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Chain-Elongation, Macrolactonization, and Hydrolysis of Natural and Reduced Hexaketide Substrates by the Picromycin/Methymycin Polyketide Synthase

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Materials and Methods

General materials and methods were as previously described.[1, 2] Phosphorimaging was carried out using a Bio Rad FX Molecular Imager and Bio-Rad K-series phosphorimager screens and the data were analyzed with the vendor's software. Radio-HPLC was carried out on a Rainin HPLC equipped with dual HPXL solvent delivery system and a Packard Radiomatic Flo-One\Beta detection system. ^{1}H NMR (300 and 400 MHz) utilized Bruker Avance AM 300 and AM 400 spectrometers. High resolution ESI Mass spectra were recorded on a Applied Biosystems QSTAR TOF mass spectrometer. Optical rotations were recorded using a Jasco P1010 polarimeter. Kinetic data were analyzed by direct fitting to the Michaelis-Menten equation using the KaleidaGraph data analysis software (Synergy Software.) Molecular mechanics energy minimizations were performed using the MM2 module of Chem 3D.

The aglycone 10-deoxymethynolide (**1**) was isolated from *Streptomyces venezuelae* inhibited with xanthotoxin, as previously described.[3] *Seco*-SNAC-thioester **5**, 7-dihydro-*seco*-SNAC-thioester **6**, and [^{14}C]-**6** were synthesized from 10-deoxymethynolide (**1**) or [1,3,5,7,9,11-^{14}C]-**1** as previously described.[4] Recombinant PICS module 6+TE was expressed in *Escherichia coli*, purified, and assayed as previously described.[5]

All non-enzymatic reactions were carried out with dry solvents under anhydrous conditions. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reagents were of the highest commercial quality and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as a visualizing agent and *p*-anisaldehyde stain and heat as developing agent. Sorbent silica gel (60, particle size 0.040-0.063 mm) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.25 mm E. Merck silica gel plates (60F-254).

Experimental Procedures

Conversion of *seco*-SNAC-thioester 5 to 10-deoxymethymycin (1) and narbonolide (2) by PICS module 6+TE. A preparative scale reaction was conducted in 3 mL of 100 mM phosphate buffer (pH 7.2, 1mM EDTA, 1 mM TCEP, 0.5 M NaCl, 10% glycerol, 8% (v/v) DMSO) with 2 mM **5**, 2 mM methylmalonyl-CoA, and 13.2 μM PICS module 6 +TE at 30 °C. After 30 min incubation, the reaction was extracted with 3 x 10 mL of ethyl acetate and the organic solvent was removed *in vacuo*. The resulting residue was dissolved in 200 μL of 50% (v/v) acetonitrile in water, and subject to reverse phase HPLC separation (Thermo Hypersil analytical column, 250 x 46 mm, Keystone) using isocratic acetonitrile in water (55% v/v). Macrolide aglycones **1** and **2** were eluted and collected at 15 min and 17 min, respectively. The product pools were dried by lyophilization after removing organic solvent *in vacuo*, ^{1}H and ^{13}C NMR and HR-ESI(+)-MS confirmed the identity of the products with authentic **1** and **2**.

Conversion of 7-dihydro-*seco*-SNAC-thioester 6 to 7, 8, and 9 by PICS module 6+TE. A 10-mL preparative scale reaction was performed following the procedure described for the isolation of compounds **1** and **2**, except that substrate **6** was used and that the products **7**, **8**, and **9** were separated by flash column chromatography. NMR and HRMS (ESI+) spectra of the product **7** are essentially the same as those reported previously. The lactones **8** and **9** were by analyzed NMR (^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC, NOESY) as well as HR-ESI(+)MS. **8**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.42 (dd, *J* = 15.4, 7.7 Hz, 1H, H-11), 5.34 (dd, *J* = 15.4, 7.8 Hz, 1H, H-10), 4.77 (td, *J* = 10.3, 3.1 Hz, 1H, H-13), 4.02 (dd, *J* = 5.4, 2,4 Hz, 1H, H-5), 3.81 (q, *J* = 7.1 Hz, 1H, H-2), 3.47 (t, *J* = 8.5 Hz, 1H, H-9), 2.89 (dq, *J* = 7.2, 5.7 Hz, 1H, H-4), 2.58 (dp, *J* = 7.2, 3.0 Hz, 1H, H-12), 1.92-2.01 (br s, 1H, H-6), 1.60-1.70 (m, 1H, H-14a), 1.48-1.60 (m, 2H, H-14b, H-8), 1.38 (d, *J* =7.1 Hz, 3H, H-16), 1.23 (d, *J* = 7.2 Hz, 3H, H-17), 1.20-1.25 (m, 1H, H-7a), 1.02 (ovlp d, *J* = 7.3, 6.0 Hz, 6H, H-20, H-19), 0.98 (d, *J* = 7.1 Hz, 3H, H-18), 0.90 (t, *J* = 7.3 Hz, 3H, H-15), 0.74 (ddd, *J* =14.4, 10.1, 4.7 Hz, 1H, H-7b); ^{13}C NMR ($CDCl_3$, 75 MHz) 209.2 (C-3), 169.7 (C-1), 134.1 (C-10), 133.6 (C-11), 80.1 (C-13), 78.8 (C-9), 71.7 (C-5), 51.2 (C-2), 47.0

(C-4), 39.4 (C-12), 37.0 (C-8), 36.2 (C-6), 36.1 (C-7), 21.9 (C-14), 17.1 (C-19 or C-20), 17.0 (C-20 or C-19), 16.2 (C-18), 14.0 (C-16 or C-17), 13.8 (C-17 or C-16), 10.9 ppm (C-15); HRMS (ESI+) *m/z* 377.2300 ($C_{20}H_{34}O_5$+Na^+ requires 377.2304); α_D (c = 0.18, MeOH) +12.57°. **9**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.69 (dd, *J* = 15.5, 7.4 Hz, 1H, H-11), 5.54 (dd, *J* = 15.5, 6.4 Hz, 1H, H-10), 4.32 (dd, *J* = 9.8, 2.0 Hz, 1H, H-5), 4.04 (t, *J* = 5.1 Hz, 1H, H-9), 3.62 (q, *J* = 6.6 Hz, 1H, H-2), 3.44 (td, *J* = 8.4, 3.5 Hz, 1H, H-13),2.69 (dq, *J* = 7.5, 2.2 Hz, 1H, H-4), 2.27-2.35 (m, 1H, H-12), 1.94-2.10 (m, 3H, H-6, H-7), 1.77-1.84 (m, 1H, H-8), 1.46-1.58 (m, 1H, H-14a), 1.35-1.46 (m, 1H, H-14b), 1.33 (d, *J* = 6.6 Hz, 3H, H-20), 1.11 (d, *J* = 7.5 Hz, 3H, H-19), 1.02 (d, *J* = 6.8 Hz, 3H, H-16), 0.89-0.97 (m, 9H, H-15, H-17, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz) 206.2 (C-3), 170.5 (C-1), 135.4 (C-11), 131.7 (C-10), 82.4 (C-5), 76.8 (C-13), 75.9 (C-9), 50.6 (C-2), 43.5 (C-4), 42.2 (C-12), 37.5 (C-7), 37.1 (C-8), 32.3 (C-6), 27.4 (C-14), 16.9 (C-15 or C-17), 16.0 (C-15 or C-17), 14.6 (C-16), 10.9 (C-18), 10.3 (C-19), 8.5 ppm (C-20); HRMS (ESI+) *m/z* 377.2315 ($C_{20}H_{34}O_5$+Na^+ requires 377.2304); α_D (c = 0.092, MeOH) +52.36°.

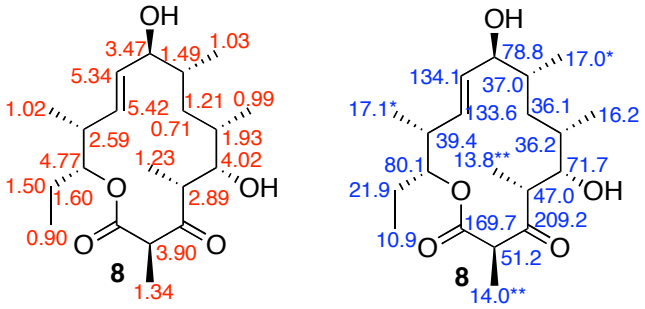

Figure S1. ^{1}H and ^{13}C NMR assignments for macrolactone **8**.

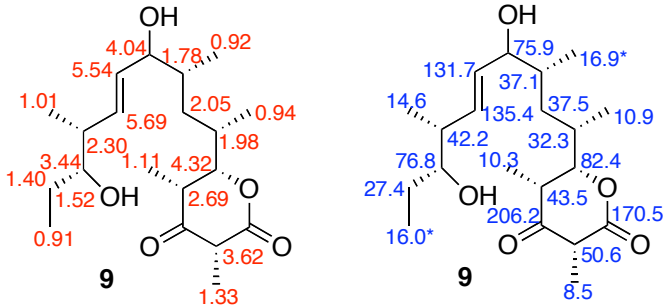

Figure S2. ^{1}H and ^{13}C NMR assignments for γ-lactone **9**.

Kinetics of the conversion of *seco*-SNAC-thioester 5 to 10-deoxymethymycin (1) and narbonolide (2) by PICS module 6+TE. The reaction of *seco*-SNAC-thioester **5** and methylmalonyl-CoA catalyzed by PICS module 6+TE was analyzed by RP-HPLC using the same

conditions as described for the preparative-scale reaction, with monitoring by a diode array detector (230 nm) and an in-line flow-scintillation. Steady-state kinetic measurements were performed by determining the initial velocities for the formation of **1** and **2** at concentrations of **5** of 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM, and 4.0 mM. The assay mixtures consisted of 100 mM phosphate buffer at pH 7.2, 1mM EDTA, 1 mM TCEP, 0.5 M NaCl, 10% glycerol, 6.6 μM PICS module 6+TE, 1 mM DL-[2-^{14}C]methylmalonyl-CoA (S. A. 0.5 mCi/mmol), variable concentrations of **5**, and 8% (v/v) DMSO in a total volume of 25 μL. The reactions were incubated at 30 °C for 5 min, then quenched by the addition of 5 μL of 1 M HCl and the mixture was analyzed by reverse phase HPLC or by phosphorimaging. The response of the HPLC-diode array detector at 230 nm was calibrated for **1**, using a stock solution of 50 mM **1** in DMSO to generate a series of dilutions of **1** in the reaction buffer (0.01 mM to 2 mM). Standard volumes of 20 μL of each dilution were injected onto the HPLC column and the areas of the peaks corresponding to **1** in the 230-nm UV trace of the HPLC chromatograph were quantitated using the vendor's software to generate a standard curve for the concentration of **1**. The area of the peaks for enzymatically generated **1** in each HPLC chromatogram was used to calculate the concentration from this standard curve. Due to the severe tailing of compound **2** in HPLC chromatography, the quantification of compound in each reaction was performed by TLC-phophorimaging. For each reaction subjected to HPLC analysis, a parallel reaction was extracted with ethyl acetate. The organic pool was then concentrated and subjected to TLC. Dilutions of [2-^{14}C]methylmalonyl-CoA were spotted on the same TLC plate and utilized as standards for phosphorimaging analysis. Initial velocities at different substrate concentrations were fit to the Michaelis-Menten equation by nonlinear least-squares regression to calculate k_{cat} and K_m (Figure S3).

The lactonization of **5** was also monitored in the absence of methylmalonyl-CoA, using identical RP-HPLC with diode array UV detection to monitor the formation of **1** as a function of variable substrate concentration (Figure S4).

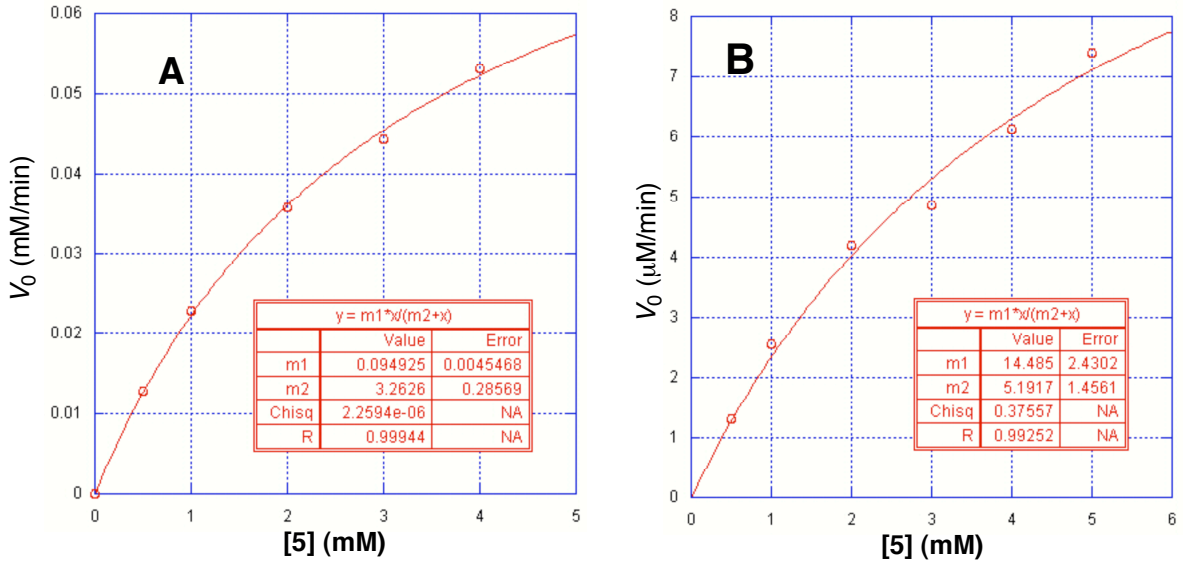

Figure S3. Steady state kinetic analysis of PICS module 6+TE-catalyzed conversion of **5** to **1** (Panel A) and to **2** (Panel B) in the presence of methylmalonyl-CoA.

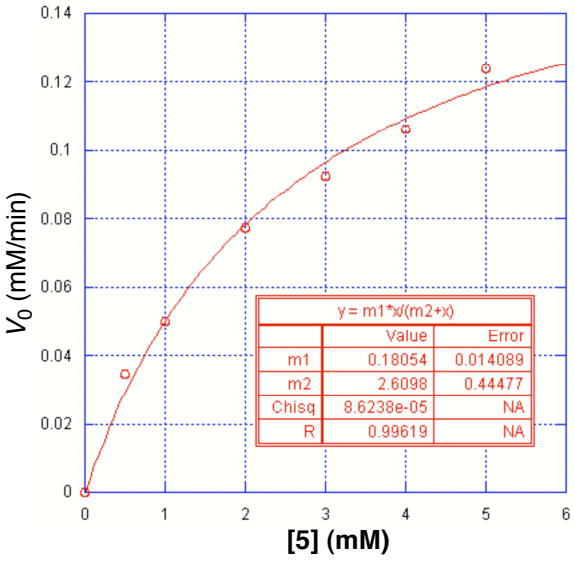

Figure S4. Steady state kinetic analysis of PICS module 6+TE-catalyzed conversion of **5** to **1** in the absence of methylmalonyl-CoA.

Kinetics of the incubation of [^{14}C]-7-dihydro-*seco*-SNAC-thioester 6 with PICS module 6+TE. Incubations with **7** were carried out in the same manner as for **5**, except that [^{14}C]-**7** and unlabeled methylmalonyl-CoA were used in the assay. Upon quenching with 5 μL of 1 M HCl, reactions were extracted with 3 X 200 μL ethyl acetate and the pooled organic extracts were

concentrated on a SpeedVac. The residues were re-dissolved in 30 μL ethyl acetate and spotted on a TLC plate, which was then developed with chloroform containing 10% methanol (R_fs: **7**, 0.08; **8**, 0.23; **9**, 0.32). After spotting radioactive standards ([2-^{14}C]methylmalonyl-CoA dilutions), the TLC plate was exposed overnight and analyzed by phosphorimager. The data were fit to the Michaelis-Menten equation (Figure S5). The rate of formation of both **8** and **9** could also be assayed using unlabeled **7** in combination with [2-^{14}C]methylmalonyl-CoA and monitoring by TLC-phosphorimaging.

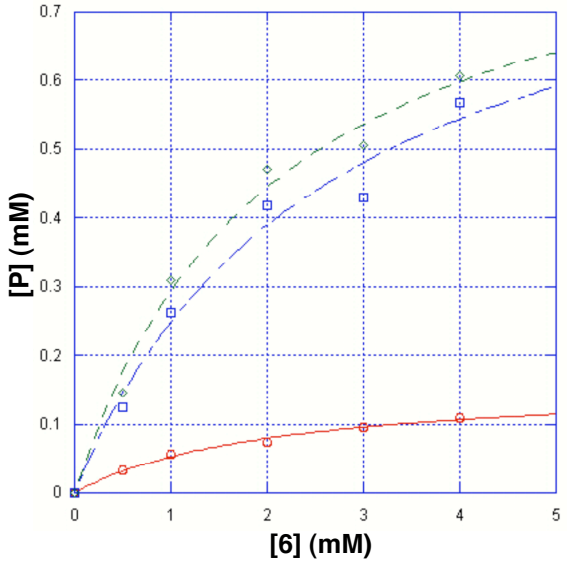

Figure S5. Steady state kinetic analysis of PICS module 6+TE-catalyzed conversion of **6** to **7** (hydrolysis) and **8** and **9** (lactonization) in the presence of methylmalonyl-CoA. **7**, green diamonds; **8**, red squares; **9**, blue squares.

References

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