Supplementary Information

Covalent Warhead Assembly in Fostriecin Biosynthesis: Malonylation-Lactonisation by a Bifunctional Thioesterase and Enzymatic Demalonylation

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Biochemistry Materials

The chemicals used for the preparation of buffers, solutions and culture media were generally purchased from CARL ROTH, GRÜSSLING, SIGMA ALDRICH and THERMO FISHER SCIENTIFIC. Plastic pipette tips and reaction vessels were purchased from SARSTEDT and GREINER BIO-ONE. Unless otherwise stated, ultrapure water was used and obtained from a B30 water purification system from ADRONA. Buffers and media used for bacterial cultivation were autoclaved at 121 °C for 20 min or sterile filtered before use. Commercial enzymes were purchased from THERMO FISHER SCIENTIFIC and used according to the manufacturer's instructions with the corresponding buffers. Sonication for cell disruption was performed using a Sonoplus HD3100 homogenzier with a MS-73 probe from BANDELIN. Protein concentration was measured on a Varioskan LUX multimode microplate reader (THERMO FISHER SCIENTIFIC). All primers used in this study are listed in Supplementary Table 2 and were purchased from MERCK. Protein purification was performed on an ÄKTA pure chromatography system (CYTIVA) with a U9-L UV detector, a C9 conductivity detector and a F9-R fraction collector. All buffers were filtered through a 0.2 µm nylon or a 0.45 µm cellulose acetate membrane (CYTIVA) before use.

Chemistry Materials

All chemicals and solvents were obtained from ABCR, ACROS ORGANICS, BLD PHARM, CARBOLUTION, CHEMIPUR, DEUTERO GMBH, EURISOTOP, FISHER CHEMICAL, FLUOROCHEM, GRÜSSING, ROTH, SIGMA-ALDRICH, THERMO FISHER, TCI and VWR and were, unless otherwise stated, used without further purification. Dry solvents were obtained from THERMO FISHER. All reactions were performed under argon gas with dry solvents and reagents. The reactions were monitored *via* TLC with Alugram SilG/UV254 TLC-foils from MACHEREY-NAGEL. The substances were detected with UV-light and a $KMnO_4$-stain (1.50 g $KMnO_4$, 10.0 g K_2CO_3, 2.50 mL 5%NaOH, 200 mL H_2O). Products were purified by flash chromatography on SiO_2 (MACHEREY-NAGEL MN Kieselgel 60, 40-63 µm).

NMR spectroscopy and optical rotation

All NMR spectra were recorded with BRUKER AVANCE III HD 500 with the residual solvent signal as internal standard: $CDCl_3$ 7.26 ppm for ^1H, 77.16 ppm for ^{13}C; acetone-d_6 2.05 ppm for ^1H, 29.84 ppm for ^{13}C; MeOH-d_4 3.31 ppm for ^1H, 49.00 ppm for ^{13}C; D_2O 4.79 ppm for ^1H. Signal multiplicities are stated, using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. For ^{13}C NMR, the following abbreviations were used: q =

quarternary, t = tertiary, s = secondary, p = primary. The chemical shifts are reported as values of the δ-scale in [ppm] and the coupling constants *J* in [Hz]. Signal assignments were made with 2D-NMR spectra (COSY, HSQC, HMBC). Optical rotation was recorded on a JASCO P-1020 polarimeter (10 cm cell) using the sodium D line (589 nm). The given value of $[\alpha]^D$ represents the average of 50 individual measurements and is stated as $deg \cdot mL \cdot g^{-1} \cdot dm^{-1}$.

Analytical UHPLC-MS

All UHPLC applications were performed with membrane-filtrated ultrapure water as well as commercially available UHPLC-grade acetonitrile. Samples were always centrifuged or filtered through a PTFE syringe filter from MACHEREY-NAGEL (0.25 µm) before application. UHPLC-MS analysis was performed on a WATERS Acquity Ultra Performance UPLC system (column: BEH C18, 1.7 µm particle size, 2.1 × 50 mm, 100 Å pore size; PDA detector; SQ detector). The column was maintained at 40 °C. Ionisation was performed by ESI in positive ion mode. The mobile phase consisted of H_2O (solvent A) and CH_3CN + 0.1% formic acid (solvent B). A linear solvent gradient of 10 → 90% or 20 → 60% solvent B in solvent A at a flowrate of 0.5 mL/min over 6 min or 8 min was applied. Prior to each run, the column was equilibrated for 2 min with the respective starting solvent composition (i.e., 10% B in A for the 10 → 90% B in A gradient, and 20% B in A for the 20 → 60% B in A gradient). Tune parameters of the mass spectrometer were as follows: 3 kV capillary voltage, 50 V cone voltage, 3 V extrator voltage, 0.10 V radio frequency (RF) lens, 150 °C source temperature, 400 °C desolvation temperature, 50 L/h cone gas flow, 650 L/h desolvation gas flow, 15 low and high mass resolution, 0.5 ion energy, −851.50 V multiplier voltage. MassLynx software version 4.1 was used for data analysis.

High resolution mass spectra were obtained using the UltiMate 3000 UHPLC system from THERMO FISHER SCIENTIFIC, coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer with an ESI ion source. A flowrate of 0.5 mL/min applied and prior to each run, the corresponding column (BEH C18, 1.7 µm particle size, 2.1 × 50 mm, 100 Å pore size (1); YMC-Triart Bio C4, 1.9 µm particle size, 2.1 × 50 mm, 300 Å pore size (2); YMC-UltraH Hydrosphere C18, 2 µm particle size, 2.0 × 50 mm, 120 Å pore size (3)) was equilibrated for 2.5 min with the respective starting solvent composition. All columns were thermostated at 40 °C. The following mobile phases were used: H_2O + 0.1% formic acid (solvent A), CH_3CN + 0.1% formic acid (solvent B), H_2O + 0.1% difluoroacetic acid (solvent C), 10 mM NH_4CO_2H, pH 3 (solvent D) and CH_3CN + 0.1% difluoroacetic acid (solvent E). For MS and MS/MS anlysis of in vitro assays and chemically synthesized compounds using column 1, the following gradient was applied: 0 – 1.5 min 10% B in A, followed by 1.5 – 8.5 min 10 → 90% B in A. Analysis of malonyl-CoA compounds was performed using column 2 with the following

gradient: 0 – 1.5 min 0% B in D, followed by 1.5 – 8.5 min 0 → 60% B in D. For analysis of protein samples using column 3, the following gradient was applied: 0 – 1.5 min 30% E in C, followed by 1.5 – 15.5 min 30 → 50% E in C. Tune parameters of the mass spectrometer were as follows: 3.70 kV spray voltage, 300 °C capillary temperature, 438 °C Aux gas heater temperature, 30 arb. units sheath gas, 15 arb. units aux gas, 100 µA max. spray current, 55 arb. units S-lens RF level. Orbitrap settings for MS of small molecules: 70000 resolution, 3e6 automatic gain control (AGC) Target, 200 ms maximum injection time. Orbitrap settings for targeted MS/MS of small molecules: 140000 resolution, 3e6 AGC target, 100 ms maximum injection time, 9 loop counts (for Selected Ion Monitoring); 35000 resolution, 5e4 AGC Target, 100 ms maximum injection time, 9 loop counts, 4.0 m/z isolation window, 30 or 35 normalised collision energy (NCE). Data analyses were perfromed using Xcalibur software version 4.5.

(Semi-)Preparative HPLC

All HPLC applications were performed with membrane-filtrated ultrapure water as well as commercially available HPLC-grade acetonitrile. Samples were always centrifuged or filtered through a PTFE syringe filter from MACHEREY-NAGEL (0.25 µm) before application. Semi-preparative purification was generally achieved using a WATERS HPLC system (600 controller, 2487 Dual wavelength absorbance detector), equipped with a PHENOMENEX Kinetex C18 (5 µm particle size, 50 × 30 mm, 100 Å pore size) or a YMC Hydrosphere C18 column (5 µm particle size, 150 × 10 mm, 120 Å pore size). The mobile phase comprised H_2O + 0.1% formic acid (solvent A) and CH_3CN + 0.1% formic acid (solvent B). Preparative HPLC purification was performed using a SHIMADZU HPLC system (CBM-20A controller, SPD-M20A PDA detector), equipped with a PHENOMENEX Gemini 5 µm NX-C18 (5 µm particle size, 250 × 21.2 mm, 110 Å pore size). The mobile phase was composed of H_2O + 0.1% formic acid (solvent A) and CH_3CN (solvent B). Unless otherwise stated, the following solvent gradient (flowrate 20 mL/min) was used for preparative HPLC purification: 0 – 20 min: 30% B, 20 – 65 min: 30% → 55% B, 65 – 67 min: 55% → 95% B, 67 – 72 min: 95% B, 72 – 74 min: 95% → 65% B, 74 – 97 min: 65% B.

Analytical Size Exclusion Chromatography

The integrity of module proteins was analyzed by size exclusion chromatography (SEC) using an AGILENT HP 1100 HPLC system equipped with a variable wavelength detector (λ = 200, 214, 254, and 280 nm; 10 mm path length; 14 µl cell volume) and a Superdex 200 Increase 10/300 GL column from CYTIVA (30 cm × 10 mm, 8.6 µm particle size). All chromatographic separations were performed isocratically at a flow rate of 75 µL/min and room temperature for

32 min, using a degassed and filtered (0.2 µm) 200 mM sodium phosphate buffer (pH 6.8) as the mobile phase. The column was always equilibrated with at least one column volume (CV) of the corresponding buffer prior to each run.

Strains, primers & DNA / protein sequences

Supplementary Table 1: Bacterial strains used in this study.

Organism	Strain	Genotype
E. coli	One Shot® TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
	BL21™(DE3)	F- *omp*T *hsdS*B (rB-. mB-) *gal dcm* (DE3)
	BAP1	BL21(DE3) Δ*prp*RBCD::T7prom-*sfp*,T7prom-*prp*E

Supplementary Table 2: Oligonucleotides used in this study.

Name	5'→3' Primer Sequence	Purpose
fosM_fwd	GCCTGGTGCCGCGCGGCAGCCACATG CTGCGTACCACACG	cloning of pET-28a(+)_*fosM*
fosM_rev	CAGTGGTGGTGGTGGTGGTGCTCGATT AGCTTGCCGGACTATCTG	cloning of pET-28a(+)_*fosM*
fosTE_fwd	ACTTTAAGAAGGAGATATACCATGGGTG GTGATGCGGG	cloning of pET-28a(+)_*fosTE*
fosTE_rev	AGTGGTGGTGGTGGTGGTGCGCCGGA CGGGTCAGACCC	cloning of pET-28a(+)_*fosTE*
fosACP7_fwd	CAGCAGCGGCCTGGTGCCGCGCGGCA GCCATGATGCTGGCACAGCGGG	cloning of pET-28a(+)_*fosACP*
fosACP7_rev	GCCGGATCTCAGTGGTGGTGGTGGTG GTGCTTAGCTCGCACCACCAACACGTT C	cloning of pET-28a(+)_*fosACP7*
fosACP8_fwd	GTTTAACTTTAAGAAGGAGATATACCATG GAAAGCGGTGCGGG	cloning of pET-28a(+)_*fosACP8*
fosACP8_rev	ATCTCAGTGGTGGTGGTGGTGGTGCGC AGGTGCTGCGTTATCC	cloning of pET-28a(+)_*fosACP8*
fosKR8_fwd	GCGGCCTGGTGCCGCGCGGCAGCCAT GAAACCGATGGTTGGCG	cloning of pET-28a(+)_*fosKR8*
fosKR8_rev	ATCTCAGTGGTGGTGGTGGTGGTGCTT AGGTATCATCAAAATCTGCAAACAG	cloning of pET-28a(+)_*fosKR8*
fosAT8_fwd	CCTGGTGCCGCGCGGCAGCCACACCG AAGCACCGGTGGG	cloning of pET-28a(+)_*fosAT8*
fosAT8_rev	CAGTGGTGGTGGTGGTGGTGCTTATTC GCGTGCCAGATCATC	cloning of pET-28a(+)_*fosAT8*
fosKS8-AT8-KR8_fwd	GTTTAACTTTAAGAAGGAGATATACCATG AGCACCAACGAAGATAAAC	cloning of pET-28a(+)_*fosKS8-AT8-KR8*

fosKS8-AT8-KR8_rev	ATCTCAGTGGTGGTGGTGGTGGTGCGC GGTATCATCAAAATCTGC	cloning of pET-28a(+)_*fosKS8-AT8-KR8*
fosMod8(S159A)_fwd	GGGTTATGCGAGTAGCGGTTGGCTGGC ACAGGCAGC	FosMod8 mutagenesis
fosMod8(S159A)_rev	CCGCTACTCGCATAACCCAGCAGTGCAT AAGGACGACCATC	
fosMod8(R227L)_fwd	GGCACCTATCTGGGTATGTTTCGTGGTT GGCAGCCTCGTC	FosMod8 and FosTE mutagenesis
fosMod8(R227L)_rev	CATTATGATGGACTGACCGCACTGGGC ACCTATCTGGGTATGTTT	
fosMod8(R198L)_fwd	CTGGAAATGCTGAAAGCAATGACCTATG AAGTTGTGGAACGTCGTATGC	
fosMod8(R198L)_rev	TGTTAGATACCTATCCGCCTGATAGCAT GACCCTGGAAATGCTGAAAGCAATG	
fosMod8_fwd	GTTTAACTTTAAGAAGGAGATATACCATG AGCACCAACGAAGATAAACTGC	cloning of pET-28a(+)_*fosMod8*
fosMod8_rev	ATCTCAGTGGTGGTGGTGGTGGTGCGC CGGACGGGTCAGACCC	
fosMod8(-TE,+jerTE)_fwd	ATTTTGTTTAACTTTAAGAAGGAGATATA CCATGAGCACCAACGAAGATAAAC	cloning of pET-28a(+)_*fosMod8(jerTE)*
fosMod8(-TE,+jerTE)_rev	AACAACTGCGCTACCTTCACCATCACCG GCAGGTGCTGCGTTATCCAG	
fosACP8_jerTE	GTTTTTGATCATCCGACGCTGGGTGCC CTGGTTACCCATTTACTGGATAACGCAG CACCTGCGAGCCAGCTGGATAGTGGCA CCCCAGCGCGTGAAGCCAGTAGTGCG CTGCGTGAT	cloning of pET-28a(+)_*fosMod8(jerTE)*
jerTE(fosMod8)_fwd	GTTACCCATTTACTGGATAACGCAGCAC CTGCCGGTGATGGTGAAGGTAG	cloning of pET-28a(+)_*fosMod8(jerTE)*
jerTE(fosMod8)_rev	GCCGGATCTCAGTGGTGGTGGTGGTG GTGCGCCAGCTGCAGGGCATGTTC	
fos^{N}DD6_(C-His$_{6}$)_fwd	AAATAATTTTGTTTAACTTTAAGAAGGA GATATACCATGGCTTCAGAAAATCAACT ATTAGATTATTTGAAGCGCG	cloning of pET-28a(+)_*fosMod7(N-His$_{6}$-^{N}DD6)* and pET-28a(+)_*fosMod7-fosTE(N-His$_{6}$-^{N}DD6)*
fos^{N}DD6_(C-His$_{6}$)_rev	ACAATAACAATCGGATCACGCGCAGCG TCCTCCACAT	
fos^{N}DD6	ACAATAACAATCGGATCACGCGCAGCG TCCTCCAC	
fosMod7(w/o_interdomain_ C-His$_{6}$)_fwd	GTGGAGGACGCTGCGCGTGATCCGATT GTTATTGTTGG	
fosMod7(w/o_interdomain_ C-His$_{6}$)_rev	TAGCAGCCGGATCTCAGTGGTGGTGGT GGTGGTGCGCGCTAATGCCAAATTCTT TTTC	
^{N}DD6-fosMod7(N-His$_{6}$)_fwd	GCGGCCTGGTGCCGCGCGGCAGCCAC ATGGCTTCAGAAAATCAAC	
^{N}DD6-fosMod7(N-His$_{6}$)_rev	ATCTCAGTGGTGGTGGTGGTGGTGCTT AGCTAATGCCAAATTCTTTTTC	
^{N}DD6-fosMod7-fosTE(N-His$_{6}$)_rev	ACCCGCATCACCACCTTCTGCCAGGGC ACTTGC	cloning of pET-28a(+)_*fosMod7-fosTE(N-His$_{6}$-^{N}DD6)*
fosTE(fosMod7)_fwd	AGTGCCCTGGCAGAAGGTGGTGATGC GGGTGC	
fosTE(fosMod7)_rev	GCCGGATCTCAGTGGTGGTGGTGGTG GTGCTTACGGACGGGTCAGACC	

Supplementary Table 3: Plasmids used in this study.

Plasmids	Fusion Tag	Selectable Marker	Source
pET-28a(+)_*fosMod7(N-His$_6$-^{N}DD6)*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod7-fosTE(N-His$_6$-^{N}DD6)*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8(R198L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8(R227L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8(R198L/R227L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8(S159A)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosMod8(jerTE)*	C-terminal His$_5$-tag	Kanamycin	This study
pET-28a(+)_*fosKR8*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosACP7*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosACP8*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosTE*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosTE(R198L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosTE(R227L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosTE(R198L/R227L)*	C-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosM*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28b(+)_*fosH*	N-terminal His$_6$-tag	Kanamycin	Plasmid with insert was purchased from GENSCRIPT
pET-28a(+)_*fosTEII*	N-terminal His$_6$-tag	Kanamycin	Plasmid with insert was purchased from GENSCRIPT
pHis8_*matB*	N-terminal His$_8$-tag	Kanamycin	Obtained from Prof. T. Gulder (Univ. / HIPS Saarland)
pET-28a(+)_*pikTE*	N-terminal His$_6$-tag	Kanamycin	Purchased from GENEWIZ[1]
pET-28a(+)_*fosAT8*	N-terminal His$_6$-tag	Kanamycin	This study
pET-28a(+)_*fosKS8-AT8-KR8*	C-terminal His$_6$-tag	Kanamycin	This study

FosMod8-His$_6$ (pET-28a(+)_*fosMod8*)

MSTNEDKLRHYVKELTGDLLRTRGRLRELEAAGNEPIALVGMACKYPGGVASPEDLWRLVAEGRDAISPFPADRG
WDLGRLPAAGGGFLHDAAEFDAGFFGISPRDAAAMDPQQRIALETCWEAVERSGISADSLRGKPVGVFMGGAVQG
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MATPYAFVEFGRQGGLSADGRCRSFSADAEGTGWSEGVGVVVLERLSDARRNGHEVLAVVRGSAVNQDGASNGLT
APNGPSQQRVIVRALAGAGLSTSDVDVMEAHGTGTRLGDPIEAQALIATYGQGRAEGRPLWLGSLKSNIGHTQAA
AGVGGVIKMVMAMRHGVLPRTLHVSQPSPHVDWSAGAVELLTRARQWPQTGRARRAGVSSFGISGTNAHVILEHE
PVESTEAPVGSAQVPVESTQALVVAGELPWVVSGRTEGAVRAQAARLAAFVAGRGGGALDVGGVGLALVSSRSVF
DHSAVVSGGSLDELLAGVGGVARGDGSAAGGVVFERRVAGGVGVAFSGQGSQRPGMGRELYGRFPVFAAALDEVC
AEVEAQTGAELLGVVFGDDAGVLEDTGVAQPALFAVEVALYRLAESFGVRADVLIGHSLGELSAAYVAGVWSLAD
AVRVVVARARLMGSLPSGGRMVAVEATEEEVAPLVADVAAAGGMVSLAAVNAPGAVVVSGQDAAVDQIADIFAGR
GRRTRALAVSHAFHSPLMEPMLAEFADVLAQVEFRAPSIPVVSNVTGTIADAEELCSPEYWVRHVREAVRFGDGV
GAVLAQGVATVVELGPDPVLTALGERVRAASAERDSAARDVAFVPTLSRRSTDTRAFLGMLARVHARGHQVDWTA
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SGEDQVALRADGARVRRLRPAGPDGAPEPVRPVAPESLVAPEGADATGRTGDPQPPAAREPWWSHGSVLITGGTG
ALGAHTARRLAEQGAPHLVLASRQGPDAPGAADLRAELAAHGATVDLVSCDVTSRDEVAALAADLAGRGAPVGAV
VHTAGVAAEHPLADLDATEFAAVVDAKVTGAVILDEVLGDGLAAFVVYSSIAGTWGSTRGGAYAAGNAFLDALVE
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GRPNHLFADFDDTESGAGHPDAGRTGAAQPGEWQRLPDLPLADQRPYVLDIVRREAARVLGHADAGTITEDQEFL
ALGFDSLAAVELRGRLTVLTGLALPSSLVFDHPTLGALVTHLLDNAAPGGDAGASPAPGVSAAPSASVAAAPPQD
SNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHVTTLGHGEAEPQLVCFPPFAPVDGSLQFA
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GGTAGAGCACGTCGTGCGGGTGTTAGCAGCTTTGGTATTTCAGGCACCAATGCACATGTGATTCTGGAACATGAA
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CGTGTGCGTGCAGCAAGCGCAGAACGTGATAGCGCAGCCCGTGATGTTGCCTTTGTTCCGACACTGAGCCGTCGT
AGCACAGATACCCGTGCATTTCTGGGTATGCTGGCACGTGTTCATGCACGTGGTCATCAGGTGGATTGGACCGCA
CTGGGTCGTGCAGATGATCTGGCACGCGAACTGCCGACCTATGCCTTTCAGCATGAACATCATTGGCTGAAAGGT
GCAAGCGTTCGTCCGGGTTCAGCAGCAAGCCGTACCGCAGGTAGTGATGGTGCCTTTTGGAAAGTTGTTCAAGAA
CAGGATCTGCAGCGTCTGGCAAGCGATCTGGGTGTTGATCCGGATGCACCGCTGCATACCGTTCTGCCTGCATTA
GGTGATTGGCATCAGACCCATATTGAAGCAAGCGAAACCGATGGTTGGCGTTATCGTGTTGCATGGGAACGTCCG
ACCGCACAGCATGCACCGGAAGGTCCGGCAACACTGCATGGCACCTGGCTGATTGTTGTTCCGGAAGGTGATCTG
CGTGCAGGTCATCTGCTGGATAATGATGGTCTGCATGATGGCCTGCACGGTGAAGTTCGTCGTGTTCTGACCGAT
GCCGGTGCAGAAGTTAAAAGCCTGAGCCTGGCTCCGGAAGATATTGATCGTCAGACCATTGCAAAACTGCTGAAT
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His$_6$-^{N}DD6-FosMod7 (pET-28a(+)_*fosMod7(N-His$_6$-^{N}DD6)*)

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TAA

His$_6$-^{N}DD6-FosMod7-FosTE (pET-28a(+)_*fosMod7(N-His$_6$-^{N}DD6) -fosTE*)

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FosMod8(JerTE)-His$_5$ (pET-28a(+)_*fosMod8*(C-His$_5$)-*jerTE*)

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FosMod8(S159A)-His$_6$ (pET-28a(+)_*fosMod8*(S159A))

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CCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosMod8(R198L/R227L)-His_6 (pET-28a(+)_*fosMod8*(R198L/R227L))

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FosMod8(R198L)-His_6 (pET-28a(+)_*fosMod8*(R198L))

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CCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosMod8(R227L)-His_6 (pET-28a(+)_*fosMod7*(R227L))

MSTNEDKLRHYVKELTGDLLRTRGRLRELEAAGNEPIALVGMACKYPGGVASPEDLWRLVAEGRDAISPFPADRG
WDLGRLPAAGGGFLHDAAEFDAGFFGISPRDAAAMDPQQRIALETCWEAVERSGISADSLRGKPVGVFMGGAVQG
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AGVGGVIKMVMAMRHGVLPRTLHVSQPSPHVDWSAGAVELLTRARQWPQTGRARRAGVSSFGISGTNAHVILEHE
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DHSAVVSGGSLDELLAGVGGVARGDGSAAGGVVFERRVAGGVGVAFSGQGSQRPGMGRELYGRFPVFAAALDEVC
AEVEAQTGAELLGVVFGDDAGVLEDTGVAQPALFAVEVALYRLAESFGVRADVLIGHSLGELSAAYVAGVWSLAD
AVRVVVARARLMGSLPSGGRMVAVEATEEEVAPLVADVAAAGGMVSLAAVNAPGAVVVSGQDAAVDQIADIFAGR
GRRTRALAVSHAFHSPLMEPMLAEFADVLAQVEFRAPSIPVVSNVTGTIADAEELCSPEYWVRHVREAVRFGDGV
GAVLAQGVATVVELGPDPVLTALGERVRAASAERDSAARDVAFVPTLSRRSTDTRAFLGMLARVHARGHQVDWTA
LGRADDLARELPTYAFQHEHHWLKGASVRPGSAASRTAGSDGAFWKVVQEQDLQRLASDLGVDPDAPLHTVLPAL
GDWHQTHIEASETDGWRYRVAWERPTAQHAPEGPATLHGTWLIVVPEGDLRAGHLLDNDGLHDGLHGEVRRVLTD
AGAEVKSLSLAPEDIDRQTIAKLLNGLDDTPAGVVSLLALSGREHTGPRGVGSGAWASVCLLQALLDTGWSATRL
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SGEDQVALRADGARVRRLRPAGPDGAPEPVRPVAPESLVAPEGADATGRTGDPQPPAAREPWWSHGSVLITGGTG
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SNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHVTTLGHGEAEPQLVCFPPFAPVDGSLQFA
RLANHFRGRRRVSVVTVPGFMAGEPLAASLEVLIETLAEAVLRAADGRPYALLGYSSSGWLAQAAATWLEERGTG
PVGVVLLDTYPPDSMTLEMRKAMTYEVVERRMRFTSMHYDGLTALGTYLGMFRGWQPRQLAVPTLFVRPDSCIPG
SPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAAAVDEWLSRTPGLTRPAHHHHHH-

ATGAGCACCAACGAAGATAAACTGCGCCATTATGTTAAAGAACTGACCGGTGATCTGCTGCGTACCCGTGGTCGT
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GCAGTTCGTGTTGTGGTTGCACGTGCCCGTCTGATGGGTAGTCTGCCGAGCGGTGGTCGTATGGTTGCAGTGGAA
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CGTGTGCGTGCAGCAAGCGCAGAACGTGATAGCGCAGCCCGTGATGTTGCCTTTGTTCCGACACTGAGCCGTCGT
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CGTCTGGCCAATCATTTTCGTGGTCGCCGTCGTGTTAGCGTTGTGACCGTTCCGGGTTTCATGGCTGGTGAACCG
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GCACTGCTGGGTTATAGCAGTAGCGGTTGGCTGGCACAGGCAGCAGCAACCTGGCTGGAAGAACGTGGCACCGGT
CCGGTTGGCGTGGTTCTGTTAGATACCTATCCGCCTGATAGCATGACCCTGGAAATGCGTAAAGCAATGACCTAT
GAAGTTGTGGAACGTCGTATGCGTTTTACCAGCATGCATTATGATGGACTGACCGCACTGGGCACCTATCTGGGT
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AGTCCGGAAGAACCGATGGCTGGTCCGGATTGGCAGGCAGCATGGCCTCTGGATCATGAAGAAACCCAGGTTCCG
GGTGATCATTGTACCATGATTGGTGAATTTAGCGAAACCACCGCAGCCGCAGTTGATGAATGGCTGAGCCGTACA
CCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosTE-His$_6$ (pET-28a(+)_*fosTE*)

MGGDAGASPAPGVSAAPSASVAAAPPQDSNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHV
TTLGHGEAEPQLVCFPPFAPVDGSLQFARLANHFRGRRRVSVVTVPGFMAGEPLAASLEVLIETLAEAVLRAADG
RPYALLGYSSSGWLAQAAATWLEERGTGPVGVVLLDTYPPDSMTLEMRKAMTYEVVERRMRFTSMHYDGLTALGT
YRGMFRGWQPRQLAVPTLFVRPDSCIPGSPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAAAVDEWL
SRTPGLTRPAHHHHHH-

ATGGGTGGTGATGCGGGTGCAAGTCCGGCTCCGGGTGTTAGCGCAGCACCGAGCGCAAGCGTTGCAGCCGCACCG
CCTCAGGATAGCAATGATAGCGTTGTTGGTATTTATCGTAAACTGAGCCTGCAGGGTCGTATGCAAGAAGTTGAA
GCATTTCTGAGCAGCGCCAGCGCACTGCGTACCCGTTTTCATGGTGCCGAAGATTTAGGTCGCGGTGCACATGTT
ACCACCTTAGGTCATGGTGAAGCAGAACCGCAGCTGGTTTGTTTTCCGCCTTTTGCACCGGTTGATGGTAGCCTG
CAGTTTGCACGTCTGGCCAATCATTTTCGTGGTCGCCGTCGTGTTAGCGTTGTGACCGTTCCGGGTTTCATGGCT
GGTGAACCGCTGGCAGCGAGCCTGGAAGTTCTGATTGAAACCCTGGCCGAAGCAGTTTTACGTGCAGCGGATGGT
CGTCCTTATGCACTGCTGGGTTATAGCAGTAGCGGTTGGCTGGCACAGGCAGCAGCAACCTGGCTGGAAGAACGT
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TATCGTGGTATGTTTCGTGGTTGGCAGCCTCGTCAGCTGGCAGTGCCGACGCTGTTTGTGCGTCCGGATAGCTGT
ATTCCGGGTAGTCCGGAAGAACCGATGGCTGGTCCGGATTGGCAGGCAGCATGGCCTCTGGATCATGAAGAAACC
CAGGTTCCGGGTGATCATTGTACCATGATTGGTGAATTTAGCGAAACCACCGCAGCCGCAGTTGATGAATGGCTG
AGCCGTACACCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosTE(R198L/R227L)-His_6

MGGDAGASPAPGVSAAPSASVAAAPPQDSNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHV
TTLGHGEAEPQLVCFPPFAPVDGSLQFARLANHFRGRRRVSVVTVPGFMAGEPLAASLEVLIETLAEAVLRAADG
RPYALLGYSSSGWLAQAAATWLEERGTGPVGVVLLDTYPPDSMTLEMLKAMTYEVVERRMRFTSMHYDGLTALGT
YLGMFRGWQPRQLAVPTLFVRPDSCIPGSPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAAAVDEWL
SRTPGLTRPAHHHHHH-

ATGGGTGGTGATGCGGGTGCAAGTCCGGCTCCGGGTGTTAGCGCAGCACCGAGCGCAAGCGTTGCAGCCGCACCG
CCTCAGGATAGCAATGATAGCGTTGTTGGTATTTATCGTAAACTGAGCCTGCAGGGTCGTATGCAAGAAGTTGAA
GCATTTCTGAGCAGCGCCAGCGCACTGCGTACCCGTTTTCATGGTGCCGAAGATTTAGGTCGCGGTGCACATGTT
ACCACCTTAGGTCATGGTGAAGCAGAACCGCAGCTGGTTTGTTTTCCGCCTTTTGCACCGGTTGATGGTAGCCTG
CAGTTTGCACGTCTGGCCAATCATTTTCGTGGTCGCCGTCGTGTTAGCGTTGTGACCGTTCCGGGTTTCATGGCT
GGTGAACCGCTGGCAGCGAGCCTGGAAGTTCTGATTGAAACCCTGGCCGAAGCAGTTTTACGTGCAGCGGATGGT
CGTCCTTATGCACTGCTGGGTTATAGCAGTAGCGGTTGGCTGGCACAGGCAGCAGCAACCTGGCTGGAAGAACGT
GGCACCGGTCCGGTTGGCGTGGTTCTGTTAGATACCTATCCGCCTGATAGCATGACCCTGGAAATGCTGAAAGCA
ATGACCTATGAAGTTGTGGAACGTCGTATGCGTTTTACCAGCATGCATTATGATGGACTGACCGCACTGGGCACC
TATCTGGGTATGTTTCGTGGTTGGCAGCCTCGTCAGCTGGCAGTGCCGACGCTGTTTGTGCGTCCGGATAGCTGT
ATTCCGGGTAGTCCGGAAGAACCGATGGCTGGTCCGGATTGGCAGGCAGCATGGCCTCTGGATCATGAAGAAACC
CAGGTTCCGGGTGATCATTGTACCATGATTGGTGAATTTAGCGAAACCACCGCAGCCGCAGTTGATGAATGGCTG
AGCCGTACACCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosTE(R198L)-His_6

MGGDAGASPAPGVSAAPSASVAAAPPQDSNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHV
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RPYALLGYSSSGWLAQAAATWLEERGTGPVGVVLLDTYPPDSMTLEMLKAMTYEVVERRMRFTSMHYDGLTALGT
YRGMFRGWQPRQLAVPTLFVRPDSCIPGSPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAAAVDEWL
SRTPGLTRPAHHHHHH-

ATGGGTGGTGATGCGGGTGCAAGTCCGGCTCCGGGTGTTAGCGCAGCACCGAGCGCAAGCGTTGCAGCCGCACCG
CCTCAGGATAGCAATGATAGCGTTGTTGGTATTTATCGTAAACTGAGCCTGCAGGGTCGTATGCAAGAAGTTGAA
GCATTTCTGAGCAGCGCCAGCGCACTGCGTACCCGTTTTCATGGTGCCGAAGATTTAGGTCGCGGTGCACATGTT
ACCACCTTAGGTCATGGTGAAGCAGAACCGCAGCTGGTTTGTTTTCCGCCTTTTGCACCGGTTGATGGTAGCCTG
CAGTTTGCACGTCTGGCCAATCATTTTCGTGGTCGCCGTCGTGTTAGCGTTGTGACCGTTCCGGGTTTCATGGCT
GGTGAACCGCTGGCAGCGAGCCTGGAAGTTCTGATTGAAACCCTGGCCGAAGCAGTTTTACGTGCAGCGGATGGT
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GGCACCGGTCCGGTTGGCGTGGTTCTGTTAGATACCTATCCGCCTGATAGCATGACCCTGGAAATGCTGAAAGCA
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TATCGTGGTATGTTTCGTGGTTGGCAGCCTCGTCAGCTGGCAGTGCCGACGCTGTTTGTGCGTCCGGATAGCTGT
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CAGGTTCCGGGTGATCATTGTACCATGATTGGTGAATTTAGCGAAACCACCGCAGCCGCAGTTGATGAATGGCTG
AGCCGTACACCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

FosTE(R227L)-His_6

MGGDAGASPAPGVSAAPSASVAAAPPQDSNDSVVGIYRKLSLQGRMQEVEAFLSSASALRTRFHGAEDLGRGAHV
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RPYALLGYSSSGWLAQAAATWLEERGTGPVGVVLLDTYPPDSMTLEMRKAMTYEVVERRMRFTSMHYDGLTALGT
YLGMFRGWQPRQLAVPTLFVRPDSCIPGSPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAAAVDEWL
SRTPGLTRPAHHHHHH-

ATGGGTGGTGATGCGGGTGCAAGTCCGGCTCCGGGTGTTAGCGCAGCACCGAGCGCAAGCGTTGCAGCCGCACCG
CCTCAGGATAGCAATGATAGCGTTGTTGGTATTTATCGTAAACTGAGCCTGCAGGGTCGTATGCAAGAAGTTGAA
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ACCACCTTAGGTCATGGTGAAGCAGAACCGCAGCTGGTTTGTTTTCCGCCTTTTGCACCGGTTGATGGTAGCCTG
CAGTTTGCACGTCTGGCCAATCATTTTCGTGGTCGCCGTCGTGTTAGCGTTGTGACCGTTCCGGGTTTCATGGCT
GGTGAACCGCTGGCAGCGAGCCTGGAAGTTCTGATTGAAACCCTGGCCGAAGCAGTTTTACGTGCAGCGGATGGT
CGTCCTTATGCACTGCTGGGTTATAGCAGTAGCGGTTGGCTGGCACAGGCAGCAGCAACCTGGCTGGAAGAACGT
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TATCTGGGTATGTTTCGTGGTTGGCAGCCTCGTCAGCTGGCAGTGCCGACGCTGTTTGTGCGTCCGGATAGCTGT
ATTCCGGGTAGTCCGGAAGAACCGATGGCTGGTCCGGATTGGCAGGCAGCATGGCCTCTGGATCATGAAGAAACC
CAGGTTCCGGGTGATCATTGTACCATGATTGGTGAATTTAGCGAAACCACCGCAGCCGCAGTTGATGAATGGCTG
AGCCGTACACCGGGTCTGACCCGTCCGGCGCACCACCACCACCACCAC**TGA**

His_6-FosTEII (pET-28a(+)_*fosTEII*)

MGSSHHHHHHSSGLVPRGSHMYAPTPKPSTDRQAWLRRYTNAPDARHRLVCLPHAGGSASFYMPLARALAPEIDI
VAVQYPGRQDRRADPFPATLQDLAAHVAEALCGEPAVPTAFFGHSMGAAVAFEVIRLLEDSTTPVTALFASGRGA
PSVNRGERVHAMSQEDVLAELRGLEGTDSRMFDDPEIIEMIMPPLRNDYRLIETYRYVPGPPVACPIRGFLGAQD
PKVDEGEMKLWADHTAGSFDLTLLPGGHFYLVQHQPEIVEAIRNTLLVAPPYV-

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGTACGCCCCGACC
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TGCCTGCCGCACGCGGGCGGTTCCGCCAGCTTCTACATGCCGCTCGCGCGTGCCCTGGCCCCGGAGATCGACATC
GTCGCCGTCCAGTACCCGGGGCGGCAGGACCGCAGGGCCGACCCGTTCCCCGCCACGCTGCAAGACCTCGCCGCC
CACGTCGCCGAGGCGCTGTGCGGAGAACCGGCCGTACCGACGGCGTTCTTCGGGCACAGCATGGGGGCGGCGGTG
GCCTTCGAGGTCATCCGCCTCCTCGAGGACTCCACGACGCCGGTGACGGCGCTCTTCGCGTCTGGCCGCGGCGCC
CCGTCGGTCAACCGCGGCGAGCGGGTGCACGCCATGTCGCAGGAAGACGTGCTGGCCGAACTGCGCGGCCTGGAG
GGCACGGACAGCCGGATGTTCGACGATCCCGAGATCATCGAGATGATCATGCCCCCGCTGCGCAACGACTACCGC
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CCGAAGGTCGACGAGGGCGAGATGAAGCTGTGGGCCGACCACACCGCCGGTTCGTTCGACCTGACACTGCTGCCG
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CCGTACGTG**TGA**

His_6-FosACP7 (pET-28a(+)_*fosACP7*)

MGSSHHHHHHSSGLVPRGSHDAGTAGATPFVARWTALSGGERRRVLVETVCTQAAAELGHASGGTIEPERPFQEL
GFDSLAAVGLRQRLEKLTGLKLPATLVFDHPTPAALAQVVASALAERVGGAS-

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATGATGCTGGCACAGCG
GGTGCAACCCCGTTTGTTGCACGTTGGACCGCACTGAGCGGTGGTGAACGCCGTCGCGTTCTGGTGGAAACCGTT
TGTACCCAGGCAGCCGCAGAACTGGGCCATGCAAGCGGTGGCACCATCGAACCGGAACGTCCGTTTCAAGAACTG
GGTTTTGATTCACTGGCAGCGGTTGGTCTGCGTCAGCGTCTGGAAAAACTGACCGGTCTGAAACTGCCTGCAACA
CTGGTTTTTGATCATCCTACACCGGCAGCGCTGGCCCAGGTTGTTGCAAGTGCCCTGGCAGAACGTGTTGGTGGT
GCGAGC**TAA**

FosACP8-His$_6$ (pET-28a(+)_*fosACP8*)

MESGAGHPDAGRTGAAQPGEWQRLPDLPLADQRPYVLDIVRREAARVLGHADAGTITEDQEFLALGFDSLAAVEL
RGRLTVLTGLALPSSLVFDHPTLGALVTHLLDNAAPAHHHHHH-

ATGGAAAGCGGTGCGGGTCATCCTGATGCAGGTCGCACAGGTGCAGCACAGCCTGGTGAATGGCAGCGCCTTCCG
GATCTGCCGCTGGCGGATCAGCGTCCGTATGTTCTGGATATTGTGCGTCGTGAAGCAGCCCGTGTTCTGGGTCAT
GCAGATGCCGGTACAATTACCGAAGATCAAGAATTTCTGGCACTGGGTTTTGATAGCCTGGCAGCCGTTGAACTG
CGTGGTCGTCTGACCGTGCTGACCGGTCTGGCCCTGCCGAGCAGCCTGGTTTTTGATCATCCGACGCTGGGTGCC
CTGGTTACCCATTTACTGGATAACGCAGCACCTGCGCACCACCACCACCACCAC**TGA**

His$_6$-PikTE (pET-28a(+)_*pikTE*)

MGSSHHHHHHSSGLVPRGSHMSGADTGAGAGMFRALFRQAVEDDRYGEFLDVLAEASAFRPQFASPEACSERLDP
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ARAILRAAGDAPVVLLGHSGGALLAHELAFRLERAHGAPPAGIVLVDPYPPGHQEPIEVWSRQLGEGLFAGELEP
MSDARLLAMGRYARFLAGPRPGRSSAPVLLVRASEPLGDWQEERGDWRAHWDLPHTVADVPGDHFTMMRDHAPAV
AEAVLSWLDAIEGIEGAGK-

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGAGTGGTGCCGAT
ACCGGTGCCGGTGCGGGTATGTTTCGTGCGCTGTTCCGCCAAGCCGTTGAAGATGACCGCTACGGCGAGTTTCTG
GACGTTCTGGCCGAAGCGAGCGCCTTTCGTCCACAGTTTGCGAGCCCGGAAGCGTGCAGCGAACGTCTCGATCCG
GTTCTGCTCGCCGGTGGTCCAACCGATCGTGCGGAAGGTCGCGCCGTTCTGGTTGGTTGCACCGGTACCGCCGCG
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CCGGGCTATGGTACCGGCACGGGTACGGGTACCGCGCTGCTGCCAGCCGATCTCGATACGGCGCTGGATGCGCAA
GCGCGTGCCATTCTCCGTGCCGCCGGTGATGCCCCAGTTGTGCTGCTCGGTCATAGCGGTGGTGCGCTGCTGGCC
CATGAACTGGCGTTTCGTCTGGAACGTGCGCATGGTGCGCCACCAGCGGGTATCGTGCTGGTTGATCCGTACCCG
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GCGCCAGTTCTGCTCGTGCGTGCCAGCGAACCGCTGGGTGACTGGCAAGAAGAGCGCGGTGATTGGCGTGCGCAC
TGGGATCTGCCACATACCGTTGCCGATGTGCCGGGCGACCATTTCACCATGATGCGCGATCATGCGCCAGCCGTT
GCCGAAGCCGTTCTGAGCTGGCTGGACGCGATCGAAGGTATTGAAGGCGCGGGCAAA**TAA**

His$_6$-FosH (pET-28b(+)_*fosH*)

MGSSHHHHHHSSGLVPRGSHMPESTDAAAAVPEAHDLPLTLWGWQDWTVEPWERLPGDEGYSSHTYLVRHDGVRH
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LGRIDSVLLHAPVPEGVPRWNEVLELFLLEQDFLKGHDWIRRTLEQAGGALSPDDLTIGLINCDAAAKEFRVLGD
TAGLLDWSEAMYAPCMLELATTLSYLEDETDGEPLVRGYFEEGPADRAELGFLADILRFRCAAEGWIYAARQNAG
DETGTTASTWSNEKLIERARQNAENADRIAARFQVF-

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GACGGCGTGCGCCACGTTGTGAAAGCTGTGCGTAAAGATATGGGTCCGAAGCTGACCGCCGGGTTGCTGGTTGCA
CAGGAGGTCGAGCGCCACGGCATCGCGGCTGGCGGTCCGCTGCCGACCACGGGCGGTGAGGTGACCGCGTATCAG
GGTGACTTCTGCTATTCGCTGTTGACGTACCTGGACGGCGAGCGTGTGGATGAGACAGATCCGGCGCACCTGCGT
GCGGTGGGTCGTACCTTAGGCCGCATCGATAGCGTTTTGCTGCACGCACCGGTTCCGGAAGGCGTTCCGCGTTGG
AATGAAGTCCTGGAGTTGTTTCTCTTGGAGCAAGACTTCCTGAAGGGTCATGACTGGATTCGTCGTACCTTGGAG
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ACCACCCTGTCCTATCTGGAAGATGAAACCGATGGTGAACCGCTGGTTCGTGGTTACTTCGAGGAAGGTCCAGCG
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CAAAACGCCGAAAACGCGGACCGTATCGCCGCGCGCTTCCAGGTTTTT**TAA**

His$_6$-FosM (pET28a(+)_*fosM*)

MGSSHHHHHHSSGLVPRGSHMLRTTRPRKSRTWRERTDMATEPMKEDISNMPISKADETADAPRTADAALRAEAA
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CGTCTGGCAGAAAGCATTCTGGCACGTGGTCTGACACCGGTTGGTCTGATTGGTGAAGCAGGTCATAGCGAAAGC
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AAACGCGCAGATAGTCCGGCAAGC**TAA**

His$_8$-MatB (pHis8_*matB*)

MKHHHHHHHHGGLVPRGSHGGSSSLFPALSPAPTGAPADRPALRFGERSLTYAELAAAAGATAGRIGGAGRVAVW
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VIERYGMTETLMNTSVRADGEPRAGTVGVPLPGVELRLVEEDGTPIAALDGESVGEIQVRGPNLFTEYLNRPDAT
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CGGGCGCTGAACCGTGAC**TGA**

His$_6$-FosKR8 (pET-28a(+)_*fosKR8*)

MGSSHHHHHHSSGLVPRGSHETDGWRYRVAWERPTAQHAPEGPATLHGTWLIVVPEGDLRAGHLLDNDGLHDGLH
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His$_6$-FosAT8 (pET-28a(+)_*fosAT8*)

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MLARVHARGHQVDWTALGRADDLARE-

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CGTACCGAAGGTGCCGTTCGTGCACAGGCAGCACGTCTGGCAGCCTTTGTTGCAGGTCGTGGTGGTGGTGCACTG
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ATGCTGGCACGTGTTCATGCACGTGGTCATCAGGTGGATTGGACCGCACTGGGTCGTGCAGATGATCTGGCACGC
GAA**TAA**

FosKS8-AT8-KR8-His$_6$ (pET-28a(+)_*fosKS8-AT8-KR8*)

MSTNEDKLRHYVKELTGDLLRTRGRLRELEAAGNEPIALVGMACKYPGGVASPEDLWRLVAEGRDAISPFPADRG
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GAVLAQGVATVVELGPDPVLTALGERVRAASAERDSAARDVAFVPTLSRRSTDTRAFLGMLARVHARGHQVDWTA
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GRPNHLFADFDDTAHHHHHH-

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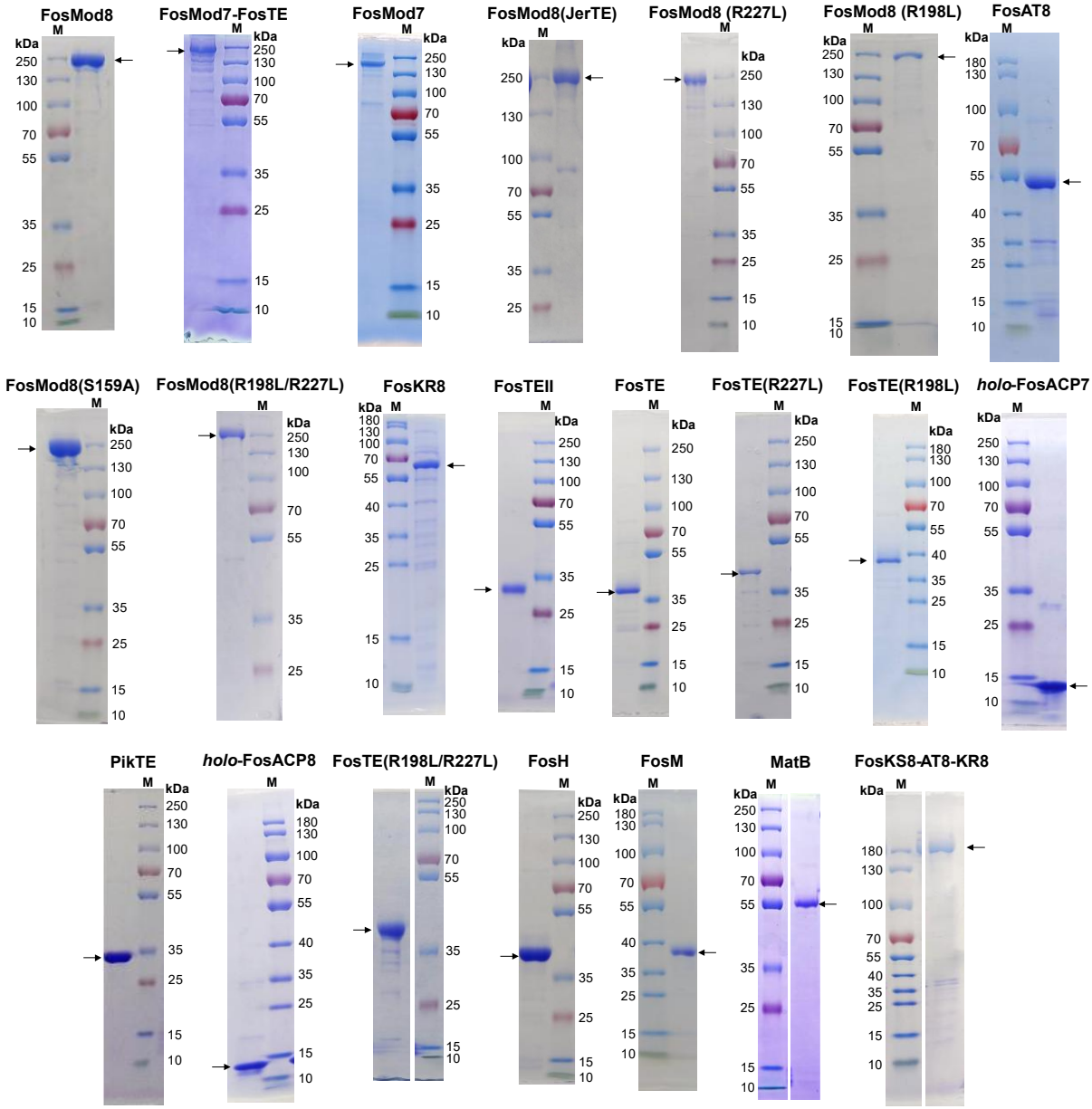
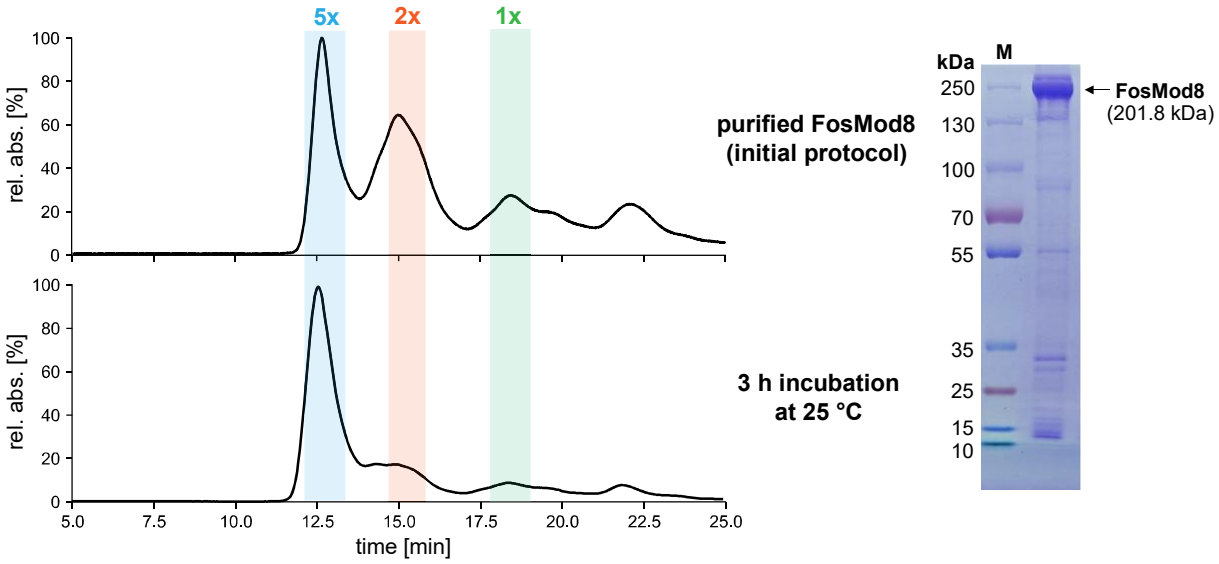
GGCCGTCCGAATCACCTGTTTGCAGATTTTGATGATACCGCGCACCACCACCACCACCAC**TGA**

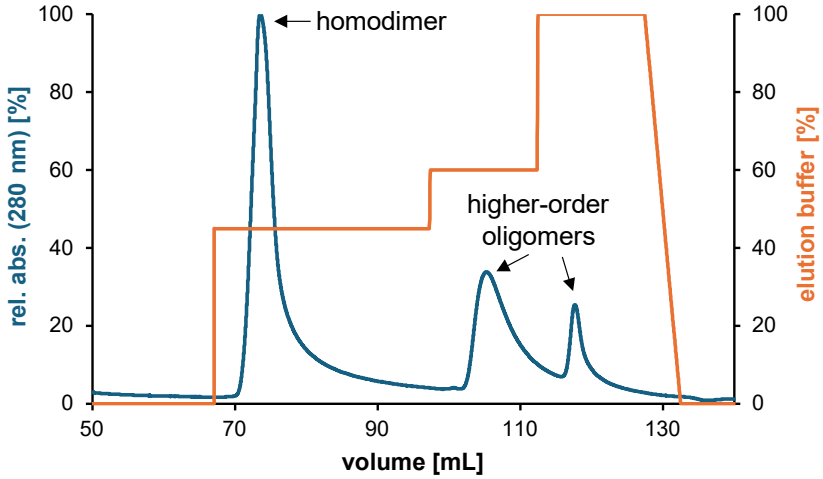
Protein production and purification



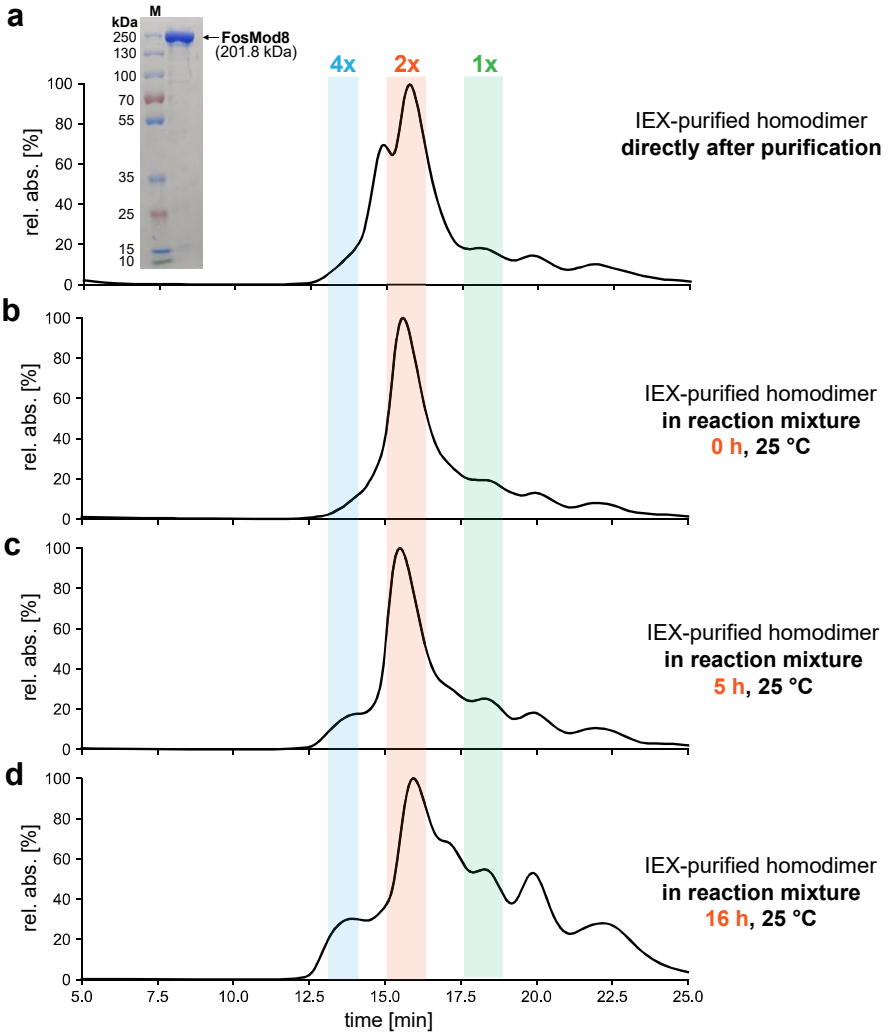
Supplementary Figure 1: SDS-PAGE analysis (6 – 15% or 8 – 15% acrylamide) of all purified proteins used in this study. FosMod8 (201.8 kDa), FosMod7-FosTE (199.5 kDa), FosMod7 (176.2 kDa), FosMod8(JerTE) (199.1 kDa), FosMod8(R227L) (201.8 kDa), FosMod8(R198L) (201.8 kDa), FosAT8 (48.6 kDa), FosMod8(S159A) (201.8 kDa), FosMod8(R198L/R227L) (201.8 kDa), FosKR8 (57 kDa), FosTEII (30.5 kDa), FosTE (34.0 kDa), FosTE(R227L) (34.0 kDa), FosTE(R198L) (34.0 kDa), *holo*-FosACP7 (13.2 kDa), PikTE (33.7 kDa), *holo*-FosACP8 (12.6 kDa), FosTE(R198L/R227L) (39.9 kDa), FosH (37.0 kDa), FosM (35 kDa), MatB (52.9 kDa) and FosKS8-AT8-KR8 (157.3 kDa). M: protein molecular weight marker (PageRuler or PageRuler Plus from THERMO SCIENTIFIC). The bands corresponding to the purified protein on the SDS-PAGE gel are marked with an arrow.



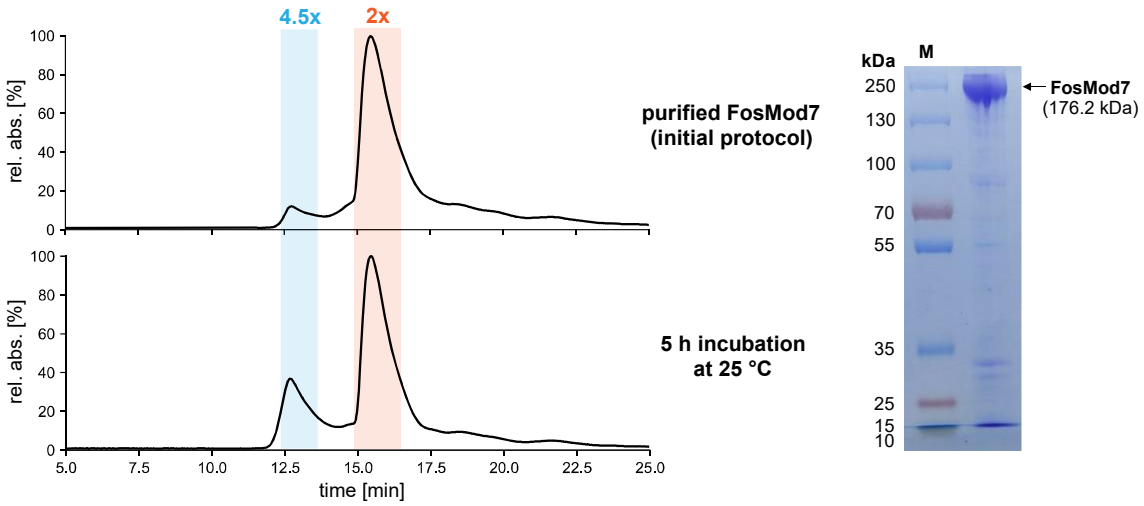
Supplementary Figure 2: SEC-UV analysis of FosMod8 (initial purification protocol). Oligomisation profiles of FosMod8 (normalised absorbance at 280 nm), directly after purification using the initial purification protocol (top left) and after incubation in reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **17**) for 3 h at 25 °C (bottom left). The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation states (5×, 2× and 1×; highlighted in color) are based on the calculated molecular weight of the FosMod8 monomer (201.8 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



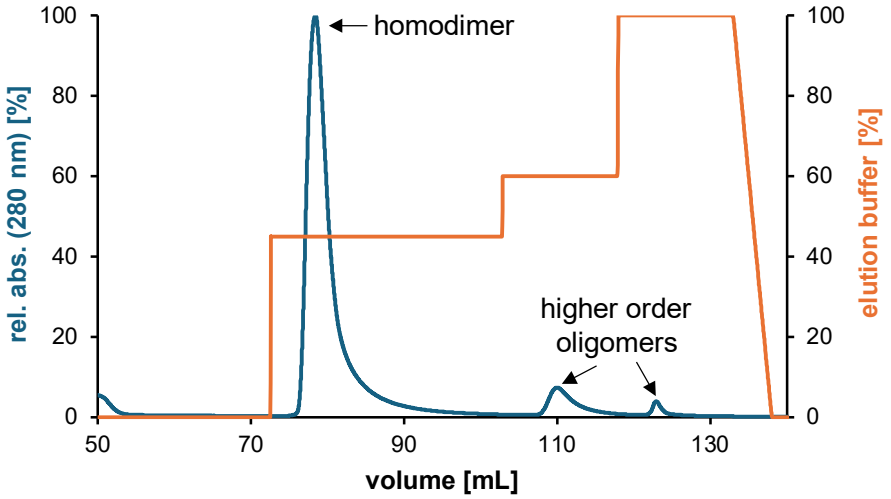
Supplementary Figure 3: UV chromatogram of FosMod8 purification by anion exchange chromatography (IEX) following Ni-NTA affinity chromatography, as part of the optimised purification protocol. Normalised absorbance at 280 nm (blue) and elution buffer concentration (orange) are shown as a function of elution volume. Peaks corresponding to isolated homodimeric protein (first elution step) and separated higher order oligomers are shown.



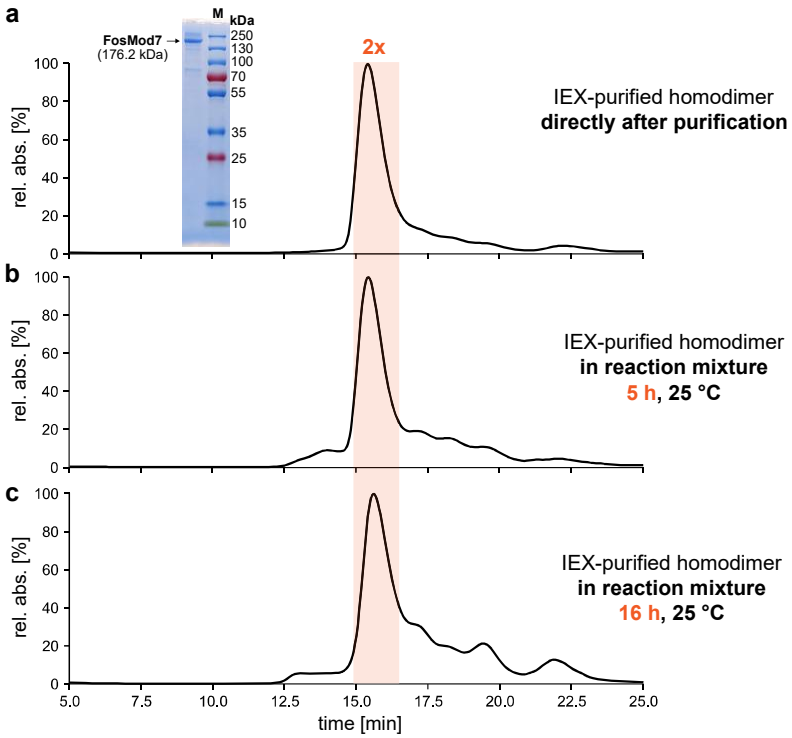
Supplementary Figure 4: SEC-UV analysis of structural integrity and stability of FosMod8 (optimised purification protocol). Oligomisation profiles of purified FosMod8 using IEX (normalised absorbance at 280 nm). **a,** Isolated FosMod8 immediately after IEX purification. **b,** The same FosMod8 sample after subjection to reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **17**) at 25 °C. **c,** The same FosMod8 sample after incubation in reaction mixture for 5 h at 25 °C. **d,** The same FosMod8 sample after incubation in reaction mixture for 16 h at 25 °C. The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation states (4×, 2× and 1×; highlighted in color) are based on the calculated molecular weight of the FosMod8 monomer (201.8 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



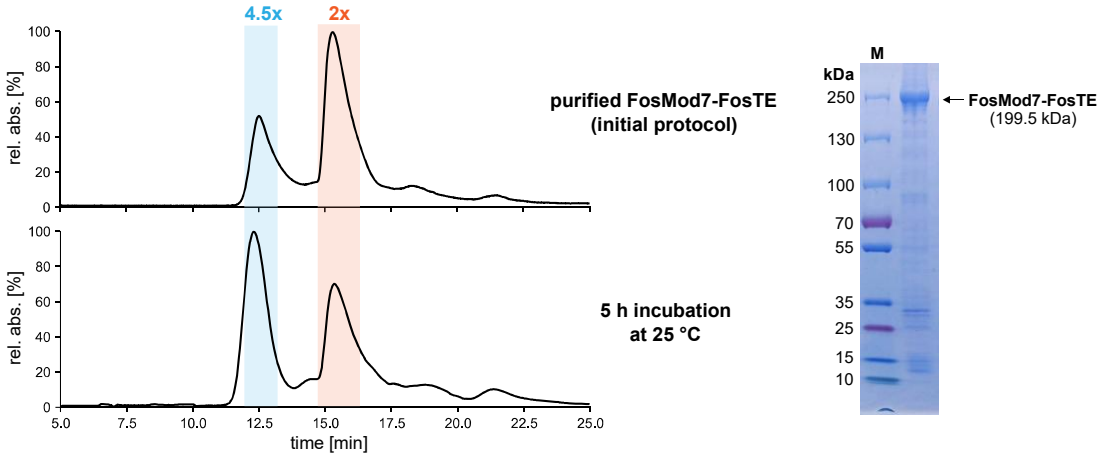
Supplementary Figure 5: SEC-UV analysis of FosMod7 (initial purification protocol). Oligomisation profiles of FosMod7 (normalised absorbance at 280 nm), immediately after purification using the initial purification protocol (top left) and after incubation in reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **16**) for 5 h at 25 °C (bottom left). The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation states (4.5× and 2×; highlighted in color) are based on the calculated molecular weight of the FosMod7 monomer (176.2 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



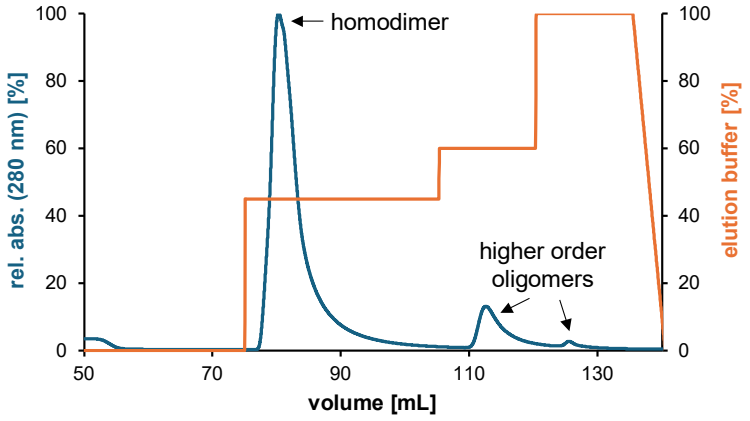
Supplementary Figure 6: UV chromatogram of FosMod7 purification by anion exchange chromatography (IEX) following Ni-NTA affinity chromatography, as part of the optimised purification protocol. Normalised absorbance at 280 nm (blue) and elution buffer concentration (orange) are shown as a function of elution volume. Peaks corresponding to isolated homodimeric protein (first elution step) and separated higher order oligomers are shown.



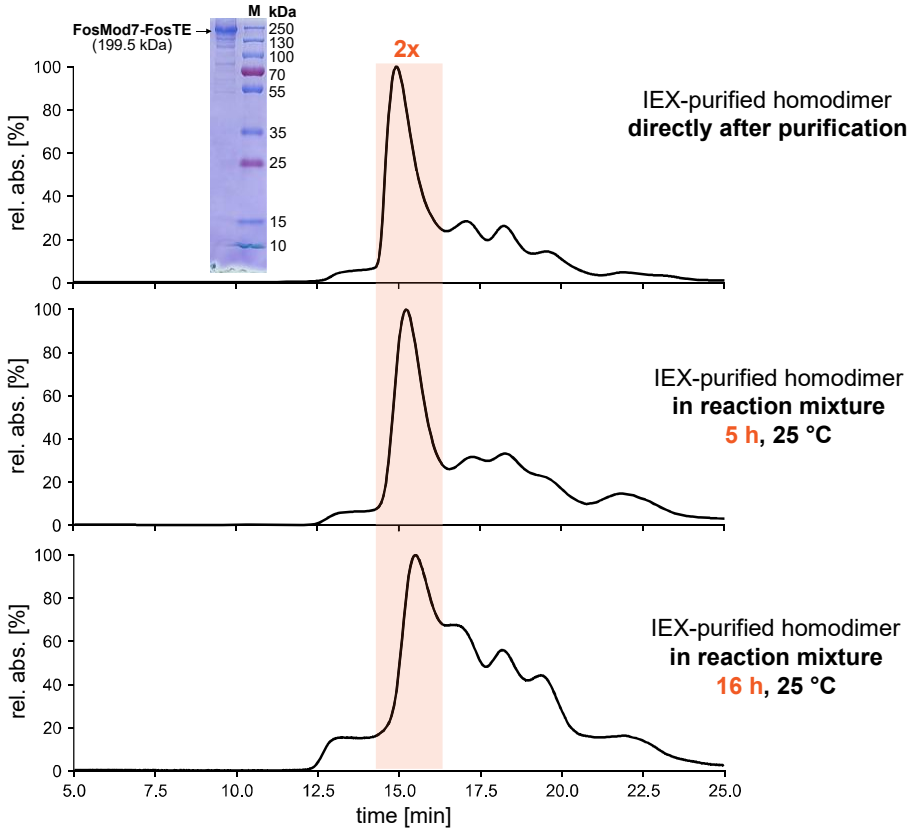
Supplementary Figure 7: SEC-UV analysis of structural integrity and stability of FosMod7 (optimised purification protocol). Oligomisation profiles of purified FosMod7 using IEX (normalised absorbance at 280 nm). **a,** Isolated FosMod7 immediately after IEX purification. **b,** The same FosMod7 sample after subjection to reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **16**) and incubation for 5 h at 25 °C. **c,** The same FosMod7 sample after incubation in reaction mixture for 16 h at 25 °C. The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation state (2×; highlighted in color) is based on the calculated molecular weight of the FosMod8 monomer (176.2 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



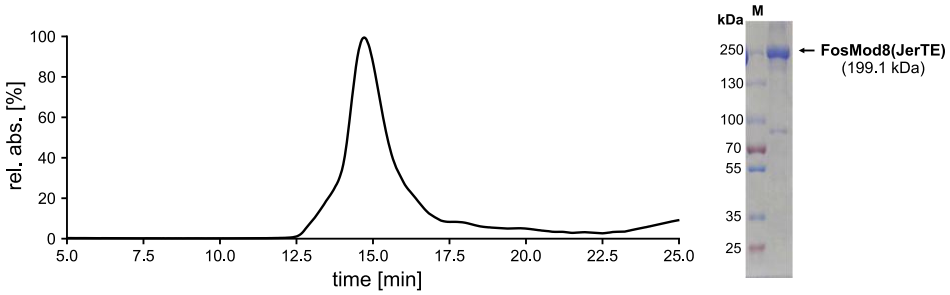
Supplementary Figure 8: SEC-UV analysis of FosMod7-FosTE (initial purification protocol). Oligomisation profiles of FosMod7-FosTE (normalised absorbance at 280 nm), immediately after purification using the initial purification protocol (top left) and after incubation in reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **16**) for 5 h at 25 °C (bottom left). The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation states (4.5× and 2×; highlighted in color) are based on the calculated molecular weight of the FosMod7-FosTE monomer (199.5 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



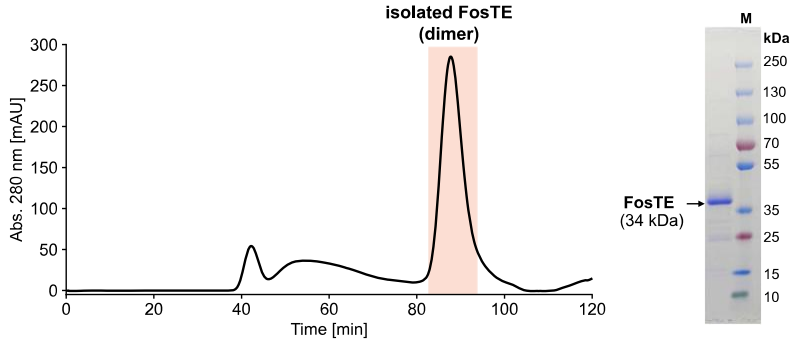
Supplementary Figure 9: UV chromatogram of FosMod7-FosTE purification by anion exchange chromatography (IEX) following Ni-NTA affinity chromatography, as part of the optimised purification protocol. Normalised absorbance at 280 nm (blue) and elution buffer concentration (orange) are shown as a function of elution volume. Peaks corresponding to isolated homodimeric protein (first elution step) and separated higher order oligomers are shown.



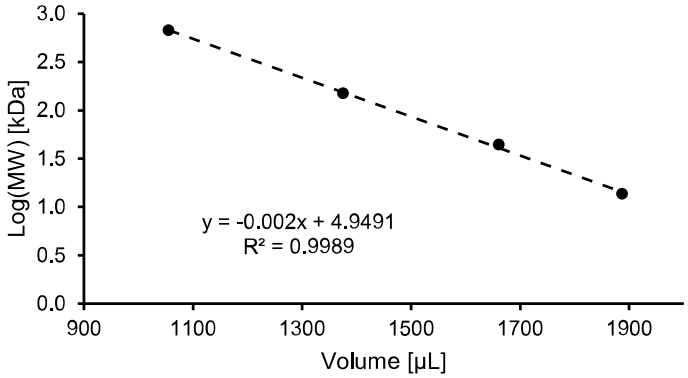
Supplementary Figure 10: SEC-UV analysis of structural integrity and stability of FosMod7 (optimised purification protocol). Oligomisation profiles of purified FosMod7 using IEX (normalised absorbance at 280 nm). **a,** Isolated FosMod7 immediately after IEX purification. **b,** The same FosMod7 sample after subjection to reaction mixture (200 mM sodium phosphate buffer (pH 6.8), 5 mM malonyl-CoA, 5 mM NADPH, and substrate **16**) and incubation for 5 h at 25 °C. **c,** The same FosMod7 sample after incubation in reaction mixture for 16 h at 25 °C. The molecular weight was determined using a protein standard (protein standard mix 15 – 600 kDa from Supelco (MERCK)) under identical chromatographic conditions. The oligomerisation state (2×; highlighted in color) is based on the calculated molecular weight of the FosMod8 monomer (176.2 kDa). The corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).



Supplementary Figure 11: SEC-UV analysis of purified FosMod8(JerTE). The UV-chromatogram (normalised absorbance at 280 nm) of homodimeric FosMod8(JerTE), isolated via preparative SEC purification is shown on the left and the corresponding SDS-PAGE gel (6 – 15% acrylamide) of the purified protein is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).

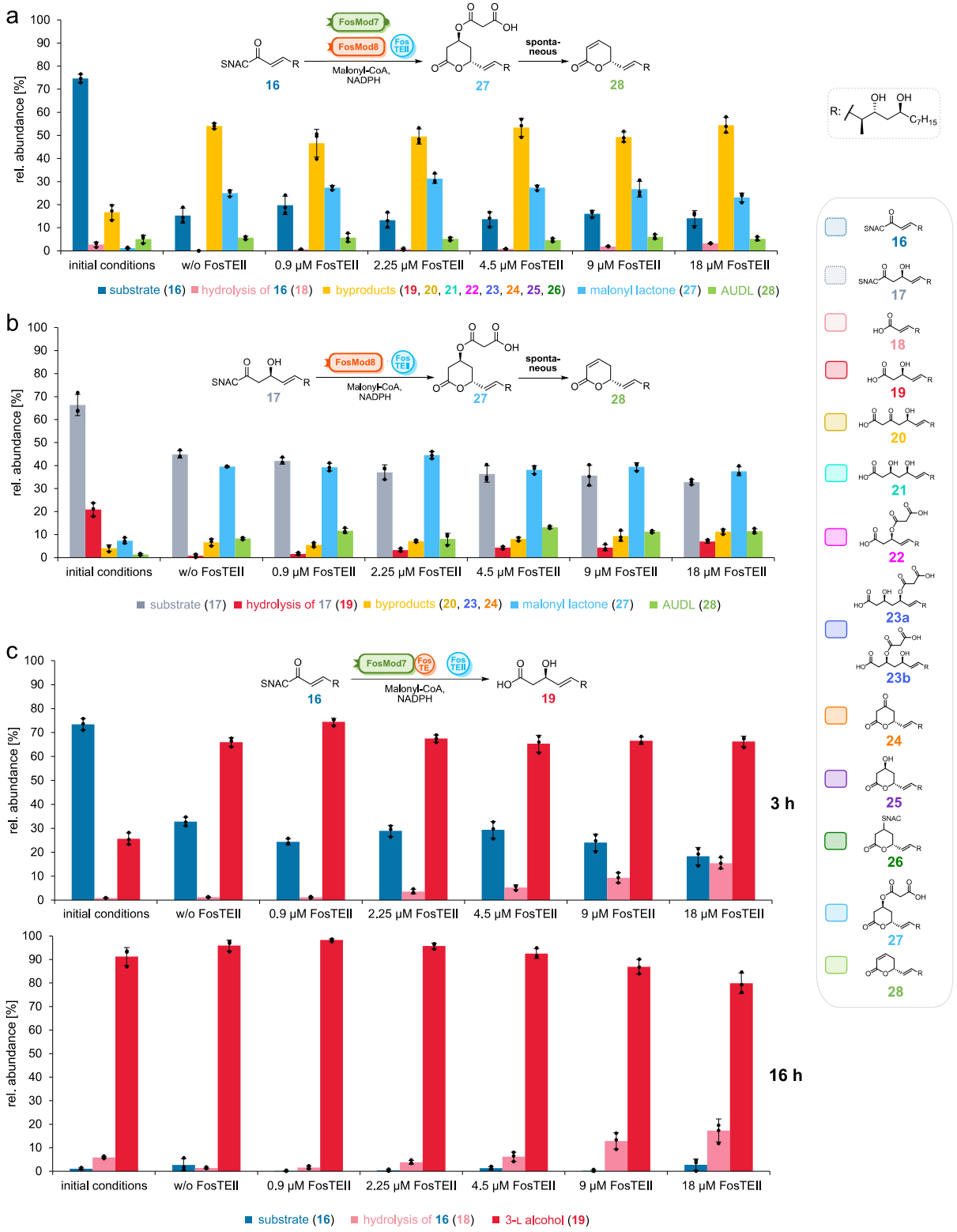


Supplementary Figure 12: Purification of FosTE via preparative SEC. The UV chromatogram of the FosTE purification is shown on the left, with the isolated fraction containing the homodimeric protein highlighted in orange. The corresponding SDS-PAGE gel (6 – 15% acrylamide) is shown on the right. M: protein molecular weight marker (PageRuler Plus from THERMO SCIENTIFIC).

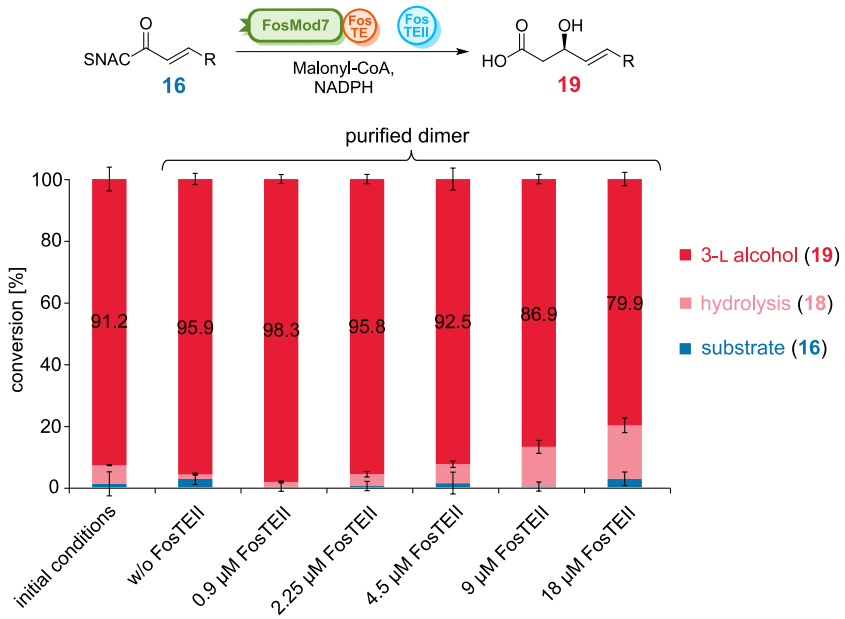


Supplementary Figure 13: Protein standard curve for SEC-UV analysis. The standard curve for the determination of the momlecular weight (MW) was created using the protein standard mix 15 – 600 kDa from Supelco (MERCK).

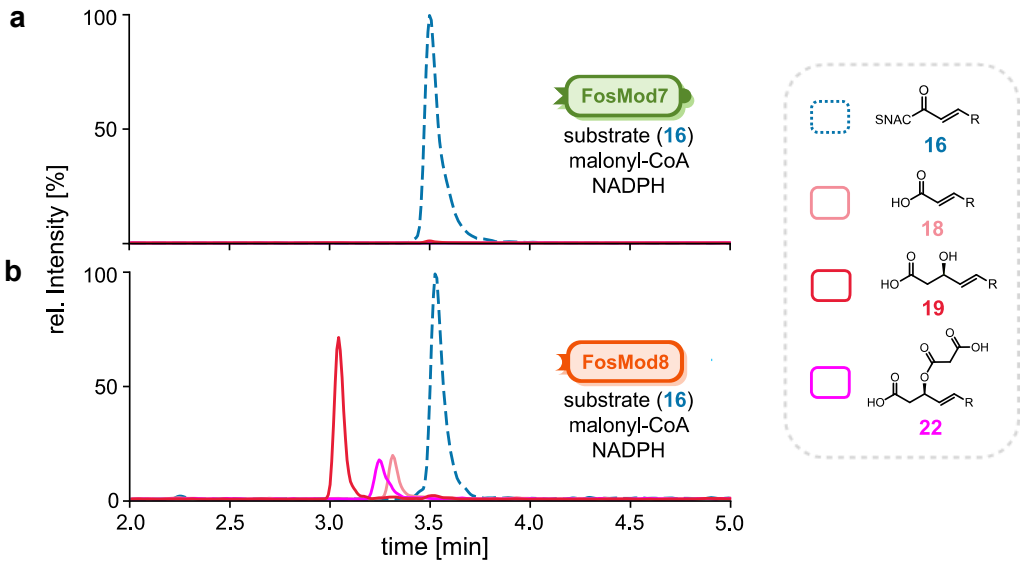
Analytical scale enzymatic assays



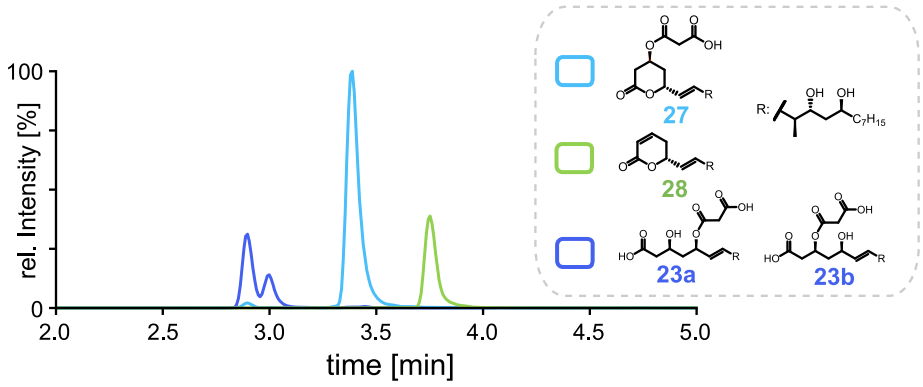
Supplementary Figure 14: Relative abundance (%) of compounds detected in module assays under varying concentrations of FosTEII with individual data points. a, Conversion of substrate **16** in reactions with FosMod7 and FosMod8 with increasing concentrations of FosTEII. **b,** Conversion of substrate **17** in reactions with FosMod8 and increasing concentrations of FosTEII. **c,** Conversion of substrate **16** in reactions with FosMod7-FosTE showing relative abundances after 3 h (top) and 16 h (bottom). Bars represent mean values ± s.d., with individual data points shown (n = 3 for each condition). The reaction schemes above each panel illustrate the enzymatic assays monitored.



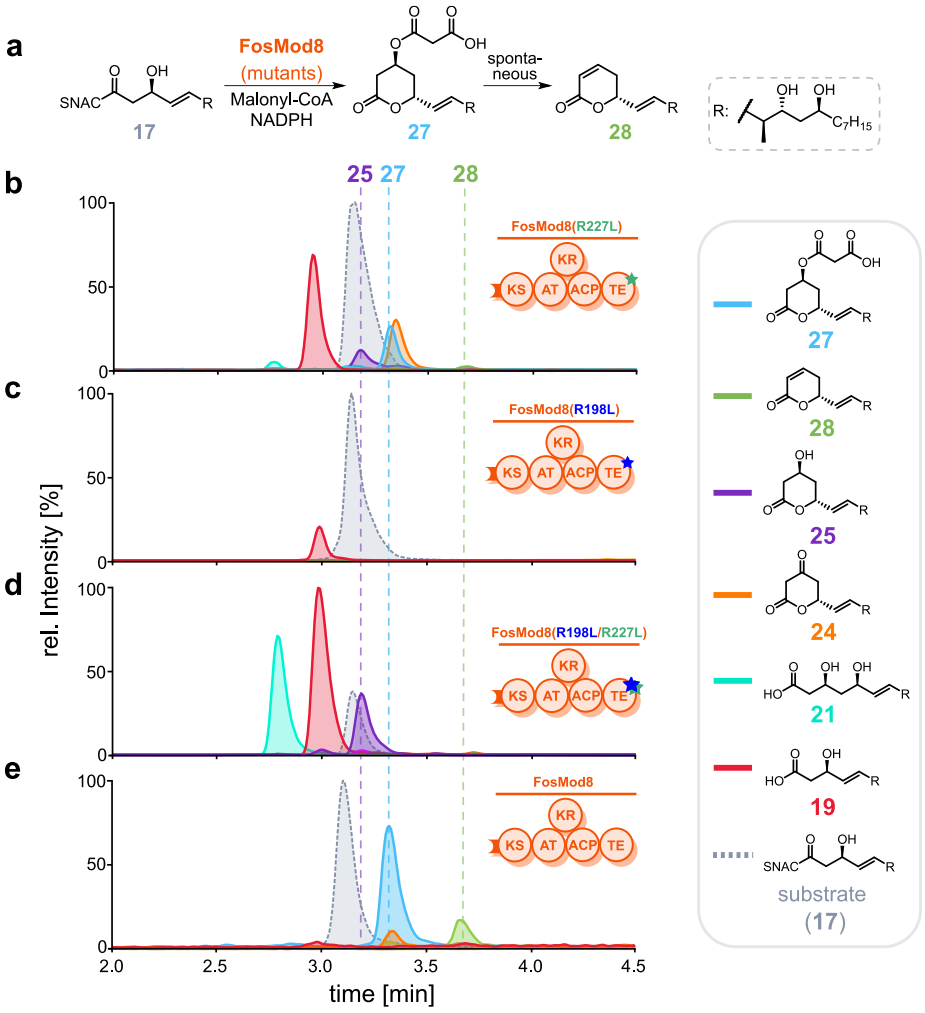
Supplementary Figure 15: Enzymatic activity of FosMod7-FosTE. Reaction scheme for activity assay with surrogate **16**, FosMod7-FosTE and FosTEII (top). Conversion for FosMod7-FosTE reactions with **16** and increasing FosTEII equivalents (right) after 16 h of incubation. **b**, Reaction scheme for activity assays with surrogate **16**, FosMod7, FosMod8 and FosTEII (top). Conversion rates were approximated from EIC data. The mean values ± s.d. from three independent, separately prepared reactions are shown.



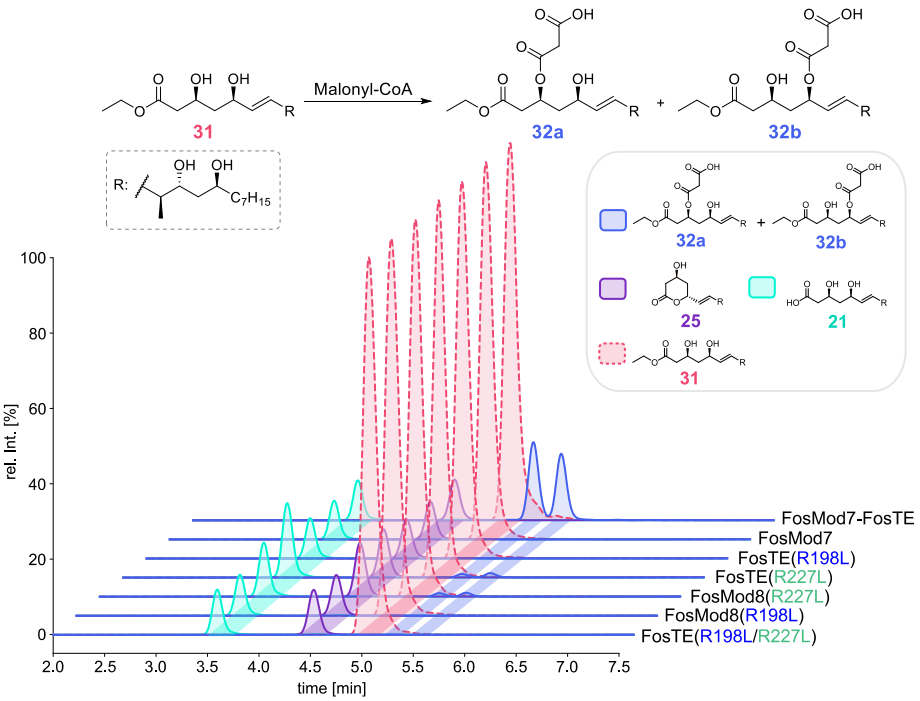
Supplementary Figure 16: LC-MS analysis (EIC) of in vitro reactions with surrogate 16. a, EIC of activity assay with FosMod7, malonyl-CoA and NADPH (3 h incubation). In the absence of a covalently fused TE domain, no hydrolysis of module-bound intermediates was detected. **b,** EIC of activity assay with FosMod8, malonyl-CoA and NADPH, showing the elongation, ketoreduction and *O*-malonylation activity of FosMod8, evidenced by the detection of **19** and **22**. Mass to charge ratios (*m/z*) used for each compound are listed in Supplementary Table 4.



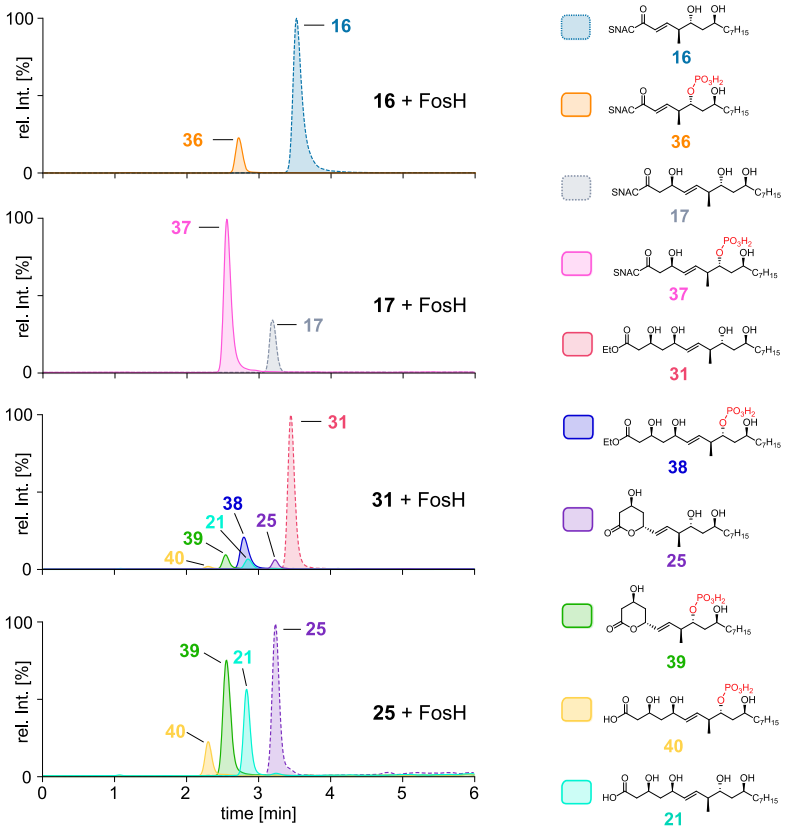
Supplementary Figure 17: LC-MS analysis (EIC) of malonyl lactone **27** upon incubation in phosphate buffer (pH 6.8) at 25 °C for 13 h. Lactone hydrolysis to **23** and demalonylation to **28** were observed as spontaneous processes.



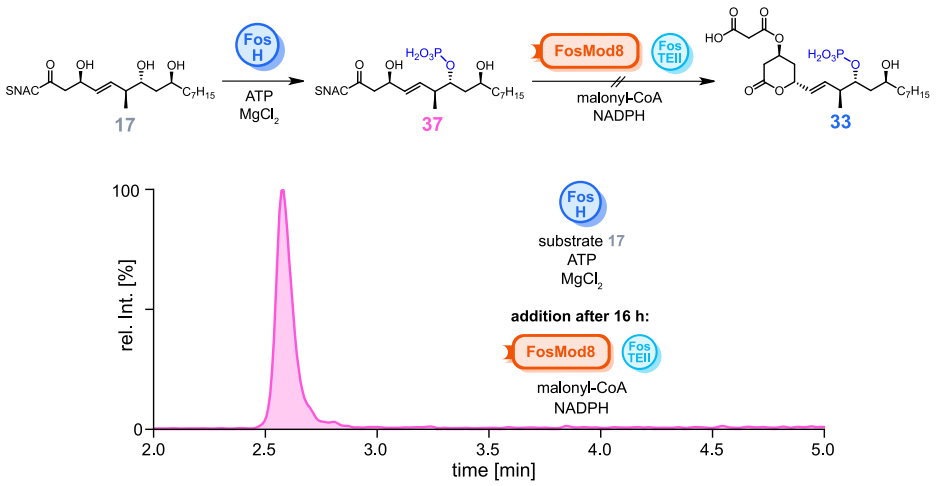
Supplementary Figure 18: LC-MS analysis of in vitro reactions of 17 with mutant variants of FosMod8. a, Reaction scheme. **b**, EIC of activity assay with FosMod8(R227L). **c**, Activity assay with FosMod8(R198L). **d**, Activity assay with FosMod8(R198L/R227L). **e,** Activity assay with wild-type FosMod8. Mass to charge ratios (*m/z*) used for each compound are listed in Supplementary Table 4.



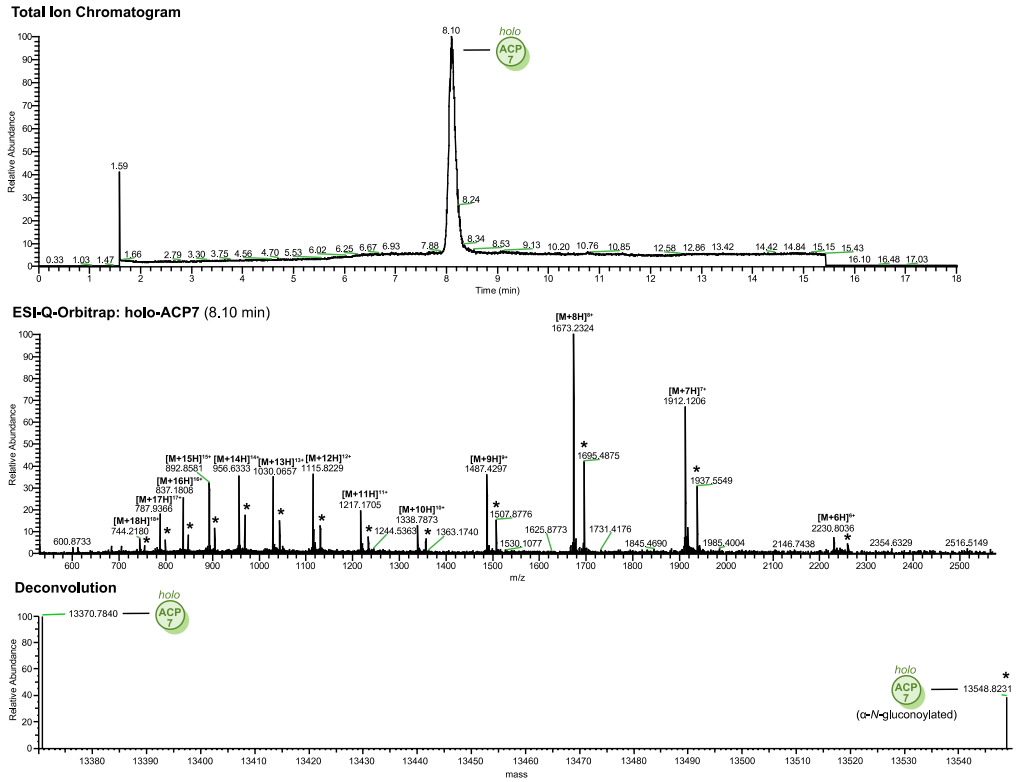
Supplementary Figure 19: LC-MS analysis of in vitro reactions with oxoester 31. The reaction scheme and EICs show the in vitro reactions of oxoester **31** with FosMod7-FosTE, FosMod7, FosTE(R198L), FosTE(R227L), FosMod8(R227L), FosMod8(R198L) and FosTE(R198L/R227L). Mass to charge ratios (*m/z*) used for each compound are listed in Supplementary Table 4.



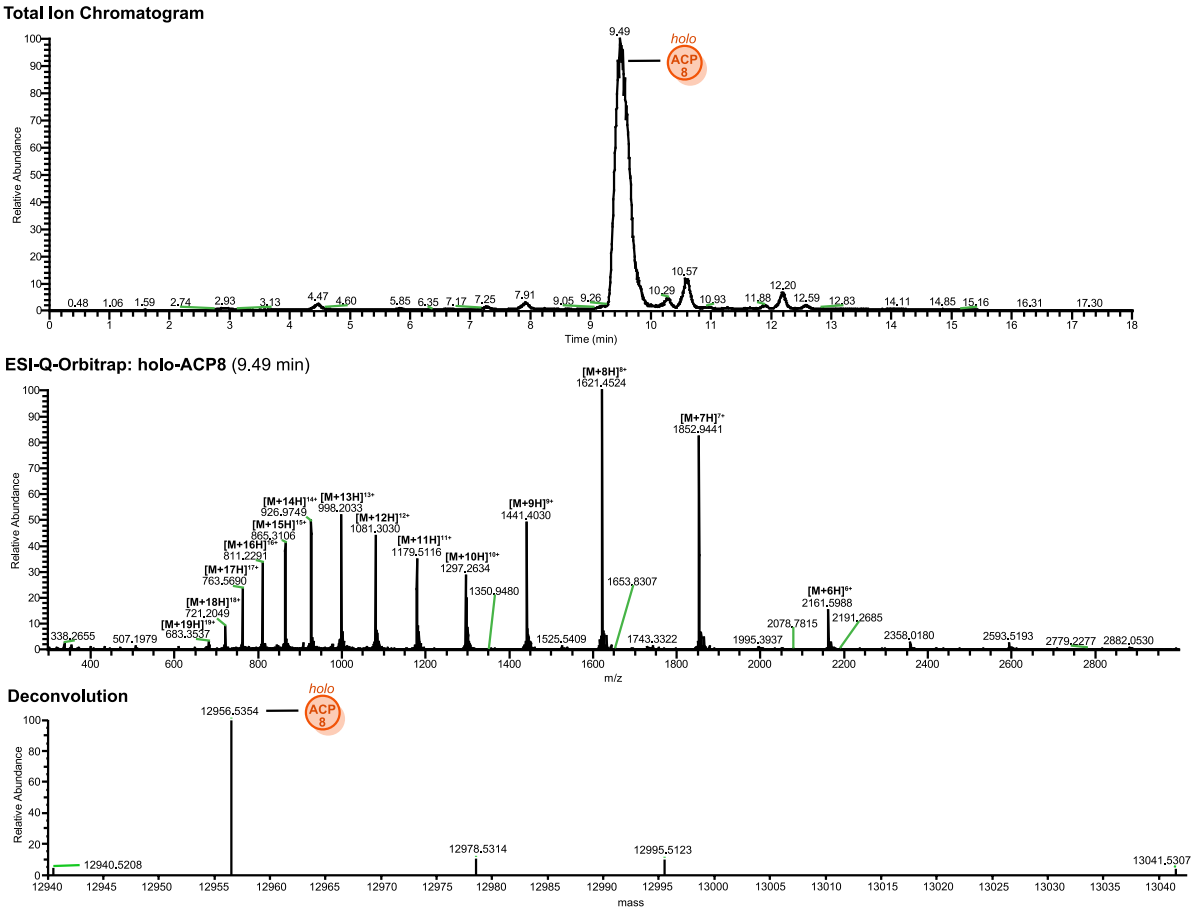
Supplementary Figure 20: LC-MS analysis of FosH activity assays using substrates 16, 17, 25 and 31. Phosphorylation was observed in all activity assays, indicating relaxed substrate specificity of FosH. All reactions were performed in the presence of ATP and $MgCl_2$ and were incubated for 1 h. Compounds arising from spontaneous lactonisation / hydrolysis were observed in the reactions with **31** and **25** as in the experiments in Supplementary Fig. 19 and Fig. 6. Mass to charge ratios (*m/z*) used for each compound are listed in Supplementary Table 4.



Supplementary Figure 21: LC-MS analysis of reaction with FosH in combination with FosMod8. FosH was incubated with substrate **17**, ATP, and $MgCl_2$ for 16 h to achieve complete conversion to **37**, after which FosH was removed from the reaction. Subsequently, FosMod8, FosTEII, malonyl-CoA, and NADPH were added, and the mixture was incubated for an additional 5 h. Mass to charge ratios (*m/z*) used for each compound are listed in Supplementary Table 4.

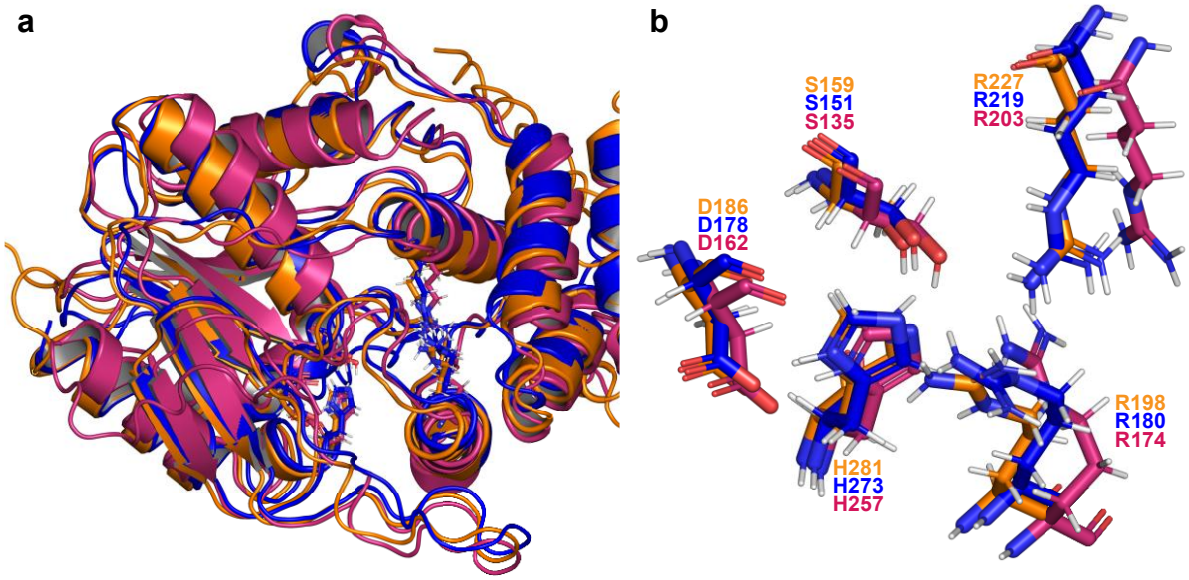


Supplementary Figure 22: LC-HRMS analysis of *holo*-FosACP7, purified from *E. coli* BAP1. Total ion chromatogram (top), mass spectrum of the peak at 8.10 min (middle) and deconvoluted mass spectrum (bottom). Calculated molecular weight of *holo*-FosACP7: 13370.8360 Da and deconvoluted mass: 13370.7840 Da (Δm/z: -3.8891 ppm); calculated molecular weight of α-*N*-gluconoylated *holo*-FosACP7 (*): 13543.8871 Da and deconvoluted mass: 13548.8231 Da (Δm/z: −4.7254 ppm).

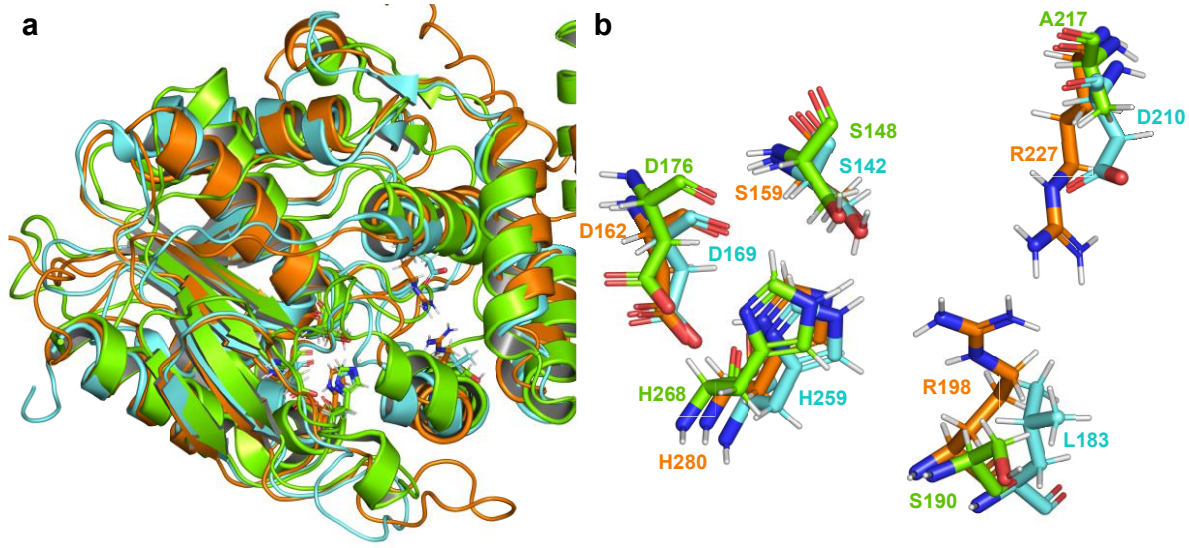


Supplementary Figure 23: LC-HRMS analysis of *holo*-FosACP8, purified from *E. coli* BAP1. Total ion chromatogram (top), mass spectrum of the peak at 9.49 min (middle) and deconvoluted mass spectrum (bottom). Calculated molecular weight of *holo*-FosACP8: 12956.5692 Da and deconvoluted mass: 12956.5354 Da (Δm/z: −2.6087 ppm).

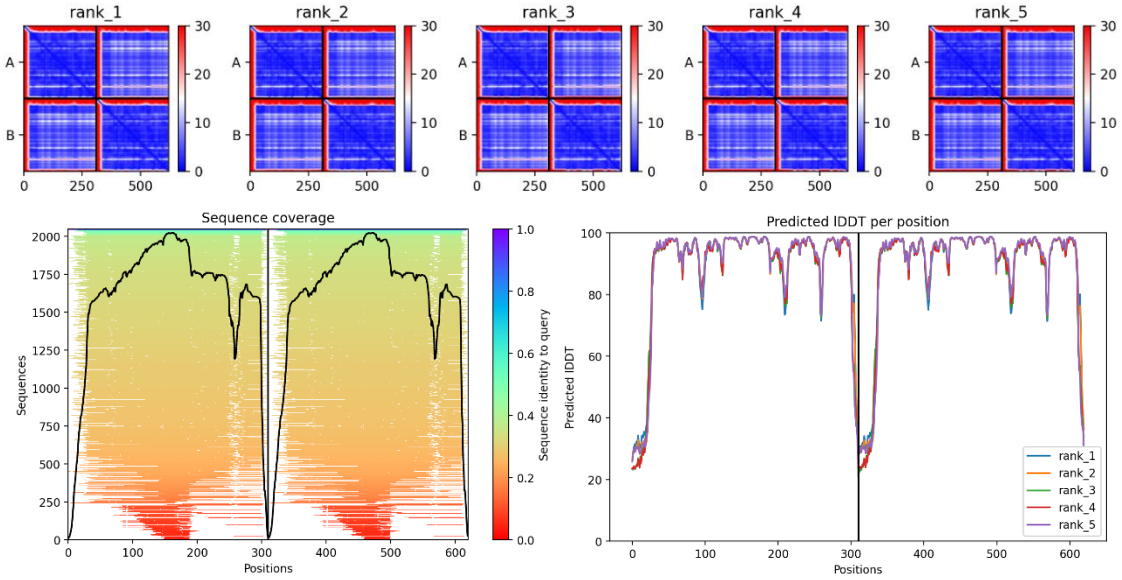
Bioinformatic analysis



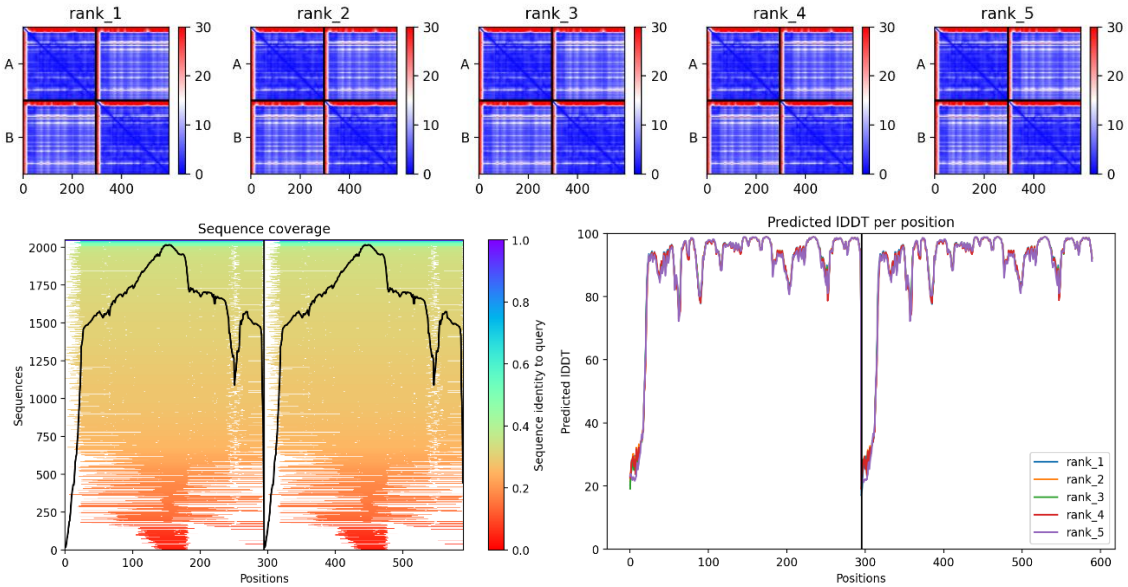
Supplementary Figure 24: Structural comparison of Alphafold2 models of FosTE (Fostriecin PKS), PlmTE (Phoslactomycin PKS) and LepTE (Leptomycin PKS). **a,** Superposition of predicted models of FosTE (orange), PlmTE (blue), and LepTE (red) highlights similarities and differences in overall structural features. All structures are featuring the canonical α/β-hydrolase fold characteristic of PKS TE domains, the N-terminal α-helices forming the dimer interface and a catalytic triad positioned at the center of a tunnel. **b,** Close-up views of the active sites, showing the characteristic catalytic triad (His, Asp, Ser) and structurally conserved arginine residues implicated in *O*-malonylation and lactonisation. Structures were rendered using PyMol.



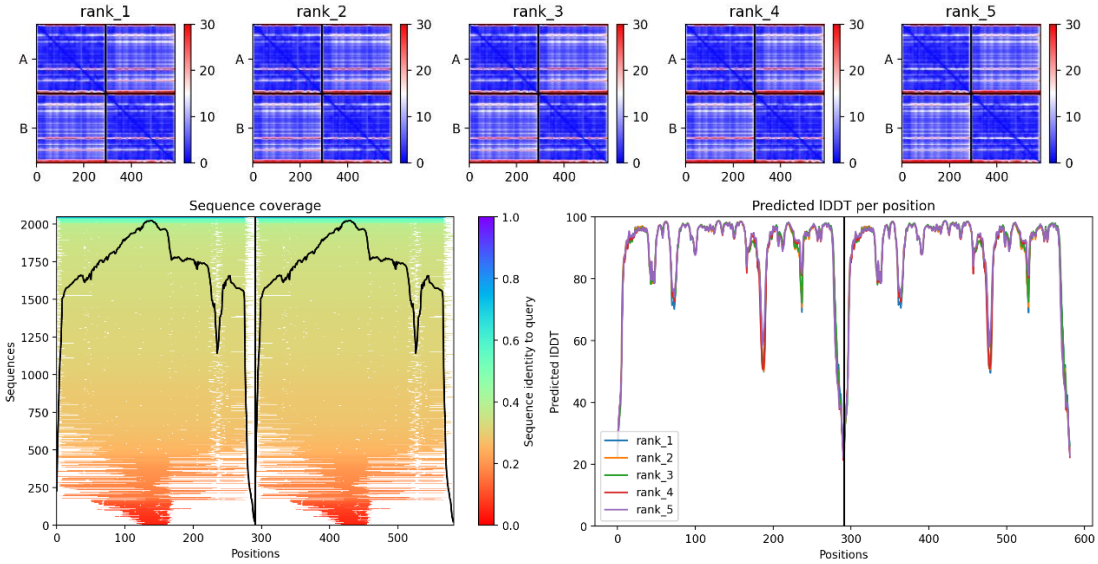
Supplementary Figure 25: Structural comparison of FosTE (Fostriecin PKS) Alphafold2 model with solved structures of EryTE (Erythromycin PKS, PDB 5D3K) and PikTE (Pikromycin PKS, PDB 1MNA). a, Superposition of the predicted FosTE model (orange) with crystal structures of EryTE (aquamarine) and PikTE (green) highlights similarities and differences in overall structural features. All structures feature the canonical α/β-hydrolase fold characteristic of PKS TE domains, with N-terminal α-helices forming the dimer interface and a catalytic triad positioned at the center of a tunnel. **b,** Close-up views of the active sites, showing the characteristic catalytic triad (His, Asp, Ser). Unlike EryTE and PikTE, which have smaller, hydrophobic (S190, L183, A217) or oppositely charged (D210) residues at these positions, FosTE features structurally conserved arginine residues (R198 and R227) implicated in *O*-malonylation and lactonisation. Structures were rendered using PyMol.



Supplementary Figure 26: Quality of FosTE (Fostriecin PKS) structure prediction with Alphafold2/ColabFold.[2] **a,** Predicted aligned error (PAE) heat map for the top five ranked relaxed structural models to measure the confidence in the relative position of two residues within the predicted structure on a scale from 0 – 30. The lower the PAE score the higher the confidence (blue = low PAE score; red = high PAE score). **b,** MSA sequence coverage. One colored bar in the plot represents a sequence within the MSA input and is arranged according to its similarity on a scale from 0.0 – 1.0. The number of matches for each position is depicted as black line. **c,** Per-residue confidence estimate of predictions (pLDDT) to measure local structure confidence on a scale from 0 – 100 (> 90: very high confidence (blue), 70 – 90: high confidence (cyan), 50 – 70: low confidence (yellow), <50: very low confidence (orange/red).



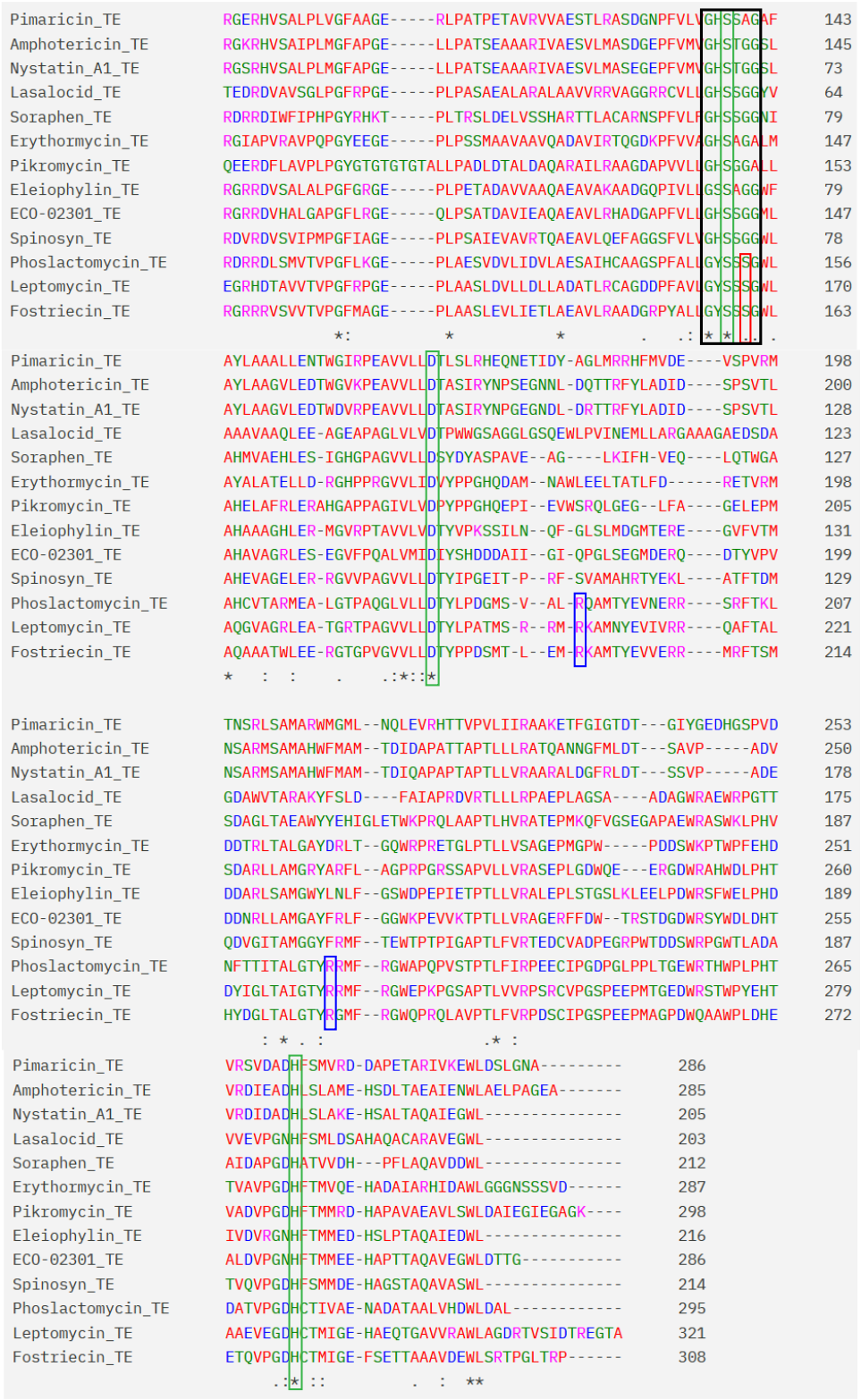
Supplementary Figure 27: Quality of PlmTE (Phoslactomycin PKS) structure prediction with Alphafold2/ColabFold.[2] **a,** Predicted aligned error (PAE) heat map for the top five ranked relaxed structural models to measure the confidence in the relative position of two residues within the predicted structure on a scale from 0 – 30. The lower the PAE score the higher the confidence (blue = low PAE score; red = high PAE score). **b,** MSA sequence coverage. One colored bar in the plot represents a sequence within the MSA input and is arranged according to its similarity on a scale from 0.0 – 1.0. The number of matches for each position is depicted as black line. **c,** Per-residue confidence estimate of predictions (pLDDT) to measure local structure confidence on a scale from 0 – 100 (> 90: very high confidence (blue), 70 – 90: high confidence (cyan), 50 – 70: low confidence (yellow), <50: very low confidence (orange/red).



Supplementary Figure 28: Quality of LepTE (Leptomycin PKS) structure prediction with Alphafold2/ColabFold.[2] **a,** Predicted aligned error (PAE) heat map for the top five ranked relaxed structural models to measure the confidence in the relative position of two residues within the predicted structure on a scale from 0 – 30. The lower the PAE score the higher the confidence (blue = low PAE score; red = high PAE score). **b,** MSA sequence coverage. One colored bar in the plot represents a sequence within the MSA input and is arranged according to its similarity on a scale from 0.0 – 1.0. The number of matches for each position is depicted as black line. **c,** Per-residue confidence estimate of predictions (pLDDT) to measure local structure confidence on a scale from 0 – 100 (> 90: very high confidence (blue), 70 – 90: high confidence (cyan), 50 – 70: low confidence (yellow), <50: very low confidence (orange/red).

```
PlmTE       -------AGHDRTAQKTGE-------GHEDLVSGADESFAAIYRKVALRGRMEEVEDLLG 46
LepTE       HETPAALAAHLADALREGVPETRPAPTAPGGAEDSNDMLGTVYRKLALLGRMDDAESLLV 60
FosTE       -------GGDAGASPAPGVSAAPSASVAAAPPQDSNDSVVGIYRKLSLQGRMQEVEAFLS 53
                   ...   :   *              ..::: .  :***::* ***::.* :*

PlmTE       SASGLRARFSGASEAPDGTRFVRLATGGQGPAVISFPPFAPVEQTLQFVRLASFFRDRRD 106
LepTE       GAAGLRQTFEDPNRLPKTPGFTRLARGPARPRVICFPPFAPVEGAIQFGRLAGTFEGRHD 120
FosTE       SASALRTRFHGAEDLGRGAHVTTLGHGEAEPQLVCFPPFAPVDGSLQFARLANHFRGRRR 113
            .*:.**  * . .       .. *. *   * ::.*******: ::** ***. *..*:

PlmTE       LSMVTVPGFLKGEPLAESVDVLIDVLAESAIHCAAGSPFALLGYSSSGWLAHCVTARMEA 166
LepTE       TAVVTVPGFRPGEPLAASLDVLLDLLADATLRCAGDDPFAVLGYSSSGWLAQGVAGRLEA 180
FosTE       VSVVTVPGFMAGEPLAASLEVLIETLAEAVLRAADGRPYALLGYSSSGWLAQAAATWLEE 173
             ::******  ***** *::**:: **::.::.* . *:*:*********: .:  :*

PlmTE       LGTPAQGLVLLDTYLPDGMSVALRQAMTYEVNERRSRFTKLNFTTITALGTYRRMFRGWA 226
LepTE       TGRTPAGVVLLDTYLPATMSRRMRKAMNYEVIVRRQAFTALDYIGLTAIGTYRRMFRGWE 240
FosTE       RGTGPVGVVLLDTYPPDSMTLEMRKAMTYEVVERRMRFTSMHYDGLTALGTYRGMFRGWQ 233
             *    *:****** *  *:  :*:**.***  **  ** :.:  :**:**** *****

PlmTE       PQPVSTPTLFIRPEECIPGDPGLPPLTGEWRTHWPLPHTDATVPGDHCTIVAENADATAA 286
LepTE       PKPGSAPTLVVRPSRCVPGSPEEPMTGEDWRSTWPYEHTAAEVEGDHCTMIGEHAEQTGA 300
FosTE       PRQLAVPTLFVRPDSCIPGSPEEPMAGPDWQAAWPLDHEETQVPGDHCTMIGEFSETTAA 293
            *:  :.***.:**. *:**.*  *    :*:: **  *  : * ******::.* :: *.*

PlmTE       LVHDWLDAL------------        295
LepTE       VVRAWLAGDRTVSIDTREGTA        321
FosTE       AVDEWLSRTPGLTRP------        308
             *  **
```

Supplementary Figure 29: Multiple sequence alignment of TE domains from FosTE (Fostriecin PKS), PlmTE (Phoslactomycin PKS) and LepTE (Leptomycin PKS). Amino acid sequences of FosTE, PlmTE, and LepTE were aligned using Clustal Omega[3] to identify conserved and functionally important residues. Conserved sequence motifs characteristic of type I PKS TE domains are highlighted, including the canonical GXSXG motif (boxed in black), which contains the active site serine residue (S, indicated by a green box). The conserved aspartate (D, boxed in green) and histidine (H, boxed in green) residues, which together with the active site serine form the catalytic triad, are also highlighted. Additionally, the alignment shows the conservation of arginine residues (R, boxed in blue) implicated in *O*-malonylation among FosTE, PlmTE, and LepTE.



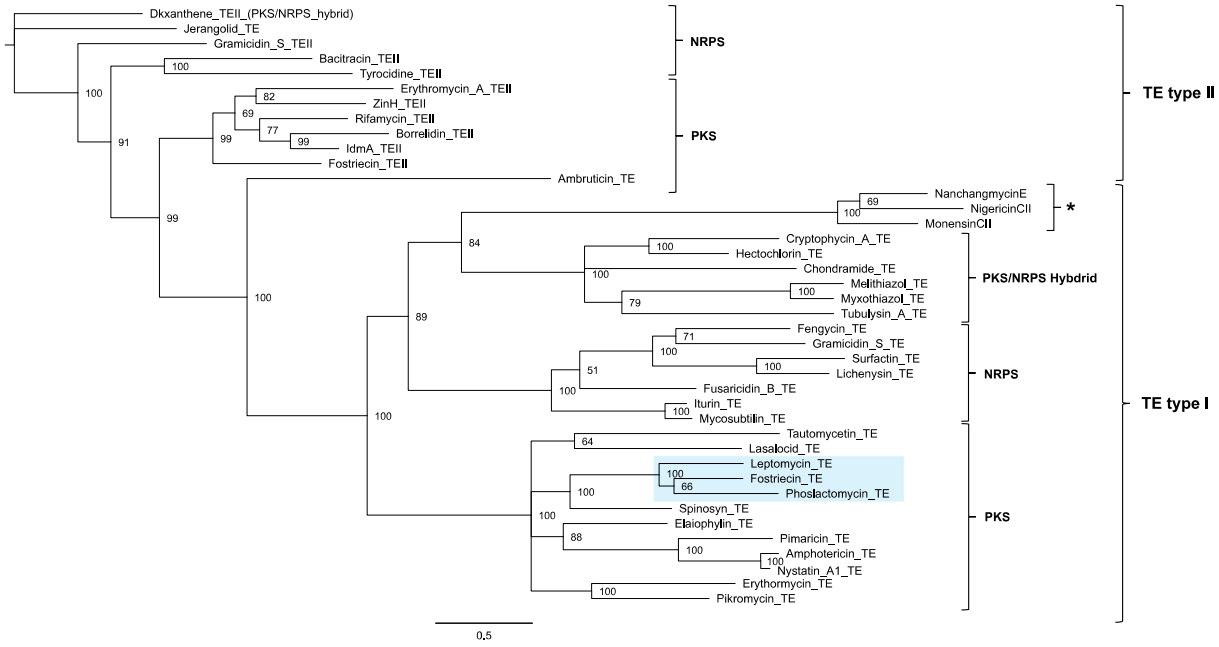
Supplementary Figure 30: Multiple sequence alignment of TE domains from selected multimodular Type I PKS systems. Amino acid sequences of TE domains from multimodular type I PKSs were aligned using Clustal Omega[3] to identify conserved and functionally important residues. Conserved sequence motifs characteristic of type I PKS TE domains are highlighted, including the canonical GXSXG motif (boxed in black), which contains the active site serine residue (S, indicated by a green box). The conserved aspartate (D, boxed in green) and histidine (H, boxed in green) residues, which together with the active site serine form the catalytic triad, are also emphasised. Additionally, the alignment shows the conservation of the arginine residues involved in *O*-malonylation among FosTE, PlmTE and LepTE (R, boxed in blue).

```
#=======================================
#
# Aligned_sequences: 2
# 1: FosTE
# 2: PlmTE
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 314
# Identity:     153/314 (48.7%)
# Similarity:   197/314 (62.7%)
# Gaps:          25/314 ( 8.0%)
# Score: 778.0
#
#
#=======================================
```

```
#=======================================
#
# Aligned_sequences: 2
# 1: FosTE
# 2: LepTE
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 324
# Identity:     167/324 (51.5%)
# Similarity:   206/324 (63.6%)
# Gaps:          19/324 ( 5.9%)
# Score: 837.5
#
#
#=======================================
```

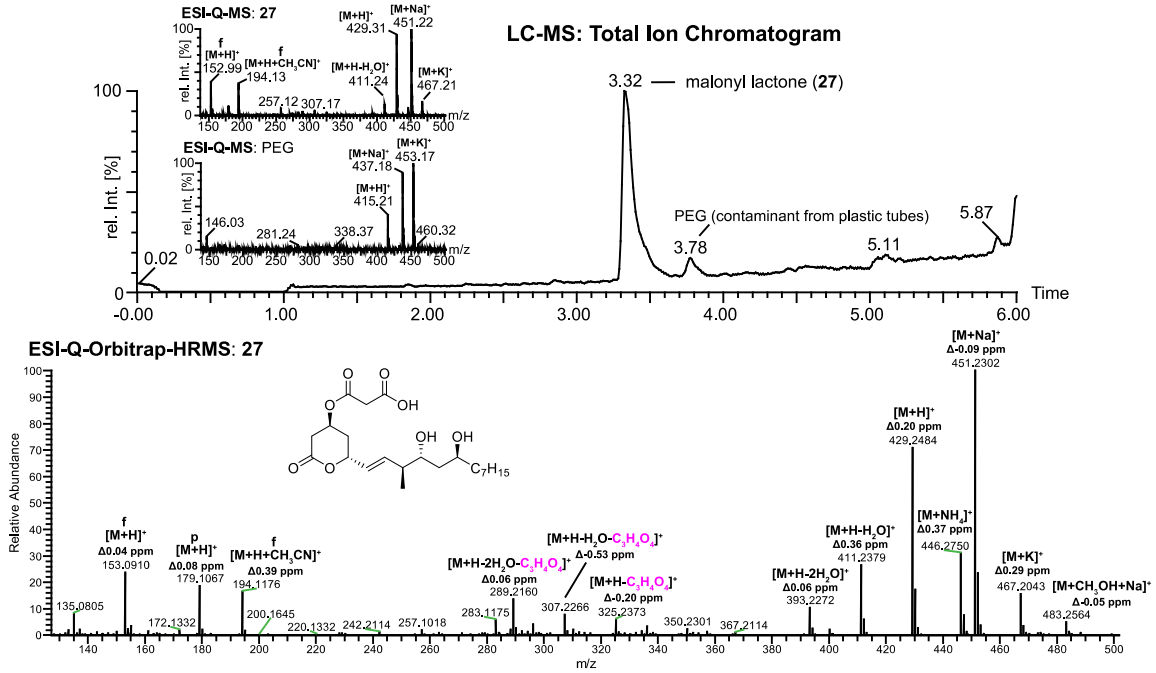
```
#=======================================
#
# Aligned_sequences: 2
# 1: PlmTE
# 2: LepTE
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 326
# Identity:     154/326 (47.2%)
# Similarity:   191/326 (58.6%)
# Gaps:          36/326 (11.0%)
# Score: 768.5
#
#
#=======================================
```

Supplementary Figure 31: Pairwise sequence alignment statistics for FosTE, PlmTE and LepTE. Pairwise alignments were performed to compare the amino acid sequences of FosTE, PlmTE, and LepTE. The results show that FosTE and PlmTE share 48.7% identical and 62.7% similar residues over 314 aligned positions, with 8.0% gaps. FosTE and LepTE are 51.5% identical and 63.6% similar over 324 positions, with 5.9% gaps. PlmTE and LepTE share 47.2% identity and 58.6% similarity over 326 positions, with 11.0% gaps. These values indicate that all three proteins are closely related, with FosTE and LepTE being the most similar pair. Alignment parameters: EBLOSUM62 substitution matrix, gap penalty 10.0, extension penalty 0.5.

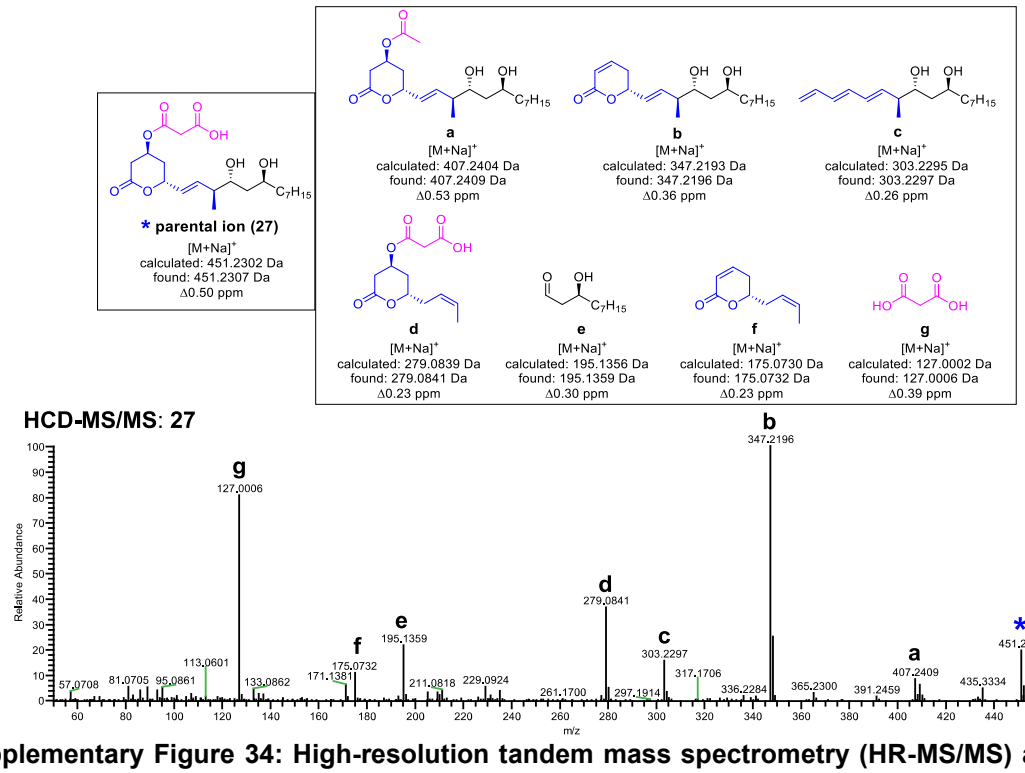


Supplementary Figure 32: Bayesian phylogenetic tree of selected type I and II TE domains from PKS and NRPS systems. The tree was constructed using Bayesian inference in MrBayes 3.2.7, with 1000000 Markov Chain Monte Carlo (MCMC) generations (four chains: three heated, one cold), sampling every 1000 generations and discarding the first 25% as burn-in prior to consensus tree construction. Posterior probabilities are indicated at the nodes. Branch lengths are proportional to the number of substitutions per site; the scale bar represents 0.5 substitutions per site. The tree was visualised using FigTree v1.4.4. The corresponding multiple sequence alignment was created using MAFFT (v7.511)[4] with the L-INS-i strategy. FosTE, PlmTE, and LepTE are highlighted in blue within a well-supported clade of type I PKS TE domains. These three TE domains cluster closely together, supported by high posterior probabilities, indicating a strong evolutionary relationship. Their placement within the type I PKS TE clade suggests that FosTE, PlmTE, and LepTE share a recent common ancestor and have likely conserved both structural features and catalytic functions. *Nanchangmycin, nigericin and monensin TEs are discrete enzymes (resembling stand-alone type II TEs) but act as type I TEs in the respective biosynthesis.[5,6]

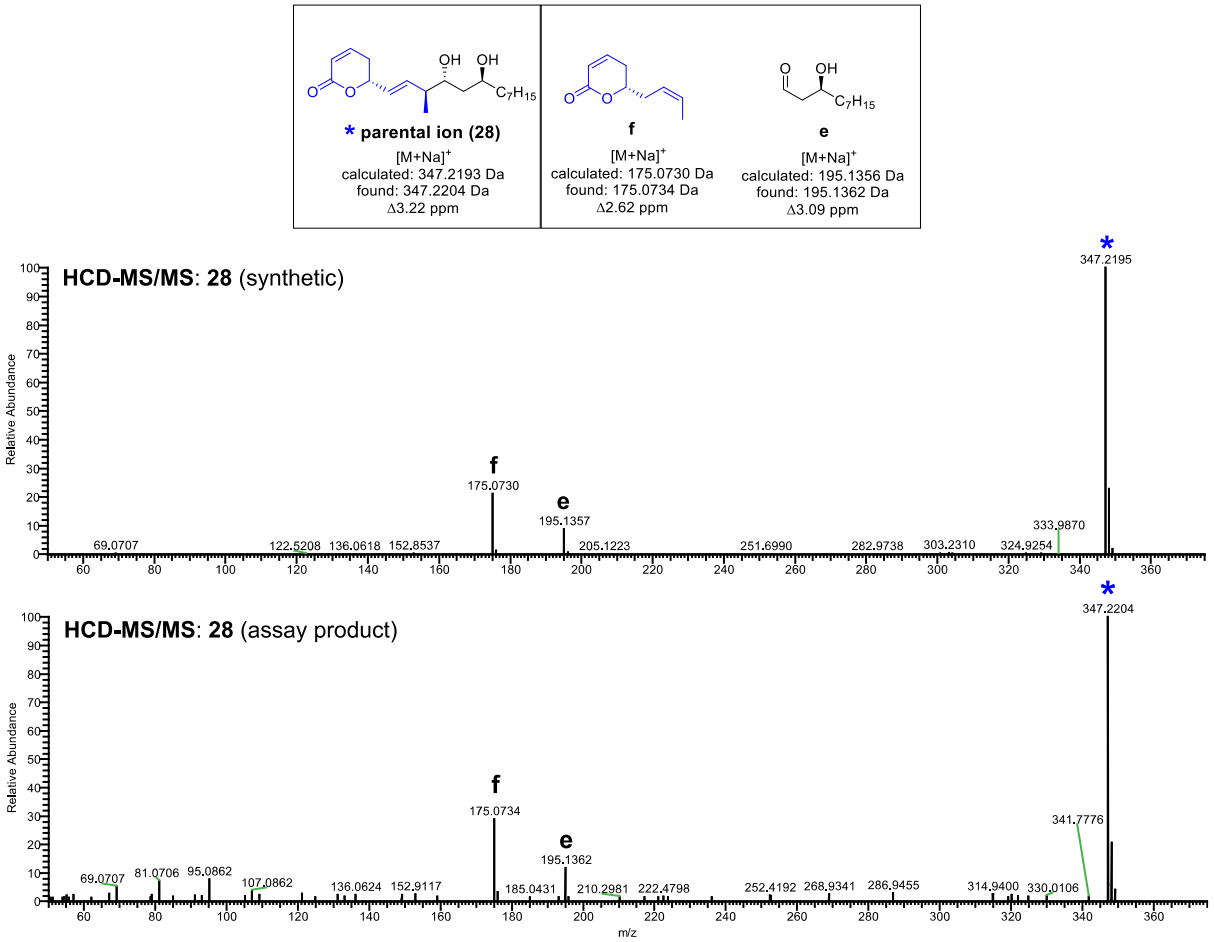
LC-MS/MS and LC-HRMS analysis of assay products



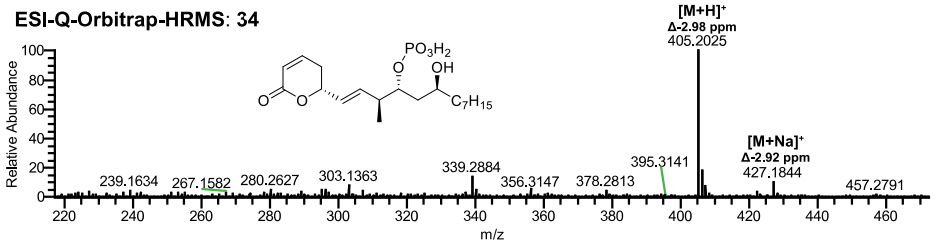
Supplementary Figure 33: LC-MS and HRMS analysis of malonyl lactone (27). The upper panel shows the LC-MS total ion chromatogram with a main peak at 3.32 min corresponding to malonyl lactone **27**. The smaller peak at 3.78 min is identified as PEG contaminant from plastic ware. The inserted spectra illustrate ESI-Q-MS data for malonyl lactone **27** and the PEG contaminant. The lower panel represents the high-resolution ESI-Q-Orbitrap-HRMS spectrum of malonyl lactone **27**, showing the most abundant adduct ions and characteristic fragments. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 34.



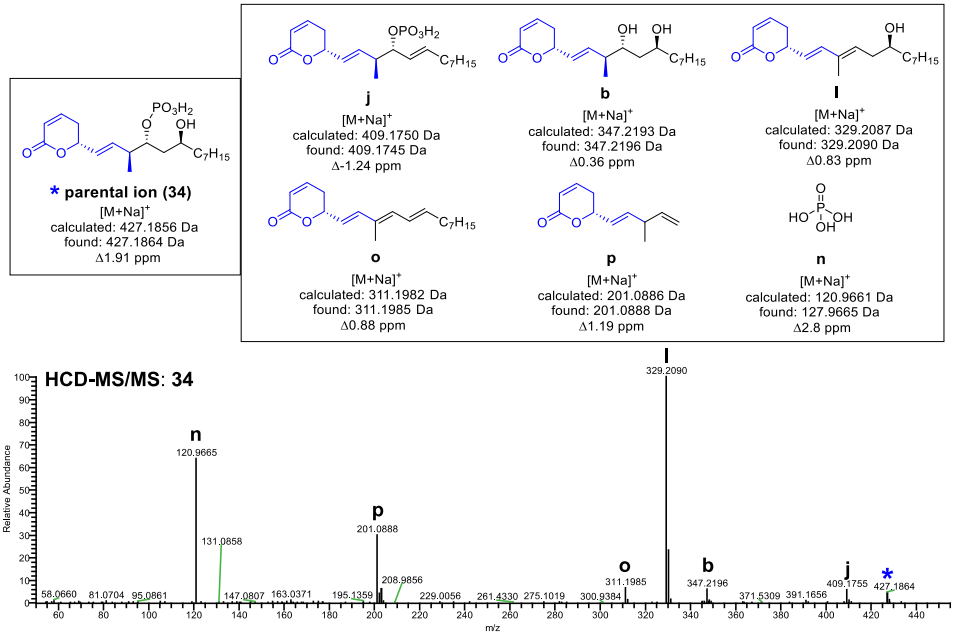
Supplementary Figure 34: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of malonyl lactone 27. The upper panel shows the proposed fragmentation scheme of malonyl lactone **27** with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 451.2307 (*), with major fragment ions corresponding to the structures above. Calculated and observed m/z values, as well as mass errors (Δ in ppm), are provided for each fragment.



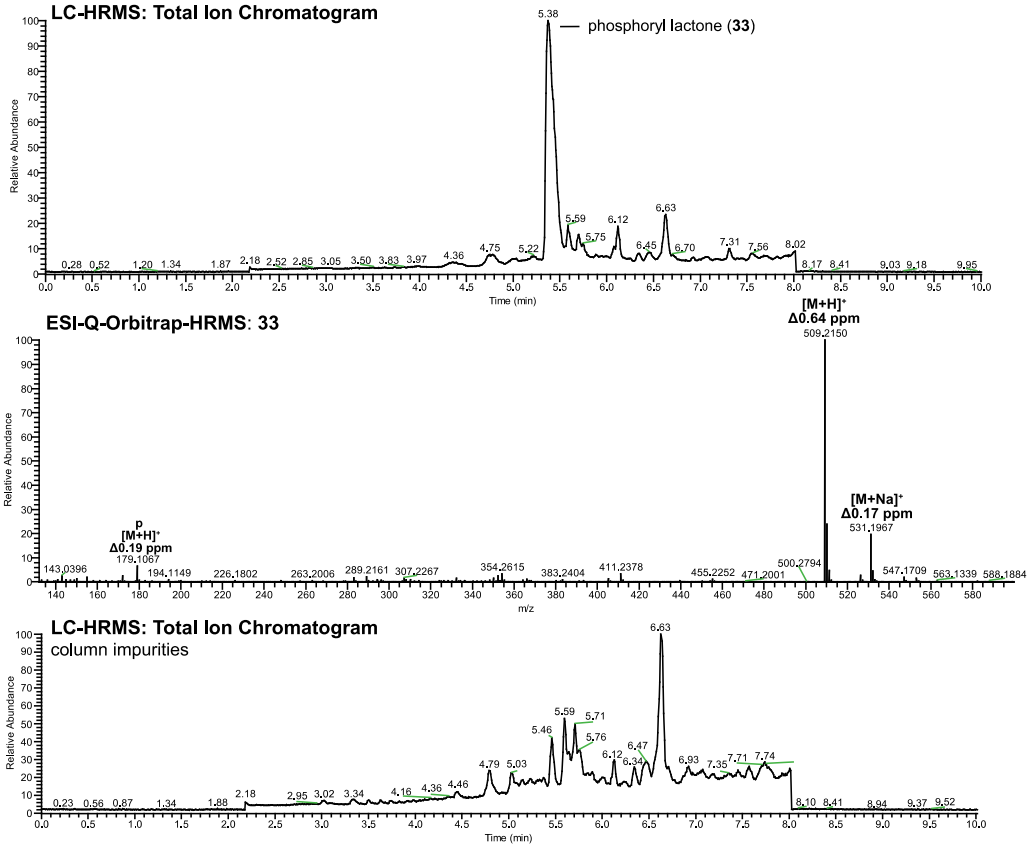
Supplementary Figure 35: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of lactone 28. The upper panel shows the proposed fragmentation pattern with assigned structures and calculated/observed m/z values, including mass errors (Δ in ppm). The lower panels display higher-energy collisional dissociation (HCD) MS/MS spectra (normalised collision energy: 35) of the parental (*) sodium adducts $[M+Na]^+$ at m/z 347.2195 (synthetic reference) and 347.2204 (assay product) for the chemically synthesised compound (top) and the enzymatic assay product (bottom), with major fragment ions corresponding to the structures above.



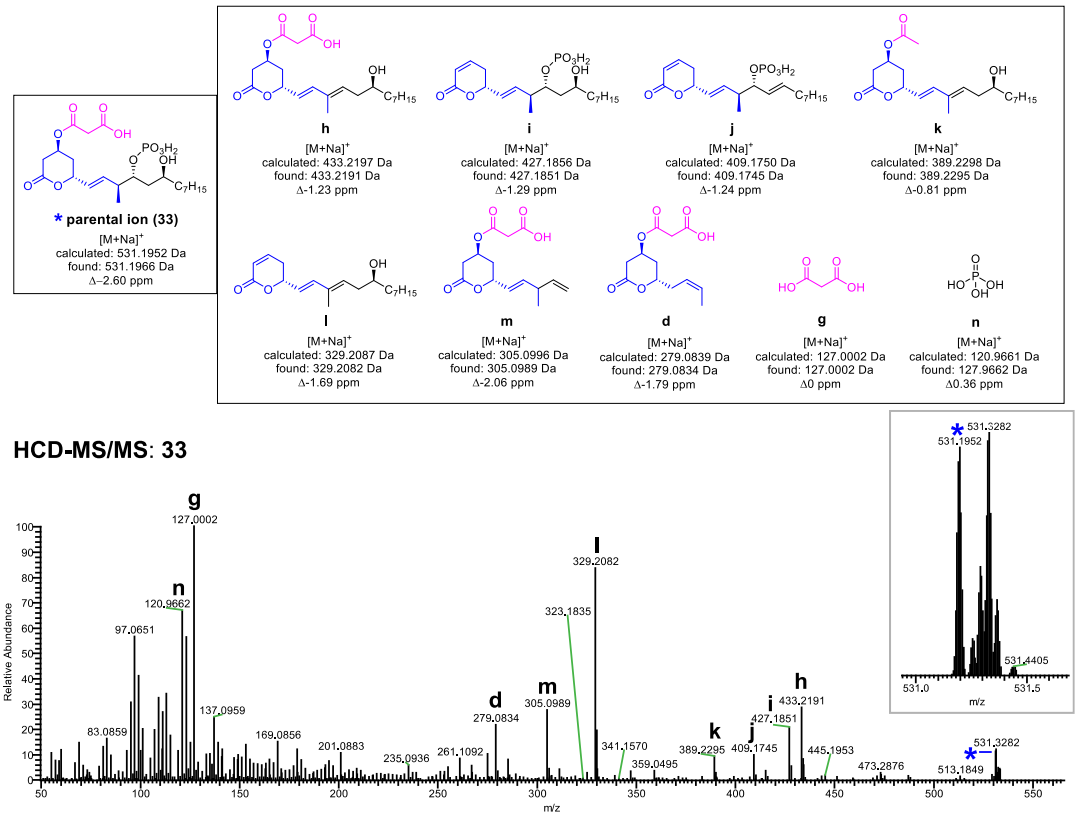
Supplementary Figure 36: HRMS analysis of phosphoryl lactone 34. High-resolution ESI-Q-Orbitrap-HRMS spectrum of malonyl lactone **34**, showing the most abundant adduct ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 37.



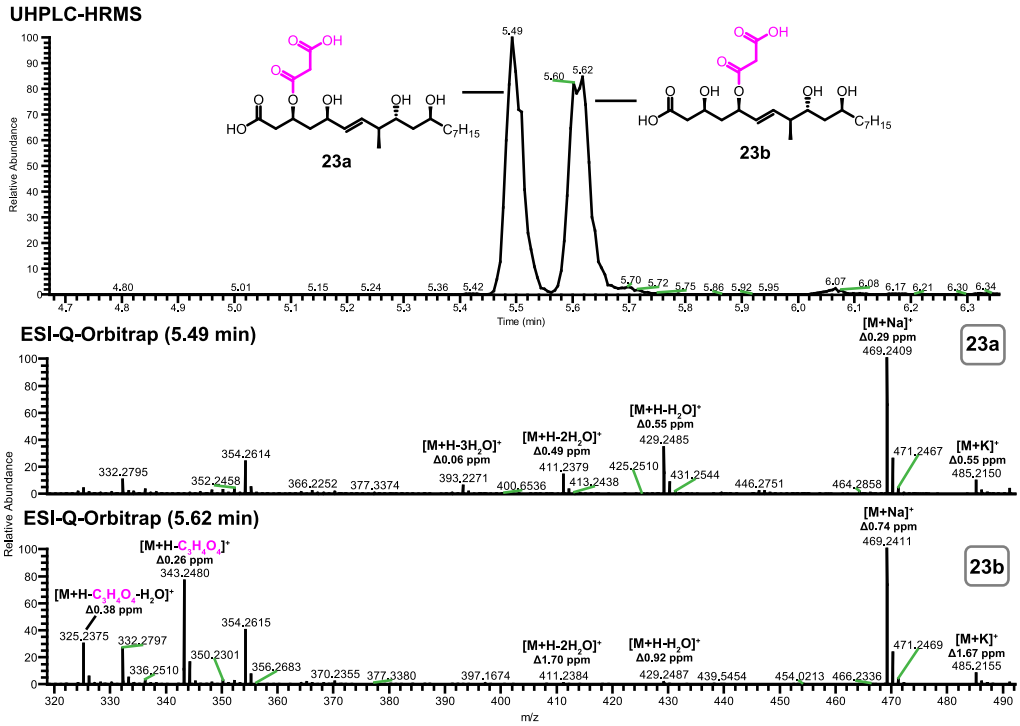
Supplementary Figure 37: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of phosphoryl lactone 34. The upper panel shows the proposed fragmentation scheme of phosphoryl lactone **34** with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 427.1864 (*), with major fragment ions corresponding to the structures above. Calculated and observed m/z values, as well as mass errors (Δ in ppm), are provided for each fragment.



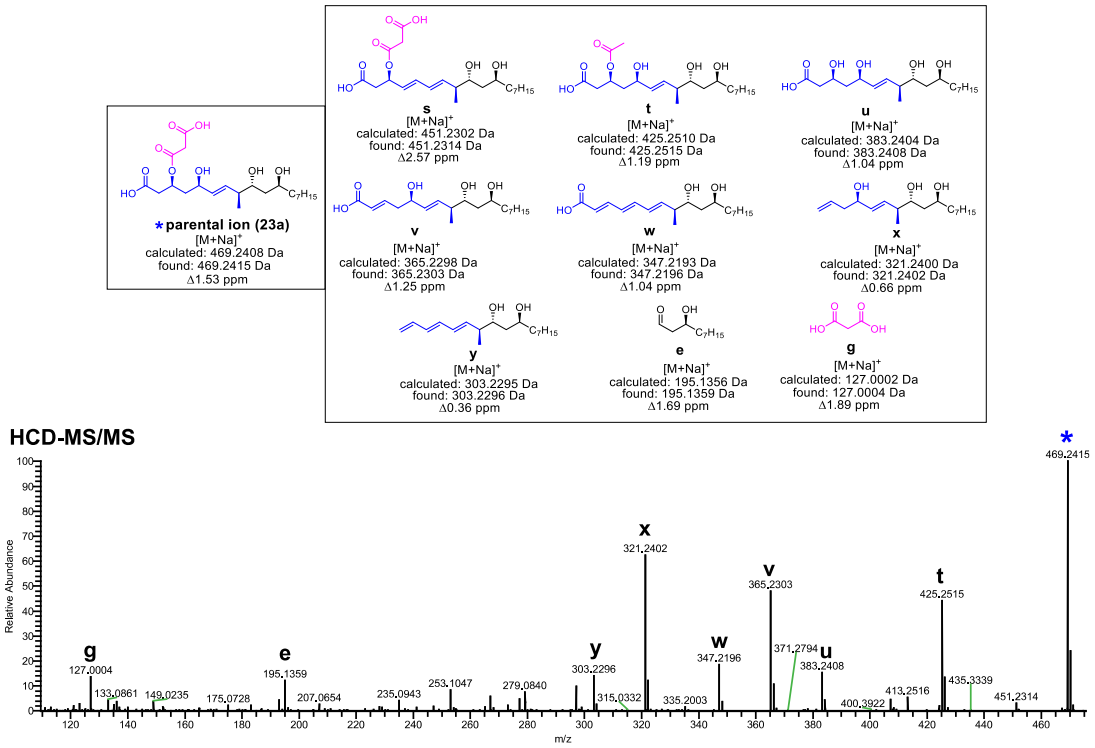
Supplementary Figure 38: LC-MS and HRMS analysis of phosphoryl lactone (33). The upper panel shows the total ion chromatogram with a main peak at 5.38 min corresponding to phosphoryl lactone **33**. The middle panel displays the ESI-Q-Orbitrap-HRMS spectrum of phosphoryl lactone **33**, showing the most abundant $[M+H]^+$ and $[M+Na]^+$ adduct ions. The lower panel presents the LC-MS total ion chromatogram of column impurities, with multiple peaks corresponding to background signals from the chromatographic system. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 39.



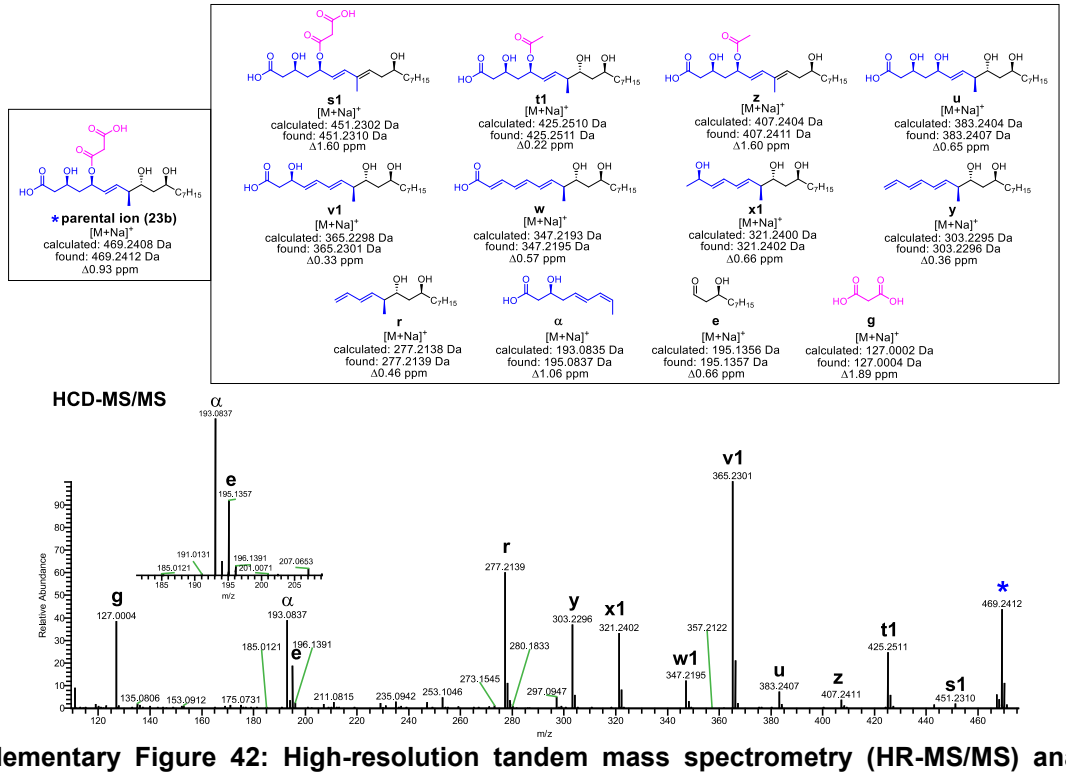
Supplementary Figure 39: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of phosphoryl lactone 33. The upper panel shows the proposed fragmentation scheme of phosphoryl lactone **33** with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 30) of the parental sodium adduct $[M+Na]^+$ at *m/z* 427.1864 (*), with major fragment ions corresponding to the structures above. Calculated and observed m/z values, as well as mass errors (Δ in ppm), are provided for each fragment.



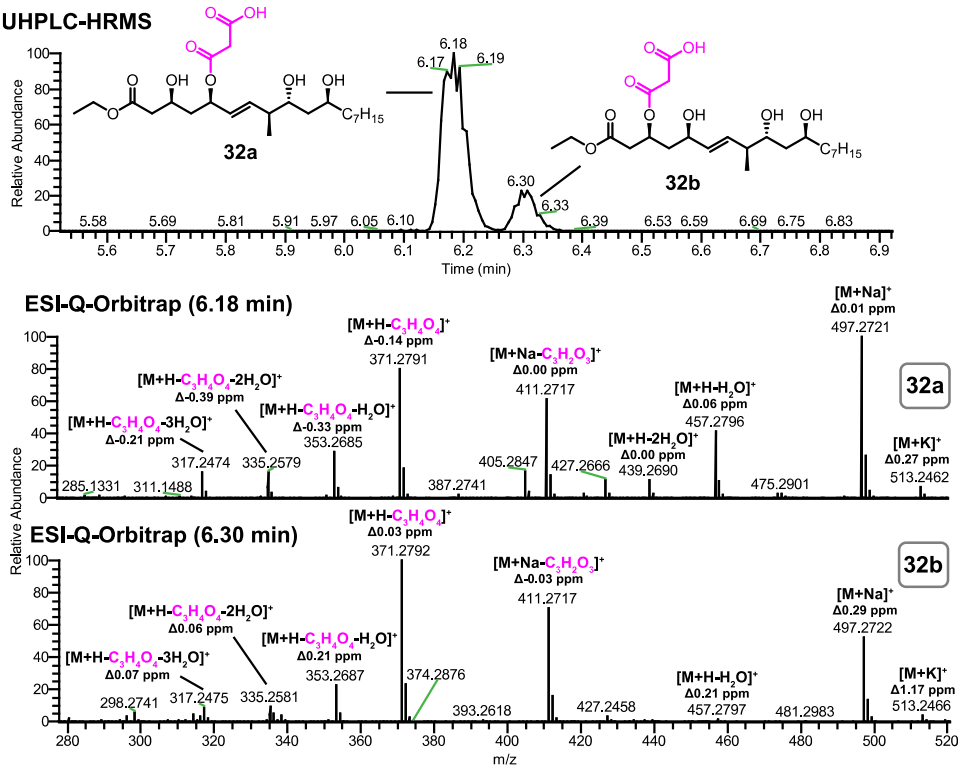
Supplementary Figure 40: LC-MS and HRMS analysis of carboxylic acid 23. The upper panel shows the LC-MS chromatogram with peaks at 5.49 and 5.52 min corresponding to **23a** and **23b**. The lower panels display the ESI-Q-Orbitrap-HRMS spectra, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectra are shown in Supplementary Figs. 41 and 42.



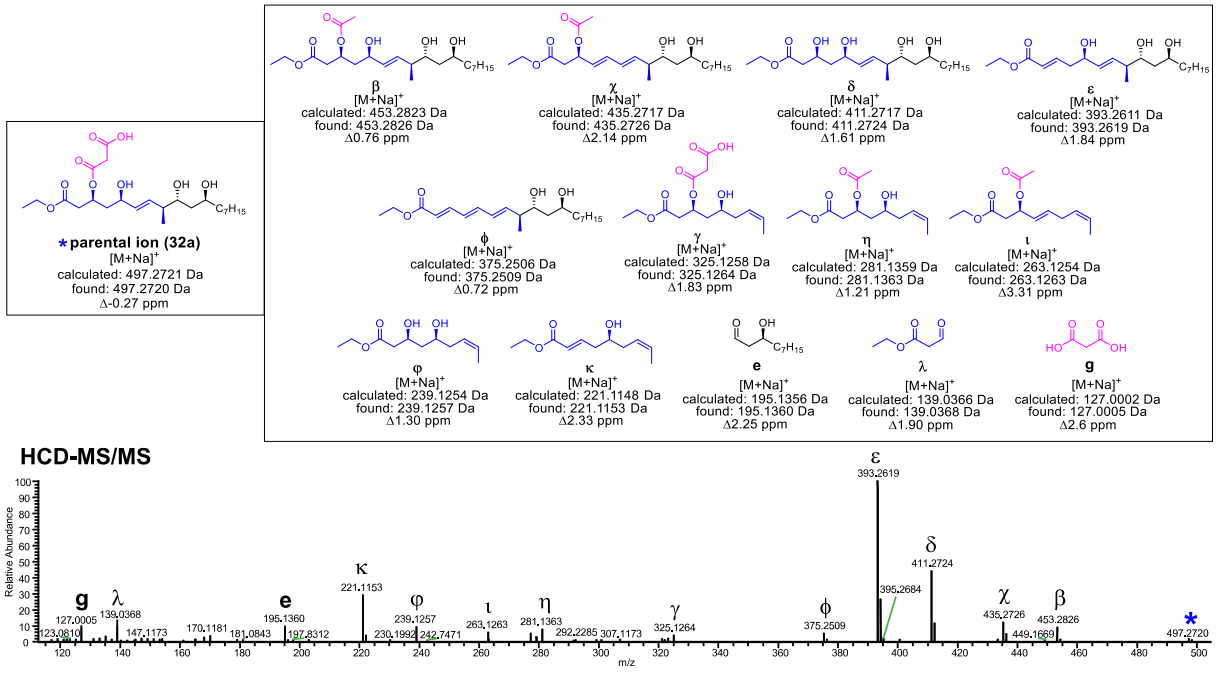
Supplementary Figure 41: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 23a. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 469.2415 (*), with major fragment ions corresponding to the structures above. Calculated and observed m/z values, as well as mass errors (Δ in ppm), are provided for each fragment.



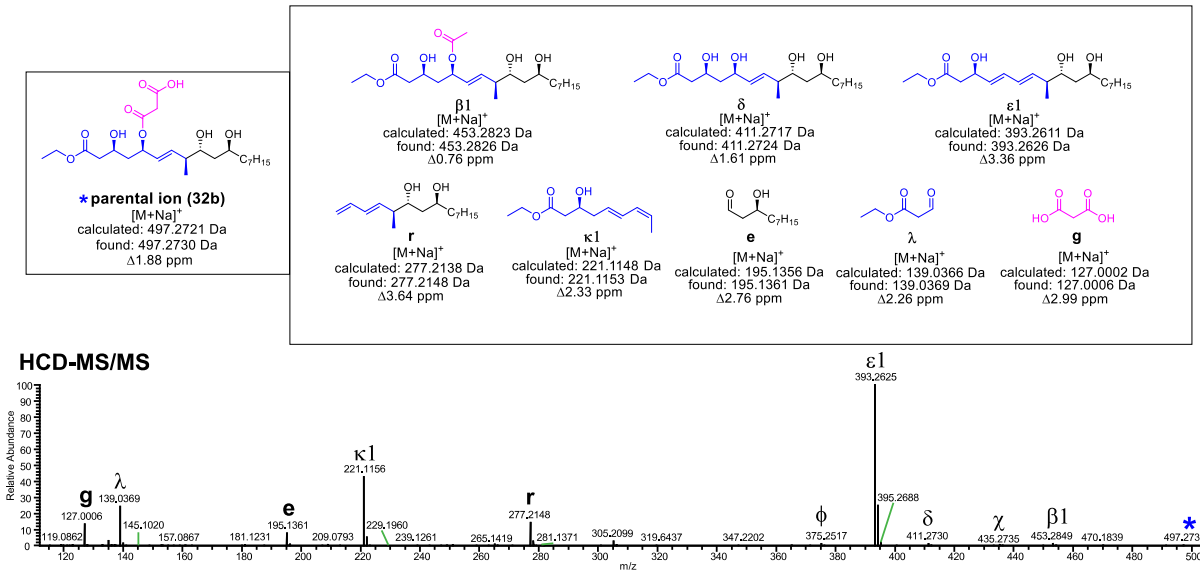
Supplementary Figure 42: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 23b. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 469.2415 (*), with major fragment ions corresponding to the structures above. Calculated and observed m/z values, as well as mass errors (Δ in ppm), are provided for each fragment.



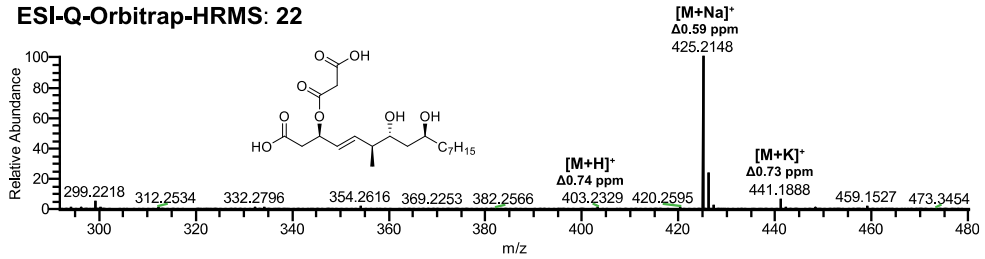
Supplementary Figure 43: LC-MS and HRMS analysis of carboxylic acid 32. The upper panel shows the LC-MS chromatogram with peaks at 5.49 and 5.52 min corresponding to **32a** and **32b**. The lower panels displays the ESI-Q-Orbitrap-HRMS spectra, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectra are shown in Supplementary Figs. 44 and 45.



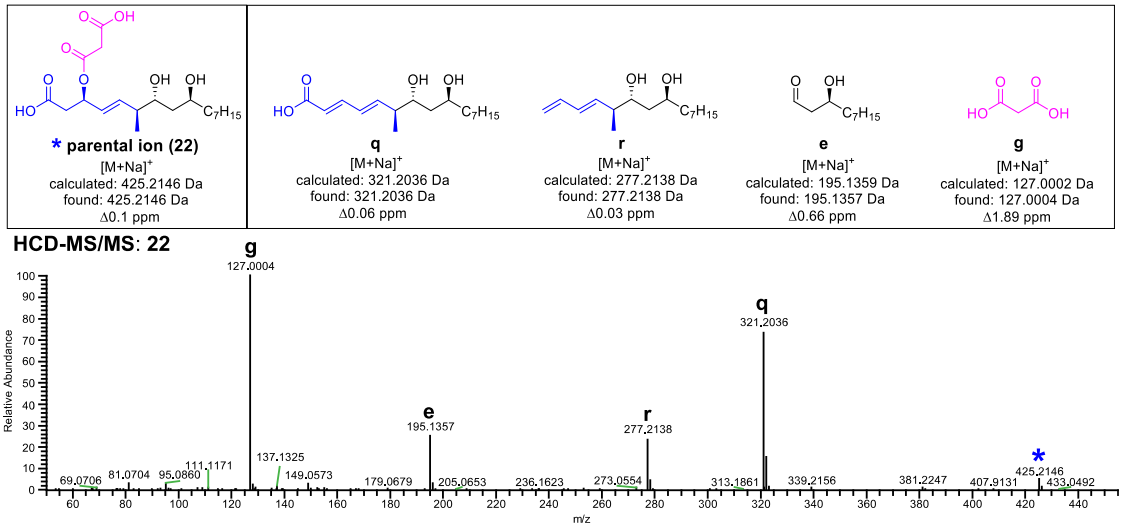
Supplementary Figure 44: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 32a. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 497.2720 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



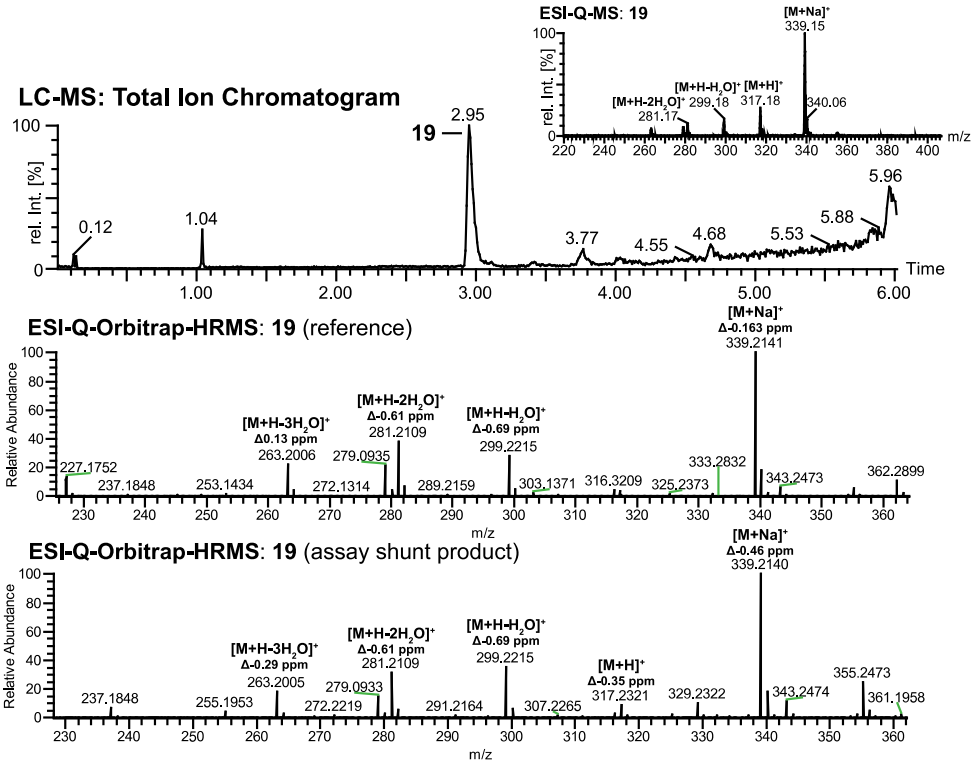
Supplementary Figure 45: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 32b. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 497.2730 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



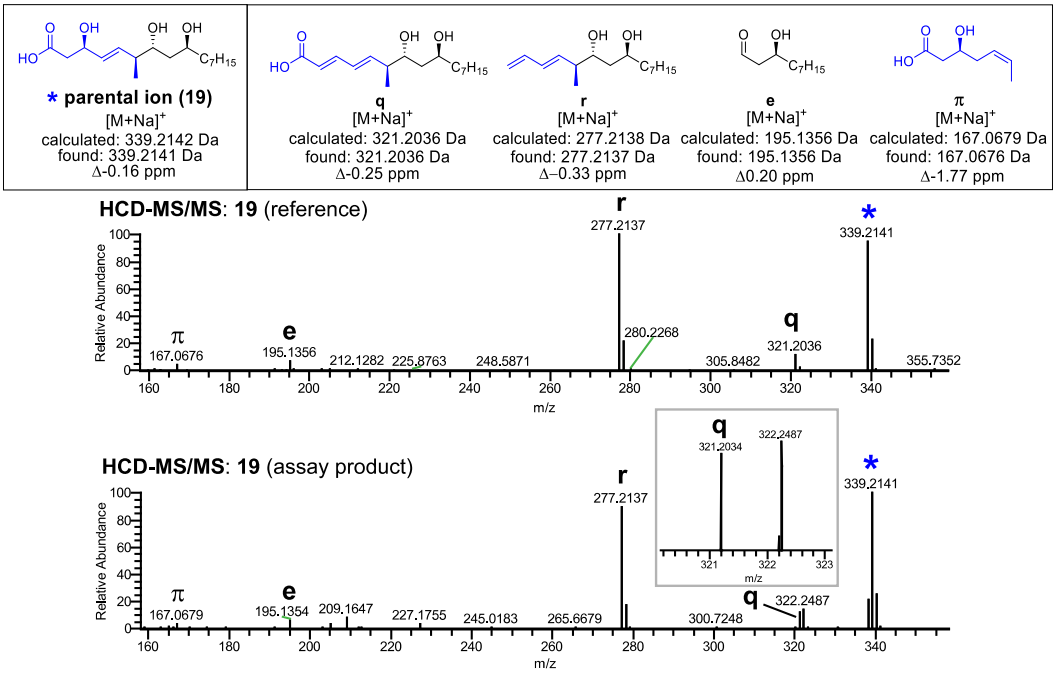
Supplementary Figure 46: HRMS analysis of carboxylic acid 22. High-resolution ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 47.



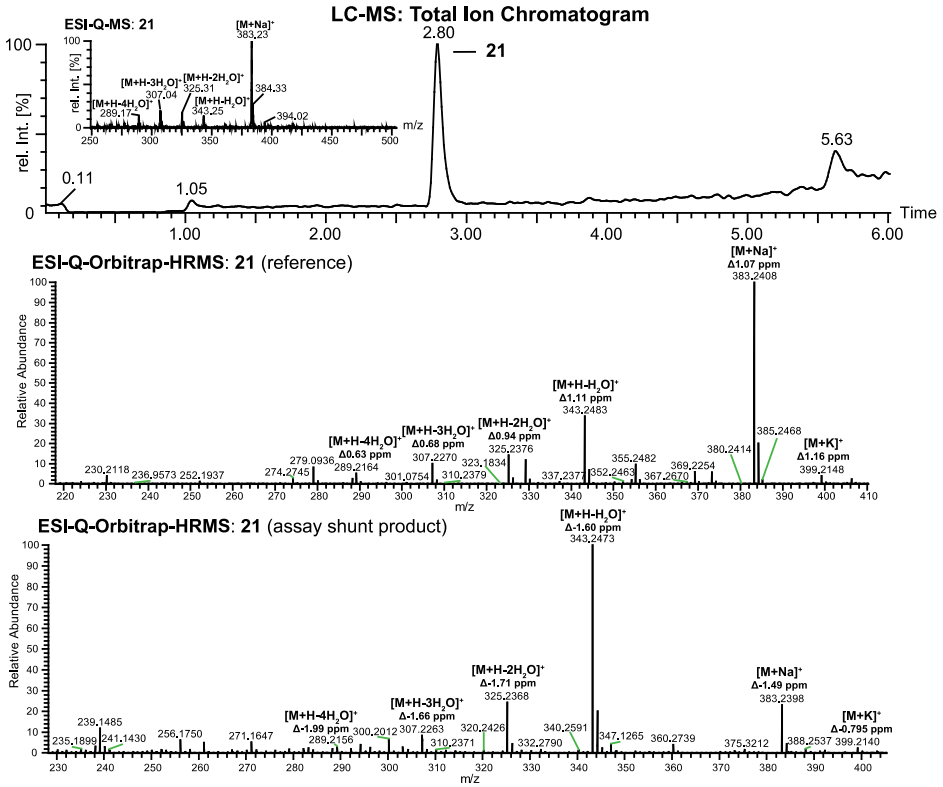
Supplementary Figure 47: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 22. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 425.2146 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



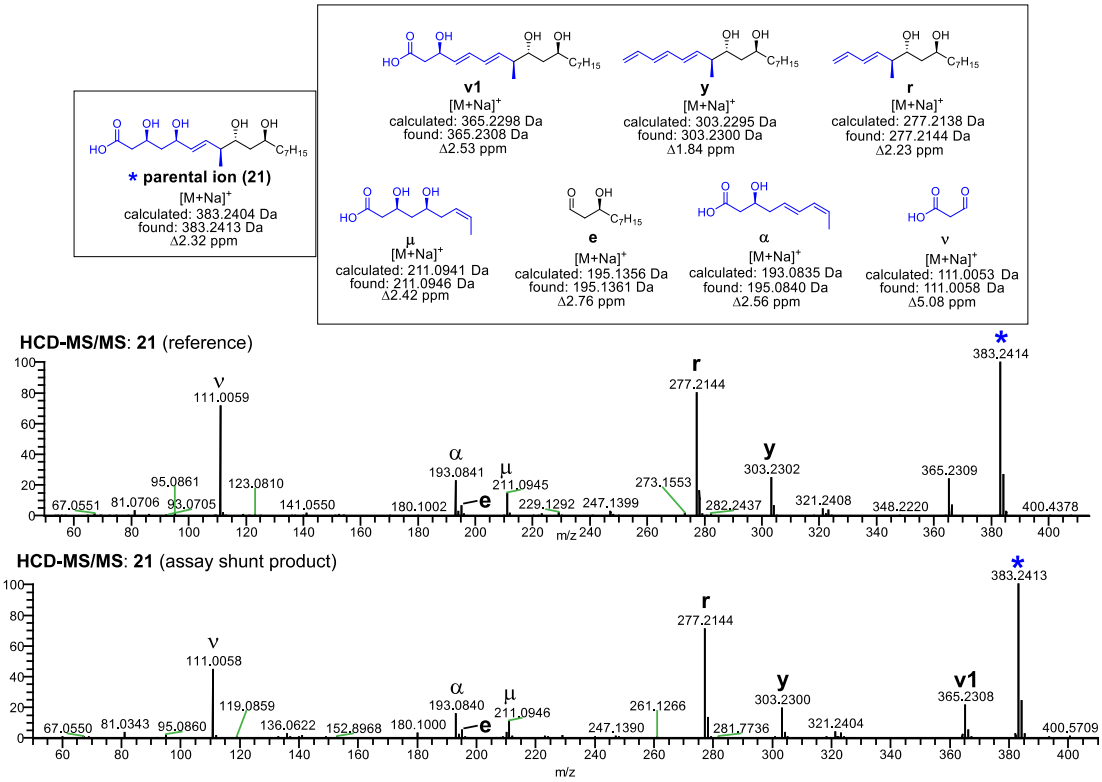
Supplementary Figure 48: LC-MS and HRMS analysis of carboxylic acid 19. The upper panel shows the total ion chromatogram with a main peak at 2.95 min corresponding to **19**. The middle panel displays the ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 49.



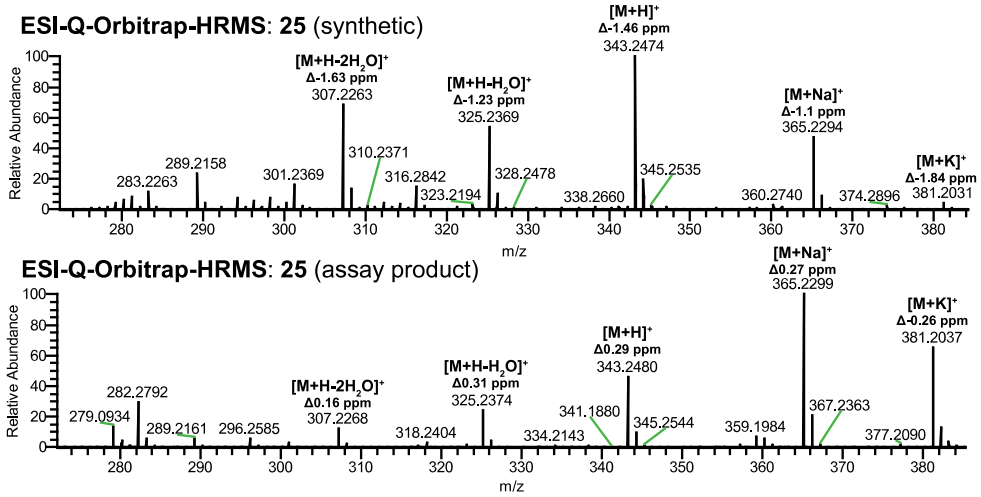
Supplementary Figure 49: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 19. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower presents the higher energy collisional dissociation (HCD) MS/MS spectra (normalised collision energy: 35) of the parental (*) sodium adduct $[M+Na]^+$ at *m/z* 339.2141 (reference and assay product), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



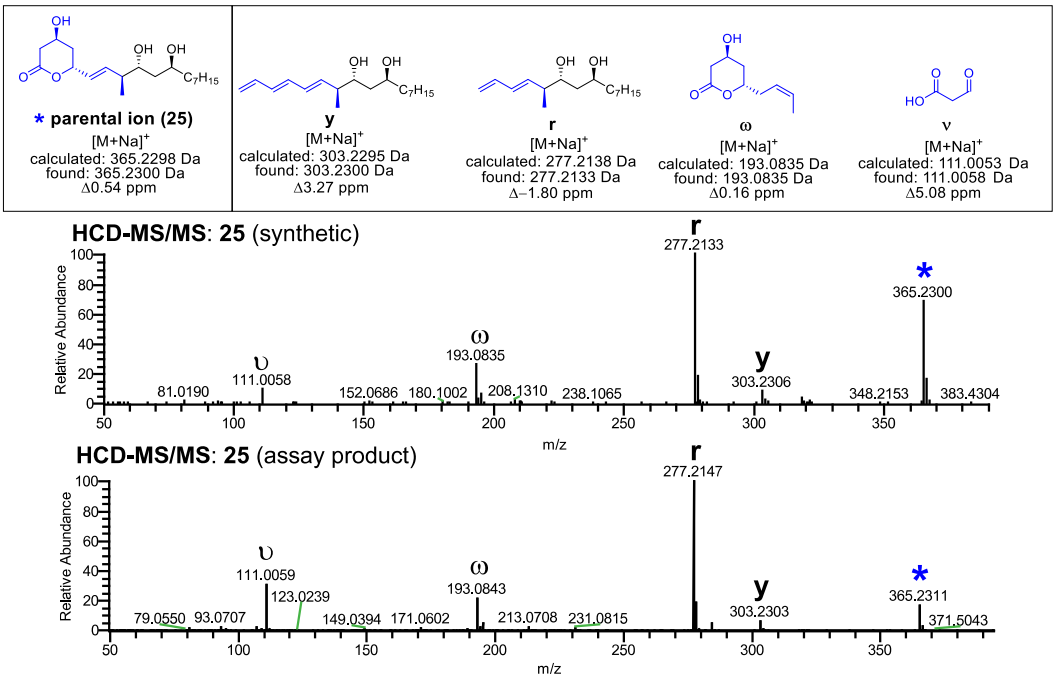
Supplementary Figure 50: LC-MS and HRMS analysis of carboxylic acid 21. The upper panel shows the total ion chromatogram with a main peak at 2.80 min corresponding to **21**. The middle panel displays the ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 51.



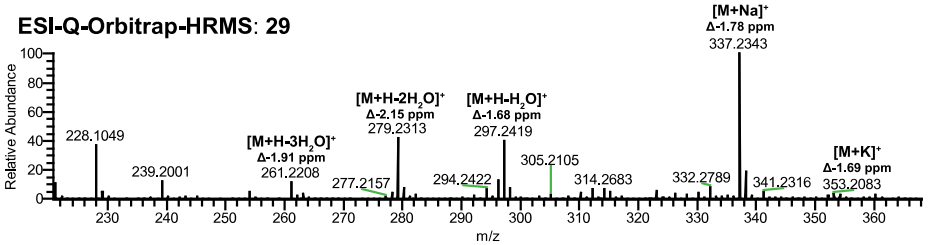
Supplementary Figure 51: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 21. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower presents the higher energy collisional dissociation (HCD) MS/MS spectra (normalised collision energy: 35) of the parental (*) sodium adduct $[M+Na]^+$ at *m/z* 383.2414 (reference) and 383.2413 (assay product), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



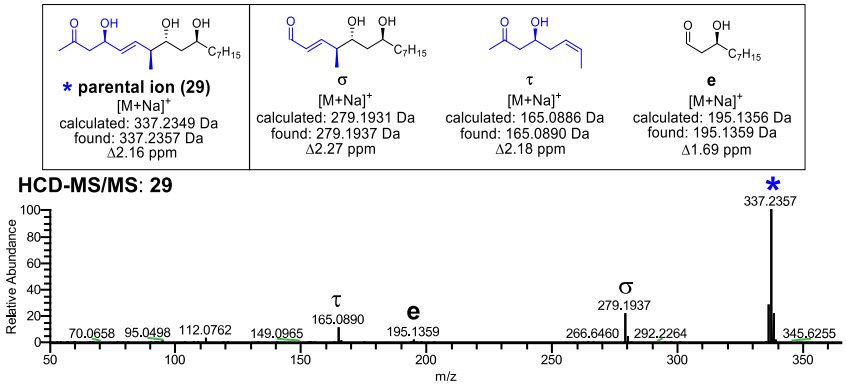
Supplementary Figure 52: HRMS analysis of lactone 25. High-resolution ESI-Q-Orbitrap-HRMS spectrum of lactone **25**, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 53.



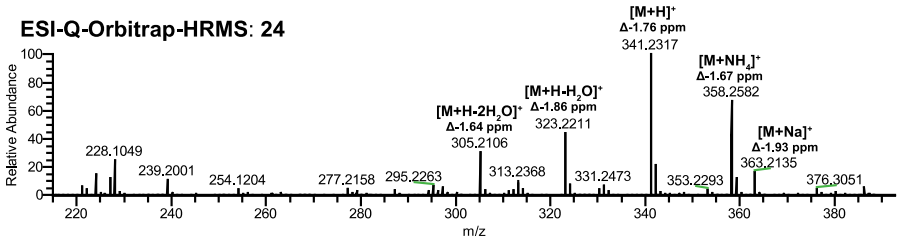
Supplementary Figure 53: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 25. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower presents the higher energy collisional dissociation (HCD) MS/MS spectra (normalised collision energy: 35) of the parental (*) sodium adduct $[M+Na]^+$ at *m/z* 365.2300 (reference) and 365.2311 (assay product), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



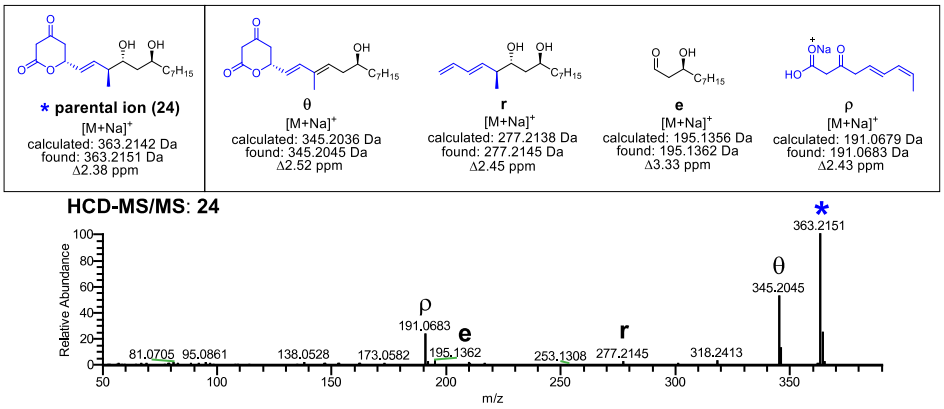
Supplementary Figure 54: HRMS analysis of carboxylic acid 29. High-resolution ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 55.



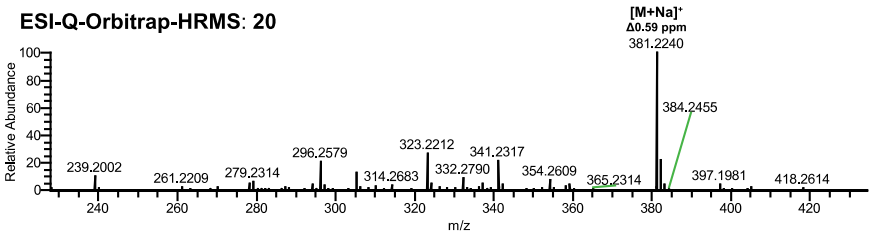
Supplementary Figure 55: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 29. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 337.2357 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



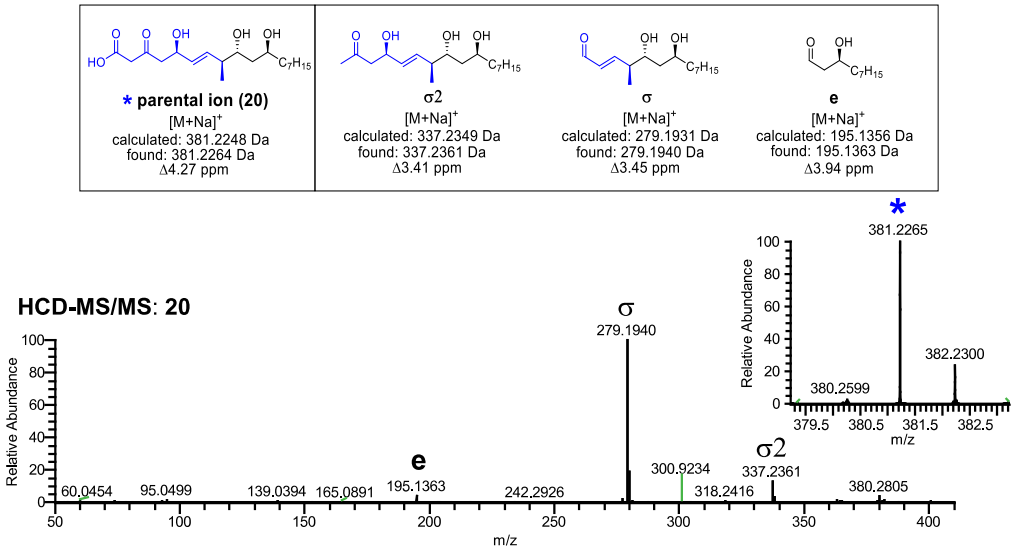
Supplementary Figure 56: HRMS analysis of lactone 24. High-resolution ESI-Q-Orbitrap-HRMS spectrum of lactone **24**, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 57.



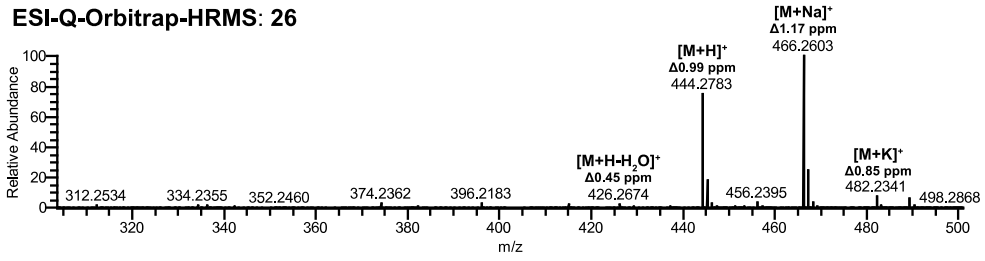
Supplementary Figure 57: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 24. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 363.2151 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



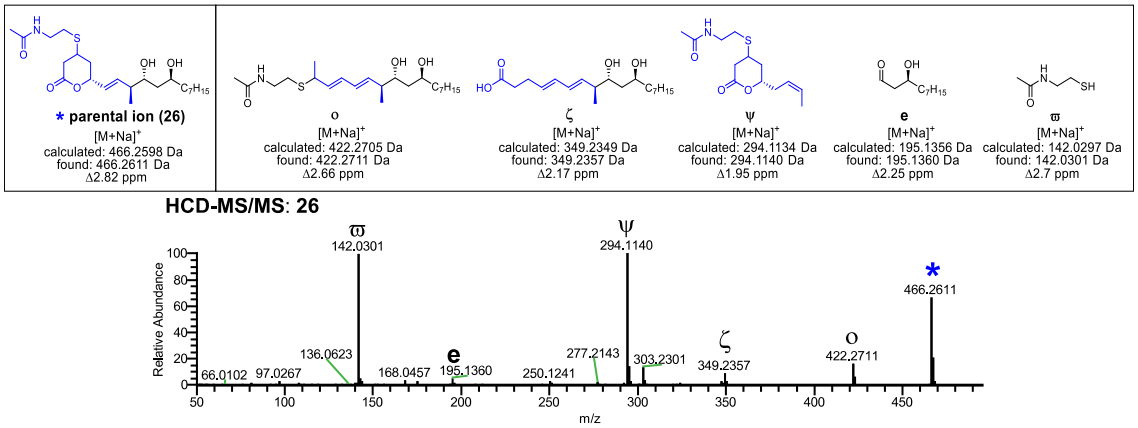
Supplementary Figure 58: HRMS analysis of carboxylic acid 20. High-resolution ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 59.



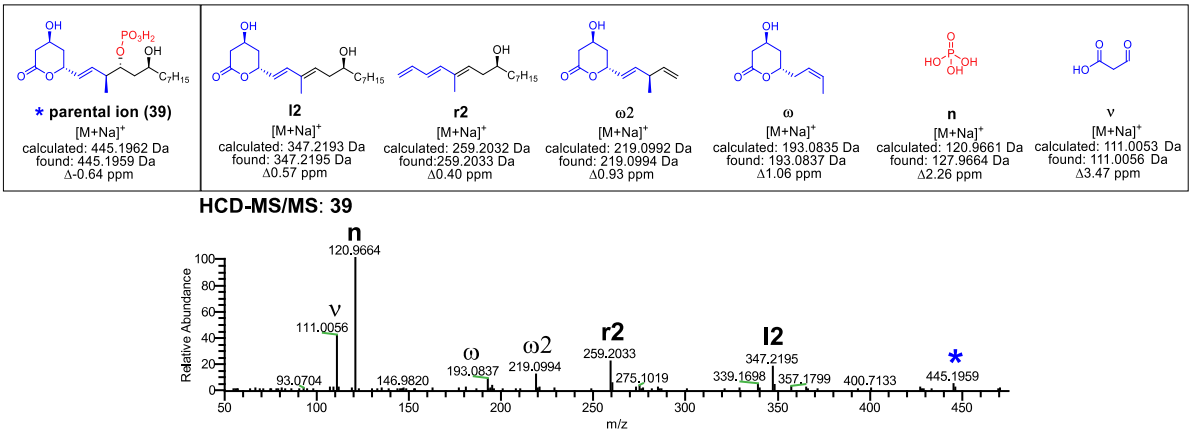
Supplementary Figure 59: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 20. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 381.2265 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



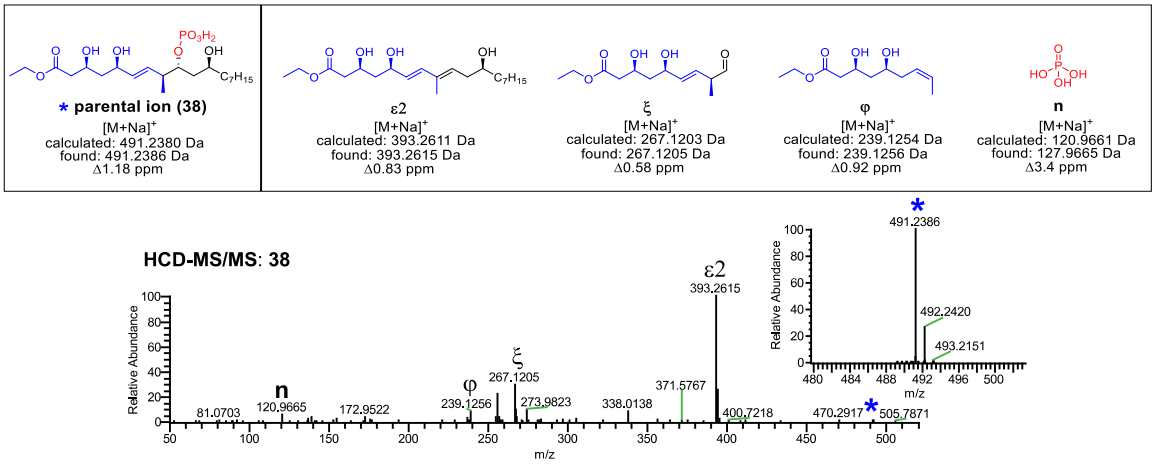
Supplementary Figure 60: HRMS analysis of carboxylic acid 26. High-resolution ESI-Q-Orbitrap-HRMS spectrum, showing the most abundant adduct and fragment ions. The corresponding HR-MS/MS spectrum is shown in Supplementary Fig. 61.



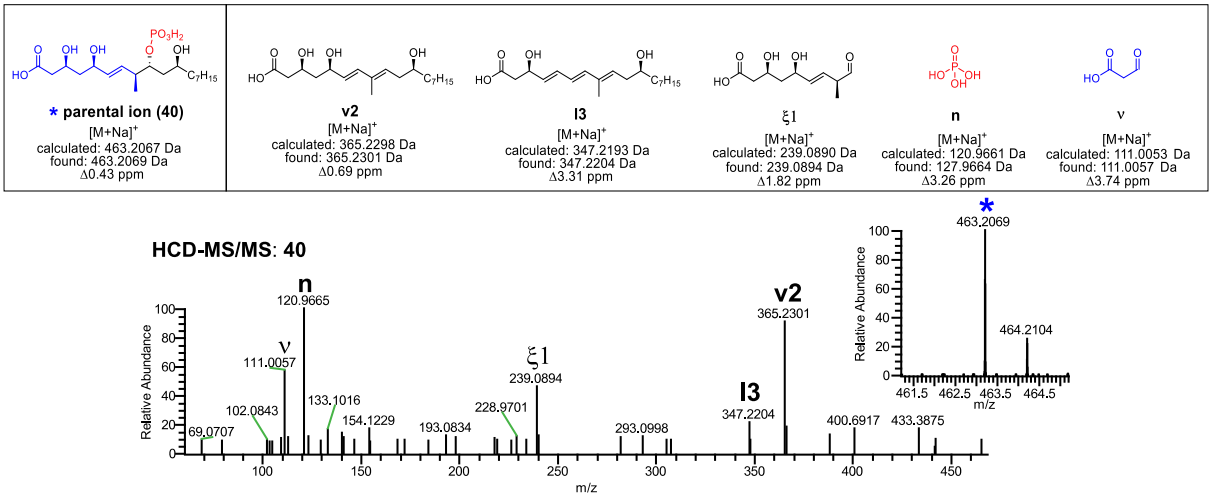
Supplementary Figure 61: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 26. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 466.2611 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



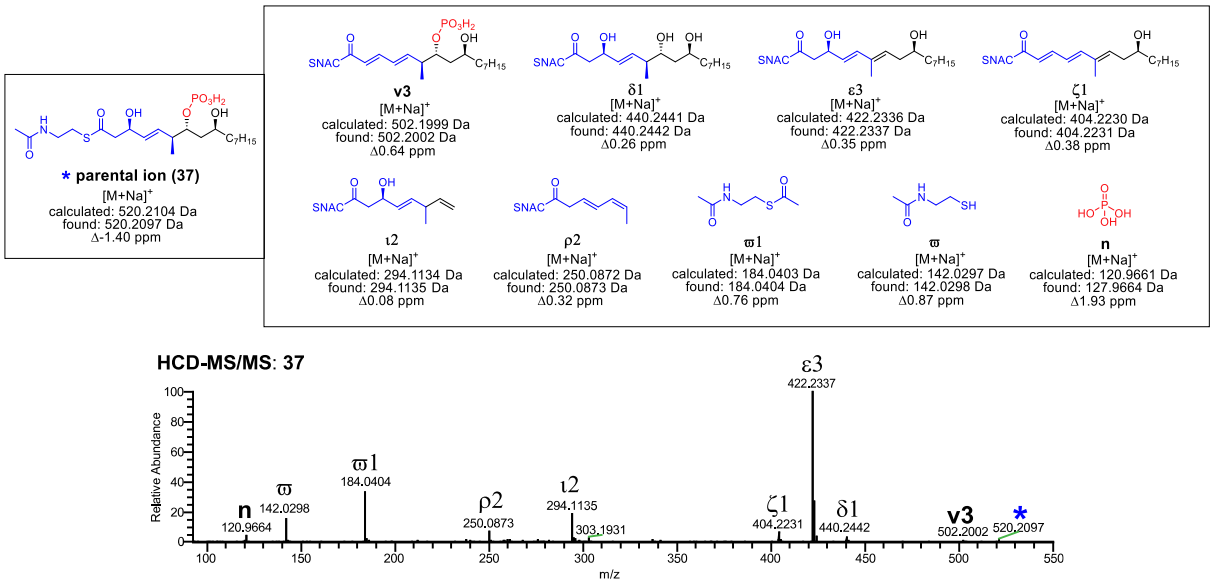
Supplementary Figure 62: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 39. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 445.1959 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



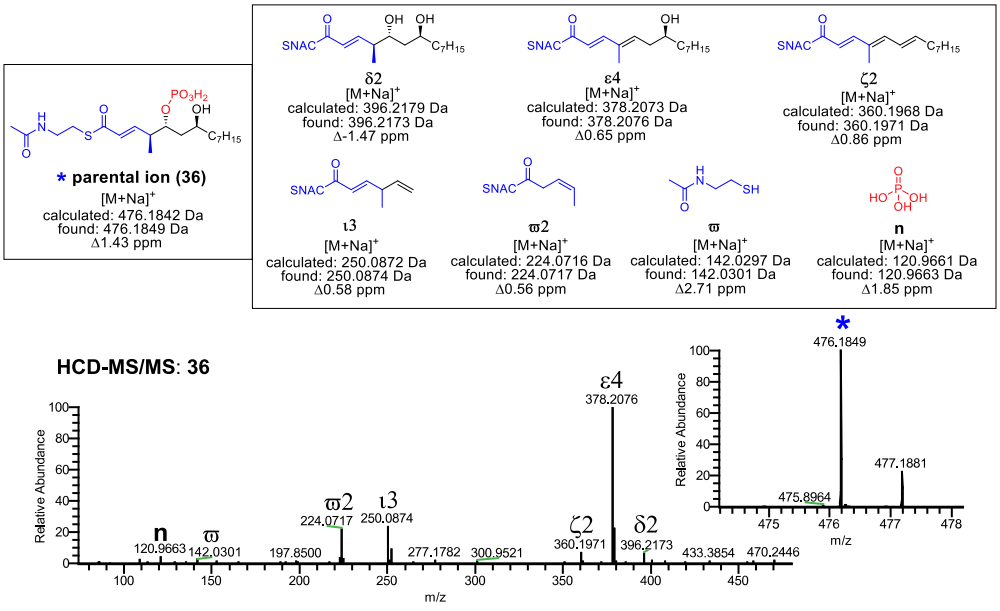
Supplementary Figure 63: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 38. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 491.2386 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



Supplementary Figure 64: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 40. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct $[M+Na]^+$ at *m/z* 463.2069 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



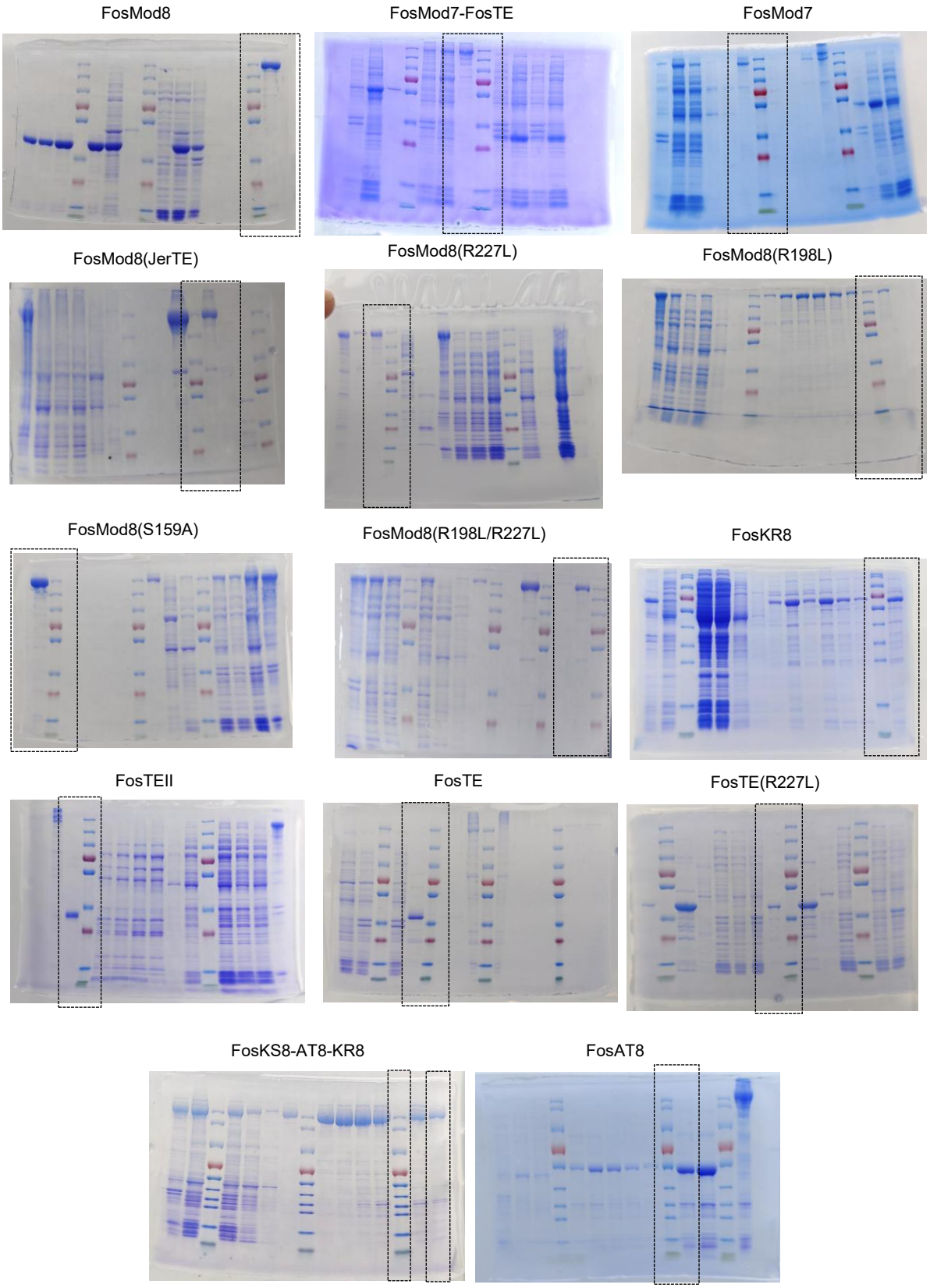
Supplementary Figure 65: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 37. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct [M+Na]⁺ at *m/z* 520.2097 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.



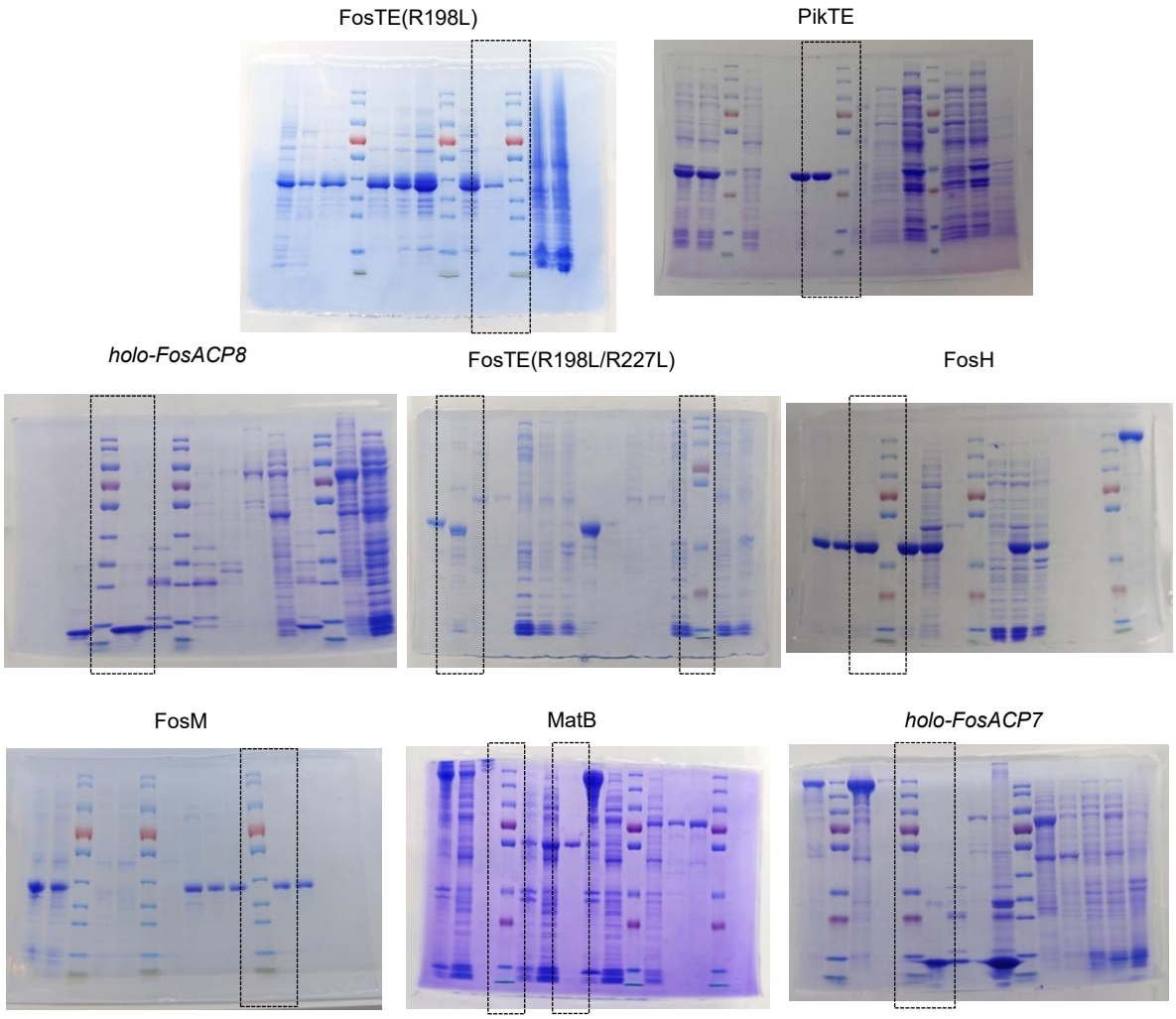
Supplementary Figure 66: High-resolution tandem mass spectrometry (HR-MS/MS) analysis of 36. The upper panel shows the proposed fragmentation scheme with labeled fragment structures. The lower panel presents the higher energy collisional dissociation (HCD) MS/MS spectrum (normalised collision energy: 35) of the parental sodium adduct [M+Na]⁺ at *m/z* 476.1849 (*), with major fragment ions corresponding to the structures above. Calculated and observed *m/z* values, as well as mass errors (Δ in ppm), are provided for each fragment.

Supplementary Table 4: Adduct and fragment ions used in LC-MS analysis for visualisation (EICs) and for semi-quantification. Adduct ions and fragment ions were selected for LC-MS analysis and visualisation based on their abundance and specificity for each compound. For visualisation, EICs were generated using the most abundant and specific adduct ions for each analyte to ensure clear chromatographic profiles and to minimise overlap from structurally similar fragments. For quantification, all observable and compound-specific fragments were included to maximise sensitivity and selectivity. Fragments shown in grey were used exclusively for quantification when detected in the respective sample and were not included in EIC visualisations.

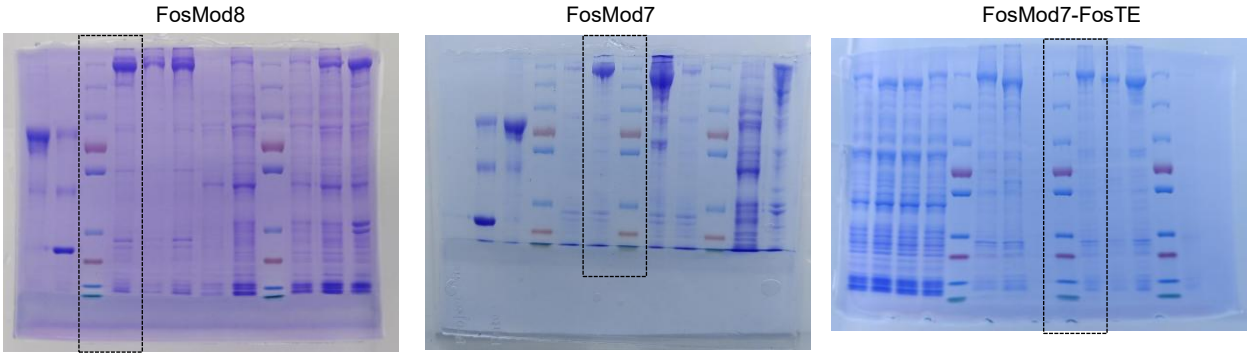
Compound	Adduct Ions (m/z)
16	374 $[M+H]^+$, 396 $[M+Na]^+$, 412 $[M+K]^+$, 356 $[M+H-H_2O]^+$, 338 $[M+H-2H_2O]^+$
17	418 $[M+H]^+$, 440 $[M+Na]^+$, 456 $[M+K]^+$, 400 $[M+H-H_2O]^+$, 382 $[M+H-2H_2O]^+$
18	273 $[M+H]^+$,295 $[M+Na]^+$, 255 $[M+H-H_2O]^+$
19	317 $[M+H]^+$, 339 $[M+Na]^+$, 355 $[M+K]^+$, 299 $[M+H-H_2O]^+$, 281 $[M+H-2H_2O]^+$
27	429 $[M+H]^+$, 451 $[M+Na]^+$, 467 $[M+K]^+$, 411 $[M+H-H_2O]^+$, 194 $[M+H+CH_3CN-C_{13}H_{24}O_6]^+$, 153 $[M+H-C_{13}H_{24}O_6]^+$
28	325 $[M+H]^+$, 347 $[M+Na]^+$, 363 $[M+K]^+$, 307 $[M+H-H_2O]^+$, 289 $[M+H-2H_2O]^+$, 153 $[M+H-C_{10}H_{20}O_2]^+$
22	403 $[M+H]^+$, 425 $[M+Na]^+$
23	447 $[M+H]^+$, 469 $[M+Na]^+$, 485 $[M+K]^+$, 429 $[M+H-H_2O]^+$
21	361 $[M+H]^+$, 383 $[M+Na]^+$, 399 $[M+K]^+$, 325 $[M+H-2H_2O]^+$, 307 $[M+H-3H_2O]^+$
20	359 $[M+H]^+$, 381 $[M+Na]^+$, 397 $[M+K]^+$
24	341 $[M+H]^+$, 363 $[M+Na]^+$, 379 $[M+K]^+$
25	343 $[M+H]^+$, 365 $[M+Na]^+$, 381 $[M+K]^+$, 325 $[M+H-H_2O]^+$, 307 $[M+H-2H_2O]^+$, 289 $[M+H-3H_2O]^+$, 171 $[M+H-C_{10}H_{20}O_2]^+$
26	444 $[M+H]^+$, 466 $[M+Na]^+$, 482 $[M+K]^+$
31	389 $[M+H]^+$, 411 $[M+Na]^+$, 427 $[M+K]^+$
32	475 $[M+H]^+$, 497 $[M+Na]^+$, 513 $[M+K]^+$, 457 $[M+H-H_2O]^+$
29	315 $[M+H]^+$, 337 $[M+Na]^+$
33	509 $[M+H]^+$, 531 $[M+Na]^+$, 547 $[M+K]^+$, 411 $[M+H-H_3PO_4]^+$, 283 $[M+H-C_8H_{19}O_5P]^+$
34	405 $[M+H]^+$, 427 $[M+Na]^+$, 443 $[M+K]^+$
36	454 $[M+H]^+$, 476 $[M+Na]^+$, 492 $[M+K]^+$
37	498 $[M+H]^+$, 520 $[M+Na]^+$, 536 $[M+K]^+$
38	469 $[M+H]^+$, 491 $[M+Na]^+$, 507 $[M+K]^+$
39	423 $[M+H]^+$, 445 $[M+Na]^+$, 461 $[M+K]^+$
40	441 $[M+H]^+$, 463 $[M+Na]^+$, 479 $[M+K]^+$



Supplementary Figure 67: Uncropped SDS-PAGE gel images used for Supplementary Fig. 1. The cropped sections of the gel images are highlighted by the dashed box.



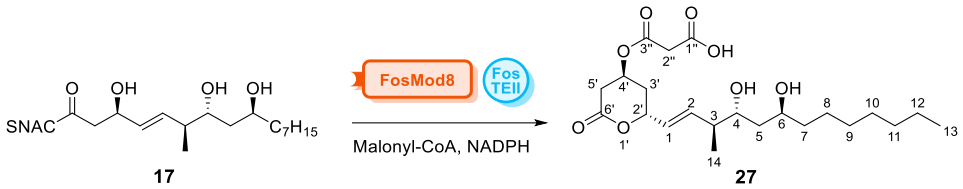
Supplementary Figure 68: Uncropped SDS-PAGE gel images used for Supplementary Fig. 1. The cropped sections of the gel images are highlighted by the dashed box.



Supplementary Figure 69: Uncropped SDS-PAGE gel images used for Supplementary Figs. 2, 5 and 8. The cropped sections of the gel images are highlighted by the dashed box.

Semi-preparative scale production of 27

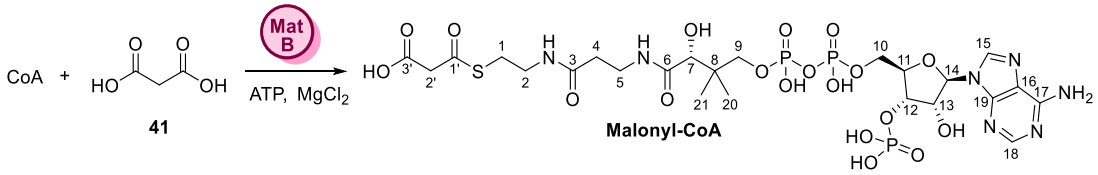
3''-(((2'*R*,4'*S*)-2''-((3*S*,4*R*,6*S*,*E*)-4,6-Dihydroxy-3-methyltridec-1-en-1-yl)-6'-oxotetrahydro-2*H*-pyran-4'-yl)oxy)-3''-oxopropanoic acid (**27**)



Reactions with SNAC thioester **17** were set up on a scale of 5 mg (1 mM, 12 µmol, 1 eq.) starting material in 200 mM sodium phosphate, pH 6.8, 2%(v/v) DMSO, 5 mM NADPH (59.9 µmol, 5 eq.), 5 mM Malonyl-CoA (59.9 µmol, 5 eq.), 9 µM FosMod8 and 0.25 µM FosTEII. After incubation at 25 °C and 300 rpm for 3 h, the enzymes were removed using a 10 kDa MWCO centrifugal filter (5000 × g). The membrane was then rinsed twice with 5 mL of water by repeated centrifugation at 5000 × g. Purification of the combined filtrates via semi-preparative HPLC (Hydrosphere C18, linear gradient: 0 → 25% B in A over 20 min, 25 → 60% B in A over 60 min, 5 mL/min; A: H_2O + 0.1% formic acid, B: CH_3CN + 0.1% formic acid) and subsequent lyophilisation yielded product **27** (1.3 mg, 3.1 µmol, 26%) as colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.85 (dd, *J* = 15.4, 8.4 Hz, 1H, 2-H), 5.56 (dd, *J* = 15.4, 7.3 Hz, 1H, 1-H), 5.39-5.36 (m, 1H, 4'-H), 5.08 (ddd, *J* = 10.8, 7.6, 2.4 Hz, 1 H, 2'-H), 3.95-3.91 (m, 1H, 6-H), 3.83-3.80 (m, 1H, 4-H), 3.43 (dd, *J* = 15.97, 36.63 Hz, 2H, 2''-H), 2.88-2.76 (m, 2H, 5'-H), 2.31-2.21 (m, 3H, 3-H, 3'-H_a), 1.89 (ddd, *J* = 14.5, 11.4, 2.8 Hz, 1H, 3'-H_b), 1.65-1.51 (m, 3H, 5-H, 7-H), 1.49-1.42 (m, 1H, 7-H), 1.37-1.33 (m, 1H, 8-H), 1.31-1.25 (m, 9H, 8-H, 9-H, 10-H, 11-H, 12-H), 1.05 (d, *J* = 6.9 Hz, 1H, 14-H), 0.88 (t, *J* = 6.9 Hz, 2H, 13-H) ppm. **^{13}C NMR** (150 MHz, $CDCl_3$): δ = 168.8 (C-6'), 168.1 (C-1''), 166.1 (C-3''), 137.6 (C-2), 128.0 (C-1), 72.6 (C-4), 69.8 (C-6), 67.0 (C-4'), 42.6 (C-3), 41.4 (C-2''), 39.7 (C-5), 37.3 (C-7), 34.9 (C-5'), 33.3 (C-3'), 31.9 (C-11), 29.7 (C-9), 29.3 (C-10), 25.9 (C-8), 22.8 (C-12), 16.7 (C-14), 14.2 (C-13) ppm. **HRMS** [ESI$^+$]: *m/z* for $C_{22}H_{36}O_8$ $[M+Na]^+$: calc. = 451.2302, found = 451.2302; $[M+H]^+$: calc. = 429.2484, found = 429.2483.

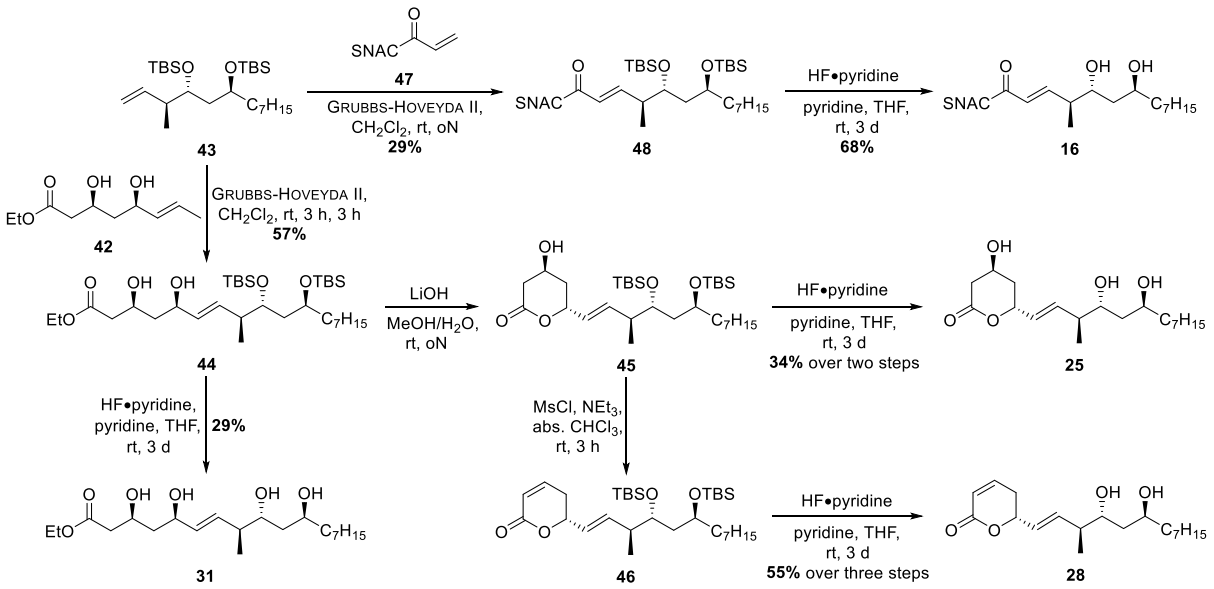
Malonyl-Coenzyme A



Coenzyme A trilithium salt (52.5 mg, 66.9 µmol, 1 eq., 5 mM) and Malonic acid **41** (26.5 mg, 255 µmol, 4 eq., 20 mM) were set up in a reaction with ATP disodium salt (129.3 mg, 255 µmol, 4 eq. 20 mM), $MgCl_2 \cdot 6\ H_2O$ (38.9 mg, 191.3 µmol, 3 eq., 15 mM) and 10 µM MatB in 100 mM sodium phosphate buffer, pH 6.8 at 25 °C and 300 rpm for 13 h. The enzyme was removed using a 10 kDa MWCO centrifugal filter (5000 × g) and the membrane was subsequently rinsed twice with 5 mL of water by repeated centrifugation at 5000 × g. The combined filtrate was purified via semi-preparative HPLC (Hydrosphere C18, linear gradient 0 → 30% B in A over 30 min; A: 10 mM NH_4HCO_2, pH 3, B: CH_3CN + 0.1% formic acid). After removal of the solvent via lyophilisation, malonyl-CoA (50 mg, 58.6 µmol, 89.5%) was obtained as white powder. The yield was calculated by considering the amount of Coenzyme A used as well as the amount of malonyl-CoA obtained as free acid.

^{1}H NMR (500 MHz, D_2O): δ = 8.50 (s, 1H, 15-H), 8.22 (s, 1 H, 18-H), 6.14-6.13 (d, *J* = 6.31 Hz, 1 H, 14-H), 4.83-4.79 (m, 2 H, 12-H, 13-H), 4.57-4.53 (m, 1 H, 11-H), 4.26-4.16 (m, 2 H, 10-H), 3.97 (s, 1 H, 7-H), 3.52 (dd, *J* = 4.71, 9.71 Hz, 1 H, 9-H), 3.81 (dd, *J* = 9.67, 4.74 Hz, 1 H, 9-H), 3.45-3.37 (m, 2 H, 5-H), 3.30 (t, *J* = 3.30 Hz, 2 H, 2-H), 2.98 (t, *J* = 6.45 Hz, 2 H, 1-H), 2.39 (t, *J* = 6.64 Hz, 2 H, 4-H), 0.84 (s, 3H, 21-H), 0.71 (s, 3H, 20-H) ppm. **^{13}C NMR** (125 MHz, D_2O): δ = 197.3 (C-1‘), 174.6 (C-6), 173.9 (C-3), 173.4 (C-3‘), 155.4 (C-17), 152.7 (C-18), 149.2 (C-19), 139.7 (C-15), 118.9 (C-16), 86.3 (C-14), 83.5 (C-11), 73.9 (C-7), 73.8 (C-12), 73.7 (C-13), 71.7 (C-9), 65.3 (C-10), 38.4 (C-2), 38.2 (C-8), 38.1 (C-2‘), 35.3 (C-5), 35.1 (C-4), 28.2 (C-1), 20.7 (C-21), 17.9 (C-20) ppm. **^{31}P-NMR** (202 MHz, D_2O): δ = 1.54 (s, 12-P), −10.85 (d, 10-P), −11.60 (d, 9-P) ppm. **HRMS** [ESI$^+$]: *m/z* for $C_{24}H_{38}N_7O_{19}P_3S$ $[M+H]^+$: calc. = 854.1229, found = 854.1223.

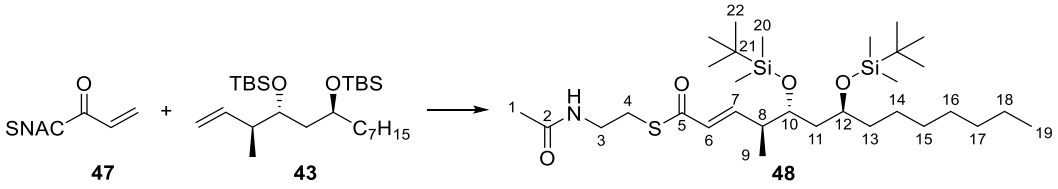
Synthesis of substrate surrogates and reference compounds



General Procedure: Silyl ether deprotection

The silyl ether (1.00 eq.) was dissolved in THF (150 mM) before a HF-containing stock-solution was added (70% HF•pyridine/pyridine/THF (1:2:8)) and the reaction was stirred at rt for 3 d. Saturated aqueous $NaHCO_3$ solution was added until no more formation of CO_2 was observed and after separation of the phases, the aqueous one was extracted three times with EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. After concentration *in vacuo* the crude product was purified *via* preparative HPLC.

S-(2-Acetamidoethyl)-(4*S*,5*R*,7*S*,*E*)-5,7-bis((*tert*-butyldimethylsilyl)oxy)-4-methyltetradec-2-enethioate (**48**)

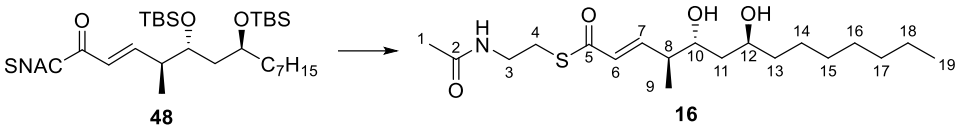


43 (100 mg, 219 µmol, 1.0 eq.) and **47** (114 mg, 657 µmol, 3.0 eq) were dissolved in CH_2Cl_2 (4.38 mL, 0.05 M), Grubbs-Hoveyda II catalyst (7.00 mg, 11.0 µmol, 5.0 mol%) added and heated to reflux overnight. The solvent was removed *in vacuo* and after purification *via* flash chromatography (SiO_2, cyclohexane/EtOAc : 3/1), **48** (38.0 mg, 63.0 µmol, 29%) was obtained as a slightly yellowish oil.

R_f = 0.15 (cyclohexane/EtOAc : 3/1). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 6.94 (dd, J = 15.7, 7.7 Hz, 1 H, 7-H), 6.13 (dd, J = 15.7, 1.0 Hz, 1 H, 6-H), 5.89 (br. s, 1 H, NH), 3.79 (td, J_t = 6.0 Hz,

J_d = 3.1 Hz, 1 H, 10-H), 3.71-3.67 (m, 1 H, 12-H), 3.46 (dt, J_d = 5.9 Hz, J_t = 6.0 Hz, 2 H, 3-H), 3.09 (t, J = 6.4 Hz, 2 H, 4-H), 2.49-2.43 (m, 1 H, 8-H), 1.96 (s, 3 H, 1-H), 1.54-1.38 (m, 4 H, 11-H, 13-H), 1.30-1.24 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 1.07 (d, J = 6.8 Hz, 3 H, 9-H), 0.90-0.88 (m, 12 H, 19-H, 22-H), 0.86 (s, 9 H, 22-H), 0.07 (s, 3 H, 20-H), 0.06 (s, 3 H, 20-H), 0.04 (s, 6 H, 2×20-H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 190.6 (C-5), 170.4 (C-2), 148.4 (C-7), 128.7 (C-6), 73.2 (C-10), 70.4 (C-12), 42.8 (C-8), 42.6 (C-11), 40.1 (C-3), 38.0 (C-13), 32.0 (C-17), 29.9 (C-15), 29.5 (C-16), 28.4 (C-4), 26.1 (C-22), 26.0 (C-22), 25.1 (C-14), 23.4 (C-1), 22.8 (C-18), 18.2 (2×C-21), 15.2 (C-9), 14.3 (C-19), -3.7 (C-20), -3.96 (C-20), -3.98 (C-20), -4.2 (C-20) ppm. $[\alpha]_{20}^{D}$: -0.01 (c = 0.60 in CH_2Cl_2). **HRMS** [ESI$^+$]: *m/z* for $C_{31}H_{63}NO_4SSi_2$ $[M+H]^+$: calc. = 602.4095, found = 602.4084.

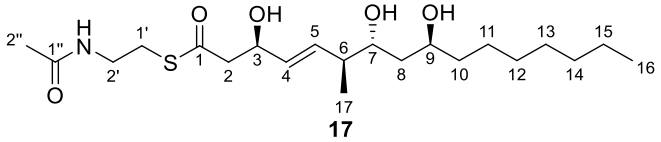
<u>*S*-(2-Acetamidoethyl)-(4*S*,5*R*,7*S*,*E*)-5,7-dihydroxy-4-methyltetradec-2-enethioate (**16**)</u>



The TBS-groups of **48** (38.0 mg, 63.0 µmol, 1.0 eq.) were removed following the general procedure for silyl ether deprotection. Purification *via* flash chromatography (SiO_2, cyclohexane/EtOAc : 1/1 → EtOAc) provided **16** (16.0 mg, 43.0 µmol, 68%) as a slightly yellowish oil.

R_f = 0.05 (cyclohexane/EtOAc : 1/1). **t_R** = 3.50 min (UHPLC-MS). **^{1}H NMR** (500 MHz, $CDCl_3$): δ = 6.96 (dd, J = 15.6, 8.2 Hz, 1 H, 7-H), 6.18 (d, J = 15.6 Hz, 1 H, 6-H), 5.95 (br. s, 1 H, NH), 3.96-3.88 (m, 2 H, 10-H, 12-H), 3.50-3.43 (dt, J_d = 6.3 Hz, J_t = 6.1 Hz, 2 H, 3-H), 3.09 (t, J = 6.3 Hz, 2 H, 4-H), 2.45-2.39 (m, 1 H, 8-H), 1.97 (s, 3 H, 1-H), 1.68-1.53 (m, 2 H, 11-H), 1.50-1.39 (m, 2 H, 12-H), 1.31-1.25 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 1.10 (d, J = 6.9 Hz, 3 H, 9-H), 0.88 (t, J = 6.8 Hz, 3 H, 19-H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 190.5 (C-5), 170.6 (C-2), 148.1 (C-7), 129.0 (C-6), 71.9 (C-10), 69.6 (C-12), 43.1 (C-8), 40.0 (C-11), 39.9 (C-3), 37.5 (C-13), 31.9 (C-17), 29.7 (C-15), 29.4 (C-16), 28.5 (C-4), 26.0 (C-14), 23.4 (C-1), 22.8 (C-18), 15.9 (C-9), 14.3 (C-19) ppm. $[\alpha]_{20}^{D}$: -0.02 (c = 1.00 in CH_2Cl_2). **HRMS** [ESI$^+$]: *m/z* for $C_{19}H_{35}NO_4S$ $[M+H]^+$: calc. = 374.2365, found = 374.2360.

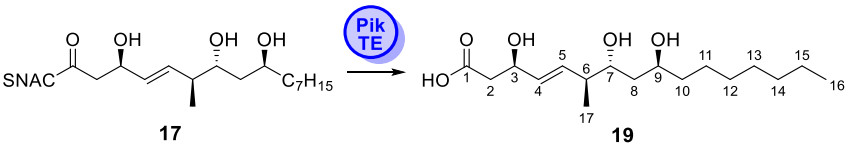
<u>*S*-(2'-Acetamidoethyl) (3*R*,6*S*,7*R*,9*S*,*E*)-3,7,9-trihydroxy-6-methylhexadec-4-enethioate (**17**)</u>



The synthesis of *S*-(2'-Acetamidoethyl) (3*S*,5*R*,8*S*,9*R*,11*S*,*E*)-3,5,9,11-tetrahydroxy-8-methyloctadec-6-enethioate (**17**) was described in a previous publication[7] and a sample of this material was used in this study. The compound was re-purified before the enzymatic assays *via* semi-preparative HPLC (Hydrosphere C18, linear gradient: 25 → 60% B in A over 60 min, 5 mL/min; A: H_2O + 0.1% formic acid, B: CH_3CN + 0.1% formic acid).

^{1}H NMR (500 MHz, CD_3CN): δ = 6.59 (br, 1H, NH), 5.63-5.59 (dd, *J* = 15.84, 8.42 Hz, 1H, 5-H), 5.49-5.44 (dd, *J* = 15.64, 6.38 Hz, 1H, 4-H), 4.46-4.42 (m, 1H, 3-H), 3.73-3.69 (m, 1H, 9-H), 3.65-3.61 (m, 1H, 7-H), 3.29-3.25 (m, 2H, 2'-H), 2.98-2.92 (m, 2H, 2'-H), 2.76-2.67 (m, 2H, 2-H), 2.15 (m, 1H, 6-H), 1.82 (s, 3H, 2"-H), 1.45-1.34 (m, 5H, 8-H, 10-H, 11-H), 1.32-1.22 (m, 9H, 11-H, 12-H, 13-H, 14-H, 15-H), 0.98-0.96 (d, *J* = 6.87 Hz, 3H, 17-H), 0.89 (t, *J* = 6.92 Hz, 3H, 16-H) ppm. **^{13}C NMR** (125 MHz, CD_3CN): δ = 198.0 (C-1), 170.9 (C-1"), 134.5 (C-5), 133.1 (C-4), 72.2 (C-7), 70.2 (C-3), 68.9 (C-9), 52.5 (C-2), 43.7 (C-6), 41.8 (C-8), 39.4 (C-2'), 38.6 (C-10), 32.6 (C-14), 30.3 (C-12), 30.0 (C-13), 29.4 (C-1'), 26.5 (C-11), 23.3 (C-11, C-12, C-13, C-14, C-15), 23.0 (C-2"), 16.8 (C-17), 14.3 (C-16) ppm.

(3*R*,6*S*,7*R*,9*S*,*E*)-3,7,9-Trihydroxy-6-methylhexadec-4-enoic acid (**19**)



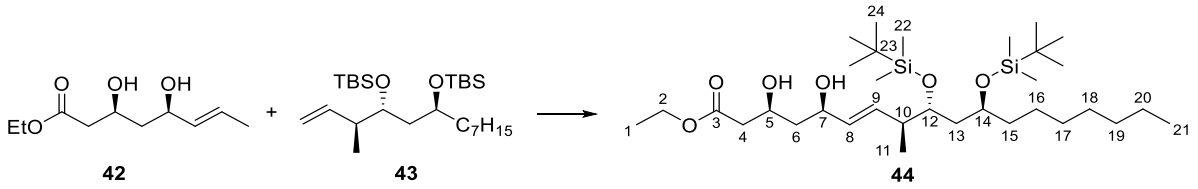
17 **19**

Thioester **17** (5.8 mg, 14.1 µmol, 1 mM) was incubated with 75 µM PikTE in 100 mM sodium phosphate buffer (pH 7.5) containing 2%(v/v) DMSO at 25 °C and 450 rpm for 24 h. Proteinase K (Carl Roth) was then added to a final concentration of 150 µg/mL, together with 2 mM $CaCl_2$ and the mixture was incubated at 37 °C for 45 min. The reaction was quenched with 1 M HCl until pH 2 was reached. After extraction with EtOAc (3 × 10 mL), the organic phases were combined and washed with brine. The organic solvent was removed *in vacuo* and the crude product was purified *via* preparative HPLC (Kinetex C18, 5% → 95% solvent B in solvent A for 20 min, 20 mL/min; A: H_2O + 0.1%FA, B: CH_3CN + 0.1%FA). Carboxylic acid **19** was obtained as colorless oil (3 mg, 9.5 µmol, 68%).

^{1}H NMR (500 MHz, MeOH-d_4): δ = 5.69 (dd, *J* = 15.5, 7.9 Hz, 1H, 5-H), 5.53 (dd, *J* = 15.5, 6.5 Hz, 1H, 4-H), 4.46 (dt, *J* = 6.7, 6.5 Hz, 1H, 3-H), 3.79-3.71 (m, 1H, 7-H), 3.79-3.71 (m, 1H, 9-H), 2.50-2.45 (m, 2H, 2-H), 2.23-2.16 (m, 1H, 6-H), 1.48-1.39 (m, 5H, 8-H, 10-H, 11-H), 1.38-1.25 (m, 9H, 11-H, 12-H, 13-H, 14-H, 15-H), 1.04 (d, *J* = 6.89 Hz, 3H, 17-H), 0.90 (t, *J* = 6.96 Hz, 3H, 16-H) ppm. **^{13}C NMR** (125 MHz, MeOH-d_4): δ = 170.9 (C-1), 134.5 (C-5), 133.5 (C-4), 72.5 (C-7), 70.3 (C-9), 69.2 (C-3), 44.2 (C-6), 43.4 (C-2), 42.5 (C-8), 39.2 (C-10),

33.0 (C-14), 30.8 (C-12), 30.4 (C-13), 26.8 (C-11), 23.7 (C-15), 16.5 (C-17), 14.4 (C-16) ppm. **HRMS** [ESI$^+$]: *m/z* for $C_{17}H_{32}O_5$ [M+Na]$^+$: calc. = 339.2142, found = 339.2141.

Ethyl-(3*S*,5*R*,8*S*,9*R*,11*S*,*E*)-9,11-bis((*tert*-butyldimethylsilyl)oxy)-3,5-dihydroxy-8-methyl-octadec-6-enoate (**44**)

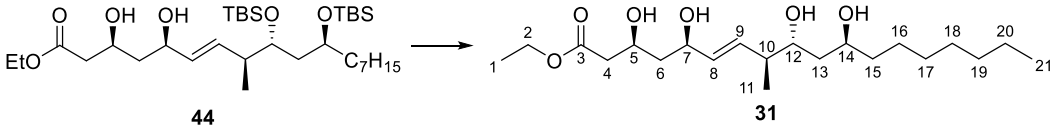


42 **43** **44**

42 (44.3 mg, 219 µmol, 1.0 eq., prepared according to literature[8] and **43** (100 mg, 219 µmol, 1.0 eq., synthesised according to literature[9]) were dissolved in CH_2Cl_2 (4.38 mL, 50 mM), GRUBBS-HOVEYDA-II-cat. (6.8 mg, 11.0 µmol, 5 mol%) added and the reaction was heated to reflux for three hours. Another portion of the catalyst (5 mol%) was added and heated to reflux for three more hours. The solvent was removed *in vacuo* and after purification *via* flash chromatography (SiO_2, cyclohexane/EtOAc : 9/1→4/1), **44** (76.9 mg, 125 µmol, 57%) was obtained as a brown oil.

R_f = 0.23 (cyclohexane/EtOAc : 4/1). **^{1}H NMR** (500 MHz, acetone-d_6): δ = 5.67 (ddd, *J* = 15.6, 7.5, 0.8 Hz, 1 H, 9-H), 5.55 (dd, *J* = 15.6, 6.1 Hz, 1 H, 8-H), 4.35-4.30 (m, 1 H, 7-H), 4.23-4.20 (m, 1 H, 5-H), 4.09 (q, *J* = 7.1 Hz, 2 H, 2-H), 3.84-3.80 (m, 2 H, 12-H, 14-H), 2.49-2.39 (m, 2 H, 4-H), 2.38-2.33 (m, 1 H, 10-H), 1.67-1.61 (m, 2 H, 6-H), 1.60-1.46 (m, 4 H, 13-H, 15-H), 1.29-1.22 (m, 10 H, 16-H, 17-H, 18-H, 19-H, 20-H), 1.22 (t, *J* = 7.1 Hz, 3 H, 1-H), 1.00 (d, *J* = 6.9 Hz, 3 H, 11-H), 0.92 (s, 9 H, 24-H), 0.90-0.87 (m, 12 H, 21-H, 24-H), 0.12 (s, 3 H, 22-H), 0.11 (s, 3 H, 22-H), 0.10 (s, 3 H, 22-H), 0.09 (s, 3 H, 22-H) ppm. **^{13}C NMR** (125 MHz, acetone-d_6): δ = 172.0 (C-3), 135.0 (C-8), 132.3 (C-9), 74.5 (C-12), 72.4 (C-7), 71.0 (C-14), 68.5 (C-5), 60.6 (C-2), 44.8 (C-6), 43.2 (C-4), 42.9 (C-10), 42.2 (C-13), 38.6 (C-15), 32.6 (C-19), 30.4 (C-17), 30.1 (C-18), 26.4 (C-24), 26.3 (C-24), 25.7 (C-16), 23.3 (C-20), 18.71 (C-23), 18.66 (C-23), 16.0 (C-11), 14.6 (C-1), 14.4 (C-21), -3.6 (C-22), -3.7 (C-22), -3.9 (C-22), -4.0 (C-22) ppm. $[\boldsymbol{\alpha}]_{\boldsymbol{20}}^{\boldsymbol{D}}$: +9.21 (c = 1.00 in CH_2Cl_2).

Ethyl-(3*S*,5*R*,8*S*,9*R*,11*S*,*E*)-3,5,9,11-tetrahydroxy-8-methyloctadec-6-enoate (**31**)

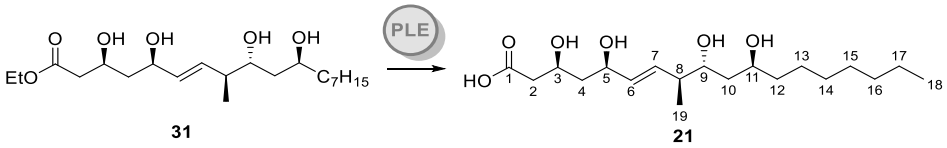


44 **31**

The TBS-groups of **44** (53.0 mg, 85.9 µmol, 1.0 eq.) were removed following the general procedure for silyl ether deprotection. Purification *via* preparative HPLC provided **31** (9.6 mg, 24.7 µmol, 29%) as a colorless oil.

$\mathbf{t_R}$ = 46.8 min (HPLC), 3.45 min (UHPLC-MS). **^{1}H NMR** (500 MHz, Acetone-d_6): δ = 5.67 (ddd, *J* = 15.5, 8.0, 0.8 Hz, 1 H, 9-H), 5.49 (ddd, *J* = 15.5, 6.7, 0.6 Hz, 1 H, 8-H), 4.33-4.28 (m, 1 H, 7-H), 4.23-4.17 (m, 1 H, 5-H), 4.09 (q, *J* = 7.1 Hz, 2 H, 2-H), 3.84-3.80 (m, 1 H, 14-H), 3.79-3.75 (m, 1 H, 12-H), 2.48-2.39 (m, 2 H, 4-H), 2.22-2.13 (m, 1 H, 10-H), 1.70-1.60 (m, 2 H, 6-H), 1.52-1.39 (m, 4 H, 13-H, 15-H), 1.34-1.25 (m, 10 H, 16-H, 17-H, 18-H, 19-H, 20-H), 1.21 (t, *J* = 7.1 Hz, 3 H, 1-H), 1.01 (d, *J* = 6.9 Hz, 3 H, 11-H), 0.88 (t, *J* = 7.0 Hz, 3 H, 21-H) ppm. **^{13}C NMR** (125 MHz, Acetone-d_6): δ = 172.1 (C-3), 134.7 (C-8), 133.4 (C-9), 72.2 (C-7), 72.1 (C-12), 68.9 (C-14), 68.0 (C-5), 60.6 (C-2), 44.8 (C-6), 43.7 (C-10), 43.3 (C-4), 42.1 (C-13), 38.8 (C-15), 32.6 (C-19), 30.5 (C-17), 30.1 (C-18), 26.7 (C-16), 23.3 (C-20), 16.8 (C-11), 14.5 (C-1), 14.4 (C-21) ppm. $[\alpha]_{20}^{D}$: -0.67 (c = 0.30 in CH_2Cl_2). **HRMS** [ESI$^+$]: *m/z* for $C_{21}H_{40}O_6$ [M+Na]$^+$: calc. = 411.2717, found = 411.2707.

(3*S*,5*R*,8*S*,9*R*,11*S*,*E*)-3,5,9,11-Tetrahydroxy-8-methyloctadec-6-enoic acid (**21**)



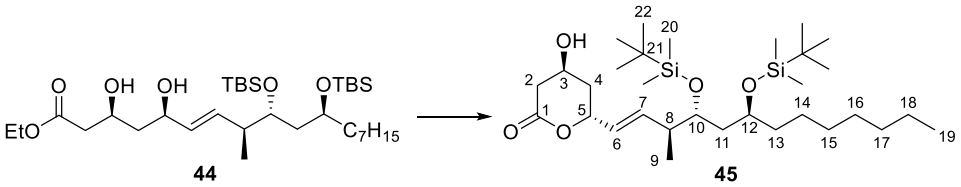
31 (2 mg, 5.1 µmol, 1.5 mM) was incubated with 1 mg/mL pig liver esterase (PLE, MERCK) in 200 mM potassium phosphate buffer, pH 8.0, supplemented with 3%(v/v) DMSO at 25 °C and 150 rpm for 16 h. Proteinase K (Carl Roth) was then added to a final concentration of 150 µg/mL alongside with 2 mM $CaCl_2$ and the mixture was incubated at 37 °C for 45 min. The reaction was quenched with 1 M HCl until pH 2 was reached. After extraction with EtOAc (3 × 6 mL), the organic phases were combined and washed with brine. The organic solvent was removed *in vacuo* and the crude product was purified *via* semi-preparative HPLC (Hydrosphere C18, linear gradient: 0 → 25% B in A over 20 min, 25 → 60% B in A over 60 min with 5 mL/min; A: H_2O + 0.1% formic acid, B: CH_3CN + 0.1% formic acid). The column temperature was set to 40 °C. Carboxylic acid **21** (1.3 mg, 3.6 µmol, 70%) was obtained as colorless oil.

^{1}H NMR (500 MHz, MeOH-d_4): δ = 5.56 (dd, *J* = 15.6, 7.9 Hz, 1H, 7-H), 5.37 (dd, *J* = 15.4, 7.3 Hz, 1H, 6-H), 4.15 (dt, *J* = 6.7, 6.6 Hz, 1H, 5-H), 4.01-3.91 (m, 1H, 3-H), 3.68-3.62 (m, 2H, 9-H, 11-H), 2.33-2.22 (m, 2H, 2-H), 2.14-2.08 (m, 1H, 8-H), 1.67-1.61 (m, 1H, 4-H), 1.54-1.47 (m, 1H, 4-H), 1.38-1.30 (m, 5H, 10-H, 12-H, 13-H), 1.27-1.15 (m, 9H, 13-H, 14-H, 15-H, 16-H,

17-H), 0.95 (d, *J* = 6.9 Hz, 3H, 19-H), 0.80 (t, *J* = 6.6 Hz, 3H, 18-H) ppm. **^{13}C NMR** (125 MHz, MeOH-d_4): *δ* = 175.2 (C-1), 134.8 (C-7), 134.4 (C-6), 72.7 (C-9), 72.0 (C-5), 69.3 (C-11), 68.0 (C-3), 45.0 (C-4), 44.6 (C-2), 44.3 (C-8), 42.7 (C-10), 39.2 (C-12), 33.1 (C-16), 30.8 (C-14), 30.5 (C-15), 26.9 (C-13), 23.8 (C-17), 16.6 (C-19), 14.5 (C-18) ppm.

HRMS [ESI$^+$]: *m/z* for $C_{22}H_{36}O_8$ [M+Na]$^+$: calc. = 383.2404, found = 383.2408.

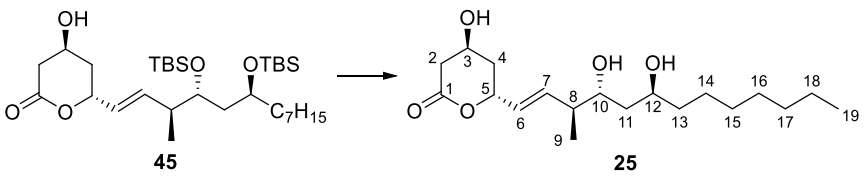
(4*S*,6*R*)-6-((3*S*,4*R*,6*S*,*E*)-4,6-Bis((*tert*-butyldimethylsilyl)oxy)-3-methyltridec-1-en-1-yl)-4-hydroxytetrahydro-2*H*-pyran-2-one (**45**)



44 (100 mg, 162 µmol, 1.0 eq.) was dissolved in MeOH/H_2O (3/1, 1.6 mL, 100 mM), LiOH (14.4 mg, 600 µmol, 3.7 eq.) was added and the reaction was stirred overnight at rt. MeOH was removed *in vacuo* and the pH of the aqueous phase was set to 1 by addition of 1 M HCl. The aqueous phase was extracted with EtOAc three times, the combined organic phases were dried over Na_2SO_4 and concentrated *in vacuo* to yield crude **45** (80 mg) as a brown oil, which was directly used in the next step without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): *δ* = 5.77 (ddd, *J* = 15.6, 7.6, 0.7 Hz, 1 H, 7-H), 5.52 (ddd, *J* = 15.6, 6.8, 0.7 Hz, 1 H, 6-H), 5.14 (ddd, *J* = 10.3, 6.8, 3.2 Hz, 1 H, 5-H), 4.42-4.39 (m, 1 H, 3-H), 3.73-3.67 (m, 2 H, 10-H, 12-H), 2.75 (dd, *J* = 17.7, 5.0 Hz, 1 H, 2-H_a), 2.63 (ddd, *J* = 17.7, 3.8, 1.6 Hz, 1 H, 2-H_b), 2.35-2.30 (m, 1 H, 8-H), 2.05-1.99 (m, 1 H, 4-H_a), 1.87 (ddd, *J* = 14.2, 10.8, 3.2 Hz, 1 H, 4-H_b), 1.42-1.38 (m, 4 H, 11-H, 13-H), 1.30-1.24 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 1.00 (d, *J* = 6.9 Hz, 3 H, 9-H), 0.90-0.88 (m, 12 H, 19-H, 22-H), 0.86 (s, 9 H, 22-H), 0.06 (s, 3 H, 20-H), 0.05 (s, 3 H, 20-H), 0.038 (s, 3 H, 20-H), 0.035 (s, 3 H, 20-H) ppm.

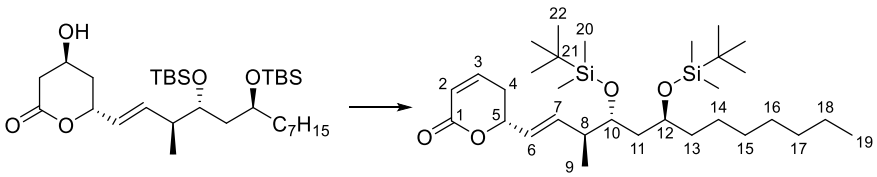
(4*S*,6*R*)-6-((3*S*,4*R*,6*S*,*E*)-4,6-Dihydroxy-3-methyltridec-1-en-1-yl)-4-hydroxytetrahydro-2*H*-pyran-2-one (**25**)



The TBS-groups of **45** (40.0 mg, 70.1 µmol, 1.0 eq.) were removed following the general procedure for silyl ether deprotection. Purification *via* preparative HPLC provided **25** (9.4 mg, 27.4 µmol, 34% over two steps) as a colorless oil.

$\mathbf{t_R}$ = 43.6 min (HPLC), 3.24 min (UHPLC-MS). **^{1}H NMR** (500 MHz, Acetone-d_6): δ = 5.69 (ddd, J = 15.6, 8.0, 1.0 Hz, 1 H, 7-H), 5.44 (ddd, J = 15.6, 6.9, 0.8 Hz, 1 H, 6-H), 4.97 (ddd, J = 10.5, 7.0, 3.4 Hz, 1 H, 5-H), 4.22-4.18 (m, 1 H, 3-H), 3.72-3.66 (m, 2 H, 10-H, 12-H), 2.55 (dd, J = 17.4, 4.7 Hz, 1 H, 2-H_a), 2.36 (ddd, J = 17.4, 3.7, 1.7 Hz, 1 H, 2-H_b), 2.13-2.09 (m, 1 H, 8-H), 1.86-1.81 (m, 1 H, 4-H_a), 1.79-1.73 (m, 1 H, 4-H_b), 1.41-1.27 (m, 4 H, 11-H, 13-C), 1.19-1.14 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 0.91 (d, J = 6.9 Hz, 3 H, 9-H), 0.75 (t, J = 7.0 Hz, 3 H, 19-H) ppm. **^{13}C NMR** (125 MHz, Acetone-d_6): δ = 170.1 (C-1), 136.5 (C-7), 129.8 (C-6), 77.0 (C-5), 72.0 (C-10), 68.8 (C-12), 63.0 (C-3), 43.8 (C-8), 42.2 (C-11), 39.4 (C-2), 38.9 (C-13), 37.2 (C-4), 32.6 (C-17), 30.5 (C-15), 30.1 (C-16), 26.7 (C-14), 23.3 (C-18), 16.8 (C-9), 14.4 (C-19) ppm. $[\alpha]_{20}^{D}$: -27.0 (c = 0.25 in CH_2Cl_2). **HRMS** [ESI$^+$]: *m/z* for $C_{19}H_{34}O_5$ $[M+H]^+$: calc. = 343.2479, found = 343.2481; $[M+Na]^+$: calc. = 365.2298, found = 365.2301.

(*R*)-6-((3*S*,4*R*,6*S*,*E*)-4,6-Bis((*tert*-butyldimethylsilyl)oxy)-3-methyltridec-1-en-1-yl)-5,6-dihydro-2*H*-pyran-2-one (**46**)



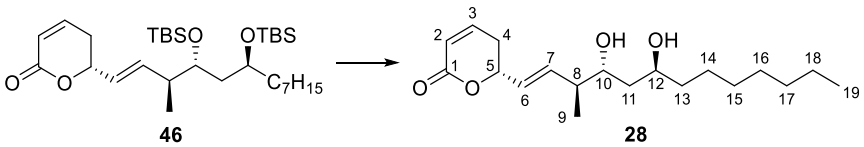
45 **46**

Following a literature procedure[10], **45** (40.0 mg, 70.1 µmol, 1.0 eq.) was dissolved in $CHCl_3$ (580 µL, 120 mM), before NEt_3 (41.8 µL, 30.4 mg, 300 µmol, 4.3 eq.) and MsCl (9.1 µL, 13.4 mg, 117 µmol, 1.7 eq.) were added and the reaction was stirred at rt for 3 h. Saturated aqueous $NaHCO_3$ solution was added and the mixture was stirred at rt for another 5 min. Subsequently, the phases were separated, and the aqueous one was extracted three times with CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 and concentrated *in vacuo* to obtain crude **46** (39 mg) as a brown oil, which was used in the next step without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.87 (m, 1 H, 3-H), 6.04 (dt, J_d = 9.8 Hz, J_t = 1.8 Hz, 1 H, 2-H), 5.79 (ddd, J = 15.7, 7.7, 0.7 Hz, 1 H, 7-H), 5.61 (ddd, J = 15.7, 6.8, 0.8 Hz, 1 H, 6-H), 4.90-4.85 (m, 1 H, 5-H), 3.73-3.67 (m, 2 H, 10-H, 12-H), 2.44-2.41 (m, 2 H, 4-H), 2.35-2.30 (m, 1 H, 8-H), 1.46-1.38 (m, 4 H, 11-H, 13-H), 1.30-1.24 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 1.01

(d, *J* = 6.9 Hz, 3 H, 9-H), 0.89-0.88 (m, 12 H, 19-H, 22-H), 0.86 (m, 9 H, 22-H), 0.06 (s, 3 H, 20-H), 0.05 (s, 3 H, 20-H), 0.039 (s, 3 H, 20-H), 0.036 (s, 3 H, 20-H) ppm.

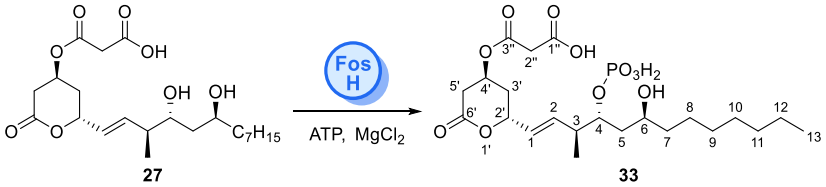
(*R*)-6-((3*S*,4*R*,6*S*,*E*)-4,6-Dihydroxy-3-methyltridec-1-en-1-yl)-5,6-dihydro-2*H*-pyran-2-one (**28**)



The TBS-groups of crude **46** (39.0 mg, <70.5 µmol, 1.0 eq.) from the previous reaction were removed following the general procedure for silyl ether deprotection. Purification *via* preparative HPLC provided **28** (14.4 mg, 44.4 µmol, 55% over three steps) as a colorless oil.

t_R = 55.2 min (HPLC), 3.79 min (UHPLC-MS). **^{1}H NMR** (500 MHz, Acetone-d_6): *δ* = 7.00 (ddd, *J* = 9.7, 5.7, 2.8 Hz, 1 H, 3-H), 5.93 (ddd, *J* = 9.8, 2.5, 1.1 Hz, 1 H, 2-H), 5.88 (ddd, *J* = 15.6, 8.0, 1.0 Hz, 1 H, 7-H), 5.64 (ddd, *J* = 15.6, 6.7, 0.9 Hz, 1 H, 6-H), 4.91 (dddd, *J* = 10.9, 6.5, 4.3, 0.8 Hz, 1 H, 5-H), 3.85-3.79 (m, 2 H, 10-H, 12-H), 2.52 (dddd, *J* = 18.5, 5.6, 4.4, 1.1 Hz, 1 H, 4-H_a), 2.43 (dddd, *J* = 18.5, 10.8, 2.7, 2.6 Hz, 1 H, 4-H_b), 2.29-2.22 (m, 1 H, 8-H), 1.54-1.40 (m, 4 H, 11-H, 13-H), 1.33-1.25 (m, 10 H, 14-H, 15-H, 16-H, 17-H, 18-H), 1.04 (d, *J* = 6.9 Hz, 3 H, 9-H), 0.88 (t, *J* = 7.0 Hz, 3 H, 19-H) ppm. **^{13}C NMR** (125 MHz, Acetone-d_6): *δ* = 164.1 (C-1), 146.4 (C-3), 137.6 (C-7), 128.6 (C-6), 121.7 (C-2), 79.0 (C-5), 71.9 (C-10), 68.8 (C-12), 43.8 (C-8), 42.2 (C-11), 38.8 (C-13), 32.6 (C-17), 30.6 (C-4), 30.5 (C-15), 30.1 (C-16), 26.6 (C-14), 23.3 (C-18), 16.7 (C-9), 14.4 (C-19) ppm. $[\alpha]_{20}^{D}$: +4.67 (c = 0.55 in CH_2Cl_2). **HRMS** [ESI$^+$]: *m/z* for $C_{19}H_{34}O_5$ $[M+H]^+$: calc. = 325.2373, found = 325.2375; $[M+Na]^+$: calc. = 347.2193, found = 347.2195.

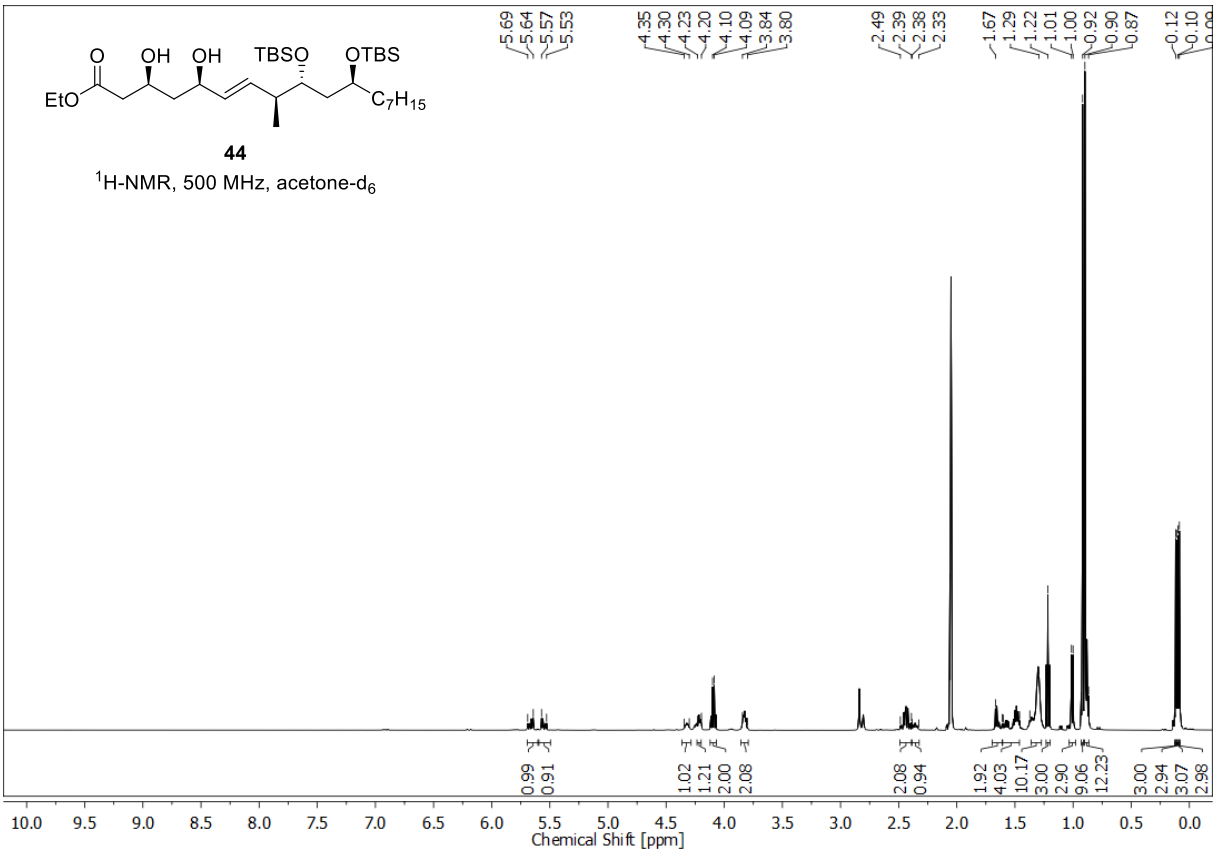
3''-(((2'*R*,4'*S*)-2''-((3*S*,4*R*,6*S*,*E*)-6-Hydroxy-3-methyl-4-(phosphonooxy)tridec-1-en-1-yl)-6'-oxotetrahydro-2*H*-pyran-4'-yl)oxy)-3''-oxopropanoic acid (**33**)



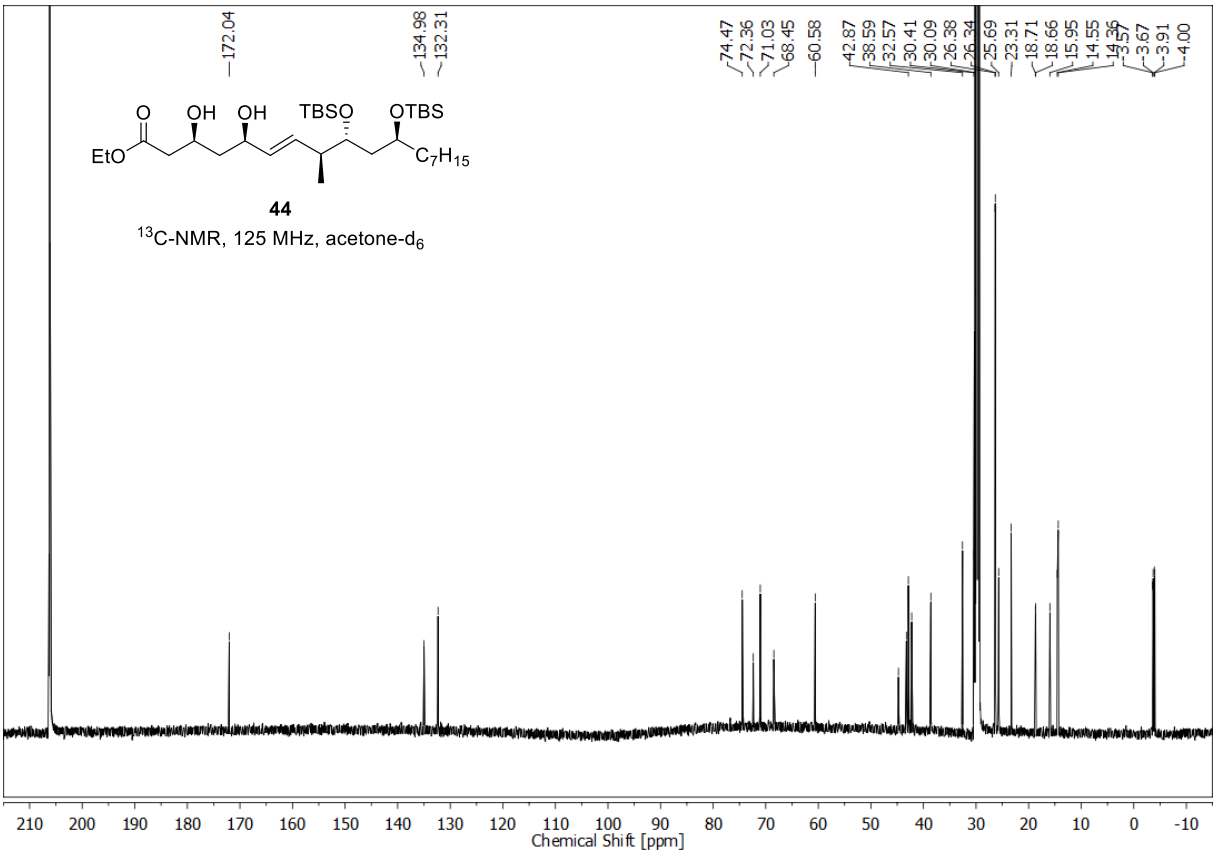
Malonyl lactone **27** (0.4 mg, 0.9 µmol, 1 eq., 1 mM) was incubated with 60 µM FosH, $MgCl_2$ (4.7 µmol, 5 eq., 5 mM), ATP (0.9 µmol, 1 eq., 1 mM) in 200 mM sodium phosphate buffer, pH 6.8 at 25 °C for 1 h. After completion of the reaction, the enzyme was removed using a 10 kDa MWCO centrifugal filter (14000 × g), and **33** (0.2 mg, 0.39 µmol, 42%) was

subsequently isolated using analytical UHPLC (BEH C18, 10 → 90% B in A over 6 min, A: H_2O, B: CH_3CN + 0.1% formic acid).

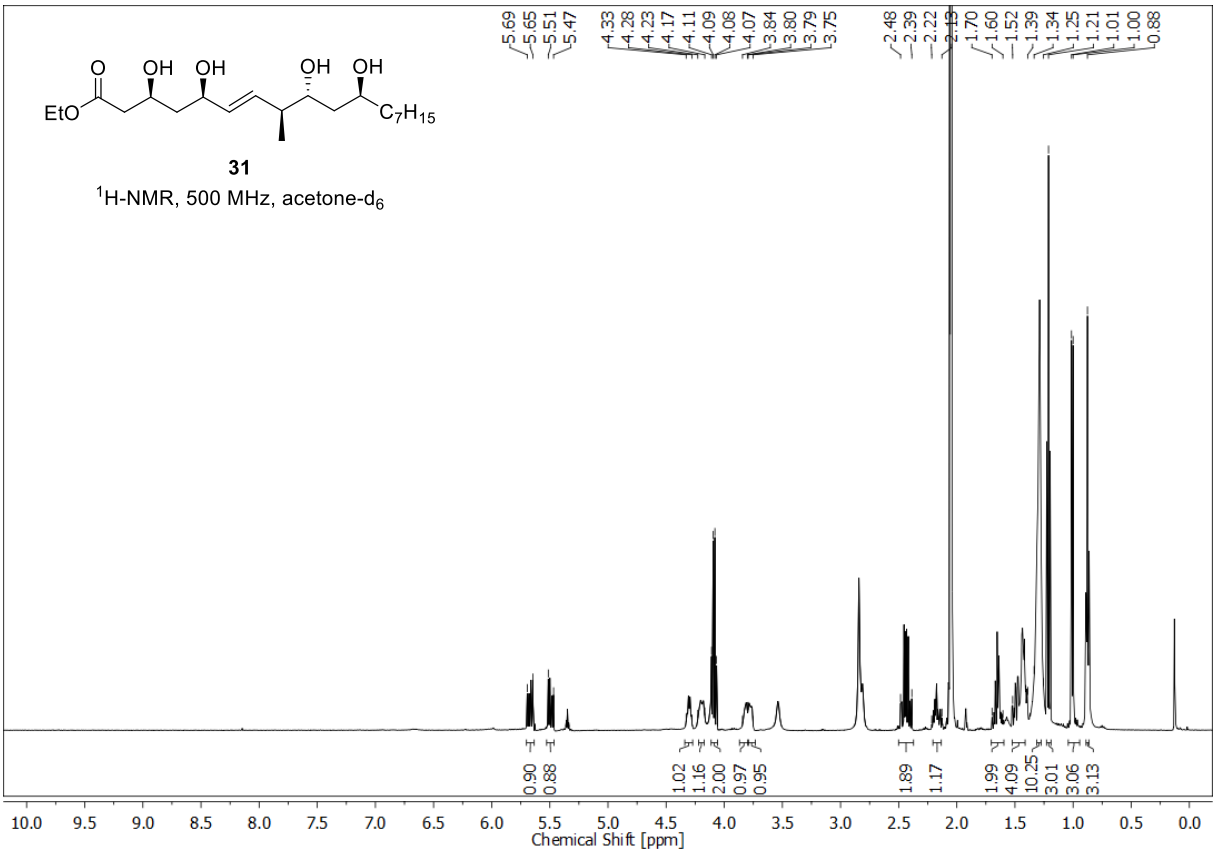
HRMS [ESI$^+$]: *m/z* for $C_{22}H_{37}O_{11}P$ $[M+H]^+$: calc. = 509.2146, found = 509.2150; $[M+Na]^+$: calc. = 531.1966, found = 531.1967.



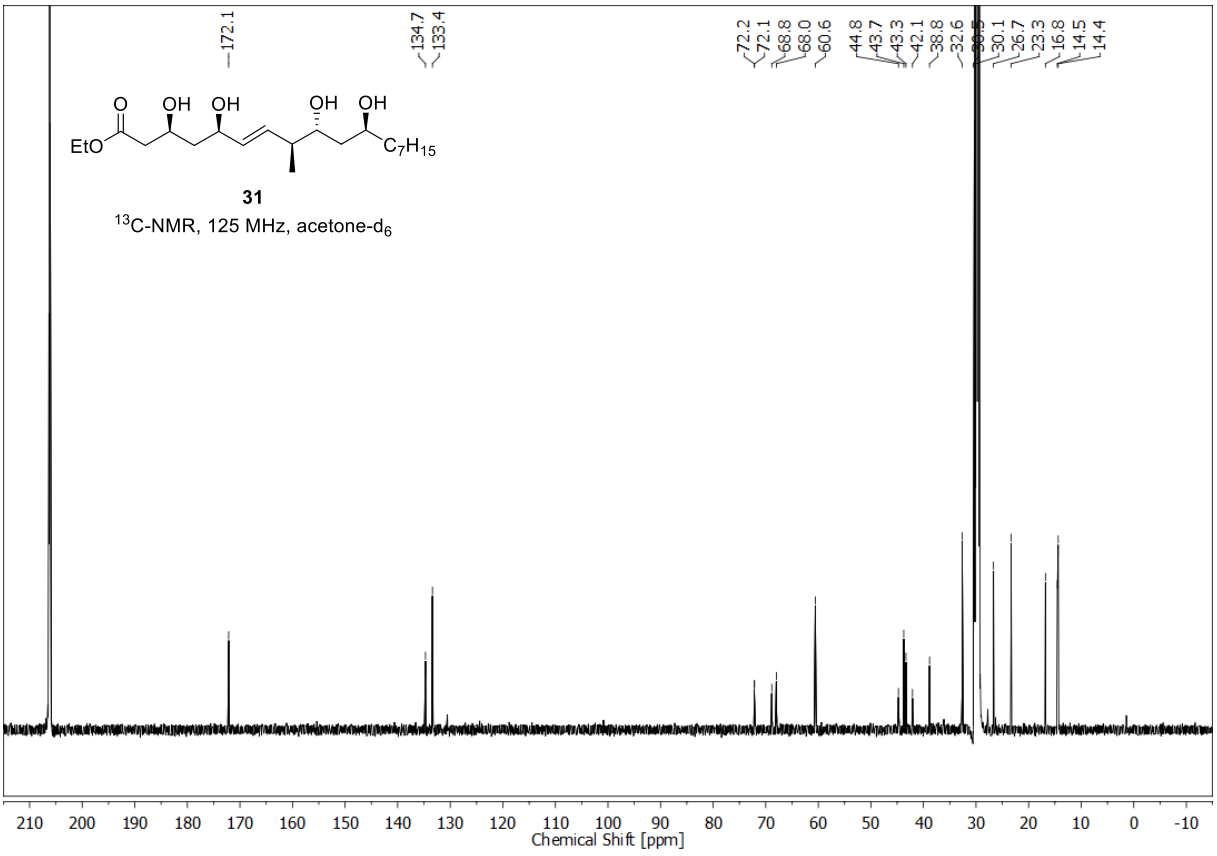
Supplementary Figure 70: ^{1}H NMR-spectrum of **44** in acetone-d_6.



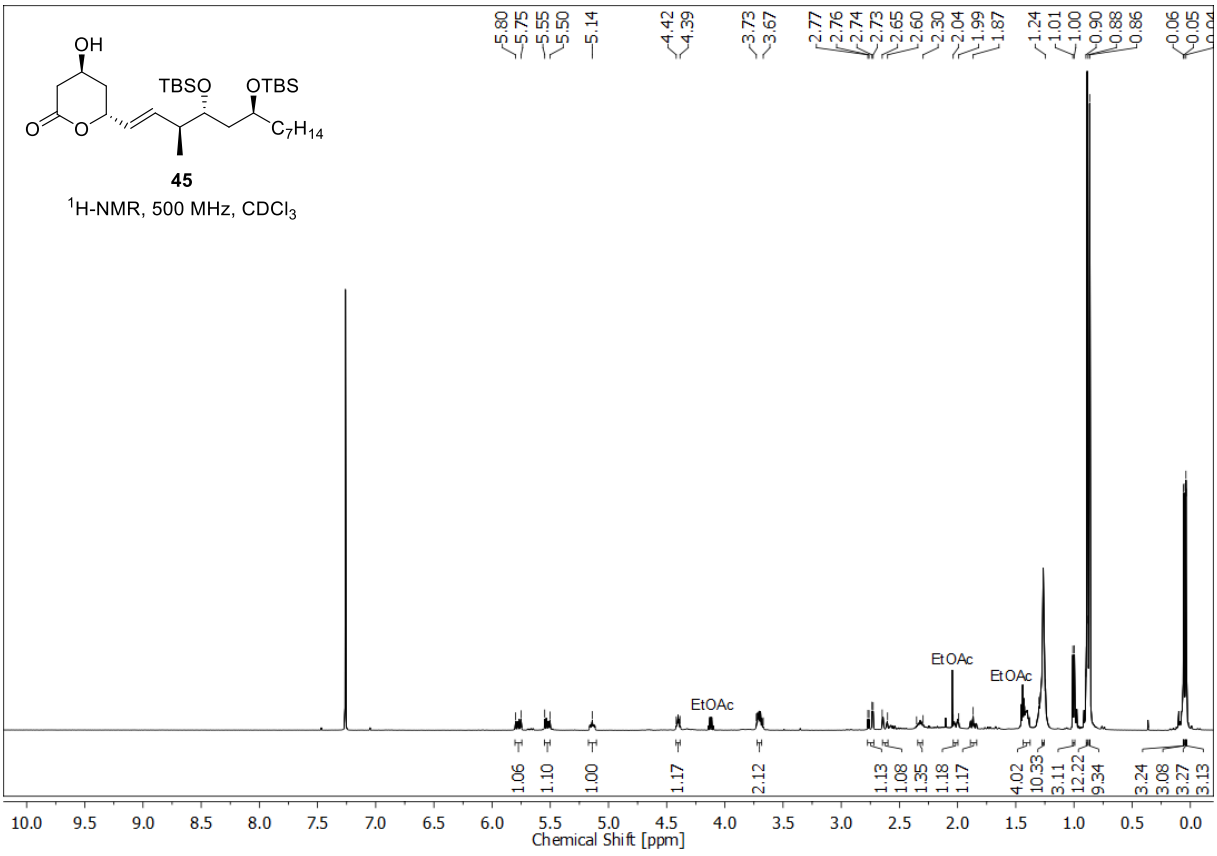
Supplementary Figure 71: ^{13}C NMR-spectrum of **44** in acetone-d_6.



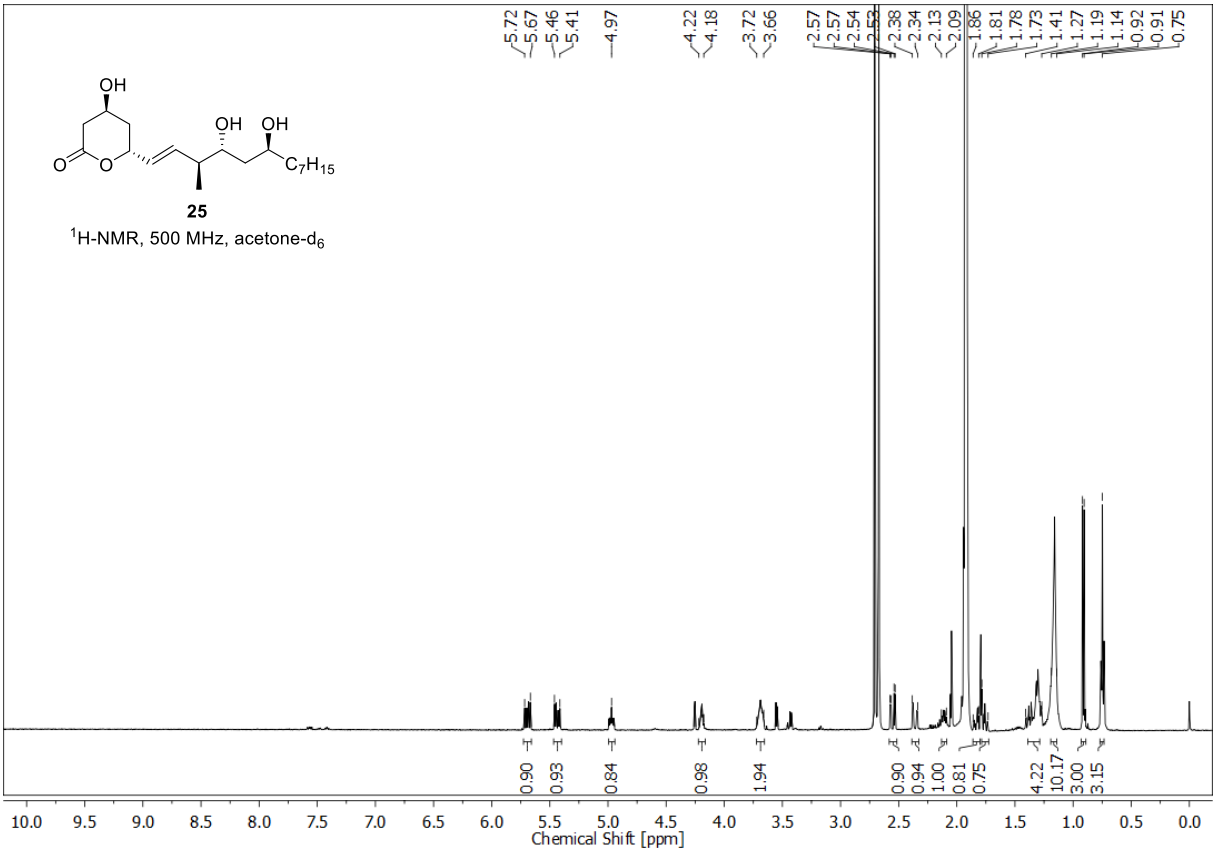
Supplementary Figure 72: ^{1}H NMR-spectrum of **31** in acetone-d_6.



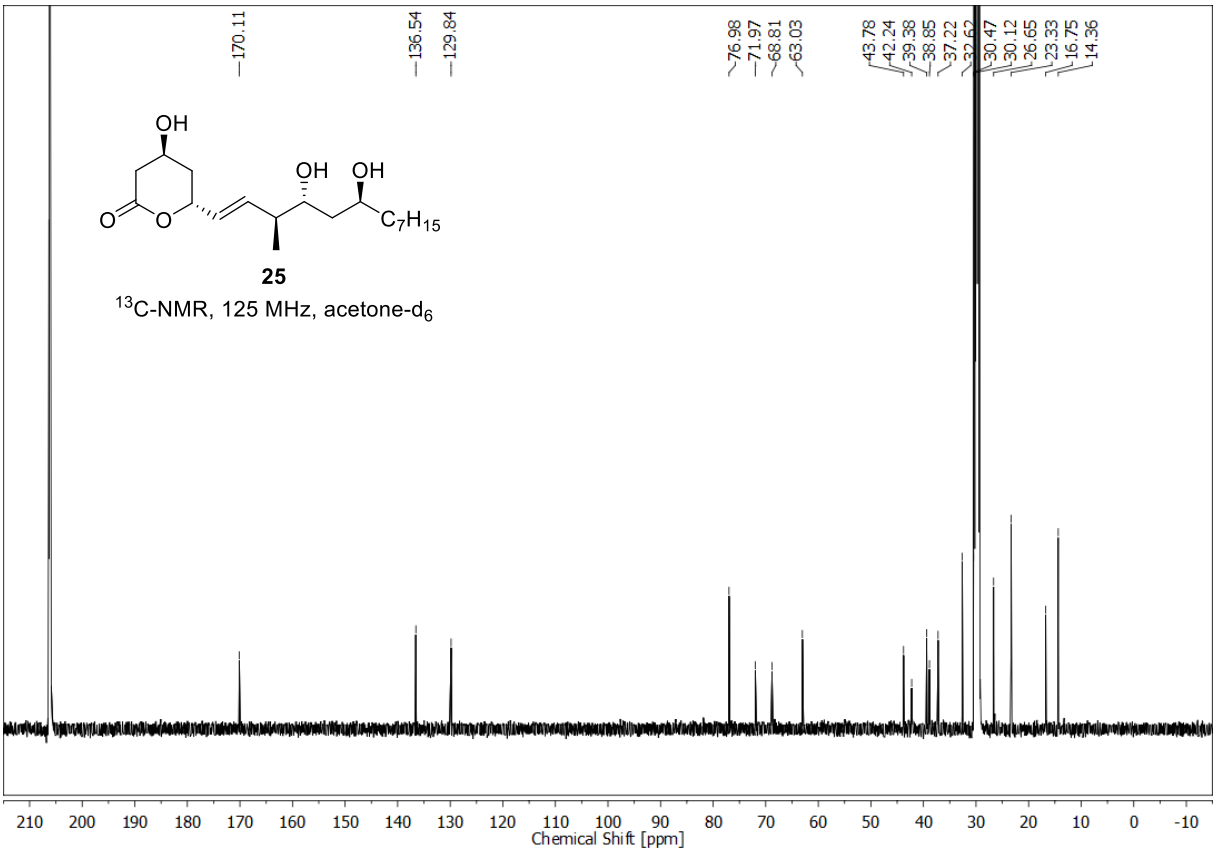
Supplementary Figure 73: ^{13}C NMR-spectrum of **31** in acetone-d_6.



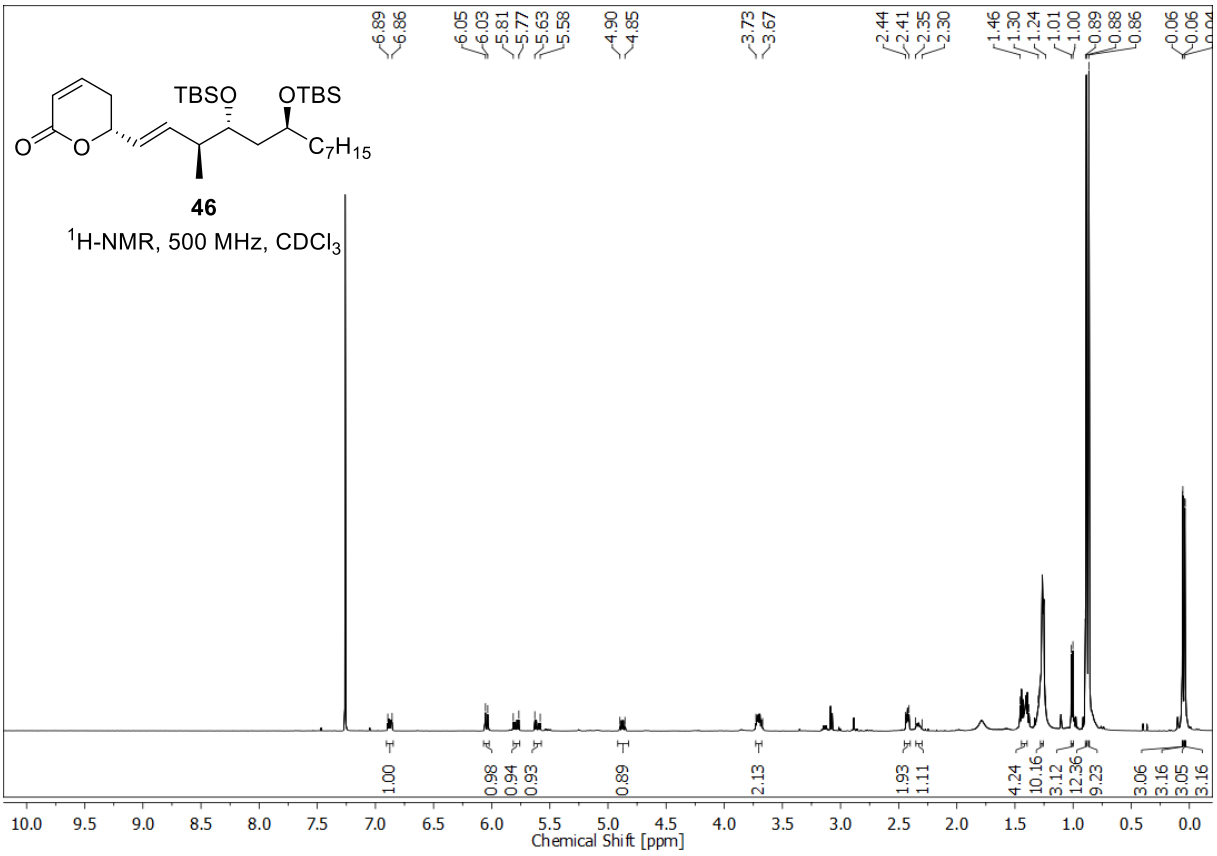
Supplementary Figure 74: Crude ^{1}H NMR-spectrum of **45** in $CDCl_3$ with traces of EtOAc.



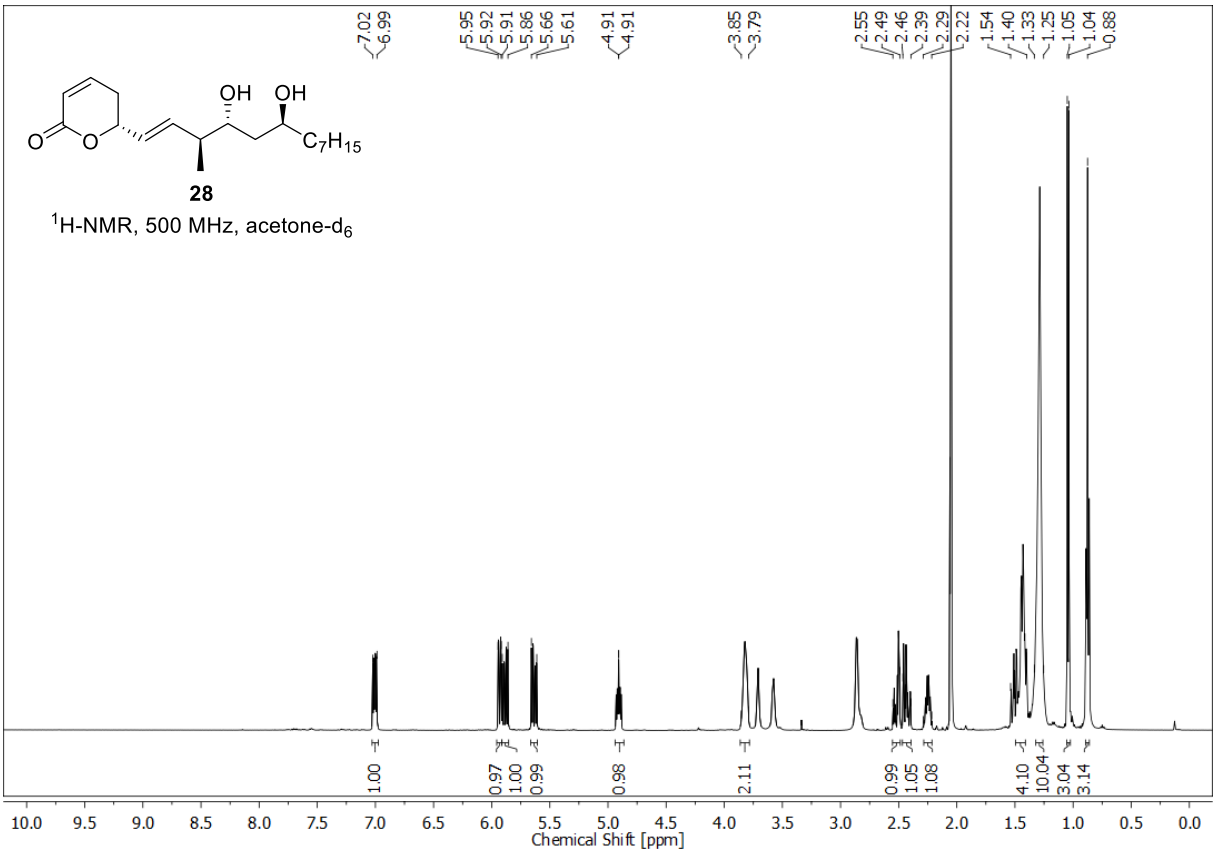
Supplementary Figure 75: ^{1}H NMR-spectrum of **25** in acetone-d_6.



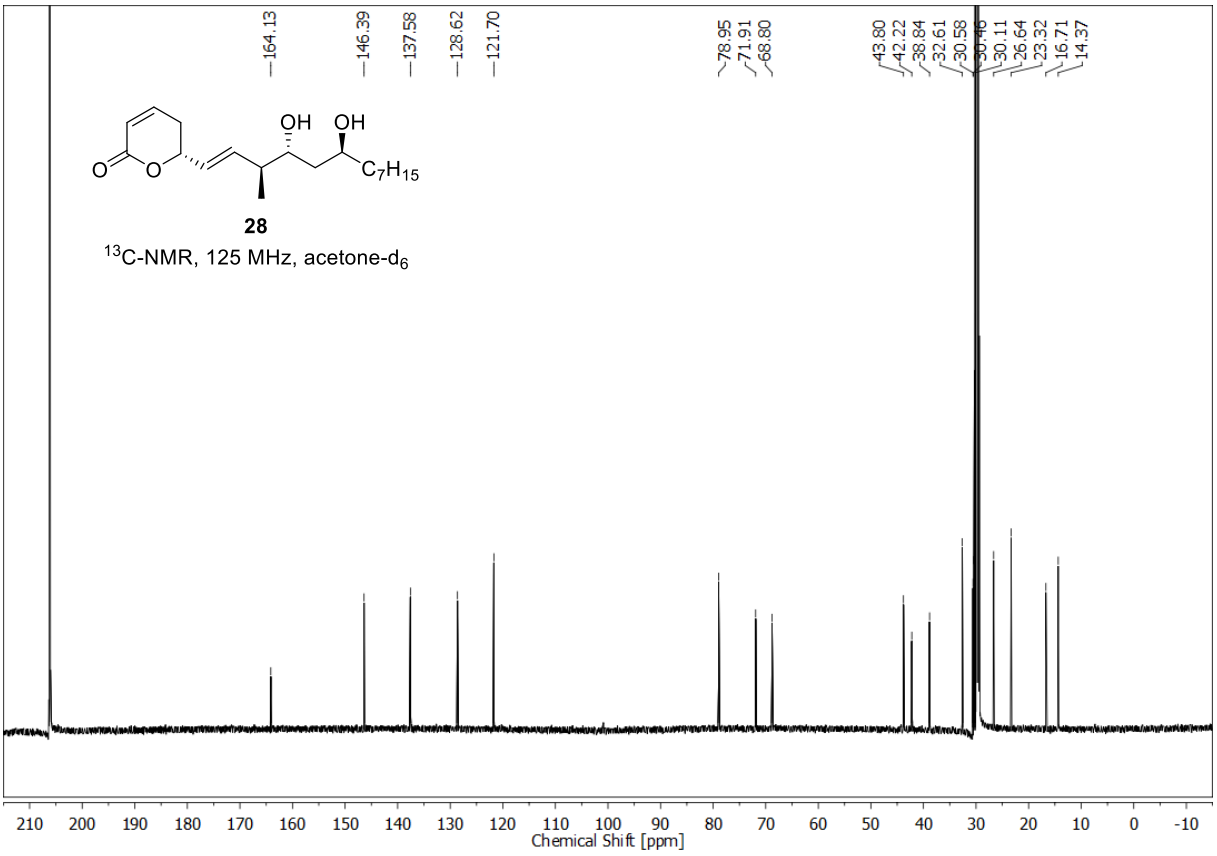
Supplementary Figure 76: ^{13}C NMR-spectrum of **25** in acetone-d_6.



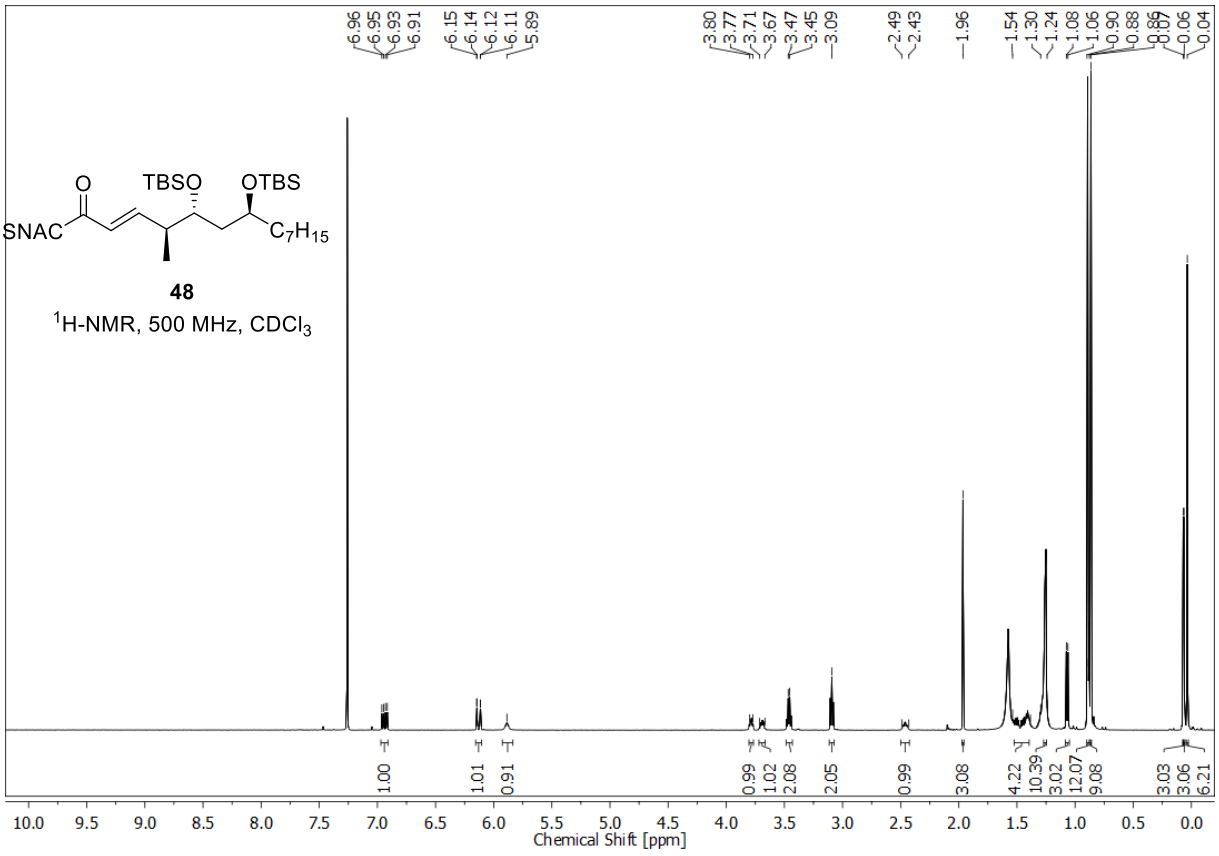
Supplementary Figure 77: Crude ^{1}H NMR-spectrum of **46** in $CDCl_3$ with minor impurities.



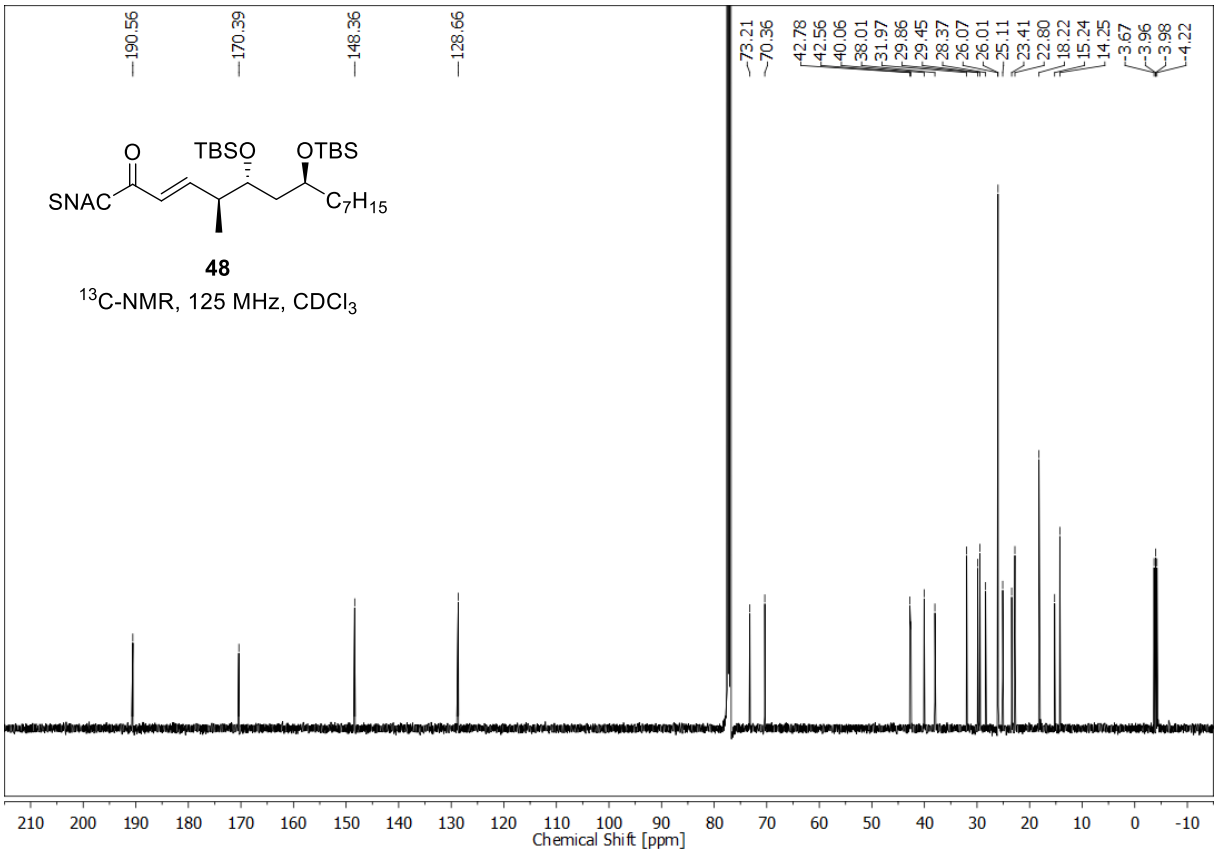
Supplementary Figure 78: ^{1}H NMR-spectrum of **28** in acetone-d_6.



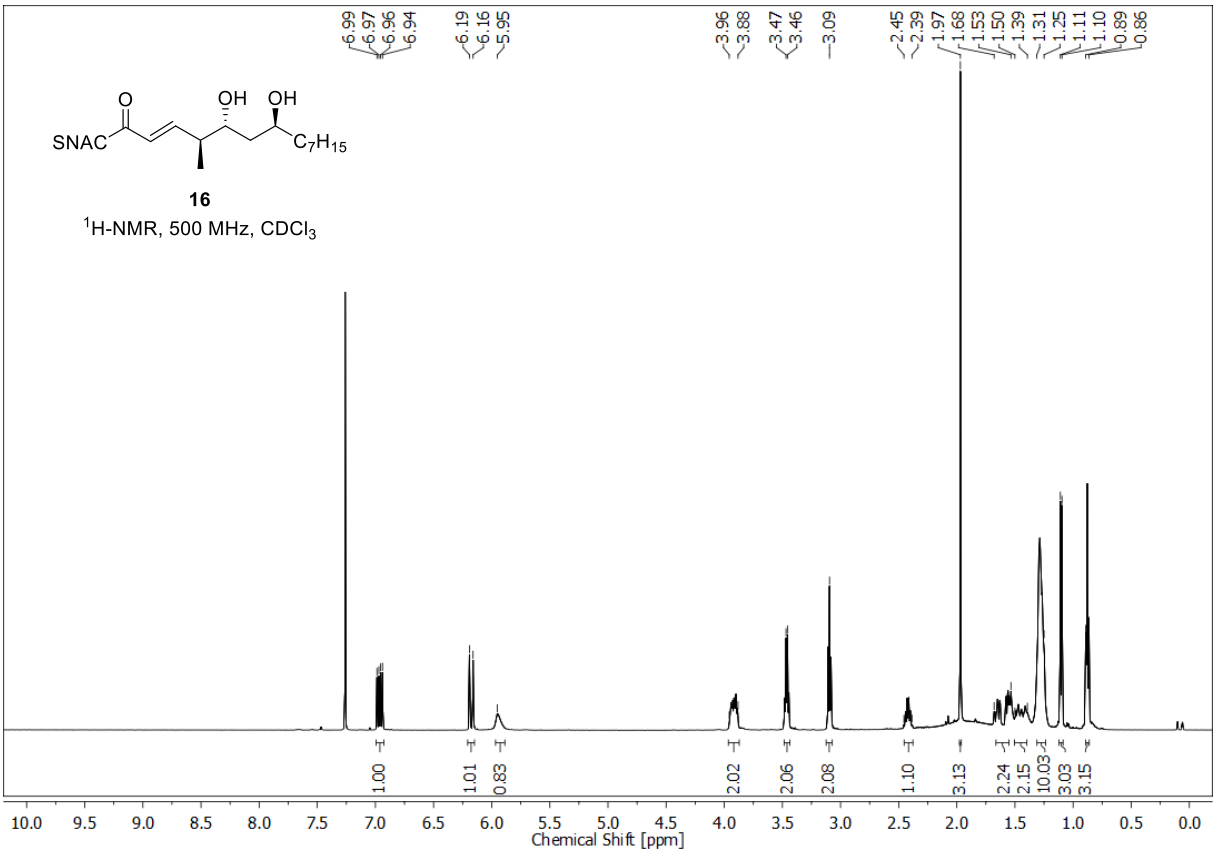
Supplementary Figure 79: ^{13}C NMR-spectrum of **28** in acetone-d_6.



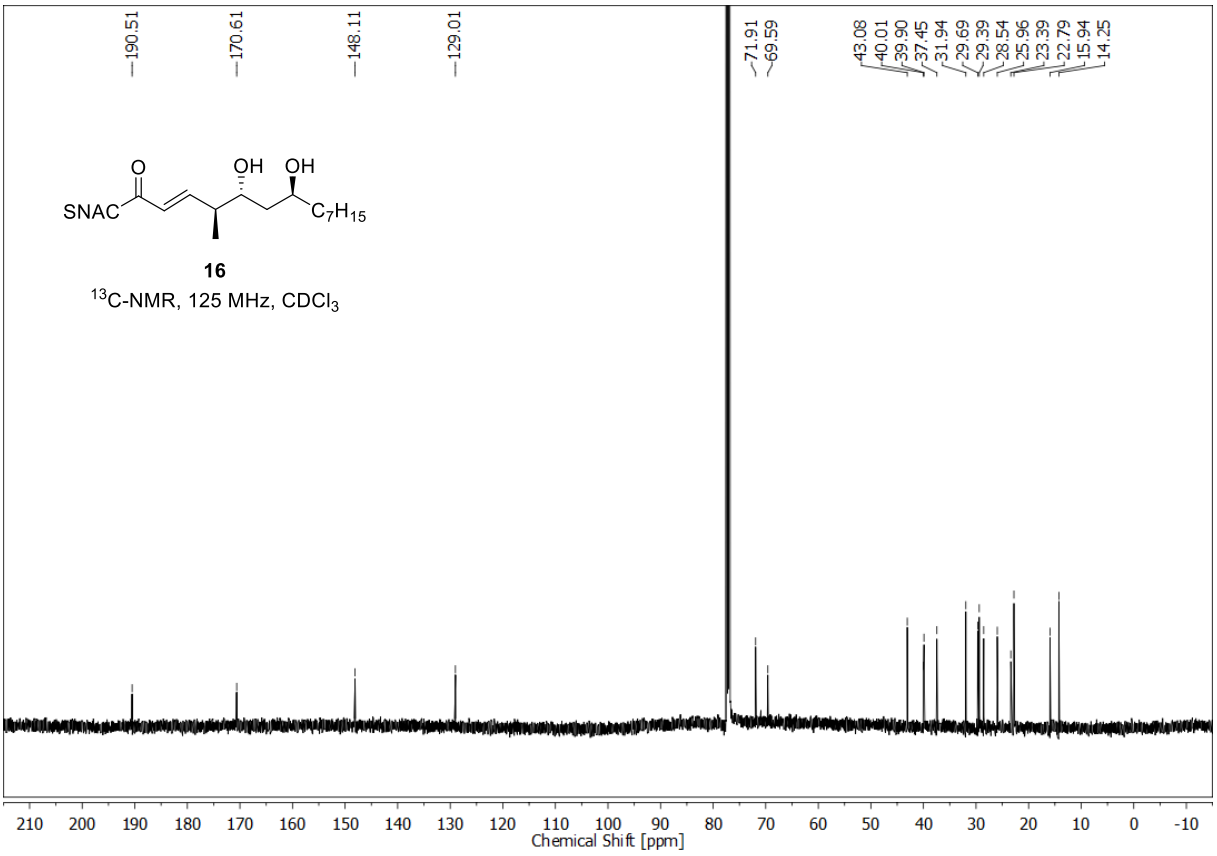
Supplementary Figure 80: ^{1}H NMR-spectrum of **48** in $CDCl_3$.



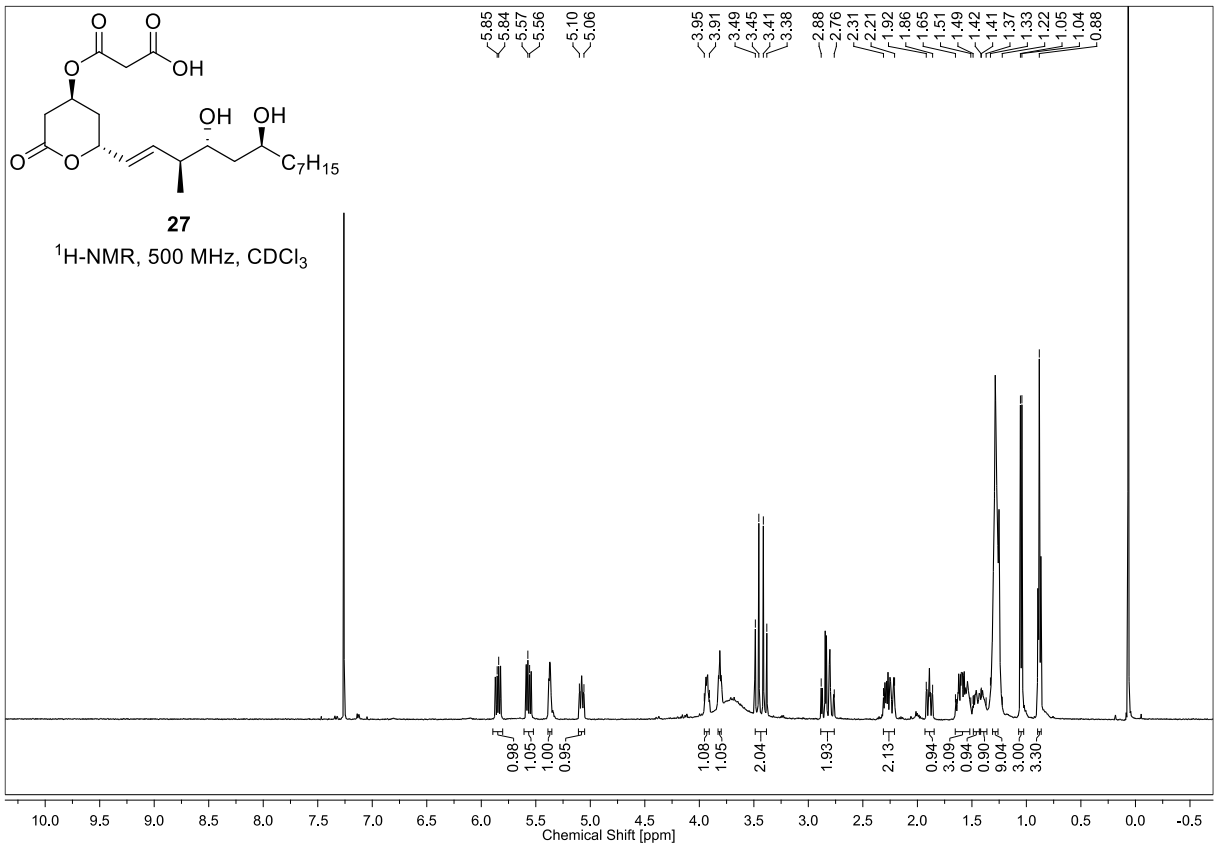
Supplementary Figure 81: ^{13}C NMR-spectrum of **48** in $CDCl_3$.



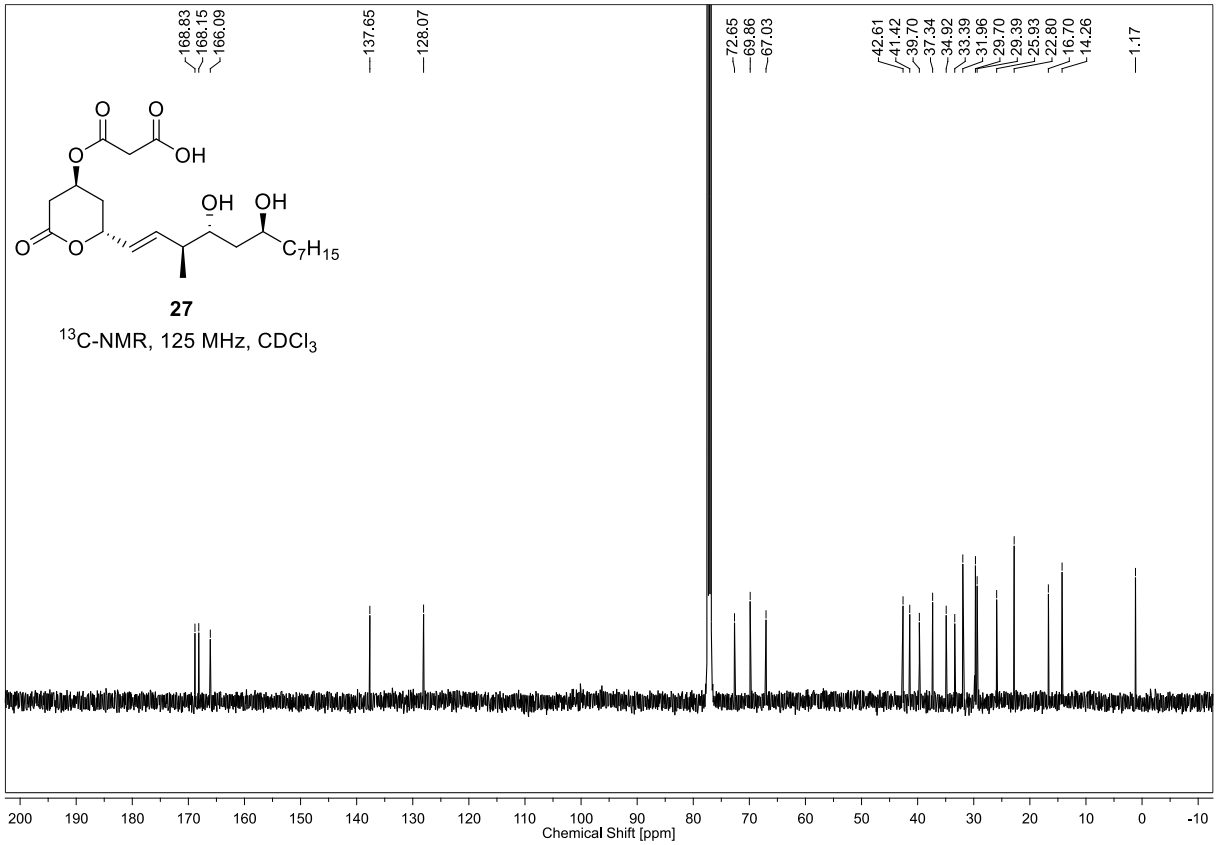
Supplementary Figure 82: ^{1}H NMR-spectrum of **16** in $CDCl_3$.



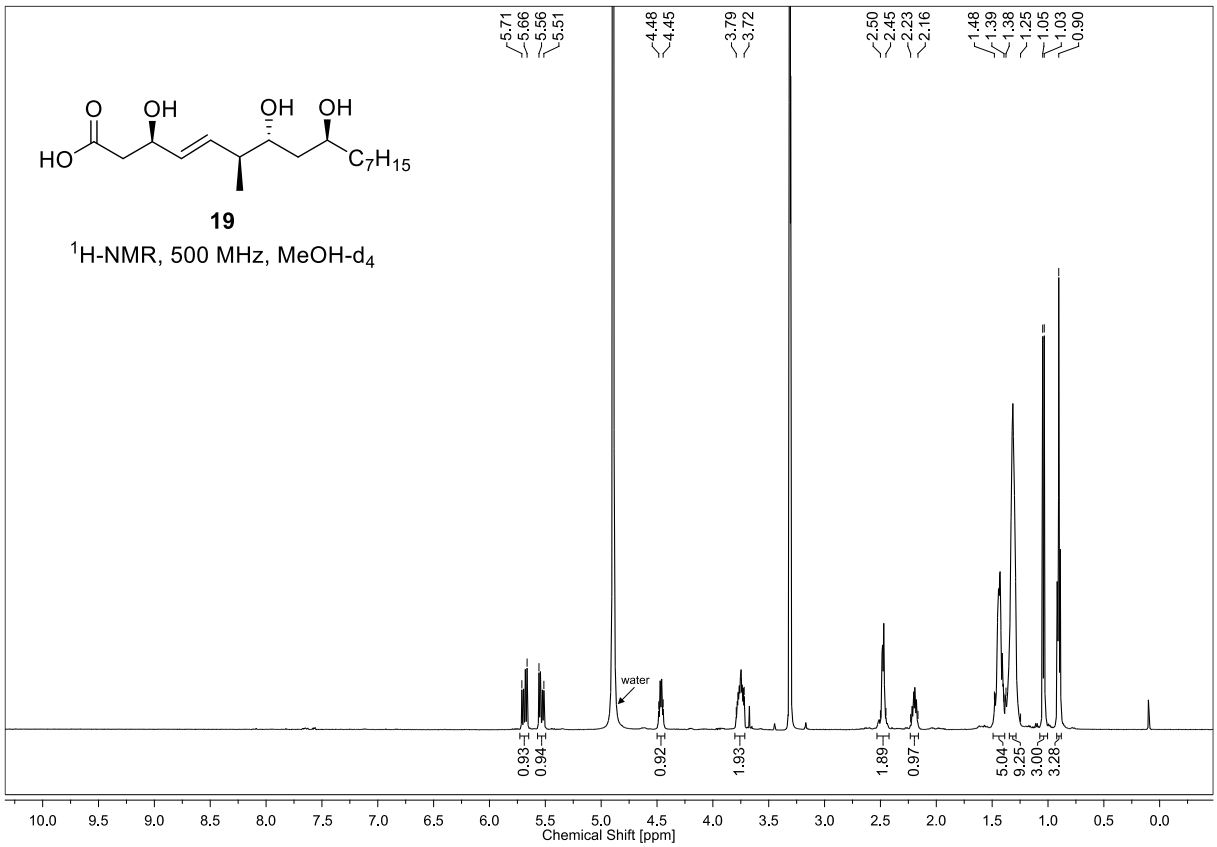
Supplementary Figure 83: ^{13}C NMR-spectrum of **16** in $CDCl_3$.



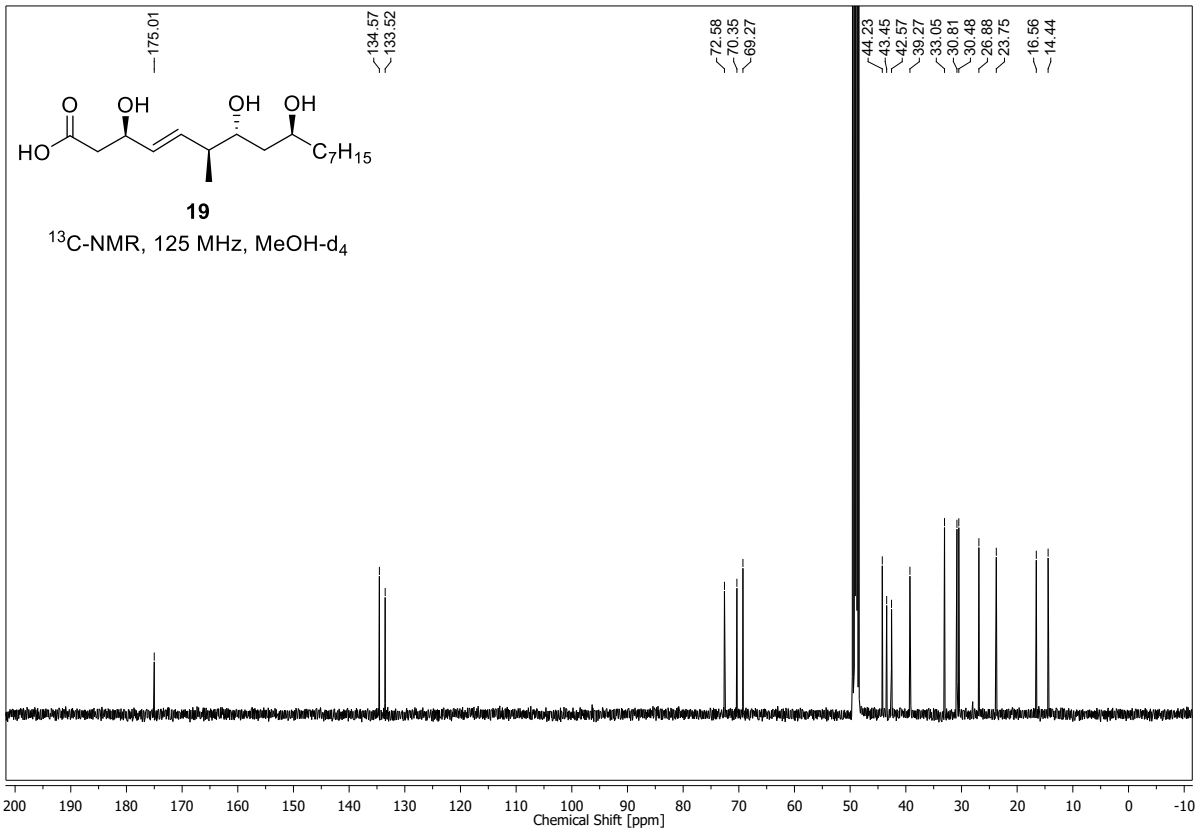
Supplementary Figure 84: ^{1}H NMR-spectrum of **27** in $CDCl_3$.



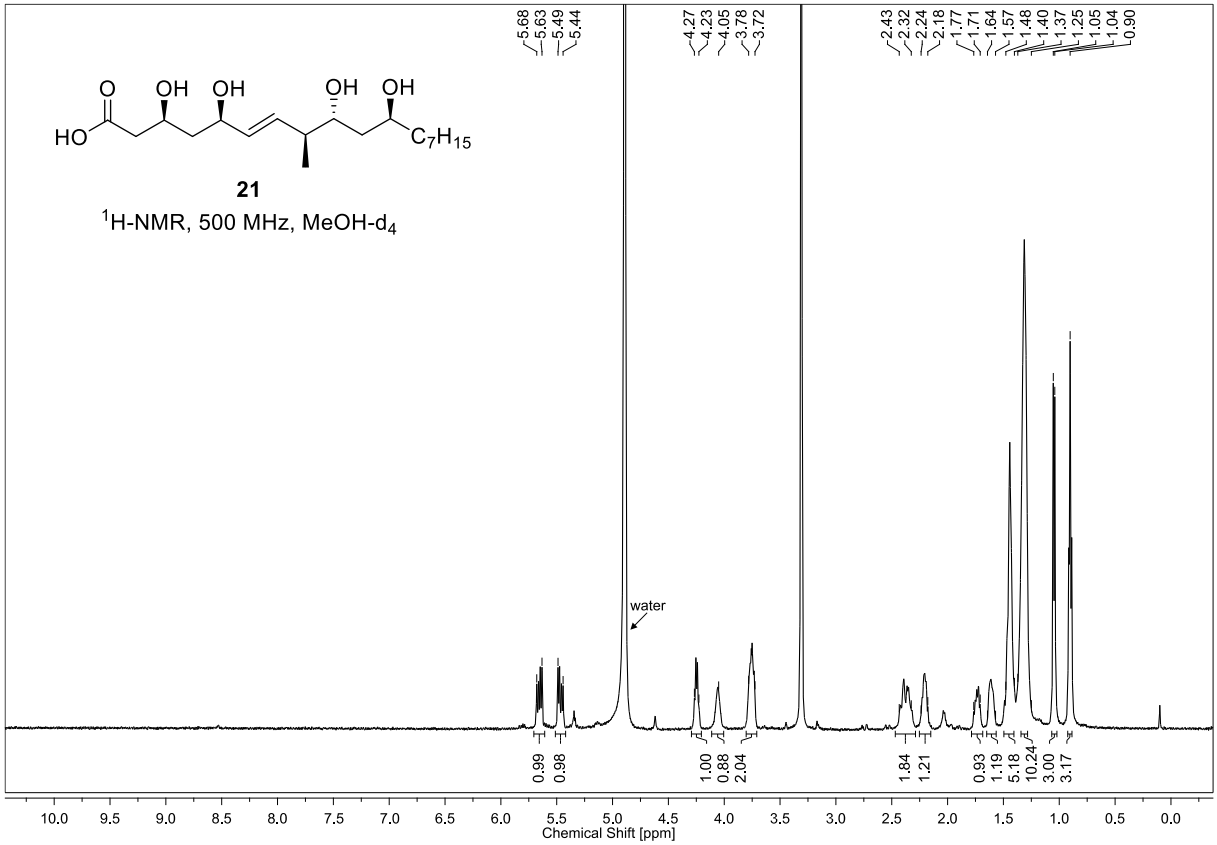
Supplementary Figure 85: ^{13}C NMR-spectrum of **27** in $CDCl_3$.



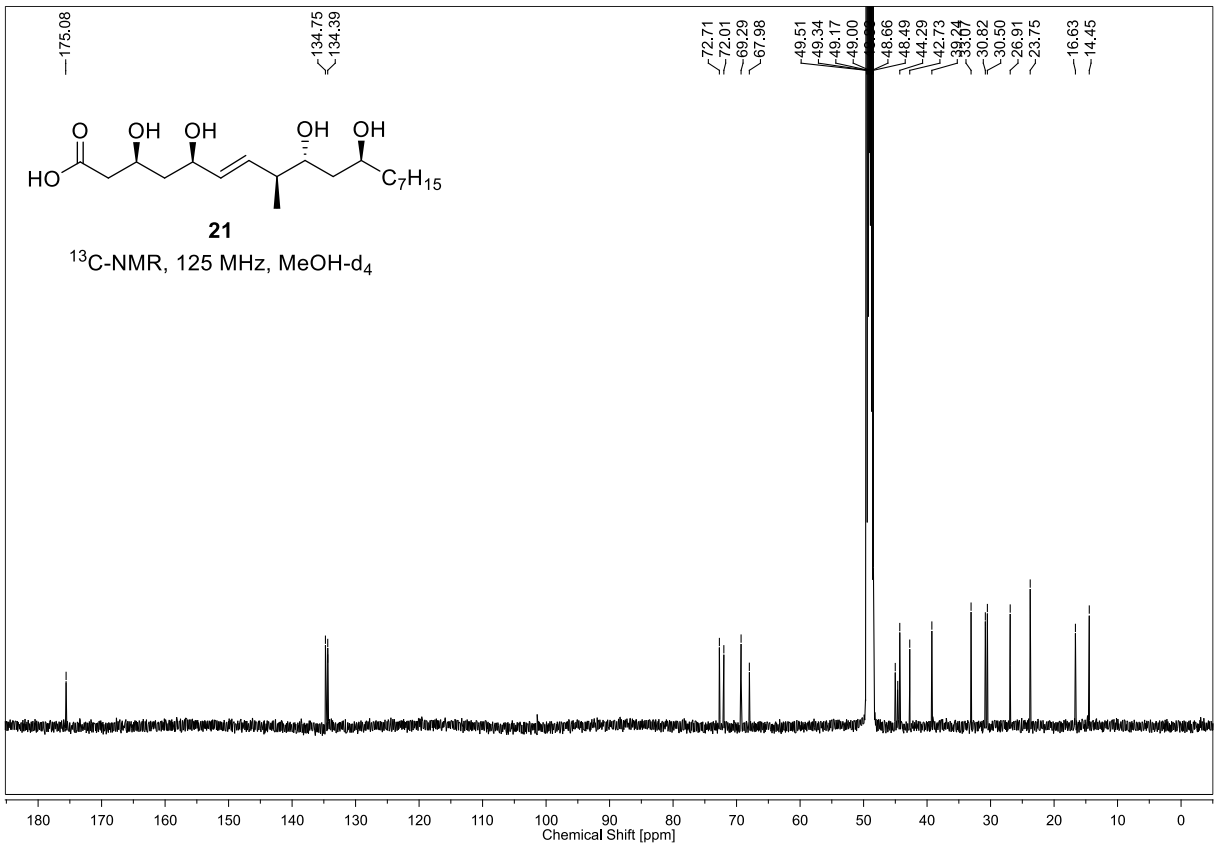
Supplementary Figure 86: ^{1}H NMR-spectrum of **19** in MeOH-d_4.



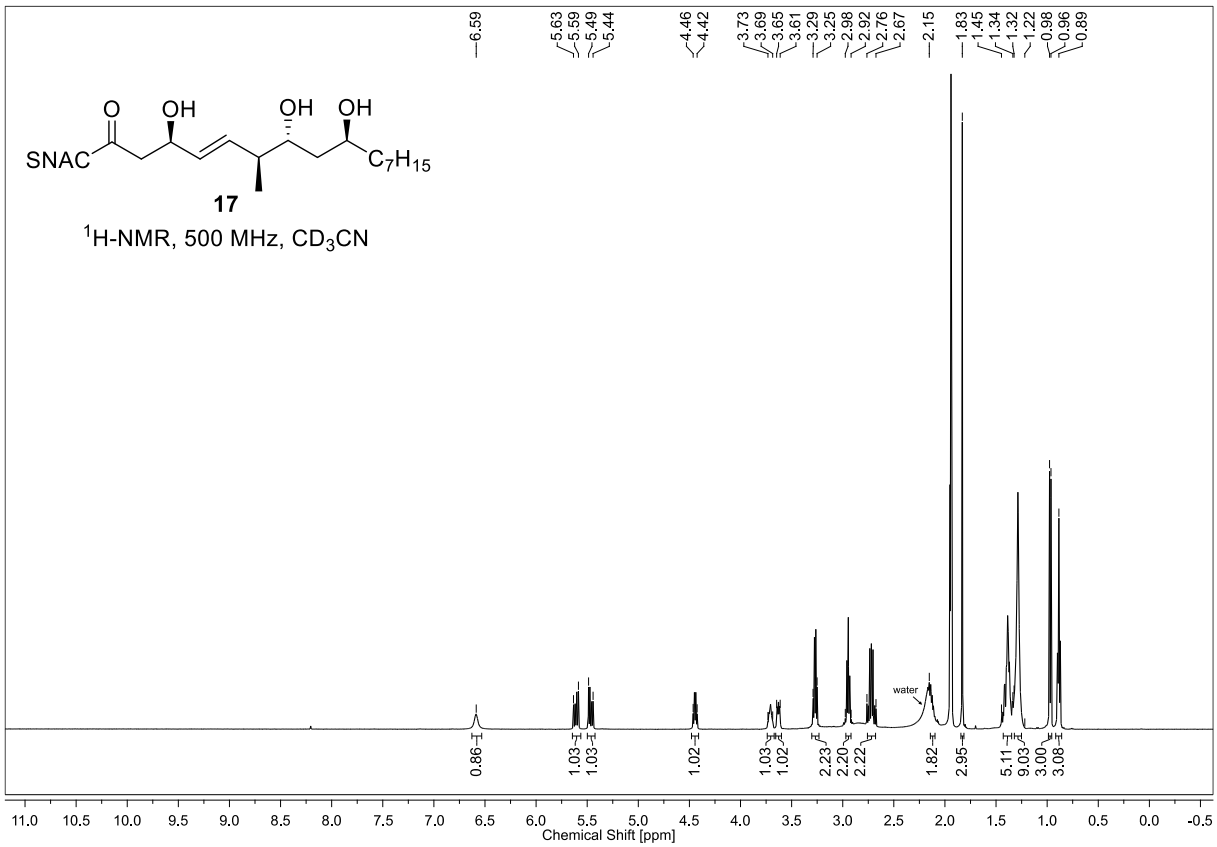
Supplementary Figure 87: ^{13}C NMR-spectrum of **19** in MeOH-d_4.



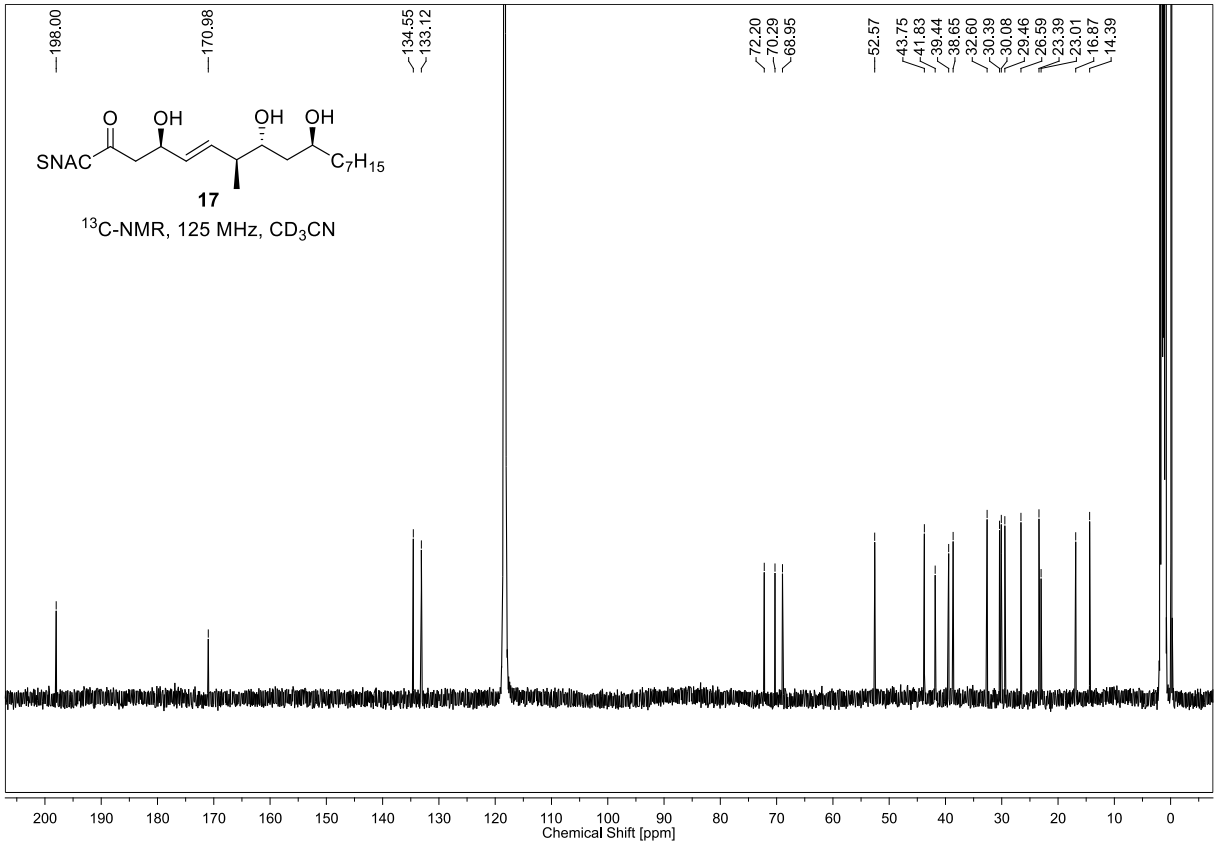
Supplementary Figure 88: ^{1}H NMR-spectrum of **21** in MeOH-d_4.



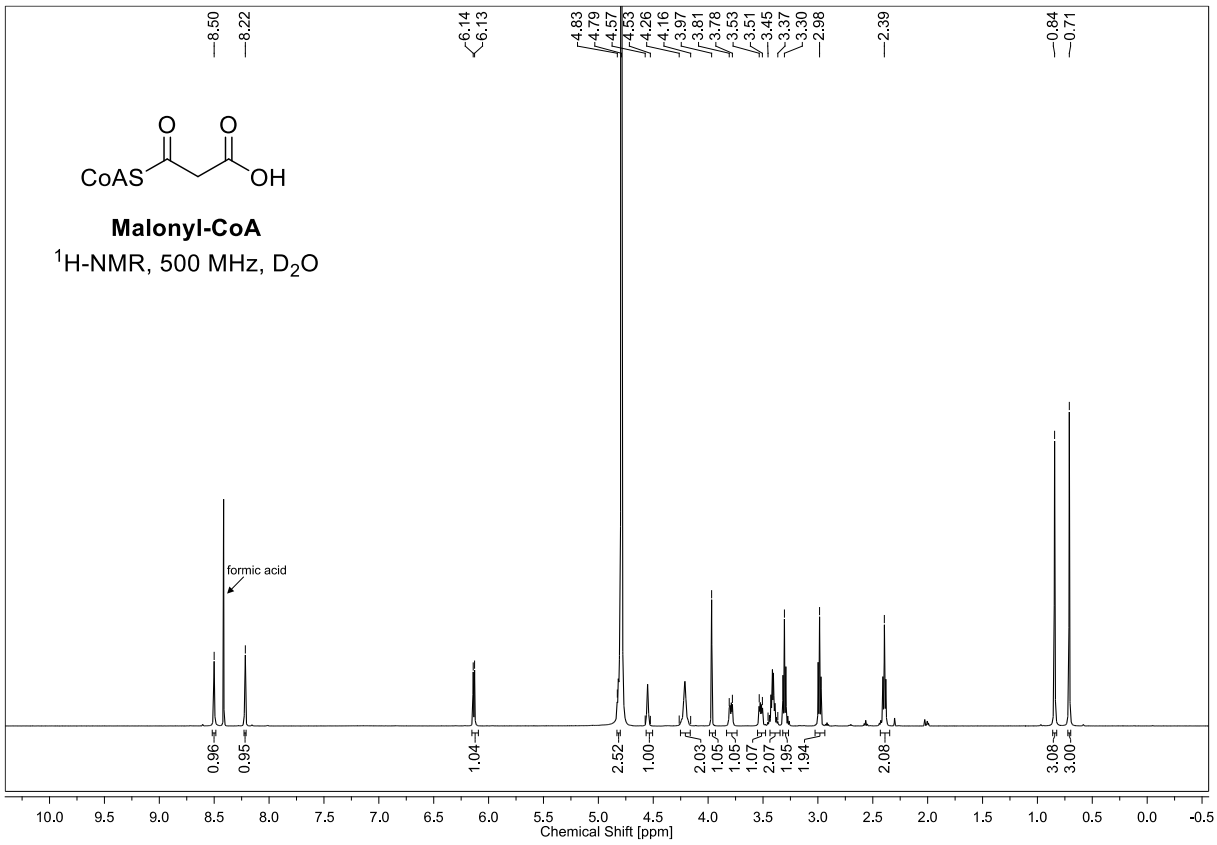
Supplementary Figure 89: ^{13}C NMR-spectrum of **21** in MeOH-d_4.



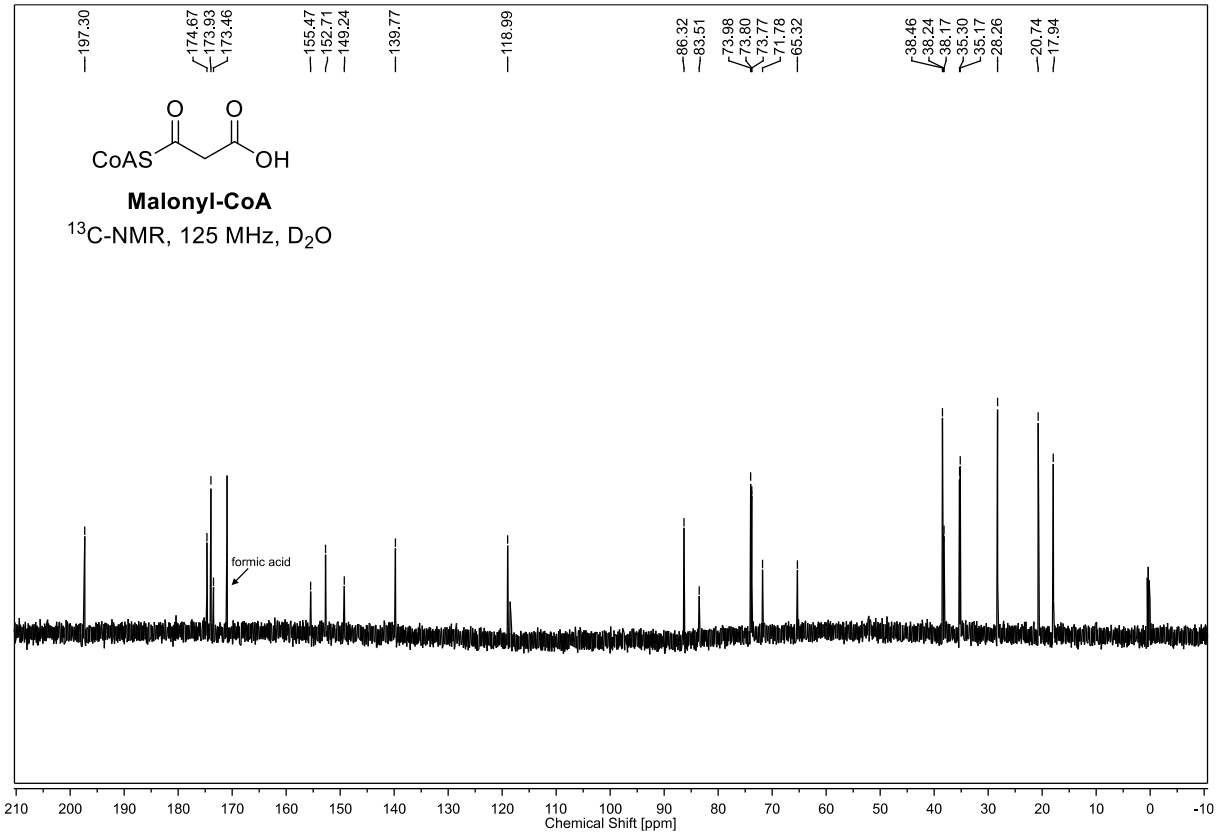
Supplementary Figure 90: ^{1}H NMR-spectrum of **17** in CD_3CN.



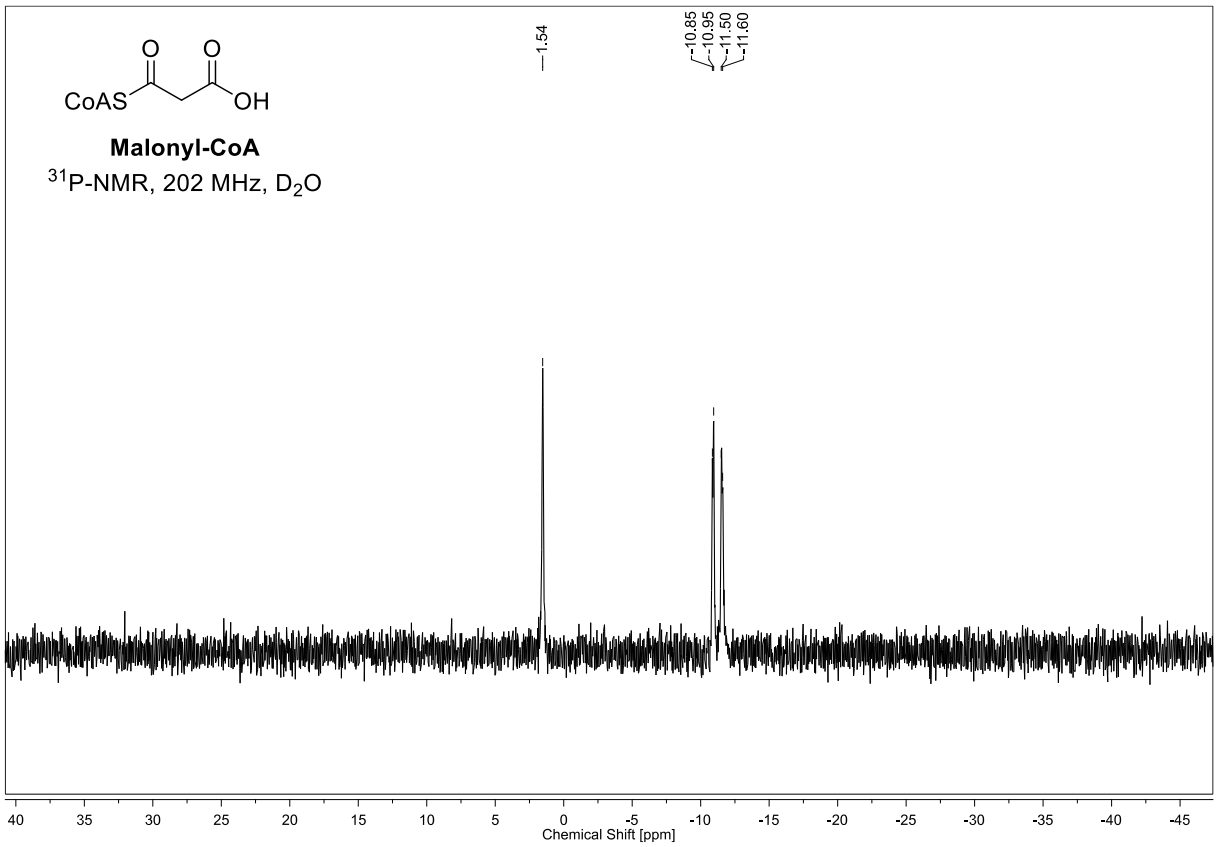
Supplementary Figure 91: ^{13}C NMR-spectrum of **17** in CD_3CN.



Supplementary Figure 92: ^{1}H NMR-spectrum of malonyl-CoA in D_2O.



Supplementary Figure 93: ^{13}C NMR-spectrum of malonyl-CoA in D_2O.



Supplementary Figure 94: ^{31}P-NMR-spectrum of malonyl-CoA in D_2O.

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