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Acknowledgements

I would like to thank my supervisors, Prof. Raphaël Frédérick and Prof. Olivier Riant. First of all, I am grateful to them for taking the time to listen to me during my pharmacy and chemistry studies and for giving me the opportunity to carry out several internships. It is thanks to them, as well as to the people I met during these Master's internships, that I developed the desire to pursue doctoral training. I would also like to thank them for the kindness and trust they have shown me throughout these four years of my PhD.

I would like to thank Prof. **Raphaël Frédérick** for the great enthusiasm he has always shown toward my project, each time I presented new results. I am grateful to him for always providing the means to move the project forward and to carry out new experiments. I also thank him for his strategic advice in medicinal chemistry and for consistently aiming to maximize the impact and value of the project.

I am grateful to Prof. **Olivier Riant** for the many times he reassured me during moments of doubt, and for the stimulating discussions during which he would share his ideas with a pen in hand. What I have learned from him about organometallic chemistry, especially concerning palladium and copper, is invaluable.

I would like to thank Professors **Jérôme Waser**, **Charlotte Martin**, **Morgane Pasco**, **Michael Singleton**, and **Jean-François Gohy** for kindly accepting to serve as members of my thesis committee.

I would like to thank **Junjie** and **Elisabetta**, two of the kindest people I have had the privilege of meeting, for the time they dedicated to me during my internships at UCLouvain and KULeuven, respectively. These organic synthesis internships went by far too quickly, and it is thanks to them.

A huge thank you goes to the colleagues from the two laboratories in which I worked during my thesis, namely the group of Olivier Riant, part of the Molecular Chemistry, Materials and Catalysis (MOST) division of the Institute of Condensed Matter and Nanosciences (IMCN) in Louvain-la-Neuve, and the Medicinal Chemistry Research Group (CMFA) of the Louvain Drug Research Institute (LDRI) in Woluwe-Saint-Lambert. It was a pleasure to work with such wonderful

colleagues, many of whom have become friends. I am grateful for their advice, their help, and their good humor on a daily basis. They made these four years truly wonderful, both in and out of the lab.

Thank you to **Yonghua**, **Sarah E.**, **Michela**, **Nathalie**, **Logan**, and **Maxime** for the first two years of my thesis, which were mainly spent in Louvain-la-Neuve. **Yonghua**, I learned so much from you, especially in practical organic chemistry, and I will miss the long afternoons and late evenings spent working alongside you in the laboratory. Your immense kindness, your generosity in effort, and your unwavering courage have been a true source of inspiration to me. **Sarah E.**, thank you for your diplomacy, your calmness, and your insight. You were an excellent English teacher, especially after our meetings. I will miss the times when we had ice cream together with **Yonghua**. **Michela**, thank you for your constant good humor and ever-present smile. Your sense of celebration often helped us unwind after work. **Nathalie**, many thanks for your positive energy, your smile, and for fostering such a supportive and pleasant working environment in the laboratory. At times, I found myself afraid of doing something you might not approve of. This will probably help me in my future work. **Logan**, thank you for the wonderful year spent side by side in the lab, sharing jokes and lighthearted moments. Thanks to that, the working days never felt difficult. **Maxime**, thank you for your help and for all the time you devoted to supporting the laboratory, without ever expecting thanks in return. **François B.**, thank you for the warm welcome you gave me from my very first day as an intern in the group, six years ago. Thank you as well for all the time you devoted to me, whether for administrative matters or questions related to the functioning of the lab. I would also like to thank the other members of the laboratory, with whom I always maintained excellent relationships: **Gilles**, **Marine L.**, **Corentin**, **Ervan**, **Jonathan**, **Simone**, **Sebastian**, and **Xavier**.

Thank you to the entire Woluwe team, with whom I spent most of the last two years of my thesis. Despite the stress that comes with the end of a PhD, I am truly happy to have met such wonderful people there. **Marine D.**, thank you for the warm welcome, for always taking the time to explain how the laboratory functioned, and for including me from the very beginning in the activities you organized after work. **Quentin**, thank you for your always insightful input and strategic advice during meetings. Beyond that, thank you for your support and for the many

runs we went on together toward the end of my thesis. They allowed me to clear my mind and remain productive while writing. **Ayham**, thank you for always being so available and willing to help your colleagues, especially with IT-related questions and modeling topics. We could always count on you, even after your departure, for instance when software needed to be installed. I am very grateful for that. **Romain**, thank you for your unfailing kindness, your calmness, and your sharp sense of humor. You were the quiet strength that so many people could always rely on. Thank you as well for your invaluable help in solving the HPLC instrument issues. Even though this was often tedious and time-consuming, you always remained calm and persistent. **Marine A.**, I am very happy to have had the chance to work alongside you. Thank you for your joyfulness and your daily smile, whose absence was truly felt after your departure. I strongly envy your current colleagues. **Caroline**, thank you for the tremendous efforts you made to keep the laboratory organized. I am very grateful for that. Thank you as well for always trusting me when discussing organometallic chemistry matters. **Esra**, it was a true comfort to be able to count on you throughout all my doctoral years. Thank you for the gentleness, kindness, and benevolence you maintain in every circumstance, even when dealing with problems or bad news in the laboratory. **Chiara**, even though we did not spend much time together in the laboratory, I would like to thank you for the warm welcome and the thoughtfulness you showed me when I arrived in Woluwe. Thank you as well for the many moments of laughter shared outside the laboratory. **Rachel**, I adored you from the moment I met you, and even more so after hearing your very first silly remarks. Even though I should probably hold a grudge against you for missing my public defense, I wish you all the very best for the PhD journey you are just beginning. **Asia**, thank you for taking such great care of us and of the laboratory in general. Thank you for all the little thoughtful gestures and the small conversations that brightened everyday life. **Alexandre**, thank you for your daily good humor. Thank you as well for frequently putting me back in my place on the badminton court. It helped me stay humble. **Benjamin**, thank you for your cheerfulness and for always being enthusiastic about activities outside the laboratory. You never hesitated to help your colleagues, and that was greatly appreciated. Thank you as well for your love of beautiful chemistry and for the interesting discussions we shared about peptides. **Perrine**, thank you

for the conversations, the support, and the warm welcome in the office. Thank you for the friendly and welcoming atmosphere you created both in the office and in the laboratory in general. **Juhans**, my dear friend, thank you for your cynical humor and for all the moments we spent bickering without restraint. I will miss you very much, and I hope the feeling is at least a little mutual. I find it hard to imagine a workplace without a grump like you to argue with. **S  raphin**, I thoroughly enjoyed your goofy humor throughout these three years. I hope I never forget some of the lines, jokes, and impressions you came up with. Beyond that, thank you for the chemistry discussions, the conversations about life, and for listening to me during the final year of my PhD. **Darius**, thank you for your kindness, and above all for the incredible atmosphere and good humor you brought throughout my thesis. Every time I ran into you in the laboratory, I could never help bursting into laughter at the outrageous things you would say just to make me laugh. Whenever my workdays stretched late into the evening, it was a real pleasure to have you around, making the work feel joyful rather than exhausting. **Ariana**, thank you for your kindness and your football talent. I would have gladly signed up for a second teammate like you at WAPA instead of Darius. **Giuditta**, thank you for your kindness, your calmness, your smile, and your understanding, especially when I monopolized the HPLC at the end of my thesis. **Karine** and **Ferran**, even though I did not work alongside you for very long, thank you for your valuable advice both in the laboratory and during meetings. Thank you as well to the new generation of PhD students whom I had the pleasure of meeting during the final year of my thesis: **Manon**, **Amandine**, **Sarah H.**, **Marie-Sophie**, **Lou-Anne**, **Rachel**, and **Camilla**. I was very happy to share conversations and laughter with you, especially during lunch breaks. I admire your courage and wish you all the very best for the rest of your PhD journey. Finally, I would like once again to thank a few people from the Woluwe laboratory, namely **Darius**, **Juhans**, **S  raphin**, and **Perrine**, who were especially present for me during the final year of my thesis. I will never thank you enough for so often helping me clear my mind, whether through the many moments of genuine laughter during lunch breaks or through activities outside the laboratory.

I would like to thank all the technicians and administrative staff members I had the chance to meet, namely **Fran  ois B.**, **Audrey**,

Marie-Claire, Esra, Asia, Hoang, and Maëlle. I truly admire your calmness and your ability to remain kind and pleasant, despite the fact that people usually come to you when they have a technical or administrative problem and need help.

Thank you as well to all the interns and master's students I met during my thesis, including **Guillaume, Anne-Sophie, Loïc, Reda, Joudy, Abiba, Vicky, and Aline.** I wish you all the best for your exams and your future careers. I grew particularly fond of three especially endearing duos: **Emilie and Alissa, Nicolas and Valentin,** and the chemistry enthusiasts **José and Bilal.** I truly appreciated your kindness, your enthusiasm, and your scientific curiosity. It was a pleasure to supervise **Amaury, Paul, and Prisca. Prisca,** thank you for your great kindness and motivation during the six months you worked alongside me in the laboratory. Your help allowed me to start focusing on the writing process while you carried out a very important part of the experiments aimed at evaluating the chemoselectivity of our Cys–Lys stapling method. It was not an easy task, especially because of my rigidity and my superstitions, but you showed remarkable adaptability and exemplary dedication.

I would like to thank once again **Yonghua, Sarah E., Michela, Darius, Marine A., Esra, Giuditta, Ariana, Benjamin, Rachel, and Camilla,** who always kept smiling and greeted me every morning with the same cheerfulness, even on more difficult days, despite the technical issues, scientific disappointments, and stress that a PhD can bring. You truly were rays of sunshine.

Beyond the colleagues from my two laboratories, I would also like to thank collaborators from other laboratories, in particular **Laurent Collard, Gabriella Barozzino** (IMCN, ASM platform), **Khadija Wahni** (VIB–VUB Center for Structural Biology), and **Nicolas Joudiou** (LDRI, NEST platform), for training me in the use of mass spectrometry, circular dichroism, and nuclear magnetic resonance instruments, or for recording spectra for me.

I would also like to thank **Bernard Ucakar** (LDRI), **Vincent Stroobant,** and **Bénédicte Tollet** (de Duve Institute) for so often allowing me to use their laboratory's lyophilizer and for introducing me to its use.

Vincent, thank you for the wonderful, and sometimes lengthy, discussions I had the chance to share with you at the de Duve Institute. I will miss you greatly. I learned so much from you about peptide synthesis, chromatography, and mass spectrometry. Without ever counting your time, you devoted so much of it to helping me and answering my questions, always with the same kindness and the same passion for science. After each of these discussions, I always returned to the laboratory wiser, with new answers and ideas. A thousand thanks.

I would like to thank Prof. **Didier Lambert** for his kindness and for the many times he shared his knowledge of medicinal chemistry. Thank you to Prof. **Raphaël Robiette** for his valuable answers to my mechanistic questions. Thank you as well to Prof. **Joris Messens** for his insightful advice during the writing of my article and for allowing me to use the circular dichroism instrument in his laboratory at the Vrije Universiteit Brussel (VUB).

Finally, I would like to thank my family, in particular my parents, my brother, my cousins, my four grandparents, my godmother, and my uncle, as well as my friends, for their unconditional support throughout this thesis.

Abbreviations

- Abu: α -aminobutyric acid
- Ac: acetyl
- Ac₅c: 1-aminocyclopentanecarboxylic acid
- ADIBO: azadibenzocyclooctyne
- Aib: α -aminoisobutyric acid
- Ala (A): alanine
- Alloc: allyloxycarbonyl
- Api: 4-aminopiperidine-4-carboxylic acid
- aq.: aqueous
- Arg (R): arginine
- AU: arbitrary unit
- azaPra: azapropargylglycine
- B5: 2,2-bis(4'-pentenyl)glycine
- BAK: Bcl-2 homologous antagonist/killer
- Bcl: B-cell CLL/lymphoma
- BH3: Bcl-2 homology 3
- BiPhePhos: 6,6'-[(3,3'-di-*tert*-butyl-5,5'-dimethoxy-1,1'-biphenyl-2,2'-diyl)bis(oxy)]bis(dibenzo[*d,f*][1,3,2]dioxaphosphepine)
- Bn: benzyl
- Boc: *tert*-butoxycarbonyl
- BOP: (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
- BSA: bovine serum albumin
- Cbz: benzyloxycarbonyl
- CD: circular dichroism
- CID: collision-induced dissociation
- COSY: correlation spectroscopy
- CuAAC: copper-catalyzed azide–alkyne cycloaddition
- Cys (C): cysteine
- Dab: 2,4-diaminobutyrate
- Dap: 2,3-diaminopropionate
- dba: dibenzylideneacetone
- DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene
- DCE: 1,2-dichloroethane
- DCM: dichloromethane
- Dha: dehydroalanine
- DIC: *N,N'*-diisopropylcarbodiimide

- DIPEA: *N,N*-diisopropylethylamine
- DMAD: dimethyl acetylenedicarboxylate
- DMAP: 4-dimethylaminopyridine
- DMF: *N,N*-dimethylformamide
- DMSO: dimethylsulfoxide
- DNA: deoxyribonucleic acid
- EBX: 1-ethynyl-1,2-benziodoxol-3(1*H*)-one
- EC₅₀: half maximal effective concentration
- EDG: electron-donating group
- EIC: extracted ion current
- equiv.: equivalent
- ESI⁺: positive electrospray ionization
- Et: ethyl
- EtOH: ethanol
- FAM: carboxyfluorescein
- Fmoc: fluoren-9-ylmethoxycarbonyl
- FP: fluorescence polarization
- Gln (Q): glutamine
- Glu (E): glutamic acid
- GSH: glutathione
- HATU: *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
- HBS: hydrogen-bond surrogate
- HBTU: *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
- hCys: homocysteine
- HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
- HIF-1: hypoxia-inducible factor 1
- His (H): histidine
- HMBC: heteronuclear multiple bond correlation
- HOAt: 1-hydroxy-7-azabenzotriazole
- HPLC: high-performance liquid chromatography
- HRMS: high-resolution mass spectrometry
- HSQC: heteronuclear single quantum correlation
- IEDDA: inverse electron demand Diels–Alder
- Ile (I): isoleucine
- Iva: isovaline
- *K*_d: equilibrium dissociation constant
- LDH: lactate dehydrogenase

- Leu (L): leucine
- LRMS: low-resolution mass spectrometry
- LTQ: linear quadrupole ion trap
- Lys (K): lysine
- Mcl-1: myeloid cell leukemia 1
- MCR: multicomponent reaction
- MDM2: murine double minute 2
- MDMX: murine double minute X
- Me: methyl
- Me-DalPhos: 2-(di-1-adamantylphosphino)-*N,N*-dimethylaniline
- MeCN: acetonitrile
- MeOH: methanol
- Met (M): methionine
- Mmt: 4-methoxytrityl
- MPAA: 4-mercaptophenylacetic acid
- MS: mass spectrometry
- MS/MS: tandem mass spectrometry
- MSD: mass-selective detector
- MST: microscale thermophoresis
- NAD: nicotinamide adenine dinucleotide
- NBS: *N*-bromosuccinimide
- NCS: *N*-chlorosuccinimide
- NHS: *N*-hydroxysuccinimide
- Nle: norleucine
- NMM: *N*-methylmorpholine
- NMP: *N*-methylpyrrolidin-2-one
- NMR: nuclear magnetic resonance
- NOE: nuclear Overhauser effect
- NOESY: nuclear Overhauser effect spectroscopy
- Nva: norvaline
- OPA: ortho-phthalaldehyde
- Orn: ornithine
- Oxyma: ethyl cyano(hydroxyimino)acetate
- Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
- PBS : phosphate-buffered saline
- PCC: pyridinium chlorochromate
- PDB: Protein Data Bank
- Pen: penicillamine
- PFP: pentafluorophenyl

- Ph: phenyl
- pic-BH₃: 2-picoline–borane
- PMB: *p*-methoxybenzyl
- PPI: protein–protein interaction
- Pra : propargylglycine
- PyBOP: (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
- R8 : (*R*)-2-(7'-octenyl)alanine
- RCM: ruthenium-catalyzed ring-closing metathesis
- Rf: retention factor
- ROESY: rotating-frame Overhauser effect spectroscopy
- RP-HPLC: reversed-phase high-performance liquid chromatography
- rpm: revolutions per minute
- rt: room temperature
- RuAAC: ruthenium-catalyzed azide–alkyne cycloaddition
- RuAtAC: ruthenium-catalyzed azide–thioalkyne cycloaddition
- RuPhos: 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl
- S5: (*S*)-2-(4'-pentenyl)alanine
- S8: (*S*)-2-(7'-octenyl)alanine
- SD: standard deviation
- Ser (*S*): serine
- S_NAr: nucleophilic aromatic substitution
- SPAAC: strain-promoted azide–alkyne cycloaddition
- SPPS: solid-phase peptide synthesis
- sSPhos: sodium 2'-dicyclohexylphosphino-2,6-dimethoxy-1,1'-biphenyl-3-sulfonate
- TAMRA: carboxytetramethylrhodamine
- TBAI: tetrabutylammonium iodide
- *t*Bu: *tert*-butyl
- *t*BuBrettPhos: 2-(di-*tert*-butylphosphino)-2',4',6'-triisopropyl-3,6-dimethoxy-1,1'-biphenyl
- *t*BuOH: *tert*-butanol
- TCEP: tris(2-carboxyethyl)phosphine
- TFA: trifluoroacetic acid
- TFE: 2,2,2-trifluoroethanol
- THF: tetrahydrofuran
- THPTA: tris(3-hydroxypropyl)triazolylmethylamine
- TIS: triisopropylsilane

- Thr (T): threonine
- TIC: total ion current
- TLC: thin-layer chromatography
- TNBS: 2,4,6-trinitrobenzenesulfonic acid
- TOCSY: total correlation spectroscopy
- TPPTS: triphenylphosphine-3,3',3''-trisulfonic acid trisodium salt
- t_R : retention time
- TRIS: tris(hydroxymethyl)aminomethane
- Trp (W): tryptophan
- Trt: trityl (triphenylmethyl)
- Tyr (Y): tyrosine
- UV: ultraviolet

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1. Introduction

1.1. Targeting Protein–Protein Interactions (PPIs): A Promising Field in Medicinal Chemistry

Protein–protein interactions (PPIs) play key roles in numerous physiological processes and pathologies, such as cancer and neurodegenerative diseases.^[1,2] Therefore, designing compounds able to interfere with PPIs has emerged as a promising strategy to tackle diseases for which no effective treatments are available. However, PPIs are difficult to target with classical small-molecule drugs because they typically involve large and flat interfaces. In contrast, larger molecules, such as peptides, have attracted increasing interest as potential PPI inhibitors, particularly since the emergence of peptide-constraining methodologies in the late 1990s.^[3,4]

Several peptide-based PPI inhibitors have progressed to clinical evaluation.^[5] Among them, sulanemadlin (ALRN-6924) and PEP-010 are representative drug candidates currently being tested in patients with solid tumors (NCT03725436 and NCT04733027, respectively), whereas enlicitide decanoate is under clinical investigation for hypercholesterolemia (NCT07058077).

1.2. α -Helical Peptides as Potential PPI Inhibitors

α -Helices are prevalent secondary structures in protein–protein interfaces. Since many PPIs occur between a short α -helical segment and a globular protein domain, helical peptides or peptidomimetics reproducing the key recognition features of the native segment could act as competitive PPI inhibitors.^[3,6]

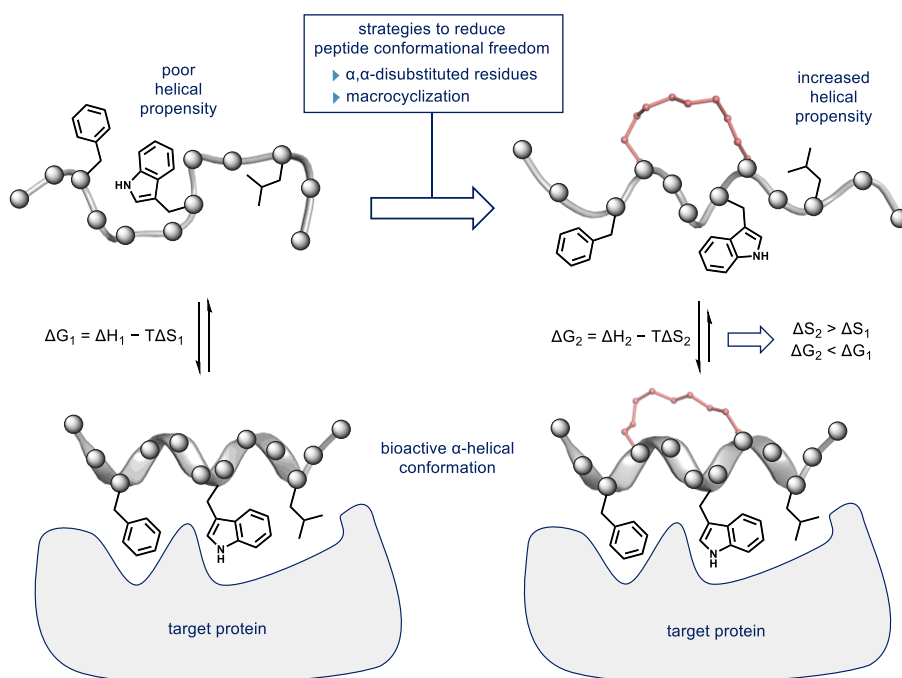


Figure 1. Enhancing the binding affinity of short peptides by reducing the entropic penalty upon binding.

ΔG : binding free energy; ΔH : enthalpy change upon binding; ΔS : entropy change upon binding.

The most straightforward approach to mimic a key α -helical protein segment involved in a PPI is to prepare a peptide with the same sequence. However, outside of protein environments, short linear peptides (typically ≤ 20 residues) usually display low helical propensities in aqueous solutions. The entropic penalty associated with adopting their bioactive helical conformation might contribute to

low binding affinity toward their target protein.^[7-10] Different chemical approaches have been developed to enhance the helical content of short peptides, which can translate into improved binding affinity (Figure 1).

This potential link between increased helicity and higher binding affinity should, however, be interpreted with caution: an enhancement of helical propensity does not necessarily result in stronger binding. From a theoretical perspective, Wilson and coworkers proposed that the assumed positive relationship between structural preorganization and binding affinity may not be valid for PPIs governed by an induced fit mechanism, as biasing a peptide toward its bioactive conformation may reduce the number of ways in which the peptide can bind to the target protein.^[10]

Nevertheless, in view of the numerous positive examples reported in the literature, increasing helicity remains an obvious strategy to explore when attempting to optimize a peptide whose bioactive conformation is α -helical.

Importantly, besides its potentially beneficial effect on binding affinity, an increase in helicity can also improve proteolytic stability and cell permeability,^[3,6,11] as the hydrogen-bonding network in helices decreases the solvent exposure of polar backbone amide groups, which can enhance resistance to proteases and facilitate penetration across cell membranes.^[11]

1.3. Structural Features of α -Helices

Before addressing the reported strategies to enhance the α -helical propensity of short peptides, it is important to outline the structural features that define an α -helix.

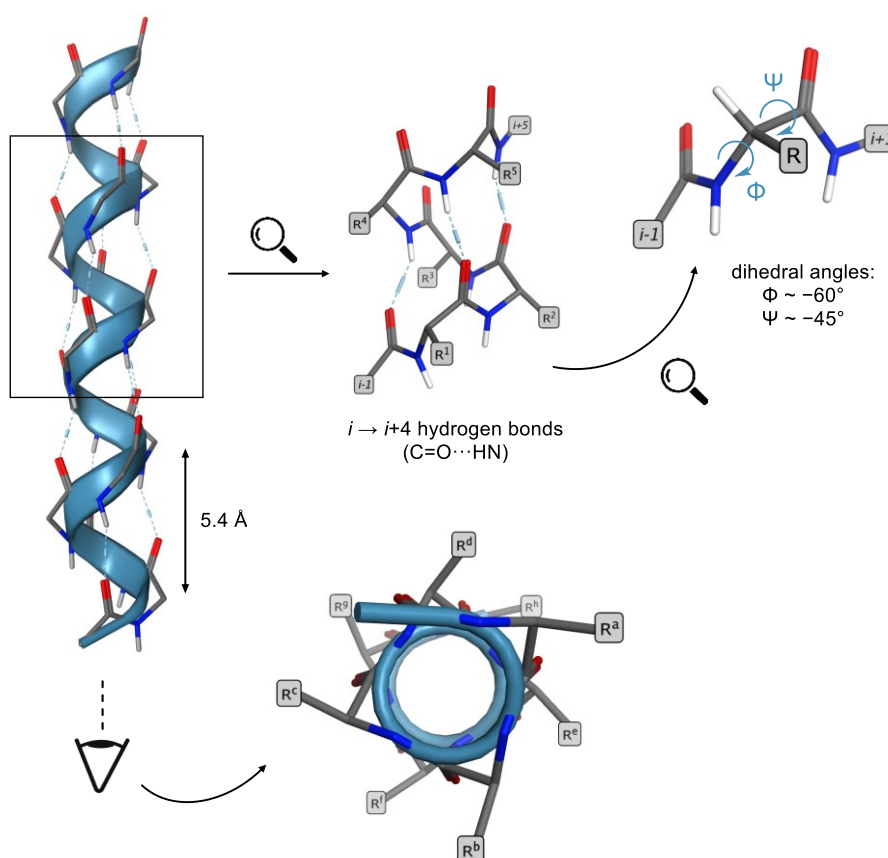


Figure 2. Structural features of α -helices.
Sides chains omitted for clarity.

α -Helices are the most common type of secondary structure in proteins. They are right-handed helices characterized by a repeating pattern of hydrogen bonds formed between the backbone amide groups of residues i and $i+4$. More precisely, the carbonyl oxygen of each residue in an α -helix accepts a hydrogen bond from the amide

NH group of the residue located four positions further along the peptide or protein sequence. This arrangement imposes characteristic dihedral angles, with Φ and Ψ values of approximately -60° et -45° , respectively. An α -helix contains 3.6 residues per turn and has a pitch of approximately 5.4 Å (Figure 2).^[3]

Despite being less frequent than α -helices, 3_{10} -helices and π -helices are additional helical secondary structures observed in proteins, characterized by $i \rightarrow i+3$ and $i \rightarrow i+5$ hydrogen-bonding networks, respectively.^[12]

Among the canonical amino acids, some residues are more prevalent in α -helices than others. Notably, alanine (Ala, A) exhibits the highest propensity for α -helix formation, whereas proline (Pro, P) and glycine (Gly, G) have the lowest and are typically considered α -helix breakers. Moreover, β -branched residues are less common in α -helices than β -unbranched residues.^[13-16]

Ionic interactions between charged side chains can stabilize the α -helical conformation, as can charge–dipole interactions involving charged side chains and the helix macrodipole. In this context, $i \rightarrow i+4$ -spaced glutamic acid (Glu, E)–lysine (Lys, K)^[7,17] and Glu–arginine (Arg, R)^[17,18] residue pairs have been shown to stabilize helical structures more efficiently than the corresponding $i \rightarrow i+3$ -spaced residue pairs, as well as more efficiently than the reverse pairwise orientation ($i \rightarrow i+4$ Lys–Glu and Arg–Glu, respectively).

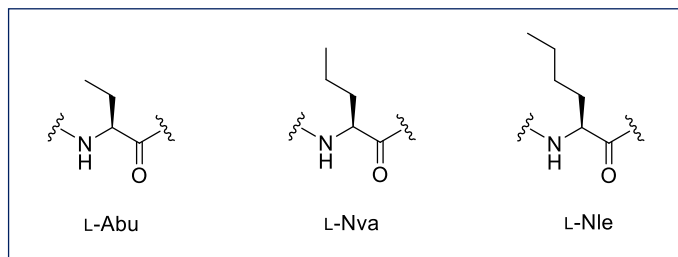
1.4. Strategies to Increase the Helical Propensity of Peptides

1.4.1. Unnatural Helicogenic Amino Acid Residues

Some unnatural amino acid residues have been shown to favor helicity (Figure 3). Notably, residues with linear aliphatic side chains such as norleucine (Nle), norvaline (Nva), and α -aminobutyric acid (Abu) may have helix-forming propensities comparable to that of alanine.^[14]

In addition, various α,α -disubstituted α -amino acids have been used as peptide-helix promoters. Among them, α -aminoisobutyric acid (Aib) is the simplest and most widely employed, and is known to favor 3_{10} -, α -, or mixed 3_{10} -/ α -helical conformations when incorporated into peptide sequences.^[12,19] Other representative α,α -disubstituted residues shown to promote helicity include acyclic quaternary amino acids such as isovaline (Iva)^[20,21] and (*S*)-2-(4'-pentenyl)alanine (S5),^[11,22] and cyclic quaternary amino acids such as 1-aminocyclopentanecarboxylic acid (Ac₅c) and its 3,4-disubstituted chiral analogues,^[23] as well as 4-aminopiperidine-4-carboxylic acid (Api) derivatives.^[24,25]

α -monosubstituted α -amino acid residues



α,α -disubstituted α -amino acid residues

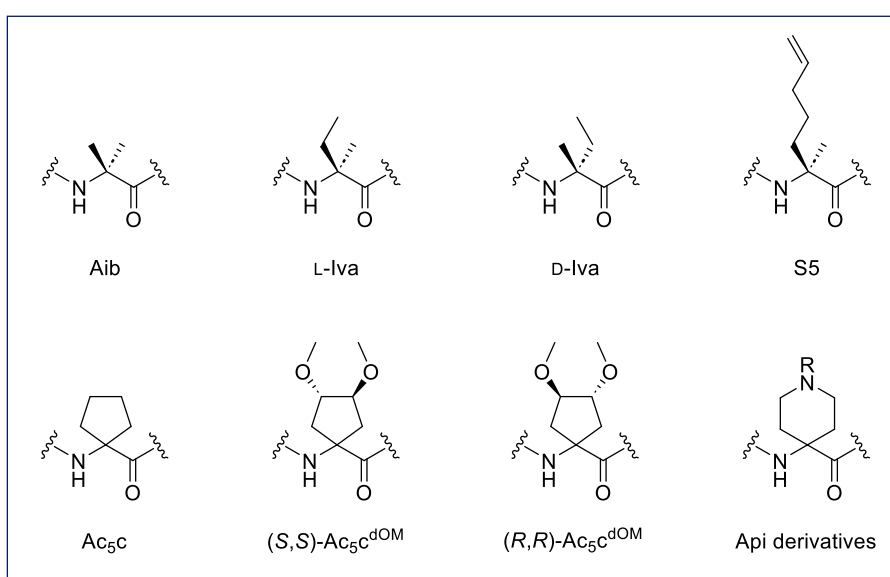
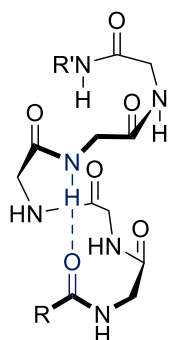


Figure 3. Non-canonical amino acid residues with high helical propensity.

1.4.2. Peptide Macrocyclization

1.4.2.1. Hydrogen-Bond Surrogates

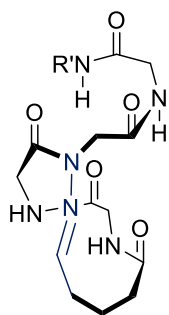
Macrocyclization probably represents the most powerful strategy to reduce the conformational flexibility of peptides, and has been extensively employed to increase the helical content of short linear peptides. Among macrocyclization approaches used to conformationally restrict peptides into helices, the hydrogen-bond surrogate (HBS) strategy consists in replacing an $i \rightarrow i+4$ main-chain hydrogen bond with a covalent bond, thereby mimicking the 13-membered hydrogen-bonded ring characteristic of α -helices (Figure 4).^[26-30] From a synthetic perspective, such a covalent linkage can be installed through several approaches, including hydrazone formation,^[26] ruthenium-catalyzed ring-closing metathesis (RCM),^[27,28] reductive amination,^[29] or thiol alkylation.^[30] However, reported HBS methodologies, despite their potential to nucleate and stabilize helical structures, only allow mimicry of the N-terminal main-chain hydrogen bond^[26-28,30] and/or require multi-step syntheses to access the building blocks needed for solid-phase peptide synthesis (SPPS), such as protected amino acids,^[26] dipeptides,^[27,28] and preformed cyclic pentapeptides,^[29] which limits their operational simplicity.



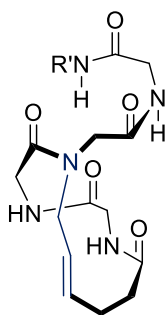
$i \rightarrow i+4$ hydrogen bond
(13-membered hydrogen-bonded ring)



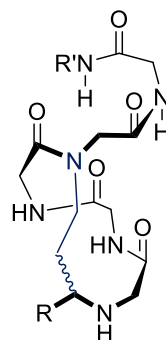
HBS strategy



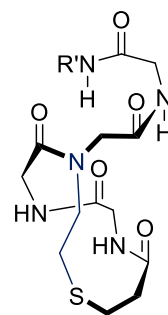
Cabezas and Satterthwait,
1999



Arora *et al.*,
2004



Allewood *et al.*,
2009

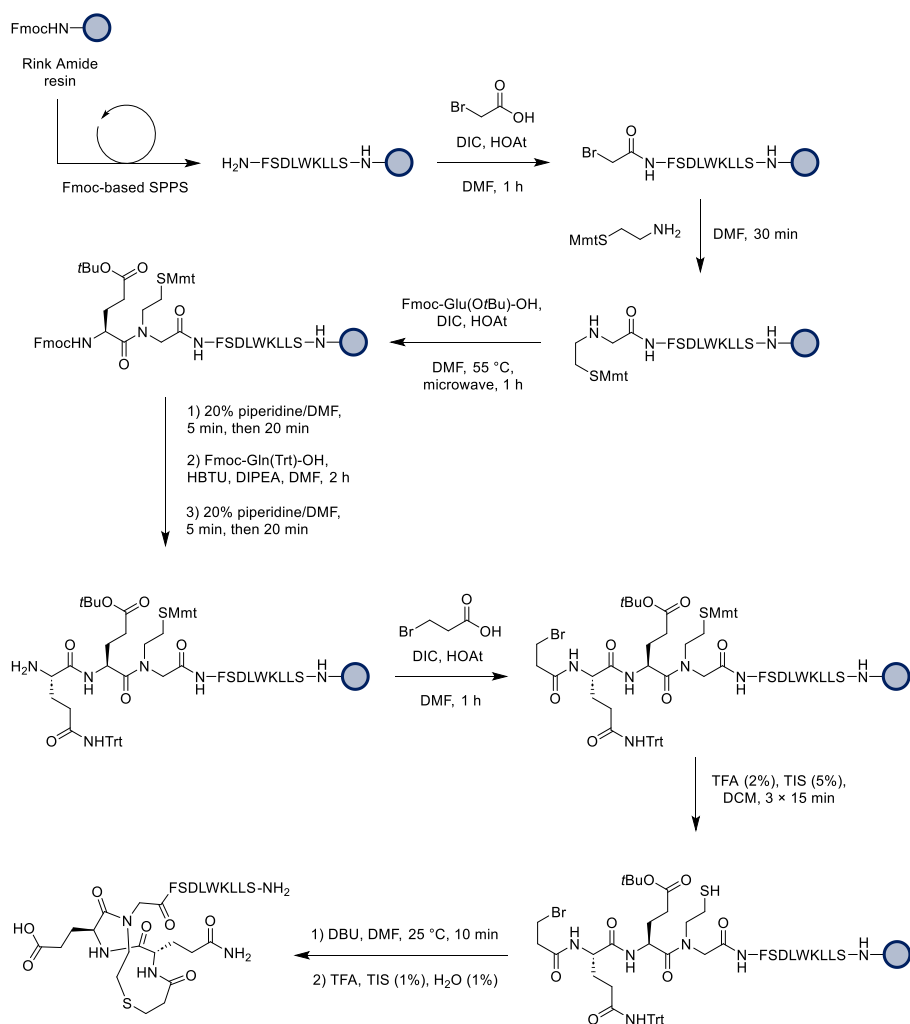


Mahon and Arora,
2012

13-membered covalent rings

Figure 4. Hydrogen-bond surrogate (HBS) strategy.
Side chains omitted for clarity.

One HBS approach developed by Arora and coworkers, consisting in replacing the N-terminal $i \rightarrow i+4$ hydrogen bond with a covalent thioether linkage, is, however, relatively direct from a synthetic standpoint and is presented here as a representative example of the HBS strategy (Scheme 1). The synthesis of the linear C-terminal segment was first carried out by Fmoc-based SPPS. The subsequent installation of the N-terminal cyclic tetrapeptide unit was also performed on-resin, beginning with the coupling of bromoacetic acid to the free α -amine of the resin-bound peptide. The bromide was then substituted with the primary amine of S-(4-methoxytrityl)-2-aminoethanethiol. The next two residues were added through two additional SPPS cycles, followed by coupling of 3-bromopropionic acid. After selective removal of the 4-methoxytrityl (Mmt) group, cyclization occurred through intramolecular S-alkylation upon treatment of the resin with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Finally, the crude cyclic peptide was obtained via standard peptide–resin cleavage and simultaneous removal of the acid-labile side-chain protecting groups.



Scheme 1. Synthesis of a thioether-based HBS helix derived from the p53 tumor suppressor protein.

Adapted from *Chem. Commun.* **2012**, *48*, 1416–1418.^[30]

1.4.2.2. Peptide Stapling

1.4.2.2.1. General Concepts

Peptide stapling consists in covalently linking two amino acids located on the same face of an α -helix (at positions i and $i+3$, $i+4$, $i+7$, or $i+11$), through side-chain-to-side-chain or side-chain-to-terminus cyclization (Figure 5).^[6] Stapling is a more widely employed approach than hydrogen-bond surrogates to design peptides with high helical propensity, notably because of its relative ease of implementation and its potential to generate structurally diverse macrocycles. Moreover, although stapling is often carried out at solvent-exposed positions to avoid interfering with key interactions between the peptide and its target, it can sometimes improve binding affinity not only through its effect on helicity but also through direct favorable interactions between the staple and the target protein.^[31-34]

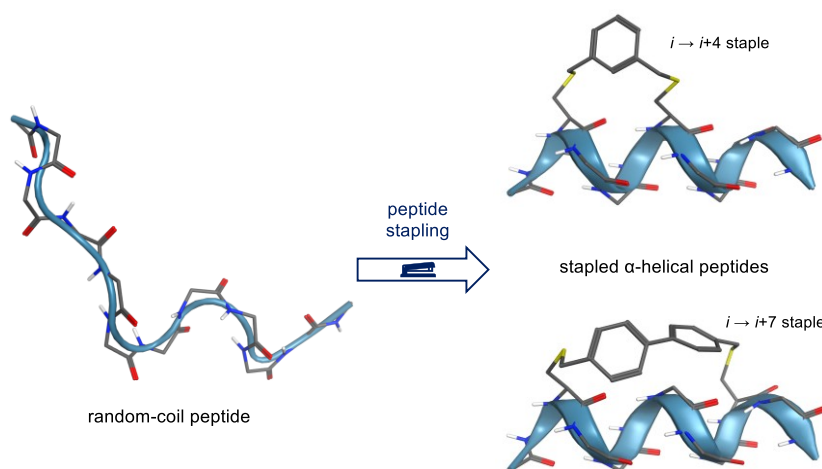


Figure 5. Peptide stapling via $i \rightarrow i+4$ or $i \rightarrow i+7$ side-chain-to-side-chain cross-links.

Side chains omitted for clarity, except those involved in the cross-links.

Peptide stapling, initially reported by Blackwell and Grubbs in 1998, was first implemented through RCM using *O*-allylserine or *O*-allylhomoserine residues.^[35] New stapling chemistries were further developed by many research groups, to the point that stapling can now

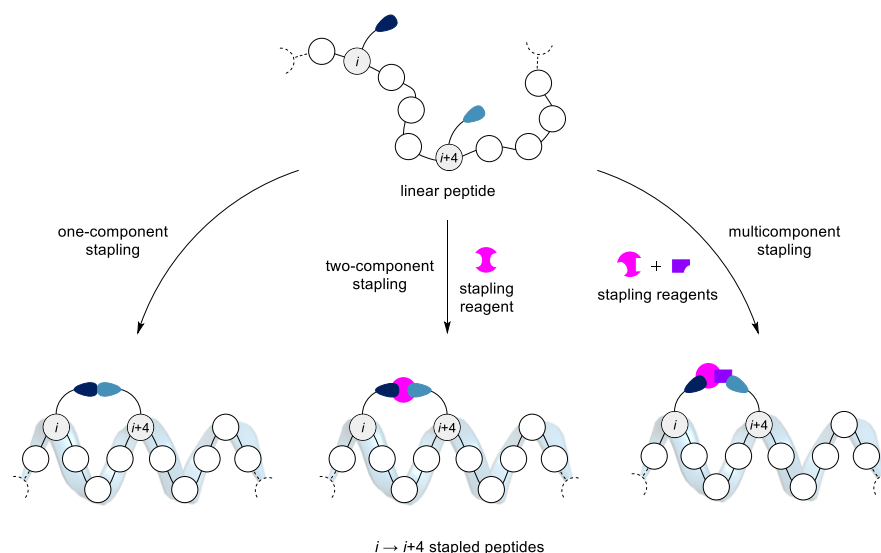
be performed using a wide variety of reactions, as illustrated by numerous reviews on the topic.^[6,36-45] The diversity of the stapling toolbox enables rapid access to various macrocyclic peptides, thereby facilitating the optimization of linear bioactive compounds.

Stapling approaches can be classified in multiple ways, such as by the nature of the anchoring residues, the spacing between them, or the number of components involved in the macrocyclization reaction.

Various amino acid residues, either natural or unnatural, can serve as anchoring residues for peptide stapling. The use of canonical amino acids may represent a more economical option owing to the accessibility of the corresponding building blocks required for SPPS. However, methodologies relying on unnatural residues are also of great interest, as they enable the installation of linkers whose structures would not be accessible otherwise, thereby conferring to the stapled peptide distinct physicochemical and biological properties compared to approaches based on canonical amino acids.

As an α -helix comprises 3.6 residues per turn, residues aligned on the same face are located at positions i , $i+4$, $i+7$, $i+11$, etc. Therefore, peptide stapling can be carried out by tethering $i \rightarrow i+4$ (or occasionally $i \rightarrow i+3$), $i \rightarrow i+7$, or $i \rightarrow i+11$ -spaced residue pairs, corresponding to macrocycles spanning one, two, or three helical turns, respectively. Nevertheless, $i \rightarrow i+4$ and $i \rightarrow i+7$ -spaced residue pairs represent by far the most commonly employed positions in peptide stapling.

Finally, stapling procedures can broadly be divided into two main categories depending on whether they involve an external non-peptidic cross-linking reagent (Scheme 2). One-component stapling occurs through formation of a cross-link between two mutually reactive side chains or termini, without incorporation of atoms from an external reagent. Conversely, two-component stapling involves the use of an external reagent that typically reacts with two functional groups of a linear peptide. This classification of stapling strategies, which is the most relevant from a synthetic perspective, will be used in this work. Accordingly, the following sections describe the most common one-component and two-component stapling methodologies. However, as a remarkably diverse set of chemistries has been applied to peptide stapling, not all of them will be presented here. Additional examples can be found in more detailed reviews.^[6,40,42-45]



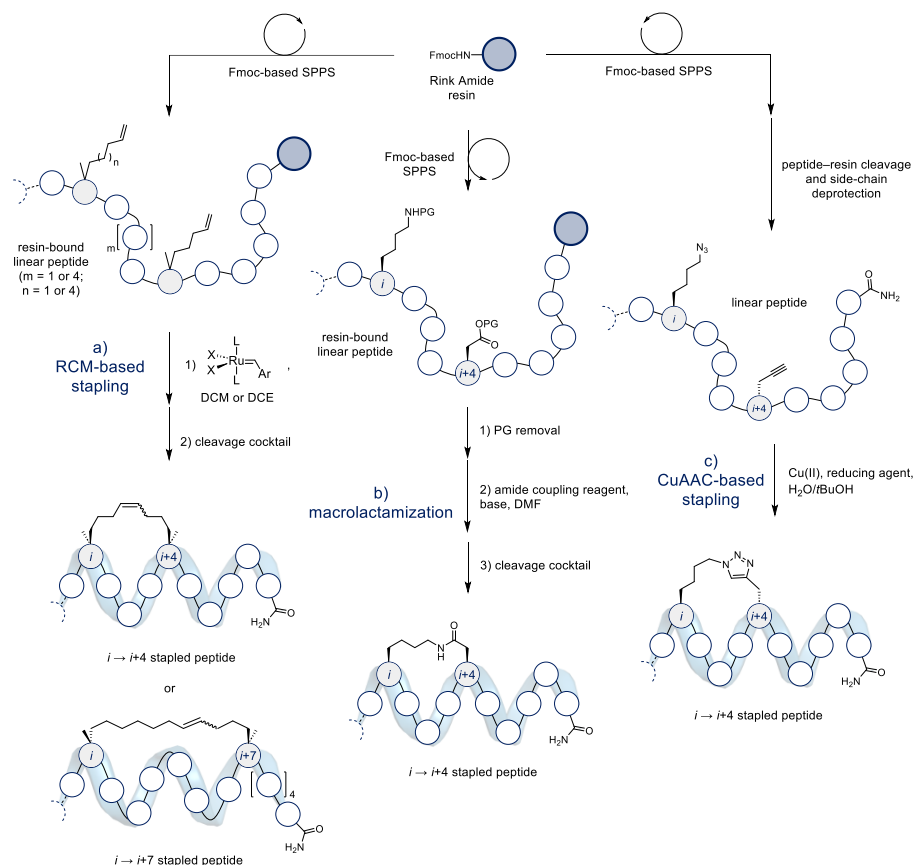
Scheme 2. One-, two-, and multicomponent stapling strategies (illustrated in the context of $i \rightarrow i+4$ cross-linking).

It should also be noted that powerful multicomponent stapling methodologies have been developed, further expanding the structural diversification achievable beyond two-component approaches.^[46-48]

1.4.2.2.2. One-Component Stapling

1.4.2.2.2.1. Ruthenium-Catalyzed Ring-Closing Metathesis

Peptides can be efficiently stapled through intramolecular olefin metathesis (Scheme 3a). The first example was reported in 1998 by Blackwell and Grubbs, who employed bis(tricyclohexylphosphine)benzylidene ruthenium(IV) dichloride (the first-generation Grubbs catalyst) in chloroform to cyclize, in solution phase, two hydrophobic peptides containing either two *O*-allylserine or *O*-allylhomoserine residues located at positions i and $i+4$. However, circular dichroism (CD) analyses revealed that the resulting constrained peptides did not display an enhanced helical content compared to their linear counterparts.^[35]



Scheme 3. One-component stapling through (a) ruthenium-catalyzed ring-closing metathesis, (b) macrolactamization, and (c) copper-catalyzed azide–alkyne cycloaddition.

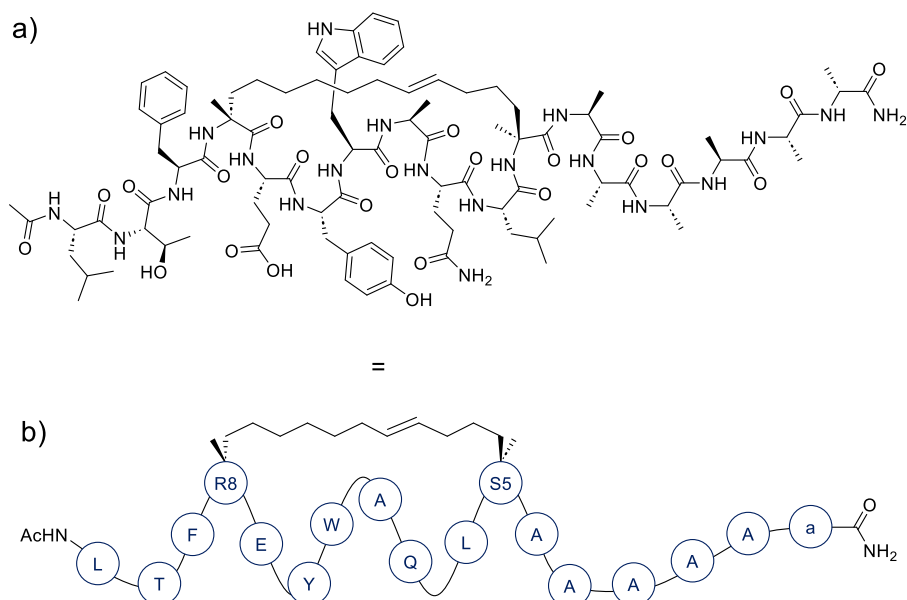
Representative examples of stapling via S5–S5, R8–S5, Lys–Asp, and L-Nle–D-Pra residue pairs are shown. L = L-type ligand. X = X-type ligand. PG = protecting groups orthogonal to Boc, *t*Bu, Trt, and Pbf.

Two years later, Verdine and coworkers pioneered the use of α,α -disubstituted α -amino acids bearing a terminal carbon–carbon double bond. They synthesized 15-mer peptides derived from ribonuclease A, in which two specific residues, located in an $i \rightarrow i+4$ or $i \rightarrow i+7$ relationship, were substituted with (*S*)- and/or (*R*)- α -alkenylalanine residues featuring alkenyl side chains of different lengths. The corresponding resin-bound peptides were also subjected to on-resin

RCM using the first-generation Grubbs catalyst in 1,2-dichloroethane (DCE), with the aim of generating $i \rightarrow i+4$ and $i \rightarrow i+7$ macrocyclic peptides equipped with staples of diverse lengths and stereochemical configurations. Importantly, they observed that RCM efficiency decreased markedly when short alkenyl side chains were used, and no metathesis occurred for substrates containing an $i \rightarrow i+4$ -spaced (*R*)-alkenylalanine–(*S*)-alkenylalanine residue pair. As expected, replacing canonical residues with certain α,α -disubstituted α -amino acids increased the intrinsic helical propensity even without cyclization. Most cyclic products, however, did not exhibit substantially greater helicity than their unmetathesized precursors. Nevertheless, the formation of an 11-atom-long staple proved particularly remarkable for cross-linking (*R*)- and (*S*)-alkenylalanine residues at positions i and $i+7$, respectively, resulting in a 26% increase in helicity relative to the corresponding unconstrained peptide.^[11]

Based on these foundations, olefin metathesis involving two α -alkenylalanine residues was subsequently applied extensively to equip short peptides with hydrocarbon staples.^[22,32,49-61] Following peptide chain assembly by Fmoc-based SPPS, stapling is typically carried out on solid support using first-^[11,32,49-53,55,58,59,61] or second-generation^[54] Grubbs catalysts, or the second-generation Hoveyda–Grubbs catalyst,^[57] in dichloromethane (DCM)^[49,61] or DCE.^[11,32,50-55,57-59] Cyclization can in principle generate *E*- and *Z*-stereoisomers. However, in most reported cases, such isomerism was either not observed or not reported, and the major (or sole) metathesized product was isolated. In rare instances, both isomers could be isolated, and these were found to display different helical propensities.^[11]

The introduction of two (*S*)-2-(4'-pentenyl)alanine residues (S5), followed by RCM-based cyclization, is the most common strategy for generating $i \rightarrow i+4$ hydrocarbon-stapled peptides with high helicity,^[22,49,51-54] whereas combining (*R*)-2-(7'-octenyl)alanine (R8) at position i with S5 at position $i+7$ represents the gold standard for $i \rightarrow i+7$ stapling.^[49,50,52] The enhanced helicity achieved through these approaches was in many cases accompanied by increased binding affinity,^[49,50] and by improvements in proteolytic resistance^[11,49,50] or cellular uptake.^[49,50,54] These combined advantages have made RCM-mediated stapling one of the most powerful and widely used strategies for the design of constrained α -helical peptides.



Scheme 4. Structure of $i \rightarrow i+7$ stapled peptide sulanemadlin. (a) Detailed structure. (b) Simplified representation.^a

^a For clarity, this simplified representation is used for all peptides throughout the manuscript. Each residue is represented by a circle containing either the one-letter code (for canonical amino acids; upper- and lowercase letters denote L- and D-amino acid residues, respectively) or a defined abbreviation (for non-canonical amino acids). Side chains are omitted, except for those of the anchoring residues.

Sulanemadlin (ALRN-6924) is an example of an RCM-stapled peptide of major clinical importance, as it has been evaluated in several cancer clinical trials and remains under investigation today. This $i \rightarrow i+7$ hydrocarbon-stapled peptide mimics the N-terminal α -helical domain of the p53 tumor suppressor protein. Its active metabolite, which can be formed in cells upon proteolytic removal of the C-terminal amide-capped D-alanine and two adjacent L-alanines, binds the target proteins MDM2 and MDMX with high affinity ($K_d = 1.4$ nM for MDM2; $K_d = 6.9$ nM for MDMX). Its optimized C-terminal polyalanine extension enables better cell permeability and allows simple aqueous formulation, overcoming the limited solubility and weaker cell activity of previously reported p53-derived stapled peptides (Scheme 4).^[60]

1.4.2.2.2. Macrolactamization

The formation of a lactam bridge between the side chains of lysine or ornithine (Orn) and glutamate or aspartate residues is another widely used one-component approach for biasing a peptide toward the α -helical conformation (Scheme 3b). From a chemical standpoint, modern methodologies involve assembly of the linear peptide by Fmoc-based SPPS, followed by selective removal of the protecting groups from the amine and carboxylic acid functionalities to be engaged in cyclization, on-resin macrolactamization using coupling reagents such as HBTU,^[62,63] BOP,^[64] and PyBOP,^[55,56,58,59,65,66] and finally cleavage of the cyclic peptide from the resin with simultaneous removal of the remaining side-chain protecting groups.

In 2005, Fairlie and coworkers produced a series of $i \rightarrow i+4$ lactam-bridged penta- and-hexapeptides, including peptides with the general sequence Ac-(cyclo-1.5)-[X¹ARAX²]-NH₂ (X¹ and X² representing the anchoring residues). Among all the combinations tested (Lys/Orn–Glu/Asp, or the reverse pairwise orientation), the $i \rightarrow i+4$ Lys–Asp lactam-bridged pentapeptide, featuring a 7-atom-long staple, displayed the highest helicity. Compared to this peptide, the Lys–Glu, Asp–Lys and Glu–Lys cross-linked peptides exhibited relative helicities of 0.08, 0.60, and 0.58, respectively, indicating that both ring size and staple orientation can influence helical folding. Further structural studies by CD and nuclear magnetic resonance (NMR) demonstrated the high conformational stability of $i \rightarrow i+4$ Lys–Asp stapled peptides as α -helices in water, which was striking given the small size of the peptides studied (5 or 6 residues).^[64] These important findings highlighted the effectiveness of $i \rightarrow i+4$ macrolactamization in significantly enhancing peptide α -helicity, an effect later corroborated by additional examples in the literature.^[55-58,65,66]

Stapling via formation of a lactam bridge has, in some cases, been shown to positively affect the binding affinity^[59,66,67] or activity^[55] of bioactive peptides, although this effect was not always clearly correlated with increased helicity.^[55] Some studies have reported enhanced proteolytic stability upon macrolactamization,^[58,59,64] whereas others have not shown such an improvement.^[55]

As a major drawback of this stapling approach, installing a lactam bridge on linear lead peptides rarely translates into higher cell

permeability, which may be attributed to the insufficient hydrophobicity of the lactam linkage.^[6,55,56]

1.4.2.2.3. Copper-Catalyzed Azide–Alkyne Cycloaddition

Copper-catalyzed azide–alkyne cycloaddition (CuAAC) is another widely adopted reaction for peptide stapling (Scheme 3c), notably due to its chemoselectivity and the ease with which it can be applied to peptide cyclization. In this context, CuAAC is most often performed for $i \rightarrow i+4$ side-chain-to-side-chain cyclization, with ϵ -azidonorleucine (Nle(ϵ -N₃)) and propargylglycine (Pra) as the anchoring residues.

After preparation of the linear peptide via Fmoc-based SPPS, CuAAC-based peptide stapling is typically carried out in solution, using an excess of copper(II) sulfate as the copper source, ascorbic acid or sodium ascorbate as the reducing agent to generate the catalytically active Cu(I) species, and a water/*tert*-butanol mixture as the solvent system.^[56,65,68-70] Alternatively, the cyclization reaction can be performed directly on solid support, as demonstrated in recent reports.^[57,59,71,72]

CuAAC-mediated peptide stapling affords cyclic peptides featuring a 1,4-disubstituted-1,2,3-triazole-based staple. Interestingly, the geometry of this staple can be modulated by varying the order of the azide- and alkyne-bearing residues, the length of their side chains,^[59,65,69,70] and their absolute configuration,^[65,70] all of which can have a significant effect on the helicity of the resulting macrocyclic peptide. These factors were systematically investigated by Wang and coworkers. They synthesized a large library of stapled 24-mer peptides derived from the Bcl9 protein, and demonstrated that combining L-Nle(ϵ -N₃) at position i with D-Pra at position $i+4$ provided the highest helical content upon $i \rightarrow i+4$ CuAAC-based stapling.^[70]

Stapling via CuAAC has been successfully applied to enhance the binding affinity^[59,70] or biological activity^[57,72] of linear peptides. Moreover, triazole-stapled peptides have in some cases exhibited substantial improvements in proteolytic stability compared with their linear counterparts.^[59,72] However, as with lactam-bridged peptides, the

weak hydrophobicity of the triazole moiety may limit the cellular uptake of peptides stapled through CuAAC.^[56]

1.4.2.2.3. Two-Component Stapling

1.4.2.2.3.1. Advantages of Two-Component Stapling Approaches

Although the one-component stapling approaches described above (RCM, macrolactamization, and CuAAC) have proven highly effective for generating peptides with strong helical propensity in aqueous solution, they also present several limitations. At the stage of peptide assembly by Fmoc-based SPPS, these methods require either the use of non-standard building blocks, which may need to be synthesized or are commercially less accessible, or orthogonal protection schemes, thereby making the synthesis less straightforward. The reliance of such methods on SPPS also limits their applicability to long peptides (>50 residues), which are generally produced by recombinant DNA technology. Moreover, regarding $i \rightarrow i+7$ stapling, while certain 11- or 12-atom staples can be installed through cross-metathesis of appropriately substituted α -alkenylalanine residues, the staples accessible through one-component approaches are generally too short to efficiently stabilize two consecutive α -helical turns. Yet, $i \rightarrow i+7$ stapling may offer advantages over $i \rightarrow i+4$ cross-linking, notably by further increasing the hydrophobic surface area of the macrocyclic peptide and thereby promoting cell membrane penetration. Finally, one-component methodologies do not allow linker diversification from a single linear precursor. Instead, they require preparation of a distinct linear peptide for each stapled analogue. As a result, generating libraries of macrocyclic peptides through these techniques demands substantial synthetic effort, which hinders rapid exploration of chemical space.

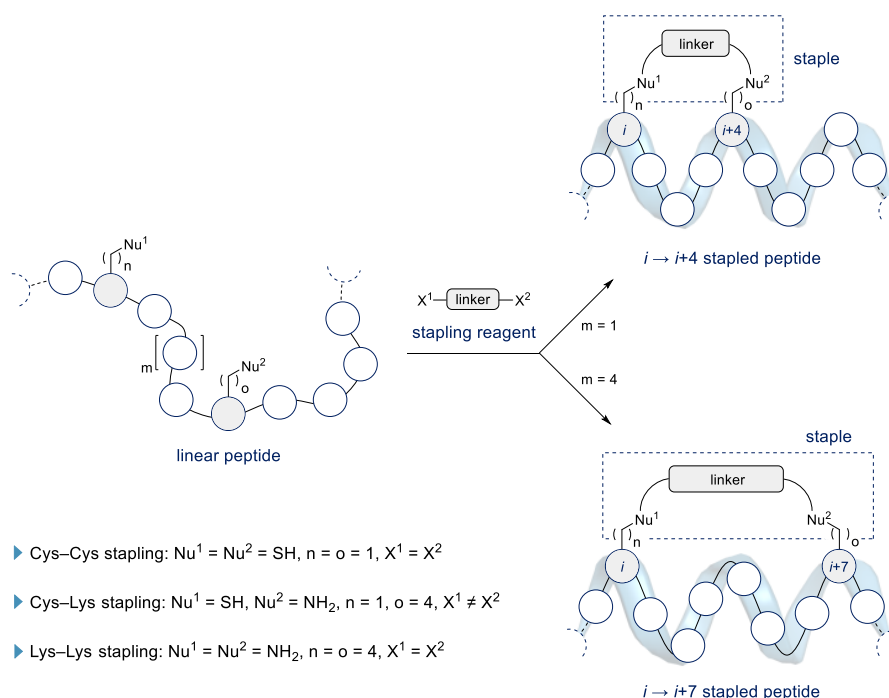
Two-component stapling methodologies, which rely on the use of an external stapling reagent, typically a bifunctional molecule, can overcome these limitations. Indeed, some of these techniques allow the use of canonical amino acids as anchoring residues by taking advantage of the reactivity of their side chains. Furthermore, regardless of the nature of the anchoring residues, the staples installed

can span a broad range of lengths and physicochemical properties, determined solely by the choice of the cross-linking reagent. In addition, the most significant advantage of two-component stapling is its ability to generate multiple macrocycles from a single linear peptide through the use of different stapling reagents. The structural diversity generated makes the two-component macrocyclization approach a powerful tool to fine-tune the chemical and biological properties of a lead peptide. This also facilitates the screening of large libraries of cyclic peptides against a target protein, as demonstrated in studies by Heinis and coworkers, among others.^[73-76] Finally, certain stapling reagents can also incorporate a third functionality that serves as a conjugation handle, thereby enabling post-stapling modifications.^[77-82] This notably allows grafting onto the stapled peptide various valuable entities, such as fluorophores,^[77-80,82] solubilizing moieties,^[79] affinity tags,^[78,79] cell-penetrating peptides,^[78,81] or drugs.^[82]

1.4.2.2.3.2. Anchoring Residues Used in Two-Component Stapling

Most stapling reagents used in two-component stapling approaches are bis-electrophiles that modify peptides by exploiting the inherent reactivity of proteinogenic nucleophiles such as thiols, amines, and thioethers. In this context, the cysteine (Cys, C) β -thiol and the lysine ϵ -amine represent the most common functional groups used for side-chain-to-side-chain macrocyclization (Scheme 5). As these side chains are also those investigated in the two main projects of this work, the following sections outline stapling methodologies involving a Cys–Cys, Cys–Lys, or Lys–Lys residue pair.

It is important to note, however, that in addition to canonical amino acids, some unnatural amino acids, such as ϵ -azidonorleucine,^[79] δ -azidonorvaline,^[83-85] O-propargyl tyrosine,^[86] and aldehyde-modified glutamate^[87] have also been successfully used as anchoring residues in two-component macrocyclization, further expanding the peptide stapling toolbox.



Scheme 5. Two-component peptide stapling via Cys–Cys, Cys–Lys, or Lys–Lys cross-linking.

The terms “stapling reagent”, “linker”, and “staple” refer to the elements highlighted in this scheme and are used accordingly throughout the manuscript. Nu = nucleophile. X = thiol- or amine-reactive group.

1.4.2.2.3.3. Cys–Cys Stapling

1.4.2.2.3.3.1. Cross-Linking Reagents for Cys–Cys Stapling

The relative scarcity of cysteine, together with the strong nucleophilicity and unique reactivity profile of its thiol group, makes cysteine the residue of choice for chemoselective covalent modification of peptides and proteins. Therefore, a widely used stapling strategy consists in mutating two appropriate residues within a linear peptide to cysteines, followed by solution-phase chemoselective macrocyclization using reagents bearing two identical thiol-reactive groups. This Cys–Cys stapling approach has been extensively developed since the 2000s: a broad range of bifunctional reagents, such as alkylating agents,^[88-94] Michael acceptors,^[75,82,95-98] arylating agents,^[58,67,77,99-101] dihalo-maleimides,^[102,103] dinitroimidazoles,^[104] alkynylating agents,^[80]

chlorooximes,^[86,105] and sulfenylating agents,^[106] has been shown to enable efficient cysteine-mediated cyclization of unprotected linear peptides. In addition to bis-electrophiles, various dienes^[81,107] and 1,8-nonadiyne^[81] have also been employed as reagents for Cys–Cys stapling through radical thiol–ene and thiol–yne processes, respectively.

The Cys–Cys cross-linking reagents are presented in Table 1, grouped by chemical class and listed in chronological order of appearance in the literature.

From a chemical perspective, Cys–Cys stapling is typically performed in solution, after preparation of the linear dithiol precursor, most commonly through Fmoc-based SPPS. Most stapling procedures, notably those involving alkylating agents, Michael acceptors, dihalomaleimides, dinitroimidazoles, and chlorooximes, are generally carried out at room temperature in water or aqueous buffer at neutral or slightly basic pH (pH 7.0–8.5), sometimes in the presence of acetonitrile as a co-solvent. A reducing agent such as tris(2-carboxyethyl)phosphine (TCEP) is often added to prevent intramolecular disulfide formation.

Among alkylating agents, azobenzene-based reagents substituted with two α -haloamides or benzyl bromide moieties (entries 1, 3, 24) have been used since 2000, following their introduction by Woolley and coworkers. Their photoisomerizable nitrogen–nitrogen double bond enables photocontrol of the conformation of $i \rightarrow i+4$, $i \rightarrow i+7$, or $i \rightarrow i+11$ Cys–Cys stapled peptides, and thus of their binding affinity. Bis(bromomethyl)-substituted aromatic reagents (entries 2, 4, 6–8, 10), notably α,α' -dibromoxylenes (entry 2), are probably the most widely used stapling reagents for $i \rightarrow i+4$ Cys–Cys stapling. Nevertheless, allylic bromides (entries 9, 19), dichloroacetone (entry 16), *N*-alkyl- α -bromoacetamides (entry 17), and allylic carbonates (which generate π -allylpalladium intermediates in the presence of a Pd(0) species, entries 20, 21) have also been employed for this purpose. Stapling between $i \rightarrow i+7$ -spaced cysteine residues has been shown to be readily achieved by treating linear peptides with 3,3'- and 4,4'-bis(bromomethyl)biphenyl derivatives (entries 5, 11, 12, 22), 6,6'-bis(bromomethyl)-3,3'-bipyridine (entry 5), 1,4-bis(3-bromoprop-1-en-1-yl)benzene (entry 13), 2,8-bis(bromomethyl)phenazine (entry 14), as

well as *N*-aryl- (entry 15) and *N*-alkyl- α -bromoacetamides (entry 17). Although they are less reactive, some procedures have also reported the use of unactivated alkyl halides as cross-linkers for $i \rightarrow i+4$ and $i \rightarrow i+7$ Cys–Cys stapling (entries 18, 23).

As demonstrated by Weil and coworkers, α -[(*p*-toluenesulfonyl)methyl]- α,β -unsaturated carbonyl compounds (which can be generated *in situ* from the corresponding bis-sulfone through β -elimination of *p*-toluenesulfinic acid) are efficient Michael acceptors for side-chain-to-side-chain cross-linking of cysteine residues, although the impact of the resulting linkers on peptide conformation has not been investigated (entries 25, 26).^[95,96] Other Michael acceptors have also been successfully employed for covalently linking two thiol-bearing side chains at different relative positions, such as a bis-allenamide-substituted azobenzene analogue (entry 27),^[97] vinyl sulfonamide derivatives (entry 28),^[82] and divinyl sulfone (entry 30).^[75] Besides classical Michael acceptors, Cys–Cys stapling has been shown to be feasible using 2,4-divinyl-1,3,5-triazine derivatives, via nucleophilic addition to their electron-poor alkene moieties (entry 29).^[98]

Arylating agents are another class of cross-linking reagents for Cys–Cys stapling. In this context, cysteine residues can be tethered either through nucleophilic aromatic substitution (S_NAr), typically employing perfluoroaromatic-based reagents, or through transition-metal-mediated *S*-arylation. The use of perfluoroarenes for Cys–Cys cross-linking was first reported by Pentelute and coworkers in 2013, who described efficient stapling of linear peptides incorporating an $i \rightarrow i+4$ Cys–Cys residue pair using an excess of hexafluorobenzene (entry 31) or decafluorobiphenyl (entry 32) in *N,N*-dimethylformamide (DMF) and in the presence of tris(hydroxymethyl)aminomethane (TRIS) as a base.^[99] Building on this work, hexafluorobenzene has since been widely used in the literature,^[56,58,59,65,67,99,103,108] particularly for $i \rightarrow i+4$ stapling. Longer perfluoroaromatic cross-linkers, based on two (entries 32–34, 37),^[59,99–101] or three connected aromatic rings (entry 34),^[100] have also been employed to link cysteine residues across various Cys–Cys spacings. 3,6-Dichloro-1,2,4,5-tetrazine (entry 35) is another convenient reagent for cross-linking two cysteine residues, as efficient Cys–Cys stapling was reported in a biphasic mixture of chloroform and sodium phosphate buffer at pH 5. Interestingly, the resulting tetrazine

linker can be functionalized through an inverse electron demand Diels–Alder (IEDDA) reaction or removed upon ultraviolet (UV) irradiation, making the dichlorotetrazine-based stapling strategy reversible.^[77] Beyond S_NAr chemistry, various organometallic palladium and gold reagents (entries 36, 38, 39), notably those developed by the Buchwald group, have been shown to enable fast $i \rightarrow i+4$ or $i \rightarrow i+7$ Cys–Cys stapling in water or water/acetonitrile mixtures, with TRIS as a base, allowing the introduction of structurally diverse linkers.^[109–112]

The rapid kinetics and chemoselectivity of photo-induced thiol–ene and thiol–yne reactions have also been exploited in the field of two-component peptide macrocyclization. Notably, Chou and coworkers reported efficient radical Cys–Cys stapling procedures relying on various dienes (entry 40) or 1,8-nonadiyne (entry 41) as stapling reagents. These reactions, carried out under UV irradiation ($\lambda = 365$ nm) in the presence of a radical initiator, in either *N*-methylpyrrolidin-2-one (NMP) or water, enabled the installation of various linkers, including purely hydrocarbon-based ones.^[81,107]

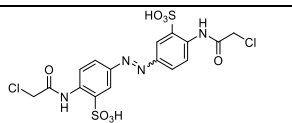
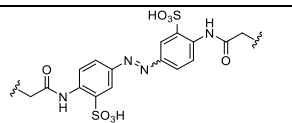
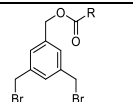
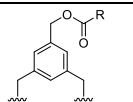
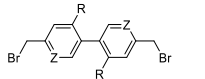
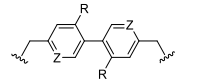
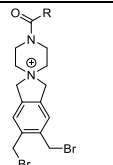
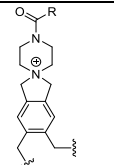
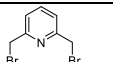
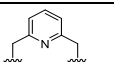
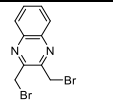
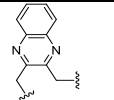
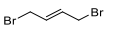
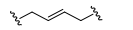
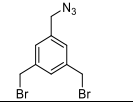
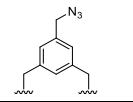
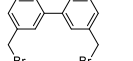
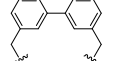
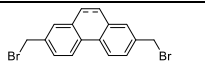
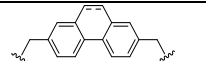
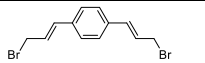
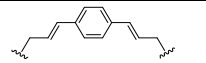
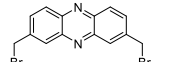
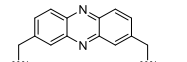
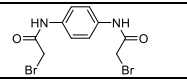
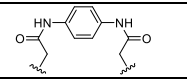
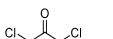
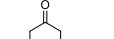
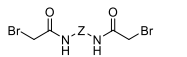
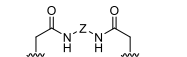
More exotic stapling reagents, such as dihalomaleimides, dinitroimidazoles, alkynylating agents, chlorooximes, and sulfenylating agents, have also been employed more recently for side-chain-to-side-chain cross-linking of cysteine residues. Dibromomaleimide and *N*-alkylated derivatives (entry 42) were shown to enable fast $i \rightarrow i+4$ and $i \rightarrow i+7$ Cys–Cys stapling in water/acetonitrile mixtures.^[102,103] However, the resulting maleimide linkage was found to be unstable in the presence of thiols,^[102] which, although allowing facile regeneration of the linear peptide dithiol (of interest for controlled drug release), indicates that peptides stapled in this manner may not retain their integrity under biological conditions. Bifunctional reagents bearing two 1,4-dinitroimidazole (entry 43) or 1-alkynyl-1,2-benziodoxole (entry 44) moieties were also recently reported as chemoselective Cys–Cys stapling reagents, proceeding via *cine*-substitution^[104] and *S*-alkynylation^[80] reactions, respectively. In recent work, reactions between the cysteine β -thiol and chlorooximes were shown to be among the fastest cysteine conjugation processes known to date.^[105] Accordingly, *N*¹,*N*³-dihydroxyisophthalimidoyl dichloride (entry 45) appears to be a very efficient Cys–Cys cross-linker,^[86,105] although no studies have yet investigated the conformation of peptides stapled using this reagent. Finally, in 2023, Jiang and coworkers prepared a

broad range of stapling reagents containing two terminal 1-chloro-2-phthalimidodisulfonyl-substituted alkene moieties (entry 46), capable of modifying cysteine residues through disulfide formation. In water/acetonitrile (1:7) mixtures, these cross-linkers enabled rapid stapling of linear peptides featuring Cys–Cys residue pairs with different spacings. Nevertheless, as expected, the resulting disulfide-based linkage displayed poor stability in serum.^[106]

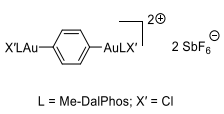
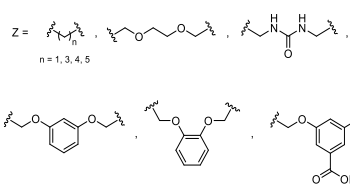
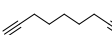
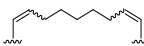
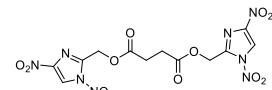
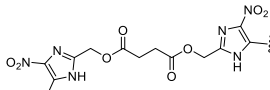
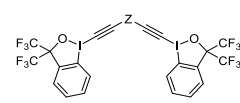
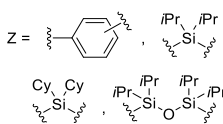
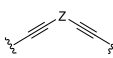
Overall, these structurally diverse Cys–Cys cross-linking reagents give access to macrocyclic peptides featuring linkers of various lengths (from 3 to 19 atoms, corresponding to total staple lengths of 7 to 23 atoms) and rigidities, thereby enabling modulation of α -helicity. Certain installed linkers also allow post-stapling functionalization, further increasing structural diversity and/or enabling the introduction of a useful group. For instance post-stapling derivatization of Cys–Cys stapled peptides has been achieved through bioorthogonal reactions such as CuAAC,^[82] strain-promoted azide–alkyne cycloaddition (SPAAC),^[113] IEDDA,^[77] oxime ligation,^[103,113] or thiol-ene processes.^[81] Furthermore, beyond linker variation, the geometric properties of staples formed via thiol-to-thiol peptide macrocyclization can also be modulated by inverting the absolute configuration of one or both cysteine residues involved,^[59,108,114,115] or by replacing them with homocysteine (hCys)^[58,59,78,103,108] or penicillamine (Pen)^[108,115] residues.

Table 1. Cross-linking reagents for Cys–Cys stapling.

Entry	Stapling Reagent	Resulting Linker	Year ^a
Alkylating agents			
1			2000 ^[88,89]
	Photoisomerizable nitrogen–nitrogen double bond		
2			2005 ^[19,56,58,59,65,67,75,76,90,93,94,103,116-118]

3			2007 ^[91,92]
	Photoisomerizable nitrogen–nitrogen double bond		
4			2009 ^[119]
5			2011 ^[76,114,116,120,121]
	R = H, OMe; Z = CH, N		
6			2012 ^[113]
7			2012 ^[59,75,93,94]
8			2012 ^[59,93]
9			2012 ^[59,75,76,93,94]
10			2014 ^[122,123]
11			2014 ^[121]
12			2014 ^[121]
13			2014 ^[121]
14			2014 ^[121]
15			2015 ^[116]
16			2015 ^[75,78,103]
17			2016 ^[76,124,125]

29			2019 ^[98]
	Z = OH, NHR		
30			2023 ^[75]
Arylating agents			
31			2013 ^[56,58,59,65,67,99,103,108]
32			2013 ^[59,99,100]
33			2014 ^[100]
34			2014 ^[100]
	Z = , , ,		
35			2015 ^[77,103]
36			2015 ^[109]
L = RuPhos; X' = Cl			
37			2016 ^[101]
38			2017 ^[110,111]
	L = RuPhos, sSPHos; X' = Cl, Br Ar = ,		

39	 L = Me-DalPhos; X' = Cl		2018 ^[112]
Alkenes (radical reactivity)			
40	 		2015 ^[81,107]
Alkynes (radical reactivity)			
41			2017 ^[81]
Dihalomaleimides			
42			2017 ^[102,103]
Dinitroimidazoles			
43			2019 ^[104]
Alkynyating agents			
44	 		2021 ^[80]

Chlorooximes			
45			2022 ^[86,105]
Sulfenylating agents			
46	 $Z =$		2023 ^[106]

X = thiol-reactive group. ^a Year of first reported use in the literature, to the best of our knowledge.

1.4.2.2.3.3.2. Effect of Cys–Cys Stapling on Helicity

Some linkers introduced through $i \rightarrow i+4$ ^[19,58,93,102,116] or $i \rightarrow i+7$ ^[58,88,98,114,116,121] Cys–Cys stapling have been reported to positively influence the helical propensity of peptides in water, although to varying extents.

A relevant geometrical consideration is that the S–S distances between cysteine residues located at positions $i \rightarrow i+4$ or $i \rightarrow i+7$ in an α -helical peptide are approximately 6–7 Å and 11–12 Å, respectively.^[6,121] Alongside hexafluorobenzene, α,α' -dibromoxylenes are the most commonly used reagents for constraining peptides via $i \rightarrow i+4$ Cys–Cys stapling, generating linkers with lengths ranging from 4 to 6 atoms (corresponding to total staple lengths of 8–10 atoms). Depending on its substitution pattern, the resulting xylylene linker can lead to differences in peptide conformation.^[93,94,118] In an influential study published in 2012, Greenbaum and coworkers reported that the *meta*-

xylylene linker had the most beneficial effect on the helicity of a 18-mer peptide model, compared to the *ortho*- and *para*-xylylene linkers.^[93] α,α' -Dibromo-*m*-xylene was widely used in the following years as a stapling reagent to stabilize the helical conformation of various peptides.^[19,67,116,117] However, in two comparative studies using a pentapeptide Ac-X¹AAAX²-NH₂ (X¹ and X² representing the anchoring residues)^[65] or a dodecapeptide targeting estrogen receptor α ^[56] as model systems, the 9-atom-long staple formed through α,α' -dibromo-*m*-xylene-mediated $i \rightarrow i+4$ Cys–Cys stapling proved less effective at increasing peptide helicity than staples generated through classical one-component $i \rightarrow i+4$ stapling approaches, namely the 8-atom-long hydrocarbon staple obtained by RCM between two S5 residues, the 7-atom-long Lys–Asp lactam bridge, and the 8-atom-long triazole-based staple formed using an Nle(ϵ -N₃)–Pra residue pair.

In terms of $i \rightarrow i+7$ cross-linking, Cys–Cys stapling using various reagents, such as 4,4'-bis(bromomethyl)biphenyl,^[58,114] 6,6'-bis(bromomethyl)-3,3'-bipyridine,^[121] *N,N'*-(1,4-phenylene)bis(2-bromoacetamide),^[116] *N*-(4,6-divinyl-1,3,5-triazin-2-yl)-*N*-methylglycine,^[98] 1,8-dibromooctane,^[58] and 4,4'-bis(bromomethyl)-2,2',3,3',5,5',6,6'-octafluoro-1,1'-biphenyl,^[58] has been reported to enhance the α -helical content of several peptides. Therefore, linker lengths of 7–10 atoms (corresponding to staple lengths of 11–14 atoms) may be considered suitable for stabilizing the helical conformation of short peptides via two-loop ($i \rightarrow i+7$) stapling.

Following stapling with Woolley's 4,4'-bis(haloacetamide)-azobenzenes, *trans*-to-*cis* photoisomerization of the nitrogen–nitrogen double bond promotes α -helix formation in $i \rightarrow i+4$ ^[89,92] and $i \rightarrow i+7$ ^[88,92] stapled peptides, while it is detrimental to the α -helicity of $i \rightarrow i+11$ cross-linked peptides.^[89,92]

Overall, Cys–Cys stapling constitutes a widely used strategy for constraining bioactive peptides in order to reduce the entropic cost associated with target binding. Many peptides, notably peptides targeting PPIs such as p53–MDM2,^[58,76,98] BAK–Bcl-x_L,^[102] and Noxa–Mcl-1,^[114,121] have been shown to exhibit enhanced binding affinity upon Cys–Cys stapling. Furthermore, numerous studies have reported increased proteolytic resistance,^[58,59,98,102,114,116] and/or cell permeability^[56,76,114,116] following Cys–Cys cross-linking.

1.4.2.2.3.4. Cys–Lys Stapling

1.4.2.2.3.4.1. Rationale for Cys–Lys Stapling Approaches

The drawback of Cys–Cys stapling lies in the necessity of using symmetrical stapling reagents to avoid the formation of mixtures of constitutional isomers, which to some extent limits the diversity of linkers that can be installed. In contrast, using one cysteine and one lysine residue as anchoring points enables the introduction of non-symmetrical linkers. This approach, referred to as Cys–Lys stapling (or more generally amino–sulfhydryl stapling), was first envisioned on resin. However, reported on-resin approaches required orthogonally protected^[33,129,130] or prefunctionalized^[33,130] cysteine and lysine (or ornithine) derivatives, and therefore involved multi-step syntheses.

In 2019, $i \rightarrow i+4$ Cys–Lys stapling of fully deprotected peptides via formation of a dithiocarbamate tether was reported by Wu and coworkers. They developed an original solution-phase strategy based on the conversion of cysteine into dehydroalanine (Dha), through cysteine bis-S-alkylation with 2,5-dibromohexanediamide and elimination of the resulting cyclic sulfonium cation, followed by nucleophilic addition of the lysine ϵ -amine to carbon disulfide and intramolecular trapping of the electrophilic Dha residue by the generated dithiocarbamate anion. However, this two-step approach involved purification of the Dha-containing intermediate, thereby limiting its operational convenience. Furthermore, the possible formation of two epimeric peptides (incorporating a modified cysteine residue with either L- or D-configuration) during the Michael addition step could not be excluded.^[131]

Over the past decade, other solution-phase stapling methodologies have been developed to exploit the difference in nucleophilicity between cysteine and lysine side chains, thereby enabling one-step cyclization of unprotected peptides (Scheme 6). Operationally, these methods are considerably simpler than the approaches mentioned above. Nevertheless, to obtain the desired Cys–Lys stapled peptide in good yield from an unprotected linear precursor, the stapling reagent employed must display high chemoselectivity toward both cysteine and lysine residues. Beyond chemoselectivity, controlling site-selectivity (i.e., the ability to target a single, specific amino acid residue among multiple residues of the same type) represents a critical consideration

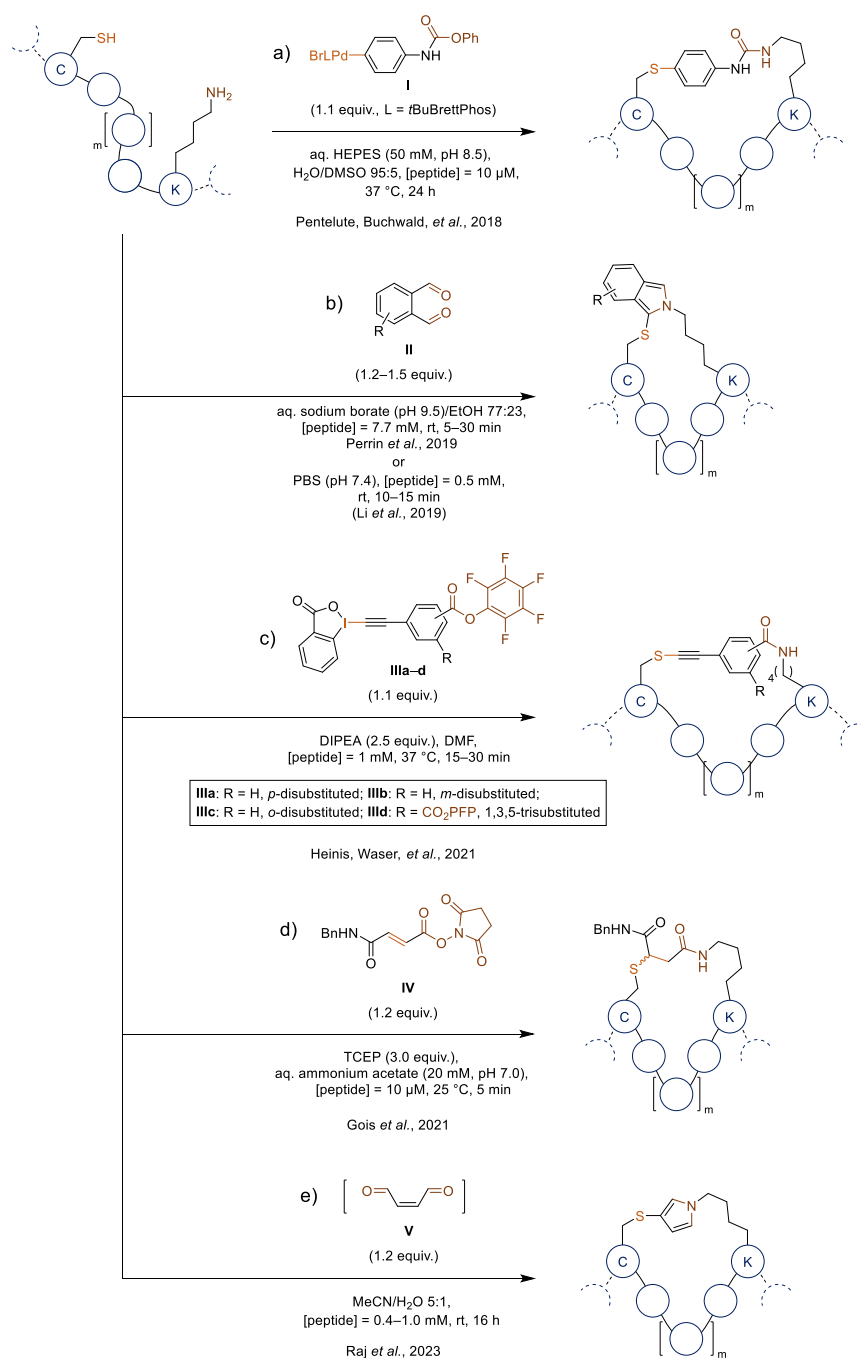
in Cys–Lys stapling processes, given the substantial abundance of lysine residues in peptides and proteins.

The following sections present the stapling reagents reported for one-step solution-phase Cys–Lys stapling, in chronological order, with emphasis on the reaction mechanisms involved and on the structural diversity of the resulting macrocycles.

1.4.2.2.3.4.2. Cross-Linking Reagents for Cys–Lys Stapling

1.4.2.2.3.4.2.1. Organometallic Reagents

In 2018, Buchwald and coworkers reported an efficient protocol for Cys–Lys stapling using a palladium oxidative addition complex bearing an *O*-phenyl carbamate moiety (Scheme 6a). The methodology, which proceeds through palladium-mediated cysteine *S*-arylation (via ligand exchange followed by reductive elimination) and subsequent intramolecular lysine *N*-acylation (via nucleophilic addition to the carbamate electrophile and phenol elimination), was first optimized using a dodecapeptide model incorporating an *i* → *i*+5-spaced Lys–Cys residue pair and several additional nucleophilic residues. The stapling reaction was carried out using 1.1 equivalents (equiv.) of palladium reagent I, in the presence of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 8.5, in a 19:1 mixture of water and dimethylsulfoxide (DMSO). After 24 h at 37 °C, the desired Lys–Cys cross-linked peptide was obtained in 94% yield as determined by high-performance liquid chromatography (HPLC), with only minor hydrolysis of the *O*-phenyl carbamate moiety.



Scheme 6. Solution-phase Cys–Lys stapling via (a) palladium oxidative addition complexes, (b) *ortho*-phthalaldehyde derivatives, (c) hypervalent iodine reagents, (d) *N*-hydroxysuccinimide-activated acrylamide derivatives, and (e) malealdehyde.

The stapling procedure was then extended to peptides with various spacings between the lysine and cysteine residues (from $i \rightarrow i+1$ to $i \rightarrow i+11$), and excellent stapling yields were again obtained. The stability of the installed staple, featuring thioether and urea linkages, was confirmed by subjecting the $i \rightarrow i+5$ stapled peptide mentioned above to basic, acidic, and oxidative conditions, as well as to glutathione (GSH) as an external thiol nucleophile. To probe the site-selectivity of this stapling approach, it was subsequently applied to Cys–Lys cross-linking within engineered sortase A, a protein containing one cysteine and 18 lysine residues. Interestingly, treatment of this protein with **I** under the optimized conditions yielded two cross-linked species, displaying either a Lys(162)–Cys(184) or a Cys(184)–Lys(198) connectivity, Lys(162) and Lys(198) being the lysine residues closest to the cysteine residue in the protein sequence.^[132]

Although this method is high-yielding and chemically original, it suffers from several limitations. It requires the preparation of organometallic reagent **I** from expensive materials and its use in stoichiometric amounts. Moreover, the stapling reactions were carried out at high dilution of peptides (10 μ M), which limits their applicability on a preparative scale. The tolerance of the method toward methionine, whose thioether side chain is known to bind to Pd(II) species,^[133] was not evaluated. Finally, only stapling reagent **I**, which enables installation of a *p*-phenylene-based linker, was investigated, thereby restricting the structural diversity accessible through this stapling approach.

1.4.2.2.3.4.2.2. *Ortho*-Phthalaldehyde Derivatives

Besides the use of bifunctional reagents combining two independent thiol- and amine-reactive groups separated by a spacer, such as the cross-linking reagent **I** described above, Cys–Lys stapling can in principle be achieved through generation of a first reactive intermediate via reaction of one side chain with an external stapling reagent, followed by trapping of this intermediate by the second side chain. Such a strategy was first reported in 2019, when the groups of Perrin^[134] and Li^[135] independently described the use of *ortho*-phthalaldehyde (OPA) for Cys–Lys cross-linking (Scheme 6b). After imine formation between the lysine ϵ -amine and one aldehyde group

of OPA, the mechanism was proposed to proceed through nucleophilic attack of the cysteine β -thiol on the imine carbon and cyclization involving the nitrogen atom and the second aldehyde group, followed by aromatization via dehydration to form a fluorescent isoindole. The stapling process proved efficient using 1.2 or 1.5 equiv. of OPA in aqueous buffers at pH values from 7.4 to 10.0, yielding the desired macrocycle in 30 min or less.

This stapling methodology enabled the synthesis of diverse stapled peptides from unprotected precursors featuring Cys–Lys residue pairs with both orientations and various spacings. Interestingly, OPA-mediated macrocyclization also proved successful for stapling peptides incorporating ornithine, diaminobutyrate (Dab), or diaminopropionate (Dap) residues instead of lysine, as well as for N-terminus-to-cysteine cross-linking. OPA-mediated bicyclization of Cys–Lys-containing cyclic peptides was also achieved. Furthermore, the structure of the isoindole-based staple was effectively modulated through the use of 3- or 4-substituted OPA analogues, although this frequently resulted in the formation of two regioisomeric stapled peptides.^[134,135]

Beyond the fact that OPA is commercially available, that it enables rapid and chemoselective Cys–Lys stapling under physiological conditions, and that structural variation of the installed linker is possible, this stapling strategy offers additional advantages. Indeed, owing to the isoindole-based linker formed (whose photophysical properties can be tuned through the choice and position of an optional substituent), peptides stapled via OPA-mediated Cys–Lys stapling can directly serve as fluorescent probes, without the need for post-stapling modification (which may in some cases alter peptide bioactivity).^[134] Nevertheless, the nucleophilicity of the isoindole cross-link also allows post-stapling derivatization, as demonstrated by Li and coworkers using dimethyl acetylenedicarboxylate (DMAD) and maleimide derivatives as electrophiles. Interestingly, efficient stapling and subsequent post-stapling modification could be performed in a one-pot fashion, with fast reaction rates for both steps.^[135]

The main drawback of this stapling approach lies in the relative instability of the isoindole linker, which was shown to be sensitive to oxidation. This was evidenced in aqueous solutions by the formation,

over time, of oxidized products such as phthalimide and 3-alkylthio-substituted phthalimidine derivatives.^[134,135]

1.4.2.2.3.4.2.3. Hypervalent Iodine Reagents

In 2013, Waser and coworkers demonstrated that hypervalent iodine compounds derived from 1-ethynyl-1,2-benziodoxol-3(1*H*)-one (EBX) are powerful reagents for thiol alkynylation.^[136] These electrophilic alkyne transfer reagents were shown to promote *S*-alkynylation of various thiols, notably cysteine thiols within peptides and proteins.^[136-138] Subsequently, in 2021, Heinis, Waser, and coworkers reported original Cys–Cys and Cys–Lys stapling procedures based on the use of EBX-derived hypervalent iodine reagents.

The bifunctional Cys–Lys cross-linking reagents (**IIIa–c**) were designed by combining an EBX moiety and a pentafluorophenyl (PFP) ester as the thiol- and amine-reactive groups, respectively, separated by an *o*-, *m*-, or *p*-phenylene spacer (Scheme 6c). Linear peptides incorporating cysteine and lysine residues at various relative positions, as well as additional nucleophilic side chains, were individually treated with 1.1 equiv. of each stapling reagent in DMF in the presence of *N,N*-diisopropylethylamine (DIPEA) as a base. This afforded the corresponding stapled products after 30 min at 37 °C. These products were generally obtained in satisfactory yields, although cyclic peptides featuring an *ortho*-disubstituted linker could not be isolated. Kinetic experiments showed that the cysteine *S*-alkynylation occurs first, followed by proximity-driven *N*-acylation of the lysine side chain.

A Cys–Lys cross-linking reagent carrying an additional pentafluorophenyl ester group (**IIId**) was then employed. Following cysteine-to-lysine macrocyclization of an appropriate linear substrate, the remaining ester group was successfully engaged in intramolecular or intermolecular amidation, enabling bicyclization or post-stapling modification, respectively. Remarkably, post-stapling modification was also possible by exploiting the alkyne moiety of the linker, which could be functionalized through ruthenium-catalyzed azide–thioalkyne cycloaddition (RuAtAC). In this context, a one-pot stapling–RuAtAC protocol was developed, enabling rapid access to variously derivatized macrocyclic peptides.^[80]

Overall, this chemoselective and efficient Cys–Lys stapling strategy holds considerable potential for generating structural diversity, either through variation of the geometry of the installed linker or through diverse post-stapling modification pathways. A limitation, however, lies in the need to prepare the stapling reagents through multi-step synthesis. This methodology may also raise sustainability issues, given the low atom economy of the stapling process and the use of DMF as a solvent, which is associated with toxicity and environmental concerns.

1.4.2.2.3.4.2.4. *N*-Hydroxysuccinimide-Activated Acrylamide Derivatives

Another major advance in the field of thiol-to-amine peptide cyclization was reported in 2021 by Gois and coworkers. Based on the high reactivity of carbonylacrylic Michael acceptors toward cysteine thiols, previously exploited for cysteine-selective protein bioconjugation,^[139] and on the well-known ability of *N*-hydroxysuccinimide (NHS) esters to promote acylation of proteinogenic amines, they developed bifunctional acrylic derivatives incorporating an *N*-hydroxysuccinimide (NHS) ester and a secondary or tertiary amide at opposite ends of the olefin. These reagents were first applied to functionalization of N-terminal cysteine residues, generating a 1,4-thiazepan-5-one moiety with very fast reaction rates, through thia-Michael addition (at the β -carbon atom of the NHS ester) followed by intramolecular *N*-acylation. The possibility of performing Cys–Lys stapling was then investigated, using NHS-activated acrylamide derivative **IV** as the cross-linking reagent (Scheme 6d). After pretreatment with TCEP, stapling of three linear model peptides (containing an $i \rightarrow i+2$ -, $i \rightarrow i+3$ -, or $i \rightarrow i+4$ -spaced Lys–Cys residue pair) using 1.2 equiv. of **IV** proved highly efficient in aqueous buffer at pH 7.0, affording the cyclic compounds in excellent HPLC yields ($\geq 88\%$) after 5 min at room temperature.

Interestingly, stapling of a 32-mer peptide featuring a C-terminal cysteine residue and 8 lysine residues (notably at the $i-1$ and $i-2$ positions relative to the cysteine) yielded the $i \rightarrow i+1$ Lys–Cys stapled peptide in 89% yield, highlighting the remarkable site-selectivity of the procedure. Although the potential for post-stapling modification was not

examined, structural diversification of the linker could be achieved using NHS-activated acrylamides bearing valuable substituents at the amide nitrogen. Notably, a stapling reagent prefunctionalized with a fluorescent dansyl moiety was successfully employed to modify bovine serum albumin (BSA), a protein featuring a single free thiol and 59 lysine residues. Again, a high degree of site-selectivity was observed, as the cyclic species resulting from cross-linking of the free cysteine (position 34) with a single lysine residue (position 136) was the major product formed.^[140]

In summary, this stapling method proved advantageous in several aspects: rapidity, compatibility with physiological conditions, and high chemoselectivity and site-selectivity. Structural variability was introduced through variation of the amide substituents, and the corresponding stapling reagents were readily accessible from a synthetic standpoint. However, two important limitations should be noted regarding the isolation of Cys–Lys stapled peptides produced via this approach.

First, nucleophilic attack of the thiol on the Michael acceptor generates a new stereocenter, and two diastereomers of the cyclic peptide can therefore be obtained, as observed during stapling of the peptide models in this study. This may be undesirable in certain cases, particularly when isolation of the individual stereoisomers is difficult or impractical, thereby complicating evaluation of their respective structural or biological properties.

Second, although reactions performed on an analytical scale (at a peptide concentration of 10 μM) proved high-yielding, increasing the peptide concentration to 250 μM under preparative conditions led to the formation of polymeric peptide species. As suggested by the authors, this may account for the low isolated yields obtained (7–35%) compared to those achieved at the analytical scale ($\geq 88\%$). Therefore, a very high dilution appears necessary to obtain the desired macrocyclic peptide in good yield, which limits the applicability of this approach at the preparative scale.

1.4.2.2.3.4.2.5. Malealdehyde

In 2023, Raj and coworkers reported that multicomponent processes involving malealdehyde (**V**), a thiol, and an amine could be applied to Cys–Lys stapling of unprotected peptides. Mechanistically, they proposed that the dialdehyde **V**, generated *in situ* from *N*-bromosuccinimide (NBS)-mediated oxidation of furan, first undergoes thia–Michael addition, followed by imine formation between the amine component and the aldehyde group distal to the newly formed thioether. Subsequent cyclization involving the nitrogen atom and the second aldehyde group, followed by dehydration and aromatization, leads to formation of an *N*-substituted-3-(alkylthio)pyrrole heterocycle.

After demonstrating that this multicomponent reaction (MCR) was chemoselective toward cysteine and lysine residues, using linear peptides incorporating either a lysine or a cysteine residue together with various other nucleophilic amino acids, the macrocyclization of three linear precursors featuring different Cys–Lys spacings ($i \rightarrow i+2$, $i \rightarrow i+4$, or $i \rightarrow i+7$) was investigated (Scheme 6e). In practice, these peptides, dissolved in a 5:1 mixture of acetonitrile and water, were treated with freshly prepared malealdehyde (1.2 equiv.). After stirring for 16 h at room temperature, moderate to high conversions of the linear precursors to the corresponding Cys–Lys stapled peptides were observed. Notably, the reaction profile of the $i \rightarrow i+7$ macrocyclization reaction proved less clean, with the lowest conversion to the target macrocycle (78%) as determined by HPLC analysis. Stapling of a heptapeptide incorporating two lysine residues and one cysteine residue (at positions 1, 4, and 7, respectively) did not appear site-selective, since both $i \rightarrow i+3$ and $i \rightarrow i+6$ Lys–Cys stapled products were formed in comparable proportions based on HPLC analysis.

The developed multicomponent strategy was further extensively applied to protein modification. Importantly, the stability of the installed pyrrole moiety under basic and acidic conditions was demonstrated through stability studies conducted on pyrrole-modified myoglobin.^[48]

Taken together, although the scope of the reported methodology remains only partially explored for Cys–Lys stapling of short peptides, the malealdehyde-mediated Cys–Lys cross-linking approach appears highly attractive owing to its chemoselectivity and operational simplicity. In particular, the stapling reagent can be rapidly generated

from an inexpensive starting material, namely furan, and directly employed for stapling in a one-pot manner. However, preparation of a fresh malealdehyde solution prior to stapling must be performed in the absence of the linear peptide to avoid oxidation of its cysteine residue.

In light of the above findings, achieving high site-selectivity in Cys–Lys stapling of peptides featuring multiple lysine residues does not appear feasible via this approach. Furthermore, structural diversification of the pyrrole-bridged macrocyclic peptides was not investigated. However, such diversification should be attainable through the use of various substituted furans or through post-stapling modification with DMAD, as previously reported for the functionalization of isoindole-bridged macrocycles.^[135] Indeed, the aldehyde generated from 2-methylfuran was shown to react efficiently with 1-butanethiol and 1-butylamine to yield the corresponding trisubstituted pyrrole, and functionalization at the C2 position of 1-butyl-3-(butylthio)-1*H*-pyrrole using DMAD was successfully achieved.^[48]

1.4.2.2.3.4.3. Effect of Cys–Lys Stapling on Helicity

The five Cys–Lys stapling approaches described above enable installation of linkers with lengths of 1 to 7 atoms and varying rigidity, resulting in total staple lengths ranging from 8 to 14 atoms. However, owing to the high flexibility of the lysine side chain, one might expect that, for a given staple length, Cys–Lys stapling strategies might generally be less effective than Cys–Cys approaches at reducing the conformational flexibility of a peptide and promoting its α -helical conformation. Nevertheless, data on the helical propensity of Cys–Lys stapled peptides remain scarce, precluding the establishment of clear structure–helicity relationships and a direct comparison between Cys–Lys and Cys–Cys stapling strategies.

Heinis, Waser, and coworkers analyzed by CD several $i \rightarrow i+4$ and $i \rightarrow i+7$ stapled Cys–Lys peptides in which the anchoring cysteine and lysine residues were connected through a 6- or 7-atom-long linker (composed of ethynylene, *m*- or *p*-phenylene, and carbonyl units), resulting in total staple lengths of 13 or 14 atoms. None of the four $i \rightarrow i+4$ stapled peptides investigated, featuring either an *m*- or *p*-phenylene spacer, exhibited increased helicity relative to their linear

precursors. Conversely, installation of the same staples in an $i \rightarrow i+7$ arrangement on a p53-derived 16-mer peptide resulted in increased helical propensity, suggesting that these relatively long staples may be more helix-stabilizing when spanning two putative α -helical turns. The highest enhancement of α -helicity was obtained with the *m*-disubstituted linker. Interestingly, both $i \rightarrow i+7$ cross-linked peptides displayed increased binding affinity toward MDM2 compared to the corresponding linear peptide.^[80]

To the best of our knowledge, no additional CD or NMR data are currently available documenting the helicity of $i \rightarrow i+4$ or $i \rightarrow i+7$ Cys–Lys stapled peptides. Further studies will therefore be required to identify the optimal linker length, rigidity, and overall geometry for stabilizing one or two α -helical turns. In particular, it would be of interest to evaluate the helical propensity of $i \rightarrow i+4$ Cys–Lys cross-linked peptides obtained using OPA, malealdehyde, or NHS-activated acrylamides as stapling reagents, since the relatively short staples installed by these approaches (8, 9, or 10 atoms in length, respectively) may be better suited than longer staples to promote α -helicity in an $i \rightarrow i+4$ cross-linking context.

Brunel and Dawson reported that $i \rightarrow i+4$ Cys–Orn stapled peptides, prepared on resin and featuring a methylenecarbonyl linker between the cysteine β -sulfur atom and the ornithine δ -nitrogen atom, exhibited increased α -helicity relative to their unconstrained analogues.^[129] The use of lysine surrogates with shorter alkylamine side chains (Orn, Dab, or Dap) in place of lysine may therefore represent an additional strategy to consider when designing stapled α -helical peptides via thiol-to-amine macrocyclization.

1.4.2.2.3.4.4. Challenges and Opportunities in Cys–Lys Stapling

The five solution-phase Cys–Lys stapling methodologies presented above (see Section 1.4.2.2.3.4.2) all enable straightforward access to macrocyclic peptides featuring non-symmetrical linkers from unprotected precursors. However, they are associated with several limitations, as discussed in the respective sections, which concern operational simplicity, sustainability, scalability, stability of the resulting

macrocycles, or opportunities for structural diversification. Additionally, the macrocyclic peptides obtained through these procedures all feature a lysine ϵ -nitrogen atom that is either acylated or incorporated into an isoindole or pyrrole ring, resulting in a loss of basicity and nucleophilicity. As a consequence, the positive charge of the lysine side chain at physiological pH is abolished, leading to significant changes in the peptide's physicochemical properties and potentially deleterious effects on its biological activity. Furthermore, the lysine side chain can no longer engage in reactions with external electrophiles, thereby eliminating a convenient route to post-stapling modification.

Beyond their respective limitations, and although some of these methodologies allow substantial structural variation, the limited number of available Cys–Lys stapling strategies currently restricts the diversity of linkers that can be installed in this context. Consequently, expanding the chemical toolbox for Cys–Lys stapling is essential. The development of chemically original methodologies would broaden the range of accessible linkers and, in combination with the approaches already reported, enable optimization of the physicochemical and biological properties of Cys–Lys stapled peptides. Moreover, the introduction of additional stapling strategies would generate valuable fundamental insights into the structural determinants governing the helicity of peptides constrained through cysteine and lysine residues, an area that remains insufficiently explored to date.

Overall, original Cys–Lys stapling methods are required to complement the existing strategies.

1.4.2.2.3.5. Lys–Lys Stapling

The use of two lysine residues as anchoring sites for two-component stapling is also feasible, and several solution-phase Lys–Lys cross-linking methodologies have been reported. These methods are associated with limitations similar to those of Cys–Cys stapling in terms of linker symmetry. Indeed, they are either restricted to the installation of symmetrical linkers, as is the case for most of the methodologies developed to date,^[104,141-143] or the use of a non-symmetrical stapling reagent results in the formation of two constitutional isomers of the stapled product.^[144]

From a synthetic standpoint, Lys–Lys stapling methodologies are less diverse than their Cys–Cys counterparts and have, to date, been far less widely employed to optimize the properties of bioactive peptides.

Some Lys–Lys stapling methodologies have been achieved using classes of stapling reagents previously exploited for Cys–Cys stapling, notably arylating agents (such as perfluoroaromatic- and dichlorotriazine-based reagents^[141] and organometallic palladium complexes^[142]) and 1,4-dinitroimidazoles.^[104] In these cases, Lys–Lys stapling necessarily required the absence of free thiols. It also sometimes involved modified reaction conditions compared to the corresponding Cys–Cys protocols (including the use of DMSO as the solvent,^[104,142] the use of a base such as sodium phenoxide^[142] or DIPEA^[104], higher temperatures,^[141] and/or longer reaction times^[104,141,142]), reflecting the lower nucleophilicity of the lysine ϵ -amine relative to the cysteine β -thiol in these contexts.

As mentioned previously, OPA is a convenient reagent that mediates efficient Cys–Lys stapling under physiological conditions. In the absence of cysteine, it was recently shown to also promote rapid Lys–Lys stapling via formation of an isoindolinimine-based linker. The stapling process was found to be efficient in 1:1 water/alcohol cosolvent mixtures, using either a buffer at pH 8.0 as the aqueous component of the solvent system or DIPEA as a base. However, this strategy resulted in the formation of two constitutional isomers of the isoindolinimine-bridged cyclic peptide, which could not be separated in most cases.^[144]

Moreover, although this methodology may be classified as either a two-component or a multicomponent stapling approach, reductive *N*-alkylation of two lysine side chains has been reported as a viable strategy for Lys–Lys stapling. Indeed, Bell and Malins showed that various macrocyclic peptides, notably lysine-to-lysine cyclized products, could be obtained upon treatment of linear peptide diamines with 2,6-pyridinedicarboxaldehyde (or 4-substituted derivatives thereof) in aqueous buffer at pH 9.0, using sodium cyanoborohydride (NaBH₃CN) as the hydride source. Unfortunately, cysteine residues were not tolerated under these conditions.^[143]

To the best of our knowledge, no Lys–Lys stapling protocol reported to date is compatible with the presence of free cysteine residues,

underscoring the progress that still needs to be made in the field of lysine-selective peptide modification and macrocyclization.

1.4.2.2.3.6. Multicomponent Reactions Applied to Two-Component Peptide Stapling

Beyond approaches involving the reaction between a peptide bearing two reactive residues and a bifunctional stapling reagent, other noteworthy two-component peptide stapling strategies proceed via three-component reactions, in which two reaction partners are provided by the linear peptide and the third is introduced by an external reagent that does not need to be bifunctional.

Stapling approaches mediated by three-component reactions enable the installation of non-symmetrical staples, thereby further increasing the structural diversity of macrocycles accessible through peptide stapling. From a synthetic standpoint, such procedures can be distinguished based on the order in which the three reactive functionalities engage with one another.

In the first scenario, the starting peptide bears two mutually reactive functional groups capable of reversibly forming, under appropriate conditions, a cyclic reactive intermediate that subsequently undergoes irreversible intermolecular reaction with the stapling reagent.

In the second scenario, reaction between one of the two reactive groups of the peptide and the external stapling reagent generates the reactive intermediate, which is then trapped intramolecularly by the second reactive group of the peptide. Examples of this second scenario include the OPA-^[135] and malealdehyde-mediated^[48] Cys–Lys stapling approaches described above.

Trapping of imine/iminium intermediates has been extensively applied in the field of peptide modification and cyclization.^[47,143,145-155] The reactivity of these intermediates has notably been exploited in two-component stapling strategies. In this context, peptides cyclized through intramolecular imine formation have been successfully trapped by external reagents such as NaBH₃CN^[145] and 2-formyl- and 2-ketopyridine derivatives.^[155] Furthermore, imine/iminium species formed intermolecularly between an amine-bearing side chain and

formaldehyde have been trapped intramolecularly by nucleophiles such as copper acetylides^[151] and phenol- and guanidine-bearing side chains (from tyrosine and arginine residues, respectively).^[150]

1.4.2.2.4. Double-Stapled, Stitched, and Other Bicyclic Peptides

As compared with monocyclic peptides, bicyclic peptides may exhibit further reduced conformational flexibility, which can be advantageous for the design of secondary structure mimics.

Peptides bearing two staples on different $i \rightarrow i+4$ -spaced residue pairs can show enhanced α -helical propensity relative to their single-stapled analogues.^[19,58,70,156-158] Such double-stapled peptides have been synthesized through RCM,^[19,58,156-158] macrolactamization,^[58,156-158] CuAAC,^[70] or Cys–Cys stapling,^[19] using either the same^[58,70,156,158] or different^[19,58,156-158] chemistries for the two staples. In certain cases, introduction of the second staple led to improved binding affinity^[156,158] and/or increased proteolytic resistance.^[58,157,158]

Another class of interesting bicyclic peptides consists of spirocyclic peptides featuring two contiguous hydrocarbon staples.^[159-161] These “stitched” peptides were introduced by Verdine and coworkers in 2014. They can be prepared through on-resin bis-RCM involving a central α,α -bis(alkenyl)-substituted residue, namely 2,2-bis(4'-pentenyl)-glycine (B5), and two α -alkenylalanine residues (with either *S*- or *R*-configuration) positioned at different sites relative to B5. A study performed on 15-mer peptides revealed that the combination of S5, B5 and (*S*)-2-(7'-octenyl)alanine (S8) at positions i , $i+4$, and $i+11$, respectively, afforded, upon bis-RCM, the highest α -helicity among the combinations tested. The $i+4+7$ (S5–B5–S8) stitched peptide notably displayed higher helicity than both the corresponding $i \rightarrow i+7$ (R5–S8) single-stapled analogue and the unmodified linear peptide (with measured helical contents of 86%, 60% and 24%, respectively), as well as increased conformational stability.^[159] Beyond enhanced α -helical stabilization relative to stapling, stitching can also provide greater improvements in proteolytic resistance^[159,160] and cellular uptake.^[159]

Although they are not performed with the aim of enhancing peptide helicity, other bicyclization strategies, which also result in increased conformational rigidity, are worth mentioning. Notably, head-to-tail cyclic peptides (obtained through a prior cyclization step) can undergo bicyclization via side-chain-to-side-chain cross-linking.^[103,134,135] In addition, linear peptides can be converted into bicyclic structures using an external trifunctional cross-linking reagent, either in a single step^[90,162-165] or through multi-step sequences.^[78,166] In this context, trifunctional reagents based on a 1,3,5-trisubstituted benzene core have been widely employed.^[86,90,162-164,166] Such bicyclization strategies may present several advantages in medicinal chemistry. From a synthetic standpoint, they can enable the generation of structurally diverse bicyclic peptide libraries through variation of the cross-linking reagent.^[163] From a biological perspective, bicyclization may confer improved metabolic stability.^[164,166] Bicyclic peptides have been proposed as potential mimics of discontinuous protein epitopes or pairs of protein loops.^[78,90,162] Moreover, incorporation of a planar 1,3,5-trisubstituted benzene scaffold may constrain the resulting peptides to adopt an overall planar geometry, thereby facilitating interactions with flat protein surfaces.^[166]

2. Objectives

This thesis focused on peptide stapling, which represents a well-established strategy for increasing the helical propensity of short peptides.

Thanks to the high nucleophilicity of cysteine residues and their low abundance within peptides and proteins, Cys–Cys stapling is by far the most widely used stapling approach. Nevertheless, such a strategy typically requires the use of symmetrical cross-linking reagents, which to some extent limits the chemical diversity of the resulting linkers. In contrast, Cys–Lys stapling enables the installation of non-symmetrical linchpins, thereby further increasing the degree of structural diversity that can be achieved. However, as explained previously (see Section 1.4.2.2.3.4.4), the number of chemical methodologies enabling one-step Cys–Lys stapling of unprotected peptides in solution remains limited. Moreover, the procedures reported to date suffer from several drawbacks, such as the need to synthesize the stapling reagent from expensive materials^[132] or through multi-step organic synthesis,^[80] the poor atom economy of the stapling process,^[80,132] the high dilution needed to avoid formation of polymers (limiting applicability on a preparative scale),^[140] or the sensitivity of isoindole-based linkers to oxidation.^[134,135] Additionally, in all these stapling processes, the lysine ϵ -amine is either acylated^[80,132,140] or incorporated into an isoindole^[134,135] or pyrrole^[48] heterocycle, which effectively suppresses its basic and nucleophilic character. These limitations highlight the lack of a Cys–Lys cross-linking strategy combining operational simplicity with preservation of the lysine side-chain's basicity and nucleophilicity.

As a consequence, new thiol-to-amine macrocyclization methods based on alternative chemistries are needed to expand the Cys–Lys stapling toolbox. This work therefore aimed to develop a new Cys–Lys stapling methodology that could complement current approaches by addressing the aforementioned drawbacks.

3. Thesis Outline

This thesis manuscript is organized into two main chapters. The first chapter served as a training chapter, with the aim of gaining the expertise required to address the main objective of the thesis as effectively as possible, which is the subject of the second chapter.

Among other research activities, the laboratory of Raphaël Frédérick is involved in the development of compounds (small molecules or peptides) capable of competitively inhibiting the tetramerization of lactate dehydrogenase. In this context, the group recently showed that macrocyclization of peptides derived from the tetramerization arm of this enzyme could increase their affinity for the tetramerization site. As this improvement in affinity may be related to increased helicity,^[67] further enhancing the helical propensity of these peptides could lead to an additional increase in binding affinity. Thus, in the first chapter of this thesis, several macrocyclization procedures previously reported in the literature for Cys–Cys and CuAAC stapling were applied to lactate dehydrogenase-derived peptides, with the aim of identifying macrocyclic peptides displaying a high helical propensity. This work enabled familiarization with solution-phase peptide chemistry at both analytical and preparative scales, as well as with the associated techniques and instruments (analytical and semi-preparative HPLC, microbalance, lyophilizer, tandem mass spectrometry, and circular dichroism).

The second chapter describes the development of an original thiol-to-amine stapling methodology based on sequential *S*-alkylation and reductive *N*-alkylation. The experience gained during the experiments described in the first chapter proved instrumental in shaping this second part in a more rigorous manner. In contrast to the first chapter, which relied on macrocyclization protocols previously described in the literature, the second chapter, focused on methodology development, required determination of the HPLC yields of the stapling reactions and estimation of isolated yields when these reactions were also performed on a preparative scale.

Finally, this thesis discusses the various perspectives that may be envisioned based on the results obtained in both chapters.

4. Application of Cys–Cys and CuAAC-Based Stapling Approaches to Lactate Dehydrogenase-Derived Peptides (Chapter 1)

4.1. Lactate Dehydrogenase 1 as a Promising Anticancer Target

Lactate dehydrogenases (LDHs) are enzymes that catalyze the interconversion of pyruvate and lactate. Functional LDHs are homo- or heterotetrameric enzymes mainly formed by the assembly of LDH-H and/or LDH-M subunits, which are encoded by the LDHB and LDHA genes, respectively. The association of LDH-H and LDH-M subunits gives rise to 5 isozymes (LDH-1 to LDH-5), which differ in their subunit composition. LDH-1 is composed of four LDH-H subunits. It preferentially catalyzes the oxidation of lactate to pyruvate, more precisely the conversion of lactate and NAD^+ into pyruvate, NADH, and H^+ . In contrast, LDH-5, which is composed of four LDH-M subunits, preferentially reduces pyruvate to lactate.^[67,167,168]

LDH-1 plays a key role in the cancer cell survival and proliferation. This enzyme is notably involved in the metabolic cooperation between oxidative cancer cells, located close to tumor-feeding blood vessels, and distant hypoxic cancer cells (“metabolic symbiosis”).

Following glycolysis and pyruvate reduction, the lactate produced in hypoxic cancer cells can be exported and diffuse along its concentration gradient toward blood vessels. It can then be internalized by oxygenated cancer cells and converted into pyruvate through LDH-1-catalyzed oxidation. The pyruvate produced in these cells fuels the tricarboxylic acid cycle, thereby generating energy. As these oxidative cells preferentially use lactate over glucose as an energy substrate, glucose is allowed to diffuse into the hypoxic tumor compartment. This enables glucose to be used by hypoxic cancer cells to sustain anaerobic glycolysis. Energy production in hypoxic cells therefore depends on lactate oxidation in oxidative cells.^[169,170]

As previously mentioned, lactate oxidation by LDH-1 generates protons. LDH-1 activity has been shown to be required for lysosomal acidification in both oxidative and glycolytic cancer cells. As lysosomal acidification is essential for autophagy, which enables cancer cells to

cope with nutrient starvation and recycle damaged cellular components, LDH-1 activity is critical for autophagy in cancer cells.^[170] In addition, the lactate-induced activation of the transcription factor HIF-1 in oxidative cancer cells, which results in pro-angiogenic activity, may also depend on the oxidation of lactate to pyruvate.^[171]

Overall, LDH-1 represents a highly compelling therapeutic target in cancer, as its inhibition would simultaneously suppress the use of lactate as an energy substrate by oxidative cancer cells, inhibit autophagy in both oxidative and glycolytic cancer cells, and impair lactate-driven pro-angiogenic signaling in oxidative cancer cells. Furthermore, LDH-H deficiency does not lead to a severe pathological phenotype in humans,^[172] suggesting that long-term pharmacological inhibition of LDH-1 may be associated with limited adverse effects.

4.2. Stapled Peptides as Potential Inhibitors of LDH-1 Tetramerization

The design of selective LDH-1 inhibitors is highly attractive. However, the active sites of LDH-M and LDH-H subunits display a high degree of structural similarity. Selective inhibition of the LDH-1 isozyme through a catalytic-site-directed approach therefore appears challenging.^[67,168] Moreover, the LDH catalytic site, which accommodates both the substrate and the NAD cofactor (NADH or NAD⁺), is poorly accessible, rich in cationic residues and highly polar. Consequently, inhibitors capable of interacting with this site with high affinity are often themselves polar, which can complicate their further optimization and can result in poor *in cellulo* and *in vivo* activity.^[67,168,173,174] In addition, the NAD cofactor utilized by LDH is also required by numerous other cellular enzymes, which are expected to display high structural homology in their NAD-binding site. As a result, inhibitors that interfere with NAD binding may lack selectivity for LDH over other NAD-dependent enzymes.^[67,168]

Instead of targeting the LDH catalytic site, Sonveaux, Frédérick, and coworkers explored an alternative inhibition strategy consisting of preventing the tetramerization of the enzyme. In 2020, they reported that a truncated variant of LDH-H lacking the 19 N-terminal residues (LDH-Htr) was a poorly active dimeric protein (Figure 6). Accordingly,

they focused on the design of compounds, notably peptides, capable of competing with the LDH N-terminal domain (also referred to as the N-terminal arm). Such compounds could competitively inhibit LDH tetramerization and disrupt preformed LDH tetramers, thereby preventing catalytic activity.

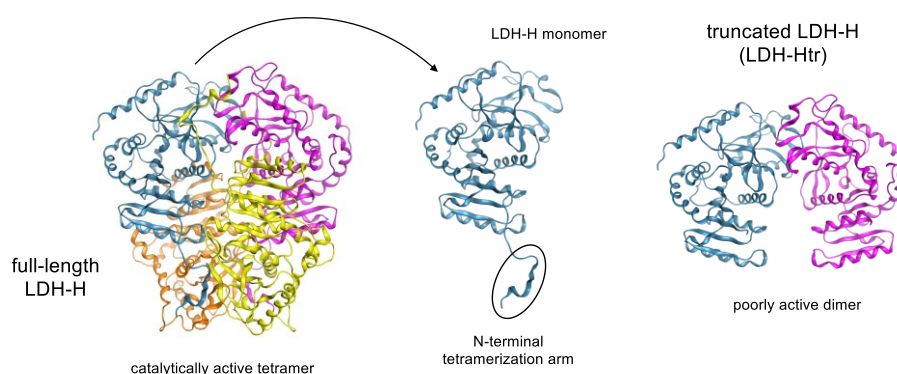


Figure 6. Catalytically active LDH-1 tetramer (composed of four LDH-H subunits) and dimeric model of LDH-1 (composed of two LDH-Htr subunits).

Molecular graphics were generated using the MOE software (Chemical Computing Group) based on the crystallographic structure of LDH-H (PDB entry 1I0Z).

The 19-residue N-terminal arm of each LDH monomer stabilizes the LDH tetramer through predominantly nonpolar contacts with a tetramerization site formed by two adjacent monomers (Figure 7). The N-terminal segment of this tetramerization arm consists of a short 8-residue α -helix. Frédéric and coworkers, in the search for potential LDH tetramer disruptors, first prepared a 19-mer peptide (**LB19**) corresponding to the full tetramerization arm. This peptide was shown to bind LDH-Htr, used as a dimeric model of LDH, with an equilibrium dissociation constant (K_d) of 1.78 mM, as determined by microscale thermophoresis (MST). The C-terminal end of **LB19** was subsequently progressively truncated in order to identify the shortest peptide capable of binding to the tetramerization site in LDH-Htr. This led to the octapeptide H-ATLKEKLI-NH₂ (**LB8**), whose sequence exactly matched that of the N-terminal α -helix of the tetramerization arm and

which exhibited a K_d of 1.05 mM for LDH-Htr. The lipophilic leucine residues at positions 3 and 7 (L3 and L7) were identified as key residues involved in the interactions with LDH-Htr.

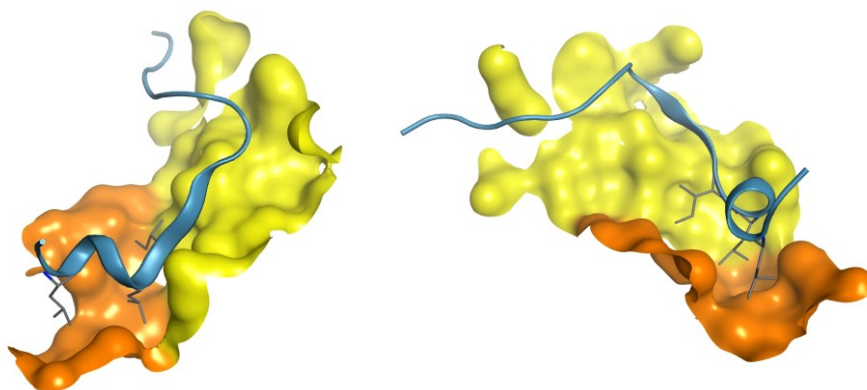


Figure 7. *In silico* model of the interaction between the 19-residue N-terminal tetramerization arm of one LDH-1 monomer (blue) and the tetramerization site formed by two adjacent monomers (orange and yellow), shown from two different viewpoints.

Side chains are omitted for clarity, except for those of residues L3, L7, and I8, which are buried in hydrophobic cavities. Molecular graphics were generated using the MOE software (Chemical Computing Group) based on the crystallographic structure of LDH-H (PDB entry 1I0Z).

Although encouraging, the affinity of **LB8** for the tetramerization site was still low. CD and NMR studies revealed that this peptide exhibited poor helical propensity in water, which may contribute to its weak binding potency. The authors therefore hypothesized that constraining the peptide conformation through macrocyclization could improve binding affinity. Accordingly, they notably performed Cys–Cys stapling after mutating the solvent-exposed alanine and glutamate residues at positions 1 and 5 (Figure 8). Among the stapling reagents tested, the use of α,α' -dibromo-*m*-xylene provided an $i \rightarrow i+4$ Cys–Cys stapled peptide (macrocycle **MC1**) with increased helicity relative to **LB8**, as evidenced by CD spectroscopy (Figure 9). Interestingly, this stapled peptide displayed a markedly improved binding affinity toward LDH-Htr, with a measured K_d of 64 μ M. In addition, an $i \rightarrow i+4$ stapled

peptide incorporating a tetrafluorophenylene linker (macrocycle **MCF**) exhibited an even greater improvement in binding affinity (K_d of 11 μM) and promising LDH tetramer-disrupting activity.^[67] However, the effect of the fluorinated linker on the peptide conformation is difficult to assess by CD.^[56]

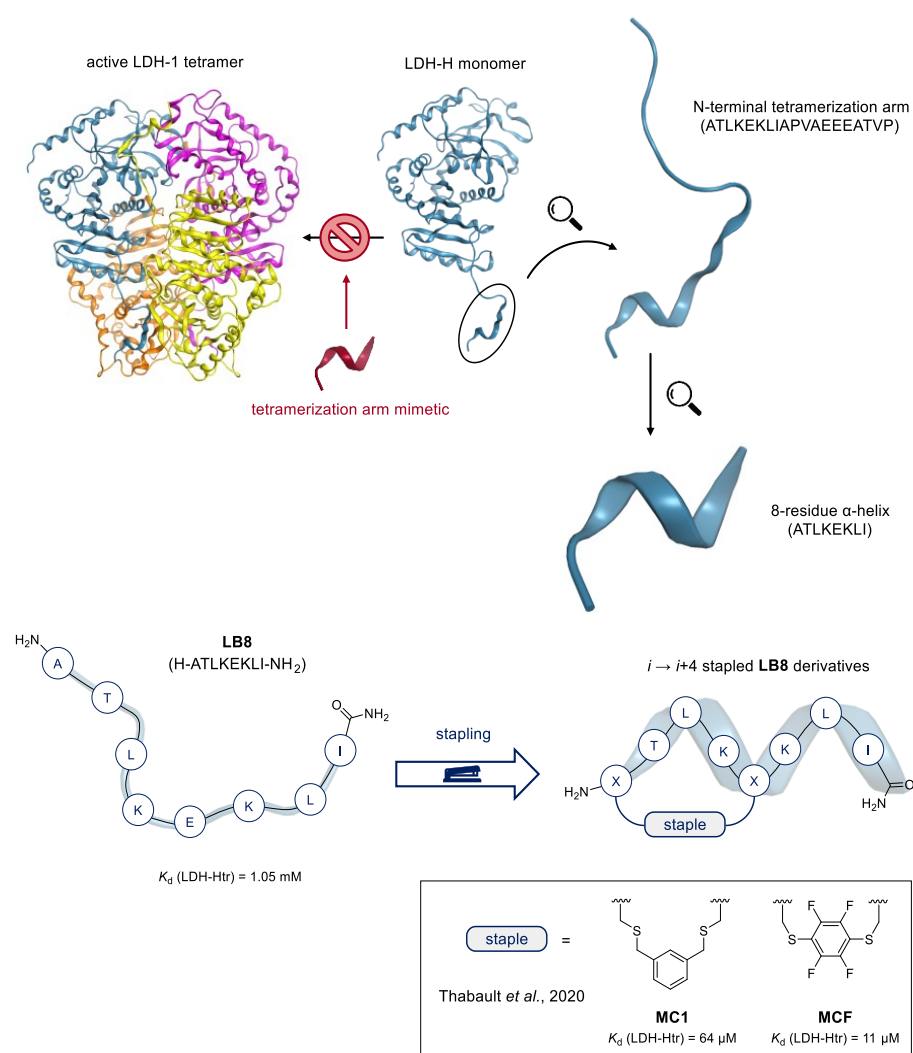


Figure 8. Design of stapled peptides mimicking the LDH-1 tetramerization arm.

These findings support the notion that increasing the helicity of LDH tetramerization arm derivatives could provide compounds with improved binding affinity for the tetramerization site. Since the enhancement in the helical content of macrocycle **MC1** compared to **LB8** was modest (particularly considering the slight increase in the CD signal intensity at 222 nm),^[67] the design of stapled peptides with higher helical content could translate into a further improvement in binding affinity. This may afford potent inhibitors of LDH-1 tetramerization. Therefore, in this work, various new stapled analogues of **LB8** were synthesized, and their helical propensity was examined.

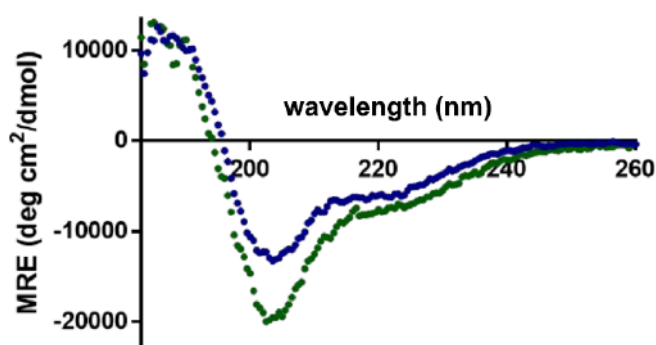


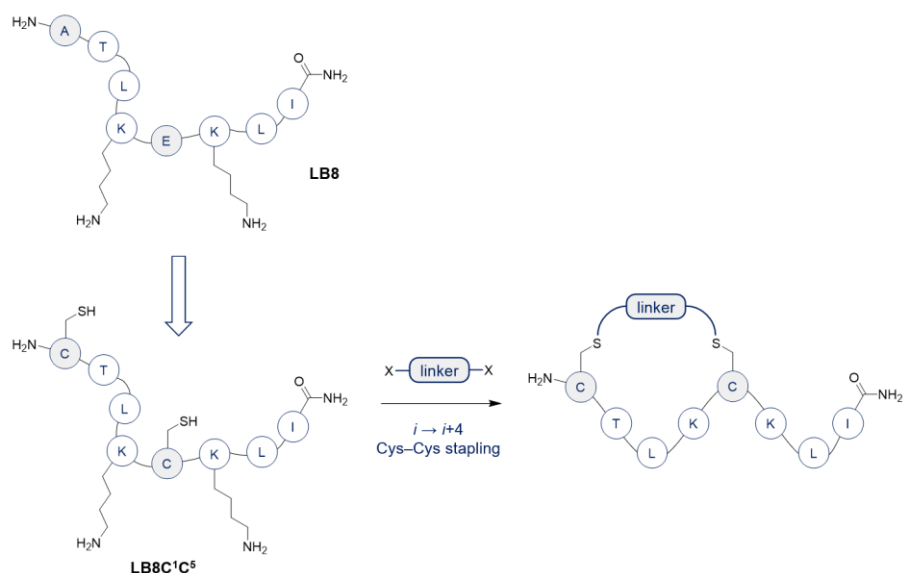
Figure 9. Circular dichroism spectra of **LB8** (blue) and **MC1** (green).

Reproduced from *J. Med. Chem. Commun.* **2020**, 63, 4628–4643.^[67] Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.6)/trifluoroethanol (4:1, v/v); T = 25 °C.

4.3. Cys–Cys Stapling of an LDH-Derived Octapeptide

In this work, the late-stage diversification enabled by Cys–Cys stapling approaches was exploited to access **LB8** derivatives with potentially increased α -helicity relative to the previously reported stapled peptide **MC1**. In other words, the linear peptide H-CTLKCKLI-NH₂ (**LB8C¹C⁵**), in which the residues at positions 1 and 5 of the tetramerization arm were replaced with cysteine residues, was macrocyclized using different stapling reagents reported in the literature, thereby providing several $i \rightarrow i+4$ stapled peptides from a single linear precursor (Scheme 7). As **LB8C¹C⁵** contains two lysine residues and a free N-

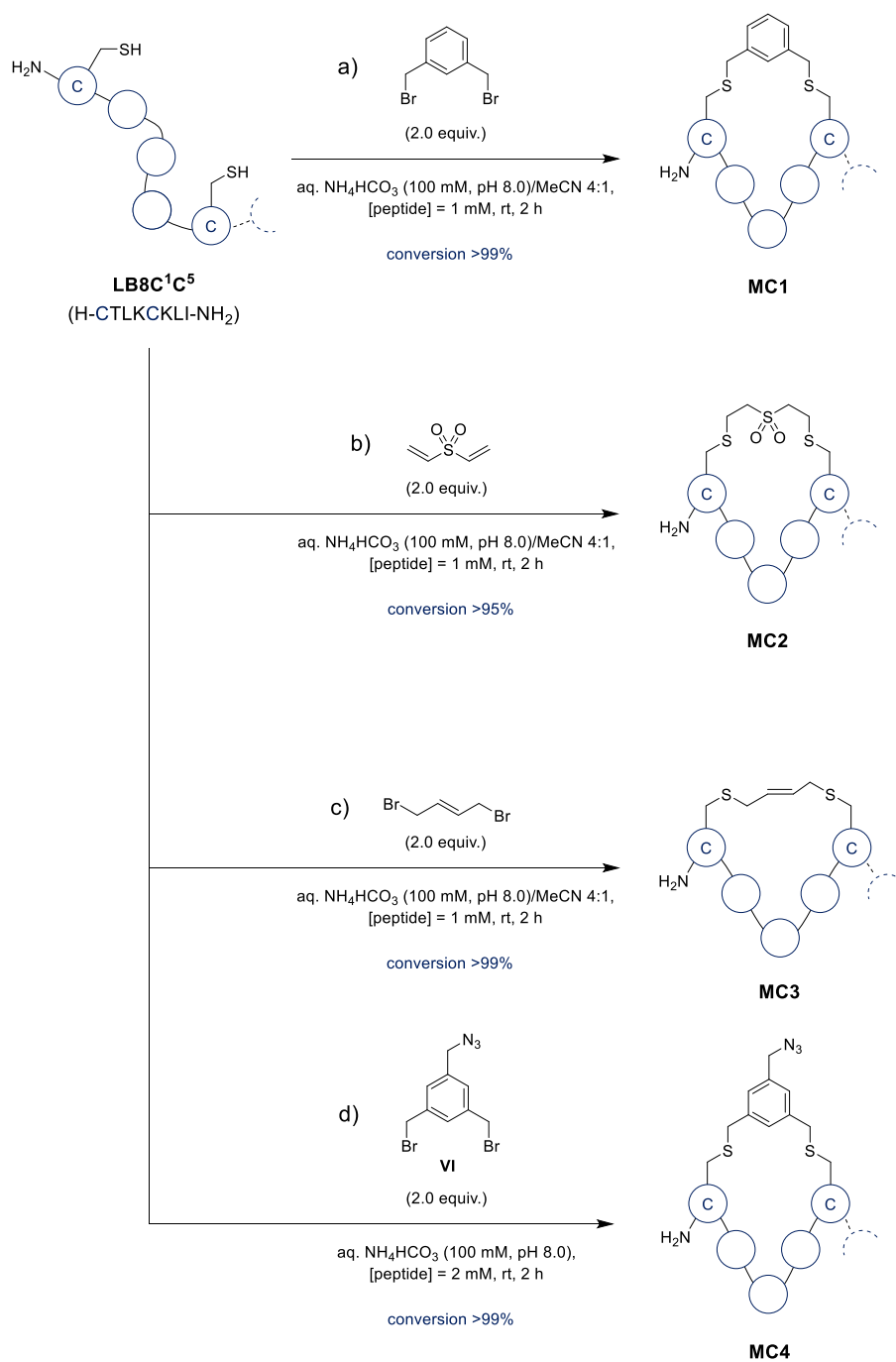
terminal α -amine, the chemoselectivity of the stapling procedures toward cysteine residues was crucial to obtain the target Cys–Cys cross-linked peptides in satisfactory amounts. Most Cys–Cys stapling reactions were carried out in slightly basic aqueous buffer (optionally with acetonitrile as a cosolvent), using an excess of cross-linking reagent, following procedures from the literature.^[67,75,78] In most cases, the use of TCEP as a reducing agent was not required, as intramolecular disulfide formation occurred only to a negligible extent.



Scheme 7. Design of Cys–Cys stapled peptides derived from the LDH-1 tetramerization arm.

X = thiol-reactive group.

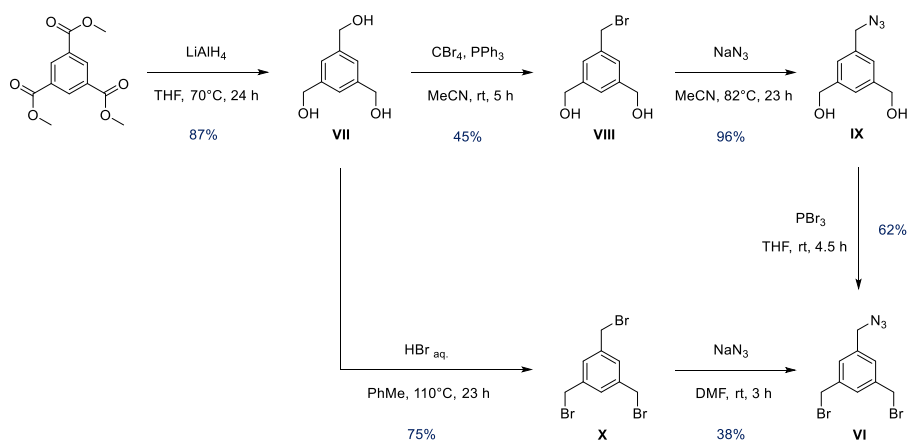
First, macrocycle **MC1**, featuring a 5-atom-long linker, was resynthesized using α,α' -dibromo-*m*-xylene as the stapling reagent (Scheme 8a). The installation of a more flexible linker was then achieved through the use of divinyl sulfone, yielding stapled peptide **MC2** (Scheme 8b). As we envisioned that shortening the linker length relative to the *m*-xylylene linker of **MC1**, while maintaining a certain degree of conformational rigidity, could have a positive impact on peptide helicity, Cys–Cys cross-linking mediated by (*E*)-1,4-dibromo-2-butene was subsequently carried out. This afforded **MC3**, featuring a rigid 4-atom-long (*E*)-2-butenylene linker (Scheme 8c). Of note, these three reactions proceeded very efficiently, with the desired stapled peptide being largely predominant in the corresponding crude mixture. As expected, **MC2** and **MC3** exhibited shorter retention times (t_R) than **MC1** in reversed-phase high-performance liquid chromatography (RP-HPLC), consistent with lower hydrophobicity (t_R = 7.4, 5.0, and 5.5 min for **MC1**, **MC2**, and **MC3**, respectively).



Scheme 8. Cys–Cys stapling of the LDH-derived octapeptide **LB8C¹C⁵**.

Next, the synthesis of a **MC1** derivative bearing a bioorthogonal handle, specifically an azide group, was investigated, as this could provide a convenient tool to introduce diverse payloads, such as fluorophores. Installing such a reactive handle on the linker of the stapled peptide could minimize the potential alteration of its binding affinity toward LDH-Htr, because the cysteine anchoring residues are expected to be solvent-exposed. Moreover, it would likely not result in a decrease in α -helicity compared to **MC1**. From a synthetic perspective, introducing an additional azide moiety on the linker of the macrocyclic peptide required the preparation, prior to stapling, of a symmetrical stapling reagent derived from α,α' -dibromo-*m*-xylene and featuring an azide handle in the *meta* position relative to the two thiol-reactive moieties. Such a reagent, namely compound **VI**, has already been described and used for Cys–Cys cross-linking (see Section 1.4.2.2.3.3.1, Table 1, entry 10).^[122,123]

Therefore, stapling reagent **VI** was prepared through multi-step organic synthesis (Scheme 9). Trimethyl 1,3-5-benzenetricarboxylate was first reduced with lithium aluminium hydride to afford triol **VII**. Compound **VII** was subjected to monobromination via an Appel reaction, and the resulting benzyl bromide derivative **VIII** then underwent nucleophilic substitution with sodium azide to give compound **IX**. Phosphorus tribromide-mediated bromination of the two remaining alcohol groups of **IX** finally yielded the desired stapling reagent **VI** in an overall yield of 23%. Alternatively, triol **VII** could be fully brominated in a biphasic mixture of aqueous hydrobromic acid and toluene, to give 1,3,5-tris(bromomethyl)benzene (**X**), whose substitution of one bromide leaving group with sodium azide led to reagent **VI** in an overall yield of 25%. Both synthetic pathways proved to be limited by one low-yielding step involving the conversion of only one among three identical functional groups. Nevertheless, the second pathway, based on tris-bromination of triol **VII**, was slightly more efficient and, above all, more straightforward (three steps instead of four).



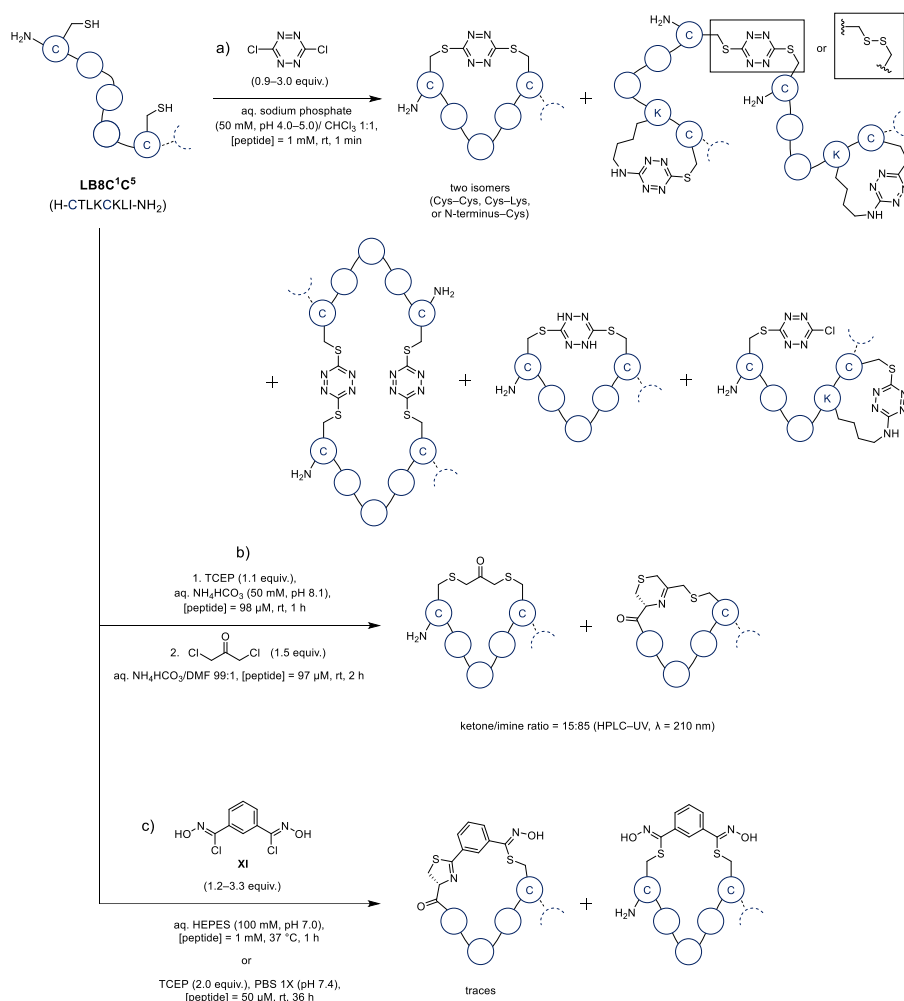
Scheme 9. Synthesis of azide-functionalized stapling reagent **VI**.

With reagent **VI** in hand, Cys–Cys stapling of **LB8C¹C⁵** was conducted, similarly to the synthesis of macrocycles **MC1–MC3**, to give stapled peptide **MC4** (Scheme 8d), whose linker is amenable to post-stapling modification.

Unfortunately, attempts to widen the library of **LB8**-derived monocyclic peptides through the use of other known Cys–Cys cross-linkers were not successful (Scheme 10). Notably, the use of 3,6-dichloro-1,2,4,5-tetrazine was investigated, as it could provide a short and rigid 4-atom-long tetrazine linker, potentially imposing a substantial restriction on conformational flexibility and enabling the installation of a more polar linker than the previously installed aromatic, sulfone-based, and alkenylene tethers. Moreover, such a tetrazine linker would be readily derivatizable through IEDDA. Thus, **LB8C¹C⁵** was reacted with dichlorotetrazine in 1:1 biphasic mixtures of sodium phosphate buffer and chloroform, based on the procedure reported by Brown and Smith (Scheme 10a).^[77] Nevertheless, despite extensive variation of the reaction conditions (from 3.0 to 0.9 equiv. of dichlorotetrazine; pH of the aqueous phase from 5.0 to 4.0; with or without quenching with L-cysteine), the desired monocyclic species (*m/z* 999) was either not formed or obtained as a mixture of two isomers. Various other byproducts were also formed, resulting in complex mixtures. The most commonly observed side-products were dimeric species (incorporating two peptide units and two or three tetrazine moieties; *m/z* 999 and

1038, respectively) and reduced (m/z 1001) and monochlorotetrazine-substituted (incorporating one peptide unit, two tetrazine moieties, and one chlorine atom; m/z 1113) monocyclic products. The major isomer featuring the target m/z value could be isolated by RP-HPLC, but reanalysis of the purified fractions after lyophilization revealed the presence of several peptidic impurities, suggesting that degradation of the desired cyclic peptide had taken place. Taken together, these findings suggested that cross-reactivity of dichlorotetrazine toward thiol and α - or ϵ -amines may have occurred. Therefore, the dichlorotetrazine-mediated Cys–Cys stapling approach may not be chemoselective enough to enable efficient thiol-to-thiol macrocyclization of **LB8C¹C⁵**, which represents a challenging substrate given that one cysteine residue is N-terminal and the second is located between two lysine residues.

Cys–Cys stapling of linear **LB8C¹C⁵** with dichloroacetone was also attempted with the aim of introducing a very short 3-atom-long linker, which would result in a total staple length of 7 atoms. Following the conditions reported by Dawson and coworkers,^[78] **LB8C¹C⁵** was first pretreated with TCEP in aqueous buffer at pH 8.1, before dichloroacetone was added (Scheme 10b). After stirring for 2 h, HPLC analysis revealed the presence of only a minor amount of the target acetone-bridged peptide (m/z 975) together with an abundant dehydrated derivative (m/z 957), in a 15:85 ratio based on their UV peak areas (λ = 210 nm). The dehydrated species presumably corresponded to a 6-membered cyclic imine resulting from 6-exo-trig addition of the N-terminal α -amine to the installed ketone. An attempt to purify this compound by RP-HPLC proved unsuccessful. This observation may indicate that the dichloroacetone-based macrocyclization approach, which has, to the best of our knowledge, only been applied to cross-linking of internal cysteine or homocysteine residues,^[75,78,103] may not be suitable for stapling peptides featuring an N-terminal cysteine.

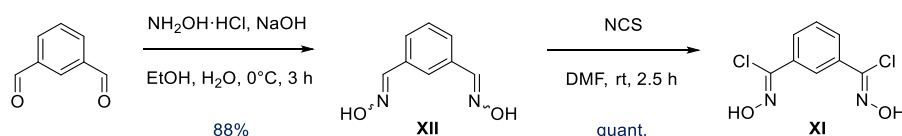


Scheme 10. Attempts at macrocyclization of **LB8C¹C⁵ using dichlorotetrazine, dichloroacetone, and bis-chlorooxime **XI**.**

The proposed structures of the byproducts are based on their *m/z* values (obtained by HPLC–MS analysis of the crude reaction mixtures). However, their connectivity was not elucidated, and constitutional isomers therefore cannot be excluded.

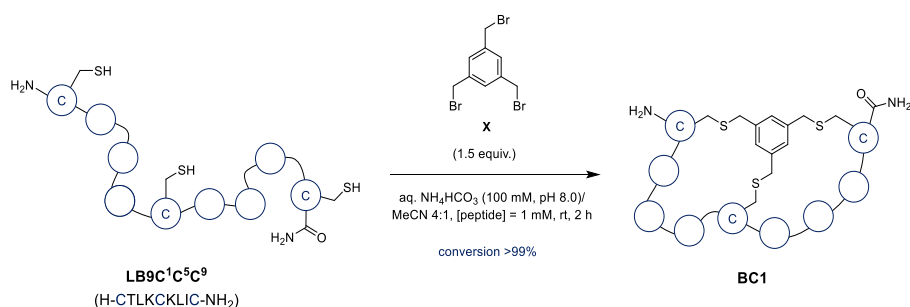
Chlorooximes have recently been reported to enable fast cysteine-selective modification, stapling, and bicyclization of peptides under physiological conditions. While reactions between chlorooximes and internal cysteine residues generate a thiohydroximate moiety, reactions involving N-terminal cysteines were shown to result in the formation of a 4,5-dihydrothiazole ring. Applied to stapling, bis-

chlorooximes therefore offer the possibility to prepare cyclic peptides with a non-symmetrical staple from linear peptide dithiols incorporating one N-terminal and one internal cysteine residues^[86,105] Bis-chlorooxime **XI**, which was prepared in two steps from isophthalaldehyde (Scheme 11), was accordingly investigated as a stapling reagent, with the aim of preparing, from **LB8C¹C⁵**, a macrocyclic peptide bearing both 4,5-dihydrothiazole and thiohydroximate linkage. In this context, experimental procedures independently reported by Wang^[86] and Gao^[105] were used. Unfortunately, none of them gave the desired cyclic peptide in satisfactory amounts (Scheme 10c). Treatment of the linear peptide with **XI** in HEPES buffer for 1 h at 37 °C afforded the Cys–Cys stapled peptide featuring two thiohydroximate functions (m/z 1081) as the major product detected by HPLC, together with the cyclic disulfide. However, following both reported procedures, the target 4,5-dihydrothiazole derivative (m/z 1048) was detected only in trace amounts, even after prolonged reaction times.



Scheme 11. Synthesis of bis-chlorooxime **XI**.

To investigate the effect of bicyclization on the conformation of **LB8** derivatives, an analogue of **LB8C¹C⁵** bearing an additional cysteine residue at the C-terminal end (**LB9C¹C⁵C⁹**) was subjected to tris-S-alkylation with compound **X** (Scheme 12). The reaction profile proved clean, and the target bicyclic peptide **BC1** was obtained as the largely predominant product.



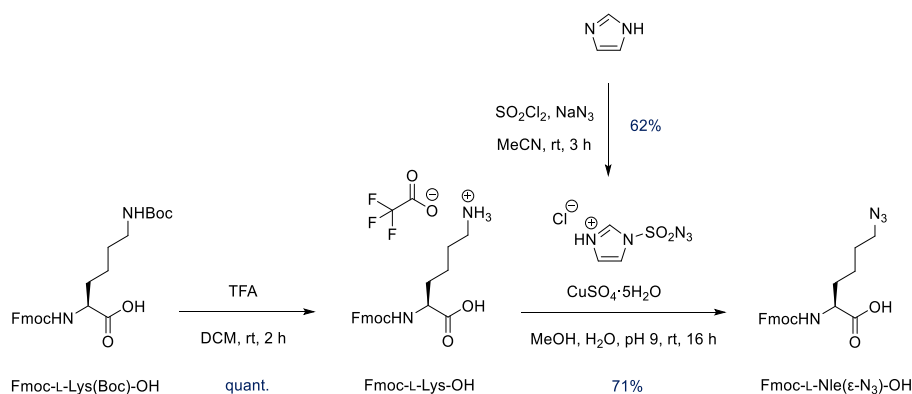
Scheme 12. Cysteine-mediated bicyclization of the LDH-derived nonapeptide **LB8C¹C⁵C⁹** using cross-linking reagent **X**.

4.4. CuAAC-Based Stapling of an LDH-Derived Octapeptide

In parallel with Cys–Cys stapling, we envisioned that alternative stapling strategies could provide access to stapled peptides with distinct geometrical features. Moreover, two comparative studies have suggested the superiority of one-component stapling approaches over cysteine-mediated stapling for promoting α -helix formation.^[56,65]

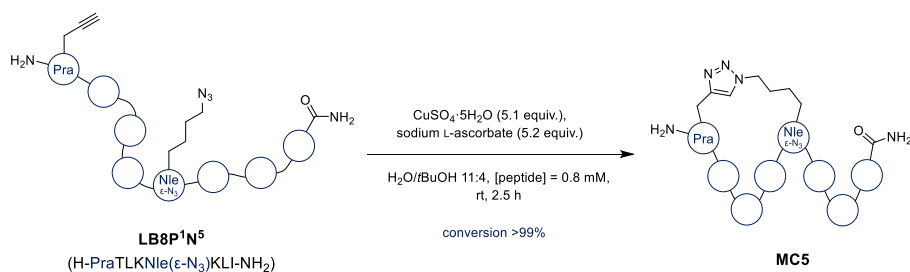
Among one-component side-chain-to-side-chain macrocyclization strategies, CuAAC-based stapling is relatively easy to implement, as a linear peptide bearing appropriately spaced azide- and alkyne-containing residues can be prepared by Fmoc-based SPPS without the need for orthogonal protection schemes and then efficiently cyclized in solution using an inexpensive Cu(II) source and a reducing agent.

In the design of G_s peptidomimetics able to stabilize the active state of the β_2 -adrenergic receptor, Ballet and coworkers reported that stapling of an $i \rightarrow i+4$ -spaced L-Pra–L-Nle(ϵ -N₃) residue pair located near the N-terminal end of the peptide sequence resulted in a macrocyclic peptide with pronounced α -helical propensity and promising active-state-stabilizing activity.^[57] Therefore, we decided to apply the CuAAC-based stapling strategy to an **LB8** analogue harboring L-Pra and L-Nle(ϵ -N₃) residues at positions 1 and 5, respectively. This required incorporation of the non-standard Fmoc-Pra-OH and Fmoc-Nle(ϵ -N₃) building blocks during SPPS. Fmoc-Pra-OH was purchased commercially, whereas Fmoc-Nle(ϵ -N₃) was prepared from Fmoc-Lys-OH through diazotransfer (Scheme 13).



Scheme 13. Synthesis of Fmoc-L-Nle($\epsilon\text{-N}_3$).

Following Fmoc-based SPPS, the linear octapeptide H-PraTLKNle($\epsilon\text{-N}_3$)KLI-NH₂ (**LB8P¹N⁵**) was stapled in solution using copper(II) sulfate as the copper source and sodium ascorbate as the reducing agent (Scheme 14). This afforded the target triazole-bridged peptide **MC5**.



Scheme 14. CuAAC-based stapling of the LDH-derived octapeptide **LB8P¹N⁵**.

4.5. Evaluation of α -Helicity of LDH-Derived Macrocyclic Peptides

Circular dichroism (CD) is a widely used technique for rapidly evaluating the secondary structure of proteins and peptides.^[175,176] α -Helical peptides containing more than 15 residues typically exhibit CD spectra featuring two characteristic minima at approximately 208 nm and 222 nm, together with a strong positive maximum near 190 nm.^[7,26,175] It is worth noting that shorter α -helices may display shifts of the two negative bands to lower wavelengths.^[64,177] A highly negative band near 222 nm is a typical signature of α -helices, and the mean peptide ellipticity ($[\theta]$) at 222 nm is commonly used to quantify α -helical propensity.^[27,117,175]

The five monocyclic peptides **MC1–MC5** and the bicyclic derivative **BC1** were analyzed by CD at a concentration of 100 μ M in a mixture of sodium phosphate buffer (pH 7.4) and trifluoroethanol (TFE). Given the weak helical content previously measured for **MC1**,^[67] TFE was used as a helix-inducing cosolvent, as it was expected to increase the likelihood of detecting differences in helicity among the various cyclic peptides analyzed. A buffer/TFE ratio of 4:1 (v/v) was employed.

The CD spectra of the four $i \rightarrow i+4$ Cys–Cys stapled analogues showed no major differences. The α -helical propensity of these compounds proved relatively low, as indicated by the weak $[\theta]_{222}$ values measured, which ranged from -4449 to -6399 $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ (Figure 10). Based on these $[\theta]_{222}$ values, **MC2** displayed the lowest α -helicity, which was attributed to the increased conformational flexibility of its 5-atom-long sulfone-containing linker compared to the aromatic and alkenylene linkers. **MC1** and **MC3** exhibited very similar CD spectra, suggesting a similar impact of the corresponding *m*-xylylene and (*E*)-2-butenylene linkers on peptide conformation. As expected, introduction of the azidomethyl handle on the *m*-xylylene tether did not lead to a significant change in peptide helicity, based on the spectra recorded for **MC1** and **MC4**.

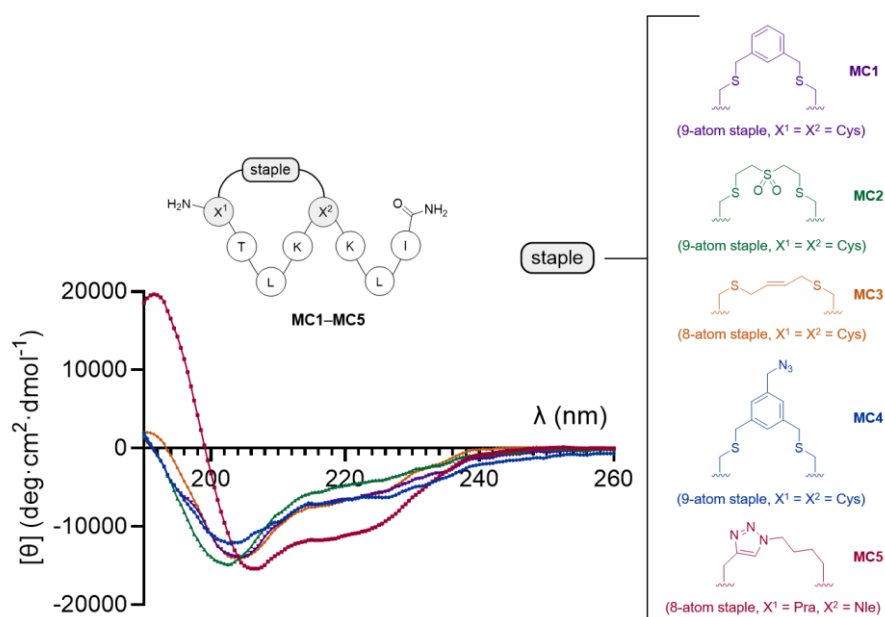


Figure 10. Circular dichroism spectra of stapled LDH-derived peptides **MC1–MC5**.

Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/trifluoroethanol (4:1, v/v); room temperature.

Compared to the spectra of the four Cys–Cys stapled peptides, the spectrum of the triazole-bridged peptide **MC5** showed the appearance of an intense positive band at 192 nm, a significant increase in the absolute mean peptide ellipticity at 222 nm ($[\theta]_{222} = -10829 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$), and a shift of the minimum from 203–204 nm to 207 nm. These three spectral features were indicative of a strong increase in α -helicity. The α -helical propensity of **MC5** in buffer (pH 7.4)/TFE 4:1 maybe roughly twofold higher than that of the Cys–Cys cross-linked peptides, based on the $[\theta]_{222}$ values. The significant differences measured between macrocycles **MC3** and **MC5**, although both bear an 8-atom-long staple, suggest that the overall geometry and electronic properties of the staple can have a major impact on the peptide conformation.

Although the α -helical preference of a peptide is difficult to quantify accurately, it is worth noting that the ellipticity ratio between the bands at 222 nm and near 208 nm ($[\theta]_{222}/[\theta]_{208}$) is sometimes used to

distinguish α -helices from 3_{10} -helices: values close to 1 are typical of α -helices, whereas values below 0.7 are indicative of 3_{10} -helices.^[12,19,28,64,175] Based on these considerations, **MC5** ($[\theta]_{222}/[\theta]_{207} = 0.70$) may adopt a mixed α -helix/ 3_{10} -helix conformation in water/TFE 4:1, whereas **MC1–MC4** ($[\theta]_{222}/[\theta]_{203-204} = 0.30\text{--}0.53$) are likely folded predominantly as 3_{10} -helices in this environment.

Overall, CuAAC-based stapling was more effective than Cys–Cys stapling for stabilizing the α -helical conformation of short **LB8**-derived peptides. In the future, the affinity of all stapled peptides **MC1–MC5** for LDH-Htr will need to be evaluated, which will make it possible to determine whether the helical propensity of these peptides correlates with their binding affinity.

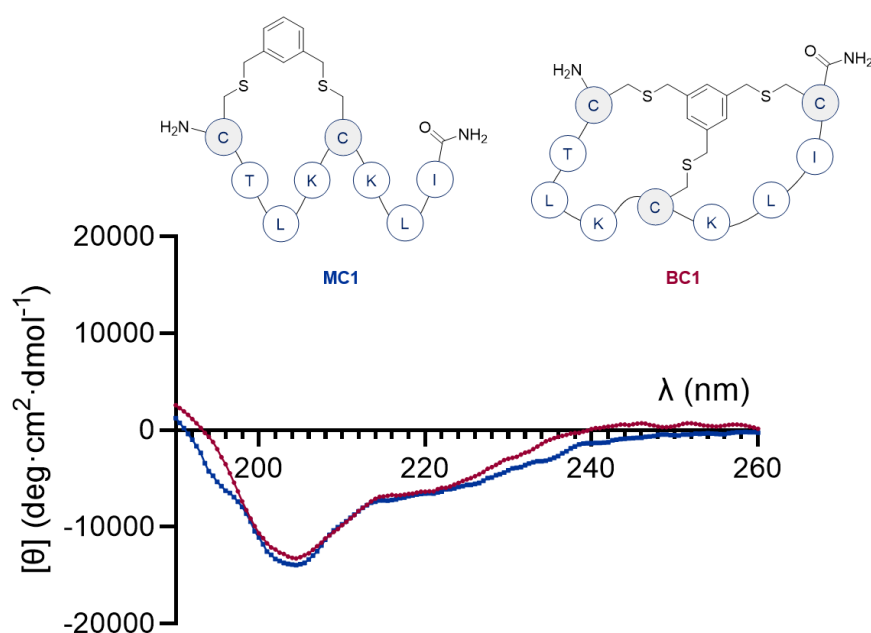


Figure 11. Circular dichroism spectra of monocyclic and bicyclic peptides **MC1** and **BC1**.

Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/trifluoroethanol (4:1, v/v); room temperature.

Little is known about the conformation of bicyclic peptides generated by linking three cysteine residues through a mesitylene scaffold. In this

context, the CD spectrum of bicyclic peptide **BC1** was compared to that of its monocyclic counterpart **MC1**. The two CD spectra were similar, with measured $[\theta]_{222}$ values of -6340 and $-5962 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ for **MC1** and **BC1**, respectively, and $[\theta]_{222}/[\theta]_{204}$ ratios of approximately 0.45, indicative of a 3_{10} -helical conformation (Figure 11). Thus, performing *i*+4+4 cysteine-mediated bicyclization using the mesitylene-derived cross-linking reagent **V** did not appear to be an effective strategy for enhancing the α -helical propensity of potential mimics of the LDH tetramerization arm.

4.6. Fluorescent Probes

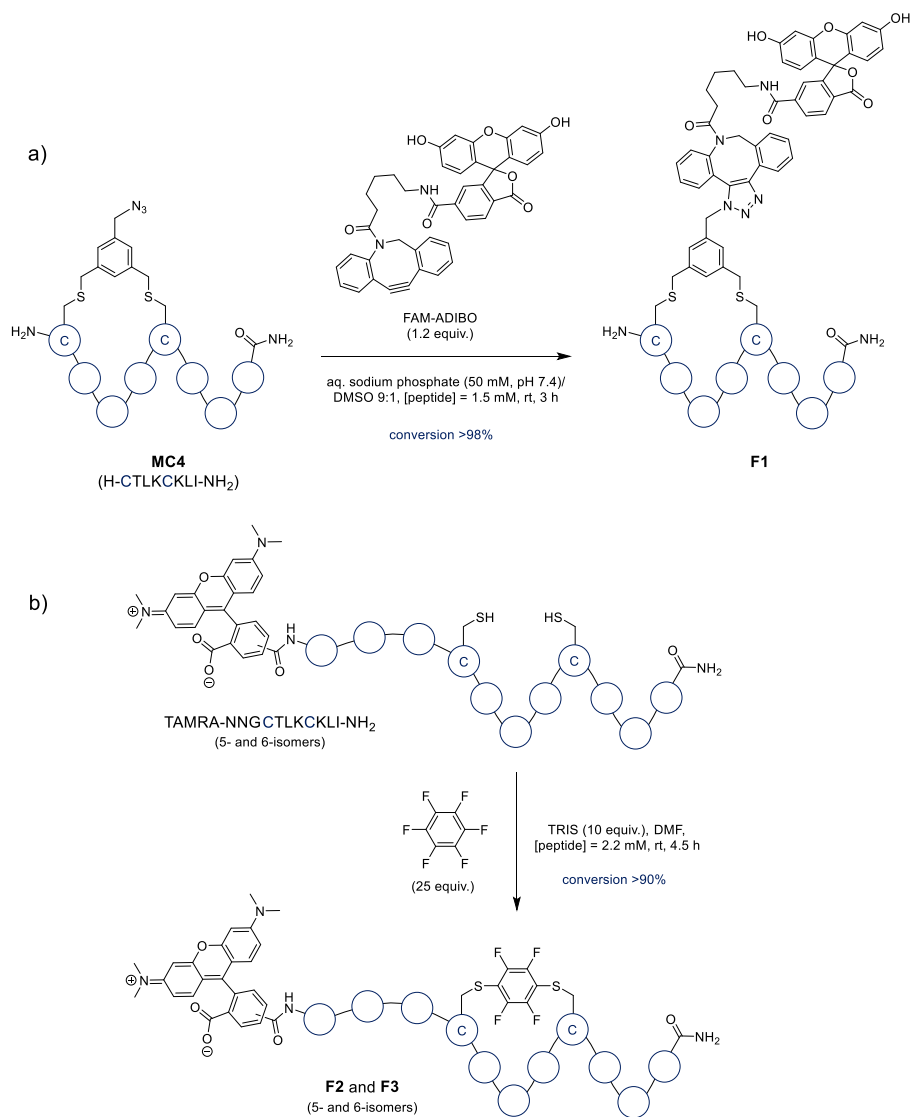
Fluorescent probes are powerful chemical biology tools in the development of bioactive peptides. Notably, the cell permeability of numerous stapled peptides has been evaluated using fluorescently labeled analogues of the corresponding peptides.^[49,50,56,114] Besides cellular uptake assays, fluorescence polarization (FP) is widely used to assess the binding affinity of stapled peptides.^[66,80,98,102,114] Direct FP experiments requires the preparation of a fluorescent analogue for each peptide to be tested. More interestingly, FP competition assays allow screening of multiple unlabeled peptides using a single fluorescent reporter peptide. In such assays, the reporter peptide can be displaced from the binding site of the target protein by the unlabeled competitors, to an extent that depends on their relative binding affinity. This approach enables rapid determination of binding affinities for compound libraries, with considerably less synthetic effort than direct FP assays.

We envisioned that the preparation of fluorescent **LB8** derivatives would be particularly useful for the development of potential LDH-1 inhibitors. First, the cell permeability of such analogues could be assessed by confocal microscopy or flow cytometry. Furthermore, designing a fluorescent probe capable of binding to the LDH-Htr tetramerization site would allow its use as a reporter in FP competition assays, in order to screen a wide range of additional compounds (peptides or small molecules) against this site. This could lead to the identification of potential LDH-1 tetramer disruptors.

Compared to **LB8**, the *m*-xylylene-bridged peptide **MC1** has previously been shown to display a marked increase in binding affinity toward LDH-Htr (K_d of **LB8** = 1.05 mM; K_d of **MC1** = 64 μ M).^[67] Because CD analysis of azide-bearing peptide **MC4** did not reveal any significant conformation change relative to **MC1**, and since the stapling reaction involved anchoring residues that are expected to be solvent-exposed, **MC4** would be anticipated to display a binding affinity similar to that of **MC1** toward the LDH-Htr tetramerization site. Likewise, derivatization of the **MC4** linker through functionalization of its azide conjugation handle would be expected to exert only a limited impact on binding affinity. Therefore, we first considered conjugating **MC4** with a fluorescent tag.

Thus, the azide-bearing stapled peptide **MC4** was subjected to SPAAC in a 9:1 (v/v) mixture of phosphate buffer and DMSO. A commercially available carboxyfluorescein (FAM) derivative equipped with an azadibenzocyclooctyne (ADIBO) moiety was used as the alkyne partner (Scheme 15a). This afforded the desired peptide–fluorophore conjugate **F1** as the sole product.

As mentioned previously, the stapled peptide **MCF**, featuring $i \rightarrow i+4$ -spaced cysteine residues connected through a *p*-tetrafluorophenylene linker, showed a higher binding affinity for LDH-Htr (K_d = 11 μ M) than **MC1** did (K_d = 64 μ M). Thus, fluorescent labeling of **MC7** could result in probes that are more potent than those obtained from **MC1** or its derivatives. However, the fluorinated linker of **MCF** may not be suitable for the efficient preparation of a single fluorescent peptide via post-stapling modification of this tether. Instead, we decided to perform hexafluorobenzene-mediated Cys–Cys cross-linking on a fluorescently labeled **LB8** derivative. Therefore, an **LB8C¹C⁵** analogue with a tetramethylrhodamine-functionalized N-terminus was prepared. This linear peptide was synthesized through Fmoc-based SPPS in the laboratory of Strømgaard at the University of Copenhagen. Since the early 2010s, Strømgaard and coworkers have produced various fluorescently labeled peptides for use in direct FP assays or as probes in FP competition experiments.^[178-181] In practice, following assembly of the resin-bound **LB8C¹C⁵** octapeptide (H-CTLKCKLI-resin), an NNG tripeptide spacer and 5/6-carboxytetramethylrhodamine (TAMRA) were incorporated through 4 additional SPPS cycles.



Scheme 15. Synthesis of fluorescently-labeled LDH-derived stapled peptides.

As TAMRA was used as a mixture of 5- and 6-regioisomers, the linear fluorescent peptide was obtained as two distinct isomers. Purification of the crude material obtained after SPPS was therefore performed to isolate the two pure regioisomers. Following reported procedures,^[99]

these two linear peptide dithiols were then individually cyclized through S-arylation with hexafluorobenzene in DMF, affording the stapled peptides **F2** and **F3** (Scheme 15b). Of note, although **F2** and **F3** were the major products formed in the corresponding reaction mixtures, the reaction profiles showed several side-products, notably dimeric species and products arising from additional arylation of the target monocyclic peptide. This was not surprising, as perfluoroaromatic reagents can react with lysine side chains under the conditions used.^[67] Therefore, the chemoselectivity of these reagents toward cysteine residues is not absolute.

Overall, several fluorescent probes derived from the LDH tetramerization arm were prepared. Based on our *in silico* model of the interaction between **LB8** and the LDH-H tetramerization site,^[67] the fluorescent tags were introduced at positions that are not predicted to contribute to binding. Nevertheless, further work will be required to determine the respective binding affinities of **F1**, **F2**, and **F3** for the tetramerization site of LDH-Htr. Depending on the results obtained, such compounds could represent valuable tools for live-cell imaging or flow cytometry, or for screening libraries of compounds against LDH-Htr.

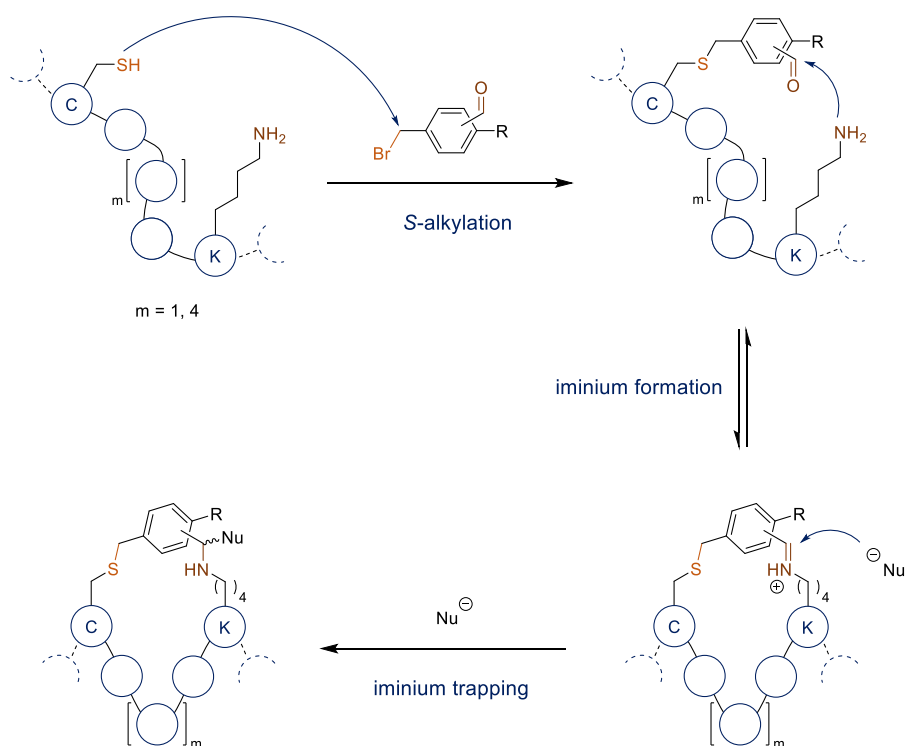
5. Cys–Lys Stapling via One-Pot S-Alkylation/Reductive Amination (Chapter 2)

5.1. Synthetic Strategy

Building on the expertise acquired during the studies described in Chapter 1, the main objective of this thesis, namely the development of a macrocyclization strategy that could complement existing Cys–Lys stapling methods, was subsequently pursued. More specifically, we sought to establish a stapling methodology that could be performed using commercially available or easily accessible cross-linking reagents and that would preserve the basicity and nucleophilicity of the lysine side chain. To this end, we considered using stapling reagents bearing an aromatic aldehyde as the amine-reactive group and a benzyl bromide moiety, whose reactivity toward cysteine thiolates is well documented.^[73,90,93] We envisioned that, following S-alkylation of a cysteine residue, the transient imine/iminium species formed between the aldehyde group and a proximal lysine residue could be trapped by an appropriate nucleophile (Scheme 16). Indeed, nucleophilic addition to lysine-derived imines has already been successfully applied in peptide macrocyclization.^[47,143,145,150] The use of a hydride donor would be preferable to C-nucleophiles such as cyanide,^[148] alkynes,^[151,182] arylboronic acids,^[47] or phenols,^[150] as it would avoid forming mixtures of two diastereomeric α,α -disubstituted amines. Moreover, reductive amination-mediated peptide modification has already been shown to be broadly compatible with all the canonical amino acids, except N-terminal cysteines.^[143,147,148,183,184] The potential of the envisioned approach for structural diversification would arise both from the variety of stapling reagents that can be employed (different substituents, substitution patterns, and possibly different thiol-reactive groups) and from post-stapling derivatization of the macrocyclic secondary amine.

From a chemical strategy perspective, performing intermolecular cysteine S-alkylation, which is known to be very fast at slightly basic pH,^[73,90,93] prior to intramolecular reductive N-alkylation appears advantageous over the reverse order, as intermolecular reductive amination reactions can be sluggish. In contrast, intramolecular reductive amination processes are considerably more efficient and have proved to be powerful reactions for peptide

cyclization,^[145,148,153,183-185] even for linear peptides that are not conformationally predisposed to macrocyclization.^[148] Moreover, we envisioned carrying out *S*-alkylation at relatively high concentration, so that this reaction would be completed even faster, and intramolecular reductive amination at lower concentration, in order to minimize undesired intermolecular reactions leading to formation of oligomers, polymers, or over-*N*-alkylated products. Although the two steps cannot be efficiently performed simultaneously, as their optimal reaction conditions (including solvent and pH) differ, this order of steps enables consideration of a one-pot protocol in which the crude intermediate obtained upon *S*-alkylation would be diluted with an appropriate buffer prior to reductive amination.



Scheme 16. Synthetic strategy for Cys–Lys stapling via *S*-alkylation and iminium trapping.

Nu = nucleophile.

5.2. Optimization of the Stapling Procedure

5.2.1. Selection of the Peptide Model

First investigations of peptide stapling via cysteine S-alkylation and lysine reductive *N*-alkylation were conducted using the octapeptide Cbz-CTLGKGLI-NH₂ as a peptide model, and *m*-(bromomethyl)benzaldehyde (**1**) as the cross-linking reagent. The octapeptide model corresponded to an **LB8** derivative in which the alanine and glutamate residues at positions 1 and 5 were mutated to cysteine and lysine, respectively, so that stapling would span one putative helical turn (similarly to the previously prepared *i* → *i*+4 Cys–Cys stapled **LB8** analogues). To avoid competition between several nucleophilic amines, the lysines at positions 4 and 6 were replaced with glycine residues, and the N-terminus was protected with a benzyloxycarbonyl (Cbz) group (Figure 12).

Optimization of the cysteine-to-lysine macrocyclization of this Cbz-protected octapeptide demonstrated that the envisioned stapling strategy was feasible. However, based on its CD spectrum, the resulting *i* → *i*+4 stapled peptide did not exhibit any signs of helical folding in water, even in the presence of up to 40% TFE. The absence of helicity was attributed to the presence of two glycine residues in the peptide sequence. The octapeptide model was therefore judged unsuitable for evaluating the impact of our stapling strategy on α -helicity.

Consequently, other model substrates were considered. Notably, the two glycine residues were mutated to alanines, and a third alanine was introduced at the C-terminal end to allow further comparison between *i* → *i*+4 monocyclic and *i*+4+4 bicyclic nonapeptides. As these modifications were expected to decrease aqueous solubility, which was undesirable given our aim to develop a water-compatible stapling protocol, the hydrophobic N-terminal Cbz group was replaced with an acetyl group, and the isoleucine at position 8 was mutated to a more polar glutamine residue. To our satisfaction, the resulting nonapeptide Ac-CTLAKALQA-NH₂ (**P1**), obtained by SPPS, was well soluble at room temperature in mixtures of 50 mM ammonium bicarbonate buffer (pH 8.0) and acetonitrile, at concentrations of at least 10 mM. Two other derivatives of **P1**, namely Ac-CTLAKALIA-NH₂ and Ac-

CTLAKQLAA-NH₂, were also synthesized, but both proved insoluble in the same solvent system.

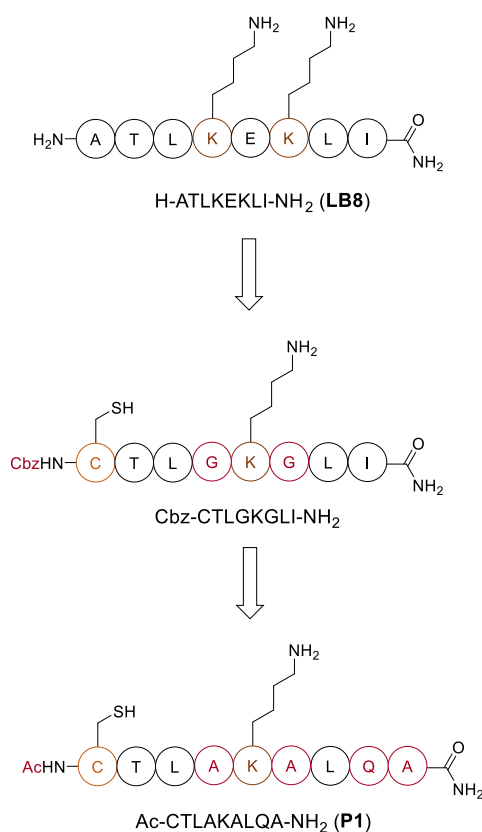


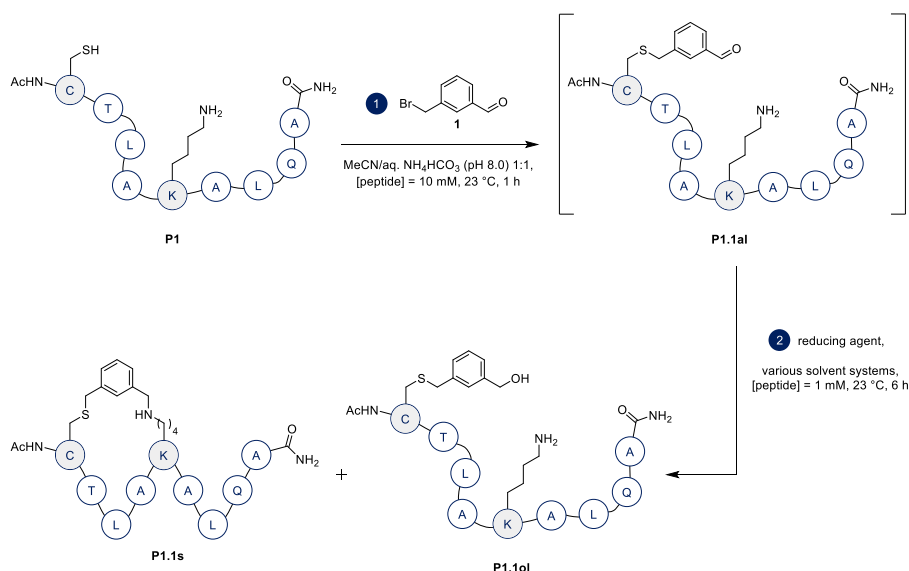
Figure 12. Evolution of the peptide model used for optimization of the Cys–Lys stapling procedure.

Although CD analysis showed that **P1** displays low helical content in aqueous solution, its large proportion of residues with high helical propensities (alanine, leucine, lysine, and glutamine)^[16] was expected to increase its ability to adopt a helical structure upon macrocyclization. **P1** was therefore selected as the peptide model for further careful optimization of the Cys–Lys cross-linking protocol. To enable these optimization studies, 500 mg of **P1** were obtained from a commercial supplier.

5.2.2. Optimization of the Stepwise S-Alkylation/Reductive Amination Protocol

5.2.2.1. Intermolecular S-alkylation

Cys–Lys cross-linking of nonapeptide model Ac-CTLAKALQA-NH₂ (**P1**) with *m*-(bromomethyl)benzaldehyde (**1**), to give the stapled peptide **P1.1s**, was selected as the model transformation for optimizing our stapling approach (Scheme 17). Compound **1** was chosen as the model stapling reagent because it is commercially available, cheap, and because the *meta*-xylylene linker (that would be generated upon S-alkylation and reductive *N*-alkylation) may provide higher helicity than the *ortho*- and *para*-xylylene linkers when installed in *i* → *i*+4 Cys–Cys stapled peptides, as suggested by a study on an 18-mer peptide by Greenbaum and co-workers.^[93] Furthermore, the *meta*-(alkylthiomethyl)benzaldehyde intermediate formed via S-alkylation of **P1** with **1** was expected to exhibit an electrophilic reactivity closer to that of unsubstituted benzaldehyde than its *ortho*- and *para*-substituted analogues, which could facilitate the interpretation of the effects of any additional substituents.



Scheme 17. Cys–Lys stapling of peptide model **P1** via sequential S-alkylation and reductive *N*-alkylation.

Based on reported procedures,^[76,183] **P1** (4.0 μmol) was first cleanly *S*-alkylated with **1** in a 1:1 (v/v) mixture of ammonium bicarbonate buffer (50 mM, pH 8.0) and acetonitrile, at a peptide concentration of 10 mM. Using only 1.2 equiv. of **1**, this transformation was complete within 1 h at 23 °C, yielding the *S*-alkylated peptide **P1.1al**, which bears a side-chain benzaldehyde moiety (and is therefore referred to hereafter as peptide aldehyde **P1.1al**), with no bis-alkylated byproducts detected by HPLC. Furthermore, no reducing agent was required, as the amount of disulfide byproduct (**P1.1ds**) formed under the conditions used was negligible. The *S*-alkylation reaction was then repeated on a larger scale (73.4 μmol), and the diluted reaction mixture was lyophilized to provide multiple crude batches of **P1.1al**, which were subsequently used to investigate the intramolecular reductive amination.

5.2.2.2. Intramolecular reductive *N*-alkylation

Sodium cyanoborohydride (NaBH_3CN) appeared to be the hydride donor of choice for cyclization of **P1.1al**, as this reagent is compatible with aqueous conditions and has been widely used as a mild and imine/iminium-selective reducing agent in the field of peptide and protein modification.^[143,145,147,148,184-186] However, the envisioned stapling methodology has a major drawback: intramolecular reductive amination can yield non-negligible amounts of the corresponding alcohol byproduct if selectivity for iminium reduction over aldehyde reduction is not properly controlled. This issue was notably highlighted in studies by the Baran and Mayer groups, in which NaBH_3CN -mediated cyclizations carried out in aqueous media on peptides bearing either a C-terminal^[148] or a side-chain aromatic^[184] aldehyde showed slow conversion and substantial alcohol formation when a lysine ϵ -amine was the only available amine. Nevertheless, we anticipated that both the reaction rate and the selectivity for iminium reduction in our model reaction could be improved through the use of organic cosolvents and/or additives.

Thus, crude peptide aldehyde **P1.1al** was subjected to reductive amination through several rounds of optimization, all reactions being conducted on an analytical scale (1 μmol). This optimization was guided by the selectivity for iminium reduction, which was assessed by HPLC–UV ($\lambda = 210 \text{ nm}$) through measurement of the ratio between the

peak area of the target stapled peptide **P1.1s** and the combined peak area of **P1.1s** and the alcohol byproduct **P1.1ol**. Based on the literature,^[143,148,149] each reductive amination reaction was carried out at a peptide concentration of 1 mM (relative to the initial amount of **P1**), as this concentration was anticipated to be sufficiently low to favor the desired intramolecular process while remaining compatible with preparative-scale synthesis of stapled peptides.

First, the use of an organic cosolvent was shown to significantly favor the reductive amination compared with an aqueous buffer alone, with methanol providing higher conversion than acetonitrile. Using 10 equiv. of NaBH₃CN in a 1:1 (v/v) mixture of acetate buffer (100 mM, pH 5.0) and methanol afforded, after incubation for 6 h at 23 °C and subsequent 10-fold dilution into 0.1% trifluoroacetic acid (TFA) in water, a **P1.1s**/(**P1.1s** + **P1.1ol**) ratio of 87%, which was already satisfactory. Doubling the amount of reducing agent lowered the selectivity to 84% (Table 2).

Table 2. Screening of solvent composition and NaBH₃CN loading for intramolecular reductive amination.

Entry	NaBH ₃ CN (equiv.)	Solvent	P1.1s (mAU·s) ^a	P1.1ol (mAU·s) ^a	s/(s + ol) (%) ^b
1	10	aq. buffer (pH 5.0) ^c	685.1	430.0	61
2		MeCN/aq. buffer (pH 5.0) ^c (~1:1)	1166.5	218.2	84
3		MeOH/aq. buffer (pH 5.0) ^c (~1:1)	1693.5	251.6	87
4	20	aq. buffer (pH 5.0) ^c	889.6	590.9	60
5		MeCN/aq. buffer (pH 5.0) ^c (~1:1)	1435.4	238.7	86
6		MeOH/aq. buffer (pH 5.0) ^c (~1:1)	1762.5	329.2	84

^a Absorbance areas measured by HPLC–UV (λ = 210 nm). ^b Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) × 100.

^c Sodium acetate buffer (100 mM, pH 5.0).

As expected, increasing the methanol/aqueous buffer ratio further reduced the formation of **P1.1ol**. A 3:1 (v/v) methanol/buffer ratio was selected for subsequent optimization steps, as it afforded the stapled peptide with 94% selectivity (Table 3). Further decreasing the aqueous content of the solvent system might provide even better results for the model reaction but could compromise the solubility of more hydrophilic peptides.

Table 3. Screening of methanol/aqueous buffer volume ratio for intramolecular reductive amination.

Entry	NaBH ₃ CN (equiv.)	Solvent	P1.1s (mAU·s) ^a	P1.1ol (mAU·s) ^a	s/(s + ol) (%) ^b
1	10	MeOH/aq. buffer ^c (~1:3, v/v)	1154.6	354.4	77
2		MeOH/aq. buffer ^c (~1:2, v/v)	1302.5	310.5	81
3		MeOH/aq. buffer ^c (~1:1, v/v)	1569.5	227.2	87
4		MeOH/aq. buffer ^c (~2:1, v/v)	1858.3	154.3	92
5		MeOH/aq. buffer ^c (~3:1, v/v)	1857.4	127.7	94

^a Absorbance areas measured by HPLC–UV (λ = 210 nm). ^b Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) × 100.

^c Sodium acetate buffer (100 mM, pH 5.0).

Buffer screening revealed that buffers with pH values of 4–5 provided the highest selectivity (Table 4). Given the similarity of the results obtained in entries 3 and 4, buffers at both pH 4.0 and 5.0 were selected for the subsequent optimization step, namely comparison of acetate and citrate buffers.

Table 4. Screening of pH for intramolecular reductive amination.

Entry	NaBH ₃ CN (equiv.)	Solvent	pH ^a	P1.1s (mAU·s) ^b	P1.1ol (mAU·s) ^b	s/(s + ol) (%) ^c
1	10	MeOH/aq. buffer (~3:1, v/v) ^d	2.1 ^e	1625.1	323.3	83
2			3.0 ^f	1802.9	179.7	91
3			4.0 ^g	1765.5	109.7	94
4			5.0 ^g	1854.0	131.3	93
5			6.0 ^h	1650.5	164.5	91
6			7.0 ^h	1497.7	172.0	90
7			8.0 ^h	1267.5	196.7	87
8			9.0 ⁱ	1024.2	162.2	86

^a pH of the aqueous buffer. ^b Absorbance areas measured by HPLC–UV (λ = 210 nm). ^c Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) $\times$ 100. ^d Buffer concentration: 25 mM in MeOH/H₂O (~3:1, v/v). ^e Phosphoric acid–sodium phosphate buffer. ^f Sodium citrate buffer. ^g Sodium acetate buffer. ^h Sodium phosphate buffer. ⁱ Cloudy reaction medium. ^j Sodium borate buffer.

Acetate and citrate buffers gave comparable results. However, citrate buffers appeared to slightly attenuate aldehyde reduction. A **P1.1s**/(**P1.1s** + **P1.1ol**) ratio of 96% was obtained using a citrate buffer at pH 4.0 and a final buffer concentration of 25 mM (Table 5).

Table 5. Comparison of acetate and citrate buffers for intramolecular reductive amination.

Entry	NaBH ₃ CN (equiv.)	Solvent	Buffer	P1.1s (mAU·s) ^a	P1.1ol (mAU·s) ^a	s/(s + ol) (%) ^b
1	10	MeOH/aq. buffer (~3:1, v/v) ^c	sodium acetate, pH 4.0	1763.3	121.2	94
2			sodium citrate, pH 4.0	1863.4	81.7	96
3			sodium acetate, pH 5.0	1860.2	148.8	93
4			sodium citrate, pH 5.0	1841.8	99.9	95

^a Absorbance areas measured by HPLC–UV (λ = 210 nm). ^b Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) $\times$ 100.

^c Buffer concentration: 25 mM in MeOH/H₂O (~3:1, v/v).

Finally, increasing the buffer concentration led to a reduction in the **P1.1ol** peak intensity, although the differences observed at 25 mM, 50 mM, and 100 mM sodium citrate might not be significant (Table 6).

Table 6. Screening of buffer concentration for intramolecular reductive amination.

Entry	NaBH ₃ CN (equiv.)	Solvent	[Buffer] (mM) ^a	P1.1s (mAU·s) ^b	P1.1ol (mAU·s) ^b	s/(s + ol) (%) ^c
1	10	MeOH/sodium acetate buffer ^d (~3:1, v/v)	12.5	1726.5	188.5	90
2			25	1766.9	130.3	93
3			50	1824.7	107.3	94
4			100	1846.3	88.5	95
5		MeOH/sodium citrate buffer ^d (~3:1, v/v)	12.5	1794.3	94.5	95
6			25	1838.6	79.7	96
7			50	1838.4	72.4	96
8			100	1872.9	72.1	96

^a Final buffer concentration in MeOH/H₂O (~3:1, v/v). ^b Absorbance areas measured by HPLC–UV (λ = 210 nm). ^c Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) × 100. ^d pH 4.0.

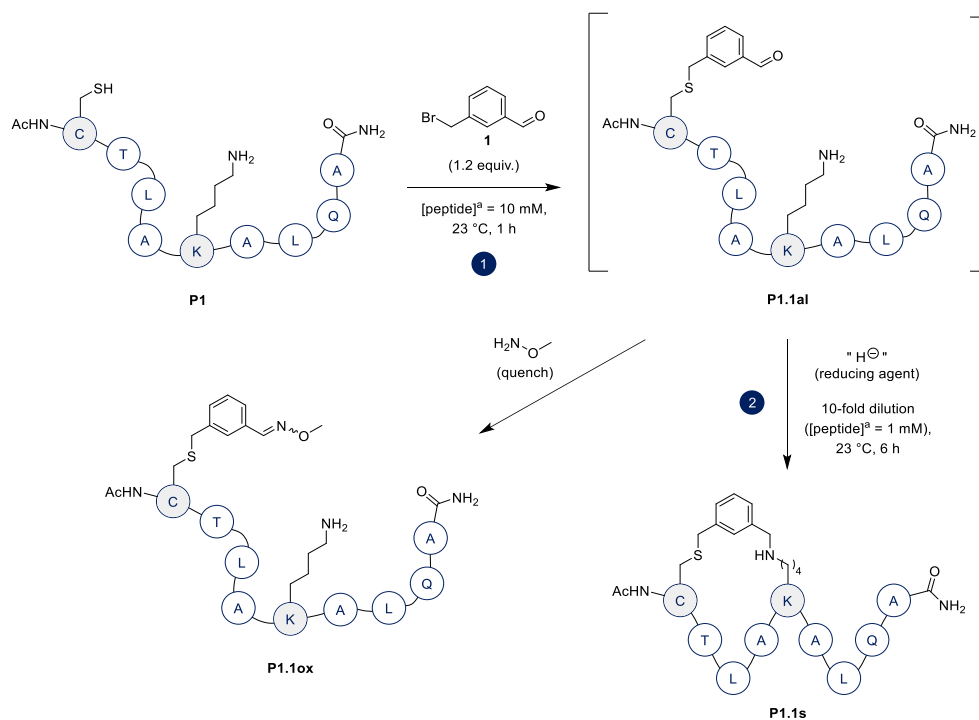
5.2.3. Optimization of the One-Pot S-Alkylation/Reductive Amination Protocol

The results of the stepwise optimization collectively showed that a protocol based on sequential intermolecular S-alkylation and intramolecular reductive N-alkylation could enable efficient Cys–Lys stapling of the peptide model, and that adjustment of the solvent system for the intramolecular step can minimize formation of the alcohol byproduct. However, the stepwise approach used so far involved lyophilization of the peptide aldehyde intermediate prior to cyclization, which is time-consuming. To improve the operational simplicity of the stapling strategy, we therefore investigated a one-pot protocol, in which cyclization would be directly performed after 10-fold dilution of the crude S-alkylated peptide. This approach was encouraged by the observation that efficient S-alkylation was achieved at an ammonium bicarbonate buffer concentration as low as 25 mM (only 2.5-fold higher than the peptide concentration), which was

expected to minimize the impact of this basic buffer on the pH of the final reaction medium after dilution.

To increase the likelihood of identifying optimal conditions for one-pot stapling, we screened a range of closely related conditions for the cyclization step, centered on those that had provided the best results in the previous optimization, namely the use of 10 equiv. of NaBH₃CN in a 3:1 (v/v) mixture of methanol and sodium citrate buffer adjusted to pH 4.0. Thus, after S-alkylation of **P1** in acetonitrile/ammonium bicarbonate buffer (1:1, v/v), crude **P1.1al** was directly diluted in various solvent systems prior to addition of the hydride donor. To allow accurate estimation of the conversion of the peptide aldehyde **P1.1al**, each reaction mixture was quenched with methoxylamine after incubation for 6 h. This reaction time was selected because it had previously been shown to allow complete or near-complete conversion of **P1.1al** and because it would result in a total reaction time of 7 h, enabling synthesis of the target stapled peptide within a single working day. The reaction yield was determined from a calibration curve obtained using purified **P1.1s**, while the selectivity for iminium reduction over aldehyde reduction was measured as previously described (Tables 7 and S1).

Table 7. Selected conditions for the optimization of the one-pot S-alkylation/reductive amination protocol (see Table S1 for the complete screening).



Step	Entry ^b	Reagent	Solvent Composition				Conversion (%) ^{e, f}	Yield (%) ^{e, g}	s/(s + ol) (%) ^{e, h}
			Organic Solvent(s) (v/v)	Aqueous Buffer	Org./Aq. (v/v) ^c	Buffer (mM) ^d			
1	/	1 (1.2 equiv.)	MeCN	NH ₄ HCO ₃ (pH 8.0)	1:1	25	>99	/	/
2	1	NaBH ₃ CN (10 equiv.)	MeOH/MeCN 14:1	sodium citrate (pH 3.0)	3:1	25	>99	84	92
	2		MeOH/MeCN 14:1	sodium citrate (pH 4.0)	3:1	25	>99	86	97
	3		MeOH/MeCN 14:1	sodium citrate (pH 4.0)	3:1	50	>99	86	97
	5		MeOH/MeCN 14:1	sodium acetate (pH 4.0)	3:1	100	>99	83	96
	6		MeOH/MeCN 14:1	sodium citrate (pH 5.0)	3:1	25	>99	85	96
	8		MeOH/MeCN 9:1	sodium citrate (pH 4.0)	1:1	50	89	72	90
	9		MeOH/MeCN 18:1	/	19:1	/	89	70	95

10		MeCN	sodium citrate (pH 4.0)	3:1	25	88	76	94
11		EtOH/MeCN 14:1	sodium citrate (pH 4.0)	3:1	25	>99	84	96
12	NaBH ₃ CN (20 equiv.)	MeOH/MeCN 14:1	sodium citrate (pH 4.0)	3:1	25	>99	84	95
14	pic-BH ₃ (10 equiv.)	MeOH/MeCN/ THF 69:5:1	sodium citrate (pH 4.0)	3:1	25	79	69	95

^a Peptide concentration relative to the initial amount of peptide model **P1**. ^b Entry numbering as in Table S1. ^c Ratio of organic solvent(s) to aqueous phase (v/v). ^d Final buffer concentration in the corresponding solvent mixture (if applicable). ^e Determined by HPLC–UV (λ = 210 nm); average of duplicate reactions. ^f Calculated from the absorbance area of **P1** (step 1) or of the oxime-trapped derivative of **P1.1al** (step 2), respectively. ^g Calculated based on the calibration curve established for **P1.1s** (see Figure S11). ^h Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) $\times$ 100.

As shown in Table 7, the use of a sodium citrate buffer at pH 4.0 and at a final buffer concentration of 25 mM provided higher cyclization yield and selectivity than the corresponding buffer at pH 3.0 (entries 1 and 2), and only marginally improved results compared with the buffer at pH 5.0 (entries 2 and 6). Increasing the citrate concentration to 50 mM resulted in a modest decrease in the UV peak area of **P1.1ol**, from 69.5 to 60.4 mAU·s (see related HPLC–UV chromatograms), even though the stapling yield and measured selectivity did not exceed 86% and 97%, respectively (entries 2 and 3). Sodium acetate buffer, when used at a final concentration of 100 mM gave a similar selectivity for iminium reduction, albeit with a slightly lower yield (entries 3 and 5).

Increasing the proportion of water from 25% to 50% (v/v) led to a decrease in conversion (from >99 to 89%) and stapling yield (from 86 to 72%), as expected (entries 3 and 8). Interestingly, dilution of intermediate **P1.1al** in non-buffered methanol prior to addition of NaBH₃CN, despite decreasing the overall water content of the solvent system, resulted in suboptimal macrocyclization efficiency (89% conversion and 70% yield, entry 9). This observation further supported the need for an acidic buffer to achieve high yields in reductive amination-mediated peptide cyclization. Using acetonitrile instead of methanol in the second step of the stapling procedure resulted in suboptimal conversion of **P1.1al** (88%), whereas ethanol afforded results comparable to those obtained with methanol (entries 2, 10, and 11). A twofold higher loading of NaBH₃CN slightly reduced the

selectivity for iminium reduction and, consequently, the yield (entries 2 and 12). Replacing NaBH₃CN with 2-picoline–borane (pic-BH₃), another iminium-selective and water-compatible reducing agent,^[187] enabled only 79% conversion of **P1.1al** after 6 h (entry 14). Notably, NaBH₃CN-mediated cyclization could be performed at peptide concentrations up to 2 mM without a significant increase in the formation of dimeric or oligomeric byproducts (Table S1, entries 15 and 16).

Overall, this screening of conditions demonstrated that high yields of the *i* → *i*+4 stapled peptide **P1.1s** (up to 86%) could be achieved through *S*-alkylation of **P1**, direct dilution of the peptide aldehyde intermediate **P1.1al** into a mixture of methanol and an acidic buffer at pH 4.0–5.0, followed by intramolecular NaBH₃CN-mediated reductive amination. Both sodium citrate and acetate buffers proved effective. Acetate buffer may represent a practical option, as the volatility of acetic acid enables its direct removal by lyophilization. However, because the lowest amount of alcohol byproduct **P1.1ol** was observed when using citrate buffer at 50 mM, this buffer was selected for exploration of the methodology's scope. Moreover, compared with acetate buffers, citrate buffers were found to further limit the undesired formation of a cyclic *N*-methylated byproduct, which can arise when the methanol employed contains trace amounts of formaldehyde (Tables 7 and S1, entries 2–5).

The best conditions identified (Table 7, entry 3) were subsequently applied to the synthesis of **P1.1s** on a preparative scale. To our delight, starting from 39 μmol of **P1**, **P1.1s** was obtained in 45% isolated yield after purification by semi-preparative HPLC. The Cys–Lys connectivity of the installed *m*-xylylene linker was confirmed by tandem mass spectrometry (MS/MS) analysis.

5.3. Peptide Scope

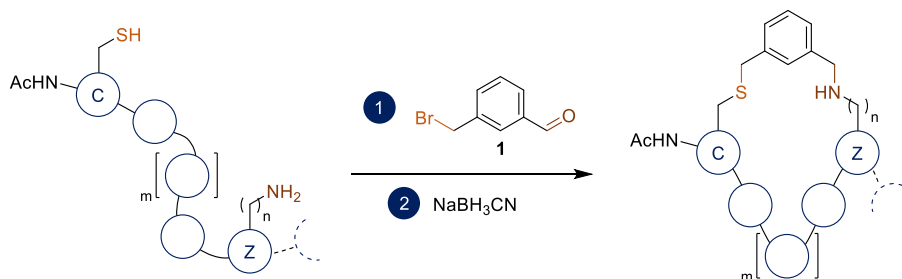
5.3.1. Variation of Ring Size

The scope of the developed one-pot stapling protocol was next investigated through its application to a series of peptides. First, the optimized conditions were applied to analogues of the peptide model

bearing shorter alkylamine side chains. The goal was twofold: to assess the influence of this structural modification on stapling efficiency and to obtain, upon preparative-scale macrocyclization, cyclic peptides featuring a shorter and more rigid staple, which could positively impact their helical propensity. The stapling procedure was applied on a 1 μ mol scale to linear nonapeptides **P2–P4** (Ac-CTLAZALQA-NH₂), incorporating an ornithine (Orn), a diaminobutyrate (Dab), or a diaminopropionate (Dap) residue in place of the lysine residue in the peptide model **P1** (Table 8, entries 1–4). Cys–Orn stapled peptide **P2.1s** and Cys–Dap stapled peptide **P4.1s** were obtained in excellent HPLC yields ($\geq 80\%$), whereas stapling of the Dab-containing peptide **P3** afforded the target macrocycle **P3.1s** in a lower 68% yield, together with a significant amount of a dimeric byproduct (**P3.1di_a**), with an absorbance ratio ($\lambda = 210$ nm) **P3.1s**/**P3.1di_a** of 4:1. Interestingly, **P2**, **P3**, and **P4** were macrocyclized with higher selectivity for iminium reduction than that observed for **P1**. Notably, no alcohol byproduct was detected by HPLC following stapling of the Dap-containing substrate **P4**.

Peptide **P5**, in which the positions of lysine and glutamine are inverted relative to **P1**, was also subjected to one-pot *S*-alkylation/reductive amination in order to assess whether the methodology could enable access to $i \rightarrow i+7$ stapled peptides. As this linear peptide was not soluble at 10 mM in acetonitrile/ammonium bicarbonate buffer (1:1, v/v), the *S*-alkylation step was conducted at a peptide concentration of 5 mM, while maintaining a 1 mM peptide concentration for the reductive amination stage. Intramolecular reductive amination of the peptide aldehyde intermediate **P5.1al** reached only 88% conversion after 6 h. Therefore, the reaction mixture was incubated overnight, which provided the target peptide **P5.1s** in 80% HPLC yield (Table 8, entry 5).

Table 8. Peptide scope.



Entry	Starting Material	m	n	Product	Yield (%) ^{a, b}	s/(s + ol) (%) ^{a, c}
1 ^d	Ac-CTLA K ALQA-NH ₂ (P1)	1	4	P1.1s	86	97
2 ^d	Ac-CTLA Orn ALQA-NH ₂ (P2)	1	3	P2.1s	80	99
3 ^d	Ac-CTLA Dab ALQA-NH ₂ (P3)	1	2	P3.1s	68	99
4 ^d	Ac-CTLA Dap ALQA-NH ₂ (P4)	1	1	P4.1s	86	>99
5 ^e	Ac-CTLAQAL K A-NH ₂ (P5)	4	4	P5.1s	80	96

Z = Lys, Orn, Dab, or Dap. ^a Determined by HPLC–UV (λ = 210 nm); average of duplicate reactions. ^b Calculated based on calibration curves established for **P1.1s**–**P5.1s** (see Figures S11–S15). ^c Calculated as (absorbance area of **s** / combined absorbance area of **s** and **ol**) $\times$ 100. ^d Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 6 h; final solvent composition: MeOH/MeCN/H₂O 14:1:5 (v/v/v). ^e Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (25 mM, pH 8.0) 2:2, [peptide] = 5 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (333.3 mM, pH 4.0) 13:3, [peptide] = 1 mM, 23 °C, 24 h; final solvent composition: MeOH/MeCN/H₂O 13:2:5 (v/v/v).

The use of a citrate buffer at pH 5.0 (rather than pH 4.0) during the reductive amination step was also evaluated, as these two pH values had previously afforded comparable cyclization yields for the peptide model. This pH change had little influence on the stapling efficiency: the yields of **P2.1s**, **P3.1s**, and **P5.1s** were only slightly lower (79% vs 80%, 62% vs 68%, and 79% vs 80%, respectively), whereas that of **P4.1s** increased marginally from 86% to 87%, a difference that may not be significant.

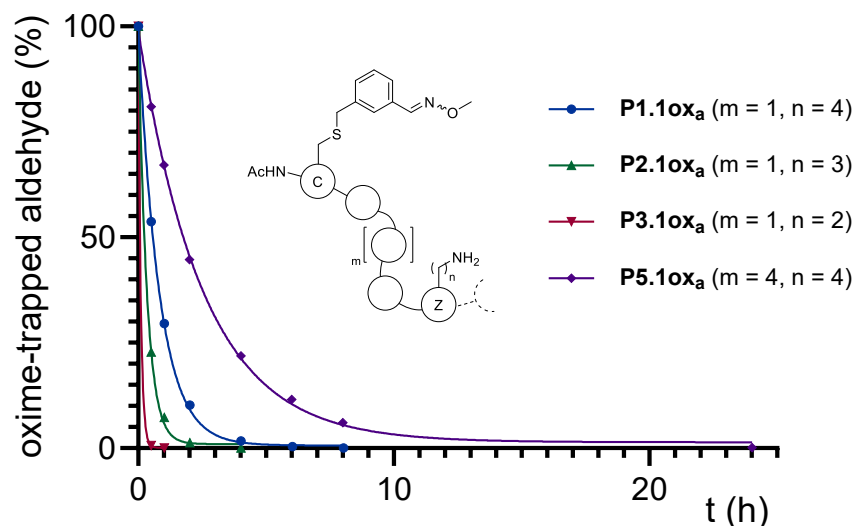


Figure 13. Kinetic profiles of intramolecular reductive amination of peptide aldehydes (**al**): normalized absorbance area of oxime-trapped species (**ox_a**) versus time. Series shown: **P1.1ox_a**; **P2.1ox_a**; **P3.1ox_a**; **P5.1ox_a**.

The differences in reactivity observed among substrates **P1–P5** prompted us to further investigate the kinetics of the intramolecular reductive amination step. Accordingly, the conversion of each peptide aldehyde intermediate, trapped as the corresponding oxime following treatment with methoxylamine, was monitored over time by HPLC–UV (Figure 13). Among peptide aldehydes **P1.1al–P4.1al**, shortening the alkylamine side chain from lysine to Dap was found to markedly accelerate reductive amination. Strikingly, cyclization of **P3.1al** was already complete within 1 h after addition of NaBH₃CN, whereas the oxime-trapped derivative of **P4.1al** was already undetectable at the first sampling time (30 min). We hypothesized that the observed reactivity trend of the alkylamine side chains (Lys < Orn < Dab < Dap) could be rationalized by a combination of reduced conformational flexibility and decreased basicity of the corresponding amine. In terms of basicity, and considering lysine and diaminopropionate as representative extremes, the Dap β-amine ($pK_a \sim 5\text{--}8$)^[188,189] is expected to be deprotonated to a greater extent than the Lys ε-amine ($pK_a \sim 10\text{--}$

11)^[147,148,184] under the conditions used, thereby facilitating intramolecular nucleophilic addition to the tethered aldehyde. Consistent with this interpretation, differences in the protonation states of amine groups have been previously invoked by the groups of Chou, Baran, and Mayer to explain the selective modification of N-terminal α -amines ($pK_a \sim 8$) by intermolecular^[147] or intramolecular^[148,184] reductive *N*-alkylation in the presence of an unprotected lysine side chain.

Compared with **P1.1al**, which features an $i \rightarrow i+4$ spacing between the cysteine side chain bearing a benzaldehyde moiety and the lysine residue, the slower cyclization of **P5.1al** can be reasonably attributed to the increased conformational flexibility imposed by its $i \rightarrow i+7$ Cys–Lys spacing, or to a reduced conformational predisposition to cyclization. The slightly lower proportion of methanol (65% rather than 70%, v/v) in the reaction medium may also have contributed to the slower cyclization rate.

Finally, within the complete series **P1–P5**, a slower rate of intramolecular reductive amination renders the undesired aldehyde reduction more competitive, with the lowest selectivity for iminium reduction (96%) observed upon treatment of **P5.1al** with NaBH_3CN .

Despite these modest differences in reactivity, peptides **P2–P5** were all successfully stapled on a larger scale (30–41 μmol) and subjected to semi-preparative HPLC to afford pure macrocycles **P2.1s–P5.1s**, with ring sizes ranging from 22 to 34 atoms. This demonstrated the synthetic utility of the *S*-alkylation/reductive amination stapling methodology.

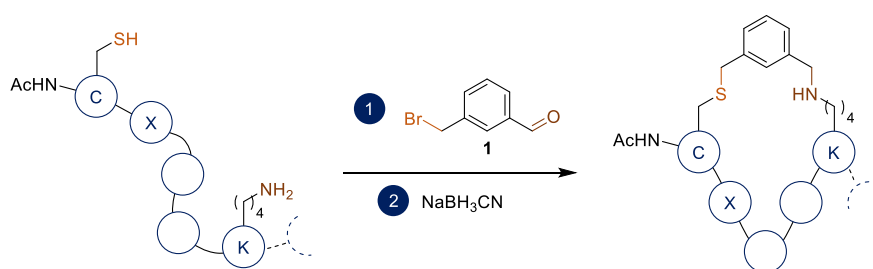
5.3.2. Chemoselectivity Studies

5.3.2.1. Tolerance toward Nucleophilic Residues

Cysteine *S*-benzylation reactions^[90,93,162] and peptide cyclization processes based on NaBH_3CN -mediated reductive amination^[143,148,184] are known to be compatible with all canonical amino acid side chains. However, to confirm the chemoselectivity of the stapling strategy, we sought to apply it to peptides incorporating nucleophilic residues, including serine (S), methionine (M), tyrosine (Y), arginine (R), histidine (H), and tryptophan (W). Accordingly, linear hexapeptides **P6–P11** (Ac-

CXLAKA-NH₂), bearing an *i* → *i*+4-spaced Cys–Lys residue pair and a variable residue at position 2 (X = S, M, Y, R, H, and W, respectively), were stapled with reagent **1** according to the optimized protocol on a 1 μmol scale. In each case, the procedure proved highly effective, with the desired stapled peptide accounting for at least 94% of the combined UV peak area (λ = 210 nm) of all relevant peptide-derived species, and only minor formation of alcohol, disulfide, dimeric, or over-alkylated byproducts (Table 9). No byproducts arising from intramolecular capture of the iminium intermediate by a nucleophilic side chain were detected.

Table 9. Macrocyclization of peptides with nucleophilic side chains.^a



Entry	Starting Material	Sequence	X	Product	Absorbance Ratio (%) ^b
1	P6	Ac-CXLAKA-NH ₂	S	P6.1s	97
2	P7		M	P7.1s	94
3	P8		Y	P8.1s	97
4	P9		R	P9.1s	96
5	P10		H	P10.1s	96
6	P11		W	P11.1s	95

^a Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 24 h. ^b Determined by HPLC–UV (λ = 210 nm); calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) × 100.

Notably, the compatibility of the stapling procedure toward methionine is highly encouraging, given that several reported thiol-to-amine macrocyclization methods either did not document such tolerance or were shown to fail in the presence of methionine.^[73,132,190]

5.3.2.2. Comparison of S-Alkylation/Reductive Amination and S,N-bis-Alkylation

Although the one-pot stapling methodology described herein has the potential to cross-link cysteine and lysine residues with non-symmetrical linkers, the *m*-xylylene linker that is generated upon peptide S-alkylation with **1** followed by reductive amination is symmetrical. In principle, the resulting stapled peptides could therefore also be accessible via direct peptide S,N-bis-alkylation, with α,α' -dibromo-*m*-xylene as the stapling reagent. Bis(bromomethyl)benzene-based reagents have indeed been successfully employed to cyclize peptides through successive alkylation of a cysteine thiol and an N-terminal α -amine.^[73,190] Applied to Cys–Lys cross-linking, this approach would offer the advantage of enabling peptide stapling without the need to modify reaction conditions between the S-alkylation and N-alkylation steps, thereby improving operational simplicity relative to sequential S-alkylation and reductive N-alkylation. However, given the high degree of protonation of lysine side chains under aqueous conditions, we anticipated that intramolecular nucleophilic attack of a lysine ϵ -amine onto a bromomethyl moiety would be less favorable than that of an N-terminal α -amine. To test this hypothesis and compare the efficiency of both macrocyclization strategies, the peptide model **P1** was reacted with α,α' -dibromo-*m*-xylene under the conditions reported by Frost and coworkers for N-terminal-proline-to-cysteine cyclization, namely using 1.2 equiv. of the bis-electrophile in a 1:1 (v/v) mixture of sodium phosphate buffer (pH 8.0) and acetonitrile (Figure 14a,b).^[190] At a peptide concentration of 1 mM, this method enabled formation of the target peptide **P1.1s**. However, after incubation for 8 h, its UV peak area reached only 49% of that obtained using the S-alkylation/reductive amination protocol. The linear dimeric byproduct **P1ld**, resulting from two successive intermolecular nucleophilic displacements of the benzyl bromide groups by a cysteine thiol, was the major byproduct observed by HPLC, with a UV peak area ($\lambda = 210$ nm) corresponding to approximately 78% of that of **P1.1s**. Notably, formation of this byproduct is precluded when using stapling reagent **1**, thereby contributing significantly to the higher stapling yield obtained with our optimized one-pot procedure. In addition, the *N*-[*m*-(bromomethyl)benzyl]-substituted tertiary amine (**P1.1Nb**), arising from intermolecular N-alkylation or reductive N-alkylation of **P1.1s** with

excess stapling reagent, was found to be formed in significantly greater amounts under *S,N*-bis-alkylation conditions than under *S*-alkylation/reductive amination conditions, representing 9% and 1% of the total peptide-related UV signal, respectively, after incubation for 8 h.

Performing *S,N*-bis-alkylation with ammonium bicarbonate buffer as the aqueous component of the solvent system, rather than sodium phosphate, also proved less efficient than *S*-alkylation/reductive amination. Indeed, the UV peak area of **P1.1s** reached only 52% of that obtained using the *S*-alkylation/reductive amination methodology.

As we anticipated the Dap residue in nonapeptide **P4** to be less basic than the lysine residue of **P1**, we hypothesized that applying the *S,N*-bis-alkylation protocol to **P4** would afford the target cyclic peptide **P4.1s** in a higher yield than **P1.1s**. Using the same conditions as for the bis-alkylation of **P1**, **P4** was incubated with 1.2 equiv. of α,α' -dibromo-*m*-xylene, and the formation of the stapled peptide **P4.1s** was monitored over time. Its highest absorbance area, obtained after 1 h of incubation, amounted to 81% of that achieved via *S*-alkylation/reductive amination. This observation indicates that the difference in efficiency between the two methodologies may be less pronounced for Cys–Dap stapling than for Cys–Lys stapling.

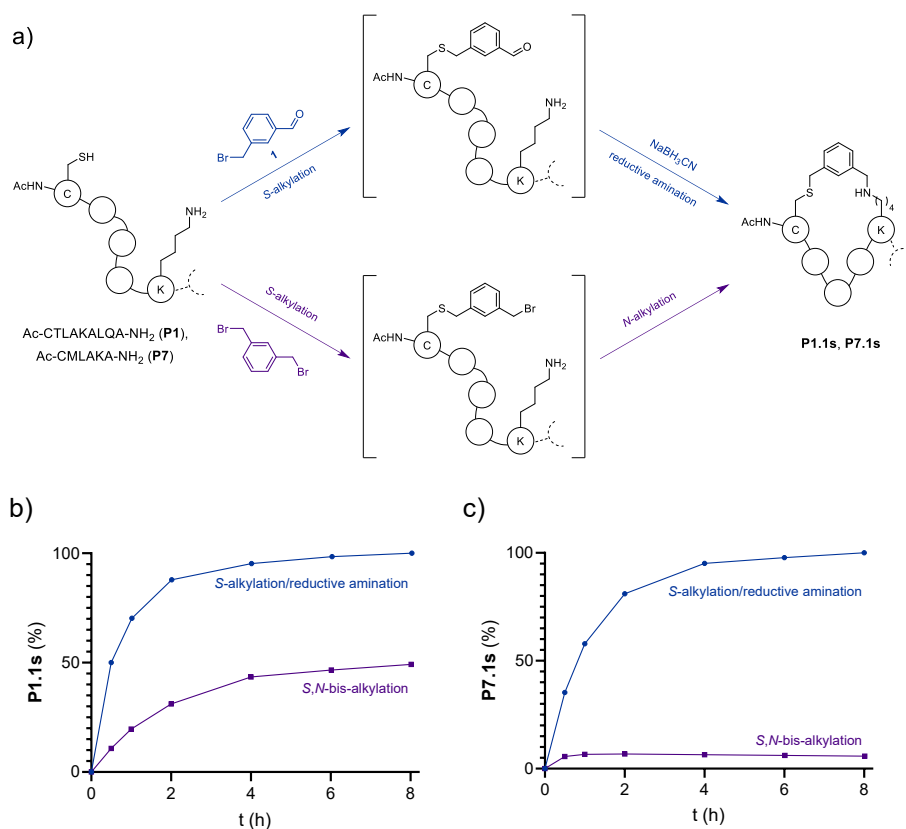


Figure 14. Comparison of two macrocyclization strategies. (a) Reaction overview for *S*-alkylation/reductive amination^a and *S,N*-bis-alkylation^b applied to peptides **P1** and **P7**. (b) Kinetic profile for formation of **P1.1s** via both methods.^{c, d} (c) Kinetic profile for formation of **P7.1s** via both methods.^{c, d}

^a Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH_4HCO_3 (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH_3CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C. ^b Reaction conditions: α,α' -dibromo-*m*-xylene (1.2 equiv.), MeCN/aq. sodium phosphate (50 mM, pH 8.0) 1:1, [peptide] = 1 mM, 23 °C. ^c Absorbance areas of macrocyclic products **P1.1s** and **P7.1s** were extracted from HPLC–UV chromatograms ($\lambda = 210$ nm), normalized to the highest value obtained across the two reactions, and plotted against the quench time. ^d Time zero (t_0) corresponds to the addition of NaBH_3CN in the *S*-alkylation/reductive amination method, and to the addition of α,α' -dibromo-*m*-xylene in the *S,N*-bis-alkylation method.

Using hexapeptide **P7** as a linear substrate, Cys–Lys stapling via *S*-alkylation/reductive amination was previously shown to tolerate the

presence of a methionine residue. In contrast, treatment of **P7** with α,α' -dibromo-*m*-xylene afforded the Cys–Lys stapled peptide **P7.1s** in very low yield. The highest UV peak area, measured after 2 h of incubation, reached only 7% of that achieved through the S-alkylation/reductive amination protocol (Figure 14a,c). This poor yield can be readily rationalized: following initial S-alkylation of the cysteine residue of **P7** with α,α' -dibromo-*m*-xylene, intramolecular N-alkylation of the lysine residue is outcompeted by two faster pathways, as evidenced by the rapid formation of three major byproducts detected in large amounts within 30 min. Intermolecular nucleophilic substitution of the remaining bromide leaving group by the cysteine residue of a second **P7** molecule generates the linear dimer **P7Id**, whereas intramolecular alkylation of the methionine thioether affords two diastereomeric cyclic sulfonium species, **P7su_a** and **P7su_b** (see related chromatograms).

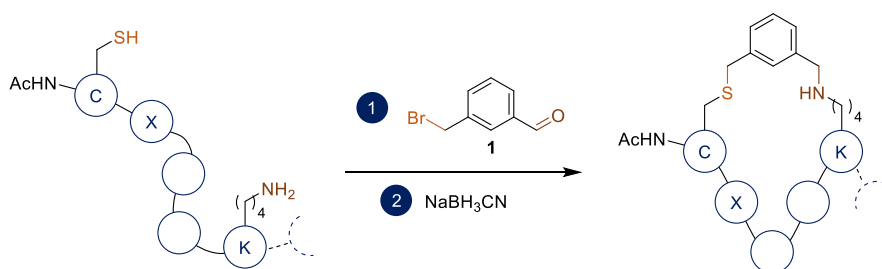
Overall, the use of *m*-(bromomethyl)benzaldehyde (**1**) proved to be superior to that of α,α' -dibromo-*m*-xylene for cross-linking cysteine and lysine residues with an *m*-xylylene tether, and that superiority was even more pronounced in the presence of a methionine residue.

5.3.2.3. Tolerance toward Protected Lysine Residues

Lysine residues are relatively prevalent within peptides and proteins.^[6,44] Site-selective installation of a single Cys–Lys staple on linear lead peptides containing multiple lysine residues represents a challenge. In such cases, incorporation of protected lysine residues into the peptide sequence to mask non-targeted ϵ -amines, followed by peptide stapling and final deprotection, constitutes a viable strategy, albeit at the cost of increased synthetic effort. To further probe the generality of the optimized S-alkylation/reductive amination method, stapling was therefore attempted on linear precursors (Ac-CXLAKA-NH₂) incorporating a protected lysine residue at position 2. In this context, hexapeptides **P12** and **P13** (X = Nle(ϵ -N₃) and Lys(N ϵ -Alloc), respectively) were examined as substrates, since the use of azides^[191-193] and O-allylcarbamates^[57,64,194] as amine masking groups represents two well-established and relatively atom-economical approaches in peptide chemistry. Gratifyingly, macrocyclization of both peptides yielded the desired macrocyclic compounds **P12.1s** and **P13.1s** as the

predominant products (Table 10). No byproducts arising from reduction of the azido moiety or from removal of the allyloxycarbonyl (Alloc) group were detected, further demonstrating the mildness of the reductive amination conditions.

Table 10. Macrocyclization of peptides with protected lysine residues.^a



Entry	Starting Material	Sequence	X	Product	Absorbance Ratio (%) ^b
1	P12	Ac-CXLAKA-NH ₂	Nle(ϵ -N ₃)	P12.1s	96
2	P13		Lys(<i>N</i> ϵ -Alloc)	P13.1s	98

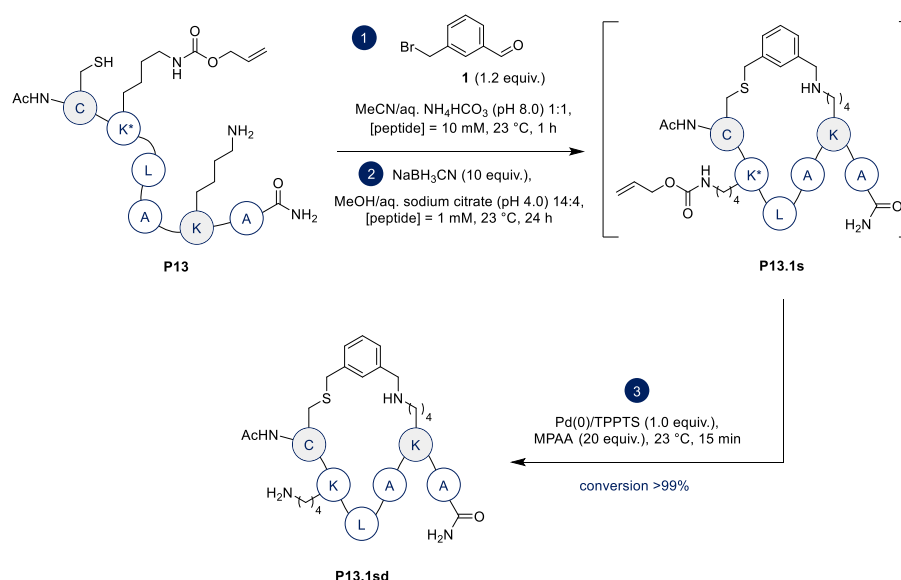
^a Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 24 h. ^b Determined by HPLC–UV (λ = 210 nm); calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) $\times$ 100.

5.3.2.4. One-Pot S-Alkylation/Reductive Amination/Pd-Mediated Deallyloxycarbonylation

Interestingly, deprotection of the Alloc-protected lysine residue of **P13.1s** could be carried out directly through palladium-mediated *N*-deallyloxycarbonylation, without the need for prior purification of **P13.1s** (Scheme 18). Complete removal of the Alloc moiety was achieved within 15 min, using one equivalent of a Pd(0) species generated from Pd(OAc)₂ and triphenylphosphine-3,3',3''-trisulfonic acid trisodium salt (TPPTS), together with an excess of 4-mercaptophenylacetic acid (MPAA) as the allyl scavenger. The deprotected cyclic peptide **P13.1sd** was obtained as the major product. The only peptidic byproduct formed during the deprotection step

exhibited an m/z value of 862 (+2 relative to that of **P13.1s**) and may have resulted from undesired hydrogenation of the carbon–carbon double bond of the Alloc moiety, thereby preventing subsequent palladium-mediated cleavage. Nevertheless, its UV peak area accounted for only 1–2% of that of the desired deprotected compound. These results highlight the potential of a one-pot *S*-alkylation/reductive *N*-alkylation/palladium-mediated *N'*-deallyloxycarbonylation sequence as a suitable chemical approach for synthesizing stapled peptides featuring a single Cys–Lys tether from linear precursors in which non-targeted lysine ϵ -amines are protected.

Of note, reducing the loading of the Pd(0)/TPPTS species below 1.0 equiv. resulted in incomplete conversion of **P13.1s**.

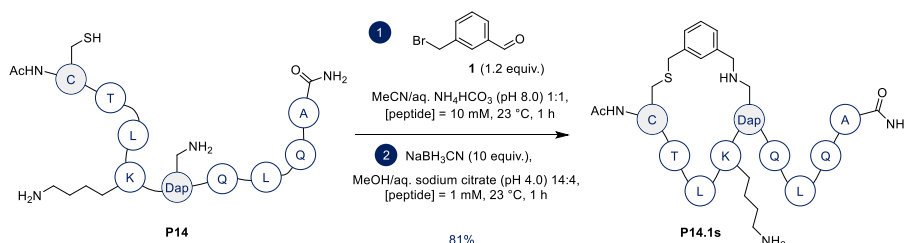


Scheme 18. One-pot *S*-alkylation/reductive *N*-alkylation/Pd-mediated *N'*-deallyloxycarbonylation.

$\text{K}^* = \text{Lys}(\text{N}\epsilon\text{-Alloc})$.

5.3.3. Cys–Dap Stapling in the Presence of a Lysine Residue

The striking difference observed in the rate of NaBH₃CN-mediated cyclization of peptide aldehydes **P1.1al** and **P4.1al** (see Section 5.3.1) prompted us to investigate whether selective Cys–Dap stapling could be achieved in peptides bearing one cysteine, one diaminopropionate, and one unprotected lysine residue. To this end, the linear peptide **P14** (Ac-CTLKDapQLQA-NH₂) was selected, as $i \rightarrow i+3$ Cys–Lys and $i \rightarrow i+4$ Cys–Dap stapling would both lead to 22-membered macrocycles, thereby minimizing the influence of conformational flexibility on the outcome of the stapling reaction in terms of selectivity. After *S*-alkylation of **P14** (1 μmol) followed by direct reductive *N*-alkylation under the optimized conditions, only a single compound with the expected *m/z* value (1134) was detected, suggesting the formation of a single stapled peptide with complete selectivity (Scheme 19). The stapled peptide was resynthesized on a larger scale (34 μmol), purified, and subjected to MS/MS analysis, which confirmed the expected Cys–Dap connectivity.



Scheme 19. Cys–Dap stapling in the presence of an unprotected lysine residue.

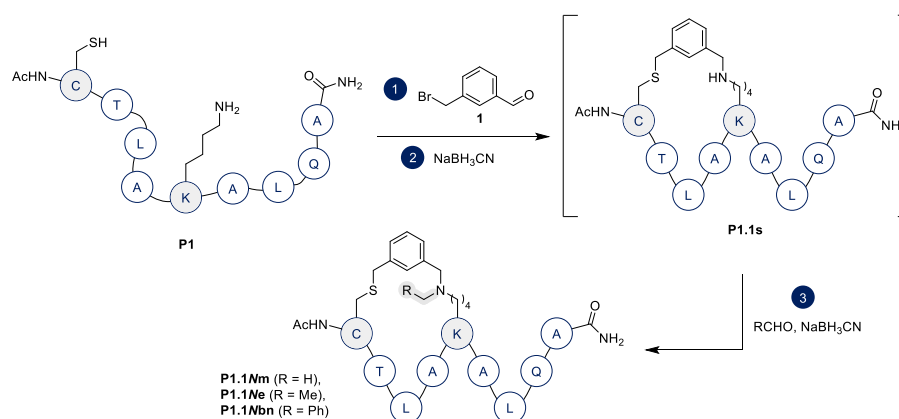
These findings are very promising, as they suggest the feasibility of rapid and selective stapling of peptides containing cysteine and diaminopropionate residues without interference from other canonical residues. Given the low natural abundance of cysteine residues and the non-proteinogenic nature of diaminopropionate, lead peptides could in principle be optimized by simple mutation of two solvent-exposed residues to cysteine and diaminopropionate, which could

subsequently be covalently linked via two-component macrocyclization. Nevertheless, although the Cys–Dap selectivity observed for **P14** was remarkably high, with no Cys–Lys cross-linked peptide detected, a decrease in selectivity cannot be excluded when applying the S-alkylation/reductive amination methodology to peptides in which cysteine and lysine residues are in closer proximity ($i \rightarrow i+1$ or $i \rightarrow i+2$ relationship), or in which cysteine and diaminopropionate residues are more distant ($i \rightarrow i+7$ relationship, for instance).

5.4. Post-Stapling Modification via Intermolecular Reductive Amination

Cyclic secondary amines generated through the stapling approach presented herein remain nucleophilic and can therefore be further modified by reaction with external or internal electrophiles. Notably, upon completion of the intramolecular reductive amination step, the stapled peptide **P1.1s** was successfully *N*-methylated or *N*-ethylated by direct addition of formaldehyde or acetaldehyde to the reaction mixture, along with an additional 10 equiv. of reducing agent (Table 11, entries 1–3).

Table 11. One-pot derivatization of **P1.1s** via intermolecular reductive amination.^a



Entry	Starting Material	External Aldehyde	Product	Time, Step 3 (h)	Conversion (%) ^b
1	P1	Formaldehyde (R = H)	P1.1Nm	12	>99
2		Acetaldehyde (R = Me)	P1.1Ne	12	97
3				18	>99
4		Benzaldehyde (R = Ph)	P1.1Nbn	12	17
5				18	26
6				36	39

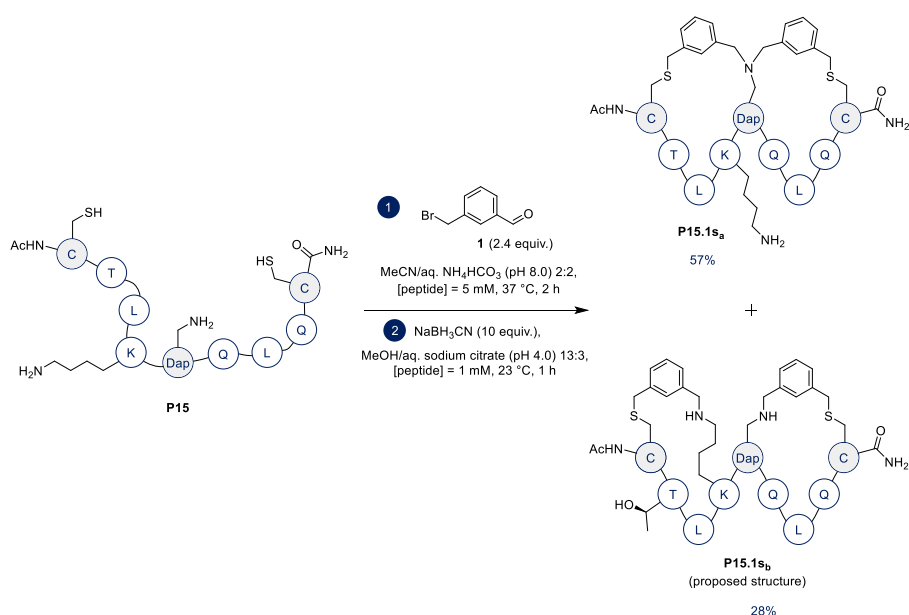
^a Reaction conditions: 1) **1** (1.2 equiv.), MeCN/aq. NH_4HCO_3 (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; 2) NaBH_3CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 6 h; 3) RCHO (10 equiv.), NaBH_3CN (10 equiv.), 23 °C. ^b Conversion of **P1.1s** during step 3 (derivatization step); determined by HPLC–UV (λ = 210 nm); calculated as $[1 - (\text{absorbance area of } \mathbf{P1.1s} \text{ at } t / \text{absorbance area of } \mathbf{P1.1s} \text{ at } t_0)] \times 100$.

Intermolecular reductive *N*-alkylation of **P1.1s** with benzaldehyde was markedly sluggish (Table 11, entries 4–6), reaching only 39% conversion after 36 h. This was substantially slower than the cyclization step of the stapling process (i.e., the intramolecular reductive amination between the primary ϵ -amine of **P1.1aI** and its tethered benzaldehyde moiety). Interestingly, this observation suggests that, under our optimized acidic stapling conditions, even a large excess of benzaldehyde-based stapling reagent would be unlikely to induce significant over-*N*-alkylation.

5.5. Bicyclization via Intramolecular bis-Reductive Amination

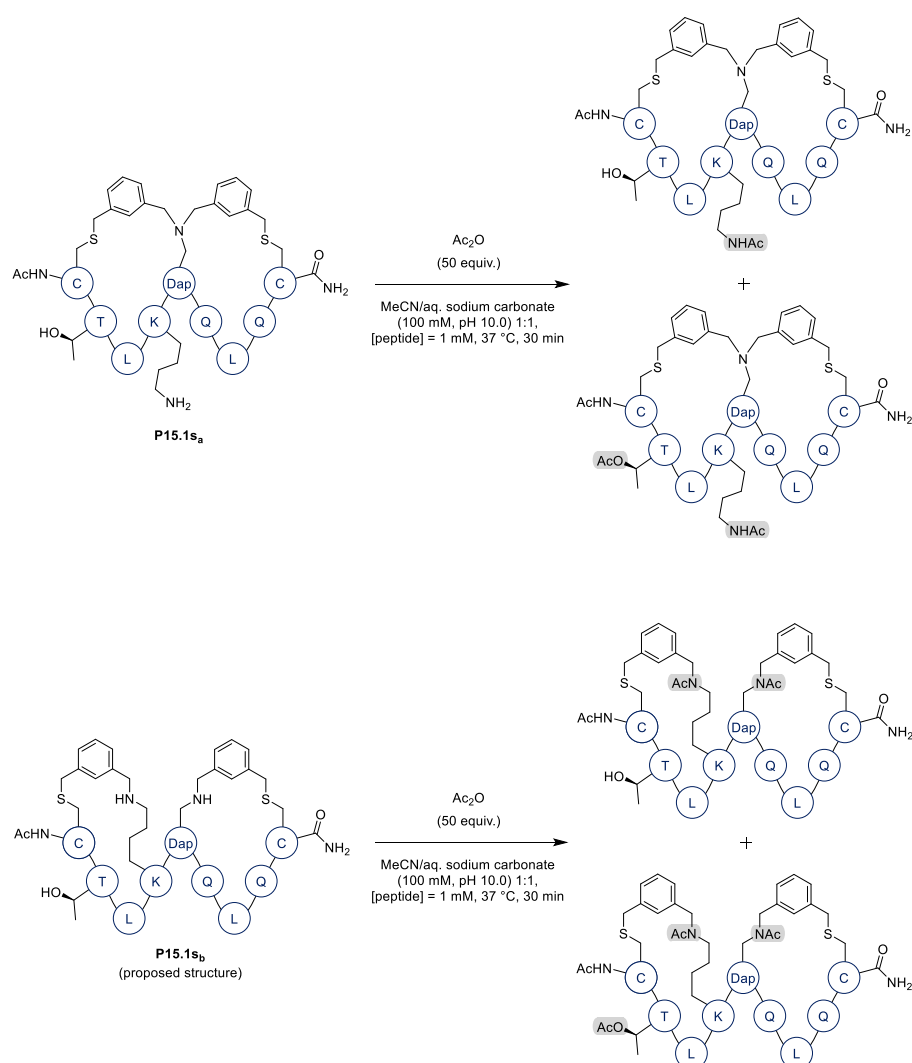
The preservation of amine nucleophilicity following our stapling procedure offers another highly attractive derivatization opportunity. In fact, performing two successive intramolecular reductive amination reactions can yield bicyclic compounds from peptides bearing one amine and two aldehyde moieties. Accordingly, we envisioned a bicyclization approach based on bis-*S*-alkylation of a peptide dithiol with two equiv. of stapling reagent **1**, followed by bis-reductive amination involving the unmodified amine side chain and the two installed benzaldehyde moieties. This strategy was investigated using peptide dithiol **P15** (Ac-CTLKDapQLQC-NH₂), featuring a second cysteine residue in place of the C-terminal alanine of **P14** (Scheme 20). In contrast to other peptide dithiols examined, **P15** had the advantage of being soluble at 5 mM in acetonitrile/ammonium bicarbonate buffer (1:1, v/v), notably owing to the presence of the unprotected lysine at position 4. The presence of this lysine residue was also intended to assess potential competition between two bicyclization pathways: the first NaBH₃CN-mediated cyclization event can be expected to selectively involve diaminopropionate rather than lysine, leading to two possible monocyclic Cys–Dap stapled peptides, whose subsequent cyclization would proceed via reaction of the remaining aldehyde group with either the *N*-alkylated diaminopropionate β -amine or the free lysine ϵ -amine. In practice, **P15** was treated with 2.4 equiv. of **1**, and the mixture was incubated for 2 h at 37 °C to ensure complete conversion of **P15** and its mono-*S*-alkylated derivatives. The crude peptide bis-aldehyde obtained (**P15.1aI**) was then subjected to the

previously optimized conditions for intramolecular reductive amination. Interestingly, 1 h after the addition of the reducing agent, HPLC analysis revealed three peaks with the expected m/z value (1268), most likely corresponding to the three possible bicyclic compounds, in a 72:25:3 ratio based on their UV peak areas ($\lambda = 210$ nm).



Scheme 20. One-pot bis-S-alkylation/bis-reductive amination.

The two predominant isomers (**P15.1s_a** and **P15.1s_b**) were isolated and treated with acetic anhydride to distinguish primary and secondary amines from tertiary amines (Scheme 21). The major compound (**P15.1s_a**), obtained in 57% HPLC yield, was identified as the bicyclic peptide featuring the desired Cys–Dap–Cys connectivity, whereas the other two isomers presumably include one Cys–Dap and one Cys–Lys linkage. One-pot bis-S-alkylation/bis-reductive amination therefore appears to be an efficient strategy for the preparation of bicyclic peptides from Dap-containing peptide dithiols.



Scheme 21. Acetylation of bicyclic peptides **P15.1s_a** and **P15.1s_b**.

Although satisfactory, the yield of the bicyclic product **P15.1s_a** (57%) was significantly lower than that of the monocyclic peptide **P14.1s** (81%). This observation led us to hypothesize that, after the first cyclization event of bis-aldehyde **P15.1al**, the desired second cyclization process, involving the secondary β-amine generated and the remaining benzaldehyde moiety, could be less kinetically favored than the initial cyclization, thereby allowing competition between the

secondary β -amine and the lysine primary ϵ -amine for reaction with the second aldehyde.

Among the two isomeric peptides featuring two secondary amine moieties, the more abundant species (**P15.1s_b**) would be expected to correspond to the compound bearing $i \rightarrow i+3$ Cys–Lys and $i+4 \rightarrow i+8$ Dap–Cys staples, whereas the minor isomer (**P15.1s_c**) presumably exhibits the alternative connectivity, corresponding to the compound featuring $i \rightarrow i+4$ Cys–Dap and $i+3 \rightarrow i+8$ Lys–Cys staples. Indeed, kinetic data obtained for the cyclizations of peptide aldehydes **P1.1al**–**P5.1al** suggest that intramolecular reductive amination is proximity-driven. The monocyclic intermediate formed through $i+4 \rightarrow i+8$ Dap–Cys stapling may face significant competition between the modified diaminopropionate β -amine and the free lysine ϵ -amine (at positions 3 and 4, respectively) for addition to the remaining benzaldehyde group at position 1, yielding either the desired bicyclic peptide **P15.1s_a** or the double-stapled derivative **P15.1s_b**. In the alternative scenario, in which the initial macrocyclization occurs between cysteine and diaminopropionate residues at positions 1 and 4, the subsequent $i+3 \rightarrow i+8$ Lys–Cys cyclization leading to **P15.1s_c** may proceed considerably more slowly, thereby favoring formation of **P15.1s_a** through reaction of the remaining aldehyde at position 9 with the secondary amine of the central diaminopropionate residue.

5.6. Scope of Stapling Reagents

5.6.1. Cys–Lys Stapling with Benzaldehyde-Based Reagents

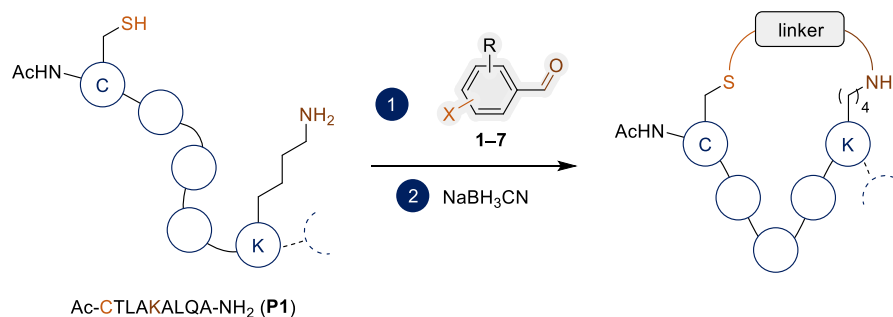
To explore the potential of the current stapling strategy, relying on cysteine modification followed by intramolecular reductive amination, to generate structurally diverse macrocyclic peptides from a single linear precursor, we investigated the stapling of peptide model **P1** using a range of benzaldehyde-based cross-linkers other than reagent **1** (Table 12, entries 2–7). Stapling reactions were carried out on a 1 μ mol scale, and the conversion of each peptide aldehyde intermediate during the cyclization step was monitored by HPLC–UV at predefined time points (30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, and 48 h) following addition of NaBH₃CN (Figure S2). The selectivity for iminium

reduction over aldehyde reduction (calculated as previously described) was used as an indicator of the performance of the stapling process, together with the earliest time point at which a conversion of at least 99% was observed.

First, **P1** was macrocyclized using stapling reagent **2**, a derivative of compound **1** bearing an additional propargyloxy substituent in the *para* position relative to the bromomethyl group (entry 2). Reagent **2** was prepared in two steps from 2-hydroxy-5-(hydroxymethyl)-benzaldehyde. The optimized stapling conditions afforded the desired cyclic peptide **P1.2s** with a 97% selectivity for iminium reduction. Interestingly, cyclization of the intermediate **P1.2al** proved significantly faster than that of **P1.1al**, indicating a positive effect of the propargyloxy moiety on the rate of the reductive amination step. Reagent **2** can thus be considered as a convenient cross-linker for anchoring a functionalizable *m*-xylylene tether between cysteine and lysine side chains.

The substitution pattern of the stapling reagent was then varied by employing commercially available *o*- (**3**) and *p*-(bromomethyl)benzaldehyde (**4**) (entries 3 and 4). The *ortho*-(alkylthiomethyl)benzaldehyde intermediate **P1.3al** underwent faster intramolecular reductive amination than its *meta* analogue **P1.1al**, as complete conversion was observed after incubation for 4 h. Conversely, macrocyclization of the *para*-substituted benzaldehyde derivative **P1.4al** was much slower, requiring more than 24 h of incubation to reach completion. This resulted in a higher proportion of the corresponding alcohol byproduct (5%).

Table 12. Scope of benzaldehyde-based stapling reagents.



Entry		Stapling Reagent	linker	Product	Time, Step 2 (h) ^{a, b}	s/(s + ol) (%) ^{a, c}
1 ^d	1			P1.1s	6	97
2 ^d	2			P1.2s	2	97
3 ^d	3			P1.3s	4	99
4 ^d	4			P1.4s	48	95
5 ^e	5			P1.5s	24	95
6 ^f	6			P1.6s	24	94
7 ^d	7			P1.7s	24	78

X = thiol-reactive group. ^a Determined by HPLC–UV ($\lambda = 210$ nm). ^b Earliest sampling time point at which a conversion >99% was observed; conversion calculated from the absorbance area of the oxime-derivatized peptide aldehyde intermediate.

^c Calculated as (absorbance area of **s** / combined absorbance area of **s** and **ol**) $\times 100$. ^d Reaction conditions: **P1**, stapling reagent (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C; final solvent composition: MeOH/MeCN/H₂O 14:1:5 (v/v/v). ^e Reaction conditions as in d, except for the temperature and duration of the first step (37 °C, 4 h). ^f Reaction conditions: **P1**, **6** (1.2 equiv.), Pd(0)/BiPhePhos (5 mol%), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 3:1, [peptide] = 5 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 12:4, [peptide] = 1 mM, 23 °C; final solvent composition: MeOH/MeCN/H₂O 12:3:5 (v/v/v).

To further demonstrate the versatility of the method, the bromomethyl moiety of reagent **1** was replaced with other thiol-reactive groups. In principle, any irreversible cysteine-modifying moiety described in the literature could be suitable, provided that the resulting linkage is stable toward the subsequent reductive amination conditions. In this study, three alternative thiol-reactive compounds were investigated, namely Michael acceptor **5**, allylating agent **6**, and arylating agent **7** (entries 5–7).

N-arylacrylamide **5** was prepared in two steps from 3-aminobenzyl alcohol before being evaluated for cross-linking of the cysteine and lysine residues of **P1**. As this compound was found to be less reactive than benzylating agent **1**, the initial *S*-modification step via thia-Michael addition required increasing the temperature to 37 °C and extending the reaction duration to 4 h. Subsequent cyclization via NaBH₃CN-mediated reductive amination was complete within 24 h, with a measured selectivity of 95% for iminium reduction.

Breinbauer and coworkers have previously demonstrated that palladium-mediated *S*-allylation reactions are powerful tools for the chemoselective modification of cysteine residues within peptides and proteins, as well as for Cys–Cys stapling.^[127] In particular, they have shown that a catalyst system generated from Pd(dba)₂ and the biphosphite ligand BiPhePhos enables the formation of unbranched products with high regioselectivity.^[127,195] Taking inspiration from their work, allylic acetate **6**, synthesized in two steps from 3-hydroxybenzaldehyde, was evaluated as a Cys–Lys stapling reagent, combining Pd-catalyzed *S*-allylation and subsequent reductive *N*-alkylation. Following a brief screening of the catalyst loading from 1 to 25 mol%, *S*-allylation of **P1** using 1.2 equiv. of **6** and 5 mol% of Pd(0)/BiPhePhos proceeded relatively cleanly in a 3:1 (v/v) mixture of acetonitrile and ammonium bicarbonate buffer, affording peptide aldehyde **P1.6aI** as the major product. Upon reductive amination for 24 h, two main peaks with the desired *m/z* value (1118) were detected by HPLC in a 9:1 ratio ($\lambda = 210$ nm). These two compounds may correspond to *E/Z* stereoisomers or unbranched/branched regioisomers.

Perfluoroaromatic-based reagents have been commonly used for the modification of cysteine residues via nucleophilic aromatic substitution

(S_NAr), particularly in the field of peptide macrocyclization.^[58,67,99-101] Moreover, S_NAr reactions of monosubstituted pentafluorobenzene derivatives are known to proceed regioselectively at the para position with respect to the substituent.^[100,101,196,197] Therefore, we envisioned that pentafluorobenzaldehyde (**7**) could enable cysteine-to-lysine macrocyclization through sequential S-arylation and reductive amination. To our delight, application of our optimized conditions for one-pot S-modification/reductive amination led to efficient formation of the stapled peptide **P1.7s** featuring a tetrafluorinated linker. Consistent with its expected higher electrophilicity compared to **P1.1al**, the *p*-(alkylthio)tetrafluorobenzaldehyde intermediate **P1.7al** (*m/z* 1136) was detected by HPLC as a mixture with its hydrated form (*m/z* 1154), and aldehyde reduction occurred to a greater extent during the cyclization step, resulting in a lower selectivity for iminium reduction (78%).

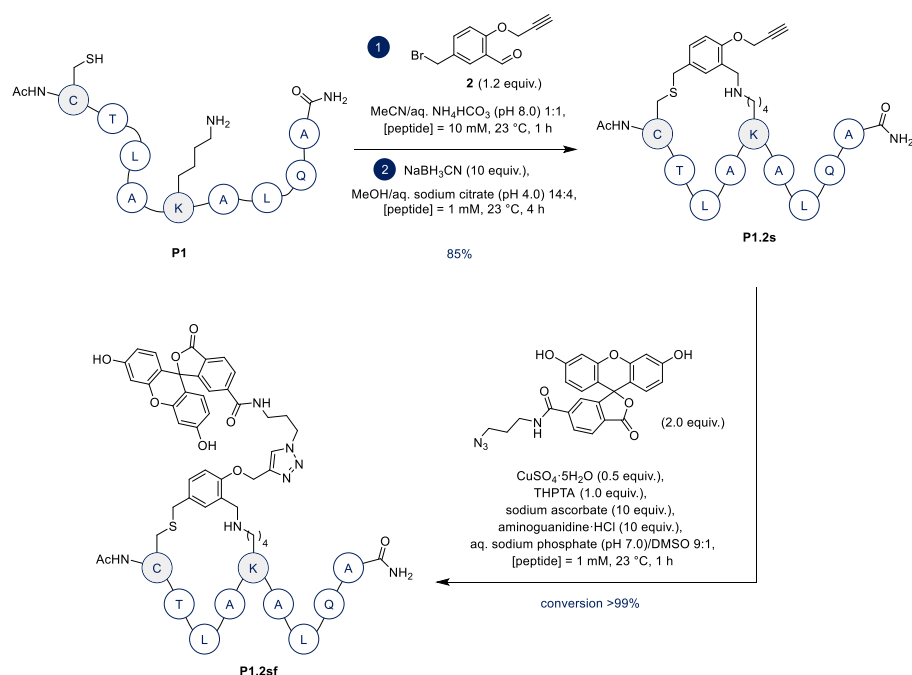
Thus, the substitution pattern of the stapling reagent appears to exert a greater influence on the cyclization rate than the size of the macrocycle to be formed, with the *para*-(alkylthiomethyl)benzaldehyde intermediate **P1.4al** being the slowest to cyclize. Among *meta*-substituted benzaldehyde derivatives, intramolecular reductive amination of intermediates with longer 8-atom and 9-atom linkers (**P1.5al** and **P1.6al**, respectively) was slower than that of **P1.1al**, which features a 5-atom-long linker, presumably due to increased conformational flexibility. However, a rigorous comparison is hampered by the lower methanol content under the conditions employed for stapling via Pd-catalyzed S-allylation/reductive amination. The reduced rate of reductive amination observed for intermediates **P1.4al**–**P1.6al** allowed the competing aldehyde reduction pathway to occur to a greater extent, resulting in a slightly increased amount of the corresponding alcohol byproduct (5–6%). The substantially higher proportion of alcohol observed (22%) upon cyclization of **P1.7al** is more likely related to its increased electrophilicity rather than to a slower reductive amination step.

Overall, the one-pot S-modification/reductive amination strategy, applied to Cys–Lys stapling of the peptide model **P1**, proved effective for the installation of aromatic linchpins, both symmetrical (entries 1, 3, and 4) and non-symmetrical (entries 2, 5–7), with linker sizes ranging from 4 to 9 atoms, affording 24- to 29-membered macrocycles. Furthermore, all benzaldehyde-derived stapling reagents were either

commercially available (reagents **1**, **3**, **4**, and **7**) or readily accessible through two-step syntheses (reagents **2**, **5**, and **6**).

5.6.2. Post-Stapling Modification via Copper-Catalyzed Azide–Alkyne Cycloaddition

Stapled peptide **P1.2s**, whose linker carries an alkyne handle, was also prepared on a preparative scale and subsequently conjugated with 6-carboxyfluorescein azide by CuAAC, yielding the fluorescent peptide **P1.2sf** (Scheme 22). This illustrates that the current strategy can provide stapled peptides bearing a linker amenable to further functionalization.



Scheme 22. Cys–Lys stapling and post-stapling modification via CuAAC.

5.6.3. Cys–Lys Stapling with Chloroacetaldehyde and Methacrolein

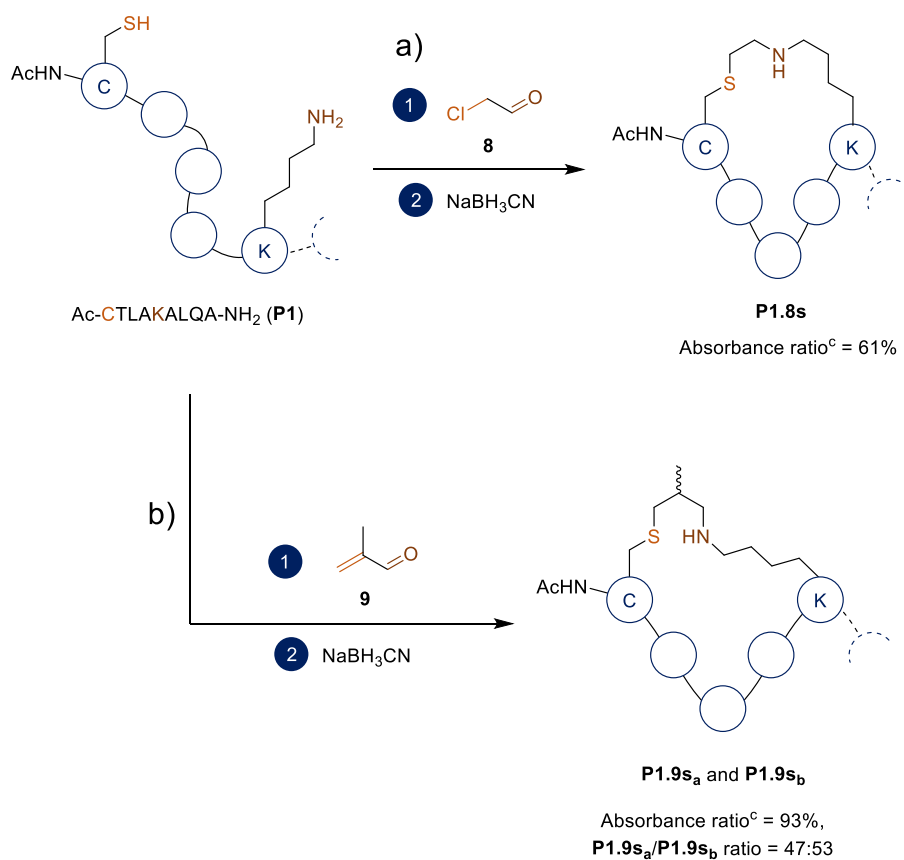
Finally, the installation of short aliphatic linkers via one-pot S-alkylation/reductive amination was explored.

Rathman and Del Valle reported an elegant approach to mimic side-chain-to-backbone hydrogen bonds. Their macrocyclization strategy involved cysteine S-alkylation with chloroacetaldehyde, intramolecular condensation between the aldehyde and a backbone *N*-amino substituent, and subsequent NaBH₃CN-mediated reduction of the resulting hydrazone. Lysine ϵ -amines and N-terminal α -amines were not modified under their cyclization conditions, namely in 1:1 (v/v) mixtures of acetonitrile and aqueous buffer adjusted to pH 2.0.^[183]

Nonetheless, we sought to investigate the use of chloroacetaldehyde (**8**) for Cys–Lys stapling by applying to the cyclization step the conditions previously optimized for intramolecular lysine reductive *N*-alkylation (Scheme 23a). Accordingly, peptide **P1** was subjected to S-alkylation with chloroacetaldehyde. Given the relatively high peptide concentration (10 mM), and the remarkable electrophilicity of this reagent, this step proved very fast. However, in addition to the expected intermediate **P1.8al**, significant amounts of a dimeric species, presumably arising from autocondensation of **P1.8al**, were detected by HPLC after incubation for 30 min–1 h. To limit its formation, the duration of the S-alkylation step was reduced to 15 min. Gratifyingly, subsequent reductive amination in methanol/sodium citrate buffer (3:1, v/v) afforded the desired ethylene-bridged peptide **P1.8s** as the predominant product.

The use of α,β -unsaturated aldehydes was then considered, as this could potentially enable the formation of stapled peptides featuring 3-atom linkers. As acrolein was not readily available, methacrolein (**9**) was selected as a model acrolein analogue. To our delight, Cys–Lys cross-linking of **P1** with methacrolein proved efficient (Scheme 23b). Specifically, thia-Michael addition of the **P1** thiol to methacrolein followed by intramolecular reductive amination, according to our optimized one-pot protocol, yielded the diastereomeric cyclic peptides **P1.9s_a** and **P1.9s_b** with a clean reaction profile.

These findings demonstrate that reductive-amination-based Cys–Lys stapling is compatible with non-aromatic aldehydes as stapling reagents, enabling the introduction of short 2- or 3-atom-long hydrocarbon tethers. To the best of our knowledge, this constitutes the first report of the installation of such linkers via one-pot Cys–Lys cross-linking.



Scheme 23. Cys–Lys stapling using (a) chloroacetaldehyde^a or (b) methacrolein^b.

Reaction conditions: 1) **8** or **9** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 15 min^a or 1 h^b; 2) NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 6 h^a or 2 h^b.

^c Determined by HPLC–UV (λ = 210 nm); calculated as (absorbance area of the target product(s) / combined absorbance area of all relevant peptidic species) × 100.

5.7. Evaluation of α -Helicity of Cys–Lys, Cys–Orn, Cys–Dab, and Cys–Dap Stapled Peptides

To evaluate the ability of the developed stapling strategy to enhance peptide helicity, macrocyclic nonapeptides **P1.1s–P5.1s**, **P14**, and **P15**, all synthesized on a preparative scale using the *meta*-disubstituted reagent **1**, were analyzed by circular dichroism (CD). The CD spectrum of each stapled peptide, recorded at 100 μ M in a 4:1 (v/v) mixture of sodium phosphate buffer (pH 7.4) and TFE, was compared with that of its linear precursor. The mean peptide ellipticity ($[\theta]$) at 222 nm was used to quantify helical propensity. More precisely, the percentage helicity of each peptide was estimated by dividing its $[\theta]_{222}$ value by the theoretical $[\theta]_{222}$ value of a fully folded α -helix of the same length (see Supporting Information for details).

Encouragingly, the $i \rightarrow i+4$ Cys–Lys stapled peptide **P1.1s** displayed a higher helical propensity than the linear peptide model **P1** (Figure 15a), although its percentage helicity remained modest (43%). We postulated that the staple in **P1.1s**, with a total length of 12 atoms (considering the side chains of cysteine and lysine as well as the *m*-xylylene linker), may be too long to provide strong stabilization of one α -helical turn.

As peptide **P1.2s** had to be synthesized on a preparative scale, it was also analyzed by CD, which revealed a slight increase in helicity compared with **P1.1s** (Figure S3). Although this increase may not be significant, we hypothesized that the additional propargyloxy group of **P1.2s** could further rigidify the staple through formation of a 6-membered hydrogen-bonded ring involving the aryl propargyl ether and the benzylic secondary amine, thereby restricting rotation about the C–C and C–N single bonds connecting the aromatic ring to the amine moiety.

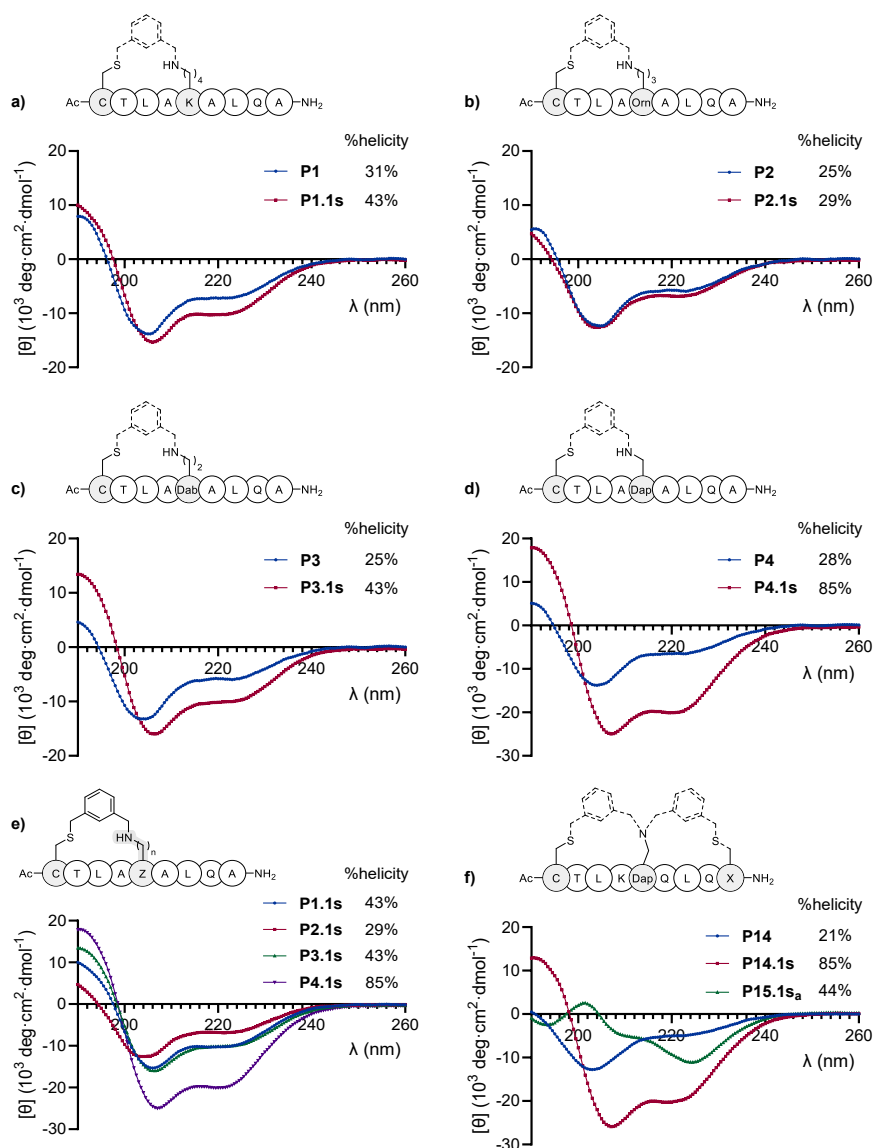


Figure 15. Circular dichroism spectra of representative linear and stapled peptides. (a) **P1** and **P1.1s**. (b) **P2** and **P2.1s**. (c) **P3** and **P3.1s**. (d) **P4** and **P4.1s**. (e) **P1.1s**, **P2.1s**, **P3.1s**, and **P4.1s**. (f) **P14**, **P14.1s**, and **P15.1s_a**.

Z = Lys (**P1.1s**), Orn (**P2.1s**), Dab (**P3.1s**), or Dap (**P4.1s**). X = Ala (**P14**, **P14.1s**) or Cys (**P15.1s_a**). Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.4)/trifluoroethanol (4:1, v/v); T = 20 $^{\circ}$ C; three replicates. %helicity calculated from $[\theta]_{222}$ (see SI for details).

Cys–Orn, Cys–Dab, and Cys–Dap stapled peptides **P2.1s**, **P3.1s**, and **P4.1s** were then investigated, with the expectation that shortening the staple might result in increased helical content. The Cys–Orn cross-linked compound **P2.1s**, featuring a 11-atom staple, displayed only a slight increase in helicity relative to the unconstrained peptide **P2**, from 25% to 29% (Figure 15b). In contrast, Cys–Dab and Cys–Dap stapling of the linear peptides **P3** and **P4**, respectively, both induced a marked enhancement of α -helical propensity, as evidenced by the increased signal intensity at 190 nm and 222 nm in the CD spectra (Figure 15c,d).

Among the four $i \rightarrow i+4$ stapled peptides **P1.1s–P4.1s**, the Dap-containing macrocycle **P4.1s** clearly emerged as the most helical, with a measured percentage helicity of 85% (Figure 15e). The geometry of the Cys–Dap staple, being the shortest evaluated herein (9-atom length), may therefore be considered suitable for stabilizing the helical conformation of $i \rightarrow i+4$ cross-linked peptides. On the other hand, the unexpectedly low helicity of the cysteine-to-ornithine cyclized peptide **P2.1s** may tentatively be attributed to hydrogen bonding between the δ -amine of the modified ornithine residue and nearby backbone amide groups, thereby disfavoring the formation of the $i \rightarrow i+4$ main-chain hydrogen bonds required for α -helicity.

Stapling involving i and $i+7$ residues is a common strategy for stabilizing two consecutive helical turns. In the context of $i \rightarrow i+7$ stapling, staples with a total length ranging from 11 to 14 atoms are generally optimal.^[6,11,58,98,114,116,121] Therefore, we anticipated that Cys–Lys stapling using cross-linker **1**, which results in a 12-atom-long staple, could represent a suitable approach to generate $i \rightarrow i+7$ stapled peptides exhibiting high helicity. Surprisingly, $i \rightarrow i+7$ Cys–Lys stapling had a detrimental impact on the helicity of peptide **P5** (Figure S7).

As for **P4**, Cys–Dap stapling of **P14** considerably enhanced its helical propensity in a 4:1 buffer/TFE mixture, with percentage helicity increasing from 21% to 85% (Figure 15f). This result is particularly noteworthy given that **P14**, which contains only one alanine residue rather than three, was less helical than **P4**. The macrocyclic peptide **P14.1s** also displayed a reasonable helicity (60%) in buffer alone, without the use of TFE as helix-inducing solvent (Figure S9).

In contrast, the bicyclic peptide **P15.1s_a**, featuring an additional *m*-xylylene tether between the *N*-alkylated diaminopropionate residue

and the ninth residue, showed no positive maximum at 190 nm and a reduced signal intensity at 222 nm, suggesting a loss of helicity compared with its monocyclic counterpart **P14.1s** (Figure 15f). Interestingly, the bicyclic compound **P15.1s_b**, which incorporates one Cys–Lys staple and one Cys–Dap staple, was found to be more helical than its isomer **P15.1s_a** (Figure S10).

Taken together, these findings suggest that $i \rightarrow i+4$ Cys–Dap stapling using *m*-(bromomethyl)benzaldehyde constitutes a promising methodology for the generation of helical peptides. Cys–Lys and Cys–Dab stapling performed in an $i \rightarrow i+4$ fashion also induced measurable helix-stabilizing effects in this study, although these effects were less pronounced than those observed for Cys–Dap stapling. This difference may be related to the greater conformational flexibility of the longer Cys–Lys and Cys–Dab staples.

Based on their $[\theta]_{222}/[\theta]_{208}$ ratios, the most helical peptides identified in this study, **P4.1s** ($[\theta]_{222}/[\theta]_{208} = 0.80$) and **P14.1s** ($[\theta]_{222}/[\theta]_{208} = 0.77$) are likely to adopt a mixed α -helix/ 3_{10} -helix conformation in water/TFE 4:1, with a relatively high α -helical contribution.

The conformation of **P14.1s**, which was more water-soluble than **P4.1s**, was also investigated via 2D NMR using TOCSY and ROESY experiments. However, due to overlapping peaks in both the amide NH and H- α regions, nuclear Overhauser effects (NOEs) characteristic of helical conformations could not be unambiguously identified, except for two sequential d_{NN} ($i, i+1$) NOEs involving the T2, L3, and K4 residues.

5.8. Strengths and Limitations of the Developed Cys–Lys Stapling Methodology

We have developed a high-yielding, methionine-compatible, and operationally simple thiol-to-amine stapling method based on one-pot S-alkylation/reductive amination, which can be implemented using commercially available aldehydes as cross-linking reagents. The macrocyclization protocol was first optimized using a peptide model bearing an $i \rightarrow i+4$ -spaced Cys–Lys residue pair, revealing that a combination of methanol and aqueous buffer at pH 4.0 is suitable for limiting the extent of aldehyde reduction. Ethanol can also be used as a greener alternative to methanol. The methodology was subsequently applied to peptides with shorter alkylamine side chains, demonstrating the feasibility of Cys–Orn, Cys–Dab, and Cys–Dap stapling, as well as to peptides bearing nucleophilic side chains or protected lysine residues, highlighting the excellent functional group tolerance of the method.

Macrocyclization of cysteine and diaminopropionate residues at positions i and $i+4$ using *m*-(bromomethyl)benzaldehyde (**1**) emerged as a powerful stapling approach, owing to the fast rate of intramolecular reductive amination, which prompted consideration of chemoselective Cys–Dap stapling in the presence of unprotected lysine residues. Furthermore, Cys–Dap stapled peptides exhibited a high helical propensity, as evidenced by CD studies.

The scope of the S-modification/reductive amination protocol was further explored by varying the stapling reagent. Cyclized products featuring a range of linkers, either symmetrical or non-symmetrical, were obtained on an analytical scale. Accordingly, the current strategy offers the potential for the rapid generation of macrocyclic peptides with different sizes and tunable linker properties, including rigidity and hydrophobicity. Notably, the helicity of a given lead peptide may be optimized by adjusting the geometry of the linker, a point that likely represents the most significant perspective arising from this work.

Post-stapling derivatization of the macrocyclic peptides could be achieved through additional intermolecular or intramolecular reductive amination, leading to *N*-alkylated derivatives or bicyclic peptides,

respectively, or via CuAAC enabled by a propargyloxy-substituted *m*-xylylene tether.

The method described herein complements existing Cys–Lys stapling approaches (based on *N*-acylation or on the formation of isoindole or pyrrole heterocycles) for several reasons: 1) the targeted lysine residue remains basic after reductive *N*-alkylation, thereby preserving its positive charge under physiological conditions, which can be desirable for solubility or when this residue participates in the PPI of interest through contacts with negatively charged or aromatic residues on the binding partner; 2) reductive amination also maintains the nucleophilicity of the amine group, enabling post-stapling diversification through reaction with external or internal electrophiles; and 3) as proposed by Baran and coworkers, intramolecular reductive *N*-alkylation, which relies on reversible imine formation, may be superior to intramolecular *N*-acylation for linear substrates that are not predisposed to macrocyclization, since competitive hydrolysis of the acylating moiety can reduce the cyclization efficiency in such cases.^[148]

Nevertheless, the thiol-to-amine stapling method reported in this work is associated with non-negligible limitations. First, the use of NaBH₃CN as the reducing agent raises safety concerns due to the potential release of HCN gas under acidic conditions. This risk was mitigated by performing all reductive amination reactions, including work-up, in a well-ventilated fume hood and by avoiding the use of NaBH₃CN in solutions with a pH below 4. A second limitation arises from the need to employ relatively high concentrations of acidic buffer to maintain the desired pH, which is critical for maximizing the selectivity for iminium reduction over aldehyde reduction. As a consequence, the crude reaction mixtures contain significant amounts of salts, and the cyclic peptides must be purified prior to further evaluation. Thus, direct screening of crude macrocyclic peptide libraries is not readily feasible.

6. Perspectives

6.1. Further Design of Stapled Peptides Targeting the LDH Tetramerization Site

In the first part of this project, five new cyclic peptides (**MC2–MC5**, **BC1**), all obtained from variants of the LDH-derived octapeptide **LB8**, were synthesized. Among them, the triazole-bridged peptide **MC5**, obtained through CuAAC-based stapling, exhibited the highest helical propensity according to CD analyses. At this stage, it would be of interest to evaluate the affinity of these five peptides for their target, namely the tetramerization site of LDH-H. This could be carried out in the laboratory of Raphael Frédérick using biophysical techniques such as MST, employing the dimeric model of LDH-H previously developed (LDH-Htr) as the target protein.

If the results obtained reveal a correlation between the binding affinity and the helical content of the cyclic peptides, it would then be reasonable to attempt to further increase the helicity of **LB8**-derived peptides using strategies other than those considered so far.

Notably, varying the geometry of the triazole-containing staple in **MC5**, which was prepared using L-Pra and L-Nle(ϵ -N₃) as the anchoring residues (at positions 1 and 5, respectively), could yield peptides with further improved helical propensities. In this context, macrocyclization between $i \rightarrow i+4$ -spaced L-Nle(ϵ -N₃) and D-Pra residues may represent a promising alternative to achieve additional helix stabilization.^[70]

Besides CuAAC-based and Cys–Cys stapling procedures, RCM-based stapling or hydrogen-bond mimicry should also be considered. Although these approaches may be more demanding experimentally, they often exert a pronounced helix-inducing effect and would therefore be worth applying to **LB8**, provided that a correlation is observed between the helicity and the binding potency of the newly prepared **LB8**-derived cyclic analogues. Indeed, RCM-mediated stapling of two S5 residues in a dodecapeptide Ac-WKX¹RILX²RLLQE-NH₂ (X¹ and X² representing the anchoring residues) produced a peptide with higher helical propensity than CuAAC-mediated cross-linking between L-Nle(ϵ -N₃) and L-Pra residues.^[56] In addition, from a future development perspective, the increased hydrophobicity conferred by a hydrocarbon staple could improve cellular uptake compared with triazole-bridged

peptides such as **MC5**. Apart from stapling, the HBS strategy is known to generate peptides with strong helical propensity in aqueous media. In this context, mimicking an N-terminal $i \rightarrow i+4$ main-chain hydrogen bond, for instance through the synthetic approaches described by Arora and coworkers,^[27,28,30] would be well suited to the design of analogues of the LDH tetramerization arm, given the N-terminal position of its α -helical segment.

As previously mentioned, the binding affinity of the fluorescently labeled peptides **F1**, **F2**, and **F3** for LDH-Htr will also need to be determined. The most potent probe could then be selected to serve as a reporter in FP competition assays. Upon optimization of the assay conditions, the implementation of such FP experiments in the laboratory would provide a powerful tool to screen various compounds, including peptides and small molecules, against the tetramerization site of LDH.

In the future, we hope that the different macrocyclization strategies envisioned will provide constrained α -helical peptides with high affinity for LDH-Htr. The most potent compounds may prove capable of disrupting the LDH tetramer. Subsequent optimization of the drug-like properties of such molecules could then yield promising LDH inhibitors, enabling the investigation of the effects of in vivo inhibition of LDH.

6.2. Improving the Developed Cys–Lys Stapling Methodology

6.2.1. Expanding the Peptide Scope

The second part of this project focused on the development of a cysteine-to-lysine macrocyclization strategy mediated by sequential *S*-alkylation and reductive *N*-alkylation reactions performed in a one-pot fashion. The scope of the methodology has already been partially explored through its application to various peptides incorporating nucleophilic residues, protected lysine side chains, and lysine surrogates bearing shorter alkylamine side chains, as well as to a range of stapling reagents. Nevertheless, further work is required to better document the synthetic applicability of this procedure and to

identify the staple characteristics that provide the greatest enhancement of α -helicity in a thiol-to-amine stapling context.

All the monocyclization reactions carried out to date involved peptides containing an N-terminal cysteine residue and an amine-bearing residue located at the $i+4$ or $i+7$ position, and were performed on peptides composed exclusively of L-amino acid residues. Therefore, the peptide scope could first be expanded by inverting the relative positions of the anchoring residues and/or the absolute configuration of one or both of them. In addition, mutating the cysteine anchoring residue to homocysteine represents another way to modulate the total length of the staple, and has notably been shown to significantly impact the conformation of cyclic peptides prepared through thiol-to-thiol two-component macrocyclization.^[58,78]

As previously mentioned, whereas free cysteine residues are rare in peptides and proteins, lysine residues are more abundant. The site-selective incorporation of a single Cys–Lys tether may be difficult in peptides containing several lysine residues, which are therefore challenging substrates. Using peptides bearing a single Cys–Lys residue pair (**P1** and **P5**), we showed that $i \rightarrow i+4$ Cys–Lys stapling was faster than $i \rightarrow i+7$ Cys–Lys cross-linking (Figure 13). Nevertheless, the degree of site-selectivity achievable through our S-alkylation/reductive amination strategy has not yet been investigated. To address this question, the stapling protocol could be applied to peptides featuring one cysteine and two or more lysine residues. For instance, peptides containing, in addition to $i \rightarrow i+4$ -spaced cysteine and lysine residues, an additional lysine at position $i+5$, $i+6$, or $i+7$ could be used as model substrates. The extent of site-selectivity could then be assessed by determining the ratios of the two possible Cys–Lys cross-linked products for each of the three substrates.

N-terminal α -amines are more reactive than lysine ϵ -amines in reductive amination processes conducted in aqueous medium.^[147,148,184] Therefore, we do not anticipate Cys–Lys stapling through S-alkylation/reductive amination to be efficient in the presence of a free N-terminus. The stapled peptide obtained from Cys–Lys stapling of the first peptide model used (Cbz-CTLGKGLI-NH₂) was subjected to several Cbz cleavage attempts. However, preliminary deprotection attempts (notably using hydrogen and palladium on

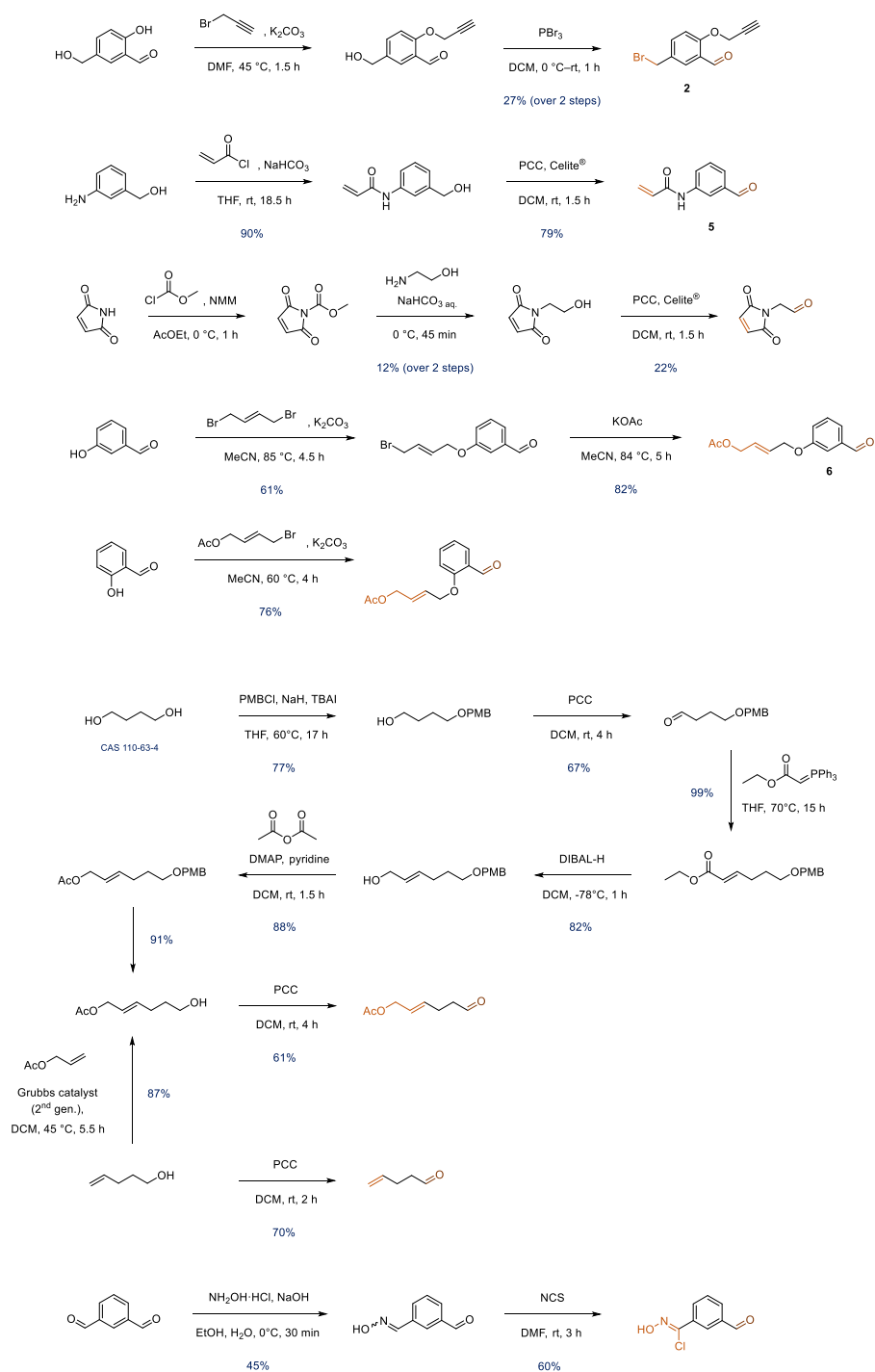
carbon or hydrogen bromide in acetic acid) proved unsuccessful, as side reactions precluded isolation of the desired deprotected cyclic peptide. Based on chromatographic analysis of the crude reaction mixtures, desulfurization occurred during the hydrogenation process, whereas acetylation (presumably O-acetylation of the threonine residue) was rapidly observed upon treatment of the protected cyclic peptide with hydrogen bromide in acetic acid. Cleaner removal of the Cbz protecting group could be achieved in a cleavage cocktail consisting of TFA/water/triisopropylsilane/thioanisole 90:2,5:2,5:5 (v/v/v/v), a mixture typically used after Fmoc-based SPPS for peptide-resin cleavage and simultaneous removal of acid-labile Boc, *t*Bu, and Trt protecting groups. However, deprotection under these conditions was slow and remained incomplete even after 7 h.

Based on the positive results obtained in the palladium-mediated deprotection of the Alloc-masked lysine in **P13.1s** (see Section 5.3.2.4), the Alloc group may represent a more suitable option than the Cbz moiety for protecting the N-terminal amine of a linear peptide that is to be subsequently cyclized via Cys–Lys stapling. In analogy to the deprotection of the non-targeted lysine residue in **P13.1s**, it is reasonable to anticipate that N-terminal deprotection could be carried out in a one-pot fashion after Cys–Lys stapling, by simply adding the Pd(0) species and the allyl scavenger to the reaction mixture.

6.2.2. Expanding the Scope of Stapling Reagents

The first year of this PhD project was largely devoted to organic synthesis. During this period, several bifunctional reagents potentially suitable for Cys–Lys stapling were prepared (Scheme 24). Some of these reagents could not be tested due to time constraints. Among them are notably non-aromatic derivatives, as well as reagents bearing substitution patterns different from those of the reagents already investigated. In addition, some of these reagents feature thiol-reactive moieties that have not yet been explored in this context, such as maleimide, unactivated alkene, and chlorooxime groups.

All these reagents may prove useful in the future, as they offer additional possibilities to modulate the conformation of Cys–Lys stapled peptides.



6.2.3. Trapping the Iminium Intermediate with Alternative Nucleophiles

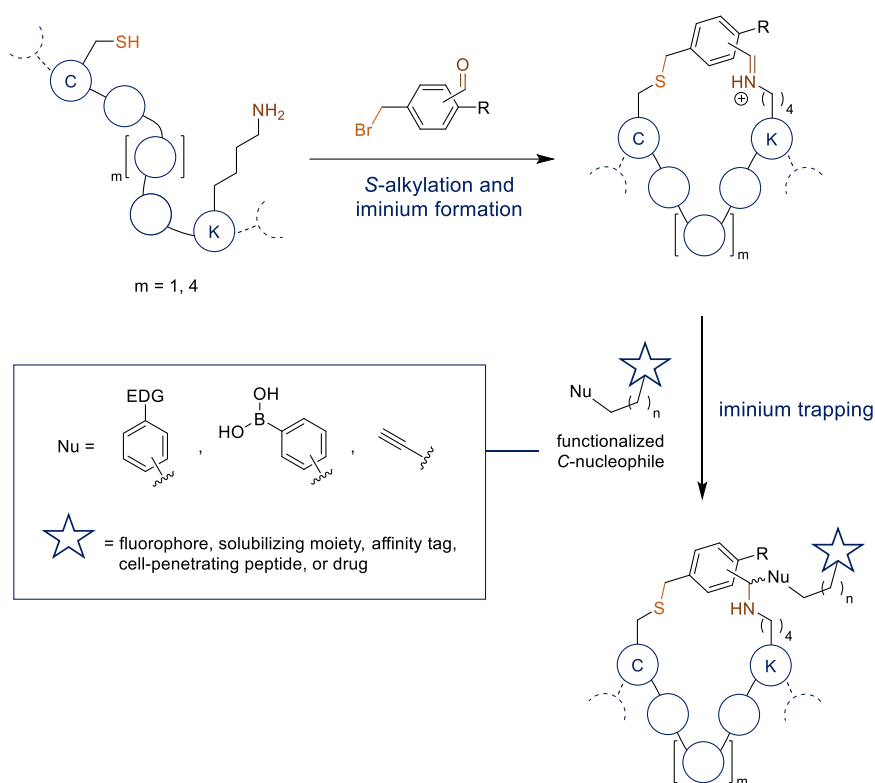
As previously mentioned (see Section 5.1), various nucleophiles can be used to trap an iminium intermediate, thereby affording a secondary amine. So far, only hydride donors have been considered in the Cys–Lys stapling strategy described herein in order to avoid formation of a mixture of diastereomers of the desired cyclic product. This choice was made both to facilitate analysis of the crude reaction mixtures during optimization and to simplify isolation of the stapled peptides as single stereoisomers, thereby facilitating interpretation of the circular dichroism results. However, a major advance could be achieved by employing a broader range of nucleophiles. Indeed, this would further expand the structural diversity accessible through this methodology.

The use of nucleophiles bearing valuable payloads could enable the combination of macrocyclization through iminium trapping and functionalization within a single step. This would eliminate the need to purify the macrocyclic peptide prior to post-stapling functionalization, which was notably required for derivatization of the stapled peptide **P1.2s** via CuAAC (see Section 5.6.2).

Trapping of the iminium intermediate formed after S-alkylation of the peptide substrate with a benzaldehyde-derived stapling reagent could be envisioned using C-nucleophiles such as electron-rich aromatic compounds, arylboronic acids, and alkynes (Scheme 25). The corresponding cyclization step would need to be optimized to obtain the desired product in good yield. This could be achieved by drawing inspiration from conditions previously reported in the literature. Indeed, trapping of imine/iminium intermediates derived from *N*-terminal α -amines^[148] or lysine ϵ -amines^[47,150,151] by the types of carbon nucleophiles listed above is a well-established strategy in the field of peptide macrocyclization. However, in most examples reported to date, nucleophilic attack on the iminium ion was intramolecular, whereas the trapping process envisioned here would be intermolecular, which would likely require adaptation of the reaction conditions.

The use of cyanide as a nucleophile for iminium trapping (Strecker reaction), although successfully employed by Baran and coworkers for cyclization of various peptide aldehydes,^[148] appears less attractive in

the present context. On the one hand, cyanide cannot be equipped with a useful chemical biology group. On the other hand, the resulting α -aminonitriles may display limited stability in aqueous acidic media.^[148]



Scheme 25. Cys–Lys stapling via S-alkylation followed by iminium trapping with functionalized C-nucleophiles.
EDG = electron-donating group.

6.2.4. Optimizing the Helicity of the Macrocycles

As mentioned above, very limited CD and NMR data are currently available to document the helicity of peptides stapled through thiol-to-amine macrocyclization (see Section 1.4.2.2.3.4.3). The CD data obtained in this work (for **P1**, **P2**, **P3**, **P4**, **P5**, **P14**, **P15**, and their stapled derivatives) therefore contribute to expanding the literature on this topic. Nevertheless, additional studies will be required to confirm

the potential of amino–sulfhydryl stapling for the generation of constrained α -helical peptides.

In particular, the highest helical propensities measured in Chapter 2 of this work were observed for the $i \rightarrow i+4$ Cys–Dap stapled peptides **P4.1s** and **P14.1s**, both bearing an *m*-xylylene linker. Although Cys–Dap stapling has the potential to enable the efficient incorporation of non-symmetrical linkers, which is not possible with Cys–Cys stapling, it would be valuable to directly compare the helicity of Cys–Dap and Cys–Cys stapled peptides incorporating the same linker, and therefore possessing a staple with the same number of atoms.

In the present context, this could be achieved by stapling a Cys–Cys derivative of **P4** or **P14** using α,α' -dibromo-*m*-xylene and comparing the helicity of the resulting macrocycle with that of its Cys–Dap stapled analogue. Such a comparison would clarify whether the differences in the electronic properties of the thioether and secondary amine functionalities, and/or the difference in bond length between C–S and C–N bonds, have a significant impact on the helicity of stapled peptides.

All thiol-to-amine cross-linked macrocycles studied by CD to date were prepared using compound **1** as the stapling reagent. In other words, only *m*-xylylene-bridged peptides have been analyzed by CD. However, as demonstrated in the scope of stapling reagents, many other linkages can be installed through S-modification/reductive amination. Owing to time constraints, the effect of linker variation on peptide helicity has not yet been investigated. Interestingly, this would provide an additional strategy to modulate peptide helicity, beyond the parameters associated with the linear peptide scaffold (namely the side-chain length and absolute configuration of the anchoring residues, as well as their relative position). Ideally, such structural variations could enable the identification of an “optimal staple” for increasing the helicity of a given linear peptide. This may constitute the most promising direction emerging from this thesis work.

This search for an “optimal staple” would require the preparative-scale synthesis of the most promising macrocyclic derivatives, followed by their purification and CD analysis. Considering the very large number of possible combinations (L- or D-configuration at each anchoring residue; relative position of the anchoring residues; Cys or hCys; Lys,

Orn, Dab, or Dap; linker structure), it would be unreasonable to attempt the preparation of all possible macrocycles for a systematic evaluation of their helicity. Molecular dynamics simulations^[102] would likely be valuable to identify the combinations most likely to translate into high α -helicity. In addition, the use of lysine as a natural anchoring residue could be considered advantageous compared with the use of ornithine, diaminobutyrate, or diaminopropionate, since these lysine surrogates cannot be incorporated using conventional recombinant DNA techniques. If one prioritizes the use of naturally occurring residues, only L-Lys and L-Cys would be employed as anchoring residues. Variation of their relative positions and of the cross-linker structure would then enable the generation of a macrocyclic peptide library which, although still substantial, would remain more reasonable in terms of the required synthetic effort.

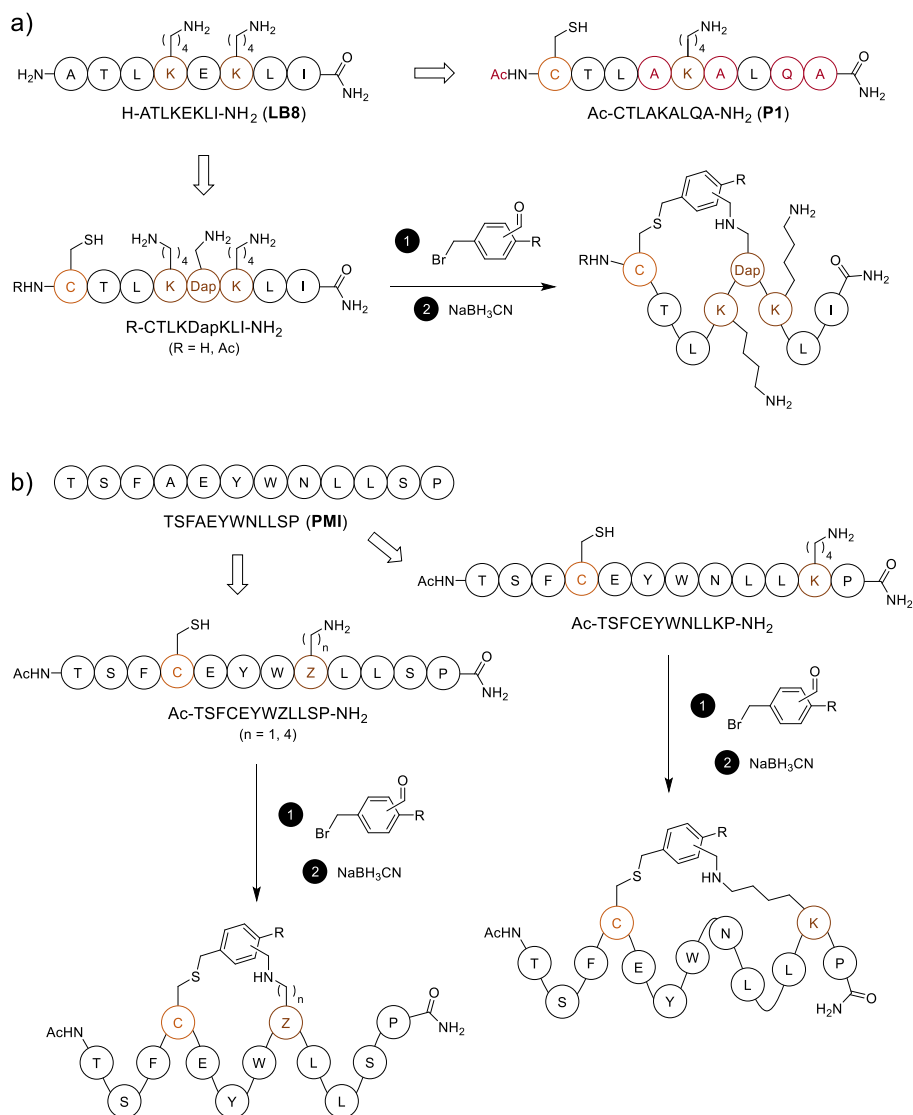
In this context, given the high flexibility of the lysine side chain, one might reasonably expect peptides bearing the shortest cross-linkers to display the highest helicity. Accordingly, Cys–Lys stapling using chloroacetaldehyde, acrolein derivatives, or *o*-(bromomethyl)benzaldehyde would likely be worth investigating first on a preparative scale. The conformation of the resulting macrocycles (featuring 2-, 3-, or 4-atom-long linkages) could then be compared with that of their *m*-xylylene-bridged counterpart (bearing a 5-atom-long linker). Such a comparison would determine whether reducing the length of Cys–Lys staples effectively translates into enhanced helix stabilization. Furthermore, in the case of Cys–Lys stapling with α -substituted acrolein derivatives, separation of the two diastereomeric peptides **P1.9s_a** and **P1.9s_b**, previously obtained in a 47:53 absorbance ratio ($\lambda = 210$ nm) upon treatment of **P1** with methacrolein, followed by individual CD analysis, could help assess the potential influence of the configuration of the methyl substituent on peptide helicity.

6.2.5. Application to Bioactive Peptides

Finally, to demonstrate that the present amino–sulfhydryl cross-linking strategy can serve as an effective tool to improve the binding affinity of short peptides through reinforcement of their α -helical structure, we aim to apply the stapling protocol to a short peptide whose bioactive conformation is expected to be α -helical.

Although peptide model **P1** is derived from **LB8**, its sequence was considerably modified for the purposes of optimization (see Section 5.2.1). Notably, **P1** does not display a free N-terminus nor an isoleucine residue at position 8, two characteristics that may contribute to the binding affinity of **LB8**-derived peptides for LDH-Htr.^[67] Nonetheless, it could be worthwhile to investigate $i \rightarrow i+4$ stapling of a peptide whose sequence more closely resembles that of **LB8**, thereby preserving as many recognition features of this octapeptide as possible (Scheme 26a). Like **P1**, such a linear peptide would need to incorporate a cysteine residue and an amine-bearing residue at positions 1 and 5, respectively, instead of the alanine and glutamate residues present in **LB8**. Rather than mutating the lysine residues at positions 4 and 6 of **LB8** to alanine, these residues could be retained by using diaminopropionate as the amine-bearing anchoring residue. Indeed, the results obtained in this work suggest that the intramolecular reductive amination step may be selective for β -amines over ϵ -amines (see Section 5.3.3). Thus, $i \rightarrow i+4$ Cys–Dap cross-linking might be achievable even in the presence of two free lysine residues. Going further, such a cyclization could also be attempted on a peptide bearing a free N-terminus, as in the case of **LB8**. This would make it possible both to document the potential competition between the β -amine of a diaminopropionate residue and an N-terminal α -amine, which has not yet been investigated, and, provided that the Cys–Dap stapling process proves efficient, to obtain a stapled **LB8** derivative bearing a free N-terminus and therefore potentially displaying higher affinity for LDH-Htr than *N* α -acetylated peptides.

Alternatively, our thiol-to-amine stapling methodology could be applied to another PPI system, such as the interaction between the tumor suppressor p53 and the ubiquitin ligase MDM2, one of the most extensively studied PPIs.



Scheme 26. Application of the developed thiol-to-amine stapling methodology to peptides derived from (a) **LB8** and (b) **PMI** for targeting LDH tetramerization and the p53–MDM2 interaction, respectively.
 Z = Lys (n = 4) or Dap (n = 1).

Loss of p53 activity, notably through overexpression of MDM2, is common in human cancers, and this PPI represents a validated target for anticancer therapy. The p53–MDM2 interaction occurs through contacts between a short α -helix of p53 ($^{17}\text{ET}\underline{\text{F}}\text{SDL}\underline{\text{W}}\text{KLL}\underline{\text{P}}\text{E}^{28}$) and a hydrophobic cleft of MDM2. F19, W23, and L26 are essential residues for binding to MDM2. Many stapled α -helical p53 derivatives displaying high affinity for MDM2, including sulanemadlin, have been developed to inhibit this PPI and thereby prevent MDM2-mediated p53 degradation.^[34,50,60,108,131]

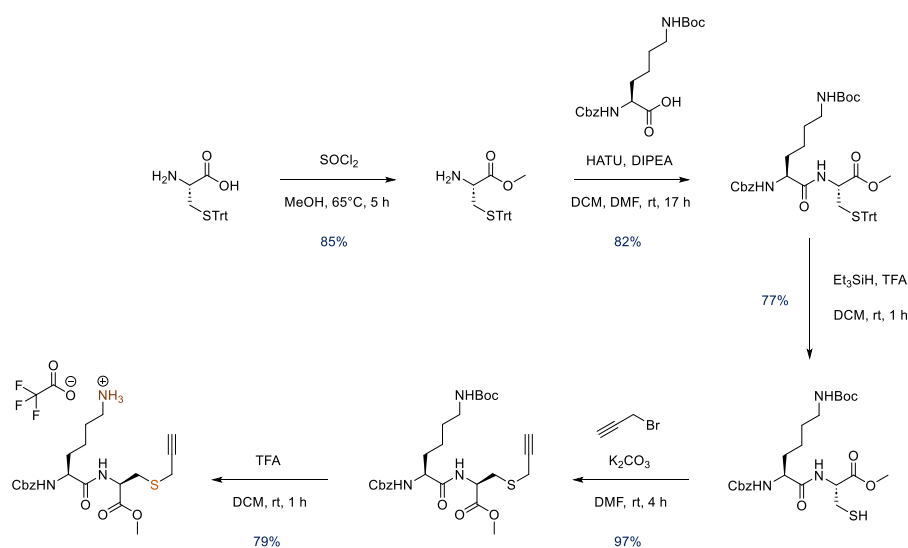
The dodecapeptide **PMI** ($\text{TS}\underline{\text{F}}\text{AEY}\underline{\text{W}}\text{NLL}\underline{\text{S}}\text{P}$), identified by phage display, exhibits relatively high affinity for MDM2 ($K_d = 3.4$ nM, as determined by a surface plasmon resonance-based competition assay).^[198] Similarly to previous reports in the literature,^[34,131] we could attempt to further improve the affinity of this dodecapeptide using our stapling approach (Scheme 26b). The staple could be introduced at various solvent-exposed positions, in either an $i \rightarrow i+4$ or an $i \rightarrow i+7$ fashion. Initially, lysine could be used as the amine-bearing residue. If the helicities and affinities obtained upon $i \rightarrow i+4$ or $i \rightarrow i+7$ Cys–Lys stapling are not satisfactory, the use of diaminopropionate instead of lysine could be envisaged. Indeed, our macrocyclization studies on nonapeptides $\text{Ac-CTLAX}^1\text{ALX}^2\text{A-NH}_2$ ($X^1 = \text{Lys, Orn, Dab, Dap, or Gln}$; $X^2 = \text{Lys or Gln}$), using stapling reagent **1**, suggested that $i \rightarrow i+4$ Cys–Dap stapling could translate into higher helical propensity than $i \rightarrow i+4$ or $i \rightarrow i+7$ Cys–Lys cross-linking.

Besides binding affinity, the effects of the thiol-to-amine stapling approach presented herein on cellular uptake and proteolytic resistance still need to be investigated.

6.3. Cys–Lys Stapling via S-modification/A³-coupling

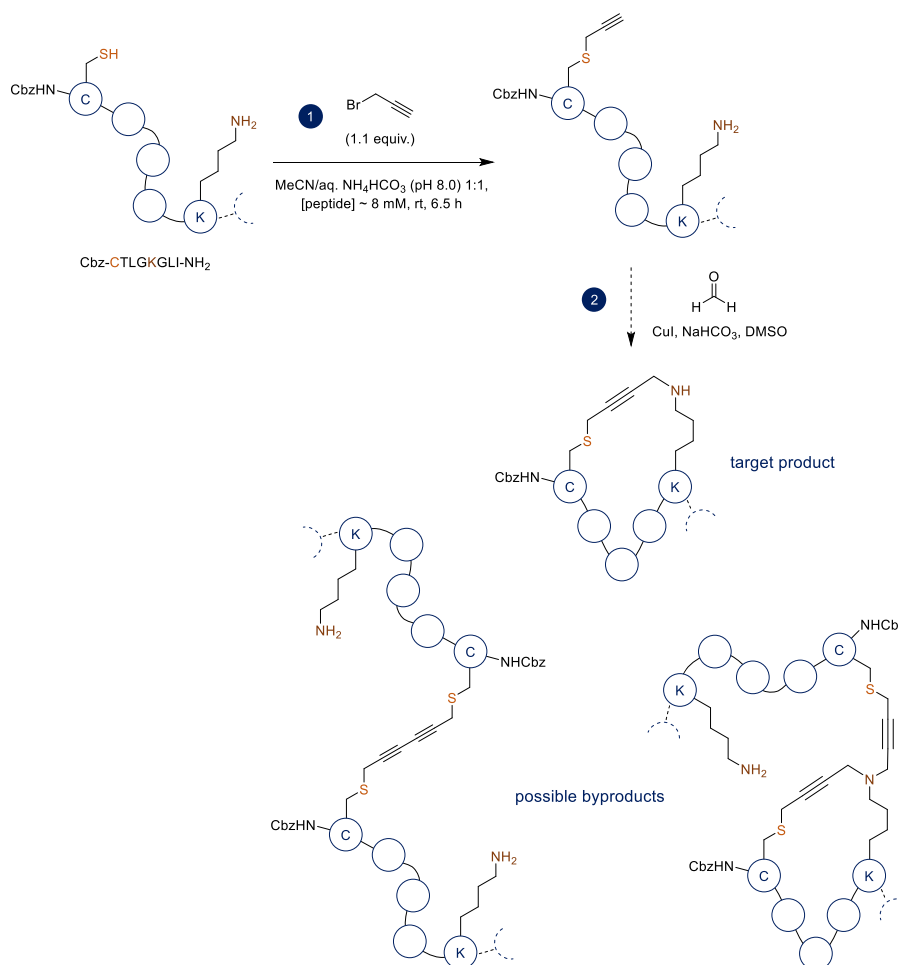
An alternative Cys–Lys stapling strategy was explored. Terminal alkyne moieties can be efficiently installed on cysteine residues within peptides either through S-ethynylation^[138] or S-propargylation.^[199,200] Moreover, in copper-catalyzed A³-coupling processes, copper acetylides react with imine/iminium species formed upon condensation of an aldehyde and an amine. Therefore, we considered a reaction sequence based on thiol ethynylation or propargylation, followed by cyclization through A³-coupling involving the installed alkyne group, a lysine ϵ -amine, and an external aldehyde reagent.

Multicomponent A³-coupling reactions have successfully been used by Lubell and coworkers to prepare cyclic propargylamine-bridged peptides. However, the reported protocols only focused on cyclization between an *N* ^{ϵ} -alkyl-lysine, ornithine, or diaminobutyrate residue, and a propargylglycine (Pra) or azapropargylglycine (azaPra) residue, using formaldehyde as the aldehyde component and copper(I) iodide as the copper source. All these procedures involved multi-step peptide assembly and on-resin cyclization of protected resin-bound peptides.^[151,182,201] Inspired by these studies, we envisioned a convenient solution-phase strategy for Cys–Lys stapling of unprotected peptides, in which installation of an alkyne handle on a cysteine residue would be followed by intramolecular copper-catalyzed A³-coupling. The intramolecular A³-coupling reaction was first investigated using a dipeptide model featuring a propargyl thioether moiety, which was obtained through multi-step synthesis (Scheme 27). Under the different reaction conditions tested, the desired cyclic secondary propargylamine was formed in variable proportions, alongside byproducts such as the 1,3-diyne formed by Glaser homocoupling and dimeric byproducts arising from sequential intra- and intermolecular A³-coupling processes (either in this order or in the reverse order). Nevertheless, the target 15-membered macrocycle was obtained as the major product when the reaction was performed in DMSO at 37 °C, using 3–6 equiv. of formaldehyde, 1 equiv. of sodium bicarbonate, and 4 equiv. of copper(I) iodide. This encouraging result prompted us to apply our envisioned S-alkylation/A³-coupling approach to Cys–Lys stapling of the octapeptide model Cbz-CTLGKGLI-NH₂ (Scheme 28).



Scheme 27. Synthesis of a dipeptide model for investigating intramolecular A³-coupling.

To our delight, *S*-propargylation of the octapeptide proved very clean using 1.1 equiv. of propargyl bromide in a 1:1 (v/v) mixture of ammonium bicarbonate buffer (50 mM, pH 8.0) and acetonitrile, with less than 5% of a bis-propargylated byproduct being formed, based on the chromatogram obtained following HPLC–UV analysis ($\lambda = 210$ nm) of the crude reaction mixture. Initial trials of intramolecular A³-coupling using either purified or crude *S*-propargylated peptide, employing conditions similar to those used for the model dipeptide, led to the detection of a species presumably corresponding to the desired Cys–Lys cross-linked peptide. However, several impurities were detected, and further optimization work needs to be conducted in order to improve the reaction profile. Indeed, although A³-coupling has been widely used for the synthesis of tertiary propargylamines and secondary *N*-arylpropargylamines, the synthesis of secondary *N*-alkylpropargylamines is less well documented, and primary alkylamines may represent challenging substrates in this context.^[202] Nevertheless, optimization of the envisioned three-component Cys–Lys strategy is particularly exciting.



Scheme 28. Cys–Lys stapling through sequential S-propargylation and intramolecular A³-coupling: preliminary experiments.

If a one-pot S-alkylation/A³-coupling procedure proves feasible, and if the chemoselectivity toward cysteine and lysine residues over other canonical residues is confirmed, this could allow convenient solution-phase synthesis of alkyne-bridged peptides from unprotected precursors. The structure of the resulting macrocycles could be readily varied through the use of different aldehydes. Notably, the use of aldehydes bearing valuable payloads could lead to useful chemical biology tools. Moreover, the installed alkyne-based tether could undergo post-stapling functionalization through ruthenium-catalyzed

azide–alkyne cycloaddition (RuAAC) or thiol-yne photo-click reaction. In terms of helicity, this rigid 4-atom-long linker could give rise to peptides displaying higher helicity than peptides bearing longer or more flexible linkers. Furthermore, its geometry could be modulated through hydrogenation, leading to the corresponding alkenylene or alkylene linkers. Finally, such a strategy would also be compatible with a bicyclization approach, similarly to that previously applied to peptide dithiols bearing one central amine-bearing residue through S-alkylation/reductive amination. In other words, after bis-S-propargylation of the peptide dithiol, bis-A³-coupling involving the primary amine, the two terminal alkynes, and two aldehyde molecules could lead to a bicyclic tertiary amine. If the secondary amine formed upon monocyclization proves more reactive in A³-coupling than the primary amine of the unmodified lysine residue, such a bicyclization strategy could be less likely to generate dimeric or oligomeric byproducts and could therefore be easier to develop compared than the corresponding stapling procedure.

7. Conclusion

Peptide stapling is a widely used macrocyclization approach for biasing short peptides toward their helical conformation and improving their binding affinity through reduction of the associated entropic penalty.

In this work, various Cys–Cys stapled octapeptides derived from the tetramerization arm of lactate dehydrogenase were synthesized. Unfortunately, the resulting macrocycles did not appear significantly more helical than the previously prepared Cys–Cys cross-linked peptide **MC1**. In contrast, a triazole-bridged peptide produced via CuAAC was shown to display a substantially increased α -helical propensity. The binding affinity of the newly prepared peptides for their target has not yet been studied. However, such studies will be necessary to determine whether a correlation exists between their affinity and their helicity, and thus whether further increasing their helicity represents a promising strategy for optimizing derivatives of the LDH tetramerization arm. New fluorescently-labeled peptides were also prepared as potentially useful chemical biology tools.

Cys–Cys stapling, which is by far the most straightforward stapling strategy from a synthetic standpoint, is limited to the introduction of symmetrical tethers. Cys–Lys stapling enables a higher degree of structural diversification, as it allows the high-yielding synthesis of macrocyclic peptides bearing non-symmetrical linkers. However, cysteine-to-lysine macrocyclization of unprotected linear peptides is challenging. Nevertheless, chemically original solution-phase Cys–Lys stapling approaches have been reported since the late 2010s. Such methods are rare, however, and new methodologies are required to expand the diversity of accessible linkers. This could facilitate the design of Cys–Lys peptides with high helical propensity, cellular permeability, and proteolytic stability.

In the second part of this PhD project, a chemically simple Cys–Lys stapling procedure based on one-pot S-modification/reductive amination was optimized. The developed protocol complements existing methodologies, notably because it preserves the nucleophilic nature of the lysine residue, thereby allowing convenient post-stapling modification. The scope of the methodology was explored by varying both the structure of the linear peptides and the stapling reagents

involved. Notably, reducing the length of the amine-bearing side chain increased the rate of the intramolecular reductive amination. One-loop ($i \rightarrow i+4$) Cys–Dap stapling, which showed the fastest cyclization rate compared with the corresponding Cys–Lys, Cys–Orn, and Cys–Dab processes, provided the *m*-xylylene-bridged peptides with the highest helical propensities. Although the effect was less pronounced than with Cys–Dap cross-linking, $i \rightarrow i+4$ Cys–Lys stapling still reinforced the helical structure of the peptide model, suggesting that the present stapling protocol may provide a promising strategy for optimizing peptides containing only canonical residues.

The effect of cross-linker variation on both the stapling kinetics and the conformation of the resulting macrocycles now remains to be investigated. Overall, given its operational simplicity and the substantial structural diversity it enables, we anticipate that the one-pot S-modification/reductive amination procedure could emerge as a useful stapling approach for fine-tuning the physicochemical and biological properties of bioactive peptides.

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S1. General information

S1.1. Materials

Commercially available materials and solvents were purchased from chemical suppliers and used without further purification (see supplier and product references below).

Small-molecule organic compounds that were not commercially available were prepared according to established procedures (see Section S1.2, *General Methods for Organic Synthesis*). Linear peptides were either purchased from GeneCust (Boynes, France) or manually synthesized via Fmoc-based solid-phase peptide synthesis (see Section S1.3, *General Methods for Solid-Phase Peptide Synthesis*). Macrocyclic peptides were obtained by solution-phase cyclization of linear peptides (see Section S1.4, *General Methods for Solution-Phase Peptide Chemistry*).

- 2,2,2-trifluoroethanol: Fluorochem (Cat. No. F001273)
- 2,4,6-trinitrobenzenesulfonic acid (~1% in DMF): TCI (Cat. No. T2024)
- 2-hydroxy-5-(hydroxymethyl)benzaldehyde: Fluorochem (Cat. No. F369874)
- 2-picoline–borane: TCI (Cat. No. B3018)
- 3-aminobenzyl alcohol: TCI (Cat. No. A2259)
- 3-hydroxybenzaldehyde: Alfa Aesar (Cat. No. A13541)
- 6-carboxyfluorescein azide: Lumiprobe (Cat. No. C5130)
- Acetaldehyde: Fluka (Cat. No. 00070)
- Acetic acid: Thermo Chemical (Cat. No. A/0400/PB15)
- Acetic anhydride: Acros Organics (Cat. No. 149490010)
- Acetonitrile (for analytical and semi-preparative HPLC): VWR (Cat. No. 83639.320)
- Acetonitrile (for solution-phase reactions): Acros Organics (Cat. No. 326811000)
- Acryloyl chloride (contains <210 ppm monomethyl ether of hydroquinone as stabilizer): Aldrich (Cat. No. 549797)
- Aminoguanidine hydrochloride: TCI (Cat. No. A1129)
- Ammonium hydrogen bicarbonate: Thermo Scientific (Cat. No. 014249.A1)
- Benzaldehyde: Aldrich (Cat. No. 418099)
- Boric acid: Thermo Scientific (Cat. No. A10896.36)

- Celite®: Roth (Cat. No. 0011.2)
- Citric acid: VWR (Cat. No. 0529)
- Copper(II) sulfate pentahydrate: Acros Organics (Cat. No. 423615000)
- Deuterium oxide: Eurisotop (Cat. No. D214FS)
- Deuterium oxide (containing 0.75 wt. % 3-(trimethylsilyl)propionic-2,2,3,3-*d*₄ acid, sodium salt): Sigma–Aldrich (Cat. No. 293040)
- α,α'-dibromo-*m*-xylene: Fluorochem (Cat. No. F237159)
- Dichloromethane (for solid-phase peptide synthesis): VWR (Cat. No. 23373.320)
- Dichloromethane (for solution-phase reactions): Sigma–Aldrich (Cat. No. 270997)
- Dimethylsulfoxide: Sigma–Aldrich (Cat. No. 276855)
- Ethane-1,2-dithiol: TCI (Cat. No. E0032)
- Ethanol: VWR (Cat. No. 20821.310)
- Ethyl cyano(hydroxyimino)acetate: Novabiochem (Cat. No. 8510860025)
- Fmoc-L-Ala-OH: Fluorochem (Cat. No. M03347)
- Fmoc-L-Arg(Pbf)-OH: Fluorochem (Cat. No. M03398)
- Fmoc-L-Cys(Trt)-OH: Fluorochem (Cat. No. M03395)
- Fmoc-L-His(Trt)-OH: Fluorochem (Cat. No. M03415)
- Fmoc-L-Gln(Trt)-OH: Fluorochem (Cat. No. M03356)
- Fmoc-L-Leu-OH: Fluorochem (Cat. No. M03365)
- Fmoc-L-Lys(Alloc)-OH: TCI (Cat. No. A3245)
- Fmoc-L-Lys(Boc)-OH: Fluorochem (Cat. No. M03419)
- Fmoc-L-Met-OH: Fluorochem (Cat. No. M03368)
- Fmoc-L-Ser(*t*Bu)-OH: Fluorochem (Cat. No. M03382)
- Fmoc-L-Thr(*t*Bu)-OH: Fluorochem (Cat. No. M03389)
- Fmoc-L-Trp(Boc)-OH: Fluorochem (Cat. No. M03376)
- Fmoc-L-Tyr(*t*Bu)-OH: Fluorochem (Cat. No. M03428)
- Formaldehyde ≥36 wt. % in H₂O (contains ~10% methanol as stabilizer): Sigma–Aldrich (Cat. No. 47608)
- Formic acid: Supelco (Cat. No. 5.43804)
- Lithium chloride: Acros Organics (Cat. No. 193370010)
- *m*-(bromomethyl)benzaldehyde: Fluorochem (Cat. No. F065125)
- Methanol: Thermo Scientific (Cat. No. 41467), Sigma–Aldrich (Cat. No. 322415)
- Methoxylamine hydrochloride: TCI (Cat. No. M0343)
- *n*-hexane: VWR (Cat. No. 24575.320)

- *N,N*-diisopropylethylamine: TCI (Cat. No. D1599)
- *N,N*-dimethylformamide (for solid-phase peptide synthesis): Biosolve (Cat. No. 0004193302BS)
- *N,N*-dimethylformamide (for solution-phase reactions): Sigma–Aldrich (Cat. No. 227056)
- *N,N'*-diisopropylcarbodiimide: Sigma–Aldrich (Cat. No. D125407)
- *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate: Fluorochem (Cat. No. F023926)
- *o*-(bromomethyl)benzaldehyde: BLDpharm (Cat. No. BD290207)
- *p*-(bromomethyl)benzaldehyde: BLDpharm (Cat. No. BD91095)
- Palladium(II) acetate: Fluorochem (Cat. No. F035785)
- Petroleum ether: VWR (Cat. No. 23826.360)
- Phosphoric acid 85 wt. % in H₂O: Sigma–Aldrich (Cat. No. 101774118)
- Phosphorus tribromide: Aldrich (Cat. No. 15,778-3)
- Piperidine: Carl Roth (A122.3)
- Potassium acetate: Janssen Chimica (Cat. No. 14.960.22)
- Potassium carbonate: VWR (Cat. No. 26726.297)
- Propargyl bromide 80 wt. % in toluene (stabilized with MgO): TCI (Cat. No. P1272)
- Pyridinium chlorochromate: TCI (Cat. No. P0930)
- Rink Amide AM resin: Novabiochem (Cat. No. 8.55004.0005)
- Sodium acetate: Sigma–Aldrich (Cat. No. S2889)
- Sodium bicarbonate: Fisher Scientific (Cat. No. BP328-1)
- Sodium carbonate decahydrate: RPL (Cat. No. 1719)
- Sodium citrate dihydrate: Aldrich (Cat. No. 85,578-2)
- Sodium cyanoborohydride: TCI (Cat. No. S0396)
- Sodium hydroxide: Supelco (Cat. No. 1.06462.5000)
- Sodium L-ascorbate: Sigma–Aldrich (Cat. No. A4034)
- Sodium phosphate dibasic: Acros Organics (Cat. No. 448140050)
- Tetrahydrofuran: Sigma–Aldrich (Cat. No. 401757)
- Trifluoroacetic acid: Thermo Scientific (Cat. No. 293811000)
- Triisopropylsilane: TCI (Cat. No. T1533)
- Tris(3-hydroxypropyltriazolylmethyl)amine: Apollo Scientific (Cat. No. OR1015384)

S1.2. General Methods for Organic Synthesis

Moisture-sensitive reactions were conducted under an inert atmosphere of argon or nitrogen.

The progress of the reactions was monitored by thin-layer chromatography (TLC), on silica gel plates (Supelco, 60 F₂₅₄). Spots were visualized under ultraviolet (UV) light.

Purification by column chromatography was performed using silica gel (ROCC, SI 1721, 60 Å, 40–61 µm). All synthesized compounds were characterized by proton nuclear magnetic resonance (¹H NMR), carbon-13 nuclear magnetic resonance (¹³C NMR), and high-resolution mass spectrometry (HRMS).

S1.3. General Methods for Solid-Phase Peptide Synthesis

Fmoc-based solid-phase peptide synthesis (SPPS) was carried out on a 0.04–0.14 mmol scale using Rink amide AM resin (substitution: 0.64 mmol/g).

The resin was swollen in dichloromethane (DCM) for 1 h prior to synthesis. For each coupling cycle, Fmoc group removal was performed using 20% piperidine in *N,N*-dimethylformamide (DMF) (1.6 mL/100 mg resin) for 2 × 5 minutes at room temperature. The resin was then washed sequentially with DMF (3 × 2 mL) and DCM (3 × 2 mL). Racemization-prone Fmoc-protected amino acids (Fmoc-L-Cys(Trt)-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Ser(*t*Bu)-OH; 3 equiv.) were activated with ethyl cyano(hydroxyimino)acetate (Oxyma, 3 equiv.) and *N,N'*-diisopropylcarbodiimide (DIC, 3 equiv.) in DMF (final amino acid concentration: 0.3 M), and coupling was performed for 1 h at room temperature. All the other Fmoc-protected amino acids (5 equivalents) were activated with *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 5 equiv.) and *N,N*-diisopropylethylamine (DIPEA, 10 equiv.) in DMF (final amino acid concentration: 0.2 M), and coupling was performed for 30 minutes at room temperature. The resin was washed with DMF (3 × 2 mL) and DCM (3 × 2 mL) before the next coupling cycle.

Coupling efficiency was monitored by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) test. A second coupling was carried out if the reaction was incomplete.

Acetylation of the *N*-terminus was performed using acetic anhydride (10 equiv.) and DIPEA (5 equiv.) in DMF (2.5 mL) for 1 h at room temperature.

Before storage of the resin at 4 °C overnight, and prior to peptide cleavage, the resin was washed with DMF (3 × 2 mL), DCM (3 × 2 mL), and methanol (3 × 2 mL), then dried under vacuum.

Side-chain deprotection and cleavage of the peptide from the resin were simultaneously achieved using a cleavage cocktail of trifluoroacetic acid (TFA)/water/triisopropylsilane/ethane-1,2-dithiol (92.5:2.5:2.5:2.5, v/v/v/v) for 2 h at room temperature, protected from light. The reaction mixture was filtered, and the resin was rinsed with 2 mL of TFA/DCM (1:1, v/v). The combined filtrate was concentrated under a nitrogen stream to remove TFA and DCM. Cold diethyl ether was added to the residue to precipitate the peptide. The suspension was centrifuged (5000 rpm, 4 °C) for 10 minutes, and the supernatant was carefully removed. The precipitation and centrifugation steps were repeated once. The crude peptide was obtained as a solid after removal of the supernatant.

Crude linear peptides were purified by semi-preparative high-performance liquid chromatography (HPLC).

S1.4. General Methods for Solution-Phase Peptide Chemistry

Solution-phase peptide modification reactions were conducted on an analytical and/or preparative scale. Reactions on an analytical scale were carried out in 1.5 mL Eppendorf tubes (ClearLine, Cat. No. 390690) or 15 mL centrifuge tubes (ClearLine, Cat. No. CL482), whereas preparative-scale reactions were performed in 50 mL centrifuge tubes (ClearLine, Cat. No. CL474, unless otherwise specified).

Before being used in solution-phase reactions, purchased and synthesized peptides were weighed using a microbalance (Mettler Toledo Excellence Plus XP2U). For all calculations involving molecular weights, one molecule of TFA was assumed per basic amino acid residue (Arg, His, Lys, Orn, Dab, and Dap). For all concentration calculations, volume contraction resulting from mixing water with

organic solvents (acetonitrile, methanol, ethanol, THF, DMF, or DMSO) was neglected.

All aqueous (aq.) buffers were adjusted to the desired pH prior to use using diluted aq. solutions of HCl and NaOH (for ammonium bicarbonate, sodium citrate, and sodium borate buffers), acetic acid and NaOH (for sodium acetate buffers), or phosphoric acid and NaOH (for sodium phosphate buffers). It should be noted that all reported pH values refer to those of the buffer solutions (adjusted prior to use), not the actual pH of the final reaction mixtures. This distinction is particularly relevant when NaBH_3CN is added, as it can induce a pH increase.

Reaction mixtures were incubated at either 23 °C or 37 °C in an incubator shaker (IKA KS 4000 ic control, set to 120 rpm) connected to a circulating water bath chiller (Thermo Scientific DC 10 K10).

Reaction yields were assessed by analytical HPLC following appropriate dilution of the reaction mixture.

When estimation of the conversion of a peptide aldehyde (peptide bearing a side-chain aldehyde) during reductive amination was required, the reaction mixture was quenched with a buffer containing methoxylamine* to trap the residual aldehyde as a stable oxime. The quenching buffer was prepared by dissolving methoxylamine hydrochloride (5 mM) in sodium acetate buffer (50 mM, pH 4.5). In HPLC analyses, two oxime stereoisomers were typically observed for each peptide aldehyde (major isomer denoted **ox_a**, minor isomer denoted **ox_b**). However, only the UV peak area of the major isomer was used to estimate the conversion, assuming a constant isomer ratio.

Macrocyclic peptides synthesized on a preparative scale were purified by semi-preparative HPLC.

* Note on the use of methoxylamine: methoxylamine was preferred over hydroxylamine for trapping peptide aldehydes in order to increase the hydrophobicity of the resulting oxime species, thereby avoiding co-elution with other peptidic species, notably the desired secondary amine product and the alcohol byproduct, which generally exhibited shorter retention times in analytical HPLC.

S1.5. NMR

NMR spectra were measured at 300 MHz for ^1H and 75 MHz for ^{13}C nuclei (Bruker Avance II 300 MHz system), at 400 MHz for ^1H and 101 MHz for ^{13}C nuclei (Bruker Avance II 400 MHz system), or at 600 MHz for ^1H and 151 MHz for ^{13}C nuclei (Bruker Ascend Avance III 600 MHz system). Chemical shifts are reported in units of ppm relative to the residual solvent signal. The following abbreviations are used to indicate multiplicities: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublets), and dt (doublet of triplets). Coupling constants are expressed in hertz (Hz). Assignment of NMR signals was performed using a combination of ^1H , ^1H -COSY, ^1H , ^1H -NOESY, ^1H , ^{13}C -HSQC and ^1H , ^{13}C -HMBC experiments.

S1.6. Analytical HPLC

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100/1260 Series system equipped with a G4225A high-performance degasser, a G1367A autosampler, a G1312A binary pump, a G1316A column compartment, a G1314A variable wavelength detector, and a G6125B single quadrupole LC/MSD detector.

Analyses were carried out using a C18 reversed-phase column (Kinetex EVO C18, 100 Å, 5 μm , 150 $\times$ 4.6 mm, Phenomenex; part no. 00F-4633-E0) as the stationary phase. The mobile phase consisted of distilled water and acetonitrile, each containing 0.1% formic acid or TFA as specified below, delivered at a flow rate of 1.0 mL/min. The injection volume was 20 μL .

HPLC methods were selected based on analyte polarity. The corresponding gradients and solvent compositions were as follows:

- Method 1: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–22.5 min, 5–50% B; 22.5–23.5 min, 50–100% B; 23.5–27.5 min, 100% B.
- Method 2: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–20.0 min, 10–40% B; 20.0–21.0 min, 40–100% B; 21.0–25.0 min, 100% B.
- Method 3: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–20.0 min, 5–35% B; 20.0–21.0 min, 35–100% B; 21.0–25.0 min, 100% B.

- Method 4: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–20.0 min, 2.5–32.5% B; 20.0–21.0 min, 32.5–100% B; 21.0–25.0 min, 100% B.
- Method 5: Solvent C = 0.1% TFA in water; Solvent D = 0.1% TFA in acetonitrile. Gradient: 0–20.0 min, 10–40% D; 20.0–21.0 min, 40–100% D; 21.0–25.0 min, 100% D.
- Method 6: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–20 min, 5–45% B; 20–21 min, 45–100% B; 21–25 min, 100% B.
- Method 7: Solvent A = 0.1% formic acid in water; Solvent B = 0.1% formic acid in acetonitrile. Gradient: 0–15 min, 5–80% B; 15–16 min, 80–100% B; 16–20 min, 100% B.

The compounds were analyzed using both UV detection ($\lambda = 210$ nm) and low-resolution mass spectrometry (LRMS). The LC/MSD detector was operated in positive electrospray ionization (ESI⁺) mode with the following parameters: *m/z* range = 100–2000; drying gas temperature = 350 °C; drying gas flow = 13 L/min; nebulizer pressure = 60 psi; capillary voltage = 3000 V; fragmentor voltage = 70 V.

Throughout the manuscript, reported absorbance areas and retention times refer to peaks observed in HPLC–UV chromatograms, whereas HPLC–MS total ion current (TIC) chromatograms were used to assign the identity of the corresponding UV peaks.

S1.7. Semi-Preparative HPLC

Semi-preparative RP-HPLC was performed on an Agilent 1260/1290 Infinity II system equipped with a G7157A preparative autosampler, a G7161A preparative binary pump, and a G7114A variable wavelength detector.

Peptides were purified using a C18 reversed-phase column (Kinetex EVO C18, 100 Å, 5 μ m, 150 $\times$ 21.2 mm, Phenomenex; part no. 00F-4633-P0-AX) as the stationary phase. Unless otherwise specified, the mobile phase consisted of 0.1% TFA in distilled water (Solvent C) and 0.1% TFA in acetonitrile (Solvent D), delivered at a flow rate of 10.0 mL/min. Focused gradients were used, as specified for each peptide. The injection volume was 200 μ L, and the UV detector was operated at 210 nm.

Solvents used to dissolve crude solids before purification are specified for each peptide. If insoluble material remained, the mixture was filtered before injection.*

Pure fractions were combined and lyophilized to obtain the desired peptides as trifluoroacetate salts, with purity >98% (unless otherwise stated), as confirmed by analytical HPLC–UV ($\lambda = 210$ nm).

* **Safety note:** Before injection, solutions of crude macrocyclic peptides obtained through NaBH_3CN -mediated reductive amination were checked using pH paper to ensure that the pH was not below 4, thereby avoiding potential generation of toxic HCN from residual NaBH_3CN .

S1.8. MS/MS

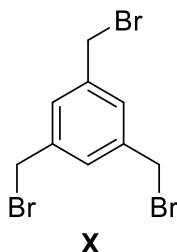
Tandem mass spectrometry (MS/MS) was performed in an LTQ XL ion-trap mass spectrometer (Thermo Scientific) fitted with a microelectrospray probe.

Prior to analysis, purified peptides, dissolved in water/acetonitrile containing 0.1% formic acid, were diluted with methanol to a final concentration of 1–20 μM . The $[\text{M}+\text{H}]^+$ ion was isolated and subjected to collision-induced dissociation (CID) using a normalized collision energy of 25–30. When relevant, fragment ions were further isolated and subjected to additional stages of fragmentation (MS^n).

For each peptide analyzed by MS/MS, the observed fragment ions, designated by their fragment type, are listed in order of decreasing relative abundance, which is indicated in parentheses. In the case of linear peptides, fragmentation typically resulted in numerous fragment ions, and only fragments with a relative abundance above 15% were reported.

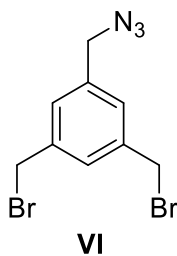
S2. Synthesis of Stapling Reagents

1,3,5-tris(bromomethyl)benzene (X)



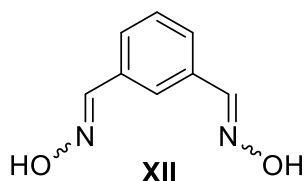
Compound **X** was prepared according to a procedure described by Mastalerz and coworkers.^[1] The NMR data were consistent with those reported in the literature.

1-(azidomethyl)-3,5-tris(bromomethyl)benzene (VI)



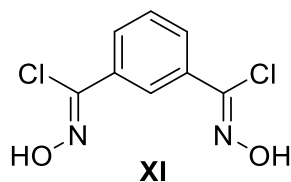
Compound **X** was prepared according to a procedure described by Manna and coworkers.^[2] The NMR data were consistent with those reported in the literature.

Isophthalaldehyde dioxime (XII)



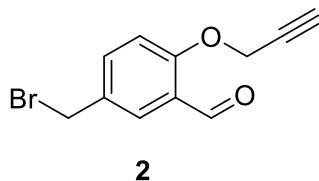
Compound **XII** was prepared according to a procedure described by Kim and coworkers.^[3] The NMR data were consistent with those reported in the literature.

(1Z,3Z)-N¹,N³-Dihydroxyisophthalimidoyl dichloride (XI)



Compound **XI** was prepared according to previously reported procedures.^[3,4] The NMR data were consistent with those reported in the literature.

5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde (2)



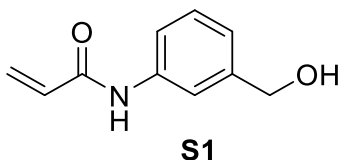
A 25 mL round-bottom flask equipped with a stirring bar was charged with 2-hydroxy-5-(hydroxymethyl)benzaldehyde (200 mg, 1.31 mmol, 1.0 equiv.) and K₂CO₃ (363 mg, 2.63 mmol, 2.0 equiv.), dried under vacuum, and flushed with nitrogen. Anhydrous DMF (6.6 mL) was added to give a bright yellow suspension. Propargyl bromide (9.2 M in toluene; 171 μ L, 1.58 mmol, 1.2 equiv.) was added dropwise to the stirring mixture, and the flask was transferred to an oil bath preheated to 45 °C. The reaction mixture was stirred under nitrogen for 1.5 h at 45 °C with TLC monitoring (mobile phase: Et₂O). EtOAc (30 mL) and 5% aq. LiCl (30 mL) were added, and the resulting mixture was transferred to a separatory funnel. The layers were separated, and the aqueous phase was extracted with EtOAc (2 $\times$ 20 mL). The combined organic layers were washed with 5% aq. LiCl (2 $\times$ 20 mL) and brine (20 mL), dried over Na₂SO₄, filtered, and concentrated under reduced pressure to a volume of 5–10 mL. The residue was transferred to a 25 mL round-bottom flask, concentrated again, and dried under

vacuum to afford crude 5-(hydroxymethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde as an oil.

The flask containing crude 5-(hydroxymethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde was equipped with a stirring bar, dried under vacuum, and flushed with nitrogen. Anhydrous DCM (13.1 mL) was added, and the resulting solution was cooled to 0 °C using an ice–water bath. PBr₃ (249 µL, 2.65 mmol, 2.0 equiv.) was added dropwise to the cold solution under stirring. The bath was removed, allowing the reaction mixture to warm to room temperature. The mixture was stirred under nitrogen for 1 h at room temperature with TLC monitoring (mobile phase: petroleum ether/EtOAc 1:1, v/v). The reaction mixture was then cooled again to 0 °C, and 5% aq. NaHCO₃ (30 mL) was added portionwise (gas evolution). The mixture was transferred to a separatory funnel, and DCM (20 mL) was added. After shaking, the layers were separated, and the aqueous phase was extracted with DCM (2 × 10 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure, and dried under vacuum. The crude product was purified by column chromatography (silica gel, 0 → 20% Et₂O in *n*-hexane) to afford **2** as a white solid (90 mg, 27%).

R_f = 0.3 (petroleum ether/Et₂O 4:1, v/v). **¹H NMR** (600 MHz, CDCl₃): δ (ppm) 10.45 (s, 1H, CHO), 7.88 (d, *J* = 2.5 Hz, 1H, H-6), 7.61 (dd, *J* = 8.6, 2.5 Hz, 1H, H-4), 7.12 (d, *J* = 8.6 Hz, 1H, H-3), 4.84 (d, *J* = 2.4 Hz, 2H, OCH₂C≡CH, H₂-1'), 4.48 (s, 2H, ArCH₂Br), 2.58 (t, *J* = 2.4 Hz, 1H, C≡CH, H-3'). **¹³C NMR** (101 MHz, CDCl₃): δ (ppm) 189.0 (CHO), 159.6 (C-2), 136.5 (C-4), 131.4 (C-5), 129.1 (C-6), 125.5 (C-1), 113.9 (C-3), 77.4 (C≡C, C-2'), 76.9 (C≡C, C-3'), 56.6 (OCH₂), 32.4 (ArCH₂Br). **HRMS** (APCI⁺) *m/z*: [M + H]⁺ calcd for C₁₁H₁₀BrO₂, 252.9859; found, 252.9856.

***N*-[3-(hydroxymethyl)phenyl]acrylamide (S1)**

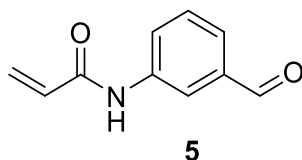


A 100 mL round-bottom flask equipped with a stirring bar was charged with 3-aminobenzyl alcohol (1.00 g, 8.12 mmol, 1.0 equiv.), dried under

vacuum, and backfilled with argon (three vacuum–argon cycles). Anhydrous tetrahydrofuran (THF; 42 mL) and NaHCO₃ (2.05 g, 24.4 mmol, 3.0 equiv.) were successively added. Acryloyl chloride (670 μ L, 8.25 mmol, 1.0 equiv.) was added dropwise to the stirring mixture, and stirring was continued under argon for 18.5 h at room temperature. Ice water (60 mL) was then added, and the resulting mixture was extracted with EtOAc (3 $\times$ 40 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give the crude product, which was purified by column chromatography (silica gel, 0 $\rightarrow$ 10% MeOH in DCM) to afford **S1** as a white solid (1.30 g, 90%).

Rf = 0.4 (DCM/MeOH 9:1, v/v). **¹H NMR** (300 MHz, DMSO-*d*₆): δ (ppm) 10.11 (s, 1H), 7.67–7.53 (m, 2H), 7.26 (t, *J* = 7.8 Hz, 1H), 7.00 (d, *J* = 7.5 Hz, 1H), 6.44 (dd, *J* = 17.0, 10.0 Hz, 1H), 6.25 (dd, *J* = 17.0, 2.1 Hz, 1H), 5.74 (dd, *J* = 10.0, 2.1 Hz, 1H), 5.20 (t, *J* = 5.7 Hz, 1H), 4.47 (d, *J* = 5.7 Hz, 2H). **¹³C NMR** (75 MHz, DMSO-*d*₆): δ (ppm) 163.1, 143.3, 138.9, 132.0, 128.5, 126.8, 121.5, 117.6, 117.3, 62.8. **HRMS** (ESI⁺) *m/z*: [M + Na]⁺ calcd for C₁₀H₁₁NO₂Na, 200.0682; found, 200.0683.

***N*-(3-formylphenyl)acrylamide (**5**)**

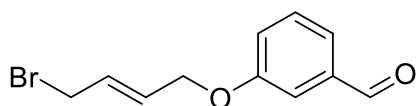


A 25 mL round-bottom flask equipped with a stirring bar was charged with **S1** (100 mg, 565 μ mol, 1.0 equiv.). Anhydrous DCM (6.0 mL), anhydrous DMF (0.5 mL), and Celite® (99.4 mg) were added. Pyridinium chlorochromate (PCC; 370 mg, 1.72 mmol, 3.0 equiv.) was added portionwise to the stirring mixture, which turned dark red. The reaction mixture was stirred at room temperature for 1.5 h with TLC monitoring (mobile phase: DCM/MeOH 9:1, v/v). The mixture was then concentrated under reduced pressure and dried under vacuum. The residue was purified by column chromatography (silica gel, 0 $\rightarrow$ 30% EtOAc in petroleum ether) to afford **5** as a white solid (78 mg, 79%).

Rf = 0.4 (petroleum ether/EtOAc 1:1, v/v). **¹H NMR** (300 MHz, CDCl₃): δ (ppm) 9.95 (s, 1H, CHO), 8.36 (s, 1H, NH), 8.10 (s, 1H, H-2), 7.98 (d,

$J = 7.8$ Hz, 1H, H-6), 7.62 (d, $J = 7.5$ Hz, 1H, H-4), 7.47 (t, $J = 7.8$ Hz, 1H, H-5), 6.47 (dd, $J = 16.9, 1.1$ Hz, 1H, H- β -*trans*), 6.34 (dd, $J = 16.9, 9.8$ Hz, 1H, H- α), 5.79 (dd, $J = 9.8, 1.1$ Hz, 1H, H- β -*cis*). **^{13}C NMR** (75 MHz, CDCl_3): δ (ppm) 192.4 (CHO), 164.3 (C=O amide), 139.0 (C-1), 137.1 (C-3), 130.9 (CH- α), 129.9 (C-5), 128.7 (CH $_2$ - β), 126.1 (C-6), 126.0 (C-4), 120.8 (C-2). **HRMS** (ESI $^+$) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{10}\text{H}_{10}\text{NO}_2$, 176.0706; found, 176.0708.

(*E*)-3-[(4-bromobut-2-en-1-yl)oxy]benzaldehyde (S2**)**

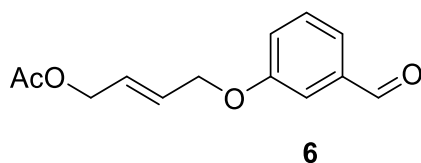


S2

Compound **S2** was prepared according to a procedure described by Cozzi and coworkers,^[5] with minor modifications. A 50 mL round-bottom flask equipped with a stirring bar was charged with 3-hydroxybenzaldehyde (398 mg, 3.26 mmol, 1.0 equiv.), dried under vacuum, and backfilled with argon (three vacuum–argon cycles). Anhydrous acetonitrile (9.0 mL) was added. K_2CO_3 (493 mg, 3.57 mmol, 1.1 equiv.) and (*E*)-1,4-dibromobut-2-ene (993 mg, 4.64 mmol, 1.4 equiv.) were successively added under stirring at room temperature. The mixture was then refluxed (oil bath temperature: 85 °C) under argon for 4.5 h. After cooling to room temperature, the mixture was filtered over a pad of Celite[®]. The filtrate was concentrated under reduced pressure and dried under vacuum. The crude product was purified by column chromatography (silica gel, 0 $\rightarrow$ 10% EtOAc in petroleum ether) to afford **S2** as a colorless oil (507 mg, 61%).

Rf = 0.3 (petroleum ether/EtOAc 9:1, v/v). **^1H NMR** (300 MHz, CDCl_3): δ (ppm) 9.97 (s, 1H, CHO), 7.50–7.42 (m, 2H, H-6, H-5), 7.40–7.36 (m, 1H, H-2), 7.23–7.15 (m, 1H, H-4), 6.16–6.05 (m, 1H, CH=CH, H-2' or H-3'), 5.99 (dt, $J = 15.3, 4.8$ Hz, 1H, CH=CH, H-2' or H-3'), 4.65–4.59 (m, 2H, $\text{OCH}_2\text{CH}=\text{CH}$, H $_2$ -1'), 4.00 (d, $J = 7.1$ Hz, 2H, $\text{BrCH}_2\text{CH}=\text{CH}$, H $_2$ -4'). **^{13}C NMR** (75 MHz, CDCl_3): δ (ppm) 192.2 (CHO), 159.0 (C-3), 137.9 (C-1), 130.3 (C-5), 129.8 (CH=CH, C-2' or C-3'), 129.5 (CH=CH, C-2' or C-3'), 124.0 (C-6), 122.2 (C-4), 113.0 (C-2), 67.5 ($\text{OCH}_2\text{CH}=\text{CH}$, C-1'), 31.6 ($\text{BrCH}_2\text{CH}=\text{CH}$, C-4'). **HRMS** (APCI $^+$) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{11}\text{H}_{12}\text{BrO}_2$, 255.0015; found, 255.0011.

(*E*)-3-[(4-acetoxybut-2-en-1-yl)oxy]benzaldehyde (6**)**



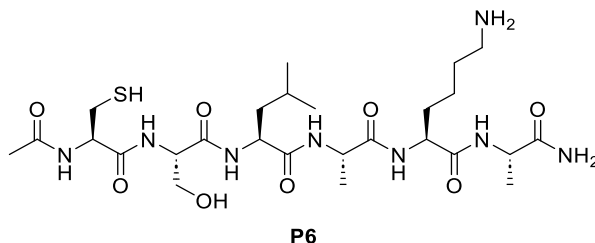
Compound **6** was prepared according to a procedure described by Cozzi and coworkers,^[5] with minor modifications. A 50 mL round-bottom flask equipped with a stirring bar was charged with potassium acetate (405 mg, 4.13 mmol, 2.2 equiv.), dried under vacuum, and backfilled with argon. Another 50 mL round-bottom flask containing **S2** (489 mg, 1.92 mmol, 1.0 equiv.) was flushed with argon, and anhydrous acetonitrile (1.0 mL) was added to dissolve its content. Anhydrous acetonitrile (9.0 mL) was added to the flask containing potassium acetate. To the resulting suspension under stirring, the solution of (*E*)-3-[(4-bromobut-2-en-1-yl)oxy]benzaldehyde was added at room temperature. The reaction mixture was then refluxed (oil bath temperature: 84 °C) under argon for 5 h with TLC monitoring (mobile phase: petroleum ether/EtOAc 5:1, v/v). After cooling to room temperature, the mixture was filtered over a pad of Celite®. The filtrate was concentrated under reduced pressure and dried under vacuum. The crude product was purified by column chromatography (silica gel, 0 → 20% EtOAc in petroleum ether) to afford **6** as a pale yellow oil (367 mg, 82%).

R_f = 0.3 (petroleum ether/EtOAc 5:1, v/v). **¹H NMR** (300 MHz, CDCl₃): δ (ppm) 9.97 (s, 1H, CHO), 7.49–7.42 (m, 2H, H-6, H-5), 7.40–7.37 (m, 1H, H-2), 7.23–7.15 (m, 1H, H-4), 6.16–6.05 (m, 2H, CH=CH, H-2', H-3'), 4.64–4.59 (m, 4H, H₂-1', H₂-4'), 2.09 (s, 3H, CH₃). **¹³C NMR** (75 MHz, CDCl₃): δ (ppm) 192.2 (CHO), 170.8 (C=O ester), 159.1 (C-3), 137.9 (C-1), 130.3 (C-5), 128.6 (CH=CH, C-2' or C-3'), 127.8 (CH=CH, C-2' or C-3'), 124.0 (C-6), 122.2 (C-4), 113.0 (C-2), 67.9 (ArOCH₂CH=CH, C-1'), 64.1 (AcOCH₂CH=CH, C-4'), 21.1 (CH₃). **HRMS** (APCI⁺) *m/z*: [M + H]⁺ calcd for C₁₃H₁₅O₄, 235.0965; found, 235.0962.

S3. Synthesis of Linear Peptides

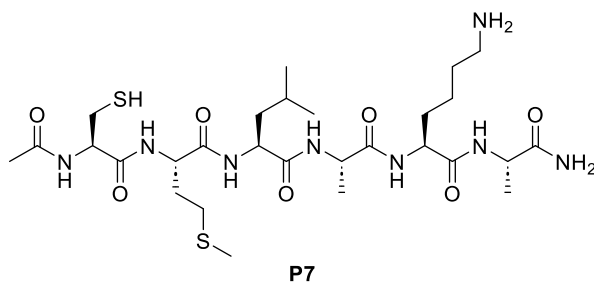
Linear hexapeptides Ac-CXLAKA-NH₂ (**P6**, X = Ser; **P7**, X = Met; **P8**, X = Tyr; **P9**, X = Arg; **P10**, X = His; **P11**, X = Trp; **P12**, X = Nle(ϵ -N₃); **P13**, X = Lys(N ϵ -Alloc)) were prepared according to the procedure described in Section S1.3, *General Methods for Solid-Phase Peptide Synthesis*. HPLC–UV chromatograms of the purified peptides (in solution in water or water/acetonitrile mixtures containing 0.1% TFA) are provided in Section S11.

Peptide **P6** (Ac-CSLAKA-NH₂)



P6 was synthesized on a 0.040 mmol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–10 min, 6–11% D; 10–11 min, 11–100% D; 11–15 min, 100% D; t_R = 9.3 min) to afford **P6** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

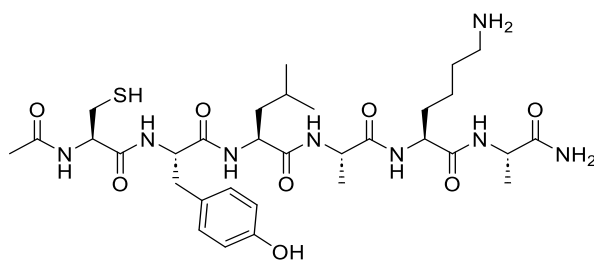
Peptide **P7** (Ac-CMLAKA-NH₂)



P7 was synthesized on a 0.087 mmol scale. The crude solid was dissolved in water/acetonitrile (2:1, v/v) containing 0.1% TFA and

purified by semi-preparative HPLC (gradient: 0–5 min, 5–14% D; 5–15 min, 14–19% D; 15–16 min, 19–100% D; 16–20 min, 100% D; t_R = 9.8 min) to afford **P7** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

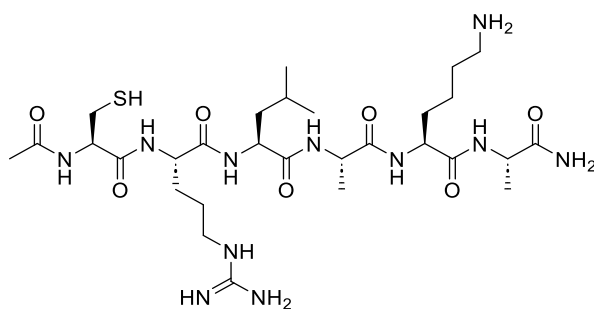
Peptide P8 (Ac-CYLAKA-NH₂)



P8

P8 was synthesized on a 0.140 mmol scale. The crude solid was dissolved in water/acetonitrile/methanol (8:8:1, v/v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–5 min, 5–15% D; 5–15 min, 15–20% D; 15–16 min, 20–100% D; 16–20 min, 100% D; t_R = 9.7 min) to afford **P8** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Peptide P9 (Ac-CRLAKA-NH₂)

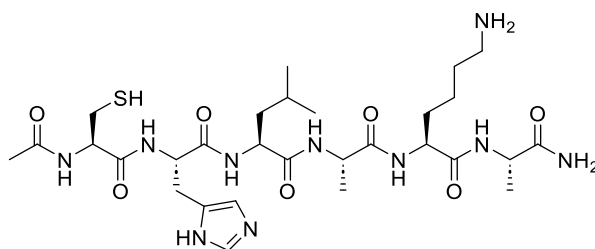


P9

P9 was synthesized on a 0.140 mmol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–10 min, 6–11% D; 10–

11 min, 11–100% D; 11–15 min, 100% D; t_R = 5.7 min) to afford **P9** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

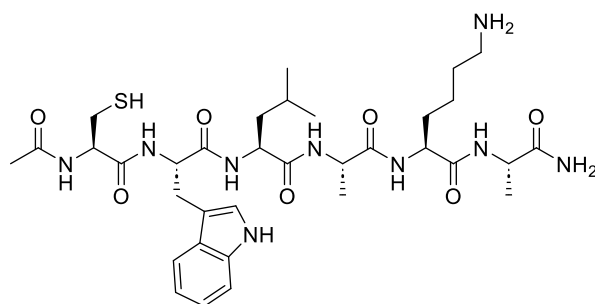
Peptide P10 (Ac-CHLAKEA-NH₂)



P10

P10 was synthesized on a 0.140 mmol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–10 min, 6–11% D; 10–11 min, 11–100% D; 11–15 min, 100% D; t_R = 5.5 min) to afford **P10** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Peptide P11 (Ac-CWLAKA-NH₂)

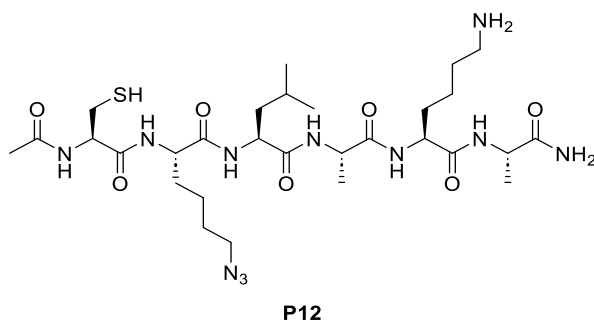


P11

P11 was synthesized on a 0.110 mmol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA. The resulting solution was purified by semi-preparative HPLC using a mobile phase consisting of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B), as the use of 0.1% TFA in the mobile phase (Solvents C and D) did not allow the isolation of **P11** with sufficient purity. The following gradient was used: 0–5 min, 5–20% B; 5–15 min, 20–25% B; 15–16 min, 25–100% B; 16–20 min, 100% B

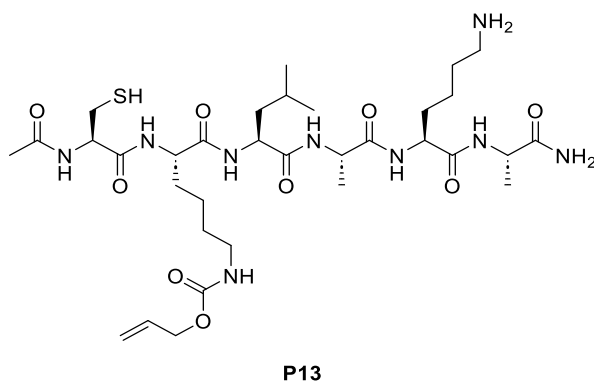
(t_R = 8.1 min). After lyophilization of the pure fractions, the solid was redissolved in 0.05% TFA in water and lyophilized again to afford **P11** (trifluoroacetate salt) as a white fluffy solid.

Peptide P12 (Ac-CNle(ϵ -N₃)LAKA-NH₂)



P12 was synthesized on a 0.110 μ mol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–5 min, 5–23% D; 5–15 min, 23–28% D; 15–16 min, 28–100% D; 16–20 min, 100% D; t_R = 8.7 min) to afford **P12** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Peptide P13 (Ac-CK(N ϵ -Alloc)LAKA-NH₂)

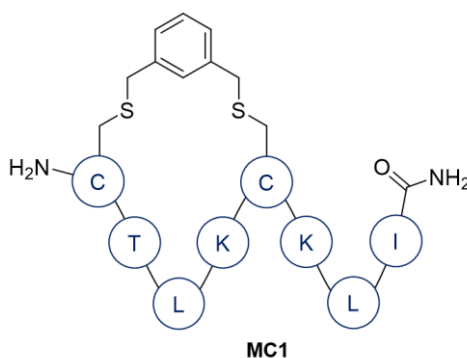


P13 was synthesized on a 0.099 mmol scale. The crude solid was dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA and purified by semi-preparative HPLC (gradient: 0–5 min, 5–23% D; 5–15 min, 23–28% D; 15–16 min, 28–100% D; 16–20 min, 100% D; t_R = 8.8

min) to afford **P13** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

S4. Cys–Cys and CuAAC–Based Stapling

Peptide MC1

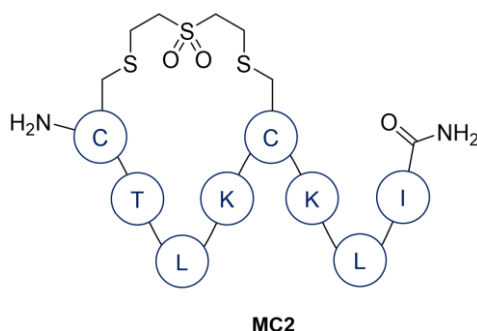


MC1 was synthesized from **LB8C¹C⁵** (16.4 μmol) according to a procedure described by Heinis and coworkers,^[6] except that 100 mM ammonium bicarbonate buffer was used instead of a 60 mM buffer

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–23% D; 5–15 min, 23–28% D; 15–16 min, 28–95% D; 16–20 min, 95% D; t_R = 9.1 min) afforded **MC1** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

A purity of 97% was determined by HPLC–UV analysis (λ = 210 nm) of a stock solution at approximately 100 μM .

Peptide MC2

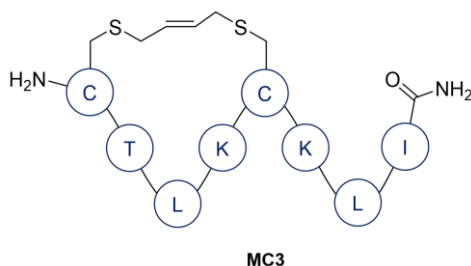


MC2 was synthesized from **LB8C¹C⁵** (23.7 μmol) according to a procedure described by Heinis and coworkers,^[6] except that 100 mM ammonium bicarbonate buffer was used instead of a 60 mM buffer.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–20% D; 5–15 min, 20–25% D; 15–16 min, 25–95% D; 16–20 min, 95% D; t_R = 8.2 min) afforded **MC2** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Purity was verified by HPLC–UV analysis (λ = 210 nm) of a stock solution at approximately 100 μM .

Peptide MC3



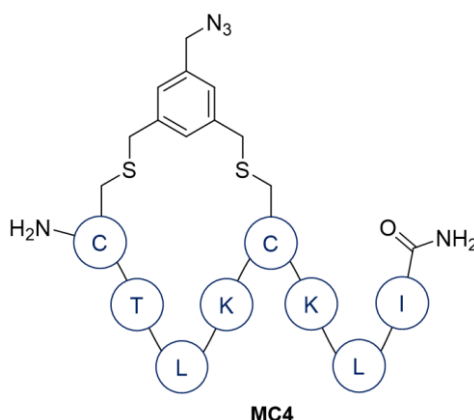
MC3 was synthesized from **LB8C¹C⁵** (13.9 μmol) according to a procedure described by Heinis and coworkers,^[6] except that 100 mM ammonium bicarbonate buffer was used instead of a 60 mM buffer.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–20% D; 5–15 min, 20–25% D; 15–16 min, 25–95% D; 16–20 min, 95% D; t_R =

8.8 min) afforded **MC3** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

A purity of 97% was determined by HPLC–UV analysis ($\lambda = 210$ nm) of a stock solution at approximately 100 μ M.

Peptide MC4

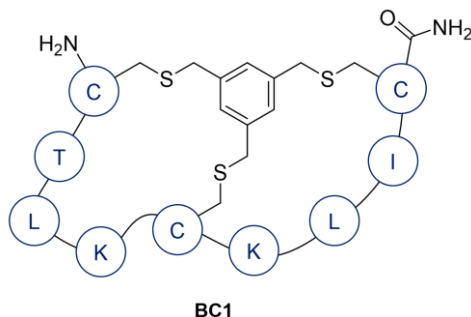


MC4 was synthesized from **LB8C¹C⁵** (27.5 μ mol) according to a procedure described by Frédérick and coworkers,^[7] except that TCEP pretreatment was not performed and cyclization was carried out at a peptide concentration of approximately 2 mM instead of 1.5 mM.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–21% D; 5–15 min, 21–27% D; 15–16 min, 27–100% D; 16–20 min, 100% D; $t_R = 8.2$ min) afforded **MC4** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Purity was verified by HPLC–UV analysis ($\lambda = 210$ nm) of a stock solution at approximately 100 μ M.

Peptide BC1

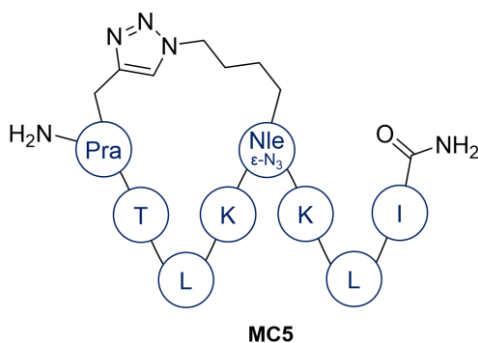


BC1 was synthesized from **LB9C¹C⁵C⁹** (13.4 μ mol) according to a procedure described by Heinis and coworkers,^[6] except that 100 mM ammonium bicarbonate buffer was used instead of a 60 mM buffer and 1.5 equivalents of cross-linking reagent (as a 15 mM solution in MeCN) was used instead of 2.0 equivalents.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–25% D; 5–15 min, 25–27% D; 15–16 min, 27–95% D; 16–20 min, 95% D; t_R = 8.6 min) afforded **BC1** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

Purity was verified by HPLC–UV analysis (λ = 210 nm) of a stock solution at approximately 100 μ M.

Peptide MC5

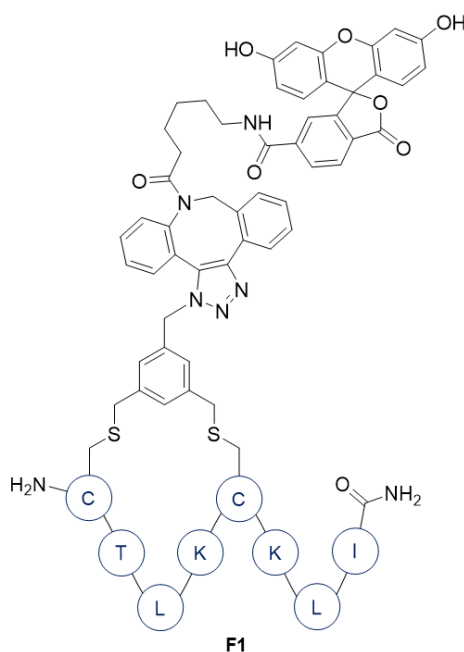


MC5 was synthesized from **LB8P¹N⁵** (8.3 μ mol) according to a procedure described by Wang and coworkers,^[8] using 5.1 equivalents of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 5.2 equivalents of sodium L-ascorbate. The reaction mixture was stirred for 2.5 h.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–20% D; 5–15 min, 20–22% D; 15–16 min, 22–100% D; 16–20 min, 100% D; t_R = 8.4 min) afforded **MC5** (trifluoroacetate salt) as a white fluffy solid following lyophilization.

A purity of 99% was determined by HPLC–UV analysis (λ = 210 nm) of a stock solution at approximately 100 μ M.

Peptide F1

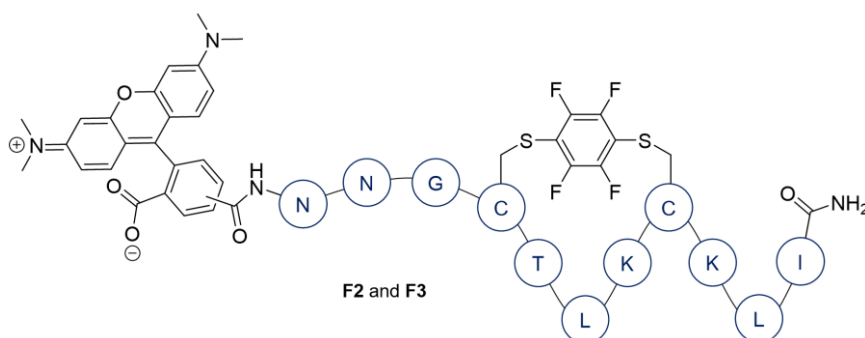


F1 was synthesized from **MC4** and FAM-ADIBO (6-isomer). **MC4** (3.9 mg, 2.7 μ mol, 1.0 equiv.) was dissolved in sodium phosphate buffer (50 mM, pH 7.4; 1650 μ L). FAM-ADIBO (6-isomer; 18 mM in DMSO; 183 μ L, 3.3 μ mol, 1.2 equiv.) was then added, resulting in a peptide concentration of approximately 1.5 mM. After incubation at room temperature for 3 h, the reaction mixture was diluted twofold with water.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–30% D; 5–15 min, 30–32% D; 15–16 min, 32–100% D; 16–20 min, 100% D; t_R = 8.5 min) afforded **F1** (trifluoroacetate salt) as a yellow fluffy solid following lyophilization.

Purity was verified by HPLC–UV analysis ($\lambda = 210$ nm) of a stock solution prepared by dissolving a small amount of the purified product in water.

Peptides **F2** and **F3**



F2 and **F3** were individually synthesized from the 5- and 6-regioisomers of the linear peptide TAMRA-NNGCTLKCKLI-NH₂, (2.2–2.9 μ mol) according to a procedure described by Pentelute and coworkers.^[9] The reaction mixtures were incubated for 4.5 h, protected from light. The crude mixtures were individually subjected to semi-preparative HPLC purification.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–24% D; 5–15 min, 24–30% D; 15–16 min, 30–100% D; 16–20 min, 100% D; $t_R = 10.2$ min) afforded **F2** (trifluoroacetate salt) as a purple fluffy solid following lyophilization.

Purification by semi-preparative HPLC (gradient: 0–5 min, 5–28% D; 5–15 min, 28–32% D; 15–16 min, 32–100% D; 16–20 min, 100% D; $t_R = 9.1$ min) afforded **F3** (trifluoroacetate salt) as a purple fluffy solid following lyophilization.

Purity was verified by HPLC–UV analysis ($\lambda = 210$ nm) of the pooled fractions obtained by semi-preparative HPLC.

S5. Cys–Lys, Cys–Orn, Cys–Dab, and Cys–Dap Stapling with *m*-(bromomethyl)benzaldehyde

S5.1. Optimization of the Stapling Procedure

S5.1.1. Large-Scale S-Alkylation of the Peptide Model

The linear peptide model Ac-CTLAKALQA-NH₂ (**P1**; purchased from GeneCust; trifluoroacetate salt; purity >95%; 78.8 mg, 73.4 μmol, 1.0 equiv.) was weighed and transferred to a 15 mL centrifuge tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0, 3.670 mL) and acetonitrile (3.494 mL) were added sequentially. A solution of *m*-(bromomethyl)benzaldehyde (**1**; 0.50 M in acetonitrile; 176 μL, 88.0 μmol, 1.2 equiv.) was then added, resulting in a peptide concentration of approximately 10 mM. The reaction mixture was briefly shaken manually and incubated at 23 °C, protected from light.

After incubation for 1 h, 100 μL of the reaction mixture was diluted 100-fold with 0.1% TFA in water and analyzed by HPLC (Method 1), which indicated >99% conversion based on the absorbance of a 100 μM solution of the starting peptide. After this analysis (approximately 100 minutes total reaction time), the remaining reaction medium was divided into ten fractions: eight 650 μL aliquots were transferred to 15 mL centrifuge tubes containing 5.850 mL of 0.1% TFA in water and two 850 μL aliquots were transferred to tubes containing 7.650 mL of 0.1% TFA in water. The diluted fractions were then lyophilized to afford ten crude samples of the S-alkylated peptide **P1.1al**, corresponding to 8 × 6.5 μmol and 2 × 8.5 μmol, calculated relative to the initial amount of peptide used and assuming quantitative conversion.

S5.1.2. Screening of Conditions for Intramolecular Reductive Amination

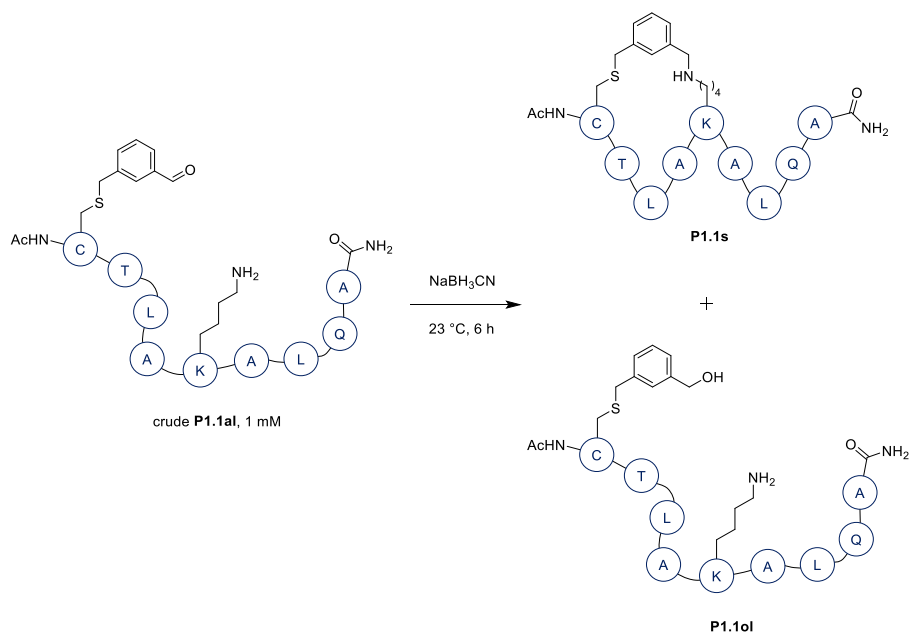
S5.1.2.1. Solvent and NaBH₃CN Loading

Sodium acetate buffer (100 mM, pH 5.0, 3.25 mL) was added to a 15 mL centrifuge tube containing approximately 6.5 μmol of crude peptide aldehyde **P1.1al** to give a peptide concentration of 2 mM. Six 500 μL aliquots of the resulting solution were transferred to 1.5 mL Eppendorf tubes, each containing 490 μL (entries 1–3) or 480 μL (entries 4–6) of sodium acetate buffer (100 mM, pH 5.0; entries 1 and 4), acetonitrile

(entries 2 and 5), or methanol (entries 3 and 6). The resulting solutions were briefly shaken manually.

Either 10 μL (10 μmol , 10 equiv.; entries 1–3) or 20 μL (20 μmol , 20 equiv.; entries 4–6) of a freshly prepared solution of NaBH_3CN (1.0 M in methanol) was added to each Eppendorf tube (peptide concentration: ~ 1 mM, relative to the initial amount of **P1**). The reaction mixtures were briefly shaken manually and incubated at 23 $^\circ\text{C}$, protected from light.

After incubation for 6 h, 500 μL of each reaction mixture was diluted 2-fold with 0.1% TFA in water and frozen. Each sample was subsequently thawed individually and further diluted 5-fold with 0.1% TFA in water (final peptide concentration: ~ 100 μM , relative to the initial amount of **P1**), then analyzed by HPLC (Method 1). The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** ($t_R = 12.8$ min) and the alcohol byproduct **P1.1ol** ($t_R = 12.3$ min) were measured to estimate the efficiency of the cyclization reaction, and more specifically, the ability of the reducing agent to selectively reduce the iminium intermediate over the aldehyde.



Scheme S1. Intramolecular reductive amination of peptide aldehyde **P1.1al**.

S4.1.2.2. Methanol/Aqueous Buffer Volume Ratio

3.25 mL of a 1:1 (v/v) mixture of methanol and sodium acetate buffer (100 mM, pH 5.0) was added to a 15 mL centrifuge tube containing approximately 6.5 μ mol of crude peptide aldehyde **P1.1al**. The tube was briefly sonicated to yield a solution with a peptide concentration of 2 mM. Five 500 μ L aliquots were transferred to 1.5 mL Eppendorf tubes, each containing 490 μ L of sodium acetate buffer (100 mM, pH 5.0; entry 1), methanol/sodium acetate buffer (1:5, v/v; entry 2), methanol/sodium acetate buffer (1:1, v/v; entry 3), methanol/sodium acetate buffer (5:1, v/v; entry 4), or methanol (entry 5). The resulting solutions were briefly shaken manually.

A freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 μ L, 10 μ mol, 10 equiv.) was added to each Eppendorf tube (peptide concentration: ~1 mM, relative to the initial amount of **P1**). The reaction mixtures were briefly shaken manually and incubated at 23 °C, protected from light.

After incubation for 6 h, 500 μ L of each reaction mixture was diluted 2-fold with 0.1% TFA in water and frozen. Each sample was subsequently thawed individually and further diluted 5-fold with 0.1% TFA in water (final peptide concentration: ~100 μ M, relative to the initial amount of **P1**), then analyzed by HPLC (Method 1). The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** (t_R = 12.7–12.8 min) and the alcohol byproduct **P1.1ol** (t_R = 12.3 min) were measured to estimate the efficiency of the cyclization reaction, and more specifically, the ability of the reducing agent to selectively reduce the iminium intermediate over the aldehyde.

S5.1.2.3. pH of the Aqueous Buffer

A 4:1 (v/v) mixture of methanol and water (6.375 mL) was added to a 15 mL centrifuge tube containing approximately 8.5 μ mol of crude peptide aldehyde **P1.1al**. The tube was briefly sonicated to yield a solution with a peptide concentration of 1.33 mM. Eight 750 μ L aliquots were transferred to 1.5 mL Eppendorf tubes, each containing 140 μ L of methanol and 100 μ L of one of the following aqueous buffers (250 mM):

- Phosphoric acid–sodium phosphate, pH 2.1 (entry 1)
- Sodium citrate, pH 3.0 (entry 2)

- Sodium acetate, pH 4.0 (entry 3)
- Sodium acetate, pH 5.0 (entry 4)
- Sodium phosphate, pH 6.0 (entry 5)
- Sodium phosphate, pH 7.0 (entry 6)
- Sodium phosphate, pH 8.0 (entry 7)
- Sodium borate, pH 9.0 (entry 8)

The resulting solutions were briefly shaken manually.

A freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 µL, 10 µmol, 10 equiv.) was added to each Eppendorf tube (peptide concentration: ~1 mM, relative to the initial amount of **P1**; buffer concentration: 25 mM). The reaction mixtures were briefly shaken manually and incubated at 23 °C, protected from light.

After incubation for 6 h, 500 µL of each reaction mixture was diluted 2-fold with 0.1% TFA in water and frozen. Each sample was subsequently thawed individually and further diluted 5-fold with 0.1% TFA in water (final peptide concentration: ~100 µM, relative to the initial amount of **P1**), then analyzed by HPLC (Method 1). The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** (*t_R* = 12.7 min) and the alcohol byproduct **P1.1ol** (*t_R* = 12.2–12.3 min) were measured to estimate the efficiency of the cyclization reaction, and more specifically, the ability of the reducing agent to selectively reduce the iminium intermediate over the aldehyde.

S5.1.2.4. Nature of the Aqueous Buffer

A 4:1 (v/v) mixture of methanol and water (4.875 mL) was added to a 15 mL centrifuge tube containing approximately 6.5 µmol of crude peptide aldehyde **P1.1al**. The tube was briefly sonicated to yield a solution with a peptide concentration of 1.33 mM. Four 750 µL aliquots were transferred to 1.5 mL Eppendorf tubes, each containing 140 µL of methanol and 100 µL of one of the following aqueous buffers (250 mM):

- Sodium acetate, pH 4.0 (entry 1)
- Sodium citrate, pH 4.0 (entry 2)
- Sodium acetate, pH 5.0 (entry 3)
- Sodium citrate, pH 5.0 (entry 4)

The resulting solutions were briefly shaken manually.

A freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 μ L, 10 μ mol, 10 equiv.) was added to each Eppendorf tube (peptide concentration: ~1 mM, relative to the initial amount of **P1**; buffer concentration: 25 mM). The reaction mixtures were briefly shaken manually and incubated at 23 °C, protected from light.

After incubation for 6 h, 500 μ L of each reaction mixture was diluted 2-fold with 0.1% TFA in water and frozen. Each sample was subsequently thawed individually and further diluted 5-fold with 0.1% TFA in water (final peptide concentration: ~100 μ M, relative to the initial amount of **P1**), then analyzed by HPLC (Method 1). The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** (t_R = 12.7 min) and the alcohol byproduct **P1.1ol** (t_R = 12.3 min) were measured to estimate the efficiency of the cyclization reaction, and more specifically, the ability of the reducing agent to selectively reduce the iminium intermediate over the aldehyde.

S5.1.2.5. Buffer Concentration

A 4:1 (v/v) mixture of methanol and water (6.375 mL) was added to a 15 mL centrifuge tube containing approximately 8.5 μ mol of crude peptide aldehyde **P1.1al**. The tube was briefly sonicated to yield a solution with a peptide concentration of 1.33 mM. Eight 750 μ L aliquots were transferred to 1.5 mL Eppendorf tubes, each containing 140 μ L of methanol and 100 μ L of one of the following aqueous buffers (pH 4.0):

- 125 mM sodium acetate (entry 1)
- 250 mM sodium acetate (entry 2)
- 500 mM sodium acetate (entry 3)
- 1 M sodium acetate (entry 4)
- 125 mM sodium citrate (entry 5)
- 250 mM sodium citrate (entry 6)
- 500 mM sodium citrate (entry 7)
- 1 M sodium citrate (entry 8)

The resulting solutions were briefly shaken manually.

A freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 μ L, 10 μ mol, 10 equiv.) was added to each Eppendorf tube (peptide concentration: ~1 mM, relative to the initial amount of **P1**). The reaction

mixtures were briefly shaken manually and incubated at 23 °C, protected from light.

After incubation for 6 h, 500 µL of each reaction mixture was diluted 2-fold with 0.1% TFA in water and frozen. Each sample was subsequently thawed individually and further diluted 5-fold with 0.1% TFA in water (final peptide concentration: ~100 µM, relative to the initial amount of **P1**), then analyzed by HPLC (Method 1). The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** (t_R = 12.7 min) and the alcohol byproduct **P1.1ol** (t_R = 12.2–12.3 min) were measured to estimate the efficiency of the cyclization reaction, and more specifically, the ability of the reducing agent to selectively reduce the iminium intermediate over the aldehyde.

S5.1.3. Optimization of the One-Pot S-Alkylation/Reductive Amination Protocol

The investigation of the intramolecular reductive amination of the crude S-alkylated peptide **P1.1al** indicated that a buffer at pH 4.0 afforded the most favorable target product-to-alcohol byproduct ratio (see Table 4). To verify whether this remains true under one-pot conditions (i.e., without lyophilization of the crude S-alkylated intermediate **P1.1al**), buffers at three different pH values (3.0, 4.0, and 5.0) were tested during the optimization of the one-pot protocol. A high buffer concentration also appeared to favor the selective reduction of the iminium species over the aldehyde (see Table 6). However, at 50 mM, citrate buffer may exhibit limited solubility at pH above 4 in 3:1 (v/v) methanol/water mixtures, with precipitation occasionally observed in Eppendorf tubes after a few hours. Therefore, a final buffer concentration of 25 mM was selected as the baseline concentration for the subsequent optimization study.

The linear peptide model Ac-CTLAKALQA-NH₂ (**P1**; purchased from GeneCust; trifluoroacetate salt; purity >95%; 46.6 mg, 43.4 µmol, 1.0 equiv.) was weighed and transferred to a 15 mL centrifuge tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 2.171 mL) and acetonitrile (2.066 mL) were added sequentially, and the mixture was vortexed for a few seconds. A solution of *m*-(bromomethyl)benzaldehyde (**1**; 0.50 M in acetonitrile; 104 µL, 52.1 µmol, 1.2 equiv.) was then added, resulting in a peptide concentration

of 10 mM. The reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, 100 µL aliquots of the reaction mixture (containing the *S*-alkylated intermediate **P1.1aI**) were transferred to 16 Eppendorf tubes that had been pre-filled with various solvent systems, as described in the table below.

Tube "P1.1 inter."	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
900 µL 0.1% TFA in H ₂ O	690 µL MeOH + 200 µL sodium citrate buffer (125 mM, pH 3.0)	690 µL MeOH + 200 µL sodium citrate buffer (125 mM, pH 4.0)	690 µL MeOH + 200 µL sodium citrate buffer (250 mM, pH 4.0)	690 µL MeOH + 200 µL sodium acetate buffer (125 mM, pH 4.0)	690 µL MeOH + 200 µL sodium acetate buffer (500 mM, pH 4.0)
Tube 6	Tube 7	Tube 8	Tube 9	Tube 10	Tube 11
690 µL MeOH + 200 µL sodium citrate buffer (125 mM, pH 5.0)	690 µL MeOH + 200 µL sodium acetate buffer (500 mM, pH 5.0)	440 µL MeOH + 450 µL sodium citrate buffer (111.1 mM, pH 4.0)	890 µL MeOH	690 µL MeCN + 200 µL sodium citrate buffer (125 mM, pH 4.0)	690 µL EtOH + 200 µL sodium citrate buffer (125 mM, pH 4.0)
Tube 12	Tube 13	Tube 14	Tube 15	Tube 16	
680 µL MeOH + 200 µL sodium citrate buffer (125 mM, pH 4.0)	680 µL MeOH + 200 µL sodium citrate buffer (250 mM, pH 4.0)	690 µL MeOH + 200 µL sodium citrate buffer (125 mM, pH 4.0)	540 µL MeOH + 150 µL sodium citrate buffer (166.7 mM, pH 4.0)	315 µL MeOH + 75 µL sodium citrate buffer (333.3 mM, pH 4.0)	

The 17 tubes were vortexed. Tube "P1.1 inter." was frozen while the others were subjected to reductive amination. To this end, a freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 µL in tubes 1–11 and 15–16, 10 µmol, 10 equiv.; 20 µL in tubes 12–13, 20 µmol, 20 equiv.) or 2-picoline–borane (pic-BH₃; 1.0 M in THF; 10 µL in tube 14, 10 µmol, 10 equiv.) was added. Final volumes were 1000 µL for Tubes "P1.1 inter." and 1–14, 800 µL for Tube 15, and 500 µL for Tube 16 (volume contraction ignored). Each reaction mixture (Tubes 1–16) was vortexed immediately after addition of the reducing agent. The mixtures were then incubated at 23 °C, protected from light.

To obtain duplicates, 100 µL aliquots of the remaining solution of *S*-alkylated intermediate **P1.1aI** were transferred to 17 additional Eppendorf tubes pre-filled in the same manner (Tubes "P1.1 inter. bis")

and 1 bis–16 bis). These duplicate samples were treated identically, as described above.

After incubation for 6 h, 100 μ L of each reaction mixture was diluted 10-fold into methoxylamine buffer. The resulting samples were each briefly mixed by manual shaking and allowed to stand at room temperature for 30–45 minutes before being frozen. These diluted samples were then thawed individually and directly analyzed by HPLC, except those obtained from Tubes 15 and 16, which were further diluted (1.25-fold and 2-fold, respectively) with methoxylamine buffer prior to injection. All analyzed samples (entries 1–16 and 1 bis–16 bis) had a final peptide concentration of approximately 100 μ M relative to the initial amount of peptide model **P1**, and were evaluated using Method 2.

To enable subsequent estimation of the conversion of the S-alkylated intermediate **P1.1al** (peptide aldehyde), Tube “P1.1 inter.” was thawed and 100 μ L aliquots were diluted 10-fold into either 0.1% TFA in water or methoxylamine buffer, yielding a final peptide concentration of approximately 100 μ M. The sample diluted in 0.1% TFA was directly analyzed by HPLC (entry A; Method 2), while the one diluted in methoxylamine buffer was allowed to stand at room temperature for about 30 minutes prior to analysis (entry O; Method 2). Tube “P1.1 inter. bis” was treated identically, giving entries A bis and O bis. The absorbance areas corresponding to the S-alkylated intermediate **P1.1al** (t_R = 12.6 min) and its oxime-trapped derivative **P1.1ox_a** (major isomer, t_R = 14.9 min) were recorded: the average absorbance area of the S-alkylated intermediate **P1.1al** was 3179.6 mAU·s (entries A and A bis), while that of its trapped derivative **P1.1ox_a** was 2687.0 mAU·s (entries O and O bis).

The chromatograms obtained from entries 1–16 and 1 bis–16 bis were processed as follows. The absorbance areas corresponding to the desired macrocyclic peptide **P1.1s** (t_R = 12.3–12.4 min), the alcohol byproduct **P1.1ol** (t_R = 11.7–11.8 min), and the trapped intermediate **P1.1ox_a** (major isomer, t_R = 14.9–15.0 min) were measured. Conversion of intermediate **P1.1al** during the reductive amination step was estimated by comparing the absorbance area of the trapped intermediate (6 h after addition of the reducing agent) with the average area measured in entries O and O bis, which represent the reaction medium prior to the start of the reductive amination step. Reaction yields were determined based on a calibration curve established using isolated macrocyclic peptide **P1.1s** (see Figure S11). Selectivity for

iminium reduction over aldehyde reduction was assessed based on the absorbance areas of the target product and the alcohol byproduct.

Table S1. Optimization of the one-pot S-alkylation/reductive amination protocol.

Step ^a	Entry	[Peptide] (mM) ^b	Reagent	Solvent Composition (v/v)	[Buffer] (mM) ^c	Conversion (%) ^{d, e}	Yield (%) ^{d, f}	s/(s + ol) (%) ^{d, g}
1	/	10	1 (1.2 equiv.)	MeCN/aq. NH ₄ HCO ₃ (pH 8.0) 1:1	25	>99	/	/
2	1	1	NaBH ₃ CN (10 equiv.)	MeOH/MeCN/aq. sodium citrate (pH 3.0) 14:1:5	25	>99	84	92
	2			MeOH/MeCN/aq. sodium citrate (pH 4.0) 14:1:5	25	>99	86	97
	3			MeOH/MeCN/aq. sodium citrate (pH 4.0) 14:1:5	50	>99	86	97
	4			MeOH/MeCN/aq. sodium acetate (pH 4.0) 14:1:5	25	>99	82	95
	5			MeOH/MeCN/aq. sodium acetate (pH 4.0) 14:1:5	100	>99	83	96
	6			MeOH/MeCN/aq. sodium citrate (pH 5.0) 14:1:5	25	>99	85	96
	7			MeOH/MeCN/aq. sodium acetate (pH 5.0) 14:1:5	100	>99	83	96
	8			MeOH/MeCN/aq. sodium citrate (pH 4.0) 9:1:10	50	89	72	90
	9			MeOH/MeCN/H ₂ O 18:1:1	/	89	70	95
	10			MeCN/aq. sodium citrate (pH 4.0) 15:5	25	88	76	94
	11			EtOH/MeCN/aq. sodium citrate (pH 4.0) 14:1:5	25	>99	84	96
	12		NaBH ₃ CN (20 equiv.)	MeOH/MeCN/aq. sodium citrate (pH 4.0) 14:1:5	25	>99	84	95
	13			MeOH/MeCN/aq. sodium citrate (pH 4.0) 14:1:5	50	>99	84	96
	14		pic-BH ₃ (10 equiv.)	MeOH/MeCN/THF/aq. sodium citrate (pH 4.0) 69:5:1:25	25	79	69	95
	15	1.25	NaBH ₃ CN (10 equiv.)	MeOH/MeCN/aq. sodium citrate (pH 4.0) 55:5:20	31.25	>99	84	96
	16	2		MeOH/MeCN/aq. sodium citrate (pH 4.0) 13:2:5	50	>99	83	95

^a Reaction time for step 1 = 1 h; reaction time for step 2 = 6 h; temperature for both steps = 23 °C.

^b Peptide concentration relative to the initial amount of peptide model **P1**.

^c Final buffer concentration in the corresponding solvent mixture (if applicable); NH₄HCO₃ concentration in step 2 not reported, being at least 10-fold lower than that of the buffer added after step 1.

^d Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μM); average of duplicate reactions.

^e Calculated as [1 – (absorbance area of **P1** at t = 1 h / absorbance area of **P1** at t₀)] × 100 (step 1), or [1 – (absorbance area of **P1.1ox**_a at t = 6 h / absorbance area of **P1.1ox**_a at t₀)] × 100 (step 2).

^f Calculated based on the calibration curve established for **P1.1s** (see Figure S11).

^g Calculated as (absorbance area of **P1.1s** / combined absorbance area of **P1.1s** and **P1.1ol**) × 100.

S5.2. General Procedures for S-alkylation/Reductive Amination on an Analytical Scale

To enable estimation of the conversion of the S-alkylated intermediate (peptide aldehyde) during the reductive amination step, an aliquot of the reaction mixture was quenched immediately after the S-alkylation step. For the intramolecular reductive amination step, a buffer at pH 4.0 was used by default. In specific cases (cyclization of intermediates **P1.1s–P5.1s**, as well as **P1.2s**), a buffer at pH 5.0 was tested in parallel, as these pH values had previously provided comparable cyclization yields for the peptide model. In addition, some reactions were carried out in duplicate. These considerations required the reaction mixture to be divided into several Eppendorf tubes.

The S-alkylation step was performed in a 1:1 (v/v) mixture of acetonitrile and ammonium bicarbonate buffer (pH 8.0), typically at a concentration of 10 mM of the starting peptide, when solubility permitted. Depending on the peptide sequence, precipitation of the S-alkylated intermediate was occasionally observed during this step, as assessed visually.

To ensure that a reproducible quantity of intermediate was subjected to the reductive amination step, two different protocols were employed depending on precipitation behavior. **General Procedure 1** was used when no precipitation had been observed in preliminary S-alkylation experiments, or when the crude reaction medium containing the S-alkylated intermediate could be homogenized by manual shaking or sonication. In this case, the reaction mixture was divided after the S-alkylation step. **General Procedure 2** was followed when preliminary S-alkylation experiments resulted in a cloudy reaction medium, when solubility of the S-alkylated intermediate was ambiguous (i.e., the visual appearance of a precipitate was unclear in earlier experiments), or when no prior solubility assessment had been conducted. In this procedure, the solution of starting peptide was divided before the S-alkylation step.

If the starting peptide was not soluble at 10 mM in the initial solvent system, **General Procedure 3** was used. In this case, the S-alkylation step was conducted at a peptide concentration of 5 mM, and the solution of starting peptide was divided before this step.

General Procedure 1:

The solid linear peptide (trifluoroacetate salt; 4–10 μmol , 1.0 equiv.) was weighed and transferred to a 15 mL centrifuge tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 $\mu\text{L}/\mu\text{mol}$ peptide) and acetonitrile (47.6 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. A solution of the appropriate stapling reagent (0.50 M in acetonitrile; 2.4 $\mu\text{L}/\mu\text{mol}$ peptide, 1.2 equiv.) was then added, resulting in a peptide concentration of 10 mM. The reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, 100 μL aliquots of the reaction mixture (containing the S-alkylated intermediate) were transferred to Eppendorf tubes that had been pre-filled with various solvent systems, as described in the table below.

Tube “inter.”	Tube 1	Tube 2 (optional)
900 μL 0.1% TFA in H_2O	690 μL MeOH + 200 μL sodium citrate buffer (250 mM, pH 4.0)	690 μL MeOH + 200 μL sodium citrate buffer (125 mM, pH 5.0)
	Tube 1 bis (optional)	Tube 2 bis (optional)
	690 μL MeOH + 200 μL sodium citrate buffer (250 mM, pH 4.0)	690 μL MeOH + 200 μL sodium citrate buffer (125 mM, pH 5.0)

Tubes “inter.”, 1, and 2 were vortexed briefly, and then treated as described below.

Post-S-modification treatment of reaction tubes and chromatogram processing (common to General Procedures 1–3):

Tube “inter.” was frozen while Tubes 1 and 2 were subjected to reductive amination. To this end, a freshly prepared solution of NaBH_3CN (1.0 M in methanol; 10 μL , 10 μmol , 10 equiv.) was added. Final volumes were 1000 μL (volume contraction ignored), yielding a peptide concentration of approximately 1 mM relative to the initial amount of starting peptide. Tubes 1 and 2 were vortexed immediately after addition of the reducing agent. The mixtures were then incubated at 23 °C, protected from light.

After incubation for a specified time, 100 μL of each reaction mixture (Tubes 1 and 2) was diluted 10-fold into methoxylamine buffer, yielding a final peptide concentration of approximately 100 μM relative to the initial amount of starting peptide. The resulting samples were each

briefly mixed by manual shaking and allowed to stand at room temperature for 15–30 minutes before being frozen. These diluted samples were then thawed individually and directly analyzed by HPLC (entries 1 and 2).

To enable subsequent estimation of the conversion of the S-alkylated intermediate (peptide aldehyde), Tube “inter.” was thawed and 100 μL aliquots were diluted 10-fold into either 0.1% TFA in water or methoxylamine buffer, yielding a final peptide concentration of approximately 100 μM . The sample diluted in 0.1% TFA was directly analyzed by HPLC (entry A), while the one diluted in methoxylamine buffer was allowed to stand at room temperature for about 30 minutes prior to analysis (entry O). The absorbance areas corresponding to the S-alkylated intermediate (**al**) and its oxime-trapped derivative (major isomer, **ox_a**) were recorded.

The chromatograms obtained from entries 1 and 2 were processed as follows. The absorbance areas corresponding to the desired stapled peptide (**s**), the alcohol byproduct (**ol**), and the trapped intermediate (major isomer, **ox_a**) were measured. Conversion of the peptide aldehyde intermediate during the reductive amination step was estimated by comparing the absorbance area of the trapped intermediate with that measured in entry O, which represents the reaction medium prior to the start of the reductive amination step. Reaction yields were determined based on a calibration curve established using the corresponding isolated macrocyclic peptide, unless otherwise specified (see Section S5.3, *Determination of Reaction Yields by HPLC*). Selectivity for iminium reduction over aldehyde reduction was assessed based on the absorbance areas of the target product and the corresponding alcohol byproduct.

General Procedure 2:

The solid linear peptide (trifluoroacetate salt; 4–10 μmol) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 $\mu\text{L}/\mu\text{mol}$ peptide) and acetonitrile (47.6 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. 97.6 μL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “inter.”, 1, and 2 when applicable), so that each tube contained 1 μmol of starting peptide. A solution of the appropriate stapling reagent (0.50 M in acetonitrile;

2.4 μL , 1.2 μmol , 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 $^{\circ}\text{C}$, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “inter.”, 1, and 2 as described in the table below.

Tube “inter.”	Tube 1	Tube 2 (optional)
900 μL 0.1% TFA in H_2O	200 μL sodium citrate buffer (250 mM, pH 4.0) + 690 μL MeOH	200 μL sodium citrate buffer (125 mM, pH 5.0) + 690 μL MeOH
	Tube 1 bis (optional)	Tube 2 bis (optional)
	200 μL sodium citrate buffer (250 mM, pH 4.0) + 690 μL MeOH	200 μL sodium citrate buffer (125 mM, pH 5.0) + 690 μL MeOH

Tubes “inter.”, 1, and 2 were vortexed briefly, and then treated as described in General Procedure 1. The resulting chromatograms were processed in the same manner.

General Procedure 3:

The solid linear peptide (trifluoroacetate salt; 4–10 μmol) was weighed and transferred to a 15 mL centrifuge tube. Acetonitrile (97.6 $\mu\text{L}/\mu\text{mol}$ peptide) and freshly prepared ammonium bicarbonate buffer (25 mM, pH 8.0; 100 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. 197.6 μL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “inter.”, 1, and 2 when applicable), so that each tube contained 1 μmol of starting peptide. A solution of the appropriate stapling reagent (0.50 M in acetonitrile; 2.4 μL , 1.2 μmol , 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 5 mM. Each reaction mixture was vortexed briefly and incubated at 23 $^{\circ}\text{C}$, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “inter.”, 1, and 2 as described in the table below.

Tube “inter.”	Tube 1	Tube 2 (optional)
800 μL 0.1% TFA in H_2O	150 μL sodium citrate buffer (333.3 mM, pH 4.0) + 640 μL MeOH	150 μL sodium citrate buffer (166.7 mM, pH 5.0) + 640 μL MeOH
	Tube 1 bis (optional)	Tube 2 bis (optional)
	150 μL sodium citrate buffer (333.3 mM, pH 4.0) + 640 μL MeOH	150 μL sodium citrate buffer (166.7 mM, pH 5.0) + 640 μL MeOH

Tubes “inter.”, 1, and 2 were vortexed briefly, and then treated as described in General Procedure 1. The resulting chromatograms were processed in the same manner.

S5.3. Determination of Reaction Yields by HPLC

Yields were determined from reactions carried out on an analytical scale. Unless otherwise stated, they were calculated using an HPLC–UV calibration curve established with the purified target peptide. The concentration of the target peptide in the diluted analytical-scale reaction mixture was obtained from its absorbance area using the calibration equation. This value was then divided by the total peptide concentration in that sample, expressed relative to the initial amount of starting peptide.

The HPLC yield value could have been affected by uncertainties arising from weighing small amounts of starting peptide, volume contraction upon mixing, and the presence of impurities (<5%) in commercial batches of the starting peptide.

The isolated yield was reported for completeness, but it was less representative of the reaction efficiency than the calibration-based yield, because non-negligible peptide losses typically occurred during semi-preparative HPLC. Moreover, since product purity was prioritized over isolated mass in this work, the isolated yield for a given target peptide often depended strongly on how readily it could be separated from impurities of similar polarity.

When the target peptide had not been produced on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yields of the corresponding analytical-scale reactions were not determined. Instead, reaction efficiency was evaluated using an absorbance ratio (%), calculated as [(absorbance area of the target peptide / combined absorbance area of all relevant peptidic species) × 100]. This ratio was less accurate and generally overestimated the actual yield, as low-abundance species could be masked by the HPLC–UV baseline noise. For instance, stapling of **P1** with reagent **1** under the optimized reaction conditions afforded **P1.1s** in an 86% HPLC yield (Table S1, entry 3) and a 95% absorbance ratio (see the corresponding HPLC–UV chromatograms).

S5.4. General Procedures for S-alkylation/Reductive Amination on a Preparative Scale

For the preparative synthesis of stapled peptides, the S-alkylation step was performed at a peptide concentration of either 10 mM (**General Procedure A**) or 5 mM (**General procedure B**), depending on the solubility of the starting peptide in a 1:1 (v/v) mixture of acetonitrile and ammonium bicarbonate buffer (pH 8.0). If the peptide was soluble at 10 mM in this solvent system, **General Procedure A** was used; otherwise, **General Procedure B** was followed.

In both procedures, intramolecular reductive amination was carried out at a peptide concentration of approximately 1 mM, relative to the initial amount of starting peptide.

General Procedure A:

The solid linear peptide (trifluoroacetate salt; 10–41 μmol , 1.0 equiv.) was weighed and transferred to a 50 mL centrifuge tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 $\mu\text{L}/\mu\text{mol}$ peptide) and acetonitrile (47.6 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. If needed, the mixture was sonicated to ensure homogeneity. A solution of the appropriate stapling reagent (0.50 M in acetonitrile; 2.4 $\mu\text{L}/\mu\text{mol}$ peptide, 1.2 equiv.) was then added, resulting in a peptide concentration of 10 mM. The reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, sodium citrate buffer (250 mM, pH 4.0; 200 $\mu\text{L}/\mu\text{mol}$ peptide) and methanol (690 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the centrifuge tube was vortexed briefly. A freshly prepared solution of NaBH_3CN (1.0 M methanol; 10 $\mu\text{L}/\mu\text{mol}$ peptide, 10 equiv.) was added. The resulting mixture was vortexed, yielding a peptide concentration of approximately 1 mM (volume contraction ignored) in a 14:1:5 (v/v/v) mixture of methanol, acetonitrile, and water. The reaction mixture was then incubated at 23 °C, protected from light.

After incubation for an appropriate period, 15 μL of the reaction medium was diluted 10-fold into methoxylamine buffer. The resulting analytical sample (final peptide concentration: $\sim 100 \mu\text{M}$, relative to the initial amount of starting peptide) was mixed, allowed to stand at room

temperature for 5–15 minutes, and then analyzed by HPLC to assess the conversion of the *S*-alkylated intermediate (peptide aldehyde).

After HPLC analysis indicated full conversion (>99%), the crude reaction medium was transferred to a 100 mL round-bottom flask, and the organic solvents were evaporated at 35 °C under reduced pressure in a well-ventilated fume hood. The remaining aqueous phase was lyophilized overnight to give the crude product, which was purified by semi-preparative HPLC to afford the desired macrocyclic peptide.

General Procedure B:

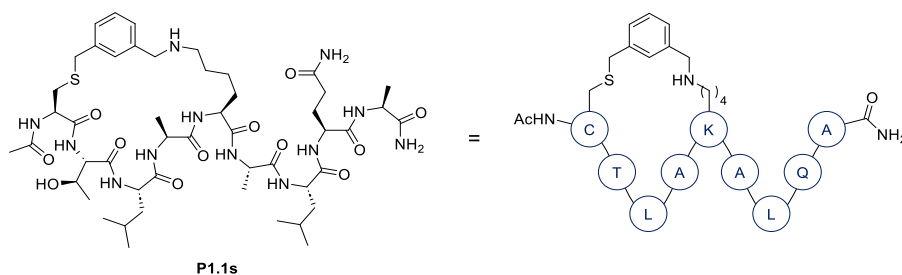
The solid linear peptide (trifluoroacetate salt; 1.0 equiv.) was weighed and transferred to a 50 mL centrifuge tube. Acetonitrile (97.6 $\mu\text{L}/\mu\text{mol}$ peptide) and freshly prepared ammonium bicarbonate buffer (25 mM, pH 8.0; 100 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. If needed, the mixture was sonicated to ensure homogeneity. A solution of the appropriate stapling reagent (0.50 M in acetonitrile; 2.4 $\mu\text{L}/\mu\text{mol}$ peptide, 1.2 equiv.) was then added, resulting in a peptide concentration of 5 mM. The reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, sodium citrate buffer (333.3 mM, pH 4.0; 150 $\mu\text{L}/\mu\text{mol}$ peptide) and methanol (640 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the centrifuge tube was vortexed briefly. A freshly prepared solution of NaBH_3CN (1.0 M methanol; 10 $\mu\text{L}/\mu\text{mol}$ peptide, 10 equiv.) was added. The resulting mixture was vortexed, yielding a peptide concentration of approximately 1 mM (volume contraction ignored) in a 13:2:5 (v/v/v) mixture of methanol, acetonitrile, and water. The reaction mixture was then incubated at 23 °C, protected from light.

After incubation for an appropriate period, the reaction medium was treated as described in General Procedure A to afford the desired macrocyclic peptide.

S5.5. Synthesis of Cys–Lys, Cys–Orn, Cys–Dab, and Cys–Dap Stapled Peptides

Peptide **P1.1s**

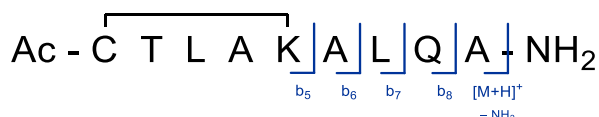


The synthesis of peptide **P1.1s** was first optimized on an analytical scale (see Section S5.1.3, *Optimization of the One-Pot S-Alkylation/Reductive Amination Protocol*). The optimized conditions provided the desired macrocyclic peptide in 86% HPLC yield.

Peptide **P1.1s** was then synthesized on a preparative scale from **P1** (purchased from GeneCust; purity >95%; 41.6 mg, 38.8 μmol), **1** (0.50 M in acetonitrile; 93.0 μL , 46.5 μmol), and NaBH_3CN (1.0 M in methanol; 388 μL , 388 μmol), according to General Procedure A. An HPLC analysis conducted 6 h after addition of the reducing agent indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 4:1 (v/v) mixture of water and methanol to a peptide concentration of 4–6 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 5–30% D; 5–15 min, 30–35% D; 15–16 min, 35–95% D; 16–20 min, 95% D; t_R = 10.2–10.3 min) afforded **P1.1s** (trifluoroacetate salt; 20.6 mg, 45%) as a white fluffy solid following lyophilization.

MS/MS analysis of stapled peptide **P1.1s**:

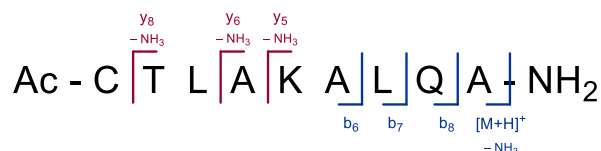
MS² of m/z 1061.70 (CID, normalized collision energy = 30)



Fragments observed: $[M+H]^+ - \text{NH}_3$ (100%); b_8 (40%); b_7 (27%); b_6 (16%); b_5 (3%).

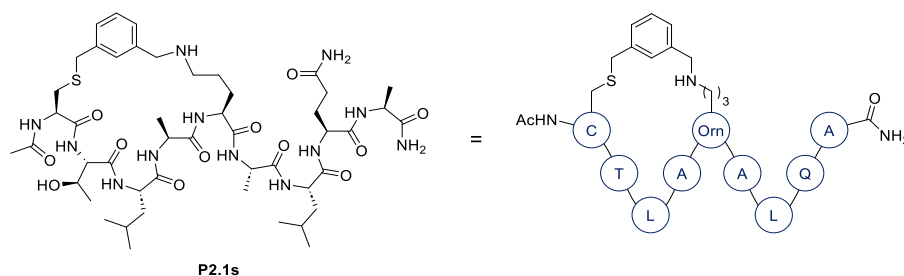
MS/MS analysis of linear peptide **P1** (for comparison):

MS² of *m/z* 959.70 (CID, normalized collision energy = 30)



Fragments observed: [M+H]⁺ – NH₃ (100%); y₅ – NH₃ (49%); y₈ – NH₃ (29%); b₈ (28%); y₆ – NH₃ (28%); b₇ (20%); b₆ (18%).

Peptide **P2.1s**



The synthesis of peptide **P2.1s** was first investigated on an analytical scale following General Procedure 1. The optimized conditions provided the desired macrocyclic peptide in 80% HPLC yield.

Table S2. Synthesis of peptide **P2.1s** on an analytical scale.

Entry	Starting Material	Reaction Conditions	Acidic Buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}	s/(s + ol) (%) ^{b, e}
1	P2^f	1 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/acidic aq. buffer 14:4, [peptide] = 1 mM, 23 °C, 6 h	Sodium citrate (50 mM, pH 4.0)	>99 ^g	80 ^g	99 ^g
2			Sodium citrate (25 mM, pH 5.0)	>99	79	98

^a Indicated concentration: final buffer concentration in MeOH/MeCN/H₂O (14:1:5, v/v/v).

^b Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μM).

^c Conversion of peptide aldehyde **P2.1al** during the reductive amination step; calculated as [1 – (absorbance area of **P2.1ox_a** at t = 6 h / absorbance area of **P2.1ox_a** at t₀)] × 100.

^d Calculated based on the calibration curve established for **P2.1s** (see Figure S12).

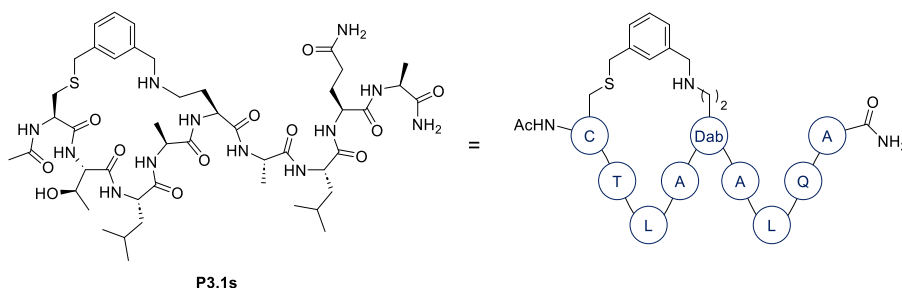
^e Calculated as (absorbance area of **P2.1s** / combined absorbance area of **P2.1s** and **P2.1ol**) × 100.

^f Purchased from GeneCust; purity >95%.

^g Average of duplicate reactions.

Peptide **P2.1s** was then synthesized on a preparative scale from **P2** (purchased from GeneCust; purity >95%; 31.6 mg, 29.9 μ mol), **1** (0.50 M in acetonitrile; 71.7 μ L, 35.8 μ mol), and NaBH₃CN (1.0 M in methanol; 299 μ L, 299 μ mol), according to General Procedure A. An HPLC analysis conducted 6 h after addition of the reducing agent indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 4:1 (v/v) mixture of water and methanol to a peptide concentration of 5–7 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 5–28% D; 5–15 min, 28–33% D; 15–16 min, 33–95% D; 16–20 min, 95% D; t_R = 10.0 min) afforded **P2.1s** (trifluoroacetate salt; 15.7 mg, 45%) as a white fluffy solid following lyophilization.

Peptide **P3.1s**



The synthesis of peptide **P3.1s** was first investigated on an analytical scale following General Procedure 2. The optimized conditions provided the desired macrocyclic peptide in 68% HPLC yield.

Table S3. Synthesis of peptide **P3.1s** on an analytical scale.

Entry	Starting Material	Reaction Conditions	Acidic Buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}	s/(s + ol) (%) ^{b, e}
1	P3 ^f	1 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/acidic aq. buffer 14:4, [peptide] = 1 mM, 23 °C, 6 h	Sodium citrate (50 mM, pH 4.0)	>99	68	99
2			Sodium citrate (25 mM, pH 5.0)	>99	62	98

^a Indicated concentration: final buffer concentration in MeOH/MeCN/H₂O (14:1:5, v/v/v).

^b Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M); average of duplicate reactions.

^c Conversion of peptide aldehyde **P3.1al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P3.1ox_a} \text{ at } t = 6 \text{ h} / \text{absorbance area of } \mathbf{P3.1ox_a} \text{ at } t_0)] \times 100$.

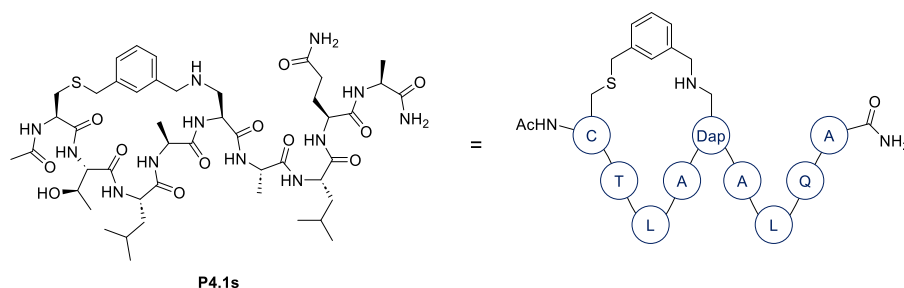
^d Calculated based on the calibration curve established for **P3.1s** (see Figure S13).

^e Calculated as (absorbance area of **P3.1s** / combined absorbance area of **P3.1s** and **P3.1ol**) $\times$ 100.

^f Purchased from GeneCust; purity >95%.

Peptide **P3.1s** was then synthesized on a preparative scale from **P3** (purchased from GeneCust; purity >95%; 42.5 mg, 40.7 μmol), **1** (0.50 M in acetonitrile; 97.6 μL , 48.8 μmol), and NaBH_3CN (1.0 M in methanol; 407 μL , 407 μmol), according to General Procedure A. An HPLC analysis conducted 6 h after addition of the reducing agent indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 5:1:1 (v/v/v) mixture of water, methanol, and acetonitrile to a peptide concentration of 4–6 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 25% D; 5–15 min, 25–45% D; 15–16 min, 45–95% D; 16–20 min, 95% D; t_R = 10.9–11.0 min) afforded **P3.1s** (trifluoroacetate salt; 14.1 mg, 30%) as a white fluffy solid following lyophilization.

Peptide **P4.1s**



The synthesis of peptide **P4.1s** was first investigated on an analytical scale following General Procedure 2. The optimized conditions provided the desired macrocyclic peptide in 86% HPLC yield.

Table S4. Synthesis of peptide **P4.1s** on an analytical scale.

Entry	Starting Material	Reaction Conditions	Acidic Buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}	s/(s + ol) (%) ^{b, e}
1	P4 ^f	1 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/acidic aq. buffer 14:4, [peptide] = 1 mM, 23 °C, 6 h	Sodium citrate (50 mM, pH 4.0)	>99	86	>99
2			Sodium citrate (25 mM, pH 5.0)	>99	87	>99

^a Indicated concentration: final buffer concentration in MeOH/MeCN/H₂O (14:1:5, v/v/v).

^b Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M); average of duplicate reactions.

^c Conversion of peptide aldehyde **P4.1aI** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P4.1ox_a} \text{ at } t = 6 \text{ h} / \text{absorbance area of } \mathbf{P4.1ox_a} \text{ at } t_0)] \times 100$.

^d Calculated based on the calibration curve established for **P4.1s** (see Figure S14).

^e Calculated as $(\text{absorbance area of } \mathbf{P4.1s} / \text{combined absorbance area of } \mathbf{P4.1s} \text{ and } \mathbf{P4.1ol}) \times 100$.

^f Purchased from GeneCust; purity >95%.

Peptide **P4.1s** was then synthesized on a preparative scale from **P4** (purchased from GeneCust; purity >95%; 34.7 mg, 33.6 μ mol), **1** (0.50 M in acetonitrile; 80.7 μ L, 40.3 μ mol), and NaBH₃CN (1.0 M in methanol; 336 μ L, 336 μ mol), according to General Procedure A. An HPLC analysis conducted 2 h after addition of the reducing agent indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 4.5:1:1 (v/v/v) mixture of water, methanol, and acetonitrile to a peptide concentration of 5–7 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 5–29% D; 5–15 min, 29–34% D; 15–16 min, 34–95% D; 16–20 min, 95% D; t_R = 10.0–10.1 min) afforded **P4.1s** (trifluoroacetate salt; 16.4 mg, 43%) as a white fluffy solid following lyophilization.

Table S5. Synthesis of peptide **P5.1s** on an analytical scale.

Entry	Starting Material	Reaction Conditions	Acidic Buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}	s/(s + ol) (%) ^{b, e}
1	P5'	1 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (25 mM, pH 8.0) 2:2, [peptide] = 5 mM, 23 °C, 1 h; then NaBH ₄ CN (10 equiv.), MeOH/acidic aq. buffer 13:3, [peptide] = 1 mM, 23 °C. 24 h	Sodium citrate (50 mM, pH 4.0)	>99	80	96
2			Sodium citrate (25 mM, pH 5.0)	>99	79	95

^f Purchased from GeneCust: purity >95%.

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S5.6. Byproducts

Figure S1 presents the tentative structures of byproducts detected in crude reaction mixtures following peptide stapling via sequential *S*-alkylation and reductive amination. These structures were proposed based on ESI⁺-LRMS data. In HPLC–UV chromatograms, peaks corresponding to specific byproducts were annotated when their absorbance area exceeded 1% of the total peptide-related UV signal (see Section S11 for processed HPLC–UV traces).

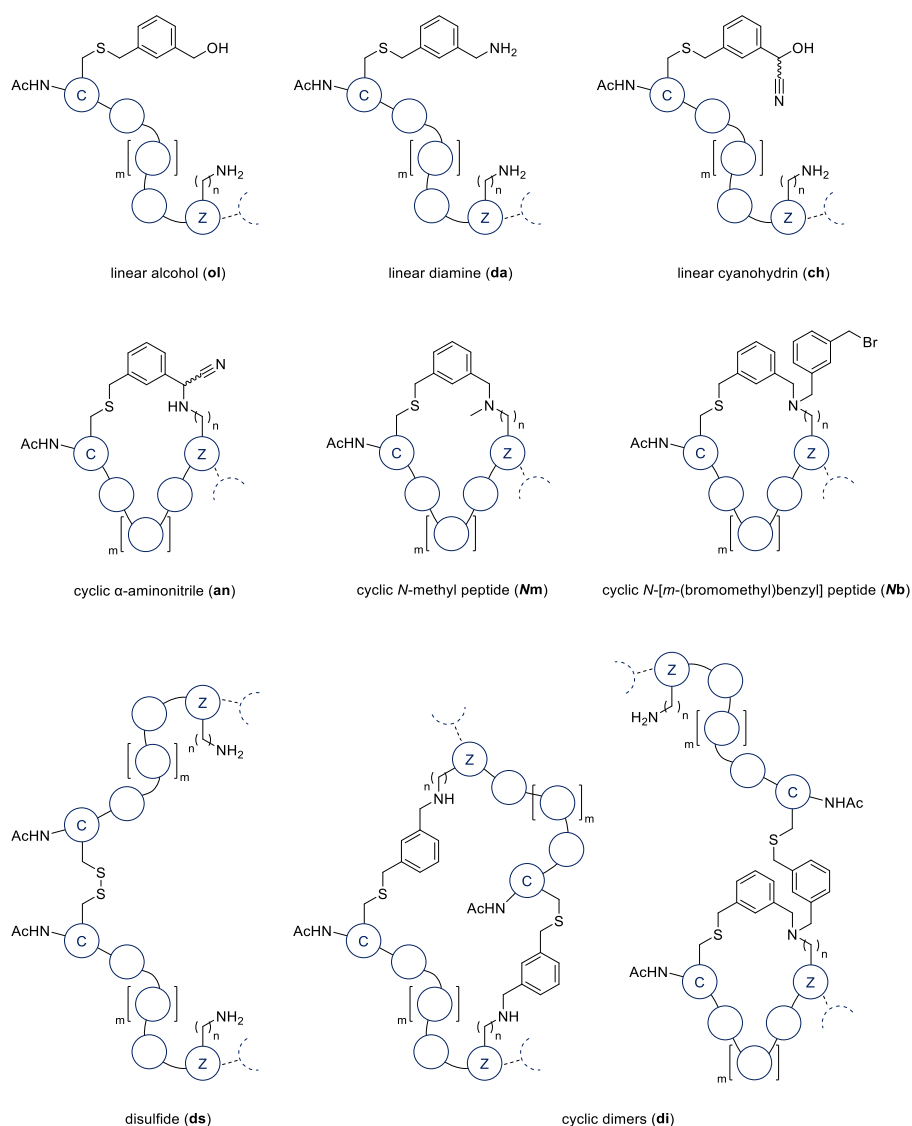


Figure S1. Byproducts formed during peptide macrocyclization through sequential *S*-alkylation and reductive amination.

Z = lysine ($n = 4$), ornithine ($n = 3$), diaminobutyrate ($n = 2$), or diaminopropionate ($n = 1$) residue. $m = 1, 4$.

Notes on byproducts observed:

- Undesired reduction of the peptide aldehyde intermediate led to a linear alcohol byproduct (**ol**). Its formation was minimized during the optimization of the stapling procedure.
- Intermolecular reductive amination of the peptide aldehyde intermediate with ammonia present in the reaction medium could occur, affording a linear diamine (**da**). However, this byproduct was observed in significant amounts only when acetonitrile was used as the major cosolvent (Table S1, entry 10).
- Linear cyanohydrin (**ch**) and cyclic α -aminonitrile (**an**) resulted from cyanide addition to the peptide aldehyde intermediate or to the imine/iminium species formed *in situ*, respectively. The **ch** byproduct was observed only when the medium contained a high proportion of water ($\geq 50\%$, v/v). Increasing the methanol/water volume ratio clearly reduced the amount of cyanohydrin formed (Table 3, entries 1–5). The **an** byproduct was observed in reaction mixtures at pH 6.0 or higher (Table 4, entries 5–8). Because cyanide addition to the imine/iminium is reversible,^[10] **an** was never detected after quenching the reaction with methoxylamine buffer.
- A cyclic *N*-methyl byproduct (**Nm**) was formed when the desired macrocyclic peptide reacted with formaldehyde present in the solvent system, undergoing an additional reductive alkylation. Trace levels of formaldehyde are known to be present in commercial methanol and can be assessed using 2,4-dinitrophenylhydrazine.^[11] Formation of **Nm** can therefore be minimized by employing methanol grades with the lowest possible formaldehyde content.
- Because the stapling reagent was used in excess (1.2 equiv.), it could further react with the desired cyclic secondary amine via an additional reductive amination, affording an undesired *N*-[*m*-(bromomethyl)benzyl]-substituted tertiary amine (**Nb**). Although this byproduct was formed in negligible amounts, this underscores the need to use only a small excess of the stapling reagent.
- Disulfide formation, arising from oxidation of the starting peptide, was observed. In most cases, the disulfide byproduct (**ds**) represented less than 1% of the total peptide-related UV signal. Nevertheless, to minimize its formation, the stapling

reagent should be added immediately upon dissolution of the starting material in acetonitrile and ammonium bicarbonate buffer (1:1, v/v).

- Cyclic dimers (**di**) resulted from intramolecular reductive amination followed by intermolecular reductive amination, or vice versa. They typically accounted for less than 1% of the total peptide-related UV signal. The two isomers could not be distinguished based on m/z alone.
- Certain additional byproducts not shown in Figure S1 were observed only in specific cases. Where possible, their tentative structures were indicated below the corresponding chromatograms.

S5.7. Kinetic Studies

Linear peptides (purchased from GeneCust; trifluoroacetate salt; purity >95%) were modified as described in General Procedure 2 (peptides **P1–P4**) or General Procedure 3 (peptide **P5**), and conversion of the peptide aldehyde intermediate was monitored to determine the rate of the intramolecular reductive amination. To this end, sampling was scheduled at nominal time points (30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h) after the addition of NaBH₃CN and, upon collection, 100 μ L aliquots of the reaction medium were quenched by 10-fold dilution into methoxylamine buffer. The actual quench time was recorded (typically within 1–2 min of the nominal time). The resulting samples were each briefly mixed by manual shaking and allowed to stand at room temperature for about 30 min before being frozen. These diluted samples were then thawed individually and analyzed by HPLC. The UV peak area of the oxime-trapped peptide (major isomer) was measured and normalized to its value prior to addition of the reducing agent (t_0). The normalized peak area was then plotted against the actual quench time (Figure 13), except for that of **P4.1ox_a**, which was already undetectable at the first sampling time.

Kinetic curves of peptide aldehydes **P1.1al**, **P2.1al**, and **P5.1al** were well described ($R^2 \geq 0.999$) by a single-exponential model consistent with first-order or pseudo-first-order behavior. The observed first-order rate constants (k_{obs}) were determined by nonlinear least-squares fitting in GraphPad Prism 10.6.1 and are reported in Table S6. Observed rate constants could not be determined for the macrocyclization of peptides **P3.1al** and **P4.1al**, as the reactions were complete by 1 h (**P3.1al**) and

30 min (**P4.1al**), respectively, leaving insufficient time points for reliable fitting.

Table S6. Kinetic parameters (k_{obs} , $t_{1/2}$) determined for the intramolecular reductive amination of peptide aldehydes **P1.1al**, **P2.1al**, and **P5.1al**.

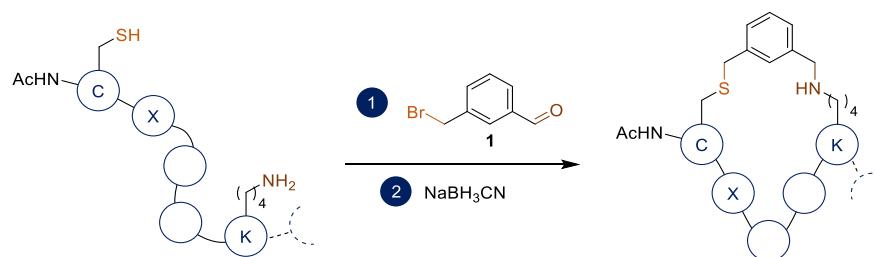
Entry	Peptide aldehyde	k_{obs} (h^{-1})	$t_{1/2}$ (h)
1	P1.1al	1.215	0.5704
2	P2.1al	2.962	0.2341
3	P5.1al	0.3962	1.750

S5.8. Chemoselectivity Studies

S5.8.1. Tolerance toward Nucleophilic Residues

Peptides **P6–P11** were cyclized on an analytical scale according to General Procedure 2, and conversion of the peptide aldehyde intermediate was monitored as described in Section S5.7 (*Kinetic Studies*). Upon completion of cyclization, reaction efficiency was evaluated using the absorbance ratio.

Table S7. Macrocyclization of peptides with nucleophilic side chains.^a



Entry	Starting Material ^b	Sequence	X	Product	Absorbance Ratio (%) ^c
1	P6	Ac-CXLAKA-NH ₂	S	P6.1s	97 ^d
2	P7		M	P7.1s	94 ^d
3	P8		Y	P8.1s	97 ^d
4	P9		R	P9.1s	96 ^e
5	P10		H	P10.1s	96 ^e
6	P11		W	P11.1s	95 ^d

^a Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 24 h.

^b Manually synthesized via Fmoc-based SPPS.

^c Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) × 100.

^d Determined by HPLC–UV (λ = 210 nm; Method 3; peptide concentration ~100 μM).

^e Determined by HPLC–UV (λ = 210 nm; Method 4; peptide concentration ~100 μM).

S5.8.2. Comparison of S-Alkylation/Reductive Amination and S,N-bis-Alkylation

The efficiency of the optimized one-pot S-alkylation/reductive amination protocol using *m*-(bromomethyl)benzaldehyde was compared with that of an S,N-bis-alkylation reaction using α,α'-dibromo-*m*-xylene. This comparison was carried out using both the peptide model **P1** and the methionine-containing peptide **P7** as linear substrates.

First, the absorbance area of the target product **P1.1s** was extracted from chromatograms obtained during kinetic studies of the cyclization of the peptide model **P1** via S-alkylation/reductive amination (see Section S5.7, *Kinetic Studies*).

Peptide model **P1** was also subjected to S,N-bis-alkylation using conditions described by Frost and coworkers.^[12] Accordingly, the linear peptide **P1** (purchased from GeneCust; trifluoroacetate salt; purity

>95%; 1.055 mg, 0.983 μmol , 1.0 equiv.) was weighed and transferred to an Eppendorf tube. Sodium phosphate buffer (50 mM, pH 8.0; 492 μL) and acetonitrile (489 μL) were added sequentially, and the mixture was vortexed for a few seconds. A solution of α,α' -dibromo-*m*-xylene (0.50 M in acetonitrile; 2.36 μL , 1.180 μmol , 1.2 equiv.) was then added, resulting in a peptide concentration of 1 mM. The reaction mixture was vortexed briefly and incubated at 23 $^{\circ}\text{C}$, protected from light. At various time points after the addition of α,α' -dibromo-*m*-xylene (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h), 100 μL aliquots of the reaction medium were quenched by 10-fold dilution into 0.1% TFA in water. The actual quench time was recorded (typically within 1–2 min of the nominal time). The resulting samples were each mixed immediately and frozen. These diluted samples were then thawed individually and analyzed by HPLC (Method 2). The absorbance area of the target product **P1.1s** was extracted from each chromatogram.

Absorbance areas of **P1.1s** from both reactions were normalized to the highest value obtained across the two reactions and then plotted against the actual quench time.

The efficiency of both macrocyclization protocols for Cys–Lys stapling of the methionine-containing peptide **P7** were then compared in the same manner.

To this end, the absorbance area of Cys–Lys stapled product **P7.1s** was extracted from chromatograms obtained during monitoring of the cyclization of the peptide **P7** via *S*-alkylation/reductive amination.

Peptide **P7** was also subjected to *S,N*-bis-alkylation.^[12] Accordingly, the linear peptide **P7** (manually synthesized via Fmoc-based SPPS; trifluoroacetate salt; 1.070 mg, 1.353 μmol , 1.0 equiv.) was weighed and transferred to an Eppendorf tube. Sodium phosphate buffer (50 mM, pH 8.0; 677 μL) and acetonitrile (673 μL) were added sequentially, and the mixture was vortexed for a few seconds. A solution of α,α' -dibromo-*m*-xylene (0.50 M in acetonitrile; 3.25 μL , 1.625 μmol , 1.2 equiv.) was then added, resulting in a peptide concentration of 1 mM. The reaction mixture was vortexed briefly and incubated at 23 $^{\circ}\text{C}$, protected from light. At various time points after the addition of α,α' -dibromo-*m*-xylene (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h), 100 μL aliquots of the reaction medium were quenched by 10-fold dilution into 0.1% TFA in water. The actual quench time was recorded (typically within 1–2 min of the nominal time). The resulting samples were each mixed immediately and frozen. These diluted samples were then

thawed individually and analyzed by HPLC (Method 3). The absorbance area of the target product **P7.1s** was extracted from each chromatogram.

Absorbance areas of **P7.1s** from both reactions were normalized to the highest value obtained across the two reactions and then plotted against the actual quench time.

S5.8.3. Tolerance toward Protected Lysine Residues

Peptides **P12** and **P13** were cyclized on an analytical scale according to General Procedure 2, and conversion of the peptide aldehyde intermediate was monitored as described in Section S5.7 (*Kinetic Studies*). Upon completion of cyclization, reaction efficiency was evaluated using the absorbance ratio.

Table S8. Macrocyclization of peptides with protected lysine residues.^a

Entry	Starting Material ^b	Sequence	X	Product	Absorbance Ratio (%) ^c
1	P12	Ac-CXLAKA-NH ₂	Nle (ε-N ₃)	P12.1s	96 ^d
2	P13		Lys(Nε-Alloc)	P13.1s	98 ^d

^a Reaction conditions: **1** (1.2 equiv.), MeCN/aq. NH₄HCO₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH₃CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 24 h.

^b Manually synthesized via Fmoc-based SPPS.

^c Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) × 100.

^d Determined by HPLC–UV (λ = 210 nm; Method 3; peptide concentration ~100 μM).

S5.8.4. One-Pot S-Alkylation/Reductive Amination/Pd-Mediated Deallyloxycarbonylation

Peptide **P13** was cyclized on an analytical scale according to General Procedure 2, using **1** as the stapling reagent. At 24 h after addition of NaBH₃CN, 200 μ L of the reaction medium (0.2 μ mol, relative to the initial amount of starting peptide) was transferred to a 500 μ L Eppendorf tube and then subjected to palladium-mediated deallyloxycarbonylation using conditions adapted from Okamoto and coworkers.^[13] Anhydrous DMF and distilled water were degassed by nitrogen bubbling under sonication for 30 min. Freshly prepared solutions of Pd(OAc)₂ (188 mM in degassed DMF; 15.9 μ L) and triphenylphosphine-3,3',3''-trisulfonic acid trisodium salt (TPPTS; 850 mM in degassed water; 14.1 μ L) were combined in a 200 μ L Eppendorf tube, and the mixture was vortexed for 30 s, affording a solution of an *in situ*-generated Pd(0)/TPPTS species ([Pd] = 100 mM; [TPPTS] = 400 mM). A 500 mM solution of 4-mercaptophenylacetic acid (MPAA) in degassed water was prepared, and 8.0 μ L of this solution (4.0 μ mol, 20 equiv.) was added to the tube containing the crude **P13.1s** solution, which was vortexed briefly. The Pd(0)/TPPTS solution (2.0 μ L, 0.2 μ mol, 1.0 equiv.) was then added, and the mixture was vortexed for a few seconds. After incubation for 15 min at 23 °C, 50 μ L of the reaction mixture was diluted 10-fold into 0.1% TFA in water, giving a final peptide concentration of approximately 95.2 μ M relative to the initial amount of **P13**. The resulting sample was briefly mixed by manual shaking, frozen, then thawed and analyzed by HPLC (Method 5), which indicated complete removal of the Alloc group. This provided the desired deprotected macrocyclic peptide **P13.1sd** in a measured HPLC yield of 97%.

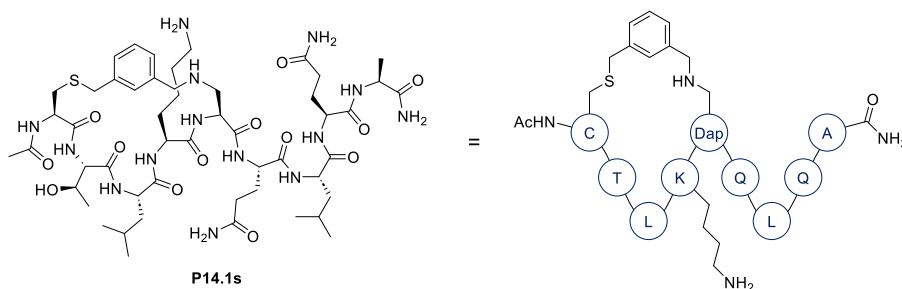
To isolate **P13.1sd**, the same protocol was applied in parallel on a larger scale, starting from 3.0 mL of a solution of crude cyclic peptide **P13.1s** (obtained according to General Procedure A and containing 3.0 μ mol of peptide relative to the initial amount of **P13**), MPAA (59.4 μ mol), and Pd(0)/TPPTS (3.0 μ mol). An HPLC analysis conducted 15 min after addition of the Pd(0)/TPPTS complex indicated full conversion of the protected peptide. The crude reaction medium was transferred to a 4 mL vial, and the organic solvents were evaporated under reduced pressure at 35 °C. A 3:1 (v/v) mixture of water and acetonitrile (2 mL) was added to the residue. The resulting mixture was filtered and directly purified by semi-preparative HPLC (gradient: 0–5 min, 5–17% D; 5–15 min, 17–22% D; 15–16 min, 22–95% D; 16–20

min, 95% D; t_R = 9.8–9.9 min) to afford **P13.1sd** (trifluoroacetate salt; 0.9 mg, 30%) as a white fluffy solid following lyophilization.

Note on HPLC analysis: In this section, crude samples and purified fractions were analyzed using TFA as the acid modifier instead of formic acid, because analyses performed with formic acid showed significant tailing of the peak corresponding to the desired deprotected peptide **P13.1sd**, which bears two amine side chains.

S5.9. Cys–Dap Stapling in the Presence of a Lysine Residue

Peptide **P14.1s**



The synthesis of peptide **P14.1s** was first investigated on an analytical scale following General Procedure 2. The optimized conditions provided the desired macrocyclic peptide in 81% HPLC yield.

Table S9. Synthesis of peptide **P14.1s** on an analytical scale.

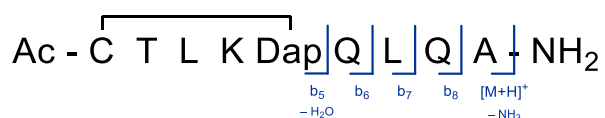
Entry	Starting material	Reaction conditions	Acidic buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}
1	P14^e	1 (1.2 equiv.), MeCN/aq. NH_4HCO_3 (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH_3CN (10 equiv.), MeOH/acidic aq. buffer 14:4, [peptide] = 1 mM, 23 °C, 1 h	Sodium citrate (50 mM, pH 4.0)	>99	81
2			Sodium citrate (25 mM, pH 5.0)	>99	76

Peptide **P14.1s** was then synthesized on a preparative scale from **P14** (purchased from GeneCust; purity >95%; 42.8 mg, 34.0 μmol), **1** (0.50 M in acetonitrile; 81.6 μL , 40.8 μmol), and NaBH_3CN (1.0 M in methanol; 340 μL , 340 μmol), according to General Procedure A. An HPLC analysis conducted 1 h after addition of the reducing agent

indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 4:1:1 (v/v/v) mixture of water, methanol, and acetonitrile to a peptide concentration of 5–7 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 7.5% D; 5–15 min, 7.5–27.5% D; 15–16 min, 27.5–95% D; 16–20 min, 95% D; t_R = 14.6 min) afforded **P14.1s** (trifluoroacetate salt; 18.7 mg, 40%) as a white fluffy solid following lyophilization.

MS/MS analysis of stapled peptide **P14.1s**:

- MS² of m/z 1133.70 (CID, normalized collision energy = 30)



Fragments observed: $[\text{M}+\text{H}]^+ - \text{NH}_3$ (100%); b_8 (38%); b_7 (27%); b_6 (8%); $\text{b}_5 - \text{H}_2\text{O}$ (3%).

- MS⁴ (m/z 1133.70 $\rightarrow$ m/z 804.60 $\rightarrow$ m/z 676.40) did not yield b_4 (m/z 590) or $\text{b}_4 - \text{H}_2\text{O}$ (m/z 572) fragment ions, which would be diagnostic of an $i \rightarrow i+3$ Cys–Lys connectivity rather than an $i \rightarrow i+4$ Cys–Dap connectivity.

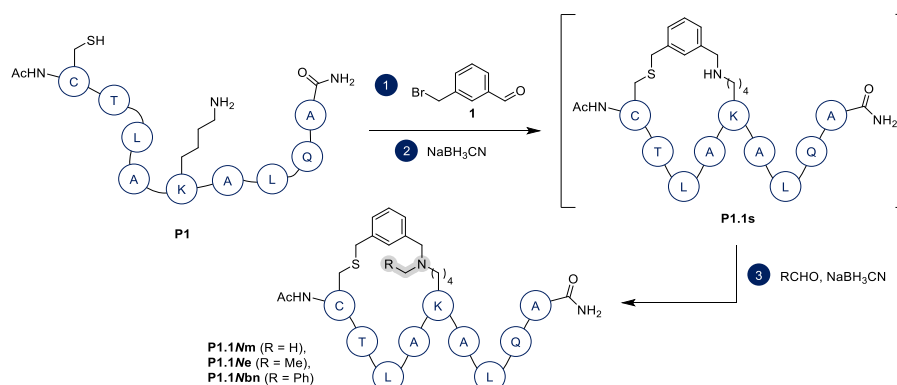
S5.10. Post-Stapling Modification via Intermolecular Reductive Amination

Peptide model **P1** (11 μmol) was cyclized on a preparative scale according to General Procedure A, using **1** as the stapling reagent. At 6 h after addition of NaBH_3CN , 15 μL of the reaction medium was diluted 10-fold into methoxylamine buffer. The resulting analytical sample (final peptide concentration: ~ 100 μM , relative to the initial amount of **P1**) was vortexed briefly, allowed to stand at room temperature for 15 min, and then analyzed by HPLC (Method 2), which indicated complete conversion ($>99\%$) of the peptide aldehyde intermediate. Three 1.00 mL aliquots of the reaction mixture were then transferred separately to 1.5 mL Eppendorf tubes. Freshly prepared solutions of formaldehyde, acetaldehyde, or benzaldehyde (0.50 M in methanol; 20 μL , 10 μmol , 10 equiv.)* were added, one to each tube.

Each tube was vortexed for a few seconds, then another freshly prepared solution of NaBH₃CN (1.0 M in methanol; 10 μ L, 10 μ mol, 10 equiv.) was added. Each mixture was vortexed briefly and incubated overnight at 23 °C, protected from light.

At defined time points after addition of the external aldehyde, aliquots from each reaction mixture were diluted 9.71-fold into methoxylamine buffer, giving a final peptide concentration of approximately 100 μ M relative to the initial amount of **P1**. The resulting samples were each briefly mixed by manual shaking and allowed to stand at room temperature for about 30 min before being frozen. These diluted samples were then thawed individually and analyzed by HPLC (Method 2). Conversion of the stapled peptide **P1.1s** to its *N*-methyl, *N*-ethyl, or *N*-benzyl derivative was estimated by comparing the absorbance area of **P1.1s** at each time point with that measured prior to addition of the external aldehyde.

Table S10. One-pot derivatization of **P1.1s** via intermolecular reductive amination.



Entry	Starting Material	Reaction Conditions	External Aldehyde	Time, Step 3 (h)	Conversion (%) ^a
1	P1^b	1) 1 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h	Formaldehyde (R = H)	12	>99
2			Acetaldehyde (R = Me)	12	97
3		2) NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 6 h	Benzaldehyde (R = Ph)	18	>99
4				12	17
5		3) RCHO (10 equiv.), NaBH ₃ CN (10 equiv.), 23 °C		18	26
6				36	39

^a Conversion of **P1.1s** during the derivatization step; determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M); calculated as $[1 - (\text{absorbance area of } \mathbf{P1.1s} \text{ at } t / \text{absorbance area of } \mathbf{P1.1s} \text{ at } t_0)] \times 100$.

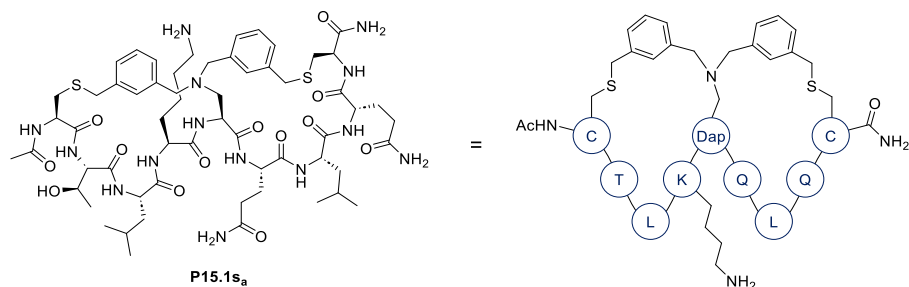
^b Purchased from GeneCust; purity >95%.

* Note on external aldehyde solutions: 0.50 M formaldehyde solution was prepared from a 36% aqueous solution by 26.6-fold dilution into methanol; 0.50 M acetaldehyde and 0.50 M benzaldehyde solutions were prepared by dissolving the neat aldehydes in methanol.

S5.11. Bicyclization via Intramolecular bis-Reductive Amination

S5.11.1. One-Pot bis-S-Alkylation/bis-Reductive Amination

Peptide **P15.1s_a**



The synthesis of peptide **P15.1s_a** was first investigated on an analytical scale. To this end, the linear peptide Ac-CTLKDapQLQC-NH₂ (**P15**; purchased from GeneCust; trifluoroacetate salt; purity >95%; 4.84 mg, 3.75 μmol, 1.0 equiv.) was weighed and transferred to an Eppendorf tube. Acetonitrile (357 μL) and freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 375 μL) were added, and the mixture was vortexed for a few seconds. 195.2 μL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P15.1 inter.”, 1, and 1 bis), so that each tube contained 1 μmol of starting peptide. A solution of *m*-(bromomethyl)benzaldehyde (**1**; 0.50 M in acetonitrile; 4.8 μL, 2.4 μmol, 2.4 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 5 mM. Each reaction mixture was vortexed briefly and incubated at 37 °C, protected from light. After incubation for 2 h, appropriate solvents were added to Tubes “P15.1 inter.”, 1, and 1 bis as described in the table below.

Tube “P15.1 inter.”	Tube 1	Tube 1 bis
800 μL 0.1% TFA in H ₂ O	150 μL sodium citrate buffer (333.3 mM, pH 4.0) + 640 μL MeOH	150 μL sodium citrate buffer (333.3 mM, pH 4.0) + 640 μL MeOH

Tubes “P15.1 inter.”, 1, and 1 bis were vortexed briefly, and then treated as described in General Procedure 1. The resulting chromatograms were processed in the same manner. The chromatograms obtained after reductive amination indicated the formation of three bicyclic peptides, all at *m/z* 1268, in a 72:25:3 absorbance ratio (λ = 210 nm; Method 3). The desired isomer (**P15.1s_a**), whose connectivity was later confirmed by acetylation (see

Section S5.11.2, *Connectivity Elucidation by Acetylation*) was the major product (57% HPLC yield), while the second-most-abundant isomer (**P15.1s_b**) was obtained in 28% HPLC yield.

Table S11. Synthesis of peptide **P15.1s_a** on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Yield (%) ^{a, c}
1	P15^d	1 (2.4 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 2:2, [peptide] = 5 mM, 37 °C, 2 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (333.3 mM, pH 4.0) 13:3, [peptide] = 1 mM, 23 °C, 1 h	>99	57

^a Determined by HPLC–UV (λ = 210 nm; Method 3; peptide concentration ~100 μ M); average of duplicate reactions.

^b Conversion of peptide bis-aldehyde **P15.1al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P15.1ox_a} \text{ at } t = 1 \text{ h} / \text{absorbance area of } \mathbf{P15.1ox_a} \text{ at } t_0)] \times 100$.

^c Calculated based on the calibration curve established for **P15.1s_a** (see Figure S18).

^d Purchased from GeneCust; purity >95%.

Peptide **P15.1s_a** was then synthesized on a preparative scale. The linear peptide Ac-CTLKDapQLQC-NH₂ (**1**; purchased from GeneCust; trifluoroacetate salt; purity >95%; 26.5 mg, 20.5 μ mol, 1.0 equiv.) was weighed and transferred to a 50 mL centrifuge tube. Acetonitrile (1954 μ L) and freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 2053 μ L) were added, and the mixture was vortexed for a few seconds. A solution of *m*-(bromomethyl)benzaldehyde (**1**; 0.50 M in acetonitrile; 98.5 μ L, 49.2 μ mol, 2.4 equiv.) was then added, resulting in a peptide concentration of 5 mM. The reaction mixture was vortexed briefly and incubated at 37 °C, protected from light. After incubation for 2 h, sodium citrate buffer (333.3 mM, pH 4.0; 3079 μ L) and methanol (13139 μ L) were added, and the centrifuge tube was vortexed briefly. A freshly prepared solution of NaBH₃CN (1.0 M methanol; 205, 10 equiv.) was added. The resulting mixture was vortexed, yielding a peptide concentration of approximately 1 mM (volume contraction ignored) in a 13:2:5 (v/v/v) mixture of methanol, acetonitrile, and water. The reaction mixture was then incubated at 23 °C, protected from light. After incubation for 1 h, 15 μ L of the reaction medium was diluted 10-fold into methoxylamine buffer. The resulting analytical sample (final peptide concentration: ~100 μ M, relative to the initial amount of **P15**) was mixed, allowed to stand at room temperature for 30 min, and then analyzed by HPLC (Method 3), which indicated complete conversion of the peptide aldehydes: neither the linear bis-oxime **P15.1ox_a** (*m/z* 1357) nor any monocyclic mono-oxime species (*m/z* 1312) were detected. The reaction medium was then transferred to a 100 mL

round-bottom flask, and the organic solvents were evaporated under reduced pressure at 35 °C. The remaining aqueous phase was lyophilized overnight.

The crude solid was dissolved in a 4:1:1 (v/v/v) mixture of water, methanol, and acetonitrile to a peptide concentration of 2–4 mM. The resulting solution was subjected to semi-preparative HPLC using a mobile phase consisting of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B), because the use of 0.1% TFA in the mobile phase (Solvents C and D) did not allow efficient separation of isomers **P15.1s_a** and **P15.1s_b**. The following gradient was used: 0–5 min, 5–18% B; 5–15 min, 18–23% B; 15–16 min, 23–95% B; 16–20 min, 95% B (t_R = 11.0–11.1 min for **P15.1s_a**; t_R = 8.5 min for **P15.1s_b**). After the first lyophilization of the pure fractions, the two isomers were each redissolved in 0.05% TFA in water and lyophilized again; this cycle was repeated once in order to obtain the compounds as trifluoroacetate salts. This afforded **P15.1s_a** (trifluoroacetate salt; 7.3 mg, 14%) and **P15.1s_b** (trifluoroacetate salt; 1.3 mg, 3%) as white fluffy solids.

S5.11.2. Connectivity Elucidation by Acetylation

Bicyclic peptide **P15.1s_a** (trifluoroacetate salt; 0.43 mg, 0.29 μ mol, 1.0 equiv.) was weighed and transferred to an Eppendorf tube. Acetonitrile (131 μ L) and sodium carbonate buffer (100 mM, pH 10.0; 145 μ L) were added, and the mixture was vortexed for a few seconds. A solution of acetic anhydride (1.0 M in acetonitrile; 14.5 μ L, 14.5 μ mol, 50 equiv.) was then added, resulting in a peptide concentration of 1 mM. The reaction mixture was vortexed briefly and incubated at 37 °C, protected from light. After incubation for 30 minutes, 30 μ L aliquots were diluted 10-fold into either 0.1% TFA in water or sodium carbonate buffer (50 mM, pH 10.0). The sample diluted in 0.1% TFA was analyzed by HPLC (Method 2), which indicated the formation of a mono-acetylated peptide and a bis-acetylated peptide in an 89:11 absorbance ratio (λ = 210 nm). The sample diluted in sodium carbonate buffer was incubated for an additional 2 h at 37 °C, protected from light, and then analyzed by HPLC (Method 2), which showed disappearance of the bis-acetylated species (presumably *N,O*-bis-acetyl- **P15.1s_a**) and an increase in the abundance of the mono-acetylated peptide (presumably *N*-acetyl-**P15.1s_a**).

Bicyclic peptide **P15.1s_b** (trifluoroacetate salt; 0.47 mg, 0.31 μ mol) was subjected to the same procedure, which gave bis- and tris-acetylated peptides as the predominant products (presumably *N,N'*-bis-acetyl-**P15.1s_b** and *N,N',O*-tris-acetyl-**P15.1s_b**, respectively) after acetylation for 30 minutes. Ten-fold dilution into sodium carbonate buffer (50 mM, pH 10.0) and incubation for an additional 2 h resulted in a decrease in the UV peak area of the tris-acetylated species from 1732.3 to 421.8 mAU·s, and an increase in that of the bis-acetylated species from 2167.0 to 3352.5 mAU·s.

Taken together, these results demonstrated that **P15.1s_a** features a primary and a tertiary amine, thereby validating its *i*+4+4 connectivity, whereas **P15.1s_b** displays two secondary amines.

S6. Cys–Lys Stapling with Other Benzaldehyde-Based Reagents

S6.1. S-Modification/Reductive Amination on an Analytical Scale

S6.1.1. Optional Deviations from General Procedure 2

Stapling of the peptide model **P1** with reagents **2–7** was conducted on a 1 μ mol scale following General Procedure 2. Cysteine S-modification using reagents **2**, **5**, and **6** required minor adjustments to the reaction conditions relative to those described in General Procedure 2, which, for stapling procedures involving reagents **5** and **6**, resulted in changes to the final solvent composition. These adaptations are highlighted in Table S12, and detailed procedures are described in the following sections. In all cases, intramolecular reductive amination was performed at 23 °C with 10 equivalents of NaBH₃CN, at a peptide concentration of approximately 1 mM relative to the initial amount of **P1**, and with a final solvent composition consisting of organic solvents (combined methanol, acetonitrile, and DMF) and aqueous sodium citrate in a 3:1 (v/v) ratio.

Only a sodium citrate buffer at pH 4.0 was investigated for the reductive amination step, except in the case of stapling using reagent **2**, for which a sodium citrate buffer at pH 5.0 was also evaluated (see Section S6.1.2, *Stapling with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde*).

Table S12. Analytical-scale stapling of **P1** using reagents **2–7**.^a

S-modification Step								Reductive Amination step			
Reaction	Stapling Reagent			[Peptide] (mM) ^b	Solvent composition (v/v)	T (°C)	t (h)	NaBH ₃ CN		[Peptide] (mM) ^b	Final Solvent Composition (v/v)
	Compound No.	Loading (equiv.)	Stock Solution					Loading (equiv.)	Stock Solution		
S-alkylation	2	1.2	0.20 M in MeCN	10	MeCN/aq. NH ₄ HCO ₃ 1:1	23	1	10	1.0 M in MeOH	1	MeOH/MeCN/aq. sodium citrate 14:1:5
	3, 4	1.2	0.50 M in MeCN	10	MeCN/aq. NH ₄ HCO ₃ 1:1	23	1	10	1.0 M in MeOH	1	MeOH/MeCN/aq. sodium citrate 14:1:5
Thia-Michael addition	5	1.2	0.50 M in DMF	10	MeCN/DMF /aq. NH ₄ HCO ₃ 47.6:2.4:50	37	4	10	1.0 M in MeOH	1	MeOH/MeCN/ DMF/aq. sodium citrate 700:47.6:2.4:250
Pd- catalyzed S-allylation ^c	6	1.2	0.10 M in MeCN	5	MeCN/aq. NH ₄ HCO ₃ 3:1	23	1	10	1.0 M in MeOH	1	MeOH/MeCN/aq. sodium citrate 12:3:5
S-arylation	7	1.2	0.50 M in MeCN	10	MeCN/aq. NH ₄ HCO ₃ 1:1	23	1	10	1.0 M in MeOH	1	MeOH/MeCN/aq. sodium citrate 14:1:5

^a Deviations from General Procedure 2 highlighted in grey.^b Peptide concentration relative to the initial amount of peptide model **P1**.^c Pd(0)/BiPhePhos (5 mol%) used as the catalyst system.

S6.1.2. Stapling with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde

Stapling of the peptide model **P1** with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde (**2**) was performed on an analytical scale following General Procedure 2, with minor modifications, owing to the need to dissolve stapling reagent **2** in acetonitrile at 0.20 M instead of 0.50 M to ensure complete dissolution.

Thus, solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 µL/µmol peptide) and acetonitrile (44 µL/µmol peptide) were added, and the mixture was vortexed for a few seconds. 94 µL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P1.2 inter.”, 1, and 2), so that each tube contained 1 µmol of **P1**. A solution of **2** (0.20 M in acetonitrile; 6.0 µL, 1.2 µmol, 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “P1.2 inter.”, 1, and 2 as described in the table below.

Tube “P1.2 inter.”	Tube 1	Tube 2
900 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH	200 μ L sodium citrate buffer (125 mM, pH 5.0) + 690 μ L MeOH
	Tube 1 bis	Tube 2 bis
	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH	200 μ L sodium citrate buffer (125 mM, pH 5.0) + 690 μ L MeOH

Tubes “P1.2 inter.”, 1, and 2 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μ L aliquots of each reaction mixture (Tubes 1 and 2) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH₃CN (~30 min, 1 h, 2 h, 4 h, 6 h), as described in Section S5.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.2 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tubes 1 and 2 at each time point (entries 1 and 2) were processed as described in General Procedure 1, enabling monitoring of the conversion of peptide aldehyde **P1.2al**. A conversion of **P1.2al** exceeding 99% was already observed 2 h after addition of NaBH₃CN. However, **P1.2ox_a** was still detectable at this time point, and reaction efficiency was therefore evaluated at a reaction time of 4 h.

As **P1.2s** had to be prepared on a preparative scale and purified (see Section S6.2, *Synthesis of a Cys–Lys Stapled Peptide with an Alkyne-Bearing Linker*) for subsequent functionalization via CuAAC, HPLC yields could be determined based on a calibration curve established with isolated macrocyclic peptide **P1.2s**.

Table S13. Stapling of peptide model **P1** with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde (**2**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Acidic Buffer ^a	Conversion (%) ^{b, c}	Yield (%) ^{b, d}	s/(s + ol) (%) ^{b, e}
1	P1 ^f	2 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/acidic aq. buffer 14:4, [peptide] = 1 mM, 23 °C, 4 h	Sodium citrate (50 mM, pH 4.0)	>99	85	97
2			Sodium citrate (25 mM, pH 5.0)	>99	85	97

^a Indicated concentration: final buffer concentration in MeOH/MeCN/H₂O (14:1:5, v/v/v).

^b Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M); average of duplicate reactions.

^c Conversion of peptide aldehyde **P1.2al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.2ox}_a \text{ at } t = 4 \text{ h} / \text{absorbance area of } \mathbf{P1.2ox}_a \text{ at } t_0)] \times 100$.

^d Calculated based on the calibration curve established for **P1.2s** (see Figure S20).

^e Calculated as $(\text{absorbance area of } \mathbf{P1.2s} / \text{combined absorbance area of } \mathbf{P1.2s} \text{ and } \mathbf{P1.2ol}) \times 100$.

^f Purchased from GeneCust; purity >95%.

S6.1.3. Stapling with *o*- and *p*-(bromomethyl)benzaldehyde

Stapling of the peptide model **P1** with *o*- (**3**) and *p*-(bromomethyl)benzaldehyde (**4**) was performed on an analytical scale following General Procedure 2.

Thus, solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 μ L/ μ mol peptide) and acetonitrile (47.6 μ L/ μ mol peptide) were added, and the mixture was vortexed for a few seconds. 97.6 μ L aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “inter.” and 1), so that each tube contained 1 μ mol of **P1**. A solution of **2** or **3** (0.50 M in acetonitrile; 2.4 μ L, 1.2 μ mol, 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “inter.” and 1 as described in the table below.

Tube “P1.3 inter.”	Tube 1 (P1.3)
900 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH
Tube “P1.4 inter.”	Tube 1 (P1.4)
900 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH

Tubes “inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μL aliquots of each reaction mixture (Tubes 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH_3CN (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h), as described in Section S5.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tubes “inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tubes 1 at each time point (entries 1) were processed as described in General Procedure 1, enabling monitoring of the conversion of peptide aldehydes **P1.3al** and **P1.4al**. Conversions exceeding 99% were observed 4 h after addition of NaBH_3CN for **P1.3al** and 48 h after addition of NaBH_3CN for **P1.4al**.

As **P1.3s** and **P1.4s** were not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yields of the corresponding analytical-scale reactions were not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

Table S14. Stapling of peptide model **P1** with *o*-(bromomethyl)benzaldehyde (**3**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	3 (1.2 equiv.), MeCN/aq. NH_4HCO_3 (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH_3CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 4 h	>99	98	99

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μM).

^b Conversion of peptide aldehyde **P1.3al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.3ox_a} \text{ at } t = 4 \text{ h} / \text{absorbance area of } \mathbf{P1.3ox_a} \text{ at } t_0)] \times 100$.

^c Calculated as $(\text{absorbance area of the target product} / \text{combined absorbance area of all relevant peptidic species}) \times 100$.

^d Calculated as $(\text{absorbance area of } \mathbf{P1.3s} / \text{combined absorbance area of } \mathbf{P1.3s} \text{ and } \mathbf{P1.3ol}) \times 100$.

^e Purchased from GeneCust; purity >95%.

Table S15. Stapling of peptide model **P1** with *p*-(bromomethyl)benzaldehyde (**4**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	4 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 48 h	>99	93	95

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M).

^b Conversion of peptide aldehyde **P1.4al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.4ox_a} \text{ at } t = 48 \text{ h} / \text{absorbance area of } \mathbf{P1.4ox_a} \text{ at } t_0)] \times 100$.

^c Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) $\times 100$.

^d Calculated as (absorbance area of **P1.4s** / combined absorbance area of **P1.4s** and **P1.4ol**) $\times 100$.

^e Purchased from GeneCust; purity >95%.

S6.1.4. Stapling via Thia-Michael Addition/Reductive Amination

Stapling of the peptide model **P1** with *N*-(3-formylphenyl)acrylamide (**5**) was performed on an analytical scale following General Procedure 2, with minor modifications. These adjustments were required because stapling reagent **5** exhibited limited solubility in acetonitrile and was therefore used as a stock solution in DMF, and because the *S*-modification step involving Michael acceptor **5** proceeded more slowly than *S*-modification using benzylating agents **1–4**.

Thus, solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 μ L/ μ mol peptide) and acetonitrile (47.6 μ L/ μ mol peptide) were added, and the mixture was vortexed for a few seconds. 97.6 μ L aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P1.5 inter.” and 1), so that each tube contained 1 μ mol of **P1**. A solution of **5** (0.50 M in DMF; 2.4 μ L, 1.2 μ mol, 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 37 °C, protected from light.

After incubation for 4 h, appropriate solvents were added to Tubes “P1.5 inter.” and 1 as described in the table below.

Tube “P1.5 inter.”	Tube 1
900 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH

Tubes “P1.5 inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μ L aliquots of the reaction mixture (Tube 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH₃CN (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h), as described in Section S5.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.5 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tube 1 at each time point (entries 1) were processed as described in General Procedure 1, enabling monitoring of the conversion of peptide aldehyde **P1.5al**. A conversion of **P1.5al** exceeding 99% was observed 24 h after addition of NaBH₃CN.

As **P1.5s** was not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yield of the corresponding analytical-scale reaction was not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

Table S16. Stapling of peptide model **P1** with *N*-(3-formylphenyl)acrylamide (**5**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	5 (1.2 equiv.), MeCN/DMF/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 47.6:2.4:50, [peptide] = 10 mM, 37 °C, 4 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 700:200, [peptide] = 1 mM, 23 °C, 24 h	>99	86	95

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M).

^b Conversion of peptide aldehyde **P1.5al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.5ox_a} \text{ at } t = 24 \text{ h} / \text{absorbance area of } \mathbf{P1.5ox_a} \text{ at } t_0)] \times 100$.

^c Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) $\times 100$.

^d Calculated as (absorbance area of **P1.5s** / combined absorbance area of **P1.5s** and **P1.5ol**) $\times 100$.

^e Purchased from GeneCust; purity >95%.

S6.1.5. Stapling via Pd-Catalyzed S-Allylation/Reductive Amination

Stapling of the peptide model **P1** with (*E*)-3-[(4-acetoxybut-2-en-1-yl)oxy]benzaldehyde (**6**) was performed on an analytical scale following General Procedure 2, with some adjustments. Notably, Pd-catalyzed S-allylation was conducted at a **P1** concentration of 5 mM in

a 3:1 (v/v) mixture of acetonitrile and ammonium bicarbonate buffer. Among catalyst loadings of 1, 5, and 25 mol%, the most satisfactory results were obtained using 5 mol% of Pd/BiPhePhos, as rapid conversion was observed together with a clean reaction profile.

Thus, solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. A 50 mM ammonium bicarbonate buffer was prepared by 5-fold dilution of a freshly prepared 250 mM buffer (adjusted to pH 8.0) with water that had been degassed by nitrogen bubbling under sonication for approximately 30 min. Ammonium bicarbonate buffer (50 mM; 50 μ L/ μ mol peptide) and anhydrous acetonitrile (50 μ L/ μ mol peptide) were added to the Eppendorf tube containing **P1**, and the mixture was vortexed for a few seconds. 100 μ L aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “first step” and 1) that had been pre-filled with anhydrous acetonitrile (78 μ L) and a solution of **6** (0.10 M in anhydrous acetonitrile; 12 μ L, 1.2 μ mol, 1.2 equiv.), so that each tube contained 1 μ mol of **P1**. A Pd/BiPhePhos catalyst solution (5.0 mM in anhydrous acetonitrile*; 10 μ L, 50 nmol, 5 mol%) was then added to each tube, resulting in a peptide concentration of approximately 5 mM. Each reaction mixture (Tubes “first step” and 1) was vortexed briefly. A 50 μ L aliquot of Tube “first step” was transferred to another Eppendorf tube (Tube “P1.6 inter.”). Tubes “P1.6 inter.” and 1 were then incubated at 23 °C, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “P1.6 inter.” and 1 as described in the table below.

Tube “P1.6 inter.”	Tube 1
200 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 590 μ L MeOH

Tubes “P1.6 inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μ L aliquots of the reaction mixture (Tube 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH₃CN (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h), as described in Section S5.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.6 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tube 1 at each time point (entries 1) were processed as described in General

Procedure 1, enabling monitoring of the conversion of peptide aldehyde **P1.6al**. A conversion of **P1.6al** exceeding 99% was observed 24 h after addition of NaBH₃CN.

As **P1.6s** was not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yield of the corresponding analytical-scale reaction was not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

* Preparation of the Pd/BiPhePhos catalyst: The Pd/BiPhePhos solution was prepared at a concentration of 5 mM according to the procedure described by Breinbauer and coworkers,^[14] with minor modifications. A flame-dried 10 mL round-bottom flask equipped with a stirring bar was charged with BiPhePhos (13.8 mg, 17.5 μmol) and Pd(dba)₂ (10.1 mg, 17.6 μmol) and flushed with nitrogen. Anhydrous acetonitrile (3.5 mL) was added, and the flask was transferred to an oil bath preheated to 60 °C. The mixture was stirred under nitrogen for 35 min at 60 °C, affording a bright yellow solution. The solution was allowed to cool to room temperature, and a 2 mL aliquot was transferred to a 4 mL vial.

Table S17. Stapling of peptide model **P1** with (*E*)-3-[(4-acetoxybut-2-en-1-yl)oxy]benzaldehyde (**6**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	6 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 3:1, [peptide] = 5 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 12:4, [peptide] = 1 mM, 23 °C, 24 h	>99	73	94

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μM).

^b Conversion of peptide aldehyde **P1.6al** during the reductive amination step; calculated as [1 – (absorbance area of **P1.6ox**_a at t = 24 h / absorbance area of **P1.6ox**_a at t₀)] × 100.

^c Calculated as (combined absorbance area of **P1.6s**_a, **P1.6s**_b, and **P1.6s**_c / combined absorbance area of all relevant peptidic species) × 100.

^d Calculated as (combined absorbance area of **P1.6s**_a, **P1.6s**_b, and **P1.6s**_c / combined absorbance area of **P1.6s**_a, **P1.6s**_b, **P1.6s**_c, and **P1.6ol**) × 100.

^e Purchased from GeneCust; purity >95%.

S6.1.6. Stapling via S-Arylation/Reductive Amination

Stapling of the peptide model **P1** with pentafluorobenzaldehyde (**7**) was performed on an analytical scale following General Procedure 2.

Thus, solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 $\mu\text{L}/\mu\text{mol}$ peptide) and acetonitrile (47.6 $\mu\text{L}/\mu\text{mol}$ peptide) were added, and the mixture was vortexed for a few seconds. 97.6 μL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P1.7 inter.” and 1), so that each tube contained 1 μmol of **P1**. A solution of **7** (0.50 M in acetonitrile; 2.4 μL , 1.2 μmol , 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “P1.7 inter.” and 1 as described in the table below.

Tube “P1.7 inter.”	Tube 1
900 μL 0.1% TFA in H_2O	200 μL sodium citrate buffer (250 mM, pH 4.0) + 690 μL MeOH

Tubes “P1.7 inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μL aliquots of the reaction mixture (Tube 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH_3CN (~30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h), as described in Section S5.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.7 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tube 1 at each time point (entries 1) were processed as described in General Procedure 1, enabling monitoring of the conversion of peptide aldehyde **P1.7al**. A conversion of **P1.7al** exceeding 99% was observed 24 h after addition of NaBH_3CN .

As **P1.7s** was not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yield of the corresponding analytical-scale reaction was not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

Table S18. Stapling of peptide model **P1** with pentafluorobenzaldehyde (**7**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	7 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 24 h	>99	75	78

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M).

^b Conversion of peptide aldehyde **P1.7al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.7ox_a} \text{ at } t = 24 \text{ h} / \text{absorbance area of } \mathbf{P1.7ox_a} \text{ at } t_0)] \times 100$.

^c Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) $\times 100$.

^d Calculated as (absorbance area of **P1.7s** / combined absorbance area of **P1.7s** and **P1.7ol**) $\times 100$.

^e Purchased from GeneCust; purity >95%.

S6.1.7. Kinetics of Intramolecular Reductive Amination of Intermediates **P1.1al**–**P1.7al**

Monitoring the conversion of peptide aldehyde intermediates **P1.2al**–**P1.7al** during intramolecular reductive amination enabled the establishment of kinetic curves, as described in Section S5.7 (*Kinetic Studies*). These kinetic curves, obtained by nonlinear regression using a single-exponential model in GraphPad Prism 10.6.1, are presented in Figure S2, together with the one obtained for peptide aldehyde **P1.1al**.*

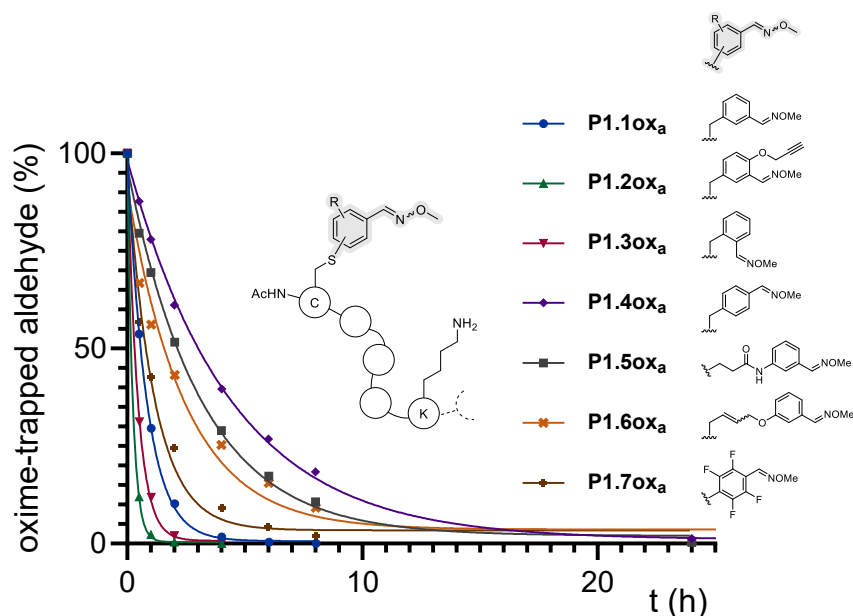


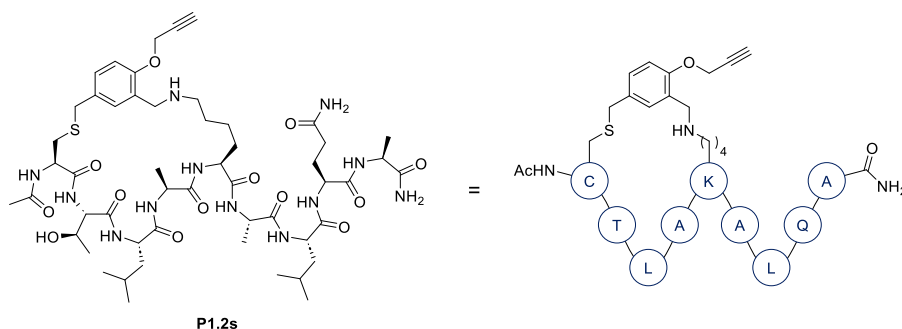
Figure S2. Kinetic profiles of intramolecular reductive amination of peptide aldehydes (**al**): normalized absorbance area of oxime-trapped species (**ox_a**) versus time. Series shown: **P1.1ox_a**; **P1.2ox_a**; **P1.3ox_a**; **P1.4ox_a**; **P1.5ox_a**; **P1.6ox_a**; **P1.7ox_a**.

* Note on the kinetic curve of **P1.6al**: The poorer quality of the fit observed for the kinetic curve of **P1.6al** is tentatively attributed to an overestimation of the initial aldehyde concentration at t_0 , which may arise from an unusually high amount of **P1.6al** present in Tube “P1.6 inter.” Indeed, the peak areas measured for **P1.6al** and its corresponding oxime derivative **P1.6ox_a** at t_0 were acquired from analytical samples resulting from dilution of Tube “P1.6 inter.” (corresponding to the reaction medium prior to the start of the reductive amination step), whereas the data points recorded from 30 min to 48 h were acquired from analytical samples obtained from a different reaction tube (Tube 1) in which the aldehyde underwent reductive amination. This hypothesis is supported by the fact that earlier *S*-allylation experiments afforded, under identical conditions, substantially lower aldehyde peak areas than that observed in chromatogram **P1.6A**. On this basis, the peak area of **P1.6ox_a** at t_0 is presumed to be overestimated, which may account for the poorer

agreement of the corresponding data set with a single-exponential model.

S6.2. Synthesis of a Cys–Lys Stapled Peptide with an Alkyne-Bearing Linker

Peptide **P1.2s**



The synthesis of peptide **P1.2s** was first investigated on an analytical scale following General Procedure 2, with minor modifications (see Section S6.1.2, *Stapling with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde*). The optimized conditions provided the desired macrocyclic peptide in 85% HPLC yield.

Peptide **P1.2s** was then synthesized on a preparative scale from **P1** (purchased from GeneCust; purity >95%; 38.9 mg, 36.2 μmol), **2** (0.20 M in acetonitrile; 217.5 μL , 43.5 μmol), and NaBH_3CN (1.0 M in methanol; 362 μL , 362 μmol), according to General Procedure A, with minor adjustments in the S-alkylation step: an acetonitrile volume of 44 $\mu\text{L}/\mu\text{mol}$ peptide (instead of 47.6 $\mu\text{L}/\mu\text{mol}$ peptide) was used to prepare the starting peptide solution, and the stapling reagent was added as a 0.20 M solution rather than 0.50 M (6 $\mu\text{L}/\mu\text{mol}$ peptide instead of 2.4 $\mu\text{L}/\mu\text{mol}$ peptide). An HPLC analysis conducted 3 h after addition of the reducing agent indicated full conversion of the peptide aldehyde intermediate. After work-up (evaporation of organic solvents followed by lyophilization), the crude solid was dissolved in a 4:1:1 (v/v/v) mixture of water, methanol, and acetonitrile to a peptide concentration of 5–7 mM. Purification by semi-preparative HPLC (gradient: 0–5 min, 5–31% D; 5–15 min, 31–36% D; 15–16 min, 36–95% D; 16–20 min, 95% D; t_R = 10.4 min) afforded **P1.2s**

(trifluoroacetate salt; 14.2 mg, 32%) as a white fluffy solid following lyophilization.

S6.3. Post-Stapling Modification via Copper-Catalyzed Azide–Alkyne Cycloaddition

Stapled peptide **P1.2s** was subjected to copper-catalyzed azide–alkyne cycloaddition (CuAAC) using conditions adapted from Finn and coworkers.^[15] Accordingly, purified* **P1.2s** (trifluoroacetate salt; 1.912 mg, 1.556 μmol) was weighed and transferred to an Eppendorf tube. Sodium phosphate buffer (100 mM, pH 7.0; 778 μL) was added, and the resulting suspension was alternately vortexed and sonicated to give a 2.00 mM solution. An aliquot (250 μL , 0.500 μmol , 1.0 equiv.) was transferred to another Eppendorf tube. Five volumes of CuSO_4 solution (20.0 mM in water) were mixed with four volumes of tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) solution (50.0 mM in water).

The following solutions were then added in this order to the tube containing 0.500 μmol of **P1.2s**, with pipette mixing between each addition: sodium phosphate buffer (100 mM, pH 7.0; 77.5 μL), 6-carboxyfluorescein azide (20 mM in DMSO; 50.0 μL , 1.00 μmol , 2.0 equiv.), Cu/THPTA (11.1 mM Cu and 22.2 mM THPTA in water; 22.5 μL , 0.250 μmol Cu and 0.500 μmol THPTA, 0.5 equiv. Cu and 1.0 equiv. THPTA), aminoguanidine hydrochloride (100 mM in water; 50.0 μL , 5.00 μmol , 10 equiv.), and sodium ascorbate (100 mM in water; 50.0 μL , 5.00 μmol , 10 equiv.). The reaction mixture (final volume: 500 μL ; final concentrations: 1.0 mM **P1.2s**, 2.0 mM 6-carboxyfluorescein azide, 0.50 mM Cu, 1.0 mM THPTA, 10 mM aminoguanidine hydrochloride, 10 mM sodium ascorbate) was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, 50 μL of the reaction mixture was diluted 10-fold into water. The resulting analytical sample (final peptide concentration: ~100 μM , relative to the initial amount of **P1.2s**) was vortexed for a few seconds and analyzed by HPLC (Method 2), which indicated complete conversion of **P1.2s**. The remaining reaction medium was frozen.

Subsequently, the crude reaction mixture was thawed and diluted with water to a peptide concentration of 0.5–0.7 mM. Purification by semi-

preparative HPLC (gradient: 0–5 min, 5–30% D; 5–15 min, 30–40% D; 15–16 min, 40–95% D; 16–20 min, 95% D; t_R = 13.0 min) afforded **P1.2sf** (trifluoroacetate salt) as a bright yellow fluffy solid following lyophilization. To check the purity, a 0.15–0.25 mM solution of **P1.2sf** was prepared by dissolving 0.138 mg of the purified solid in water/acetonitrile/TFA (1:1:0.002, v/v/v; 328 μ L). This solution was then diluted 5-fold into 0.1% TFA in water and analyzed by HPLC (Method 2), which indicated a purity >95% (λ = 210 nm).

* Note on CuAAC from crude **P1.2s**: Attempts to perform the CuAAC reaction on crude **P1.2s** obtained after S-alkylation/reductive amination and work-up (evaporation of organic solvents followed by lyophilization, without purification) resulted in slow conversion and formation of impurities, which may correspond to methylated or otherwise modified derivatives of **P1.2s** and **P1.2sf** (m/z +14). Similar results were obtained when CuAAC was attempted directly after the intramolecular reductive amination, with or without dilution of the reaction mixture in sodium phosphate buffer (100 mM, pH 7.0), and without evaporation of acetonitrile and methanol.

S7. Cys–Lys Stapling with Chloroacetaldehyde and Methacrolein

P1 was macrocyclized on an analytical scale using chloroacetaldehyde (**8**) or methacrolein (**9**) as stapling reagents (Scheme 23). Methacrolein-mediated Cys–Lys stapling was carried out according to General Procedure 2. For chloroacetaldehyde-mediated Cys–Lys stapling, the duration of the S-alkylation step was reduced from 1 h to 15 min. Chloroacetaldehyde was used as a freshly prepared 0.50 M stock solution in water (prepared from a 55 wt.% solution) rather than as a solution in acetonitrile, which required slight adaptations of the protocol relative to General Procedure 2.

Chloroacetaldehyde-mediated Cys–Lys stapling:

Solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (52.5 mM, pH 8.0; 47.6 μ L/ μ mol peptide) and acetonitrile (50 μ L/ μ mol peptide) were added, and the mixture was vortexed for a few seconds.

97.6 μL aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P1.8 inter.” and 1), so that each tube contained 1 μmol of **P1**. A solution of chloroacetaldehyde (**8**, 0.50 M in water; 2.4 μL , 1.2 μmol , 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 15 min, appropriate solvents were added to Tubes “P1.8 inter.” and 1 as described in the table below.

Tube “P1.8 inter.”	Tube 1
900 μL 0.1% TFA in H_2O	200 μL sodium citrate buffer (250 mM, pH 4.0) + 690 μL MeOH

Tubes “P1.8 inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μL aliquots of the reaction mixture (Tube 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH_3CN (~30 min, 1 h, 2 h, 4 h, 6 h), as described in Section S4.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.8 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tube 1 at each time point (entries 1) were processed as described in General Procedure 1.

Ten-fold dilution into methoxylamine buffer, followed by standing at room temperature for 30 min, did not result in complete trapping of the **P1.8al** intermediate, as evidenced by chromatogram P1.8O. Thus, the conversion of peptide aldehyde **P1.8al** during the reductive amination step could not be efficiently monitored. As the absorbance area of the target product **P1.8s** ceased to increase after 6 h, the reaction was considered complete at this time point, and the reaction efficiency was evaluated at that time.

As **P1.8s** was not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yield of the corresponding analytical-scale reaction was not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

Table S19. Stapling of peptide model **P1** with chloroacetaldehyde (**8**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Absorbance Ratio (%) ^{a, b}	s/(s + ol) (%) ^{a, c}
1	P1 ^d	8 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 15 min; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 6 h	61	99

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M).

^b Calculated as (absorbance area of the target product / combined absorbance area of all relevant peptidic species) $\times$ 100.

^c Calculated as (absorbance area of **P1.8s** / combined absorbance area of **P1.8s** and **P1.8ol**) $\times$ 100

^d Purchased from GeneCust; purity >95%.

Methacrolein-mediated Cys–Lys stapling:

Solid **P1** (trifluoroacetate salt) was weighed and transferred to an Eppendorf tube. Freshly prepared ammonium bicarbonate buffer (50 mM, pH 8.0; 50 μ L/ μ mol peptide) and acetonitrile (47.6 μ L/ μ mol peptide) were added, and the mixture was vortexed for a few seconds. 97.6 μ L aliquots of the resulting solution were transferred to Eppendorf tubes (Tubes “P1.9 inter.” and 1), so that each tube contained 1 μ mol of **P1**. A solution of methacrolein (**9**, 0.50 M in acetonitrile; 2.4 μ L, 1.2 μ mol, 1.2 equiv.) was then added to each tube, resulting in a peptide concentration of approximately 10 mM. Each reaction mixture was vortexed briefly and incubated at 23 °C, protected from light.

After incubation for 1 h, appropriate solvents were added to Tubes “P1.9 inter.” and 1 as described in the table below.

Tube “P1.9 inter.”	Tube 1
900 μ L 0.1% TFA in H ₂ O	200 μ L sodium citrate buffer (250 mM, pH 4.0) + 690 μ L MeOH

Tubes “P1.9 inter.” and 1 were vortexed briefly, and then treated as described in General Procedure 1.

During the reductive amination step, 100 μ L aliquots of the reaction mixture (Tube 1) were quenched by 10-fold dilution into methoxylamine buffer at various time points after addition of NaBH₃CN (~30 min, 1 h, 2 h, 4 h, 6 h), as described in Section S4.7 (*Kinetic Studies*). The chromatograms obtained following treatment of Tube “P1.9 inter.” (entries A and O) were processed as described in General Procedure 1. The chromatograms obtained following treatment of Tube 1 at each time point (entries 1) were processed as described in General

Procedure 1, enabling monitoring of the conversion of peptide aldehyde **P1.9al**. A conversion of **P1.9al** exceeding 99% was observed 2 h after addition of NaBH₃CN.

As **P1.9s_a** and **P1.9s_b** were not prepared on a preparative scale and purified, no calibration curve was available. Therefore, the HPLC yield of the corresponding analytical-scale reaction was not determined. Instead, reaction efficiency was evaluated using the absorbance ratio and the selectivity for iminium reduction.

Table S20. Stapling of peptide model **P1** with methacrolein (**9**) on an analytical scale.

Entry	Starting Material	Reaction Conditions	Conversion (%) ^{a, b}	Absorbance Ratio (%) ^{a, c}	s/(s + ol) (%) ^{a, d}
1	P1 ^e	9 (1.2 equiv.), MeCN/aq. NH ₄ HCO ₃ (50 mM, pH 8.0) 1:1, [peptide] = 10 mM, 23 °C, 1 h; then NaBH ₃ CN (10 equiv.), MeOH/aq. sodium citrate (250 mM, pH 4.0) 14:4, [peptide] = 1 mM, 23 °C, 2 h	>99	93	98

^a Determined by HPLC–UV (λ = 210 nm; Method 2; peptide concentration ~100 μ M).

^b Conversion of peptide aldehyde **P1.9al** during the reductive amination step; calculated as $[1 - (\text{absorbance area of } \mathbf{P1.9ox} \text{ at } t = 2 \text{ h} / \text{absorbance area of } \mathbf{P1.9ox} \text{ at } t_0)] \times 100$.

^c Calculated as (combined absorbance area of **P1.9s_a** and **P1.9s_b** / combined absorbance area of all relevant peptidic species) $\times$ 100.

^d Calculated as (combined absorbance area of **P1.9s_a** and **P1.9s_b** / combined absorbance area of **P1.9s_a**, **P1.9s_b**, and **P1.9ol**) $\times$ 100.

^e Purchased from GeneCust; purity >95%.

S8. Circular Dichroism Spectroscopy

Each linear or stapled peptide was dissolved in a 10 mM sodium phosphate buffer (pH 7.4) or in a 4:1 (v/v) mixture of the same buffer and trifluoroethanol (TFE) to afford a 500 μ M stock solution. The stock solution was further diluted 5-fold with the corresponding solvent to give a 100 μ M analytical solution.

An aliquot of 350–400 μ L of the analytical solution was transferred to a 1 mm path length quartz cell (Hellma®), and analyzed by circular dichroism (CD) at 20 °C using a MOS-500 spectrometer equipped with a Peltier temperature controller. Ellipticity was recorded from 190 to 260 nm using the following parameters: 5 scans, 0.5 nm step, 0.5 s acquisition period. The photomultiplier tube voltage was monitored and did not exceed 700 V.

Each analytical solution of thiol-to-amine stapled peptide was analyzed by HPLC, and sample concentrations were corrected using the

corresponding HPLC–UV calibration curves (see Section S10, *HPLC–UV Calibration Curves*).

Ellipticity data were collected in millidegrees (mdeg), baseline-subtracted, and converted to mean peptide ellipticity ($[\theta]$) using the following equation:^[16]

$$[\theta] = \frac{\theta}{10 \times C \times N_p \times l}$$

$[\theta]$: mean peptide ellipticity ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$); θ : ellipticity (mdeg); C : concentration (M); N_p : number of peptide units ($N_p = 8$ for the **LB8**-derived octapeptides and $N_p = 10$ for the *N*-acetylated nonapeptides); l : cell path length (cm).

For each *N*-acetylated nonapeptide, three independent solutions were analyzed. Data shown in the graphs (Figures S3–S10) represent the average of these three measurements.

The percentage helicity (%helicity) of each peptide was calculated from its mean peptide ellipticity at 222 nm ($[\theta]_{222}$) using the following equations:^[16,17]

$$[\theta]_{\max} = (-44000 + 250 T)(1 - k/N_p)$$

$$\% \text{helicity} = \frac{[\theta]_{222}}{[\theta]_{\max}} \times 100$$

%helicity: percentage helicity (%); $[\theta]_{\max}$: theoretical ellipticity value of a fully folded α -helix at 222 nm ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$); T : temperature ($^{\circ}\text{C}$); k : finite length correction ($k = 4$)*;^[16-18] N_p : number of peptide units; $[\theta]_{222}$: mean peptide ellipticity measured at 222 nm ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$; average of three values).

* Note on the finite length correction (k): the value of k , which is difficult to define for short peptides, strongly affects the $[\theta]_{\max}$ value and therefore the calculated percentage helicity.^[16,17,19] Values of k ranging from 2.5 to 4.5 have been used in the literature.^[8,16-18,20-22] We used $k = 4$, as this has been found appropriate notably by Fairlie^[16] and by Arora.^[17] However, using $k = 2.5$ would give percentage helicity values 1.25-fold lower, highlighting the need for caution when interpreting these percentage helicity values. Because k may vary significantly with helix length for short helices,^[19] the percentages calculated for the nonapeptides in this work should not be directly compared with those reported for peptides of different lengths.

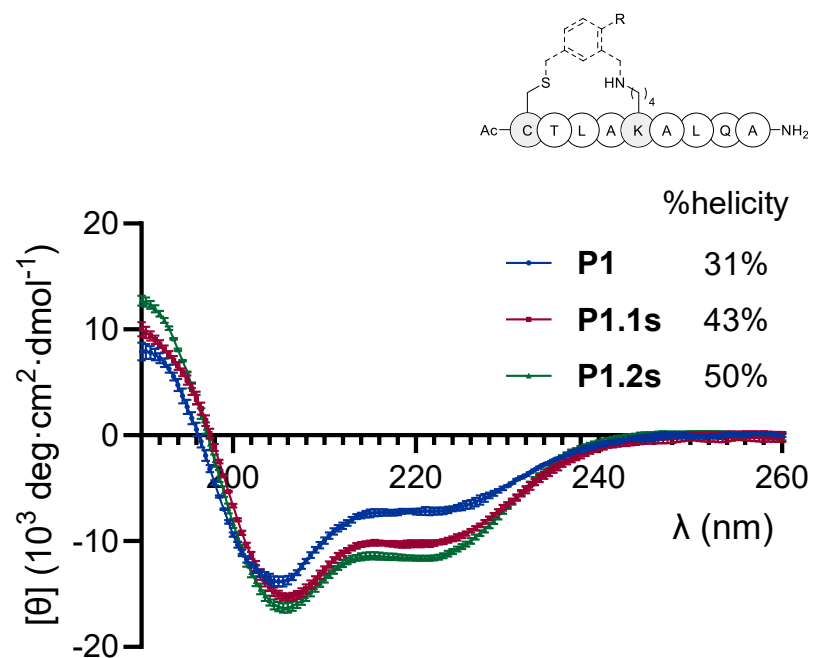


Figure S3. Circular dichroism spectra of **P1**, **P1.1s**, and **P1.2s**. *R* = H (**P1.1s**) or OCH₂C≡CH (**P1.2s**). Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); *T* = 20 °C. Error bars represent ±1 SD (*n* = 3).

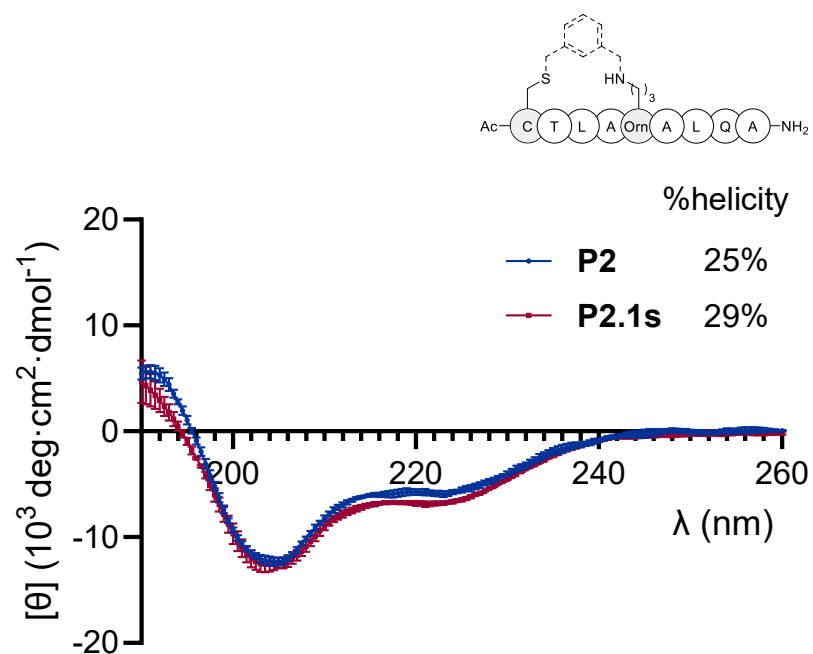


Figure S4. Circular dichroism spectra of **P2** and **P2.1s**.

Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 $^{\circ}\text{C}$. Error bars represent ± 1 SD (n = 3).

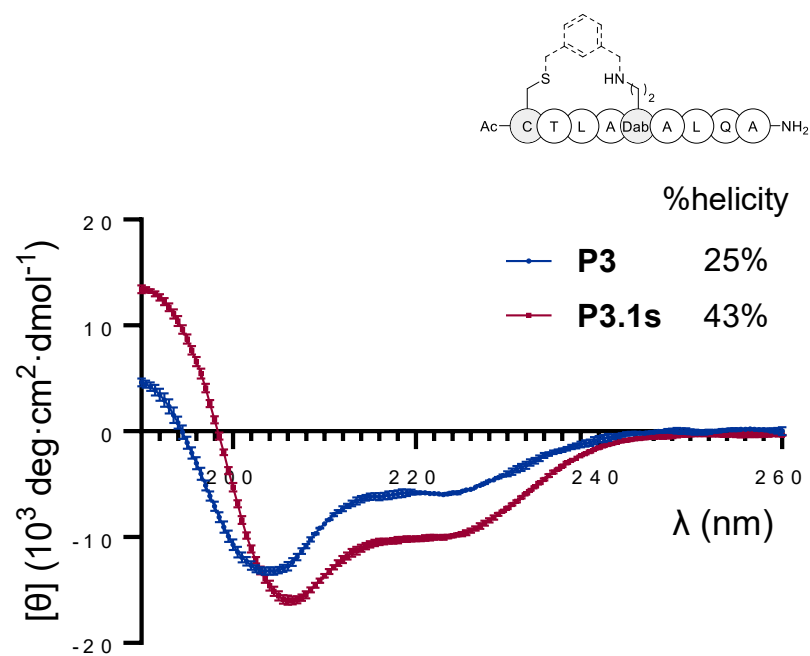


Figure S5. Circular dichroism spectra of **P3** and **P3.1s**.
 Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 °C. Error bars represent ± 1 SD (n = 3).

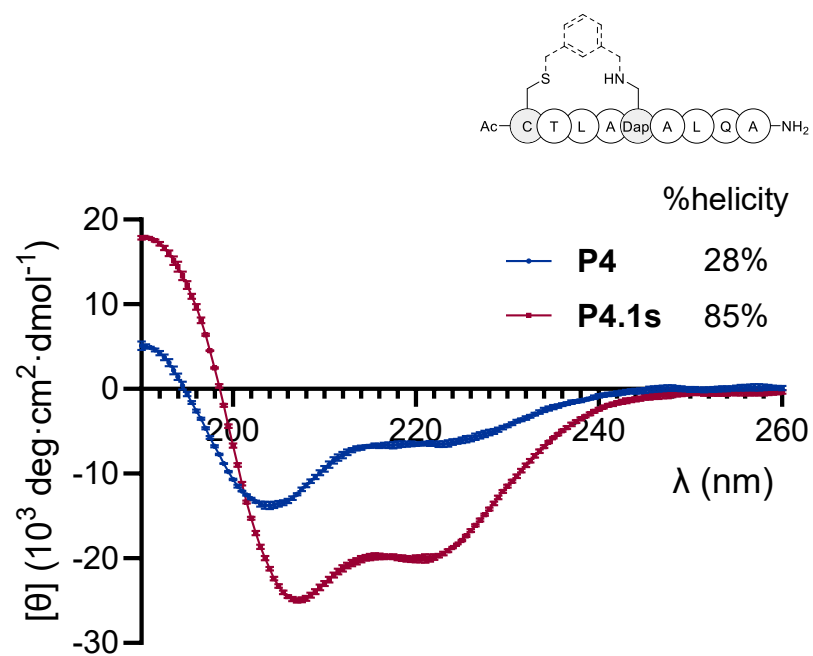


Figure S6. Circular dichroism spectra of **P4** and **P4.1s**.
 Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 °C. Error bars represent ± 1 SD (n = 3).

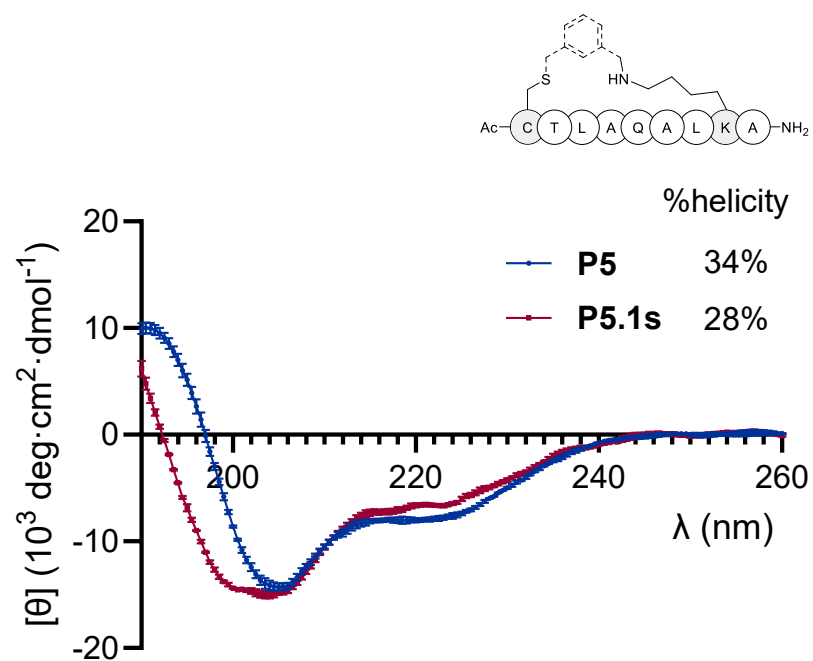


Figure S7. Circular dichroism spectra of **P5** and **P5.1s**.

Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 °C. Error bars represent ± 1 SD (n = 3).

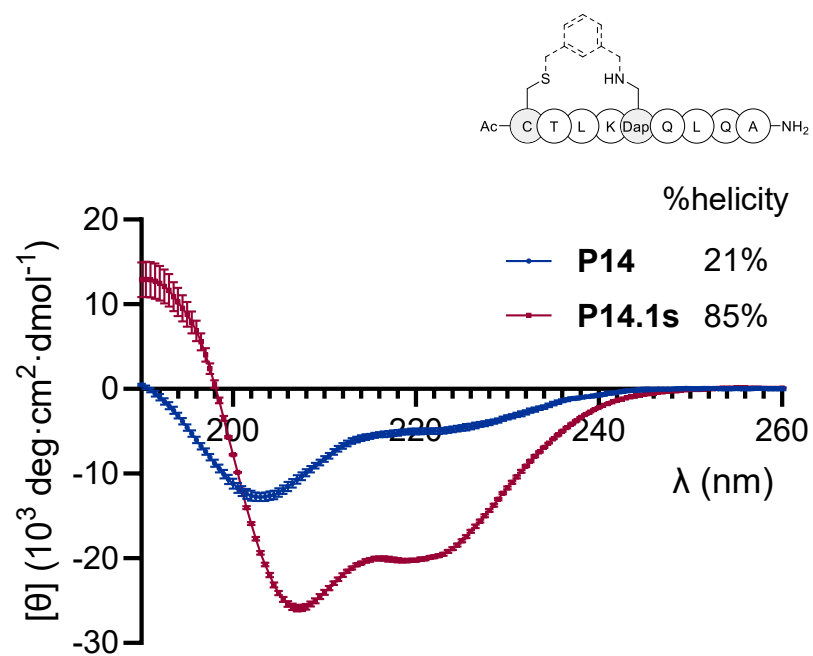


Figure S8. Circular dichroism spectra of **P14** and **P14.1s**.
 Conditions: [peptide] = 100 μM in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 °C. Error bars represent ± 1 SD (n = 3).



Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.4) or in a 4:1 (v/v) mixture of sodium phosphate buffer (10 mM, pH 7.4) and TFE; T = 20 $^{\circ}$ C. Error bars represent ± 1 SD (n = 3).

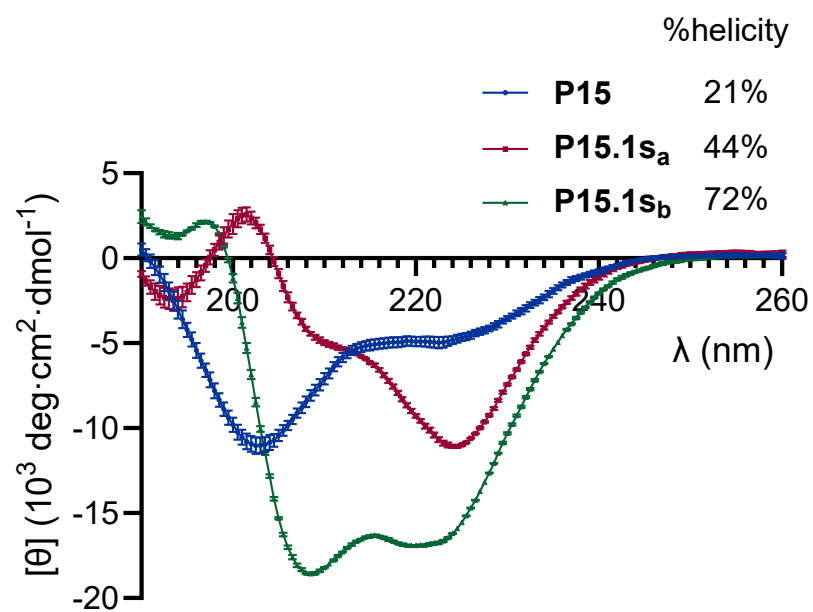
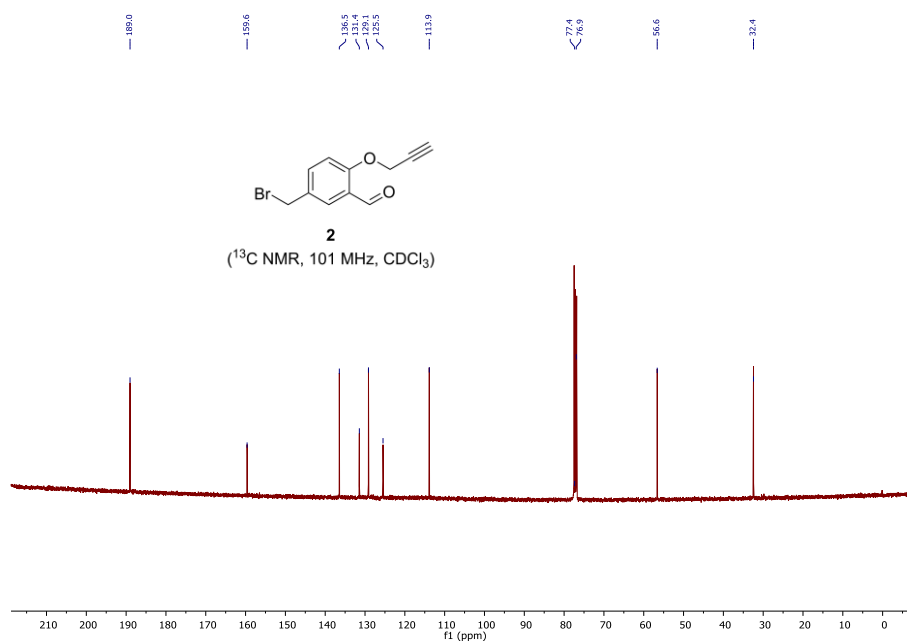
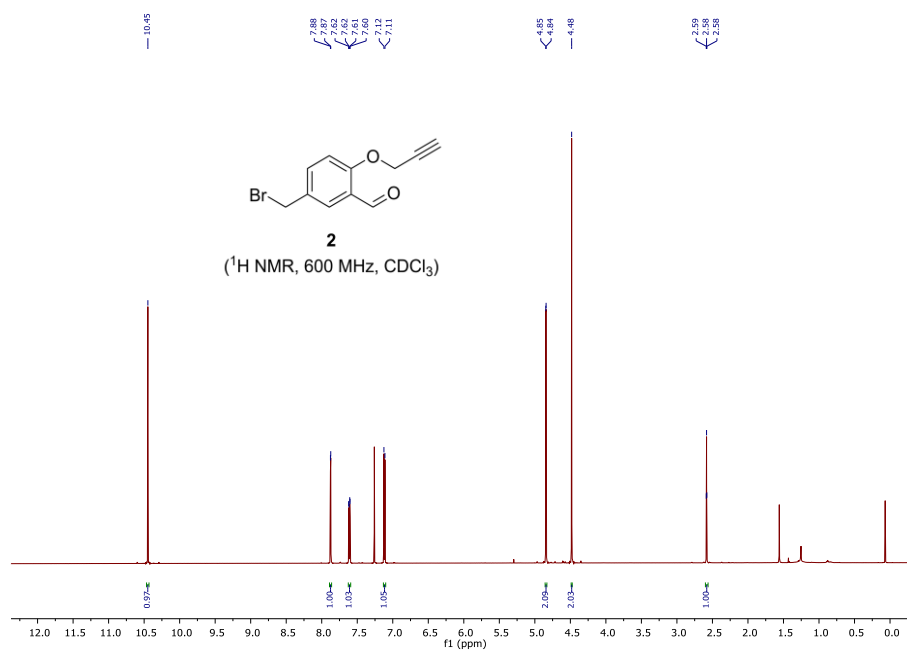
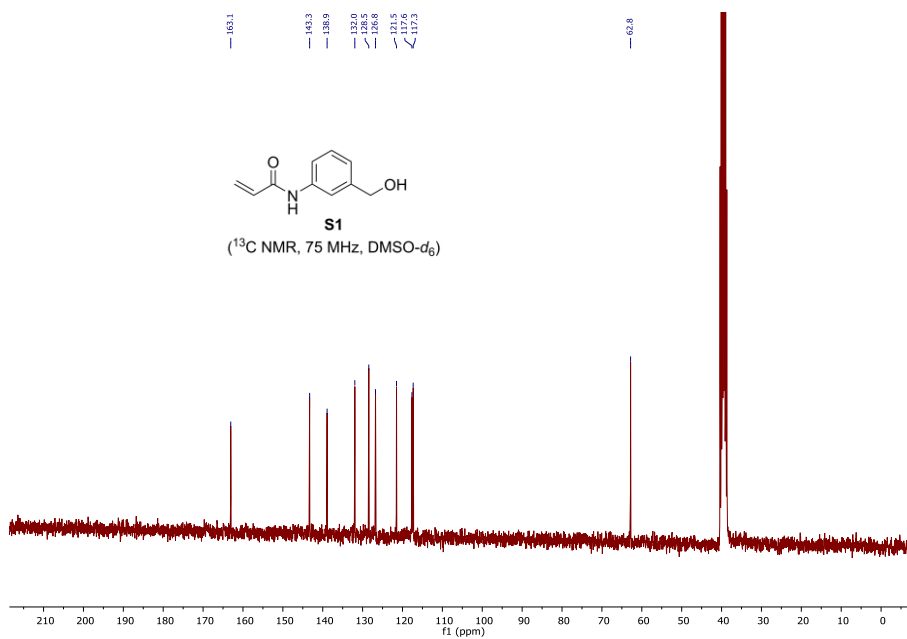
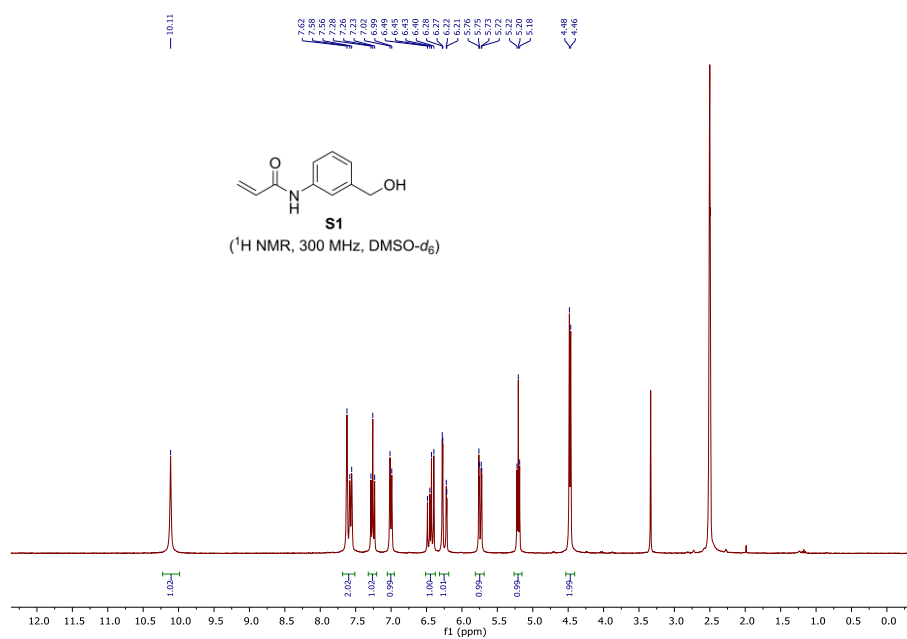
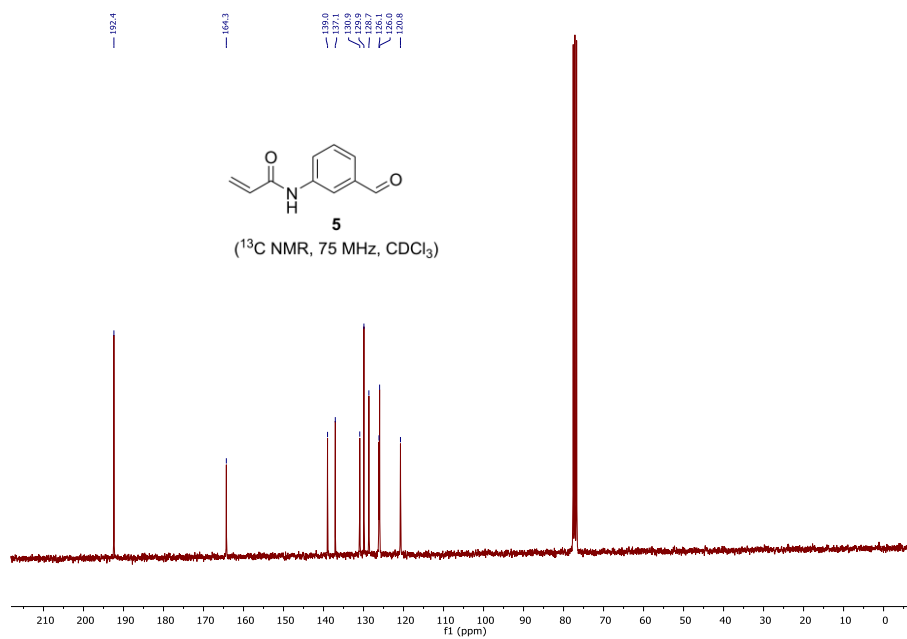
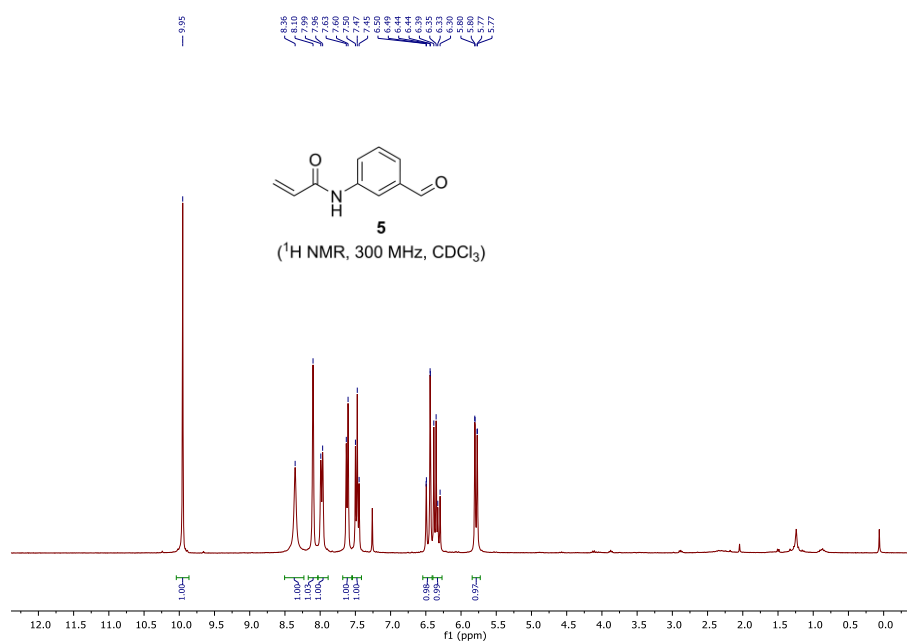


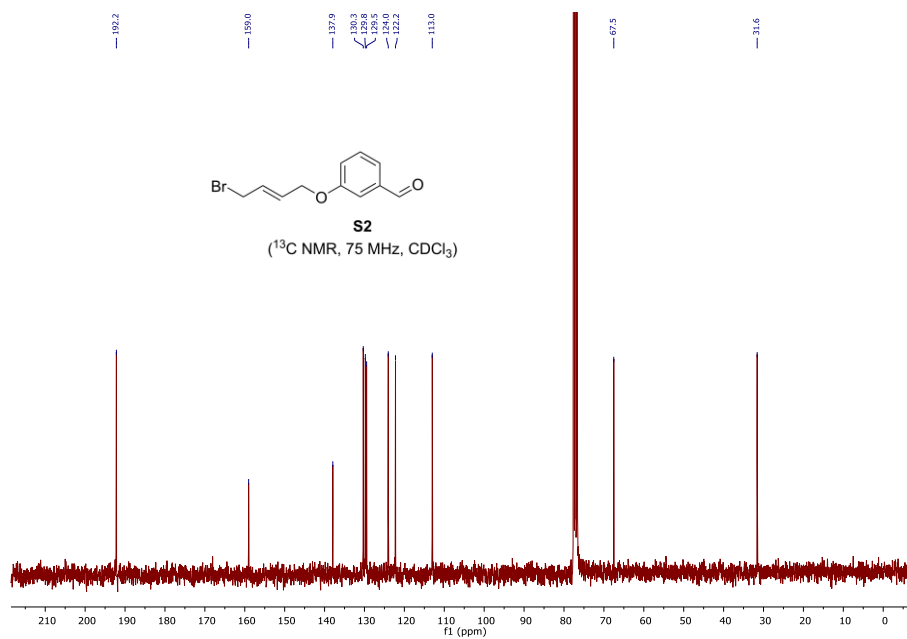
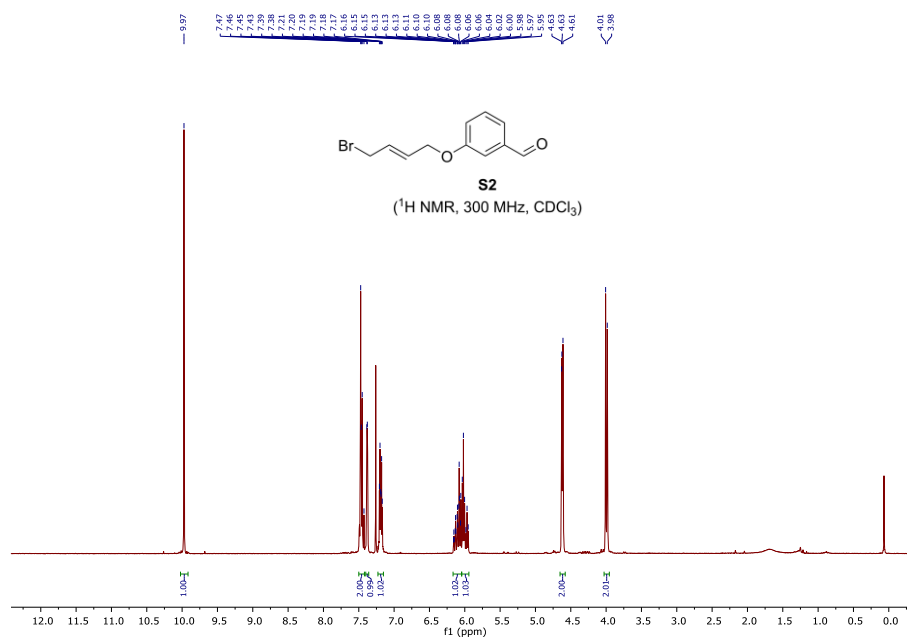
Figure S10. Circular dichroism spectra of **P15**, **P15.1s_a** and **P15.1s_b**.
 Conditions: [peptide] = 100 μ M in sodium phosphate buffer (10 mM, pH 7.4)/TFE (4:1, v/v); T = 20 $^{\circ}$ C. Error bars represent ± 1 SD (n = 3).

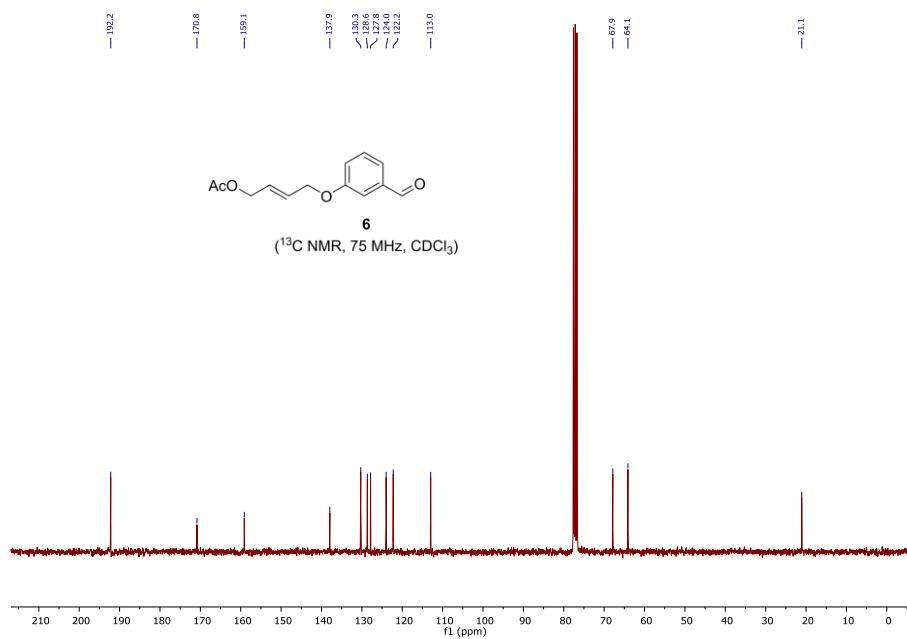
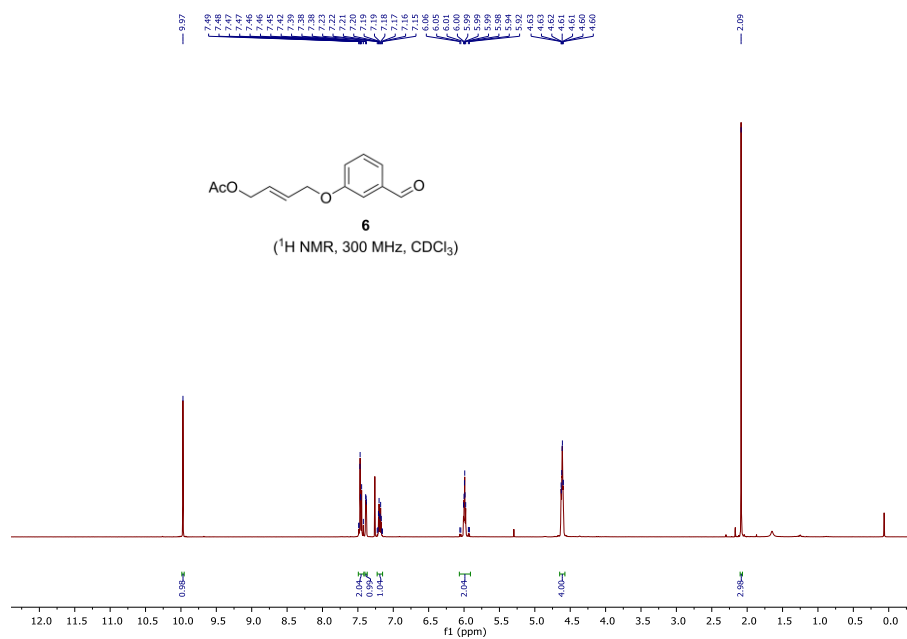
S9. NMR Spectra











S10. HPLC–UV Calibration Curves

Purified peptides were dissolved in water/acetonitrile (1:1, v/v) containing 0.1% TFA to obtain stock solutions at a concentration of 500 μM . These stock solutions were diluted with 0.1% TFA in water to afford standard solutions at the following concentrations: 10, 20, 40, 60, 80, and 100 μM . Each standard solution was analyzed by analytical HPLC ($\lambda = 210 \text{ nm}$), and the corresponding UV peak area was recorded to establish a calibration curve. Calibration of **P1.1s** was specifically performed in duplicate using independently prepared standard solutions.

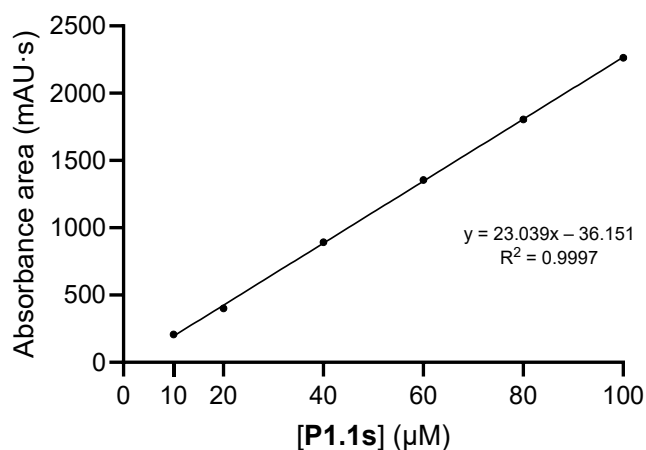


Figure S11. HPLC–UV calibration curve of **P1.1s** ($\lambda = 210 \text{ nm}$; Method 2; duplicate measurements).

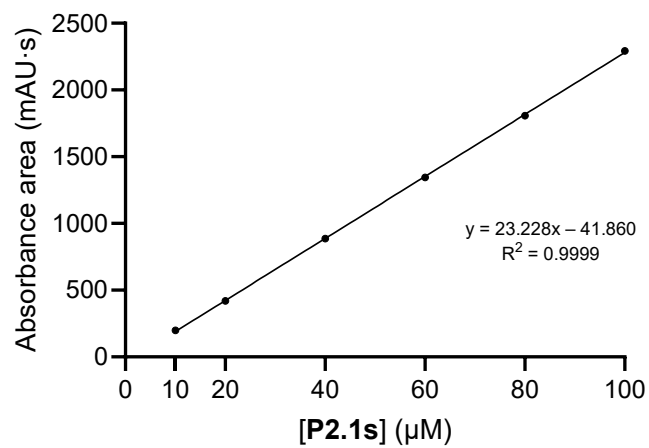


Figure S12. HPLC–UV calibration curve of **P2.1s** ($\lambda = 210$ nm; Method 2).

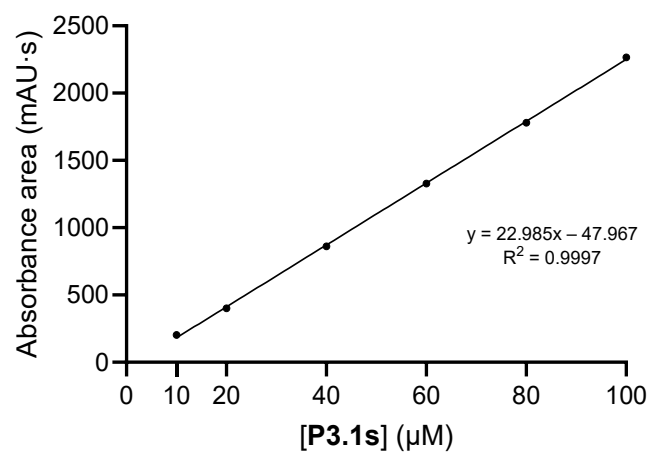


Figure S13. HPLC–UV calibration curve of **P3.1s** ($\lambda = 210$ nm; Method 2).

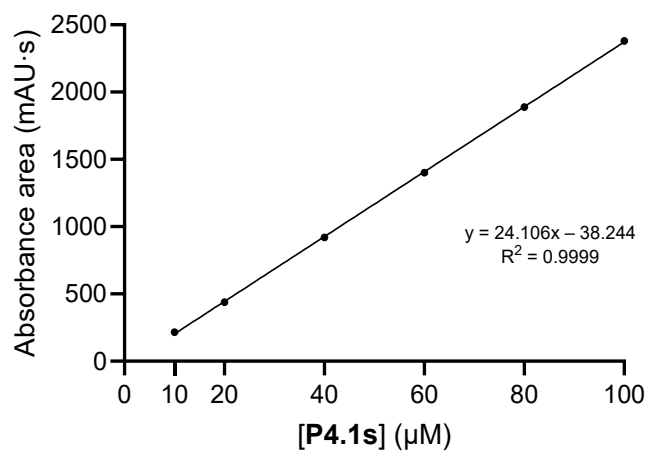


Figure S14. HPLC–UV calibration curve of **P4.1s** ($\lambda = 210$ nm; Method 2).

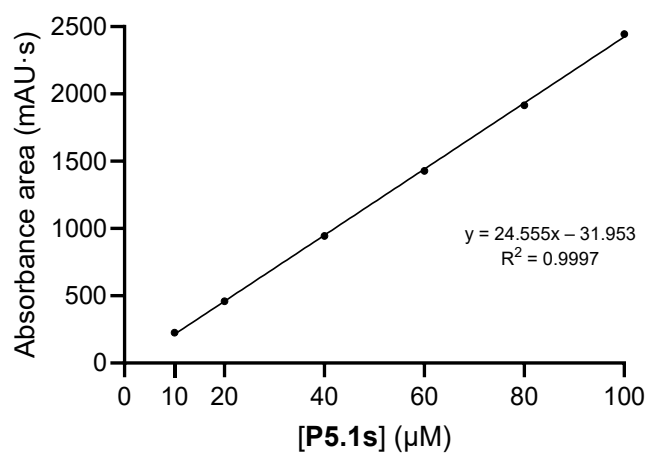


Figure S15. HPLC–UV calibration curve of **P5.1s** ($\lambda = 210$ nm; Method 2).

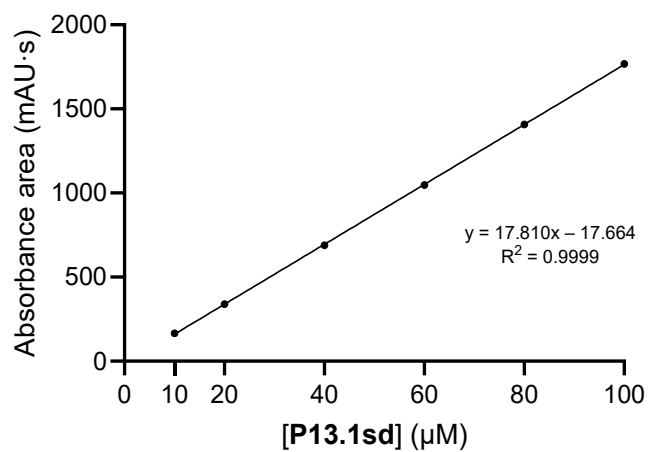


Figure S16. HPLC–UV calibration curve of **P13.1sd** ($\lambda = 210$ nm; Method 5).

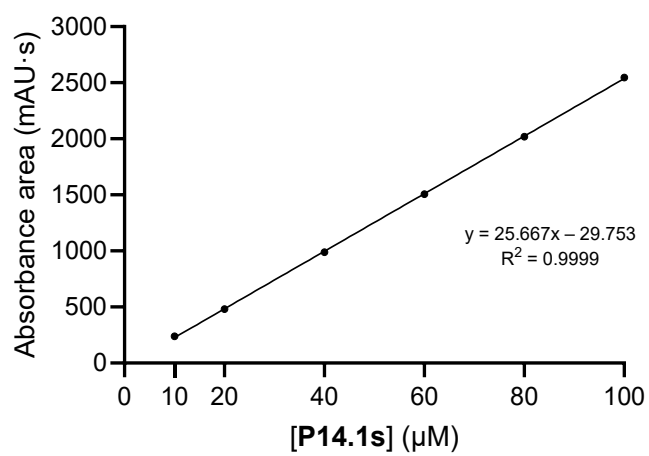


Figure S17. HPLC–UV calibration curve of **P14.1s** ($\lambda = 210$ nm; Method 3).

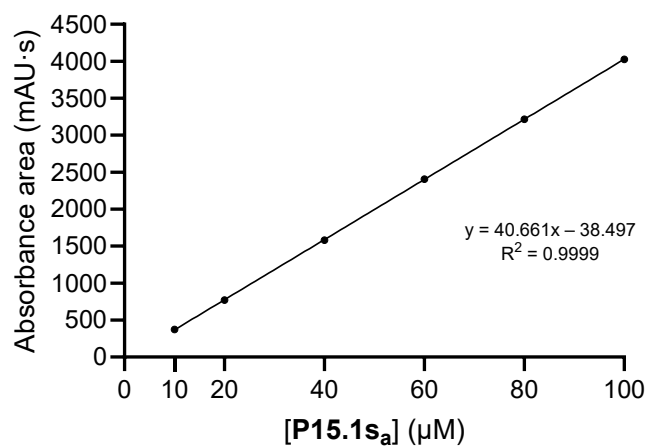


Figure S18. HPLC–UV calibration curve of **P15.1s_a** ($\lambda = 210$ nm; Method 3).

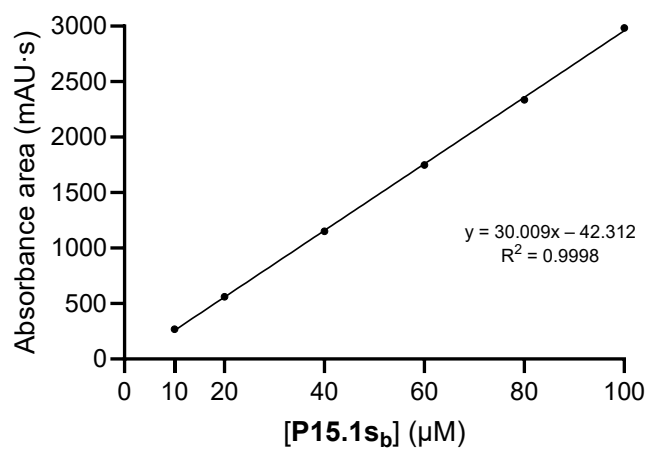


Figure S19. HPLC–UV calibration curve of **P15.1s_b** ($\lambda = 210$ nm; Method 3).

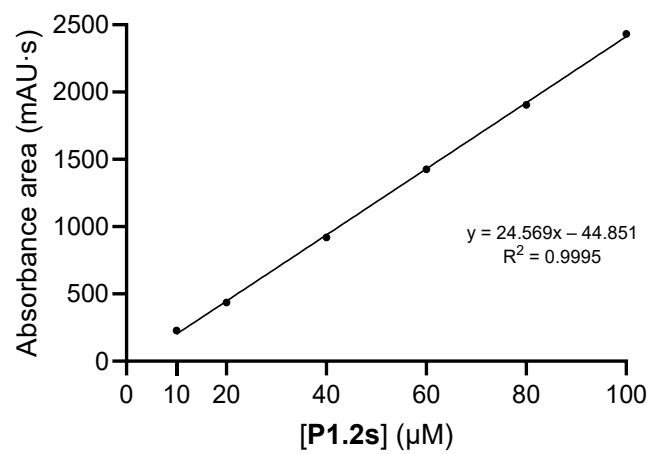


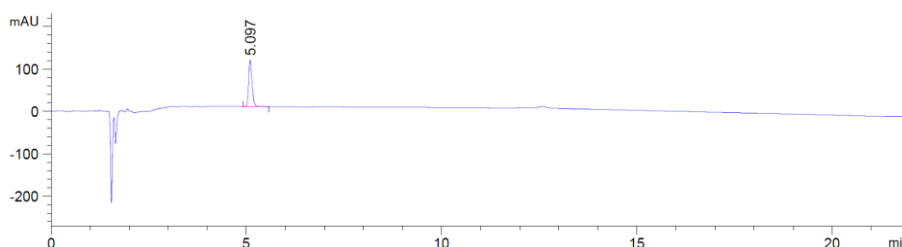
Figure S20. HPLC–UV calibration curve of **P1.2s** ($\lambda = 210$ nm; Method 2).

S11. HPLC–UV Chromatograms

This section presents the analytical HPLC–UV chromatograms of selected reaction mixtures and purified products. Retention times and UV absorbance areas were used to characterize the compounds detected. Peak assignments were supported by low-resolution ESI⁺ mass spectrometry whenever possible. Unless otherwise specified, minor peaks with absorbance areas below 1% of the total peptide-related UV signal were not annotated. In cases where the peak corresponding to the target product also included a significant proportion of a co-eluting species (>2%), the contribution of the target product to the peak signal was estimated based on the ratio of the integrals of the extracted ion current (EIC) chromatograms for the two species. When a commercial peptide was used as the starting material, peaks resulting from impurities already present in the starting material were not considered.

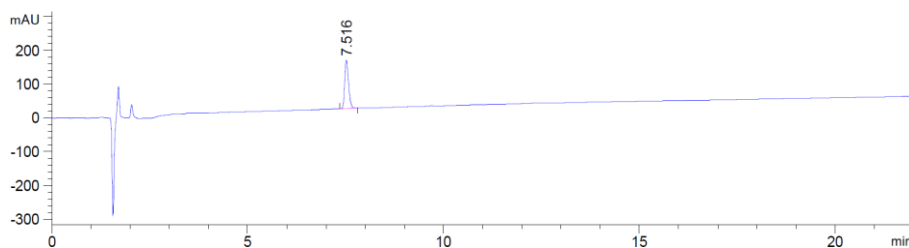
Chromatograms related to Section S3 (*Synthesis of Linear Peptides*)

Purified P6 ($\lambda = 210$ nm; Method 3; [P6] = 50–100 μ M)



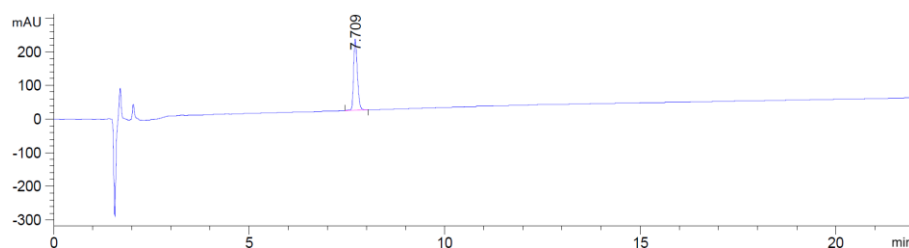
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
5.1	P6	681.6	633.3	633.3

Purified P7 ($\lambda = 210$ nm; Method 3; [P7] ~100 μ M)



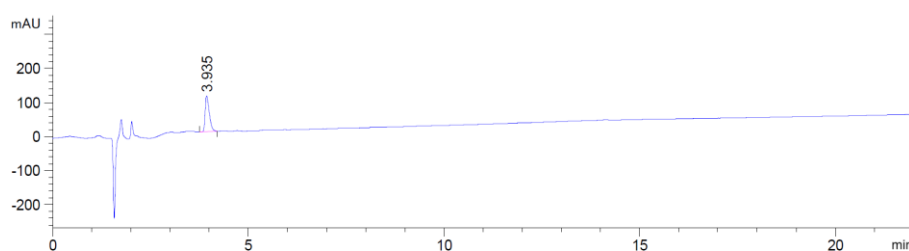
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
7.5	P7	981.6	677.3	677.3

Purified P8 ($\lambda = 210$ nm; Method 3; [P8] ~100 μ M)



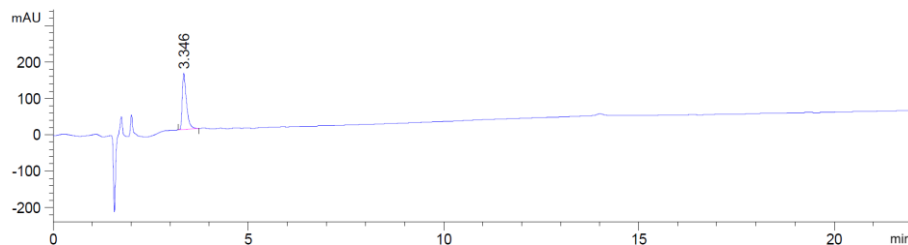
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
7.7	P8	1423.8	709.4	709.3

Purified P9 ($\lambda = 210$ nm; Method 4; [P9] ~100 μ M)



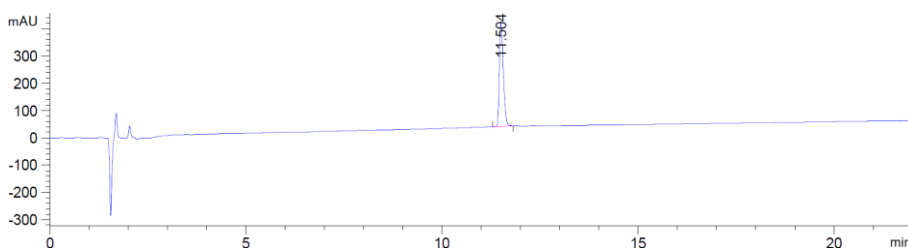
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.9	P9	766.4	702.4	702.3

Purified P10 ($\lambda = 210$ nm; Method 4; [P10] ~100 μ M)



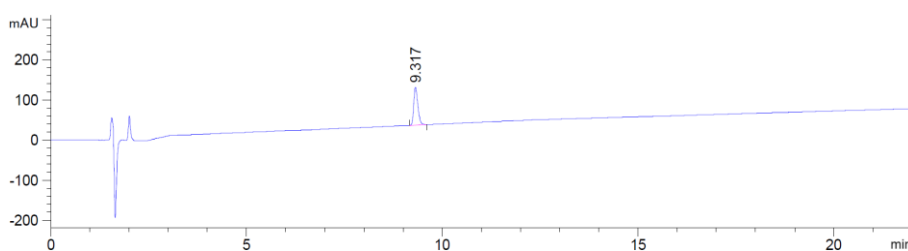
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.3	P10	1154.5	683.4	683.4

Purified P11 ($\lambda = 210$ nm; Method 3; [P11] ~100 μ M)



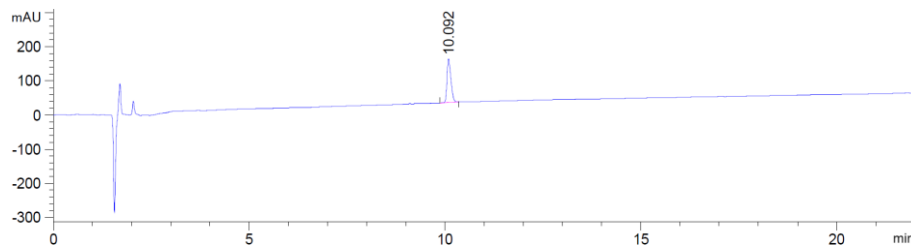
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.5	P11	2692.8	732.4	732.3

Purified P12 ($\lambda = 210$ nm; Method 3; [P12] = 50–100 μ M)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.3	P12	685.9	700.4	700.3

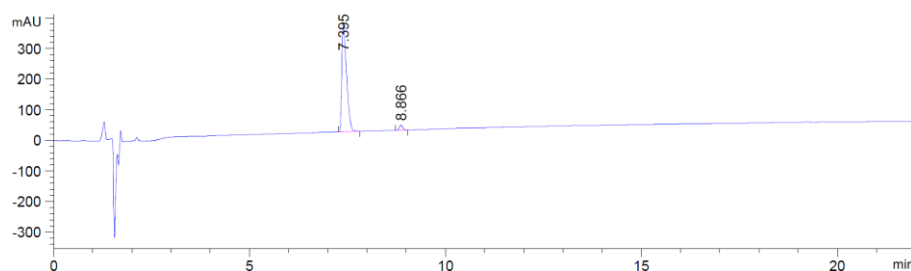
Purified P13 ($\lambda = 210$ nm; Method 3; [P13] ~100 μ M)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
10.1	P13	887.1	758.4	758.4

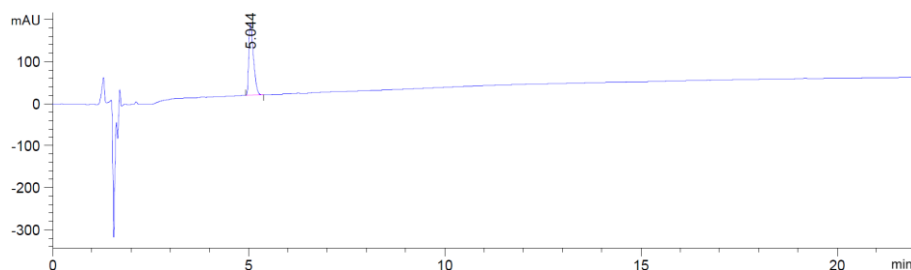
Chromatograms related to Section S4 (Cys–Cys and CuAAC-Based Stapling)

Purified MC1 ($\lambda = 210$ nm; Method 6; [MC1] ~100 μ M)



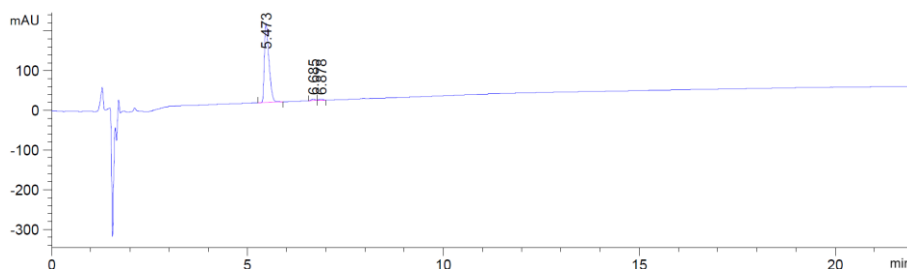
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
7.4	MC1	2963.9	1022.6	1022.5
8.9	x (not identified)	94.8	—	1366.6

Purified MC2 ($\lambda = 210$ nm; Method 6; [MC2] ~100 μ M)



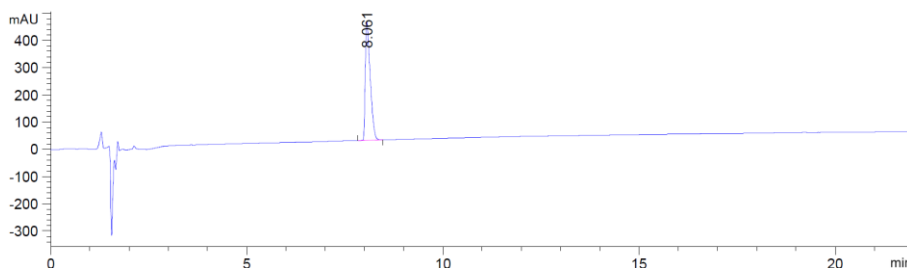
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
5.0	MC2	1393.0	1038.6	1038.5

Purified MC3 ($\lambda = 210$ nm; Method 6; **[MC3]** ~100 μ M)



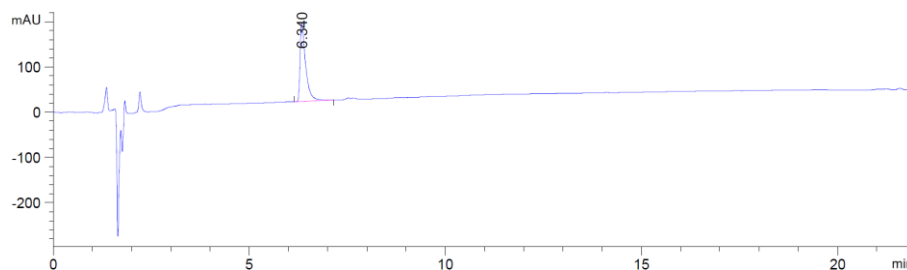
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
5.5	MC3	1622.7	972.6	972.5
6.7	x (not identified)	23.7	—	1038.5
6.9	y (not identified)	18.8	—	1038.5

Purified MC4 ($\lambda = 210$ nm; Method 6; **[MC4]** ~100 μ M)



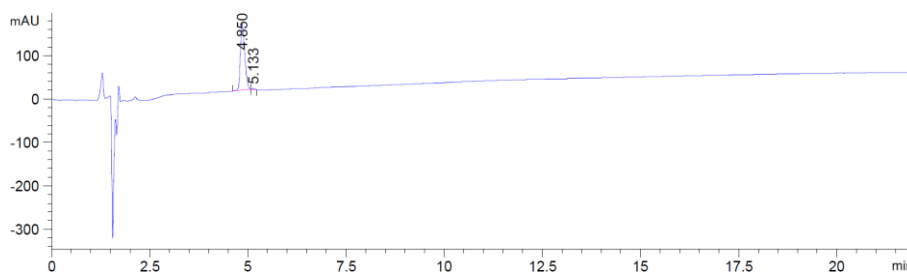
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.1	MC4	3770.2	1077.6	1077.5

Purified BC1 ($\lambda = 210$ nm; Method 6; [BC1] ~100 μ M)



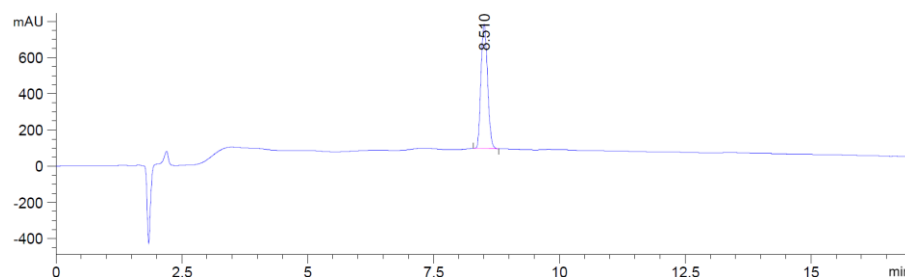
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
6.3	BC1	1722.0	1137.6	1137.5

Purified MC5 ($\lambda = 210$ nm; Method 6; [MC5] ~100 μ M)



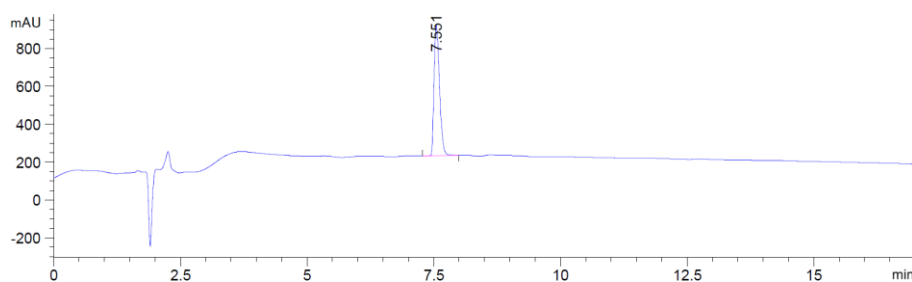
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
4.8	MC5	1174.7	963.6	963.6
5.1	x (not identified)	11.9	—	979.6

Purified F1 ($\lambda = 210$ nm; Method 7)



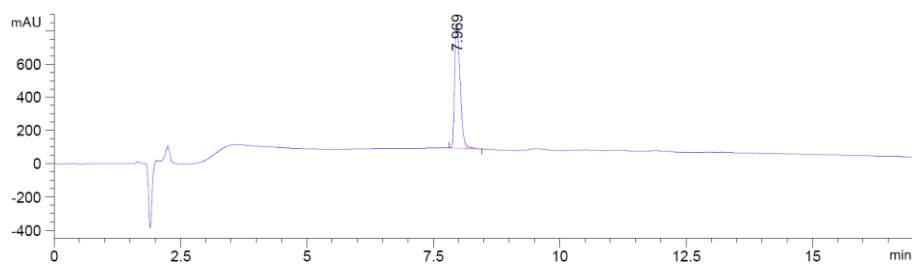
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.5	F1	6239.6	1753.8	1753.8

Purified F2 ($\lambda = 210$ nm; Method 7)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
7.6	F2	5219.3	1763.8	1763.7

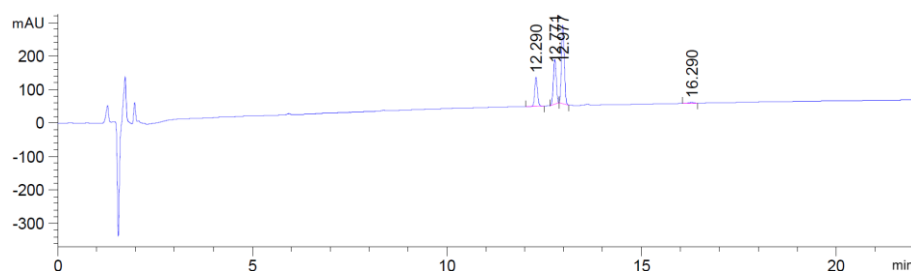
Purified F3 ($\lambda = 210$ nm; Method 7)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	F3	5662.1	1763.8	1763.7

Chromatograms related to Section S5.1.2.1 (Solvent and NaBH₃CN loading)

Entry 1, Table 2 ($\lambda = 210$ nm; Method 1)



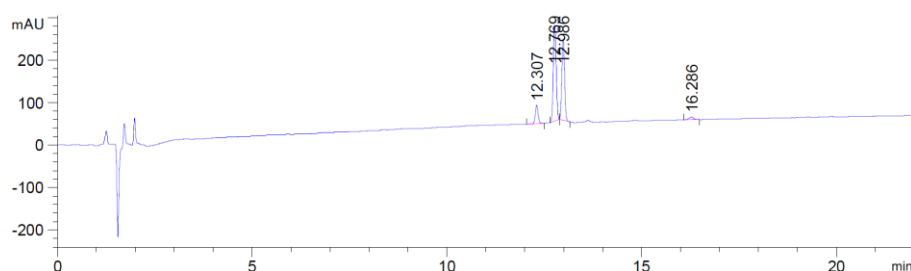
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	430.0	1079.6	1079.4
12.8 ^a	P1.1s	685.1	1061.6	1061.4
13.0	P1.1al	1190.8	1077.6	1077.4
16.3	—	24.6	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~93:7 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] $\times$ 100 = 30%

Entry 2, Table 2 ($\lambda = 210$ nm; Method 1)



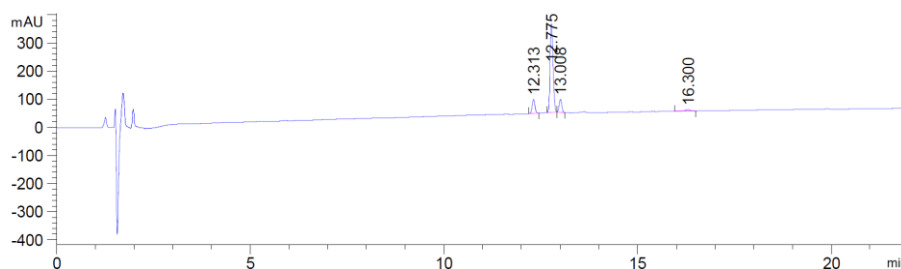
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	218.2	1079.6	1079.4
12.8 ^a	P1.1s	1166.5	1061.6	1061.4
13.0	P1.1al	950.6	1077.6	1077.4
16.3	—	53.1	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~95:5 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] × 100 = 50%

Entry 3, Table 2 ($\lambda = 210$ nm; Method 1)



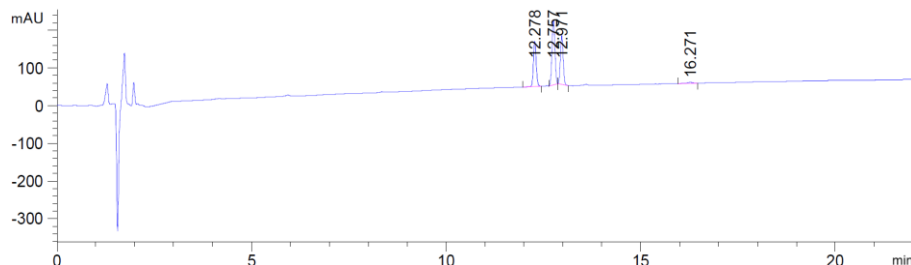
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	251.6	1079.6	1079.4
12.8 ^a	P1.1s	1693.5	1061.6	1061.4
13.0	P1.1al	219.3	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	47.6	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~97:3 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 78%

Entry 4, Table 2 ($\lambda = 210$ nm; Method 1)



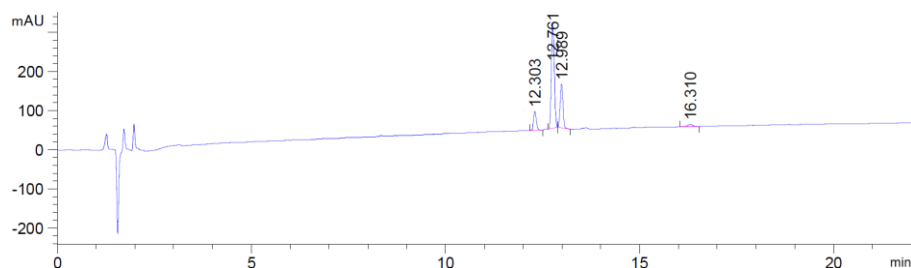
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	590.9	1079.6	1079.4
12.8 ^a	P1.1s	889.6	1061.6	1061.4
13.0	P1.1al	638.0	1077.6	1077.4
16.3	—	25.8	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s**/**P1.1ch** ratio ~95:5 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] $\times$ 100 = 42%

Entry 5, Table 2 ($\lambda = 210$ nm; Method 1)



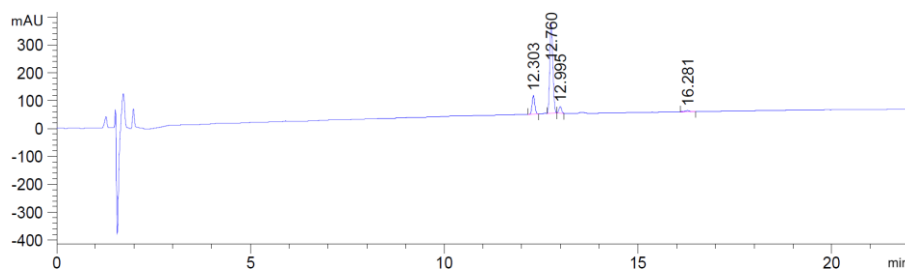
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	238.7	1079.6	1079.4
12.8 ^a	P1.1s	1435.4	1061.6	1061.4
13.0	P1.1al	553.7	1077.6	1077.4
16.3	—	55.2	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s**/ **P1.1ch** ratio ~96:4 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] × 100 = 64%

Entry 6, Table 2 ($\lambda = 210$ nm; Method 1)



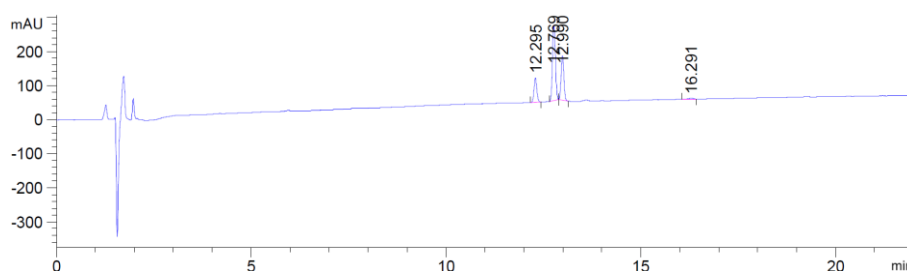
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	329.2	1079.6	1079.4
12.8	P1.1s	1762.5	1061.6	1061.4
13.0	P1.1al	108.0	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	33.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 80%

Chromatograms related to Section S5.1.2.2 (Methanol/Aqueous Buffer Volume Ratio)

Entry 1, Table 3 ($\lambda = 210$ nm; Method 1)



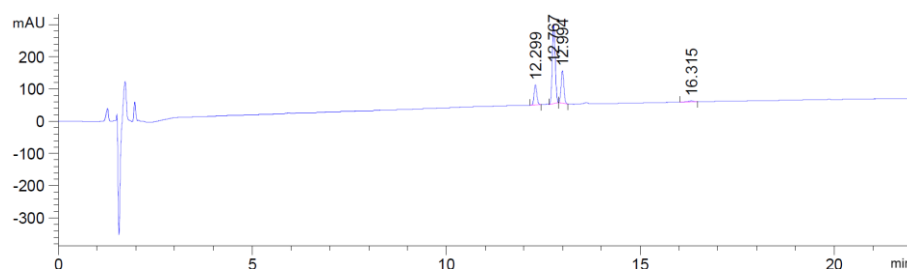
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	354.4	1079.6	1079.4
12.8 ^a	P1.1s	1154.6	1061.6	1061.4
13.0	P1.1al	636.6	1077.6	1077.4
16.3	—	25.0	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~95:5 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] $\times$ 100 = 54%

Entry 2, Table 3 ($\lambda = 210$ nm; Method 1)



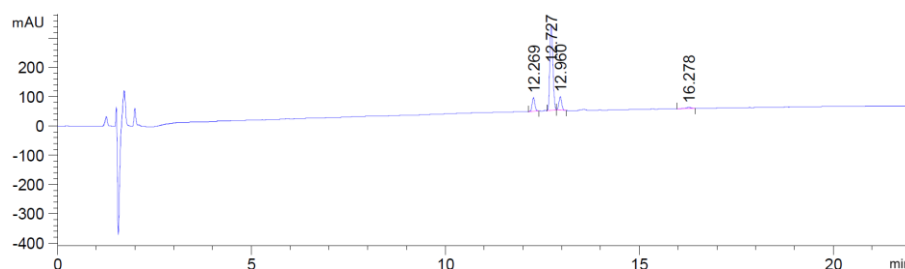
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	310.5	1079.6	1079.4
12.8 ^a	P1.1s	1302.5	1061.6	1061.4
13.0	P1.1al	496.2	1077.6	1077.3
16.3	—	34.7	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~96:4 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al**)] × 100 = 62%

Entry 3, Table 3 ($\lambda = 210$ nm; Method 1)



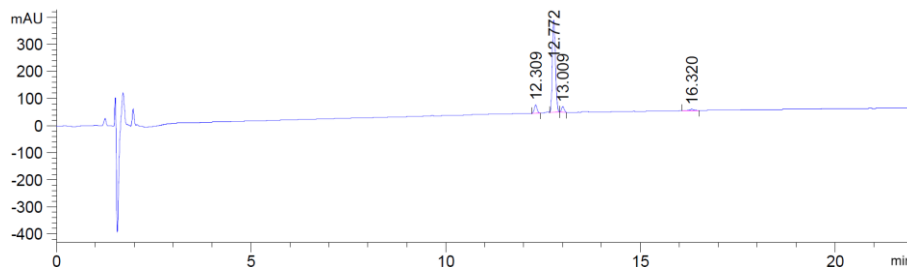
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	227.2	1079.6	1079.4
12.7 ^a	P1.1s	1569.5	1061.6	1061.4
13.0	P1.1al	221.2	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	38.1	— ^b	— ^b

^a Peak revealing a co-eluting byproduct **P1.1ch**, likely the cyanohydrin derived from **P1.1al** (m/z calcd for $[M+H]^+ = 1104.6$; found = 1104.4); estimated **P1.1s/P1.1ch** ratio ~97:3 based on EIC peak areas for **P1.1s** (m/z 1061.3–1061.5) and **P1.1ch** (m/z 1104.3–1104.5).

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 78%

Entry 4, Table 3 ($\lambda = 210$ nm; Method 1)

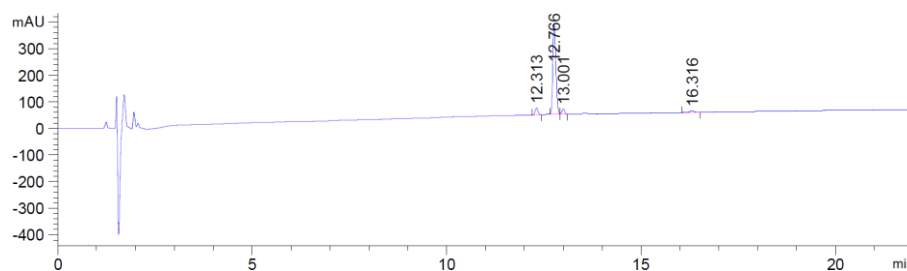


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	154.3	1079.6	1079.4
12.8	P1.1s	1858.3	1061.6	1061.4
13.0	P1.1al	93.8	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	41.8	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] $\times$ 100 = 88%

Entry 5, Table 3 ($\lambda = 210$ nm; Method 1)



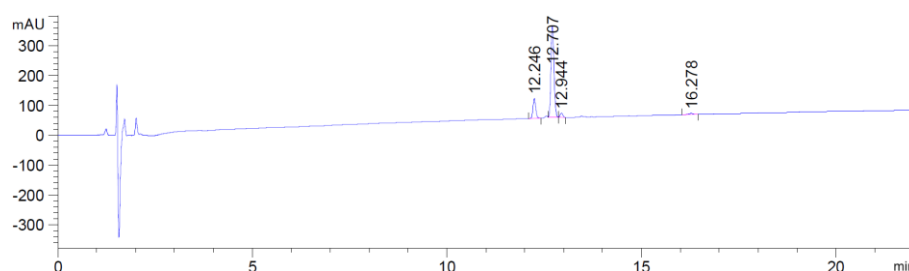
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	127.7	1079.6	1079.4
12.8	P1.1s	1857.4	1061.6	1061.4
13.0	P1.1al	85.0	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	46.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1al + P1.1Nm)] × 100 = 90%

Chromatograms related to Section S5.1.2.3 (pH of the Aqueous Buffer)

Entry 1, Table 4 (λ = 210 nm; Method 1)

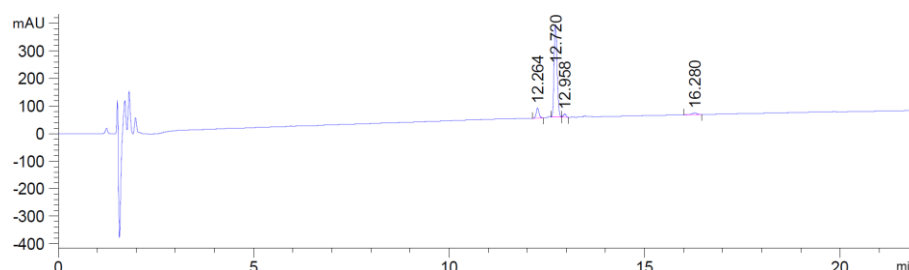


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.2	P1.1ol	323.3	1079.6	1079.4
12.7	P1.1s	1625.1	1061.6	1061.4
12.9	P1.1al	63.0	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	35.8	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1al + P1.1Nm)] × 100 = 81%

Entry 2, Table 4 (λ = 210 nm; Method 1)

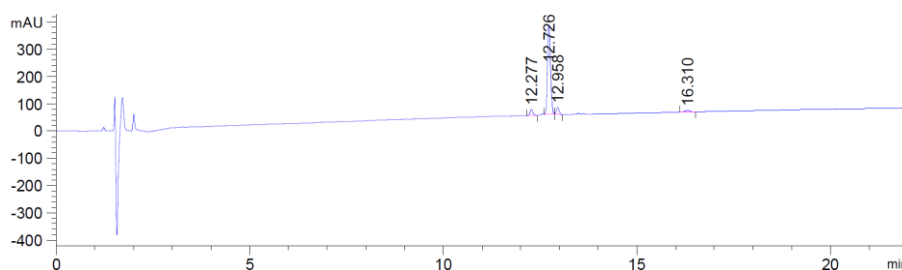


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	179.7	1079.6	1079.4
12.7	P1.1s	1802.9	1061.6	1061.4
13.0	P1.1al	47.8	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	50.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 89%

Entry 3, Table 4 (λ = 210 nm; Method 1)

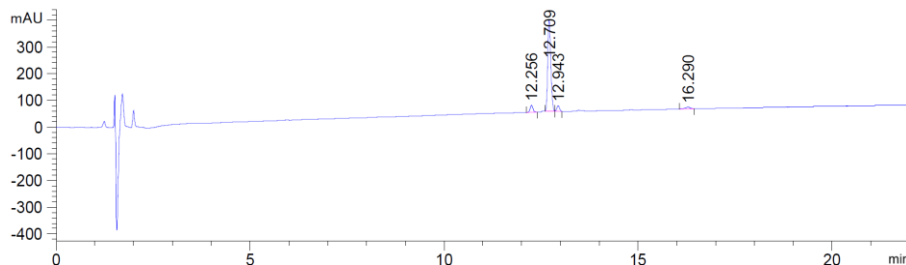


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	109.7	1079.6	1079.4
12.7	P1.1s	1765.5	1061.6	1061.4
13.0	P1.1al	121.1	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	50.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 88%

Entry 4, Table 4 ($\lambda = 210$ nm; Method 1)

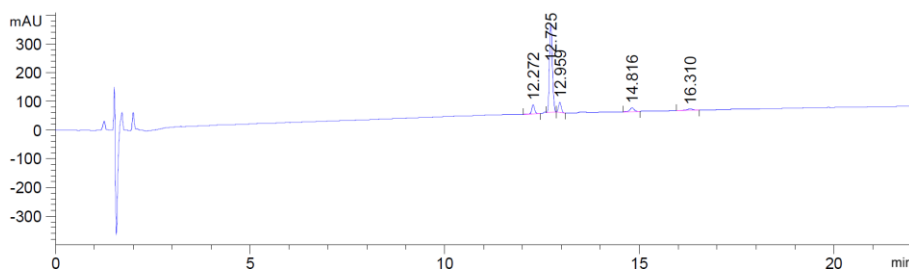


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	131.3	1079.6	1079.4
12.7	P1.1s	1854.0	1061.6	1061.4
12.9	P1.1al	91.4	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.3	—	53.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] $\times$ 100 = 89%

Entry 5, Table 4 ($\lambda = 210$ nm; Method 1)

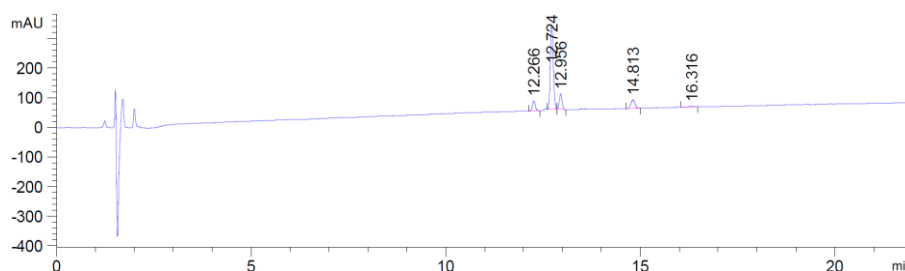


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	164.5	1079.6	1079.4
12.7	P1.1s	1650.5	1061.6	1061.4
13.0	P1.1al	174.1	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
14.8	P1.1an	97.7	1086.6	1086.4
16.3	—	49.3	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm** + **P1.1an**)] × 100 = 79%

Entry 6, Table 4 (λ = 210 nm; Method 1)

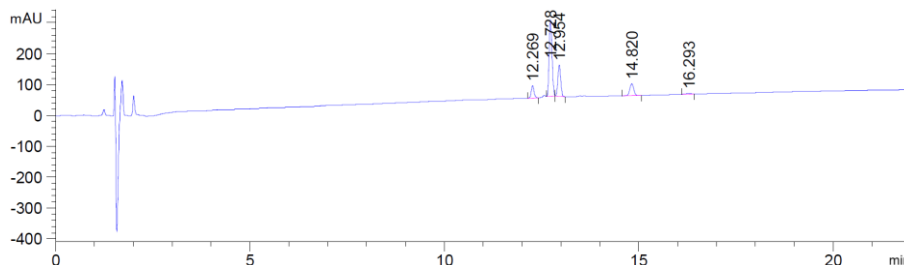


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	172.0	1079.6	1079.4
12.7	P1.1s	1497.7	1061.6	1061.4
13.0	P1.1al	250.6	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
14.8	P1.1an	204.1	1086.6	1086.4
16.3	—	31.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm** + **P1.1an**)] × 100 = 70%

Entry 7, Table 4 ($\lambda = 210$ nm; Method 1)

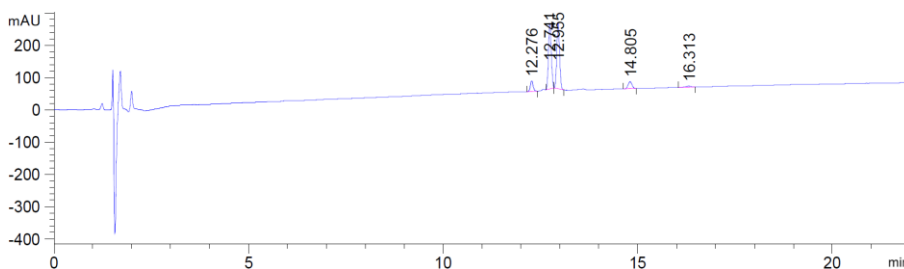


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	196.7	1079.6	1079.4
12.7	P1.1s	1267.5	1061.6	1061.4
13.0	P1.1al	506.2	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
14.8	P1.1an	269.5	1086.6	1086.4
16.3	—	20.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm** + **P1.1an**)] $\times$ 100 = 57%

Entry 8, Table 4 ($\lambda = 210$ nm; Method 1)



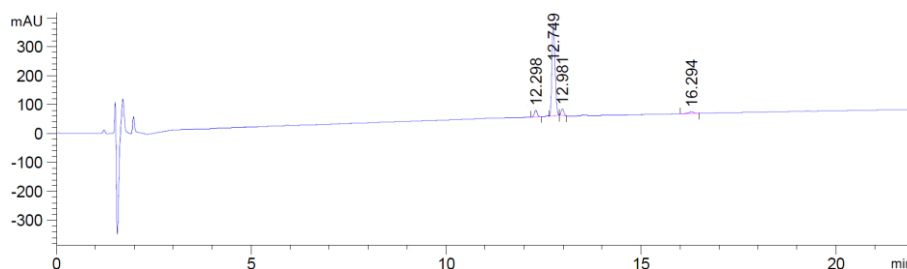
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	162.2	1079.6	1079.4
12.7	P1.1s	1024.2	1061.6	1061.4
13.0	P1.1al	1031.8	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
14.8	P1.1an	150.9	1086.6	1086.4
16.3	—	30.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm** + **P1.1an**)] × 100 = 43%

Chromatograms related to Section S5.1.2.4 (*Nature of the Aqueous Buffer*)

Entry 1, Table 5 (λ = 210 nm; Method 1)

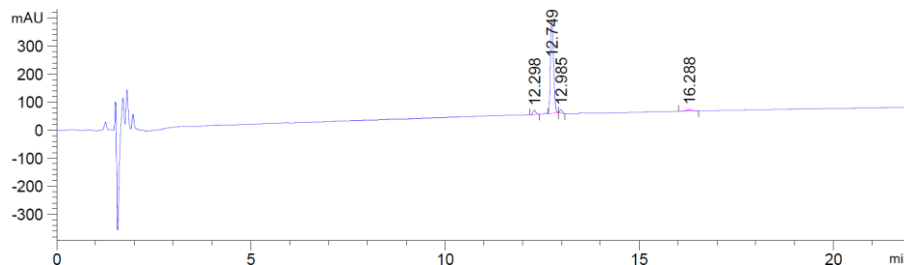


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	121.2	1079.6	1079.4
12.7	P1.1s	1763.3	1061.6	1061.4
13.0	P1.1al	110.5	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	52.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 88%

Entry 2, Table 5 ($\lambda = 210$ nm; Method 1)

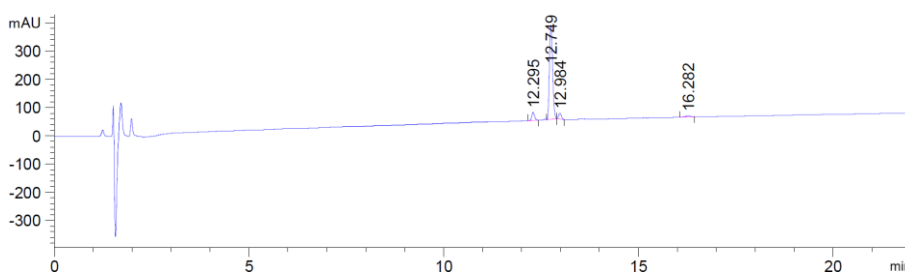


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	81.7	1079.6	1079.4
12.7	P1.1s	1863.4	1061.6	1061.4
13.0	P1.1al	59.7	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.3	—	63.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] $\times$ 100 = 93%

Entry 3, Table 5 ($\lambda = 210$ nm; Method 1)

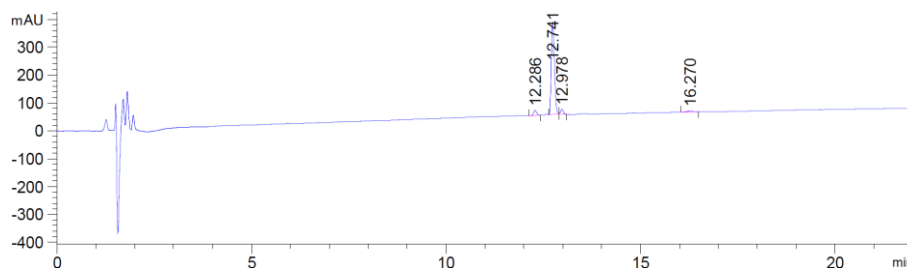


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	148.8	1079.6	1079.4
12.7	P1.1s	1860.2	1061.6	1061.4
13.0	P1.1al	99.1	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.3	—	45.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 88%

Entry 4, Table 5 (λ = 210 nm; Method 1)



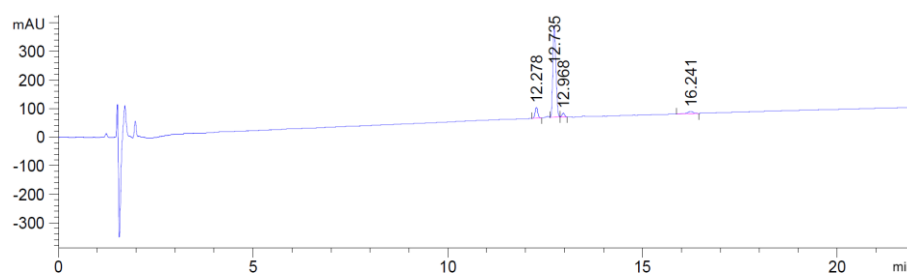
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	99.9	1079.6	1079.4
12.7	P1.1s	1841.8	1061.6	1061.4
13.0	P1.1al	79.9	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.3	—	49.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 91%

Chromatograms related to Section S5.1.2.5 (*Buffer Concentration*)

Entry 1, Table 6 ($\lambda = 210$ nm; Method 1)

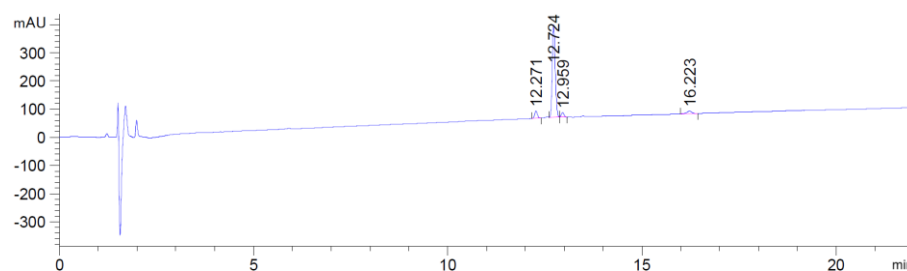


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	188.5	1079.6	1079.4
12.7	P1.1s	1726.5	1061.6	1061.4
13.0	P1.1al	60.1	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.2	—	75.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] $\times$ 100 = 87%

Entry 2, Table 6 ($\lambda = 210$ nm; Method 1)

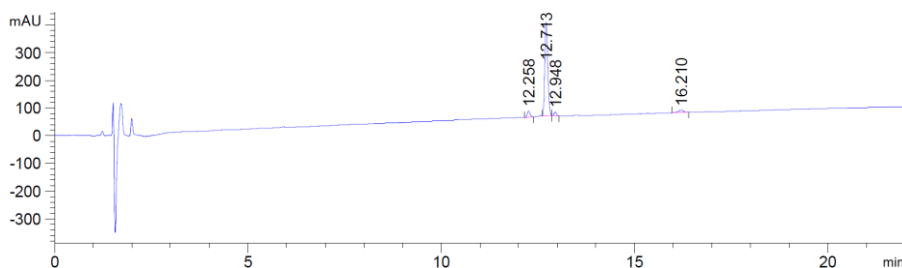


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	130.3	1079.6	1079.4
12.7	P1.1s	1766.9	1061.6	1061.4
13.0	P1.1al	74.2	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.2	—	84.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 90%

Entry 3, Table 6 (λ = 210 nm; Method 1)

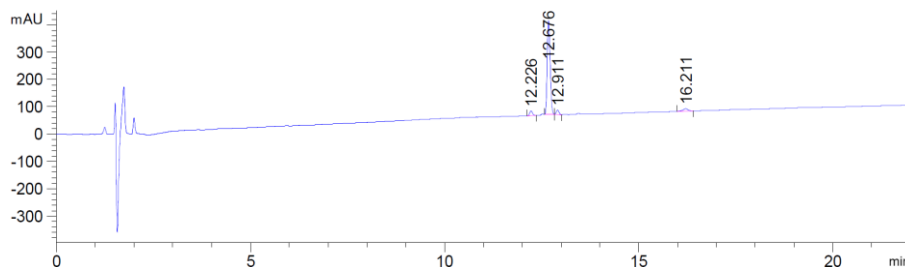


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	107.3	1079.6	1079.3
12.7	P1.1s	1824.7	1061.6	1061.4
12.9	P1.1al	63.4	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.2	—	85.5	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 91%

Entry 4, Table 6 ($\lambda = 210$ nm; Method 1)

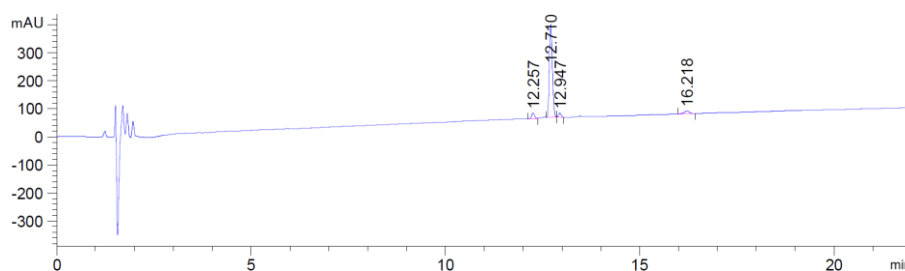


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.2	P1.1ol	88.5	1079.6	1079.4
12.7	P1.1s	1846.3	1061.6	1061.4
12.9	P1.1al	77.8	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.2	—	81.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] $\times$ 100 = 92%

Entry 5, Table 6 ($\lambda = 210$ nm; Method 1)

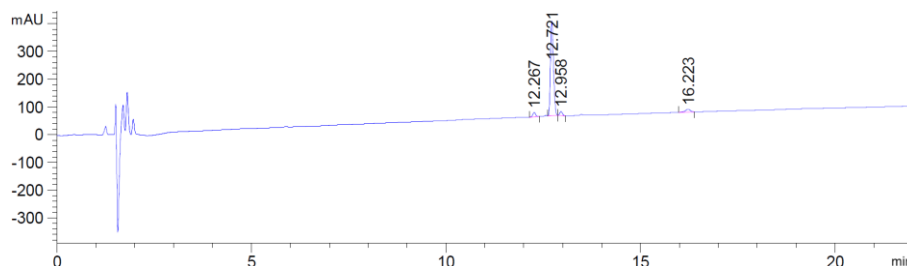


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	94.5	1079.6	1079.4
12.7	P1.1s	1794.3	1061.6	1061.4
12.9	P1.1al	63.7	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.2	—	85.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1al + P1.1Nm)] × 100 = 92%

Entry 6, Table 6 (λ = 210 nm; Method 1)

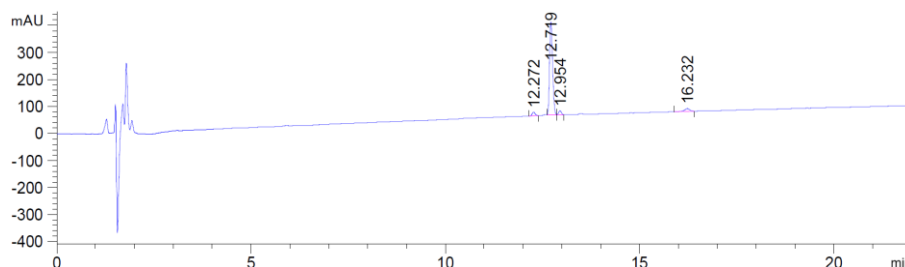


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	79.7	1079.6	1079.4
12.7	P1.1s	1838.6	1061.6	1061.4
13.0	P1.1al	68.0	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.2	—	87.8	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1al + P1.1Nm)] × 100 = 93%

Entry 7, Table 6 (λ = 210 nm; Method 1)

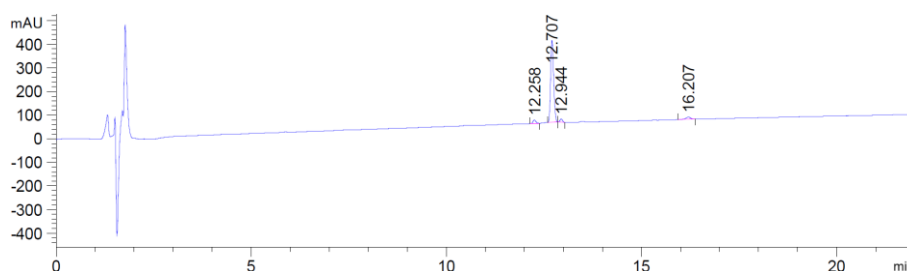


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	72.4	1079.6	1079.4
12.7	P1.1s	1838.4	1061.6	1061.3
13.0	P1.1al	68.0	1077.6	1077.4
	P1.1Nm		1075.6	1075.4
16.2	—	95.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 93%

Entry 8, Table 6 (λ = 210 nm; Method 1)



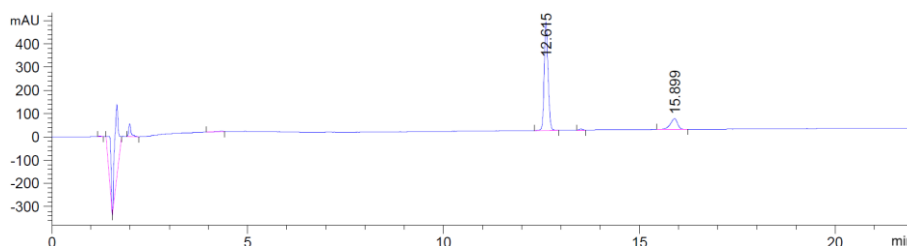
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1ol	72.1	1079.6	1079.4
12.7	P1.1s	1872.9	1061.6	1061.4
12.9	P1.1al	64.6	1077.6	1077.3
	P1.1Nm		1075.6	1075.4
16.2	—	87.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1al** + **P1.1Nm**)] × 100 = 93%

Chromatograms related to Section S5.1.3 (*Optimization of the One-Pot S-Alkylation/Reductive Amination Protocol*)

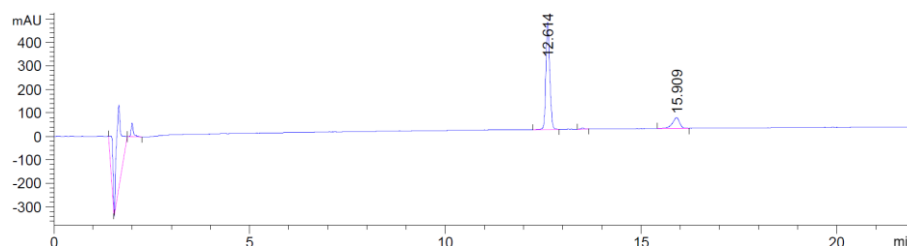
Entry A ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.6	P1.1al	3216.9	1077.6	1077.3
15.9	1	595.6	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

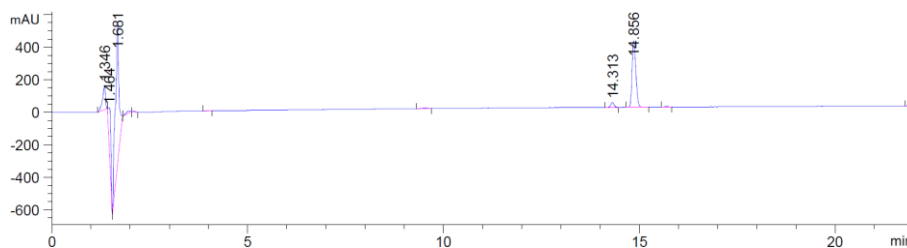
Entry A bis ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.6	P1.1al	3142.2	1077.6	1077.3
15.9	1	587.3	— ^a	— ^a

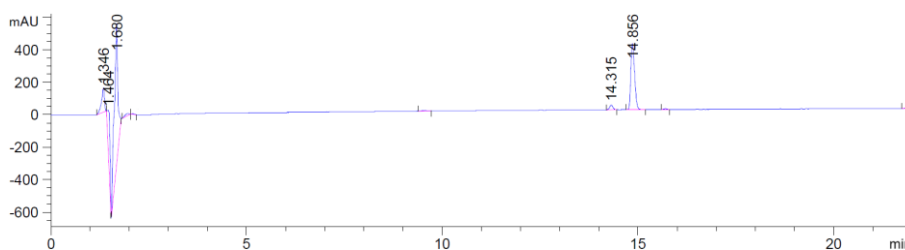
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry O ($\lambda = 210$ nm; Method 2)



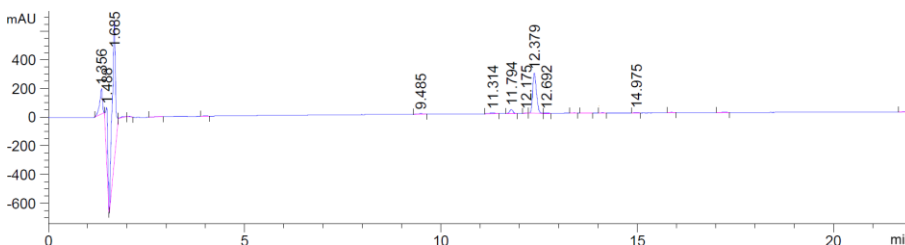
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.3	P1.1ox_b	169.0	1106.6	1106.4
14.9	P1.1ox_a	2690.7	1106.6	1106.4

Entry O bis ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.3	P1.1ox_b	167.2	1106.6	1106.4
14.9	P1.1ox_a	2683.4	1106.6	1106.4

Entry 1, Table S1 ($\lambda = 210$ nm; Method 2)

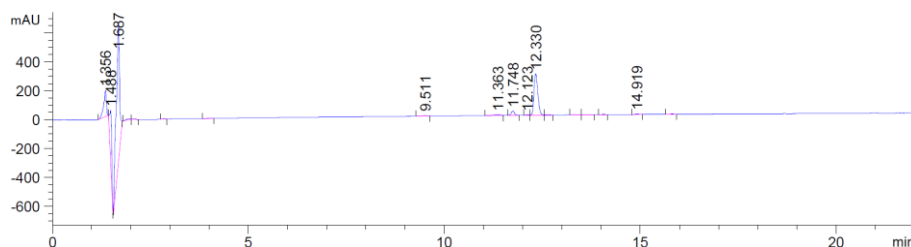


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	28.5	— ^a	— ^a
11.3	—	50.0	— ^a	— ^a
11.8	P1.1ol	159.7	1079.6	1079.4
12.4	P1.1s	1883.7	1061.6	1061.4
15.0	P1.1ox_a	21.8	1106.6	1106.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1ox_a**)] × 100 = 91%

Entry 1 bis, Table S1 ($\lambda = 210$ nm; Method 2)

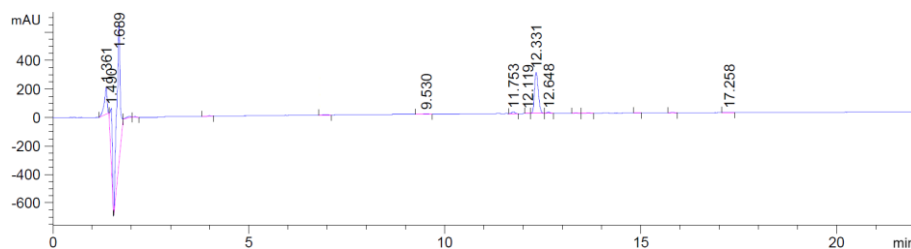


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	30.7	— ^a	— ^a
11.4	—	54.1	— ^a	— ^a
11.7	P1.1ol	167.4	1079.6	1079.3
12.3	P1.1s	1921.6	1061.6	1061.4
14.9	P1.1ox_a	25.1	1106.6	1106.3

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = $[P1.1s / (P1.1ol + P1.1s + P1.1ox_a)] \times 100 = 91\%$

Entry 2, Table S1 ($\lambda = 210$ nm; Method 2)

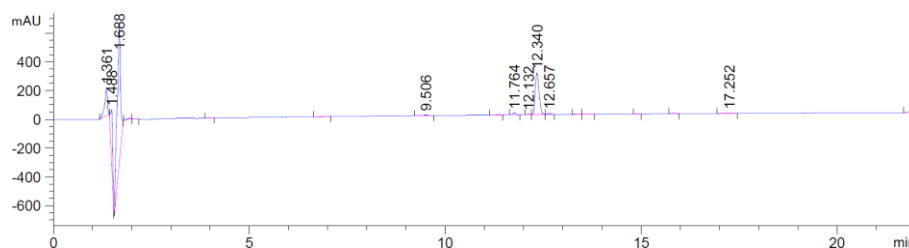


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	37.3	— ^a	— ^a
11.8	P1.1ol	68.1	1079.6	1079.3
12.3	P1.1s	1941.9	1061.6	1061.3
12.6	P1.1Nm	49.0	1075.6	1075.4
17.3	—	21.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] × 100 = 94%

Entry 2 bis, Table S1 (λ = 210 nm; Method 2)

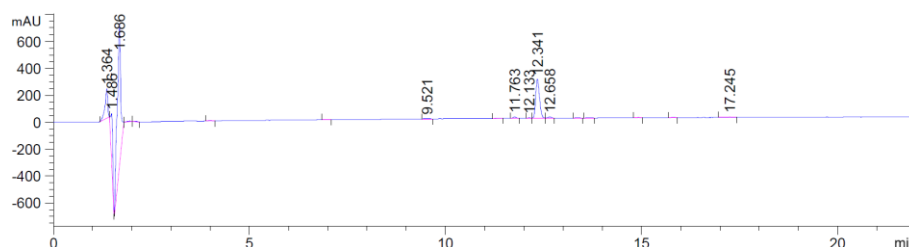


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	36.9	— ^a	— ^a
11.8	P1.1ol	70.9	1079.6	1079.4
12.3	P1.1s	1947.7	1061.6	1061.4
12.7	P1.1Nm	47.4	1075.6	1075.4
17.3	—	32.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] × 100 = 94%

Entry 3, Table S1 (λ = 210 nm; Method 2)

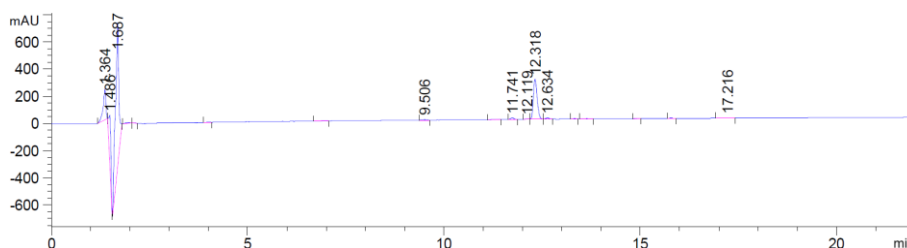


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	29.1	— ^a	— ^a
11.8	P1.1ol	59.6	1079.6	1079.4
12.3	P1.1s	1937.2	1061.6	1061.4
12.7	P1.1Nm	48.2	1075.6	1075.4
17.2	—	30.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] × 100 = 95%

Entry 3 bis, Table S1 (λ = 210 nm; Method 2)

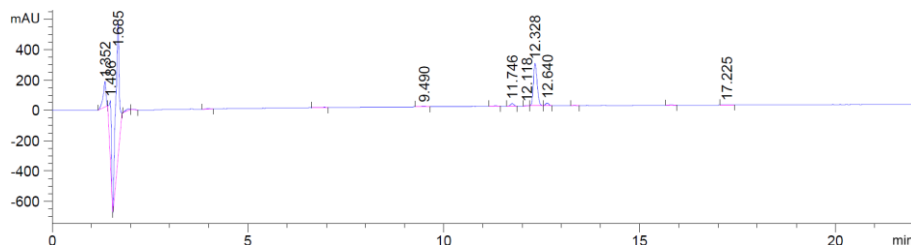


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	30.4	— ^a	— ^a
11.7	P1.1ol	61.3	1079.6	1079.4
12.3	P1.1s	1950.7	1061.6	1061.4
12.6	P1.1Nm	46.2	1075.6	1075.4
17.2	—	30.5	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] × 100 = 95%

Entry 4, Table S1 ($\lambda = 210$ nm; Method 2)

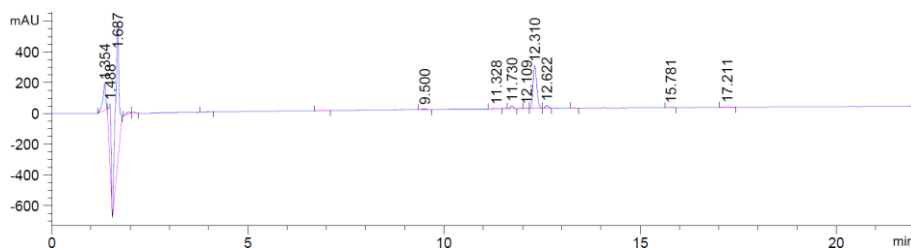


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	32.9	— ^a	— ^a
11.7	P1.1ol	97.5	1079.6	1079.4
12.3	P1.1s	1854.4	1061.6	1061.4
12.6	P1.1Nm	104.1	1075.6	1075.4
17.2	—	23.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] $\times$ 100 = 90%

Entry 4 bis, Table S1 ($\lambda = 210$ nm; Method 2)

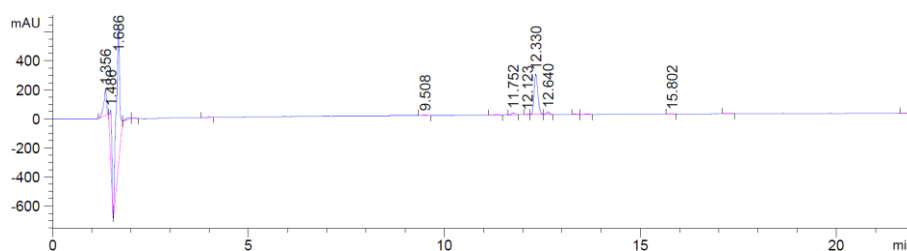


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	31.1	— ^a	— ^a
11.3	—	22.8	— ^a	— ^a
11.7	P1.1ol	97.9	1079.6	1079.4
12.3	P1.1s	1838.6	1061.6	1061.4
12.6	P1.1Nm	108.5	1075.6	1075.4
15.8	P1.1Nb	21.7	1243.5	1243.4
17.2	—	26.7	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 89%

Entry 5, Table S1 (λ = 210 nm; Method 2)

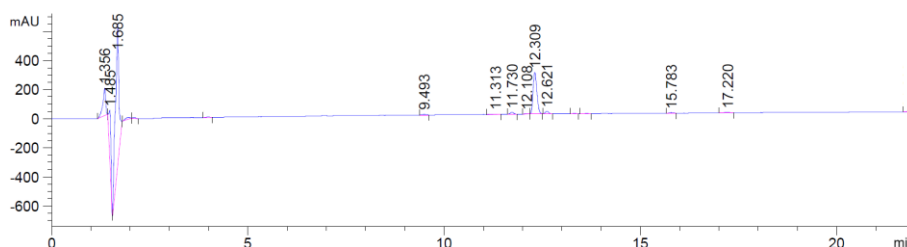


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	28.2	— ^a	— ^a
11.8	P1.1ol	70.4	1079.6	1079.3
12.3	P1.1s	1850.7	1061.6	1061.4
12.6	P1.1Nm	95.1	1075.6	1075.4
15.8	P1.1Nb	21.0	1243.5	1243.3

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 91%

Entry 5 bis, Table S1 (λ = 210 nm; Method 2)

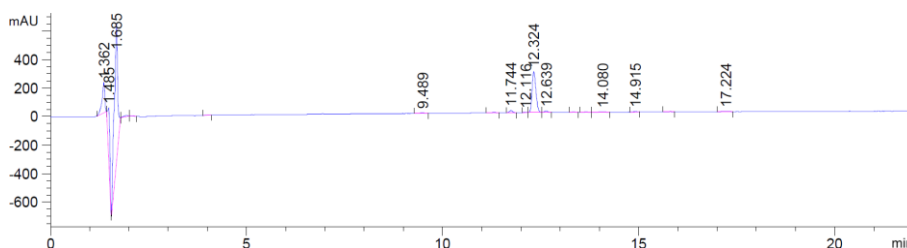


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	27.6	— ^a	— ^a
11.3	—	21.7	— ^a	— ^a
11.7	P1.1ol	72.9	1079.6	1079.4
12.3	P1.1s	1896.5	1061.6	1061.4
12.6	P1.1Nm	98.3	1075.6	1075.4
15.8	P1.1Nb	23.2	1243.5	1243.3
17.2	—	23.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 91%

Entry 6, Table S1 (λ = 210 nm; Method 2)

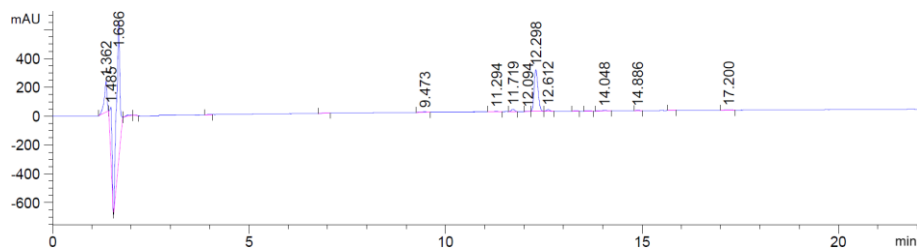


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	29.6	— ^a	— ^a
11.7	P1.1ol	83.7	1079.6	1079.3
12.3	P1.1s	1906.1	1061.6	1061.4
12.6	P1.1Nm	60.8	1075.6	1075.4
14.1	—	40.9	— ^a	— ^a
14.9	P1.1ox_a	22.6	1106.6	1106.4
17.2	—	25.8	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1ox_a**)] × 100 = 92%

Entry 6 bis, Table S1 ($\lambda = 210$ nm; Method 2)

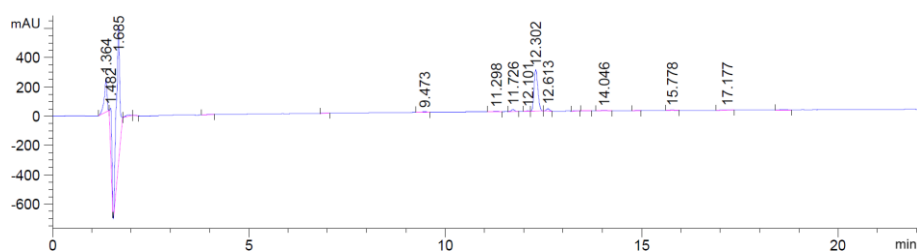


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	31.8	— ^a	— ^a
11.3	—	23.1	— ^a	— ^a
11.7	P1.1ol	85.6	1079.6	1079.3
12.3	P1.1s	1927.9	1061.6	1061.4
12.6	P1.1Nm	62.6	1075.6	1075.4
14.0	—	41.2	— ^a	— ^a
14.9	P1.1ox_a	16.6	1106.6	1106.4
17.2	—	25.3	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1ox_a**)] $\times$ 100 = 92%

Entry 7, Table S1 ($\lambda = 210$ nm; Method 2)

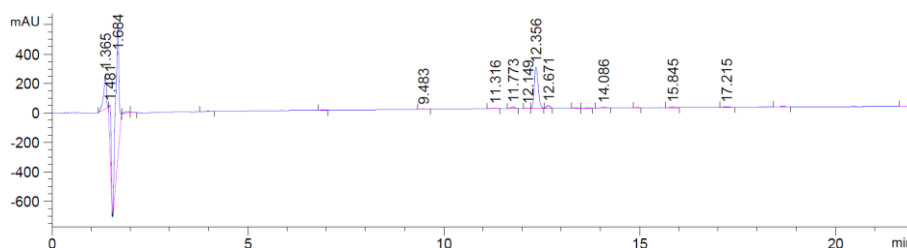


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	30.2	— ^a	— ^a
11.3	—	22.3	— ^a	— ^a
11.7	P1.1ol	74.8	1079.6	1079.4
12.3	P1.1s	1887.1	1061.6	1061.4
12.6	P1.1Nm	86.7	1075.6	1075.4
14.0	—	44.9	— ^a	— ^a
15.8	P1.1Nb	25.4	1243.5	1243.3
17.2	—	28.5	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 91%

Entry 7 bis, Table S1 (λ = 210 nm; Method 2)

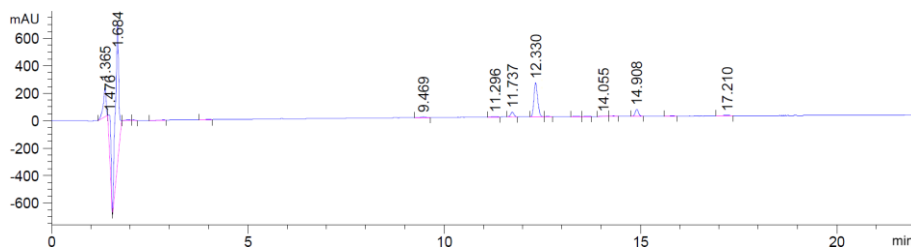


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	24.5	— ^a	— ^a
11.3	—	24.6	— ^a	— ^a
11.8	P1.1ol	71.7	1079.6	1079.4
12.4	P1.1s	1880.2	1061.6	1061.4
12.7	P1.1Nm	100.7	1075.6	1075.4
14.1	—	53.1	— ^a	— ^a
15.8	P1.1Nb	31.4	1243.5	1243.3
17.2	—	26.7	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 90%

Entry 8, Table S1 ($\lambda = 210$ nm; Method 2)

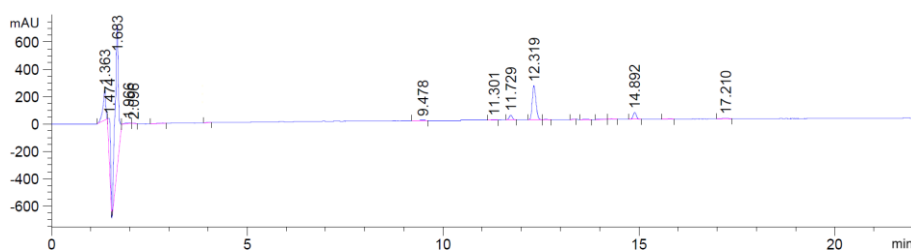


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	44.2	— ^a	— ^a
11.3	—	30.3	— ^a	— ^a
11.7	P1.1ol	191.0	1079.6	1079.4
12.3	P1.1s	1614.1	1061.6	1061.4
14.1	x (not identified)	22.3	—	1123.4
14.9	P1.1ox_a	291.7	1106.6	1106.4
17.2	—	48.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **x** + **P1.1ox_a**)] $\times$ 100 = 76%

Entry 8 bis, Table S1 ($\lambda = 210$ nm; Method 2)

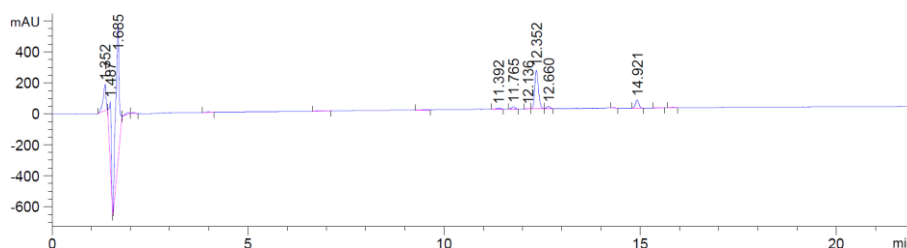


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	41.0	— ^a	— ^a
11.3	—	31.6	— ^a	— ^a
11.7	P1.1ol	187.0	1079.6	1079.4
12.3	P1.1s	1619.2	1061.6	1061.4
14.9	P1.1ox_a	289.7	1106.6	1106.4
17.2	—	47.5	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1ox_a**)] × 100 = 77%

Entry 9, Table S1 (λ = 210 nm; Method 2)



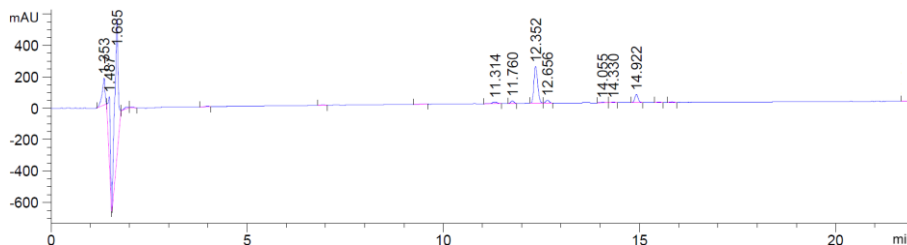
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.4	—	39.9	— ^b	— ^b
11.8	P1.1ol	81.0	1079.6	1079.4
12.4	P1.1s	1615.3	1061.6	1061.4
12.7	P1.1Nm	80.7	1075.6	1075.4
13.6	P1.1di	22.5	1061.6 ($[M+2H]^{2+}$)	1061.6 ($[M+2H]^{2+}$)
14.9	P1.1ox_a	298.5	1106.6	1106.4

^a $[M+H]^+$ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1di** + **P1.1ox_a**)] × 100 = 77%

Entry 9 bis, Table S1 ($\lambda = 210$ nm; Method 2)



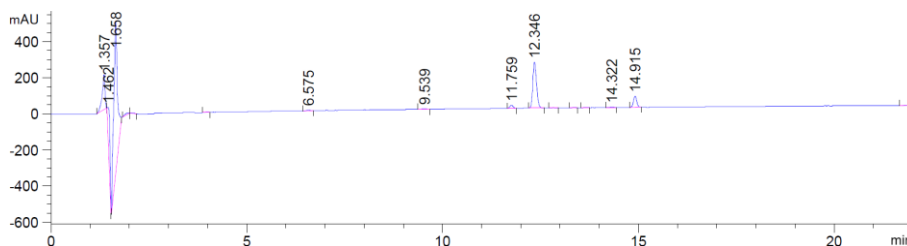
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.3	—	85.1	— ^b	— ^b
11.8	P1.1ol	87.4	1079.6	1079.4
12.4	P1.1s	1516.0	1061.6	1061.4
12.7	P1.1Nm	106.4	1075.6	1075.4
13.6	P1.1di	27.6	1061.6 ([M+2H] ²⁺)	1061.6 ([M+2H] ²⁺)
14.1	—	22.3	— ^b	— ^b
14.3	P1.1ox_b	28.2	1106.6	1106.4
14.9	P1.1ox_a	302.3	1106.6	1106.4

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1di** + **P1.1ox_b** + **P1.1ox_a**)]
 × 100 = 73%

Entry 10, Table S1 ($\lambda = 210$ nm; Method 2)

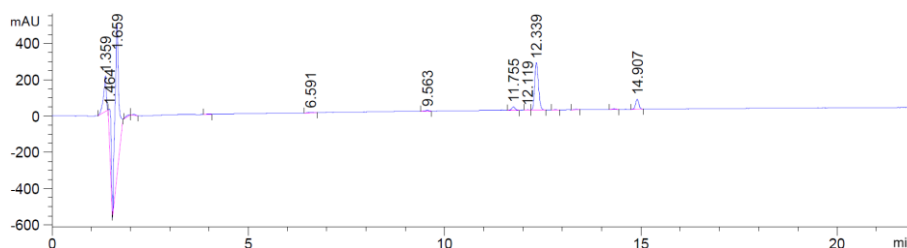


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
6.6	P1.1da	25.6	1078.6	1078.4
9.5	—	24.2	— ^a	— ^a
11.8	P1.1ol	98.4	1079.6	1079.4
12.3	P1.1s	1680.2	1061.6	1061.4
14.3	P1.1ox_b	22.9	1106.6	1106.4
14.9	P1.1ox_a	355.3	1106.6	1106.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1da** + **P1.1ol** + **P1.1s** + **P1.1ox_b** + **P1.1ox_a**)] × 100 = 77%

Entry 10 bis, Table S1 (λ = 210 nm; Method 2)

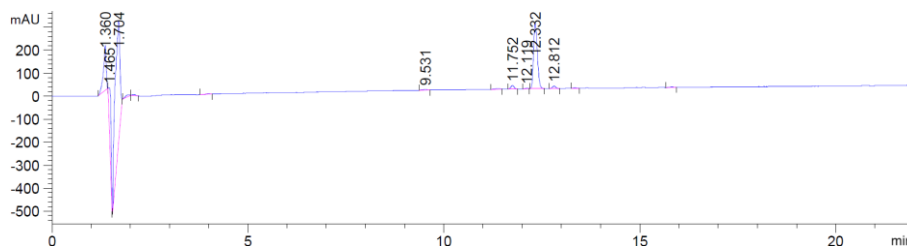


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
6.6	P1.1da	30.7	1078.6	1078.3
9.6	—	24.8	— ^a	— ^a
11.8	P1.1ol	105.8	1079.6	1079.4
12.3	P1.1s	1736.6	1061.6	1061.4
14.9	P1.1ox_a	311.3	1106.6	1106.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1da** + **P1.1ol** + **P1.1s** + **P1.1ox_a**)] × 100 = 79%

Entry 11, Table S1 ($\lambda = 210$ nm; Method 2)

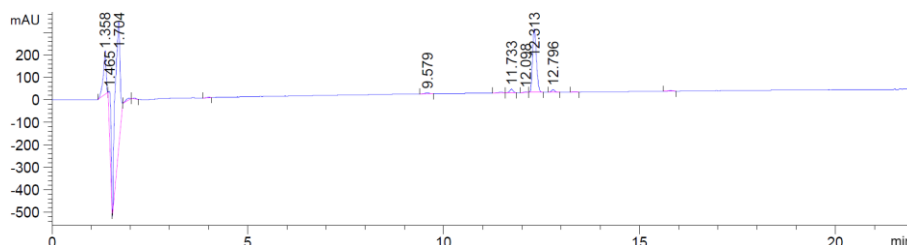


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	26.5	— ^a	— ^a
11.8	P1.1ol	83.5	1079.6	1079.4
12.3	P1.1s	1921.2	1061.6	1061.4
12.8	P1.1Ne	67.0	1089.6	1089.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Ne**)] $\times$ 100 = 93%

Entry 11 bis, Table S1 ($\lambda = 210$ nm; Method 2)

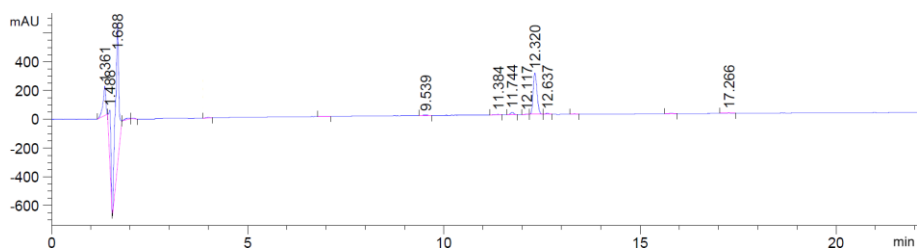


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	—	34.3	— ^a	— ^a
11.7	P1.1ol	89.4	1079.6	1079.4
12.3	P1.1s	1898.1	1061.6	1061.3
12.8	P1.1Ne	68.9	1089.6	1089.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1Nm)] × 100 = 92%

Entry 12, Table S1 (λ = 210 nm; Method 2)

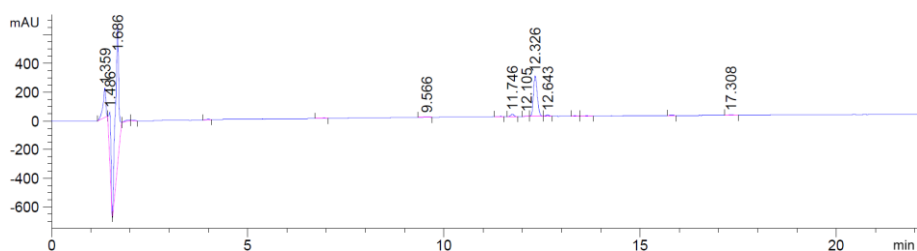


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	24.9	— ^a	— ^a
11.4	—	22.1	— ^a	— ^a
11.7	P1.1ol	93.1	1079.6	1079.4
12.3	P1.1s	1934.7	1061.6	1061.4
12.6	P1.1Nm	45.9	1075.6	1075.4
17.3	—	24.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1Nm)] × 100 = 93%

Entry 12 bis, Table S1 (λ = 210 nm; Method 2)

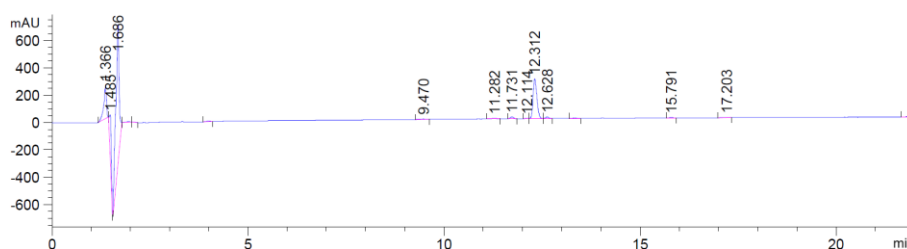


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	—	30.2	— ^a	— ^a
11.7	P1.1ol	89.9	1079.6	1079.3
12.3	P1.1s	1869.4	1061.6	1061.4
12.6	P1.1Nm	49.0	1075.6	1075.4
17.3	—	20.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm**)] × 100 = 93%

Entry 13, Table S1 (λ = 210 nm; Method 2)

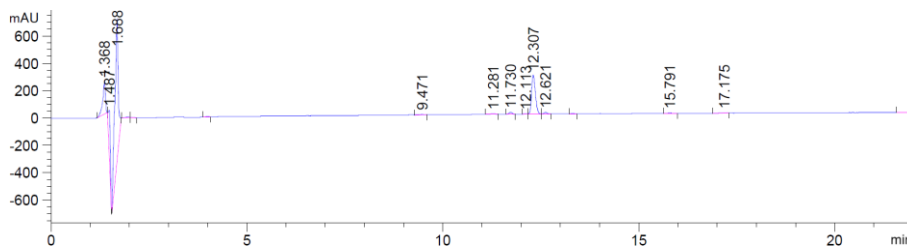


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	26.4	— ^a	— ^a
11.3	—	37.2	— ^a	— ^a
11.7	P1.1ol	79.6	1079.6	1079.4
12.3	P1.1s	1926.4	1061.6	1061.4
12.6	P1.1Nm	59.4	1075.6	1075.4
15.8	P1.1Nb	23.9	1243.5	1243.2
17.2	—	27.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 92%

Entry 13 bis, Table S1 ($\lambda = 210$ nm; Method 2)

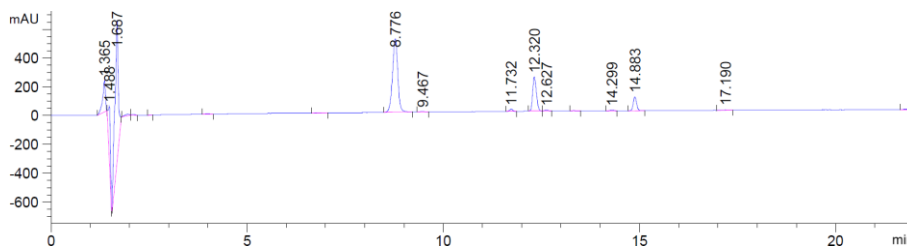


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	24.1	— ^a	— ^a
11.3	—	32.0	— ^a	— ^a
11.7	P1.1ol	76.3	1079.6	1079.3
12.3	P1.1s	1889.8	1061.6	1061.3
12.6	P1.1Nm	59.9	1075.6	1075.4
15.8	P1.1Nb	24.0	1243.5	1243.2
17.2	—	23.3	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] $\times$ 100 = 92%

Entry 14, Table S1 ($\lambda = 210$ nm; Method 2)



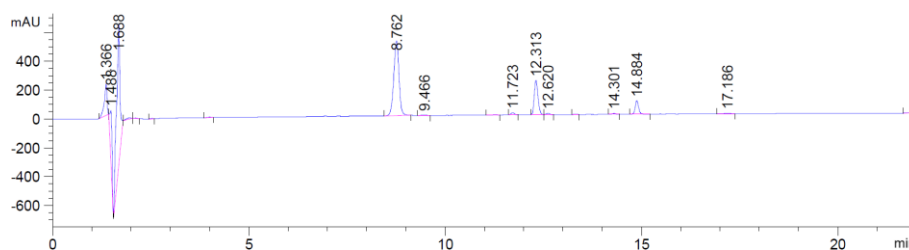
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
8.8	pic-BH ₃	4747.9	106.1 ([M-H] ⁺)	106.1 ([M-H] ⁺)
9.5	—	27.3	— ^b	— ^b
11.7	P1.1ol	85.5	1079.6	1079.4
12.3	P1.1s	1544.5	1061.6	1061.3
12.6	P1.1Nm	47.0	1075.6	1075.4
14.3	P1.1ox_b	36.7	1106.6	1106.4
14.9	P1.1ox_a	578.9	1106.6	1106.4
17.2	—	32.5	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1Nm + P1.1ox_b + P1.1ox_a)] × 100 = 67%

Entry 14 bis, Table S1 (λ = 210 nm; Method 2)



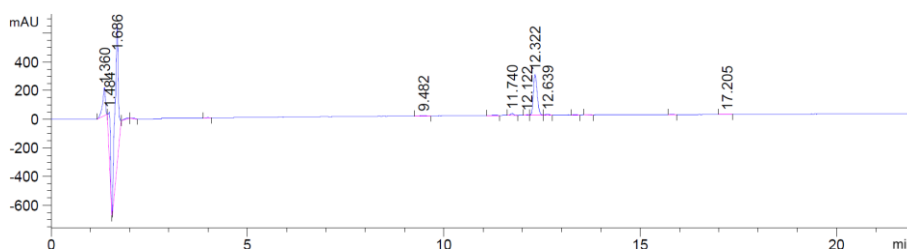
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
8.8	pic-BH ₃	4790.0	106.1 ([M-H] ⁺)	106.1 ([M-H] ⁺)
9.5	—	26.5	— ^b	— ^b
11.7	P1.1ol	82.8	1079.6	1079.4
12.3	P1.1s	1547.4	1061.6	1061.3
12.6	P1.1Nm	52.4	1075.6	1075.4
14.3	P1.1ox_b	36.1	1106.6	1106.4
14.9	P1.1ox_a	567.1	1106.6	1106.4
17.2	—	36.8	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1Nm + P1.1ox_b + P1.1ox_a)] × 100 = 68%

Entry 15, Table S1 (λ = 210 nm; Method 2)

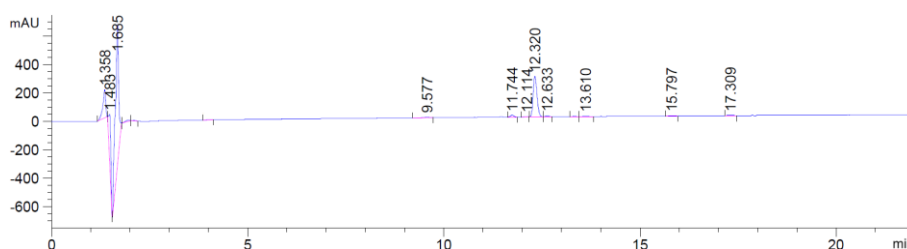


<i>t_R</i> (min)	Assigned compound	Absorbance area (mAU·s)	<i>m/z</i> calcd for [M+H] ⁺	<i>m/z</i> found for [M+H] ⁺
9.5	—	34.2	— ^a	— ^a
11.7	P1.1ol	72.7	1079.6	1079.4
12.3	P1.1s	1889.1	1061.6	1061.4
12.6	P1.1Nm	41.1	1075.6	1075.4
17.2	—	23.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1.1Nm)] × 100 = 94%

Entry 15 bis, Table S1 (λ = 210 nm; Method 2)



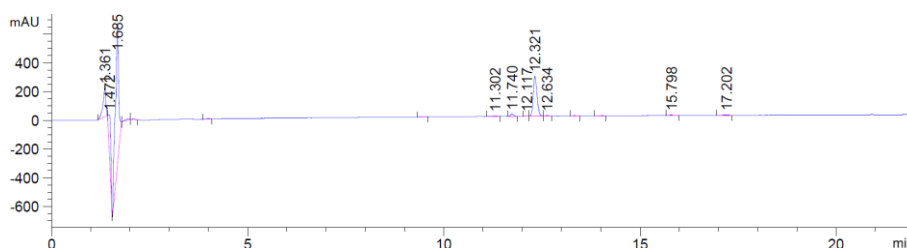
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.6	—	40.4	— ^b	— ^b
11.7	P1.1ol	73.2	1079.6	1079.4
12.3	P1.1s	1905.0	1061.6	1061.4
12.6	P1.1Nm	39.3	1075.6	1075.4
13.6	P1.1di	22.8	1061.6 ([M+2H] ²⁺)	1061.9 ([M+2H] ²⁺)
15.8	P1.1Nb	21.5	1243.5	1243.3
17.3	—	20.7	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1di** + **P1.1Nb**)] × 100 = 92%

Entry 16, Table S1 (λ = 210 nm; Method 2)

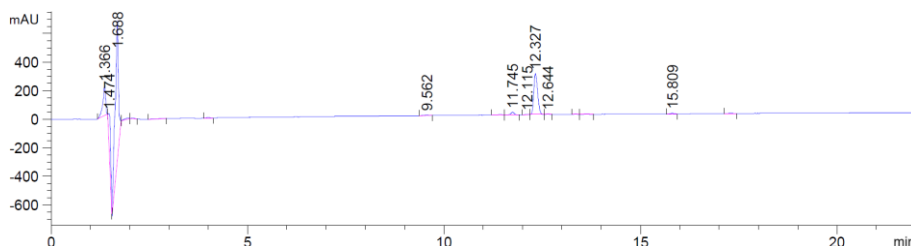


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
11.3	—	24.8	— ^a	— ^a
11.7	P1.1ol	82.2	1079.6	1079.4
12.3	P1.1s	1835.6	1061.6	1061.4
12.6	P1.1Nm	21.1	1075.6	1075.4
15.8	P1.1Nb	27.4	1243.5	1243.2
17.2	—	23.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] × 100 = 93%

Entry 16 bis, Table S1 ($\lambda = 210$ nm; Method 2)



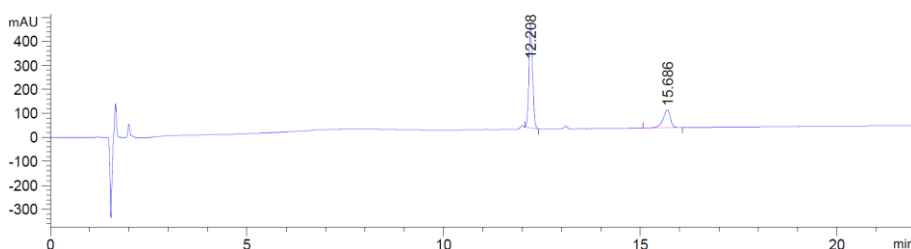
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	—	33.8	— ^a	— ^a
11.7	P1.1ol	103.7	1079.6	1079.4
12.3	P1.1s	1936.3	1061.6	1061.3
12.6	P1.1Nm	21.5	1075.6	1075.4
15.8	P1.1Nb	23.9	1243.5	1243.4

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1s** / (**P1.1ol** + **P1.1s** + **P1.1Nm** + **P1.1Nb**)] $\times$ 100 = 93%

Chromatograms related to Section S5.5 (Synthesis of Cys–Lys, Cys–Orn, Cys–Dab, and Cys–Dap Stapled Peptides)

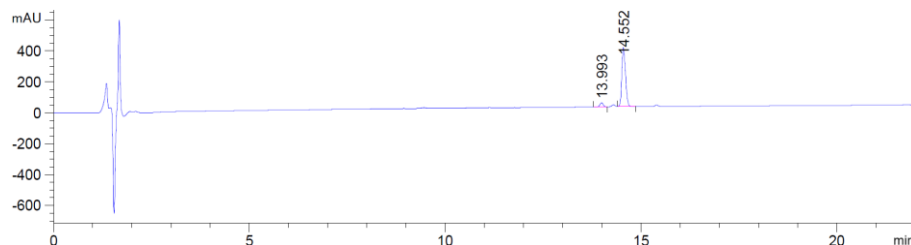
Entry P2.1A ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.2	P2.1al	2840.6	1063.6	1063.4
15.7	1	955.6	— ^a	— ^a

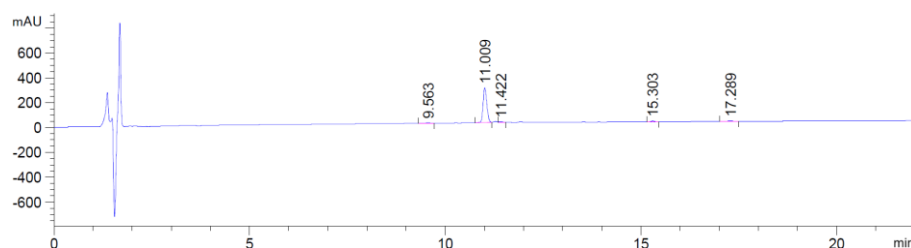
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry P2.1O ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.0	P2.1ox_b	155.1	1092.6	1092.4
14.6	P2.1ox_a	2466.3	1092.6	1092.4

Entry 1, Table S2 ($\lambda = 210$ nm; Method 2)

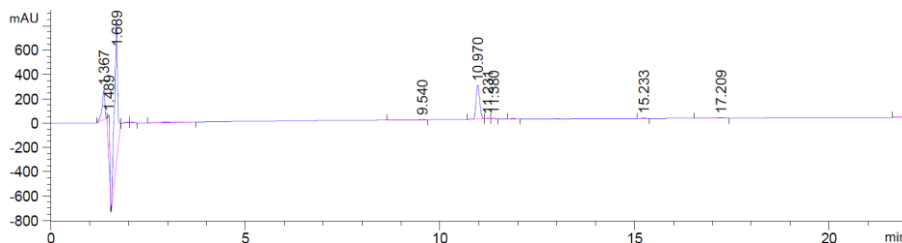


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	—	26.1	— ^a	— ^a
11.0	P2.1s	1827.0	1047.6	1047.3
11.4	P2.1ol	24.8	1065.6	1065.3
15.3	P2.1Nb	36.3	1229.5	1229.3
17.3	—	33.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P2.1s** / (**P2.1s** + **P2.1ol** + **P2.1Nb**)] $\times$ 100 = 97%

Entry 1 bis, Table S2 ($\lambda = 210$ nm; Method 2)

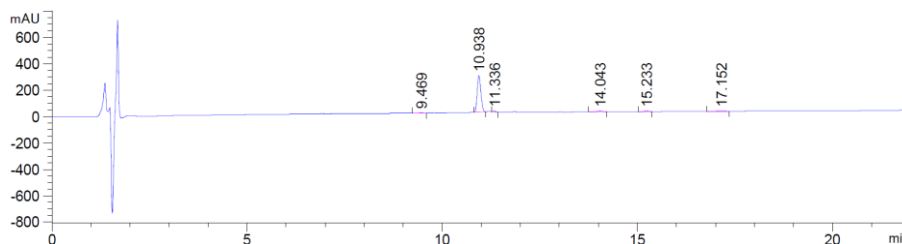


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	27.7	— ^a	— ^a
11.0	P2.1s	1815.1	1047.6	1047.3
11.4	P2.1ol	26.3	1065.6	1065.3
15.2	P2.1Nb	35.9	1229.5	1229.3
17.2	—	43.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [P2.1s / (P2.1s + P2.1ol + P2.1Nb)] $\times$ 100 = 97%

Entry 2, Table S2 ($\lambda = 210$ nm; Method 2)

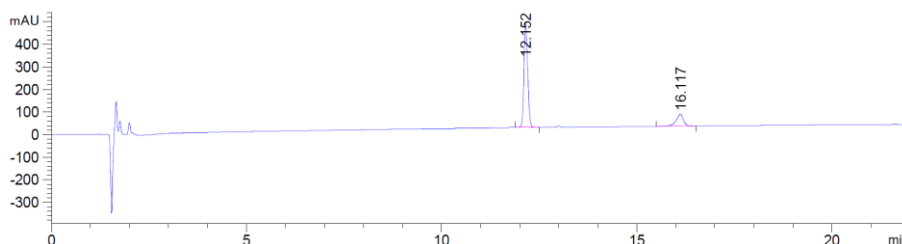


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	19.2	— ^a	— ^a
10.9	P2.1s	1789.8	1047.6	1047.3
11.3	P2.1ol	29.9	1065.6	1065.3
14.0	—	61.1	— ^a	— ^a
15.2	P2.1Nb	37.0	1229.5	1229.3
17.2	—	35.6	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P2.1s / (P2.1s + P2.1ol + P2.1Nb)] × 100 = 96%

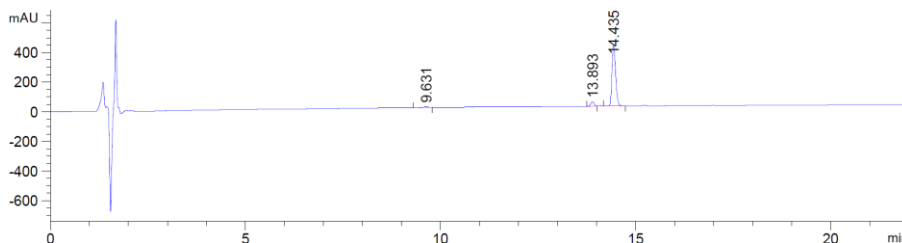
Entry P3.1A (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.2	P3.1al	3157.1	1049.5	1049.4
16.1	1	672.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

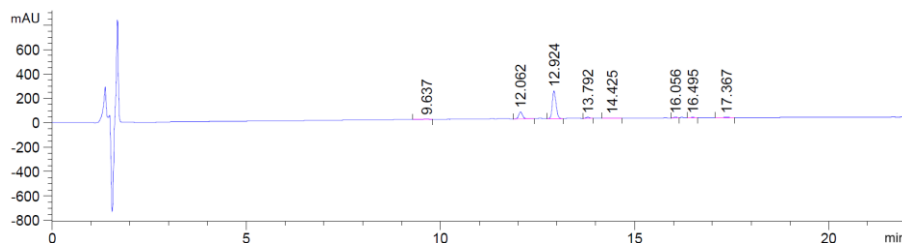
Entry P3.1O (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	—	37.0	— ^a	— ^a
13.9	P3.1ox_b	163.4	1078.6	1078.5
14.4	P3.1ox_a	2668.6	1078.6	1078.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry 1, Table S3 ($\lambda = 210$ nm; Method 2)



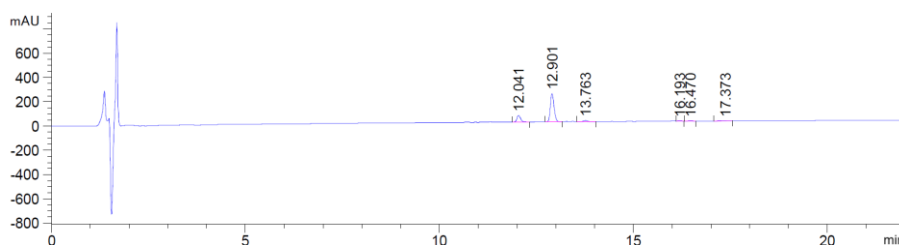
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.6	—	30.6	— ^b	— ^b
12.1	P3.1di_a	378.6	1033.5 ([M+2H] ²⁺)	1034.0 ([M+2H] ²⁺)
12.9	P3.1s	1490.9	1033.5	1033.4
13.8	P3.1di_b	55.2	1033.5 ([M+2H] ²⁺)	1033.6 ([M+2H] ²⁺)
14.4	—	20.7	— ^b	— ^b
16.1	P3.1Nb	29.0	1215.5	1215.4
16.5	x (not identified)	27.8	—	1053.2 ([M+2H] ²⁺)
17.4	—	36.9	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P3.1s** / (**P3.1di_a** + **P3.1s** + **P3.1di_b** + **P3.1Nb** + **x**)] × 100 = 75%

Entry 1 bis, Table S3 ($\lambda = 210$ nm; Method 2)



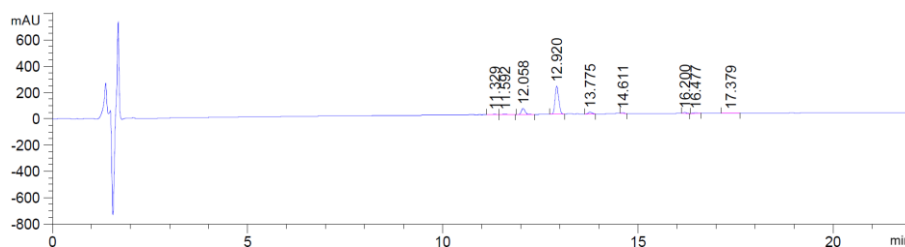
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
12.0	P3.1di_a	363.5	1033.5 ([M+2H] ²⁺)	1034.0 ([M+2H] ²⁺)
12.9	P3.1s	1530.7	1033.5	1033.4
13.8	P3.1di_b	65.5	1033.5 ([M+2H] ²⁺)	1034.1 ([M+2H] ²⁺)
16.2	y (not identified)	20.5	—	1053.1 ([M+2H] ²⁺)
16.5	x (not identified)	26.9	—	1053.1 ([M+2H] ²⁺)
17.4	—	24.2	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P3.1s / (P3.1di_a + P3.1s + P3.1di_b + y + x)] × 100 = 76%

Entry 2, Table S3 (λ = 210 nm; Method 2)



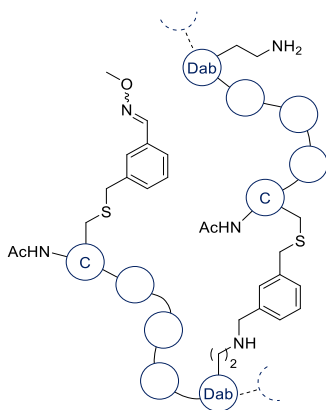
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.3	P3.1ol	23.3	1051.6	1051.5
11.6	—	31.4	— ^b	— ^b
12.1	P3.1di_a	308.9	1033.5 ([M+2H] ²⁺)	1033.8 ([M+2H] ²⁺)
12.9	P3.1s	1391.8	1033.5	1033.4
13.8	P3.1di_b	93.2	1033.5 ([M+2H] ²⁺)	1033.7 ([M+2H] ²⁺)
14.6	P3.1do	32.2	1056.1 ([M+2H] ²⁺)	1056.5 ([M+2H] ²⁺)
16.2	y (not identified)	39.2	—	1053.1 ([M+2H] ²⁺)
16.5	x (not identified)	40.1	—	1053.2 ([M+2H] ²⁺)
17.4	—	23.6	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

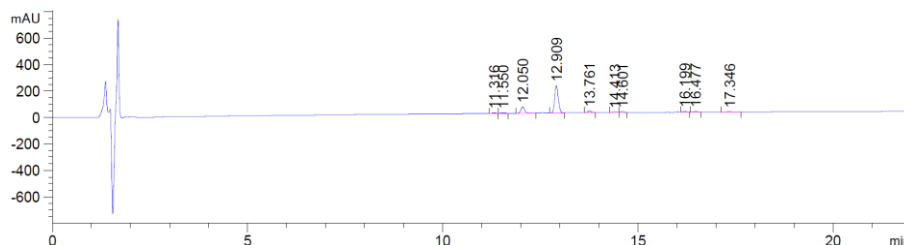
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100 = [P3.1s / (P3.1ol + P3.1di_a + P3.1s + P3.1di_b + P3.1do + y + x)] × 100 = 72%

Proposed structure of **P3.1do**:



Entry 2 bis, Table S3 ($\lambda = 210$ nm; Method 2)



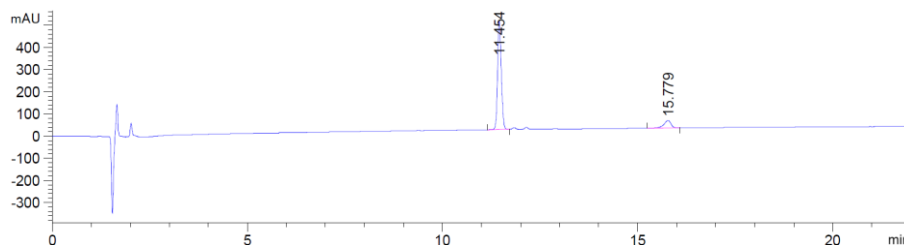
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.3	P3.1ol	22.1	1051.6	1051.4
11.5	—	20.0	— ^b	— ^b
12.0	P3.1di_a	320.9	1033.5 ([M+2H] ²⁺)	1033.9 ([M+2H] ²⁺)
12.9	P3.1s	1350.9	1033.5	1033.4
13.8	P3.1di_b	90.8	1033.5 ([M+2H] ²⁺)	1033.8 ([M+2H] ²⁺)
14.4	—	20.6	— ^b	— ^b
14.6	P3.1do	39.7	1056.1 ([M+2H] ²⁺)	1056.3 ([M+2H] ²⁺)
16.2	y (not identified)	43	—	1053.0 ([M+2H] ²⁺)
16.5	x (not identified)	40.1	—	1053.1 ([M+2H] ²⁺)
17.3	—	21.3	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [P3.1s / (P3.1ol + P3.1di_a + P3.1s + P3.1di_b + P3.1do + y + x)]
 × 100 = 71%

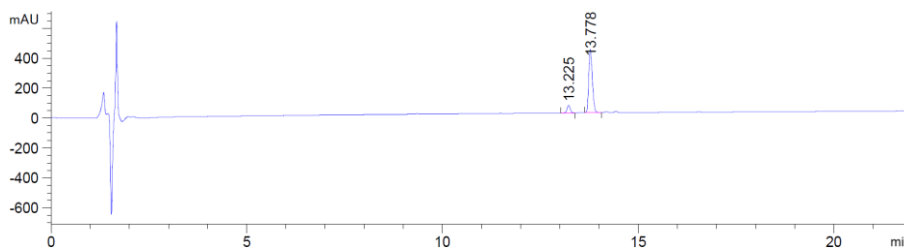
Entry P4.1A ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.5	P4.1aI	3266.9	1035.5	1035.4
15.8	1	414.5	— ^a	— ^a

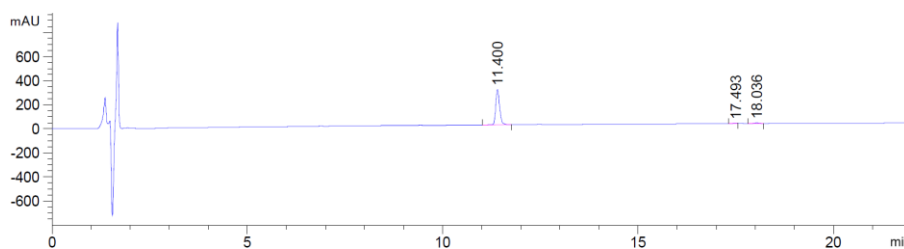
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry P4.1O ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.2	P4.1ox_b	285.3	1064.6	1064.5
13.8	P4.1ox_a	2725.5	1064.6	1064.5

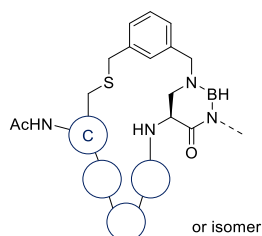
Entry 1, Table S4 ($\lambda = 210$ nm; Method 2)



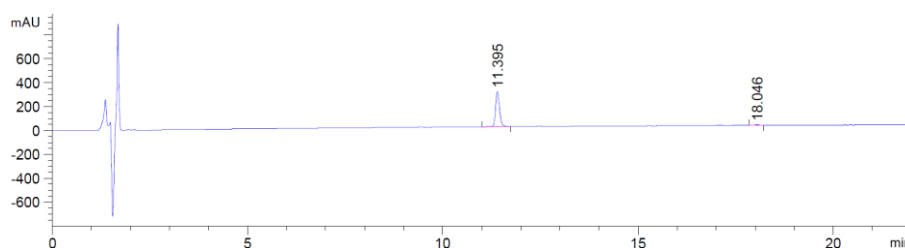
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	2049.4	1019.5	1019.5
17.5	P4.1B	22.2	1029.5	1029.4
18.0	P4.1Nb	40.3	1201.5	1201.4

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P4.1s** / (**P4.1s** + **P4.1B** + **P4.1Nb**)] $\times$ 100 = 97%

Proposed structure of **P4.1B**:



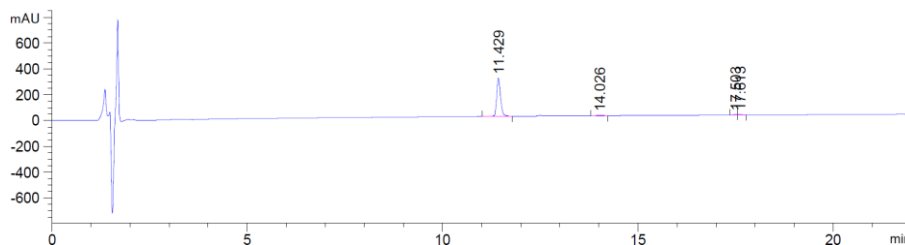
Entry 1 bis, Table S4 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	2035.0	1019.5	1019.4
18.0	P4.1Nb	43.3	1201.5	1201.4

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P4.1s** / (**P4.1s** + **P4.1Nb**)] $\times$ 100 = 98%

Entry 2, Table S4 ($\lambda = 210$ nm; Method 2)

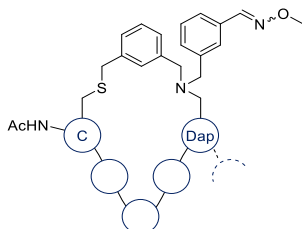


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	2043.3	1019.5	1019.4
14.0	—	35.1	— ^a	— ^a
17.5	P4.1B	22.3	1029.5	1029.5
17.6	P4.1No	31.8	1166.6	1166.5

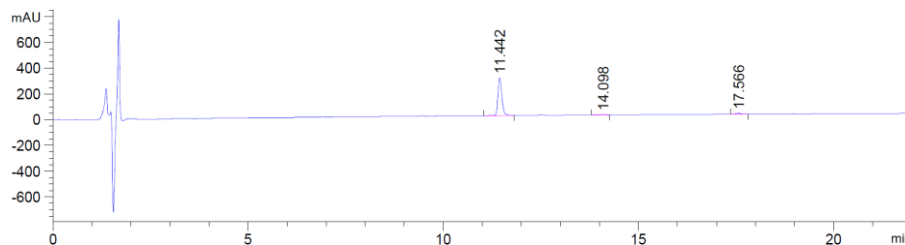
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P4.1s** / (**P4.1s** + **P4.1B** + **P4.1No**)] $\times$ 100 = 97%

Proposed structure of **P4.1No**:



Entry 2 bis, Table S4 ($\lambda = 210$ nm; Method 2)

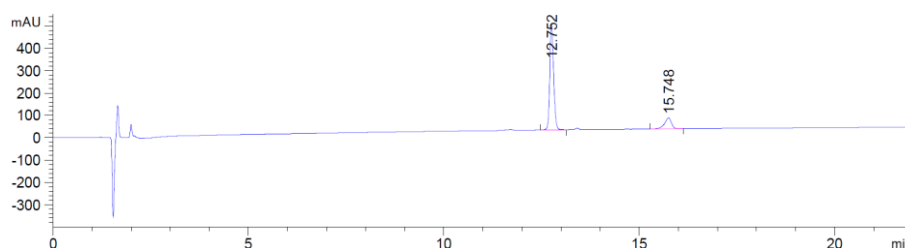


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	2052.6	1019.5	1019.5
14.1	—	37.8	— ^a	— ^a
17.6	P4.1B	62.6	1029.5	1029.4
	P4.1No		1166.6	1166.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P4.1s** / (**P4.1s** + **P4.1B** + **P4.1No**)] × 100 = 97%

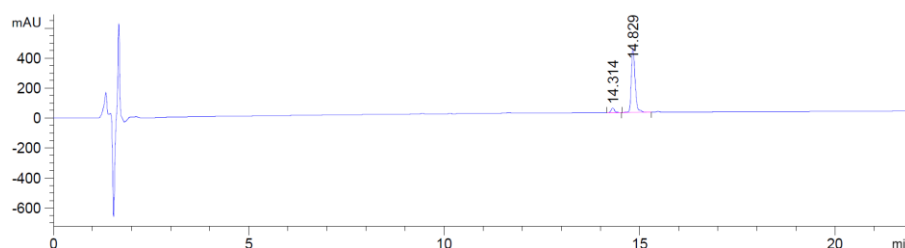
Entry P5.1A (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P5.1aI	3243.3	1077.6	1077.5
15.7	1	600.9	— ^a	— ^a

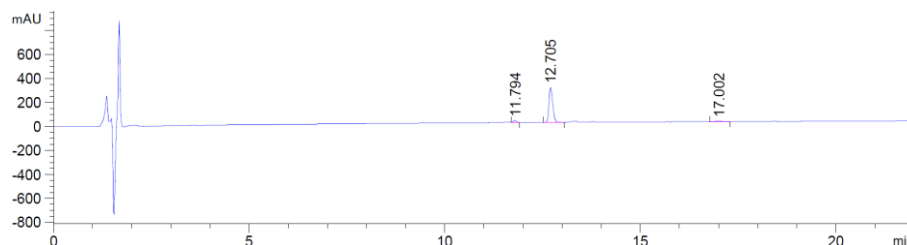
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry P5.1O (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.3	P5.1ox_b	169.5	1106.6	1106.5
14.8	P5.1ox_a	2850.5	1106.6	1106.5

Entry 1, Table S5 ($\lambda = 210$ nm; Method 2)

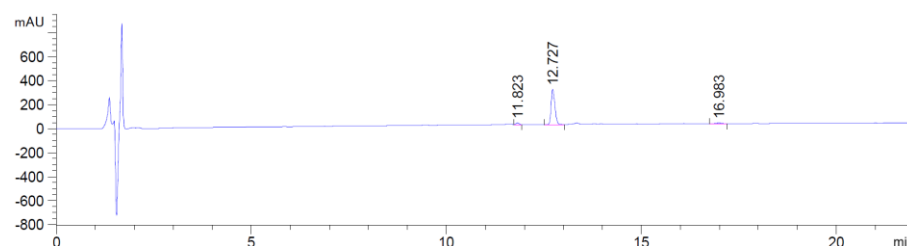


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P5.1ol	80.0	1079.6	1079.5
12.7	P5.1s	1930.4	1061.6	1061.5
17.0	—	53.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P5.1s** / (**P5.1ol** + **P5.1s**)] $\times$ 100 = 96%

Entry 1 bis, Table S5 ($\lambda = 210$ nm; Method 2)

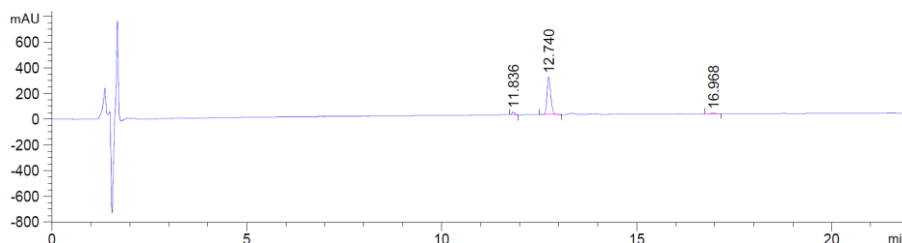


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P5.1ol	72.5	1079.6	1079.5
12.7	P5.1s	1922.8	1061.6	1061.5
17.0	—	63.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P5.1s** / (**P5.1ol** + **P5.1s**)] $\times$ 100 = 96%

Entry 2, Table S5 ($\lambda = 210$ nm; Method 2)

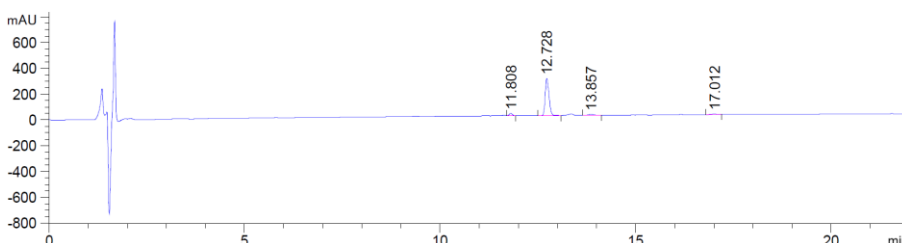


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P5.1ol	98.3	1079.6	1079.5
12.7	P5.1s	1914.4	1061.6	1061.5
17.0	—	52.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [P5.1s / (P5.1ol + P5.1s)] $\times$ 100 = 95%

Entry 2 bis, Table S5 ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.8	P5.1ol	94.8	1079.6	1079.5
12.7	P5.1s	1923.1	1061.6	1061.5
13.9	P5.1di	65.3	1061.6 ($[M+2H]^{2+}$)	1061.5 ($[M+2H]^{2+}$)
17.0	—	43.4	— ^b	— ^b

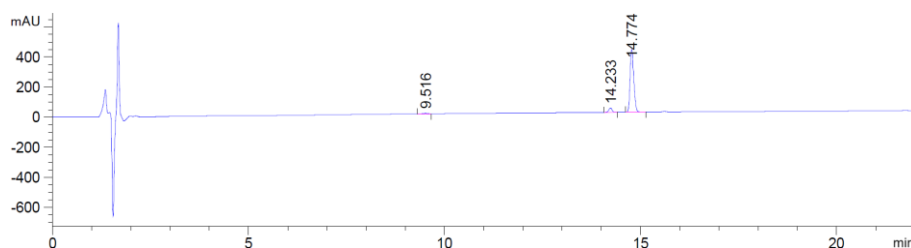
^a $[M+H]^+$ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [P5.1s / (P5.1ol + P5.1s + P5.1di)] × 100 = 92%

Chromatograms related to Section S5.7 (*Kinetic Studies*)

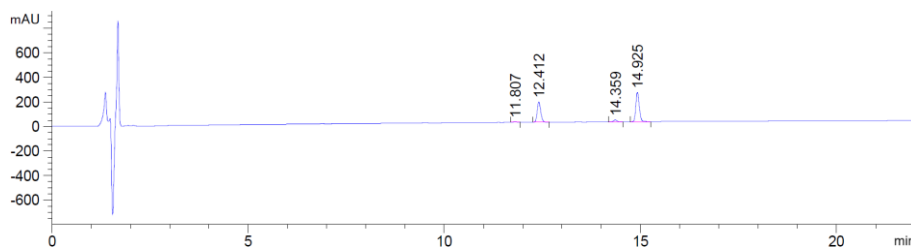
Intramolecular reductive amination of P1.1al, t = 0 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	31.8	— ^a	— ^a
14.2	P1.1ox_b	165.0	1106.6	1106.5
14.8	P1.1ox_a	2702.8	1106.6	1106.5

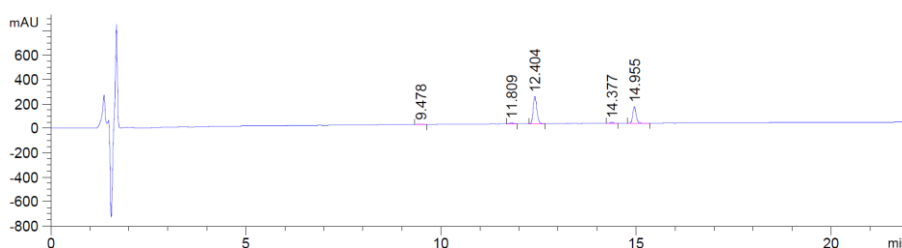
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P1.1al, t = 0.5 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P1.1ol	38.6	1079.6	1079.5
12.4	P1.1s	1030.0	1061.6	1061.5
14.4	P1.1ox_b	88.8	1106.6	1106.5
14.9	P1.1ox_a	1452.5	1106.6	1106.5

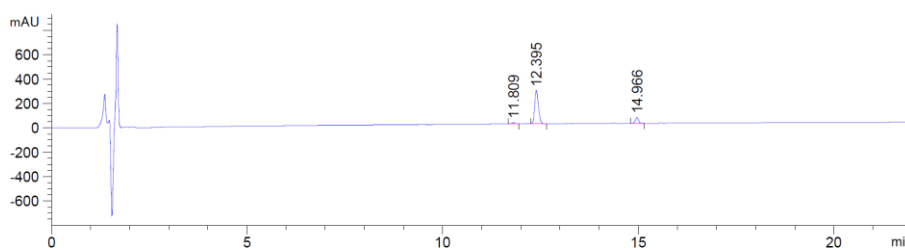
Intramolecular reductive amination of P1.1al, t = 1 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	29.1	— ^a	— ^a
11.8	P1.1ol	51.2	1079.6	1079.5
12.4	P1.1s	1447.2	1061.6	1061.5
14.4	P1.1ox_b	47.6	1106.6	1106.5
15.0	P1.1ox_a	798.5	1106.6	1106.5

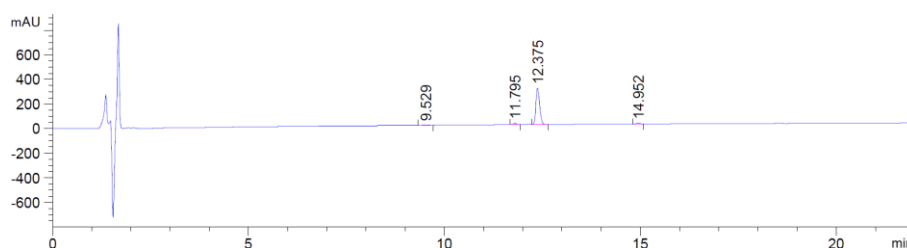
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P1.1al, t = 2 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P1.1ol	59.3	1079.6	1079.5
12.4	P1.1s	1809.8	1061.6	1061.5
15.0	P1.1ox_a	276.6	1106.6	1106.5

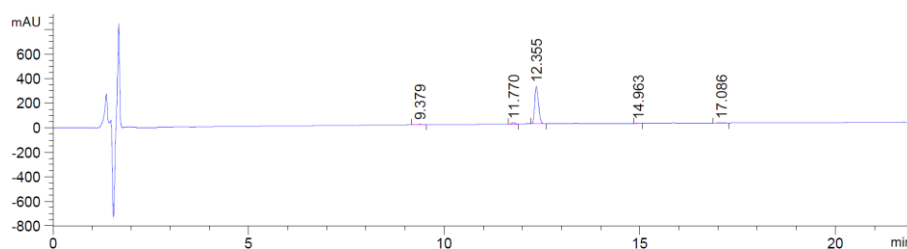
Intramolecular reductive amination of P1.1al, t = 4 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	25.4	— ^a	— ^a
11.8	P1.1ol	59.7	1079.6	1079.5
12.4	P1.1s	1961.8	1061.6	1061.5
15.0	P1.1ox_a	46.1	1106.6	1106.6

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P1.1al, t = 6 h (λ = 210 nm; Method 2)

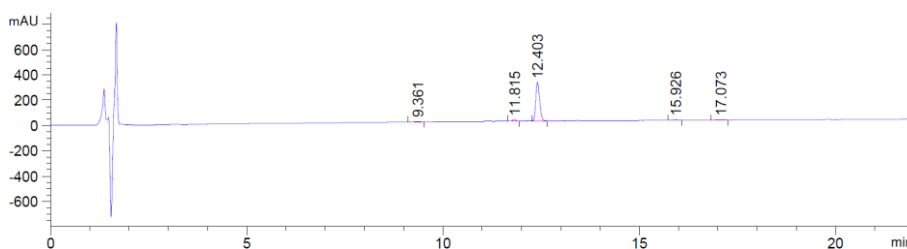


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	—	26.4	— ^a	— ^a
11.8	P1.1ol	64.4	1079.6	1079.5
12.4	P1.1s	2028.1	1061.6	1061.5
15.0	P1.1ox_a	9.1 ^b	1106.6	1106.5
17.1	—	24.8	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

^b <1% of the total peptide-related UV signal.

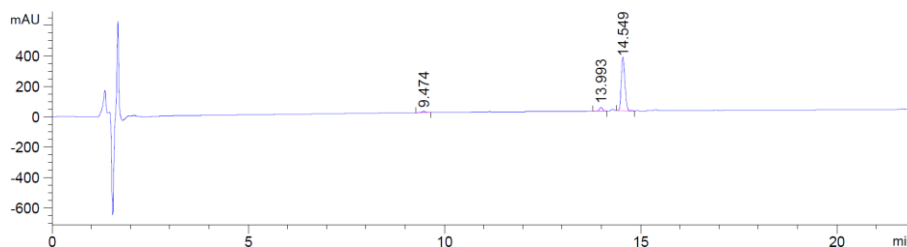
Intramolecular reductive amination of P1.1al, t = 8 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	—	28.2	— ^a	— ^a
11.8	P1.1ol	70.4	1079.6	1079.5
12.4	P1.1s	2059.0	1061.6	1061.5
15.9	P1.1Nb	23.5	1243.5	1243.4
17.1	—	31.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

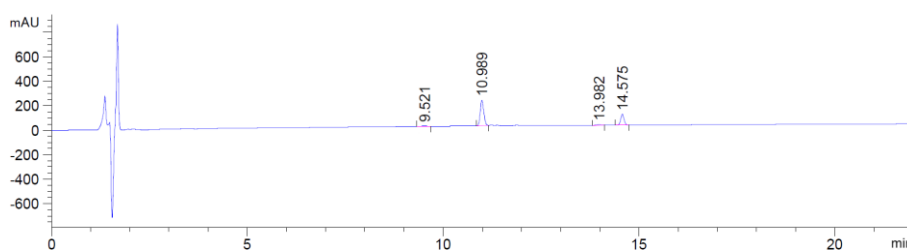
Intramolecular reductive amination of P2.1al, t = 0 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	53.5	— ^a	— ^a
14.0	P2.1ox_b	141.1	1092.6	1092.5
14.5	P2.1ox_a	2243.2	1092.6	1092.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode

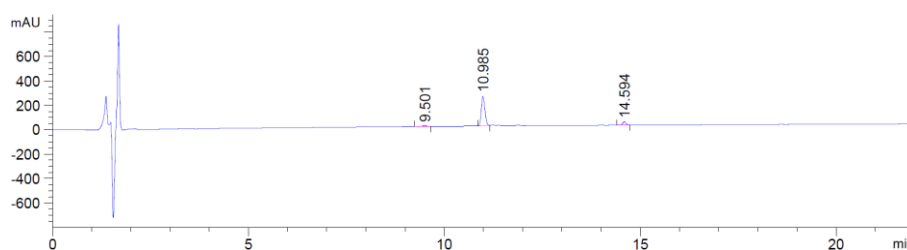
Intramolecular reductive amination of P2.1aI, t = 0.5 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	29.7	— ^a	— ^a
11.0	P2.1s	1285.8	1047.6	1047.5
14.0	P2.1ox_b	32.8	1092.6	1092.4
14.6	P2.1ox_a	511.9	1092.6	1092.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

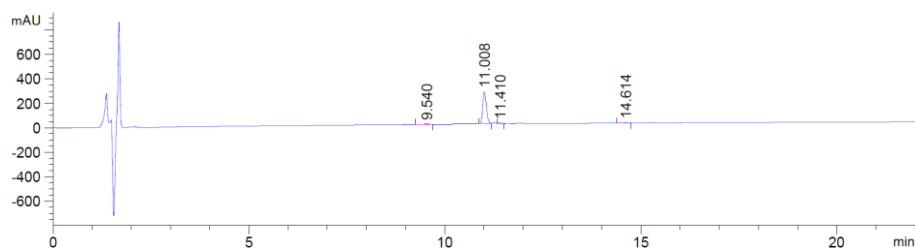
Intramolecular reductive amination of P2.1aI, t = 1 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	40.1	— ^a	— ^a
11.0	P2.1s	1518.4	1047.6	1047.5
14.6	P2.1ox_a	164.0	1092.6	1092.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

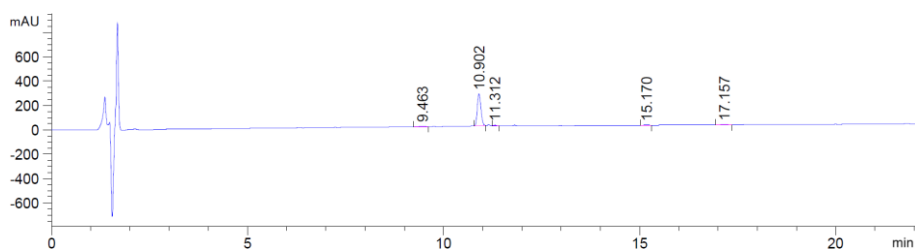
Intramolecular reductive amination of P2.1aI, t = 2 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	34.3	— ^a	— ^a
11.0	P2.1s	1653.1	1047.6	1047.5
11.4	P2.1ol	19.9	1065.6	1065.5
14.6	P2.1ox_a	28.6	1092.6	1092.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

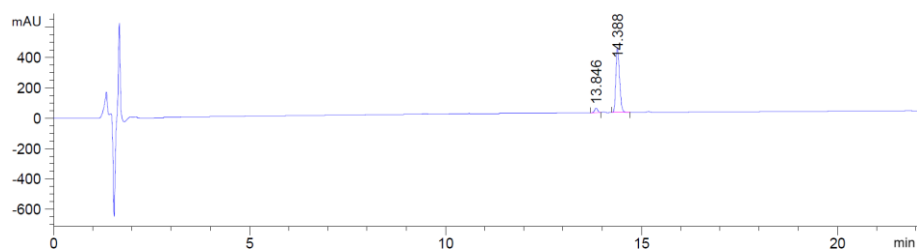
Intramolecular reductive amination of P2.1aI, t = 4 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.5	—	33.6	— ^a	— ^a
10.9	P2.1s	1665.8	1047.6	1047.5
11.3	P2.1ol	26.3	1065.6	1065.4
15.2	P2.1Nb	20.7	1229.5	1229.3
17.2	—	23.5	— ^a	— ^a

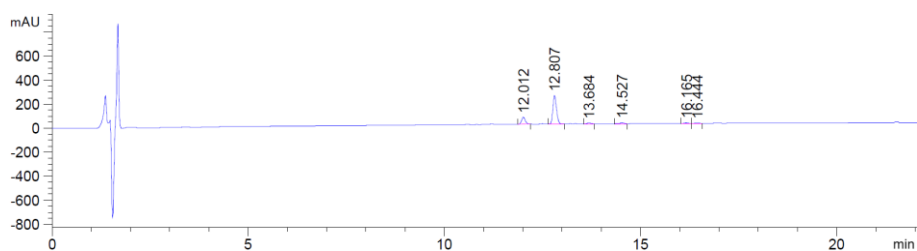
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P3.1aI, t = 0 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.8	P3.1ox_b	163.6	1078.6	1078.5
14.4	P3.1ox_a	2775.8	1078.6	1078.5

Intramolecular reductive amination of P3.1aI, t = 0.5 h (λ = 210 nm; Method 2)

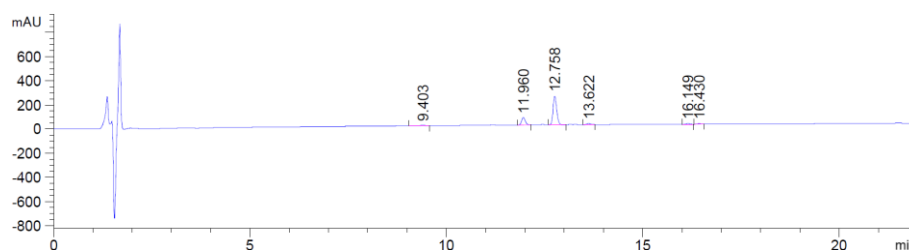


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
12.0	P3.1di_a	378.5	1033.5 ([M+2H] ²⁺)	1033.9 ([M+2H] ²⁺)
12.8	P3.1s	1529.1	1033.5	1033.4
13.7	P3.1di_b	44.6	1033.5 ([M+2H] ²⁺)	1033.8 ([M+2H] ²⁺)
14.5 ^b	P3.1do	57.6	1056.1 ([M+2H] ²⁺)	1056.4 ([M+2H] ²⁺)
	P3.1ox_a		1078.6	1078.5
16.2	y (not identified)	34.3	—	1053.1 ([M+2H] ²⁺)
16.4	x (not identified)	25.7	—	1053.0 ([M+2H] ²⁺)

^a [M+H]⁺ ion, unless otherwise specified.

^b Ratio of EIC peak areas for **P3.1do** (m/z 1056.0–1057.0, [M+2H]²⁺) and **P3.1ox_a** (m/z 539.2–540.2, [M+2H]²⁺) ~69:31.

Intramolecular reductive amination of P3.1al, t = 1 h (λ = 210 nm; Method 2)

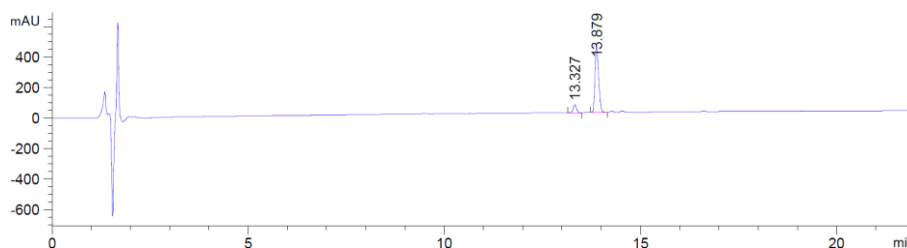


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.4	—	37.8	— ^b	— ^b
12.0	P3.1di_a	407.2	1033.5 ([M+2H] ²⁺)	1033.9 ([M+2H] ²⁺)
12.8	P3.1s	1546.8	1033.5	1033.4
13.6	P3.1di_b	60.4	1033.5 ([M+2H] ²⁺)	1033.9 ([M+2H] ²⁺)
16.1	y (not identified)	35.2	—	1053.1 ([M+2H] ²⁺)
16.4	x (not identified)	30.1	—	1053.0 ([M+2H] ²⁺)

^a [M+H]⁺ ion, unless otherwise specified.

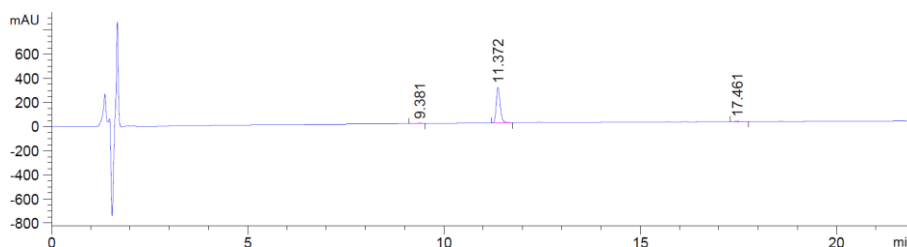
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P4.1al, t = 0 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
13.3	P4.1ox_b	294.3	1064.6	1064.4
13.9	P4.1ox_a	2819.3	1064.6	1064.5

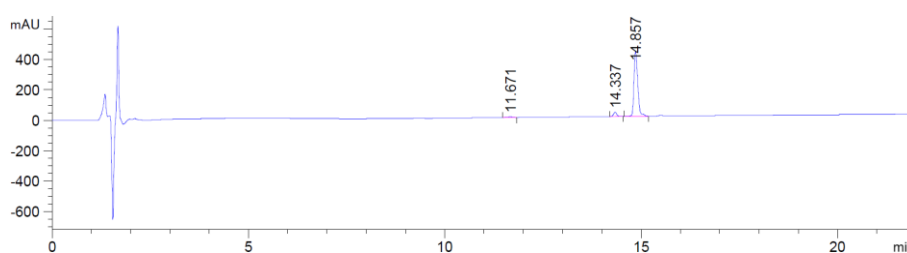
Intramolecular reductive amination of P4.1al, t = 0.5 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	—	21.0	— ^a	— ^a
11.4	P4.1s	2015.1	1019.5	1019.5
17.5	P4.1B	34.6	1029.5	1029.4
	P4.1No		1166.6	1166.5

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

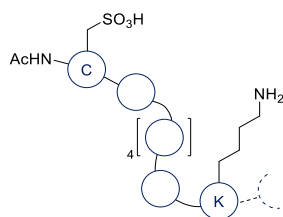
Intramolecular reductive amination of **P5.1aI**, $t = 0$ h ($\lambda = 210$ nm; Method 2)



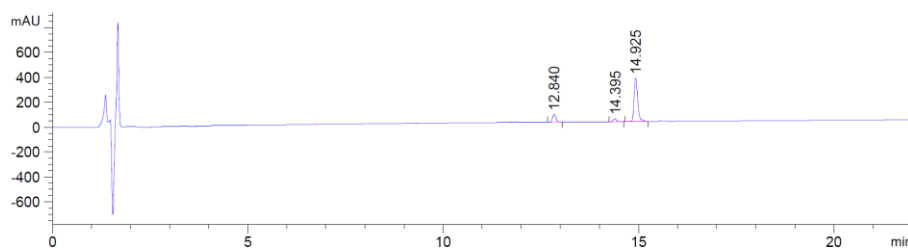
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.6	P5sua	38.1	1007.5	1007.5
	P5ds		958.5 ($[M+2H]^{2+}$)	958.8 ($[M+2H]^{2+}$)
14.3	P5.1ox_b	159.5	1106.6	1106.5
14.9	P5.1ox_a	2798.7	1106.6	1106.5

^a $[M+H]^+$ ion, unless otherwise specified.

Proposed structure of **P5sua**:

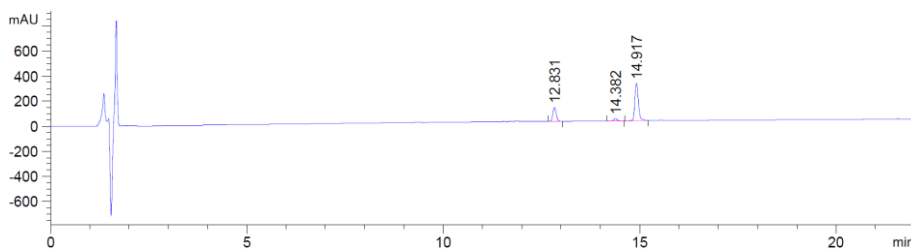


Intramolecular reductive amination of P5.1aI, t = 0.5 h ($\lambda = 210$ nm; Method 2)



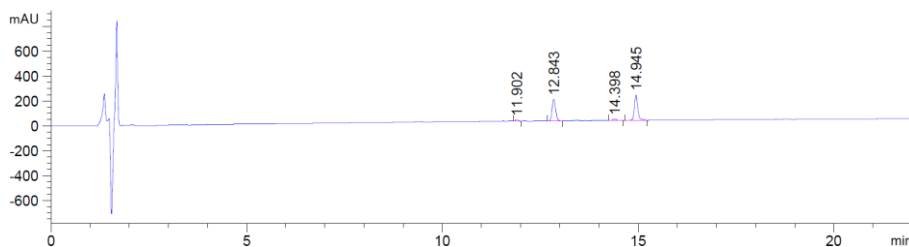
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P5.1s	384.9	1061.6	1061.5
14.4	P5.1ox_b	135.8	1106.6	1106.5
14.9	P5.1ox_a	2264.8	1106.6	1106.5

Intramolecular reductive amination of P5.1aI, t = 1 h ($\lambda = 210$ nm; Method 2)



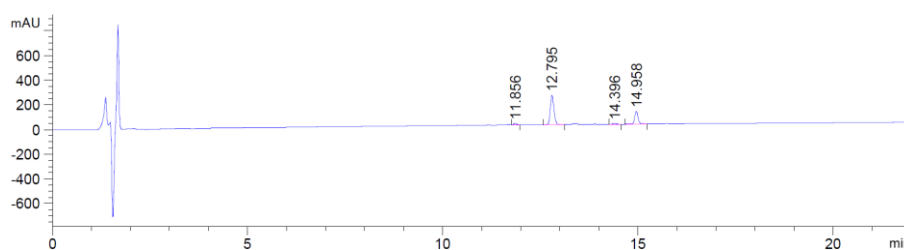
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P5.1s	664.2	1061.6	1061.5
14.4	P5.1ox_b	113.9	1106.6	1106.5
14.9	P5.1ox_a	1877.8	1106.6	1106.5

Intramolecular reductive amination of P5.1aI, t = 2 h ($\lambda = 210$ nm; Method 2)



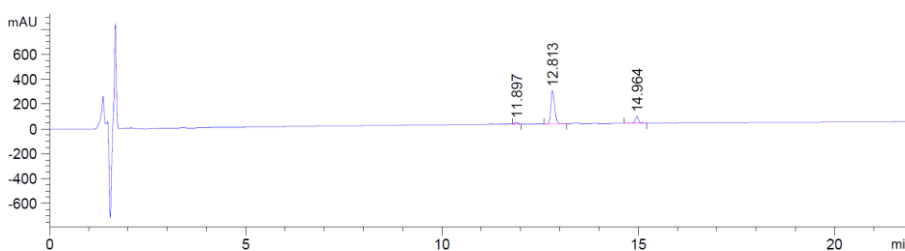
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.9	P5.1ol	43.1	1079.6	1079.5
12.8	P5.1s	1080.3	1061.6	1061.5
14.4	P5.1ox_b	75.8	1106.6	1106.5
14.9	P5.1ox_a	1250.5	1106.6	1106.5

Intramolecular reductive amination of P5.1al, t = 4 h (λ = 210 nm; Method 2)



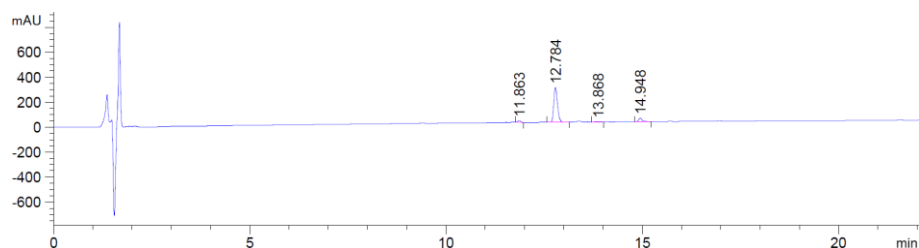
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.9	P5.1ol	60.9	1079.6	1079.5
12.8	P5.1s	1536.6	1061.6	1061.5
14.4	P5.1ox_b	35.5	1106.6	1106.5
15.0	P5.1ox_a	611.5	1106.6	1106.5

Intramolecular reductive amination of P5.1al, t = 6 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.9	P5.1ol	67.8	1079.6	1079.5
12.8	P5.1s	1735.4	1061.6	1061.5
15.0	P5.1ox_a	323.8	1106.6	1106.5

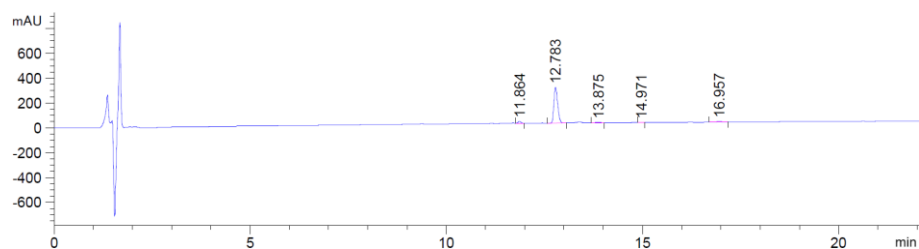
Intramolecular reductive amination of P5.1al, t = 8 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.9	P5.1ol	72.0	1079.6	1079.5
12.8	P5.1s	1836.9	1061.6	1061.5
13.9	P5.1di	24.4	1061.6 ([M+2H] ²⁺)	1062.0 ([M+2H] ²⁺)
14.9	P5.1ox_a	169.6	1106.6	1106.5

^a [M+H]⁺ ion, unless otherwise specified.

Intramolecular reductive amination of P5.1al, t = 24 h (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.9	P5.1ol	74.0	1079.6	1079.5
12.8	P5.1s	1913.1	1061.6	1061.5
13.9	P5.1di	37.5	1061.6 ([M+2H] ²⁺)	1062.0 ([M+2H] ²⁺)
15.0	P5.1ox_a	4.1 ^b	1106.6	1106.6
17.0	—	44.9	— ^c	— ^c

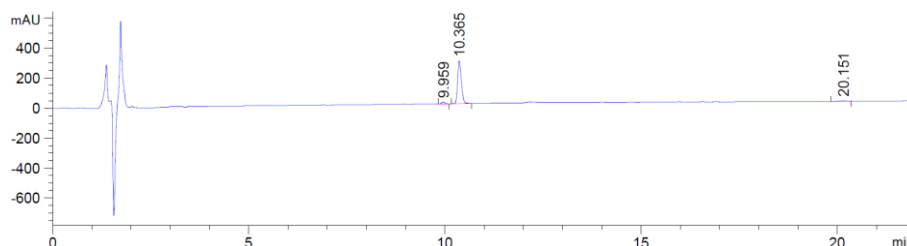
^a [M+H]⁺ ion, unless otherwise specified.

^b <1% of the total peptide-related UV signal.

^c Compound exhibiting low ionization efficiency in ESI⁺ mode.

Chromatograms related to Section S5.8.1 (*Tolerance toward Nucleophilic Residues*)

Entry 1, Table S7 ($\lambda = 210$ nm; Method 3)

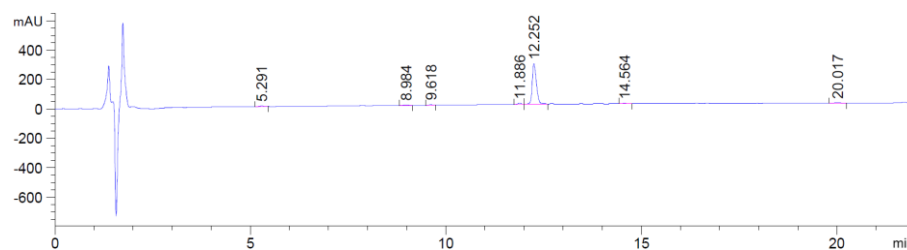


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
10.0	P6.1ol	55.0	753.4	753.3
10.4	P6.1s	1854.9	735.4	735.3
20.2	—	38.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P6.1s** / (**P6.1ol** + **P6.1s**)] $\times$ 100 = 97%

Entry 2, Table S7 ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
5.3	—	33.0	— ^b	— ^b
9.0	P7.1NS'	27.7	881.4	881.6
9.6	P7ds	22.3	1351.7	1351.8
11.9	P7.1ol	49.9	797.4	797.5
12.3	P7.1s	2010.2 ^c	779.4	779.4
14.6	P7.1di	25.7	779.4 ([M+2H] ²⁺)	779.4 ([M+2H] ²⁺)
20.0	—	36.8	— ^b	— ^b

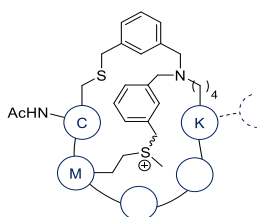
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

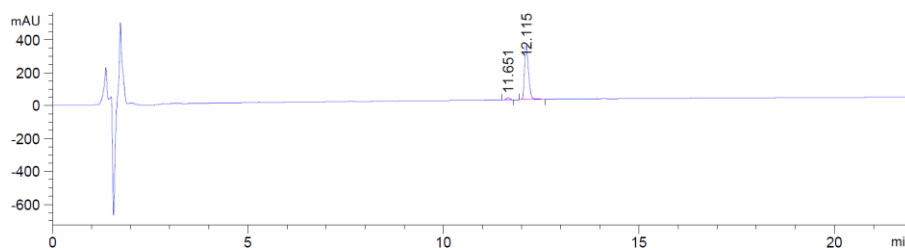
^c Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1869.8 mAU·s.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P7.1s** / (**P7.1NS** + **P7ds** + **P7.1ol** + **P7.1s** + **P7.1di**)] × 100 = 94%

Proposed structure of **P7.1NS'**:



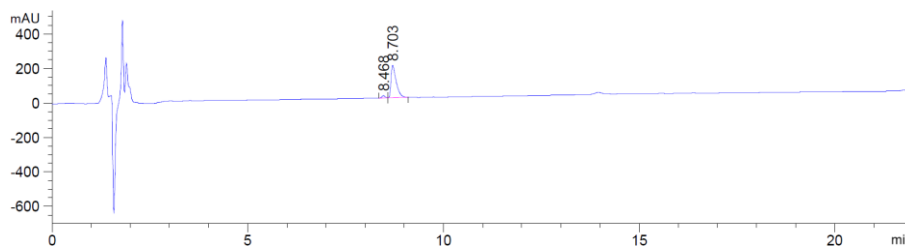
Entry 3, Table S7 (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
11.7	P8.1ol	66.2	829.4	829.2
12.1	P8.1s	2303.7	811.4	811.3

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P8.1s** / (**P8.1ol** + **P8.1s**)] × 100 = 97%

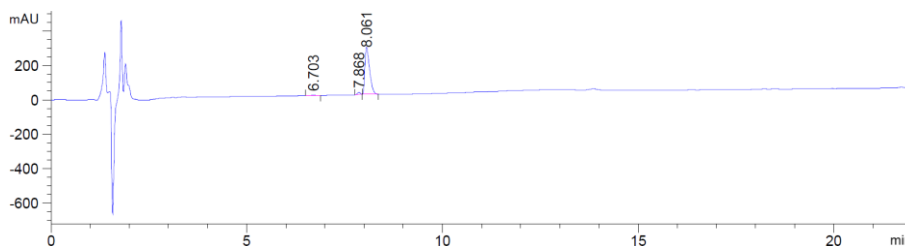
Entry 4, Table S7 (λ = 210 nm; Method 4)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.5	P9.1ol	72.9	822.5	822.3
8.7	P9.1s	1702.1	804.4	804.3

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P9.1s** / (**P9.1ol** + **P9.1s**)] × 100 = 96%

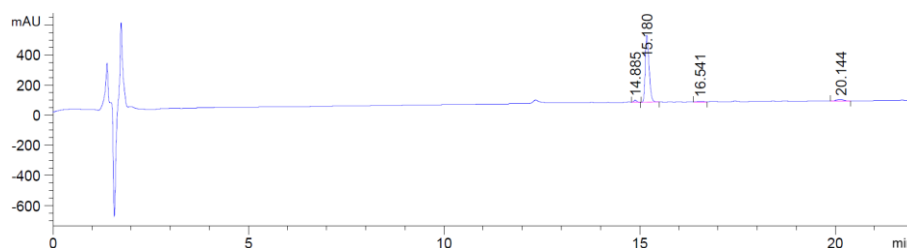
Entry 5, Table S7 (λ = 210 nm; Method 4)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
6.7	x (not identified)	23.9	—	785.4
7.9	P10.1ol	65.8	803.4	803.4
8.1	P10.1s	2253.1	785.4	785.3

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P10.1s** / (**x** + **P10.1ol** + **P10.1s**)] $\times$ 100 = 96%

Entry 6, Table S7 (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
14.9	P11.1ol	69.9	852.4	852.4
15.2	P11.1s	3026.9	834.4	834.4
16.5	x (not identified)	33.3	—	783.3 ($[M+2H]^{2+}$)
	P11.1di		834.4 ($[M+2H]^{2+}$)	834.4 ($[M+2H]^{2+}$)
20.1	P11.1Nb	111.1	1016.4	1016.3
	—		— ^b	— ^b

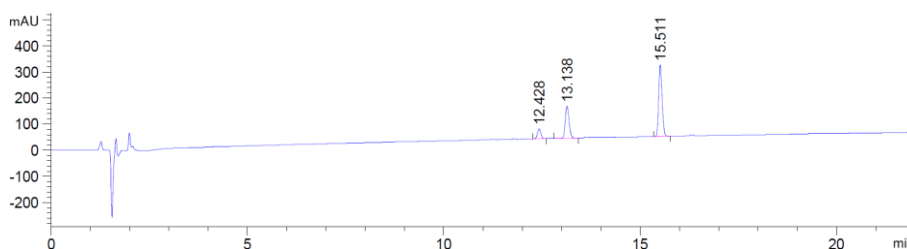
^a $[M+H]^+$ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P11.1s** / (**P11.1ol** + **P11.1s** + **x** + **P11.1di** + **P11.1Nb**)] $\times$ 100 = 95%

Chromatograms related to Section S5.8.2 (Comparison of *S*-Alkylation/Reductive Amination and *S,N*-bis-Alkylation)

***S,N*-bis-Alkylation of P1, t = 0.5 h (λ = 210 nm; Method 2)**

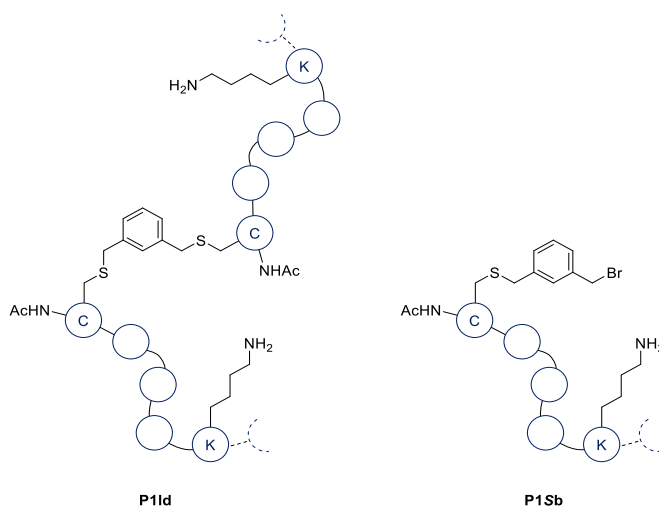


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
12.4	P1.1s	221.7	1061.6	1061.4
13.1	P1Id	823	1010.6 ([M+2H] ²⁺)	1010.6 ([M+2H] ²⁺)
15.5	P1Sb	1657.3	1141.5	1141.3

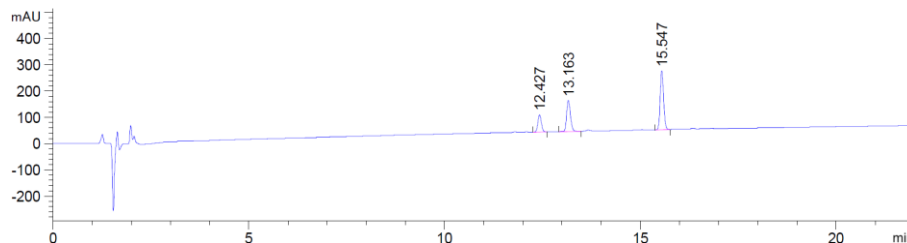
^a [M+H]⁺ ion, unless otherwise specified.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.1s** / (**P1.1s** + **P1Id** + **P1Sb**)] × 100 = 8%

Proposed structures of **P1Id** and **P1Sb**:



S,N-bis-Alkylation of P1, t = 1 h ($\lambda = 210$ nm; Method 2)

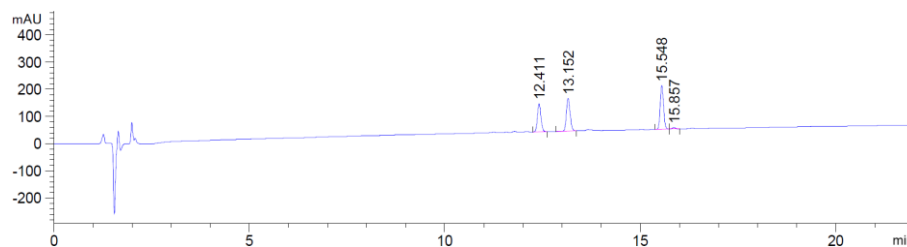


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
12.4	P1.1s	403.6	1061.6	1061.4
13.2	P1Id	797.3	1010.6 ([M+2H] ²⁺)	1010.7 ([M+2H] ²⁺)
15.5	P1Sb	1346.6	1141.5	1141.3

^a [M+H]⁺ ion, unless otherwise specified.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.1s** / (**P1.1s** + **P1Id** + **P1Sb**)] × 100 = 16%

S,N-bis-Alkylation of P1, t = 2 h ($\lambda = 210$ nm; Method 2)

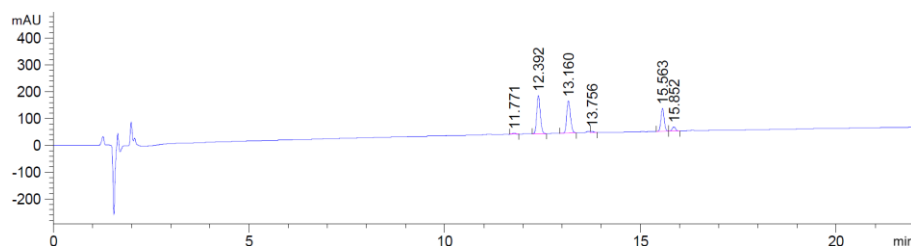


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
12.4	P1.1s	641.6	1061.6	1061.4
13.2	P1Id	800.7	1010.6 ([M+2H] ²⁺)	1010.6 ([M+2H] ²⁺)
15.5	P1Sb	945.6	1141.5	1141.3
15.9	P1.1Nb	32.4	1243.5	1243.2

^a [M+H]⁺ ion, unless otherwise specified.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1s + P1Id + P1Sb + P1.1Nb)] × 100 = 27%

S,N-bis-Alkylation of P1, t = 4 h (λ = 210 nm; Method 2)

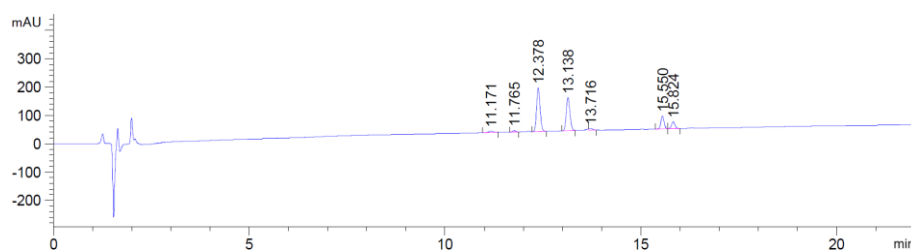


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.8	P1.1ol	25.3	1079.6	1079.4
12.4	P1.1s	894.3	1061.6	1061.4
13.2	P1Id	790.4	1010.6 ([M+2H] ²⁺)	1010.5 ([M+2H] ²⁺)
13.8	P1.1di	25.5	1061.6 ([M+2H] ²⁺)	1062.0 ([M+2H] ²⁺)
15.6	P1Sb	483.9	1141.5	1141.3
15.9	P1.1Nb	91.4	1243.5	1243.3

^a [M+H]⁺ ion, unless otherwise specified.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1Id + P1.1di + P1Sb + P1.1Nb)] × 100
= 39%

S,N-bis-Alkylation of P1, t = 6 h (λ = 210 nm; Method 2)



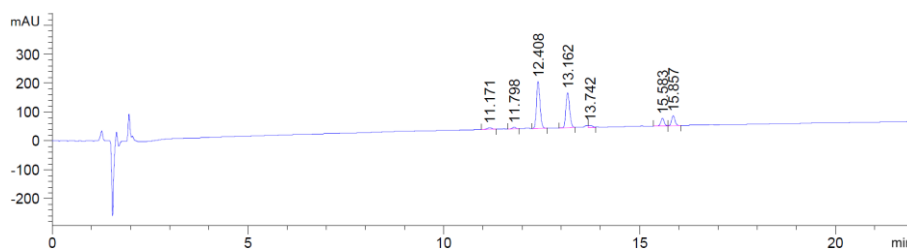
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.2	—	42.3	— ^b	— ^b
11.8	P1.1ol	28.7	1079.6	1079.4
12.4	P1.1s	960.0	1061.6	1061.4
13.1	P1ld	768.3	1010.6 ([M+2H] ²⁺)	1010.6 ([M+2H] ²⁺)
13.7	P1.1di	40.9	1061.6 ([M+2H] ²⁺)	1061.9 ([M+2H] ²⁺)
15.5	P1Sb	252.2	1141.5	1141.3
15.8	P1.1Nb	148.1	1243.5	1243.3

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1ld + P1.1di + P1Sb + P1.1Nb)] × 100
= 43%

S,N-bis-Alkylation of P1, t = 8 h (λ = 210 nm; Method 2)



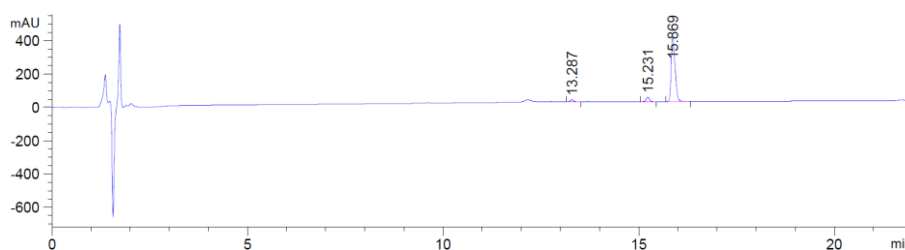
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.2	—	47.9	— ^b	— ^b
11.8	P1.1ol	32.1	1079.6	1079.4
12.4	P1.1s	1013.9	1061.6	1061.4
13.2	P1ld	786.3	1010.6 ([M+2H] ²⁺)	1010.6 ([M+2H] ²⁺)
13.7	P1.1di	40.4	1061.6 ([M+2H] ²⁺)	1062.0 ([M+2H] ²⁺)
15.6	P1Sb	153.7	1141.5	1141.3
15.9	P1.1Nb	209.3	1243.5	1243.3

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

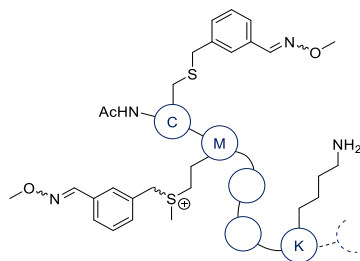
Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1s / (P1.1ol + P1.1s + P1ld + P1.1di + P1Sb + P1.1Nb)] × 100
= 45%

Intramolecular reductive amination of P7.1al, t = 0 h (λ = 210 nm; Method 3)

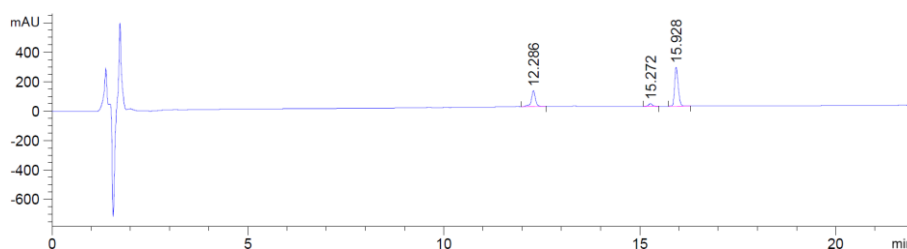


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
13.3	P7.1SS'ox	77.1	971.5	971.5
15.2	P7.1ox_b	153.9	824.4	824.4
15.9	P7.1ox_a	2645.9	824.4	824.4

Proposed structure of **P7.1SS'ox**:



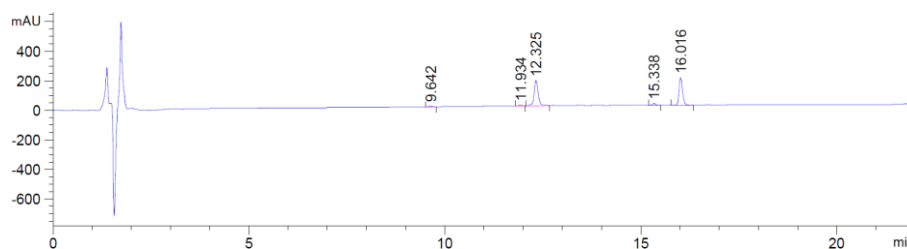
Intramolecular reductive amination of P7, t = 0.5 h (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P7.1s	782.8 ^a	779.4	779.5
15.3	P7.1ox_b	102.0	824.4	824.5
15.9	P7.1ox_a	1682.1	824.4	824.5

^a Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 655.3 mAU·s.

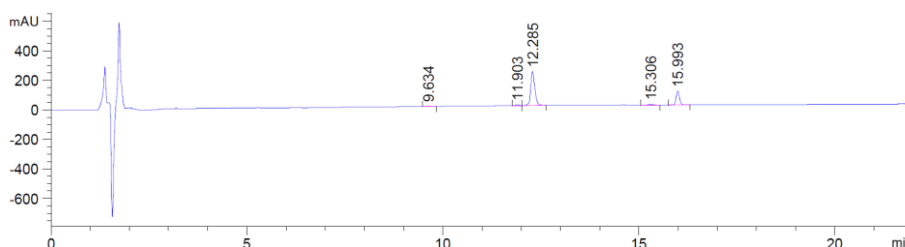
Intramolecular reductive amination of P7.1aI, t = 1 h (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	P7ds	24.2	1351.7	1351.7
11.9	P7.1ol	31	797.4	797.4
12.3	P7.1s	1200.3 ^a	779.4	779.5
15.3	P7.1ox_b	69.2	824.4	824.5
16.0	P7.1ox_a	1147.9	824.4	824.5

^a Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1075.4 mAU·s.

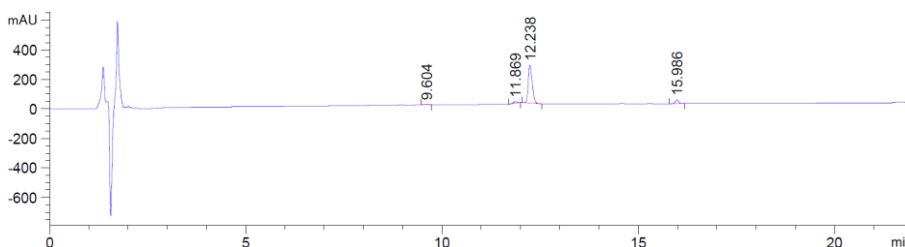
Intramolecular reductive amination of P7.1al, t = 2 h (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	P7ds	26.5	1351.7	1351.7
11.9	P7.1ol	40.3	797.4	797.4
12.3	P7.1s	1661.5 ^a	779.4	779.5
15.3	P7.1ox_b	35.3	824.4	824.5
16.0	P7.1ox_a	542.8	824.4	824.5

^a Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1505.4 mAU·s.

Intramolecular reductive amination of P7.1al, t = 4 h (λ = 210 nm; Method 3)



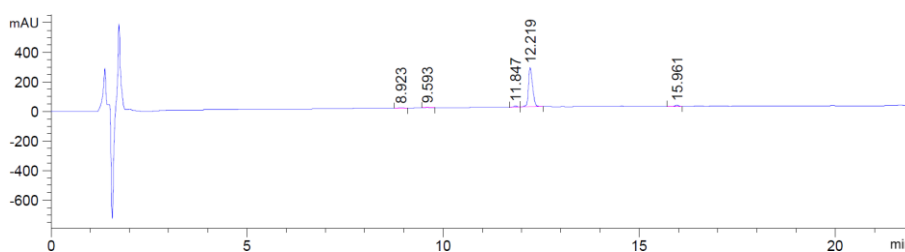
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.6	P7ds	23.9	1351.7	1351.6
11.9	P7.1ol	83.6 ^{a, b}	797.4	797.5
12.2	P7.1s	1797.8 ^{a, c}	779.4	779.5
16.0	P7.1ox_a	146.6	824.4	824.5

^a Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample).

^b Absorbance area from a second analysis after contaminant removal: 46.9 mAU·s.

^c Absorbance area from a second analysis after contaminant removal: 1767.3 mAU·s.

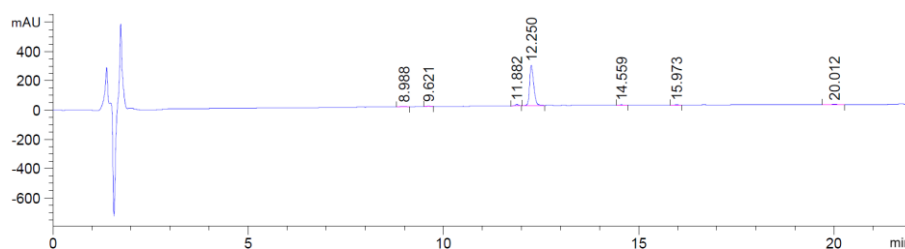
Intramolecular reductive amination of P7.1al, $t = 6$ h ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.9	P7.1NS'	20.9	881.4	881.5
9.6	P7ds	24.5	1351.7	1351.6
11.8	P7.1ol	45.5	797.4	797.4
12.2	P7.1s	1946.6 ^a	779.4	779.4
16.0	P7.1ox_a	43.5	824.4	824.5

^a Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1817.1 mAU·s.

Intramolecular reductive amination of P7.1al, $t = 8$ h ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.0	P7.1NS'	20.5	881.4	881.5
9.6	P7ds	22.3	1351.7	1351.7
11.9	P7.1ol	49.6	797.4	797.5
12.2	P7.1s	1991.6 ^b	779.4	779.5
14.6	P7.1di	19.9	779.4 ([M+2H] ²⁺)	779.5 ([M+2H] ²⁺)
16.0	P7.1ox_a	14.8 ^c	824.4	824.4
20.0	—	21.8	— ^d	— ^d

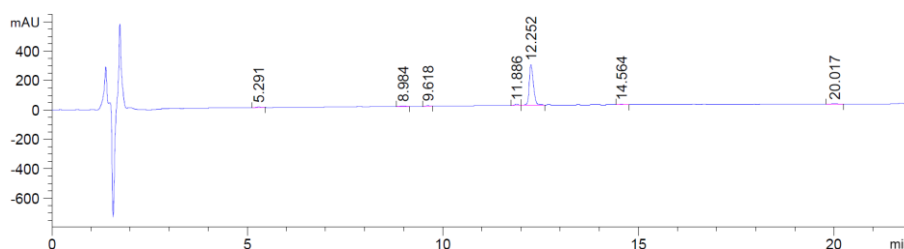
^a [M+H]⁺ ion, unless otherwise specified.

^b Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1858.2 mAU·s.

^c <1% of the total peptide-related UV signal.

^d Compound exhibiting low ionization efficiency in ESI⁺ mode.

Intramolecular reductive amination of P7.1al, t = 24 h (λ = 210 nm; Method 3)



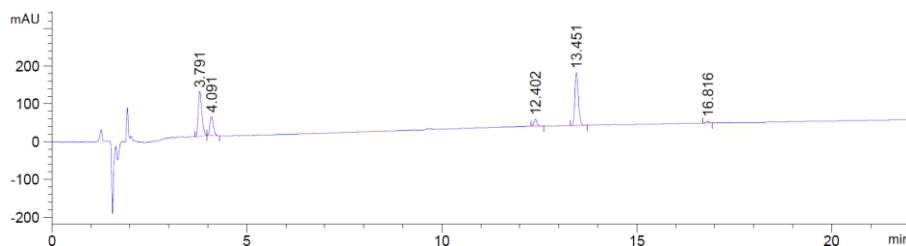
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
5.3	—	33.0	— ^b	— ^b
9.0	P7.1NS'	27.7	881.4	881.6
9.6	P7ds	22.3	1351.7	1351.8
11.9	P7.1ol	49.9	797.4	797.5
12.3	P7.1s	2010.2 ^c	779.4	779.4
14.6	P7.1di	25.7	779.4 ([M+2H] ²⁺)	779.4 ([M+2H] ²⁺)
20.0	—	36.8	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

^c Absorbance area overestimated due to a co-eluting non-peptidic contaminant (observed in the HPLC analysis of a blank sample); absorbance area from a second analysis after contaminant removal: 1869.8 mAU·s.

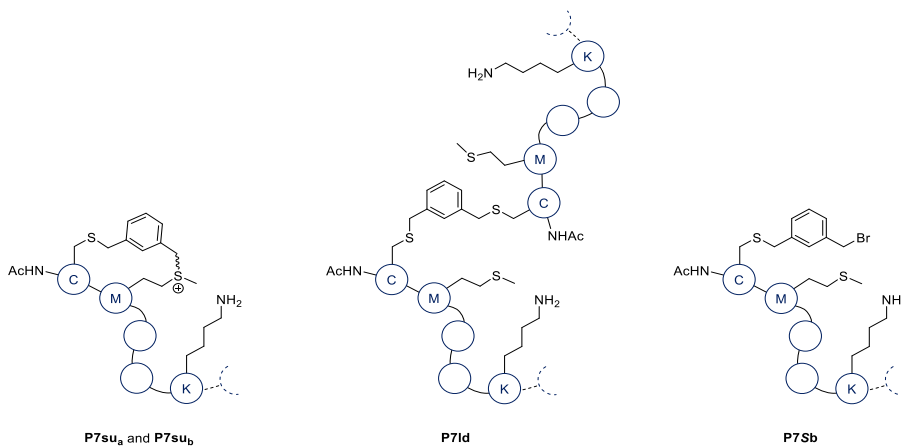
S,N-bis-Alkylation of P7, t = 0.5 h ($\lambda = 210$ nm; Method 3)



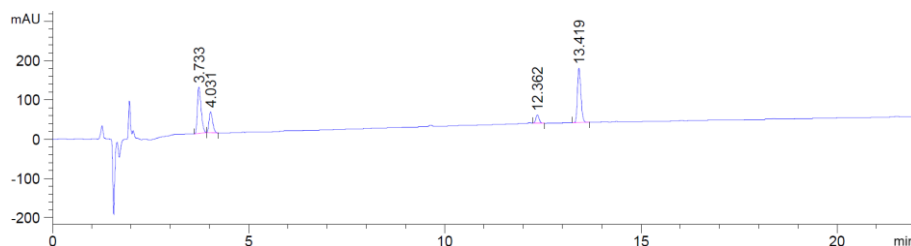
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.8	P7su_a	783.4	779.4	779.3
4.1	P7su_b	324.7	779.4	779.3
12.4	P7.1s	104.4	779.4	779.3
13.5	P7ld	870.6	1455.7	1455.6
16.8	P7Sb	24.3	859.3	859.2

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P7.1s** / (**P7su_a** + **P7su_b** + **P7.1s** + **P7ld** + **P7Sb**)] $\times$ 100 = 5%

Proposed structures of **P7su_a**, **P7su_b**, **P7ld**, and **P7Sb**:



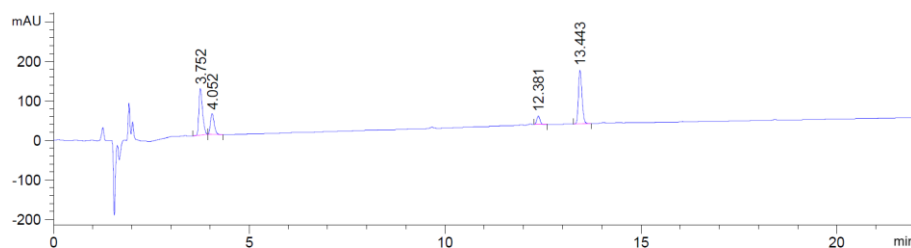
S,N-bis-Alkylation of P7, t = 1 h ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.7	P7su_a	781.1	779.4	779.3
4.0	P7su_b	334.1	779.4	779.3
12.4	P7.1s	122.6	779.4	779.3
13.4	P7ld	856.7	1455.7	1455.6

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P7.1s** / (**P7su_a** + **P7su_b** + **P7.1s** + **P7ld**)] $\times$ 100 = 6%

S,N-bis-Alkylation of P7, t = 2 h ($\lambda = 210$ nm; Method 3)

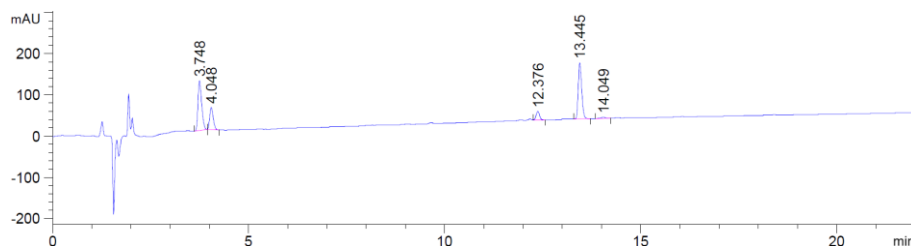


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.8	P7su_a	769.2	779.4	779.3
4.1	P7su_b	350.5	779.4	779.3
12.4	P7.1s	125.6	779.4	779.4
13.4	P7ld	850.6	1455.7	1455.6

^a $[M+H]^+$ ion, unless otherwise specified.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P7.1s** / (**P7su_a** + **P7su_b** + **P7.1s** + **P7ld**)] $\times$ 100 = 6%

S,N-bis-Alkylation of P7, t = 4 h ($\lambda = 210$ nm; Method 3)

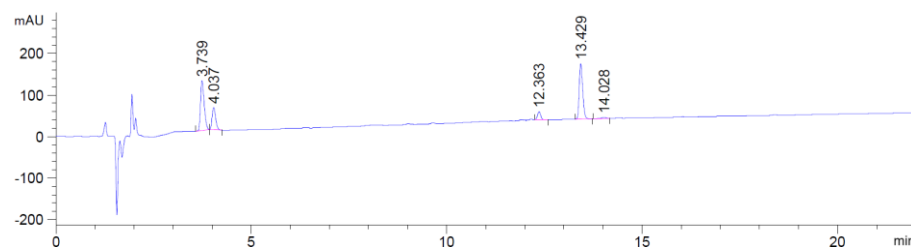


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.7	P7su_a	788.7	779.4	779.3
4.0	P7su_b	342.0	779.4	779.3
12.4	P7.1s	119.8	779.4	779.3
13.4	P7ld	834.9	1455.7	1455.5
14.0	—	22.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [P7.1s / (P7su_a + P7su_b + P7.1s + P7ld)] $\times$ 100 = 6%

S,N-bis-Alkylation of P7, t = 6 h ($\lambda = 210$ nm; Method 3)

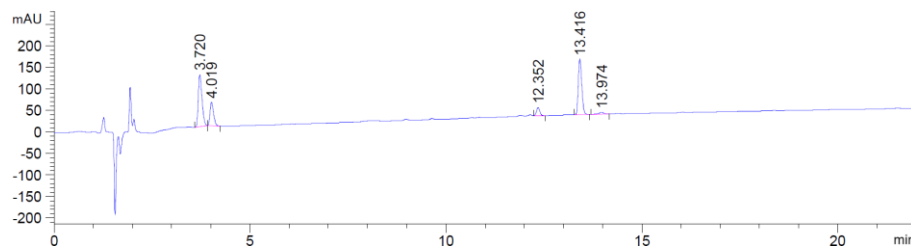


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.7	P7su_a	779.7	779.4	779.3
4.0	P7su_b	336.9	779.4	779.3
12.4	P7.1s	113.6	779.4	779.3
13.4	P7ld	820.2	1455.7	1455.6
14.0	—	27.7	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P7.1s / (P7su_a + P7su_b + P7.1s + P7Id)] × 100 = 6%

S,N-bis-Alkylation of P7, t = 8 h (λ = 210 nm; Method 3)

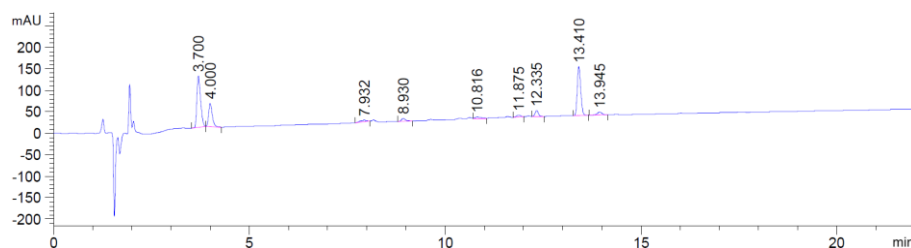


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
3.7	P7su_a	791.1	779.4	779.3
4.0	P7su_b	345.9	779.4	779.3
12.4	P7.1s	107.4	779.4	779.3
13.4	P7Id	788.9	1455.7	1455.6
14.0	—	35.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P7.1s / (P7su_a + P7su_b + P7.1s + P7Id)] × 100 = 5%

S,N-bis-Alkylation of P7, t = 24 h (λ = 210 nm; Method 3)



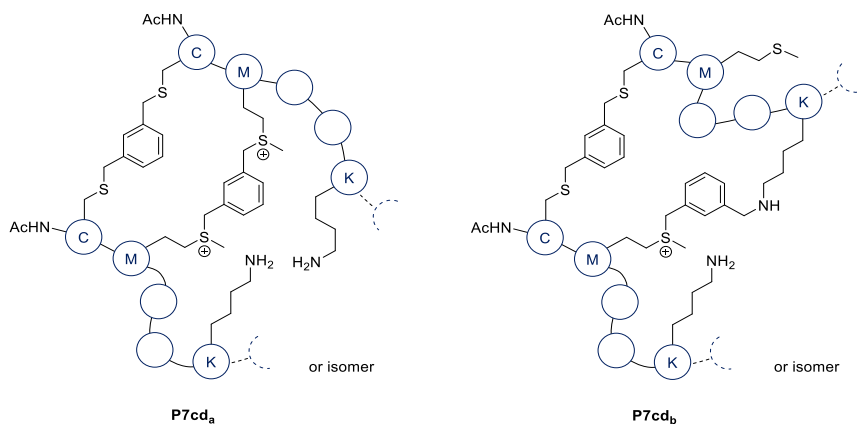
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
3.7	P7su_a	776.8	779.4	779.3
4.0	P7su_b	347.4	779.4	779.4
7.9	P7cd_a	42.9	779.4 ([M+2H] ²⁺)	779.3 ([M+2H] ²⁺)
8.9	P7.1NS'	48.2	881.4	881.4
10.8	P7cd_b	24.5	1557.8	1557.5
11.9	P7.1ol	30.7	797.4	797.3
	x (not identified)		915.4	915.4
12.3	P7.1s	78.2	779.4	779.3
13.4	P7ld	686.5	1455.7	1455.6
13.9	—	60.7	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

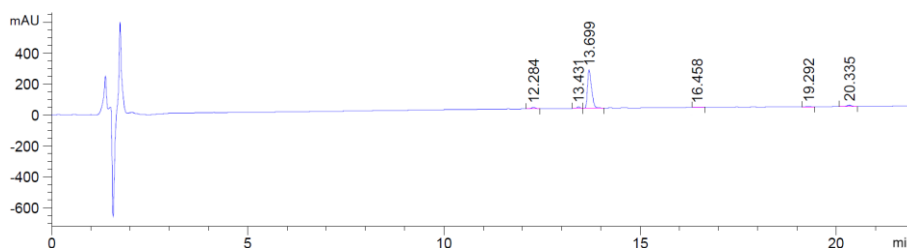
Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P7.1s / (P7su_a + P7su_b + P7cd_a + P7.1NS' + P7cd_b + P7.1ol + x + P7.1s + P7ld)] × 100 = 4%

Proposed structures of **P7cd_a** and **P7cd_b**:



Chromatograms related to Section S5.8.3 (*Tolerance toward Protected Lysine Residues*)

Entry 1, Table S8 ($\lambda = 210$ nm; Method 3)

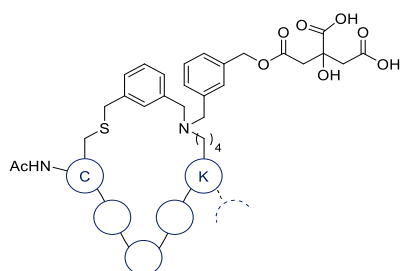


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	—	58.9	— ^a	— ^a
13.4	P12.1ol	50.4	820.4	820.4
13.7	P12.1s	1783.4	802.4	802.4
16.5	—	23.5	— ^a	— ^a
	P12.1Nc		1096.5	1096.3
19.3	P12.1Nb	22.7	984.4	984.3
20.3	—	72.8	— ^a	— ^a

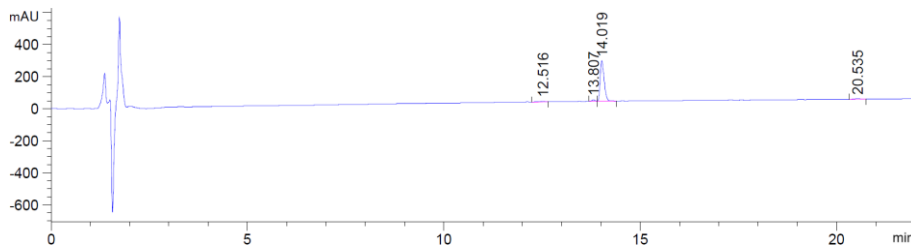
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P12.1s** / (**P12.1ol** + **P12.1s** + **P12.1Nc** + **P12.1Nb**)] $\times$ 100 = 96%

Proposed structure of **P12.1Nc**:



Entry 2, Table S8 ($\lambda = 210$ nm; Method 3)



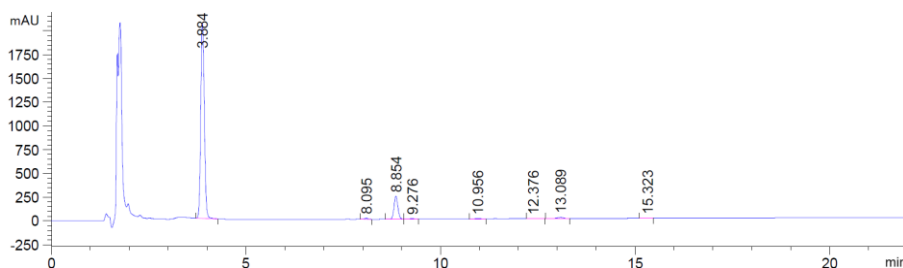
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.5	—	29.4	— ^a	— ^a
13.8	P13.1ol	43.5	878.5	878.5
14.0	P13.1s	1758.6	860.5	860.4
20.5	—	41.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P13.1s** / (**P13.1ol** + **P13.1s**)] $\times$ 100 = 98%

Chromatograms related to Section S5.8.4 (*One-Pot S-Alkylation/Reductive Amination/Pd-Mediated Deallyloxycarbonylation*)

Diluted reaction mixture ($\lambda = 210$ nm; Method 5; 15 min after addition of Pd(0)/TPPTS)

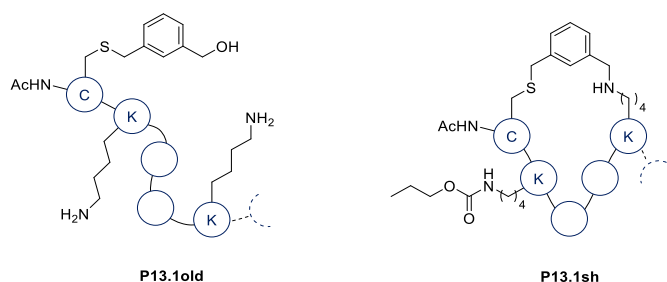


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
3.9	MPAA	13070.6	— ^a	— ^a
8.1	P13.1old	60.7	794.5	794.3
8.9	P13.1sd	1633.0	776.4	776.3
9.3	—	37.4	— ^a	— ^a
11.0	—	22.7	— ^a	— ^a
12.4	—	20.5	— ^a	— ^a
13.1	—	135.6	— ^a	— ^a
15.3	P13.1sh	21.9	862.5	862.3

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

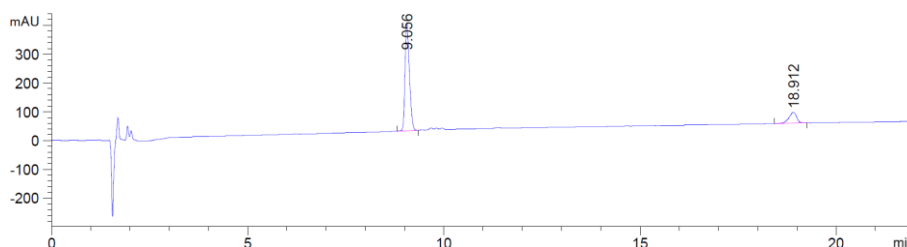
Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P13.1sd** / (**P13.1old** + **P13.1sd** + **P13.1sh**)] × 100 = 95%

Proposed structures of **P13.1old** and **P13.1sh**:



Chromatograms related to Section S5.9 (*Cys–Dap Stapling in the Presence of a Lysine Residue*)

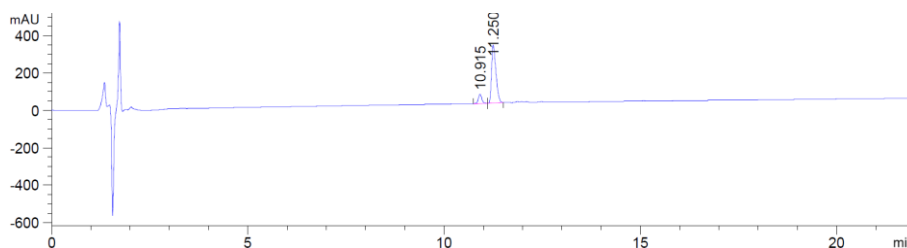
Entry **P14.1A** (λ = 210 nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.1	P14.1al	2897.3	1149.6	1149.5
18.9	1	522.7	— ^a	— ^a

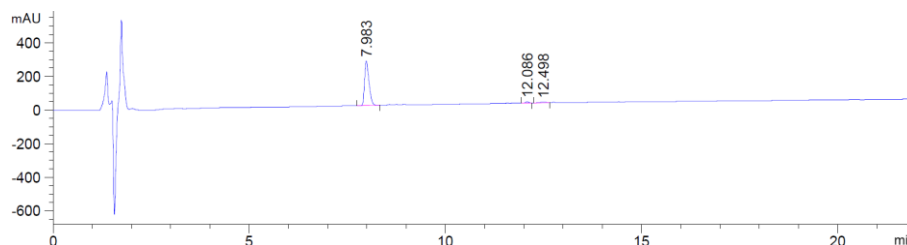
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry P14.10 ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
10.9	P14.1ox_b	289.9	1178.6	1178.5
11.2	P14.1ox_a	2374.8	1178.6	1178.5

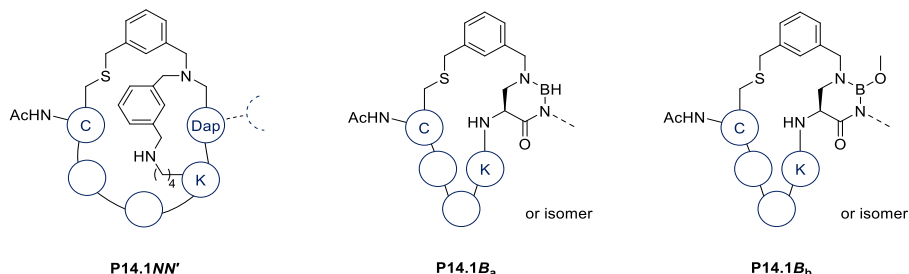
Entry 1, Table S9 ($\lambda = 210$ nm; Method 3)



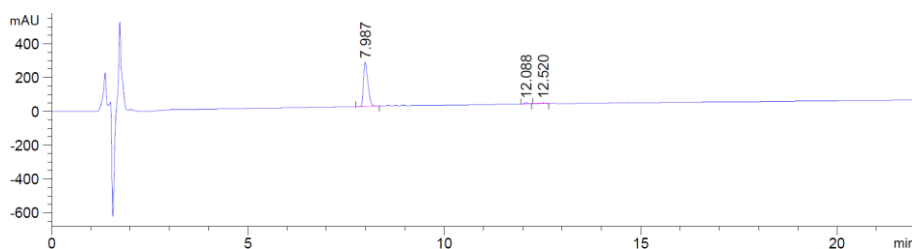
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	2073.5	1133.6	1133.4
12.1	P14.1B_a	42.9	1143.6	1143.5
12.5	P14.1NN'	46.2	1235.7	1235.5
	P14.1B_b		1173.6	1173.4

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P14.1s** / (**P14.1s** + **P14.1B_a** + **P14.1NN'** + **P14.1B_b**)] $\times$ 100 = 96%

Proposed structures of **P14.1NN'**, **P14.1B_a**, and **P14.1B_b**:



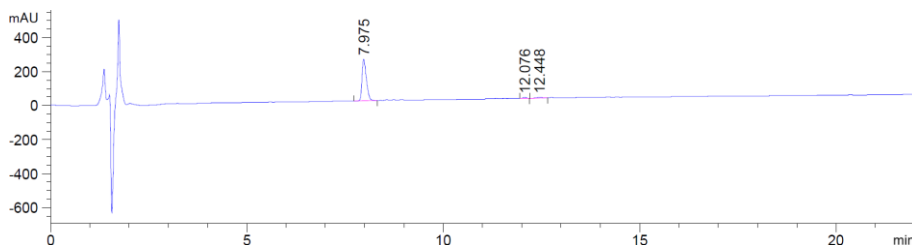
Entry 1 bis, Table S9 ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	2041.1	1133.6	1133.4
12.1	P14.1B_a	43.5	1143.6	1143.6
12.5	P14.1NN'	41.3	1235.7	1235.6
	P14.1B_b		1173.6	1173.6

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P14.1s** / (**P14.1s** + **P14.1B_a** + **P14.1NN'** + **P14.1B_b**)] $\times$ 100 = 96%

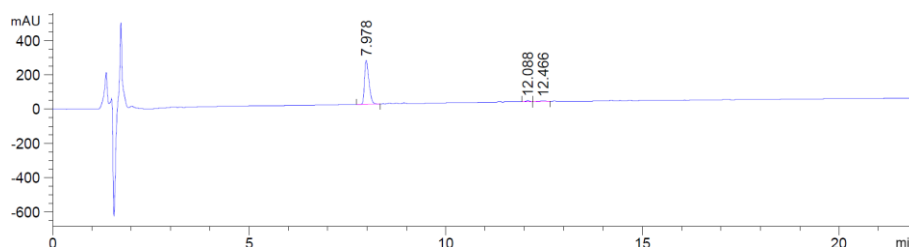
Entry 2, Table S9 ($\lambda = 210$ nm; Method 3)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	1870.0	1133.6	1133.5
12.1	P14.1B_a	35.3	1143.6	1143.5
12.4	P14.1NN'	41.2	1235.7	1235.5
	P14.1B_b		1173.6	1173.5

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P14.1s** / (**P14.1s** + **P14.1B_a** + **P14.1NN'** + **P14.1B_b**)] × 100 = 96%

Entry 2 bis, Table S9 (λ = 210 nm; Method 3)

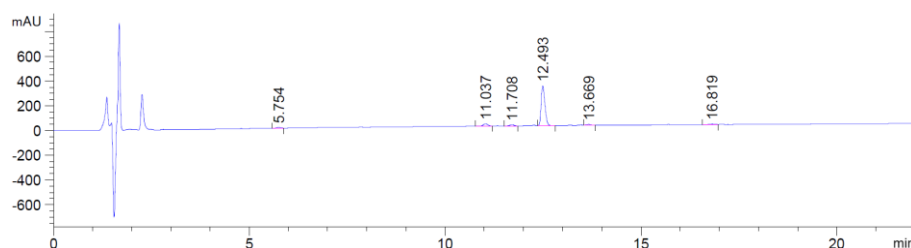


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	1977.6	1133.6	1133.5
12.1	P14.1B_a	39.5	1143.6	1143.4
12.5	P14.1NN'	41.3	1235.7	1235.4
	P14.1B_b		1173.6	1173.5

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P14.1s** / (**P14.1s** + **P14.1B_a** + **P14.1NN'** + **P14.1B_b**)] × 100 = 96%

Chromatograms related to Section S5.10 (*Post-Stapling Modification via Intermolecular Reductive Amination*)

Entry 1, Table S10 ($\lambda = 210$ nm; Method 2)



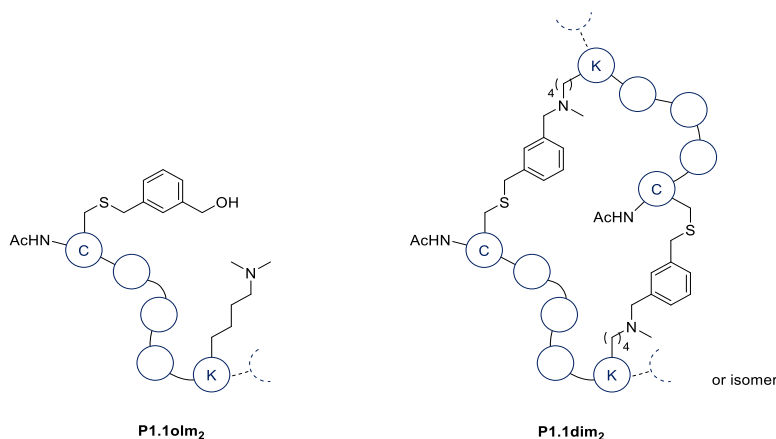
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
5.8	—	41.6	— ^b	— ^b
11.0	—	150.3	— ^b	— ^b
11.7	P1.1olm₂	70.4	1107.6	1107.5
12.5	P1.1Nm	2135.3	1075.6	1075.5
13.7	P1.1dim₂	50.1	1075.6 ([M+2H] ²⁺)	1075.6 ([M+2H] ²⁺)
16.8	—	36.4	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

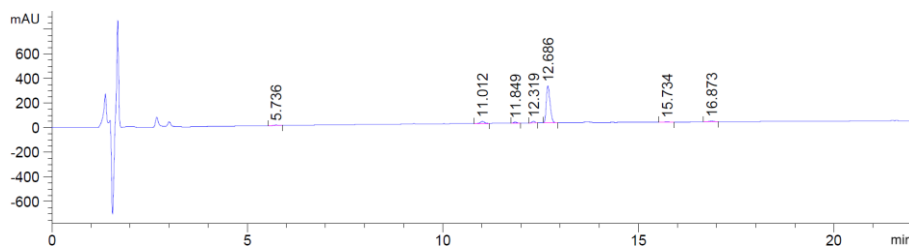
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.1Nm** / (**P1.1olm₂** + **P1.1Nm** + **P1.1dim₂**)] × 100 = 95%

Proposed structures of **P1.1olm₂** and **P1.1dim₂**:



Entry 2, Table S10 ($\lambda = 210$ nm; Method 2)

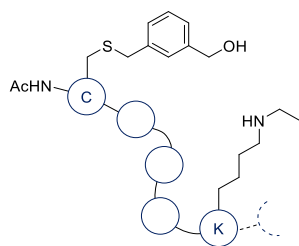


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
5.7	—	26.9	— ^a	— ^a
11.0	—	120.6	— ^a	— ^a
11.8	P1.1ole	39.5	1107.6	1107.5
12.3	P1.1s	67.9	1061.6	1061.5
12.7	P1.1Ne	1986.5	1089.6	1089.5
15.7	P1.1Nb	25.6	1243.5	1243.4
16.9	—	55.8	— ^a	— ^a

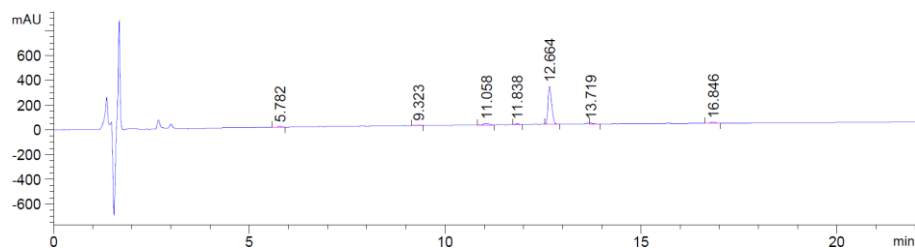
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1Ne** / (**P1.1ole** + **P1.1s** + **P1.1Ne** + **P1.1Nb**)] $\times$ 100 = 94%

Proposed structure of **P1.1ole**:



Entry 3, Table S10 ($\lambda = 210$ nm; Method 2)



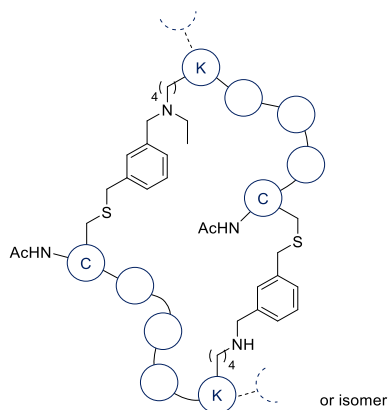
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
5.8	—	36.5	— ^b	— ^b
9.3	—	22.3	— ^b	— ^b
11.1	—	116.2	— ^b	— ^b
11.8	P1.1ole	43.9	1107.6	1107.5
12.7	P1.1Ne	2011.1	1089.6	1089.6
13.7	P1.1die	44.8	1075.6 ([M+2H] ²⁺)	1075.8 ([M+2H] ²⁺)
16.8	—	65.5	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

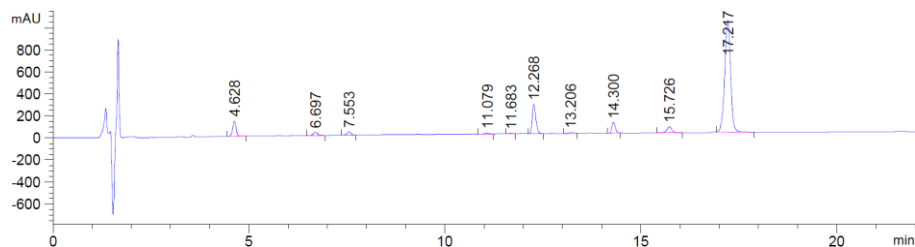
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.1Ne** / (**P1.1ole** + **P1.1Ne** + **P1.1die**)] × 100 = 96%

Proposed structure of **P1.1die**:



Entry 4, Table S10 ($\lambda = 210$ nm; Method 2)

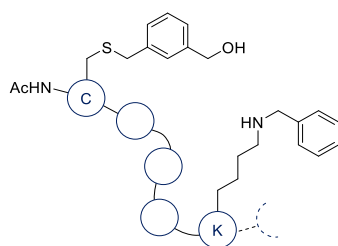


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
4.6	—	829.9	— ^a	— ^a
6.7	—	222.6	— ^a	— ^a
7.6	—	217.6	— ^a	— ^a
11.1	—	74.4	— ^a	— ^a
11.7	P1.1ol	32.3	1079.6	1079.5
12.3	P1.1s	1700.9	1061.6	1061.5
13.2	P1.1olbn	59.9	1169.6	1169.6
14.3	P1.1Nbn	598.6	1151.6	1151.5
15.7	—	513.8	— ^a	— ^a
17.2	—	10552.0	— ^a	— ^a

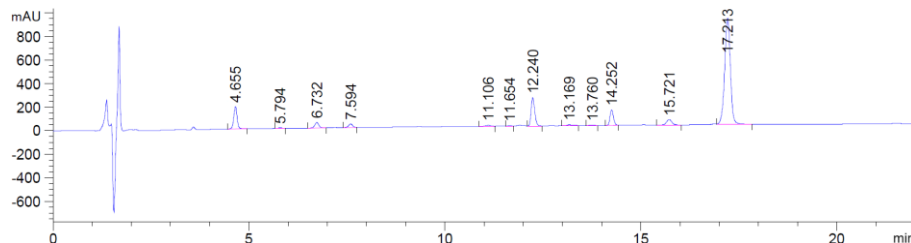
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1Nbn** / (**P1.1ol** + **P1.1s** + **P1.1olbn** + **P1.1Nbn**)] $\times$ 100 = 25%

Proposed structure of **P1.1olbn**:



Entry 5, Table S10 ($\lambda = 210$ nm; Method 2)

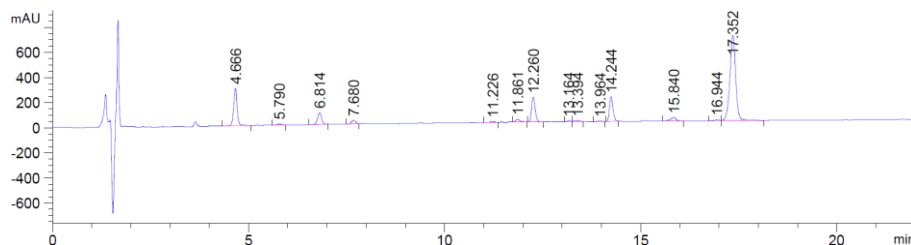


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
4.7	—	1117.9	— ^a	— ^a
5.8	—	32.2	— ^a	— ^a
6.7	—	341.0	— ^a	— ^a
7.6	—	214.9	— ^a	— ^a
11.1	—	73.1	— ^a	— ^a
11.7	P1.1ol	24.6	1079.6	1079.5
12.2	P1.1s	1524.4	1061.6	1061.5
13.2	P1.1olbn	66.4	1169.6	1169.6
13.8	—	32.3	— ^a	— ^a
14.3	P1.1Nbn	798.5	1151.6	1151.5
15.7	—	442.5	— ^a	— ^a
17.2	—	9032.9	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.1Nbn** / (**P1.1ol** + **P1.1s** + **P1.1olbn** + **P1.1Nbn**)] $\times$ 100 = 33%

Entry 6, Table S10 ($\lambda = 210$ nm; Method 2)



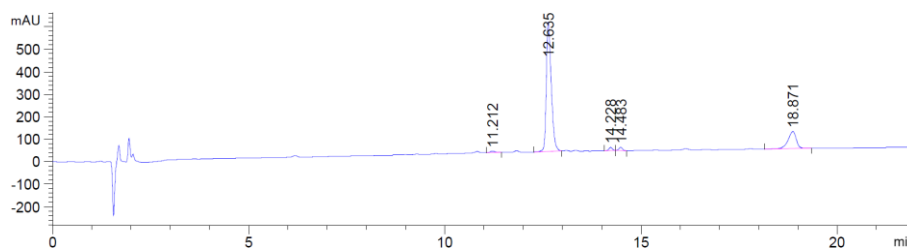
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
4.7	—	1812.5	— ^a	— ^a
5.8	—	65.7	— ^a	— ^a
6.8	—	683.6	— ^a	— ^a
7.7	—	208.6	— ^a	— ^a
11.2	—	54.2	— ^a	— ^a
11.9	x (not identified)	177.5	—	288.2
12.3	P1.1s	1258.2	1061.6	1061.6
13.2	P1.1olbn	31.5	1169.6	1169.6
13.4	—	49.7	— ^a	— ^a
14.0	—	43.2	— ^a	— ^a
14.2	P1.1Nbn	1237.4	1151.6	1151.6
15.8	—	295.1	— ^a	— ^a
16.9	—	48.6	— ^a	— ^a
17.4	—	6926.0	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.1Nbn / (P1.1s + P1.1olbn + P1.1Nbn)] × 100 = 49%

Chromatograms related to Section S5.11.1 (*One-Pot bis-S-Alkylation/bis-Reductive Amination*)

Entry P15.1A (λ = 210 nm; Method 3)

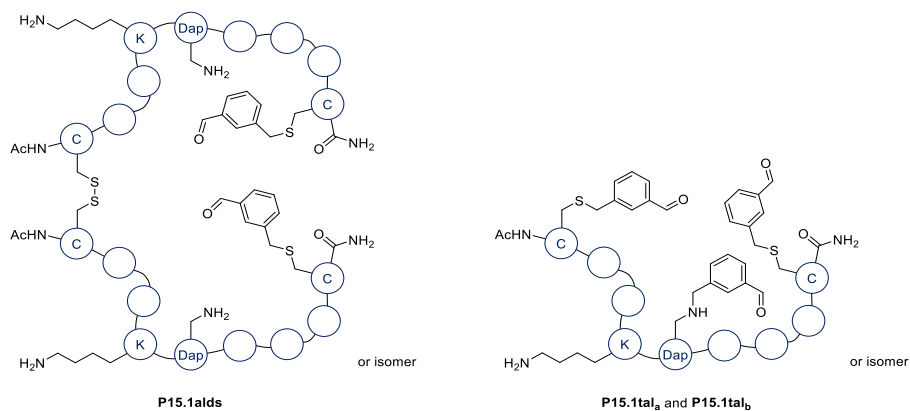


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
11.2	P15.1alds	57.4	787.4 ([M+3H] ³⁺)	787.6 ([M+3H] ³⁺)
12.6	P15.1al	5049	1299.6	1299.5
14.2	P15.1tal_a	89.8	1417.7	1417.5
14.5	P15.1tal_b	85.5	1417.7	1417.6
18.9	1	1114.8	— ^b	— ^b

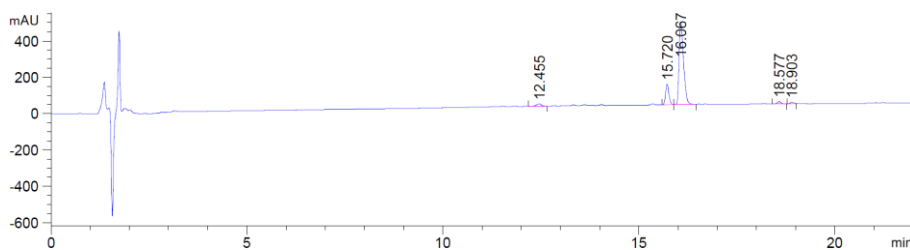
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structures of **P15.1alds**, **P15.1tal_a**, and **P15.1tal_b**:



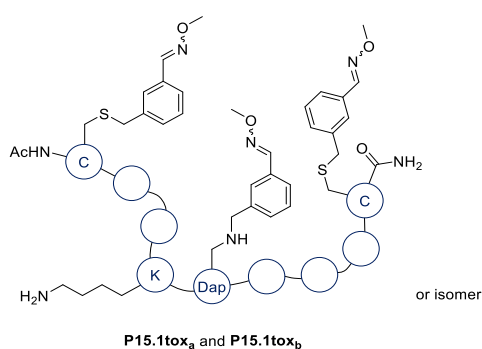
Entry P15.10 ($\lambda = 210$ nm; Method 3)



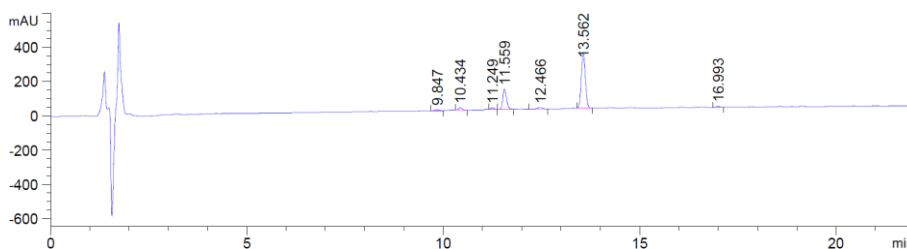
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.5	—	115.1	— ^a	— ^a
15.7	P15.1tox_b	670.9	1357.7	1357.5
16.1	P15.1tox_a	3606.9	1357.7	1357.5
18.6	P15.1tox_a	87.3	1504.7	1504.6
18.9	P15.1tox_b	49.9	1504.7	1504.6

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structure of **P15.1tox_a** and **P15.1tox_b**:



Entry 1, Table S11 ($\lambda = 210$ nm; Method 3)

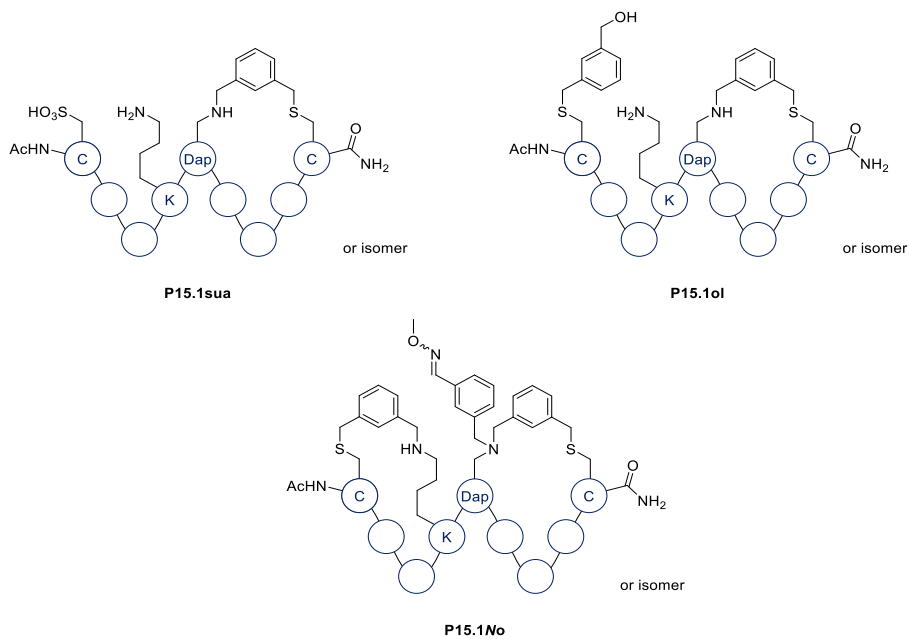


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.8	P15.1sua	48.4	1213.6	1213.5
10.4	P15.1s_c	91.9	1267.6	1267.5
11.2	P15.1ol	54.9	1285.6	1285.5
11.6	P15.1s_b	782.7	1267.6	1267.5
12.5	—	87.4	— ^a	— ^a
13.6	P15.1s_a	2296.4	1267.6	1267.5
17.0	P15.1No	39.2	1414.7	1414.5

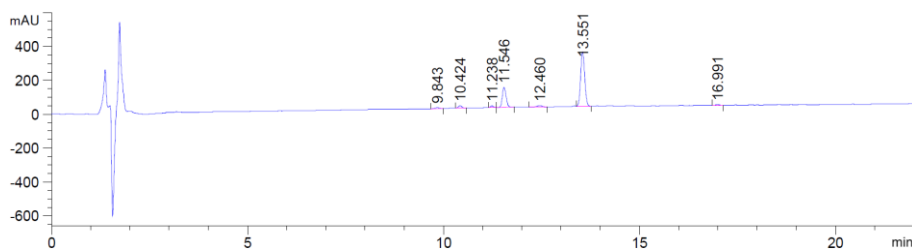
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [P15.1s_a / (P15.1s_{ua} + P15.1s_c + P15.1ol + P15.1s_b + P15.1s_a + P15.1No)] × 100 = 69%

Proposed structures of P15.1s_{ua}, P15.1ol, and P15.1No:



Entry 1 bis, Table S11 (λ = 210 nm; Method 3)



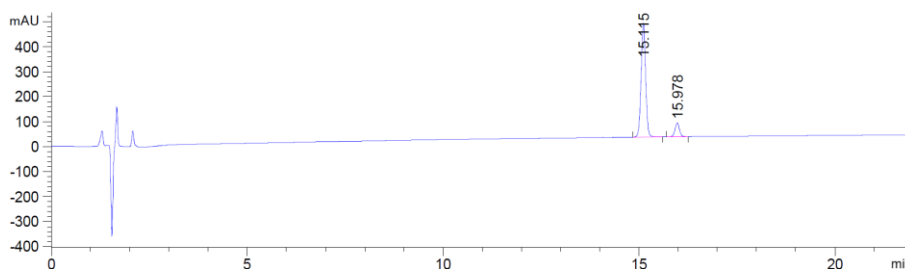
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.8	P15.1sua	47.9	1213.6	1213.4
10.4	P15.1sc	90.9	1267.6	1267.5
11.2	P15.1ol	53.1	1285.6	1285.5
11.5	P15.1sb	787.2	1267.6	1267.5
12.5	—	83.3	— ^a	— ^a
13.6	P15.1sa	2275.7	1267.6	1267.5
17.0	P15.1No	36.4	1414.7	1414.6

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P15.1sa** / (**P15.1sua** + **P15.1sc** + **P15.1ol** + **P15.1sb** + **P15.1sa** + **P15.1No**)] × 100 = 69%

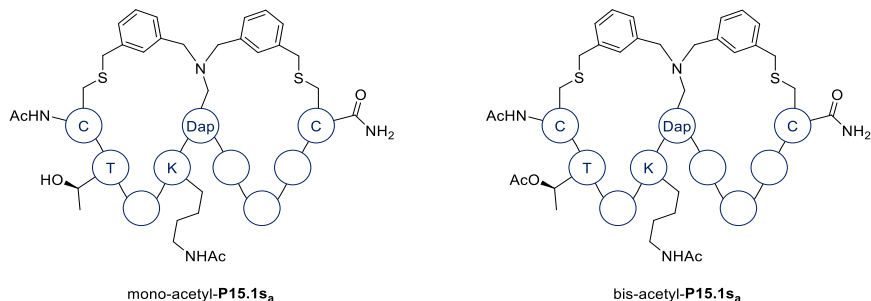
Chromatograms related to Section S5.11.2 (*Connectivity Elucidation by Acetylation*)

Acetylation of P15.1sa ($\lambda = 210$ nm; Method 2; 30 min after addition of Ac₂O)

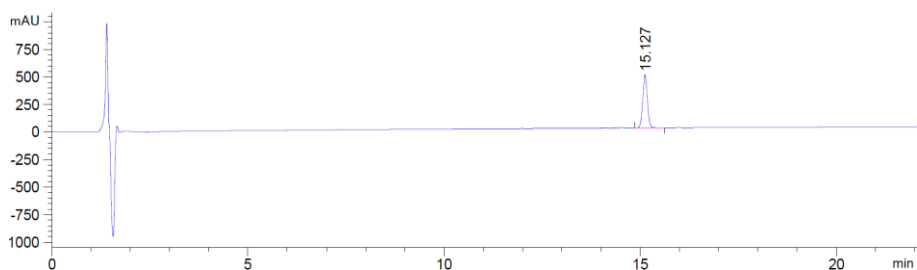


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
15.1	mono-acetyl- P15.1sa	3644.1	1309.6	1309.5
16.0	bis-acetyl- P15.1sa	428.0	1351.6	1351.5

Proposed structures of mono-acetyl-**P15.1s_a** and bis-acetyl-**P15.1s_a**:

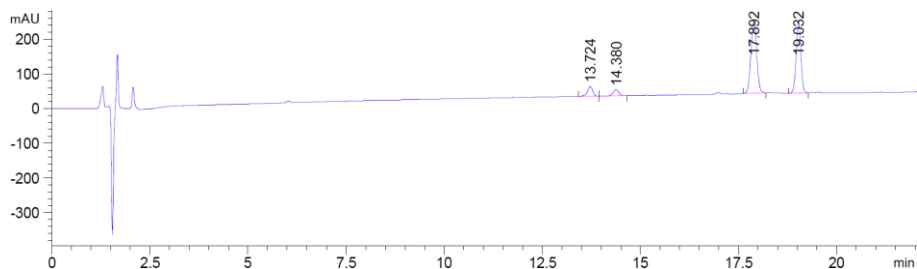


Acetylation of P15.1s_a ($\lambda = 210$ nm; Method 2; 2 h after 10-fold dilution into sodium carbonate buffer, 50 mM, pH 10.0)

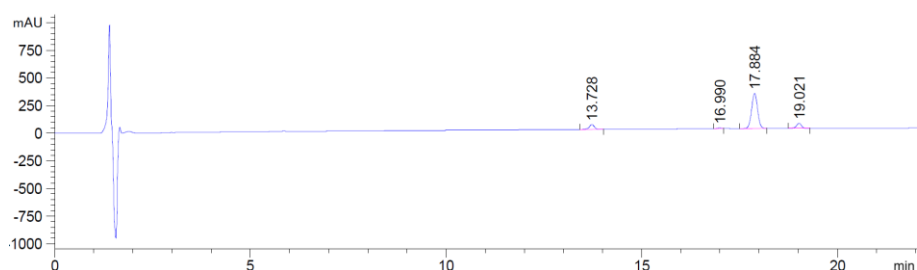


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
15.1	mono-acetyl- P15.1s_a	3867.2	1309.6	1309.5

Acetylation of P15.1s_b ($\lambda = 210$ nm; Method 2; 30 min after addition of Ac_2O)



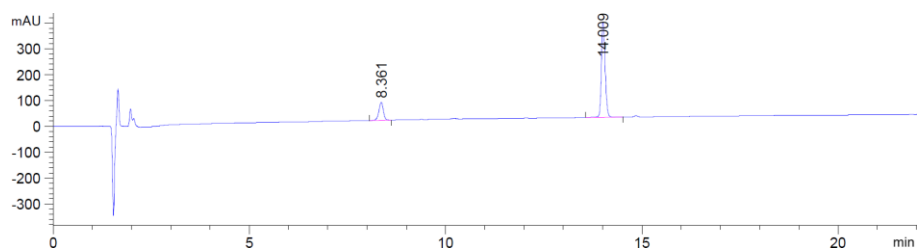
Acetylation of P15.1s_b ($\lambda = 210$ nm; Method 2; 2 h after 10-fold dilution into sodium carbonate buffer, 50 mM, pH 10.0)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.7	mono-acetyl- P15.1s_b	419.2	1309.6	1309.3
17.0	x (not identified)	44.3	—	1479.6
	y (not identified)		—	1369.4
17.9	bis-acetyl- P15.1s_a (isomer 2)	3352.5	1351.6	1351.4
19.0	tris-acetyl- P15.1s_b	421.8	1393.7	1393.4

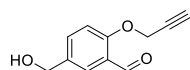
Chromatograms related to Section S6.1.2 (Stapling with 5-(bromomethyl)-2-(prop-2-yn-1-yloxy)benzaldehyde)

Entry P1.2A ($\lambda = 210$ nm; Method 2)

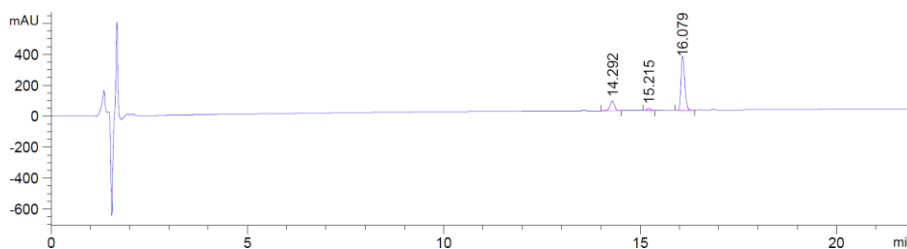


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.4	2ol	540.8	191.1	191.1
14.0	P1.2al	2462.0	1131.6	1131.5

Proposed structure of **2ol**:

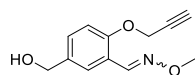


Entry P1.2O (λ = 210 nm; Method 2)

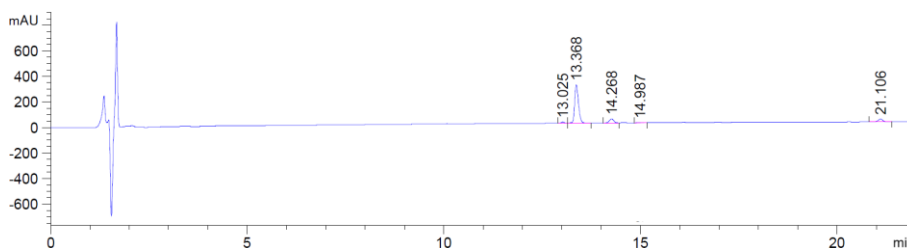


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.3	2ox	486.0	220.1	220.1
15.2	P1.2ox_b	78.8	1160.6	1160.5
16.1	P1.2ox_a	2249.0	1160.6	1160.6

Proposed structure of **2ox**:



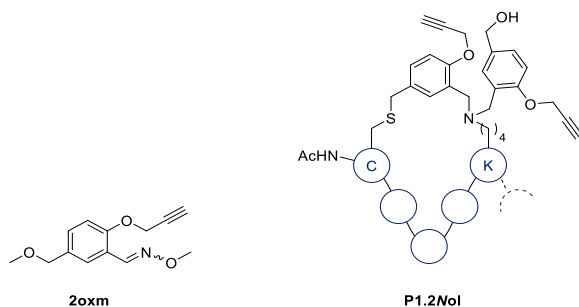
Entry 1, Table S13 (λ = 210 nm; Method 2)



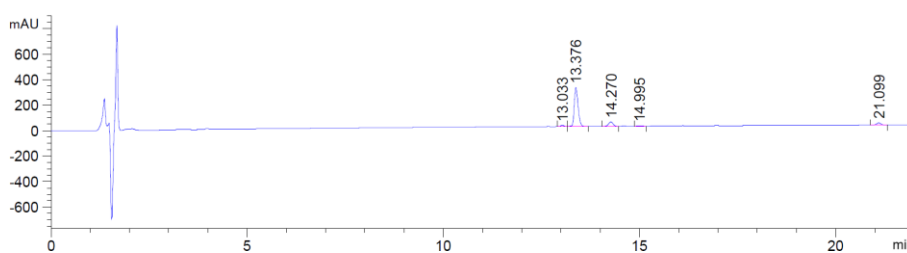
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.0	P1.2ol	52.1	1133.6	1133.5
13.4	P1.2s	2031.4	1115.6	1115.5
14.3	2ox	237.5	220.1	220.1
15.0	P1.2Nol	23.2	1289.7	1289.5
21.1	2oxm	186.1	234.1	234.1

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
= [**P1.2s** / (**P1.2ol** + **P1.2s** + **P1.2Nol**)] $\times$ 100 = 96%

Proposed structures of **2oxm** and **P1.2Nol**:



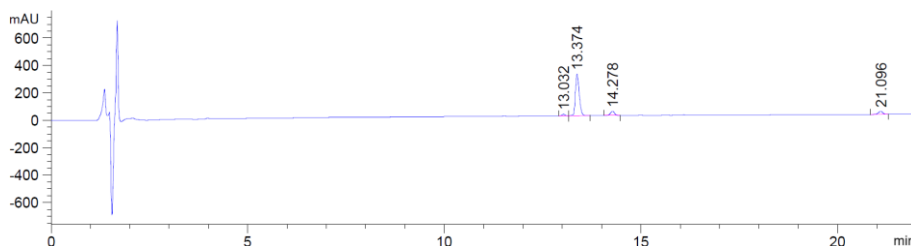
Entry 1 bis, Table S13 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.0	P1.2ol	54.4	1133.6	1133.5
13.4	P1.2s	2061.4	1115.6	1115.5
14.3	2ox	263.5	220.1	220.1
15.0	P1.2Nol	21.4	1289.7	1289.6
21.1	2oxm	150.1	234.1	234.1

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.2s / (P1.2ol + P1.2s + P1.2Nol)] × 100 = 96%

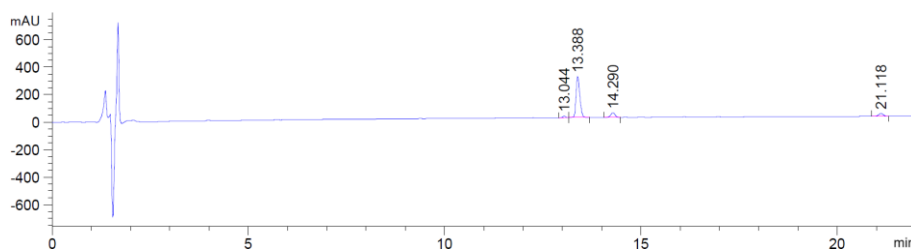
Entry 2, Table S13 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.0	P1.2ol	59.7	1133.6	1133.5
13.4	P1.2s	2059.1	1115.6	1115.5
14.3	2ox	235.8	220.1	220.1
21.1	2oxm	184.7	234.1	234.1

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.2s / (P1.2ol + P1.2s)] × 100 = 97%

Entry 2 bis, Table S13 (λ = 210 nm; Method 2)

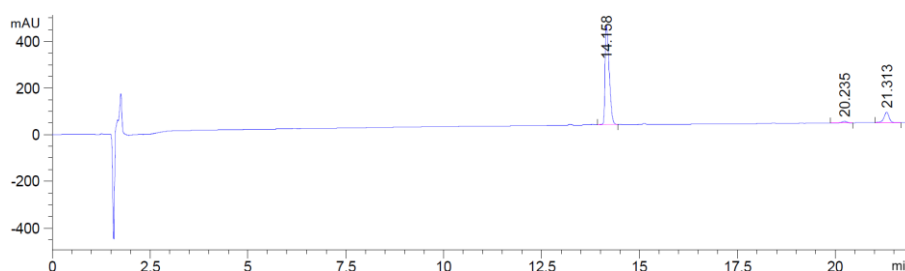


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.0	P1.2ol	60.2	1133.6	1133.5
13.4	P1.2s	2031.7	1115.6	1115.5
14.3	2ox	263.6	220.1	220.1
21.1	2oxm	168.3	234.1	234.1

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [P1.2s / (P1.2ol + P1.2s)] × 100 = 97%

Chromatograms related to Section S6.1.3 (Stapling with *o*- and *p*-(bromomethyl)benzaldehyde)

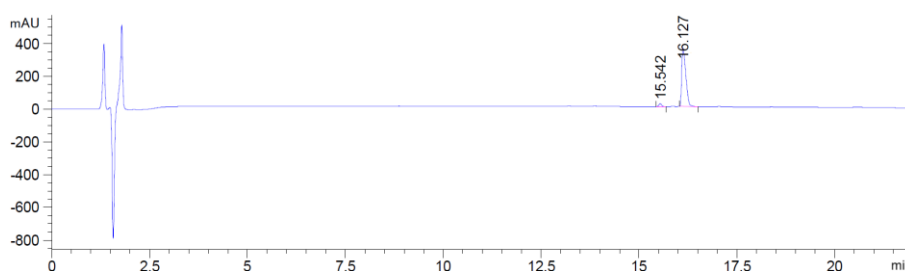
Entry P1.3A (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.2	P1.3al	3061.8	1077.6	1077.4
20.2	—	67.6	— ^a	— ^a
21.3	—	395.2	— ^a	— ^a

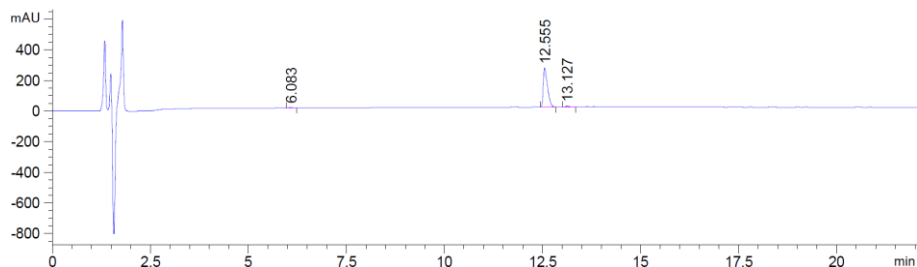
^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry P1.3O (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
15.5	P1.3ox_b	88.7	1106.6	1106.4
16.1	P1.3ox_a	2564.4	1106.6	1106.4

Entry 1, Table S14 ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
6.1	—	20.9	— ^b	— ^b
12.6	P1.3s	1912.9	1061.6	1061.4
13.1 ^c	P1.3ol	45.5	1079.6	1079.4
	P1ds		958.5 ([M+2H] ²⁺)	958.5 ([M+2H] ²⁺)

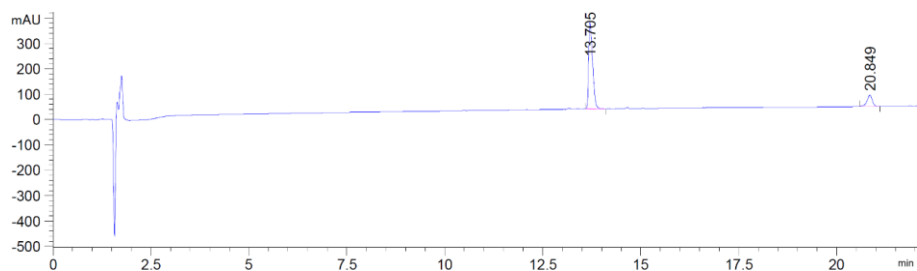
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

^c Ratio of EIC peak areas for **P1.3ol** (m/z 539.8–540.8, [M+2H]²⁺) and **P1ds** (m/z 958.1–959.1, [M+2H]²⁺) ~61:39.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = [**P1.3s** / (**P1.3s** + **P1.3ol** + **P1ds**)] $\times$ 100 = 98%

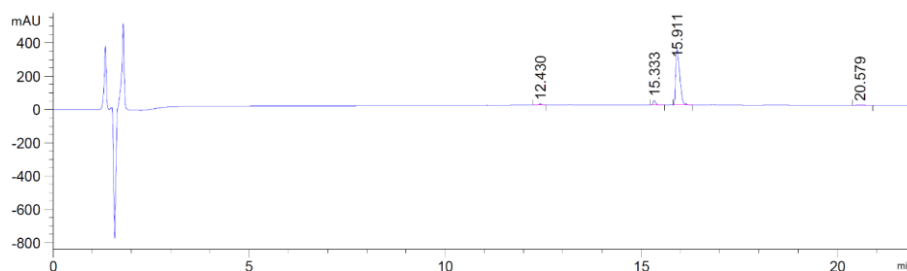
Entry P1.4A ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.7	P1.4al	2373.7	1077.6	1077.4
20.8	—	399.2	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

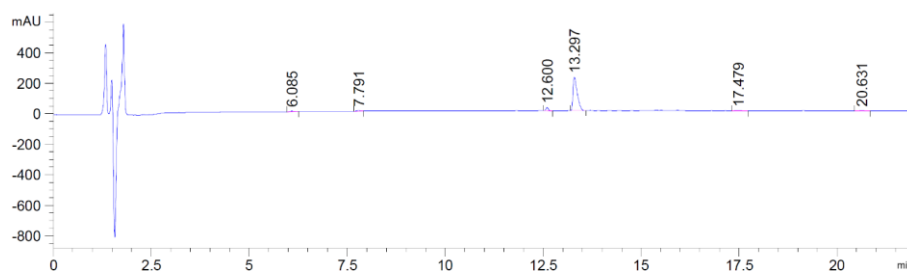
Entry P1.4O (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.4	—	40.4	— ^a	— ^a
15.3	P1.4ox_b	130.4	1106.6	1106.4
15.9	P1.4ox_a	2388.1	1106.6	1106.5
20.6	—	32.7	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry 1, Table S15 (λ = 210 nm; Method 2)



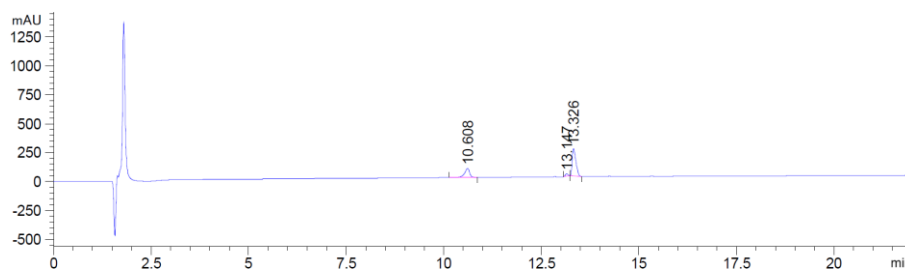
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
6.1	—	20.3	— ^a	— ^a
7.8	—	22.3	— ^a	— ^a
12.6	P1.4ol	94.9	1079.6	1079.5
13.3	P1.4s	1644.8	1061.6	1061.4
17.5	P1.4Nb	24.5	1243.5	1243.4
20.6	—	21.4	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.4s** / (**P1.4ol** + **P1.4s** + **P1.4Nb**)] × 100 = 93%

Chromatograms related to Section S6.1.4 (Stapling via Thia-Michael Addition/Reductive Amination)

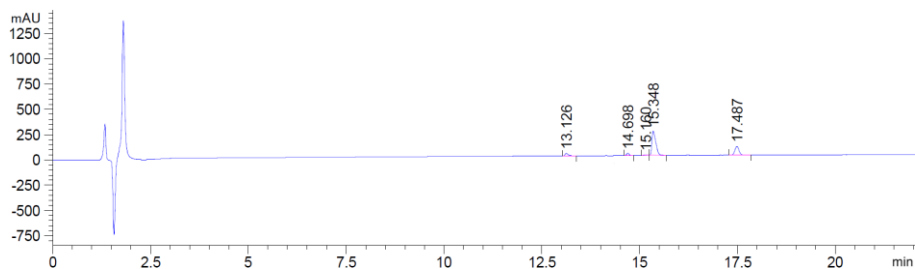
Entry **P1.5A** (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
10.6	5	691.8	176.1	176.1
13.1	P1ds	94.2	958.5 ($[M+2H]^{2+}$)	958.6 ($[M+2H]^{2+}$)
13.3	P1.5al	1523.5	1134.6	1134.4

^a $[M+H]^+$ ion, unless otherwise specified.

Entry P1.5O ($\lambda = 210$ nm; Method 2)

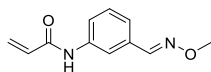


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
13.1	P1ds	141.4	958.5 ([M+2H] ²⁺)	958.5 ([M+2H] ²⁺)
14.7	P1.5ox_b	106.3	1163.6	1163.5
15.2	—	29.7	— ^b	— ^b
15.3	P1.5ox_a	1616.8	1163.6	1163.4
17.5	5ox_a	640.0	205.1	205.1

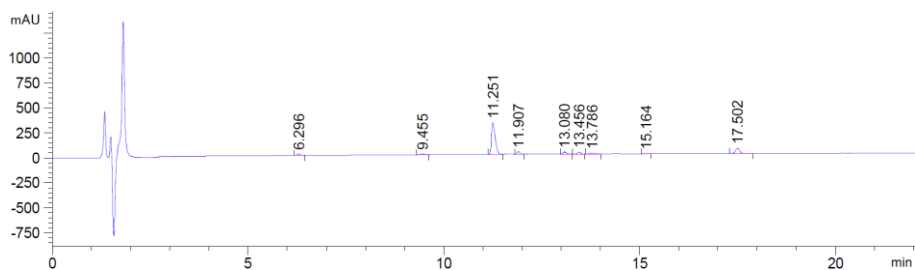
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structure of **5ox_a**:



Entry 1, Table S16 ($\lambda = 210$ nm; Method 2)



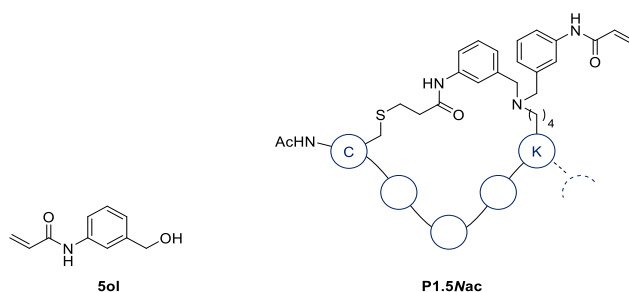
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
6.3	5ol	79.9	178.1	178.1
9.5	—	44.2	— ^b	— ^b
11.3	P1.5s	2057.9	1118.6	1118.4
11.9	P1.5ol	116.1	1136.6	1136.5
13.1	P1ds	118.0	958.5 ([M+2H] ²⁺)	958.6 ([M+2H] ²⁺)
13.5	P1.5Nac	103.6	1277.7	1277.5
13.8	x (not identified)	60.4	—	190.1
15.2	5ox_b	25.6	205.1	205.1
17.5	5ox_a	402.6	205.1	205.1

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

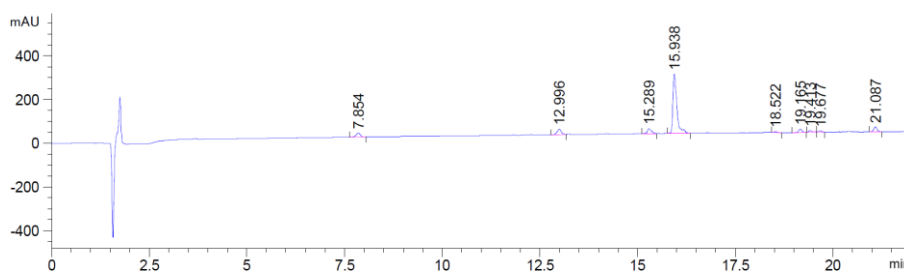
Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [**P1.5s** / (**P1.5s** + **P1.5ol** + **P1ds** + **P1.5Nac**)] × 100 = 86%

Proposed structures of **5ol** and **P1.5Nac**:



Chromatograms related to Section S6.1.5 (Stapling via Pd-Catalyzed S-Allylation/Reductive Amination)

Entry **P1.6A** ($\lambda = 210$ nm; Method 2)



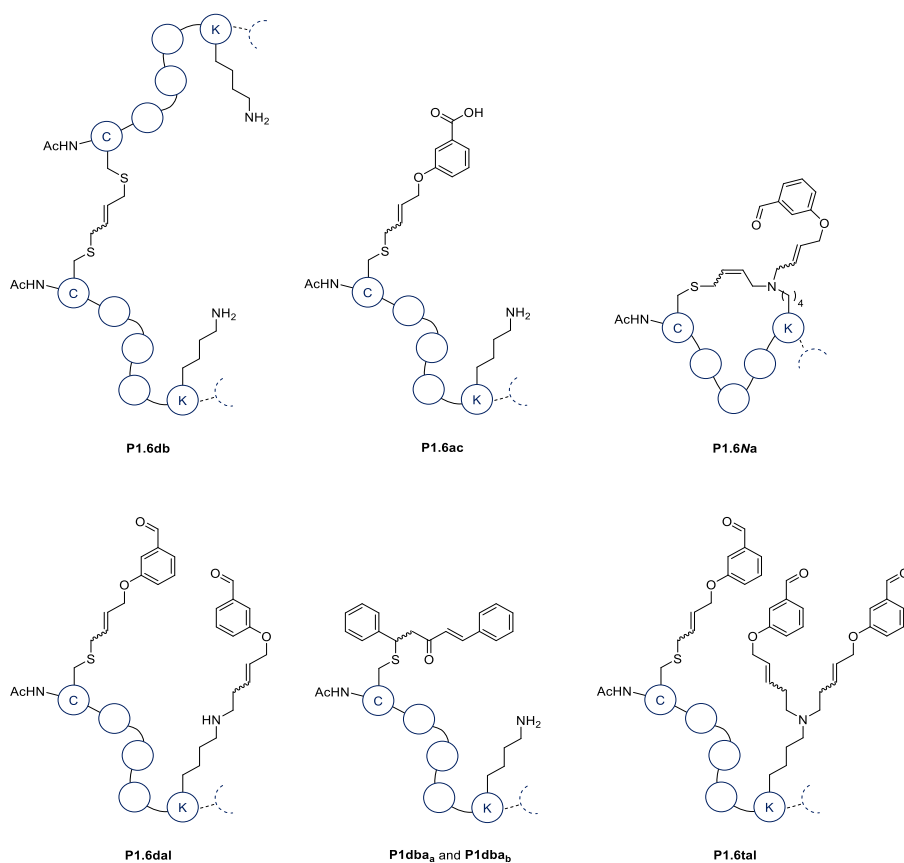
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
7.9	—	137.4	— ^b	— ^b
13.0	P1.6db	180.2	985.5 ([M+2H] ²⁺)	985.8 ([M+2H] ²⁺)
	P1.ds		958.5 ([M+2H] ²⁺)	959.0 ([M+2H] ²⁺)
15.3	P1.6ac	155.9	1149.6	1149.4
	P1.6Na		1185.6	1185.4
15.9 ^c	P1.6al	1898.2	1133.6	1133.4
18.5	P1.6dal	20.9	1307.7	1307.5
19.2	P1.dba_a	91.9	1193.6	1193.4
19.4	P1.dba_b	47.8	1193.6	1193.5
19.7	x (not identified)	28.4	—	540.2
21.1	P1.6tal	144.3	1481.7	1481.5

^a [M+H]⁺ ion, unless otherwise specified.

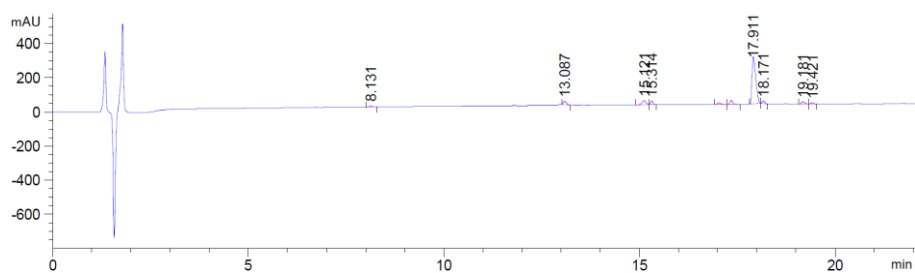
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

^c Peak exhibiting a shoulder.

Proposed structures of **P1.6db**, **P1.6ac**, **P1.6Na**, **P1.6dal**, **P1dba_a**, **P1dba_b**, and **P1.6tal**:



Entry P1.60 ($\lambda = 210$ nm; Method 2)

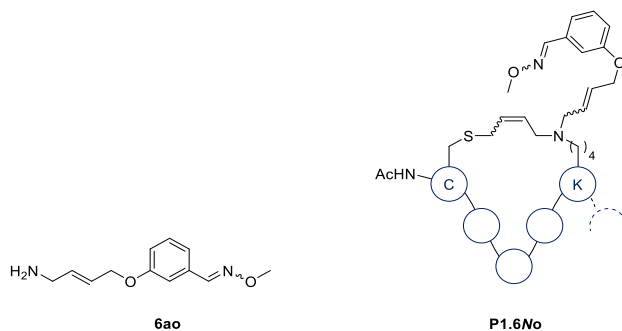


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
8.1	6ao	29.3	221.1	221.1
13.1	P1ds	96.8	958.5 ([M+2H] ²⁺)	958.6 ([M+2H] ²⁺)
15.1	—	155.1	— ^b	— ^b
15.3	P1.6ac	88.3	1149.6	1149.4
17.0	P1.6No	61.4	1214.7	1214.5
17.3	P1.6ox_b	121.3	1162.6	1162.5
17.9	P1.6ox_a	1840.0	1162.6	1162.5
18.2	P1.6ox_c	70.9	1162.6	1162.5
19.2	P1dba_a	81.9	1193.6	1193.4
19.4	P1dba_b	40.1	1193.6	1193.5

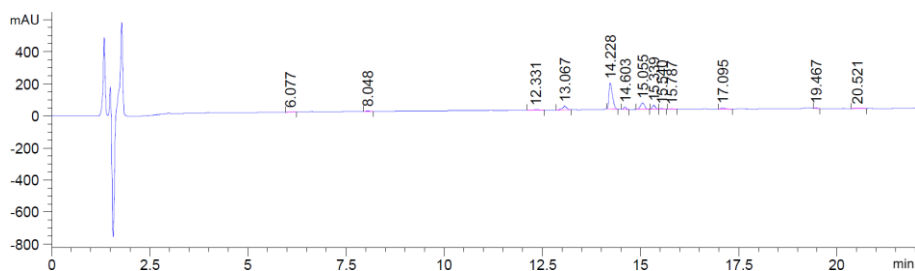
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structures of **6ao** and **P1.6No**:



Entry 1, Table S17 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
6.1	—	16.6	— ^b	— ^b
8.0	6ao	18.9	221.1	221.1
12.3	x (not identified)	24.8	—	1011.4
13.1	P1.6db	172.6	985.5 ([M+2H] ²⁺)	986.0 ([M+2H] ²⁺)
	P1ds		958.5 ([M+2H] ²⁺)	958.6 ([M+2H] ²⁺)
14.2	P1.6s_a	998.7	1117.6	1117.4
14.6	P1.6ol	64.9	1135.6	1135.5
15.1	—	291.7	— ^b	— ^b
15.3 ^c	P1.6ac	120.1	1149.6	1149.4
	P1.6s_b		1117.6	1117.4
15.5	P1.6s_c	18.4	1117.6	1117.4
15.8	y (not identified)	16.5	—	1223.5
17.1	P1.6No	46.3	1214.7	1214.4
19.5	P1dba_b	19.3	1193.6	1193.4
20.5	—	27.5	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

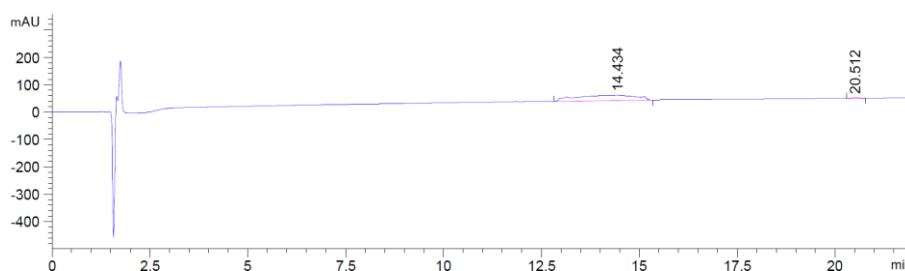
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

^c Ratio of EIC peak areas for **P1.6ac** (m/z 1148.9–1149.9) and **P1.6s_b** (m/z 1116.9–1117.9) ~43:57.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [(**P1.6s_a** + **P1.6s_b** + **P1.6s_c**) / (**x** + **P1.6db** + **P1ds** + **P1.6s_a** + **P1.6ol** + **P1.6ac** + **P1.6s_b** + **P1.6s_c** + **y** + **P1.6No** + **P1dba_b**)] × 100 = 73%

Chromatograms related to Section S6.1.6 (Stapling via S-Arylation/Reductive Amination)

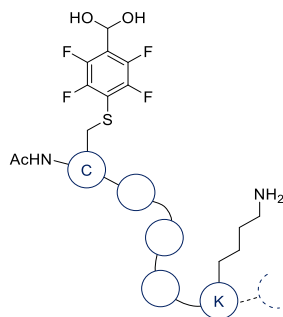
Entry **P1.7A** ($\lambda = 210$ nm; Method 2)



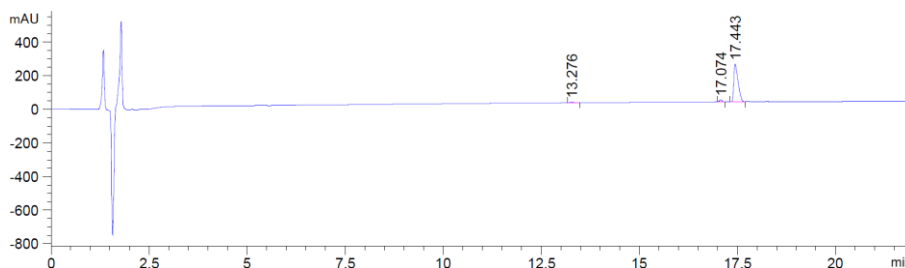
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
14.4	P1.7al	2034.3	1135.5	1135.4
	P1.7hy		1153.5	1153.4
20.5	—	21.1	— ^a	— ^a

^a Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structure of **P1.7hy**:



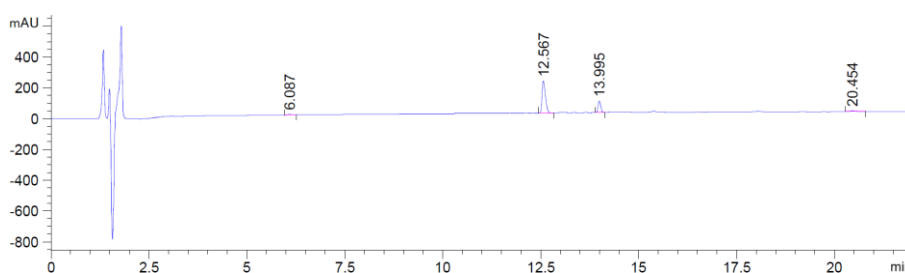
Entry **P1.7O** ($\lambda = 210$ nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
13.3	P1ds	25.8	958.5 ([M+2H] ²⁺)	959.0 ([M+2H] ²⁺)
17.1	P1.7ox_b	49.0	1164.5	1164.4
17.4	P1.7ox_a	1687.3	1164.5	1164.4

^a [M+H]⁺ ion, unless otherwise specified.

Entry 1, Table S18 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
6.1	—	24.7	— ^b	— ^b
12.6	P1.7s	1244.8	1119.5	1119.3
14.0	P1.7ol	357.6	1137.5	1137.3
20.5	P1.7di	58.8	1119.5 ([M+2H] ²⁺)	1119.6 ([M+2H] ²⁺)

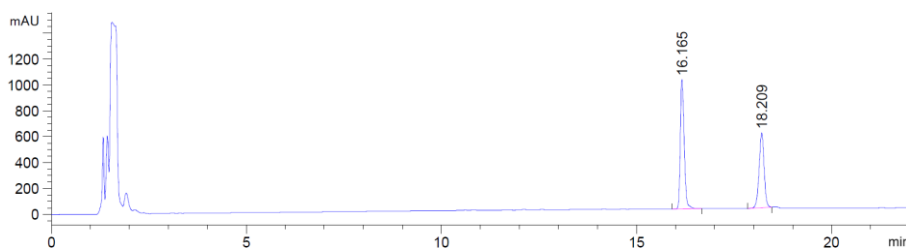
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
= [P1.7s / (P1.7s + P1.7ol + P1.7di)] × 100 = 75%

Chromatograms related to Section S6.3 (*Post-Stapling Modification via Copper-Catalyzed Azide–Alkyne Cycloaddition*)

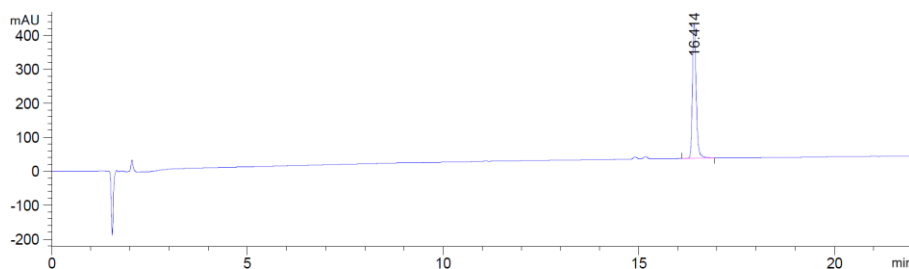
Diluted reaction mixture ($\lambda = 210$ nm; Method 2; 1 h after addition of sodium ascorbate)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
16.2	P1.2sf	6933.3	1573.7	1573.6
18.2	6-carboxyfluorescein azide	5125.7	459.1	459.1

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] $\times$ 100
 = (**P1.2sf** / **P1.2sf**) $\times$ 100 = 100%

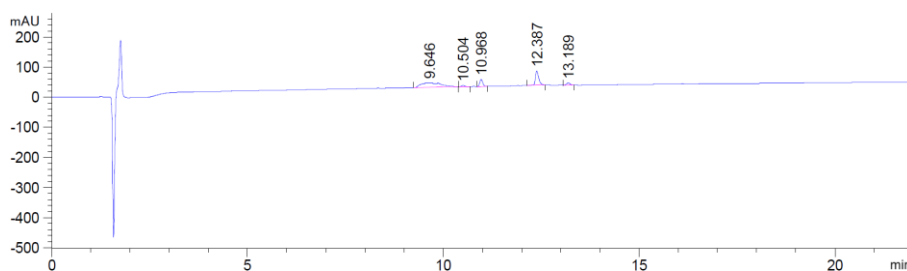
Purified P1.2sf ($\lambda = 210$ nm; Method 2; [**P1.2sf**] = 30–50 μ M)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
16.4	P1.2sf	2618.7	1573.7	1573.6

Chromatograms related to Section S7 (Cys–Lys Stapling with Chloroacetaldehyde and Methacrolein)

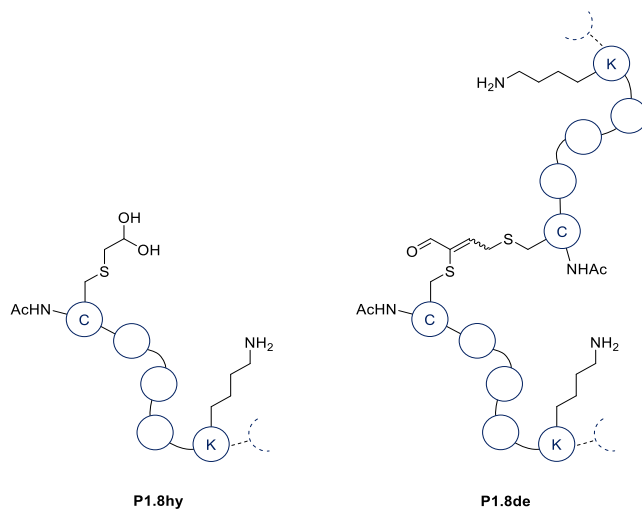
Entry P1.8A ($\lambda = 210$ nm; Method 2)



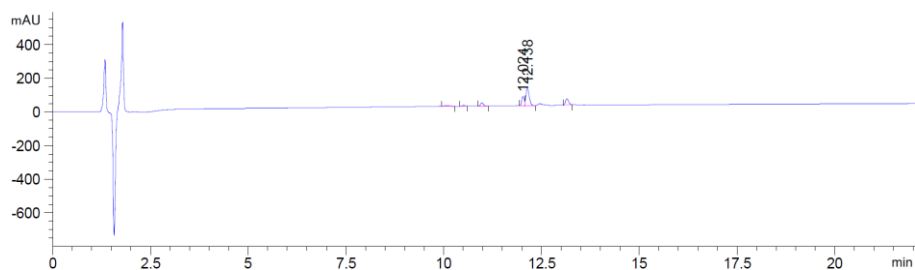
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.6	P1.8al (or tautomer)	528.9	1001.5	1001.4
	P1.8hy		1019.6	1019.4
10.5	P1	24.1	959.5	959.4
11.0	P1.8al (or tautomer)	122.8	1001.5	1001.4
12.4	P1.8de	283.4	992.5 ($[M+2H]^{2+}$)	992.5 ($[M+2H]^{2+}$)
13.2	P1ds	45.0	958.5 ($[M+2H]^{2+}$)	958.6 ($[M+2H]^{2+}$)

^a $[M+H]^+$ ion, unless otherwise specified.

Proposed structures of **P1.8hy** and **P1.8de**:



Entry P1.80 ($\lambda = 210$ nm; Method 2)

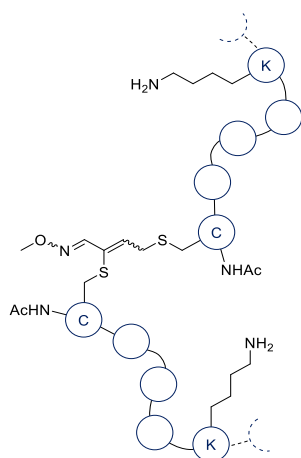


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
10.1	—	39.6	— ^b	— ^b
10.5	P1	27.1	959.5	959.4
11.0	P1.8al (or tautomer)	100.0	1001.5	1001.4
12.0	P1.8ox_b	245.8	1030.6	1030.4
12.1	P1.8ox_a	589.9	1030.6	1030.4
13.1	x (not identified)	202.6	—	1030.7 ([M+2H] ²⁺)
	P1.8deo		1007.1 ([M+2H] ²⁺)	1007.2 ([M+2H] ²⁺)
	P1ds		958.5 ([M+2H] ²⁺)	958.8 ([M+2H] ²⁺)

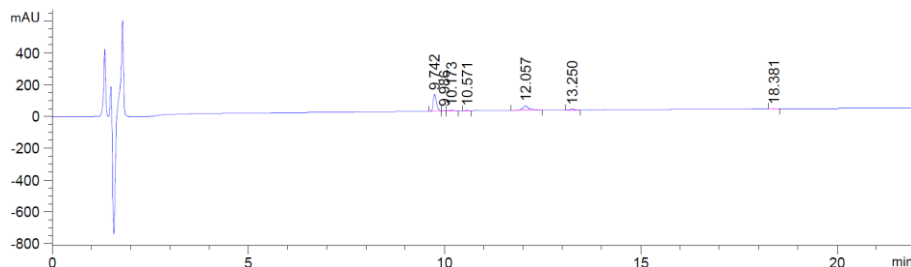
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structure of **P1.8deo**:



Entry 1, Table S19 ($\lambda = 210$ nm; Method 2)



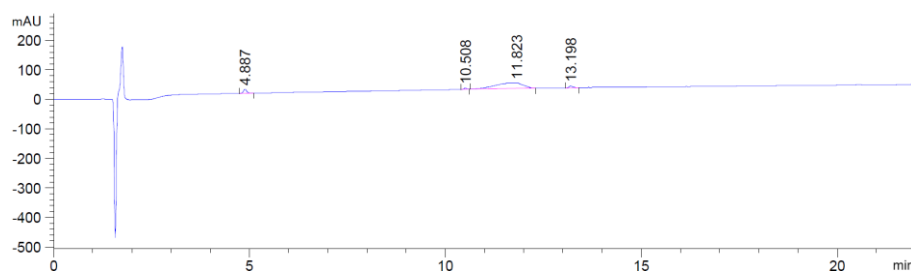
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
9.7	P1.8s	620.6	985.5	985.4
10.0	P1.8ol	8.7	1003.6	1003.5
10.2	—	38.1	— ^b	— ^b
10.6	P1	20.1	959.5	959.5
12.1	P1.8di	330.1	985.5	986.0 ([M+2H] ²⁺)
13.2	P1ds	39.6	958.5 ([M+2H] ²⁺)	958.7 ([M+2H] ²⁺)
18.4	—	10.9	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Absorbance ratio (%) = [absorbance area of the target product / combined absorbance area of all relevant peptidic species] × 100
 = [**P1.8s** / (**P1.8s** + **P1.8ol** + **P1** + **P1.8di** + **P1ds**)] × 100 = 61%

Entry P1.9A ($\lambda = 210$ nm; Method 2)

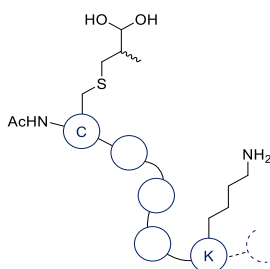


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
4.9	—	78.2	— ^b	— ^b
10.5	P1	20.6	959.5	959.5
11.8	P1.9al	949.4	1029.6	1029.5
	P1.9hy		1047.6	1047.5
13.2	P1ds	40.8	958.5 ([M+2H] ²⁺)	958.9 ([M+2H] ²⁺)

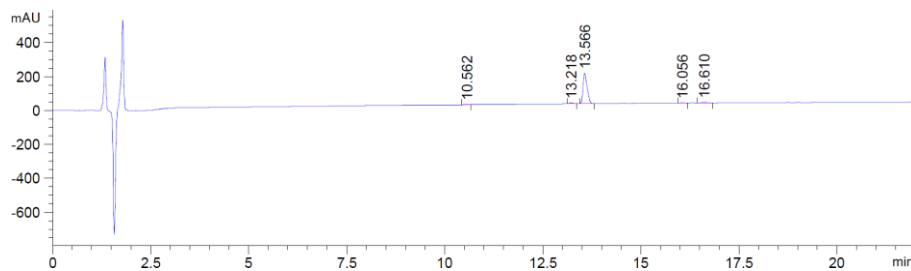
^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Proposed structure of **P1.9hy**:



Entry P1.90 (λ = 210 nm; Method 2)

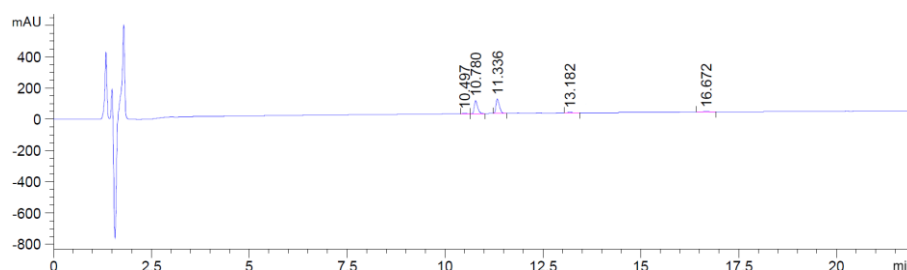


t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
10.6	P1	23.8	959.5	959.5
13.2	P1ds	31.9	958.5 ([M+2H] ²⁺)	958.6 ([M+2H] ²⁺)
13.6	P1.9ox	1275.7	1058.6	1058.5
16.1	x (not identified)	18.3	—	1157.5
16.6	—	39.6	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

Entry 1, Table S20 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd ^a	m/z found ^a
10.5	P1	21.0	959.5	959.5
10.8	P1.9s_a	479.1	1013.6	1013.5
11.3 ^c	P1.9s_b	548.3	1013.6	1013.5
	P1.9ol		1031.6	1031.5
13.2	P1ds	39.5	958.5 ([M+2H] ²⁺)	958.7 ([M+2H] ²⁺)
16.7	—	41.7	— ^b	— ^b

^a [M+H]⁺ ion, unless otherwise specified.

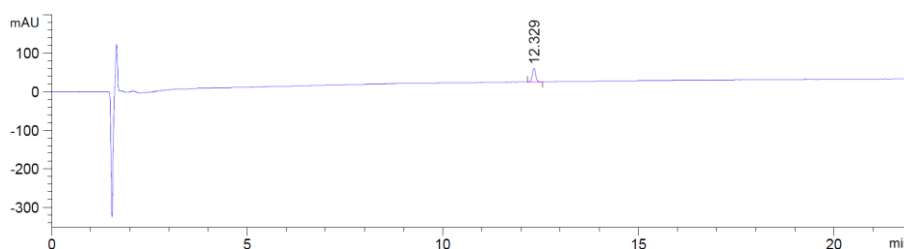
^b Compound exhibiting low ionization efficiency in ESI⁺ mode.

^c Ratio of EIC peak areas for **P1.9s_b** (m/z 1113.0–1114.0) and **P1.9ol** (m/z 1031.0–1032.0) ~97:3.

Absorbance ratio (%) = [absorbance area of the target products / combined absorbance area of all relevant peptidic species] × 100
= [(**P1.9s_a** + **P1.9s_b**) / (**P1** + **P1.9s_a** + **P1.9s_b** + **P1.9ol** + **P1ds**)] × 100
= 93%

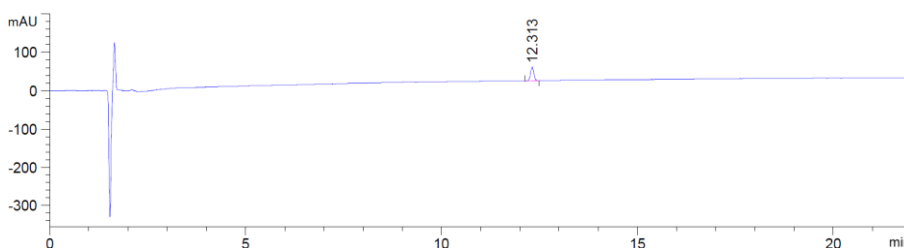
**Chromatograms related to Section S10 (HPLC–UV
Calibration Curves)**

10 μ M P1.1s (replicate 1), Figure S11 (λ = 210 nm; Method 2)



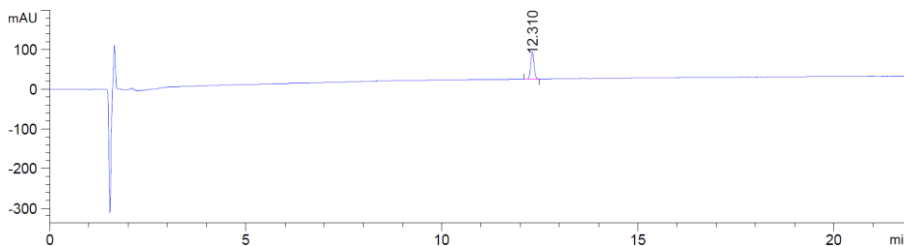
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	206.8	1061.6	1061.4

10 μ M P1.1s (replicate 2), Figure S11 (λ = 210 nm; Method 2)



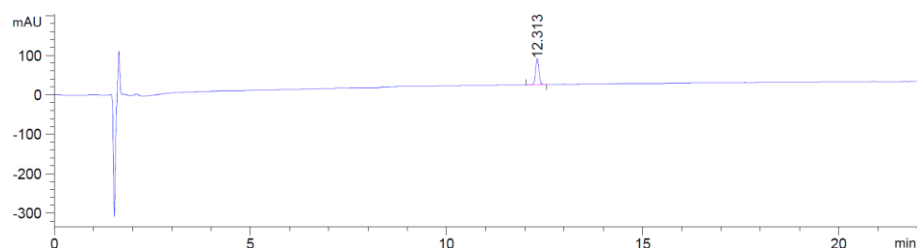
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	207.2	1061.6	1061.4

20 μ M P1.1s (replicate 1), Figure S11 (λ = 210 nm; Method 2)



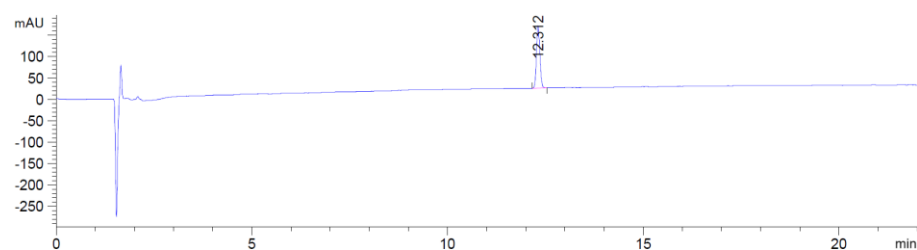
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	407.2	1061.6	1061.4

20 μ M P1.1s (replicate 2), Figure S11 (λ = 210 nm; Method 2)



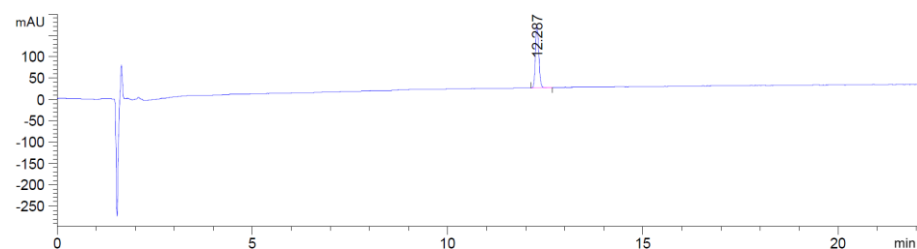
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	396.3	1061.6	1061.4

40 μ M P1.1s (replicate 1), Figure S11 (λ = 210 nm; Method 2)



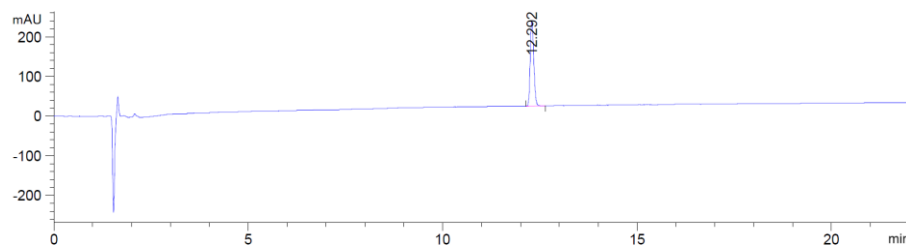
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	887.2	1061.6	1061.4

40 μ M P1.1s (replicate 2), Figure S11 (λ = 210 nm; Method 2)



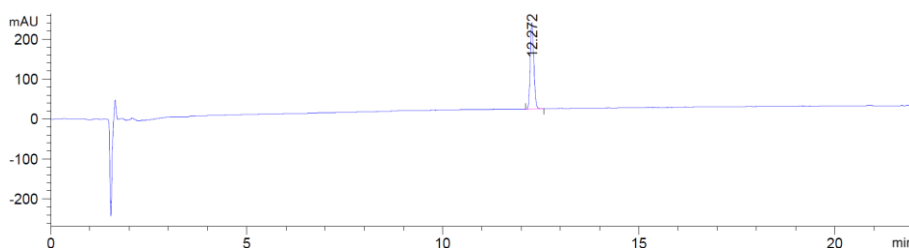
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	896.3	1061.6	1061.4

60 μ M P1.1s (replicate 1), Figure S11 (λ = 210 nm; Method 2)



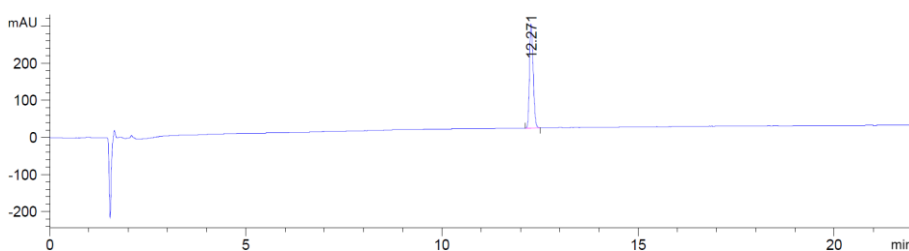
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	1349.6	1061.6	1061.4

60 μ M P1.1s (replicate 2), Figure S11 (λ = 210 nm; Method 2)



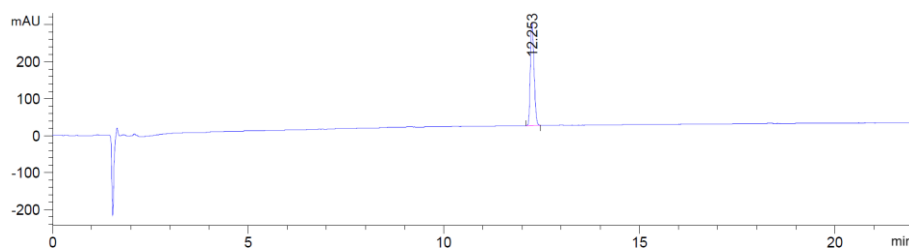
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	1358.9	1061.6	1061.4

80 μ M P1.1s (replicate 1), Figure S11 (λ = 210 nm; Method 2)



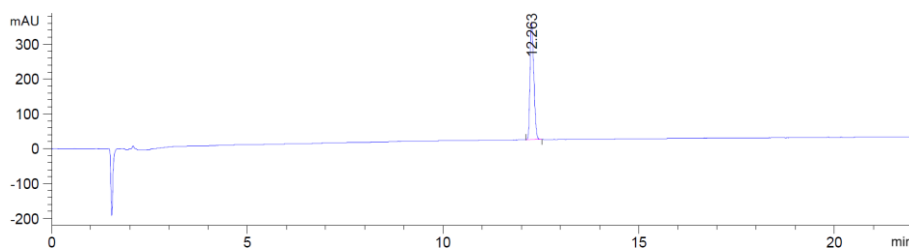
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	x	1813.3	1061.6	1061.4

80 μ M P1.1s (replicate 2), Figure S11 ($\lambda = 210$ nm; Method 2)



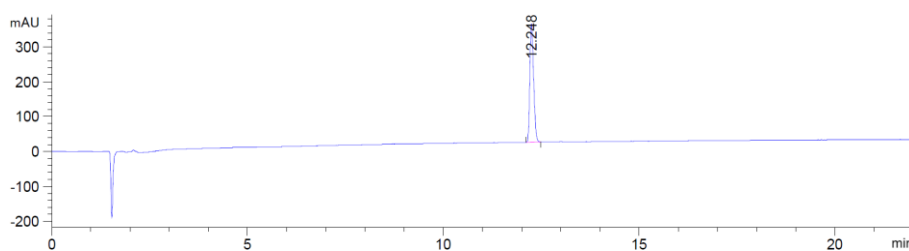
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	1798.5	1061.6	1061.4

100 μ M P1.1s (replicate 1), Figure S11 ($\lambda = 210$ nm; Method 2)



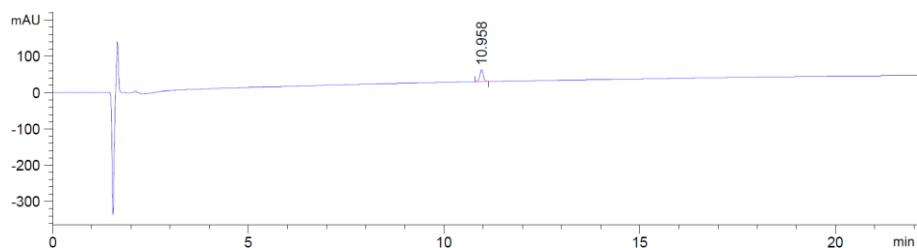
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.3	P1.1s	2258.4	1061.6	1061.4

100 μ M P1.1s (replicate 2), Figure S11 ($\lambda = 210$ nm; Method 2)



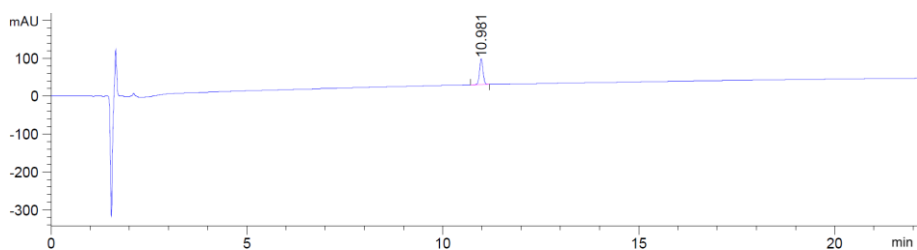
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.2	P1.1s	2270.7	1061.6	1061.4

10 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



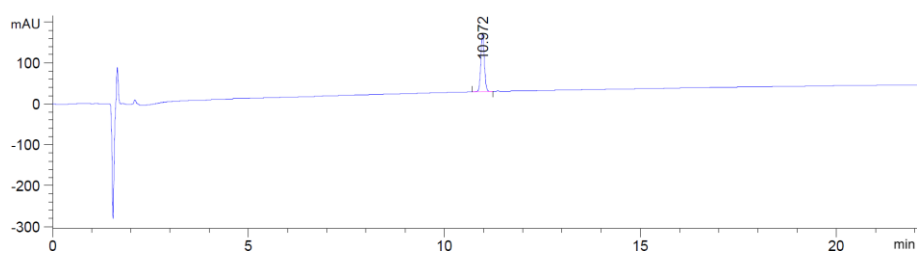
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P2.1s	199.1	1047.6	1047.5

20 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



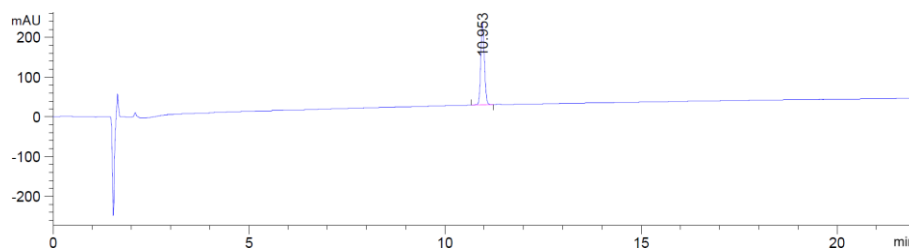
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P2.1s	420.2	1047.6	1047.5

40 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



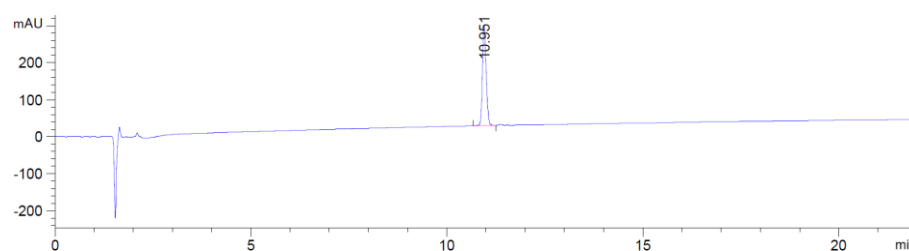
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P2.1s	885.7	1047.6	1047.5

60 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



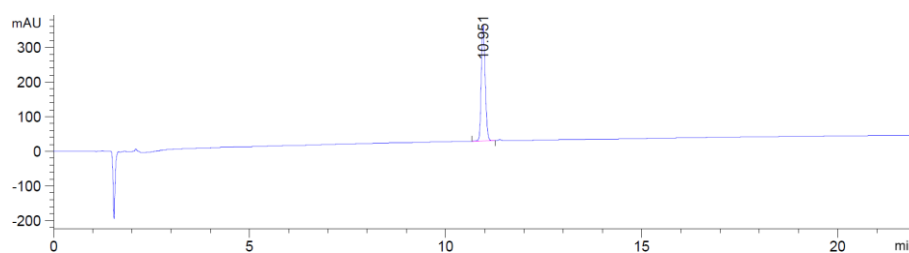
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P2.1s	1344.5	1047.6	1047.5

80 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



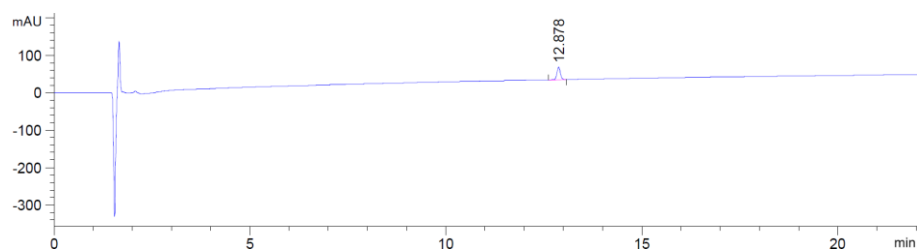
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P2.1s	1806.8	1047.6	1047.5

100 μ M P2.1s, Figure S12 (λ = 210 nm; Method 2)



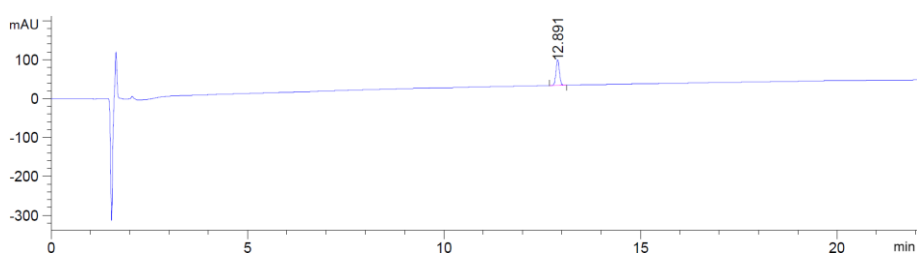
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.0	P3.1s	2293.3	1047.6	1047.5

10 μ M P3.1s, Figure S13 ($\lambda = 210$ nm; Method 2)



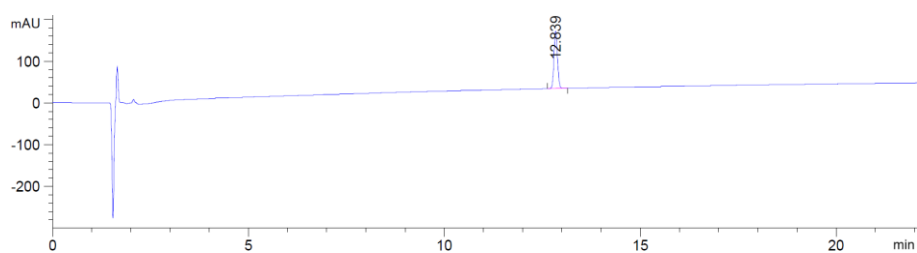
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.9	P3.1s	202.1	1033.5	1033.5

20 μ M P3.1s, Figure S13 ($\lambda = 210$ nm; Method 2)



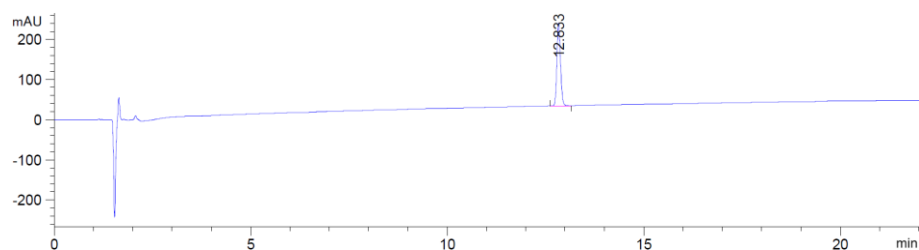
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.9	P3.1s	401.2	1033.5	1033.5

40 μ M P3.1s, Figure S13 ($\lambda = 210$ nm; Method 2)



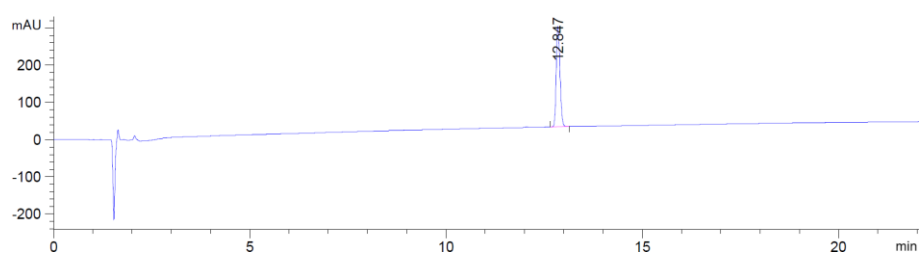
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P3.1s	861.0	1033.5	1033.4

60 μ M P3.1s, Figure S13 (λ = 210 nm; Method 2)



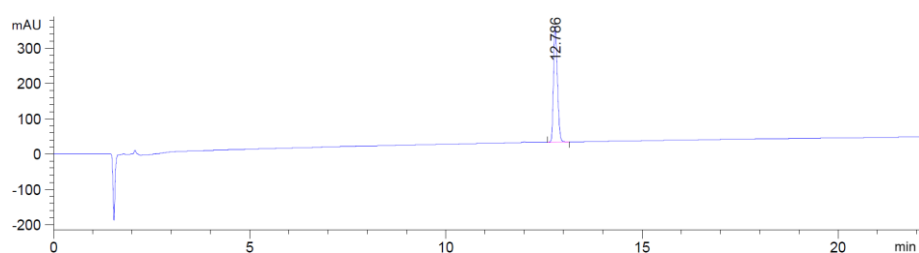
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P3.1s	1327.3	1033.5	1033.5

80 μ M P3.1s, Figure S13 (λ = 210 nm; Method 2)



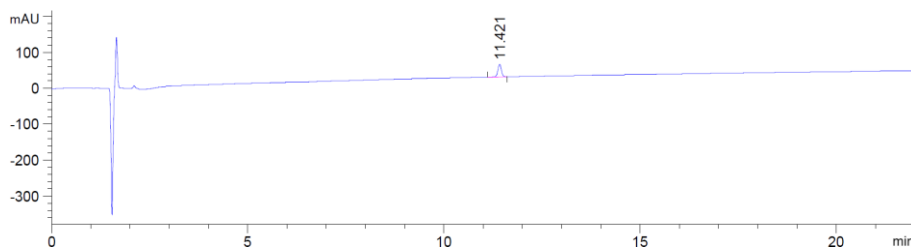
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P3.1s	1780.9	1033.5	1033.5

100 μ M P3.1s, Figure S13 (λ = 210 nm; Method 2)



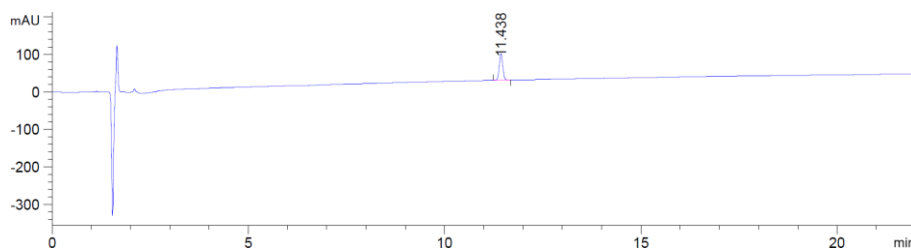
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P3.1s	2265.0	1033.5	1033.4

10 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



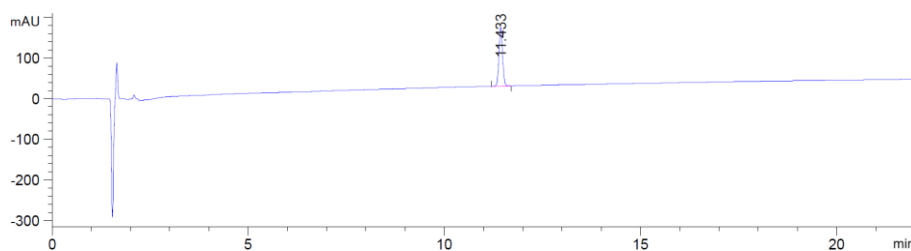
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	216.4	1019.5	1019.5

20 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



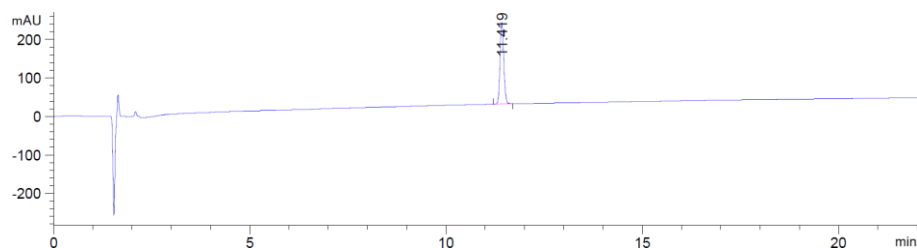
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	437.7	1019.5	1019.4

40 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



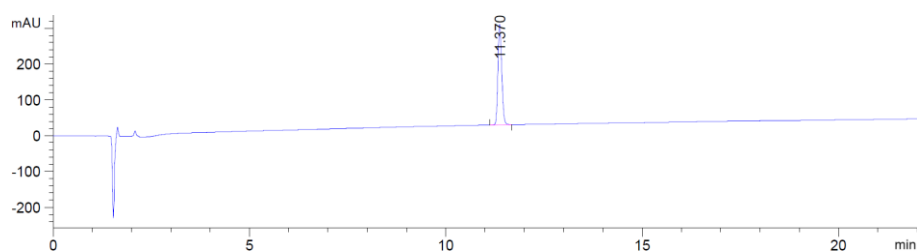
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	919.3	1019.5	1019.5

60 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



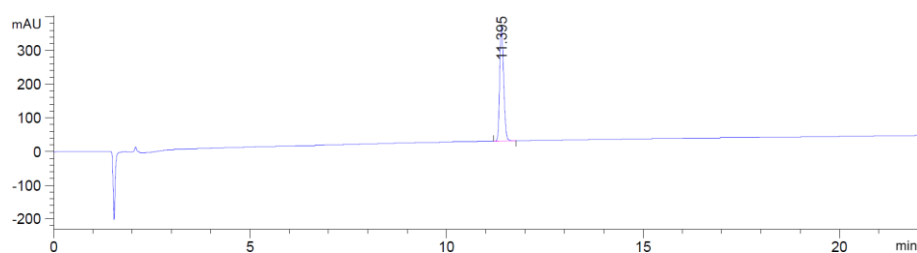
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	1400.7	1019.5	1019.4

80 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



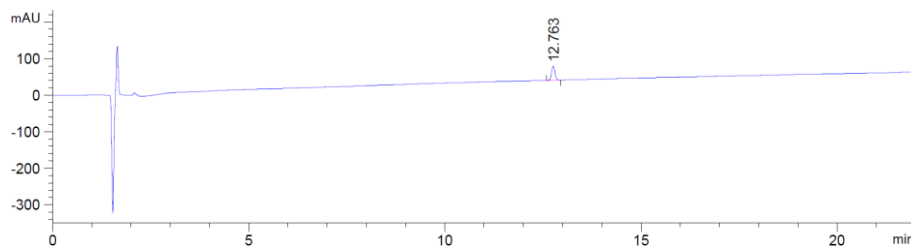
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	1888.7	1019.5	1019.5

100 μ M P4.1s, Figure S14 (λ = 210 nm; Method 2)



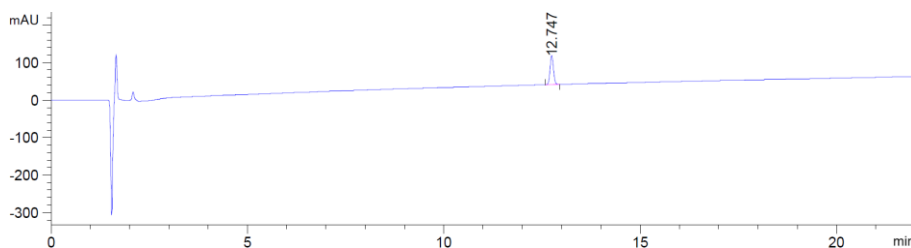
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.4	P4.1s	2380.6	1019.5	1019.5

10 μ M P5.1s, Figure S15 (λ = 210 nm; Method 2)



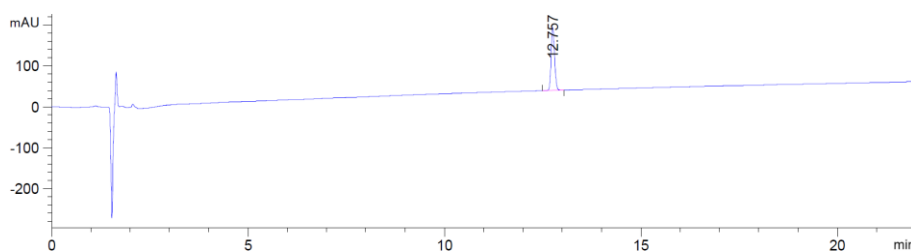
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P5.1s	226.8	1061.6	1061.5

20 μ M P5.1s, Figure S15 (λ = 210 nm; Method 2)



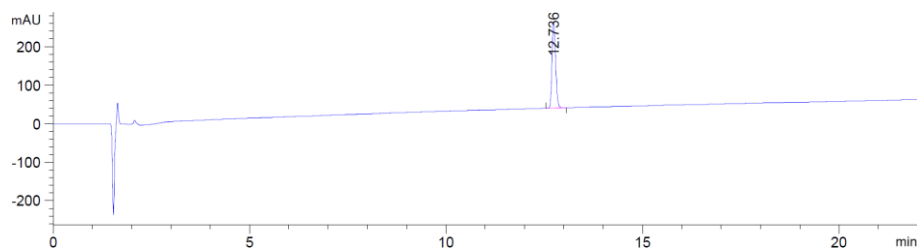
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.7	P5.1s	459.4	1061.6	1061.5

40 μ M P5.1s, Figure S15 (λ = 210 nm; Method 2)



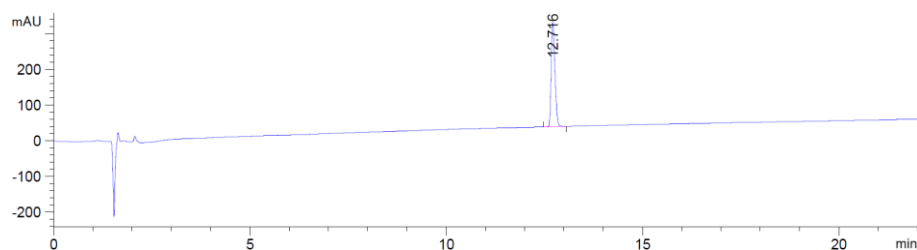
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.8	P5.1s	943.8	1061.6	1061.5

60 μ M P5.1s, Figure S15 ($\lambda = 210$ nm; Method 2)



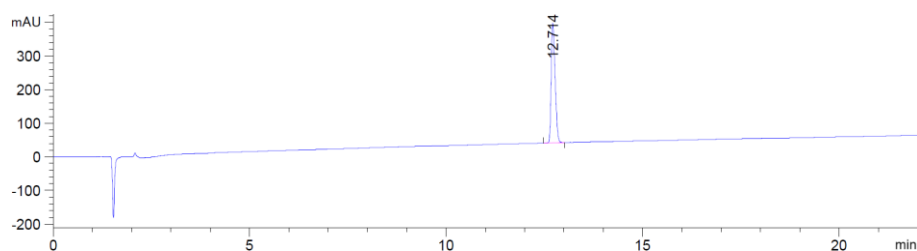
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.7	P5.1s	1428.5	1061.6	1061.5

80 μ M P5.1s, Figure S15 ($\lambda = 210$ nm; Method 2)



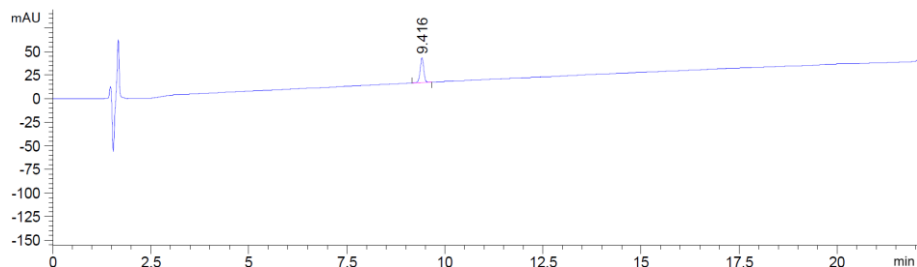
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.7	P5.1s	1916.5	1061.6	1061.5

100 μ M P5.1s, Figure S15 ($\lambda = 210$ nm; Method 2)



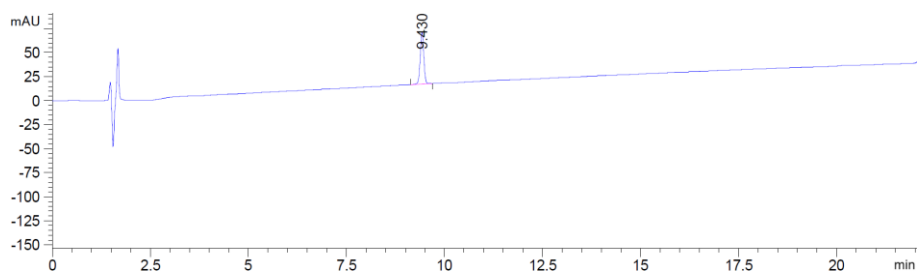
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
12.7	P5.1s	2445.1	1061.6	1061.5

10 μ M P13.1sd, Figure S16 (λ = 210 nm; Method 5)



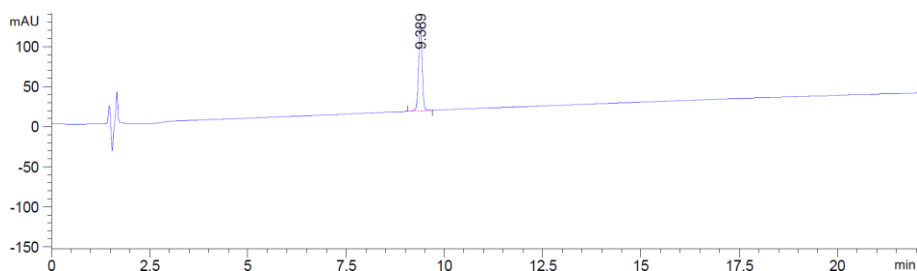
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	165.5	776.4	776.4

20 μ M P13.1sd, Figure S16 (λ = 210 nm; Method 5)



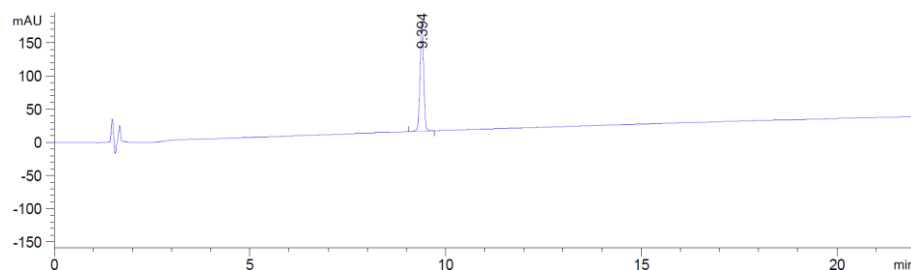
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	339.0	776.4	776.4

40 μ M P13.1sd, Figure S16 (λ = 210 nm; Method 5)



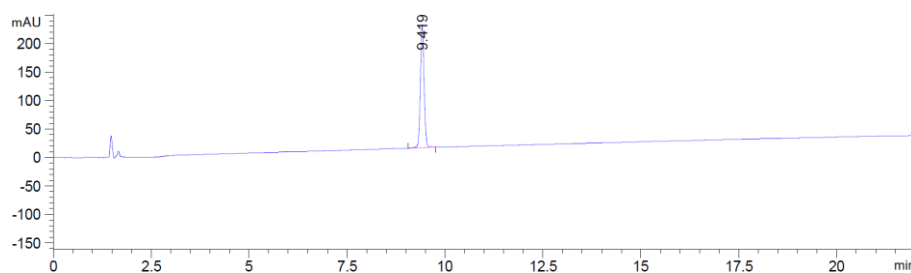
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	688.4	776.4	776.4

60 μ M P13.1sd, Figure S16 ($\lambda = 210$ nm; Method 5)



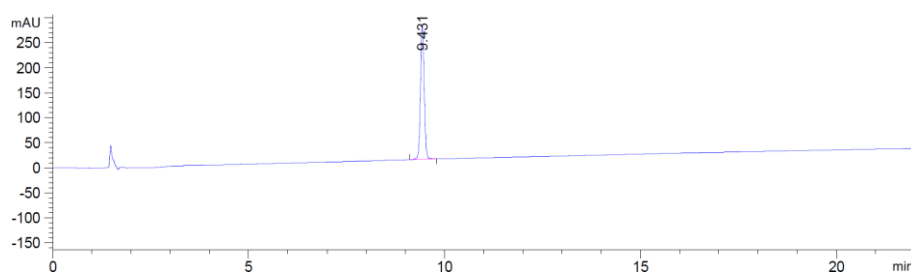
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	1048.0	776.4	776.4

80 μ M P13.1sd, Figure S16 ($\lambda = 210$ nm; Method 5)



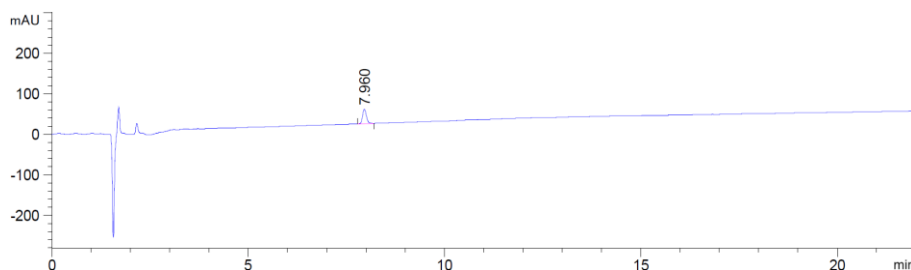
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	1407.6	776.4	776.4

100 μ M P13.1sd, Figure S16 ($\lambda = 210$ nm; Method 5)



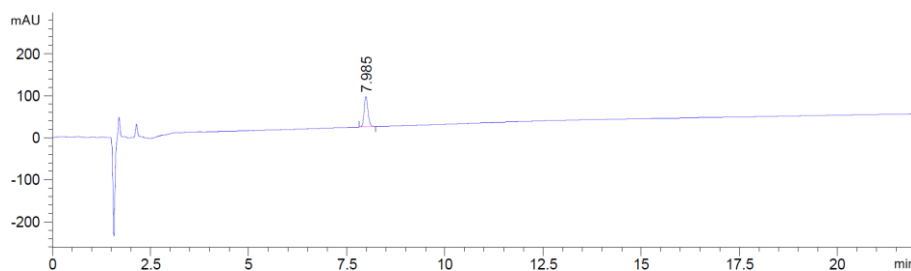
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
9.4	P13.1sd	1766.8	776.4	776.4

10 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



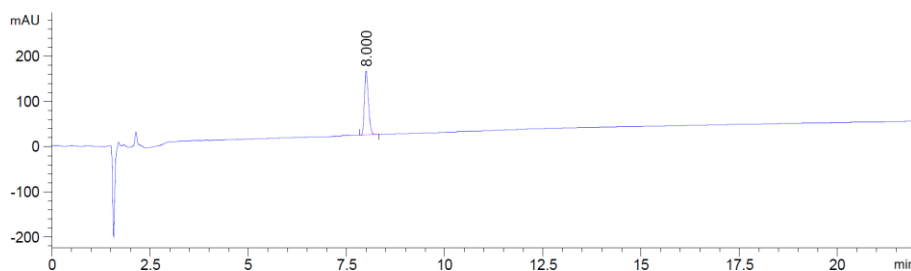
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	237.7	1133.6	1133.5

20 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



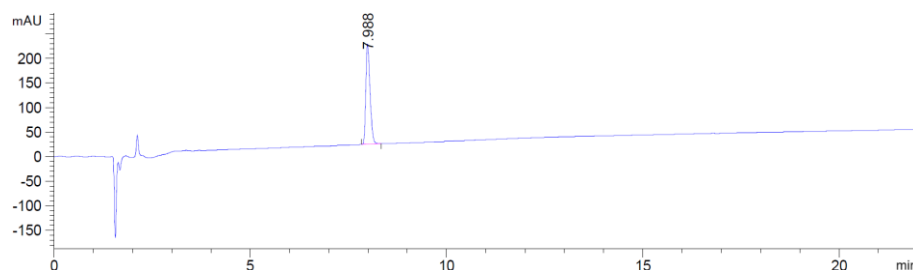
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	481.7	1133.6	1133.5

40 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



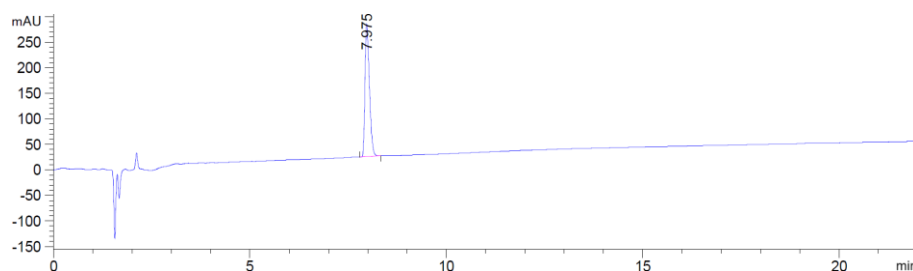
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	988.5	1133.6	1133.5

60 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



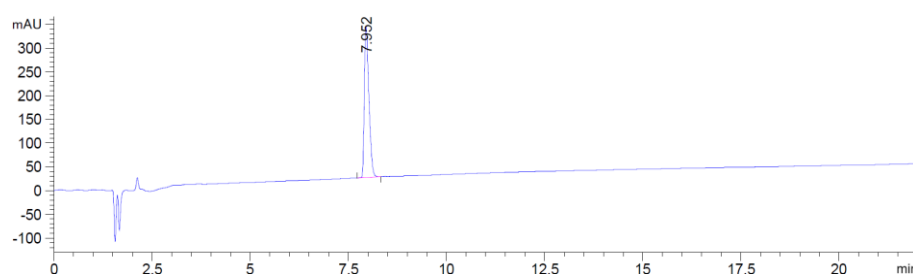
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	1505.2	1133.6	1133.5

80 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



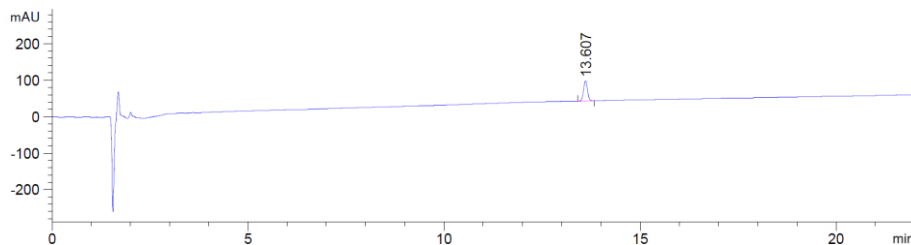
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	2018.1	1133.6	1133.5

100 μ M P14.1s, Figure S17 (λ = 210 nm; Method 3)



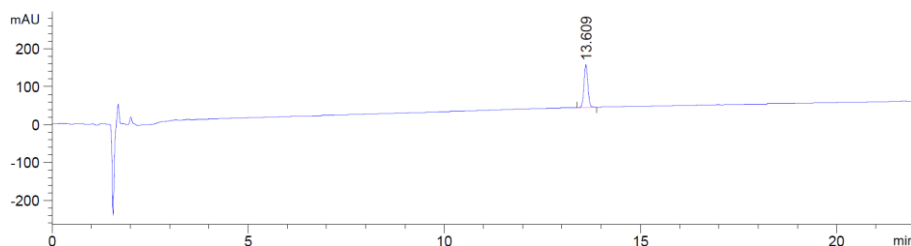
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
8.0	P14.1s	2547.0	1133.6	1133.5

10 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



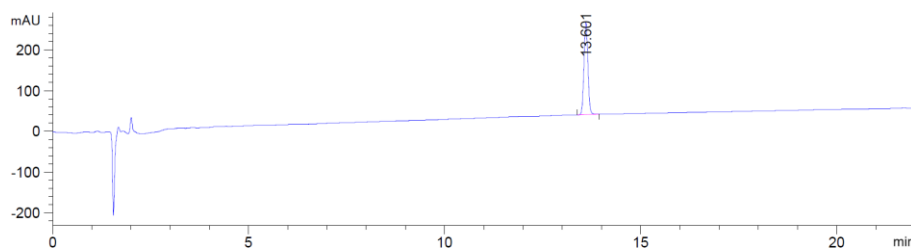
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	374.8	1267.6	1267.5

20 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



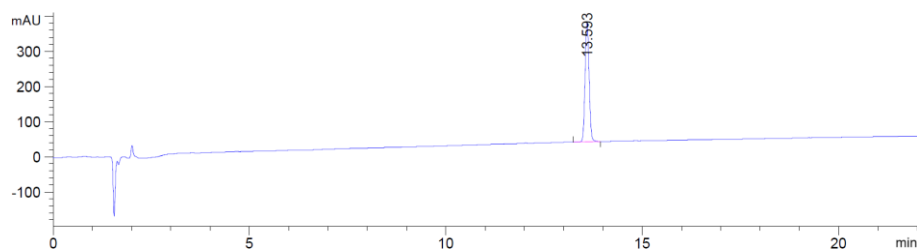
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	769.4	1267.6	1267.5

40 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



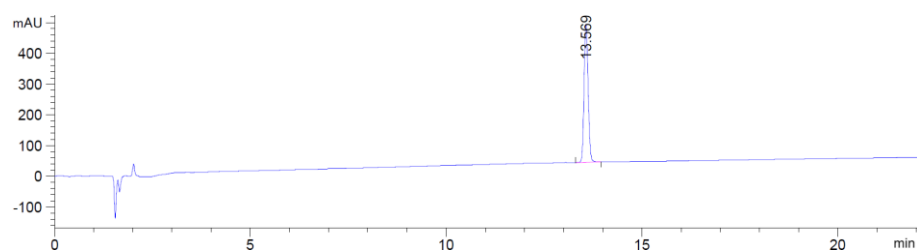
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	1580.1	1267.6	1267.5

60 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



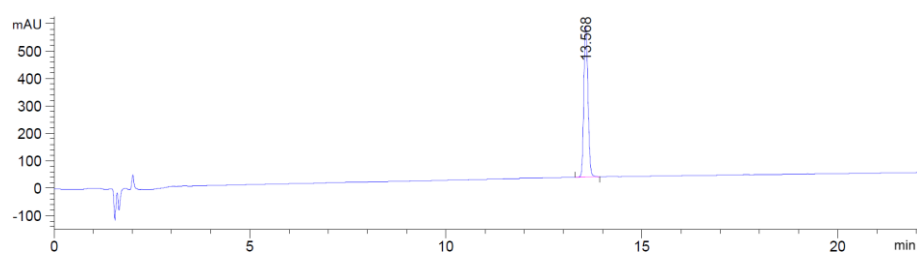
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	2407.1	1267.6	1267.5

80 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



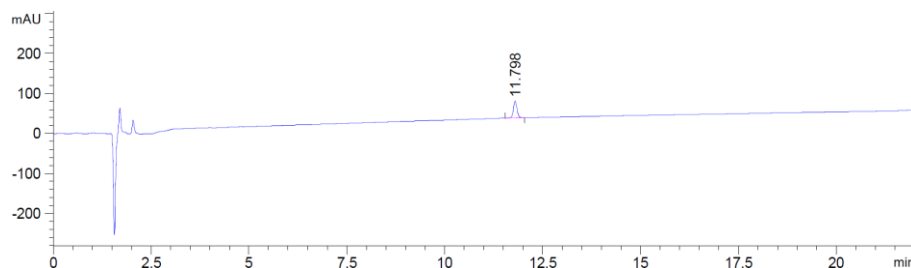
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	3217.0	1267.6	1267.5

100 μ M P15.1s_a, Figure S18 (λ = 210 nm; Method 3)



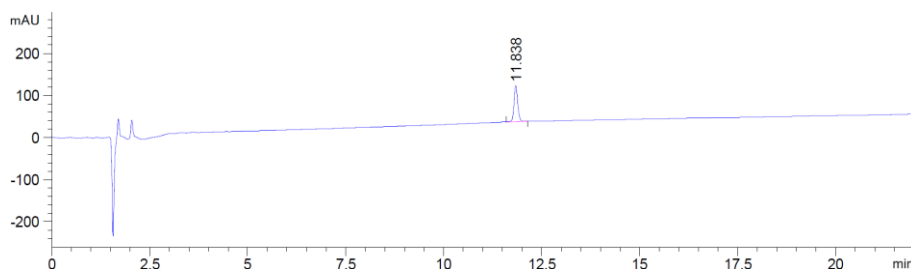
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.6	P15.1s_a	4025.4	1267.6	1267.5

10 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



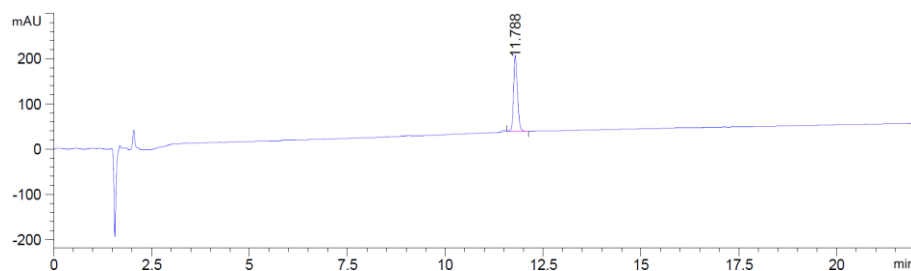
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P15.1s_b	269.4	1267.6	1267.5

20 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



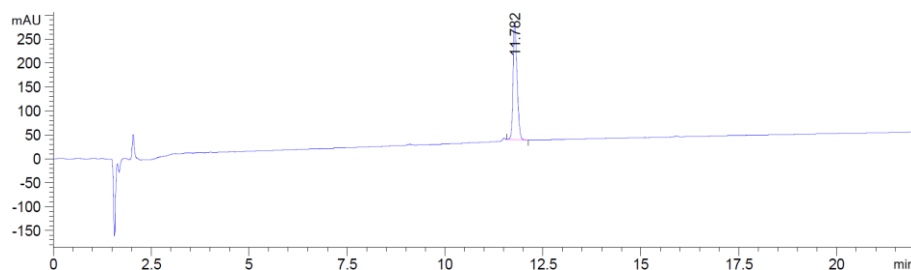
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P15.1s_b	560.8	1267.6	1267.5

40 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



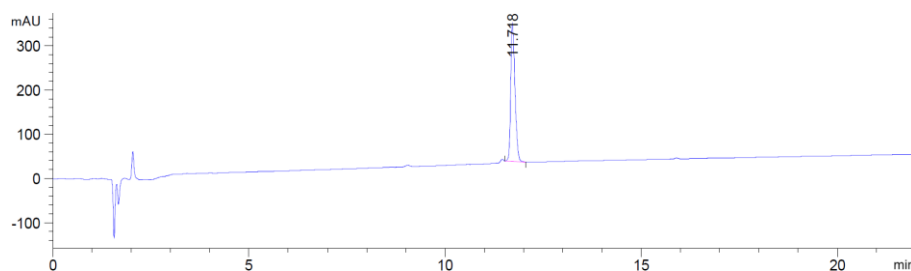
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
11.8	P15.1s_b	1150.1	1267.6	1267.5

60 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



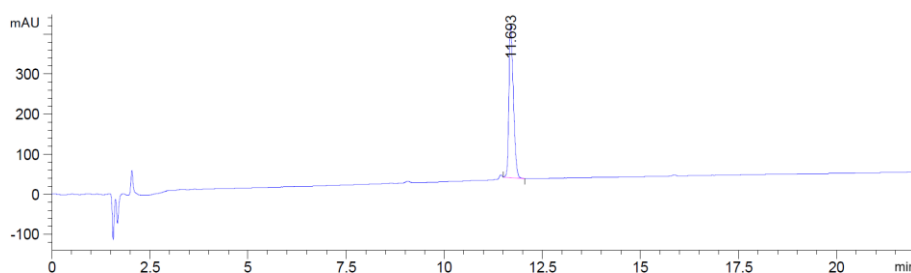
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
11.8	P15.1s_b	1748.8	1267.6	1267.5

80 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



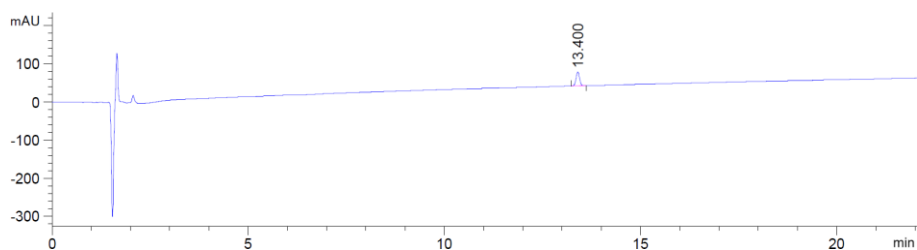
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
11.7	P15.1s_b	2337.0	1267.6	1267.5

100 μ M P15.1s_b, Figure S19 (λ = 210 nm; Method 3)



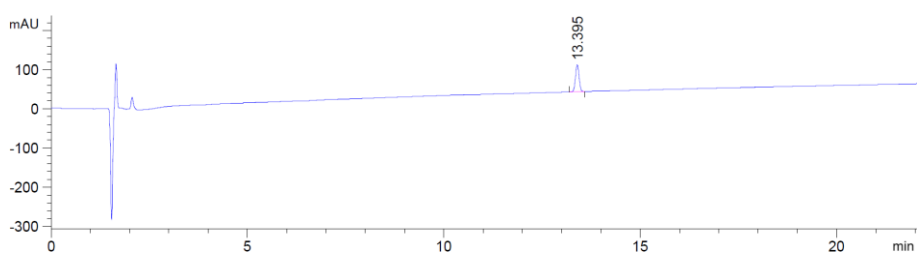
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for [M+H] ⁺	m/z found for [M+H] ⁺
11.7	P15.1s_b	2982.7	1267.6	1267.5

10 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



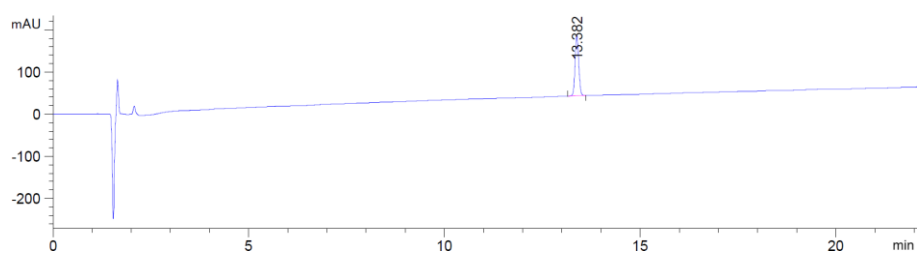
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.4	P1.2s	227.2	1115.6	1116.0

20 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



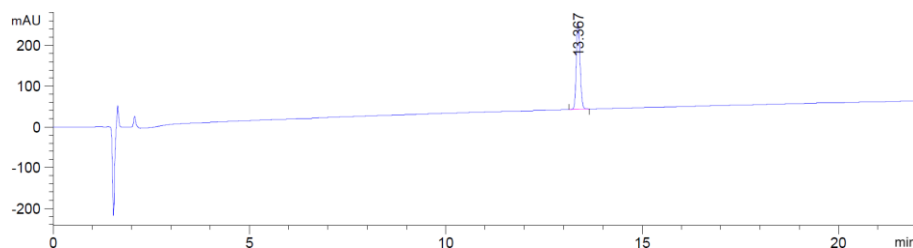
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.4	P1.2s	436.0	1115.6	1115.8

40 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



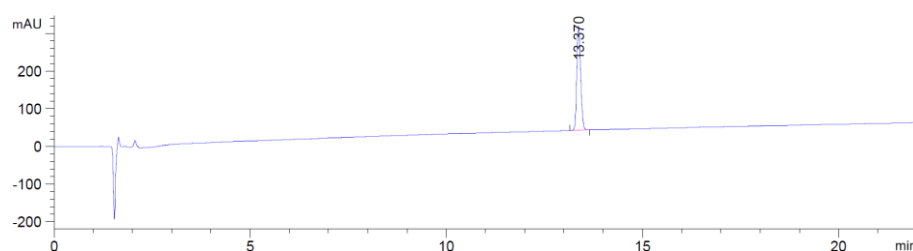
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.4	P1.2s	919.3	1115.6	1115.6

60 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



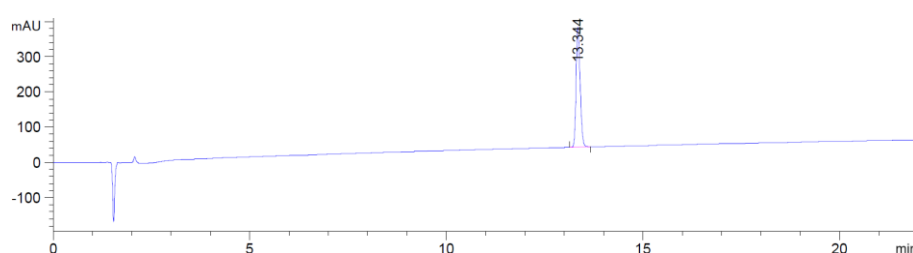
t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.4	P1.2s	1426.9	1115.6	1115.5

80 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.4	P1.2s	1905.0	1115.6	1115.5

100 μ M P1.2s, Figure S20 (λ = 210 nm; Method 2)



t_R (min)	Assigned compound	Absorbance area (mAU·s)	m/z calcd for $[M+H]^+$	m/z found for $[M+H]^+$
13.3	P1.2s	2433.0	1115.6	1115.5

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