



# Bicyclic peptides: types, synthesis and applications

Shahrzad Ahangarzadeh<sup>1</sup>, Mohammad M. Kanafi<sup>4</sup>, Simzar Hosseinzadeh<sup>4</sup>,  
Ahad Mokhtarzadeh<sup>5</sup>, Mahmood Barati<sup>6</sup>, Javad Ranjbari<sup>2,3</sup> and Lobat Tayebi<sup>7</sup>



<sup>1</sup>Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

<sup>2</sup>Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>3</sup>Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>4</sup>Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>5</sup>Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

<sup>6</sup>Department of Medical Biotechnology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran

<sup>7</sup>Marquette University School of Dentistry, Milwaukee, WI 53233, USA

**Bicyclic peptides form one of the most promising platforms for drug development owing to their biocompatibility, similarity and chemical diversity to proteins, and they are considered as a possible practical tool in various therapeutic and diagnostic applications. Bicyclic peptides are known to have the capability of being employed as an effective alternative to complex molecules, such as antibodies, or small molecules. This review provides a summary of the recent progress on the types, synthesis and applications of bicyclic peptides. More specifically, natural and synthetic bicyclic peptides are introduced with their different production methods and relevant applications, including drug targeting, imaging and diagnosis. Their uses as antimicrobial agents, as well as the therapeutic functions of different bicyclic peptides, are also discussed.**

## Introduction

Small chemical molecules with molecular weight (MW) <500 Da are the most usable FDA-approved drugs with the ability to regulate a biological process. However, small molecules are generally limited to targeting proteins with well-defined binding pockets (e.g., enzymes, receptors and ion channels). Small molecules cannot affect proteins involved in protein–protein interactions (PPIs) because these proteins are not easily available. To overcome the limitations of small molecules, many macromolecules with MW >5000 Da, such as antibodies, have arrived in the clinic as therapeutics since the 1980s. These macromolecules can interact with challenging targets but, unlike small molecules, they are too large to enter the cell and are thus limited to extracellular targets [1]. Peptides, as the next-generation therapeutics, represent unique features – molecularly they are between small molecules

and macromolecules, but are biochemically and therapeutically distinct from both [2].

Peptides have a more specific function compared with small molecules. Therapeutic peptides are categorized as linear or cyclic. Cyclic peptides do not have amino and carboxyl termini and are resistant to hydrolysis by exopeptidases. Also, owing to the conformational rigidity, cyclic peptides usually have a better biological activity compared with linear ones. The rigidity of cyclic peptides decreases the Gibbs free energy, thus allowing the enhanced binding toward targets [3]. Cyclic peptides can be used as a therapeutic, drug delivery tool, surfactant, imaging agent, biosensor or antimicrobial agent. Bicyclic peptides, compared with monocyclic peptides, have additional advantages, including higher conformational rigidity, target binding affinity and selectivity, improved metabolic stability and better membrane permeability [4].

Natural bicyclic peptides are produced by different organisms. Romidepsin, actinomycin D, moroidin and celogentin C are some

Corresponding authors: J. Ranjbari, (ranjbarijavad@sbmu.ac.ir),  
L. Tayebi, (lobat.tayebi@marquette.edu)

examples of natural bicyclic peptide that have different activities – like inhibition of tubulin polymerization, prevention the polarization of actin filaments, inhibition of RNA polymerase II, among others [4–6]. Bicyclic peptide libraries include many bicyclic peptides with high diversity. Combinatorial library technologies have recently been developed to rapidly synthesize and screen large bicyclic peptide libraries for ligands against specific targets. Two categories can be mentioned for bicyclic peptide libraries: biologic and synthetic. Some peptide libraries are restricted to peptidogenic amino acids, whereas other libraries can contain nonpeptidogenic amino acids, such as  $\beta$ - or  $\gamma$ -amino acids, D-amino acids or non-natural amino acids. Nowadays, different methods like phage display method, split-intein circular ligation of peptides and proteins (SICLOPPS) and mRNA display are used for bicyclic peptide library fabrication [7]. In this review, we will discuss types, preparation and application of bicyclic peptides as next-generation therapeutics.

### Bicyclic peptide features and types

Bicyclic peptides are a class of polypeptides with two macrocyclic rings that have advantages over linear and monocyclic peptides. Their size, structure, functionality and increased conformational rigidity improve their proteolytic stability in the blood circulation, binding properties and offer better tissue penetration, widening their application routes [1]. In addition, each ring in this bicyclic structure can function independently, allowing these peptides to be bifunctional [8]. Bicyclic peptides potentially have some of the favorable properties of monoclonal antibodies (high target specificity and affinity) and small molecules (stability and good tissue penetration) and, therefore, might be appropriate candidates for the progress of therapeutics with low toxic profiles against a diverse set of targets [9]. These molecules are classified into two groups: natural and synthetic. There are different types of bicyclic peptides in nature, exhibiting various biological activities. Moroidin, celogentins A–K, phallotoxins and amatoxins are typical examples of natural bicyclic peptides. Also, the various activities of naturally occurring bicyclic peptides suggest that synthetic bicyclic peptides can mimic some biological activities [1].

#### Natural bicyclic peptides

Many bicyclic peptides with various biological activities are naturally produced by different organisms (Table 1). One of these naturally occurring peptides is romidepsin, which is extracted from the bacterium *Chromobacterium violaceum* [10]. Because this bicyclic peptide inhibits histone deacetylase, it is used as an anticancer agent in T cell lymphoma [11]. There is also a class

of bicyclic heptapeptides consisting of at least seven compounds (phalloidin, prophalloin, phalloin, phallisin, phallacidin, phallacin and phallisacin) isolated from *Amanita phalloides* (the death cap mushroom) [12]. Phallotoxins bind actin, specifically at the interface between F-actin subunits, and inhibit the depolarization of actin filaments [13,14]. Fluorescent derivatives of phalloidin have mainly been used to localize and visualize actin filaments in cells [15]. Vitilevuamide, a cytotoxic compound isolated from two marine ascidians, is a bicyclic 13-amino-acid peptide. This naturally occurring bicyclic peptide is very toxic in mammalian cell lines and inhibits tubulin polymerization [5]. Owing to the appealing features of bicyclic peptides, several strategies have been examined to generate synthetic bicyclic peptides for various applications.

#### Synthetic bicyclic peptides

The diverse biological functions of naturally occurring bicyclic peptides and their potential in the development of novel therapeutics have encouraged scientists to generate synthetic bicyclic peptides with desired activities. Consequently, the first synthetic bicyclic heptapeptide was synthesized in 1978, which was structurally similar to phallotoxins [1,16]. Vancomycin, a branched tricyclic glycopeptide antibiotic, is produced by the actinomycete *Amycolaptosis orientalis* and is commonly used for the treatment of serious bacterial infections. Recently, Yang *et al.* reported a bicyclic tripeptide synthesis as a mimic of the ABC ring system of vancomycin using the ruthenium-based cyclization chemistries [17]. In another study, Rodriguez and co-workers designed and synthesized a polymyxin-based bicyclic peptide by developing a new solid-phase synthetic approach and showed that this synthetic bicyclic peptide has the potential to be used as a source for the design and generation of novel and more-effective carbohydrate receptors for bioanalytical or medicinal applications [18].

Also, Dehua Pei and colleagues improved the cell permeability of protein tyrosine phosphatase 1B (PTP1B) inhibitor 2 by a bicyclic system in which the cyclo(F $\Phi$ RRRRQ) (cF $\Phi$ R4, where  $\Phi$  is L-naphthylalanine) as a novel class of cell-penetrating peptide (CPP) is placed in one ring and the target-binding sequence constitutes the other ring. Fusion of cyclic PTP1B inhibitor 2 with cF $\Phi$ R4 as the cyclic cell-penetrating peptide produces bicyclic peptides that are cell permeable and retain the ability to recognize specific intracellular targets [19].

#### Chemical methods for cyclization of peptides

To date, several strategies have been used to form synthetic bicyclic peptides. The first strategy was reported to generate a bicyclic

**TABLE 1**  
Examples of naturally occurring bicyclic peptides

Name	Source	Mechanism of action	Activity	Refs
Actinomycin D	<i>Streptomyces parvulus</i>	Interfering with mRNA synthesis	An antineoplastic antibiotic	[6]
Moroidin	<i>Celosia argentea</i>	Inhibitor of tubulin polymerization	Antimitotic activity	[57]
Celogentin C	<i>C. argentea</i>	Inhibitor of tubulin polymerization	Antimitotic activity	[58]
Phalloidin	Death cap mushroom ( <i>Amanita phalloides</i> )	Preventing the polarization of actin filaments	Inhibition of cytokinesis and cytotoxicity	[15]
$\alpha$ -Amanitin	<i>A. phalloides</i>	Inhibition of RNA polymerases, in particular RNA polymerase II	Cytotoxicity	[59]
Theonellamide F	Marine sponge ( <i>Theonella</i> )	Inhibits growth of various pathogenic fungi	Cytotoxicity	[60]

tryptathionine heptapeptide in 1978 by Zanotti *et al.* [16]. In this method, the first step of cyclization involved formation of a thioether bond between a cysteine residue and a derivative of L-tryptophan, which was generated by oxidation with peroxyacetic acid. The second cyclization (head-to-tail cyclization) was performed by the mixed anhydride method.

Kemp and McNamara described syntheses of the conformationally restricted cyclic nonapeptides cyclo-(Gly-L-Cys(Bzl)-Gly)<sub>3</sub>, cyclo-(L-Pr-Gly-L-Cys(Meb))<sub>3</sub> and cyclo-(L-Cys(Meb)-LL-Acp)<sub>3</sub>, in which Meb = *p*-methoxybenzyl and Acp = 3-amino-2-piperidone-6-carboxylic acid. In this method, a ring-forming reaction was performed by 1,3,5-tris(bromomethylene)benzene after removal of the S-blocking groups from each compound [20].

Sun *et al.* reported an efficient approach to synthesize amphipathic bicyclic peptides in 2001. The linear peptide precursors were produced using the Boc/BOP protocol [21]. The sequences of these precursors contained an N-terminal cysteine, a C-terminal thioester and an internal cysteine residue. First, cyclization of unprotected peptides was performed using an on-resin intramolecular thioester ligation reaction between N-terminal Cys and C-terminal thioester (N-to-C cyclization). Finally, bicyclic peptides were obtained by forming an off-resin intramolecular disulfide bond between N-terminal cysteine and an internal cysteine residue by DMSO oxidation (Fig. 1a).

In another strategy, Ghalit *et al.* synthesized bicyclic mimics of the lantibiotic nisin by ring-closing metathesis. In these alkene-/alkane-bridged bicyclic peptides, the disulfide bond and thioether bridge were replaced with alkene staples (Fig. 1b) [22]. Later, Mendive-Tapia *et al.* reported a new stapling methodology for the synthesis of peptides containing a C–C bond between tryptophan and phenylalanine or tyrosine residues via a selective palladium-catalyzed C–H arylation process (Fig. 1c) [23]. Grandas and colleagues reported a straightforward synthesis of bicyclic peptides from cysteine-containing peptides. In this approach, they formed bicyclic peptides via an intramolecular Michael addition reaction between thiols and maleimides [24].

Also, Teixeira *et al.* described solid-phase synthesis and characterization of *N*-methyl-rich peptides. Synthesis of high *N*-methylamino acid content peptides is challenging because the coupling of protected *N*-methylamino acids with *N*-methylamino acids generally occurs with low yields. They described (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) or PyBOP/1-hydroxy-7-azabenzotriazole (HOAt) as the most promising coupling reagents for these couplings. Also, they showed that the time of cleavage of the peptide from the resin affected loss of *Ac-N*-methylamino acid when a peptide contains an acetylated *N*-methylamino acid at the N-terminal position [25].

Bartoloni *et al.* showed that bicyclic homodetic peptides can be obtained by solid-phase peptide synthesis and two subsequent cyclizations. The first on-resin cyclization was performed through the formation of an amide bond between the  $\alpha$ - or  $\gamma$ -carboxyl group of glutamate and the N terminus. The resulting monocyclic peptides were then subjected to amide-bond formation conditions under high dilution to perform the second cyclization and to form bicyclic peptides (Fig. 1e) [26]. This strategy was also used to prepare bridged bicyclic peptides (BBPs) corresponding to the topology of bridged bicyclic alkanes, such as norbornane [27]. Recently, in 2016, Cromm and co-workers applied a chemically

orthogonal ring-closing olefin (RCM) and alkyne metathesis (RCAM) system to generate GTPase-targeting bicyclic peptides (Fig. 1d) [28]. In addition to the above methods, a simple way to synthesize bicyclic peptides is the linking of two monocyclic peptides by different linkers. For example, Oh *et al.* synthesized two different bicyclic peptides through linking monocyclic peptides using triazole and  $\beta$ -alanine ( $\beta$ -Ala) linkers [29].

### Screening of bicyclic peptides from a random library

A bicyclic peptide library contains a great number of bicyclic peptides and provides a powerful tool for the isolation of bicyclic peptides against a target of interest for various applications. To build large libraries and increase the chances of identifying bicyclic peptides with desired properties, various chemical and biological methods have been developed.

#### Chemical methods

As mentioned in the previous section, the first chemically synthesized library of nine bicyclic peptides was created by Sun *et al.* using intramolecular thioester ligation and DMSO-mediated disulfide formation [21]. To synthesize larger libraries and increase the likelihood of finding peptides with desired properties, other methods should be developed. The one-bead-two-compound (OBTC) approach is a simple method used to design bicyclic libraries, in which each cyclic peptide is encoded on the same bead by the corresponding linear peptide [9].

One of the main advantages of chemical synthesis of bicyclic peptides is the possibility of the incorporation of nonproteinogenic amino acids and nonpeptidic moieties into a peptide structure, which can increase the diversity and stability of the peptide structure. Upadhyaya and co-workers devised an approach for generating and screening large bicyclic peptide libraries displayed on rigid small-molecule scaffolds. Bicyclic peptides produced by this method contained random residues of 24 different amino acids (including ten proteinogenic amino acids, four nonproteinogenic  $\alpha$ -L-amino acids and ten  $\alpha$ -D-amino acids) in each ring. After screening of a chemically synthesized library against tumor necrosis factor (TNF) $\alpha$ , they recognized an element that inhibits the TNF $\alpha$ –TNFR interaction [9]. In another study, Upadhyaya *et al.* synthesized a bicyclic peptide library in OBTC format according to the previous approach, screened against the K-Ras G12V mutant and finally discovered several direct Ras inhibitors [30].

#### Biological methods

Another method for producing bicyclic peptides is biological production through ribosomal synthesis, which can generate large libraries with enormous diversity. Heinis *et al.* developed a phage strategy for generation of combinatorial chemical bicyclic peptide libraries and affinity selection [31]. In this strategy, they constructed a library of linear peptides using phage display. According to the phage display method, a library of peptides, including two sequences of six random amino acids flanked by three cysteines [Cys-(Xaa)<sub>6</sub>-Cys-(Xaa)<sub>6</sub>-Cys], was expressed on the surface of a filamentous phage. These linear phage-encoded peptides were tethered to the trifunctional compound tris(bromomethyl)benzene (TBMB) in a nucleophilic substitution reaction that alkylated the three Cys residues in the peptide sequences and converted the linear sequences into bicyclic peptides. Finally, they isolated

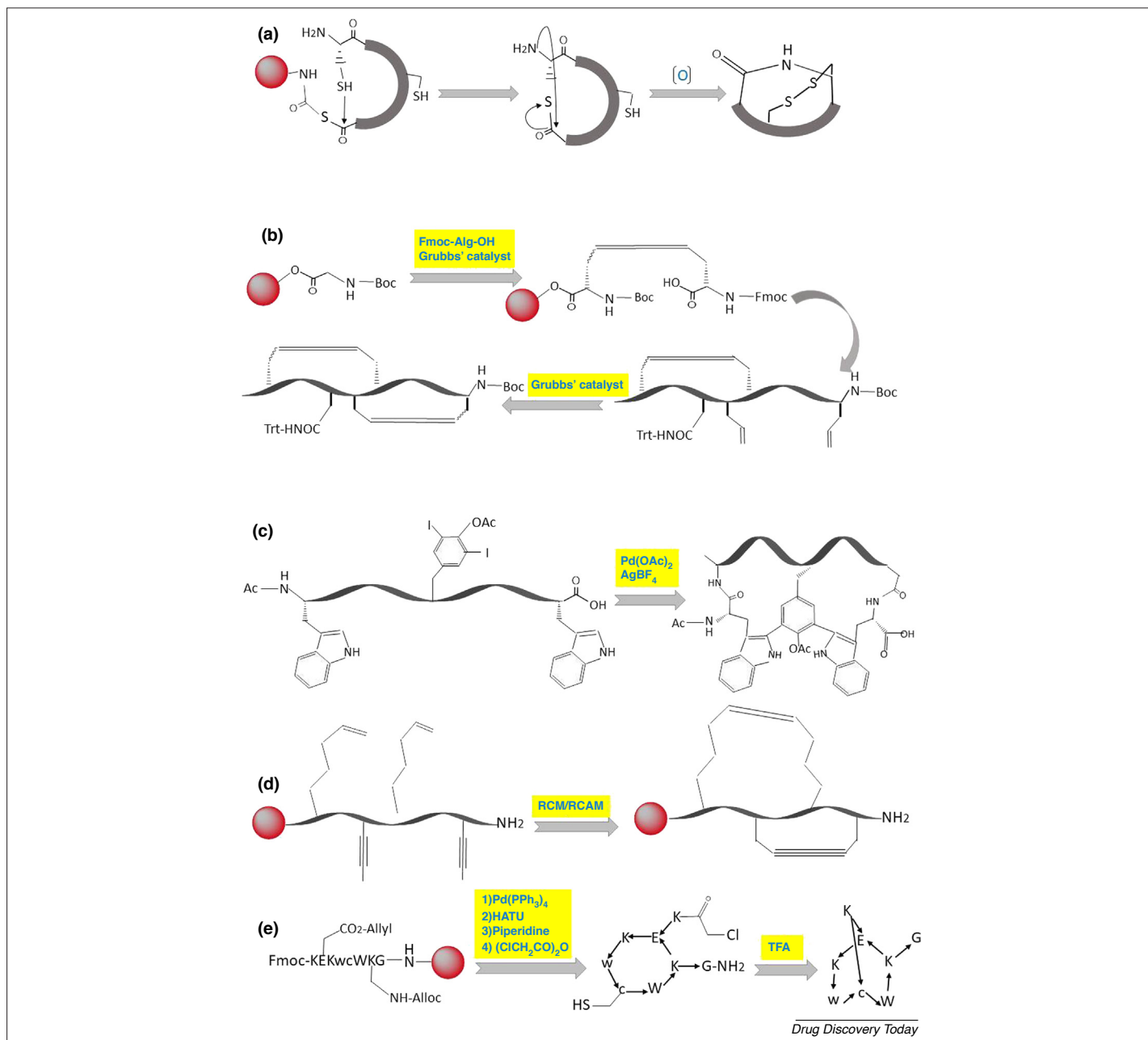


FIGURE 1

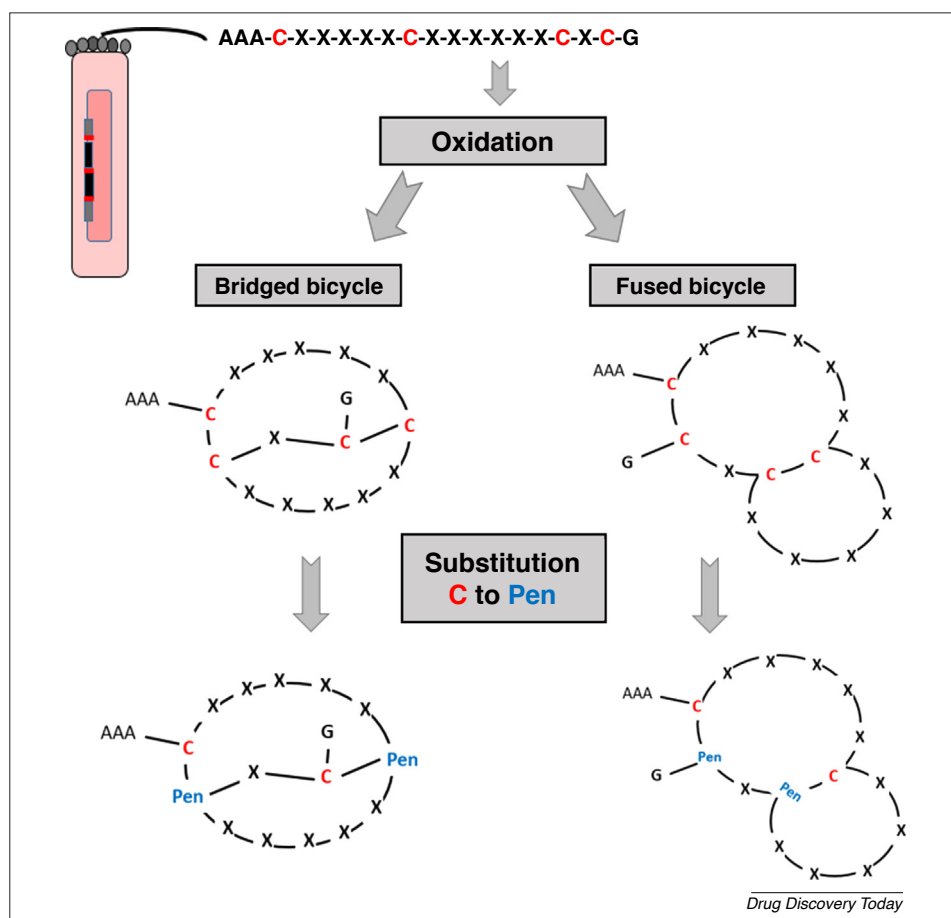
Various strategies for chemical synthesis of bicyclic peptides. (a) Synthesis through an on-resin intramolecular thioester ligation and an off-resin DMSO-mediated disulfide formation. (b) Synthesis by ring-closing metathesis. (c) Synthesis by formation of C–C bond (through an intramolecular palladium-catalyzed C–H activation process) between tryptophan and iodinated tyrosine or phenylalanine. (d) Synthesis through ring-closing alkyne metathesis. (e) Synthesis through the formation of an amide bond between the  $\alpha$ - or  $\gamma$ -carboxyl group of glutamate and the N terminus, then subjected to amide-bond formation conditions to perform the second cyclization. Abbreviations: RCM, ring-close metathesis; RCAM, ring-close alkyne metathesis.

potent human plasma kallikrein inhibitors ( $K_i = 1.5 \text{ nM}$ ) from a library of  $>10^9$  members [31].

In another study, Rebollo *et al.* produced bicyclic peptide phage libraries by combining dissimilar sized macrocyclic rings (Fig. 2). They cloned 14 phage-coded peptide libraries of the format Cys (Xaa)*m*-Cys-(Xaa)*n*-Cys, wherein Xaa are random amino acids and the numbers '*m*' and '*n*' were 3, 4, 5 or 6. The libraries were exposed to three rounds of affinity assortments against the cancer-associated protease uPA [32]. The phage display bicyclic peptide can also be generated through the formation of two disulfide bonds [33]

but, in peptides with several cysteine residues, disulfide isomerization can lead to multiple peptides with various biological actions. Chen *et al.* developed a dithiol amino acid (Dtaa) that can make two disulfide bridges at a single amino acid site and showed that substitution of two cysteines by Dtaas can prevent the formation of different disulfide bond isomers [34].

Very recently, Zheng *et al.* presented the *de novo* design of cysteine–penicillamine (Cys–Pen)-mixed peptide scaffolds, which can be folded into specific isomers [35]. The resulting sequence-independent peptide scaffolds, which were constrained with two

**FIGURE 2**

Construction of a phage-encoded peptide library of the format CXC(X)5C(X)5C. The CXC motif can direct the folding of randomized peptides on the phage surface toward either a fused or bridged bicyclic structure. After iterative affinity selections, the selected peptide sequence can then be structurally reshaped into a specific bicyclic structure (either fused or bridged) through orthogonal Cys–Pen disulfide pairing technology (only two of the four Pen-substituted analogs were present) [35].

noncanonical disulfide bonds, were isomerically pure and topologically formidable. Another phage-screening strategy for developing bicyclic peptides was reported by Zha *et al.*, in which a phage-encoded peptide library of the format CXC(X)5C(X)5C was synthesized. The CXC motif can direct the folding of phage-displayed peptides toward either a fused or bridged bicyclic structure. Finally, the selected peptide sequences were structurally reshaped into bicyclic structures using orthogonal Cys–Pen disulfide pairing technology. They obtained a library of  $4.5 \times 10^9$  independent transformants by transformation of the phagemid vector into *Escherichia coli* [36]. This strategy can be useful to develop bicyclic peptide ligands (Fig. 2).

Another display technique used for biological production of bicyclic peptides is mRNA display. In this cell-free technique, a covalent bond between each protein or peptide and its own encoding mRNA is formed using puromycin as a mimic of aminoacyl-tRNA [37]. The mRNA–protein complex is reverse transcribed to cDNA and employed for *in vitro* selection. The DNA sequences encoding selected peptides can be analyzed by sequencing [38]. Sako *et al.* developed a novel approach to construct bicyclic peptide scaffolds by modification of the mRNA display method. They reprogrammed the genetic code to construct a peptide scaffold containing a cysteine (Cys) at the N-terminus and three nonproteinogenic amino acids

[4-(2-chloroacetyl) aminobutyric acid (Cab), Aha, azidohomoalanine (Aha) and *Propargylglycine* (Pgl)]. Post-translational cyclizations performed by crosslinking between two pairs of amino acids, Cys–Cab and Aha–Pgl [39]. In a method recently described by Hacker *et al.*, two orthogonal cyclization steps (bisbromomethylbenzene cysteine alkylation and copper-mediated azide–alkyne cyclo addition) were used to make highly constrained bicyclic peptide libraries. In this strategy, the attachment points for each loop are independent, thereby enabling the formation of either manacle or theta-bridged bicyclic peptides, and *in vitro* selection of bicyclic peptides with desired characteristics can be done using mRNA display (Fig. 3) [40]. SICLOPPS is a split-intein-based technique for the intracellular construction of cyclic peptides [41]. The method of Bionda and Fasan: ribosomal production of bicyclic peptides in *E. coli*, involved the SICLOPPS method (for initial N-to-C circularization) combined with genetic incorporation of *O*-(2-bromoethyl)-tyrosine (O2beY) (for an inter-side-chain thioether bond-forming reaction between a cysteine residue and O2beY to form the bicyclic peptide) (Fig. 4) [42].

### Applications of bicyclic peptides in medicine

Diverse biological activities of naturally occurring bicyclic peptides have inspired scientists to identify and synthesize new bicyclic

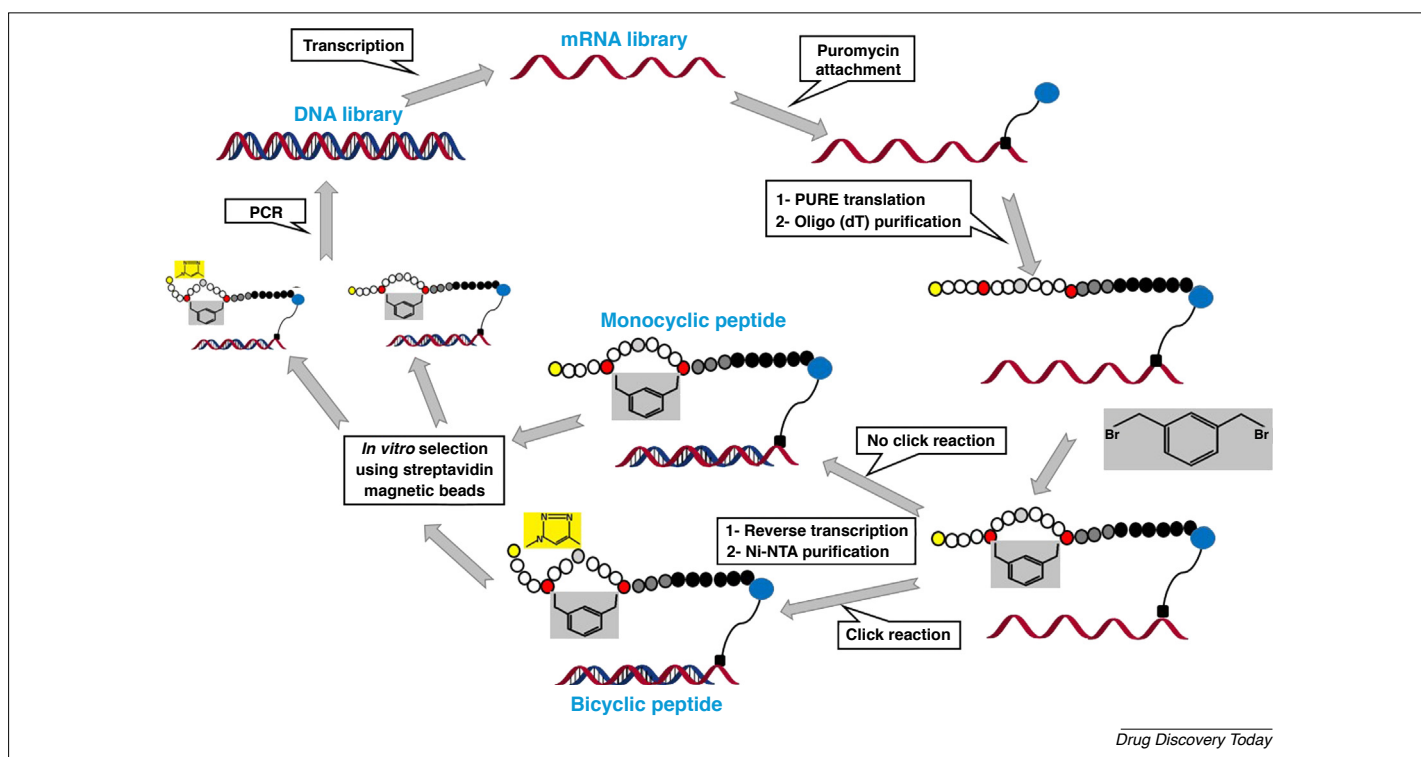


FIGURE 3

mRNA display scheme. Comparative *in vitro* selections against streptavidin. PURE translation is started with puromycin-linked mRNA. mRNA–peptide fusions are bis-alkylated with  $\alpha,\alpha'$ -dibromo-m-xylene (green box) and immobilized on oligo (dT) resin to form the monocyclic peptides. The monocyclic fusion pool is split after reverse transcription and bound to nickel resin, where the CuAAC reaction is performed on the portion of the pool targeted for bicyclic peptide formation (blue box). The monocyclic and bicyclic fusion pools are then used to select for high-affinity binders to streptavidin. Following competitive elution with biotin, functional fusions were amplified via PCR to generate the enriched round 2 DNA library. All steps were performed independently for subsequent rounds [39].

clic peptides for various applications, such as therapeutic agents, drug targeting, imaging and diagnostics.

### Therapeutic agents

#### Enzyme inhibitors

Some bicyclic peptides can act as enzyme inhibitors and be used as therapeutic agents in many diseases. For example, Jaulent and Leatherbarrow designed two potent bicyclic protease inhibitors by combining two advantageous features of two naturally occurring inhibitors: Bowman Birk inhibitor (BBI) and sunflower trypsin inhibitor (SFTI)-1. The resulting bicyclic and bifunctional protease inhibitors showed the potent inhibitory properties of the parent structures that are also relatively resistant to proteolysis; but they cannot inhibit two proteases at the same time [43].

In another study, two bicyclic peptide inhibitors of the serine protease urokinase-type plasminogen activator (uPA) were designed rationally by conversion of an established monocyclic peptide (upain-2) to bicyclic peptides. The affinities of two bicyclic peptide inhibitors were similar to upain-2, and isothermal titration calorimetry (ITC) data indicated that the bicyclic peptides reduced loss of entropy upon binding to uPA [44]. Islam *et al.* synthesized bicyclic tetrapeptide histone deacetylase (HDAC) inhibitors by designing and synthesizing CHAP31-based bicyclic tetrapeptide hydroxamic acids by altering the aliphatic loop length. The potent HDAC inhibitory activity of these nonaromatic bicyclic HDAC inhibitors was evaluated and confirmed *in vivo* and *in vitro* [45]. More recently, Teufel *et al.* discovered stable and long-lasting

bicyclic peptide inhibitors against plasma kallikrein (PKal) using a combination of phage display and chemical cyclization. The efficacy of these peptides in inhibition of PKal activity was confirmed by different *in vitro* assays and in different biological matrices and demonstrated that bicyclic constrained peptides act as potent and highly specific PKal inhibitors that can be useful in the treatment of diabetic retinopathy and diabetic macular edema (DME) [46].

#### PPI inhibitors

Targeting and inhibition of PPIs has appeared as a novel approach for discovering and developing new therapeutics for complex and multifactorial diseases. Therefore, one of the main goals of developing bicyclic peptides is the development of inhibitors against PPIs; and, in this regard, several bicyclic peptide PPI inhibitors have been synthesized. For example, Grb2, a signaling adaptor, has a key role in receptor tyrosine kinase signaling pathways, and Grb2 inhibitors could be useful as anticancer drugs. A bicyclic peptide inhibitor of the SH2 domain of Grb2 was synthesized by conversion of a monocyclic peptide into bicyclic peptide, enhancing its potency and selectivity for Grb2 SH2 [8].

In another study, Lian *et al.* identified a bicyclic peptide inhibitor of the TNF $\alpha$ –TNFR interaction by screening of large combinatorial libraries of bicyclic peptides. The resulting bicyclic peptide PPI inhibitor can protect from cell death induced by TNF $\alpha$  [9]. In a recent study, Guardiola *et al.* designed metabolically stable bicyclic peptides targeting the epidermal growth factor (EGF)–EGFR interaction. EGF contributes to tumor progression and performs its

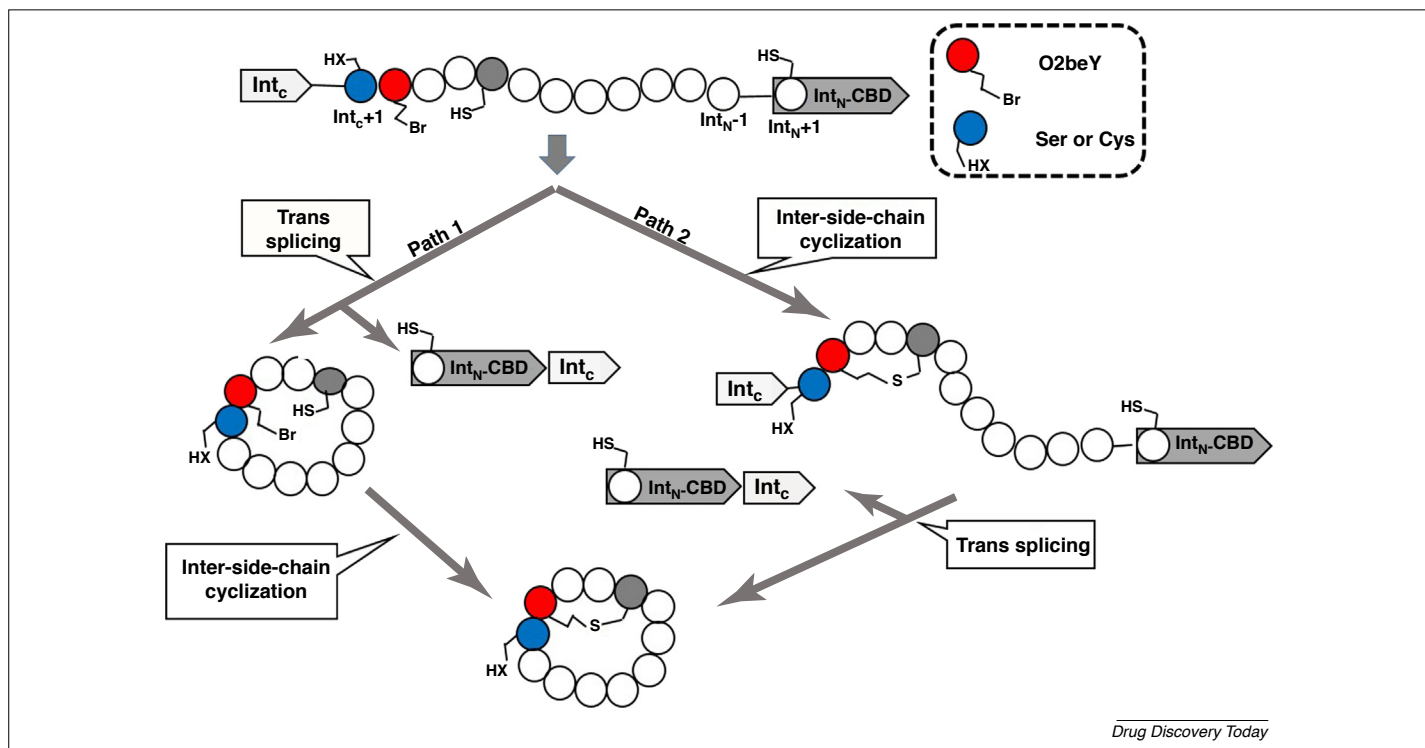


FIGURE 4

The approach to ribosomal synthesis of thioether-bridged bicyclic peptides in *Escherichia coli*. From N to C, the linear precursor polypeptide includes the split intein DnaE C-terminal domain (IntC), a Ser or Cys residue at IntC++1 site, the unnatural amino acid O-(2-bromoethyl)-tyrosine (O2beY or 'Z'), a variable target sequence comprising the reactive cysteine and a DnaE N-terminal domain attached to a chitin-binding domain (IntN-CBD). The two intended paths to make the bicyclic product are indicated [41].

function by binding to its receptor (EGFR), which initiates intracellular events leading to tumor development. A series of bicyclic-constrained peptides mimicking one of the interacting domains of EGFR was designed using a combination of *in silico* and biophysical methods. The inhibitory effect of these peptides on the EGF-EGFR interaction was established in a specific PPI-disruption assay [47]. These peptides also selectively decrease the viability of human cancer cells expressing a high level of EGFR, with mid-micromolar IC<sub>50</sub> values [47]. The efficacy of these peptides, in addition to their metabolic stability, makes them suitable as complementary therapeutics in cancer therapy.

#### Receptor agonist and antagonist

Some bicyclic peptides act as agonists or antagonists for cell surface receptors, which are either naturally occurring or rationally designed (Fig. 5a). For example, BI-32169 is a naturally occurring bicyclic 19-peptide isolated from *Streptomyces* sp. (DSM 14996) and is a potent human glucagon receptor antagonist [48]. Jia *et al.* developed a specific bicyclic peptide antagonist (EG3287) of vascular endothelial growth factor (VEGF)-A165 binding to neuropilin (NP)-1, which is a receptor for VEGF in endothelial cells. By characterization of this antagonist, they reported that the exon 8 of VEGF is very important for NP-1 binding, and NP-1 is needed for optimum kinase insert domain receptor (KDR) signaling [49].

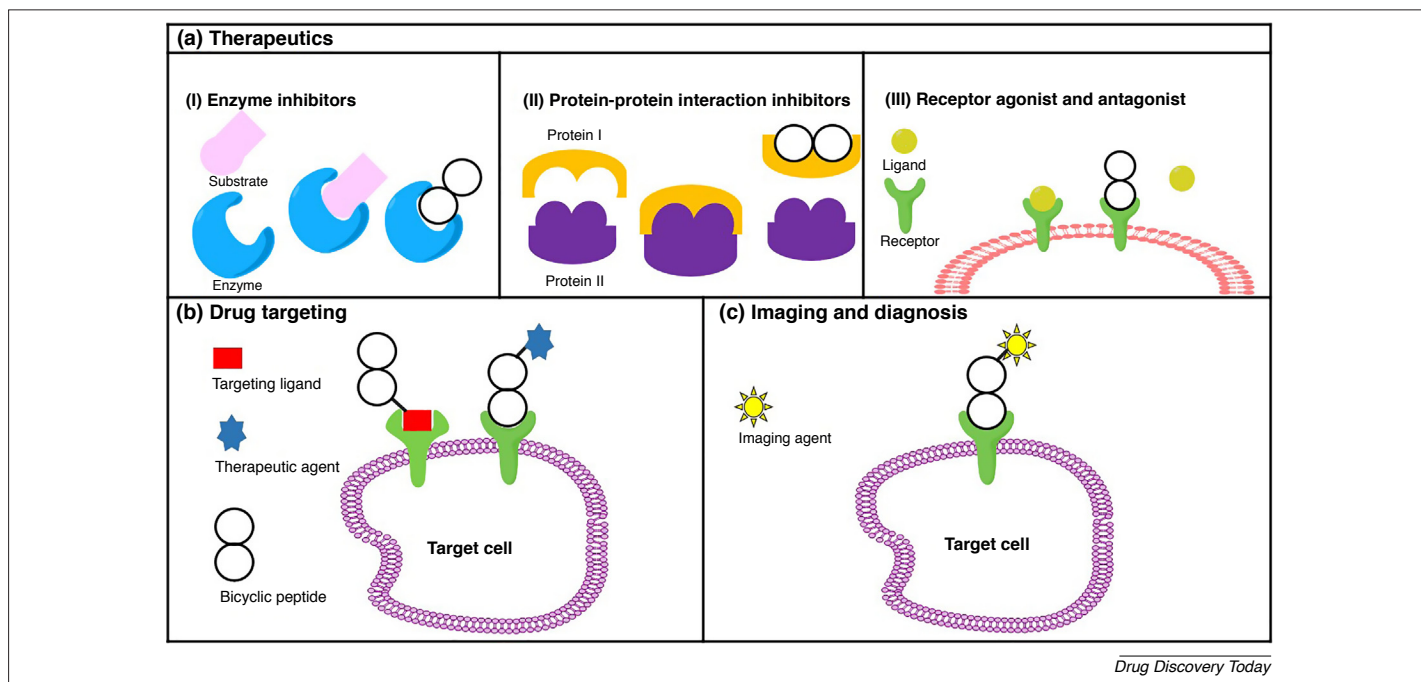
#### Drug targeting

Bicyclic peptides can be ideal candidates for drug targeting owing to their small size and their ability to bind to different protein targets with good affinity and specificity (Fig. 5b). Moreover, they also exhibit

better proteolytic stability and improved plasma stability compared with linear peptides [50,51]. Paclitaxel (PTX), a mitotic inhibitor, binds to tubulin and prevents the disassembly of microtubules and is used as a microtubule-stabilizing drug in cancer treatment. Chen *et al.* conjugated paclitaxel with a bicyclic peptide E[c(RGDyK)]<sub>2</sub> (RGD) and evaluated its antitumor activity in a metastatic breast cancer cell line [52]. Because RGD peptide can selectively bind to  $\alpha_v$  integrin receptors, conjugation of PTX to RGD allowed its targeted delivery to tumor cells expressing  $\alpha_v\beta_3$  integrin. Bicycle Therapeutics (Cambridge, MA) developed the bicyclic peptide platform based on bicyclic-peptide-drug conjugates (BDCs) for cell-specific targeting of chemotherapeutic agents. BT1718 is a Bicycle drug conjugate developed by Bicycle Therapeutics to target tumors [53]. BT1718 binds selectively to membrane type 1 matrix metalloprotease (MT1-MMP) with high affinity and is a promising compound to treat solid tumors. MT1-MMP is a tumor-associated membrane-anchored endopeptidase overexpressed in a variety of cancer cells. The results of recent studies show that BDCs can be developed as alternatives to antibody-drug conjugates and a superior strategy for targeted drug delivery [53].

#### Imaging and diagnosis (PET scan imaging)

Bicyclic peptides can develop into diagnostic imaging agents, especially as positron emission tomography (PET) tracers (Fig. 5c). The main advantages of bicyclic peptides over other protein binders (particularly monoclonal antibodies) are their faster clearance from the blood circulation owing to their smaller sizes, resulting in shorter imaging times delay, and better signal-to-noise ratio (SNR) [1]. Park *et al.* synthesized two bicyclic RGD peptides: c(RGD-ACP-K) and



**FIGURE 5**  
Applications of bicyclic peptides in medicine.

c(RGD-ACH-K), by incorporating aminocyclopentane (ACP) or aminocyclohexane (ACH) into a monocyclic RGD peptide. After conjugation to DOTA and radiolabeling with radiometal  $^{64}\text{Cu}$ , the efficiency of these peptides as PET radiotracers was evaluated for cancer imaging. Both bicyclic peptides and their DOTA conjugates exhibited high affinities toward U87MG glioblastoma cells, which are better than the monocyclic c(RGDyK). The corresponding  $^{64}\text{Cu}$  complexes showed excellent stability in human and mouse serum and high tumor uptake, as observed by PET, which demonstrated their promise as PET radiotracers for tumor imaging [54]. In another example, Eder *et al.* identified a bicyclic peptide targeting MT1-MMP (tumor-overexpressed matrix metalloproteinase) with subnanomolar affinity and showed the power of its radioconjugation as a tumor-targeting probe in an HT1080 xenograft mouse model. *In vivo* studies using different mouse xenograft models showed high affinity to MT1-MMP ( $K_d$  at 1 nM) and, compared to radiolabeled antibodies directed against the same target, showed rapid tumor penetration characteristics and high potency and chemical versatility [55].

#### As antimicrobial agents

Di Bonaventura *et al.* discovered that antimicrobial bridged bicyclic peptides (AMBPs) were active against *Pseudomonas aeruginosa* and its biofilm. Screening of all AMBPs for antibiofilm activity led to the identification of two potent *P. aeruginosa* biofilm inhibitors that were

enhancers of the activity of polymyxin. They used the concept of chemical space to discover their AMBPs and calculated their chemical space using 2DP—a 136-dimensional molecular fingerprint for peptides. These results suggest that bridged bicyclic peptides can be useful as a new class of antimicrobial peptides [56]. In a recent study, this group identified an antimicrobial bicyclic peptide with potent activity against several multidrug-resistant Gram-negative bacteria. They explored a virtual bicyclic peptide library using the concept of chemical space [57].

#### Concluding remarks

Bicyclic peptides (with two macrocyclic rings) have advantages over linear and monocyclic peptides owing to their size, structure, functionality and increased conformational rigidity. This class of polypeptides has some of the favorable properties of monoclonal antibodies and small molecules and, therefore, can have different therapeutic and diagnostic applications. Various chemical, biological and combinatorial methods have been developed to synthesize large bicyclic peptide libraries and increase the chances of identifying peptides with desired properties. Owing to the similarity of antibodies and peptides in the affinity and specificity to various proteins, it is anticipated that, in the future, peptides will play an important part as therapeutics, diagnostics agents and research tools rather than antibodies, because they have a less complex structure, greater metabolic stability and lower cost of production.

#### References

- Rhodes, C.A. and Pei, D. (2017) Bicyclic peptides as next-generation therapeutics. *Chemistry* 23, 12690–12703
- Lau, J.L. and Dunn, M.K. (2018) Therapeutic peptides: historical perspectives, current development trends, and future directions. *Bioorg. Med. Chem.* 26, 2700–2707
- Joo, S.H. (2012) Cyclic peptides as therapeutic agents and biochemical tools. *Biomol. Ther.* 20, 19–26
- Saijo, K. *et al.* (2012) Romidepsin (FK228) and its analogs directly inhibit phosphatidylinositol 3-kinase activity and potentially induce apoptosis as histone deacetylase/phosphatidylinositol 3-kinase dual inhibitors. *Cancer Sci.* 103, 1994–2001
- Edler, M.C. *et al.* (2002) Inhibition of tubulin polymerization by vitilevuamide, a bicyclic marine peptide, at a site distinct from colchicine, the vinca alkaloids, and dolastatin 10. *Biochem. Pharmacol.* 63, 707–715
- Liu, X.F. *et al.* (2016) Actinomycin D enhances killing of cancer cells by immunotoxin RG7787 through activation of the extrinsic pathway of apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* 113, 10666–10671



- 7 Foster, A.D. *et al.* (2015) Methods for the creation of cyclic peptide libraries for use in lead discovery. *J. Biomol. Screen.* 20, 563–576
- 8 Quartararo, J.S. *et al.* (2014) A bicyclic peptide scaffold promotes phosphotyrosine mimicry and cellular uptake. *Bioorg. Med. Chem.* 22, 6387–6391
- 9 Lian, W. *et al.* (2013) Screening bicyclic peptide libraries for protein–protein interaction inhibitors: discovery of a tumor necrosis factor- $\alpha$  antagonist. *J. Am. Chem. Soc.* 135, 11990–11995
- 10 Sun, W.J. *et al.* (2017) Romidepsin induces G2/M phase arrest via Erk/cdc25C/cdc2/cyclinB pathway and apoptosis induction through JNK/c-Jun/caspase3 pathway in hepatocellular carcinoma cells. *Biochem. Pharmacol.* 127, 90–100
- 11 Reddy, S.A. (2016) Romidepsin for the treatment of relapsed/refractory cutaneous T-cell lymphoma (mycosis fungoides/Sezary syndrome): use in a community setting. *Crit. Rev. Oncol. Hematol.* 106, 99–107
- 12 Wieland, T. and Faulstich, H. (1978) Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms. *CRC Crit. Rev. Biochem.* 5, 185–260
- 13 Bukatina, A.E. and Sik, G.S. (2003) [Phallotoxins as an instrument for studying the intramyofibrillar regulation of muscle contraction]. *Biofizika* 48, 1086–1093
- 14 Anderl, J. *et al.* (2012) Chemical modification allows phallotoxins and amatoxins to be used as tools in cell biology. *Beilstein J. Org. Chem.* 8, 2072–2084
- 15 Thiede, B.R. and Corwin, J.T. (2014) Permeation of fluorophore-conjugated phalloidin into live hair cells of the inner ear is modulated by P2Y receptors. *J. Assoc. Res. Otolaryngol.* 15, 13–30
- 16 Zanotti, G. *et al.* (1978) Syntheses of monocyclic and bicyclic peptides of tryptathionine and glycine. *Int. J. Pept. Protein Res.* 12, 204–216
- 17 Yang, X. *et al.* (2017) Synthesis of bicyclic tripeptides inspired by the ABC-ring system of vancomycin through ruthenium-based cyclization chemistries. *Tetrahedron Lett.* 58, 4542–4546
- 18 Rodriguez, M.C. *et al.* (2017) Bicyclic peptide based lectinomimic. *Croat. Chem. Acta* 90, 699–705
- 19 Lian, W. *et al.* (2014) Cell-permeable bicyclic peptide inhibitors against intracellular proteins. *J. Am. Chem. Soc.* 136, 9830–9833
- 20 Kemp, D. and McNamara, P.E. (1985) Conformationally restricted cyclic nonapeptides derived from L-cysteine and  $\beta$ -3-amino-2-piperidone-6-carboxylic acid ( $\beta$ -Acp), a potent  $\beta$ -turn-inducing dipeptide analog. *J. Org. Chem.* 50, 5834–5838
- 21 Sun, Y. *et al.* (2001) A thioester ligation approach to amphipathic bicyclic peptide library. *Org. Lett.* 3, 1681–1684
- 22 Ghalit, N. *et al.* (2007) Synthesis of bicyclic alkene/alkane-bridged nisin mimics by ring-closing metathesis and their biochemical evaluation as lipid II binders: toward the design of potential novel antibiotics. *Chembiochem* 8, 1540–1554
- 23 Mendive-Tapia, L. *et al.* (2015) New peptide architectures through C–H activation stapling between tryptophan-phenylalanine/tyrosine residues. *Nat. Commun.* 6, 7160
- 24 Elduque, X. *et al.* (2013) Straightforward synthesis of cyclic and bicyclic peptides. *Org. Lett.* 15, 2038–2041
- 25 Teixidó, M. *et al.* (2005) Solid-phase synthesis and characterization of N-methyl-rich peptides. *J. Peptide Res.* 65, 153–166
- 26 Bartoloni, M. *et al.* (2011) Expanding the accessible chemical space by solid phase synthesis of bicyclic homodetic peptides. *Chem. Commun.* 47, 12634–12636
- 27 Pollaro, L. *et al.* (2015) Bicyclic peptides conjugated to an albumin-binding tag diffuse efficiently into solid tumors. *Mol. Cancer Ther.* 14, 151–161
- 28 Cromm, P.M. *et al.* (2016) Orthogonal ring-closing alkyne and olefin metathesis for the synthesis of small GTPase-targeting bicyclic peptides. *Nat. Commun.* 7, 11300
- 29 Oh, D. *et al.* (2014) Amphiphilic bicyclic peptides as cellular delivery agents. *ChemMedChem* 9, 2449–2453
- 30 Upadhyaya, P. *et al.* (2014) Direct Ras inhibitors identified from a structurally rigidified bicyclic peptide library. *Tetrahedron* 70, 7714–7720
- 31 Heinis, C. *et al.* (2009) Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nat. Chem. Biol.* 5, 502–507
- 32 Rebollo, I.R. *et al.* (2013) Phage display libraries of differently sized bicyclic peptides. *Med. Chem. Commun.* 4, 145–150
- 33 Urech-Varenne, C. *et al.* (2015) Phage selection of bicyclic peptide ligands of the Notch1 receptor. *ChemMedChem* 10, 1754–1761
- 34 Chen, S. *et al.* (2014) Dithiol amino acids can structurally shape and enhance the ligand-binding properties of polypeptides. *Nat. Chem.* 6, 1009–1016
- 35 Zheng, Y. *et al.* (2018) De novo design of constrained and sequence-independent peptide scaffolds with topologically-formidable disulfide connectivities. *Chem. Sci.* 9, 569–575
- 36 Zha, M. *et al.* (2018) A phage display-based strategy for the de novo creation of disulfide-constrained and isomer-free bicyclic peptide affinity reagents. *Chem. Commun.* 54, 4029–4032
- 37 Josephson, K. *et al.* (2014) mRNA display: from basic principles to macrocycle drug discovery. *Drug Discov. Today* 19, 388–399
- 38 Takahashi, T.T. *et al.* (2003) mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.* 28, 159–165
- 39 Sako, Y. *et al.* (2008) Ribosomal synthesis of bicyclic peptides via two orthogonal inter-side-chain reactions. *J. Am. Chem. Soc.* 130, 7232–7234
- 40 Hacker, D.E. *et al.* (2017) Highly constrained bicyclic scaffolds for the discovery of protease-stable peptides via mRNA display. *ACS Chem. Biol.* 12, 795–804
- 41 Osher, E.L. and Tavassoli, A. (2017) Intracellular production of cyclic peptide libraries with SICLOPPS. *Methods Mol. Biol.* 1495, 27–39
- 42 Bionda, N. and Fasan, R. (2015) Ribosomal synthesis of natural-product-like bicyclic peptides in *Escherichia coli*. *Chembiochem* 16, 2011–2016
- 43 Jaulent, A.M. and Leatherbarrow, R.J. (2004) Design, synthesis and analysis of novel bicyclic and bifunctional protease inhibitors. *Protein Eng. Des. Sel.* 17, 681–687
- 44 Roodbeen, R. *et al.* (2013) Bicyclic peptide inhibitor of urokinase-type plasminogen activator: mode of action. *Chembiochem* 14, 2179–2188
- 45 Islam, N.M. *et al.* (2010) Bicyclic peptides as potent inhibitors of histone deacetylases: optimization of alkyl loop length. *Bioorg. Med. Chem. Lett.* 20, 997–999
- 46 Teufel, D.P. *et al.* (2018) Stable and long-lasting, novel bicyclic peptide plasma kallikrein inhibitors for the treatment of diabetic macular edema. *J. Med. Chem.* 61, 2823–2836
- 47 Guardiola, S. *et al.* (2018) Toward a novel drug to target the EGF–EGFR interaction: design of metabolically stable bicyclic peptides. *Chembiochem* 19, 76–84
- 48 Potterat, O. *et al.* (2004) BI-32169, a bicyclic 19-peptide with strong glucagon receptor antagonist activity from *Streptomyces* sp. *J. Nat. Prod.* 67, 1528–1531
- 49 Jia, H. *et al.* (2006) Characterization of a bicyclic peptide neuropilin-1 (NP-1) antagonist (EG3287) reveals importance of vascular endothelial growth factor exon 8 for NP-1 binding and role of NP-1 in KDR signaling. *J. Biol. Chem.* 281, 13493–13502
- 50 Diderich, P. and Heinis, C. (2013) Directed evolution of bicyclic peptides for therapeutic application. *Chimia* 67, 910–915
- 51 Baeriswyl, V. and Heinis, C. (2013) Polycyclic peptide therapeutics. *ChemMedChem* 8, 377–384
- 52 Chen, X. *et al.* (2005) Synthesis and biological evaluation of dimeric RGD peptide-paclitaxel conjugate as a model for integrin-targeted drug delivery. *J. Med. Chem.* 48, 1098–1106
- 53 Bennett, G. *et al.* (2016) Development of BT1718, a Bicycle Drug Conjugate® (BDC) targeting MT1–MMP for treatment of solid tumours. *Eur. J. Cancer* 69, S21
- 54 Park, J.A. *et al.* (2014) Cyclic RGD peptides incorporating cycloalkanes: synthesis and evaluation as PET radiotracers for tumor imaging. *ACS Med. Chem. Lett.* 5, 979–982
- 55 Eder, M. *et al.* (2019) Bicyclic peptides as a new modality for imaging and targeting of proteins overexpressed by tumors. *Cancer Res.* <http://dx.doi.org/10.1158/0008-5472.CAN-18-0238>
- 56 Di Bonaventura, I. *et al.* (2017) Chemical space guided discovery of antimicrobial bridged bicyclic peptides against *Pseudomonas aeruginosa* and its biofilms. *Chem. Sci.* 8, 6784–6798
- 57 Di Bonaventura, I. *et al.* (2018) An antimicrobial bicyclic peptide from chemical space against multidrug resistant Gram-negative bacteria. *Chem. Commun.* 54, 5130–5133
- 58 Ma, B. *et al.* (2010) Total synthesis of the antimitotic bicyclic peptide celogentin C. *J. Am. Chem. Soc.* 132, 1159–1171
- 59 Matinkhoo, K. *et al.* (2018) Synthesis of the death-cap mushroom toxin alpha-amanitin. *J. Am. Chem. Soc.* 140, 6513–6517
- 60 Wada, S. *et al.* (1999) Theonellamide F, a bicyclic peptide marine toxin, induces formation of vacuoles in 3Y1 rat embryonic fibroblast. *Mar. Biotechnol. (NY)* 1, 337–341