SUPPLEMENTARY METHODS:

The chemical synthesis of the butylamide derivatives as GC-MS authentic standards is outlined in Supplementary Figure 16. Butylamide derivatives, 8, 10, 12 and 14 were synthesized from carboxylic acids 7, 9, 11 and 13, respectively.

General Protocol A. Chemical synthesis and spectroscopic data of N-butyl-3-methylbutanamide (8). A solution of carboxylic acid 7 (200 mg, 1.96 mmol) in 20 mL of CH₂Cl₂ was treated with butylamine (160 mg, 2.19 mmol), HOBt (340 mg, 2.5 mmol), EDC hydrochloride (480 mg, 2.5 mmol) and i-Pr₂NEt (1.1 mL, 6.5 mmol). The reaction mixture was stirred at room temperature for 15 h, concentrated in vacuo, and partitioned between cold (0°C) 1 N HCl (100 mL) and Et₂O (100 mL). The aqueous phase was further extracted with Et₂O (2 x 100 mL). The combined organic extracts were washed with brine (150 mL), dried (MgSO₄), concentrated in vacuo, and purified by chromatography on SiO₂ (1:1, hexane/EtOAc) to provide 8 (250 mg, 80%) as a white powder: ¹H NMR (400 MHz, CDCl₃) δ 3.15 (dd, J = 12.9, 7.1 Hz, 2 H), 1.96-2.09 (m, 1 H), 1.93 (d, J = 7.6 Hz, 2 H), 1.35-1.41 (m, 2 H), 1.22-1.28 (m, 2 H), 0.85 (d, J = 6.5 Hz, 6 H), 0.83 (t, J = 7.3 Hz, 3 H); MS (ESI) calculated for [M+H]⁺ 158.15, found 158.14.

Spectroscopic data for N-butyl-3-methylbut-2-enamide (10). ¹H NMR (400 MHz, CDCl₃) δ 5.46-5.50 (m, 1 H), 3.18 (dd, J = 13.5, 6.3 Hz, 2 H), 2.06 (s, 3 H), 1.74 (s, 3 H), 1.37-1.44 (m, 2 H), 1.22-1.31 (m, 2 H), 0.84 (t, J = 6.5 Hz, 3 H); MS (ESI) calculated for [M+H]⁺ 156.13, found 156.12.

Spectroscopic data for N-butyl-3-methylbut-3-enamide (12). ¹H NMR (400 MHz, CDCl₃) δ 4.87-4.89 (m, 1 H), 4.79-4.81 (m, 1 H), 3.15 (dd, J = 13.6, 6.5 Hz, 2 H), 2.86 (s, 2 H), 1.70 (s, 3 H), 1.34-1.42 (m, 2 H), 1.21-1.29 (m, 2 H), 0.83 (t, J = 7.3 Hz, 3 H); MS (ESI) calculated for [M+H]⁺ 156.13, found 156.12.

Spectroscopic data for (1R,2S)-N-butyl-2-methylcyclopropane-1-carboxamide (14) and its racemic mixture of cis- and trans-isomers (cis,trans-14). The enantiomerically pure acid 13 was a gift from Timothy M. Ramsey (Novartis Institutes for Biomedical Research, Inc.). The racemic mixture of cis- and trans-isomers of 14 was generated from 2-methylcyclopropanecarboxylic acid. Data for 14: ¹H NMR (400 MHz, CDCl₃) δ 3.15 (dd, J = 13.7, 6.6 Hz, 2 H), 1.36-1.44 (m, 2 H), 1.21-1.31 (m, 2 H), 0.96-1.04 (m, 1 H), 0.99 (d, J = 6.1 Hz, 3 H), 0.84 (t, J = 7.3 Hz, 3 H), 0.78-0.85 (m, 1 H), 0.44 (ddd, J = 7.7, 6.1, 3.6 Hz, 1 H); MS (ESI) calculated for [M+H]⁺ 156.13, found 156.13.

Spectroscopic data and chemical synthesis of (E)-N-butyl-4-chloro-3-methylbut-2-enamide (18). (E)-4-Bromo-3-methylbut-2-enoic acid (16): To a solution of 3,3-dimethylacrylic acid (200 mg, 2.0 mmol) in CCL₄ (10 mL) was added freshly recrystallized NBS (391 mg, 2.2 mmol, 1.1 equiv) followed
by benzoyl peroxide (24.2 mg, 0.05 equiv). The reaction mixture was heated at reflux for 1 h, cooled to room temperature, and filtered to remove succinimide. An aliquot was removed for $^1$H NMR analysis, and the remainder of the solution was poured into a separatory funnel and washed with H$_2$O (2 x 20 mL) and brine (1 x 20 mL). The organic layer was dried (Na$_2$SO$_4$) and the solvent was removed in vacuo to afford a mixture (371 mg) of isomeric allylic bromides as a pale yellow oil. A solution of the crude oil (371 mg, 2.07 mmol) in water (2 mL) containing 82.8 mg (2.07 mmol) of NaOH was stirred at room temperature for 1 h and was then extracted with CH$_2$Cl$_2$ (2 x 2 mL). The aqueous layer was acidified with 6 N HCl (1 mL) and extracted again with CH$_2$Cl$_2$ (2 x 5 mL). The combined organic extracts were washed with brine (5 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo to afford the desired carboxylic acid 16 (88 mg, 24%) as a pale yellow oil. This compound was carried on to the next step without further purification.

(E)-4-Chloro-3-methylbut-2-enoic acid (17): A solution of bromo acid 16 (360 mg, 2.01 mmol, 1.0 equiv) in CH$_2$Cl$_2$ (5 mL) was cooled to 0˚C, treated with t-butyl ammonium chloride (1.12 g, 4.02 mmol, 2.0 equiv) and stirred at 0˚C for 24 h. The mixture was concentrated in vacuo to afford a viscous pale yellow oil. Purification by chromatography on SiO$_2$ (2% MeOH/CH$_2$Cl$_2$ containing 0.5% AcOH) provided the desired acid 17 (215 mg, 79%) as a colorless oil.

(E)-N-Butyl-4-chloro-3-methylbut-2-enamide (18): To a solution of carboxylic acid 17 (40.0 mg, 0.297 mmol) in CH$_2$Cl$_2$ (0.5 mL) was added oxalyl chloride (0.377 g, 2.97 mmol, 10.0 equiv). The reaction mixture was heated at a gentle reflux for 2 h, allowed to cool to room temperature, and concentrated in vacuo to afford a viscous pale yellow oil. After addition of THF (0.5 mL), the solution was cooled to 0˚C, treated with a mixture of triethylamine (45 mg, 0.45 mmol, 1.5 equiv), butylamine (24 mg, 0.37 mmol, 1.1 equiv) and DMAP (1.8 mg, 0.015 mmol, 0.05 equiv), stirred at 0˚C for 15 min, and warmed to RT. The reaction mixture was stirred at room temperature for an additional 45 min and poured into a separatory funnel containing 1 M HCl (2 mL) and ether (2 mL). The organic layer was washed with water (1 x 2 mL) and brine (1 x 2 mL), dried (Na$_2$SO$_4$), concentrated and purified by chromatography on SiO$_2$ to afford the desired butylamide derivative 18 (35 mg, 62%) as a pale yellow oil: IR (neat) 3296, 1633, 1548, 1266, 1182 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) δ 5.88-5.86 (m, 1 H), 5.73 (br s, 1 H), 4.00 (s, 2 H), 3.29 (q, 2 H, $J = 8.0$ Hz), 2.21 (bs, 3 H), 1.50 (app p, 2 H, $J = 7.5$ Hz), 1.35 (app s, 2 H, $J = 7.4$ Hz), 0.92 (t, 3 H, $J = 8.0$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 165.9, 146.6, 121.4, 50.2, 39.1, 31.6, 20.1, 16.3, 13.7; MS (ESI) calculated for [M+H]$^+$ 189.09, found 189.10.

Spectroscopic data and chemical synthesis of (Z)-N-butyl-4-chloro-3-methylbut-2-enamide (21).

(Z)-4-Chloro-3-methylbut-2-enoic acid (20): A solution of an (E,Z)-mixture of allylic bromides 19 (0.67 g, 3.74 mmol) in CH$_2$Cl$_2$ was treated at 0˚C with tetrabutyl ammonium chloride (2.08 g, 7.49 mmol), stirred at 0˚C for 24 h, and concentrated to afford a pale yellow viscous oil. Purification by chromatography on SiO$_2$ (1:99, MeOH/CH$_2$Cl$_2$ containing 0.5% AcOH) provided the chloroacid (0.402
mg, 79%) as a 1.2:1 mixture of (E,Z)- isomers. Further purification by SFC (SiO2 column, 7% MeOH/CO2) led to pure (Z)-isomer 20 (retention time = 3.12-3.17 min) (77 mg) as a colorless oil. (Z)-N-Butyl-4-chloro-3-methylbut-2-enamide (21): To a solution of the (Z)-acid 20 (40.0 mg, 0.297 mmol) in CH2Cl2 (0.5 mL) was added oxalyl chloride (0.377 g, 2.97 mmol, 10.0 equiv). The reaction mixture was heated at reflux for 2 h, cooled to room temperature, and concentrated in vacuo. A solution of the viscous pale yellow oily residue in THF (0.5 mL) was cooled to 0˚C, treated with a mixture of triethylamine (45 mg, 0.45 mmol, 1.5 equiv), butylamine (24 mg, 0.33 mmol, 1.1 equiv) and DMAP (1.8 mg, 0.015 mmol, 0.05 equiv), stirred at 0˚C for 15 min and then allowed to warm to RT. The solution was stirred at room temperature for 45 min, and poured into a separatory funnel containing 1 M HCl (2 mL) and ether (2 mL). The organic layer was washed with water (1 x 2 mL) and brine (1 x 2 mL), dried (Na2SO4) and concentrated. The residue was purified by chromatography on SiO2 (1% MeOH/CH2Cl2) to afford butylamide 21 (33 mg, 59%) as a pale yellow oil: IR (neat) 3424, 1691, 1631 cm⁻¹; ¹H NMR (300 MHz, CDCl3) δ 5.69 (br s, 2 H) 4.69 (br s, 2 H), 3.29 (br t, 2 H, J = 6.6 Hz), 1.97 (d, 3 H, J = 1.2 Hz), 1.56-1.46 (m, 2 H), 1.42-1.29 (m, 2 H), 0.93 (t, 3 H, J = 7.2 Hz); ¹³C NMR (75 MHz, CDCl3) δ 165.4, 146.8, 122.7, 42.8, 39.1, 31.6, 22.4, 20.1, 13.7; MS (ESI) calculated for [M+H]+ 189.09, found 189.10.

Spectroscopic data and chemical synthesis of (E)-N-butyl-4-chloro-3-methylbut-3-enamide (25). (E)-4-Chloro-3-methylbut-3-enoic acid (24): A solution of Cp2ZrCl2 (1.46 g, 5.0 mmol, 1.0 equiv) in CH2Cl2 (5 mL) in a 50 mL 3-necked flask was stirred at 0˚C for 15 min and then treated with a solution of AlMe3 (1.08 g, 15.0 mmol, 3.0 equiv) in CH2Cl2 (5 mL). The resultant pale yellow reaction mixture was stirred at 0˚C for 15 min, treated over 15 min with a solution of 3-butyn-1-ol (350 mg, 5 mmol) in CH2Cl2 (2 mL), slowly warmed to room temperature, stirred for 14 h, and cooled to -23˚C for 15 min before addition of NCS (800 mg, 6.0 mmol, 1.2 equiv). The mixture was stirred at -23˚C for 30 min, slowly warmed to room temperature and stirred for 15 min before the reaction was quenched by the slow, dropwise addition of 1 M HCl (20 mL). The solution was extracted with CH2Cl2 (2 x 20 mL). The organic layer was washed with water (1 x 20 mL) and brine (1 x 20 mL), dried (Na2SO4) and concentrated in vacuo to afford the alcohol 23 as a pale yellow oil. Purification by chromatography on SiO2 (10% EtOAc/hexanes, followed by 20% EtOAc/hexanes) gave 23 (362 mg, 60%) as a colorless oil. A solution of alcohol 23 (30.1 mg, 0.25 mmol, 1.0 equiv) in acetone (1 mL) was cooled to 0˚C in an ice-bath for 15 min, treated with Jones reagent (0.25 mL, 0.63 mmol) and stirred at 0˚C for 20 min. After the addition of 2-propanol (400 μL), the reaction mixture was poured into a separatory funnel containing ether (5 mL) and water (5 mL). The aqueous layer was re-extracted with ether (2 x 5 mL). The combined organic layers were washed with brine (1 x 20 mL), dried (Na2SO4), and concentrated in vacuo. The resulting brown oil was purified by chromatography on SiO2 (1% MeOH/CH2Cl2 containing 0.5% AcOH) to give the desired acid 24 (22.8 mg, 68%) as a pale yellow oil.
(E)-N-Butyl-4-chloro-3-methylbut-3-enamide (25): To a solution of the chloro acid 24 (34 mg, 0.25 mmol) in CH₂Cl₂ (0.5 mL) was added EDC•HCl (58 mg, 0.30 mmol, 1.2 equiv), triethylamine (38.4 mg, 0.379 mmol, 1.5 equiv) and butylamine (22.2 mg, 0.303 mmol, 1.2 equiv). The reaction mixture was stirred at room temperature for 14 h, poured into a separatory funnel containing 1 M HCl (1 mL) and ether (2 mL), and the organic layer was washed with water (1 x 2 mL) and brine (1 x 2 mL) dried (Na₂SO₄) and concentrated in vacuo. The yellow oily residue was purified by chromatography on SiO₂ (10% EtOAc/hexanes, followed by 20% EtOAc/hexanes) to afford the desired amide 25 (20 mg, 41%) as a colorless oil: IR (neat) 3429, 1636, 1556 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 6.03-6.00 (m, 1 H), 5.61 (br s, 1 H), 3.30-3.19 (m, 2 H), 2.98 (s, 2 H), 1.85 (br d, 3 H, J = 1.2 Hz), 1.54-1.43 (m, 2 H), 1.39-1.29 (m, 2 H), 0.92 (t, 3 H, J = 7.2 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 169.1, 133.5, 116.6, 44.8, 39.4, 31.6, 20.0, 16.7, 13.7; MS (ESI) calculated for [M+H]+ 189.09, found 189.10.

Spectroscopic data and chemical synthesis of (Z)-N-buty1-4-chloro-3-methylbut-3-enamide (28).

(Z)-4-Chloro-3-methylbut-3-enolic acid (27). A solution of Cp₂ZrCl₂ (0.292 g, 1.0 mmol, 1.0 equiv) in 1,2-DCE (1 mL) in a 50 mL 3-necked flask was stirred at 0˚C for 15 min, treated with a solution of AlMe₃ (0.216 g, 3.0 mmol, 3.0 equiv) in 1,2-DCE (2 mL) and stirred at 0˚C for 15 min. To the reaction mixture was added a solution of 3-butyn-1-ol (70 mg, 1.0 mmol, 1 equiv) in 1,2-DCE (1 mL) over 15 min. The mixture was slowly warmed to room temperature, stirred for 14 h, slowly heated to reflux and maintained at a reflux temperature for 3 d. The solution was cooled to 0˚C and subsequently to -23˚C for 15 min before addition of NCS (127 mg, 1.20 mmol, 1.2 equiv). The reaction mixture was stirred at -23˚C for an additional 30 min, slowly warmed to room temperature, stirred for 15 min, and quenched by a slow, dropwise addition of 1 M HCl (10 mL). The solution was extracted with ether (5 x 20 mL), and the combined organic layers were washed with brine (1 x 50 mL), dried (Na₂SO₄) and concentrated in vacuo to afford the desired alcohol 26 as a pale yellow oil. Purification of the crude compound by chromatography on SiO₂ (10% EtOAc/hexanes, followed by 20% EtOAc/hexanes) provided alcohol 26 (61 mg, 51%) as a pale yellow oil. A solution 26 (61 mg, 0.51 mmol, 1.0 equiv) in acetone (2 mL) was cooled to 0˚C for 15 min, treated with Jones reagent (0.51 mL, 1.28 mmol) and stirred for 20 min at 0˚C. The mixture was diluted with 2-propanol (400 µL), poured into a separatory funnel containing ether (5 mL) and water (5 mL), and the aqueous layer was washed with ether (2 x 5 mL). The combined organic layers were washed with brine (1 x 20 mL), dried (Na₂SO₄) and concentrated in vacuo. The brown oily residue was purified by chromatography on SiO₂ (1% MeOH/CH₂Cl₂ containing 0.5% AcOH) to give the desired acid 27 (44.3 mg, 65%) as a pale yellow oil.

(Z)-N-Butyl-4-chloro-3-methylbut-3-enamide (28). To a solution of acid 27 (11.0 mg, 0.08 mmol, 1.0 equiv) in THF (1 mL) were added sequentially at 0˚C diphenylphosphinic chloride (19.3 mg, 0.082 mmol, 1.0 equiv), N-methylmorpholine (34.7 mg, 0.343 mmol, 4.2 equiv) and butylamine (5.9 mg, 0.08 mmol, 1.0 equiv). The reaction mixture was stirred at 0˚C for 1 h, concentrated and purified by
chromatography on SiO2 (20% EtOAc/hexanes) to give amide 28 (5.0 mg 32%) as a colorless oil: IR (neat) 3428, 1649, 1440 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 6.00 (app d, 1 H, \(J = 1.5\) Hz), 5.61 (br s, 1 H), 3.28-3.21 (m, 2 H), 3.16 (s, 2 H), 1.86 (d, 3 H, \(J = 1.2\) Hz), 1.53-1.43 (m, 2 H), 1.40-1.25 (m, 2 H), 0.92 (t, 3 H, \(J = 7.2\) Hz); \(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 168.6, 134.0, 114.9, 40.4, 39.4, 31.6, 21.4, 20.0, 13.7; MS (ESI) calculated for [M+H]\(^+\) 189.09, found 189.10.

**Spectroscopic data and chemical synthesis of \(N\)-butyl-4-chloro-3-methylbutanamide (31).**

\(N\)-Butyl-4-chloro-3-methylbutanamide (31). To a solution of lactone 29 (100 mg, 1.0 mmol, 1.0 equiv) in thionyl chloride (0.13 g, 1.0 equiv) was added freshly fused ZnCl\(_2\) (6.8 mg, 0.05 mmol, 0.05 equiv). The reaction mixture was slowly heated to reflux, maintained at reflux for 8 h, and then slowly cooled to room temperature. The mixture was concentrated in vacuo and a solution of the brown oily residue in THF (0.5 mL) was cooled to 0°C, treated with a mixture of triethylamine (0.15 g, 0.15 mmol, 1.5 equiv) and butylamine (80 mg, 1.1 mmol, 1.1 equiv), stirred at 0°C for 30 min, warmed to room temperature, stirred for 30 min, and poured into a separatory funnel containing 1 M HCl (2 mL) and CH\(_2\)Cl\(_2\) (2 mL). The organic layer was washed with brine (1 x 2 mL), dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo to afford a pale brown oil that was purified by chromatography on SiO2 (20% EtOAc/hexanes followed by 50% EtOAc/hexanes) to give amide 31 (70.7 mg, 37%) as a yellow oil: IR (neat) 3422, 1644, 1559, 1459, 1379 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.92 (br s, 1 H), 3.59-3.48 (m, 2 H), 3.24 (app q, 2 H, \(J = 6.6\) Hz), 2.50-2.30 (m, 2 H), 2.10 (dd, 1 H, \(J = 14.1, 7.2\) Hz), 1.52-1.43 (m, 2 H), 1.37-1.27 (m, 2 H), 1.04 (d, 3 H, \(J = 6.6\) Hz), 0.91 (t, 3 H, \(J = 7.2\) Hz); \(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 171.3, 50.6, 40.6, 39.3, 32.6, 31.6, 20.0, 17.6, 13.7; MS (ESI) calculated for [M+H]\(^+\) 191.11, found 191.10.

**Chemical synthesis of 6-CoA.** The chemical synthesis of 6-CoA was described in our previous work\(^1\).

**Enzymatic synthesis of 1-CoA.** 1-CoA was enzymatically generated by HMG-CoA reductase as previously described\(^1\)\(^2\). The reaction mixture was separated by a self-packed DEAE Sepharose column (1 cm x 30 cm, GE Healthcare)) equilibrated with 0.1 M HCO\(_2\)NH\(_4\), pH 4.4. 1-CoA was eluted with 0.4 M HCO\(_2\)NH\(_4\), pH 4.4\(^2\). 1-CoA was further purified using XBridge Preparative C18 column (10 x 250 mm, 5 \(\mu\)m) and an elution gradient of 3-60% CH\(_3\)OH/H\(_2\)O (10 mM NH\(_4\)OAc). The fractions were pooled and lyophilized, and 1-CoA purity was measured by HPLC to be >98%.

**Enzymatic synthesis of 2-CoA.** 2-CoA was enzymatically generated from 1-CoA by Cur ECH\(_1\) as previously described\(^3\).

**Bacterial strains, media and culture conditions.** *Escherichia coli* DH5\(\alpha\) MCR (Invitrogen) was used for DNA propagation. *Escherichia coli* BL21 (DE3) transformed with the derivatives of pET20b, pET24b, pET28b, and pET41a (Novagen) were used for protein overexpression in Luria-Bertani (LB) medium.
Ampicillin (100 μg/mL), carbenecillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), and apramycin (50 μg/mL) were used for the corresponding plasmid construct resistance marker selection in *E. coli* cultures.

**Plasmid construction and site mutagenesis.** The primers for the plasmid construction were listed in Supplementary Table 1. The expression plasmids for CurB ACPIV, CurE ECH1 and CurF ECH2 were constructed in our previous work¹. CurA Hal, Hal-ACP3 and CurF ER genes were amplified from the cosm id pLM54⁴. JamI ECH1, JamJ ECH2 and JamJ ER genes were amplified from cosmid pJam3⁵. CurA Hal and Hal-ACP3 genes were inserted into pET28b plasmid using NdeI/XhoI restriction sites. JamI ECH1 gene was inserted into pET24b plasmid using NdeI/XhoI restriction sites. JamJ ECH2 gene was inserted into pET20b plasmid using NdeI/XhoI restriction sites. CurF ER and JamJ ER genes were inserted into pET41a plasmid using SacII/XhoI restriction sites. The CurA ACPI, ACPI, ACPII and ACPIII expression plasmids derived from a synthetic ACP3 gene were provided by Professor Christopher Walsh and Dr. Christopher Calderone at Harvard Medical School. CurA ACPI (1946-2031), ACPII (2057-2146) and ACPIII (2161-2248) genes were inserted into pET29a plasmid using NdeI/XhoI restriction sites. CurF ECH2 mutants, H240A, H240Q, K86A, K86Q and Y82F, were made in our previous work³. All the constructs and mutations were verified by DNA sequencing.

**Protein overexpression.** The protein expression conditions for CurB ACPIV, CurE ECH1, CurF ECH2 and the corresponding mutants were described in our previous work¹³. **A)** *CurA* (apo) ACPI, (apo) ACPI, (apo) ACPII, (apo) ACPIII, JamI ECH1, and JamJ ECH2: *E. coli* BL21 (DE3) was transformed by the pET28a::ACP3 (pCC111) plasmid to overexpress the N-terminal His-tagged protein, and by pET29a::ACP1 (pCC112), pET29a::ACP1 (pCC113), pET29a::ACP1II (pCC114), pET24b::Jam ECH1 and pET20b::Jam ECH2 to overexpress the C-terminal His-tagged proteins. Cells were grown at 35°C to an OD (590 nm) = 0.5-0.6, and then cooled to 18°C prior to addition of 1 mM isopropyl-β-D-galactopyranoside (IPTG). The cultures were grown at 18°C for another 12-15 h before harvesting. **B)** *CurA Hal* and (apo) Hal-ACP3: *E. coli* BL21 (DE3) was transformed by pET28b::Cur Hal and pET28b::Cur Hal-ACP3 plasmids to overexpress the N-terminal His-tagged proteins. Cells were grown at 30°C to an OD (590 nm) = 0.5-0.6, and then cooled to 15°C prior to the addition of 1 mM IPTG. The cultures were grown at 15°C for another 18-20 h before harvesting. **C)** *CurF ER* and *JamJ ER*: *E. coli* BL21 (DE3) was transformed by pET41b::Cur ER and pET41b::Jam ER to overexpress the N-terminal GST-tagged and C-terminal His-tagged proteins. Cells were grown at 30°C to an OD (590 nm) = 0.5-0.6, and then cooled to 18°C prior to the addition of 1 mM IPTG. The cultures were grown at 18°C for another 12-15 h before harvesting.

**Protein purification.** Protein purifications were performed at 4°C. In general, the first step Ni-affinity purifications for all His-tagged proteins were performed under the same conditions. *E. coli* cells were
harvested by centrifugation (5,000 g, 20 min, 4°C), resuspended in ice cold lysis buffer A (50 mM PBS buffer, pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol) and disrupted by sonication on ice. The cell debris was removed by centrifugation at 15,000 g for 50 min. The supernatant was gently removed and loaded onto the 5 ml HisTrap column (GE Healthcare) preequilibrated with lysis buffer A. The resin was washed successively with ~10 column volumes of washing buffer B (50 mM PBS buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol) to remove nonspecifically bound contaminants. Bound proteins were eluted with imidazole by a linear gradient of the elution buffer C (50 mM PBS buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole, 20% glycerol). The fractions of the eluate were checked for purity by SDS-PAGE, pooled and concentrated using Amicon Ultra-15 (30 kDa, 10 kDa or 5 kDa) centrifugal devices (Millipore). The follow-up purification and buffer exchange for different proteins are described below:

A) CurA (apo) ACP3, (apo) ACP1, (apo) ACP11, (apo) ACP11, CurB (apo) ACP1V, CurF ECH2 WT and the mutants, CurF ER, JamI ECH1, JamJ ECH2 and JamJ ER: the concentrated eluate was loaded onto HiPrep 26/10 Desalting column (GE Healthcare) equilibrated with the storage buffer D (50 mM PBS buffer, pH 7.5, 200 mM NaCl, 20% glycerol), The fractions were pooled, concentrated, flash-frozen in 50-200 µl aliquots by liquid N2, and stored at -80°C. B) CurE ECH1: CurE ECH1 is prone to precipitate after its elution from HisTrap column. So the eluted protein was immediately desalted by PD10 column (GE Healthcare) equilibrated with the storage buffer D. The protein was flash-frozen in 100 µl aliquots by liquid N2 and stored at -80°C.

Anaerobic purification of Cur Hal and (apo) Hal-ACP3. The FeII-, α-KG- and O2-dependent halogenases were reported to be sensitive to O2, and anaerobic purification was performed to remain their activities. The purification was performed under inert atmosphere by using ÄKTA FPLC (GE Healthcare) accompanied with a glove box (Coy Laboratory Products), which is similar to the system previously described. All the buffers were sparged with argon and equilibrated in glove box for two days. E. coli cells were resuspended in buffer A and disrupted by sonication on ice. Then the cell lysate was equilibrated with argon for ~1 hour, transferred to gas-tight tubes in glove box, and centrifuged at 15,000 g for 50 min. The Ni-affinity purification for Hal and Hal-ACP3 was performed as described above. After elution from HisTrap column, the proteins were exchanged into buffer D using HiPrep 26/10 Desalting column. The N-terminal His-tag of Hal was removed by thrombin for metal analysis. The biotinylated thrombin (Novagen) was added into Hal in buffer D, and the reaction was incubated at room temperature for overnight to achieve a complete His-tag cleavage. The biotinylated thrombin was removed from the reaction mixture by using 1ml HiTrap Streptavidin HP column (GE Healthcare). The flow-through was loaded onto HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with the storage buffer D. Cur Hal was eluted as a dimer from size-exclusion columns. The fractions were pooled, concentrated, flash-frozen in 50 µl aliquots by liquid N2, and stored at -80°C.
Metal analysis of Cur Hal. To determine which metal bound to Hal, His-tag cleaved Cur Hal was anaerobically reconstituted with 1 mM α-KG and a metal mixture containing 25 μM Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ for 10 min before desalted by PD10 column equilibrated with buffer E (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 10% glycerol). The metal content of Cur Hal was measured by ICP-MS (Finnigan). The buffer E was applied as blank for analysis. For ~2.0 μM Cur Hal, the metal concentrations were measured to be 1.81 ± 0.09 μM Fe (blank 0.09 ± 0.02), < 0.0031 μM Co (blank < 0.0027), < 0.0017 μM Ni (blank < 0.0015), < 0.0019 μM Cu (blank < 0.0017), and 0.067 ± 0.003 μM Zn (blank 0.039 ± 0.003). Based on this result, the reconstitution of with α-KG and Fe(NH₄)₂(SO₄)₂ were performed for its activity assay described below.

Substrate loading onto (apo) ACPs. The ACP-linked substrates were generated by loading the corresponding CoA substrates onto (apo) ACPs by using recombinant Bacillus subtilis Sfp¹⁹. Typically, 500 μM acyl-CoA and 50 μM (apo) ACP were incubated with 2 μM Sfp, and 10 μM MgCl₂ in 50 mM Tris-HCl buffer, pH 8.1, at room temperature for ~2 h. Reaction mixtures were desalted by PD10 column equilibrated with the buffer E. The desalted (holo) ACPs were concentrated by using Amicon Ultra-4 (5 kDa, Millipore), flash-frozen in 10-50 μl aliquots in liquid N₂, and stored at -80°C. For substrate loading onto (apo) Hal-ACP₃, the reagents were equilibrated with argon prior to use. The substrate loading onto (apo) Hal-ACP₃ was manipulated in glove box. The substrate-loaded ACP samples were analyzed by reverse-phase HPLC using a Jupiter C4 column (250 x 2.0 mm, 5 μm, 300 Å, Phenomenex), and a linear elution gradient from 5% to 90% of CH₃CN (0.1% CF₃CO₂H)/H₂O (0.1% CF₃CO₂H).

Cur Hal activity assays & enzymatic generation of γ-Cl-1-ACP₈. Cur Hal activity was tested by using ACPIV or ACPII-linked substrates including malonyl-ACP₄, acetyl-ACP₄, 1-ACP₈, 2-ACP₈, 3-ACP₈, 4-ACP₈ and 6-ACP₈. The chlorination product was only detected for 1-ACP₈. As such, the follow-up chlorination assays were performed by incubating the enzyme with 1-ACP₈ or 1-ACP₃. Typically, 200 μl reaction mixture containing 50 μM 1-ACP₈ or 1-ACP₃, 5 μM Cur Hal, 50 μM fresh Fe(NH₄)₂(SO₄)₂, and 0.5 mM α-KG in 50 mM Tris-HCl buffer (pH 7.5) was prepared in glove box. The reaction was initiated by exposing the reaction mixture to air, and incubated at 30°C for 2 h to achieve a full conversion to γ-Cl-1-ACP₈. The γ-Cl-1-ACP₈ was served as substrate for ECH₁/ECH₂/ER reactions. FTICR-MS and IRMPD were employed to detect the products.

α-KG and O₂ dependence of Hal chlorination. The α-KG and O₂ dependence of Cur Hal chlorination was investigated by incubating the enzyme with 1-ACP₈ in the absence of α-KG or O₂ under the uniform reaction condition described above. For Hal chlorination without O₂, the reaction mixture was prepared in glove box, and capped during its incubation.
**ECH₁ and ECH₂ activity assays.** ECH₁ and ECH₂ assays were performed as previously described². Briefly, ~50 μM 1-ACP₉ or γ-Cl-1-ACP₉ was added with 1 μM ECH₁ or ECH₁/ECH₂ in 50 mM Tris-HCl buffer (pH 7.5), and incubated at 30°C. After quenched by addition of 10% formic acid, the reactions were analyzed by reverse-phase HPLC using Jupiter C4 column. FTICR-MS and IRMPD were applied to detect the products.

**ER activity assays.** The chlorinated substrate, γ-Cl-3-ACP₉, was generated from γ-Cl-1-ACP₉ by Cur ECH₁/ECH₂, and the non-chlorinated substrate, 3-ACP₉ was generated by loading (apo) ACP₉ with 3-CoA. Typically, ER reactions were performed by incubating ~50 μM γ-Cl-3-ACP₉ or 3-ACP₉ with 1 μM ER and 0.5 mM NADPH in 50 mM Tris-HCl buffer (pH 7.5) at 30°C. Alternatively, ER reaction was coupled with ECH₁/ECH₂ dehydration and decarboxylation by serving γ-Cl-1-ACP₉ or 1-ACP₉ as the substrate. FTICR-MS and IRMPD were applied to detect the products. When NADH was substituted for NADPH, no saturation product was detected in ER assays.

**One-pot reactions with Cur Hal-ACP₃ or ACP₃-linked substrate.** The one-pot reactions were conducted to confirm the products of ECH₁/ECH₂, Hal/ECH₁/ECH₂, ECH₁/ECH₂/ER, and Hal/ECH₁/ECH₂/ER coupled reactions. The ACP₃-linked HMG substrates were generated by loading Cur (apo) Hal-ACP₃ and ACP₃ with 1-CoA as described above. Substrate loading of (apo) Hal-ACP₃ was performed in an anaerobic glove box. Typically, the one-pot reactions were performed by incubating ~50 μM ACP₃ or Hal-ACP₃-linked HMG substrate with ~10 μM enzymes and their corresponding cofactors at 30°C for 5 min. The reactions were initiated by exposing the reaction mixture to O₂, and quenched by addition of 10% formic acid. Hal-ACP₃ and ACP₃ were purified from their reaction mixtures by reverse-phase HPLC prior to butylamine aminolysis as described below.

**Butylamine aminolysis.** To determine the structures of products linked to the ACP₃ and Hal-ACP₃ PPant arms, the acyl groups were cleaved by butylamine aminolysis to generate the butylamide derivatives, which were analyzed by GC/MS¹⁰ and compared with the authentic standards. To remove contaminants, the ACP₃ and Hal-ACP₃ were purified from their reaction mixtures by reverse-phase HPLC using the Source 15PRC column. The proteins were eluted using a linear gradient from 30% to 70% of CH₃CN (0.1% CF₃COOH)/H₂O (0.1% CF₃COOH), and pooled in glass vials before lyophilization. The aminolysis reaction was performed by addition of 160 μl H₂O, 100 μl hexane and 40 μl butylamine to ~5 nmol lyophilized ACP₃ or Hal-ACP₃. The reaction mixture was incubated at 30°C for 30 min, quenched by 66 μl 12 M HCl, and extracted with 2 x 2 ml ethyl acetate. The extracts were dried under nitrogen, and the butylamides were redissolved in 100 μl hexane before GC/MS analysis.

**GC/EI-MS analysis.** The samples and authentic standards were analyzed by a Hewlett Packard 6890
gas chromatograph equipped with a 5973 mass selective quadrupole detector. The butylamides were separated on a DB-VRX (Agilent J&W) capillary column (60 m x 253 μm x 1.40 μm), which was operated with helium-carrier gas and splitless injection. Both the injector and detector temperatures were set as 250°C. After initial 6 min at 45°C, the oven temperature was raised to 140°C at 8°C/min and held for 10 minutes, and then to 225°C at 30°C/min and held for 25 minutes. Total ion chromatograms were recorded using a mass range of 35-270 amu, and the selective ion chromatograms were recorded by monitoring 2-3 most abundant masses plus the parent masses of target compounds.

**Analysis of ACP samples by electrospray ionization (ESI)-FTICR-MS.** The observed and calculated masses for all ACPH samples were listed in Supplementary Table 2. The ESI-FTICR-MS samples were prepared by separating ACPH from the reaction mixtures using the Source 15PRC reverse phase column as previously described. All samples were freshly prepared and analyzed with an actively shielded 7 Tesla quadrupole-FTICR mass spectrometer (APEX-Q, Bruker Daltonics, Billerica, MA). Target analytes in electrospray solution (1:1 CH3CN:H2O with 0.1% HCOOH) were directly infused into an electrospray ionization (ESI) source (Apollo II, Bruker Daltonics) operating in positive ion mode at a flow rate of 70 μL/h and a voltage of -3.8 kV. A counterflow of hot (240 °C) nitrogen gas was applied to assist desolvation of ESI droplets. Multiply protonated ions generated by ESI were externally accumulated in a hexapole and transferred via high voltage ion optics to the ICR cell for analysis. For IRMPD, precursor ions were mass-selectively accumulated in the hexapole with a 5-10 m/z quadrupole isolation window, transferred to the ICR cell, and irradiated for 100-300 ms by 10.6 μm photons at 10 W laser power (25 W CO2 laser, Synrad, Mukilteo, WA). All data were acquired with XMASS software (version 6.1, Bruker Daltonics) in broadband mode from m/z = 200 to 2000 with 512k data points and summed over 10-30 scans. Mass spectra were analyzed with the MIDAS analysis software. For accurate mass determination, apomyoglobin (Sigma, St. Louis, MO) peaks with charge state of 19-20 (apomyoglobin was spiked into the ESI solution prior to analysis), or known y-type product ions (y22) generated from IRMPD of the same protein were used as internal calibrants. Once the exact mass of a protein had been determined, its two most abundant charge states were selected as internal standards for further calibration (apomyoglobin was not spiked into all reactions). All frequency-to-m/z calibrations were performed with a two-term calibration equation.

**IRMPD-based quantification.** Peak abundances of PPant ejection products (PEPs) generated by IRMPD were measured to quantify the yields of the ER catalyzed α,β C=C saturation and cyclopropanation reactions for time-course studies, or site mutagenesis assays. In ER α,β C=C saturation reaction, an addition of two hydrogens to the substrate is unlikely to affect ESI efficiency. Furthermore, products and substrates were observed with the same charge state distribution patterns, allowing a single charge state to be used for analysis. For Cur ER cyclopropanation, quantification of 5-ACP was
achieved by using 4-ACP_EII as an internal standard. No desaturation product of 4-ACP_EII, which would interfere with 5-ACP_EII measurement, was detected. The ratio of these acyl-ACP_EII species with 2 Dalton mass difference can be straightforwardly measured by analyzing their PEPs. With IRMPD, quantification results from a single charge state (+12) were identical to those based on an average of the entire charge state distribution. Thus, we chose to utilize the +12 charge state for IRMPD quantification analysis. The ratio of PEPs with 2 Dalton mass difference was calculated by measuring the abundance of the n and n+2 product ion peaks (the n+2 peak abundance was adjusted by subtracting the natural n+2 isotopic abundance for peak n). For saturation reactions, yields were calculated by measuring the ratios of substrates with respect to products whereas, for cyclopropanation reactions, yields were calculated by measuring the ratios of products with respect to the internal standard. Signal/noise ratios were typically ~10 but varied from 2-100 depending on the time point. All peak assignments are based on accurate mass.

**Time-course studies of ER saturation and cyclopropanation.** The time-course studies were performed to compare the catalytic efficiencies of Cur ER cyclopropanation and saturation, and Jam ER saturation. γ-Cl-3-ACP_EII was applied as the substrate for Cur ER cyclopropanation and Jam ER saturation, and 3-ACP_EII as the substrate for Cur and Jam ER saturation. All the reactions were performed in triplicate under uniform reaction conditions. ~200 μM γ-Cl-3-ACP_EII or 3-ACP_EII was incubated with 2 μM Jam or Cur ER, and 1 mM NADPH in 50 mM Tris-HCl buffer (pH 7.5) at 30˚C. For Cur ER cyclopropanation, ~100 μM 4-ACP_EII, which was generated from (apo) ACPII and 4-CoA, was added as the internal standard for 5-ACP_EII. No desaturation product of 4-CoA was observed under the tested conditions. After certain amounts of reaction time, ~20 μl aliquots were removed from the reaction mixture, added with 50 μl 10% formic acid to quench the reaction, and flash-frozen by liquid N2. The ACPII samples were purified by reverse-phase HPLC, and immediately subjected to the IRMPD-based quantification analysis.

**Quantification of regiochemical products of the ECH2 WT and mutants.** The ratios of α,β and β,γ C=C products of the ECH2 decarboxylation were measured for Cur ECH2 WT and mutants, and Jam ECH2 WT. The products were generated from γ-Cl-1-ACP_EII by ECH1/ECH2 coupled dehydration and decarboxylation. 50 μM γ-Cl-1-ACP_EII was incubated with 2 μM Cur ECH1 and 2 μM Cur or Jam ECH2 in 50 mM Tris-HCl buffer (pH 7.5) at 30˚C for 45 min. Next, the reaction mixtures were treated with 2 μM Jam ER with 0.5 mM NADPH for 45 min before an addition of 10% formic acid to quench the reactions. The ratio of γ-Cl-4-ACP_EII and γ-Cl-6-ACP_EII were measured by IRMPD-based quantification as described above.

**Modeling the PPant arm of (4S)-γ-Cl-2-ACP_EII into Cur ECH2 structure.** Initial atomic coordinates
and topology files for the PPant arm of (4S)-γ-Cl-2-ACP$_{II}$ were generated using the PRODRG2 server$^{15}$. The PPant arm was initially positioned into Cur ECH$_2$ (PDB code 2Q34) as previously described$^{3}$. Any overlapping water molecules were removed before energy minimization after the addition of hydrogens using the program CNS 1.2$^{16}$ in 1080 steps of conjugate gradient minimization using no experimental energy terms and full harmonic restraints.
Supplementary Figure 1. PKS hybridization mediated by a “di-AT replacement”. a. Comparative sequence identities of the enzymes encoded by the two highly similar regions in the Cur and Jam pathways. The aligned DNA sequences are located at the boundaries of these two regions. b. Alignment of AT domains of the sequenced pathways from L. majuscula, including curacin, jamaicamide, barbamide and putative carminabin pathways. As previously reported^{17,18}, the AT N-terminus and “post-AT linker” regions are highly conserved, which can promote AT domain replacement by homologous recombination.
Supplementary Figure 2. Phylogenetic trees for a, AT, b, KS and c, DH domain sequences of curacin, jamaicamide, barbamide and putative carmabin pathways from *L. majuscula*. The phylogram trees with distances were constructed by ClustalW2. **a**, CurA and JamE ATs as well as CurF and JamJ ATs reveal extraordinarily high similarity (86% and 79% sequence identities, respectively). **b**, the CurF and JamJ KSs within the proposed “di-AT replacement” region are most closely related, but in contrast the CurA and JamE KSs outside this region are relatively distant. **c**, the CurF and JamJ DHs, which are outside the “di-AT replacement” region, show a relatively distant relationship, with the JamJ DH being most similar to DHs from the putative carmabin pathway (~70% sequence identity). Overall, this phylogenetic analysis is consistent with the “di-AT replacement” hypothesis.
Supplementary Figure 3. Coomassie blue-stained SDS-PAGE of purified Cur and Jam enzymes. JamJ ER and CurF ER were expressed as N-terminal GST-tagged proteins to increase their solubility. CurE ECH1 was coexpressed with chaperones to increase its solubility and coeluted with them from the Ni-affinity column. His-tag cleaved CurA Hal was prepared for metal content analysis. The target enzymes are indicated by red arrows. Due to nearly identical amino acid sequences of the ACPI, ACPII and ACPIII, only ACPII was purified for assays.
Supplementary Figure 4. HPLC analysis of HMG substrate loading onto a, (apo) ACP₃, b, (apo) ACP₁₁. Multiple peaks were observed for (apo) ACP₃ substrate loading due to mono-, di- and tri-loading with (S)-HMG-CoA. See Supplementary Methods for detailed assay protocols.
Supplementary Figure 5. a-c, HMG substrate chirality preference of Cur Hal and ECH1; d, O2 and α-KG dependence of Cur Hal. Partial FTICR mass spectra for ECH1 and Hal reactions with a, pure (S)-HMG-ACP II, b, 1:1 (S)-HMG-ACP II and (R)-HMG-ACP II mixture, and c, <1:5 (S)-HMG-ACP II and (R)-HMG-ACP II mixture. Cur ECH1 was proved to accept HMG-CoA substrate, and (R)-HMG-CoA was enriched by removing (S)-HMG-CoA from (R,S)-HMG-CoA mixture via Cur ECH1-catalyzed dehydration for multiple times. The enriched (R)-HMG-CoA was loaded onto (apo) ACP II to produce the (R)-HMG-ACP II enriched substrate. Asterisks denote unidentified species. d, Partial FTICR mass spectra for Hal reactions without O2 or α-KG. For Hal reaction without O2, the reaction mixture was prepared in glove box and capped during its incubation.
**Supplementary Figure 6. IRMPD spectra.**
a, IRMPD spectra for Figure 2a-2h; b, IRMPD spectra for Figure 3a-3f. The structures of PPant ejection products (PEPs) are also shown.
Supplementary Figure 7. Feeding experiment to demonstrate that Hal chlorination precedes ECH$_2$ decarboxylation. The $\beta$-branching carbon forming the cyclopropane was labeled with only one deuterium atom by [2H$_3$,2-13C]acetate supplied in the growth media of *L. majuscula*. If Hal chlorination happens after ECH$_2$ decarboxylation, the $\beta$-branching carbon would be labeled randomly with either one or two deuterium atoms in a 2:1 ratio.
Supplementary Figure 8. HPLC analysis of ECH\textsubscript{1}/ECH\textsubscript{2} coupled dehydration and decarboxylation to compare the rates for the chlorinated and non-chlorinated substrate. The reactions were performed under a uniform reaction condition, and quenched after the indicated periods of time. See Supplementary Methods for detailed protocols.
Supplementary Figure 9. IRMPD-based quantification to measure the yields of a, Cur ER cyclopropanation, b, Jam ER saturation, and c, the ratio of $\alpha,\beta$ and $\beta,\gamma$ C=C products of ECH$_2$s. Because the ACPII species with 2 Dalton mass difference are equally distributed over different charge states, we focused on the +12 charge state to record IRMPD spectra. a, To measure the yield of Cur ER cyclopropanation, 4-ACP$\Pi$ was added as internal standard for 5-ACP$\Pi$. b, The yield of Jam ER saturation was measured by the ratio of $\gamma$-Cl-3-ACP$\Pi$ substrate and $\gamma$-Cl-4-ACP$\Pi$ product. c, To measure the ratio of $\alpha,\beta$ and $\beta,\gamma$ C=C products, the $\alpha,\beta$ C=C product was reduced by Jam ER by treating the mixtures of $\alpha,\beta$ and $\beta,\gamma$ C=C products with Jam ER for 30 min before IRMPD analysis. See Supplementary Methods for detailed protocols.
**Supplementary Figure 10. Validation of IRMPD-based quantification methods.** 3-ACP\textsubscript{II}, 4-ACP\textsubscript{II}, and 5-ACP\textsubscript{II} were prepared by loading the corresponding CoA thioesters onto (apo) ACP\textsubscript{II}. γ-Cl-4-ACP\textsubscript{II} and γ-Cl-6-ACP\textsubscript{II} were enzymatically generated from 1-ACP\textsubscript{II} as described in Supplementary Methods. The pairs of ACP standards were mixed with known ratios before the IRMPD analysis. Peak abundances of the PPant ejection products generated from the 12+ charge state of the pair of ACP standards were measured to calculate their ratio. The ratios of IRMPD products were compared with the known ratios of ACP standards. The assays were performed in triplicate, and standard deviation error bars are shown.
Supplementary Figure 11. Functional differentiation of ERs. a, Comparison of catalytic efficiencies for cyclopropanation and saturation by Cur and Jam ERs. 3-ACP$_i$ was used as substrate for Cur ER saturation, and γ-Cl-3-ACP$_i$ was used as substrate for Cur ER cyclopropanation and Jam ER saturation. b, Comparison of Cur and Jam ER saturation by using 3-ACP$_i$ substrate. It was not possible to measure enzyme kinetic parameters ($k_{cat}$ and $K_M$) due to the tendency of ER to aggregate and the solubility limits of ACP-tethered substrates. Thus, time-course studies were performed in triplicate (standard deviation error bars are shown) under uniform reaction conditions. Product yields were measured by IRMPD-based quantification. See Supplementary Methods for detailed protocols.

Jam ER saturation and Cur ER cyclopropanation of γ-Cl-3-ACP$_i$ are faster by ~400-fold and ~50-fold, respectively, than is Cur ER saturation of 3-ACP$_i$ under identical experimental conditions. For 3-ACP$_i$, Jam ER saturation is ~240-fold faster than is Cur ER saturation. This comparison confirmed that Jam ER has retained canonical function as an α,β-enoxy reductase, in contrast to the Cur ER as a cyclopropanase. Given the proposed hydride-transfer step for both Cur and Jam ERs, their mechanisms are likely differentiated after formation of the α-carbanion intermediate, which functions as an intramolecular nucleophile (Cur ER) or is protonated (Jam ER) (Fig. 4b).
Supplementary Figure 12. FTICR mass spectra and IRMPD spectra for Jam enzyme reactions. a-h, Partial FTICR mass spectra for Jam ECH₁, ECH₂ and ER reactions excluding (a-d) or including (e-h) the Hal chlorination step. 1-ACP₃ was chlorinated by Cur Hal for 1 h to generate γ-Cl-1-ACP₃ substrate. The reactions were incubated at 30°C for 2 h for the 1-ACP₃ substrate and 30 min for the γ-Cl-1-ACP₃ substrate. i-j, IRMPD spectra for the PEPs of 6-ACP₃ (i) and γ-Cl-6-ACP₃ (j) before Jam ER saturation (upper) and after Jam ER saturation (lower). A small amount of saturated product with its PEP indicated by red arrow was observed to be mixed with the 6-ACP₃. Based on the butylamine cleavage analysis, 3-ACP₃ was inferred to be the minor α,β C=C product of Jam ECH₂ when using the non-chlorinated substrate.
Supplementary Figure 13. The UV absorption difference for Cur and Jam ECH₂ decarboxylation products. a, HPLC traces showing the separation of γ-Cl-1-ACP$_{II}$ and its decarboxylation products catalyzed by Cur and Jam ECH$_1$/ECH$_2$. b, UV spectra (220-310 nm) of Cur (red) and Jam (blue) ECH$_2$ decarboxylation products.
Supplementary Figure 14. Natural products with different β-branching-associated C=C positions via ECH_2 regiochemical control. a, Two groups of natural products with the α,β and β,γ C=Cs adjacent to their β-branching carbons. The C=Cs of curacin A and myxovirescin A have been modified by ERs. b, The mechanism for the formation of α,β and β,γ C=C products.
Supplementary Figure 15. Loss of Cur ECH2-mediated regiochemical control by site-directed mutagenesis. a, The hypervariable region (in magenta) of Cur ECH2 and the active site chamber modeled with the chlorinated substrate. The S-configuration of the HMG γ-carbon is preferred based on modeling results. b, Activity and regiochemical control of ECH2 WT and Cur ECH2 mutants. γ-Cl-1-ACP II was used as the substrate for all reactions. (Left) HPLC analysis for ECH1/ECH2 coupled dehydration and decarboxylation. All reactions were quenched after 10 min incubation at 30°C. (Right) IRMPD-based quantification to measure the percentage of β,γ C=C products (see Supplementary Methods). The coupled ECH1/ECH2 reactions were incubated for 45 min before treated with Jam ER for 45 min at 30°C. Assays were performed in triplicate, and standard deviation error bars are shown.

Site-directed experiments to understand Cur ECH2-mediated regiochemical control:

Similar to our previous assays, ECH1/ECH2-coupled dehydration and decarboxylation of γ-Cl-1-ACP II was analyzed to compare the catalytic efficiencies of WT and mutant Cur ECH2s. The catalytic activities of WT and mutant Cur ECH2s were significantly increased with the chlorinated substrate, possibly due to γ-Cl stabilization of the carbanion intermediate. However, their relative catalytic activities are similar to our previous results for the non-chlorinated substrate. Under same reaction conditions, the K86Q, K86A,
H240Q and H240A variants had significantly decreased activities (b, left panel). This suggests that His\textsuperscript{240} and/or Lys\textsuperscript{86} may draw the γ-carboxyl group away from the substrate in the transition state of ECH\textsubscript{2} decarboxylation through hydrogen-bond formation (a, right panel). The alanine substitutions at Lys\textsuperscript{86} and His\textsuperscript{240} had higher activity than the corresponding glutamine substitutions (b, left panel), possibly due to the small side chain of alanine allowing space for water molecules to stabilize the γ-carboxyl group or to donate a proton to the presumed enolate intermediate. Cur ECH\textsubscript{2} Y82F and Jam ECH\textsubscript{2} WT had similar activities to Cur ECH\textsubscript{2} WT, indicating that Tyr\textsuperscript{82} is not essential to the decarboxylation step.

Changes in the ratio of α,β and β,γ C=C products were assessed by measuring UV absorbance ratios (\(A_{280\text{nm}}/A_{250\text{nm}}\), Supplementary Fig. 13) for HPLC peaks corresponding to ECH\textsubscript{2} decarboxylation products (b, left panel). Measured peak ratios for Cur ECH\textsubscript{2} WT, K86Q, K86A, H240Q and H240A are ~1.75, for Jam ECH\textsubscript{2} WT the ratio is 2.23, but for Cur ECH\textsubscript{2} Y82F it is 1.85. The intermediate value for Cur ECH\textsubscript{2} Y82F suggests a mixture of α,β and β,γ C=C products. These products can be distinguished directly by using Jam ER as a reagent to selectively reduce α,β C=C followed by IRMPD to quantify product ratios (Supplementary Fig. 9c). The level of β,γ C=C product (γ-Cl-6-ACP\textsubscript{II}) for Cur ECH\textsubscript{2} WT, K86Q, K86A, H240Q and H240A was ~3% of the total product formed, but was ~30% of total product generated by Cur ECH\textsubscript{2} Y82F (b, right panel). Thus, replacing the Tyr\textsuperscript{82} hydroxyl group with a hydrogen atom resulted in substantially reduced regioselectivity during Cur ECH\textsubscript{2} decarboxylation. As proposed, product regiochemistry is controlled by a protonation step leading to collapse of the presumed enolate intermediate. While the proton donor has not been identified, we propose that a hydrogen bond from the Tyr\textsuperscript{82} hydroxyl group to the Glu\textsuperscript{92} backbone carbonyl stabilizes the hypervariable loop, thereby blocking the entrance of water to protonate the α-C of the substrate to generate the β,γ C=C product (γ-Cl-6-ACP\textsubscript{II}). Regiochemical control of the protonation step for Jam ECH\textsubscript{2} remains unclear since its crystal structure is not yet available. However, site-directed mutagenesis experiments described above suggest that this hypervariable region (a, left panel, α2–loop–α3, in magenta) in ECH\textsubscript{2}s plays an important role for regiochemical control.
Supplementary Figure 16. Synthesis of butylamide derivatives as GC-MS authentic standards.
Supplementary Table 1. Primers for expression constructs.

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The restriction sites are underlined.
Supplementary Table 2. ESI-FTICR-MS analysis.

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Masses are in Daltons. Calc., calculated; Obs., observed.

PEP, PPant ejection product.
REFERENCES:


