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

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Vitamin K-dependent carboxylase (VKDC) enzymes modify glutamate residues in mammalian vitamin K-dependent proteins, generating γ -carboxyglutamic acids with malonate moieties that mediate important physiological responses such as blood coagulation. Proteins with sequence similarity to mammalian VKDC are also found in bacteria; however, their function remains unknown. The antibiotic malonomycin from *Streptomyces rimosus* contains an unusual malonate group, of unknown origin, that is essential for its biological activity. Here, we show that a bacterial VKDC orthologue (MloH) is responsible for the malonic acid moiety in malonomycin. Using clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 gene editing, complementation and mutagenesis experiments, this VKDC-like enzyme was shown to α -carboxylate an aspartyl residue within a hybrid polyketide–non-ribosomal peptide intermediate during malonomycin biosynthesis. This study reveals a highly unusual biosynthetic pathway to malonic acid-containing metabolites, providing a functional role for VKDC-like proteins in prokaryotes and a vitamin K-dependent carboxylation reaction with a non-proteinogenic substrate.

Malonomycin (antibiotic K16) **1** (Fig. 1a) is a secondary metabolite with antiprotozoal and antifungal activity, isolated from *Streptomyces rimosus paromomycinus*¹. Malonomycin is the only known bacterial natural product with an intact aminomalonic acid moiety. While the structure of malonomycin was established some time ago, with L-Ser and L-diaminopropionate (L-Dap) as probable precursors^{1–3}, the biosynthetic pathway, including the origin of the unique aminomalonate unit, has remained unexplained. Malonates are key precursors in the biosynthesis of fatty acids and polyketides (Fig. 1b), and are produced by either biotin-dependent acetyl-CoA carboxylases, crotonyl-CoA carboxylase/reductase enzymes or oxidation of glyceryl/serinyl-acyl carrier protein precursors to hydroxy/aminomalonyl-acyl carrier protein thioesters in polyketide synthase (PKS) assembly lines⁴. In these examples, decarboxylation of the malonyl thioester intermediates provides the thermodynamic driving force for subsequent carbon–carbon bond formation. It is difficult to envisage how this common biosynthetic logic could account for the aminomalonate moiety of malonomycin.

Another metabolite possessing an intact malonic acid moiety is γ -carboxyglutamic acid, derived from carboxylation of Glu residues in vitamin K-dependent (VKD) proteins^{5–8} (Fig. 1c). The mammalian VKD carboxylase (VKDC), catalysing this transformation, is suggested to deprotonate and oxidize the vitamin K hydroquinone (KH₂) co-factor, driving the formation of a strong base (K[–])^{9–11} that deprotonates the non-acidic γ -methylene group of Glu, which then attacks CO₂ to give γ -carboxyglutamic acid (Fig. 1c). The resulting vitamin K epoxide (KO) is recycled back to KH₂ by the KO reductase enzyme—the target of clinically important anticoagulant drugs including warfarin^{12,13}. Proteins with sequence similarity to the mammalian VKDC are found within other domains of life, including bacteria. For example, a *Leptospira* VKDC orthologue was shown to convert KH₂ to KO, but no carboxylation activity was observed¹⁴. The function of this and other putative bacterial VKDC remains unknown.

Here, we describe the discovery of a highly unusual biosynthetic pathway to the antibiotic malonomycin. We identified the malonomycin (*mlo*) biosynthetic gene cluster (BGC) in two *S. rimosus* strains. Using a combination of bioinformatics, gene editing, complementation and in vitro experiments, we show that a hybrid non-ribosomal peptide synthetase (NRPS)–PKS assembly line generates a precursor premalonomycin, possessing an aspartyl residue that is carboxylated by a bacterial VKDC orthologue (MloH), generating the malonic acid moiety of malonomycin. VKDC-like enzymes are shown to be present in other bacterial species, opening the way for the discovery of new carboxylase enzymes and metabolites containing malonic acid.

Results

Identification and analysis of the *mlo* BGC. First, we sequenced the genomes of two *S. rimosus* strains, *paromomycinus* NRRL 2455 and *paramyceticus* R2374, and located two almost identical BGCs containing the genes *mloF* and *mloG*, encoding proteins with similarity to SbnA and SbnB known to transform L-Ser into L-Dap¹⁵—a precursor of the malonomycin **1** tetramic acid group³. The *mlo* BGC (Fig. 2a and Supplementary Tables 1 and 2) possesses a gene *mloI*, encoding a dual-module NRPS. The first adenylation (A₁) domain of MloI is predicted to activate L-Ser, but bioinformatics tools fail to predict the specificity of the second adenylation domain (A₂). We propose that MloI A₂ activates L-Asp via the side-chain carboxyl group to generate the Ser–Asp dipeptide **2** (Fig. 2b). A hybrid PKS–NRPS MloJ is then proposed to introduce malonyl-CoA to give the β -keto thioester **3**. The MloJ adenylation domain is predicted to activate L-Dap, which on condensation with **3** would generate β -ketoacyl-Dap thioester **4**. Dieckmann cyclization and chain release would afford the tetramic acid-containing precursor **5** (premalonomycin). Finally, α -carboxylation of the aspartyl moiety of **5** would generate **1**. Analysis revealed *mloH*, encoding a putative VKDC-like enzyme, as a candidate for this challenging carboxylation (Supplementary Table 1). MloH exhibits low but significant similarity (23% identity and 38% similarity) to the human

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Fig. 1 | Biosynthesis of malonic acid derivatives. **a**, Malonomycin **1** contains an intact aminomalonate moiety. **b**, Biosynthesis of malonates in primary and secondary metabolism ($R = H$ or alkyl). **c**, Proposed mechanism of the mammalian VKDC. Phyloquinones (vitamin K1) are utilized in mammalian VKDC as cofactors, while menaquinones (vitamin K2) with variable isoprenoid side-chain lengths are produced by bacteria, including *Streptomyces*, and may serve a similar role in bacterial VKDC-like enzymes. ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; CCR, crotonyl-CoA carboxylase/reductase enzymes; Gla, γ -carboxyglutamic acid; K, vitamin K quinone form; K^- , vitamin K oxygenated intermediate; VKOR, KO reductase.

VKDC. Key catalytic residues within the human VKDC, including K218 and H160, which are proposed to be involved in vitamin K co-factor deprotonation and substrate glutamyl carboxylation, respectively^{16,17}, are also conserved in MloH (Supplementary Fig. 1). Whereas the human VKDC utilises vitamin K phyloquinone (vitamin K1), bacteria including *Streptomyces* possess vitamin K menaquinones (vitamin K2), which are mainly used in respiration and ATP generation (Fig. 1c)^{18–20}. Similar to the human enzyme, MloH is predicted to possess multiple transmembrane domains centred within a conserved horizontally transferred transmembrane (HTTM) domain characteristic of VKDC (Supplementary Fig. 1). This HTTM domain is horizontally transferred from metazoan VKDCs to bacteria and, to date, the only known function for proteins possessing this HTTM domain is as VKDC enzymes²¹.

This suggests that MloH is an integral membrane protein within the *S. rimosus* cell membrane, and thus carboxylation probably occurs as a final biosynthetic step before malonomycin is exported.

Characterization of the *mlo* BGC. Both *S. rimosus* strains were cultivated and analysed for production of malonomycin **1**. R2374 gave a much higher yield of **1** (67 mg l⁻¹) than NRRL 2455 (2 mg l⁻¹), as confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography–high resolution mass spectrometry (LC-HRMS) and NMR (Supplementary Fig. 2 and Supplementary Table 3), and was chosen for further studies. To investigate the biosynthesis of **1**, *mloABDLMN* genes were inactivated individually by clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) gene editing²². Although MloI appears to possess all of the domains required for premalonomycin **5** assembly, *mloAB* encodes two additional NRPS modules, the deletion of which significantly impaired production of **1** (Supplementary Fig. 3a). This, coupled with the presence of a condensation domain, rather than the usual adenylation domain, at the amino (N) terminus of MloI, suggests that MloAB may be a functional component of the assembly line (Supplementary Fig. 3b,c), initiating peptide assembly and installing amino acids at the Ser residue of malonomycin precursors that are subsequently cleaved by a protease, as seen for some other NRPS products^{23–25}. To investigate this, the N-terminal MloI condensation domain was deactivated using CRISPR–Cas9 to mutate the catalytic His residue (H146A), which is known to be essential for the activity of condensation domains^{26,27}. The resultant strain produced only a trace amount of **1**, similar to the $\Delta mloA$ strain (Supplementary Fig. 4). An *mloA* S546A mutation was similarly introduced onto the chromosome, blocking phosphopantetheinylation of the MloA peptidyl carrier protein domain, which also resulted in only a trace amount of **1**. In addition, complementation of the $\Delta mloA$ strain with the *mloA*(S546A) mutant gene failed to restore malonomycin production, whereas complementation of $\Delta mloA$ with *mloA* restored production to wild-type levels (Supplementary Fig. 4). Given that phosphopantetheinylation of MloA and a catalytically active MloI C₁-domain are both required for efficient production of malonomycin, it is likely that MloA fulfils a catalytic function, transferring an aminoacyl or peptidyl precursor to MloI via its N-terminal condensation domain, generating a propeptide that is subsequently processed. The observations that the MloA and MloI C₁ domain mutants can still produce trace quantities of **1** suggest that MloI can initiate peptide assembly with L-Ser, albeit very inefficiently.

As malonomycin is exported out of the cell in the wild-type strain, the putative ABC transporter *mloD* was targeted for inactivation. In the absence of the transporter, it was envisaged that precursors, including propeptides, might accumulate within the cell, allowing their detection. While the resultant $\Delta mloD$ strain showed no evidence of malonomycin or premalonomycin in the fermentation media, LC-HRMS analysis of cellular extracts from $\Delta mloD$, and also wild-type strains, has so far failed to identify the presence of malonomycin propeptides. However, the possibility that propeptide precursors exist cannot be ruled out.

Of the remaining genes in the *mlo* cluster, inactivation of either *mloL* or *mloN* had no effect on malonomycin production; however, deletion of *mloM*—a putative LuxR-type transcriptional regulator—abolished production of **1**. Complementation of $\Delta mloM$ with constitutively expressed *mloM* resulted in 5.5-fold enhanced production of **1** (370 mg l⁻¹) over the wild-type R2374. Expression of the R2374 *mloM* in NRRL 2445 also increased titres of **1** 140-fold (267 mg l⁻¹) over the parent strain (Table 1), confirming MloM is a positive regulator of the *mlo* BGC.

MloI activates L-Asp via the side-chain carboxyl group. To further elucidate the biosynthesis of malonomycin **1**, we explored the

Fig. 2 | Malonomycin (*mlo*) gene cluster and biosynthetic pathway. **a, Organization of genes in the malonomycin (*mlo*) cluster. **b**, Proposed pathway for malonomycin **1** biosynthesis. The dipeptide **2** from MloI is condensed with a malonate unit introduced by a *trans*-AT enzyme encoded by either *mloK*, or a gene elsewhere in the genome, to generate **3**. Condensation with L-Dap gives **4** and subsequent Dieckmann cyclization catalysed by MloJ yields premalonomycin **5**. Finally, premalonomycin is carboxylated by MloH, a VKDC-like enzyme that likely resides in the cell membrane due to its predicted multiple transmembrane domains, to give malonomycin **1**. A, adenylation domain; ACP, acyl carrier protein; AT, acyltransferase domain; C, condensation domain; Dap, L-2,3-diaminopropionic acid; KS, ketosynthase domain; T, thioesterase domain.**

specificity and regioselectivity of the MloI A₂ domain, to establish whether L-Asp activation occurs via the side-chain carboxyl (Fig. 3a). MloI A₂T₂ didomain was overproduced in *Escherichia*

coli and assayed for adenylation activity²⁸. Activity was observed with L-Asp and at a lower level with L-Asn, while the 18 other amino acids including D-Asp showed little or no activity (Fig. 3b).

Table 1 | Titres of compounds from *S. rimosus* strains generated in this study

Strain	Calculated compound titres (mg l ⁻¹)			Production of 1 as % of wild type
	1	5	12	
Wild-type R2374	67.2 ± 4.2	3.1 ± 2.0	1.0 ± 0.4	100.0
Δ <i>mloH</i>	0	6.9 ± 2.7	8.7 ± 3.7	0
Δ <i>mloH</i> :: <i>mloH</i>	59.8 ± 10.9	3.6 ± 0.7	1.2 ± 0.2	89.0
Δ <i>mloH</i> :: <i>Ng orf44</i>	35.9 ± 9.6	5.8 ± 2.4	3.9 ± 1.5	53.4
Δ <i>mloH</i> :: <i>mloH</i> (K182A)	3.1 ± 0.5	3.0 ± 0.7	4.4 ± 0.9	4.5
Δ <i>mloH</i> :: <i>mloH</i> (K182H)	1.3 ± 0.6	5.0 ± 2.9	6.1 ± 2.9	1.9
Δ <i>mloH</i> :: <i>mloH</i> (K182R)	0	3.6 ± 1.5	3.9 ± 1.9	0
Δ <i>mloH</i> :: <i>mloH</i> (H119A)	0	2.6 ± 0.1	3.3 ± 0.4	0
Δ <i>mloH</i> :: <i>mloH</i> (H119K)	0	4.5 ± 2.2	6.1 ± 2.6	0
Δ <i>mloH</i> :: <i>mloH</i> (H119R)	0.2 ± 0.1	3.2 ± 0.9	3.4 ± 0.6	0.4
Δ <i>mloM</i>	0	0	0	0
Δ <i>mloM</i> :: <i>mloM</i>	369.3 ± 88.1	0	0	549.2
Wild-type NRRL 2455	1.9 ± 0.3	0	0	100.0
NRRL 2455:: <i>mloM</i>	266.7 ± 10.7	0	0	13818.7

Quantification of malonomycin **1**, premalonomycin **5** and *N*-succinylated-premalonomycin **12** was based on 50 ml cultures in at least triplicate. Culture supernatants were partially purified to generate crude extracts that were analysed by RP-HPLC. Peak areas were calculated and compared with a standard curve to calculate titres. Values represent means, and errors denote s.d. values, with *n* ≥ 3.

Fig. 3 | Substrate selectivity of MloI A₂T₂ didomain and L-Asp substrate side-chain activation. **a**, Reaction scheme for monitoring the substrate selectivity and regiospecificity of MloI. Hydrolysis of L-Asp O-methyl esters **6** and **7** with Na¹⁸OH generates ¹⁸O-labelled L-Asp isotopomers **8** and **9** with around 90% ¹⁸O enrichment. Cognate substrates forming an aminoacyl-adenylate intermediate **10** lead to PPi release, which is monitored in real time using a 7-methyl-6-thio-guanosine-based spectrophotometric assay²⁸. Transfer of an ¹⁸O label onto AMP **11** or retention of the ¹⁸O label in the Asp-SNAC thioester **12** is dependent on the isotopomer used and the regioselectivity of the MloI A₂T₂ enzyme. **b**, Adenylation domain activity assays of MloI A₂T₂ show significant activation of L-Asp. Values represent means, and error bars denote s.d., with *n* = 3. **c**, LC-HRMS analysis of AMP generated from assays of MloI with ¹⁸O L-Asp isotopomers reveals transfer of the ¹⁸O label to AMP with L-[4-¹⁸O]-Asp **9** (increased *m/z* 350), but not with L-[1-¹⁸O]-Asp **8**. Values represent means, and error bars denote s.d., with *n* = 3. **d**, LC-HRMS of Asp-SNAC thioesters generated from L-[1-¹⁸O]-Asp **8** and L-[4-¹⁸O]-Asp **9**. Taking into account that both isotopomers **8** and **9** were around 90% ¹⁸O enriched at the outset, the SNAC thioester derived from **8** retained >99% of the ¹⁸O label, whereas thiolation of **9** resulted in around 50% loss of ¹⁸O. Given that only one of the two oxygen atoms of the carboxyl group is labelled and only half of the ¹⁸O can be lost on thiolation, these assays show that MloI A₂ is highly selective (around 99% selective) for the C4 carboxyl group. LC-HRMS data: Asp-SNAC [M + H]⁺ *m/z*: calculated 235.0747, observed 235.0742. ¹⁸O-Asp-SNAC [M + H]⁺ *m/z*: calculated 237.0789, observed 237.0783.

¹⁸O-labelled L-[1-¹⁸O]-Asp **8** and L-[4-¹⁸O]-Asp **9** were then synthesized and incubated with MloI A₂T₂ and ATP. Transfer of the ¹⁸O label from the C4 (side-chain) carboxyl of **9** to adenosine monophosphate (AMP) was observed, but not from the C1 carboxyl of **8** (Fig. 3c). Although MloI A₂T₂ shows clear regioselectivity for the C4 carboxyl of L-Asp, the incorporation of ¹⁸O into AMP was low. This may be due to background ATPase activity of the adenylation domain²⁹ when removed from its native assembly line. For additional confirmation of the MloI A₂ regioselectivity, a second thiolation assay was developed using *N*-acetylcysteine (SNAC) to capture Asp from the Asp-AMP intermediate **10** (Fig. 3a,d). Analysis of the Asp-SNAC thioesters produced from incubating MloI A₂T₂ with ATP, SNAC and isotopomers **8** or **9** confirmed that the A₂ domain is

highly selective for the L-Asp side-chain carboxyl group, consistent with the proposed pathway (Fig. 2b). Control experiments carried out with a standard L-Asp-activating adenylation domain (A₂) from the surfactin NRPS (SrfAB)³⁰ showed the reverse isotopic labelling patterns of what was expected (Supplementary Fig. 5).

Characterization of the bacterial VKDC orthologue MloH. Next, we deleted the putative VKDC-like enzyme MloH (Supplementary Fig. 6a,b). Production of malonomycin **1** was abolished in the Δ *mloH* strain, and two new compounds **5** and **12** were produced at significant titres (Fig. 4a and Table 1). Compound **5** was shown to be identical, as judged by RP-HPLC, LC-HRMS and mass spectrometry² (Supplementary Fig. 6c,d and Supplementary

Fig. 4 | Characterization of compounds from wild-type and engineered strains. a, RP-HPLC analysis of extracts from R2374 wild type, $\Delta mloH$, $\Delta mloH::mloH$ and $\Delta mloH::Ng\ orf44$ strains. Production of malonomycin **1** is observed in the wild-type strain, but is abolished in the VKDC deletion strain $\Delta mloH$, which produces compounds premalonomycin **5** and N-succinylated premalonomycin **12**. Complementation of $\Delta mloH$ with either the wild-type *mloH* or *orf44* from *N. gamkensis* restores production of **1**. **b**, RP-HPLC analysis of acid hydrolysates of decarboxylated malonomycin (8R/S)-**5** and premalonomycin **5**, following derivatization with Marfey's reagent. Premalonomycin **5** hydrolysis gives L-Ser and L-Asp, indicating the (8S) configuration of **5**.

Table 3), to thermally decarboxylated malonomycin (8R/S)-**5**. Complementation of the $\Delta mloH$ strain with *mloH* restored production of **1** to wild-type levels. Thus, MloH is essential for the carboxylation of the precursor premalonomycin **5**, to generate malonomycin **1**. NMR experiments confirmed that **5** isolated from $\Delta mloH$ is a single diastereoisomer, whereas the decarboxylated malonomycin (8R/S)-**5** is a 1:1 mixture of diastereoisomers (Supplementary Figs. 7–9). Derivatization of the amino acids, derived from hydrolysis of **5**, using Marfey's reagent³¹, revealed that **5** from $\Delta mloH$ possesses both L-Ser and L-Asp, confirming the (8S) configuration of **5** (Fig. 4b and Supplementary Fig. 6e), consistent with the activation of L-Asp by MloI A₂T₂ (Fig. 3b). The other metabolite **12** was shown by LC-HRMS, mass spectrometry² and NMR analysis to be an N-succinylated premalonomycin derivative (Fig. 4 and Supplementary Figs. 10–14). Succinylation of peptide natural products has been reported, and may modify the biological activity as part of a host self-defence mechanism^{32–35}. However, as no detectable quantity of succinylated **1**, and only trace quantities (1 mg l⁻¹) of **12** are evident in the wild-type strain, we suggest that premalonomycin **5** accumulates in $\Delta mloH$, and is adventitiously succinylated by the abundant cellular pool of succinyl-CoA to give the side product **12**.

Further evidence that MloH shares a similar catalytic function to the mammalian VKDC was obtained by site-directed mutagenesis. In the human enzyme, K218 and H160 are important for activity^{16,17}.

A K218A mutation results in the loss of co-factor oxidation and γ -Glu carboxylation, suggesting that K218 deprotonates the KH₂ co-factor¹⁶ (Fig. 1c). Conversely, a H160A mutant showed minimal carboxylation activity, but oxidation of KH₂ to KO was unaffected, suggesting that H160 may help stabilize the reactive γ -glutamyl enolate intermediate during carboxylation¹⁷. Mutagenesis of the corresponding residues in *mloH* generated K182A/H/R or H119A/K/R mutants that were used to complement the $\Delta mloH$ strain. Whereas the $\Delta mloH::mloH$ strain produces **1** at wild-type levels, $\Delta mloH::mloH$ (H119A) failed to restore the production of **1**, suggesting that this residue is involved in carboxylation activity (Table 1). The K182A mutant reduced the production of **1** to less than 5% of the wild type. The human K218A mutant abolished enzyme activity, but could be rescued with the addition of exogenous amines, which are suggested to deprotonate the KH₂ co-factor in the absence of the general base K218 (ref. 16). It is therefore possible that the residual activity of MloH K182A could be a consequence of an alternative cellular amine or water functioning as a base. Substitutions by larger amino acids further reduced (K182H) or abolished (K182R) production (Table 1). Presumably, in K182R, the side chain is unable to adopt the geometry required to deprotonate KH₂ and also blocks another molecule from functioning as a base. The MloH H116 residue, given its close proximity to H119, was also subjected to mutagenesis. In this case, H116A/K/R mutations all resulted in significant malonomycin production (Supplementary Fig. 15). These results suggest that the conserved K182 and H119 residues of MloH provide the same catalytic functions as the corresponding residues in the related human VKDC.

From BLAST analysis, it is apparent that there are a number of other VKDC-like proteins in bacteria exhibiting high similarity to MloH. One such protein is encoded by *orf44* from *Nocardia gamkensis* NBRC 108242 (52% identity and 64% similarity). The *N. gamkensis orf44* is present in a BGC that resembles the *mlo* BGC (Supplementary Figs. 3d and 16). Unlike the NRRL 2455 and R2374 *mlo* BGCs, which share >93% identity, the proteins in the *Nocardia* BGC exhibit between 40 and 60% identity to the corresponding *mlo*-encoded proteins. Efforts to isolate malonomycin-like metabolites from *N. gamkensis*, and from expression of the *Nocardia* BGC in the heterologous hosts *Streptomyces coelicolor* and *Staphylococcus albus*, have so far been unsuccessful. However, ORF44 exhibits 52% identity to MloH, so we examined this protein for VKDC-like activity using *orf44* to complement $\Delta mloH$. The resulting strain $\Delta mloH::Ng\ orf44$ produced good quantities of **1** (53% of that of the wild-type R2374 (Fig. 4a and Table 1)), confirming that two VKDC-like enzymes from distinct bacterial genera have the ability to α -carboxylate aspartyl precursors to generate intact aminomalonic acid structures in secondary metabolism.

Conclusion

In summary, a genomics-driven approach has been used to identify the BGC for malonomycin, revealing a bacterial VKDC orthologue (MloH). Through gene knockout and complementation experiments, we showed that MloH is responsible for the α -carboxylation of an aspartyl precursor, generating the intact aminomalonic acid moiety of malonomycin. Site-directed mutagenesis of conserved active-site residues in MloH abolished activity, in line with previously reported effects in the human enzyme, as expected if MloH is a functional orthologue of VKDC. A second bacterial VKDC-like protein from *Nocardia* was also shown to share functionality with MloH. Additionally, ¹⁸O-labelling experiments showed that L-Asp is activated via its side-chain carboxyl group in an NRPS-PKS assembly line to deliver the premalonomycin precursor for subsequent carboxylation. This work provides functional characterization of VKDC-like enzymes of bacterial origin and reveals a highly unusual pathway to aminomalonic acid-containing metabolites.

Methods

Materials. Chemical reagents and solvents were purchased from commercial sources (Sigma–Aldrich and Fisher Scientific) and used without further purification unless otherwise stated. Molecular biology enzymes were purchased from New England Biolabs unless otherwise stated. Oligos were synthesized by Sigma–Aldrich.

Nucleotide sequencing and assembly of the *S. rimosus* R2374 and NRRL 2455 genomes. Sequencing was carried out on an Illumina MiSeq platform using the Illumina V2 500 cycle kit run in 2 × 250 base pair (bp) mode, using around one-eighth of the full-run capacity for each library type. Nextera Shotgun and Nextera Matepair libraries were constructed from high-molecular-weight genomic DNA following the manufacturer's protocols. Reads were processed using an in-house Illumina adapter trimming tool (fastq_miseq_trimmer). Read pairs were then pre-assembled using FLASH version 1.2.11 (<https://ccb.jhu.edu/software/FLASH/>). The Nextera Matepair linker sequence was used for splitting of matepair reads (fastq_miseq_trimmer). Processed reads were assembled de novo using Newbler version 3 (Roche). Several assemblies were carried out using either all or subsets of the input dataset, and the best assembly was selected using a score calculated from the scaffold N50, edge count and total number of contigs.

Genome annotation. Coding sequences were predicted ab initio using fgeneSB version 2 (<http://www.softberry.com/>). Annotation was transferred by a set of in-house PERL scripts from the most representative hits to KEGG and Non-Redundant databases. Coding sequences were colour coded according to the hits to the Clusters of Orthologous Groups functional database. National Center for Biotechnology Information BLAST version 2.2.17 (ref. 36) was used for protein database searches. The final annotation results were manually curated using Artemis (version 16)³⁷.

Production of malonomycin and derivatives. Procedures for the production of malonomycin and malonomycin derivatives were adapted from Schipper et al.³ as follows. A seed culture consisting of 20 ml Tryptone Soy Broth (TSB) media in an Erlenmeyer flask containing a spring was inoculated with 50 µl of a stock suspension of *S. rimosus* subspecies *paramyceticus* R2374 spores. The seed culture was cultivated at 28 °C with 150 r.p.m. agitation for 48 h and subsequently used to inoculate 70 ml malonomycin production medium (malt extract (Oxoid) 18 g l⁻¹, peptone (Formedium) 7 g l⁻¹ and NaCl 5 g l⁻¹; pH 8.5) at 1% v/v in 250 ml flasks containing springs. The fermentation cultures were cultivated for 65 h at 28 °C with 150 r.p.m. agitation.

Isolation of malonomycin and derivatives. Fermentation cultures were centrifuged at 5,000g to pellet the cells. The fermentation supernatant was reduced in volume tenfold under reduced pressure, adjusted to pH 2 with HCl and centrifuged (5,000g for 10 min) to pellet the precipitated media components and organic matter.

Procedures for the isolation of malonomycin and malonomycin derivatives were adapted from Schipper et al.³ as follows. For the isolation of malonomycin **1** from the wild-type R2374, the supernatant was adjusted to the isoelectric point of malonomycin (pH 2.7) and 4 volumes of methanol were added, before incubating at –20 °C for 2 h. The precipitated malonomycin was pelleted by centrifugation (5,000g for 10 min at 4 °C), redissolved in H₂O and adjusted to pH 8.5. The resultant crude extract was loaded over an anion exchange column consisting of DEAE–Sephadex A25 resin pre-swollen in 0.5 M ammonium acetate (pH 8.5). Malonomycin **1** was eluted with a gradient of 0–1 M acetic acid in stepwise 0.1 M increments of 1.5 column volumes each. Fractions were collected and analysed by RP–HPLC (Phenomenex Kinetex XB–C18 column; 4.6 mm × 250 mm; 5 µm particle size) using the following method: 240, 280 nm ultraviolet absorbance, a 1 ml min⁻¹ flow rate and a gradient of 0–4 min 3% B, 4–8 min 3–80% B, 8.1–10 min 95% B, 10–11 min 95–3% B and 11–14 min 3% B, with mobile phase A consisting of H₂O + 0.1% formic acid and mobile phase B consisting of methanol + 0.1% formic acid. Fractions containing **1** were pooled and dried under reduced pressure. The resultant pale yellow–brown residue was washed with ice-cold pH 7.0 H₂O, and **1** precipitated as a white crystalline solid. The liquid was removed after centrifugation and the wet pellet was frozen and lyophilised, yielding **1** (76 mg isolated from 1 l culture).

Isolation of premalonomycin **5** from the *ΔmloH* strain was carried out as described above, except anion exchange fractions containing premalonomycin **5** were pooled and dried under reduced pressure, redissolved in H₂O and passed through a C18 Bondelut (Agilent). The flowthrough and water wash were pooled and lyophilised, yielding a light yellow–brown solid of **5** (46 mg isolated from 1 l culture).

Compound **12** was purified from the *ΔmloH* strain by semi-preparative RP–HPLC from the crude extract obtained after precipitation with methanol. A Varian ProStar HPLC with a Phenomenex Gemini C18 column (10 mm × 250 mm; 5 µm particle size) was used, with the following method: 240, 280 nm ultraviolet absorbance and a gradient of 0–5 min 3% B, 5–8 min 3–70% B, 8–8.5 min 70–95% B, 8.5–10 min 95% B, 10–10.5 min 95–3% B and 10.5–12 min 3% B, with mobile phase A consisting of H₂O + 0.1% formic acid and mobile phase B consisting of

methanol + 0.1% formic acid. Fractions containing compound **12** were pooled and dried under reduced pressure, redissolved in water and loaded over a cation exchange column consisting of Dowex 50WX8 resin pre-swollen in H₂O. The column was washed with H₂O and **12** was eluted with 1 M NH₄OH.

Characterization of malonomycin and derivatives. LC–HRMS characterization of all malonomycin-related compounds was carried out using a Thermo Dionex UltiMate 3000 UHPLC coupled to a Thermo Q Exactive spectrometer with the following method: (Thermo Accucore C18 column, 2.1 mm × 100 mm; 2.6 µm particle size), a 0.3 ml min⁻¹ flow rate and a gradient of 0–3 min 3% B, 3–6.5 min 3–95% B, 6.5–8.5 min 95% B, 8.5–9 min 95–3% B and 9–12 min 3% B, with mobile phase A consisting of H₂O + 0.1% formic acid and mobile phase B consisting of methanol + 0.1% formic acid. Multiple-stage mass spectrometry analysis of malonomycin and related compounds was carried out on a Waters ACQUITY UPLC coupled to an LTQ Orbitrap XL with a Phenomenex Kinetex C18 column (2.1 mm × 100 mm; 2.6 µm particle size) and a gradient as described previously. Fragmentation settings were set as targeted multiple-stage mass spectrometry (iso width = 0.5 m/z; CID = 20; 30 eV). For NMR analysis, compounds were dissolved in D₂O/DCI (**1**), D₂O (**5**) and (8*R*/*S*)-**5**) or D₂O:H₂O 1:9 (**12**) (for further details of NMR assignments; see the Supplementary Methods).

Stereochemistry of premalonomycin (5). A thermally decarboxylated malonomycin standard (8*R*/*S*)-**5** was generated by suspending malonomycin **1** in H₂O and heating at 100 °C for 10 min. Premalonomycin **5** and decarboxylated malonomycin (8*R*/*S*)-**5** were hydrolysed in 6 M HCl at 98 °C for 3 h before derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)³¹ using standard procedures. Standards of L–Asp, D–Asp, L–Ser and D–Ser were directly derivatized without previous HCl treatment. The samples were analysed on an Agilent Infinity 1200 series HPLC (Phenomenex Kinetex XB–C18 column; 4.6 mm × 250 mm; 5 µm particle size) with the following method: 340 nm ultraviolet absorbance, a 1 ml min⁻¹ flow rate and a gradient of 0–1 min 5% B, 1–19 min 5–70% B, 19–20 min 95% B, 20–22 min 95% B, 22–23 min 95–5% B and 23–25 min 5% B, with mobile phase A consisting of H₂O + 0.05% trifluoroacetic acid and mobile phase B consisting of acetonitrile + 0.05% trifluoroacetic acid. They were also analysed by LC–HRMS on a Thermo Dionex UltiMate 3000 UHPLC coupled to a Thermo Q Exactive spectrometer with the following method: (Thermo Accucore C18 column; 2.1 mm × 100 mm; 2.6 µm particle size), a 0.3 ml min⁻¹ flow rate and a gradient of 0–2 min 5% B, 2–10 min 5–70% B, 10–10.5 min 70–95% B, 10.5–12.5 min 95% B, 12.5–13 min 95–5% B and 13–15 min 5% B, with mobile phase A consisting of H₂O + 0.1% formic acid and mobile phase B consisting of methanol + 0.1% formic acid. In addition to RP–HPLC and LC–HRMS analysis, the derivatized hydrolysates of **5** and (8*R*/*S*)-**5** were run with co-injections of derivatized L– and D–Asp and Ser standards to identify the component amino acids present in each sample.

Synthesis of N-succinylated regioisomers and characterization of N-succinyl-premalonomycin (12). Premalonomycin **5** (1 mg; 3 µmol) was treated with succinic anhydride (2.5 mg; 25 µmol) in 10 mM phosphate buffer pH 6.5 at 21 °C for 16 h, generating two regioisomers of N-succinylated premalonomycin, with succinylation occurring on either the tetramic acid aminomethyl side chain or the amine of the serinyl moiety. The reaction was analysed by RP–HPLC, with co-injection of **12** isolated from the *S. rimosus* *ΔmloH* strain identifying the earlier-eluting regioisomer as the natural product **12**. Characterization of the two regioisomers by mass spectrometry² and two-dimensional NMR (COSY and NOESY) revealed the early-eluting peak **12** to be succinylated on the serinyl moiety and the later eluting peak to be succinylated on the tetramic acid moiety.

CRISPR–Cas9 inactivation of *mloH* and other open reading frames (ORFs). Inactivation of *mloH*, encoding a putative bacterial VKDC orthologue, was carried out by in-frame deletion using CRISPR–Cas9 (ref. 23). A 102-bp region, encoding putative active-site residues, was targeted for deletion to prevent polar effects on flanking ORFs. A single protospacer insert was generated using primers ZY56F (5′-ACGCATCAGGCGGGAGAGCCACGG-3′) and ZY56R (5′-AAACCCGTGGCTCTCCGCTGAT-3′), and subsequently inserted into pCRISPOmyces-2 using Golden Gate assembly. A 996-bp thiostrepton resistance cassette (*tsr*) amplified from pJ6021 using primers ZY59F (5′-GGAGGAAAGTGCAATGTACTGATCAAGGCGAATACCTC-3′) and ZY59R (5′-CTGGAGGGAAACCAGCGTACTGATCATCACTGACGAATCG-3′) was then inserted using the *Sna*BI site using HiFi assembly (New England Biolabs), as a selectable marker due to the inherent resistance of *S. rimosus* R2374 against apramycin (pCRISPOmyces-2 selective marker). Finally, homologous regions flanking the 102-bp deletion site were amplified using primers ZY57F (5′-TGCCGCCGGGCGT TTTTATGGCGGTGTGACGGCGAGAT-3′), ZY57R (5′-CATGTTCCGCCGCGGAGTGGTGATGCTGGACAGCCGGTC-3′), ZY58F (5′-GACCGGGCTGTCCAGCATCAACCATCCGCGGCGGAACATG-3′) and ZY58R (5′-TTACGGTTCTGGCCTCTAGGCGAGGACTCCGTGCTGGTC-3′), inserted at the *Xba*I site using HiFi assembly (generating the construct pCm2-*tsr*-*mloH*), and verified by restriction digestion and nucleotide sequencing. To generate the *mloH* deletion in *S. rimosus*, pCm2-*tsr*-*mloH* was used to transform

E. coli ET12567 (pUZ8002) and subsequently introduced into *S. rimosus* R2374 by conjugation. Exconjugants were picked to SFM agar plates containing thiostrepton (30 µg ml⁻¹) and cultivated at 30 °C for 2–3 d. Deletion of 102 bp from *mloH* was verified by colony PCR amplification of the internal region of *mloH* using primers ZY70F (5'-CCAGTAGCGGGCAGCATCATC-3') and ZY70R (5'-ACGACTTCTCCCTCGACGGC-3'). Colonies showing deletion of 102 bp from *mloH* were further cultivated on SFM at high temperature (37–39 °C) to promote loss of the plasmid. Replica plating on SFM with and without thiostrepton was used to screen for *mloH* mutants without plasmid. Gene inactivation of other *mlo* ORFs was carried out similarly, using the primers and plasmids detailed in Supplementary Tables 4 and 5. For point mutations introduced to the chromosome using CRISPR–Cas9, the target codons were altered on the homologous flanking arms used for DNA repair after Cas9 cleavage. The plasmids were constructed as described previously.

Complementation of the $\Delta mloH$ strain and other ORFs. A *tsr* amplified from pJ16021 using primers ZY65F (5'-TGAAAAACGCTCACTGGTACCTGATCAAGGCGAATACTTC-3') and ZY65R (5'-CAAGCTTAGATCTATGCATGTGATCATCACTGACGAATCG-3') was inserted into the Φ BT1-integrative plasmid pAV11b (a gift from M. Smith at the University of York) at the *KpnI* site using HiFi assembly, regenerating the *KpnI* site. The constitutive promoter *ermE** was amplified from pJ186 using primers ZY72F (5'-TGAAGTATTCGCCTTGATCAGTACCAGCCGACCCGAGCA-3') and ZY72R (5'-AGCCACCAAGTGCCTGATCATATGTCCGTACCTCCGTGCT-3'), and *mloH* (1.5 kilobases) was amplified from *S. rimosus* R2374 genomic DNA using primers ZY73F (5'-AGCAACGGAGGTACGGACATATGATCAGCACTGGTGGCT-3') and ZY73R (5'-GAAAAACGCTCACTGGTACCTACCCGACGGGCTCCCGA-3'). Both fragments were inserted into the *KpnI* site of the pAV11b-*tsr* plasmid using HiFi assembly (New England Biolabs), generating construct pAV11b-*tsr*-*mloH*, carrying the wild-type *mloH* gene under control of the *ermE** constitutive promoter. The plasmid was subsequently introduced into the *S. rimosus* R2374 $\Delta mloH$ strain by conjugation using *E. coli* ET12567 (pUZ8002). Thiostrepton-resistant exconjugants after overlay with thiostrepton (30 µg ml⁻¹) and nalidixic acid (25 µg ml⁻¹) were picked to fresh SFM agar plates supplemented with thiostrepton and subsequently used to inoculate Tryptone Soy Broth liquid cultures. Genomic DNA was extracted, and successfully complemented colonies were verified by PCR using primers ZY70F and R. Other complementation plasmids for *mlo* knockout strains were constructed similarly using the primers and plasmids detailed in Supplementary Tables 4 and 5.

Site-directed mutagenesis of MloH and other Mlo proteins. To generate point mutations of putative catalytic residues of the *S. rimosus* R2374 VKDC, plasmid pAV11b-*tsr*-*mloH* used to complement the $\Delta mloH$ strain was subjected to site-directed mutagenesis using a Q5 site-directed mutagenesis kit (New England Biolabs) with primers K182X F (5'-GGAGTGGCCNNNGTCCGCTACGGCTGG-3'), where NNN is GCC (K182A), CAC (K182H) or CGC (K182R), and K182X R (5'-GCCGGCGAACATGGTGTACGCGAGG-3'); H116X F (5'-GGGGGCAANNNGTCCAGCACTC-3'), where NNN is GCC (H116A), CAC (H116K) or CGC (H116R), and R1 (H116A/K) (5'-CAGCGCACCCGCGGTGACCGC-3') or R2 (H116R) (5'-CAGCGCACCCGCGGTGACCG-3'); and H119X F (5'-CACTCCACGNNNTCCGCGCTCTACGC-3'), where NNN is GCC (H116A), CAC (H116K) or CGC (H116R), and R (5'-GTTGGCCCCAGCGCACCGG-3'), to introduce a single codon change at positions K182, H116 or H119, respectively. Mutations were verified by nucleotide sequencing (GATC Biotech) and the plasmids were conjugated into *S. rimosus* R2374 $\Delta mloH$ using standard procedures. Single-colony exconjugants were picked and selected for on thiostrepton-containing SFM agar plates, and integration of the mutant plasmids was verified by PCR. Construction of the *mloA* (S546A) complementation plasmid was carried out similarly, except primers S546A F (5'-GGTACGCGCTGCTGGCGTTCCGC-3') and S546 R (5'-GCCAGGCCGAAGAAGTCTCGTC-3') were used to generate the point mutation, with pAV11b-*tsr*-*mloA* as a template. Additional non-catalytic point mutations to *mloH* to confirm that mutations did not affect MloH protein structure and activity were made using pAV11b-*tsr*-*mloH* as a template and the primers listed in Supplementary Tables 4 and 5.

Cloning and purification of recombinant MloA, MloI A₂T₂ and SrfAB A₂ proteins. The *mloA* gene was PCR amplified from *S. rimosus* R2374 genomic DNA using primers *mloA*-AT F (5'-AAAAACATATGACAA GCACCCAGGCGTCGG-3') and *mloA*-AT R (5'-AAAAAAGCTCGAGAC GCGCGCGCTCGACAGCC-3'). The PCR product was doubly digested with *NdeI* and *XhoI* restriction enzymes and ligated into a similarly digested pET21b vector. The resultant construct pET21b-*mloA* was used to transform *E. coli* BL21 for protein production.

The gene encoding the MloI A₂T₂ didomain was PCR amplified from *S. rimosus* R2374 genomic DNA using the primers *mloI*-A₂T₂ F (5'-AAAAAATATGTACTCGCTGCTCGCTCGCC-3') and *mloI*-A₂T₂ R (5'-AAAAAAGCTCGAGGAAGTCATGCTCGGAAACCTCGTCTTCC-3'). The resultant DNA product was doubly digested with *NdeI* and *XhoI* and ligated into a similarly

digested pET21b vector, generating construct pET21b-*mloI*-A₂T₂. The resultant plasmid was used to transform *E. coli* BL21 for protein production.

The gene encoding the SrfAB A₂ domain³⁰ was PCR amplified from genomic DNA extracted from *Bacillus subtilis* strain 168 using primers SrfAB-A₂ F (5'-AAC TTTAAGAAGGAGATATAATGAACGTTTCGGCTGCTGCTGATGTAG-3') and SrfAB-A₂ R (5'-CAGTGGTGGTGGTGGTGGTGGGCCAAGGCCTTGCCTCC-3'). The DNA fragment was inserted into pET21b (*NdeI* and *XhoI* digested) using HiFi assembly (New England Biolabs), generating construct pET21b-srfAB-A₂, and used to transform *E. coli* BL21 for protein production.

For production of recombinant proteins (MloA, MloI A₂T₂ and SrfAB A₂), a single BL21 colony was used to inoculate an LB seed culture containing ampicillin (50 µg ml⁻¹) and cultivated at 37 °C overnight, before dilution 100-fold into fresh LB supplemented with ampicillin (50 µg ml⁻¹). Cultures were incubated at 37 °C until an OD₆₀₀ of 0.6 was reached, whereupon isopropyl β-D-1-thiogalactopyranoside (0.5 mM) was added to induce protein production. The cultures were cultivated for a further 4 h at 30 °C before the cells were harvested by centrifugation (4000g for 10 min). The cell pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl and 10% glycerol) and lysed by sonication (Bandelin Sonopuls; 50% power, 30% cycle, 8 min). Lysates were clarified by centrifugation (10,000g). The supernatants were loaded over Ni-NTA columns and washed with 5 column volumes of wash buffer 1 (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole and 10% glycerol) and 3 column volumes wash buffer 2 (50 mM Tris pH 8.0, 300 mM NaCl, 60 mM imidazole and 10% glycerol), and the protein was eluted with 5 column volumes of elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole and 10% glycerol). Protein eluates were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, buffer exchanged into 50 mM Tris pH 8.0, 300 mM NaCl and 10% glycerol, and stored at -80 °C.

Adenylation domain activity assays. The MloI A₂T₂ didomain was assayed against a range of substrates including the proteinogenic amino acids, D-Asp, β-Ala and succinic acid. Activity was measured in real time using an EnzChek pyrophosphate (PPi) detection assay (Thermo Fisher Scientific), with assays set up as follows: 5 mM dithiothreitol, 1 mM ATP, 10 mM MgCl₂, 10 µM MloI, 1 U ml⁻¹ purine nucleoside phosphorylase, 0.03 U ml⁻¹ inorganic pyrophosphatase, 0.2 mM 7-methyl-6-thio-guanosine, 10% glycerol v/v, 50 mM Tris HCl pH 7.5 and 0.1 mM NaN₃. Reaction mixtures were set up without amino acid substrates and pre-incubated at 25 °C for 2 min, whereupon substrate (1 mM) or H₂O and PPi (0.5 mM) controls were added and the reaction mixtures were incubated for a further 25 min with agitation in a Synergy HT microplate reader monitoring the absorbance at 360 nm. Relative rates of PPi release were calculated as ΔA₃₆₀ min⁻¹, and compensated for background absorbance due to enzyme-mediated ATP hydrolysis by subtracting the absorbance of the negative controls (H₂O added in place of the substrate). Assays with MloA and SrfAB A₂ were carried out similarly.

¹⁸O-transfer assays. The L-[1-¹⁸O]-Asp 8 and L-[4-¹⁸O]-Asp 9 isotopomer substrates were generated by hydrolysis of the corresponding methyl esters 6 and 7 with Na¹⁸OH in H₂¹⁸O (Fig. 3a). The reactions were dried and redissolved to approximately 25 mM in buffer (50 mM Tris pH 7.5). Assays with MloI A₂T₂ or SrfAB A₂ were carried out as above. For analysis of ¹⁸O-labelled AMP, reactions were quenched with methanol and centrifuged, and ion-suppressing buffer salts were removed from the reaction mixtures by ion exchange using Phenomenex strata SAX SPE cartridges. Briefly, the supernatants were loaded over SAX SPE cartridges, washed with H₂O and eluted with 5% formic acid in 10% methanol. Reactions were analysed by LC-HRMS and isotopic ratios were calculated by measuring the ion count for each of the isotope peaks for AMP [M + H]⁺ (*m/z* 348, 349 and 350). For thiolation assays involving the capture of Asp by SNAC, reactions were set up as follows: 50 mM Tris HCl pH 7.5, 0.1 mM NaN₃, 10 mM MgCl₂, 0.5 mM ATP, 10% glycerol v/v, 10 µM MloI A₂T₂ or SrfAB A₂, 2 mM L-Asp substrate and 2 mM SNAC. Reactions were incubated at 25 °C for 30 min, then quenched with an equal volume of methanol. The reactions were then centrifuged and the supernatants were analysed by LC-HRMS.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the plots within this paper and other findings of this study are available from the corresponding author upon reasonable request. Nucleotide sequences for the malonomycin BGCs are deposited in GenBank (accession numbers MH104948 (*S. rimosus paramyceticus* R2374) and MH104947 (*S. rimosus paramomycinus* NRRL 2455). The whole genome shotgun sequence for *N. gamkensis* NBRC 108242, used for the identification of the malonomycin-like cluster from this strain, was obtained from GenBank National Center for Biotechnology Information reference sequence NZ_LBDBM01000044.1. Details on nucleotide sequences and proposed annotations are detailed in the Supplementary Information.

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Author contributions

B.J.C.L., Y. Zhuo, M.W. and J.M. designed the experiments. B.J.C.L., Y.Z., M.W., D.F., Y. Zhang, A.M. and L.R. carried out the experiments. M.S. and P.F.L. sequenced and annotated the genomes. B.J.C.L. and J.M. wrote the manuscript. All authors analysed the data and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Data analysis

Thermo Xcalibur v2.2 (mass spectrometry), Agilent ChemStation (HPLC), Shimadzu LabSolutions Lite v5 (HPLC), BioTek Gen5 v2 (96-well plate reader spectrophotometer), antiSMASH v3.0.5 and Artemis v16 (gene cluster analysis), fgeneSB v2 (genome annotation), Clustal OMEGA, T Coffee, Mega7 (sequence alignments and phylogeny), MestReNova v11 (NMR), ChemDraw Professional v15 (chemical structures and exact mass calculations)

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Nucleotide sequences for the malonomycin biosynthetic gene clusters are deposited in GenBank; accession numbers MH104948 (*Streptomyces rimosus* paramyceticus R2374) and MH104947 (*Streptomyces rimosus* paramomycinus NRRL 2455). All data are included in the manuscript or in the supplementary information. The whole genome shotgun sequence for *Nocardia gamkensis* NBRC 108242, used for the identification of the malonomycin-like cluster from this strain, was obtained from GenBank NCBI reference sequence: NZ_BDBM01000044.1. Details on individual nucleotide sequences and proposed annotation are detailed in the supplementary.

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