

Supporting Information

Expanding the Structural Diversity of Polyketides by Exploring the Cofactor Tolerance of an
Inline Methyltransferase Domain

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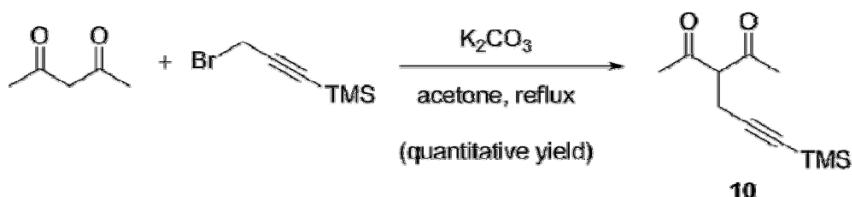
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Experimental Procedures

Chemicals and spectral analysis

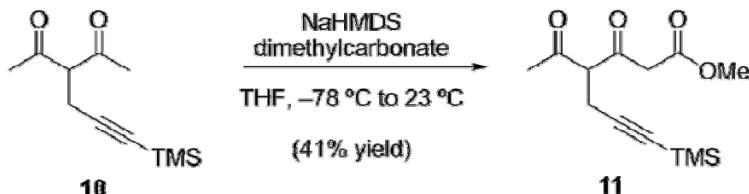
All solvents and other chemicals used were of analytical grade. Azide-PEG₃-5(6)-carboxytetramethylrhodamine and the click chemistry protein reaction buffer kit was purchased from Click Chemistry Tools (Scottsdale, AZ). Acetoacetyl-S-N-acetyl cysteamine (acetoacetyl-SNAC) was a gift from Dr. Kangjian Qiao (UCLA). ProSeAM and Keto-SAM were prepared as previously described.¹ All ¹H NMR spectra were obtained on a 500 MHz Bruker AV500 spectrometer with a 5 mm dual cryoprobe.

Synthesis of dione (**10**)



To a flask containing 2,4-pentanedione (18.5 mL, 80.0 mmol, 5.0 equiv) in acetone (32 mL) was added 3-bromoprop-1-yn-1-yl-trimethylsilane (3.0 g, 16.0 mmol, 1.0 equiv). Potassium carbonate (11.0 g, 80.0 mmol, 5.0 equiv) was then added. The flask was topped with a reflux condenser and the system placed under N₂. The reaction mixture was heated to reflux for 2 h. After cooling, the reaction was filtered and the filter cake was washed with acetone (3 x 20 mL). The filtrate was then concentrated under reduced pressure. The resultant oil was purified by flash chromatography (10:1 hexanes:EtOAc) to afford dione **10** (3.3 g, quantitative yield) as a pale yellow oil in a 1:1 mixture of keto:enol tautomers. R_f 0.8 (6:1 hexanes:EtOAc); ¹H NMR (500 MHz, CDCl₃) (Figure S1): Enol tautomer: δ 16.52 (s, 1H), 3.14 (s, 2H), 2.21 (s, 6H), 0.14 (s, 9H); Keto tautomer: δ 3.84 (t, J = 7.65 Hz, 1H), 2.71 (d, J = 7.65 Hz, 2H), 2.40 (s, 6H), 0.13 (s, 9H); ¹³C NMR (125 MHz, CDCl₃): δ 202.4, 190.9, 106.7, 103.8, 102.6, 87.6, 85.1, 66.7, 29.5, 23.2, 18.9, 18.8, 0.1, 0.01; IR (film): 2960, 2176, 1703, 1357, 1249 cm⁻¹; HRMS-ESI (m/z) [M - H] calcd for C₁₁H₁₇O₂Si, 209.1076; found, 209.0998.

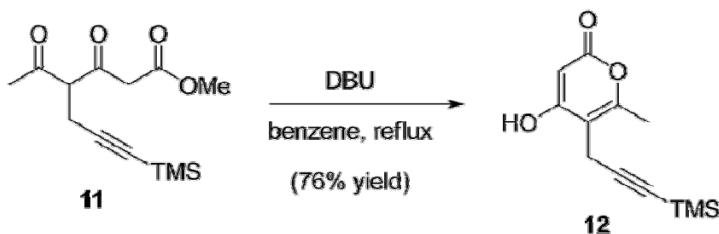
Synthesis of methyl ester (**11**)



To a flask containing a solution of **10** (198.0 mg, 0.942 mmol, 1.0 equiv) in THF (4.71 mL) at -78 °C was added a freshly made 1M solution of NaHMDS in THF (2.82 mL, 2.82 mmol, 3.0 equiv). After stirring at -78 °C for 1 h, dimethylcarbonate (95 µL, 1.03 mmol, 1.1 equiv) was added. The resulting mixture was removed from the cooling bath and warmed until the internal temperature of the reaction reached -10 °C. The reaction was then quenched by the addition of 1 M HCl (10 mL). The reaction mixture was transferred to a separatory funnel containing EtOAc

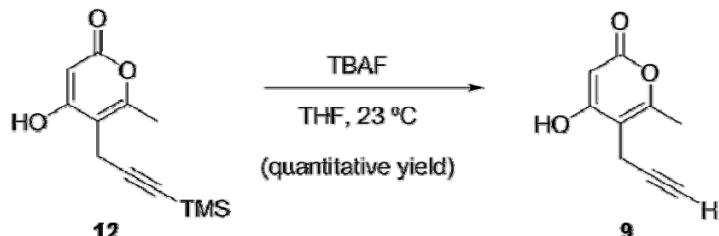
(20 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (30 mL), dried over MgSO₄, and concentrated under reduced pressure. The resultant yellow oil was purified by flash chromatography (10:1 hexanes:EtOAc) to afford methyl ester **11** (120.2 mg, 45% yield) as a clear oil. *R*_f 0.28 (6:1 hexanes:EtOAc); ¹H NMR (500 MHz, DMSO-*d*6) (Figure S2): δ 4.21 (t, *J* = 7.25, 1H), 3.77 (d, *J* = 16.9, 1H), 3.70 (d, *J* = 16.9, 1H), 3.63 (s, 3H), 2.64 (dd, *J* = 7.25, 1.95, 2H), 2.21 (s, 3H), 0.10 (s, 9H); ¹³C NMR (125 MHz, DMSO-*d*6): δ 202.5, 198.6, 166.9, 104.0, 86.6, 63.45, 52.0, 48.9, 30.0, 18.1, -0.11; IR (film): 2958, 2177, 1727, 1707, 1626, 1249 cm⁻¹; HRMS-ESI (m/z) [M + H]⁺ calcd for C₁₃H₂₁O₄Si, 269.1209; found 269.1201.

Synthesis of pyrone (**12**)



To a vial containing a solution of **11** (126.2 mg, 0.447 mmol, 1.0 equiv) in benzene (844 μL) was added DBU (134 μL, 0.894 mmol, 2.0 equiv) in one portion. The resulting solution was stirred at 60 °C for 3 h. After cooling, the reaction was quenched with saturated aqueous NH₄Cl (5 mL). The layers were separated and the aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over MgSO₄, and concentrated under reduced pressure. The resultant solid was purified by flash chromatography (20:1 CH₂Cl₂:MeOH) to afford pyrone **12** (79.8 mg, 76% yield) as a white solid. Mp: 184–186 °C; *R*_f 0.5 (10:1 CH₂Cl₂:MeOH); ¹H NMR (500 MHz, CDCl₃) (Figure S3): δ 5.64 (s, 1H), 3.36 (s, 2H), 2.34 (s, 3H), 0.13 (s, 9H); ¹³C NMR (125 MHz, CDCl₃): δ 171.2, 167.2, 161.6, 109.4, 102.2, 90.1, 85.5, 17.8, 14.9, 0.12; IR (film): 2958, 2176, 1727, 1645, 1608, 1560, 1248 cm⁻¹; HRMS-ESI (m/z) [M + H]⁺ calcd for C₁₂H₁₇O₃Si, 237.0947; found, 237.0955.

Synthesis of pyrone (**9**)



To a vial containing a solution of **12** (16.6 mg, 0.058 mmol, 1.0 equiv) in THF (588 μL) was added a solution of TBAF (1.0M in THF, 88 μL, 0.088 mmol, 1.5 equiv). The reaction mixture was stirred at 23 °C for 14 h and then transferred to a separatory funnel containing EtOAc (10 mL) and 1 M HCl (25 mL). The layers were separated and the resulting aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine (40 mL), dried over MgSO₄, and concentrated under reduced pressure to afford the crude product. The resultant solid was purified by flash chromatography (2:1 EtOAc:hexanes) to afford **9** (9.2

mg, 97% yield) as a white solid. Mp: 209–210 °C; R_f 0.25 (100% EtOAc); ^1H NMR (500 MHz, acetone-*d*6) (Figure S4): δ 10.70 (br s, 1H), 5.37 (s, 1H), 3.33 (d, J = 2.60, 2H), 2.42 (t, J = 2.60, 1H), 2.28 (s, 3H); ^{13}C NMR (125 MHz, acetone-*d*6): δ 169.5, 163.6, 161.6, 108.1, 90.0, 81.6, 69.8, 17.5, 13.9; IR (film): 2959, 2924, 1706, 1674, 1561, 1259 cm⁻¹; HRMS-ESI (m/z) [M – H]⁻ calcd for C₉H₇O₃, 163.0395; found, 163.0395.

CazF *in vitro* activity assays

CazF was expressed from *S. cerevisiae* BJ5464-NpgA² and purified by gravity-flow column chromatography as previously described.³ The specificity of CazF's MT domain toward its natural SAM cofactor and unnatural analogs was analyzed by incubating 5 μM CazF with 2 mM acetoacetyl-SNAC (**4**) and 5 μM , 10 μM , 25 μM , 50 μM and 1 mM of SAM (**1**), ProSeAM (**2**), or Keto-SAM (**3**) in 100 mM phosphate buffer pH 7.4 in a total volume of 10 μL for one hour at room temperature. The reaction was quenched by adding 90 μL of MeOH and then centrifuged for ten minutes at room temperature. A 10 μL aliquot was analyzed on a Shimadzu 2010 EV LC-MS with a Phenomenex Luna 5 μ 2.0 x 100 mM C18 column using a linear gradient of 5–95% MeCN/H₂O containing 0.1% formic acid over 30 minutes with a flow rate of 0.1 mL/min. The corresponding *m/z* peaks for **5** and **6** were integrated and the amount of alkylated-SNAC product was quantified using a standard curve. Kinetic constants were calculated by fitting initial velocity data at various concentrations of **1** or **2** to Michaelis-Menten parameters using nonlinear least squares curve fitting in GraphPad Prism (Figure S5).

To assess whether the KS domain of CazF would perform another round of elongation after the attachment of the propargyl group, 25 μM of protein was incubated at room temperature with 2 mM malonyl-CoA and 0.2 mM of **1** or **2** in 100 mM phosphate buffer pH 7.4 in a total volume of 100 μL . After 1 hour, the reaction was treated with base to release the polyketide product by adding 20 μL 1M NaOH, incubated at 65 °C for 10 minutes, followed by addition of 40 μL 1N HCl. The reaction was then extracted two times with 200 μL of 99% ethyl acetate:1% acetic acid and the organic layer was dried using a Speedvac. The extract was resuspended in 20 μL MeOH and analyzed by LC-MS as mentioned above. The production of the propargyl- α -pyrone in the *in vitro* assay was confirmed using the synthetic standard **9** (Figure S6). The *in vitro* production of **8** was previously confirmed.³

In vitro biosynthesis of 4'propargyl-chaetoviridin A (**17**) using CazF and CazE

In a 100 μL reaction, 25 μM CazF; 25 μM CazE, which was expressed and purified as previously described;³ 2 mM NADPH; 2 mM malonyl-CoA; and 2 mM cazonochromene (**14**) were incubated with **1** or **2** in 100 mM phosphate buffer pH 7.4 for one hour at room temperature. Due to the instability of **1** and **2** at neutral conditions and at room temperature, 1 mM of **1** and **2** were added at the start of the reaction and after 30 minutes. The final concentration of the cofactors was 2 mM. The reaction was extracted twice with 300 μL ethyl acetate containing 1% acetic acid. The organic layer was dried using a Speedvac and resuspended in 20 μL MeOH. The extract was analyzed by LC-MS using the same conditions as in the CazF *in vitro* activity assays. The *in vitro* biosynthesis of chaetoviridin A (**15**) using **1** as

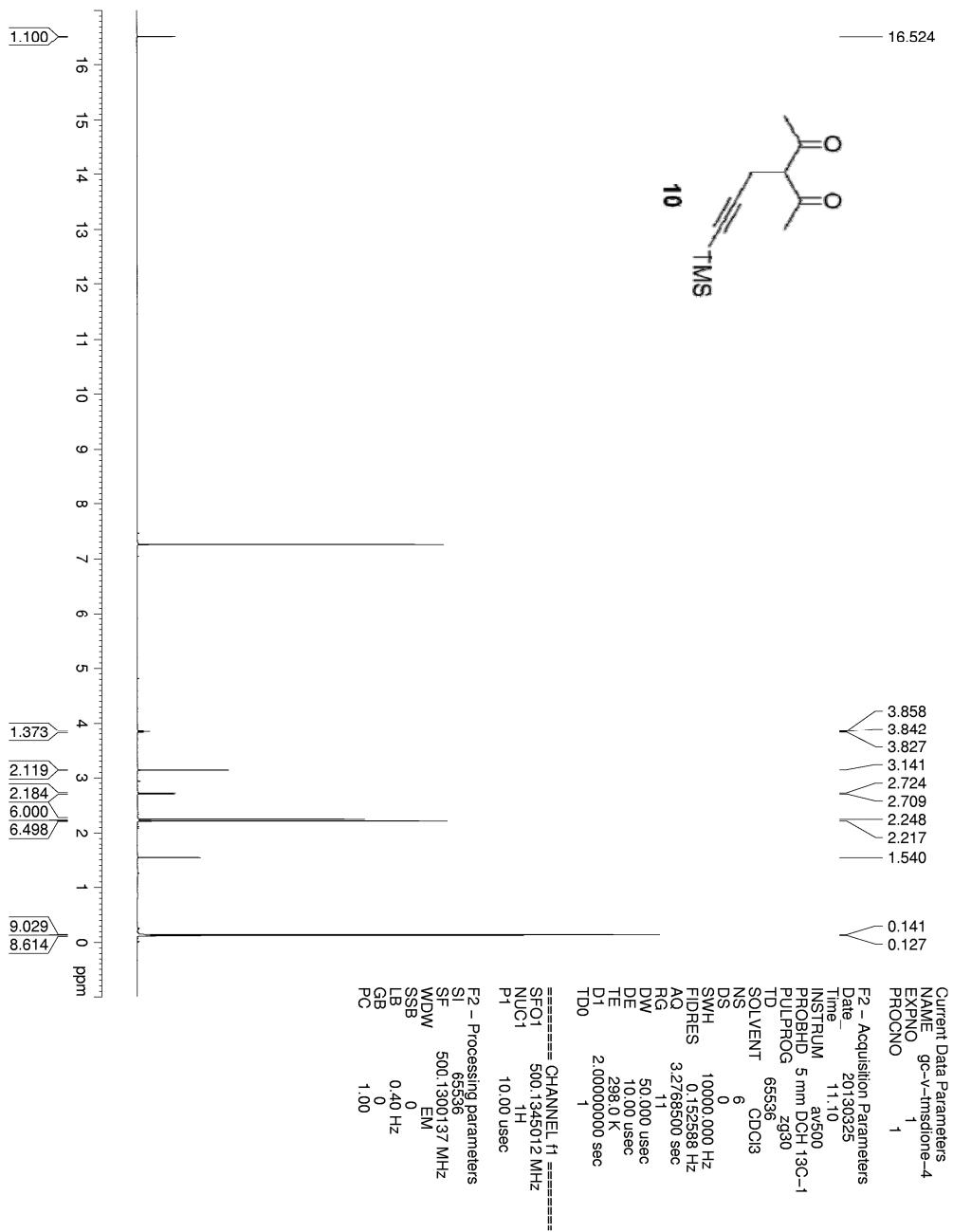
the alkyl donor was confirmed previously.³ The structure of 4'-propargyl-chaetoviridin A (**17**), which was formed using **2** as the alkyl donor, was proposed based on its retention time, UV profile and observed *m/z* isotopic ratio (Figure S7).

Copper-catalyzed azide-alkyne cycloaddition reaction

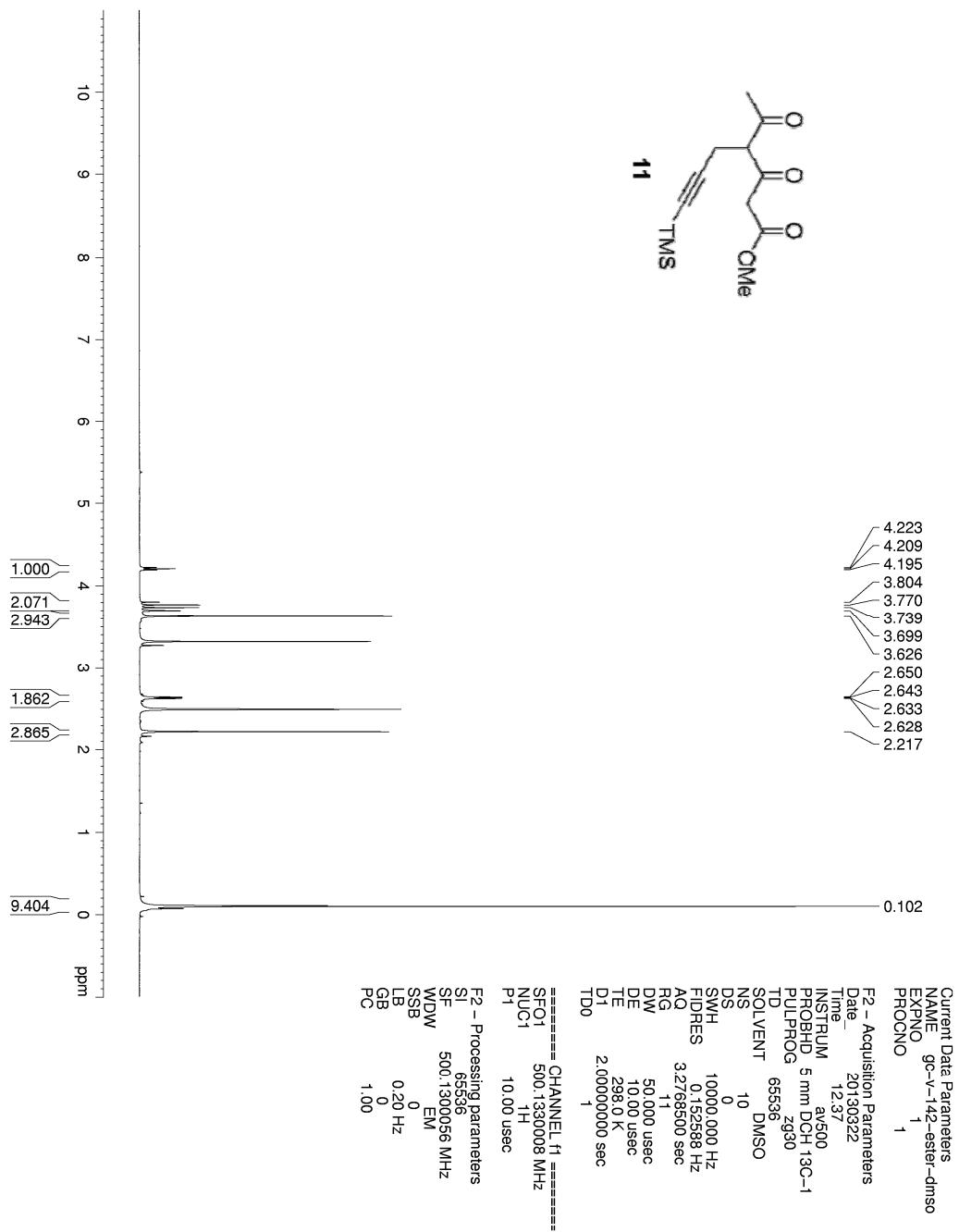
The in vitro reaction for the enzymatic synthesis of **17** was repeated using the same conditions mentioned above. After incubating at room temperature for one hour, the reaction was extracted twice with 300 μ L ethyl acetate containing 1% acetic acid and the organic layer was dried using a Speedvac. The crude extract was then resuspended in 50 μ L MeOH. Azide-PEG3-5(6)-carboxytetramethylrhodamine was dissolved in DMSO to a final concentration of 2.5 mM and the click labeling reaction was carried out using 2 μ L of the 2.5 mM rhodamine-azide according to the manufacturer's instructions. After a four hour incubation at room temperature, the reaction was extracted twice with 250 μ L ethyl-acetate containing 1% acetic acid and the organic layer was dried using a Speedvac. The extract was resuspended in 20 μ L MeOH and analyzed by LC-MS using the same conditions as mentioned previously in the CazF in vitro activity assay.

Experimental Figures

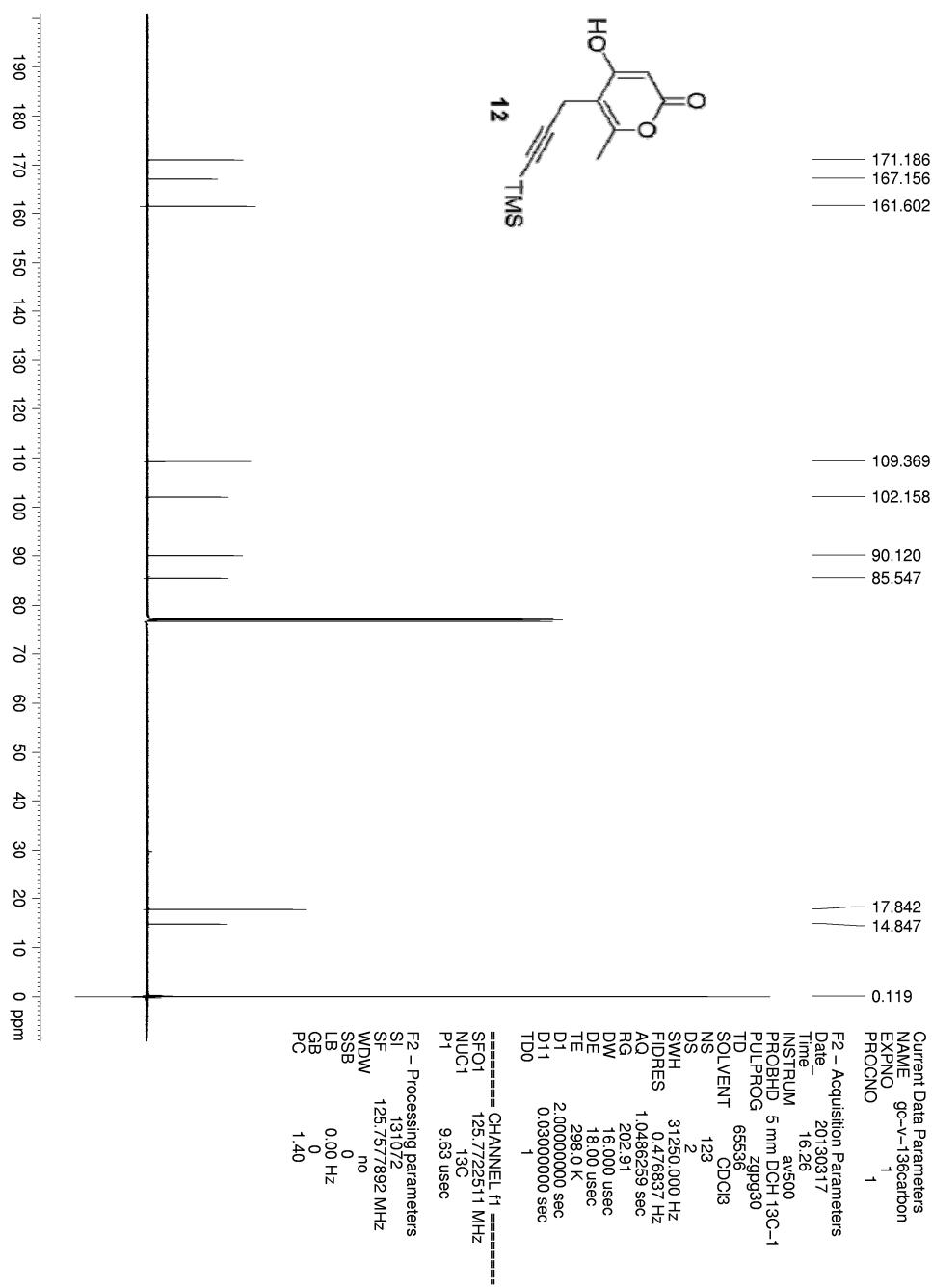
Supplementary Figure 1. ^1H NMR spectrum (500 MHz) of dione (**10**) in CDCl_3



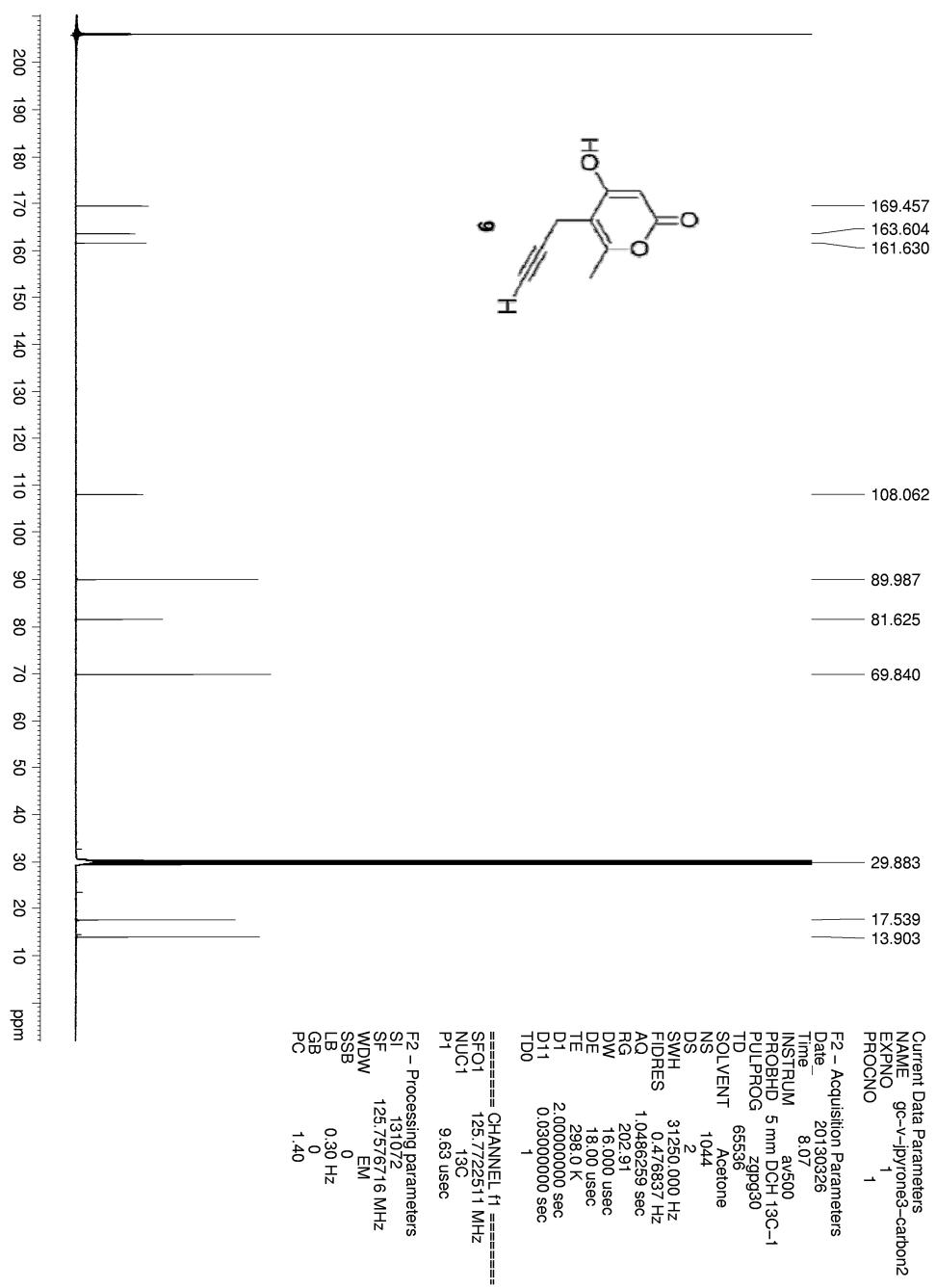
Supplementary Figure 2. ^1H NMR spectrum (500 MHz) of methyl ester (**11**) in DMSO



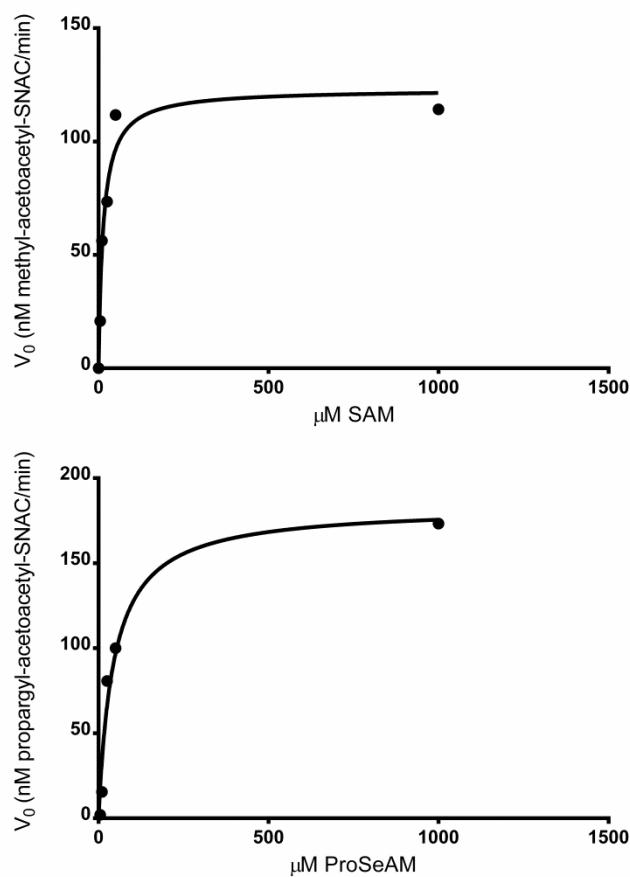
Supplementary Figure 3. ^1H NMR spectrum (500 MHz) of pyrone (**12**) in CDCl_3



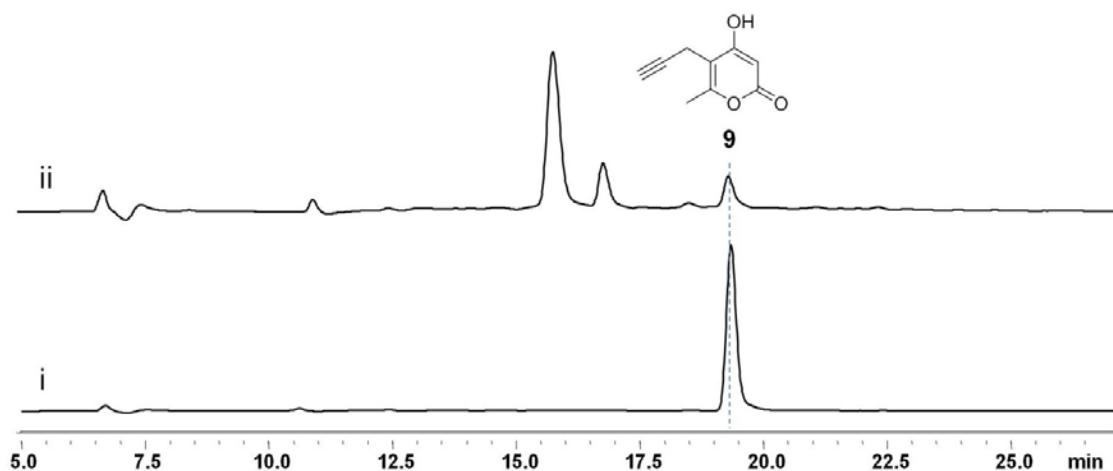
Supplementary Figure 4. ^1H NMR spectrum (500 MHz) of pyrone (**9**) in acetone



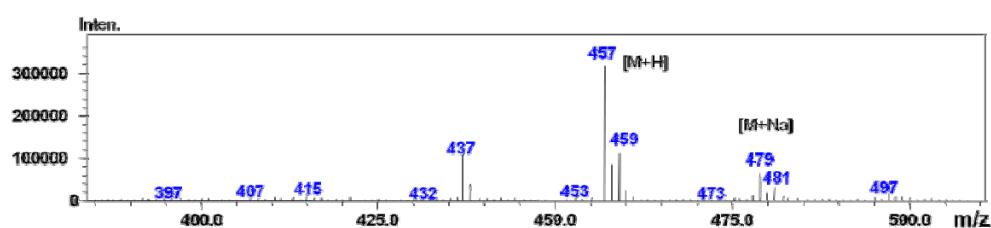
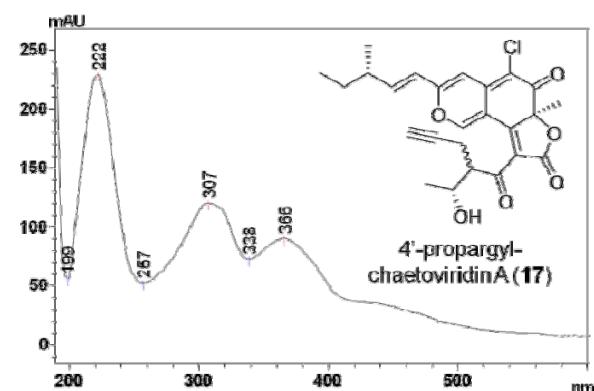
Supplementary Figure 5. Steady-state kinetic analysis of the methyltransferase domain in CazF using acetoacetyl-SNAC as the substrate. The solid lines represent best fits using the Michaelis-Menten equation for initial rate data (black circles).



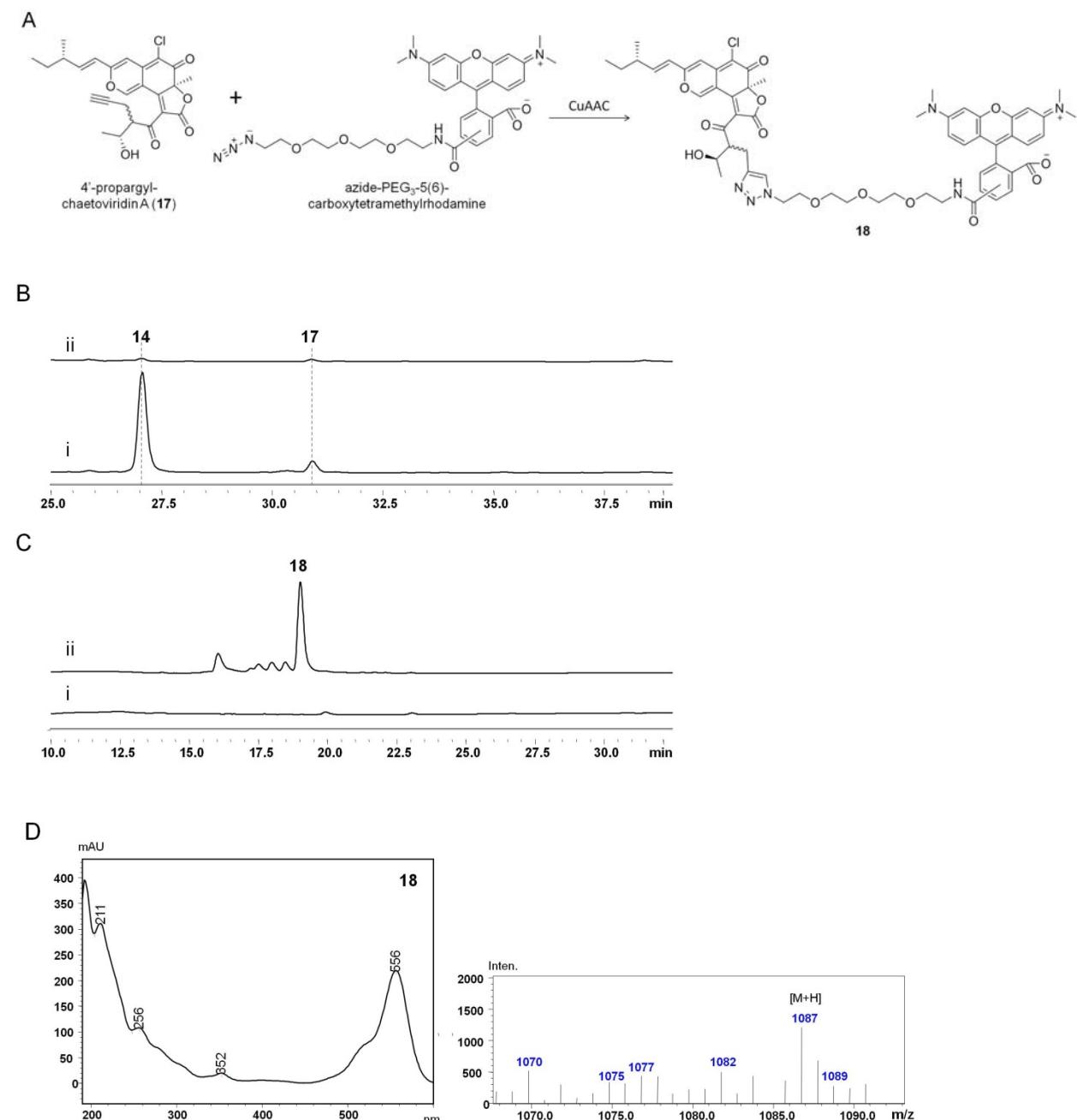
Supplementary Figure 6. Formation and verification of propargyl- α -pyrone. i) Synthetic standard of the propargyl- α -pyrone **9**; ii) Compound **9** produced in the CazF in vitro assay with malonyl-CoA and ProSeAM. HPLC traces are shown at $\lambda = 280$ nm.



Supplementary Figure 7. UV profile and m/z of 4'-propargyl-chaetoviridin A (**17**)



Supplementary Figure 8. Copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC) between **17** and azide-PEG₃-5(6)-carboxytetramethylrhodamine and detection of the triazole-containing conjugate **18**. A) Coupling of the terminal alkyne in **17** with the rhodamine-azide to form the triazole-containing molecule **18**. B) HPLC analysis (360 nm) of the organic extract from the in vitro enzymatic synthesis of **17** (i) before the CuAAC reaction and (ii) after the CuAAC reaction. C) HPLC analysis (550 nm) of the organic extract from the in vitro enzymatic synthesis of **17** (i) before the CuAAC reaction and (ii) after the CuAAC reaction. D) UV profile and *m/z* for compound **18**.



Supplementary References

1. Bothwell, I. R.; Islam, K.; Chen, Y.; Zheng, W.; Blum, G.; Deng, H.; Luo, M. *J. Am. Chem. Soc.* **2012**, *134*, 14905.
2. Lee, K. K., Da Silva, N. A., Kealey, J. T. *Anal. Biochem.* **2009**, *394*, 75.
3. Winter, J.M., Sato, M., Sugimoto, S., Chiou, G., Garg, N. K., Tang, Y., Watanabe, K. *J. Am. Chem. Soc.* **2012**, *134*, 17900.