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Disulfides are a common functional group observed in biological systems, serving a variety of purposes ranging from structural rigidification of proteins to taking part in cell signalling and control of metabolic pathways.\(^1\)\(^2\) Coupled with recent interest in the development of macrocyclic scaffolds as modulators of protein-protein interactions,\(^3\)\(^4\) disulfide-bridged peptide bicycles (DBPBs) represent an underexplored class of molecules with respect to their synthesis, conformational properties, and biological activity. This review highlights known disulfide-bridged peptide bicycles (DBPBs) and the studies on their unique structural and biological features.

Structural Features of the Disulfide Functional Group

In peptides and proteins, disulfides arise primarily from oxidation of thiols, often originating from (but not limited to) cysteine residues in biological systems. A variety of oxidants can effect this transformation, including air and hydrogen peroxide. In the laboratory setting, iodine and various metals (Cu(II), Fe(III), Tl(III)) are employed most frequently. Since cellular compartments generally offer a reducing environment, disulfide bonds are often believed to be unstable in the cytosol but there are exceptions to this.\(^5\) In fact, disulfides are integral in the folding and stability of various proteins. The S-S bond is quite strong, with a bond dissociation energy of roughly 60 kcal mol\(^{-1}\) compared to ~80 – 100 kcal mol\(^{-1}\) of alkyl C-H and C-C bonds. The structure of the disulfide functional group is described by its χ\(_{3}\) dihedral angle (χ\(_3\) in cystines) within the C\(^\alpha\)-S\(^\gamma\)-S\(^\gamma\)′-C\(^\beta\)′ sequence of atoms, with a ground state configuration of ±90°. Deviation from this value results in strain of the disulfide system. The value of ±90° is unusual, especially in the context of peptides, where side-chain dihedral angles tend to adopt trans/anti (180°) or gauche (±60°) conformations. Disulfides also possess axial chirality, where a positive dihedral angle indicates a P (plus) or right-handed helix, while a negative angle indicates an M (minus) or left-handed helix. Examination of the S-S stretching region using Raman spectroscopy offers structural insights into the magnitude of the dihedral angle in disulfide compounds.

Naturally Occurring Disulfide-Bridged Peptide Bicycles

Ulithiacyclamides

![Ulithiacyclamides](image1)

Ulithiacyclamide A (1)   Ulithiacyclamide B (2)

Ulithiacyclamide E (3)   Ulithiacyclamide F (4)

Ulithiacyclamide G (5)

**Bicycles**

Ulithiacyclamides

Fig. 1 Structure of Ulithiacyclamides.
Ulithiacyclamides (Fig. 1) are a class of DBPBs isolated from *Lissoclinum patella*, a species of marine tunicate. Ulithiacyclamide A (1), isolated in 1980, is characterized by a symmetrical dimeric structure consisting of oxazoline and thiazole rings in addition to a transannular disulfide. Ulithiacyclamide B (2), discovered in 1989, is similar in structure to ulithiacyclamide A, but with one of the two D-Leu residues substituted with D-Phe, resulting in an asymmetric dimeric structure. The isolation of ulithiacyclamides E (3), F (4) and G (5) was reported in 1990, and all three compounds are related in structure to ulithiacyclamide B, but with either both (E) or just one of the two (F and G) oxazoline rings existing as their hydrolyzed L-Thr counterpart. As ulithiacyclamides A and B were the earliest discovered among the ulithiacyclamides, in-depth studies to date have focused on the structure and activities of these two compounds.

Temperature dependence of the amide protons and nuclear Overhauser enhancement between backbone and side-chain protons demonstrated that in non-polar solvents such as CDCl₃ and C₆D₆, ulithiacyclamide A adopts a Type 1 conformation. However in a polar aprotic solvent such as (CD₃)₂SO, ulithiacyclamide A adopts a Type 2 conformation instead. Energy minimization calculations were carried out with a molecular mechanics force field by inputting torsional angles derived from NMR to yield models of the two conformational structures. As observed by VT NMR, all four amide protons are directed toward the interior of the macrocycle in type 1, while in type 2 the amide protons adjacent to the thiazole are directed toward the solvent. Moreover, the disulfide adopts an M helical conformation in type 1, while it is a P helix in type 2.

The most intriguing aspect of this work is apparent when compared to the solution structure of the ascidiacyclamide. Ascidiacyclamide (9, Fig. 2) has a similar macrocyclic backbone to the ulithiacyclamides, but does not contain the transannular disulfide bond. In contrast to ulithiacyclamide A, ascidiacyclamide adopts a type 1 structure irrespective of solvent. This suggests that the disulfide in ulithiacyclamide, rather than acting as a rigidifying structural element, confers greater conformational flexibility to the molecule. The disulfide is required to access an unprecedented type 2 conformation, and the authors suggest that this key feature may be closely related to its biological activity.

**Quinoxaline bis-Intercalators**

Following the discovery of echinomycin in 1957, eighteen additional compounds have been added to this family of bisintercalator natural products. The characteristic feature of this family is the modification of the macrocyclic core with planar heteroaromatic units in a two-fold symmetrical manner, which is necessary for their activity as duplex DNA bisintercalators and is the origin of their categorization as quinoxaline antibiotics. A subset of these, namely the thioacoralines (10) (isolated from the *Micromonospora* genus) and the BE-22179 (11), triostins (12), and SW-163C (13) (all isolated from the *Streptomyces* genus) are DBPBs (Fig. 3), in contrast to other members of the family, which are either monocyclic or bridged by a thioacetal (as in echinomycin (14), Fig. 4).
The discovery of the bisintercalator natural products has inspired the preparation of many synthetic analogues, including the 
aza[14] and oxathiocoralines,[15] which replace the backbone thioester with the amide and ester functionalities, and the 
aza-triostins,[16] the ester to amide analogues of the triostins.

[Image]

**Fig. 4** Structure of echinomycin, a quinoxaline bis-intercalator with a transannular thioacetal.

In a study comparing the DNA binding of triostin A (12) and echinomycin (14), it was determined that the nature of the transannular linker influences DNA binding specificity.[17] Triostin A and echinomycin differ only in the identity of the transannular linker, and triostin A with its transannular disulfide displays a binding preference for AT-rich sequences, while echinomycin with its transannular thioacetal binds preferably to GC-rich sequences.

NMR studies on triostin A have revealed that in CDCl₃, the molecule exhibits slow interconversion between two conformations (approximately 1:1),[18] attributed to cis/trans isomerization of the Ala-MeCys amide. However, upon addition of adenine to the NMR sample, the triostin A-dinucleobase complex favours the all-trans conformation of triostin A, therefore implicating all-trans as the active DNA binding conformation.[19]

**Malformin**

[Image]

**Fig. 5** Structure of Malformins.

Malformins represent another class of DBPBs isolated from the fungus *Aspergillus niger*. They are cyclic pentapeptides that are characterized by the general sequence cyclo-[ccV-(i/l/v)-(i/l/v/L)]. The vicinal d-Cys residues form the characteristic disulfide bridge, a key feature of this class of molecules. Many members of this family have been reported, differing only by variation of two of the three branched-chain amino acids (15-22, Fig. 5).

Malformin A₁ (15) was first isolated in 1961, named for its ability to induce the curvature and malformation of bean plants and corn roots.[20] However, its amino acid connectivity was only correctly determined over a decade later, when it was established that the initially proposed structure of cyclo-[d-Cys-L-Val-d-Cys-L-Leu-l-Ile] was actually cyclo-[d-Cys-d-Cys-L-Val-d-Leu-l-Ile].[21] Since then, malformin A has been shown to possess antibiotic activity[22] and inhibit mitosis and the pre-mitotic phase in animal cultures.[23,24]

The eight-membered ring consisting of the atoms from the disulfide of the vicinal d-Cys residues is a particularly noteworthy feature of the malformins because the energetic barrier to cis/trans-isomerization of the d-Cys-d-Cys amide could be reduced by the eight-membered ring strain. Theoretical calculations[25,26], IR studies[27], and x-ray crystallography[28,29] of l-Cys-l-Cys disulfides suggest that the amide in the dipeptide is cis, but it was unknown if this is maintained in bicyclic systems such as the malformins. Several studies have been published on the conformation of malformin A using a combination of CD,[30] NMR,[31,32] and Raman spectroscopy.[33] Because of an incorrect proposed structure, these experiments were later revisited[34,35] when the revised structure was reported. In particular, exchange experiments with D₂O revealed that the d-Cys-l-Cys amide is not exchanged and is the only amide that is buried within the interior of the molecule.

Mitra et al. considered six different conformations of Malformin A that were in agreement with the torsion angles generated from previously reported NMR data. These six conformations were grouped into three pairs in which both M and P-helical models at the disulfide were considered. The first pair contains a cis-configured d-Cys-d-Cys amide (authors denote M1 and P1), while the remaining two pairs are in the trans-configuration (authors denote M2, P2, M3, and P3). Of the six conformations, the trans P3 conformation was found to be most energetically stable, which is in contrast to the cis-amide configuration seen in isolated l-cystine dipeptides. The authors attribute this to fewer bond angle distortions (V₉, N-C-C' bond angle) and fewer non-planar deviations (V₅) in the trans peptide conformers than in the cis-containing conformers.

Hall et al. performed a similar analysis in which they considered both M and P-helical chirality at the disulfide but only trans-configuration at the d-Cys-d-Cys amide. Analysis of the CD data suggests that the disulfide exists as a P helix, which is in accordance with the conclusions made by Mitra et al. Additionally, the eight-membered disulfide ring is highly strained, apparent in the amide torsion angle within the ring that deviates significantly from planarity (160° vs. planar 180°), which is in accordance with previous reports on the ease of Malformin A reduction at cystine[36] and the frequency of the S-S vibration.[37]

**Sunflower Trypsin Inhibitor-1**

Sunflower trypsin inhibitor (SFTI-1, 23, Fig. 6) is a 14-residue DBPB isolated from sunflower seeds in 1999.[38] SFTI-1 inhibits β-trypsin with a subnanomolar Kᵅ of 0.1 nM, with great selectivity...
over related serine proteases such as chymotrypsin, elastase, and thrombin. Its structure was characterized by NMR spectroscopy and by co-crystallization with bovine-β-trypsin, and is one of the smallest naturally-occurring plant protein inhibitors. Its total synthesis was reported in 2001.\(^{19}\)

**SFTI-1** shows both sequence and conformational homology with the trypsin-reactive loop of the Bowman-Birk family of serine proteinase inhibitors, but unlike other members in the family, it is monofunctional, bicyclic, and significantly shorter (14 vs 60 – 70 residues). The amino acid sequence of SFTI-1 is cyclo-[GRCTKIPICFPD], and its structure is characterized by two anti-parallel β-strands connected on one end by an extended loop region, and by a hairpin turn at the opposite end. The strands are further constrained by a disulfide, dividing the macrocycle into nine-residue and five-residue loop regions, known as the reactive loop and cyclic loop, respectively. The Ile-Pro amide adopts a cis conformation which produces a sharp turn in the peptide backbone. There have been extensive studies on the synthesis of SFTI-1 analogues, including the preparation of a bifunctional SFTI-1 derivative based on the combination of a dual reactive loop and selenocysteine analogues.\(^{42}\) In the latter study, although Guo et al. found that the seleno-analogue had lower activity, they also established that the disulfide is necessary for SFTI-1 activity when a Cys to Ser mutant peptide completely abolished activity. Redox-stable analogues in which the disulfide was replaced with an alkyl or alkenyl linker, or by a triazole have also been prepared, but these did not display improved potency compared to the native disulfide.

Studies on replacement of certain residues of SFTI-1 to improve activity have led to mixed results. A report by Li et al. looked at the effect of replacing the residues at positions 2, 5, 10, and/or 12. While the authors did not find a more potent inhibitor, they found a derivative (Ile10 to Gln) that was over 1000-fold more selective for matriptase than for thrombin. They also investigated the effect of homocysteine at the disulfide linker or a methylene dithioether bridge but neither compound was more effective than SFTI-1.\(^{45}\) A separate report by Fittler et al. looked at the replacement of SFTI-1 residues with triazolyl side chains, and their most potent analogues have actually abolished the N-to-C-terminal cyclic scaffold to achieve selective and improved inhibition of matriptase compared to SFTI-1. Most notably, all of the analogues that were prepared still retain the cystine, underscoring the importance of the disulfide constraint.\(^{46}\)

**Romidepsin**

Romidepsin (24, Isthodax\(^*\), codenamed FK228 and FR901228, Fig. 7) is a DBPB marketed by Celgene Corporation and approved by the FDA for the treatment of cutaneous T-cell lymphoma (CTCL). The compound was isolated in 1994 from *Chromobacterium violaceum* obtained in a soil sample, and demonstrated antitumor activity in mice.\(^{47,48,49}\) The total synthesis of romidepsin was first reported in 1996.\(^{50}\)

Romidepsin acts as a prodrug, where reductive cleavage of the disulfide by glutathione yields the active monocyclic dithiol.\(^{51,52}\) One of the released thiol groups (position 13) forms a reversible complex with the active site zinc atom in the binding pocket of Zn-dependent histone deacetylases (HDAC),\(^{53}\) with stronger inhibition of class 1 HDACs (HDAC1 and HDAC2) over class 2 HDACs (HDAC4 and HDAC6). HDAC inhibition prevents deacetylation from N-terminal Lys residues of histones, maintaining the transcriptionally active chromatin state.\(^{54}\)

While romidepsin is only active in its reduced form, administration of the reduced drug results in rapid inactivation in serum, likely due to sequestration by serum proteins. Interestingly, an SAR study revealed that the role of the d-Cys thiol is only to act as a protecting group for the Zn-binding thiol, as masking the Zn-binding thiol as a thioester (enzymatically hydrolyzed in vivo) still yields an active compound.\(^{55}\) However, a macrocyclic scaffold, whether constrained via backbone or disulfide\(^{56}\) cyclization, remains essential for activity.

There are several structurally related DBPBs to romidepsin that all contain a macrocyclic depsipeptide scaffold bridged by a disulfide between a 3-hydroxy-7-mercaptohept-4-enolic acid and d-Cys residue. These include the spiruchostatins (A – D)\(^{57}\) isolated from the *Pseudomonas* genus, and thailandepsins (A – F, some of the members were formerly known as the burkholdacs)\(^{58}\) isolated from *Burkholderia thailandensis* E264 (spiruchostatin A (25) as representative, Fig. 7).\(^{59}\) Collectively, these compounds...
are biosynthesized by a hybrid “assembly-line” of nonribosomal peptide synthetase-polyketide synthase systems, and in particular, a few of the thailandepsin analogues were discovered by genome mining for sequence homology with the romidepsin gene cluster. As a consequence of the structural similarity to romidepsin, all of these compounds also exhibit HDAC inhibition through Zn-binding, speaking to the importance of the conserved disulfide-bridged bicyclic scaffold in conferring biological activity.

**Contrypphan-Vn**

Contrypphan-Vn (26) is an interesting example of a disulfide-constrained macrocycle. While contrypphan-Vn does not exist as a head-to-tail cyclic peptide, Asp and Lys side chains within the compound are in close proximity such that they form a salt bridge, approximating the molecule as a DBPB. Isolated from the marine snail *Conus ventricosus* in 2001 and shown to be a modulator of Ca-dependent K channels, the sequence of Contrypphan-Vn is H-Gly-Asp-Cys-Pro-0-Trp-Lys-Pro-Trp-Cys-NH₂ with a disulfide between the Cys residues (Fig. 8a). The structure of Contrypphan-Vn shows two chain reversals, with a type IV turn between Gly1 and Lys6, as well as a type I β-turn between Lys6 and Cys5.62

![Contrypphan-Vn (26)](image)

The RP-HPLC profile of Contrypphan-Vn suggests that it exists in an equilibrium between two stable conformers, attributed to the cis-trans isomerization of the Cys3-Pro4 amide, favoring the cis configuration as determined by NMR (cis:trans = 7:1, 298 K). The stability of the cis conformer was attributed to the presence of the Asp2-Lys6 salt bridge, observed through specific nOe’s between the Asp and Lys side chain protons.63 Additionally, molecular dynamics simulations in vacuum show that the indole of Trp8 is sterically limited in motion by the Asp2-Lys6 salt bridge, indicating that Trp8 acts as a hydrophobic shield to protect the salt bridge from solvent (Fig. 8b). To evaluate the role of Trp8 as a hydrophobic shield, a Trp-to-Ser mutant was prepared. [W8S]Contrypphan-Vn displayed a markedly different cis:trans ratio for the Cys3-Pro4 amide of approximately 1:1 at 298 K.64 Additionally, the Asp2-Lys6 salt bridge is absent in [W8S]Contrypphan-Vn, highlighting the fundamental role of Trp8 in the preservation of the ionic interaction. Thus, the structure of contrypphan-Vn represents a fine-tuned balance in which Trp8 stabilizes an Asp2-Lys6 salt bridge, which in turn reinforces a cis-amide configuration at Cys3-Pro4.

**Synthetic Disulfide-Bridged Peptide Bicycles Inspired by Nature**

Synthetic chemists have also explored the possibility of improving upon nature’s peptidic scaffolds by generating bicyclic variants of monocyclic compounds. This approach typically starts with disulfide-constrained peptides and linking free N- and C-termini to generate DBPBs. Researchers have been able to discover compounds with even greater potency than the peptides from which they were derived, but these successes require a comprehensive understanding of the original peptide configuration such that an appropriate linker is chosen prior to head-to-tail cyclization in order to minimize perturbations of the active conformation. The following are a few examples illustrating this idea.

**Somatostatin**

Somatostatin is a 14-residue peptide hormone that is responsible for regulating the endocrine and gastrointestinal systems. It is a monocyclic peptide constrained by a single disulfide, with the sequence AGCKNNFWKFTFTSC. Based on the biologically active conformation of somatostatin65,66, and with the intention of conformationally constraining the somatostatin scaffold, Veber *et al*. reported the syntheses of several bicyclic analogues in the late seventies.67,68 These constrained bicyclic analogues were found to possess either comparable or improved potency relative to somatostatin (Fig. 9). The most potent of the first generation series (27) retains the –FWKT– portion of somatostatin (the authors found that d-Trp enhanced activity relative to l-Trp). The authors posited that by constraining these...
key residues in the proper conformation is key to its activity. Additionally, and perhaps unsurprisingly, the bicyclic analogues were found to be more resistant to enzymatic cleavage by trypsin than their monocyclic counterparts. To evaluate the role of the disulfide in compound III, Nutt et al. prepared non-reducible analogues by replacement of the disulfide with an ethylene unit. These carba analogues (28) demonstrated reduced potency relative to compound III, as well as a reduced duration of action at comparable doses. CD spectra of the analogues suggested a different conformation than for compound III, where a weaker CD band at 200 – 220 nm for the analogues point to a less rigid conformation of the peptide backbone.

To further optimize compound III, Veber et al. investigated replacing the Cys-Aha-Cys portion with a variety of known dipeptide turn-inducing elements to yield cyclic hexapeptides with two β-turns and ultimately improving the ligand efficiency of the resulting compound. Cyclo[Pro-Phe-6-Trp-Lys-Thr-Phe] (29) was identified as a slightly more potent analogue compared to 27 and with a similar duration of action. A later study revisited these results by looking at replacement of the Phe-Pro with a vicinal cystine bridge to generate bicyclic analogues of the cyclic hexapeptide. Modelling demonstrated that a vicinal disulfide could adopt a similar cis-amide configuration of the Phe-Pro dipeptide, suggesting that a cystine could potentially act as an effective surrogate. Gratifyingly, the resulting cystine analogues (30, 31) proved to be two- to ten-fold more potent than the Pro-Phe hexapeptide analogues, which the authors attribute to an increased preference for a cis-amide configuration at the disulfide-containing eight-membered ring. Indeed, the cis-amide topology of the vicinal disulfide was established by a ROESY experiment, where nOe’s were observed between the two Ca-protons of the two Cys residues.

Conotoxins are a class of venomous compounds isolated from marine snails of the Conus genus used for the rapid immobilization of prey. They range from 12 – 30 residues long and target membrane receptors with great potency and selectivity, and have proven to be useful neurophysiological probes and drug leads. They are characterized by extensive disulfide bond networks within their secondary structure, containing up to four disulfides linked in a specific manner. In particular, Clark et al. investigated the possibility of head-to-tail cyclization of MI, a 16-residue conotoxin from Conus magus, in order to improve its biological stability.

The authors prepared three head-to-tail cyclized analogues, termed cMI-5, cMI-6, and cMI-7, where the number refers to the number of A/G residues added to the N-terminus of the peptide to act as a linker between the N- and C-termini (Fig. 10). Structural and biological characterization revealed that the cMI-6 and cMI-7 analogues resemble the structure of MI, and this is reflected in their similar activity profiles as well. In addition, the cMI-6 and cMI-7 analogues displayed increased stability against cleavage by a protease, EndoGluC, compared to MI. However, the five-residue linker in cMI-5 proved to be too short and as a result. Its structure was severely perturbed from MI and this is reflected in the loss of activity of cMI-5. These studies collectively illustrate that a thorough understanding of conformation is required before backbone cyclization is undertaken in order to preserve the potency and activity of the starting peptide.

**Synthetic DBPBs to Explore Methods to Understand Conformational Properties**

As part of our research program, we have been interested in exploring the types of macrocyclic topologies that can be accessed using aziridine aldehydes and isocyanides to stitch together peptide termini. The initial report by Hili et al. in 2010 described the use of amphoteric aziridine aldehydes and isocyanides in a multicomponent reaction with peptides to generate peptide macrocycles.

We were interested in exploring if this technology could be used to make disulfide-bridged bicycles, where incorporation of two Cys residues within the peptide sequence and subsequent late-stage oxidative deprotection following macrocyclization would yield the DBPB architecture. Gratifyingly, we have successfully prepared a series of DBPB ranging from five to eight amino acids (32-34, Fig. 11). These compounds contain an azide functionality, introduced through aziridine ring-opening, which could potentially act as a handle for click chemistry.
The disulfide-containing cyclic octapeptide cyclo(CGPF)$_2$ (35, Fig. 12a) was prepared by Kopple et al. to understand its conformational properties and intramolecular mobility by NMR. The authors describe the use of proton rotating frame relaxation ($T_{1\rho}$) as a reporter of internal peptide motion. Additionally, the authors reported a crystal structure for cyclo(CGPF)$_2$, which is characterized by two Pro-Phe $\beta$-turns. One of these is a type I, and the other is type II, resulting in an asymmetric structure. Furthermore, the dihedral angles at the two Cys residues are different, reinforcing the asymmetrical structure. In contrast, the solution NMR structure exhibits $C_2$ symmetry, with temperature-dependent peak broadening corresponding to fast exchange between the two halves of the molecule that appear as conformationally distinct in the crystal structure. Proton $T_{1\rho}$ measurements indicate that the internal motion of the peptide has two components — the first involves movement of the disulfide and adjacent groups, while the second involves rotation of amide planes.

Another study looked at the NMR conformations of decapetide DBPBs compared with their monocyclic counterparts. For monocycle cyclo-[IAYPGHDSPG] (36), amide Ser8-Pro9 was found to exist as a mixture of cis/trans configurations with a ratio of 30/70 (Fig. 12b). However, in the bicyclic disulfide variant cyclo-[ICYPGHCSPG] (37), NMR reveals a trans only configuration at Ser8-Pro9. This demonstrates that transannular disulfides are capable of rigidifying macrocyclic structures by favouring one conformer over another.

However, this example should not be interpreted as a steadfast rule, as we have also discussed ulithiacyclamide in which the presence of a disulfide allows access to an otherwise inaccessible conformation, suggesting an increase in flexibility of the macrocyclic system. While the effect of a disulfide bridge on bicyclic conformations has not yet been thoroughly explored, Rabenstein et al. have looked at how disulfide bridged monocycles can influence peptide conformation. A collection of acyclic dithiol and cyclic disulfide tetrapeptides (Ac-CPXC-NH$_2$) were prepared and evaluated for cis/trans isomerization about the Xaa-Pro amide, and it was found that the disulfide-constrained variants underwent cis/trans isomerization at a two- to 13-fold enhancement compared to their linear dithiol counterparts (Fig. 13).
Additionally, Rabenstein et al. discovered that the cyclic variants display periodicity with respect to the number of residues in the monocyle. In a study looking at cyclic disulfide peptides of various lengths (Ac-Cys-Pro-(Xaa)ₘ-Cys-NH₂, m = 0 – 4) where an odd number of residues between the cysteines dramatically increases the %cis conformation at the Cys-Pro amide bond (Fig. 14). In concert, these findings demonstrate rather counter-intuitively that conformational flexibility can be engineered into macrocycles through disulfide incorporation, and should be taken into account when considering bicyclic systems.

### Identifying Active Disulfide-Bridged Peptide Bicyclic Scaffolds

From the aforementioned examples, it is apparent that existing DBPBs possess interesting structural and biological features. However, a question that remains is how we can discover novel DBPBs as potential therapeutic agents. Recent studies have demonstrated the power of selecting for function from compound libraries, whether derived from synthesis or from phage display.

Kasher et al. proposed a synthetic library approach where, given the structure of a peptide to target protein interaction, modelling of binding site residues and constraining by backbone cyclization can yield compounds with μM binding affinities. As proof of concept, the researchers investigated the bovine pancreatic trypsin inhibitor (BPTI):trypsin complex, where the binding region is defined by seven residues on BPTI (-Pro13-Cys-Lys-Ala-Arg-Ile-Ile19-). Additionally, Cys 14 forms a disulfide with a distal Cys38. Using this as a starting sequence, the authors joined the Arg-Gly amide nitrogen to the C-terminus of the distal Cys to create ring A of the bicycle, and linked the N-terminal Pro of the seven-residue peptide with the N-terminus of the distal Cys to create ring B of the bicycle, using alkyl chain linkers of variable lengths (Fig. 16).

This backbone cyclization approach ultimately yielded a 20-member DBPB library with Kᵢ values in the ten- to hundred-μM range. However, the authors noted that as these are only leads, optimization of these structures could yield improved binding profiles. A more in-depth study of one particular binder yielded a low-resolution x-ray structure which showed a large difference in the spatial orientation of the disulfide when compared to the native BPTI:trypsin interaction. The orientation of the disulfide is integral to position the Lys-Ala of BPTI for maximum inhibition, and the authors suggest that future iterations to optimize this in the synthetic peptide could improve its binding affinity to trypsin.
When structural information of a ligand-protein interaction is unavailable, phage display can be used as a method to elucidate the sequence of a potential binder. Phage display is a powerful method that links the binding properties of a phage-presented peptide to the DNA that encodes for it, with over $10^{9}$ theoretical members within a phage display library that represent combinations of all naturally-occurring amino acids. Disulfide-constrained peptides can be encoded into a phage library to obtain sequences that mimic a cyclic topology, and in combination with peptidomimetic chemistry, an even greater degree of structural diversity can be screened for activity as a potential ligand. Encapsulating this idea, Dias et al. elaborated upon Fc-III (38), a disulfide-constrained monocyclic peptide discovered by phage display, by generating two backbone-cyclized derivatives, capped by the D-Pro-L-Pro β-turn inducing element (Fig. 17). In the first derivative, FcBP-1 (39) truncates the N- and C-terminal of Fc-III and replaces the disulfide with D-Pro-L-Pro to yield a monocyclic macrocycle. For the second, FcBP-2 (40) extends the full-length Fc-III with the D-Pro-L-Pro cap to yield a DBPB. While FcBP-1 demonstrated poor binding affinities for the Fc domain, FcBP-2 exhibited a ten-fold improvement in IC$_{50}$ relative to that of Fc-III. Additionally, reduction of FcBP-2 to the dithiol resulted in a ten-fold drop in IC$_{50}$ relative to the disulfide, suggesting that the disulfide is integral in constraining the peptide in its biologically active conformation.

Phage libraries can even be encoded with more than one pair of Cys residues. Chen et al. investigated phage libraries displaying peptides of the format X$_{m}$CX$_{n}$CX$_{o}$CX$_{p}$, where a fourth Cys residue can appear in any of the (m + n + o + p) randomized positions to yield peptides containing two cystines. Depending on where the fourth Cys is randomized, different peptide topologies could be obtained. Screening of these libraries against streptavidin and a serine protease uPa yielded enriched libraries that were heavily biased toward bis-cystine scaffolds, after only two rounds of phage panning.

Since a given cysteine can form a disulfide with any of the other three Cys residues, three other bis-cystine topologies can be obtained, but intriguingly, these topoisomers did not hinder phage display selections as hits were still obtained. As a demonstration of binding, the authors were able to obtain a crystal structure of one of their bicyclic peptides (UK504, 41) in complex with uPa (Fig. 18). Consistent selection for DBPBs demonstrates the viability and utility of this class of molecules as modulators of protein-protein interactions.

**Conclusions**

The transannular disulfide bridge within DBPB scaffolds have been shown to have nuanced but integral effects on the conformational properties of the resulting peptides. While often assumed to be a labile functional group, these studies have demonstrated that overall peptide stability is more influenced by the presence of free termini and susceptibility to hydrolytic cleavage by proteases rather than disulfide reduction. In addition, the disulfide variant of these bicyclic scaffolds, when compared to other analogues to replace the disulfide, often possesses greater activity. This is apparent in SFTI-1 and somatostatin analogues, where replacement of the disulfide with alkyl, alkenyl, or triazolyl linkers often results in reduced activity, speaking to the very specific geometry that the disulfide adopts.
to influence bicyclic conformation and its importance in attributing to potency.

While there are many instances of biologically active DBPB scaffolds that are naturally-occurring, there is a lack of understanding of how synthetic bicycles could be rationally designed to use the disulfide in a biologically meaningful manner. However, this is not necessarily problematic, as rational design can be cumbersome. A screening approach is much more desirable, and phage display has been established as a powerful tool to discover DBPB topologies with great structural diversity. We also have a structural understanding of conformations of DBPB and their modes of binding through NMR and x-ray crystallography, which means that synthetic chemists are poised to take phage-selected binders and develop them into even more potent molecules through incorporation of unnatural components. We therefore envision future DBPB compounds that are discovered through a synergy of biology and chemistry, by combining the high-throughput technology of phage display to obtain sequences that can then be elaborated and improved upon through synthesis.

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Notes and references

Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Citations should appear here in the format A. Name, B. Name, C. Name, Journal Title, 2000, 35, 3523. A. Name and C. Name, Journal Title, 2000, 35, 3523.


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