Isonitriles: Versatile Handles for the Bioorthogonal Functionalization of Proteins

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ABSTRACT: The property of the isonitrile group to enable the simultaneous \( \alpha \)-addition of a strong electrophile and a nucleophile has always attracted the attention of organic chemists. Its versatility is augmented when recognizing that its high structural compactness, the inertia to most of the naturally occurring functional groups, and relatively prolonged physiological and metabolical stability, convert it into the smallest bioorthogonal group. The discovery and optimization of the isonitrile-tetrazine \([4+1]\) cycloaddition as an alternative tool for the development of ligation and decaging strategies and the recently reported reaction of isonitriles with chlorooximes bring new opportunities for the utilization of this functional group in biological systems. Although several approaches have been reported for the synthesis of isonitrile-modified carbohydrates and polysaccharides, its incorporation in proteins has been barely explored. Besides compiling the reported methods for the assembly of isonitrile-modified proteins, this Mini-Review aims at calling attention to the real potential of this modification for protein ligation, decaging, immobilization, imaging, and many other applications at a low structural and functional cost.

INTRODUCTION

Bioorthogonal reactions are of utmost importance for the modification of biomolecules in their native environment, enabling the study of relevant biological processes, including their localization and spatial resolution. The development of such molecular tools has found a plethora of applications in glycan and protein selective ligation and decaging and \textit{in vivo} imaging, among others. These reactions should take place under relevant physiological conditions with fast kinetics, without interfering with the naturally occurring functional groups and having no source of toxic input. To date, few functional groups meet the bioorthogonal criteria; these include strained alkenes and alkynes, azides, and tetrazines as their counterparts. In this context, the search for new bioorthogonal reagents and reactions is a demanding need at exploring a wider chemical space in biological systems. The present Mini-Review is intended to call attention to the advantages of isonitriles as bioorthogonal reporters for the functionalization of proteins. Isonitrile-functionalized proteins and their very recent advances on ligation and decaging will be discussed as well as a brief outlook on the perspectives of such strategies. A more general review from the Franzini’s group compiling the chemical insights on isonitriles as bioorthogonal reporters has been recently published.

ISONITRILES: THE SMALLEST BIOORTHOGONAL FUNCTIONAL GROUPS

Isonitriles or isocyanides are unique compounds bearing a relatively stable divalent carbon with a partial carbeneon character. Although the isonitrile group is not found in mammals and vertebrates, the evolution of specific biosynthetic pathways has ensured their presence in many terrestrial and marine natural compounds. The reactivity of isonitriles as weak nucleophiles is limited to highly electrophilic species such as resonance-stabilized carbocations, iminium, \(N\)-alkylquinolinium, and tropolonyl ions, which are often accompanied by a second nucleophile for a double \( \alpha \)-addition. Thus, isonitriles are mostly unreactive in the presence of biologically occurring functional groups such as alcohols, amines, thiols, carboxylic acids, aldehydes, and ketones. Their stability—measured as the hydrolysis rate to the corresponding formamidines—is almost negligible around neutral pH values and can be further avoided by employing \(\alpha,\alpha\)-disubstituted isonitriles. Recent studies also demonstrate that secondary and tertiary isonitriles are metabolically stable, only exhibiting a slow NADPH-dependent microsomal oxidation. However, probably the most outstanding feature of the isonitrile group, when compared with other bioorthogonal handles, is its structural compactness. The isonitrile is the smallest known bioorthogonal group, and a potential solution for the study of biological processes in which the structural perturbations introduced by the reactive handle affect the functionality of the biomolecule of interest.

ISONITRILES FOR THE BIOORTHOGONAL MODIFICATION OF PROTEINS

These interesting features make isonitriles perfectly suitable for the modification of biomolecules such as carbohydrates and...
proteins (Figure 1). In organic synthesis, isonitrile-based multicomponent reactions (iMCRs) such as the Passerini 3-component reaction (P-3CR), consisting of the condensation of an isonitrile, a carbonyl component, and a carboxylic acid and the Ugi 4-component reaction (Ugi-4CR) additionally involving an amine, have been extensively studied by the Wessjohann and Rivera groups and proven excellent for the assembly of cyclic peptides, lipo- and glyco-peptides and very recently for the conjugation of large biomolecules such as proteins and polysaccharides. The isonitrile group is a carbon monoxide type acceptor and coordinates late transition metals such as Ag, Fe, Cu, Ni, Pd, Pt, among others11, a feature that can be exploited in strategies comprising protein self-assembly, immobilization, purification and imaging, flow biocatalysis, and so on. Although the reaction of isonitriles with tetrazines, one of the most described bioorthogonal reactants, has been known since 1982,12 isonitriles have been rarely employed as bioorthogonal reporters, a scenario that is about to change with the recent studies on isonitrile-tetrazine ligation as a unique tool for bioconjugation and also the development of the isonitrile-tetrazine ligation as a unique tool for bioconjugation. Carbohydrates and proteins bear a high amount of solvent-accessible reactive groups, which contribute to the formation of bioconjugates lacking a defined composition and molecular mass. Nevertheless, glycoconjugate and polymer research fields have benefited from these processes. Isonitriles have also been widely employed in coordination chemistry. The isonitrile group is a carbon monoxide type σ-donor and π-acceptor and coordinates late transition metals such as Ag, Fe, Cu, Ni, Pd, Pt, among others,11 a feature that can be potentially exploited in strategies comprising protein self-assembly, immobilization, purification and imaging, flow biocatalysis, and so on. Although the reaction of isonitriles with tetrazines, one of the most described bioorthogonal reactants, has been known since 1982,12 isonitriles have been rarely employed as bioorthogonal reporters, a scenario that is about to change with the recent studies on isonitrile-tetrazine click and release strategies.13 Moreover, a bioorthogonal reaction between isonitriles and chlorooximes has been recently described and employed for the live cell labeling of surface glycans.17

PROTEIN FUNCTIONALIZATION WITH ISONITRILES

Although extensively employed in carbohydrate and polysaccharide modification,18,19 few reports exist describing the modification of proteins with isonitriles. In organic synthesis, isonitriles are mainly prepared via the dehydration of a formamide under reaction conditions that cannot be directly employed with proteins or other complex biomolecules.20 Instead, the isonitrile should be introduced through a heterobifunctional linker and a preferably site-selective reaction.

A pioneering work by Leeper and co-workers comprised the development of the isonitrile-tetrazine ligation as a unique tool for bioconjugation and also the first report of protein functionalization with isonitriles (Scheme 1A).21 The authors developed an elegant procedure to prepare a maleimide-isonitrile bifunctionalized linker, which was employed subsequently for the functionalization of a mutant of the C2A domain of synaptotagmin-I bearing a single cysteine residue at position 78.

Recently, Chaubert and co-workers reported on the utilization of iMCRs for the modification of Trastuzumab, taking advantage of the spatial proximity of lysins and glutamic/aspartic acids as Ugi-4CR counterparts (Scheme 1B). Interestingly, by employing homo-bifunctional isonitriles in high excess, it was possible to access isonitrile-functionalized proteins.22 A totally different but highly valuable approach was followed by Xiao and co-workers, incorporating for the first time isonitrile-containing amino acids to the genetic code and inserting them into sfGFP and EGP proteins expressed in E. coli and mammalian HeLa cells (Scheme 1C). To this end, the authors employed a pyrrolysyl-tRNA synthetase (PylRS)/tRNA<sub>CUA</sub> pair, which allows the insertion of noncanonical synthetic amino acids containing the isonitrile moiety.23

ISONITRILE-TETRAZINE LIGATION FOR PROTEIN LABELING AND DECAGING

In 1982, Seitz and co-workers first described the inverse-electron demand [4+1]-cycloaddition of isonitriles and 1,2,4,5-tetrazines, that upon N<sub>2</sub> release affords a tetraaza-norbornadien-imine derivative (2). This imine undergoes tautomerization to generate a new imine intermediate (3) comprising an aromatic pyrazole, that in the presence of H<sub>2</sub>O hydrolyses to the corresponding 4-aminopyrazole (5) and an aldehyde (4) (Scheme 2 A).12 Although this reaction showed potential for masking carbonyl groups as isonitriles, the lack of interest toward this discovery was, most probably, determined by the formation of an unstable conjugate. Only 30 years after this seminal work, Leeper and co-workers were able to expand the scope of isonitriles as bioorthogonal reporters, with the design of isonitriles affording stable adducts with tetrazines. They
described that the adducts from tertiary (α,α-disubstituted) isonitriles with tetrazines persisted for many hours in buffer, because the imine tautomerization step (II to III) was prohibited. On the other hand, propionate-derived isonitriles formed stable adducts by further tautomerizing to a vinylogous urethane derivative. Then, the authors combined a C2Am-tert-isonitrile adduct and a tetrazine-rhodamine and obtained the corresponding 4H-pyrazol-4-imine adduct accompanied by traces of the tert-amine hydrolysis product (Scheme 2B).21 Furthermore, Leeper and co-workers expanded the utilization of this ligation platform for metabolic glycan imaging,24 including dual metabolic glycan imaging with different isonitrile- and azide-labeled sugars, which further demonstrated the orthogonality between azides and isonitriles toward their corresponding azide-strained-alkyne [3+2]- and isonitrile-tetrazine [4+1]-cycloadditions.25 The isonitrile-tetrazine re-
action is, however, characterized by modest rates \( (k_2 = 0.05−0.6 \text{ M}^{-1} \text{ s}^{-1}) \). The influence of different experimental parameters was further studied by Franzini and co-workers, demonstrating that under physiological conditions (buffer, 37 °C) and in the presence of bulky tetrazines, the reactions take place significantly faster. Computational modeling demonstrated that dispersion forces between bulky substituted tetrazines and the isonitrile group, rather than triggering repulsion, were able to lower the transition state energy and enhance kinetics. This report was followed by the remarkable experimental evidence that bulky tetrazines were able to stabilize the isonitrile-tetrazine adducts, thus circumventing the utilization of tertiary isonitriles. These findings are of high interest for protein labeling, as they allow to take advantage of the compact structure of the isonitrile group.

Franzini’s group has been highly active on the assessment of the isonitrile-tetrazine ligation as a bioorthogonal release method (Scheme 3). To this end, they have employed 3-isocyanopropyl (ICPr) groups \( (7) \), which upon reactions with the tetrazines afford aldehydes \( (8) \) that then experience a spontaneous \( \beta \)-elimination. The rationality behind this process is that 3-oxopropyl groups eliminate different leaving groups under physiological conditions, a reaction that can be catalyzed by serum albumin. The authors demonstrated that the reaction of ICPr groups with tetrazines is fast \( (k_2 = 4 \text{ M}^{-1} \text{ s}^{-1}) \) and that the release of phenols and amines (from carbamates) \( (9) \) is quantitative in diluted serum. Furthermore, they demonstrated that the elimination step is independent from hydrolysis and can also occur from the imine intermediate.

The capacity of spontaneous elimination from the benzylc position of five-membered heterocycles such as 4-aminopyrazoles \( (11) \) was further exploited as a release strategy. NMR and DFT studies suggested that the elimination can also occur from the intermediate imine \( (10) \). Thus, tetrazylmethyl (TzMe) and tetrazylmethoxy carbonyl (Tzmoc) groups were designed to protect phenols and amines (as carbamates) \( (12) \), respectively, to be released upon addition of isonitriles. A combined strategy, employing isonitrile and tetrazine caged fluorophores, proved also to be efficient for their simultaneous release in zebrafish. Besides activating different tetrazine-caged fluorophores, the click and release strategy offers many opportunities for drug delivery applications and site-specific protein decaging. With the incorporation of isonitrile-containing amino acids to the genetic code, Xiao and co-workers also explored their reaction with tetrazines to either label or activate modified peptides and proteins such as lipoyc acid ligase-acceptor (LAP) and LAP-tagged neurexin1β in living cells.

### ISONITRILE-CHLOROOXIME LIGATION

Very recently, Wennemers and co-workers reported on a new bioorthogonal ligation consisting on the reaction of isonitriles with chlorooximes \( (13) \) under physiological conditions. The water-assisted generation of nitrile oxide \( (14) \) from the chlorooxime is followed by the reaction of this species with the isonitrile to form an intermediate nitrium ion \( (15) \), that then reacts with water to afford an \( \alpha \)-hydroxyimino amide \( (16) \) (Scheme 4). As in the case of the isonitrile-tetrazine ligation, this reaction is governed by a second-order kinetic law and occurs with moderate rates under room and physiological temperatures \( (0.74 \text{ (r.t.)}/1.04 \text{ (37 °C)} \text{ M}^{-1} \text{ s}^{-1}) \). Furthermore, the authors explored the chemoselectivity of the reaction in the presence of naturally occurring functional groups, discovering that only reduced thiols were able to interfere, which could be avoided by performing the reactions at pH 5. To demonstrate the utility of this method, metabolic labeling of cell surface glycans with the addition of peracetylated \( N \)-isocyanopropyl mannosamine and further imaging with a biotinsulfon-fucosylated chlorooxime/avidin-Alexa Fluor 488 system was successfully performed.

### CONCLUSIONS AND FUTURE PERSPECTIVES

The isonitrile group stands as one of the most versatile and unique functional moieties in organic chemistry, with a synthetic potential to be further explored. Introducing isonitriles as functional groups in proteins—whether chemically or recombinantly—opens up an avenue of possibilities toward different protein bioconjugation, ligation, labeling, immobilization, and decaging approaches.

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Notes
The authors declare no competing financial interest.

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Dr. Aldrin V. Vasco received his Diploma and Master degrees in Chemistry from the University of Havana, Cuba in 2013 and 2015, respectively (Prof. D. G. Rivera). From 2013 to 2015 he worked as lecturer assistant at the Department of Chemistry at the Technical University of Havana. He moved to the Leibniz-Institute of Plant Biochemistry (Halle, Germany) for his Ph.D. as part of a DAAD scholarship, and he obtained his Ph.D. degree in 2020 from the Martin Luther University Halle-Wittenberg under the supervision of Profs L. A. Wessjohann and D. G. Rivera. From April 2020, he has been a postdoctoral researcher at the Bioorganic Chemistry Department at the Leibniz-Institute of Plant Biochemistry.

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DEDICATION

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