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Current development of bicyclic peptides

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ABSTRACT

Bicyclic peptides, a class of polypeptides with two loops within their structure, have emerged as powerful tools in the development of new peptide drugs. They have the potential to bind to challenged drug targets, with antibody-like affinity and selectivity. Meanwhile, bicyclic peptides possess small molecule-like access to chemical synthesis, which is conducive to large-scale synthesis and screening. In the last five years, bicyclic peptide technology has been increasingly developed, and researchers have carried out a variety of studies to elucidate the potential functions of bicyclic peptides. With the continuous development of synthetic methods and the advances of new technology to build bicyclic peptide libraries, bicyclic peptides are now becoming widely used in the development of new drugs for various diseases. This perspective provides an overview of the structure types, synthesis and applications of bicyclic peptides in current drug development, and our own views on future challenges of bicyclic peptides.

1. Introduction

Over the last century, small molecules (molecular weight, MW < 500 Da) have been mainstream in the development of drugs for treating diverse diseases due to their stability, oral bioavailability and easy preparation [1,2]. However, their inherent small size endows them with restricted surface area to interact with targets, which severely limits their ability to effectively occupy flat and broad binding sites of targeted receptors. More than approximately one-third of drug targets have proven to be undruggable with small molecules, especially for targeting protein–protein interactions (PPIs) [3]. In addition, some binding regions or binding characteristics of small-molecule drugs are highly conserved among proteins [4], leading to the issue of low selectivity; such defects inevitably can cause various side effects. In recent decades, protein therapeutics (such as antibodies) have shown high affinity and specificity for targets, making them a significant component of the pharmaceutical market [1].

To discover novel drugs that have advantages over both small molecules and protein therapeutics, special attention has been recently given to peptide-based therapeutics. There are two types of peptides, linear peptides and cyclic peptides. In many cases, linear peptides are considered inferior drug candidates due to their poor oral absorption, instability in metabolism, and unfavorable cell permeability [5]; however, a few of them have been approved by the FDA for use as oral drugs [6,7]. After cyclization and modification, such as Cys-disulfides and thioether bridges, constrained peptides are resistant to hydrolysis by exopeptidases due to the lack of amino and carboxyl termini, making it possible to be more stable in systemic circulation [7]. Some cyclic peptides, although not all, are able to cross cell membranes [8]. Cyclic peptides possess a large surface area and size, leading to a higher binding capacity to a specific target with better selectivity compared to small molecules. Moreover, they have a smaller MW and better pharmacokinetic properties than antibodies. Inspired by the clinical success of several monocyclic peptides (ring size < 10 amino acids), cyclic peptides have been regarded as next-generation therapeutics [5,9,10]. However, as the ring size increases, monocyclic peptides become more conformationally flexible and tend to undergo proteolytic degradation by endopeptidases. In contrast to monocyclic peptides, bicyclic peptides have stronger conformational restrictions, improving their target binding ability and specificity [11].

At present, compared with small molecule compounds, the definition of bicyclic peptides has not been strictly regulated. In particular, the determination of the number of rings often easily causes confusion.

To facilitate understanding and discussion, we briefly summarized the classification basis for bicyclic peptides here.

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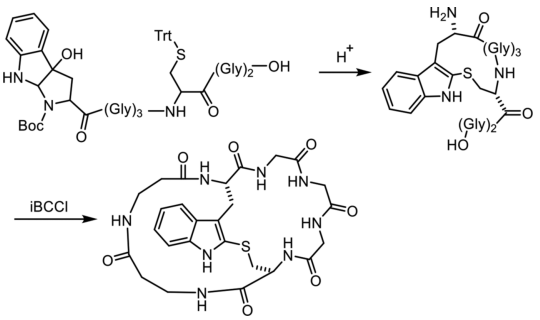


Fig. 1. First synthesis of bicyclic heptapeptides.

(1) The parent chain should have the maximum number of amino acid residues.
(2) Search down the main chain until there is a side ring. Side rings are the ring chains that are not in the parent chain but are branched off from it and back to the parent through the least number of atoms in a ring. It should be noted that the starting point of one branch chain cannot coincide with the end point of another chain.
(3) Repeat the above process until all atoms on the main chain have been retrieved.
(4) When a linear peptide has two side rings or a cyclic peptide has one side ring, we define it as a bicyclic peptide in this review.

To date, various usages of bicyclic peptides have been reported, including targeting PPI [12–15] as well as developing enzyme inhibitors [16,17], antagonists/agonists [18,19] and new tools for molecular imaging [20,21]. In recent years, bicyclic peptides have made great progress in the research and development of drugs for major diseases [22–25], and several bicyclic peptide drugs have entered clinical trials. In this review, we will summarize the advances of bicyclic peptides and present the perspectives on the strategic application of bicyclic peptides. We hope that this review will serve as a potential reference for research focusing on the development of new peptide drugs.

2. Structural types and synthesis of bicyclic peptides

In 1978, Zanotti *et al.* reported the synthesis of bicyclic heptapeptides and decapeptides. Drawing on the method of Savige *et al.*, they firstly constructed the first ring by forming a thioether bond between cysteine and oxidized tryptophan residues, and then synthesized a bicyclic peptide *via* connecting the peptide chain head to tail (Fig. 1) [26].

In the recent past, the intramolecular Savige-Fontana reaction is still used for the synthesis of bicyclic peptides. In 2018, Perrin used this method to complete the total synthesis of death-cap mushroom toxin α-amanitin (Fig. 2) [27].

After continuous development by researchers, there are already abundant methods to manage the construction of bicyclic structures. In addition to the traditional amide or ester condensation reaction, at present, researchers have developed many new methods such as native chemical ligation (NCL) [28], olefin/alkyne metathesis [29] and palladium-catalyzed C-C bond formation [30]. In 2015, Reymond and colleagues obtained cyclic peptides by cross-linking lysine and glutamate residues, bicyclic peptides are finally obtained by the reaction of cysteine residues with the N-terminal chloroacetyl group [31]. In addition, the method of aspartic acid ligation as a fairly mature method also has great potential in synthesizing bicyclic structures [32]. Among the methods for the construction of bicyclic peptides, the methods utilizing thioether bonds and disulfide bonds are particularly prominent. Cyclization *via* sulfhydryl groups is common in the peptides from natural origin [33], and this is a common strategy for artificial synthesis of bicyclic peptides. Thanks to the development of organic chemistry methodology, increasing thiol-based bicyclic peptide synthesis methods have been reported in recent years.

In 2017, Chou and his collaborators successfully applied thiol–yne/thiol–ene reactions to the synthesis of bicyclic peptides [34]. This method can be well applied to the synthesis of bicyclic stapled peptides. After their improvement, this method works well in aqueous systems. In 2019, Wu and his collaborators developed a synthetic method for topologically controlled bicyclic and tricyclic peptides [35]. Unlike other methods, this one is based on the thiol group while primed by selenoether formation. In general, as the bicyclic peptide sequence grows, the rigidity of the molecule becomes difficult to maintain, which is often detrimental for target binding. Wu and his collaborators designed a bicyclic peptide backbone structure with a conserved cysteine/proline framework, using cystine bridges and proline turns to maintain the order of the secondary structure [36]. In 2021, Chen and collaborators constructed monocyclic peptides and bicyclic peptides through palladium-catalyzed intramolecular *S*-arylation [37]. This method has good substrate adaptability and is compatible with loop structures of different sizes. In addition, the researchers completed the construction of the DNA-encoded compound libraries (DELs) through this method, verifying the potential of this method in constructing large-scale libraries. Parallel to the advancement in thiol-based bicyclic peptide synthesis methods, there were new developments in substrate-directed formation of peptide bonds. In 2019, a seminal contribution to peptide synthesis was reported by Yamamoto and coworkers. Their tantalum-catalyzed method addresses some critical issues such as racemization [38].

On the one hand, the emerging synthetic methods provide researchers with synthetic tools, and on the other hand, they also inspire and prompt researchers to explore possible bicyclic structures.

Based on the bicyclic structure, bicyclic peptides can be roughly divided into two parts, the main chain of amino acids and the connecting chain. According to the position and type of the connecting chain, they can be further divided into four types (Fig. 3A).

The first type of bicyclic peptide is the simplest motif for which a further linkage connecting two side chains (R_1 of type I, Fig. 3A) was formed inside of a head-to-tail cyclized macrocyclic peptide (type I, Fig. 3A). This type of bicyclic peptide can be successfully constructed by a two-step cyclization reaction. Native chemical ligation (NCL) was first reported as an independent method in 1994 [28]. It is an important synthesis method to construct a peptide bond between two large polypeptides, which has been widely applied for the synthesis of bicyclic peptides. Using NCL, researchers first used off-resin and on-resin to synthesize a type I bicyclic peptide library [39].

In the second type of bicyclic peptide (type II, Fig. 3A), there is a partial overlap between the two loops. When the linker is an amide bond, the structure is often similar in form to some of the first type of bicyclic peptide. The third motif is a linear backbone linking two separate macrocyclic peptides (Fig. 3A). In the synthesis of type I and type III bicyclic peptides, if the connection site is improperly designed or inappropriate protective groups are used, the second type may also appear as impurities in the reaction system. Meanwhile, there are also some methods with good selectivity can be taken into account the synthesis of the second type and third type of bicyclic peptides. In 2021, Cheung and cooperators reported a synthetic strategy for constructing diverse peptide *via* chemoselective peptide ligation [40]. It is worth noting that the benzofuran moiety was utilized as the peptide salicylaldehyde ester surrogate, and Dap–Ser/Lys–Ser dipeptide was applied as the hydroxyl amino functionality. By applying this strategy, many cyclic

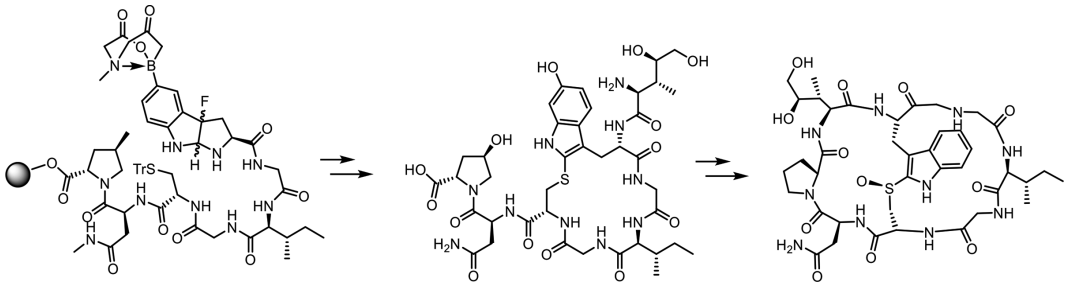


Fig. 2. Synthesis of death-cap mushroom toxin α-amanitin.

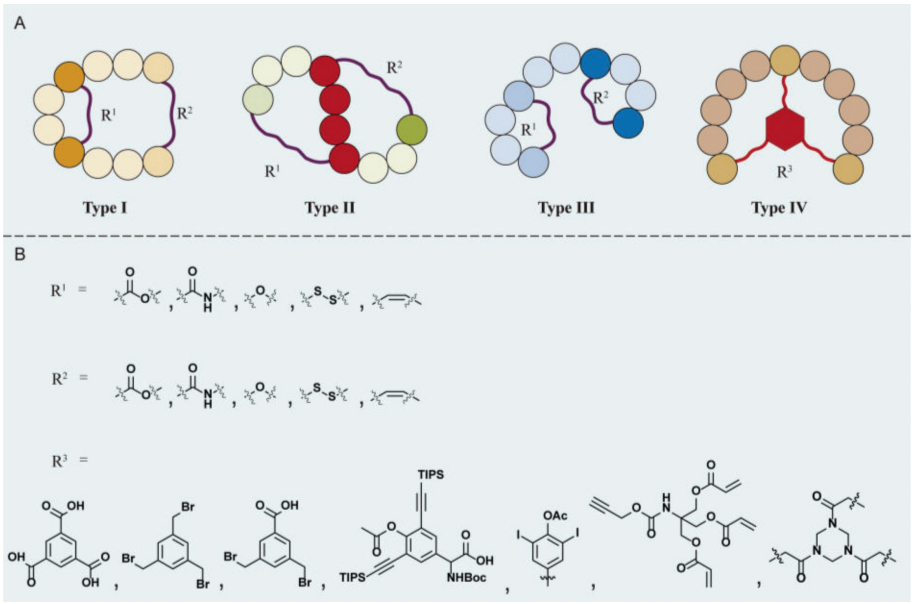


Fig. 3. Various types of bicyclic peptide structures. (A) Four common types of bicyclic peptides. (B) General forms of link chains.

peptide structures can be efficiently constructed included bicyclic peptides.

Elduque *et al.* developed a method to prepare bicyclic peptides by using maleimide and cysteine, which undergoes an intramolecular Michael-type reaction to complete cyclization [41]. By selectively deprotecting cysteines at different positions of the main chain, the type III bicyclic peptide with separated rings or the type II bicyclic peptide with partial overlap can be effectively obtained. When Ghalit *et al.* developed a synthesis method for a series of antibiotic analogs, they used olefin bridges to construct bicyclic peptides [29]. Distinguished from Liskamp's work, Cromm *et al.* developed a method using ring-closing olefin/alkyne metathesis (RCM/RCAM) to complete peptide cyclization [42]. They first used solid-phase peptide synthesis (SPPS) to introduce two α-methyl-α-alkynyl building blocks and two olefin building blocks at different positions on the peptide chain. The use of a first-generation Grubbs' catalyst leads to the selective formation of alkyne or alkene macrocycles, and a further RCM/RCAM reaction furnishes the bicyclic peptide. If the sequence of the two building blocks on the main chain is changed, this method can also be successfully applied to obtain the fused type II bicyclic peptide.

The fourth type of bicyclic peptide involves small molecule compounds that are used as a backbone to connect the peptide chain to form a handcuff-shaped structure (type IV, Fig. 3A) [25,30,43–46]. More conveniently, there are a variety of choices for small molecules (Fig. 3B), such as tris-(bromomethyl) benzene [43] and 3,5-bis(mercaptomethyl)benzoic acid (BMB) [25]. Since the construction of the fourth type is relatively fast, convenient, and more suitable for the rapid synthesis of a large quantity, it has been widely used in the construction of bicyclic peptide libraries. In 2014, Lavilla *et al.* developed a method to synthesize bicyclic peptides by using an intramolecular palladium-catalyzed C–H activation reaction [30]. The method utilized the palladium-catalyzed coupling reaction between the iodine-substituted aryl of an amino acid in the polypeptide sequence and the indole ring of the tryptophan residue to complete the construction of the bicyclic ring. Click chemistry has also been reported to synthesize type IV bicyclic peptides. Zhang *et al.* applied Ru(II)-catalyzed click chemistry to synthesize a hexapeptide to simulate the structure of vancomycin [44].

At the same time, studies on multi-cyclic peptides, such as tricyclic peptides, are also emerging. Similar to bicyclic peptides, tricyclic peptides achieve a more restricted 3D conformation by reducing the flexibility of the peptide. In general, adding appropriate conformational constraints can improve the binding ability of peptides to targets, which means that tricyclic peptides may have greater advantages and potentiality, but it also brings greater difficulty in the research of de novo design and more investment.

With the increasingly improved construction methods of bicyclic peptide libraries, it is possible to quickly obtain a large number of bicyclic peptide molecules, which is undoubtedly beneficial to peptide drug discovery. However, after finding the lead bicyclic

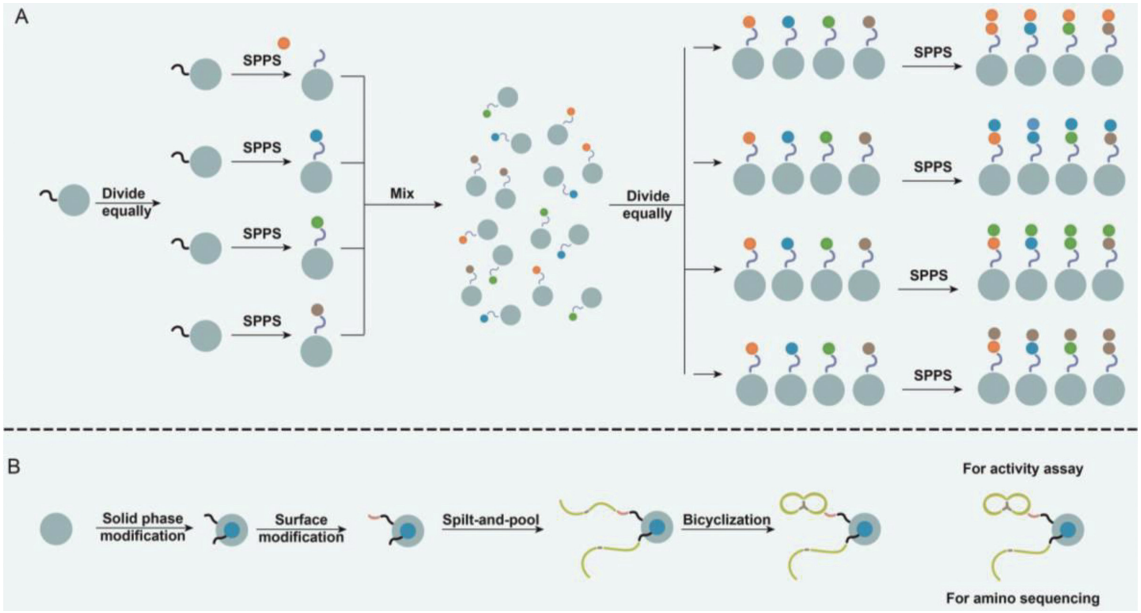


Fig. 4. (A) Split-and-pool. This method consists of four steps: (1) Divide the solid phase carrier into several parts; (2) coupling one different amino acids block to each part by solid-phase peptide synthesis (SPPS); (3) mix the compounds; (4) repeat 1–3 steps until peptide chains of the target length are obtained. (B) OBTC, modify the carrier microspheres (usually using H_2O and DCM/ether) to contain two layers, then the outer layer is selectively modified. The SPPS method is used to synthesize the same sequence on the inner and outer layers, and only the outer layer is cyclized. The outer layer of the microspheres thus obtained is connected with bicyclic peptides for activity testing, and the inner layer contains linear peptide sequences for sequencing.

peptide, further conformational restriction and modification may be a more efficient method for designing of multi-cyclic peptides. This review, however, mainly focuses on the progress of bicyclic peptides, other multi-cyclic peptides will not be described in detail herein.

3. Synthesis of bicyclic peptide libraries

The chemical synthesis technology developed for the synthesis of a single molecule has difficulty meeting the needs of large-scale screening of bicyclic peptides. With the demand for large-scale screening being gradually increased, the synthesis technology of bicyclic peptide libraries has emerged. According to its technical basis, the construction of bicyclic peptide libraries can be categorized as chemical synthesis and biosynthesis.

3.1. Chemical synthesis of bicyclic peptide libraries

The first bicyclic peptide library was established by Sun *et al.* in 2001 [39], which contains nine different bicyclic peptide molecules. Radioactivity detection-HPLC (RD-HPLC) and matrix-assisted laser desorption/ionization-MS (MALDI-MS) methods were applied to identify and analyze each molecule. Their work created a precedent for the construction of a bicyclic peptide library, but at the same time, there were also some problems in the complicated synthesis process, including a lack of diversity and difficulties in molecular identification.

The diversity of peptides in the library and the identification of cyclic peptides were the two primary problems faced by peptide molecular libraries in the application.

In 1984, Fodor *et al.* utilized 96-well plates to synthesize thousands of peptides, which were applied to form a peptide library based on permutation and combination [47]. In 1982, Furka *et al.* developed the split-and-pool method (Fig. 4A) [48], which greatly improved the synthesis efficiency of compounds. This method has become the mainstream choice for the chemical synthesis of bicyclic peptide libraries. Through this method, researchers can efficiently obtain a huge variety of peptide libraries.

Researchers made use of the one-bead-one-compound (OBOC) method to synthesize peptides earlier [49]. However, in the synthesis of cyclic peptides, due to the existence of the cyclic structure, it was difficult to use the Edman method or mass spectrometry for structural identification. Lam *et al.* pioneered the one-bead-two-compound (OBTC) technology based on modified microbeads (Fig. 4B) [50]. Through modification, the microbeads could be divided into two layers. This technology enabled the experimentally obtained microspheres to provide peptides with testing activity on the surface, and there were linear peptides inside the microspheres for sequencing. This technology has been applied to synthesize cyclic peptides [51].

The use of DNA-encoded compound libraries technology to screen for potential compounds is one of the most frontier technologies for new drug discovery, and has received intense attention from academia and industry. For peptides containing non-proteinogenic amino or other highly diverse chemical structures, DEL technology has good potential. As early as 2004, Liu and collaborators used DNA-Templated Organic Synthesis to complete the DEL construction of cyclic polypeptides [52]. It is worth mentioning that most of the amino acids are non-proteinogenic.

The realization of this structural diversity gives researchers more inspiration. For example, inspired by polyketide and mixed peptide-polyketide natural products, Gillingham and coworkers constructed cyclic peptide libraries with rich backbone diversity [53]. In 2018, scientists from GSK successfully applied this technology to the screening of cyclic peptide inhibitors against VHL and RSV N protein [54].

Recently, the improved technology of DEL called YO-DEL has also been applied to the synthesis of cyclic peptide libraries [55], and it has been fused with new cyclic peptide synthesis methods [37].

In general, although there are few reports on the application of DEL technology in the literatures of construction of bicyclic peptide libraries, as a technology that can greatly improve the struc-

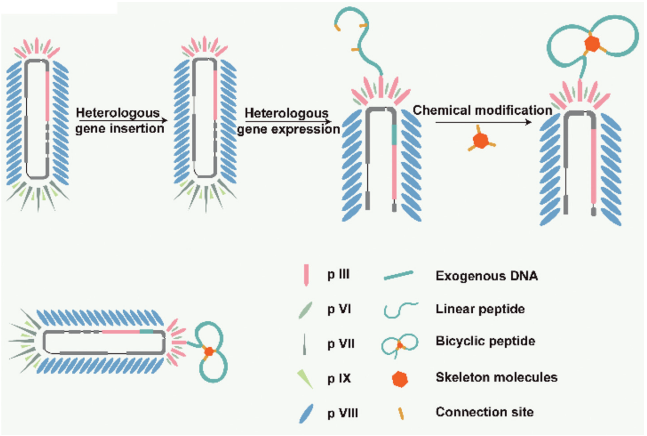


Fig. 5. Bicyclic peptides synthesis based on phage display technology. By modifying the G3P gene of filamentous bacteriophages, a specific peptide chain can be expressed on the PIII protein. Then the bicyclic structure is construed by chemistry modification.

tural diversity of drugs, DEL has great application potential in the synthesis of bicyclic peptides and in peptide drug development.

3.2. Biological synthesis of bicyclic peptide libraries

Bicyclic peptides can be synthesized biologically through ribosomal synthesis, including phage display [56], mRNA display [57], split-intein circular ligation of peptides and proteins (SICLOPPS) [58], and other processes. Among them, phage display has emerged as one of the most important approaches for the synthesis of bicyclic peptides, and the filamentous bacteriophage "M13" is the most commonly used for this application. In general, as shown in Fig. 5, the corresponding DNA sequences of linear peptides containing three residues are inserted into the phase's gene sequence near gene III, which encodes the filamentous coat protein pIII. After expression, the target peptides are displayed on the phage coat protein pIII. The linear peptides are subsequently cyclized by reaction with trivalent agents, such as 1,3,5-tris(bromomethyl)benzene (TBMB).

Interestingly, Camarero *et al.* developed a new method for constructing a library of intracellular cyclic peptides. This method allows researchers to utilize NCL in combination with a modified protein-splicing unit or intein to complete the synthesis of cyclic peptide in cells [59]. In 2009, they successfully applied this method to the synthesis of sunflower trypsin inhibitor-1 (SFTI-1) and a small library containing multiple Ala mutants [60]. In addition, Suga and colleagues reprogrammed genetic code to enable unnatural amino acids to be added to bicyclic peptide sequences *via* modified mRNA display [61].

These reports made us realize that the construction of bicyclic peptide libraries *in vivo* is completely feasible and increasingly mature.

4. Research progress on natural bicyclic peptides

Natural products have always been a vast source of novel structures and a continuous inspiration to advances in modern medicinal chemistry. Recent studies have found that bicyclic peptides have a wide distribution in nature (Scheme 1).

Theonellamide F derived from a marine sponge (Theonella) has shown good cytotoxicity and the ability to inhibit the growth of a variety of fungi [62].

BI-32169 is a bicyclic peptide (DSM 14996) isolated from *Streptomyces* sp., which has a moderate antagonistic effect on human glucagon receptors in functional cell-based tests [63].

Some of the natural polypeptides isolated from living organisms have delicate ring structures and diverse biological activities. Meanwhile, defensive peptides isolated from mammals have also been shown to have antibacterial and antiviral activities, such as rhesus theta-defensin (RTD-1) and retrocyclin-1 [64]. Trulance is an endogenous gastrointestinal (GI) peptide produced by the human body that has activity against chronic idiopathic constipation. It was approved for marketing by the FDA in 2017. In 2018, Matinkhoo *et al.* reported a synthesis of α-amanitin [27]. α-Amanitin is a potent inhibitor of RNA polymerase II isolated from *Amanita phalloides*.

In 2018, Kim *et al.* isolated and identified six Seongsanamides from the culture broth of *Bacillus safensis* KCTC 12796BP, including four bicyclic peptides and two monocyclic peptides. Interestingly, when studying their biological activity, the researchers found that although these peptides contain similar sequences, only the bicyclic peptides had antiallergic activity. The antiallergic activity of Seongsanamides A was even comparable with that of fexofenadine-HCl (Fexo) [65].

Another natural bicyclic peptide, bouvardin, is a translation inhibitor. It has been patented for anticancer drugs and used in combination with radiotherapy or other chemotherapeutics [66].

There is a long history of using the seeds of *Celosia argentea* to treat liver and eye diseases in China and Japan. According to modern studies, a variety of bicyclic peptides can be detected and extracted from the seeds of *Celosia argentea* [67], some of which show good antitumor effects even higher than that of vinblastine [68]. The more representative peptide is Celogentin C.

Sunflower trypsin inhibitor-1 is a bicyclic peptide found in sunflower seeds. It consists of 14 amino acids, combining a head-to-tail cyclized backbone and a disulfide bond between Cys3 and Cys11. It is known as the smallest Bowman-Birk family of serine protease inhibitors [60]. SFTI-1 has antibacterial effects and serves as a radiopharmaceutical and proangiogenic peptide [69]. In 2021, Durek *et al.* designed new potent bivalent melanocortin receptor ligands based on SFTI-1. One of the designed compounds even displayed low picomolar agonist activity at hMC1R *in vitro* [70].

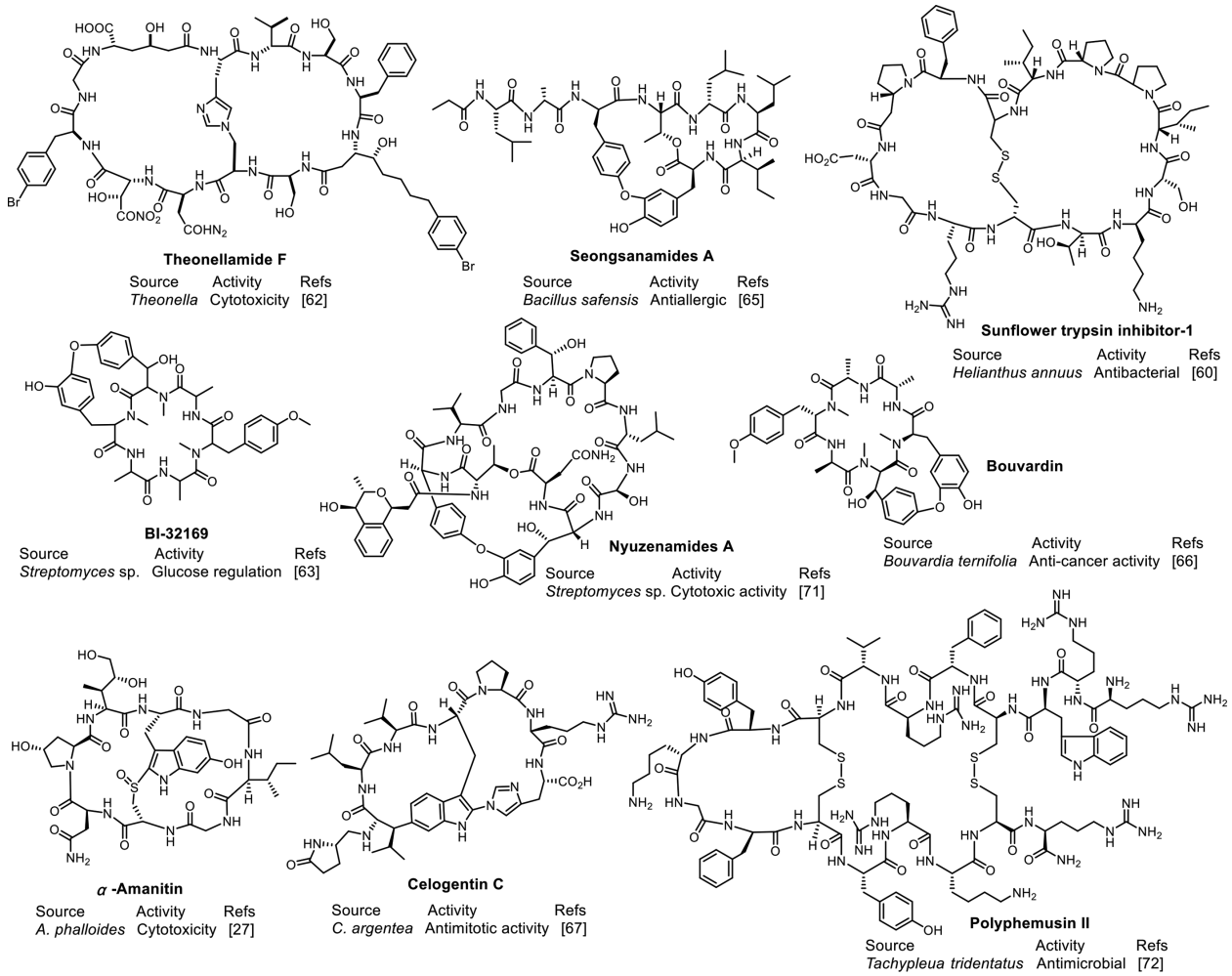
Researchers have isolated nearly 9000 compounds from Streptomyces. In 2021, Igarashi *et al.* isolated and identified two bicyclic peptides, Nyuzenamides A and B, from *Streptomyces*. Both compounds exhibited antifungal activity and cytotoxicity against mouse P388 leukemia cells in subsequent experiments [71]. In general, bicyclic peptides, similar to other peptides, also have a wide distribution. It is likely that a growing number of natural bicyclic peptides will be discovered.

Although they have interesting structures and rich potential for biological activity, the structures of most defense peptides do not meet the definition of bicyclic peptides, so they will not be discussed in this review. Interestingly, researchers have also isolated biologically active bicyclic peptide structures from animals, such as polyphemusin II isolated from horseshoe crab hemocytes in 1989, which was shown to have antimicrobial activity [72]. It has functional similarities to vertebrate defensins.

5. Bicyclic peptides in clinical trials

Similar to the development of other drugs, the progress of bicyclic peptide technology is also inseparable from the participation of pharmaceutical companies. Bicycle Therapeutics, a biopharmaceutical company, takes bicyclic peptides as a platform technology and has developed several bicyclic peptides into clinical trials.

BT1718 is another bicycle toxin conjugate developed by Bicycle Therapeutics [73]. BT1718 consists of a bicyclic peptide and a toxin. The bicyclic peptide targets membrane type 1 matrix metalloproteinase (MT1-MMP), also known as MMP-14, with high affinity and selectivity. Since MMP-14 was identified as a protease in tumor



Scheme 1. Examples of natural bicyclic peptides.

cells [74], researchers have found that it is associated with the development and poor prognosis of many tumors [75]. BT1718 is currently undergoing a phase I/IIa clinical trial sponsored by Cancer Research UK's Centre for Drug Development [76].

BT5528 is a bicyclic peptide-conjugated toxin developed by a bicyclic therapeutic company that targets EphA2 (or Ephrin type-A receptor 2). The researchers initially used the ADC strategy to develop antitumor drugs targeting the EphA2 protein. ADCs have promising activity but unfortunately often exhibit unacceptable multiorgan toxicity in preclinical experiments.

Cleverly combining bicyclic peptides with cytotoxic drugs, researchers developed a bicycle toxin conjugate. This conjugate showed an excellent tumor-killing effect and acceptable results in preclinical toxicology and drug metabolism studies. BT5528 has now entered the clinical phase I/II evaluation stage and has shown positive anticancer activity in phase 1 clinical trials. It achieved partial remission in treated urothelial cancer patients and an 80% disease control rate in ovarian cancer patients.

BT8009 is another bicycle toxin conjugate that targets Nectin-4 (Nectin cell adhesion molecule 4). BT8009 consists of a bicyclic peptide through a sarcosine chain coupled to the cytotoxin MMAE. It has been reported that Nectin-4 is overexpressed in a variety of human cancers and is associated with tumor cell growth and proliferation [77]. In 2019, researchers announced that in preclinical experiments, BT8009 successfully released toxin at the tumor site in animal models and had good efficacy in several mouse xenograft models [78]. In 2020, Bicycle Therapeutics advanced BT8009 to the clinical trial stage and will initially verify its safety and activity data in severe cancer patients. In patients with Nectin-4-expressing advanced malignancies, preliminary verification of its safety and efficacy is ongoing.

Elevated levels of plasma kallikrein (PKal) are considered to be one of the causative factors of diabetic macular edema (DME), and targeting PKal may offer a vascular endothelial growth factor (VEGF)-independent mechanism for inhibiting DME. THR-149, a bicyclic peptide discovered by Bicycle Therapeutics through phage display and chemical optimization, is a potent and specific inhibitor of PKal with stability. Currently, THR-149 is being developed by Oxurion N.V. for the treatment of diabetic macular edema. In 2019, it was reported that THR-149 achieved positive results in a phase 1 clinical trial, and in 2020, Oxurion announced positive results from a phase 2 clinical trial of THR-149 in patients with DME. All dose levels of THR-149 had favorable safety profiles, all adverse events were mild to moderate in severity, and no inflammation was observed. The highest dose of THR-149 (0.13 mg) produced the greatest improvement in the BCVA measure of visual acuity in patients. Patients in the highest dose group had an average improvement in best-corrected visual acuity of 6.1 letters at month 3.

Based on the existing technology, scientists at Bicycle Therapeutics recently synthesized a conjugated bicyclic peptide that acts on both CD137 and Nectin-4, which provides a multipronged anti-

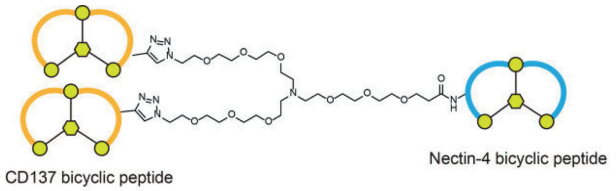


Fig. 6. Structure of BT7480.

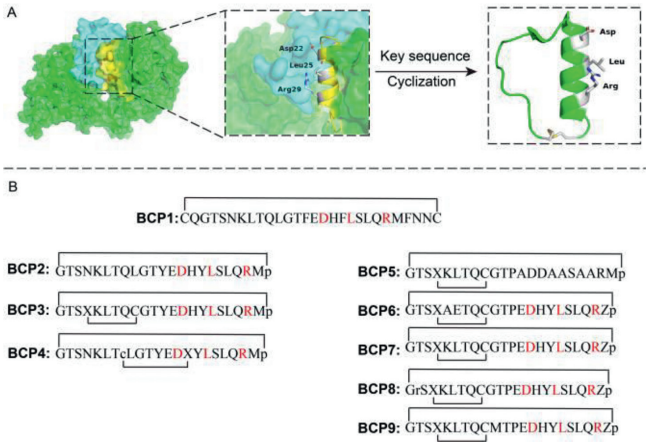


Fig. 7. (A) binding interface of EGF(cyan) and EGFR(green, the key domains are highlighted in yellow, PDB ID: 1IVO). Cyclic peptide BCP1 is shown on the right. (B) Sequences of peptides BCP1-9.

tumor approach. After *in vitro* proof-of-concept, linker selection and valence optimization, the researchers obtained BT7480 (Fig. 6) [79].

By linking two bicyclic peptides through PEG chains, the newly obtained bicyclic peptide conjugates can act on two target proteins simultaneously, effectively activate CD137 and exert anti-tumor effects. Most recently, a combined phase I/II study of BT7480 have been launched [80].

At present, the enthusiasm of pharmaceutical companies for bicyclic peptides is still growing, including AstraZeneca and Genentech, and many companies are involved in the field of bicyclic peptides.

6. Progress in fundamental research on bicyclic peptides

In the last five years, bicyclic peptide technology has been increasingly developed, and researchers have carried out a variety of studies to elucidate their potential functions.

6.1. Using bicyclic peptides to target "difficult to drug" targets

There are as many as 650,000 protein–protein interactions (PPIs) in the human interactome, which regulate various functions of cells and may be considered new therapeutic targets. However, PPIs are typically characterized by large, flat and featureless interfaces [81]. It is difficult to use small molecule drugs as inhibitors.

EGF-EGFR was validated to play important roles in tumor cell proliferation, invasion and metastasis and has also become the focus of cancer therapy. In 2017, using a rational design approach, Ernest Giralt and his coworkers successfully targeted EGF-EGFR [82]. Crystallographic studies demonstrated that the α-helical motif in the EGFR domain features hotspots (Asp22, Leu25, Arg29) that establish key contacts with EGF (Fig. 7). BCP1 was chosen as the lead compound, which is able to mimic the binding of the receptor to EGF with a K_d of 286 μmol/L. In BCP1, Asp22, Leu25 and Arg29 are the key amino acids for interaction, and these three sites are mostly retained in the derivatives.

In the first round of design, three peptides were designed. For all three peptides, they used *d*-Pro-Gly to replace the original closure part of BCP1 and mutated the Phe residues at the helix for Tyr. In particular, they introduced a bicyclic structure using cysteine and L-homocysteine in BCP3 and BCP4. This move resulted in a slight decrease in activity (BCP2: $K_d = 773$ μmol/L; BCP3: $K_d = 575$ μmol/L; BCP4: $K_d = 1860$ μmol/L) but made the peptides more in line with their design philosophy, "mimic the bioactive conformation of the receptor with constrained peptides, which are endowed with superior stability and drug-like properties. The researchers chose BCP3 as the preferred compound and performed virtual mutation for the second round of design. By design and evaluation in silico, they selected three of the best compounds, named BCP5, BCP6, and BCP7, for wet experiments. For all three analogs, they replaced Tyr12 with Pro to achieve better ΔG values in the interaction. The best analog of three, BCP7, improved the potency to 280 μmol/L. In the final cycle of the in silico design, they still aim to improve the ΔG score. Although most mutations of BCP7 lead to a detrimental effect on the ΔG values, they found that the substitution of Gly10 for Met leads to a better ΔG score. Finally, BCP9 targeted EGFR at 115 μmol/L, which was superior to that of BCP1.

The kallikrein–kinin system (KKS) has been verified to modulate a variety of physiological processes, including blood pressure, inflammation, and cell proliferation. PKal is an important member of the KKS, and preclinical evidence has implicated PKal as strongly associated with vision loss in patients suffering from diabetes mellitus.

In 2018, Teufel *et al.* described the design, optimization, and *in vitro/in vivo* experiments of a series of highly potent, stable, and long-lasting PKal inhibitory bicyclic peptides [83].

Like many researchers, Vanhove and coworkers found lead compounds by screening bicyclic peptide libraries. All bicyclic peptides in the library contain three cysteine residues. Three thiols react with 1,3,5-tris(bromomethyl)benzene (TBMB) to form a molecular scaffold. The initially obtained bicyclic peptide was BCP10 (Fig. 8A), which showed strong inhibitory activity ($K_i = 2.3 \pm 2.7$ and 0.40 ± 0.24 nmol/L to both human and rat PKal). However, its poor stability and solubility forced researchers to synthesize its derivative, BCP11 (Fig. 8A), which was synthesized by replacing the N-terminal alanine with an acetyl group and adding 3 sarcosines and 2 D-arginines at the C-terminus.

A 5*5 phage library was screened, and BCP12 (Fig. 8A), with good activity against human and mouse PKal ($K_i = 0.07 \pm 0.02$ and 0.90 ± 0.33 nmol/L, respectively), was obtained. Variant BCP13 (Fig. 8A), which latched the protease labile terminal alanine residues, was subsequently synthesized. However, the half-lives of BCP13 in mouse, rat, and human plasma are only 1.5 h, 2.8 h, and 7 h, respectively. MALDI-TOF analysis of BCP13 found that Arg5 and His7 may be important sites for the sensitivity of BCP13 to plasma proteases. To enhance the stability of BCP13 and maintain its biological activity, extensive amino acid substitutions were carried out in BCP13, wherein two residues, Trp2 and Ala4, were changed in the derivative BCP14 (Fig. 8A). To enhance the stability of BCP13 and maintain the biological activity of its derivatives, the amino acids of BCP13 were extensively substituted. Under this goal, the researchers finally completed two rounds of modification and completed amino acid substitutions at multiple sites to obtain the derivative BCP15 (Fig. 8B), which has good biological activity and stability.

In the screening of another library, the researchers found a bicyclic peptide with good stability without chemical modification. The analysis suggests that the source of its stability is equivalent since the His7 proteolytic recognition site is absent in the loop. Unfortunately, this bicyclic peptide showed poor water solubility. To

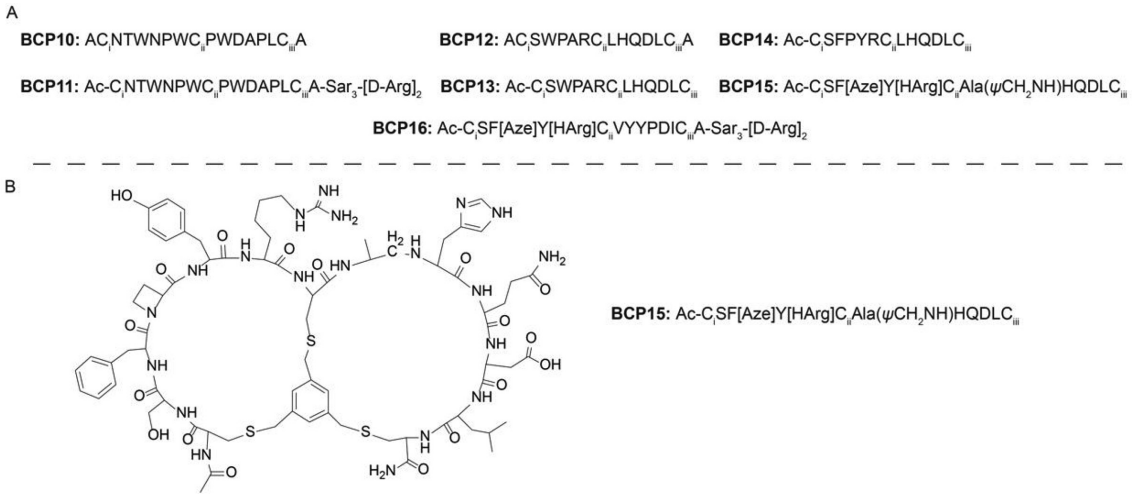


Fig. 8. (A) Sequences of BCP10-16. (B) Structure of BCP15.

improve the solubility, the author extended the solubilizing group to the bicyclic peptide sequence by a similar method to BCP11 and obtained the derivative BCP16.

Later, studies on the selectivity of various enzymes and the plasma and vitreous stability were carried out. BCP15 excelled in pharmacokinetic and pharmacodynamic experiments with good safety, and no overt toxicological effect was observed at the highest tested systemic dose of 125 μg (0.43−0.60 mg/kg) in the rat and 1.25 mg (0.42−0.49 mg/kg) in the cynomolgus macaque.

Clinical trials demonstrate that PCSK9 is a target of importance for hypercholesterolemia and coronary artery disease. Direct blockade of the LDL-receptor–PCSK9 interaction may be an effective strategy for the treatment of coronary heart disease [84].

However, there are currently no small molecule drugs available that can achieve this goal, and currently, two commercial therapeutic antibodies (Alirocumab and Evococumab) [85,86] are difficult to produce, expensive, and limited in clinical use.

In 2020, Alleyne *et al.* used bicyclic peptides to challenge PCSK9. mRNA display screening is used to rapidly obtain large numbers of cyclic peptides and perform activity screening, as the results have demonstrated that macrocyclic peptides have superior stability and are suitable for binding to the large, flat surfaces in PPIs [87].

Modifications were carried out immediately after the screening of the hit polypeptides. The original idea of modification was to reduce redundant groups and the molecular weight of the polypeptide in exchange for higher stability. By removing the N-terminal amino acid, the researchers obtained BCP17, which they used as a template for earlier studies. The protease panel study of BCP17 identified its major metabolic site. After further structural optimization of BCP17 and replacement of proline at key metabolic positions with α-methyl proline, BCP18 was obtained which had an 8-fold increase in activity and improved stability compared to BCP17. Comprehensive and in-depth SAR analysis was the best feature of this study. Utilizing cocrystal technology to aid structure-based drug design, the activity of the peptide was ultimately increased by approximately 1000-fold. The use of SAR analysis also enabled researchers to gradually gain a deeper understanding of the off-target issue of mast cell degranulation of bicyclic peptides. Researchers have used the classic structure-based design approach to explore novel orally bioavailable cyclic peptide PCSK9 inhibitors step by step. Although the final series of compounds still needs to be improved, this work undoubtedly provides a novel idea for targeting PCSK9 protein with bicyclic peptides. Among a series of active molecules, BCP19 is the most active, with $K_i = 1.5 \pm 0.5$ nmol/L. According to crystallography data, BCP19 has a strong interaction with Ser381 and Phe379 of PCSK9 (Fig. 9).

It is worth noting that in 2021, Tucker further explored and designed a peptide with a tricyclic structure on the basis of their previous work [88]. The new molecule maintained the original interaction mode, exhibited acceptable pharmacokinetic properties and astonishing activity ($K_i = 0.00239$ nmol/L), which is almost 100,000 folds over the initial lead structure. It is not difficult to find that the process of transitioning from bicyclic peptides to tricyclic peptides is the result of "extensive use and interpretation of crystallographic data". We believe that with the support of crystallographic data, researchers can analyze the binding conformation of the molecule exactly, and modify the molecule at the appropriate site to optimize the binding capacity of the molecule.

Because of the strict restriction of molecular exchange by the blood−brain barrier (BBB), drug discovery studies to target proteins in the central nervous system (CNS) are challenging. Although bicyclic peptides have demonstrated excellent targeting of refractory proteins over the past decade, designing bicyclic peptides for targets within the nervous system is often not the first choice. In 2021, Sakamoto and coworkers reported the ingenious work of using bicyclic peptide technology to target the receptor VIPR2 in the brain to intervene in psychiatric disorders in a mouse model. Research began with the design of derivatives of VIpep-3, a known VIP2 inhibitor [89].

In the analysis of the binding model of VIPR2, the researchers concluded that Val71, Val73, and Pro74 of VIPR2 were likely to form hydrophobic interactions with VIP (Fig. 10), and these interactions were thought to be critical. As there was no crystal structure of the VIpep-3/VIPR2 complex available at the time, researchers used a predictive binding model to design the structure. After analyzing the predicted binding model, the team proposed substituting Arg with Tyr and removing Leu14, Arg15 and Ser16 to improve the binding capacity. Furthermore, two strategies were proposed. One was introducing the positively charged amino acid L-2,3-diaminopropionic acid (Dap) to the 7th and 11th positions, from which KS132 was designed and synthesized. The other was cyclization, in which the disulfide bonds of Cys1 and Cys10 and the amide bonds of Lys7 and Asp11 together form a bicyclic structure. Based on this strategy, KS133 and KS133 (monocyclic) were designed and synthesized.

In the follow-up experiments, KS132 and KS133 showed biological activity exceeding that of VIpep-3. KS132 and KS133 had IC_{50} values of 33.4 nmol/L and 24.8 nmol/L, respectively, which were stronger antagonistic activities than their parental peptide VIpep-

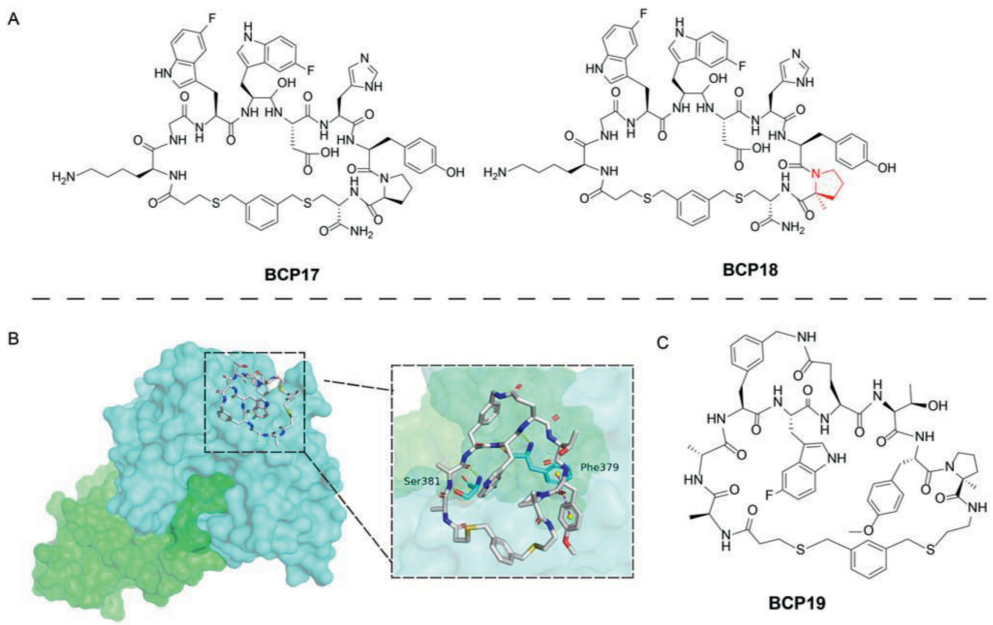


Fig. 9. (A) Structures of BCP17 and BCP18. (B) Details of the BCP19–PCSK9 binding interface (PDB ID: 6XIE). (C) Structure of BCP19.

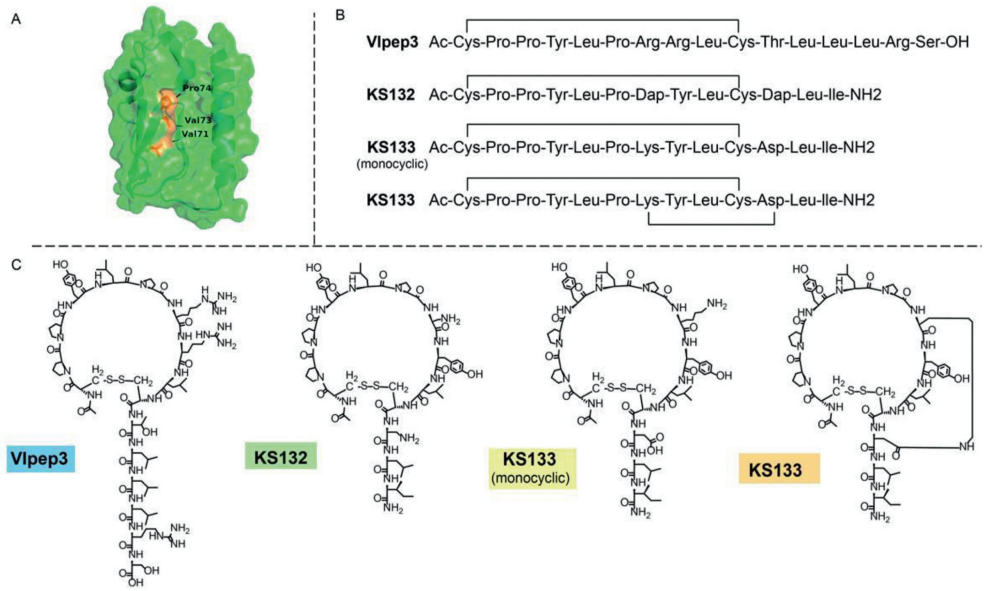


Fig. 10. (A) Extracellular domain of VIPR2 (hotspots of interaction are highlighted in orange, PDB ID: 2 × 57). (B) Sequences of Vipep3, KS132, KS133(monocyclic) and KS133. (C) Structures of Vipep3, KS132, KS133 (monocyclic) and KS133.

3 (40.6 nmol/L). KS133 and KS133 (monocyclic) showed good resistance to protease degradation. While VIpep-3 and KS132 were significantly degraded within 24 h, KS133 was highly stable for at least up to 24 h. KS133 (monocyclic) exhibited anomalous stability, which the authors believed was due to the formation of pseudo-bicycles by intramolecular hydrogen bonds. The level of KS133 in plasma and brain was analyzed, and it was found that the levels of KS133 were 2.78 ± 0.08 μmol L^{-1} g^{-1} in plasma and 0.035 ± 0.005 nmol/g in brain at 80 min after intravenous injection of adult male ICR mice. These results indicate that KS133 can cross the BBB, which is also supported by positive ethological results.

Due to the extensive binding of bicyclic peptides to the surface of the target protein, these bindings exist not only at the active site of the target protein but also in the vicinity of the active site. This is often favorable for the bicyclic peptide to exert its biological activity, which also explains the strong binding affinity of the bicyclic peptide.

6.2. Using bicyclic peptides as tool drugs

An increasing number of research tools need to be designed to meet the needs of protein function research. Compared with the development of medicine, tool drugs have lower requirements on the properties of the compounds. Inspired by previous studies using bicyclic peptides to target proteins [90], Slavoff *et al.* believed that cell-permeable, selective chemical inhibitors of DCP2 could be achieved by bicyclic peptides. In 2020, they reported a high-affinity and selective DCP2 bicyclic peptide inhibitor obtained by phage display [91]. This is in accordance with the research

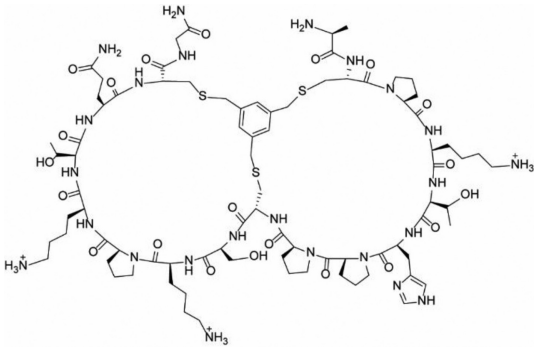


Fig. 11. Structure of bicyclic peptide BCP20.

ideas proposed by Rentero Rebollo and Heinis *et al.* in 2013 [92]. Slavoff and coworkers conducted three rounds of affinity screening for immobilized DCP2 with site-specific biotinylation. Screening started with 10^{10} purified TBMB-modified phage particles. After the screening, the results were analyzed, and the amino acids of some sites were selectively fixed. The final activity assay identified the optimal active sequence, which resulted in the bicyclic peptide BCP20 (Fig. 11) after recyclization with the addition of alanine to the N-terminus and the addition of aminoglycine to the C-terminus. The K_d of BCP20 for the catalytically active DCP2 truncation construct was 116 ± 31 nmol/L, and the membrane permeability of BCP20 was further determined in subsequent activity experiments at the cellular level. BCP20 was subsequently used as a tool drug in biological studies to identify substrates of DCP2. BCP20 played an important role as a tool drug in the identification of previously undiscovered DCP2 substrates.

The work of Slavoff *et al.* drew on the work of Rentero Rebollo and Heinis in the screening phase of bicyclic peptides and even used the initial peptide library. This not only affirms the use of bicyclic peptides as a platform for rapid screening of tool drugs but also shows that a single bicyclic peptide library has great development potential.

In 2020, Machida *et al.* used a G-actin-binding bicyclic peptide to analyze and manipulate nuclear actin in cells [93].

Because actin is involved in a wide range of cellular functions [94–96], the tools required for studying actin have become extremely desirable. There are many molecular tools obtained through extraction from natural sources or complex chemical synthesis procedures, but their inaccessibility makes their application less than ideal. It is difficult for bicyclic peptide molecules to enter the nucleus to perform their functions. If bicyclic peptides function on nuclear actin, smooth entry into the cell nucleus is an essential requirement. After obtaining the bicyclic peptide BCP21 (Fig. 12), which has good binding ability and selectivity for globular actin (G-actin), how to promote smooth cell entry and nuclear entry has become an important issue. The membrane permeability experiment in cell culture conditions showed that BCP21 could not smoothly penetrate the cell membrane. Subsequently, researchers used electroporation to introduce the peptide into living cells. Upon entry into cells, BCP21 was observed predominantly in the cytoplasm even though the BCP21 molecule was small enough to penetrate nuclear pores. To solve this problem, the authors tried to fuse a nuclear localization signal (NLS) to the N- or C-terminus of BCP21, thus obtaining NLS-BCP21 or BCP21-NLS bicyclic peptides. NLS-BCP21 and BCP21-NLS (Fig. 12) significantly accumulated in the nucleus compared to BCP21 bicyclic peptides without an NLS. Further experiments confirmed that the NLS-BCP21 bicyclic peptide can be seen to accumulate more efficiently in the nucleus, and it can effectively repress the formation of nuclear F-actin and impair the function of nuclear F-actin. Therefore, NLS-BCP21 is expected to be used as a tool compound to study manipulating nuclear and chromatin functions through the modification of nuclear actin dynamics.

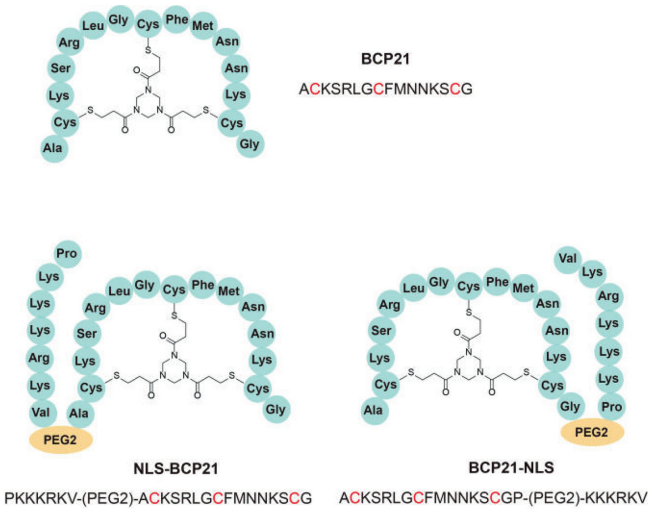


Fig. 12. Structures of BCP21, NLS-BCP21 and BCP21-NLS.

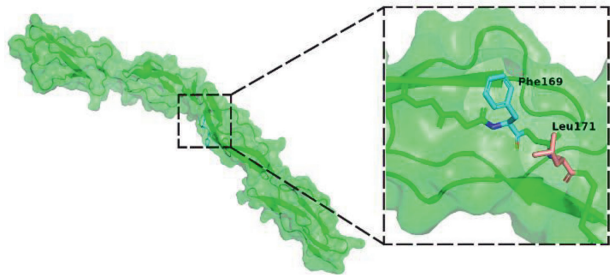


Fig. 13. The large and complex surface of CD55 (PDB: 1OJV, key amino acid residues predicted by docking are highlighted).

Many practices have proven that bicyclic peptides have advantages in targeting macromolecules, and bioactive bicyclic peptides have been designed for many undruggable targets. At this point, determining whether they can exert the same activity as antibodies is the desired goal. In 2021, Moreira and coworkers developed a miniaturized anti-CD55 antibody using bicyclic peptides [97].

As a major regulator of the complement system, CD55 overexpression is associated with many diseases, including cancer. The current view is that using cd55-targeting antibodies to interfere with CD55-mediated complement regulation is a potential therapeutic approach. However, the expensive development cost of antibody drugs and their poor properties limit their development and application. Based on previous studies, the neutralizing minibody MB55 can efficiently block the inhibitory action of CD55 on the complement system. Information obtained through paratope prediction and docking analysis revealed the key to the interaction between CD55 and MB55. According to the docking study results, Phe169 and Leu171 of CD55 played key roles in the interaction with MB55 (Fig. 13). Based on the results of these analyses, the authors designed and synthesized the bicyclic peptide miniAB55 as a miniaturized antibody targeting CD55. It was found that miniAB55 bound to recombinant CD55 with an affinity constant in the nanomolar range ($K_d = 166$ nmol/L). Although the miniAB55 molecule was referred to as a miniaturized antibody in the article, its chemical structure is not fundamentally different from the bicyclic peptide described above, and the core structure is formed by three thiol groups and TBMB molecules. The common bicyclic peptide paradigm can exert similar activity to the neutralizing mini-

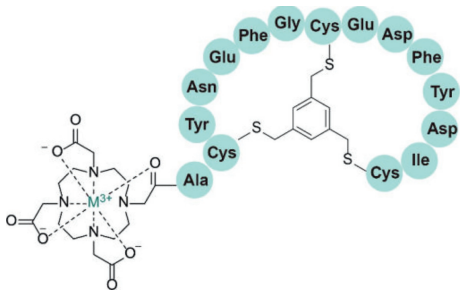


Fig. 14. Structure of BCP22.

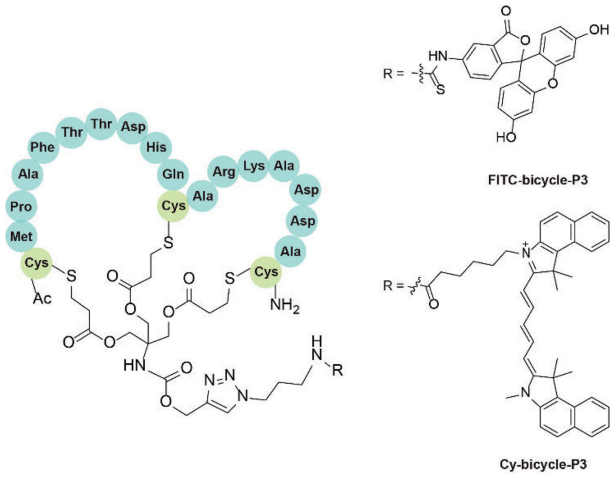


Fig. 15. Structures of FITC-bicycle-P3 and Cy-bicycle-P3.

body MB55. This example further develops the function of the bicyclic peptide and demonstrates the potential of the bicyclic peptide structure. An antibody substitute that can be chemically synthesized, easily modified, and has good permeability; therefore, such substitutes are undoubtedly the common pursuit of drug researchers.

6.3. Application of bicyclic peptides in molecular imaging

In 2019, Li *et al.* reported a strategy for the first time using bicyclic peptides as a platform for diagnostic imaging and targeting. In this study, researchers chose tumor-associated membrane type 1 matrix metalloproteinase (MT1-MMP), which is overexpressed in a variety of tumors and is associated with poor prognosis, as the target [98].

They used phage display technology to construct a library of bicyclic peptides. Target affinity screening was carried out, and a bicyclic peptide with strong binding ability and selectivity to the target, stable to protease at the same time, was obtained. The research team used ^{177}Lu to label the bicyclic peptide and finally completed the construction of the molecular probe BCP22 (Fig. 14). Activity testing and imaging experiments verified its affinity ($K_d = 0.51$ nmol/L) and imaging capabilities *in vivo* [20].

In 2020, Li *et al.* adopted similar strategies. Matrix metalloproteinase-2 (MMP2) uses the triacryl-tris-alkyne skeleton group, which is relatively rare in bicyclic peptides, to complete the construction of the bicyclic structure and click chemistry to introduce FITC and Cy5.5 into the bicyclic peptide molecule separately (Fig. 15). Further experiments were carried out to verify its cell uptake ability and *in vivo* positioning imaging ability [21].

Compared with macromolecular probes possessing strong targeting effect (especially monoclonal antibodies), peptide molecular probes are smaller in size and often have better pharmacokinetic properties. In addition to reducing the loss of entropy and enhancing the binding ability to the target, the structure of the bicyclic ring can also function as a targeting platform because of its three-dimensional structure, which is convenient for the modification and further research of fluorescent molecules.

6.4. Bicyclic peptides are effective against multidrug-resistant bacteria

Antibiotic resistance is one of the biggest threats to human health, and many infections are becoming more difficult to treat due to the presence of multidrug-resistant (MDR) bacteria.

Overcoming multidrug-resistant bacteria is currently a major problem in the field of medicinal chemistry. In 2017, Di Bonaventura and his coworkers first used bicyclic peptides as antimicrobial bicyclic peptides (AMBPs) against *P. aeruginosa* [99].

By analyzing pharmacophores and molecular shape using chemical space encoding, Reymond and his coworkers were able to explore as much compound diversity as possible while testing fewer molecules. According to the common amino acid types and sequence characteristics of antimicrobial peptides (AMPs), a peptide library at the astronomical level was obtained. Current antimicrobial peptides demonstrate that the ratio of lysine to leucine has a significant effect on antimicrobial activity. Therefore, subsequent researchers decided to focus on lysine and leucine and adjust the ratio of lysine and leucine to make them more in line with the requirements of antibacterial peptides. Ultimately, 28 bicyclic peptides were synthesized for activity testing. Although no structures effective against *P. aeruginosa* were found in this batch of compounds, many peptides showed inhibitory activity against *Bacillus subtilis*. Among them, BCP23 (Fig. 16A) showed the best inhibitory activity (MIC against *Bacillus subtilis* = 8 μg/mL).

Chemical space was used to search for the most similar structure to BCP23. Nineteen of them were used for the second round of activity testing, and many of them showed inhibition of *P. aeruginosa*. The BCP24 (Fig. 16B) with the best activity was selected as the leader of the next round of screening, and the third round of screening conducted virtual screening, synthesis and activity testing for the most similar polypeptides to BCP24. To explore more possibilities, some amino acid positions were changed greatly when designing BCP24 analogs, but the results were not satisfactory. BCP25 (Fig. 16B) was obtained by adding a hydrophobic side chain to the original sequence C-terminus. The activity experiment confirmed that its inhibitory activity against *P. aeruginosa* was improved, with the best *P. aeruginosa* inhibitory activity (MIC = 1 μg/mL) and the best Bacillus subtilis inhibitory activity (MIC = 16–8 μg/mL).

It is undoubtedly of great significance to apply the chemical space-guided approach widely used in small molecule design to peptide design. Prior to this, researchers mostly performed tireless screening of large peptide libraries, which often resulted in relatively low efficiency and high scientific research costs. Obviously, the application of this method can improve the development efficiency and accuracy of bicyclic peptides. However, the bicyclic peptides obtained by Reymond *et al.* in this work only exhibited resistance to the laboratory strain *P. aeruginosa* but not to MDR strains.

In 2018, Di Bonaventura *et al.* reapplied a chemical space-guided approach to the design of bicyclic peptides, with the goal of obtaining an antimicrobial bicyclic peptide (AMBP) with activity against MDR strains [22].

Inspired by the work of previous researchers, in this work, they adopted cyclic AMPs containing a xylene linker. Performing cluster analysis on a library comprised of 4,685,090 bicyclic peptides, the researchers tested 31 bicyclic peptides in the first round of synthesis. Three polypeptides, numbered BCP26, BCP27 and BCP28 (Fig. 17A), were found to have potential against the laboratory strains

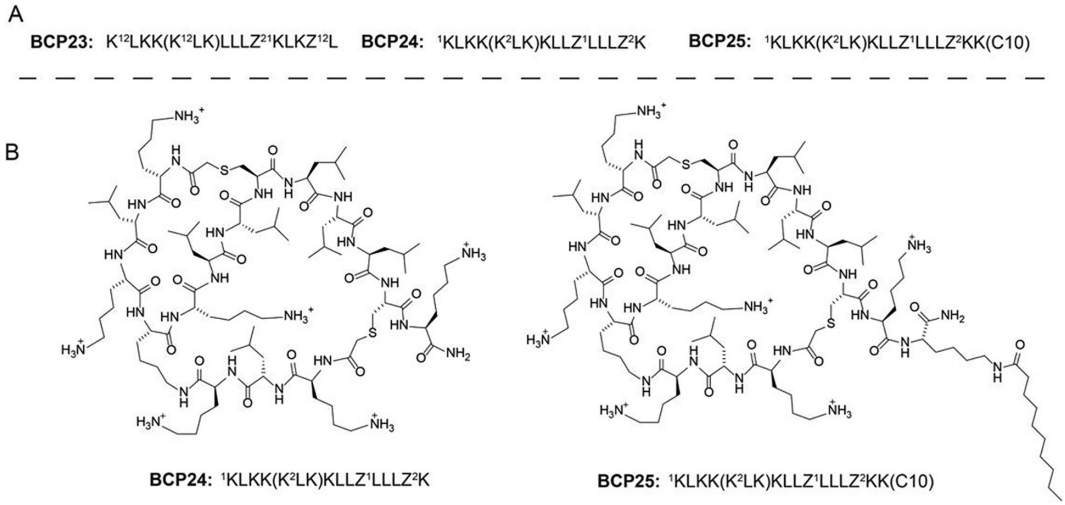


Fig. 16. (A) Sequences of BCP23-25. (B) Structures of BCP24 and BCP25.

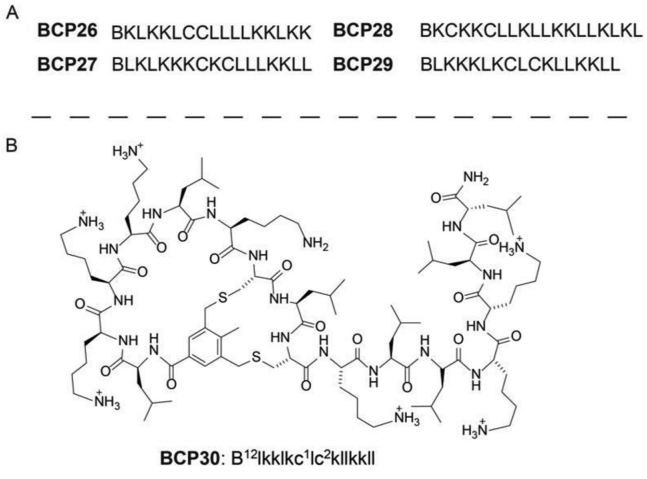


Fig. 17. (A) Sequences of BCP26-29. (B) Structure of BCP30.

P. aeruginosa PAO1 and *Bacillus subtilis* BR151. According to the research method of the chemical space strategy, the 20 bicyclic peptides closest to BCP26 and BCP27 in chemical space were synthesized. Improvement occurred with analogs of hit BCP27, and derivative BCP29 (Fig. 17A) enhanced PAO1 activity. Replacing some amino acids with D-amino acids is a common method for antimicrobial peptide development. BCP30 (Fig. 17B) is the D-enantiomer of BCP29 and shows similar antibacterial activity but is completely stable in serum. Further experiments demonstrated that BCP30 also showed weak activity against some clinically isolated strains.

Compared to their initial research in 2017, this study provides a more rapid and convenient screening method for antibacterial bicyclic peptides and showed preliminary inhibitory activity against clinical strains. This also hints at the potential of applying chemical space approaches to peptide design, especially to bicyclic peptide design.

When a few clinical drugs are currently not effective against multidrug-resistant bacteria, antimicrobial peptide strategies have become a hot field. Compared with linear antibacterial peptides, the advantages of bicyclic peptides as AMPs are obvious. Cyclization can make the peptide more difficult to be destroyed by proteases, which brings a longer half-life and has the potential to exert a stronger antibacterial effect. However, from the current research, bicyclic peptides as AMPs do not seem to solve people's concerns about toxicity, especially hemolysis.

In addition to the above fields, bicyclic peptides are also used in anti-AD and antitumor research. In 2021, Ikenoue *et al.* rationally designed a bicyclic peptide molecule based on known protein A fibrosis, which has therapeutic potential for Alzheimer's disease [100]. In 2021, Tang *et al.* designed stapled α-helical peptides with a bicyclic structure. Experiments revealed that this bicyclic peptide could induce selective death of $\alpha_V\beta_3$ integrin-positive B16F10 cells by interfering with mitochondrial bioenergy function [101].

7. Conclusions and perspectives

After a thorough review of bicyclic peptides, we briefly summarized the following sections – achievements and future challenges of bicyclic peptides.

7.1. Achievements

With the continuous study of targets, researchers have gradually realized that undruggable targets are more like hard-to-drug targets. The development of bicyclic peptide technology seems promising to turn these thorny targets into low-hanging fruits. We were surprised to find that many potential but challenging targets, such as PCSK9 and VIPR2, can be targeted using bicyclic peptides. We think that bicyclic peptides provide more explorable chemical space for new drug design, especially peptide drug design. We believe that there will be more proteins with broad binding sites targeted by novel bicyclic peptides.

Clearly, this advantage of bicyclic peptides will not only be favored by medicinal chemists; several reports have also demonstrated the potential of bicyclic peptides as a tool platform. We believe that tools for understanding biological systems may shine in chemical biology. Moreover, the design of a new modality for imaging has provided powerful proof that bicyclic peptides can be used as a platform for the loading of other functional groups.

In addition, the rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, therefore, antimicrobial peptide strategies will become the more hot filed of drug research. AMPs, which have unique membrane-disrupting mechanisms, have attracted significant enthusiasm in recent decades. Especially, in the past five years, some bicyclic peptides from natural products and laboratories have been shown to have antibacterial potential. Bicyclic peptides with different properties compared to traditional AMPs have been shown to be capa-

ble of improving linear AMPs in the form of restricted peptides. The new progress has also shown us the progress of chemical space-guided discovery in the design of novel antimicrobial peptides. These achievements are undoubtedly interesting for the field of antimicrobial agents.

In the more than 20 years since the first ADC drug, Mylotarg, was approved, the progress of PDC drugs has been relatively slow. Only two drugs, Lutathera and Pepaxto, have been approved (Pepaxto was withdrawn 8 months after approval). However, recently, the positive results of BTC molecules in clinical trials have shown that BTCs based on bicyclic peptide technology may be a new breakthrough point in drug development.

High activity, strong modifiability, good stability, and strong membrane permeability make it difficult to say which is the reason for the progress of bicyclic peptides in the aforementioned fields. Although its core advantages are difficult to quantify, its progress is real.

7.2. Challenges

When discussing the advantages of bicyclic peptides over monocyclic peptides, it is common to say that conformational constraints on cyclic peptides can effectively reduce the entropy penalty, thereby increasing the activity of bicyclic peptide molecules *in vivo*. However, another well-known fact is that a higher degree of confinement means that the molecule will exhibit lower degrees of freedom in the environment. This means that the dominant conformation of the molecule may be in a disadvantageous energetic and steric position when it binds to the receptor. In other words, if the polypeptide is incorrectly constrained, the wrong conformation may be locked, and the ability to bind to the target could be eliminated. This makes bicyclic peptide technology often limited to the modification of known polypeptide leads, as this approach has an apparently high success rate. Since the current virtual screening method is suitable for peptides, it is often necessary to screen an astronomical entity library to find the first-in-class bicyclic peptide molecules. Even though pioneers have provided a variety of methods to construct bicyclic peptide libraries, for many pharmacologists with a chemical background, picking the "low-hanging fruit" still faces considerable difficulties.

At present, there are far more than 20 kinds of amino acids available for polypeptide design; however, the design of their sequences is more complicated. For bicyclic peptides, the selection of cyclization sites has become a new problem. How to efficiently determine the space to be explored is an issue that should be considered before exploring. However, unreasonable library selection may lead to considerable meaningless consumption.

Most of the bicyclic peptides reported thus far still belong to the category of polypeptides, and while they inherit the advantages of peptides, however, they still cannot completely eliminate the common defects of peptides. For peptide researchers, the solubilization of peptides is often an important issue. As a cyclized polypeptide, will its solubility become worse, or will there be a rapid decrease in solubility? From a larger perspective, will the changes in the bicyclic peptide structure introduce new defects different from the linear morphology while bringing about excellent properties? How can these deficiencies be resolved? These are many interesting topics that researchers need to explore. From previous reports, we have already known that linear peptides constructed from appropriate intramolecular interactions can rival bicyclic peptide molecules in activity. After all, the bicycle is only a method, not an end. Under the appearance of the bicycle structure, there may be deeper rules and more challenges to be explored. We believe that bicyclic peptides, as a promising peptide format for new drug developments, will received more intense attention in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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