Metabolic alkene labeling and in vitro detection of histone acylation via the aqueous oxidative Heck reaction

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Contents

1. General

Chemicals were obtained from commercial suppliers (Sigma Aldrich, Acros Organics, Axon Medchem) and used without further purification, unless stated otherwise. Aluminum sheets of Silica Gel 60 F254 were used for Thin layer chromatography (TLC). Spots were visualized under ultraviolet light or stained with $KMnO_4$ solution. MP Ecochrom Silica Gel 32-63 60 Å was used for column chromatography. Nuclear magnetic resonance spectra were recorded on a Bruker Avance 500 spectrometer (^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz)). Chemical shift values are reported in ppm (δ) relative to tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet.

The 4-OT R61C gene was purchased from DNA2.0, Inc. (Menlo Park, CA). The technique for transformation was based on a method reported in literature.[1] For purification of the 4-OT enzyme pre-packed PD-10 Sephadex G-25 gelfiltration columns were used. Fermentas PageRuler™ Prestained Protein Ladder was used as a ladder during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The loading buffer (4x) consists of 20% of 0.2 M Tris-HCl pH 6.8, 8.9 % of SDS, 40% of glycerol, 10% of 0.05 M EDTA, 0.09% of bromophenol blue, 21% of deionized H_2O. Gels were stained with the coomassie based gel stain InstantBlue™ (Expedeon Ltd, Harston, Cambridgeshire, UK). Concentrations of the unmodified proteins were determined using the method of Waddell,[2] using the absorbance as measured on a V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). The Bradford assay was used to determine the concentration of the chemically modified proteins[3] using Coomassie Protein Assay Reagent (950 mL) from Thermo Scientific and the absorbance was measured on a SPECTROstar Omega – UV/Vis absorbance spectrophotometer microplate reader from BMG Labtech. Chemiluminescence imaging was performed in G:BOX from Syngene under no light, no filter and after 5 min exposure.

Protein mass spectrometry was performed using a Shimadzu LC system, consisting of a LC-20AD gradient pumps and a SIL-20AC autosampler. Chromatographic separation was achieved on an Alltima C18 column (2.1x150 mm, 5 µm, Grace Davison Discovery Sciences). The injection volume was 50 µL. Elution was performed by a linear gradient from 5% to 60% eluent B mixed with eluent A in 30 min, followed by an increase to 90% eluent B in 1 min, where it was kept 4 min, after which it returned to the starting conditions. Eluent A was 99.5% H_2O / 0.5% formic acid and eluent B was 95.5% acetonitrile/0.5% formic acid. The flow rate was 0.3 mL/min. The UV signal was recorded at 220 nm. The HPLC system was coupled to an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) via a TurboIonSpray source. The ionization was performed by electrospray in the positive ion mode. Data acquisition and processing was performed using the Analyst software version 1.4.2 and 1.5 (Applied Biosystems/MDS Sciex). Multiply charged peak envelopes of proteins were deconvoluted in Analyst; all reported mass spectra show the reconstructed, uncharged peaks. Mass spectra were recorded in profile mode with 0.1 amu step size, and the mass accuracy of reconstructed protein masses is estimated at 100 ppm.

RAW 264.7 Cells were purchased from American Type Culture Collection, Manassas, USA. Dulbecco's Modified Eagle Medium (DMEM) and Penicillin-Streptomycin (10,000 U/mL) from Life Technologies and Fetal Bovine Serum (FBS) from Biowest were used for the cell culture. Triton™ X-100 for molecular biology and Protease Inhibitor Cocktail were purchased from Sigma Aldrich, Netherlands. The samples were centrifuged in an Eppendorf centrifuge 5810 R and 5415 R. Dulbecco's Phosphate-Buffered Saline (DPBS) (10X), no calcium, no magnesium was purchased from Life Technologies and

Tween® 20, Molecular Biology Grade was purchased from Promega. Immun-Blot® PVDF Membranes from BIO-RAD were used for Western blotting. The membranes were blocked using Campina Elk skimmed milk powder. Streptavidin HRP (ab7403) was purchased from Abcam and Western Lightning® Plus-ECL from PerkinElmer was used for the Enhanced Chemiluminescence assay.

2. Synthesis of starting materials

2.1 Synthesis of Bis(aryl)acenaphthequinonediimine (BIAN) (S1)

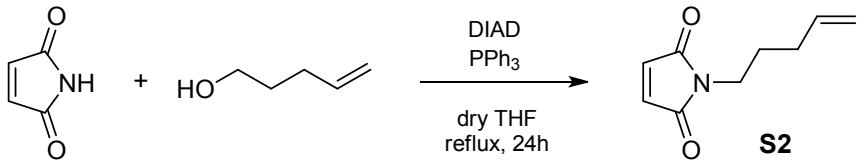
BIAN was synthesized according to literature procedures.[4]

TLC: R_f = 0.5 (5:1 pentane/ether)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.89 (d, *J* = 8.3 Hz, 2H), 7.39 (t, *J* = 7.7 Hz, 2H), 7.08 (t, *J* = 7.5 Hz, 2H), 7.16 (d, *J* = 7.5 Hz, 4H), 6.72 (d, *J* = 7.2 Hz, 2H), 2.14 (s, 12H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 161.0, 149.4, 140.8, 131.2, 129.7, 129.1, 128.5, 128.4, 125.0, 123.8, 122.7, 17.9 ppm.

LC-MS: 389.29 $[M+H]^+$.

2.2 Synthesis of 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione (S2) (Mitsunobu reaction)



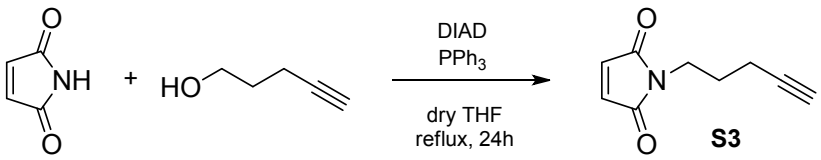
The synthesis was performed as described previously (78% yield).[5]

TLC: R_f = 0.11 (EtOAc/ heptane 1:10).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.58 (s, 2H), 5.62 (m, 1H), 4.85-4.90 (m, 1H), 4.80-4.83 (m, 1H), 3.37 (t, *J* = 7.2 Hz, 2H), 1.90 (m, 2H), 1.52 (m, 2H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ= 179.0 (x2), 136.2 (x2), 133.0, 114.2, 35.7, 29.2, 25.9 ppm.

LC-MS: 166.1 $[M+H]^+$.

2.3 Synthesis of 1-(pent-4-yn-1-yl)-1H-pyrrole-2,5-dione (S3)



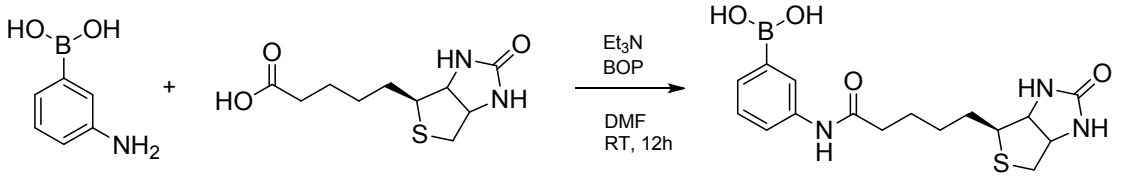
In a 10 mL round bottom flask equipped with a magnetic stirring bar, the maleimide (0.58 g, 6.0 mmol) and PPh_3 (1.6 g, 6.0 mmol) were dissolved in dry THF (4.0 mL). Pent-4-yn-1-ol (0.56 ml, 6.0 mmol) and diisopropylazodicarboxylate (DIAD) (1.2 mL, 6.0 mmol) were added, the flask was equipped with a condenser and the mixture was left stirring for 24 h under reflux. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (EtOAc/ petroleum ether 1:5) to afford the product as white crystals (0.79 g, 81%).

TLC: R_f = 0.76 (EtOAc/ petroleum ether 1:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.67 (s, 2H), 3.59 (t, *J* = 7.1 Hz, 2H), 2.19-2.16 (m, 2H), 1.94 (t, *J* = 7.1 Hz, 1H), 1.82-1.77 (m, 2H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ= 170.7 (x2), 134.2 (x2), 83.0, 69.1, 37.0, 27.2, 16.1 ppm.

LC-MS: 164.1 $[M+H]^+$.

2.4 Synthesis of 3-(biotinylamino)phenylboronic acid (1) (Axon 2256)



1

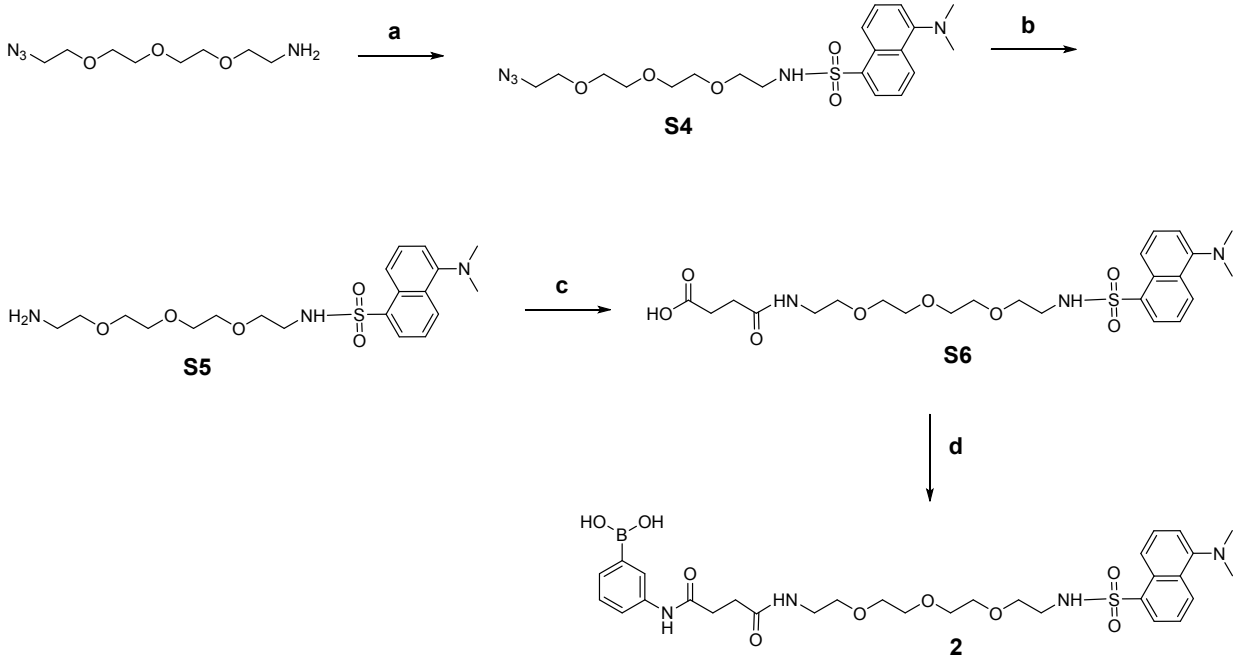
In a 10 mL round bottom flask equipped with a magnetic stirring bar, biotin (0.24 g, 1.0 mmol) was dissolved in DMF (2.0 mL). Et_3N (0.14 mL, 1.0 mmol) was added and the solution was stirred for 15 min. Following the addition of (Benzotriazol-1-yloxy)tris(dimethylamino phosphonium hexafluorophosphate (BOP) (0.44 g, 1.0 mmol) at 0 ^{o}C, the mixture was stirred for 30 min and a solution of 3-aminophenylboronic acid (0.14 g, 1.0 mmol) in DMF (2.0 mL) was added. The reaction mixture was left stirring overnight at room temperature. Ether was added (10x the volume of the reaction mixture) to form a white precipitate. The mixture was centrifuged at 4000 rpm for 20 min. The supernatant was discarded and the residue was purified by column chromatography (CH_2Cl_2:MeOH 15:1) to afford the product as a white solid (0.25 g, 70%).

TLC: R_f =0.25 (CH_2Cl_2:MeOH 4:1).

^{1}H NMR (500 MHz, CD_3OD): δ = 7.81 (s, 1H), 7.61 (d, *J* = 7.3 Hz, 1H), 7.47 (s, 1H), 7.28 (t, *J* = 7.3 Hz, 1H), 4.47-4.46 (m, 1H), 4.28-4.27 (m, 1H), 3.19-3.17 (m, 1H), 2.91-2.88 (dd, *J* = 12.8 Hz, 4.9 Hz, 1H), 2.69 (d, *J* = 12.7 Hz, 1H), 2.38 (t, *J* = 7.3 Hz, 2H), 1.79-1.67 (m, 3H), 1.65-1.55 (m, 1H), 1.52-1.40 (m, 2H) ppm. **^{13}C NMR** (125 MHz, CD_3OD): δ = 174.3, 165.9, 138.9, 135.3, 130.6, 128.9, 126.7, 123.4, 63.1, 61.4, 56.8, 40.9, 37.5, 29.6, 29.3, 26.6 ppm.

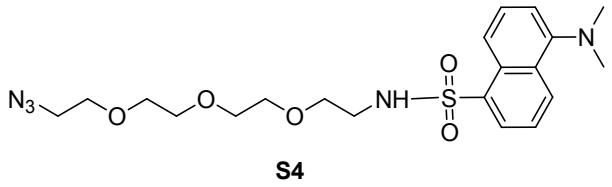
LC-MS: 364.3 $[M+H]^+$.

2.5 Synthesis of 3-(Dansyl-PEG-amino)phenylboronic acid (2) (Axon 2257)



Scheme S1: Synthesis of (3-(1-(5-(dimethylamino)naphthalene-1-sulfonamido)-13-oxo-3,6,9-trioxa-12-azahexadecanamido)phenyl)boronic acid **(2)**. a) Et_3N, dansylchloride, CH_2Cl_2, 83% yield, b) H_2, Pd/C 10%, MeOH, 99% yield, c) Et_3N, succinic anhydride, THF, CH_3CN, 99% yield, d) Et_3N, BOP,3-aminophenylboronic acid, DMF, 51% yield.

2.5.1 Synthesis of N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-(dimethylamino)naphthalene-1-sulfonamide (S4)



S4

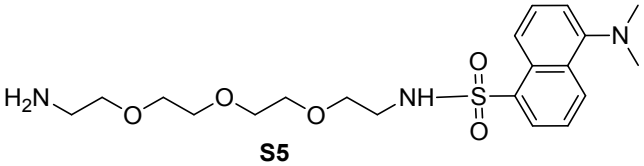
In a 50 mL round bottom flask equipped with a magnetic stirring bar, 11-azido-3,6,9-trioxaundecan-1-amine (0.95 mL, 4.4 mmol) was dissolved in CH_2Cl_2 (10 mL). Triethylamine (2.0 mL, 14 mmol) was added and the solution was stirred for 10 min. A solution of dansylchloride (1.1 g, 4.0 mmol) in CH_2Cl_2 (10 mL) was added dropwise and the reaction mixture was left stirring for 12 h at room temperature. The solvent was evaporated and the residue was purified by column chromatography (heptane:ethyl acetate 3:1) to afford the product as a yellow oil (1.5 g, 83%).

TLC: R_f =0.34 (heptane:ethyl acetate 1:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.48 (d, *J* = 8.7 Hz, 1H), 8.28 (d, *J* = 8.7 Hz, 1H), 8.19 (d, *J* = 7.2 Hz, 1H), 7.53-7.50 (m, 1H), 7.48-7.45 (m, 1H), 7.14 (d, *J* = 7.6 Hz, 1H), 5.64 (t, *J* = 5.8 Hz, 1H), 3.61-3.58 (m, 4H), 3.55-3.53 (m, 2H), 3.43-3.42 (m, 2H), 3.33-3.30 (m, 6H), 3.08-3.05 (m, 2H), 2.83 (s, 6H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 151.8, 135.1, 130.2, 129.8, 129.6, 129.1, 128.2, 123.1, 119.0, 115.1, 70.5, 70.4, 70.3, 70.1, 69.9, 69.1, 50.5, 45.3 (x2), 43.0 ppm.

LC-MS: 452.5 $[M+H]^+$.

2.5.2 Synthesis of N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-5-(dimethylamino)naphthalene-1-sulfonamide (6)



S5

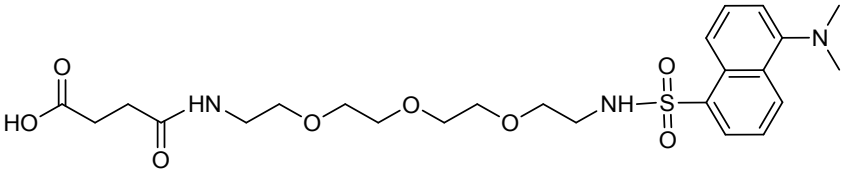
S4 (1.5 g, 3.3 mmol) was dissolved in MeOH (20 mL). Pd on activated carbon 10% (35 mg, 0.33 mmol in Pd) was added and the mixture was left shaking under hydrogen atmosphere (3 atm) for 5 h. After filtration through a pad of celite, the solvent was evaporated to afford the product as a yellow viscous oil (1.39 g, 99%).

TLC: R_f =0.13 (CH_2Cl_2:MeOH 5:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ =8.48 (d, *J* = 8.7 Hz, 1H), 8.35 (d, *J* = 8.7 Hz, 1H), 8.20 (d, *J* = 7.2 Hz, 1H), 7.55-7.51 (m, 1H), 7.50-7.46 (m, 1H), 7.16 (d, *J* = 7.6 Hz, 1H), 4.58 (bs, 2H), 3.62-3.57 (m, 4H), 3.51-3.49 (m, 4H), 3.41-3.37 (m, 6H), 3.11-3.09 (m, 2H), 2.86 (s, 6H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ =152.3, 136.2, 130.4, 130.3, 130.2, 129.4, 128.4, 123.6, 119.7, 115.4, 73.1, 70.6, 70.4, 70.2, 70.1, 69.8, 45.3 (x2), 42.9, 41.3 ppm.

LC-MS: 426.4 $[M+H]^+$.

2.5.3 Synthesis of 1-(5-(dimethylamino)naphthalene-1-sulfonamido)-13-oxo-3,6,9-trioxa-12-azahexadecan-16-oic acid (S6)



S6

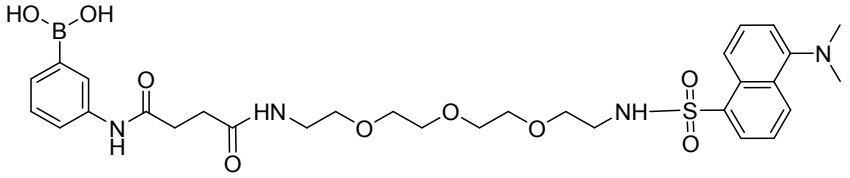
In a 25 mL round bottom flask equipped with a magnetic stirring bar, **S5** (1.3 g, 3.0 mmol) was dissolved in THF (7.0 mL). Et_3N (0.42 mL, 3.0 mmol) was added and the mixture was left stirring for 15 min. A solution of succinic anhydride (0.30 g, 3.0 mmol) in CH_3CN (3.0 mL) was added dropwise at 0 ^oC. The mixture was left stirring at room temperature overnight. The solvents were evaporated to afford the residue as a yellow oil (1.57 g, 99%).

TLC: R_f =0.09 (CH_2Cl_2:MeOH 15:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ =10.95 (bs, 1H), 8.48 (d, *J* = 8.4 Hz, 1H), 8.29 (d, *J* = 8.4 Hz, 1H), 8.18 (d, *J* = 7.2 Hz, 1H), 7.53-7.50 (m, 1H), 7.49-7.46 (m, 1H), 7.15 (d, *J* = 7.6 Hz, 1H), 6.90 (bs, 1H), 6.20 (bs, 1H), 3.60-3.55 (m, 4H), 3.54-3.49 (m, 2H), 3.46-3.42 (m, 2H), 3.40-3.32 (m, 6H), 3.10-3.06 (m, 2H), 2.84 (s, 6H), 2.53 (t, *J* = 6.7 Hz, 2H), 2.43 (t, *J* = 6.7 Hz, 2H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ =176.7, 173.2, 151.9, 135.4, 130.3, 129.9, 129.7, 129.2, 128.3, 123.2, 119.1, 115.3, 70.4, 70.3, 70.2, 70.0, 69.8, 69.4, 45.5 (x2), 43.0, 39.3, 31.7, 31.2 ppm.

LC-MS: 526.6 $[M+H]^+$.

2.5.4 Synthesis of 3-(Dansyl-PEG-amino)phenylboronic acid (2)



2

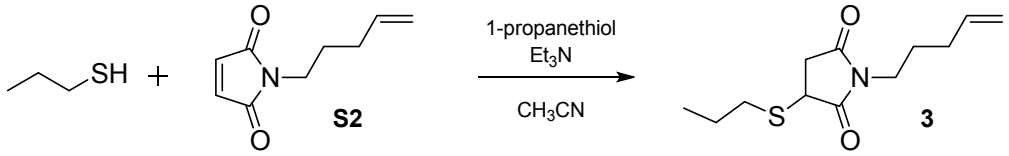
In a 10 mL round bottom flask equipped with a magnetic stirring bar, **S6** (1.6 g, 3.0 mmol) was dissolved in DMF (7.0 mL). Et_3N (0.42 mL, 3.0 mmol) was added and the solution was stirred for 15 min. Following the addition of benzotriazol-1-yloxy)tris(dimethylamino phosphonium hexafluorophosphate (BOP) (1.3 g, 3.0 mmol) at 0 °C, the mixture was stirred for 30 min and a solution of 3-aminophenylboronic acid (0.42 g, 3.0 mmol) in DMF (3.0 mL) was added. The reaction mixture was left stirring overnight at room temperature. Diethyl ether was added (10x the volume of the reaction mixture) to form a white precipitate. The mixture was centrifuged at 4000 rpm for 20 min. The supernatant was discarded and the residue was purified by column chromatography (CH_2Cl_2:MeOH 10:1) to afford the product as a brown oil (0.97 g, 51%).

TLC: R_f = 0.8 (CH_2Cl_2:MeOH 4:1).

^{1}H NMR (500 MHz, CD_3OD): δ =8.46 (d, J = 8.4 Hz, 1H), 8.31 (d, *J* = 8.4 Hz, 1H), 8.15 (d, *J* = 7.4 Hz, 1H), 7.89 (s, 1H), 7.73 (d, *J* = 7.1 Hz, 1H), 7.57 (d, *J* = 7.8 Hz, 1H), 7.51 (m, 1H), 7.49 (m, 1H), 7.22 (m, 1H), 7.18 (d, *J* = 7.6 Hz, 1H), 3.47-3.44 (m, 6H), 3.35-3.33 (m, 2H), 3.32-3.29 (m, 2H), 3.26-3.24 (m, 4H), 2.99 (m, 2H), 2.78 (s, 6H), 2.63 (t, *J* = 6.8 Hz, 2H), 2.53 (t, *J* = 6.8 Hz, 2H) ppm. **^{13}C NMR** (125 MHz, CD_3OD): δ =174.7, 172.8, 153.1, 137.2, 131.1, 130.9, 130.6, 130.1, 130.0, 129.1, 126.7, 126.1, 124.3, 123.3, 120.5, 116.3, 71.4, 71.2, 71.1, 71.1, 70.9, 70.4, 45.8 (x2), 43.7, 40.5, 33.0, 31.9 ppm.

LC-MS: 645.5 $[M+H]^+$.

2.6 Synthesis of 1-(pent-4-en-1-yl)-3-(propylthio)pyrrolidine-2,5-dione (3) (Michael addition)



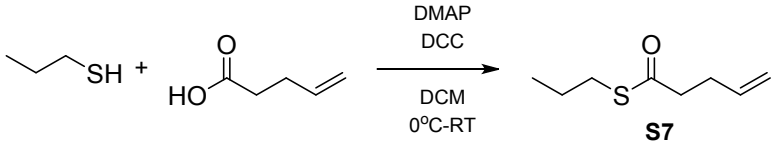
The synthesis was performed as described previously (69% yield).[5]

TLC: R_f = 0.11 (EtOAc/ heptane 1:10).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.79 (m, 1H), 5.06-4.97 (m, 2H), 3.68 (dd, *J* = 9 Hz, *J* = 3.7 Hz, 1H), 3.52 (t, *J* =7.4 Hz, 2H), 3.09 (dd, *J* = 18.7 Hz, *J* = 9 Hz, 1H), 2.87 (m, 1H), 2.72 (m, 1H), 2.52 (dd, *J* = 18.7 Hz, *J* = 3.7 Hz, 1H), 2.06 (m, 2H), 1.68 (m, 4H), 1.01 (t, *J* = 7.4 Hz, 3H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 176.0, 174.1, 136.3, 114.4, 37.5, 37.1, 34.6, 32.2, 29.3, 25.0, 20.8, 11.7 ppm.

GC-MS: m/z 241.1.

2.7 Synthesis of *S*-propyl pent-4-enethioate (S7)



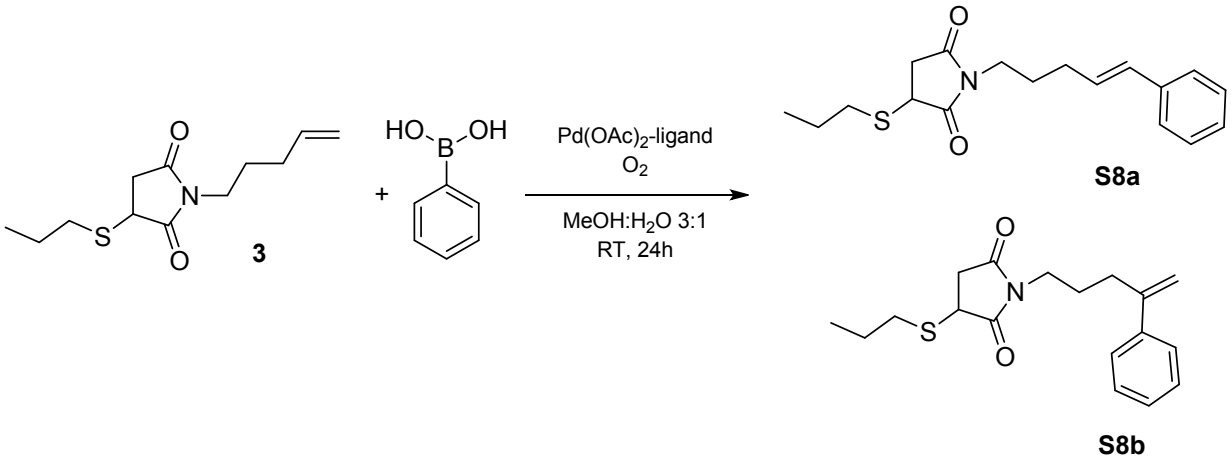
In a 10 mL round bottom flask equipped with a magnetic stirring bar, 4-pentenoic acid (0.51 mL, 5 mmol) was dissolved in CH_2Cl_2 (5.0 mL). Following the addition of 4-dimethylaminopyridine (41 mg, 0.25 mmol) and propane-1-thiol (0.54 mL, 6.0 mmol), *N,N'*-dicyclohexylcarbodiimide (1.2 g, 6.0 mmol) was added at 0°C and the mixture was left stirring overnight at room temperature. The urea formed (white precipitate) was filtered off through a pad of celite. The filtrate was concentrated and purified by flash column chromatography (heptane:ethyl acetate: 10:1) to afford the product as a colorless oil (0.63 g, 80%).

TLC: R_f =0.78 (heptane:ethyl acetate 2:1) (stained with $KMnO_4$).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.82-5.74 (m, 1H), 5.06-4.98 (m, 2H), 2.84 (t, *J* = 7.2 Hz, 2H), 2.62 (t, *J* = 7.2 Hz, 2H), 2.41-2.37 (m, 2H), 1.61-1.54 (m, 2H), 0.95 (t, *J* = 7.3 Hz, 3H) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$): δ = 198.9, 136.4, 115.8, 43.3, 30.9, 29.6, 23.1, 13.4 ppm.

LC-MS: 157.1 $[M-H]^-$.

3. Oxidative Heck reactions on 1-(pent-4-en-1-yl)-3-(propylthio)pyrrolidine-2,5-dione (3)



The Oxidative Heck reaction conditions on small molecules-terminal alkenes, reported previously by Minnaard *et al.* (olefin (1.0 equiv.), palladium acetate (5.0 mol %, 0.05 equiv.), and BIAN (7.0 mol %, 0.07 equiv., phenylboronic acid 1.5 equiv. and reaction time 30 h))[4],[5] were modified in order to imitate the conditions applied for the protein conjugation.

BIAN ligand

The two isomeric products **S8a** and **S8b** were obtained according to a previously described procedure (ratio 2.3:1, 82% yield).[5]

2-amino-4,6-dihydroxypyrimidine ligand

The catalyst was prepared according to literature procedure.[6]

In a 10 mL double neck round bottom flask, equipped with a magnetic stirring bar and a septum, was added 2-amino-4,6-dihydroxypyrimidine (0.13 g, 1.0 mmol, 2.0 equiv.) and NaOH (2.0 mL, 1.0 M). The pyrimidine ligand was dissolved completely by stirring for 2 min in a water bath preheated to 65 °C. To the resulting solution was added $Pd(OAc)_2$ (0.11 g, 0.50 mmol, 1.0 equiv.). The flask was equipped with an oxygen balloon on the side arm. Oxygen was immediately flushed through the flask and the mixture was left stirring vigorously at 65 °C for 30 min to give a homogenous yellow-orange solution. After cooling to room temperature, the olefin (0.12 g, 0.50 mmol, 1.0 equiv.) and the phenylboronic acid (0.61 g, 5.0 mmol, 10 equiv.) were added as solutions in MeOH (3.0 mL) and the reaction mixture was allowed to stir at room temperature under oxygen atmosphere for 24 h. MeOH was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (5.0 mL) and washed with H_2O (3x10 mL). The organic phase was dried over $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/ heptane 1:10) to afford **S8a** and **S8b** in a ratio 3.8: 1 as a yellowish oil (0.1 g, 64%).

TLC: R_f = 0.11 (EtOAc/ heptane 1:10).

S8a: ^{1}**H NMR** (500 MHz, $CDCl_3$): δ = 7.39-7.18 (m, 5H), 6.39 (d, *J* = 15.8 Hz, 1H), 6.21-6.15 (m, 1H), 3.67 (dd, *J* = 9.0 Hz, *J* = 3.7 Hz, 1H), 3.58 (t, *J* = 7.4 Hz, 2H), 3.06 (dd, *J* = 18.7 Hz, *J* = 9.0, 1H), 2.90-2.84 (m, 1H), 2.73-2.68 (m, 1H), 2.51 (d, *J* = 3.7 Hz, 1H), 2.26-2.22 (m, 2H), 1.71-1.60 (m, 4H), 1.00 (t, *J* = 7.4 Hz, 3H) ppm. ^{13}**C NMR** (125 MHz, $CDCl_3$): δ = 176.9, 175.0, 137.6, 130.9, 129.2, 128.7, 127.2, 126.1, 39.1, 38.8, 36.3, 33.9, 30.4, 27.1, 22.5, 13.5 ppm.

S8b: ^{1}**H NMR** (500 MHz, $CDCl_3$): δ = 7.39-7.18 (m, 5H), 5.29 (s, 1H), 5.09 (s, 1H), 3.67 (dd, *J* = 9 Hz, *J* = 3.7 Hz, 1H), 3.56 (t, *J* = 7.4 Hz, 2H), 3.06 (dd, *J* = 18.7 Hz, *J* = 9 Hz, 1H), 2.90-2.84 (m, 1H), 2.73-2.68 (m, 1H), 2.48 (d, *J* = 3.7 Hz, 1H), 1.82-1.76 (m, 6H), 1.00 (t, *J* = 7.4 Hz, 3H) ppm. ^{13}**C NMR** (125 MHz, $CDCl_3$): δ = 176.8, 174.9, 147.3, 141.0, 128.5, 127.6, 126.2, 113.0, 39.1, 38.9, 38.5, 32.6, 29.8, 26.3, 22.5, 13.5 ppm.

EDTA ligand

EDTA stock solution (0.26 M, pH 7.0)

Ethylenediaminetetraacetic acid disodium salt dihydrate (48 g, 0.13 mol) was dissolved in deionized H_2O (50 mL) and the pH was set at 7.0 using a concentrated solution of NaOH.

To a 10 mL double neck round bottom flask equipped with a magnetic stirring bar and a septum were added palladium acetate (0.11 g, 0.50 mmol, 1.0 equiv.), and EDTA (1.9 mL, 0.26 M, 0.50 mmol, 1.0 equiv.). The flask was equipped with an oxygen balloon on the side arm. Oxygen was immediately flushed through the flask and the mixture was left stirring vigorously for 1 h at 66 °C and under oxygen atmosphere until it became an homogenous yellow solution. The olefin (0.12 g, 0.50 mmol, 1.0 equiv.) and the phenylboronic acid (0.61 g, 5.0 mmol, 10 equiv.) were dissolved in MeOH (3.0 mL) each and then added to the flask. The reaction mixture was allowed to stir at room temperature under oxygen atmosphere for 24 h. MeOH was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (5.0 mL) and washed with H_2O (3x10 mL). The organic phase was dried

over $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure. ^1H NMR showed peaks of the starting material along with peaks of the two isomeric products (ratio 3.3:1, 50% conversion).

4. Oxidative Heck reactions on protein-bound terminal alkene 4-OT R61C-1

4.1 Preparation of 4-OT R61C-1

4.1.1 Sequence of 4-Oxalocrotonate tautomerase (4-OT) R61C mutant

PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKV**C**R

4.1.2 Expression and purification 4-OT R61C

The 4-OT R61C mutant enzyme was produced in E. coli BL21 (DE3) as a native protein without His-tag using the Pj Express 414 expression system. The purification was performed according to literature procedures.[7]

4-OT R61C was stored in 0.5 mL cups as a 10 mg/mL solution (determined by Waddell method) in ammonium formate buffer (pH 8.0, 50 mM). An aliquot of this protein sample was directly analysed by ESI-MS.

The cups containing the protein solution were snapfrozen in liquid nitrogen and kept in -20°C.

ESI-MS spectrum **4-OT R61C**: mass expected= 6757, mass found= 13513 (dimer)

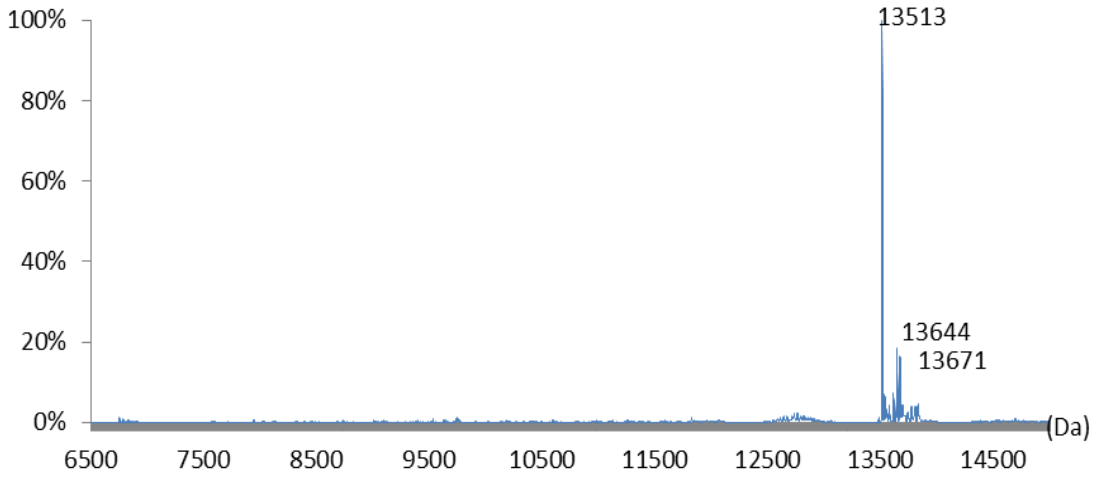
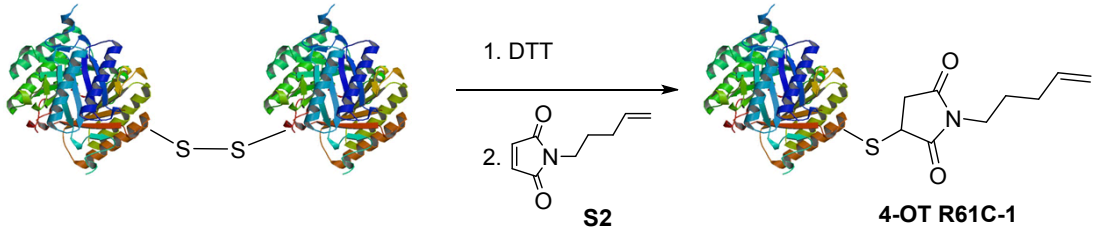


Figure S1. Expressed and purified **4-OT R61C**. ESI-MS spectrum **4-OT R61C**: mass expected= 6757, mass found= 13513 (dimer).

Note: The mass of 13644 shows that, in a small extent, during the expression of the enzyme the amino acid methionine was not cleaved. The mass of 13671 corresponds to the formylated methionine.

4.1.3 Reduction of 4-OT R61C dimer and coupling with 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione (4-OT R61C-1)



The protein solution was warmed to room temperature. A 0.13 mL aliquot (96 nmol) was added to a 1.5 mL cup along with sodium phosphate buffer (0.30 mL, pH 8.0, 50 mM). A stock solution of dithiothreitol (DTT) was prepared by dissolving DTT (3.1 mg, 0.020 mmol) in sodium phosphate buffer (0.32 mL, pH 8.0, 50 mM). In order to reduce the disulfide, 30 µL of the DTT stock solution was added to the protein solution and the mixture was shaken at room temperature for 10 min. A stock solution of 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione was prepared by dissolving **S2** (34 mg, 0.21 mmol) in CH_3CN (0.33 mL). The final concentration of the stock was 0.63 M. 30 µL of the stock solution of **S2** was added to the protein solution and the mixture was shaken at room temperature for 10 min. The protein was purified by using a PD-10 size exclusion column. The column was washed 3 times with deionized water and 3 times with ammonium formate buffer (pH 7.0, 50 mM). The sample was loaded onto the column and elution was conducted with the same buffer. The fractions containing 4-OT were identified by polyacrylamide gel electrophoresis and the concentration of the protein was determined using the Bradford assay and a solution of known concentration of reduced 4-OT R61C in DTT as a reference.

The protein sample was diluted to reach a final concentration 0.65 mg/mL. An aliquot of this protein solution was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with a mass corresponding to the mass of **4-OT R61C-1**.

ESI-MS spectrum: mass expected 6922, mass found 6923 (7054: modified enzyme with methionine, 7081: modified enzyme with formylated methionine).

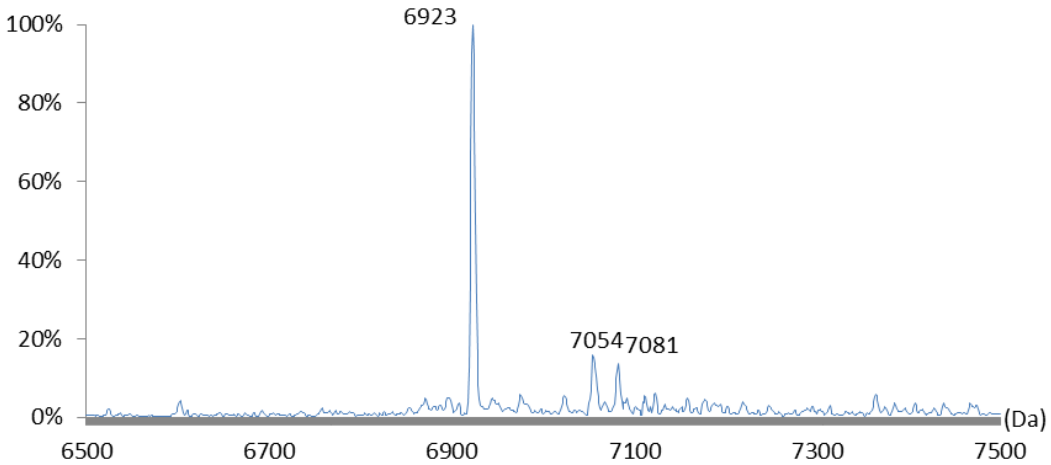


Figure S2. Alkene labeling of **4-OT R61C** to give **4-OT R61C-1**. ESI-MS spectrum: mass expected 6922, mass found 6923 (7054: modified enzyme with methionine, 7081: modified enzyme with formylated methionine).

4.2 Preparation of stock solutions

BIAN-Pd (II)catalyst stock solution

BIAN (4.3 mg, 11 μmol) and $Pd(OAc)_2$ (1.8 mg, 8.0 μmol) were dissolved in DMF (1.0 mL) in a 10 mL double neck round bottom flask equipped with a magnetic stirring bar and a septum. The flask was equipped with an oxygen balloon on the side arm. Oxygen was immediately flushed through the flask and the mixture was left stirring for 30 min at room temperature under oxygen atmosphere. The final concentration in Pd was 8.0 mM and in BIAN 11 mM.

2-amino-4,6-dihydroxypyrimidine-Pd (II) catalyst stock solution

The catalyst was prepared according to literature a procedure.[6]

In a 10 mL double neck round bottom flask, equipped with a magnetic stirring bar and a septum, was added 2-amino-4,6-dihydroxypyrimidine (13 mg, 0.10 mmol) and NaOH (2.0 mL, 0.10 M). The pyrimidine ligand was dissolved completely by stirring for 2 minutes in a water bath preheated to 65 ^oC. To the resulting solution was added $Pd(OAc)_2$ (11 mg, 0.05 mmol). The flask was equipped with an oxygen balloon on the side arm. Oxygen was immediately flushed through the flask and the mixture was left stirring vigorously at 65 ^oC for 30 min to give a homogenous yellow-orange solution. After cooling to room temperature, the stirring bar was removed and the solution was diluted to 5.0 mL with ammonium formate buffer (pH 7.0, 50 mM). The final concentration in Pd was 0.01 M.

Note: When BIAN and 2-amino-4,6-dihydroxypyrimidine were used as ligands, mass spectrometric analysis revealed protein peaks with an additional mass of 105, corresponding to Pd, presumably due to the fact that palladium (II) binds to aminoacids such as histidine.[8] In order to resolve this problem, EDTA was used to chelate palladium after the completion of the reaction.

EDTA stock solution (1.0 M, pH 7.0)

Ethylenediaminetetraacetic acid disodium salt dihydrate (18.6 g, 50 mmol) was dissolved in deionized H_2O (50 mL) and the pH was set at 7.0 using a concentrated solution of NaOH.

EDTA-Pd (II) catalyst solution

EDTA stock solution (8.0 mM, pH 7.0)

Ethylenediaminetetraacetic acid disodium salt dihydrate (0.15 g, 0.40 mmol) was dissolved in deionized H_2O (50 mL) and the pH was set at 7.0 using a concentrated solution of NaOH.

$Pd(OAc)_2$ (1.8 mg, 8.0 μmol) was dissolved in EDTA solution (1.0 mL, 8.0 mM, pH 7.0) in a 10 mL double neck round bottom flask equipped with a magnetic stirring bar and a septum. The flask was equipped with an oxygen balloon on the side arm. Oxygen was immediately flushed through the flask and the mixture was left stirring vigorously for 1 h at 66 ^oC and under oxygen atmosphere until it becomes an homogenous yellow solution. The final concentration in Pd was 8.0 mM.

3-(biotinylamino)phenylboronic acid stock solution

1 (5.0 mg, 14 μmol) was dissolved in MeOH (0.14 mL). The final concentration in phenylboronic acid was 0.1 M.

3-(Dansyl-PEG-amino)phenylboronic acid stock solution

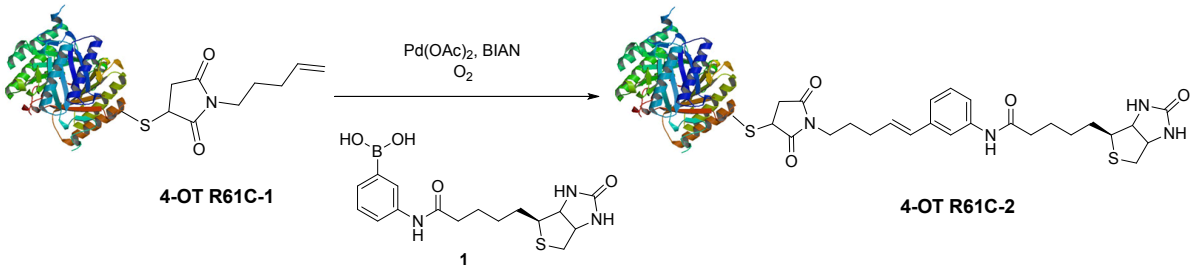
2 (5.0 mg, 7.8 µmol) was dissolved in MeOH (70 µL). The final concentration was 0.1 M.

Phenylboronic acid 40 mM stock solution

Phenylboronic acid (4.0 mg, 33 µmol) were dissolved in ammonium formate buffer (0.83 mL, 0.4 M, pH= 3, 5, 7, 9 and 11).

4.3 Coupling 3-(biotinylamino)phenylboronic acid to protein-bound terminal alkene 4-OT R61C-1

BIAN ligand



4-OT R61C-1 (0.21 mL, 0.65 mg/mL, 20 nmol), **1** (20 µL, 0.1 M, 2.0 µmol) and BIAN-Pd (II) (50 µL, 8.0 mM, 0.4 µmol in Pd, 0.55 µmol in BIAN) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and EDTA (0.2 mL, 8.0 mM) was added. The solution was left rotating for 3 h at room temperature. Afterwards, it was transferred in a 10 mL tube and DMF (5.0 mL) was added and the mixture was centrifuged at 13300 rpm for 5 min. The supernatant was removed and the pellet was redissolved in deionized H_2O (0.4 mL). LC-MS analysis showed 54% conversion of the protein **4-OT R61C-1** to protein **4-OT R61C-2**.

ESI-MS spectrum: mass expected 7240, mass found 7241

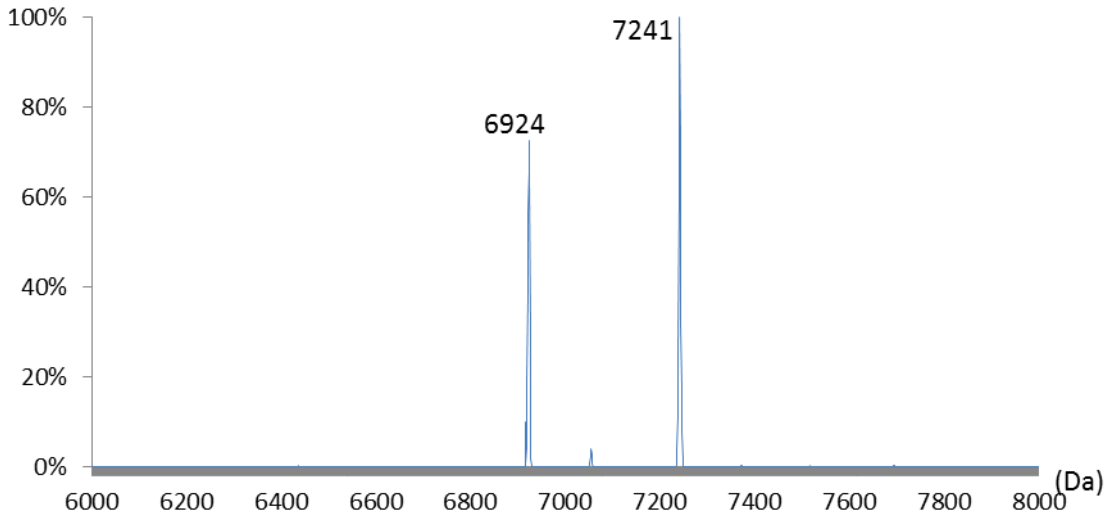
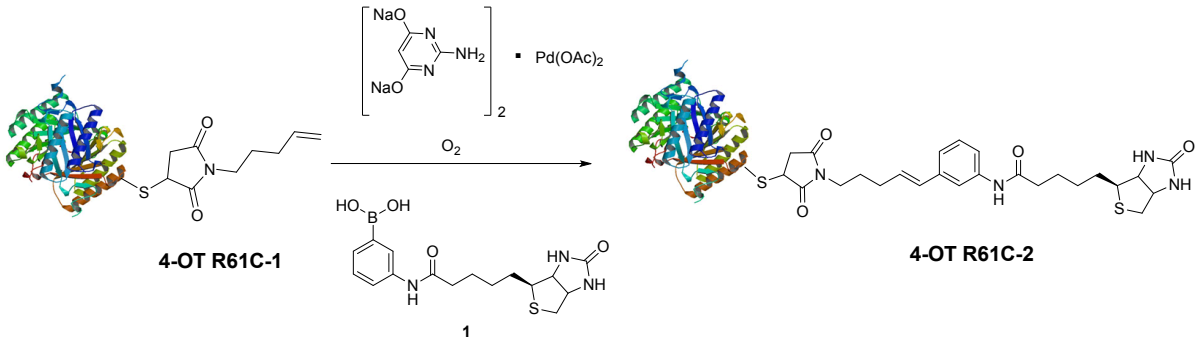


Figure S3. Coupling of **1** to **4-OT R61C-1** via the oxidative Heck reaction using BIAN as ligand. ESI-MS spectrum: mass expected 7240, mass found 7241.

2-amino-4,6-dihydroxypyrimidine ligand



4-OT R61C-1 (0.21 mL, 0.65 mg/mL, 20 nmol), **1** (20 µL, 0.1 M, 2.0 µmol) and 2-amino-4,6-dihydroxypyrimidine-Pd (II) (40 µL, 0.01 M, 0.4 µmol in Pd) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and EDTA (0.2 mL, 8.0 mM) was added. The solution was left rotating for 3 h at room temperature. Afterwards, it was transferred in a 10 mL tube and DMF (5.0 mL) was added and the mixture was centrifuged at 13300 rpm for 5 min. The supernatant was removed and the pellet was redissolved in deionized H_2O (0.4 mL). LC-MS analysis showed around 84% conversion of the protein **4-OT R61C-1** to protein **4-OT R61C-2**.

ESI-MS spectrum: mass expected 7240, mass found 7241

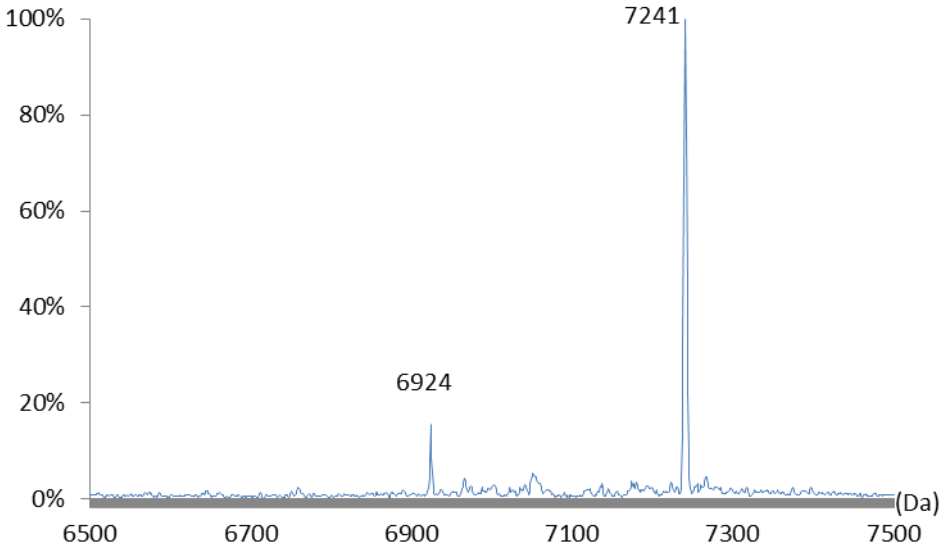
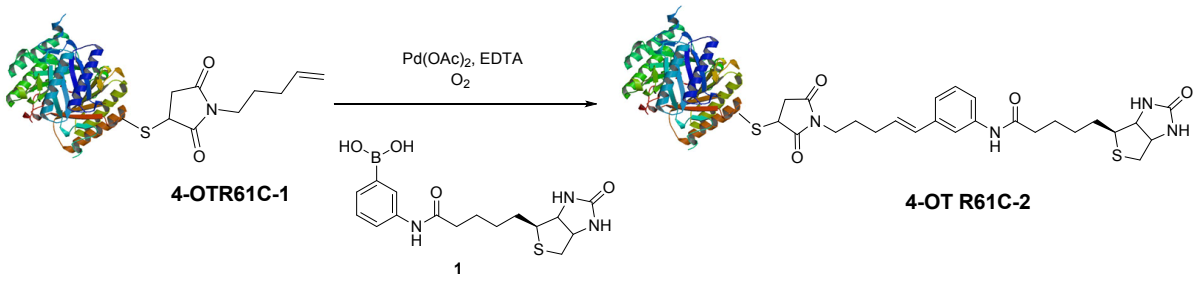


Figure S4. Coupling of **1** to **4-OT R61C-1** via the oxidative Heck reaction using 2-amino-4,6-dihydroxypyrimidine as ligand. ESI-MS spectrum: mass expected 7240, mass found 7241.

EDTA ligand

71 µM scale



4-OT R61C-1 (0.21 mL, 0.65 mg/mL, 20 nmol), **1** (20 µL, 0.1 M, 2.0 µmol) and EDTA-Pd (II) (50 µL, 8.0 mM, 0.4 µmol in Pd, 0.4 µmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred in a 10 mL tube and DMF (5.0 mL) was added and the mixture was centrifuged at 13300 rpm for 5 min. The supernatant was removed and the pellet was redissolved in deionized H_2O (0.4 mL). LC-MS analysis showed full conversion of the protein **4-OT R61C-1** to protein **4-OT R61C-2**.

ESI-MS spectrum: mass expected 7240, mass found 7241

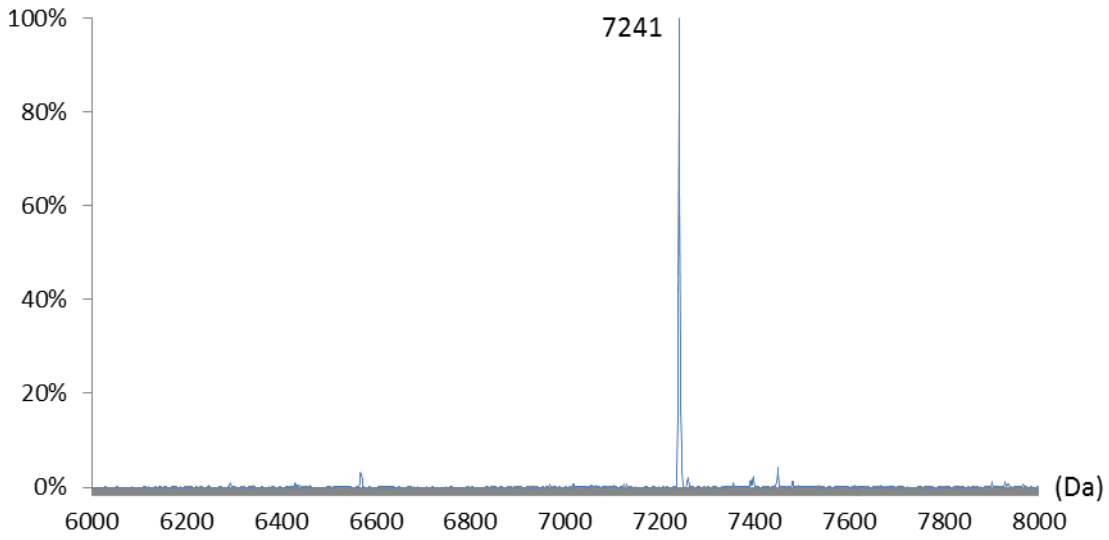


Figure S5. Coupling of **1** to **4-OT R61C-1** via the oxidative Heck reaction using EDTA as ligand. ESI-MS spectrum: mass expected 7240, mass found 7241.

5 µM scale

4-OT R61C-1 (21 µL, 0.65 mg/mL, 2.0 nmol), **1** (2.0 µL, 0.1 M, 0.2 µmol) and EDTA-Pd (II) (5.0 µL, 8.0 mM, 40 nmol in Pd, 40 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum along with ammonium formate buffer (0.37 mL, 50 mM, pH 7.0). The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h the reaction was stopped and stored at -20 °C. (see SDS-PAGE below at 4.6.2 "Oxidative Heck reaction on **4-OT R61C-1** in presence of a cell lysate (ratio 1:10)").

3 µM scale

3-(biotinylamino)phenylboronic acid 0.01 M

For smaller scale reactions a 10 times dilution of the 0.1 M stock of **1** was needed: 10 µL of the 0.1 M solution were added in 90 µL of MeOH.

4-OT R61C-1 (6.4 µL, 0.65 mg/mL, 0.6 nmol), **1** (6.0 µL, 0.01 M, 60 nmol) and EDTA-Pd (II) (1.5 µL, 8.0 mM, 12 nmol in Pd, 12 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum along with ammonium formate buffer (0.19 mL, 50 mM, pH 7.0). The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h the reaction was stopped and stored at -20 °C.

1 µM scale

EDTA-Pd (II) solution 4.0 mM

To the solution of 8.0 mM, prepared as mentioned above, were added ammonium formate buffer (1.0 mL, 50 mM, pH 7.0).

4-OT R61C-1 (2.1 µL, 0.65 mg/mL, 0.2 nmol), **1** (2.0 µL, 0.01 M, 20 nmol) and EDTA-Pd (II) (1.0 µL, 4.0 mM, 4 nmol in Pd, 4 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum along with ammonium formate buffer (0.20 mL, 50 mM, pH 7.0). The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h the reaction was stopped and stored at -20 °C.

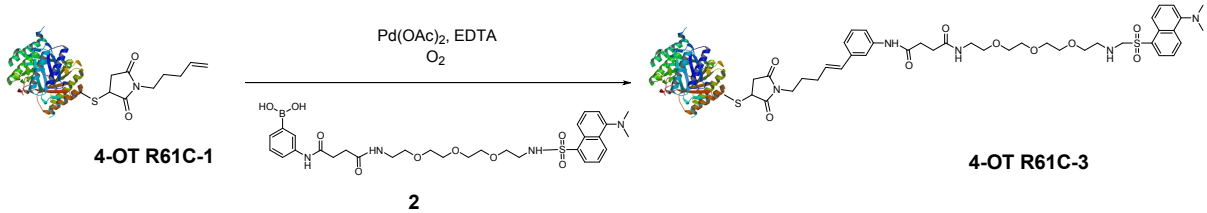
0.5 µM scale

EDTA-Pd (II) solution 2.0 mM

To the solution of 8.0 mM, prepared as mentioned above, were added ammonium formate buffer (3.0 mL, 50 mM, pH 7.0).

4-OT R61C-1 (1.1 µL, 0.65 mg/ml, 0.1 nmol), **1** (1.0 µL, 0.01 M, 10 nmol) and EDTA-Pd (II) (1.0 µL, 2.0 mM, 2 nmol in Pd, 2 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum along with ammonium formate buffer (0.20 mL, 50 mM, pH 7.0). The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h the reaction was stopped and stored at -20 °C.

4.4 Coupling of 3-(Dansyl-PEG-amino)phenylboronic acid to protein-bound terminal alkene 4-OT R61C-1



4-OT R61C-1 (0.21 mL, 0.65 mg/mL, 20 nmol), **2** (18 µL, 0.1 M, 2.0 µmol) and EDTA-Pd (II) (50 µL, 8.0 mM, 0.4 µmol in Pd, 0.4 µmol in EDTA) were added in a 10 mL double neck round bottom flask, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, the solution was transferred in a 10 mL tube and DMF (5.0 mL) was added and the mixture was centrifuged at 13300 rpm for 5 min. The supernatant

was removed and the pellet was redissolved in deionized H_2O (0.4 mL). LC-MS analysis showed more than 90% conversion of the protein **4-OT R61C-1** to protein **4-OT R61C-3**.

ESI-MS spectrum: mass expected 7252, mass found 7251

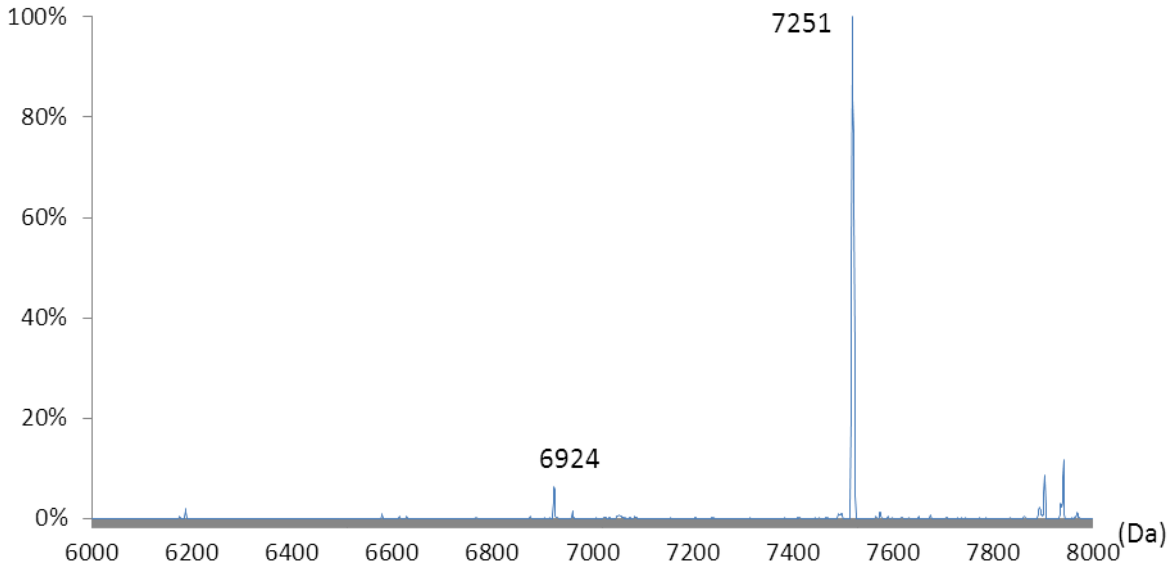


Figure S6. Coupling of **2** to **4-OT R61C-1** using the oxidative Heck reaction with EDTA as ligand. ESI-MS spectrum: mass expected 7252, mass found 7251.

4.5 Detection limit using the biotinylated protein 4-OT R61C-2

Enhanced chemiluminescence assay-ECL

A series of solutions of **4-OT R61C-2** was prepared at the indicated protein concentrations: 12 µM, 6 µM, 4 µM, 2 µM, 1.5 µM, 750 nM, 500 nM, 300 nM and 166 nM.

SDS-PAGE

Note: Prior to use, for each 0.5 mL of (4x) loading buffer, 80 µL of betamercaptoethanol were added.

10 µL of each solution of **4-OT R61C-2** mixed with 10 µL of the loading buffer were boiled for 5 min and loaded on the gel (12%) along with 5.0 µL of the protein ladder. The gel was left running for 1.5 h at 150 V.

Western blot

The proteins were transferred to a PVDF membrane by Western blot (300 mA, 2 h). The membrane was then left shaking in 5% milk in PBS-T buffer (10 mL) (1x PBS containing 0.1% Tween 20) for 1 h and washed three times with the PBS-T buffer every 10 min. The buffer was discarded and 10 µL of HRP-Conjugated Streptavidin (1.0 mg/mL) in PBS-T buffer (10 mL) were added. The membrane was then left shaking for 1 h and washed three times with the PBS buffer every 10 min. Enhanced Luminol Reagent (1.0 mL) and oxidizing reagent (1.0 mL) were mixed and applied onto the membrane. The proteins were detected by luminescence.

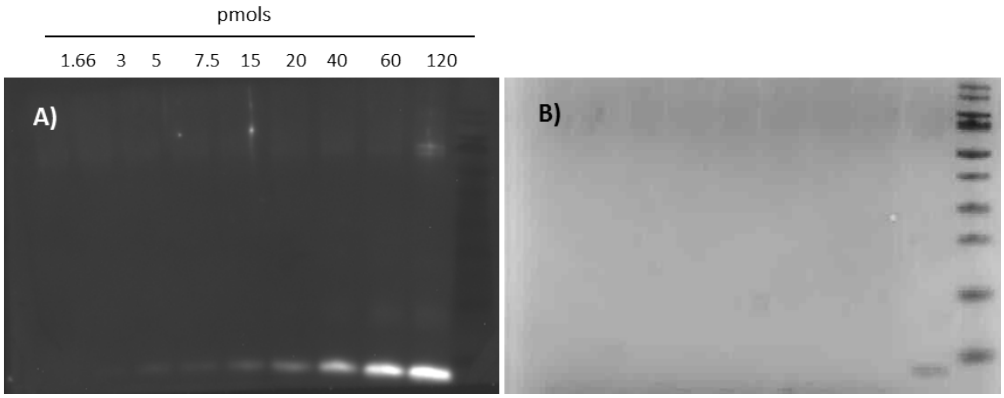


Figure S7: Detection limit using the fully biotinylated alkene-labeled protein **4-OT R61C-2**. A) Luminescence imaging on PVDF membrane of different concentrations of **4-OT R61C-2**. B) Coomassie staining of the SDS-PAGE loaded with the same protein concentrations as A).

4.6 Coupling of 3-(biotinylamino)phenylboronic acid to protein-bound terminal alkene 4-OT R61C-1 in the presence of cell lysates

Preparation of cell lysates

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 °C and 5% CO_2 to afford approximately $3x10^7$ cells. Cells were collected by scrapping, transferred in a 10 mL tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1x PBS (1.0 mL). Then, the solution was transferred into a 1.5 mL cup and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was removed and the pellet was dissolved in lysis buffer (1.0 mL) (1.94 mL of 0.5% Triton X-100 in 1xPBS, 20 µL of Protease Inhibitor Cocktail, 20 µL of 0.1 M phenylmethylsulfonyl fluoride (PMSF)and 20 µL of 0.1 M sodium butyrate). The solution was sonicated three times for 30 sec and centrifuged at 10000 rpm and 4 °C for 5 min. The supernatant, containing the cell lysates, was transferred into a plastic tube of 5.0 mL and diluted to afford 6.0 mg/mL protein concentration (determined by Waddell method) and stored at -20 °C until needed.

4.6.1 Oxidative Heck reaction on 4-OTR61C-1 in presence of a cell lysate (ratio 1:1)

4-OT R61C-1 (0.21 mL, 0.65 mg/mL, 20 nmol), cell lysates (23 µL, 6.0 mg/mL, 0.14 mg), **1** (20 µL, 0.1 M, 2.0 µmol) and EDTA-Pd (II) (50 µL, 8.0 mM, 0.4 µmol in Pd, 0.4 µmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. A blank reaction on the cell lysate in absence of **4-OT R61C-1** was also set up. After 24 h, the solution was transferred in a 10 mL tube and DMF (5.0 mL) was added and the mixture was centrifuged at 13300 rpm for 5 min. The supernatant was removed and the pellet was redissolved in deionized H_2O (0.4 mL).

SDS-PAGE

2.5 µL of the reaction mixture mixed with 10 µL of the loading buffer were boiled for 5 min and the total 12.5 µL were loaded on a SDS PAGE (12%) along with 5.0 µL of the protein ladder. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The blotting was performed as described above.

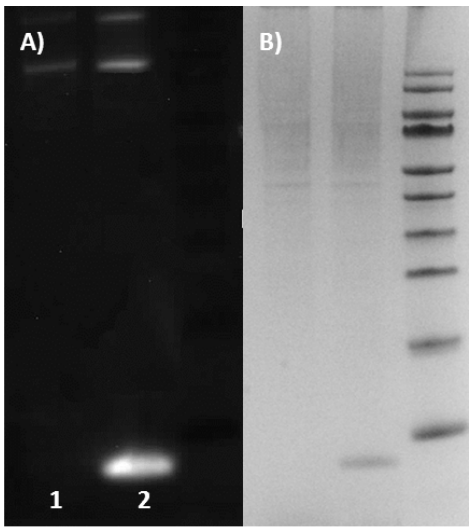


Figure S8: A) Luminescence imaging on PVDF membrane of **4-OT R61C-1** labeled in presence of a cell lysate (protein ratio 1:1). B) Coomassie staining of A). 1) The reaction in absence of **4-OT R61C-1** and 2) in presence of **4-OT R61C-1**.

In order to ensure that the luminescent signal of the higher molecular weight proteins is due to endogenous biotinylation, a sample of unreacted cell lysates (approximately 1.0 µg) was loaded on a SDS-PAGE (15%). Electrophoresis was performed for 2 h at 150 V.

The western blotting was performed as described previously.

Figure S9: A) Luminescence imaging on PVDF membrane of a cell lysate from RAW 264.7 cells. B) Coomassie staining of A).

Preparation of the sample for LC-MS analysis

The 4-OT band was cut out of the SDS-PAGE, chopped in small pieces and put in a 2.0 mL cup containing deionized water (50 µL) and left overnight at 4 °C in order to let the protein diffuse out of the gel. Subsequently, the sample was centrifuged at 13300 rpm for 10 min at 4 °C and the supernatant was subjected to LC-MS analysis. The analysis showed full conversion of **4-OT R61C-1** to **4-OT R61C-2**.

ESI-MS spectrum: mass expected 7240, mass found 7241

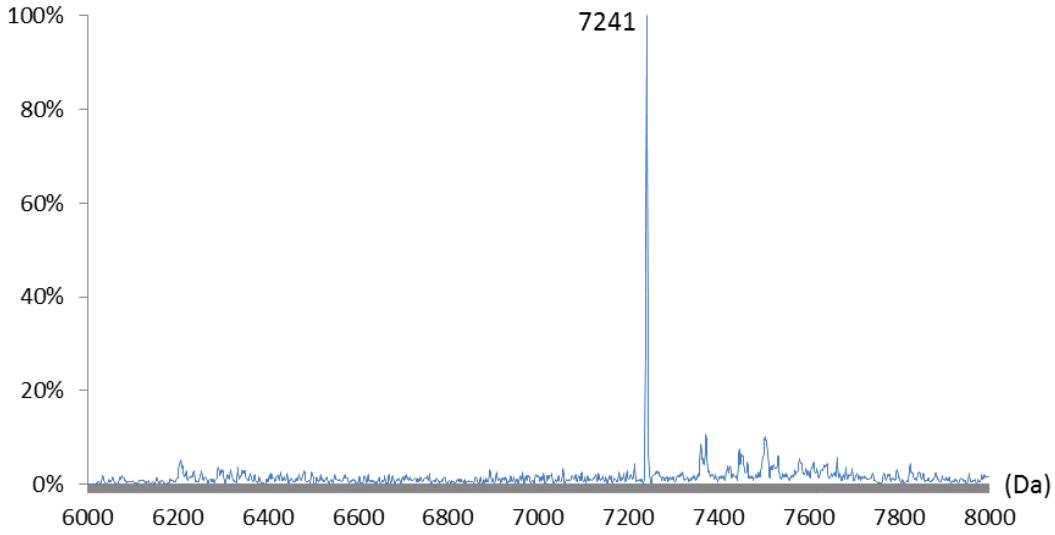


Figure S10. Coupling of **1** to **4-OT R61C-1** via the oxidative Heck reaction in presence of a cell lysate. ESI-MS spectrum: mass expected 7240, mass found 7241.

4.6.2 Oxidative Heck reaction on 4-OTR61C-1 in presence of a cell lysate (ratio 1:10)

4-OT R61C-1 (21 µL, 0.65 mg/mL, 2.0 nmol), cell lysates (23 µL, 6.0 mg/mL, 0.14 mg), **1** (2.0 µL, 0.1 M, 0.2 µmol) and EDTA-Pd (II) (5.0 µL, 8.0 mM, 40 nmol in Pd, 40 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum along with ammonium formate buffer (0.35 mL, 50 mM, pH 7.0). The reaction mixture was left stirring at room temperature under oxygen atmosphere. A blank reaction on the cell lysate in absence of **4-OT R61C-1** was also set up. After 24 h the reaction was stopped and stored at -20 °C.

SDS-PAGE

2.5 µL of the reaction mixture mixed with 10 µL of the loading buffer were boiled for 5 min and the total 12.5 µL were loaded on a SDS PAGE (12%) along with 5.0 µL of the protein ladder. Also 2.5 µL of the 5 µM scale reaction between the protein-bound alkene **4-OT R61C-1** and **1** were loaded after mixing and boiling with 10 µL of the loading buffer**.** In order to estimate the conversion of **4-OT R61C-1** to the product **4-OT R61C-2**, we also loaded the equivalent amount of a reference solution of **4-OTR61C-2**. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The blotting was performed as described above.

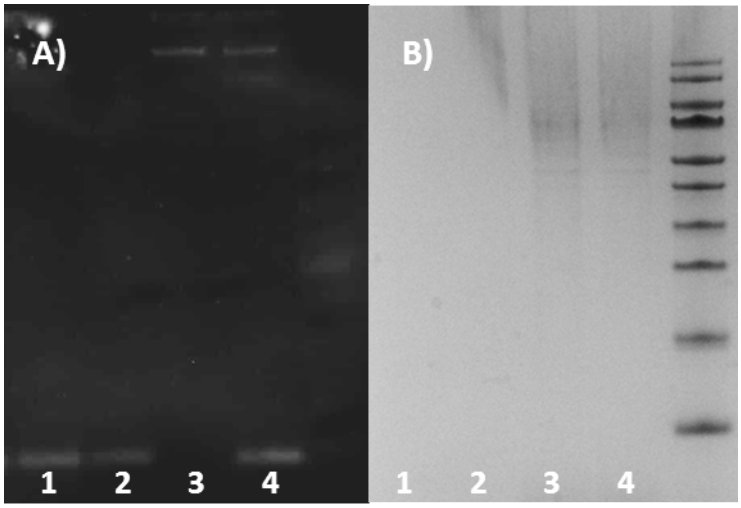
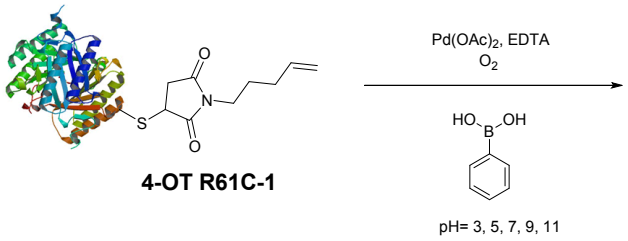


Figure S11. A) Luminescence imaging on PVDF membrane of **4-OT R61C-1** labeled in small scale as well as in presence of a cell lysate (protein ratio 1:10). B) Coomassie staining of A). 1) 5 µM scale reaction of **4-OT R61C-1**, 2) **4-OT R61C-2** (reference), 3) the reaction in absence of **4-OT R61C-1** and 4) in presence of **4-OT R61C-1** (cell lysate ratio 1:10).

4.7 Oxidative Heck reactions on protein-bound terminal alkene 4-OT R61C-1 at various pHs



4-OT R61C-1 (43 µL, 0.65 mg/mL, 4.0 nmol), phenylboronic acid (10 µL, 40 mM, 0.4 µmol) and EDTA-Pd (II) (10 µL, 8.0 mM, 80 nmol in Pd, 80 nmol in EDTA) were added in a 4 mL vial, equipped with a magnetic stirring bar and a septum, along with ammonium formate buffer (0.14 mL, 50 mM, pH 3, 5, 7, 9 or 11). The reaction mixture was left stirring at room temperature under oxygen atmosphere for 24 h. Each sample was subjected for LC-MS analysis.

PH 3

ESI-MS spectrum: mass expected 7000, mass found 7000 (6923 unreacted protein, 7054 unreacted protein with methionine)

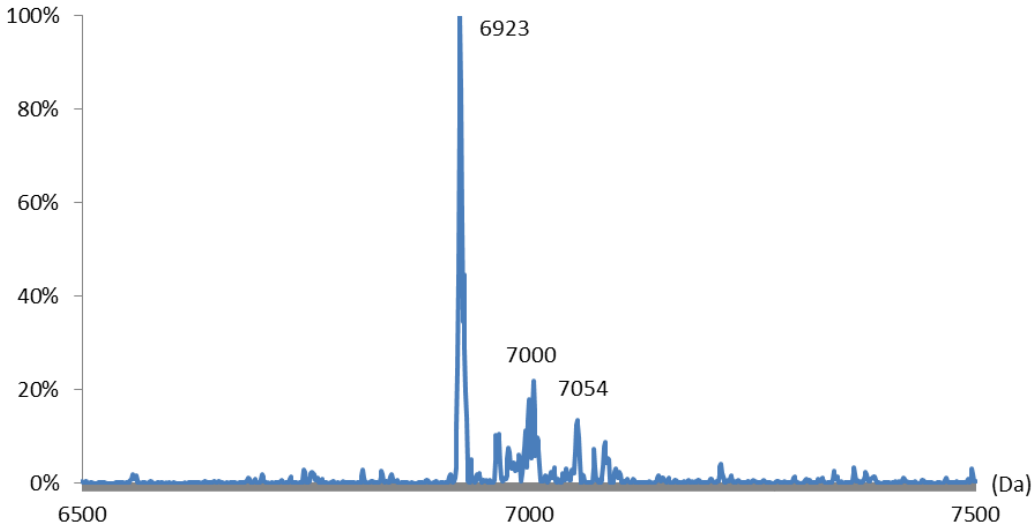


Figure S12. Oxidative Heck reaction on **4-OT R61C-1** at pH 3. ESI-MS spectrum: mass expected 7000, mass found 7000 (6923 unreacted protein, 7054 unreacted protein with methionine).

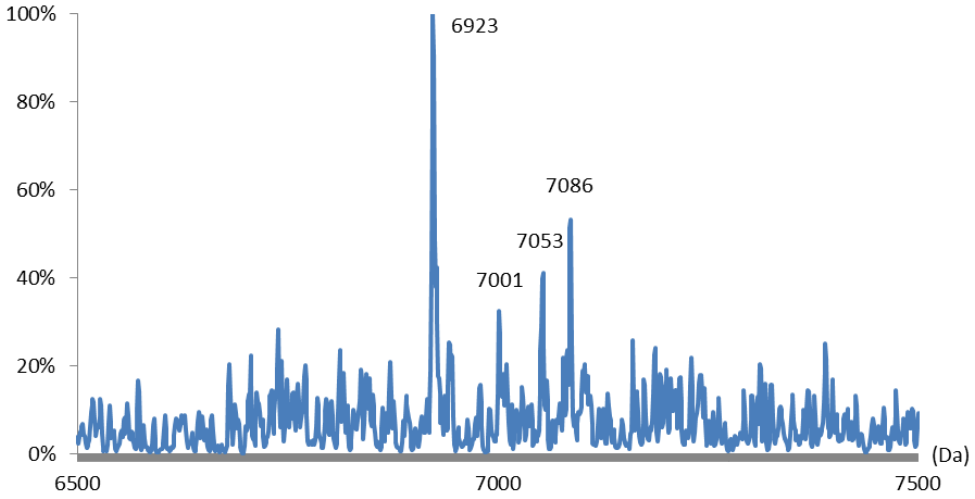


Figure S13. Oxidative Heck reaction on **4-OT R61C-1** at pH 5. ESI-MS spectrum: mass expected 7000, mass found 7001 (6923 unreacted protein, 7053 unreacted protein with methionine, 7086 unreacted protein with formylated methionine).

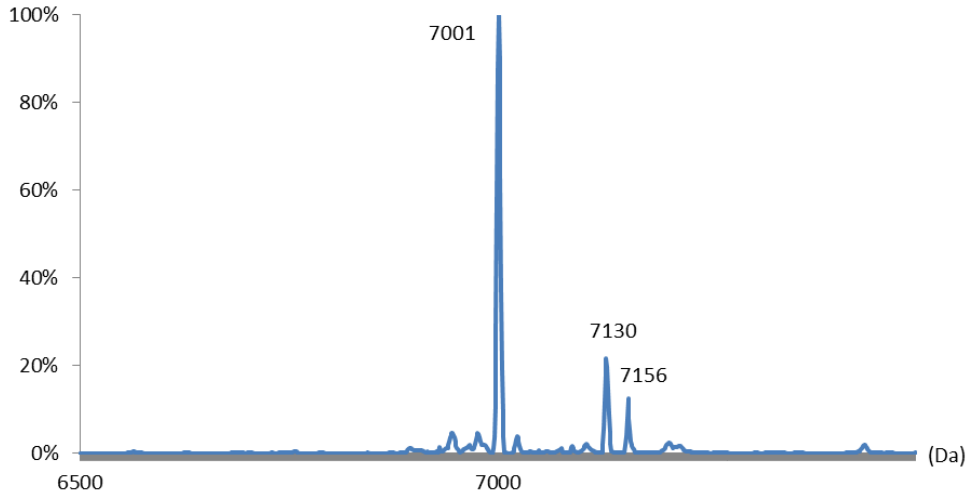


Figure S14. Oxidative Heck reaction on **4-OT R61C-1** at pH 7. ESI-MS spectrum: mass expected 7000, mass found 7001 (7130 reacted protein with methionine, 7156 reacted protein with formylated methionine).

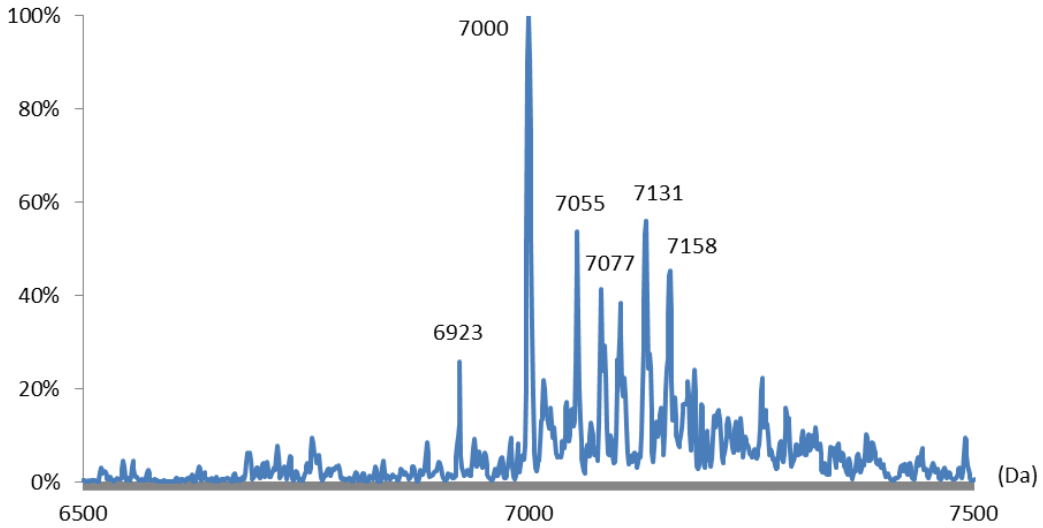


Figure S15. Oxidative Heck reaction on **4-OT R61C-1** at pH 9. ESI-MS spectrum: mass expected 7000, mass found 7000 (6923 unreacted protein, 7055 unreacted protein with methionine, 7077 double phenylated protein, 7131 phenylated protein with methionine, 7158 phenylated protein with formylated methionine).

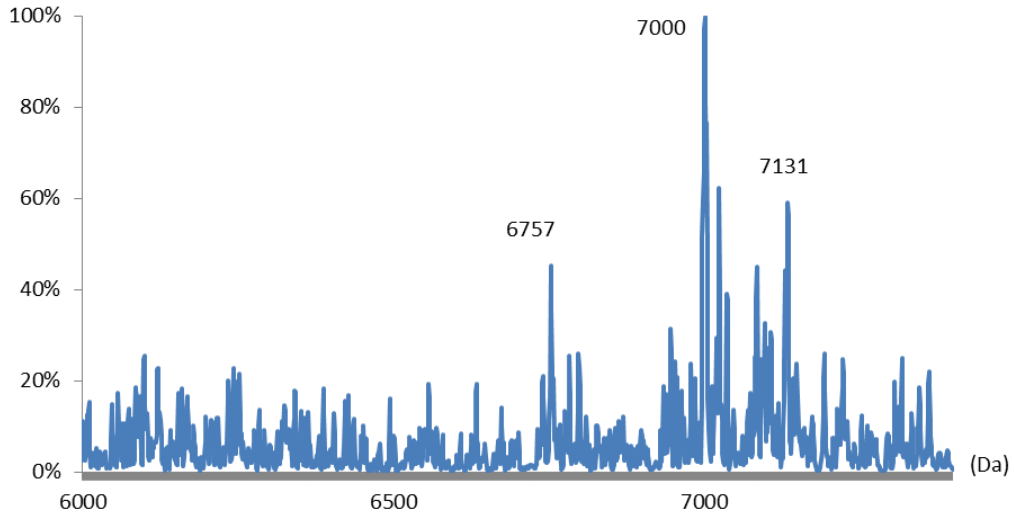


Figure S16. Oxidative Heck reaction on **4-OT R61C-1** at pH 11. ESI-MS spectrum: mass expected 7000, mass found 7000 (6757 reduced **4-OT R61C**, 7131 phenylated protein with methionine).

5. Oxidative Heck reaction conditions on 4-OT wild type at various pHs

Expression and purification of **4-OT** wild type was performed as described above for **4-OTR61C**. The protein was stored in 0.5 mL cups as a 17.21 mg/mL solution (determined by Waddell method) in ammonium formate buffer (pH 3.0, 50 mM). An aliquot of this protein sample was directly analysed by ESI-MS.

The cups containing the protein solution were snapfrozen in liquid nitrogen and kept in -20°C.

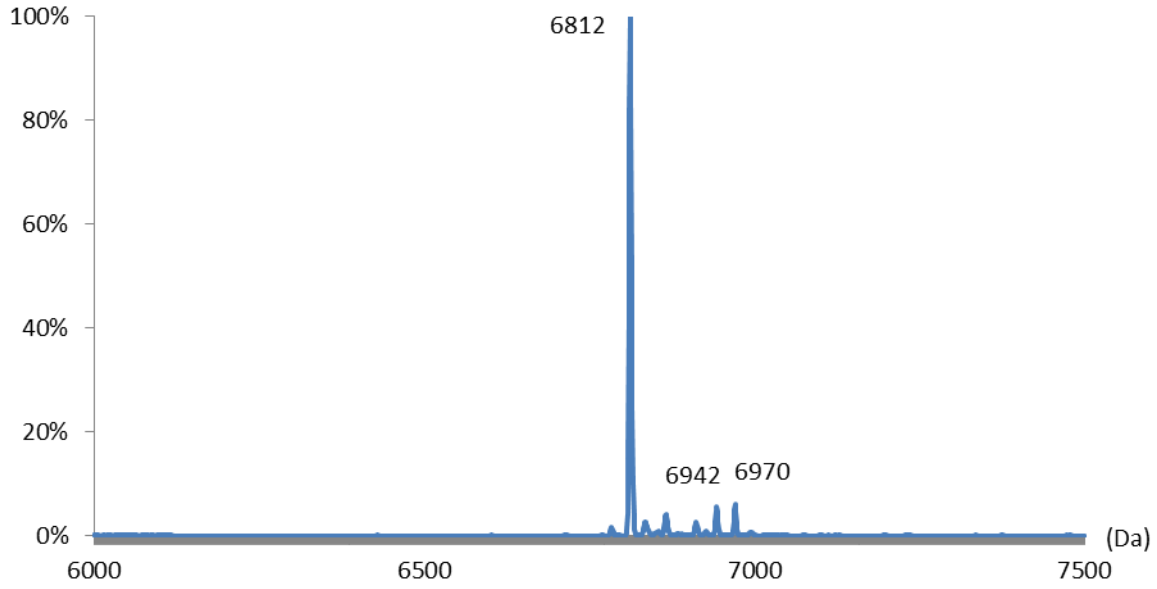
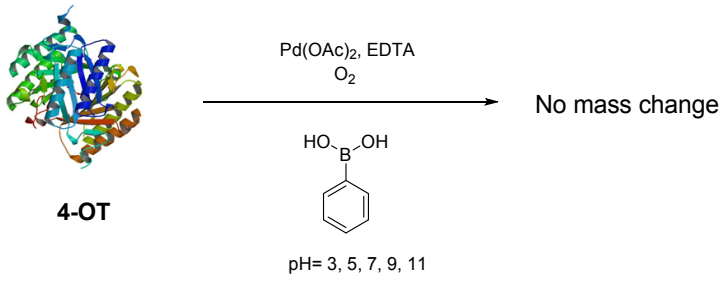


Figure S17. Wild type **4-OT** for control reactions on the stability of the enzyme. ESI-MS spectrum **4-OT**: mass expected= 6811, mass found= 6812 (6942: enzyme with methionine, 6970: enzyme with formylated methionine).



In a 4 mL vial equipped with a stirring bar and a septum were added sequentially ammonium formate buffer (0.2 mL, 0.4 M, pH 3, 5, 7, 9 or 11), 4-OT (8.0 µL, 17.21 mg/mL, 20 nmol), phenylboronic acid (50 µL, 40 mM, 2.0 µmol) and EDTA-Pd (II) (50 µL, 8.0 mM, 0.4 µmol in Pd, 0.4 µmol in EDTA). The reaction mixture was left stirring at room temperature under oxygen atmosphere for 24 h. LC-MS analysis showed no mass change.

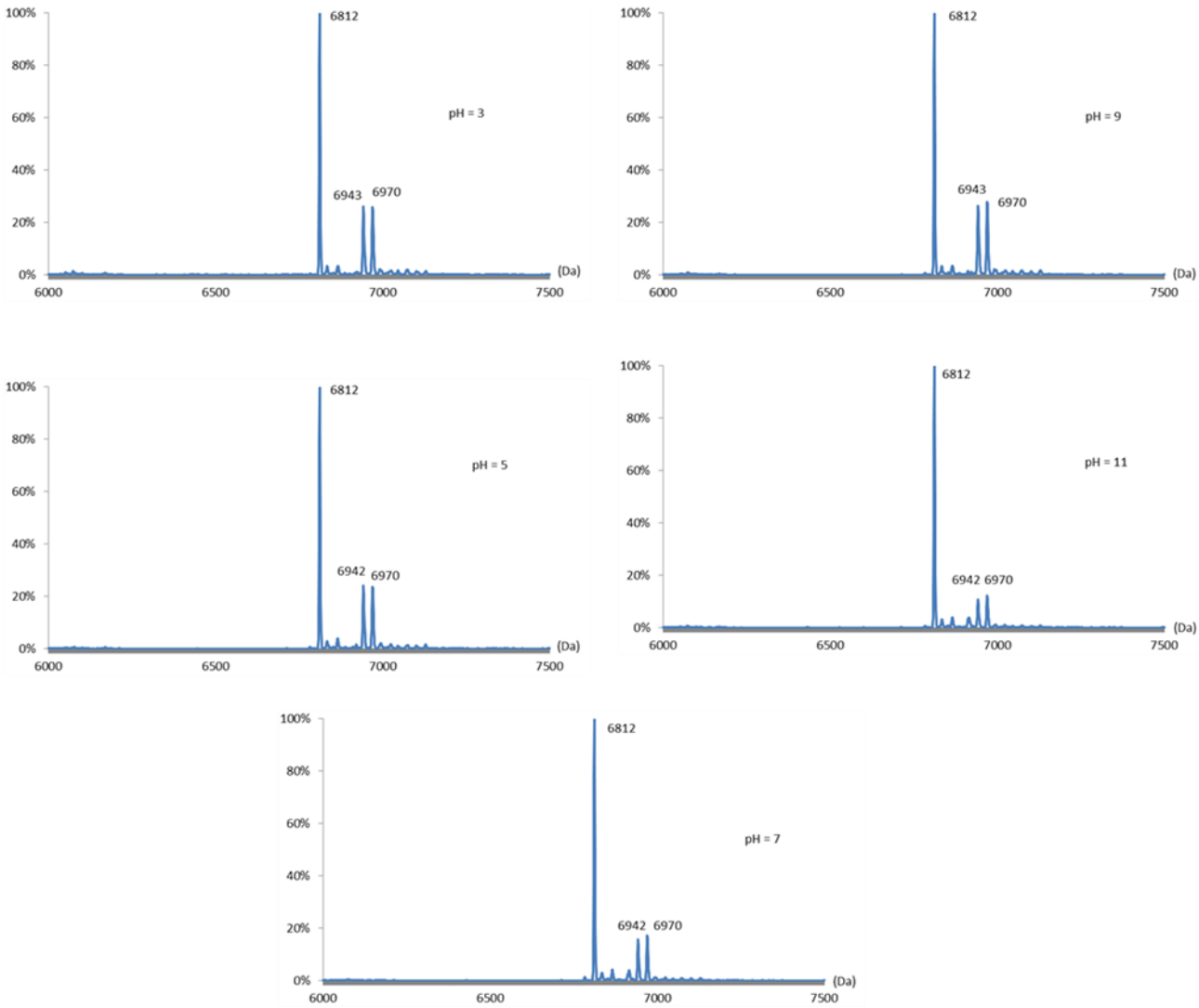
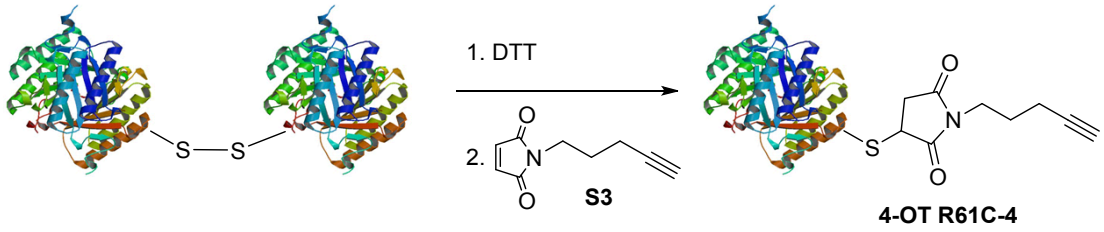


Figure S18. Control reactions at various pHs for stability of wild type 4-OT.

6. Click reactions on protein-bound terminal alkyne 4-OT R61C-4

6.1 Reduction of 4-OT R61C dimer and coupling with 1-(pent-4-yn-1-yl)-1H-pyrrole-2,5-dione (4-OT R61C-4)



A stock solution of 1-(pent-4-yn-1-yl)-1H-pyrrole-2,5-dione was prepared by dissolving **S3** (34 mg, 0.21 mmol) in CH_3CN (0.33 mL). The final concentration of the stock was 0.63 M. The reduction of **4-OT R61C** dimer and the coupling with **S3** was performed as described above. The protein concentration of the fraction containing 4-OT was found to be 0.39 mg/mL. An aliquot of this protein

solution was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with a mass corresponding to the mass of **4-OT R61C-4**.

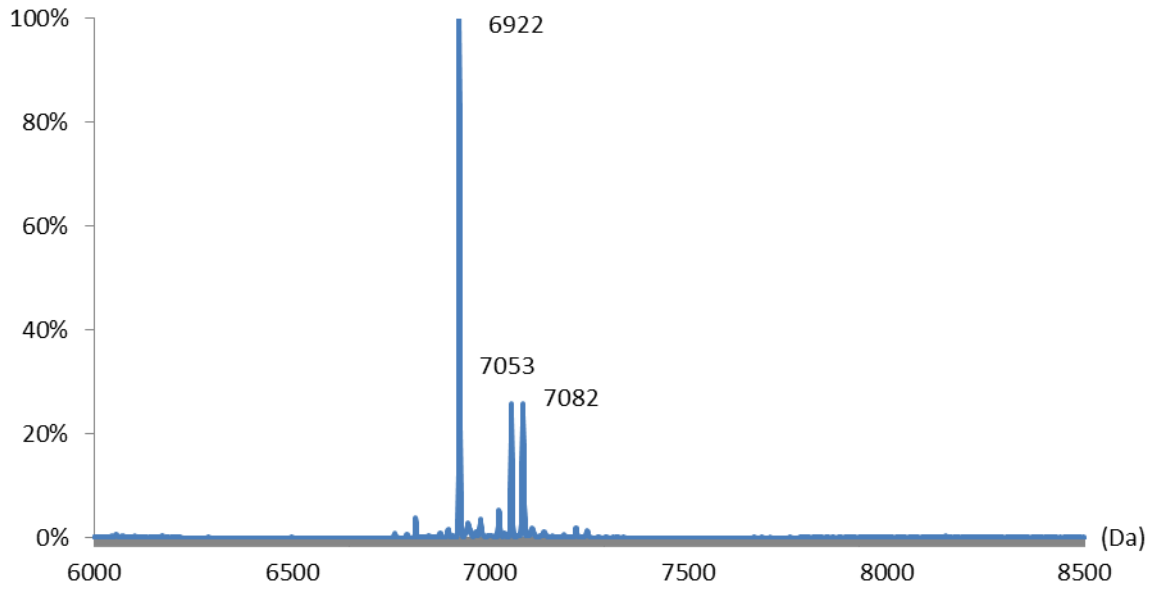


Figure S19. Alkyne labelling of **4-OT R61C** to give **4-OT R61C-4**. ESI-MS spectrum: mass expected 6920, mass found 6922 (7053: modified enzyme with methionine, 7082: modified enzyme with formylated methionine).

6.2 Preparation of stock solutions

$CuSO_4$ 5.0 mM stock solution

$CuSO_4$ (8.0 mg, 5.0 µmol) were dissolved in d.H_2O (10 mL).

TABTA 20 mM stock solution

(3-(4-((Bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)propyl)trimethylammonium trifluoroacetate was synthesized as reported previously. [9]

For the preparation of the stock solution TABTA (5.6 mg, 12 µmol) were dissolved in d.H_2O (0.59 mL).

Sodium ascorbate 0.1 M stock solution

(+)-Sodium L-ascorbate (0.2 g, 1.0 mmol) were dissolved in d.H_2O (10 mL).

Azide-PEG3-biotin 10 mM stock solution

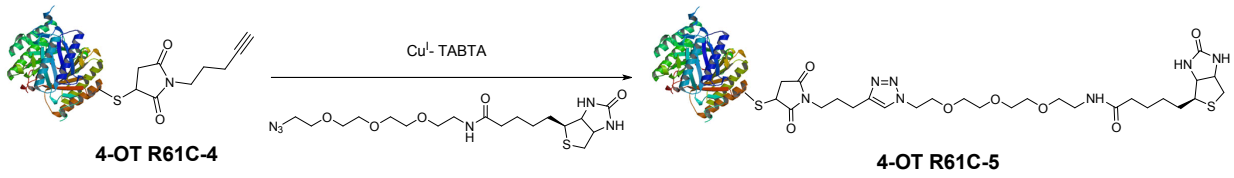
Azide-PEG3-biotin (2.0 mg, 4.5 µmol) were dissolved in d.H_2O (0.45 mL).

Catalyst solution

Before use, 25 µL of $CuSO_4$ and TABTA stock solutions were mixed**.**

6.3 Coupling of biotinylated azide to protein-bound terminal alkyne 4-OT R61C-4

The click reaction was performed according to literature procedures.[9]



20 µM scale

In a 4 mL vial equipped with a stirring bar were added sequentially d.H_2O (0.12 mL), **4-OT R61C-4** (89.3 µL, 0.39 mg/mL, 5.0 nmol), azide-PEG3-biotin (Sigma Aldrich, product number 762024) (5.0 µL, 10 mM, 50 nmol), premixed catalyst (20 µL, 50 nmol in $CuSO_4$, 200 nmol in TABTA) and sodium ascorbate (10 µL, 0.1 M, 1.0 µmol). The reaction mixture was stirred for 2 h at room temperature. LC-MS analysis showed full conversion of **4-OT R61C-4** to **4-OT R61C-5**.

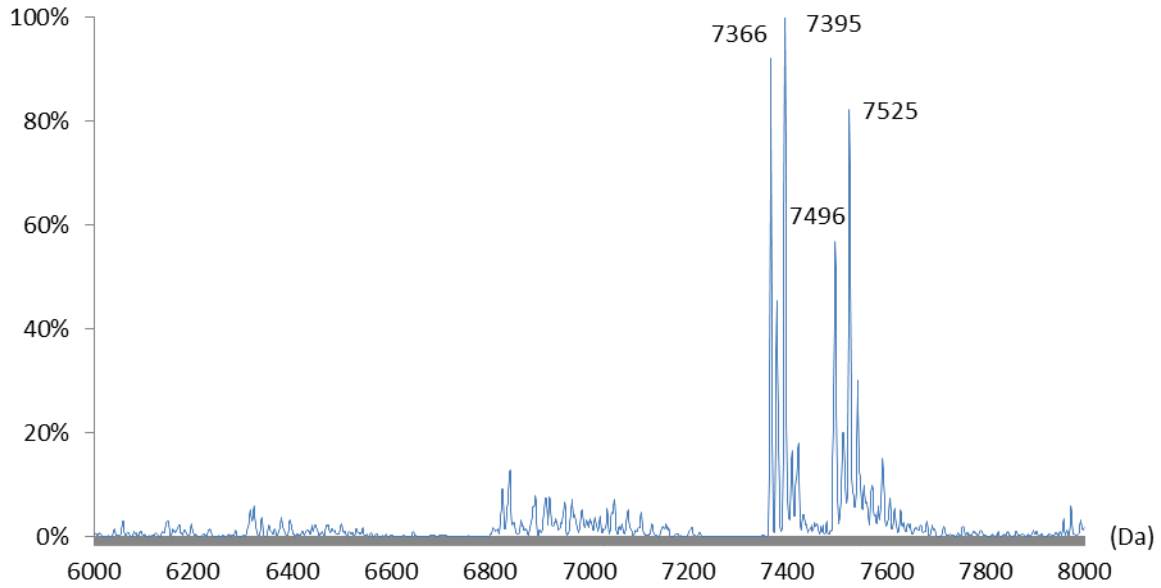


Figure S20. Click reaction on **4-OT R61C-4**. ESI-MS spectrum: mass expected 7367, mass found 7366 (7496: modified enzyme with methionine, 7525: modified enzyme with formylated methionine).

3 µM scale

Azide-PEG3-biotin 1.0 mM

10 µL of the 10 mM solution were added in 90 µL of d.H_2O.

In a 4 mL vial equipped with a stirring bar were added sequentially d.H_2O (0.12 mL), **4-OT R61C-4** (16 µL, 0.39 mg/mL, 0.9 nmol), azide-PEG3-biotin (9.0 µL, 1.0 mM, 9.0 nmol), premixed catalyst (3.6 µL, 9.0 nmol in $CuSO_4$, 36 nmol in TABTA) and sodium ascorbate (1.8 µL, 0.1 M, 0.18 µmol). After 2h stirring at room temperature, the reaction was stopped and stored at -20 °C.

1 µM scale

$CuSO_4$ 0.5 mM solution

10 µL of the 5.0 mM solution were added in 90 µL of d.H_2O.

TABTA 2.0 mM solution

10 µL of the 20 mM solution were added in 90 µL of d.H_2O.

Sodium ascorbate 10 mM solution

10 µL of the 0.1 M solution were added in 90 µL of d.H_2O.

Catalyst solution

Before use, 25 µL of $CuSO_4$ 0.5 mM and TABTA 2.0 mM solutions were mixed**.**

In a 4 mL vial equipped with a stirring bar were added sequentially d.H_2O (0.27 mL), **4-OT R61C-4** (5.3 µL, 0.39 mg/mL, 0.3 nmol), azide-PEG3-biotin (3.0 µL, 1.0 mM, 3.0 nmol), premixed catalyst (12 µL, 3.0 nmol in $CuSO_4$, 12 nmol in TABTA) and sodium ascorbate (6.0 µL, 10 mM, 60 nmol). After 2 h stirring at room temperature, the reaction was stopped and stored at -20 ^oC.

0.5 µM scale

In a 4 mL vial equipped with a stirring bar were added sequentially d.H_2O (0.29 mL), **4-OT R61C-4** (2.7 µL, 0.39 mg/mL, 0.15 nmol), azide-PEG3-biotin (1.5 µL, 1.0 mM, 1.5 nmol), premixed catalyst (6.0 µL, 1.5 nmol in $CuSO_4$, 6.0 nmol in TABTA) and sodium ascorbate (3.0 µL, 10 mM, 30 nmol). After 2 h stirring at room temperature, the reaction was stopped and stored at -20 ^oC.

7. Click vs Oxidative Heck reaction

7.1 Comparison at low concentrations

SDS-PAGE

5.0 µL of the 3 µM scale reactions and 15 µL of 1 µM scale reactions were mixed with 10 µL of (4x) loading buffer. 30 µL of 0.5 µM scale reactions were mixed with 5.0 µL of (4x) loading buffer and the samples were boiled for 5 min. Then, the total was loaded on a SDS PAGE (12%) along with 6.0 µL of the protein ladder. An equivalent amount of **4-OTR61C-2** was also loaded on the gel as a reference.

Western blot

The blotting was performed as described above and the proteins were detected after 30 min of exposure of the PVDF membrane.

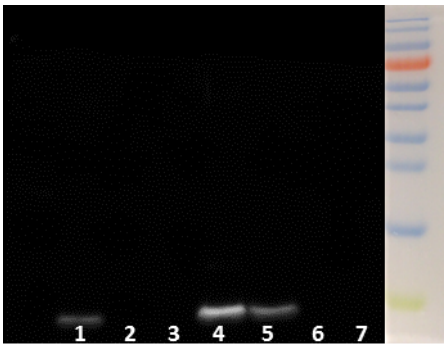
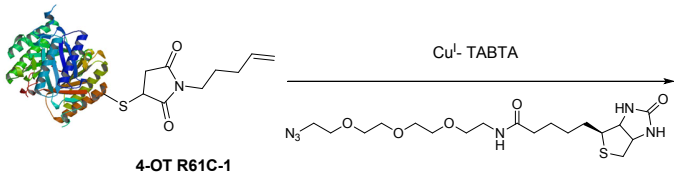


Figure S21. Luminescence imaging on PVDF membrane of biotinylation of **4-OT R61C-1** and **4-OT R61C-4** in small scale via the oxidative Heck (1, 2, 3) and the Click reaction (5, 6, 7). 1), 5) 3 µM scale reaction, 2), 6) 1 µM scale reaction, 3), 7) 0.5 µM scale reaction and 4) **4-OT R61C-2** (reference).

7.2 Checking bioorthogonality

7.2.1 Click reaction conditions on protein-bound alkene 4-OT R61C-1



In a 4 mL vial equipped with a stirring bar were added sequentially d.H_2O (0.16 mL), **4-OT R61C-1** (53 µL, 0.65 mg/mL, 5.0 nmol), azide-PEG3-biotin (5.0 µL, 10 mM, 50 nmol), premixed catalyst (20 µL, 50 nmol in $CuSO_4$, 200 nmol in TABTA) and sodium ascorbate (10 µL, 0.1 M, 1.0 µmol). The reaction mixture was stirred for 2 h at room temperature. LC-MS analysis revealed the mass peak of the starting protein **4-OT R61C-1**.

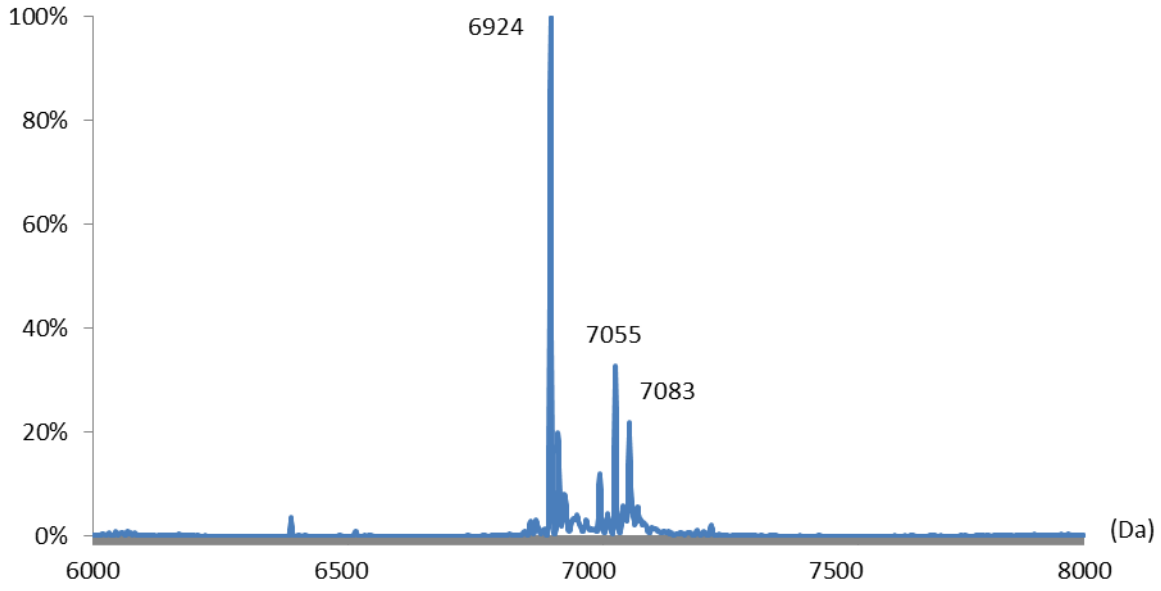
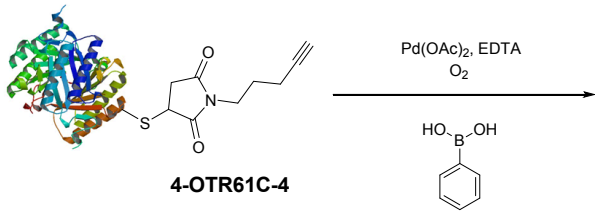


Figure S22. Click reaction conditions on **4-OT R61C-1**. ESI-MS spectrum 6924 **4-OT R61C-1**, 7055 **4-OT R61C-1** with methionine, 7083: **4-OT R61C-1** with formylated methionine).

7.2.2 Oxidative Heck reaction conditions on protein-bound alkyne 4-OT R61C-4



Phenylboronic acid 40 mM stock solution

4.0 mg of phenylboronic acid were dissolved in 0.83 mL of d.H_2O.

In a 4 mL vial equipped with a stirring bar and a septum were added sequentially ammonium formate buffer (0.14 mL, 50 mM, pH 7.0), **4-OT R61C-4** (89.3 µL, 0.39 mg/mL, 5.0 nmol), phenylboronic acid (12.5 µL, 40 mM, 0.5 µmol) and EDTA-Pd (II) (12.5 µL, 8.0 mM, 0.10 µmol in Pd, 0.10 µmol in EDTA). The reaction mixture was left stirring at room temperature under oxygen atmosphere for 24 h. LC-MS analysis revealed the mass peak of the starting protein **4-OT R61C-4**, along with other peaks corresponding to modified enzyme.

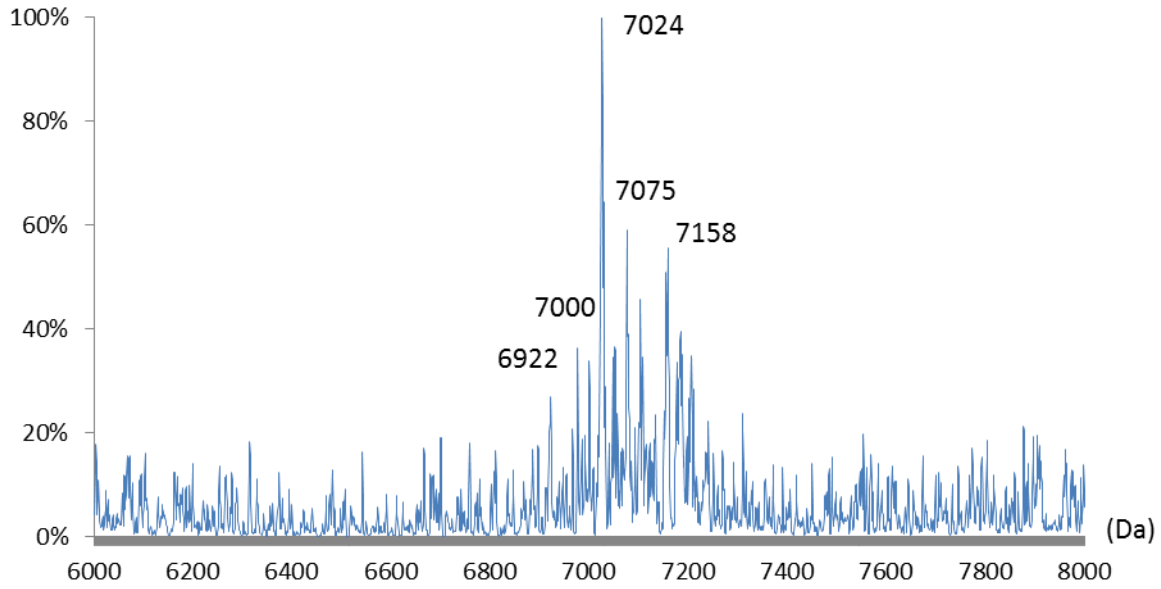


Figure S23. Oxidative Heck reaction conditions on **4-OT R61C-4**. ESI-MS spectrum: 6922 **4-OT R61C-4**, 6998 **4-OT R61C-4** phenylated, 7074: **4-OT R61C-4** double phenylated)

8. Detection of histone acylation using the oxidative Heck reaction

8.1 Preparation of stock solutions of the chemical reporters and HAT/HDAC inhibitors

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit from Promega was used to test the viability of RAW 264.7 cells after 6 h incubation with different concentrations of sodium pent-4-enoate, S-propyl pent-4-enethioate and methyl 4-pentenoate (olefinic chemical reporters) at 37 °C and 5% CO_2. 10 mM was chosen as the maximum concentration where the cell viability was not affected at the indicated incubation time. The cell toxicity of the HAT inhibitor C646 and HDAC

inhibitor SAHA was tested using the same assay after 20 h incubation. Concentrations of 30 μM and 0.41 μM correspondingly were chosen for inhibition studies.

1.0 M Sodium pent-4-enoate solution

4-Pentenoic acid (Sigma Aldrich, Netherlands) (60 μL, 0.59 mmol) were added in deionized H_2O (0.54 mL) and the pH was set at 8.0 using a concentrated solution of NaOH.

1.0 M *S*-propyl pent-4-enethioate

S7 (0.13 mL, 0.76 mmol) were added in filter-sterilized DMF (0.63 mL).

1.0 M Methyl 4-pentenoate

The methyl ester (Sigma Aldrich, Netherlands) (48 μL, 0.6 mmol) were added in filter-sterilized DMF (0.55 mL).

10 mM C646

C646 (Axon MedChem, Netherlands) (8.9 mg, 20 μmol) were dissolved in filtersterilized DMF (2.0 mL).

10 mM SAHA

N-Hydroxy-*N'*-phenyl-octanediamide (SAHA) (5.3 mg, 20 μmol) were dissolved in filter-sterilized DMF (2.0 mL).

8.2 Metabolic labeling and oxidative Heck reaction on alkene-labeled extracted core of histones

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 ^oC and 5% CO_2 to afford approximately $2x10^7$ cells. The medium was aspirated and replaced by new DMEM containing the chemical reporters at concentrations of 5 and 10 mM. The incubation was performed at 37 ^oC and 5 % CO_2 for 6 h. Untreated cells (only dH_2O or DMF) were used for negative control experiments. Cells were collected by scrapping and lysis was performed as described at 4.6 "Preparation of cell lysates". The supernatant, containing the cell lysates, was discarded and the pellet was resuspended in ice-cold Tris-EDTA buffer (10 and 13 mM correspondingly, 1.0 mL, pH 7.4) and centrifuged at 10000 rpm and 4 ^oC for 10 min. Afterwards, the supernatant was discarded and the pellet was resuspended in of ice-cold water (0.2 mL).

Histone extraction

H_2SO_4 was added to reach a final concentration of 0.4 M. The solution was spinned down and the samples were incubated at least for 1 h on ice with gentle agitation. Then, they were centrifuged at 10000 rpm and 4 ^oC for 10 min and the supernatant was transferred into a 2.0 mL cup containing acetone (1.5 mL). After an overnight incubation at -20 ^oC, the samples were centrifuged at 10000 rpm and 4 ^oC for 10 min, the supernatant was discarded and the pellets were allowed to air-dry. In each sample, the histones were resuspended in ammonium formate buffer (0.5 mL, 50 mM, pH 7.0). The samples may be placed in an ultrasonic waterbath for 10 min in order to help the dissolution of the

histones. The protein concentration was determined to be 0.8 mg/mL, after performing the Bradford assay.

Oxidative Heck reactions using 3-(biotinylamino)phenylboronic acid (1)

The samples were transferred in 4 mL vials equipped with a magnetic stirring bar and a septum and SDS (2.0 µL, 4% solution in d. H_2O) solution were added. **1** (5.0 µL, 0.1 M) and EDTA-Pd (II) (5.0 µL, 8.0 mM) were added and the reaction mixture was left stirring at room temperature under oxygen atmosphere for 24 h. For control reactions on histones extracted from untreated cells, **1** (5.0 µL, 0.1 M) was added in presence and absence of Pd-EDTA catalyst (5.0 µL, 8.0 mM).

SDS-PAGE

7.0 µL of each reaction mixture mixed with 10 µL of the loading buffer were boiled for 5 min and the total 17 µL were loaded on a SDS PAGE (12.5%) along with 5.0 µL of the protein ladder. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The blotting was performed as described above.

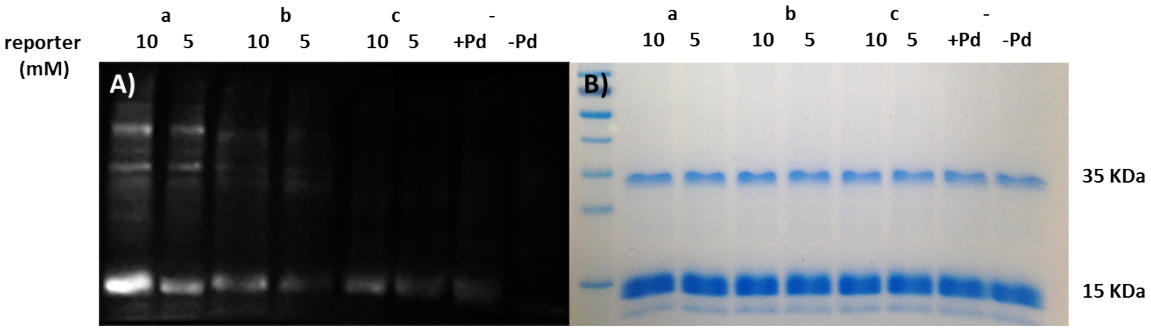


Figure S24. Detection of alkenylated histones extracted from RAW 264.7 cells via the oxidative Heck reaction using a biotinylated phenylboronic acid. A) Luminescence imaging on PVDF membrane of histones after 6 h incubation with 5 and 10 mM of a) sodium pent-4-enoate, b) *S*-propyl pent-4-enethioate and c) methyl 4-pentenoate. Control reactions in presence and absence of Pd are also shown. B) Coomassie staining of A).

In order to understand more clearly which histone types are labeled, 1.0 µg of histones treated with 10 mM of sodium 4-pentenoate mixed with 5.0 µL of the loading buffer were loaded on a SDS PAGE (15%). Electrophoresis was performed for 2 h at 150 V.

Western blot

The blotting was performed as described above.

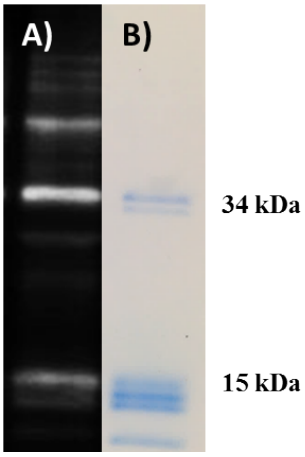


Figure S25. Determination of histone types labeled after 6 h incubation of RAW cells with 10 mM of sodium pent-4-enoate and subsequent biotinylation via the oxidative Heck reaction. A) Luminescence imaging on PVDF membrane. B) Coomassie staining of A).

8.3 Treatment with C646 and SAHA

For inhibition studies, 30 µM of C646 and 0.41 µM of SAHA were first incubated with $1.4x10^7$ RAW 264.7 cells grown in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 ^oC and 5% CO_2 for 14 h. Then, the reporter sodium pent-4-enoate was added at a final concentration 10 mM for 6 h. Untreated cells (no inhibitor-only reporter) were used as positive control. Cell lysis and histone extraction were performed as described above. The histone pellets were dissolved in ammonium formate buffer (pH 7.0, 50 mM) and diluted up to 0.5 mL with same buffer to reach a final concentration of 0.8 mg/mL.

The oxidative Heck reaction was performed as described above.

SDS-PAGE

5.0 µL of each reaction mixture mixed with 10 µL of the loading buffer were boiled for 5 min and the total 15 µL were loaded on a SDS PAGE (12.5%) along with 6.0 µL of the protein ladder. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The blotting was performed as described above.

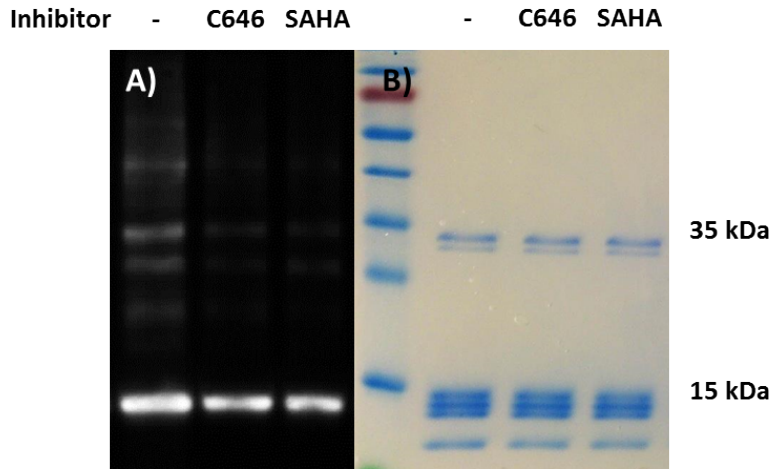


Figure S26. Detection of alkenylated histones extracted from RAW 264.7 cells after incubation with HAT and HDAC inhibitors via the oxidative Heck reaction. A) Luminescence imaging on PVDF membrane of histones after 14 h incubation with 30 µM of C646 and 0.41 µM of SAHA and subsequent 6 h incubation with 10 mM of the reporter sodium pent-4-enoate. B) Coomassie staining of A).

9. Detection of histone acylation using the click reaction

Preparation of sodium 4-pentynoate 1.0 M

4-pentynoic acid (41.7 mg, 42.5 mmol) were dissolved in an aqueous solution of NaOH (1.0 M, 0.43 µL).

Metabolic labeling

For metabolic labeling and cell lysis, a procedure described elsewhere was followed.[10]

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 °C and 5% CO_2 to afford approximately $2.5x10^7$ cells. The medium was aspirated and replaced by new DMEM containing 10 mM of sodium 4-pentynoate. The incubation was performed at 37 °C and 5 % CO_2 for 6 h. Untreated cells (only dH_2O) were used as negative control experiment. Cells were collected by scrapping, transferred in a 50 mL tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1x PBS (1.0 mL). Then, the solution was transferred into a 1.5 mL cup and centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet was dissolved in “click” lysis buffer (0.88 mL) (10 mM triethanolamine pH 7.4, 1.0 mM KCl, 1.5 mM $MgCl_2$) supplemented with 0.1 mL of 10x EDTA-free Protease Inhibitor Cocktail, 10 µL of 0.1 M phenylmethylsulfonyl fluoride (PMSF)and 10 µL of 0.1 M sodium butyrate. The solution was then sonicated three times for 30 sec and centrifuged at 10000 rpm and 4 °C for 5 min. The supernatant was discarded and the pellet was resuspended in ice-cold Tris buffer (10 mM, 1.0 mL, pH 7.4) and centrifuged at 10000 rpm and 4 °C for 10 min. Afterwards, the supernatant was discarded and the pellet was resuspended in of ice-cold water (0.2 mL).

Histone extraction

The histone extraction was performed as described above. The histone pellet was redissolved in buffer (0.2 mL, 0.15 M NaCl, 50 mM triethanolamine pH 7.4).

Click reaction

For the preparation of the click reagents see 6.2 “Preparation of stock solutions“ above.

To the protein samples were added 8.0 µL of premixed catalyst, 2.0 µL of azide-PEG-biotin and 4.0 µL of sodium ascorbate. The mixtures were left stirring at room temperature for 2 h.

SDS-PAGE

5.0 µL of each reaction mixture mixed with 5.0 µL of the loading buffer were boiled for 5 min and the total 10 µL were loaded on a SDS PAGE (12.5%). 6.0 µL of the protein ladder, 9.0 µL of the histone sample treated with 10 mM of sodium 4-pentenoate as well as 9.0 µL of the untreated sample were also loaded. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The blotting was performed as described above.

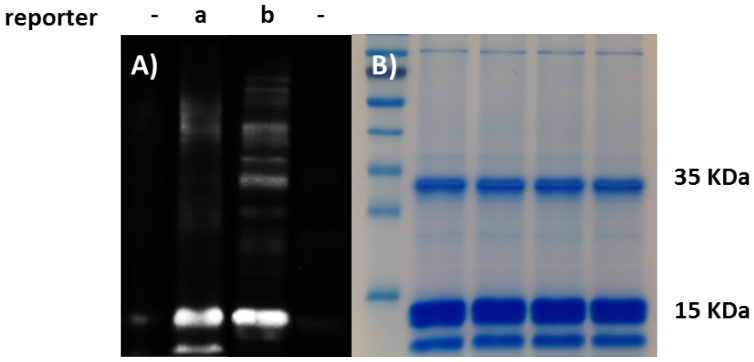


Figure S27. Detection of alkynylated histones extracted from RAW 264.7 cells via the click reaction and comparison with the oxidative Heck reaction. A) Luminescence imaging on PVDF membrane of histones after 6 h incubation with 10 mM of a) sodium pent-4-ynoate and b) sodium pent-4-enoate. Negative control experiments are also shown next to the corresponding reaction. B) Coomassie staining of A).

10. Detection of histone acetylation using the Anti-Acetyl Lysine Antibody

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 ^{o}C and 5% CO_2 to afford approximately 1.3x10^{7} cells. The medium was aspirated and replaced by new containing 30 µM of C646 and 0.41 µM of SAHA. The incubation was performed at 37 ^{o}C and 5 % CO_2 for 20 h. Untreated cells (only DMF) were used for negative control experiments. Cell lysis and histone extraction were performed as described above. The histone pellets were dissolved in ammonium formate buffer (0.4 mL, 50 mM, pH 7.0,) and the protein concentration was found to be 1.2 mg/mL, using the Bradford assay.

SDS-PAGE

2.5 µL of each protein solution mixed with 10 µL of the loading buffer were boiled for 5 min and the total 12.5 µL were loaded on a SDS PAGE (12.5%) along with 6.0 µL of the protein ladder. Electrophoresis was performed for 1.5 h at 150 V.

Western blot

The proteins were transferred to a PVDF membrane by Western blot (300 mA, 2 h). The membrane was then left shaking in 5% BSA in PBS-T 0.05% buffer (20.0 mL) (1x PBS containing 0.05% Tween 20) at room temperature for 1 h and washed quickly with $d.H_2O$. Afterwards, the membrane was placed into a 50 mL tube containing 5% BSA and 10 µL of Anti-Acetyl Lysine Antibody (Rabbit polyclonal, Millipore, AB3879) in 1x PBS buffer (5.0 mL) and it was left shaking at 4°C overnight. Then, it was washed three times with PBS-T 0.1 % buffer (10.0 mL) (1x PBS containing 0.1% Tween 20) every 10 min, placed into a 50 mL tube containing 5% BSA and 5 µL of Polyclonal Swine Anti-Rabbit Immunoglobulins-HRP (Dako) in PBS-T 0.1 % (10 mL) and it was left shaking at at room temperature for 1 h. Finally the membrane was washed three times with the 1x PBS buffer every 10 min. Enhanced Luminol Reagent (1.0 mL) and oxidizing reagent (1.0 mL) were mixed and applied onto the membrane. The proteins were detected by luminescence.

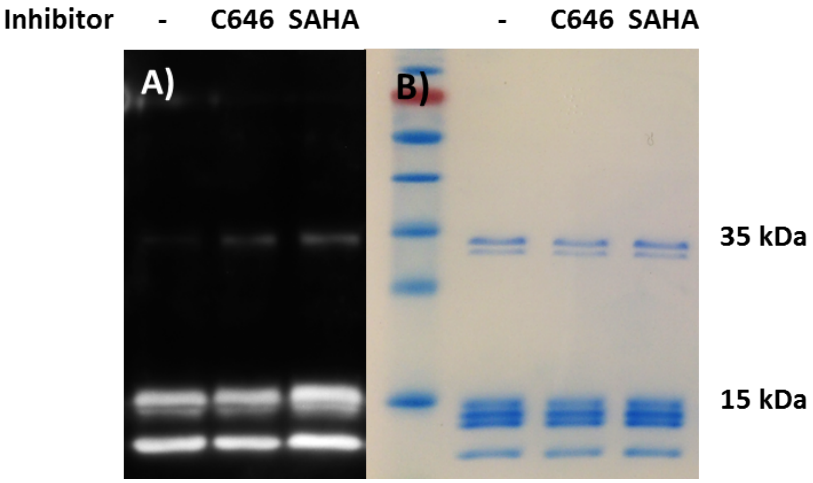


Figure S28. Detection of acetylated histones extracted from RAW 264.7 after incubation with HAT and HDAC inhibitors using the Anti-Acetyl Lysine Antibody. A) Luminescence imaging on PVDF membrane of histones after 20 h incubation with 30 µM of C646 and 0.41 µM of SAHA. B) Coomassie staining of A).

11. Detection of alkene or alkyne metabolically labeled cell lysate from RAW264.7

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and maintained at 37 °C and 5% CO_2 to afford approximately 10^7 cells. The medium was aspirated and replaced by new DMEM containing 10 mM of sodium 4-pentenoate or sodium 4-pentenoate. The incubation was performed at 37 °C and 5% CO_2 for 6 h. Untreated cells (only dH_2O) were used as negative control experiment. Cells were collected by scrapping and for the alkene-treated cells lysis was performed as described at 4.6 “Preparation of cell lysates”, whereas the alkyne-treated cells were lysed as described in section 9 “Detection of histone acylation using the

click reaction-Metabolic labeling". The protein concentration was found to be 3.0 mg/mL (determined by Bradford assay).

Biotinylation of alkene or alkyne-labeled cell lysates

The cell lysates were diluted to 1.0 mg/mL using the appropriate buffer and the click or oxidative Heck reaction was performed on the alkyne or alkene-labeled proteins accordingly, as described previously. Afterwards, DMF (up to 2.0 mL) was added and the samples were stored at -20 ^{o}C overnight. The mixtures were then centrifuged at 13300 rpm and 4 ^{o}C for 5 min. The supernatant was removed and the protein pellet was redissolved in deionized H_2O (0.2 mL).

SDS-PAGE

3.0 µg of cell lysates from each sample were loaded on a SDS PAGE (15%) along with 6.0 µL of the protein ladder. Electrophoresis was performed for 2 h at 150 V.

Western blot

The Western blotting was performed as described previously.

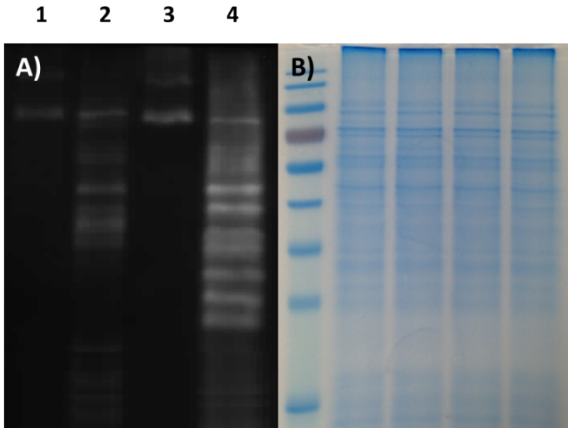


Figure S29. Detection of alkene or alkyne metabolically labeled cell lysate from RAW264.7 via the oxidative Heck and the click reaction. A) Luminescence imaging on PVDF membrane of a cell lysate after 6 h incubation with 10 mM of 2) sodium 4-pentenoate and 4) sodium 4-pentynoate. Negative control experiments are also shown next to the corresponding reaction. B) Coomassie staining of A).

12. NMR spectra

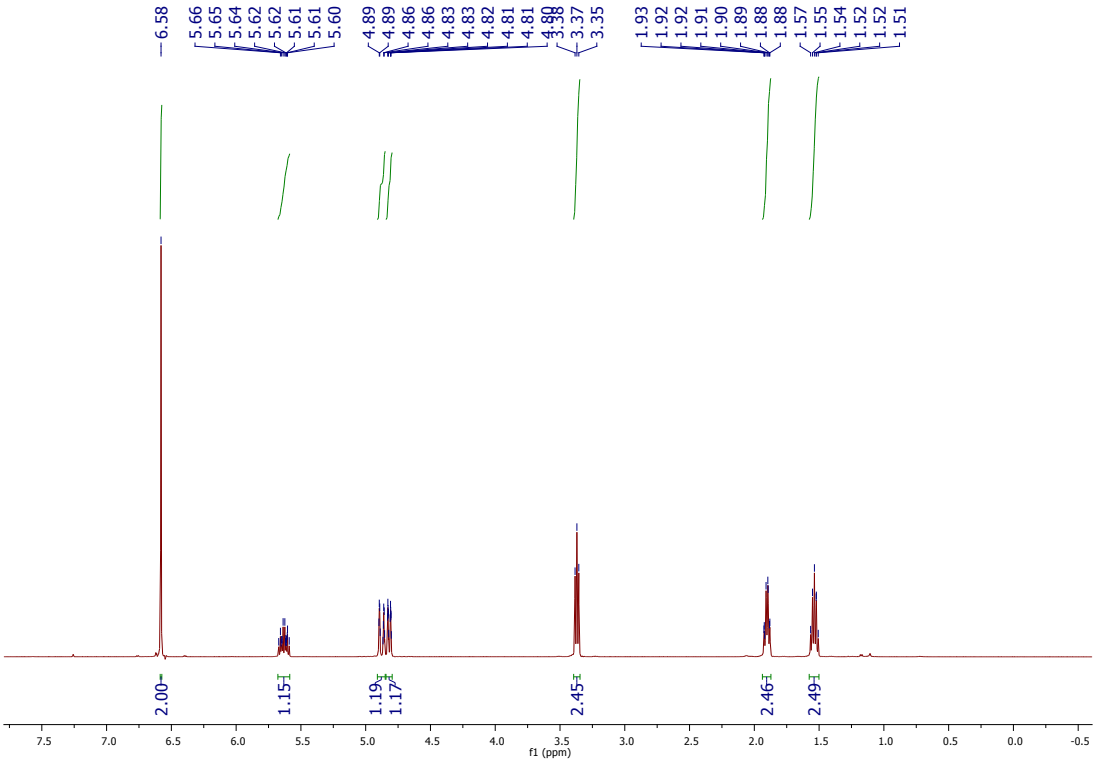


Figure S30. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S2**.

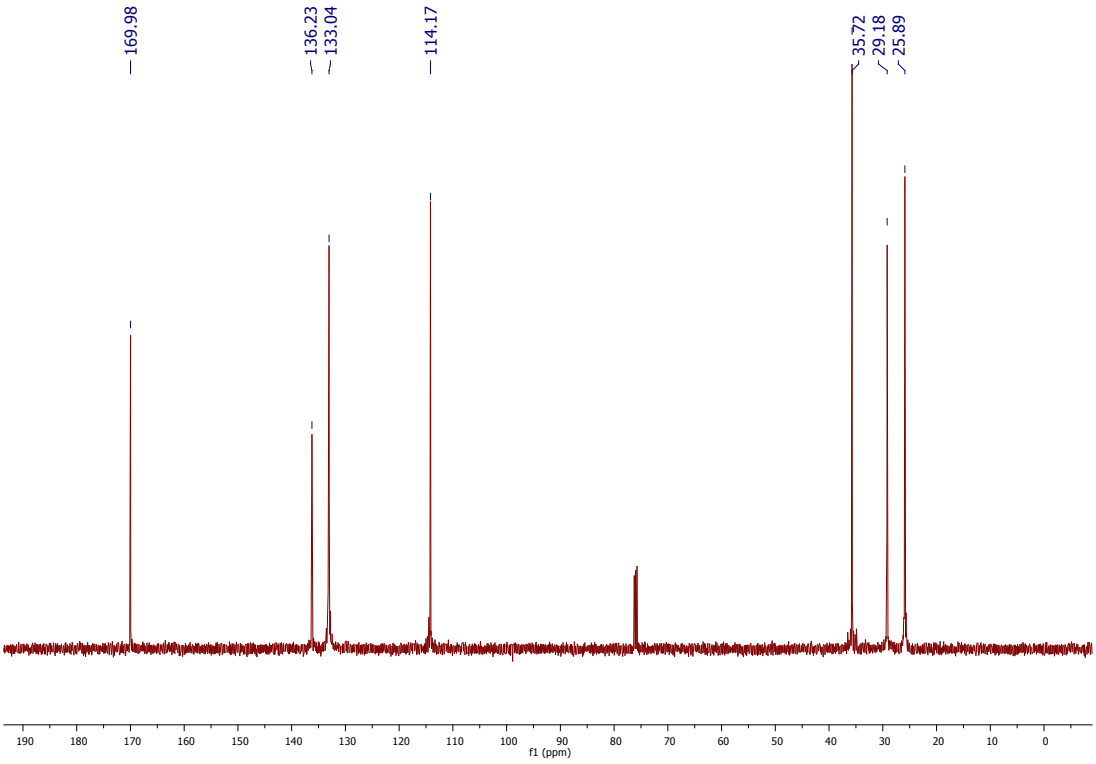


Figure S31. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S2**.

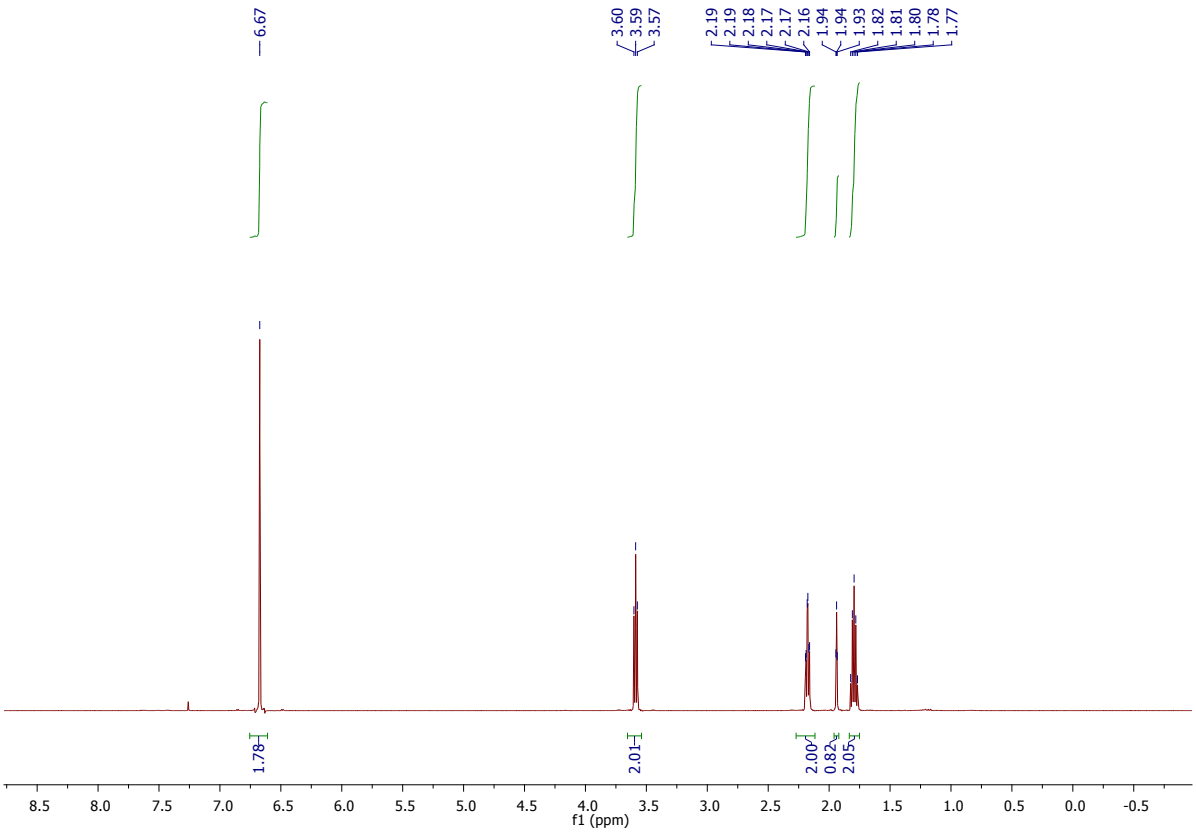


Figure S32. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S3**.

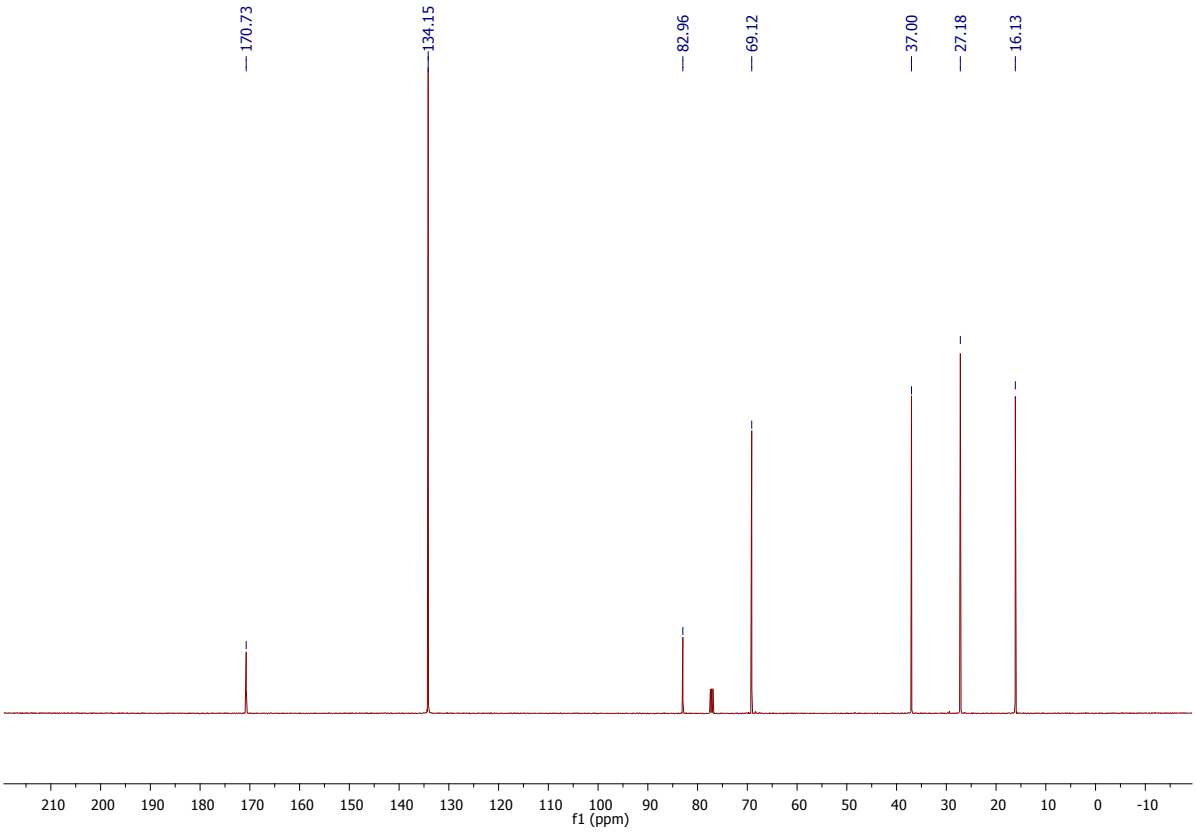


Figure S33. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S3**.

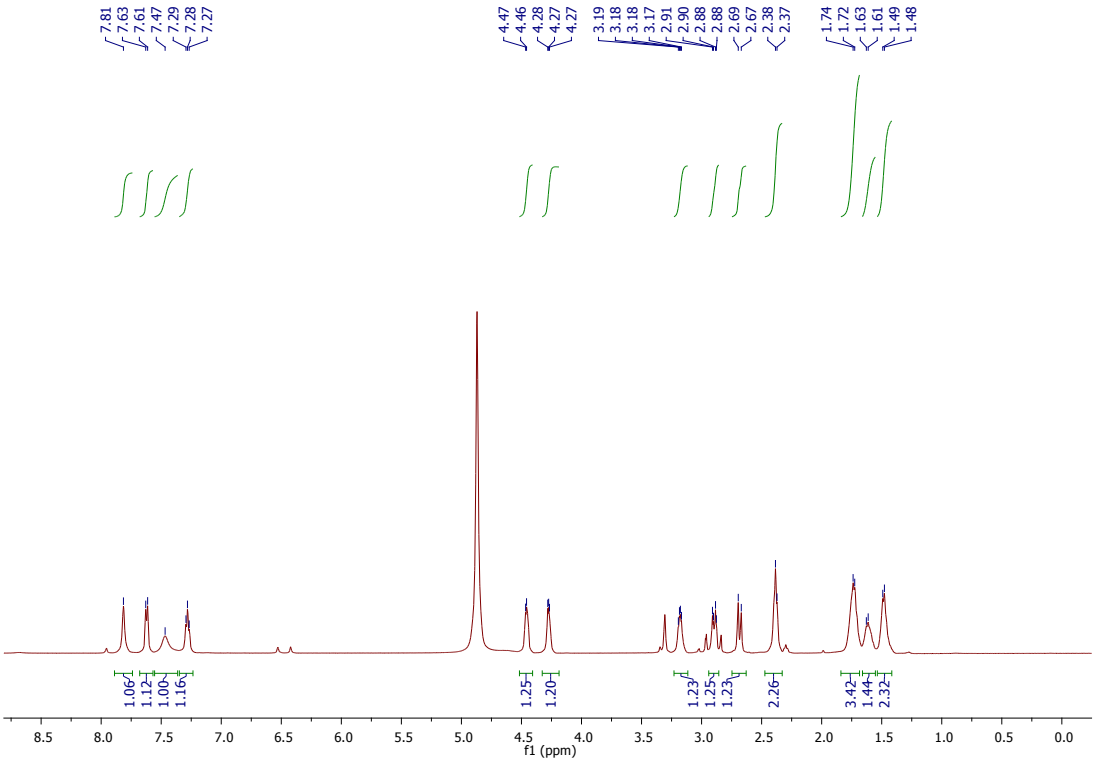


Figure S34. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of **1**.

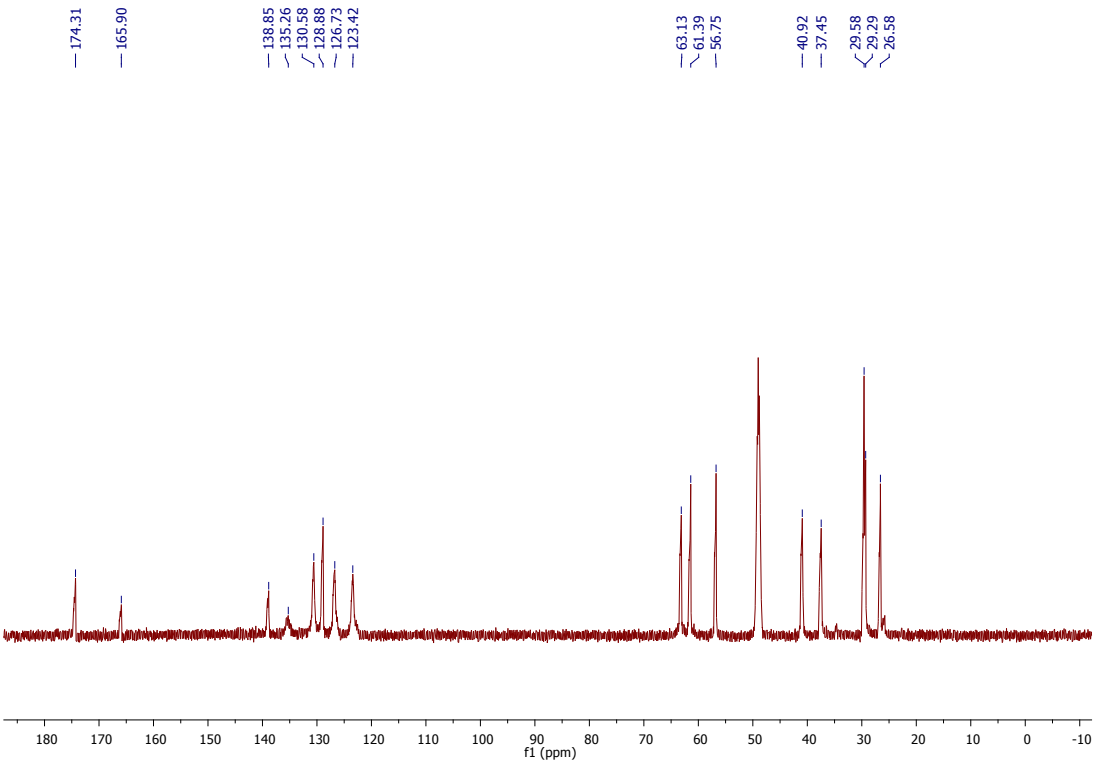


Figure S35. ^{13}C NMR spectrum (CD_3OD, 125 MHz) of **1**.

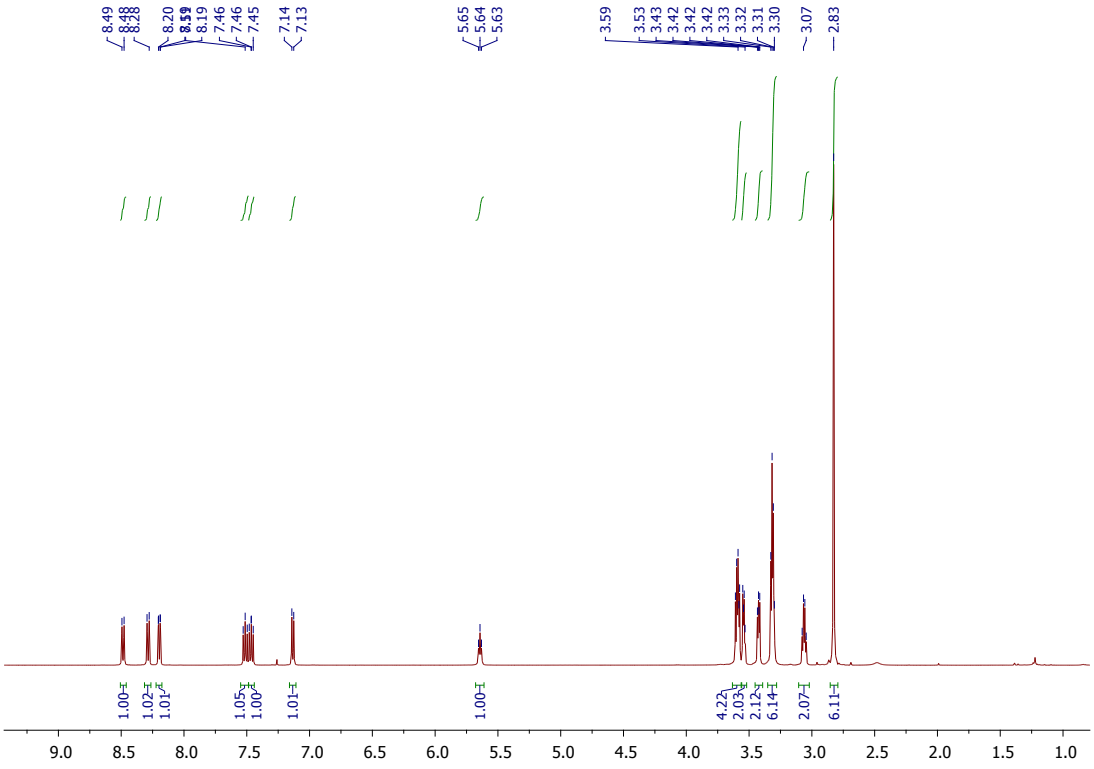


Figure S36. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S4**.

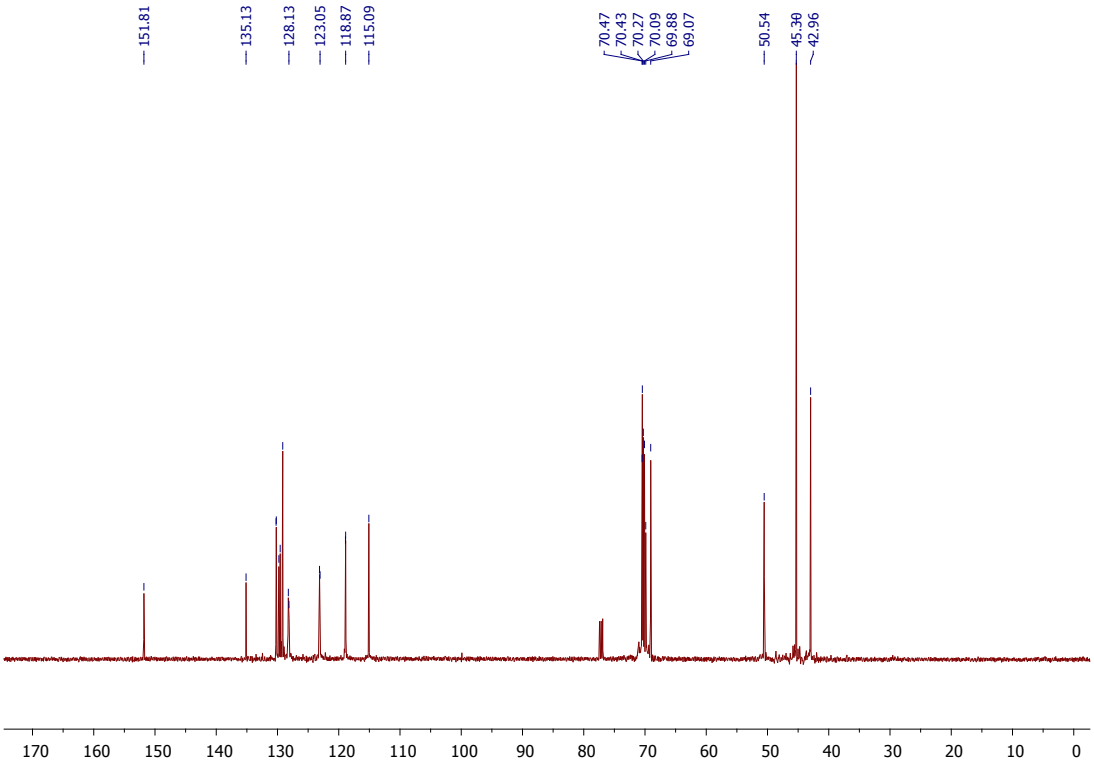


Figure S37. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S4**.

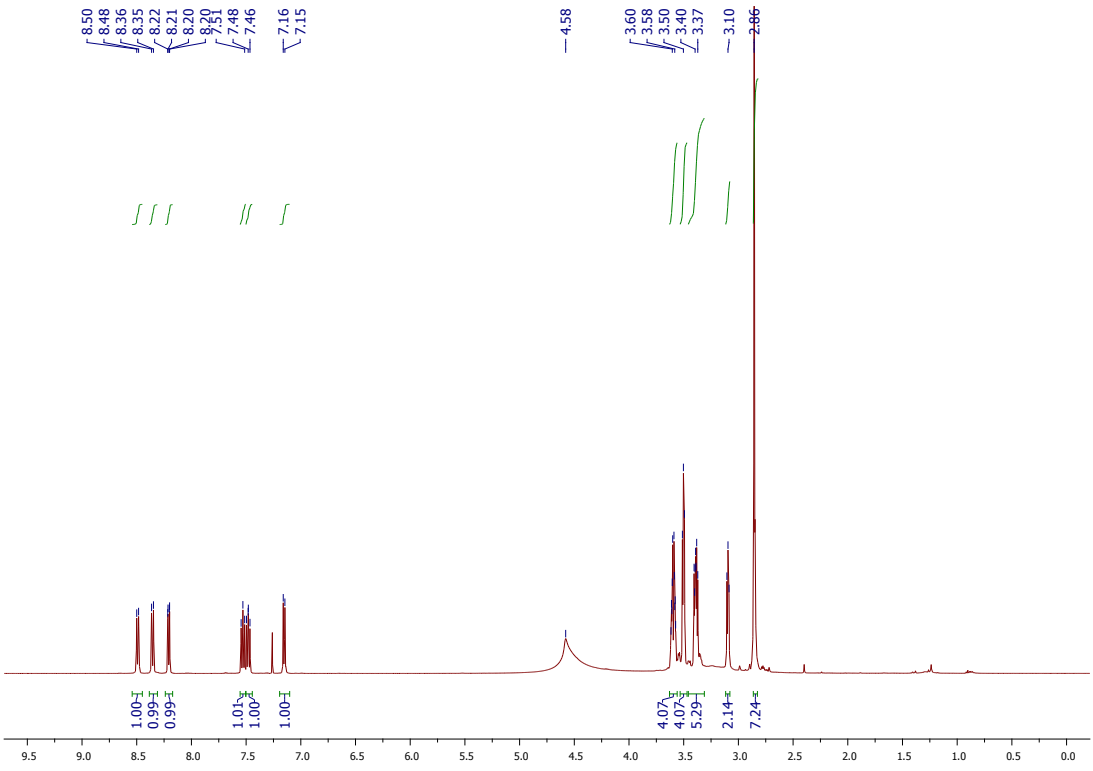


Figure S38. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S5**.

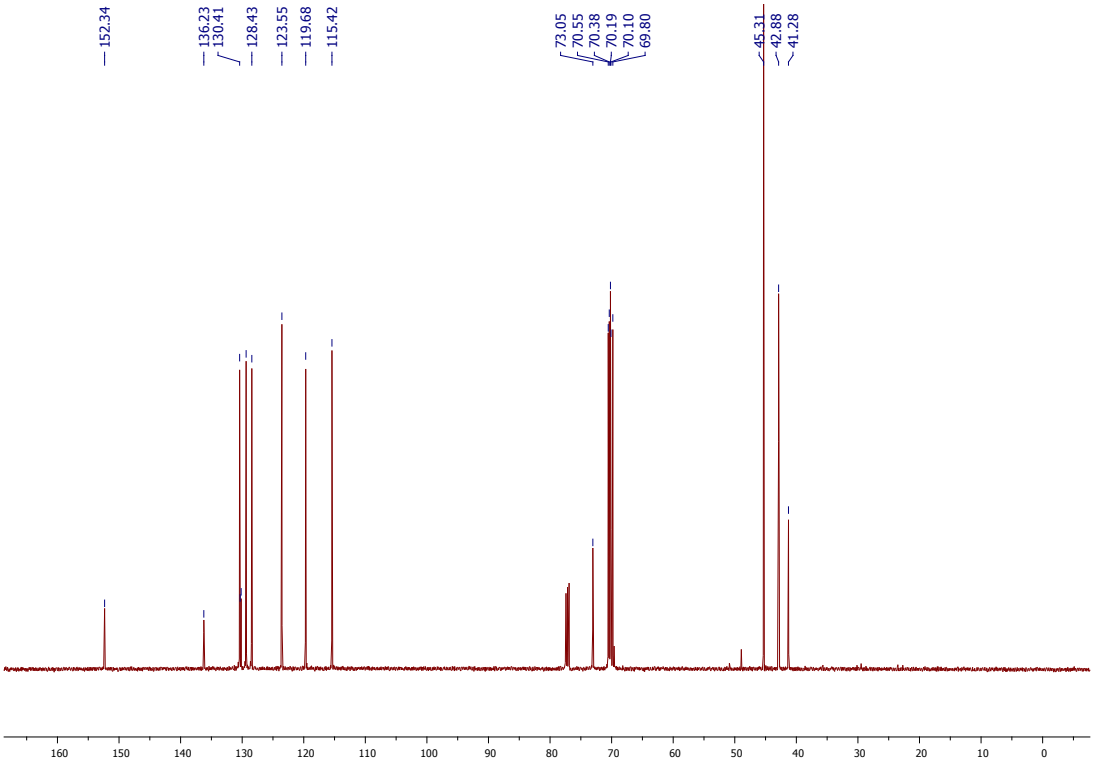


Figure S39. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S5**.

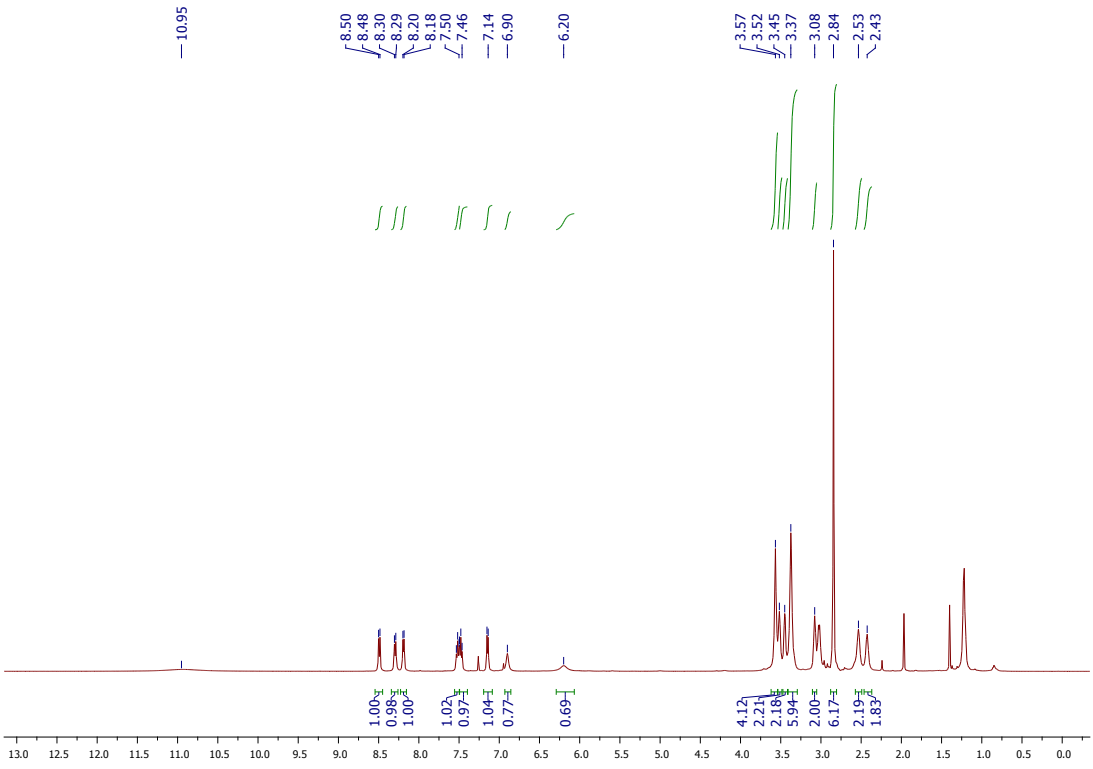


Figure S40. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S6**.

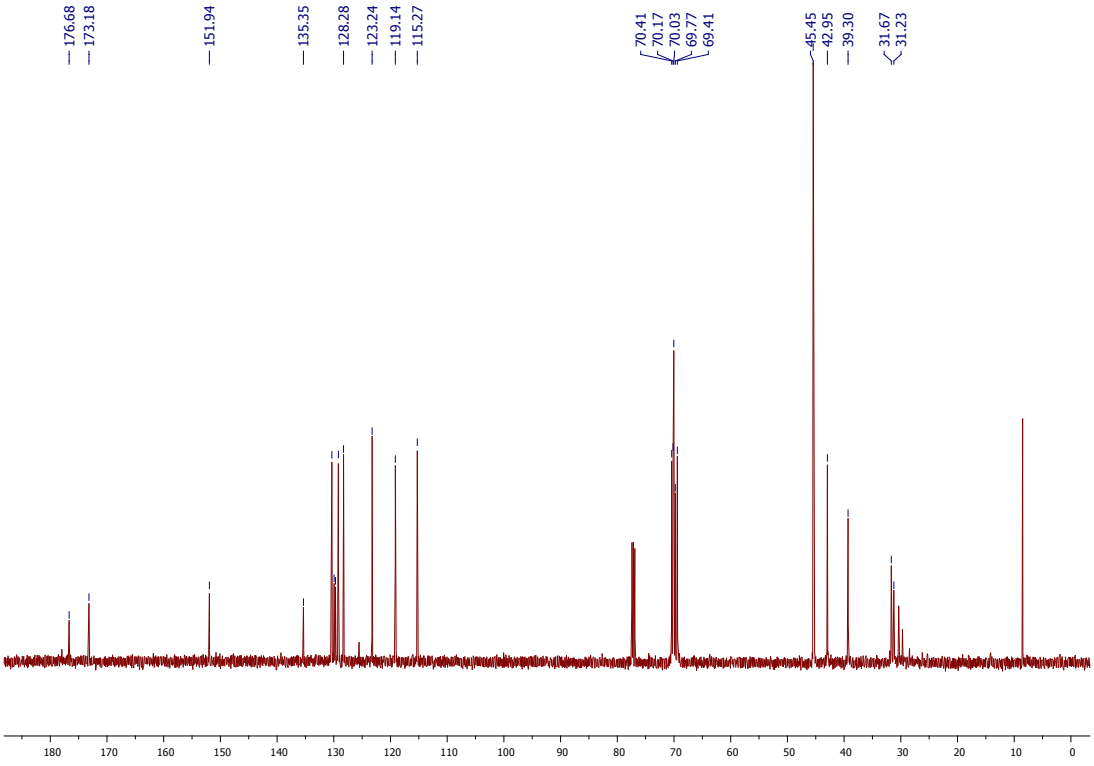


Figure S41. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S6**.

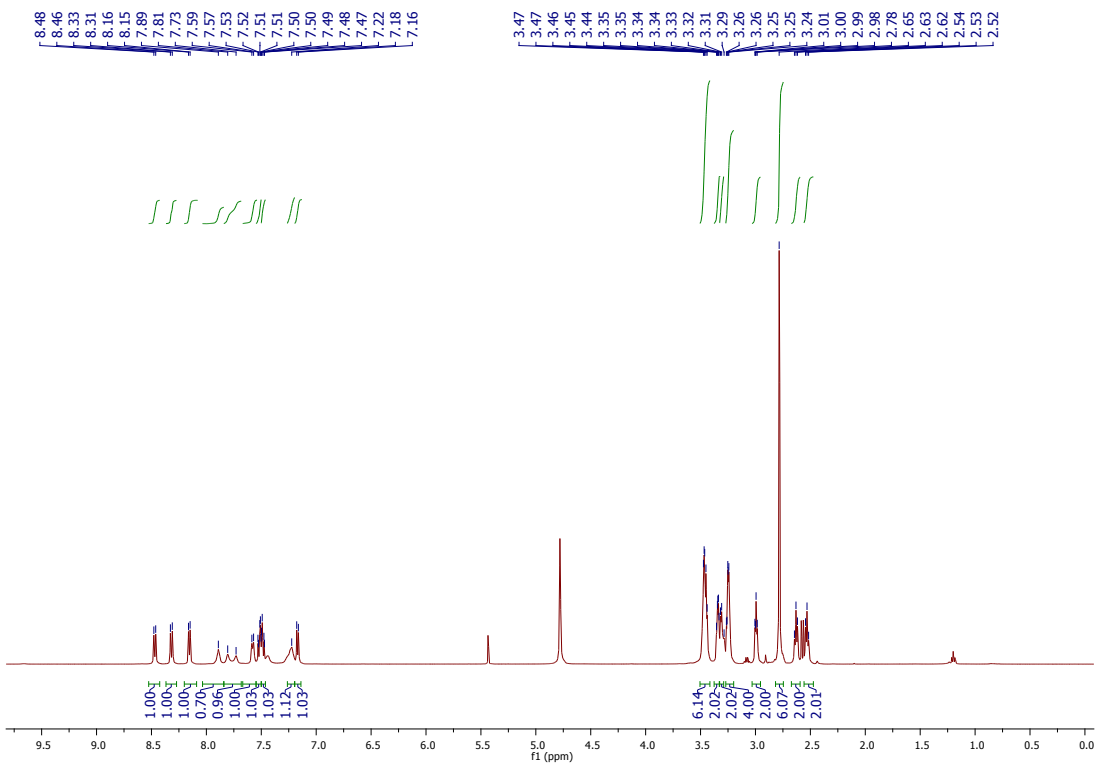


Figure S42. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of **2**.

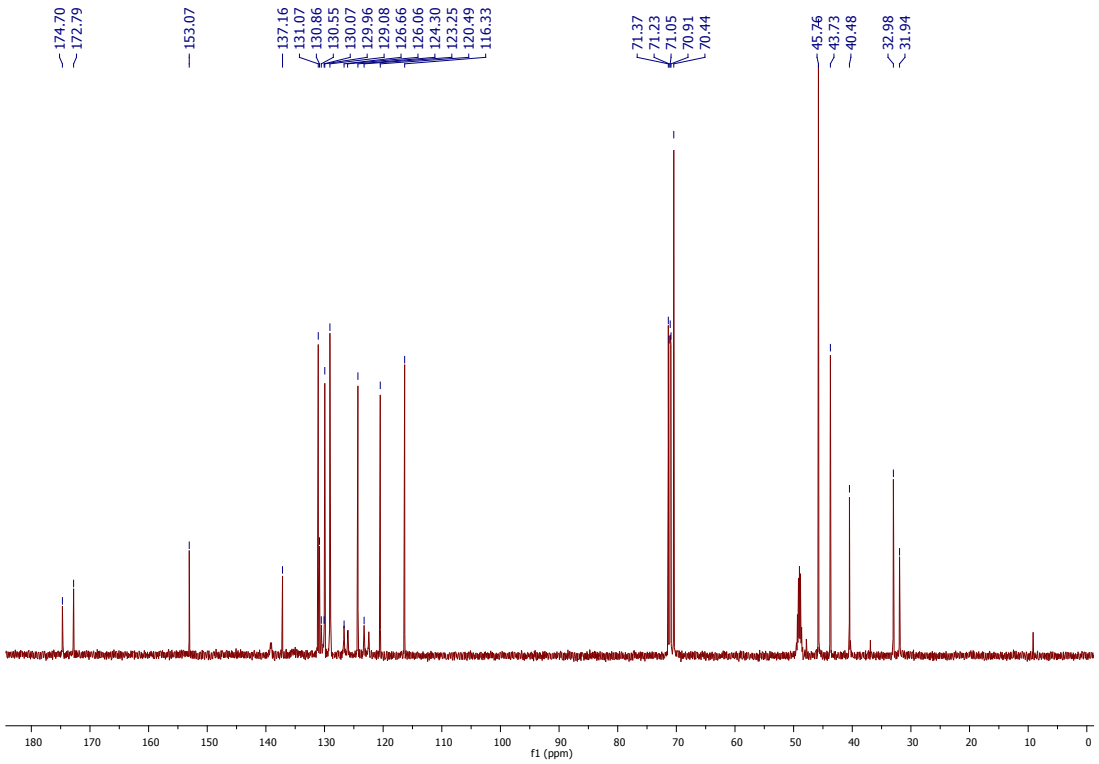


Figure S43. ^{13}C NMR spectrum (CD_3OD, 125 MHz) of **2**.

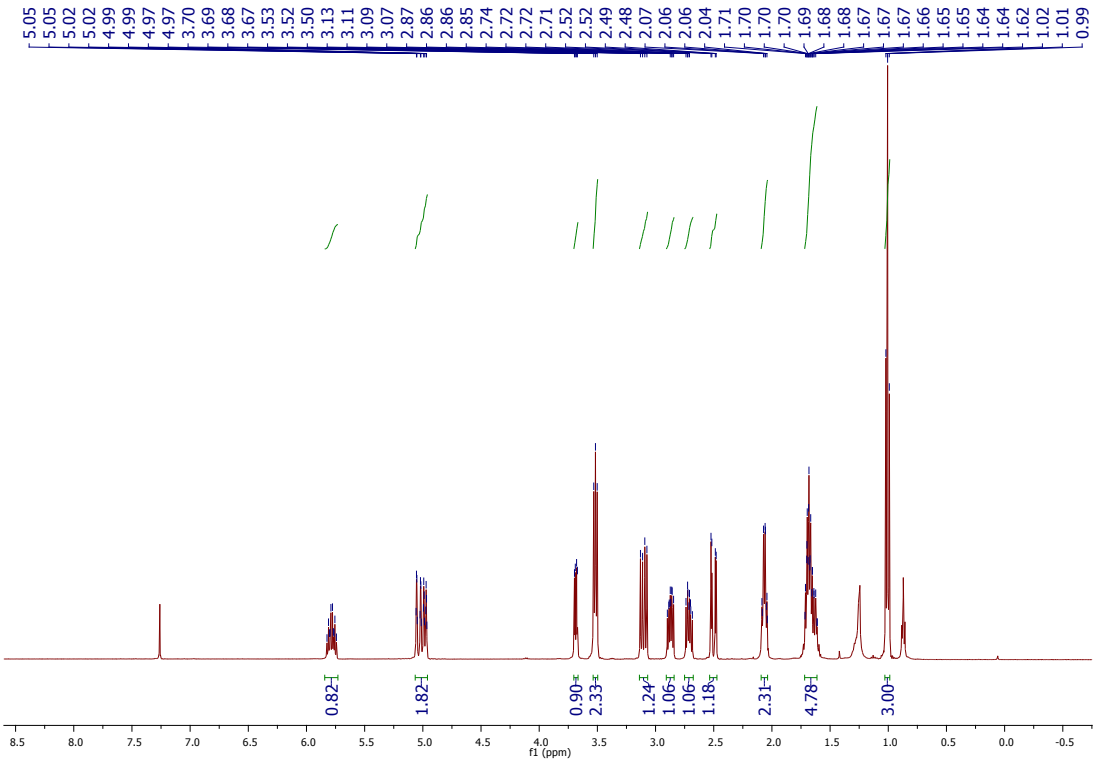


Figure S44. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **3**.

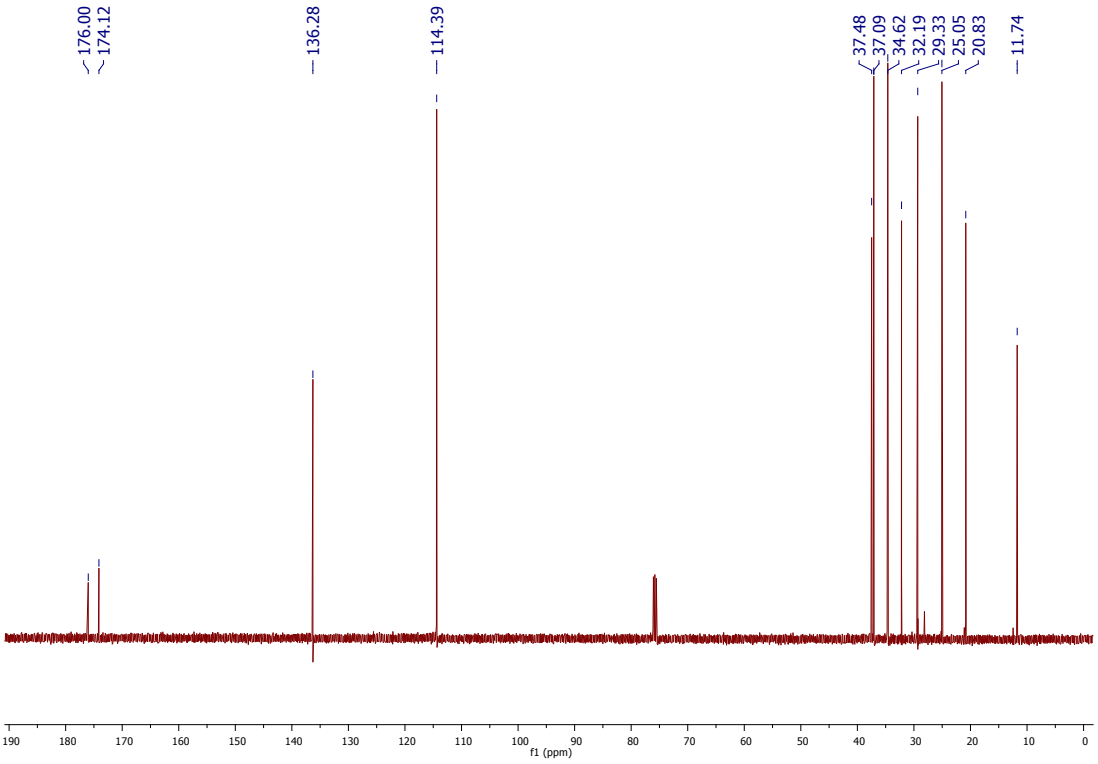


Figure S45. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **3**.

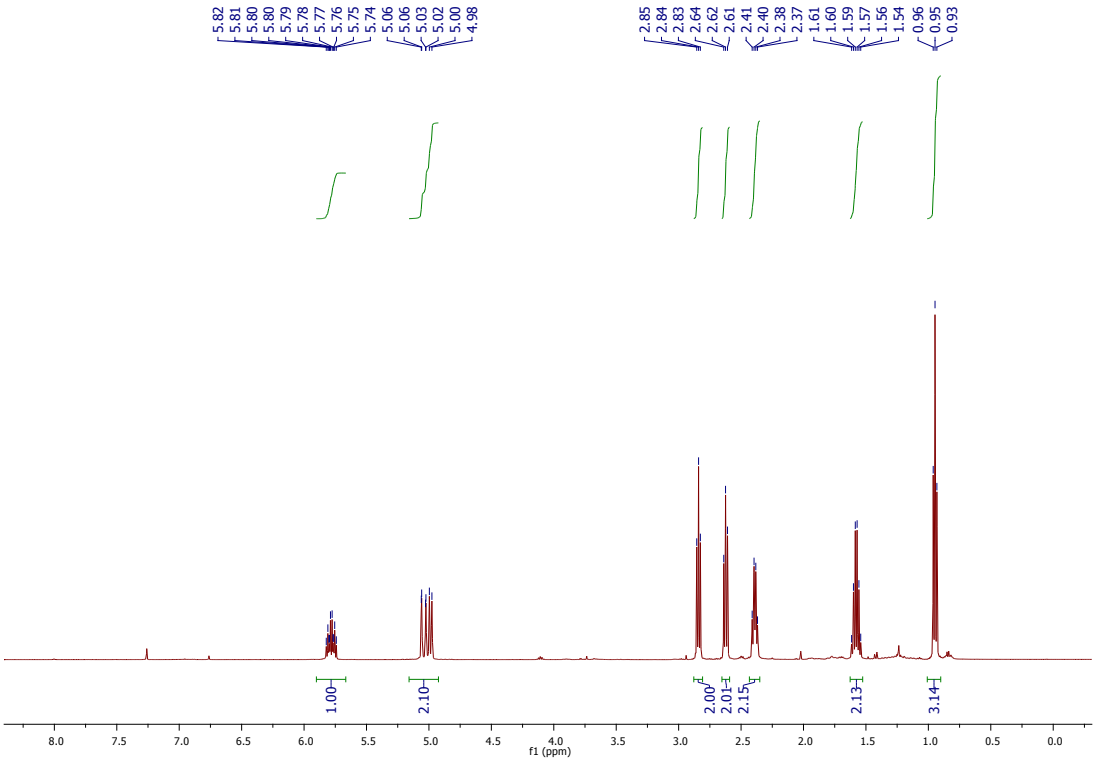


Figure S46. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S7**.

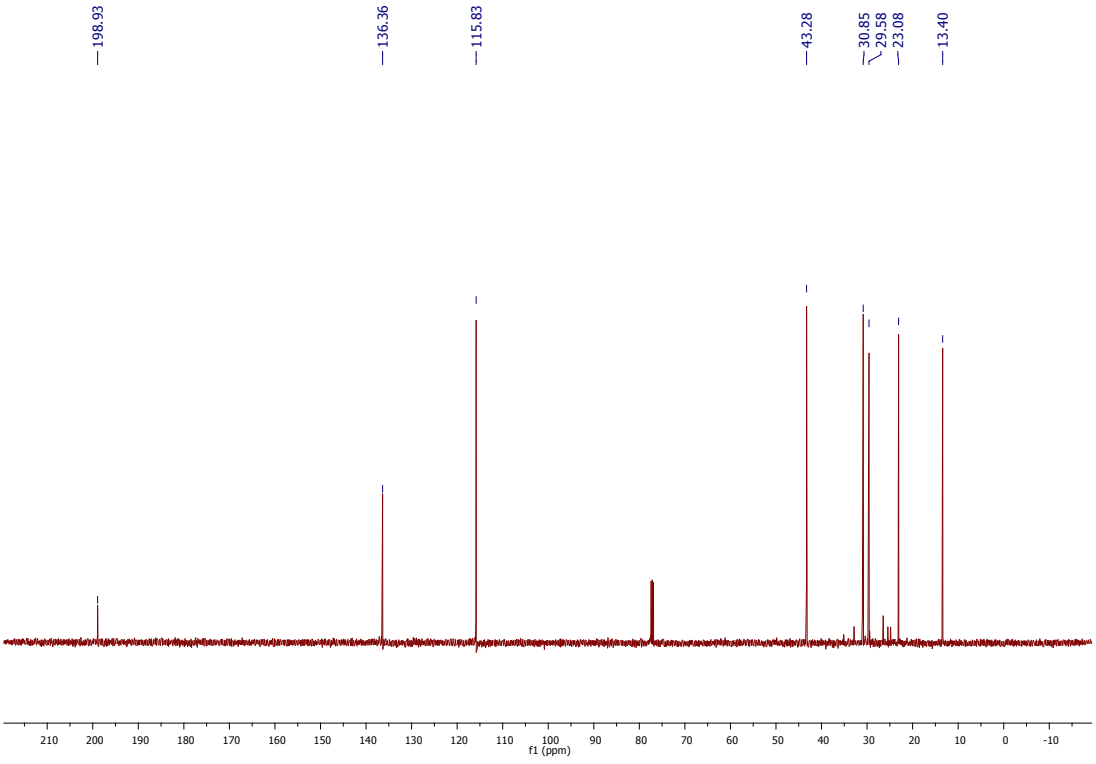


Figure S47. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S7**.

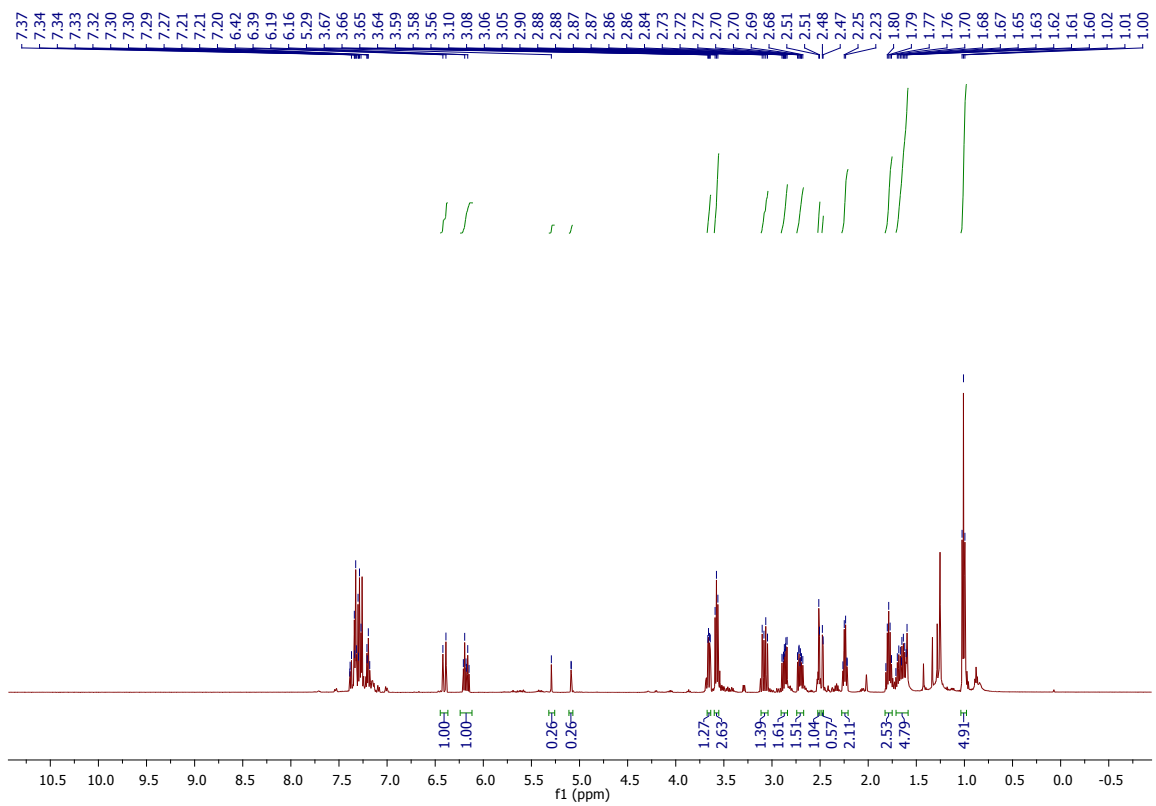


Figure S48. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S8a** and **S8b** (2-amino-4,6-dihydroxypyrimidine ligand).

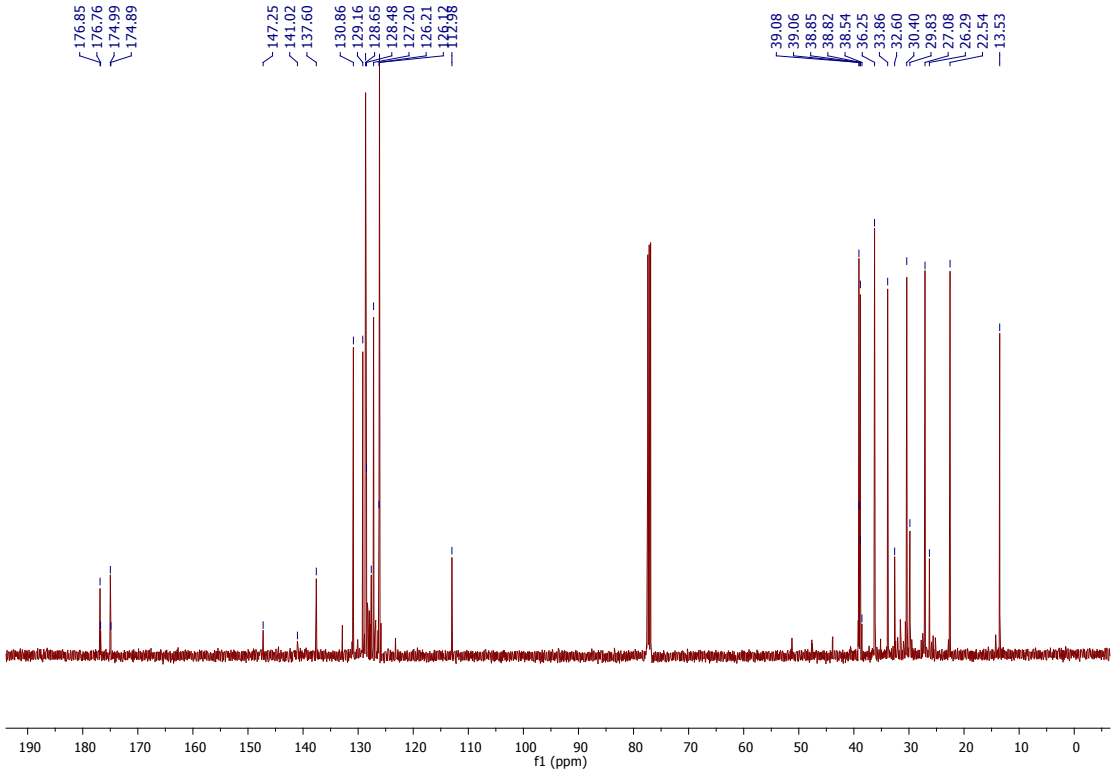


Figure S49. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **S8a** and **S8b** (2-amino-4,6-dihydroxypyrimidine ligand).

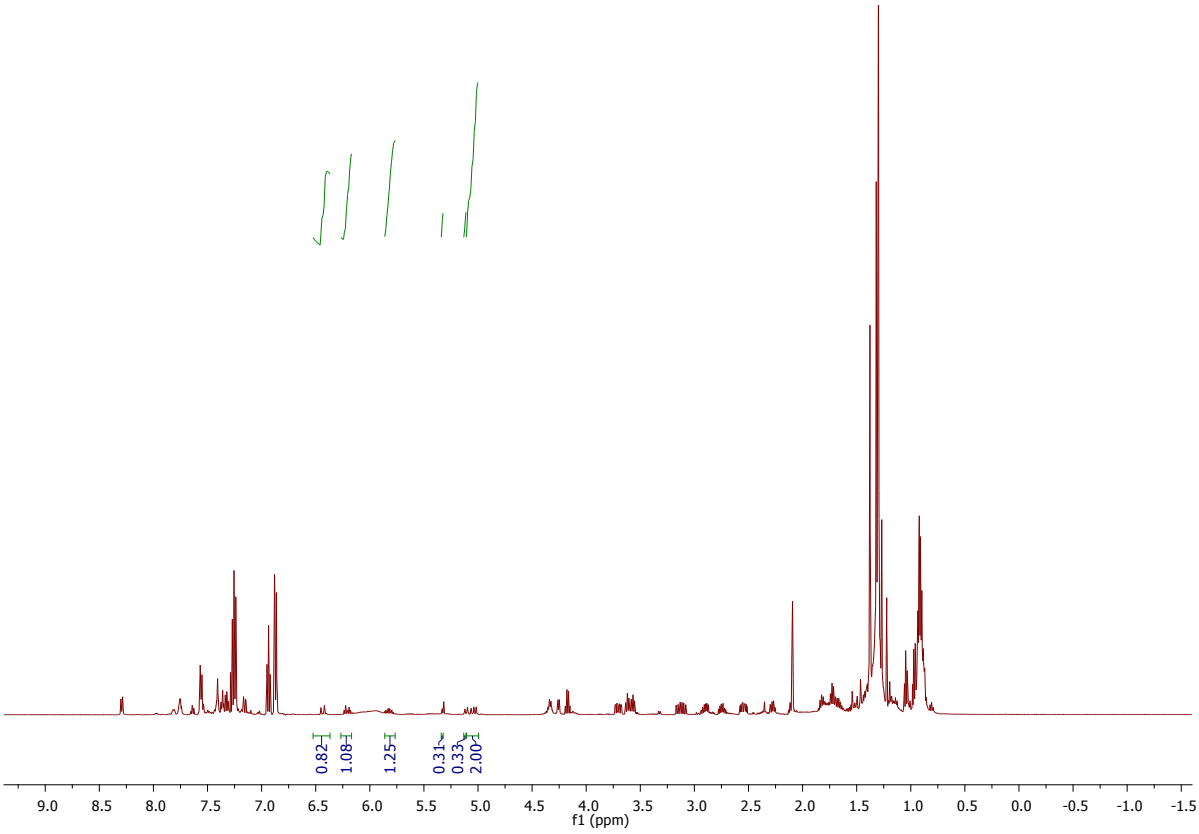


Figure S50. Crude ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S8a** and **S8b** (EDTA ligand).

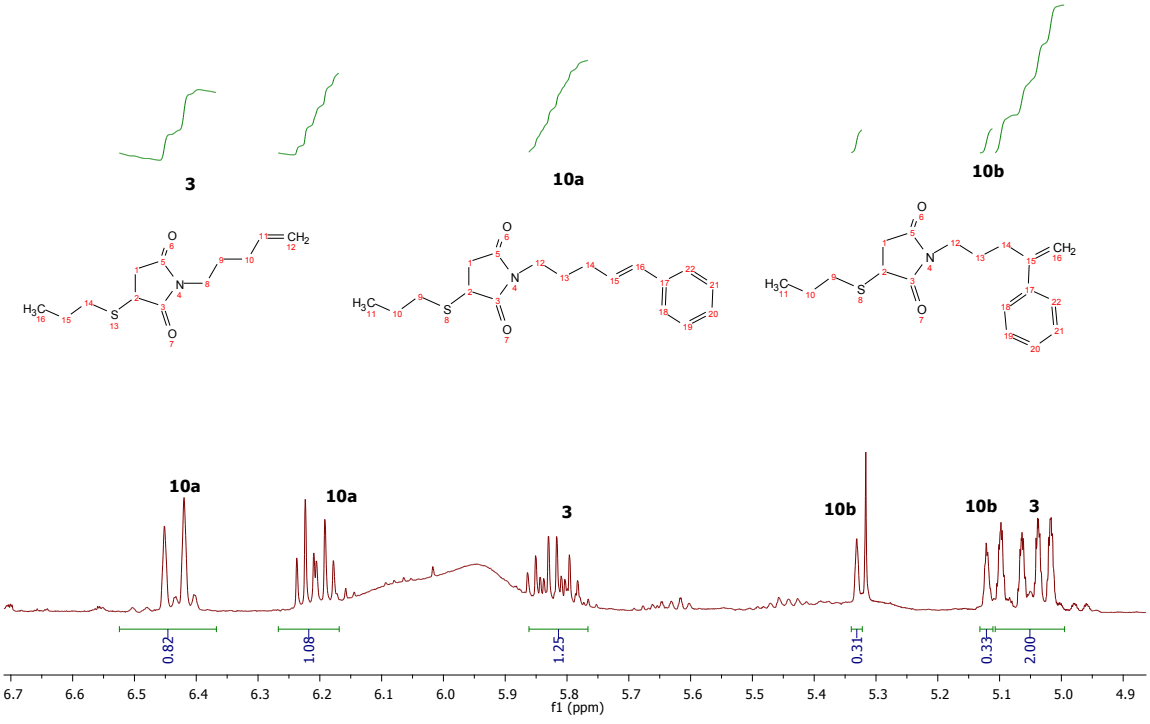


Figure S51. Olefinic area of the crude ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **S8a** and **S8b** (EDTA ligand).

13. References

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