SUPPORTING INFORMATION FOR

Comparative analysis of the substrate specificity of *trans*- versus *cis*-acyltransferases of assembly line polyketide synthases

Briana J. Dunn[‡,†], Katharine R. Watts[‡,#], Thomas Robbins[#], David E. Cane[§], and Chaitan Khosla[†,#,‖,*]

[‡]These authors contributed equally.
[†]Departments of Chemical Engineering, [#]Chemistry, and [‖]Biochemistry, Stanford University, Stanford, California 94305, United States
[§]Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912-9108, United States

*To whom correspondence should be addressed. E-mail: khosla@stanford.edu. Telephone: (650) 723-6538. Fax: (650) 725-7294.

Experimental Procedures

Synthesis of Ethylmalonyl-CoA

Synthesis of ethylmalonic acid:

Diethyl ethylmalonate (1 g, 5.3 mmol) in a solution of ethanol (2.4 mL) and 6 M sodium hydroxide (2.4 mL) was allowed to reflux with stirring for 5 hours. The solution was then acidified to pH 2 with 1 M hydrochloric acid (15 mL) and the resulting diacid was extracted with three 20 mL washes of diethyl ether. The combined extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to produce a white solid (596 mg, 4.5 mmol, yield=85%). ^{1}H NMR (D_2O): δ 0.92 (t, 3H), 1.85 (m, 2H), 3.39 (t, 1H).

Note that the following synthetic steps were modified from Taoka et. al.[1]

Synthesis of monothiophenyl ethylmalonate:

Ethylmalonic acid (132 mg, 1 mmol) was dissolved in a solution of dimethylformamide (DMF, 10 mL) and thiophenol (0.1 mL, 0.91 mmol) at 0°C. A solution of dicyclohexylcarbodiimide (DCC, 252 mg, 1.22 mmol) in DMF (15 mL) was added dropwise over a period of two hours, and stirring was then continued for three hours. The reaction was quenched with water (10 mL) and gravity filtered to remove precipitated dicyclohexyl urea. This process was repeated three times. The filtrate was then acidified to pH~2.5 using 1M hydrochloric acid, and the monothiophenyl ethylmalonate was extracted with four 100 mL washes of diethyl ether. The combined extracts were then washed with 50 mL 0.1 M hydrochloric acid and concentrated to ~50 mL *in vacuo*. This concentrated extract was washed twice with 0.2 M sodium bicarbonate, and the aqueous phase was washed three times with diethyl ether (50 mL) to remove traces of thiophenol and dithiophenyl ethylmalonate. The pH of the aqueous layer was adjusted to ~2 with cold 1 M hydrochloric acid, and was then extracted with two 100 mL washes of diethyl ether. The organic extracts were combined and washed with 50 mL brine and dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to produce a

white solid. This solid was then dissolved in dichloromethane and placed at -20°C overnight to allow residual dicyclohexyl urea to precipitate. The supernatant was concentrated *in vacuo* to produce a white solid (49 mg, 0.22 mmol, yield=24%). ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H), 1.88 (m, 2H), 3.47 (t, 1H), 7.09 (s, 5H).

Synthesis of Ethylmalonyl-CoA:

Ethylmalonyl-CoA was synthesized by reaction of free coenzyme A (CoASH) with monothiophenyl ethylmalonate. CoASH (30 mg, 36 μmol) dissolved in 0.1 M sodium bicarbonate (pH 8.0, 500 μL) was stirred at 0°C, and a solution of monothiophenyl ethylmalonate (44 mg, 196 μmol), also dissolved in 0.1 M sodium bicarbonate (pH 8.0, 1.5 mL), was added. The pH was adjusted back to 8.0 using cold 0.2 M sodium hydroxide and the reaction was continued for 6 hours. The pH was then lowered to ~4 using 0.5 M hydrochloric acid to quench the reaction. Water was added to increase the volume of the reaction mixture to ~20 mL, and this solution was washed 5 times with diethyl ether (20 mL) and 5 times with ethyl acetate (20 mL). The aqueous phase was lyophilized to produce a white solid. The absence of CoASH in the sample was confirmed by reaction with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)), and the concentration of the final ethylmalonyl-CoA stock for use in fluorometric assays was confirmed by comparison of the absorbance at 254nm to the absorbance of CoASH and malonyl-CoA standards. The overall yield of the final synthetic step was determined to be ~99%. ^{1}H NMR (D_2O): δ 0.72 (s, 3H, H-b), 0.86 (m, 6H, H-b' and H-j), 1.75 (m, 2H, H-i), 2.38 (t, 2H, H-e), 2.99 (m, 3H, H-g and H-h), 3.28 (m, 2H, H-f), 3.40 (m, 2H, H-d), 3.52 (m, 1H, H-a), 3.78 (m, 1H, H-a), 3.97 (s, 1H, H-c), 4.19 (s,2H, H-5'), 6.14 (d,1H, H-1'), 8.23 (s, 1H, H-2), 8.52 (s, 1H, H-8). The unassigned ribose protons overlapped with the solvent peak. The CoA proton nomenclature is as described by Taoka et al.[1] and previously by Patel et al.[2] Ethylmalonyl-CoA fragment ions at m/z=428.0 and 375.2 were observed by LC-ESI-MS in positive mode, as reported by Park et al.[3]

Supplemental Tables

Gene	Synthetic DNA Sequence (5' – 3')
kirCII	ATGACGGTTCGTCTGTTTGCAGTCGCAGCAGGTACCGAAGAAGGTCTGCTGGCAGCTCTGCGCGGCCAT GCAGATCGCATTCGTGCTGGTCGTGACCTGCCGACCCTGGCACGTTATTGCCATGATGCAGCAGCACGT ACGCCGGGTCTGGCACACCGTGCTGCACTGACCGCAGACTCCTACGATGACCTGGCGGAAGGTCTGGAT AAACTGGTGCGCGAATGGGCGGACCGTATCCCGCCGCCGAGCCCGTCTCGTCGCCCGGGTCTGGTGTTT GTTTTCGCTGGCCAGGGTGCGCAATGGGATGGCATGGGTCTGGAACTGCTGGACACCGAACCGGTTTTT GGCGCCGCACTGCGTCGCTGTGATGAACGCGTGCGTGAACTGGCCGGTTTCTCAGTTATTCAGCAACTG CGTGCTGGTCCGGCAATGTCGCGTCTGGGTGAAATTGATGTTCTGCAGCCGACCATGGTCAGTCTGCAA ATCGCCCTGGTGGCACTGTGGCGTTCCTGGCGTGTGGAACCGGACGCAGTTACGGGTCATAGTATGGGT GAAATCTCCGCAGGTTATGCTGCAGGTGCACTGACCCTGGATGACGCACTGCTGATTGCATGCCGTCGC AGCGCACTGCTGCGTCGCATCGCTGGTCGTGGTGCACTGGCAACCACGGAACTGTCTCCGGAAGCAGCA CACGCACTGGCTGCAAGCTCTGGCGGTCGTATTTGCGTTGCAGGCGAAAACTCACCGCGCTCGACGGTC CTGGCTGGTGATACCGCGACGCTGACCGCCCTGGTCGAAGATCTGGACCGTCGCGGCGTGTATTGTCGC ATGGTTCGTGGTACCGTCGCGAGTCATTCCCACTACGTGGATGAACTGCGTGATGACCTGGCTGGTGCA CTGCGTCCGCTGAGCCCGGTGCCGTCTCGCGTTCCGTTTTATAGTACGGTCACCGCAGCACCGGTGCCG GGTACGGATCTGGGTCCGGCATACTGGATGCGCAATCTGCGTGAACCGGTTCGTCTGGCTGCAGCAACC GGTCGTCTGGCAGAAGATGGTCATGAAATCTTCGTCGAAGTGAGCACGCACCCGGTGCTGCTGAGTTCC CTGCGTCAGACCCTGGAAAGCGCAGGTCGTCCGGGTGAAGTTCTGCCGTCTGGTCGTCGCCGTACCGAA CGCCGTGCCATGCTGTCATCGCTGGGCACGCTGTTCACCTATGGTCGTGATCCGCACTGGCCGACGTCT GCACCGCCGGCACCGGCACTGACCCCGTACCAGGCAGCTGTCCTGGCGGCCGTGCGCCGTCCGCGCCCGT CACCGGCAGCTGCGGGCTCGGGTGCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACC ACCACCACCACCACTGA
kirACP4	ATGGGCATTGGTGTTCCGACGCCGGATCCGGAACCGGCACCGCCGGCTGTCACCAGTGCAGTGGTTCAG CCGGTGGCTCAACCGGTTCCGCCGTCCGCAGAACCGTGGCGTGCAGCAGTGGCAGCTGACCTGCTGGCA CTGGTTCGTGGTGTCGCAGGTCTGGGTGCAGCCGAACTGACGGAAAACGATCAGCTGAGCCGTTTTGGT TTCGACTCTATTATGTATACCCGTCTGAGCCATCAAGTCAATGTGCGTTGGGATCTGGACGCAACCCCG GCAGCGTTTTTCGGTGTCGCAACGGCTGGTGAACTGGTGGCGAAAATCACCGCCGAATATGGCGAAGA ACTGGCCCGTCACTACCGCCCGGCGGCCAGCACGGCGCCGGCCGTTTGA
kirACP5	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCCGGAA CCGGCAGCAGCAGTCCCGGCACCGCCGGCAGAAAGTGCCCCGGCACCGGCTGCAACCGCAGCAGGCGGT GATCCGGAATCCGCAGTCCGTGACCATGTGCGTACGCTGCTGGCTGCACACCTGGGTATGGCACCGGAT CGTCTGCCGCCGGACCGTGTTCTGAGCGATGTGGGCGTTGACTCTCTGGGTCTGCGTCGCCTGAGCCGT CGCCTGGGCGCAACCTANGGTGTGGATATTCCGGCTCGTATGTTTGGCGTTGGTCAGACGGTCCGTGCT CTGGCACGTGCAGTTCATGATAAATACGGCCCGCTGCCGGCAACCGCTACGCCGGAACCGTGAAAGCTT GCGGCCGCACTCGAGCACCACCACCACCACTGA

Table S1. Codon-optimized sequences used for expression of AT and ACP proteins from the kirromycin synthase.

Supplemental Figures

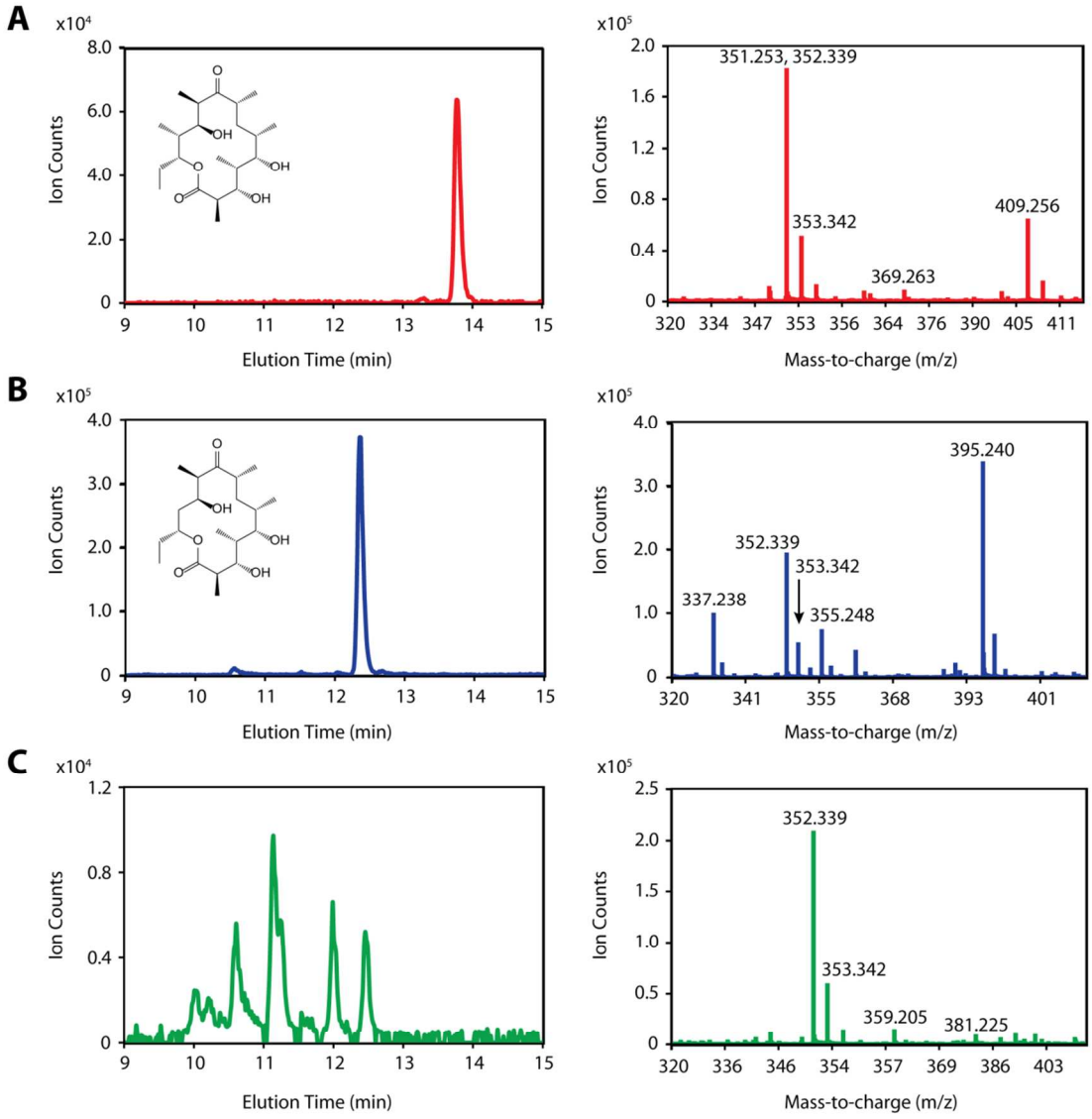

Figure S1. LC-MS analysis of 6-deoxyerythronolide B (6-dEB) and desmethyl analogs produced by complementation of an AT-null module 1 DEBS assembly line with 1 μM DSZS AT. (A) Extracted ion chromatogram (EIC) (left) and full mass spectrum (right) of 6-dEB ($C_{21}H_{38}O_6$; calculated MW 386.267). EIC was obtained by extraction of the $[M+Na]^+$ species. The major peaks shown correspond to the $[M+H-2H_2O]^+$ (351.253), $[M+H-H_2O]^+$ (369.263), and $[M+Na]^+$ (409.256) peaks. Peaks at m/z=352.339 and 353.342 are contaminants present in all mass spectra obtained on the instrument. (B) EIC and full mass spectrum of putative 12-desmethyl-6-dEB analog ($C_{20}H_{36}O_6$; calculated MW 372.25). EIC was obtained by extraction of the $[M+Na]^+$ species. The major peaks shown correspond to the $[M+H-2H_2O]^+$ (337.238), $[M+H-H_2O]^+$ (355.248), and $[M+Na]^+$ (395.240) peaks. (C) EIC and full mass spectrum of putative didesmethyl-6-dEB analogs ($C_{19}H_{34}O_6$; calculated MW 358.24). EIC was obtained by extraction of the $[M+Na]^+$ species and was integrated from approximately 9.5-12.5 minutes to obtain values reported in Figure 4 of the main text. The major peaks shown correspond to the $[M+H]^+$ (359.205) and $[M+Na]^+$ (381.225) peaks. Note that mass spectra have nonlinear x-axes.

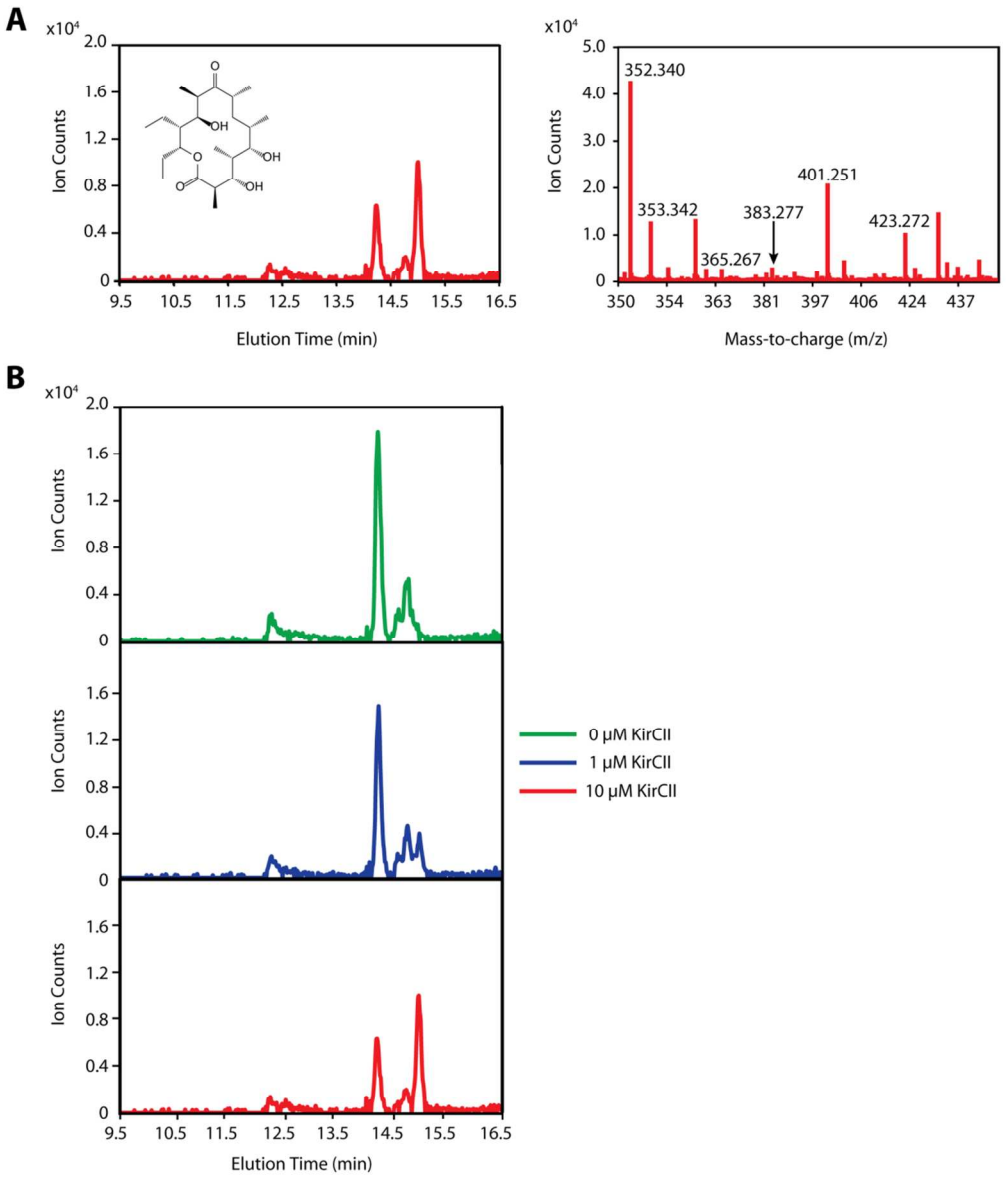

Figure S2. LC-MS analysis of putative 12-desmethyl-12-ethyl-6-dEB analog and other ethyl-6-dEB analogs produced by complementation of an AT-null module 1 DEBS assembly line with KirCII. (A) Extracted ion chromatogram (EIC) (left) and full mass spectrum (right) of the putative 12-desmethyl-12-ethyl-6-dEB analog ($C_{22}H_{40}O_6$; calculated MW 400.28) obtained with 10 μM KirCII. EIC was obtained by extraction of the $[M+Na]^+$ species and was integrated from 12-15 minutes to obtain values reported in Figure 5 of the main text. The EIC values in Figure 5 thus account for all ethyl-6-dEB analogs. Mass spectrum at right was obtained from the species eluting at approximately 15 minutes (likely the 12-desmethyl-12-ethyl-6-dEB analog, see below). The major peaks shown correspond to the $[M+H-2H_2O]^+$ (365.267), $[M+H-H_2O]^+$ (383.277), $[M+H]^+$ (401.251), and $[M+Na]^+$ (423.272) peaks. Peaks at m/z=352.340 and 353.342 are contaminants present in all mass spectra obtained on the instrument. Note that the mass spectrum has a nonlinear x-axis. (B) Representative EIC for 0, 1, and 10 μM KirCII complementation experiments. The ratio of different ethyl-6-dEB analogs appears to depend on the KirCII concentration, as evidenced by a corresponding change in relative peak heights. The peak at approximately 15 minutes likely corresponds to the 12-desmethyl-12-ethyl-6-dEB analog.

References

(1) Taoka, S., Padmakumar, R., Lai, M., Liu, H., and Banerjee, R. (1994) Inhibition of the human methylmalonyl-CoA mutase by various CoA-esters. *J. Biol. Chem. 269*, 31630–31634.

(2) Patel, S. S., and Walt, D. R. (1987) Substrate specificity of acetyl coenzyme A synthetase. *J. Biol. Chem. 262*, 7132–4.

(3) Park, J. W., Jung, W. S., Park, S. R., Park, B. C., and Yoon, Y. J. (2007) Analysis of intracellular short organic acid-coenzyme A esters from actinomycetes using liquid chromatography-electrospray ionization-mass spectrometry. *J. Mass Spectrom. 42*, 1136–1147.