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Supplementary Figure 1: Sequence alignment of CCRC homologues identified in *Streptomyces ambofaciens* ATCC23877 with well characterized CCRCs. The residues highlighted by the stars and red boxes are essential in defining the substrate specificity of CCRCs.¹⁻³ The specificity-conferring residues in the CCRC homologues in *S. ambofaciens* ATCC23877 do not resemble those of RevT, PteB, and CinF, all of which are known to be responsible for the biosynthesis of longer alkylmalonyl-CoA extender units. The sequence names highlighted in red are those from *S. ambofaciens* ATCC23877. The sequences designated CCRcc and CCR_S are both specific for crotonyl-CoA and the three CCRC homologues in *S. ambofaciens* have very similar specificityconferring residues to these enzymes. Accession numbers: CCRcc, 3HZZ; CCR_S, 3KRT; AntE, AGG37751; SpnE, AKA54628; PteB, WP_010981851; SalG, ABP73651; RevT, BAK64636; CinF, CBW54676; SamL0374 (SAM23877_0426), AKZ53475; Srm4*c (SAM23877_5633), AKZ58678; SAM23877_6076, AK59112



Supplementary Figure 2: Mass spectra from UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the [U-²H]L-valine 11 incorporation experiments. Spectra resulting from addition of (**a**) 5 mM [U-²H]L-valine 11 and (**b**) no [U-²H]L-valine to the growth medium. The signal at m/z = 692.9594 corresponds to [M+Na+H]²⁺ for stambomycins C/D 3/4 and the signal at m/z = 696.4787 corresponds to [M+Na+H]²⁺ for stambomycin C 3 derived from incorporation of [U-²H]L-valine 11. Comparison of the spectrum (**c**) measured for labeled stambomycin C 3 derived from incorporation of [U-²H]L-valine 11 with (**d**) the spectrum calculated for the [C₇₂H₁₂₅D₇NNaO₂₂]²⁺ ion. Spectra for stambomycins A/B 1/2 resulting from addition of (**e**) 5 mM [U-²H]L-valine 11 and (**f**) no [U-²H]L-valine to the growth medium. No specific incorporation of the deuterium-labeled precursor is observed.



Supplementary Figure 3: Mass spectra from UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the [U-²H]butyric acid 12 incorporation experiments. Spectra resulting from addition of (a) 5 mM [U-²H]butyric acid 12 and (b) no [U-²H]butyric acid to the growth medium. The signal at m/z = 692.9583 corresponds to [M+Na+H]²⁺ for stambomycins C/D 3/4 and the signal at m/z = 696.4803 corresponds to [M+Na+H]²⁺ for stambomycin D 4 derived from incorporation of [U-²H]butyric acid 12 (calculated for [C₇₂H₁₂₅D₇NNaO₂₂]²⁺:696.4784). Spectra for stambomycins A/B 1/2 resulting from addition of (c) 5 mM [U-²H]butyric acid 12 and (d) no [U-²H]butyric acid to the growth medium. No specific incorporation of the deuterium-labeled precursor is observed.



Supplementary Figure 4: Mass spectra from UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the [U-²H]L-isoleucine 9 incorporation experiments. Spectra resulting from addition of (a) 5 mM [U-²H]L-isoleucine 9 and (b) no [U-²H]L-isoleucine to the growth medium. The signal at m/z = 700.4757 corresponds to [M+Na+H]²⁺ for stambomycins A/B 1/2 and the signal at m/z = 704.5016 corresponds to [M+Na+H]²⁺ for stambomycins A/B 1/2 and the signal at m/z = 704.5016 corresponds to [M+Na+H]²⁺ for stambomycin A 1 derived from incorporation of [U-²H]L-isoleucine 9 (calculated for [C₇₃H₁₂₅D₉NNaO₂₂]²⁺:704.4925;). Spectra for stambomycins C/D 3/4 resulting from addition of (c) 5 mM [U-²H]L- isoleucine 9 and (d) no [U-²H]L-isoleucine to the growth medium. No specific incorporation of the deuterium-labeled precursor is observed.



Supplementary Figure 5: Mass spectra from UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the [U-2H]L-leucine 10 incorporation experiments. Spectra resulting from addition of (a) 5 mM [U-2H]Lleucine 10 and (b) no $[U^{-2}H]_{L}$ -leucine to the growth medium. The signal at m/z = 699.9645 corresponds to $[M+Na+H]^{2+}$ for stambomycins A/B 1/2 and the signal at m/z = 704.4945 corresponds to $[M+Na+H]^{2+}$ for stambomycin В 2 derived from incorporation of [U-2H]L-isoleucine 10 (calculated for [C₇₃H₁₂₅D₉NNaO₂₂]²⁺:704.4925). Spectra for stambomycins C/D **3/4** resulting from addition of (c) 5 mM [U-²H]Lleucine 10 and (d) no [U-2H]L-leucine to the growth medium. No specific incorporation of the deuterium-labeled precursor was observed. The regions of the spectra highlighted by the blue boxes result from the incorporation of deuterium labeled acetyl-CoA derived from the catabolism of [U-2H]L-leucine 10.



Supplementary Figure 6: UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the *n*-heptanoic acid incorporation experiments. Extracted ion chromatogram at m/z = 685.9, corresponding to $[M+Na+H]^{2+}$ for stambomycin analogue 22, from UHPLC-ESI-Q-TOF-MS analyses of methanolic mycelial extracts *S. ambofaciens* W130 grown in the absence (a) and (b) presence of 5 mM *n*-heptanoic acid. (c) Mass spectrum of 22 from UHPLC-ESI-Q-TOF-MS analyses of *S. ambofaciens* W130 fed with *n*-heptanoic acid. (d) Simulated spectrum for C₇₁H₁₃₀NNaO₂₂²⁺.



stambomycins C/D 3/4.



Supplementary Figure 8: ¹³C-APT NMR spectrum (175 MHz, d₄-MeOH) of 22.



Supplementary Figure 9: Overlaid COSY NMR spectra (700 MHz, d₄-MeOH) of 22 (black) and stambomycins C/D 3/4 (pink).



Supplementary Figure 10: Overlaid TOCSY NMR spectra (700 MHz, d₄-MeOH) of 22 (black) and stambomycins C/D 3/4 (pink).



Supplementary Figure 11: Overlaid HMBC NMR spectra (700 MHz / 175 MHz, d₄-MeOH) of 22 (black) and stambomycins C/D 3/4 (pink).



Supplementary Figure 12: Comparison of the ¹H NMR spectra of [3-²H₂]heptanoic acid 23 and unlabeled *n*-heptanoic acid 21.



Supplementary Figure 13: Comparison of the ¹³C NMR spectra of [3-²H₂]heptanoic acid 23 and unlabelled *n*- heptanoic acid 21. The splitting of the C-3 signal as a result of the attached deuterium atoms is shown in the inset.



Supplementary Figure 14: Mass spectra from UHPLC-ESI-Q-TOF-MS analysis of the stambomycin analogue 22 produced as a result of feeding [3-²H₂]heptanoic acid to *S. ambofaciens* W130. (a) Measured spectrum. (b) Simulated spectrum for C₇₁H₁₂₈D₂NNaO₂₂²⁺ corresponding to [M+Na+H]⁺ for doubly labeled 22.



Supplementary Figure 15: Mass spectra from ESI-TOF-MS analysis of partially purified reveromycin D 32 produced from $[3-{}^{2}H_{2}]$ heptanoic acid incorporation experiments. Comparison of ESI-TOF mass spectra of partially purified reveromycin D 32 from cultures of *Streptomyces* sp. SN-593 $\Delta revR$ mutant grown in the absence (a) and presence (b) of $[3-{}^{2}H_{2}]$ heptanoic acid. Simulated isotope distributions for unlabeled (c), singly-labeled (d) and doubly-labeled reveromycin D (e). The data are consistent with 20% incorporation of singly-labeled heptanoic acid into 32.



Supplementary Figure 16: LC-MS analysis of partially purified extracts from the $[3-^{2}H_{2}]$ heptanoic acid incorporation experiments. Mass spectra of reveromycin D 32 from LC-MS analyses of partially purified extracts from the cultures of (a) the $\Delta revT$ mutant, (b) the $\Delta revT$::samR0483 mutant to which $[3-^{2}H_{2}]$ heptanoic acid 23 has been fed, and (c) wild type *Streptomyces* sp. SN-593.



Supplementary Figure 17: Superimposition of PccB from *S. coelicolor* (cyan) with MccB from *S. ambofaciens* (green). The structures are displayed as colored ribbons.



Supplementary Figure 18: Structural comparison of MccB (PDB ID: 5INI), PccB (PDB ID: 1XNY), and AccD5 (PDB ID: 2A7S) active site residues. Colors are green, cyan, and magenta, respectively.



Supplementary Figure 19: Stereo view of hexanoyl-CoA bound to MccB with 2F0-FC SA omit map contoured 1.0 σ at 2.85 Å. The surrounding protein has been removed for clarity.





Supplementary Figure 20: Sequence alignment of MccB from *S. ambofaciens* with other ACC βsubunits. The enzymes denoted 2-4C use acetyl-, propionyl or butyryl-CoA as a substrate. MccB in *Streptomyces azurea* isproposed to assemble butylmalonyl-CoA and other 6-8 carbon extender units incorporated by PriA6 into the primycins.⁹



Supplementary Figure 21: UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the 6-azidohexanoic acid incorporation experiments. EICs at m/z = 699.4, corresponding to $[M+H+Na]^{2+}$ for stambomycin analogue 37 (green), at m/z = 692.9, corresponding to $[M+H+Na]^{2+}$ for stambomycins C/D 3/4 (orange), and at m/z = 699.9, corresponding to $[M+H+Na]^{2+}$ for stambomycins A/B 1/2 (purple), from UHPLC-ESI-Q-TOF-MS analyses of methanolic mycelial extracts of *S. ambofaciens* W130 grown in the absence (**a**) and presence (**b**) of 5 mM 6-azidohexanoic acid. (**c**) Measured mass spectrum for peak corresponding to stambomycin analogue 37 with a retention time of 25 minutes (calculated for C₇₀H₁₂₇N₄NaO₂₂⁺: 699.4414).



Supplementary Figure 22: UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the 6-heptynoic acid incorporation experiments. Base peak chromatograms from UHPLC-ESI-Q-TOF-MS analyses of mycelial extracts of *S. ambofaciens* W130 grown in the absence (**a**) and presence (**b**) of 5 mM 6-heptynoic acid. The peaks with retention times of 10.3 minutes and 10.7 minutes correspond to stambomycins C/D **3/4** and stambomycins A/B **1/2**, respectively. A new peak with a retention time of 9.8 minutes, corresponding to a novel stambomycin analogue is observed. (**c**) Mass spectrum of the stambomycin analogue with a retention time of 9.8 minutes observed in UHPLC-ESI-Q-TOF-MS analyses of *S. ambofaciens* W130 fed with 6-heptynoic acid. (**d**) Simulated spectrum for C₇₁H₁₂₆NNaO₂₂²⁺, corresponding to the stambomycin analogue expected to result from direct incorporation of 6-heptynoic acid. (**e**) Simulated spectrum for C₇₃H₁₃₀NNaO₂₂²⁺, corresponding to stambomycin analogue **39**.



Supplementary Figure 23: UHPLC-ESI-Q-TOF-MS analysis of methanolic mycelial extracts from the 8-nonynoic acid incorporation experiments. EICs at m/z = 697.9, corresponding to $[M+H+Na]^{2+}$ for stambomycin analogue 39 (green), at m/z = 692.9, corresponding to $[M+H+Na]^{2+}$ for stambomycins C/D 3/4 (blue), and at m/z = 699.9, corresponding to $[M+H+Na]^{2+}$ for stambomycins A/B 1/2 (black), from UHPLC-ESI-Q-TOF-MS analyses of methanolic mycelial extracts of *S. ambofaciens* W130 grown in the absence (a) and presence (b) of 5 mM 8-nonynoic acid. (c) Measured and (d) simulated mass spectra for the $[M+Na+H]^{2+}$ ion of stambomycin analogue 39.



39 and (b) stambomycins C/D 3/4 .



Supplementary Figure 25: ¹H-NMR (400 MHz, MeOD) spectrum of biotinylated stambomycin analogue derived from the click reaction of stambomycin analogue 39 with the azide-PEG3-biotin conjugate. The signal at 7.68 ppm corresponds to the triazole proton and the signals at 4.2 ppm correspond to the protons around the fused thiolane ring of biotin.



Supplementary Figure 26: Stereo view of the apo-MccB hexamer with 2F0-FC map contoured to 1.0 σ at 2.45 Å.



Supplementary Figure 27: Stereo views of the MccB hexamer bound to four molecules of hexanoyl-CoA. (a) The overall architecture of MccB bound to four molecules of hexanoyl-CoA. (b) The active site pocket at the dimer interface of opposing MccB monomers. 2F0-FC maps are contoured to 1.0 σ at 2.85 Å.



Supplementary Figure 28: Stereo views of the MccB hexamer bound to one molecule of hexanoyl-CoA. (a) The overall architecture of MccB bound to one molecule of hexanoyl-CoA. (b) The active site pocket at the dimer interface of opposing MccB monomers. 2F0-FC maps are contoured to 1.0 σ at 2.75 Å.

Strain/plasmid	Relevant characteristics		
Strain			
S. ambofaciens ATCC	Wild-type strain, in which stambomycin cluster is poorly expressed	4	
23877			
S. ambofaciens W130	Stambomycin producing strain in which samR0484 is integrated	This work	
	into the chromosome and is under control of the <i>erm</i> E* promoter		
<i>E. coli</i> ET12567/pUZ8002	Strain used for conjugal transfer of pOSV556t derivatives into S.	5	
	ambofaciens ATCC 23877		
Streptomyces sp. SN-593	Wild-type reveromycin-producing strain	6	
E. coli GM2929 hsdS::	Strain used for conjugal transfer of pTYM19 derivatives into	7	
Tn10 / pUB307- <i>aph</i> ::Tn7	Streptomyces sp. SN-593		
∆revT	revT disruptant of Streptomyces sp. SN-593	8	
∆revR	revR disruptant of Streptomyces sp. SN-593		
∆revT::samR0483	$\Delta revT$ mutant complemented with pTYM19-P _{aph} -samR0483	This work	
Plasmid			
pOSV556t	Integrative E. coli-Streptomyces shuttle vector	9	
pOSV556t-samR0484	samR0484 inserted between the HindIII and Clal sites of pOSV556t	This work	
pOSV556t-samR0483	samR0483 inserted between the HindIII and Clal sites of pOSV556t	This work	
pTYM19	Integrative Streptomyces-E. coli shuttle vector	10	
pTYM19-P _{aph}	Derivative of pTYM19 in which the aphII promoter fragment is	11	
	inserted into the EcoRI and BamHI sites.		
pTYM19-P _{aph} -samR0483	samR0483 inserted into BamHI and HindIII sites of pTYM19-P _{aph}	This work	
pET151- <i>samR04</i> 83	MccB overproduction construct	This work	
pET28a- <i>samR04</i> 83	MccB overproduction construct	This work	

Supplementary Table 1: Bacterial strains and plasmids

Supplementary Table 2: Oligonucleotide primers

Target gene (template)	Primers	Ref.	
samR0484	pOSV_samR0484_FW:		
	5'-AAAGGG <u>AAGCTT</u> AGGAGGCCAGTCATTGCTGGTCC-3'		
(for cloning into pOSV556t	pOSV_samR0484_RV:		
between HindIII and Clal)	5'-CCCTTT <u>ATCGAT</u> CGTGGGCAGGCTCTGCTC-3'		
samR0483	pOSV_samR0483_FW:	This work	
	5'-AAGGG <u>AAGCTT</u> GCTGGTATGTCGTCCCA-3'		
(for cloning into pOSV556t	pOSV_samR0483_RV:		
between HindIII and Clal)	5'- CCCTTT <u>ATCGAT</u> ACGACGTCACA -3'		
samR0483	0483_BamHI:	This work	
	5'-CGC <u>GGATCC</u> ATGTCGCTCCAGGA-3'		
	0483_HindIII:		
(pOSV556t- <i>samR0483</i>)	5'-GGG <u>AAGCTT</u> TCACAGGGGAATGT-3'		
samR0483	TOPO_483_FW:	This work	
	5'-CACCATGTCGCTCCAGGAGCCTGTCTC-3'		
(For TOPO cloning into	TOPO_483_R:		
pET151)	5'-TCACAGGGGAATGTTCCCGTGTT-3'		
samR0483 in pET151	pET28a-samR0483_FW:	This work	
	5'- ATGCGC <u>GCTAGC</u> GATTCTACGGAAAAC-3'		
(For cloning into pET28a	pET28a-samR0483_RV:		
between Nhel and Notl)	5'-TATATA <u>GCGGCCGC</u> TCACAGGGGAATGTT-3'		

Position	δ _c (ppm) in 4	δ _c (ppm) in 22	δн (ppm) in 4	δн (ppm) in 22	Jн₋н (Hz)
1	173.3	173.1	-	-	
2	44.3	44.3	2.52, 2.83	2.53, 2.76	14.5
3	100.0	100.1	-	-	
4	46.2	46.2	1.56	1.57	
4-Me	12.4	12.5	1.04	1.11	
5	81.0	81.0	3.66	3.66	
1'	104.6	105.8	4.44	4.44	7.0
2'	70.0	69.8	3.54	3.54	7.5, 10.5
3'	72.0	72.0	3.23	3.23	10.5
4'	70.8	70.5	3.38	3.39	10.0
5'	74.0	74.0	3.42	3.42	6.0, 9.0
6'	18.1	17.8	1.29	1.29	
3'-NMe ₂	42.2	42.3	3.03	3.00	
6	39.1	38.9	1.61, 1.90	1.91	
7	70.0	69.9	3.83	3.83	
8	40.8	41.0	1.56	1.58	
8-Me	9.3	9.4	0.93	0.89	
9	79.9	79.9	3.90	3.90	7.5
10	137.1	136.9	-	-	
10-Me	12.4	12.4	1.65	1.65	
11	128.0	127.8	6.06	6.04	11.0
12	129.9	129.5	6.35	6.36	11.0, 14.5
13	131.7	131.6	5.81	5.80	
14	39.8	39.8	2.34	2.34	6.5
15	75.8	75.7	3.77	3.78	
16	42.6	42.9	1.52	1.52	
16-Me	7.4	7.4	0.95	0.96	
17	75.5	75.5	3.72	3.72	
18	36.1	36.1	1.48	1.47	
19	23.5	23.3	1.32, 1.34	1.32	
20	38.5	38.5	1.47	1.45	
21	71.1	70.9	3.55	3.55	
22	43.1	43.0	1.61, 1.67	1.61, 1.68	
23	77.5	77.4	4.23	4.23	7.0
24	139.4	138.5	-	-	
24-Me	12.2	12.4	1.64	1.64	
25	129.3	129.9	5.23	5.21	10.5

Supplementary Table 3: Comparison of ¹H and ¹³C NMR data for stambomycin D¹ (**4**) and stambomycin analogue **22**. The atom numbering is shown in Figure 3 of the main manuscript.

26	41.9	41.8	2.45	2.44	
1"	32.4	32.4	1.18, 1.79	1.19, 1.81	7.0
2"	26.6	30.6	1.37	1.38	
3"	35.8	34.9	1.31, 1.44	1.29	
4"	34.0	33.0	1.43, 1.49	1.28	
5"	29.2	14.4	1.29	0.88	
6"	11.5	-	0.87	-	
27	79.7	79.6	3.36	3.36	
28	74.2	74.0	3.47	3.47	
29	27.8	27.8	1.43, 1.68	1.42, 1.66	
30	36.1	36.0	1.43, 1.70	1.69	
31	70.1	69.9	3.83	3.83	
32	46.4	46.2	1.50, 1.57	1.51, 1.57	
33	66.8	66.7	4.06	4.07	
34	42.0	41.8	1.48, 1.50	1.49, 1.50	
35	72.9	72.8	3.74	3.74	
36	40.8	40.6	1.48	1.47	
36-Me	15.6	15.6	0.92	0.93	
37	29.8	29.6	1.28, 1.12	1.12, 1.56	
38	36.0	36.0	1.50	1.49	
39	76.3	76.3	3.72	3.72	
40	42.3	42.3	1.51	1.51	
40-Me	7.0	6.9	0.91	0.92	
41	75.9	75.9	3.71	3.71	
42	33.8	33.6	1.43, 1.62	1.43, 1.62	
43	22.9	22.9	1.43	1.45	
44	38.8	38.9	1.45, 1.46	1.45, 1.46	
45	70.6	70.5	3.67	3.67	
46	44.8	44.9	1.60, 1.65	1.60	
47	71.1	71.0	4.28	4.27	6.5, 13.0
48	136.7	136.8	5.80	5.77	5.0, 10.5
49	130.8	130.9	5.72	5.73	6.0, 15.5
50	72.8	72.8	5.43	5.42	6.0
51	20.8	20.8	1.33	1.35	

Supplementary Table 4: Crystallographic statistics

	<i>аро</i> -МссВ	MccB + 1 Hex-CoA	MccB + 4 Hex-CoA
	5ING	5INF	5INI
Data collection	ALS BL821	ALS BL822	ALS BL821
Wavelength (Å)	0.9999	0.9998	1.000
Space group	P212121	P212121	P212121
Cell dimensions			
a, b, c (Å)	120.56, 163.44,	110.072 165.453	110.978,161.526,
	186.55	190.261	187.294
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	58.12 - 2.45	42.22 - 2.75	48.44 - 2.85
R _{merge}	0.112 (0.966)	0.208 (0.837)	0.206 (0.731)
l/σl	11.8 (1.6)	14.4 (3.5)	10 (3.6)
Completeness (%)	99 (97.3)	100 (99.9)	100 (99.4)
Multiplicity	6.1 (5.4)	7.4 (7.3)	6.9 (6.7)
CC(1/2)	(0.662)	(0.796)	(0.820)
Refinement			
Resolution (Å)	58.12 - 2.45	42.22 - 2.75	48.44 - 2.85
No. reflections	133794 (12965)	90300 (8650)	79173 (7792)
Rwork/ Rfree	0.195/0.223	0.184/0.242	0.173/0.230
No. atoms			
Protein	22424	21976	22284
Ligand/ion	N/A	55	220
Water	956	1091	392
B-factors			
Protein	48.57	27.71	24.59
Ligand/ion	N/A	52.88	54.39
Water	47.12	26.09	20.27
R.M.S deviations			
Bond lengths (Å)	0.007	0.013	0.007
Bond angles (°)	1.07	1.17	0.90

All structures were determined from a single crystal

*Highest resolution shell is shown in parenthesis.

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