

**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.<sup>1-3</sup> 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-<sup>2</sup>H]L-valine 11 incorporation experiments**. Spectra resulting from addition of (**a**) 5 mM [U-<sup>2</sup>H]L-valine **11** and (b) no [U-<sup>2</sup>H]L-valine to the growth medium. The signal at  $m/z = 692.9594$  corresponds to [M+Na+H]<sup>2+</sup> 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-<sup>2</sup>H]L-valine **11**. Comparison of the spectrum (**c**) measured for labeled stambomycin C **3**  derived from incorporation of [U-<sup>2</sup>H]L-valine 11 with (**d**) the spectrum calculated for the [C<sub>72</sub>H<sub>125</sub>D<sub>7</sub>NNaO<sub>22</sub>]<sup>2+</sup> ion. Spectra for stambomycins A/B **1**/**2** resulting from addition of (**e**) 5 mM [U-<sup>2</sup>H]L-valine **11** and (**f**) no [U-<sup>2</sup>H]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-<sup>2</sup>H]butyric acid 12 incorporation experiments.** Spectra resulting from addition of (**a**) 5 mM [U-<sup>2</sup>H]butyric acid **12** and (**b**) no [U-<sup>2</sup>H]butyric acid to the growth medium. The signal at *m/z* = 692.9583 corresponds to  $[M+Na+H]^2$ <sup>+</sup> for stambomycins C/D 3/4 and the signal at  $m/z = 696.4803$  corresponds to  $[M+Na+H]^2$ <sup>+</sup> for stambomycin D **4** derived from incorporation of [U-<sup>2</sup>H]butyric acid **12** (calculated for [C72H125D7NNaO22] 2+:696.4784). Spectra for stambomycins A/B **1**/**2** resulting from addition of (**c**) 5 mM [U-<sup>2</sup>H]butyric acid **12** and (**d**) no [U-<sup>2</sup>H]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-<sup>2</sup>H]L-isoleucine 9 incorporation experiments.** Spectra resulting from addition of (**a**) 5 mM [U-<sup>2</sup>H]Lisoleucine **9** and (**b**) no [U-<sup>2</sup>H]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 stambomycin A 1 derived from incorporation of [U-<sup>2</sup>H]L-isoleucine 9 (calculated for [C<sub>73</sub>H<sub>125</sub>D<sub>9</sub>NNaO<sub>22</sub>]<sup>2+</sup>:704.4925;). Spectra for stambomycins C/D **3**/**4** resulting from addition of (**c**) 5 mM [U-<sup>2</sup>H]L- isoleucine **9** and (**d**) no [U-<sup>2</sup>H]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-<sup>2</sup>H]L-leucine 10 incorporation experiments.** Spectra resulting from addition of (**a**) 5 mM [U-<sup>2</sup>H]Lleucine **10** and (**b**) no [U-<sup>2</sup>H]L-leucine to the growth medium. The signal at *m/z* = 699.9645 corresponds to  $[M+Na+H]^2$ <sup>+</sup> for stambomycins A/B 1/2 and the signal at  $m/z = 704.4945$  corresponds to  $[M+Na+H]^2$ <sup>+</sup> for stambomycin B 2 derived from incorporation of <sup>2</sup>H]L-isoleucine **10** (calculated for [C73H125D9NNaO22] 2+:704.4925). Spectra for stambomycins C/D **3**/**4** resulting from addition of (**c**) 5 mM [U-<sup>2</sup>H]Lleucine **10** and (**d**) no [U-<sup>2</sup>H]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-<sup>2</sup>H]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 C<sub>71</sub>H<sub>130</sub>NNaO<sub>22</sub><sup>2+</sup>.



**stambomycins C/D 3/4.**



**Supplementary Figure 8**: **<sup>13</sup>C-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 <sup>1</sup>H NMR spectra of [3- <sup>2</sup>H2]heptanoic acid 23 and unlabeled** *n***heptanoic acid 21.**



**Supplementary Figure 13**: **Comparison of the <sup>13</sup>C NMR spectra of [3- <sup>2</sup>H2]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- <sup>2</sup>H2]heptanoic acid to** *S. ambofaciens* **W130.** (**a**) Measured spectrum. (**b**) Simulated spectrum for C<sub>71</sub>H<sub>128</sub>D<sub>2</sub>NNaO<sub>22</sub><sup>2+</sup> corresponding to [M+Na+H]<sup>+</sup> for doubly labeled 22.



**Supplementary Figure 15: Mass spectra from ESI-TOF-MS analysis of partially purified reveromycin D 32 produced from [3- <sup>2</sup>H2]heptanoic acid incorporation experiments.** Comparison of ESI-TOF mass spectra of partially purified reveromycin D **32** from cultures of *Streptomyces* sp. SN-593 Δ*revR* mutant grown in the absence (**a**) and presence (**b**) of [3- <sup>2</sup>H2]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- <sup>2</sup>H2]heptanoic acid incorporation experiments.** Mass spectra of reveromycin D **32** from LC-MS analyses of partially purified extracts from the cultures of (**a**) the Δ*revT* mutant, (**b**) the Δ*revT::samR0483* mutant to which [3- <sup>2</sup>H2]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 subunits**. 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  $[M+H+Na]<sup>2+</sup>$  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 C<sub>70</sub>H<sub>127</sub>N<sub>4</sub>NaO<sub>22</sub><sup>+</sup>: 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 C<sub>71</sub>H<sub>126</sub>NNaO<sub>22</sub><sup>2+</sup>, 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  $[M+H+Na]<sup>2+</sup>$  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]<sup>2+</sup> ion of stambomycin analogue 39.



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



**Supplementary Figure 25**: **<sup>1</sup>H-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.



## **Supplementary Table 1**: Bacterial strains and plasmids







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



## **Supplementary Table 4**: Crystallographic statistics



All structures were determined from a single crystal

\*Highest resolution shell is shown in parenthesis.

## **Supplementary References**

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