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A Fluorogenic Dye Activated by the Staudinger Ligation

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In the emerging field of proteomics, the demands of parallel purification, quantitation, and identification of proteins from complex cellular extracts has necessitated the development of methods for their selective labeling with detectable probes.[1−3] Posttranslational modifications of proteins such as glycosylation and lipidation introduce significant additional complexity into the proteome.[4] While the protein itself may be labeled with gene-encoded tags such as fluorescent proteins[5] and FLAsH tetracysteine motifs,[6] there are few general tools available for labeling post-translational modifications.

Previous work in our lab has established that unnatural sugar substrates bearing bio-orthogonal functional groups can be metabolically incorporated into cellular glycoproteins.[7−10] Selective covalent reaction of the functional groups with biochemical or biophysical probes permits the detection and isolation of proteins bearing the corresponding sugar as a posttranslational modification. The azide is a sterically undemanding, bio-orthogonal electrophile that can be introduced into myriad metabolic precursors.[7] The Staudinger ligation between azides and engineered phosphine reagents (Figure 1A) has proven particularly useful for bio-orthogonal labeling of glycans on mammalian glycoproteins,[9] and unnatural amino acids within proteins expressed in *Escherichia coli*.[10] The primary labeling reagents used in these experiments, biotin[9] and FLAG peptide−phosphines,[10] were designed for detection using fluorescent secondary reagents (FITC−avidin and FITC−anti-FLAG antibody, respectively). The two-step procedure permitted removal of excess unbound primary label before the detection step, and as a result, minimized the background signal that can otherwise frustrate covalent detection experiments.

For some applications, however, it is either inconvenient or simply impossible to use such two-step labeling procedures. For example, excess primary labeling reagents cannot be easily washed away from intracellular compartments or from tissues of living organisms. If the ligation product could be readily distinguished from the excess primary detection agent, the unbound agent would not contribute to the background signal in an assay. A chemoselective reaction with this feature potentially obviates the need for a secondary reagent since detection of the product could be achieved in a single covalent labeling step.

Here we report a fluorogenic coumarin−phosphine dye (**1**, Figure 1B) that is activated by the Staudinger ligation with azides. Compound **1** comprises a triaryl phosphine in which one phosphorus ligand is a 2-substituted methyl benzoate that traps the intermediate iminophosphorane to generate, after hydrolysis, an amide-linked product.[9] A second ligand on phosphorus is a 7-amino coumarin attached via the 3-position. Substituents at this position of 7-amino coumarin dyes are known to strongly influence their fluorescence properties.[11] Before the Staudinger ligation, the lone pair of electrons on phosphorus quenches the excited state of the fluorophore,

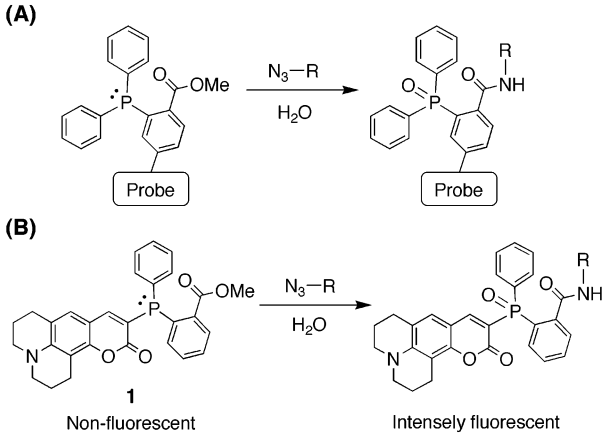

Figure 1. (A) The Staudinger ligation. (B) A coumarin−phosphine fluorogenic dye (**1**) activated by the Staudinger ligation.

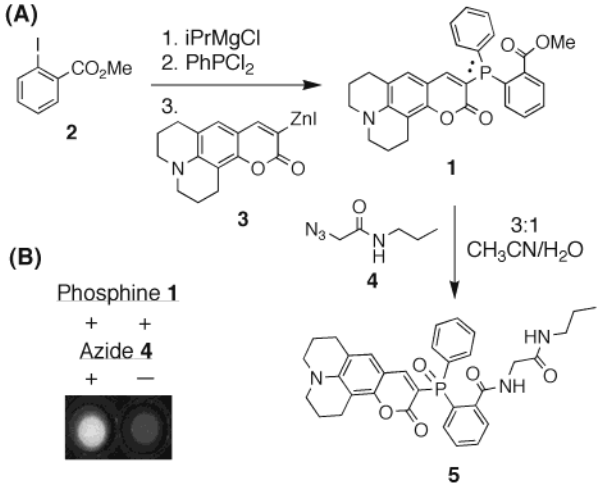

Figure 2. (A) Synthesis of compound **1**. (B) Detection of the fluorescence of ligation product **5** in a microtiter plate excited at 365 nm.

rendering the molecule nonfluorescent. The oxidation of the phosphine that occurs during the reaction eliminates quenching and activates fluorescence.

The synthesis of compound **1** is described in detail in the Supporting Information and is outlined in Figure 2A. Briefly, methyl 2-iodobenzoate (**2**) was converted to the Grignard reagent and then condensed with one equivalent of dichlorophenylphosphine. Subsequently, coumarin−zinc reagent **3** was added to generate compound **1**.

To test its reactivity and fluorescence properties, we reacted compound **1** with model azide **4** in an acetonitrile/water[12] mixture, affording the ligated phosphine oxide **5** as the exclusive product (Figure 2A). The increase in fluorescence during the reaction of **1**

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Table 1. Photophysical Parameters of Coumarin Analogues

	ϵ (M^{-1} cm^{-1})[a]	λ_{abs} (nm)[a]	λ_{ex} (nm)[b]	λ_{em} (nm)[b]	quantum yield
1	33,000 ± 2000	421	432	507	0.011 ± 0.001
5	47,000 ± 2000	430	443	495	0.65 ± 0.04

[a] Measured in ethanol. [b] Measured in phosphate-buffered saline (PBS).

and **4** was immediately evident and did not occur in control reactions lacking **4** (Figure 2B).

The photophysical parameters of phosphine **1** and phosphine oxide **5** are quite distinct and are summarized in Table 1. The maximum absorbance of the ligated product **5** was slightly red-shifted compared to phosphine **1**. In addition, the molar absorptivity of **5** was greater. The excitation spectra measured in phosphate-buffered saline were observed to have maxima at 432 and 443 nm for compounds **1** and **5**, respectively. The most profound difference between the two compounds was observed in their quantum yields of fluorescence. Compound **5** showed intense emission with a maximum at 495 nm and a quantum yield relative to quinine sulfate of 0.65 ± 0.04. In contrast, phosphine **1** exhibited a very weak fluorescence with a quantum yield of 0.011 ± 0.001 and an emission maximum at 507 nm. Thus, reaction of phosphine **1** with azides produces a product easily distinguishable from the reactants by its intense fluorescence.

To demonstrate the utility of compound **1** for biomolecule labeling, we reacted the phosphine dye with recombinant murine dihydrofolate reductase bearing azidohomoalanine residues in place of native methionine residues.[10] This azido protein (azido-mDHFR) was generated by metabolic incorporation of azidohomoalanine during overexpression in a methionine auxotrophoic *E. coli* strain, as previously reported.[10] Azido-mDHFR and native mDHFR were incubated with 200 μM phosphine **1**, and the crude reactions were loaded directly onto a gel with no separation of unreacted dye. Analysis of the gel by silver stain (lanes 1 and 2) and fluorescence imaging (lanes 3 and 4) showed specific labeling of the azido protein by compound **1** and no detectable labeling of the native protein lacking azides (Figure 3). Unlike previous experiments using FLAG and biotin conjugates, the labeled protein could be directly observed without need for Western blotting, washing, or secondary labeling steps.

We determined the rate of reaction between phosphine **1** and azide **4**, exploiting fluorescence as a measure of product formation, to optimize conditions for biomolecule labeling (Figure 4**)**. In the absence of azide **4** there was no significant change in the fluorescence intensity with time, an indication that background oxidation (presumably with dissolved molecular oxygen) occurs at a relatively low rate. The rate data obtained in these experiments were used to calculate an apparent second-order rate constant of 0.015 M^{-1} s^{-1}.[13] This value suggests that relatively high concentrations (i.e., 100 μM to mM) of compound **1** will be required to label azides present at low levels within metabolically labeled biomolecules at an appreciable rate. Therefore, the low background fluorescence of compound **1** will be particularly important. Efforts are underway to increase the rate of reaction by modulating the substituents on phosphorus. The application of this tool in the detection and quantitation of azide-labeled post-translational modifications in vitro and in vivo is also of significant future interest.

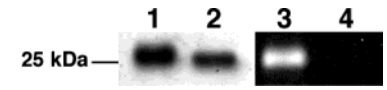

Figure 3. Specific labeling of azido-mDHFR with phosphine **1**. Purified azido-mDHFR (lanes 1 and 3) and native mDHRF (lanes 2 and 4) were incubated overnight with 200 μM **1** in 40% EtOH/PBS, pH 7.1, and the crude reaction was analyzed by 12% SDS-PAGE. Lanes 1 and 2 show the silver-stained gel, indicating total protein content. Lanes 3 and 4 show the same gel analyzed by fluorescence imaging (excitation at 457 nm, emission collected with a 520 nm band-pass filter).

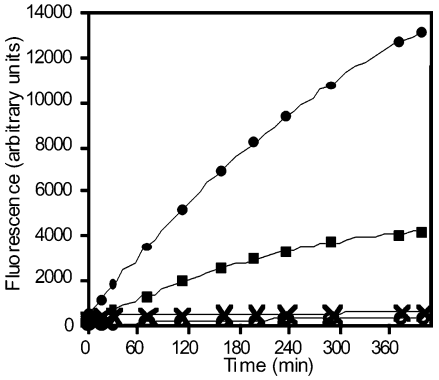

Figure 4. Kinetics of the reaction between compounds **1** and **4** under pseudo-first-order conditions: (●) 5 mM **4**, 0.011 mM **1**; (■) 5 mM **4**, 0.0022 mM **1**; (×) 0.011 mM **1**; (○) 0.0022 mM **1**. Fluorescence intensities were determined at λ_{ex} = 443 nm and λ_{em} = 493 nm.

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Supporting Information Available: Synthetic procedures and analytical data for compound **1** and its precursors, experimental details for fluorescence quantum yield and kinetic measurements, excitation and emission spectra for compounds **1** and **5**, and procedures for protein labeling (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The reaction can also be performed in strictly aqueous media, but the solubility of phosphine **1** therein has a limit around 60 μM.
(13) This value is similar to that reported for the reaction of triphenylphosphine with ethyl 2-azidoacetate in benzene (0.01 M^{-1} s^{-1}). Leffler, J. E.; Temple, R. D. *J. Am. Chem. Soc.* **1967**, *89*, 5235−5246.

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