Combining lipid-mimicking-enabled transition metal and enzyme mediated catalysis at the cell surface of *E. coli*

Tristan Wegner[§[a]], Alexander Dombovski[§[b]], Katrin Gesing[[b]], Alexander Köhrer[[c]], Matthias Elinkmann[[c]], Uwe Karst[[c]], Frank Glorius[*[a]], Joachim Jose[*[b]]

[a] University of Münster, Institute of Organic Chemistry, 48149 Münster, Germany.

[b] University of Münster, Institute of Pharmaceutical and Medical Chemistry, 48149 Münster, Germany.

[c] University of Münster, Institute of Inorganic and Analytical Chemistry, 48149 Münster, Germany.

§These authors contributed equally.

*Corresponding authors: Email: joachim.jose@uni-muenster.de (J. J.); glorius@uni-muenster.de (F. G.)

CONTENT

SUPPORTING METHODS

I. Synthesis of imidazolium derivatives and caged chromophores

1.1 General information

All reactions were performed under argon. Reaction temperatures are referred to the ones of the heating/cooling media (heating block, cryogenic bath). Reagents were purchased from Sigma-Aldrich, Merck, ACROS Organics, Alfa Aesar, Fluorochem, Combiblocks, TCI and used without further purification. Reactions were stirred using PTFE-coated magnetic stirring bars at ~ 1000 rpm. Low boiling solvents (<110 °C) were removed by rotary evaporation under reduced pressure, heating the solution with a water bath at 50 °C. High boiling solvents (>110 °C) were removed in vacuo (<1 mbar) at room temperature or under mild heating (<60 °C). Identity and purity of synthesized compounds was verified by means of ^{1}H NMR, ^{13}C NMR or ESI-MS (accurate mass determination). Literature known compounds were compared with the available analytical data to confirm their identity.

Thin layer chromatography (TLC) was performed using Merck silica gel 60 F254 aluminum plates and visualization was accomplished with UV light (254 nm) and/or staining with basic $KMnO_4$ solution (4 g of $KMnO_4$, 10 g K_2CO_3, 1 g NaOH in 200 ml of distilled water). GC samples for reaction control were filtered over a short plug of silica (length: 2-3 cm, diameter: 4 mm) eluting with EtOAc before analysis, if not stated otherwise. GC-MS spectra were recorded on an Agilent Technologies 7890A GC-system (Agilent 5975C VL MSD or an Agilent 5975 MSD) or Agilent Intuvo 9000 (Agilent 5977B MSD), and a HP-5MS column (0.25 mm · 30 m, film: 0.25 µm) and analyzed using MSD ChemStation E.02.02.1431. ^{1}H and ^{13}C spectra were recorded at room temperature on a Bruker Avance 400 (^{1}H: 400.13 MHz; ^{13}C: 100.62 MHz), Avance Neo 400 (^{1}H: 400.23 MHz; ^{13}C: 100.65 MHz), Varian 500 MHz INOVA (^{1}H: 499.83 MHz; ^{13}C: 125.70 MHz) or Varian Unity plus 600 (^{1}H: 599.31 MHz; ^{13}C: 150.71 MHz) in deuterated solvent (> 99.5 Deuteration) purchased from Sigma-Aldrich ($CDCl_3$). Chemical shifts (δ) for ^{1}H and ^{13}C NMR spectra are given in parts per million (ppm) relative to tetramethylsilane (TMS) using the residual solvent signals as references for ^{1}H and ^{13}C NMR spectra ($CDCl_3$: δ_H= 7.26 ppm, δ_C= 77.16 ppm). NMR signals multiplicities that can be analyzed as first order multiplets are reported using the following abbreviations (or combination thereof): s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad signal. All spectra were processed using MestReNova 14 using standard phase and baseline correction automations. ESI accurate mass spectra were recorded on a TQ Orbitap LTQ XL (Thermo-Fisher Scientific, Bremen) with loop injection or an Orbitrap Velos Pro (Thermo-Fisher Scientific, Bremen) with nanospray injection.

Unless otherwise noted, compounds were purified by flash column chromatography using ACROS Organics silica (0.035-0.070 mm, 60 Å) and the specified solvent system under 0.3-0.5 bar argon overpressure. Pentane, dichloromethane and ethyl acetate used for chromatography were purchased of technical grade and preliminary purified by atmospheric pressure distillation. Dry (H_2O content < 50 ppm) reaction solvents were used to perform reactions. Acetone (ACROS ExtraDry solvents with ACROSeal® cap) was purchased from ACROS Organics, stored under 3 or 4Å activated molecular sieves and collected under positive argon pressure. Tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were purchased from ACROS Organics, Sigma-Aldrich and Fischer Scientific (HPLC

grade) and purified using a custom SPS with activated alumina columns (built by the "Feinmechanische Werkstatt des Organisch-Chemischen Instituts, WWU Münster") and collected under positive argon pressure.

1.2 Synthesis

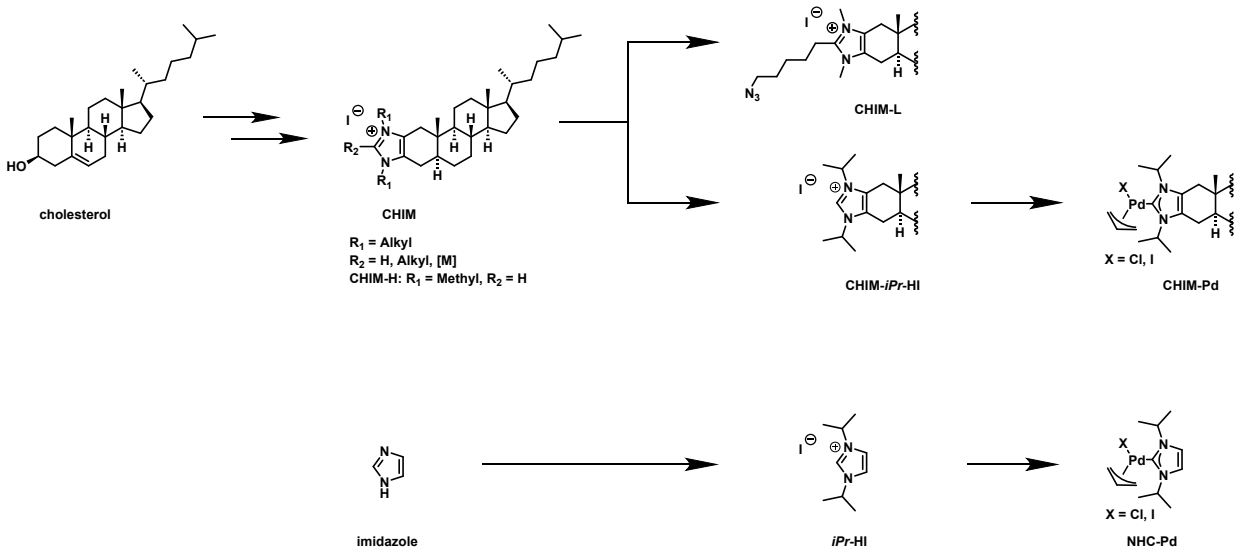


Figure S1. Synthetic routes to the herein investigated imidazolium salt CHIM-L and the palladium complexes CHIM-Pd and NHC-Pd.

CHIM-L and **CHIM-H** were synthesized as previously reported by Rakers *et al*.[1] **CHIM-*iPr*-HI** was synthesized as previously reported by Wegner *et al.*[2]

***iPr*-HI**

Imidazole (335 mg, 4.99 mmol, 1 equiv) and K_2CO_3 (830 mg, 6.01 mmol, 1.2 equiv) were added to an oven-dried Schlenk tube and THF (45 mL) was added. 2-Isopropyl iodide (2.5 mL, 25 mmol, 5 equiv) was added and the reaction mixture was stirred at 85 °C overnight. The solvent was removed under reduced pressure and the crude product was subjected to flash column chromatography (DCM/MeOH = 100/0 → 90/10) to yield ***iPr*-HI** as a white solid (630 mg, 2.25 mmol, 45%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 10.14 (t, *J* = 1.7 Hz, 1H), 7.52 (t, *J* = 1.7 Hz, 2H), 4.97–4.84 (m, 2H), 1.60 (d, *J* = 6.8 Hz, 12H).

^{13}C{^{1}H}-NMR (101 MHz, $CDCl_3$): δ (ppm) = 134.3, 120.3, 53.4, 23.3.

ESI-MS: *m/z* calculated $[C_9H_{17}N_2]^+$: 153.13863, found: 153.13826.

NHC-Pd

***iPr*-HI** (90 mg, 0.32 mmol, 1 equiv), $Allyl_2Pd_2Cl_2$ (94 mg, 0.26 mmol, 0.8 equiv) and KO*t*Bu (43 mg, 0.38 mmol, 1.2 equiv) were added to an oven-dried Schlenk tube. THF (8 mL) was added and the reaction mixture was stirred at room temperature overnight. Then, the solvent was removed under reduced pressure and the crude product

was subjected to flash column chromatography (pentane/EtOAc = 9/1) to yield **NHC-Pd** as an off-white solid (32 mg, 0.075 mmol, 23%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 6.99 (s, 2H), 5.28–5.13 (m, 1H), 5.03 (bs, 1H), 4.75 (bs, 1H), 4.37–4.27 (m, 1H), 3.88–3.78 (m, 1H), 3.06 (d, *J* = 13.5 Hz, 1H), 2.58 (d, *J* = 12.2 Hz, 1H), 1.56–1.25 (m, 12H) .

ESI-MS: *m/z* calculated $[(C_{12}H_{21}N_2Pd)_2I]^+$: 727.05279, found: 727.05254.

CHIM-Pd

CHIM-*iPr*-HI (23 mg, 0.037 mmol, 1 equiv), $Allyl_2Pd_2Cl_2$ (6.8 mg, 0.019 mmol, 0.5 equiv) and KO*t*Bu (5.0 mg, 0.044 mmol, 1.2 equiv) were added to an oven-dried Schlenk tube. THF (800 µL) was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was directly subjected to flash column chromatography (pentane/EtOAc = 9/1) to yield **CHIM-Pd** as a yellow solid (15 mg, 0.019 mmol, 51%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 5.31–5.03 (m, 2H), 4.91–4.69 (m, 1H), 4.27 (d, *J* = 7.6 Hz, 1H), 3.86–3.74 (m, 1H), 3.04 (d, *J* = 13.4 Hz, 1H), 2.73–2.47 (m, 3H), 2.29–2.15 (m, 2H), 2.08–2.00 (m, 1H), 1.87–1.78 (m, 1H), 1.74–1.67 (m, 1H), 1.59–0.98 (m, 46H), 0.91 (d, *J* = 6.4 Hz, 3H), 0.86 (dd, *J* = 6.6, 1.9 Hz, 10H), 0.78–0.75 (m, 3H), 0.68 (s, 3H).

ESI-MS: *m/z* calculated $[C_{37}H_{63}N_2IPdNa]^+$: 791.29768, found: 791.29897; *m/z* calculated $[(C_{37}H_{63}N_2Pd)_2I]^+$: 1411.71250, found: 1411.71143.

Propargylated coumarine

The synthesis was performed based on a literature procedure.[3] 7-Hydroxycoumarine (1.0 g, 6.2 mmol, 1 equiv) and K_2CO_3 (1.0 g, 7.4 mmol, 1.2 equiv) were added to an oven-dried Schlenk tube. Acetone (40 mL) and propargyl bromide (80% in toluene, 0.85 mL, 7.4 mmol, 1.2 equiv) were added and the reaction mixture was stirred at 60 °C overnight. The reaction mixture was filtered hot, the filtrate was concentrated under reduced pressure and pentane was added which resulted in precipitation of an off-white solid. The solid was filtered off and dried under vacuum to yield **propargylated coumarine** (800 mg, 4.00 mmol, 65%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.65 (d, *J* = 9.2 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 1H), 6.96–6.87 (m, 2H), 6.27 (d, *J* = 9.5 Hz, 1H), 4.76 (d, *J* = 2.4 Hz, 2H), 2.58 (t, *J* = 2.4 Hz, 1H).

Dipropargylated fluorescein

The synthesis was performed based on a literature procedure.[4] Fluorescein (1.0 g, 3.0 mmol, 1 equiv) and K_2CO_3 (2.93 g, 21.0 mmol, 7 equiv) were added to an oven-dried Schlenk tube. DMF (8 mL) and propargyl bromide (80% in toluene, 3.40 mL, 30.0 mmol, 10 equiv) were added and the reaction mixture was stirred at 70 °C overnight. The resulting dark brown reaction mixture was diluted with EtOAc (50 mL) and consecutively washed with 1M aqueous HCl and saturated aqueous $NaHCO_3$ solution (3x 60 mL each). The washed organic phase was dried over

$MgSO_4$ and concentrated under reduced pressure. The resulting crude was subjected to flash column chromatography (DCM/MeOH = 50/1) to yield **dipropargylated fluorescein** as an off-white solid (271 mg, 0.664 mmol, 22%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.06–7.98 (m, 1H), 7.71–7.57 (m, 2H), 7.19–7.13 (m, 1H), 6.90–6.85 (m, 1H), 6.75–6.64 (m, 4H), 4.72 (d, *J* = 2.4 Hz, 4H), 2.56 (t, *J* = 2.4 Hz, 2H).

Propargylated 2,6-DMP

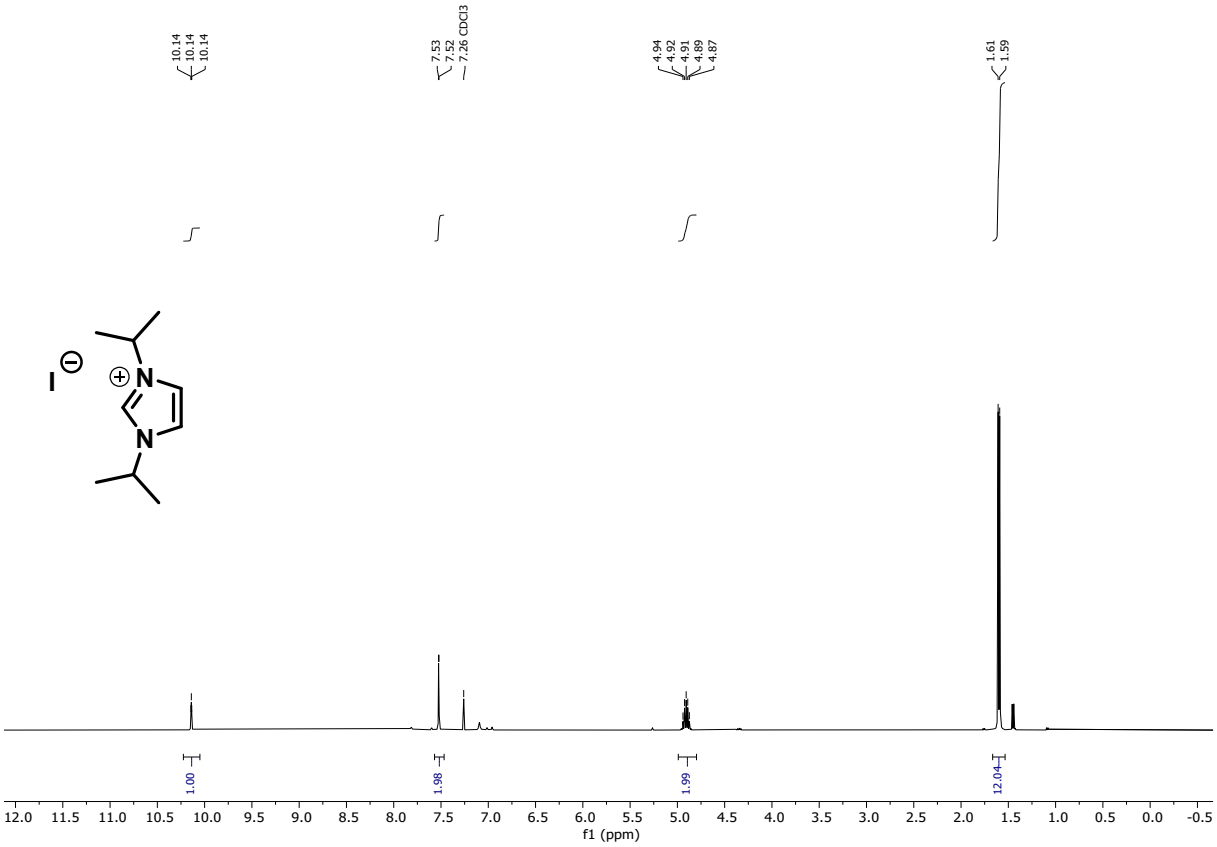
2,6-Dimethoxyphenol (250 mg, 1.62 mmol, 1 equiv) and K_2CO_3 (275 mg, 1.94 mmol, 1.2 equiv) were added to an oven-dried Schlenk tube. Acetone (10 mL) and propargyl bromide (80% in toluene, 225 µL, 1.94 mmol, 1.2 equiv) were added and the reaction mixture was stirred at 60 °C overnight. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The crude was subjected to flash column chromatography (pentane/EtOAc = 7/1) to yield **propargylated 2,6-DMP** as an orange/yellow oil (72 mg, 0.37 mmol, 23%).

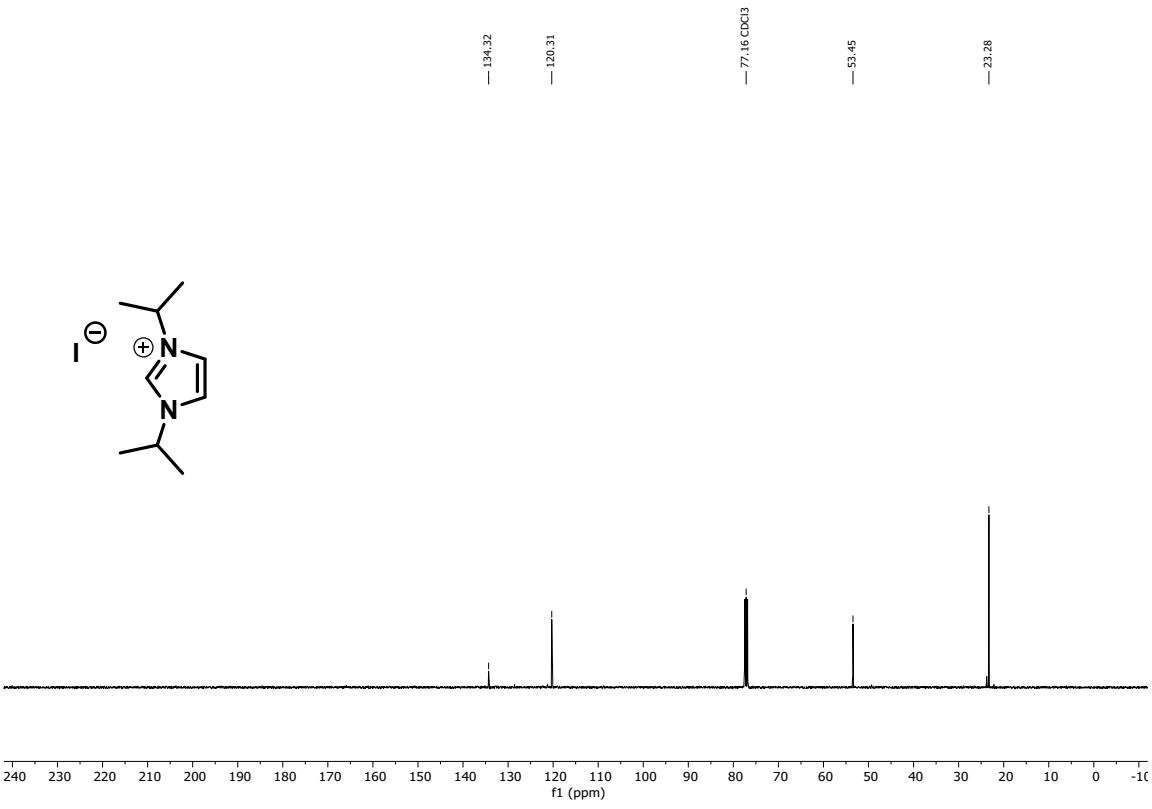
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.02 (t, *J* = 8.4 Hz, 1H), 6.58 (d, *J* = 8.4 Hz, 2H), 4.72 (d, *J* = 2.4 Hz, 2H), 3.85 (s, 6H), 2.43 (t, *J* = 2.4 Hz, 1H).

^{13}C{^{1}H}-NMR (101 MHz, $CDCl_3$): δ (ppm) = 153.9, 135.8, 124.5, 105.4, 79.6, 74.8, 60.0, 56.3.

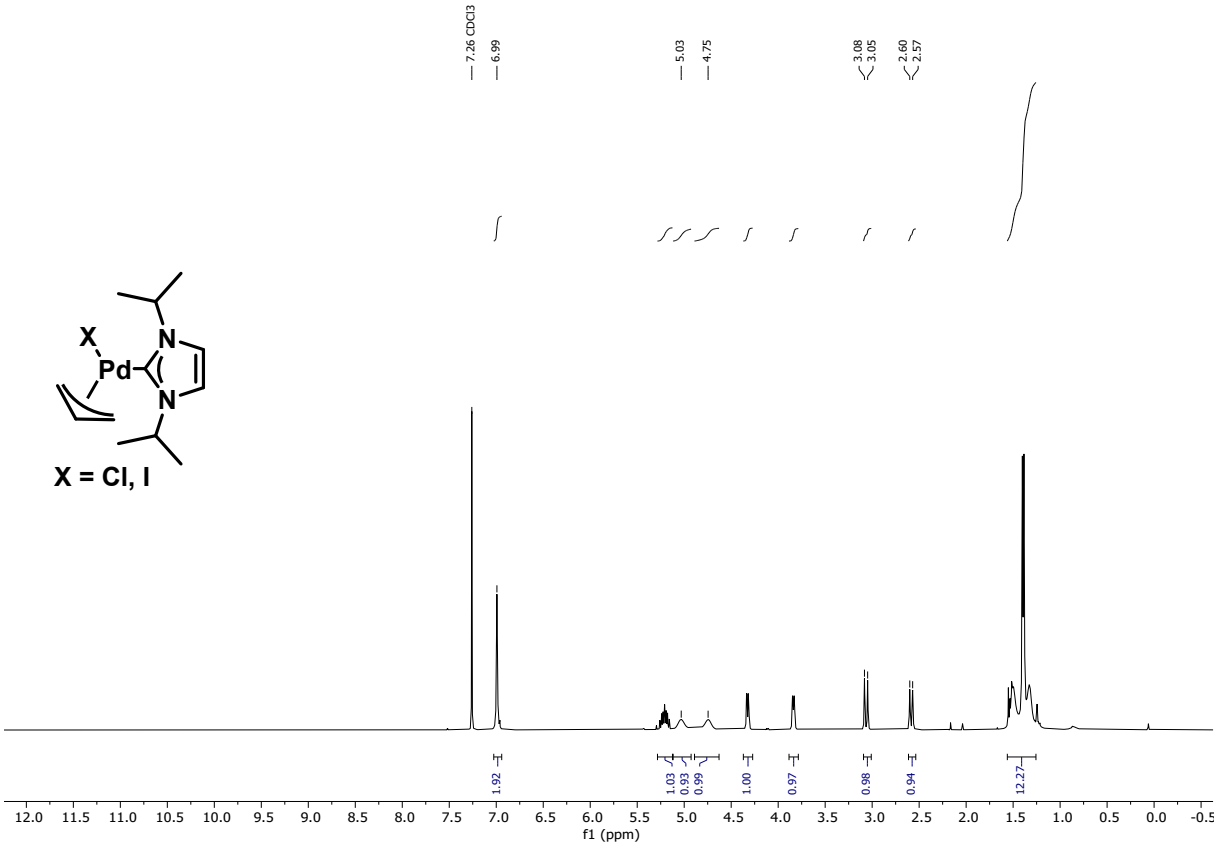
1.3 NMR spectra

***iPr*-HI**

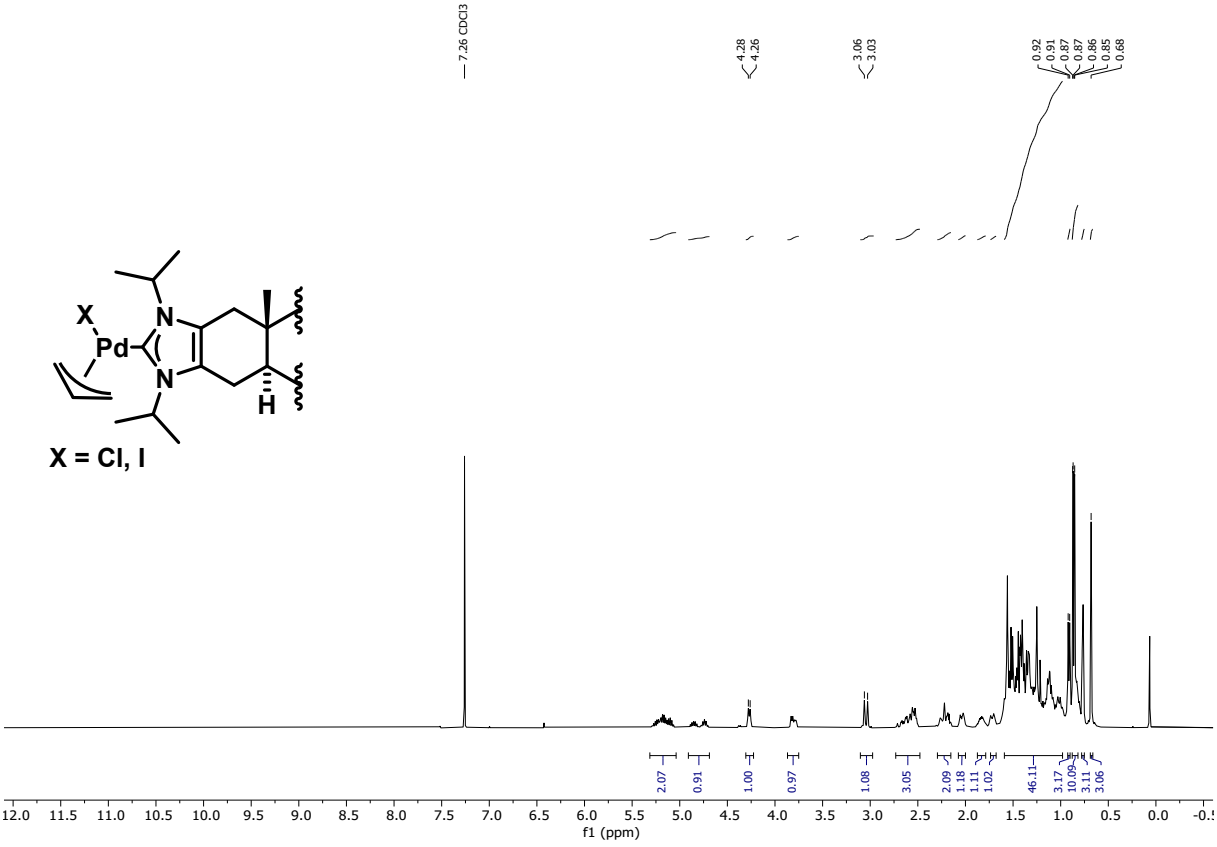




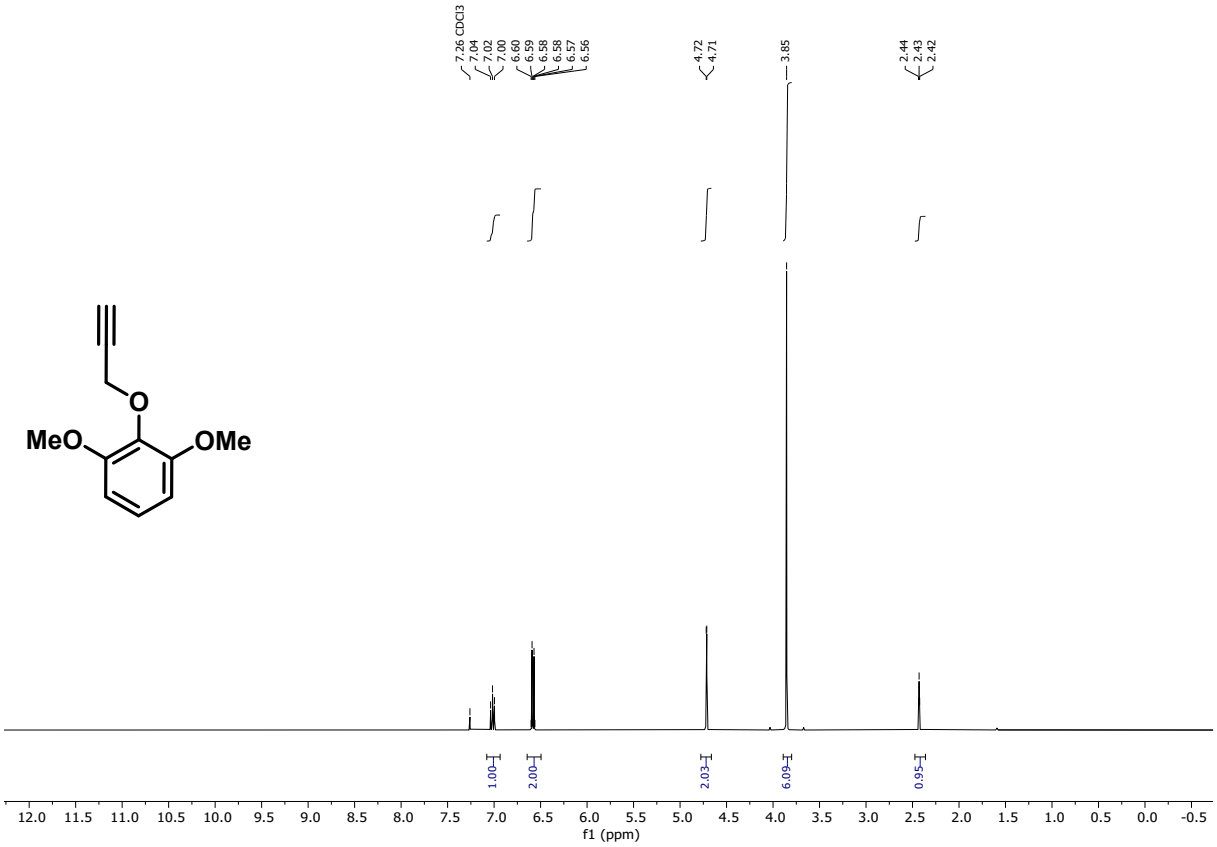
NHC-Pd

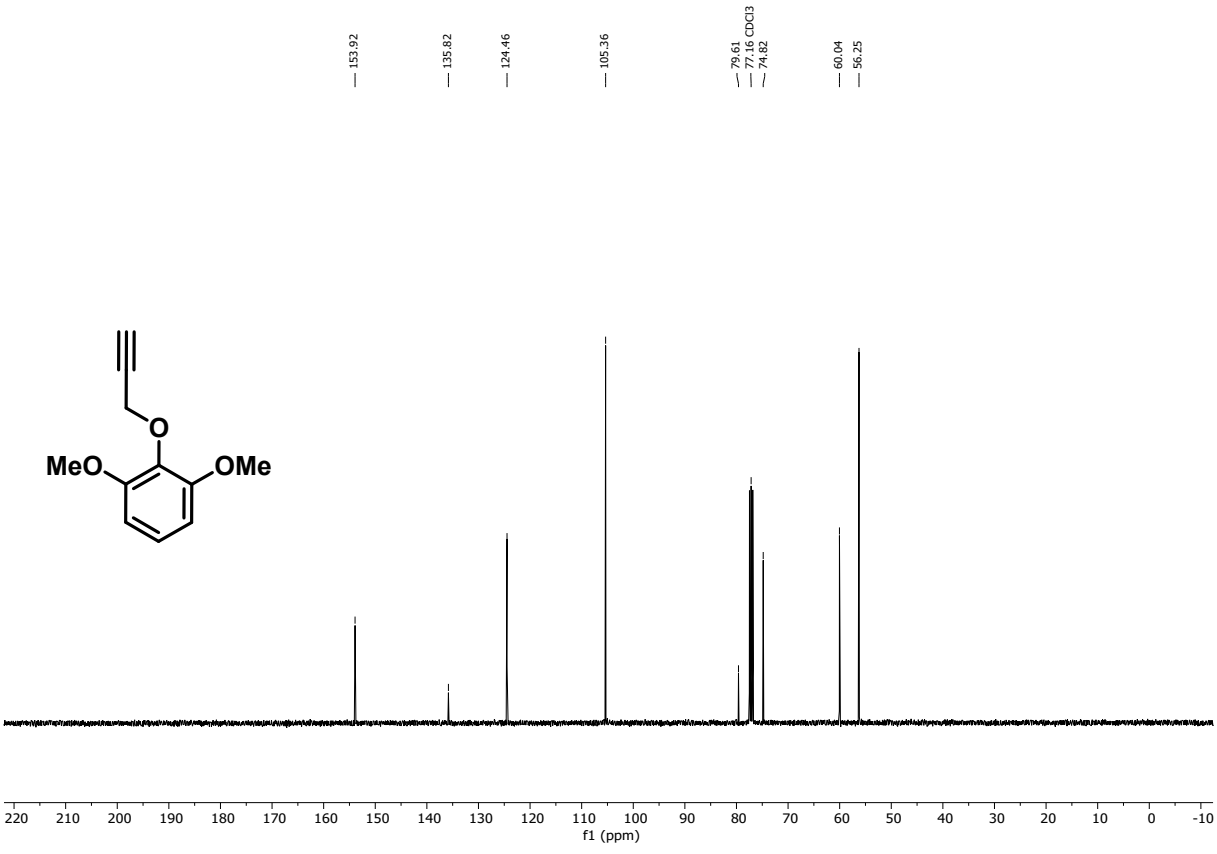


CHIM-Pd

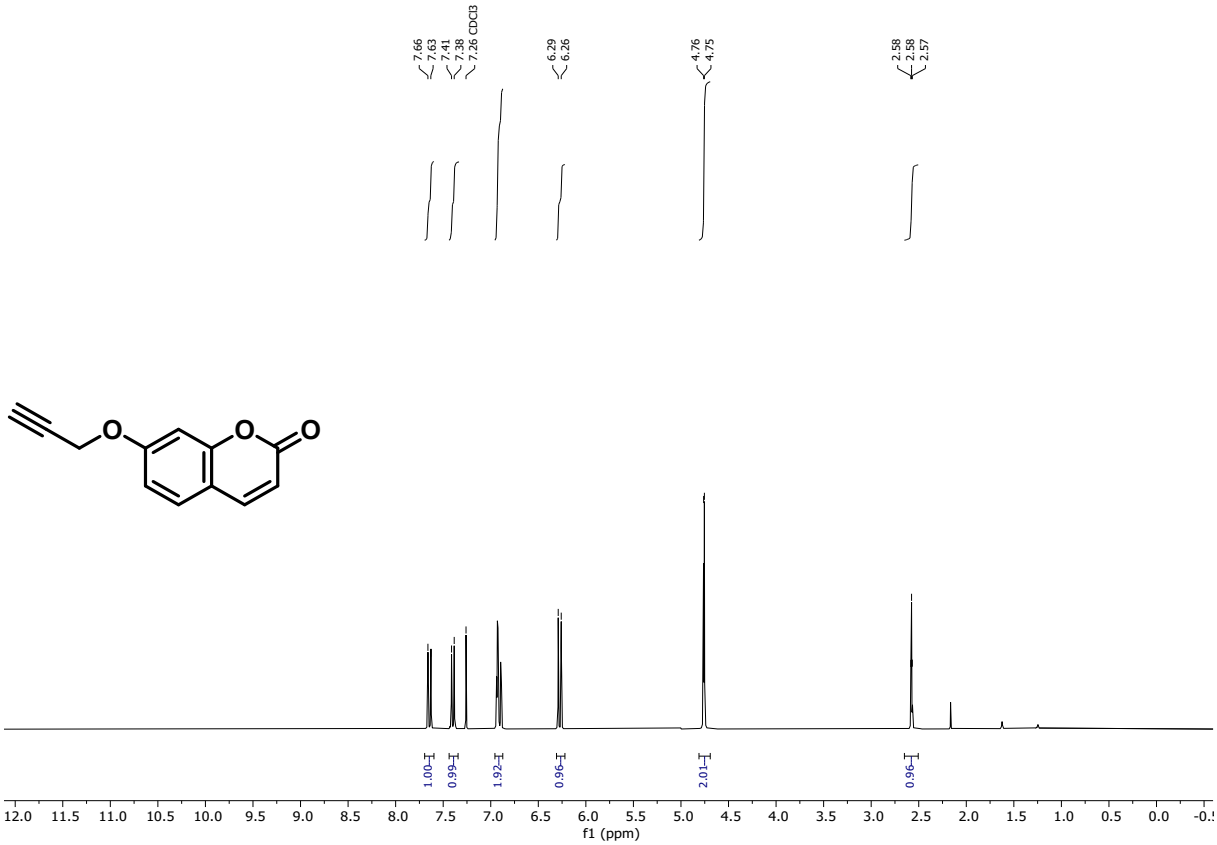


Propargylated 2,6-DMP

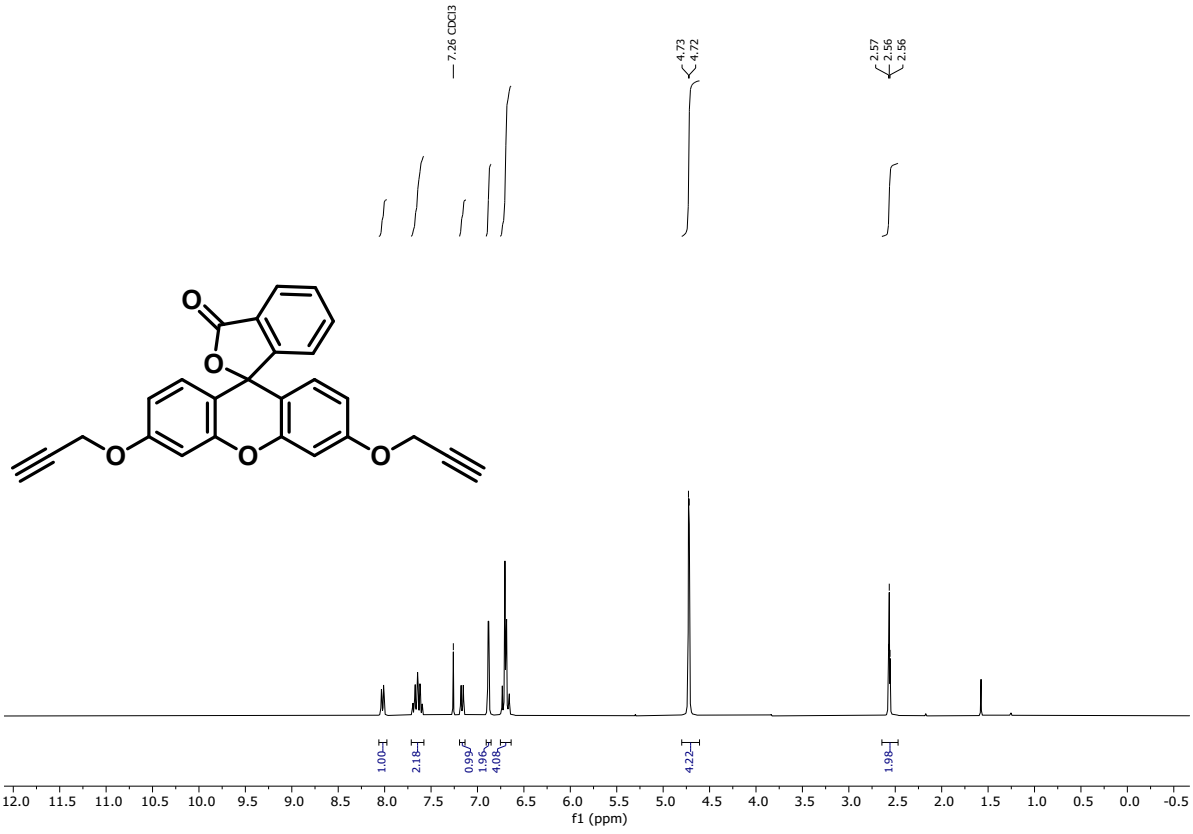




Propargylated coumarine



Dipropargylated fluorescein



II. Biological Procedures

2.1 General information

Kanamycin, L-arabinose, Tris(hydroxymethyl)aminomethane (Tris), tryptone and yeast extract were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). NaCl, KCl, and methanol were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Na_2HPO_4 (dihydrate) was purchased from Merck KGaA (Darmstadt, Germany). KH_2PO_4 (anhydrous) was purchased from AppliChem GmbH (Darmstadt, Germany). DMSO and 2,6-dimethoxyphenol (2-6-DMP) were purchased from VWR International GmbH (Darmstadt, Germany). Dibenzocycloocyne (DBCO)-PEG4-Fluor 545 was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and prepared as a 500 µM stock solution in MeOH/DMSO (1:1). Cholesterol was purchased from Acros Organics (Geel, Belgium) and prepared as 5 mM stock solution in DMSO. CHIMs and NHC-Pd were synthesized as described and prepared as 5 mM stock solutions in DMSO. All Pd-containing DMSO stocks and aqueous solutions were always freshly prepared as soon as they were required and never stored for later use. Fluorogenic substrates pro-umbelliferone and pro-fluorescein were synthesized as described and prepared as 5 mM stock solutions in DMSO. 2,6-DMP was freshly prepared as a 10 mM solution in Tris buffer pH 8.0. Propargylated 2,6-DMP (pro-2,6-DMP) was synthesized as described and prepared as a 10 mM solution in Tris buffer pH 8.0. Kanamycin was prepared as an aqueous stock solution of 50 g/L. Kanamycin stock solution was diluted 1:1000 into the medium if required. L-arabinose was prepared as an aqueous stock solution of 20% (w/v). For induction of protein expression L-arabinose stock solution was diluted 1:100 into the medium. Lysogeny broth (LB) medium (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) was prepared by dissolving the components in ddH_2O and autoclaving. Tris buffer pH 8.0 (0.5 mol/L Tris) was prepared by dissolving the components in ddH_2O and adjusting pH to 8.0 with HCl. Phosphate-buffered saline (PBS) X10 concentrate (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4) was prepared by dissolving the components in ddH_2O, adjusting pH to 7.4 with HCl and sterile filtering through a 0.22 µM pore filter. PBS was prepared by diluting PBS X10 concentrate 1:10 with ddH_2O and sterile filtering through a 0.22 µM pore filter.

2.2 Bacterial strains

E. coli BL21(DE3) (B, F^-, dcm, ompT, lon, hsdS (rB^- mB^-), gal, λ(DE3)) was routinely used for all experiments if not stated otherwise. *E. coli* BL21 *ΔcueO* (B, F^-, dcm, ompT, lon, hsdS (rB^- mB^-), gal, *ΔcueO*) was used for experiments requiring cells with surface displayed CotA laccase.

2.3 Plasmids

For the surface display of CotA laccase *E. coli* BL21 *ΔcueO* was transformed with the plasmid pMATE-CotA by heat shock method. The plasmid encoded the sequence of a fusion protein consisting of (N- to C-terminal) cholera toxin B subunit signal peptide, CotA laccase from *Bacillus coagulans* and EhaA autotransporter translocation domain. The expression of the fusion protein was controlled by the L-arabinose inducible P_{BAD} promoter. Additionally, the plasmid carried a kanamycin resistance cassette as selectable marker and pBBR1 origin of replication derived from *Bordetella bronchiseptica*.

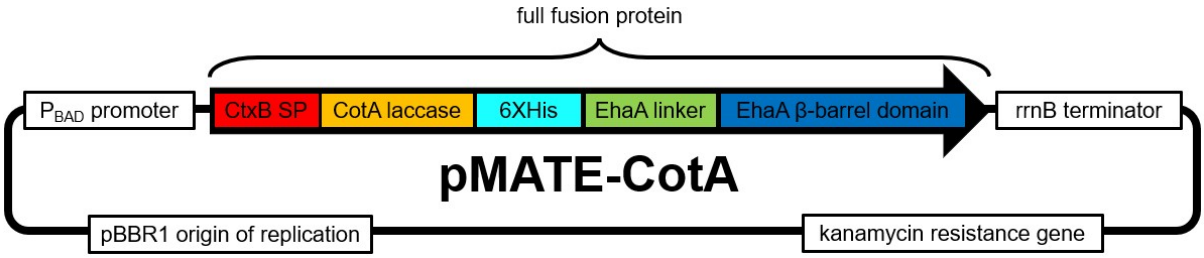


Figure S2: Schematic plasmid map representation of pMATE-CotA that was used for the surface display of CotA laccase on *E. coli.* The full fusion protein consists of cholera toxin B subunit signal peptide (CtxB SP) (red), CotA laccase (orange), 6XHis tag (cyan), EhaA linker (green) and EhaA β-barrel domain (blue). The signal peptide (red) directs the nascent fusion protein into the periplasm and is cleaved off afterwards. The β-barrel domain (blue) is inserted into the outer membrane and the adjacent linker (green) and CotA laccase are translocated from the periplasm through the β-barrel pore to the exterior of the cell. On the cell surface CotA laccase is folding to adopt its functional tertiary structure. Gene expression is under the control of L-arabinose inducible P_{BAD} promoter. Additionaly, the plasmid carries a rrnB terminator region, pBBR1 origin of replication and a kanamycin resistance gene.

2.4 Cultivation

Pre-cultures were prepared by inoculating 5 mL LB medium in culture tubes with a single colony picked from agar dishes. If required, the medium and agar dishes were supplemented with 50 mg/L kanamycin as selection antibiotic. Pre-cultures were routinely grown at 37 °C, 200 rpm for 16 h. Depending on the experimental setup the main cultures were cultivated differently and the conditions are described in detail in the respective experimental setup.

2.5 Fluorescent labeling of E. coli membranes by dye-lipid conjugate

Main cultures were prepared by inoculating 20 mL LB medium with 200 µL of a pre-culture in 200 mL flasks. Cultivation proceeded at 37 °C, 200 rpm, until an OD578 nm of 0.5. The cultures were then pelleted by centrifuging 5 min, 3850 rcf at 4 °C. The supernatant was discarded and the cell pellet washed thrice by repeatedly resuspending in sterile filtered PBS, centrifuging 5 min, 3850 rcf at 4 °C and discarding the supernatant. After the washing steps the cell pellet was resuspended in sterile filtered PBS to an OD578 nm of 0.4. A cell aliquot was prepared by transferring 1 mL of the cell suspension in a reaction tube and pelleting the cells by centrifuging 1 min, 11.000 rcf at room temperature. The supernatant was entirely discarded by pipetting. DBCO-PEG4-Fluor 545 was used as a fluorescent dye to confirm successful strain promoted alkyne-azide cycloaddition (SPAAC) reaction and incorporation of the lipid-dye conjugate into *E. coli* membranes. The SPAAC reaction was performed by the addition of 2 µL of 5 mM CHIM-L stock solution and 20 µL of 500 µM DBCO-PEG4-Fluor 545 stock solution to 78 µL ddH_2O. This resulted in an equimolar ratio of lipid to dye of 100 µM and the reaction was allowed to proceed for 24 h, 1200 rpm, at room temperature in a light protected (brown) 1.5 mL reaction tube. The reaction solution was then supplied with 100 µL PBS X10 concentrate and 800 µL ddH_2O resulting in 1 mL of the final labeling incubation solution (optionally the labeling medium additionally contained 5 % bovine serum albumin). 1 mL of labeling incubation solution was used to label one pelleted cell aliquot by resuspending the pellet and incubation for 90 min, 800 rpm at 37 °C in a light protected (brown) 1.5 mL reaction

tube. After the incubation the cells were pelleted by centrifuging 1 min, 11.000 rcf at room temperature and the supernatant was discarded by pipetting. To remove unspecifically bound dye and lipid the cell pellet was washed thrice by repeatedly resuspending in 100 µL sterile filtered PBS, centrifuging 1 min, 11.000 rcf at room temperature and discarding the supernatant. After washing, the cell pellet was resuspended in 1 mL of PBS. Control samples were prepared by replacing CHIM-L with non-clickable lipids CHOL and CHIM-H.

2.6 Flow cytometry

A cell suspension that was obtained in "Fluorescent labeling of *E. coli* cell membrane by dye-lipid conjugate" was directly analyzed with a FACS Aria III flow cytometer (BD Biosciences, USA). In case the event count exceeded 10.000 s-1 the sample was further diluted with sterile filtered PBS. 50.000 events were analyzed per sample. The sample was excited with a yellow-green laser at 561 nm. Emitted fluorescence intensity was detected with a 582/15 nm bandpass filter and photomultiplier tube detector voltage value of 540.

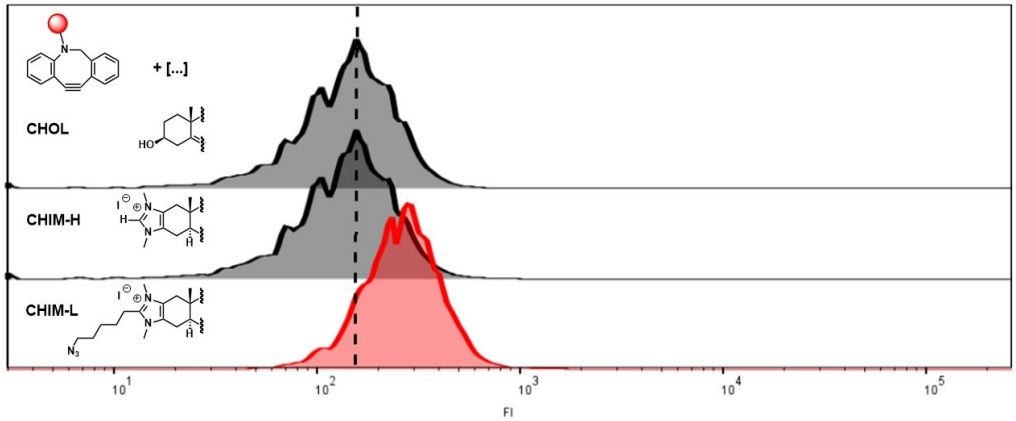
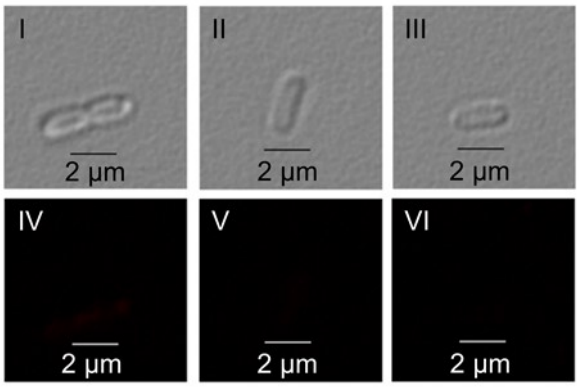


Figure S3. Histogram overlay from flow cytometer analysis of *E. coli* cells after membrane labeling in presence of bovine serum albumin (BSA). The labeling procedure was identical to the procedure as previously described with the exception that 5 % BSA was added during the incubation step with the cells. An identical degree of fluorescence labeling of the cells with the CHIM-L-dye conjugate was observed, as in the experiment without the addition of BSA. In accordance to the previous experiment, a clear differentiation between the clickable lipid CHIM-L and non-clickable control compounds CHIM-H and CHOL was possible. Dotted line approximates median fluorescence intensity of unlabeled samples. FI = fluorescence intensity [a.u.] (561 nm laser, 582/15 nm bandpass filter).

2.7 Fluorescence microscopy

2 µL of a cell suspension that was obtained in "Fluorescent labeling of *E. coli* cell membrane by dye-lipid conjugate" was directly transferred onto a microscope slide and carefully spread by covering with a cover slip without fixation. Keyence BZ-9000 (Keyence Deutschland GmbH, Germany) fluorescence microscope was used with a Texas Red fluorescence imaging filter cube (excitation 562/40 nm, longpass dichroic mirror 593 nm,



emission 624/40 nm), 100X optical magnification objective and oil immersion.

Figure S4. Fluorescence microscopy of *E. coli* cells incubated with CHIM-H that is not capable of forming a clicked conjugate with the DBCO-PEG4-Fluor 545 dye (100X optical magnification, excitation 562/40 nm, emission 624/40 nm). I–VI: Examples of control cells that were incubated with non-conjugated CHIM-H after it was allowed to react with the DBCO-PEG4-Fluor 545 dye. The cells did not show any fluorescence.

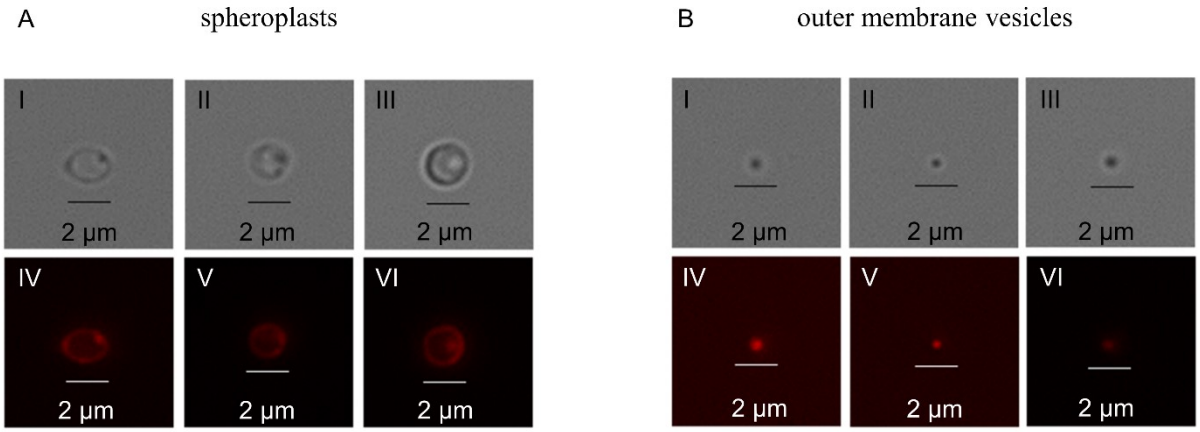


Figure S5. *E. coli* cells were fluorescently labeled with CHIM-L-dye conjugate as described previously and subjected to an osmotic shock/lysozyme treatment to separate the outer membrane from the rest of the cell. This was done by resuspending the cells in 37.5 µL 0.2 M Tris/HCl pH 8.0 buffer and adding (in the following order) 2.5 µL sucrose 1 mol/L, 2.5 µL EDTA 10 mM, 2.5 µL lysozyme 10 mg/mL and 80 µL water. The suspension was carefully mixed by titling, incubated for 1 min at room temperature, 2 µL transferred onto a microscope slide and covered with a cover slip without fixation. The presence of roundish spheroplasts (A) with an approximate diameter of 2 µm indicated successful stripping of the outer membrane from

the cells (left). Other than the weakly fluorescent spheroplasts, the sample contained small and highly fluorescent particles, presumably outer membrane vesicles (B) released by osmotic shock/lysozyme treatment.

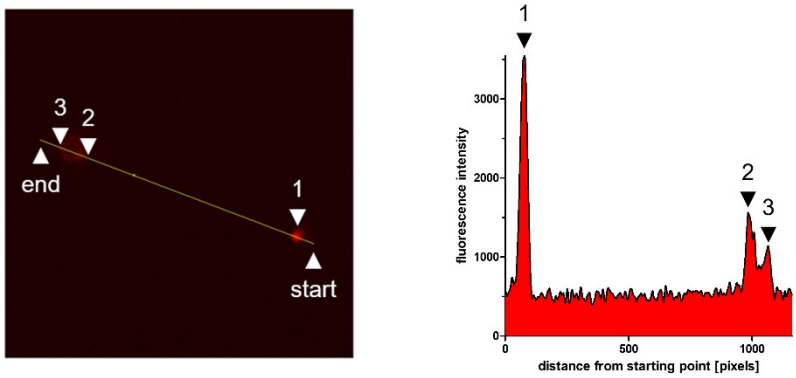


Figure S6. Fluorescence intensity line profile of a spheroplast and an outer membrane vesicle after osmotic shock/lysozyme treatment. Comparison of fluorescence intensities of the spheroplast and the outer membrane vesicle indicates that the concentration of the CHIM-L-dye conjugate is higher in the outer membrane vesicle than in the spheroplast.

2.8 Palladization of non-displaying E. coli with CHIM-Pd

Main cultures were prepared in 100 mL flasks by inoculating 10 mL LB medium with 10 µL of a pre-culture. Cultivation proceeded for 16 h at 30 °C, 200 rpm. The cultures were then pelleted by centrifuging 5 min, 3850 rcf at 4 °C. The supernatant was discarded and the cell pellet washed thrice by repeatedly resuspending in Tris buffer pH 8.0, centrifuging 5 min, 3850 rcf at 4 °C and discarding the supernatant. After the washing steps the cell pellet was resuspended in Tris buffer pH 8.0 to an OD578 nm of 1.0. A cell aliquot was prepared by transferring 100 µL of the cell suspension in a 1.5 mL reaction tube and pelleting the cells by centrifuging 2 min, 20.000 rcf at 4 °C. The supernatant was discarded by pipetting. An incubation solution for palladization was prepared by transferring 4 µL of a freshly prepared 5 mM CHIM-Pd stock solution into 196 µL Tris buffer pH 8.0 and gently mixing by pipetting to a final concentration of 100 µM CHIM-Pd (200 µL per cell aliquot). Vortexing was avoided. 200 µL of 100 µM CHIM-Pd in Tris buffer pH 8.0 was used to resuspend a pelleted cell aliquot and incubated for 90 min, 1200 rpm at 37 °C in a 1.5 mL reaction tube. Thereafter, the cells were pelleted by centrifuging 2 min, 20.000 rcf at 4 °C and the supernatant discarded. To remove unspecifically bound compounds the cell pellet was thoroughly washed thrice by repeatedly resuspending in 100 µL Tris buffer pH 8.0 by pipetting, centrifuging 2 min, 20.000 rcf at 4 °C and discarding the supernatant. Control samples were prepared by substituting CHIM-Pd with NHC-Pd (no lipophilic cholestane anchor). For the quantification of Pd per cell CHIM-Pd and NHC-Pd was used at a concentration of 100 µM. For the decaging of fluorogenic substrates CHIM-Pd was used at a concentration of 100 µM and NHC-Pd used at a concentration of 1 mM.

2.9 Microplate reader assays

Tecan Infinite 200Pro (Tecan Deutschland GmbH, Crailsheim, Germany) was used for absorption and fluorescence measurements in 96 well plates.

2.10 Pd-mediated uncaging of fluorogenic compounds

Pro-umbelliferone: Substrate buffer was prepared by transferring 4 µL of 5 mM pro-umbelliferone stock solution and 4 µL DMSO into 192 µL of Tris buffer pH 8.0 to a final concentration of 100 µM pro-umbelliferone (200 µL substrate buffer per cell aliquot). A pelleted cell sample that was obtained in "Palladization of non-displaying *E. coli* with CHIM-Pd" was resuspended in 200 µL substrate buffer and transferred into a well of a 96 well plate. Measurement was performed at 30 °C, excitation at 370/9 nm, emission at 460/20 nm and data points were acquired in cycles of 5 min over a period of 24 h. Pro-fluorescein: Substrate buffer was prepared by transferring 2 µL of a 5 mM pro-fluorescein stock solution and 6 µL DMSO into 192 µL of Tris buffer pH 8.0 to a final concentration of 50 µM pro-fluorescein (200 µL substrate buffer per cell aliquot). A pelleted cell sample that was obtained in "Palladization of non-displaying *E. coli* with CHIM-Pd" was resuspended in 200 µL substrate buffer and transferred into a well of a 96 well plate. Measurement was performed at 30 °C, excitation at 488/9 nm, emission at 520/20 nm and data points were acquired in cycles of 5 min over a period of 24 h.

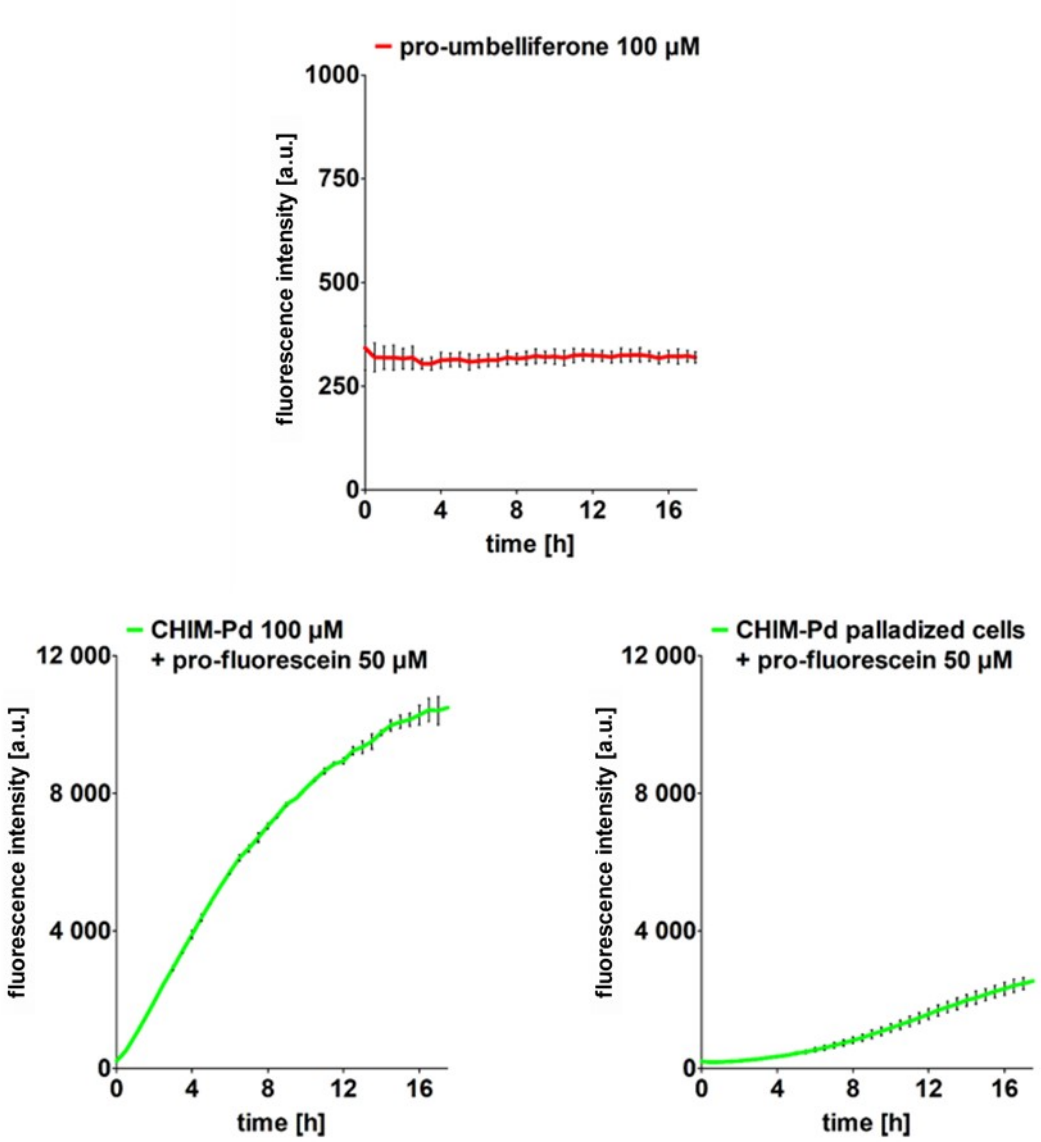


Figure S7. Spectrofluorometric measurement of the Pd-mediated uncaging reaction of fluorogenic compounds pro-umbelliferone (red) and pro-fluorescein (green). Pro-umbelliferone in the presence of cells and under exclusion of Pd remained caged throughout the investigated time frame of 17 h (top). CHIM-Pd was directly added into the cell-free substrate buffer and enabled the Pd-mediated uncaging reaction of pro-fluorescein (bottom left). 100 µM CHIM-Pd was used to palladize *E. coli* cells. Palladized cells were resuspended in substrate buffer to enable the Pd-mediated uncaging reaction by membrane-integrated CHIM-Pd, thus confirming successful palladization of the cells (bottom right).

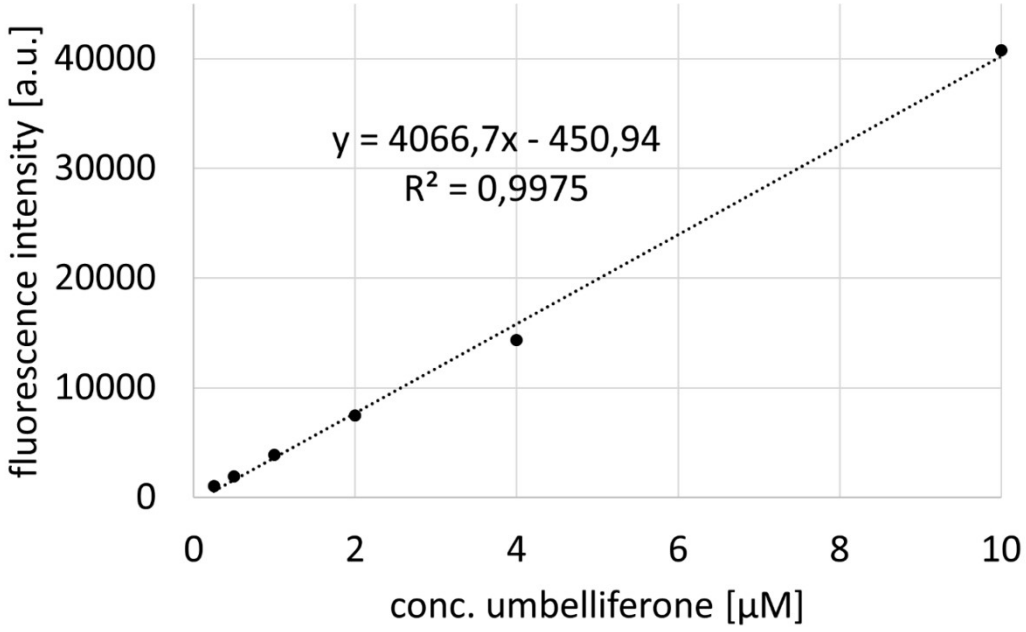


Figure S8. Calibration curve of umbelliferone in Tris buffer pH 8.0. A linear correlation of concentration to fluorescence intensity is observed in the concentration range between 0.25 µM and 10 µM.

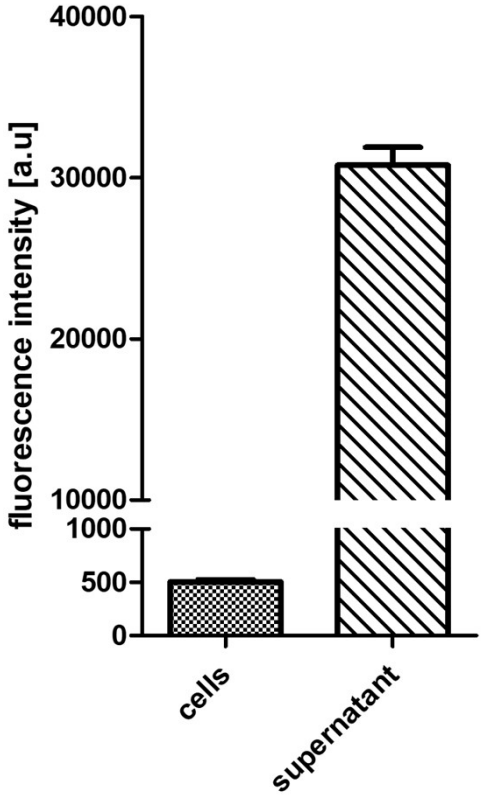


Figure S9. Localization of uncaged umbelliferone after depropargylation with palladized *E. coli* cells. The depropargylation reaction with palladized cells was performed as described for 18 h. Subsequently, the cell suspension was centrifugated (20.000 rcf, 10 min, 4 °C) to separate the cells from the supernatant. The supernatant (200 µL) was removed and transferred into a well plate. The cells were resuspended in fresh substrate buffer (200 µL of 100 µM pro-umbelliferone in 0.5 Tris/HCl pH 8.0) and transferred into a well plate. Notably, the fluorescence intensity of the cells (503 ± 21 a.u.) accounts only for 1.6 % of the fluorescence intensity of the cell-free supernatant (30762 ± 1109 a.u.) (mean and SEM values of triplicates are given).

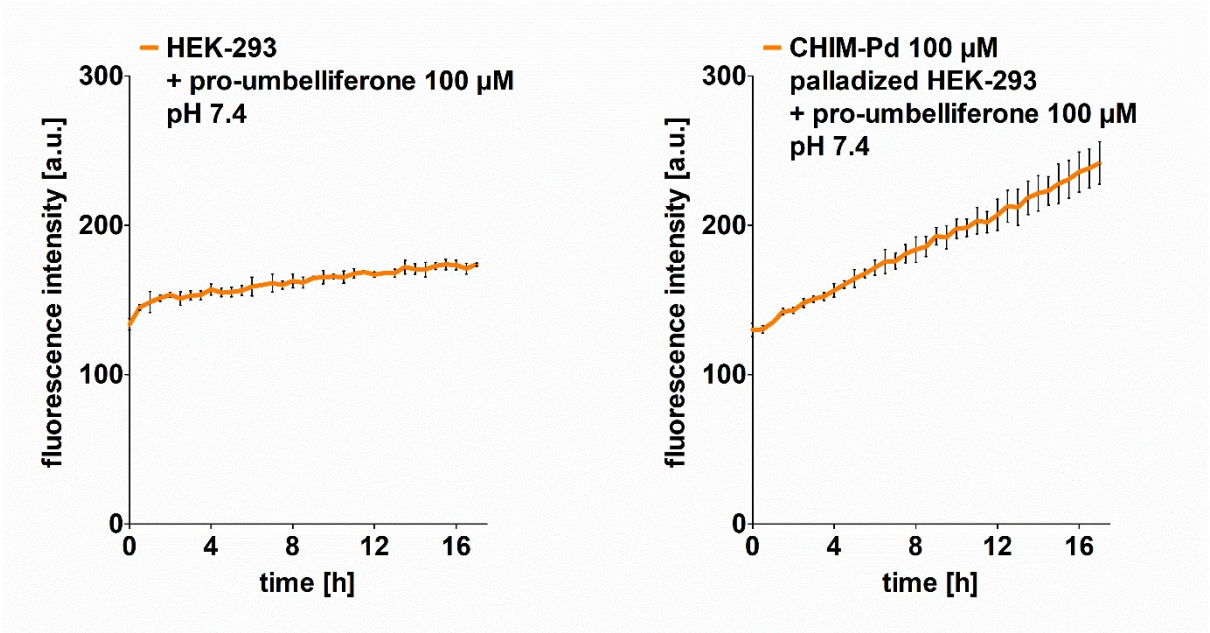


Figure S10. HEK-293 cells were grown in 75 mL culture flasks, detached by trypsinization, suspended in DMEM (+10 % FCS, +1% penicillin/streptomycin) and adjusted to an OD of 1.0. 100 µL were taken per aliquot, centrifuged (500 rcf, 5 min), the supernatant discarded and the cells resuspended in 200 µL CHIM-Pd 100 µM in PBS. After an incubation for 90 min at 37 °C and 600 rpm shaking the cells were washed three times by repeatedly centrifuging (500 rcf, 5 min), discarding the supernatant and resuspending in 200 µL PBS. After the last washing step, the cells were transferred into 200 µL pro-umbelliferone 100 µM in PBS and the formation of umbelliferone was tracked spectrometrically, as described. Palladized HEK-293 cells can be distinguished from non-palladized cells but the increase in fluorescence intensity is considerably small in comparison to palladized *E. coli* cells treated with the same amount of CHIM-Pd.

2.11 Surface display of CotA laccase on E. coli

Main cultures were prepared in 100 mL flasks by supplementing 50 mL LB medium with 50 mg/mL kanamycin, 400 µM $CuCl_2$ and inoculation with 500 µL pre-cultures of *E. coli* BL21 *ΔcueO* transformed with pMATE-CotA. The cultures were incubated at 37 °C, 200 rpm. When an OD_{578nm} of 0.5 was reached the cultures were supplemented with L-arabinose resulting in a final concentration of 0.2% (w/v) and transferred into sterile 50 mL falcons. The falcons were sealed with screw caps and stationary incubated at 37 °C for 21 h. Thereafter the cultures were pelleted by centrifugation 5 min, 3850 rcf at 4 °C and the supernatant discarded. The cell pellet was washed once by resuspending in 20 mL Tris buffer pH 8.0, centrifuging 5 min, 3850 rcf at 4 °C and discarding the supernatant. The washed cell pellet was then resuspended in Tris buffer pH 8.0 and the cell suspension adjusted to an OD_{578nm}

of 1.0. A cell aliquot was prepared by transferring 100 µL of the cell suspension in a 1.5 mL reaction tube and pelleting the cells by centrifuging 2 min, 20.000 rcf at 4 °C and discarding the supernatant by pipetting. Control samples were prepared by omitting $CuCl_2$ supplementation or using *E. coli* BL21 *ΔcueO* without plasmid pMATE-CotA.

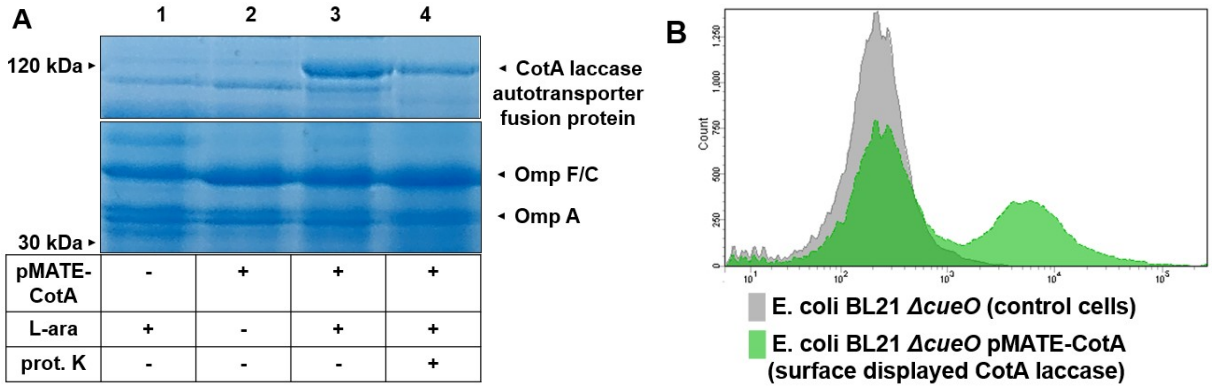


Figure S11. Proof of surface display of CotA laccase by A) proteinase K accessibility assay and B) flow cytometer analysis after antibody labeling. A) Outer membrane protein isolates of different cultures of *E. coli* BL21 *ΔcueO,* analyzed by SDS PAGE. A protein band corresponding to the CotA laccase autotransporter fusion protein (calculated molecular weight of approximately 120 kDa) was only detectable in samples of *E. coli* BL21 *ΔcueO* pMATE-CotA, that have been additionally supplied with inducer L-arabinose (L-ara) (lane 3). A diminishment of the corresponding band was observed, when the cells were additionally treated with proteinase K (prot. K) before outer membrane protein isolation (lane 4). Proteinase K is not membrane permeable, confirming that the digested protein was (partially) located on the exterior of the cell. Outer membrane proteins (Omp) A, C and F served as indicators of membrane integrity and loading control. B) Histogram overlay from flow cytometer analysis of *E. coli* cells after antibody labeling. CotA laccase displaying cells (*E. coli* BL21 *ΔcueO* pMATE-CotA) and control cells (*E. coli* BL21 *ΔcueO*) were incubated 30 min at room temperature with (primary) THE^{TM} His Tag mouse antibody (1:50 in PBS, GenScript, USA), washed, incubated 45 min at room temperature with (secondary) anti-IgG-mouse-DyLight633 antibody (1:50 in PBS, ThermoFisher), washed and subjected to flow cytometer analysis (50.000 events, excitation 633 nm, emission 660/20 nm). *E. coli* BL21 *ΔcueO* pMATE-CotA cells showed a higher mean fluorescence intensity in comparison to control cells due to the labeling of antibody accessible 6XHis tag located within the full fusion protein between CotA laccase and EhaA linker.

2.12 Chromogenic laccase activity assay

Substrate buffer was freshly prepared by dissolving 2,6-DMP or pro-2,6-DMP in Tris buffer pH 8.0 to a final concentration of 10 mM. A cell sample that was obtained in "Surface display of CotA laccase on *E. coli*" was resuspended in 200 µL substrate buffer and transferred into a well of a 96 well plate. Measurement was performed at 30 °C. Coerulignone formation was tracked by absorption at 468/9 nm. Data points were acquired in cycles of 2 min over the course of 1 h.

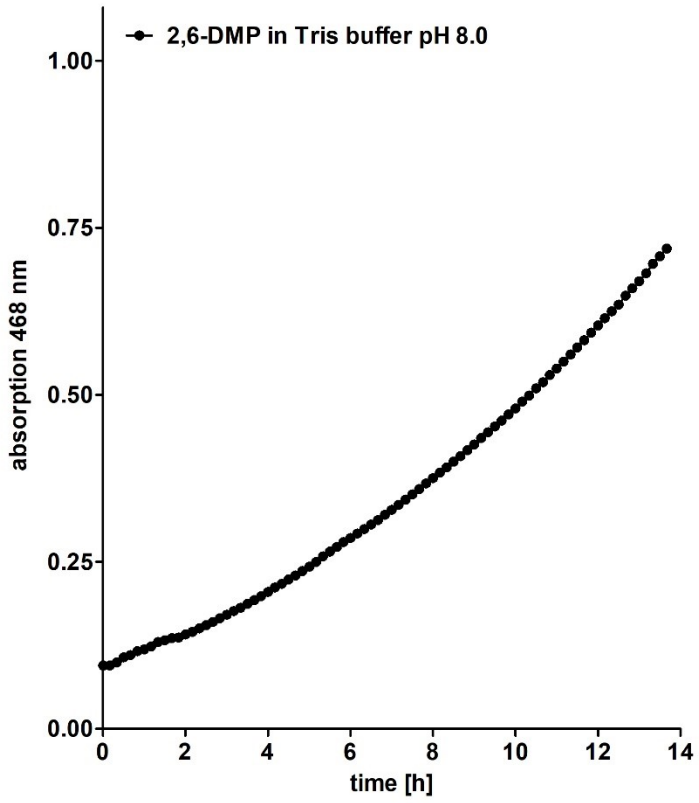


Figure S12. Autoxidation of 2,6-DMP in cell-free Tris buffer (pH 8.0). Due to the alkaline pH an autoxidation of 2,6-DMP to coerulignone is occurring without biocatalytic contribution of the CotA laccase in cell-free conditions.

2.13 Palladization of CotA laccase displaying E. coli

A cell pellet that was obtained in "Surface display of CotA laccase on *E. coli*" was used to additionally load the cells with Pd. An incubation solution for palladization was prepared by transferring 4 µL of a freshly prepared 5 mM CHIM-Pd stock solution into 196 µL Tris buffer pH 8.0 and gently mixing by pipetting to a final concentration of 100 µM CHIM-Pd (200 µL per cell aliquot). Vortexing was avoided. 200 µL of 100 µM CHIM-Pd in Tris buffer pH 8.0 was used to resuspend a pelleted cell aliquot and incubated for 90 min, 1200 rpm at 37 °C in a 1.5 mL reaction tube. Thereafter, the cells were pelleted by centrifugation 2 min, 20.000 rcf at 4 °C and the supernatant discarded. To remove unspecifically bound compounds the cell pellet was thoroughly washed thrice by repeatedly resuspending in 100 µL Tris buffer pH 8.0 by pipetting, centrifuging 2 min, 20.000 rcf at 4 °C and discarding the supernatant.

2.14 Combined Pd- and CotA laccase-mediated cascade reaction of pro-2,6-DMP to coerulignone

Substrate buffer was prepared by dissolving pro-2,6-DMP in Tris buffer pH 8.0 to a final concentration of 10 mM pro-2,6-DMP. Complete dissolution required vigorous shaking and heating to 37 °C. A cell pellet that was obtained in "Palladization of CotA laccase displaying *E. coli*" was resuspended in 200 µL of 10 mM pro-2,6-DMP in Tris buffer pH 8.0 and transferred into a well of a 96 well plate. Before starting the measurement, the cell suspensions in the well plate were allowed to equilibrate for 30 min at 30 °C. Measurement was performed at 30 °C. Data points for the absorption at 468/9 nm were acquired in cycles of 5 min over the course of 24 h with 1 sec orbital shaking at the start of each cycle.

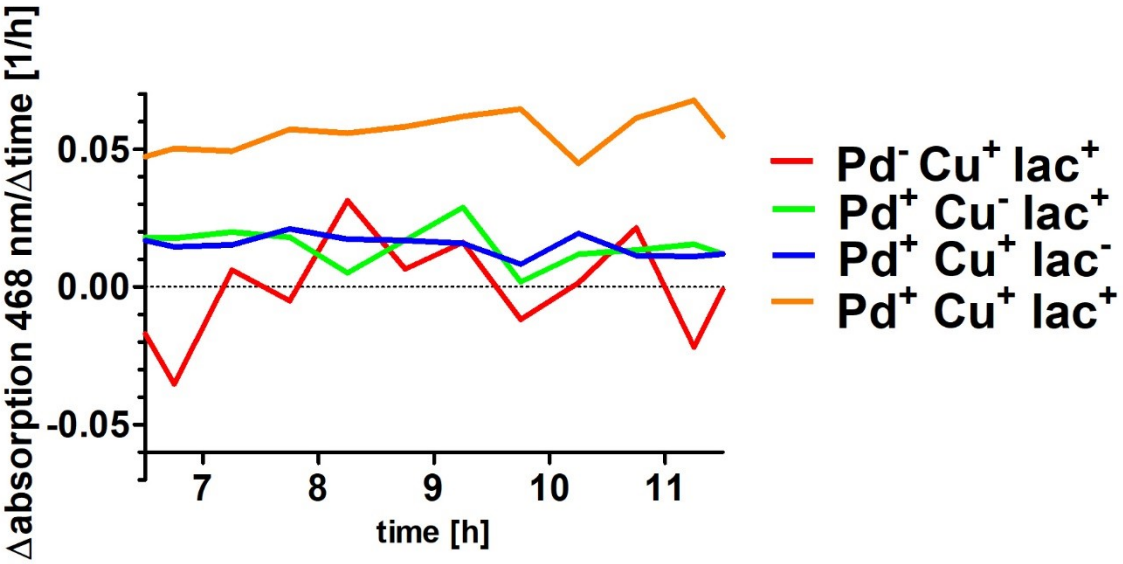


Figure S13. First derivative of the data obtained in the combined laccase oxidation and Pd uncaging assay with pro-2,6-DMP as the substrate. Only the timeframe with almost linear increase of absorption and therefore constant reaction rate is depicted. While the coerulignone formation rate is virtually zero under Pd-free conditions (red), a minimal rate due to autoxidation is observed in palladized samples lacking either the laccase (blue) or copper supplementation (green). In comparison to the control samples, the rate of coerulignone formation is remarkably higher in the samples with functional, copper supplemented laccase and palladization (orange).

2.15 scICP-MS

Table S1. Overview of statistical parameters. Each incubation culture was measured in quatruplicates (n = 4).

Compound	NHC-Pd	SD	RSD	CHIM-Pd	SD	RSD

Number of Events	2104	141	7%	1273	83	7%
Mean [fg/cell]	7	0	3%	824	47	6%
Median [fg/cell]	6	0	3%	276	8	3%

Certified ICP Pd standard (1.000 mg/L) was purchased from Honeywell, Inc. (Fluka; Seelze, Germany). 100 nm Au nanospheres (citrate-coated) were obtained from nanoComposix, Inc. (NanoXact; San Diego, US). For single cell ICP-MS, a single quadrupole ICP-MS (PlasmaQuant Elite MS, Analytik Jena, Jena, Germany) was used, equipped with a specialized combination of a low-flow nebulizer (CytoNeb 50) and a total consumption on-axis spray chamber (CytoSpray; both Elemental Scientific, Inc., Omaha, US), as well as a nickel sampler and skimmer. A pelleted cell sample that was obtained in "Palladization of non-displaying *E. coli* with CHIM-Pd" was resuspended in 100 µM water after the last washing step. Cell suspensions were introduced immediately after 1:10 dilution with H_2O using a syringe pump at a flow rate of 10 µL/min. RF power was set to 1500 W. Sheath and nebulizer gas flows were used at 0.93 L/min and 0.20 L/min, respectively. Using a dwell time of 100 µs, the isotope ^{105}Pd was monitored. For quantification, external calibration was performed using Pd standard solutions in H_2O. The transport efficiency of the system was determined using 100 nm Au nanoparticles via the particle frequency method by Pace *et al*.[5]

Cell events were extracted from time-resolved raw data using in-house software, developed by Matthias Elinkmann (Karst group, University of Münster, Münster, Germany). Herein, a detection threshold is employed based on Poisson statistics, as proposed by Currie[6] and further discussed by Tanner[7] and Mozhayeva and Engelhard[8]. Afterwards, a split correction is performed, combining adjacent dwell times with a signal reading above the threshold to a single event. Signal intensities were converted to mass per-cell values based on calibration data, using OriginPro (ver. 2021b; OriginLab Corporation, Northampton, US) and MS Excel (ver. 2108; Microsoft, Inc., Redmond, US).

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