Bioconjugate Chemistry

Communication

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Hydrazino-Pictet-Spengler Ligation as a Biocompatible Method for the Generation of Stable Protein Conjugates

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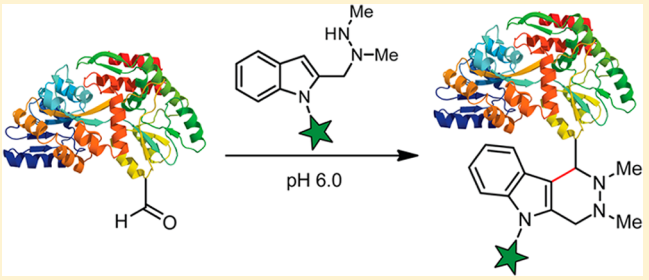
Supporting Information

ABSTRACT: Aldehyde- and ketone-functionalized biomolecules have found widespread use in biochemical and biotechnological fields. They are typically conjugated with hydrazide or aminooxy nucleophiles under acidic conditions to yield hydrazone or oxime products that are relatively stable, but susceptible to hydrolysis over time. We introduce a new reaction, the hydrazino-Pictet-Spengler (HIPS) ligation, which has two distinct advantages over hydrazone and oxime ligations. First, the HIPS ligation proceeds quickly near neutral pH, allowing for one-step labeling of aldehyde-functionalized proteins under mild conditions. Second, the HIPS ligation product is very stable (>5 days) in human plasma relative to an oxime-linked conjugate (~1 day), as demonstrated by monitoring protein-fluorophore conjugates by ELISA. Thus, the HIPS ligation exhibits a combination of product stability and speed near neutral pH that is unparalleled by current carbonyl bioconjugation chemistries.



The introduction of aldehydes and ketones into proteins has emerged as a powerful strategy for conjugation methods. The reactivity of these moieties as electrophilic functional groups renders them bioorthogonal, allowing for selective reactivity with nucleophiles in the presence of native amino acids and a variety of post-translational modifications (PTMs). As a result, the number of tools available to incorporate aldehydes and ketones into proteins, particularly using site-selective approaches, has grown dramatically in the past decade. There are now many ways to incorporate these functional groups into proteins using chemical,[1−6] enzymatic,[7−10] and chemoenzymatic[11−14] methods. The introduction of aldehydes and ketones into proteins has facilitated glycoproteomic studies,[15−18] protein imaging in live cells,[11,19] single-molecule imaging studies,[20] and protein purification,[21] as well as preparation of chemically modified therapeutic and heterobifunctional proteins,[22−24] functional protein-based materials,[25,26] and protein nucleic acid conjugates and glycoconjugates.[27,28] In most of these applications, a protein aldehyde or ketone is treated with a molecule of interest bearing an aminooxy nucleophile to generate an oxime-linked conjugate that is used in a downstream application.

One major drawback to oxime conjugation chemistry is the requirement for acidic conditions to enable the reaction to proceed at an appreciable rate. While many biomolecules can tolerate extended incubation under typical oxime conjugation conditions (pH 4.5), some types of proteins and PTMs are susceptible to conformational changes or degradation under acidic conditions. For example, histidine phosphorylation, a PTM increasingly appreciated for its biological relevance, is unstable to mildly acidic conditions;[29] large protein assemblies such as viral capsids are prone to acid-induced disruption of quaternary structure;[30] proteins mediating viral infection such as influenza hemagglutinin undergo irreversible acid-triggered conformational changes;[31] and a variety of proteins implicated in amyloid diseases undergo acid-induced aggregation.[32,33] To prevent loss of function, the labeling of these types of proteins through oxime-based chemistry would need to be carried out close to neutral pH, where oxime formation is very slow. The use of aniline as a nucleophilic catalyst for oxime conjugations has obviated this problem to a degree,[34] but its efficiency varies according to the system. For some conjugations aniline catalysis works well, while for others it has a neutral or detrimental impact on reaction kinetics and conjugation efficiencies.[17,22] Furthermore, for sensitive biological applications it may prove problematic to use millimolar quantities of aniline as a catalyst due to concerns about its toxicity.[35]

As an alternative to aminooxy nucleophiles, hydrazines are attractive functional groups for bioconjugation reactions with aldehydes and ketones because of their nucleophilicity near neutral pH.[36] However, reactions of aliphatic hydrazines to form hydrazones suffer from low equilibrium constants in water, requiring a large excess of hydrazine reagent to achieve good conversions in conjugation reactions.[37] Furthermore, alkylhydrazones are readily hydrolyzed under aqueous conditions, severely limiting the utility of these conjugates due to

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the high likelihood of hydrolysis after purification.[38] As a result, hydrazine derivatives such as hydrazides and semicarbazides have found use in bioconjugation reactions with aldehydes and ketones because the resulting acylhydrazone conjugates hydrolyze less readily than alkylhydrazones. However, hydrazide and semicarbazide reagents suffer from slower reaction kinetics because of the presence of electron-withdrawing groups adjacent to the nucleophilic hydrazine moiety.

At the outset of our study, we sought to design a reactive group that would (i) retain the intrinsic nucleophilicity of alkylhydrazines at neutral pH, and (ii) provide a stable conjugate, ideally without the necessity for catalysis or auxiliary reagents. Thus, we developed a strategy for generating stable conjugates between hydrazine-bearing probes and aldehyde-containing proteins that does not depend on the incorporation of electron-withdrawing groups to impart stability. Our design was based on the recently reported Pictet-Spengler ligation, which uses aminooxy-functionalized indoles that react with aldehydes to generate an oxyiminium ion intermediate. This intermediate rearranges to the hydrolytically stable reaction product[39] containing a very stable C−C bond. Because the original Pictet-Spengler ligation uses an aminooxy nucleophile to form the C═N intermediate, the optimal reaction conditions are acidic (pH less than 5.0) rather than the preferable neutral reaction conditions for most bioorthogonal and bioconjugation reactions. We reasoned that the reaction kinetics of the ligation could be significantly improved near neutral pH by replacement of the aminooxy nucleophile with an alkylhydrazine nucleophile. Thus, we designed a reactive partner for aldehydes and ketones that would generate an intermediate hydrazonium ion followed by intramolecular alkylation with a nucleophilic indole (Figure 1). We anticipated

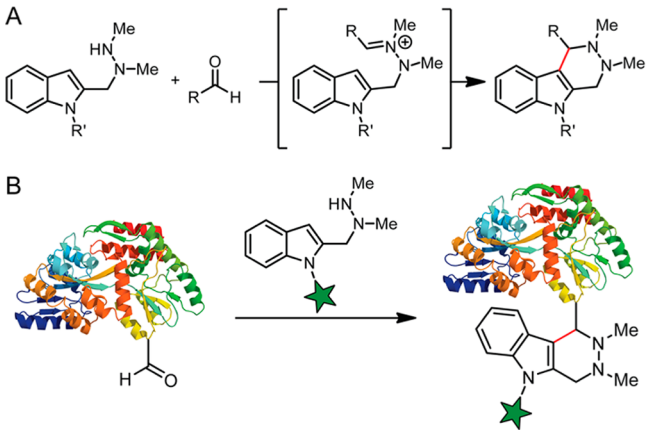

Figure 1. Overview of the hydrazino-Pictet-Spengler ligation. In reactions with (A) small molecule and (B) protein aldehydes, a C−C bond (highlighted in red) is formed between the indole and the aldehyde of interest.

that such indoles would engage in a hydrazino-Pictet-Spengler (HIPS) ligation, combining the speed and bioorthogonality of hydrazine conjugation chemistry with the stability of a C−C bond to the biomolecule of interest.

To test our hypothesis, we first prepared a model HIPS ligation reagent (Scheme 1). Oxidation of alcohol **1** with Dess-Martin periodinane followed by saponification with lithium hydroxide provided indole **2**. Next, we installed the hydrazine moiety by reductive amination with an Fmoc-protected *N,N′*-dimethylhydrazine. We chose to use a dimethylhydrazine rather than a monomethylhydrazine moiety to avoid the conjugate heterogeneity that could result from a mixture of 5- and 6-*endo-trig* cyclizations in the latter case. Removal of the Fmoc group from **3** provided the model hydrazine-functionalized indole **4** as its piperidinium salt. We found that **4** reacts smoothly in 1:1 water:acetonitrile with benzyloxyacetaldehyde, which we chose as a model small molecule aldehyde for its UV absorption and aqueous solubility properties, to produce the desired azacarboline product **5**.

Next, we studied the reactivity of HIPS reagent **4** relative to model aminooxy, hydrazide, and Pictet-Spengler ligation reagents with benzyloxyacetaldehyde (Figure 2 and Figure S1). Buffered aqueous solutions containing 50 μM amine and aldehyde were incubated at room temperature for 2 h prior to analysis by HPLC. Within a pH range encompassing conditions commonly used for aldehyde and ketone bioconjugation reactions, aminooxy indole **6** (a model Pictet-Spengler ligation reagent) and model aminooxy compound **7** reacted more quickly under acidic conditions, as expected. The reaction of hydrazide **8** did not proceed appreciably under any conditions, probably because of the low equilibrium constant for hydrazone formation. Notably, above pH 6, indole **4** outperformed the other amine nucleophiles, suggesting that the HIPS ligation might be particularly useful for labeling proteins near neutral pH. The observed bell-shaped pH-rate curve is typical of amine-carbonyl addition reactions;[40] the decreased reaction rate at lower pH likely reflects rate-limiting carbonyl addition due to protonation of the hydrazine moiety of **4**, since trialkylhydrazines such as the one in **4** ($pK_a \sim 6.6$ for trialkylhydrazinium ions) are more basic than *N,O*-dialkylaminooxy moieties such as the one in **6** ($pK_a \sim 4.8$ for alkoxyammonium ions).[41,42] Finally, to yield a more robust comparison of the relative reactivity of compounds **4** and **7**, we determined the second-order rate constants for these reactions at pH 6 in aqueous solution (Figure S2). We found that in reactions with benzyloxyacetaldehyde, HIPS indole **4** ($k = 4.17 \pm 0.19$ M^{-1} s^{-1}) reacted more than three times faster than aminooxy compound **7** ($k = 1.13 \pm 0.09$ M^{-1} s^{-1}).

The promising reactivity of reagent **4** with model small molecule aldehydes suggested its potential for the selective labeling of aldehyde-containing proteins at near-neutral pH. As a model substrate we selected an aldehyde-tagged maltose binding protein (FGly-MBP), which is a variant of MBP that bears a C_α-formylglycine (FGly) residue near its C terminus.[7] As described previously, the FGly residue is installed by inclusion of the short peptide sequence LCTPSR at the C-terminus of the protein.[19,43] Coexpression of MBP with the *Mycobacterium tuberculosis* formylglycine generating enzyme results in cotranslational oxidation of the consensus sequence cysteine residue to formylglycine, which is selectively reactive with the hydrazine-containing reagent **4**. We treated FGly-MBP with indole **4** overnight and then trypsinized the resulting conjugate. Analysis of the tryptic digest by ESI-MS showed that **4** reacted with the FGly residue as expected (Figure S3).

Next, we sought to confirm the speed of the HIPS ligation under mild reaction conditions by assessing labeling kinetics on proteins at pH 6.0. To facilitate the analysis of protein conjugation experiments by SDS-PAGE, we prepared a fluorophore-functionalized HIPS indole (**9**) by coupling **3** with Alexa Fluor 488 (AF488) cadaverine followed by Fmoc deprotection with piperidine. In these experiments, we compared the relative conjugation efficiencies of HIPS reagent **9** to both the commercially available AF488 hydrazide (**10**) and

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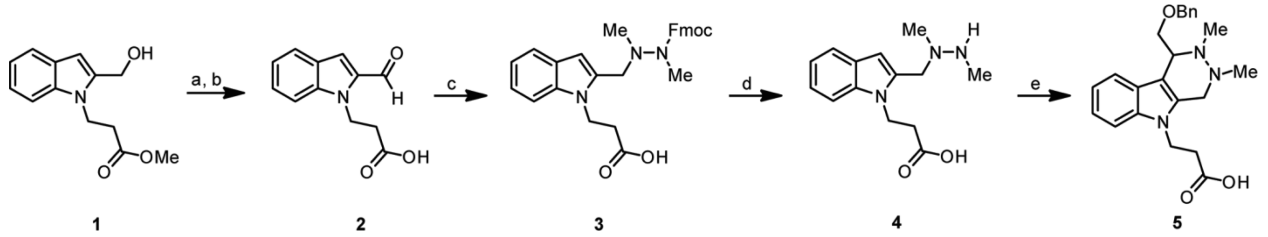
Scheme 1. Synthesis of Model Hydrazino-Pictet-Spengler Ligation Reagent 4 and Its Reaction with a Model Aldehyde to Produce Azacarboline 5[a]



[a]Reagents and conditions: (a) Dess-Martin Periodinane, 84%; (b) LiOH, 84%; (c) FmocN(Me)NHMe, $NaB(OAc)_3H$, 62%; (d) piperidine, 62%; (e) benzyloxyacetaldehyde, 88%.

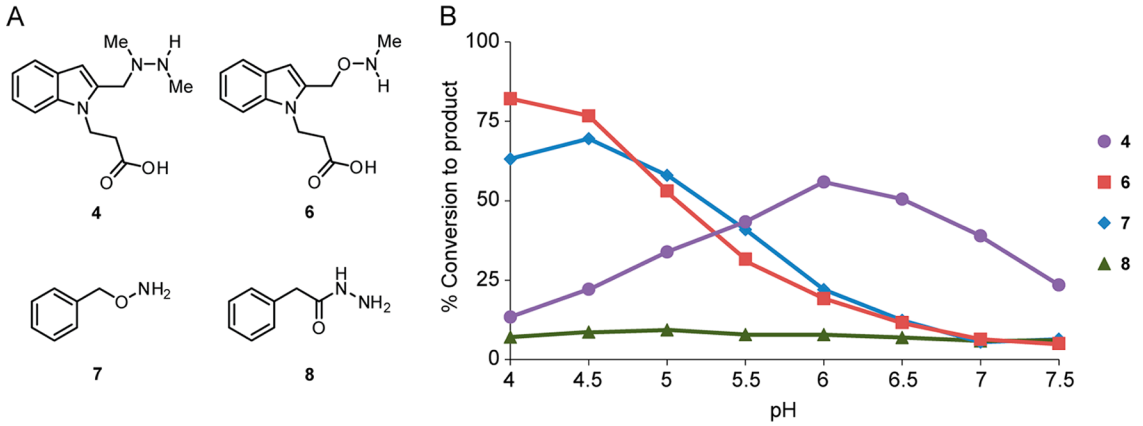


Figure 2. Percent conversion to products in condensation reactions of model amine compounds with benzyloxyacetaldehyde. (A) Panel of amines used in the experiment. (B) Percent conversion to product in sodium citrate (pH 4.0–5.5) or sodium phosphate (pH 6.0–7.5) buffers. Reactions contained 50 μM amine and benzyloxyacetaldehyde and proceeded at room temperature for 2 h prior to HPLC analysis.

the aminooxy reagent **11**. This experimental design allowed for a comparison of the HIPS ligation to the previously reported Pictet-Spengler ligation as well as commercially available aminooxy AF488 (**12**, Figure 3A). We assessed the relative labeling of two formylglycine-bearing proteins at pH 6.0: FGly-MBP and FGly-α-HER2, a variant of the therapeutic monoclonal antibody Herceptin that contains a formylglycine residue at the C terminus of each of its heavy chains.[22,39] To show the generality of the method with an aldehyde other than formylglycine, we also assessed the labeling of chemically modified myoglobin (Mb) containing an N-terminal glyoxamide installed by pyridoxal phosphate-mediated transamination.[3] In all three cases, treatment of the aldehyde-functionalized protein with 400 μM of the fluorophore reagents **9**–**12** at pH 6.0 for 2 h showed that labeling with HIPS reagent **9** was faster than labeling with the aminooxy and hydrazido-fluorophore panel (Figure 3B–D). These results were further substantiated by a comparative analysis of HIPS and oxime ligations by ESI-MS (Figure S4). Control experiments with the FGly-MBP C390A mutant (which lacks the requisite cysteine residue for conversion to formylglycine), α-HER2 without the aldehyde tag sequence, and wild-type Mb, all of which lack aldehyde functionality, showed negligible labeling with **9**. Notably, our experiment using FGly-α-HER2 demonstrates a method for the site-specific conjugation of a monoclonal antibody with a small molecule under mild conditions.

Having shown the superior speed of the HIPS ligation on several proteins near neutral pH, we next verified the hydrolytic stability of the linkage on FGly-MBP relative to oximes, which are generally regarded as the most hydrolytically stable linkage for aldehydes and ketones in bioconjugation chemistry.[38] Purified azacarboline- and oxime-linked AF488 conjugates of FGly-MBP were incubated at 10 μg/mL in human plasma at 37 °C for five days. Over this time the MBP was monitored for the loss of AF488 by ELISA (Figure 4). Our sandwich ELISA procedure entailed MBP-AF488 capture on an α-MBP-coated microtiter plate followed by detection of covalently bound AF488 using an α-AF488 antibody and an HRP-conjugated secondary antibody. Over the course of five days, we observed no appreciable hydrolysis of the azacarboline conjugate. In contrast, the oxime conjugate of AF488 quickly decomposed within one day.

The results of our MBP-AF488 conjugate stability experiments, which are consistent with a previous experiment showing hydrolysis of the oxime in phosphate-buffered saline,[39] are particularly important in light of the use of hydrazone and oxime linkages for the generation of therapeutic protein conjugates; their unintended hydrolysis in circulation is problematic from a toxicity standpoint.[44,45] In aqueous buffers, ketoximes are quite stable,[24,46,47] in contrast to the relative instability of protein-derived aldoximes.[39] However, the serum stability of protein-derived oximes has not been studied as thoroughly.[48] A recent study showed that cysteine-maleimide conjugates, which are generally regarded as stable, can be remarkably susceptible to decomposition when incubated in human plasma and *in vivo*.[49] Thus, new methods are needed to generate conjugates that are stable under the more demanding conditions in serum. When used to generate a C–C bond between a site-specifically introduced aldehyde and a small

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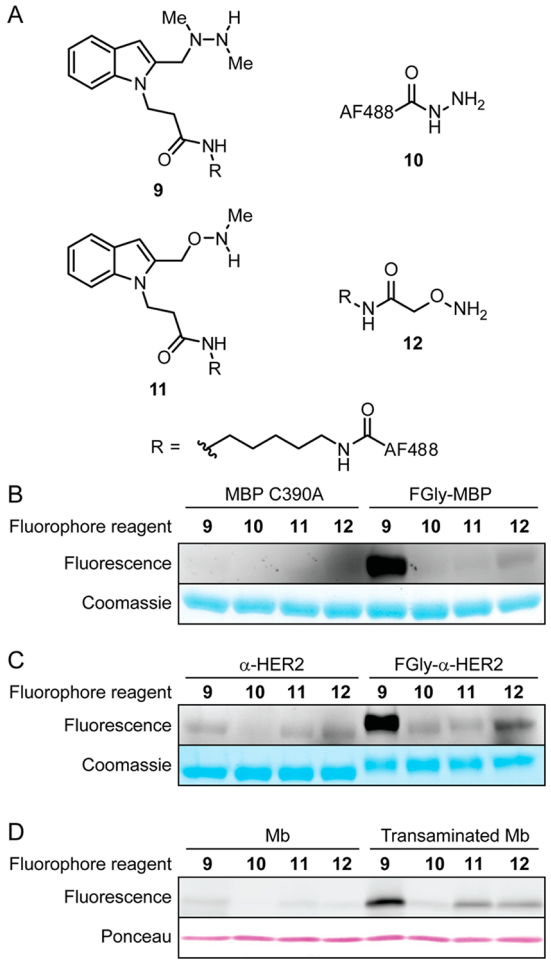

Figure 3. Fluorescent labeling of aldehyde-bearing proteins with the HIPS ligation and other common aldehyde bioconjugation chemistries. (A) Structures of the HIPS ligation reagent **9**, the hydrazide reagent **10**, the Pictet-Spengler ligation reagent **11**, and the aminooxy reagent **12**. Gel scans show the relative labeling of (B) FGly-MBP (0.67 mg/mL, 15 μM), (C) FGly-α-HER2 (0.37 mg/mL, 2.5 μM), and (D) N-terminally transaminated Mb (0.51 mg/mL, 30 μM) with reagents **9**–**12**. In all cases, buffered protein solutions at pH 6.0 were treated with 400 μM fluorophore for 2 h at 37 °C prior to analysis by SDS-PAGE.

molecule of interest, the HIPS ligation is a rare example of a stable and site-selective conjugation reaction.

In conclusion, we have shown that for small molecule and protein conjugations, the HIPS ligation exhibits reaction kinetics faster than those of aminooxy compounds at near-neutral pH. Additionally, the resulting azacarboline conjugate demonstrates vastly improved hydrolytic stability compared to an oxime conjugate. When combined with the aldehyde tag technology allowing for site-specific introduction of aldehydes into proteins, the HIPS ligation promises to be a powerful method for the scalable and reliable preparation of homogeneous protein conjugates. Potential conjugation partners include fluorophores, affinity handles, drugs, and other small molecules endowing therapeutic properties, as well as biomaterials requiring site-specific protein conjugation.[50] More generally, the HIPS ligation can be incorporated into the many workflows that now routinely make use of carbonyl-functionalized biomolecules, and the modular synthesis of HIPS reagents allows for easy incorporation of a moiety of

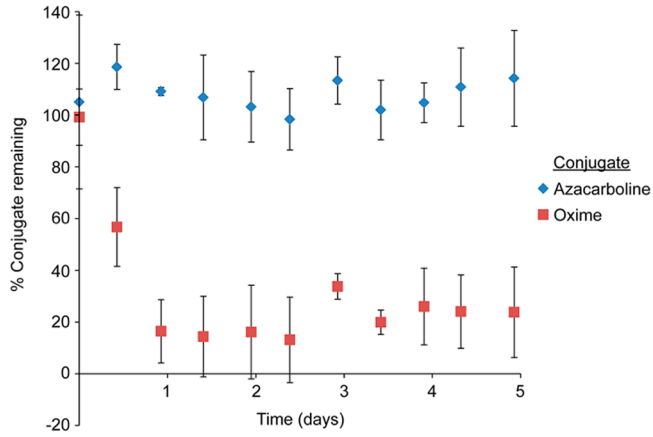

Figure 4. MBP-AF488 conjugate hydrolysis over 5 days. Human plasma containing 10 μg/mL MBP-AF488 conjugate, linked by either an azacarboline or an oxime, was incubated at 37 °C. Aliquots taken approximately 12 h apart were analyzed by ELISA. Error bars represent standard deviation of six replicate samples.

interest. We expect that the ability to generate truly stable conjugates near neutral pH without the necessity of optimizing multicomponent reactions requiring auxiliary reagents and catalysts will prove useful to researchers seeking a simple and biocompatible conjugation protocol.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and characterization of new compounds as well as detailed protocols for HPLC conversion assay, kinetics experiments, protein conjugation experiments, and conjugate stability ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The corresponding author is a founder of Redwood Bioscience and is a stakeholder in the company.

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