Supporting Information

Biosynthesis of Salinosporamides from α,β-Unsaturated Fatty Acids: Implications for Extending Polyketide Synthase Diversity

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General procedure for the feeding experiments. The *S. tropica* CNB-440 *salL*$^-$ mutant[1] was cultured in Fernbach flasks containing A1 seawater-based media supplemented with Amberlite XAD-7 resin as previously described.[2] Compounds were added aseptically after 24 h of growth. Salinosporamides were obtained by acetone elution from the polymer resin and analyzed by HPLC-MS as described.[3]

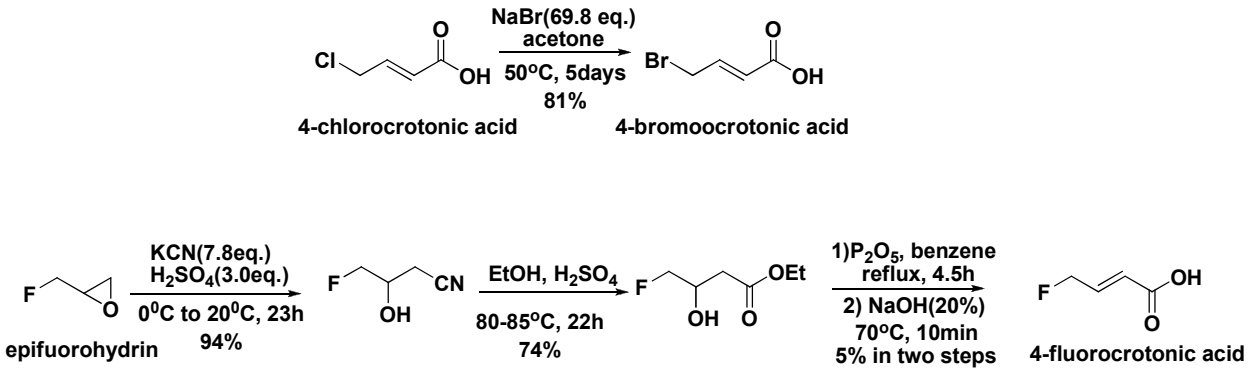
Feeding experiment of sodium [1-^{13}C]propionate to the *S. tropica salL*-deficient mutant. Following the general procedure, sodium [1-^{13}C]propionate was added as a sterilized aqueous solution (100 mg/L) to the mutant. [1-^{13}C]Salinosporamide D (2.0 mg/L) and [12-^{13}C]salinosporamide E (4.5 mg/L) were purified via RP HPLC (Luna C-18, 5 μm, 250 x 10 mm, flow rate 2.5 mL/min, detection at 210 nm) using a gradient method (38% MeCN in H_2O for 18 min, 38% → 100% within 22 min). Salinosporamides D and E eluted at 15 and 29 min, respectively.

[1-^{13}C]Salinosporamide D: white solid; ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.0 (C-1, 5% enriched), 169.0 (C-14), 128.5 (C-7), 127.8 (C-8), 86.1 (C-3), 78.4 (C-4), 69.1 (C-5), 43.1 (C-2), 37.7 (C-6), 25.3 (C-11), 24.6 (C-9), 21.0 (C-10), 18.7 (C-13), 7.4 (C-12). See Supplementary Figure S1.

[12-^{13}C]Salinosporamide E: white solid; ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.0 (C-1), 158.9 (C-16), 128.6 (C-7), 127.7 (C-8), 86.1 (C-3), 78.6 (C-4), 69.1 (C-5), 47.5 (C-2), 37.7 (C-6), 26.9 (C-12, 40% enriched), 25.3 (C-11), 24.6 (C-9), 21.0 (C-10), 20.4 (C-13), 20.0 (C-15), 14.1 (C-14). See Supplementary Figure S2.

Feeding experiments with unlabeled precursors to the *S. tropica salL*-deficient mutant. Following the above general procedure, pentanoic acid (8.1 mg per 100 ml culture), *trans*-2-pentenoic acid (8.0 mg per 100 ml culture), 4-bromocrotonic acid (1.0 mg per 50 ml culture), and 4-flourocrotonic acid (0.8 mg per 50 ml culture) in EtOH were added to the SalL$^-$ mutant in triplicate. Six days after the inoculations, salinosporamides were obtained by acetone elution from the polymer resin and analyzed by HPLC-MS as described.[3]

Synthesis of 4-bromocrotonic acid and 4-fluorocrotonic acid. These compounds were prepared according to known or slightly modified literature procedures as summarized in Supplementary Scheme 1. 4-Bromocrotonic acid[4] was obtained from the reaction of 4-chlorocrotonic acid[5] with sodium bromide in acetone. 4-Fluorocrotonic acid[6] was synthesized from epifluorohydrin in four steps involving nucleophilic attack of cyanide ion upon the epoxide ring[7] followed by the ethanolysis[8] and dehydration to form the α,β-unsaturated ester[9,10] and hydrolyzed to the acid.[6]



Supplementary Scheme 1. Summary of the synthesis of 4-bromo- and 4-fluorocrotonic acid.

(E)-4-bromocrotonic acid. To a solution of 4-chlorocrotonic acid (0.41 g, 3.40 mmol)[5] in acetone (180 ml) was added NaBr (24.42 g, 237.35 mmol, 69.8 eq.) and stirred at 50 °C for 5 days. After the salt was filtered off, the solution was concentrated and purified by flash chromatography (3:1 hexane: ethyl acetate) to afford the white solid (0.46 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.03 (dd, *J* =7.6, 1.2 Hz, 2H), 6.05 (dt, *J* = 15.2, 1.2 Hz, lH), 7.10 (dt, *J* = 15.2, 7.2 Hz, 1H).

Characterization of SalG. Recombinant SalG was prepared and assayed as previously described.[3] Assay conditions involved SalG (21 nM) incubated with various concentrations of trans-2-pentenyl-CoA in reaction buffer (100 mM Tris-HCl, pH 7.9) under saturating levels of NADPH and $NaHCO_3$. Data were collected in triplicate, averaged and apparent k_{cat} and K_M determined by nonlinear regression with GraFit 4.012 (Middlessex, U.K.). See Supplementary Figure S3 for kinetic plots. Confirmation of the propylmalonyl-CoA product from 5 mL of SalG-catalyzed reactions was carried out by LC-MS analysis. LC conditions comprised a H_2O to MeOH gradient containing 5 mM NH_4OAc with a flow rate of 0.3 ml/min on an Agilent 1200 system using a 2.1 μm Discovery HS C18 reversed phase column (Supelco). Mass spectra were collected on a micrOTOF_Q (Bruker Daltonics) mass spectrometer equipped with an electrospray ion source operating in positive mode. ESI-MS *m/z* 850.1 (trans-2-pentenyl-CoA), *m/z* 896.1 (propylmalonyl-CoA).

Trans-2-pentenyl-coenzyme A. To a solution of *trans*-2-pentenoic acid (52 mg, 0.514 mmol, 80 equiv.) in anhydrous THF (8 ml), cooled to 0 °C and under argon, was added triethylamine (72 μL, 0.514 mmol. 80 equiv.) followed by ethyl chloroformate (10 μL, 0.103 mmol, 6 equiv). The

resulting was stirred at 0 °C for 45 min and then centrifuged. The THF supernatant was added slowly to a solution of hydrated coenzyme A (5 mg, 0.006 mmol, 1 equiv.) and $NaHCO_3$ (30 mg, 0.36 mmol) in dd H_2O (3 ml). The reaction mixture was stirred at room temperature for 4 hr after which the THF was removed in vacuo. The aqueous solution was acidified to pH 3 using 1 N HCl and then extracted with EtOAc (3 x 3 ml) to remove excess trans-2-pentenoic acid. The aqueous solution was then lyophilized and the resulting solid was washed with MeOH to recover trans-2-pentenyl-CoA as a white solid. LC-MS analysis was used to confirm formation of the pentenyl-CoA substrate.

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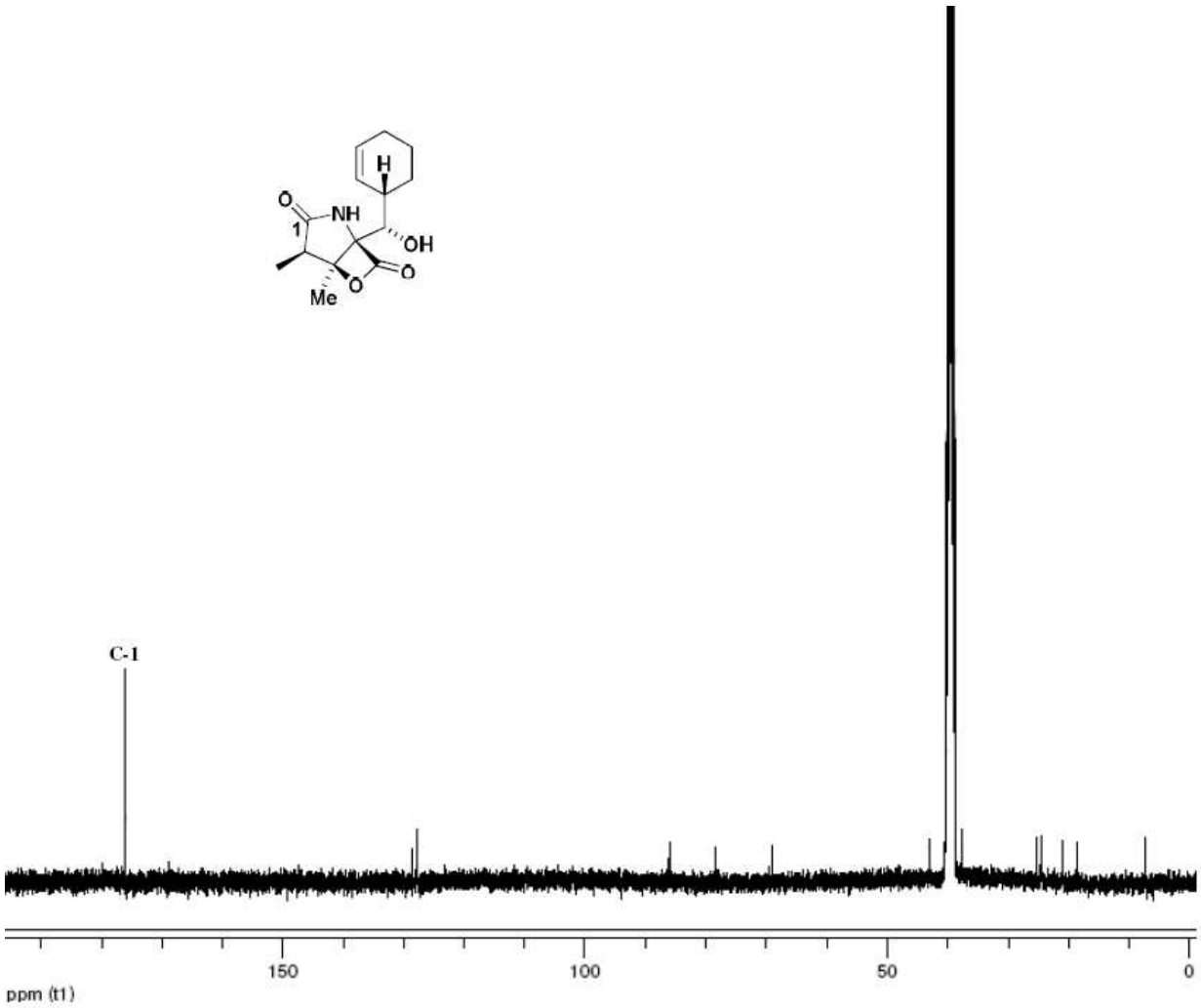
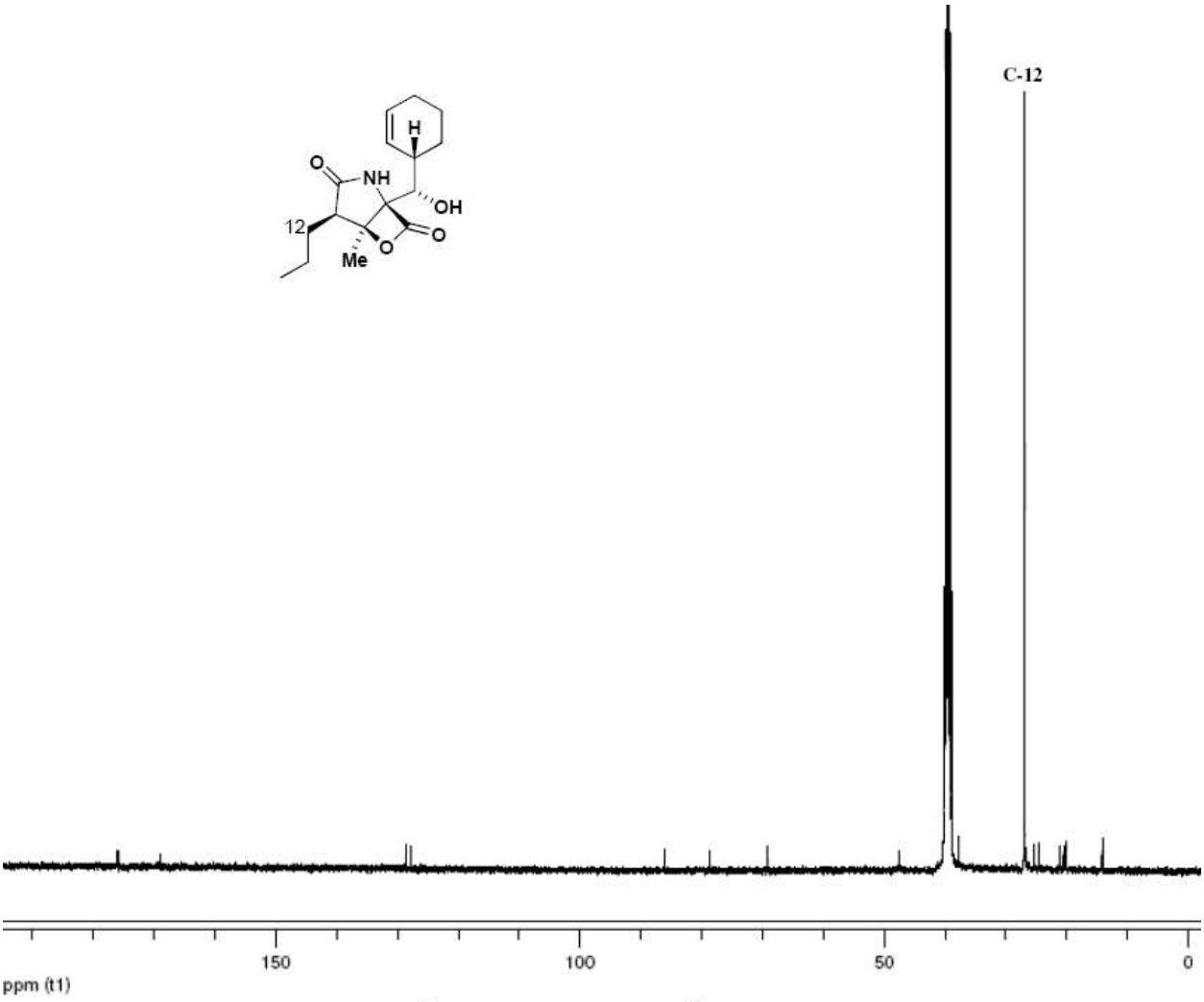
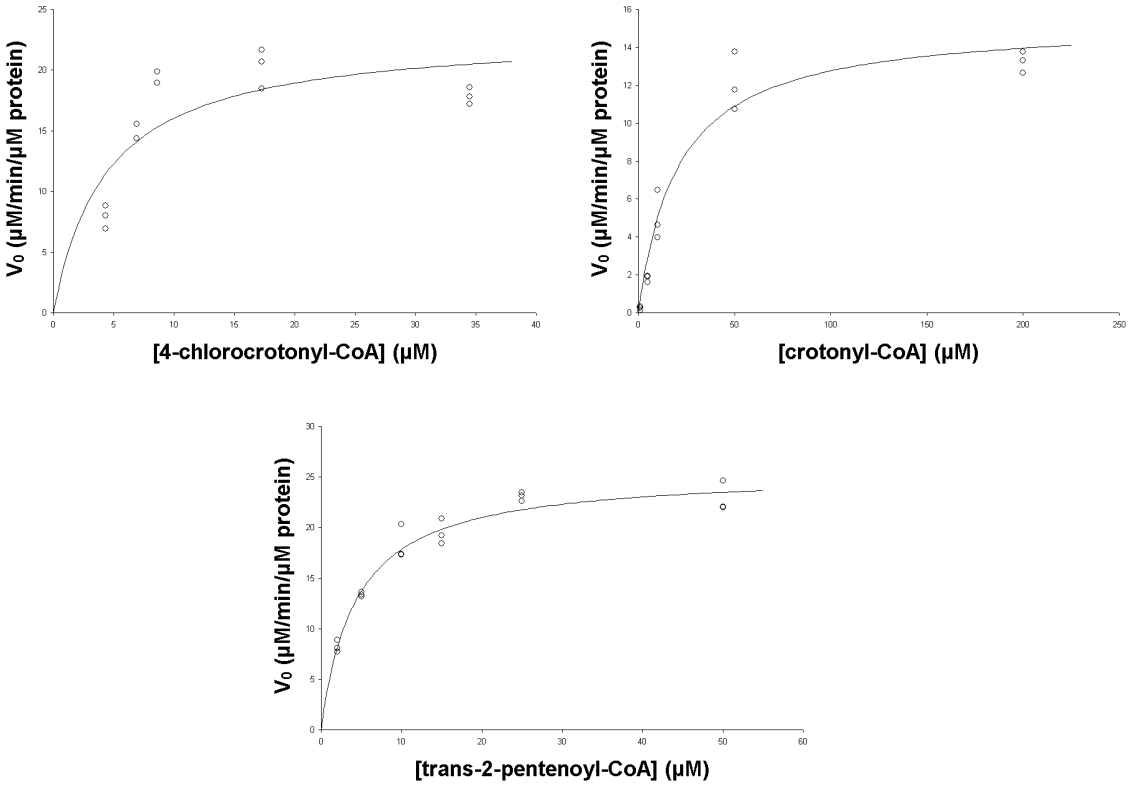


Supplementary Figure S1. ^{13}C NMR spectrum of [1-^{13}C]salinosporamide D in DMSO-d_6



Supplementary Figure S2. ^{13}C NMR spectrum of [12-^{13}C]salinosporamide E in DMSO-d_6



Supplementary Figure S3. Kinetic plots showing rate of SalG-catalyzed reductive carboxylation of substrates (4-chlorocrotonyl-CoA, crotonyl-CoA, and trans-2-pentenoyl-CoA) versus substrate concentrations.