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Hydrophilic α-Aryl-α-Diazoamides for Protein Esterification

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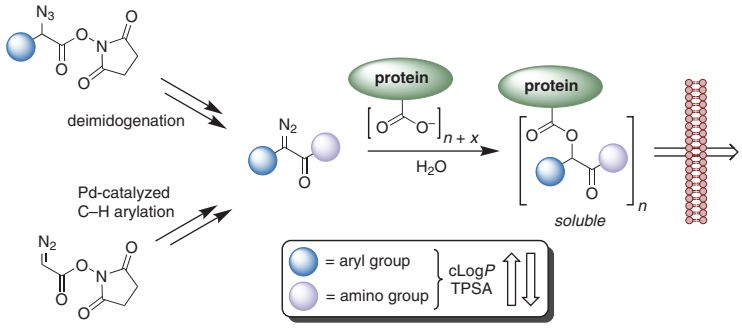

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Abstract Bioreversible protein esterification is a simple, customizable, and traceless strategy for the exogenous delivery of proteins into mammalian cells. Enabling this protein delivery strategy are α-aryl-α-diazoamides bearing a tolyl moiety. The aqueous solubility of the ensuing esterified protein is, however, often compromised, which can result in the loss of soluble esterified protein for downstream applications. Here, we undertook a structure–activity relationship campaign to generate hydrophilic diazoamides for use as protein esterification and cellular delivery agents. We find that the careful adjustment of the hydrogen-bond basicity of α-aryl-α-diazoamides is sufficient to engender soluble esterified proteins, as high hydrogen-bond basicity correlates with high aqueous solubility. Importantly, enhancing aqueous solubility of diazoamides should proceed *pari passu* with preserving their lipophilicity and reactivity towards esterification of carboxylic acids, as the best-performing diazoamide from our study contains an *N*-acetyl piperazine while retaining the tolyl moiety. Our efforts can inspire new generations of esterified proteins with better solubility.

Key words cell, delivery, diazo compounds, esterification, hydrophilicity, hydrophobicity, proteins, solubility

Innovations in intracellular delivery technologies have greatly expanded the repertoire of exogenously delivered proteins that can serve as modulators of biological function, imaging tools, and enzymes for diverse biotechnological applications. Notable strategies to facilitate protein delivery into the cytosol of mammalian cells include covalent modification of proteins with cell-penetrating vectors (such as cationic cell-penetrating peptides,[1] pH-responsive peptides,[2] and cell-permeant proteins[3]), encapsulation with nanoscale carriers (nanoparticles,[4] polycationic synthetic polymers,[5] poly(di)sulfide cationic polymers,[6] and liposomes[7]), formation of coacervate microdroplets,[8] coadministration with endosomolytic peptides,[9] and mechanical disruption of the cell membrane (microinjection and electroporation).

Exogenous protein delivery via esterification is added to the list of strategies for the vectorial delivery of protein into cells. Conceptually, protein esterification seeks to reduce the anionic character of a protein through the conversion of otherwise anionic carboxylate groups from aspartic acid, glutamic acid, and the C terminus into neutral esters.[10] To date, the bioreversible protein esterification strategy – using α-aryl-α-diazoamides – has been deployed to deliver green fluorescent protein (GFP),[10c] ribonuclease A,[11] and cytochrome c (Cyt c)[12] into the cytosol of mammalian cells, and for the on-demand triggering of enzymic catalysis.[13]

Unsurprisingly, esterifying anionic proteins has a deleterious effect on protein solubility in aqueous buffers, as the ensuing esterified protein product is typically more hydrophobic than the native protein.[14] In addition, protein esterification increases the isoelectric point (pI) of the protein product – a consequence of the net removal of anionic charge. For anionic proteins (pI < 7), esterification can raise the pI to physiological pH, diminishing solubility.[15] Such abrupt changes in hydrophobicity and pI can lead to the precipitation of proteins during esterification or during postreaction purification protocols that entail the transfer of an esterified protein into a buffer such as phosphate-buffered saline (PBS).

Current strategies for enhancing the acquisition of an esterified protein are restricted to (1) beginning with large quantities of the native protein, (2) reducing the number of pendant esters through the careful optimization of reaction conditions,[14b] and (3) formulating with an excipient-supplemented buffer.[14a] In the last strategy, protein precipitation that occurs during postreaction exchange into near-physiological pH buffers is best mitigated with β-cyclodextrin (β-CD).[14a] Such formulations might not, however, be

appropriate for all applications. For example, β-CD can affect the integrity of cellular membranes.[16] Moreover, cyclodextrin can encapsulate α-aryl-α-diazoamides and sterically diminish their reactivity with a protein.[14a]

We sought a different, general strategy for enhancing the aqueous solubility of esterified protein products during and after an esterification reaction. We were aware that the aqueous solubility of a small molecule is related to its lipophilicity or partition coefficient, log*P*, which refers to the ratio of its solute concentration in octanol to its solute concentration in water.[17] Compounds with low log*P* values tend to be more hydrophilic than those with high log*P* values. We reasoned that the problematic insolubility incurred upon protein esterification could be alleviated by increasing the hydrophilicity of the nascent ester. Herein, we demonstrate that incorporating hydrophilic functional groups in α-aryl-α-diazoamides increases the yield of soluble esterified proteins.

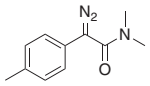
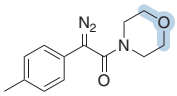
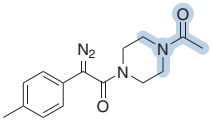
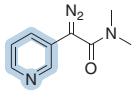
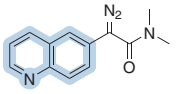
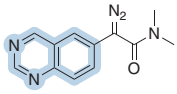
To begin, we sought to increase the aqueous solubility of an esterified protein by increasing the number of hydrogen-bond acceptors in the chemical structure of α-aryl-α-diazoamides. The hydrogen-bond acceptor strength of a functional group can be expressed as a measure of its hydrogen-bond basicity (pK_{BHX}) based on its complexation with 4-fluorophenol as in the equation $pK_{BHX} = -\log_{10}K_{BHX} = \log_{10}K$, where K is the equilibrium association constant of the hydrogen-bond acceptor with 4-fluorophenol. Hydrogen acceptors with a high value of K have a high value of pK_{BHK} and typically exhibit greater aqueous solubility.[18]

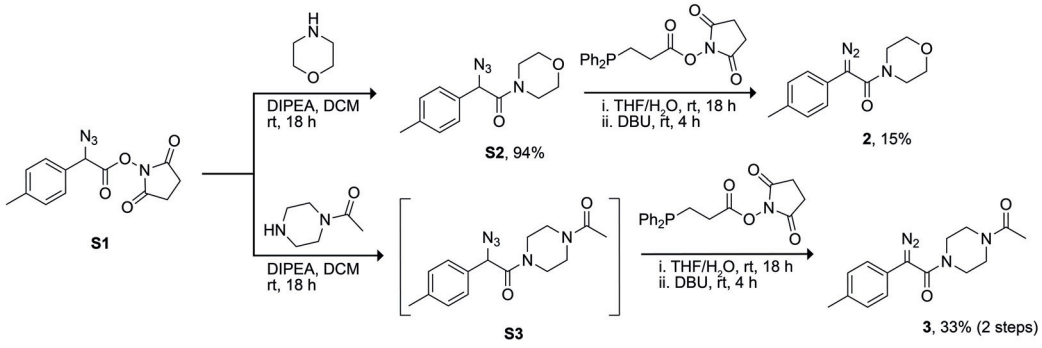
With diazoamide **1**[10c] as a starting scaffold, we elected to replace the dimethyl amide group with either a morpholino group or a 1-acetylpiperazine, to generate diazoamides **2** and **3**, respectively. Using software from Molinspiration Cheminformatics,[19] the cLog*P* values of diazoamides **2** and **3** were determined to be 1.61 and 1.02, respectively (Table 1).[20] The morpholino and 1-acetylpiperazine substitutions allowed for sizeable changes in the lipophilicity of the synthesized esterification reagents in comparison to diazoamide **1** (cLog*P* = 1.32) because the hydrogen-bond capacity of ethers is typically pK_{BHX} 1.00–1.50, and that of amides is typically pK_{BHX} 2.00–2.55.[18] In addition, these substitutions yielded compounds with increased topological polar surface area (TPSA[19]) when compared to diazoamide **1**. Interestingly, diazoamide **2** has a higher cLog*P* value than diazoamide **1**, even though it has a larger TPSA (Table 1).

Diazoamides **2** and **3** were synthesized via deimidogenation[21] of their respective azido precursors with a phosphinoester (Scheme 1). The synthetic yields were compromised by product degradation during chromatography on silica gel. Using GFP (29 kDa, pI ca. 6.2) as a model, we evaluated the ability of the synthesized diazo compounds to yield a soluble esterified protein, both during the esterification reaction and during exchange into PBS. Esterification of GFP to similar levels with either diazoamide **1** or **2** and subsequent exchange into PBS resulted in protein precipitation and complete loss of the esterified protein.[22] It is important to note that for all esterification reactions involving GFP herein, we used a small quantity of the protein (5–10 nmol) to mimic situations with limited protein availability. Esterification of GFP with diazoamide **3** yielded soluble esterified proteins with only slight precipitation during exchange into PBS (Figure 1). Though able to afford soluble esterified proteins, diazoamide **3** was a less efficient esterification reagent than diazoamide **1** and required more equivalents for esterification. This reduced reactivity towards carboxylates could be due to a steric effect from the 1-acetylpiperazine moiety compared to the dimethylamino group in diazoamide **1**. Taken together, these results suggest that modulating the hydrogen-bond acceptor capacity of diazo compounds is potentially sufficient for enhancing the solubility of esterified proteins.

Next, we explored the impact of modifying the aryl ring in α-aryl-α-diazoamides. Replacing the toluene moiety of diazoamide **1** with pyridine yielded diazoamide **4**, which is

Table 1 Physiochemical Properties and Outcome for the Esterification of GFP (10.0 nmol; 200 μM) by Diazoamides **1**–**6**

Diazoamide (equiv)	cLog*P*	TPSA	Median number of esters
 **1** (100)	1.32	46.61	(precipitation)
 **2** (150)	1.61	55.85	(precipitation)
 **3** (400)	1.02	66.92	12
 **4** (600)	–0.36	59.51	12
 **5** (400)	0.86	59.51	(precipitation)
 **6** (400)	0.45	72.40	10



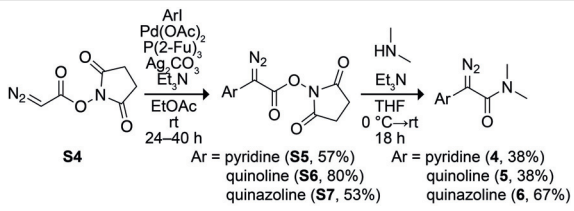
Scheme 1 Synthetic route to diazoamides **2** and **3**

markedly more hydrophilic (Table 1), attributable to the strong hydrogen-bond acceptor capacity of pyridine (pK_{BHX} = 1.86) compared to benzene (pK_{BHX} –0.49).[18] The pyridyl derivative was synthesized by the palladium-catalyzed C–H arylation[23] of *N*-succinimidyl 2-diazoacetate (**S4**) with 3-iodopyridine to generate *N*-succinimidyl pyridyl diazoacetate (**S5**). Subsequent aminolysis with dimethylamine yielded the desired pyridyl diazoamide **4** (Scheme 2). With diazoamide **4** in hand, we assessed its ability to generate a soluble esterified GFP. Diazoamide **4** yielded extensive labeling of GFP with no observable precipitation of the esterified protein product during the reaction or during exchange into PBS (Table 1). Diazoamide **4** is, however, an inefficient protein esterification reagent compared to the tolyl derivatives (**1**–**3**) and requires a large molar excess (800 equiv) to achieve substantial esterification. Moreover, esterification with diazoamide **4** did not proceed at ambient temperature and was achievable only at 37 °C and pH 5.5. The attenuated reactivity of diazoamide **4** is not surprising, given the electron-withdrawal by the pyridyl ring. The ensuing reduction in the basicity of the diazo group diminishes its ability to deprotonate the carboxylic acid, as is required in the mechanism for esterification.[24]

Moving on, we reasoned that the hydrophilicity of the pyridyl ring could be combined with the hydrophobic tolyl group to generate amphiphilic diazo compounds that yield protein conjugates with high aqueous solubility. Indeed, the installation of pyridyl ester tags on GFP prior to esterification with diazoamide **1** lessened protein precipitation during the workflow compared to esterification GFP with diazoamide **1** alone. Specifically, a median label of two or three pyridyl tags delivered soluble esterified GFP with ca. 9–10 total ester tags as observed by mass spectrometry (Figure 2).[26] Overall, these results demonstrate the aqueous solubility enhancement provided by diazoamide **4**.

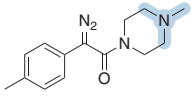
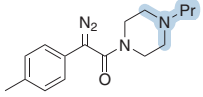
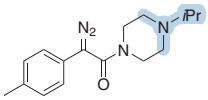
Next, we prepared diazoamides **5** and **6** by a similar synthetic route to diazoamide **4** (Scheme 2). Diazoamide **5** is a quinoline derivative of diazoamide **1** with a moderate cLog*P* value of 0.86. Attempts to esterify GFP with diazoamide **5** resulted precipitation during the reaction as well as during postesterification exchange into PBS (Table 1). In contrast, quinazoline diazoamide **6** (cLog*P* = 0.45) yielded soluble esterified GFP (Table 1). That is surprising because the pyrimidine moiety in a quinazoline has a lower hydrogen-bond capacity (pK_{BHX} ca. 1.1) than does the pyridine moiety in a quinolone (pK_{BHX} 1.86).[18]

Finally, we asked whether incorporating an ionizable center in the design of α-aryl-α-diazoamides would enhance the solubility of an esterified protein. We were specifically interested in *N*-alkyl piperazines as they are widely



Scheme 2 Synthetic route to diazoamides **4**–**6**

Table 2 Physicochemical Properties and Outcome for the Esterification of GFP (10.0 nmol; 200 μM) by *N*-Alkyl Piperazine Diazoamides **7**–**9**

Diazoamide (equiv)	cLog*P* ($-NR_2H^+$)	TPSA ($-NR_2H^+$)	Median number of esters
 **7** (400)	1.66 (–1.50)	49.85 (51.05)	6–7
 **8** (400)	2.54 (–0.62)	49.85 (51.05)	6
 **9** (500)	2.33 (–0.76)	49.85 (51.05)	7

used as ionizable derivatives of moieties in nanoparticles[27] and small-molecule drugs.[28] To explore this strategy, we designed a set of *N*-alkyl piperazine derivatives of diazoamide **1** with pK_a values 6–7.[29] Notably, the neutral *N*-alkyl piperazine diazoamides have higher cLog*P* values when compared to diazoamide **1**, which could compromise the solubility of esterified proteins near neutral pH (Table 2). Nonetheless, the cLog*P* values of the ionized species are lower than those of pyridyl diazoamide **4**, suggestive of high protein solubility, especially during the esterification reaction. The *N*-alkyl diazoamides were obtained after aminolysis of the *N*-succinimidyl diazoacetate **S8** with the appropriate *N*-alkyl piperazine, as in Scheme 2.

With diazoamides **7**–**9** in hand, we carried out esterification reactions with GFP. All three diazo compounds produced esterified GFP products that were soluble in the reaction mixture (Table 2). We did, however, observe precipitation upon transfer into PBS. Nevertheless, these diazoamides did yield soluble esterified proteins, albeit with a low number of ester tags (median: 6–7 ester tags) compared to esterification with diazoamide **1**. The ability to yield soluble esterified GFP in PBS could be due to the strong hydrogen-bond basicity of the alkyl nitrogen atom, despite the expected high cLog*P* values of the esters at neutral pH. As with diazoamide **3**, a large molar excess of diazoamides **7**–**9** was required to engender substantial esterification of GFP. Taken together, these results suggest that incorporating an ionizable nitrogen atom in the design of α-aryl-α-diazoamides can generate soluble esterified proteins at neutral conditions.

We then assessed the ability to select diazoamides that yielded soluble protein after buffer exchange to vectorially deliver GFP into mammalian cells. Esterified GFP was prepared from 10 nmol of starting native protein with diazoamides **3**, **4**, **7**, **8**, and **9**. Live CHO-K1 cells were treated with either GFP–**3** or GFP–**4**, followed by live-cell fluores-

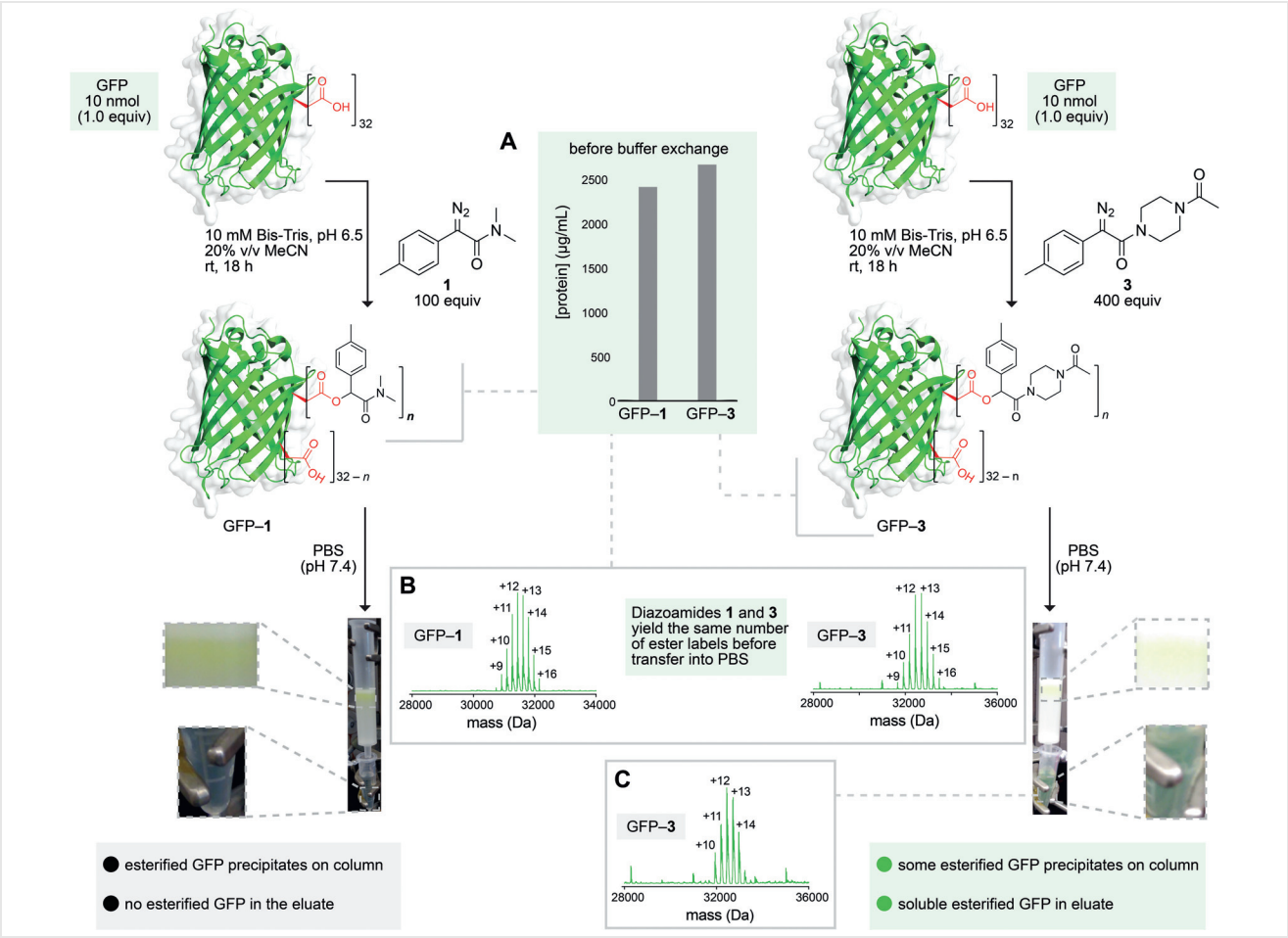


Figure 1 Comparative evaluation of GFP esterification with diazoamides **1** and **3**. GFP (10 nmol) was esterified in 10 mM Bis-Tris buffer, pH 6.5, containing MeCN (20% v/v). (A) Concentration of soluble esterified proteins in reaction mixtures. (B) Deconvoluted ESI-MS characterization of GFP–**1** and GFP–**3** at the end of the reaction before buffer exchange. (C) Deconvoluted ESI-MS characterization of soluble GFP–**3** after exchange into PBS. Images of GFP were created with PyMOL software and PDB entry 2b3p.[25]

cence microscopy. The obtained images showed that GFP–**3** accumulates within the cells at both 37 °C and 4 °C, which suggests an energy-independent mode of cellular uptake (Figure 3).[30] Cellular uptake of GFP–**4** was not observed, suggesting that heavily solvated esters deter productive interactions between the esterified protein and the lipophilic plasma membrane of mammalian cells. Esters generated with *N*-alkyl piperazine diazoamides were inefficient at delivering GFP to human melanoma cells (M21 cell line; Figure S1). The inefficient cellular uptake could be due to the low number of pendant esters generated by these diazoamides, as a high number of ester tags has been shown to parallel cellular delivery efficiency.[10c]

To further demonstrate the utility of a hydrophilic diazoamide for the cellular delivery of proteins, we elected to deploy diazoamide **3** for exogenous delivery of ubiquitin into live cells. Ubiquitin is a small neutral protein (8.6 kDa; pI ca. 6.8) that is involved in maintaining cellular proteostasis.[31] Here, we elected to deliver a truncated, nonfunctional ubiquitin variant, ubiquitin(1–74) (Ub^{74}), which is not used by live cells for ubiquitin-mediated protein degradation.[32] To enable visualization in live cells, an alanine-to-cysteine substitution at position 46 ($UbA46C^{74}$) was introduced to endow the protein with a unique bioorthogonal handle for functionalization with a fluorescent dye. $UbA46C^{74}$ was produced by heterologous expression in *Escherichia coli*. An azide handle was first installed on $UbA46C^{74}$ through a reaction with iodoacetamide **S9**. The ensuing azide-functionalized $UbA46C^{74}$ (azido–Ub^{74}) was esterified with diazoamide **3** to give azido–Ub^{74}–**3**, which was subsequently conjugated via strain-promoted azide–alkyne cycloaddition (SPAAC) with TAMRA–DBCO to give TAMRA–Ub^{74}–**3** (Figure 4 and Figures S2, and S3). Live human adenocarcinoma cells (HeLa cell line) were incubated with TAMRA–Ub^{74}–**3** followed by live-cell microscopy, which revealed the accumulation of TAMRA–Ub^{74}–**3** within HeLa cells (Figure 4). Taken

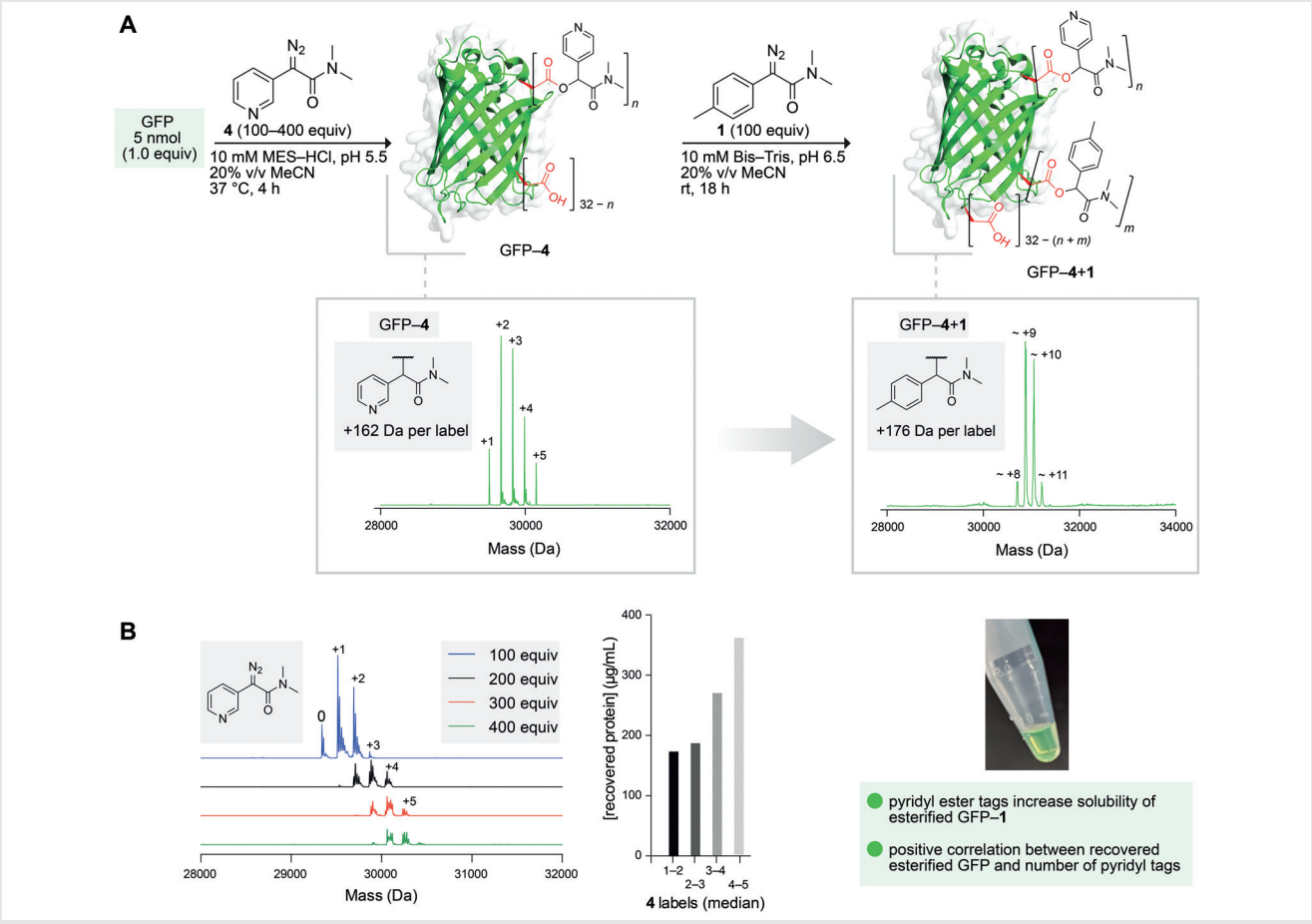


Figure 2 Pyridyl diazoamide **4** enhances aqueous solubility of GFP esterified with diazoamide **1**. (A) GFP is first esterified with pyridyl diazoamide **4** in 10 mM MES–HCl buffer, pH 5.5, containing MeCN (20% v/v) at 37 ℃ for 4 h, then exchanged into 10 mM Bis-Tris buffer, pH 6.5. Subsequent esterification of GFP–**4** with diazoamide **1** was performed in the presence of MeCN (20% v/v) at room temperature for 18 h before exchange into PBS. Deconvoluted ESI-MS characterization of each step of the workflow after buffer exchange. (B) Yields of soluble esterified GFP–**4**+**1** parallel the number of preinstalled pyridyl esters. Images of GFP were created with PyMOL software and PDB entry 2b3p.[25]

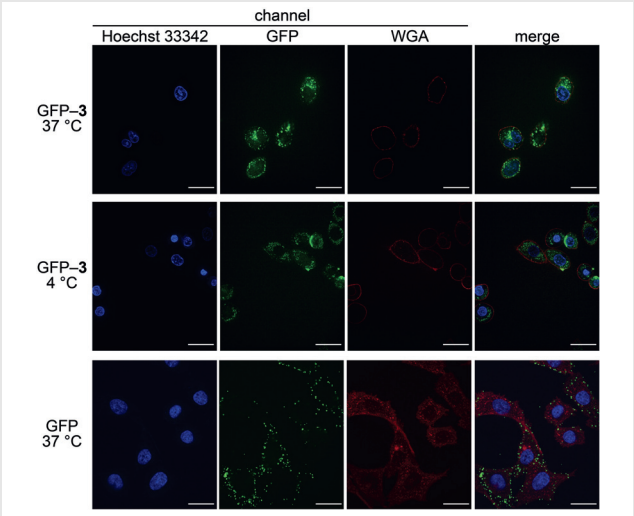


Figure 3 Live-cell fluorescence confocal images of CHO-K1 cells treated with GFP–**3** or GFP (8 μM) in serum-free medium for 2 h at 37 °C or 4 °C. Cells were washed and allowed to recover for 2 h in serum-supplemented medium at 37 °C before staining with Hoechst 33342 and wheat germ agglutinin (WGA)–Alexa Fluor 647. Identical imaging settings were used for both experimental conditions. Scale bars: 25 μm.

together, these results establish diazoamide **3** as an especially useful esterification reagent for exogenous protein delivery.

In summary, we have presented a strategy to alleviate the vexing issue of the precipitation of esterified proteins. Through a limited structure–activity relationship campaign, we have demonstrated that the aqueous solubility of esterified proteins can be enhanced by tuning the hydrogen-bond capacity of functional groups on pendant esters. Whereas improved aqueous solubility of esterified proteins is desirable, the reactivity of diazo compounds towards esterification of carboxylic acids and lipophilicity of ester tags are also important parameters that should be optimized for efficient cellular delivery applications. We believe that the strategy outlined here serves as a foundational study for the further development of amphiphilic diazo compounds for use as protein delivery reagents.

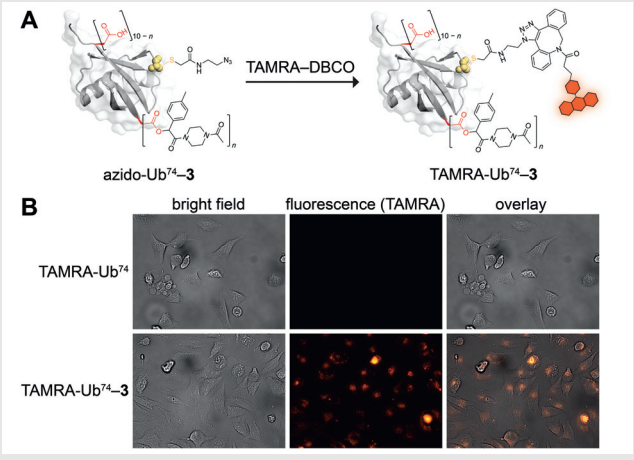


Figure 4 Delivery of TAMRA–Ub^{74} into HeLa cells. (A) Preparation of TAMRA–Ub^{74}–**3** by an SPAAC reaction. Images of Ub^{74} were created with PyMOL software and PDB entry 1ubq.[33] (B) Live-cell fluorescence confocal images of HeLa cells treated with TAMRA–Ub^{74}–**3** (10 μM) in serum-free medium for 2 h at 37 °C, then transferred into serum-supplemented medium to recover for 2 h before imaging. Identical imaging settings were used for both experimental conditions.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0043-1775499.

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