2-Cyanoisonicotinamide conjugation: A facile approach to generate potent peptide inhibitors of the Zika virus protease

Nitin A. Patil,[a,*] Jun-Ping Quek,[b,#] Barbara Schroeder,[c,d,#] Richard Morewood,[d] Jörg Rademann,[c] Dahai Luo,[b] Christoph Nitsche[d,*]

[a] Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia

[b] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 636921, Singapore

[c] Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Straße 2+4, 14195 Berlin, Germany

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

ABSTRACT

The rapid generation and modification of macrocyclic peptides in medicinal chemistry is an ever-growing area that can present various synthetic challenges. The reaction between N-terminal cysteine and 2-cyanoisonicotinamide is a new biocompatible click reaction that allows rapid access to macrocyclic peptides. Importantly, 2-cyanoisonicotinamide can be attached to different linkers directly during solid-phase peptide synthesis. The synthesis involves only commercially available precursors, allowing for a fully automated process. We demonstrate the approach for four cyclic peptide ligands of the Zika virus protease NS2B-NS3. Although all peptides display the substrate recognition motif, the activity strongly depends on the linker length, with the shortest cyclization linker corresponding to highest activity (K_i = 0.64 μM). The most active cyclic peptide displays 78 times higher affinity than its linear analog. We solved a crystal structure of the proteolytically cleaved ligand and synthesized it by applying the presented chemistry to peptide ligation.

KEYWORDS

Macrocyclization, peptides, biocompatible, protease inhibitors, Zika

Combining the best attributes of small molecules and antibodies, macrocyclic peptides are promising next-generation ligands for drug discovery.[1] Constraining a peptide ligand by cyclization can pre-organize its binding interactions and thus reduce the entropic penalty, and improve crucial pharmacokinetic parameters like bioavailability and metabolic stability.[2] Despite these great advantages for drug discovery, macrocyclic peptides remain underexplored as lead compounds in medicinal chemistry, which may relate to a lack of facile and broadly applicable synthetic methods to rapidly access macrocyclic peptide derivatives. Fundamental changes in synthetic methodology have always impacted on the character of lead compounds pursued by medicinal chemists.[3] Thus, advances in peptide chemistry shall further enhance the consideration of constrained peptides in drug discovery campaigns.

While Fmoc solid-phase peptide synthesis has revolutionized the access to linear peptides by affordable automated synthesis on demand,[4] selective modifications such as cyclization remain more exclusive.[5-7] Recently, we developed a biocompatible method to rapidly cyclize peptides using 2-cyanopyridine and 1,2-aminothiol functional groups, which has been proven to be fully compatible with all canonical amino acids including non-terminal cysteine residues.[8, 9] However, the synthesis of the core amino acid 3-(2-cyano-4-pyridyl)-alanine (Cpa) requires advanced synthetic chemistry set-ups, involving the handling of potentially explosive peroxides, toxic trimethylsilyl cyanide and carcinogenic dimethylcarbamoyl chloride.[9] Here we report the selective reaction between 2-cyanoisonicotinamide (CINA) and N-terminal cysteine as an alternative strategy for rapid peptide macrocyclization fully amenable to automation. In contrast to previous methods, CINA can be installed directly on the peptide chain during automated solid-phase synthesis from commercially available precursors, overcoming the necessity for advanced synthetic laboratory set-ups (Scheme 1). In addition, this strategy also allows for the installation of different cyclization linkers without additional effort (Scheme 1).

We demonstrate the approach for cyclic peptide inhibitors of the Zika virus protease NS2B-NS3 (ZiPro). The Zika virus is a flavivirus closely related to other health-threatening pathogens of the same genus like dengue and West Nile viruses.[10] During the unprecedented outbreak in Latin America in 2016, the WHO declared Zika a public health emergency of international concern and called for intensified research and development efforts. Most Zika infections are asymptomatic. Major health-concerns during the 2015/2016 epidemic arose from congenital malformations such as microcephaly and an increased risk of fetal loss associated with Zika infections during pregnancy.[11, 12] A direct link between Zika infections and increased

risk of the autoimmune Guillain-Barré syndrome is also concerning.[13] No Zika virus vaccines or specific antivirals have been approved yet.[14]

Flaviviruses comprise a single-stranded RNA genome that is translated into a single polyprotein in the host cell. Proteolytic cleavage of this polyprotein by host cell proteases and the viral protease NS2B-NS3 into individual structural and non-structural proteins is essential for viral replication.[15] Thus, the serine protease NS2B-NS3 from flaviviruses has been suggested as a promising drug target for infections with Zika, dengue, West Nile and other flaviviruses.[16, 17] The protease's high degree of conservation among flaviviruses may facilitate the discovery of pan-flaviviral drug candidates. The full protease complex comprises NS3 bearing the catalytic triad (serine, histidine, aspartate) and the small co-factor NS2B. The C-terminal domain of NS2B needs to wrap around the active site for sufficient substrate recognition and catalytic activity.[18] This crucial interaction has informed the design of various NS2B-NS3 constructs for drug screenings, mainly differing in the way NS2B and NS3 are connected. In case of Zika, a commonly used covalently linked protease construct might favor the dissociation of the crucial C-terminal NS2B domain from NS3.[19, 20] Therefore, a more natural unlinked construct was developed (referred to as bZiPro), which has been used in this study.[21]

Previously explored inhibitors of ZiPro were either small compounds or substrate-derived peptides.[22] Drug-like small molecules suffered from low affinity, whereas simple substrate analogs displayed high affinity but limited activity in cellular assays. ZiPro recognizes charged basic amino acids (arginine, lysine) in P_1 and P_2, which has further challenged the discovery of drug-like peptidomimetics.[22] Cyclic peptides are one promising avenue to improve pharmacokinetic parameters as well as the affinity of linear substrate analogs. Recent studies highlighted that cyclic peptides can indeed generate high-affinity ligands of the active and allosteric sites of ZiPro.[9, 23, 24] However, these cyclic analogs require multi-step synthetic procedures, limiting extensive structure-activity relationships. In this study, we show that high-affinity cyclic peptide inhibitors of ZiPro can be generated by fully automated solid-phase peptide synthesis.

Our design of constrained ZiPro inhibitors focused on the non-prime site substrate recognition sequence GKRK of ZiPro spanning residues P_4 to P_1 (Table 1).[8, 9, 22] We installed a cysteine at the N-terminus and the CINA motif at the C-terminus of the peptide (Scheme 1). In order to study the effect of macrocyclic ring size on the enzyme inhibition, we designed four analogs **1b**-**4b** with different CINA linkers, containing either Dap, Dab, Orn or Lys (Scheme 1). We chose commercially available derivatives of Dap, Dab, Orn and Lys bearing N-terminal

Fmoc and side-chain ivDde protection groups. Employing the more stable ivDde protection group instead of conventional Dde allows for orthogonal side-chain deprotection using 3% hydrazine with minimal intramolecular migration during solid-phase peptide synthesis.[25, 26] The linear precursor peptides **1a**-**4a** were obtained by sequential standard solid-phase peptide synthesis, followed by selective removal of ivDde and subsequent coupling to 2-cyanoisonicotinic acid (CINA) on the solid support (Scheme 1). Inspired by our recent efforts,[8, 9, 27] we developed a facile and biocompatible cyclization protocol to generate constrained peptides **1b**-**4b** from linear precursors **1a**-**4a** (Scheme 1) directly in aqueous buffer within 30 minutes. We observed that the macrocyclic ring size did not affect the efficiency of cyclization and compounds **1b**-**4b** were obtained in excellent isolated yields between 53% and 74% (estimated conversions determined by HPLC are 76-92%, Table S1).

We assessed the inhibition potential of compounds **1b**-**4b** using the unlinked construct bZiPro and the fluorescent substrate Bz-Nle-KKR-AMC (Nle, norleucine).[28] We monitored dose-response curves at three different substrate concentrations and calculated inhibition constants (K_i) from three IC_{50} values following the Cheng-Prusoff relationship (Figures S15-S18).[29] Unsurprisingly, all cyclic peptides **1b**-**4b** bearing the substrate recognition motif GKRK are competitive inhibitors of ZiPro (Table 1). However, we observed a remarkable structure-activity relationship with regard to the chosen cyclization linker. For instance, compound **1b** with a Dap-based linker inhibited more than 20 times stronger than analog **2b** with a Dab-based linker. Remarkably, both compounds differ only in one methylene group, clearly highlighting the importance of the cyclization linker for pre-organization, which has been neglected in previous studies.[9] Derivative **1b** with the shortest linker displays significant affinity with a K_i of 0.64 μM. A Dixon plot for compound **1b** confirms a competitive inhibition mode (Figure S20).

It has been suggested that most proteases recognize extended β-strands in their active sites.[30, 31] Our small SAR may support previous observations that macrocyclization of substrate-derived inhibitors can pre-organize the peptide in the extended conformation and thus reduce the entropic penalty.[32] In addition, our study suggests that in order to maximize affinity, the right linker geometry is crucial. In our SAR, the most constrained peptide with the shortest linker gave best results. It should be noted that Dap used in **1b** is an isostere of serine, which is recognized by ZiPro in P_1',[22] potentially contributing to the outstanding affinity of **1b**.

Encouraged by these results, we set out to solve the crystal structure of **1b** in complex with bZiPro to better understand the SAR and the pronounced specificity for the Dap-based

linker. Instead of **1b**, we observed the proteolytically digested linear derivative **5** in complex with bZiPro (Figure 1). When compound **1b** was built and refined (Figure S23), the resulting electron density maps indicate that the cyclic peptide had been cleaved during the crystallization process yielding the complex structure of bZipro and compound **5**. Derivative **5** represents the selective hydrolysis product between P_1 Lys and P_1' Dap(CINA) of **1b**, yet again indicating that the Dap-based residue might have the capability to act as a serine isostere in P_1'.

To confirm that the stronger inhibition of **1b** is driven by the cyclic peptide and not by the proteolysis product **5**, we analyzed the proteolytic stability of **1b** in presence of 0.1% bZiPro. After 8 h of incubation, we observe only minor cleavage product **5** (Figure 1b), clearly indicating that compound **1b** is the active species in the ZiPro inhibition assay relating to a K_i value of 0.64 μM. The observation of **5** in the crystal structure is likely due to the high protease concentration and prolonged incubation times under co-crystallization conditions, favoring proteolytic digest. This effect has previously been observed with a related cyclic peptide inhibitor of ZiPro.[9] The very slow proteolysis compared to the linear substrate analog Bz-Nle-KKR-AMC used in the assay also highlights that peptide cyclization cannot only increase affinity but also guard against proteolytic digest.

In the crystal structure, four bZiPro molecules were observed in a single asymmetric unit. One was occupied by **5** while another one was bound to residues K14, K15, G16 and E17 from the neighboring NS3 molecule in reverse orientation, occupying the S_4 to S_1 pockets of bZiPro. Comparison of the complex with previously reported bZiPro−ligand co-crystal structures containing substrate-derived ligands reveal similar P_1 and P_2 interactions and P_1−P_3 backbone conformations (Figure S22). Similar to a structure published previously,[9] the P_3 lysine side chain of **5** is flipped while the rest of the bulky main chain exits bZiPro at a nearby hydrophobic groove.

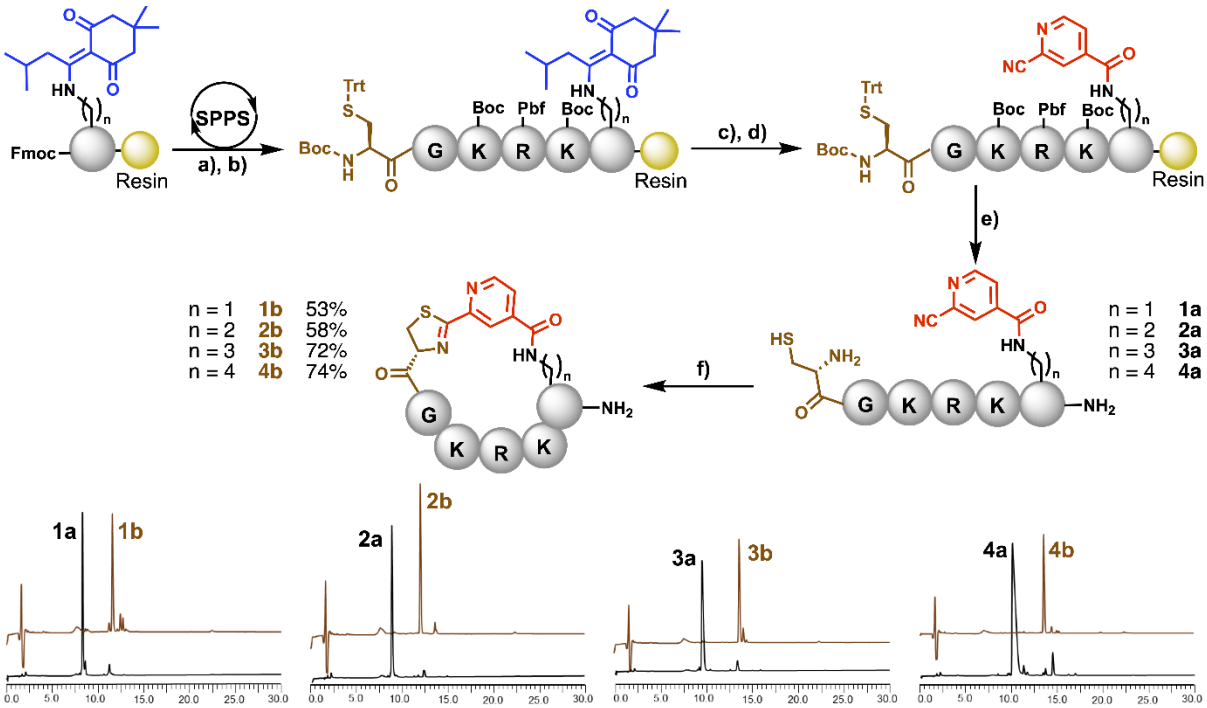
To further investigate the relevance of the proteolysis product, we synthesized ligand **5** through a CINA ligation strategy (Scheme 2). Similar to the CINA cyclization approach, both precursor fragments **5a** and **5b** were accessed from standard solid-phase peptide synthesis. To fully account for the different terminal amide and acid groups in **5**, precursor fragment **5a** was assembled on Rink amide resin, while the precursor fragment **5b** was assembled on 2-chlorotrityl resin. Unlike cyclization, the ligation was performed for 3 hours with crude linear fragments **5a** and **5b** in aqueous PBS (pH 7.4) containing 1 mM tris(2-carboxyethyl) phosphine (TCEP) (Scheme 2). Ligation product **5** was obtained with an overall isolated yield of 36%

(estimated conversion determined by HPLC is 45%, Table S1), demonstrating the effectiveness of bimolecular ligation between CINA and N-terminal cysteine.

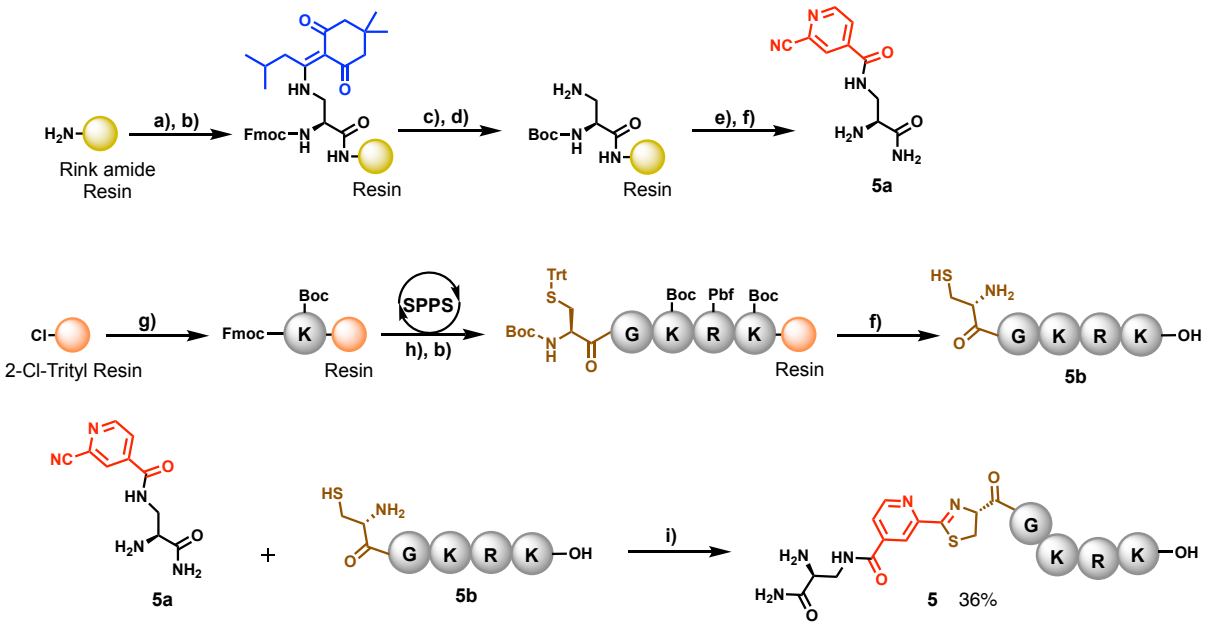
We assessed proteolysis product **5** for its ZiPro inhibition and found a K_i value of 50.2 μM, which is 78 times higher than for the cyclic analog **1b**. This observation proves that cyclic peptide **1b** is the active species and not the proteolysis product **5**. It also confirms that **1b** is almost stable against proteolysis under standard assay conditions and that proteolysis originates mainly from prolonged incubation time and high protease concentration required for crystallization.

The data presented in this study indicate a large affinity gap between cyclic compound **1b** and linear analog **5**. Apart from the flipped orientation of P_3 lysine, the co-crystal structure between **5** and ZiPro displays the most important interactions between a substrate-derived ligand and ZiPro commonly observed in other co-crystal structures. Despite this, the inhibition constant of **5** is only 50 μM, which is 78 times higher than the inhibition constant of **1b**. Although a model of ligand **1b** with ZiPro indicates a more common and thus favorable interaction with P_3 lysine (Figure S23), a lower entropic barrier of binding might be an important contributor to the significantly higher affinity of **1b**.

In summary, we present a cost-effective synthetic strategy to rapidly access macrocyclic peptides, which is fully amenable to automation. The process produces peptides in high yield, allowing for direct structure-activity relationships without the need for purification. Utilizing CINA as a non-toxic, economical and readily available building block may allow transformation to large scale industrial production of cyclic peptide ligands. CINA-based cyclization facilitates the screening of various ligation linkers, which is crucial to identify the optimal linker geometry. Using this technology, it required only a small number of compounds to identify a high-affinity ligand of the Zika virus protease. We are therefore confident that the strategy will be equally successful for alternative drug targets.



Scheme 1. Synthesis of linear peptides **1a-4a** and constrained cyclic analogs **1b-4b**. Isolated yields and LC-MS chromatograms (214 nm; retention time in minutes) are reported for the cyclization of **1a-4a** to **1b-4b**. a) Standard SPPS in the order Fmoc-AA(ivDde)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Boc-Cys(Trt)-OH. b) Piperidine, DMF. c) Hydrazine hydrate, DMF. d) 2-Cyanoisonicotinic acid (CINA), HCTU, DIPEA, DMF. e) TFA/TIPS/DODT/H_2O (94:2:2:2); DODT, 2,2′-(ethylenedioxy)diethanethiol. f) PBS pH 7.5, 1 mM TCEP, 30 min.



Scheme 2. Synthesis of **5** using peptide ligation (isolated yield indicated). a) Fmoc-Dap(ivDde)-OH, HCTU, DIPEA, DMF. b) Piperidine, DMF. c) Boc_2O, DIPEA, DMF. d) Hydrazine hydrate, DMF. e) 2-Cyanoisonicotinic acid (CINA), HCTU, DIPEA, DMF. f) TFA/TIPS/DODT/H_2O (94:2:2:2); DODT, 2,2′-(ethylenedioxy)diethanethiol. g) Fmoc-Lys(Boc)-OH, DIPEA, DMF. h) Standard SPPS in the order Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Boc-Cys(Trt)-OH. i) PBS pH 7.5, 1 mM TCEP, 3 h.

Table 1. Inhibitors of the Zika virus protease NS2B-NS3.

Cpd.[a]	Sequence[b]	K_i (bZiPro)[c]
1b	Cys-Gly-Lys-Arg-Lys-Dap(CINA)-NH_2	0.64 ± 0.01 μM
2b	Cys-Gly-Lys-Arg-Lys-Dab(CINA)-NH_2	14.3 ± 0.7 μM
3b	Cys-Gly-Lys-Arg-Lys-Orn(CINA)-NH_2	1.76 ± 0.31 μM
4b	Cys-Gly-Lys-Arg-Lys-Lys(CINA)-NH_2	7.24 ± 0.34 μM
5	[H-Dap(CINA)-NH_2] Cys-Gly-Lys-Arg-Lys-OH	50.2 ± 1.2 μM

[a] Peptides cyclized or ligated from their linear precursors **1a**-**5a** and **5b**.

[b] CINA = 2-cyanoisonicotinamide

[c] Zika virus NS2B-NS3 protease (bZiPro) inhibition. Substrate: Bz-Nle-Lys-Lys-Arg-AMC. Inhibition constants (K_i) were calculated from measurements at three different substrate concentrations.

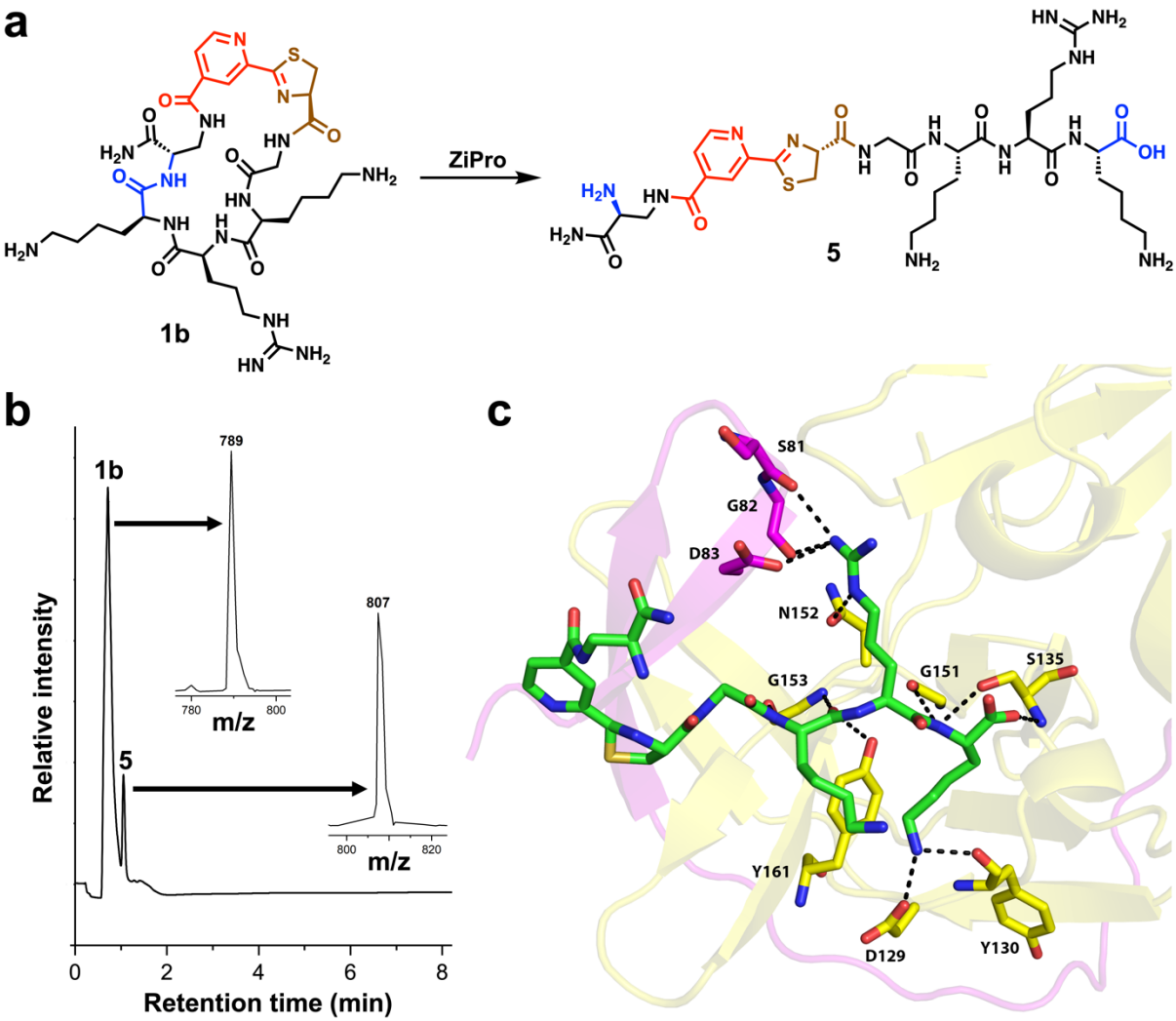


Figure 1. Macrocyclic peptide **1b** interacting with the Zika virus protease NS2B-NS3 (ZiPro). a) Scheme of proteolytic digest of **1b** to **5** by bZiPro. The cleavage site is highlighted in blue. b) LC-MS chromatogram (254 nm) and corresponding mass spectra after 8 h of incubation of **1b** in the presence of 0.1% bZiPro in 10 mM Tris-HCl pH 8.5 indicating partial proteolysis to **5**. c) Crystal structure of bZiPro in complex with **5** at a resolution of 1.9 Å (PDB code: 7DOC). NS2B (magenta) and NS3 (yellow) are shown as cartoon representations and compound **5** (green) is represented as sticks. The bZiPro residues involved in interactions with **5** are labelled and shown as sticks.

ASSOCIATED CONTENT

Crystallographic Data

Atomic coordinates of the bZiPro:**5** co-crystal structure have been deposited in the Protein Data Bank (PDB) under the accession code 7DOC.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details for Zika protease construct and preparation, synthesis and screening of macrocyclic and linear peptides, molecular structure of all peptides, LC-MS data and chromatograms of all peptides, inhibition assay, dose-response curves, Dixon plot, co-crystallization protocol, proteolytic stability assay, X-ray data collection and refinement statistics, description of interactions of **5** and bZiPro, Figures S1-S23, Tables S1-S2.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: nitin.patil@monash.edu

*E-mail: christoph.nitsche@anu.edu.au

ORCID

Nitin A. Patil: 0000-0003-0422-9411

Jun-Ping Quek: 0000-0002-3827-8580

Barbara Schroeder: 0000-0003-4523-8189

Richard Morewood: 0000-0002-1047-3691

Jörg Rademann: 0000-0001-6678-3165

Dahai Luo: 0000-0001-7637-7275

Christoph Nitsche: 0000-0002-3704-2699

Author Contributions

#These authors contributed equally. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

N. A. P thanks the National Health and Medical Research Council, Australia for the Peter Doherty Early Career Research Fellowship (APP1158171). J. P. Q. is supported by the Nanyang Presidential Graduate Scholarship. B. S. acknowledges a travel fellowship from the Ernst-Reuter-Gesellschaft and Research Alumni program, Berlin, Germany. D. L. acknowledges the support from Singapore National Research Foundation grant NRF2016NRF-CRP001-063. C. N. thanks the Australian Research Council for a Discovery Early Career Research Award (DE190100015). We gratefully acknowledge the beamline staff at TPS 05A beamline in National Synchrotron Radiation Research Center, Hsinchu, Taiwan, MXII beamline in Australian Light Source, Melbourne, Australia and PSIII beamline in Swiss Light Source (SLS) Paul Scherrer Institut, Switzerland for providing us outstanding support during the data collection.

REFERENCES

1. Morrison, C. Constrained peptides' time to shine? *Nat. Rev. Drug Discov.* **2018,** *17*, 531-533.

2. Cary, D. R.; Ohuchi, M.; Reid, P. C.; Masuya, K. Constrained peptides in drug discovery and development. *J. Synth. Org. Chem., Jpn.* **2017,** *75*, 1171-1178.

3. Walters, W. P.; Green, J.; Weiss, J. R.; Murcko, M. A. What do medicinal chemists actually make? A 50-year retrospective. *J. Med. Chem.* **2011,** *54*, 6405-6416.

4. Behrendt, R.; White, P.; Offer, J. Advances in Fmoc solid-phase peptide synthesis. *J. Pept. Sci.* **2016,** *22*, 4-27.

5. White, C. J.; Yudin, A. K. Contemporary strategies for peptide macrocyclization. *Nat. Chem.* **2011,** *3*, 509-524.

6. Reguera, L.; Rivera, D. G. Multicomponent reaction toolbox for peptide macrocyclization and stapling. *Chem. Rev.* **2019,** *119*, 9836-9860.

7. Wills, R.; Adebomi, V.; Raj, M. Site-selective peptide macrocyclization. *ChemBioChem* **2021,** *22*, 52-62.

8. Morewood, R.; Nitsche, C. A biocompatible stapling reaction for *in situ* generation of constrained peptides. *Chem. Sci.* **2021,** *12*, 669-674.

9. Nitsche, C.; Onagi, H.; Quek, J.-P.; Otting, G.; Luo, D.; Huber, T. Biocompatible macrocyclization between cysteine and 2-cyanopyridine generates stable peptide inhibitors. *Org. Lett.* **2019,** *21*, 4709-4712.

10. Pierson, T. C.; Diamond, M. S. The continued threat of emerging flaviviruses. *Nat. Microbio.* **2020,** *5*, 796-812.

11. Musso, D.; Gubler, D. J. Zika virus. *Clin. Microbiol. Rev.* **2016,** *29*, 487-524.

12. Musso, D.; Ko, A. I.; Baud, D. Zika virus infection — after the pandemic. *N. Engl. J. Med.* **2019,** *381*, 1444-1457.

13. Cao-Lormeau, V.-M.; Blake, A.; Mons, S.; Lastère, S.; Roche, C.; Vanhomwegen, J.; Dub, T.; Baudouin, L.; Teissier, A.; Larre, P.; Vial, A.-L.; Decam, C.; Choumet, V.; Halstead, S. K.; Willison, H. J.; Musset, L.; Manuguerra, J.-C.; Despres, P.; Fournier, E.; Mallet, H.-P.; Musso, D.; Fontanet, A.; Neil, J.; Ghawché, F. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *The Lancet* **2016,** *387*, 1531-1539.

14. Castanha, P. M. S.; Marques, E. T. A. Vaccine development during global epidemics: the Zika experience. *The Lancet Infect. Dis.* **2020,** *20*, 998-999.

15. Barrows, N. J.; Campos, R. K.; Liao, K.-C.; Prasanth, K. R.; Soto-Acosta, R.; Yeh, S.-C.; Schott-Lerner, G.; Pompon, J.; Sessions, O. M.; Bradrick, S. S.; Garcia-Blanco, M. A. Biochemistry and molecular biology of flaviviruses. *Chem. Rev.* **2018,** *118*, 4448-4482.

16. Nitsche, C. Proteases from dengue, West Nile and Zika viruses as drug targets. *Biophys. Rev.* **2019,** *11*, 157-165.

17. Nitsche, C. Strategies towards protease inhibitors for emerging flaviviruses. *Adv. Exp. Med. Biol.* **2018,** *1062*, 175-186.

18. Nitsche, C.; Holloway, S.; Schirmeister, T.; Klein, C. D. Biochemistry and medicinal chemistry of the dengue virus protease. *Chem. Rev.* **2014,** *114*, 11348-11381.

19. Li, Y.; Phoo, W. W.; Loh, Y. R.; Zhang, Z.; Ng, E. Y.; Wang, W.; Keller, T. H.; Luo, D.; Kang, C. Structural characterization of the linked NS2B-NS3 protease of Zika virus. *FEBS Lett.* **2017,** *591*, 2338-2347.

20. Mahawaththa, M. C.; Pearce, B. J. G.; Szabo, M.; Graham, B.; Klein, C. D.; Nitsche, C.; Otting, G. Solution conformations of a linked construct of the Zika virus NS2B-NS3 protease. *Antiviral Res.* **2017,** *142*, 141-147.

21. Zhang, Z.; Li, Y.; Loh, Y. R.; Phoo, W. W.; Hung, A. W.; Kang, C.; Luo, D. Crystal structure of unlinked NS2B-NS3 protease from Zika virus. *Science* **2016,** *354*, 1597-1600.

22. Voss, S.; Nitsche, C. Inhibitors of the Zika virus protease NS2B-NS3. *Bioorg. Med. Chem. Lett.* **2020,** *30*, 126965.

23. Braun, N. J.; Quek, J. P.; Huber, S.; Kouretova, J.; Rogge, D.; Lang-Henkel, H.; Cheong, E. Z. K.; Chew, B. L. A.; Heine, A.; Luo, D.; Steinmetzer, T. Structure-based macrocyclization of substrate analogue NS2B-NS3 protease inhibitors of Zika, West Nile and dengue viruses. *ChemMedChem* **2020,** *15*, 1439-1452.

24. Nitsche, C.; Passioura, T.; Varava, P.; Mahawaththa, M. C.; Leuthold, M. M.; Klein, C. D.; Suga, H.; Otting, G. *De novo* discovery of nonstandard macrocyclic peptides as noncompetitive inhibitors of the Zika virus NS2B-NS3 protease. *ACS Med. Chem. Lett.* **2019,** *10*, 168-174.

25. Augustyns, K.; Kraas, W.; Jung, G. Investigation on the stability of the Dde protecting group used in peptide synthesis: migration to an unprotected lysine. *J. Peptide Res.* **1998,** *51*, 127-133.

26. Chhabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C. An appraisal of new variants of Dde amine protecting group for solid phase peptide synthesis. *Tetrahedron Lett.* **1998,** *39*, 1603-1606.

27. Patil, N. A.; Karas, J. A.; Turner, B. J.; Shabanpoor, F. Thiol-cyanobenzothiazole ligation for the efficient preparation of peptide–PNA conjugates. *Bioconjugate Chem.* **2019,** *30*, 793-799.

28. Lei, J.; Hansen, G.; Nitsche, C.; Klein, C. D.; Zhang, L.; Hilgenfeld, R. Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor. *Science* **2016,** *353*, 503-505.

29. Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973,** *22*, 3099-3108.

30. Fairlie, D. P.; Tyndall, J. D. A.; Reid, R. C.; Wong, A. K.; Abbenante, G.; Scanlon, M. J.; March, D. R.; Bergman, D. A.; Chai, C. L. L.; Burkett, B. A. Conformational selection of inhibitors and substrates by proteolytic enzymes: Implications for drug design and polypeptide processing. *J. Med. Chem.* **2000,** *43*, 1271-1281.

31. Tyndall, J. D. A.; Nall, T.; Fairlie, D. P. Proteases universally recognize beta strands in their active sites. *Chem . Rev.* **2005,** *105*, 973-1000.

32. Tyndall, J. D.; Fairlie, D. P. Macrocycles mimic the extended peptide conformation recognized by aspartic, serine, cysteine and metallo proteases. *Curr. Med. Chem.* **2001,** *8*, 893-907.