Supporting Information

Lomaiviticin Biosynthesis Employs a New Strategy for Starter Unit Generation

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Supplementary Materials

1. General materials and methods

Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Recombinant plasmid DNA was purified with a Qiaprep Kit from Qiagen. Purification of PCR reactions and gel extraction of DNA fragments for restriction endonuclease clean up were performed using an Illustra GFX PCR DNA and Gel Band Purification Kit from GE Healthcare. DNA sequencing was performed by Genewiz (Boston, MA) and Beckman Coulter Genomics (Danvers, MA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) resin was purchased from Qiagen. SDS-PAGE gels were purchased from BioRad. Protein concentrations were determined according to the method of Bradford using bovine serum albumin (BSA) as a standard (for Lom60 and Lom63).[1] For Lom62, the concentration obtained using the Bradford method was averaged with the concentration obtained by measuring absorbance at 280 nm using a ND-1000 NanoDrop (Thermo Scientific). The molar extinction coefficient of Lom62 (M^{-1} cm^{-1}) was determined using ExPASy ProtParam (http://web.expasy.org/protparam/). Optical densities of *E. coli* cultures were determined with a DU 730 Life Sciences UV/Vis spectrophotometer (Beckman Coulter) by measuring absorbance at 600 nm. Coenzyme A, propionyl-CoA, and methylmalonyl-CoA were purchased from Sigma (St. Louis, MO).

Analytical HPLC of Lom60 and Lom63 standards and assay mixtures was performed on a Dionex Ultimate 3000 instrument (Thermo Scientific) with monitoring at 220 nm. HPLC analysis was carried out using a Kinetex C8 column (2.6 µM, 100 Å, 100 x 3.0 mm, Phenomenex) at a flow rate of 0.5 mL/min. The HPLC assay conditions were: 0% B for 5 min, a gradient increasing to 20% B over 2 min, a gradient increasing to 50% B over 31 min, a gradient increasing to 100% B over 2 min, 100% B for 5 min, a gradient decreasing to 0% B over 1 min, and 0% B for 4 min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile).

High-resolution LC/ESI-TOF analyses of Lom60 and Lom63 standards and assay mixtures were performed in the Small Molecule Mass Spectrometry Facility at Harvard University on an Agilent 1200 series HPLC and 6220 TOF mass spectrometer (Agilent

Technologies, Palo Alto, CA) using an electrospray ionization (ESI) source. 5 µL samples were injected onto an Agilent PLRP-S polymeric reversed phase column (50 x 2.1 mm, 5 µm, 1000 Å) at the flow rate of 0.25 mL/min. The HPLC assay conditions were: a gradient from 5% B increasing to 50% B over 10 min, 50% B for 15 min, a gradient decreasing to 5% B over 1 min, 5% B for 5 min (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile). Acetonitrile, isopropanol, water, and formic acid used for LC/ESI-TOF were high purity solvents from EMD MILLIPORE, J.T. Baker, and Sigma-Aldrich. The ESI mass spectra data were recorded on a positive ionization mode for a mass range of m/z 400 to 3000 with the m/z scale externally calibrated using Agilent ESI-L Low Concentration Tuning Mix (P/N: G1969-85000); drying gas (N_2) temperature, 325 ºC; drying gas (N_2) flow, 8.0 mL/min; nebulizer, 40 psi; capillary voltage, 3500 V; fragmentor, 180V; and elution before 2 min was diverted to waste. Deconvolution of protein raw mass spectra was performed using Agilent MassHunter protein deconvolution software (version B.06.00).

S. pacifica DPJ-0019 was routinely cultured on M1 artificial sea water-based agar (10 g/L Difco soluble starch, 4 g/L Bacto™ yeast extract, 2 g/L Bacto™ peptone, 18 g/L Bacto™ agar, 500 mL deionized water, 500 mL artificial seawater). *Escherichia coli* strains were cultured in Luria-Bertani (LB) media supplemented with the appropriate antibiotics.

2. Cloning, overexpression, and purification of Lom60, Lom62, and Lom63.

Cloning of Lom60, Lom62, and Lom63

Table S1. Oligonucleotides used for cloning. Restriction sites are underlined.

Primer Name	ORF Amplified	Primer Sequence (5' to 3')
Lom60-f	*lom60*	GAATTACATATGAAGCAGATGACACTGTCG
Lom60N-r	*lom60*	GAATTACTCGAGTCAGGCGGCCGACAG
Lom62-f	*lom62*	GAATTACATATGACCTACCAACCGGCC
Lom62C-r	*lom62*	GAATTACTCGAGACAGTTCGCTGGATGTGTGA
Lom63-f	*lom63*	GAATTACATATGCGATCCGAGAACGGT
Lom63N-r	*lom63*	GAATTACTCGAGTCACGACCCGAGATGCTC

Lom60, lom62, and *lom63* were PCR amplified from *Salinispora pacifica* DPJ-0019 genomic DNA (*S. pacifica* DPJ-0019 was obtained from Seth Herzon, Yale University) using the primers shown in Table S1. All PCR reactions contained 25 µL of Q5 High-Fidelity 2X Master Mix (New England Biolabs), 50 ng of genomic DNA template, and 500 pmoles of each primer in a total volume of 50 µL. The PCR reactions contained formamide at the following concentrations: 4% v/v for *lom60* amplification, 2% v/v for *lom62* amplification, and 4% v/v for *lom63* amplification. Thermocycling was carried out in a MyCycler gradient cycler (Bio-Rad) using the following parameters: denaturation for 30 sec at 98 °C, followed by 45 cycles of 15 sec at 98 °C; 30 sec at 71 °C (*lom62*), 68 °C (*lom60*), or 69 °C (*lom63*); 30 sec (*lom62*) or 15 sec (*lom60* and *lom63*) at 72 °C; and a final extension time of 2 min at 72 °C.

PCR reactions were analyzed by agarose gel electrophoresis with ethidium bromide staining and purified. Amplified fragments for *lom60* and *lom62* were digested with *Nde*I and *Xho*I (New England Biolabs) for 2.5 h at 37 °C. Digests contained 1 µL of water, 3 µL of NEB Buffer 4 (10x), 3 µL of BSA (10x), 20 µL of PCR product, and 1.5 µL each of *Nde*I (20 U/µL) and *Xho*I (20 U/µL). These restriction digests were purified using the enzyme clean up protocol from the Illustra GFX kit. The *lom63* gene contained a *Xho*I restriction site within its sequence, and it was found that two separate digests were required to obtain the correctly digested full-length *lom63* gene. Amplified *lom63* was first digested with *Nde*I for 2.5 h at 37 °C. The digest contained 3.75 µL of water, 4.5 µL of NEB Buffer 4 (10x), 4.5 µL of BSA (10x), 30 µL of PCR product, and 2.25 µL of *Nde*I (20 U/µL). The first digest was purified using the Illustra GFX kit. Next, a partial restriction digest was performed with *Xho*I for 30 min at 37 °C. The digest contained 7.75 µL of water, 7.5 µL of NEB Buffer 4 (10x), 7.5 µL of BSA (10x), 50 µL of *Nde*I-digested *lom63* DNA, and 2.25 µL of *Xho*I (20 U/µL). The second digest was purified using gel electrophoresis and the appropriate gel band was further purified using the Illustra GFX kit.

The digests were ligated into linearized expression vectors using T4 DNA ligase (New England Biolabs). *Lom60* and *lom63* were ligated into the pET-28a vector to encode N-

terminal His_6-tagged constructs and *lom62* was ligated into the pET-29b vector to encode a C-terminal His_6-tagged construct. Ligations were incubated at room temperature for 2 h and contained 3 µL water, 1 µL of digested vector, 3 µL of digested insert DNA, 1 µL of T4 ligase buffer (10x), and 2 µL of T4 DNA Ligase (400 U/µL). 5 µL of each ligation was used to transform 50 µL of chemically competent *E. coli* TOP10 cells. The identities of the resulting pET-28a-Lom60, pET-29b-Lom62, and pET-28a-Lom63 constructs were confirmed by sequencing of purified plasmid DNA. These constructs were transformed into chemically competent BL21(DE3) Tuner cells (Invitrogen) and stored at –80 ºC as frozen cells stocks in LB/glycerol.

Large scale overexpression of Lom62

A 50 mL starter culture of pET-29b-Lom62 BL21(DE3) Tuner *E. coli* was inoculated from a frozen cell stock and grown overnight at 37 ºC in LB medium supplemented with 50 µg/mL kanamycin. The overnight culture was diluted 1:100 into 2 L of LB medium supplemented with 50 µg/mL kanamycin. The culture was incubated at 37 ºC with shaking at 175 rpm for 3 h until OD_{600} = 0.5–0.6 and was then transferred to a 15 ºC incubator without shaking and allowed to cool for 30 min. The culture was induced with 500 µM IPTG and incubated at 15 °C with shaking at 175 rpm for 16 h.

Cells from 2 L of culture were split into two 1 L batches and pelleted by centrifugation (6,000 rpm x 10 min). One cell pellet was frozen at –80 °C for storage and the other was used for purification. This cell pellet was resuspended in 40 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, pH 8.5). The cells were lysed by passage through a cell disruptor (Avestin EmulsiFlex-C3) twice at 5,000 – 6,000 psi and the lysate was clarified by centrifugation (13,000 rpm x 30 min). The supernatant was incubated with 2 mL of Ni-NTA resin and 5 mM imidazole for 2 h at 4 ºC on a nutating mixer. The mixture was centrifuged (3,000 rpm x 5 min) and the unbound fraction was discarded. The Ni-NTA resin was resuspended in 2 mL of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, 5 mM imidazole, pH 8.5), loaded onto a glass column, and washed with 10 mL of elution buffer. Protein was eluted from the column

using a stepwise imidazole gradient in elution buffer (25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, and 200 mM), collecting 2 mL fractions. SDS–PAGE analysis (4–15% Tris-HCl gel) was used to determine the presence and purity of protein in each fraction. Fractions containing Lom62 were combined and dialyzed twice against 2 L of storage buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM DTT, pH 8.5). The protein solution was concentrated to 1 mL using a 10,000 Da molecular weight cut-off Spin-X UF Concentrator (Corning), frozen in liquid N_2, and stored at –80 ºC. This procedure afforded a yield of 0.4 mg/L for C-His_6-tagged Lom62. The concentration of Lom62 protein stock solution was 11 µM.

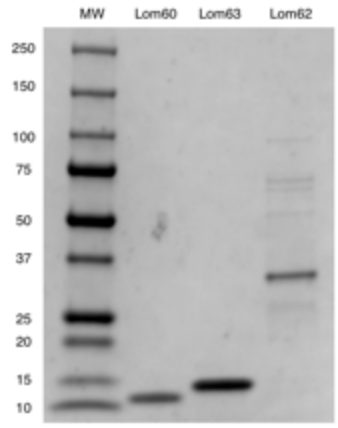
Large scale overexpressions of Lom60 and Lom63

A 50 mL starter culture of pET-28a-Lom60 or pET-28a-Lom63 BL21(DE3) Tuner *E. coli* was inoculated from a frozen cell stock and grown overnight at 37 °C in LB medium supplemented with 50 µg/mL kanamycin. Overnight cultures were diluted 1:100 into 2 L of LB medium supplemented with 50 µg/mL kanamycin. The cultures were incubated at 37 °C with shaking at 175 rpm until OD_{600} = 0.2 – 0.3 and were then transferred to an incubator at 15 °C with shaking at 175 rpm. At OD_{600} = 0.5 – 0.6 the cultures were induced with 500 µM IPTG and incubated overnight for ~ 16 h.

Cells from 2 L of culture were pelleted by centrifugation (6,000 rpm x 10 min) and resuspended in 40 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, pH 7.5 (Lom60) or pH 7.8 (Lom63)). The cells were lysed by passage through a cell disruptor (Avestin EmulsiFlex-C3) twice at 5,000 – 6,000 psi and the lysate was clarified by centrifugation (13,000 rpm x 30 min). The supernatant was incubated with 2 mL of Ni-NTA resin and 5 mM imidazole for 2 h at 4 °C. The mixture was centrifuged (3,000 rpm x 5 min) and the unbound fraction was discarded. The Ni-NTA resin was resuspended in 2 mL of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, 5 mM imidazole, pH 7.5 (Lom60) or pH 7.8 (Lom63)), loaded onto a glass column, and washed with 10 mL of elution buffer. For Lom60, protein was eluted from the column using a stepwise imidazole gradient in elution buffer collecting the following volumes: 10 mM (4 mL), 15 mM (2 mL), 25 mM (2 mL), 50 mM (2 mL), 75 mM (2 mL), 100 mM (2

mL), 125 mM (3 mL), 150 mM (3 mL), and 200 mM (3 mL). For Lom63, protein was eluted from the column using a stepwise imidazole gradient in elution buffer collecting the following volumes: 25 mM (2 mL), 50 mM (2 mL), 75 mM (2 mL), 100 mM (2 mL), 125 mM (3 mL), 150 mM (3 mL), and 200 mM (3 mL). SDS-PAGE analysis (4 – 15% Tris-HCl gel) was used to determine the presence and purity of protein in each fraction. Fractions containing the desired protein were combined and dialyzed twice against 2 L of storage buffer (25 mM Tris-HCl, 50 mM NaCl, pH 7.5 (Lom60) or pH 7.8 (Lom63)). The protein solutions were concentrated to 1.0 mL (Lom60) and 2.0 mL (Lom63) using a 5,000 Da molecular weight cut-off Spin-X UF Concentrator (Corning), frozen in liquid N_2, and stored at –80 ºC. This procedure afforded a yield of 4.98 mg/L for N-His_6-tagged Lom60 and 7.00 mg/L for N-His_6-tagged Lom63. Concentrations of protein stock solutions were: 880 μM for N-His_6-tagged Lom60 and 490 μM for N-His_6-tagged Lom63.

Figure S1. SDS-PAGE of purified N-His_6-tagged Lom60, N-His_6-tagged Lom63, and C-His_6-tagged Lom62. 4-15% Tris-HCl gel (Bio-Rad). (MW = Precision Plus Protein All Blue Molecular Weight Standards (Bio-Rad)).



3. Biochemical characterization of Lom60 and Lom63

HPLC and LC-MS analysis

Purified Lom60 (0.2 μg) and Lom63 (0.3 μg) were analyzed by HPLC (Figure S2) and LC-MS (Table S2 and Figure S3) as described in the General Materials and Methods. Protein samples were centrifuged (13,000 rpm x 10 min at 4 °C) before submission for HPLC or LC-MS analysis. Both Lom60 and Lom63 purified almost entirely in the inactive apo-form. Both proteins lacked an N-terminal *N*-formylmethionine (fMet). Additionally, Lom60 and Lom63 were partially glucuronidated at the N-terminus as evidenced by the presence of a +178 Da mass.[2]

Figure S2. HPLC traces of apo-Lom60 and apo-Lom63 standards (220 nm).

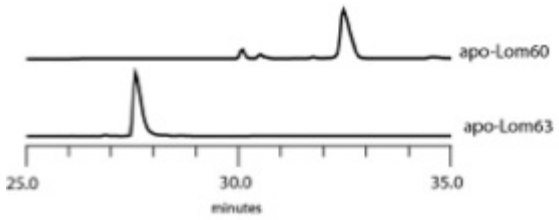
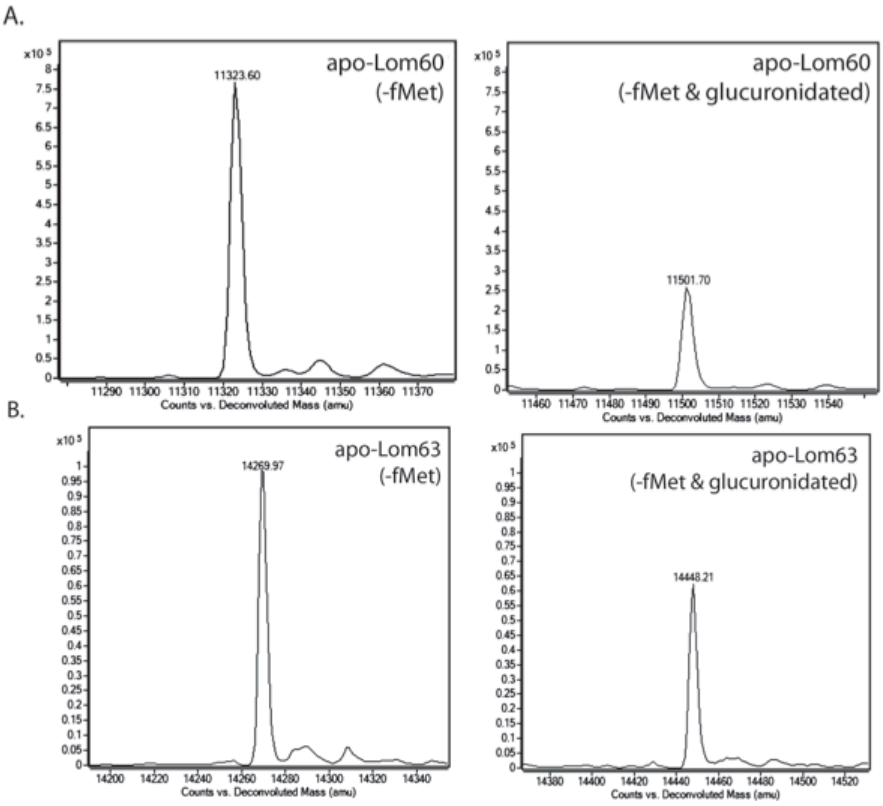


Table S2. LC-MS analysis of purified apo-Lom60 and apo-Lom63.

Observed Product	Chemical Formula	Calculated Mass (Da)	Observed Mass (Da)
apo-Lom60-N-His_6 (-fMet)	$C_{485}H_{757}N_{145}O_{161}S_4$	11323.4	11323.6
apo-Lom60-N-His_6 (-fMet & glucuronidated)	$C_{491}H_{767}N_{145}O_{167}S_4$	11501.5	11501.7
apo-Lom63-N-His_6 (-fMet)	$C_{627}H_{962}N_{188}O_{192}S_2$	14269.7	14270.0
apo-Lom63-N-His_6 (-fMet & glucuronidated)	$C_{633}H_{972}N_{188}O_{198}S_2$	14447.8	14448.2

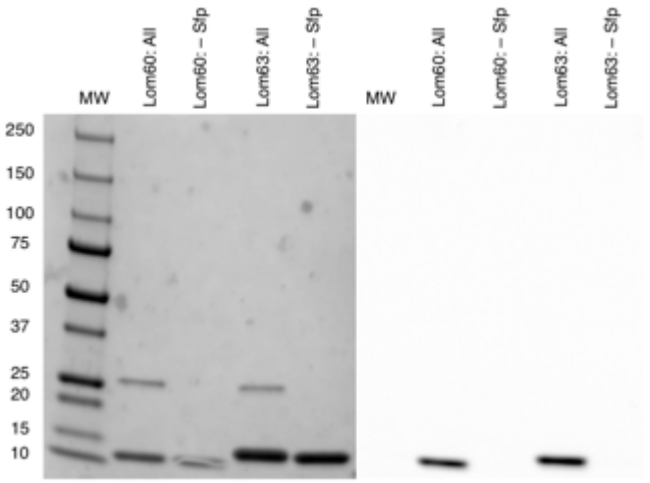
Figure S3. Deconvoluted spectra from LC-MS analysis of (A) apo-Lom60 and (B) apo-Lom63.



BODIPY-CoA fluorescent phosphopantetheinylation assays

BODIPY-CoA[3] and Sfp[4] were prepared using previously reported procedures. Assay mixtures (50 µL) contained 50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.5 mM DTT, 25 µM Lom60 or Lom63, 5 µM Sfp, and 25 µM BODIPY-CoA. Reaction mixtures were incubated in the dark at room temperature for 1 h. Reaction mixtures were then diluted 1:1 in 2x Laemmli sample buffer (Bio-Rad), heated at 90 °C for 10 min, and separated by SDS-PAGE (4-15% Tris-HCl gel). The gel was first imaged at λ = 365 nm, then stained with Coomassie and imaged again (Figure S4).

Figure S4. Loading of Lom60 and Lom63 with BODIPY-CoA by phosphopantetheinyl transferase Sfp. MW = Precision Plus Protein All Blue Molecular Weight Standards (Bio-Rad).



4. Biochemical characterization of Lom62

Preparation of holo- and propionyl-Lom63 standards

Holo-Lom63 and propionyl-Lom63 were prepared in separate 43 µL reactions. The reactions contained 50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM CoA (for holo-Lom63) or 1 mM propionyl-CoA (for propionyl-Lom63), 100 µM apo-Lom63, and 5 µM Sfp. Reactions were incubated at room temperature for 1.5 h and then 7 µL of water was added to give a final volume of 50 µL. Reaction mixtures were quenched with 50 µL of acetonitrile and centrifuged (13,000 rpm x 10 min at 4 °C) before HPLC (Figure S5) and LC-MS (Table S3 and Figure S6) analysis was performed as described in the General Materials and Methods. The reaction that generated propionyl-Lom63 (prop-rxn) did not go to completion, as evidenced by the presence of residual holo-Lom63 in the HPLC and LC-MS data.

Figure S5. HPLC traces of holo-Lom63 and propionyl-Lom63 standards (220 nm).

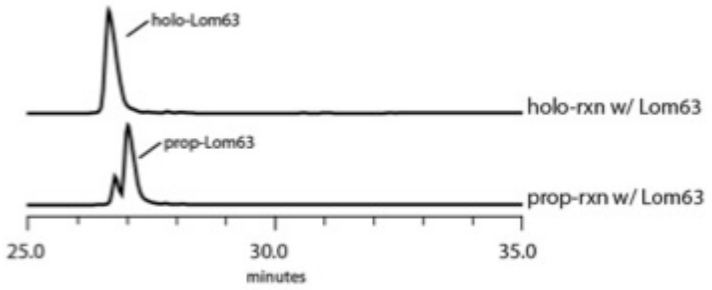
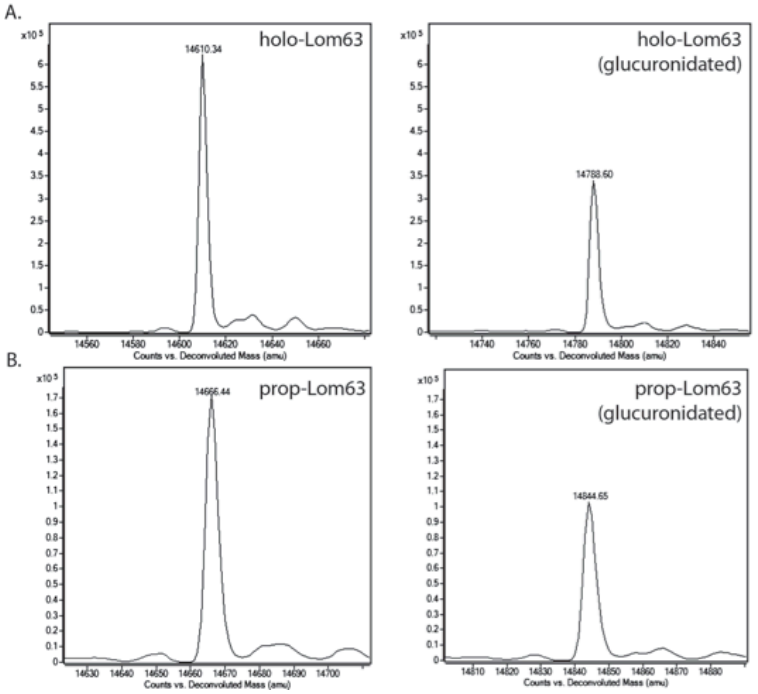


Table S3. LC-MS analysis of holo-Lom63 and propionyl-Lom63 standards.

Reaction	Observed Product	Formula	Calculated Mass (Da)	Observed Mass (Da)
holo-rxn (+ CoA)	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.3
holo-rxn (+ CoA)	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.6
prop-rxn (+ prop-CoA)	propionyl-Lom63	$C_{641}H_{987}N_{190}O_{199}S_3P$	14666.1	14666.4
prop-rxn (+ prop-CoA)	propionyl-Lom63 (glucuronidated)	$C_{647}H_{997}N_{190}O_{205}S_3P$	14844.2	14844.7
prop-rxn (+ prop-CoA)	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.3
prop-rxn (+ prop-CoA)	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.6

Figure S6. Deconvoluted mass spectra from LC-MS analysis of (A) holo-Lom63 from holo-rxn and (B) prop-Lom63 from prop-rxn.



Preparation of holo- and propionyl-Lom60 standards

Holo-Lom60 and propionyl-Lom60 were prepared in separate 43 µL reactions. The reaction mixtures contained 50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM CoA (for holo-Lom60) or 1 mM propionyl-CoA (for propionyl-Lom60), 100 µM apo-Lom60, and 5 µM Sfp. The reaction mixtures were incubated at 37 °C for 2 h and then 7 µL of water was added to give a final volume of 50 µL. Reaction mixtures were quenched with 50 µL of acetonitrile and centrifuged (13,000 rpm x 10 min at 4 °C) before HPLC (Figure S7) and LC-MS (Table S4 and Figure S8) analysis was performed as described in the General Materials and Methods. The reaction that generated propionyl-Lom63 (prop-rxn) did not go to completion, as evidenced by the presence of residual holo-Lom63 in the HPLC and LC-MS data.

Figure S7. HPLC traces of holo-Lom60 and propionyl-Lom60 standards (220 nm).

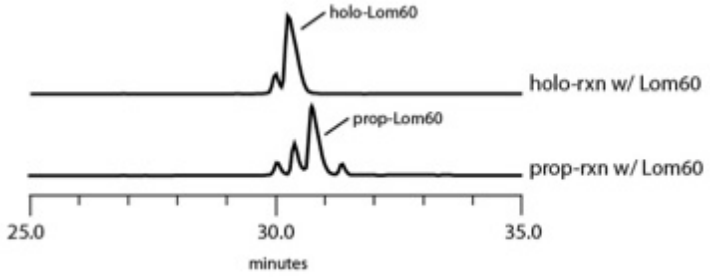
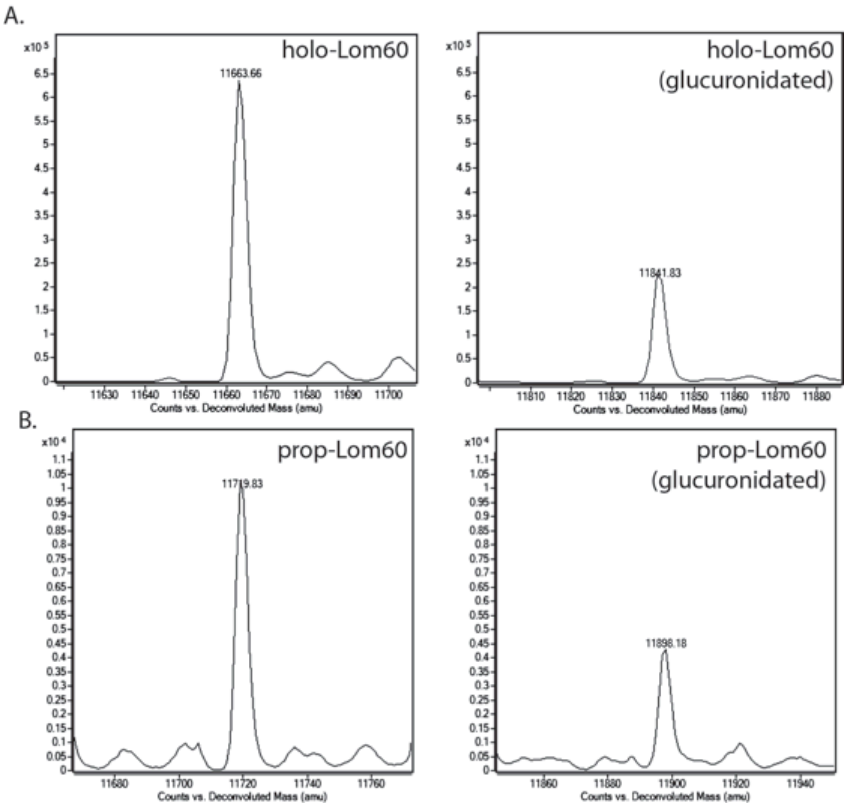


Table S4. LC-MS analysis of holo-Lom60 and propionyl-Lom60 standards.

Reaction	Observed Product	Formula	Calculated Mass (Da)	Observed Mass (Da)
holo-rxn (+ CoA)	holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.7
holo-rxn (+ CoA)	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11841.8
prop-rxn (+ prop-CoA)	propionyl-Lom60	$C_{499}H_{782}N_{147}O_{168}S_5P$	11719.8	11719.8
prop-rxn (+ prop-CoA)	propionyl-Lom60 (glucuronidated)	$C_{505}H_{792}N_{147}O_{174}S_5P$	11897.9	11898.2
prop-rxn (+ prop-CoA)	holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.4
prop-rxn (+ prop-CoA)	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11841.3

Figure S8. Deconvoluted mass spectra from LC-MS analysis of (A) holo-Lom60 from holo-rxn and (B) prop-Lom60 from prop-rxn.



HPLC assay for methylmalonyl-CoA loading and decarboxylation

Phosphopantetheinylation reactions of apo-Lom60 and apo-Lom63 using Sfp and CoA were carried out in a total volume of 43 µL and contained 50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM CoA, 100 µM apo-ACP, and 5 µM Sfp. For Lom60 the reaction mixture was incubated at 37 °C for 2 h and then at room temperature for 15 min; for Lom63 the reaction mixture was incubated at room temperature for 1.5 h. Methylmalonyl-CoA or propionyl-CoA (final concentration 500 µM) and Lom62 (final concentration 1 µM) were added separately to each reaction mixture to give a final volume of 50 µL. The reaction mixture was further incubated at room temperature for 1 h, quenched with 50 µL of acetonitrile, and centrifuged (13,000 rpm x 10 min at 4 °C) before HPLC and LC-MS (Tables S5 and S6) analysis was performed as described in the General Materials and Methods.

Table S5. LC-MS analysis of Lom62-catalyzed acyl loading reactions with Lom63.

Reaction	Observed Product	Formula	Calculated Mass (Da)	Observed Mass (Da)
+ mMal-CoA + Lom62	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.4
+ mMal-CoA + Lom62	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.6
+ mMal-CoA + Lom62	propionyl-Lom63	$C_{641}H_{987}N_{190}O_{199}S_3P$	14666.1	14666.5
+ mMal-CoA + Lom62	propionyl-Lom63 (glucuronidated)	$C_{647}H_{997}N_{190}O_{205}S_3P$	14844.2	14844.8
+ mMal-CoA – Lom62	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.4
+ mMal-CoA – Lom62	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.6
+ prop-CoA + Lom62	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.4
+ prop-CoA + Lom62	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.7
+ prop-CoA – Lom62	holo-Lom63	$C_{638}H_{983}N_{190}O_{198}S_3P$	14610.0	14610.4
+ prop-CoA – Lom62	holo-Lom63 (glucuronidated)	$C_{644}H_{993}N_{190}O_{204}S_3P$	14788.1	14788.6

Table S6. LC-MS analysis of Lom62-catalyzed acyl loading reactions with Lom60.

Reaction	Observed Product	Formula	Calculated Mass (Da)	Observed Mass (Da)
+ mMal-CoA + Lom62	holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.9
+ mMal-CoA + Lom62	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11842.1
+ mMal-CoA + Lom62	propionyl-Lom60	$C_{499}H_{782}N_{147}O_{168}S_5P$	11719.8	11720.2
+ mMal-CoA + Lom62	propionyl-Lom60 (glucuronidated)	$C_{505}H_{792}N_{147}O_{174}S_5P$	11897.9	11898.3
+ mMal-CoA – Lom62	holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.9
+ mMal-CoA – Lom62	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11842.1
+ prop-CoA + Lom62	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11842.1
+ prop-CoA – Lom62	holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.8
+ prop-CoA – Lom62	holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11842.0

HPLC competition assay for evaluating Lom62 substrate specificity

Solutions of holo-Lom60 and holo-Lom63 were prepared as described above, and after the appropriate incubation period (Lom63, room temperature for 1.5 h; Lom60, 37 °C for 2 h) the reaction mixtures were combined at room temperature. Methylmalonyl-CoA (final concentration 500 μM) and Lom62 (final concentration 1 μM) were added separately to the reaction mixture to give a final volume of 100 μL. The reaction mixture was incubated at room temperature for 1 h, quenched with 100 μL of acetonitrile, and centrifuged (13,000 rpm x 10 min at 4 °C). The supernatants were analyzed using HPLC and LC-MS (Table S7) procedures described in the General Materials and Methods.

Table S7. LC-MS analysis of Lom62 competition assay.

Observed Product	Formula	Calculated Mass (Da)	Observed Mass (Da)
holo-Lom60	$C_{496}H_{778}N_{147}O_{167}S_5P$	11663.7	11663.9
holo-Lom60 (glucuronidated)	$C_{502}H_{788}N_{147}O_{173}S_5P$	11841.8	11842.1
propionyl-Lom63	$C_{641}H_{987}N_{190}O_{199}S_3P$	14666.1	14666.6
propionyl-Lom63 (glucuronidated)	$C_{647}H_{997}N_{190}O_{205}S_3P$	14844.2	148444.9

5. Bioinformatic analyses of Lom62

Multiple sequence alignments of Lom62, Strop_2227, LnmK, and DpsC

The amino acid sequences of LnmK (AAN85524.1), Lom62 from *Salinispora pacifica* DPJ-0019, and Strop_2227 from *Salinispora tropica* CNB-440 (ABP54678.1) were aligned using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/).[5] The resulting alignment demonstrates that LnmK has 44% amino acid sequence identity with both Lom62 and Strop_2227. It also shows that both the essential tyrosine hypothesized to function in catalysis of acyl transfer and multiple residues that contact substrate are conserved in all three proteins (Figure S9).[6] Lom62 and Strop_2227 were also aligned separately with DpsC (AAA65208.1), the KSIII from *Streptomyces peucetius* involved in generating the propionyl starter unit used in daunorubicin biosynthesis. This alignment showed that DpsC shares only 20 and 19% amino acid sequence identity with Lom62 and Strop_2227, respectively (Figure S10).

Figure S9. Multiple sequence alignment of LnmK, Lom62, and Strop_2227.
The red asterisk indicates the tyrosine active site residue required for acyltransferase activity. Black asterisks indicate active site residues that contact bound coenzyme A.

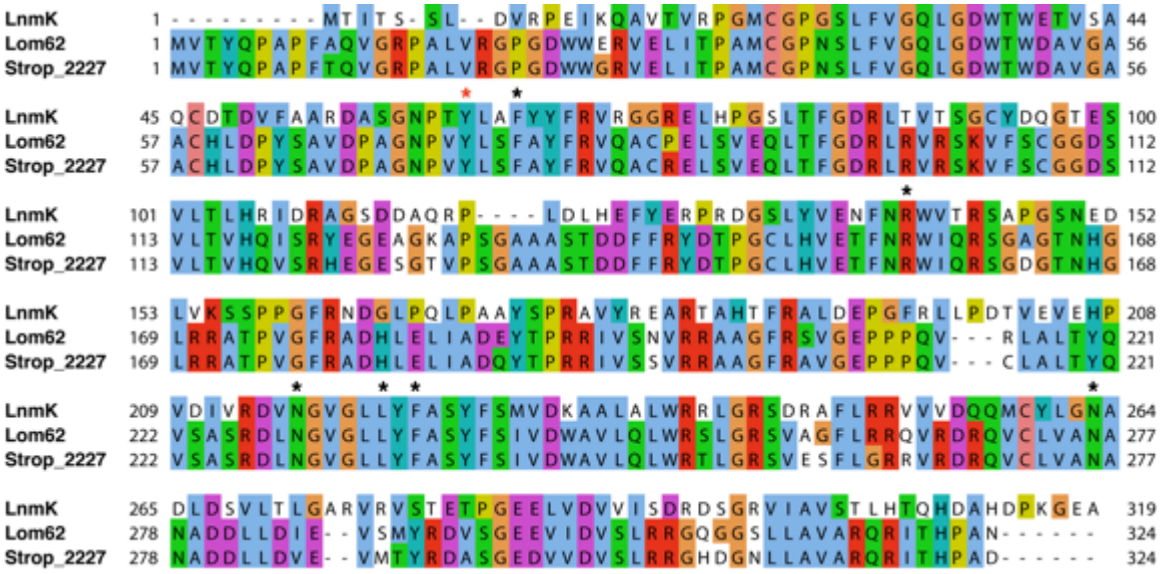
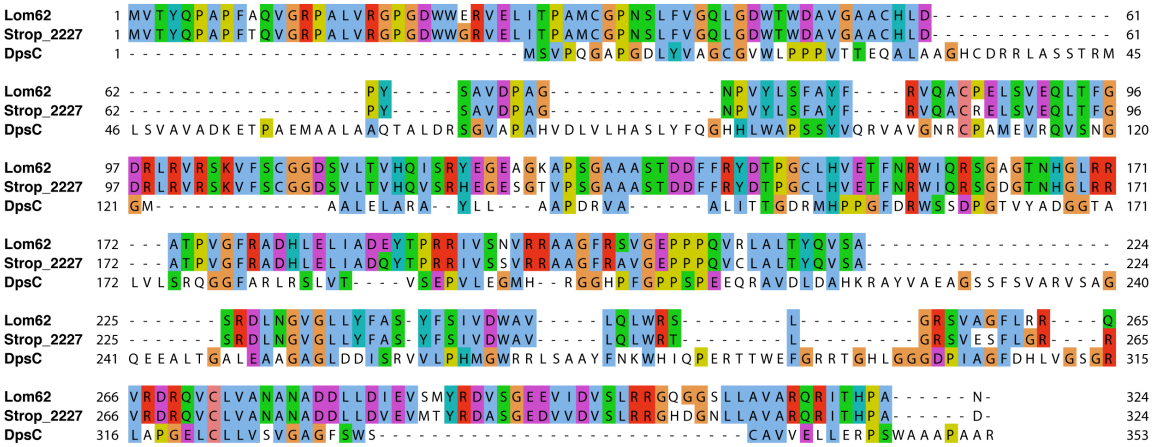


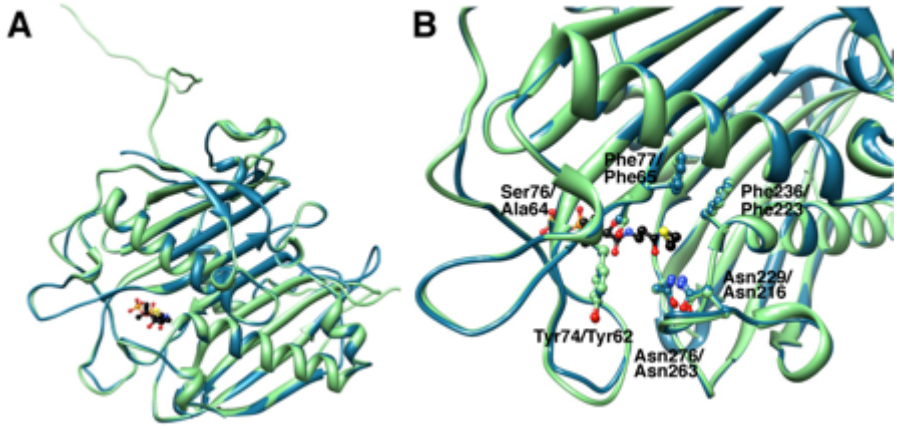
Figure S10. Multiple sequence alignment of Lom62, Strop_2227, and DpsC.



Construction of a Lom62 homology model

A Lom62 structure prediction and homology model was generated with the HHPred interactive server (http://toolkit.tuebingen.mpg.de/hhpred)[7] using the crystal structure of LnmK[6] as a template (PDB ID = 1R9D). Overlay of the LnmK homology model with the LnmK crystal structure revealed conservation of active site architecture and the essential tyrosine residue (Figure S11).

Figure S11. Homology model of Lom62. A) Overlay of the Lom62 homology model (green) with the crystal structure of LnmK (blue). B) Active site residues in the putative substrate binding site are conserved between Lom62 and LnmK. Amino acid numbering follows: Lom62/LnmK.



Identification of Lom62 homologs in sequenced bacterial genomes

Table S8. Homologs of acyltransferase/decarboxylase Lom62 in other sequenced bacterial genomes.

Organism	Gene Name	Accession #	% identity, % similarity to Lom62 (aa)	Associated PKS machinery
Salinispora tropica CNB-440	Strop_2227	YP_001159056	91, 95	Type II
Salinispora pacifica CNR942	C549DRAFT_03455	WP_018723047.1	100, 100	Type II
Salinispora pacifica CNT569	B116DRAFT_00913	-	99, 99	Type II
Salinispora pacifica CNY202	K228DRAFT_01213	-	94, 96	Type II
Salinispora arenicola CNR416	H929DRAFT_01265	-	91, 95	Type II
Salinispora tropica CNT261	H301DRAFT_01269	-	91, 95	Type II
Salinispora tropica CNR699	C589DRAFT_01301	-	91, 95	Type II
Salinispora tropica CNB536	B099DRAFT_03282	-	91, 95	Type II
Salinispora tropica CNB476	B098DRAFT_00834	-	91, 95	Type II
Salinispora tropica CNS416	C590DRAFT_01286	-	91, 95	Type II
Salinispora tropica CNS197	B123DRAFT_01569	-	91, 95	Type II
Salinspora tropica CNH898	B037DRAFT_02736	-	91, 95	Type II
Salinispora pacifica CNS055	C550DRAFT_00482	WP_018725035.1	92, 95	Type II
Salinispora pacifica CNY239	H304DRAFT_00437	-	91, 94	Type II
Salinispora pacifica CNT131	B172DRAFT_00450	-	91, 94	Type II
Salinispora pacifica CNR894	B119DRAFT_00187	-	91, 94	Type II
Salinispora pacifica CNT603	B175DRAFT_00600	-	91, 94	Type II
Salinispora pacifica CNT001	B169DRAFT_00206	-	91, 94	Type II
Salinispora pacifica CNR114	B118DRAFT_00607	-	91, 94	Type II
Salinispora pacifica CNS103	B117DRAFT_01176	-	91, 94	Type II
Salinispora pacifica CNT851	C585DRAFT_01116	WP_018821085.1	91, 95	Type II
Salinispora pacifica CNT796	B120DRAFT_00957	-	91, 95	Type II
Salinispora pacifica DSM 45544	Salpac3DRAFT_0636	WP_018219817.1	91, 94	Type II
Salinispora pacifica CNQ768	C581DRAFT_02820	WP_018811720.1	91, 94	Type II

Salinispora pacifica CNT003	B167DRAFT_01034	-	91, 94	Type II
Salinispora pacifica CNT609	C584DRAFT_03447	WP_018818745.1	91, 94	Type II
Salinispora pacifica CNT084	B171DRAFT_01452	-	91, 94	Type II
Salinispora pacifica CNT855	B113DRAFT_00239	-	90, 94	Type II
Salinispora pacifica CNY331	C588DRAFT_00998	-	91, 95	Type II
Salinispora pacifica CNY330	C587DRAFT_01997	WP_018827311.1	91, 95	Type II
Salinispora pacifica CNT148	B159DRAFT_00692	-	90, 93	Type II
Salinispora pacifica DSM 45548	Salpac2DRAFT_0954	WP_018215382.1	90, 93	Type II
Amycolatopsis alba DSM 44262	AmyalDRAFT_5878	WP_020634866.1	42, 57	Type II
Actinoplanes missouriensis 431	AMIS_50400	BAL90260	38, 52	Type II
Hamadaea tsunoensis DSM 44101	G401DRAFT_02977	-	42, 60	Type I modular, Type II
Streptomyces pristinaespiralis ATCC 25486	SSDG_05667	ICBW45697	43, 56	Type I modular, Type II
Catenulispora acidiphila DSM 44928	Caci_2599	ACU71517	47, 62	Type I modular
Myxococcus xanthus DK 1622	MXAN_3947	ABF88380	45, 59	Type I modular
Streptomyces atroolivaceus S-140	LnmK	AAN85524	44, 60	Type I modular
Saccharothrix espanaensis DSM 44229	BN6_46160	CCH31895	45, 58	Type I modular
Micromonospora sp. L5	ML5_4523	ADU09999	47, 61	Type I modular
Micromonospora aurantiaca ATCC 27029	Micau_3894	ADL47418	47, 60	Type I modular
Myxococcus xanthus DZ2	MXAN_02625	-	45, 59	Type I modular
Salinispora arenicola CNP105	C572DRAFT_01756	WP_018792598.1	46, 59	Type I modular
Salinispora arenicola CNH964	B164DRAFT_01276	-	46, 59	Type I modular
Micromonospora sp. CNB394	C569DRAFT_00140	WP_018783895.1	46, 59	Type I modular
Streptomyces rapamycinicus NRRL 5491	M271_00340	AGP51707.1	39, 54	Type I modular
Micromonospora sp. ATCC 39149	MCAG_05228	IEEP74901	38, 53	none

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