There is a growing interest for using macrocyclic peptides in drug discovery as they provide unique opportunities to target sites of protein–protein interactions. In this issue of ACS Central Science, Rudi Fasan and co-workers outlined an integrated phage display platform for finding nonreducible genetically encoded cyclic peptides (Figure 1) with inhibitory functions,1 which is a promising approach to find macrocyclic compounds for inhibitor development.

Macrocyclic peptides are well suited for targeting protein–protein interactions.2 Due to their constrained structures, macrocyclic peptides benefit from lower entropic penalty upon binding and often show high affinity and specificity. They further display increased stability and cell permeability in comparison to linear peptides. Well-known natural-product-derived macrocyclic compounds such as cyclosporin A have inspired research in the field.

Genetically encoded libraries of cyclic peptides are increasingly used to find macrocyclic compounds. Such libraries can be generated through a variety of approaches like phage display, mRNA display, and split-intein circular ligation.3 Bicyclic peptide phage libraries can further be generated by chemical cross-linking.4 Phage displays benefit from large library sizes and ease of experiments. Highly diverse cyclic M13 peptide-phage display libraries can be generated by designing randomized peptide sequences flanked by cysteine residues. However, the reversible nature of the disulfide bond limits the applicability of these macrocycles in an intracellular milieu. Fasan and co-workers outline a strategy for the generation of combinatorial libraries of macrocyclic peptides (macrocyclic organo-peptide hybrids or MOrPHs) constrained by a nonreducible thioether bridge (Figure 2).1

The authors used a modified version of the minor coat protein pIII of the M13 phage. The pIII protein was genetically linked to the macrocycle precursor. The precursor peptide sequence contains a cysteine residue, a variable region, and the noncanonical cysteine-reactive amino acid O-(2-bromoethyl)-tyrosine (O2beY). Given that O2beY is in close proximity to a cysteine, the peptide is cyclized through the formation of an inter-side-chain-to-side-chain thioether bridge. The noncanonical amino acid is encoded using amber stop codon suppression together with an engineered aminoacyl-tRNA synthetase and its cognate tRNA. The authors first confirmed that the linear O2beY containing peptides could be displayed on the phage and that the alkyl-bromide group O2beY was intact. They then took an affinity maturation approach to

Figure 1. Macrocyclic peptides for the win. Cyclization renders peptides more resistant to proteolysis, increases their permeability, and can augment their affinities while preserving their selectivity. This is why macrocyclic peptides represent an increasingly interesting scaffold for studying and modulating protein–protein interactions.
test if the method could be used to select for high-affinity streptavidin ligands. To this end, they generated two phage libraries that displayed a low-affinity streptavidin motif flanked by randomized sequences and an O2beY/Cys or a Cys/O2beY pair. The libraries were used in phage display selections against immobilized streptavidin, and the binding-enriched phage pools were analyzed through next-generation sequencing. Based on sequencing results, a set of ligands were selected for affinity determinations, which revealed that the affinities were in the nanomolar range.

After the initial validation, the authors employed a similar affinity-maturation approach to generate high-affinity macrocyclic peptide ligands for other bait proteins. The authors successfully identified nanomolar affinity macrocyclic ligands that compete for binding with the natural ligands. The affinity-maturation approach allowed the authors to use small library sizes for their initial validations ($10^5$–$10^6$ sequences). However, the strength of phage display is the potential of generating much larger libraries and thereby exploring a broader sequence space. The authors generated a library displaying randomized hexameric peptides with a diversity of about $10^8$ unique peptide sequences. They showed that the fully randomized library could be used to identify nanomolar concentrations of macrocyclic ligands for bait proteins. The genetically encoded randomized library could thus be used as a general platform for the discovery of high-affinity macrocyclic ligands of peptide binding proteins. The authors further demonstrated that the orientation of the thioether linkage directly affects the functionality of the macrocyclic peptides, and that the effect was different for distinct bait proteins. Hence, the possibility of encoding libraries with distinct orientation of the thioether linkage increases the versatility of the technique as compared to many other methods.

The authors’ previous studies have shown the feasibility of generating genetically encoded cyclic peptides and screening for ligands in a smaller scale. The novelty of their recent study lies in the scale up of the approach through the combination with phage display. A drawback of the approach is the relatively low phage titer. The relatively short peptide length may further limit the general applicability of the approach. Nevertheless, the genetically encoded cyclic peptide libraries could find a general use for finding macrocyclic inhibitors of peptide-binding proteins and parallel the proteomic peptide phage that is used as a general approach to chart potential endogenous ligands. This represents a large search space given that there are more than 200 known families of peptide binding domains and proteins and thousands of individual domains. It could for example be envisioned to use the MOrPH peptide phage display to find high-affinity ligands of specific E3 ligases. In a further

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perspective, identified macrocyclic peptide scaffolds could be used as a part of the development of protein degradation tags (also known as PROTACs). However, to be useful for such an application there is a need to overcome some of the main challenges in the field, such as making the macrocyclic compounds orally available and cell permeable.

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