Supporting Information

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

Solymar Negretti‡,[1] Alison R. H. Narayan‡,[2] Karoline C. Chiou,[3] Petrea M. Kells,[4] Jessica L. Stachowski,[3] Douglas A. Hansen,[1,2] Larissa M. Podust,[4*] John Montgomery,[1,3*] David H. Sherman[1,2,3,5*]

[1]*Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109*

[2]*Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109*

[3]*Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109*

[4]*Department of Pathology and Center for Discovery and Innovation in Parasitic Diseases, University of California, San Francisco, California 94158*

[5]*Department of Microbiology & Immunology, University of Michigan, Ann Arbor, Michigan 48109*

‡Denotes equal contribution

[**]To whom correspondence should be addressed. Email: davidhs@umich.edu, jmontg@umich.edu, larissa.podust@ucsf.edu

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 precoated plates (0.25 mm) which were visualized using UV light, *p*-anisaldehyde, $KMnO_4$, 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. ^{1}H and ^{13}C spectra were obtained in $CDCl_3$ 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 ^{1}H 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 ^{13}C NMR spectra were recorded in ppm from the central peak of $CDCl_3$ (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:

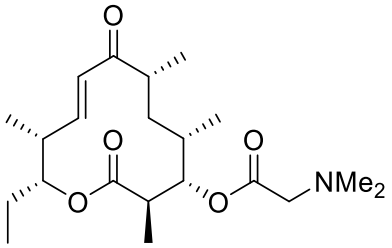
10-deoxymethynolide (10-dml): A mutant strain of *S. venezuelae* ATCC 15439 in which *des1* was knocked out was grown in 1 L of media containing 20 g glucose, 15 g soybean flour, 5 g $CaCl_2$, 1 g NaCl, 2 mg $CoCl_2 \bullet 6H_2O$, pH = 7.2. After 72 h, the culture was extracted with chloroform (3x). The organic layers were combined, washed with brine, dried over Na_2SO_4 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 4-((dimethylamino)methyl)benzoic acid were synthesized according to the procedure described by Wang et al.[1]

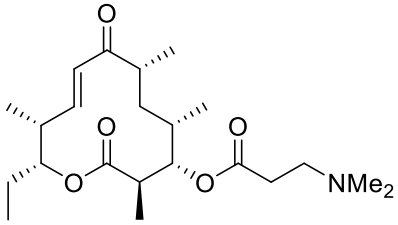
2-((dimethylamino)methyl)benzoic acid: A 40 wt % solution of $HNMe_2$ in H_2O (10 equiv) was added to 2-((bromo)methyl)benzoic[2] acid (1 equiv) in EtOH (0.8 M). The reaction mixture was stirred at ambient temperature for 12 h at which point the mixture was concentrated and partitioned between EtOAc and 1N HCl. The aqueous layer was basified to pH 8-9 with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc (3x). The organic layers were combined, dried over Na_2SO_4, 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.

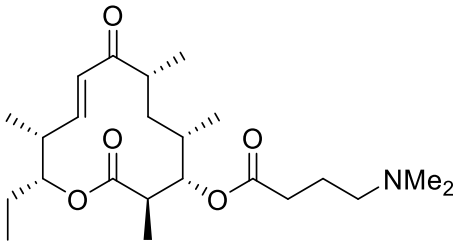


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 2-(dimethylamino)acetate (8a): 110 mg of 10-deoxymethynolide (0.35 mmol, 1.0 equiv) were dissolved in 5.0 mL of dichloromethane along with 74 mg *N*,*N*-dimethylglycine (0.53 mmol, 1.5 equiv), 84 µL of 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 $NaHCO_3$ 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**. ^{1}H NMR (500 MHz, $CDCl_3$) 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); ^{13}C NMR (175 MHz, $CDCl_3$) 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.

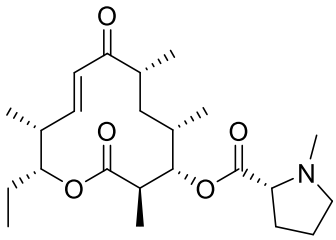


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-(dimethylamino)propanoate (8b): 29 mg of 10-deoxymethynolide (0.098 mmol, 1.0 equiv) were dissolved in 2.0 mL of dichloromethane along with 23 mg 3-*N,N*-dimethylaminopropanoic acid hydrochloride (0.15 mmol, 1.5 equiv), 23 µL 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 $NaHCO_3$ 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**. ^{1}H NMR (500 MHz, $CDCl_3$) 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); ^{13}C NMR (125 MHz, $CDCl_3$) 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.

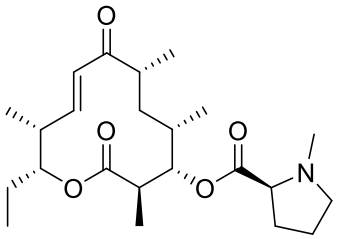


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-(dimethylamino)butanoate (10): Following the procedure employed to synthesize **8b**, 10-dml (100 mg, 0.34 mmol), 3-*N*,*N*-dimethylaminobutyric acid hydrochloride (88 mg, 0.51 mmol), triethylamine (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). ^{1}H NMR (400 MHz, $CDCl_3$) 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); ^{13}C NMR (175 MHz, $CDCl_3$) 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.

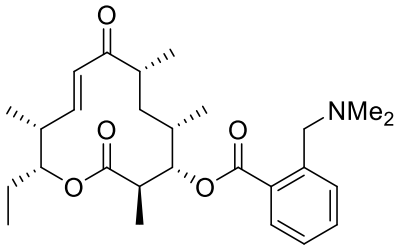


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate (12a): 10-dml (40 mg, 0.13 mmol) was dissolved in 1.8 mL of dichloromethane along with D-*N*-methylproline (26 mg, 0.20 mmol), DCC (42 mg, 0.20 mmol), DMAP (25 mg, 0.20 mmol). The 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. ^{1}H NMR (700 MHz, $CDCl_3$) 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 $[M+H]^+$ 408.2744, found 408.2738.

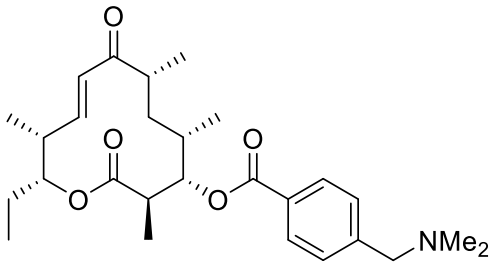


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-L-prolinate (12b): Following the procedure for (3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate, 10-dml (60 mg, 0.20 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). ^{1}H 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 $[M+H]^+$ 408.2744, found 408.2748.

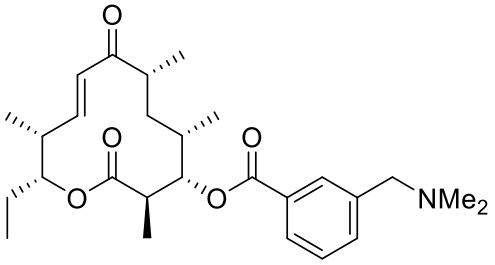


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 2-((dimethylamino)methyl)benzoate (14): 2-((dimethylamino)methyl)benzoic acid (30 mg, 0.16 mmol) was dissolved in 0.50 mL of dichloromethane. To this mixture was added one drop of *N,N*-dimethylformamide 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. ^{1}H 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, 1 H), 6.79 (dd, *J* = 15.8, 5.3 Hz, 1 H), 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 $[M+H]^+$ 458.2901, found 458.2910.

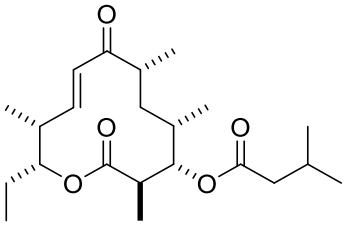


(*3R*,*4S*,*5S*,*7R*,*11R*,*12R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-((dimethylamino)methyl)benzoate (16a): 10-deoxymethynolide (40 mg, 0.14 mmol), 36 mg of 4-((dimethylamino)methyl)benzoic acid (0.20 mmol, 1.5 equiv), 42 mg DCC (0.20 mmol, 1.5 equiv) and 25 mg DMAP (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). ^{1}H 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 $[M+H]^+$ 458.2901, found 458.2911.

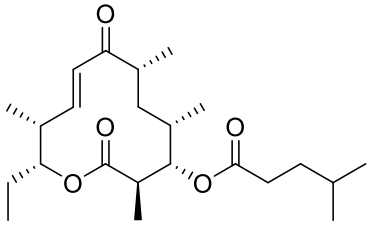


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-((dimethylamino)methyl)benzoate (16b): Following the procedure employed to synthesize **16a**, 10-deoxymethynolide (120 mg, 0.40 mmol), 110 mg of 3-((dimethylamino)methyl)benzoic acid (0.61 mmol, 1.5 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). ^{1}H 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 $[M+H]^+$ 458.2901, found 458.2911.



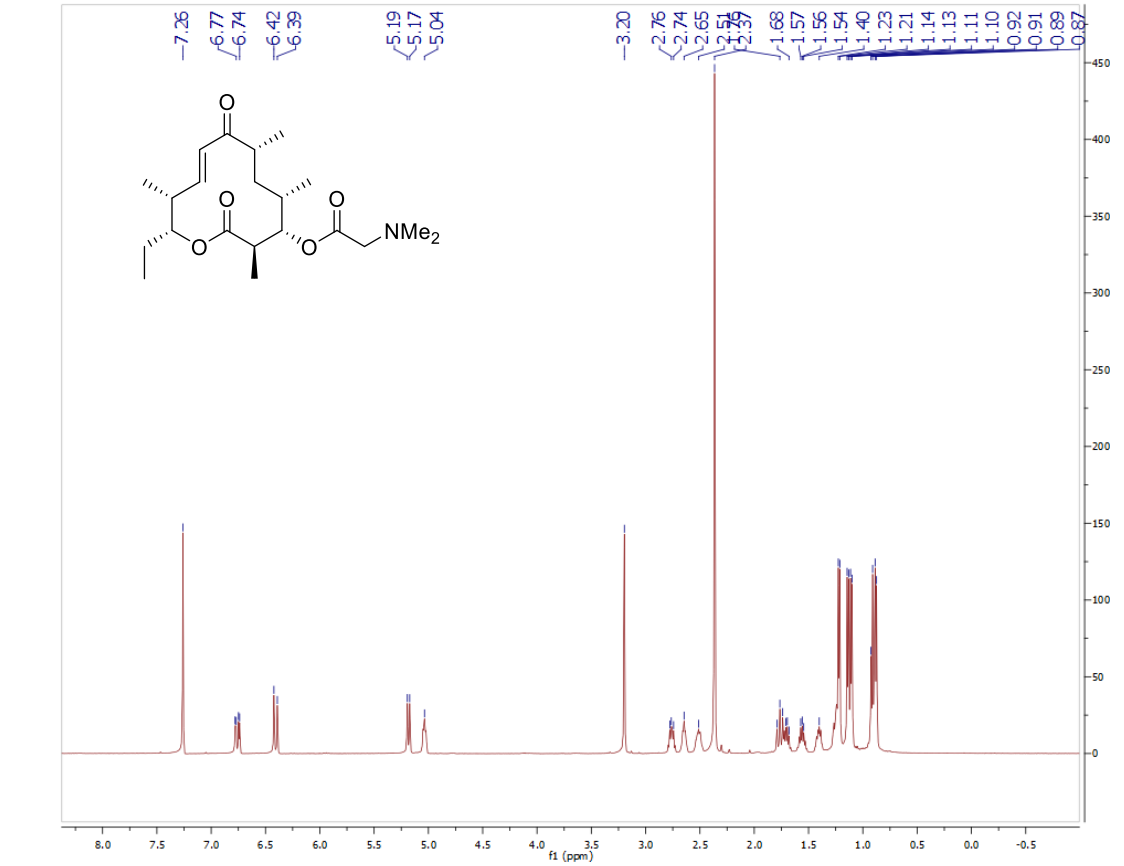
(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 3-methylbutanoate (S1): (3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate, 10-dml (6.1 mg, 0.021 mmol), DCC (6.4 mg, 0.031 mmol), 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). ^{1}H 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 $[M+H]^+$ 381.2641, found 381.2673.

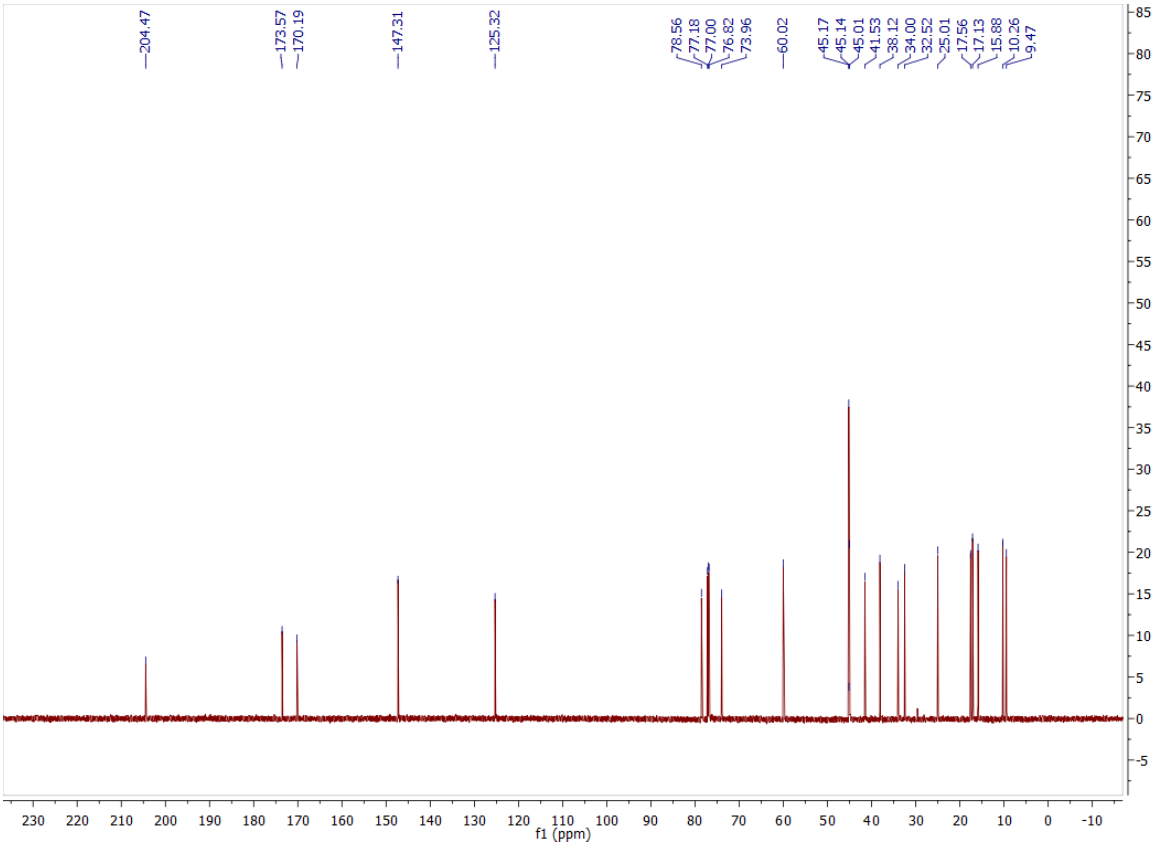


(3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-Ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl 4-methylpentanoate (S2): (3*R*,4*S*,5*S*,7*R*,11*R*,12*R*,*E*)-12-ethyl-3,5,7,11-tetramethyl-2,8-dioxooxacyclododec-9-en-4-yl *N*-methyl-D-prolinate,10-dml (11 mg, 0.038 mmol), DCC (12 mg, 0.057 mmol), DMAP (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). ^{1}H 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 $[M+H]^+$ 395.2797, found 395.2773.

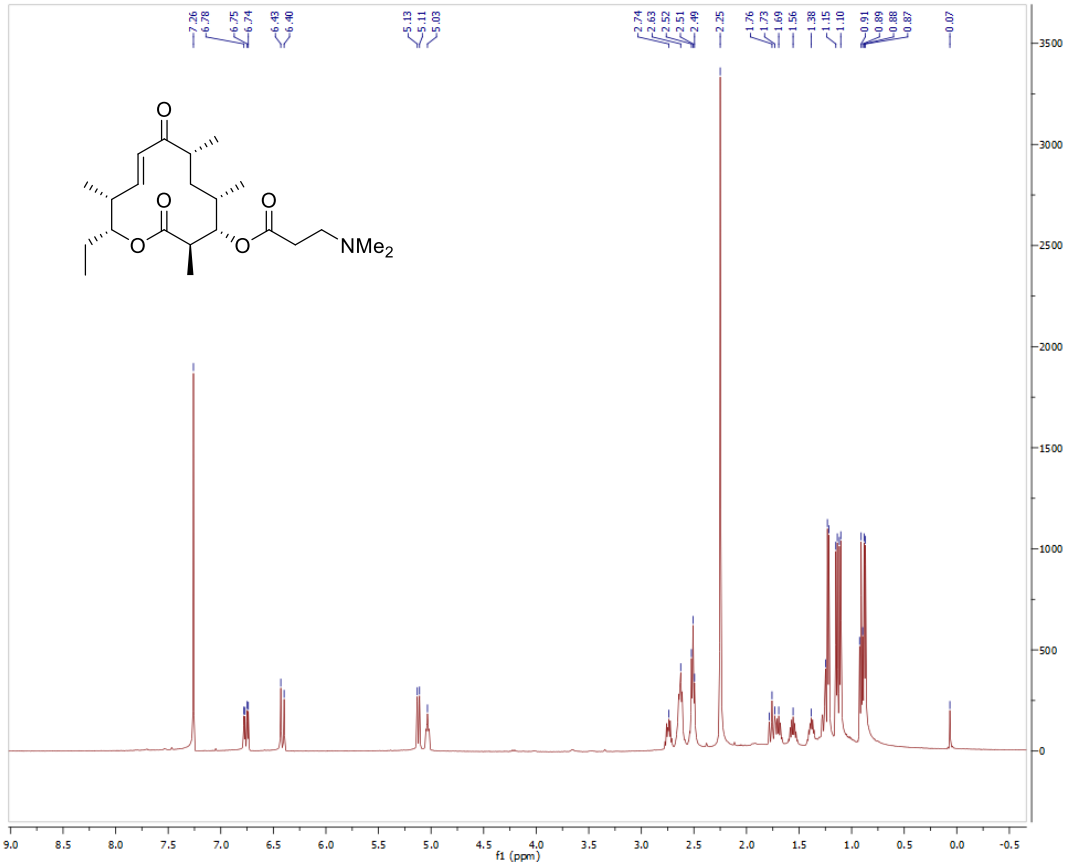
^{1}H NMR and ^{13}C NMR Spectra of substrates

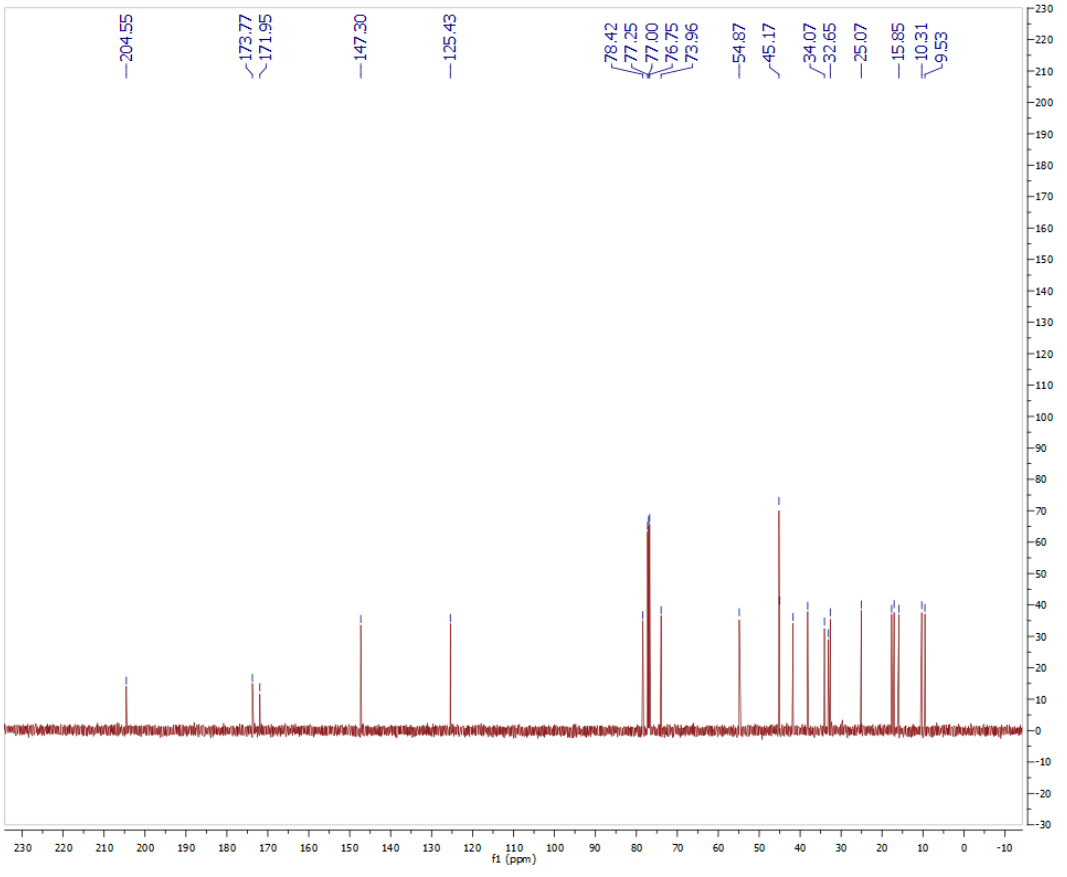
Substrate **8a**



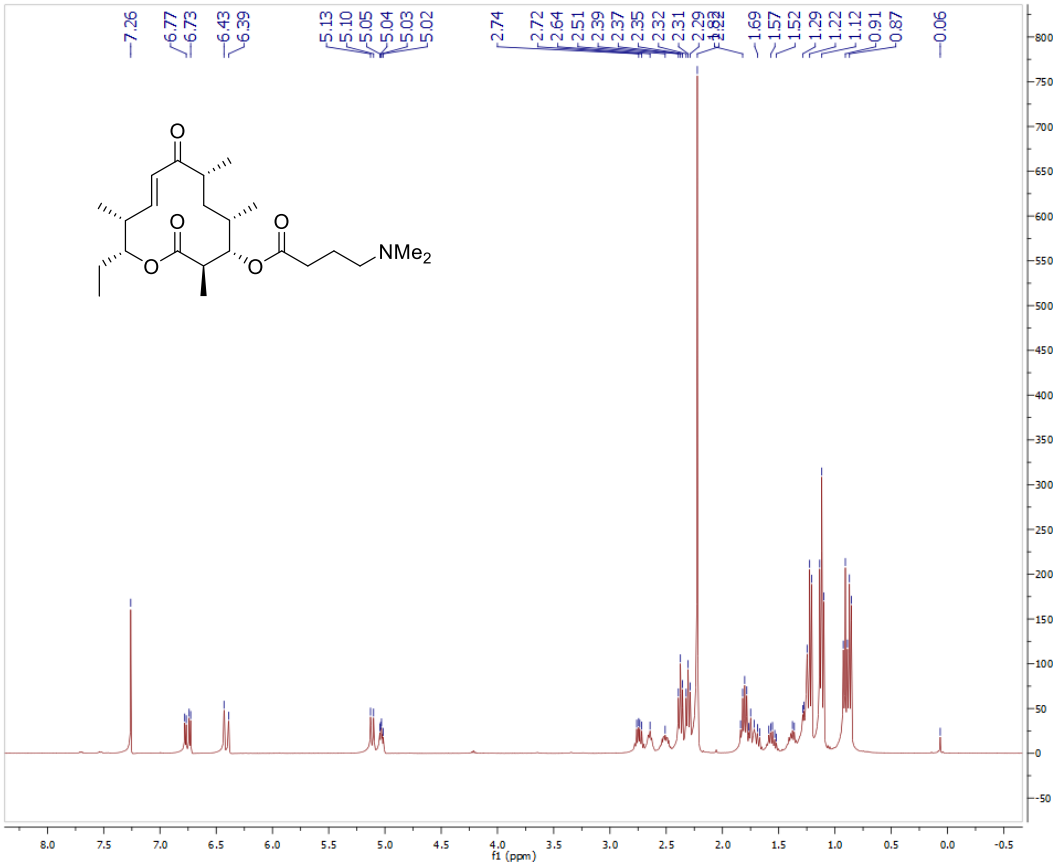


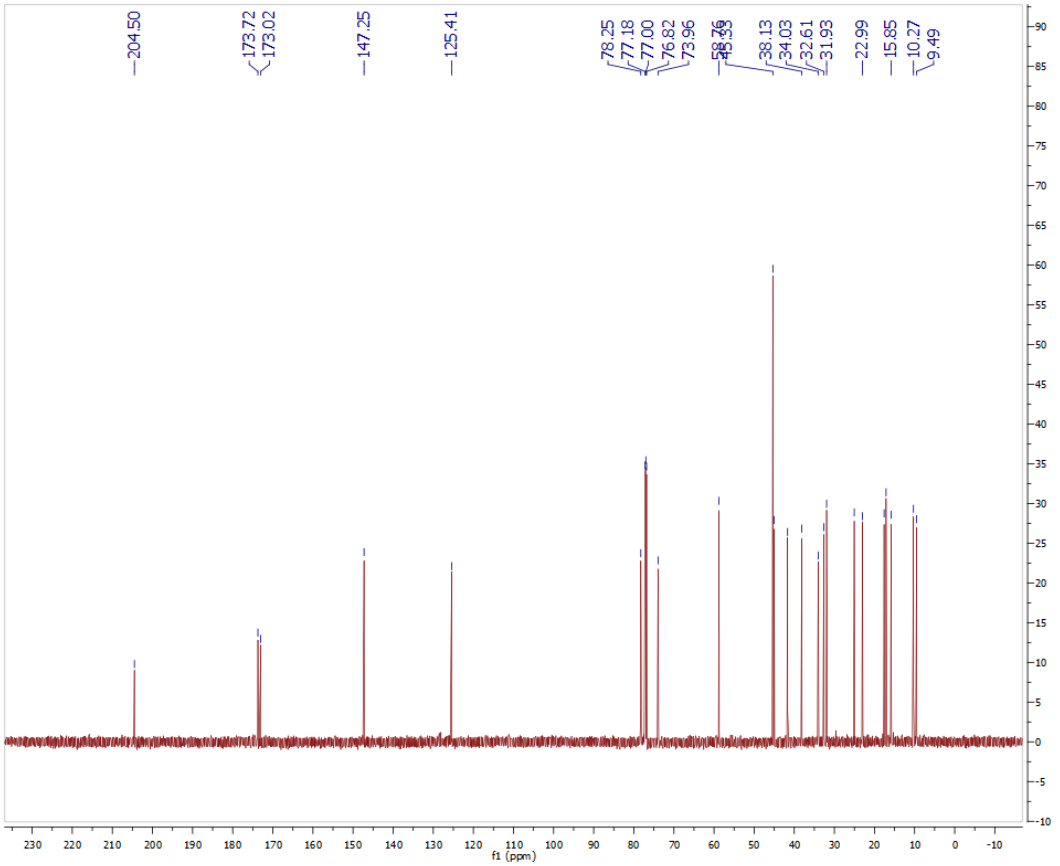
Substrate **8b**



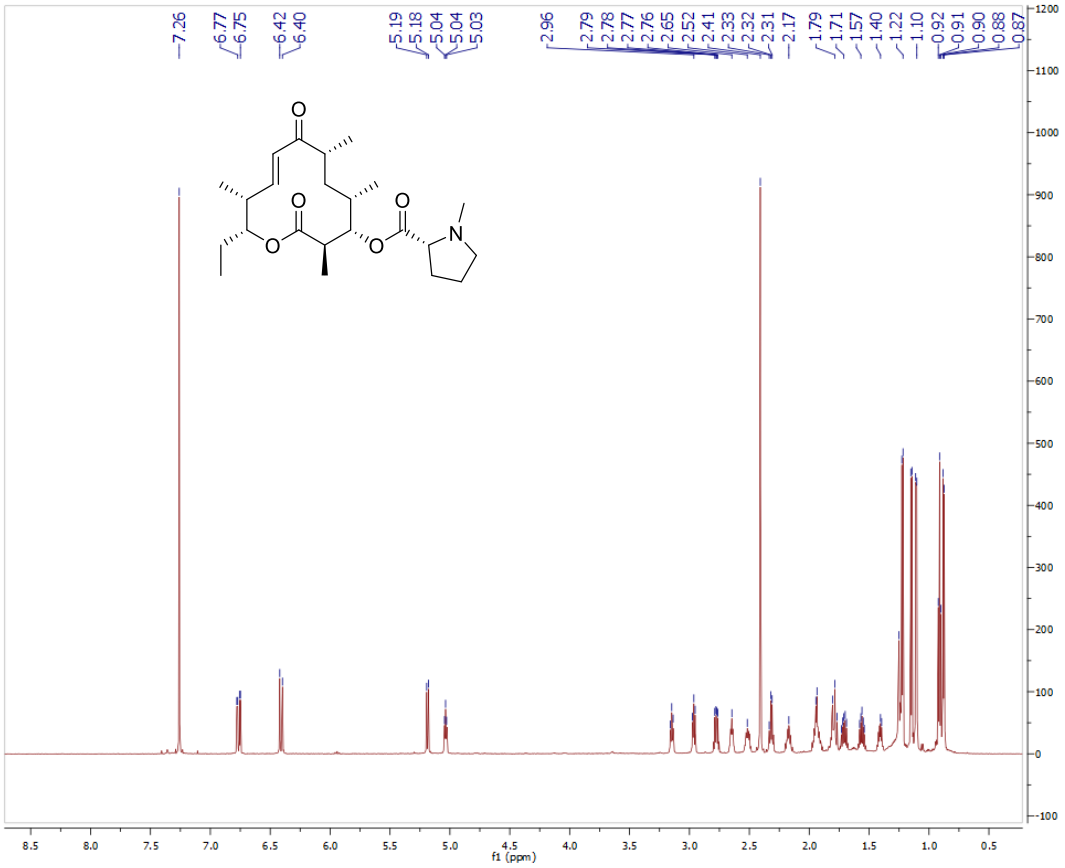


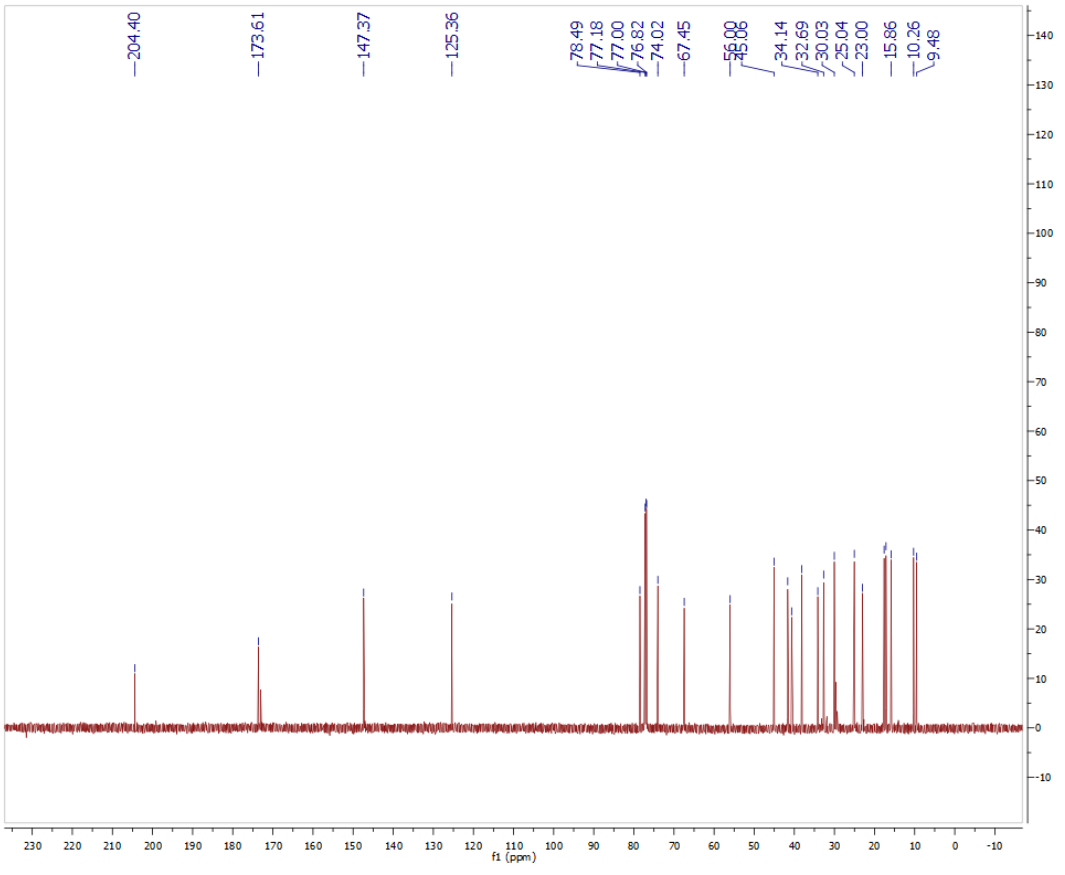
Substrate **10**



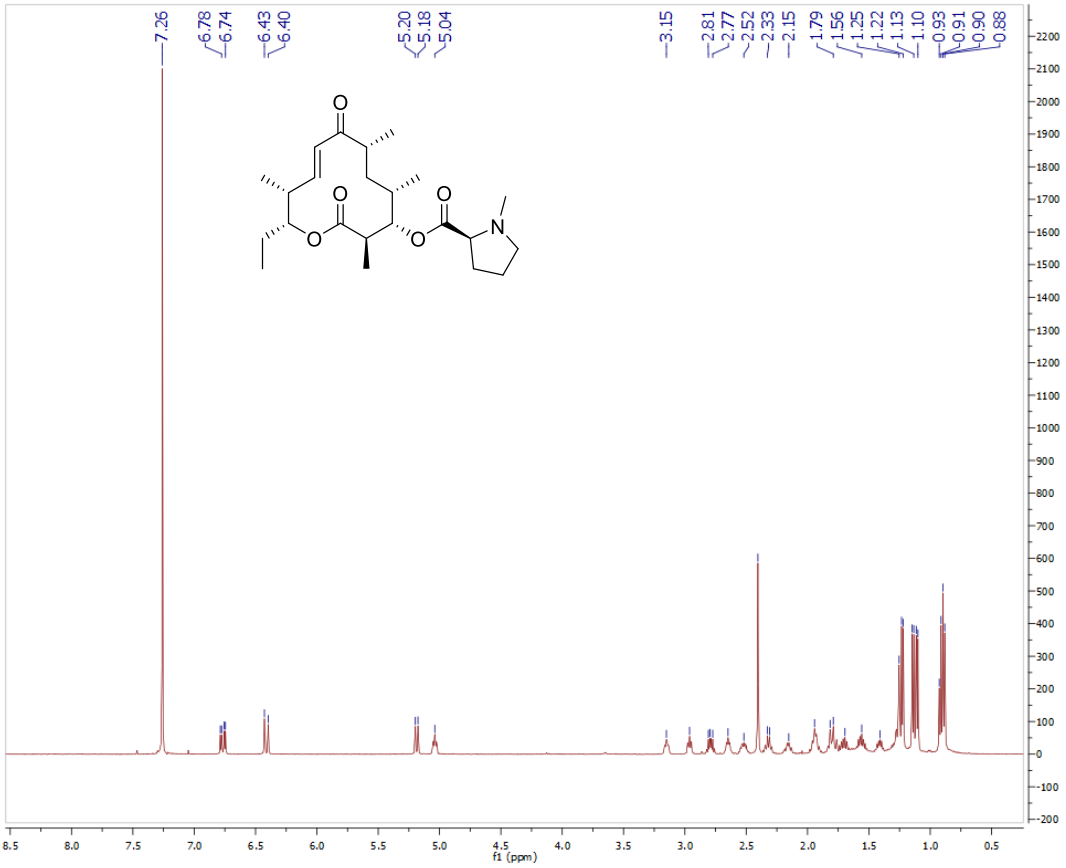


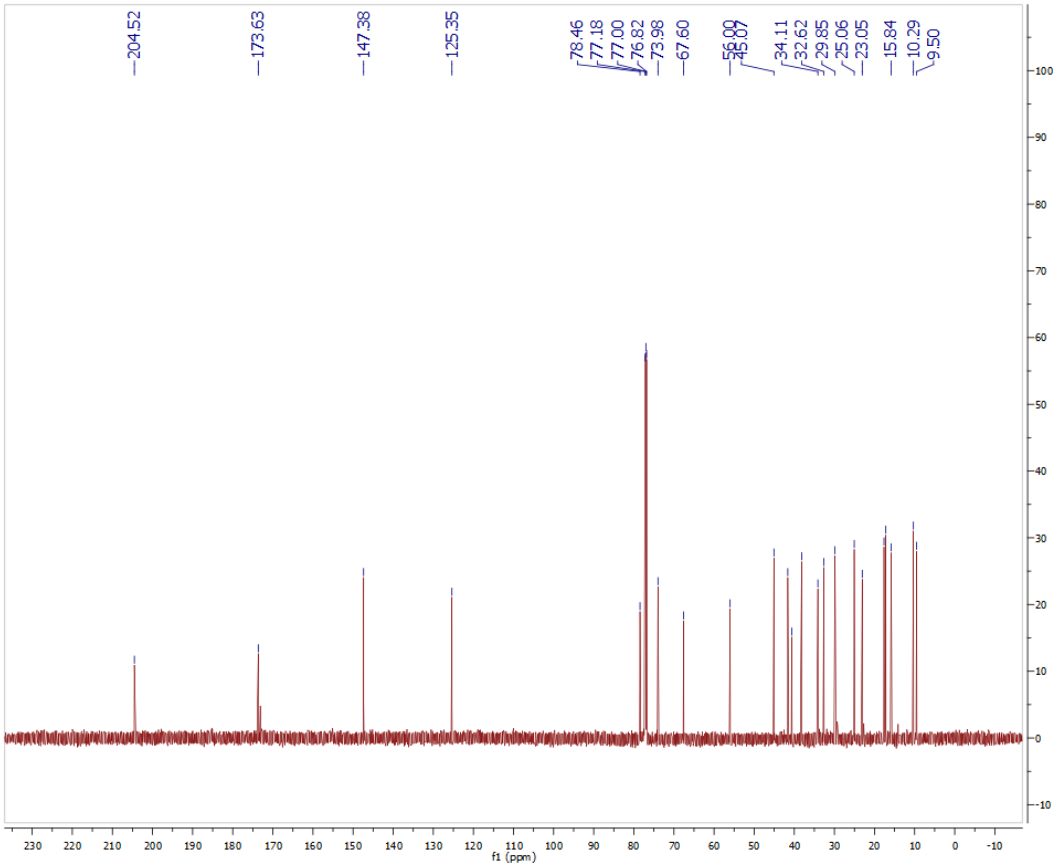
Substrate **12a**



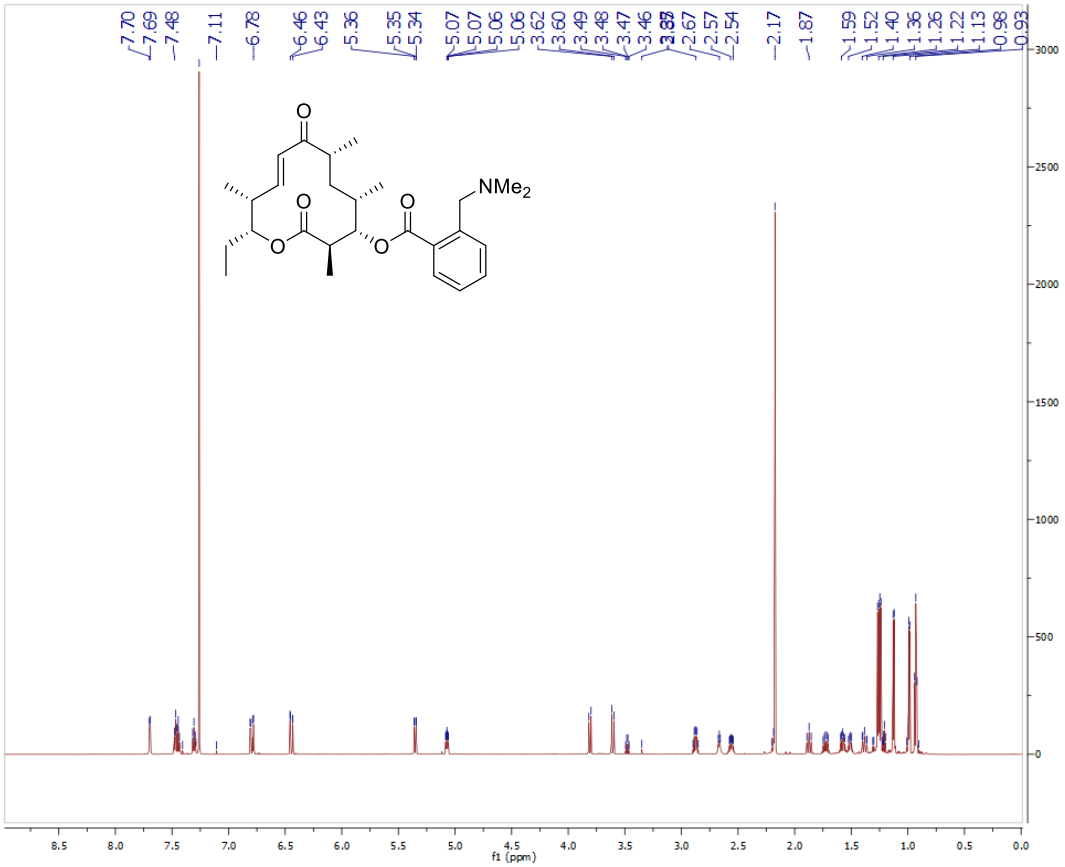


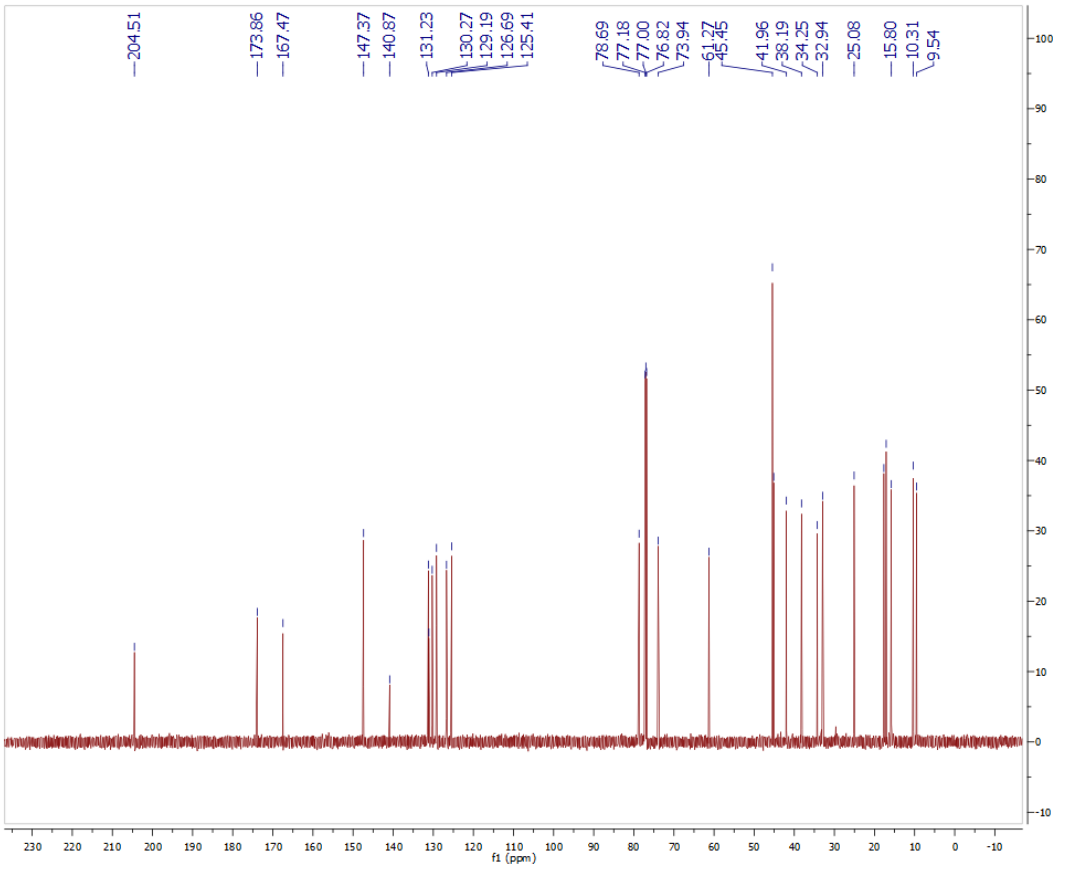
Substrate **12b**



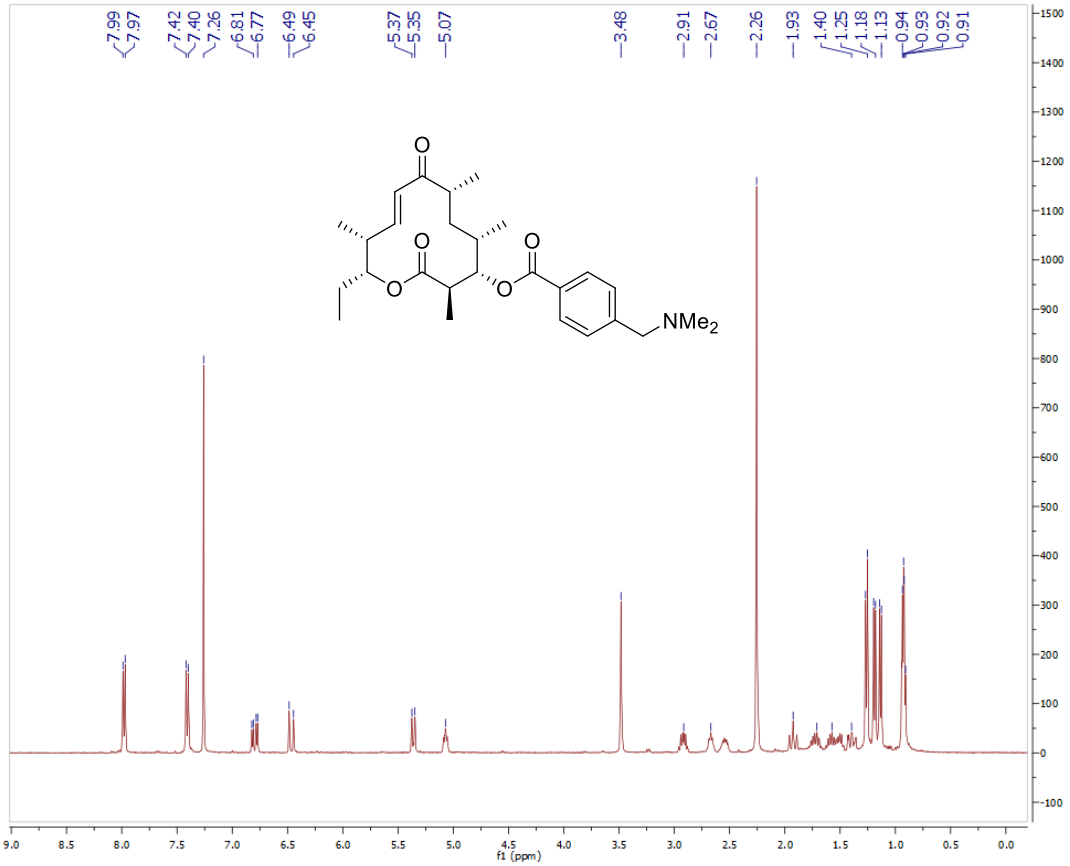


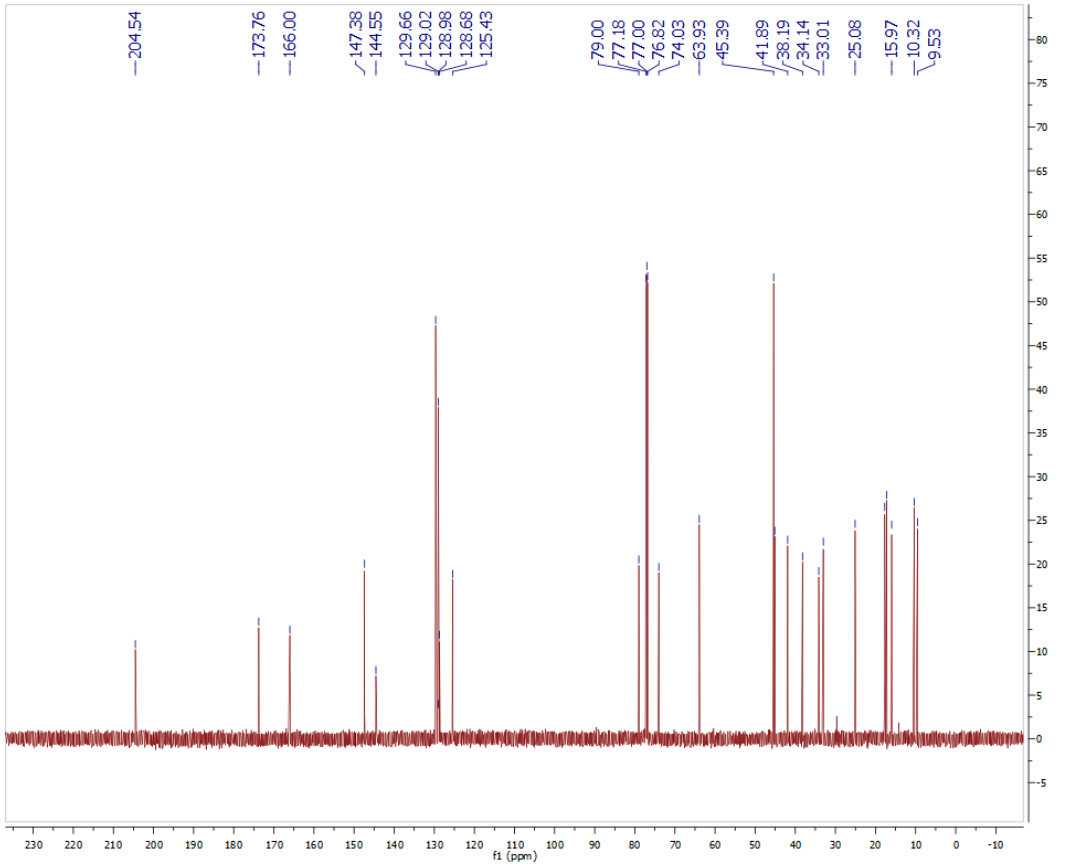
Substrate **14**


7.70
7.69
7.48
7.11
6.78
6.46
6.43
5.36
5.35
5.34
5.07
5.07
5.06
5.06
3.62
3.60
3.49
3.48
3.47
3.46
2.67
2.57
2.54
2.17
1.87
1.59
1.52
1.40
1.36
1.26
1.22
1.13
0.98
0.93
NMe2
O
O
O
O
O
f1 (ppm)

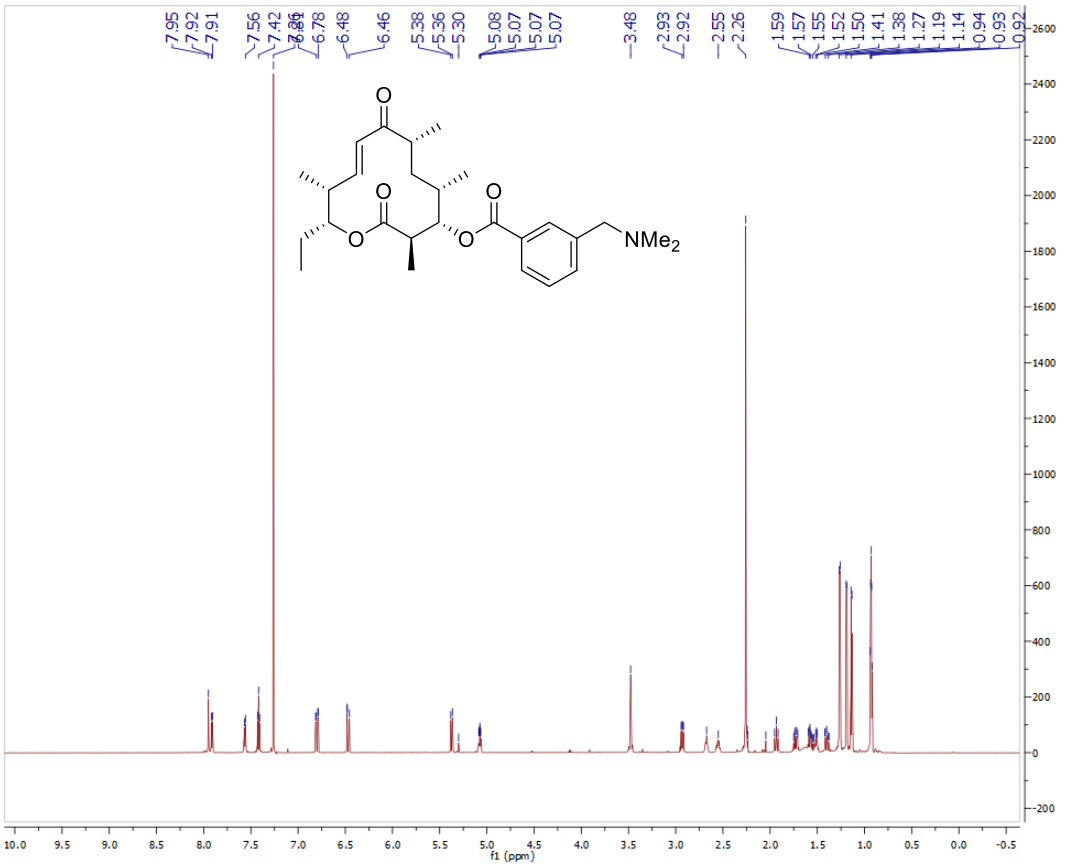

204.51
173.86
167.47
147.37
140.87
131.23
130.27
129.19
126.69
125.41
78.69
77.18
77.00
76.82
73.94
61.27
45.45
41.96
38.19
34.25
32.94
25.08
15.80
10.31
9.54
f1 (ppm)

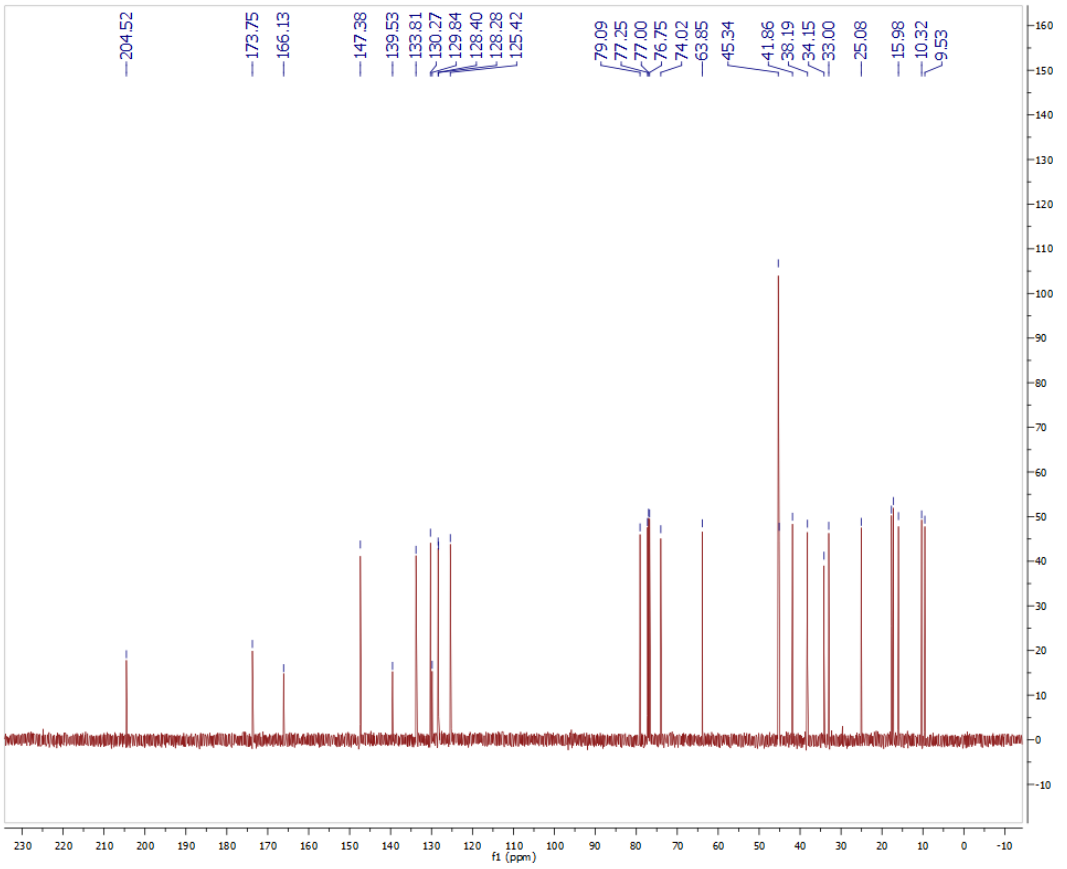
Substrate **16a**



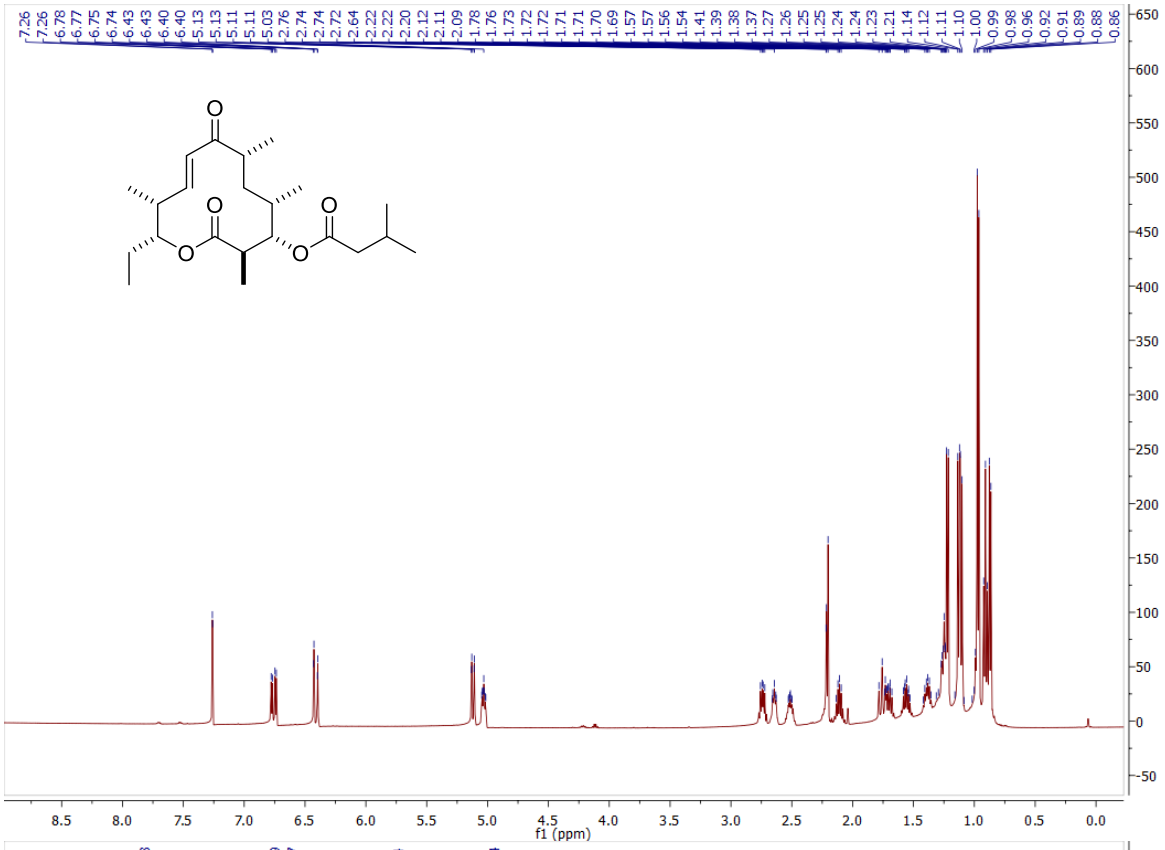


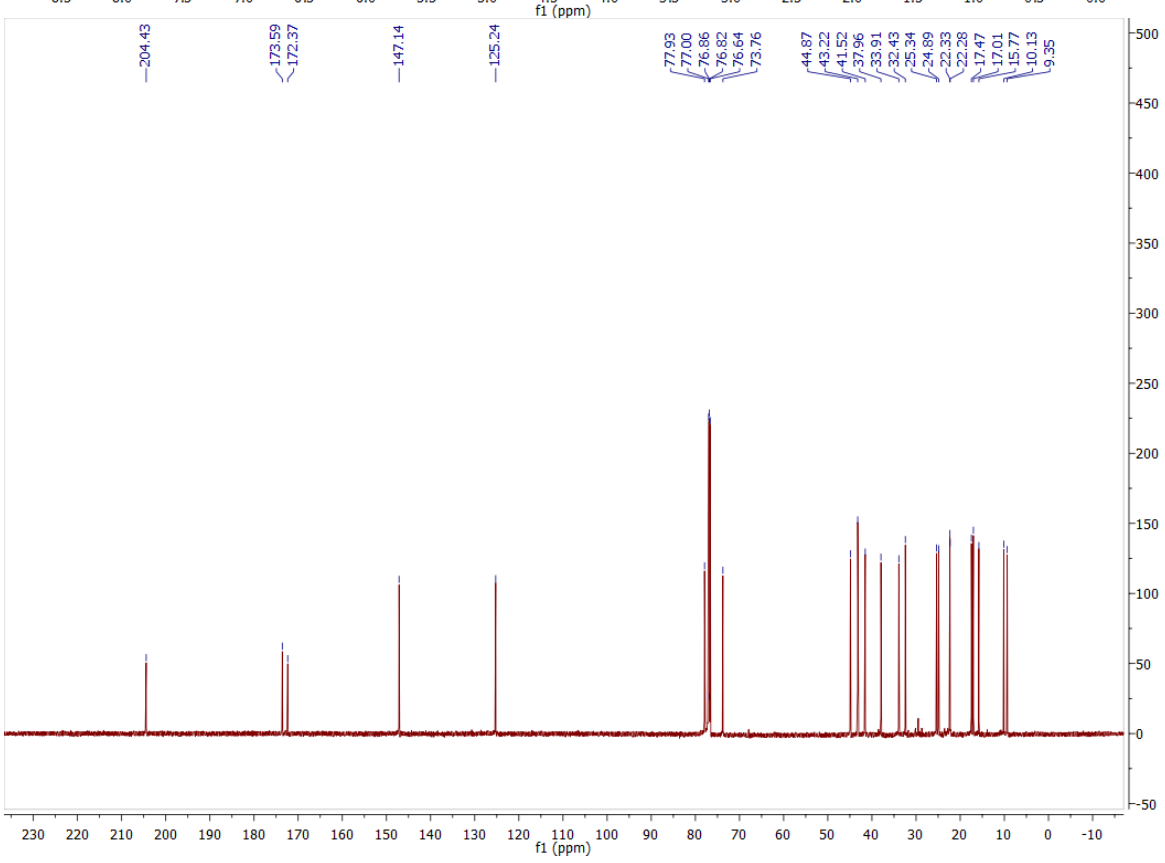
Substrate **16b**


7.95
7.92
7.91
7.56
7.42
6.78
6.48
6.46
5.38
5.36
5.30
5.08
5.07
5.07
5.07
3.48
2.93
2.92
2.55
2.26
1.59
1.57
1.55
1.52
1.50
1.41
1.38
1.27
1.19
1.14
0.94
0.93
0.92
O
O
O
O
O
NMe2
f1 (ppm)

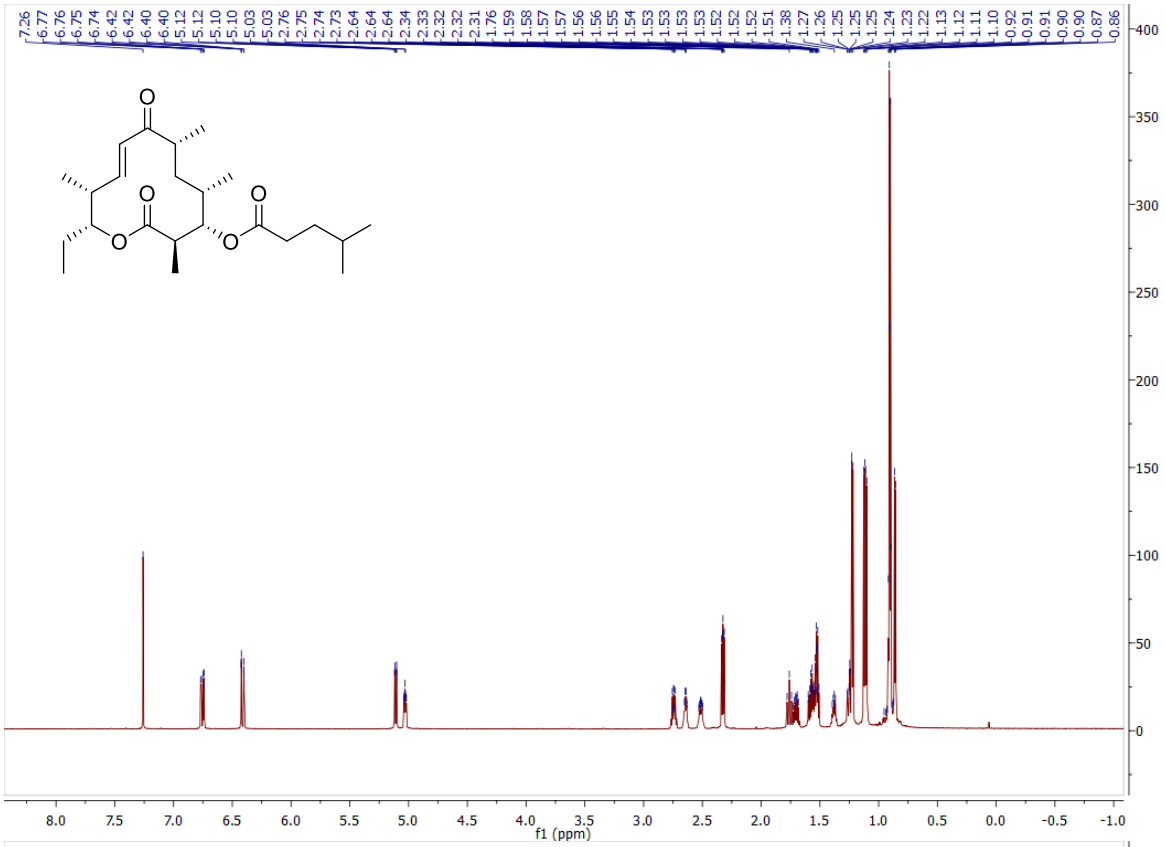

204.52
173.75
166.13
147.38
139.53
133.81
130.27
129.84
128.40
128.28
125.42
79.09
77.25
77.00
76.75
74.02
63.85
45.34
41.86
38.19
34.15
33.00
25.08
15.98
10.32
9.53
f1 (ppm)

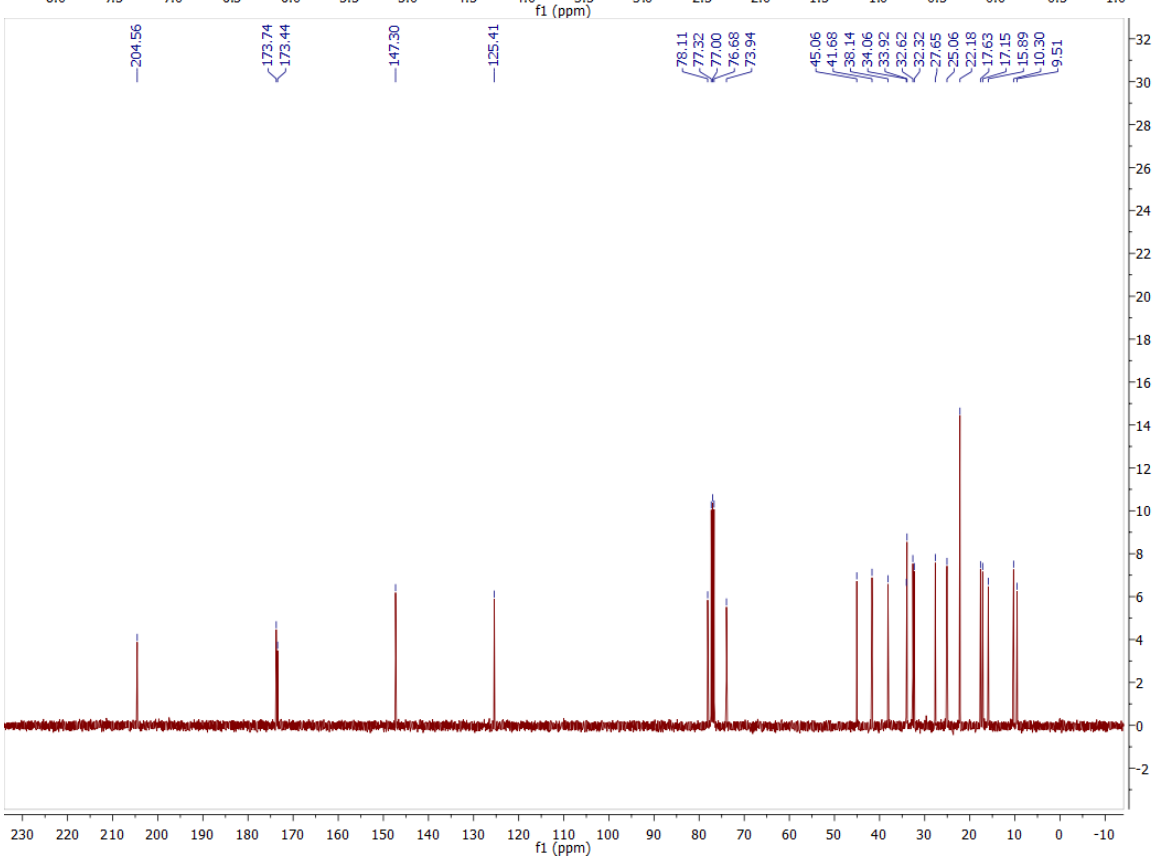
Control **S1**


f1 (ppm)


204.43
173.59
172.37
147.14
125.24
77.93
77.00
76.86
76.82
76.64
73.76
44.87
43.22
41.52
37.96
33.91
32.43
25.34
24.89
22.33
22.28
17.47
17.01
15.77
10.13
9.35
f1 (ppm)

Control **S2**


7.26 6.77 6.76 6.75 6.74 6.42 6.42 6.40 6.40 5.12 5.12 5.10 5.10 5.03 5.03 2.76 2.75 2.74 2.73 2.64 2.64 2.64 2.34 2.33 2.32 2.32 2.31 1.76 1.59 1.58 1.57 1.57 1.56 1.56 1.55 1.54 1.53 1.53 1.53 1.53 1.52 1.52 1.52 1.51 1.38 1.27 1.26 1.25 1.25 1.25 1.24 1.23 1.22 1.13 1.12 1.11 1.10 0.92 0.91 0.91 0.90 0.90 0.87 0.86
O
O
O
O
O
f1 (ppm)


204.56 173.74 173.44 147.30 125.41 78.11 77.32 77.00 76.68 73.94 45.06 41.68 38.14 34.06 33.92 32.62 32.32 27.65 25.06 22.18 17.63 17.15 15.89 10.30 9.51
f1 (ppm)

Part II: Enzymatic reactions

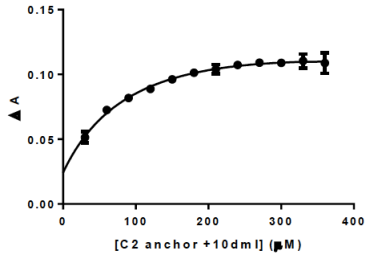
Expression and purification of $PikC_{D50N}$-RhFRED: Protein expression and purification followed a previously described procedure.[4]

$PikC_{D50N}$-RhFRED analytical-scale enzymatic reactions: The standard assay contained 5 μM $PikC_{D50N}$-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 NaH_2PO_4, pH 7.3, 1 mM EDTA, 0.2 mM dithoerythritol, and 10% glycerol) total volume 50 μL. The reaction was carried out at 30 °C for 3 h and quenched by extraction with $CHCl_3$ (2 × 200 μL). The organic extracts were combined, concentrated to dryness under N_2, and redissolved in 100 μL 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 μm, 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] (monohydoxylation) 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.

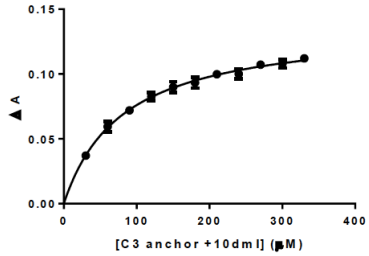
Determination of total turnover number (TTN): Total turnover number was determined by analyzing (# of moles of starting material consumed)/(# of moles of enzyme) under the following conditions: 1 μM $PikC_{D50N}$-RhFRED, 1 mM substrate, 1 mM NADP+, 0.05 units of glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate for NADPH regeneration in 50 μL of reaction buffer (50 mM NaH_2PO_4, pH 7.3, 1 mM EDTA, 0.2 mM dithoerythritol, and 10% glycerol). The reaction was carried out at 30 °C for 24 h and quenched by extraction with $CHCl_3$ (2 × 200 μL). The organic extracts were combined, concentrated to dryness under N_2, and redissolved in 100 μL 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 μm, 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 μM was titrated with the substrate dissolved in Me_2SO (30 mM) in 1-μl aliquots. The same amounts of Me_2SO alone were added to the protein in the reference cuvette followed by recording of the difference spectra. Absorbance differences ΔA ($A_{\text{peak 390 nm}} - A_{\text{trough 420 nm}}$) were plotted *versus* substrate concentration, and data from duplicated experiments were fitted to the hyperbolic function $\Delta A = (A_{max}(S/K_D + S)$, 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.

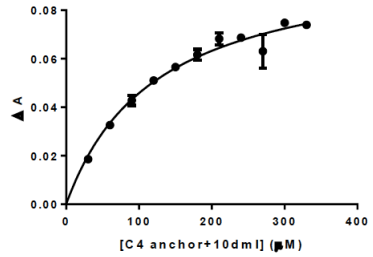
Compound **8a**: K_D = 118 μM



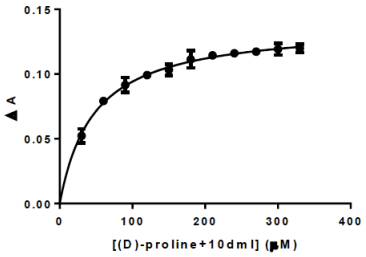
Compound **8b**: K_D = 81 μM



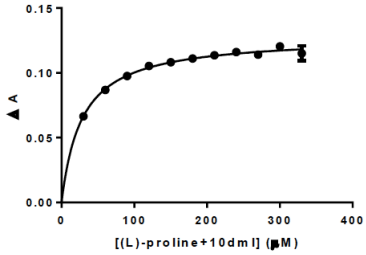
Compound **10**: K_D = 123 μM



Compound **12a**: K_D = 47 μM



Compound **12b**: K_D = 28 μM

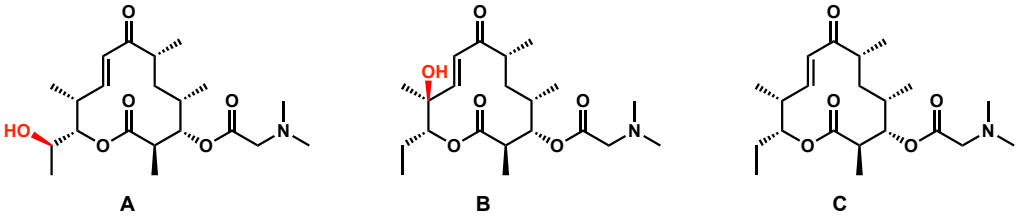


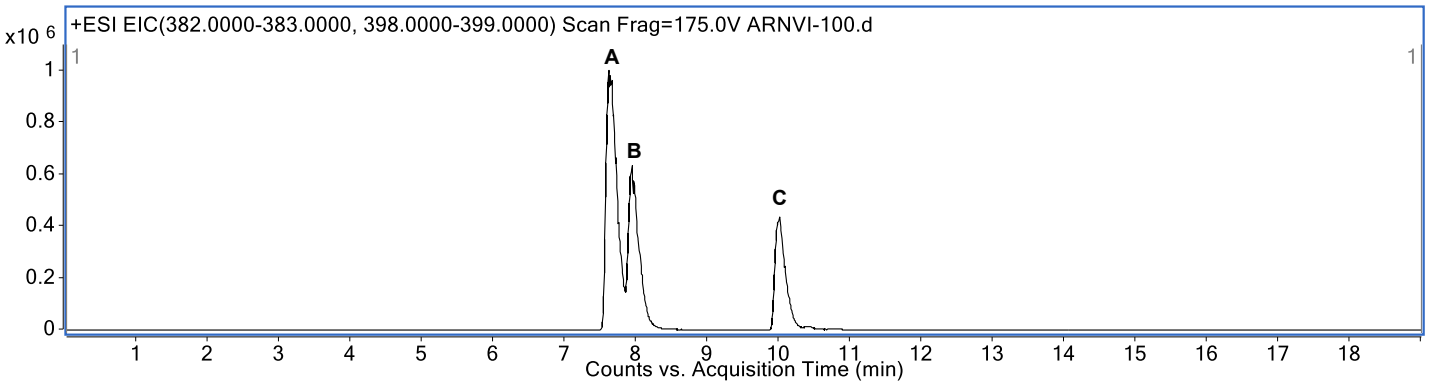
PikC$_{D50N}$-RhFRED preparative-scale enzymatic reactions: Preparative –scale enzymatic reactions were conducted on 20 mg of each substrate under the following conditions: 5 μM PikC$_{D50N}$-RhFRED, 1 mM substrate, 1 mM NADP+, 1 U/mL glucose-6-phosphate dehydrogenase, and 5 mM glucose-6-phosphate for NADPH generation in reaction buffer (50 mM NaH_2PO_4, pH 7.3, 1 mM EDTA, 0.2 mM dithoerythritol, 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 Na_2SO_4 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.

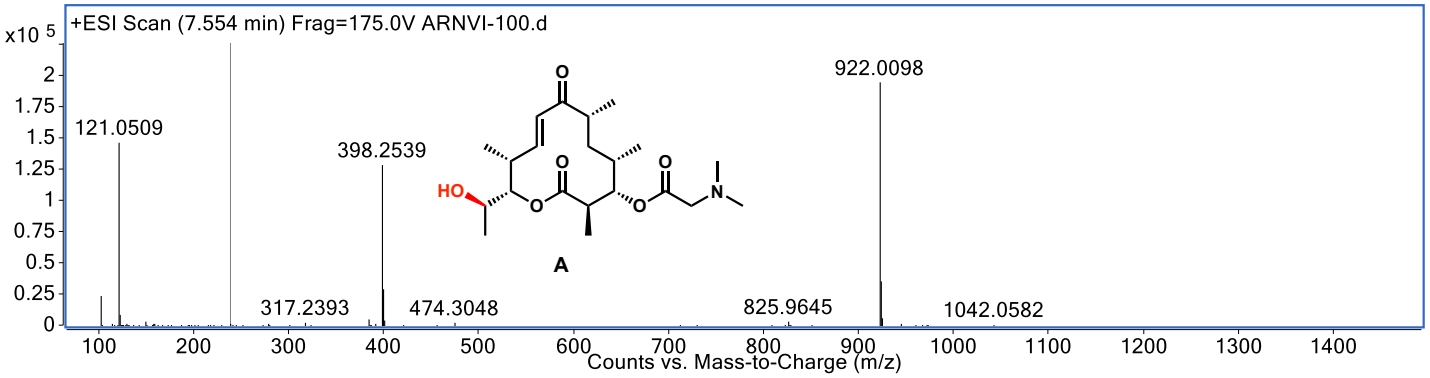
Preparation of PikC$_{D50NH238A}$-RhFRED: Using previously prepared pET28b-PikC$_{D50N}$-RhFRED as a template, site-directed mutagenesis was performed with the primer: 5'-gaggagctgctcggtatggccgcgatcctgctcgtcgcggggcac-3'. Protein expression and purification of PikC$_{D50NH238A}$-RhFRED followed the procedure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

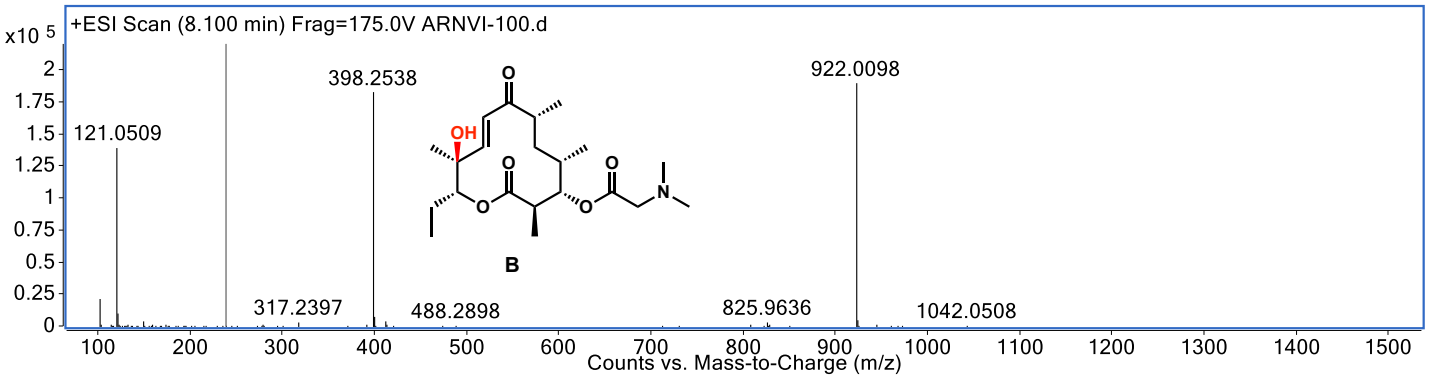
Hydroxylation product analysis and identification

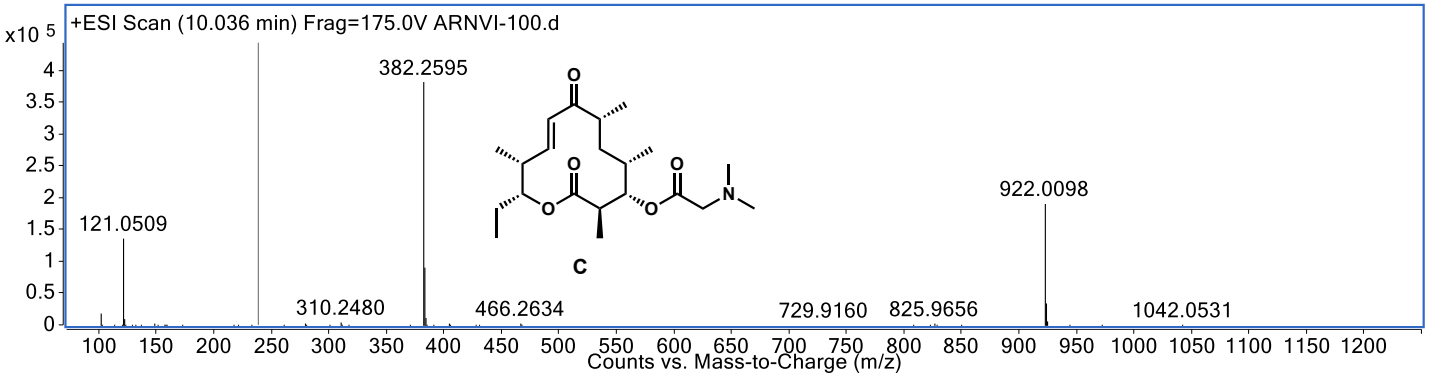
A. LC-MS traces of enzymatic reactions

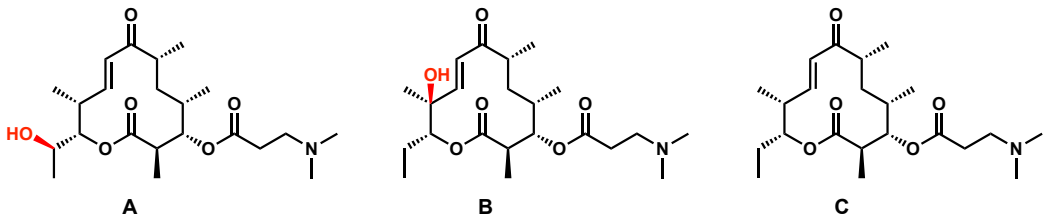

A
B
C

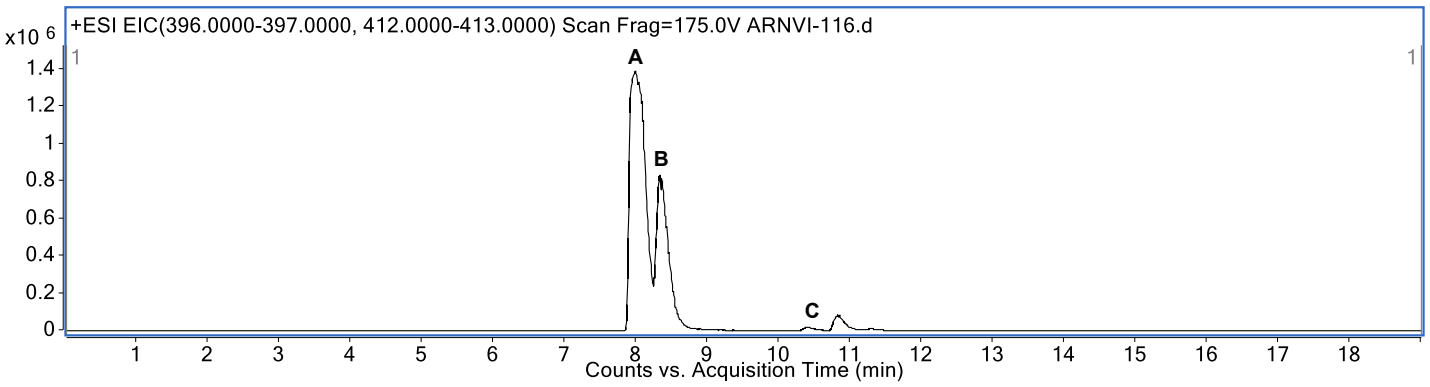

+ESI EIC(382.0000-383.0000, 398.0000-399.0000) Scan Frag=175.0V ARNVI-100.d
x10 6
A
B
C
Counts vs. Acquisition Time (min)

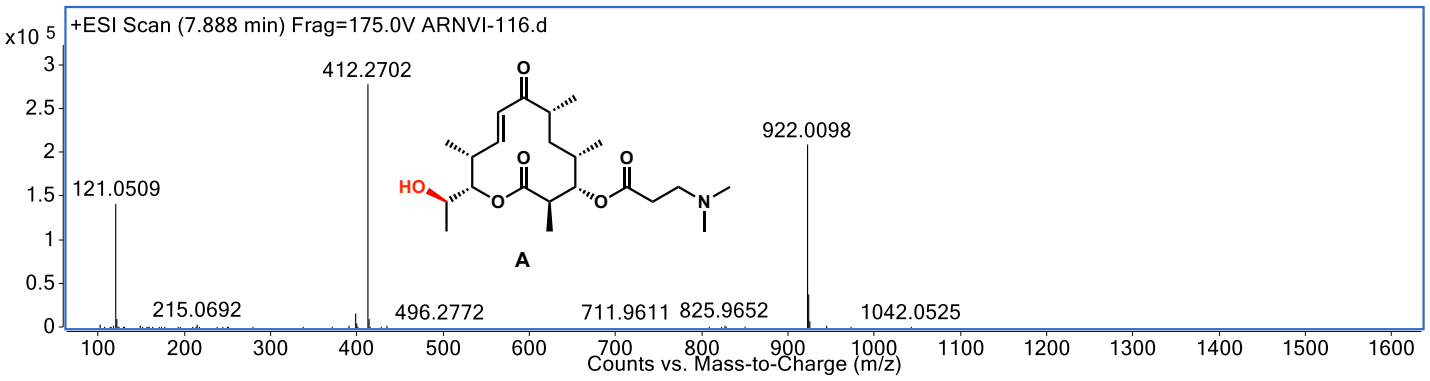

+ESI Scan (7.554 min) Frag=175.0V ARNVI-100.d
x10 5
121.0509
398.2539
922.0098
317.2393
474.3048
825.9645
1042.0582
A
Counts vs. Mass-to-Charge (m/z)

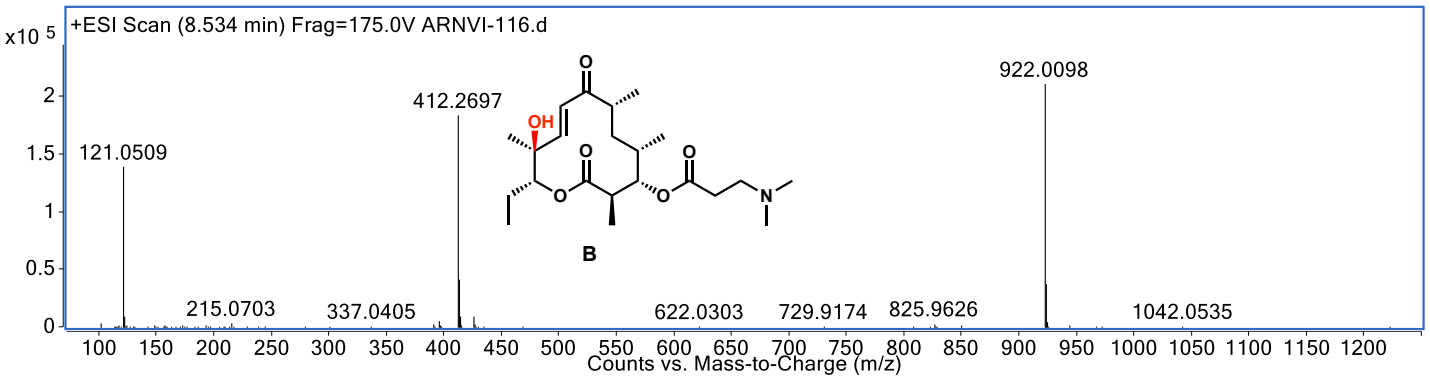

+ESI Scan (8.100 min) Frag=175.0V ARNVI-100.d
x10 5
121.0509
398.2538
922.0098
317.2397
488.2898
825.9636
1042.0508
B
Counts vs. Mass-to-Charge (m/z)

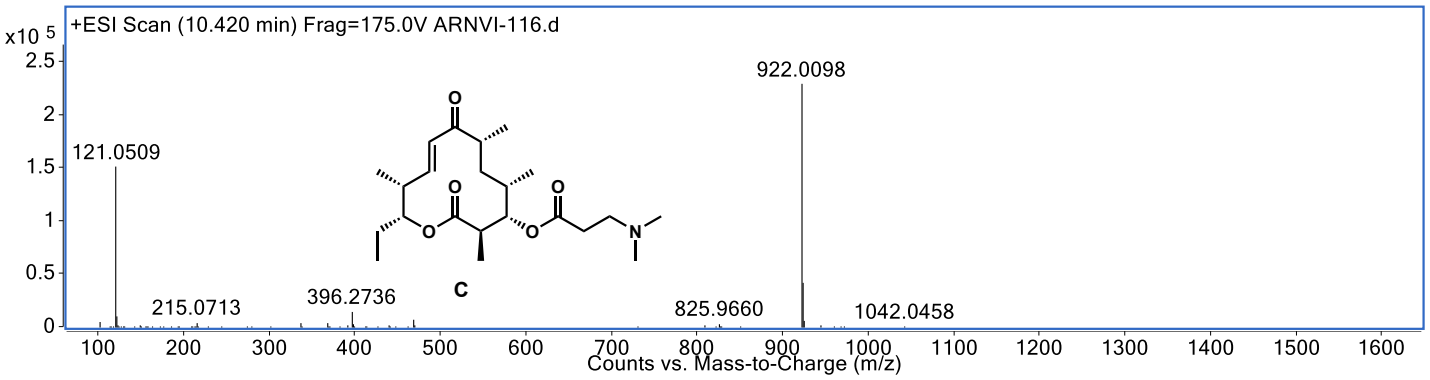

+ESI Scan (10.036 min) Frag=175.0V ARNVI-100.d
x10 5
121.0509
382.2595
922.0098
310.2480
466.2634
729.9160
825.9656
1042.0531
C
Counts vs. Mass-to-Charge (m/z)

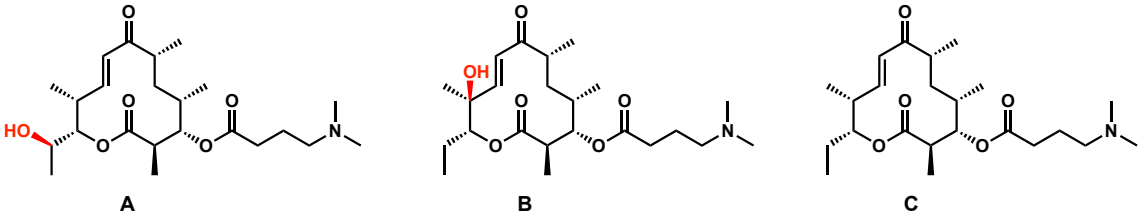

A
B
C

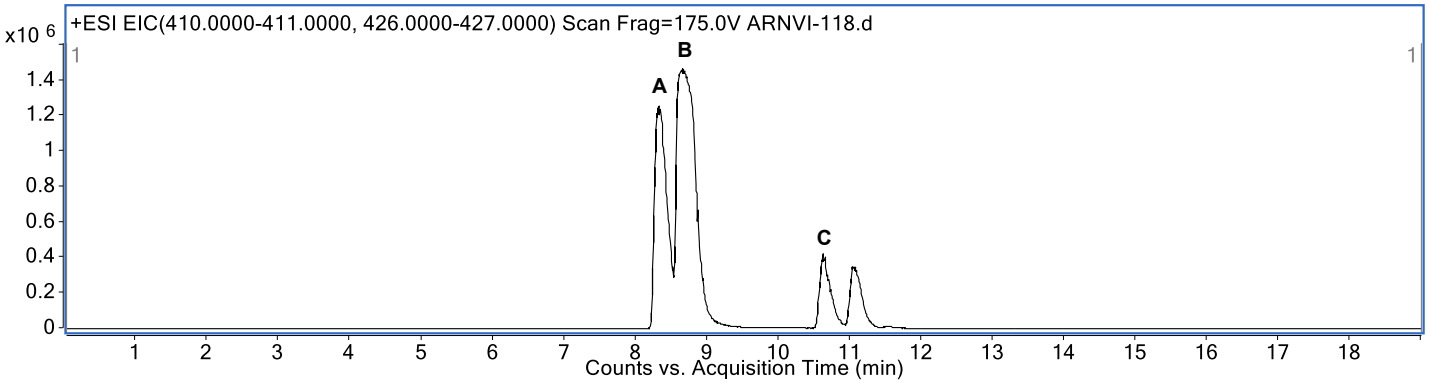

+ESI EIC(396.0000-397.0000, 412.0000-413.0000) Scan Frag=175.0V ARNVI-116.d
x10 6
A
B
C
Counts vs. Acquisition Time (min)

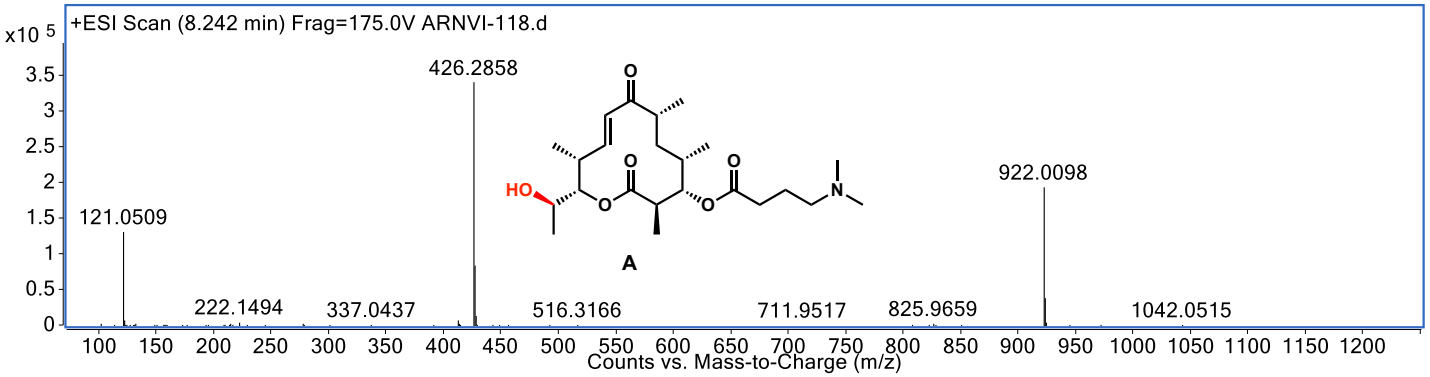

+ESI Scan (7.888 min) Frag=175.0V ARNVI-116.d
x10 5
412.2702
922.0098
121.0509
215.0692
496.2772
711.9611
825.9652
1042.0525
A
Counts vs. Mass-to-Charge (m/z)

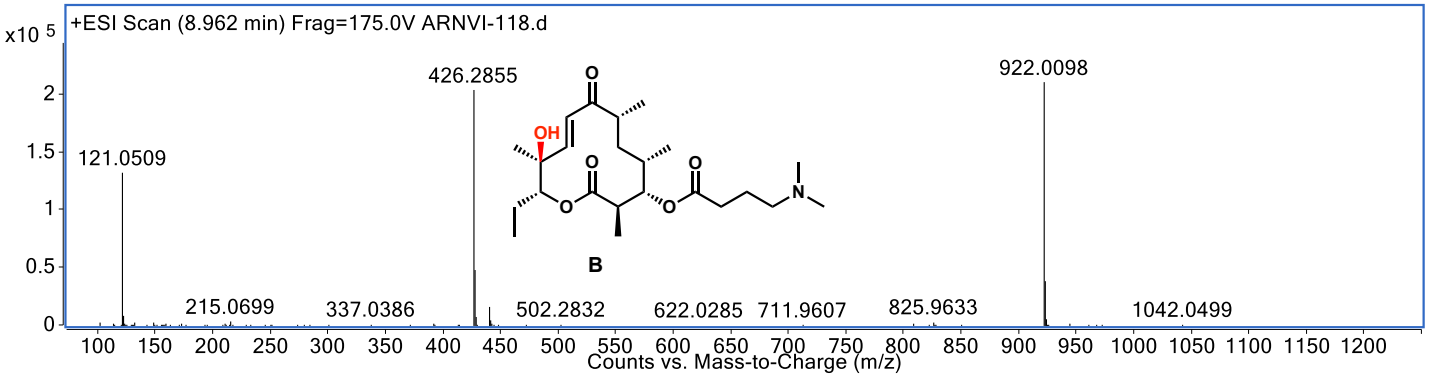

+ESI Scan (8.534 min) Frag=175.0V ARNVI-116.d
x10 5
412.2697
922.0098
121.0509
215.0703
337.0405
622.0303
729.9174
825.9626
1042.0535
B
Counts vs. Mass-to-Charge (m/z)

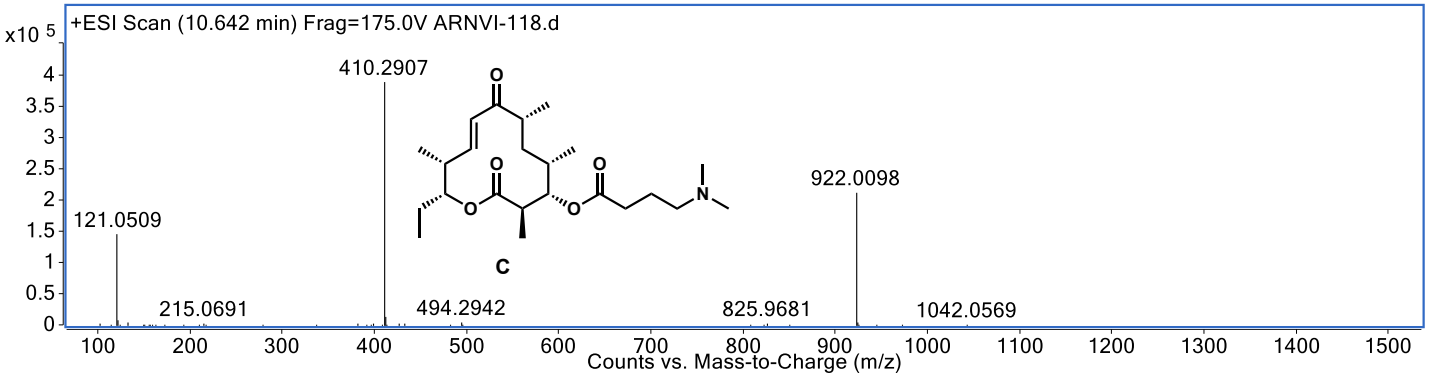

+ESI Scan (10.420 min) Frag=175.0V ARNVI-116.d
x10 5
922.0098
121.0509
215.0713
396.2736
825.9660
1042.0458
C
Counts vs. Mass-to-Charge (m/z)

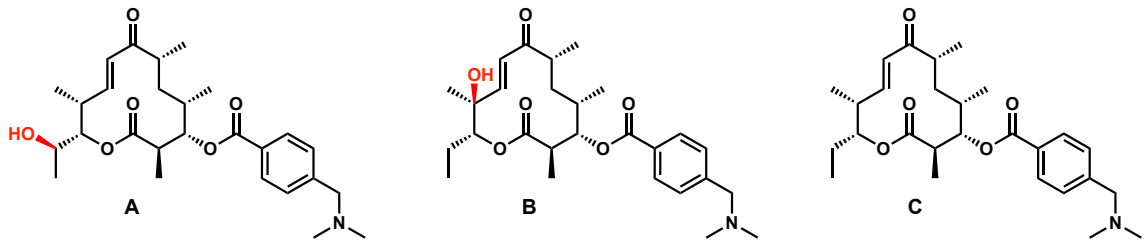

A
B
C

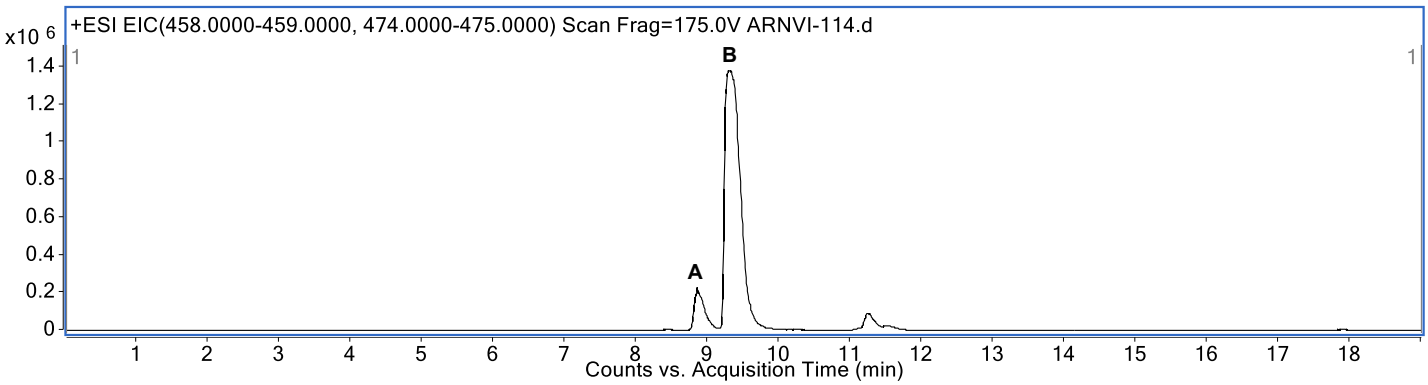

+ESI EIC(410.0000-411.0000, 426.0000-427.0000) Scan Frag=175.0V ARNVI-118.d
x10 6
A
B
C
Counts vs. Acquisition Time (min)

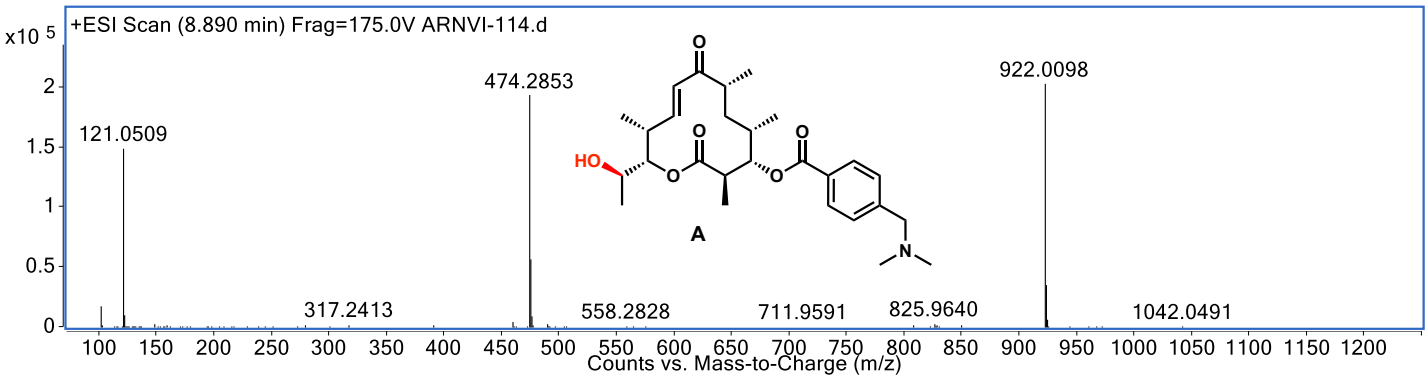

+ESI Scan (8.242 min) Frag=175.0V ARNVI-118.d
x10 5
426.2858
121.0509
922.0098
222.1494
337.0437
516.3166
711.9517
825.9659
1042.0515
A
Counts vs. Mass-to-Charge (m/z)

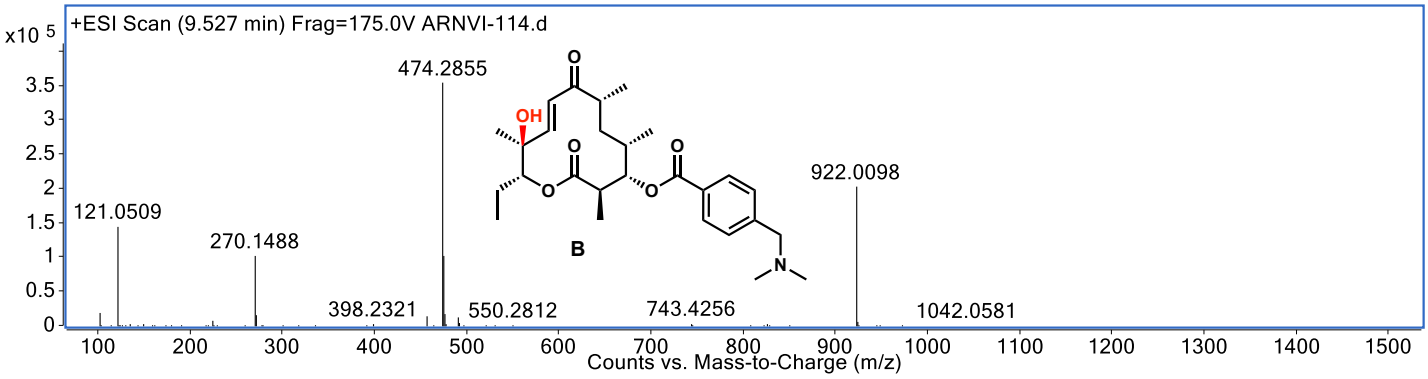

+ESI Scan (8.962 min) Frag=175.0V ARNVI-118.d
x10 5
426.2855
121.0509
922.0098
215.0699
337.0386
502.2832
622.0285
711.9607
825.9633
1042.0499
B
Counts vs. Mass-to-Charge (m/z)

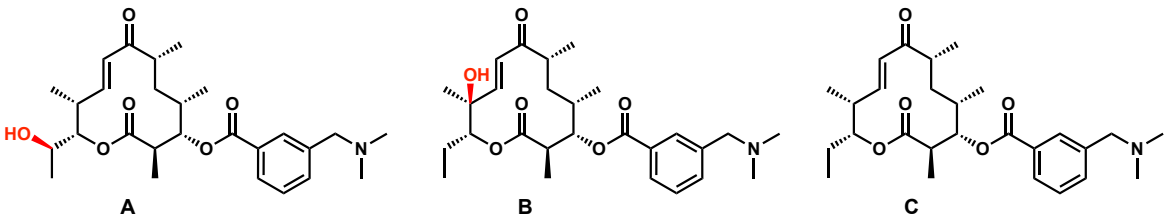

+ESI Scan (10.642 min) Frag=175.0V ARNVI-118.d
x10 5
410.2907
121.0509
922.0098
215.0691
494.2942
825.9681
1042.0569
C
Counts vs. Mass-to-Charge (m/z)

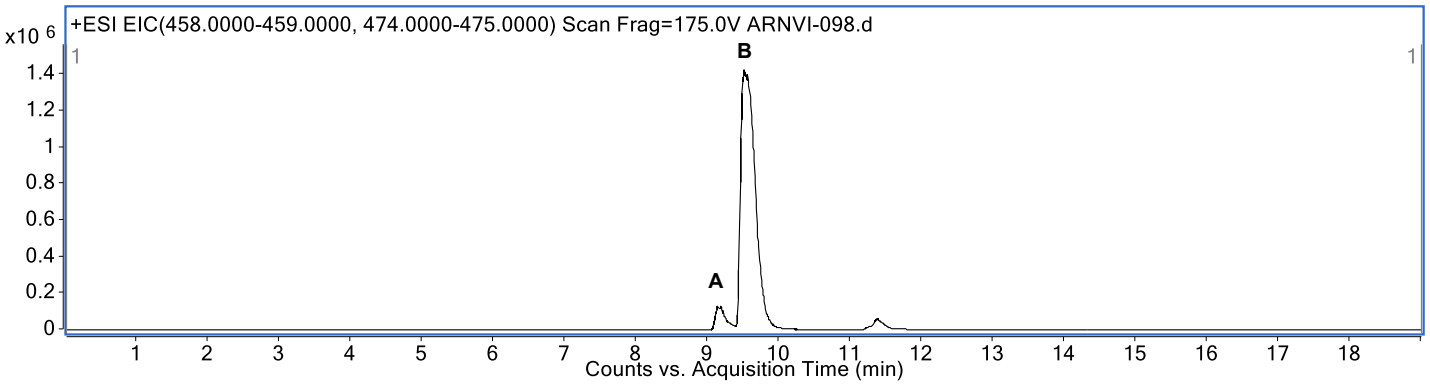

A
B
C

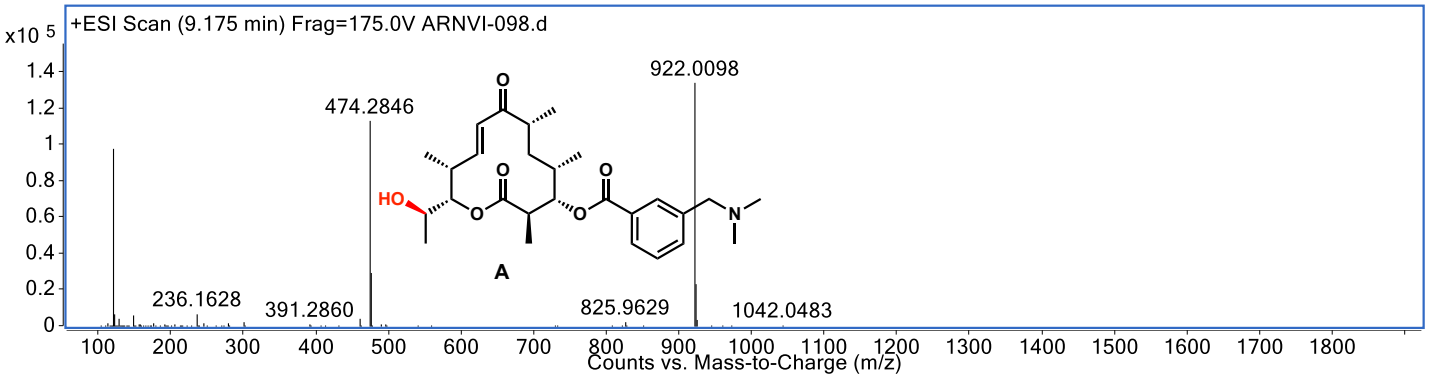

+ESI EIC(458.0000-459.0000, 474.0000-475.0000) Scan Frag=175.0V ARNVI-114.d
x10 6
B
A
Counts vs. Acquisition Time (min)

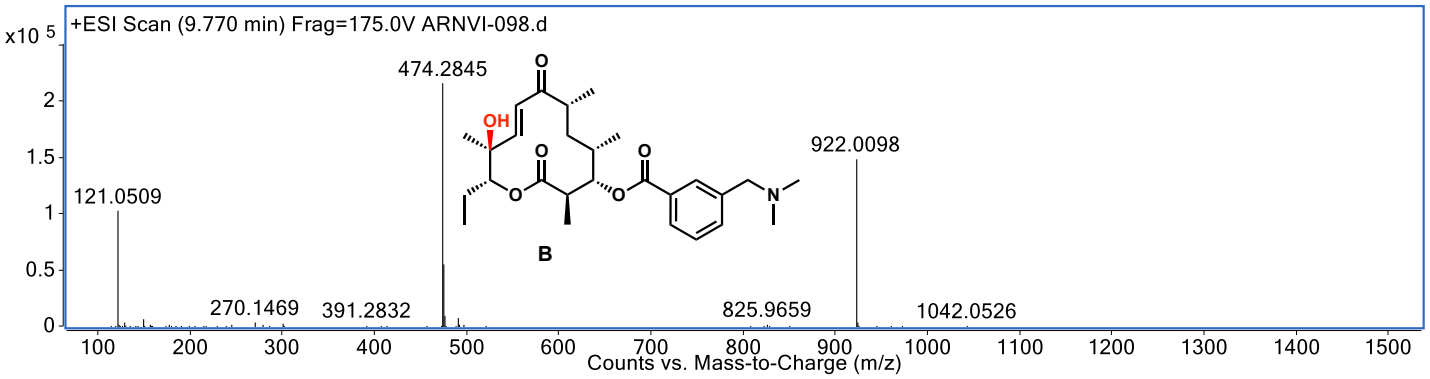

+ESI Scan (8.890 min) Frag=175.0V ARNVI-114.d
x10 5
474.2853
922.0098
121.0509
317.2413
558.2828
711.9591
825.9640
1042.0491
A
Counts vs. Mass-to-Charge (m/z)

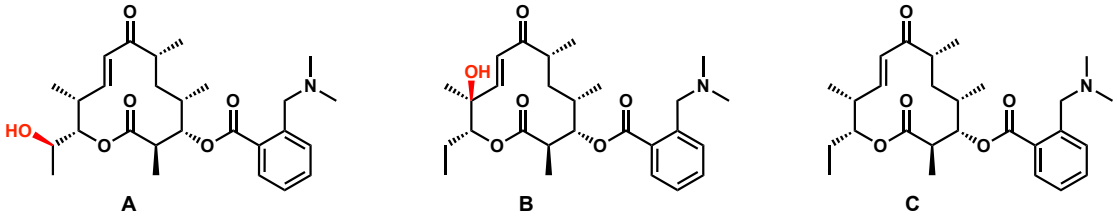

+ESI Scan (9.527 min) Frag=175.0V ARNVI-114.d
x10 5
474.2855
922.0098
121.0509
270.1488
398.2321
550.2812
743.4256
1042.0581
B
Counts vs. Mass-to-Charge (m/z)

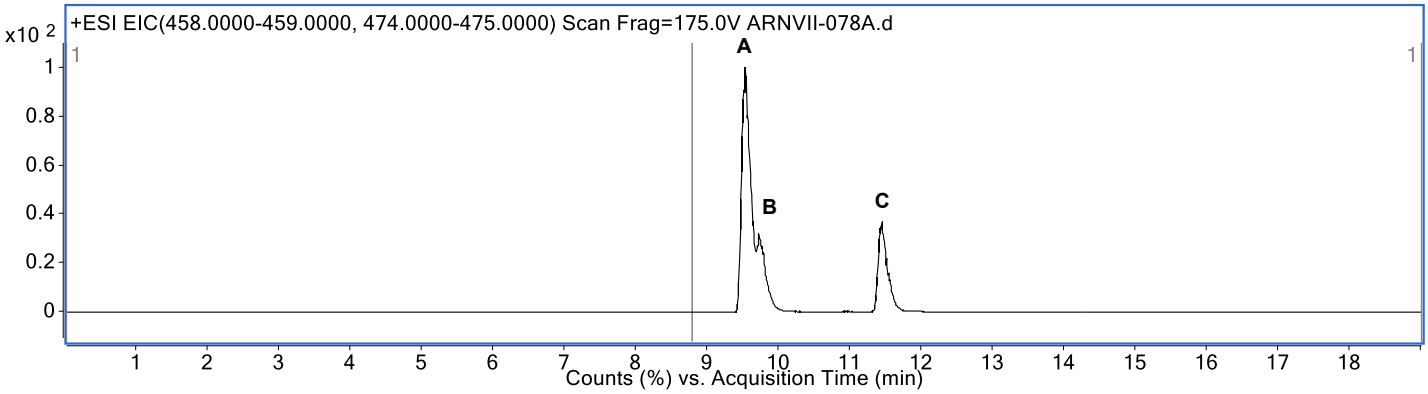

A
B
C

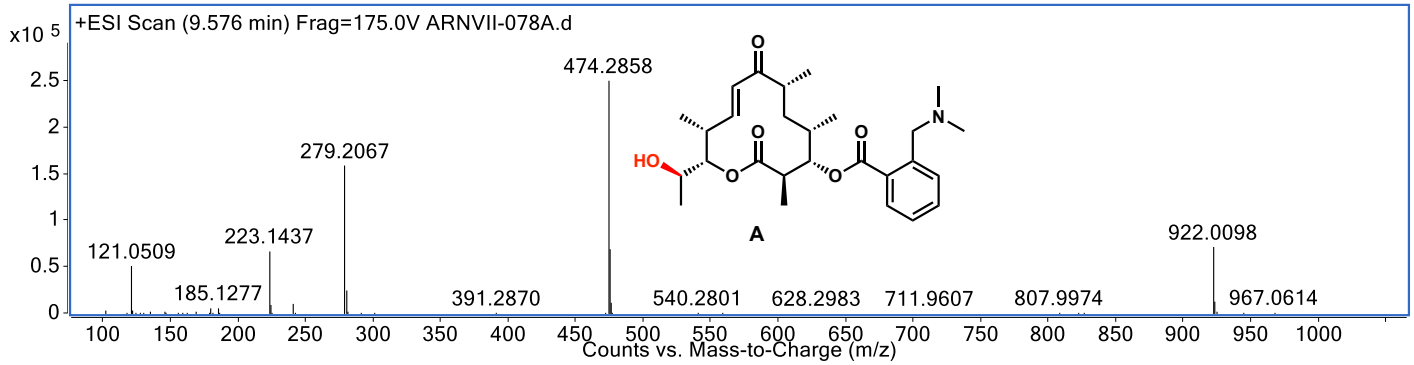

+ESI EIC(458.0000-459.0000, 474.0000-475.0000) Scan Frag=175.0V ARNVI-098.d
x10 6
B
A
Counts vs. Acquisition Time (min)

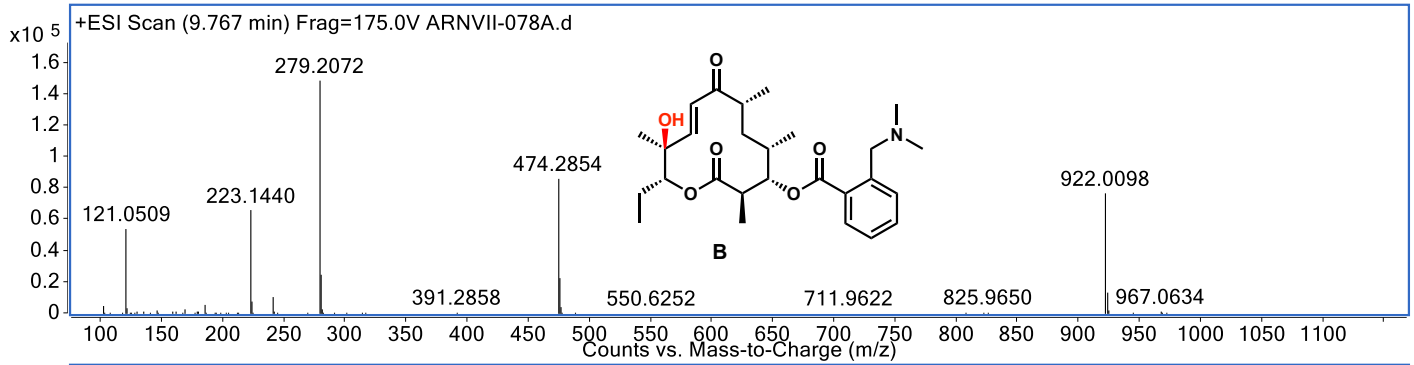

+ESI Scan (9.175 min) Frag=175.0V ARNVI-098.d
x10 5
474.2846
922.0098
236.1628
391.2860
825.9629
1042.0483
A
Counts vs. Mass-to-Charge (m/z)

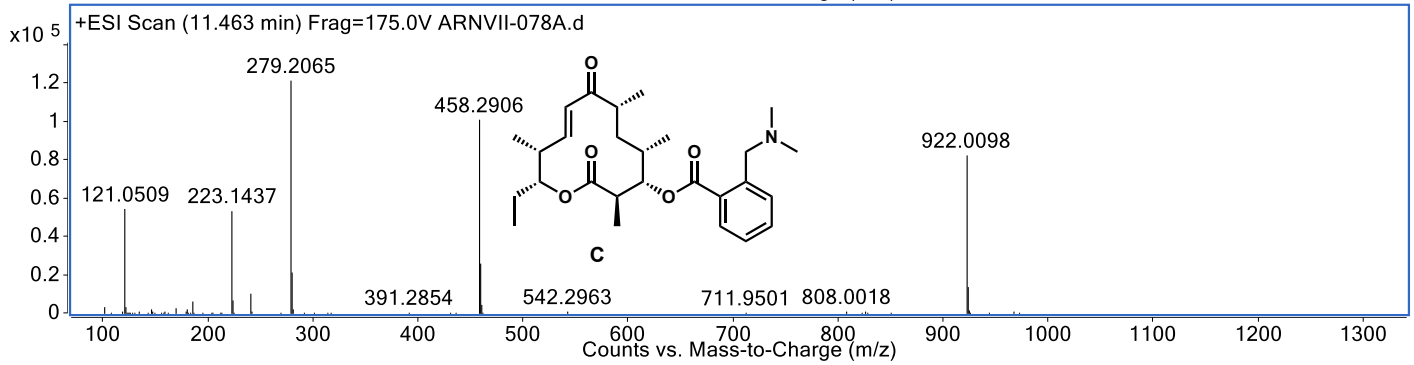

+ESI Scan (9.770 min) Frag=175.0V ARNVI-098.d
x10 5
474.2845
922.0098
121.0509
270.1469
391.2832
825.9659
1042.0526
B
Counts vs. Mass-to-Charge (m/z)

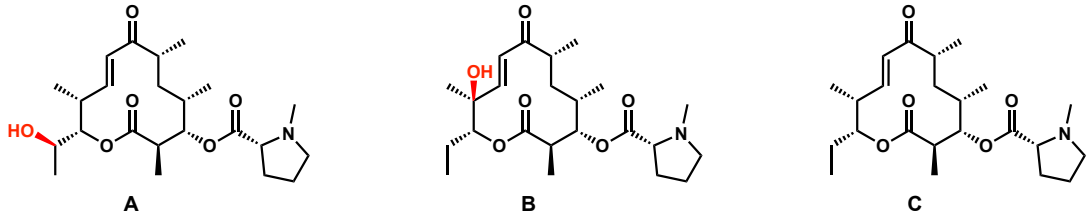

A
B
C

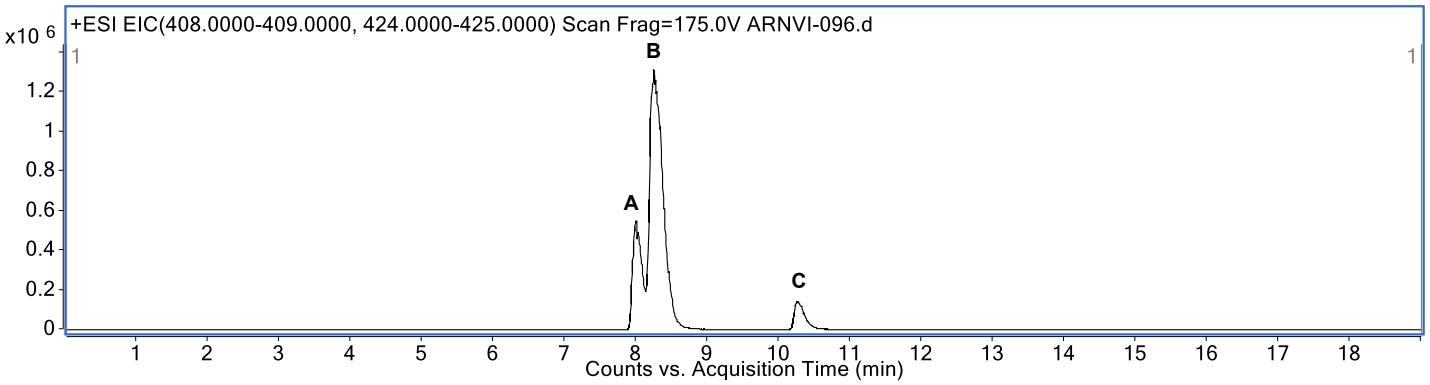

+ESI EIC(458.0000-459.0000, 474.0000-475.0000) Scan Frag=175.0V ARNVII-078A.d
x10 2
A
B
C
Counts (%) vs. Acquisition Time (min)

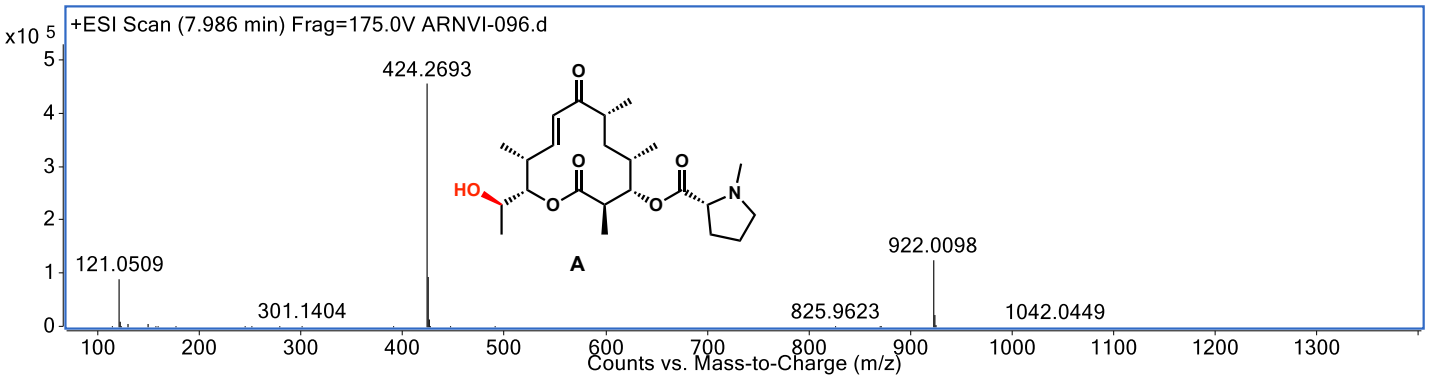

+ESI Scan (9.576 min) Frag=175.0V ARNVII-078A.d
x10 5
474.2858
279.2067
223.1437
121.0509
185.1277
391.2870
540.2801
628.2983
711.9607
807.9974
922.0098
967.0614
A
Counts vs. Mass-to-Charge (m/z)

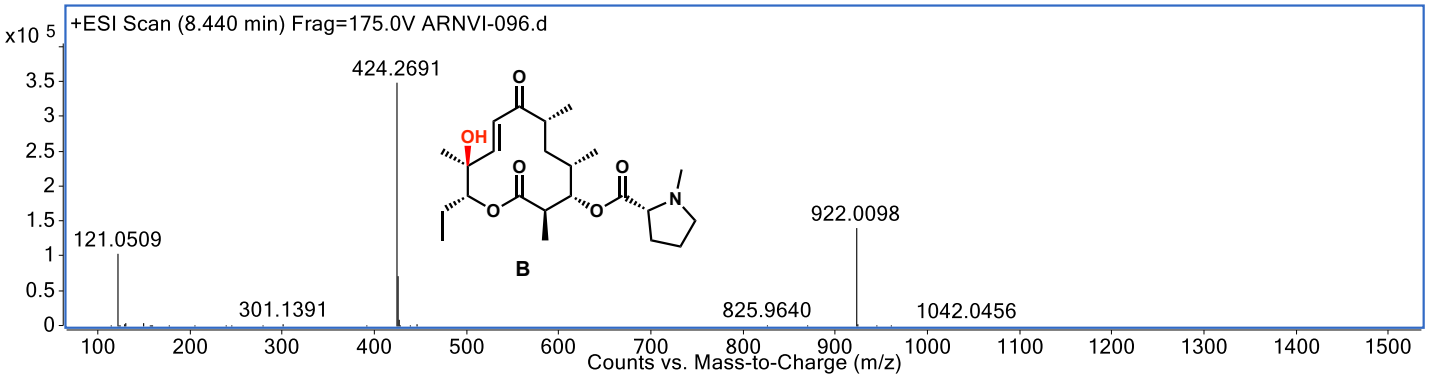

+ESI Scan (9.767 min) Frag=175.0V ARNVII-078A.d
x10 5
279.2072
474.2854
223.1440
121.0509
391.2858
550.6252
711.9622
825.9650
922.0098
967.0634
B
Counts vs. Mass-to-Charge (m/z)

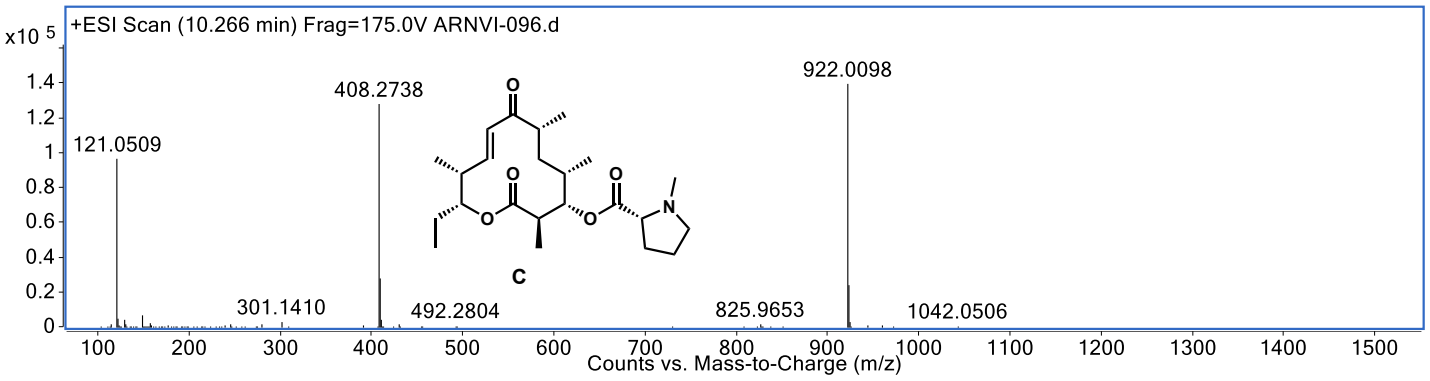

+ESI Scan (11.463 min) Frag=175.0V ARNVII-078A.d
x10 5
279.2065
458.2906
121.0509
223.1437
391.2854
542.2963
711.9501
808.0018
922.0098
C
Counts vs. Mass-to-Charge (m/z)

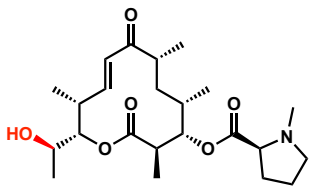

HO
A
OH
B
C


+ESI EIC(408.0000-409.0000, 424.0000-425.0000) Scan Frag=175.0V ARNVI-096.d
x10 6
A
B
C
Counts vs. Acquisition Time (min)

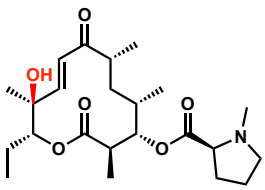

+ESI Scan (7.986 min) Frag=175.0V ARNVI-096.d
x10 5
424.2693
121.0509
301.1404
922.0098
825.9623
1042.0449
A
Counts vs. Mass-to-Charge (m/z)


+ESI Scan (8.440 min) Frag=175.0V ARNVI-096.d
x10 5
424.2691
121.0509
301.1391
922.0098
825.9640
1042.0456
B
Counts vs. Mass-to-Charge (m/z)

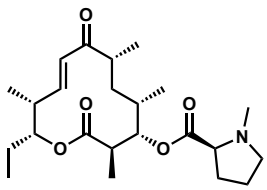

+ESI Scan (10.266 min) Frag=175.0V ARNVI-096.d
x10 5
408.2738
922.0098
121.0509
301.1410
492.2804
825.9653
1042.0506
C
Counts vs. Mass-to-Charge (m/z)


HO
O
O
O
O
O
N

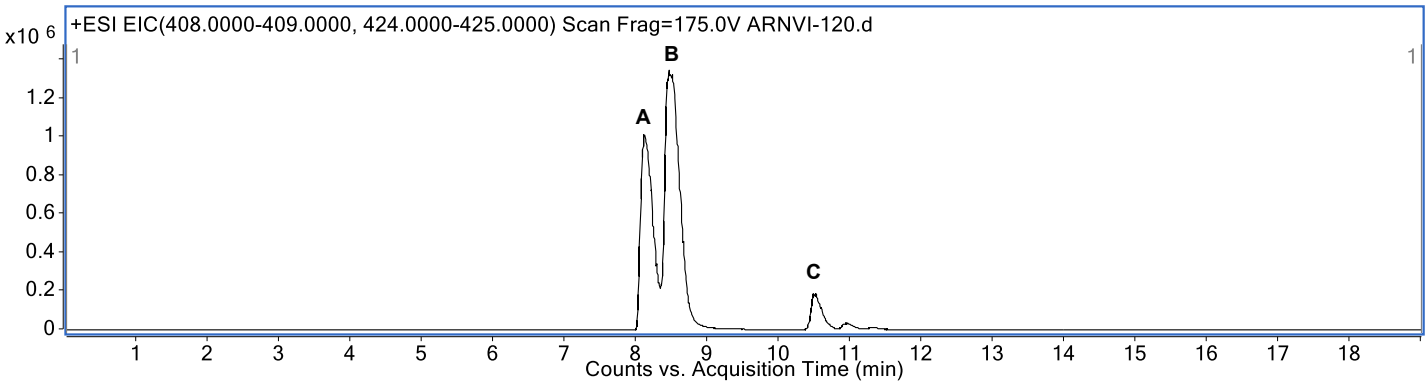
A

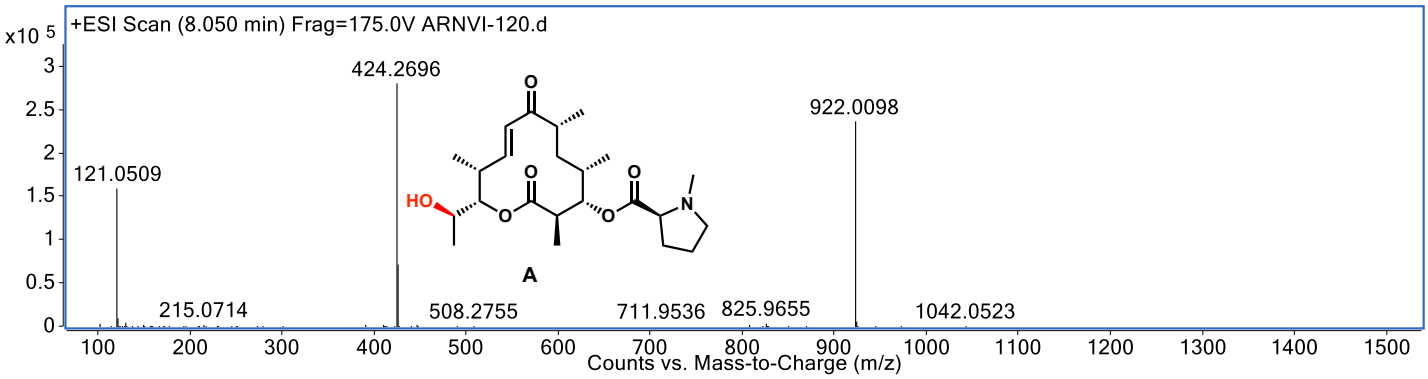

OH
O
O
O
O
O
N

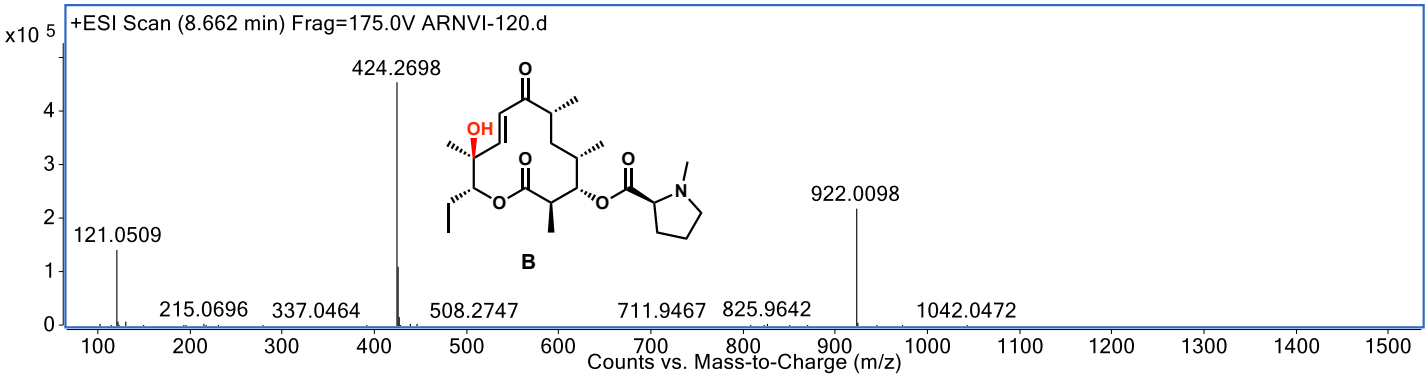
B

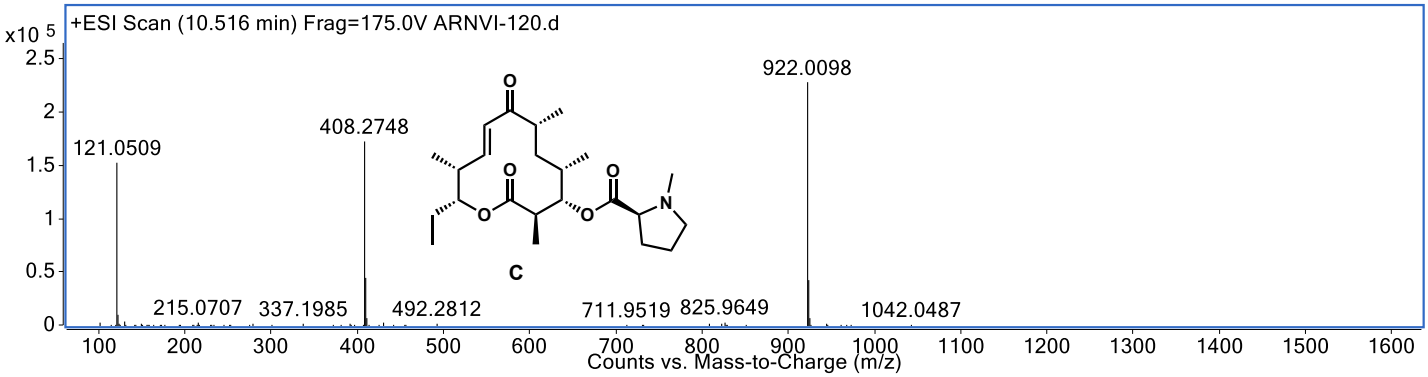

O
O
O
O
O
N

C


+ESI EIC(408.0000-409.0000, 424.0000-425.0000) Scan Frag=175.0V ARNVI-120.d
x10 6
A
B
C
Counts vs. Acquisition Time (min)


+ESI Scan (8.050 min) Frag=175.0V ARNVI-120.d
x10 5
424.2696
922.0098
121.0509
215.0714
508.2755
711.9536
825.9655
1042.0523
A
Counts vs. Mass-to-Charge (m/z)

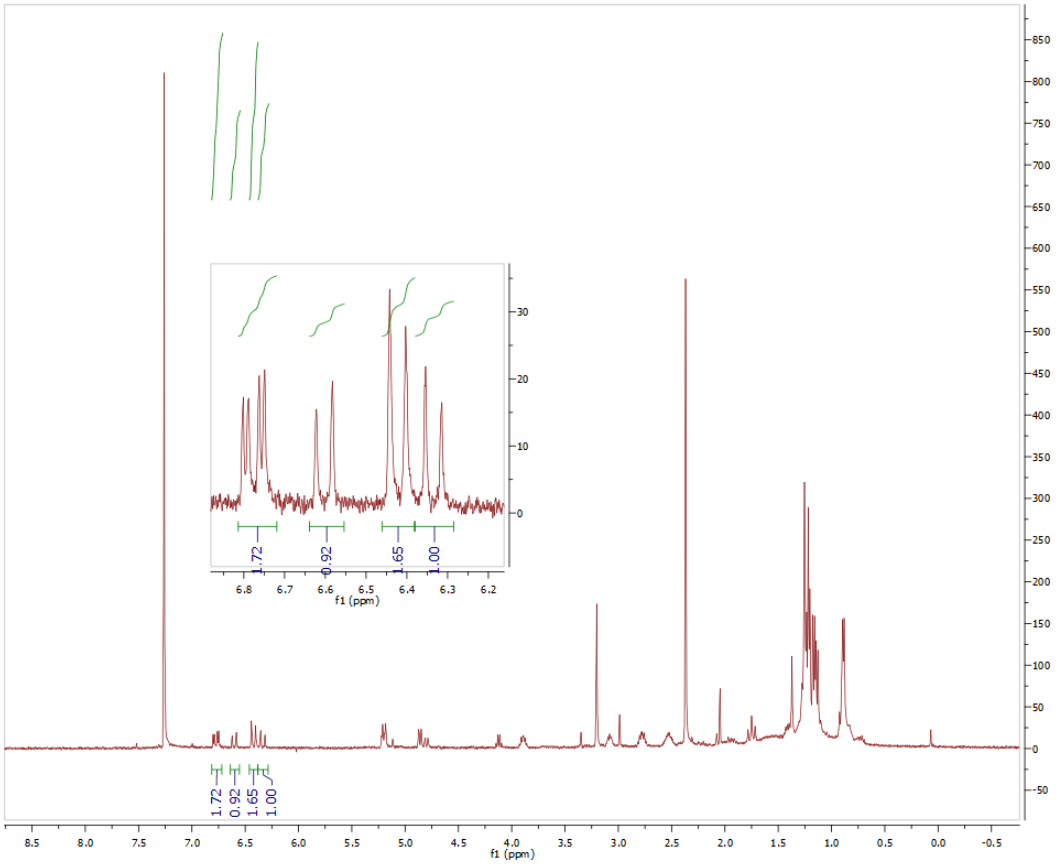

+ESI Scan (8.662 min) Frag=175.0V ARNVI-120.d
x10 5
424.2698
922.0098
121.0509
215.0696
337.0464
508.2747
711.9467
825.9642
1042.0472
B
Counts vs. Mass-to-Charge (m/z)


+ESI Scan (10.516 min) Frag=175.0V ARNVI-120.d
x10 5
922.0098
408.2748
121.0509
215.0707
337.1985
492.2812
711.9519
825.9649
1042.0487
C
Counts vs. Mass-to-Charge (m/z)

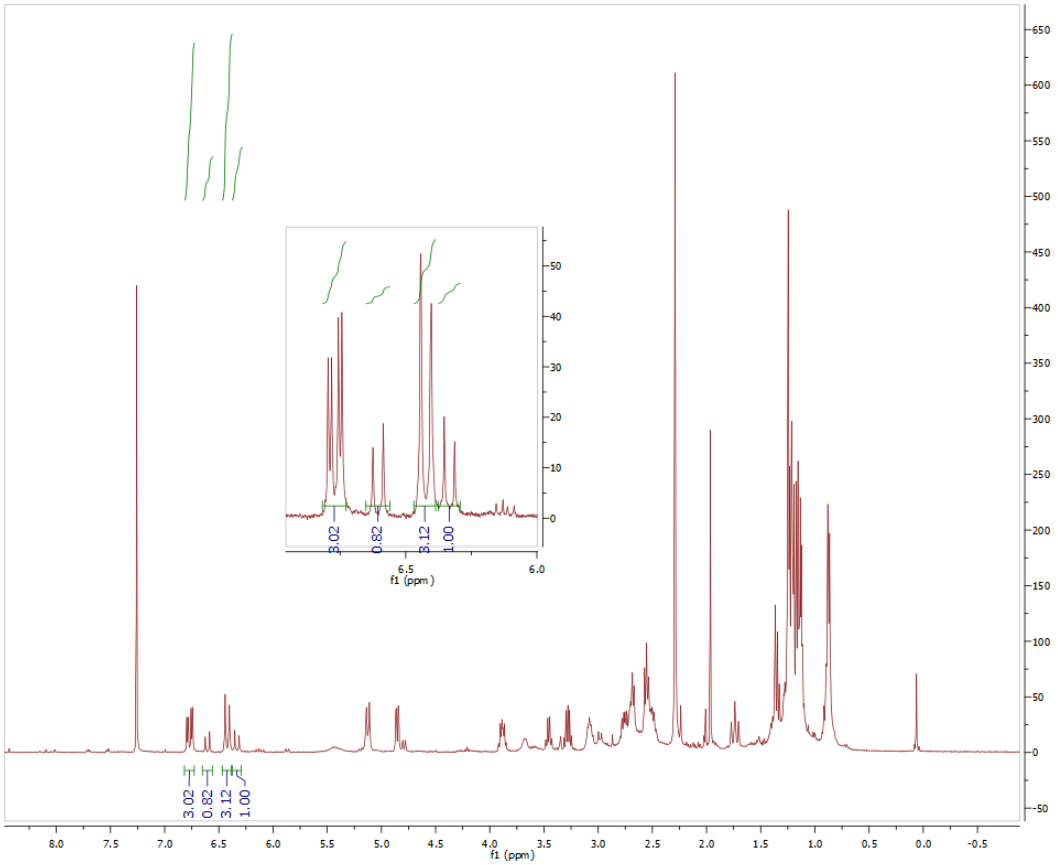
B. ^{1}H NMR data for pre-hydrolysis enzymatic reactions

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

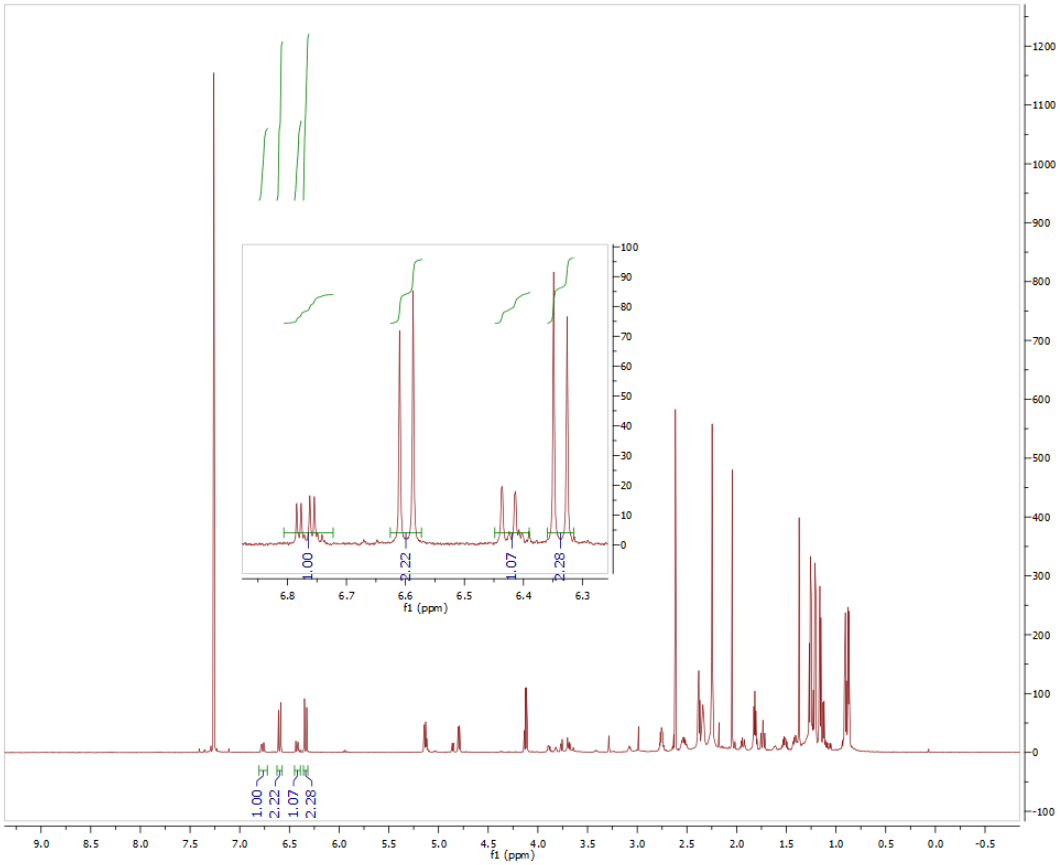
Post-column chromatography mixture of products of enzymatic reaction of substrate **8a**



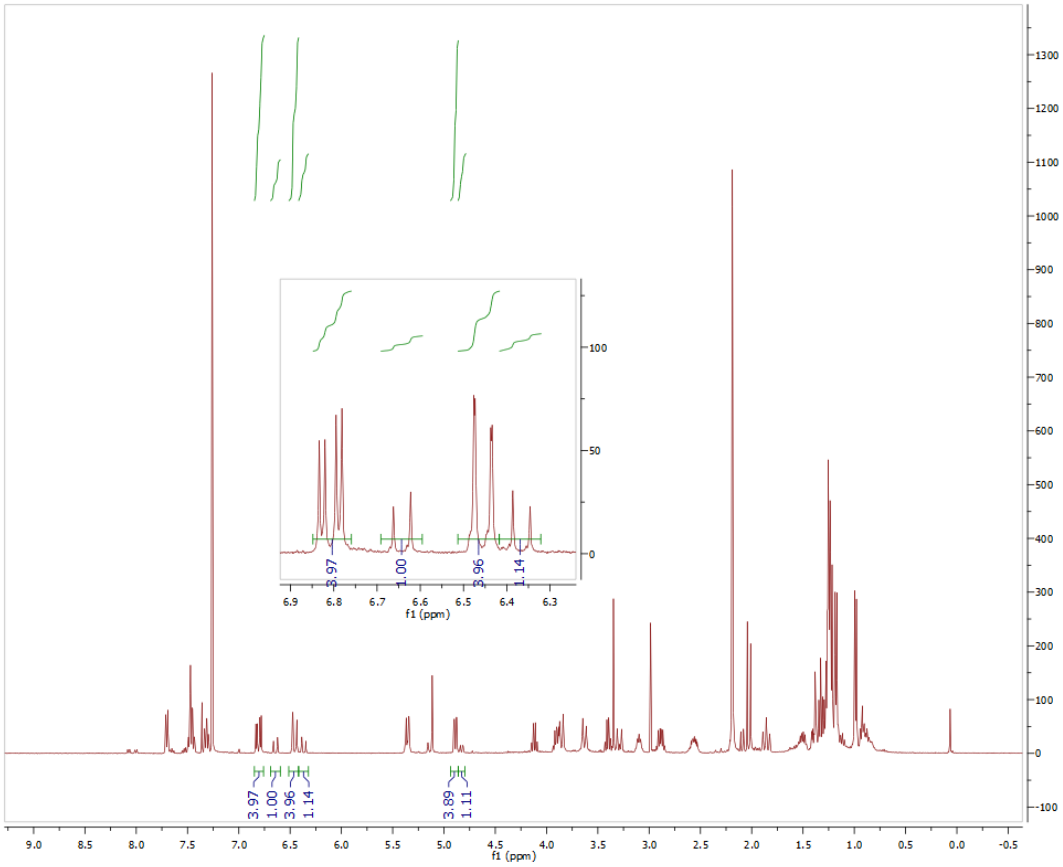
Post-column chromatography mixture of products of enzymatic reaction of substrate **8b**



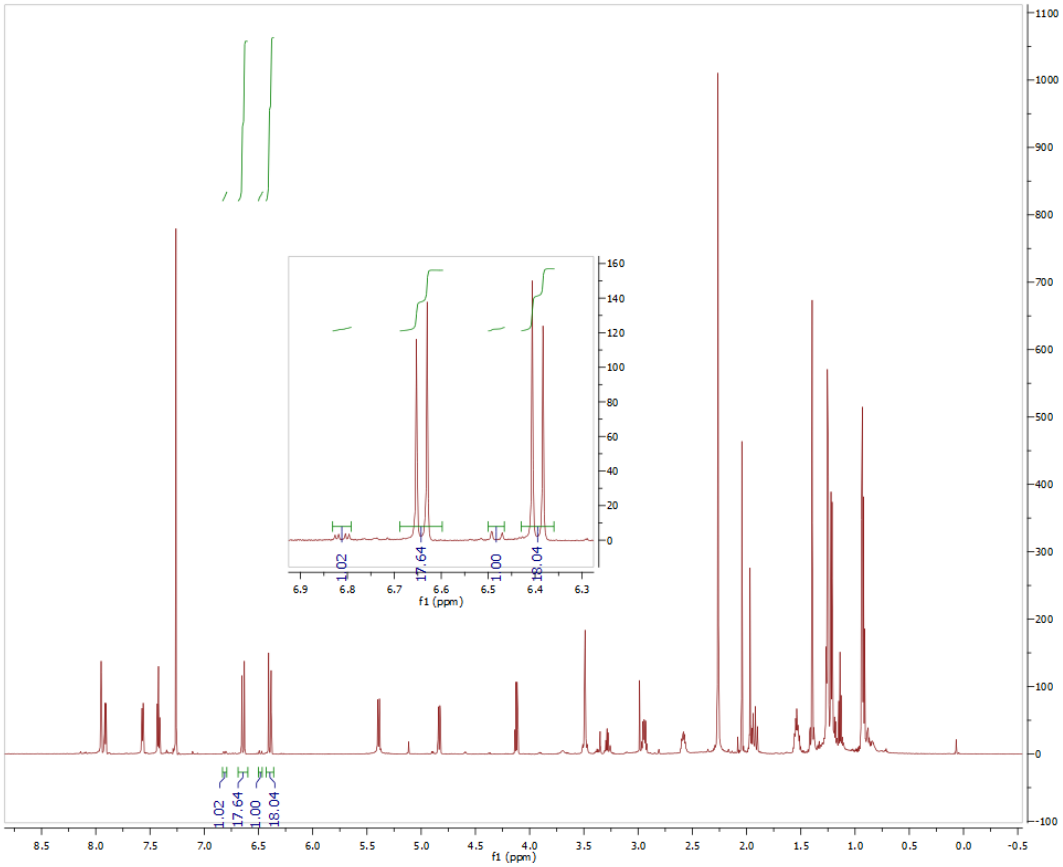
Post-column chromatography mixture of products of enzymatic reaction of substrate **10**



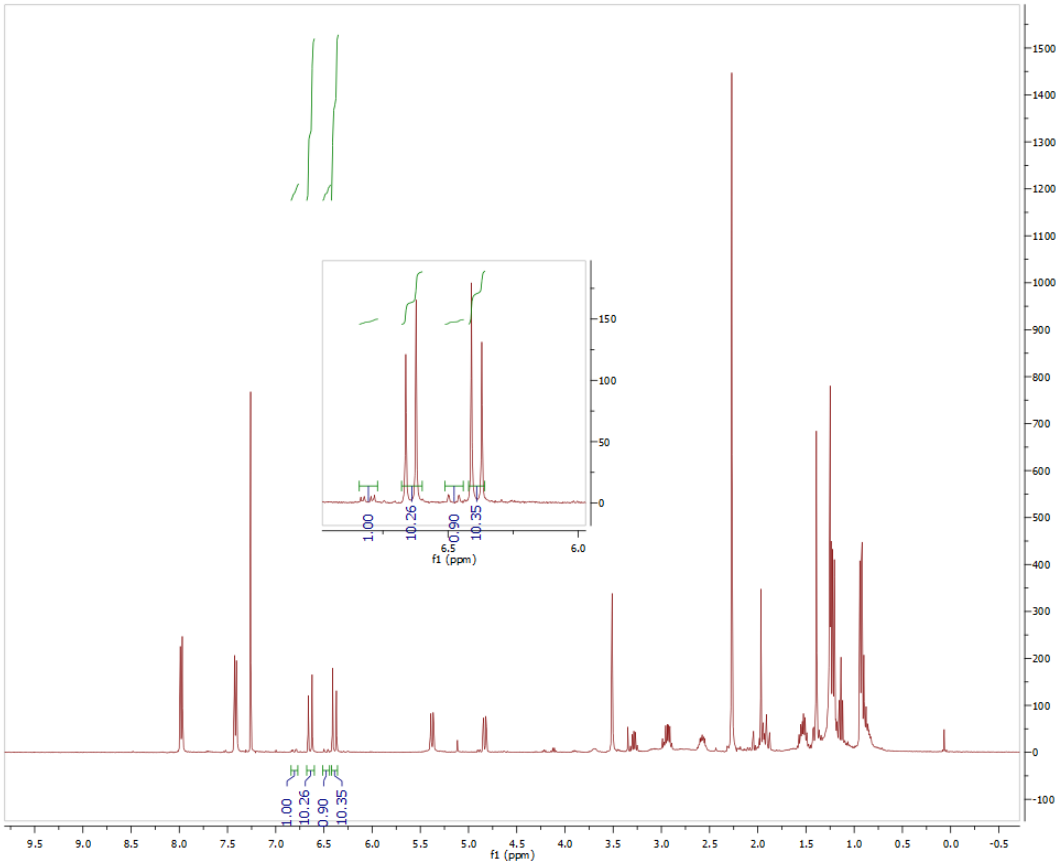
Post-column chromatography mixture of products of enzymatic reaction of substrate **14**



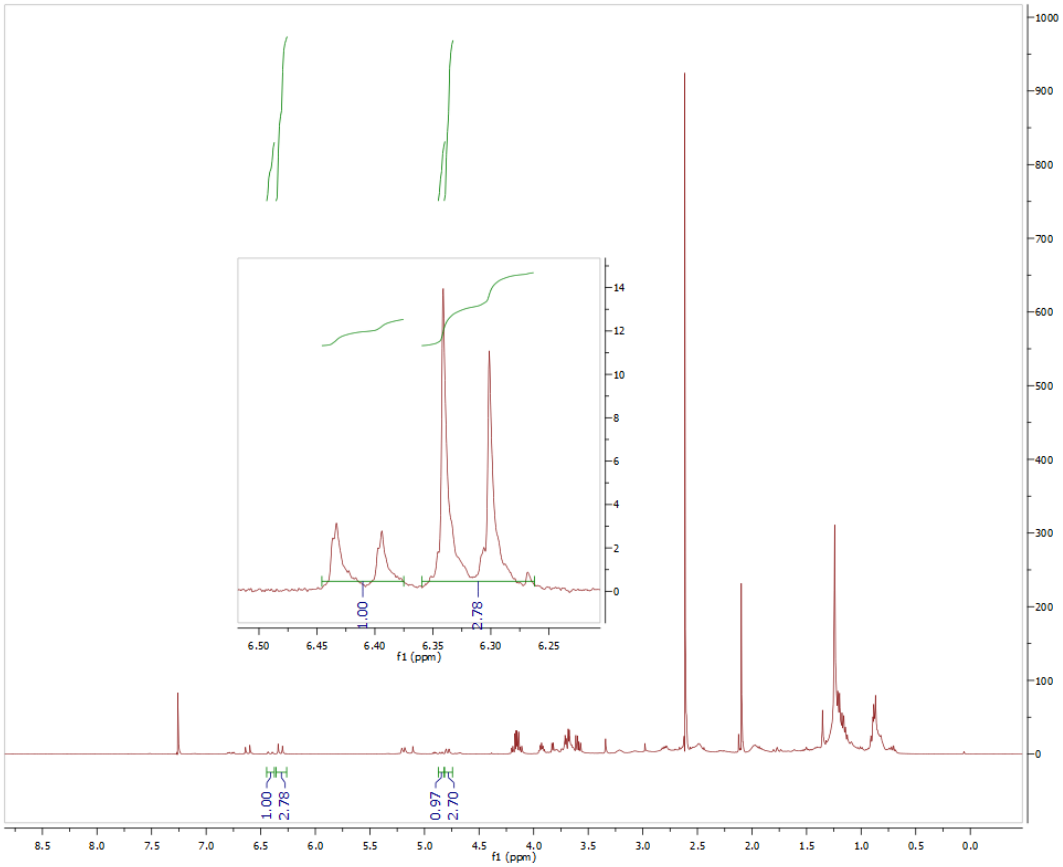
Post-column chromatography mixture of products of enzymatic reaction of substrate **16a**


1.02
17.64
1.00
18.04
f1 (ppm)

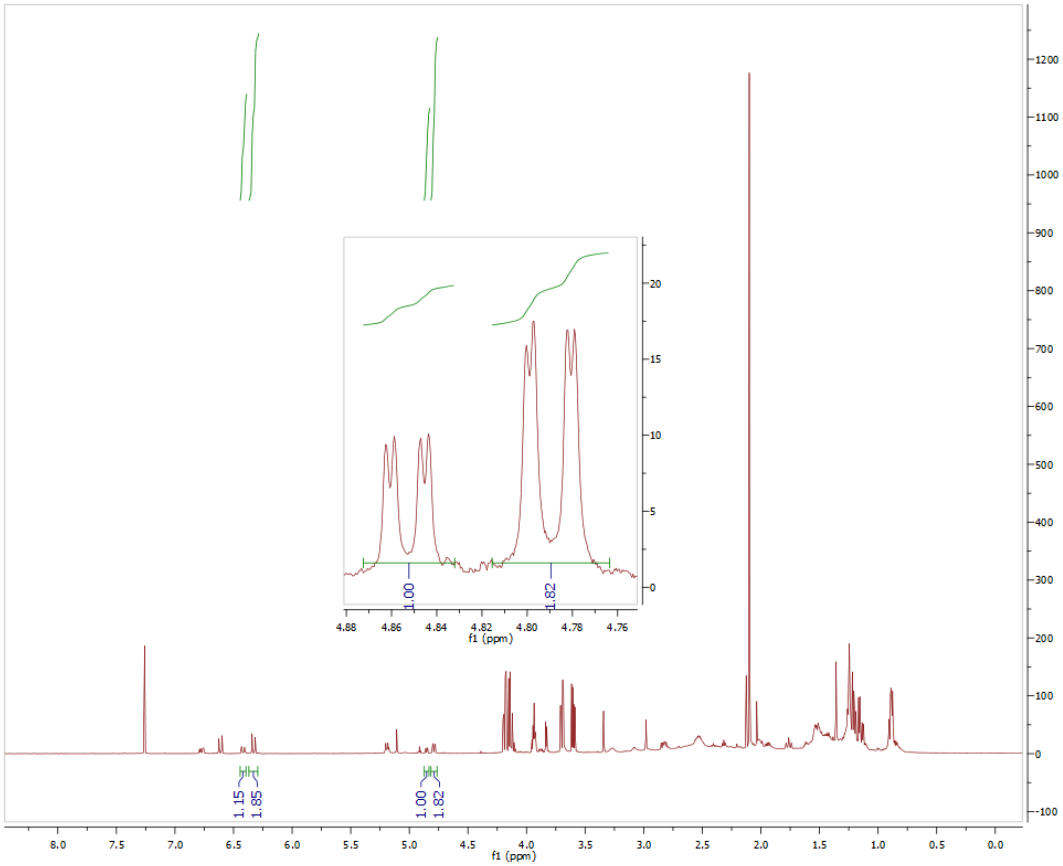
Post-column chromatography mixture of products of enzymatic reaction of substrate **16b**



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



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



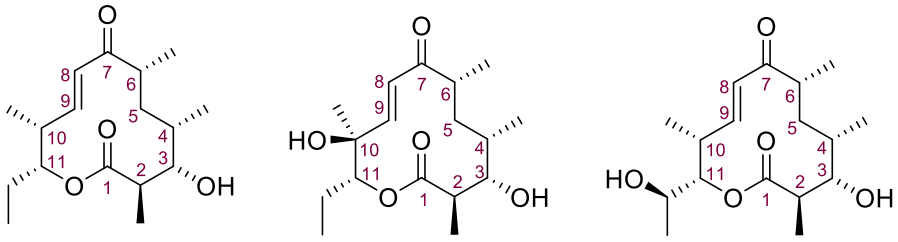
Part III: Product characterization

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:$CHCl_3$ (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 Na_2SO_4, and filtered through a plug of silica gel and concentrated under reduced pressure. The products were analyzed by ^{1}H 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 ^{1}H 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.



10-dml (**18**) methynolide (**19**) neomethynolide (**20**)

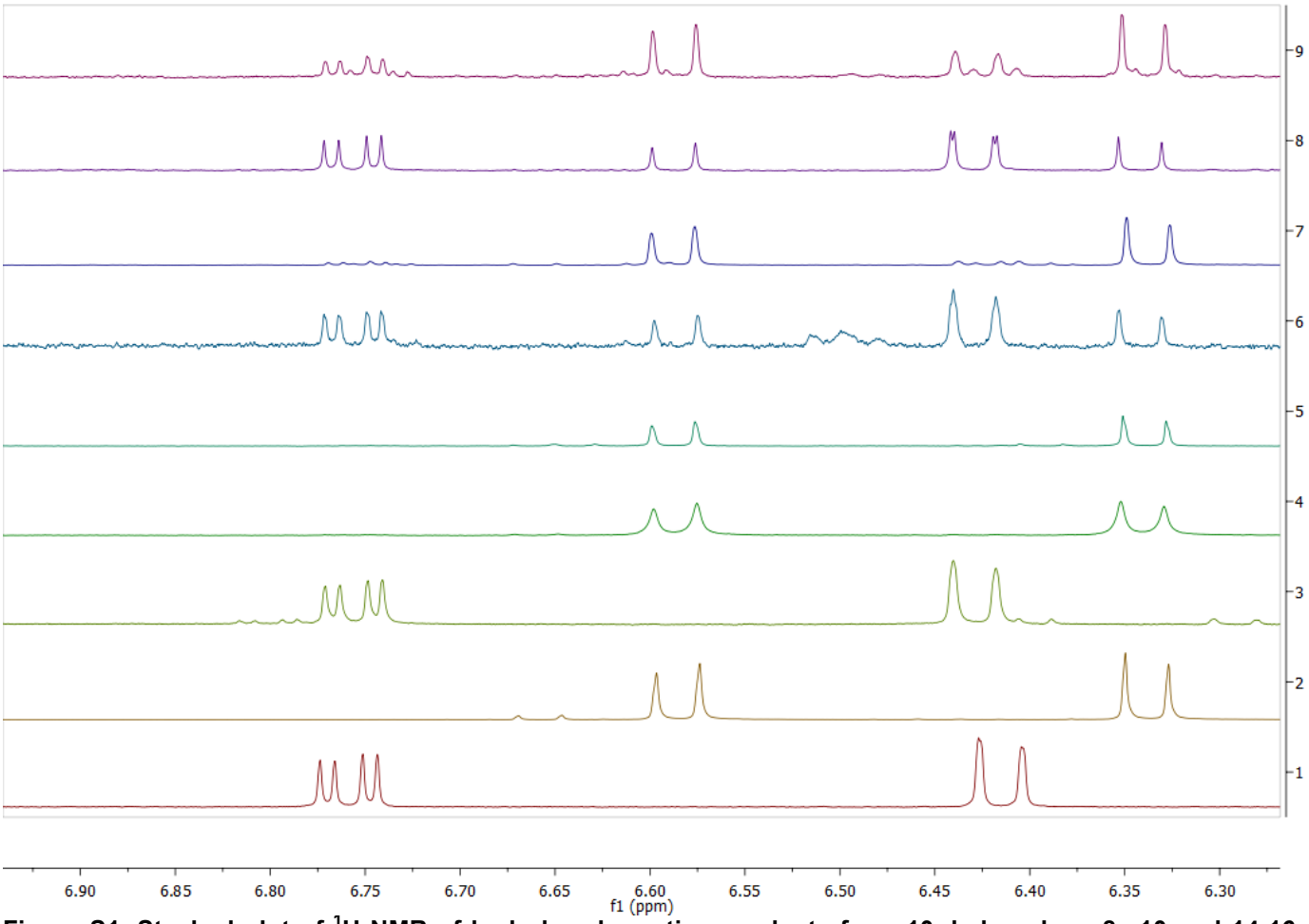
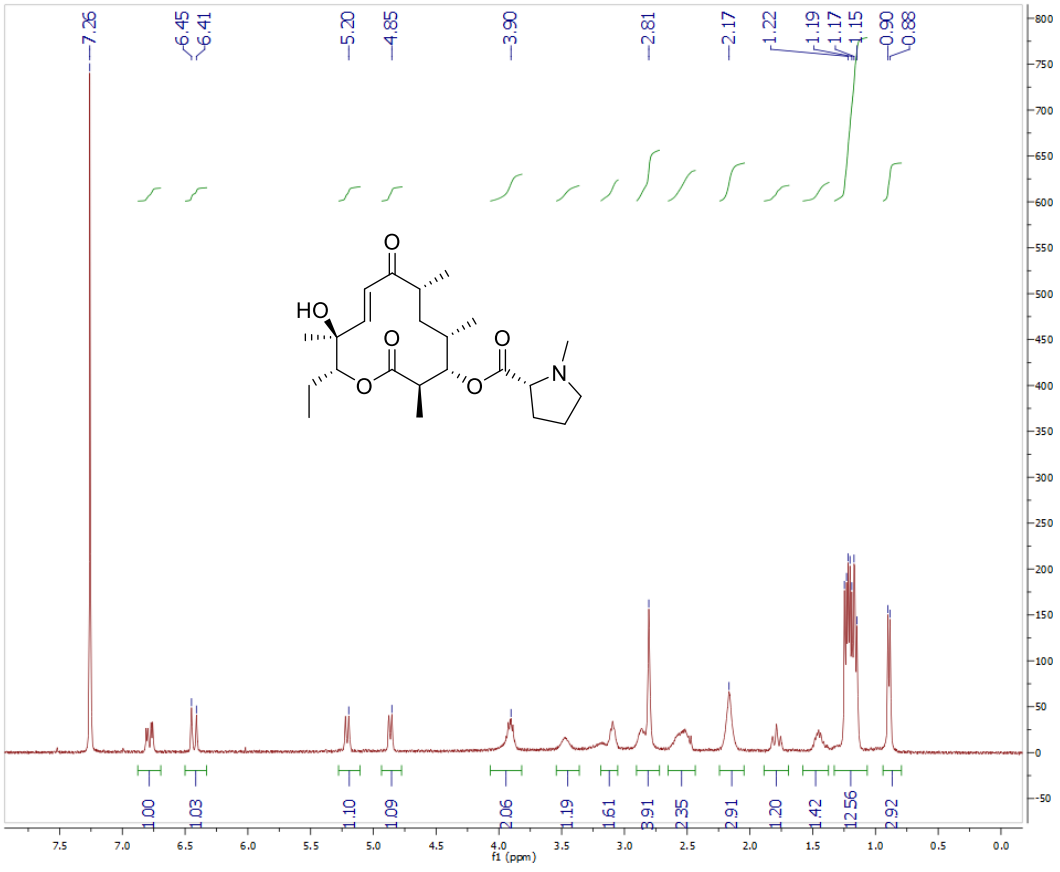
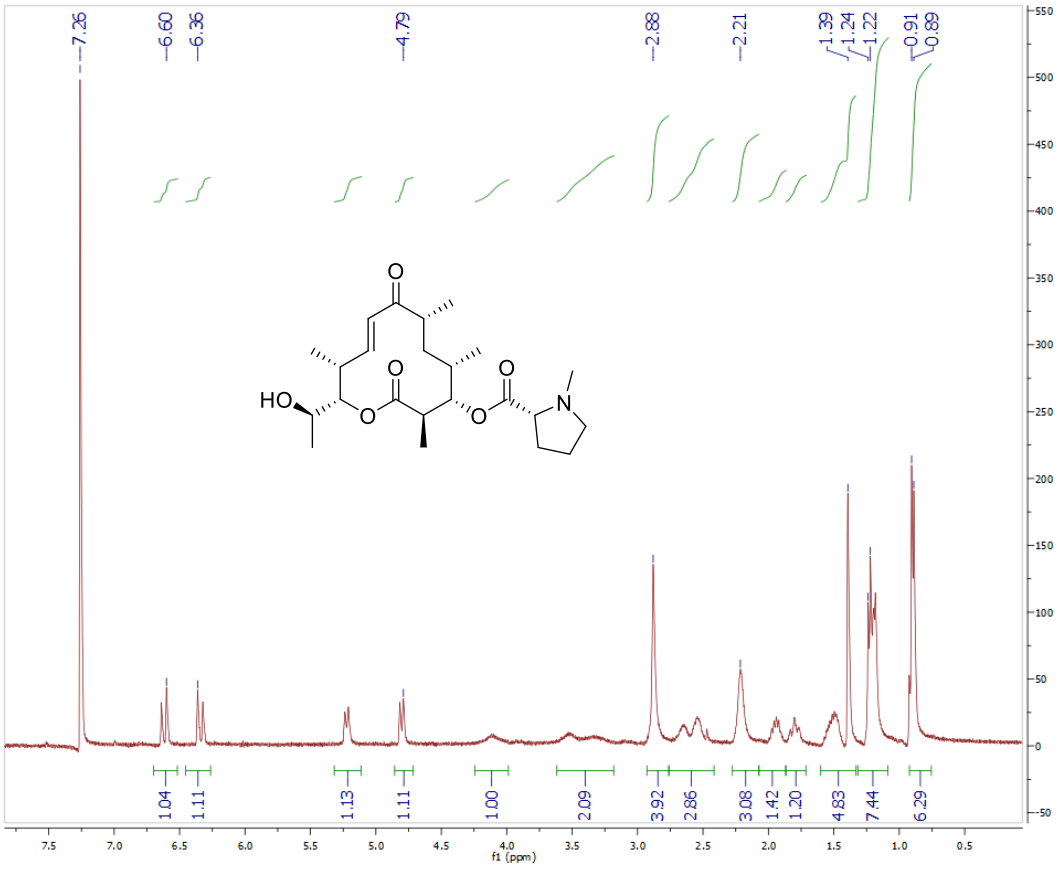


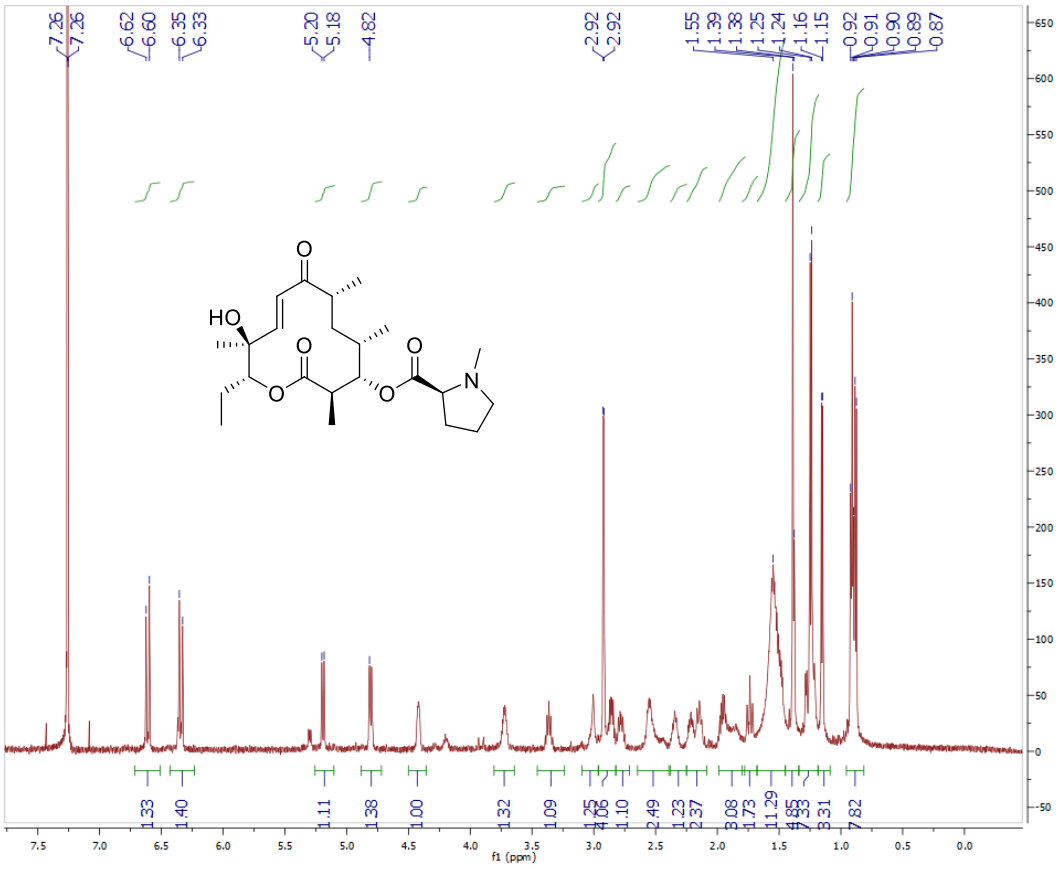
Figure S1. Stacked plot of ^{1}H NMR of hydrolyzed reaction products from 10-dml analogs 8a-10 and 14-16. From bottom to top (1 to 9): 1.10-deoxymethynolide, 2. Methynolide, 3. Neomethynolide, 4. Hydrolyzed PikC$_{D50N}$-RhFRED Reaction Products from substrate **16a**, 5. Hydrolyzed PikC$_{D50N}$-RhFRED Reaction Products from substrate **16b**, 6. Hydrolyzed PikC$_{D50N}$-RhFRED Reaction Products from substrate **14**. 7. Hydrolyzed PikCD50N-RhFRED Reaction

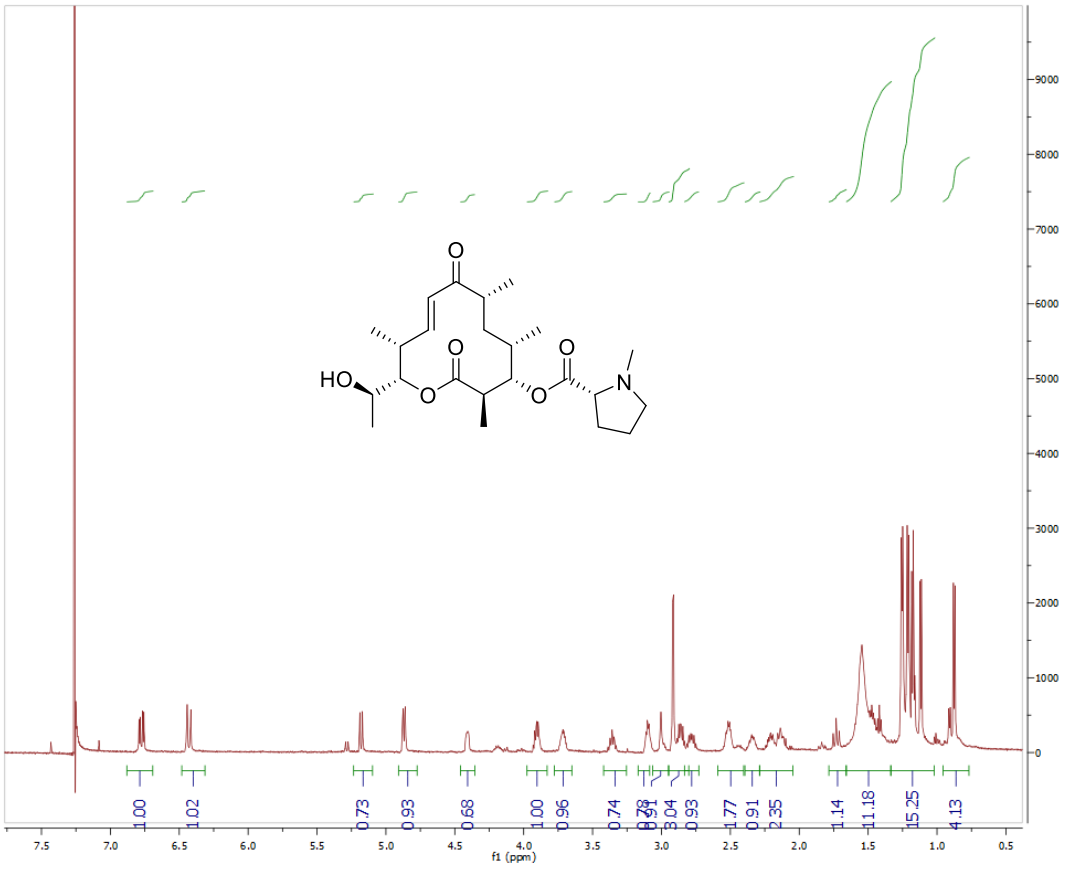
Products from substrate **10**, 8. Hydrolyzed PikC_{D50N}-RhFRED Reaction Products from substrate **8b**, 9. Hydrolyzed PikCD50N-RhFRED Reaction Products from substrate **8a**. From left to right: C_9–H signal, C_8–H signal.

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




7.26
6.60
6.36
4.79
2.88
2.21
1.39
1.24
1.22
0.91
0.89
HO
O
O
O
O
O
N
1.04
1.11
1.13
1.11
1.00
2.09
3.92
2.86
3.08
1.42
1.20
4.83
7.44
6.29
f1 (ppm)


7.26
7.26
6.62
6.60
6.35
6.33
5.20
5.18
4.82
2.92
2.92
1.55
1.39
1.38
1.25
1.24
1.16
1.15
0.92
0.91
0.90
0.89
0.87
HO
O
O
O
O
O
N
1.33
1.40
1.11
1.38
1.00
1.32
1.09
1.25
1.06
1.10
2.49
1.23
2.37
3.08
1.73
11.29
4.85
3.33
3.31
7.82
f1 (ppm)


HO
O
O
O
O
O
N
1.00
1.02
0.73
0.93
0.68
1.00
0.96
0.74
0.78
0.91
3.04
0.93
1.77
0.91
2.35
1.14
11.18
15.25
4.13
f1 (ppm)

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		
a, *b*, *c* (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
R_{sym} or R_{merge} (%)	11.4 (63.7)	9.2 (61.3)
I / σI	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
R_{work} / R_{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
B-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

[1]Values in parentheses are for highest-resolution shell.

Table S1. Crystallographic Data and Statistics.

References

(1) Wang, Y.; Wei, C.; Liu, Q.; Xiang, J.-N. (Glaxo Group Limited) Preparation of thiazole derivatives as modulators of retinoid-related orphan receptor gamma. International patent WO2012027965, March 08, 2012.

(2) Huang, J.; Xi, Z. Tetrahedron Lett. **2012**, *53*, 3654.

(3) Daughtry, K. D.; Xiao, Y.; Stoner-Ma, D.; Cho, E.; Orville, A. M.; Liu, P.; Allen, K. N. *J. Am. Chem. Soc.* **2012**, *134*, 2823

(4) Li, S.; Podust, L. M.; Sherman, D. H. *J. Am. Chem. Soc.* **2007**, *129*, 12940.

(5) Li, S.; Chaulagain, M. R.; Knaff, A. R.; Podust, L. M.; Montgomery, J.; Sherman, D. H. *Prod. Natl. Acad. Sci. USA* **2009**, *106*, 18463.

(6) Methynolide: Oh, H-S.; Xuan, R.; Kang, H-Y. *Org. Biomol. Chem.* **2009**, *7*, 4458-4463

(7) Neomethynolide: Oh, H-S.; Kang, H-Y. *Tetrahedron*. **2010**, *66*, 4307-4317

(8) Holton, J.; Alber, T. *Proc. Natl. Acad. Sci. USA*. **2004**, *101*, 1537.

(9) Emsley, P.; Cowtan, K. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60*, 2126.

(10) Murshudov, G.; Vagin, A.; Dodson, E. *Acta Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 240.

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