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Communication

Palladium–Protein Oxidative Addition Complexes by Amine-Selective Acylation

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ABSTRACT: Palladium oxidative addition complexes (OACs) are traditionally accessed by treating an aryl halide-containing substrate with a palladium(0) source. Here, a new strategy to selectively prepare stable OACs from amino groups on native proteins is presented. The approach relies on an amine-selective acylation reaction that occurs without modification of a preformed palladium(II)-aryl group. Once transferred onto a protein substrate, the palladium(II)-aryl group facilitates conjugation by undergoing reaction with a second, cysteine-containing protein. This operationally simple method is applicable to native, nonengineered enzymes as well as antibodies and is carried out in an aqueous setting and open to air. The resulting Pd–protein OACs are stable, storable reagents that retain biological activity and can be used to achieve protein–protein cross-coupling at nanomolar concentrations within hours.

Protein–protein conjugates provide a useful means of accessing novel biological reporters, heterobifunctional enzymes, and new therapeutic agents.[1,2] In each of these examples, the actions/properties of two or more different protein domains are combined into a single molecule. While covalently fused proteins are traditionally accessed via coexpression, an alternative approach is the direct bioconjugation of the two proteins. However, such reactions, due to the large molecular weights (>10 kDa) of the reactants, must operate efficiently at low micromolar concentrations. For instance, a bimolecular reaction with 1:1 stoichiometry at a 100 μM concentration of each reactant requires second-order rate constants greater than 10 M^{-1} s^{-1} to give >90% yield in hours.[3] Thus, to expand the classes of molecules that can be effectively conjugated to proteins, new, mild, and aqueous coupling conditions are needed.

Current approaches for the chemical ligation of two proteins include azide–alkyne click chemistry,[4] photocaged *o*-quinone methides,[5] and the use of maleimide functional groups.[6] While these methods are practical, limitations exist, including the requirement to engineer both coupling partners with noncanonical groups, chemoselectivity, and/or relative instability of the conjugate.[7] Seminal contributions from a variety of groups have demonstrated the use of palladium for protein-cross-linking,[8] tyrosine-selective alkylation,[9] and Suzuki–Miyaura cross-coupling.[10,11] Cross-coupling reactions using palladium complexes are suitable for modifying proteins, as they tolerate a broad range of functional groups while forming stable carbon–carbon and carbon–heteroatom bonds.[10−14] Thiol arylation with palladium(II) oxidative addition complexes (OACs) is of particular interest due to the high reaction rates and the stability of resulting *S*-aryl bonds (Figure 1A, top).[12,13] To enable the covalent attachment of two proteins via Pd-mediated thiol arylation, new strategies are necessary to access Pd–protein OACs (**2**).

The conditions used to prepare small-molecule OACs (**1**) are largely incompatible with the manipulation of proteins.[15] Adapting strategies used on small molecules to access electrophilic OACs of protein substrates is challenging (Figure 1B, blue) and hampered predominantly by three factors: (1) a two-step process that first necessitates installation of an aryl halide prior to oxidative addition;[10] (2) ineffective oxidative addition due to nonspecific metal chelation, which often requires a large excess of Pd reagents to overcome;[14] and (3) the requirement to use an inert atmosphere and organic solvents. Recently, we reported an intramolecular oxidative addition strategy from cysteine residues to gain access to Pd–protein OACs,[16] validating that these organometallic proteins are stable intermediates that can engage in large biopolymer conjugations. However, the necessity for the protein to contain a free cysteine thiol limits the scope of this first technique. We set out to develop an alternative approach to access Pd–protein OACs from proteins lacking free cysteine thiols.

Here, we show the successful preparation and characterization of functional Pd–protein OACs by a means that eliminates many of the challenges associated with the need to perform an oxidative addition directly on the substrate. Previously, we reported on the use of palladium oxidative addition complexes for peptide and protein cross-linking via a bifunctional palladium reagent.[17] It was only in the presence of cysteine thiol groups that S–C(sp^2) bonds were formed to provide *S*-arylated conjugates. Thus, we considered an

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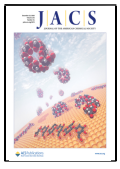

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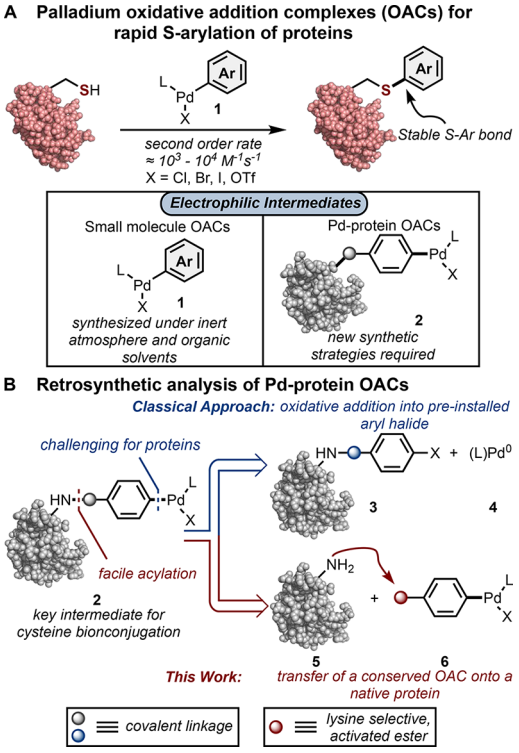

Figure 1. Strategy for formation of palladium-oxidative addition complexes of protein substrates. (A) Palladium oxidative addition complexes (OACs) demonstrate rapid reaction rates suitable for bimolecular reactions at μM concentrations while forming stable *S*-aryl bonds. (B) As an alternative to oxidative addition directly on the substrate (blue), we employ a facile acylation reaction involving an NHS ester to transfer a conserved, preformed Pd-OAC onto lysine residues of the protein (red) to circumvent many of the challenges associated with classical approaches.

alternative retrosynthetic disconnection that would involve the use of a Pd-functionalized NHS (*N*-hydroxysuccinimidyl) ester reagent (Figure 1B, red pathway). NHS esters are electrophilic groups capable of acylating numerous types of small molecules and materials containing exposed nucleophilic residues,[18] including side chains of amino acids.[19] Therefore, a bifunctional reagent containing both an NHS ester and a biarylphosphine-supported Pd-OAC would provide a means to install a Pd-OAC functional group onto the surface of a protein.

We began by preparing bifunctional reagents **10** (Figure 2A). Treatment of 3-(4-bromophenyl)propionic acid (**7**) with disuccinimidyl carbonate (**8**) gave NHS ester **9** in 87% yield. Subsequent treatment with $(COD)Pd(CH_2TMS)_2$ and biarylphosphine ligand SPhos or sSPhos provided **10** in 87% and 89% yield, respectively. To minimize NHS ester hydrolysis, which occurred upon prolonged exposure to ambient atmosphere, **10** was stored in a desiccator under a nitrogen atmosphere.[20] Acylation of ribonuclease A (RNase A, Figure 2B) with OAC transfer reagent **10** (L = sSPhos; 1.5 equiv) gave rise to a mixture of products comprising 66% unmodified RNase A (**11**), 30% singly modified RNase A, and 4% of doubly and triply modified RNase A as determined by deconvolution after liquid chromatography mass spectrometry (LC-MS) analysis.[21] The crude reaction mixture was purified by reversed phase high-performance liquid chromatography (RP-HPLC) using water/acetonitrile/0.1% TFA as eluent followed by lyophilization to provide 14.6 mg of the corresponding Pd-RNase A-OAC (**12**) from 48 mg of starting material.

The ability to isolate and store these Pd−protein OACs as stable solids for months at −20 °C demonstrates that the stability of these complexes is not affected by a variety of amino acid side chains known to coordinate palladium, notably imidazolyl, guanidino, primary amine, disulfide, and thioether (the side chains of histidine, arginine, lysine, cystine, and

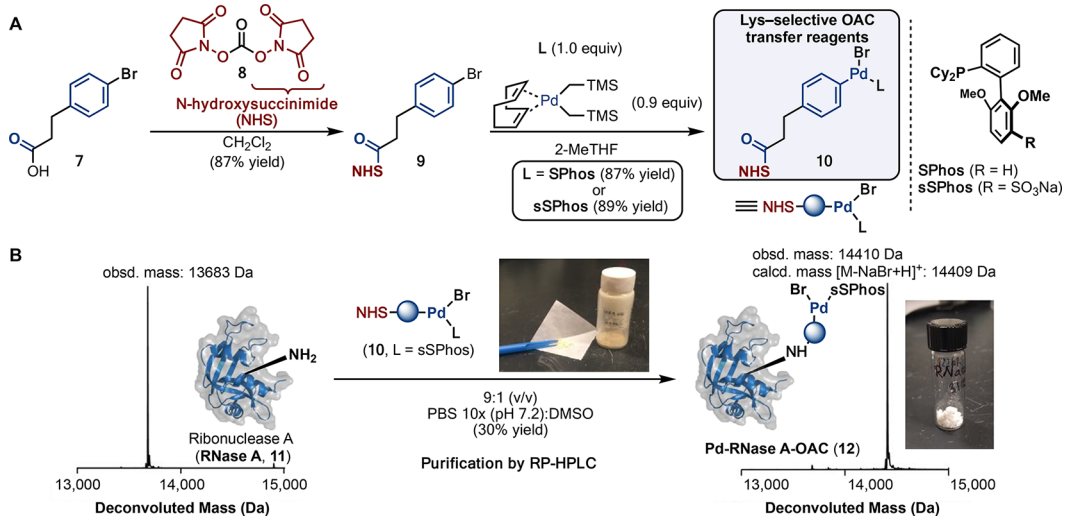

Figure 2. Pd-RNase A-oxidative addition complex (OAC) (**12**) was synthesized and isolated with amine selective palladium transfer reagent **10**. (A) Synthesis of an amine-selective OAC transfer reagents **10**. (B) Preparation and purification of palladium−protein OAC from ribonuclease A (RNase A, **11**, calculated mass [M]: 13682 Da) utilizing OAC transfer reagent **10** (L = sSPhos). Reaction conditions: RNase A (**11**) (70 μM), complex **10** (L = sSPhos) (105 μM), 10× PBS (pH 7.2), 10% DMSO, room temperature, 60 min. Left, deconvoluted LC-MS mass spectrum of the full protein, peaks are shown for the starting RNase A (**11**) and Pd-RNase A-OAC (**12**) purified by reversed phase-high performance liquid chromatography (RP-HPLC) (calculated mass $[M - NaBr + H]^+$ = 14409 Da, contains <5% of the parent RNase A). We note that **12** is isolated as a mixture of regioisomeric compounds containing a single modification at different sites.[21]

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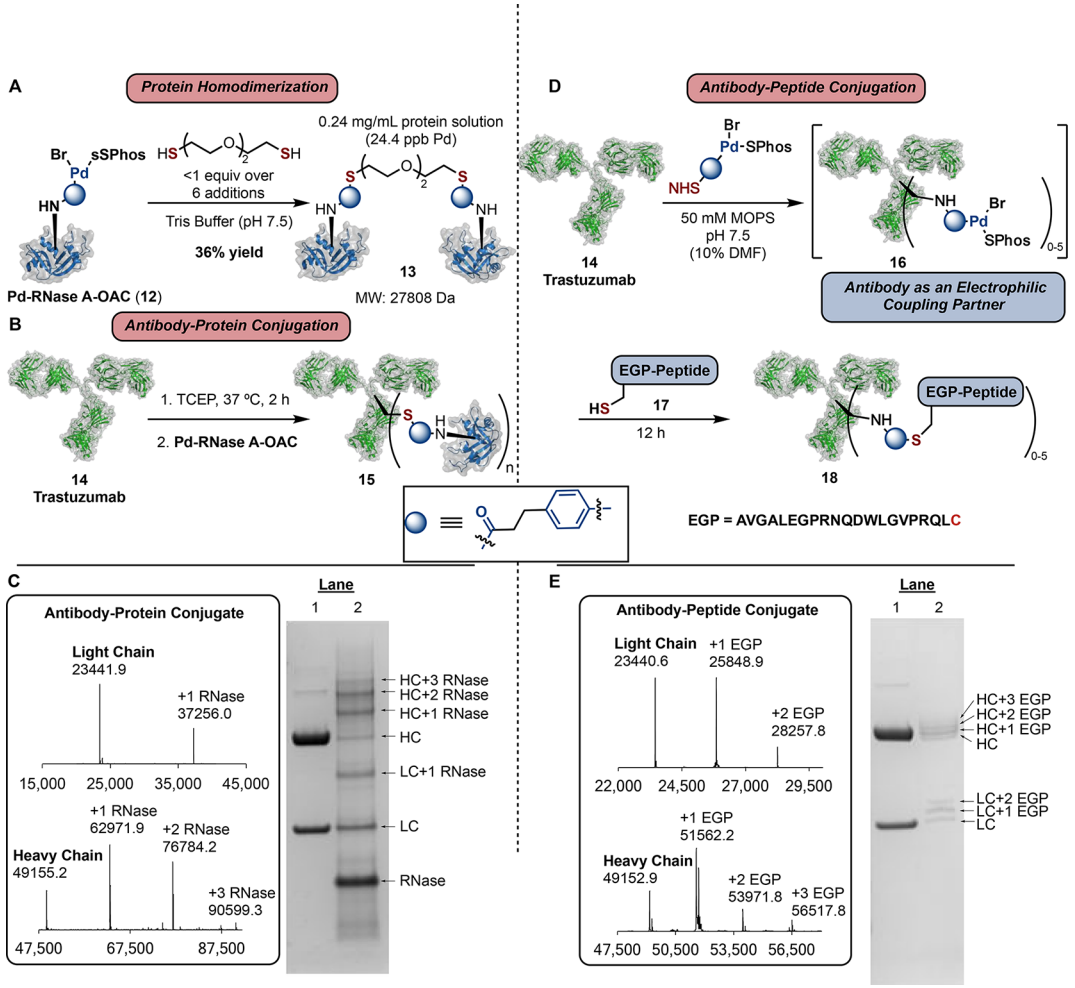

Figure 3. Cross-couplings of Pd-protein OACs enables protein homodimerization, antibody-protein conjugation and antibody-peptide conjugation. (A) Homodimerization of Pd-RNase A-OAC **12**. Conditions: Pd-RNase A-OAC (**11**) [70 μM], Tris Buffer (pH 7.5, 100 mM Tris, 150 mM NaCl). 2,2′-(ethylenedioxy)diethanethiol (1.0 equiv) was added in six portions, each with a 5 min incubation time, and then purified by size-exclusion chromatography. Calculated mass: 27806 Da, observed mass: 27808 Da. (B) Synthesis of antibody−protein conjugates via partial reduction of trastuzumab (**14**) with TCEP followed by treatment with Pd-RNase A-OAC (**12**). Reaction conditions: Trastuzumab (**14**) [5 μM], Pd-RNase A-OAC (**12**) [100 μM], Tris Buffer (50 mM, pH 8.0). (C) Left, deconvoluted mass spectra of **15** after TCEP reduction. Right, analysis of conjugates by SDS-PAGE. Lane 1: reduced trastuzumab; Lane 2: reduced conjugate. (D) Synthesis of Pd-trastuzumab-OAC **16** and subsequent conjugation with EGP peptide **17**. (E) Left, deconvoluted mass spectra of **18** after TCEP reduction. Right, analysis of conjugates by SDS-PAGE. Lane 1: reduced trastuzumab; Lane 2: reduced conjugate.

methionine respectively).[22] In buffered solutions with the pH range 5.0−8.0, these OACs were stable for days at room temperature with no detectable decomposition (Figures S6−S9). However, in unbuffered water with pH < 3.5, Pd-RNase A-OAC showed ∼20% decomposition after 24 h (Figure S6).

It is important to consider the effect of the Pd species on the enzymatic activity of the parent protein. Catalytic activity is a highly sensitive measure of the native structure of an enzyme. We found that the Pd-RNase A-OAC **12** isolated after RP-HPLC retained 56% of its activity relative to unmodified RNase A **11**, and the product of conjugation with 3-mercaptopropionic acid had 85% of the native protein's activity (Figure S11). The OAC derived from lysozyme exhibited a similar trend: the OAC manifested 38% and conjugated product 89% of the activity of unmodified lysozyme (Figure S10). The retention of enzymatic activity after bioconjugation suggests that derivatization via the Pd-OAC does not have a major effect on the structure or function of the protein target.

Pd-RNase A-OAC **12** underwent efficient conjugation with thiol containing substrates. For example, the homodimerization of **12** with a small-molecule dithiol proceeded with 68% conversion as judged by SDS-PAGE and the homodimer protein product **13** was isolated in 36% yield after size-exclusion chromatography (Figure 3A). Analysis of the purified homodimer by inductively coupled plasma-mass spectrometry (ICP-MS) indicated the product contained 0.01% palladium by mass with respect to protein (see Supporting Information).[23]

We next determined whether we could expand the types of molecules that can be accessed with our method by preparation of immunotoxin (IT) analogues.[24] Like antibody−drug conjugates (ADCs), immunotoxins take advantage of the cell-targeting ability of antibodies, but instead of employing toxic small molecules, ITs combine the antibody with the cell-killing power of cytotoxic proteins.[25] Trastuzumab (**14**) is a monoclonal antibody used in the treatment of HER2 positive breast cancer and employed in the preparation

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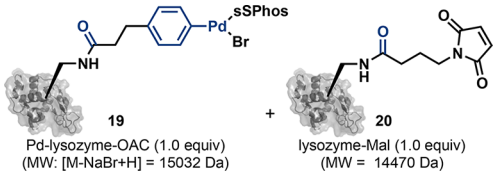

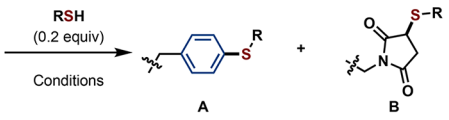

Entry	pH	Buffer	Ratio A:B Observed
1	3.0	100 mM Citric Acid	>9:1
2	5.0	100 mM Citric Acid	>9:1
3	7.0	100 mM Sodium Phosphate	>9:1
4	7.5	20 mM Tris, 150 mM NaCl	>9:1
5	8.0	100 mM Tris	3:1

Figure 4. Treatment of an equimolar mixture of Pd-lysozyme-OAC (**19**) and lysozyme-maleimide (lysozyme-Mal, **20**) with substoichiometric quantities of 3-mercaptopropionic acid preferentially gives A, the product derived from **19** at nearly all pH's tested as determined by LC-MS (see Supporting Information).

of the FDA-approved ADCs Kadcyla and Adcetris.[26] Given that homologues of RNase A have shown promise as anticancer therapeutics,[27] we envisioned preparing an IT that combined the targeting ability of trastuzumab with the RNA degrading enzyme RNase A **11**. In the preparation of Adcetris, trastuzumab participates as a nucleophile after partial reduction of interchain disulfides to prepare them for subsequent conjugation with a maleimide-bearing substrate. While we similarly employ a partial reduction of the trastuzumab antibody to prime cysteines for conjugation, as an alternative to the maleimide functional group, we employed a Pd-OAC covalently tethered to the enzyme RNase A, thus providing immunotoxin **15** showing an RNase A/trastuzumab ratio of 2.8:1, as determined by mass spectrometry and consistent with densitometry analysis (Figure 3B,C).

We also looked to target Lys residues on the surface of the antibody to preinstall multiple copies of an electrophilic handle, a strategy utilized in the preparation of Kadcyla. For this, we employed our biarylphosphine-supported aryl palladium complex; for Kadcycla, a maleimide group was employed. Initial attempts utilizing the sulfonated ligand sSPhos to improve the water solubility of the resulting Pd-OAC **10** led to the formation of visible amounts of a precipitate in the reaction mixture. Switching to the non-sulfonated SPhos as the supporting ligand and changing to MOPS buffer eliminated this problem. The antibody was then exposed to the transfer reagent **10** (L = SPhos) to give the corresponding intermediate **16** that serves as an electrophilic coupling partner for conjugation with thiol-containing nucleophiles. Combination with a 21-mer cysteine-containing peptide variant of EGP,[28] a peptide studied as a possible cancer vaccine, gave an average of three copies of the EGP peptide bound to a single copy of trastuzumab (e.g., a peptide/trastuzumab ratio of 3.4:1) (Figure 3D,E).

Thiol-containing nucleophiles react preferentially with Pd–protein OAC (**19**, Figure 4) over maleimide electrophiles (**20**) in competition experiments. These OACs were shown to outcompete maleimide for conjugation with small-molecule thiols and retain enzymatic activity. While effective, thiol–maleimide reactions are known to generate chemically unstable linkages, and much effort to alleviate this limitation continues.[7] In addition, the maleimide reaction operates within a narrow pH range (6.0–8.0). OACs, on the other hand, were reactive over the pH range 3.0–8.0. In this competition experiment, electrophilic variants of lysozyme were prepared with either a maleimide or OAC reactive group (lysozyme-Mal and Pd-lysozyme-OAC). Relative rates were evaluated using an equimolar mixture of both electrophilic protein variants and treated with a substoichiometric quantity of 3-mercaptopropionic acid (Figure 4). In these competition experiments, only

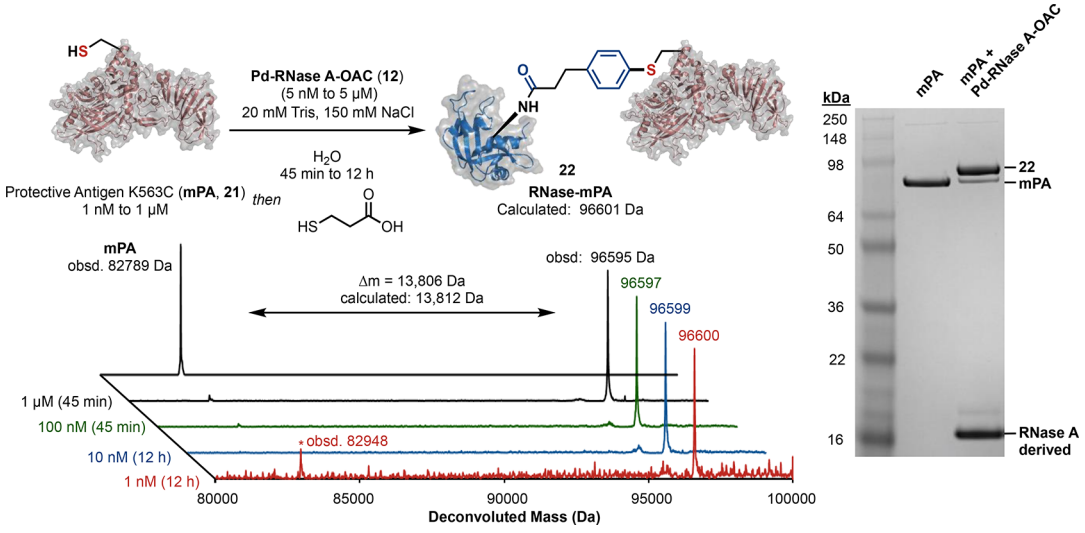

Figure 5. Protein–protein cross-coupling at submicromolar concentrations utilizing palladium-protein OACs. Protein–protein cross-coupling with protective antigen-K563C (mPA, **21**), calculated mass: 82800 Da and Pd-RNase A-OAC (**12**) reaches maximal conversion within 45 min at 1 μM of mPA and within 12 h at 1 nM. The deconvoluted mass spectra are shown for the starting mPA and all PAC derived products at initial mPA concentrations of 100 nM, 10 nM, and 1 nM. The reaction was analyzed by SDS-PAGE and Coomassie staining. Left, molecular weight standard; middle, starting mPA (**21**); right, crude reaction mixture; reaction conditions: 5 μM mPA (**21**), 25 μM Pd-RNase A-OAC (**12**), pH 7.5, 20 mM Tris, 150 mM NaCl. Densitometry analysis gives a conversion of 77%.

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Pd-lysozyme-OAC conjugation was observed with no evidence of thiol addition to lysozyme-Mal in a variety of aqueous buffered conditions (pH 3.0−7.5). However, at pH 8.0 in Tris buffer, a 3:1 ratio of OAC conjugate/maleimide conjugate was observed. At this pH, the maleimide and its conjugate showed ∼50% and 25% hydrolysis within 1 h, respectively, whereas the OAC derived conjugate was a single species. To assess the reaction conversion relative to the maleimide conjugation, a variant of protective antigen [K563C], henceforth termed mPA, containing a single surface exposed cysteine residue was independently treated with lysozyme-OAC or lysozyme-Mal under otherwise identical conditions (pH 7.5, 20 mM Tris, 150 mM NaCl) (see Supporting Information). Conversion of lysozyme-OAC and lysozyme-Mal occurred to similar extents, 82% and 93%, respectively, when treated with mPA (Figure S14). Given successful conjugation of Pd−protein OACs at a broad range of pH's with the accompanying rapid reaction rates described above, we tested Pd-mediated cross-coupling with a larger coupling partner and at more dilute conditions.

Protein−protein conjugation between a cysteine mutant of the 83-kDa protein mPA and Pd-RNase A-OAC occurs within 45 min at the 1 μM concentration of mPA as judged by the deconvoluted mass spectrum of the full protein peaks along with a minimal amount of byproducts (Figure 5). Densitometry analysis after SDS-PAGE of the crude reaction mixture showed a 77% yield of the mPA-RNase A protein−protein conjugate. Even at 1 nM of mPA, the reaction gave a similar deconvoluted mass spectrum as that at 1 μM after a 12 h reaction time. Alternatively, with Pd-RNase A-OAC as the limiting reagent, 2.7 equiv of mPA gave 90% conversion of the OAC within 1 h (Figures S3, S4).

In summary, we have demonstrated that when no free thiols are present, Pd-OACs are a relatively inert functional group. This enables the formation of OACs on native proteins via nonregiospecific, amine-selective acylation by the use of a reagent containing both a Pd-OAC and an activated NHS ester. This strategy, which circumvents the need to perform a difficult oxidative addition sequence directly on the protein substrate, enables the following: (1) access to Pd-OACs in air under aqueous conditions on complex substrates using a relatively low number of equivalents of Pd, and (2) the ability to capitalize on the rapid reaction rates and high selectivity of the resultant products in the arylation of thiols, a consequence of the high affinity of palladium for thiols for the Pd-OAC functional group. This approach permits access to a variety of Pd-OACs derived from biomolecules and polymers that are otherwise difficult to achieve, enabling Pd-mediated cross-coupling chemistry on these challenging substrates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c09180.

Experimental details and additional characterization data (PDF)

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Notes

The authors declare the following competing financial interest(s): MIT has obtained patents for some of the ligands that are described in this work from which S.L.B. and former/current co-workers receive royalty payments.

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(20) Initially, compound **10** was stored on the bench without any effort to exclude air. Subsequent reactions (e.g., the conversion of RNase A to Pd-RNase A-OAC) gave diminished conversion which we attributed to the hydrolysis of the NHS ester functionality of **10**. Storage of **10** in a desiccator or at 4 °C under a nitrogen atmosphere gave no diminished reactivity profile even after 6 months. See Supporting Information for LC data before and after 6 months which indicated an ∼90:10 ratio of peak areas corresponding to reagent **10** (L = sSPhos) and its hydrolyzed form respectively.
(21) Minimal equivalents of Pd reagent **10** were employed in an effort to produce only singly modified RNase A or lysozyme C. In typical proteins, the N-terminal amino group has a pK_a value that is 2.8 ± 1.2 units lower than those in lysine side chains and, if sterically accessible, is likely to be the most readily acylated amino group. See: Grimsley, G. R.; Scholtz, J. M.; Pace, C. N. A summary of the measured p*K* values of the ionizable groups in folded proteins. *Protein Sci.* **2009**, *18*, 247−251. See Supporting Information for additional discussion and MS/MS information. Protocols for the modification of these protein substrates with NHS ester bearing reagents were adapted from: Chen, X.; Muthoosamy, K.; Pfisterer, A.; Neumann, B.; Weil, T. Site-selective lysine modification of native proteins and peptides via kinetically controlled labelling. *Bioconjugate Chem.* **2012**, *23*, 500−508. In addition, we note that excess equivalents of Pd-reagent **10** led to observable precipitation.
(22) The Pd−protein OACs were stored at −20 °C either as lyophilized solids or as ∼0.5 mg/mL solutions in aqueous buffer (see Supporting Information for additional details).
(23) The observed palladium content in protein is comparable to reported values after the treatment of proteins with palladium-based reagents. See ref 8 above, as well as: Maity, S. K.; Mann, G.; Jbara, M.; Laps, S.; Kamnesky, G.; Brik, A. Palladium-assisted removal of a solubilizing tag from a Cys side chain to facilitate peptide and protein synthesis. *Org. Lett.* **2016**, *18*, 3026−3029.
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