncated biosynthetic intermediates (Fig. 3, path B). These chemical tools have proved successful for the identification and characterisation of intermediate species from modular PKSs, in vitro and in vivo, allowing the gathering of otherwise inaccessible information on the timing and the mechanism of single catalytic events, and also unveiling novel chemoenzymatic opportunities for the generation of unnatural polyketide derivatives. In order to shed light on the nature of the biosynthetic intermediates involved in the thiotetronate assembly and their in vivo processing, we have utilised a range of chain termination probes available in our labs. We initially employed the N-(d3) acetyl methyl ester probes 3a-b, which hydrolyse in vivo to the corresponding ‘active’ carboxylate probes 7a-b (Fig. 2), in microbial fermentations of the wild type thiolactomycin producing Lentzea sp. at variable probe concentrations. In organic extracts of cultures we observed diminished production of thiolactomycin with increasing concentrations of 3a-b, strongly suggesting that the probes were interfering in the thiotetronate assembly. However, only a handful of putative captured intermediates were detected from these substrates (Table S2, ESI†). We therefore turned to second-generation probes featuring N-decanoyl chains, which have recently proved to be more efficient tools in capturing polyketide intermediates in vivo due to their enhanced bioavailability and ease of intermediate isolation. In addition to substrates 4-5 previously reported, we also synthesised and utilised the pseudo-methylmalonate ester 6 (Scheme S1, ESI†), in order to probe the extender unit substrate specificity of the putative iterative PKSs. In parallel to wild-type strains, mutant strains carrying specific deletions in both Lentzea sp. (ΔtmA, ΔtmD1 and ΔCy) and S. thiolactonus NRRL 15439 (ΔsuB and ΔΔAKAs), as well as Streptomyces avermitilis heterologously expressing the Tü 3010 cluster, were utilised in control experiments.

An overview of the outcome of feeding experiments of Lentzea sp. and S. thiolactonus strains with compounds 7-10 is given in Fig. 3 and detailed in Tables S2-S5 (ESI†). Although malonate and methylmalonate-mimic probes 8 and 10 preferentially intercepted early stage condensation intermediates, the use of the fluoromalonate-based probe 9 in wild-type Lentzea sp. fermentations captured diketides and triketides presenting various degrees of reduction, as well as putative tetraketides (Table S2, ESI†). These species, directly mirroring the nature of ACP-bound species, were characterised by high resolution mass spectrometry tandem experiments, with diagnostic peaks corresponding to the loss of the N-decanoyl moiety and loss of ammonia, and/or cyclic imine formation (Fig. S7, S10 and S20, ESI†). Although diketide species may be potentially captured from the fatty acid biosynthetic pathway (Table S3, ESI†), the observed triketides and tetraketides were associated exclusively with TLM formation in vivo. In addition to purely polyketide species, a putative S- and O-containing tetraketide species was also captured from Lentzea sp. via 9 (Fig. 3C). A parallel investigation of S. thiolactonus wild-type, deletion mutant strains and S. avermitilis heterologously expressing the Tü 3010 cluster with methyl ester substrates 4-5 led to the capture of similar putative intermediates, albeit with lower efficiency (Tables S4 and S5, ESI†).

These results taken together constitute the first direct evidence of polyketide chain building and processing involved in thiotetronate bio-assembly. By utilising a variety of chemical probes as pseudo-malonate substrates, preliminary information on substrate specificity and the kinetics of polyketide assembly has been obtained. The pseudo-fluoromalonate 9, generated from in vivo hydrolysis of the corresponding methyl ester 5, proved to be the most efficient tool in capturing almost every intermediate involved in the polyketide TLM assembly. Compared to the malonate and methylmalonate inspired-substrates 4 and 6, we observed enhanced in vivo ester hydrolysis followed by decarboxylation for 5 (Fig. S7, S10 and S20, ESI†): this is possibly due to the electron-withdrawing effect of fluorine at the α-ester carbonyl position, or to the increased probe bioavailability, or to a likely combination of both. On the other
hand, 9 did not prove equally efficient in sampling the polyketide chain assembly for Tü 3010, suggesting a much stricter substrate specificity for StuB. By analysing the relative amounts of all the characterised species from *Lentzea* sp., it appears that, in general, early stage substrate processing is relatively slow, as previously observed for other partially reducing iterative type I synthases (iPKSs), and that the presence of TlmD1, the TlmB Cy domain and J, K, S proteins is essential for polyketide chain growth and processing beyond the triketide stage. Most type I iPKSs utilise malonate extender units, and methyl groups are introduced into their polyketide products by integral SAM-dependent methyltransferases. In this respect, TlmB and StuB display unusual substrate specificity in utilising methylmalonate and ethylmalonate extender units, dictated by their respective AT domains. We have shown here that these unusual iPKSs can also process pseudomalonate, methylmalonate and fluoromalonate substrates at different stages and to various extents. These findings have been corroborated by parallel *in vitro* investigations with recombinant TlmB in our laboratories. We have recently proposed a mechanism for sulphur insertion following the formation of the PKS-bound tetraketide, involving the nonribosomal peptide synthase (NRPS) functionalities encoded in the C-terminal part of TlmB: tetraketide epoxidation (catalysed by TlmD1/StuD1) of the PKS-bound tetraketide, involving the nonribosomal peptide synthase (NRPS) functionalities encoded in the C-terminal part of TlmB: tetraketide epoxidation (catalysed by TlmD1/StuD1) may precede the formation of a tetraketide–thiirane intermediate from PCP-bound cysteine desulphuration (Tlm/Stu J and S catalysed), followed by heterocyclisation and product release.

It is tempting to speculate that the putative S- and O-containing tetraketide species characterised from *Lentzea* sp. and *S. thiolactonus* wild-type strains may be related to this pathway. Further work will be required to establish the true nature of S-containing intermediates leading to thiotetronate formation. Nevertheless, the deconvolution of the steps of chain assembly reported here has set the stage for the investigation of these post-PKS events.

In summary, we have utilised chemical probes to gather the first information on the intermediates in polyketide assembly for thiotetronate formation *in vivo*. This represents an important step towards the full elucidation of thiotetronate assembly and the development of synthetic biology-based routes for novel thiotetronate production.

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**Notes and references**


