Bisthioether Stapled Peptides Targeting Polycomb Repressive Complex 2 Gene Repression

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Bisthioether Stapled Peptides Targeting Polycomb Repressive Complex 2 Gene Repression

By

Gan Zhang

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This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Bisthioether Stapled Peptides Targeting Polycomb Repressive Complex 2 Gene Repression

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Adviser: Professor Guillermo Gerona-Navarro

Abstract

Interactions between proteins play a key role in nearly all cellular process, and therefore, disruption of such interactions may lead to many different types of cellular dysfunctions. Hence, pathologic protein-protein interactions (PPIs) constitute highly attractive drug targets and hold great potential for developing novel therapeutic agents for the treatment of incurable human diseases. Unfortunately, the identification of PPI inhibitors is an extremely challenging task, since traditionally used small molecule ligands are mostly unable to cover and anchor on the extensive flat surfaces that define those binary protein complexes. In contrast, large biomolecules such as proteins or peptides are ideal fits for these so-called “undruggable” sites. However, their poor pharmacokinetic properties have limited their application as therapeutics. In this context, peptidomimetic molecules have emerged as an alternative and viable solution to this problem, since they conserve the architectural and structural features of peptides and also exhibit substantially improved pharmacokinetic profiles. Given the great promise of this class of compounds as therapeutics, new protocols granting easy access to them continue to be of great interest. This thesis describes the development of an efficient solid phase methodology for the chemoselective synthesis of bisthioether stapled peptides of multiple architectures and its application to discovering three families of potent allosteric inhibitors of the polycomb repressive complex 2 (PRC2) of proteins.
PRC2 is a multimeric complex consisting of four core proteins: EZH2, EED, SUZ12 and RBAP46/RBAP48, which is involved in the initiation of gene repression through its methyltransferase activity, specific for lysine 27 on Histone H3 (H3K27). The enzymatic activity of PRC2 is conferred by the catalytic SET domain of EZH2, but also to the other core components of the complex. Hence, a catalytically active PRC2 complex must contain EZH2 and at least EED and SUZ12, which underscores the role of the latter proteins as scaffolds for the proper assembly of PRC2 into its bioactive conformation. The biological relevance of PRC2 proteins is highlighted by their known role in the development and progression of different types of cancers, and thus targeting them has emerged as a high-priority strategy in the field of cancer epigenetics.

The thesis first describes the development of an innovative solid phase approach for the preparation of bisthioether stapled peptides of multiple architectures, including single-, double-turn and double stapled peptides. This methodology allows for ligation with all-hydrocarbon linkers of various lengths, avoiding the use of unnatural amino acids and expensive catalysts, and affords cyclopeptides with improved bioactive conformation and remarkable resistance to proteolytic degradation. Next, we describe the rational design, synthesis and biological evaluation of three new families of allosteric inhibitors of PRC2 function, targeting for the first time three protein interfaces in PRC2 that are crucial for its proper assembly and function: the intramolecular SANT1L/SBD interaction of EZH2, the SUZ12-VEFS/EZH2-SANT2 binary complex and the interaction between SUZ12-NBE domains. Remarkably, these inhibitors have demonstrated cell permeability, potent activity in vitro and in physiological conditions, as well as strong antiproliferative effects on Caki-1 renal cancer cells, which highlights their potential as novel therapeutics for the treatment of PRC2-dependent human cancers.
DEDICATION

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&

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Chapter I. Peptidomimetics as Chemical Probes to Target Intracellular Protein-Protein Interactions

1.1 Background and Significance

Protein-protein interactions (PPIs) are crucial for a plethora of cellular processes and thus for the proper functioning of living cells and organisms. Their dysregulation can lead to many types of cellular dysfunctions which can further develop into diseases, hence, targeting specific PPIs is currently one of the most attractive approaches for designing potential therapeutics\textsuperscript{1-4}. The “undruggable” nature of these attractive macromolecular interactions, however, have made them particularly challenging targets. PPIs involve large, flat and featureless surfaces, ranging from 1500 to 3000 Å\textsuperscript{2} and lacking well-defined architectural features such as hydrophobic pockets or clefts\textsuperscript{5-7}. Thus, these recognition events result from a collection of scattered smaller interactions or hot-spots, which are structurally unsuitable for small molecules ligands, traditionally used by medicinal chemists\textsuperscript{8-11}. Indeed, effectively disrupting PPIs requires the use of larger molecules, capable of covering extensive molecular areas and establishing contacts with multiple hot spots simultaneously. Peptides are great candidates for such purposes since they are large enough to cover lengthy contact surfaces and also have the potential to recapitulate the shape and conformation of natural binding sites\textsuperscript{12-14}. In addition, they are easily accessible, offer high structural diversity and low toxicity. Unfortunately, high susceptibility to protease degradation and often poor cell permeability and binding affinities, have limited their therapeutic applications, especially for targeting intracellular PPIs\textsuperscript{15-16}. 
Extensive efforts have been made to overcome the poor pharmacokinetic properties of peptides and to transform them into more ideal drug candidates. As a result of this work, several approaches yielding peptidomimetic molecules have been discovered. These synthetically modified peptides, with improved pharmacokinetic profiles, are designed to mimic their bioactive parent sequences and have encountered applications in a wealth of biological targets, showing great promise as potential modulators of undruggable intracellular PPIs. Several reviews in the literature have discussed the progress in the field, primarily focusing on either specific families of compounds or in their biological applications. Since the number of relevant reports covering this attractive research area continues to grow, we present here a wider comprehensive updated overview focused on both the synthesis and the biological evaluation of two major groups of biologically active peptidomimetic derivatives: “stapled peptides”, designed to mimic alpha helical motifs of protein domains; and cyclopeptides resulting from long range macrocyclizations, aimed at mimicking beta sheets and other turn motifs. Some relevant biophysical properties of such compounds, including their cellular uptake and proteolytic stability are discussed as well.

1.2 Stapled Peptides

Peptide stapling has emerged as one of the most successful strategies to produce potent peptidomimetic inhibitors of intracellular PPIs. This methodology aims primarily at mimicking native α-helical domains, a recurrent protein motif that serve as a fundamental recognition unit in over 40% of naturally occurring PPIs. The idea behind stapling a peptide lies in tethering the side chain of a residue i with that of another residue at position i+4 or i+7 in the sequence, since these residues protrude on the same face of the helix (Figure 1.2.1). By incorporating a covalent “staple” the peptide is induced into its bioactive α-helical conformation,
that in addition, is stabilized by intramolecular hydrogen bonding between the amide bonds on opposite sides of the helix. This conformational restriction effectively improves not only the helical character of a given peptide sequence, and thus, its binding affinity to the protein target, but also its resistance to proteolysis and cell permeability properties. Several factors impact the extent of such improvement, particularly the length of the peptide sequence, as well as the position and physicochemical properties of the staple. A series of chemical strategies to accomplish peptide stapling have been reported to date. Next, we summarize them and further discuss the pharmacokinetic properties of the corresponding likers used for ligation, as well as some examples demonstrating the wide range of biological applications for which this promising new class of compounds have been successfully applied.

**Figure 1.2.1** The stapled peptide approach (A) Single turn stapled peptide (i, i+4) (B) Double turn stapled peptide (i, i+7).
1.2.1 Synthetic Methods Yielding Stapled Peptides

The incorporation of all-hydrocarbon staples by means of the ring-closing metathesis (RCM) reaction is the most widely used method for stabilizing α-helical peptides. This approach was first developed by Blackwell and Grubbs, who established an efficient solid phase procedure for the macrocyclization of linear peptides containing O-allylserine residues, via the ruthenium-catalyzed RCM reaction\textsuperscript{24}. An extension of this work by Verdine and colleagues further allowed for ligation of alkenyl side chains of α,α-disubstituted unnatural amino acids, previously incorporated at positions \((i, i+3)\), \((i, i+4)\) and \((i, i+7)\) in the corresponding linear sequences, under the same RCM stapling conditions\textsuperscript{25} (Scheme 1.2.1). This latter methodology constituted a major breakthrough in the field since it yielded macrocyclic α-helical peptides with remarkable resistance to proteolytic degradation, significantly improved alpha-helical character and enhanced affinity to their protein targets. Verdine’s pioneer stapling technology has been applied to generate multiple all-hydrocarbon stapled peptide inhibitors of intracellular PPIs with a wide variety of biological applications\textsuperscript{22, 26-28}. Moreover, it has been recently applied to generate the first stapled peptide drugs entering clinical trials\textsuperscript{29}.

**Scheme 1.2.1** Schematic representation for the synthesis of stapled peptides using the olefin metathesis reaction. Incorporation of α-methyl, α-alkenyl amino acids followed by stapling through ring closing metathesis affords all-hydrocarbon stapled peptides of various architectures.
Several other synthetic approaches allow for peptide stapling using alternative chemistries and linkers with diverse topologies, including ligation of cysteine-thiols, disulfide bridges, lactamization reactions, nitrogen arylation, formation of oxime or hydrazones linkers, Cu(I) catalyzed azide-alkyne cycloadditions and metal-catalyzed arylation reactions\textsuperscript{30}.

The ligation of cysteine residues exploits the nucleophilicity of the thiol functional group to facilitate substitution reactions over highly reactive electrophiles, affording bithioether stapled peptides tethered mainly by different aromatic ring scaffolds\textsuperscript{31-32}. Several bis-aromatic electrophiles have been successfully used for this purpose, including photosensitive 4,4’-bis(iodoacetamide)azobenzene\textsuperscript{33-34}, α,α’-dibromo-m-xylene\textsuperscript{35-36}, 4,4’-bis(chloroacetamide) biphenyl-acetyl-ene\textsuperscript{37} and 4,4’-bis(bromomethyl)biphenyl (Scheme 1.2.2)\textsuperscript{38}. Other approaches have used 1,3-dichloro-acetone\textsuperscript{39}, perfluoryl\textsuperscript{40}, tetrazine\textsuperscript{41} and bis-palladium aryl derivatives\textsuperscript{42}.

\textbf{Scheme 1.2.2} Solution phase approaches for the synthesis of bithioether stapled peptides using highly reactive dihaloelectrophiles.

On the other hand, only a small number of protocols allow cysteine ligation with all-hydrocarbon linkers, primarily due to the poor electrophilicity of aliphatic dihaloalkanes. Thus,
for example, Jo et al. reported that the direct bis-alkylation of cysteine thiols with dibromo and diiodoalkanes in solution phase only affords the corresponding unreacted linear precursors mixed with disulfide-bridged peptides. An extension of this solution protocol including TCEP as reducing agent, longer reaction times and higher temperatures has allowed the preparation of $i+4$ and $i+7$ bishthioether stapled peptides linked through eight and nine methylene groups, respectively. A similar solution phase approach, using commercially available 3,3-bis(bromomethyl)oxetane as electrophile, has been recently applied to the efficient stapling of cysteine residues on peptides and proteins under biocompatible aqueous conditions.

An elegant alternative to the two-component bis-alkylation of cysteine thiols with poorly electrophilic aliphatic cross-linkers has been recently reported by Wang et al., who have applied a photoinduced thiol-ene radical reaction with $\alpha,\omega$-dienes to produce bis-thioether stapled macrocycles bearing hydrocarbon braces with five, seven and nine methylene groups (Scheme 1.2.3).

**Scheme 1.2.3** Solution synthesis of bishthioether stapled peptides by means of a two-component thiol-ene reaction between a fully deprotected peptides and various dienes.

Although this solution protocol avoids using unnatural amino acids, its scope is somewhat limited by its required use of a high boiling point organic solvent, UV-light activation and a radical
initiator as reaction catalyst. Thiol/yne stapling can also be carried out through a similar one-component radical reaction with alkynes, affording vinyl sulfide linked cyclopeptides\textsuperscript{46}.

Recently, the more reactive selenocysteine (as compared to cysteine) unnatural amino acids have been successfully used for peptide stapling through a selenoether linkage. This solution phase protocol allows for ligation with a wide range of alkylating reagents including poorly electrophilic dihaloalkanes (Scheme 1.2.4)\textsuperscript{47}. Likewise, stapling can also be achieved by means of disulfide bridges\textsuperscript{48-49}. Indeed, this was one of the first reported ligation techniques. However, due to the instability of the disulfide linkage in reducing environments this method has encountered only limited application. A recent example uses a reversible reaction for stapling BID and RNase S modified peptides through either oxidized disulfides or a dibromomaleimide crosslinker\textsuperscript{50}. The resulting macrocycles show resistance to proteolysis, enhanced $\alpha$-helical conformation and improved biological activity as compared to their respective linear counterparts.

\textbf{Scheme 1.2.4} Solution synthesis of bis-selenoether stapled peptides by means of a two-component reaction between fully deprotected selenocysteine-substituted peptides and various electrophiles.
Lactamisations have also been extensively applied for peptide stapling at positions \((i, i+4)\) through amide-bond forming intramolecular cyclization of, for example, Lys/Asp, Orn/Lys and Asp/Glu residues (Scheme 1.2.5a)\(^{31-53}\). An advantage of this approach is that it uses natural amino acids, however, it also requires orthogonal protection of the residues participating in the ligation step. Nonetheless, several groups have applied this technique to generate cyclopeptides with superior \(\alpha\)-helicity and biological activity, mostly targeting extracellular or membrane-bound PPIs. More recently, lysine-based substituted peptides have also been used as scaffolds to generate aryl-tethered stapled peptides, through an N-arylation intramolecular macrocyclization and using several aromatic bis-electrophiles (Scheme 1.2.5b)\(^{54}\).

**Scheme 1.2.5** (a) Peptide stapling through lactamisation, a polar amide bond can be chemoselectively formed by orthogonally protecting both the amino and the carboxylate functional groups used for the macrocyclization (b) Nitrogen arylation of unprotected peptides allows cross-linking through lysine-based nucleophiles, using highly reactive aryl electrophiles.

The well-known click cycloaddition between an azide and an alkyne is another example of a widely-used organic reaction applied to this field. This solution phase approach requires the incorporation of two azide functionalized amino acids at positions \((i, i+6), (i, i+7)\) or \((i, i+8)\) in the linear sequence. The ligation is next achieved by means of a double-click stapling step with
dialkynyl linkers under Cu(I) catalysis, affording bis-triazole tethered peptides (Scheme 1.2.6)\textsuperscript{28, 55-58}. Similarly, peptide stapling has also been accomplished by popular metal catalyzed C-C bond formation reactions. Thus, using the Suzuki-Miyaura methodology Meyer and colleagues have prepared bi-aryl linked cyclopeptides, from linear sequences containing borylated phenyalanine residues\textsuperscript{59}. Likewise, Mendive-Tapia et al. have reported an intramolecular cross coupling ligation between iodinated phenylalanine or tyrosine precursors and tryptophan residues, through a C-H activation process catalyzed by Pd(II). This approach yields aryl-tryptophan linked macrocycles of various sizes and is carried out in both solution and on solid phase\textsuperscript{60}.

**Scheme 1.2.6** (a) Cu(I)-catalyzed cycloaddition between bis-alkynes and a bis-azide functionalized peptide allows for peptide cross-linking through a bis-triazole linker (b) Suzuki-Miyaura coupling affords stapled peptides with a biphenyl ring spacer.
Table 1.1 Summary of Stapling Methodologies

<table>
<thead>
<tr>
<th>Stapling Reaction</th>
<th>Linkers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Hydrocarbon linkers (X= S or OMe)</strong></td>
<td><img src="image" alt="All Hydrocarbon linkers" /></td>
</tr>
<tr>
<td>Ring closing metathesis(^{15})</td>
<td><img src="image" alt="Ring closing metathesis" /></td>
</tr>
<tr>
<td>Thiol-ene/ynne reaction(^{15,46})</td>
<td><img src="image" alt="Thiol-ene/ynne reaction" /></td>
</tr>
<tr>
<td><strong>Miscellaneous linkers (X= S, OMe, NH or Se)</strong></td>
<td><img src="image" alt="Miscellaneous linkers" /></td>
</tr>
<tr>
<td>Thiol bis-alkylation(^{32-42})</td>
<td><img src="image" alt="Thiol bis-alkylation" /></td>
</tr>
<tr>
<td>Thiol-ene Thiol-yne reactions(^{45,46})</td>
<td><img src="image" alt="Thiol-ene Thiol-yne reactions" /></td>
</tr>
<tr>
<td>Azide-Alkyne Cycloaddition(^{55-59})</td>
<td><img src="image" alt="Azide-Alkyne Cycloaddition" /></td>
</tr>
<tr>
<td>Suzuki-Miyaura reaction(^{60})</td>
<td><img src="image" alt="Suzuki-Miyaura reaction" /></td>
</tr>
<tr>
<td>Selenocysteine Crosslinking(^{7})</td>
<td><img src="image" alt="Selenocysteine Crosslinking" /></td>
</tr>
<tr>
<td>Oxidation(^{48,50})</td>
<td>Disulfide bridge (S-S)</td>
</tr>
<tr>
<td>Lactamation(^{51-53})</td>
<td>Amide bonds (-CONH-)</td>
</tr>
<tr>
<td>Nitrogen Arylation(^{49})</td>
<td><img src="image" alt="Nitrogen Arylation" /></td>
</tr>
</tbody>
</table>
Overall, the number of approaches applied to stabilize peptide $\alpha$-helical structures has expanded significantly in the last few years and continues to grow fast. The versatility of these methods has undoubtedly facilitated the identification of multiple bioactive cyclopeptides, particularly useful to target intracellular intractable PPIs. Each of them has their own strengths but they also have some associated weaknesses. For example, most protocols require the use of expensive unnatural amino acids which increases significantly the overall cost of the process. An exception to this problem is to exploit the reactivity of native cysteine residues. Other significant drawbacks include the need for metal catalysis, high boiling point organic solvents (for solution phase methods) and photo-induced activation as in the case of the thiol-ene/yn e ligations. Another major difference comes from the topology of the staple resulting from the ligation step, which may have a significant impact on the induction of the $\alpha$-helical conformation of the macrocycle. This feature has been recently explored by De d. Araujo et al. who tested the degree of helical induction in a model pentapeptide ligated with six different linkers$^{61}$. These studies showed that lactamisation led to the greatest increase in $\alpha$-helicity closely followed by the all-hydrocarbon staple and triazole spacers, whereas mono- and bis-thioether linkages showed lower ability to stabilize the peptide $\alpha$-helical conformation. More comprehensive studies in larger macrocycles and using other stapling chemistries will provide additional valuable insights into this critical factor for stapled peptide’s design.

In summary, the field of stapled peptide synthesis has attracted significant interest since the discovery of the RCM stapling technology. The success and potential of this class of compounds has been already extensively demonstrated. Therefore, novel synthetic methodologies that expand the scope of the current approaches and grant easy access to stapled peptides continue to be of great interest in the field.
1.2.2 Cell Permeability of Stapled Peptides

Over 95% of the peptides entering clinical trials in 2016 were directed at GPCRs and other extracellular targets, including receptor tyrosine kinases and ion channels, whereas only the remaining 5% were designed for intractable intracellular hosts. A key factor for the low success in the latter group of peptide drugs is the overall poor cell permeability of these compounds. In this context, stapled α-helical peptides have emerged as an effective solution to this problem, since when properly designed, they show significantly improved cell penetration capabilities. Several reports have investigated in depth the internalization of this class of compounds as well as their mechanism of cellular uptake. Thus, Verdine’s laboratory developed an effective epifluorescence microscopy assay to evaluate the cell penetration of over 200 FITC-labeled peptides derived from several sequences and tethered with different staples that were placed at various positions in the parent sequence. This quantitative study confirmed the substantial superior cell penetrating power of stapled peptides versus their linear counterparts, even when comparing them to natural unmodified cell penetrating sequences. This work also indicated that both the stapling type and the formal charge of the resulting macrocycle are key for peptide translocation, while other physical parameters didn’t have a significant effect. The former is especially favored by the incorporation of all-hydrocarbon staples and the latter by net positive charges below +7 at physiological pH. Interestingly, the correlation between cell penetration and net charge observed in this work is not in agreement with other studies carried out with linear peptides and mini-proteins, in which a higher net positive charge translates into better internalization likely due to higher interaction with the negatively charged phospholipid membrane. These researchers also provided mechanistic insights for the cellular uptake of such stapled...
peptides, which occurred by an endocytosis pathway and through the interaction with sulfated proteoglycans located in surface of the cell membrane.

The Walensky laboratory has also investigated extensively both the biophysical parameters impacting the internalization of all-hydrocarbon stapled peptides and their import mechanism. Thus, in an early work carried out on leukemia cells, this group showed that BID BH3 stapled peptides were internalized through a micropinosomal mechanism followed by pinosomal release\textsuperscript{19}. This result was consistent with a later report that used electron microscopy assays to confirm vesicular cellular uptake, without plasma membrane disruption\textsuperscript{64}. The latter mechanism has also been demonstrated by high resolution fluorescence correlation microscopy studies, performed with stapled p53 peptides capable of restoring p53 function by dissociating both p53-HDM2 and p53-HDMX interactions dose-dependently\textsuperscript{27}. Likewise, an extensive study published by this laboratory in 2016 focused on determining the biophysical parameters favoring the cell penetration properties of these macrocycles\textsuperscript{65}. This report studied the internalization of a staple-scanning library of FITC-labeled stapled peptides designed to target BCL-X, an anti-apoptotic transmembrane protein that regulates mitochondrial biomolecules involved in programmed cell death mechanisms. The results identified the degree of hydrophobicity of the peptide as a major factor for enhanced cellular uptake, which is particularly favored when the hydrophobic all-hydrocarbon brace is placed at the amphipathic boundary of the peptide binding face. Notably, an excess of hydrophobicity and/or net positive charge are also undesired since cyclopeptides with such properties are more prone to cause membrane lysis at high concentrations.

To date most of the comprehensive permeability studies have been carried out with all-hydrocarbon stapled peptides. It is likely that the rules governing the cell penetrating power of such compounds also apply to the internalization of macrocycles bearing other types of staples.
This hypothesis has been partially demonstrated by Zigang Li et al. who investigated the impact of different stapling architectures on the physicochemical and cell permeability properties of a model peptide, targeting the estrogen receptor coactivator\textsuperscript{66}. Notably, the authors observed that polar linkers such as lactam or triazole diminished substantially the cellular uptake, whereas hydrophobic braces improved significantly cell permeability. Overall, they concluded that hydrophobicity correlates well with peptide translocation, in contrast with helical character that was not a major factor. More comprehensive studies using other model peptides and staples with different chemistries will contribute to a better understanding of the biophysical determinants for the effective cellular uptake of stapled peptides.

1.2.3 Staple Peptide’s Stability to Proteolysis

The ability of stapled peptides to evade protease degradation is one of the most beneficial properties validated for this class of peptidomimetics. Although stapling is typically applied to induce a desired bioactive $\alpha$-helical conformation\textsuperscript{67,67,67}, such conformational restriction also limits the ability of enzymes to recognize their natural peptidyl substrate. By “blinding” proteases through macrocyclizations or with the incorporation of unnatural building blocks, \textit{in vitro} proteolysis is decreased and serum stability is significantly improved\textsuperscript{22, 68}. This translates into enhanced pharmacokinetic properties and thus, into a more favorable drug profile. Indeed, the strong resistance of several families of stapled peptides to proteolytic degradation has been substantially validated \textit{in vitro}, by subjecting them to enzymatic reactions using proteases with broad substrate specificity such as $\alpha$-Chymotrypsin, Pepsin and Proteinase K\textsuperscript{60, 67}. Likewise, their stability in plasma, liver microsomes and \textit{in vivo} has also been well-demonstrated\textsuperscript{69}. All these studies have shown that the lifetime of stapled peptides usually ranges from several hours to days, far exceeding that of their linear counterparts, which are mostly degraded within minutes. This
remarkable resistance to proteolytic degradation has been observed in stapled peptides derived from large sequences and in macrocycles tethered by staples of various chemical nature. A representative example of the former was reported by Walensky and coworkers who introduced two all-hydrocarbon braces via the RCM reaction into a 36-residue long peptide to afford a double-stapled or “stitched peptide” (SAH-gp41), which effectively targets the HIC-1 fusion apparatus. Notably, this compound showed enhanced stability both \textit{in vitro} and \textit{in vivo} and improved oral bioavailability, as compared to its linear parent sequence. As mentioned before, macrocycles stapled via methodologies other than RCM reaction also show strong protection to proteases, indicating that this feature is not highly dependent on the structure of the brace used for ligation. Thus, cyclopeptides containing bis-triazole, vinyl sulfide, bi-aryl or aryl-tryptophan linkers have all shown significantly improved stability to protease degradation.

The ultimate validation of the stability achieved by means of properly introducing a stapling linker into a bioactive peptide sequence is given by the development of the first stapled peptide drugs entering clinical trials. Aileron Therapeutics, founded in 2005, announced its first all-hydrocarbon stapled peptide (ALRN-5281) entering human Phase I trials in 2013, as a potent agonist of the growth hormone releasing factor (GRF) currently tested for orphan endocrine disorders. The same company has recently developed a second stapled peptide drug, ATSP-7041, which targets an intracellular interaction in between MDM2 and MDMX and thus is capable of activating the function of the human tumor suppressor transcription factor p53 \textit{in vitro} and \textit{in vivo}.

\subsection*{1.2.4 Stapled Peptides: Improved Target Binding Affinity and Diversity of Binding Strategies}

The potential of stapled peptides as effective chemical modulators of intracellular protein-protein interactions was first demonstrated by Walensky \textit{et al.} in 2004. In this breakthrough
discovery, these researchers demonstrated that introducing all-hydrocarbon braces into unfolded BH3 peptides induced α-helical conformations in such sequences, which showed helicities of up to 90% in solution. Moreover, the resulting all-hydrocarbon stapled peptides were remarkably resistant to proteolytic degradation in both in vitro and in vivo studies, and in addition, exhibited in vitro nanomolar binding affinity for their BCL-2 family targets. These cell penetrant peptidomimetics, referred to as “stabilized” alpha-helix of BCL-2 domains (or SAHBs), were also able to trigger cellular stress and apoptosis in vivo.

After this original report, a large number of research groups have applied the RCM technology, and also most of the newly-developed stapling approaches, to generate a wide array of potent inhibitors of intracellular and extracellular PPIs. These modulators of protein function have shown potential applications in several diseases including cancer, metabolic and infectious diseases as well as neurological disorders (Table 2). Thus, for example, the Walensky laboratory has identified a series of BH3 mimetics that directly bind to MCL-1, and drive cancer cells to caspase-dependent apoptosis72. Other critical cancer-related mechanisms that have been successfully targeted by all-hydrocarbon stapled peptides include the Wnt/Beta-catenin and the NOTCH signaling pathways73-74. Likewise, Pellegrini et al. have used the two-component Azide-yne stapling reaction to develop a potent inhibitor of the Ctf4-DNA Polymerase α interaction28. Ctf4/AND-1, a core component of the replisome progression complex, is an adaptor protein that bridges the helicase with Polymerase α complexes, and thus plays a key role in DNA replication, repairs and chromosome segregation. Despite its biological relevance, Ctf4 has been an elusive target possibly because it lacks a well-defined active site, being thus unfit for small molecule design. Notably, these investigators have successfully developed an \((i, i+6)\) macrocycle (Sld5) that shows sub-micromolar binding affinity to Ctf4. Sld5 is capable of effectively displacing the Ctf4
partner, DNA polymerase, and thus acts as a potent DNA-damaging cancer therapeutic agent. It is worth noting that although this cyclopeptide shows poor cellular uptake, it is expected that further modifications to the topology of the tethering linker will contribute to improve its overall cell permeability.

The incorporation of a “staple” into a linear peptide sequence is now a proven and powerful tool for inducing bioactive α-helical conformations, and thus for the rational design of targeted peptide therapeutics. Interestingly, the spacer used for ligation can also provide additional binding benefits that extend beyond precise structural mimicry. This idea has been introduced by Popowicz and coworkers, who reported an X-ray structure of a stapled p53 peptide, SAH-p53-8, in complex with its protein target MDM2. This structural analysis revealed that the hydrocarbon brace of SAH-p53-8 contributes to the binding energy by establishing hydrophobic interactions with some residues in the protein surface, thus expanding the role of the staple from a purely structural stabilizer or permeability enhancer, to an element that can be exploited for increased binding affinity.

Overall, the progress achieved in the stapled peptide technology has allowed the identification of novel therapeutic agents targeting intractable protein surfaces, inadequate to be tackled by small molecule ligands. Such success is illustrated by the discovery of the first staple peptides entering human clinical trials. ALRN-6924, a second-generation macrocycle developed by Aileron Therapeutics, effectively disrupts the interaction between the p53 tumor suppression protein and both the murine double minute 2 (MDM2) and murine double minute X (MDMX) proteins, and is currently being tested in patients with peripheral T-cell lymphoma (PTCL), acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). The potential success of this
compound, and of others currently in development, could certainly transform the future of PPI’s inhibitors development.

**Table 1.2 Applications of the Stapled Peptide Technology in a Variety of Human Diseases**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Protein Target</th>
<th>Stapling Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<tr>
<td>BCL-2 Proteins</td>
<td>BAK/SAHB/BAX</td>
<td>RCM</td>
<td>Walensky et al. 19, 70</td>
</tr>
<tr>
<td></td>
<td>MCL-2/BH3/BAX</td>
<td>RCM</td>
<td>Walensky et al. 5`</td>
</tr>
<tr>
<td></td>
<td>BAK/BID-BH3</td>
<td>RCM</td>
<td>Moldoverneau et al. 76</td>
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<tr>
<td>MDM2/MDMX</td>
<td>p53/(MDM2/MDMX)</td>
<td>RCM</td>
<td>Bautista et al. 77</td>
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<tr>
<td></td>
<td>p53/(MDM2/MDMX)</td>
<td>RCM</td>
<td>Chang et al. 26</td>
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<td>RCM</td>
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<td>Azide-yne</td>
<td>Spring et al. 78</td>
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<td></td>
<td>p53/MDM2</td>
<td>Thiol-ene</td>
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<tr>
<td>NOTCH</td>
<td>SAHM1/NOTCH</td>
<td>RCM</td>
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<td>Hao, Y. et al. 29</td>
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<td>EED/EZH2</td>
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<td>Kim W et al. 80</td>
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<td>Moellering et al. 81</td>
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<td>Walensky et al. 22</td>
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<td>Dithiol bisalkylation</td>
<td>Weijun Shen et al. 83</td>
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<td>Sviridov et al. 84</td>
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<td>Neurotensin receptor</td>
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<td>Jieping Zhu et al. 87</td>
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<td>rAps III/Na+ Channels</td>
<td>Disulfide Linkage</td>
<td>King et al. 88</td>
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</table>
1.3 **Long Range Macrocyclization Methodologies**

Long-range macrocyclization strategies are one of the earliest methods used to successfully modify peptide’s secondary structures (Figure 1.3.1). Macrocyclization differs from cyclization based on the relative length of residues to be joined or linked, relative to the length of the peptide. Thus, in general terms 12 or more membered rings are considered “macrocycles”. Due to their large size, and thus the ability to interact with extended surfaces, macrocyclic peptides are also ideal candidates for disrupting protein-protein interactions. Indeed, over the last several decades, macrocyclizations have attracted a great deal of attention by medicinal chemists\(^7\).

![Figure 1.3.1](image1.png)

**Figure 1.3.1** Structural representation of long range macrocyclization.

The strategies used for macrocyclizing peptides are diverse and escape any simple classification scheme\(^7\). Most broadly, methods are categorized based on what functional regions of the peptide are involved in the eventual linkage. The possibilities include head-to-tail, head/tail-to-side chain, or side chain-to-side chain macrocyclic strategies. Within these broad descriptions of overall architecture, one can subcategorize based on the reaction pathway or the class of the resulting product. Several reactions have been applied to achieve macrocyclizations. The most popular ones include lactamization, lactonization, the RCM reaction, transition metal-catalyzed cross couplings and the one-pot azide-alkyne click chemistry cycloaddition\(^7\). Lactamization is likely the most frequently utilized, since it can exploit the reactivity between head and tail, basic side chains and tail, acidic side chains and head, or basic and acidic side chains. Evidently,
lactamization can be applied to nearly every architectural class, largely due to the diversity of amino acid side chains.

When combined with the use of non-natural amino acids, orthogonal protecting group strategies, and biosynthetic pathways, the methods and potential products of macrocyclization increase in diversity. Thus, in addition to standard reactions above-cited, other chemistries applied to macrocyclizing peptides include oxadiazole grafting, sulfur-mediated cyclizations, isocyanides and other multicomponent reactions, and intein-catalyzed S/N acyl transfer. All these approaches have been extensively reviewed and include both internal modifications to the linear peptide, and external template modifications. It is worth noting that for any macrocyclization, it is crucial to control the factors favoring such transformation over other intermolecular reaction pathways. One highly effective strategy to achieve such a goal is by reducing the concentration of the reacting species, i.e., to perform reactions at high dilution and/or to control the rate of addition of either the substrate or reagents. Similarly, through conformational control of the linear precursor it is possible to lower the entropy of the substrate, and thus induce a “turned conformer” in which both reacting ends are brought in close spatial proximity. These strategies are also discussed in the previously mentioned reviews.

1.3.1 Cell Permeability of Macrocyclic Peptides

Cell permeability remains a major challenge for macrocyclic peptides. While they have been demonstrated as highly effective and specific modulators of many extracellular PPIs, few macrocyclic peptides demonstrate high cell permeability. A recent review by Walport and colleagues discusses potential paths forward for transforming macrocyclic peptides into cell permeable molecules. Unlike α-helices, where the characteristic secondary structure allows for
the internalization of hydrogen bond-participating side chains, the secondary structures assumed by macrocyclic peptides do not necessarily possess this same helpful characteristic. Instead, the method of using N-methylated residues is frequently employed to reduce the number of hydrogen bond donors and increase overall hydrophobicity of macrocyclic peptides to increase cell permeability\textsuperscript{85} and bioavailability\textsuperscript{85-86}.

A novel alternative strategy for conferring cell permeability to macrocyclic peptides, involves coupling them with known cell permeable peptides (CPPs). Using this method, Trinh and co-workers tested the impact of incorporating cyclo(FΦRRRRQ) (cFΦR\textsubscript{4}, where Φ is L-2-naphthylalanine), a CPP with known cell internalization properties, into macrocycles designed to target K-Ras, an oncogene that plays a key role in cell differentiation and apoptosis\textsuperscript{87}. Indeed, this strategy yielded cell-penetrating bicyclic peptides capable of inducing apoptosis in cancer cells, thus validating the applicability of this approach. The CPP used in this study is thought to gain intracellular access by binding directly to phospholipids of the cellular membrane and internalizing via endocytosis\textsuperscript{88}.

1.3.2 **Macrocyclic Peptides as inhibitors of PPIs**

Macrocyclic peptides have been effective at targeting PPIs involved in a variety of biological pathways, including cancer, infectious and autoimmune diseases and regenerative medicine. Some representative applications of such inhibitors are presented in Table 1.3.

An elegant and highly novel example showing the potential of this class of compounds have been recently published by Ito and co-workers\textsuperscript{89}. Notably, the authors have developed dimeric macrocyclic peptides capable of inducing dimerization of the hepatocyte growth factor (HGF) receptor, also known as Met or c-Met, and thus activate downstream Met signaling cascades. These compounds underwent a first macrocyclization by a head-to-tail thioether-linkage forming reaction,
between an N-terminal chloroacetyl group and thiol functionality. Dimerization of the monomers was next achieved by coupling C-terminal cysteine residues with bis-maleimide linkers of different lengths. The resulting dimeric macrocycles were capable of promoting cellular responses such as migration, proliferation and branching morphogenesis at low nanomolar concentration. This example demonstrates the unique cellular mechanisms accessible to macrocyclic peptide therapeutics.

Table 1.3 Representative examples of macrocyclic peptides disrupting biologically relevant PPIs.

<table>
<thead>
<tr>
<th>Disease / Pathway</th>
<th>Protein Target</th>
<th>Macrocyclization Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td>MM401-WDR5</td>
<td>RCM</td>
<td>Dou, Y. et al.92</td>
</tr>
<tr>
<td>Leukemia</td>
<td>MM589-WDR5</td>
<td>Lactamization (Internal)</td>
<td>Wang, S. et al.93</td>
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<tr>
<td>Ebola</td>
<td>VP24-KPNA5</td>
<td>Thioether</td>
<td>Suga, H. et al.102</td>
</tr>
<tr>
<td>Wound healing</td>
<td>Met-hHGF</td>
<td>Thioether</td>
<td>Suga, H. et al.103</td>
</tr>
<tr>
<td>Immune response</td>
<td>PB1m6-Sema4D</td>
<td>Thioether</td>
<td>Suga, H. et al.104</td>
</tr>
<tr>
<td>Cancer, Organogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>AKT2-Paki L1</td>
<td>Thioether</td>
<td>Suga, H. et al.105</td>
</tr>
<tr>
<td>Cancer</td>
<td>Ras-Cyclorasin 9A5</td>
<td>Lactamization (Internal)</td>
<td>Pei, D. et al.106</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>PTP1B</td>
<td>lactamization</td>
<td>Pei, D. et al.107</td>
</tr>
<tr>
<td>Cis–trans isomerization</td>
<td>Pin1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identification and isolation of potent</td>
<td>Streptavidin</td>
<td>Intein-catalyzed S, N acyl transfer</td>
<td>Fasan, R. et al.96</td>
</tr>
</tbody>
</table>

1.4 Peptidomimetic Molecules for Therapeutic Development: Significance

Unquestionably, the methodologies applied to peptide stabilization have made a significant contribution to therapeutic development. In particular, the discovery of the stapled peptide
technology by Verdine and colleagues in 2000 has triggered an exponential and growing number of relevant publications in the field. Considerable progress has been made in the synthetic approaches allowing access to these compounds. Likewise, the data generated so far have enhanced our overall understanding of basic rational design principles, mechanisms of cellular uptake and factors impacting the pharmacokinetic properties of these macrocycles. More importantly, this technology has allowed the identification of potent inhibitors of biologically relevant PPIs that were inaccessible using traditional medicinal chemistry approaches. Such remarkable advances, achieved in a relatively short period of time, have led to the development of the first stapled peptides entering human phase 1/2 clinical trials by Aileron Therapeutics, the leading company in the field, whereas several others compounds are currently in the pipeline. Altogether, these studies demonstrate the tremendous potential of peptidomimetic molecules for the generation of novel and effective therapies to treat incurable human diseases.

In this regard, the work described in these doctoral thesis focuses, first, on developing a new and an efficient synthetic methodology for the chemoselective solid phase synthesis of single- and double-turn bis-thioether stapled peptides. The scope of this approach is demonstrated by synthesizing macrocycles derived from several sequences, validating its compatibility with different amino acids and SPPS. This method allows access to cyclopeptides tethered by all-hydrocarbon linkers of different lengths, avoiding the use of unnatural amino acids and expensive catalysts. Moreover, it affords macrocycles with marked resistance to proteolytic degradation. We expect this feature to result in an improved pharmacokinetic profile that makes this family of compounds suitable for use in biological studies.

Furthermore, we also present the application of our synthetic approach to generate stapled peptides that effectively disrupt biologically relevant intracellular PPIs. More specifically, we
describe three families of stapled peptides as allosteric inhibitors of PRC2 methyltransferase activity. Compared to their linear counterparts, these compounds showed enhanced $\alpha$-helical structure and inhibition of target function. Our lead cyclopeptides were found to be potent inhibitors of H3K27 trimethylation in both \textit{in vitro} and cellular assays, demonstrating their cell permeability and activity in physiological conditions. Inhibition of PRC2 catalytic activity by these compounds was correlated with a marked dose-dependent antiproliferative effect in metastatic Cakis-1 cells. Remarkably, in these experiments our three lead compounds were virtually as potent as GSK126, an EZH2-SET domain inhibitor currently in clinical development. The structure of our novel stapled peptides highlight the relevance of our crosslinking reaction, since such macrocycles are tethered by a three-methylene hydrocarbon brace that can only be efficiently introduced using our solid phase synthetic approach. The allosteric PRC2 inhibitors described herein could be of great value to address the resistance profiles found after extended dosing with the PRC2 inhibitors currently undergoing clinical trials, all of which target the catalytic SET domain of EZH2.

1.5 Acronyms and Abbreviations

All the abbreviations used for amino acids and peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in \textit{J. Biol. Chem.} 247, 977-983 (1982). In addition, the following abbreviations are used: SPPS: Solid Phase Peptide Synthesis, DMF: $N,N$-dimethylformamide, DCM: dichloromethane, Fmoc: $9H$-fluorenlymethoxy carbonyl, DIEA: $N,N$-diisopropylethylamine, PyBOP: (benzotriazol-1-yl)tripyrrolidinophosphonium hexafluorophosphate, TFA: trifluoroacetic acid, Oxyma: Ethyl cyano(hydroxyimino)acetate, NaI: Sodium Iodide, TCEP: Tris(2-carboxyethyl)phosphine hydrochloride, Boc: \textit{tert}-Butyloxycarbonyl, Trt: trityl, Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Mmt: 4-methoxytrityl, EDT:
PARPs: Poly (ADP-ribose) polymerases, TNKS: Tankyrase proteins, SAH-gp41: Stabilized α-helix of GP4
Chapter II. A Solid Phase Approach to Accessing Bisthioether Stapled Peptides

2.1 Introduction

Several synthetic approaches yielding stapled peptides have been developed in the last few years, mostly in solution phase, each one having its own advantages and drawbacks. Thus peptide stapling can be achieved today with a variety of spacers, however, the number of methods affording macrocycles tethered by all-hydrocarbon spacers remains scarce. Given the great promise of this class of compounds as therapeutics and their favorable pharmacokinetic properties, new protocols granting easy access to them continue to be of great interest. In this chapter, we report the development of an efficient solid phase method for the chemoselective ligation of cysteine residues applicable to the preparation of single-, double-turn and double-stapled macrocycles. This approach allows for ligation with all-hydrocarbon linkers of various lengths, avoiding the use of unnatural amino acids and expensive catalysts, and affords cyclopeptides with remarkable resistance to proteolytic degradation.

2.2 Optimization of the Stapling Reaction.

Our goal was to develop a solid phase approach that can be applicable to both hydrophilic and poorly soluble hydrophobic peptides. In addition, in contrast to the currently available cysteine stapling methods, we envisioned that such protocol could also allow the chemoselective ligation of sequences containing several cysteine residues and to prepare macrocycles containing multiple bisthioether staples.
For our initial optimization studies were chose a 10-mer sequence containing two cysteines at positions \(i\) and \(i+4\). (Scheme 2.2.1). The peptide was elongated on a low substituted resin to avoid undesired intermolecular crosslinking during the ligation step. To allow selective deprotection on beads, both Cys were orthogonally protected with 4-methoxytrityl (Mmt). We first attempted the cyclization using the conditions reported for a similar reaction in solution phase, however, in our case it only afforded small amounts of the desired cyclic peptide, mixed with unreacted linear precursor(-SH, -SH) and the disulfide-bridged (S-S) macrocycle (Table 2.1, Entry 1).\(^{43}\)

To push the reaction toward the formation of the stapled peptide, we then designed a series of 30 combinatorial experiments, with variation of one parameter at a time, and tested the use of different solvents, non-nucleophilic organic and inorganic bases, microwave activation and length of reaction time. We also explored including sodium iodide, to increase the electrophilicity of the dibromoalkane by inducing \textit{in situ} trans-halogenation, and TCEP to prevent the disulfide oxidation (Table 2.1). From these experiments, we identified optimal conditions (DIEA 35 eq., TCEP 0.5 eq., linker 3.5 eq. microwave 125 °C, 2 min) that led to 98% conversion to the desired bisthioether cyclopeptide, in high purity, as determined by both LC/MS and HPLC analysis (Table 2.1, Entry 7J). Subsequently, the reaction was scaled up. However, further efforts to complete peptide elongation to a 19-mer proved unsuccessful, primarily due to inefficient amino acid couplings after cyclization.
Scheme 2.2.1 Synthetic scheme initially designed to study the stapling reaction on a 10mer linear Peptide.
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<th>Solvent</th>
<th>Rxn conditions</th>
<th>Rxn time (min)</th>
<th>N2 (eq)</th>
<th>TCEP (eq)</th>
<th>NaI (100eq)</th>
<th>Linker (20 eq)</th>
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<td>C3</td>
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<td>C3</td>
<td>43</td>
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</table>
We then study the reverse approach, i.e., elongating the peptide first and performing the stapling reaction thereafter. The full sequence was assembled using pseudoproline dipeptides to avoid potential aggregation problems that may have caused the incomplete couplings observed before. Subsequent Cys-Mmt deprotection and stapling of this substrate, using the previously optimized conditions, afforded the desired bis-thioether stapled peptide in high overall purity as discussed in details in the following section.

2.3 Solid Phase Synthesis of Single-Turn Stapled Peptides (i, i+4).

Single-turn peptide stapling was investigated using dibromoalkyl electrophiles containing three different hydrocarbon chains and following the synthetic scheme depicted below (Scheme 2.3.1). The solid phase synthesis of the Cys-mutated linear precursor 1 was accomplished using pseudoproline dipeptides and afforded a highly pure crude material (Figure S2.1, S2.2).
Scheme 2.3.1 Solid phase synthesis of single-turn (i, i+4) stapled peptides bearing all-hydrocarbon linkers of different lengths.
Next, we carried out Cys-Mmt deprotection, which was accurately quantitated by means of a “maleimide test” developed in our laboratory (Scheme 2.3.2). Subsequent cyclization with 1,3-dibromopropane yielded only the expected stapled peptide with an overall purity for the total synthesis greater than 80% (Table 2.2, Scheme 2.3.1, Figure S2.1 and S2.2). This reaction can be scaled up to produce 75% pure stapled peptide from 5 g of resin.

Scheme 2.3.2 Maleimide test developed to assess the efficiency of the Mmt-deprotection reaction. Since bulky protecting groups in the vicinity of Cys-Mmt could cause incomplete deprotection despite the sensitivity of Mmt to acid media, we developed conditions for quantitatively assessing the efficiency of Mmt deprotection. Free thiols generated after deprotection, are covalently blocked through a well-known thiol-maleimide addition step. Cleavage of an aliquot of the reacted peptide, and LC/MS analysis allow to unequivocally assign fully deprotected products, partially or non-deprotected sequences, as well as mixtures containing all of them.
Table 2.2 Synthesis of single- \((i,i+4)\), double-turn \((i,i+7)\) and double-stapled peptides

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<th>Entry</th>
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<th>Linker (X) (eq)</th>
<th>Rxn</th>
<th>Conversion To Cyclic (%)</th>
<th>% Purity Cyclic (HPLC)</th>
<th>% Purity Cyclic (MS)</th>
<th>% Linear (HPLC)</th>
<th>% Dha (HPLC)</th>
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<td>S1C</td>
<td>C3 (3.5)</td>
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<td>98</td>
<td>85</td>
<td>83</td>
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<td>-</td>
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<td>(i,i+4)</td>
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<td>19mer: Resin-ILS(PP)IL[CS(Bu)LS(Bu)C]3T(PP)VK(Boc)K(Boc)S(PP)AE(Bu)FI-Ac</td>
<td>92%</td>
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<td>-</td>
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</tr>
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<td>II</td>
<td>2</td>
<td>C3 (3.5)</td>
<td>A</td>
<td>84</td>
<td>80</td>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>C3 (3.5)</td>
<td>B</td>
<td>88</td>
<td>84</td>
<td>80</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>C4 (3.5)</td>
<td>A</td>
<td>52</td>
<td>49</td>
<td>43</td>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>V</td>
<td>4</td>
<td>C5 (3.5)</td>
<td>A</td>
<td>85</td>
<td>81</td>
<td>78</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>VI</td>
<td>5</td>
<td>C4ore (5)</td>
<td>A</td>
<td>59</td>
<td>56</td>
<td>65</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>(i,i+7)</td>
<td>6</td>
<td>19mer: Resin-IL[LILN(Bu)S(PP)LS(Trt)C]3T(Bu)VK(Boc)K(Boc)S(PP)AE(Bu)FI-Ac</td>
<td>94%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>7</td>
<td>C7(3.5)</td>
<td>A</td>
<td>48+20(S-S)</td>
<td>48</td>
<td>50</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>7</td>
<td>C7 (20)</td>
<td>C</td>
<td>99</td>
<td>95</td>
<td>80</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IX</td>
<td>8</td>
<td>C6 (20)</td>
<td>C</td>
<td>99</td>
<td>94</td>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X</td>
<td>9</td>
<td>C8 (20)</td>
<td>C</td>
<td>64</td>
<td>70</td>
<td>57</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>((i,i+4, +Cys))</td>
<td>S2a</td>
<td>13mer: Resin-ILD(Bu)K(Boc)[CLR(Pbf)S(Trt)C]3T(Bu)K(Boc)N(Trt)Q(Trt)Ac</td>
<td>92%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XI</td>
<td>10</td>
<td>C3 (3.5)</td>
<td>A</td>
<td>90</td>
<td>91</td>
<td>85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XII</td>
<td>11</td>
<td>C4 (3.5)</td>
<td>A</td>
<td>61</td>
<td>56</td>
<td>55</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>XIII</td>
<td>12</td>
<td>C5 (3.5)</td>
<td>A</td>
<td>70</td>
<td>68</td>
<td>65</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>([2 \times (i,i+4)])</td>
<td>13</td>
<td>19mer: Resin-N(Trt)Q(Trt)PV[C(E(Bu)ALC)]3S(PP)IL[CS(PP)AIL]3D(Bu)VT(Bu)-Ac</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XIV</td>
<td>14</td>
<td>C3, C3 (20)</td>
<td>A</td>
<td>85</td>
<td>68</td>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PP= (psiMe, Mepro), C3: 1,3-dibromopropane, C4: 1,4-dibromobutane, C5: 1,5-dibromopentane, C4mix: trans-1,4-dibromobutane, C6: 1,6-dibromohexane, C7: 1,7-dibromohexane, C8: 1,8-dibromoocantane. A: DIEA (35 eq), TCEP (0.5 eq), NaI (17.5 eq), MW, 125°C, 2 min. B: DIEA (35 eq), TCEP (0.5 eq), NaI (17.5 eq), room temp., 12h. C: DIEA (35 eq), TCEP (3 eq), NaI (100 eq), MW, 125°C, 2 min. HPLC and HRMS chromatograms of all crude reaction mixtures are provided in 1.7.2 (Figures 5.1-5.8). The methods used for calculating conversion and purity by HPLC and HRMS are described below, in the General Experimental Information section of this document.
We also applied this method for the cyclization with 1,4-dibromobutane and 1,5-dibromopentane. These reactions also led to the expected bisthioether macrocycles, however, HRMS analysis of the reaction crudes also showed a significant amount of a side product of smaller molecular mass in both reactions (Figure S2.1 and S2.2). The structure of this compound was assigned to a bis-dehydroalanine substituted peptide (Scheme 2.3.3). Conversion of cysteine to dehydroalanine (Dha), by means of a cyclo-bis-alkylation-elimination mechanism, has been reported to be particularly effective when 1,4-diodo or 1,4-dibromobutane are used as electrophiles. This is likely due to the stability of the cyclic S-bialkylated sulfonium intermediate needed for the elimination step.

Scheme 2.3.3 Mechanism for the formation of bis-dehydroalanine substituted peptides as a side product in the direct alkylation of cysteine residues. Cysteine conversion to dehydroalanines (Dha) was recently reported by Chalker et al. to take place through a bis-alkylation-elimination mechanism, which is particularly effective when using 1,4-diodo or 1,4-dibromobutane as electrophiles.

To avoid this unwanted transformation, we used trans-1,4-dibromo-2-butene as electrophile since this rigid substrate would not form the key cyclic sulfonium intermediate. As
expected, this reaction only afforded the desired bis-thioether stapled peptide, thus confirming the previous structural assignment (Table 2.2, Figure S2.1 and S2.2).

2.4 Solid Phase Synthesis (SPPS) of Double-Turn Stapled Peptides (i, i+7)

We expanded our method to the preparation of double-turn stapled peptides (i, i+7) over the same 19-mer sequence, containing cysteines at positions 10 and 17 respectively (Scheme 2.4.1). The synthesis of the linear precursor included the use of pseudoproline dipeptides and yielded a highly pure crude material (Figure S2.3 and S2.4). After quantitative Cys-Mmt deprotection, as determined by our maleimide test, we proceed to cyclization using 1,7-dibromoheptane as electrophile under the previously optimized conditions.

The cyclization with 1,7-dibromoheptane under the previously optimized conditions afforded the expected macrocycle, but also the linear unreacted substrate and the disulfide oxidized product (Table 2.2). This result was not surprising since macrocyclization to form this larger ring is likely to be more challenging due to an increase in the conformational entropy of both the linear peptide (both thiols are further apart) and the electrophile (longer hydrocarbon chain). A second round of optimization was then conducted to improve efficiency of this stapling reaction. From those studies, we concluded that increasing the number of equivalents of both the electrophile and TCEP results in a 99% conversion to the desired cyclic product (Figure S2.3 and S2.4). The same reaction conditions allowed the successful preparation of (i, i+7) stapled peptides bearing all-hydrocarbon linkers with six and eight methylene groups (Table 2.2, Figure S2.3 and S2.4).
Scheme 2.4.1 Solid phase synthesis of single-turn (i, i+7) stapled peptides bearing all-hydrocarbon linkers of different lengths. Full characterization and experimental details are given in the Appendixes.

2.5 Solid Phase Synthesis of Single-Turn Stapled Peptides with Additional Cysteines in the Sequence (i, i+4, +Cys).

We further extended our approach to the cyclization of peptides containing additional cysteine(s) residues in the sequence. The chemoselective ligation of such peptides requires orthogonal protection of the extra thiol(s), to avoid undesired crosslinking with the Cys used for ligation. Two orthogonal protecting groups were studied, tert-butylthio (S-1Bu) and tert-Butyl...
(tBu), since they are both compatible with Fmoc and Mmt chemistries and are commercially available.\textsuperscript{5,6} Macrocyclization was carried out on a 13-mer linear precursor containing a total of three cysteines, two Mmt-protected used for stapling, and the third one blocked as either S-tBu or tBu (Scheme 2.5.1). After selective Mmt-deprotection and quantitative cyclization, we proceed to investigate the reaction conditions for the removal of both orthogonal protecting groups. Cys-S\textsuperscript{t}Bu was effectively deprotected using a mild reducing agent in basic conditions\textsuperscript{93}. In contrast, Cys-tBu removal was more effective when performed in solution, after the stapled peptide had been cleaved off the resin \textsuperscript{94}. Overall, both protocols yielded the corresponding selectively stapled macrocycle in good yields and high purity (Figures S2.5 and S2.6). However, we concluded that orthogonal protection with Cys-tBu is preferred due to shorter reaction times and overall simplicity of the reaction (Table 2, Entry XI-XIII).
Scheme 2.5.1 Synthetic scheme for the preparation of single-turn stapled peptides containing an additional cysteine residue in the sequence (i, i+4, +Cys).

2.6 Solid Phase Synthesis of Double Stapled Peptides [2 x (i, i+4)].

Having optimized the use of orthogonal-cysteine protection in our synthetic method, next we explored its feasibility for the chemoselective preparation of “double-stapled” peptides. These
studies were carried out with a different 19-mer sequence, containing two pairs of cysteine orthogonally protected as Cys-S’Bu and Cys-Mmt (Scheme 2.6.1). The total synthesis included (1) elongation of the linear precursor (2) Cys-S’Bu deprotection (3) incorporation of first peptide staple, (4) Cys-Mmt deprotection and (5) incorporation of second peptide staple. Both Cys deprotection steps were monitored by our maleimide test (Scheme 2.3.2). The tethering steps were monitored by LC/MS, after cleaving an aliquot of the ligated peptidyl resin. Our synthetic procedure allowed the chemoselective preparation of a double stapled peptide, in high overall purities (Table 2, Entry XIV, Figures S2.7 and S2.8).

Scheme 2.6.1 Synthetic scheme for the chemoselective preparation of double-turn stapled [2 x (i, i+4)].
2.7 Conclusion

In summary, we report a versatile synthetic method for the chemoselective preparation of all-hydrocarbon bis-thioether stapled peptides on solid phase, which allows access to single \((i+4)\) and double \((i+7)\) turn tethered peptides, as well as to double stapled peptides. Although the latter class of compounds has been prepared using the bis-RCM reaction, our protocol is the only cysteine-crosslinking method that allows for the incorporation of two hydrocarbon braces of any length chemoselectively at any position of the sequence. Our synthetic method affords staple peptides bearing all-hydrocarbon linkers of different lengths, an unprecedented feature for any of the reported stapling techniques. In addition, it avoids the use of unnatural amino acids and expensive catalysts, which makes it affordable and easier to be scaled up. The latter remains a concern for the current methods, particularly given the potential application of these class of compounds in clinical studies. We demonstrated the scope of this approach by synthesizing cyclopeptides derived from several peptide sequences and stapled at various positions, which also validates its compatibility with different amino acids and SPPS. This synthetic methodology affords macrocyclic peptides with marked resistance to proteolytic degradation, and thus improved pharmacokinetic profile, making them suitable to be used in biological studies.

2.8 Experimental Section

2.8.1 Acronyms and Abbreviations

All the abbreviations used for amino acids and peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* 247, 977-983 (1982). In addition, the following abbreviations are used: SPPS: Solid Phase Peptide Synthesis, DMF: \(N,N\)-dimethylformamide, DCM: dichloromethane, Fmoc: 9H-fluorenylemethoxycarbonyl, DIEA: \(N,N\)-diisopropylethylamine, PyBOP: \((\text{benzotriazol-1-yl oxy})\text{tripyrrolidinophosphonium}\)

PARPs: Poly (ADP-ribose) polymerases, TNKS: Tankyrase proteins, SAH-gp41: Stabilized $\alpha$-helix of GP4

2.8.2 General experimental information

Microwave reactions were carried out in a Biotage Initiator+ microwave reactor, featured with an infrared temperature sensor, and automatic power adjustment to keep the reaction temperature constant throughout the entire reaction time. The reactions were performed in sodium free glass vials, sealed with a cap equipped with a rubber septum, and under magnetic stirring (stirring rate: 300 rpm). Cooling is performed automatically using compressed air, after the reaction time ends.

Reactions were monitored by both HPLC and HPLC-MS.

- HPLC Characterization. HPLC chromatograms were obtained using an Agilent 1200 instrument equipped with a pump, degasser, an autosampler, a diode array detector and automatic collector.
Samples were run at four different wavelengths (220 nm, 254 nm, 280 nm and 590 nm), in an Eclipse XDB-C18 5 µm column, using as solvents: Solvent A: H₂O:CH₃CN:TFA (95:5:0.1%); Solvent B: CH₃CN:H₂O:TFA (95:5:0.087%), and either of the following methods:

- Method 1: Flow rate: 1.0 mL/min., Gradient 10-40% of B in 25min
- Method 2: Flow rate: 1.0 mL/min., Gradient 15-65% of B in 25min
- Method 3: Flow rate: 1.0 mL/min., Gradient 35-85% of B in 25min
- Method 4: Flow rate: 1.0 mL/min., Gradient 10-99% of B in 25min

- HPLC-MS Characterization. Samples were first injected into an HPLC (Agilent 1200) equipped with a pump, degasser, an autosampler and a diode array detector. The flow from the column was directed into the Mass Spectrometer (TOF, Agilent 6220) configured with an electrospray ionization source (ESI), using nitrogen as nebulizer gas. The acquired data was analyzed with Agilent MassHunter qualitative analysis software. Samples were run at four different wavelengths (220 nm, 254 nm, 280 nm and 590 nm), in an Eclipse XDB-C18 5 µm column, using as solvents: Solvent A: H₂O:CH₃CN:FA (95:5:0.1%); Solvent B: CH₃CN:H₂O:FA (95:5:0.1%), and the following method: Flow rate: 0.5 mL/min., Gradient 10-99% of B in 10min.

- Calculation of Conversion to Cyclic Peptides and Purity of the Stapling Reactions % of conversion to the desired macrocycles was calculated by HPLC. Purities were determined by both HPLC and MS (direct injection).

Quantitative Analysis by HPLC:

Reaction crudes were injected into the HPLC, after dilution with H₂O:CH₃CN (same ratio as the one used for initial running conditions in the method applied). The threshold of the spectra was set to 5%, using as reference the peak with the highest absorbance at 220 nm. Thus, only peaks with an absorbance above 5% of the highest peak were taken into account for the calculations, in order
to avoid errors resulting from minor peaks due to noise or residual peaks remaining in the column from previous injections.

Conversion was calculated according to the following equation:

\[
\text{% Conversion} = \frac{A_{SP}}{A_{LP}} \times 100
\]

where ASP is the area underneath the peak correspondent to the stapled peptide (identified and characterized by MS, after analyzing the collected sample correspondent to that retention time), and ALP the area underneath the peak correspondent to the linear peptide (identified and characterized by MS, after analyzing the collected sample correspondent to that retention time).

The purity of the cyclic peptide in the reaction crude was calculated according to the following equation:

\[
\text{% Purity} = \frac{A_{SP}}{A_{TP}} \times 100
\]

where \(A_{TP}\) is the sum of the areas underneath all the peaks above the threshold, excluding the injection peaks within first 2 min of the run, and \(A_{SP}\) the area underneath the peak correspondent to the desired stapled peptide (identified and characterized by MS, as previously indicated).

All original HPLC chromatograms used for these calculations are showed in this material.

Quantitative Analysis by Mass Spectrometry:

Samples were diluted into H2O:ACN (1:1) 1% formic acid. Reaction crudes were directly injected into the MS, bypassing the HPLC column, in order to determine accurately the ratio in between the components of the mixture. The threshold of the spectra was set to 5%, using as reference the peak with the highest intensity. Thus, only peaks with an intensity above 5% of the highest peak were considered for the calculations, in order to avoid minor peaks due to noise or residual peaks from previous injections.

Purity was calculated according to the following equation:
% Purity = \frac{I_{SP}}{I_{TP}} \times 100

where I_{TP} is the sum of the intensity of all the peaks above the threshold, excluding reference peaks, and I_{SP} the sum of the intensity values for all the peaks correspondent to the desired stapled peptide (including all the cyclopeptide peaks with different ionization states).

All original HRMS chromatograms used for these calculations are showed in this material. Spectra were zoomed in some cases; irrelevant peak was omitted in any of the zoomed chromatograms.

All peptides were synthesized manually using polystyrene syringes equipped with a frit and following standard Fmoc strategy. Coupling and deprotection steps were carried out as described below. Solvents and excess of soluble reagents were removed by vacuum filtration. All the peptides were synthesized using Pal (4-Alkoxy-2,6-dimethoxybenzylamine) resin (Bachem D-2125, 0.5 mmol/g).

- Peptide Elongation

0.500 g of the resin (PAL resin, BACHEM, f=0.5 mmol/g), were first pre-swelled in DCM (10 mL, 30 min) and then washed with DMF (5 mL, 3X) and DCM (5 mL, 3X). Next, a coupling solution containing the correspondent Fmoc-AA-OH (2.5 eq.), Oxyrna (2.5 eq.), PyBop (2.5 eq.) and DIEA (5 eq.) in 10 mL DMF was added to the resin. The suspension was carefully transferred into a 20-mL microwave vial containing a magnetic stir bar, capped, and reacted under microwave irradiation at 85 °C for 7 min. Next, the mixture was transferred back to the solid phase synthesis vessel to be subsequently washed with DMF (5 mL, 3X), DCM (5 mL, 3X), EtOH (5 mL 3X) and DCM (5 mL 3X).

The efficiency of the coupling step was assessed by the colorimetric Kaiser test. If a positive Kaiser test is obtained, indicative of an incomplete coupling reaction, the resin is treated again with the same coupling solution (freshly prepared) and reacted under the conditions described above. After
the coupling is complete, the N-terminus of the peptide is Fmoc-deprotected by treating the resin with 20 % Piperidine in DMF, 10 min, twice. Next, the resin is properly washed with DMF (5 mL, 3X) and DCM (5 mL, 3X), and the efficiency of the deprotection tested by the Kaiser test. This iterative process of coupling and deprotection is repeated after the incorporation of each amino acid residue, until the full peptide sequence is elongated. Finally, the N-terminus of the peptide is acetylated (after Fmoc deprotection, following the same conditions described previously), with a solution of acetic anhydride (5 eq.) and pyridine (5 eq.) under microwave irradiation at 85 °C for 5 min. For peptides sequences containing serine and/or threonine, these residues were introduced as pseudoproline dipeptides containing also the corresponding subsequent amino acid in the target peptide sequence. The identity and purity of all the synthesized peptides was assessed by LC/MS and HPLC analysis of the solution of a small peptide sample obtained after cleavage, precipitation and washed with Et₂O.

- 4-methoxytrityl (Mmt) Deprotection and Maleimide test

Mmt deprotection was carried out with a deprotection solution (15mL per 0.5g of resin, 2% TFA, 10% TIS, 88% DCM) at room temperature for 5 min. The resin was then washed with DCM (5X) and treated repeatedly with the same solution until its color changed from orange to light yellow. Maleimide test was next performed (Scheme S1.4). To this end, a small aliquot of Mmt deprotected resin was neutralized with 3mL of 5% DIEA in DMF for 5 min, and washed with DMF (2 mL, 3X), DCM (3 mL, 3X) and DMF (3 mL, 2X). The neutralized clean resin was subsequently mixed with N-methylmaleimide (5 eq. from a 20 mg/mL DMF stock solution), and DIEA (5 eq.) in a 2-mL microwave vial with a stir-bar, and reacted under microwave irradiation at 85 °C for 15 min. Next, the suspension was transferred into a SPPS vessel, washed (DMF 2 mL
3X, DCM 2 mL 3X) and treated with the proper cleavage cocktail. The resulting peptide is finally precipitated/washed with cold diethyl ether and analyzed by LC/MS to quantitate the extent of the deprotection step (Scheme S1.4). If the Mmt-deprotection is incomplete, additional deprotection rounds are repeated until completion of the reaction. After total Mmt removal is achieved, the resin is neutralized with 5% DIEA in DMF for 5 min, washed with DMF (3X) and DCM (3X), and dried under vacuum for storage or used directly for the cyclization step.

- **Stapling Reaction**

The resin containing the precursor Cys-thiol free linear peptide was transferred into a microwave reaction vial containing a stir-bar, to which NaI (17.5 eq for i+4 sequences, 100 eq for i+7 sequences) and DMF (1mL per 10mg of resin for i+4 sequences, 1 mL per 20 mg of resin for i+7 sequences) were added subsequently, while keeping the mixture stirring at all times, followed by TCEP (0.5 eq for i+4 sequences, 3 eq for i+7 sequences, from a stock solution of 300 mg/mL in water). The vial was then capped with a MW vial cap equipped with a rubber septum and the resulting suspension bubbled under N₂ for 15 min. Next, DIEA (35 eq.) was added (by syringe), keeping the stirring under N₂ for another 30 min, after which, the dibromoalkyl electrophile was injected (3.5 eq for i+4 sequences, 20 eq for i+7 sequences). The suspension is finally reacted under microwave irradiation for 2 min at 125°C, transferred into the SPPS reaction vessel and washed with water (5 X, soak if necessary to remove residual NaI), DMF (5 X) and DCM (5 X).

The efficiency of the stapling step was confirmed by cleaving a small aliquot of resin and analyzing the precipitated/washed peptide pellet by LC/MS and HPLC.
- **Final Cleavage**

The peptidyl resin is mixed with the proper TFA cleavage cocktail (TFA/Water/TIS=95/2.5/2.5 for non-cysteine containing peptides and TFA/Water/TIS/EDT=95/2.5/2.5 for cysteine containing sequences). The suspension is then allowed to react on an orbital shaker for 1 h at room temperature or under microwave irradiation at $85 \, ^\circ C$ for 5 min. Next, TFA is evaporated under a stream of nitrogen and the peptide precipitated with ice cold Et$_2$O. The resulting pellet is finally washed with ice cold Et$_2$O (5X) and isolated by centrifugation. In a typical experiment, 5 mL cleavage cocktail were used per each 100 mg of resin, which yielded about 300 mg of crude stapled peptide, and overall 75% yield of pure stapled peptide.

- **Cleavage Protocol for Sequences Containing S-t-butyl protected cysteine(s)**

S'Bu deprotection was carried out with a deprotection solution (15mL per 0.5g of resin, 25% β-Mercaptoethanol) at room temperature overnight. Maleimide test was next performed to test reaction efficiency (Scheme S1.3). To this end, a small aliquot of Mmt deprotected resin was neutralized with 3mL of 5% DIEA in DMF for 5 min, and washed with DMF (2 mL, 3X), DCM (3 mL, 3X) and DMF (3 mL, 2X). The neutralized clean resin was subsequently mixed with N-methylmaleimide (5 eq. from a 20 mg/mL DMF stock solution), and DIEA (5 eq.) in a 2-mL microwave vial with a stir-bar, and reacted under microwave irradiation at $85 \, ^\circ C$ for 15 min. Next, the suspension was transferred into a SPPS vessel, washed (DMF 2 mL 3X, DCM 2 mL 3X) and treated with the proper cleavage cocktail. The resulting peptide is finally precipitated/washed with cold diethyl ether and analyzed by LC/MS to quantitate the extent of the deprotection step (Scheme S1.3). If the deprotection is incomplete, additional deprotection rounds are repeated until
completion of the reaction. After total S\textsuperscript{Bu} removal is achieved, the resin washed with DMF (3X) and DCM (3X), and dried under vacuum for storage or used directly for the cyclization step.

- \textit{Cleavage Protocol for Sequences Containing t-butyl protected cysteine(s)}

The peptide is cleaved, precipitated and properly washed, following the protocol described above (Final Cleavage Protocol). The resulting dry pellet, still containing t-butyl protected cysteine(s), is then dissolved in a cocktail composed by thiolanisole (4\%), EDT (12\%) and TFA (84\%) (960 \textmu L of cocktail per 100mg of peptidyl resin). Next, the mixture is cooled down in an ice bath to 5 \textdegree C, after which, TFMSA (10\% v/v of TFA) is added dropwise (avoiding generation of excessive heat). The solution is kept in the ice bath for another 10 min, and then allowed to react at room temperature for 1 hour. Finally, the deprotected peptide is precipitated by adding ice cold Et\textsubscript{2}O to the mixture (5x sample volume). To achieve high precipitation yields, the sample may be left at 0\textdegree C for another 30 min. The crude is isolated by centrifugation and subsequently washed with ice cold Et\textsubscript{2}O (5X).
2.9 Appendix: Supplementary Figures

**Figure S 2.1** HPLC analysis of the crude reaction mixtures obtained after cleavage of the stapling reactions for the (i, i+4) stapled peptides 2-5, and their correspondent linear precursor 1. Experimental conditions used for each HPLC analysis are given in the peptide characterization section.
**Figure S 2.2** HRMS analysis of crude reaction mixtures obtained after cleavage of the stapling reactions for the \((i, i+4)\) stapled peptides 2-5 and their correspondent linear precursor 1. Experimental conditions used for each HRMS analysis are given in the general experimental procedure section. Observed 121.05 Da and 922.01 peaks correspond to internal references used in the analysis.
Figure S2.3 HPLC analysis of the crude reaction mixtures obtained after cleavage of the stapling reactions for the \((i, i+7)\) stapled peptides 7-9 and their correspondent linear precursor 6. Experimental conditions used for each HPLC analysis are given in the peptide characterization section.
Figure S 2.4 HRMS analysis of crude reaction mixtures obtained after cleavage of the stapling reactions for the \((i, i+7)\) stapled peptides 7-9 and their correspondent linear precursor 6. Experimental conditions used for each HRMS analysis are given in the general experimental procedure section. Peaks observed at 121.05 Da and 922.01 Da in the HRMS spectra correspond to internal references used in the analysis.
Figure S 2.5 HPLC analysis of the crude reaction mixtures obtained after cleavage of the stapling reactions for the \((i, i+4)\) stapled peptides containing an additional Cys residue 10-12 and their correspondent linear precursor S2a. Experimental conditions used for each HPLC analysis are given in the peptide characterization section.
Figure S 2.6 HRMS analysis of crude reaction mixtures obtained after cleavage of the stapling reactions for the \((i, i+4)\) stapled peptides containing an additional Cys residue 10-12 and their correspondent linear precursor S2a. Experimental conditions used for each HRMS analysis are given in the general experimental procedure section. Observed 121.05 Da and 922.01 peaks correspond to internal references used in the analysis.
Figure S 2.7 HPLC analysis of the crude reaction mixtures obtained after cleavage of the second stapling reaction for the double stapled peptide [2 x (i, i+4)] 14 and its correspondent linear precursor 13. Experimental conditions used for each HPLC run are given in the peptide characterization section.
Figure S 2.8 HRMS analysis of crude reaction mixtures obtained after cleavage of the first and second stapling reactions for the double stapled [2 x (i, i+4)] 14 and its correspondent linear precursor 13. Experimental conditions used for each HRMS analysis are given in the general experimental procedure section. Observed 121.05 Da and 922.01 peaks correspond to internal references used in the analysis.
2.10 Appendix: Peptide’s characterization

**Ac-IFEASKKVTCSLSCSLL-I-NH$_2$ (1)**

![Peptide 1 Structure]

*Isolated from S2, after TFA cleavage, Scheme 1.1 (Main text)*

**HRMS (ESI) (m/z):** [M] cacld. for C$_{94}$H$_{162}$N$_{22}$O$_{27}$S$_2$, 2095.1421

[M+2H]$^{2+}$ found 1048.5774 (monoisotopic peak)

**HPLC:** Method 2, t$_R$ = 18.74 min.

**Ac-IFEASKKVT[CSCSLL]-NH$_2$ (2)**

![Peptide 2 Structure]

*Synthesis:* Using 1,3-dibromopropane as electrophile, Scheme 1.1 (Main text)

**HRMS (ESI) (m/z):** [M] cacld. for C$_{97}$H$_{166}$N$_{22}$O$_{27}$S$_2$, 2135.1734

[M+2H]$^{2+}$ found 1068.5921 (monoisotopic peak)

**HPLC:** Method 2, t$_R$ = 16.33 min.
Ac-IFEASKKVT[CSLSC]C₄LISLI-NH₂ (3)

**Synthesis:** Using 1,4-dibromobutane as electrophile, Scheme 1.1 (Main text)

**HRMS (ESI) (m/z):** [M] cacld. for C₉₈H₁₆₈N₂₂O₂₇S₂, 2149.1891

[M+2H]²⁺ found 1075.6048 (monoisotopic peak)

**HPLC:** Method 2, tₚ = 18.82 min.

Ac-IFEASKKVT[CSLSC]C₅LISLI-NH₂ (4)

**Synthesis:** Using 1,5-dibromopentane as electrophile, Scheme 1.1 (Main text)

**HRMS (ESI) (m/z):** [M] cacld. for C₉₉H₁₇₀N₂₂O₂₇S₂, 2163.2047

[M+2H]²⁺ found 1082.6081 (monoisotopic peak)

**HPLC:** Method 2, tₚ = 19.41 min.
**Ac-IFEASKKVT[CSLSC]C4ensoLISLI-NH₂ (5)**

*Synthesis:* Using trans-1,4-dibromo-2-butene as electrophile, Scheme 1.1 (Main text)

**HRMS (ESI) (m/z):** [M] cacld. for C₉₈H₁₆₆N₂₂O₂₇S₂, 2147.1734

[M+2H]²⁺ found 1074.5926 (monoisotopic peak)

**HPLC:** Method 2, tᵣ = 16.21 min.

---

**Ac-IFIASKKVTCSLSNILCIL-NH₂ (6)**

*Isolated from S5, after TFA cleavage, Scheme 1.2 (Main text)*

**HRMS (ESI) (m/z):** [M] cacld. for C₉₆H₁₆₇N₂₃O₂₅S₂, 2122.1530

[M+2H]²⁺ found 1062.0926 (monoisotopic peak)

**HPLC:** Method 3, tᵣ = 8.86 min.
Ac-IFIASKKVT[CSLSNILC]c7IL-NH2 (7)

**Synthesis:** Using 1,7-dibromoheptane as electrophile, Scheme 1.2 (Main text)

**HRMS (ESI) (m/z):** [M] caclld. for C_{103}H_{179}N_{23}O_{25}S_{2}, 2218.2469

[M+2H]^2+ found 1110.1274 (monoisotopic peak)

**HPLC:** Method 3, t_R = 10.67 min.

Ac-IFIASKKVT[CSLSNILC]c6IL-NH2 (8)

**Synthesis:** Using 1,6-dibromohexane as electrophile, Scheme 1.2 (Main text)

**HRMS (ESI) (m/z):** [M] caclld. for C_{102}H_{177}N_{23}O_{25}S_{2}, 2204.2313

[M+2H]^2+ found 1103.1186 (monoisotopic peak)

**HPLC:** Method 3, t_R = 9.26 min.
Ac-IFIASKKVT[CSLSNILC]c8IL-NH₂ (9)

*Synthesis:* Using 1,8-dibromooctane as electrophile, Scheme 1.2 (Main text)

*HRMS (ESI) (m/z):* [M] calcd. for C₁₀₄H₁₈₁N₂₃O₂₅S₂, 2232.2626

  [M+2H]²⁺ found 1117.1367 (monoisotopic peak)

*HPLC:* Method 3, tᵣ = 10.48 min.
Ac-QNKCCSRLCKDLL-NH₂ (S4a)

Isolated from S7, after subsequent TFA and either S⁻⁴Bu or t⁻⁴Bu cleavages, Scheme S1.4

**HRMS (ESI) (m/z)**: [M] cacl. for C₆₃H₁₁₃N₂₁O₁₉S₃, 1563.7684

[M+2H]²⁺ found 782.8891 (monoisotopic peak)

**HPLC**: Method 1, t<sub>R</sub> = 14.98 min.

**HPLC**: Crude Reaction Mixture After

**HRMS**: Crude Reaction Mixture After
Ac-QNKC[CSRLC]c3KDLL-NH₂ (S4d)

**Synthesis:** Using 1,3-dibromopropane as electrophile, Scheme S1.4

**HRMS (ESI) (m/z):** [M] cacl. for C₆₆H₁₁₇N₂₁O₁₉S₃, 1603.7997

[M+2H]^{2+} found 802.9039 (monoisotopic peak)

**HPLC:** Method 1, tᵣ = 18.81 min.
Ac-QNKC[CSRLC]_4KDLL-NH₂ (S4e)

Synthesis: Using 1,4-dibromobutane as electrophile, Scheme S1.4

HRMS (ESI) (m/z): [M] cacl. for C₆₇H₁₁₉N₂₁O₁₉S₃, 1617.8153

[M+2H]²⁺ found 809.9116 (monoisotopic peak)

HPLC: Method 1, t_R = 16.89 min
Ac-QNKC[CSRLC]C5KDLL-NH₂ (S4f)

**Synthesis**: Using 1,5-dibromopentane as electrophile, Scheme S1.4

**HRMS (ESI) (m/z)**: [M] cacld. for C₆₈H₁₂₁N₂₁O₁₉S₃, 1631.8310

[M+2H]²⁺ found 816.9188 (monoisotopic peak)

**HPLC**: Method 1, tᵣ = 17.36 min.

**HPLC**: Crude Reaction Mixture After

**HRMS**: Crude Reaction Mixture After
Ac-TVDCIASCLSC(S'Bu)LAEC(S'Bu)VPQN -NH₂ (10)

Isolated from 10, after TFA cleavage, Scheme 1.3 (Main Text)

HRMS (ESI) (m/z): [M] cacld. for C98H161N23O31S₆, 2348.0053

[M+2H]²⁺ found 1175.0092 (monoisotopic peak)

HPLC: Method 4, tᵣ = 13.67 min.

Ac-TVD[CIASC]₃LS[CLAEC]₃VPQN-NH₂ (12)

Synthesis: Using 1,3-dibromopropane as electrophile, Scheme 1.3 (Main Text)

HRMS (ESI) (m/z): [M] cacld. for C96H153N23O31S₄, 2251.9986

[M+2H]²⁺ found 1127.0076 (monoisotopic peak)

HPLC: Method 4, tᵣ = 15.96 min.
Chapter III. Stapled Peptide Inhibitors Polycomb

Repressive 2 Gene Repression

3.1 Background

In eukaryote cells the genetic information is highly compacted inside the nucleus in the form of chromatin, which are complexes of DNA and small proteins called histones. The primary function of chromatin is not only to make DNA fit into the nucleus but also to dictate how this genetic information is used. Thus, chromatin regulates transcription by controlling DNA accessibility to the transcriptional cellular machinery. This is achieved through its three-dimensional architecture, consisting of two main conformational interphases: a highly condensed and repressive form, heterochromatin, in which DNA is tightly wrapped around the core histone proteins and thus inaccessible; and a loosely packed permissive state, euchromatin, in which DNA is available for transcription. The dynamic interplay between these two structures is mainly controlled by epigenetic modifications, including DNA methylation at CpG islands and N-covalent post-translational chemical modifications on the histone proteins. A balanced pattern of epigenetic modifications ensures cell identity and proper function, whereas alterations in the epigenome lead to aberrant patterns of gene expression that contribute to the development and progression of diseases.95-96

Cancer is second to cardiac diseases as the most common cause of death in the United States and it is expected to become the front-runner by 2030.97 Over the past two decades,
reprogramming cancer cells by targeting the cancer epigenome and reorganization of chromatin architecture to regulate gene expression has emerged as a promising strategy against this yet incurable disease.\textsuperscript{95} One of the most cancer-relevant epigenetic modifications is the site-specific addition of methyl groups into lysine residues on the histone tails. In particular, trimethylation of lysine 27 at histone 3 (H3K27me3) has been extensively studied and characterized as a highly repressive histone mark, whose dysregulation is strongly linked to a wide array of metastatic malignancies. This post-translational modification is installed by the polycomb repressive complex 2 (PRC2), a multimeric complex of proteins consisting of four core subunits: EZH2, EED, SUZ12 and Rbp46/48. PRC2 transfers one to three methyl groups from S-adenosyl-L-methionine (SAM) onto the ε-amino group of H3K27, generating H3K27me3, thus subsequently silencing downstream genes through chromatin compaction. The enzymatic activity of PRC2 is conferred by the catalytic SET domain of EZH2, but also to the other core components of the complex, since EZH2 does not show methyltransferase activity on its own, neither is structurally stable. Hence, a catalytically active PRC2 complex must contain EZH2 and at least EED and SUZ12, which highlights the role of the latter proteins as scaffolds for the proper assembly of PRC2 into its bioactive conformation.\textsuperscript{98-100}

The physiological relevance of the PRC2 is highlighted by the early embryonic lethality observed in mice after genetic ablation of genes encoding for PRC2-proteins. Moreover, extensive evidences show that PRC2 is dysregulated in multiple human cancers\textsuperscript{101-103}. For example, gain of function mutations of EZH2 at residues within the SET domain (A677 and Y641), with higher catalytic power yielding to increased H3K27me3 levels, have been found in melanoma and B-cell lymphoma. PRC2 proteins, and in particular EZH2, have also been found overexpressed in solid cancers including colorectal, breast, ovarian, kidney, lung, bladder, prostate, pancreatic and
sarcomas, and their upregulation correlates with advance stages of the disease and poor prognosis.\textsuperscript{104-106} Furthermore, EZH2 methylates several cytosolic non-histone proteins in a PRC2-dependent manner such as STAT3 in glioblastoma promoting tumorigenesis.\textsuperscript{104}

The pivotal role of PRC2 in multiple oncogenic processes has made PRC2 proteins highly attractive molecular targets for developing promising anticancer agents. Hence, the discovery of such compounds is an area of active research for both the pharmaceutical industry and academic laboratories. Several potent PRC2 inhibitors have been discovered in the last few years. Some of them are already undergoing clinical investigation with promising results. All these first-generation compounds are competitors of the methyl donating cofactor SAM and target the catalytic SET domain of EZH2. They are also structurally related since most share as central feature a pyridine scaffold that serve as key pharmacophore to recognize the SET-domain binding pocket. These small molecules have proven to be highly effective at reducing H3K27me3 levels \textit{in vitro} and \textit{in vivo}, and potent inhibitors of cancer cell proliferation and tumor growth. Indeed, Tazemotstat (Epizyme), GSK126 (GlaxoSmithKline), and CPIC-1205 (Constellation Pharmaceuticals) are some examples of EZH2 inhibitors under evaluation in phase 1/2 clinical trials for a variety of cancers, including advanced solid tumors.\textsuperscript{107} However, recent reports indicate that extended dosing with these drugs leads to secondary EZH2 mutants resistant to treatment.\textsuperscript{108-111} Such resistance profiles raise concerns about the potential of these compounds, especially since they share the same mechanism of action.\textsuperscript{107} Hence, novel PRC2 inhibitors targeting the PRC2 complex at other crucial protein-protein interphases are of great interest, since they constitute a potential solution to address this problem. Indeed, this alternative strategy has been recently validated by the discovery of a second generation of PRC2 modulators, designed to block the H3K27me3 binding pocket in EED.\textsuperscript{112} Notably, these compounds efficiently block H3K27me3
levels in both cell lines sensitive and resistant to EZH2-SET domain inhibitors, and result in strong efficacy against mice and human tumor xenograft models. In this chapter, we describe the design, synthesis and biological evaluation of three different families of cyclopeptide inhibitors of PRC2 catalytic function, targeting for the first time three protein interfaces in PRC2 that are crucial for its proper assembly and function: the intramolecular SANT1L/SBD interaction of EZH2, the SUZ12-VEFS/EZH2-SANT2 binary complex and the interaction between SUZ12-NBE domains. All the compounds described herein have been generated using the solid phase synthetic approach described in Chapter 1. Our inhibitors have demonstrated cell permeability, potent activity in vitro and in physiological conditions, as well as strong antiproliferative effects on Caki-1 renal cancer cells.

3.2 Bisthioether Stapled Peptides as Allosteric Inhibitors of PRC2 Targeting the SANT1L-SBD Interaction in EZH2.

A recently published crystal structure of an active PRC2 ortholog from yeast revealed several interactions among its core components thought to be crucial for both proper PRC2 assembly and function (PDB ID 5KJH)\(^9\). Based on this structure, we designed a series of stapled peptides inhibitors of PRC2 methyltransferase activity, targeting a key intramolecular interaction in EZH2 formed by its SANT1L-like domain (Figure 3.2.1a, left, orange alpha-helix) and its SANT1L-binding domain (SBD, Figure 3.2.1a, left, grey alpha-helix). This binary alpha-helical complex surrounds EED, another core PRC2 protein, forming a belt-like structure around it, and thus plays a crucial role in stabilizing the bioactive catalytic conformation of PRC2. Our design focused on mimicking an alpha helix formed by the SBD domain in EZH2 in order to generate macrocycles that could potentially bind to SANT1L, and thus disrupt the key SBD-SANT1L intramolecular interaction in PRC2 (Figure 3.2.1a, right).
Based on these structural considerations we successfully prepared five macrocycles, in which solvent-exposed residues were replaced by cysteines for the planned stapling reaction. The synthesis was accomplished successfully, following the synthetic methodology previously described in Chapter 1 (Schemes 3.2.1 and 3.2.2). The crude of the corresponding cyclopeptides were purified by HPLC, affording the final products with purities >95%.
Scheme 3.2.1 Synthetic scheme for the chemoselective preparation of single turn stapled peptides $(i, i+4)$ inhibitors of the intramolecular SANT1L-SBD interaction in EZH2.
Scheme 3.2.2 Synthetic scheme for the chemoselective preparation of double turn stapled peptides \((i, i+7)\) inhibitors of the intramolecular SANT1L-SBD interaction in EZH2.

We first investigated the impact of the staple on the secondary structure of our synthesized
macrocycles by circular dichroism (CD). The analysis of the linear wild type peptide revealed that it was only 4.8% helical in solution, indicating that this sequence is almost disordered when it is removed from the full protein structure. Interestingly, incorporation of the hydrocarbon brace resulted in enhanced helical character for both single- and double-turn stapled peptides. Remarkably, compound 2 showed the best result, with an overall improvement of 18% in helicity (Figure 3.2 a,b). Next, we tested our cyclopeptides for their ability to inhibit H3K27 trimethylation in vitro. To this end, we optimized a histone methyltransferase colorimetric assay using as catalytic complex endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1). A well-characterized EZH2-SET domain inhibitor (GSK126) was used as a positive control for determining optimal assay conditions. Both single- and double-turn stapled peptides showed concentration dependent inhibition of PRC2 catalytic function. In line with the CD data, the best inhibitor was compound 2 with an IC\textsubscript{50} value of 0.17 ± 0.02 μM. This staple peptide was virtually as potent as GSK126 in the same assay conditions and over 500 times better than its linear wild type counterpart 1 (Figure 3.2.2 a). Aiming at gaining insights about the mechanism of action of cyclopeptide 2 for PRC2 inhibition, we decided to also explore its potential synergy with the EZH2-SET domain inhibitor GSK126. Hence, we performed the previously optimized enzymatic in vitro assay, using an equimolar combination of both compounds. To quantitatively assess synergy, we used the combination index (CI) equation of Chou et al., for which a value of CI<1 is indicative of a more than additive outcome. Notably, the equimolar mixture of 2 and GSK126 yielded an IC\textsubscript{50} value of 0.031 ± 0.001μM, which corresponded to a CI of 0.53. This data suggest that our compound may works synergistically when applied in combination with an inhibitor of the catalytic SET domain of EZH2, and therefore, that it may likely function by blocking a different PRC2 domain, key for its function, i.e, in an allosteric
fashion (Figure 3.2.2c).

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Sequence</th>
<th>Linker</th>
<th>Helicity (%)</th>
<th>IC₅₀(µM)</th>
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<tbody>
<tr>
<td>1</td>
<td>Ac-TVDKIASALSVLAEVPQNV-CONH₂</td>
<td>-</td>
<td>4.8</td>
<td>89</td>
</tr>
<tr>
<td>2</td>
<td>Ac-TV[DIASC][LSVLAEVPQNV-CONH₂ CH₃CH₂CH₂</td>
<td>23</td>
<td>0.17 ± 0.02</td>
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</tr>
<tr>
<td>3</td>
<td>Ac-TV[DIASC][LSVLAEVPQNV-CONH₂ CH₂CH₂CH₂</td>
<td>9</td>
<td>7.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ac-TVDKIASA[CSVLAECC][QPQ-CONH₂ CH₂CH₂CH₂</td>
<td>8.7</td>
<td>10.1 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ac-TVDKIASA[CSVLAECC][QPQ-CONH₂ CH₂CH₂CH₂</td>
<td>13.7</td>
<td>27.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Ac-TVDKIASA[CSVLAECC][QPQ-CONH₂ CH₂CH₂CH₂</td>
<td>12.1</td>
<td>17 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSK126 (control)</td>
<td>-</td>
<td>-</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

**Figure 3.2.2 Biochemical Assays** (a) Structure of the synthesized stapled peptides, and their correspondent helicity and IC₅₀ values for inhibition of H3K27 trimethylation in vitro, as determined by a histone methyltransferase colorimetric assay optimized using endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1). (b) CD spectra of stapled peptides and their linear counterparts measured in water at 20°C. (c) Plot of H3K27 trimethylation inhibition using the enzymatic in vitro assay described in a, with varying concentrations of 2, positive control GSK126, and using an equimolar combination of both compounds to test their synergistic effects. The IC₅₀ values obtained when using recombinant PRC2 and when using a high concentration of SAM (110 X) are also shown. A summary of the IC₅₀ values for inhibition of H3K27me3 determined from the experiments shown in plot c. is also presented. Calculation of the combination index using the equation described by Chou et al. (CI= 0.53) indicated a marked synergistic effect between 2 and GSK126.

In order to get more insights into the potential allosteric nature of inhibitor 2, we performed the same H3K27me3 inhibition assay but using instead a larger concentration of SAM (100 folds, Figure 3.2.2c). Since the methyl-donor cofactor has been proven to bind directly to the EZH2-SET domain, using a larger concentration of it would directly impact the potency of any other molecule targeting the same binding site, as it has been shown previously with other well characterized orthosteric PRC2 inhibitors. As expected, in our case, the IC₅₀ obtained in such experimental
conditions was similar to the original value, which demonstrates that our cyclopeptide is not a SAM-competitor inhibitor, thus reinforcing the previous data that suggested the allosteric nature of peptide 2.

Aiming at obtaining more evidences toward the specific target site of 2 within the PRC2 complex, we performed a pull-down assay over nuclear extracts isolated from caki-1 cells and using a biontinylated derivative of it (Figure 3.2.3). The assay was performed according to the manufacture’s protocol. Briefly, the biotinylated peptide 2 was incubated with streptavidin coated beads in a fritted syringe overnight. Next, after thoroughly washing of the beads, nuclear extract was applied to the syringe and incubated with the beads for 1 hour. The beads were then washed thoroughly in order to remove the non-binding proteins, followed by isolation of the prey-proteins by carrying out washes with the elution buffer. As shown in Figure 3.2.3, western blot analysis of the prey-protein solutions indicated the presence of EZH2 in the mixture previously treated with biotinylated 2, whereas no EED or SUZ12 were detected in this sample. Neither of these proteins were found in the solution derived from the vehicle treatment (control). Thus, this data suggests that our cyclopeptide 2 does bind to EZH2 selectively.
Figure 3.2.3 (a) Structure of the biotinylated derivative of cyclopeptide 2, synthesized on solid phase (b,c) In vitro pull down assay using biotinylated cyclopeptide 2, visualized by SDS-PAGE and followed by western blot analysis using antibodies against human EZH2 (b) and human EED and SUZ12 (c).

Altogether, the results from these biochemical assays show, first, that cyclopeptide 2 is a potent inhibitor of PRC2 catalytic function, and more importantly that it may function allosterically. Further evidences indicate that this compound targets EZH2, not as a SAM-competitor inhibitor, and thus it may likely mimic the EZH2-SBD domain and therefore act by disrupting the crucial SBD-SANT1L intramolecular interaction.

Next, we tested the proteolytic stability of these compounds by subjecting two representative examples to a chymotrypsin-based assay previously validated to evaluate the stability of stapled peptides. For comparison, we also studied their linear counterparts. HPLC and LC/MS analysis of the enzymatic reactions showed complete protection of both $i+4$ and $i+7$
cyclopeptides against enzymatic degradation after 24h, whereas its linear analogue underwent full hydrolytic cleavage one hour after the reaction started (Figure 3.2.4). In addition, we tested the stability of cyclopeptide 2 and its parent linear sequence 1 in plasma. In line with the in vitro assay results, our macrocycle showed high resistance to proteolysis in these conditions, resulting also markedly superior to peptide 1, which was fully degraded after 5 minutes. Overall, we conclude that our stapling reaction leads to significant protection against enzymatic degradation, thus making this family of constrained peptides suitable for cellular and biological assays.

Figure 3.2.4 The Chymotrypsin-based proteolytic degradation assays as well as the plasma stability assay show remarkable stability of our i, i+4 and i, i+7 bisthioether stapled peptides. The rate of peptide proteolysis was monitored by HPLC and HPLC-MS analysis.

Given the encouraging in vitro H3K27me3 inhibition data and the proteolytic stability shown by our stapled peptides, we decided to explore the cellular uptake of compound 2 by confocal microscopy, using an FITC-labeled derivative of it. Confocal imaging after incubation of caki-1 cells with fluorescently-tagged 2 showed significant membrane translocation of the stapled peptide. More importantly, these experiments also confirmed that this compound is able to reach the nuclei, where the targeted PRC2 complex is localized (Figure 3.2.5).
To further explore the ability of cyclopeptide 2 to target and functionally block PRC2-mediated H3K27 methylation, we treated Caki-1 cells for 72h with varying concentrations of 2 and analyzed all degrees of methylation on K27 by western blot. We used GSK126 (1 µM) and the linear wild type peptide sequence 1 (25 µM, 100 µM) as positive and negative control, respectively (Figure 3.2.6).
Figure 3.2.6 Cellular Assays with lead bisthioether stapled peptide 2 (a) Western blot analysis shows dose-dependent response of H3K27me3, H3K27me2 and H3K27me1 within caki-1 cells, after treatment with stapled peptide 2 once daily for 72h. Single concentration treatment with GSK126 (positive control) and linear wild type sequence (1, negative control) in the same experimental conditions are also shown. Quantitation of H3K27me3 using and absorbance-based colorimetric assay yielded an IC50 value of 0.4 ± 0.2 μM. Protein loading was accurately corrected by measuring total H3 using an absorbance-based colorimetric assay. (b) Selectivity of H3K27 trimethylation inhibition over a broad panel of histone post-translational modifications. Cells were treated with stapled peptide 2 (5 μM) or vehicle, once daily for 72h. (c) Treatment of Caki-1 cells with cyclopeptide 2, control compound GSK126, and an equimolar combination of both molecules significantly inhibits cell proliferation. Proliferation was measured after 72h of daily treatment with the correspondent compound. The data is presented as a mean of two independent experiments each with triplicate measurements. (d) Plot of IC50 values obtained when testing our lead compound 2, GSK126 and a combination of both in: (d.1) an enzymatic in vitro assay monitoring H3K27me3 inhibition, using endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1), (d.2) a proliferation colorimetric assay with Caki-1 cells, (d.3) in non-cancerous human fibroblast IMR90 cells. Calculation of the combination index using the equation described by Chou et al. indicated a marked synergistic antiproliferative effect (CI= 0.61) when both compounds are used together.

The results showed a clear concentration-dependent inhibition of 2, most pronounced for trimethylation but also significant for H3K27me2 and H3K27 monomethylation (Figure 3.2.6a).
Further quantitation of H3K27me3 by an absorbance-based colorimetric assay yielded an IC$_{50}$ of 0.4 ± 0.2 μM, similar to that obtained in our in vitro enzymatic functional assay (Figure 3.2.6a). We conclude that our stapled peptide is cell permeable and a potent inhibitor of relevant physiological methylation of the PRC2 substrate. Remarkably, compound 2 also shows exceptional selectivity for H3K27 trimethylation inhibition. Thus, western blot analysis of histones isolated from cells treated with either high concentration of our cyclopeptide 2 (5 μM) or vehicle control showed no effect on H3K4me3, H3K9me3, H3K36me2, H3K79me2 and H3K27Ac, whereas total inhibition of H3K27me3 was observed in the same experimental conditions (Figure 3.2.6b).

Finally, we investigated the impact of H3K27m3 inhibition on cell growth and proliferation of Caki-1 cells, using healthy lung fibroblast cells IMR90 as a control. Cells were treated with compound 2, GSK126 as a positive control, and an equimolar combination of both compounds for 72h. Proliferation assays indicated that both cyclopeptide 2 and GSK126 are capable of significantly arresting cell growth, with IC$_{50}$ values of 0.25 ± 0.03 μM and 0.15 ± 0.06 μM, respectively, as quantified by a colorimetric method (Figure 3.2.6c). More notably, a combination of both compounds yielded an IC$_{50}$ value of 0.06 ± 0.01μM with a combination index of 0.61, indicative of a marked synergistic antiproliferative effect. Similar results were obtained in the correspondent viability assays. The compounds displayed no cytotoxicity below 15μM in non-cancerous human fibroblast IMR90 cells (Figure 3.6d).

In summary, we have applied our previously developed synthetic approach to generate macrocycles that effectively disrupt a biologically relevant intracellular protein-protein interaction in the PRC2 complex. In particular, we describe a series of cyclopeptide inhibitors of PRC2 methyltransferase activity, designed to target the intramolecular SANT1L-SBD interaction in
EZH2. Compared to their linear counterparts, these compounds showed enhanced \(\alpha\)-helical structure and inhibition of target function. Our lead cyclopeptide 2 showed potent inhibition of H3K27 trimethylation in both \textit{in vitro} and cellular assays, demonstrating that it is cell permeable and active in physiological conditions. Inhibition of PRC2 catalytic activity by 2 produced a marked dose-dependent antiproliferative effect in metastatic Cakis-1 cells. Notably, in these experiments our compound was almost as effective as GSK126, an EZH2 inhibitor currently in clinical development.\textsuperscript{113}

3.3 Bisthioether Stapled Peptides as Allosteric Inhibitors of the SUZ12-VEFS/EZH2-
SANT2 Interaction

The rational design of this family of stapled peptides focused on an interaction between the VEFS domain of SUZ12 (SUZ12-VEFS) and the catalytic subunit EZH2. In particular, we aimed at mimicking two different \(\alpha\)-helical bundles of SUZ12-VEFS, which had been documented to play a critical role in PRC2 proper assembling and function, through their interaction with EZH2 (Figure 3.3.1).\textsuperscript{98, 117-118} These evidences have also been confirmed by the resolution of a crystal structure of an active PRC2 ortholog from yeast\textsuperscript{5}, and more recently, of the cryo-electron microscopy structures of a complete human PRC2 complex (PDB ID 6C23)\textsuperscript{98}. Based on these structural considerations, we designed a series of stapled peptides derived from the human sequence of the SUZ12-VEFS domain, residues 590-603 and 652-669, respectively.
Figure 3.3.1 Left: The cryo-electron microscopy structure of a complete human PRC2 complex (PDB ID 6C23)\textsuperscript{98}. Top Right: Zoomed \(\alpha\)-helical intramolecular interaction between the targeted VEFS-SUZ12 (colored purple, yellow and gray) and EZH2 domains (colored Cyan). Bottom Right: Structure of the designed stapled peptide inhibitors, with the Cys residues incorporated for stapling labelled in green.

A total of four macrocycles, in which solvent-exposed residues were replaced by cysteines for the planned stapling reaction, were successfully prepared following a synthetic methodology previously presented in chapter 1 (Schemes 3.3.1 and 3.3.2).
Scheme 3.3.1 Synthetic scheme for the preparation of single turn stapled peptides (i, i+4) mimetics of the SUZ12-VEFS domain, residues 590-603, as inhibitors of PRC2 methyltransferase activity.
The synthesis of all the compounds was accomplished successfully as planned. The crude of the corresponding cyclopeptides were purified by HPLC, affording the final products with purities >95%.

Scheme 3.3.2 Synthetic scheme for the preparation of single turn stapled peptides (i, i+4) mimetics of the SUZ12-VEFS domain, residues 652-669, as inhibitors of PRC2 methyltransferase activity.
Figure 3.3.2 (a) Structure of the designed stapled peptides and summary of results obtained in the biochemical assays (b) Both Chymotrypsin-based proteolytic degradation and the plasma assay show enhanced stability of $i, i+4$ bisthioether stapled peptides. (c) CD spectra of stapled peptides and their linear counterparts measured in water at $20^\circ$C. (d) Inhibition of PRC2 catalytic function (H3K27Me3) as determined in an enzymatic assay using endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1), at peptide concentration of 10 $\mu$M (e) Plot of H3K27me3 inhibition obtained in an enzymatic assay using endogenous PRC2 extracted from Caki-1 cells and recombinant PRC2 complex, 100×SAM, and equimolar combination with GSK126 (for compound 11).

We first investigated the proteolytic stability of these compounds by subjecting them to both a chymotrypsin and a plasma stability assay$^{69,116}$. For comparison, we also studied their linear counterparts. HPLC and LC/MS analysis showed in both assays marked protection of our $i, i+4$ cyclopeptides to proteolysis, in contrast to their respective linear analogues, which were fully degraded after a few minutes in both conditions (Figure 3.3.2a, b). Next, we measured the impact of the staple on the secondary structure of our synthesized macrocycles by circular dichroism (CD).
This analysis indicated that stapling through a three-methylene hydrocarbon brace resulted in remarkable enhancement of the helical character for both series, more pronounced for compound 11, which showed an overall helicity of 95% (Figure 3.6a, c).

To test if the observed induction of helical conformation correlated with biological activity, we investigated our cyclopeptide’s ability to inhibit H3K27me3 in an enzymatic assay, using as catalytic complex endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1). A well-characterized EZH2-SET domain inhibitor (GSK126) was used as a positive control for determining optimal assay conditions. A first screening at 10 μM peptide concentration showed that all compounds were capable of inhibiting H3K27me3. In line with the CD results, cyclopeptide 11 resulted the more potent of both series (Figure 3.3.2a, d). Further testing of this compound showed concentration dependent inhibition of PRC2 catalytic function with an IC50 value of 0.32 ± 0.10 μM, resulting virtually as potent as GSK126 in the same assay conditions.

The specificity of 11 (GN-ZW11) for targeting PRC2 complex assembly and function was then confirmed by performing the same enzymatic assay with pure recombinant PRC2 complex, which yielded almost the same result (Figure 3.3.2e). We further confirmed the allosteric nature of this inhibitor by performing the same inhibition assay using a much higher SAM concentration (100 folds) than the in original conditions, which led to similar IC50 value of 0.28 ± 0.10 μM (Figure 3.3.2e). In line with this result, we also observed strong synergy between compound 11 and GSK126, a SAM-competitor inhibitor, when carrying out the same H3K27me3 inhibition assay using equimolar amount of both molecules. Thus, the combination index calculated as described before resulted in a value of 0.28, well below the limit set for a synergistic and not additive effect (CI < 1).
We next explored the cellular uptake of **11** by confocal microscopy, using an FITC-labeled derivative of it. Confocal imaging after incubation of caki-1 cells with fluorescently-tagged **11** showed that it penetrates the cell membrane, and more importantly that it reaches the nuclei, where the targeted PRC2 complex is localized (Figure 3.3.3a). Next, encouraged by these results, we studied the ability of **11** to target and functionally block PRC2-mediated H3K27me3 in Caki-1 cells. We also evaluated, as controls, the cyclopeptides of both series **7-9, 10** and **12**. In line with the results previously obtained in the enzymatic assay, western blot analysis after cell treatment for 72h at 10 µM peptide concentration indicated that only **11** was able to efficiently block H3K27me3 (Figure 3.3.3b). Further testing with varying concentration of this compound showed a clear dose-response inhibition of PRC2 catalytic function with an IC<sub>50</sub> of 1.04 ± 1.34 µM, which is also similar to that obtained in our enzymatic assay (Figure 3.3.3c). To assess the selectivity of our compound for targeting the PRC2 complex, we investigated its effect on different post-translational modifications. Remarkably, cyclopeptide **11** showed exceptional selectivity for H3K27Me3 inhibition. Thus, western blot analysis of histones isolated from cells treated with either high concentration of **11** (5 µM) or vehicle control showed no effect on H3K4me3, H3K9me3, H3K36me2, H3K79me2 and H3K27Ac, whereas total inhibition of H3K27me3 was observed in the same experimental conditions (Figure 3.3.3d). Altogether, this data confirmed that our stapled peptide is cell permeable and a potent selective inhibitor of the relevant physiological methylation of the PRC2 substrate.
**Figure 3.3.3** (a) Confocal microscopy images of Caki-1 cells, scale bar 10 μm. Cells were treated with FITC-labeled 11 (5 μM) and control peptide (β-Ala-β-Ala-FITC, 5 μM) at 37°C for 5h. (b) Western blotting after treating caki-1 cells with both series of peptides at 10 μM shows marked inhibition of H3K27me3 by compound 11, in contrast to the control peptides (c) Western blot analysis after treatment of caki-1 cells with varying concentration of stapled peptide 11, once daily for 72h, indicates a clear dose-dependent response of H3K27me3. Single concentration treatment with GSK126 (positive control) and linear wild type sequence 16 (negative control) are also shown. (d) Selectivity of H3K27 trimethylation inhibition over a broad panel of histone post-translational modifications. Cells were treated with stapled peptide 11 (5 μM) or vehicle, once daily for 72h.

In summary, our structure-based approach has allowed the identification of a series of cyclopeptide inhibitors of PRC2 methyltransferase activity, designed to target the interaction between the VEFS domain of SUZ12 and the SANT2 domain of EZH2, the catalytic subunit of PRC2. In particular, one of our stapled peptides (11) showed potent inhibition of H3K27

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trimethylation in both *in vitro* and cellular assays, which demonstrate that it is cell permeable and active in physiological conditions.

### 3.4 Bisthioether Stapled Peptides as Allosteric Inhibitors of the SUZ12-NBE/Nurf55 Domain Interaction

The rational design of this family of stapled peptides focused on targeting an α-helical interaction between Nurff55 and the Nurf55 binding epitope (NBE) domain of SUZ12 (SUZ12-NBE), which have been shown to play a key role in the PRC2 recruitment onto nucleosomes and thus in its methyltransferase activity. More specifically, these compounds are mimetics of the helix α1 of Nurf55, residues 20-35, which interacts with the SUZ12-NBE domain, and therefore constitute a new family of peptidomimetic modulators of SUZ12-NBE function.¹¹⁹

We designed a total of 21 macrocycles, aiming at testing four different families of stapled peptides cross-linked at four different positions in the native linear peptide sequence (Figure 3.4.1). The synthesis was accomplished successfully, following the solid phase protocol described in Chapter 1 via orthogonal Fmoc strategy. The all-hydrocarbon staples were installed chemoselectively by forming a bisthio-ether linkage, after selectively deprotecting the Mmt (4-methoxytrityl) protecting groups from the corresponding cysteines residues. A representative example of the synthesis of the compounds of this series is given in Scheme 3.4.1
Figure 3.4.1 Top: Crystal structure of Nurf55 in complex with SUZ12-NBE (PDB 2YB8)\textsuperscript{119}. Center: Zoomed $\alpha$-helical intramolecular interaction between Nurf55 and the Nurf55 binding epitope (NBE) domain of SUZ12 (SUZ12-NBE). Bottom: Structure of the designed stapled peptide inhibitors, mimetics of the helix $\alpha1$ of Nurf55, residues 20-35, with the Cys residues incorporated for stapling labelled in purple.
Scheme 3.4.1 Synthetic scheme for the preparation of single turn stapled peptides 24-28 \(i, i+4\), as mimetics of the helix α1 of Nurf55, residues 20-35, targeting the SUZ12-NBE domain.
Following cyclization, the resulting macrocycles were cleaved off the resin with a trifluoroacetic acid (TFA) cocktail (TFA/EDT/Water/TIS=94/2.5/2.5/1) and precipitated from diethyl ether. Finally, the cyclopeptide crudes were purified by semipreparative HPLC, yielding the desired products in good overall yields and with purities >95%. Characterization was performed by analytical HPLC and LC/MS.

We first measured the effect of incorporating different bis-thioether staples at different positions in the sequence, on the helicity of the resulting macrocycles by circular dichroism (CD). As expected, both the position and the length of the linker resulted key factors on stabilizing the bioactive alpha-helical conformation. Each family of stapled peptides showed several candidates with improved α-helicity, compared with linear parent sequence 13 (Figure 3.4.2). Compound 24 showed the highest helical character of the whole series, with an improvement of 20% over 13 (Figure 3.4.2a, b).

To determine if the conformational effects induced by the incorporation of the staples would translate into a potent inhibition of PRC2 methyltransferase activity, we next subjected all the synthesized macrocycles to an enzymatic assay specific for H3K27Me3, using as catalytic complex endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1). A well-characterized EZH2-SET domain inhibitor (GSK126) was used as a positive control for determining optimal assay conditions. An initial screening of all the compounds at a concentration of 10 μM showed a high correlation between helical character and inhibitory activity (Figure 3.4.2). Interestingly, the best inhibitor of each series was the cyclopeptide resulting from stapling through a three-methylene hydrocarbon linker.
Figure 3.4.2 (a) Structure, helicity and % of inhibition of H3K27me3 at 10 μM for all the synthesized stapled peptides (b) Bar graph showing the helicities and percentages of H3K27me3 inhibition at 10 μM peptide concentration, for all the synthesized stapled peptides and the linear analogue 13 (c) CD spectra of each of the peptides that showed the higher helical character from each family, measured in water at 20°C.

Further analysis of these compounds under the same enzymatic assay confirmed that they were able to efficiently block PRC2 catalytic activity, with IC₅₀ values in the low micromolar range (Figure 3.4.3a,b). In particular, stapled peptide 24 was the most potent compound with an IC₅₀ of 1.33 ± 1.36 μM, markedly superior to that of its linear parent peptide 13. To unequivocally demonstrate that the inhibition of H3K27me3 shown by compound 24 is specifically due to inactivation of PRC2 methyltransferase activity, we carried out the same enzymatic assay using instead pure recombinant PRC2. The IC₅₀ obtained in such conditions (1.78 ± 0.32 μM) was almost identical to that previously determined when using the caki-1 cell’s nuclear extract as complex source, which confirmed the molecular target of our stapled peptide (Figure 3.4.3c).
Figure 3.4.3 (a) Inhibition of PRC2 catalytic function (H3K27Me3) by the most potent stapled peptides of each series, as determined in an enzymatic assay using endogenous PRC2 extracted from a human clear cell renal carcinoma cell line (Caki-1) (b) Plot of H3K27 trimethylation inhibition using the enzymatic in vitro assay described in a (c) Plot of H3K27 trimethylation inhibition using the enzymatic in vitro assay described in a, with varying concentrations of 24 (using both endogenous PRC2 extracted from a human clear cell renal carcinoma cell line and recombinant PRC2), positive control GSK126, and using an equimolar combination of both compounds to test their synergistic effects. (d) Plot of H3K27 trimethylation inhibition using the enzymatic in vitro assay described in a, with varying concentrations of SAM. (e) A summary of the IC50 values for inhibition of H3K27me3 determined from these experiments is shown in plot b, c and d.

We further examined the potential synergy of 24 with GSK126, a well-characterized EZH2-SET domain inhibitor currently in clinical development. To this end, we performed the previously described inhibition assay, using an equimolar combination of both 24 and GSK126, which yielded an IC50 value of 0.08 ± 0.09 µM. This value corresponds to a combination index (CI) of 0.55, indicative of a marked synergistic effect between the two compounds (Figure 3.4.3c). The synergy observed strongly suggests that our stapled peptide targets PRC2 allosterically. To finally confirm this hypothesis, we carried out our H3K27me3 inhibition assay at different
concentrations of S-adenosyl-L-methionine (SAM). These experiments showed that the binding of 24 is independent of SAM concentration, and thus that our cyclopeptide does not compete with it for the binding to the SET domain of EZH2, and therefore targets PRC2 allosterically (Figure 3.4.3d).

**Figure 3.4.4 (a)** Structure of the biotinylated derivative of cyclopeptide 24, synthesized on solid phase (b,c) In vitro pull down assay using biotinylated cyclopeptide 2, visualized by SDS-PAGE and followed by western blot analysis using antibodies against human EZH2 (b) and human EED and SUZ12 (c).

The results of a pull-down assay performed similarly to that previously described in 3.3, using a biotinylated derivative of 24 synthesized on solid phase, also showed that our stapled peptide binds to Suz12 specifically and selectively, which strengthen the validation of our initial hypothesis, thus strongly suggesting that compound 24 does target SUZ12 and likely inhibits PRC2 function by prevent its colocalization to the nucleosomes.
Given such encouraging results, we next investigated the feasibility of using compound 24 in functional assays, by exploring both its proteolytic stability and its cell penetrating properties. Proteolytic stability was studied by subjecting peptide 24 to a chymotrypsin stability assay. For comparison, we also studied its linear counterpart 13 in the same conditions. HPLC and LC/MS analysis showed marked protection of 24 to proteolysis ($t_{1/2} = 20.5$ h), in contrast to its respective linear analogue, which was fully degraded in half an hour ($t_{1/2} = 0.5$ h). We further tested the stability of cyclopeptide 24 and its parent linear sequence 13 in plasma. In line with the chymotrypsin assay results, our macrocycle showed high resistance to proteolysis in these conditions, resulting also markedly superior to peptide 13, which was fully degraded after 30 minutes (Figure 3.4.5).

Figure 3.4.5 Both Chymotrypsin-based proteolytic degradation and the plasma assay show enhanced stability of our $i, i+4$ bisthioether stapled peptide 24, as compared to its linear parent peptide 13.

<table>
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<tr>
<th>Assay</th>
<th>Half life</th>
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<tr>
<td>13 Chymotrypsin</td>
<td>0.35 h</td>
</tr>
<tr>
<td>13 Plasma</td>
<td>0.28 h</td>
</tr>
<tr>
<td>24 Chymotrypsin</td>
<td>20.5 h</td>
</tr>
<tr>
<td>24 Plasma</td>
<td>6.78 h</td>
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Cellular uptake was studied by live confocal microscopy using a fluorescently labeled derivative of 24. Notably, confocal imaging after incubation of Caki-1 cells with FITC-tagged 24 showed its favorable cell penetrating properties, and more importantly that it localizes mainly in the nuclei, where the targeted PRC2 complex is located (Figure 3.4.6).

**Figure 3.4.6** Confocal microscopy images of Caki-1 cells, scale bar 10 μm. Cells were treated with FITC-labeled 24 (24-FITC, 5 μM) and control peptide (β-Ala-β-Ala-FITC, 5 μM) at 37°C for 5h.

We next demonstrated the ability of cyclopeptide 24 to target and functionally block PRC2-mediated H3K27 methylation in cells. For this purpose, we treated Caki-1 cells for 72h with varying concentrations of 24 and analyzed all degrees of methylation on H3K27 by western blot from histone extracts. We used GSK126 (1 μM, 10 nM) and the linear wild type peptide sequence 13 (10 μM, 50 μM) as positive and negative control, respectively. The results showed a clear concentration-dependent inhibition of H3K27me3 (Figure 3.4.7a). Further quantitation by an absorbance-based colorimetric assay yielded an IC$_{50}$ of 1.36 ± 1.14 μM, similar to that obtained in
our enzymatic functional assay (Figure 3.4.7b). This data confirmed that our stapled peptide is cell permeable and a potent inhibitor of relevant physiological methylation of the PRC2 substrate.

We also tested the selectivity of compound 24 for PRC2 methyltransferase activity, by monitoring its effect on several others post-translational modifications. Our stapled peptide showed exquisite selectivity for H3K27Me3 inhibition. Thus, treatment of caki-1 cells with either high concentration of 24 (5 μM) or vehicle, and subsequent western blot analysis of histones isolated from the cell lysate, indicated no effect on H3K4me3, H3K9me3, H3K36me2, H3K79me2 and H3K27Ac. On the other hand, total inhibition of H3K27me3 was observed in the same experimental conditions (Figure 3.4.7c). Altogether, this data demonstrates that our stapled peptide 24 is resistant to proteolysis, cell permeable and a potent selective inhibitor of the relevant physiological methylation substrate of PRC2.

Finally, we studied the impact of H3K27m3 inhibition on proliferation and growth of Caki-1 cells, using healthy lung fibroblast cells IMR90 and GSK126 as a negative and positive controls, respectively. Proliferation was determined after treating the cells with compound 24, GSK126 or an equimolar mixture of both compounds for 72h, adding a fresh dose of compound every 24h. Remarkably, these assays indicated that our compound is able to significantly arrest Caki-1 cells growth, with an IC50 value of 2.06 ± 1.26 μM (Figure 3.4.7d). More notably, a combination of both compounds yielded an IC50 value of 0.82 ± 0.17μM, which also suggest a marked synergistic anti-proliferative effect. This result is in line with the data previously obtained from our H3K27me3 enzymatic inhibition assay. Importantly, no cytotoxicity was observed below 100μM in non-cancerous human fibroblast IMR90 cells.
Figure 3.4.7 (a) Western blot analysis shows dose-dependent response of H3K27me3 within metastatic human clear-cells renal carcinoma cells (Caki-1), after treatment with stapled peptide 24 once daily for 72h. Single concentration treatment with GSK126 (positive control) and linear wild type sequence 13 (negative control) in the same experimental conditions are also shown. (b) Quantitation of H3K27me3 using and absorbance-based colorimetric assay yielded an IC50 value of 1.36 ± 1.14 μM. Protein loading was accurately corrected by measuring total H3 using an absorbance-based colorimetric assay. (c) Selectivity of H3K27 trimethylation inhibition over a broad panel of histone post-translational modifications. Cells were treated with stapled peptide 24 (5 μM) or vehicle, once daily for 72h. (d) Treatment of Caki-1 cells with cyclopeptide 24 significantly inhibits cell proliferation. Proliferation was measured after 72h of daily treatment with the correspondent compound. The data is presented as a mean of two independent experiments each with triplicate measurements.

In summary, we have exploited our solid phase synthetic approach to generate a series of stapled peptide capable of effectively disrupt a biologically relevant intracellular PPI. In particular, we describe the design, synthesis and biological evaluation of stapled peptide inhibitors of PRC2
methyltransferase activity, designed to target a crucial interface in PRC2, formed by the nurf55 and the SUZ12-NBE domains. Compared to their linear counterpart, most of these compounds showed enhanced α-helical structure and inhibition of target function. In particular, our lead cyclopeptide 24 resulted a potent inhibitor of H3K27 trimethylation in both in vitro and cellular assays, demonstrating that it is cell permeable and active in physiological conditions. Inhibition of PRC2 catalytic activity by this compound produced a marked dose-dependent antiproliferative effect in metastatic Cakis-1 cells. Confocal microscopy has further illustrated that cyclopeptide 24 is not only cell permeable, but also able to reach the nuclei, where the PRC2 complex is localized. Our studies also provide strong evidences suggesting that our stapled peptide 24 is an allosteric inhibitor of PRC2 function, and therefore, this compound may well be the first inhibitor of SUZ12-NBE described to date.

3.5 EXPERIMENTAL SECTION

3.5.1 General experimental information

Solid Phase Peptide Synthesis.

All peptides were manually synthesized on 4-alkoxy-2,6-dimethoxybenzyl- amine resin (PAL, 0.5mmol/g). Coupling steps were performed under Microwave irradiation, using a combination of PyBOP/Oxyme as coupling reagents. Fmoc deprotection was accomplished at room temperature with a piperidine solution, 20% in DMF.

4-methoxytrityl (Mmt) Deprotection and Maleimide test.

Mmt deprotection was carried out with a deprotection solution (15mL per 0.5g of resin, 2% TFA, 10% TIS, 88% DCM) at room temperature for 5 min. The resin was then washed with DCM
(5X) and treated repeatedly with the same solution until its color changed from orange to light yellow. Maleimide test was next performed (Scheme S4). To this end, a small aliquot of Mmt deprotected resin was neutralized with 3mL of 5% DIEA in DMF for 5 min, and washed with DMF (2 mL, 3X), DCM (3 mL, 3X) and DMF (3 mL, 2X). The neutralized clean resin was subsequently mixed with N-methylmaleimide (5 eq. from a 20 mg/mL DMF stock solution), and DIEA (5 eq.) in a 2-mL microwave vial with a stir-bar, and reacted under microwave irradiation at 85 °C for 15 min. Next, the suspension was transferred into a SPPS vessel, washed (DMF 2 mL 3X, DCM 2 mL 3X) and treated with the proper cleavage cocktail. The resulting peptide is finally precipitated/washed with cold diethyl ether and analyzed by LC/MS to quantitate the extent of the deprotection step (Scheme S4). If the Mmt-deprotection is incomplete, additional deprotection rounds are repeated until completion of the reaction. After total Mmt removal is achieved, the resin is neutralized with 5% DIEA in DMF for 5 min, washed with DMF (3X) and DCM (3X), and dried under vacuum for storage or used directly for the cyclization step.

**Stapling Reaction.**

The resin containing the precursor Cys-thiol free linear peptide was transferred into a microwave reaction vial containing a stir-bar, to which NaI (100 eq for \(i+7\) sequences, 17.5 eq for \(i+4\) sequences) and DMF (1 mL per 20 mg of resin for \(i+7\) sequences, 1mL per 10mg of resin for \(i+4\) sequences) were added subsequently, while keeping the mixture stirring at all times, followed by TCEP (3 eq for \(i+7\) sequences, 0.5 eq for \(i+4\) sequences, from an aqueous stock solution of 300 mg/mL). The vial was then capped with a MW vial cap equipped with a rubber septum and the resulting suspension bubbled under N\(_2\) for 15 min. Next, DIEA (35 eq.) was added (by syringe), keeping the stirring under N\(_2\) for another 30 min, after which, the dibromoalkyl electrophile was
injected (20 eq for i+7 sequences, 3.5 eq for i+4 sequences). The suspension is finally reacted under microwave irradiation for 2 min at 125°C, transferred into the SPPS reaction vessel and washed with water (5 X, soak if necessary to remove residual NaI), DMF (5 X) and DCM (5 X). The efficiency of the stapling step was confirmed by cleaving a small aliquot of resin and analyzing the precipitated/washed peptide pellet by LC/MS and HPLC.

3.5.2 Biochemical and Cellular Studies

Circular Dichroism (CD)

Circular dichroism spectra were recorded on a Chirascan spectrometer. Peptides were dissolved in deionized water, at a concentration of 25 μM. CD signal was monitored at 1nm intervals from 180 to 260 nm with a 1-mm path quartz cuvette. The data reported is the average of three scans, subtracting the background. The helical content of each peptide was determined using prism 7 software, with molar ellipticity [θ] [deg·cm²·dmol⁻¹] being calculated using the following formula: $[\theta] = 1000000 \times \theta / (C \times \# \text{of residues} \times l)$, where $\theta$ is the ellipticity read out from Chirascan, C is the concentration of peptide samples in μM, l is the pathlength of the cuvette measured in mm. The percentage helicity was calculated with formula: %Helicity = 100 × $[\theta]_{222}/[\theta]_{\text{max}222}$, where $[\theta]_{\text{max}222} = -31500 \times [1 - (2.5 \times \# \text{of residues})]$.²

Chymotrypsin Stability Test

Assays were performed using freshly prepared α-chymotrypsin from bovine pancreas type II, ≥ 40 U/mg. The enzyme was initially reconstituted in HCl 1M (1 μg/μL), and kept in ice until used. Peptides stock solutions were prepared in assay buffer (50 mM Tris·HCl pH=8.0, 560 μM calcium chloride, 0.1% Tween-20) containing <3% of DMSO. The reactions were performed at a
200 μL scale, in assay buffer, at final concentrations of 50 μM for peptide and 60 μM for α-chymotrypsin (10 μL from the reconstitution solution). The mixture was incubated at 37 °C and aliquots were withdrawn in portions of 25 μL at different time points for analysis. The removed aliquots were mixed with 25 uL of water 1% TFA, and then treated with a column for detergent removal following the manufacturer’s protocol (Pierce, Catalog #:87776). The rate of peptide proteolysis was monitored by HPLC and HPLC-MS analysis. Control experiments were carried out under the same conditions but using BSA instead of α-chymotrypsin.

**Plasma Stability Test**

1mM stock solution of the corresponded peptide was prepared in PBS buffer (pH 7.4). Next, 50 μL from this solution were added to 1 mL of human plasma (Sigma-Aldrich, P9523). The mixture was incubated at 37 °C with mechanical shaking at 300 rpm. Aliquots were withdrawn in portions of 150 μL at different time points for analysis and mixed with 60 μL ACN/EtOH (1:1 v/v) for plasma protein precipitation. After centrifugation at 12000 rpm for 5 min, the supernatant was collected and analyzed by HPLC and HPLC-MS.

**Enzymatic Functional Assay to Quantify Inhibition of H3K27me3.**

All peptide candidates were evaluated for their ability to inhibit H3K27 trimethylation in vitro with an EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit (H3K27) (Epigentek, P-3005). The enzymatic reactions were performed using PRC2 complex extracted as a component of the nuclear extracts from a human clear cell renal carcinoma cell line (Caki-1). Protein concentration in the nuclear extracts was first optimized in order to have the suitable concentration of functional PRC2 to produce a strong signal (Control, A=0.9) with a reliable assay window
(Blank, A=0.15). The assays were validated by using GSK-126, a well-characterized Ezh2 inhibitor, as a positive control. The trimethylation reaction was performed in vitro using immobilized recombinant oligonucleosomes and then quantified by absorbance, following the manufacturer’s protocol. Experiments were carried out using several concentrations of inhibitors, always including a 100% inhibition control of 0.1 μM GSK126. The data were fit into a standard Langmuir isotherm for inhibition, and are the result of the average and standard deviation of three independent experiments.

**Cell Culture**

Metastatic human clear cell renal carcinoma cell line (Caki-1) were used to extract the active PRC2 complex used in the enzymatic assays. Caki-1 cells and healthy control lung fibroblast cell (IMR90) cell lines were used to study the cytotoxic activity of compounds 9, 10, GSK126 and 24. The cells were all obtained from the American Type Culture Collection (ATCC) (Manassas, VA). All of the cells were grown adherently. The Caki-1 cells were cultured in Roswell Park Memorial Institute (RPMI-1640) (Mediatech Inc., Manassas, VA) medium, while IMR90 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech Inc., Manassas, VA); all media were supplemented with 10% foetal bovine serum (FBS, Life Technologies, Grand Island, NY), 1% Minimum Essential Media (MEM) nonessential amino acids (Mediatech), and 1% penicillin–streptomycin (Mediatech). All cells were cultured at 37 °C under 5% CO₂ and 95% air in a humidified incubator.

**Nuclear Extraction**
Active PRC2 was isolated as a component of the nuclear extracts from Caki-1 cells, using EpiQuik Nuclear Extraction kit (Epigentek OP-0002-1) and following the manufacturer’s protocol.

*Inhibition of H3K27me3 in Caki-1 Cells.*

The ability of our cyclopeptides to inhibit H3K27me3 in cells was studied by treating Caki-1 cells with different concentrations of the corresponding stapled peptide containing a maximum DMSO concentration of 1%. The cells were treated for 72h, fresh cyclopeptide solution was provided every 24h. Wild type linear peptides (100 μM and 25 μM), GSK126 (1 μM) and media containing 1% DMSO were used as controls. After treatment, the cells were lysed and the histone extracted using the EpiQuik Total Histone Extraction Kit (Epigentek OP-0006-100), following the instructions provided by the manufacturer. Total H3 concentrations were then measured for all the extracted samples using the EpiQuik Total Histone H3 Quantification Kit (Colorimetric, P-3062). Finally, trimethylation levels were evaluated by Western blot. To this end, 10 ng of each histone extract lysate were added on a 12% Mini-PROTEAN® TGX™ Precast Gels (Biorad). Electrophoresis was then carried out with Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Biorad) at 200 V for 30 min. Next, protein bands were transferred onto Nitrocellulose Membrane (Biorad, 0.2 μm) with Trans-blot SD Cell (Biorad) at 150 mA for 45 min. Downstream process of the membrane included blocking (50% Li-COR Odyssey® Blocking Buffer PBS, 60 min at RT on orbital shaker), incubation with primary antibody (Cell Signaling: Tri-methyl-H3K27 Rabbit mAb #9733, and Histone H3 mouse mAb #3638, overnight at 4 °C), washing with PBST buffer, and incubation with secondary antibody (IRDye® 800CW Donkey anti-Mouse IgG, and IRDye® 800CW Donkey anti-Rabbit IgG) at RT for 60 min on an orbital shaker. Blotting imagines were
obtained with Odyssey® FC Imaging System (700 nm channel for H3K27me3 and 800 nm channel for H3).

Alternatively, the levels of H3K27me3 in the extracted histones were quantified using the EpiQuick Global Tri-methyl Histone H3K27 Quantification Kit (Colorimetric, Epigentek P-3042). The calculated values were normalized to the previously determined values for the corresponding total H3. The data were fit into a standard Langmuir isotherm for inhibition, and are the result of the average and standard deviation of three independent experiments.

Confocal Microscopy

Caki-1 cells (0.8 x 10^5 cells/well) were cultured for 24h on Millicell EZ SLIDE (Milliporesigma, PEZGS0416) in 1 mL complete RPMI supplemented with 10% FBS and 2 mM L-glutamine, 1% Minimum Essential Media (MEM) nonessential amino acids (Mediatech), and 1% penicillin–streptomycin (Mediatech). The cells were then seeded, on fresh medium, with fluorescent peptide (final concentration 5 µM, 1% DMSO) or vehicle (1% DMSO) and incubated for 5h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. Next, the medium was removed, and each well washed with 1 mL PBS (3X, 5min each time), followed by fixation with 4% paraformaldehyde in PBS (500 µL, Alfa Aesar), at room temperature for 15 min. After removal of the fixation solution, the slides were washed with PBS (3X, 5min each time) and the nucleus of the cells stained with 250 µL of DAPI solution (1µg/mL in PBS, Sigma-Aldrich, 28718-90-3) at room temperature for 10 min. The DAPI solution was next aspirated, the wells were washed with PBS (3X, 5min each time), and the cellular skeleton was stained with Alexa Fluor 555 Phalloidin following the manufacturer protocol. Subsequently, the slides were washed with PBS (3X, 5min each time), before adding 20 µL of mounting medium (Prolong Gold, Invitrogen, P10144) into
each well, following the manufacturer’s protocol. The slides were kept in the dark for 24 hours before imaging. The cells were visualized with an Olympus Fluoview microscope (model number: FV10i).

**Antiproliferative assays.**

Cells were seeded at a concentration of $0.15 \times 10^6$ cells/well in 3 mL of RPMI supplemented with 10% FBS and 2 mM L-glutamine, 1% Minimum Essential Media (MEM) nonessential amino acids (Mediatech), and 1% penicillin–streptomycin (Mediatech) into tissue culture grade 6-well flat bottom plates (Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO$_2$ and 95% air in a humidified incubator. Following seeding, the cyclopeptides were dissolved in DMSO and diluted in media before addition to cell culture medium at a maximum DMSO concentration of 1%, at cyclopeptide concentrations ranging from $0.01 \mu$M to 1.5 μM. For the following 72h, every 24 h the cell culture media was changed and fresh media containing the compounds was added, 1% DMSO was used as control. After 72h of treatment, both floating and attached cells were collected and analysed. NucleoCounter NC-3000™ chamber (Chemometec, Allerod, Denmark) was used to determine total cell number and viability according to the manufacturer’s protocol.
3.6 APPENDIX

(1) Ac-TVDKIASALSVLAEVDPQN-COH₂

HRMS (ESI) (m/z): [M] cacl. for C₈₈H₁₄₉N₂₃O₃₁, 2024.0790

[M+2H]²⁺ found 1013.0458 (monoisotopic peak)

HPLC: Method 4, tᵣ = 12.03 min.
(2) Ac-TVD[CIASC]$_3$LSVLAEEVPQN-CONH$_2$

**HRMS (ESI) (m/z):** [M] cacl. for C$_{88}$H$_{146}$N$_{22}$O$_{31}$S$_2$, 2070.9966

[M+2H]$^{2+}$ found 1036.5039 (monoisotopic peak)

**HPLC:** Method 4, $t_R$ = 15.73 min.
(3) Ac-TVD[CIASC]c5LSVLAEEVPQN-CNH₂

HRMS (ESI) (m/z): [M] cacld. for C₉₀H₁₅₀N₂₂O₃₁S₂, 2099.0279

[M+2H]²⁺ found 1050.5099 (monoisotopic peak)

HPLC: Method 4, tᵣ = 17.11 min.
(4) Ac-TVDKIASA[CSVLAEEC]C6PQN-CONH₂

HRMS (ESI) (m/z): [M] cacld. for C₈₉H₁₄₉N₂₃O₃₁S₂ 2100.0231

[M+2H]²⁺ found 1051.0099 (monoisotopic peak)

HPLC: Method 4, t_R = 14.14 min.
(5) Ac-TVDKIASA[CSVLAEEC]$_7$PQN-$\text{CONH}_2$

**HRMS (ESI) (m/z):** [M] cacld. for C$_{90}$H$_{151}$N$_{23}$O$_{31}$S$_2$ 2114.0388

[M+2H]$^{2+}$ found 1058.0263 (monoisotopic peak)

**HPLC:** Method 4, $t_R = 15.53$ min.
(6) Ac-TVDKIASA[CSVLAEEC]C8PQN-CNH₂

**Synthesis:** Using 1,8-dibromo-octane as electrophile

**HRMS (ESI) (m/z):** [M] calcd. for C₉₁H₁₅₅N₂₃O₃₁S₂ 2128.0544

[M+2H]²⁺ found 1065.0344 (monoisotopic peak)

**HPLC:** Method 4, t<sub>R</sub> = 16.47 min.
(7) Ac-EWLREKTITQIEEF-CONH₂

**Synthesis:** Isolated from 13, after subsequent TFA cleavage, Scheme S2.1

**HRMS (ESI) (m/z):** [M] cacl. for C₈₅H₁₃₁N₂₁O₂₆, 1861.9574

\[\text{[M+2H]}^{2+} \text{ found 931. (monoisotopic peak)}\]

**HPLC:** Method 4, \(t_R = 12.22 \text{ min.}\)
(8) Ac-EWL[CEKTC]₃TQIEEF-CONH₂

**Synthesis:** Using 1,3-dibromopropane as electrophile, Scheme S2.1

**HRMS (ESI) (m/z):** [M] cacl. for C₈₂H₁₂₂N₁₉O₂₆S₂, 1838.8219

[M+1H]⁺ found 1839.8307 (monoisotopic peak)

**HPLC:** Method 4, tᵣ = 13.67 min.
(9) Ac-EWL[CEKTC]c5TQIEEF-CONH₂

**Synthesis:** Using 1,5-dibromopentane as electrophile, Scheme S2.1

**HRMS (ESI) (m/z):** [M] cacld. for C₈₄H₁₂₆N₁₈O₂₆S₂ 1866.8532

[M+1H]⁺ found 1867.8811 (monoisotopic peak)

**HPLC:** Method 4, tᵣ = 14.51 min.
(10) Ac-LCRNFLHLVSMHDFLI-CNH₂

Isolated from 16 after subsequent TFA and T-Bu cleavages, Scheme S2.2

HRMS (ESI) (m/z): [M] calcd. for C₉₇H₁₅₂N₂₆O₂₂S₃, 2129.0737

[M+2H]²⁺ found 1065.5472 (monoisotopic peak)

HPLC: Method 4, tᵣ = 15.14 min.
(11) Ac-LCRNFMHLVSMHDFLI-CONH₂

**Synthesis:** Using 1,3-dibromopropane as electrophile, *Scheme S2.2*

**HRMS (ESI) (m/z):** [M] cacl. for C91H146N26O22S5, 2114.9709

[M+2H]²⁺ found 1058.4900 (monoisotopic peak)

**HPLC:** Method 4, \( t_R = 13.14 \text{ min.} \)

**HRMS: Crude Reaction Mixture After**
FITC-β-Ala-β-Ala-LCRN[CMLHC]$_3$VSMHDFLI-CONH$_2$ (FITC-17)

*Synthesis:* Using 1,3-dibromopropane as electrophile, Scheme S1.3

**HRMS (ESI) (m/z):** [M] cacld. for $C_{117}H_{167}N_{29}O_{28}S_6$, 2604.0703.

[M+2H]$^+$ found 1303.0373 (monoisotopic peak)

**HPLC:** Method 4, $t_R = 15.79$ min.

FITC-β-Ala-β-Ala-CONH$_2$

**HRMS (ESI) (m/z):** [M] cacld. for $C_{27}H_{24}N_4O_7S$, 548.1366

[M+2H]$^+$ found 548.1646 (monoisotopic peak)
(12) Ac-LCRNFMLHLVSMHDFLI-CONH₂

**Synthesis:** Using 1,5-dibromopentane as electrophile, Scheme S2.2

**HRMS (ESI) (m/z):** [M] calcd. for C₉₃H₁₅₀N₂₆O₂₂S₅ 2143.0022

[M+1H]+ found 1072.5054 (monoisotopic peak)

**HPLC:** Method 4, tᵣ = 13.98 min.

**HPLC:** After

**HRMS: Crude Reaction Mixture After**
(13) Ac-VINEYKIWKKNTPFL-NH₂

HRMS (ESI) (m/z): [M] cacl. for C₉₉H₁₅₁N₂₃O₂₅, 2062.1251

[M+2H]²⁺ found 1032.0691 (monoisotopic peak)

HPLC: Method 1, tᵣ = 10.81 min.

(14) Ac-VI[CEEYC]c₃IWKKNTPLF-NH₂

HRMS (ESI) (m/z): [M] cacl. for C₉₈H₁₄₇N₂₁O₂₄S₂, 2066.0369

[M+2H]²⁺ found 1034.0256 (monoisotopic peak)

HPLC: Method 4, tᵣ = 12.73 min.

(15) Ac-VI[CEEYC]c₄IWKKNTPLF-NH₂

HRMS (ESI) (m/z): [M] cacl. for C₉₉H₁₄₉N₂₁O₂₄S₂, 2080.0526

[M+2H]²⁺ found 1041.0312 (monoisotopic peak)

HPLC: Method 2, tᵣ = 16.56 min.
(16) Ac-V[CEEY]CeneIWKKNTPFL-NH₂

**HRMS (ESI) (m/z):** [M] cacld. for C₉₉H₁₄₇N₂₁O₂₄S₂, 2078.0369

[M+2H]²⁺ found 1040.0259 (monoisotopic peak)

**HPLC:** Method 1, tᵣ = 14.75 min.

(17) Ac-V[CEEY]C₅IWKKNTPFL-NH₂

**HRMS (ESI) (m/z):** [M] cacld. for C₁₀₀H₁₅₁N₂₁O₂₄S₂, 2094.0682

[M+2H]²⁺ found 1048.0977 (monoisotopic peak)

**HPLC:** Method 2, tᵣ = 16.94 min.

(18) Ac-V[CEEY]C₆IWKKNTPFL-NH₂

**HRMS (ESI) (m/z):** [M] cacld. for C₁₀₁H₁₅₂N₂₁O₂₄S₂, 2108.0839

[M+2H]²⁺ found 1055.5736 (monoisotopic peak)

**HPLC:** Method 1, tᵣ = 13.96 min.
(19) Ac-VINEY[CIWKC]$_3$NTPFL-NH$_2$

**HRMS (ESI) (m/z):** [M] calcd. for C$_{96}$H$_{141}$N$_{21}$O$_{25}$S$_2$, 2051.9849

[M+2H]$^{2+}$ found 1026.9989 (monoisotopic peak)

**HPLC:** Method 2, t$_R$ = 15.10 min.

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(20) Ac-VINEY[CIWKC]$_4$NTPFL-NH$_2$

**HRMS (ESI) (m/z):** [M] calcd. for C$_{97}$H$_{143}$N$_{21}$O$_{25}$S$_2$, 2066.0005

[M+2H]$^{2+}$ found 1034.5075 (monoisotopic peak)

**HPLC:** Method 4, t$_R$ = 12.30 min.

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(21) Ac-VINEY[CIWKC]$_{C_{4}ene}$NTPFL-NH$_2$

**HRMS (ESI) (m/z):** [M] calcd. for C$_{97}$H$_{141}$N$_{21}$O$_{25}$S$_2$, 2063.9849

[M+2H]$^{2+}$ found 1032.9973 (monoisotopic peak)

**HPLC:** Method 1, t$_R$ = 13.68 min.
(22) Ac-VINEY[CIWKC]c5NTPFL-NH₂

HRMS (ESI) (m/z): [M] calcd. for C₉₈H₁₄₅N₂₁O₂₅S₂, 2080.0162

[M+2H]²⁺ found 1041.0148
(monoisotopic peak)

HPLC: Method 2, tₐₐ = 16.01 min.

(23) Ac-VINEY[CIWKC]c₆NTPFL-NH₂

HRMS (ESI) (m/z): [M] calcd. for C₉₉H₁₄₇N₂₁O₂₅S₂, 2094.0318

[M+2H]²⁺ found 1048.0480
(monoisotopic peak)

HPLC: Method 1, tₐₐ = 16.94 min.

(24) Ac-V[CNEEC]c₃KIWKKNTPFL-NH₂

HRMS (ESI) (m/z): [M] calcd. for C₉₃H₁₄₅N₂₃O₂₄S₂, 2032.0274

[M+2H]³⁺ found 1017.0219
(monoisotopic peak)

HPLC: Method 2, tₐₐ = 10.91 min.
(25) Ac-V[CNEEC]_4KIWKNTPL-NH_2

HRMS (ESI) (m/z): [M] cacld. for C_{94}H_{147}N_{23}O_{24}S_{2}, 2046.0431
[M+2H]^2+ found 1024.0316 (monoisotopic peak)

HPLC: Method 2, t_R = 11.64 min.

(26) Ac-V[CNEEC]_{C4ene}KIWKNTPL-NH_2

HRMS (ESI) (m/z): [M] cacld. for C_{94}H_{145}N_{23}O_{24}S_{2}, 2044.0274
[M+2H]^2+ found 1023.0231 (monoisotopic peak)

HPLC: Method 1, t_R = 9.81 min.

(27) Ac-V[CNEEC]_{C5}KIWKNTPL-NH_2

HRMS (ESI) (m/z): [M] cacld. for C_{95}H_{149}N_{23}O_{24}S_{2}, 2046.0431
[M+2H]^2+ found 1031.0359 (monoisotopic peak)

HPLC: Method 2, t_R = 12.11 min.
(28) Ac-V[CNEEC]c6KIWKKNTPFL-NH₂

**HRMS (ESI) (m/z):** [M] cacl. for C₉₆H₁₅₁N₂₃O₂₄S₂, 2046.0431

[M+2H]²⁺ found 1038.0455 (monoisotopic peak)

**HPLC:** Method 2, tᵣ = 13.28 min.

(29) Ac-VINEEYKIWKKN[CPFLC]c₃-NH₂

**HRMS (ESI) (m/z):** [M] cacl. for C₁₀₄H₁₅₈N₂₄O₂₅S₂, 2207.1271

[M+2H]²⁺ found 1104.5690 (monoisotopic peak)

**HPLC:** Method 1, tᵣ = 10.90 min.

(30) Ac-VINEEYKIWKKN[CPFLC]c₄-NH₂

**HRMS (ESI) (m/z):** [M] cacl. for C₁₀₅H₁₅₈N₂₄O₂₅S₂, 2221.1428

[M+2H]²⁺ found 1111.5768 (monoisotopic peak)

**HPLC:** Method 1, tᵣ = 15.21 min.
(31) Ac-VINEYKIWKNN[CPFLC]C4ene-NH2

HRMS (ESI) (m/z): [M] calcd. for C$_{105}$H$_{158}$N$_{24}$O$_{25}$S$_{2}$, 2219.1271

[M+2H]$^{2+}$ found 1110.5683 (monoisotopic peak)

HPLC: Method 1, $t_R = 13.25$ min.

(32) Ac-VINEYKIWKNN[CPFLC]C$_5$-NH$_2$

HRMS (ESI) (m/z): [M] calcd. for C$_{106}$H$_{162}$N$_{24}$O$_{25}$S$_{2}$, 2219.1271

[M+2H]$^{2+}$ found 1118.5822 (monoisotopic peak)

HPLC: Method 1, $t_R = 11.00$ min.

(33) Ac-VINEYKIWKNN[CPFLC]C$_6$-NH$_2$

HRMS (ESI) (m/z): [M] calcd. for C$_{107}$H$_{164}$N$_{24}$O$_{25}$S$_{2}$, 2249.1741

[M+2H]$^{2+}$ found 1125.5899 (monoisotopic peak)

HPLC: Method 1, $t_R = 13.48$ min.
Chapter IV. Summary

4.1 Summary

- A new method to accessing bisthioether stapled peptides on solid support was discovered and optimized. Its versatility and efficiency were demonstrated by applying it to the synthesis of stapled peptides of multiple architectures. This approach allows for crosslinking of natural cysteine residues with a variety of hydrocarbon spacers and without harsh reaction conditions and expensive/toxic transition metal catalysts or radical initiators. It is also the only available synthetic method that allows bisthioether stapling on sequences containing several cysteine residues and the chemoselective incorporation of multiple bisthioether staples over the same sequence.

- We have identified three families of novel allosteric inhibitors of PRC2 function. The first series was designed to target a key intramolecular interaction in EZH2, the catalytic subunit of the complex, taking place between its SBD and SANT1L-1 domains. The second family of cyclopeptides has been designed to mimic a VEFS helix of SUZ12 and therefore disrupt its binding with the EZH2-SANT2 domain, another crucial binary complex needed for the proper assembly of PRC2 in its catalytically bioactive conformation. The third series targets an alpha helical interaction between Nurff55 and SUZ12, which plays a key role in the complex recruitment onto nucleosomes and thus in its H3K27 methyltransferase activity. These three families of stapled peptides constitute the first allosteric inhibitors of PRC2 activity targeting such protein interfaces.

- The biological data presented herein demonstrate that our lead cyclopeptides 2, 11 and 24 are potent inhibitors of H3K27 trimethylation in both in vitro and cellular assays, and hence
cell permeable and active in physiological conditions. In addition, inhibition of PRC2 catalytic activity by these compounds produces a marked dose-dependent antiproliferative effect in metastatic Cakis-1 cells. Notably, in these experiments our stapled peptides were almost as effective as GSK126, an EZH2 inhibitor currently in clinical development.\textsuperscript{113} The allostERIC nature of our inhibitors could make them highly valuable to address the resistance profiles recently reported in clinical trials of three EZH2-SET domain inhibitors, in which extended dosing of these drugs have led to secondary EZH2 mutants resistant to treatment.\textsuperscript{113} Our compound’s unique mechanism of PRC2 inhibition, together with its potency, remarkable H3K27me3 inhibition selectivity, and low cytotoxicity to non-cancerous cells demonstrate these stapled peptide’s potential for future development of novel epigenetic cancer therapies.

- The success achieved in the development of allostERIC PRC2 inhibitors validate the utility of the synthetic methodology initially presented herein, and also the potential of bisthioether stapled peptides as peptidomimetic molecules useful to target intracellular undruggable protein-protein interactions of biological relevance.


76. Yu, X.; Sun, D., Macroyclic Drugs and Synthetic Methodologies toward Macrocycles. *Molecules* 2013, 18 (6), 6230.


