

# Copper-Free Sonogashira Cross-Coupling for Functionalization of Alkyne-Encoded Proteins in Aqueous Medium and in Bacterial Cells

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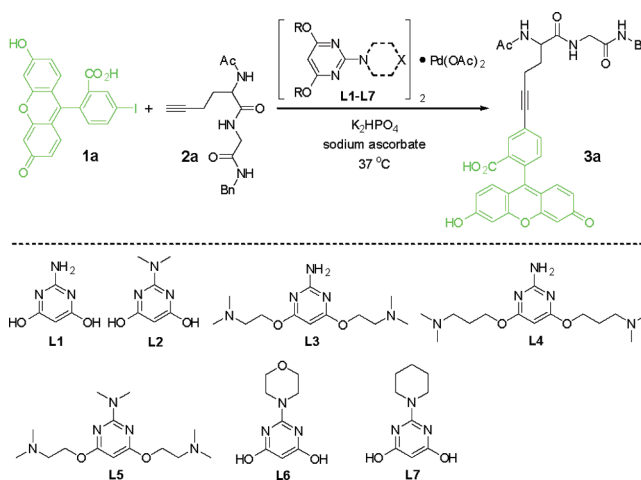
**S** Supporting Information

**ABSTRACT:** Bioorthogonal reactions suitable for functionalization of genetically or metabolically encoded alkynes, for example, copper-catalyzed azide–alkyne cycloaddition reaction (“click chemistry”), have provided chemical tools to study biomolecular dynamics and function in living systems. Despite its prominence in organic synthesis, copper-free Sonogashira cross-coupling reaction suitable for biological applications has not been reported. In this work, we report the discovery of a robust aminopyrimidine–palladium(II) complex for copper-free Sonogashira cross-coupling that enables selective functionalization of a homopropargylglycine (HPG)-encoded ubiquitin protein in aqueous medium. A wide range of aromatic groups including fluorophores and fluorinated aromatic compounds can be readily introduced into the HPG-containing ubiquitin under mild conditions with good to excellent yields. The suitability of this reaction for functionalization of HPG-encoded ubiquitin in *Escherichia coli* was also demonstrated. The high efficiency of this new catalytic system should greatly enhance the utility of Sonogashira cross-coupling in bioorthogonal chemistry.

Because of their small sizes and ease in the genetic/metabolic incorporation, simple organic groups such as azide, aldehyde, terminal alkyne, and terminal alkenes are attractive bioorthogonal reporters for studying biomolecular dynamics and functions in their native environment.<sup>1</sup> One of the key driving forces for this approach has been the emergence of a growing repertoire of bioorthogonal reactions<sup>2</sup> through which designed small-molecule probes can be selectively conjugated to pretagged biomolecules. For tracking biological dynamics, bioorthogonal functionalization needs to be rapid, highly selective, and high yielding. Indeed, an aldehyde can be selectively functionalized with the hydrazide or alkoxyamine-based probes via nucleophilic addition;<sup>3</sup> an azide can be functionalized with the alkyne probes via copper-catalyzed click chemistry,<sup>4</sup> strained-promoted cycloaddition,<sup>5</sup> or Staudinger ligation;<sup>6</sup> and terminal alkenes can be functionalized via either photoclick chemistry<sup>7</sup> or ruthenium-catalyzed olefin metathesis.<sup>8</sup> While many aqueous reactions involving terminal alkynes have been reported,<sup>9</sup> the viable option for functionalization of terminal alkynes in biological systems remains to be click chemistry.<sup>10</sup>

In an effort to apply palladium-catalyzed reactions to modify proteins in vitro, Yokoyama and co-workers previously reported a Sonogashira cross-coupling reaction between *N*-(prop-2-yn-1-yl)-biotinamide and an iodophenyl-containing Ras protein in an

**Table 1. Ligand Screen and Reaction Condition Optimization<sup>a</sup>**



entry	1a: 2a	Pd:ligand	ligand	time (min)	yield (%) <sup>b</sup>
1	1.05:1.00	20%	L1	30	NR
2	1.05:1.00	20%	L2	30	59
3	1.05:1.00	20%	L3	30	<5
4	1.05:1.00	20%	L4	30	<5
5	1.05:1.00	20%	L5	30	29
6	1.05:1.00	20%	L6	30	<5
7	1.05:1.00	20%	L7	30	13
8	1.00:2.40	30%	L2	40	91

<sup>a</sup> Reactions were carried out under ambient conditions without the need of inert gas protection. <sup>b</sup> Yields were derived by comparing the integration area of 3a in their respective HPLC traces to that of a standard curve. See Supporting Information for details.

aqueous medium containing 18% DMSO.<sup>11</sup> However, a low yield of 25% was achieved after 80-min incubation at 6 °C under N<sub>2</sub> protection; in addition, copper(I) salt was used as a cocatalyst, which further limits its potential in cellular applications because of the known cytotoxicity associated with copper.<sup>12</sup> Inspired by the recent report of a robust Suzuki–Miyaura cross-coupling with protein substrates in aqueous medium,<sup>13</sup> we envisioned that with a suitable palladium catalyst, efficient copper-free Sonogashira cross-coupling with protein substrates could be realized.

**Received:** July 18, 2011

**Published:** September 08, 2011

Table 2. Aryl Iodides in Aqueous Copper-Free Sonogashira Cross-Coupling Reactions<sup>a</sup>

Entry	Ar-I	Pd • L2	Conversion (%) <sup>b</sup>
1		30%	84
2		30%	95
3		30%	77
4		30%	88
5		30%	70
6		30%	72

<sup>a</sup> Reactions were carried out on a 4  $\mu$ mol scale at 37  $^{\circ}$ C with 1:2a ratio of 1:2.4. <sup>b</sup> Conversions were calculated based on the disappearance of aryl iodides in the reaction mixtures in the HPLC. See Supporting Information for details.

Here, we report the discovery of a new water-soluble palladium complex, and its utility in copper-free Sonogashira cross-coupling reactions in clean and rapid formation of new carbon–carbon bonds between a terminal alkyne-containing peptide/protein and a wide range of aryl iodides in aqueous medium. Furthermore, we demonstrate the suitability of this reaction for fluorescent labeling of an alkyne-encoded protein in *Escherichia coli* cells.

Our initial study focused on the Sonogashira cross-coupling between fluorescein iodide **1a** and a homopropargylglycine (HPG)-containing peptide **2a** using a water-soluble 2-amino-4,6-dihydropyrimidine (ADHP) as the palladium ligand because ADHP has been previously used successfully in the copper-free Sonogashira reactions in organic solvents.<sup>14</sup> However, no cross-coupling product was detected after 30 min (Table 1, entry 1); similar result was obtained when the reaction was extended to 3 h. We attributed this lack of reactivity to the hydration of ADHP at either the two hydroxy groups or the amino group in aqueous medium. To alter the hydration shell of ADHP and fine-tune the electron density of the pyrimidine ring, a series of pyrimidine-based ligands (**L2**–**L7**) were synthesized and their catalytic activities were examined in the model reaction in potassium phosphate buffer (Table 1). To our delight, we found that ligands **L2** and **L5** with the dimethylamino substituent gave the desired product **3a** in 59% and 29% yield, respectively (entries 2 and 5). Blocking the two hydroxyl groups led to trace amount of product (entries 3 and 4). Replacement of the amino group with bulkier morpholine or piperidine group resulted in substantially lower yields (entries 6 and 7). Using the palladium–ligand **L2** complex, we obtained the cross-coupled product **3a** in 91% yield when palladium complex usage was

Table 3. Aryl Iodide Substrates for Cross-Coupling with HPG–Ub<sup>a</sup>

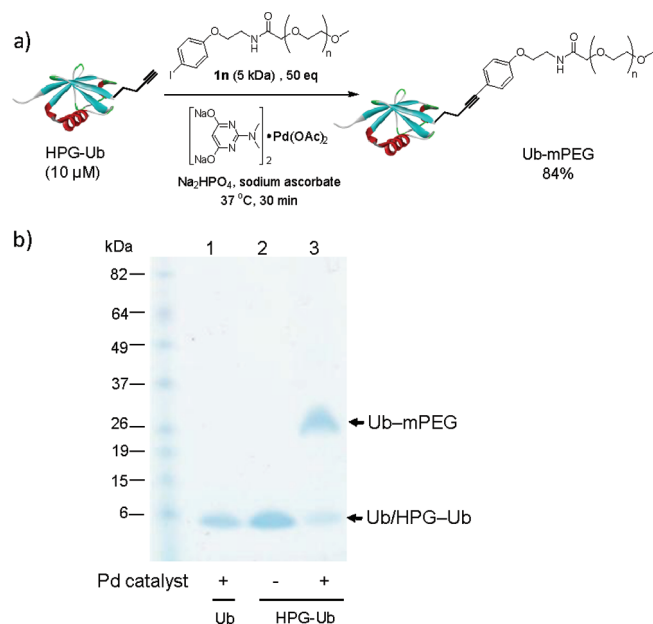
Entry	Ar-I	Time	Yield (%) <sup>b</sup>
1		30 min	93
2		30 min	83
3		30 min	55
4		30 min	86
5		240 min	73 <sup>c</sup>
6		30 min	83
7		180 min	73
8		30 min	82
9		30 min	85
10		30 min	78
11		30 min	79
12		180 min	66
13		180 min	60

<sup>a</sup> Reactions were carried out with 5–10  $\mu$ M of HPG–Ub and 50 equiv of aryl iodide and palladium complex at 37  $^{\circ}$ C under ambient conditions.

<sup>b</sup> Yields were determined based on LC–MS analysis of the reaction mixtures based on the equation: yield % =  $I_{\text{product}} / (I_{\text{HPG-Ub}} + I_{\text{product}})$ . When side products were detected, the yield was calculated using the equation: yield % =  $I_{\text{product}} / (I_{\text{HPG-Ub}} + I_{\text{product}} + I_{\text{side product}})$ , where  $I_{\text{HPG-Ub}}$ ,  $I_{\text{product}}$ , and  $I_{\text{side product}}$  represent the ion counts of the remaining HPG–Ub, product, and side product, respectively. See Supporting Information for details. <sup>c</sup> Hydrolysis of the lactone ring was observed in mass spectrum.

increased to 30% along with an excess of **2a** and a slightly longer reaction time (entry 8).

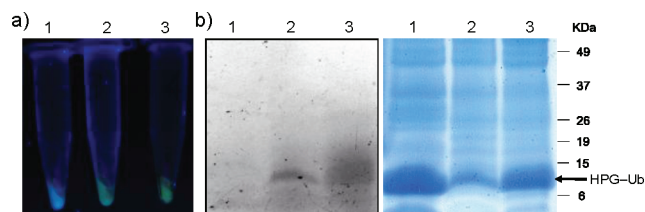
With the optimal conditions in hand, we then investigated the generality of this protocol for various aryl iodide (Table 2). Both *ortho*- or *para*-substituted aryl iodides gave good conversions (entries 1, 3, and 5). Sterically hindered 2,4,6-trimethylphenyl



**Figure 1.** Selective PEGylation of HPG-Ub with an mPEG-linked phenyl iodide **1n** via copper-free Sonogashira cross-coupling. (a) Reaction scheme. (b) Coomassie blue staining of SDS-PAGE gel showing selective formation of the PEGylated Ub (Ub-mPEG): lane 1, control reaction with wild-type ubiquitin; lane 2, control reaction with HPG-Ub in the presence of 50 equiv of **1n** but absence of palladium complex; lane 3, reaction with HPG-Ub in the presence of 50 equiv of **1n** and palladium complex.

iodide also participated in the reaction and gave a 72% conversion (entry 6). Heterocyclic aryl iodide, 2-iodothiophene, underwent successful reaction with **2a** to afford the cross-coupled product in 95% conversion (entry 2). A fluorogenic 4-iodo-7-methoxy-coumarin was also a good substrate (entry 4). However, electron-deficient aryl iodides such as 4-nitrophenyl iodide gave poor conversions under these conditions.

The encouraging results with peptide substrate **2a** prompted us to examine whether these conditions can be employed to modify a HPG-encoded protein. We selected ubiquitin as a protein model because of its small size and robust fold.<sup>15</sup> Since HPG has been shown in the literature to be an efficient methionine surrogate for metabolic incorporation,<sup>16</sup> we appended a Met-Gly-Gly sequence to the C-terminus of ubiquitin on a pQE-80L construct, and expressed the HPG-containing ubiquitin in M15A, a methionine auxotroph, in the presence of HPG. After Ni-NTA affinity chromatography, the N-terminal His-tag, together with the first Met or HPG in the sequence, was cleaved by treating the protein with PreScission protease. The resulting ubiquitin was purified by FPLC to afford either wild-type ubiquitin (Ub) or HPG-encoded ubiquitin (HPG-Ub) with one HPG at its C-terminus. The HPG occupancy was determined to be 92% based on the LC-MS analysis. To test Sonogashira reaction with the HPG-containing proteins, we incubated HPG-Ub with 50 equiv of **1a** and 50 equiv of palladium complex using a two-step addition procedure.<sup>17</sup> Gratifyingly, the reaction reached completion in 30 min (Table 3, entry 1) as monitored by LC-MS. Control experiments showed that the reaction did not proceed in the absence of palladium complex. By taking advantage of the intrinsic fluorescence of **1a**, we subjected the reaction mixture to sodium dodecyl sulfate polyacrylamide gel electrophoresis



**Figure 2.** Fluorescent labeling of HPG-Ub by fluorescein iodide **1a** in *E. coli* cells via copper-free Sonogashira cross-coupling. (a) Fluorescence image of the cell pellets upon excitation at 365 nm. Pellet 1, cells collected after treatment with 100  $\mu$ M of fluorescein iodide **1a** but not palladium complex; Pellet 2, cells collected after treatment with 100  $\mu$ M **1a**, 1 mM palladium complex, 5 mM sodium ascorbate in  $\text{Na}_2\text{HPO}_4$  buffer at 37  $^\circ\text{C}$  for 4 h; Pellet 3, cells collected after treatment with the preactivated mixture of palladium complex (1 mM) and **1a** (100  $\mu$ M). See Supporting Information for the treatment details. (b) In-gel fluorescence (left panel) and Coomassie blue staining (right panel) of SDS-PAGE analysis of the proteins captured by Ni-NTA-agarose beads from the lysates of the three cell pellets.

(SDS-PAGE) followed by in-gel fluorescence analysis. A clear green fluorescent band was detected only for HPG-Ub but not for Ub (Figure S2). We then examined a range of aryl iodides containing various functional groups including hydroxy (**1i**), carboxylic acid (**1h**), fluorine (**1j**, **1k**), nitro (**1l**), and thiophene (**1c**) groups (Table 3). In general, Sonogashira cross-coupling reactions with HPG-Ub gave the modified proteins in good yields (55–93%) independent of the electronic structures of aryl iodides. Sterically hindered substrates such as **1g** and **1m** gave lower yields (entries 7 and 13), even with extended reaction times. To verify the yield and selectivity of the reaction, HPG-Ub was incubated with 50 equiv of phenyl iodide covalently linked to monodisperse polyethylene glycol (**1n**, MW  $\approx$  5 kDa) in a sodium phosphate buffer at 37  $^\circ\text{C}$  for 30 min, and the mixture was then analyzed by SDS-PAGE (Figure 1). A distinct higher molecular weight band corresponding to Ub-mPEG with an estimated 84% yield was observed, matching closely the yield for analogous substrate, 1-iodo-4-methoxy benzene (**1f**; Table 3, entry 6).<sup>18</sup> Importantly, no PEGylated products were detected in the absence of the palladium complex, and the reaction proceeded only with HPG-Ub and not with Ub (lane 1 in Figure 1), indicating that PEGylation occurs selectively with HPG via the Sonogashira cross-coupling.

Since many enzyme-mediated biochemical transformations are facilitated by transition metals,<sup>19</sup> selective palladium-catalyzed transformations inside living cells may offer an organometallic tool to manipulate biological processes. To assess whether the copper-free Sonogashira cross-coupling reaction can be employed to modify HPG-Ub in bacterial cells, we added 1 mM of palladium complex, 100  $\mu$ M of fluorescein iodide **1a**, and 5 mM of sodium ascorbate in sodium phosphate buffer to HPG-Ub-overexpressing M15A cells. After growing at 37  $^\circ\text{C}$  for 4 h, cells were collected and washed extensively with 0.9% NaCl solution to remove the excess reagents. The palladium complex-treated cells showed green fluorescence upon 365-nm excitation while the untreated cells did not (compare cell pellets 2 and 3 to 1 in Figure 2a). Subsequently, cells were lysed and HPG-Ub proteins in the cell lysates were captured with Ni-NTA-agarose beads. The beads were re-suspended in a protein elution buffer and heated to 95  $^\circ\text{C}$  for 5 min. After centrifugation, the supernatants were analyzed by SDS-PAGE. In the in-gel fluorescence



analysis, clear fluorescent bands were observed for samples derived from cell pellets 2 and 3, but not for the sample derived from pellet 1 (left panel, Figure 2b), indicating selective cross-coupling of **1a** with HPG–Ub inside *E. coli* cells. The identities of the fluorescent HPG–Ub bands were confirmed by Coomassie blue staining of the same gel (right panel, Figure 2b). The lower abundance of HPG–Ub protein seen in sample 2 was probably due to the inhibition of protein expression by the palladium complex and **1a** when they were added separately.<sup>20</sup>

In conclusion, we have discovered a new palladium complex for the aqueous copper-free Sonogashira cross-coupling reactions. This palladium complex promoted the efficient cross-coupling of a wide range of aryl iodides with the terminal alkyne-containing peptide and protein substrates in good to excellent yields. The capability of this cross-coupling reaction in functionalizing a metabolically encoded alkyne-containing protein in *E. coli* was also demonstrated, indicating a potential utility of this reaction in monitoring and manipulating proteins in cellular systems. Efforts to further optimize this palladium-catalyzed cross-coupling reaction for cellular applications, for example, by immobilizing the complex onto nanoparticles to improve cell penetration and reduce complex loading,<sup>21</sup> are currently underway.

## ■ ASSOCIATED CONTENT

**S** Supporting Information. Synthetic schemes for new ligands, experimental procedures, and characterization of all compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

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## ■ ACKNOWLEDGMENT

We gratefully acknowledge the National Institutes of Health (R01 GM 085092) for financial support.

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(17) (a) Our preliminary studies indicated that excess amount of the palladium complex was necessary to achieve high conversions due to nonspecific sequestration of the palladium complex by the proteins; see Figure S1 in Supporting Information for details. Similar phenomena were also observed in the palladium-catalyzed Suzuki–Miyaura cross-coupling reactions with protein substrates; see ref 13. To further alleviate this problem, we also pre-incubated the palladium complex with the aryl iodide (“pre-activation”) to minimize the effect of non-specific binding. (b) 2.4% DMSO was used in the reaction to help dissolve fluorescein iodide. (c) We found that adding the palladium complex/**1a** mixture pre-incubated for 1 h (“pre-activated”) into the HPG–Ub solution in two portions instead of one improved the reaction yield.

(18) The greater than 5 kDa increase in apparent MW is typically observed in electrophoresis of PEGylated proteins; see ref 7c as well as Lim, R. K.; Lin, Q. *Chem. Commun.* **2010**, *46*, 7993. Deiters, A.; Cropp, T. A.; Summerer, D.; Mukherji, M.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743.

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(20) In our cytotoxicity studies based on colony formation assay and growth curve measurement, the pre-activated palladium complex/**1a** mixture and **1a** itself did not show toxicity to *E. coli* cells while palladium complex and palladium complex/**1a** mixture added separately showed some level of cytotoxicity. See Figure S4 in Supporting Information for details.

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