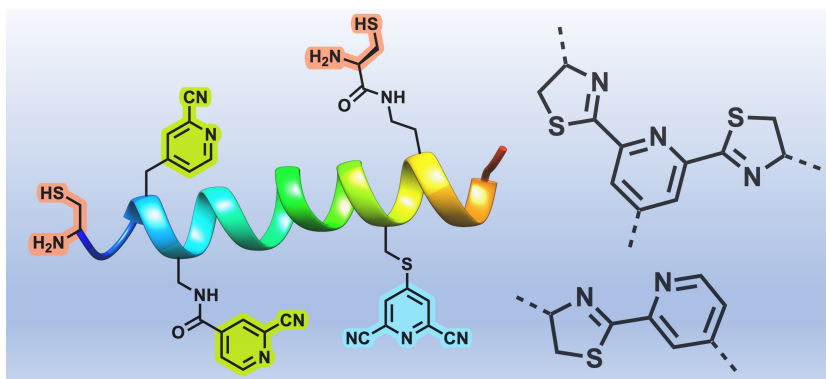


The Cyanopyridine–Aminothiols Click Reaction: Expanding Horizons in Chemical Biology

Christoph Nitsche*^a

^a Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

christoph.nitsche@anu.edu.au



Received:
Accepted:
Published online:
DOI:

Abstract Bioorthogonal reactions hold significant promise for applications in chemical biology. Despite their potential, nitriles have often been overlooked as reactive functional groups for selective bioconjugation. The condensation reaction between cyanopyridines and 1,2-aminothiols stands out as a particularly favorable nitrile modification strategy that proceeds under biocompatible conditions. Cyanopyridines can be seamlessly incorporated into peptides and proteins through both chemical and biotechnological approaches. Similarly, the selective integration of 1,2-aminothiols into peptides and proteins is achievable, leveraging the uniquely reactive N-terminal cysteine functional group.

Key words click chemistry, bioconjugation, peptides, proteins, bioorthogonal, nitrile, cysteine

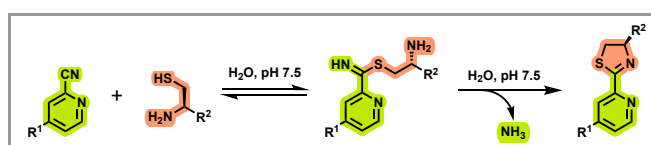
1 Introduction

The 2022 Nobel Prize in Chemistry honoured Carolyn Bertozzi, Morten Meldal and Barry Sharpless for their pioneering contributions to click chemistry and bioorthogonal chemistry.¹ This recognition underscores the pivotal role synthetic methodology plays in biomedical sciences, enabling precise and targeted modifications within living systems. This recognition further underscores the essential contributions of organic chemists in shaping innovative approaches at the interface of chemistry and biology. Undoubtedly, the click reaction between azides and alkynes stands out as the most renowned and extensively used bioorthogonal transformation.² However, alongside this widely adopted reaction, other transformations, such as tetrazine-based cycloadditions for example, are gaining prominence.³ As science evolves, these alternative reactions provide researchers with expanded options for precise and selective chemical modifications in complex biological systems.

The condensation reaction between activated nitriles and 1,2-aminothiols offers an alternative bioconjugation method that satisfies numerous criteria for bioorthogonality. The reaction proceeds efficiently in water at physiological pH and ambient temperature. It exhibits excellent chemoselectivity and the resulting thiazoline reaction product is stable under redox conditions and only hydrolyses in highly acidic, non-physiological environments. Furthermore, this reaction is orthogonal to most endogenous reactants, except for cysteine itself and peptides or proteins with N-terminal cysteine residues, which are relatively rare. It also demonstrates orthogonality to all canonical amino acid side chains, making it exceptionally valuable for selective peptide and protein modifications.

2 The biocompatible condensation reaction

As depicted in Scheme 1, the two-step reaction mechanism involves the reversible formation of a thioimide intermediate, followed by an irreversible cyclocondensation with the release of ammonia. As the initial reaction step depends on the nucleophilicity of the thiol, the reaction is usually performed in buffer at pH 7–8, which is close to the pK_a range of the thiol in N-terminal cysteine (1,2-aminothiol) in peptides and proteins.⁴



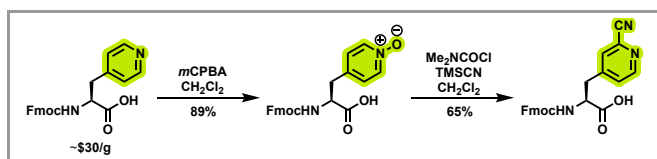
Scheme 1 Two-step mechanism of the cyanopyridine–aminothiols reaction.

The reactivity of the nitrile component is dependent on its electrophilicity, which can be finely adjusted to enable tailored applications.⁵ The reactivity of benzonitrile is typically considered insufficient for applications in bioconjugation,

necessitating the incorporation of heterocycles to enhance nitrile reactivity. For instance, 2-cyanopyridine reacts approximately 20 times faster with cysteine than benzonitrile.^{5a} Cyanopyridine demonstrates sufficient reactivity for applications in peptide macrocyclisation and bioconjugation, as highlighted below, while also maintaining stability to suppress nitrile hydrolysis in aqueous solutions or during acidic cleavage/deprotection, which is a concern with highly reactive nitrile species.⁶ An alternative larger heterocycle frequently used to activate the nitrile is benzothiazole. Taking inspiration from the biosynthesis of firefly luciferin, 2-cyanobenzothiazole (CBT) forms a thiazoline conjugate upon reacting with N-terminal cysteine.⁷ Additionally, it has been demonstrated that CBT can react with non-terminal cysteines, capturing the thioimide intermediate,⁸ whereas 2-cyanopyridine exhibits selectivity for N-terminal cysteine.

3 Peptide macrocyclisation

While CBT as well as 2-cyanothiophene have recently found application in peptide macrocyclisation with reaction rates comparable to 2-cyanopyridine,^{6, 9} it is worth noting that the synthesis of Fmoc-CBT and nitrile hydrolysis provide challenges.⁶ The use of a linked CBT-thiazoline amino acid in solid-phase peptide synthesis has recently been reported as a more practical alternative to synthesise macrocycles.⁹ An Fmoc-protected 2-cyanopyridyl-alanine (Cpa) derivative can be readily synthesised from affordable precursors in two steps (Scheme 2).¹⁰

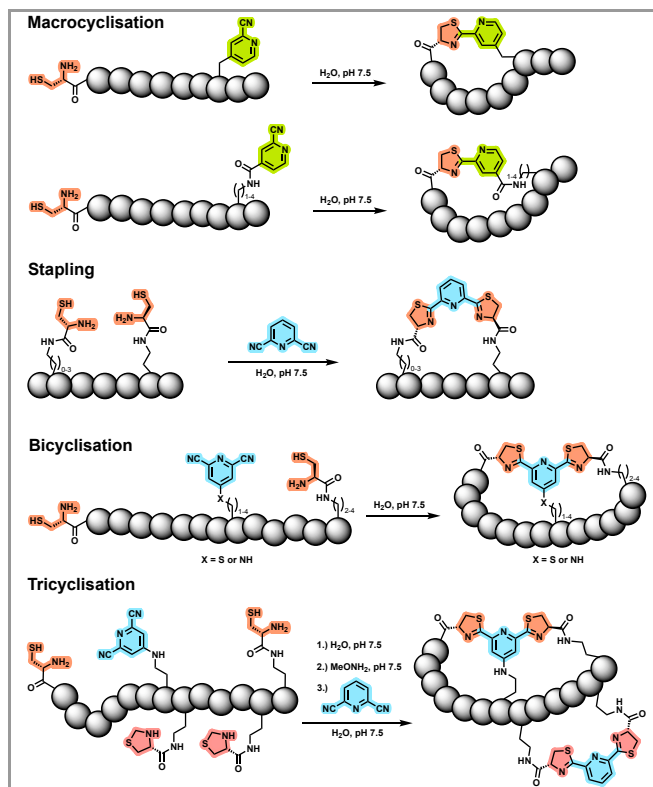


Scheme 2 Two-step synthesis of Fmoc-L-3-(2-cyano-4-pyridyl)alanine (Cpa).

Alternatively, 2-cyanopyridine-based amino acids with various linkers can be directly synthesised on the solid phase by coupling 2-cyanoisonicotinic acid to orthogonally protected amine side chains.¹¹ Similarly, Fmoc-protected 2,6-dicyanopyridine amino acids can be synthesised and employed in solid-phase peptide synthesis.¹² Commercially available 2,6-dicyanopyridine can react with peptides displaying 1,2-aminothiol functional groups, either at their N-terminus or within side chains of unnatural amino acids.¹³ Protected thiazolidine versions of these 1,2-aminothiol side chains can be deprotected under biocompatible conditions using methoxyamine in buffer, adding an additional layer of orthogonality.¹² Utilising this toolbox of amino acids and reagents, we have harnessed the cyanopyridine–aminothiol click reaction to construct macrocyclic, stapled, bicyclic and tricyclic peptides under biocompatible conditions (Scheme 3).^{10–13}

Thanks to the biocompatible nature of these transformations, they can be effectively employed for the *in-situ* modification of peptides, especially during screening campaigns. We used the stapling reaction with 2,6-dicyanopyridine to identify stapled peptide inhibitors of the Zika virus protease.¹³ The stapling reaction proceeds within the biochemical inhibition assay, allowing for the direct identification of hit compounds without the need for prior synthesis of the constrained peptide.

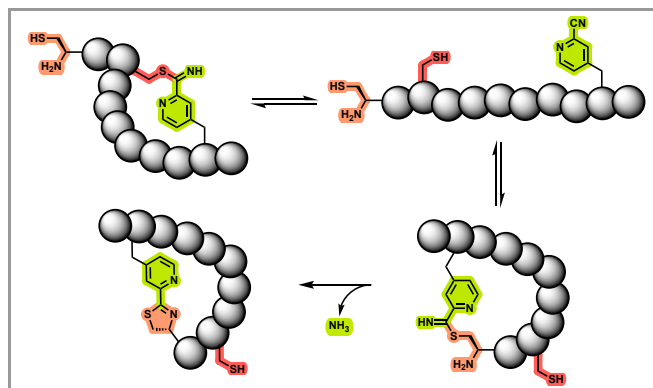
Similarly, we applied the stapling reaction to create helical variants of the antimicrobial peptide aurein 1.2.¹⁴ Remarkably, an unconventional *i, i + 8* stapling pattern resulted in enhanced helicity compared to the wild-type aurein. Overall, peptides constrained by the cyanopyridine–aminothiol click reaction were investigated as inhibitors of viral proteases,^{10–13, 15} influenza hemagglutinin,¹⁶ deubiquitinase USP15,¹⁷ as well as for the generation of large DNA-encoded libraries (DEL).¹⁸



Scheme 3 Biocompatible strategies for constraining peptides based on the cyanopyridine–aminothiol click reaction.

4 Orthogonality to cysteine

An important characteristic of cyanopyridine is its distinct selectivity for N-terminal cysteine over non-terminal cysteines. While CBT has been documented to form stable thioimides with cysteines,⁸ this is not observed for cyanopyridine (Scheme 4).



Scheme 4 Selective thiazoline formation between cyanopyridine and 1,2-aminothiols in presence of competing thiol nucleophiles.

Consequently, additional cysteine residues are well tolerated within the peptide macrocycle, as demonstrated for cyclic, stapled and bicyclic peptides (Figure 1).^{10, 12–13}

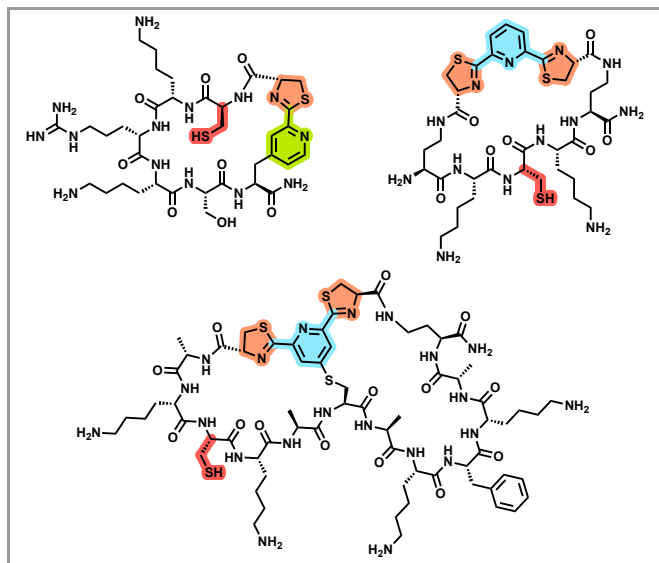
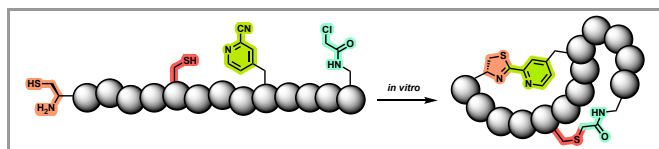


Figure 1 Macrocyclic, stapled and bicyclic peptides with additional cysteine residues that remain unaffected by the cyanopyridine–nitrile click reaction.

As recently demonstrated, this chemoselectivity can be harnessed for the synthesis of bicyclic peptides by incorporating an additional chloroacetamide side chain.^{9, 16} Given that the reaction between nitrile and N-terminal cysteine proceeds more rapidly than the alkylation reaction between chloroacetamide and cysteine, this chemistry predominantly yields the desired bicyclic product (Scheme 5).



Scheme 5 Selective bicycle formation during *in vitro* translation.

5 Combination with genetic encoding

The linkage of macrocyclic peptides to a DNA barcode presents significant advantages for screening campaigns. The creation of DNA-encoded libraries (DEL) involves the synthetic linkage of DNA and peptides.¹⁹ A recent study from Merck (MSD) demonstrates that macrocyclisation through the cyanopyridine–aminothiol click reaction is remarkably compatible with this approach, employing N-terminal cysteine, Cpa and a C-terminal DNA.¹⁸ Notably, DNA-conjugated macrocycles exhibited resilience when subjected to standard DNA ligation, purification, and DEL screen elution conditions. Furthermore, they maintained stability after one month of storage at -20°C . The robustness of this chemistry has enabled the design of a macrocyclic peptide DEL estimated to contain one billion compounds.¹⁸

Another powerful strategy involves the direct genetic encoding of unnatural amino acids, facilitating the selective

bioconjugation of proteins or the generation of macrocyclic peptide libraries from mRNA display. Aminoacyl-tRNA synthetases capable of selectively incorporating Cpa have been independently discovered to facilitate such applications.^{17, 20} It should be noted that synthetases for two different structural isomers of Cpa have been used, which are referred to as *mCpa* and *pCpa* (Figure 2). The incorporation of Cpa into small model proteins enabled macrocyclisation of peptides fused to the protein N-terminus as well as protein stapling after the incorporation of two Cpa residues.²⁰ The selective incorporation and deprotection of Lys(Thz) was also reported (Figure 2).²¹

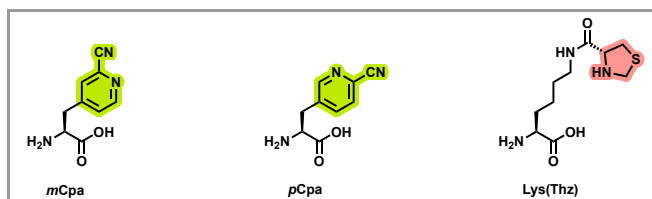


Figure 2 Amino acids for which aminoacyl-tRNA synthetases are available.

Recently, the flexizyme strategy has been employed to charge activated esters of Cpa directly onto tRNA for their application in Random Nonstandard Peptides Integrated Discovery (RaPID).¹⁶ The RaPID system is designed to accommodate various unnatural amino acids, making it possible to successfully implement the selective bicycle formation depicted in Scheme 5 for *in vitro* translation.

6 Conclusion

The cyanopyridine–aminothiol click reaction has emerged as a robust tool for macrocyclisation and conjugation. While the initial thioimide formation is reversible, the subsequent reaction leading to thiazoline formation, which exclusively occurs with N-terminal cysteine (1,2-aminothiol), is irreversible. This characteristic establishes the reaction as exceptionally selective for N-terminal cysteine over other cysteine residues, effectively rendering it bioorthogonal. Very recently, the reaction has garnered substantial attention in the creation of DNA-encoded libraries. This includes both libraries generated through the conjugation of DNA barcodes as well as the direct encoding of macrocyclic peptides. While most current applications focus on constrained peptides, future research is essential to investigate the cyanopyridine–aminothiol click reaction for broader bioconjugation purposes.

Funding Information

C.N. thanks the Australian Research Council for a Future Fellowship (FT220100010).

Conflict of Interest

The author declares no conflict of interest.

References

- (a) Zaia, J. *Anal. Bioanal. Chem.* **2023**, *415*, 527. (b) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem. Int. Ed.* **2009**, *48*, 6974. (c) Bird, R. E.; Lemmel, S. A.; Yu, X.; Zhou, Q. A. *Bioconjugate Chem.* **2021**, *32*,

2457. (d) Scinto, S. L.; Bilodeau, D. A.; Hincapie, R.; Lee, W.; Nguyen, S. S.; Xu, M.; am Ende, C. W.; Finn, M. G.; Lang, K.; Lin, Q.; Pezacki, J. P.; Prescher, J. A.; Robillard, M. S.; Fox, J. M. *Nat. Rev. Methods Primers* **2021**, *1*, 30.
- (2) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046. (b) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596. (c) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057.
- (3) (a) Wu, H.; Devaraj, N. K. *Acc. Chem. Res.* **2018**, *51*, 1249. (b) Richter, D.; Lakis, E.; Piel, J. *Nat. Chem.* **2023**, *15*, 1422.
- (4) Maguire, O. R.; Zhu, J.; Brittain, W. D. G.; Hudson, A. S.; Cobb, S. L.; O'Donoghue, A. C. *Chem. Commun.* **2020**, *56*, 6114.
- (5) (a) Berteotti, A.; Vacondio, F.; Lodola, A.; Bassi, M.; Silva, C.; Mor, M.; Cavalli, A. *ACS Med. Chem. Lett.* **2014**, *5*, 501. (b) Maltsev, O. V.; Walter, V.; Brandl, M. J.; Hintermann, L. *Synthesis* **2013**, *45*, 2763. (c) Oballa, R. M.; Truchon, J.-F.; Bayly, C. I.; Chauret, N.; Day, S.; Crane, S.; Berthelette, C. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 998. (d) Toyama, T.; Saitoh, T.; Takahashi, Y.; Oka, K.; Citterio, D.; Suzuki, K.; Nishiyama, S. *Chem. Lett.* **2017**, *46*, 753.
- (6) Tamura, T.; Inoue, M.; Yoshimitsu, Y.; Hashimoto, I.; Ohashi, N.; Tsumura, K.; Suzuki, K.; Watanabe, T.; Hohsaka, T. *Bull. Chem. Soc. Jpn.* **2022**, *95*, 359.
- (7) (a) Ren, H.; Xiao, F.; Zhan, K.; Kim, Y.-P.; Xie, H.; Xia, Z.; Rao, J. *Angew. Chem. Int. Ed.* **2009**, *48*, 9658. (b) Proj, M.; Strašek, N.; Pajk, S.; Knez, D.; Sosič, I. *Bioconjugate Chem.* **2023**, *34*, 1271.
- (8) Ramil, C. P.; An, P.; Yu, Z.; Lin, Q. *J. Am. Chem. Soc.* **2016**, *138*, 5499.
- (9) Liu, M.; Yoshisada, R.; Amedi, A.; Hopstaken, A. J. P.; Pascha, M. N.; de Haan, C. A. M.; Geerke, D. P.; Poole III, D. A.; Jongkees, S. A. K. *Chem. Eur. J.* **2023**, *29*, e202203923.
- (10) Nitsche, C.; Onagi, H.; Quek, J.-P.; Otting, G.; Luo, D.; Huber, T. *Org. Lett.* **2019**, *21*, 4709.
- (11) Patil, N. A.; Quek, J.-P.; Schroeder, B.; Morewood, R.; Rademann, J.; Luo, D.; Nitsche, C. *ACS Med. Chem. Lett.* **2021**, *12*, 732.
- (12) Ullrich, S.; George, J.; Coram, A.; Morewood, R.; Nitsche, C. *Angew. Chem. Int. Ed.* **2022**, e202208400.
- (13) Morewood, R.; Nitsche, C. *Chem. Sci.* **2021**, *12*, 669.
- (14) Coram, A. E.; Morewood, R.; Voss, S.; Price, J. L.; Nitsche, C. *J. Pept. Sci.* **2024**, *30*, e3551.
- (15) Ullrich, S.; Sasi, V. M.; Mahawaththa, M. C.; Ekanayake, K. B.; Morewood, R.; George, J.; Shuttleworth, L.; Zhang, X.; Whitefield, C.; Otting, G.; Jackson, C.; Nitsche, C. *Bioorg. Med. Chem. Lett.* **2021**, *50*, 128333.
- (16) Liu, M.; Morewood, R.; Yoshisada, R.; Pascha, M. N.; Hopstaken, A. J. P.; Tarcoveanu, E.; Poole, D. A.; de Haan, C. A. M.; Nitsche, C.; Jongkees, S. A. K. *Chem. Sci.* **2023**, *14*, 10561.
- (17) Iskandar, S. E.; Pelton, J. M.; Wick, E. T.; Bolhuis, D. L.; Baldwin, A. S.; Emanuele, M. J.; Brown, N. G.; Bowers, A. A. *J. Am. Chem. Soc.* **2023**, *145*, 1512.
- (18) Fan, L.; Yu, Y.; Jayne, C.; Frost, J. R.; Scott, J. D. *Org. Lett.* **2023**.
- (19) Shi, Y.; Wu, Y.-r.; Yu, J.-q.; Zhang, W.-n.; Zhuang, C.-l. *RSC Advances* **2021**, *11*, 2359.
- (20) Abdelkader, E. H.; Qianzhu, H.; George, J.; Frkic, R. L.; Jackson, C. J.; Nitsche, C.; Otting, G.; Huber, T. *Angew. Chem. Int. Ed.* **2022**, *61*, e202114154.
- (21) Nguyen, D. P.; Elliott, T.; Holt, M.; Muir, T. W.; Chin, J. W. *J. Am. Chem. Soc.* **2011**, *133*, 11418.

Biosketch



Christoph Nitsche studied chemistry and business administration. He completed his PhD at Heidelberg University in 2014 for which he received the PhD prize for Medicinal Chemistry by the German Chemical Society. He received a Feodor Lynen Research Fellowship by the Alexander von Humboldt Foundation to work at the Australian National University (ANU) from 2015 to 2018. After a short period as Rising Star Fellow at the Free University of Berlin, he returned to the ANU with an ARC Discovery Early Career Research Award (2019). He was appointed Senior Lecturer in 2020 and promoted to Associate Professor in 2023. He is the recipient of an ARC Future Fellowship (2022), the John Wade Early Career Researcher Award (2022), the Peter Schwerdtfeger Award (2022), the RSC Medicinal Chemistry Emerging Investigator Lectureship (2023), the Thieme Chemistry Journals Award (2023), and the Rennie Memorial Medal (2023). He was named top researcher in Medicinal Chemistry for 2023 and 2024 by The Australian Research Magazine.