Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Mechanistic analysis of aliphatic β-lactones in *Vibrio harveyi* reveals a quorum sensing independent mode of action

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General: Reactions were carried out under argon in flame dried glassware. Solvents were used from commercial sources in p.a. grade and stored over molecular sieves. All reagents and chemicals purchased from commercial sources were used without further purification. Thin-layer chromatography was performed on silica gel 60 F_{254} plates from *Merck* and silica gel 60 (0.032–0.063 mm, *Merck*) was used for column chromatography. Compounds were visualized by UV detection or staining via $KMnO_4$ stain. ^{1}H and ^{13}C-NMR spectra were recorded with Bruker 250, Bruker Avance 360 and Avance 500 spectrometers. Chemical shifts (δ) were referenced to the residual proton and carbon signal of the deuterated solvent. Coupling constants (*J*) were reported in Hertz (Hz). High-resolution mass spectra were obtained on a Thermo Scientific LTQ-FT Ultra via electrospray ionization (ESI-MS) or atmospheric-pressure chemical ionization (APCI-MS). Bioluminescence assay was conducted with an infinite M200Pro plate reader (Tecan) with Corning® 96 well plates (clear flat bottom). For SDS gel electrophoresis, fluorescence was recorded in a Fujifilm LAS-4000 luminescent image analyzer with a Fujinon VRF43LMD3 lens and a 575DF20 filter. All proteome experiments were performed on Orbitrap Fusion™ Tribrid™ mass spectrometer or LTQ Orbitrap XL mass spectrometer.

1. Supplementary Figures

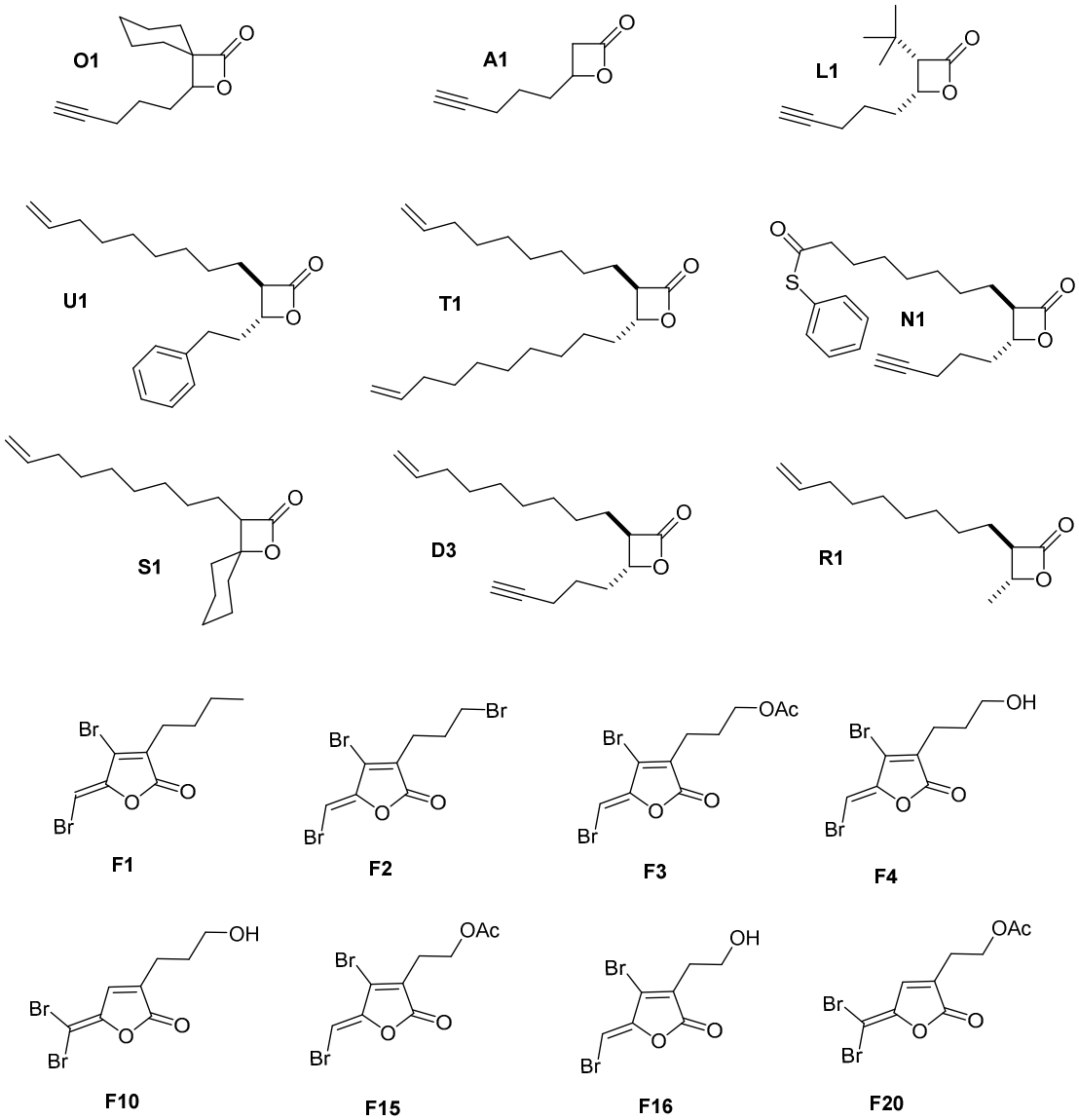


Figure S1: Structures of selected β-lactones and fimbrolide compounds.[1, 2]

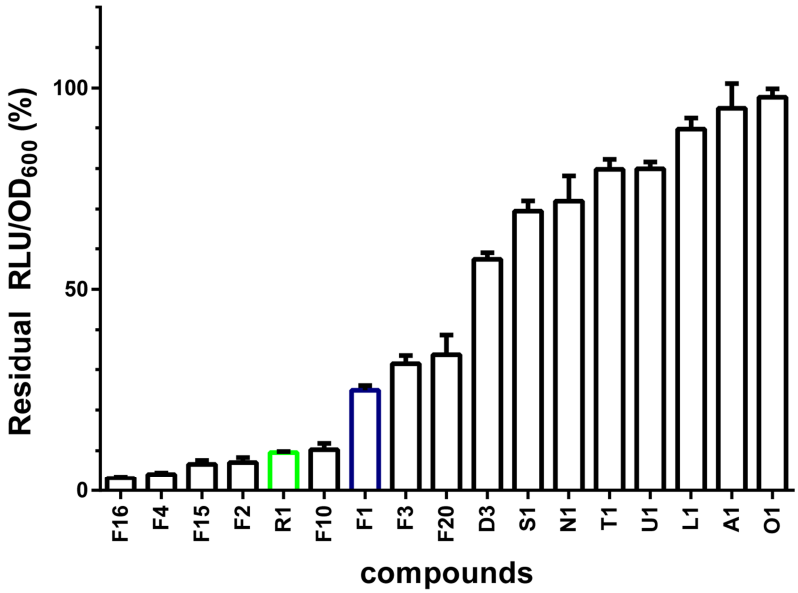


Figure S2: Residual bioluminescence production activity of *V. harveyi* NBRC 15634 cells after 30 min incubation with 50 µM selected β-lactones and fimbrolide compounds. Here the blue column signifies natural fimbrolide **F1** while the green column denotes **R1**. Relative Luminescence Units (RLU) were normalized to cell density (OD_{600}) and to the DMSO control. The data was based on three biological experiments with technical triplicates.

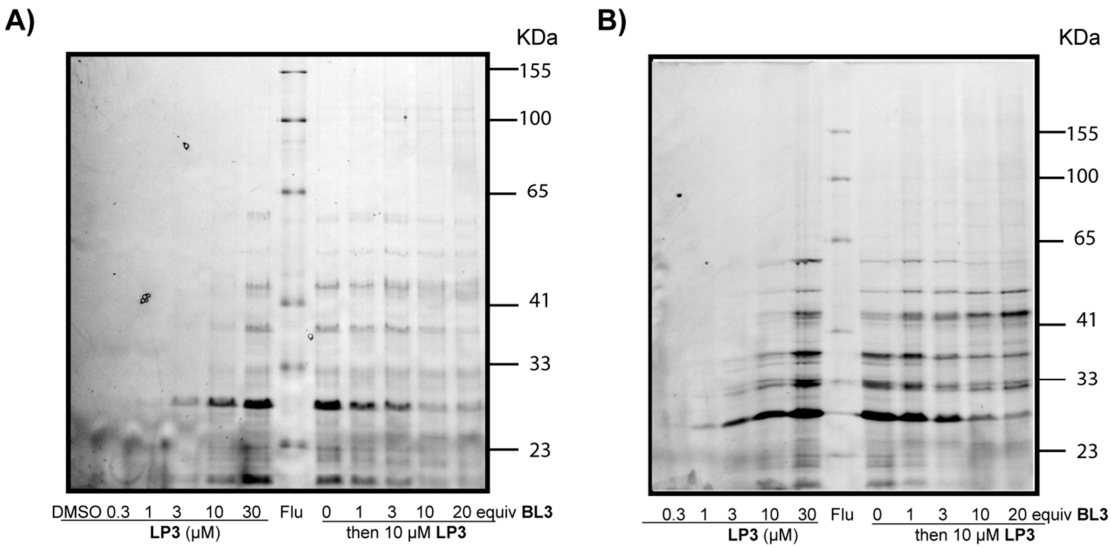


Figure S3: Fluorescent SDS-gel of *in situ* labeled *V. harveyi* BAA-1116 cells with **LP3** (various concentrations) and competitive *in situ* labeling of 10 µM **LP3** versus a various fold excess of **BL3**. A) Soluble fraction and B) insoluble fraction. DMSO denotes addition of DMSO instead of compounds. Flu = fluorescent marker.

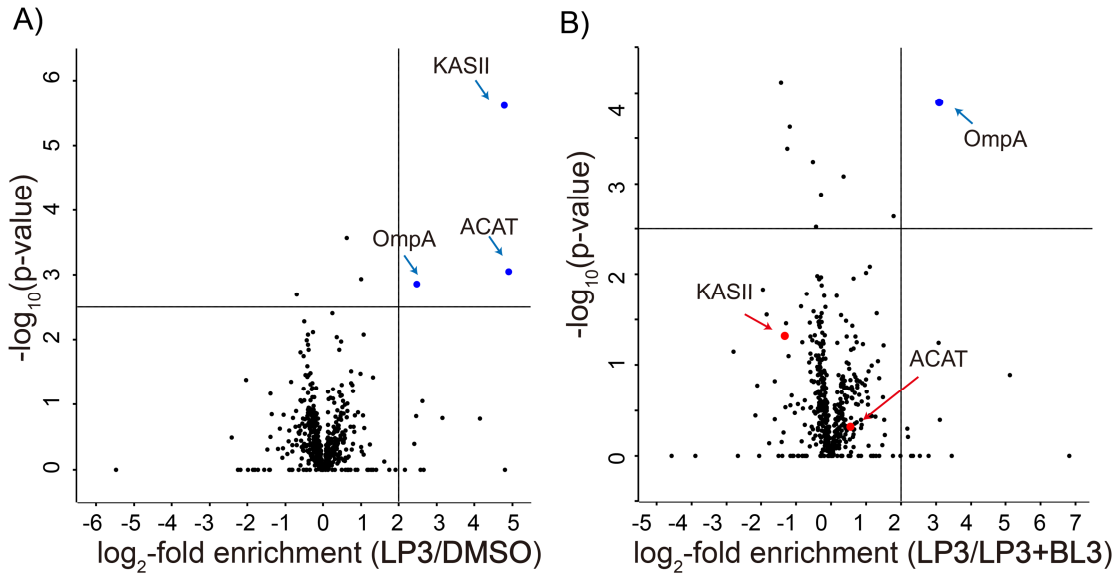


Figure S4: A) Volcano plot of gel-free quantitative ABPP experiments with 10 µM **LP3** vs. DMSO in *V. harveyi* BAA-1116 soluble fraction. Blue dots depict enriched targets (criteria: $\log_2$-fold enrichment ≥ 2 and $-\log_{10}$(p-value) ≥ 2.5). B) Volcano plot of gel-free competitive ABPP experiment in *V. harveyi* soluble fraction treated with 10 µM **LP3** vs. a 20-fold excess of **BL3** (**LP3**+**BL3**). Blue dots depict selected targets that are competed by **BL3** (criteria: $\log_2$-fold enrichment ≥ 2 and $-\log_{10}$(p-value) ≥ 2.5). The red dots denote those targets enriched by **LP3** but not competed by **BL3**. Both results were derived from three biological replicates with technical triplicates and $-\log_{10}$(p-value) were calculated using two sided one sample Student's t-test based on Z scores with Perseus.

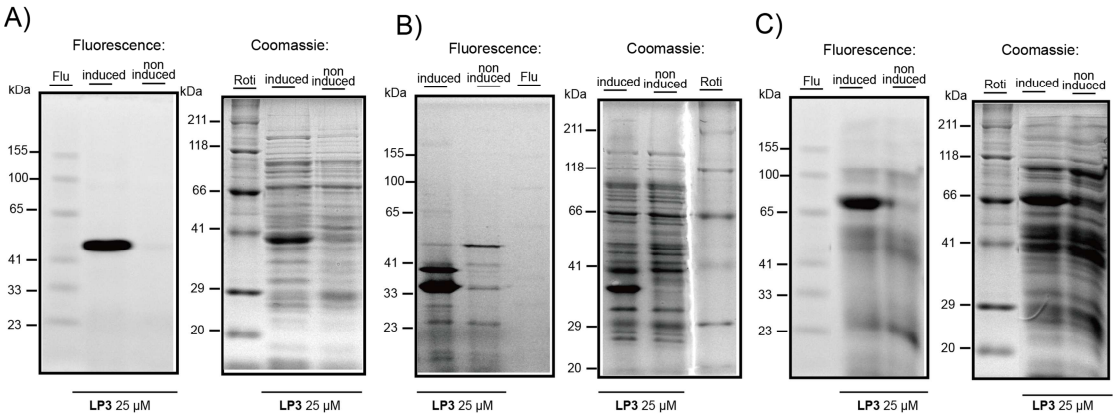


Figure S5: Analytical *in situ* labeling of recombinant protein targets expressed in *E. coli* with 25 µM **LP3** (A: ACAT; B: OmpA; C: DGC). Here induced denotes induction of protein overexpression. Flu = fluorescent marker. Roti denotes where Roti®-Mark is used.

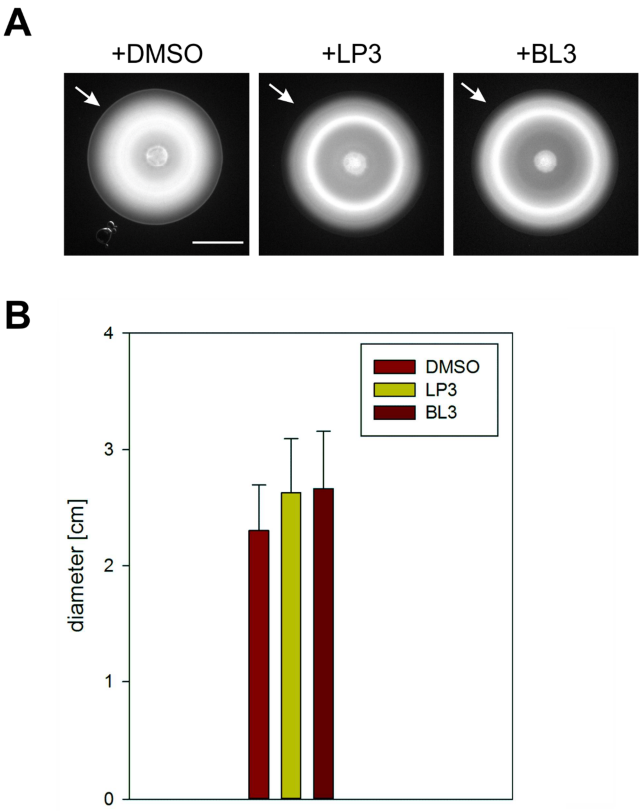


Figure S6: Effects of **LP3** and **BL3** supplementation to the plates on the motility and colony size of *V. harveyi* ATCC BAA-1116. A) Swimming motility on AB plates [0.3% (w/v) agar] supplemented with DMSO (left), 50 µM **LP3** (middle) or 50 µM **BL3** (right). Arrows indicate the differences in the outer ring formation (sharp vs. diffuse). The scale bar represents 1 cm. B) Diameter (cm) of the colony after growth of cells on AB plates [0.3% (w/v) agar] with DMSO/ 50 µM **LP3**/ 50 µM **BL3** and incubation at 30 °C. The experiment was performed in triplicate, and error bars indicate standard deviation of the mean.

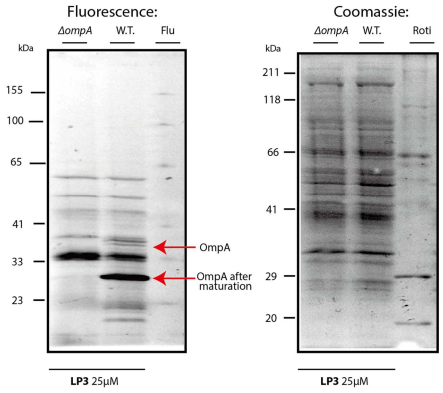


Figure S7: Comparison of analytical *in situ* labeling of 25 µM **LP3** in *V. harveyi* ATCC BAA-1116 *ΔompA* and the wild type. OmpA or its matured form was signified with the red arrow. Flu = fluorescent marker is. Roti denotes where Roti®-Mark is used. The absence of OmpA was also shown in Figure S12 in this mutant.

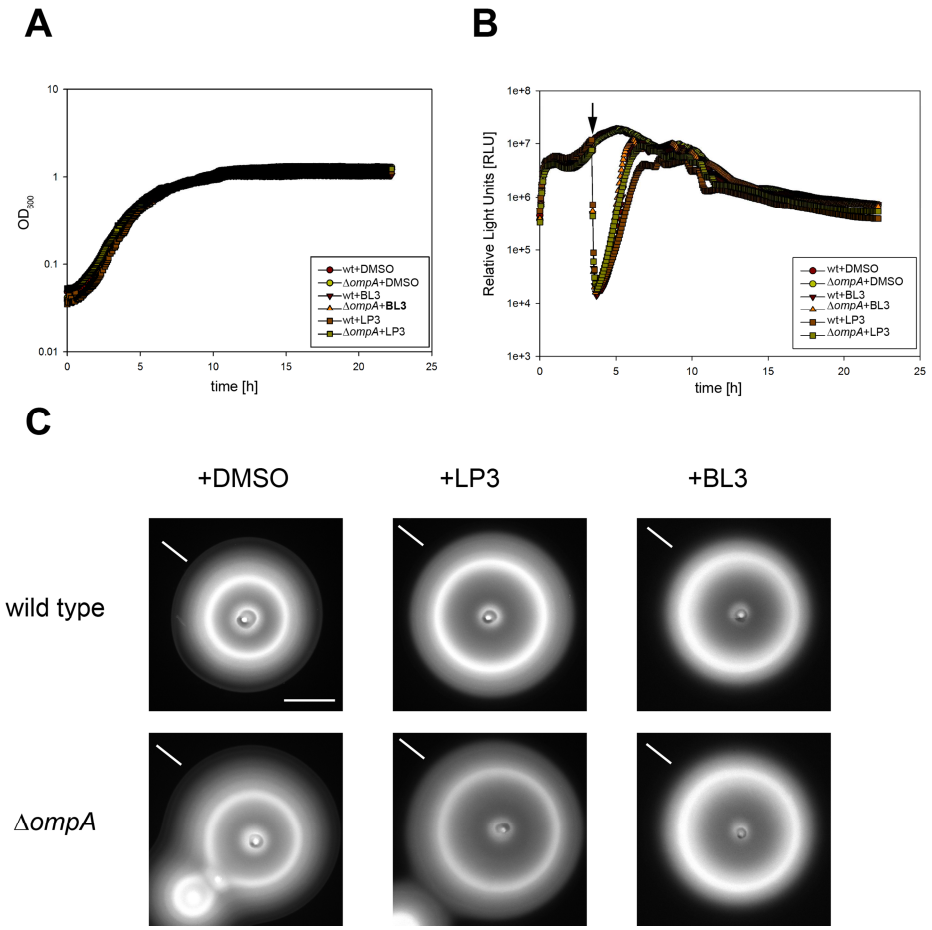


Figure S8: A) Growth curve of wild type (wt) *V. harveyi* ATCC BAA-1116 and the *ΔompA* mutant in AB medium. B) Inhibition of bioluminescence production of wild type *V. harveyi* ATCC BAA-1116 and the *ΔompA* mutant by **LP3** and **BL3**. Cells from an overnight culture were inoculated in fresh AB medium and grown aerobically at 30 °C in microtiter plates using a Tecan infinite F500 system. Bioluminescence and growth (OD_{600}) were recorded every 20 min. **LP3** and **BL3** were added in the exponential growth phase to a final concentration of 10 µM. As control the corresponding volume of DMSO was added to the cells. RLU, relative light units in counts per second per milliliter per OD_{600}. The Arrow indicates when β-lactones were added. C) Effects of **LP3** and **BL3** on swimming motility of wild type *V. harveyi* ATCC BAA-1116 and the *ΔompA* mutant. Swimming motility was analyzed after incubation of cells on AB plates [0.3% (w/v) agar] supplemented with DMSO, 50 µM **LP3** or 50 µM **BL3** at 30 °C for 20 h. Arrows indicate the differences in the outer ring formation (sharp vs. diffuse). Scale bar represents 1 cm. All experiments were performed at least three times, the standard deviation was less than 15%.

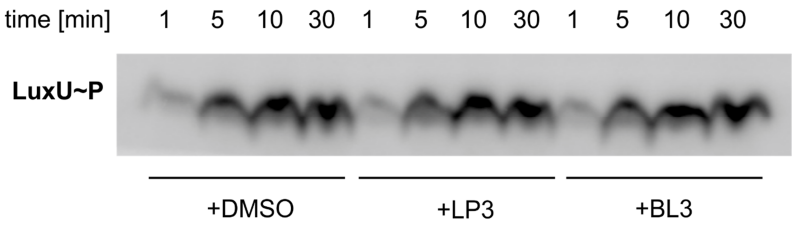


Figure S9: Effect of **LP3** and **BL3** on LuxN mediated phosphotransfer to the HPt protein LuxU *in vitro*. Inverted membrane vesicles containing the hybrid histidine kinase LuxN were mixed with LuxU, and the reaction was started with [γ-^{32}P] Mg^{2+}ATP. When indicated **LP3** or **BL3** (10 µM final concentration) or the corresponding volume of DMSO was added. At the indicated times (1, 5, 10 and 30 min) the phosphorylation reaction was stopped, proteins were separated by SDS-polyacrylamide gel electrophoresis followed by exposure to a phosphoscreen. Autoradiographs of LuxU parts of the gels are shown, which are representative for three independent experiments.

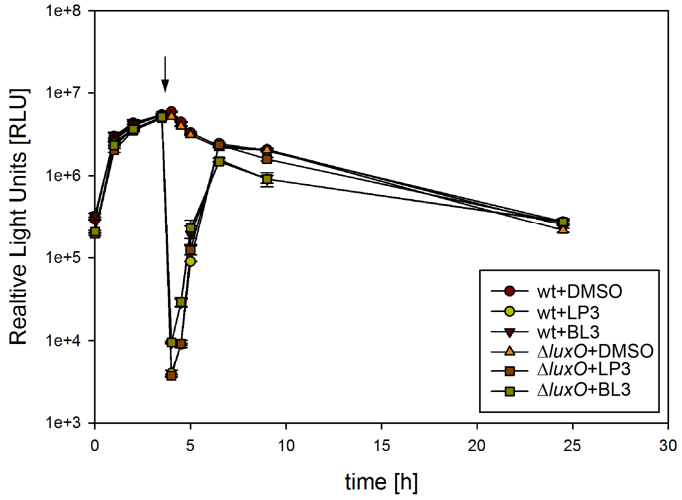


Figure S10: Effect of **LP3** and **BL3** on bioluminescence of wild type (wt) *V. harveyi* ATCC BAA-1116 and the *ΔluxO* mutant (constitutively QS-ON). Cells from an overnight culture were inoculated in fresh AB-medium and grown aerobically at 30 °C in microtiter plates using a Tecan infinite F500 system. Bioluminescence and growth (OD_{600}) were recorded every hour. **LP3** and **BL3** were added in the exponential growth phase to a final concentration of 10 µM. As a control, the corresponding volume of DMSO was added to the cells. RLU, relative light units in counts per second per milliliter per OD_{600}. The experiment was performed in triplicate, and error bars indicate standard deviation of the mean. The Arrow indicates when β-lactones were added.

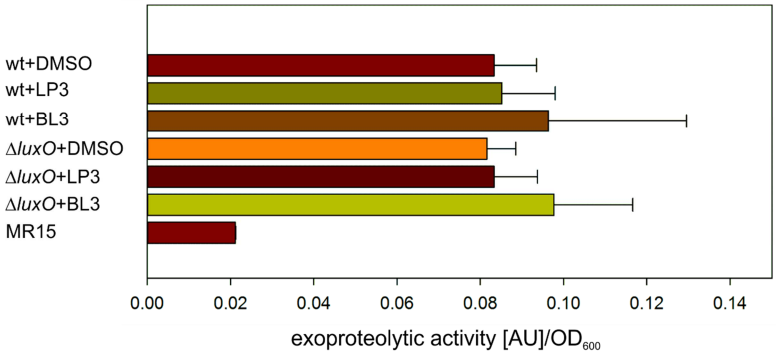


Figure S11: Influence of **LP3** and **BL3** on the exoproteolytic activity of wild type (wt) *V. harveyi* ATCC BAA-1116, the *ΔluxO* mutant (constitutively QS-ON) and the MR15 mutant (*ΔcqsA, ΔluxS, ΔluxM*, constitutively QS-OFF). Exoproteolytic activity was determined in cell-free culture fluids in all strains. When indicated 50 µM **LP3**, 50 µM **BL3** or the appropriate volume of DMSO was added prior to cultivation. Culture fluids were obtained from cells grown for 16 h. The experiment was performed in triplicate, and error bars indicate standard deviation of the mean.

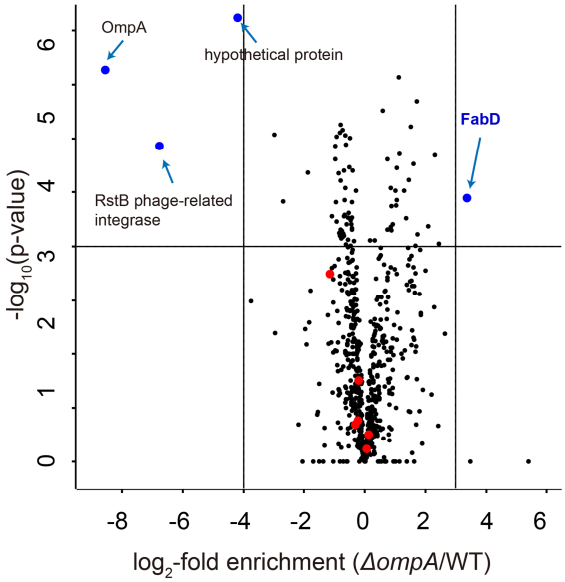
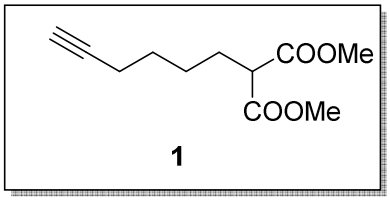


Figure S12: Volcano plot of whole proteome comparison between *V. harveyi ΔompA* mutant and the wild type. Blue dots depict selected targets that are significantly up-regulated or down-regulated (criteria: $\log_2$-fold enrichment ≥ 3 or ≤ - 4 and $-\log_{10}$(p-value) ≥ 3). Red dots represent proteins expressed from *luxCDABEG* operon which are essential for bioluminescence production in *V. harveyi*. WT denotes *V. harveyi* ATCC BAA-1116 wild type. The targets discussed in the text are highlighted in blue. Data was derived from three biological replicates with technical triplicates and $-\log_{10}$(p-value) were calculated using two sided one sample Student's t-test based on Z scores with Perseus.

2. Chemical Synthesis

dimethyl 2-(hex-5-yn-1-yl)malonate (1)



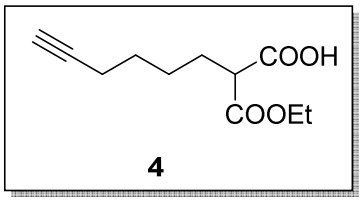
Dimethylmalonate (4.35 mL, 5.01 g, 38 mmol) was added dropwise to an ice cold suspension of NaH (720 mg, 30 mmol, 95% purity) in THF (85 mL) at 0 °C over 15 min under inert (Ar) atmosphere. The resulting slurry was stirred for 10 min at 0 °C and then 20 min at room temperature. After addition of 6-iodohex-1-yne (3.3 mL, 5.3 g, 25.5 mmol), the reaction mixture was refluxed for 3 hours and then cooled to room temperature. Brine (50 mL) was added and the resulting biphasic mixture was partitioned between Et_2O (50 mL) and H_2O (60 mL). The layers were separated and the aqueous layer was extracted three times with Et_2O (50 mL). The organic layer was combined and dried over Na_2SO_4. After removal of the organic solvent, the residue was purified by silica gel column chromatography (elution, hexane/EtOAc = 4/1) to give dimethyl 2-(hex-5-yn-1-yl)malonate (R_f = 0.60, 3.9 g, 73% yield).

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.70 (s, 6H), 3.33 (t, *J* = 7.5 Hz, 1H), 2.16 (td, *J* = 6.9, 2.6 Hz, 2H), 1.97 – 1.79 (m, 3H), 1.51 (m, *J* = 7.1 Hz, 2H), 1.46 – 1.32 (m, 2H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 169.70, 83.92, 68.53, 52.43, 51.47, 28.25, 27.93, 26.31, 18.07.

HRMS (pos. ESI) calcd for $C_{11}H_{16}O_4$ $(M+H)^+$: 213.1127, found 213.1124.

2-(ethoxycarbonyl)oct-7-ynoic acid (4)



KOH (615 mg, 11 mmol) in EtOH (5.8 mL) was added dropwise via syringe to dimethyl 2-(hex-5-yn-1-yl)malonate (1866 mg, 8.8 mmol) in ice bath under 0 °C over 50 min. The mixture was allowed to warm up to room temperature and stirred overnight for 18 hours. Then H_2O (40 mL) was added and the aqueous layer was acidified with HCl to pH 1.0. The mixture was extracted three times with AcOEt (40 mL) and the organic layer was combined. The combined organic layer was further washed with saturated brine (60 mL) and dried with Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (elution, DCM/MeOH = 20/3) to give 2-(ethoxycarbonyl) oct-7-ynoic acid (R_f = 0.67, 1.5 g, 82% yield).

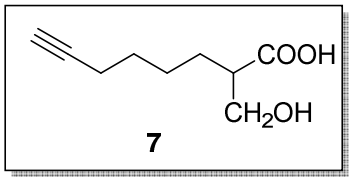
^{1}H NMR (300 MHz, $CDCl_3$): δ 11.01 (s, 1H), 4.22 (q, *J* = 7.0 Hz, 2H), 3.39 (s, 1H), 2.22 – 2.18 (m, 2H), 1.96 – 1.92 (m, 3H), 1.59 – 1.46 (m, 4H), 1.28 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 175.09, 170.37, 84.05, 68.58, 61.71, 52.05, 28.60, 27.98,

26.39, 18.12, 14.03.

HRMS (pos. ESI) calcd for $C_{11}H_{16}O_4$ $(M+H)^+$: 213.1127, found 213.1123.

2-(hydroxymethyl)oct-7-ynoic acid (7)



7

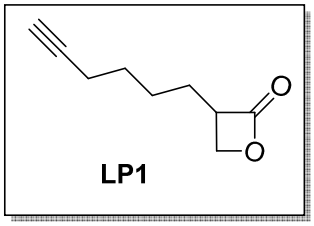
2-(ethoxycarbonyl)oct-7-ynoic acid (1050 mg, 5 mmol) was dissolved in dried i-PrOH (9 mL) and THF (4 mL) under inert (Ar) atmosphere. Then $LiBH_4$ (220 mg, 10 mmol) dissolved in THF (2 mL) was added dropwise under 0 °C. The reaction mixture was then further stirred 3 hours at room temperature. The reaction was quenched with aqueous 3 M HCl (3 mL) and then diluted with H_2O (50 mL). The aqueous layer was extracted three times with AcOEt (50 ml) and then the organic layer was combined. After dryness of the organic layer with Na_2SO_4, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (elution, DCM/MeOH = 20/3) to give 2-(hydroxymethyl)oct-7-ynoic acid (R_f = 0.35, 383 mg, 45% yield).

^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 3.75 (dd, *J* = 10.5, 7.3 Hz, 1H), 3.66 (dd, *J* = 10.5, 5.5 Hz, 1H), 2.57 – 2.48 (m, 1H), 2.33 (t, *J* = 2.7 Hz, 1H), 2.22 – 2.17 (m, 2H), 1.68 – 1.39 (m, 6H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 179.93, 84.23, 68.63, 62.93, 47.67, 28.23, 27.74, 26.27, 18.17.

HRMS (pos. ESI) calcd for $C_9H_{14}O_3$ $(M+H)^+$: 171.1021, found 171.1017.

3-(hex-5-yn-1-yl)oxetan-2-one (LP1)



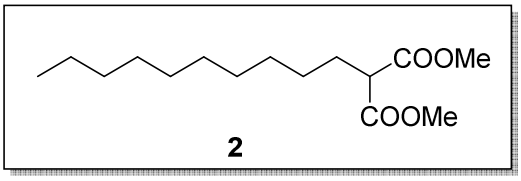
LP1

2-(hydroxymethyl)oct-7-ynoic acid (354 mg, 2.1 mmol) dissolved in DCM (5 mL) was added to a solution of HBTU (915 mg, 2.4 mmol) and $EtN(i\text{-}Pr)_2$ (968 mg, 1300 µL, 7.5 mmol) in DCM (25 mL). The mixture was stirred 5 hours at room temperature and then it was quenched by addition of saturated brine (25 mL). The reaction mixture was extracted three times with DCM (50 mL) and organic layer was combined. After dryness of the organic layer over Na_2SO_4, the organic solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (elution, hexane/AcOEt = 5/1) to give 3-(hex-5-yn-1-yl) oxetan-2-one (R_f = 0.48, 73 mg, 23% yield).

^{1}H NMR (300 MHz, $CDCl_3$): δ 4.31 (dd, *J* = 6.3, 5.2 Hz, 1H), 3.98 – 3.94 (m, 1H), 3.66 (dtd, *J* = 8.4, 6.5, 4.5 Hz, 1H), 2.16 (td, *J* = 6.7, 2.7 Hz, 2H), 1.89 (t, *J* = 2.7 Hz, 1H), 1.87 – 1.61 (m, 2H), 1.59 – 1.39 (m, 4H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 171.59, 83.81, 68.77, 64.95, 51.96, 27.91, 27.64, 25.85, 18.15.

dimethyl 2-decylmalonate (2)



2

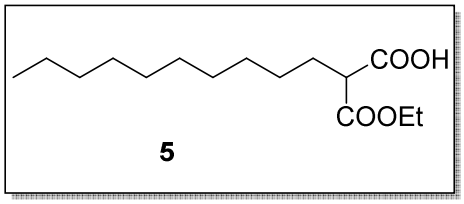
Dimethylmalonate (4.35mL, 5.01g, 38 mmol) was added dropwise to an ice cold suspension of NaH (720 mg, 30 mmol, 95% purity) in THF (85 mL) at 0 °C over 15 min under inert (Ar) atmosphere. The resulting slurry was stirred for 10 min at 0 °C and then 20 min at room temperature. After addition of 1-iododecane (6.8 g, 25.5 mmol), the reaction mixture was refluxed for 3 hours and then cooled to room temperature. Brine (50 mL) was added and the resulting biphasic mixture was partitioned between Et_2O (50 mL) and H_2O (60 mL). The layers were separated and the aqueous layer was extracted three times with Et_2O (50 mL). The organic layer was combined and dried over Na_2SO_4. After removal of the organic solvent, the residue was purified by silica gel column chromatography (elution, DCM) to give dimethyl 2-decylmalonate (R_f = 0.60, 6.3 g, 91% yield).

^{1}H NMR (360 MHz, $CDCl_3$): δ 3.67 (s, 6H), 3.29 (t, *J* = 7.6 Hz, 1H), 1.86 – 1.79 (m, 2H), 1.53 -1.50 (m, 2H), 1.20 (m, 14H), 0.81 (t, *J* = 6.6 Hz, 3H).

^{13}C NMR (63 MHz, $CDCl_3$): δ 169.94, 52.36, 51.70, 31.85, 29.49 ($2CH_2$), 29.25 ($2CH_2$), 29.14, 28.82, 27.30, 22.62, 14.04.

HRMS (pos. ESI) calcd for $C_{15}H_{28}O_4$ $(M+H)^+$: 273.2066, found 273.2061.

2-(ethoxycarbonyl)dodecanoic acid (5)



5

KOH (1.6 g, 28.7 mmol) in EtOH (16 mL) was added dropwise via syringe to dimethyl 2-decylmalonate (6.0 g, 22 mmol) in ice bath under 0 °C over 50 min. The mixture was allowed to warm up to room temperature and stirred overnight for 18 hours. Then H_2O (50 mL) was added and the aqueous layer was acidified with HCl to pH 1.0. The mixture was extracted three times with AcOEt (50 mL) and the organic layer was combined. The combined organic layer was further washed with saturated brine (50 mL) and dried with Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (elution, DCM/MeOH = 20/3) to give 2-(ethoxycarbonyl)dodecanoic acid (R_f = 0.52, 5.1 g, 85% yield).

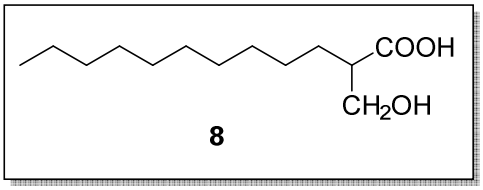
^{1}H NMR (360 MHz, $CDCl_3$) δ 9.03 (s, 1H), 4.26 – 4.11 (m, 2H), 3.38 (t, *J* = 7.4 Hz, 1H), 2.03 – 1.82 (m, 2H), 1.37 – 1.20 (m, 19H), 0.89 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (91 MHz, $CDCl_3$): δ 175.33, 169.43, 61.63, 51.74, 31.86, 29.52, 29.48, 29.27,

29.25, 29.15, 28.85, 27.23, 22.64, 14.05, 14.00.

HRMS (pos. ESI) calcd for $C_{15}H_{28}O_4$ $(M+H)^+$: 273.2066, found 273.2061.

2-(hydroxymethyl)dodecanoic acid (8)

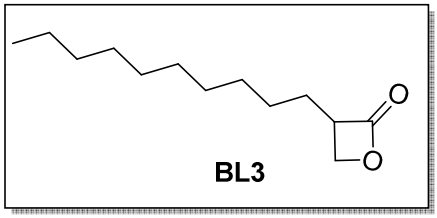


8

2-(ethoxycarbonyl)dodecanoic acid (1360 mg, 5 mmol) was dissolved in dried i-PrOH (9 mL) and THF (4 mL) under inert (Ar) atmosphere. Then $LiBH_4$ (220 mg, 10 mmol) dissolved in THF (2 mL) was added dropwise under 0 °C. The reaction mixture was then further stirred 3 hours at room temperature. Then it was quenched with aqueous 3 M HCl (3 mL) and diluted with H_2O (50 mL). The reaction mixture was extracted three times with AcOEt (50 ml) and then the organic layer was combined. After dryness of the organic layer with Na_2SO_4, the residue was purified by silica gel column chromatography (elution, DCM/MeOH = 25/1) to give 2-(hydroxymethyl)dodecanoic acid (R_f = 0.55, 298 mg, 26% yield).

HRMS (pos. ESI) calcd for $C_{13}H_{26}O_3$ $(M+H)^+$: 231.1960, found 231.1957.

3-decyloxetan-2-one (BL3)



BL3

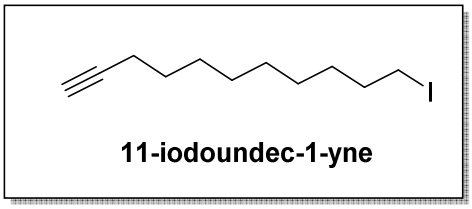
2-(hydroxymethyl)dodecanoic acid (230 mg, 1 mmol) dissolved in DCM (2.5 mL) was added to a solution of HBTU (458 mg, 1,2 mmol) and $EtN(i\text{-}Pr)_2$ (484 mg, 650 µL, 3.75 mmol) in DCM (12 mL). The mixture was stirred 5 hours at room temperature and then it was quenched by addition of saturated brine (12 mL). The aqueous fraction was extracted three times with DCM (25 mL) and organic layer was combined. After dryness of the organic layer over Na_2SO_4, the organic solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (elution, hexane/AcOEt = 5/1) to give 3-(hex-5-yn-1-yl) oxetan-2-one (R_f = 0.35, 34 mg, 16% yield).

^{1}H NMR (360 MHz, $CDCl_3$): δ 4.37 (dd, *J* = 6.3, 5.1 Hz, 1H), 4.08 – 3.98 (m, 1H), 3.72 (dtd, *J* = 8.5, 6.5, 4.5 Hz, 1H), 1.95 – 1.70 (m, 2H), 1.29 (d, *J* = 7.9 Hz, 16H), 0.93 – 0.88 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (91 MHz, $CDCl_3$): δ 171.73, 64.97, 52.15, 31.86, 29.53, 29.48, 29.30, 29.26, 29.21, 28.12, 26.80, 22.64, 14.06.

HRMS (pos. ESI) calcd for $C_{13}H_{24}O_2$ $(M+Na)^+$: 235.1674, found 235.1687.

11-iodoundec-1-yne



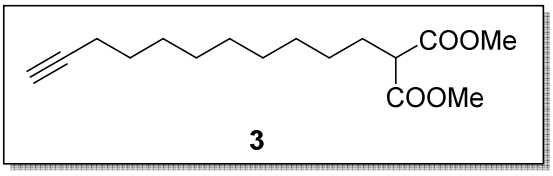
11-iodoundec-1-yne

To a solution of imidazole (5.2 g, 76 mmol) and triphenylphosphane (12 g, 46 mmol) in dried DCM (120 mL) was added I_2 (11.7 g, 46 mmol) in portions at 0 °C over the course of one hour. The reaction mixture was then stirred at room temperature for 1 hour after addition of undec-10-yn-1-ol (5.1 g, 30.5 mmol). The reaction was quenched by addition of saturated $Na_2S_2O_3$ aqueous solution (120 mL). The reaction mixture was diluted with DCM (200 mL), washed three times with saturated brine (100 mL). Then the organic layer was dried with Na_2SO_4. After removal of solvents, the product was purified by silica gel column chromatography (elution, hexane) to give 11-iodoundec-1-yne (R_f = 0.25, 4.6 g, 54% yield).

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.21 (t, *J* = 7.0 Hz, 2H), 2.20 (td, *J* = 7.0, 2.7 Hz, 2H), 1.96 (t, *J* = 2.6 Hz, 1H), 1.84 (m, 2H), 1.65 – 1.47 (m, 2H), 1.46 – 1.25 (m, 10H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 84.74, 68.12, 33.54, 30.48, 29.27, 29.00, 28.69, 28.49, 28.45, 18.40, 7.37.

dimethyl 2-(undec-10-yn-1-yl)malonate (3)



3

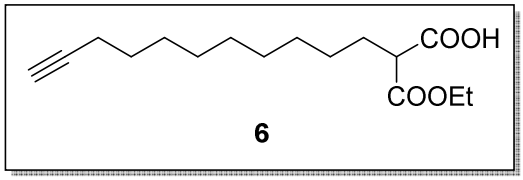
Dimethylmalonate (2.17 mL, 2.5 g, 19 mmol) was added dropwise to an ice cold suspension of NaH (360 mg, 15 mmol, 95% purity) in THF (40 mL) at 0 °C over 15 min under inert (Ar) atmosphere. The resulting slurry was stirred for 10 min at 0° C and then 20 min at room temperature. After addition of 11-iodoundec-1-yne (3.6 g, 13 mmol), the reaction mixture was refluxed for 3 hours and then cooled to room temperature. Brine (60 mL) was added and the resulting biphasic mixture was partitioned between Et_2O (50 mL) and H_2O (50 mL). The layers were separated and the aqueous layer was extracted three times with Et_2O (50 mL). The organic layer was combined and dried over Na_2SO_4. After removal of the organic solvent, the residue was purified by silica gel column chromatography (elution, hexane/AcOEt = 4/1) to give dimethyl 2-(undec-10-yn-1-yl)malonate (R_f = 0.45, 2.3 g, 64% yield).

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.75 (s, 6H), 3.37 (t, *J* = 7.6 Hz, 1H), 2.19 (td, *J* = 7.0, 2.7 Hz, 2H), 1.95 (t, *J* = 2.7 Hz, 1H), 1.93 – 1.83 (m, 2H), 1.60 – 1.46 (m, 2H), 1.42 – 1.24 (m, 12H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 169.97, 84.75, 68.08, 52.45, 51.70, 29.32, 29.22, 29.15, 29.02, 28.84, 28.70, 28.45, 27.31, 18.38.

HRMS (pos. ESI) calcd for $C_{16}H_{26}O_4$ $(M+H)^+$: 283.1909, found 283.1909.

2-(ethoxycarbonyl)tridec-12-ynoic acid (6)



6

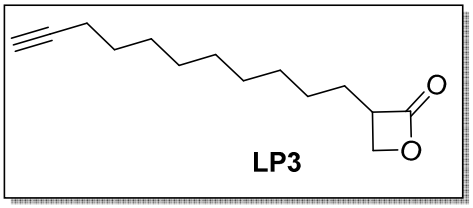
KOH (307 mg, 5.5 mmol) in EtOH (3 mL) was added dropwise via syringe to dimethyl 2-(undec-10-yn-1-yl)malonate (1240 mg, 4.4 mmol) in ice bath under 0 °C over 50 min. The mixture was allowed to warm up to room temperature and stirred overnight for 18 hours. Then H_2O (30 mL) was added and the aqueous layer was acidified with HCl to pH 1.0. The mixture was extracted three times with AcOEt (40 mL) and the organic layer was combined. The combined organic layer was further washed with saturated brine (40 mL) and dried with Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (elution, DCM/MeOH = 10/1) to give 2-(ethoxycarbonyl)tridec-12-ynoic acid (R_f = 0.45, 682 mg, 55% yield).

^{1}H NMR (300 MHz, $CDCl_3$): δ 4.23 (q, *J* = 7.0 Hz, 2H), 3.34 (t, *J* = 7.0 Hz, 1H), 2.20 (td, *J* = 7.0, 2.6 Hz, 2H), 1.96 (t, *J* = 2.7 Hz, 1H), 1.91 – 1.89 (m, 2H), 1.60 – 1.47 (m, 2H), 1.46 – 1.22 (m, 15H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 175.58, 170.89, 84.64, 68.11, 61.53, 52.28, 29.40, 29.30 ($2CH_2$), 29.23, 29.05, 28.71, 28.45, 27.40, 18.36, 14.03.

HRMS (pos. ESI) calcd for $C_{16}H_{26}O_4$ $(M+H)^+$: 283.1909, found 283.1905.

3-(undec-10-yn-1-yl)oxetan-2-one (LP3)



LP3

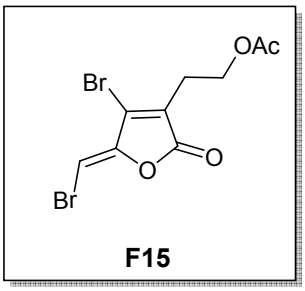
2-(ethoxycarbonyl)tridec-12-ynoic acid (564 mg, 2 mmol) was dissolved in dried i-PrOH (4.5 mL) and THF (2 mL) under inert (Ar) atmosphere. Then $LiBH_4$ (88 mg, 4 mmol) dissolved in THF (1 mL) was added dropwise under 0 °C. The reaction mixture was then further stirred 3 hours at room temperature. Afterwards, the reaction was quenched with aqueous 3 M HCl (1.5 mL) and diluted with H_2O (30 mL). The aqueous layer was extracted three times with AcOEt (40 ml) and then the organic layer was combined. After dryness of the organic layer with Na_2SO_4, the residue was used for the next step without further purification.

The residue suspended in DCM (5 mL) was added to a solution of HBTU (916 mg, 2.4 mmol) and $EtN(i\text{-}Pr)_2$ (968 mg, 1300 µL, 7.5 mmol) in DCM (25 mL). The mixture was stirred 5 hours at room temperature and then it was quenched by addition of saturated brine (30 mL). The reaction mixture was extracted three times with DCM (50 mL) and organic layer was combined. After dryness of the organic layer over Na_2SO_4, the organic solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (elution, hexane/AcOEt = 6/1) to give

3-(undec-10-yn-1-yl)oxetan-2-one (R_f = 0.4, 48.8 mg, 11% yield over two steps).
^{1}H NMR (300 MHz, $CDCl_3$): δ 4.44 – 4.32 (m, 1H), 4.05 – 4.01 (m, 1H), 3.83 – 3.63 (m, 1H), 2.23 – 2.17 (m, 2H), 1.96 (t, *J* = 2.5 Hz, 1H), 1.92 – 1.72 (m, 2H), 1.53 – 1.28 (m, 14H).
^{13}C NMR (75 MHz, $CDCl_3$): δ 171.85, 84.75, 68.10, 65.03, 52.11, 29.34, 29.27, 29.21, 29.03, 28.69, 28.45, 28.12, 26.80, 18.39.

HRMS (pos. APCI) calcd for $C_{14}H_{22}O_2$ $(M+H)^+$: 223.1698, found 223.1692.

(Z)-2-(4-bromo-5-(bromomethylene)-2-oxo-2,5-dihydrofuran-3-yl)ethyl acetate (F15)

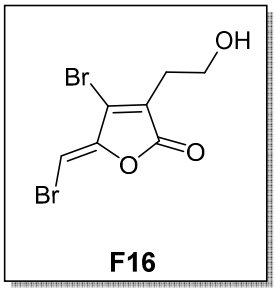


F15

F15 was synthesized according to a similar synthetic route published before with α-Bromo-γ-butyrolactone as the starting material.[2] The residue was purified by silica gel column chromatography (elution, pure DCM) to give (Z)-2-(4-bromo-5-(bromomethylene) -2-oxo-2,5-dihydrofuran-3-yl)ethyl acetate (R_f = 0.4) as a light yellow solid.
^{1}H NMR (360 MHz, $CDCl_3$) δ 6.35 (s, 1H), 4.30 (t, *J* = 6.2 Hz, 2H), 2.76 (t, *J* = 6.2 Hz, 2H), 2.04 (s, 3H).
^{13}C NMR (91 MHz, $CDCl_3$) δ 170.71, 165.77, 149.95, 132.16, 130.13, 92.26, 60.55, 25.19, 20.86.

HRMS (pos. ESI) calcd for $C_9H_8Br_2O_4$ $(M+H)^+$: 340.8847, found 340.8838.

(Z)-4-bromo-5-(bromomethylene)-3-(2-hydroxyethyl)furan-2(5H)-one (F16)



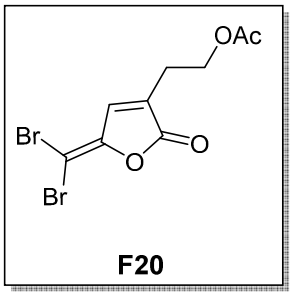
F16

F16 was synthesized according to a similar synthetic route published before with α-Bromo-γ-butyrolactone as the starting material.[2] The residue was purified by silica gel column chromatography (elution, hexane/AcOEt = 1/1) to give (Z)-4-bromo-5-(bromomethylene)-3-(2-hydroxyethyl)furan-2(5H)-one (R_f = 0.7) as a light yellow oil.
^{1}H NMR (360 MHz, $CDCl_3$) δ 6.35 (s, 1H), 3.89 (t, *J* = 6.2 Hz, 2H), 2.69 (t, *J* = 6.2 Hz, 2H).

^{13}C NMR (91 MHz, $CDCl_3$) δ 166.57, 150.08, 132.11, 130.95, 92.09, 59.48, 29.05.

HRMS (pos. ESI) calcd for $C_7H_6Br_2O_3$ $(M+H)^+$: 298.8741, found 298.8733.

2-(5-(dibromomethylene)-2-oxo-2,5-dihydrofuran-3-yl)ethyl acetate (F20)



F20

F20 was synthesized according to a similar synthetic route published before with α-Bromo-γ-butyrolactone as the starting material.[2] The residue was purified by silica gel column chromatography (elution, pure DCM) to give (Z)-2-(4-bromo-5-(bromomethylene) -2-oxo-2,5-dihydrofuran-3-yl)ethyl acetate (R_f = 0.45) as a light yellow solid.

^{1}H NMR (360 MHz, $CDCl_3$) δ 7.42 (s, 1H), 4.32 (t, *J* = 6.3 Hz, 2H), 2.72 (t, *J* = 6.3 Hz, 2H), 2.08 (s, 3H).

^{13}C NMR (91 MHz, $CDCl_3$) δ 170.75, 168.29, 149.55, 135.66, 133.64, 80.32, 61.05, 25.36, 20.86.

HRMS (pos. ESI) calcd for $C_9H_8Br_2O_4$ $(M+H)^+$: 340.8847, found 340.8839.

3. Biochemical Procedures

3.1 Bacterial strains and media

Vibrio haveryi NBRC 15634 was purchased from *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* and cultivated in BACTO Marine Broth at 28 °C with agitation (DIFCO 2216, for per liter: Bacto peptone 5.00 g, Bacto yeast extract 1.00 g, Fe(III) citrate 0.10 g ,NaCl 19.45 g, $MgCl_2$ (dried) 5.90 g, Na_2SO_4 3.24 g, $CaCl_2$ 1.80 g, KCl 0.55 g, Na_2CO_3 0.16 g, KBr 0.08 g, $SrCl_2$ 34.00 mg, H_3BO_3 22.00 mg, Na-silicate 4.00 mg, NaF 2.40 mg, $(NH_4)NO_3$ 1.60 mg, Na_2HPO_4 8.00 mg, Agar if required 15.0-18.0 g. Final pH should be 7.6 ± 0.2 at 25 °C. If using the complete medium from Difco, 37.4 g add to 1 L water.). *Vibrio harveyi* ATCC BAA-1116 and *V. harveyi* ATCC BAA-1116 *ΔompA* were generally cultivated in autoinducer bioassay (AB) medium at 30 °C with agitation. AB medium contained 17.5 g NaCl, 12.3 g $MgSO_4$, 2.0 g casamino acids, 970 mL distilled water. The pH was adjusted to 7.5 with 3 N NaOH. Fresh AB medium was made before its use by addition of 1 mL 1M potassium phosphate (pH 7.0), 1 mL 0.1 M L-arginine and 1 mL glycerol to 100 mL AB medium.
The recombinant proteins were expressed in different *Escherichia coli* strains but all of them were cultivated in lysogeny broth (LB) (10.0 g peptone ex casein, 5.00 g NaCl, 5.00 g yeast extract in 1 L distilled water, pH 7.5). The Super Optimal broth with Catabolite repression (SOC) was prepared for transformation experiments as follows: 20.0 g yeast extract, 5.00 g tryptone, 0.50 g NaCl, 0.20 g KCl, 1.00 g $MgCl_2$, 1.20 g $MgSO_4$, 3.60 g glucose in 1 L distilled water, pH 7.3.

Overnight cultures
5 mL of the particular medium for cultivation were inoculated with 5 µL of the desired bacterial cryostock (1:1,000) with a sterile pipette tip in a plastic culture tube. The culture was then incubated overnight (16 h, 200 rpm) in an Innova incubator shaker at desired degree. Overnight cultures were always prepared freshly to avoid genetic variations. A sterile control (medium containing no bacteria) was added each time.

Cryostocks
1 mL of an overnight culture of the desired bacteria were harvested by centrifugation (10 min, 4 °C, 6,000 rpm) and the pelletized cells were resuspended in 250 µL fresh, sterile medium. 250 µL of sterilized glycerin were added, the stocks were mixed, frozen in liquid nitrogen and stored at - 80 °C in 20 µL aliquots prior to use. After inoculating fresh media with the aliquot, the leftover amount of the cryostock was discarded.

Strain construction
Molecular methods were carried out according to standard protocols[3] or according to the manufacturer's instructions. Kits for the isolation of plasmids and purification of PCR products were purchased from Suedlabor (Gauting, Germany). Enzymes were purchased from New England BioLabs (Frankfurt, Germany) and Fermentas (St. Leon-Rot,

Germany). In frame deletion mutant of *Vibrio harveyi* ATCC-BAA 1116 were constructed as previously described, leaving terminal sections of the target gene.[4-6] For this purpose, upstream and downstream fragments (1,000 bp) of *ompA* gene region was amplified by using the corresponding primers (available upon request). After PCR product purification, the fragments were fused by overlap PCR.[7] The overlap product was isolated from an agarose gel, digested with the corresponding restriction enzymes, and ligated into the suicide vector pNPTS138-R6KT.[4] The resulting plasmid was introduced into *V. harveyi* ATCC-BAA 1116 by conjugative mating using *E. coli* WM3064 as the donor in LB medium containing 2,6-diaminopimelic acid (DAP). Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking DAP. Single colonies were grown over a day without antibiotics and plated onto LB plates containing 10% (w/v) sucrose to select for plasmid excision. Kanamycin-sensitive colonies were checked for targeted deletion by colony PCR using primers bracketing the site of the deletion.

3.2 Activity based protein profiling experiments

Bacterial strains were cultivated under defined growth conditions to stationary phase otherwise specified and OD_{600} was measured on Novaspec Plus visible spectrophotometer. Cultures were collected in a 50 mL falcon tube, pelletized at 6,000 rpm for 5 min at 4 °C and washed with PBS. The pellet was then resuspended in PBS to a final OD_{600} = 40.

3.2.1 Analytical gel-based ABPP

100 µL aliquots of bacterial suspension were treated with various concentrations of compounds, probes or DMSO at RT for 1 h (for the competitive experiments: the suspension was first treated with the parent compound used for competition or DMSO for 1 hour at RT and then treated sequentially with the corresponding probe at RT for another hour). After incubation, the samples were pelletized and washed twice with 1 mL PBS to remove the residual compounds or probes. The pellet was resuspended in 120 µL PBS and then lysed by sonication with a Bandelin Sonopuls with 3 x 15 sec. pulsed at 70% max. power on ice. After that, the samples were pelletized (13,000 rpm, 4 °C, 30 min). 88 µL supernatant was transferred to a new Eppendorf tube while the pellet was washed twice with 500 µL PBS and resuspended in 88 µL PBS. Additional 20% sodium dodecyl sulfate (SDS) in PBS to a final concentration of 0.8% to denature the proteins. The samples were then used to append a reporter tag via click chemistry (CC). For each 88 µL sample, add 2 µL RhN_3 (5 mM rhodamine-azide in DMSO), 2 µL freshly made TCEP (52 mM tris(2-carboxyethyl)phosphine in dd H_2O), 6 µL TBTA ligand (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, 1.667 mM in t-BuOH/DMSO = 4:1). Samples were gently vortexed and 2 µL $CuSO_4$ (50 mM $CuSO_4$ in dd H_2O) was added to initiate the cycloaddition reaction. After the addition of CC reagents, the total reaction volume was 100 µL. The samples were incubated at RT for 1 h and then 100 µL 2× SDS

loading buffer were added. The samples were mixed and stored at – 20 °C after 1 h incubation at RT. 50 µL of the samples was applied on the analytical gel and 10 µL fluorescent marker was used as ladder in parallel (BenchMark Flourescent protein standard was diluted with 4-fold ddH_2O and 5 fold 2× SDS loading buffer). Fluorescence was recorded in a Fujifilm Las-3000 Fluoreszenz Darkbox with a Fujinon VRF 43LMD Lens, 605DF40 filter and 520 nm EPI excitation wavelength.

3.2.2 Gel free ABPP and dimethyl labeling

500 µL bacteria suspension were incubated first with **BL3** (final conc. = 200 µM) for competition or with DMSO for enrichment at RT for 1 h and then incubated sequentially with the corresponding probe **LP3** (final conc. = 10 µM) at RT for another 1 h. Also, one sample was consistently treated with DMSO as control.
Technical duplicates were performed with the same bacteria suspension in parallel while biological replicates were performed with bacteria harvested on different days. After incubation, the samples were pelletized and washed twice with 1 mL PBS to remove the residual probe. The pellet was resuspended in 500 µL PBS and then lysed by sonication with a Bandelin Sonopuls with 3 x 15 sec. pulsed at 70% max. power on ice. After centrifugation at 13,000 rpm at 4 °C for 30 min to separate the soluble and insoluble fractions, the insoluble fractions were resuspended in 500 µL PBS while the supernatant was transferred to a new Protein LoBind Eppendorf tube. 20% sodium dodecyl sulfate (SDS) in PBS was added into the samples to a final concentration of 0.8%. The samples were then used to append a reporter tag via CC. For each 500 µL sample, add 3 µL azide-PEG3-biotin conjugate (Jena Bioscience, bifunctional linker with biotin and azide groups; 10 mM in DMSO), 10 µL freshly made TCEP (52 mM tris(2-carboxyethyl)phosphine in ddH_2O), 30 µL TBTA ligand (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, 1.667 mM in t-BuOH/DMSO = 4:1). The cycloaddition was initiated by 10 µL $CuSO_4$ (50 mM $CuSO_4$ in dd H_2O) and incubated at RT for 1 h. After CC, proteins were precipitated with two fold volumes of pre-chilled (- 80 °C) acetone. The samples were stored at – 20 °C overnight to get sufficient protein precipitation. Then they were pelletized at 13,000 rpm for 30 min at 4 °C. The supernatant was discarded and the pellet was washed twice with pre-chilled (- 80 °C) methanol. Subsequently, the pellet was dissolved in 500 µL PBS with 0.4% (w/v) SDS by sonication at RT and the protein concentration was measured with BCA assay. All samples were adjusted to same protein amount accordingly. They were incubated under gentle mixing with 50 µL of avidin-agarose beads from Sigma-Aldrich (avidin-agarose beads were pre-washed with 1 mL PBS with 0.4% (w/v) SDS three times) overnight at RT. After that, the beads were washed three times with 3 mL of 0.4% (w/v) SDS in PBS, twice with 1 mL of 6 M urea and three times with 1 mL PBS (collect at 1,500 rpm, 2 min, RT after each washing step). The beads were resuspended with 200 µl denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer). Dithiothreitol (DTT, 100 mM, 2 µL) was added and the tubes were mixed by vortexing shortly and incubated in a thermomixer (450 rpm, 45 min, RT). Then 2-Iodoacetamide (IAA, 550 mM, 2 µL) was added. The tubes were mixed again by vortexing shortly and incubated in the darkness (450 rpm, 30 min, RT).

The excess of IAA was quenched by the addition of dithiothreitol (DTT, 100 mM, 8 µL). The tubes were shortly mixed by vortexing and incubated in a thermomixer (450 rpm, 30 min, RT). 1 µL LysC (0.5 µg/µL) which was thawed on ice was added to each microcentrifuge tube. The tubes were mixed by short vortexing and incubated in a thermomixer (450 rpm, 4 h, RT, in the darkness). 600 µL TEAB solution (tetraethylammonium bromide, 50 mM in water) and 1.5 µL typsin (0.5 µg/µL in 50 mM acetic acid) were added to the tubes. The microcentrifuge tubes were incubated in a thermomixer overnight (450 rpm, 13 -15 h, 37 °C).

The digestion was stopped by adding 6 µL formic acid (FA) and the solution was collected by centrifugation (3,000 rpm, 2 min, RT). The trypsin digest solution was transferred to a new Protein LoBind Eppendorf tube. Further extraction was done with 50 µL 0.1% (v/v) FA (aqueous solution) twice (3,000 rpm, 2 min, RT). Finally, the tubes were centrifuged for 3 min at 13,000 rpm to collect the residual supernatant. All the supernatant was combined to the trypsin digest solution. The pH values of the samples were checked and more 1% (v/v) FA was added if the pH was above 3. 50 mg SepPak C18 columns were pre-equilibrated by gravity flow with 1 mL acetonitrile, 0.5 mL elution buffer (80% ACN, 0.5%FA) and 1 mL 0.1% TFA aqueous solution. Subsequently the samples were loaded and washed with 500 µL 0.1% TFA aqueous solution twice, 500 µL 0.5% FA aqueous solution once. The peptides were then eluted into new 2.0 mL Protein LoBind Eppendorf tubes twice with 250 µL elution buffer (80% ACN, 0.5% FA) under reduced pressure until no liquid comes out from the SepPak C18 columns.

The filtrates were dried in a lyophilizer and resuspended in 100 µL TEAB buffer (tetraethylammonium bromide, 100 mM TEAB, 0.36% FA, pH = 6) by pipetting up and down. The solution was vortexed, sonicated and spinned down afterwards. 8 µL respective fresh isotope labeling solution (light labeling solution: 2% CH_2O, 0.3 M $NaBH_3CN$ in ddH_2O; medium labeling solution: 2% CD_2O, 0.3 M $NaBH_3CN$ in ddH_2O; heavy labeling solution: 2 % $^{13}CD_2O$, 0.3 M $NaBD_3CN$ in ddH_2O) was added and briefly vortexed. The mixture was incubated 1 h (450 rpm, 25 °C). After incubation, the samples were cooled on ice for 3 min and quenched with 16 µL pre-chilled 1% ammonia solution, 8 µL pre-chilled 5 % FA solution with short vortexing and centrifugation after final addition. Differentially labeled peptide solutions were mixed in a new Protein LoBind Eppendorf tube, lyophilized and the peptides were stored at - 20°C afterwards. Before MS measurement the samples were dissolved in 35 µL 1% FA (formic acid) in ddH_2O by pipetting up and down, vortexing and sonication for 15 min. Then, the supernatants were collected by centrifugation. The VWR 0.45 µm centrifugal filter were pre-equilibrated with 500 µL dd H_2O twice, 500 µL 0.05 N NaOH once and 500 µL 1% FA twice (centrifugation of the filters: 13,000 rpm, 1 min, RT). The peptide solutions were filtered through the equilibrated filters (centrifugation: 13,000 rpm, 2 min, RT).

The digested peptides were analyzed on an UltiMate 3000 nano HPLC system (Dionex, Sunnyvale, California, USA) coupled to a Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Samples were loaded on a Acclaim C18 PepMap100 75 µm ID x 2 cm trap and transferred to a Acclaim C18 PepMap RSLC, 75 µM ID x 15 cm separation column (0.1% FA, 5% DMSO, gradient 10 min 3% ACN, 120 min from 3% to 25% ACN, 5 min to 40% ACN, 0.1 min to 90% ACN and

4.9 min hold at 90% ACN, 0.1 min to 3% ACN and 9.9 min 3% ACN). The mass spectrometer was operated in data dependent top speed mode selecting the most intense precursors with a minimal threshold of 5E3. Precursors were measured in the orbitrap at a resolution of 120,000 and an ion target of 4E5 (max inj. time of 50 ms) in a scan range from 300 to 1700 m/z. Monoisotopic precursor selection was enabled. Charge states from 1 to 7 were triggered. Dynamic exclusion duration was set to 60 s with a mass tolerance of 10 ppm. Precursors were isolated in the quadrupole (isol. window 1.6 m/z) and fragmentation was performed using higher-energy collisional dissociation (HCD). Resulting fragments were measured in the ion trap using a rapid scan rate (AGC target: 1E4 and max inj. time 40 ms). Peptide and protein identifications were performed using MaxQuant 1.4.0.8 software with Andromeda as search engine using following parameters: Carbamidomethylation of cysteines as fixed and oxidation of methionine as well as acetylation of N-termini as dynamic modifications, trypsin/P as the proteolytic enzyme, 4.5 ppm for precursor mass tolerance (main search ppm) and 0.5 Da for fragment mass tolerance (ITMS MS/MS tolerance). Searches were done against the PATRIC database sequence (www.patricbrc.org) for *V. harveyi* ATCC BAA-1116 and *V. harveyi* NBRC 15634 (downloaded on 28.07.2014). Quantification was performed using dimethyl labeling with the following settings: light: DimethLys0, DimethNter0; medium: DimethLys4, DimethNter4 and heavy: DimethLys8, DimethNter8. Variable modifications were included for quantification. I = L and requantify options were used. Identification was done with at least 2 unique peptides and quantification only with unique peptides. Statistical analysis was performed with Perseus 1.5.1.6. Putative contaminants, reverse peptides and peptides only identified by site were omitted from further processing. Dimethyl labeling ratios obtained from MaxQuant 1.5.2.8 were first transformed with $\log_2(x)$ and then normalized using z-score. $-\log_{10}$(p-value) were obtained by a two sided one sample t-test over 9 gel-free ABPP experiment results (three biological replicates with technical triplicates) in our β-lactone research.

3.3 Proteome analysis among *V. harveyi* ATCC BAA-1116 and its chemical knockdown by β-lactone and the *ΔompA mutant.*

Two sets of *V. harveyi* ATCC BAA-1116 and one set of the *ΔompA mutant* cultures were cultivated in AB medium at 30 °C to OD_{600} = 0.80. The OD_{600} was monitored on Novaspec Plus visible spectrophotometer. Then one set of *V. harveyi* ATCC BAA-1116 cultures was treated with **BL3** with a final concentration 25 μM by addition of concentrated stocks in DMSO. The other set and the *ΔompA mutant* were treated with equivalent volume of DMSO. These cultures were then allowed to grow for another two hours before harvest.
Cultures were collected in a 50 mL falcon tube and pelleted at 6,000 rpm for 5 min at 4 °C and washed with PBS. The pellet was then resuspended in cold lysis buffer (8 M urea, 1 mM EDTA, one tablet of protease inhibitor cocktail per 5 mL (complete, EDTA-free, Roche), 75 mM NaCl, 50 mM Tris-HCl, pH 8.2 at 4 °C) to a final OD_{600} = 300. The suspension was lysed by homogenization with 5 x 20 sec. pulsed at 80% max. power on

ice. The debris was removed by centrifugation (13,000 rpm, 30 min, 4°C) and the supernatant was filtered through 0.2 µm filter. The filtrate was collected in a new Protein LoBind tube from Eppendorf and the concentration of the proteins was measured by BCA assay. Adjust the samples to the same protein amount accordingly and add urea, thiourea to make a final concentration 7 M and 2 M respectively (add lysis buffer to make the right final concentration). Transfer 500 µg proteins to a new 1.5 mL tube, reduce with 1 mM DTT (500 rpm, 1h, 37 °C) and alkylate with 5.5 mM iodoacetamide (500 rpm, 30 min, RT, in the darkness). The reaction was quenched with 4 mM DTT (500 rpm, 30 min, RT) and the solution was pre-digested with Lys-C (1:200, 25 °C, 4 h). After a 1:4 dilution with 50 mM TEAB in ddH_2O (tetraethylammonium bromide), the solution was digested with trypsin (1:100, 37 °C, 12 h). The digestion was stopped by addition of FA to a final concentration of 1%. The pH value had to be below 3 (if not, more formic acid was added). 50 mg SepPak C18 columns were pre-equilibrated by gravity flow with 1 mL acetonitrile, 0.5 mL elution buffer (80% ACN, 0.5% FA) and three times 1 mL aqueous 0.5% FA solution. Subsequently, the samples were loaded and washed with 1 mL aqueous 0.5% FA solution five times. Flush each of the columns with 1 mL of the respective isotope labeling reagent five times over 20 min (light labeling solution: 0.2% CH_2O, 0.03 M $NaBH_3CN$ in 50 mM sodium phosphate, pH 7.5; medium labeling solution: 0.2% CD_2O, 0.03 M $NaBD_3CN$ in ddH_2O in 50 mM sodium phosphate, pH 7.5; heavy labeling solution: 0.2% $^{13}CD_2O$, 0.03 M $NaBD_3CN$ in 50 mM sodium phosphate, pH 7.5). The columns were washed twice with 1 mL 0.5% FA aqueous solution. The peptides were then eluted into 2 mL Protein LoBind tubes with 250 µL elution buffer twice (80% ACN, 0.5% FA) under reduced pressure until no liquid came out from the SepPak C18 columns. The differentially labeled peptide solutions were mixed and lyophilized to get the dried peptides which can be stored at –20 °C.

The peptides were dissolved in 35 µL 1% FA in ddH_2O before MS measurement by pipetting up and down, vortexing and sonication for 15 min. The samples were spin down with centrifugation. The VWR 0.45 µm centrifugal filter were pre-equilibrated with 500 µL dd H_2O twice, 500 µL 0.05 N NaOH and 500 µL 1 % FA twice (centrifugation of the filters: 13,000 rpm, 1 min, RT). Then the peptide solutions were filtered through the equilibrated filters (centrifugation: 13,000 rpm, 2 min, RT) and analyzed on LTQ Orbitrap XL mass spectrometer. Statistical analysis was performed with Perseus 1.5.1.6. Putative contaminants, reverse peptides and peptides only identified by site were omitted from further processing. Dimethyllabeling ratios obtained from MaxQuant 1.5.2.8 were transformed with $\log_2(x)$ and then normalized using z-score. $-\log_{10}$(p-value) were obtained by a two sided one sample t-test over three biological experiments with technical triplicates against *V. harveyi* ATCC BAA-1116 sequence from PATRIC database (www.patricbrc.org, downloaded on 31.08.2015).

3.4 Recombinant proteins

V. harveyi ATCC BAA-1116 ACAT was recombinantly expressed in *E. coli* Arctic Express using the Invitrogen™ Gateway® Cloning Technology. The *ACAT* gene was amplified

from the corresponding genomic DNA (*V. harveyi* ATCC BAA-1116) by PCR via a Phusion® High-Fidelity DNA Polymerase kit (New England BioLabs® Inc.). 40 ng *V. harveyi* ATCC BAA-1116 genomic DNA was prepared by standard protocols (Quiagen DNeasy® Blood & Tissue Kit 50). The *att*B1 forward primer and *att*B2 reverse primer were designed to yield *attB*-PCR Products needed for Gateway® Technology. PCR products were identified on agarose gels and extracted with an Omega Bio-tek Inc. E.Z.N.A.® MicroElute® Gel Extraction Kit. The DNA concentrations were recorded by a Tecan Infinite® M200 PRO plate reader. Then 100 fmol *attB*-PCR product and 50 fmol *attP*-containing donor vector pDONR™ 201 (Invitrogen™) in TE buffer were mixed for BP recombination reaction with BP Clonase™ II enzyme mix (Invitrogen™) to give the *attL*-containing entry clone. The resulting entry clone was then transformed into chemically competent One Shot® *E. coli* TOP10 (Invitrogen™) and then cells were selected on LB agar with 25 µg/mL kanamycin. The selected clones were cultivated in LB media with kanamycin and the cells were harvested to isolate the plasmids with Omega Bio-tek Inc. E.Z.N.A.® MicroElute® Plasmid Mini Kit. 50 fmol isolated plasmids and 50 fmol *attR*-containing destination vector pDest 007 were subsequently used in LR recombination reaction with LR Clonase™ II enzyme mix (Invitrogen™) in TE buffer. The clone was then transformed into chemically competent *E. coli* Arctic Express cells by heat shock and then selected with LB agar plates with 100 µg/mL ampicillin. The selected colonies were grown in LB media with ampicillin and the culture was used to make working stocks for future protein over-expression and isolation of the expression plasmids. The plasmid sequence was confirmed by sanger-sequencing service of GATC Biotech. The recombinant *E. coli* Arctic Express was grown at 37 °C in LB media until an OD_{600} ≈ 0.5 and then expression was induced by 432 nM anhydrotetracycline at 15 °C for 12 hours. The bacteria were harvested and washed with PBS. The resulting pellet was resuspended in binding buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl), lysed by sonication. The protein was then purified with StrepTrap™ HP columns (GE Healthcare Life Sciences) and stored at – 80 °C in the elution buffer (100mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 2.5 mM desthiobiotin, pH 8.0). The protein purity was determined by MS and SDS-gel.
forward primer:
5’-GGGACAAGTTTGTACAAAAAAGCAGGCTTTGAGAATCTTTATTTTCAGGGCGAAAA
AGTATTTATTGTTGC-3’
reverse primer:
5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGCTATTTAACTGCTTTTACAA-3’

Recombinant expression of GGDEF family protein in *E. coli*

V. harveyi ATCC BAA-1116 GGDEF family protein was recombinantly expressed in pDest 007 plasmid in *E. coli* BL21DE(3) using the Invitrogen™ Gateway® Cloning Technology similar to what described in ACAT section. Nevertheless, the bacteria were grown at 37 °C until OD_{600} ≈ 0.5 and then induced by 432 nM anhydrotetracycline at 37 °C for 4 hours. The bacteria were harvested and washed with PBS. After pelletizing, the pellet of cells was stored in - 80 °C freezer before being used.
forward primer:
5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGCTCATGAACAAAAAAGTATCCAGT

TA-3’
reverse primer:
5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATGCGCTCTTCATTTGTTTGTCAT
T-3’

Recombinant expression of OmpA in *E. coli*

V. harveyi ATCC BAA-1116 OmpA was recombinantly expressed in pDest 007 plasmid in *E. coli* BL21DE(3) using the Invitrogen™ Gateway® Cloning Technology similar to what described in ACAT section. Nevertheless, the bacteria were grown at 37 °C until OD_{600} ≈ 0.5 and then induced by 432 nM anhydrotetracycline at 37 °C for 4 hours. The bacteria were harvested and washed with PBS. After pelletizing, the pellet of cells was stored in -80 °C freezer before being used.
forward primer:
5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCAATCAATACAAGGAAACACTATG-3
’
reverse primer:
5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTCTGGTTGTACAAGTTCTTC-3’

3.5 Binding site identification by MS-MS of OmpA

E. coli BL21 DE(3) cells with a pDest 007 plasmid containing *V. harveyi* ATCC BAA-1116 *ompA* gene was cultivated in LB medium at 37 °C to OD_{600} ≈ 0.5 and then induced by 432 nM anhydrotetracycline for overexpression at 37 °C for 4 hours. Afterwards, the bacterial cells were harvested and pelletized. The pellet was resuspended in PBS with a final OD_{600} ≈ 40. The bacterial cells were then incubated with **LP3** (final concentration: 25 µM) at room temperature for 1 hour. After removal of the excess of **LP3** by centrifugation and washing the pellet with PBS, the pellet was then resuspended in cold lysis buffer (8 M urea, 1 mM EDTA, one tablet of protease inhibitor cocktail per 5 mL (complete, EDTA-free, Roche), 75 mM NaCl, 50 mM Tris-HCl, pH 8.2 at 4 °C) to a final OD_{600} = 300. The suspension was lysed by homogenization with 5 x 20 sec. pulsed at 80% max. power on ice. The debris was removed by centrifugation (13,000 rpm, 30 min, 4 °C) and the supernatant was filtered through 0.2 µm filter. The filtrate was collected in a new Protein LoBind tube from Eppendorf and the concentration of the proteins was measured by BCA assay. Adjust the samples to the same protein amount accordingly and add urea, thiourea to make a final concentration 7 M and 2 M respectively (add lysis buffer to make the right final concentration). Transfer 500 µg proteins to a new 1.5 mL tube, reduce with 1 mM DTT (500 rpm, 1h, 37 °C) and alkylate with 5.5 mM iodoacetamide (500 rpm, 30 min, RT, in the darkness). The reaction was quenched with 4 mM DTT (500 rpm, 30 min, RT) and the solution was pre-digested with Lys-C (1:200, 25 °C, 4 h). After a 1:4 dilution with 50 mM TEAB in dd H_2O (tetraethylammonium bromide), the solution was digested with trypsin (1:100, 37 °C, 12 h). The digestion was stopped by addition of FA to a final concentration of 1%. The pH value had to be below 3 (if not, more formic acid was added). 50 mg SepPak C18 columns were pre-equilibrated by gravity flow with 1 mL acetonitrile,

0.5 mL elution buffer (80% ACN, 0.5% FA) and three times 1 mL aqueous 0.5% FA solution. Subsequently, the samples were loaded and washed with 1 mL aqueous 0.5% FA solution five times. The peptides were then eluted into 2 mL Protein LoBind tubes with 250 µL elution buffer twice (80% ACN, 0.5% FA) under reduced pressure until no liquid came out from the SepPak C18 columns. The peptide solutions were lyophilized to get the dried peptides and stored at – 20 °C.
The peptides were dissolved in 300 µL 1% FA in ddH_2O before MS measurement by pipetting up and down, vortexing and sonication for 15 min. The samples were spin down with centrifugation. The VWR 0.2 µm centrifugal filters were pre-equilibrated with 500 µL 1% FA twice (centrifugation of the filters: 13,000 rpm, 1 min, RT). Then the peptide solutions were filtered through the equilibrated filters (centrifugation: 13,000 rpm, 2 min, RT) and analyzed on LTQ Orbitrap XL™ mass spectrometer. Analysis was performed on 1.5.2.8 against *V. harveyi* ATCC BAA-1116 sequence from PATRIC database (www.patricbrc.org, downloaded on 31.08.2015).

3.6 Biological assays

3.6.1 Phosphorylation assay

E. coli TKR2000 was transformed with plasmid pNKN encoding wild-type LuxN. Inside-out membrane vesicles were prepared as described before.[8] LuxU was overproduced using *E. coli* JM109 transformed with pQE30LuxU-6His, and purified as described.[8] All proteins were stored at - 80 °C.
Phosphorylation reactions were performed in phosphorylation buffer (50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 2 mM DTT) at room temperature. The hybrid histidine kinase LuxN was used as full-length membrane integrated protein in inverted membrane vesicles. LuxN containing membrane vesicles were added at final concentrations of 5.5 mg/ml and LuxU 0.36 mg/ml. The β-lactones (**LP3**, **BL3**) were added in a final concentration of 50 µM. The phosphorylation reaction was started by adding radiolabelled Mg^{2+}-ATP, typically 100 µM [γ-^{32}P] ATP (0.94 Ci/mmol; Perkin-Elmer, Rodgau-Jügesheim, Germany) and 110 µM $MgCl_2$, and stopped at various time points by the addition of SDS loading buffer, followed by fractionation of the reaction on SDS polyacrylamide gels. Gels were dried at 80 °C on filter paper, exposed to a phosphoscreen for at least 24 h and scanned using a Typhoon Trio variable mode imager (GE Healthcare, Munich, Germany).

3.6.2 Swimming assay

Swimming behavior of the bacteria was analyzed by using autoinducer bioassay (AB)

medium[9] plates containing 0.3% (w/v) agar and supplemented with 50 µM LP3 or BL3, respectively. As control the appropriate volume of DMSO was added to the plates. 1 µl of bacterial culture (OD_{600} = 1) was dropped in the middle of the plate. After 20 - 24 h of incubation at 30 °C the diameter of the bacterial lawn was measured and a chemiluminescence picture was taken (10 s exposure time).

3.6.3 Exoprotease assay

Exoproteolytic activity of *V. harveyi* strains was measured by incubating hide powder azure (Sigma-Aldrich, Darmstadt, Germany) in phosphate buffered saline (pH 7.2) with cell-free culture fluids at 37 °C.[10, 11] The reaction was stopped with trichloroacetic acid [6.7% (v/v)] after 2 h, and the absorbance at 600 nm was measured. The activity was calculated by the difference between initial and final absorption after 2 h (AU), and normalized by the OD_{600} (AU/OD_{600}).

3.6.4 Bioluminescence assay

An overnight culture of *Vibrio* strains, grown in Marine broth or AB medium at 30 °C or 28 °C, was diluted 1:100 and grown to an OD_{600} of ~1. At this point, OD_{600} was measured and the culture was diluted to a final OD_{600} = 0.5 with fresh medium. 100 µL aliquots of the culture were added to a Corning® 96 well plates (clear flat bottom) which contain 1 µL various concentrations of compounds or DMSO per well. After incubation at 30 °C or 28 °C with agitation for 30 min, luminescence and OD_{600} were measured in an infinite M200Pro plate reader (Tecan). After deduction of the background (fresh medium), luminescence units were normalized to cell density. DMSO treated control samples were normalized to 100% activity and the residual activity of inhibitor treated samples was determined. The average values of technical triplicates were calculated and then the standard deviation of the means was calculated over three independent experiments. Residual activities for the respective compound concentration were fitted to Y = 100/(1+10^((logIC_{50}-X)*HillSlope)) (Y: residual activity in %; X: $\log_{10}$(concentration)) and then IC_{50} was calculated in GraphPad Prism 6.0.

3.6.5 Growth and luminescence comparison of *V. harveyi* strains

Wild type *V. harveyi* ATCC BAA-1116, the *ΔompA* and *ΔluxO* mutants were grown overnight in LM medium (20 g/L NaCl, 10 g/L tryptone, 5 g/L). Subsequently, cultures were diluted 1:5,000 in AB medium, and growth (OD_{600}) and luminescence were determined every hour or every 20 min in microtiter plates with a Tecan Infinite F500 system (Männedorf, Switzerland) for 0.1 s. Data are reported as relative light units (RLU = Luminescence/OD_{600}).

4. Reference

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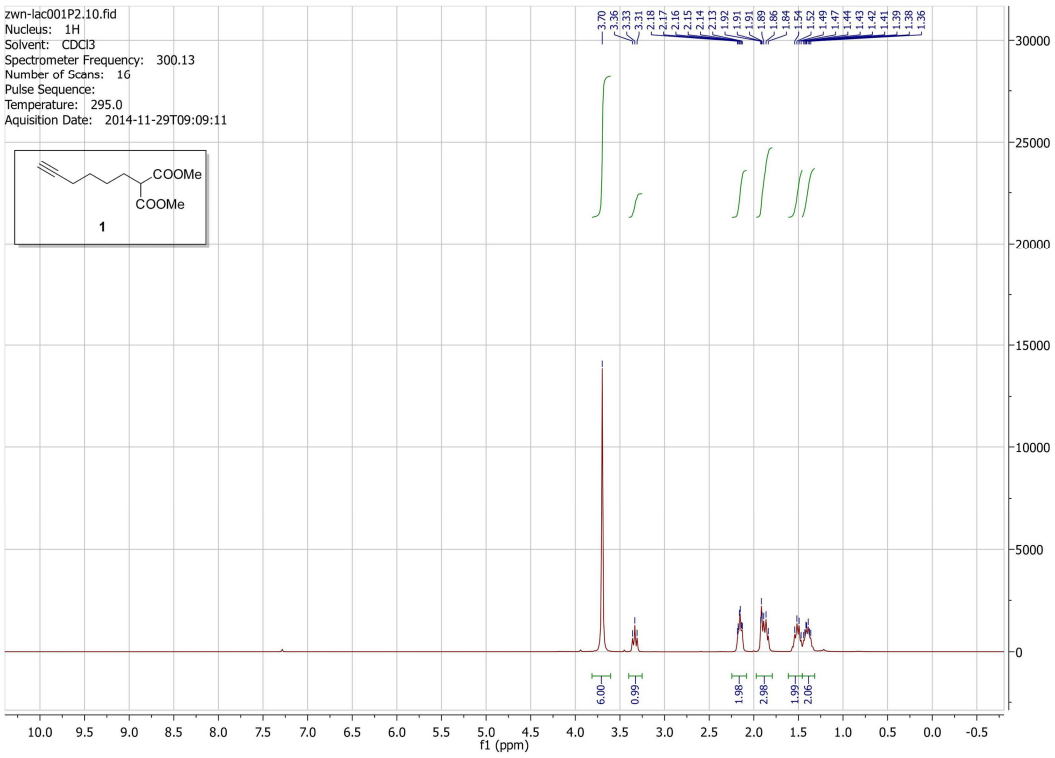
5. Appendix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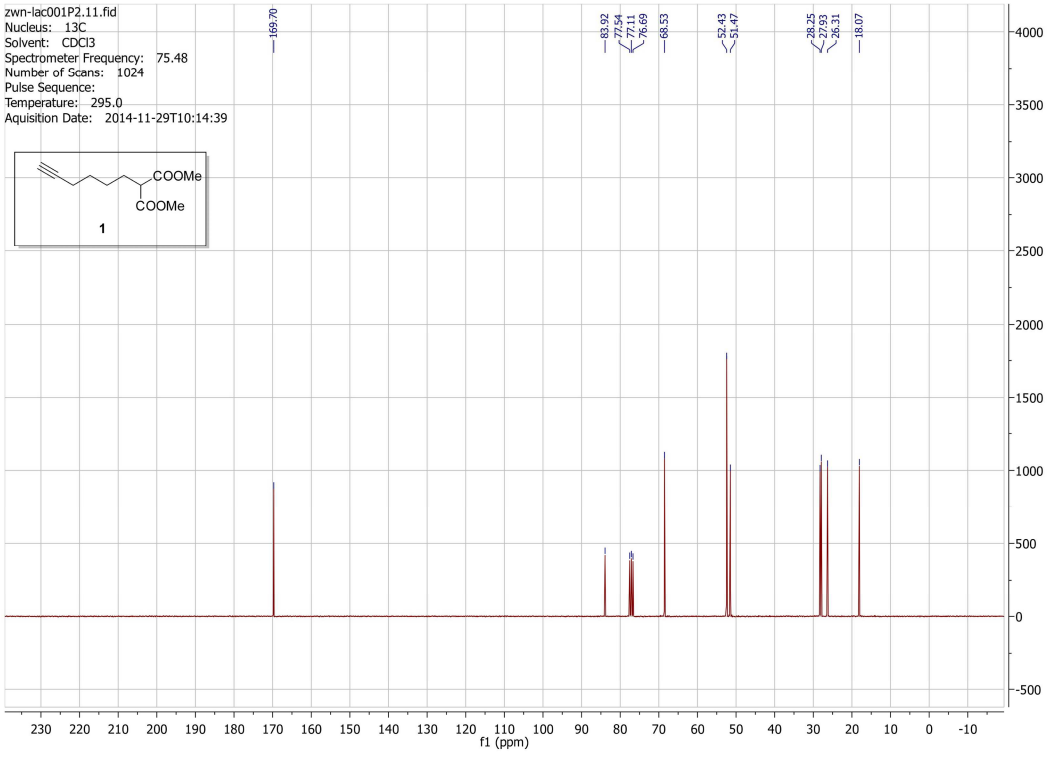
f1 (ppm)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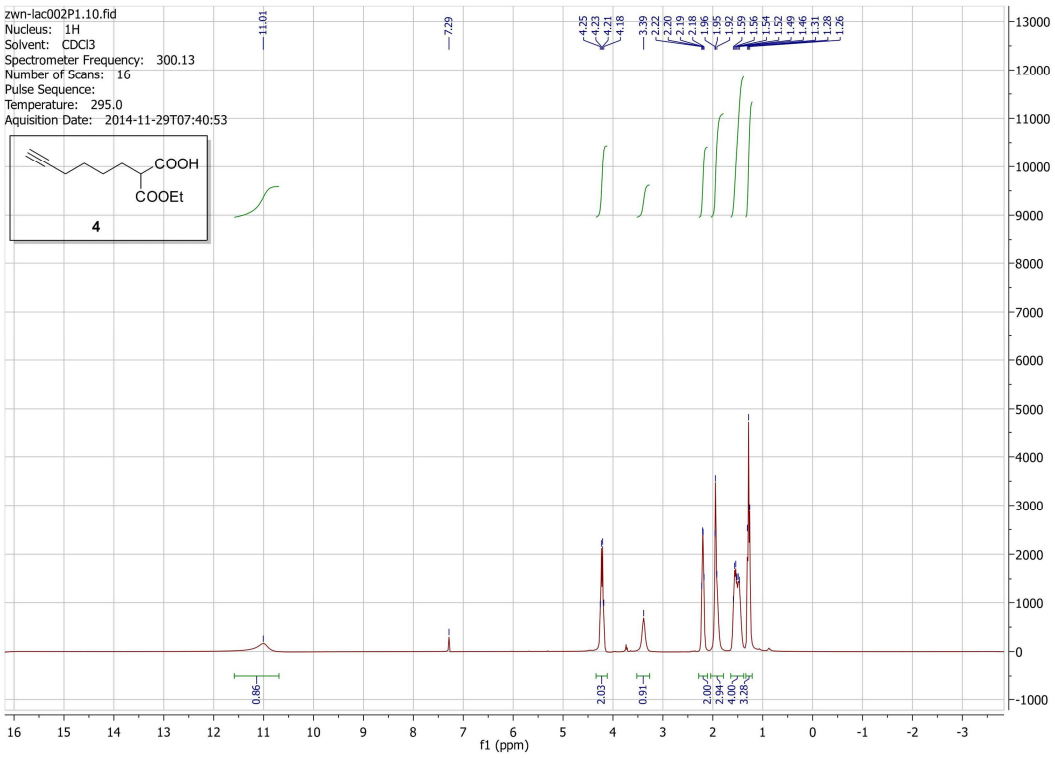
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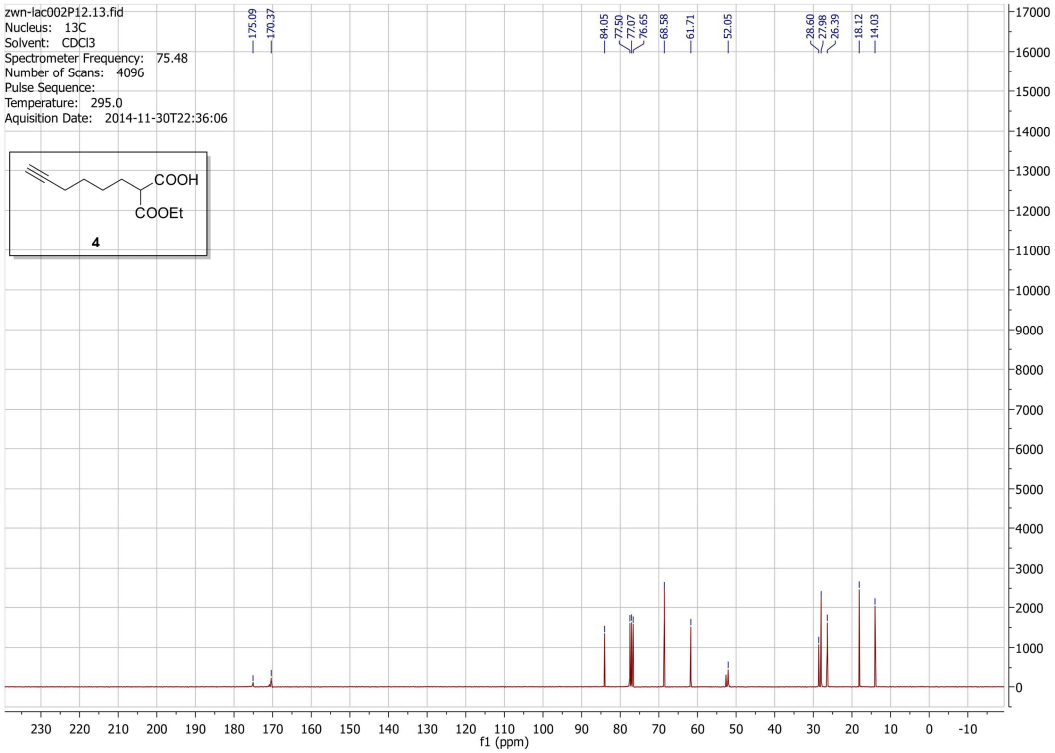
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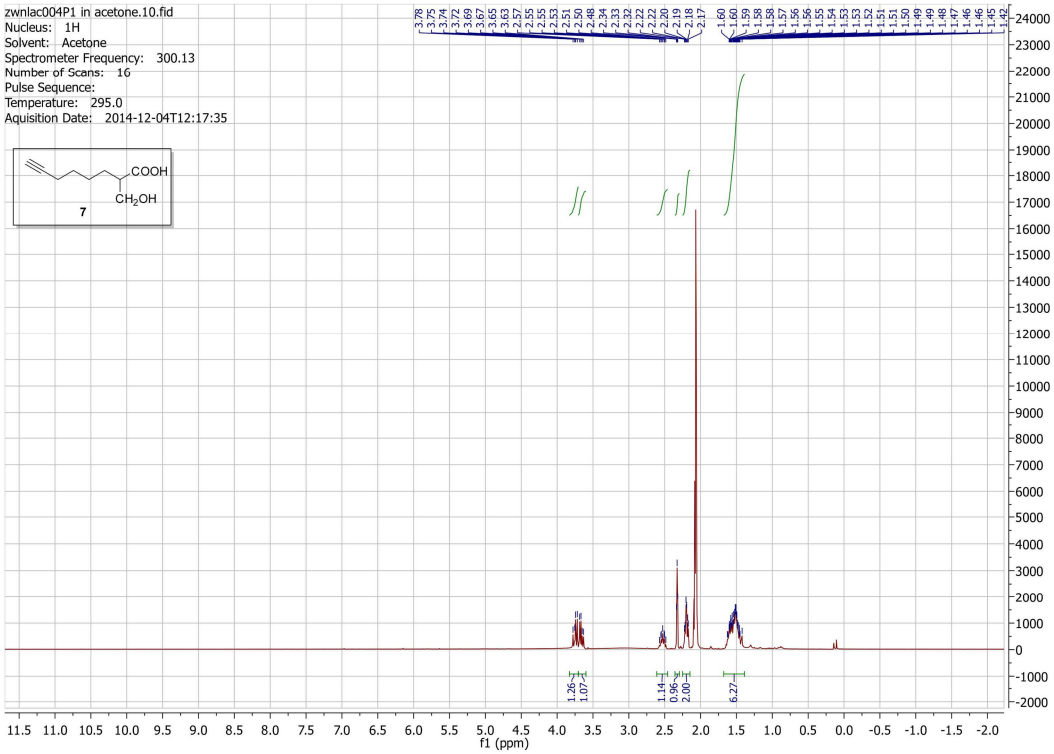
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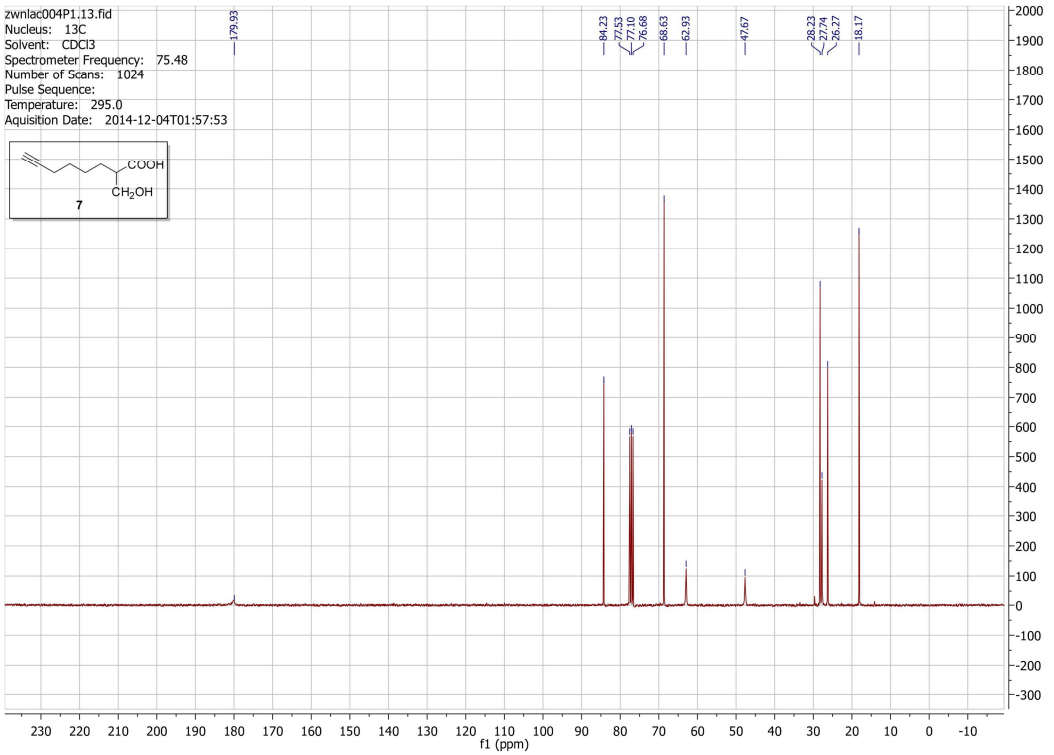
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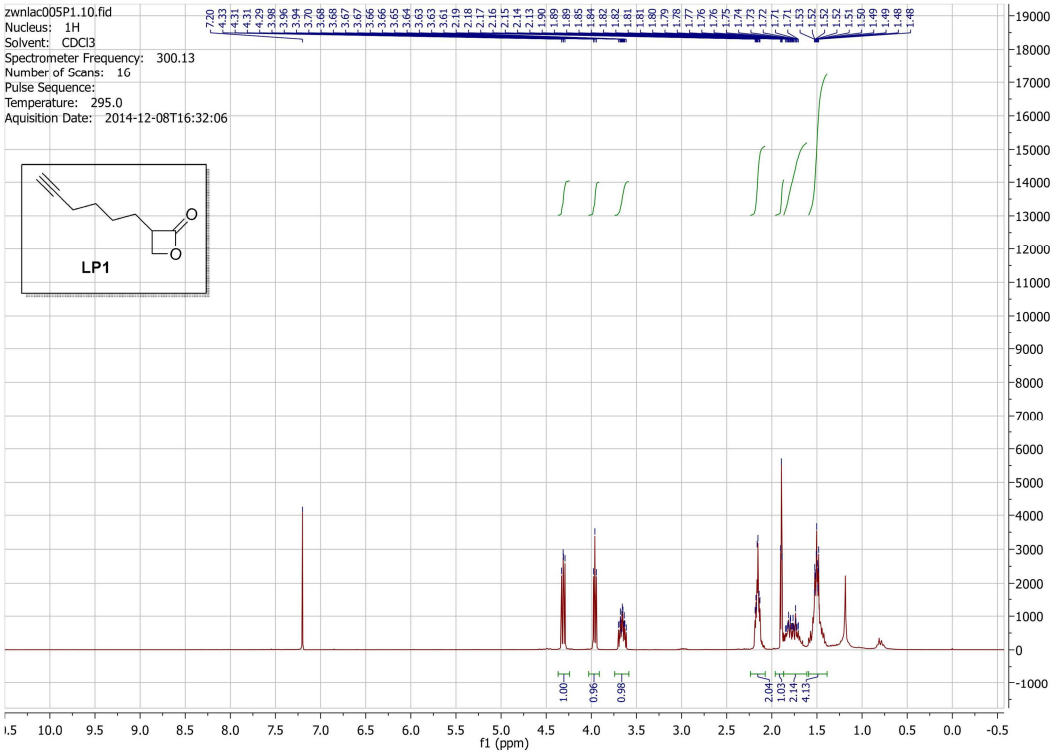
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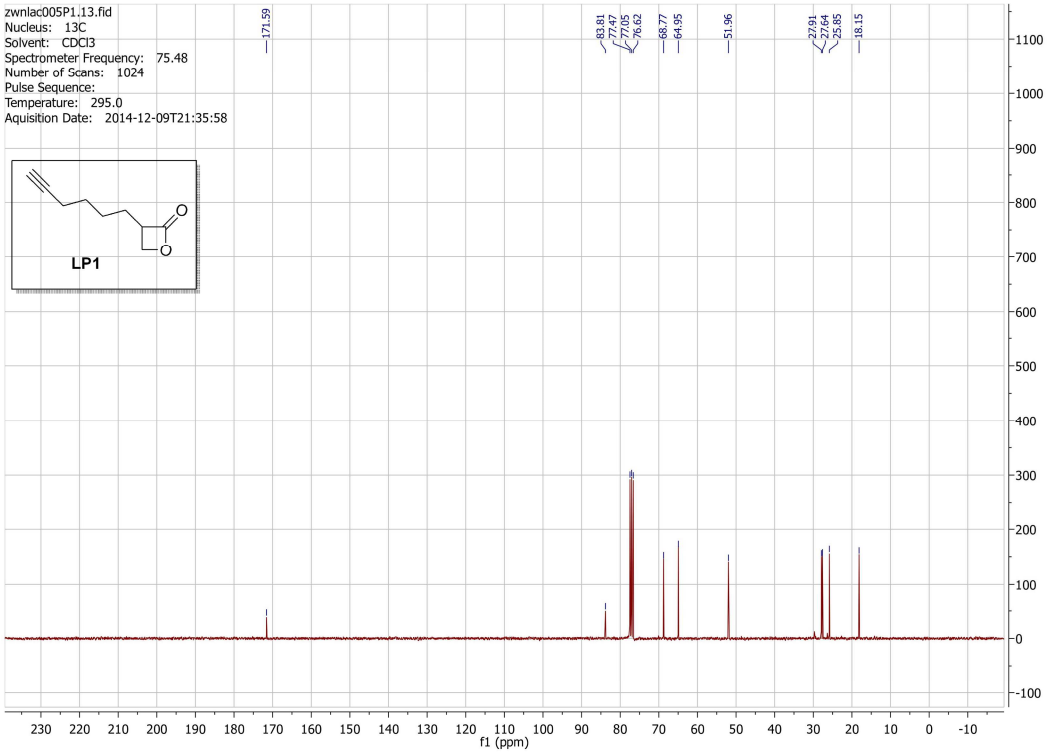
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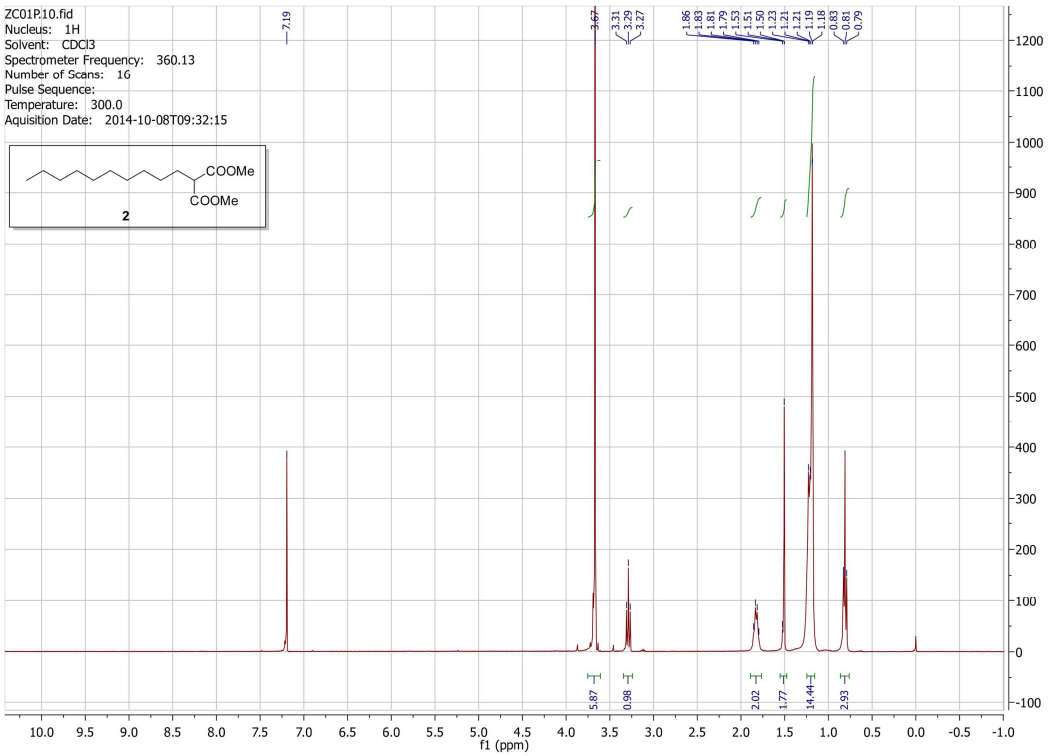
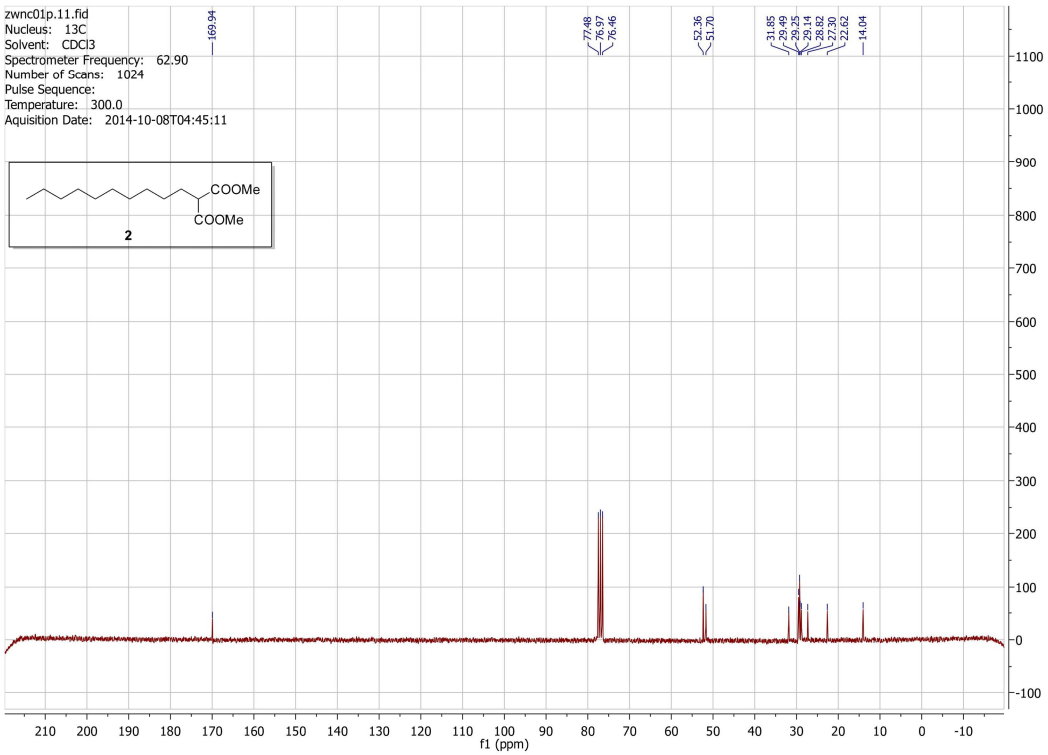
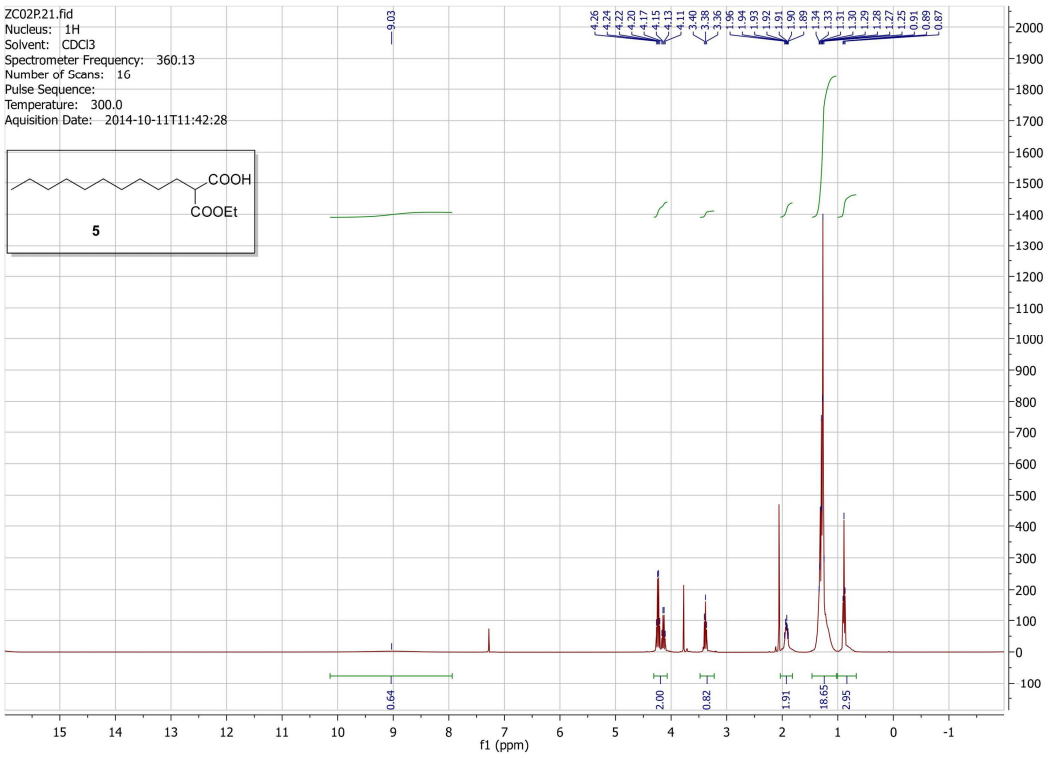
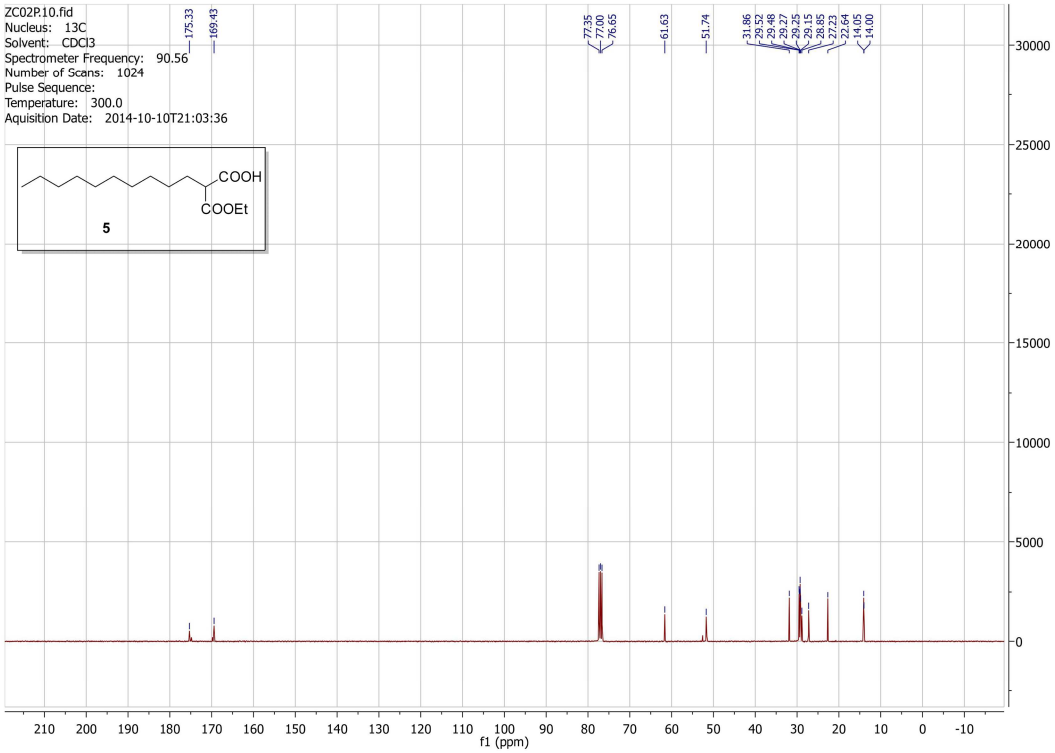
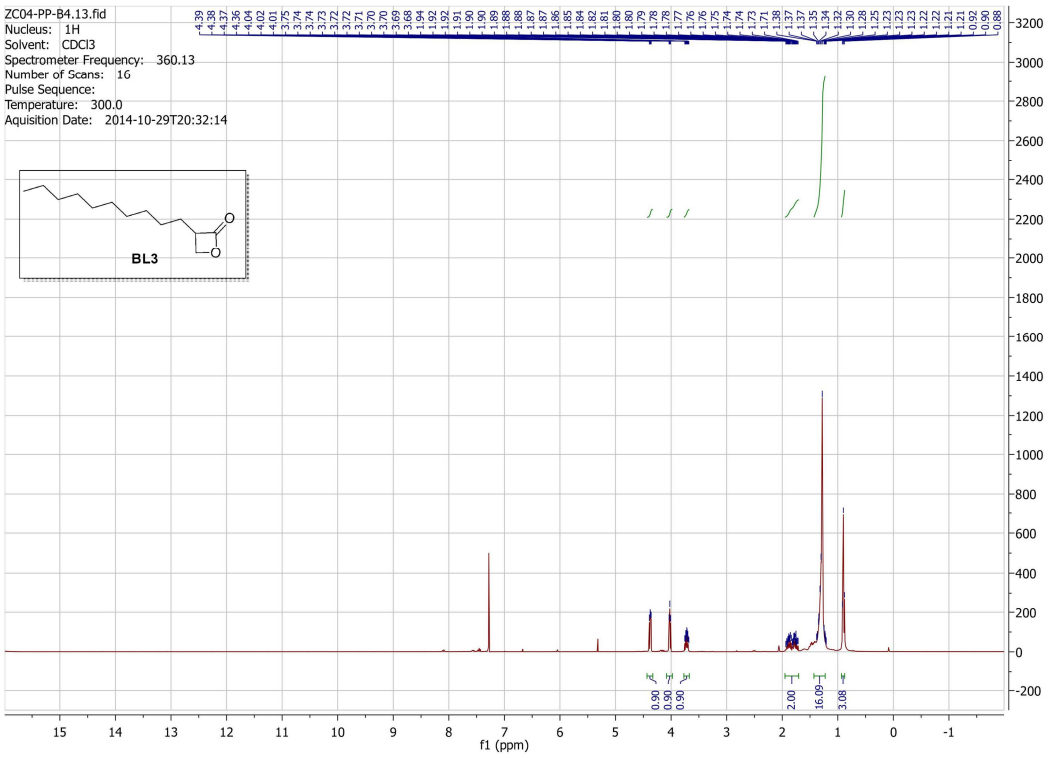
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 75.48
Number of Scans: 1024
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2014-12-09T21:35:58
LP1
f1 (ppm)

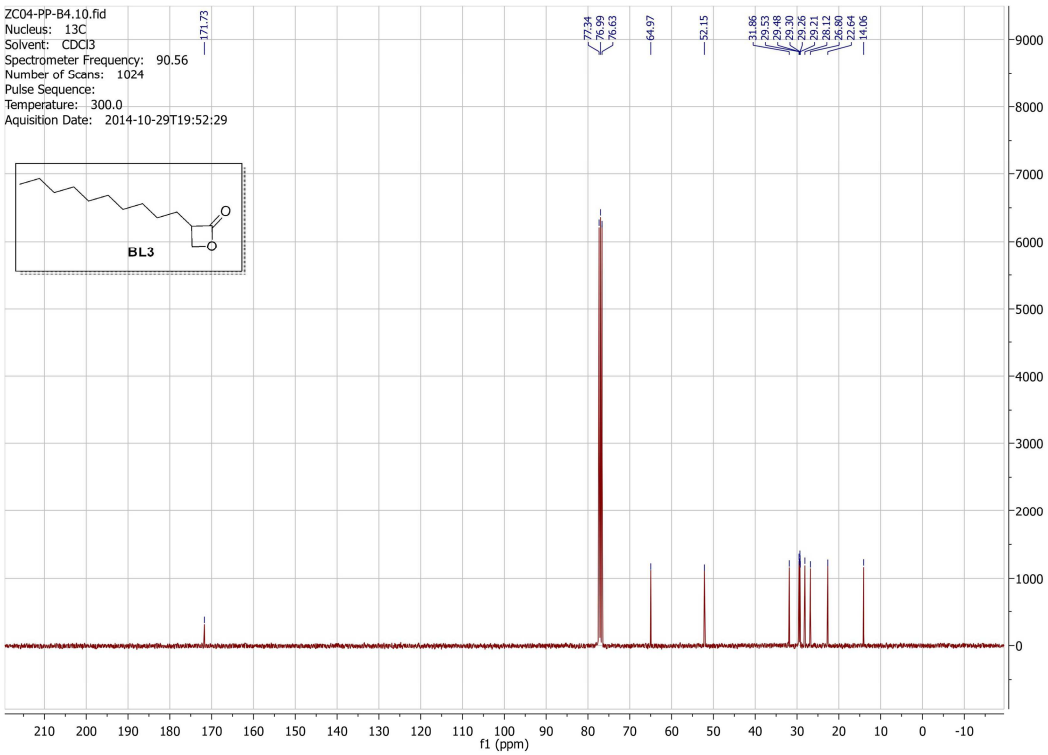

ZC01P.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 16
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-08T09:32:15
COOMe
COOMe
2
7.19
3.67
3.31
3.29
3.27
1.86
1.83
1.81
1.79
1.53
1.51
1.50
1.23
1.21
1.21
1.19
1.18
0.85
0.81
0.79
5.87
0.98
2.02
1.77
14.44
2.93
f1 (ppm)

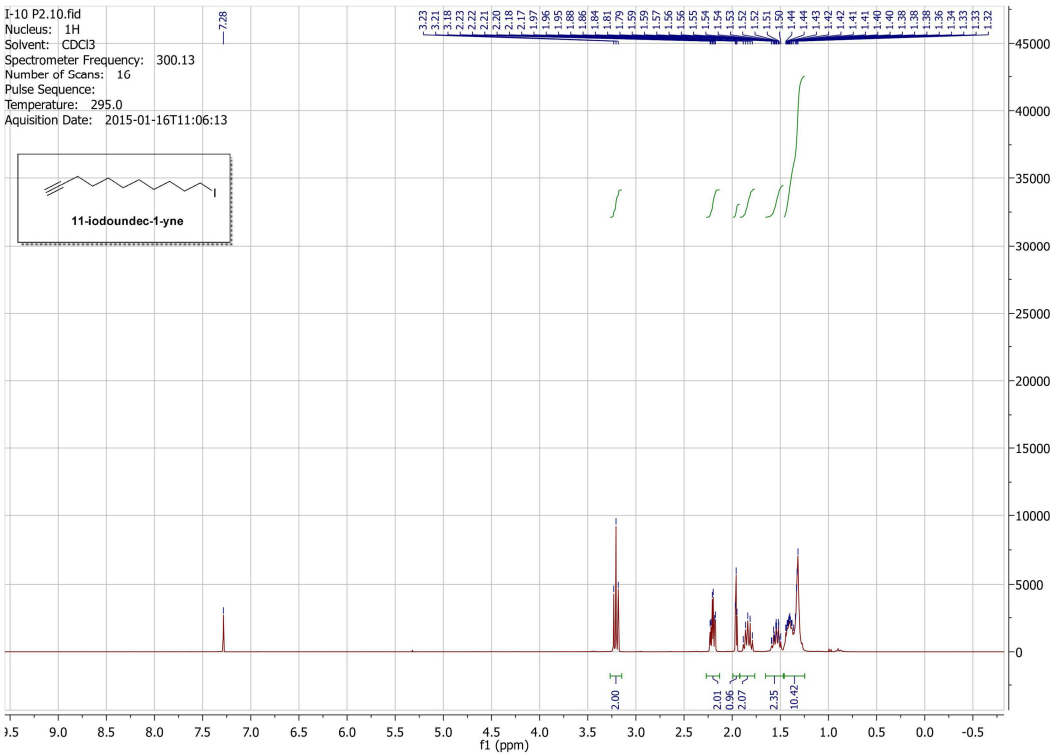

zwnc01p.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 62.90
Number of Scans: 1024
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-08T04:45:11
COOMe
COOMe
2
169.94
77.48
76.97
76.46
52.36
51.70
31.85
29.49
29.25
29.14
28.82
27.30
22.62
14.04
f1 (ppm)

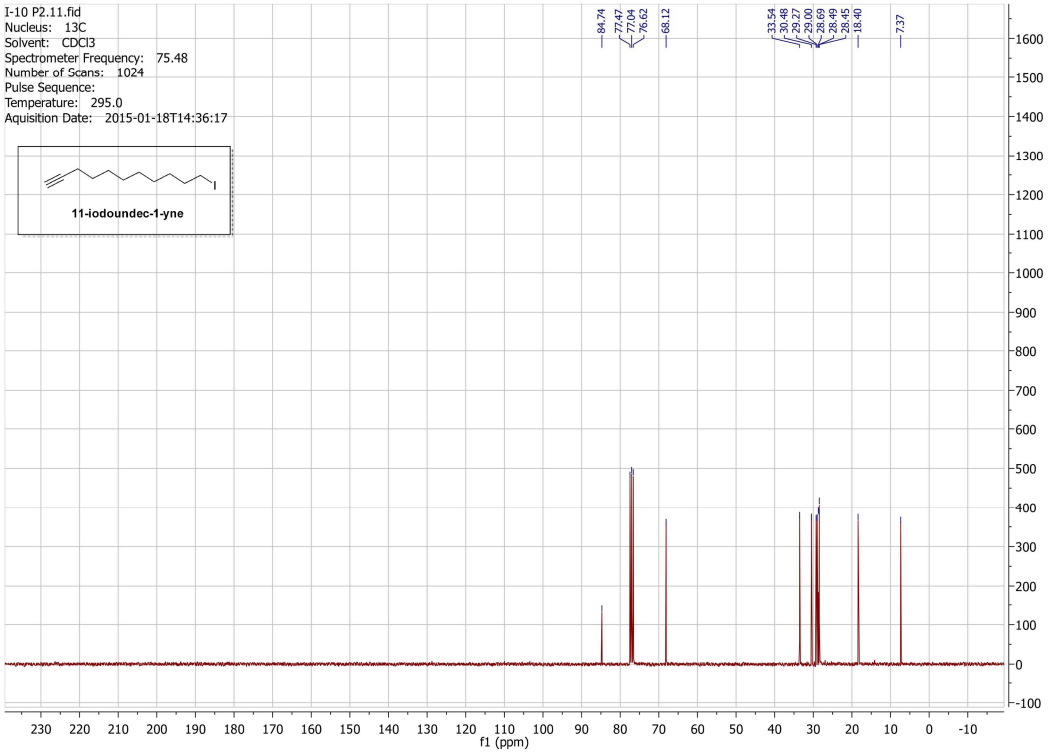

ZC02P.21.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 16
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-11T11:42:28
COOH
COOEt
5
f1 (ppm)

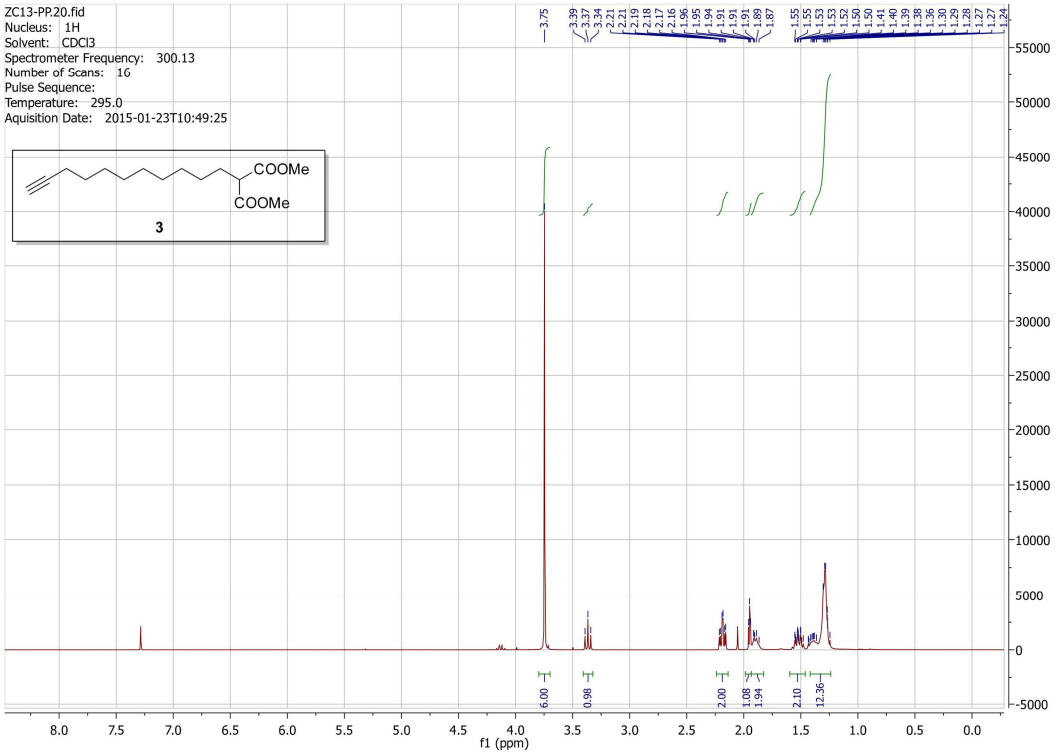

ZC02P.10.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 90.56
Number of Scans: 1024
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-10T21:03:36
COOH
COOEt
5
f1 (ppm)

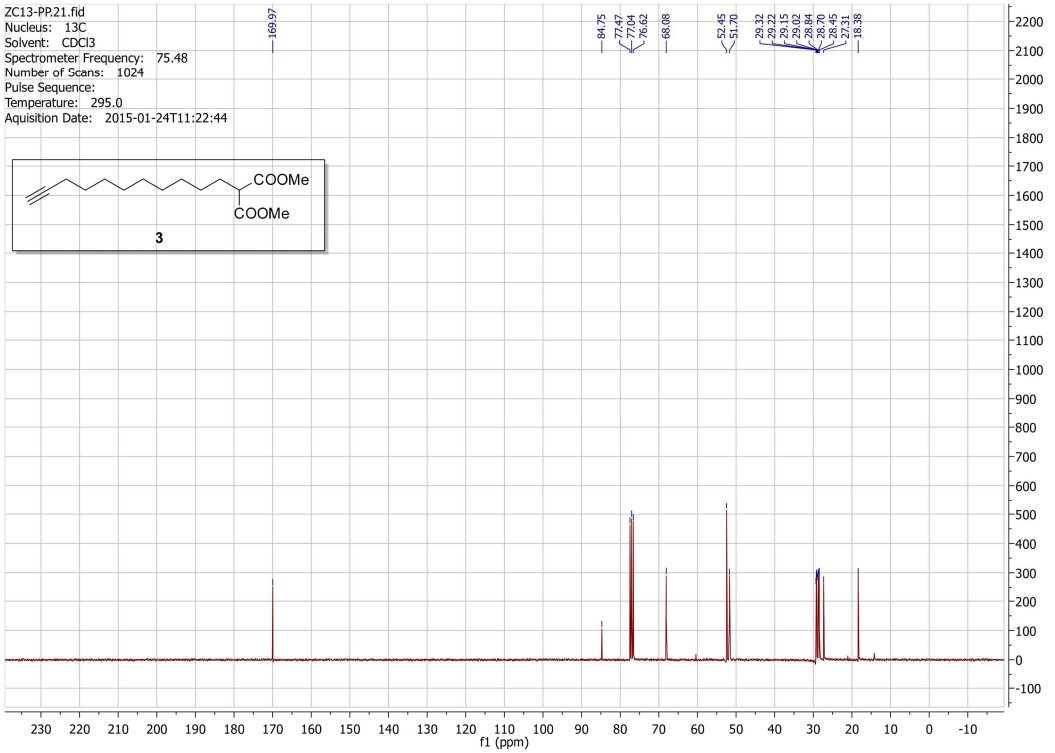

ZC04-PP-B4.13.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 16
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-29T20:32:14
BL3
f1 (ppm)

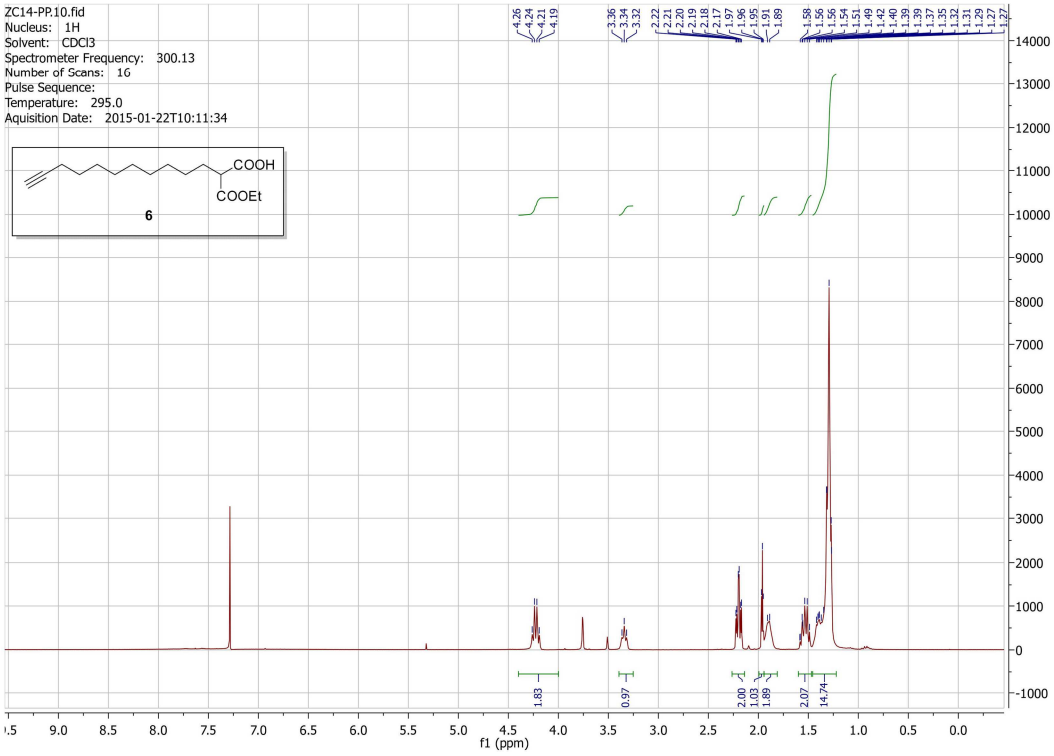

ZC04-PP-B4.10.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 90.56
Number of Scans: 1024
Pulse Sequence:
Temperature: 300.0
Aquisition Date: 2014-10-29T19:52:29
BL3
f1 (ppm)

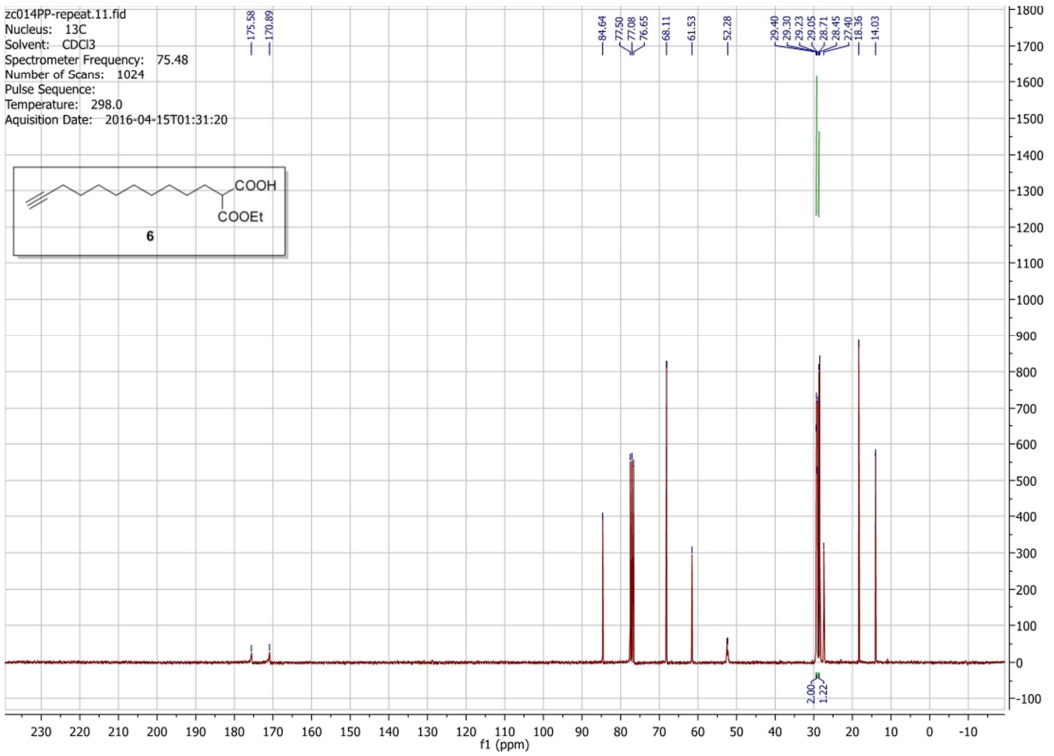

I-10 P2.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 300.13
Number of Scans: 16
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-01-16T11:06:13
11-iodoundec-1-yne
f1 (ppm)

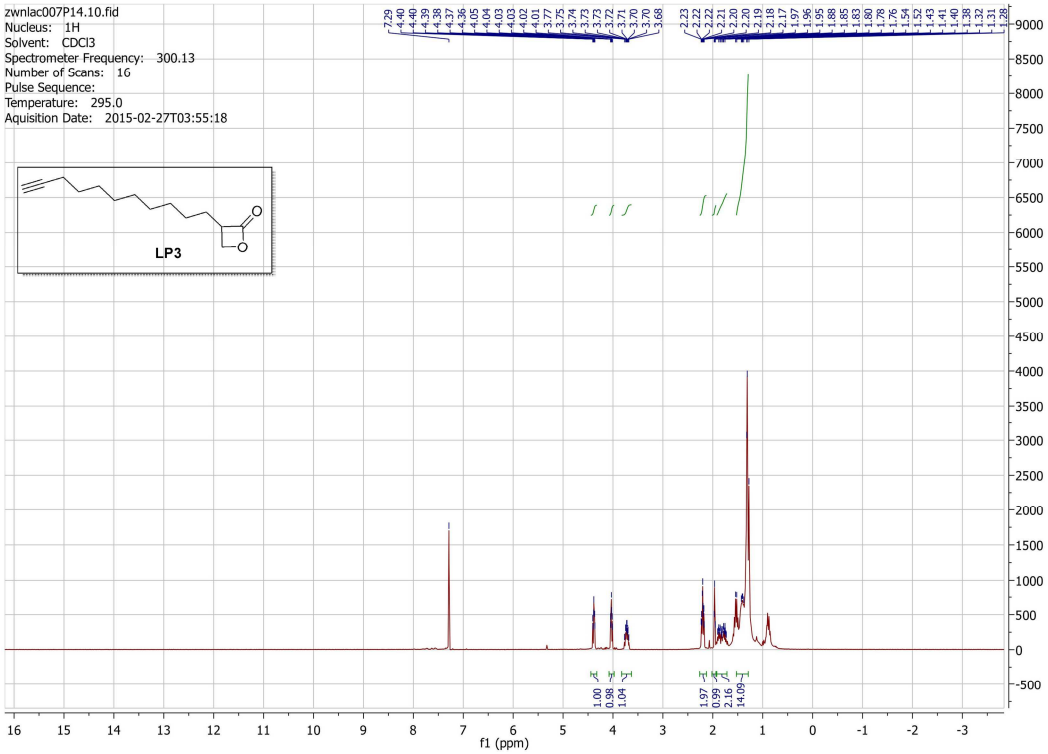

I-10 P2.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 75.48
Number of Scans: 1024
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-01-18T14:36:17
11-iodoundec-1-yne
f1 (ppm)

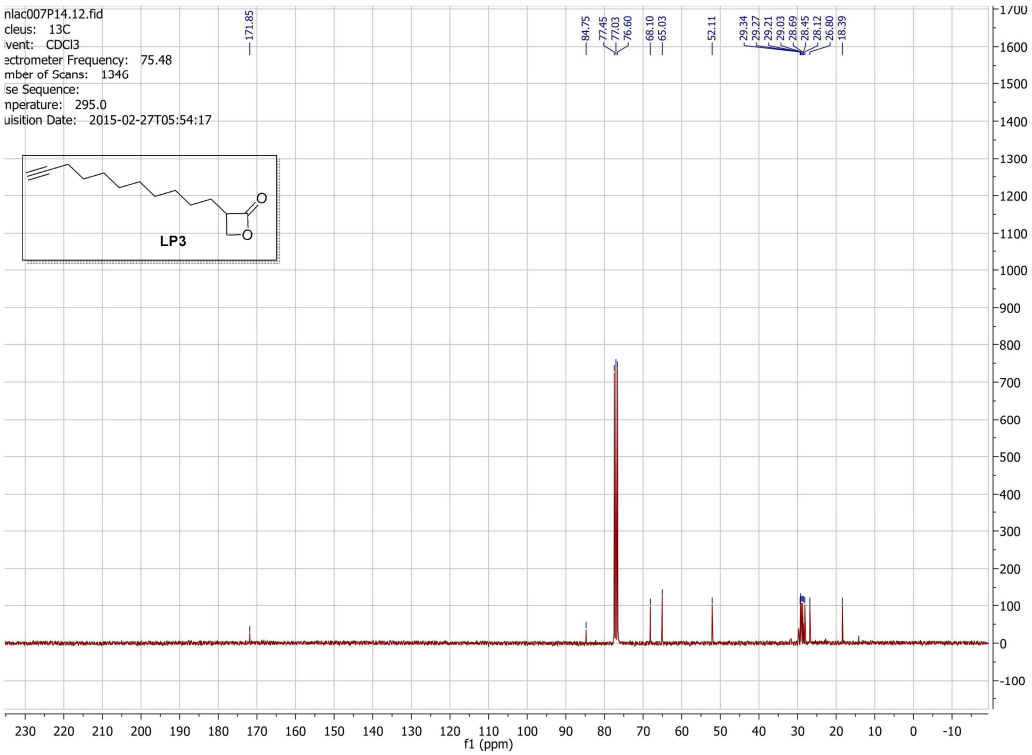

ZC13-PP.20.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 300.13
Number of Scans: 16
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-01-23T10:49:25
COOMe
COOMe
3
f1 (ppm)

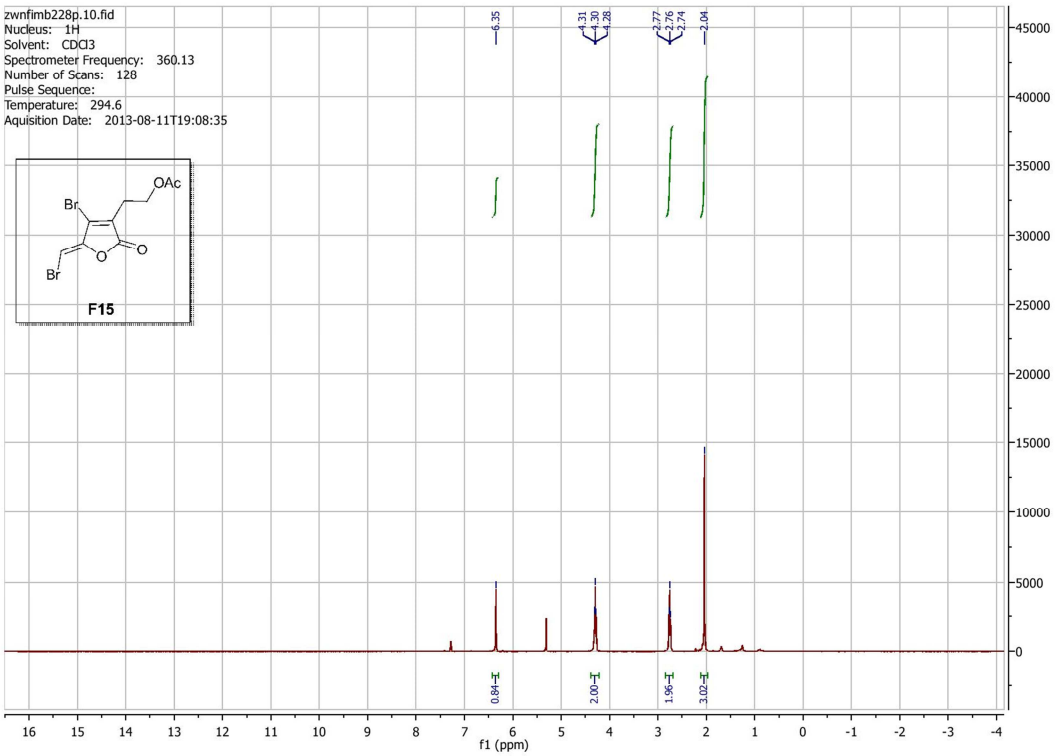

ZC13-PP.21.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 75.48
Number of Scans: 1024
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-01-24T11:22:44
COOMe
COOMe
3
f1 (ppm)

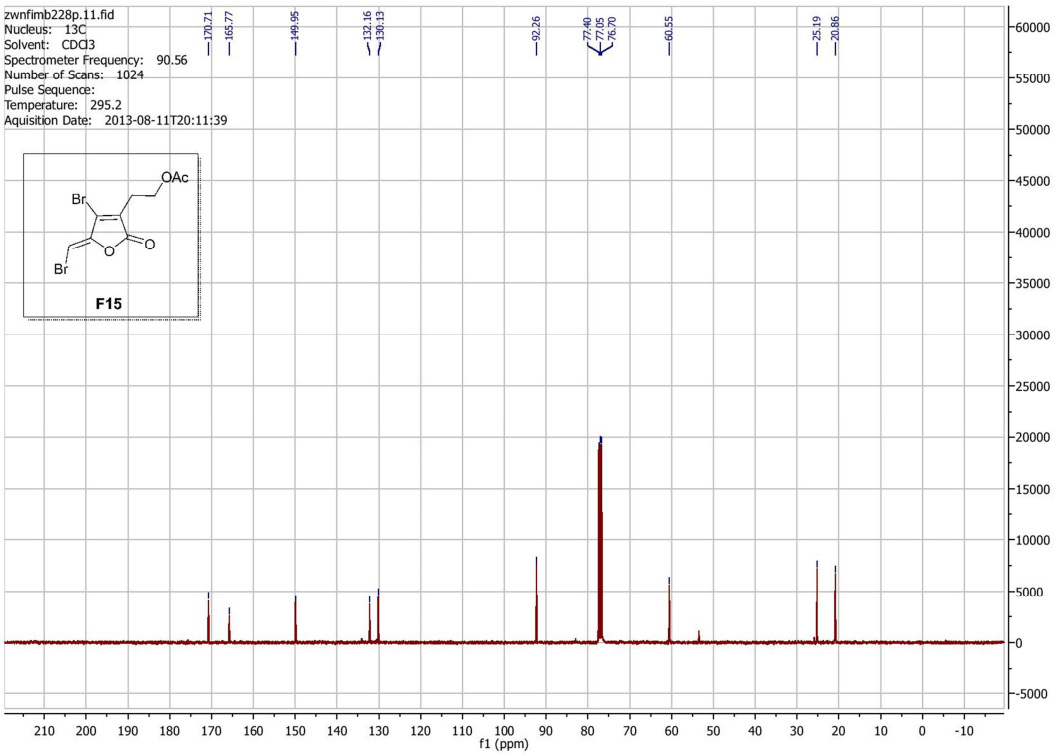

ZC14-PP.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 300.13
Number of Scans: 16
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-01-22T10:11:34
COOH
COOEt
6
f1 (ppm)

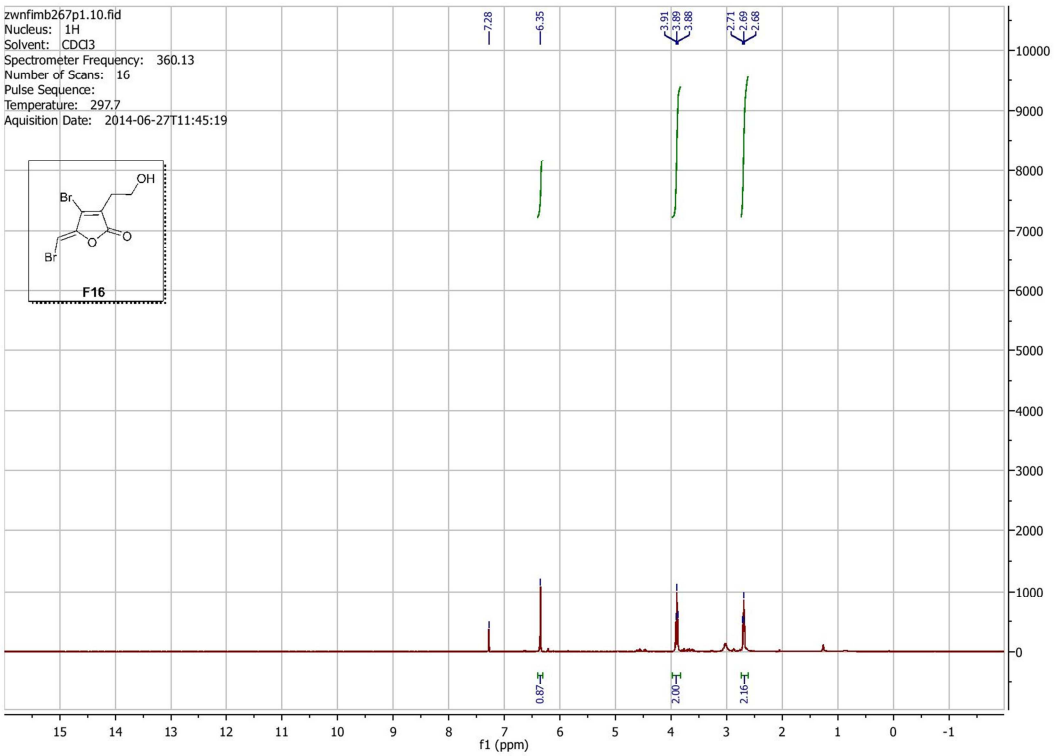

zc014PP-repeat.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 75.48
Number of Scans: 1024
Pulse Sequence:
Temperature: 298.0
Aquisition Date: 2016-04-15T01:31:20
COOH
COOEt
6
f1 (ppm)

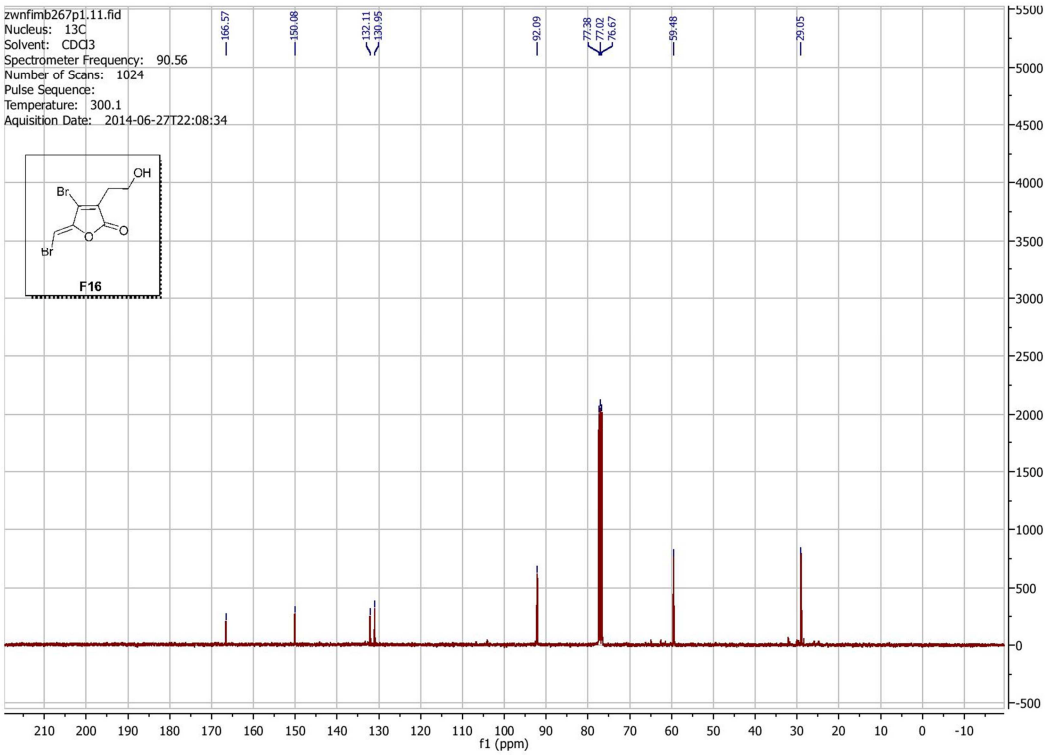

zwnlac007P14.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 300.13
Number of Scans: 16
Pulse Sequence:
Temperature: 295.0
Aquisition Date: 2015-02-27T03:55:18
LP3
f1 (ppm)

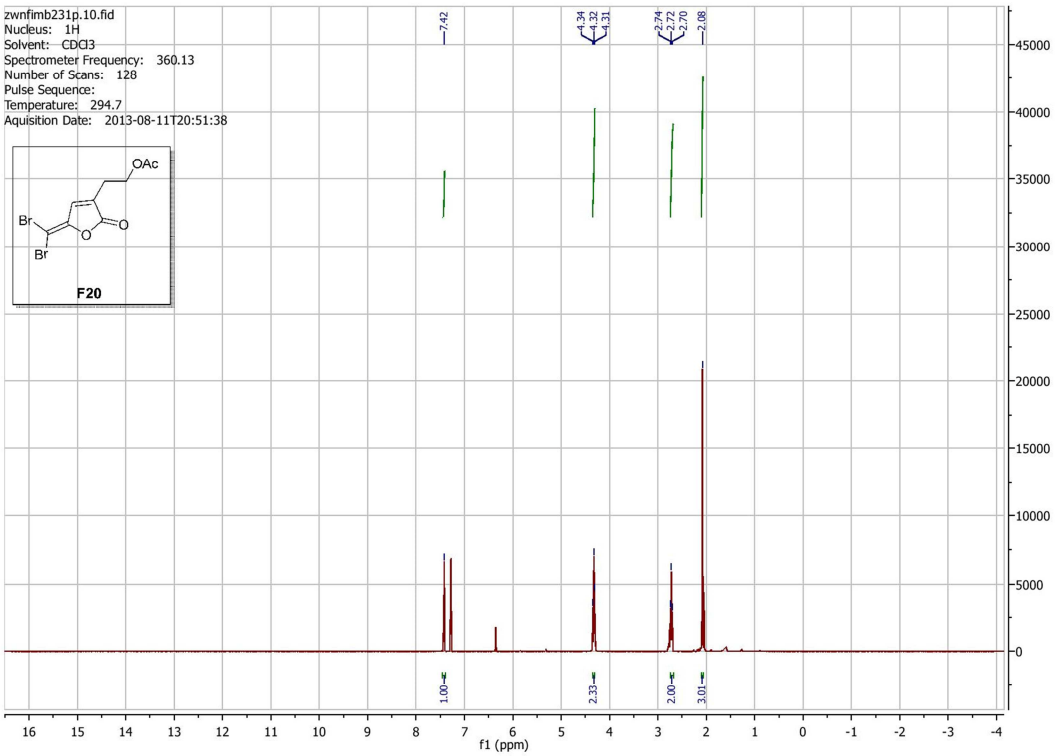

nlac007P14.12.fid
cleus: 13C
vent: CDCl3
ectrometer Frequency: 75.48
mber of Scans: 1346
se Sequence:
nperature: 295.0
uisition Date: 2015-02-27T05:54:17
LP3
f1 (ppm)

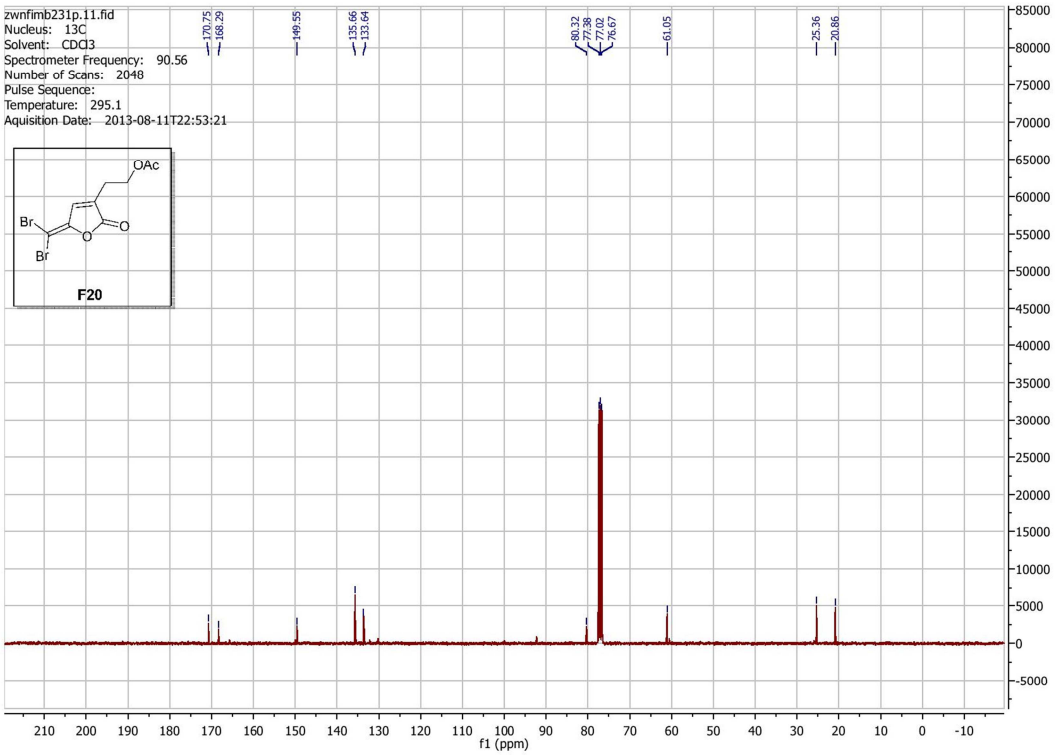

zwnfimb228p.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 128
Pulse Sequence:
Temperature: 294.6
Aquisition Date: 2013-08-11T19:08:35
6.35
4.31
4.30
4.28
2.77
2.76
2.74
2.04
OAc
Br
O
O
Br
F15
0.84
2.00
1.96
3.02
f1 (ppm)


zwnfimb228p.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 90.56
Number of Scans: 1024
Pulse Sequence:
Temperature: 295.2
Aquisition Date: 2013-08-11T20:11:39
170.71
165.77
149.95
132.16
130.13
92.26
77.40
77.05
76.70
60.55
25.19
20.86
OAc
Br
O
O
Br
F15
f1 (ppm)


zwnfimb267p1.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 16
Pulse Sequence:
Temperature: 297.7
Aquisition Date: 2014-06-27T11:45:19
F16
7.28
6.35
3.91
3.89
3.88
2.71
2.69
2.68
0.87
2.00
2.16
f1 (ppm)


zwnfimb267p1.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 90.56
Number of Scans: 1024
Pulse Sequence:
Temperature: 300.1
Aquisition Date: 2014-06-27T22:08:34
F16
166.57
150.08
132.11
130.95
92.09
77.38
77.02
76.67
59.48
29.05
f1 (ppm)


zwnfimb231p.10.fid
Nucleus: 1H
Solvent: CDCl3
Spectrometer Frequency: 360.13
Number of Scans: 128
Pulse Sequence:
Temperature: 294.7
Aquisition Date: 2013-08-11T20:51:38
OAc
Br
Br
O
O
F20
7.42
4.34
4.32
4.31
2.74
2.72
2.70
2.08
1.00
2.33
2.00
3.01
f1 (ppm)


zwnfimb231p.11.fid
Nucleus: 13C
Solvent: CDCl3
Spectrometer Frequency: 90.56
Number of Scans: 2048
Pulse Sequence:
Temperature: 295.1
Aquisition Date: 2013-08-11T22:53:21
OAc
Br
Br
O
O
F20
170.75
168.29
149.55
135.66
133.64
80.32
77.38
77.02
76.67
61.05
25.36
20.86
f1 (ppm)