Supplemental Information

Recycling of over-activated acyls by a Type II Thioesterase during Calcimycin biosynthesis in *Streptomyces chartreusis* NRRL 3882

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Supplemental Methods

Calcimycin production time course experiments

S. chartreusis NRRL 3882 WT (200mL culture in 500mL baffled conical flasks) was grown at 30 ℃ for up to 9 days. 20 ml of culture was removed aseptically every 24 hours and assayed for calcimycin as described in the **Material and Methods** section. All experiments were repeated triplicate.

Quantification of *calG* mRNA present in *S. chartreusis* NRRL 3882 WT cultures by qPCR

Using the results obtained from the calcimycin production time course experiments described above (Fig. S8), we selected the collection time points of 48 and 120 h for additional assay of the culture for *calG* transcript. These two time points were chosen because at 48 h calcimycin starts to be present in the culture and at 120 h it begins to be produced at high level. In addition the effect of supplementation of growth medium with divalent cations, 10 mM $MnCl_2$, $CaCl_2$, $MgCl_2$, or 1 mM $FeCl_2$, on *calG* transcript level was also investigated. These concentrations were selected for study on the basis of data presented in Table S5. Effectively they represent ion concentrations at which calcimycin production by *S. chartreusis* NRRL 3882 WT was greatest whilst cezomycin was lowest. All experiments were conducted in triplicate and followed the general protocol described above for the time course experiments with the exception that cultures were supplemented with the relevant metal ions. To ensure data consistency half the culture was sampled at 48 and the remaining half at 120 h and subjected to total cellular RNA extraction, reverse transcription and qPCR.

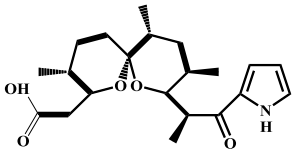
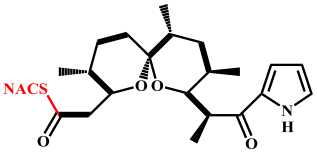
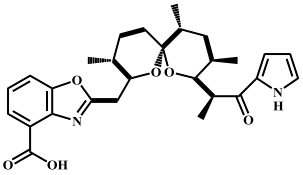
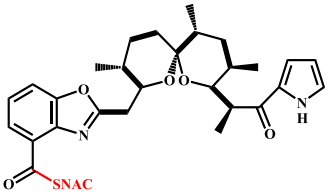
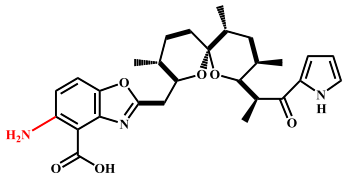
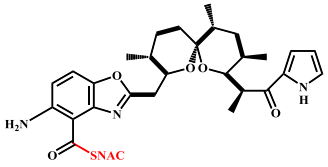
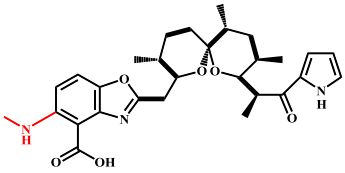
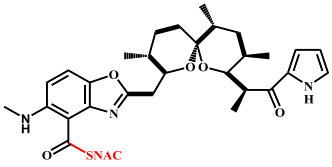
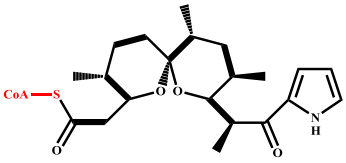
Total cellular RNA was prepared using Isogen reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. RNA concentrations were measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) and to ensure the complete removal of DNA each total RNA sample (1 mg) was treated with 1U of DNAse I (Invitrogen, Carlsbad, CA, USA) for 15min at 25 ℃. The absence of DNA was confirmed by PCR using primers G-QF1and G-QF2 (Table 3). Only total RNA samples which lacked the characteristic 200 bp band produced by the primers after agarose gel electrophoresis were used in subsequent experiments.

Reverse transcription of the total RNA to cDNA was carried out using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix (AT341-01, Transgen Biotech, http://www.transgenbiotech.com/) according to the manufacturer's instructions. Briefly, this kit uses 9 nt length primers with random sequences

in the reverse transcription of mRNA to short single stranded cDNA whose copy number reflects original mRNA abundance. The resulting cDNA pool is then suitable for qPCR using gene specific primers in which the first cycle is the synthesis of short double stranded cDNA fragments. cDNA product concentrations were assayed using Nanodrop 2000 (Thermo Fisher Scientific Inc.) and cDNA samples stored at -80 ℃ until required. qPCR reaction mixtures (25 µL) contained 150 ng cDNA, 12.5 µl SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc.) and 50 nM of the forward and reverse *calG* primers G-QF1 and G-QF2 (Table 3). *calG* transcript levels are represented relative to that of the house-keeping gene, *hrdB,* used as an internal standard, which was co-amplified with *calG* cDNA using the *hrdB* primers shown in Table 3. qPCR reactions were cycled at 95 ˚C for 10 min followed by 40 cycles of 95 ˚C for 15 sec and 60 ˚C for 1 min. A dissociation curve from 55 ˚C to 90 ˚C at 0.5 ˚C increments with a dwelling time of 30 seconds was constructed to confirm product identity and reaction specificity. 7500 software V 2.0.1 (Applied Biosystems) was used for results calculations.

Supplemental tables and figures

Table S1. Physicochemical data relating to compound 3, cezomycin, N-demethyl-calcimycin, calcimycin and related SNAC and CoA variants.

		Formula	Calculated Mass	$[M+H]^+$
Compound 3		$C_{21}H_{31}NO_5$	377.2389	378.2392
Compound 3-SNAC		$C_{25}H_{38}N_2O_5S$	478.2501	479.2574
Cezomycin		$C_{28}H_{34}N_2O_6$	494.2417	495.249
Cezomycin-SNAC		$C_{32}H_{41}N_3O_6S$	595.2716	596.2789
N-demethyl-calcimycin		$C_{28}H_{35}N_3O_6$	509.2526	510.2523
N-demethyl-calcimycin-SNAC		$C_{32}H_{42}N_4O_6S$	610.2825	611.2898
Calcimycin		$C_{29}H_{37}N_3O_6$	523.2682	524.2534
Calcimycin-SNAC		$C_{33}H_{44}N_4O_6S$	624.2982	625.3054
Compound 3-CoA		$C_{42}H_{65}N_8O_{20}P_3S$	1126.3234	1127.3321

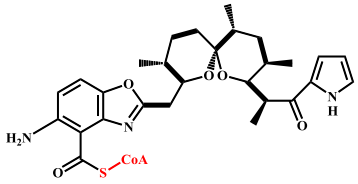
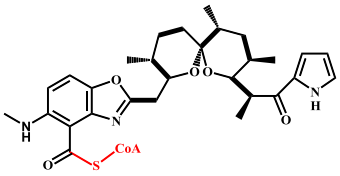
N-demethyl-calcimycin-CoA		$C_{49}H_{69}N_{10}O_{21}P_3S$	1258.3572	1259.3645
Calcimycin-CoA		$C_{50}H_{71}N_{10}O_{21}P_3S$	1272.3729	1273.3802

Fig. S1. Q-TOF MS charaterisation of compound 3, cezomycin, N-demethyl-calcimycin and calcimycin related SNAC derivatives. Pyrrole moiety (m/z=94) and benzoxazole moiety of various calcimycin related compounds. (A) Spiroketal ring of compound 3 (m/z=360). (B) Benzoxazole moiety of cezomycin (m/z=160). (C) Benzoxazole moiety of N-demethyl-calcimycin (m/z=175). (D) Benzoxazole moiety of calcimycin (m/z=189).

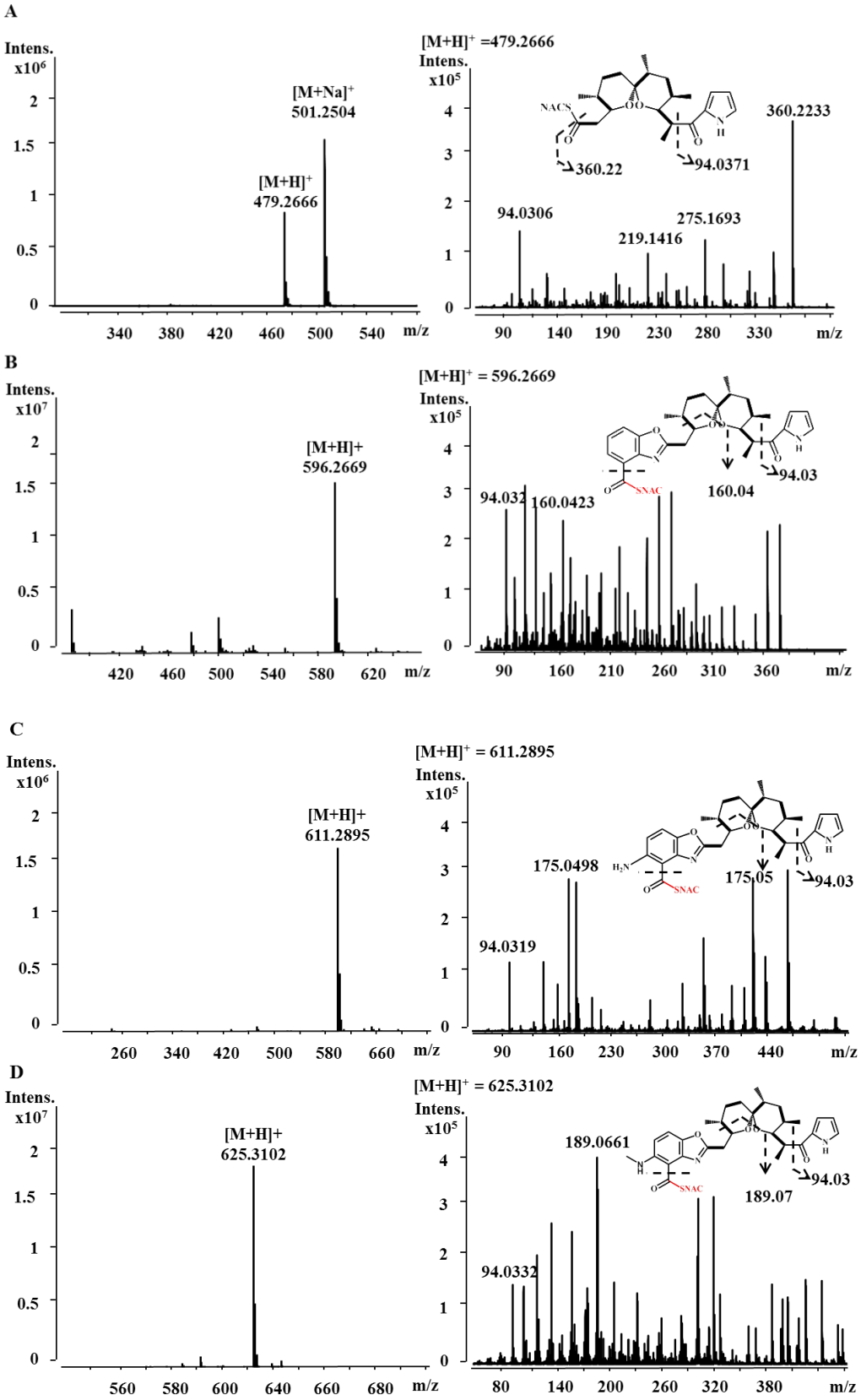


Fig. S2. Q-TOF MS charaterisation of compound 3, cezomycin, N-demethyl-calcimycin, calcimycin related CoA derivatives. Pyrrole moiety (m/z=94) and benzoxazole moiety of various calcimycin related compounds were detected. (A) Spiroketal ring of compound 3 (m/z=360). (B) Benzoxazole moiety of N-demethyl-calcimycin (m/z=175). (C) Benzoxazole moiety of calcimycin (m/z=189).

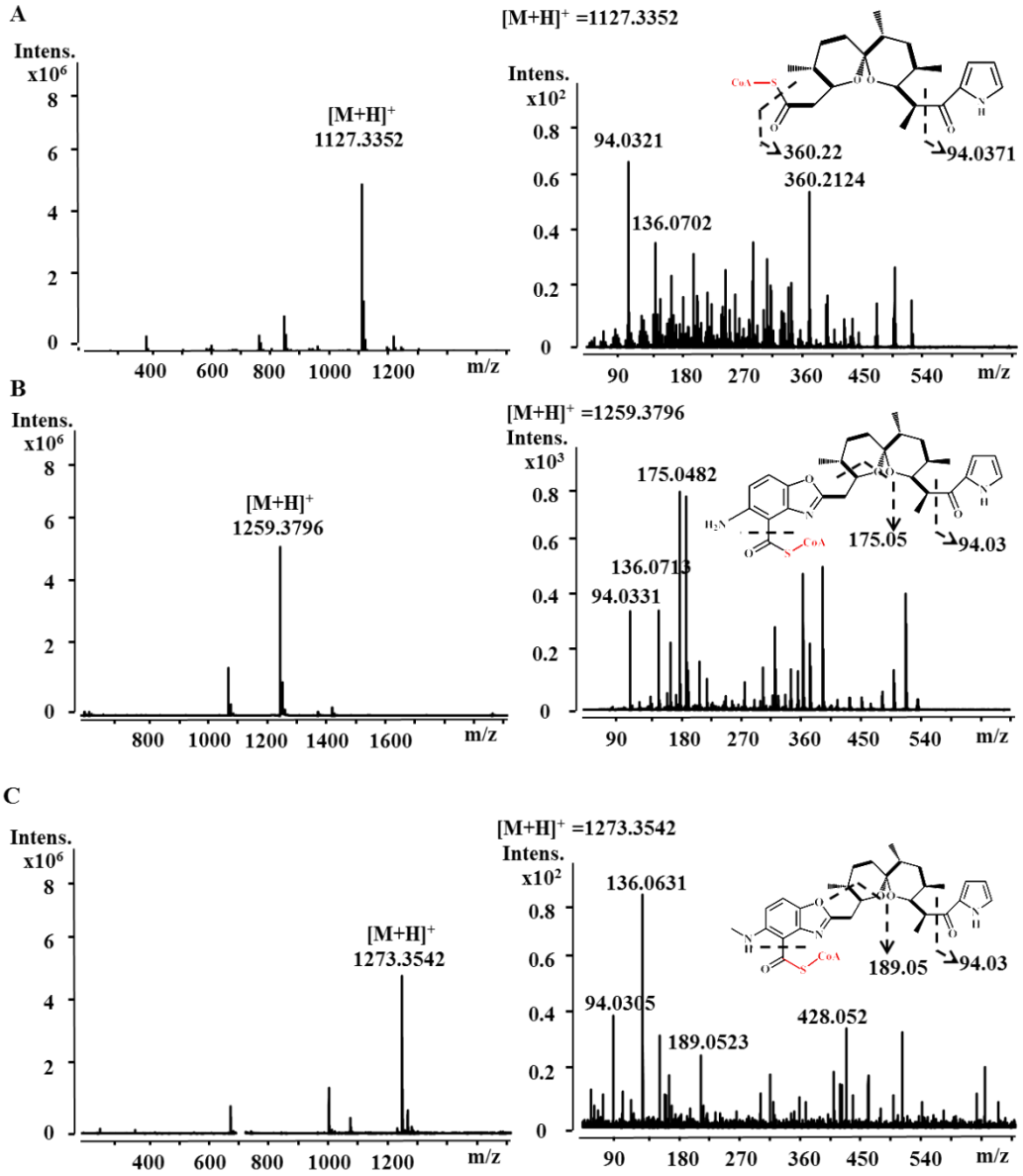


Fig. S3. Protein sequence alignments of CalG with other typical TEIIs.

CalG protein sequence was aligned with TycF from *Brevibacillus brevis* (accession number BAH43770), GrsT from *Paenibacillus mucilaginosus* (accession number AEI41826), TylO from *Streptomyces bikiniensis* (accession number AAS79448) and FscTE from *Streptomyces sp.* FR-008 (accession number AAQ82559). Identical amino acids are shaded black, while similar amino acids are shaded gray. The highly conserved GHSXG motif is boxed and consensus residues are marked with asterisk.

Fig. S4. Construction of the *⊿ calG* strain by PCR-targeting.

(A) Double crossover replacement generated strain GLX16 (*⊿calG*). The black rectangles represent the positions of the primers used to generate the PCR products. (B) Ethidium bromide-stained agarose gel. The size of the 1.69 kb band from GLX16 confirmed successful gene replacement.

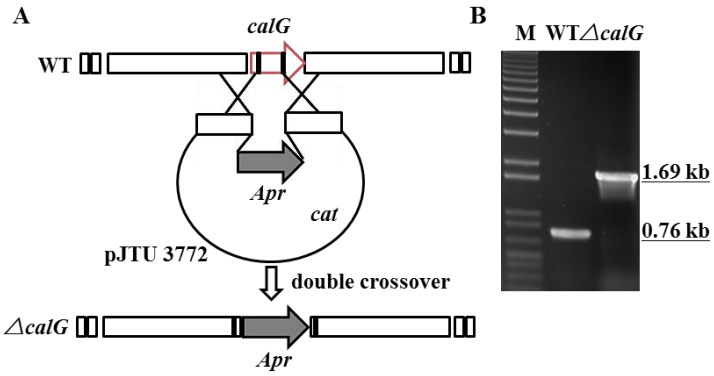


Table S2. HPLC assay of calcimycin and cezomycin production by *S. chartreusis* NRRL 3882, GLX 16 and GLX 17 strains grown in SFM liquid medium (see **Materials and Methods** for a description of the procedures involved).

Strain	Cezomycin (mM)	Calcimycin (mM)	Total (mM)
WT	0.039 ±0.002	0.009 ±0.001	0.048
GLX 16	Not detected	0.044 ±0.005	0.044
GLX 17	0.042 ±0.002	0.011 ±0.0005	0.053

Fig. S5. CalG SNAC derivative substrate usage; HPLC analysis

Traces (i) Standard compounds 1- compound 3, 2 - N-demethyl-calcimycin, 3 - cezomycin and 4 - calcimycin for reference, (ii) to (v) Substrate usage calcimycin-SNAC cezomycin-SNAC, N-demethyl-calcimycin-SNAC and compound 3-SNAC respectively (see the **Materials and Methods** for a full description of the experimental procedure employed).

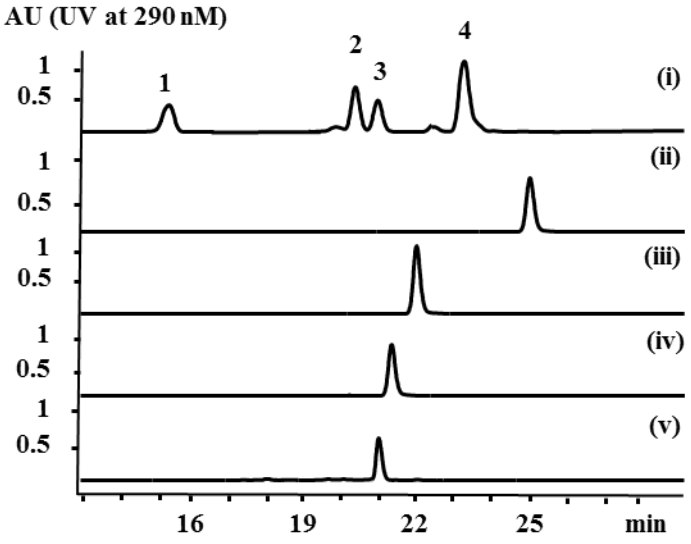


Fig. S6. CalG CoA derivative substrate usage; HPLC analysis Traces (i) CoA reference standard, (ii) to (vi) substrate usage acetyl-CoA, benzoyl-CoA, butyryl-CoA, malonyl-CoA, palmitoyl-CoA respectively. (see **Materials and Methods** for a full description of the experimental procedure employed).

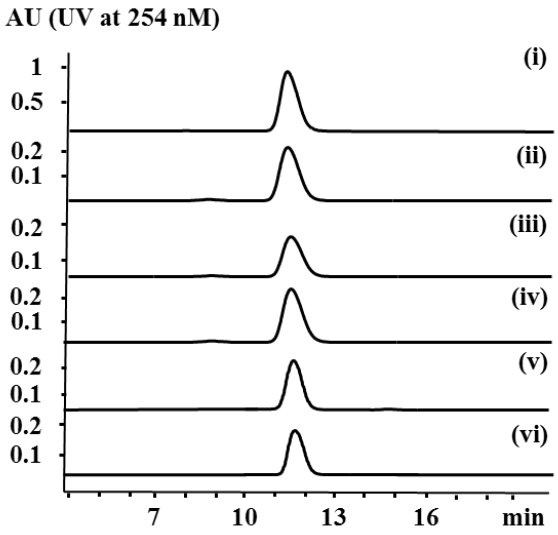


Fig. S7. Saturation curves for CalG with cezomycin-CoA and other CoA derivatives (see **Materials and Methods** for a description of the CalG activity assay procedure involved).

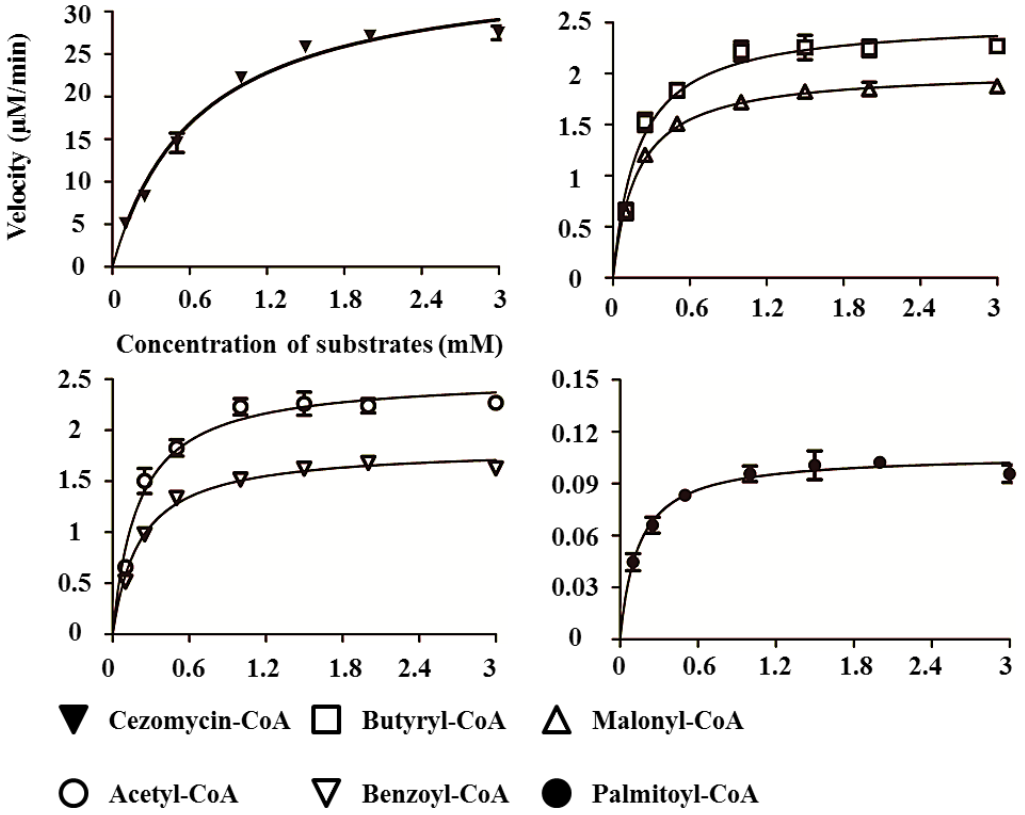


Table S3. CalG substrate usage kinetics; compound 3, cezomycin, N-demethyl-calcimycin and calcimycin related CoA derivatives as substrates. (For a full description of the procedures used see the **Materials and Methods.)**

substrate	K_m(μM)	k_{cat} (min^-)	k_{cat}/K_m($M^{-1}S^{-1}$)	Relative k_{cat}/K_m
Cezomycin-CoA	670 ±61	17.77 ±0.55	442	1
Compound3-CoA	160 ±24	0.053 ±0.002	5.52	0.012
N-demethyl-calcimycin-CoA	167 ±29	0.052 ±0.002	5.19	0.011
Calcimycin-CoA	150 ±20	0.054 ±0.001	6	0.013

Table S4. Effect of divalent cations on the production of calcimycin and cezomycin by *S. chartreusis* NRRL3882 WT in SFM liquid medium. (For a full description of the procedure employed see the **Materials and Methods** describing LC/MS analysis of molecules of interest from fermentation cultures)

	WT control	Ca^{2+} 1mM	Ca^{2+} 5mM	Ca^{2+} 10mM	Mg^{2+} 1mM	Mg^{2+} 5mM	Mg^{2+} 10mM	Mn^{2+} 1mM	Mn^{2+} 5mM	Mn^{2+} 10mM	Fe^{2+} 0.5mM	Fe^{2+} 1mM	Zn^{2+} 0.5mM	Zn^{2+} 1mM
Calcimycin (mM)	0.009	0.024	0.03	0.032	0.024	0.034	0.033	0.028	0.039	0.04	0.035	0.039	0.008	0.009
Standard error	0.001	0.0006	0.0006	0.002	0.001	0.002	0.004	0.002	0.002	0.003	0.003	0.003	0.0005	0.0006
Cezoimycin (mM)	0.039	0.025	0.022	0.01	0.023	0.008	0.009	0.019	0.009	0.009	0.015	0.007	0.038	0.039
Standard error	0.002	0.002	0.0006	0.0008	0.001	0.001	0.0006	0.001	0.0003	0.001	0.0005	0.0004	0.0005	0.003
Total (mM)	0.048	0.049	0.052	0.042	0.047	0.042	0.042	0.047	0.048	0.049	0.050	0.046	0.046	0.048

Fig. S8. Calcimycin production by *S. chartreusis* NRRL 3882 WT cultured in SFM liquid medium over nine days (A) total amount of calcimycin present, (B) ratios of the amount of calcimycin to cezomycin present (For a full description of the procedure employed see the **Supplemental Methods**).

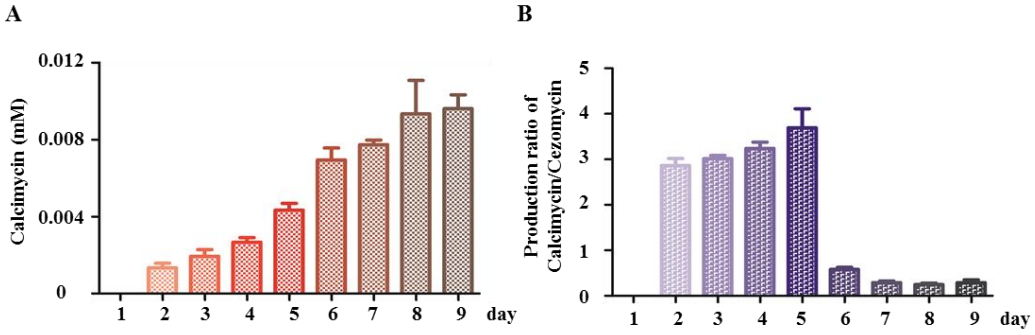


Fig. S9. *calG* transcript profiling.

RNAs were extracted from cells of *S. chartreusis* NRRL 3882 grown in the presence of either 10 mM $MnCl_2$, $CaCl_2$, $MgCl_2$, or 1 mM $FeCl_2$, reverse transcribed and quantified as described in the **Supplemental Methods** section, the relative transcript level for *calG* after 120 h incubation only is shown. No *calG* transcripts were detected at 48h incubation. Error bars indicate the standard deviations of the means derived from three experiments. .

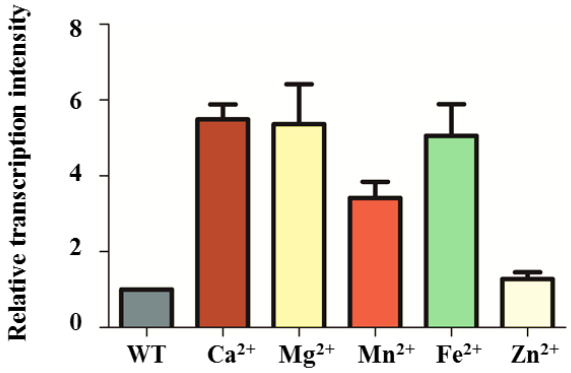


Fig. S10. Initial velocities of CalG hydrolysis of acetyl-CoA in the presence of divalent cations (see **Materials and Methods** for a full description of the CalG protein activity assay procedure involved).