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Organometallic Palladium Reagents for Cysteine Bioconjugation

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Abstract

Transition-metal based reactions have found wide use in organic synthesis and are used frequently to functionalize small molecules.^{1,2} However, there are very few reports of using transition-metal based reactions to modify complex biomolecules^{3,4}, which is due to the need for stringent reaction conditions (for example, aqueous media, low temperature, and mild pH) and the existence of multiple, reactive functional groups found in biopolymers. Here we report that palladium(II) complexes can be used for efficient and highly selective cysteine conjugation reactions. The bioconjugation reaction is rapid and robust under a range of biocompatible reaction conditions. The straightforward synthesis of the palladium reagents from diverse and easily accessible aryl halide and trifluoromethanesulfonate precursors makes the method highly practical, providing access to a large structural space for protein modification. The resulting aryl bioconjugates are stable towards acids, bases, oxidants, and external thiol nucleophiles. The broad utility of the new bioconjugation platform was further corroborated by the synthesis of new classes of stapled peptides and antibody-drug conjugates. These palladium complexes show potential as a new set of benchtop reagents for diverse bioconjugation applications.

Post-translational modifications greatly expand the function of proteins.⁵ Chemists aim to mimic Nature's success through the development of chemo- and regioselective reactions of proteins. The diversity of potentially reactive functional groups present in biomolecules (e.g., amides, acids, alcohols, amines) combined with the requirement for fast kinetics and mild reaction conditions (e.g., aqueous solvent, pH 6–8, T < 37 °C) set a high bar for the development of new techniques to functionalize proteins. Nevertheless methods have emerged for bioconjugation with natural and unnatural amino acids in protein molecules.^{6,7} Cysteine is a key residue for the chemical modification of proteins owing to the unique

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reactivity of the thiol functional group and the low abundance of cysteine residues in naturally occurring proteins.^{8,9} Michael addition to maleimides and S_N2 reaction with alkyl halides are commonly used for cysteine modification. The resulting conjugates tend to decompose in the presence of external bases or thiol nucleophiles,¹⁰ which prompted the recent development of advanced cysteine bioconjugations for the improved stability of the conjugates.¹¹

The ability to achieve high levels of chemo- and regioselectivity through the judicious choice of metal and ligand design suggest metal-mediated processes could be very attractive for the development of new bioconjugations. Existing metal based transformations often rely on the use of functional linkers¹² such as 4-iodophenylalanine, aldehyde- or alkyne-containing amino acids,^{3,4,13} and require high concentrations (mM) of derivatizing agents, which can cause off-target reactivity or purification problems. We hypothesized that palladium complexes resulting from the oxidative addition of aryl halides or trifluoromethanesulfonates¹⁴ could be used for the transfer of aryl groups to cysteine residues in proteins (Fig. 1a).¹⁵ The efficiency and selectivity of the proposed reaction with the highly active palladium species may be hampered by the presence of a variety of functional groups within complex biopolymers.¹⁷ However, we envisioned that careful choice of ligand would provide stable, yet highly reactive reagents for the desired transformations (Fig. 1b), while the interaction between the soft nucleophile cysteine thiol and the aryl palladium(II) species would guide its selectivity.

We began our study with a palladium-tolyl complex (**1A-OTf**) using 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl (RuPhos) as the ligand and trifluoromethanesulfonate as the counterion. A model peptide (**P1**) was used for the optimization of the reaction conditions and for exploration of the substrate scope. Full conversion of the starting peptide to the corresponding aryl product was observed in less than 5 minutes at low micromolar concentrations of reagents (Fig. 1c). Further, the reaction was selective for cysteine. No reaction was observed using a control peptide with cysteine mutated to serine (Supporting Information), in contrast to the palladium-mediated protein allylation, which is selective for tyrosine (*O*-allylation) over lysine and cysteine (*N*- and *S*-allylation).¹⁸ These results highlight the importance of the ligand choice to facilitate *C*-*S* reductive elimination together with the overall electrophilicity of the palladium center to tune the selectivity of the transformation.

Most cysteine conjugation reactions operate at nearly neutral to slightly basic pH values. Further evaluation of the reaction conditions using palladium reagents revealed quantitative conversion of the starting peptide to the corresponding *S*-aryl cysteine conjugate within a broad pH range (5.5 – 8.5) using common organic cosolvents (5% of *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (CH_3CN)) in various buffers (Supporting Information, Table S1). Remarkably, even in 0.1% trifluoroacetic acid (TFA) solution (pH 2.0) the reaction yielded 59% of the *S*-arylated product after 7 hours. The process was also compatible with the protein disulfide reducing agent *tris*(2-carboxyethyl)phosphine (TCEP) that has been shown to hamper bioconjugations by reacting with maleimide and α -haloacyl groups.¹⁹

The palladium mediated conjugation is fast and complete product formation occurs within 15 seconds at 4 °C. The reaction rate was estimated by competition experiments against the commonly used *N*-methyl maleimide cysteine ligation.²⁰ At pH 7.5 the rate of the palladium-mediated reaction was comparable to that of the maleimide ligation, where 70% of the products resulted from the reaction with palladium-tolyl complex (**1A-OTf**). Notably, the palladium-mediated conjugation outperformed the maleimide ligation at pH 5.5 where only the arylated product was formed.

The optimized conditions (0.1 M Tris buffer, 5% CH₃CN, pH 7.5, room temperature) were used for further evaluation of the substrate scope. Palladium complexes containing chloride, bromide and iodide counterions were all found to produce the desired product (Fig. 2, **1A-Cl**, **1A-Br**, and **1A-I**). This method can be used to functionalize unprotected peptides with a variety of important groups including fluorescent tags (**1C**, **1D**), affinity labels (**1E**), bioconjugation handles (aldehyde **1F**, ketone **1G**, and alkyne **1I**), photochemical crosslinkers (**1H**), as well as complex drug molecules (**1J**). Vinyl palladium complexes were also shown to be competent in this transformation (Supporting Information). Importantly, the palladium(II) complexes are stable under ambient conditions, and can be stored in closed vials under air at 4 °C for over four months. Long-term stability of **1A-I**, **1A-Br**, **1A-Cl**, and **1A-OTf** was evaluated, where only the complex bearing the trifluoromethanesulfonate counterion (**1A-OTf**) showed some degradation (15%) after 20 weeks (Supporting Information). Nevertheless, the “aged” reagents still exhibited reactivity comparable to the freshly made complexes.

The stability of our arylated peptides was compared to that of conjugates formed from reactions with reagents including *N*-ethyl maleimide, 2-bromoacetamide, and benzyl bromide. The *S*-arylated peptide was shown to be stable toward acids, bases, and external thiol nucleophiles (Supporting Information). In contrast, the corresponding acetamide derivative was unstable under acidic and basic conditions and the maleimide conjugate decomposed in the presence of base and exogenous thiol. Finally, comparable stability of both aryl and benzyl conjugates to treatment with the periodic acid oxidant at 37 °C was observed. However, additional tuning of the electronic properties of the aromatic ring of the arylated peptide by installing a *para*-electron withdrawing cyano-group could be achieved. This modification significantly decreased the amount of oxidation producing the most stable peptides across all the evaluated conjugates. Notably, installing the *para* cyano-group in the benzyl conjugates did not have any effect toward oxidation (Supporting Information).

We further explored this reaction with proteins. Three antibody mimetic proteins²¹ (**P4-P6**) were expressed that contained a cysteine at structurally distinct positions including the *N*-terminus, *C*-terminus, and a loop. The same proteins without cysteine were used as controls to confirm the selectivity of the reaction (**P7-P9**). All three proteins (**P4-P6**) were quantitatively tagged with either coumarin (Fig. 3) or a drug molecule (Supporting Information) within 30 minutes at 1 μM protein concentration. No arylated product was generated for proteins lacking a cysteine. The presence of small amounts of organic co-solvents is required for the efficient bioconjugation and lower product yields were observed when <5% of the co-solvent was used (Supporting Information). The fast kinetics and high efficiency of the reactions at low micromolar and low nanomolar (Supporting Information)

protein concentrations are in contrast to reported bioconjugation methods using organometallic reagents, where longer reaction times were needed and generally lower conversions were observed.^{3,22}

The developed protocol was used to arylate an engineered cysteine residue in the C-terminal region of Diphtheria toxin A-chain (DTA) fused to the lethal factor N-terminal domain (LF_N-DTA-Cys, see Supporting information).²³ The modified LF_N-DTA-Cys variant was readily separated from the remaining palladium species, ligands, and other small molecules using commercially available size-exclusion chromatography (SEC) columns (91% of palladium was removed as determined by ICP-MS analysis of the purified protein sample, see Supporting Information). The modified and purified LF_N-DTA-Cys variant displayed similar activity ($EC_{50} = 0.40 \pm 0.09$ nM) in a cell-based protein synthesis inhibition assay compared to the control (a serine mutant LFN-DTA-Ser showed $EC_{50} = 0.25 \pm 0.05$ nM) (Supporting Information).

Stapled peptides have shown significant promise as next generation therapeutics.^{24,25} However, there are limited methods for the synthesis of these bioconjugates with structurally diverse linkers,²⁶ which hinders the systematic investigation of the effect of the linker on the properties of stapled peptides.²⁷ We envisioned that palladium reagents containing two electrophilic metal centers could be efficiently used to crosslink two cysteine residues on a peptide chain, thereby providing access to stapled peptides with various aryl linkers (Fig. 4a). Indeed, running the reaction at 10 μ M concentration of peptide in a 1:1 (v/v) acetonitrile/water solvent mixture at pH 7.5 using a two-fold excess of the bis-palladium complex (**2A**) resulted in quantitative formation of the target stapled peptide within 10 minutes (Fig. 3a and Supporting Information). Considering the availability of commercially or otherwise easily accessible diarylhalide reagents, this approach provides facile access to a diverse aryl-linker space for stapled peptides.²⁸

Antibody-drug conjugates (ADCs) are a promising class of biotherapeutics, which combine the potency of cytotoxic drugs with the target specificity of monoclonal antibodies.²⁹ We aimed to attach drug molecules directly to cysteine residues in antibodies through the developed palladium conjugation chemistry. The drug payload Vandetanib was used to form palladium complex **1J** (Fig. 2) by making use of the aryl bromide present in its structure. Treating partially reduced Trastuzumab antibody³⁰ with **1J** readily produced ADCs with a 4.4 drug to antibody ratio (DAR) (Fig. 4b). The purified arylated ADCs (94% of palladium was removed as determined by the ICP-MS analysis of the purified ADCs, see Supporting Information) retained binding affinity ($K_D = 0.3 \pm 0.2$ nM) to recombinant HER2 compared to the unmodified trastuzumab antibody (Supporting Information). While traditional ADCs use various linkers to attach drug molecules to antibodies, our method significantly expands the structural space of ADCs by providing the capability to directly attach drug molecules containing native or pre-installed aryl halide or phenol functional groups. The therapeutic potential of this class of “linker-free” ADCs will be investigated in the future.

In conclusion, we have introduced a new approach for cysteine bioconjugation and have shown for the first time that palladium(II) complexes can be used for fast and chemoselective arylation of cysteine residues in complex biomolecules. The versatility of

the method is particularly notable, since a large variety of aryl halides or trifluoromethanesulfonates are commercially available or readily accessible from simple precursors. Finally, the ease of preparation, storage, and use of the palladium reagents make them particularly attractive for routine application in chemistry, biology, medicine, and materials science. Further evolution of the metals and ligands employed will likely provide an extended set of organometallic bioconjugation reagents with altered selectivity and efficiency, allowing for functionalization of other amino acid residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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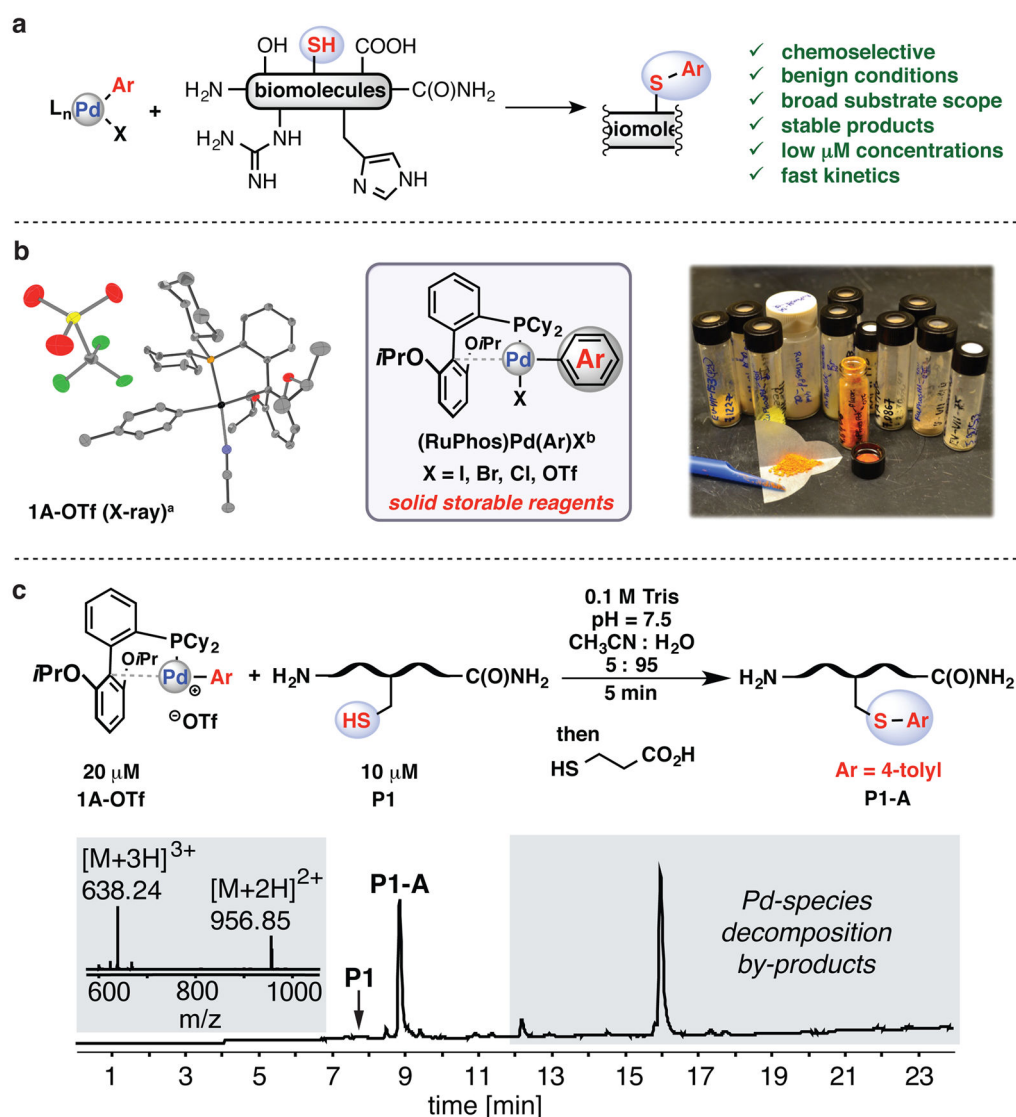
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**Figure 1.**

Organometallic palladium reagents for cysteine modification: strategy and model studies. a) Proposed cysteine bioconjugation using palladium reagents; b) Top, the reaction studied. Bottom, a selection of palladium reagents was used to test the effect of the leaving group (X) on the reactivity and explore the substrate scope with regard to biologically relevant groups (fluorescent tags, bioconjugation handles, affinity tag and a drug molecule). Full conversion of starting peptide P1 into the corresponding arylated products was observed in all the cases shown, as confirmed by LC-MS. For exact reaction procedures and conditions, see Supporting Information; c) Model reaction with a peptide substrate and the LC-MS trace of the crude reaction mixture after 5 min. The mass spectrum of the arylated product is shown in the inset. Peptide P1 sequence: $\text{NH}_2\text{-RSNFYLGACGLAHDKAT-C(O)NH}_2$. The reaction was quenched by the addition of 3-mercaptopropionic acid (3 equivalents to 1A-OTf) before LC-MS analysis. At high reaction concentrations (~ 100 mM) a cloudy precipitate formed after the addition of palladium reagent presumably due to low solubility

of the complex in the aqueous solvent. These reactions still produced the desired bioconjugate in high yields (Supporting Information).

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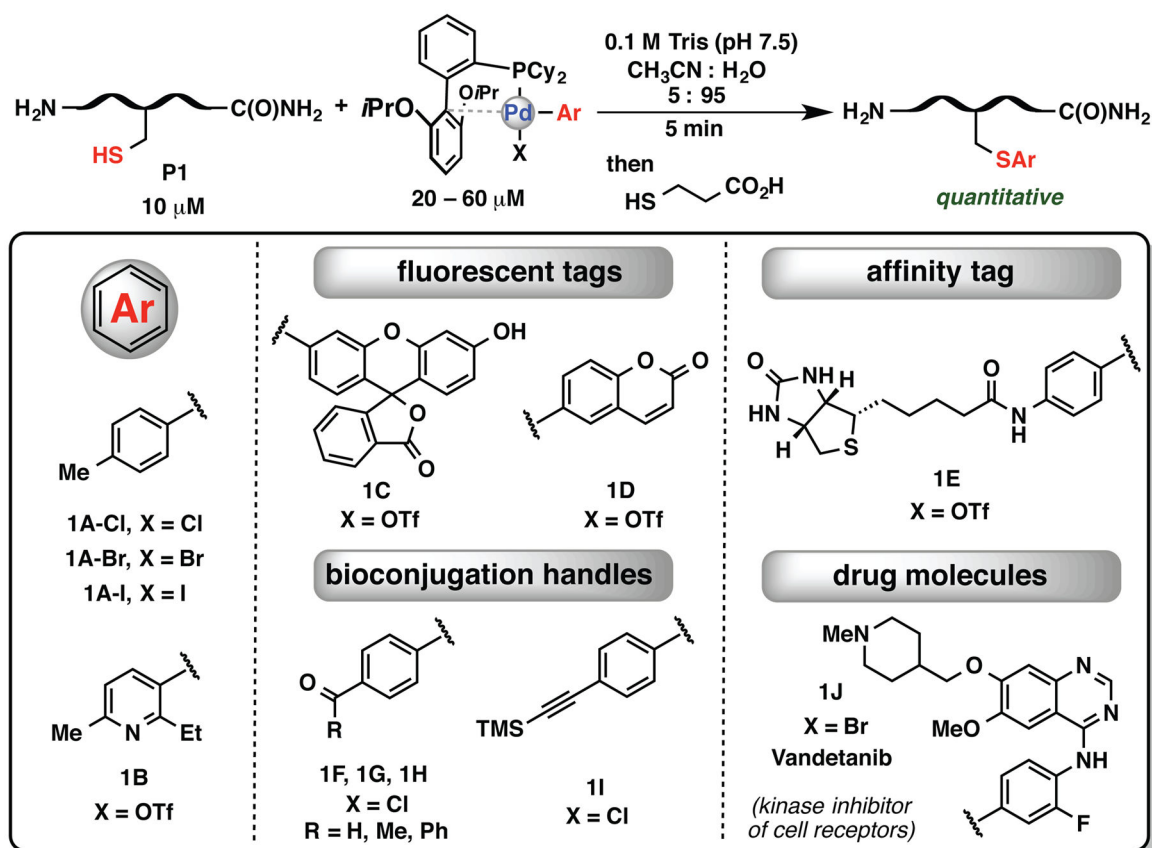


Figure 2. The substrate scope of cysteine arylation using organometallic palladium reagents
 Top, the reaction studied. Bottom, a selection of palladium reagents was used to test the effect of the leaving group (X) on the reactivity and explore the substrate scope with regard to biologically relevant groups (fluorescent tags, bioconjugation handles, affinity tag and a drug molecule). Full conversion of starting peptide **P1** into the corresponding arylated products was observed in all the cases shown, as confirmed by LC-MS. For exact reaction procedures and conditions, see Supporting Information.

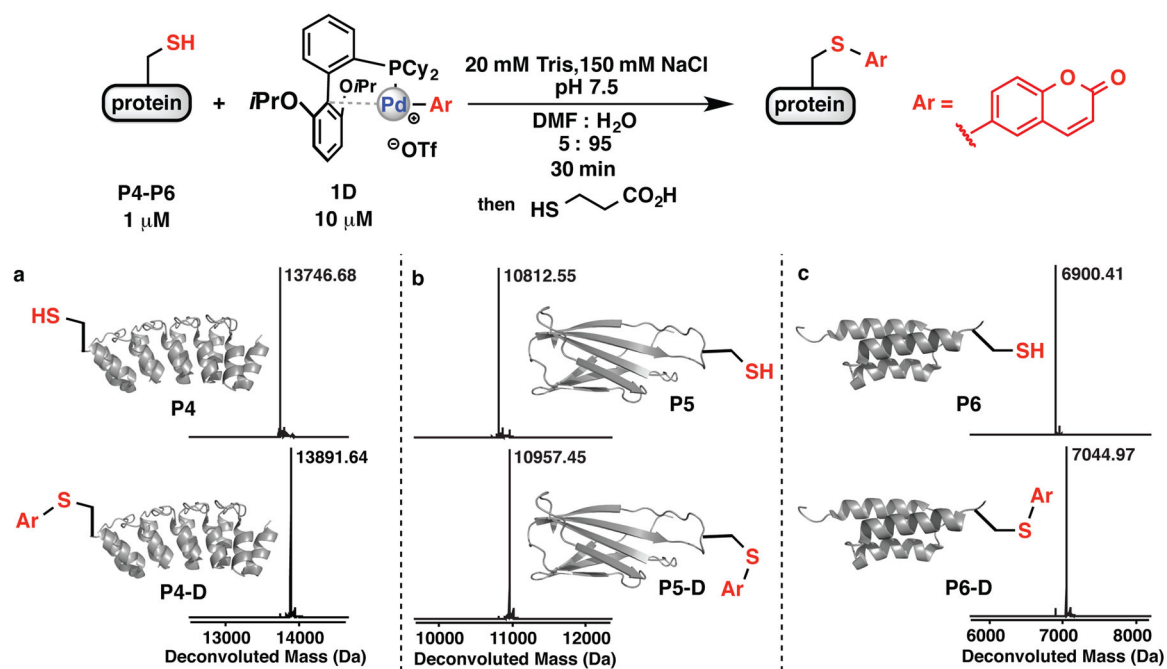


Figure 3. Protein modification using the developed palladium reagents
 Cysteine residues at the N-terminus (**P4**; a), a loop (**P5**; b) and the C-terminus (**P6**; c) of proteins are quantitatively modified with coumarin after the reaction with palladium complex **1D** (top). Deconvoluted mass spectra of the full protein peaks are shown for the starting proteins (**P4–P6**) and reactions with coumarin-palladium complexes after 30 min (bottom). Three-dimensional structures of proteins **P4–P6** and the arylated products (**P4-D**, **P5-D** and **P6-D**) are presented next to the corresponding mass spectra.

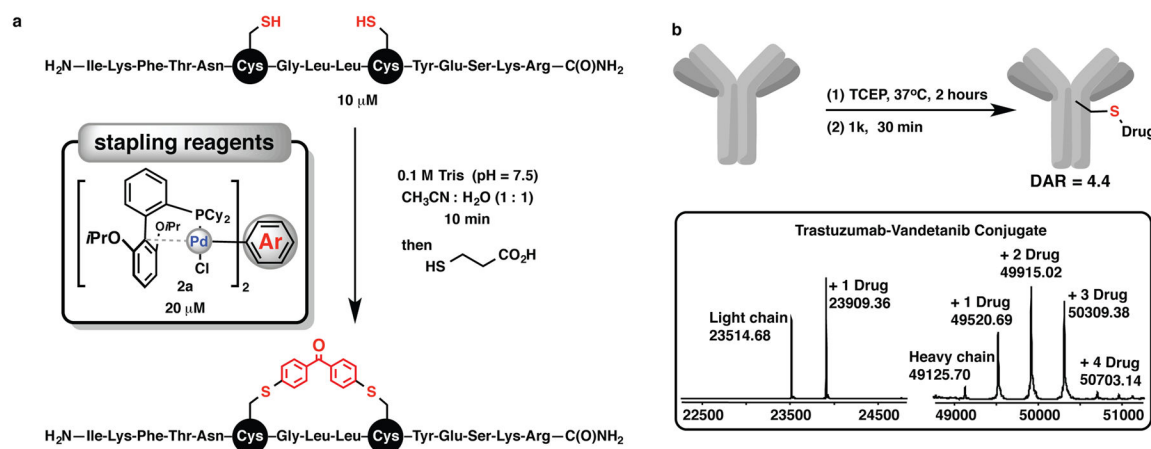


Figure 4. Peptide stapling and antibody drug conjugate formation using palladium-based reagents

a) Stapling of a model peptide using a bis-palladium reagent **2A**. Reaction conditions: **P3** (10 μM), complex **2A** (20 μM), Tris buffer (100 mM; pH 7.5), CH₃CN : H₂O = 1 : 1, rt, 10 min. The reaction was quenched by the addition of 3-mercaptopropionic acid (6 equivalents to **2A**) before the LC-MS analysis. b) Synthesis of the “linker-free” antibody-drug conjugate (ADCs). Trastuzumab antibody is represented as the two-tone grey structure at the top. Deconvoluted mass spectra of the fully reduced and deglycosylated ADCs were shown. Drug-to-Antibody Ratio (DAR) represents an average number of drugs per antibody. See Supporting Information for details.