

Supporting Information

Directing group-controlled regioselectivity in an enzymatic C-H bond oxygenation

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Part I: Substrate synthesis

General Protocols

All reagents were used as received unless otherwise noted. Solvents were purified under nitrogen using a solvent purification system (Innovative Technology, inc., Model # SPS-400-3 and PS-00-3). Reactions were monitored by thin layer chromatography using SiliCycle silica gel 60 F254 pre-coated plates (0.25 mm) which were visualized using UV light, *p*-anisaldehyde, KMnO₄, PMA or CAM stain. Flash column chromatography was performed using Kieselgel 60 (230-400 mesh) silica gel. Eluent mixtures are reported as v:v percentages of the minor constituent in the major constituent. All compounds purified by column chromatography were sufficiently pure for use in further experiments unless otherwise indicated. ¹H and ¹³C spectra were obtained in CDCl₃ at rt (25 °C), unless otherwise noted, on a Varian vnmrs (500 MHz or 700 MHz), Varian MR400 or Varian Unity 500 MHz. Chemical shifts of ¹H NMR spectra were recorded in parts per million (ppm) on the δ scale from an internal standard of residual chloroform (7.26 ppm). Chemical shifts of ¹³C NMR spectra were recorded in ppm from the central peak of CDCl₃ (77.0 ppm) on the δ scale. Low resolution electrospray mass spectra were obtained on a Micromass LCT spectrometer and high resolution electrospray mass spectra were obtained on a Micromass AutoSpec Ultima spectrometer at the University of Michigan Mass Spectrometry Laboratory.

List of reagents prepared or purified:

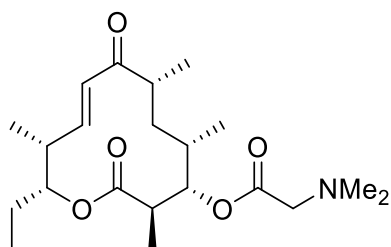
10-deoxymethynolide (10-dml): A mutant strain of *S. venezuelae* ATCC 15439 in which *des1* was knocked out was grown in 1 L of media containing 20 g glucose, 15 g soybean flour, 5 g CaCl₂, 1 g NaCl, 2 mg CoCl₂•6H₂O, pH = 7.2. After 72 h, the culture was extracted with chloroform (3x). The organic layers were combined, washed with brine, dried over Na₂SO₄ and concentrated to afford the crude macrolactone. The crude oil was purified via silica gel chromatography (4:1 to 1:1 hexanes/ethyl acetate) to afford 10-dml as a colorless foamy oil.

3-((dimethylamino)methyl)benzoic acid and **4-((dimethylamino)methyl)benzoic acid** were synthesized according to the procedure described by Wang et al.¹

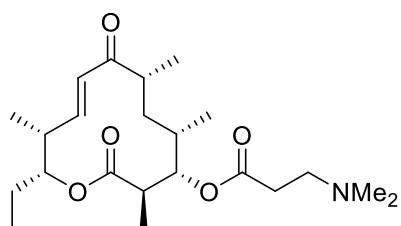
2-((dimethylamino)methyl)benzoic acid: A 40 wt % solution of HNMe₂ in H₂O (10 equiv) was added to 2-((bromo)methyl)benzoic² acid (1 equiv) in EtOH (0.8 M). The reaction mixture was stirred at ambient temperature for 12 h at which point the mixture was concentrated and partitioned between EtOAc and 1N HCl. The aqueous layer was basified to pH 8-9 with saturated aqueous NaHCO₃. The aqueous layer was extracted with EtOAc (3x). The organic layers were combined, dried over Na₂SO₄, filtered and concentrated to yield a mixture of methyl 2-((dimethylamino)methyl)benzoate and ethyl 2-((dimethylamino)methyl) benzoate. The ester mixture was hydrolyzed according to the procedure described by Wang et al.¹

N-methyl-D-proline was synthesized according to the procedure described by Daughtry et al.³

Oxalyl chloride was distilled prior to use.

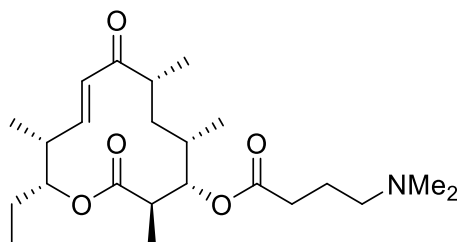


(3R,4S,5S,7R,11R,12R,E)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 2-(dimethylamino)acetate (8a): 110 mg of 10-deoxymethynolide (0.35 mmol, 1.0 equiv) were dissolved in 5.0 mL of dichloromethane along with 74 mg *N,N*-dimethylglycine (0.53 mmol, 1.5 equiv), 84 μ L of freshly distilled triethylamine (0.60 mmol, 1.7 equiv), 110 mg of *N,N'*-dicyclohexylcarbodiimide (DCC) (0.53 mmol, 1.5 equiv) and 66 mg 4-(dimethylamino)pyridine (DMAP) (0.51 mmol, 1.5 equiv). The reaction mixture was allowed to stir at room temperature for 6 d, and then, the reaction mixture was filtered through cotton and evaporated to dryness in vacuo. The crude residue was transferred to a separatory funnel by washing the flask with 0.1 M HCl and 30% EtOAc in hexanes. The acidified aqueous phase was extracted thrice with 30% EtOAc in hexanes. The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo. The crude residue was chromatographed over silica gel (30% EtOAc in hexanes) to recover the unreacted 10-deoxymethynolide. Then the aqueous layer was basified with saturated aqueous NaHCO₃ solution until it reached a pH of 8-9, measured by pH paper. The basified aqueous layer was extracted with EtOAc (3x). The EtOAc organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo. The crude residue was chromatographed over silica gel (gradient from 30% EtOAc in hexanes to 100% EtOAc) to afford 54 mg (40% yield) of the 10-dml ester derivative **8a**. ¹H NMR (500 MHz, CDCl₃) δ 6.75 (dd, *J* = 15.7, 5.4 Hz, 1H), 6.40 (d, *J* = 15.8 Hz, 1H), 5.18 (d, *J* = 10.2 Hz, 1H), 5.05 – 5.01 (m, 1H), 3.22 (s, 2H), 2.76 (dd, *J* = 10.9, 6.9 Hz, 1H), 2.67 – 2.62 (m, 1H), 2.51 (d, *J* = 1.8 Hz, 1H), 2.38 (s, 6H), 1.76 (t, *J* = 13.3 Hz, 1H), 1.70 (dd, *J* = 15.2, 7.3 Hz, 1H), 1.60 – 1.52 (m, 1H), 1.44 – 1.37 (m, 1H), 1.25 (dd, *J* = 13.1, 2.7 Hz, 1H), 1.22 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.10 (d, *J* = 6.8 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.88 (d, *J* = 6.7 Hz, 3H); ¹³C NMR (175 MHz, CDCl₃) δ 204.5, 173.6, 170.0, 147.4, 125.4, 78.8, 74.0, 59.9, 45.1, 45.1, 41.6, 38.2, 34.1, 32.6, 25.1, 17.6, 17.2, 15.9, 10.3, 9.5; IR (thin film, cm⁻¹) 2965, 2936, 2764, 1733, 1688, 1627, 1456; HRMS (ESI) *m/z* calculated for [M+H]⁺ 382.2588, found 382.2595.

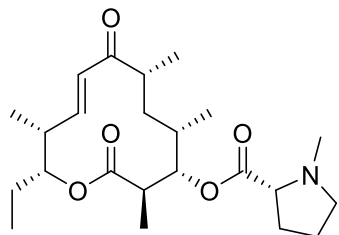


(3R,4S,5S,7R,11R,12R,E)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-(dimethylamino)propanoate (8b): 29 mg of 10-deoxymethynolide (0.098 mmol, 1.0 equiv) were dissolved in 2.0 mL of dichloromethane along with 23 mg 3-*N,N*-dimethylaminopropanoic acid hydrochloride (0.15 mmol, 1.5 equiv), 23 μ L of freshly distilled triethylamine (0.17 mmol, 1.7 equiv), 31 mg of DCC (0.15 mmol, 1.5 equiv) and 18 mg DMAP (0.15 mmol, 1.5 equiv). The reaction mixture was allowed to stir at room temperature for 6 d, and then the reaction mixture was filtered through cotton and evaporated to dryness in vacuo. The crude residue was transferred to a separatory funnel by washing the flask with 0.1 M HCl and 30% EtOAc in hexanes. The acidified aqueous phase was extracted thrice with 30% EtOAc in hexanes. The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo. The crude residue was

chromatographed over silica gel (30% EtOAc in hexanes) to recover the unreacted 10-deoxymethynolide. Then the aqueous layer was basified with saturated aqueous NaHCO₃ solution until it reached a pH of 8-9, measured by pH paper. The basified aqueous layer was extracted with ethyl acetate (3x). The ethyl acetate organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo. The crude residue was chromatographed over silica gel (gradient from 30% EtOAc in hexanes to 20% MeCN, 10% MeOH in EtOAc) to yield 13 mg (34% yield) of the 10-dml ester derivative **8b**. ¹H NMR (500 MHz, CDCl₃) δ 6.76 (dd, *J* = 15.7, 5.5 Hz, 1H), 6.41 (d, *J* = 15.6 Hz, 1H), 5.12 (d, *J* = 11.1, 1H), 5.07 – 5.00 (m, 1H), 2.74 (dq, *J* = 10.8 Hz, 6.8 Hz, 1H), 2.68 – 2.58 (m, 3H), 2.57 – 2.46 (m, 3H), 2.25 (s, 6H), 1.80 – 1.64 (m, 2H), 1.60 – 1.51 (m, 1H), 1.44 – 1.35 (m, 1H), 1.28 – 1.22 (m, 3H), 1.14 (d, *J* = 6.5 Hz, 3H), 1.11 (d, *J* = 7.0 Hz, 3H), 0.91 (t, *J* = 7.5 Hz, 3H), 0.88 (d, *J* = 7.0 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 204.6, 173.8, 171.9, 147.3, 125.4, 78.5, 74.0, 54.8, 45.1, 45.1, 41.7, 38.2, 34.1, 33.0, 32.7, 25.1, 17.7, 17.1, 15.9, 10.3, 9.5; IR (thin film, cm⁻¹) 2965, 2930, 1728, 1689, 1625, 1457, 1380, 1324; HRMS (ESI) *m/z* calculated for [M+H]⁺ 396.2750, found 396.2735.

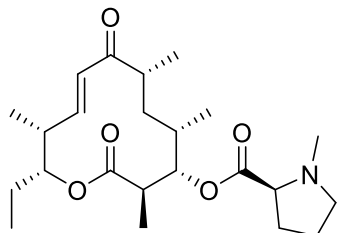


(3R,4S,5S,7R,11R,12R,E)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-(dimethylamino)butanoate (10): Following the procedure employed to synthesize **8b**, 10-dml (100 mg, 0.34 mmol), 3-*N,N*-dimethylaminobutyric acid hydrochloride (88 mg, 0.51 mmol), triethylamine (80 μL, 0.57 mmol), DCC (110 mg, 0.51 mmol), DMAP (63 mg, 0.51 mmol) in 5.0 mL of dichloromethane were employed to yield 65 mg (47% yield) of **10** after purification over silica gel (gradient from 30% EtOAc in hexanes to 10% MeOH in EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 6.75 (dd, *J* = 15.8, 5.4 Hz, 1H), 6.41 (dd, *J* = 15.4, 1.0 Hz, 1H), 5.11 (dd, *J* = 10.8, 1.2 Hz, 1H), 5.03 (ddd, *J* = 8.4, 5.6, 2.4 Hz, 2H), 2.75 (dq, *J* = 11.0, 6.8 Hz, 1H), 2.68 – 2.59 (m, 1H), 2.58 – 2.45 (m, 1H), 2.37 (t, *J* = 7.4 Hz, 2H), 2.31 (t, *J* = 7.2 Hz, 1H), 2.22 (s, 6H), 1.86 – 1.63 (m, 4H), 1.63 – 1.49 (m, 1H), 1.42-1.34 (m, 1H), 1.31-1.20 (m, 4H), 1.13 (d, *J* = 7.2 Hz, 3H), 1.11 (d, *J* = 7.2 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.86 (d, *J* = 6.4 Hz, 3H); ¹³C NMR (175 MHz, CDCl₃) δ 204.6, 173.8, 173.1, 147.3, 125.5, 78.3, 74.0, 58.8, 45.3, 45.1, 41.7, 38.2, 34.1, 32.7, 32.0, 25.1, 23.0, 17.6, 17.2, 15.9; IR (thin film, cm⁻¹) 2965, 2936, 2764, 1733, 1688, 1627, 1456; HRMS (ESI) *m/z* calculated for [M+H]⁺ 410.2906, found 410.2907.

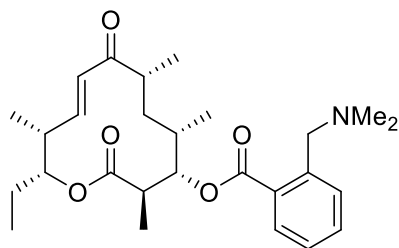


(3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl N-methyl-D-prolinate (12a): 10-dml (40 mg, 0.13 mmol) was dissolved in 1.8 mL of dichloromethane along with *D-N*-methylproline (26 mg, 0.20 mmol), DCC (42 mg, 0.20 mmol), DMAP (25 mg, 0.20 mmol). The reaction mixture was stirred at rt for 6 d, then it was filtered through cotton and the filtrate concentrated. The crude residue was purified over silica gel (gradient from 10% EtOAc in hexanes to 70% EtOAc in hexanes) to yield 24 mg (44% yield) of **12a** after. ¹H NMR (700 MHz, CDCl₃) δ 6.76 (dd, *J* = 16.1, 5.3 Hz, 1H), 6.41 (d, *J* = 16.1 Hz, 1H), 5.18 (d, *J* = 10.5 Hz, 1H), 5.04 (ddd, *J* = 8.1, 5.3,

2.1 Hz, 2H), 3.15 (td, $J = 7.2, 2.1$ Hz), 2.96 (t, $J = 7.7$ Hz), 2.78 (dq, $J = 11.2, 7.0$ Hz, 1H), 2.68 – 2.62 (m, 1H), 2.55 – 2.48 (m, 1H), 2.41 (s, 3H), 2.32 (dd, $J = 16.8, 8.4$ Hz, 1H), 2.21 – 2.13 (m, 2H), 1.84 – 1.76 (m, 2H), 1.75 – 1.67 (m, 1H), 1.60 – 1.50 (m, 1H), 1.42 – 1.38 (m, 1H), 1.27 – 1.21 (m, 4H), 1.15 (d, $J = 6.3$ Hz, 3H), 1.11 (d, $J = 7.0$ Hz, 3H), 0.91 (t, $J = 7.4$ Hz, 3H) 0.88 (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 204.5, 173.6, 173.1, 147.3, 125.5, 78.4, 74.0, 67.5, 56.1, 45.0, 41.6, 40.7, 38.1, 34.1, 32.6, 30.0, 25.0, 23.0, 17.6, 17.1, 15.9, 10.3, 9.5; IR (thin film, cm^{-1}) 2922, 2851, 1727, 1689, 1626, 1455, 1379, 1323; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 408.2744, found 408.2738.

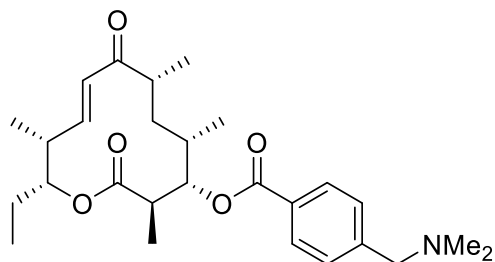


(3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl N-methyl-L-prolinate (12b): Following the procedure for (3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl N-methyl-D-prolinate, 10-dml (60 mg, 0.20 mmol), L-N-methylproline (39 mg, 0.30 mmol), DCC (63 mg, 0.30 mmol), DMAP (37 mg, 0.30 mmol) in 3.0 mL of dichloromethane were employed to yield 46 mg (56% yield) of **12b** after purification over silica gel (gradient from 10% EtOAc in hexanes to 70% EtOAc in hexanes). ^1H NMR (700 MHz, CDCl_3) δ 6.76 (dd, $J = 15.8, 5.4$ Hz, 1H), 6.41 (dd, $J = 16.0, 0.8$ Hz, 1H), 5.18 (d, $J = 10.8$ Hz, 1H), 5.04 (ddd, $J = 8.2, 5.8, 2.4$ Hz, 1H), 3.20 – 3.12 (m, 1H), 2.98 (t, $J = 7.2$ Hz), 2.79 (dq, $J = 10.8, 2.8$ Hz, 1H), 2.70 – 2.60 (m, 1H), 2.58 – 2.46 (m, 1H), 2.41 (s, 3H), 2.33 (dd, $J = 15.4, 7.8$ Hz), 2.22 – 2.10 (m, 1H), 2.00 – 1.89 (m, 2H), 1.88 – 1.50 (m, 4H), 1.46 – 1.36 (m, 1H), 1.32 – 1.20 (m, 4H), 1.14 (d, $J = 7.2$ Hz, 3H), 1.10 (d, $J = 6.8$ Hz, 3H), 0.91 (t, $J = 7.2$ Hz, 3H) 0.88 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (175 MHz, CDCl_3) δ 204.6, 173.7, 173.1, 147.4, 125.4, 78.5, 74.0, 67.6, 56.0, 45.1, 41.6, 40.6, 38.2, 34.1, 32.6, 29.9, 25.0, 23.0, 17.6, 17.2, 15.8, 10.3, 9.5; IR (thin film, cm^{-1}) 2970, 2935, 1220, 2773, 1749, 1732, 1716, 1682, 1625, 1454, 1323; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 408.2744, found 408.2748.

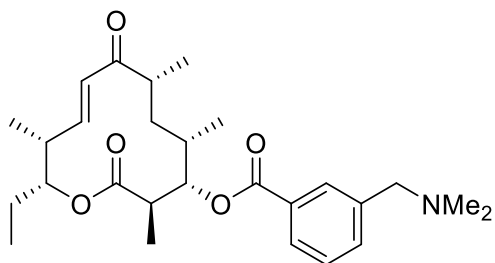


(3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 2-((dimethylamino)methyl)benzoate (14): 2-((dimethylamino)methyl)benzoic acid (30 mg, 0.16 mmol) was dissolved in 0.50 mL of dichloromethane. To this mixture was added one drop of *N,N*-dimethylformamide followed by oxalyl chloride (17 μL , 0.18 mmol). The reaction was allowed to stir at rt for 1.5 h before the solvent was evaporated and the crude acid chloride resuspended in 1.0 mL of dichloromethane. The acyl chloride suspension was added dropwise to an ice cooled solution of 10-deoxymethynolide (54 mg, 0.18 mmol), DMAP (0.082 mmol, 10 mg) and triethylamine (120 μL , 0.82 mmol) in 1.2 mL of dichloromethane. The reaction mixture was allowed to stir as it was warmed to rt. After 12 h, the reaction was quenched by the addition of saturated sodium bicarbonate solution. The aqueous phase was washed with EtOAc (3x), dried over sodium sulfate, filtered and concentrated. The crude residue was chromatographed over silica gel (gradient from 5% EtOAc in hexanes to 100% EtOAc) to yield 37 mg (49%) of the desired ester **14** as a colorless oil. ^1H NMR (700 MHz, CDCl_3) δ 7.70 (d, $J = 7.7$ Hz, 1H), 7.46 (m, 2H), 7.31 (t, $J = 7.4$ Hz, 1H), 6.79 (dd, $J = 15.8, 5.3$ Hz, 1H), 6.45 (dd, $J = 15.4, 1.4$ Hz, 1H), 5.35 (dd, $J = 11.2, 1.4$ Hz, 1H), 5.07 (ddd, $J = 9.1, 4.9, 2.1$ Hz, 1

H), 3.81 (d, $J = 14.0$ Hz, 1H), 3.61 (d, $J = 13.3$ Hz, 1H), 2.88 (dq, $J = 11.2, 7.0$ Hz, 1H), 2.69-2.64 (m, 1H), 2.59-2.53 (m, 1H), 2.18 (s, 6H), 1.87 (t, $J = 13.0$ Hz, 1H), 1.76-1.69 (m, 1H), 1.60-1.54 (m, 1H), 1.54-1.48 (m, 1H), 1.41-1.36 (m, 1H), 1.27-1.23 (m, 6H), 1.13 (d, $J = 7.0$ Hz, 3H), 0.99 (d, $J = 6.3$ Hz, 3H), 0.93 (t, 7.4 Hz, 3H); ^{13}C NMR (175 MHz, CDCl_3) δ 204.6, 173.9, 167.4, 147.4, 131.3, 131.0, 130.3, 129.2, 126.7, 125.4, 78.7, 74.0, 61.3, 45.5, 45.1, 42.0, 38.2, 34.3, 33.0, 25.1, 17.1, 17.1, 15.8, 10.3, 9.6; IR (thin film, cm^{-1}) 2918, 2850, 1729, 1691, 1628, 1457; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 458.2901, found 458.2910.

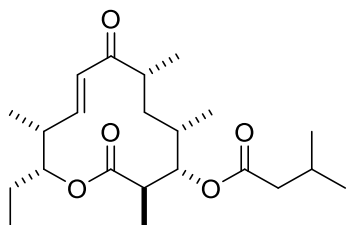


(3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-((dimethylamino)methyl)benzoate (16a): 10-deoxymethynolide (40 mg, 0.14 mmol), 36 mg of 4-((dimethylamino)methyl)benzoic acid (0.20 mmol, 1.5 equiv), 42 mg DCC (0.20 mmol, 1.5 equiv) and 25 mg DMAP (0.20 mmol, 1.5 equiv) were combined in dichloromethane. The reaction mixture was allowed to stir at room temperature for 6 d, and then, the reaction mixture was filtered through cotton and evaporated to dryness in vacuo. The crude residue was transferred to a separatory funnel by washing the flask with 0.1 M HCl and 30% EtOAc in hexanes. The acidified aqueous phase was extracted thrice with 30% EtOAc in hexanes. The organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo to yield 37 mg (60%) of the desired ester **16a** after chromatography over silica gel (gradient from 30% EtOAc in hexanes to 100% EtOAc). ^1H NMR (400 MHz, CDCl_3) δ 7.98 (d, $J = 8.0$ Hz, 2H), 7.41 (d, $J = 8.0$ Hz, 2H), 6.80 (dd, $J = 15.6, 5.2$ Hz, 1H), 6.47 (d, $J = 15.6$ Hz, 1H), 5.36 (d, $J = 10.8$ Hz, 1H), 5.07 (ddd, $J = 8.4, 5.6, 2.4$ Hz, 1H), 3.48 (s, 2H), 2.92 (dq, $J = 10.8, 6.8$ Hz, 1H), 2.72 – 2.62 (m, 1H), 2.60 – 2.49 (m, 1H), 2.26 (s, 6H), 1.93 (t, $J = 13.0$ Hz), 1.80 – 1.66 (m, 1H), 1.64 – 1.46 (m, 2H), 1.44 – 1.34 (m, 1H), 1.26 (d, $J = 7.2$ Hz, 3H), 1.19 (d, $J = 6.8$ Hz, 3H), 1.13 (d, $J = 6.8$ Hz, 3H), 0.96 – 0.89 (m, 6H); ^{13}C NMR (175 MHz, CDCl_3) δ 204.5, 173.8, 166.0, 147.4, 144.6, 129.7, 129.0, 128.7, 125.4, 79.0, 74.0, 63.9, 45.4, 45.1, 41.9, 38.2, 34.1, 33.0, 25.1, 17.7, 17.2, 16.0, 10.3, 9.5; IR (thin film, cm^{-1}) 2965, 2936, 2771, 1725, 1688, 1627, 1456; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 458.2901, found 458.2911.

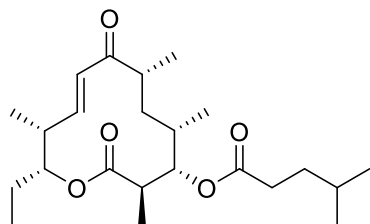


(3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-((dimethylamino)methyl)benzoate (16b): Following the procedure employed to synthesize **16a**, 10-deoxymethynolide (120 mg, 0.40 mmol), 110 mg of 3-((dimethylamino)methyl)benzoic acid (0.61 mmol, 1.5 equiv), 130 mg of DCC (0.61 mmol, 1.5 equiv) and 75 mg DMAP (0.61 mmol, 1.5 equiv) to yield 40 mg (22%) of the desired ester **16b** after chromatography over silica gel (gradient from 30% EtOAc in hexanes to 100% EtOAc). ^1H NMR (700 MHz, CDCl_3) δ 7.95 (s, 1H), 7.92 (d, $J = 7.7$ Hz, 1H), 7.57 (d, $J = 7.7$ Hz, 1H), 7.42 (t, $J = 7.7$ Hz, 1H), 6.80 (dd, $J = 15.4, 5.6$ Hz, 1H), 6.47 (dd, $J = 15.4, 1.1$ Hz, 1H), 5.37 (dd, $J = 11.2, 1.1$ Hz, 1H), 5.07 (ddd, $J = 8.4, 5.6, 2.1$ Hz, 1H), 3.52 (s, 2H), 2.93 (dq, $J = 10.5, 7.0$ Hz, 1H), 2.70 – 2.64 (m, 1H), 2.58 – 2.51 (m, 1H), 2.28 (s, 6H), 1.93 (t, $J =$

14.0 Hz, 1H), 1.77 – 1.68 (m, 1H), 1.66 – 1.57 (m, 3H), 1.42 – 1.34 (m, 1H), 1.31 – 1.22 (m, 4H), 1.19 (d, $J = 7.0$ Hz, 3H), 1.14 (d, $J = 7.0$ Hz, 3H), 0.95 – 0.91 (m, 6H); ^{13}C NMR (125 MHz, CDCl_3) δ 204.5, 173.8, 166.1, 147.4, 139.5, 133.8, 130.3, 129.8, 128.4, 128.3, 125.4, 79.1, 74.0, 63.8, 45.3, 45.1, 41.9, 38.2, 34.1, 33.0, 25.1, 17.7, 17.2, 16.0, 10.3, 9.5; IR (thin film, cm^{-1}) 2967, 2937, 2818, 2771, 1724, 1688, 1625, 1456; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 458.2901, found 458.2911.



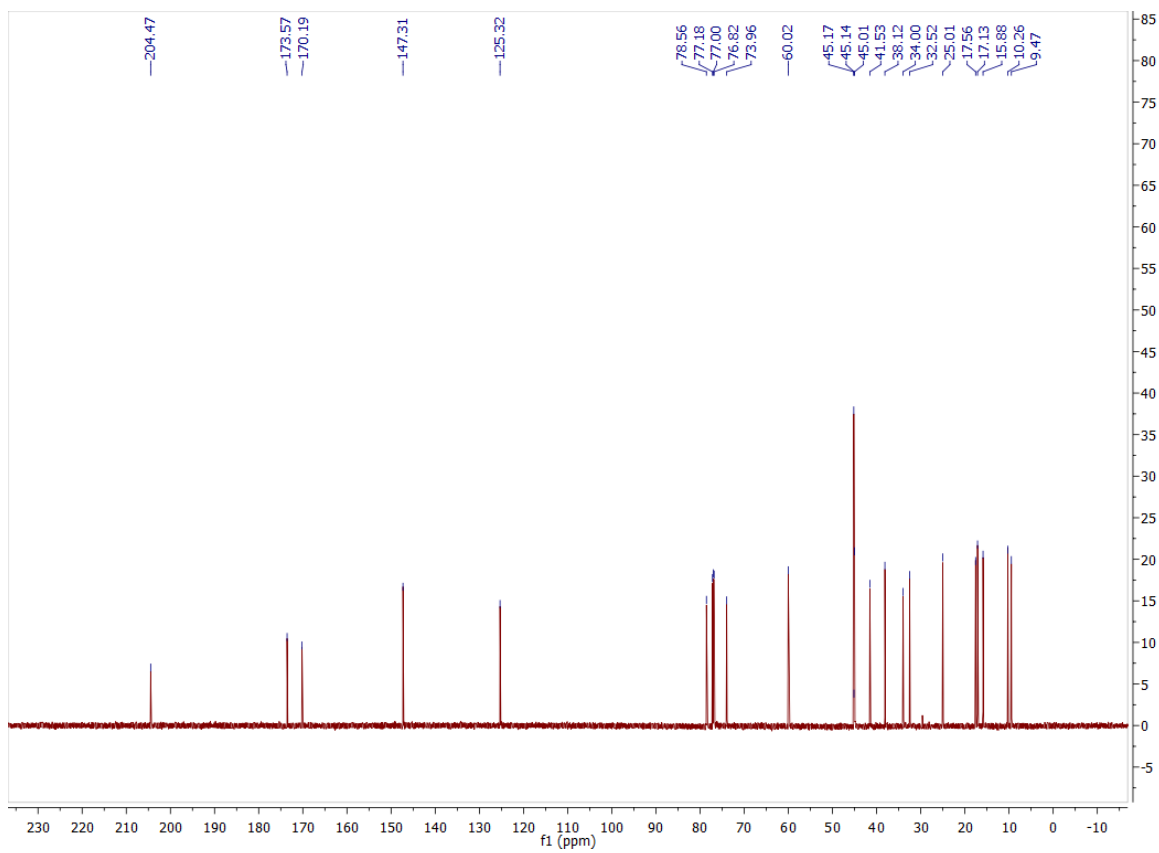
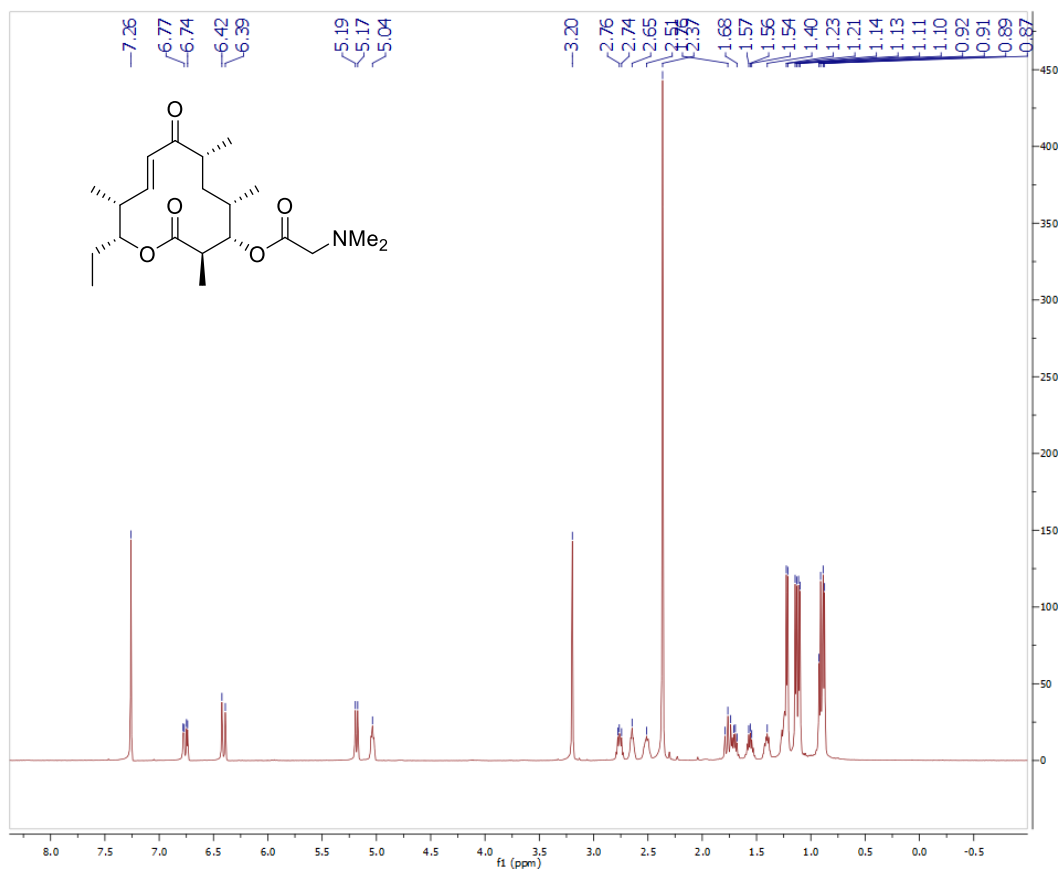
(3R,4S,5S,7R,11R,12R,E)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-methylbutanoate (S1): (3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate, 10-dml (6.1 mg, 0.021 mmol), DCC (6.4 mg, 0.031 mmol), DMAP (3.8 mg, 0.031 mmol), isovaleric acid (3.4 μL , 0.031 mmol) in 0.60 mL of dichloromethane were employed to yield 7.0 mg (88%) of the desired product (**S1**) after it was chromatographed over silica gel (eluent 20% EtOAc in hexanes). ^1H NMR (500 MHz, CDCl_3) δ 6.76 (dd, $J = 15.5, 5.5$ Hz, 1H), 6.41 (d, $J = 16.0$ Hz, 1H), 5.12 (d, $J = 11.0$ Hz, 1H), 5.03 (ddd, $J = 8.3, 5.5, 2.3$ Hz, 1H), 2.74 (dq, $J = 11.0$ Hz, 7.0 Hz), 2.68 – 2.61 (m, 1H), 2.56-2.47 (m, 1H), 2.26 – 2.18 (m, 2H), 2.11 (sept., $J = 6.8$ Hz, 1H), 1.80 – 1.66 (m, 2H), 1.65 – 1.49 (m, 1H), 1.43-1.34 (m, 1H), 1.28 – 1.20 (m, 4H), 1.18 (d, $J = 7.0$ Hz), 1.11 (d, $J = 7.0$ Hz), 0.97 (d, $J = 6.5$ Hz), 0.91 (t, $J = 7.3$ Hz), 0.87 (d, $J = 6.5$ Hz); ^{13}C NMR (175 MHz, CDCl_3) δ 204.6, 173.8, 172.6, 147.3, 125.4, 78.1, 74.0, 45.1, 43.4, 41.7, 38.1, 34.1, 32.6, 25.5, 25.1, 22.5, 17.6, 17.2, 15.9, 10.3, 9.5; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 381.2641, found 381.2673.



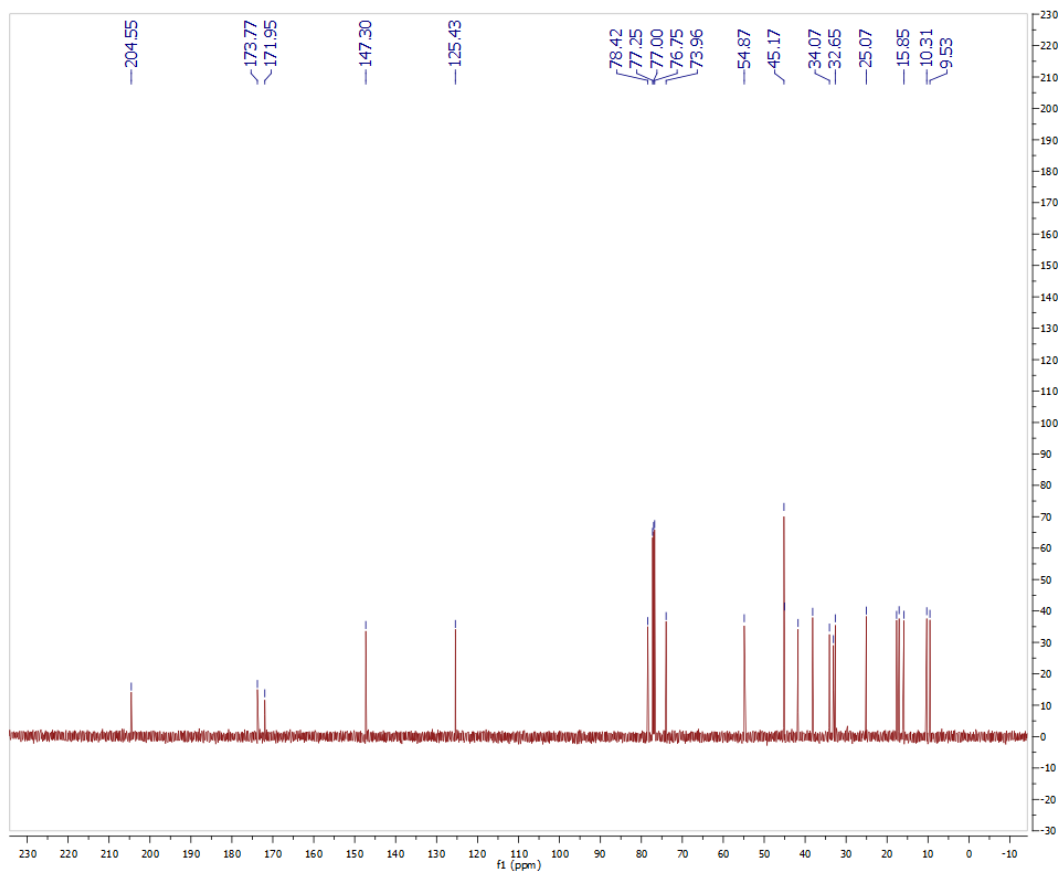
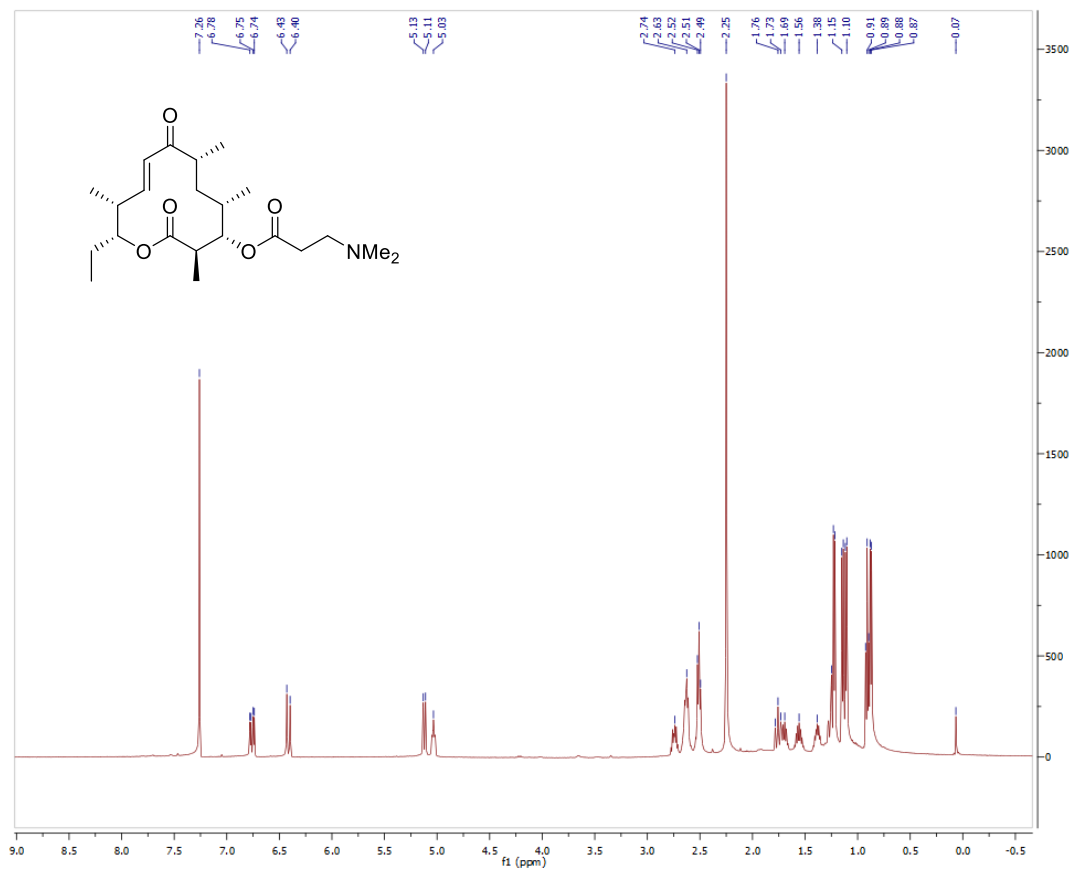
(3R,4S,5S,7R,11R,12R,E)-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-methylpentanoate (S2): (3R,4S,5S,7R,11R,12R,E)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate, 10-dml (11 mg, 0.038 mmol), DCC (12 mg, 0.057 mmol), DMAP (7.0 mg, 0.057 mmol), 4-methylvaleric acid (7.2 μL , 0.057 mmol) in 0.80 mL of dichloromethane were employed to yield 8.7 mg (58%) of **S2** after it was chromatographed over silica gel (eluent 20% EtOAc in hexanes). ^1H NMR (700 MHz, CDCl_3) δ 6.76 (dd, $J = 15.4, 5.3$ Hz, 1H), 6.41 (d, $J = 15.4$ Hz, 1H), 5.11 (d, $J = 10.5$ Hz, 1H), 5.03 (ddd, $J = 8.4, 5.6, 2.5$ Hz, 1H), 2.74 (dq, $J = 14.0, 11.2$ Hz, 1H), 2.67-2.62 (m, 1H), 2.51 (qd, $J = 6.7, 4.2$ Hz, 1H), 2.33 (t, $J = 7.7$ Hz, 2H), 1.76 (t, $J = 13.3$ Hz, 1H), 1.73-1.67 (m, 1H), 1.60-1.50 (m, 4H), 1.42-1.35 (m, 1H), 1.27-1.21 (m, 4H), 1.12 (d, $J = 7.0$ Hz, 3H), 1.11 (d, $J = 7.0$ Hz, 3H), 0.92-0.89 (m, 9H), 0.86 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ 204.6, 147.3, 125.4, 78.1, 73.9, 45.1, 41.7, 38.1, 34.1, 33.9, 32.6, 32.3, 27.8, 25.1, 22.2, 17.6, 17.2, 15.9, 10.3, 9.5; HRMS (ESI) m/z calculated for $[\text{M}+\text{H}]^+$ 395.2797, found 395.2773.

^1H NMR and ^{13}C NMR Spectra of substrates

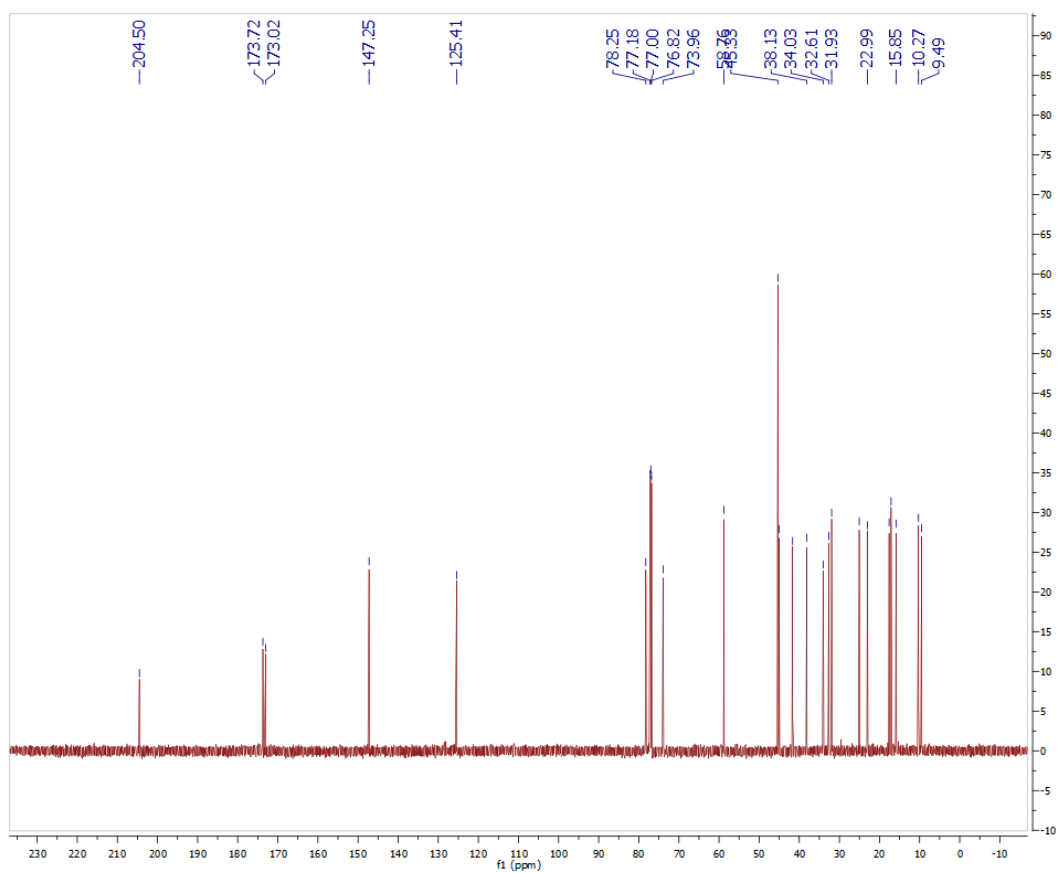
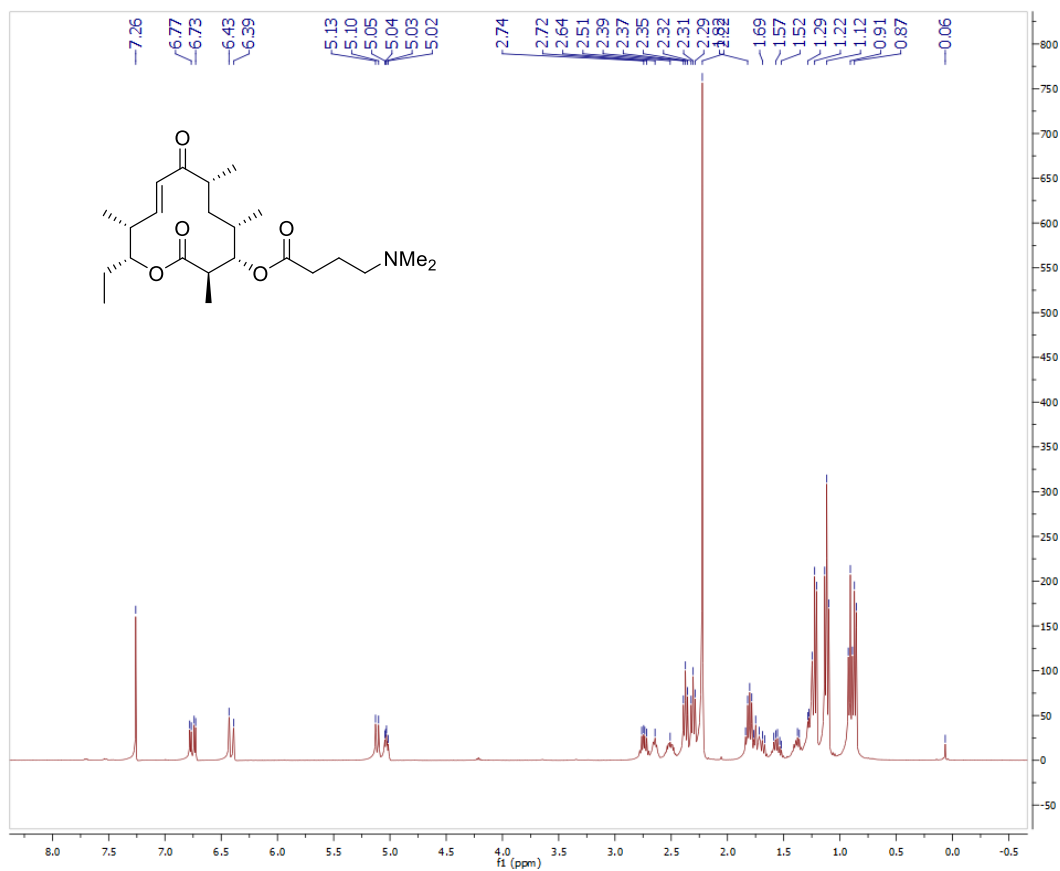
Substrate 8a



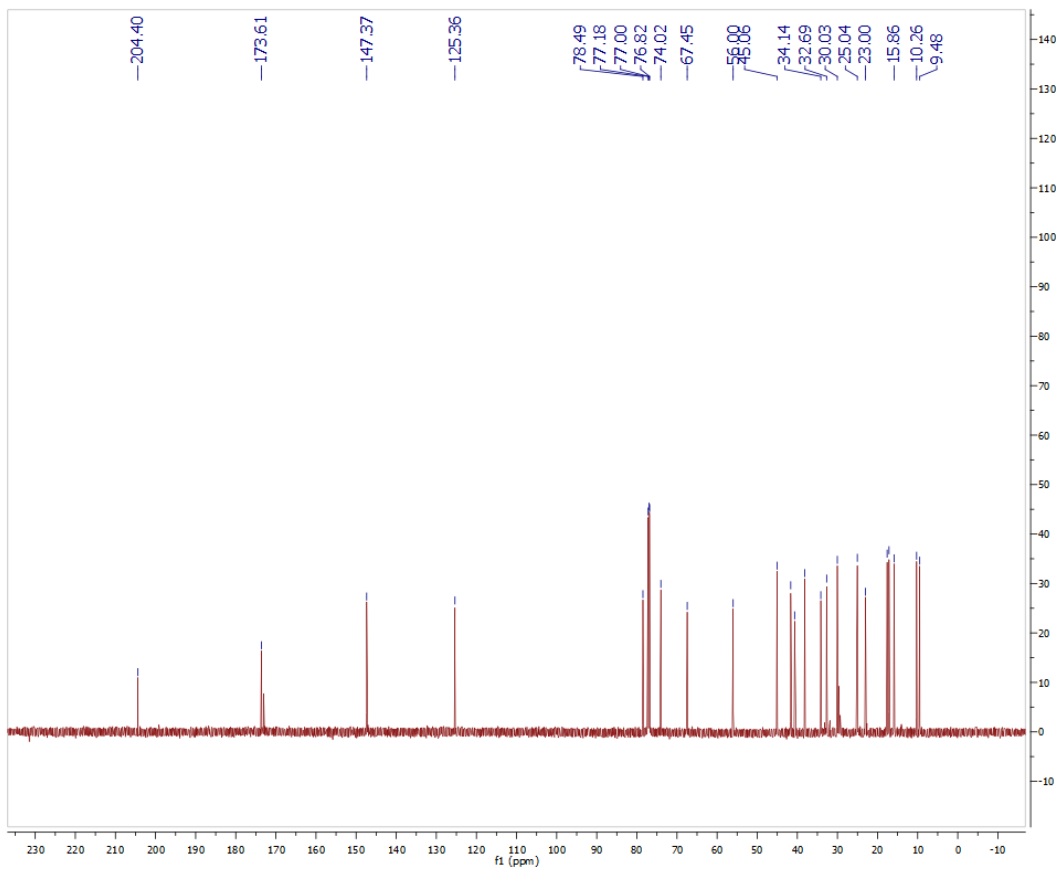
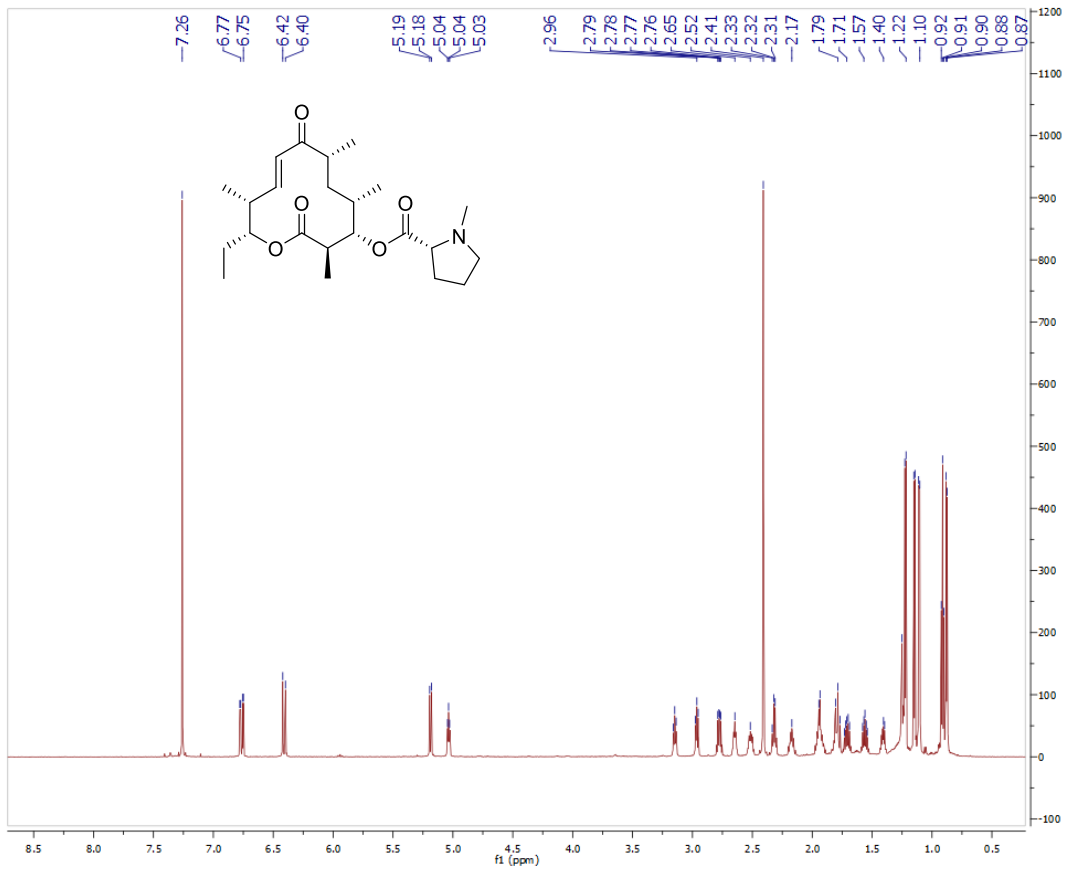
Substrate 8b



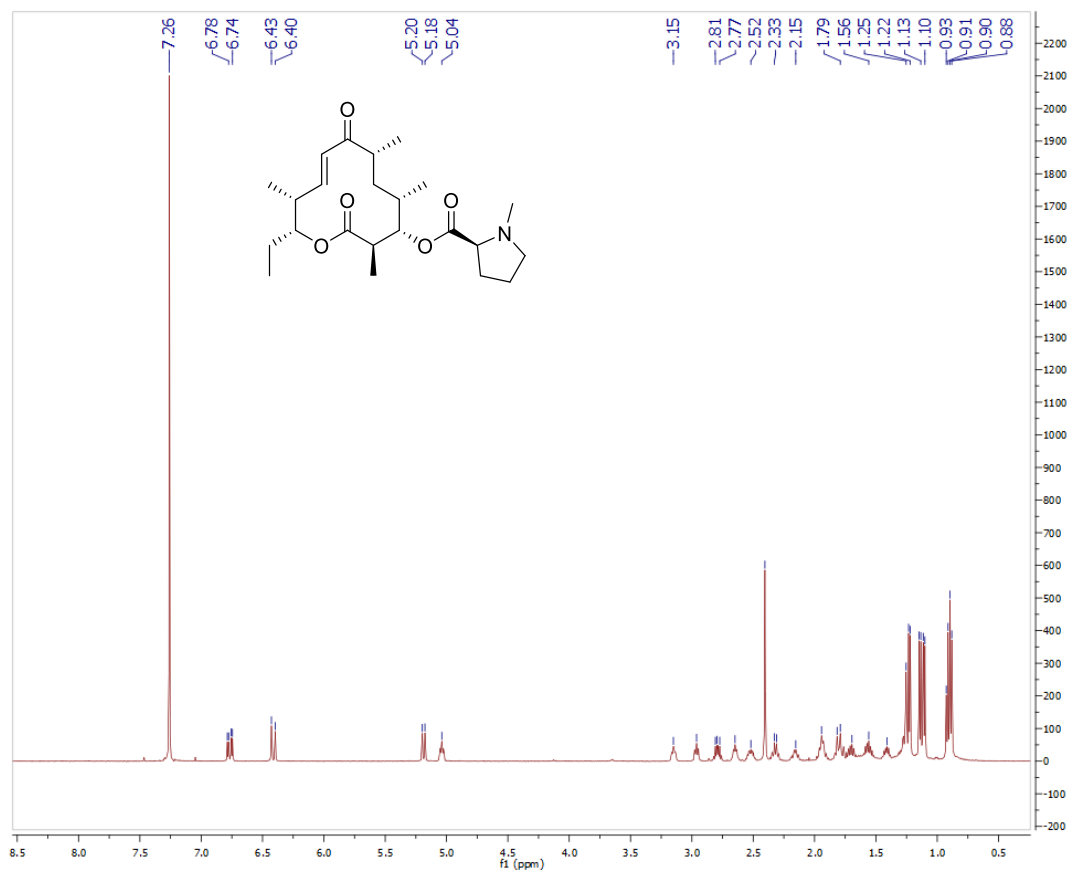
Substrate 10

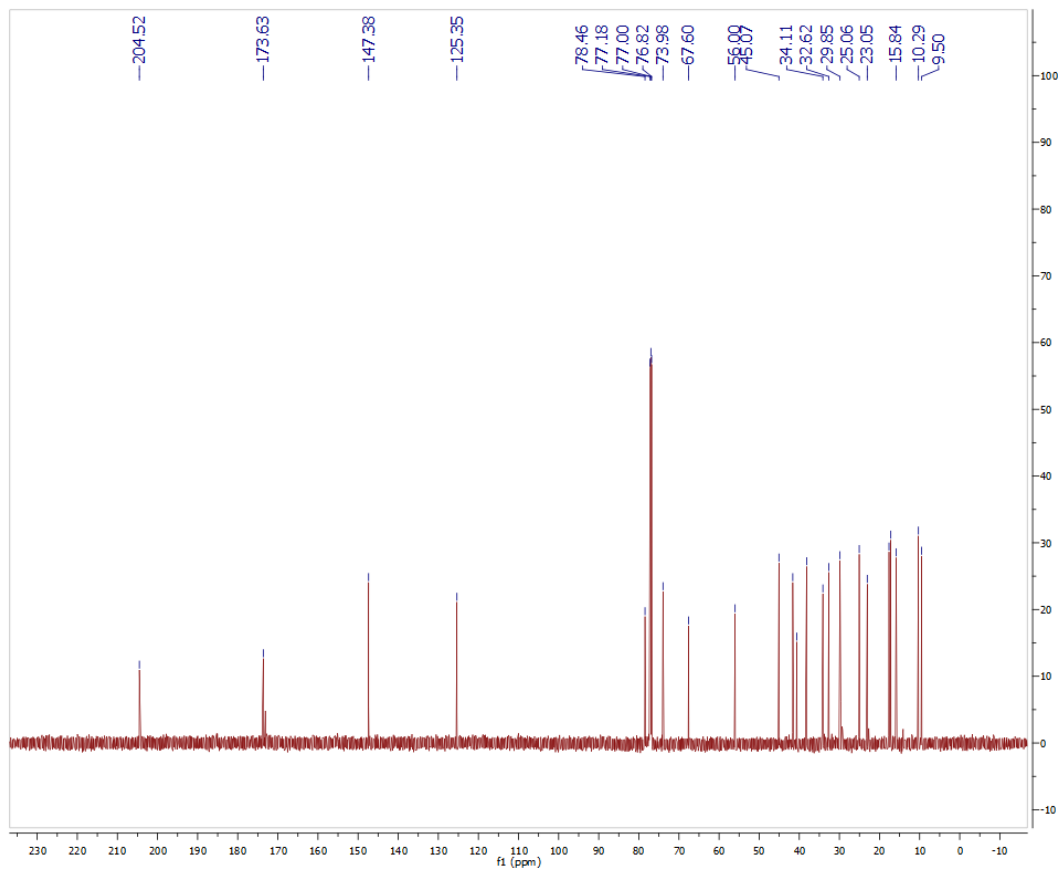


Substrate 12a

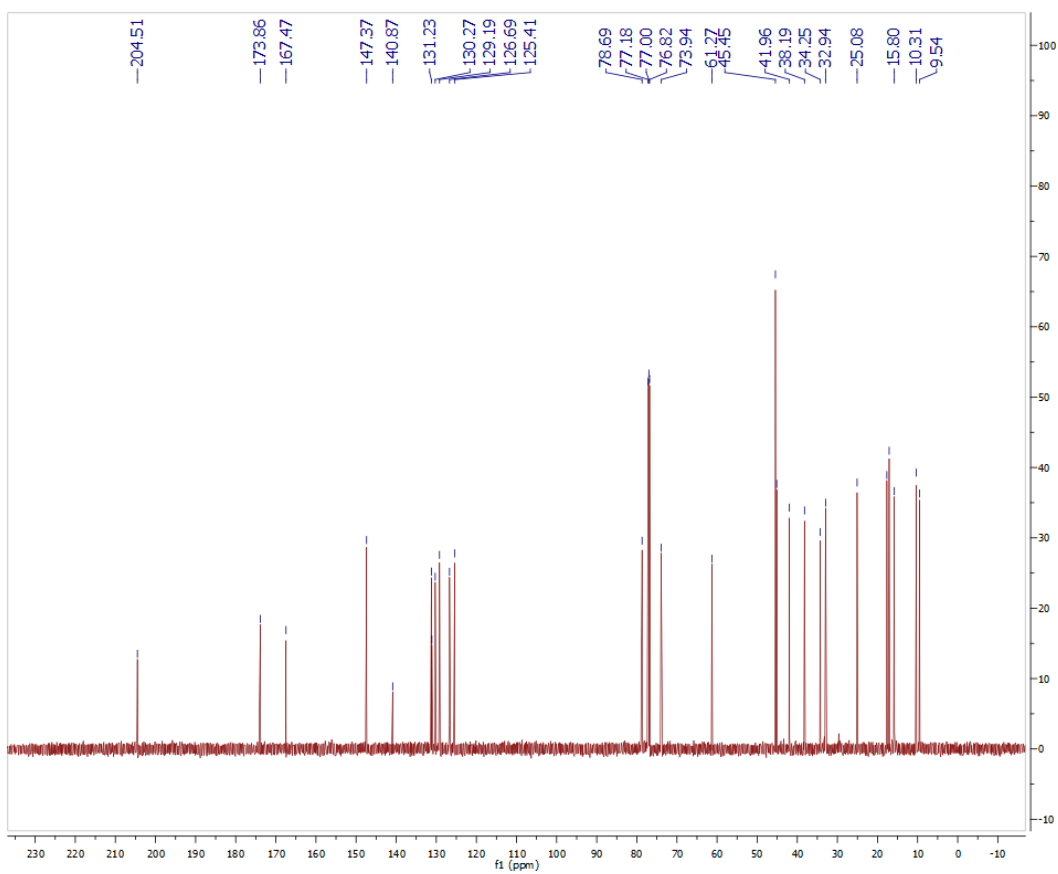
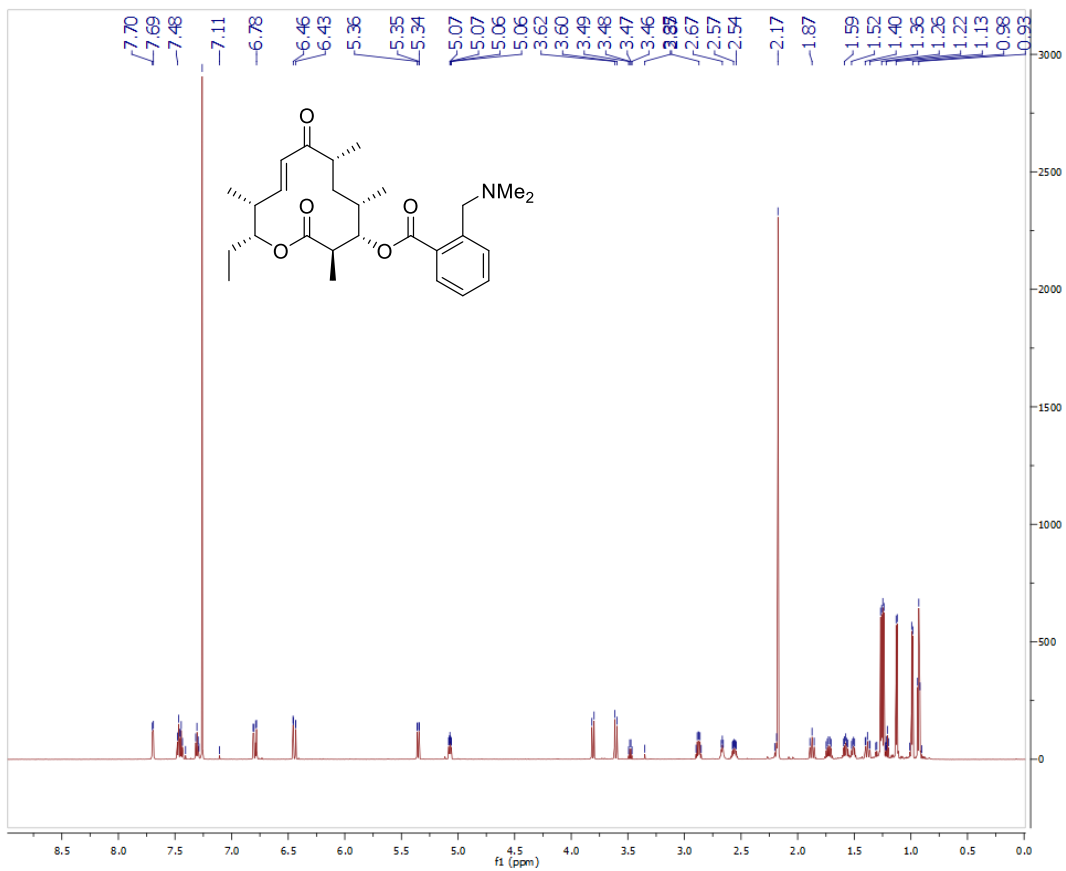


Substrate 12b

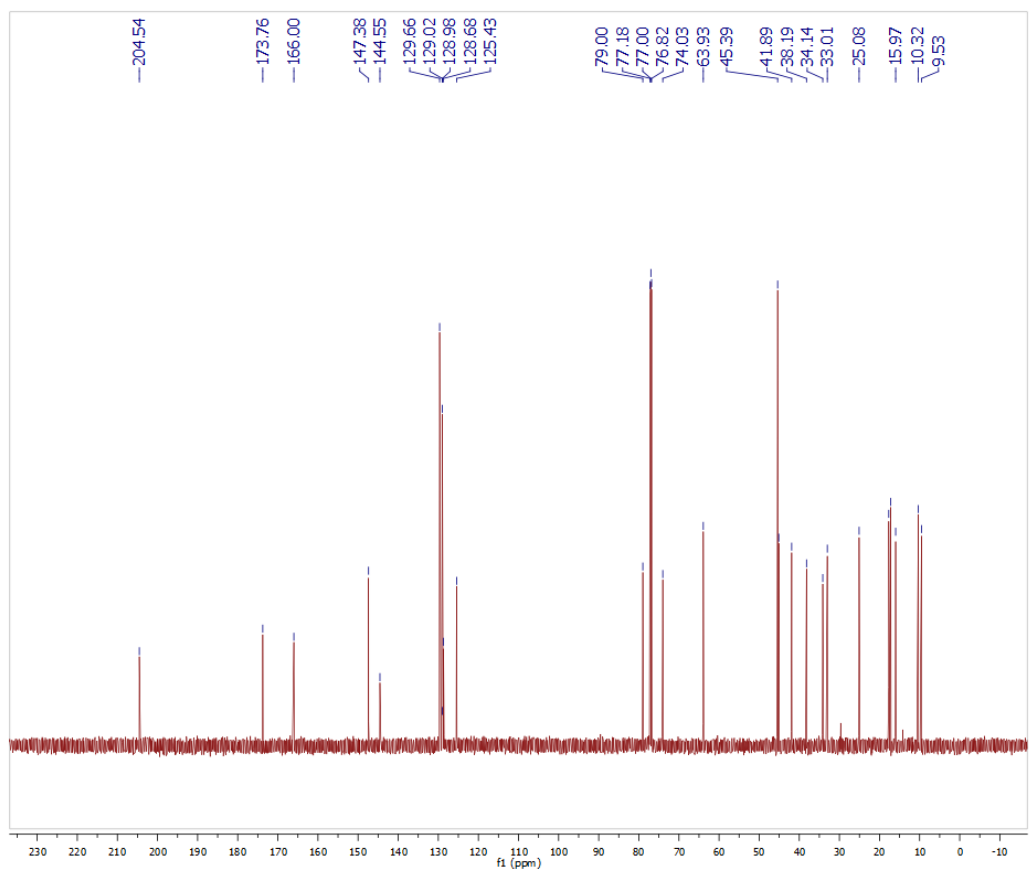
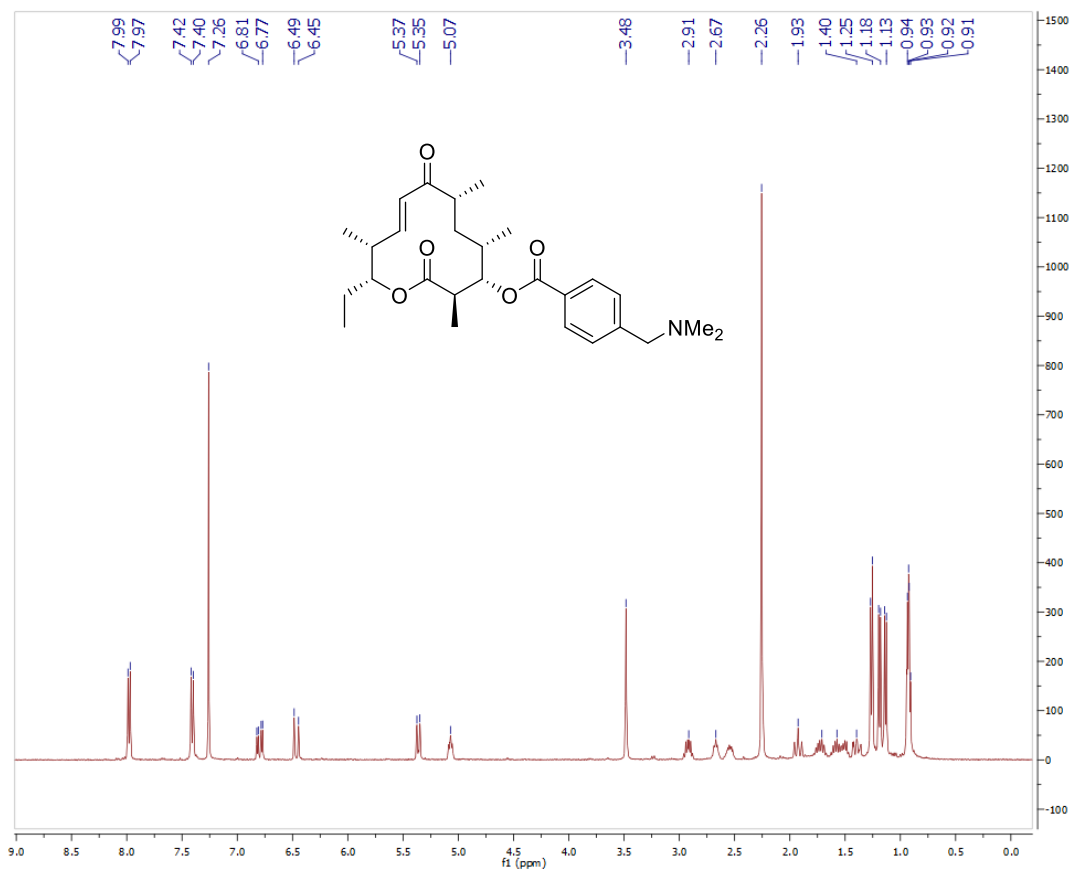




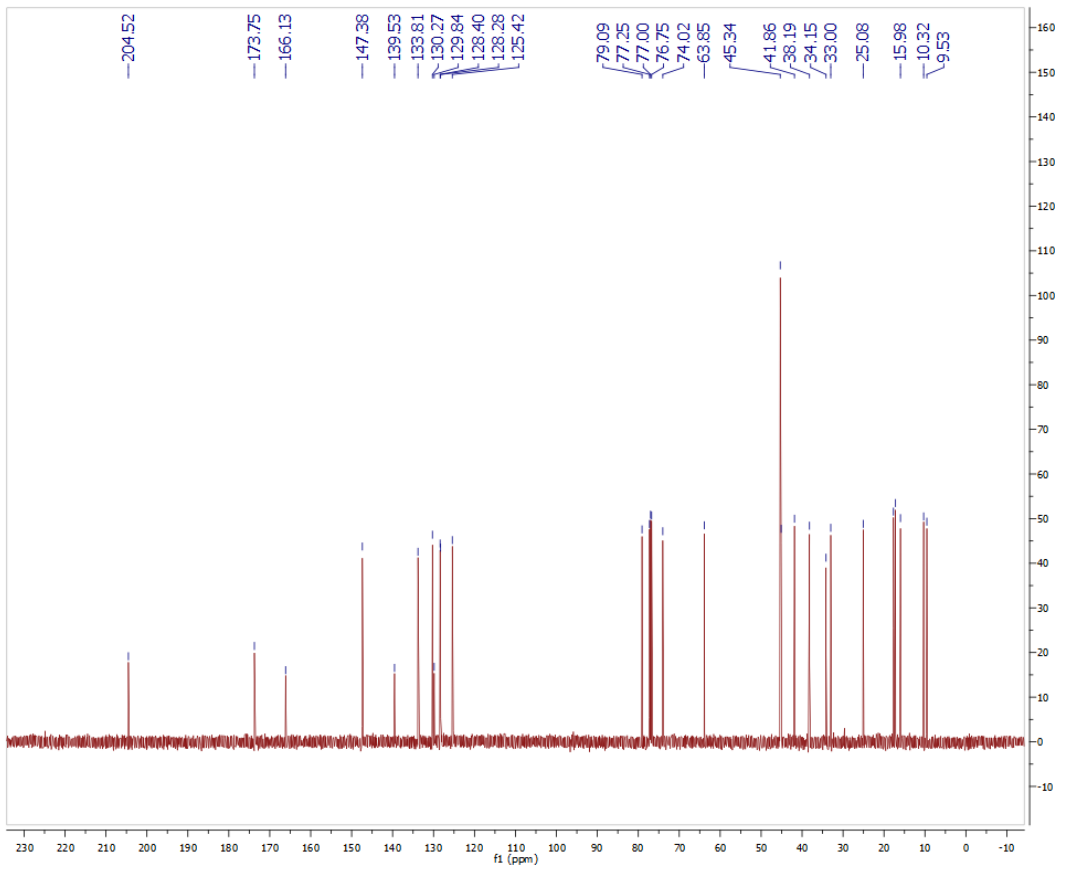
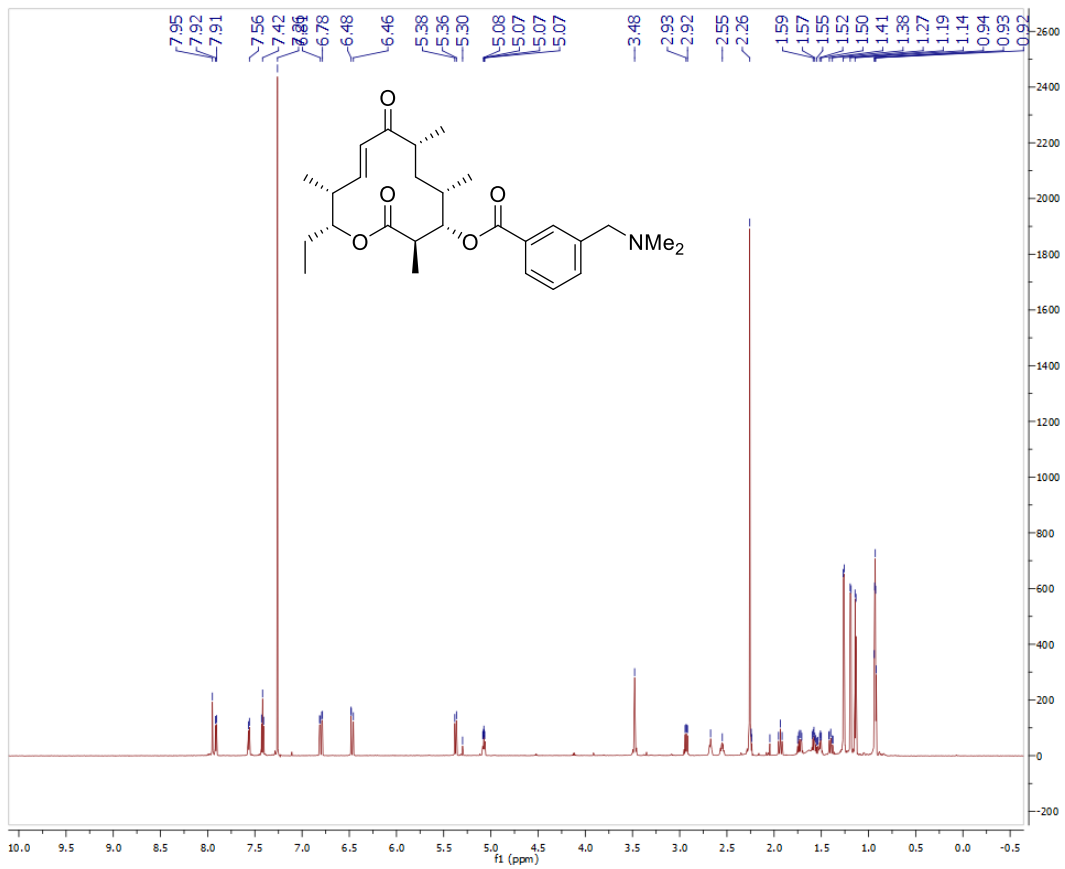
Substrate **14**



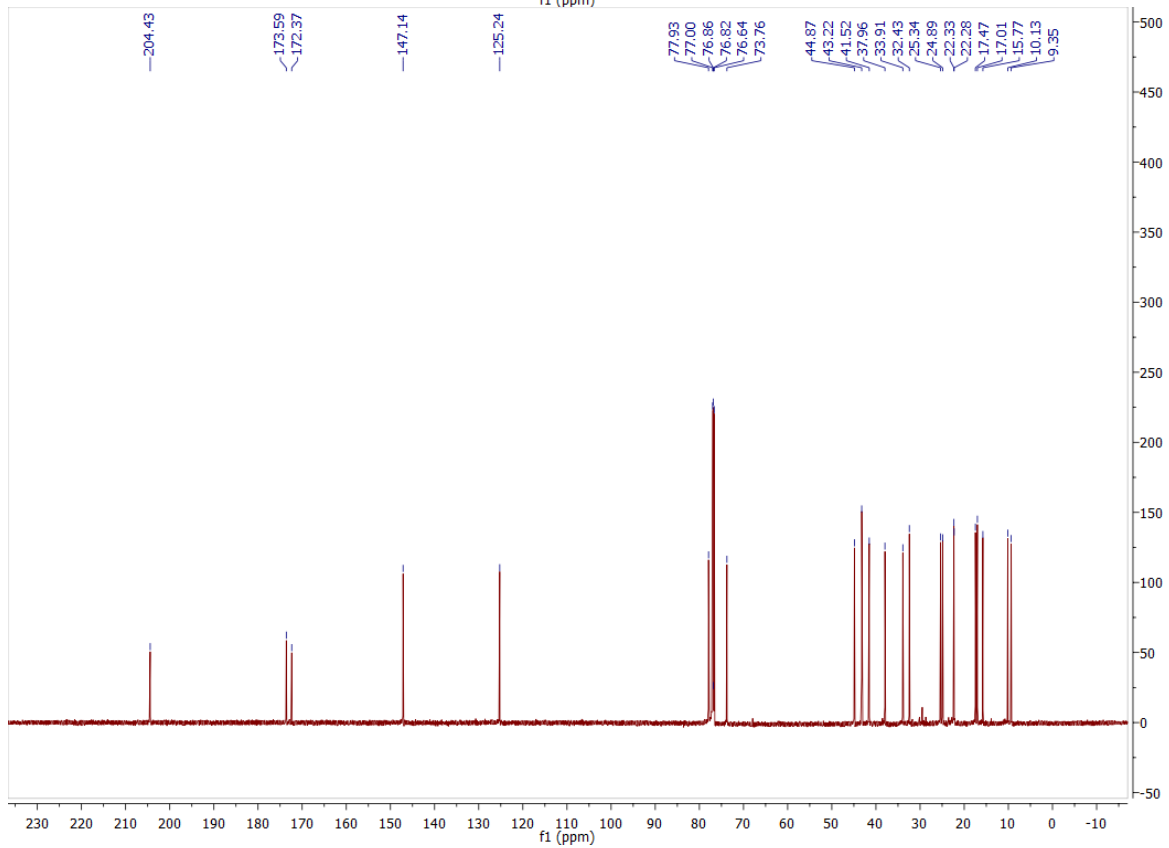
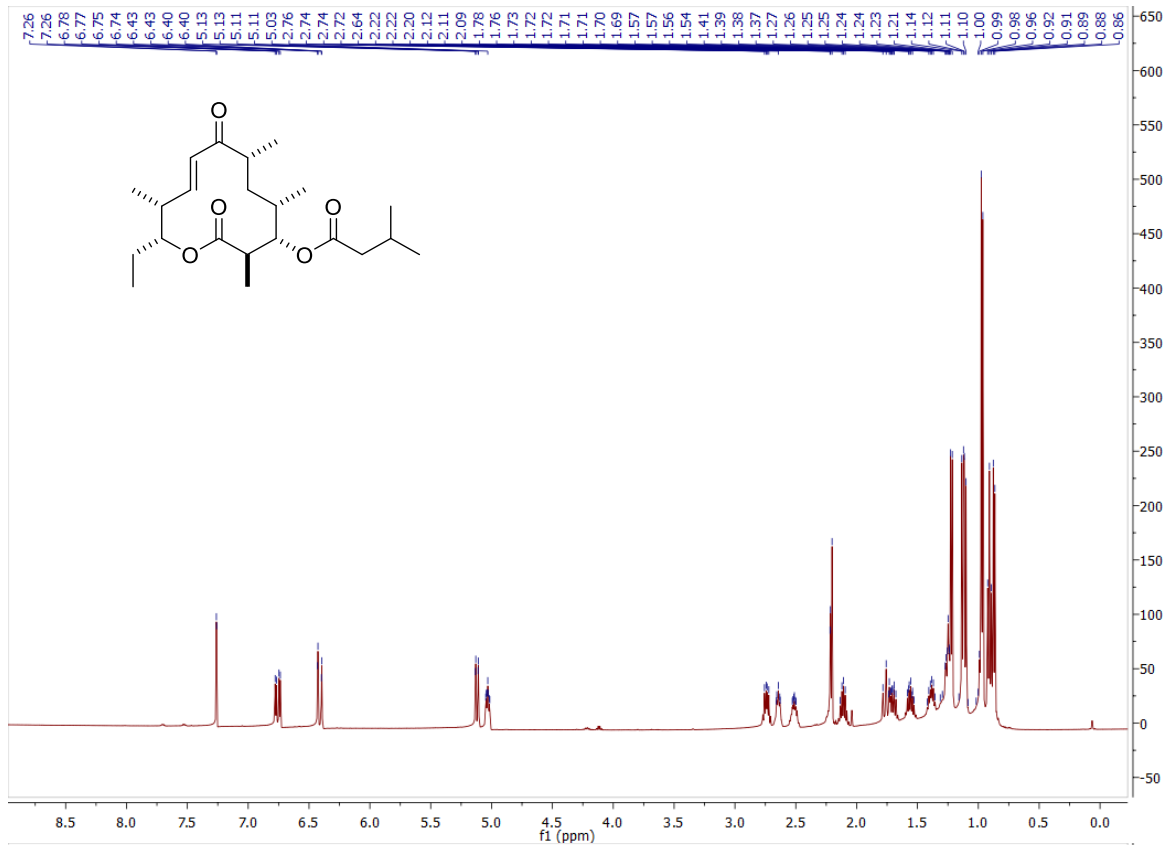
Substrate **16a**



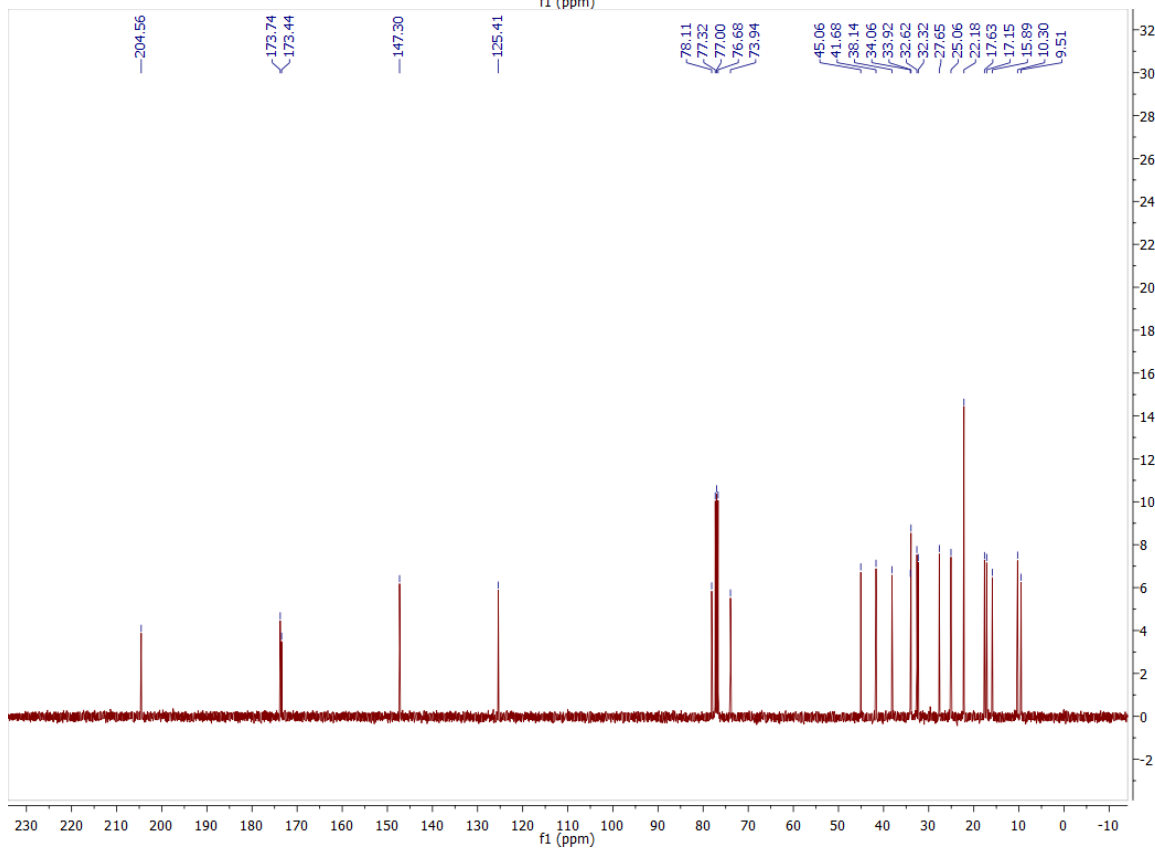
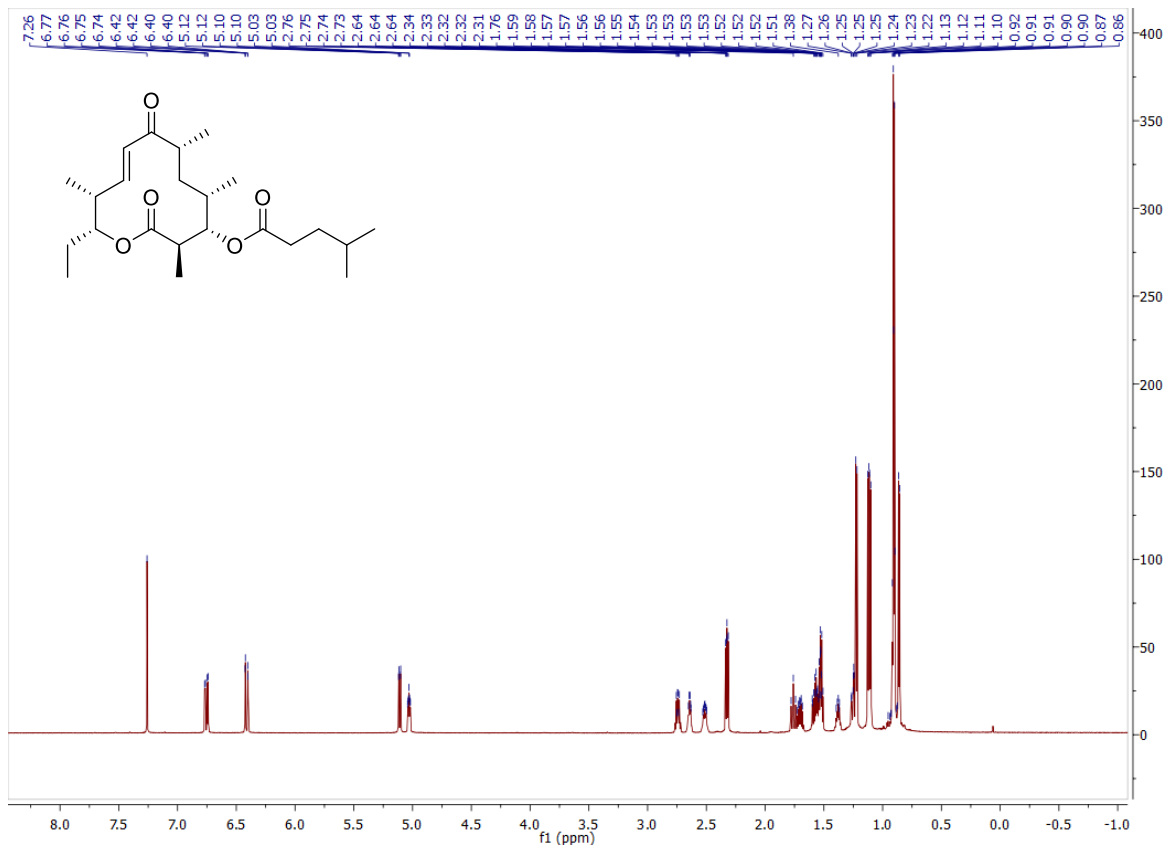
Substrate **16b**



Control S1



Control S2



Part II: Enzymatic reactions

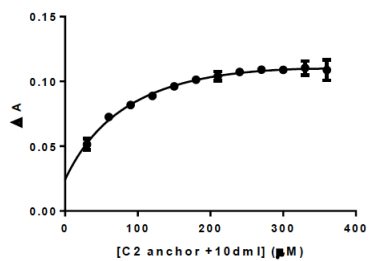
Expression and purification of PikC_{D50N}-RhFRED: Protein expression and purification followed a previously described procedure.⁴

PikC_{D50N}-RhFRED analytical-scale enzymatic reactions: The standard assay contained 5 μ M PikC_{D50N}-RhFRED, 1 mM substrate, 1 mM NADP⁺, 0.05 units of glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate for NADPH regeneration in of reaction buffer (50 mM NaH₂PO₄, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, and 10% glycerol) total volume 50 μ L. The reaction was carried out at 30 °C for 3 h and quenched by extraction with CHCl₃ (2 \times 200 μ L). The organic extracts were combined, concentrated to dryness under N₂, and redissolved in 100 μ L of methanol. The subsequent liquid chromatography mass spectrometry (LC-MS) analysis was performed on an Agilent Q-TOF HPLC-MS (Department of Chemistry, University of Michigan) equipped with an high resolution electrospray mass spectrometry (ESI-MS) source and a Beckmann Coulter reverse-phase HPLC system using an Waters XBridge C18 3.5 μ m, 2.1x150 mm under the following conditions: mobile phase (A = deionized water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid), 10% to 100% B over 15 min, 100% B for 4 min; flow rate, 0.2 mL/min. Reactions were scanned for [M+16] (monohydroxylation) and [M+32] (dihydroxylation). Minor amounts of dihydroxylation were observed only for **16a** and **16b**. The percent conversion was determined as outlined in Li et al.⁵ Briefly, the percent conversion was calculated with $AUC_{\text{total products}}/(AUC_{\text{total products}} + AUC_{\text{unreacted substrate}})$ by assuming ionization efficiency of substrate and hydroxylated products are the same, because the ionization site of this series of compounds should be the dimethylamino group.

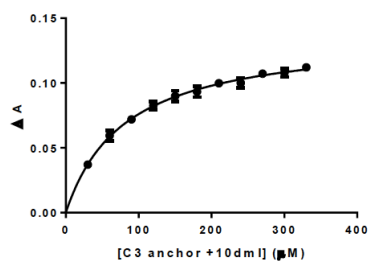
Determination of total turnover number (TTN): Total turnover number was determined by analyzing (# of moles of starting material consumed)/(# of moles of enzyme) under the following conditions: 1 μ M PikC_{D50N}-RhFRED, 1 mM substrate, 1 mM NADP⁺, 0.05 units of glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate for NADPH regeneration in 50 μ L of reaction buffer (50 mM NaH₂PO₄, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, and 10% glycerol). The reaction was carried out at 30 °C for 24 h and quenched by extraction with CHCl₃ (2 \times 200 μ L). The organic extracts were combined, concentrated to dryness under N₂, and redissolved in 100 μ L of methanol. The subsequent liquid chromatography mass spectrometry (LC-MS) analysis was performed on an Agilent Q-TOF HPLC-MS (Department of Chemistry, University of Michigan) equipped with an high resolution electrospray mass spectrometry (ESI-MS) source and a Beckmann Coulter reverse-phase HPLC system using an Waters XBridge C18 3.5 μ m, 2.1x150 mm under the following conditions: mobile phase (A = deionized water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid), 10% to 100% B over 15 min, 100% B for 4 min; flow rate, 0.2 mL/min. All reactions were performed in duplicate.

The substrate-binding assays were performed as previously described.⁴ *Equilibrium Binding Assay*—Spectroscopic substrate binding assay was carried out at room temperature using a UV-visible spectrophotometer 300 Bio (Cary). PikC_{D50N}-RhFRED dissolved in 50 mM sodium phosphate, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, and 10% glycerol at concentrations ranging from 1 to 2 μ M was titrated with the substrate dissolved in Me₂SO (30 mM) in 1- μ L aliquots. The same amounts of Me₂SO alone were added to the protein in the reference cuvette followed by recording of the difference spectra. Absorbance differences $\Delta A (A_{\text{peak } 390 \text{ nm}} - A_{\text{trough } 420 \text{ nm}})$ were plotted *versus* substrate concentration, and data from duplicated experiments were fitted to the hyperbolic function $\Delta A = (A_{\text{max}}(S/K_D + S))$, where S is the total ligand concentration, A_{max} is the maximal absorption shift at saturation, and K_D is the apparent dissociation constant for the enzyme-ligand complex.

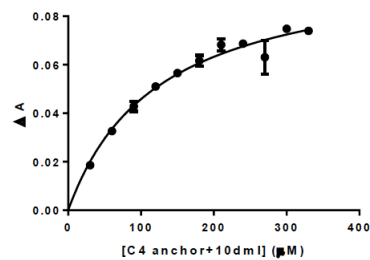
Compound **8a**: $K_D = 118 \mu\text{M}$



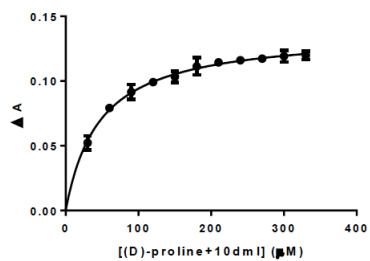
Compound **8b**: $K_D = 81 \mu\text{M}$



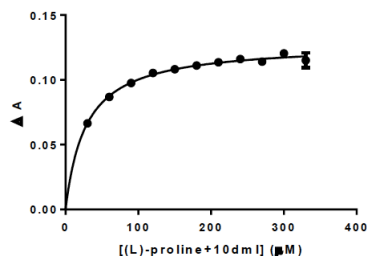
Compound **10**: $K_D = 123 \mu\text{M}$



Compound **12a**: $K_D = 47 \mu\text{M}$



Compound **12b**: $K_D = 28 \mu\text{M}$

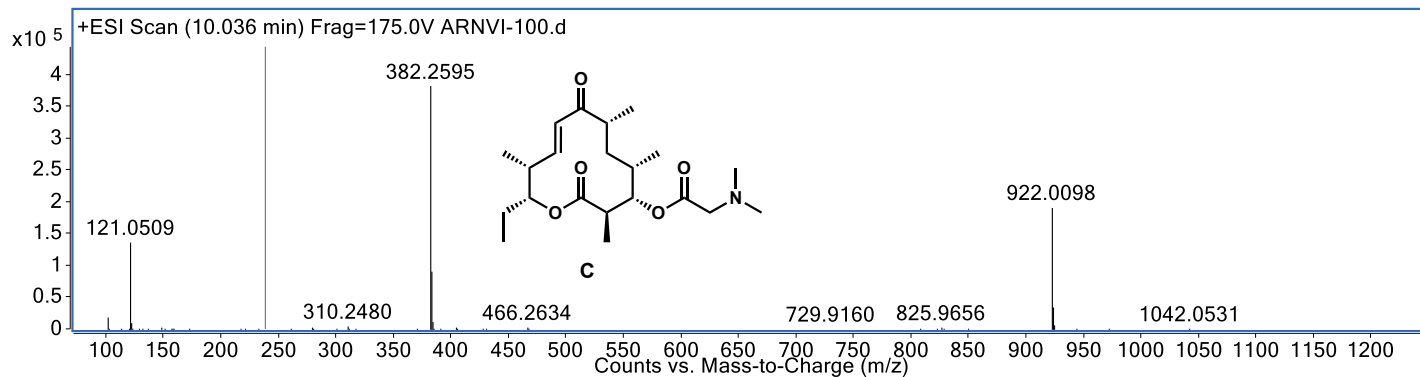
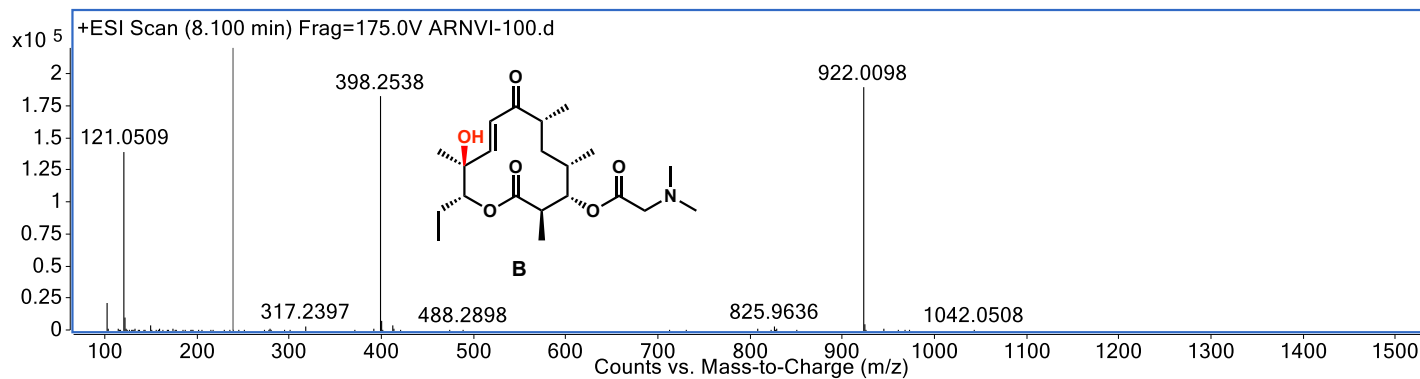
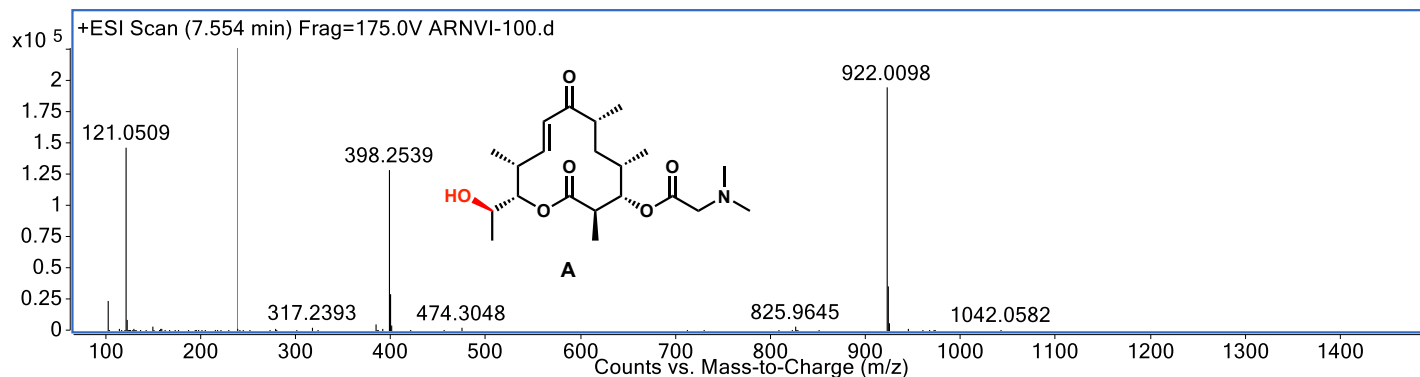
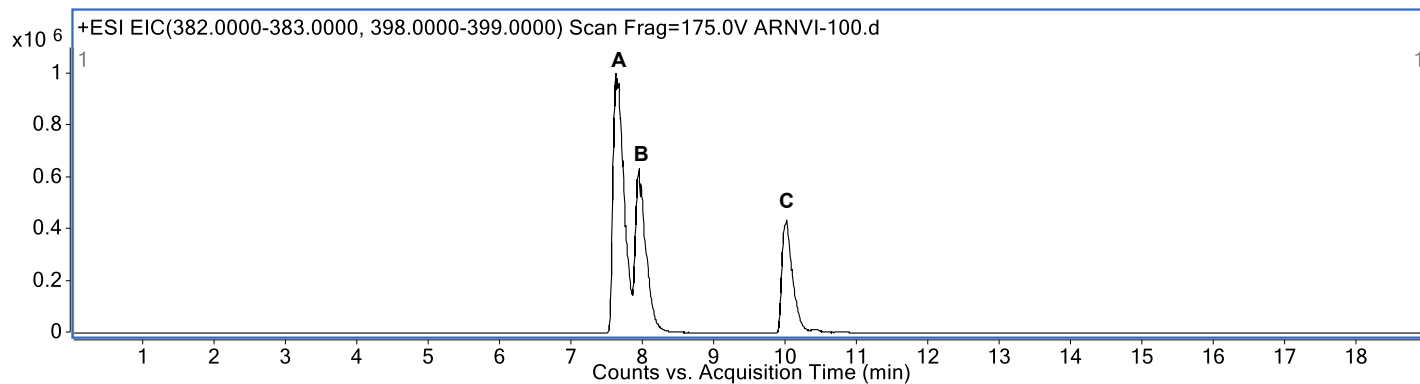
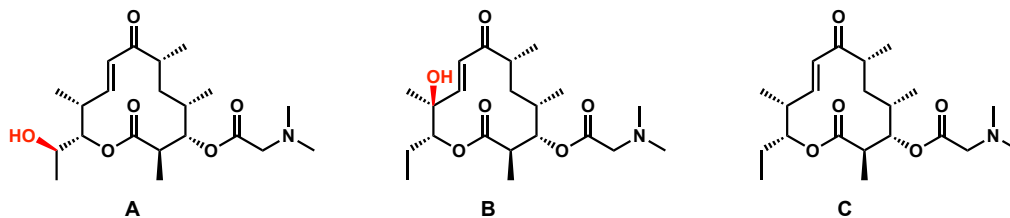


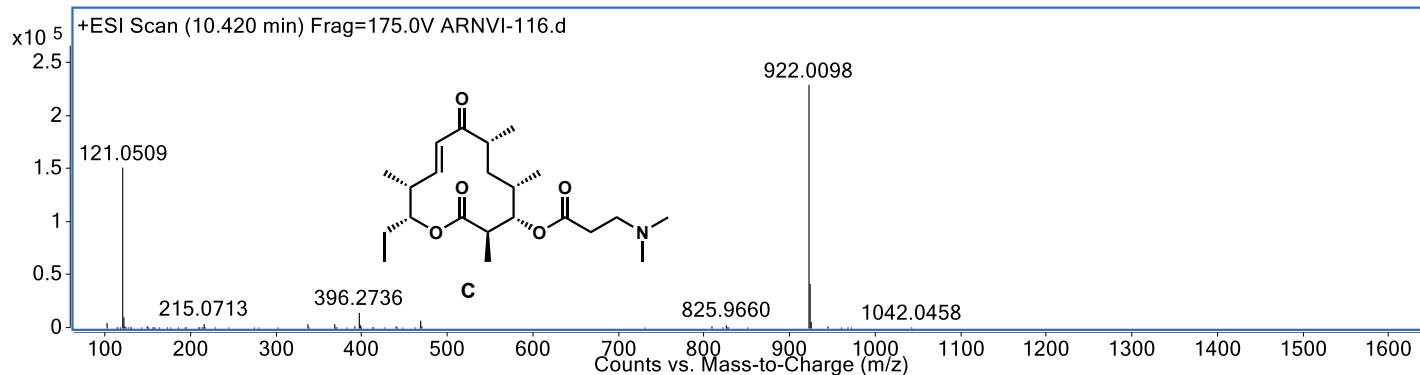
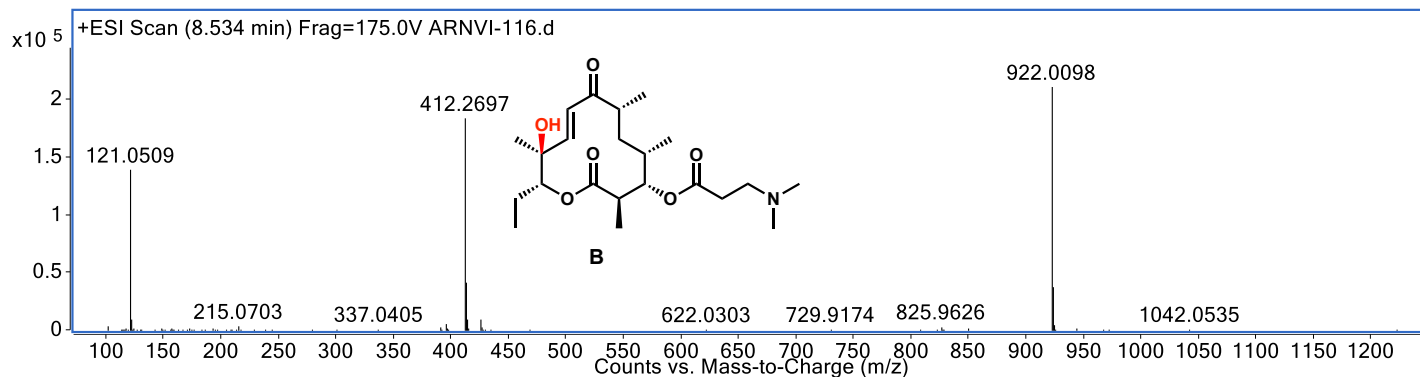
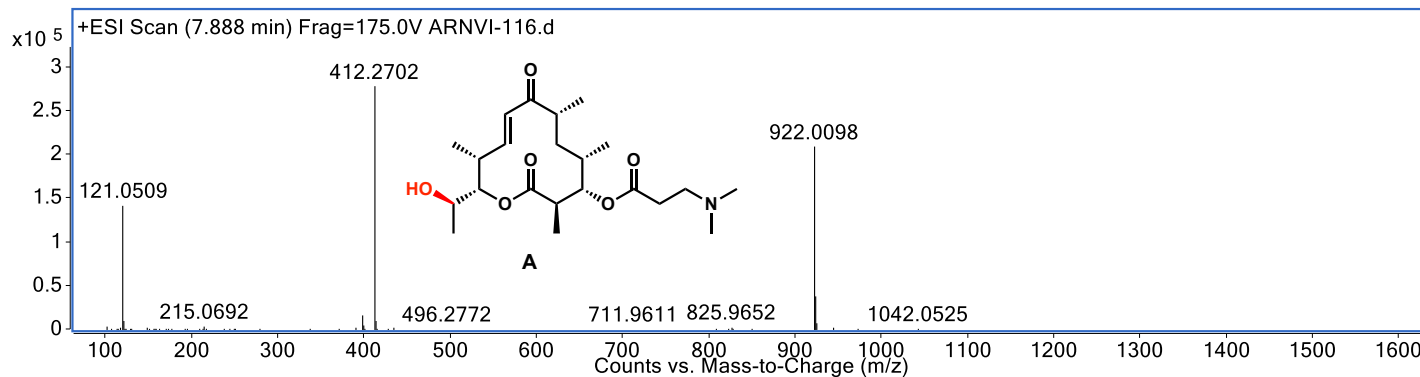
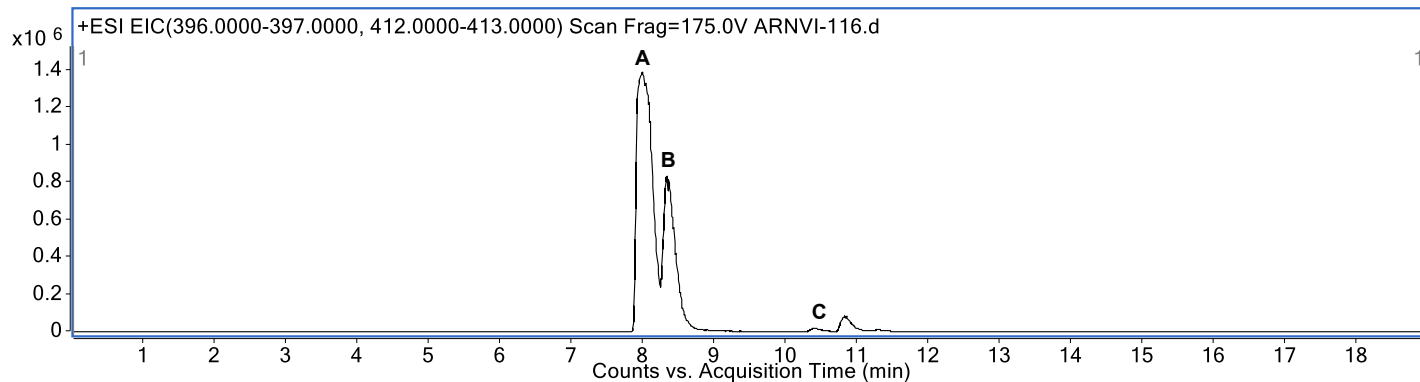
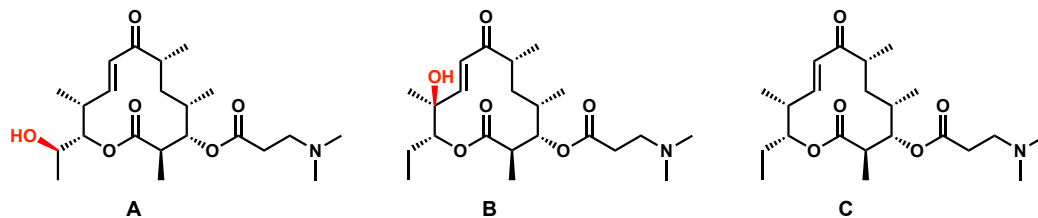
PikC_{D50N}-RhFRED preparative-scale enzymatic reactions: Preparative –scale enzymatic reactions were conducted on 20 mg of each substrate under the following conditions: 5 µM PikC_{D50N}-RhFRED, 1 mM substrate, 1 mM NADP⁺, 1 U/mL glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate for NADPH generation in reaction buffer (50 mM NaH₂PO₄, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, and 10% glycerol). The reaction mixture was divided into 50 mL conical tubes in 7 mL aliquots. Each conical tube was loosely capped and transferred to a shaking incubator. The reaction was carried out at 30 °C for 12 h at 160 rpm. After 12 h, a 50 µL aliquot was removed and processed in an identical manner to the analytical-scale reactions described above to access the outcome of the reaction via LCMS. The remaining reaction mixture was diluted with acetone (2 x total reaction volume) and cooled to 4 °C for two hours. The mixture was then filtered through celite and concentrated under reduced pressure until the acetone had been removed. The remaining solution was adjusted to pH 9, brined and extracted with EtOAc (3 x 200 mL). The organic layers were combined, dried over Na₂SO₄ and concentrated to afford a crude yellow oil. The crude oil was purified through silica flash column chromatography (column conditions from the starting material purification) to afford the mixture of hydroxylated products.

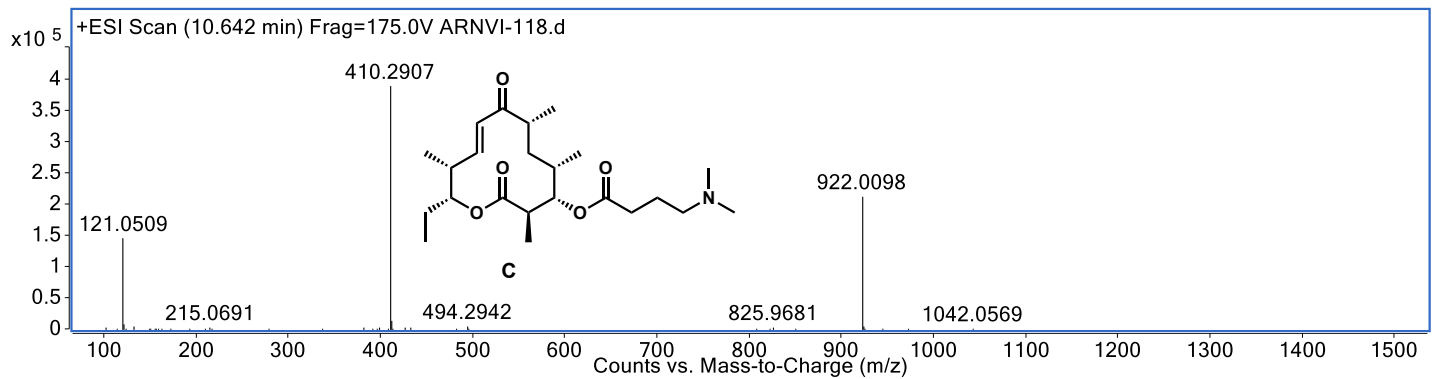
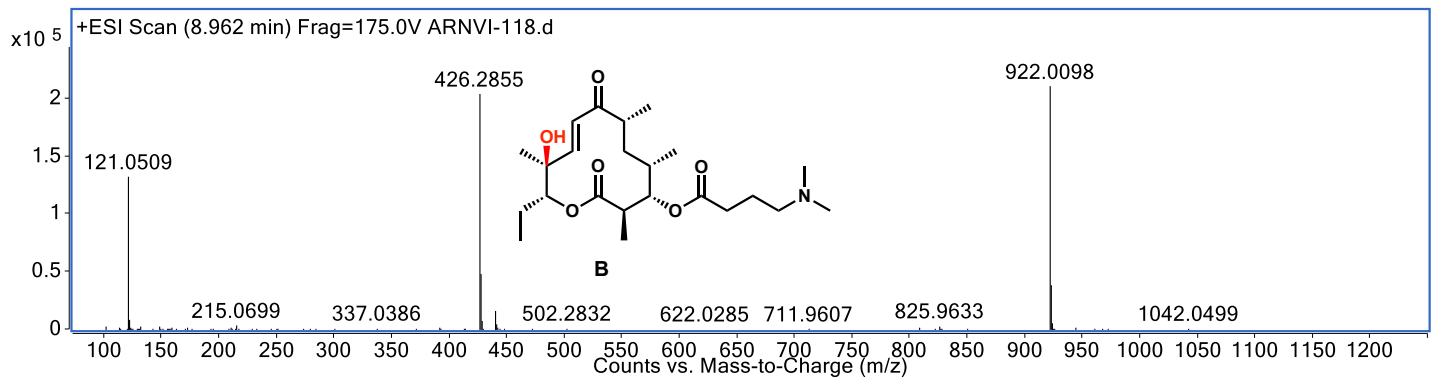
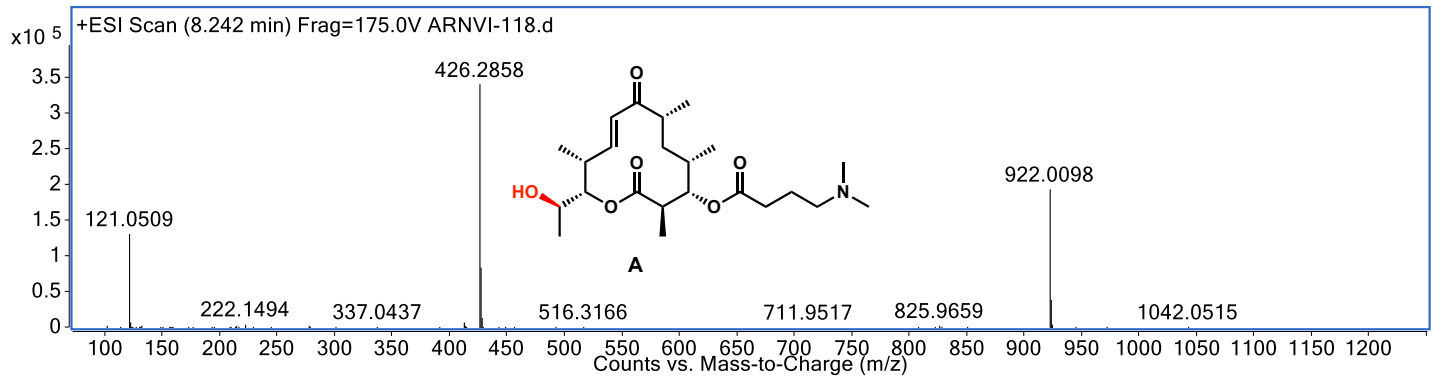
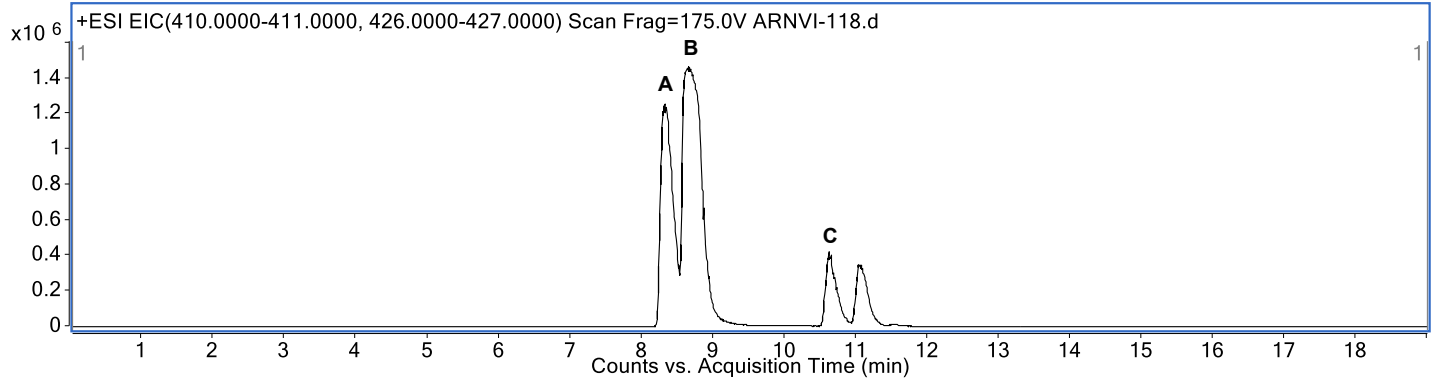
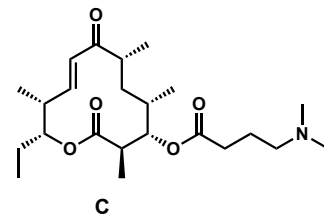
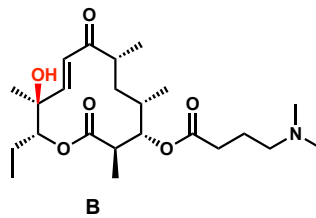
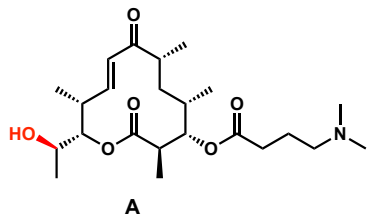
Preparation of PikC_{D50NH238A}-RhFRED: Using previously prepared pET28b-PikC_{D50N}-RhFRED as a template, site-directed mutagenesis was performed with the primer: 5'-gaggagctgctcggtatggccgcatcctgctcgtcgcggggcac-3'. Protein expression and purification of PikC_{D50NH238A}-RhFRED followed the procedure developed previously.⁴

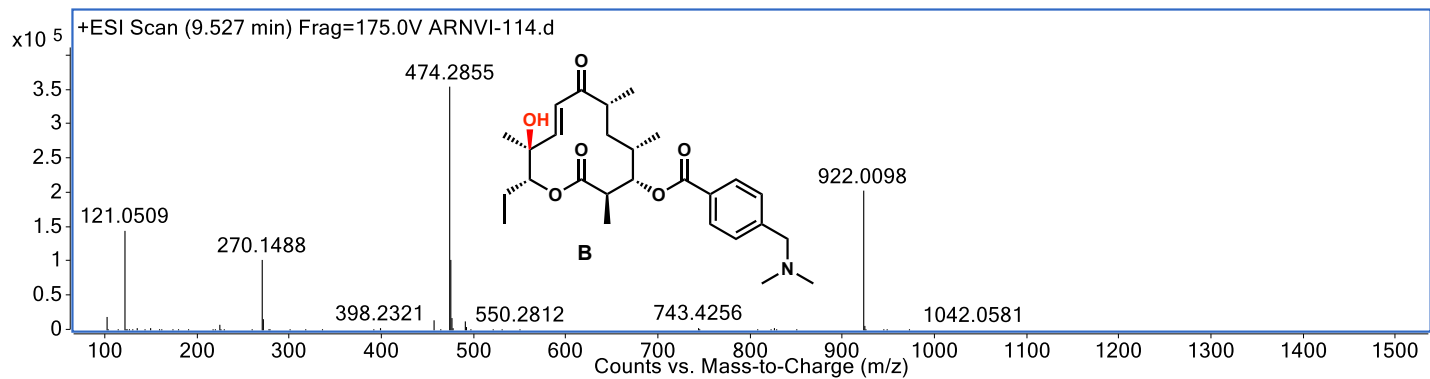
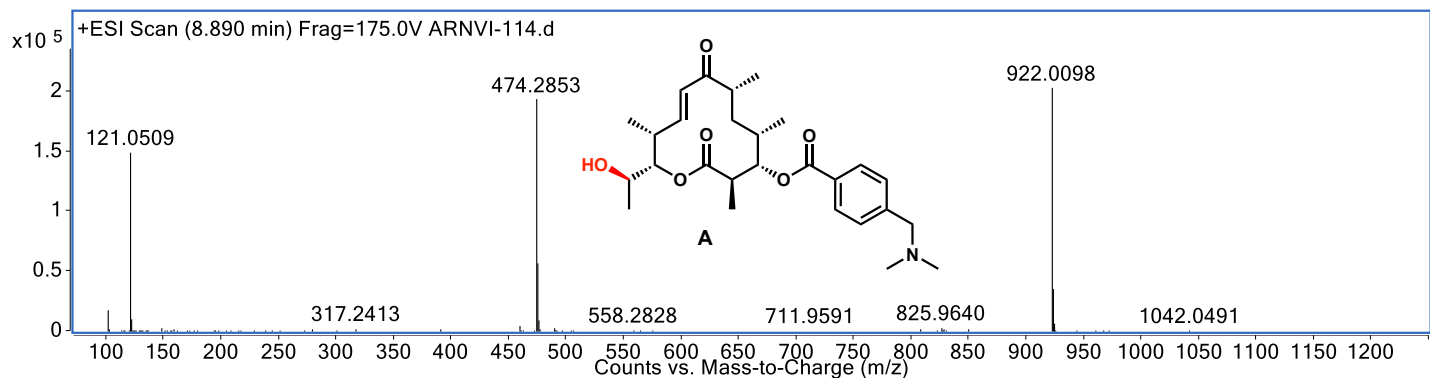
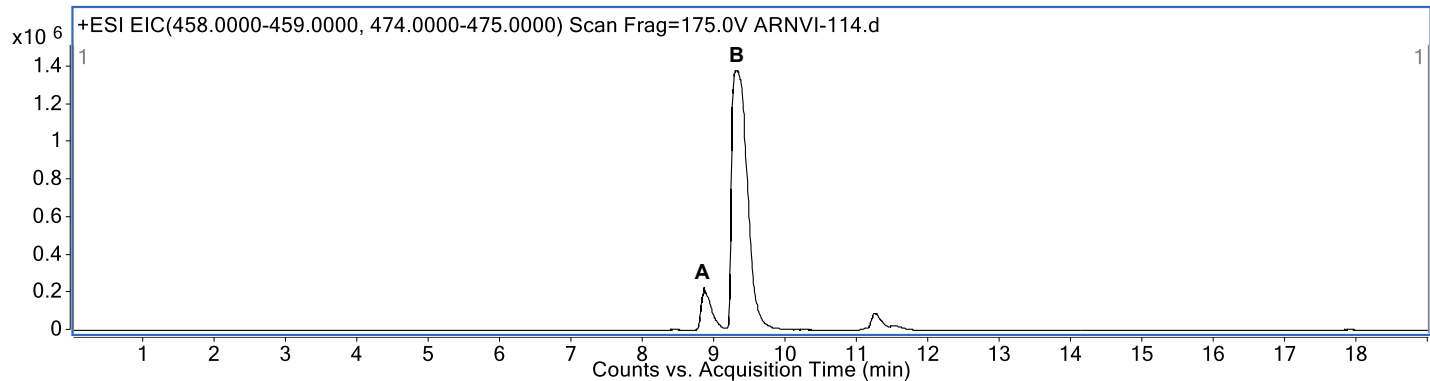
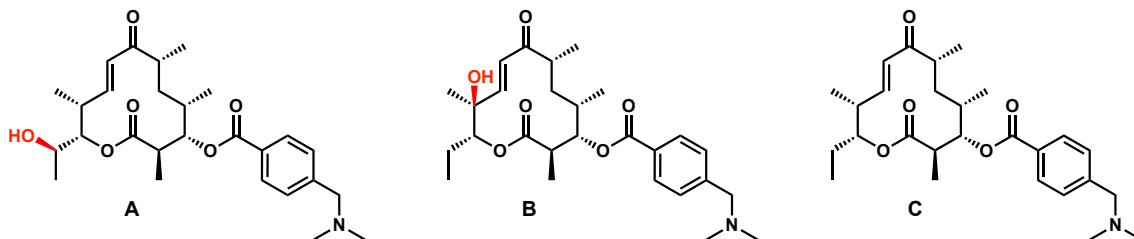
Hydroxylation product analysis and identification

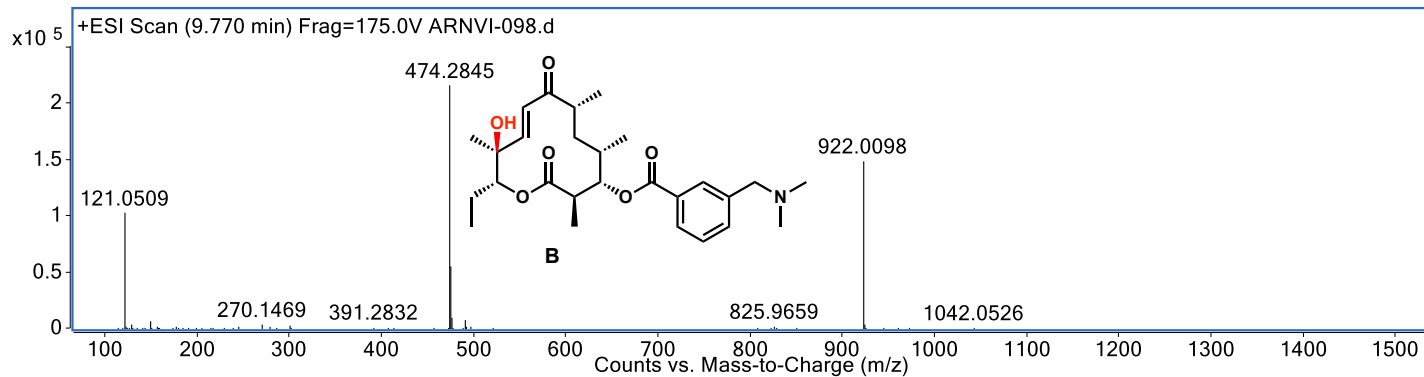
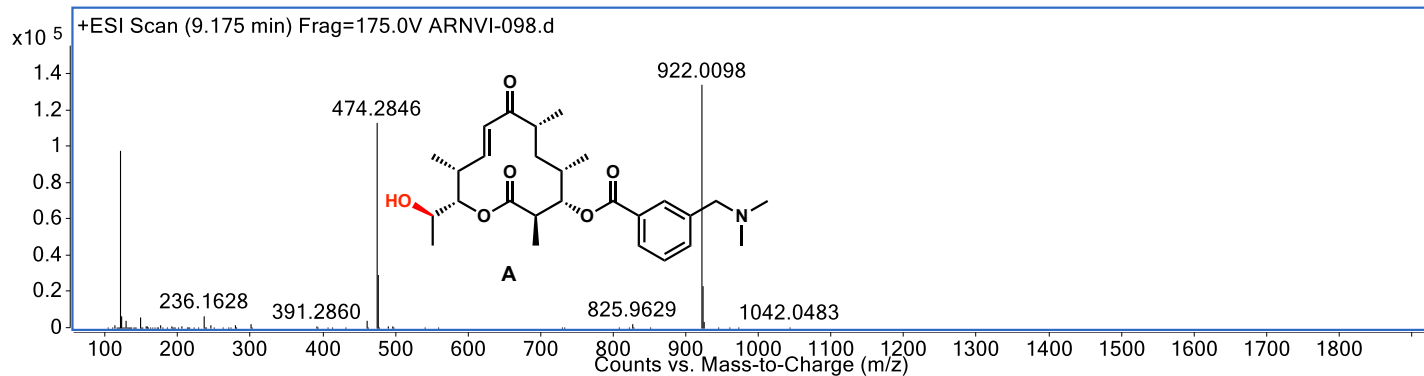
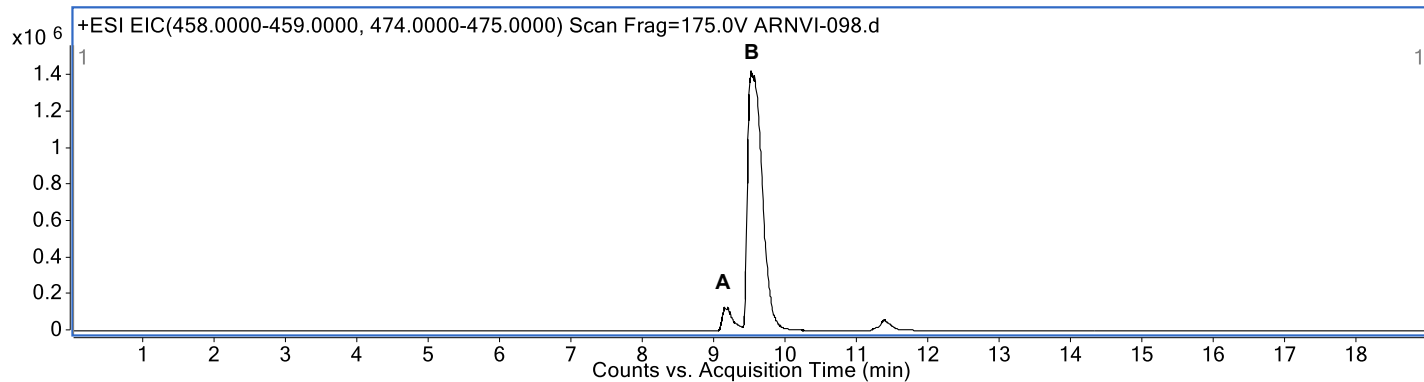
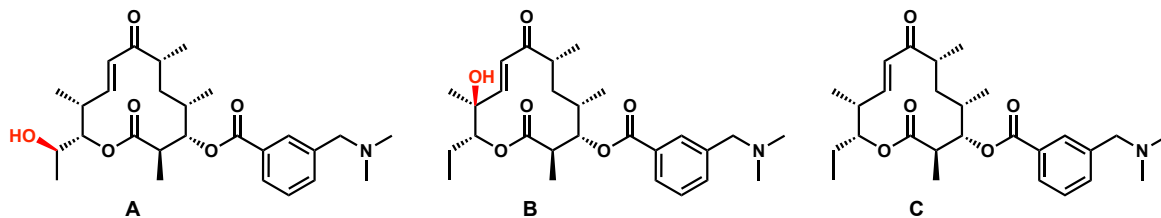
A. LC-MS traces of enzymatic reactions

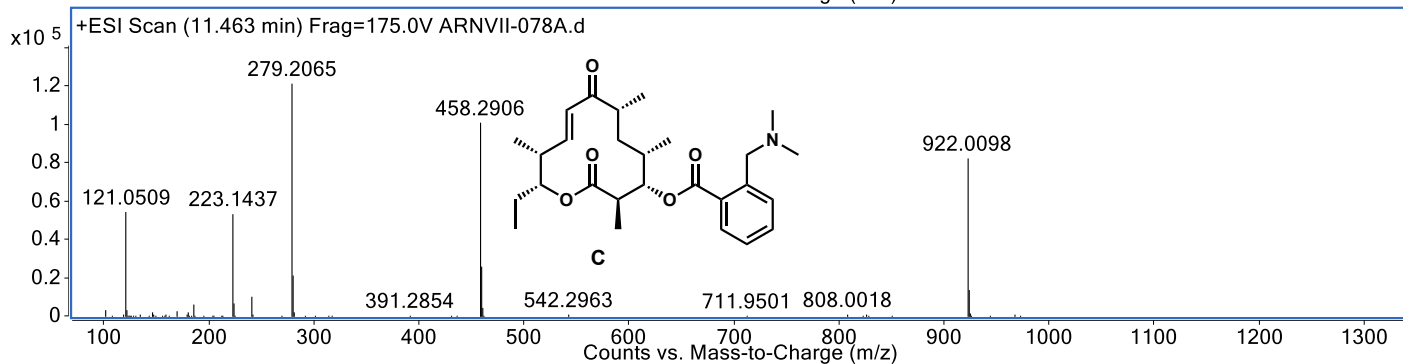
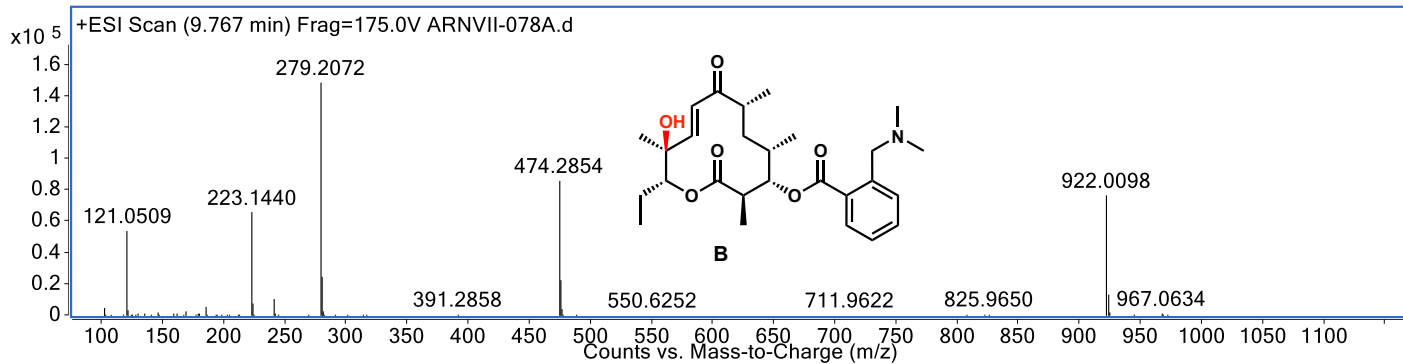
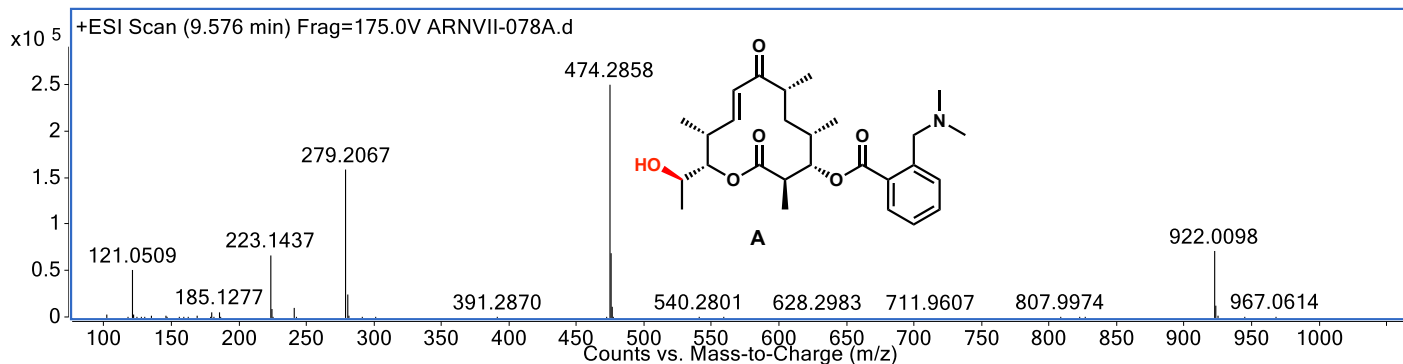
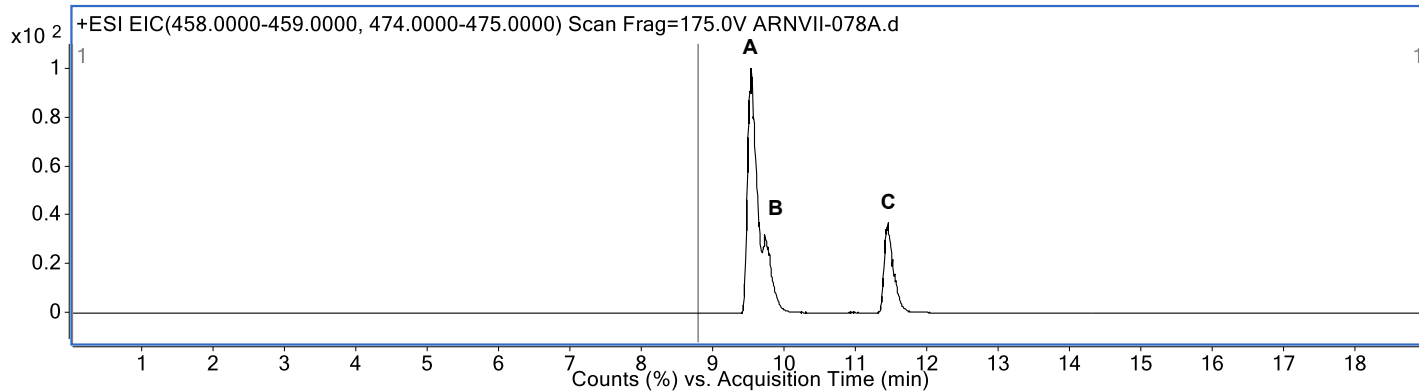
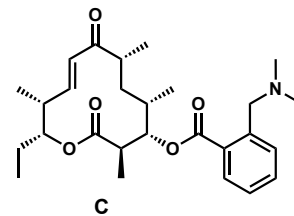
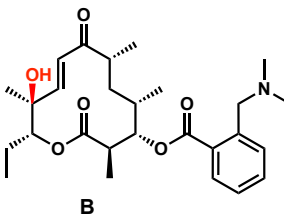
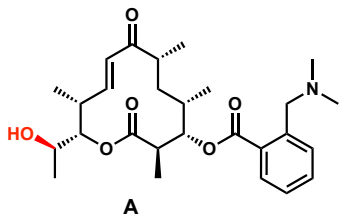


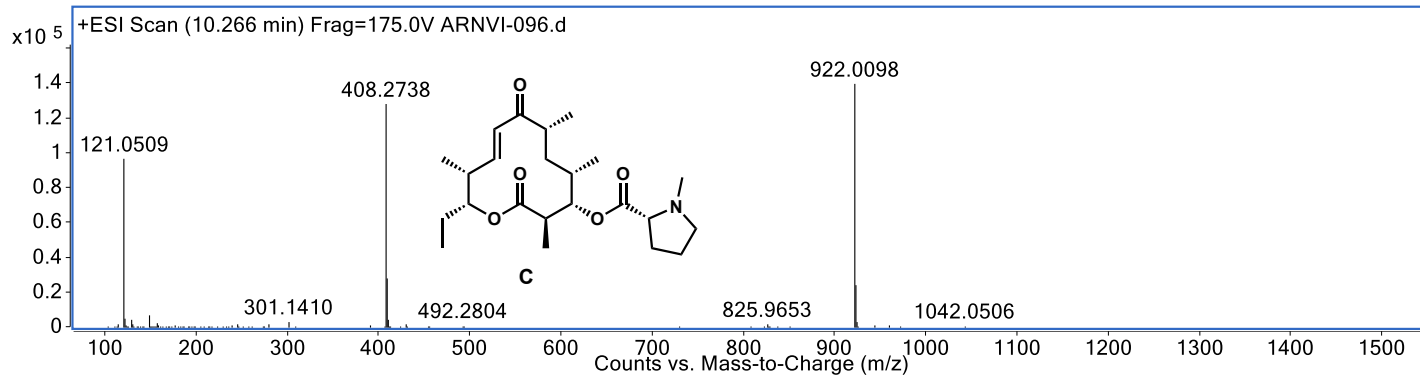
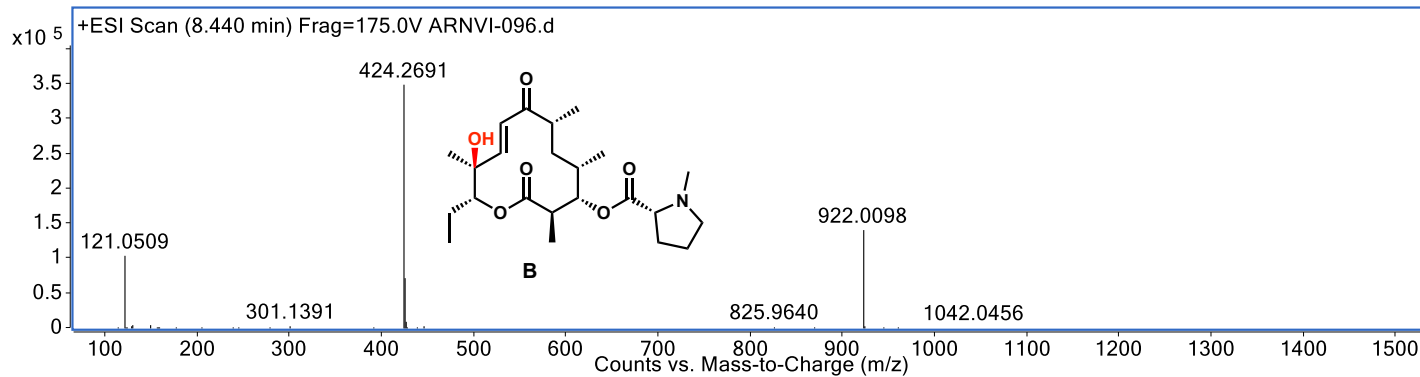
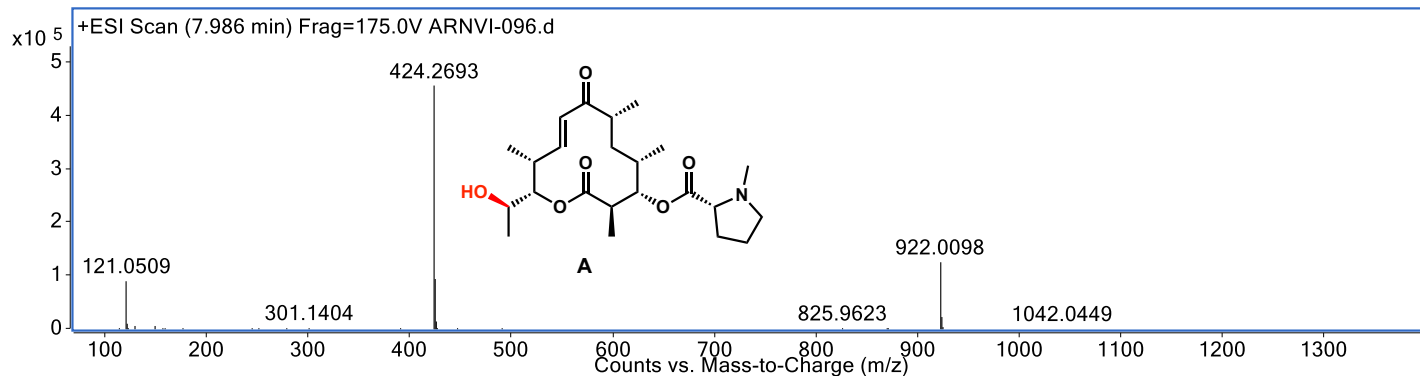
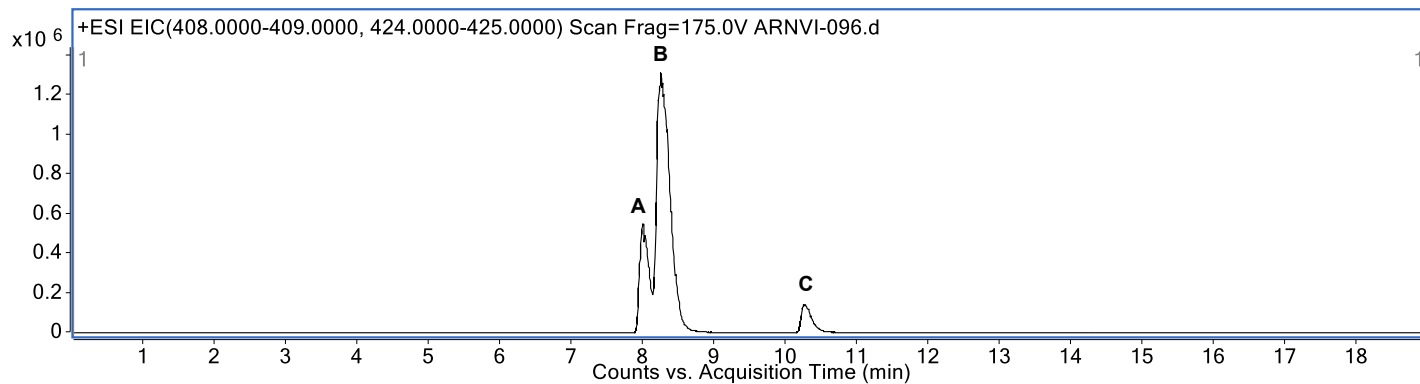
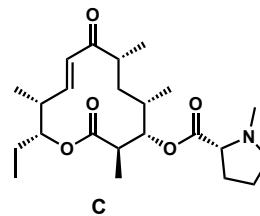
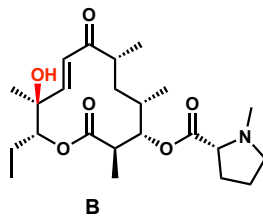
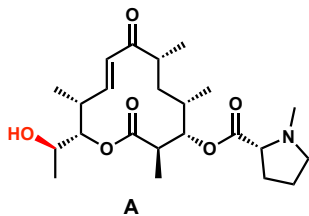


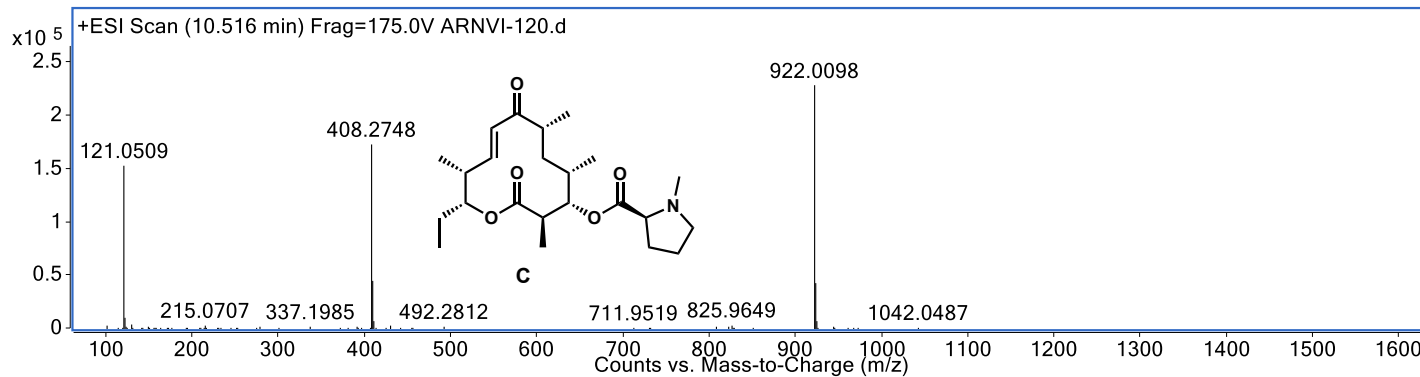
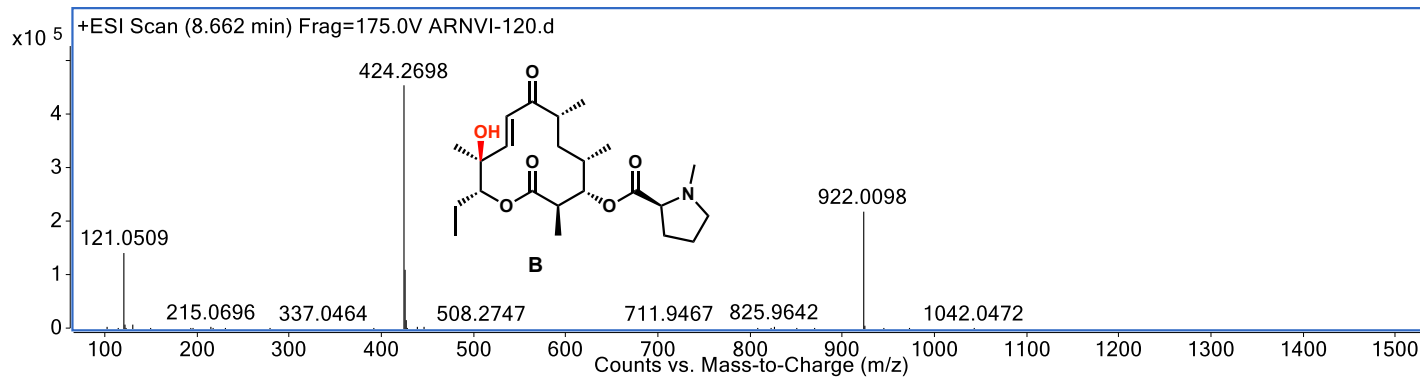
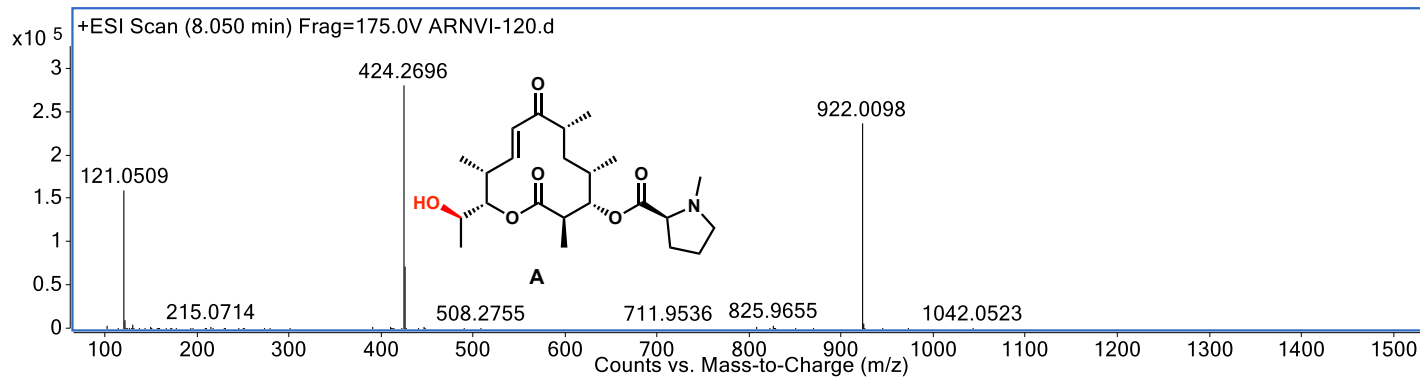
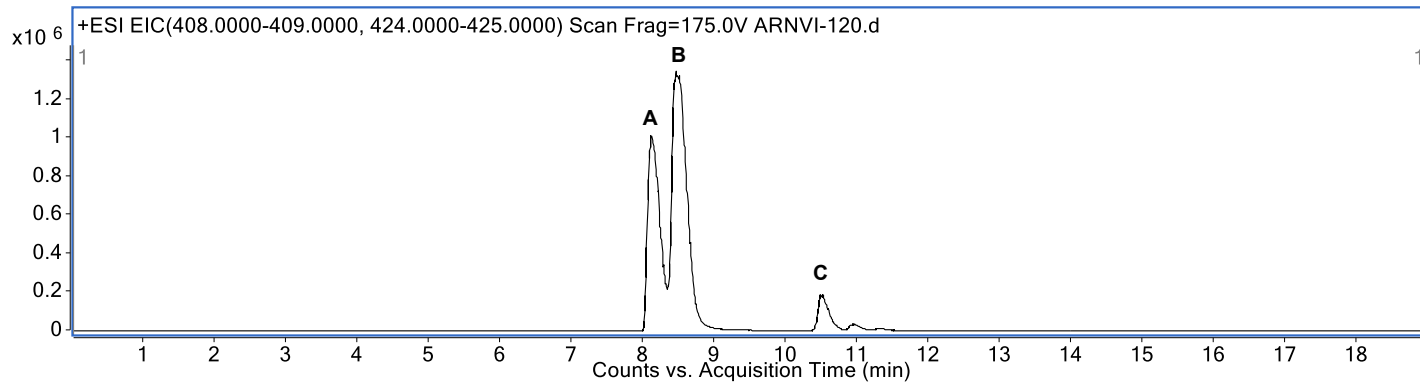
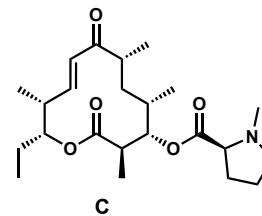
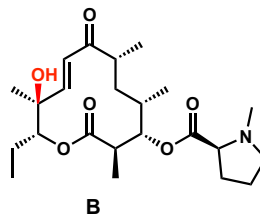
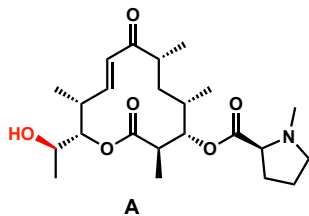








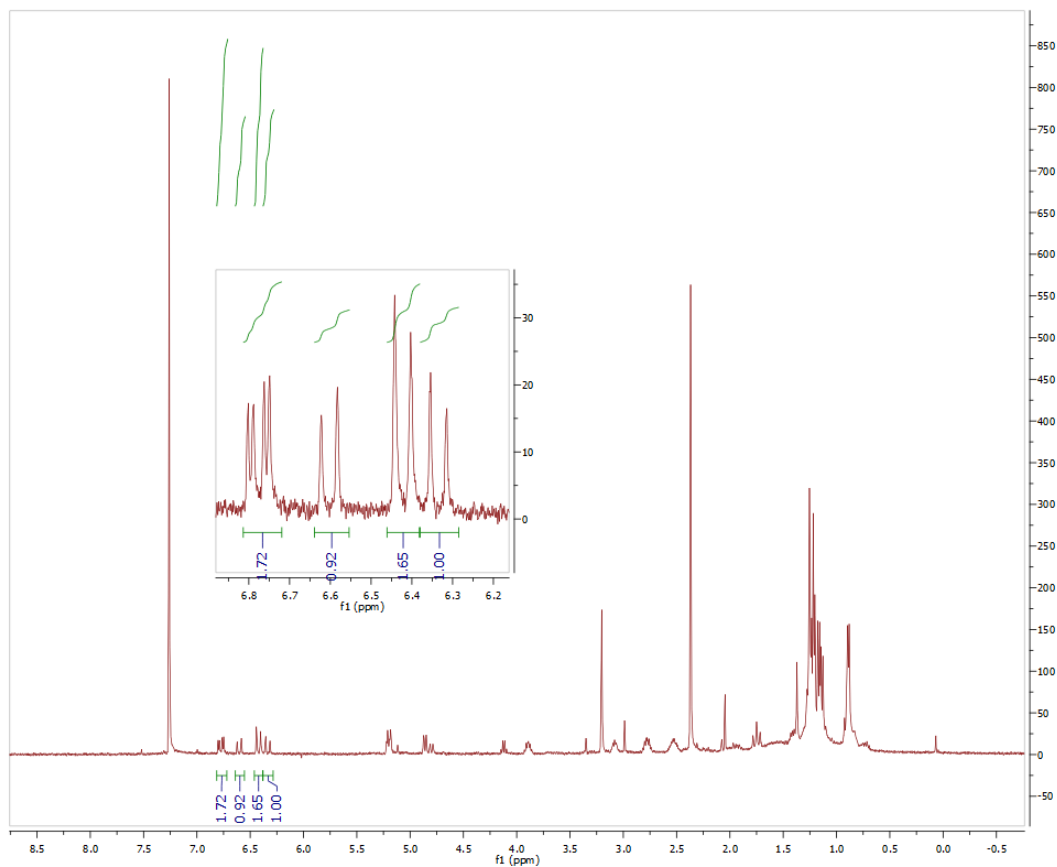




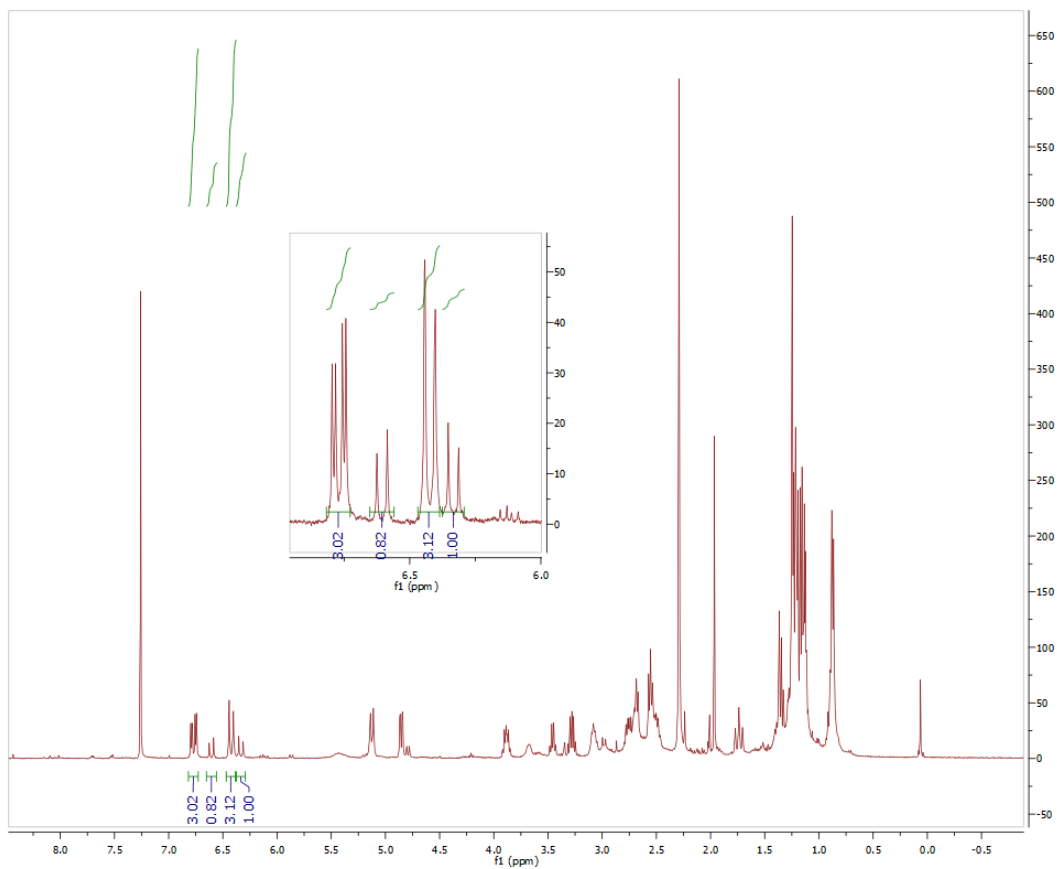
B. ^1H NMR data for pre-hydrolysis enzymatic reactions

Product ratios from LC-MS analysis were confirmed by comparison of signals corresponding to alkene protons C8 and C9 for both monohydroxylated products.

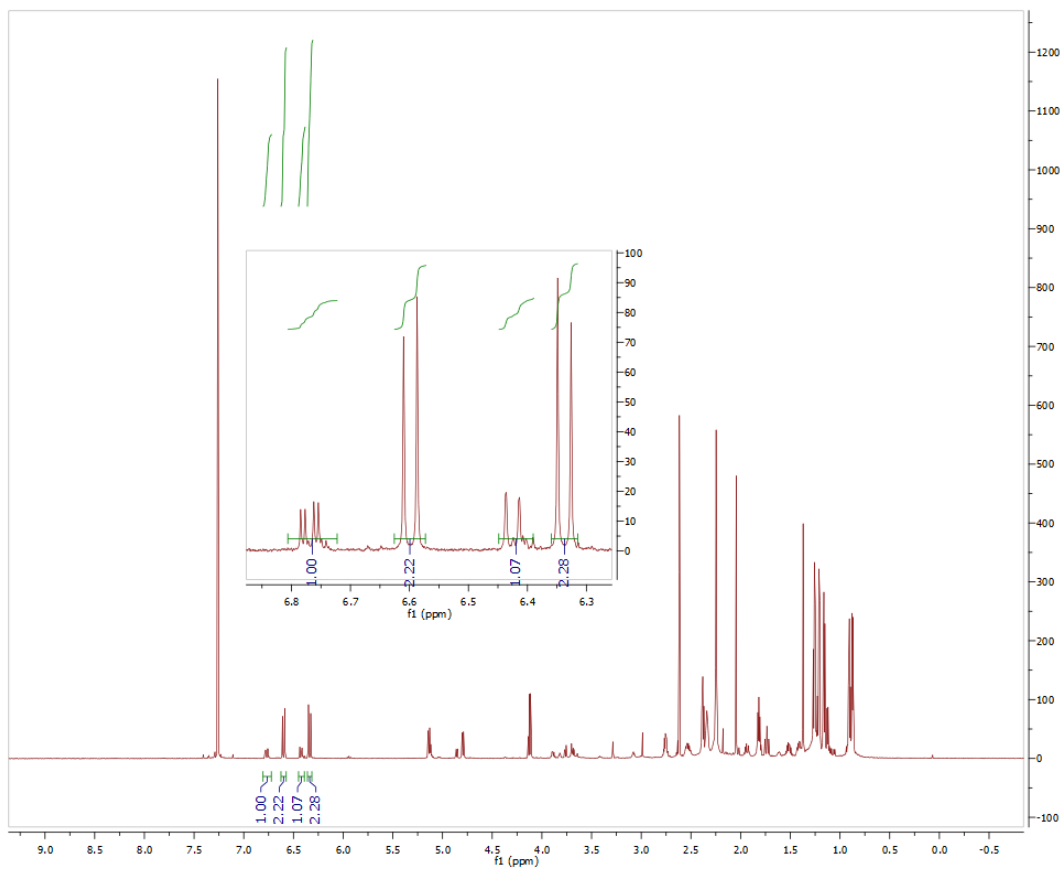
Post-column chromatography mixture of products of enzymatic reaction of substrate **8a**



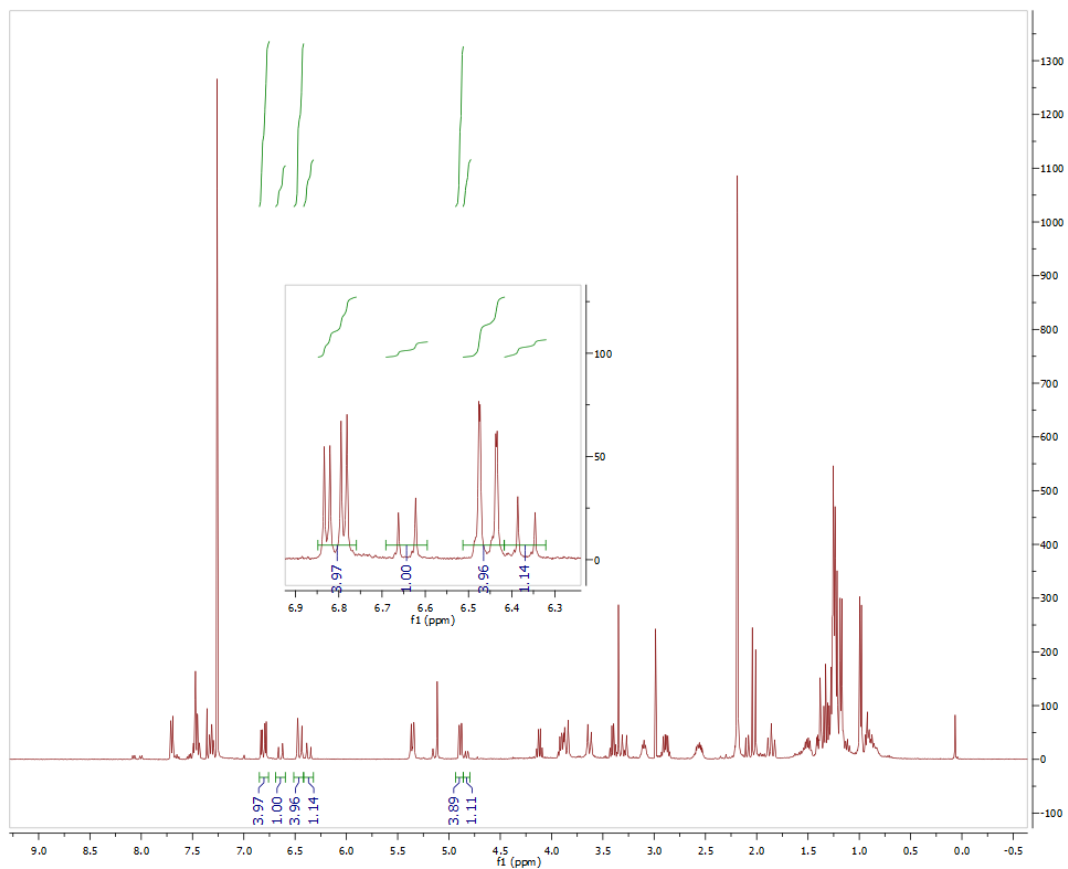
Post-column chromatography mixture of products of enzymatic reaction of substrate **8b**



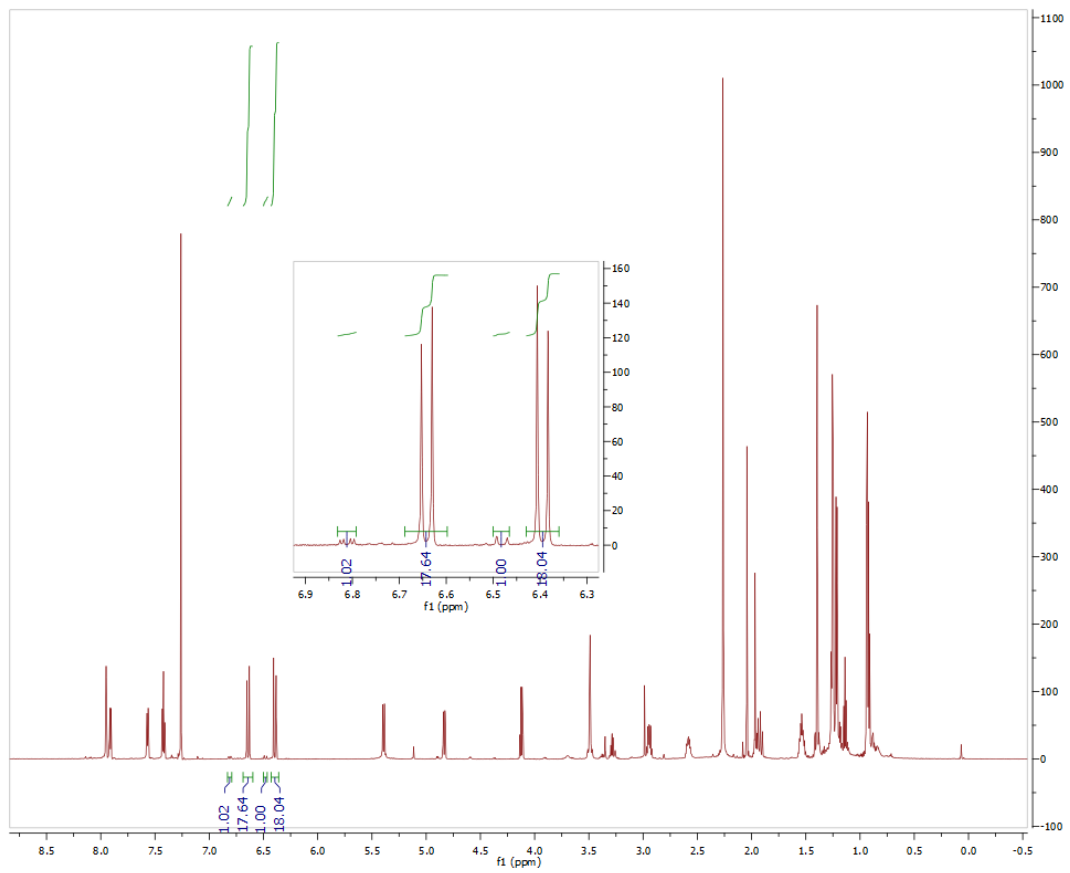
Post-column chromatography mixture of products of enzymatic reaction of substrate **10**



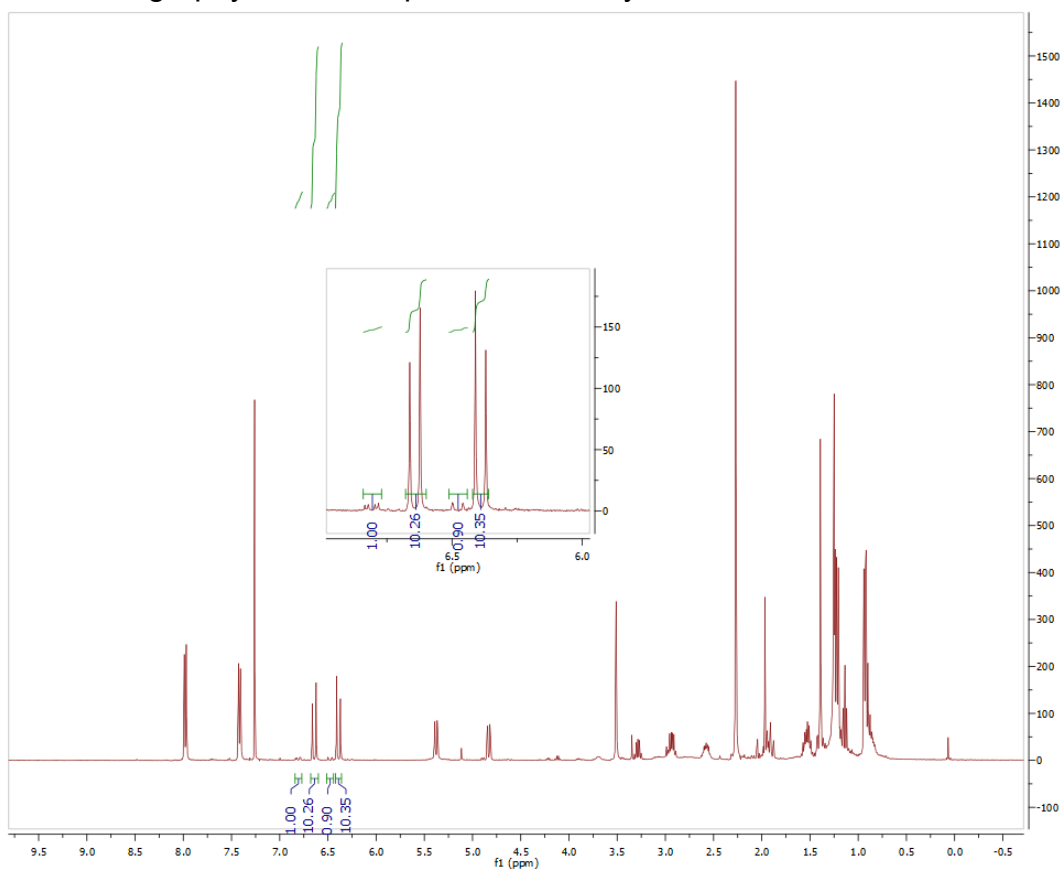
Post-column chromatography mixture of products of enzymatic reaction of substrate **14**



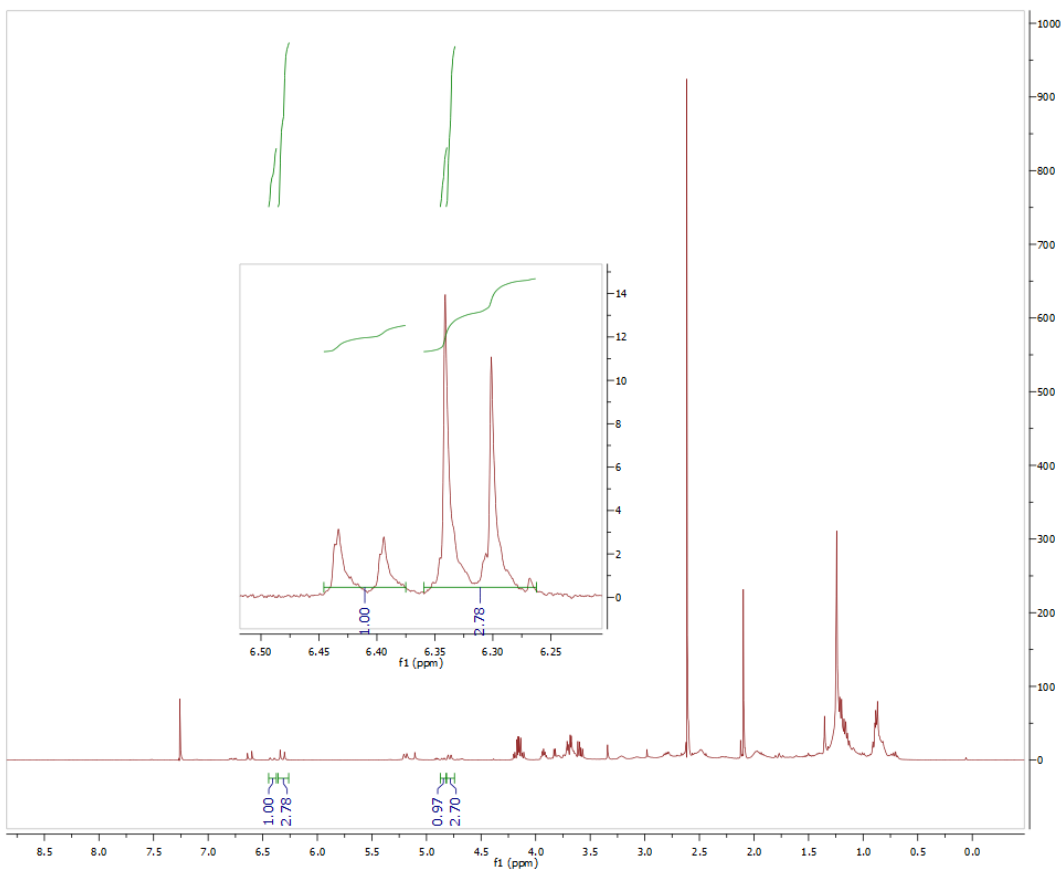
Post-column chromatography mixture of products of enzymatic reaction of substrate **16a**



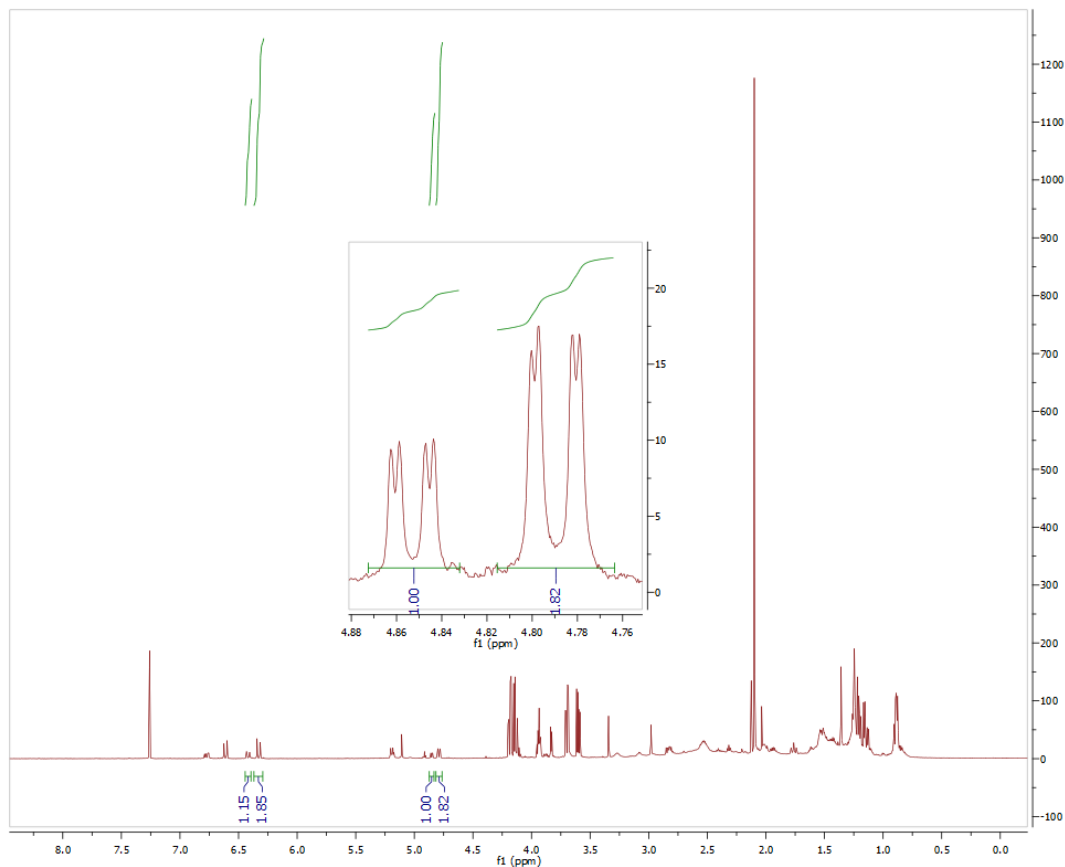
Post-column chromatography mixture of products of enzymatic reaction of substrate **16b**



Post-column chromatography mixture of products of enzymatic reaction of substrate **12a**



Post-column chromatography mixture of products of enzymatic reaction of substrate **12b**



Part III: Product characterization

A. Hydrolysis of enzymatic product mixtures:

Rapid analysis of the enzymatic reactions was enabled by the correlation of hydrolysis products of the 10-dml amino esters to known natural products methynolide (**19**) and neomethynolide (**18**, Fig. S1). Preparative-scale enzymatic reactions were carried out for each of the 10-dml analogs. The product mixtures from each reaction were subjected to hydrolysis conditions to remove anchoring groups. The hydroxylated products were compared to the authentic standards of methynolide (**19**) and neomethynolide (**18**). These macrolactones were employed in this study as authentic standards to determine sites of hydroxylation, and have been previously described as products of *Streptomyces venezuelae* ATCC 15439, through chemical degradation of methymycin and neomethymycin, and by total synthesis.

General Hydrolysis Procedure: The PikC_{D50N}-RhFRED product mixture was dissolved in a 4:1 mixture of MeOH:CHCl₃ (0.015 M) and 2.5 equiv of potassium carbonate were added. The reaction mixture was allowed to stir at rt for 72 h or until all starting material had been consumed. Additional portions of potassium carbonate were added to accelerate reaction progress as necessary. The reaction mixture was concentrated under reduced pressure, quenched by addition of saturated ammonium chloride and extracted thrice with portions of EtOAc. The combined organic layers were dried over Na₂SO₄, and filtered through a plug of silica gel and concentrated under reduced pressure. The products were analyzed by ¹H NMR and matched data reported in the literature for methynolide and neomethynolide.⁶⁻⁷ For substrates **8a**, **8b**, **10** and **14**, the ratio of the two components depended on the length of the ester anchoring group in the substrate. Hydrolysis of substrates **16a** and **16b** afforded almost exclusively methynolide.

The ^1H NMR spectra stacked plot (Fig. S1) showed signals corresponding to alkene protons (C-8/C-9), while the characteristic doublet of doublets from C-9 disappeared following PikC catalyzed oxidation at the C-10 tertiary allylic position observed in the methynolide analog. In the neomethynolide analog, the C-9 signal remained as a doublet of doublets. The C-8 signal corresponded to the C-H adjacent to the C-7 carbonyl group and was split into a doublet in both methynolide and neomethynolide. This strategy was effective for the analysis of products from compounds **8a-10** and **14-16**; however, hydrolysis of the proline anchoring groups from **12a** and **12b** proved challenging and the product mixtures were characterized with the anchoring groups in place.

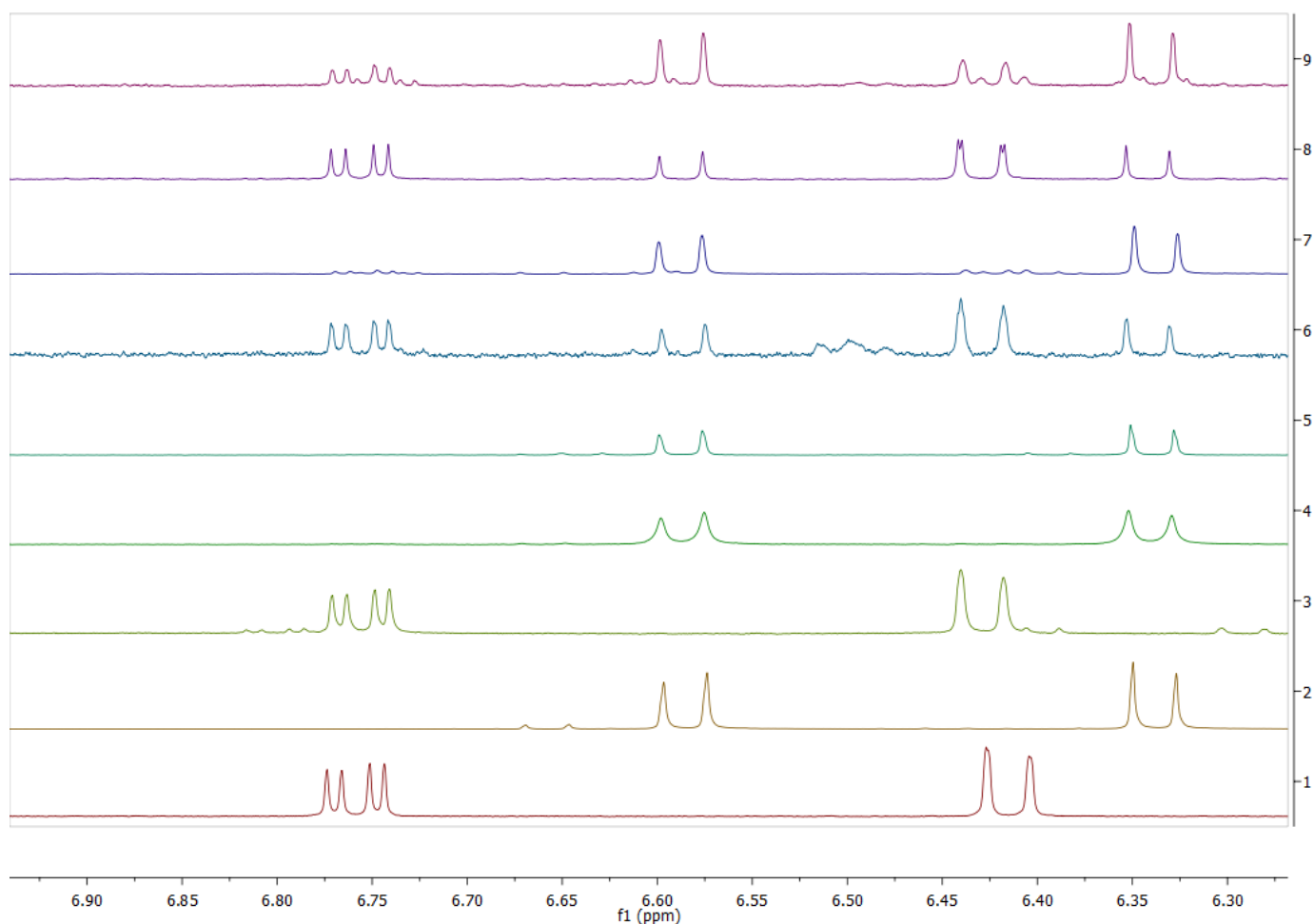
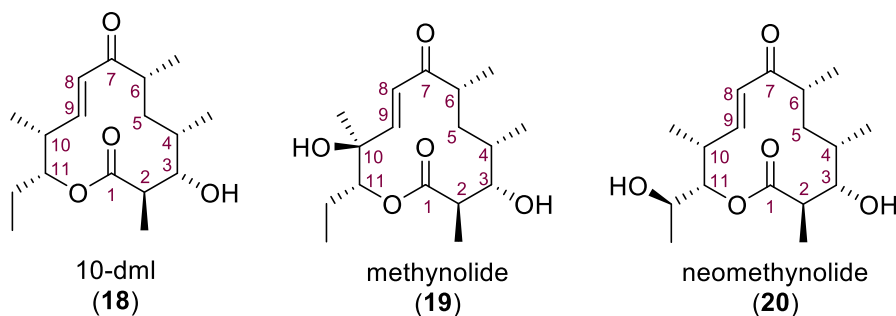
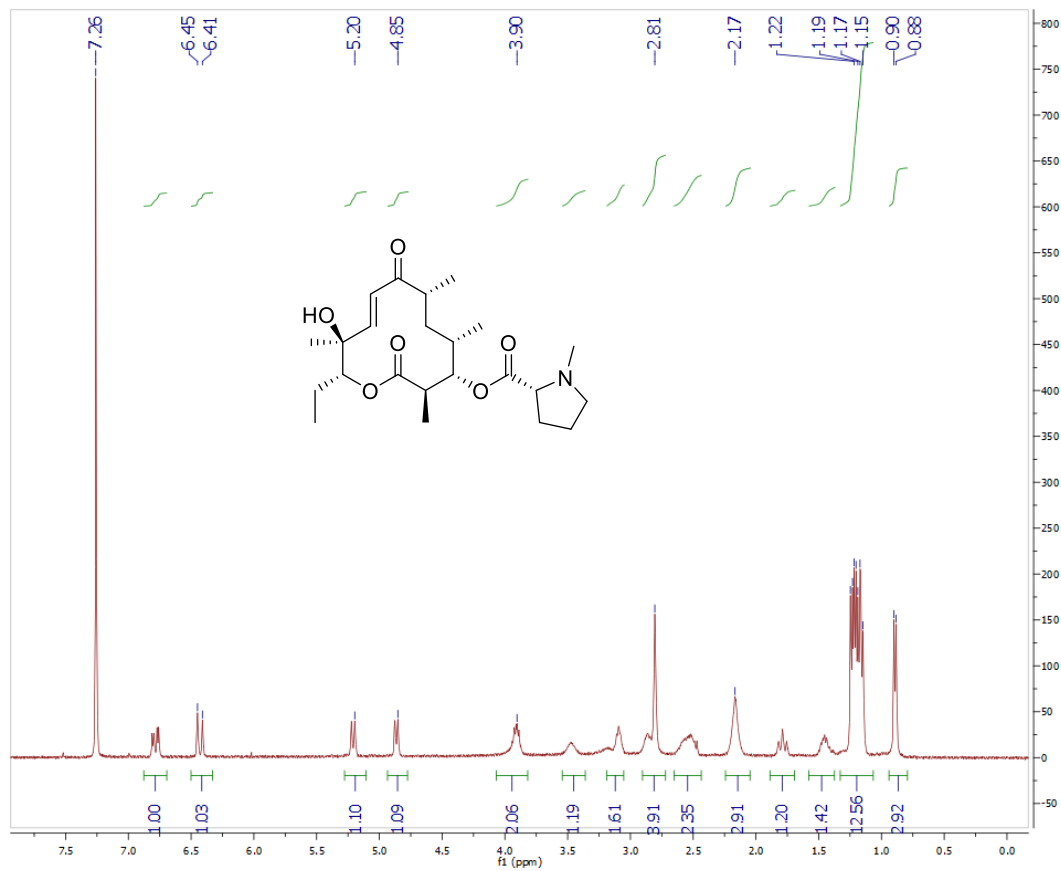
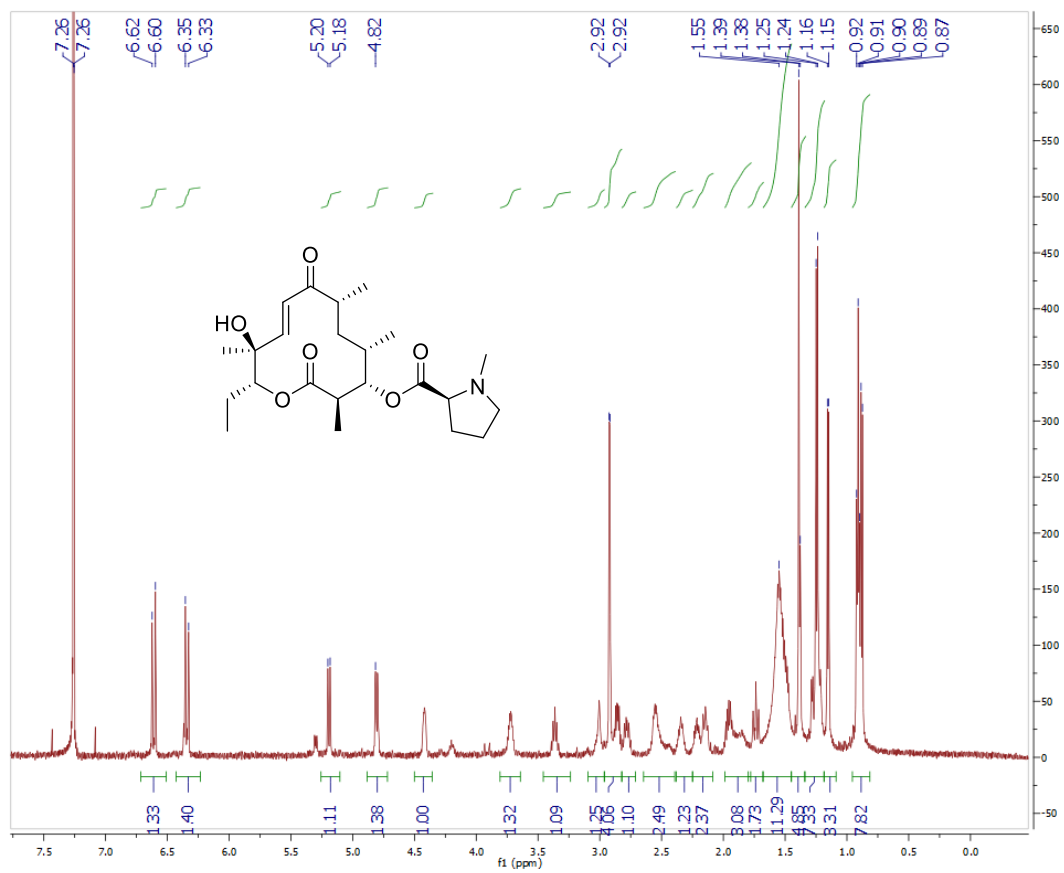


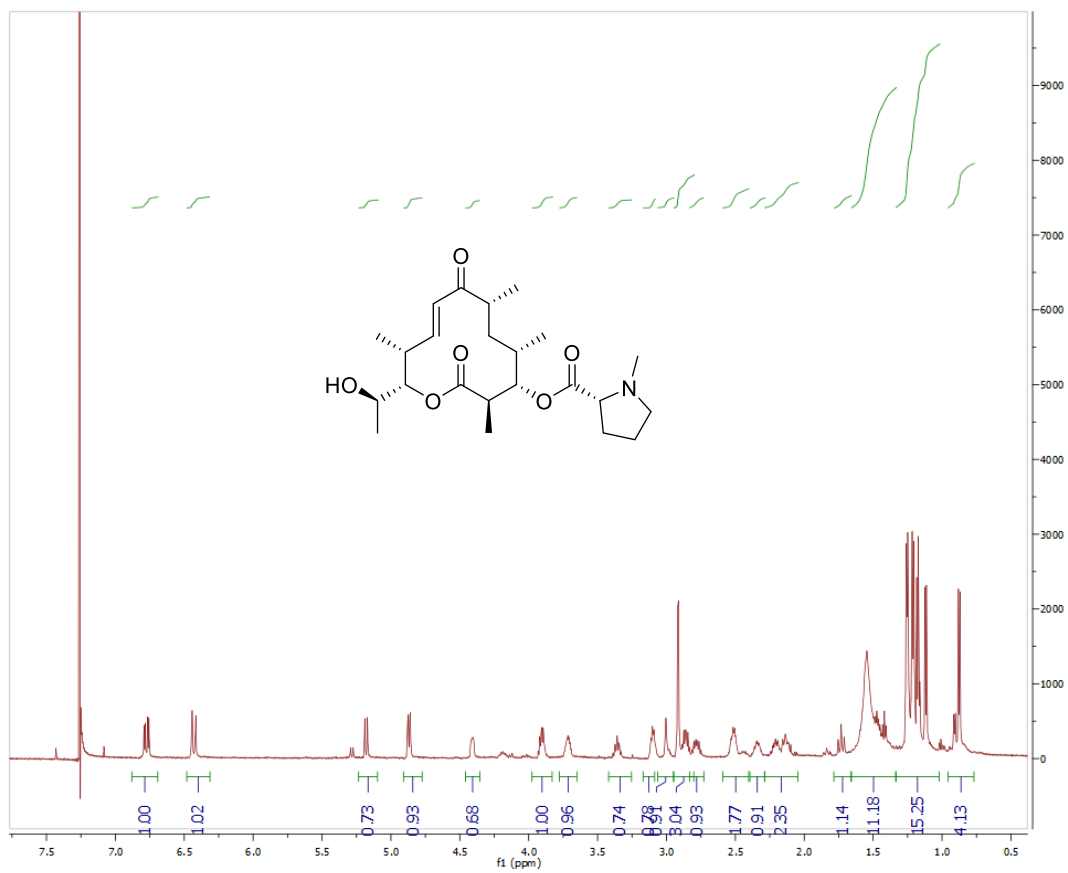
Figure S1. Stacked plot of ^1H NMR of hydrolyzed reaction products from 10-dml analogs **8a-10 and **14-16**.** From bottom to top (1 to 9): 1. 10-deoxymethynolide, 2. Methynolide, 3. Neomethynolide, 4. Hydrolyzed Pik_{D50N} -RhFRED Reaction Products from substrate **16a**, 5. Hydrolyzed Pik_{D50N} -RhFRED Reaction Products from substrate **16b**, 6. Hydrolyzed Pik_{D50N} -RhFRED Reaction Products from substrate **14**, 7. Hydrolyzed Pik_{D50N} -RhFRED Reaction

Products from substrate **10**, 8. Hydrolyzed PikC_{D50N}-RhFRED Reaction Products from substrate **8b**, 9. Hydrolyzed PikCD50N-RhFRED Reaction Products from substrate **8a**. From left to right: C₉-H signal, C₈-H signal.

B. HPLC separation of hydroxylated products from substrates 12a and 12b:







Part IV. Crystallization, data collection and structure determination

Prior to crystallization, the PikC and PikC_{D50N} protein stocks solutions stored at -80°C were diluted to 0.2 mM in 10 mM Tris-HCl, pH 7.5 buffer supplemented with 1 mM compound of interest. Crystallization conditions in each case were determined using commercial high-throughput screening kits available in deep-well format (Hampton Research), a nanoliter drop-setting Mosquito robot (TTP LabTech) operating with 96-well plates, and a hanging drop crystallization protocol. Crystals were further optimized in 96-well or 24-well plates for diffraction data collection. Prior to data collection, all crystals were cryo-protected by plunging them into a drop of reservoir solution supplemented with 20-25% ethylene glycol or glycerol, then flash frozen in liquid nitrogen. Diffraction data were collected at 100-110 K at beamline 8.3.1, Advanced Light Source, Lawrence Berkeley National Laboratory, USA. Data indexing, integration, and scaling were conducted using MOSFLM and the programs implemented in the ELVES software suite.⁸ The crystal structures were initially determined by molecular replacement using the structures of PikC (PDB ID 2BVJ) as search models. The final structures were built using COOT and refined using REFMAC5 (Collaborative Computational Project, 1994; software.⁹⁻¹¹ Data collection and refinement statistics are shown in Table S1.

Protein	PikC _{D50N}	PikC _{D50N}
Ligand	018C (7)	Sn-263 (8)
PDB ID	3ZK5	4B7S
Data collection		
Space group	P2 ₁	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	60.4, 92.4, 69.3	61.3, 92.0, 69.6
α , β , γ (°)	90, 90.3, 90	90, 90.5, 90
Molecules in AU	2	2
Wavelength	1.11587	1.11587
Resolution (Å)	1.89	1.84
<i>R</i> _{sym} or <i>R</i> _{merge} (%)	11.4 (63.7)	9.2 (61.3)
<i>I</i> / σ <i>I</i>	6.7 (1.5)	8.3 (1.5)
Completeness (%)	89.8 (84.1)	94.4 (69.3)
Redundancy	3.7 (3.6)	3.9 (3.1)
Crystallization conditions	20% PEG 3350 0.2 M MgCl ₂	2 M ammonium sulfate 0.1 M Bis-Tris, pH 5.5
Refinement		
No. reflections	51515	59932
<i>R</i> _{work} / <i>R</i> _{free} (%)	19.7/26.7	16.6/22.4
No. atoms		
Protein	6157	6212
Heme	86	86
Substrate	54	56
Solvent	524	809
Mean B value	21.2	22.6
<i>B</i> -factors		
Protein	20.9	21.5
Heme	13.5	13.1
Substrate	24.8	24.1
Solvent	27.1	33.4
R.m.s deviations		
Bond lengths (Å)	0.021	0.022
Bond angles (°)	1.928	1.847

¹Values in parentheses are for highest-resolution shell.

Table S1. Crystallographic Data and Statistics.

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- (11) Collaborative Computational Project, Number 4 *Acta Crystallogr. D* **1994**, *50*, 760.