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TITLE: Synthesis of an electronically-tuned minimally interfering photo-affinity label

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ABSTRACT: We report the synthesis of an electronically-tuned minimally interfering photo-affinity label (MI-PAL), a compact five-carbon tag functionalized with an alkyl diazirine and alkyne handle. MI-PAL is compatible with protein photo-conjugation, click chemistry and mass spectrometry and readily installed to complex molecules for biological target identification.

KEYWORDS: photochemistry, diazirine, photo-affinity labeling, chemical proteomics, CuAAC, mass spectrometry

In cellulo target identification of non-covalent ligands has been accelerated by the development of small multifunctional chemical tags embedded to the ligand. These chemical tags typically possess two primary functions: first, photo-conjugation chemistry to covalently capture the protein target and second, a reporter group for characterization of the liganded proteome.^{1,2} Photo-affinity labels (PALs) like the aryl azide, benzophenone, and diazirine generate a short-lived highly reactive carbene intermediate that inserts to nearby biomolecules.³⁻⁵ The covalent conjugation event allows for subsequent ligand-dependent detection of the biomolecule. To allow for the greatest flexibility in detection, a biocompatible handle (e.g., alkyne) is commonly embedded to the chemical tag for subsequent functionalization with a reporter molecule via copper-catalyzed azide–alkyne cycloaddition (CuAAC).⁴

Equally important is the facile integration of the chemical tag to the ligand in a minimally perturbative manner in order to preserve the native interactions of the ligand with the proteome. Thus, of the available PALs, the diazirine has recently been applied in numerous target identification studies due to its small size.⁶⁻⁸ Integration of the diazirine with an alkyne reporter handle have yielded chemical tags like the aryl diazirine **1**,⁹ 10.1 Å in length, or the “minimalist” tag (**2**),¹⁰ 9.4 Å in length (Figure 1). The minimalist tag (**2**) has now seen application in drug on-

target¹¹ and off-target identification,^{11, 12} fragment based screening,¹³ and binding site mapping.^{13,}
14

We hypothesized that synthesis of an electronically-tuned five carbon tag, such as **3**, would afford a smaller tag scaffold (6.9 Å)¹⁵ with universally improved photo-conjugation and CuAAC properties (Figure 1). Photochemical carbene intermediates and CuAAC reaction kinetics are strongly dependent on electronic substituent effects. Electronic stabilization of the reactive carbene by fluorine was first demonstrated with 3-trifluoromethyl-3-phenyldiazirine, which possessed superior stability and selectivity as compared to alkyl diazirines.¹⁶ Electronic tuning of the alkyne with fluorine likewise accelerates strain-promoted azide–alkyne cycloaddition¹⁷ and CuAAC, with measured improvements in reaction rates of 18-fold greater for the difluoropropyne relative to the dihydropropyne.¹⁸ Based on these data, we hypothesized that strategic placement of fluorine adjacent to the alkyne and diazirine functional groups would improve the essential properties required for the chemical tag and provide synthetic access to a minimally-interfering photo-affinity label (MI-PAL, **3**). The tag would be accessed by coupling the ester **4** to a monobromodifluoroalkyne **5**, followed by installation of the diazirine.

Synthesis of MI-PAL (**3**) commenced from the ester **4** and the monobromodifluoroalkyne **5** (Scheme 1). The nucleophilic addition of the monobromodifluoroalkyne **5** to the alpha-hydroxyethyl ester **4** afforded the desired difluoroketone **6** and the hydrolysis product **7** in 77% yield as a 1:1 mixture. The hydrate **7** was formed in situ due to the electrophilicity of the difluoropropyne. A brief investigation of the scope of the initial coupling step revealed that the desired reaction was promoted by chelation control imparted by the methoxymethyl ester functional group at low temperatures. The equimolar mixture of ketone **6** and hydrate **7** was then treated with hydroxylamine, followed by a sequence of tosylchloride in pyridine and ammonia in ether to install the diaziridine **8**. Elaboration of the ketone **6** to the diaziridine **8** was enabled by the difluoropropyne protected with triisopropylsilane (TIPS) to prevent undesired nucleophilic or deprotonation pathways promoted by basic ammonia. Oxidation (iodine, trimethylamine) of the diaziridine **8** afforded the diazirine **9** in 65% overall yield from the mixture of **6** and **7**. Acid deprotection (TMSCl, MeOH) of the diazirine **9** revealed the alcohol **10** (99%). The alcohol **10** could be further desilylated to yield MI-PAL (**3**) itself (66%). However, we found that MI-PAL (**3**) was relatively volatile and thus in practice rarely removed the TIPS protecting group until after incorporation of MI-PAL (**3**) to a small molecule of interest. The advanced alcohol intermediate **10** was thus prepared by a four-step sequence in high overall yields (49% overall).

With MI-PAL (**3**) in hand, we next evaluated its photochemical and CuAAC properties by mass spectrometry and Western blot. MI-PAL (**3**, 10 μ M) was incubated with an isolated protein, alpha-crystallin, and photo-irradiated (365 nm) for 15 min (Figure 2A). The conjugated protein was trypsin digested and analyzed by liquid chromatography–tandem mass spectrometry on an Orbitrap Elite by collision induced dissociation (CID). Spectra of MI-PAL (**3**) conjugated-peptides obtained by CID displayed characteristic fluorine ion losses as diagnostic markers. An example peptide conjugated to MI-PAL (**3**) is shown in Figure 2B. To test both photo-conjugation and CuAAC properties, 100 μ M of MI-PAL (**3**) was incubated with MM.1S or K562 whole cell lysates and photo-irradiated (30 min). The labeled lysates were treated by CuAAC with biotin–azide as a reporter and visualized by anti-biotin Western blot. A UV-specific anti-biotin signal from samples photo-conjugated by MI-PAL (**3**) was observed (Figure 2C).

MI-PAL (**3**) was readily incorporated to a range of small molecules (Scheme 2, 3). The alcohol **10** was activated with carbonyldiimidazole (CDI) in quantitative yield to afford the carbamate **11** (Scheme 2). The carbamate **11** was treated with several coupling partners of increasing complexity followed by deprotection of the silyl protecting group. The MI-PAL-tagged glycine methylester **12** was formed in 69% yield over two steps. Daunorubicin was readily modified by the carbamate **11** to afford the MI-PAL-tagged daunorubicin **13** in 53% yield over two steps. The alcohol **10** was additionally activated with 4-nitrophenyl chloroformate and installed to mitomycin to prepare the tagged mitomycin analog **14**. In all cases, desilylation with TBAF proceeded smoothly following conjugation of MI-PAL to a small molecule.

We additionally tested the participation of MI-PAL in direct S_N2 displacement (Scheme 3). The alcohol **10** was transformed to the iodide **15** in the presence of iodine and triphenylphosphine in excellent yield (99%). The iodide **15** was then elaborated to naproxen methylester in the presence of cesium carbonate in dimethylformamide. In situ deprotection of the alkyne by cesium carbonate afforded the MI-PAL-tagged naproxen methylester **16** in 81% yield. Mixtures of the iodide **15** and cesium carbonate with β -estradiol additionally provided the MI-PAL-tagged β -estradiol **17** in 74% yield. Thus, MI-PAL is readily functionalized to a range of complex ligands for target identification.

Photo-conjugation chemistry is transforming the study of noncovalent interactions between small molecules and their biomolecular targets. Integration of photo-activated functional groups with a handle for CuAAC in a small chemical space is critical for preserving the native interactions of the small molecule with the protein targets. Here we report the development of an

electronically-tuned five carbon tag **3** as a novel minimally-interfering photo-affinity label. MI-PAL (**3**) possesses a diazirine appended directly to a difluoropropyne that enables facile synthetic access to the tag and its essential functions in photo-conjugation and CuAAC. We demonstrated the photo-conjugation with a single protein and whole proteome, selective CuAAC with a biotin–azide reporter, and measurement by mass spectrometry, as well as incorporation of MI-PAL (**3**) into several complex small molecules. MI-PAL (**3**) thus constitutes the smallest multifunctional chemical tag for application in non-covalent ligand target identification studies.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found, in the online version.

Competing Interests

The authors declare no competing interests.

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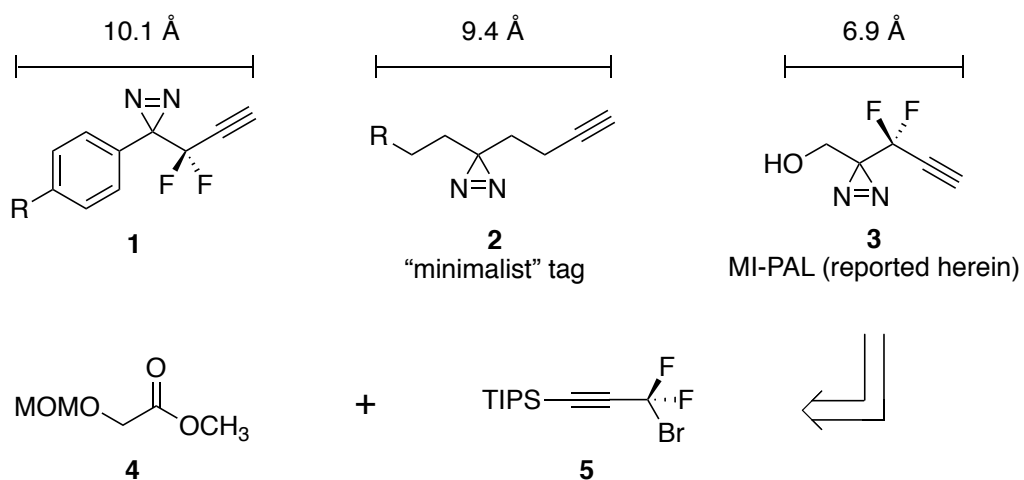
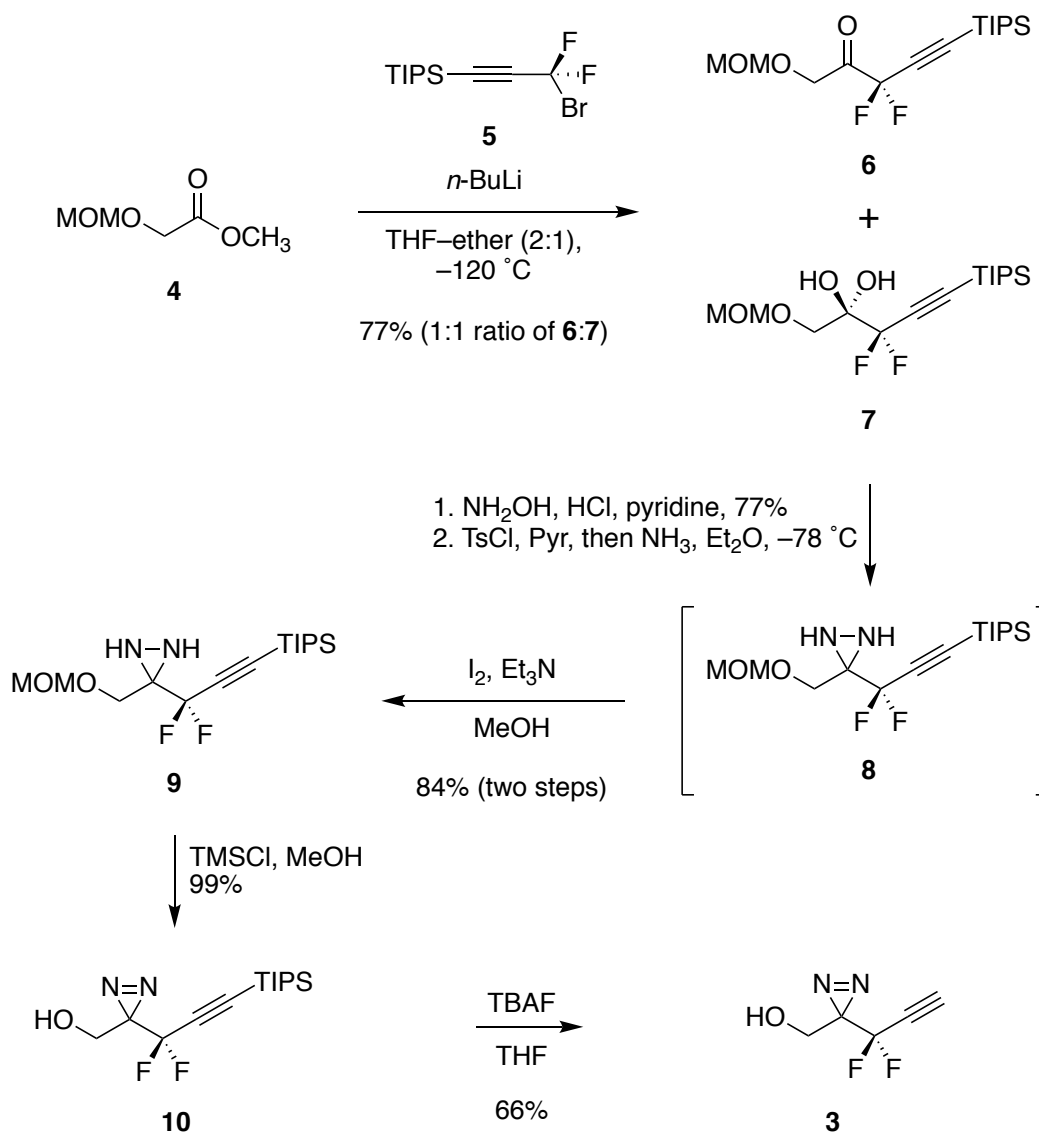


Figure 1. Structures of arylalkyne **1**, "minimalist" tag **2**, and MI-PAL (**3**), chemical tags that possess a diazirine functional group and alkyne reporter handle. Retrosynthesis of **3** proceeds through the ester **4** and alkyne **5**.



Scheme 1. Synthesis of MI-PAL (**3**) from the ester **4** and the alkyne **5**.

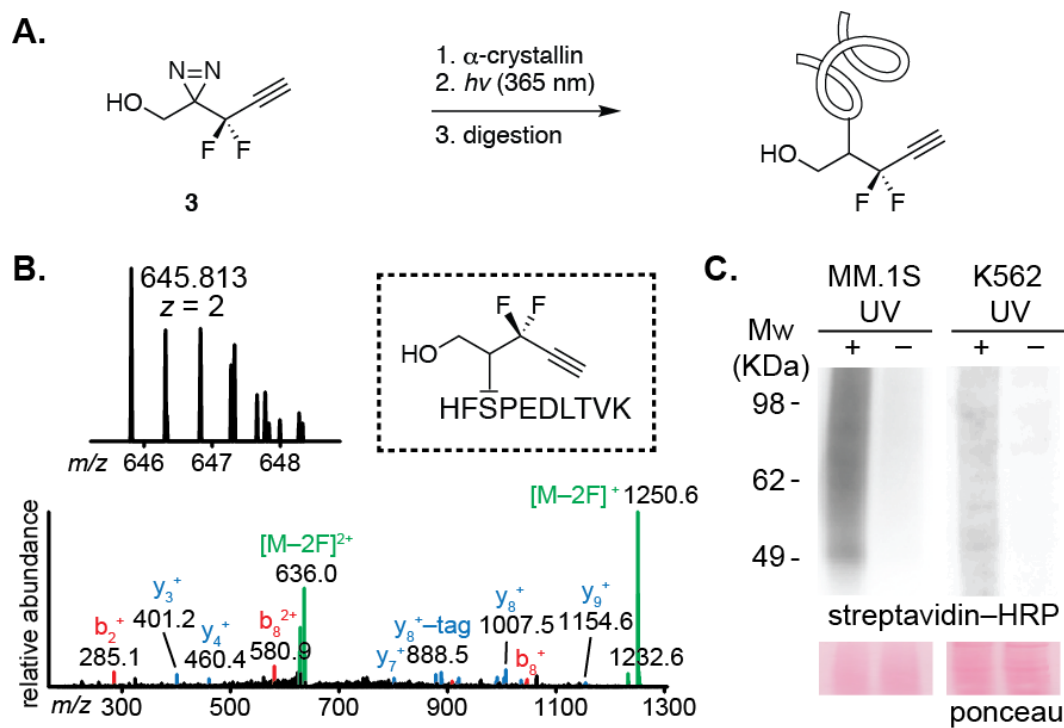
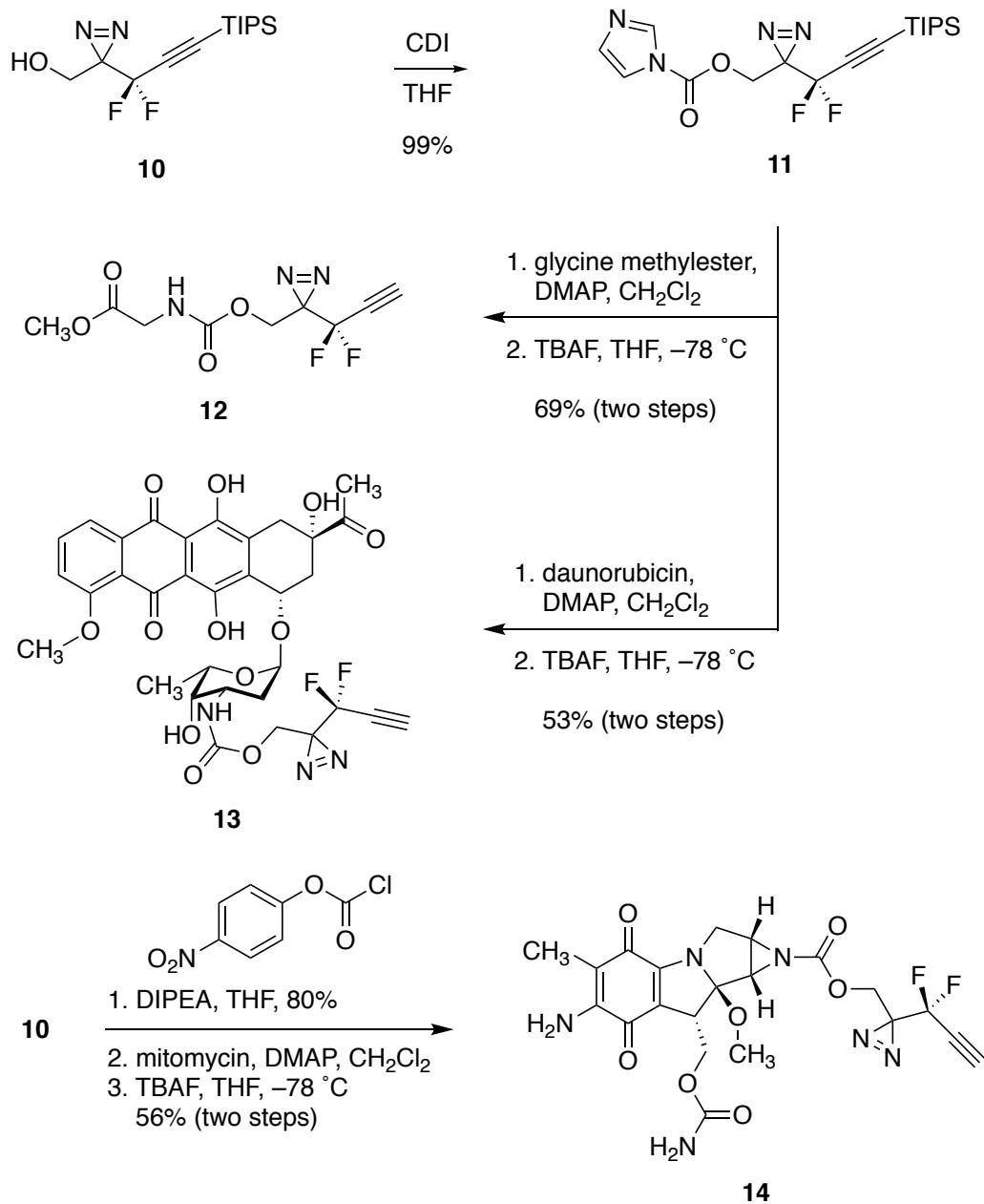
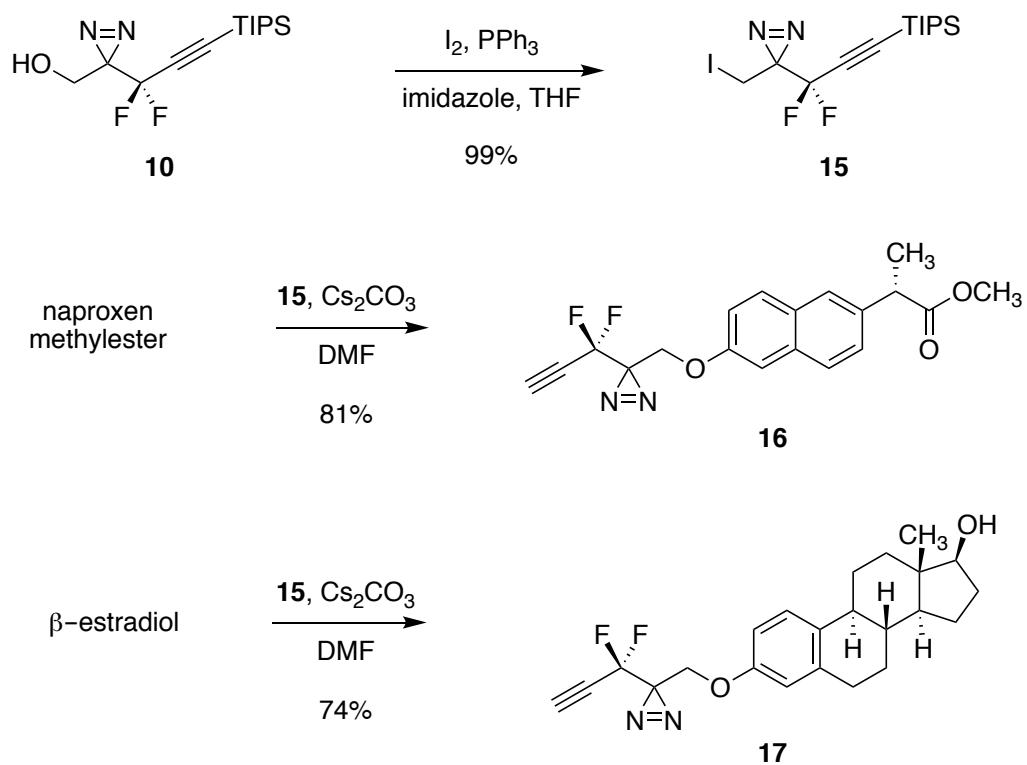


Figure 2. Photo-conjugation and CuAAC with MI-PAL (**3**) to the proteome. A. Photo-conjugation of MI-PAL (**3**) to α -crystallin. B. Example mass spectra of a peptide from α -crystallin conjugated to MI-PAL (**3**). C. Whole cell lysates from MM.1S or K562 were incubated with MI-PAL (**3**) and photoirradiated. Photo-conjugated lysates were treated with biotin-azide and visualized by anti-biotin Western blot.



Scheme 2. Synthesis of the MI-PAL-tagged glycine methylester **12**, MI-PAL-tagged daunorubicin **13** and the MI-PAL-tagged mitomycin **14**.



Scheme 3. Synthesis of the MI-PAL-tagged naproxen methylester **16** and the MI-PAL-tagged β -estradiol **17** from the iodide **15**.

Graphical Abstract

