Letter

Intracellular deprotection reactions mediated by palladium complexes equipped with designed phosphine ligands

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ACS Catal., **Just Accepted Manuscript** • DOI: 10.1021/acscatal.8b01606 • Publication Date (Web): 01 Jun 2018

Intracellular deprotection reactions mediated by palladium complexes equipped with designed phosphine ligands

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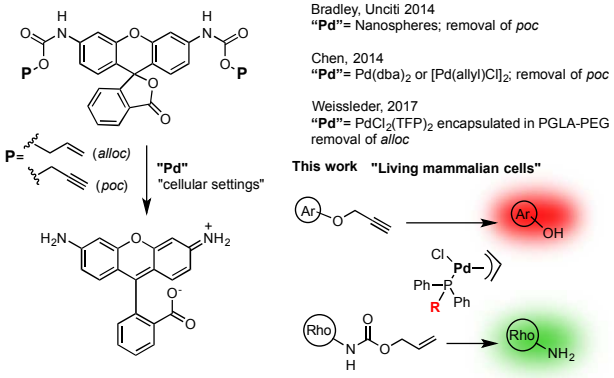
ABSTRACT: Discrete palladium (II) complexes featuring purposely designed phosphine ligands can promote depropargylation and deallylation reactions in cell lysates. These complexes perform better than other palladium sources, which apparently are rapidly deactivated in such hostile complex media. This good balance between reactivity and stability allows the use of these discrete phosphine palladium complexes in living mammalian cells, whereby they can mediate similar transformations. The presence of a phosphine ligand in the coordination sphere of palladium also provides for the introduction of targeting groups, such as hydrophobic phosphonium moieties, which facilitate the accumulation of the complexes in mitochondria.

KEYWORDS: Palladium • discrete complexes • bioorthogonal • intracellular catalysis • mitochondrial confinement.

The functioning of the cell depends on the regulated action of thousands of enzymes, many of which require metal cofactors for their activity. In most of the cases, the metal works either as a Lewis acid or as an electron-transfer centre.[1] In recent years, there have been impressive advances towards the development of artificial metalloenzymes capable of achieving transformations that do not occur in nature,[2–4] with a special emphasis on those promoted by late transition metals.[5,6] However, translating these metalloprotein catalysts to living settings is far from obvious, and has only been proved in isolated cases, and in bacterial periplasms.[7,8]

More success has been attained with discrete, small transition metal complexes which are capable of crossing cell membranes, and thereby able to promote intracellular transformations.[9–13] This is the case for several ruthenium(II) complexes that can mediate the uncaging of allylcarbamate (*alloc*) protected amines in living mammalian cells.[14–17]

Particularly exciting is the possibility of using Pd-catalysts in biological contexts, owing to the well-known transformative power of palladium in organometallic chemistry.[18,19] However, the use of palladium complexes in the complex environment of living cells is seriously compromised by a number of issues including solubility, stability, biorthogonal reactivity or toxicity. Therefore, most applications so far described involve the use Pd nano- or mesostructures, rather than discrete complexes. Hence, Bradley, Unciti-Broceta and coworkers have claimed that small Pd(0) microspheres can induce depropargylation reactions or even Suzuki-Miyaura cross-coupling in biological settings;[20,21] and Chen reported that freshly made palladium nanoparticles can promote similar reactions.[22]



Scheme 1. Previous examples of *in cellulo* uncaging of *poc* and *alloc* probes mediated by Pd catalysts and reactions described in this work.

Discrete palladium complexes have also been used, but only sporadically, and with somewhat differing results. Therefore, Chen and coworkers have shown that commercial Pd precursors like $Pd(dba)_2$ and $Pd_2(allyl)_2Cl_2$ can cleave propargyloxy groups (*poc*) in PBS at 37 °C, and even in HeLa cells.[23] However, a recent study by the Weissleder group reveals that these commercial Pd sources perform poorly when using standard tissue culture (HBSS and MEM) as reaction milieu. Remarkably, these authors were able to obtain good yields in alloc-removing reactions by using *bis*[*tris*(2-furyl)phosphine]palladium(II) dichloride as catalyst; although stability and solubility issues precluded a direct use of the complex in living settings. This problem was solved by encapsulating the metal complex within a biocompatible polymer (Scheme 1).[24]

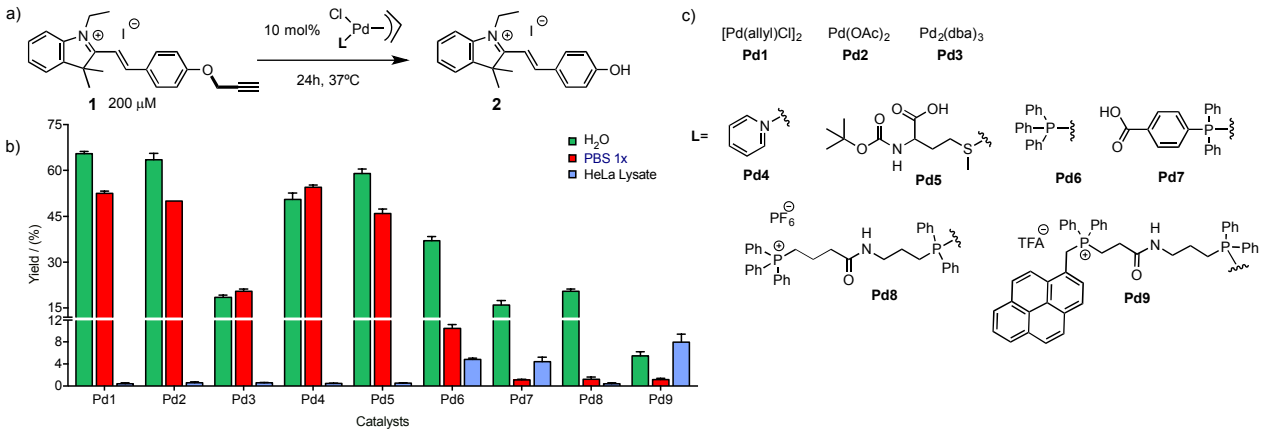


Figure 1. a) Representation of the uncaging reaction of **1**, b) bars diagram representation of the yields obtained for each catalyst under biological compatible conditions in water, PBS buffer and cell lysates. Reaction conditions: **1** (0.2 mmol, 1 eq.), Pd (0.02 mmol, 0.1 eq.) in 1 mL, 37 °C, 24 h (mean ± s.e.m, n=2); c) Pd catalysts used in this work for the study of their catalytic performance in biological compatible media and in living cells. Yields were calculated by RP-HPLC-MS using coumarin as internal standard.

The above results raise doubts on whether discrete palladium complexes can indeed be used as catalysts in complex biological media and in living cells. Such small-sized palladium complexes are very attractive because of their well-defined nature, and the possibility of playing with the ligands to tune solubility, stability and reactivity properties, and eventually favour cell transport and intracellular targeting.

Herein, we demonstrate that discrete palladium complexes with designed phosphine ligands can promote propargyl and alloc cleavage reactions in cell lysates, and even in living mammalian cells. Importantly, appropriate engineering of the ligand allows the mitochondria accumulation of an active palladium catalyst.

Given that previous studies did not establish clearly the in vitro reactivity of different palladium sources to cleave propargyloxy groups, we investigated the catalytic performance of several complexes in PBS and cell lysates.

In addition to standard commercial species $[Pd(allyl)Cl]_2$ (**Pd1**), $Pd(OAc)_2$ (**Pd2**) and $Pd_2(dba)_3$ (**Pd3**),[23–25] we purposely made a series of Pd(II) complexes (**Pd4-9**) exhibiting designed pyridine, thioether or phosphine ligands (Fig. 1 and SI pg. S3-8). In the case of the phosphine containing derivatives, we built complexes with neutral (**Pd6**), anionic (**Pd7)** or positively charged phosphonium tethers (**Pd8** and **Pd9**).

Instead of the typical assay consisting of the cleavage of a propargylic carbamate, we studied the depropargylation of phenol derivative **1.** We chose this probe because of its good aqueous solubility, ease of synthesis and good separation by HPLC-MS. Furthermore, in contrast to standard Rhodamine probes, it presents only one propargylic appendage, therefore simplifying the analysis.

The reactions were carried out using a 200 μM solution of substrate **1** and 10 mol% of the Pd sources in either water, PBS (phosphate buffered saline, pH 7.2) or HeLa cell lysates for 24 hours at 37 °C (Fig. 1 and SI pg. S9-S13). **Pd1** and **Pd2,** and the complexes bearing pyridine and methionine ligands (**Pd4-Pd5**) provided good yields of **2,** in both, water and PBS; however, in cell lysates the conversion was extremely low. Likely, these palladium complexes do not survive to the complex mixture of components present in a cell lysate, and decompose to form inactive Pd species.[26] The palladium complexes with phosphine ligands **Pd6-Pd9** gave lower conversions in water, with yields ranging from *ca.* 40% for **Pd6** to *ca.* 15 % for **Pd7-9**, and performed very poorly in PBS, likely because of the low solubility of the complexes in high ionic strength media.

However, complexes **Pd6**, **Pd7** and **Pd9** were able to promote the depropargylation reaction in cell lysates. Although the yields were low, their performance is better than that of the other palladium sources (**Pd1-Pd5**). Possibly, albeit a phosphine ligand is not the best one from a reactivity point of view, the corresponding palladium complexes are more stable, and can better resist hostile media like that present in cell lysates.

We also investigated the ability of these latter phosphine palladium reagents to remove *alloc* protecting groups, a process that likely involves Pd(0) reagents. The uncaging reaction was evaluated in substrate **3**, in absence or presence of added reducing agents (Fig. 2a and SI pag. S9-13). The reactions were performed using 200 μM solutions of **3** in water, 10 mol% of the palladium complexes, at 37 °C, in absence or presence of 2 mM of NaAsc or 0.4 mM of GSH, and monitored by HPLC-MS at 1, 3, 6 and 24 hours. In the absence of the additives, the yields were very poor, however in presence of NaAsc, we obtained yields of over 50 and 30% using **Pd7** and **Pd9** respectively (24 hours). With GSH the reaction with **Pd7** was more efficient, and hence, we observed 60% yield after 1h, and 94% after 24h. When the experiments were performed in presence of cell lysates, **Pd7** and **Pd9** led to 21% and 33% yields of the product after 24 hours.

With further addition of GSH (0.4 mM) to the lysate the yields were not very different (24% and 22% respectively, Fig. 2).

The above results confirm that phosphine palladium(II) complexes can promote depropargylation and deallylation

reactions in water with good yields. While in cell lysates the efficiency is considerably lower, we were intrigued to know whether the reactions could be achieved inside living cells, as the intracellular environment does not necessarily

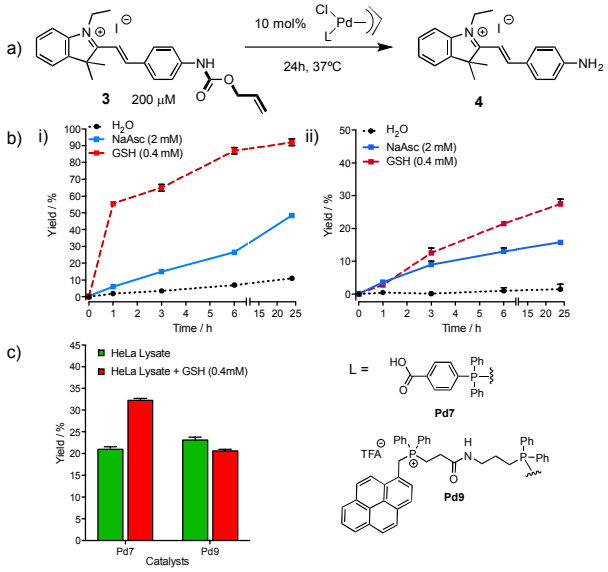


Figure 2. a) Uncaging of **3**, b) plot of the yields of reaction obtained with catalysts i) **Pd7** and ii) **Pd9** at 1, 3, 6 and 24 h in only water, or with additives: NaAsc (2 mM) or GSH (0.4 mM). Reaction conditions: **3** (0.2 mmol, 1 eq.), Pd complex (0.02 mM, 0.1 eq.) in 1 mL, 37 °C, 24 h, and c) Uncaging reaction in cell lysates (green column) and in cell lysate + 0.4 mM of GSH (red column) promoted by catalysts **Pd7** and **Pd9**. Reaction conditions: 10 (0.2 mmol, 1 eq.), Pd (0.02 mM, 0.1 eq.) in 1 mL, 37 °C, 24 h. Inset: **Pd7** and **Pd9**. Yields were calculated by RP-HPLC-MS using coumarine as internal standard (mean ± s.e.m., n=2).

parallel that of a cell lysate. A key element to take into account before moving to intracellular reactions is uptake of the potential catalysts. ICP-MS analysis of cellular extracts (Vero cells) obtained after extensive washing and lytic treatment, revealed a higher amount of palladium after the addition of complexes **Pd6-9** than of species **Pd1-5** (Fig S12 and S13). Importantly, cell viability tests (MTT in Vero cells, 24 h) demonstrated that the phosphine-containing complexes are essentially non toxic below 50 μM, and some of them can be even used at higher concentrations (Figure S14). While substrate **1** was good for *in vitro* studies, the change in fluorescence after the reactions is not strong enough for an appropriate monitoring in cellular settings. Therefore, we changed to the propargylated probe **5**, which after uncaging generates a product **6** which emits at 635 nm when excited in the far UV region (Fig. 3a).[27]

The experiments were carried out by incubation of Vero cells at a final concentration of 50 μM of the probe **5** in culture medium containing 5% fetal bovine serum (FBS-DMEM) for 30 min at 37º C. The milieu was removed, and cells were washed twice with FBS-DMEM to ensure removal of extracellular probe. Pd reagents (50 μM) were then added in fresh media (0.1 % DMSO), and after 30 min, cells were imaged by widefield fluorescence microscopy.

While in the experiments with Pd sources **Pd1-Pd3** and complexes **Pd4-Pd5** we observed almost negligible red emission (Fig. S15), with species **Pd6-Pd8**, equipped with phosphine ligands, we detected strong red emission due to the release of the product **6** inside Vero cells (Fig. 3b). The overlap between the absorption of the pyrene unit in **Pd9** and that of product **6**, precluded the analysis of the reaction with this complex. Overall, these results, are consistent with the *in vitro* studies, and confirm that the phosphine ligands are beneficial for observing "in cell" reactivity, likely because the complexes present a good balance between reactivity and stability in the cellular milieu.

Considering the amount of palladium that was present in cells, as measured by ICP-MS of cell extracts after extensive washing steps and associating the normalized fluorescence intensity with the amount of product, using calibration curves (See SI, page S18), it is possible to estimate whether there is some turnover. Indeed, for **Pd7** we calculated an average TON of 10, while for **Pd8** it is over 5, suggesting that catalytic cycles can operate in cellular settings.

The cellular reactions can also be carried out in mammalian cells other than Vero, such as HeLa (Fig S17). We also checked an inverse protocol in which cells are first treated with the palladium reagents, and the probe is added after 30 min, after the corresponding washing steps. But in these cases, we did not observe fluorescence, likely because the palladium complexes, upon time, are converted to species that are not enough active in the depropargylation process.

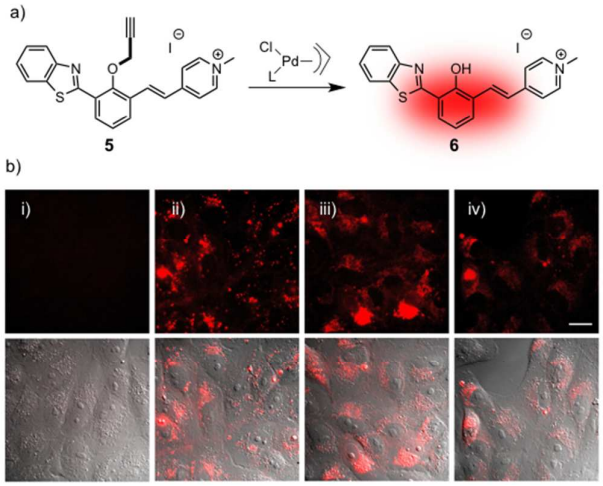


Figure 3. a) Schematic representation of the uncaging reaction of the propargylated probe **5**, and b) imaging of the reaction in Vero cells. First row – red channel; second row – brightfield and red channels merged (i: mock, ii: **Pd6**; iii: **Pd7** and iv: **Pd8**). Reaction conditions: **5** (50 μM) was incubated in DMEM with 5% Fetal Bovine Serum for 30 min. Culture medium was removed and cells were washed twice before addition of the Pd catalyst (50 μM). Cells were then incubated for another 30 min, washed twice and observed under the microscope (Scale bar = 20 μm).

We next explored the removal of alloc groups inside living HeLa cells, in this case using the caged Rhodamine 110 derivative **7**, which is essentially non-fluorescent, but emits green light after removing the alloc protecting groups. Initial experiments were carried out by mixing HeLa cellswith **7** (50 μM in FBS-DMEM) for 30 minutes, twofold washing with FBS-DMEM, and addition of the Pd complexes. After 30

min at 37ºC, cells were visualized under the microscope. As in the case of the depropargylation reaction, the complexes **Pd1-Pd5** raised only a very low intracellular fluorescence, however, with species **Pd6-8** we could observe strong intracellular green fluorescence (Fig. S18).

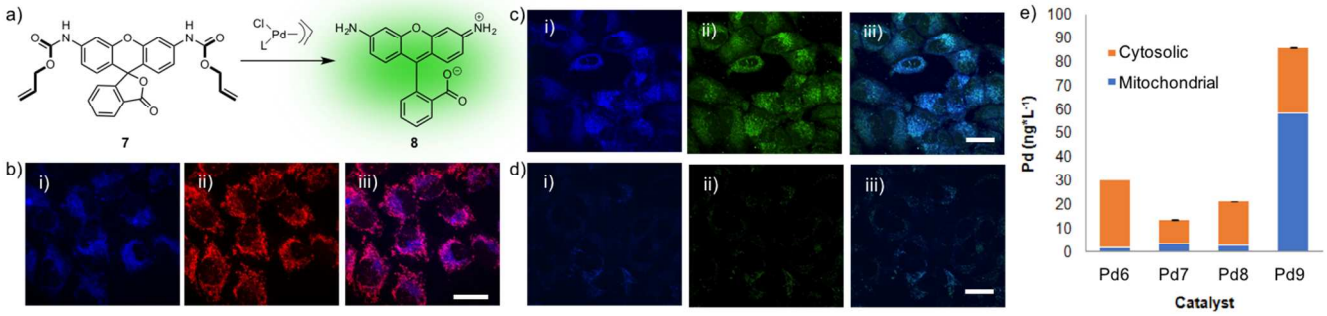


Figure 4. a) Representation of the uncaging reaction. b) Co-localization of **Pd9** with TMRE in HeLa cells, observed 2 h after incubation with the palladium complex: i) blue light emission of **Pd9**; ii) red light emission from the mitochondrial dye TMRE, iii) merged image of i) and ii). c) Reaction promoted by **Pd9**: i) blue light emission of **Pd9**; ii) green light emission from released **8, iii)** merged image of i) and ii). d) Reaction promoted by **Pd7**: i) blue channel; ii) green light emission from released **8**, iii) merged image of i) and ii). e) Graphical representation of the ICP-MS results from mitochondrial isolation of fractioned Vero cells using complexes **Pd6-9** (see supporting information for details). Reaction conditions: Pd complexes (50 μM) were incubated with HeLa cells in DMEM with 5% Fetal Bovine Serum for 2h at 37 °C before substrate **7** (50 μM) was added; the cells were incubated for another 30 min and observed under the microscope (Scale bar = 20 μm).

Inverting the order of addition, first the palladium complexes, and after 30 min, washing and addition of the probe **7**, we detected intracellular fluorescence, but only with complexes **Pd8** (Fig. S19) and **Pd9** (Fig. 4). Similar results were obtained in Vero cells (Fig S20).

In the case of **Pd9**, the presence of a pyrene group in the ligand structure allowed a direct monitoring of the species in living cells. As shown in Figure 4, the blue emission of living cells after addition of this complex shows a very good colocalization with the red color of a mitotracker (Fig. 4b, i-iii). This was further confirmed by ICP-MS analysis of cells lysed under conditions that allowed the isolation of the mitochondria from the rest of cytoplasmic mass. As indicated in Figure 4e, **Pd9** presents a substantial higher mitochondrial accumulation than the other complexes. Likely, the presence of the pyrene and phosphonium groups in the ligand allows the right combination of charge and hydrophobicity to favour a significant mitochondrial accumulation of the complex.

Importantly, in cells treated with **Pd9** we observed an efficient transformation of **7** into **8**, as deduced from the quite strong build-up of green fluorescence, that also accumulates in the mitochondria surroundings (Fig. 4c, i-iii). Other phosphine-containing catalyst, such as **Pd7** or **Pd8** were less active (Fig. 4d, i-III and Fig. S21).

Given the positive results with the rhodamine alloc probe (**7**) and **Pd8** and **Pd9** using the inverse protocol (palladium complexes added first), we also tested this protocol with the rhodamine derivative having poc instead of alloc protecting groups (**Rho-poc**).[23] The results, detailed in the supporting information, indicated that while **Pd8** failed to raise a significant fluorescence, **Pd9** was able to promote the depropargylation. This might be associated to a protective effect of the mitochondria sourroundings on the stability of the palladium complex (Fig. S22).

Conclusions

Our results confirm that while many Pd(0) and Pd(II) sources can effect depropargylation and deallylation reactions in water and PBS, they are much less effective in complex cellular media or living cells. However, palladium complexes featuring designed phosphine ligands can promote the transformations in cell lysates with variable efficiencies, which depend on the characteristics of the ligands. Despite this modest activity, these palladium phosphine complexes can be translated into living mammalian cells and promote both depropagylation and alloc cleavage reactions.

We have also demonstrated that using a phosphine ligand tethered to phosphonium and hydrophobic pyrene groups, the palladium complex presents a preferencial accumulation in mithochondria wherein it remains active. This type of subcellular targeting might open new opportunites for promoting enhanced and selective biological effects in prodrug activation processes. Our results confirm the viability of engineering well-defined, discrete palladium complexes to promote relevant reactions in cellular milieu. The small-size and ligand tunability of these complexes offer advantages with respect to other alternatives based on metalloenzymes or nanoconstructs in terms of simplicity and synthetic access, as well as of controlling properties such as reactivity, targetability and cellular uptake.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally. (match statement to author names with a symbol)

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website http://pubs.acs.org." Supplementary Figures S1–S22, experimental procedures, and cell studies.

ACKNOWLEDGMENT

We are thankful for the financial support from the Xunta de Galicia (Centro Singular de Investigación de Galicia Acreditación 2016–2019, ED431G/09) and the European Union (European Regional Development Fund – ERDF). We also thank support given by the Spanish grant SAF2016-76689-R, the Xunta de Galicia (grants 2015-CP082, ED431C 2017/19), and the European Research Council (Advanced Grant No. 340055). MMC thanks the Ministerio de Economía y Competitividad for the Postdoctoral fellowship (IJCI-2014-19326). JR and JM thank to Xunta de Galicia and Ministerio of Educacion for predoctoral fellowships, respectively. The authors thank R. Menaya-Vargas for technical assistance.

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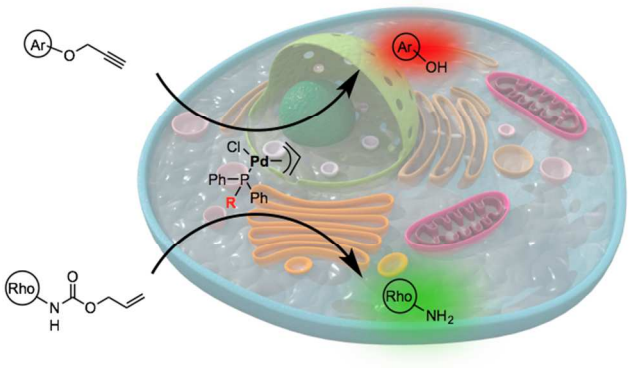

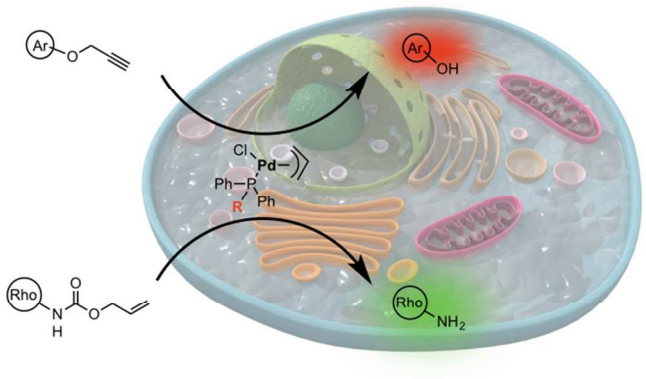

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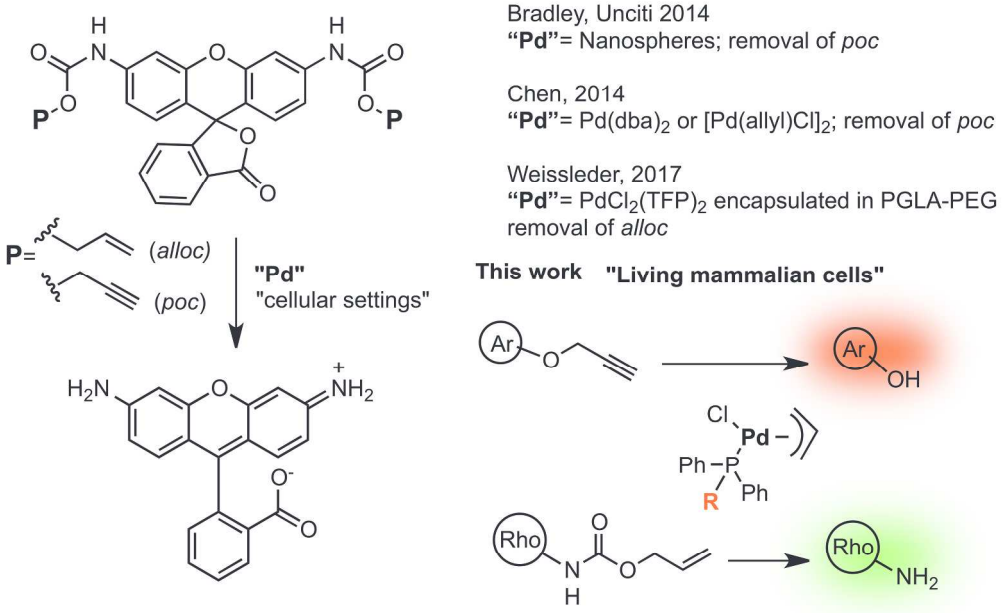

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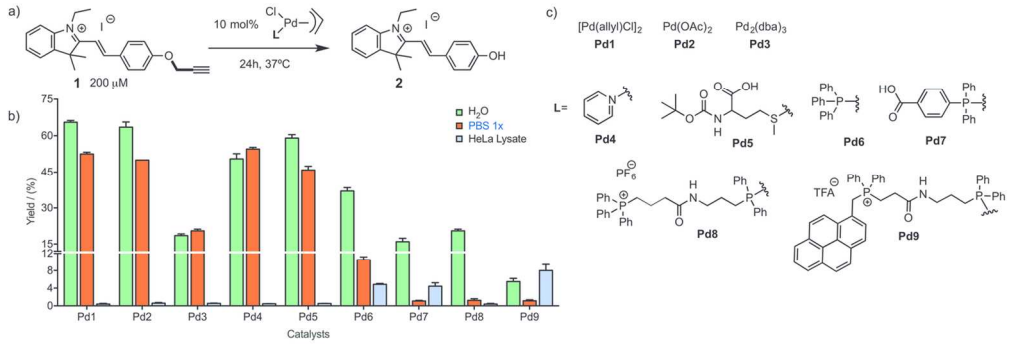

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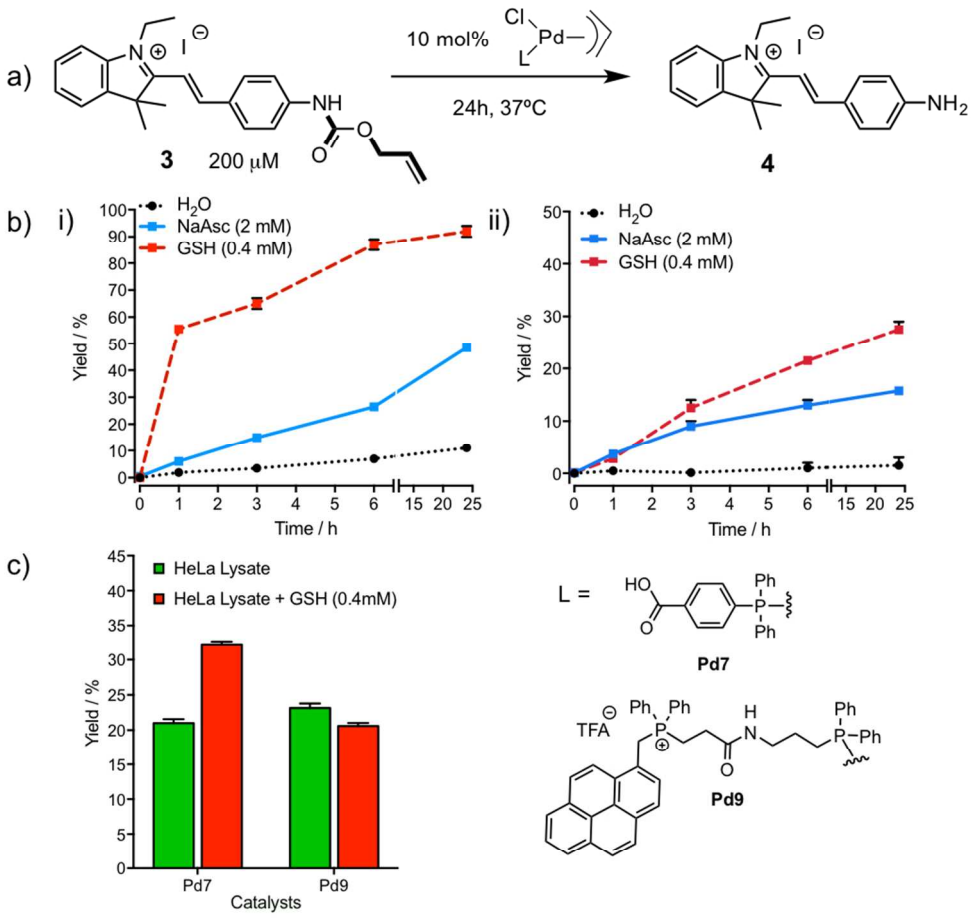
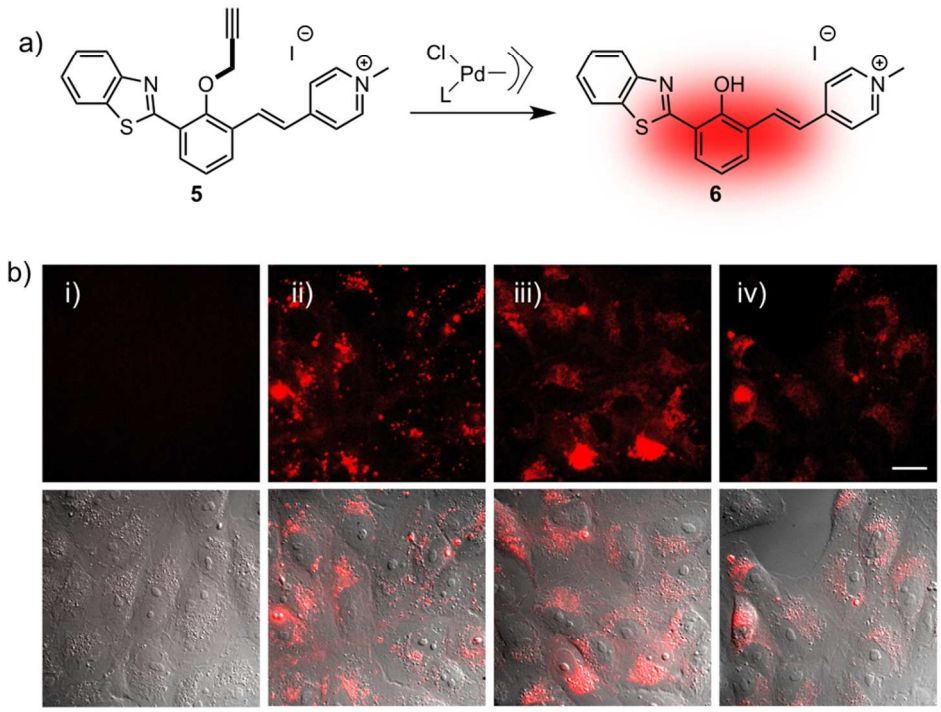
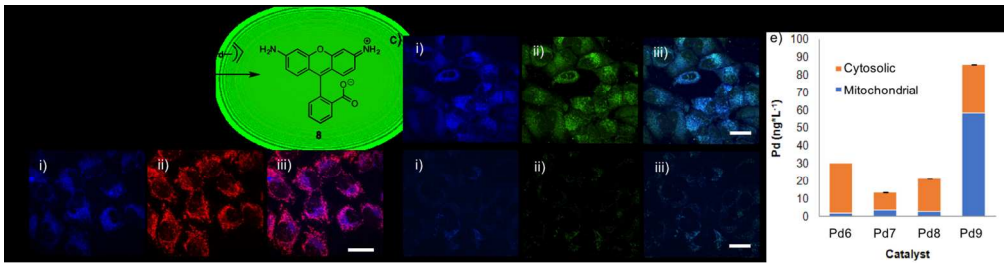


124x126mm (216 x 216 DPI)



125x97mm (216 x 216 DPI)



257x65mm (150 x 150 DPI)