# Peptide Carbocycles: From –SS– to –CC– via a Late-Stage "Snip-and-Stitch"

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"Now more than ever, peptide medicinal chemists exploit synthetic functional groups to turn ordinary peptides into next-generation biopharmaceuticals. These "groups" often take the form of non-proteinogenic amino acids (NPAAs), which, unlike their biogenic counterparts, impart increased stability, binding affinity, and target specificity to the peptide," said Professor Steven Bloom, from the University of Kansas (Lawrence, USA), whose group is active in the area of NPAAs research. "Accordingly, a slew of technologies has been introduced to streamline the incorporation of NPAAs into peptides, one route being the use of a dehydroalanine (Dha) residue as a synthetic linchpin," he continued. "A dehydroalanine residue is a competent Michael acceptor, and once placed into the peptide, can accept a wide variety of one- and two-electron nucleophiles to insert one of any number of new amino acid side chains at the former Dha site. Applied over each position in the peptide, this approach enables its user to optimize peptide sequences with enhanced physiochemical properties and bioactivity."

Professor Bloom went on by explaining to SYNFORM that besides inserting NPAAs, cyclizing the peptide can be an effective strategy for developing it as a drug: "Most cyclic peptides take advantage of disulfide bonds, formed between two cysteine residues. While the disulfide bond can be easily installed, it is a metabolic lability. Hence, medicinal chemists aim to replace labile disulfide bonds with chemically benign carbon-rich groups." To do this, most cyclization platforms substitute the two cysteine residues with two tailor-made amino acids and then use synthetic chemistry to link the two amino acid fragments together. Each pair of synthetic amino acids produces exactly one *carbo*cyclic peptide derivative. Importantly, each carbon-bridged variant gives the cyclic peptide a unique structure and this alters its bioactivity. According to Professor Bloom, finding a carbon-based group that improves physiochemical properties and retains bioactivity can, therefore, require numerous individual syntheses with every amino acid pair needing to be made and inserted prior to the key cyclization step. Such an approach is less conducive to modern peptide drug discovery campaigns, where producing many peptides and testing them *in parallel* is desirable. Professor Bloom said: "To us, this presented an unmet challenge in cyclic peptide medicinal chemistry, one that we thought could be addressed using Dha residues. We reasoned that a cysteine disulfide could be cleaved into a pair of Dha residues. From here, a carbon-based fragment having two nucleophilic sites could be used to re-cyclize the peptide, each nucleophilic position reacting with one of the two Dha units. This general format would allow a wide variety of carbocyclic peptide variants to be made from a single bis-Dha peptide progenitor. Below is the first-hand account of our studies. We detail our initial plans, rationale, and those unexpected surprises that added immense value to our manuscript."

In previous years, Professor Bloom's lab discovered that organic molecules having a single boronic acid can be transformed into carbon-centered radicals that add to a Dha residue in a peptide through aqueous flavin photoredox catalysis. "We reasoned that under similar conditions, a diboronic acid could be converted into a diradical that inserts nicely between two Dha residues affording new carbocyclic peptides," said Professor Bloom. He continued: "Making peptides that have two Dha residues was straightforward, as prior studies showed that this was possible from disulfides using an easily prepared dibromo ester reagent. Unfortunately, our light-driven approach was not fruitful, only a trace amount of the cyclic peptide being made by LC-MS when a test peptide $Dha_2$-terlipressin was used. This result suggested to us that both the identity of the bis-nucleophile and the chemistry for activating it would be *incredibly* important to the success of this project and we decided to increase our chances by taking a combinatorial approach to the problem. We made seven different linker chemotypes and tested them across three different synthetic platforms, namely, electrochemistry, photoredox catalysis, and transition-metal catalysis. We found *only* two sets of conditions that worked, one of which used a cocktail of zinc metal and organodiiodides." In addition to the desired cyclic peptide, an appreciable amount of an uncyclized product wherein each Dha separately reacted with a different diiodide, hereafter termed the diaddition product, was formed under the conditions initially used by the group. Biasing cyclization to a useable level (yield) was the immediate challenge facing this project. "While most of our observations were noted in our original manuscript, a few additional points are worth mentioning," said Professor Bloom. He went on to list these: "**One**, we found that copper carbonate was *essential*

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for improving the conversion to peptide products, both linear dialkylated and cyclic peptide. Other copper salts were ineffective. This is not the case in related works where the identity of the copper salt appears to be less important. **Two**, the rate of stirring is *very* important. For best results, the stir rate needed to be kept high. Failure to use adequate stirring resulted in significantly lower yields, likely due to the heterogeneous nature of the reaction. **Three**, although we imagine that the mixture of zinc metal and copper carbonate spontaneously forms a zinc-copper couple in situ, the identity of this composite is unknown. However, we do know that commercial zinc-copper couple is not nearly as effective and should be avoided in our reaction. And **Four**, the inclusion of a hydrosilane additive biases the selectivity of our reaction for cyclization, as opposed to diaddition products, and can enforce diastereoselectivity. Admittedly, while we expected that a hydrosilane might help to encourage cyclization, the observed change in diastereoselectivity was *serendipitous*. The reason that different hydrosilanes cause different amounts of the four possible cyclic peptide diastereomers to be formed is still unclear, even with our experimental evidence that they can quench α-carbonyl radicals generated at Dha positions. We fully intend to follow up on this result and expect that chiral silanes could be important to look at for Generation-II systems."

Although the group noted some surprising results while optimizing their reaction, more unexpected findings were to come during product isolation. "It is worth noting that our lab tries to avoid HPLC purification when possible," explained Professor Bloom. He continued: "Not every lab has an HPLC, and we believe that relying on one greatly limits the ability of others to use (translate) our technologies. Not discussed in our original publication is that we explored many methods to purify our cyclic peptides to avoid HPLC before finding a successful route. We examined solid-phase extraction (SPE), liquid-phase extraction (LPE), and preparative thin-layer chromatography (TLC). With SPE, we could not separate diaddition products from cyclic peptide products. For LPE, all peptide products co-extracted, no matter the solvent choice. Perhaps most unique is what happened in TLC. The diaddition and cyclic peptide products separated when normal- or reverse-phase TLC plates were used; however, the peptides impregnated the solid matrix. We could not dissolve the peptides from the matrix with any solvent including strong acids like trifluoroacetic acid. The inability to release the peptides off the solid matrix prevented us from using preparative TLC, and no further attempts were made in this direction. At this point, we decided to try flash chromatography. Saying that we had little faith in this approach would be an understatement. Separating peptide diastereomers (and peptide mixtures in general) typically requires higher pressures than what flash chromatography can achieve. Indeed, when we first attempted this approach using reverse- or normal-phase columns, no separation between any peptides was found with combinations of MeCN, MeOH, and $H_2O$ as eluent. The incorporation of fluorine-rich acids did little to improve separation. As a "last ditch effort" we replaced MeOH with EtOH in our elution mixture. To our amazement, this change caused all the peptides – diaddition and cyclic peptides and their respective diastereomers – to separate on the column. Our best hypothesis is that EtOH displaces water molecules from the peptides, causing diaddition products to appear more linear and cyclic peptides to look more spherical. Reinforcing these innate 3-D structures causes the various peptide products to separate. This unexpected result was *critical,* as we feared we would have to resort to HPLC if unsuccessful."

Professor Bloom commented that the remainder of their manuscript is "comparatively uneventful", saying that they evaluated the physiochemical properties of select cyclic peptides using laboratory assays that are more conducive to high-throughput experimentation. He admitted: "To be honest, the use of ethylene glycol–heptane partitioning as a measure of blood-brain penetration was met with some speculation among our peers despite being well documented for peptides in the literature. Thus, we would like to formally acknowledge that while cell-based measurements might be a better predictor of results in vivo, our approach is still very useful for surveying many peptide analogues and quickly identifying those that have suitable CNS penetrating properties. It is also less expensive than cell-based assays and might be more useful when cost is a limiting factor. In all, our approach allows many peptide variants to be triaged at the early stages of CNS peptide drug discovery campaigns and should be used as such."

"In conclusion, our studies resulted in a brand-new platform wherein one cyclic disulfide peptide becomes many different carbocyclic analogues using a two-step "Snip-and-Stitch" approach," said Professor Bloom. He continued: "By this we mean that our technology is like a "chemical sewing kit", allowing the disulfide bond to be 'snipped' out and a new functional group 'stitched' in its place. Our work yielded several interesting findings along the way, at least some of which open new doors for peptide medicinal chemistry and others that highlight still unsolved problems for the field." Professor Bloom concluded: "It is our hope that relating these additional points will help others to use our technology and to drive peptide medicinal chemistry ever forward."

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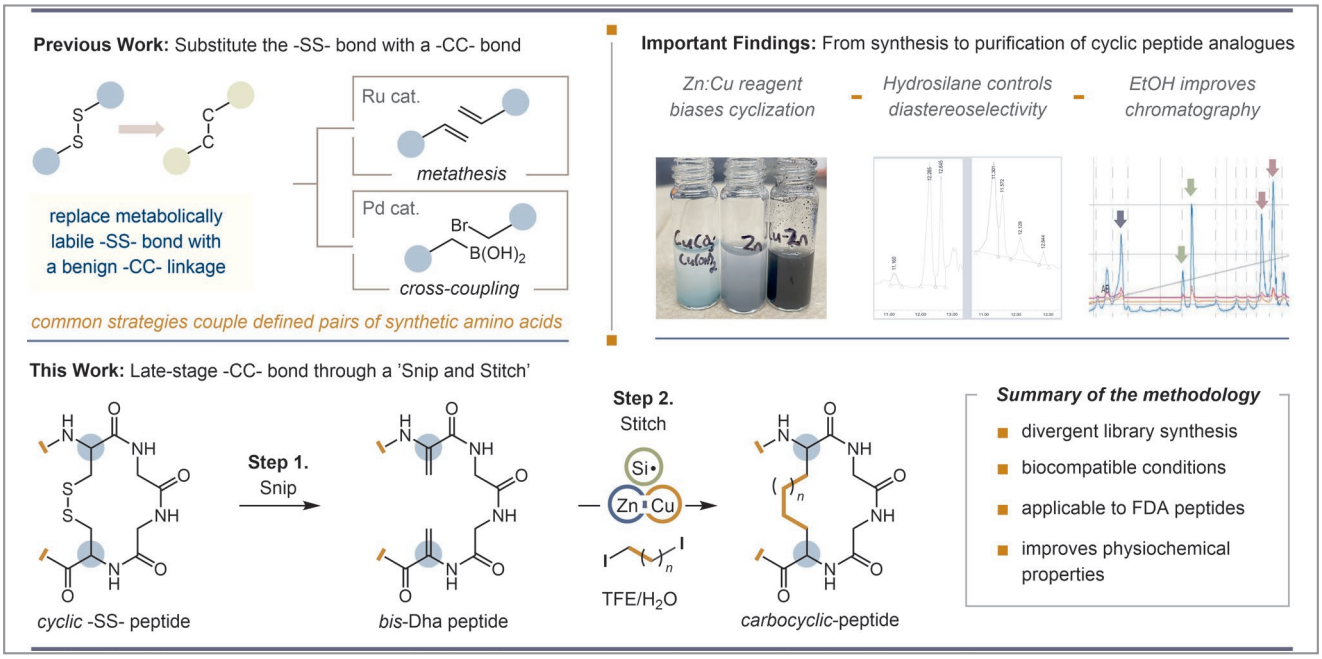


**Figure 1** Summary of the work and key findings. Previous technologies for making carbocyclic peptides relied heavily on uniting two synthetic amino acids in the peptide to give a single product. The title work takes advantage of native disulfides to access a pair of endogenous dehydroalanine residues that can be tethered together using an organodiiodide, zinc-copper couple and a hydrosilane, affording a divergent route to many cyclic peptide analogues. Judicious choice in hydrosilane can bias the stereochemical outcome of the cyclization step and EtOH greatly improves the separation of the products by flash chromatography.

## About the authors

*S. Gary*

**Samuel Gary** was born and raised in West Virginia (USA). He earned his B.S. in biochemistry from West Virginia University (USA) where he completed undergraduate research with Dr. Nik Kovinich, studying anticancer natural products. Sam began graduate studies at the University of Kansas (USA) in 2019, under the guidance of Prof. Steven Bloom. His research focuses on designing new platforms to construct libraries of cyclic peptides and to insert non-proteinogenic amino acids into peptides. His work aims to streamline structure–activity relationship studies and the hit-to-lead development of novel peptide therapeutics.

*Prof. S. Bloom*

**Steven Bloom** is from Harford County, Maryland (USA). He attended McDaniel College, located in western Maryland (USA), where he earned his B. A. in chemistry and biochemistry in 2010. He then completed graduate studies at Johns Hopkins University (USA) under the tutelage of Prof. Thomas Lectka in organofluorine chemistry. After earning his Ph.D. in 2015, Steve moved to Princeton University (USA) where he completed postdoctoral studies with Prof. David W. C. MacMillan as an NIH Ruth L. Kirschstein Fellow. There, Steve worked in bioorganic chemistry, developing photocatalyzed strategies to selectively modify proteins. Steve began his independent career at the University of Kansas (USA) in 2018. His group focuses on designing new synthetically divergent technologies to accelerate peptide medicinal chemistry and drug discovery.

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