Biocompatible and selective generation of bicyclic peptides

Sven Ullrich, Josemon George, Alexandra E. Coram, Richard Morewood, Christoph Nitsche*

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

* christoph.nitsche@anu.edu.au

Abstract

Bicyclic peptides possess superior properties for drug discovery; however, their chemical synthesis is not straightforward and often neither biocompatible nor fully orthogonal to all canonical amino acids. The selective reaction between 1,2-aminothiols and 2,6-dicyanopyridine allows direct access to complex bicyclic peptides in high yield. The process can be fully automated using standard solid-phase peptide synthesis. Bicyclization occurs in water at physiological pH within minutes and without the need for a catalyst. The use of various linkers allows tailored bicyclic peptides with qualities such as plasma stability, conformational preorganization, and high target affinity. We demonstrate this for a bicyclic inhibitor of the Zika virus protease NS2B-NS3 as well as for bicyclic versions of the α-helical antimicrobial peptide aurein 1.2.

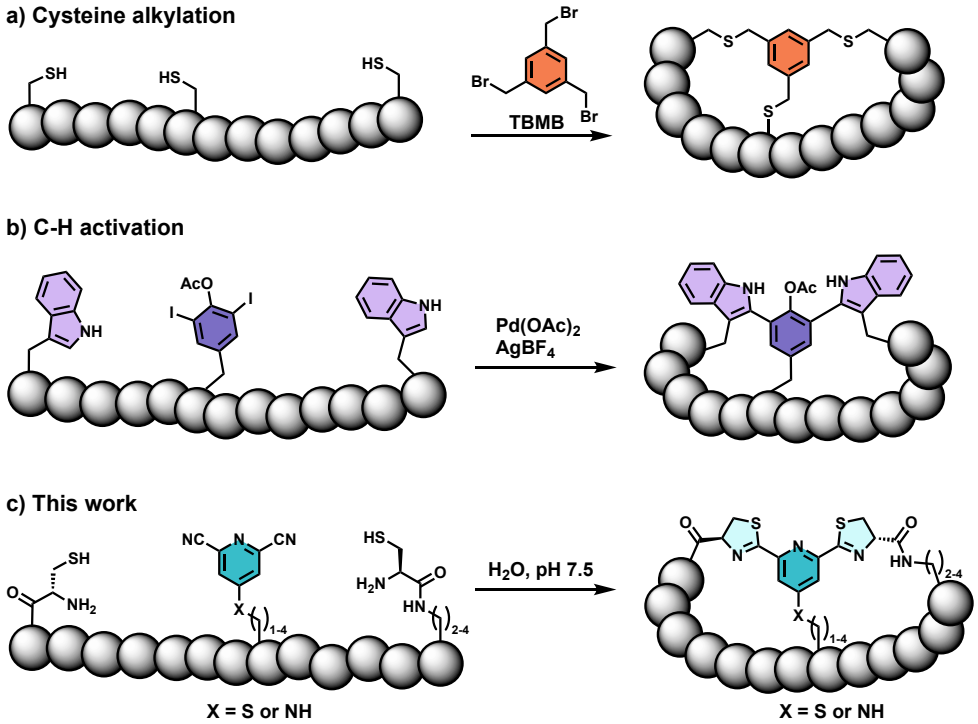
Keywords

Peptides, bicycles, aminothiol-nitrile reaction, macrocyclization, cyanopyridine

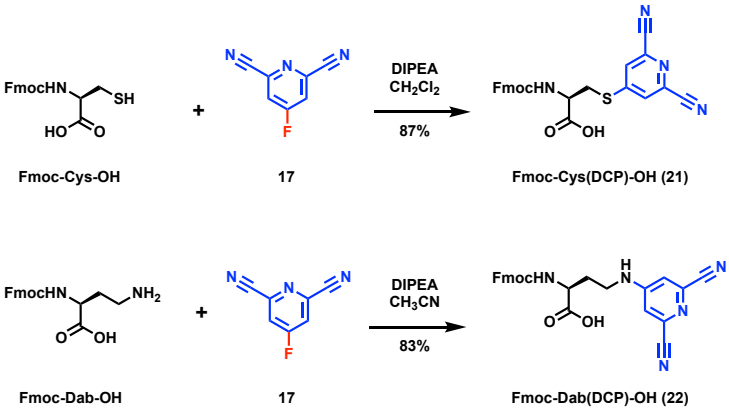
Bicyclic peptides are considered next-generation pharmaceuticals, as they combine properties of antibodies with those of small molecules.[1] The high conformational constraint of bicycles leads to enhanced metabolic stability and antibody-like affinity and specificity.[1-2] Hence, bicyclic peptides are even capable of disrupting protein-protein interactions (PPIs) which were formerly deemed undruggable.[2b, 3] The virtue of genetically encoded peptide libraries allows the identification of constrained peptide ligands for targets of interest using state-of-the-art display screenings.[4] While these techniques are routinely used for the identification of linear and monocyclic peptides, challenges in biocompatible and selective chemistry complicate the application of bicyclic peptide libraries. Available methods to access bicyclic peptides are barely sufficient to keep pace with the current shift toward bicycles as highly anticipated drugs.

Despite numerous synthetic strategies to generate monocyclic peptides with high chemical diversity,[5] conventional approaches to access bicyclic peptides are mainly based on cysteine modification, using alkylating agents like TBMB (Scheme 1a).[6] Irrespective of the outstanding success of these reagents in phage display and other encoded chemical libraries, they are neither fully selective nor biocompatible as they may modify other nucleophilic residues in peptides or target proteins.[7] Alternative late-stage modifications such as C-H activation between tryptophan and phenylalanine/tyrosine residues (Scheme 1b) require metal catalysts and organic solvents, rendering them bioincompatible.[8]

We present a peptide bicyclization technique that is catalyst-free, biocompatible, and orthogonal to all canonical amino acids including cysteine (Scheme 1c). The strategy is based on the condensation reaction between 1,2-aminothiols and cyanopyridine to furnish monocyclic and stapled peptides with superior bioactivity.[9] The introduction of unnatural amino acids bearing a 2,6-dicyanopyridine moiety in the side chain enables the selective and biocompatible synthesis of much more structurally complex bicyclic peptides. The 1,2-aminothiol counterpart can be introduced as side chain in unnatural amino acids or by using N-terminal cysteine in the peptide sequence.[9c]



Scheme 1. Unlike the strategy described in this study, conventional methods to access bicyclic peptides are neither fully selective nor biocompatible. a) Connection of three cysteine residues with 1,3,5-tris(bromomethyl)benzene (TBMB). b) Conjugation between tryptophan and 4-acetoxy-3,5-diiodophenylalanine side chains. c) Biocompatible and selective conjugation between 1,2-aminothiols and 2,6-dicyanopyridine side chains.



Scheme 2. Facile synthesis of Fmoc-Cys(DCP)-OH (**21**) and Fmoc-Dab(DCP)-OH (**22**) suitable for standard Fmoc solid-phase peptide synthesis.

To introduce 2,6-dicyanopyridine (DCP) in the side chain of nucleophilic amino acids, we developed the reagent 4-fluoro-2,6-dicyanopyridine (4F-DCP, **17**), which is readily accessible from commercially available 4-chloro-2,6-dicyanopyridine and cesium fluoride (Scheme S1). Reaction of **17** with Fmoc-protected cysteine or side-chain amines (e.g., Fmoc-Dab-OH),

furnishes amino acids with DCP side chains (Scheme 2). Despite the highly electron deficient aromatic system, these amino acids (e.g., **21** and **22**) can be used directly in automated Fmoc solid-phase peptide synthesis under standard coupling and cleavage conditions. Subsequently, spontaneous bicyclization is triggered in water at physiological pH and the bicyclic peptide can be purified using standard HPLC methods. We first demonstrated this approach for model peptide **1b** (Figure 1), which contains five amino acids in each cycle. DCP was introduced in the peptide center as Fmoc-Cys(DCP)-OH (**21**). N-terminal cysteine and C-terminal Fmoc-Dab($Cys^{Boc,Trt}$)-OH (**23**) served as the two 1,2-aminothiol functional groups necessary for bicycle formation. Following standard cleavage with 91% TFA, the crude peptide was exposed to aqueous buffer at pH 7.5 to furnish the bicyclic peptide after less than 10 minutes in 81% yield (Figure 1). We isolated **1b** using standard HPLC purification and characterized it by high-resolution mass spectrometry and NMR spectroscopy. Assignment of all ^{1}H and ^{13}C resonances in water (10% D_2O) confirmed a single species of bicyclic peptide without diastereomers (Figure S9).

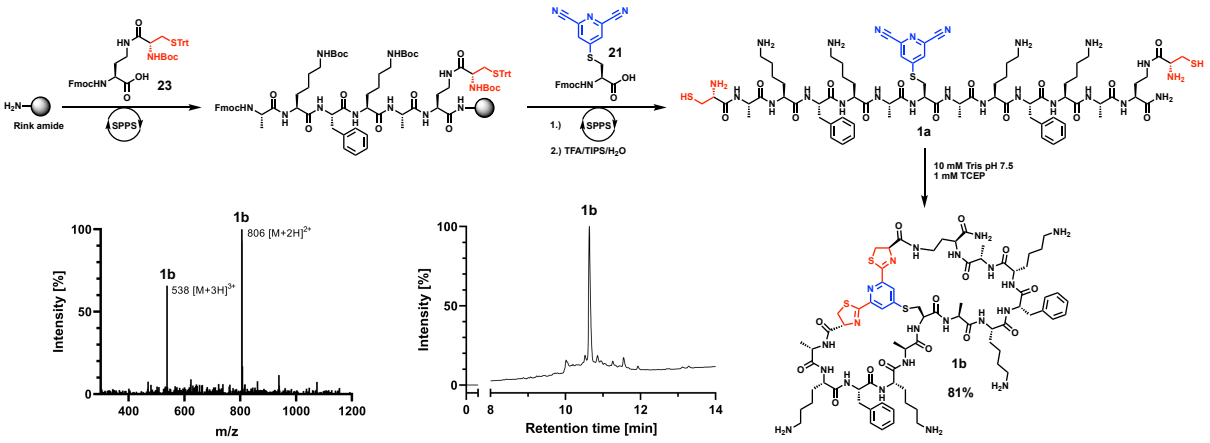
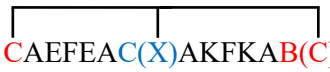
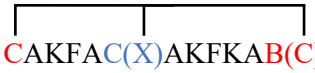
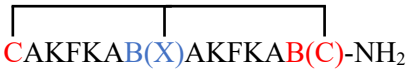


Figure 1. Synthesis of model peptide **1a** *via* standard Fmoc solid-phase peptide synthesis and subsequent formation of bicycle **1b** in aqueous buffer. LC-MS chromatogram (254 nm) and associated ESI mass spectrum of crude **1b** are shown.

Studying the scope of bicycle formation using this strategy, we explored various derivatives of **1b**, which were accessed in yields ranging from 66% to 98% (**2b**–**7b**, Table 1). These include highly basic sequences (**2b**), negatively charged residues (**3b**), contracted (**5b**) and expanded (**6b**) cycle sizes. Most importantly, derivative **4b** contains an additional cysteine residue within the peptide sequence, clearly demonstrating that the presented strategy is truly selective and orthogonal to all canonical amino acids, *including* cysteine. As cysteine is commonly used in conjugation of peptides and proteins,[10] our method offers clear orthogonality to these

conventional approaches, allowing for selective cysteine modification in bicyclic peptides. We further demonstrated that bicycle formation occurs irrespective of the position of DCP and 1,2-aminothiol functional groups in the peptide sequence. Compound **7b** is a constitutional isomer of **1b** with differing connectivity, as **7b** contains the DCP residue at the C-terminus (Table 1), highlighting the versatility of the approach. Peptides **8b**–**10b** demonstrate that the DCP linker can be incrementally increased to the most flexible lysine-based amino acid, allowing for a broad diversity of available linkers for various applications.

Table 1. Bicyclic peptides synthesized in this study.

Cpd.	Sequence[a]	Yield (%)[b]
1b		81[c], 69[d]
2b		66[c]
3b		71[c]
4b		69[c]
5b		96[c]
6b		75[c]
7b		98[c]
8b		62[c], 62[e]
9b		73[e], 52[f]
10b		68[e]

[a] X = 2,6-dicyanopyridine, B = L-2,4-diaminobutyric acid (Dab). Each linkage between X and C represents a thiazoline (Figure 1). [b] Ratio of bicyclic peptide after total synthesis according to LC-MS. [c] Synthesized using Fmoc-Cys(DCP)-OH (**21**) or Fmoc-Dab(DCP)-OH (**22**). [d] Synthesized using Fmoc-Cys(Stmp)-OH followed by deprotection on the solid support and substitution with 4F-DCP (**17**). [e] Synthesized using Fmoc-Dab(Ivdde)-OH, Fmoc-Orn(Ivdde)-OH or Fmoc-Lys(Ivdde)-OH followed by deprotection on the solid support and substitution with 4F-DCP (**17**). [f] Synthesized using Fmoc-Orn(Alloc)-OH followed by deprotection on the solid support and substitution with 4F-DCP (**17**).

To make our protocol fully applicable to laboratories with limited access to synthetic chemistry set-ups, we established a protocol that is fully amenable to automation. Previously, we reported a protocol to assemble amino acids with 1,2-aminothiol groups, such as Dab(Cys), Orn(Cys) and Lys(Cys), directly during solid-phase peptide synthesis.[9c] Here, we expand this strategy towards DCP-containing amino acids, enabling the fully programmable synthesis of bicyclic peptides (Schemes S11–13). Orthogonal protecting groups for cysteine (Stmp)[11] and amine side chains (Alloc, Ivdde)[12] were selectively deprotected on the solid support and subsequently reacted with **17**. In case of cysteine, this enabled the direct synthesis of **1b** on solid support in 69% yield. In case of amine side chains (Dab, Orn, Lys), this facilitated the synthesis of **8b**–**10b** in good yield, demonstrating the compatibility of the method with frequently used and commercially available amino acid building blocks.

To demonstrate that our approach can rapidly deliver bicyclic peptides with biological activity, we validated this for an inhibitor of the Zika virus protease NS2B-NS3 (ZiPro). We synthesized and isolated compound **2b** (Figure S7) which contains the tribasic substrate recognition motif KRK of ZiPro.[13] Indeed, the bicycle displayed a low half-maximal inhibitory concentration (IC_{50}) of 260 nM (Figure S4) in our established *in vitro* ZiPro inhibition assay, showcasing desirable binding affinity[14] even without applying a display screening. In addition, **1b** showed pronounced plasma stability with no detectable degradation over 90 minutes compared to the control compound propantheline bromide (Figure S5). High affinity and plasma stability are two crucial parameters that render bicyclic peptides excellent drug candidates.

An important application of peptide cyclisation, often referred to as ‘stapling’, is the stabilization of helical peptide conformations necessary for their biological activity.[15] Although bicyclic peptides offer superior immobilization and rigidification, only a few examples of bicyclic helical peptides exist.[16] Therefore, we set out to demonstrate that the presented strategy can also be applied to bicyclic peptides with helical conformation. We chose the short peptide aurein 1.2, which is one of various antimicrobial peptides secreted by the Australian frogs *Litoria aurea* and *Litoria raniformis*.[17] Aurein 1.2 exhibits its antimicrobial activity when forming an amphipathic α-helix upon association with bacterial membranes.[17-18] We synthesized peptides **11b** and **12b** in which the hydrophobic residues I5, I9, and F13 of wild-type aurein 1.2 were replaced by our unnatural amino acids, representing stapling in *i, i + 4, i – 4* (Figure 2). Notably, the bicyclization reaction was performed in 60% trifluoroethanol containing aqueous buffer to promote the helical conformation. Both bicycles,

11b and **12b**, were successfully isolated (Figure S8) and subsequently analyzed by circular dichroism (CD) spectroscopy with varying proportions of trifluoroethanol (TFE, 0–80%) at physiological pH (Figure 2). Despite the tremendous constraint imposed by the bicyclization, both compounds clearly display a helical conformation when triggered with trifluoroethanol.

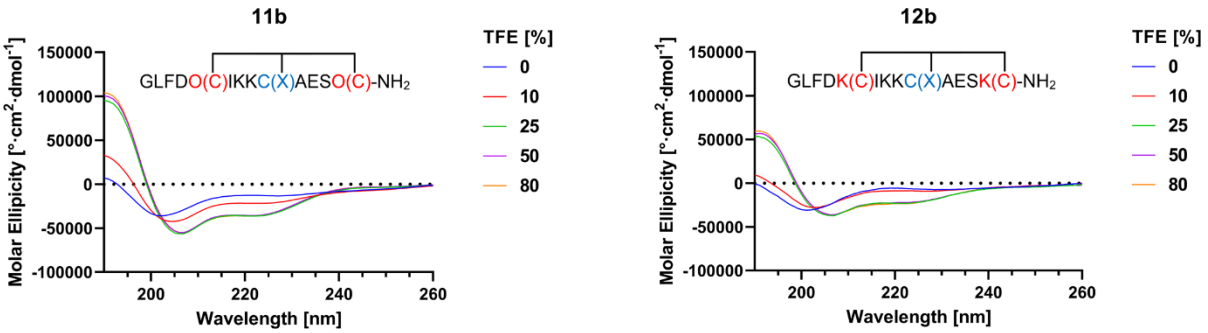


Figure 2. Sequences and circular dichroism spectra of peptide bicycle derivatives **11b** and **12b** of the antimicrobial peptide aurein 1.2 at varying concentrations (v/v) of trifluoroethanol (TFE).

While the presented methodology is already chemoselective and orthogonal to conventional methods, the use of masked 1,2-aminothiols in form of thiazolidines allows for further orthogonality and the selective formation of tricyclic peptides. We introduced thiazolidines in amino acid side chains in lieu of the 1,2-aminothiols and employed them directly in standard Fmoc solid-phase synthesis (Scheme S10). After peptide synthesis and purification, the thiazolidines can be selectively deprotected with methoxyamine under biocompatible conditions.[19] Using this approach, we were able to demonstrate the synthesis of tricyclic peptide **13c** from bicycle **13b** (Figure 3, Scheme S14).

In conclusion, we have harnessed biocompatible and selective chemistry to readily access bicyclic peptides. The approach is highly versatile, with several unnatural 1,2-aminothiol and DCP-containing amino acids available for the design of tailored bicyclic peptides. The combination of up to four amino acids with 1,2-aminothiol side chains (and the opportunity for N-terminal cysteine) combined with up to four DCP amino acids provides 80 conceivable options to construct any given bicyclic peptide sequence, representing an extraordinarily large chemical linker space. Combination with thiazolidine-based amino acids further expands the opportunities toward the selective generation of tricyclic peptides and beyond. The presented synthesis is also fully compatible with automation using commercially available building blocks for peptide synthesis. Furthermore, the method is compatible with all canonical amino

acids, most notably cysteine, enabling further modification through universally employed conjugation methods. The great opportunity of this methodology for drug discovery was demonstrated by the discovery of a nanomolar protease inhibitor with significant plasma stability and bicyclic helical peptides as potential future antimicrobial agents.

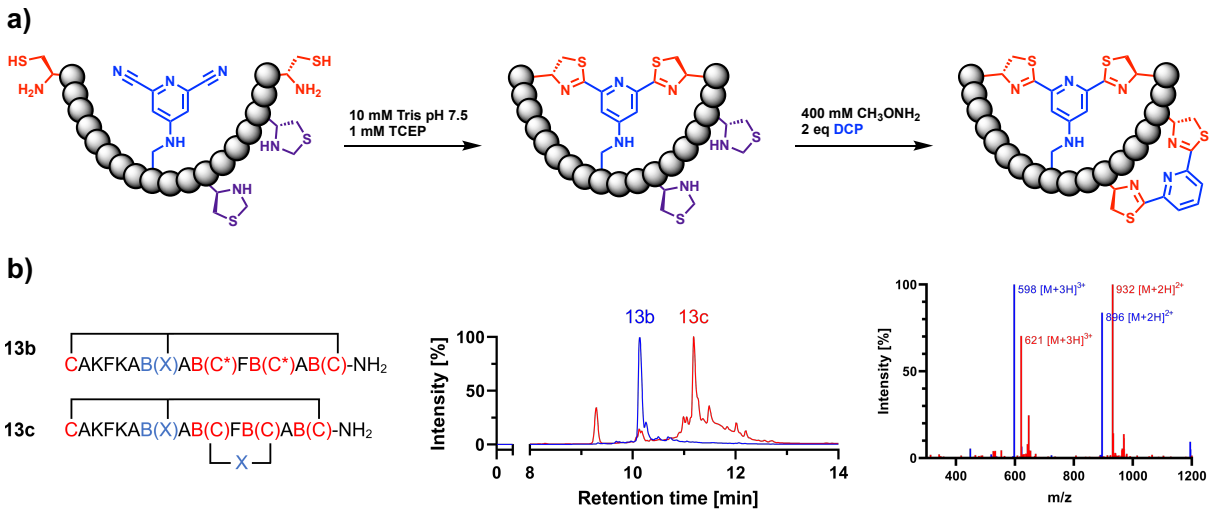


Figure 3. a) General strategy for the biocompatible synthesis of tricyclic peptides. b) Example bicyclic and tricyclic peptides **13b** and **13c**, respectively, with superimposed LC-MS chromatograms and ESI mass spectrometry data (**13b** blue, **13c** red). X = 2,6-dicyanopyridine, C* = L-thiazolidine-4-carboxylic acid.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by an Australian Research Council DECRA (DE190100015) and Discovery Project funding (DP200100348) awarded to CN. Support by a RAMR (MAWA) grant awarded to SU and CN is gratefully acknowledged. SU acknowledges a Researcher Development Grant by the Royal Society of Chemistry. We thank Prof. Gottfried Otting (Australian National University) for support in recording and analyzing peptide NMR spectra.

References

[1] a) C. A. Rhodes, D. Pei, *Chem. Eur. J.* **2017**, *23*, 12690–12703; b) C. Morrison, *Nat. Rev. Drug. Discov.* **2018**, *17*, 531–533.

[2] a) A. A. Vinogradov, Y. Yin, H. Suga, *J. Am. Chem. Soc.* **2019**, *141*, 4167–4181; b) N. Tsomaia, *Eur. J. Med. Chem.* **2015**, *94*, 459–470.

[3] S. Rubin, N. Qvit, *Crit. Rev. Eukaryot. Gene Expr.* **2016**, *26*, 199–221.

[4] a) C. Sohrabi, A. Foster, A. Tavassoli, *Nat. Rev. Chem.* **2020**, *4*, 90–101; b) T. Bosma, R. Rink, M. A. Moosmeier, G. N. Moll, *ChemBioChem* **2019**; c) A. Angelini, C. Heinis, *Curr. Opin. Chem. Biol.* **2011**, *15*, 355–361.

[5] a) L. Reguera, D. G. Rivera, *Chem. Rev.* **2019**, *119*, 9836–9860; b) Y. Wu, H. F. Chau, W. Thor, K. H. Y. Chan, X. Ma, W. L. Chan, N. J. Long, K. L. Wong, *Angew. Chem. Int. Ed.* **2021**, *60*, 20301–20307; c) C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509–524.

[6] a) C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.* **2009**, *5*, 502–507; b) S. Ahangarzadeh, M. M. Kanafi, S. Hosseinzadeh, A. Mokhtarzadeh, M. Barati, J. Ranjbari, L. Tayebi, *Drug Discov. Today* **2019**, *24*, 1311–1319; c) C. Heinis, G. Winter, *Curr. Opin. Chem. Biol.* **2015**, *26*, 89–98; d) S. Chen, D. Bertoldo, A. Angelini, F. Pojer, C. Heinis, *Angew. Chem. Int. Ed.* **2014**, *53*, 1602–1606.

[7] C. Ernst, J. Heidrich, C. Sessler, J. Sindlinger, D. Schwarzer, P. Koch, F. M. Boeckler, *Front. Chem.* **2018**, *6*.

[8] L. Mendive-Tapia, S. Preciado, J. García, R. Ramón, N. Kielland, F. Albericio, R. Lavilla, *Nat. Commun.* **2015**, *6*, 7160.

[9] a) C. Nitsche, H. Onagi, J.-P. Quek, G. Otting, D. Luo, T. Huber, *Org. Lett.* **2019**, *21*, 4709–4712; b) N. A. Patil, J.-P. Quek, B. Schroeder, R. Morewood, J. Rademann, D. Luo, C. Nitsche, *ACS Med. Chem. Lett.* **2021**, *12*, 732–737; c) R. Morewood, C. Nitsche, *Chem. Sci.* **2021**, *12*, 669–674; d) S. Ullrich, V. M. Sasi, M. C. Mahawaththa, K. B. Ekanayake, R. Morewood, J. George, L. Shuttleworth, X. Zhang, C. Whitefield, G. Otting, C. Jackson, C. Nitsche, *Bioorg. Med. Chem. Lett.* **2021**, *50*, 128333.

[10] a) O. Boutureira, G. J. L. Bernardes, *Chem. Rev.* **2015**, *115*, 2174–2195; b) E. Baslé, N. Joubert, M. Pucheault, *Chem. Biol.* **2010**, *17*, 213–227; c) J. M. Chalker, G. J. L. Bernardes, Y. A. Lin, B. G. Davis, *Chem. Asian J.* **2009**, *4*, 630–640; d) S. B. Gunnoo, A. Madder, *ChemBioChem* **2016**, *17*, 529–553; e) L. Xu, S. L. Kuan, T. Weil, *Angew. Chem. Int. Ed.* **2021**, *60*, 13757–13777; f) R. V. J. Chari, M. L. Miller, W. C. Widdison, *Angew. Chem. Int. Ed.* **2014**, *53*, 3796–3827; g) K. Yamada, Y. Ito, *ChemBioChem*

2019, *20*, 2729–2737; h) P. Ochtrop, C. P. R. Hackenberger, *Curr. Opin. Chem. Biol.* **2020**, *58*, 28–36; i) A. C. Conibear, E. E. Watson, R. J. Payne, C. F. W. Becker, *Chem. Soc. Rev.* **2018**, *47*, 9046–9068.

[11] T. M. Postma, M. Giraud, F. Albericio, *Org. Lett.* **2012**, *14*, 5468–5471.

[12] P. G. M. Wuts, in *Greene's protective groups in organic synthesis*, 5th ed. (Ed.: P. G. M. Wuts), John Wiley & Sons, Hoboken, NJ, United States, **2014**, pp. 895–1193.

[13] a) C. Nitsche, *Biophys. Rev.* **2019**, *11*, 157–165; b) S. Voss, C. Nitsche, *Bioorg. Med. Chem. Lett.* **2020**, *30*, 126965; c) S. Voss, J. Rademann, C. Nitsche, *Angew. Chem. Int. Ed.* **2021**, *61*, e202113857.

[14] F. F. Hefti, *BMC Neurosci.* **2008**, *9*, S7.

[15] a) Y. H. Lau, P. de Andrade, Y. Wu, D. R. Spring, *Chem. Soc. Rev.* **2015**, *44*, 91–102; b) Y. S. Tan, D. P. Lane, C. S. Verma, *Drug Discov. Today* **2016**, *21*, 1642–1653; c) M. T. J. Bluntzer, J. O'Connell, T. S. Baker, J. Michel, A. N. Hulme, *Pept. Sci.* **2020**, *113*.

[16] a) G. J. Hilinski, Y.-W. Kim, J. Hong, P. S. Kutchukian, C. M. Crenshaw, S. S. Berkovitch, A. Chang, S. Ham, G. L. Verdine, *J. Am. Chem. Soc.* **2014**, *136*, 12314–12322; b) A. D. de Araujo, J. Lim, K.-C. Wu, Y. Xiang, A. C. Good, R. Skerlj, D. P. Fairlie, *J. Med. Chem.* **2018**, *61*, 2962–2972; c) K. Chen, Y. Tang, M. Wu, X.-C. Wan, Y.-N. Zhang, X.-X. Chen, F.-Q. Yu, Z.-H. Cui, J.-M. Ma, Z. Zhou, G.-M. Fang, *Org. Lett.* **2021**, *24*, 53–57.

[17] T. Rozek, K. L. Wegener, J. H. Bowie, I. N. Olver, J. A. Carver, J. C. Wallace, M. J. Tyler, *Eur. J. Biochem.* **2000**, *267*, 5330–5341.

[18] M. A. Apponyi, T. L. Pukala, C. S. Brinkworth, V. M. Maselli, J. H. Bowie, M. J. Tyler, G. W. Booker, J. C. Wallace, J. A. Carver, F. Separovic, J. Doyle, L. E. Llewellyn, *Peptides* **2004**, *25*, 1035–1054.

[19] D. P. Nguyen, T. Elliott, M. Holt, T. W. Muir, J. W. Chin, *J. Am. Chem. Soc.* **2011**, *133*, 11418–11421.