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Chemistry-based enzyme detection and inhibition in epigenetics

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CHAPTER 2

Aqueous oxidative Heck reaction as a protein-labeling strategy

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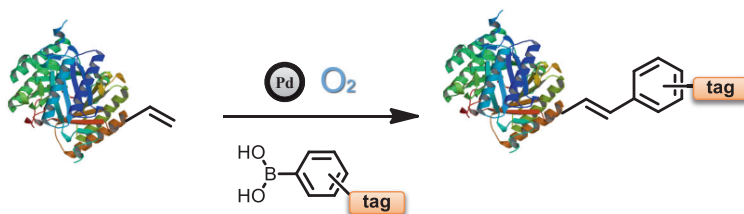
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Abstract

An increasing number of chemical reactions are being employed for bioorthogonal coupling of detection labels to protein-bound functional groups. Several of these strategies, however, are limited in their application to pure proteins and are ineffective in complex biological samples such as cell lysates. Here we present the palladium-catalyzed oxidative Heck reaction as a new and robust bioorthogonal strategy for linking functionalized arylboronic acids to protein-bound alkenes in high yields and with excellent chemoselectivity even in the presence of complex protein mixtures from living cells. Advantageously, this reaction proceeds under aerobic conditions, whereas most other metal-catalyzed reactions require inert atmosphere.



Introduction

Bioorthogonal coupling of protein-bound small organic molecules is a long-standing challenge with many implications for cell biology.¹ In combination with metabolic labeling, it has enabled the detection of post-translational modifications for which no antibodies are available.¹ In addition, bioorthogonal conjugations have shown excellent selectivity for the detection of specific endogenous protein modifications.² Regrettably, though, the wealth of organic chemistry reactions is poorly translated into new methods that are applicable in cell biology. In this study we present the oxidative Heck reaction as a strategy for bioorthogonal coupling reactions between arylboronic acids and protein-bound alkenes.

The most widely used strategy for bioconjugation is the linkage of azides to alkynes through copper-catalyzed Huisgen cycloaddition, known as the 'click reaction'.³ Despite their success as chemical reporters, however, terminal alkyne systems suffer from a lack of stability under physiological conditions, most probably due to the relatively acidic alkyne hydrogen atom.⁴ Side reactions are alkyne homocoupling,⁵ covalent inactivation of specific oxidative enzymes,⁶ and the covalent binding of terminal alkynes to active site cysteine residues (thiol-yne reaction).⁷ Some of these problems are avoided by use of the recently developed strain-promoted 'click reaction',⁸ but the relatively large sizes of the strained alkynes limit their application in metabolic labeling.⁸

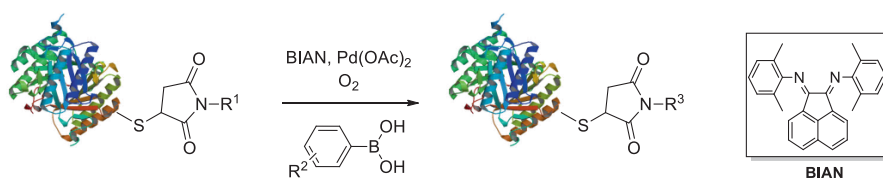
Non-conjugated alkenes appear to be ideal protein labels because of their low chemical reactivity and their low abundance in cellular proteins. Alkenes have been employed as protein labels for linkage to proteins through the photochemical thiol-ene reaction.⁹ In addition, they can be ligated by reaction with tetrazoles or tetrazines after photochemical conversion of these reagents into highly reactive nitrile imine intermediates.¹⁰ However, no metal-catalyzed couplings have been described so far. This is remarkable because the potential of metal-catalyzed conjugations in protein labeling reactions has been demonstrated by recently developed bioorthogonal strategies employing ruthenium-catalyzed cross-metathesis of allyl-substituted cysteines,¹¹ palladium-catalyzed Suzuki–Miyaura reactions of phenyl iodides,^{12,13} and other reactions.¹⁴

We anticipated that the Pd-catalyzed oxidative Heck reaction—that is, coupling of an arylboronic acid to an alkene— could be developed into an excellent tool in protein probing, provided that an active catalyst applicable in water could be identified.^{15–17} Importantly, the oxidative Heck reaction, unlike most other metal-catalyzed reactions, does not require oxygen-free conditions; this makes this reaction robust for applications in cell lysates.

Although many previous studies focused on oxidative Heck reactions of activated alkenes such as cyclohexenones¹⁶ and acrylate esters,¹⁵ several recent studies have reported high yields and in several cases excellent regioselectivities in oxidative Heck reactions of isolated alkenes.^{17–19} This sets the stage for development of this reaction into a bioconjugation strategy for isolated alkene systems.

Results and discussion

We selected the enzyme 4-oxalocrotonate tautomerase (4-OT) as a model protein for the generation of protein-bound alkenes in order to establish the oxidative Heck reaction for bioorthogonal conjugation. This enzyme contains no cysteine residues, and so we selected the mutant R61C (4-OT R61C) to include a cysteine residue in the solvent-exposed C terminus region of the protein.²⁰ Upon expression the enzyme proved to be a dimer, due to formation of disulfide bonds (see Supplementary information). After reduction, alkenes were coupled through a maleimide linker to give the protein-bound terminal alkene 4-OT R61C-1, the *cis* internal alkene 4-OT R61C-2, the *trans* internal alkene 4-OT R61C-3, and, as a control, the protein 4-OT R61C-4 without the olefinic functional group (Scheme 1, Table 1).



Scheme 1. Oxidative Heck reactions with alkenes linked to the protein 4-OT R61C. Substitutions R¹, R² and R³ are defined in Table 1.

In an attempt to develop a water soluble catalyst for cross-coupling of arylboronic acids to protein-bound alkenes we considered application of the highly water soluble 2-amino-4,6-dihydroxypyrimidine ligands in combination with Pd(OAc)₂ as reported by Davis *et al.* in the context of Pd-catalyzed coupling reactions.¹² In our hands, however, the use of 2-amino-4,6-dihydroxypyrimidine as a ligand was not effective for this type of oxidative Heck reaction and led to low yields (20%) in test reactions on small molecules. We therefore employed the bis-imine of acenaphthenequinone and mesitylamine (BIAN) which we had developed earlier as a superior ligand for oxidative Heck reactions of challenging substrates.¹⁶

Because of its limited water solubility the Pd(OAc)₂/BIAN catalyst was prepared in an organic solvent (DMF), with the final buffer:DMF ratio in the reaction mixture being 6:1. This proved to be compatible with protein solutions. All reactions were performed at room temperature and at neutral pH under oxygen for a 24h period. Conversion was monitored by LC-MS after removal of palladium by EDTA chelation in order to provide suitable mass spectra.

Gratifyingly, we found full conversion of the protein-bound alkene 4-OT R61C-1 into its arylated product with use of 50 or 20 equiv. of the Pd(OAc)₂/BIAN catalyst and 300 or 100 equiv. of the phenylboronic acid (Table 1). Application of 10 equiv. of the catalyst and 50 equiv. of the phenylboronic acid did not result in full conversion (85%). As a result, 20 equiv. of Pd(OAc)₂/BIAN and 100 equiv. of the arylboronic acid were chosen as optimal for further experimentation.

Subsequently, *cis* and *trans* alkenes were also subjected to the same reaction conditions. Interestingly, 4-OT R61C-2 showed significant conversion (70%)

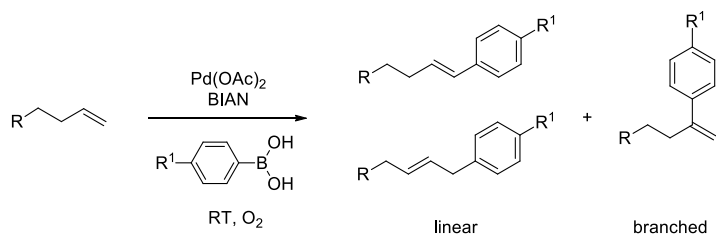
whereas 4-OT R61C-3, in contrast, hardly reacted (5%; Table 1). This demonstrates that the oxidative Heck reaction can also be employed to label *cis* internal alkenes and discriminates between *cis* and *trans* alkenes. This finding presents exciting opportunities for selective labeling and detection of *cis* (in the presence of *trans*) unsaturated fatty acids in biological systems.

To investigate the selectivity of the reaction for protein-bound alkenes, control experiments were performed with omission either of the catalyst or of the boronic acid (control experiments 1 and 2, Supplementary information). In both cases the protein 4-OT R61C-1 did not react. Importantly, protein 4-OT R61C-4, which does not contain an alkene system, did not react under any of the reaction conditions (Table 1). This demonstrates that the phenyl group selectively reacts with the protein-bound alkene and not with other functional groups in the protein.

Table 1. Aqueous oxidative Heck reactions with protein-bound alkenes according to Scheme 1 monitored by LC-MS.

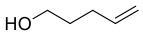

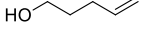
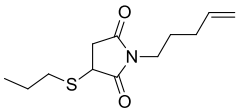
R ¹	R ²	R ³	Catalyst/ boronic acid	Conversion
	H		50:300	full
	H		20:100	full
	H		10:50	≈85%
	4-OMe		20:100	full
4-OT R61C-1	4-COOMe		20:100	≈85%
	3-NH-dansyl		20:100	full
	H		20:100	≈70%
4-OT R61C-2	H		20:100	≈5%
	H		20:100	≈5%
4-OT R61C-3	H		20:100	no
4-OT R61C-4	H		20:100	no

Next, the influence of the electronic properties of arylboronic acid derivatives on their reactivity with protein-bound alkenes was investigated. It was found that 4-methoxyphenylboronic acid gave full conversion, whereas 4-methoxycarbonylphenylboronic acid gave only 85% conversion. The same phenomenon was observed in reactions with small-molecule alkenes in the presence of the same catalyst (Scheme 2, Table 2, entries 1-3). Apparently the reaction is disfavored by the presence of electron-withdrawing groups on the phenylboronic acid. Little *et al.* reported that, for Pd-catalyzed Suzuki cross-coupling reactions, certain catalytic systems proved to be tolerant to the electronic character of the arylboronic acid.^{21,22} However, Larhed and co-workers investigated the influence of the nature of the arylboronic acid in an attempt to discover an efficient catalyst for oxidative Heck vinylation. Using 2,9-dimethyl-1,10-phenanthroline (dmphen) as a ligand, they found that electron-rich boronic acids afforded higher yields than the electron-poor ones and attributed their observation in the faster transmetalation of such partners with palladium.²³



Scheme 2. Oxidative Heck reactions with small-molecule alkenes and the isomers formed. The employed terminal alkenes and R¹ substitution are defined in Table 2.

Although irrelevant in most applications as bioorthogonal coupling strategy, multiple regio- and stereoisomers can be formed in oxidative Heck reactions of terminal alkenes (i.e. linear versus branched, Scheme 2). However, the positions of the resulting double bonds could not be determined from the mass spectrometric analysis in the protein tagging reactions. In order to estimate which isomers are formed under Pd/BIAN catalysis conditions, small-molecule alkenes were linked to arylboronic acids (Table 2), and the product ratios were investigated. The ratio between the linear and branched isomers in the reactions with phenyl- and 4-methoxyphenylboronic acids varies between 2:1 and 1:1. In order to mimic the conditions used for the protein labeling further, the reaction was performed with the alkene part of protein 4-OT R61C-1 (Table 2, entry 4), with use of a stoichiometric amount of catalyst and a tenfold excess of the boronic acid in DMF for a 24h period. The ratio between the linear and branched products was about 2:1, which suggests that similar isomers are formed in bioconjugation reactions. Apparently, chelate control as reported by White *et al.*¹⁸ in the case of their regioselective oxidative Heck reactions does not play a significant role here. Despite its limited relevance for most protein couplings, chelate control to achieve higher regioselectivity remains an interesting challenge for further development of this reaction.

Table 2. Oxidative Heck reactions between arylboronic acids and small-molecule alkenes according to Scheme 2.			
Alkene	Boronic acid; R ¹	Isolated yield [%]	Linear/ branched
1 	H	67 ^a	2.1:1
2 	-OCH ₃	64 ^a	1:1.1
3 	-COOCH ₃	-	-
4 	H	81 ^b	2.3:1

^aPd(OAc)₂ (5 mol%), BIAN (7 mol%), phenylboronic acid (1.5 equiv.), room temperature, 30h. ^bPd(OAc)₂ (1 equiv.), BIAN (1.4 equiv.), phenylboronic acid (10 equiv.), room temperature, 24h.

To enable detection of the protein-bound alkenes by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) we moved on to the linkage of the fluorescent 3-(dansylamino)phenylboronic acid to 4-OT R61C-1 (Table 1). Full conversion was observed by LC-MS, and the protein-bound fluorophore was visualized by fluorescence detection on SDS-PAGE (Figure 1C). Furthermore, the same reaction was performed with the soluble fraction of lysates from RAW264.7 macrophages enriched with 4-OT R61C-1. We were pleased to find that 4-OT R61C-1 was the only fluorescently labeled protein (Figure 1E and F). LC-MS analysis of the fluorescently labeled protein band from the gel in Figure 1E demonstrated more than 90% attachment to 4-OT R61C-1 (6924 Da), giving the fluorescently labeled product of 7248 Da (Figure 1B). In order to reduce the contrast between the labeled band and the background, less 4-OT R61C-1 was added to the same cell lysate, and the oxidative Heck reaction was performed. Pleasingly, also in this experiment no fluorescence in the other proteins was observed (Figure 1G and 1H).

In order to estimate the conversion, we loaded an equivalent amount of 4-OT R61C that was directly fluorescently labeled with *N*-[2-(dansylamino)ethyl] maleimide as a reference. The intensity of fluorescently labeled 4-OT R61C-1 produced by the oxidative Heck reaction (Figure 1G, lane 1) was slightly lower than that of the reference (Figure 1G, lane 3). This indicates that coupling at low alkene concentrations is qualitative rather than quantitative.

These experiments demonstrate that the oxidative Heck reaction is very selective and suitable for bioorthogonal coupling to protein-bound alkenes even in the presence of a complex protein mixture from cells.

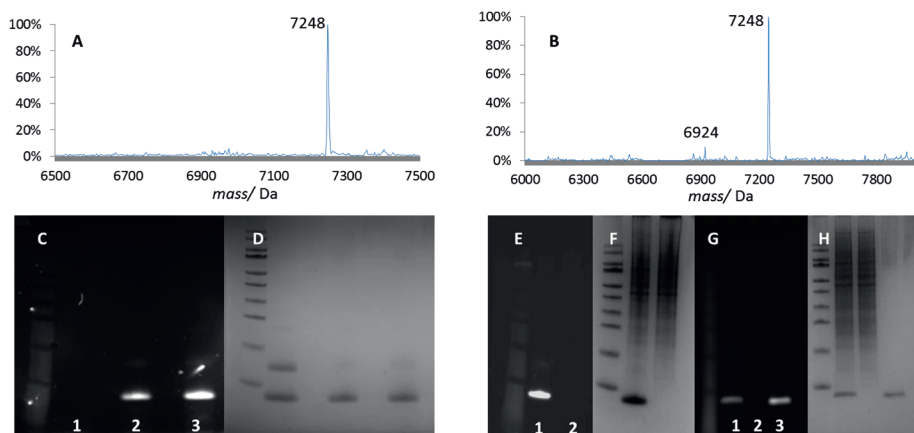


Figure 1. Fluorescent labeling of protein 4-OT R61C-1 with the aid of the oxidative Heck reaction. A) Mass spectrum demonstrating full conversion of pure 4-OT R61C-1. B) Mass spectrum demonstrating the conversion of 4-OT R61C-1 mixed with a complex protein mixture from cells (ratio 1:1). C) Fluorescence imaging on SDS-PAGE of labeled 4-OT R61C-1. 1) 5 μ g of unlabeled protein 4-OT R61C, 2) 2 μ g and 3) 3 μ g of labeled 4-OT R61C-1. D) Coomassie staining of C. E) Fluorescence imaging on SDS-PAGE of 4-OT R61C-1 labeled in the presence of a cell lysate (protein ratio 1:1). Reaction 1) in the presence of 4-OT R61C-1 and 2) in the absence of 4-OT R61C-1. F) Coomassie staining of E. G) Fluorescence imaging on SDS-PAGE of 4-OT R61C-1 labeled in the presence of a cell lysate (protein ratio 1:10). Reaction 1) in the presence of 4-OT R61C-1, 2) in the absence of 4-OT R61C-1 and 3) an equivalent amount of fluorescently labeled 4-OT R61C after direct coupling with *N*-[2-(dansylamino ethyl)]maleimide. H) Coomassie staining of G.

Conclusion

In conclusion, the oxidative Heck reaction has been added to the toolkit of bioorthogonal conjugation methods. Importantly, this reaction runs to completion with protein-bound alkenes and proceeds under aerobic conditions. Control experiments demonstrate exclusive linkage to protein-bound alkenes and not to other protein functional groups. Pleasingly, this coupling works effectively in cell lysates and is selective for alkene-labeled proteins, which demonstrates its excellent performance in biological samples.

Contributions

The experimental work was performed mainly by M. E. Ourailidou. J.-Y. van der Meer and B.-J. Baas contributed in the expression and purification of 4-OT R61C. M. Jeronimus-Stratingh performed the mass spectrometric analysis and A. L. Gottumukkala contributed in the optimization of the oxidative Heck reaction on small molecules using BIAN as a ligand. Supervision of the project was done by F. J. Dekker, G. J. Poelarends and A. J. Minnaard. All authors contributed in the revision of the manuscript.

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Supplementary information

Preparation of protein-bound alkenes

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 gelfiltration 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 enzyme³ 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. 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. 15 and 50 mL centrifuge tubes, serological pipettes and cell culture flasks were purchased from Greiner bio-one, The Netherlands.

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.

Sequence of 4-oxalocrotonate tautomerase (4-OT) R61C mutant

PIAQIHILEGRSDEQKETLIREVSEAIRSLDAPLTSVRVIITEMAKGHFGIGGELASKVCR

Expression and purification of 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 analyzed by ESI-MS. The cups containing the protein solution were snapfrozen in liquid nitrogen and kept in -20 °C.

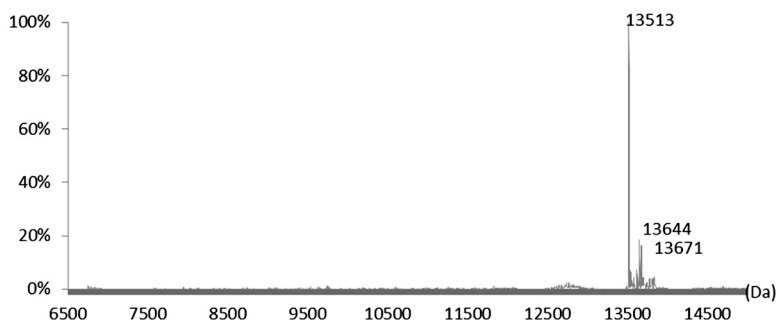
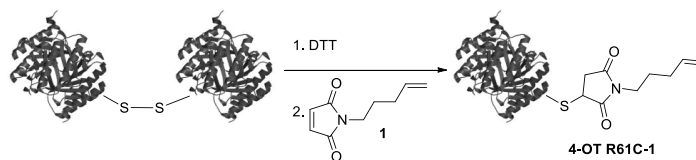


Figure S1. ESI-MS spectrum of expressed and purified 4-OT R61C. Mass expected 6757, mass found 13513 (dimer) (13644: modified enzyme with methionine, 13671: modified enzyme with formylated methionine).

Preparation of 4-OT R61C conjugates



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 CH_3CN (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.

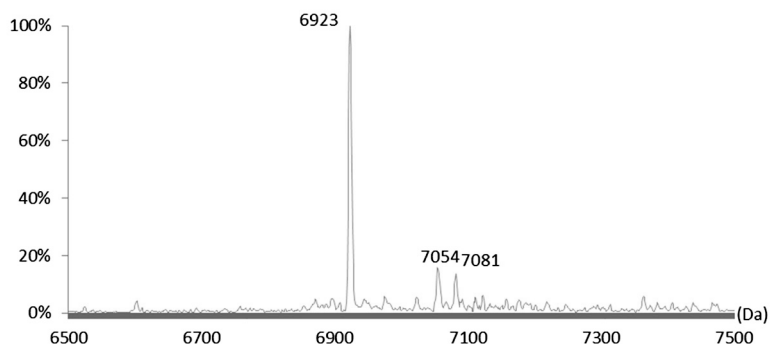
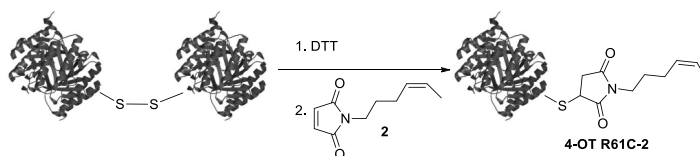


Figure S2. Coupling of **1** to 4-OT R61C. ESI-MS spectrum: mass expected 6922, mass found 6923 (7054: modified enzyme with methionine, 7081: modified enzyme with formylated methionine).



The reduction of the dimer and subsequent coupling were performed as described above.

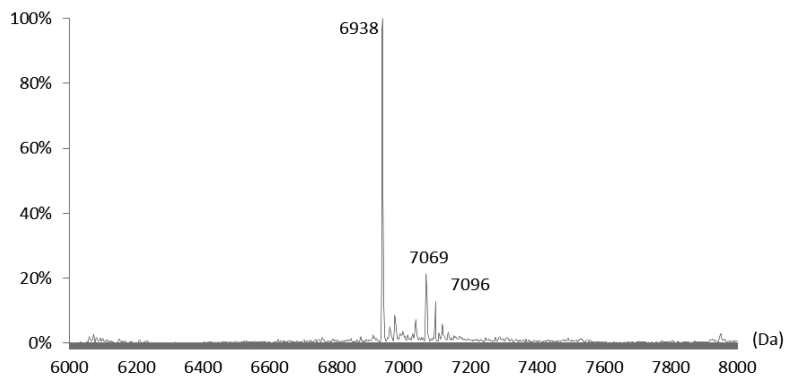


Figure S3. Coupling of **2** to 4-OT R61C. ESI-MS spectrum: mass expected 6936, mass found 6938 (7069: modified enzyme with methionine, 7096: modified enzyme with formylated methionine).

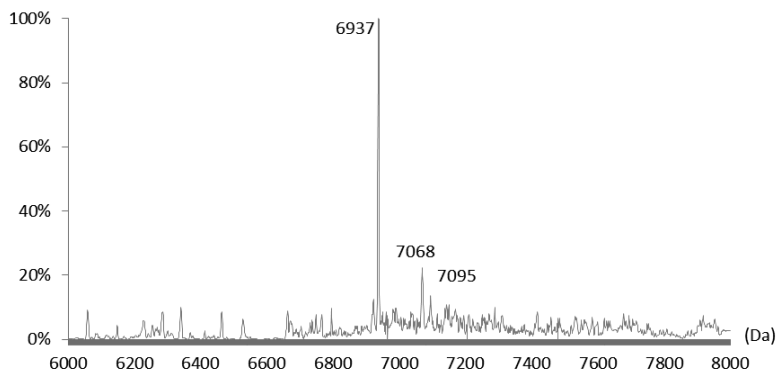
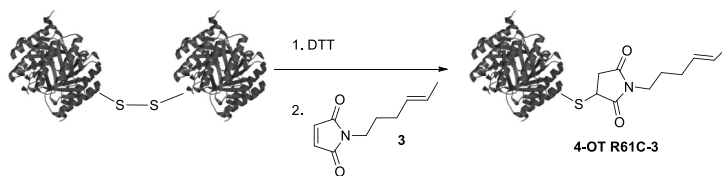


Figure S4. Coupling of **3** to 4-OT R61C. ESI-MS spectrum: mass expected 6936, mass found 6937 (7068: modified enzyme with methionine, 7095: modified enzyme with formylated methionine).

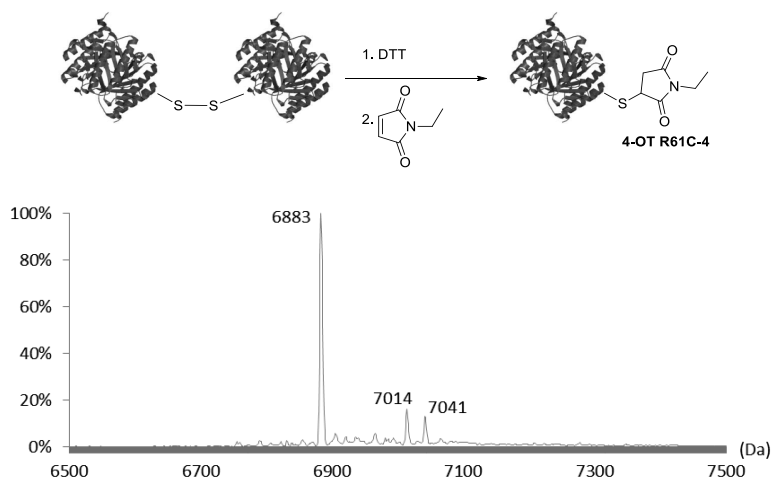


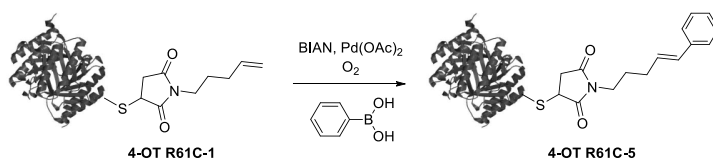
Figure S5. Coupling of *N*-ethylmaleimide to 4-OT R61C. ESI-MS spectrum: mass expected 6882, mass found 6883 (7014: modified enzyme with methionine, 7041: modified enzyme with formylated methionine).

Oxidative Heck reaction for bioorthogonal protein coupling

Preparation of stock solutions

The palladium(II)-BIAN catalyst stock solution was prepared by dissolving BIAN (4.3 mg, 11 μmol) and $\text{Pd}(\text{OAc})_2$ (1.8 mg, 8 μmol) 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. 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. The EDTA stock solution (1.0 M, pH 7.0) was prepared by dissolving ethylenediaminetetraacetic acid disodium salt dihydrate (18.6 g, 50 mmol) in deionized H_2O (50 mL) and the pH was set at 7.0 using a concentrated solution of NaOH.

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



A 40 mM 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.

50 equiv. catalyst/300 equiv. phenylboronic acid

2

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 24h, it was transferred into a 2 mL cup and 500 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). 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.

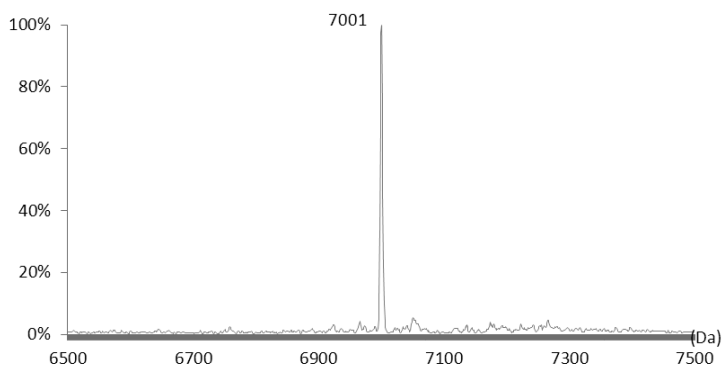


Figure S6. Coupling of phenylboronic acid to 4-OT R61C-1 (50 equiv. catalyst/300 equiv. phenylboronic acid). ESI-MS spectrum: mass expected 6999, mass found 7001.

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 mixed and the same procedure was followed as described above with the only difference that 200 μL of the EDTA stock solution were used for chelation of the catalyst.

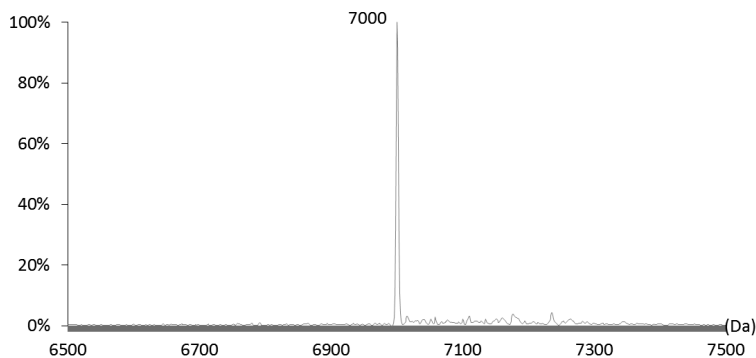


Figure S7. Coupling of phenylboronic acid to 4-OT R61C-1 (20 equiv. catalyst/100 equiv. phenylboronic acid). ESI-MS spectrum: mass expected 6999, mass found 7000.

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 mixed and 100 μL of the EDTA stock solution were used for chelation.

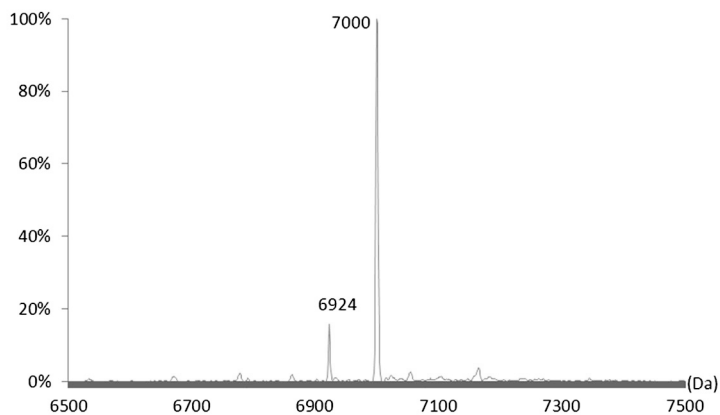


Figure S8. Coupling of phenylboronic acid to 4-OT R61C-1 (10 equiv. catalyst/50 equiv. phenylboronic acid). ESI-MS spectrum: mass expected 6999, mass found 7000.

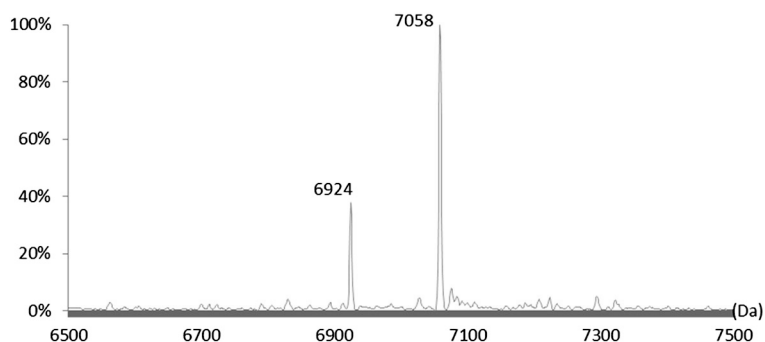
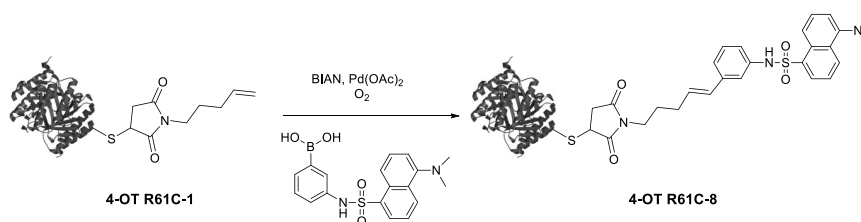


Figure S10. Coupling of 4-(methoxycarbonyl)phenylboronic acid to 4-OT R61C-1. ESI-MS spectrum: mass expected 7057, mass found 7058.

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



A 40 mM 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).

Fluorescence imaging

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%). 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 150V, 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.

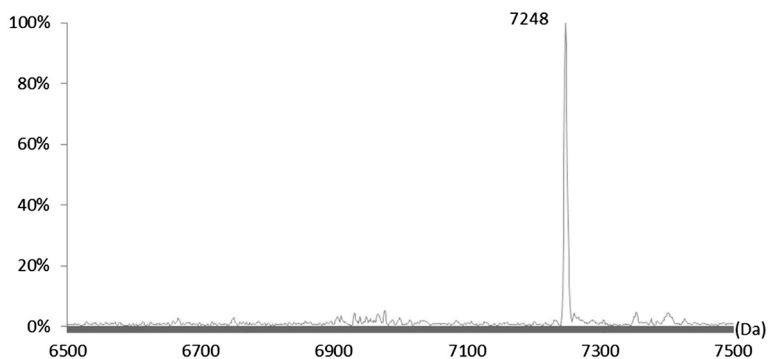
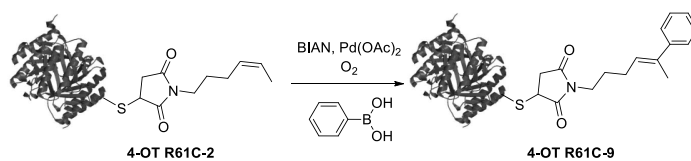


Figure S11. Coupling of 3-(dansylamino)phenylboronic acid to 4-OT R61C-1. ESI-MS spectrum: mass expected 7247, mass found 7248.

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.

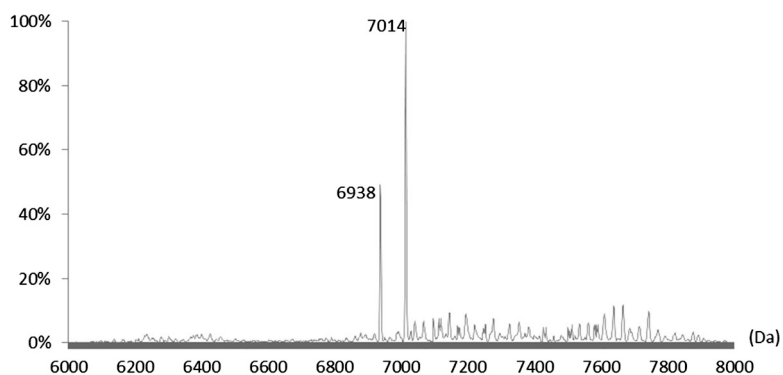


Figure S12. Coupling of phenylboronic acid to 4-OT R61C-2. ESI-MS spectrum: mass expected 7014, mass found 7014.

Coupling of phenylboronic acid to protein-bound *trans* internal alkene 4-OT R61C-3

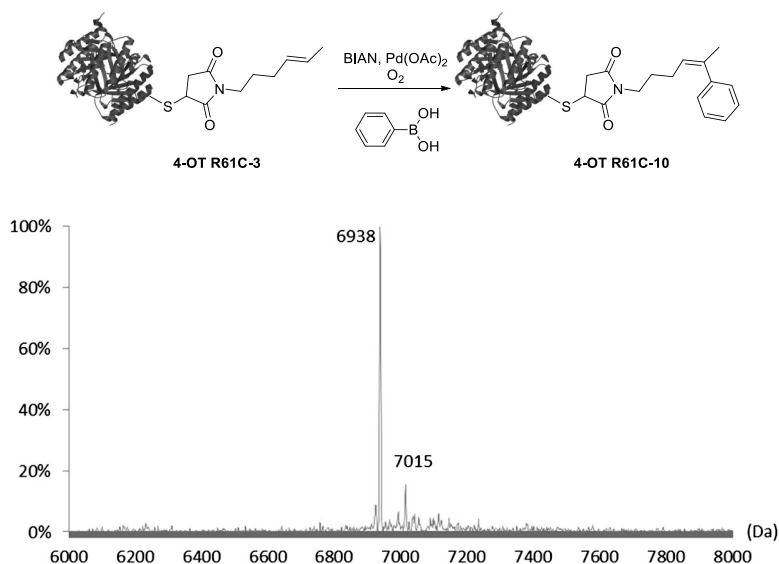


Figure S13. Coupling of phenylboronic acid to 4-OT R61C-3. ESI-MS spectrum: mass expected 7014, mass found 7015.

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

RAW264.7 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 1xPBS. 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 coupling 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. 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 (protected from light). 360 μL of this stock was added to 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 $^{\circ}\text{C}$. After the addition of ammonium formate buffer (15 mL, pH 7.0, 1 M), it was centrifuged again for 1h 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 $^{\circ}\text{C}$ until needed.

2

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) 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 24h, 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 3h 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). For fluorescence imaging 12 μL of each reaction mixture were used. For estimation of the conversion *via* MS analysis, 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%). 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.

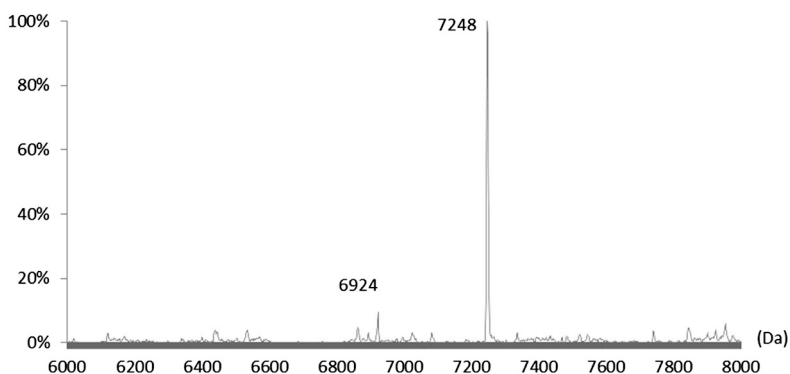
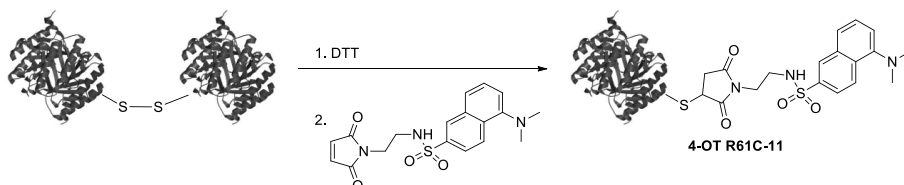


Figure S14. Coupling of 3-(dansylamino)phenylboronic acid to 4-OT R61C-1 in presence of cell lysates (protein ratio 1:1). ESI-MS spectrum: mass expected 7247, mass found 7248.

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 mixed and the same procedure was followed as mentioned above in case of protein ratio 1:1. 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. For fluorescence imaging 6 μL of each reaction mixture and 1 μL of 4-OT R61C-11 were used.

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



The usual procedure was followed (see preparation of 4-OT conjugates).

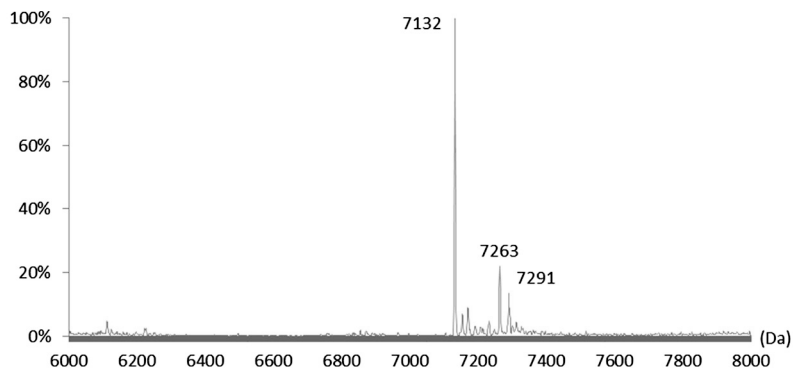


Figure S15. Coupling of *N*-[2-(dansylamino)ethyl]maleimide to 4-OT R61C. ESI-MS spectrum: mass expected 7130, mass found 7132 (7263: modified enzyme with methionine, 7291: modified enzyme with formylated methionine).

Control reactions

Control reaction 1; oxidative Heck in absence of catalyst

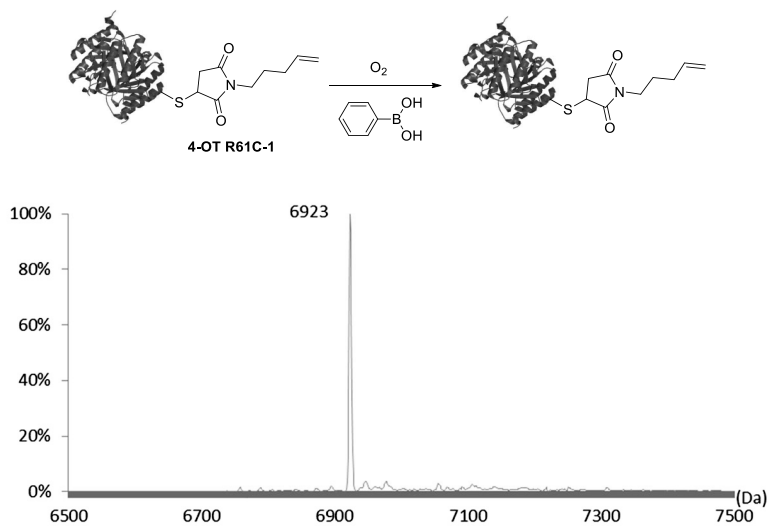
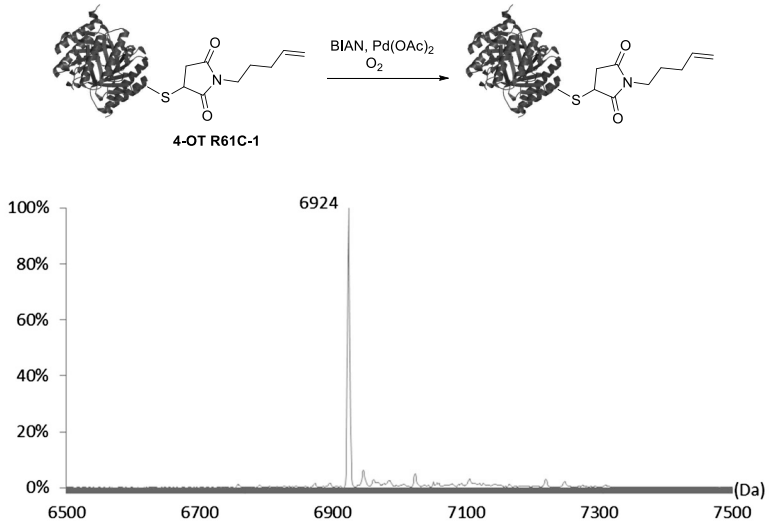
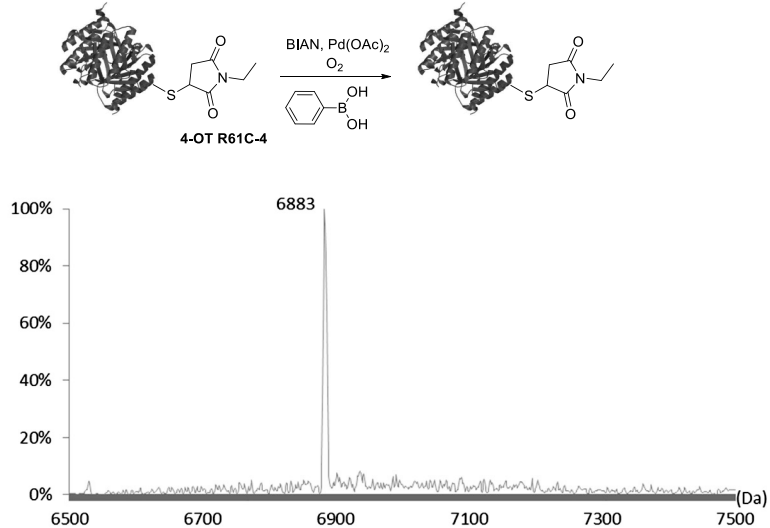
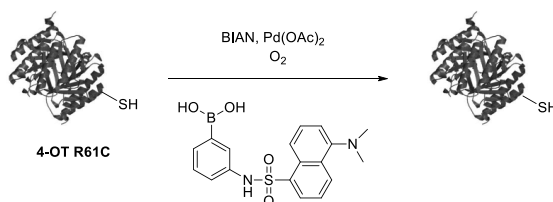


Figure S16. ESI-MS spectrum of control reaction 1. Mass expected 6923, mass found 6923.

Control reaction 2; oxidative Heck in absence of boronic acid**Figure S17.** ESI-MS spectrum of control reaction 2. Mass expected 6923, mass found 6924.**Control reaction 3; oxidative Heck in absence of alkene****Figure S18.** ESI-MS spectrum of control reaction 3. Mass expected 6883, mass found 6883.

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



The reduction of 4-OT R61C dimer was performed as described before. The protein was subjected to usual oxidative Heck reaction conditions. After chelation, 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 μ L). LC-MS analysis showed a protein mass corresponding to the mass of the unreacted 4-OT R61C.

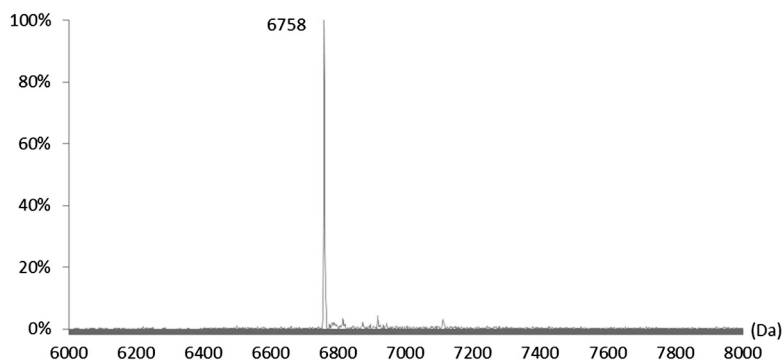


Figure S19. ESI-MS spectrum of control reaction 4. Mass expected 6757, mass found 6758.

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 and the coupling with the fluorescent 3-(dansylamino)phenylboronic acid in presence and absence of the protein-bound alkene 4-OT R61C-1 were performed as described before. For fluorescence imaging, 9 μ L of each reaction mixture were used.

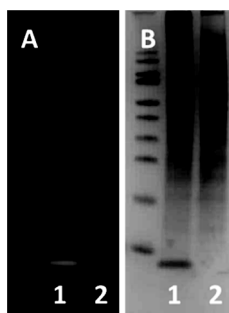


Figure S20. A) Fluorescence imaging on SDS-PAGE of 4-OT R61C-1 labeled in presence of a cell lysate (protein ratio 1:10). B) Coomassie staining of A. 1) The reaction in presence of 4-OT R61C-1 and 2) in absence of 4-OT R61C-1.

Oxidative Heck reaction on small molecules

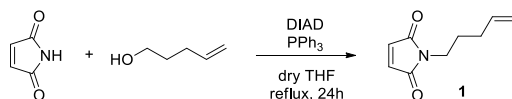
Materials and Methods

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.

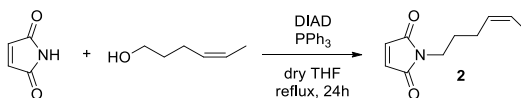
Synthesis of starting materials

Bis(aryl)acenaphthequinonediimine (BIAN)

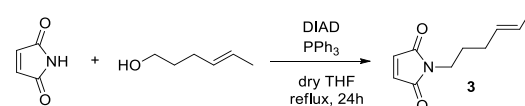
BIAN was synthesized according to literature procedures.⁶ $R_f = 0.5$ (5:1 pentane/ether). ^1H NMR (500 MHz, CDCl_3): $\delta = 7.89$ (d, $J = 8.3$ Hz, 2H), 7.39 (t, $J = 7.7$ Hz, 2H), 7.08 (t, $J = 7.5$ Hz, 2H), 7.16 (d, $J = 7.5$ Hz, 4H), 6.72 (d, $J = 7.2$ Hz, 2H), 2.14 (s, 12H) ppm. ^{13}C NMR (125 MHz, CDCl_3): $\delta = 161.0, 149.4, 140.8, 131.2, 129.7, 129.1, 128.5, 128.4, 125.0, 123.8, 122.7, 17.9$ ppm. LC-MS: m/z 389.29.

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 PPh₃ (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%). R_f = 0.11 (EtOAc/ heptane 1:10). ¹H NMR (500 MHz, CDCl₃): 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. ¹³C NMR (125 MHz, CDCl₃): δ = 179.0, 136.2, 133.0, 114.2, 35.7, 29.2, 25.9 ppm. GC-MS: m/z 165.0.

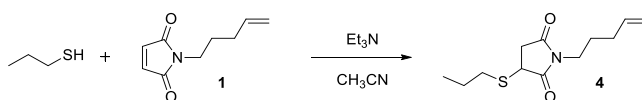
(Z)-1-(hex-4-en-1-yl)-1H-pyrrole-2,5-dione (2)

In a 10 mL round bottom flask equipped with a magnetic stirring bar, the maleimide (97 mg, 1.0 mmol) and PPh₃ (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%). R_f = 0.5 (EtOAc/ heptane 1:1). ¹H NMR (500 MHz, CDCl₃): 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. ¹³C NMR (125 MHz, CDCl₃): δ = 170.7, 134.0, 128.9, 124.8, 37.5, 28.2, 24.1, 12.7 ppm. GC-MS: m/z 179.1.

(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%). $R_f = 0.5$ (EtOAc/ heptane 1:1). $^1\text{H NMR}$ (500 MHz, CDCl_3): $\delta = 6.52$ (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): $\delta = 170.3, 133.7, 129.4, 125.2, 36.9, 29.3, 27.7, 17.4$ ppm. GC-MS: m/z 179.1.

1-(Pent-4-en-1-yl)-3-(propylthio)pyrrolidine-2,5-dione (4) (Michael addition)



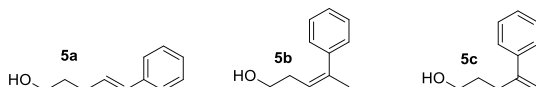
In a 10 mL round bottom flask equipped with a magnetic stirring bar were added sequentially a solution of 1-(pent-4-en-1-yl)-1H-pyrrole-2,5-dione (0.17 g, 1.0 mmol) in 2.0 mL CH_3CN , 1-propanethiol (90 μL , 1.0 mmol) and triethylamine (60 μL , 0.40 mmol) and the mixture was left stirring at room temperature for 2h. The solvent was then evaporated and the residue was dissolved in EtOAc (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 as a yellow oil (0.17 g, 69%). $R_f = 0.11$ (EtOAc/ heptane 1:10). $^1\text{H NMR}$ (500 MHz, CDCl_3): $\delta = 5.79$ (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): $\delta = 176.0, 174.1, 136.3, 114.4, 37.5, 37.1, 34.6, 32.2, 29.3, 25.0, 20.8, 11.7$ ppm. GC-MS: m/z 241.1.

General procedure for the oxidative Heck reaction

A procedure reported previously by Minnaard *et al.* was followed 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 EtOAc (5.0 mL) and washed with H_2O (5x20 mL). The organic phase

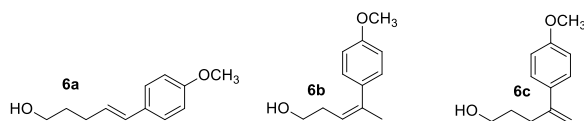
was dried over MgSO_4 , filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography. The ratio of the isomers formed was estimated from the integration of the olefinic peaks of each isomeric product that could be distinguished in the ^1H NMR spectrum.

(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). 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, 109 mg, 67%). Ratio of the isomers: **5a** : **5b** : **5c** 4.5 : 1.1 : 1. 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, 32.4, 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.

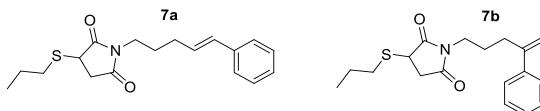
(E)-5-(4-methoxyphenyl)pent-4-en-1-ol (6a), 4-(4-methoxyphenyl)pent-3-en-1-ol (6b), 4-(4-methoxyphenyl)pent-4-en-1-ol (6c)



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). 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, 125 mg, 64%). Ratio of the isomers: **6a** : **6b** : **6c** 2.6 : 2 : 1. R_f = 0.10 (EtOAc/ heptane 1:5). **6a**: ^1H 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, 2H), 2.30 (m, 2H), 1.74 (m, 2H) ppm. ^{13}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**: ^1H 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}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**: ^1H 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}C NMR (125 MHz, CDCl_3): $\delta = 158.7, 140.8, 129.8, 127.5, 114.3, 113.8, 62.5, 55.3, 29.6, 25.6$ ppm. GC-MS: m/z 192.

(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 **4** (0.24 g, 1.0 mmol). 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 conditions 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%). $R_f = 0.11$ (EtOAc/ heptane 1:10). **7a**: ^1H NMR (500 MHz, CDCl_3): $\delta = 7.12\text{--}7.32$ (m, 5H), 6.32 (d, $J = 15.8$ Hz, 1H), 6.13 (m, 1H), 3.59 (dd, $J = 9.0$ Hz, $J = 3.7$ Hz, 1H), 3.51 (t, $J = 7.4$ Hz, 2H), 3.00 (dd, $J = 18.7$ Hz, $J = 9.0$ Hz, 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}C NMR (125 MHz, CDCl_3): $\delta = 176.8, 175.0, 137.6, 130.9, 129.2, 128.6, 127.2, 126.1, 39.1, 28.8, 36.3, 33.9, 30.4, 27.1, 22.5, 13.5$ ppm. **7b**: ^1H NMR (500 MHz, CDCl_3): $\delta = 7.12\text{--}7.32$ (m, 5H), 5.23 (s, 1H), 5.02 (s, 1H), 3.59 (dd, $J = 9.0$ Hz, $J = 3.7$ Hz, 1H), 3.00 (dd, $J = 18.7$ Hz, $J = 9.0$ 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}C NMR (125 MHz, CDCl_3): $\delta = 176.8, 175.0, 147.3, 141.0, 128.5, 127.6, 126.2, 113.0, 39.1, 38.8, 38.7, 32.6, 29.8, 26.3, 22.5, 13.5$ 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.

Supplementary references

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