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Storable palladacycles for selective functionalization of alkyne-containing proteins†‡

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We report the facile preparation of palladacycles as storable arylpalladium(II) reagents from acetanilides *via* cyclopalladation. The palladacycles exhibit good stability in PBS buffer and are capable of functionalizing a metabolically encoded HPG-containing protein, thus providing a new type of biocompatible organometallic reagent for selectively functionalizing the alkyne-encoded proteins.

Bioorthogonal reactions have provided chemical tools to study biomolecular dynamics and function in living systems.[1–4] Similar to azides, terminal alkynes have become attractive bioorthogonal chemical reporters due to their small size, excellent biocompatibility, and ease of incorporation into proteins. Importantly, the alkynes readily react with the azide probes *via* Cu-catalyzed azide–alkyne cycloaddition[5–8] or strain-promoted cycloaddition reaction.[9] Recently, we developed a new protein bioconjugation strategy through a palladium-mediated cross-coupling reaction to label an alkyne-encoded protein *in vitro* and in *E. coli*.[10] In this strategy, we employed a two-step procedure in which we generate the 'preactivated' arylpalladium(II) complex first followed by the cross-coupling reaction with the alkyne-encoded protein. The reaction was clean and efficient, however, the *in situ* generated arylpalladium(II) complex gradually loses its reactivity in phosphate buffered saline (PBS) over time. Hence, excess amount of reagent is typically required in order to achieve high conversion. At about the same time, Myers and co-workers reported the preparation of storable arylpalladium(II) reagents[11] through the decarboxylative palladation and showed that these reagents were capable of labeling the olefinic substrates *via* stoichiometric Heck-type reaction in aqueous media. Inspired by this report, we envisioned that by introducing a weak-coordination directing group[12,13] in our palladium complex, we may achieve an optimum balance between reactivity and stability. Herein, we describe the preparation of the storable palladacycles with an *ortho*-directing group, the characterization of their stability in PBS buffer, and their use in selective functionalization of an alkyne-encoded protein in PBS under mild conditions.

We first prepared a series of palladacycles with varying substituents on the aromatic ring from the acetanilide derivatives by following Yu's cyclopalladation protocol.[14] We found that substrates with methoxy substituents (except at the *ortho* position which is probably due to the *ortho*-effect[14]) provided the desired palladacycles in excellent yields (Table 1, entries 2 and 12) whereas substrates with methyl substituents afforded modest yields[15] (entries 8 and 9) similar to **2a** without any substituents.[14,16,17] Interestingly, fluorine substitution gave rise to high yields (entries 6 and 10) compared to chlorine which gave much lower yields (entries 5 and 7). BODIPY substrate **1k** served as a poor substrate for the cyclopalladation reaction, giving only 35% yield (entry 11). Notably, high regioselectivity was observed in the formation of palladacycle **2d** from 1-acetamidonaphthalene **1d** (see ESI‡). Using $Pd(O_2CCF_3)$ as a palladium source,[18] we can also readily generate palladacycles containing a fluorescein or a PEG (MW $\approx$ 5 kDa) group (entries 14 and 15).

With the palladacycles in hand, we examined their stability in PBS buffer. Gratifyingly, most palladacycles exhibited high tolerance to the PBS buffer–DMSO (19 : 1) mixture as monitored by ^{1}H NMR within 24 h (see Table S1 in ESI‡). Next, we assessed whether palladacycle **2a** can be employed to modify a metabolically encoded homopropargylglycine (HPG)-containing ubiquitin (HPG-Ub)[10] *via* a cross-coupling reaction process (Scheme 1). Treating 2.5 μM of HPG-Ub with 4 equiv. of palladacycle **2a** in PBS buffer at 37 °C for 30 min afforded the ligation product **3a** in 70% yield, based on LC-MS analysis. Prolonging the reaction time to 4 h increased the yield to 93%. Likewise, addition of a large excess of palladacycle **2a** (25 equiv.) increased the yield to 92% (Scheme 1). This encouraging result led us to investigate the rest of the palladacycles (**2b–2o**) in the series using 4 equiv. at three time points, namely, 30 min, 2 h, and 4 h, and the results are summarized in Table 2. In the palladacycle series, **2d** with the naphthyl ring showed the highest ligation efficiency, reaching essentially quantitative yield within 30 min. Palladacycles with methyl, methoxy, or fluorine substituents on the aromatic ring (Table 2, entries 2, 3, 6, 8, 9, 10, and 12) also gave high yields. However, the reaction with chloro-substituted palladacycles

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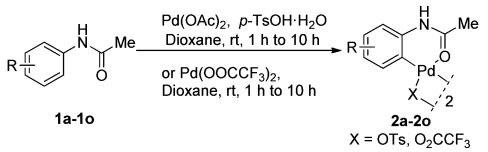
† This manuscript is dedicated to Professor Andrew Hamilton on the occasion of his 60th birthday.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c3cc43479f

Table 1 Synthesis of palladacycles[a]



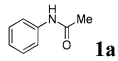
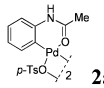
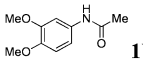
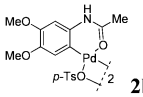
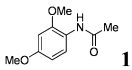
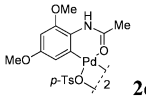
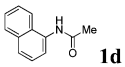
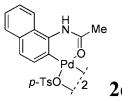
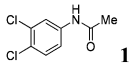
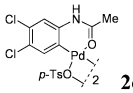
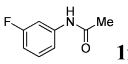
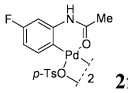
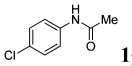
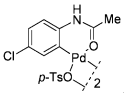
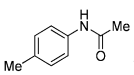
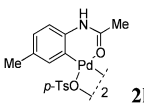
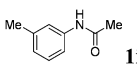
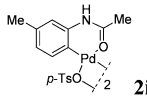
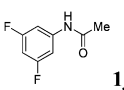
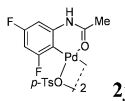
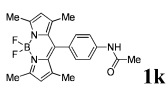
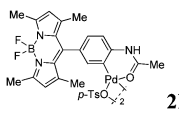
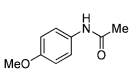
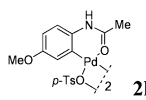
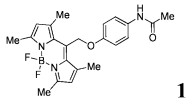
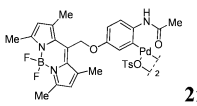
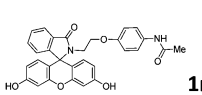
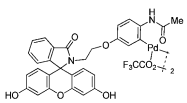
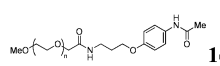
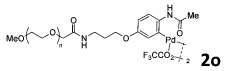
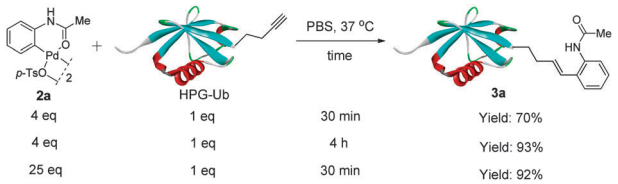
Entry	Aryl acetanilide	Palladacycles	Yield[b] (%)
1	 **1a**	 **2a**	75
2	 **1b**	 **2b**	93
3	 **1c**	 **2c**	68
4	 **1d**	 **2d**	87
5	 **1e**	 **2e**	54
6	 **1f**	 **2f**	95
7	 **1g**	 **2g**	67
8	 **1h**	 **2h**	70
9	 **1i**	 **2i**	71
10	 **1j**	 **2j**	73
11	 **1k**	 **2k**	35
12	 **1l**	 **2l**	92
13	 **1m**	 **2m**	72
14	 **1n**	 **2n**	60

Table 1 (*continued*)

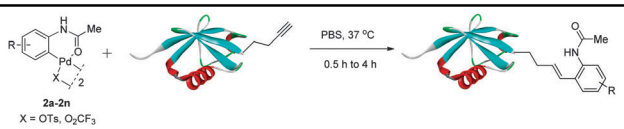
Entry	Aryl acetanilide	Palladacycles	Yield[b] (%)
15	 **1o**	 **2o**	85[c]

[a] Reactions were carried out with 0.1 mmol acetanilide, 1 equiv. of $Pd(OAc)_2$ and 1 equiv. of *p*-TsOH·H_2O in 1 mL of dioxane at room temperature for 1–10 h. [b] Isolated yields. [c] Estimated based on ^1H NMR.



Scheme 1 Optimization of reaction conditions.

Table 2 Reactions of palladacycles with HPG-Ub[a]



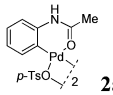
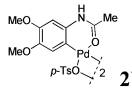
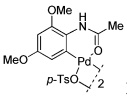
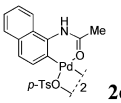
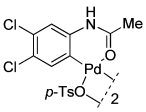
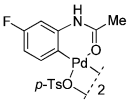
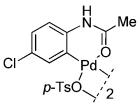
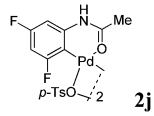
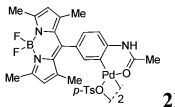
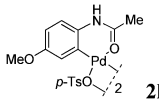
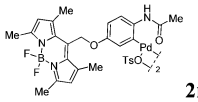
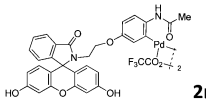
Entry	Palladacycles	Yield[b] (%) 0.5 h	2 h	4 h
1	 **2a**	70	96	93
2	 **2b**	65	84	93
3	 **2c**	49	70	80
4	 **2d**	96	97	99
5	 **2e**	20	33	42
6	 **2f**	68	85	89
7	 **2g**	45	56	61
8	**2h**	82	90	98
9	**2i**	83	88	92

Table 2 (*continued*)

Entry	Palladacycles	Yield[b] (%) 0.5 h	2 h	4 h
10	 **2j**	59	70	80
11	 **2k**	30[c]	ND	ND
12	 **2l**	60	76	85
13	 **2m**	14[c]	ND	ND
14	 **2n**	60[c]	ND	ND

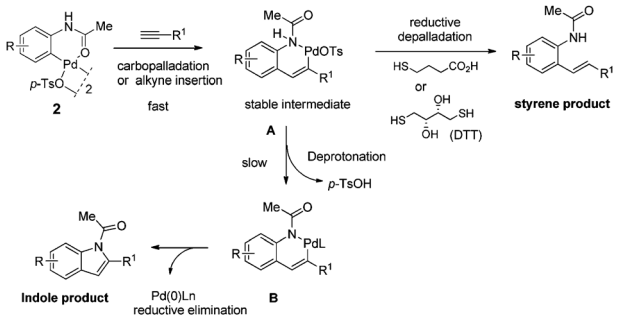
[a] Reactions were carried out using 2.5 μM of HPG-Ub and 4 equiv. of palladacycle at 37 °C. [b] Yields were determined based on LC-MS analysis: yield% = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$, where $I_{product}$ and $I_{HPG\text{-}Ub}$ represent the ion counts of the ligated product and HPG-Ub, respectively. [c] 25 equiv. of palladacycle was used. ND, not determined.

2e and **2g** proceeded sluggishly, giving lower yields (Table 2, entries 5 and 7). In addition, palladacycles **2k** and **2m** carrying a BODIPY group gave only 30% and 14% yields, respectively, when 25 equiv. of palladacycles were employed (Table 2, entries 11 and 13). The lower reactivity can be attributed to their poor solubility in PBS buffer. Palladacycle **2n** with a fluorescein group gave a relatively higher yield (60%) under the same conditions (Table 2, entry 14).

To elucidate the structure of the ligation product, the adduct of HPG-Ub with palladacycle **2d** was digested with trypsin and subsequently analysed by LC-MS. The mass of the naphthyl acetanilide-modified C-terminal pentapeptide is consistent with a structure in which the linkage between naphthalene and the peptide fragment is a double bond (Fig. S1, Tables S2 and S3, ESI‡).

To verify the selectivity of this type of ligation, we analysed the reaction mixture of HPG-Ub with the BODIPY-functionalized palladacycle **2k** by SDS-PAGE. In-gel fluorescence analysis revealed that only HPG-Ub was fluorescently labelled, while no fluorescent band was detected with wild-type ubiquitin under identical conditions (Fig. S2, ESI‡). Similarly, the reaction of HPG-Ub with PEG-containing palladacycle **2o** led to the concentration-dependent formation of a distinct higher molecular weight band by SDS-PAGE, indicating the selective formation of the PEGylated HPG-Ub adduct (Fig. S3, ESI‡).

To provide insights into the reaction mechanism, we tested the reaction of **2d** with *N*-acylated HPG-dipeptide **3** (ref. 10) under dilute condition (2.5 μM) in PBS buffer for 30 min (Fig. S4, ESI‡). LC-MS analysis showed the formation of a complex mixture containing styrene, alkyne (or indole), and some uncharacterized side products. Compared with the HPG-dipeptide substrate, HPG-Ub reacts much cleaner with the palladacycles. To explain this discrepancy, we propose that palladacycle **2** would undergo carbopalladation with alkyne (alkyne insertion) to afford a vinylpalladium(II) intermediate **A** (Scheme 2), which could be stabilized by coordination with nearby residues such as Arg in the protein substrate, thus preventing a path to indole formation *via* intermediate **B** or other side reactions such as multi-alkyne-insertion. Upon treatment with a reducing reagent such as 3-mercaptopropanoic acid or dithiothreitol, intermediate **A** would undergo reductive depalladation to generate the styrene product.



Scheme 2 Proposed mechanism for the generation of a styrene product.

In conclusion, we have synthesized stable palladacycles that are suitable for protein labelling in aqueous medium. These palladacycles were used to modify a terminal alkyne-encoded protein in PBS buffer at 37 °C to form the styrene adducts with moderate to high yields. Because of their superior stability and reactivity, these palladacycles may be useful for functionalizing the terminal alkyne-encoded proteins in cellular systems.

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