nature catalysis

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41929-018-0178-2

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A vitamin K-dependent carboxylase orthologue is involved in antibiotic biosynthesis

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Supplementary Information

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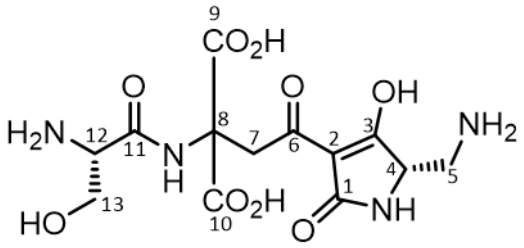
Supplementary Methods

Characterisation of compounds

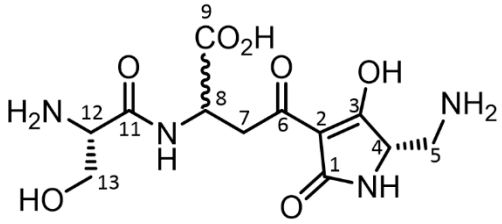
Malonomycin **1**



1

δ_H (400 MHz, D_2O/DCl) 4.16 (1 H, dd, J 6.0, 4.4, *H12*), 4.01 – 3.97 (1 H, m, *H4*) 3.97 – 3.93 (1 H, m, *H13*), 3.89 (1 H, dd, *J* 12.4, 6.0, *H13*), 3.79 (1 H, d, J 16.8, *H7*), 3.64 (1 H, d, J 16.8, *H7*), 3.30 (1 H, dd, J 13.4, 4.0, *H5*), 3.24 (1 H, dd, J 13.4, 4.6, *H5*); δ_C (201 MHz, D_2O) 193.5 (*C6*), 191.2 (*C3*), 177.8 (*C1*), 172.7 (*C9*), 172.6 (*C10*), 166.3 (*C11*), 102.5 (*C2*) , 65.1 (*C8*), 60.0 (*C13*), 56.2 (*C4*), 54.3 (*C12*), 43.4 (*C7*), 39.9 (*C5*); *m/z* (ESI) calculated: 375.1147 $[M+H]^+$; observed: 375.1146.

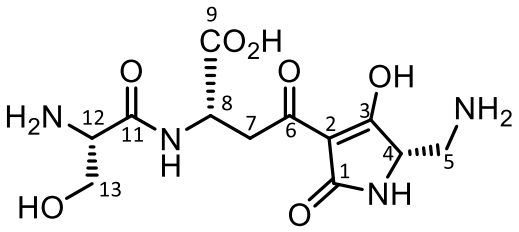
Decarboxylated malonomycin (8*R/S*)-**5**



(8*R/S*)-**5**

δ_H (800 MHz, D_2O) 4.53 (1 H, dd, *J* 9.4, 4.4, *H8'*), 4.50 (1 H, dd, *J* 9.0, 4.2, *H8*), 4.04 (1 H, dd, *J* 5.6, 4.2, *H12*), 4.01 (1 H, t, *J* 4.8, *H12'*), 3.96 (2 H, td, *J* 4.5, 1.6, *H4*, *H4'*), 3.91 (2 H, d, *J* 4.8, *H13*), 3.83 (1 H, dd, *J* 12.6, 4.6, *H13'*), 3.76 (2 H, dd, *J* 12.6, 5.8, *H13'*), 3.25 – 3.20 (4 H, m, *H5, H5'*), 3.20 – 3.14 (2 H, m, *H7 , H7'*), 3.11 – 3.07 (2 H, m, *H7, H7'*); δ_C (201 MHz, D_2O) 193.6 (C6/ C3), 193.6 (C6/C3), 193.5 (C6/C3), 178.2 (C1/C1'), 178.0 (C1/C1'), 177.8 (C9, C9'), 166.9 (C11/C11'), 166.9 (C11/C11'), 102.4 (C2, C2'), 60.2 (C13/C13'), 60.1 (C13/C13') , 56.2 (C4/C4'), 56.2 (C4/C4'), 54.7 (C12/C12'), 54.7 (C12/C12'), 52.1 (C8/C8'), 51.9 (C8/C8'), 42.1 (C7/C7'), 42.1 (C7/C7'), 39.9 (C5).; *m/z* (ESI) calculated: 331.1248 $[M+H]^+$; observed: 331.1247.

Premalonomycin **5**



5

δ_H (400 MHz, D_2O) 4.46 (1 H, dd, *J* 8.2, 4.8, *H8*), 4.00 – 3.97 (1 H, m, *H12*), 3.92 (1 H, t, *J* 4.4, *H4*), 3.87 (2 H, d, *J* 4.8, *13*), 3.18 (2 H, t, *J* 4.4, *H5*), 3.16 – 3.01 (2 H, m, *H7*); δ_C (201 MHz, D_2O) 193.5 (C6/3), 193.4 (C6/3), 178.1 (C1), 177.7 (C9), 166.9 (C11), 102.3 (C2), 60.1 (C13), 56.2 (C4), 54.6 (C12), 51.9 (C8), 42.0 (C7), 39.9 (C5); *m/z* (ESI) calculated: 331.1248 $[M+H]^+$; observed: 331.1249.

N-succinyl-premalonomycin **12**

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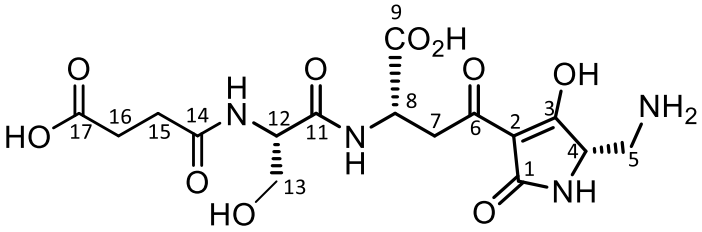
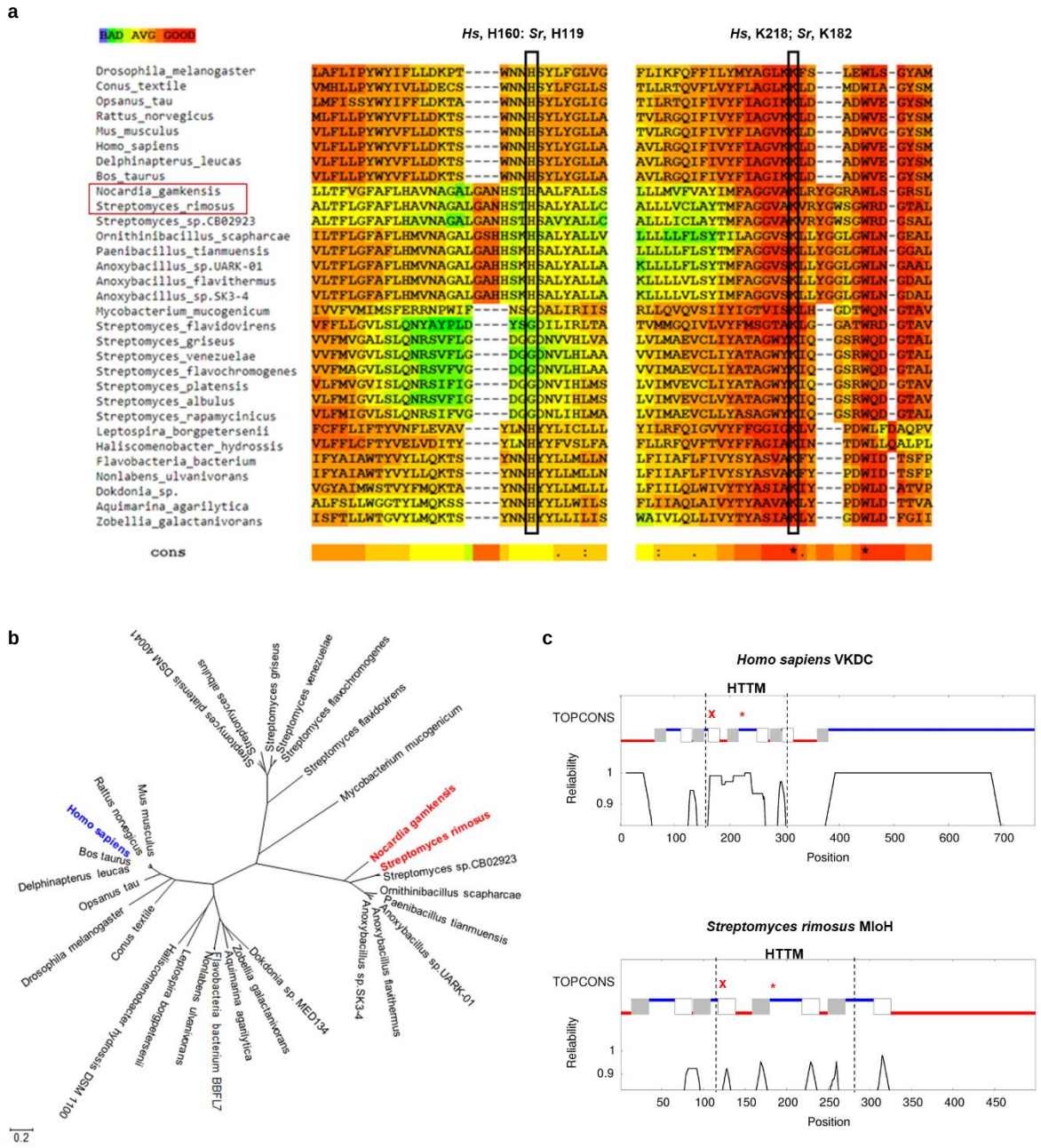


12

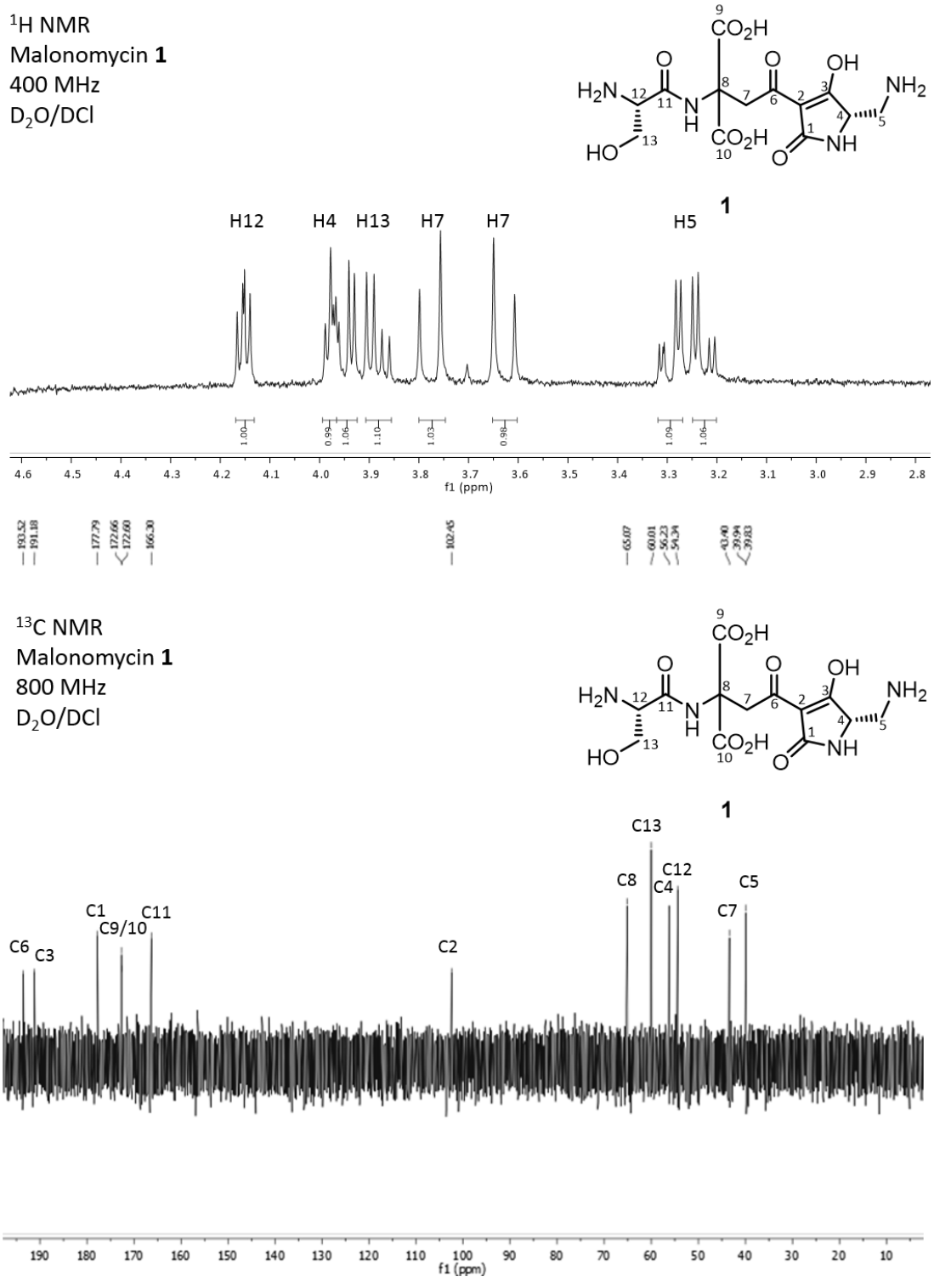
δ_H (800 MHz, H_2O:D_2O 9:1) 4.47 – 4.41 (1 H, m, H8), 4.39 – 4.34 (1 H, m, H12), 3.97 (1 H, d, *J* 5.1, H4), 3.78 – 3.71 (2 H, m, H13), 3.28 – 3.18 (3 H, m, H5+H7), 3.01 – 2.92 (1 H, m, H5), 2.52 – 2.45 (2 H, m, H16), 2.42 (2 H, t, *J* 7.3, H17); δ_C (201 MHz, D_2O) 193.7 (C6/3), 193.5 (C6/3), 180.2 (C1), 178.2 (CO_2R), 178.0 (CO_2R), 175.9 (CO_2R), 171.0 (CO_2R), 102.7 (C2), 61.4 (C13) , 56.4 (C4), 55.4 (C12), 52.5 (C8), 41.7 (C5), 40.1 (C7), 32.0 (C16), 31.7 (C17); *m/z* (ESI) calculated: 431.1409 $[M+H]^+$; observed: 431.1409.

Supplementary Figures

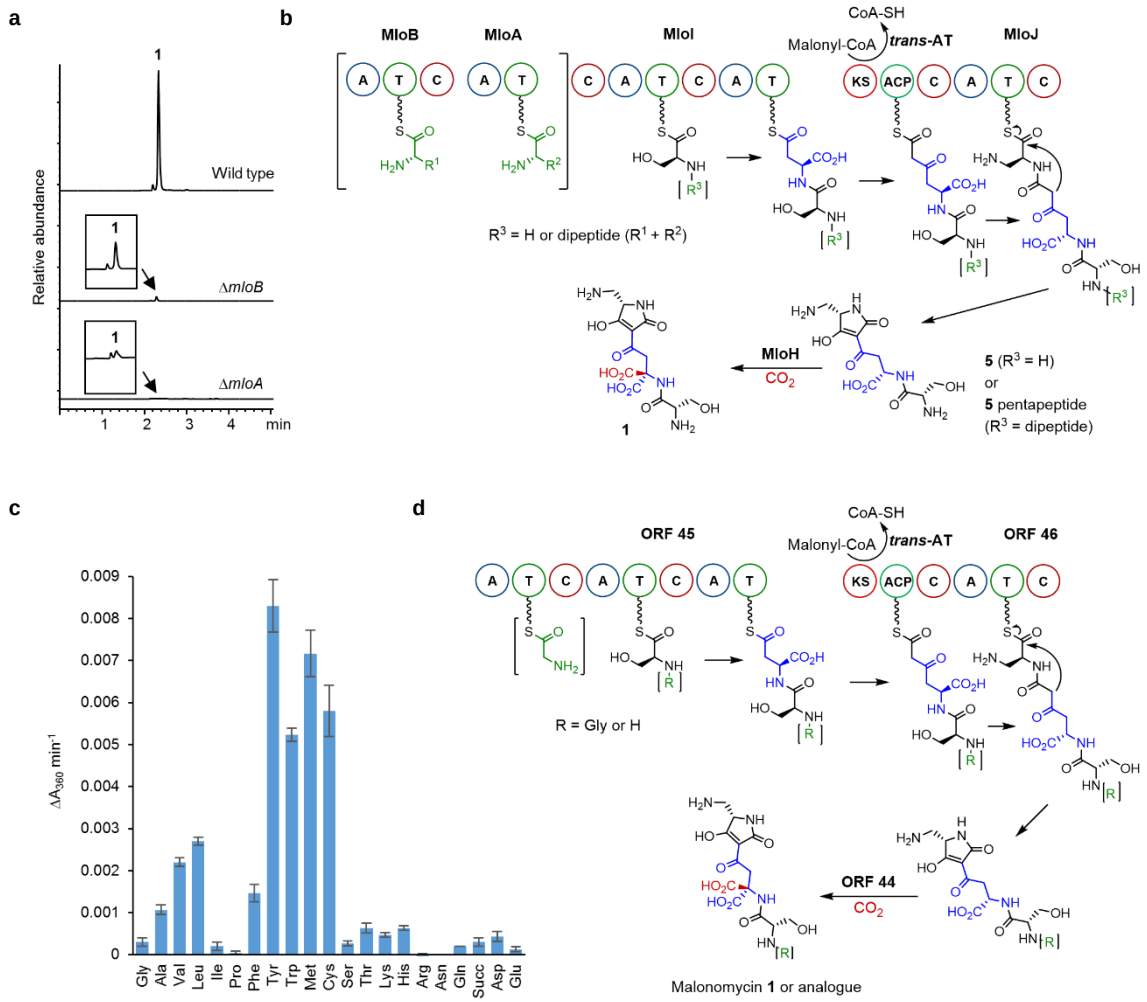


Supplementary Figure 1. Bioinformatics analyses of VKDC. a, Protein alignment of VKDC with partial sequences shown. Sequence homology between VKDC is centred on a horizontally transferred transmembrane (HTTM) domain that is conserved in VKDC[1]. This HTTM domain, containing conserved residues important for activity (*Hs*, H160 and K218; *Sr*, H119 and K182 – highlighted with boxes) is presumed to have transferred from metazoan VKDC to bacteria[1]. Whereas *Hs* K218, predicted

to deprotonate the vitamin K co-factor to generate a reactive oxyanion (strong base), is an invariant residue found also in *S. rimosus*, Hs H160 is conserved in VKDC orthologues including *S. rimosus* except a subset of HTTM-containing proteins in Streptomycetes. **b**, Phylogenetic tree of VKDC from different taxa. **c**, Predicted membrane topology of VKDC orthologues from *Homo sapiens* and *Streptomyces rimosus*. Topology predictions were performed using TOPCONS[2]. Predicted transmembrane helices are shown as white and grey boxes. The HTTM domain conserved in VKDC is found within the region marked with dashed lines. The locations of the conserved Lys and His residues are denoted by * and x, respectively. HTTM regions were determined by multiple protein sequence alignments using blastp. The predicted location of MloH in the *S. rimosus* cell membrane suggests that carboxylation by MloH occurs as a final step in the biosynthetic pathway. Abbreviations: *Hs*, *Homo sapiens*; *Sr*, *Streptomyces rimosus* sp. *paramyceticus* R2374.

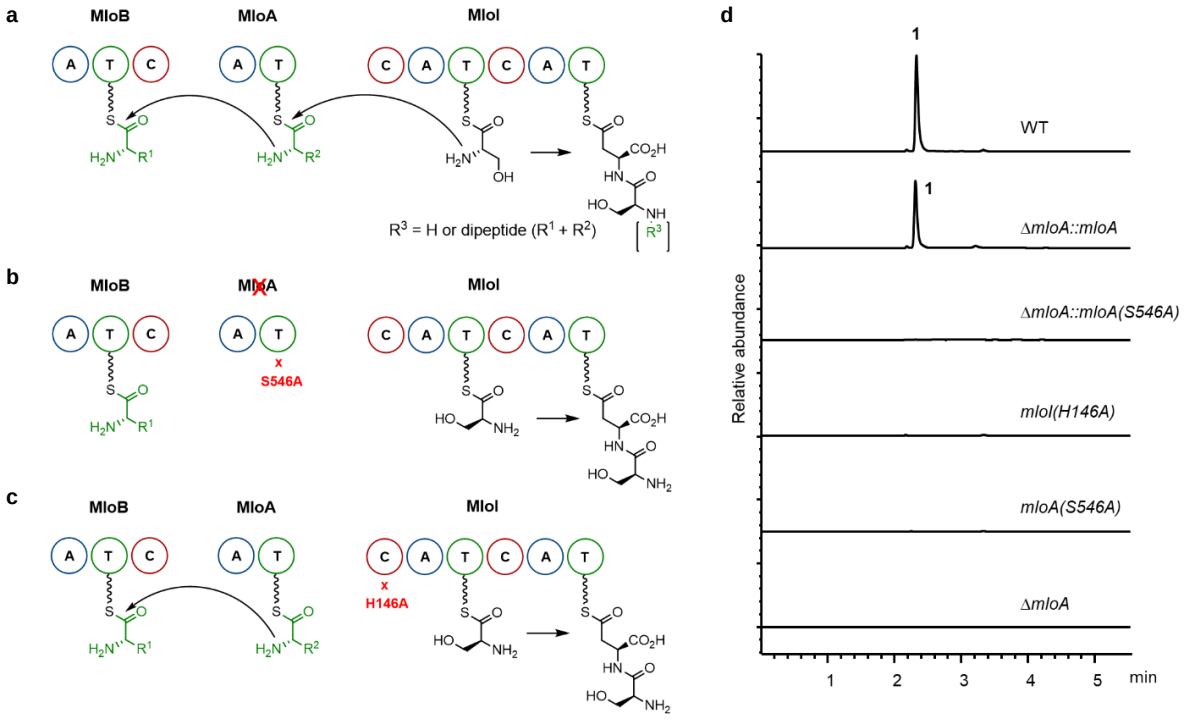


Supplementary Figure 2. ^{1}H and ^{13}C NMR of 1 isolated from *S. rimosus* R2374.

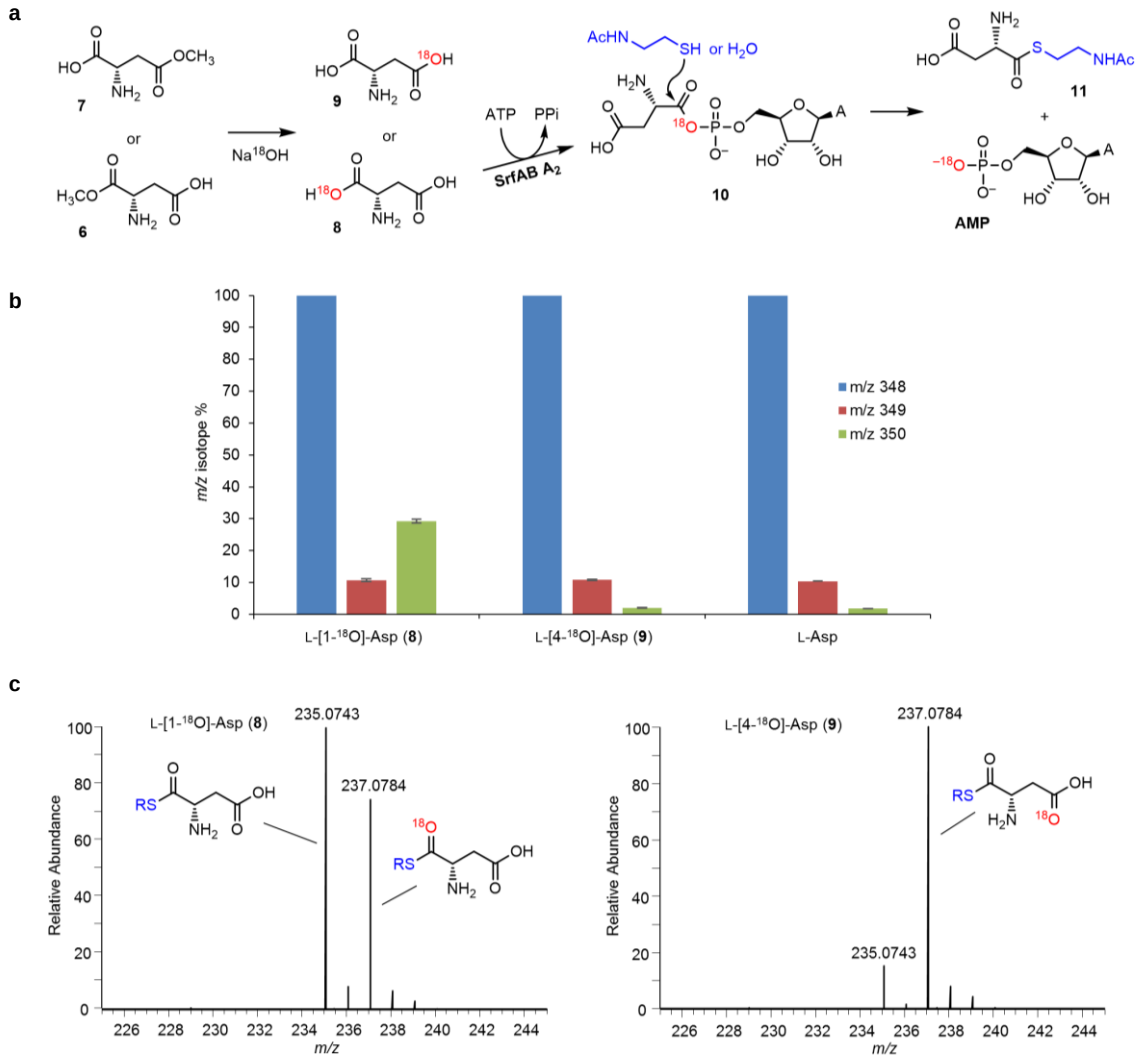


Supplementary Figure 3. Comparison of the NRPS from the *mlo* BGC with a related NRPS from *N. gamkensis*. **a**, RP-HPLC analysis showing CRISPR/Cas9 inactivation of *mloA* and *mloB* in R2374 leads to only trace amounts of malonomycin **1** being produced. **b**, Proposed extended biosynthetic pathway of malonomycin **1** in *S. rimosus*. The minimal assembly-line enzymes MloI and MloJ may require MloA and MloB (predicted to activate hydrophobic substrates, panel **c**) to form a longer, more hydrophobic pentapeptide intermediate to allow for efficient targeting to the membrane where the predicted multiple transmembrane protein MloH resides. **c**, Adenylation domain activity assays with MloA. The MloA A-T didomain was assayed with the twenty proteinogenic amino acids and succinic acid, showing a preference for activation of hydrophobic amino acids. Values represent means, and error bars denote standard deviations, with $n = 3$. **d**, Proposed pathway in *N. gamkensis*. ORF 45 encodes a tri-module NRPS with an N-terminal A-T module (predicted to activate Gly: NRPSpredictor2, 80% identity score) that is not present in MloI. This additional module may lead to formation of a tetrapeptide

malonomycin-like metabolite. This N-terminal module of the ORF 45 NRPS may functionally replace MloA and MloB found in *S. rimosus*.

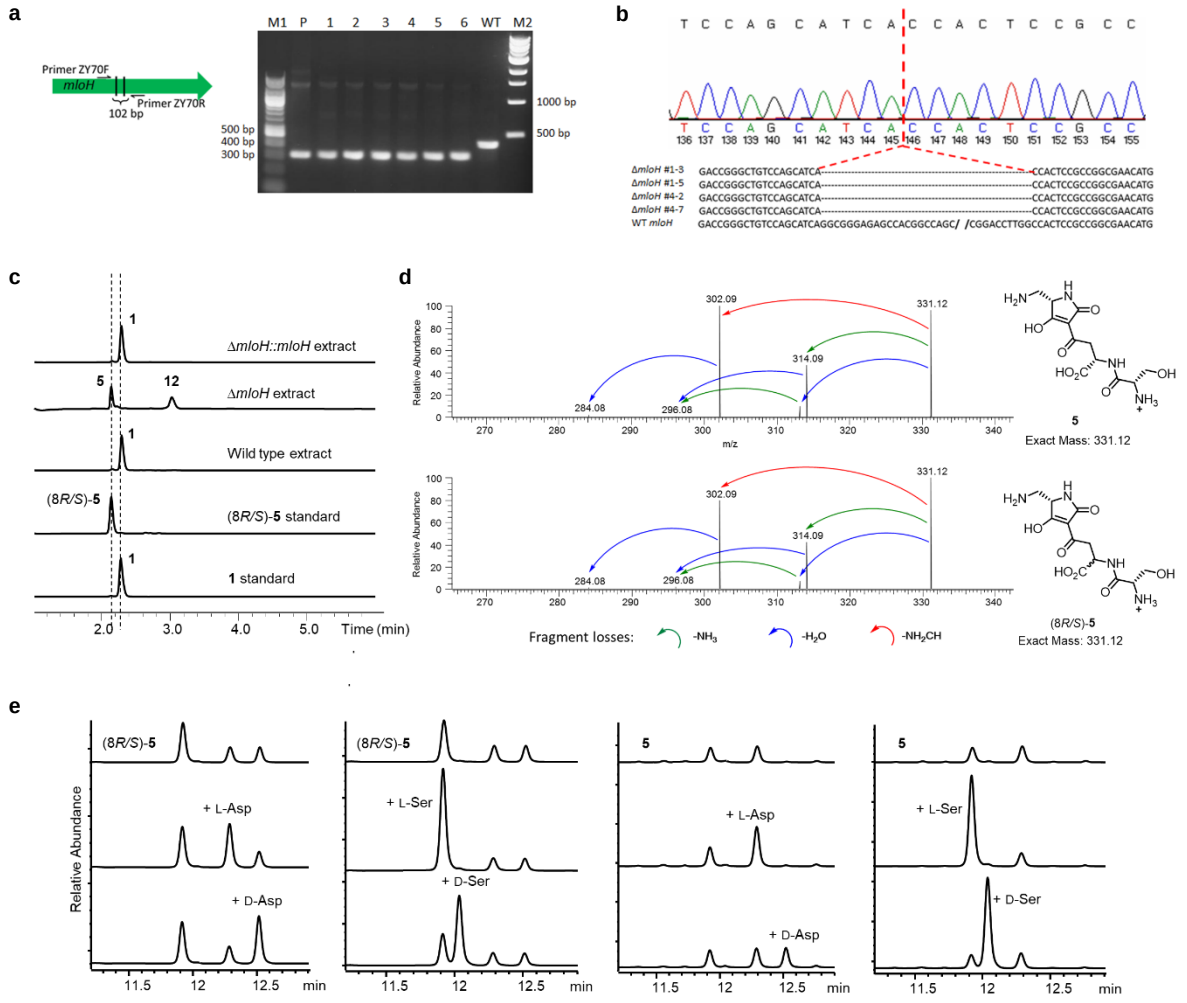


Supplementary Figure 4. Investigation of the catalytic role of MloA in malonomycin (1) biosynthesis. a, Possible pathway involving malonomycin propeptide formation with MloAB adding additional residues that are subsequently cleaved to generate the final product **1**. **b**, Either deletion of *mloA*, or point mutation of the conserved Ser residue (S546A) of the MloA PCP domain results in very low level production of **1** (only evident from high sensitivity LC–HRMS). **c**, Point mutation of the catalytic histidine residue (H146A) of the MloI C_1 domain also leads to only trace amounts of **1**, only evident from LC-HRMS, similar to the Δ*mloA* strain. **d**, RP-HPLC analysis of strains generated in schemes **a-c**. Complementation of the Δ*mloA* strain with *mloA* restores production of malonomycin, whereas complementation with the inactive *mloA*(S546A) does not. This indicates that MloA is likely to fulfil a catalytic role transferring an amino acyl- or peptidyl-precursor to MloI. Disrupting the function of MloA or the MloI C_1 domain leads to trace amounts of malonomycin (detectable by sensitive LC-HRMS), suggesting that peptide assembly can be initiated by MloI with L-Ser, albeit inefficiently. Furthermore, the production of malonomycin in MloA and MloI C_1 mutant strains indicates that the VKDC-like enzyme MloH is also capable of carboxylating a tripeptide-derived precursor (premalonomycin **5**).



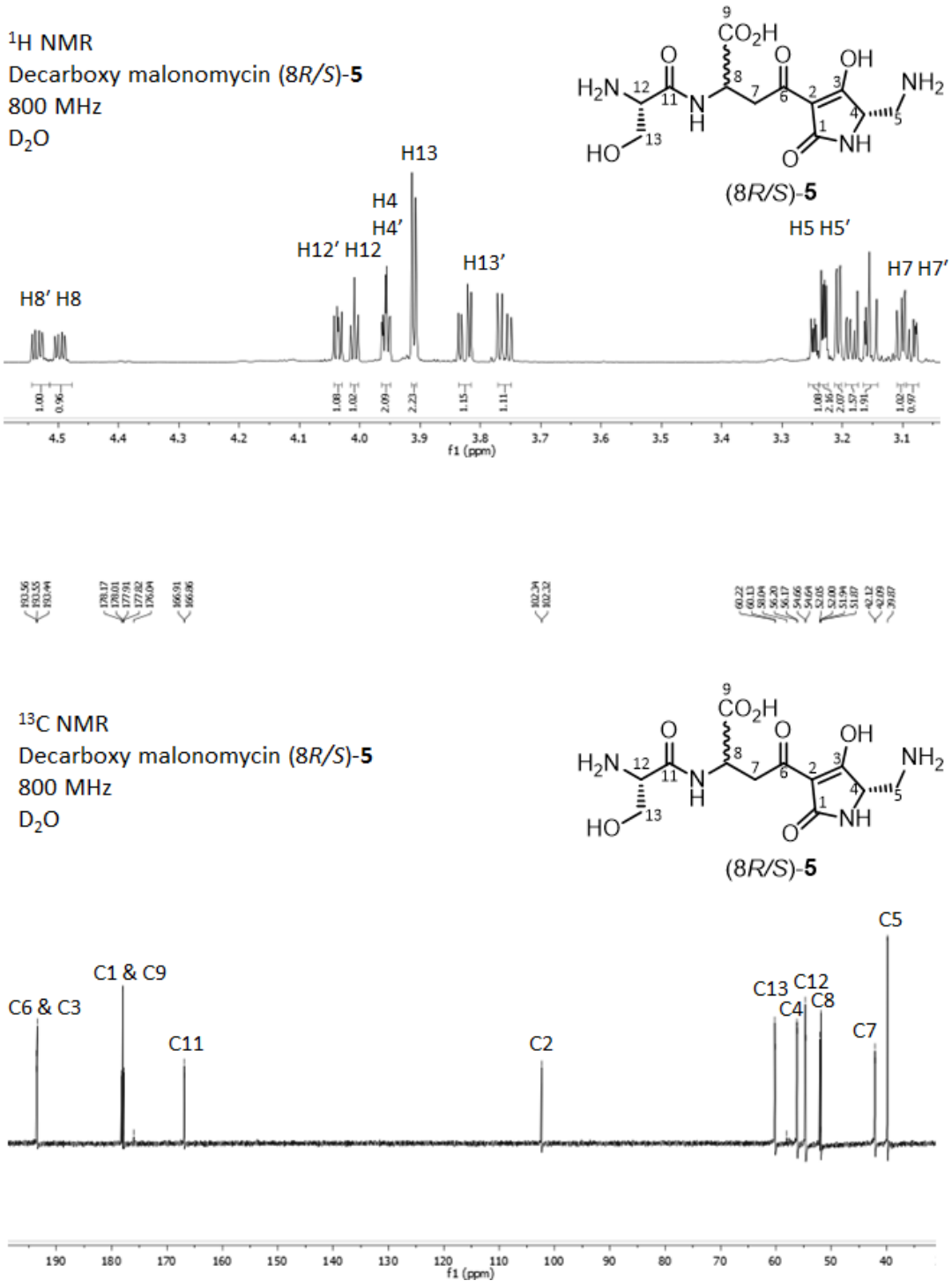
Supplementary Figure 5. Reactions of SrfAB module 2 Asp-activating A domain with ^{18}O Asp isotopomers. a, Reaction scheme for monitoring substrate selectivity and regiospecificity of SrfAB A_2. **b**, LC-HRMS analysis of AMP generated from assays of SrfAB A_2 with ^{18}O L-Asp isotopomers and ATP. Standard activation of L-Asp via the α-carboxyl group by SrfAB A_2 from the surfactin BGC in *Bacillus subtilis* results in ^{18}O enrichment of AMP (increased *m/z* 350) only when L-[1-^{18}O]-Asp **8** is used as a substrate, whereas no enrichment occurs when L-[4-^{18}O]-Asp **9** or L-Asp are used. Conversely, MloI A_2T_2 which installs L-Asp via the side chain carboxyl, showed enrichment of AMP when L-[4-^{18}O]-Asp **9** was used as a substrate (Fig. 3c). *m/z* 348: monoisotopic mass of AMP $[M+H]^+$; *m/z* 350: monoisotopic mass of AMP (^{18}O) $[M+H]^+$. Values represent means, and error bars denote standard deviations, with $n = 3$. **c**, LC-HRMS of Asp-SNAC thioesters generated from SrfAB A_2 assays with L-[1-^{18}O]Asp **8** and L-[4-^{18}O]Asp **9**, ATP and SNAC, showing that the SrfAB A_2-domain is highly

selective for the C1-carboxyl group of Asp. HRMS data: Asp-SNAC $[M+H]^+$ *m/z*: cal'd 235.0747, obs'd 235.0743. ^{18}O-Asp-SNAC $[M+H]^+$ *m/z*: cal'd 237.0789, obs'd 237.0784.

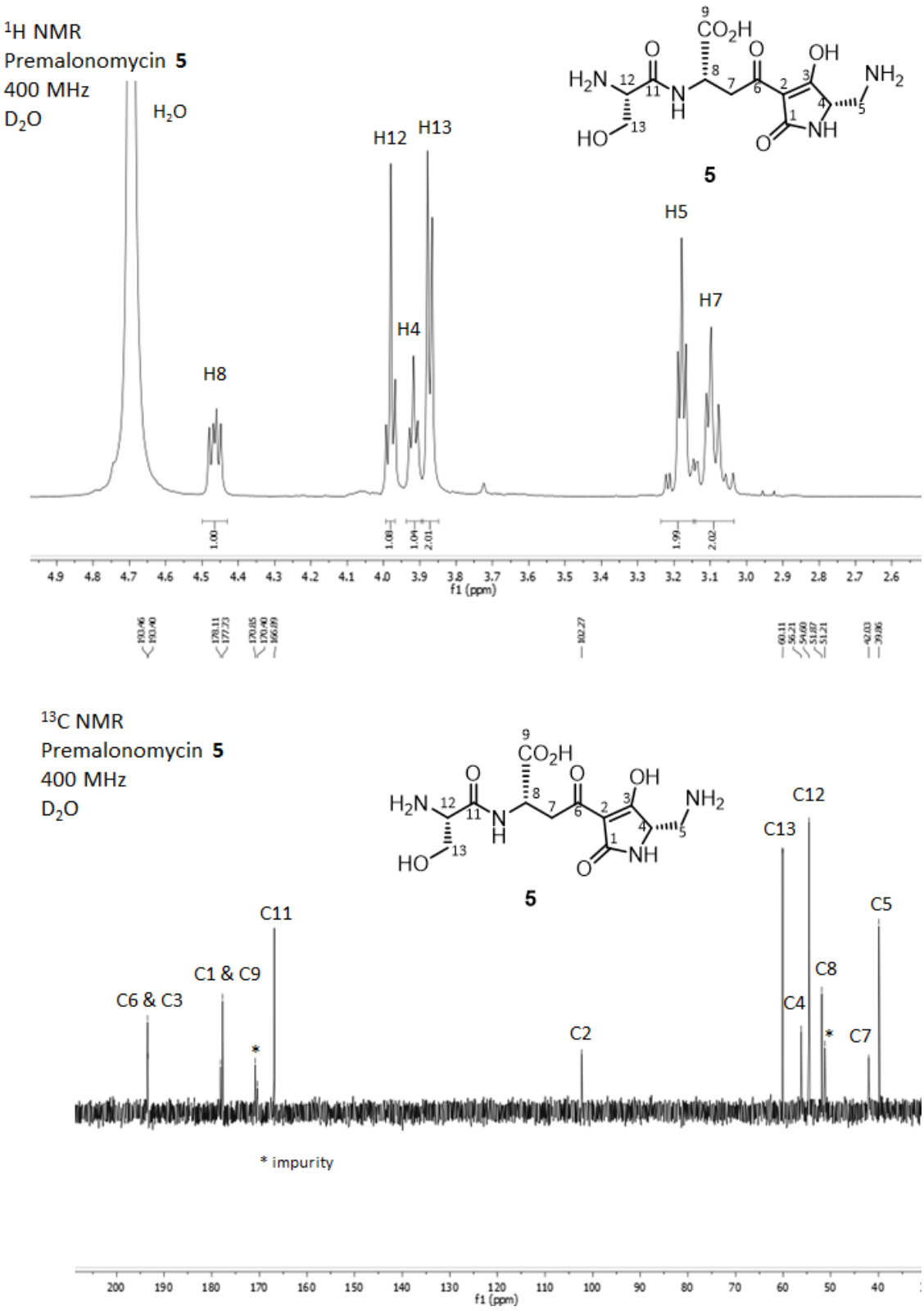


Supplementary Figure 6. CRISPR/Cas9 inactivation of *mloH* and compounds produced by the Δ*mloH* strain. a, Amplification of an internal region of the *mloH* gene using primers ZY70F&R that flank the site of deletion; the wild type strain produces a 411 bp product and in-frame deletion of 102 bp from the *mloH* gene via CRISPR/Cas9 results in a 309 bp product. Lanes: M1, 100 bp DNA ladder; P, plasmid pCm2-tsr-mloH (pCRISPomyces-2 based construct carrying a repair cassette and protospacer for *mloH* 102 bp deletion); 1-6, colonies screened for *mloH* inactivation; WT, *S. rimosus* R2374 wild type. **b**, Nucleotide sequencing of the amplified PCR product from R2374 Δ*mloH* strain. Confirmation of the internal 102 bp deletion from *mloH*. **c**, RP-HPLC of compound **5** (premalonomycin, isolated from the Δ*mloH* strain) shows an identical retention time to decarboxylated malonomycin (8*R/S*)-**5**. **d**, MS/MS analysis of **5** and (8*R/S*)-**5** show that both compounds possess identical fragmentation profiles, indicative of a shared structure. **e**, Derivatisation of amino acids from acid

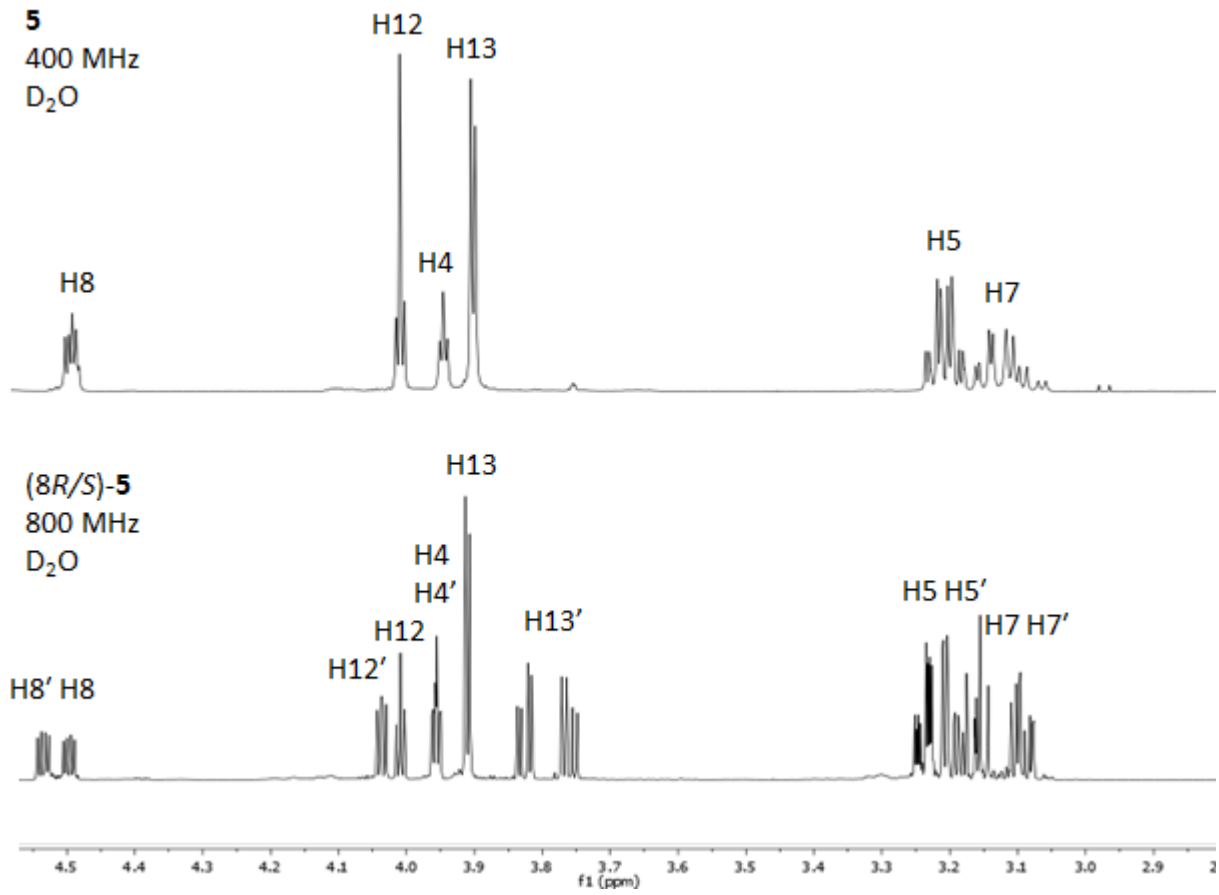
hydrolysates of decarboxylated malonomycin (8*R/S*)-**5** and premalonomycin **5** with Marfey's reagent (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide, FDAA). (Top traces) FDAA-derivatised acid hydrolysates; (middle traces) with co-injections of L-Asp or L-Ser; (bottom traces) with co-injections of D-Asp or D-Ser.



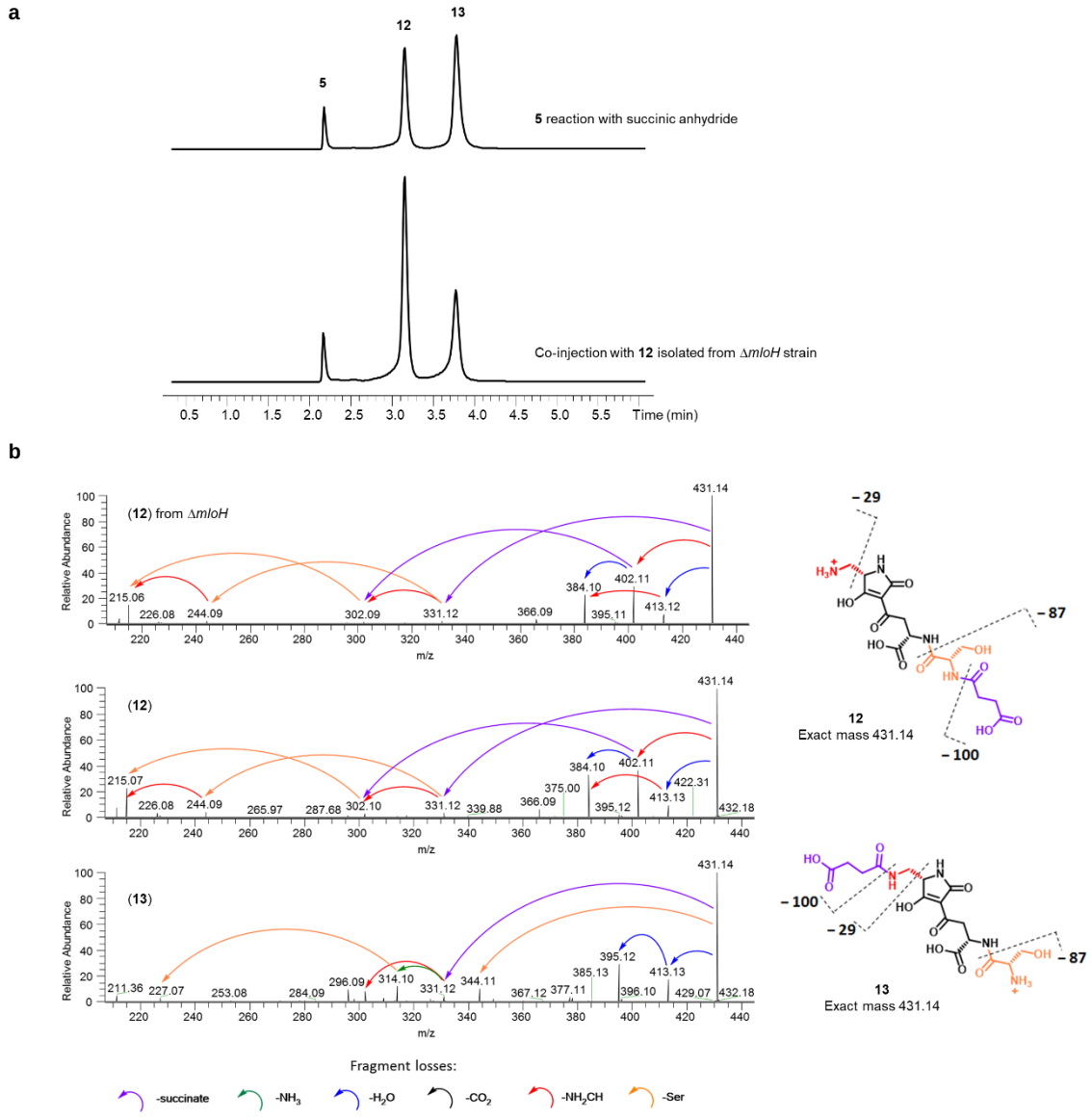
Supplementary Figure 7. ^{1}H and ^{13}C NMR of (8*R*/*S*)-5.



Supplementary Figure 8. ^{1}H and ^{13}C NMR of 5 from Δ*mloH*.

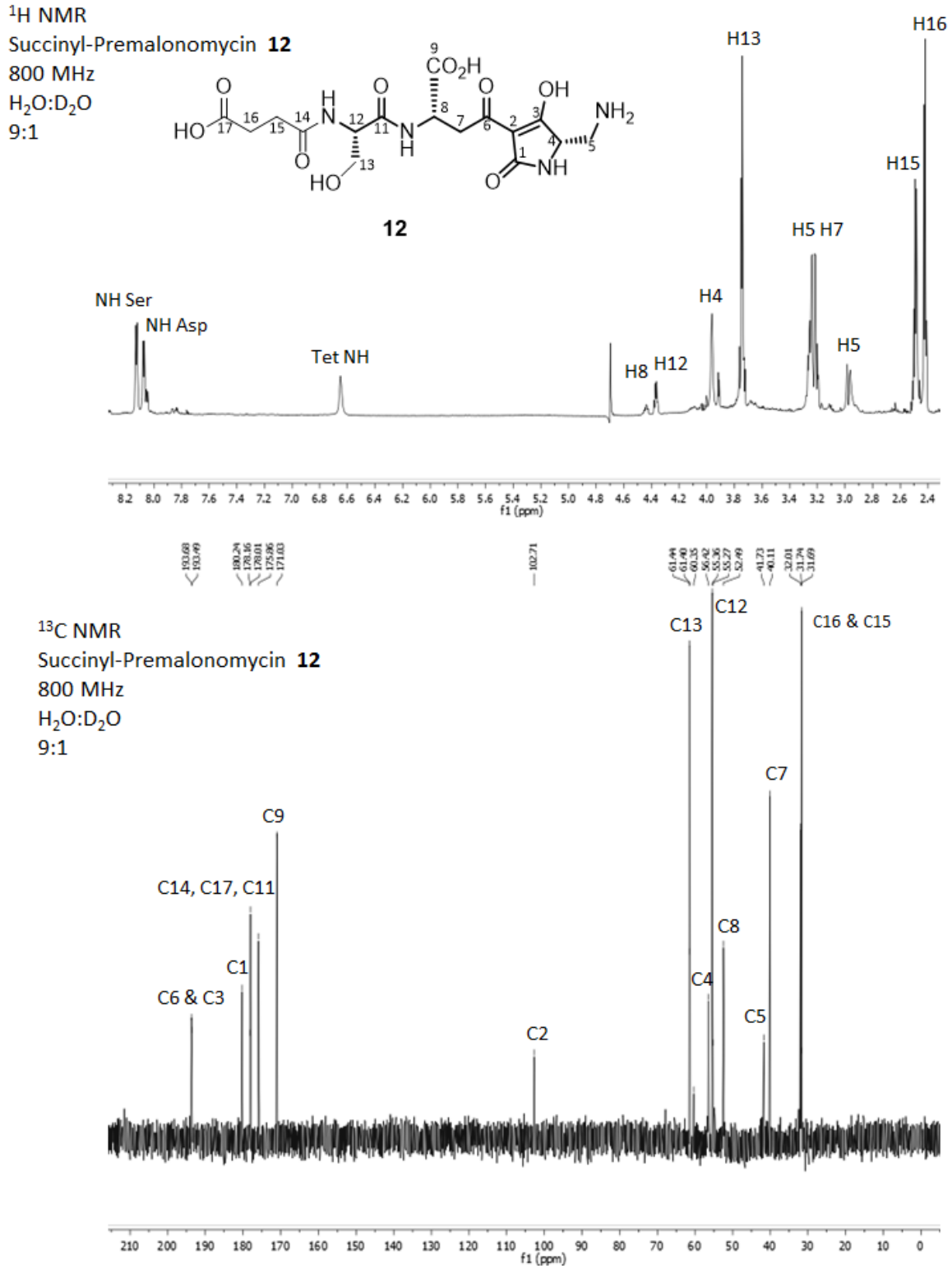


Supplementary Figure 9. Comparison of ^{1}H spectra of 5 and (8*R/S*)-5. Decarboxylated malonomycin (8*R/S*)-**5** is a mixture of diastereomers, showing two sets of signals when compared with the single diastereomer premalonomycin **5**.

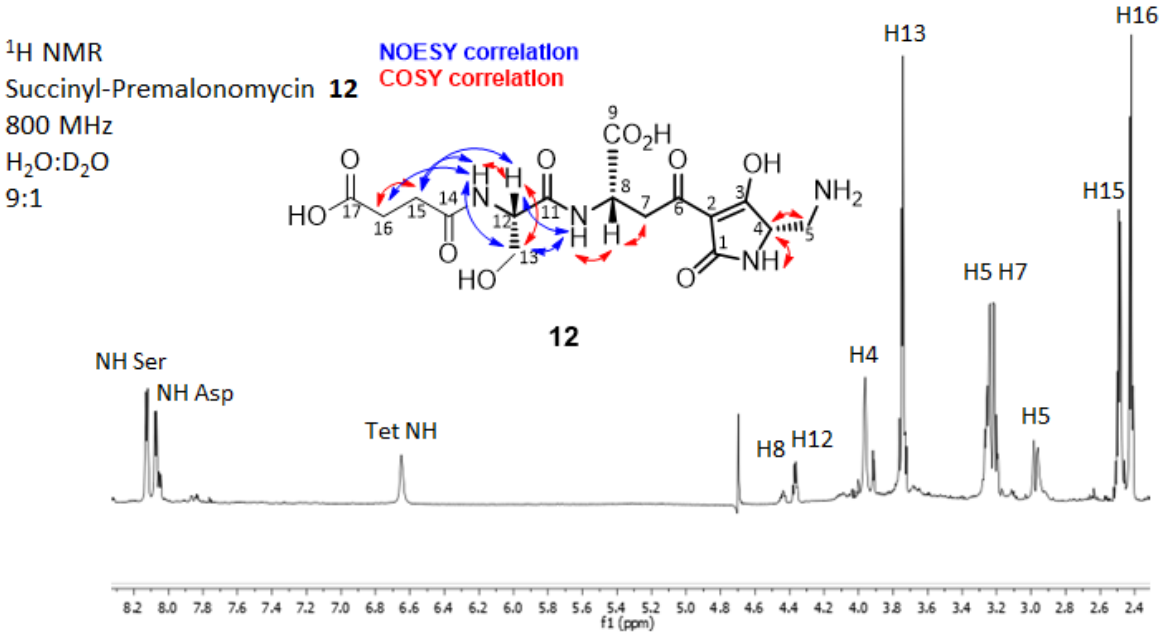


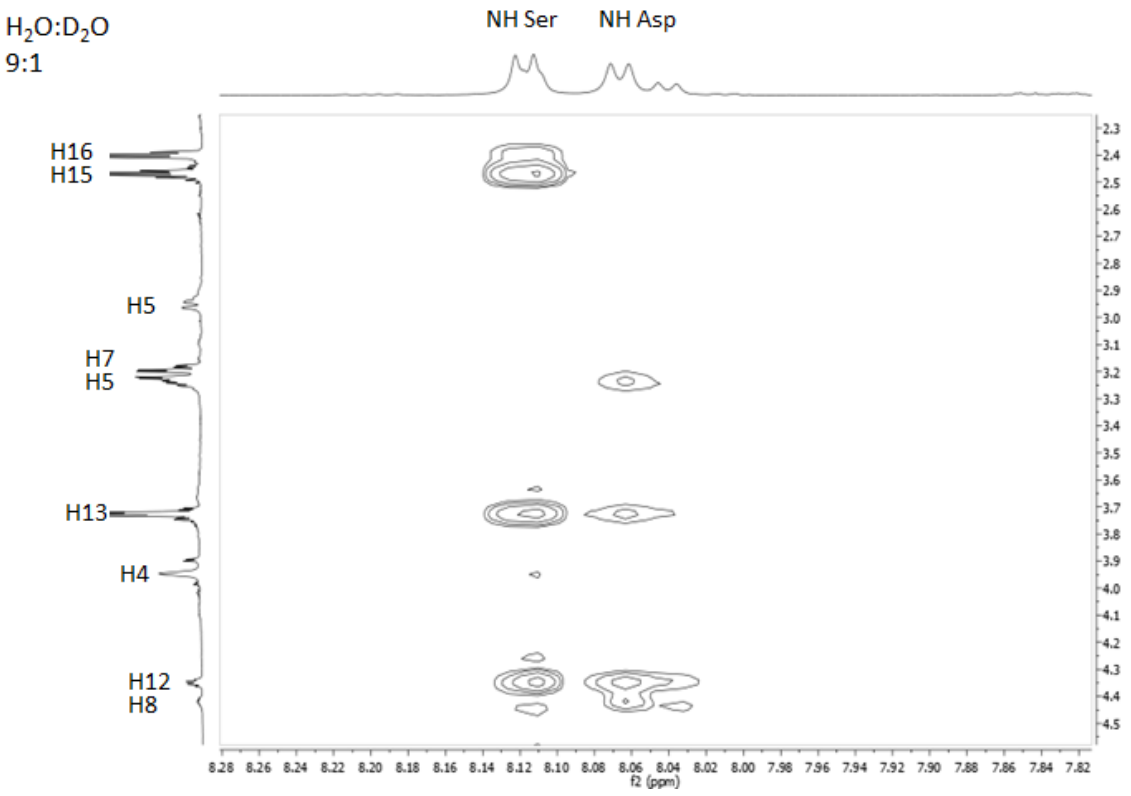
Supplementary Figure 10. Compound 12 isolated from Δ*mloH* is *N*-(serinyl)-succinylated premalonomycin. a, (Top) reaction of premalonomycin **5** with succinic anhydride, yielding two regioisomeric products **12** and 1**3**. (Bottom) co-injection of putative succinylated-**5** isolated from Δ*mloH* strain with *m/z* 431.14, with the increase in peak area of the earlier-eluting regioisomer showing that the natural product is regioisomer **12**. **b**, LC-MS/MS analysis of succinylated premalonomycin. (Top) MS/MS fragmentation of compound **12** isolated from the Δ*mloH* strain**.** (Middle) MS/MS fragmentation of **12** generated from reaction of **5** with succinic anhydride. Direct loss of succinate and NH_2CH from the parent ion indicate a free aminomethyl moiety on the tetramic acid and succinylation on the serinyl amine. Loss of the serine occurs only from fragment ions that have already lost the

succinate group. (Bottom) MS/MS fragmentation of **13** generated from reaction of **5** with succinic anhydride. Direct loss of succinate and serine from the parent ion indicate that both moieties must be terminal residues. Loss of the NH_2CH occurs only from fragment ions that have lost the succinate, indicating the attachment of the succinyl group on the amino group of the tetramic acid. Comparison of the MS/MS spectra of the compound from the Δ*mloH* strain (top) and regioisomer **12** (middle) reveal identical fragmentation patterns, showing that the compound from Δ*mloH* is succinylated on the serinyl amine. This was confirmed by NMR, including NOESY and COSY experiments.

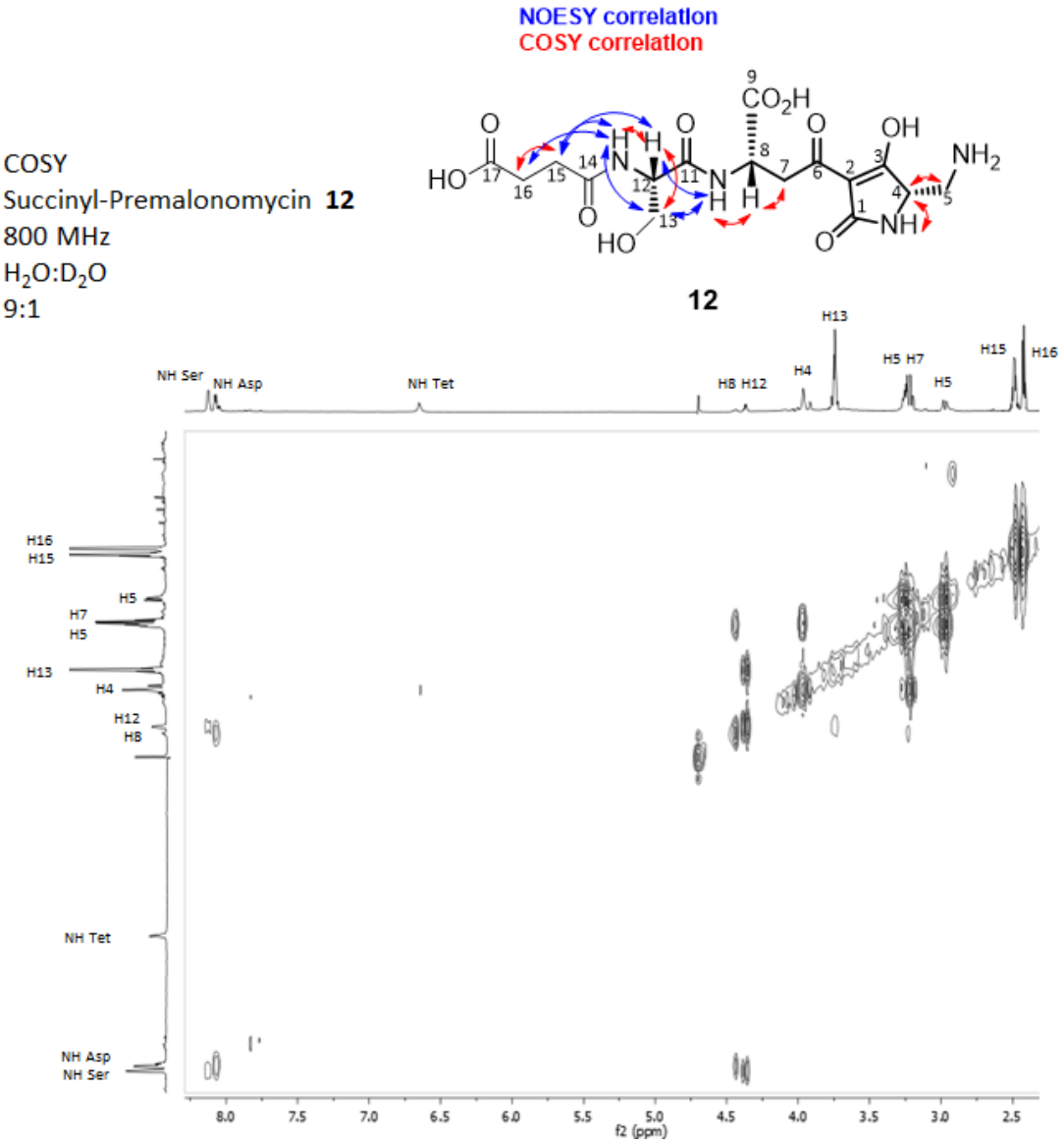


Supplementary Figure 11. ^{1}H and ^{13}C NMR of 12 from Δ*mloH*.

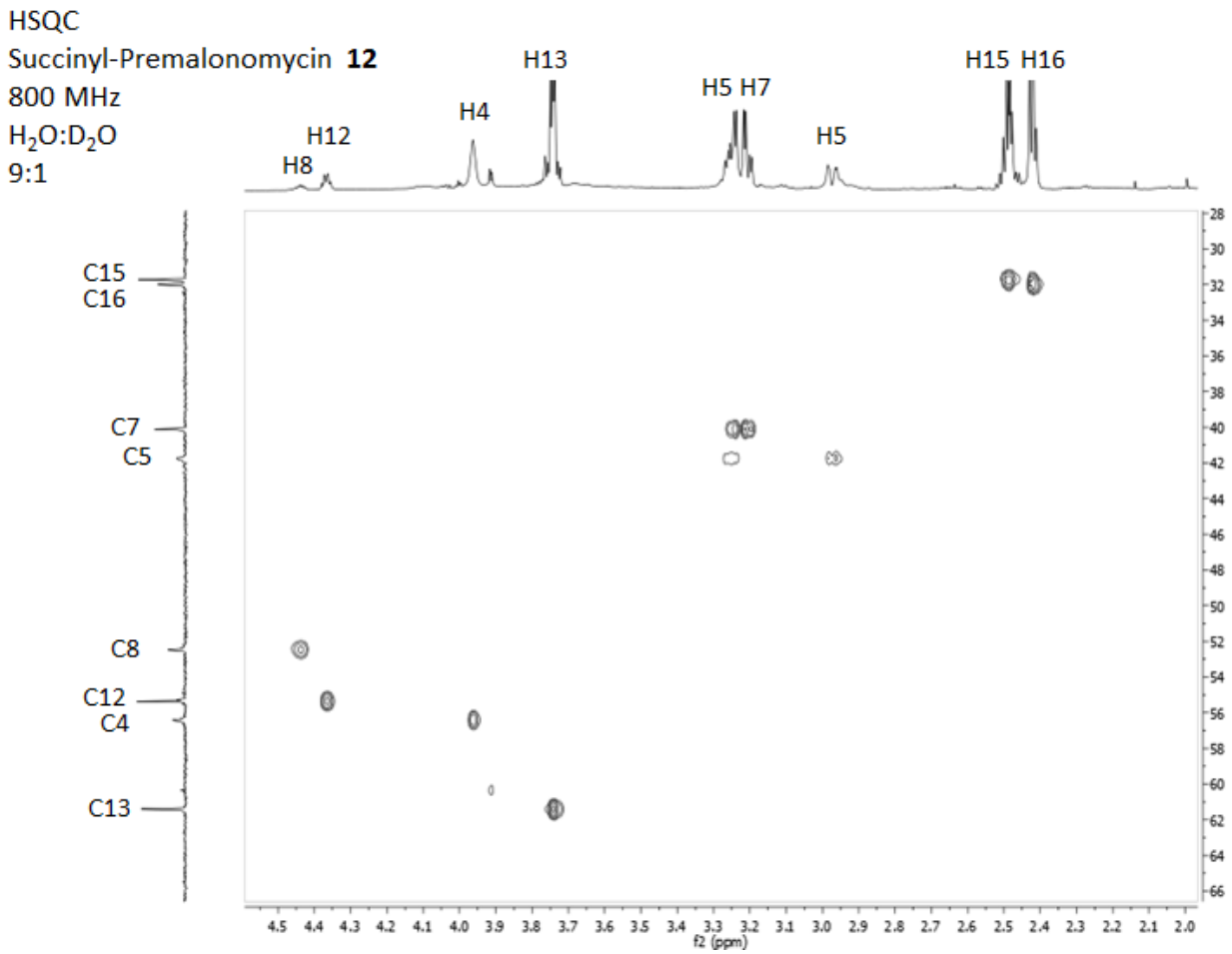




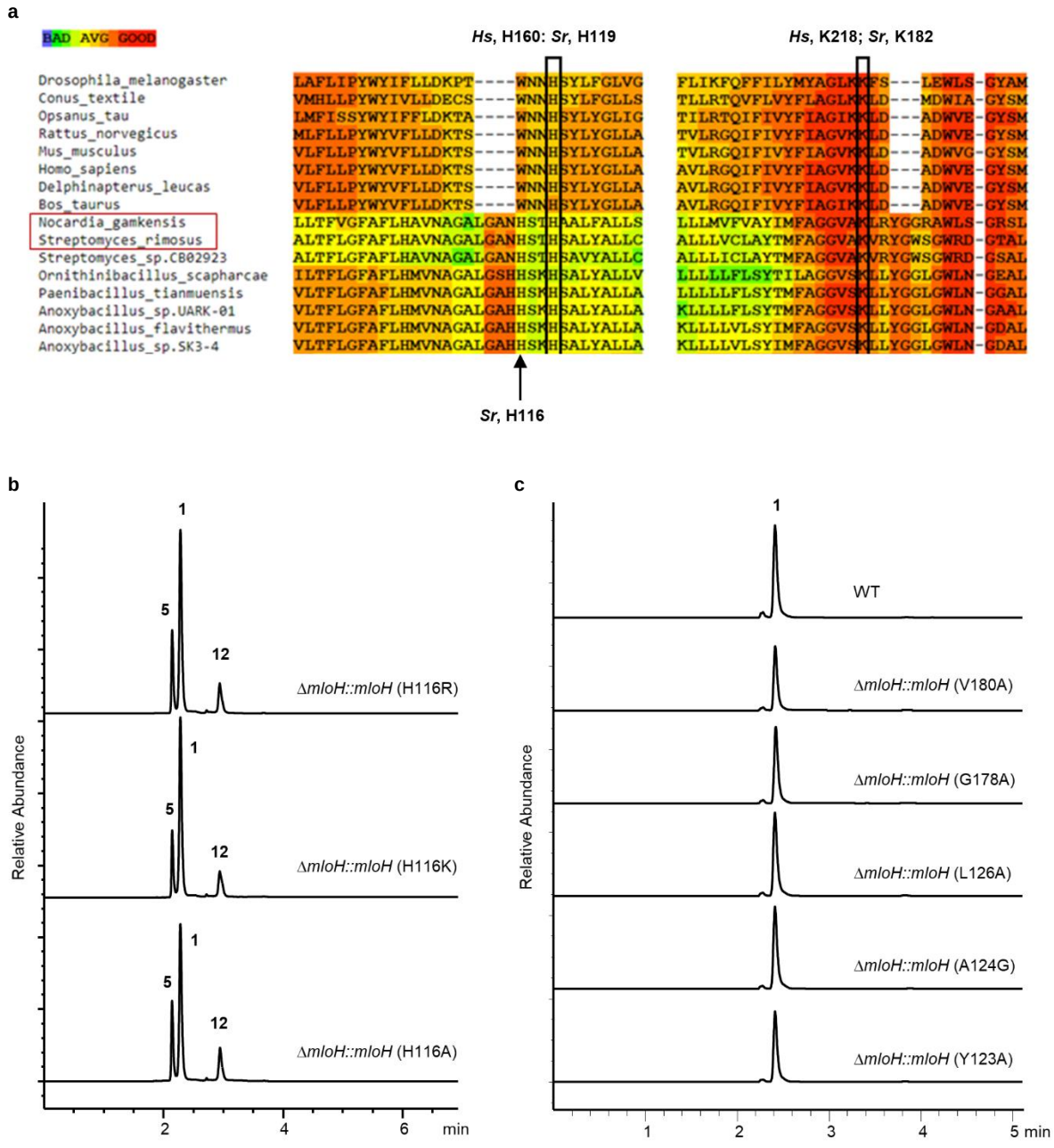
Supplementary Figure 12. NOESY NMR of 12 from Δ*mloH*.



Supplementary Figure 13. COSY NMR of 12 from Δ*mloH*.



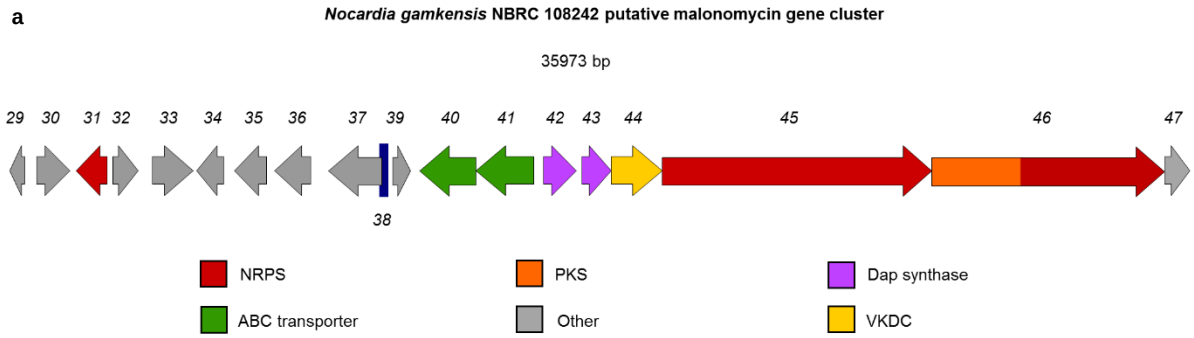
Supplementary Figure 14. HSQC NMR of 12 from Δ*mloH*.



Supplementary Figure 15. Additional point mutations to MloH. a, Protein alignment showing conservation of the *Hs* H160 residue in *S. rimosus* (H119). The proximal H116 residue was also mutated. Whereas complementation of the Δ*mloH* strain with H119A/K/R mutants failed to rescue production of **1**, H116 mutants (panel **b**) were able to restore production of malonomycin to ~25-35% of wild type MloH. Thus, while H119 is essential in *S. rimosus*, and likely plays a key role in stabilising the enolate intermediate (from deprotonation of the Asp residue) allowing it to attack CO_2, H116 is not essential for catalysis. **c**, Amino acid residues located around positions H119 and K182 (Y123, A124, L126, G178 and V180) were also mutated to confirm the effects of point mutations on the VKDC

protein integrity. Only mutation of the proposed catalytic residues H119 and K182 (Table 1) led to abolished VKDC activity, whereas mutation of the other residues did not affect malonomycin production, indicating unperturbed VKDC function and protein folding.

a



b

S. rimosus R2374		*N. gamkensis* NBRC 108242		Identity/Similarity (%)	Proposed function
Gene	**Size (aa)**	**Gene**	**Size (aa)**		
mloA	586	-	-	-	Nonribosomal peptide synthetase
mloB	1022	-	-	-	Nonribosomal peptide synthetase
mloC	267	*orf 31*	310	25/38	Thioesterase
mloD	579	*orf 41*	569	56/71	ABC-type multidrug transport system
mloE	569	*orf 40*	560	56/69	ABC transporter
mloF	342	*orf 42*	328	61/74	L-Dap biosynthesis
mloG	333	*orf 43*	293	66/78	L-Dap biosynthesis
mloH	499	*orf 44*	500	52/64	vitamin k-dependent gamma-carboxylase
mloI	2140	*orf 45*	2694	49/60	Nonribosomal peptide synthetase
mloJ	2368	*orf 46*	2316	50/62	Polyketide synthase/Nonribosomal peptide synthetase
mloK	252	*orf 47*	250	44/55	Acetyltransferase
mloL	495	*	-	-	Phytoene dehydrogenase
mloM	342	*	-	-	LuxR regulator
mloN	312	*	-	-	Oxidoreductase

Supplementary Figure 16. Malonomycin-like gene cluster in *Nocardia gamkensis*. a, Relative organisation of genes in the cluster. Abbreviations: nonribosomal peptide synthetase (NRPS); polyketide synthase (PKS); vitamin K dependent carboxylase (VKDC). **b**, Table showing comparison of genes in the *mlo* cluster from *S. rimosus paramyceticus* R2374 and putative *mlo*-like cluster from *Nocardia gamkensis* NBRC 108242.

Supplementary Tables

Supplementary Table 1. Proposed functions of genes in the malonomycin (*mlo*) biosynthetic gene cluster from *Streptomyces rimosus paramyceticus* R2374.

Gene	Size (aa)	Predicted function	Protein homologue	Accession No.	Identity/ Similarity (%)
mloA	586	NRPS	*Streptomyces scabiei*	WP_079087242.1	72/82
mloB	1026	NRPS	*Streptomyces lavendulae*	WP_030008440.1	49/62
mloC	266	Type II thioesterase	*Micromonospora rifamycinica*	WP_067307207.1	56/70
mloD	580	ABC-type multidrug transport system, ATPase and permease component	*Streptomyces clavuligerus* ATCC 27064	EDY49101.1	57/73
mloE	569	ABC transporter	*Streptomyces clavuligerus*	WP_003954584.1	62/74
mloF	342	Cysteine synthase (L-Dap biosynthesis)	*Streptomyces uncialis*	WP_073792447.1	69/80
mloG	333	Amino acid dehydrogenases and ornithine cyclodeaminase (L-Dap biosynthesis)	*Streptomyces platensis*	WP_085923602.1	72/80
mloH	499	Membrane protein/vitamin K-dependent gamma-carboxylase	*Paenibacillus tianmuensis*	WP_090674101.1	58/71
mloI	2138	NRPS	*Nocardia gamkensis*	WP_062975541.1	49/60
mloJ	2370	PKS-NRPS	*Nocardia gamkensis*	WP_062975543.1	50/62
mloK	252	GNAT family N-acetyltransferase	*Streptomyces albireticuli*	WP_095580451.1	84/89
mloL	489	NAD(P)/FAD-dependent oxidoreductase	*Streptomyces* sp. NRRL F-6131	WP_078881900.1	78/86
mloM	342	Transcriptional regulator, LuxR family	*Streptomyces roseochromogenus*	WP_023549576.1	52/66
mloN	312	Gfo/Idh/MocA family oxidoreductase, hypothetical protein	*Streptomyces roseochromogenus*	WP_023549582.1	46/59

Supplementary Table 2. Comparison of genes in the *mlo* clusters from *S. rimosus paramyceticus* R2374 and *paromomycinus* NRRL 2455.

Gene	*S. rimosus R2374* Size (aa)	*S. rimosus NRRL 2455* Size (aa)	Identity/Similarity (%)	Predicted function
mloA	586	586	98/98	NRPS
mloB	1026	1054	93/95	NRPS
mloC	266	267	94/95	Type II thioesterase
mloD	580	579	97/98	ABC-type multidrug transport system
mloE	569	569	97/97	ABC transporter
mloF	342	342	97/97	L-Dap biosynthesis
mloG	333	333	98/99	L-Dap biosynthesis
mloH	499	499	97/97	Vitamin K-dependent gamma-carboxylase
mloI	2138	2140	95/96	NRPS
mloJ	2370	2368	95/97	PKS/NRPS
mloK	252	252	95/96	Acetyltransferase
mloL	489	495	97/98	Phytoene dehydrogenase
mloM	342	342	97/98	LuxR regulator
mloN	312	312	95/95	Oxidoreductase

Supplementary Table 3. LC-HRMS analysis of malonomycin compounds.

Compound	**1** *m/z* $[M+H]^+$ 375.1147 $[M+Na]^+$ 397.0966	(8*R/S*)-**5**/**5** *m/z* $[M+H]^+$ 331.1248 $[M+Na]^+$ 353.1068 $[M-H+2Na]^+$ 375.0887	**12** *m/z* $[M+H]^+$ 431.1409
1 (wild type)	$[M+H]^+$ 375.1146 (Δ 0.3 ppm) $[M+Na]^+$ 397.0966 (Δ 0.0 ppm)		
(8*R/S*)-**5**		$[M+H]^+$ 331.1247 (Δ 0.3 ppm) $[M+Na]^+$ 353.1065 (Δ 0.3 ppm)	
5 (Δ*mloH)*		$[M+H]^+$ 331.1249 (Δ 0.3 ppm) $[M+Na]^+$ 353.1069 (Δ 0.3 ppm) $[M-H+2Na]^+$ 375.0888 (Δ 0.3 ppm)	
12 (Δ*mloH)*			$[M+H]^+$ 431.1409 (Δ 0.0 ppm)
Δ*mloH::mloH* extract	$[M+H]^+$ 375.1149 (Δ 0.5 ppm) $[M+Na]^+$ 397.0968 (Δ 0.5 ppm	$[M+H]^+$ 331.1252 (Δ 1.2 ppm) $[M+Na]^+$ 353.1071 (Δ 0.8 ppm)	
Δ*mloH::Ng orf44* extract	$[M+H]^+$ 375.1141 (Δ 1.6 ppm) $[M+Na]^+$ 397.0959 (Δ 1.8 ppm)	$[M+H]^+$ 331.1244 (Δ 1.2 ppm) $[M+Na]^+$ 353.1063 (Δ 1.4 ppm)	$[M+H]^+$ 431.1406 (Δ 0.7 ppm)

Purified standards of **1**, **5** and **12** were obtained from wild type R2374 (**1**) or Δ*mloH* (**5**, **12**) strains and characterised by LC-HRMS, MS/MS, ^{1}H and ^{13}C NMR. Compound (8*R/S*)-**5** was generated by thermal decarboxylation of **1**.

Supplementary Table 4. List of primers used in this study

Primer	Sequence 5'-3'	Description
ZY56F	ACGCATCAGGCGGGAGAGCCACGG	*mloH* spacer for pCRISPomyces-2 guide RNA
ZY56R	AAACCCGTGGCTCTCCCGCCTGAT	
ZY57F	TGCCGCCGGGCGTTTTTTATGGCGGTGTTGACGGGCAGAT	amplification of left flanking arm for *mloH* gene knockout
ZY57R	CATGTTCGCCGGCGGAGTGGTGATGCTGGACAGCCCGGTC	
ZY58F	GACCGGGCTGTCCAGCATCACCACTCCGCCGGCGAACATG	amplification of right flanking arm for *mloH* gene knockout
ZY58R	TTACGGTTCCTGGCCTCTAGGCGAGGACTCCGTGCTGGTC	
ZY70F	CCCAGTAGGCGGGCATCATC	PCR check for *mloH* gene deletion
ZY70R	ACGACTTCTCCCTCGACGGC	
ZY59F	GGAGGGAAAGTGCAATGTACCTGATCAAGGCGAATACTTC	introduction of *tsr* cassette into pCRISPomyces-2
ZY59R	CTGGAGGGAAACCAGCGTACTGATCATCACTGACGAATCG	
ZY65F	TGAAAAACGCTCACTGGTACCTGATCAAGGCGAATACTTC	introduce *tsr* cassette into pAV11b
ZY65R	CAAGCTTAGATCTATGCATGTGATCATCACTGACGAATCG	
ZY72F	TGAAGTATTCGCCTTGATCAGTACCAGCCCGACCCGAGCA	amplification of the ermE*p for pAV11b insertion
ZY72R	AGCCACCAGTGCGTGATCATATGTCCGTACCTCCGTTGCT	
ZY73F	AGCAACGGAGGTACGGACATATGATCACGCACTGGTGGCT	amplification of *mloH* gene for complementation
ZY73R	GAAAAACGCTCACTGGTACCTCACCGGACGGGCTCCCCGA	
K182A F	GGAGTGGCCGCCGTCCGCTACGGCTGG	K→A codon change in *mloH*
K182H F	GGAGTGGCCCACGTCCGCTACGGCTGG	K→H codon change in *mloH*
K182R F	GGAGTGGCCCGCGTCCGCTACGGCTGG	K→R codon change in *mloH*
K182 R	GCCGGCGAACATGGTGTACGCGAGG	Reverse primer for K182
H116A F	GGGGCCAACGCGTCCACGCACTC	H→A codon change in *mloH*
H116K F	GGGGCCAACAAATCCACGCACTC	H→K codon change in *mloH*
H116R F	GGGGCCAACCGCTCCACGCACTC	H→R codon change in *mloH*
H116 R1	CAGCGCACCGGCGTTGACCGC	Reverse primer for H116A/K
H116 R2	CAGCGCACCGGCGTTGACC	Reverse primer for H116R
H119A F	CACTCCACGGCGTCGGCGCTCTACGC	H→A codon change in *mloH*
H119K F	CACTCCACGAAATCGGCGCTCTACGC	H→K codon change in *mloH*
H119R F	CACTCCACGCGCTCGGCGCTCTACGC	H→R codon change in *mloH*
H119 R	GTTGGCCCCCAGCGCACCGG	Reverse primer for H119
Y123A F	TCGGCGCTCGCGGCGCTGCTGTGCAT	Y→A codon change in *mloH*
Y123A R	GTGCGTGGAGTGGTTGGCCCCCAGCG	Reverse primer for Y123
A124G F	TCGGCGCTCTACGGCCTGCTGTGCATGAGCTT	A→G codon change in *mloH*
A124G R	GTGCGTGGAGTGGTTGGCCCCCAGCG	Reverse primer for A124
L126A F	TACGCGCTGGCGTGCATGAGCTTTTCGG	L→A codon change in *mloH*
L126A R	GAGCGCCGAGTGCGTGGAGTGGTTGG	Reverse primer for L126
G178A F	ATGTTCGCCGCGGGAGTGGCCAAGGTC	G→A codon change in *mloH*
G178A R	GGTGTACGCGAGGCAGACCAGCAGCAGC	Reverse primer for G178
V180A F	GCCGGCGGAGCGGCCAAGGTCCGCTA	V→A codon change in *mloH*
V180A R	GAACATGGTGTACGCGAGGCAGACCAGCAGCAGC	Reverse primer for V180
ZY74F	ACGCGGCCGTAGCAGTTGTAGAGG	*mloA* spacer for pCRISPomyces-2 guide RNA
ZY74R	AAACCCTCTACAACTGCTACGGCC	
ZY75F	TGCCGCCGGGGCGTTTTTTATAGCGCCACACCGTCACCGCC	amplification of left flanking arm for *mloA* gene knockout
ZY75R	CGTCGTGCACGAGGACCAGGCCGCGGAGATCACCACCGGG	
ZY76F	CCCGGTGGTGATCTCCGCGGCCTGGTCCTCGTGCACGACG	amplification of right flanking arm for *mloA* gene knockout
ZY76R	TTACGGTTCCTGGCCTCTAGCGTGACGGCCATGACCGATT	
ZY77F	AACAGCTCGCCGATCTCGC	PCR check for *mloA* gene deletion
ZY77R	CGAGATGTGGAGCACCCTGG	
ZY211F	ACGCGCTCACGATTGATGCGGTGG	*mloA* S546A spacer for pCRISPomyces-2 guide RNA
ZY211R	AAACCCACCGCATCAATCGTGAGC	
ZY212F	TGCCGCCGGGGCGTTTTTTATCGGCTTCGAGGTCCTGTCCA	amplification of left flanking arm for *mloA* S546A point mutation
ZY212R	CATGCGGAACGCCAGCAGCGCATGCCCGCCCAGGCCGAAGAAG	
ZY213F	CTTCTTCGGCCTGGGCGGGCATGCGCTGCTGGCGTTCCGCATG	amplification of right flanking arm for *mloA S546A* point mutation
ZY213R	TTACGGTTCCTGGCCTCTAGTCTGCCCGAGCAGGTCGC	
ZY214F	GAGGTCCTGGAAGCGGTGGT	PCR check for *mloA* S546A point mutation
ZY214R	GAGCCCGAGGTGTAGAAGATGC	
ermE-mloA F	TGAAGTATTCGCCTTGATCAGTACCAGCCCGACCCGAGCA	amplification of ermE*p for pAVIIb insertion
ermE-mloA R	ACGCCTGGGGTGCTTGTCATATGTCCGTACCTCCGTTGCT	
mloA HiFi F	AGCAACGGAGGTACGGACATATGACAAGCACCCCAGGCGT	amplification of *mloA* for pAVIIb insertion
mloA HiFi R	GAAAAACGCTCACTGGTACCCTAACGCGCGGCGTCGACAG	
mloA S546A F	GGTCACGCGCTGCTGGCGTTCCGC	S→A codon change in *mloA*
mloA S546 R	GCCCAGGCCGAAGAAGTCCTCGTC	Reverse primer for S546
ZY124F	ACGCCGGAGCGGATGACGTGATGG	*mloB* spacer for pCRISPomyces-2 guide RNA
ZY124R	AAACCCATCACGTCATCCGCTCCG	

ZY125F	TGCCGCCGGGCGTTTTTTATTCCACGATCAGCTCGACGGC	amplification of left flanking arm for *mloB* gene knockout
ZY125R	GGAACGGCTGGACCTCTTCGCGGACGTGGCCCAGGACAG	
ZY126F	CTGTCCTGGGCCACGTCCGCGAAGAGGTCCAGCCGTTCC	amplification of right flanking arm for *mloB* gene knockout
ZY126R	TTACGGTTCCTGGCCTCTAGAGCAGATGGAGCGGTACGGC	
ZY127F	AAGAAGCGGCGCTCGGTCT	PCR check for *mloB* gene deletion
ZY127R	ACGCCGAGCAGCTGGAACA	

ZY198F	ACGCACGATCAGCAGGCTGTACGG	*mloD* spacer for pCRISPomyces-2 guide RNA
ZY198R	AAACCCGTACAGCCTGCTGATCGT	
ZY199F	TGCCGCCGGGCGTTTTTTATGGTTCAGGCCCTCGCACTTC	amplification of left flanking arm for *mloD* gene knockout
ZY199R	CTGGGAGGTGCTGGAGCAGCTGATCGTGGACGAGGCGG	
ZY200F	CCGCCTCGTCCACGATCAGCTGCTCCAGCACCTCCCAG	amplification of right flanking arm for *mloD* gene knockout
ZY200R	TTACGGTTCCTGGCCTCTAGTCGAACGGCACCACGAACC	
ZY201F	CCGGCCTCGGTGATCTCCTT	PCR check for *mloD* gene deletion
ZY201R	CGGTCGTCAACCTGCTCTGC	

ZY207F	ACGCGCGCGGCGACCGCCAAGAGG	*mloI* H146A spacer for pCRISPomyces-2 guide RNA
ZY207R	AAACCCTCTTGGCGGTCGCCGCGC	
ZY208F	TGCCGCCGGGCGTTTTTTATGGCCTCCTGGTCATCTGTCTCG	amplification of left flanking arm for *mloI* H146A point mutation
ZY208R	CAGCCGTCCACCGCGATCGCGTGCGCGGCGACCGCCAAGA	
ZY209F	TCTTGGCGGTCGCCGCGCACGCGATCGCGGTGGACGGCTG	amplification of right flanking arm for *mloI* H146A point mutation
ZY209R	TTACGGTTCCTGGCCTCTAGGATGACGTAGAGGCGGTTGCC	
ZY210F	CGAAGAGTGAGGAAGCCGTGAG	PCR check for *mloI* H146A point mutation
ZY210R	GTGCCTTGAGTGCCTGGAGC	

ZY99F	ACGCCCGGAAGAACGGGGAGGCGG	*mloL* spacer for pCRISPomyces-2 guide RNA
ZY99R	AAACCCGCCTCCCCGTTCTTCCGG	
ZY100F	TGCCGCCGGGCGTTTTTTATTGCCTGGGGATGCTGAGTTC	amplification of left flanking arm for *mloL* gene knockout
ZY100R	GGTCACCATGGCACGGGCCTTCCGGGAGTTCGACCTGC	
ZY101F	GCAGGTCGAACTCCCGGAAGGCCCGTGCCATGGTGACC	amplification of left flanking arm for *mloL* gene knockout
ZY101R	TTACGGTTCCTGGCCTCTAGCGTCCTGCTGACGTCGGTGC	
ZY102F	CGGCGTCCAGGTCGTGATG	PCR check for *mloL* gene deletion
ZY102R	GACCTCCGAACCCACCTTGC	

ZY95F	ACGCAGCCGGCTGAGCGTCCGCGG	*mloM* spacer for pCRISPomyces-2 guide RNA
ZY95R	AAACCCGCGGACGCTCAGCCGGCT	
ZY96F	TGCCGCCGGGCGTTTTTTATGGAGGCGTTGAGCATCCACA	amplification of left flanking arm for *mloM* gene knockout
ZY96R	CTTCGTCCCAGCGGCTGAGGATCTCCACCCTGCTCGACC	
ZY97F	GGTCGAGCAGGGTGGAGATCCTCAGCCGCTGGGACGAAG	amplification of right flanking arm for *mloM* gene knockout
ZY97R	TTACGGTTCCTGGCCTCTAGGGACTCGCAGGTGAGGGAGA	
ZY98F	GGCATGGCCGGCTACCTG	PCR check for *mloM* gene deletion
ZY98R	GAGAGCAGCACGCAGGGTTTC	
ZY108F	AGCAACGGAGGTACGGACATATGGTGAGGGGAAACCCGAC	amplification of *mloM* gene for complementation
ZY108R	GAAAAACGCTCACTGGTACCTCAGGCCGCTCCTTCTTGTG	

ZY91F	ACGCGCGGGAGACAGTACCGGTGG	*mloN* spacer for pCRISPomyces-2 guide RNA
ZY91R	AAACCCACCGGTACTGTCTCCCGC	
ZY92F	TGCCGCCGGGCGTTTTTTATCCAAGTCGGGCCACAGTCG	amplification of left flanking arm for *mloN* gene knockout
ZY92R	CAAGGCCGTGTTGCTGGAGGCGGACGTGTGGCACGGAC	
ZY93F	GTCCGTGCCACACGTCCGCCTCCAGCAACACGGCCTTG	amplification of right flanking arm for *mloN* gene knockout
ZY93R	TTACGGTTCCTGGCCTCTAGCTGCGCGGGGTGTATCTGTG	
ZY94F	CGGGCCGAGCAACTGACAG	PCR check for *mloN* gene deletion
ZY94R	CCGCCGCCGAATACCTCTC	

mloA-AT F	AAAAAACATATGACAAGCACCCCAGGCGTCGG	*Nde*I & *Xho*I sites for *mloA* ligation into pET21b
mloA-AT R	AAAAAACTCGAGACGCGCGGCGTCGACAGCC	

mloI-A_2T_2 F	AAAAAACATATGTACTCGCTGGTCCTGCCCTCGCC	*Nde*I & *Xho*I sites for *mloI* ligation into pET21b
mloI-A_2T_2 R	AAAAAACTCGAGGAAGTCATGCTCGGAAACCTCGTCTTCC	

SrfAB-A_2 F	AACTTTAAGAAGGAGATATAATGAACGTTCGGCTGTCTGATGTAG	HiFi assembly of SrfAB A_2 into pET21b
SrfAB-A_2 R	CAGTGGTGGTGGTGGTGGTGGGCCAAGGCCTTGCCTCC	

Supplementary Table 5. List of plasmids used in this study

Plasmid	Description	Source
pCRISPomyces-2	*E.coli-Streptomyces* shuttle vector for gene inactivation in Streptomyces species	(*3*)
pAV11b	*E.coli-Streptomyces* shuttle vector for complementation in *Streptomyces*, phiBT1 integrase	
pCm2-tsr-mloH	pCRISPomyces-2 derivative (+*tsr*): for *mloH* in-frame deletion	
pAV11b-tsr-mloH	pAV11b derivative containing ermE*p, *mloH* and *tsr* for complementation	
pCm2-tsr-mloM	pCRISPomyces-2 derivative (+*tsr*): for *mloM* in-frame deletion	
pAV11b-tsr-mloM	pAV11b derivative containing ermE*p, *mloM* and *tsr* for complementation	
pAV11b-tsr-Ng_orf44	pAV11b derivative containing ermE*p, *Ng orf44* and *tsr* for complementation	
pCm2-tsr-mloA	pCRISPomyces-2 derivative (+*tsr*): for *mloA* in-frame deletion	
pAV11b-tsr-mloA	pAV11b derivative containing ermE*p, *mloA* and *tsr* for complementation	
pAV11b-tsr-mloA(S546A)	pAV11b derivative containing ermE*p, *mloA*(S546A) and *tsr* for complementation	
pCm2-tsr-mloA(S546A)	pCRISPomyces-2 derivative (+*tsr*): for chromosomal point mutation of *mloA*	
pCm2-tsr-mloB	pCRISPomyces-2 derivative (+*tsr*): for *mloB* in-frame deletion	
pCm2-tsr-mloD	pCRISPomyces-2 derivative (+*tsr*): for *mloD* in-frame deletion	
pCm2-tsr-mloI(H146A)	pCRISPomyces-2 derivative (+*tsr*): for chromosomal point mutation of *mloI*	
pCm2-tsr-mloL	pCRISPomyces-2 derivative (+*tsr*): for *mloL* in-frame deletion	
pCm2-tsr-mloN	pCRISPomyces-2 derivative (+*tsr*): for *mloN* in-frame deletion	
pET21b-mloA	Production of MloA A-T in *E. coli*	
pET21b-mloI-A_2T_2	Production of MloI A_2-T_2 in *E. coli*	
pET21b-srfAB-A_2	Production of SrfAB A_2 in *E. coli*	

Supplementary References

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