Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

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

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1. Synthetic methods

Solvents and reagents: Unless specified otherwise, chemicals were purchased from Sigma Aldrich, Fisher Scientific, Carbosynth, Acros Organics or Alfa Aesar and were used without further purification. Anhydrous dichloromethane and tetrahydrofurane were purchased from VWR International (AR grade) and dried using solvent towers. Anhydrous ethyl acetate and methanol were purchased from Fisher Scientific or Sigma Aldrich. Reagent grade dichloromethane, ethyl acetate, methanol, acetonitrile, cyclohexane and chloroform were purchased from Fisher Scientific.

Chromatography: Analytical thin-layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel 60 (F254, Merck) and visualized under ultra-violet light (short and long-wave) and using potassium permanganate ($KMnO_4$) or vanillin stains. Silica gel was purchased from Sigma Aldrich (Tech Grade, pore size 60 Å, 230-400 mesh).

NMR Spectroscopy: ^{1}H and ^{13}C NMR spectra were recorded in d^4-MeOD, $CDCl_3$ or D_2O on the following Bruker Avance instruments: DPX-400 400 MHz, DRX-500 500 MHz, AV III-500 HD 500 MHz or AV-600 600 MHz.

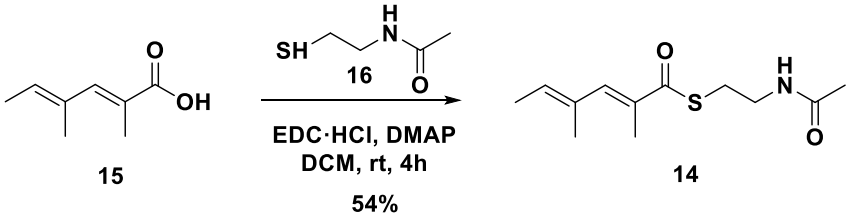
LC-HRMS: High-resolution mass spectra (HRMS) of newly made synthetic compounds were obtained using electrospray ionization (ESI) on a MaXis UHR-TOF (Bruker Daltonics) or on Bruker MaXis (ESI-HR-MS).

IR: All IR data were recorded on a Bruker ALPHA FT-IR spectrometer as thin films or neat.

Methyl ester probes **4-5** were prepared as previously reported. [1]

[1] (a) E. Riva, I. Wilkening, S. Gazzola, W. M. A. Li, L. Smith, P. F. Leadlay and M. Tosin, *Angew. Chem. Int. Ed*., **2014**, *53*, 11944-11949; (b) I. Wilkening, S. Gazzola, E. Riva, J. S. Parascandolo, L. Song and M. Tosin, *ChemComm*, **2016**, *52*, 10392-10395.

1.1 Synthesis of S-(2-acetamidoethyl) (2E,4E)-2,4-dimethylhexa-2,4-dienethioate (14)



Scheme 1S

To a solution of (2E,4E)-2,4-dimethylhexa-2,4-dienoic acid[2] (**15**, 40 mg, 0.29 mmol) in dry dichloromethane (DCM, 2.5 mL) under N_2 and at 0°C, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, 60 mg, 0.31 mmol) and 4-(Dimethylamino)pyridine (DMAP, 2 mg, 0.02 mmol) were added and the solution was stirred at 0°C for 15 mins. *N*-acetylcysteamine (**20**, 28 µL, 0.29 mmol) was then added and the reaction was stirred at room temperature for further 4 hours. The mixture was diluted with DCM (10 mL) and washed with diluted aqueous HCl (0.01 M, 2 x 5 mL) and brine (2 x 5 mL). The organic layer was separated and dried over $MgSO_4$, filtered and concentrated to afford the crude product. Purification by silica gel chromatography with a gradient of 0-90 % EtOAc in cyclohexane gave pure **14** as a white solid (37 mg, 54 %, R_f = 0.16 in 1:1 EtOAc : cyclohexane): **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 1.77 (3H, d, *J* = 6.9 Hz, C*H*$_3$CH), 1.87 (3H, s, C*H*$_3$CCHCCSO), 1.96 (3H, s, C*H*$_3$CONH), 2.06 (3H, s, C*H*$_3$CCSO), 3.08 (2H, t, *J* = 6.3 Hz, SC*H*$_2$), 3.46 (2H, q, *J* = 6.1 Hz, C*H*$_2$NH), 5.82 (1H, q, *J* = 6.7 Hz, C*H*CH$_3$), 5.91 (1H, br s, N*H*), 7.12 (1H, s, C*H*CCSO); **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 14.1 (*C*H$_3$CCHCCSO), 14.3 (*C*H$_3$CH), 16.0 (*C*H$_3$), 23.3 (*C*H$_3$CONH), 28.7 (S*C*H$_2$), 39.9 (*C*H$_2$NH), 132.5 (*C*CSO), 132.9 (*C*CHCCSO), 133.2 (*C*HCH$_3$), 142.8 (*C*HCCSO), 170.3 (NH*C*O), 194.9 (O*C*S); **IR** (thin film) υ_{max} = 1547, 1649, 2855, 2925, 3077, 3277 cm^{-1}; ***m/z* (HR-ESI-MS)**: found $[M+Na]^+$ 264.1039, $C_{12}H_{19}NNaO_2S^+$ requires 264.1029.

[2] K. Ohata and S. Terashima, *Tetrahedron*, **2009**, *65*, 2244-2253.

2. Heterologous expression of the *tlm* cluster

Construction of pTLM and intergeneric conjugal transfer: For heterologous expression of the *tlm* cluster the plasmid pTLM was constructed. The 13.7 kbp *tlm* fragment was amplified using *Lentzea* sp. genomic DNA and the primer pairs: Tlm_het_F and Tlm_het_R. The vector pIB139 was digested with *Nde*I and *Eco*RI (ThermoFisher Scientific) and purified by gel electrophoresis. Ligation of cut pIB139 and the amplified fragment was accomplished by the isothermal Gibson assembly method according to the manufacturer's protocol (New England Biolabs). The assembly mixture was incubated at 50°C for 60 min, and then was used to transform *E. coli* DH10B. Plasmid pTLM was confirmed by Sanger sequencing and introduced through conjugal transfer into the *S. coelicolor* M1154 and *S. lividans* TK24 genomes through the donor strain *E. coli* ET12567/pUZ8002. After incubation at 30°C for 16 hours, exconjugants were selected with 5 µg/mL apramycin and 25 µg/mL nalidixic acid. Antibiotic resistance was confirmed through transfer of exconjugants to a SFM plate containing 50 µg/mL apramycin and 25 µg/mL nalidixic acid. The integrity of the M1154::pTlm and TK24::pTlm mutants was checked by PCR analysis using the primer pairs: Tlm_conf_1, Tlm_conf_2, and Tlm_conf_3 (Fig. 1S).

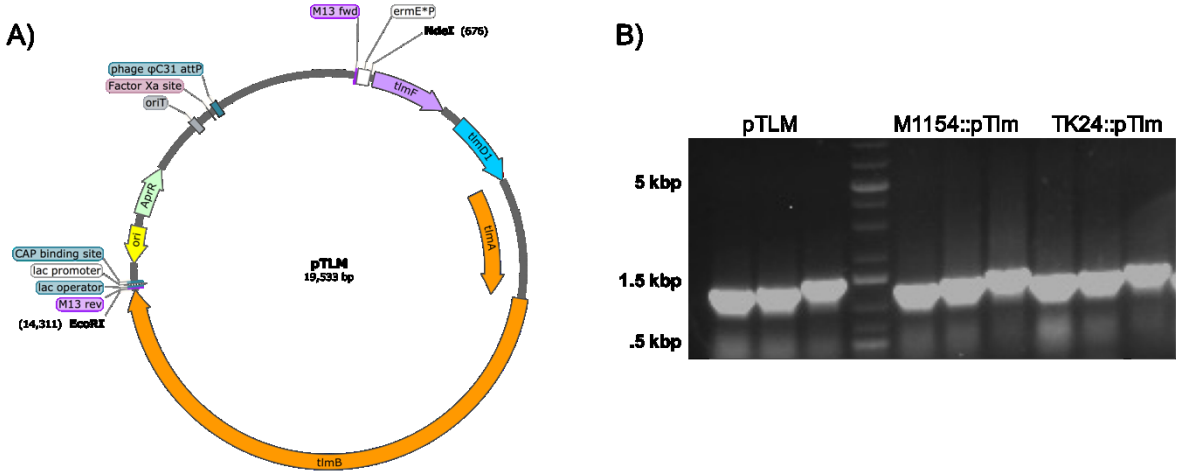


Fig. 1S. Plasmid map of pTLM (**A**) and agarose gel (**B**) confirming successful integration of pTLM into heterologous host strains. The primer sets Tlm_conf_1 (1168 bp), Tlm_conf_2 (1225 bp), Tlm_conf_3 (1327) were used, in order, with the template indicated. GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) was used as a molecular DNA size marker.

Chemical analysis of TLM: Fermentation broths (see culture conditions below) were adjusted to pH 3, and shaken vigorously with an equal volume of ethyl acetate for 2 h (37°C, 250 rpm), then clarified by centrifugation (3,000 x *g*, 10 min). The resulting supernatant was then evaporated to dryness under reduced pressure in a rotary evaporator, the crude extract was dissolved in 600 mL of methanol, and clarified by centrifugation (12,000 x *g*, 10 min). Low resolution HPLC-MS analysis was performed using an HPLC (Hewlett Packard, Agilent Technologies 1200 series) coupled to a Finnigan MAT LTQ mass spectrometer (Thermo Finnigan) fitted with an electrospray ionisation (ESI) source. The mass spectrometer was run in positive ionisation mode, scanning from m/z 150 to 1800 with fragmentation at normalised

collision energy 12%. For analysis of thiolactomycin the system was fitted with a Phenomenex Prodigy C18 column (4.6 x 250 mm, 5 mm). Separation was achieved using a solvent system of acetonitrile and Milli- Q water (both containing 0.1% formic acid) with a linear gradient of 3 – 100% of acetonitrile over 42 min at a flow rate of 0.7 mL/min. UV absorption was monitored at 238 nm and 303 nm.

Culture conditions: All *E. coli* strains were grown in liquid or solid Luria-Bertani (LB) medium at 37°C with appropriate antibiotic selection (apramycin 50 µg/mL, chloramphenicol 25 µg/mL, kanamycin 50 µg/mL). *Lentzea* sp., *S. lividans* TK24, *S. coelicolor* M1154 and related mutant strains were grown in TSB medium[1] for isolation of chromosomal DNA, and on SFM medium[3] for sporulation and conjugation. For production of TLM in *Lentzea* sp., TK24::pTlm, and M1154::pTlm strains were cultured in TSB[2] or TLM PM (1% soytone; 1% soluble starch; 2% D-maltose (w/v); pH 6.7). For liquid cultures, the strains were grown at 30°C on a rotary shaker incubator at 200 rpm for 4 days, and for solid cultures, the strains were grown at 30°C for 5-7 days. Bacterial strains and plasmids used in this study are summarised in Tables 1S and 2S respectively.

Table 1S. Bacterial strains used in this study.

Strains	Characteristics	Reference
Escherichia coli		
DH10B	F^- *mcrA* Δ(*mrr*$^-$ *hsd* RMS$^-$ *mcrBC*), Φ80*lacZ* ΔM15, Δ*lacX74 recA1* endA1 *araD139* Δ (*ara leu*)7697 *galU* galK *rpsL nupG* λ^-. Host for general DNA manipulation	Invitrogen
BL21(DES)	F^- *ompT hsdS*$_B$(r_B^-, m_B^-) *gal dcm* (DE3). Host for protein expression.	Novagen
ET12567/pUZ8002	(F^- *dam*$^-$ 13::Tn9 *dcm*$^-$ 6 *hsdM hsdR recF143* zjj-202::Tn10 *galK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*). Donor strain for conjugation between *E. coli* and *Streptomyces.*	[4]
***Lentzea* sp.**		
Lentzea sp. (ATCC 31319)	TLM producing wild-type strain	[5]
Streptomyces		
S. lividans TK24	Heterologous host strain.	[6]
S. coelicolor M1154	Heterologous host strain.	[7]
TK24::pTlm	TK24 with the *tlm* cluster heterologously	This study

[3] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater and D. A. Hopwood, *Practical Streptomyces Genetics*; John Innes Foundation, **2000**.

[4] D. J. Macneil, K. M. Gewain, C. L. Ruby, G. Dezeny, P. H. Gibbons and T. Maeneil, *Gene,* **1992**, *111*, 61-68.

[5] H. Oishi, T. Noto, H. Sasaki, K. Suzuki, T. Hyashi, O. Hiroshi, K. Ando and M. Sawada, *J. Antibiot.* **1982**, *35*, 771-777.

[6] D. A. Hopwood, T. Kieser, H. A. Wright and M. J. Bibb, *J. Gen. Microbiol.* **1983**, *3*, 2257-2269.

[7] J. P. Gomez-Escribano and M. J. Bibb, *Micro. Biotechnol.* **2011,** *4*, 207-215.

	transferred	
M1154::pTlm	M1154 with the *tlm* cluster heterologously transferred.	This study

Table 2S. Plasmids used in this study.

Plasmid	Characteristics	Reference
pIB139	*aac(3)IV, oriT, attP*(FC31), *int*, P_{ermE*}, ori_{pUC}, FC31 site integrative vector with P*erm*E* promoter	[8]
pET28a(+)	*kan*, *lacI,* P_{T7}, ori_{pBR322}, ori_{F1}, N-terminal His_6-tag, for protein expression	Novagen
pTLM	Thiolactomycin heterologous expression construct in which the *tlm* cluster is cloned into pIB139 (*Nde*I/*Eco*RV)	This study
pTlmB	A protein expression construct containing *tlmB* from *Lentzea* sp. between *Nde*I and *Eco*RI of pET28a(+) with an N-terminal His_6-tag	This study
pSUSfp	A protein expression construct containing *sfp* from *Bacillus subtilis* between *Nde*I and *Sal*I of pSUMtaA, Cam^R, used for co-expression	[9]

DNA Manipulation: oligonucleotides used in this work were custom synthesized by Eurofins Genomics and supplied in lyophilised form (listed in Table 3S). Polymerase chain reactions were carried out using PrimeSTAR GXL polymerase from Takara or *Phusion* High-Fidelity PCR Master Mix from New England Biolabs (for cloning) and BioMix Red from Bioline (for screening). Isolation of plasmid DNA from an overnight culture was performed using the E.Z.N.A. HP Plasmid Mini Kit I (Omega Bio-Tek). High-molecular weight genomic DNA from *Streptomyces* and *Lentzea* strains was isolated using the salting out procedure.[3] Purification of DNA fragments from agarose gels was performed using GeneJet Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacture's instruction. DNA sequencing was carried out by the DNA Sequencing Facility in the Department of Biochemistry, University of Cambridge. Genome sequence DNA was analysed and annotated using Artemis Release 13.0. Functional analysis of each gene product was carried out using BlastP[10] and multiple protein alignments were compiled by Clustal Omega.[11] All plasmid construction and visualisation and analysis of DNA sequence data was carried out using the programme SnapGene® 3.1.4 (GSL Biotech).

[8] C. J. Wilkinson, Z. A. Hughes-Thomas, C. J. Martin, I. Böhm, T. Mironenko, M. Deacon, M. Wheatcroft, G. Wirtz, J. Staunton and P. F. Leadlay, *J. Mol. Microbiol. Biotechnol.* **2002**, *4*, 417-426.

[9] C. Kanchanabanca, PhD Thesis, University of Cambridge, **2014**.

[10] S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, *J. Mol. Biol.* **1990**, ***215***, 403-410.

[11] F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson and D. G. Higgins, *Mol. Syst. Biol.* **2001**, *7*, 1-6.

Table 3S. Oligonucleotide primers used in this study.

Primer	Oligonucleotide sequence (5'-3')	Restriction site
Tlm_het_F	ACATAATAAAGGAGGACCACCATATGATTGCTTGAG GTTGCCAACCAACC	*Nde*I
Tlm_het_R	AGCTATGACATGATTACGAATTCCCTCGTTCGCCCTT CTCAGCTCAGC	*Eco*RI
Tlm_conf_1_F	CATATGGTGTACGCCGCCATCAGGAAGG	
Tlm_conf_1_R	AAGAATTCGATCGCGCTCATCGGGTCAC	
Tlm_conf_2_F	AAATGCCGACGAACGCGGGCGTGTG	
Tlm_conf_2_R	TTCTCACACCGCCGGTCCGACGACCAGC	
Tlm_conf_3_F	TCGACGACGGCAGGTACG	
Tlm_conf_3_R	CTGGCATTCCACGCATCG	
TlmB_pet_F	AACATATGGACGACGCGATCGCAGTGG	*Nde*I
TlmB_pet_R	AAGAATTCTCAGCTCAGCAGCTGCCGC	*Eco*RI

3. *In vitro* experiments with recombinant TlmB and mass spectrometry analyses

Construction of pTlmB: The 8979 bp *tlmB* gene was amplified using *Lentzea* sp. genomic DNA and the primer pair TlmB_pet_F and TlmB_pet_R. The amplified fragment and pET28a(+) vector were digested with *Nde*I and *Eco*RI and purified by gel electrophoresis. The digested fragment was ligated into the linearized vector using T4 DNA ligase (New England Biolabs) as according to the manufactures protocol. Following transformation into *E. coli* DH10B pTlmB was confirmed by Sanger sequencing.

Protein expression and purification: Expression plasmids pTlmB and pSUSfp were introduced into chemically competent BL21(DE3) cells through transformation. A single colony was scraped and transferred into 10 mL of LB medium and was incubated overnight at 37°C and 220 rpm. This starter culture was then used to inoculate 1 L of LS medium (1% tryptone; 1% yeast extract; 0.5% NaCl; pH 7.5) containing 25 µg/mL of chloramphenicol and 50 µg/mL of kanamycin. The culture was incubated at 37°C, 220 rpm until the A_{600} reached 0.6 - 1.0, at which point gene expression was induced by adding 200 µL of 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM then incubated for a further 16 h at 20˚C, 200 rpm. Cells were harvested by centrifugation at 12,000 x *g* for 5 min and resuspended in 40 mL buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.6) and lysed by sonication (Vibra-Cell™ system, Sonics & Materials Inc.). During sonication the cells were kept on ice and the following program was used: at 40% power level 2 s pulse on, 5 s pulse off for a total 5 min pulse on. The sonicated lysate was centrifuged at 35,000 x *g*, 20 min, 4˚C, after which the supernatant fraction was removed and loaded on a PVP (polyvinylpyrrolidone)-treated 100 kDa cut-off Amicon Ultra-4 concentrators (Millipore) and centrifuged at 3,000 x *g*, 4˚C, to remove the bulk of *E. coli* proteins. The concentrated lysate (typically about 20 mL) was transferred to a small centrifuge tube and placed on a rolling shaker (IKA®) at 4˚C. Solid $(NH_4)_2SO_4$ was slowly added to 20% saturation, left to stir for approximately 30 min, followed by centrifugation at 15,000 x *g* for 20 min (4˚C). The supernatant was then transferred to a new small centrifuge tube (the previous pellet stored on ice) and the same procedure was carried out for 40%, 60%, and 80% $(NH_4)_2SO_4$ precipitation fractions. A small amount of each pellet was subject to SDS-PAGE analysis (Fig. 2A, main text) and the fraction containing pure TlmB (typically the 20% saturation fraction) was dissolved in a small amount of storage buffer (20 mM Tris-HCl, 0.1 M NaCl, 10% glycerol, pH 7.6) and used immediately in enzymatic assays.

Protein analysis: Recombinant TlmB was characterised using trypsin digestion and matrix-assisted laser desorption/ionisation (MALDI) mass fingerprinting (Fig. 2S and 3S), carried out by the PNAC Facility, Department of Biochemistry, University of Cambridge.

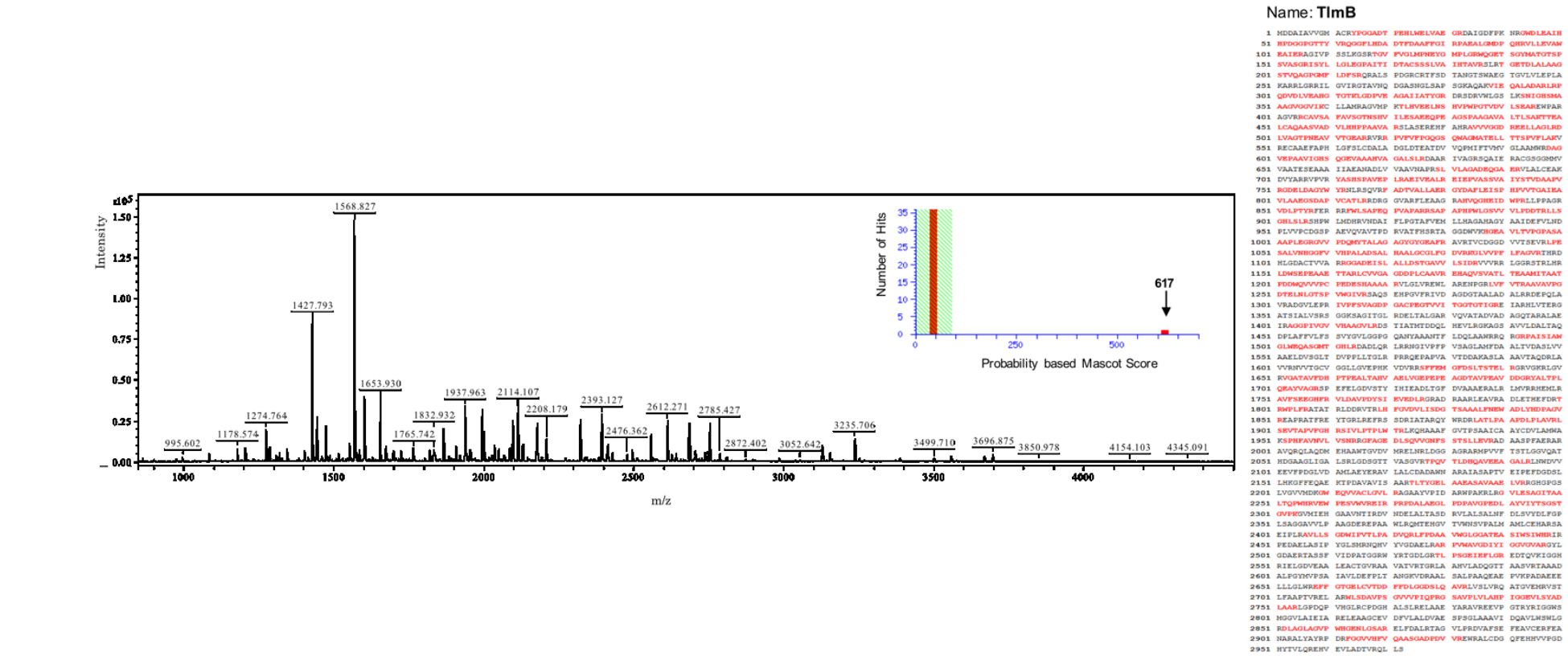


Fig. 2S. MALDI-TOF MS fragment pattern following trypsin digestion and corresponding matched residues (represented in red) from the TlmB protein sequence. The Mascot score histogram shows that TlmB was identified, as a single hit, with a score of 617 (a protein score is represented as -10*Log(P) where P is the probability the observed match is a random event) indicating high quality and unambiguous identification. The cross-hatched area represents the low-significance region of hits.

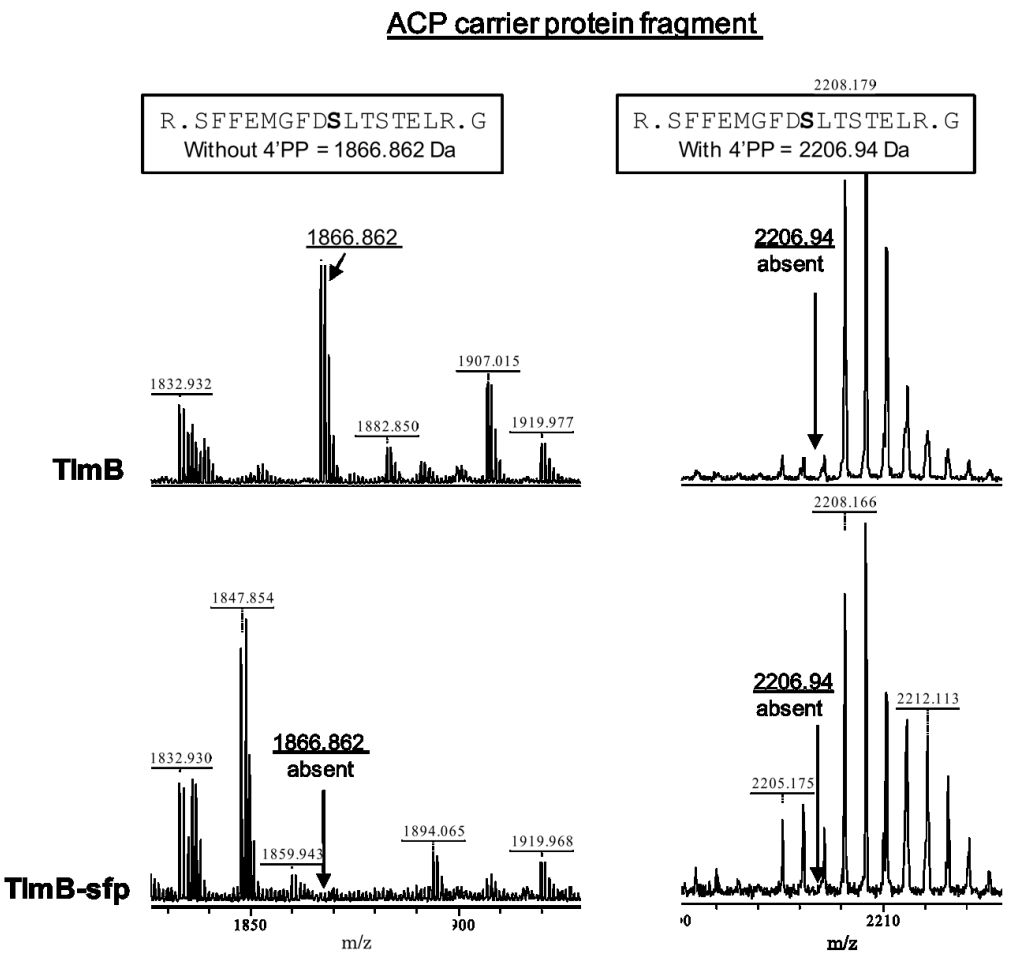


Fig. 3S. MALDI-TOF MS fingerprinting analysis for 4'-phosphopantetheine (4'PP) arm addition. Peptide analysis indicating the disappearance of the ACP fragment containing the carrier protein consensus motif Gx(D/H)**S**(L/I)) in the TlmB coexpressed with Sfp sample (TlmB-Sfp).

PLE hydrolysis of ester probes: To generate the active carboxylates **6** and **7** from the ester probes **4** and **5**, esterase from porcine liver (PLE), purchased from Sigma-Aldrich, was used. All reactions were set up in TLM buffer (10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.2) with the intended final terminator concentration. Reactions were incubated at 37˚C for 2 h and included 3.2 mM of **4** or **5** (dissolved in 90 µL DMSO), 1 µL PLE, and TLM buffer up to a final volume of 1 mL. Following reaction completion, the buffer containing carboxylates **6** or **7** was used directly for TlmB activity assays.

TlmB *in vitro* enzymatic assay using the carboxylate probes: To probe the activity of TlmB-Sfp, reactions were set up with 2 mM DTT, 2 mM NADPH, 2 mM acetyl-CoA, 2 mM methylmalonyl-CoA, 250 µM CoA, 5 µM purified Sfp, 5 µL of ammonium sulfate purified TlmB-Sfp suspension (approximately 10 µM concentration), and TLM buffer, which contained 3.2 mM of either **6** or **7** up to a final volume of 100 µL. Boiled TlmB-Sfp (at 98˚C for 15 min) and reactions that did not include either probe were used as negative controls. Reactions were consistently preformed as triplicates and incubated in a room temperature water bath overnight. Following overnight incubation each assay mixture was extracted twice with 100 µL ethyl acetate. The organic layer was then transferred to a new tube and evaporated under nitrogen.

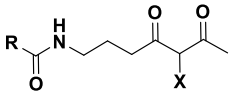
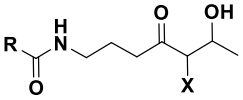
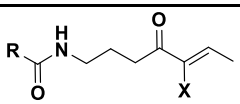
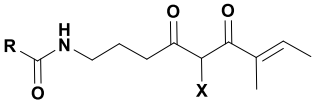
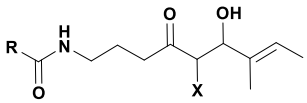
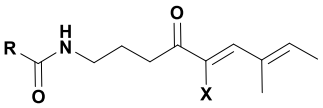
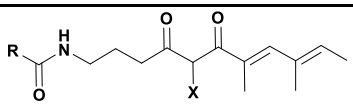
TlmB *in vitro* enzymatic assay using the synthetic SNAc triketide 14: Reactions were similarly set up with 2 mM of the synthetic triketide **14**, 2 mM DTT, 2 mM NADPH, 2 mM methylmalonyl-CoA, 250 µM CoA, 5 µM purified Sfp, 5 µL of ammonium sulfate purified TlmB-Sfp suspension (approximately 10 µM concentration), and TLM buffer (containing 3.2 mM of either **6** or **7**) to a final volume of 100 µL. Boiled TlmB-Sfp (at 98 ˚C for 15 min) and reactions that did not include **14** were used as negative controls. Reactions were consistently preformed as triplicates and incubated in a room temperature water bath overnight. Following overnight incubation each assay mixture was extracted twice with 100 µL ethyl acetate. The organic layer was then transferred to a new tube and evaporated under nitrogen.

HPLC-HR-ESI-MS analyses of assay organic extracts were performed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo) instrument as previously reported.[1, 12] Reversed phase chromatography was used to separate the mixtures prior to MS analysis. Two columns were utilized: an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5 µm 100 Å and an Acclaim PepMap RSLC 75 µm x 15 cm 2 µm 100 Å (Thermo Scientific). The columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was composed of 0.1% aqueous formic acid and mobile phase B was composed of 100% acetonitrile containing 0.1% formic acid. Samples were loaded onto the µ-precolumn equilibrated in 2% aqueous acetonitrile containing 0.1% trifluoroacetic acid for 8 min at 10 µL min^{-1} after which compounds were eluted onto the analytical column following a 45 min gradient for which the mobile phase B concentration was increased from 50% B to 99.5% over 15 min, then maintained at 99.5% B for 5 min, then decreased to 50% over 16 min, followed by a 9 min wash at 50% B. Eluting cations were converted to gas-phase ions by electrospray ionization and analyzed. Survey scans of precursors from 150 to 1500 *m*/*z* were performed at 60K resolution (at 200 *m*/*z*) with a 5×10^5 ion count target. Tandem MS was performed by isolation at 0.7 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS^2 ion count target was set to 10^4 and the maximum injection time was 35 ms. A filter targeted inclusion mass list was used to select the precursor ions. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 5 s cycles, meaning the instrument would continuously perform MS^2 events until the list of nonexcluded precursors diminishes to zero or 5 s, whichever is shorter. Fusion runs were performed with Survey scans of precursors from 150 to 1500 *m*/*z* 60K resolution (at 200 *m*/*z*) with a 1×10^6 ion count target. Tandem MS was performed by isolation at 1.8 Th with the ion-trap, CAD fragmentation with normalized collision energy of 32, and 15K resolution scan MS analysis in the Orbitrap. The data dependent top 20 precursors were selected for MS^2. MS^2 ion count target was set to 4×10^6 and the max injection time was 50 ms. The dynamic exclusion duration was set to 40 s with a 10 ppm tolerance around the selected precursor and its isotopes.

[12] (a) J. S. Parascandolo, J. Havemann, H. K. Potter, F. Huang, E. Riva, J. Connolly, I. Wilkening, L. Song, P. F. Leadlay and M. Tosin, *Angew. Chem. Int. Ed.*, **2016**, *55*, 3463-3467; (b) H. Kage, E. Riva, J. S. Parascandolo, M. F. Kreutzer, M. Tosin and M. Nett, *Org. Biomol. Chem.*, **2015**, *13*, 11414-11417.

3.1 Capture of putative biosynthetic intermediates from TlmB *via* probes 6 and 7

Table 4S: Overview of intermediates captured and characterized from enzymatic assays of recombinant TlmB incubated with acetyl-CoA, methylmalonyl-CoA and NADPH *via* probes **6** and **7**

intermediate	**putative structure**[a]	**probe 6** R= $(CH_2)_8CH_3$ X= H	**probe 7** R= $(CH_2)_8CH_3$ X= F
Diketides		✓	✓
		✓ low abundance	✓
		✓	✓
Triketides		✓ low abundance	n.d.
		✓	✓
		✓ traces	n.d.
Tetraketides [b]		✓	✓

[a] Exact stereochemistry to be confirmed; [b] also detected from the incubation of TlmB with SNAc triketide **14**, methylmalonyl-CoA and NADPH.

3.1.1 Intermediate capture *via* probe 6

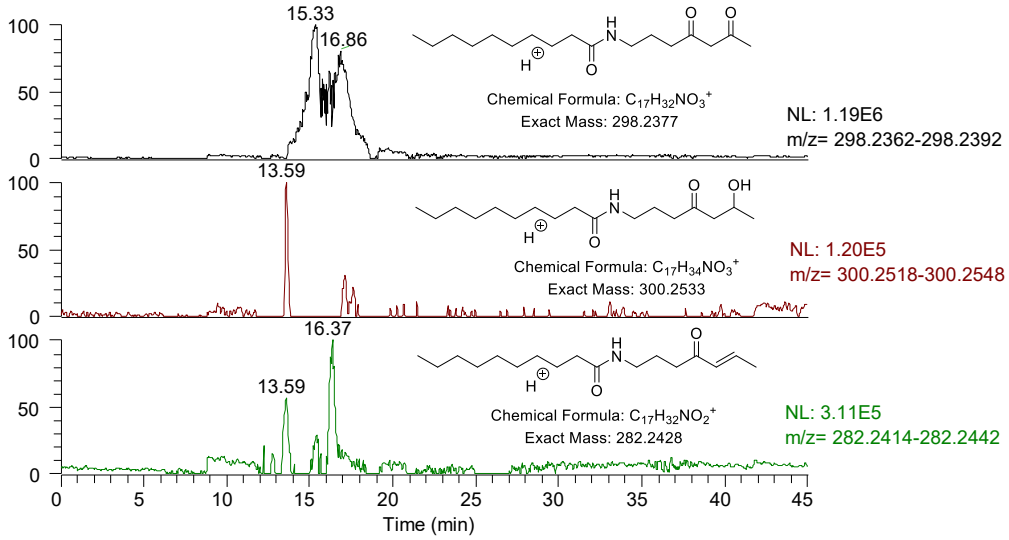


Figure 4S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB assays (incubated with acetyl-CoA, methylmalonyl-CoA and NADPH) in the presence of **6**: extracted ion chromatograms (EICs) for putative intercepted diketides ($[M+H]^+$ adducts).

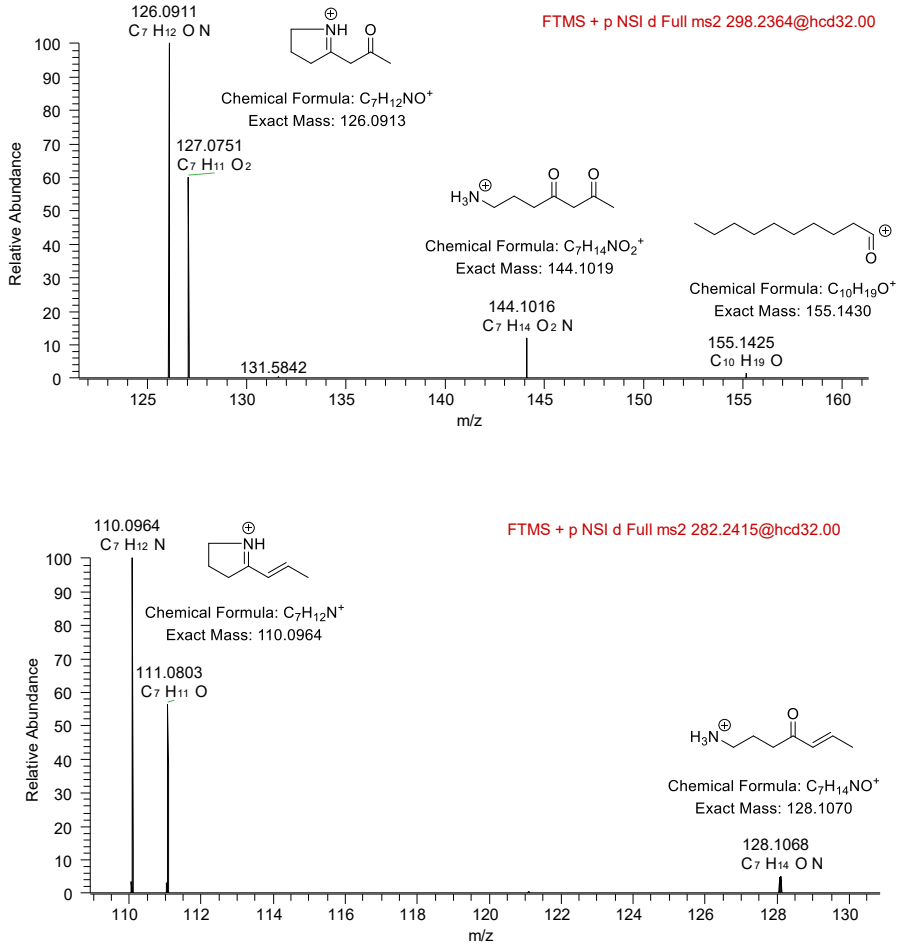


Figure 5S: HR-MS^2 analyses (Orbitrap Fusion) of the putative intercepted diketides (see also Fig. 4S).

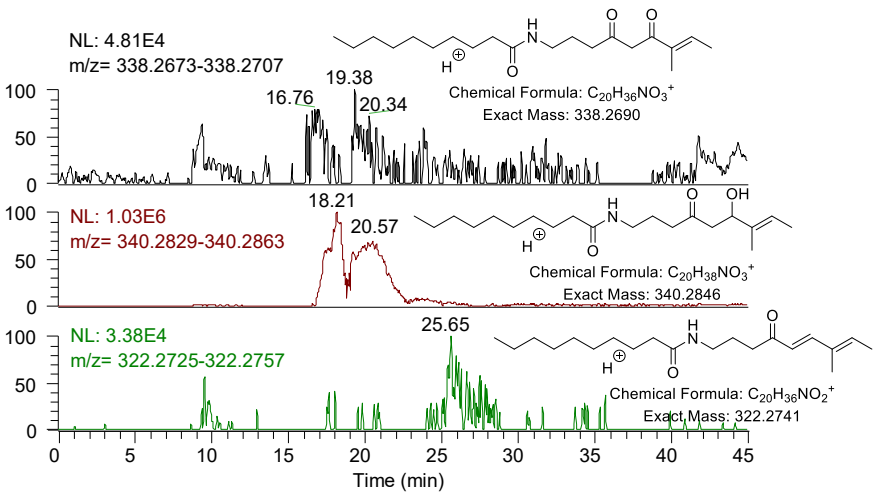


Figure 6S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB assays (incubated with acetyl-CoA, methylmalonyl-CoA and NADPH) in the presence of **6**: EICs for putative intercepted triketides ($[M+H]^+$ adducts).

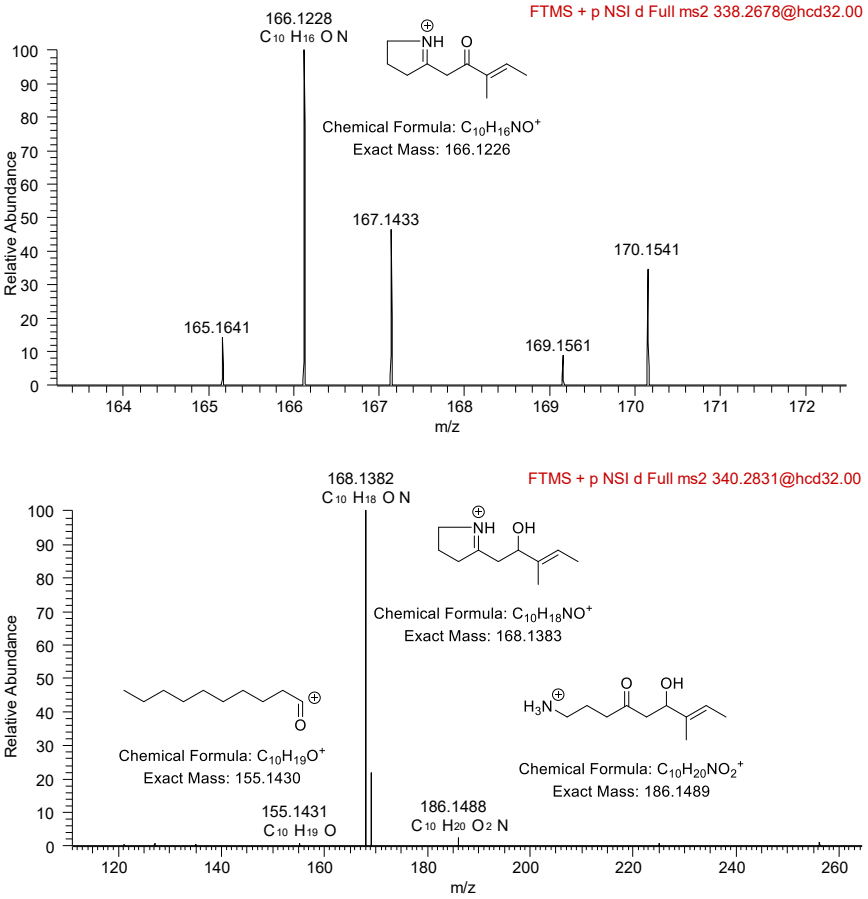


Figure 7S: HR-MS^2 analyses (Orbitrap Fusion) of putative intercepted triketides (see also Fig. 6S).

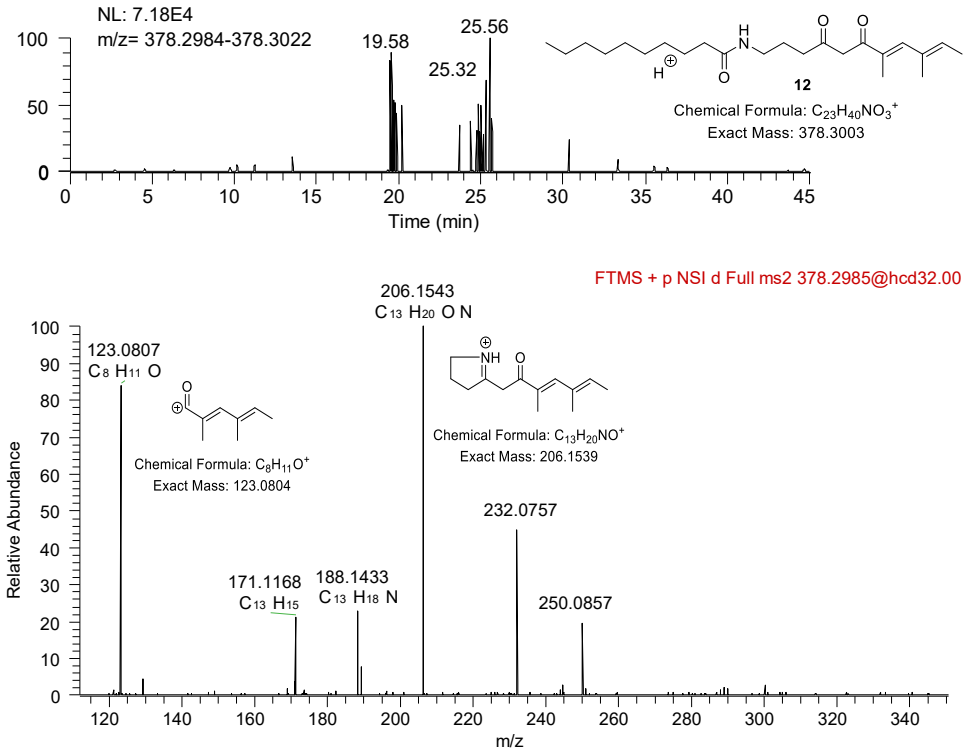


Figure 8S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB assays (incubated with acetyl-CoA, methylmalonyl-CoA and NADPH) in the presence of **6**: EIC (top) and MS^2 fragmentation (bottom) for the putative tetraketide **12** ($[M+H]^+$ adduct).

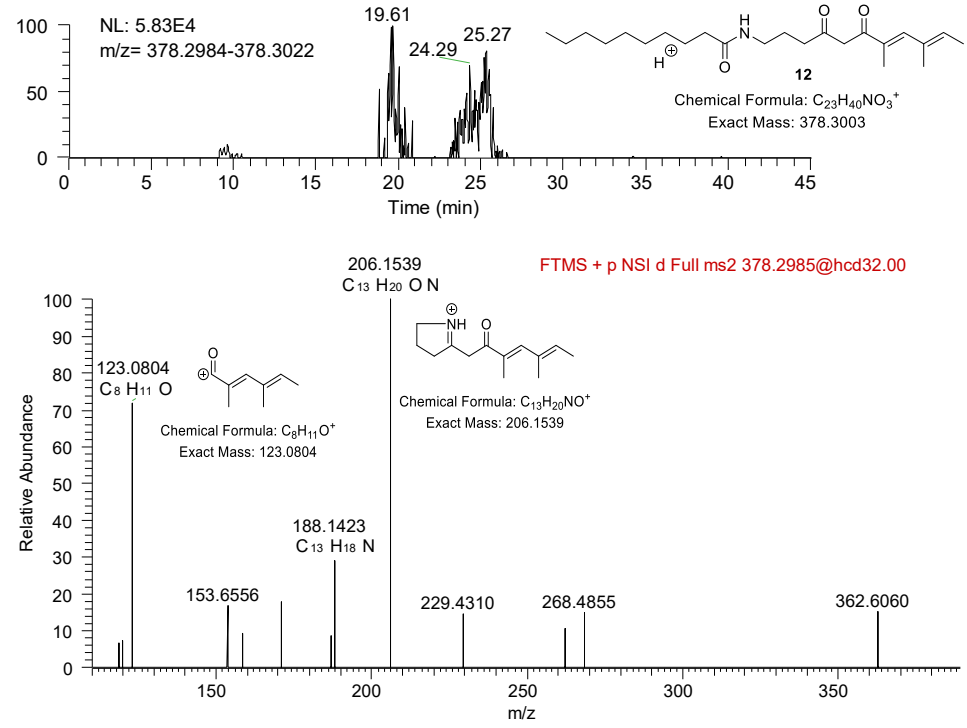


Figure 9S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB incubated with SNAc triketide **14** , methylmalonyl-CoA and NADPH in the presence of **6**: EIC (top) and MS^2 fragmentation (bottom) for the putative tetraketide **12** ($[M+H]^+$ adduct), which are identical to those previously obtained (Fig. 8S).

3.1.2 Intermediate capture *via* probe 7

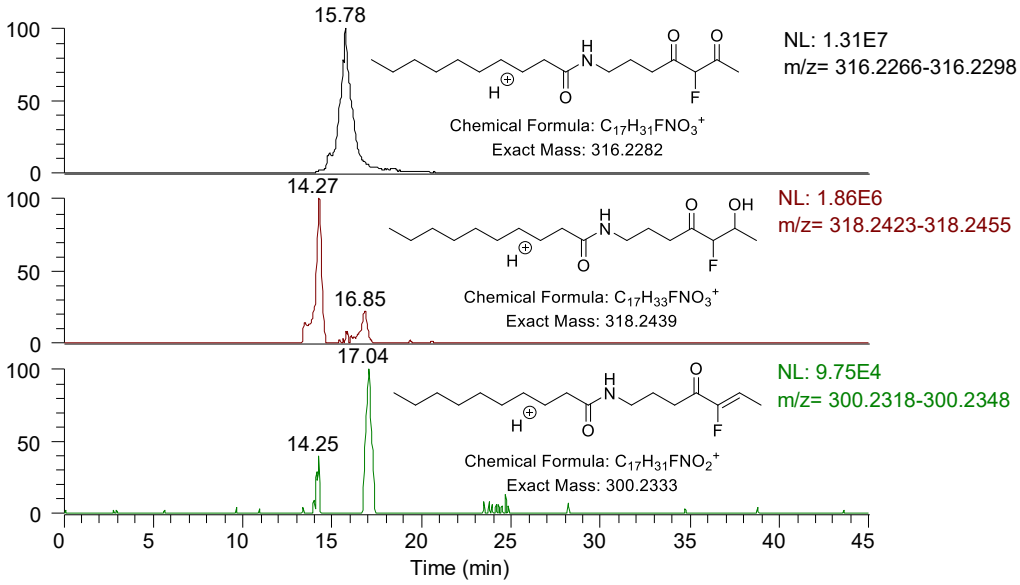


Figure 10S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB assays (incubated with acetyl-CoA, methylmalonyl-CoA and NADPH) in the presence of **7**: EICs for putative intercepted diketides ($[M+H]^+$ adducts).

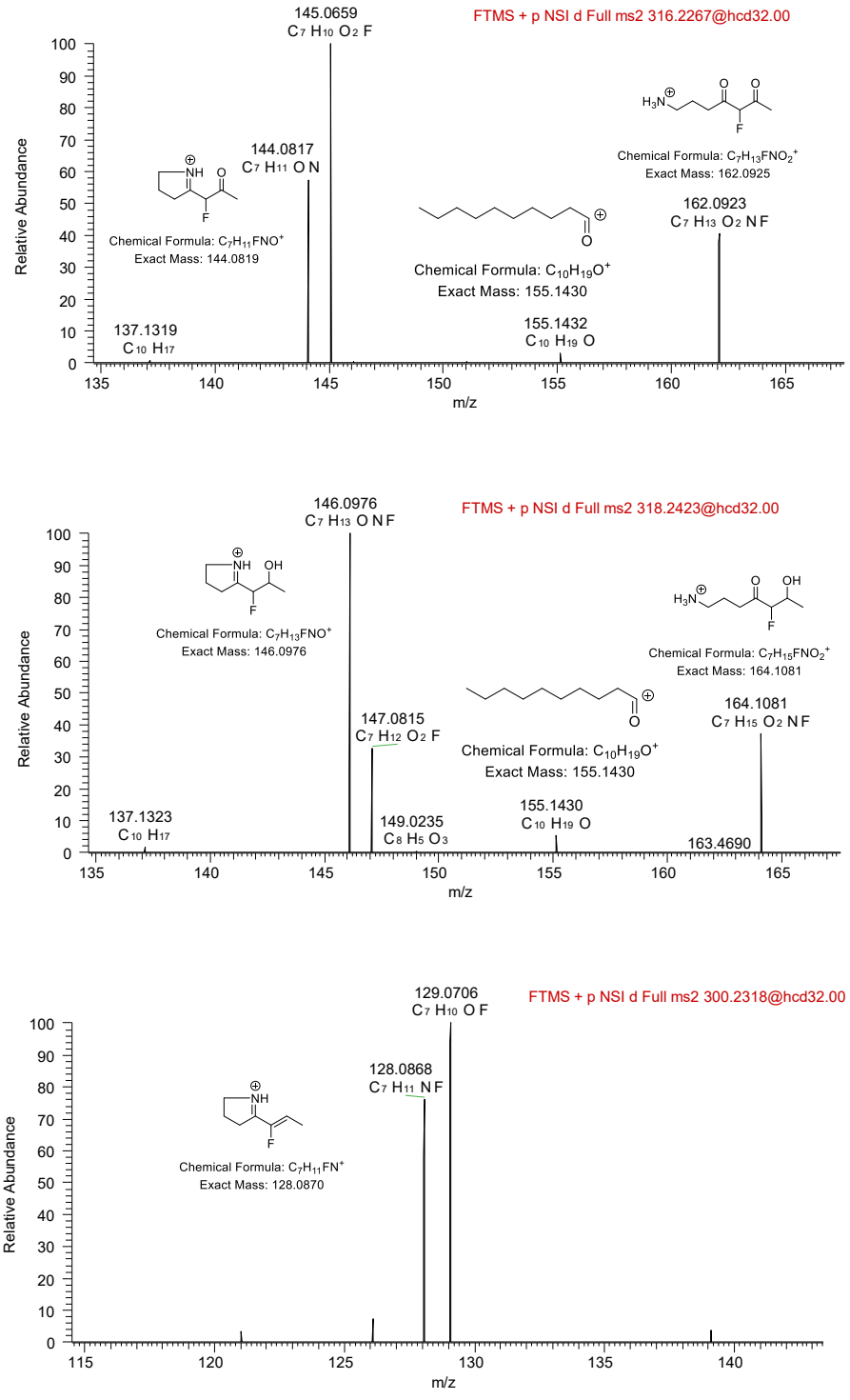


Figure 11S: HR-MS^2 analyses (Orbitrap Fusion) of the putative intercepted diketides (see also Fig. 10S).

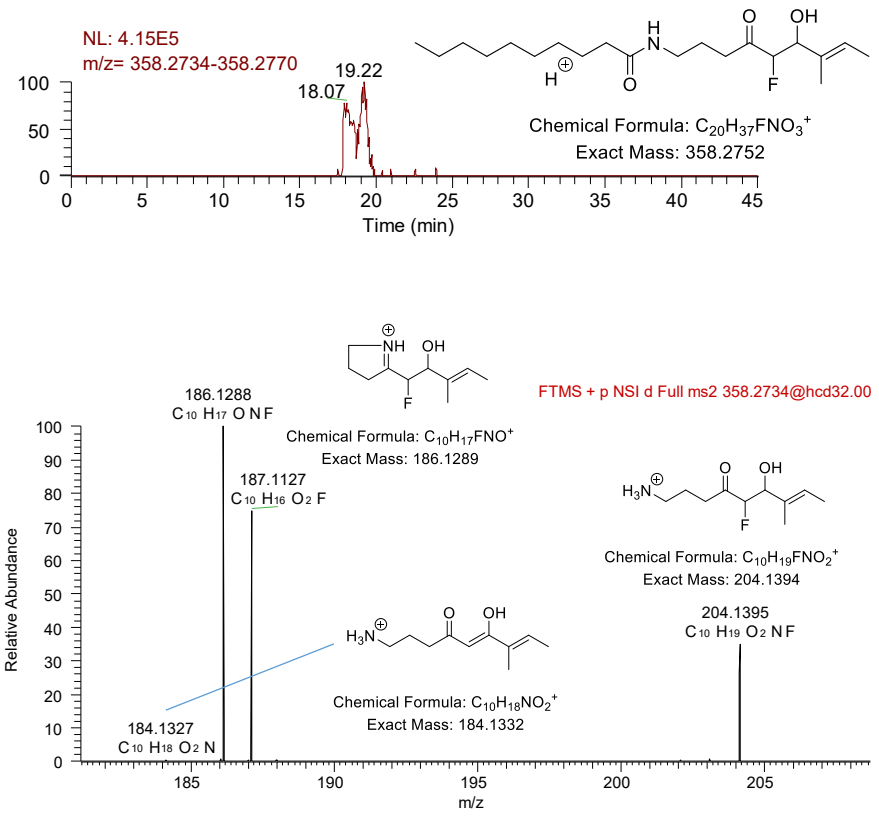


Figure 12S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB assays (incubated with acetyl-CoA, methylmalonyl-CoA and NADPH) in the presence of **7**: EIC (top) and MS^2 (bottom) for a putative intercepted triketide ($[M+H]^+$ adducts).

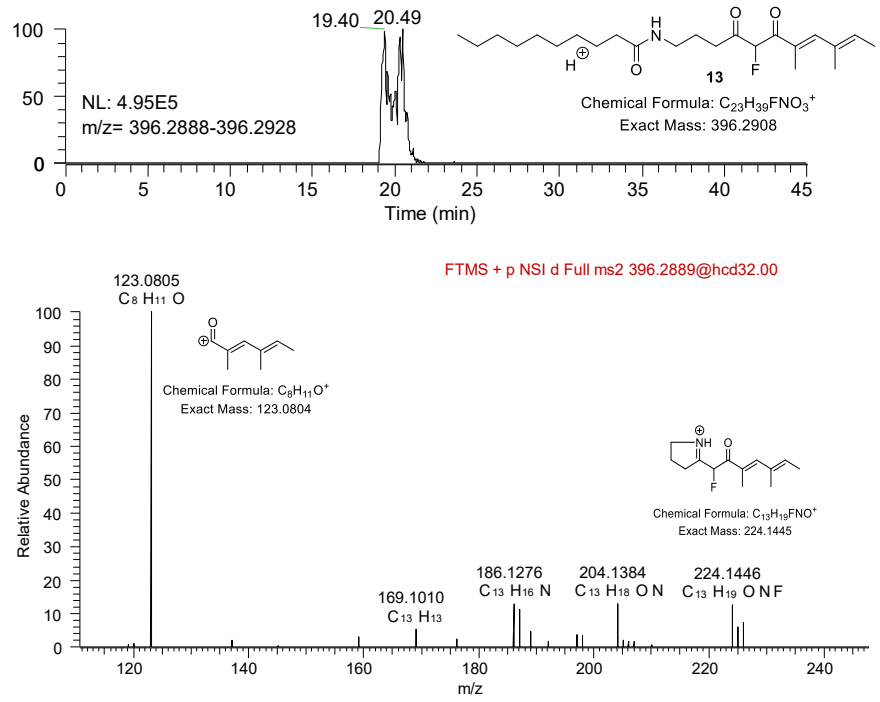


Figure 13S: LC-HRMS analyses (Orbitrap Fusion) of the organic extracts of recombinant TlmB incubated with SNAc triketide **14** , methylmalonyl-CoA and NADPH in the presence of **7**: EIC (top) and MS^2 fragmentation (bottom) for the putative tetraketide **13** ($[M+H]^+$ adduct).

4. Spectra

4.1. ^{1}H- and ^{13}C-NMR of 14 (500 MHz, $CDCl_3$)