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Supporting Information

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Aqueous Oxidative Heck Reaction as a Protein-Labeling Strategy

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1. Oxidative Heck reaction on protein bound alkenes

1.1 Preparation of protein bound alkenes

1.1.1 General

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 gel filtration columns were used. The protein was analyzed using precasted 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (NuPAGE® 10% Bis-Tris gel, Invitrogen™, Carlsbad, CA). As a ladder Fermentas PageRuler™ Prestained Protein Ladder was used. Gels were stained with the coomassie based gel stain InstantBlue™ (Expedeon Ltd, Harston, Cambridgeshire, UK). Protein concentrations of the unmodified enzyme 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 enzyme^[3] using Coomassie Protein Assay Reagent (950 mL) from Thermo Scientific from which the absorbance was measured on a SPECTROstar Omega – UV/Vis absorbance spectrophotometer microplate reader from BMG Labtech. Gel imaging was performed on a Chemi Genius² Bio Imaging System. The EtBr/UV emission filter (550-640nm) was used for fluorescence imaging of the gels.

RAW264.7 Cells were purchased from American Type Culture Collection, Manassas, USA and Dulbecco's Modified Eagle Medium (DMEM) from Life Technologies.

The 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% H₂O / 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 TurbolonSpray 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.

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

PIAQIHILEGRSDEQKETLIREVSEAIRSLDAPLTSVRVIITEMAKGHFGIGGELASKVCR

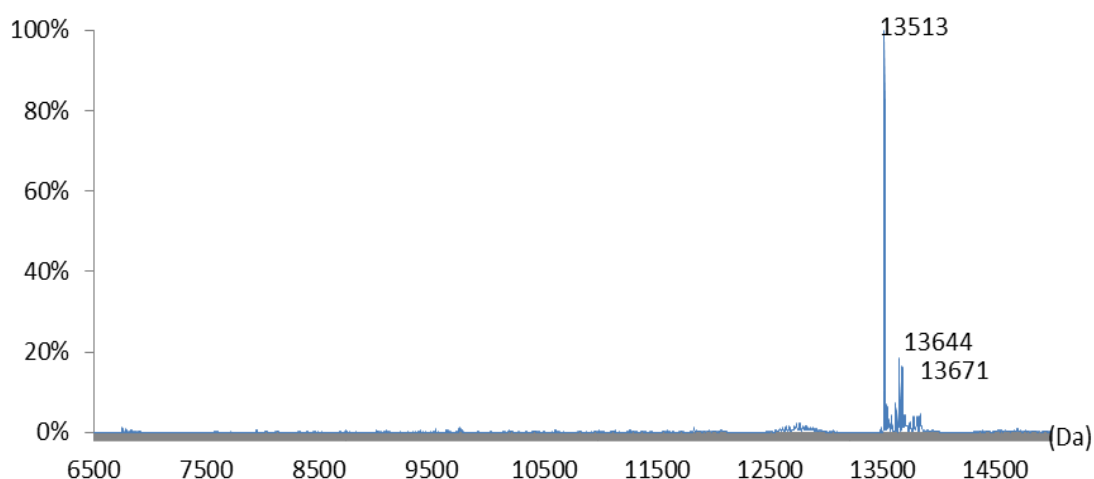
1.1.3 Expression and purification 4-OT R61C

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.^[4]

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.

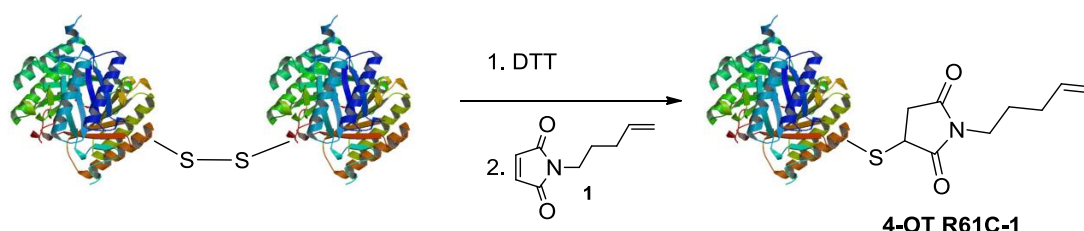
ESI-MS spectrum **4-OT R61C**: mass expected= 6757, mass found= 13513 (dimer)



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.

1.2 Preparation of the 4-OT conjugates

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

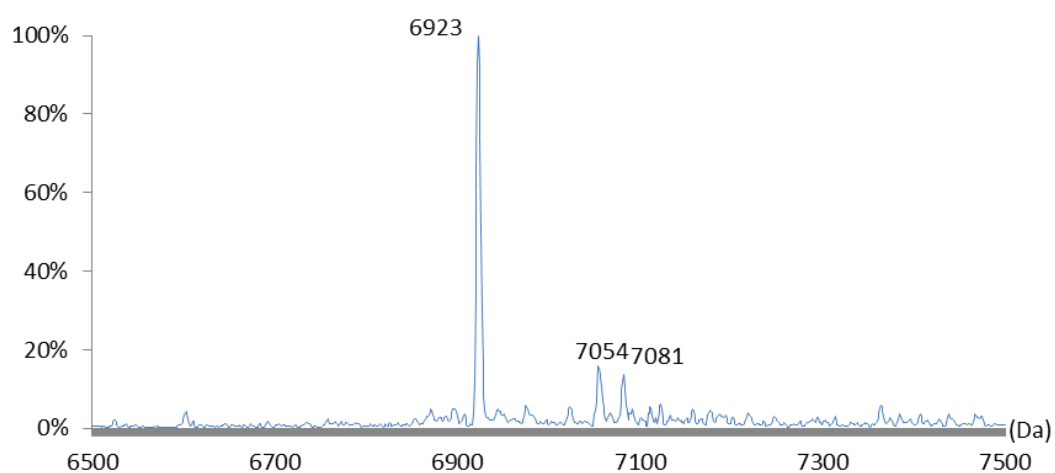


The protein solution was warmed to room temperature. A 130 μ L aliquot (96 nmol) was added to a 1.5 mL cup along with sodium phosphate buffer (300 μ L, pH 8.0, 50 mM). A stock solution of dithiothreitol (DTT) was prepared by dissolving DTT (3.1 mg, 0.02 mmol) in sodium phosphate buffer (320 μ L, pH 8.0, 50 mM). In order to reduce the disulfide, 30 μ L of

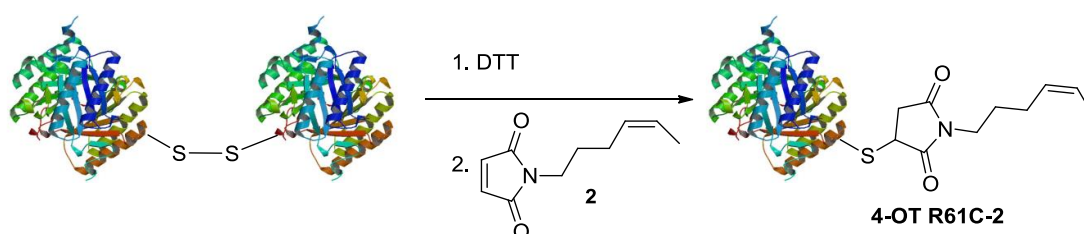
the DTT stock solution was added to the protein solution and the mixture was shaken at room temperature for 10 min. A stock solution of 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione (**1**) was prepared by dissolving **1** (34 mg, 0.21 mmol) in CH₃CN (334 μ L). The final concentration of the stock was 0.63 M. 30 μ L of the stock solution of **1** was added to the protein solution and the mixture was shaken at room temperature for 10 min. The protein was purified by using PD-10 size exclusion. 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.

A 0.65 mg/ml protein solution was prepared. 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).



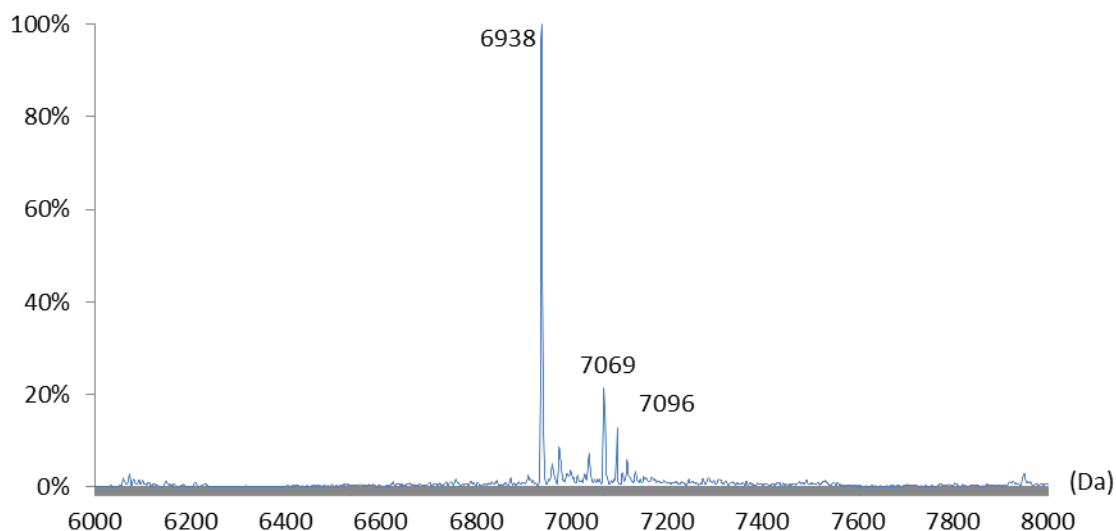
1.2.2 Reduction of 4-OT R61C dimer and coupling with (Z)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (4-OT R61C-2)



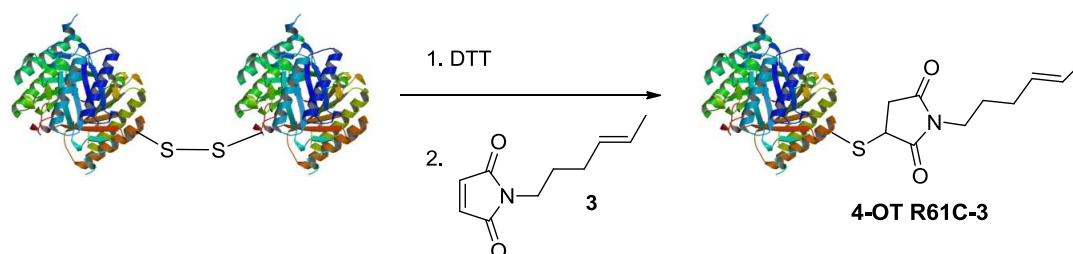
The reduction of the dimer was done according to the procedure described for **4-OT R61C-1**. A stock solution of (Z)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (**2**) was prepared by dissolving **2** (38 mg, 0.21 mmol) in CH₃CN (334 μ L). The final concentration of the stock was 0.63 M. 30 μ L of the stock solution of **2** was added to the protein solution and the mixture was shaken at room temperature for 10 min. The purification of the protein and the concentration assessment were done as described for **4-OT R61C-1**. A solution containing 0.65 mg/mL was

prepared in ammonium formate buffer (pH 7.0, 50 mM). An aliquot of this protein sample was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with mass corresponding to the mass of **4-OT R61C-2**.

ESI-MS spectrum: mass expected 6936, mass found 6938 (7069: modified enzyme with methionine, 7096: modified enzyme with formylated methionine)

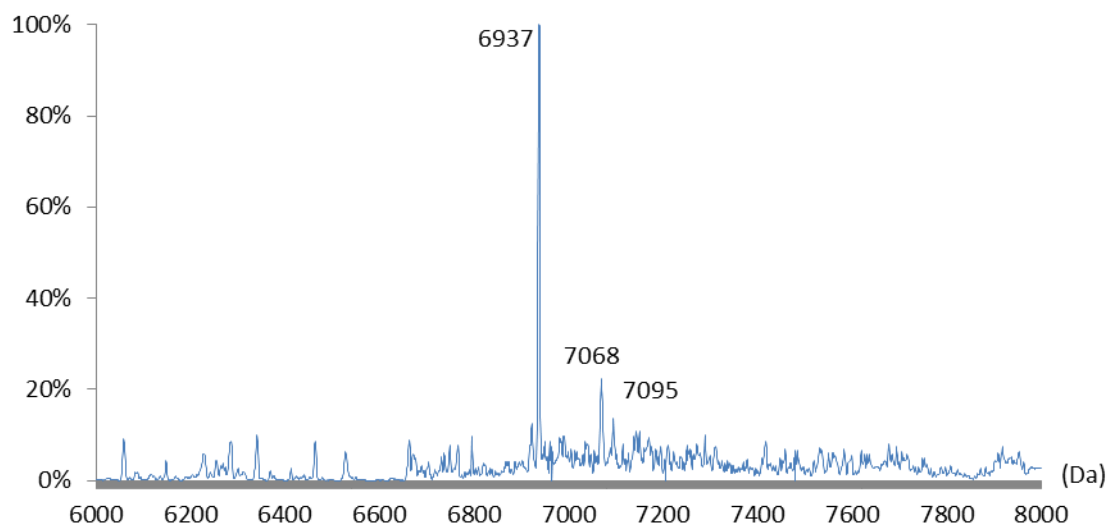


1.2.3 Reduction of 4-OT R61C dimer and coupling with (*E*)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (**4-OT R61C-3**)

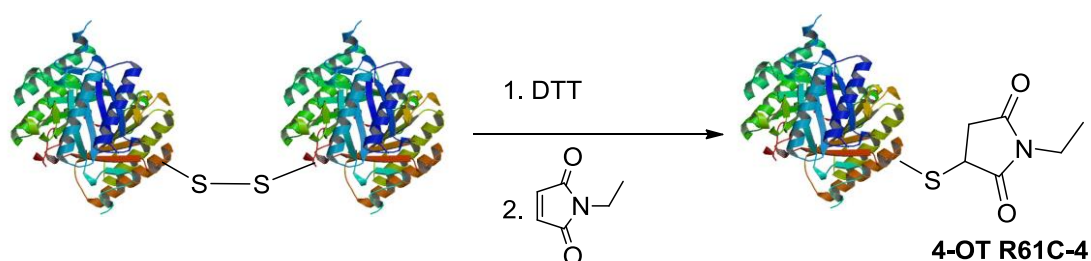


The reduction of the dimer was done according to the procedure described for **4-OT R61C-1**. A stock solution of (*E*)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (**3**) was prepared by dissolving **3** (38 mg, 0.21 mmol) in CH₃CN (334 μL). 30 μL of this solution was added to the reduced **4-OT R61C-1** and the reaction mixture was shaken at room temperature for 10 min. The purification of the protein and the concentration assessment were done as described for **4-OT R61C-1**. A solution containing 0.65 mg/mL was prepared in ammonium formate buffer (pH 7.0, 50 mM). An aliquot of this protein sample was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with a mass corresponding to the mass of **4-OT R61C-3**.

ESI-MS spectrum: mass expected 6936, mass found 6937 (7068: modified enzyme with methionine, 7095: modified enzyme with formylated methionine)

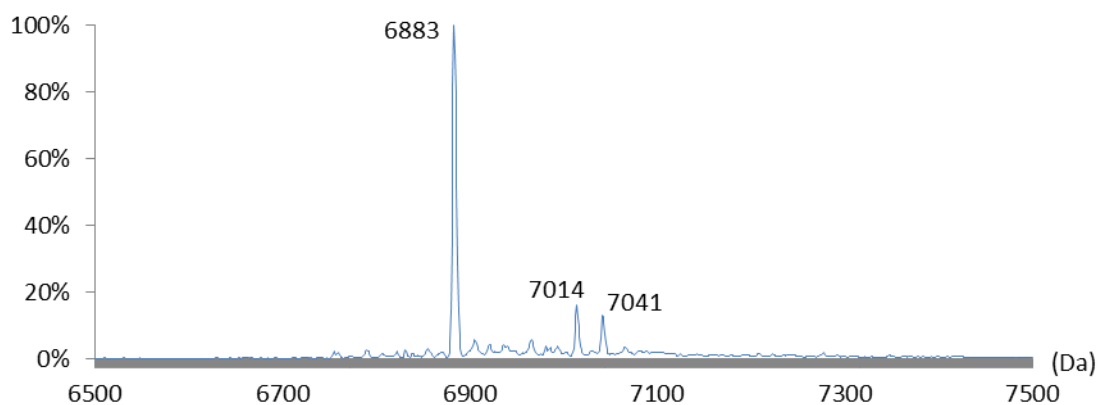


1.2.4 Reduction of 4-OT dimer and coupling with *N*-ethylmaleimide (4-OT R61C-4)



The reduction of the dimer was done according to the procedure described for **4-OT R61C-1**. A stock solution of *N*-ethylmaleimide was prepared by dissolving the *N*-ethylmaleimide (31 mg, 0.25 mmol) in CH₃CN (0.4 ml). 30 μL of this solution was added to the reduced **4-OT R61C-1** and the reaction mixture was shaken at room temperature for 10 min. The purification of the protein and the concentration assessment were done as described for **4-OT R61C-1**. A solution containing 0.65 mg/mL was prepared in ammonium formate buffer (pH 7.0, 50 mM). An aliquot of this protein sample was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with mass corresponding to the mass of **4-OT R61C-4**.

ESI-MS spectrum: mass expected 6882, mass found 6883 (7014: modified enzyme with methionine, 7041: modified enzyme with formylated methionine)



1.3 Oxidative Heck reaction for bioorthogonal protein ligation

1.3.1 Preparation of stock solutions

Palladium (II)-BIAN catalyst stock solution

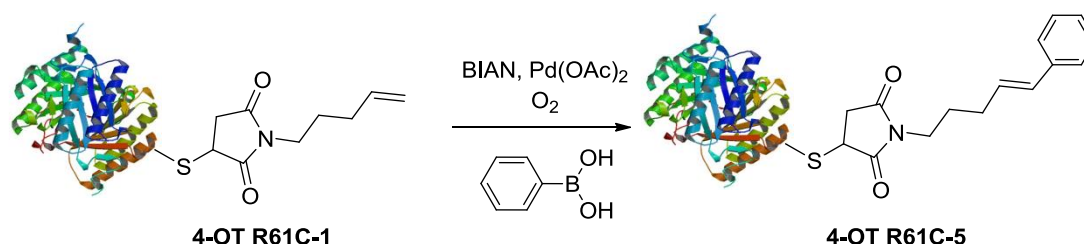
BIAN (4.3 mg, 11 μmol) and $\text{Pd}(\text{OAc})_2$ (1.8 mg, 8 μmol) were dissolved in DMF (1 mL) in a 10 mL double neck round bottom flask equipped with a magnetic stirring bar and a septum. The flask was equipped with an oxygen balloon on the side arm. Oxygen was flushed through the flask and the mixture was left stirring for 30 min at room temperature. The final concentration in Pd was 8 mM and in BIAN 11 mM.

Note: Mass spectrometric analysis proved to be difficult, presumably, due to the fact that palladium (II) binds to aminoacids such as histidine.^[5] 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.

1.3.2 Coupling of phenylboronic acid to protein bound terminal alkene 4-OT R61C-1

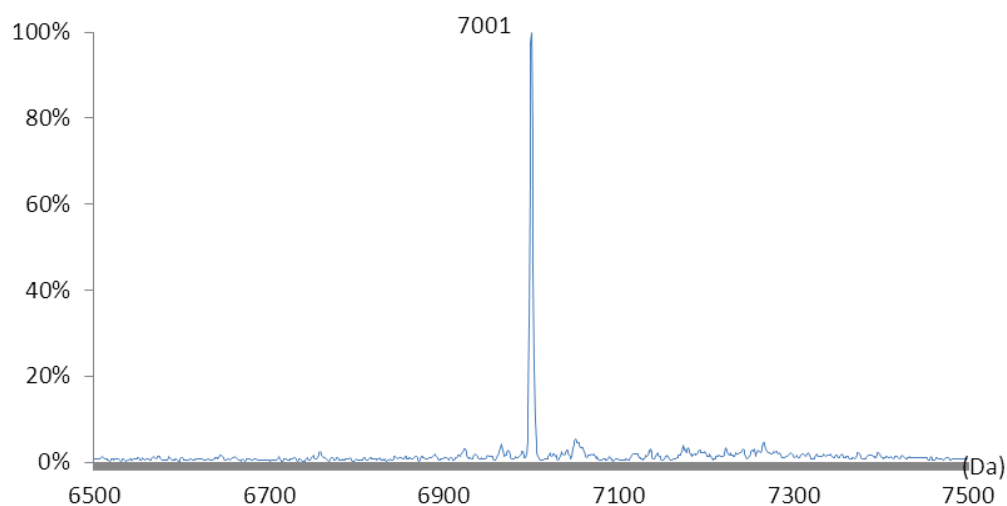


A stock solution was prepared by dissolving phenylboronic acid (4.0 mg, 33 μmol) in $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ buffer (825 μL , pH 7.0, 0.5 M), after shaking at room temperature for 10 min. The final concentration in phenylboronic acid was 40 mM.

50 equiv. catalyst/300 equiv. phenylboronic acid

210 μL of the solution of protein 4-OT R61C-1 (20 nmol), 150 μL of the phenylboronic acid stock solution (6.0 μmol) and 125 μL of the catalyst stock solution (1.0 μmol in Pd, 1.4 μmol in BIAN) 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, it was transferred into a 2 mL cup and 500 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). Each time the sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed full conversion of the protein 4-OT R61C-1 to protein 4-OT R61C-5.

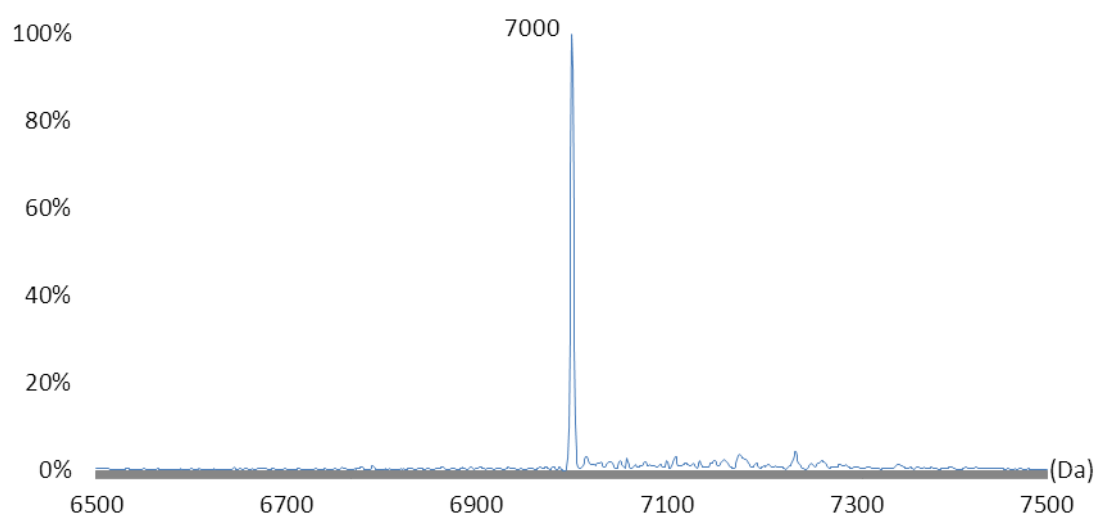
ESI-MS spectrum: mass expected 6999, mass found 7001



20 equiv. catalyst/100 equiv. phenylboronic acid

210 μL of the solution of protein **4-OT R61C-1** (20 nmol), 50 μL of the phenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) 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, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). Each time the sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed full conversion of the protein **4-OT R61C-1** to protein **4-OT R61C-5**.

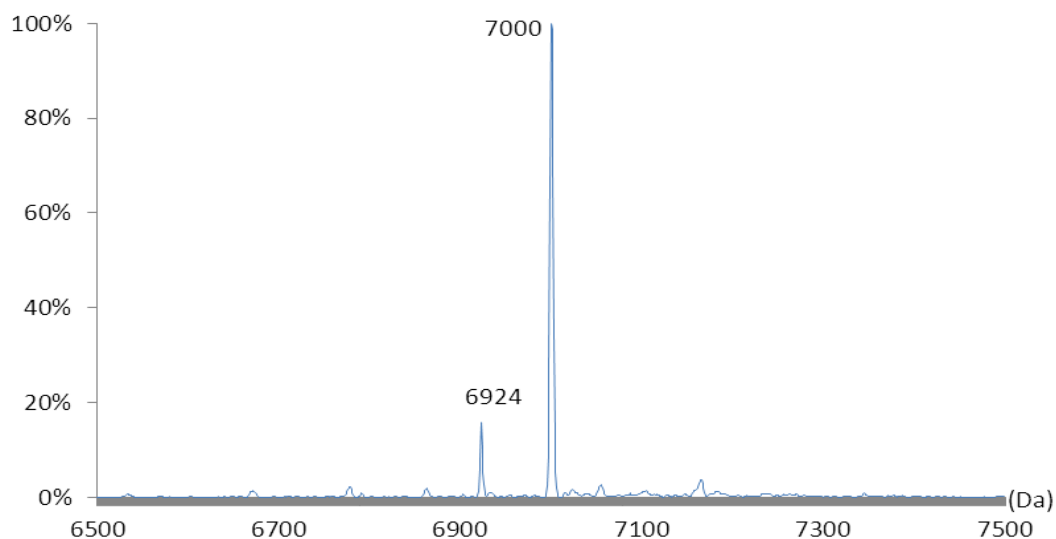
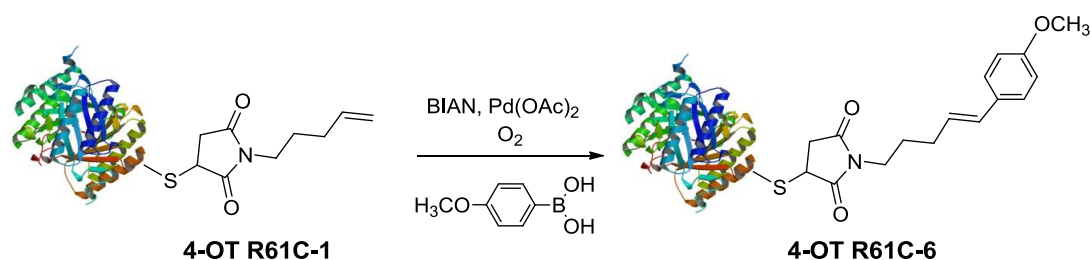
ESI-MS spectrum: mass expected 6999, mass found 7000



10 equiv. catalyst/50 equiv. phenylboronic acid

210 μL of the solution of protein **4-OT R61C-1** (20 nmol), 25 μL of the phenylboronic acid stock solution (1.0 μmol) and 25 μL of the catalyst stock solution (200 nmol in Pd, 275 nmol in BIAN) 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, it was transferred into a 2 mL cup and 100 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). Each time the sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed a protein mass corresponding to the mass of product **4-OT R61C-5** but also a peak with a mass corresponding to protein **4-OT R61C-1**.

ESI-MS spectrum: mass expected 6999, mass found 7000

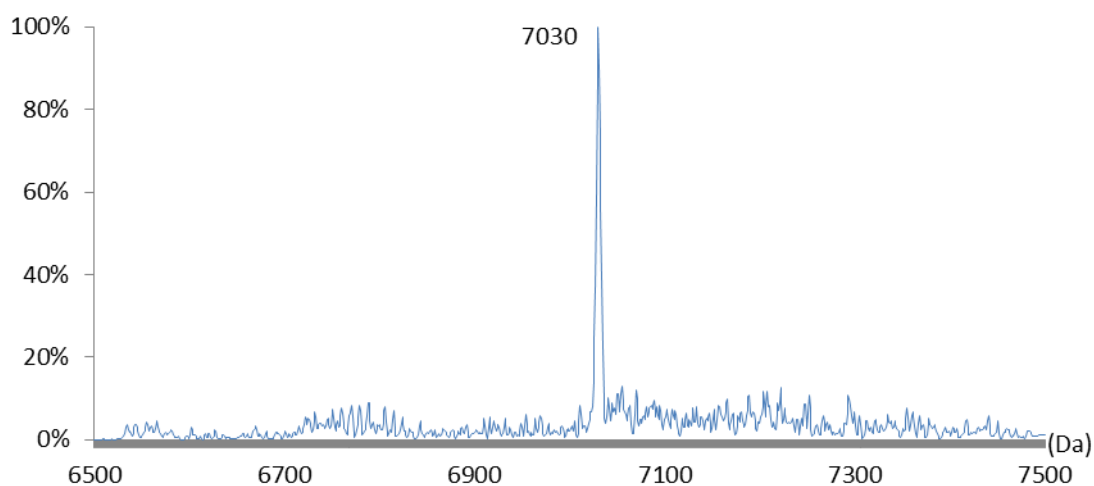
**1.3.3 Coupling of 4-methoxyphenylboronic acid to protein bound terminal alkene 4-OT R61C-1**

A stock solution was prepared by dissolving 4-methoxyphenylboronic acid (4.9 mg, 32 μmol) in $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ buffer (400 μL , pH 7.0, 0.5 M) and DMF (400 μL). The final concentration in 4-methoxyphenylboronic acid was 40 mM.

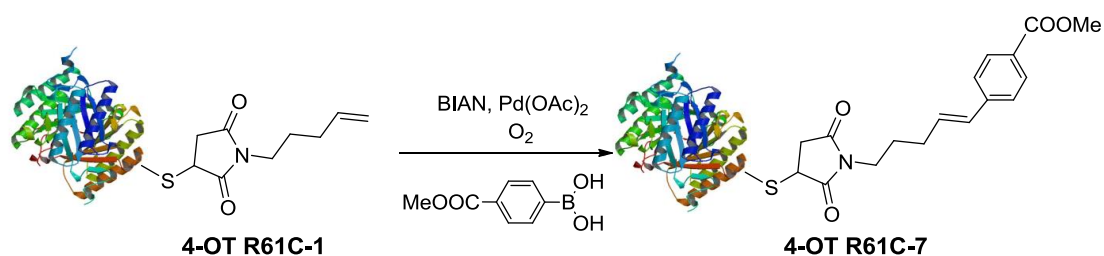
210 μL of the solution of protein **4-OT R61C-1** (20 nmol), 50 μL of the 4-methoxyphenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask,

equipped with a 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 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. Subsequently, a microextraction was performed using *n*-heptane (3x500 μL). The sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed a protein mass corresponding to the mass of product **4-OT R61C-6**.

ESI-MS spectrum: mass expected 7029, mass found 7030



1.3.4 Coupling of 4-(methoxycarbonyl)phenylboronic acid to protein bound terminal alkene **4-OT R61C-1**

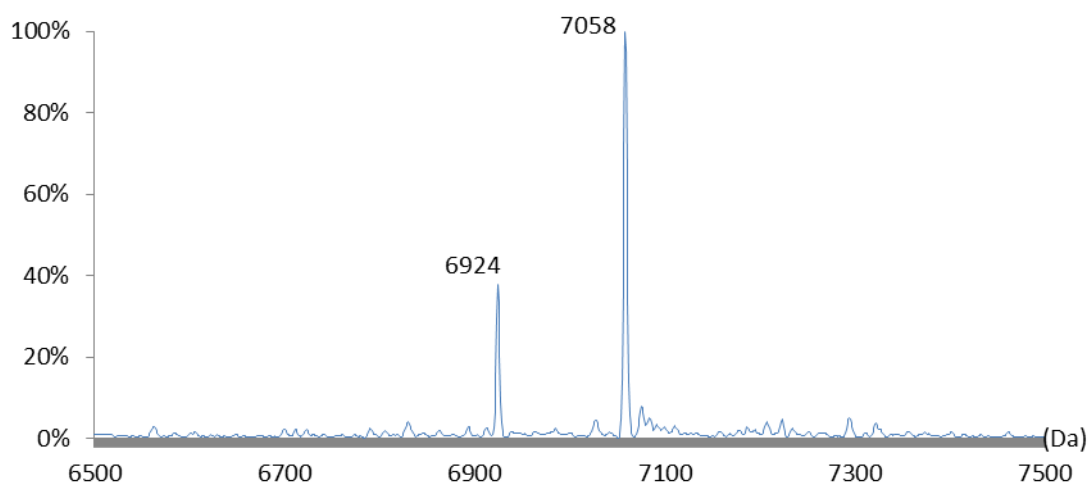


A stock solution was prepared by dissolving 4-(methoxycarbonyl)phenylboronic acid (5.8 mg, 32 μmol) in $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ buffer (400 μL , pH 7.0, 0.5 M) and DMF (400 μL). The final concentration in 4-(methoxycarbonyl)phenylboronic acid was 40 mM.

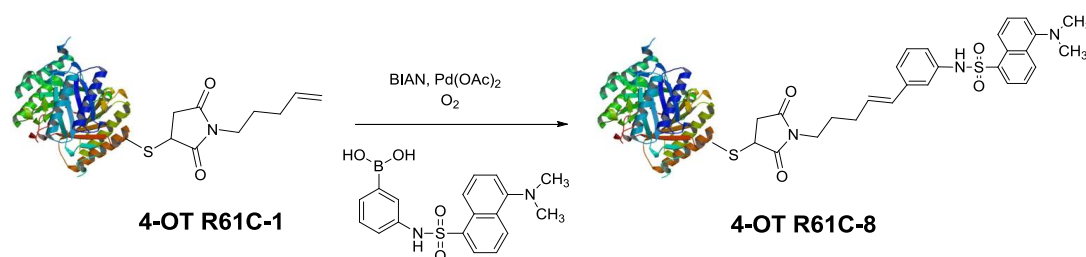
210 μL of the solution of protein **4-OT R61C-1** (20 nmol), 50 μL of the 4-(methoxycarbonyl)phenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum. The reaction mixture was left stirring at room temperature under an oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). The sample was homogenized by vortexing carefully and the organic

phase was removed. LC-MS analysis showed a protein mass corresponding to the mass of product **4-OT R61C-7** but also a peak with mass corresponding to protein **4-OT R61C-1**.

ESI-MS spectrum: mass expected 7057, mass found 7058



1.3.5 Coupling of 3-(dansylamino)phenylboronic acid to protein bound terminal alkene **4-OT R61C-1**



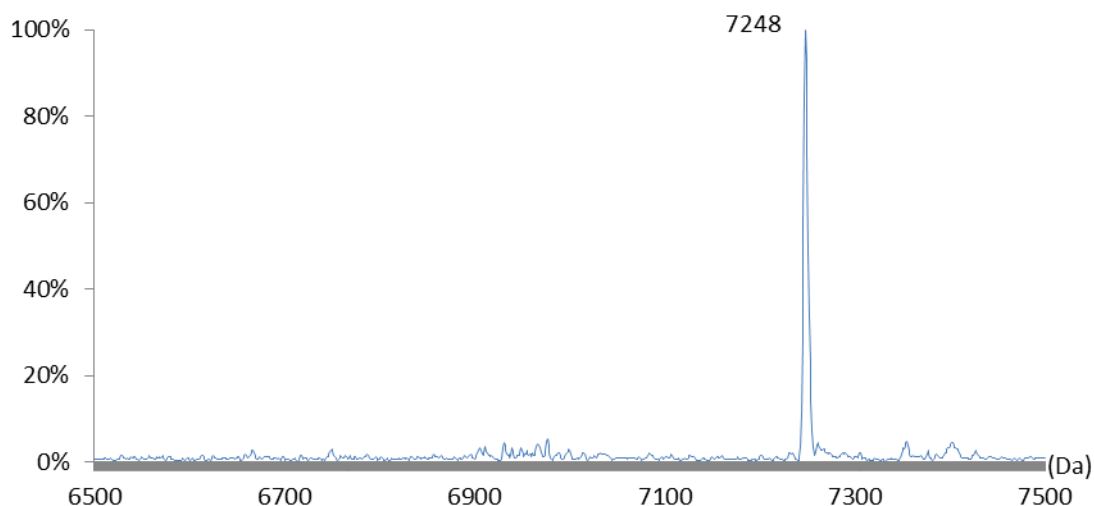
A stock solution of the fluorophore was prepared by dissolving 3-(dansylamino)phenylboronic acid (3.7 mg, 10 μmol) in $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ buffer (125 μL , pH 7.0, 0.5 M) and DMF (125 μL). The final concentration in fluorophore was 40 mM.

210 μL of the solution of protein **4-OT R61C-1** (20 nmol), 50 μL of the fluorophore stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a 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 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. DMF (1.0 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 (400 μL). LC-MS analysis showed a protein mass corresponding to the mass of product **4-OT R61C-8**.

Fluorescence imaging of SDS PAGE

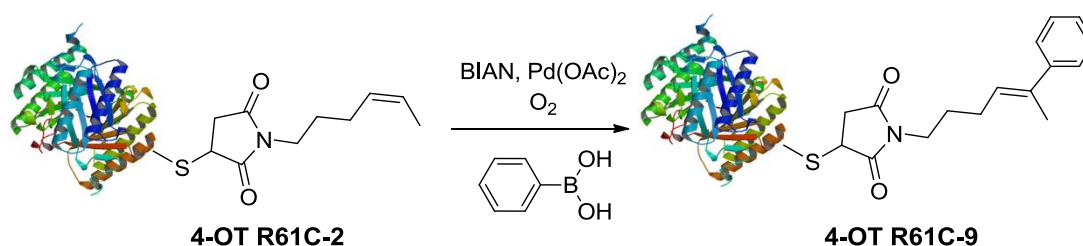
5 μg of **4-OT R61C**, 6 μL or 9 μL of **4-OT R61C-8** all mixed with 10 μL of the sample buffer and 5 μL of the ladder were loaded on SDS-PAGE (12% crosslinking). Bromophenol blue was excluded from the protein sample buffer because it quenches the fluorescence of the Dansyl fluorophore. Following the running of the gel for 50 min at 150 V, a picture was taken under UV light (exposure time 4 sec). The gel was then stained with Coomassie and a new picture was taken under visible light.

ESI-MS spectrum: mass expected 7247, mass found 7248



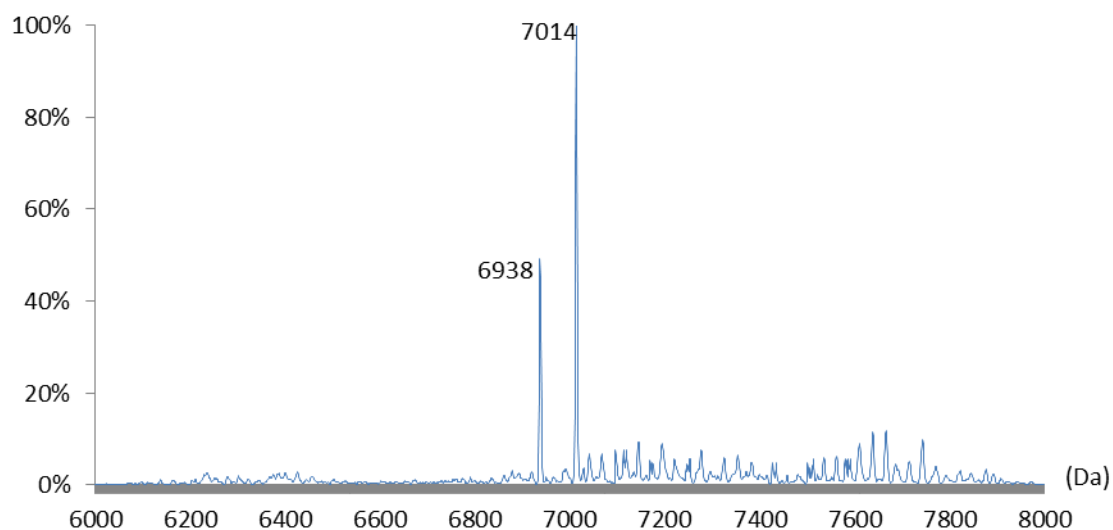
1.3.6 Coupling of phenylboronic acid to protein bound cis internal alkene 4-OT R61C-2

The stock solutions of the catalyst, EDTA and phenylboronic acid were prepared as described previously.

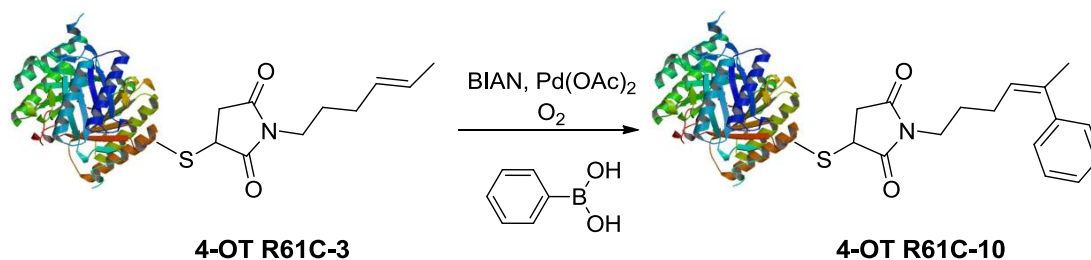


210 μL of the solution of protein **4-OT R61C-2** (20 nmol), 50 μL of the phenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a magnetic stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). Each time the sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed substantial conversion of the protein **4-OT R61C-2** to protein **4-OT R61C-9**.

ESI-MS spectrum: mass expected 7014, mass found 7014

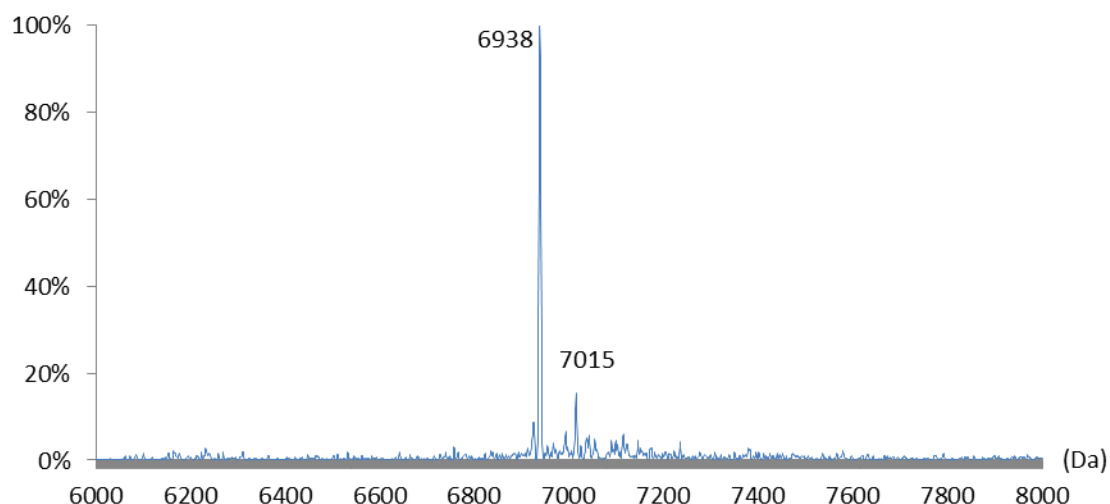


1.3.7 Coupling of phenylboronic acid to protein bound trans-internal alkene 4-OT R61C-4



210 μL of the solution of protein **4-OT R61C-3** (20 nmol), 50 μL of the phenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) 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, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). Each time the sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis showed very limited conversion of the protein **4-OT R61C-3** to protein **4-OT R61C-10**.

ESI-MS spectrum: mass expected 7014, mass found 7015



1.3.8 Coupling of 3-(dansylamino)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 % CO₂. Cells were collected by scrapping, and washed with 1x PBS. Then, they were 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 1 mL lysis buffer (1980 µL of 0.5 % triton in PBS and 20 µL of Protease Inhibitor Cocktail (Sigma Aldrich)). The solution was sonicated three times for 3 sec and centrifuged at 5000 rpm and 4 °C for 5 min. The supernatant, containing the cell lysates, was transferred into a plastic tube of 5 mL. To the protein sample were added 2 % SDS (360 µL), ammonium bicarbonate buffer (3.25 mL, pH 8.0, 200 mM) and TCEP (360 µL, pH 8.0, 200 mM). The sample was then incubated at 55 °C for 1h.

Under the conditions of the oxidative Heck reaction, we observed no ligation to cysteine residues in cell lysate proteins and **4-OT R61C** (see Control reactions below). Blocking with iodoacetamide was performed as a standard procedure to prevent the formation of disulfide bonds.

Blocking with iodoacetamide

Immediately before use, 25 mg of iodoacetamide were dissolved in ammonium bicarbonate buffer (360 µL, pH 8.0, 200 mM) to make a stock solution of a concentration of 375 mM (protect from light). 360 µL of this stock was added and the cell lysate sample, which was subsequently incubated for 30 min (protected from light). The sample was then loaded into a viva spin column (5000 Da cut) and centrifuged at 4000 rpm for 45 min at 4 °C. After the addition of ammonium formate buffer (15 mL, pH 7.0, 1 M), it was centrifuged again for 1 h and transferred into a 2 mL cup. The cell lysates were diluted to 6 mg/mL protein concentration (determined by Waddell method) and stored at -20 °C until needed.

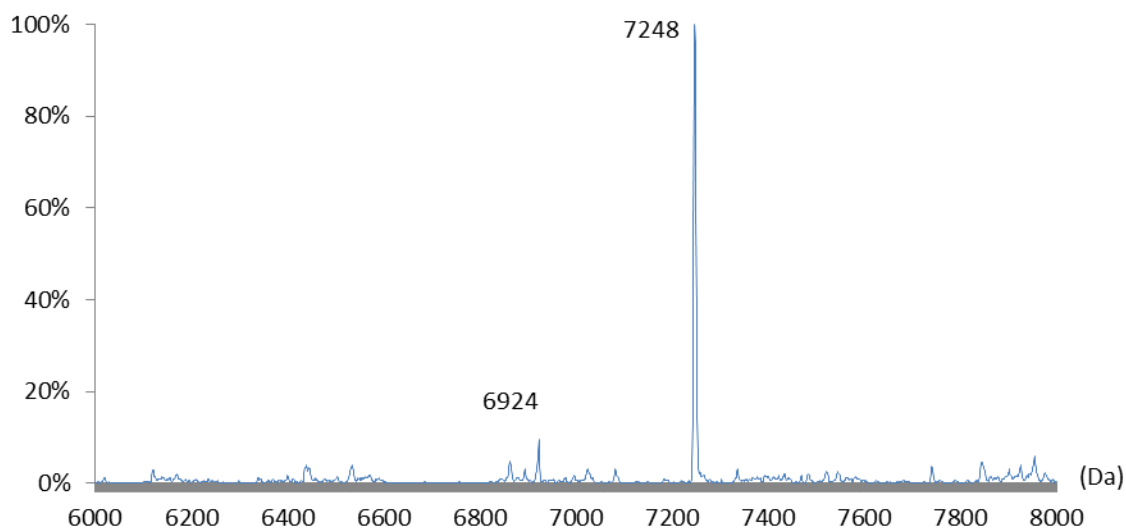
Fluorescent labeling of 4-OT R61C-1 in presence of cell lysates (protein ratio 1:1)

210 μL of the solution of protein **4-OT R61C-1** (20 nmol, 138 μg), 23 μL of the cell lysates (138 μg), 50 μL of the fluorophore stock solution (2.0 μmol , see p.S11) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum. A control reaction on the cell lysate in absence of **4-OT R61C-1** was also set up. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added and the solution was left rotating for 3 h at room temperature. DMF (1.0 mL) was added and the suspension was centrifuged at 13300 rpm for 5 min. The supernatant was removed and the pellet was redissolved in deionized H_2O (400 μL). 30 μL of the reaction mixture was mixed with 30 μL of the sample buffer (without bromophenol blue) and 20 μL of this mixture were loaded three times on a SDS PAGE (12 %) along with 5 μL of the protein ladder. Electrophoresis was performed for 50 min at 150 V.

Preparation of the sample for MS

The 4-OT bands were cut out of the SDS-PAGE, chopped in small pieces and put in a 2 mL cup containing 50 μL of deionized water and left overnight at 4 $^\circ\text{C}$ in order to let the protein diffuse out of the gel. Subsequently, the sample was centrifuged at 13300 rpm for 10 min at 4 $^\circ\text{C}$ and the supernatant was subjected to LC-MS analysis. The analysis showed more than 90% conversion of **4-OT R61C-1** to **4-OT R61C-8**.

ESI-MS spectrum: mass expected 7247, mass found 7248



Fluorescence imaging of SDS PAGE

5 μL of the protein ladder, 12 μL of the reaction mixture of **4-OT R61C-1** in presence of cell lysate mixed with 8 μL of the sample buffer and 12 μL of the blank reaction on cell lysate mixed with 8 μL of the sample buffer were loaded on the gel (12%). Bromophenol blue was excluded from the protein sample buffer because it quenches the fluorescence of the Dansyl fluorophore. Following the running of the gel for 50 min at 150 V, a picture was taken under

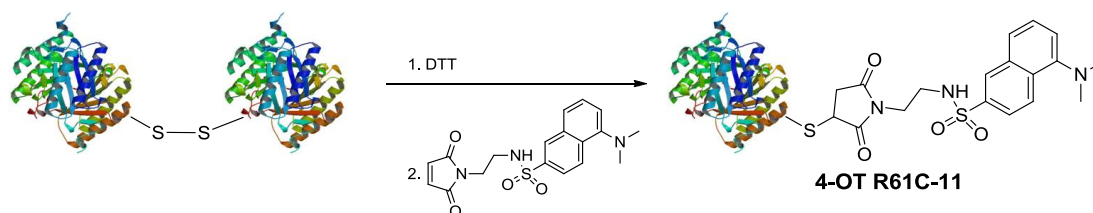
UV light (exposure time 4 sec). The gel was then stained with Coomassie and a new picture was taken under visible light.

Fluorescent labeling of 4-OT R61C-1 in presence of cell lysates (protein ratio 1:10)

21 μL of the solution of protein **4-OT R61C-1** (2 nmol, 13.8 μg), 23 μL of the cell lysates (138 μg protein content), 5 μL of the fluorophore stock solution (200 nmol) and 5 μL of the catalyst stock solution (40 nmol in Pd, 55 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum along with 200 μL ammonium formate buffer (pH 7.0, 50 mM). A blank reaction on the cell lysate in absence of **4-OT R61C-1** was also set up. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24 h, it was transferred into a 2 mL cup and 20 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. DMF (1.0 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 (200 μL).

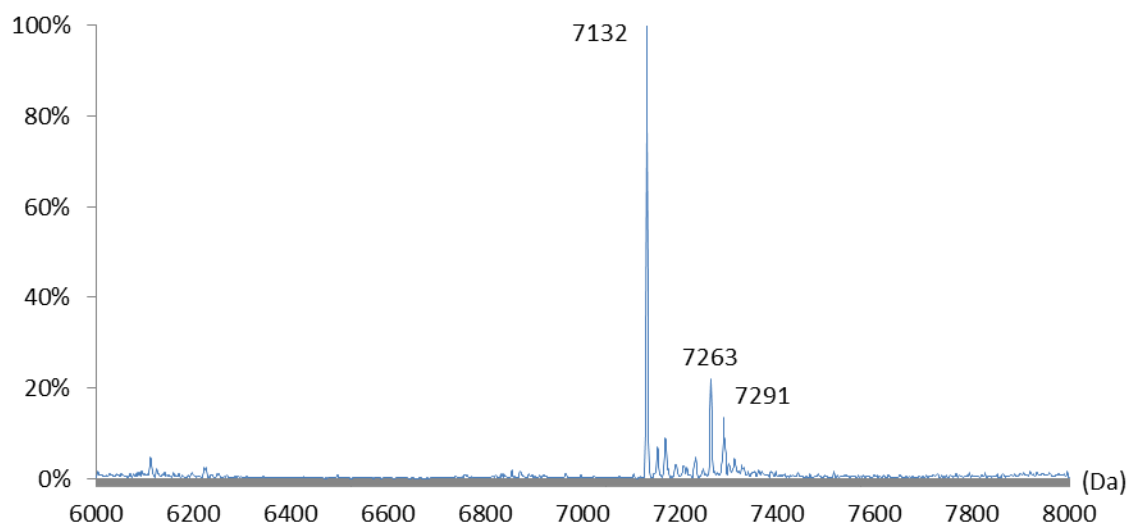
In order to estimate the conversion of the protein-bound alkene **4-OT R61C-1** to the product **4-OT R61C-8**, we coupled the fluorescently labeled maleimide directly to **4-OT R61C** monomer and used the product **4-OT R61C-11** as a reference sample.

Coupling of N-[2-(Dansylamino)ethyl]maleimide to 4-OT R61C (Michael addition)



The reduction of the dimer was done according to the procedure described above. A stock solution of N-[2-(Dansylamino)ethyl]maleimide was prepared by dissolving the maleimide (11 mg, 0.03 mmol) in MeOH (43 μL). 30 μL of the stock solution was added to the protein solution and the mixture was shaken at room temperature for 10 min. The purification of the protein and the concentration assessment were done as described for **4-OT R61C-1**. A solution containing 0.4 mg/mL was prepared in ammonium formate buffer (pH 7.0, 50 mM). An aliquot of this protein sample was directly analyzed by ESI-MS. Mass spectrometry revealed a protein peak with mass corresponding to the mass of **4-OT R61C-11**.

ESI-MS spectrum: mass expected 7130, mass found 7132 (7263: modified enzyme with methionine, 7291: modified enzyme with formylated methionine)

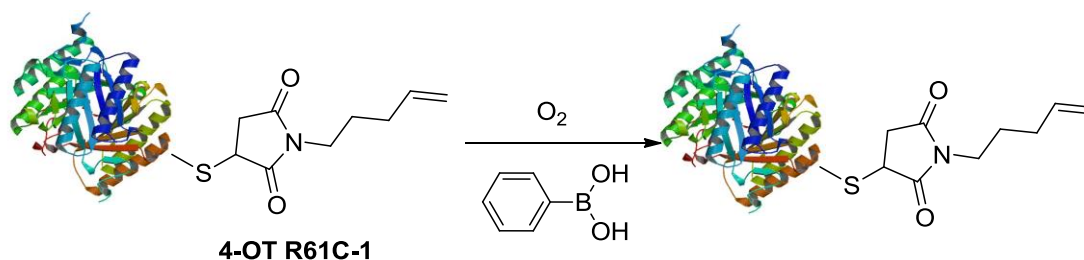


Fluorescence imaging of SDS PAGE

5 μL of the protein ladder, 6 μL of the reaction mixture of **4-OT R61C-1** in presence of cell lysate mixed with 10 μL of the sample buffer, 6 μL of the blank reaction on cell lysate mixed with 10 μL of the sample buffer and 1 μL of **4-OT R61C-11** mixed with 10 μL of the sample buffer were loaded on the gel (12%). Bromophenol blue was excluded from the protein sample buffer because it quenches the fluorescence of the Dansyl fluorophore. Following the running of the gel for 50 min at 150 V, a picture was taken under UV light (exposure time 4 sec). The gel was then stained with Coomassie and a new picture was taken under visible light.

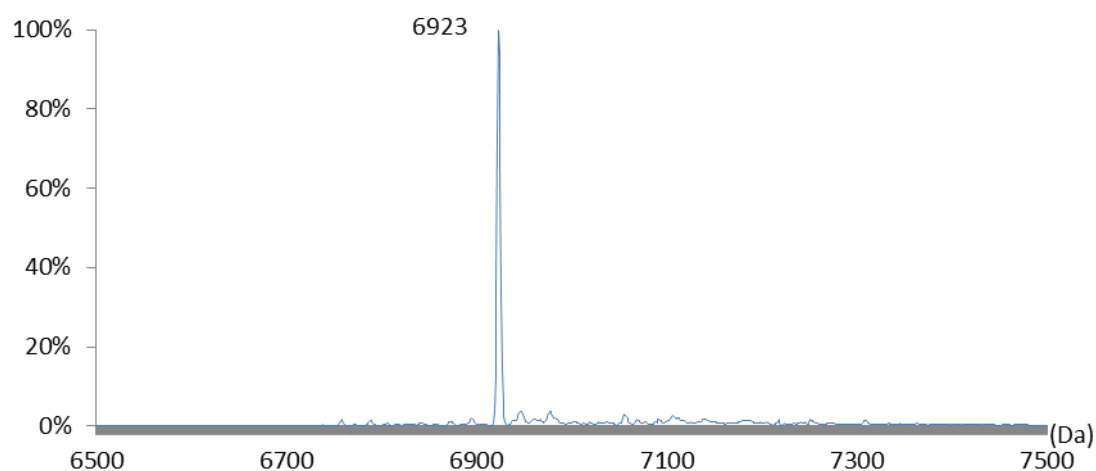
1.4 Control reactions

1.4.1 Control reaction 1; Oxidative Heck in absence of catalyst

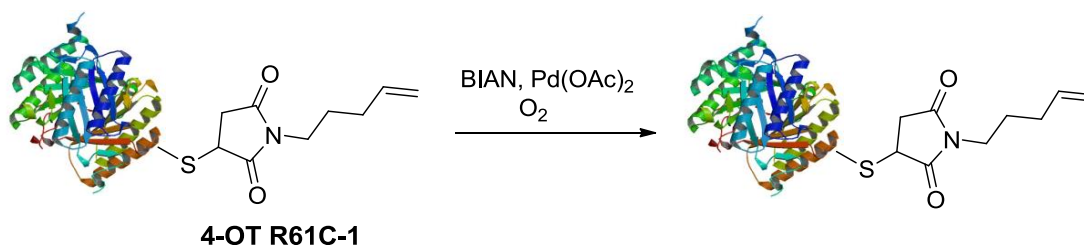


210 μL of the protein solution of **4-OT R61C-1** (20 nmol), 50 μL of the phenylboronic acid stock solution (2.0 μmol) were added sequentially in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24h, it was analyzed directly by LC-MS. The protein mass found to correspond to the unreacted **4-OT R61C-1**.

ESI-MS spectrum: mass expected 6923, mass found 6923



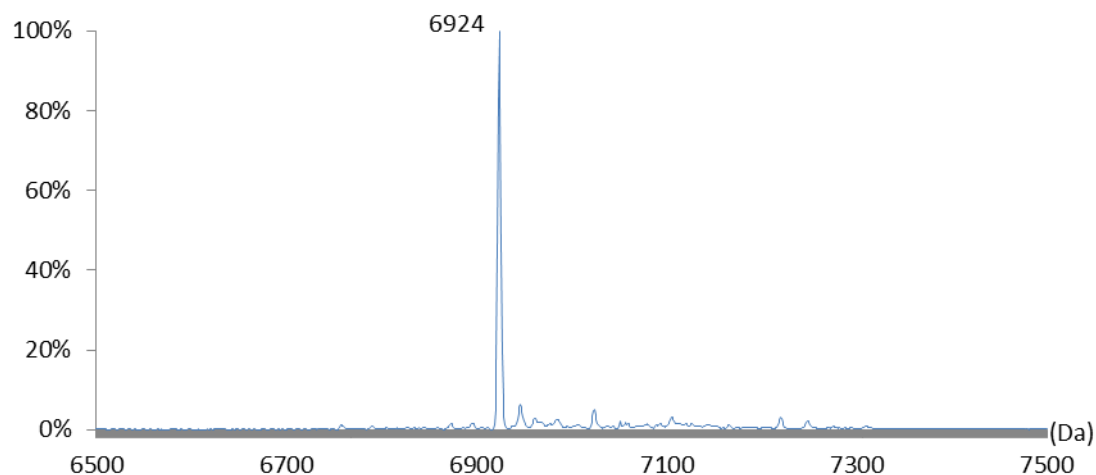
1.4.2 Control reaction 2; Oxidative Heck in absence of boronic acid



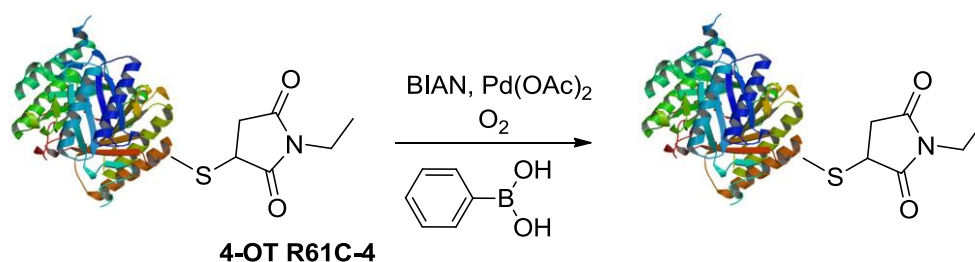
210 μL of the solution of protein **4-OT R61C-1** (20 nmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum. The reaction mixture was left stirring at room temperature under an oxygen atmosphere. After 24h, it was transferred

into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). The sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS of the aqueous phase showed a protein mass corresponding to the unreacted **4-OT R61C-1**.

ESI-MS spectrum: mass expected 6923, mass found 6924

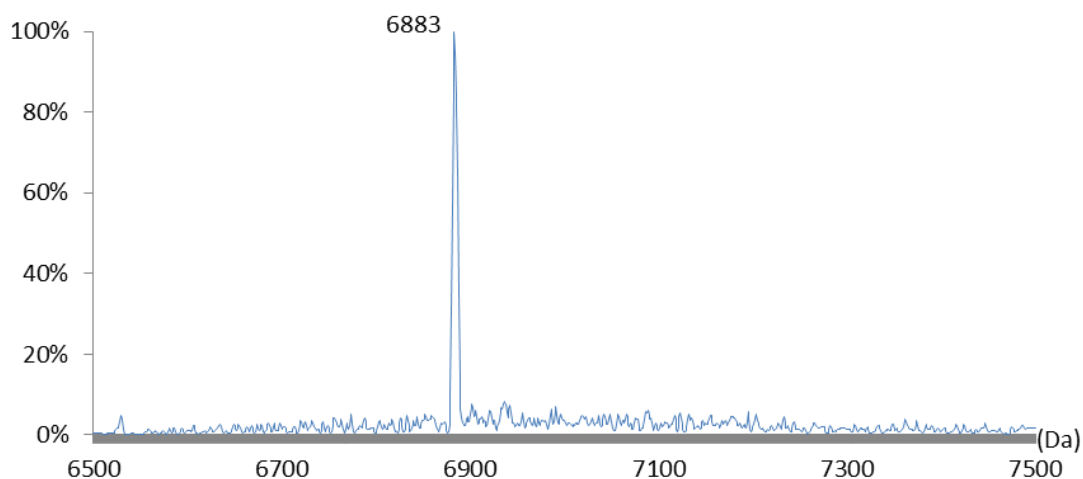


1.4.3 Control reaction 3; Oxidative Heck in absence of alkene



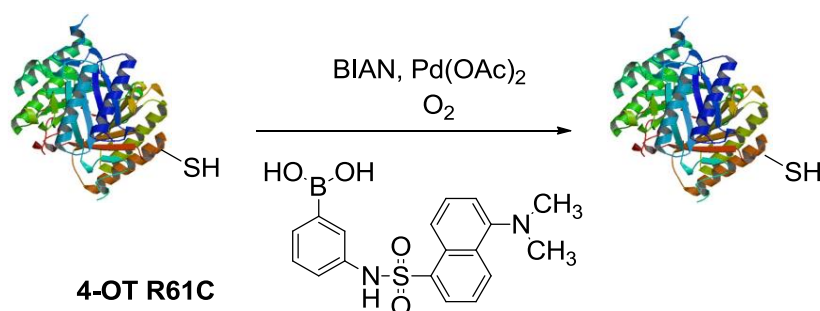
210 μL of the solution of protein **4-OT R61C-4** (20 nmol), 50 μL of the 4-methoxyphenylboronic acid stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a stirring bar and a septum. The reaction mixture was left stirring at room temperature under oxygen atmosphere. After 24h, it was transferred into a 2 mL cup and 200 μL of the EDTA stock solution was added. The solution was left rotating for 3h at room temperature. A microextraction was then performed using *n*-heptane (3x500 μL). The sample was homogenized by vortexing carefully and the organic phase was removed. LC-MS analysis of the aqueous phase showed a protein mass corresponding to the mass of the unreacted **4-OT R61C-4**.

ESI-MS spectrum: mass expected 6883, mass found 6883



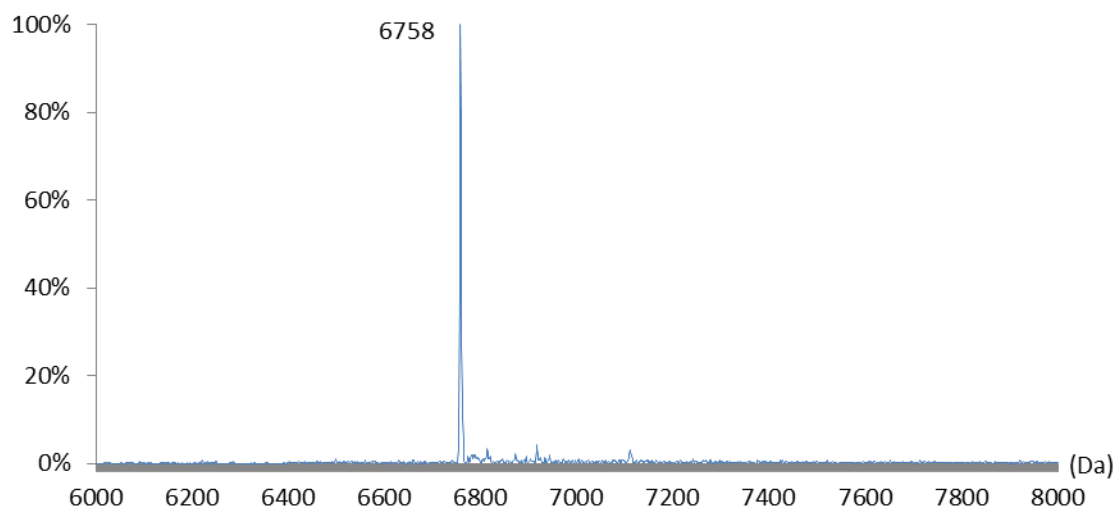
1.4.4 Control reaction 4; Coupling of cysteine thiols to 3-(dansylamino)phenylboronic acid using the optimized conditions of the oxidative Heck reaction as a possible side reaction

Coupling of 4-OT R61C



The reduction of **4-OT R61C** dimer was performed as described before. 51 μL of the protein solution of reduced **4-OT R61C** (20 nmol), 50 μL of the fluorophore stock solution (2.0 μmol) and 50 μL of the catalyst stock solution (400 nmol in Pd, 550 nmol in BIAN) were added in a 10 mL double neck round bottom flask, equipped with a 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 200 μL of the EDTA stock solution was added. The solution was left rotating for 3 h at room temperature. DMF (1.0 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 H₂O (400 μL). LC-MS analysis showed a protein mass corresponding to the mass of the unreacted **4-OT R61C**.

ESI-MS spectrum: mass expected 6757, mass found 6758



Coupling of the cysteine residues of a cell lysate in presence (ratio 1:10) and in absence of 4-OT R61C-1

The preparation of the cell lysate was done as described above, without treatment with iodoacetamide. The procedure followed for the coupling of the fluorescent 3-(dansylamino)phenylboronic acid in presence and absence of the protein-bound alkene **4-OT R61C-1** was the same as described before.

Fluorescence imaging of SDS PAGE

5 μ L of the protein ladder, 9 μ L of the reaction mixture of **4-OT R61C-1** in presence of cell lysate mixed with 10 μ L of the sample buffer and 9 μ L of the blank reaction on cell lysate mixed with 10 μ L of the sample buffer were loaded on the gel (12%). Bromophenol blue was excluded from the protein sample buffer because it quenches the fluorescence of the Dansyl fluorophore. Following the running of the gel for 50 min at 150 V, a picture was taken under UV light (exposure time 4 sec). The gel was then stained with Coomassie and a new picture was taken under visible light.

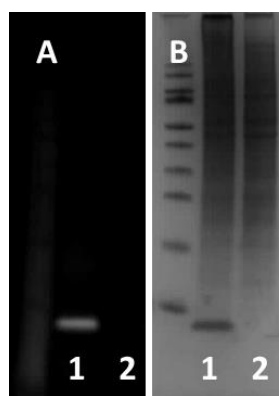


Figure S1: A) Fluorescence imaging on SDS-PAGE of 4-OT R61C-1 labeled in presence of a cell lysate (protein ratio 1:10). B) Coomassie staining of A. 1) the reaction in presence of **4-OT R61C-1** and 2) in absence of **4-OT R61C-1**.

2. Oxidative Heck reaction on small molecules

2.1 Materials and Methods

Chemicals were obtained from commercial suppliers (Sigma Aldrich, Acros Organics) and used without further purification. 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 (^1H 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. GC-MS spectra were recorded on a GCMS-QP5000 Gas Chromatograph Mass Spectrometer. HP-5MS was used as a column. The temperature was set at 250.00 °C, the column inlet pressure was 92.3 kPa and the column flow was 1.5 mL/min.

2.2 Synthesis of starting materials

2.2.1 Synthesis of Bis(aryl)acenaphthequinonediimine (BIAN)

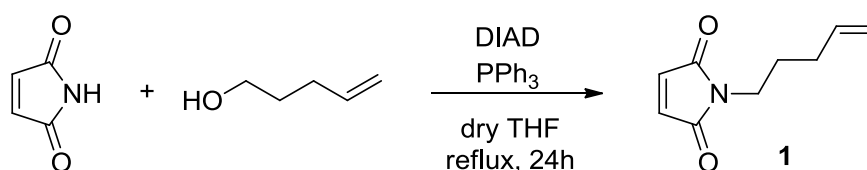
BIAN was synthesized according to literature procedures.^[6]

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

^1H 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: m/z 389.29.

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



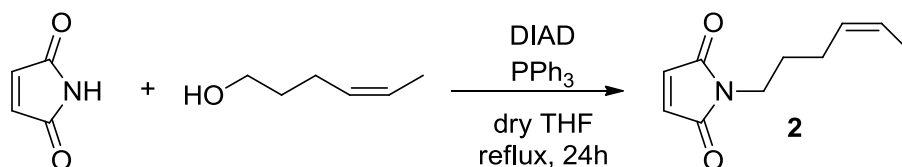
In a 10 mL round bottom flask equipped with a magnetic stirring bar, the maleimide (97 mg, 1.0 mmol) and PPh_3 (0.26 g, 1.0 mmol) were dissolved in dry THF (2.0 mL). Pent-4-en-1-ol (0.10 ml, 1.0 mmol) and diisopropylazodicarboxylate (DIAD) (0.20 mL, 1.0 mmol) were added, the flask was equipped with a condenser and the mixture was left stirring for 24h under reflux. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (EtOAc/ heptane 1:10) to afford the product as a yellowish oil (0.13 g, 78%).

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

$^1\text{H NMR}$ (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. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 179.0, 136.2, 133.0, 114.2, 35.7, 29.2, 25.9 ppm.

GC-MS: m/z 165.0.

2.2.3 Synthesis of (Z)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (2)



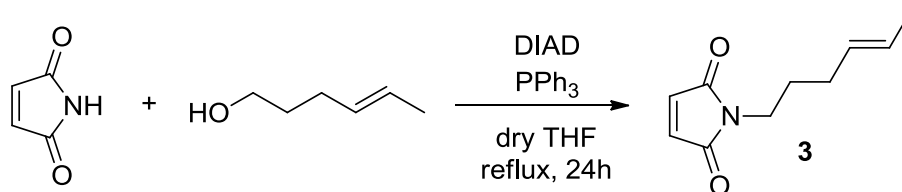
In a 10 mL round bottom flask equipped with a magnetic stirring bar, the maleimide (97 mg, 1.0 mmol) and PPh_3 (0.26 g, 1.0 mmol) were dissolved in dry THF (2.0 mL). Cis-4-hexen-1-ol (0.12 ml, 1.0 mmol) and diisopropylazodicarboxylate (DIAD) (0.20 mL, 1.0 mmol) were added subsequently, the flask was equipped with a condenser and the mixture was left stirring for 24h under reflux. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (EtOAc/ heptane 1:10) to afford the product as a yellowish oil (0.14 g, 78%).

TLC: R_f = 0.5 (EtOAc/ heptane 1:1).

$^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 6.62 (s, 2H), 5.40-5.34 (m, 1H), 5.28-5.23 (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. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 170.7, 134.0, 128.9, 124.8, 37.5, 28.2, 24.1, 12.7 ppm.

GC-MS: m/z 179.1.

2.2.4 Synthesis of (E)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (3)



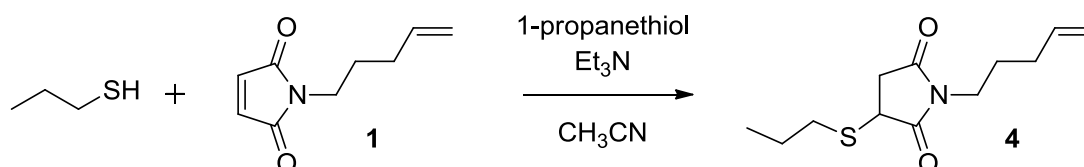
In a 10 mL round bottom flask equipped with a magnetic stirring bar, the maleimide (97 mg, 1.0 mmol) and PPh_3 (0.26 g, 1.0 mmol) were dissolved in dry THF (2.0 mL). Trans-4-hexen-1-ol (0.12 ml, 1.0 mmol) and diisopropylazodicarboxylate (DIAD) (0.20 mL, 1.0 mmol) were added, the flask was equipped with a condenser and the mixture was left stirring for 24h under reflux. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (EtOAc/ heptane 1:10) to afford the product as a yellowish oil (0.13 g, 76%).

TLC: R_f = 0.5 (EtOAc/ heptane 1:1).

$^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 6.52 (s, 2H), 5.22-5.11 (m, 2H), 3.25 (t, J = 7.4 Hz, 2H), 1.72 (m, 2H), 1.38 (m, 2H), 1.38 (d, J = 5.9 Hz, 3H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 170.3, 133.7, 129.4, 125.2, 36.9, 29.3, 27.7, 17.4 ppm.

GC-MS: m/z 179.1.

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



In a 10 mL round bottom flask equipped with a magnetic stirring bar were added sequentially a solution of 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione (0.17 g, 1.0 mmol) in 2.0 mL CH_3CN , 1-propanethiol (90 μL , 1.0 mmol) and triethylamine (60 μL , 0.40 mmol) and the mixture was left stirring at room temperature for 2h. The solvent was then evaporated and the residue was dissolved in ethyl acetate (3.0 mL). The organic phase was washed 3 times with 10 mL of NaOH 1N, dried over MgSO_4 and evaporated under reduced pressure to afford the product **6** as a yellow oil (0.17 g, 69%).

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

$^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 5.79 (m, 1H), 4.97-5.06 (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. $^{13}\text{C NMR}$ (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.

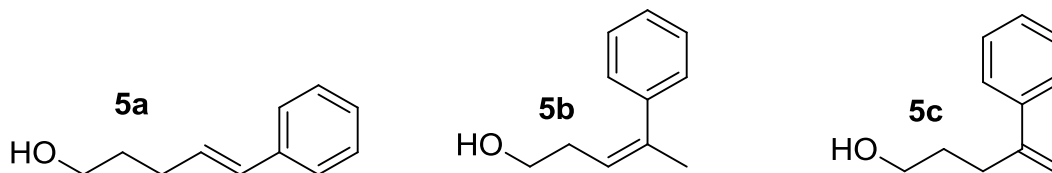
2.3 General procedure for the oxidative Heck reaction

The general procedure for the oxidative Heck reaction was performed as reported previously by Minnaard *et al.* with some modifications.^[6]

To a 10 mL double neck round bottom flask equipped with a magnetic stirring bar and a septum was added palladium acetate (11 mg, 5.0 mol %, 0.05 equiv.), and BIAN (28 mg, 7.0 mol %, 0.07 equiv.) and the mixture was dissolved in DMF (2.0 mL) (99+%, extra pure, Acros Organics). The flask was equipped with an oxygen balloon on the side arm and immediately after the addition of DMF, oxygen was flushed through the flask and the mixture was stirred for 30 min at room temperature. The olefin (1.0 equiv.) and the phenylboronic acid (1.5 equiv.) were then added to the flask and the reaction mixture was allowed to stir at room temperature under oxygen atmosphere. After 30h, the reaction mixture was diluted with ethyl acetate (5.0 mL) and washed with H_2O (5x20 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.

Note: The ratio of the isomers formed was estimated from the integration of the olefinic peaks of each isomeric product that could be distinguished in the ^1H NMR spectrum.

2.3.1 Synthesis of (*E*)-5-phenylpent-4-en-1-ol (**5a**), 4-phenylpent-3-en-1-ol (**5b**) and 4-phenylpent-4-en-1-ol (**5c**)



The title compounds were prepared from pent-4-en-1-ol (0.10 mL, 1.0 mmol) and phenylboronic acid (0.18 g, 1.5 mmol) according to general procedures A and B. The residue was purified by flash column chromatography (EtOAc/ heptane 1:20) to afford **5a**, **5b** and **5c** as a mixture of isomers (yellowish solid).

Procedure A: 109 mg, 67%. Ratio of the isomers: **5a** : **5b** : **5c** 4.5 : 1.1 : 1.

TLC: R_f = 0.10 (EtOAc/ heptane 1:5).

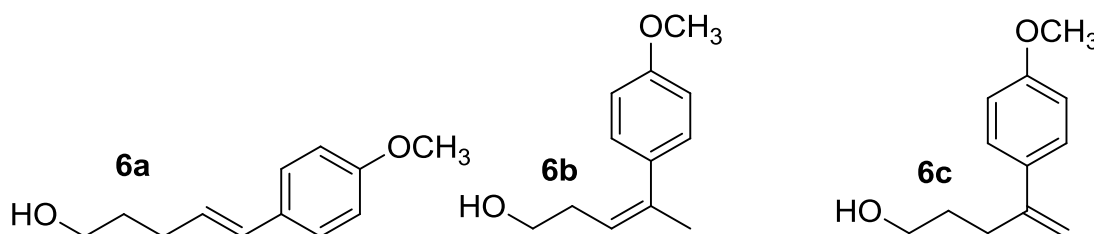
5a: ^1H NMR (500 MHz, CDCl_3): δ = 7.18-7.42 (m, 5H), 6.40 (d, J = 15.7 Hz, 1H), 6.23 (m, 1H), 3.70 (t, J = 6.4 Hz, 2H), 2.31 (m, 2H), 1.75 (m, 2H) ppm. ^{13}C NMR (125 MHz, CDCl_3): δ = 137.8, 130.2, 128.6, 127.1, 126.3, 126.1, 62.6, 3a2.4, 29.5 ppm.

5b: ^1H NMR (500 MHz, CDCl_3): δ = 7.18-7.42 (m, 5H), 5.78 (m, 1H), 3.66 (t, J = 6.4 Hz, 2H), 2.51 (m, 2H), 2.07 (s, 3H) ppm. ^{13}C NMR (125 MHz, CDCl_3): δ = 143.7, 133.4, 128.3, 127.0, 125.8, 123.9, 62.5, 31.3, 16.2 ppm.

5c: ^1H NMR (500 MHz, CDCl_3): δ = 7,18-7,42 (m, 5H), 5,29 (s, 1H), 5,09 (s, 1H), 3,75 (t, J = 6,4 Hz, 2H), 2,61 (t, J = 7,5 Hz, 2H), 1,73 (m, 2H). ^{13}C NMR (125 MHz, CDCl_3): δ = 148.1, 137.9, 130.5, 128.6, 127.6, 112.7, 62.6, 32.5, 31.7 ppm.

GC-MS: m/z 162.2.

2.3.2 Synthesis of (*E*)-5-(4-methoxyphenyl)pent-4-en-1-ol (**6a**), 4-(4-methoxyphenyl)pent-3-en-1-ol (**6b**), 4-(4-methoxyphenyl)pent-4-en-1-ol (**6c**)



The title compounds were prepared from pent-4-en-1-ol (0.10 mL, 1.0 mmol) and 4-methoxyphenylboronic acid (0.23 g, 1.5 mmol) according to general procedure A. The residue was purified by flash column chromatography (EtOAc/ heptane 1:20) to afford **6a**, **6b** and **6c** as a mixture of isomers (yellowish solid).

Procedure A: 125 mg, 64 %. Ratio of the isomers: **6a** : **6b** : **6c** 2.6 : 2 : 1.

TLC: R_f = 0.10 (EtOAc/ heptane 1:5).

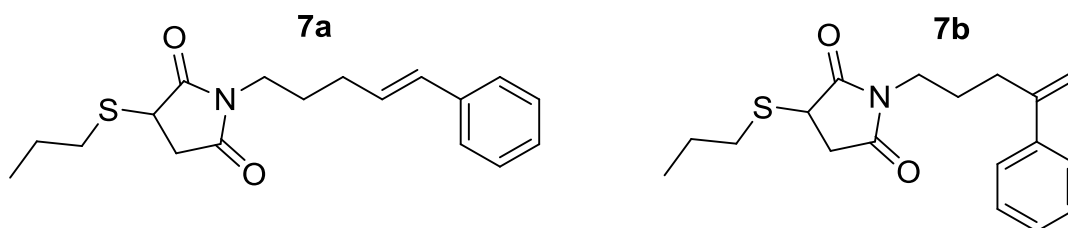
6a: $^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 7.26 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.8 Hz, 2H), 6.35 (d, J = 15.7 Hz, 1H), 6.10 (m, 1H), 3.80 (s, 3H), 3.71 (t, J = 6.4 Hz, 2.30 (m, 2H), 1.74 (m, 2H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 159.4, 130.9, 128.3, 127.5, 126.3, 114.3, 62.5, 55.3, 32.3, 29.6 ppm.

6b: $^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 7.32 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.8 Hz, 2H), 5.71 (m, 1H), 3.81 (s, 3H), 3.74 (t, J = 6.5 Hz, 2H), 2.49 (m, 2H), 2.05 (s, 3H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 159.4, 130.9, 129.8, 127.1, 126.3, 113.8, 67.5, 55.3, 39.4, 29.2 ppm.

6c: $^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 7.35 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H), 5.23 (s, 1H), 5.01 (s, 1H), 3.81 (s, 3H), 3.67 (t, J = 6.6 Hz, 2H), 2.58 (t, J = 7.4 Hz, 2H), 1.74 (m, 2H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 158.7, 140.8, 129.8, 127.5, 114.3, 113.8, 62.5, 55.3, 29.6, 25.6 ppm.

GC-MS: m/z 192.

2.3.3 Synthesis of (*E*)-1-(5-phenylpent-4-en-1-yl)-3-(propylthio)pyrrolidine-2,5-dione (**7a**), 1-(4-phenylpent-4-en-1-yl)-3-(propylthio)pyrrolidine-2,5-dione (**7b**)



The title compounds were prepared from compound **7** (0.24 g, 1.0 mmol) according to general procedure A. However, for this reaction the amount of the catalyst (palladium acetate 0.23 g, 1.0 equiv), the ligand (BIAN 0.55 g, 1.4 equiv.) and phenylboronic acid (1.2 g, 10 mmol) were increased in order to imitate the condition applied for the protein conjugation. After 24h, DMF was removed by extractions with H_2O and the residue was purified by flash column chromatography (EtOAc/ heptane 1:10) to afford **7a** and **7b** in a ratio 2.3 : 1 as a yellowish oil (0.26 g, 82%).

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

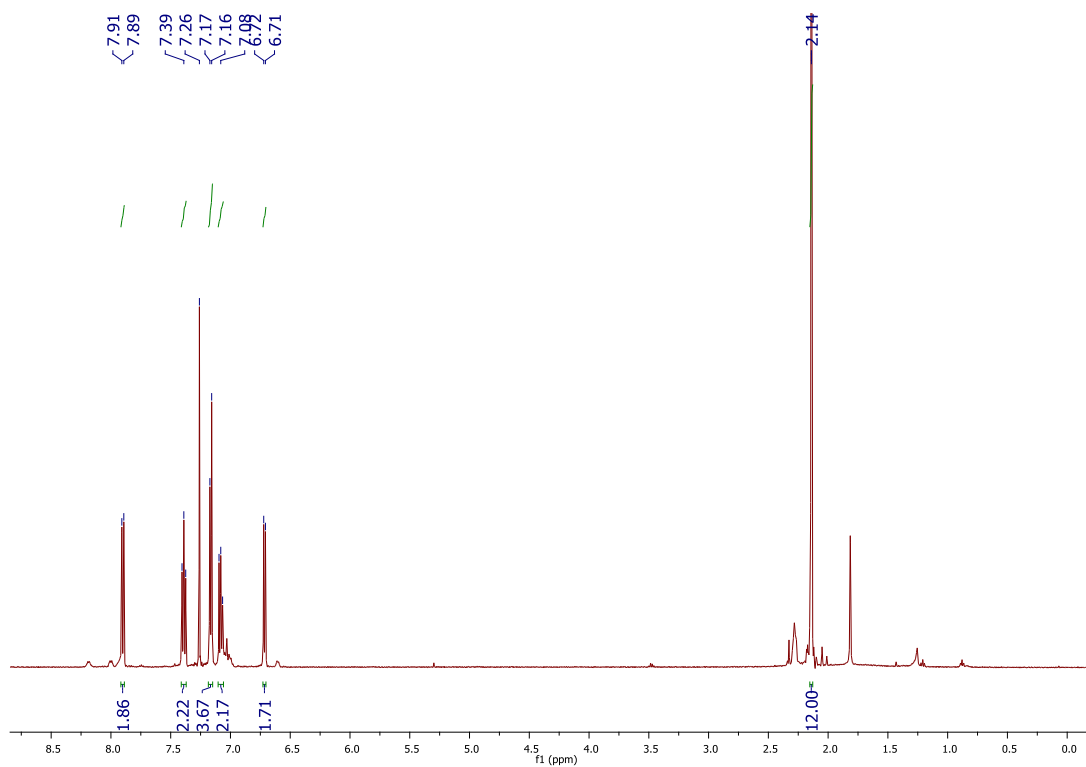
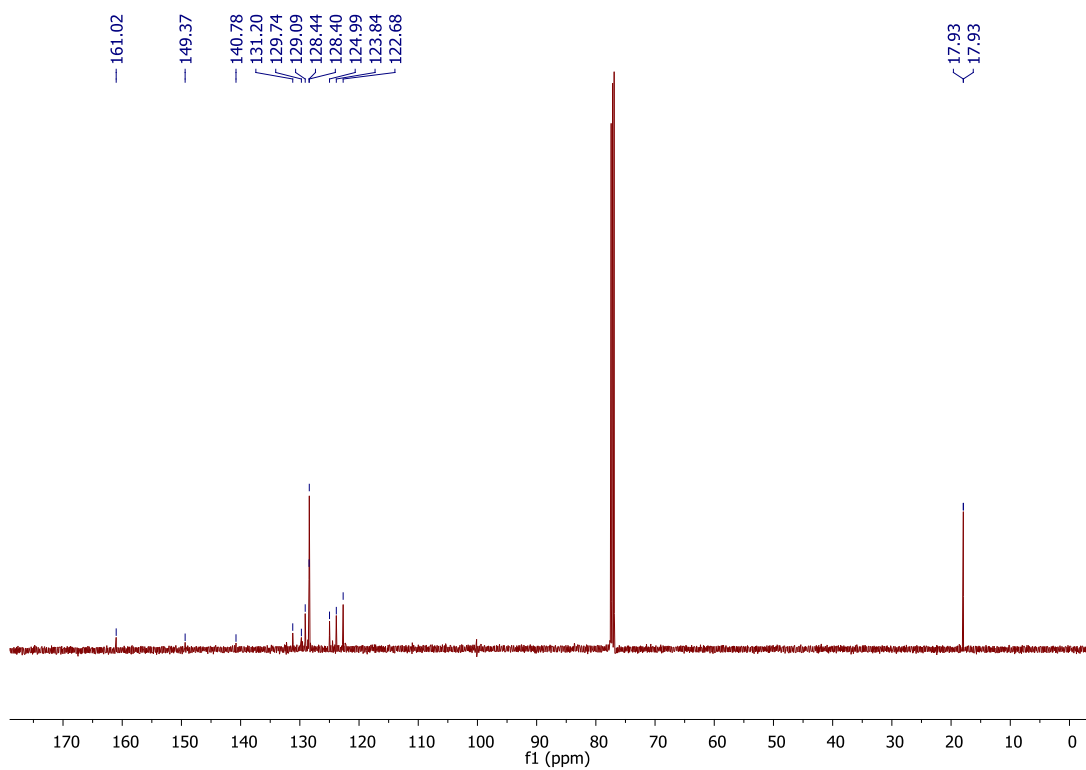
7a: $^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 7.12-7.32 (m, 5H), 6.32 (d, J = 15.8 Hz, 1H), 6.13 (m, 1H), 3.59 (dd, J = 9 Hz, J = 3.7 Hz, 1H), 3.51 (t, J = 7.4 Hz, 2H), 3.00 (dd, J = 18.7 Hz, J = 9.0, 1H), 2.79 (m, 1H), 2.66 (m, 1H), 2.45 (dd, J = 18.7 Hz, J = 3.7 Hz, 1H), 2.17 (m, 2H), 1.53-1.63 (m, 4H), 0.95 (t, J = 7.4 Hz, 3H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ = 176.8, 175.0, 137.6, 130.9, 129.2, 128.6, 127.2, 126.1, 39.1, 28.8, 36.3, 33.9, 30.4, 27.1, 22.5, 13.5 ppm.

7b: $^1\text{H NMR}$ (500 MHz, CDCl_3): δ = 7.12-7.32 (m, 5H), 5.23 (s, 1H), 5.02 (s, 1H), 3.59 (dd, J = 9 Hz, J = 3.7 Hz, 1H), 3.00 (dd, J = 18.7 Hz, J = 9 Hz, 1H), 2.79 (m, 1H), 2.66 (m, 1H), 2.45 (dd, J = 18.7 Hz, J = 3.7 Hz, 1H), 1.72 (m, 6H), 0.95 (t, J = 7.4 Hz, 3H) ppm. $^{13}\text{C NMR}$ (125 MHz, CDCl_3):

δ = 176.8, 175.0, 147.3, 141.0, 128.5, 127.6, 126.2, 113.0, 39.1, 38.8, 38.7, 32.6, 29.8, 26.3, 22.5, 13.5 ppm.

GC-MS: m/z 43.0, 54.9, 60.0, 77.0, 84.0, 91.0, 100.1, 115.1, 129.1, 143.1, 144.1, 158.1, 174.0, 184.0, 243.0.

3. NMR spectra

Figure S2: ¹H NMR spectrum (CDCl₃, 500 MHz) of BIAN.Figure S3: ¹³C NMR spectrum (CDCl₃, 125 MHz) of BIAN.

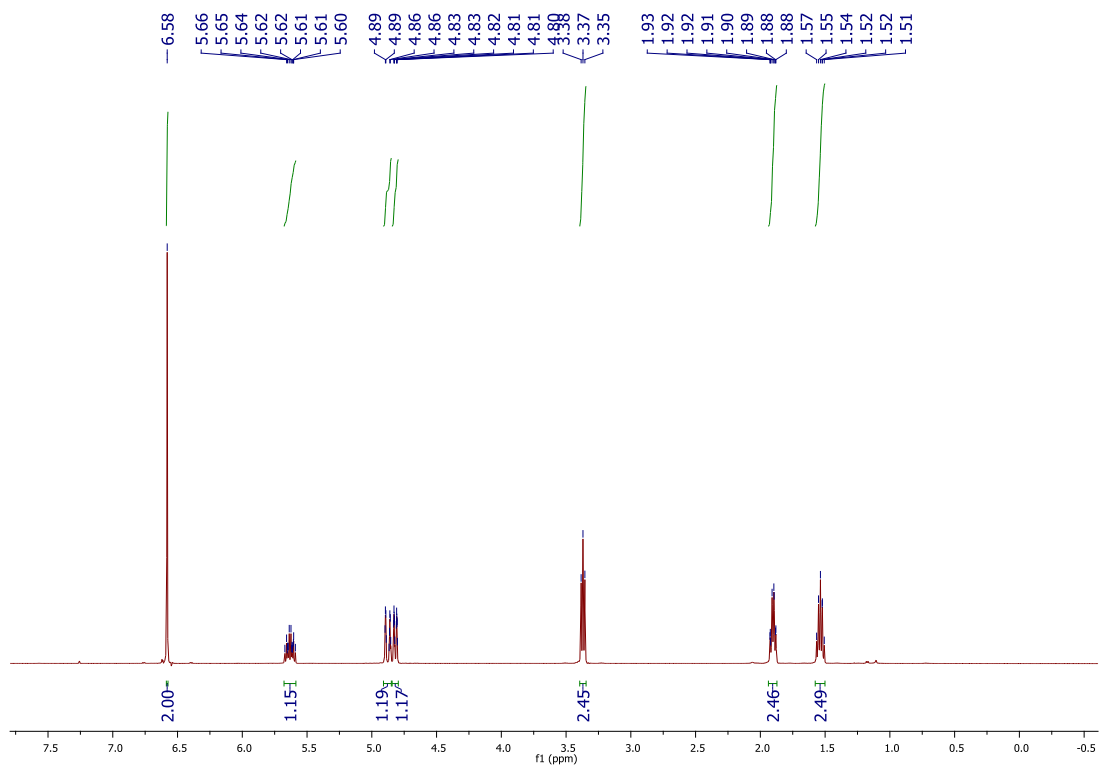


Figure S4: ^1H NMR spectrum (CDCl₃, 500 MHz) of **1**.

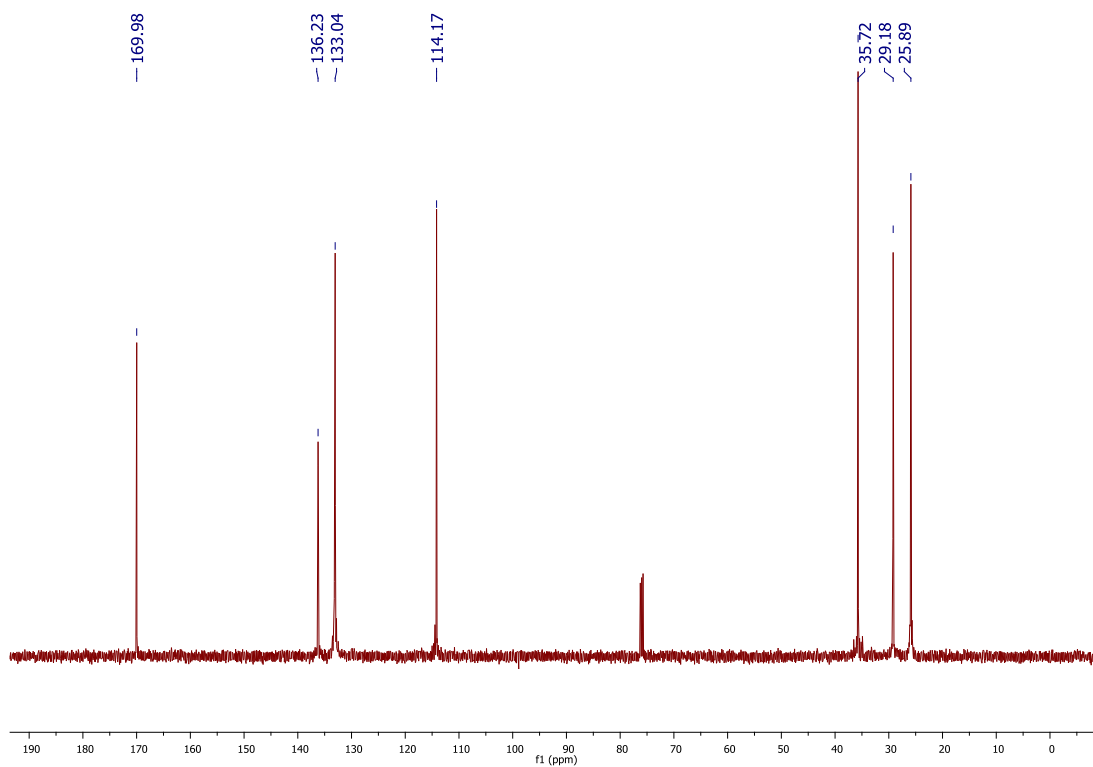


Figure S5: ^{13}C NMR spectrum (CDCl₃, 125 MHz) of **1**.

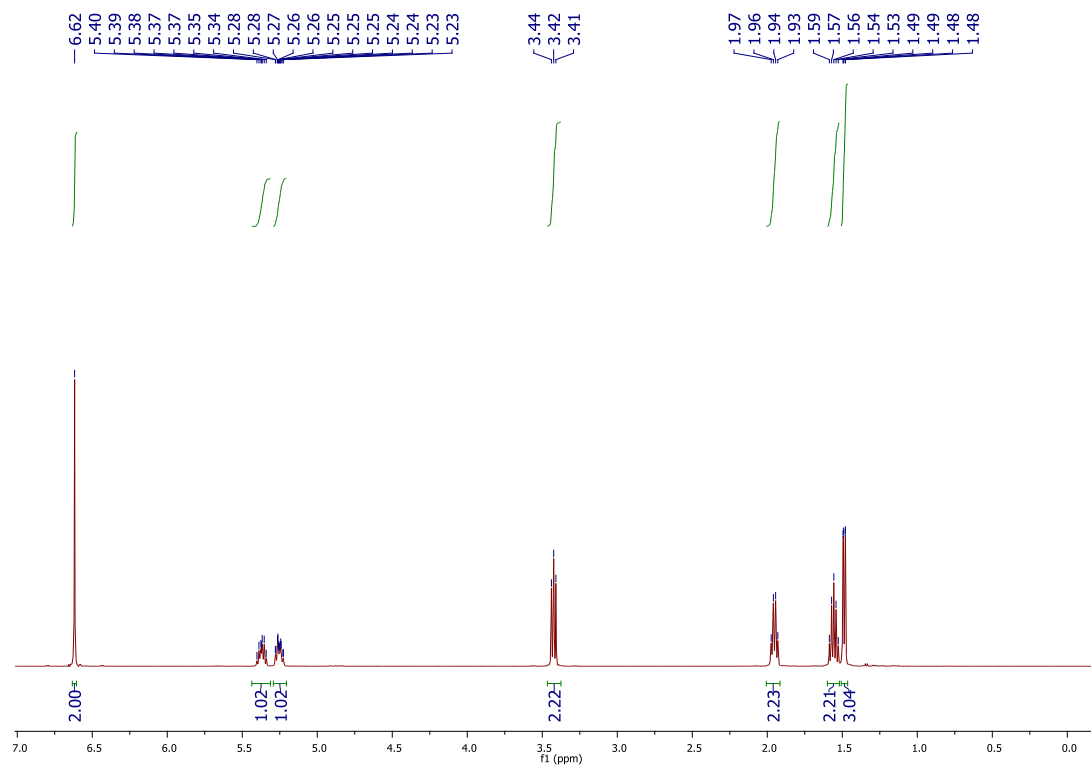


Figure S6: ¹H NMR spectrum (CDCl₃, 500 MHz) of **2**.

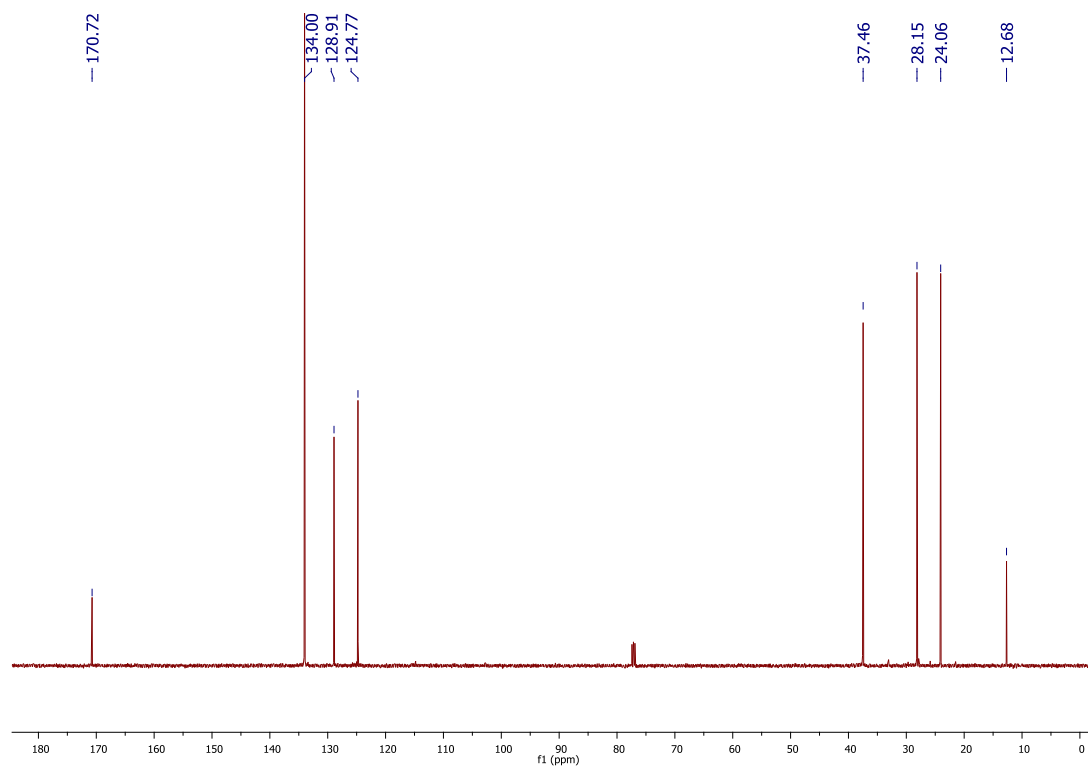


Figure S7: ¹³C NMR spectrum (CDCl₃, 125 MHz) of **2**.

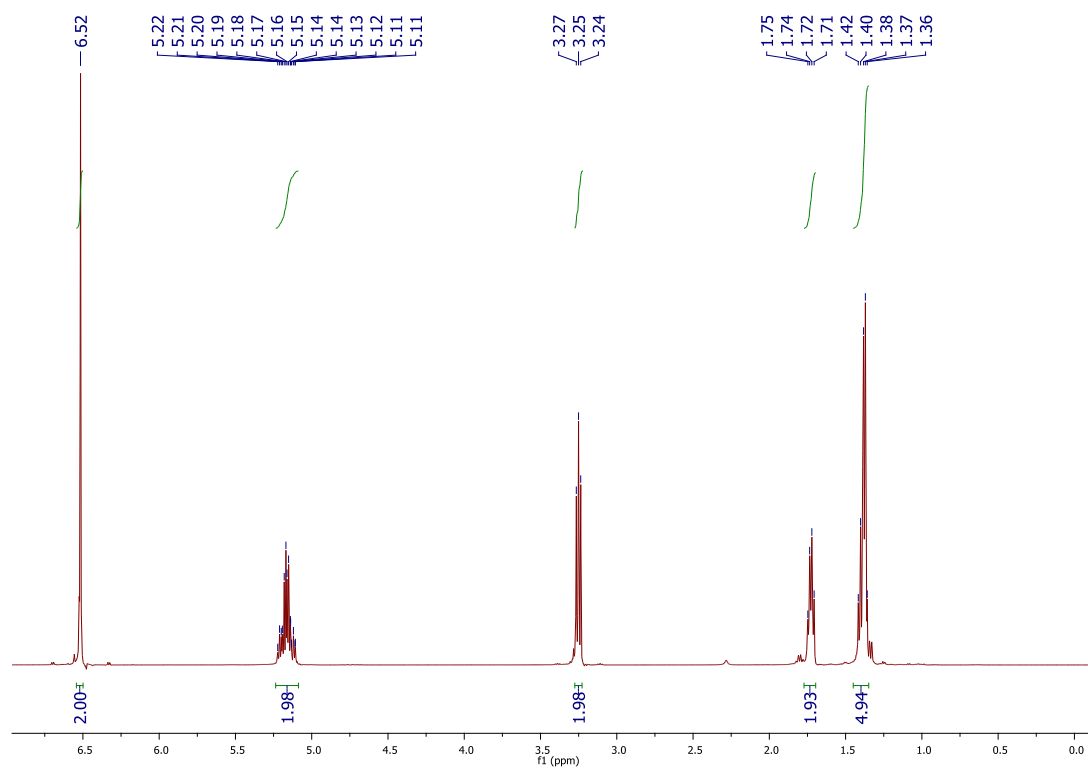


Figure S8: ^1H NMR spectrum (CDCl_3 , 500 MHz) of **3**.

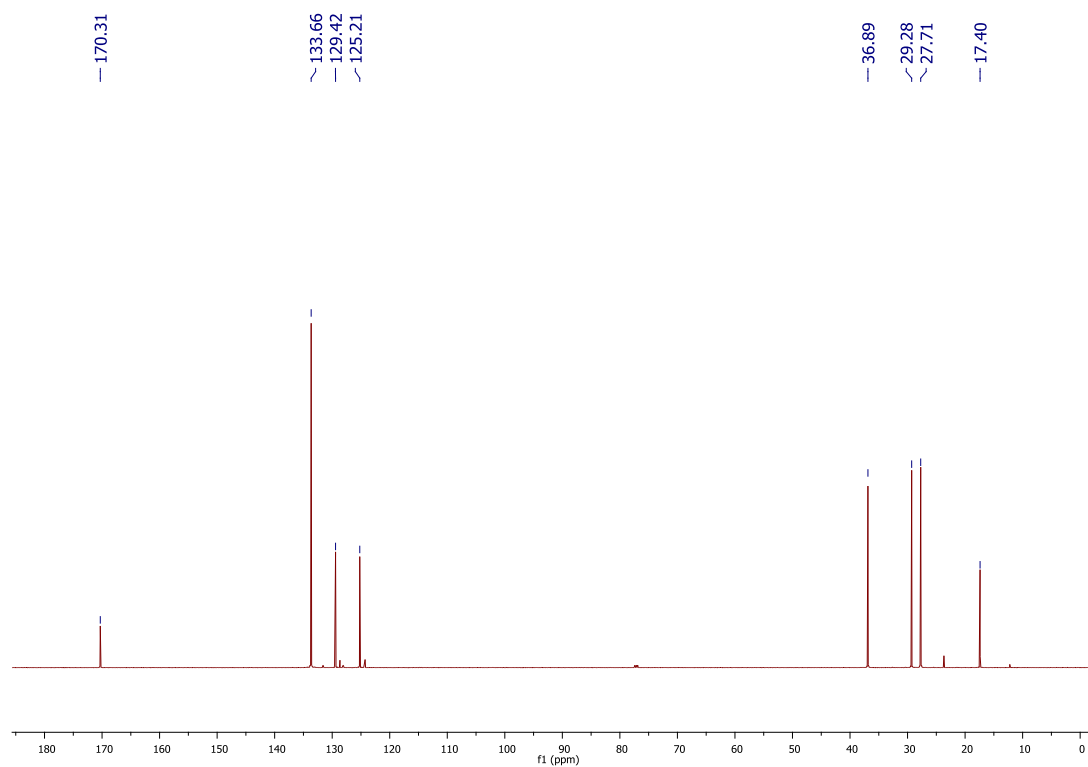


Figure S9: ^{13}C NMR spectrum (CDCl_3 , 125MHz) of **3**.

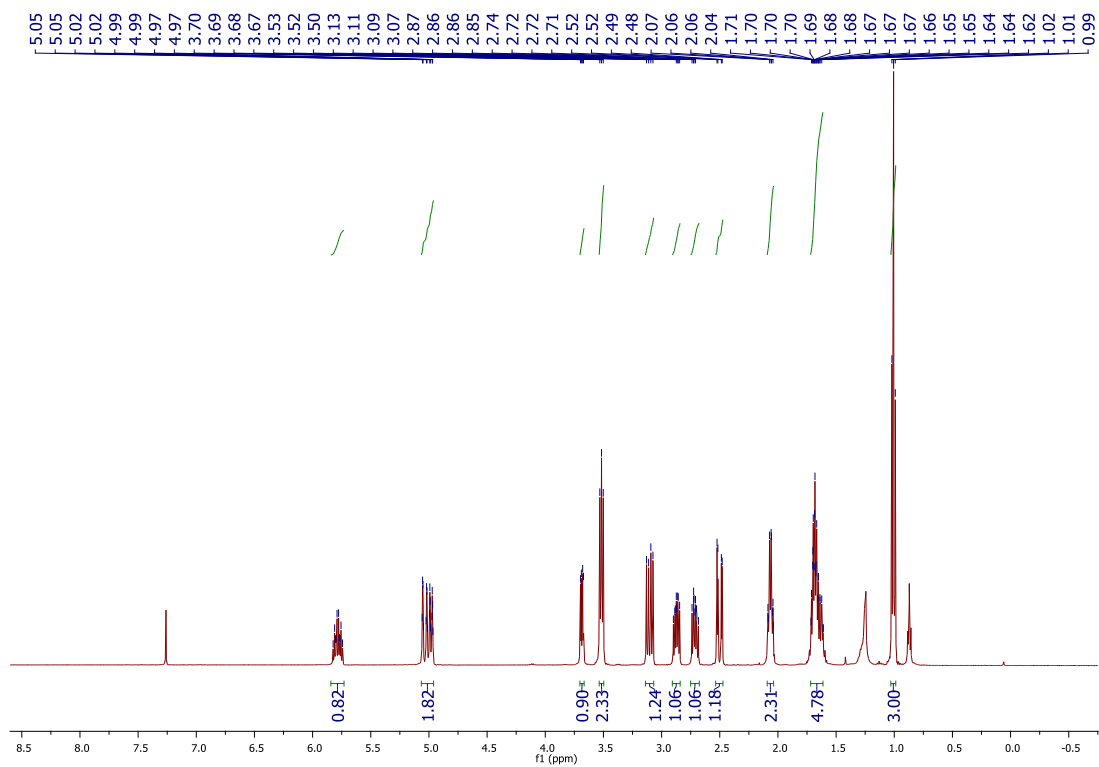


Figure S10: ^1H NMR spectrum (CDCl_3 , 500 MHz) of **4**.

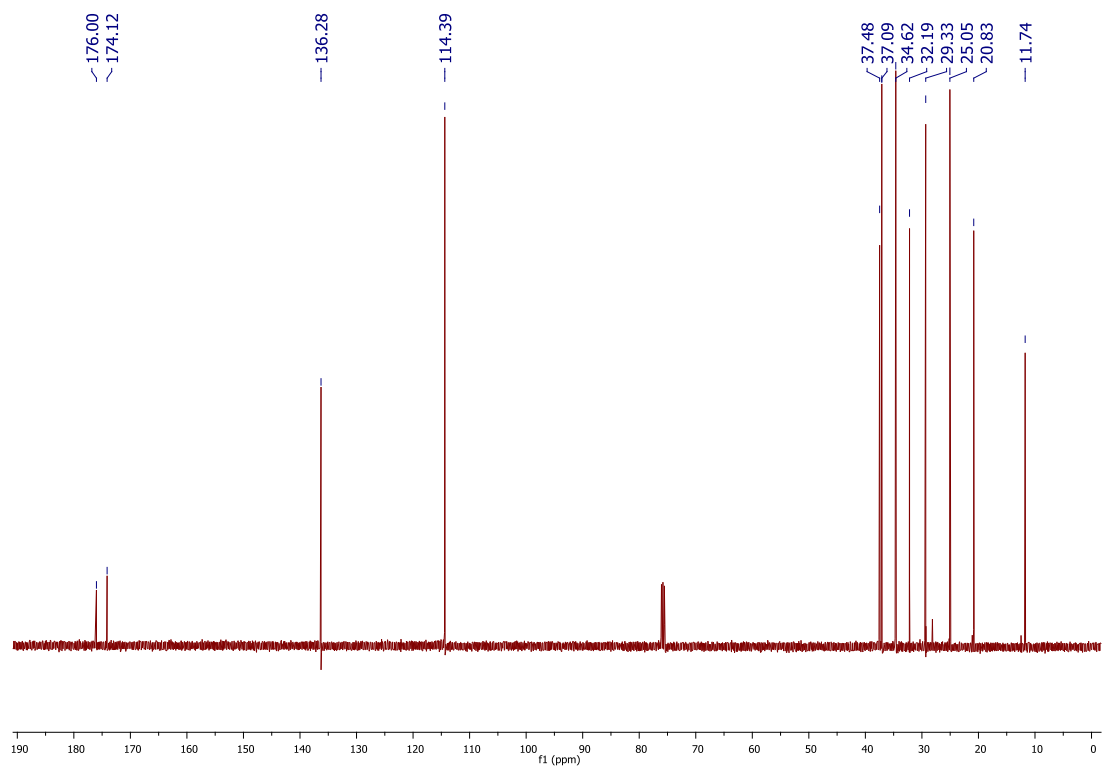


Figure S11: ^{13}C NMR spectrum (CDCl_3 , 125MHz) of **4**.

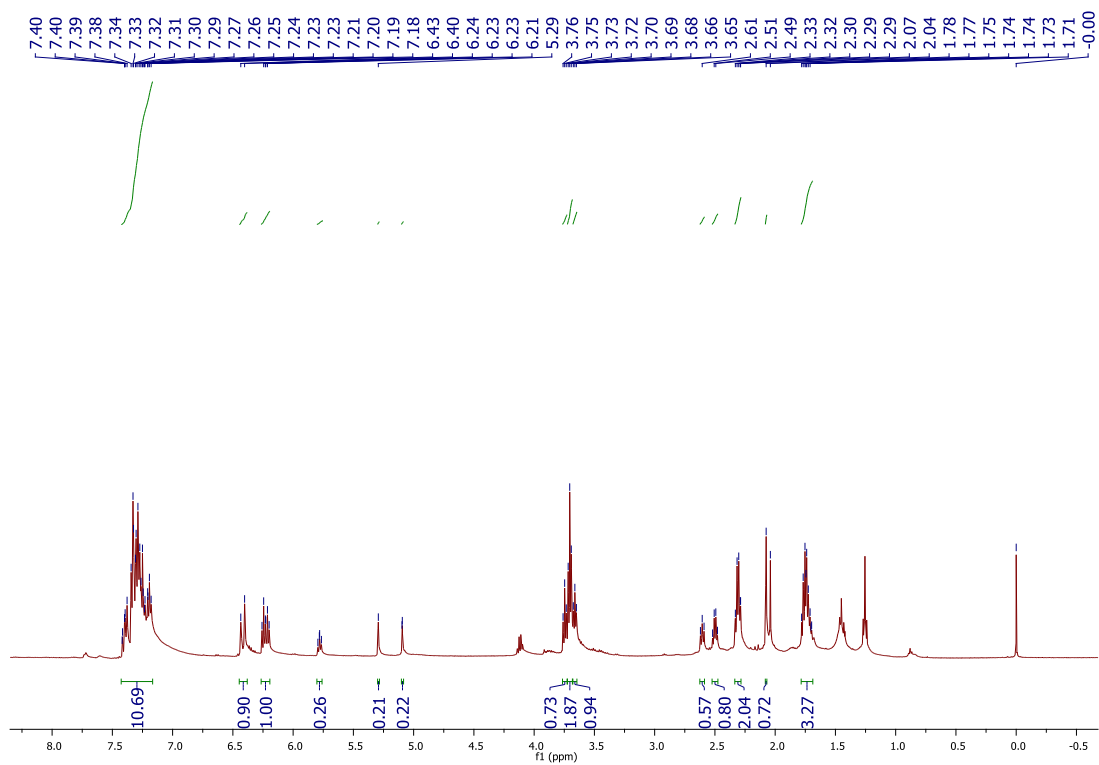


Figure S12: ¹H NMR spectrum (CDCl₃, 500 MHz) of 5a, 5b and 5c.

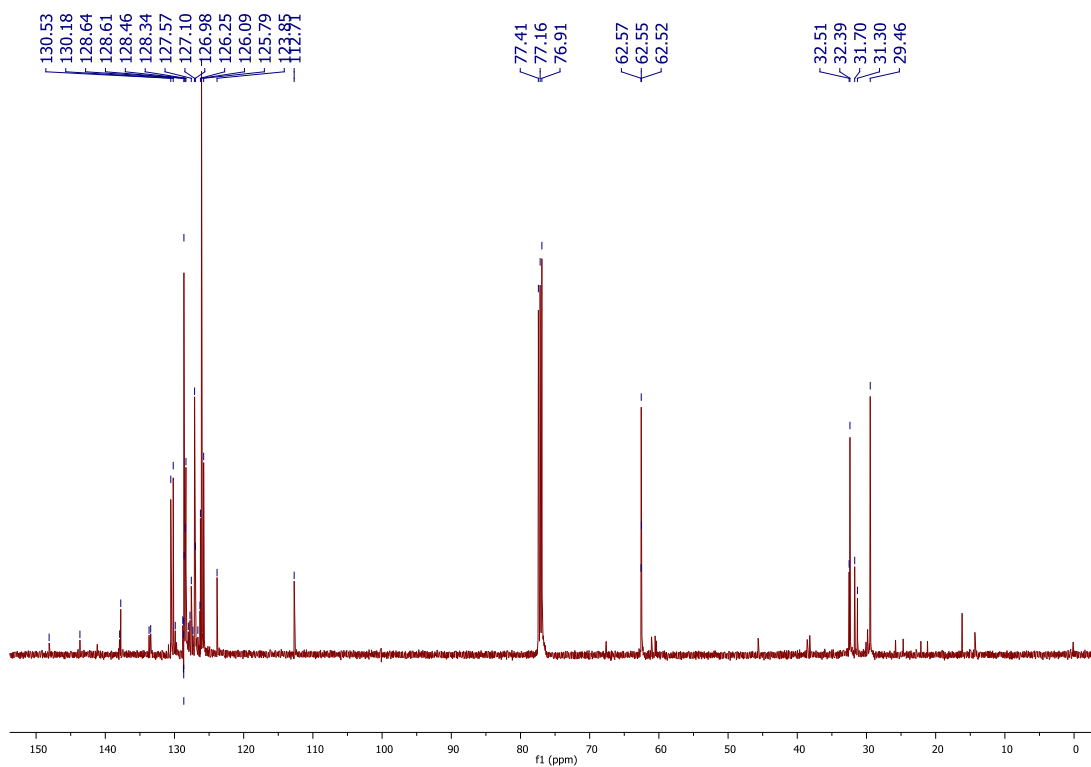


Figure S13: ¹³C NMR spectrum (CDCl₃, 125 MHz) of 5a, 5b and 5c.

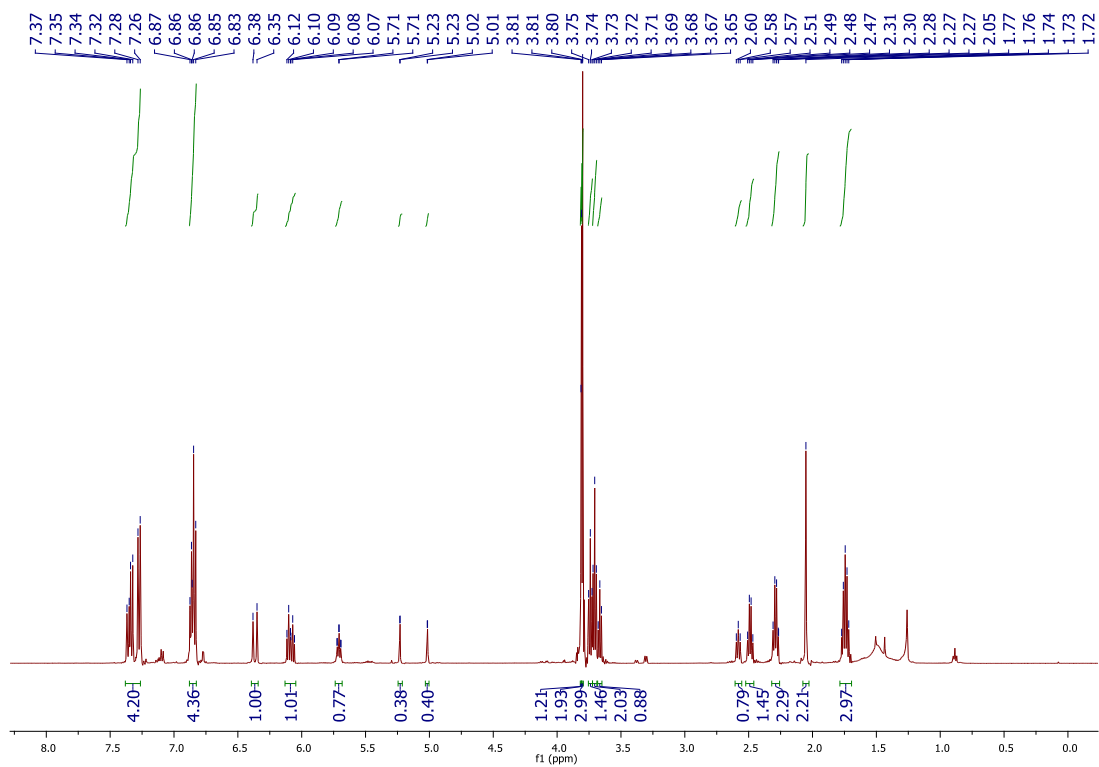


Figure S14: ^1H NMR spectrum (CDCl_3 , 500 MHz) of **6a**, **6b** and **6c**.

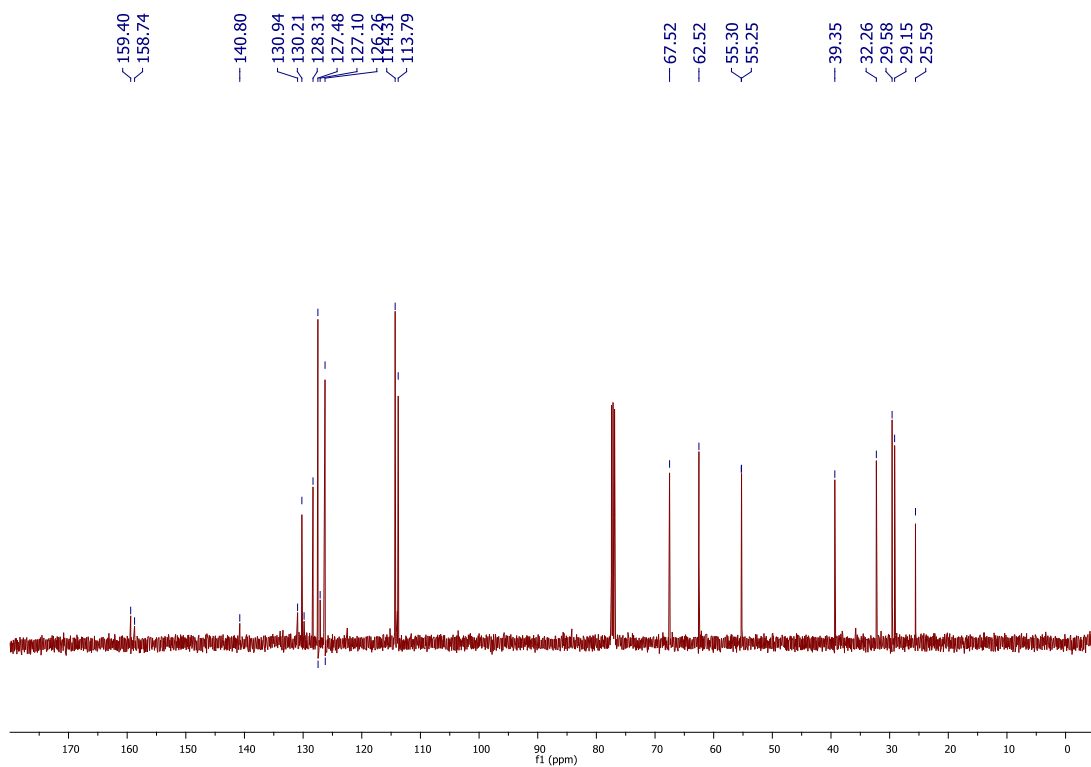


Figure S15: ^{13}C NMR spectrum (CDCl_3 , 125MHz) of **6a**, **6b** and **6c**.

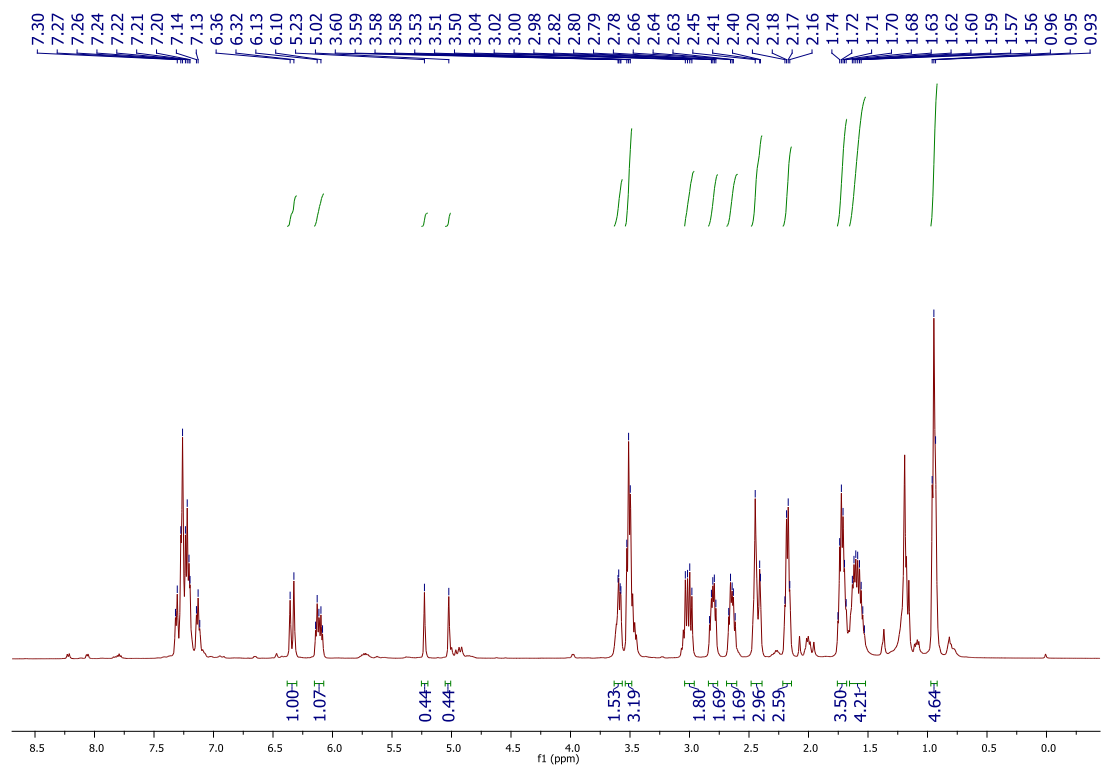


Figure S16: ^1H NMR spectrum (CDCl_3 , 500 MHz) of **7a** and **7b**.

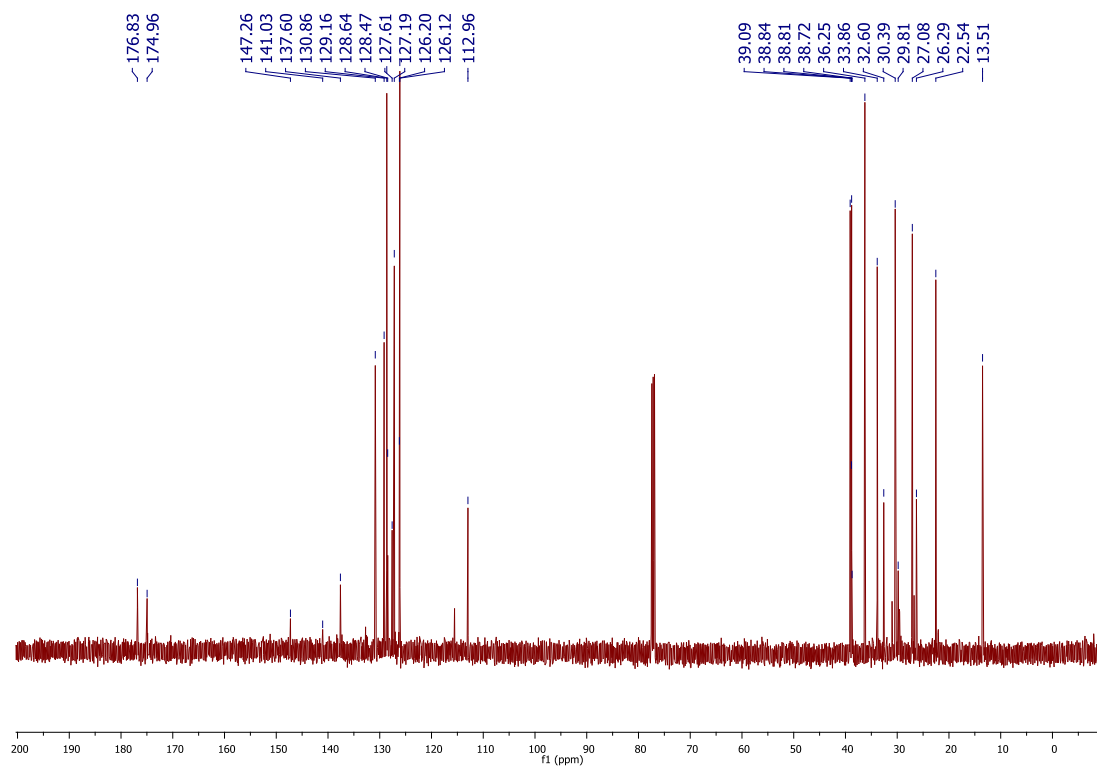
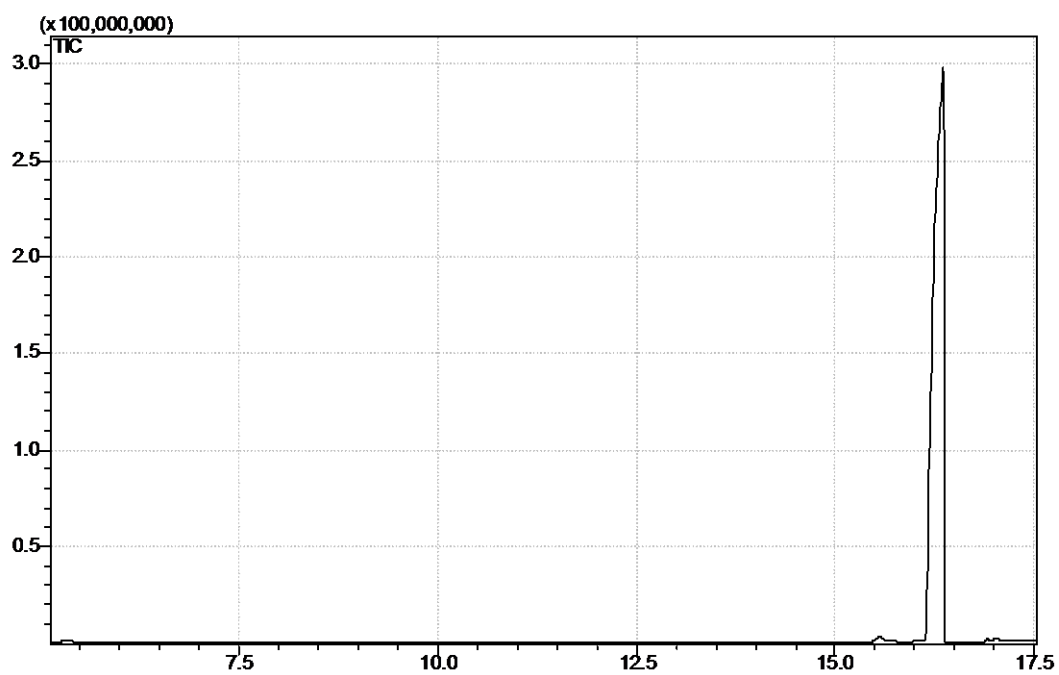


Figure S17: ^{13}C NMR spectrum (CDCl_3 , 125 MHz) of **7a** and **7b**.

4. GC-MS spectra

A)



B)

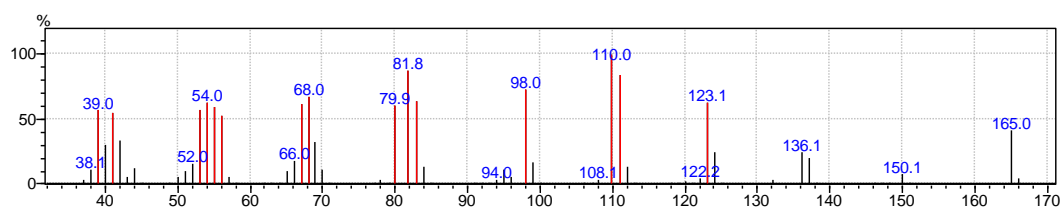
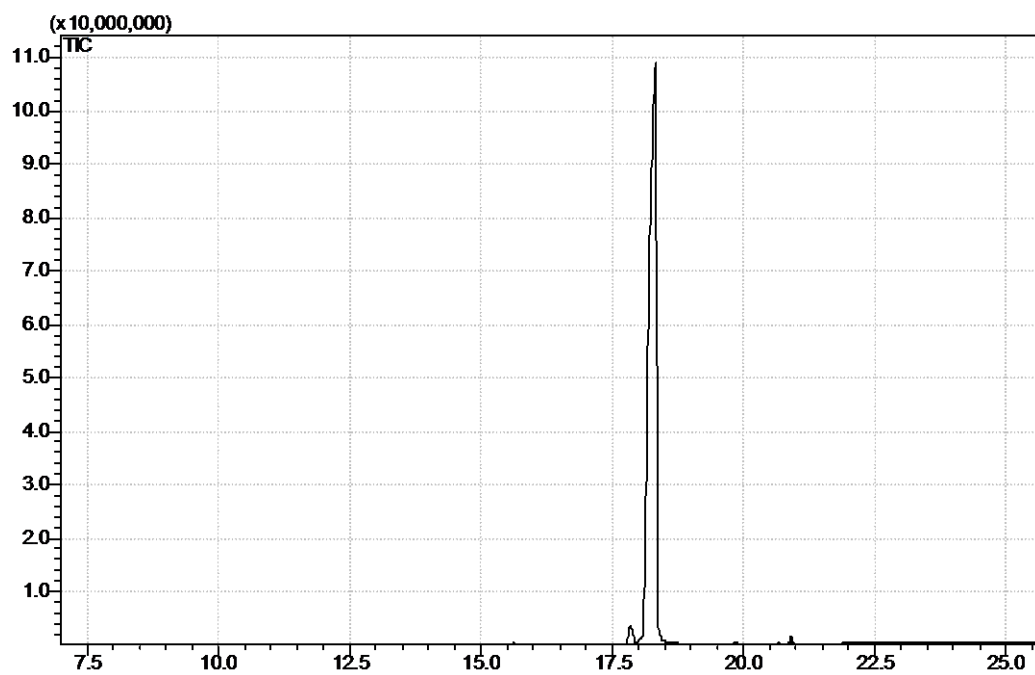


Figure S18: A) Gas chromatogram of **1** (retention time in min). **B)** The mass spectrum (m/z) at 15.25 min of **1**.

A)



B)

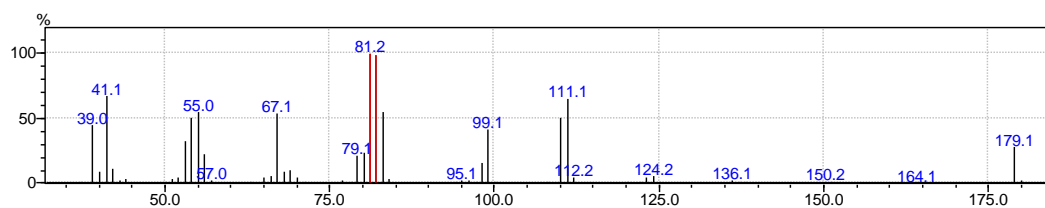
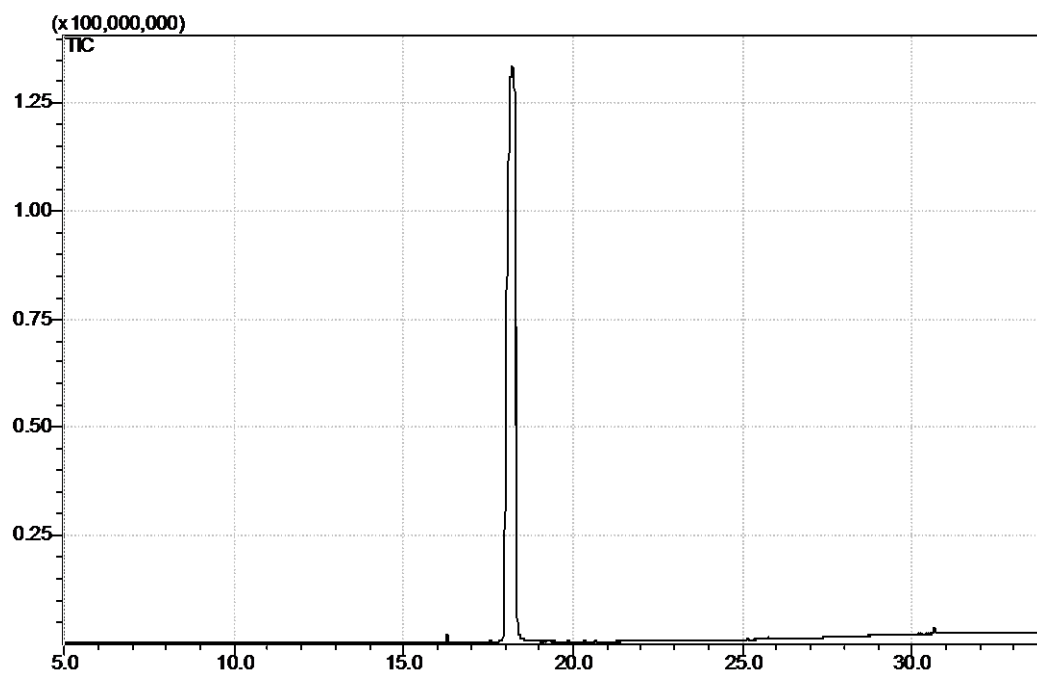


Figure S19: A) Gas chromatogram of **2** (retention time in min). **B)** The mass spectrum (m/z) at 18.25 min of **2**.

A)



B)

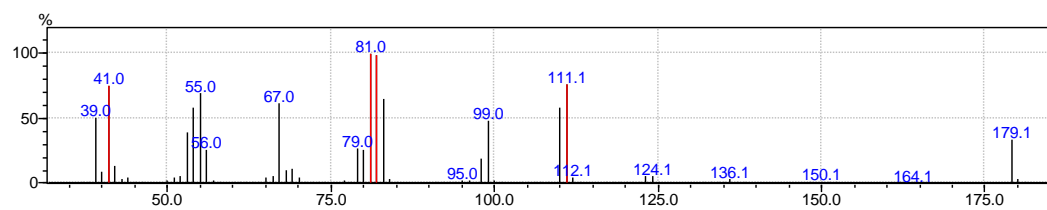
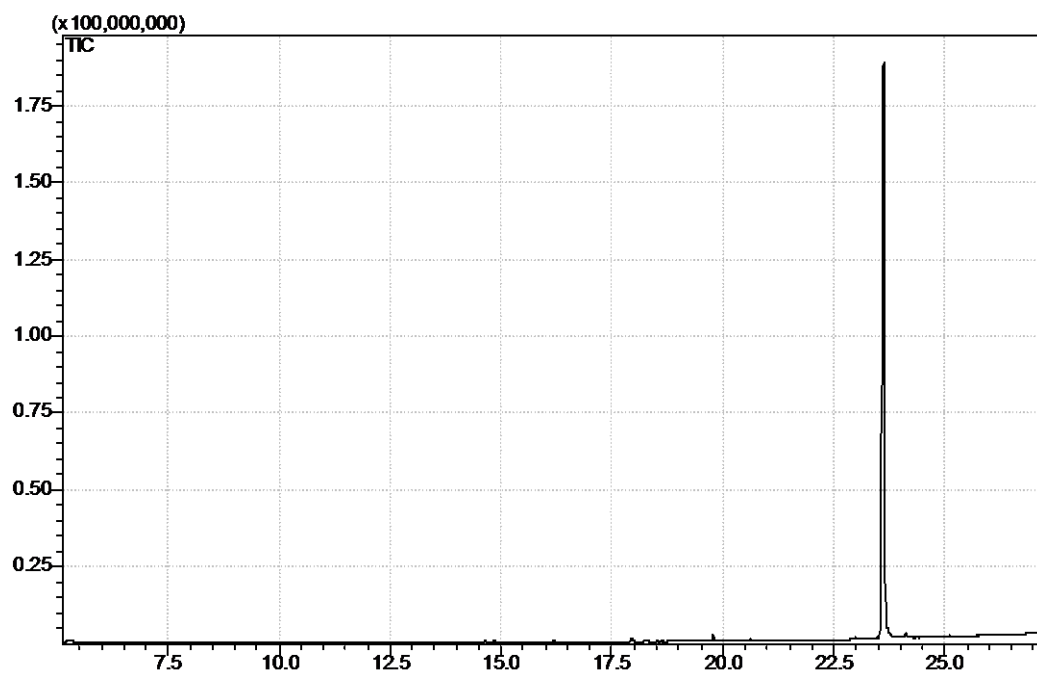


Figure S20: A) Gas chromatogram of **3** (retention time in min). **B)** The mass spectrum (m/z) at 18.25 min of **3**.

A)



B)

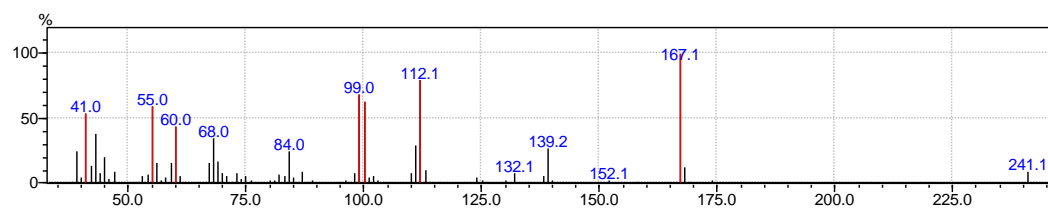
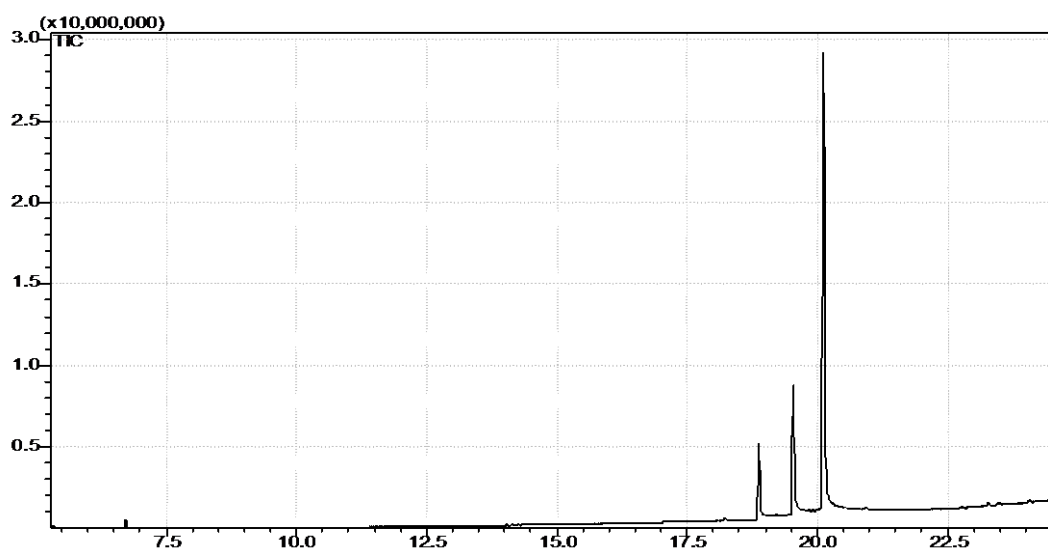
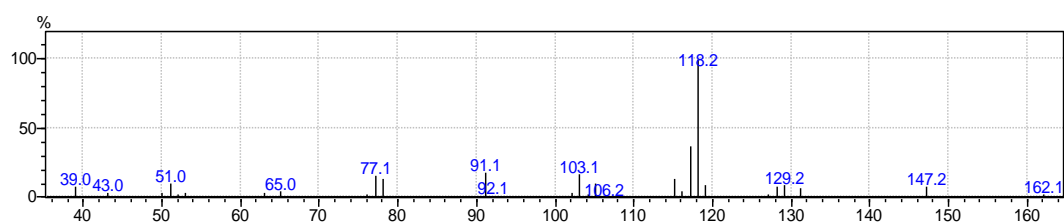


Figure S21: A) Gas chromatogram of **4** (retention time in min). **B)** The mass spectrum (m/z) at 22.75 min of **4**.

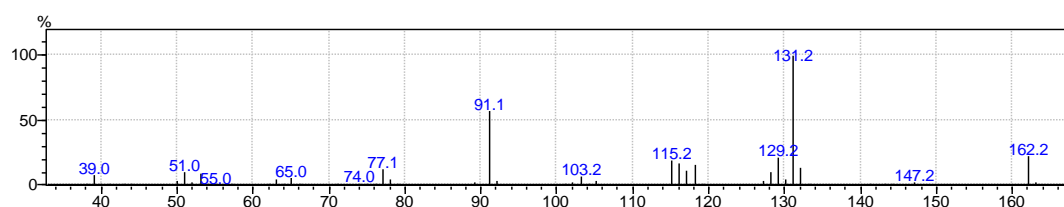
A)



B)



C)



D)

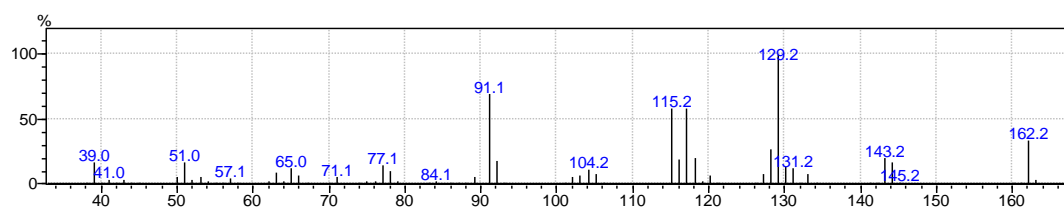
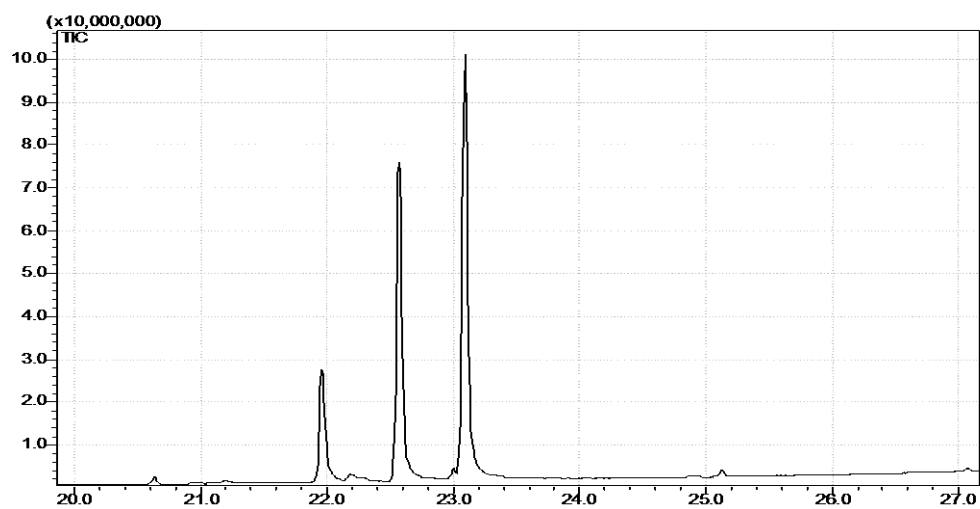
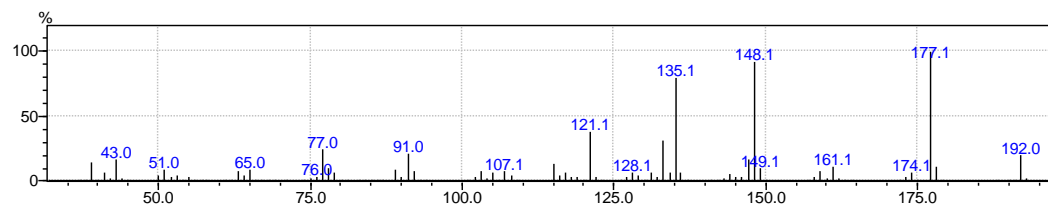


Figure S22: A) Gas chromatogram of the isomers **5a**, **5b** and **5c** (retention time in min), B) the mass spectrum (m/z) at 18.8 min, C) the mass spectrum (m/z) at 19.1 min and D) the mass spectrum (m/z) at 20.2 min of the isomers **5a**, **5b** and **5c**.

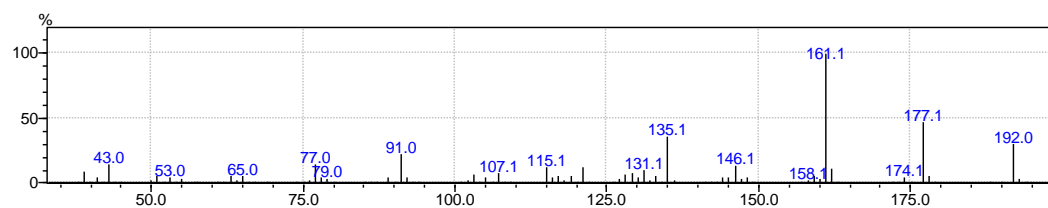
A)



B)



C)



D)

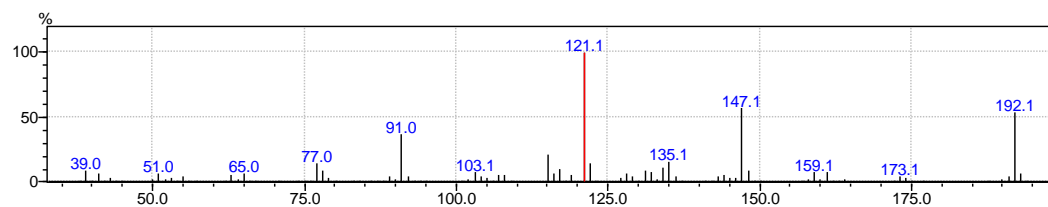
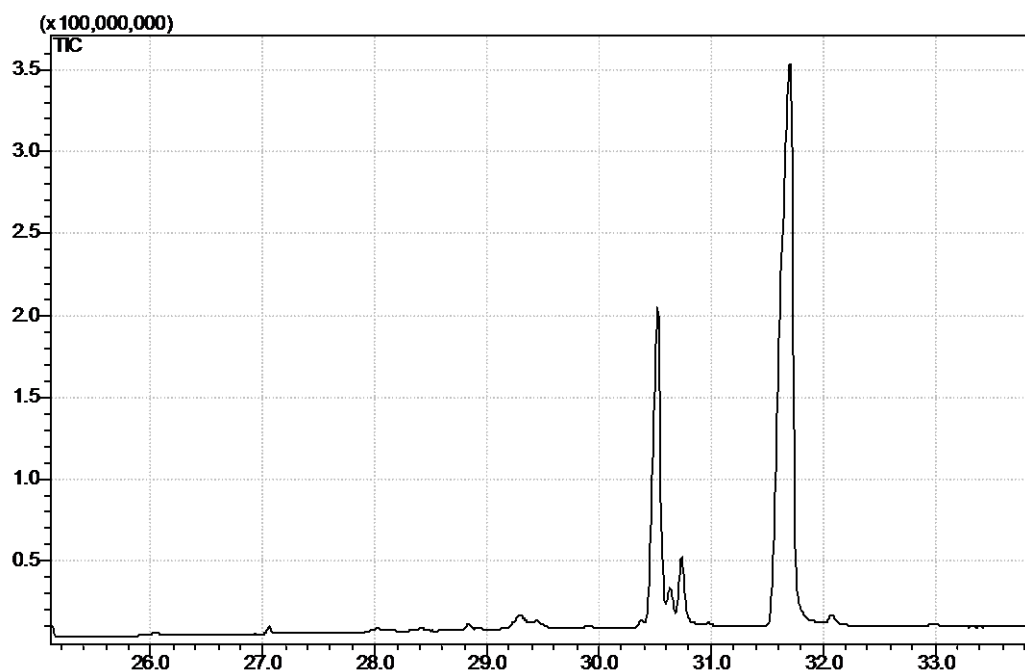
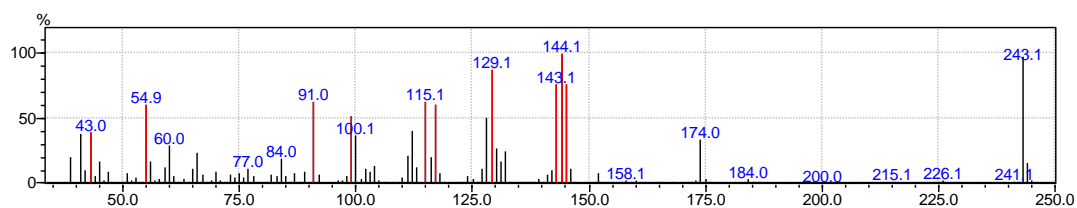


Figure S23: A) Gas chromatogram of the isomers **6a**, **6b** and **6c** (retention time in min), B) the mass spectrum (m/z) at 21.9 min, C) the mass spectrum (m/z) at 22.6 min and D) the mass spectrum (m/z) at 23.15 min of the isomers **6a**, **6b** and **6c**.

A)



B)



C)

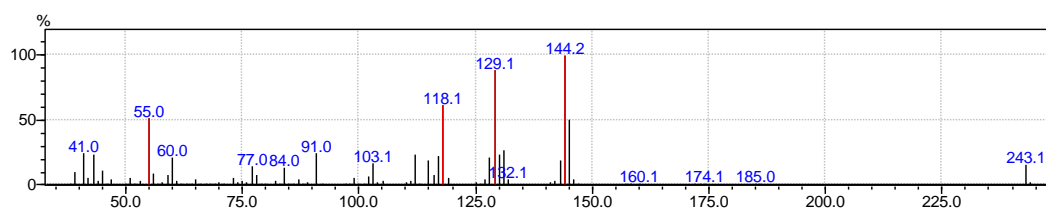


Figure S24: **A)** Gas chromatogram of the isomers **7a** and **7b** (retention time in min), **B)** the mass spectrum (m/z) at 30.45 min and **C)** the mass spectrum (m/z) at 31.6 min of the isomers **7a** and **7b**.

5. References

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