Supporting Information

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SI Materials and Methods

Construction of Gene Replacement and Complementation Mutants. The *napB* and *napD* genes were inactivated with the application of the REDIRECT Technology system. A spectinomycin resistance gene *aadA*/*oriT* cassette was used to replace an internal region of each target gene (amplified with primers *napB*-For/Rev and *napD*-For/Rev; Table S2). Mutant cosmids Δ*napB* and Δ*napD* for gene inactivation were constructed (Fig. S2) and introduced into *Streptomyces lusitanus* NRRL 8034 by conjugation from *Escherichia coli* S17-1. The double-crossover mutants found to be spectinomycin-resistant and apramycin-sensitive were selected. The genotypes of the resultant mutants were confirmed by PCR (amplified with primers *napB*-E-For/Rev and *napD*-E-For/Rev; Table S2) and named *S. lusitanus* TG3001 (Δ*napB*) and *S. lusitanus* TG3002 (Δ*napD*).

To complement *napB* and *napD* in mutant *S. lusitanus* TG3001 and *S. lusitanus* TG3002, a 4.6-kb HindIII/XbaI fragment containing the intact *napB–napC–napD* gene cassette together with a 0.45-kb EcoRI/HindIII promoter *PermE** fragment were cloned to pSET152, giving rise to plasmid pTG3012. The result plasmid was introduced into *S. lusitanus* TG3001 (Δ*napB*) and TG3002 (Δ*napD*) to yield *S. lusitanus* TG3003 and *S. lusitanus* TG3004, respectively. As to heterologous complementation of *qncL* and *qncN*, a 4.5-kb HindIII/XbaI fragment of the *qncN–qncM–qncL* gene cassette from *Streptomyces melanovinaceus* with the same promoter *PermE** fragment was cloned into pSET152 to yield pTG3013, which was introduced into *S. lusitanus* TG3001 (Δ*napB*) and TG3002 (Δ*napD*) to give rise to *S. lusitanus* TG3005 and *S. lusitanus* TG3006. The colonies with apramycin resistance were identified as complementation mutants with a PCR test (amplified with primers *napB*-E-For/Rev, *napD*-E-For/Rev, *qncN*-For/Rev, and *qncL*-For/Rev; Table S2).

Production and Analysis of Naphthyridinomycin (NDM). *S. lusitanus* NRRL 8034 WT and recombinant strains were grown in a seed culture (0.4% yeast extract, 1.0% malt extract, and 1.0% glucose) at 28 °C for 48 h. For fermentation, 3 mL of seeding culture suspension of each strain was transferred into a 500-mL flask containing 100 mL of broth [0.3% trypticase soy broth, 0.5% cotton seed meal, 0.3% yeast extract, 2.0% (wt/vol) molasses, 2.0% (wt/vol) glucose, and 0.4% $CaCO_3$] and incubated at 25 °C for 96 h. Each 100 mL of the culture broth was filtered and extracted twice with 100 mL of methylene chloride, and the combined extract was finally concentrated to 500 μL. HPLC analysis was carried out on a Kromasil 100-C18 column (5μ, 4.6 × 250 mm). The column was equilibrated with 50% solvents A (H_2O and 0.1% HCOOH) and B (CH_3CN and 0.1% HCOOH) and developed with the following program: 0–15 min, a linear gradient from 10% B to 12% B; 15–20 min, a linear gradient from 12% B to 55% B; 22–28 min constant 55% B. This process was carried out at a flow rate of 1 mL/min and UV detection at 270 nm using an Agilent 1100 HPLC system. The identity of compound was confirmed by liquid chromatography/MS (LC-MS) analysis performed on a Thermo Scientific LCQ FLEET system under the same conditions. NDM showed $[M + H]^+$ ion at m/z = 418.3, consistent with the molecular formula $C_{21}H_{27}N_3O_6$.

Precursor Feeding and Isolation of NDM. For preparation of cyanonaphthyridinomycin (CN-NDM), 1 mM potassium cyanide was added to the filtered broth after a 96-h fermentation of *S. lusitanus* NRRL 8034. Thus, NDM can be converted to its cyanide derivative, which is stable in concentrated organic solvent, and used for isolation and spectral studies. After stirring at room temperature for 3 h, the antibiotic was extracted with 2 × 1 L of methylene chloride from 1 L of filtered fermentation medium, and the extracts were concentrated in vacuo to give a dark brown residue. This residue was further purified by silica gel column chromatography (ethyl acetate/petroleum ether 30:70 as eluent) to give the pure cyanide derivative CN-NDM. The spectrum of this bright orange compound in ^{13}C NMR (100.6 MHz, $CDCl_3$) was the same as that reported in ref. 1.

To figure out the possible origin of the two-carbon unit C9–C9′, we fed isotope-labeled precursors (500 mg of [2-^{13}C]pyruvate, 200 mg of [2-^{13}C]glycine, 800 mg of [2-^{13}C]glucose, and 500 mg of [1-^{13}C]fructose; Sigma-Aldrich) to the NDM producer (*S. lusitanus* NRRL 8034). Each isotope-labeled precursor was added to 10 × 500-mL flasks, containing 10 × 100 mL of broth cultures after 48 h, 60 h, and 72 h of incubation under sterile conditions. After fermentation, the cultures were extracted and treated as described above. The pure labeled cyanide derivative CN-NDM was isolated and spectral-analyzed by ^{13}C NMR (100.6 MHz, $CDCl_3$).

Protein Expression and Purification. The *qncM* gene encoding the acyl carrier protein (ACP) domain was amplified by PCR from cosmid pTG3401 using the primers shown in Table S2. PCR products were purified, cloned, and confirmed by sequencing, then an NdeI/HindIII fragment was cloned into the same sites of pET28a to make the expression plasmid pTG3014. To produce the corresponding holo-protein, *E. coli* BL21 (DE3) was cotransformed with pTG3014 and pSU20-Sfp, which harbors the gene encoding Sfp, the phosphopantetheinyl transferase from *Bacillus subtilis*. Cells were grown in LB medium supplemented with 50 μg/mL kanamycin and 25 μg/mL chloramphenicol (chloramphenicol for pSU20-Sfp selection). Cultures (1 L) were incubated to an OD_{600} of 0.4 to ∼0.5 at 37 °C and then cooled to 16 °C for 30 min. The cultures were induced with 50 μM isopropyl-β-D-thiogalactopyranoside and grown for an additional 24 h. The purification of the His-tagged fusion protein with Ni-NTA affinity resin was performed according to the manufacturer's manual (Qiagen). After desalting with a PD-10 column (GE Healthcare) and exchange into storage buffer [50 mM Mops (pH 7.8), 50 mM NaCl, and 10% glycerol], the purified recombinant holo-ACP was concentrated with an Amicon Ultra-4 (10 kDa; GE Healthcare) and stored at −80 °C.

qncL and *qncN* were amplified by PCR from cosmid pTG3401 using the primers shown in Table S2. PCR products were purified, cloned, and confirmed by sequencing, then the NdeI/XhoI fragment for *qncL* and the NdeI/HindIII fragment for *qncN* were cloned into the same sites of pET37b and pET28a separately to make the expression plasmid pTG3015 (for QncL) and pTG3016 (for QncN). The NdeI/BlnI fragment from pTG3015 and the NcoI/HindIII fragment from pTG3016 were cloned into pCDFDuet-1 to yield pTG3017 for coexpression of QncL and QncN. Overexpression in *E. coli* BL21 (DE3) and purification of the recombinant QncN/QncL proteins were performed following the previously described procedures.

HPLC and LC-MS Analysis of Enzymatic Reaction. HPLC analysis was carried out on a GRACE/Vydac protein and peptide C8 column (W. R. Grace & Co.) with detection at 220 nm. The column was equilibrated with 80% solvent A [H_2O and 0.1% trifluoroacetic acid (TFA)] and 20% solvent B (CH_3CN and 0.1% TFA) and developed with the following program: 0–3 min, constant 70%

A/30% B; 3–20 min, a linear gradient to 50% A/50% B; 20–25 min, a linear gradient to 19% A/81% B; 25–27 min, a linear gradient to 5% A/95% B; 27–29 min, a linear gradient to 5% A/95% B; and 29–33 min, constant 80% A/20% B. It was carried out at a flow rate of 1 mL/min with UV detection at 220 nm, using a SHIMADZU HPLC system. Product analysis was carried out by the Agilent 6530 Accurate-Mass Quadrupole–Time of Flight (Q-TOF) LC-MS.

Further Biochemical Assay to Determine the Time Courses and Kinetics for the Donor Substrates. The time courses of QncN/QncL-catalyzed two-carbon glycolaldehyde unit transformation were carried out in 50 μL of reaction with 2 mM xylulose-5-phosphate (X-5-P), 2 mM fructose-6-phosphate (F-6-P), 2 mM sedoheptulose-7-phosphate (S-7-P), 2 mM hydroxypyruvate (HPA), and 10 mM dihydroxyacetone (DHA) as the donor substrates. The reactions containing 50 mM Mops (pH 7.5), 2.5 mM $MgCl_2$, 400 μM thiamin diphosphate (ThDP), 100 μM holo-ACP, 50 μg (1 mg/mL) QncN/QncL, 5 μM pyruvate dehydrogenase (PDH) E3, 250 μM FAD, and 1 mM NAD were initiated by addition of donor substrates and incubated at 30 °C. The reactions were quenched with 10% TFA at different time points (5, 10, 15, 25, 45, and 120 min for X-5-P, F-6-P, and S-7-P and 5, 25, and 120 min for HPA and DHA). After centrifugation, the clarified supernatant was analyzed by HPLC following the conditions described above. HPLC analysis showed that the enzymatic reaction was time-dependent, enabling the determination of single-substrate kinetic constants.

To determine the kinetics for the donor substrates for X-5-P, F-6-P, and S-7-P, 50 μL of reaction mixtures contained 50 mM Mops (pH 7.5), 2.5 mM $MgCl_2$, 400 μM ThDP, 100 μM holo-ACP, 50 μg (0.1 mg/mL) QncN/QncL, 5 μM PDH E3, 250 μM FAD, 1 mM NAD, and varying concentrations of donor substrates (0.04 mM to 4 mM for X-5-P and 0.1 mM to 4 mM for F-6-P and S-7-P). The reactions were initiated by the addition of donor substrates and carried out in triplicate. The reactions were quenched with 10% TFA after being incubated at 30 °C for a few minutes (3 min for X-5-P and 5 min for F-6-P and S-7-P). After centrifugation, the resulting clarified supernatant was subjected to HPLC analysis. To quantify the amount of product formed from each reaction, a calibration curve based on the HPLC peak area with UV detection at 220 nm was generated with a known amount of holo-ACP. The Michaelis–Menten equation was fit to plots of initial rate of product formation versus substrate concentration to extract values for the K_m and k_{cat} parameters.

1. Zmijewski M, Jr., Mikolajczak M, Viswanatha V, Hruby VJ (1982) Biosynthesis of the antitumor antibiotic naphthyridinomycin. *J Am Chem Soc* 104:4969–4971.

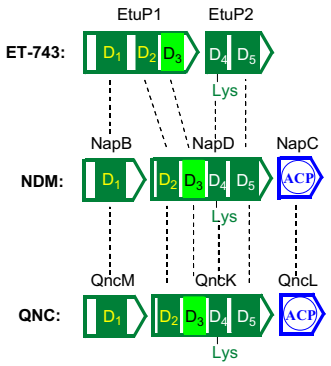


Fig. S1. Comparison between the transketolase (TKase) systems in NDM, quinocarcin (QNC), and ecteinascidin 743 (ET-743) biosynthesis. D1, ThDP binding domain; D2, PDH/TKase pyrimidine binding domain; D3, TKase C-terminal domain; D4, lipoyl attachment domain; D5, acyltransferase catalytic domain.

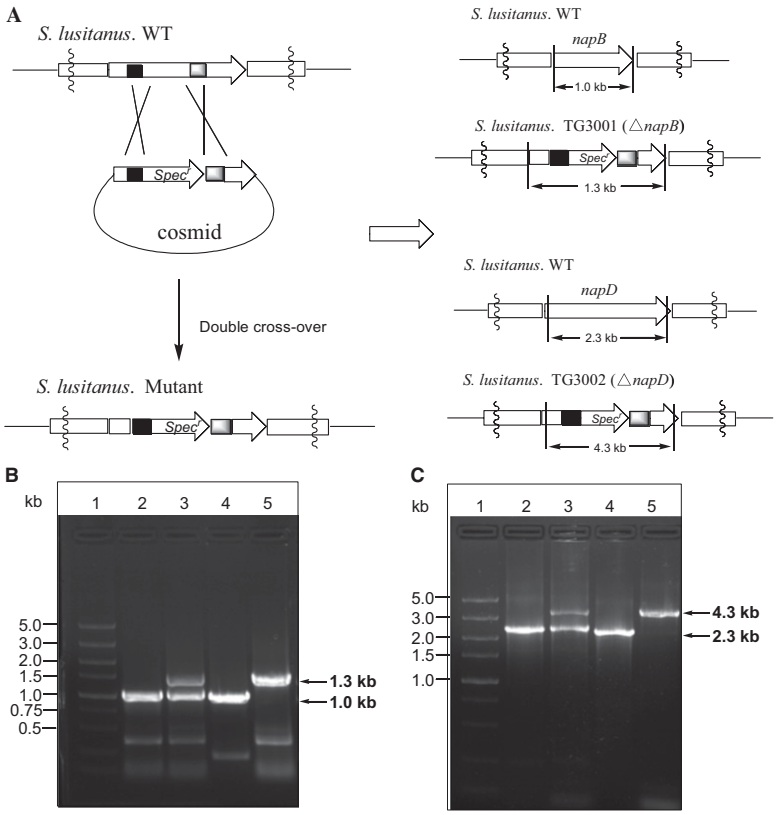
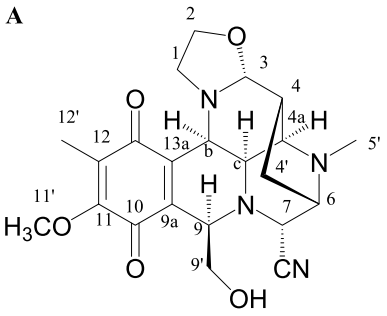


Fig. S2. Identification of the genotype of gene replacement mutants. (*A*) Construction of gene replacement strains via homologous recombination. (*B*) PCR analysis of the genotype of mutant strain *S. lusitanus* TG3001 (Δ*napB*) and respective complementary mutants. The genome DNA was extracted from the mutant strains *S. lusitanus* TG3001 (lane 5), *S. lusitanus* TG3003 (lane 3), and WT *S. lusitanus* NRRL 8034 (lane 2) for PCR amplification as template using *napB*-E-For and *napB*-E-Rev as primers. A 1.3-kb signal can be detected with the genome DNA of correct mutant strains, whereas the WT strain gives a 1.0-kb signal. Lane 4 is the PCR result using genome DNA from *S. lusitanus* TG3005 as template and *qncN*-For and *qncN*-Rev as primers, which give a 1.0-kb signal. (*C*) PCR analysis of the genotype of mutant strain *S. lusitanus* TG3002 (Δ*napD*) and respective complementary mutants. The genome DNA was extracted from the mutant strains *S. lusitanus* TG3002 (lane 5), *S. lusitanus* TG3004 (lane 3), and WT *S. lusitanus* NRRL 8034 (lane 2) for PCR amplification as template using *napD*-E-For and *napD*-E-Rev as primers. A 4.3-kb signal can be detected with the genome DNA of correct mutant strains, whereas the WT strain gives a 2.3-kb signal. Lane 4 is the PCR result using genome DNA from *S. lusitanus* TG3006 as template and *qncL*-For and *qncL*-Rev as primers, which give a 2.2-kb signal.

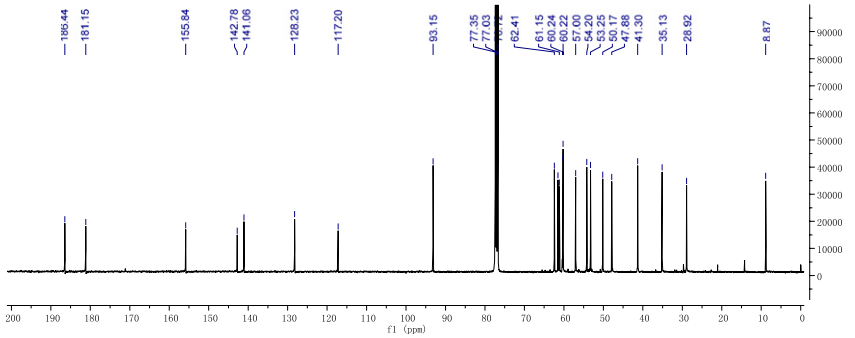
A



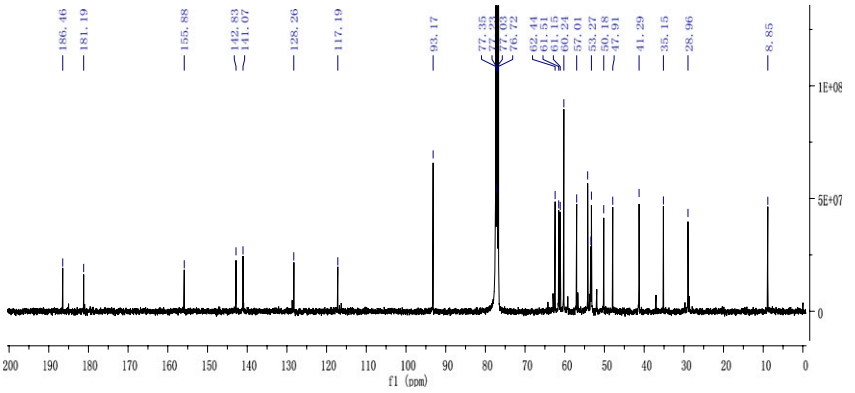
$C_{22}H_{26}N_4O_5$

No. of C	δ/C-13	No. of C	δ/C-13
1	50.1	10	181.1
2	61.5	11	155.8
3	93.1	12	128.2
4	35.1	13	186.4
4a	60.18	13a	142.7
4'	28.9	13b	47.9
6	62.4	13c	53.2
7	54.2	5'	41.3
9	57.0	11'	61.1
9'	60.21	12'	8.8
9a	141.0	-CN	117.2

B



C



D

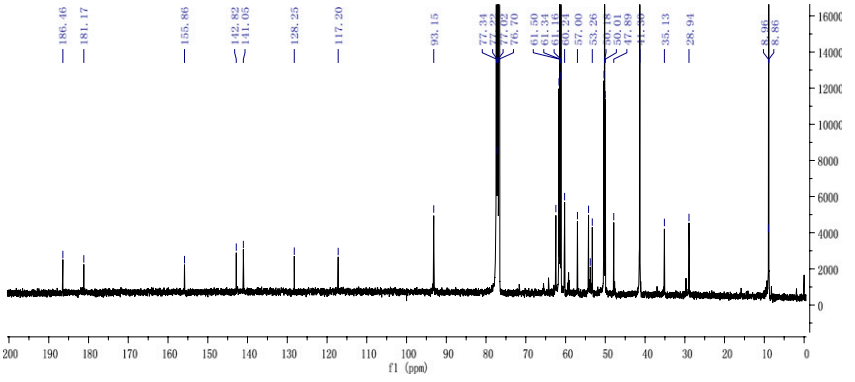


Fig. S3. Investigation of the precursors by feeding experiments. (*A*) The structure of CN-NDM and summary of chemical shift (ppm) of every carbon. (*B–D*) ^{13}C NMR spectra of CN-NDM without labeled precursor feeding (*B*), with [2-^{13}C]pyruvate feeding (*C*), and with [2-^{13}C]glycine feeding (*D*) as positive control.

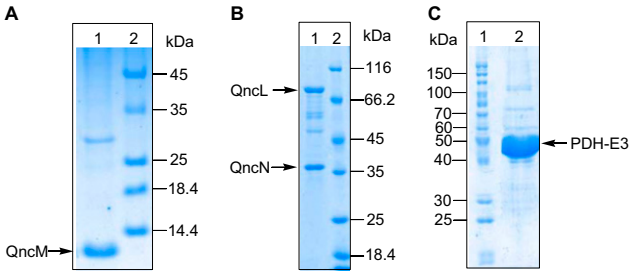


Fig. S4. Purity of recombinant His-tagged proteins monitored by SDS/PAGE gels. (*A*) QncM (ACP). (*B*) QncN and QncL. (*C*) PDH E3 component purified from *E. coli* BL21 (DE3) by expressing the gene from *Streptomyces lividans*.

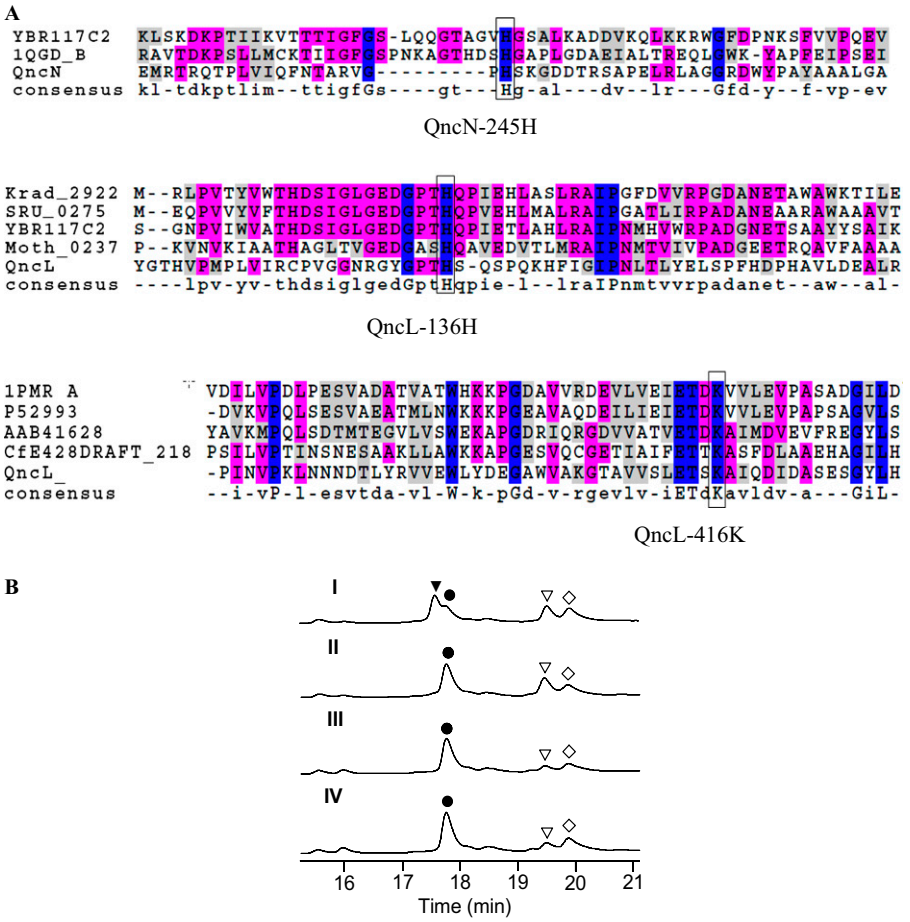


Fig. S5. In vitro enzymatic assay with QncN H245A, QncL H134A, and QncL K416A mutants. (*A*) Multiple sequence alignment of QncN/QncL and its analogs to identify the key active sites. (*B*) Biochemical characterization of glycolicacyl transfer to ACP catalyzed by TKase. HPLC analysis of enzymatic reaction using X-5-P as donor substrate when incubated with QncN/QncL WT (I), QncN/QncL-K416A (II), QncN-H134A/QncL (III), and QncN-H245A/QncL (IV) for 15 min. ●, holo-ACP; ▼, glycolicacyl-*S*-ACP; ◆, glycolicacyl-*O*-glycolicacyl-*S*-ACP; ▽, QncM or QncK; ◇, PDH E3.

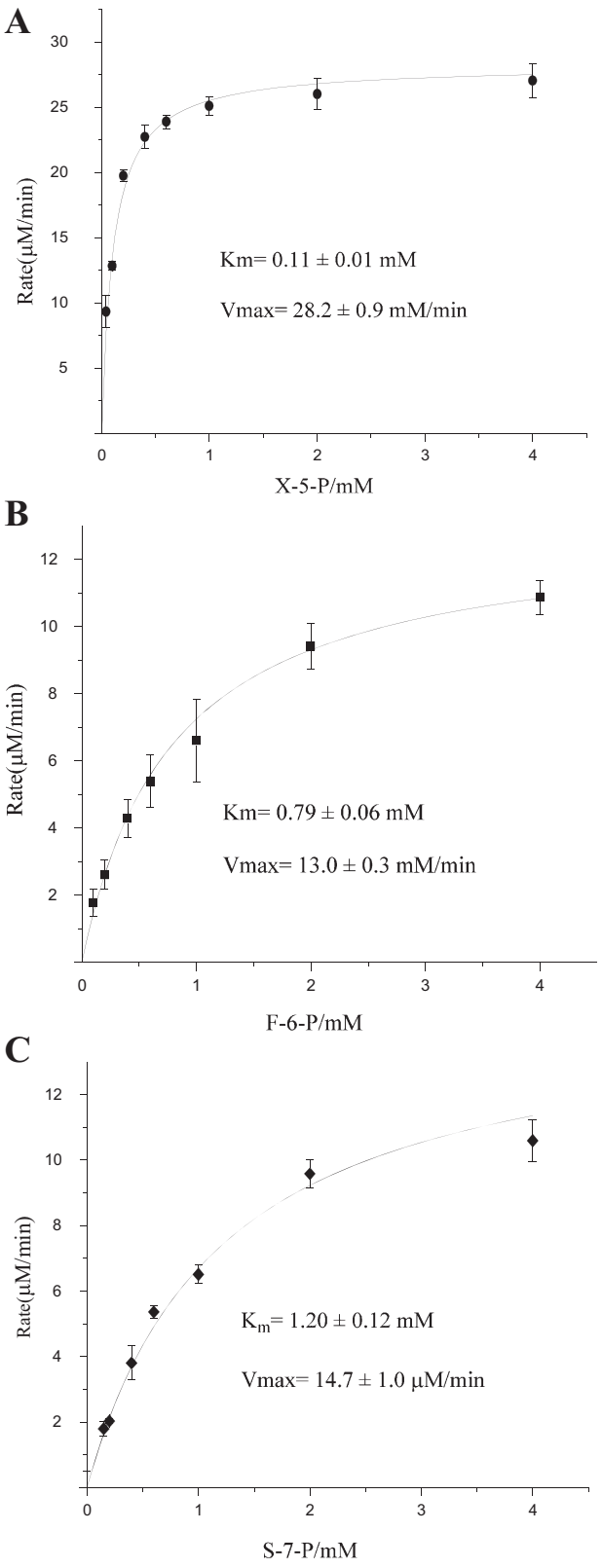


Fig. S6. Kinetic analysis of the glycolicacylation of QncM catalyzed by QncN/QncL with different donor substrates. (*A*) X-5-P as substrate. (*B*) F-6-P as substrate. (*C*) S-7-P as substrate.

Table S1. Strains and plasmids used in this study

Strain/plasmid	Characteristics	Source
Strains		
E. coli DH5α	Host for general cloning	Invitrogen
E. coli S17-1	Donor strain for conjugation between *E. coli* and *Streptomyces*	(1)
E. coli BL21 (DE3)	Host for protein expression	Invitrogen
S. lusitanus NRRL 8034	WT strain, NDM producing	NRRL
S. lusitanus TG3001	Δ*napB* gene replacement mutant, NDM nonproducing	This work
S. lusitanus TG3002	Δ*napD* gene replacement mutant, NDM nonproducing	This work
S. lusitanus TG3003	Δ*napB* gene complementation mutant with pTG3012 NDM producing	This work
S. lusitanus TG3004	Δ*napD* gene complementation mutant with pTG3012, NDM producing	This work
S. lusitanus TG3005	Δ*napB* gene complementation mutant with pTG3013, NDM producing	This work
S. lusitanus TG3006	Δ*napD* gene complementation mutant with pTG3013, NDM producing	This work
Plasmids		
pSP72	Ap^R, *E. coli* subcloning vector	Promega
pANT841	Ap^R, *E. coli* subcloning vector	AF438749
pET28a	Km^R, heterologous expression vector in *E. coli*	Invitrogen
pET37b	Km^R, heterologous expression vector in *E. coli*	Invitrogen
pCDFDuet-1	Sp^R, heterologous coexpression vector in *E. coli*	Invitrogen
pCC1FOS-1	Fosmid vector for genomic library construction	Epicentre
pOJ260	Am^R, *E. coli–Streptomyces* shuttle vector for gene inactivation	(1)
pSET152	Am^R, *E. coli–Streptomyces* shuttle vector for gene complementation	(1)
pTG3001	pCC1FOS-1–based, *S. lusitanus* NRRL 8034 fosmid containing NDM gene cluster (right part)	This work
pTG3401	pJTU2463-based, *S. melanovinaceus* NRRL 12388 cosmid containing QNC gene cluster (right part)	This work
pTG3010	pTG3001 derivative for gene replacement of *napB*	This work
pTG3011	pTG3001 derivative for gene replacement of *napD*	This work
pTG3012	4.6-kb fragment containing *napB–napC–napD* operon under the control of *PermE** in pSET152	This work
pTG3013	4.5-kb fragment containing *qncN–qncM–qncL* operon under the control of *PermE** in pSET152	This work
pTG3014	pET28a derivative containing *qncM* gene for protein expression	This work
pTG3015	pET37b derivative containing *qncL* gene for protein expression	This work
pTG3016	pET28a derivative containing *qncN* gene for protein expression	This work
pTG3017	pCDFDuet-1 derivative containing *qncN* and *qncL* gene for protein coexpression	This work
pTG3018	pET28a derivative containing *PDH E3* gene from *S. lividans* for protein expression	This work

Am^R, apramycin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Sp^R, spectinomycin resistance.

1. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics* (John Innes Foundation, Norwich, UK).

Table S2. PCR primers used in this study

Primers	Sequence (5′ → 3′)	Restriction enzyme
napB-For	CGTGAGGACCTGGAATCCCTGCTGACCATCCGCCACTTCATTCCGGGGATCCGTCGACC	—
napB-Rev	GGGCGGCCGTGCCTCCACCTCACGGGCCATTTCCTCCAGTGTAGGCTGGAGCTGCTTC	—
napB-E-For	ATAGAATTCCATATGACCGCGCGTCCGCCG	EcoRI/NdeI
napB-E-Rev	TATAAGCTTTTACTCGAGGTTCAGCCATCGGTCCTC	HindIII/XhoI
napD-For	GGCGACTTCGCCGCGCTCGCCTTCGACCAGATACTCAAC ATTCCGGGGATCCGTCGACC	—
napD-Rev	GTAGAGGACCTTGTCCTCGAAGAGCATCGCGGGTCCGCG TGTAGGCTGGAGCTGCTTC	—
napD-E-For	ATAGAATTCCATATGTGTCGAACGCGCCTA	EcoRI/NdeI
napD-E-Rev	TATAAGCTTTTACTCGAGCTGCGGGCCGGCGCATGC	HindIII/XhoI
napBCD-For	ATAAGCTTGCACACGCAGTCCTGCCCGC	HindIII
napBCD-Rev	AATCTAGATGGTCAGCACTGGAGGATCGC	XbaI
qncNML-For	ATAAGCTTACGGCCAGCTCTTTGCGC	HindIII
qncNML-Rev	AATCTAGAGCCCACCACTTCCCTGTC	XbaI
QncL-For	ATAGAATTCCATATGACCCGCACCCGGACCTCCGCG	EcoRI/NdeI
QncL-Rev	ATAAAGCTTACTCGAGGCTGTCGAGGAGACGGGCGAG	HindIII/XhoI
QncN-For	ATAGAATTCCATATGGCAGCACCGACCCACGGACC	EcoRI/NdeI
QncN-Rev	ATAAAGCTTACTCGAGCGCCGCTGTGGCTCCTCCCG	HindIII/XhoI
QncM-For	ATATCTAGACATATGACCACCGTCGAGAATCTCGTC	EcoRI/NdeI
QncM-Rev	ATAAAGCTTACTCGAGTGAATTCAATCCCTTTGCCGAC	HindIII/XhoI
PDH E3-For	ATAGAATTCCATATGGCGAACGACGCCAGCACCGT	EcoRI/NdeI
PDH E3-Rev	ATAAAGCTTACTCGAGCTTCTTCTTCGCTTTCGGGTTCG	HindIII/XhoI

Underline indicates the recognization sequence of restriction enzyme. For, forward; Rev, reverse.