Introducing Azomethine Imines to Chemical Biology: Bioorthogonal Reaction with Isonitriles

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ABSTRACT: Azomethine imines are valuable substrates for chemical synthesis in organic solvents, often requiring anhydrous conditions. Here, we introduce *C,N*-cyclic-*N′*-acyl azomethine imines (AMIs) to bioorthogonal reactions in aqueous environment. These AMIs are stable under physiological conditions and react rapidly (k_2 = 0.1–250 M^{-1} s^{-1}, depending on pH) and chemoselectively with isonitriles in the presence of biological nucleophiles, including thiols. Live-cell imaging of cell surface-bound isonitriles underlines the biocompatibility of the AMI-isonitrile ligation, and simultaneous one-pot triple protein labeling demonstrates its orthogonality to commonly used bioorthogonal reactions such as the SPAAC and iEDDA ligations.

Azomethine imines (AMIs) are readily accessible 1,3-dipoles that are widely used in synthetic chemistry.[1,2] Since Huisgen introduced AMIs as prototypes of allylic 1,3-dipoles in the early 1960s,[3,4] they have been extensively used for the synthesis of nitrogen-containing heterocycles, in asymmetric catalysis,[5–9] and, more recently, for the late-stage functionalization of peptides.[10] AMIs react with a broad range of dipolarophiles and C-nucleophiles, reactions that require organic solvents and often even anhydrous conditions to prevent AMI hydrolysis (Figure 1A).[1–10] Here, we expand the utility of AMIs to bioorthogonal reactions in complex aqueous environments.

Bioorthogonal ligations are useful for, *e.g.*, labeling and imaging of biomolecules or for the engineering of therapeutics, such as antibody-drug conjugates.[11] Such reactions need to be chemoselective and fast under physiological conditions, form stable products, and ideally employ a small reaction partner ("chemical reporter") that can be installed at a biomolecule.[12–16] To allow for simultaneous multitarget labeling, any new bioorthogonal reaction should, ideally, also be orthogonal to common cycloaddition-based ligations, *e.g.*, the strain-promoted azide-alkyne cycloaddition (SPAAC) and the inverse electron demand Diels-Alder (iEDDA) cycloaddition.[17–23]

Whereas AMIs with *N*'-alkyl or *N*'-aryl substituents are susceptible to hydrolysis and have to be generated in situ under anhydrous conditions, *N*'-acyl groups, as well as *C,N*- and *N,N*-cyclization stabilize AMIs.[9] We, therefore, reasoned that appropriately substituted AMIs could be stable in water and chemoselectively react with a suitable partner. Specifically, we envisioned that *N'*-acyl substituted AMIs would react with isonitriles[24,25] and, in an aqueous solution, provide ligation product **I** via hydrolysis of the initially formed oxadiazine **II** (Figure 1B). The isonitrile group is small and stable under physiological conditions and, thus, an attractive chemical reporter.[26–28]

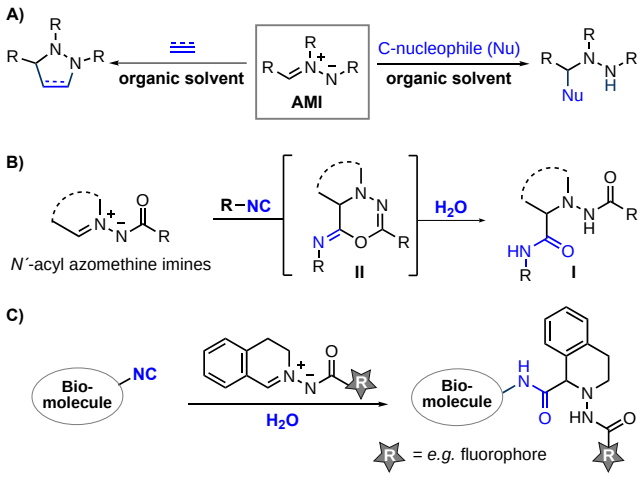
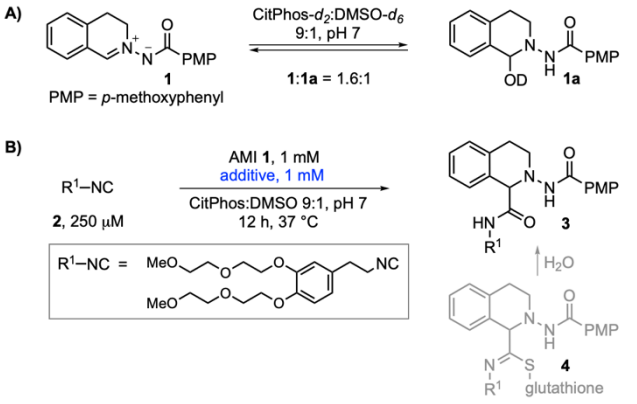


Figure 1. A) Reactions of azomethine imines in organic solvents. B) Envisioned reactivity of azomethine imines with isonitriles in aqueous solution. C) Bioorthogonal AMI-isonitrile ligation.

Here, we show that *C,N*-cyclic-*N'*-acyl AMIs are exceptionally stable 1,3-dipoles in aqueous environment and react chemoselectively with isonitriles (Figure 1C). This AMI-isonitrile ligation is fast (k_2 = 0.1–250 M^{-1} s^{-1}), and orthogonal to established SPAAC and iEDDA ligations.

We initiated our studies by evaluating the reactivity and stability of AMI **1** (Figure 2). This *C,N*-cyclic-*N'*-acyl AMI was readily obtained by reaction of 2-(2-bromoethyl)benzaldehyde with 4-methoxybenzhydrazide in 82% yield (Scheme S1). In water, AMI **1** is in equilibrium



entry	additive	conv.[a]
1	–	> 95%
2	H-Lys-OH, H-His-OH, H-Ser-OH, H-Trp-OH	> 95%
3	glucose	> 95%
4	NADH	> 95%
5	glutathione (GSH)	> 95%[b]
6	glutathione (GSH)	> 95%[b,c]

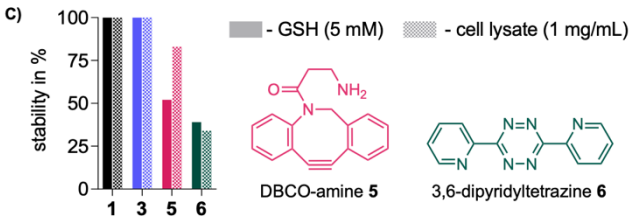


Figure 2. A) Equilibrium between AMI **1** and its hemiaminal **1a** in CitPhos buffer-*d2*:DMSO-*d6* 9:1. B) Chemoselectivity of the reaction between AMI **1** and isonitrile **2**. [a]Conversion to **3** determined by HPLC. [b]Conversion after 24 h. [c]Conversion after incubation of **1** with GSH for 24 h at 37 °C prior to addition of **2**. C) Stability of AMI **1**, ligation product **3**, DBCO-amine **5**, and 3,6-dipyridyltetrazine **6** in an aqueous solution (PBS, pH 7.4) of GSH (5 mM) and in cell lysate (1 mg/mL protein content) after 24 h incubation at 37 °C, monitored by HPLC.

with its hemiaminal (**1**:**1a** = 1.6:1 in citrate-phosphate (CitPhos) buffer:DMSO 9:1, pH 7; Figure 2A, S1), and no hydrolysis to the aldehyde and the hydrazide was observed. Reaction with water-soluble isonitrile **2** in aqueous solution (CitPhos buffer:DMSO 9:1) at pH 7 provided ligation product **3** quantitatively within 12 h (Figure 2B, S5). This reaction proceeded cleanly, also in the presence of biomolecules, including carboxylic acids, amines, alcohols, imidazoles, and indoles (Figure S9, S10). Even the reductant NADH and glutathione (GSH), a thiol that is present at a millimolar concentration in most cells,[29] did not interfere with the ligation (Figure 2B, S11, S12). In the presence of GSH, the three-component reaction product **4** was observed, which hydrolyzed to ligation product **3** over time. The ligation even proceeded when AMI **1** was incubated with GSH for 24 h at 37 °C prior to the addition of isonitrile **2** (Figure 2B, entry 6, S13). These results demonstrate the exquisite chemoselectivity of the AMI-isonitrile ligation.

We further studied the stability of AMI **1** and ligation product **3** under physiological conditions and compared it with that of the widely used bioorthogonal reagents DBCO-amine **5** and 3,6-dipyridyltetrazine **6** (Figure 2C). AMI **1** and ligation product **3** were stable in cell lysate (protein concentration 1 mg/mL) and in a solution of GSH (5 mM) in PBS (pH 7.4) at 37 °C for 24 h as revealed by HPLC analysis. Under the same conditions, alkyne **5** and tetrazine **6** decomposed to a considerable extent (Figure 2C, S14-S21).

Next, we studied the kinetics of the reaction between AMI **1** and isonitrile **2**. Unique absorption bands of AMIs (340–390 nm) allowed us to monitor the AMI-isonitrile ligation via UV-Vis spectroscopy (Figure S22). Reaction progress kinetic analysis revealed a linear relationship of the reciprocal of the concentration of AMI **1** over time, and thus second–order kinetics (Figure 3A). At pH 7 and 25 °C, the rate constant is k_2 = 0.10 ± 0.01 M^{-1} s^{-1} (Figure 3A, S23). This reaction rate is comparable to that of the SPAAC ligation.[30] Lowering the pH to pH 4 increased the reaction rate tenfold per pH unit. At pH 4, the reaction takes place with a rate constant of k_2 = 110 ± 6 M^{-1} s^{-1}. Further lowering of the pH resulted in a non-linear rate enhancement, indicating general acid catalysis (Figure 3A).

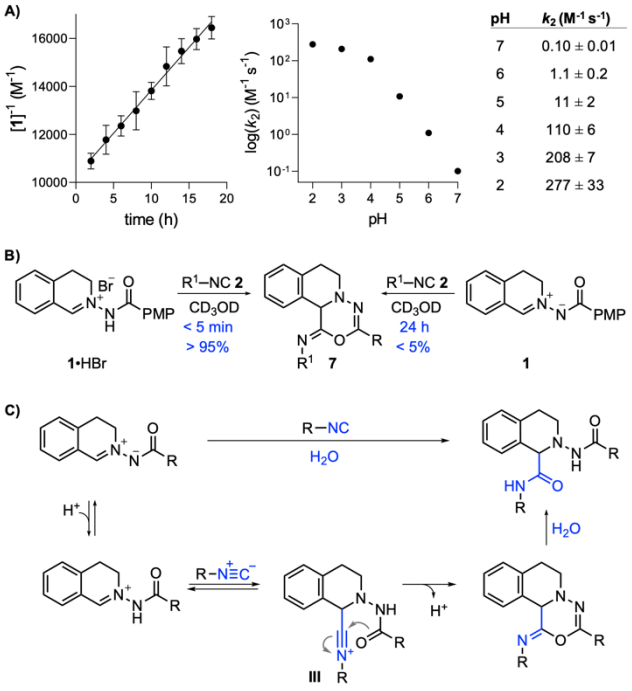


Figure 3. A) Kinetic analysis of the AMI-isonitrile ligation at different pH. B) Reaction of hydrobromide salt **1**•HBr (5 mM) and AMI **1** (5 mM) with isonitrile **2** (5 mM) in methanol-*d4*. C) Plausible mechanism of the AMI-isonitrile ligation.

These findings suggest that protonation of the *N'*-acyl nitrogen initiates the AMI–isonitrile ligation. To corroborate this hypothesis, we synthesized hydrobromide salt **1**•HBr and examined its reactivity towards isonitrile **2** in methanol-*d4*. Complete conversion to oxadiazine **7** was observed in less than 5 minutes, whereas AMI **1** did not react under the same conditions, even after 24 hours (Figure 3B, S30, S31). Oxadiazine **7** was also observed as an intermediate by HPLC monitoring of the reaction between

1 and **2** in aqueous solution. A plausible mechanism of the AMI-isonitrile ligation, therefore, starts with protonation of the *N'*-acyl nitrogen, followed by isonitrile addition to form nitrilium intermediate **III**, which is intramolecularly trapped (Figure 3C). The thus formed oxadiazine **7** then hydrolyzes in a second acid-catalyzed step to the final amide ligation product.

To assess the utility of the AMI-isonitrile ligation in a more complex environment, we first chose protein labeling as a testing ground. We derivatized a recombinant version of the small, globular protein ubiquitin (Ub) bearing a single cysteine at its C-terminus with a maleimide bearing an isonitrile group (**Ub-NC**; Figure 4A, Scheme S4). **Ub-NC** (20 μM) reacted quantitatively with AMI **1** (50 equiv.) to conjugate **8** in CitPhos buffer (pH 5) within one hour at 37 °C without side reactions as monitored by intact protein liquid chromatography-mass spectrometry (LC-MS; Figure 4A, S37). Quantitative labeling was also achieved at pH 6 and pH 7 within 3 h and 13 h, respectively (Figure S38, S39). Next, we probed the compatibility of the AMI-isonitrile ligation with other commonly used bioorthogonal labeling methods. We reasoned that the unique reactivity of AMIs with isonitriles should allow for the simultaneous one-pot triple protein labeling in combination with SPAAC and iEDDA ligations. Amber codon suppression enabled the site-specific incorporation of the previously described *trans*-cyclooctene (TCO)- and azide-bearing non-canonical amino acids (TCOK and AzGGK) into SUMO2 bearing an amber codon at position K42 and into sfGFP with an amber codon at position 150 to yield SUMO2-K42TCOK (**SUMO2-TCO**) and sfGFP-N150AzGGK (**sfGFP-N$_3$**; Figure S32, S34, S35).[15,31–34] These functionalized proteins were, firstly, exposed individually and then simultaneously in one-pot to their corresponding ligation partners bearing different dyes: **Ub-NC** to fluorescein-AMI **9** (green), **SUMO2-TCO** to Cy7-tetrazine **10** (blue) and **sfGFP-N$_3$** to AF647-DBCO **11** (red; Figure 4B lanes 1–3). Tetrazine **10** was used due to its low reactivity towards isonitriles.[26,35] Coomassie and in-gel fluorescence imaging, and LC-MS analysis in the case of the AMI-isonitrile ligation, revealed quantitative conversion to the ligation products (Figure 4B, lane 1, S40). In the simultaneous one-pot labeling experiments, each protein was selectively labeled with the matching fluorophore with minimal cross-reactivity, as demonstrated by in-gel fluorescence imaging (Figure 4B, lanes 4–6). The similar intensity of the in-gel fluorescence and Coomassie stain of the AMI-isonitrile ligation in the single target and multitarget labeling experiments underscores the high chemoselectivity of the AMI-isonitrile ligation and its complementarity to the two most widely employed bioorthogonal reactions.

Finally, we investigated the biocompatibility of the AMI-isonitrile ligation by live-cell surface labeling. We, therefore, labeled amino groups on the cell surface of CHO-K1 cells with isonitriles by incubation with the *N*-hydroxysuccinimide ester of 3-isocyanopropionic acid (NHS-NC **12**; Figure 5A).[36] Addition of fluorescein-AMI **9** and analysis by confocal microscopy revealed intense green fluorescence localized at the plasma membrane (Figure 5B, top, S41, S42). Control experiments with native CHO-cells showed hardly any fluorescence, indicating that fluorescein-AMI **9** does not bind non-specifically to the cell surface (Figure 5B, bottom, Figure S41, S42). Labeling of the cells with more NHS-NC **12** (300 μM instead of 100 μM) also increased the fluorescence intensity, as revealed by flow cytometry (Figure 5C).

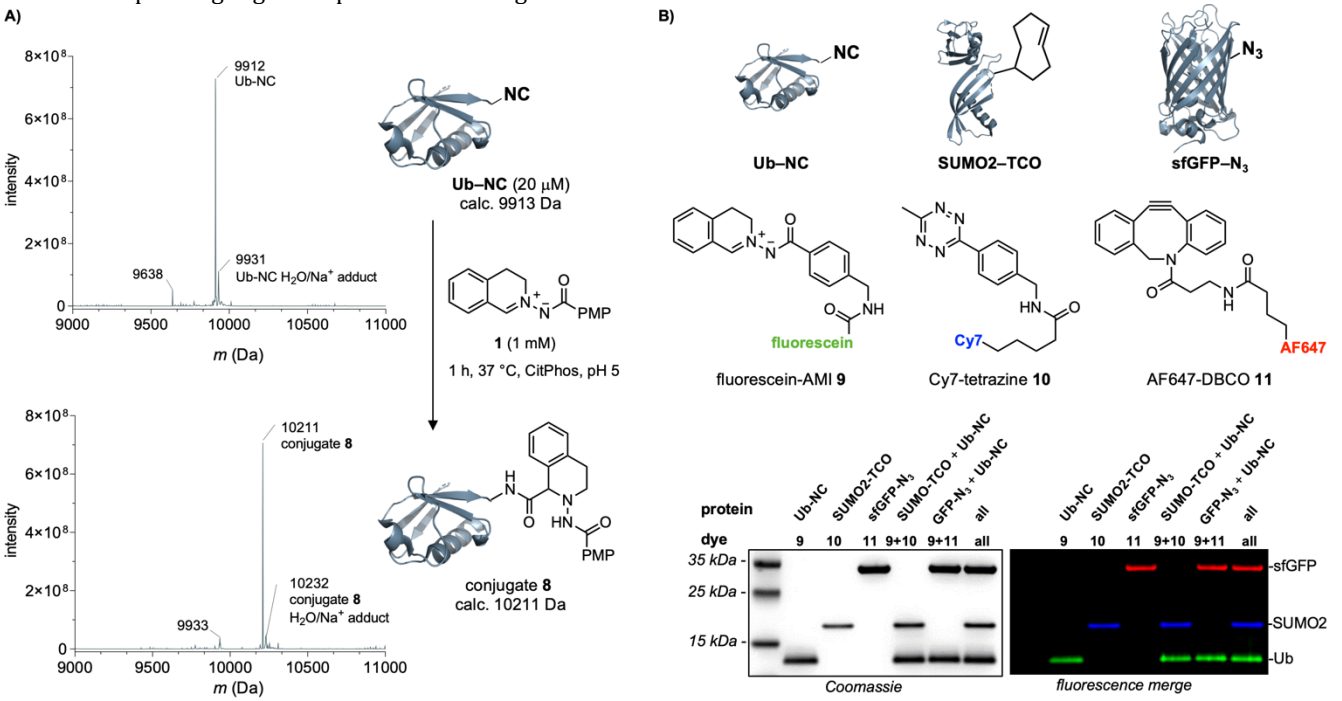


Figure 4. Protein labeling experiments. A) Mass spectrometry (ESI) analysis of **Ub-NC** and the reaction between **Ub-NC** and AMI **1**. B) Coomassie and in-gel fluorescence analysis of single and triple orthogonal labeling of **Ub-NC** (20 μM), **SUMO2-TCO** (20 μM) and **sfGFP-N$_3$** (20 μM) in CitPhos buffer pH 7, 13 h, at 37 °C with fluorescein-AMI **9** (1 mM), Cy7-tetrazine **10** (50 μM), and AF647-DBCO **11** (500 μM), respectively.

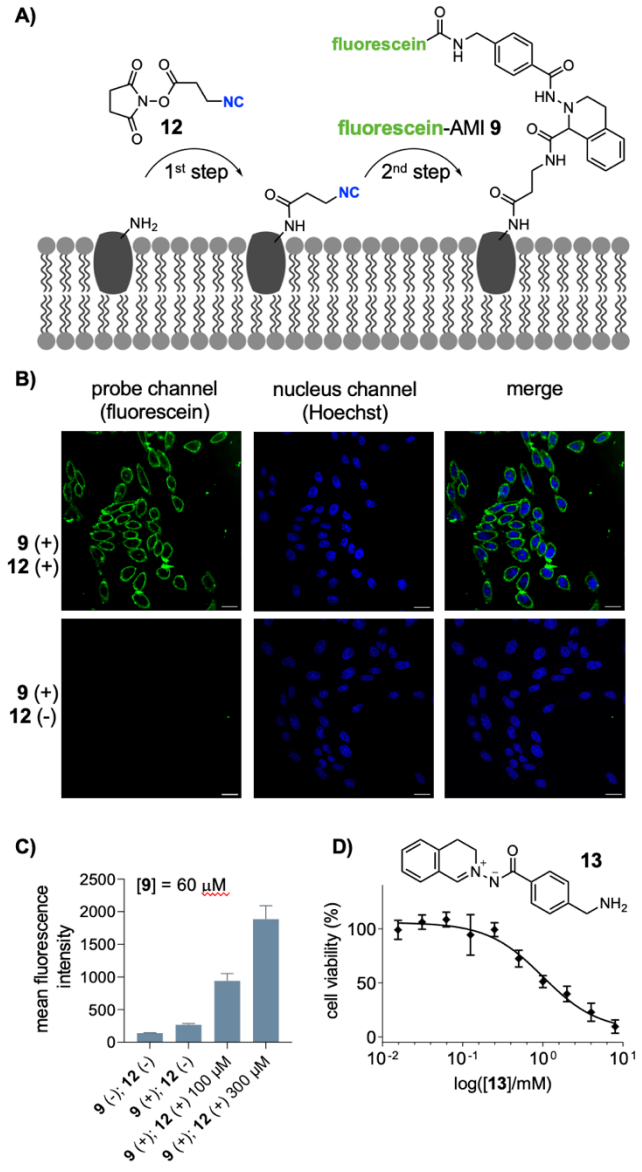


Figure 5. Cell surface labeling of CHO-K1 cells by the AMI-isonitrile ligation. A) Schematic representation of the installation of an isonitrile and subsequent labeling. B) Confocal microscopy images of CHO-K1 cells. Top row: Cells incubated with **12** (100 µM) in live cell imaging solution for 30 min, labeled with **9** (60 µM) for 5 min in CitPhos buffer pH 5; bottom row: control experiment without incubation with **12**; all cell images are overlaid with the blue channel (Hoechst 33342 nuclei stain), scale bar 20 µM. For labeling at pH 6 and 7, see Figure S41, S42. C) Flow cytometry analysis of fluorescence intensity of CHO-K1 at various concentrations of **12** and labeling with **9** (60 µM). Error bars represent standard deviation of three repeats, 10 000 events per repeat. D) Viability of CHO-K1 cells (MTT assays) at various concentrations of **13**.

Furthermore, MTT cell viability assays showed no toxicity of AMI **13** up to a concentration of 200 µM (Figure 5D). These results showcase the value of the AMI-isonitrile ligation for applications on live cells.

In summary, *C,N*-cyclic-*N´*-acyl AMIs are effective reagents for the bioorthogonal ligation with isonitriles. The reaction rate is, at physiological pH, comparable to that of SPAAC and very fast under acidic conditions (k_2 = 100 M^{-1} s^{-1} at pH 4). We anticipate this higher reactivity at lower pH to be beneficial for the selective labeling and targeting of, for example, tumor cells or organelles with low local pH values. The AMI-isonitrile ligation is orthogonal to the established SPAAC and iEDDA reactions and compatible with live cells. These features open exciting new opportunities for the use of azomethine imines beyond classical organic synthesis. We envision that the AMI-isonitrile ligation will be a valuable tool for chemical biology, material sciences, and drug discovery.

ASSOCIATED CONTENT

Supporting Information

Details on the syntheses and analyses of the presented compounds and the cellular experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no conflict of interest.

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