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Cyclization of peptides with two chemical bridges affords large scaffold diversities

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Successful screening campaigns depend on large and structurally diverse collections of compounds. In macrocycle screening, variation of the molecular scaffold is important for structural diversity, but so far it has been challenging to diversify this aspect in large combinatorial libraries. Here, we report the cyclization of peptides with two chemical bridges to provide rapid access to thousands of different macrocyclic scaffolds in libraries that are easy to synthesize, screen and decode. Application of this strategy to phage-encoded libraries allowed for the screening of an unprecedented structural diversity of macrocycles against plasma kallikrein, which is important in the swelling disorder hereditary angioedema. These libraries yielded inhibitors with remarkable binding properties (subnanomolar K_i, >1,000-fold selectivity) despite the small molecular mass (~1,200 Da). An interlaced bridge format characteristic of this strategy provided high proteolytic stability ($t_{1/2}$ in plasma of >3 days), making double-bridged peptides potentially amenable to topical or oral delivery.

Macrocycles have emerged as an interesting therapeutic class because they can bind to challenging targets that are not easily accessible to traditional small-molecule compounds[1,2]. They owe their favourable binding properties to their larger size, which enables interactions with extended surfaces where small molecules cannot normally bind, and a ring-shaped structure that limits the conformational flexibility, and thus the entropic penalty, upon target binding[3]. The vast majority of macrocyclic drugs are natural products or derivatives thereof[4]. For many targets, there are no available natural-product-derived macrocycles, or the high complexity of the natural product hampers their synthetic modification and optimization. Thus, there is a need for synthetic macrocycles developed in a combinatorial fashion, which can greatly reduce the overall complexity of the discovery process. The necessary library diversity required to make these combinatorial macrocycles can be acquired by incorporating variable backbone structures (macrocyclic scaffold), by decorating the scaffolds with diverse functional groups, and by attaching peripheral substituents outside the main ring. Variation of the macrocyclic scaffold is considered to be the most important determinant of structural diversity[5]. Therefore, a key step to achieve high structural diversity in combinatorial macrocyclic libraries is to find a way to incorporate these variable scaffolds into the screening system. To this end, a range of elegant diversity-oriented synthesis strategies have been published in recent years[6]. However, the development of strategies to diversify the scaffolds in large combinatorial libraries, ideally comprising millions of macrocycles, remains an important challenge.

Display technologies such as phage and mRNA display have enabled the generation and screening of enormously large numbers of peptide macrocycles, typically reaching several billion compounds at a time[7–10]. Efficient chemical reactions allow for the transformation of genetically encoded linear peptides into cyclic structures, which can provide access to variably cyclized peptides while retaining the benefits of the genetically encoded library[11,12]. Typically, two or three amino acids are ligated to generate mono- or bicyclic peptide libraries. By combining orthogonal reactions, more complex macrocycle structures have also been generated. Recent technology innovations have enabled the incorporation of unnatural amino acids into mRNA display, expanding the diversity of chemical groups that can be used to build these types of libraries[13–15]. To this point, however, a major limitation of phage or mRNA display macrocyclic libraries is the low scaffold diversity. Due to the dependence on ribosomal translation, the backbones of the macrocycles are required to be polypeptidic in nature. In most of the reported libraries, the macrocycles contain a single polypeptide scaffold, and the diversity is based only on variation of the amino-acid side chains. By varying the number of amino acids in the random peptides[14,16,17] or the chemical cyclization linkers[18], libraries comprising several different scaffolds were generated, but none of the reported libraries were designed to contain more than a dozen different scaffolds.

Here we report an efficient and robust strategy for generating
r(c)-3(9000001(d(e)-5(i0001 s-)-5(i-6()11.8999996 1.4)12(ra)-9(o)-16(y)8(c)6.0
amino acids, respectively (highlighted in red, orange and green in Fig. 1a). Altering the number of random amino acids in each seg

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ment in a combinatorial fashion allows for the generation of a much larger scaffold diversity than when peptides are divided by only two or three cysteines into fixed segments. Additional structural diversity stems from the connectivity between the cysteines, due to the fact that four cysteines can be connected by two chemical linkers in three different ways. The greater number of different bicyclic peptide scaffolds that can be generated with two chemical bridges as opposed to only one is exemplified for nine amino-acid peptides in Fig. 1b. Cyclizing nonapeptides with one bridge (three cysteines, six random amino acids) yields only seven different bicyclic scaffolds, while cyclizing with two bridges (four cysteines, five random amino acids) yields 63 different bicyclic scaffolds. This already provides a ninefold improvement in diversity, and this will increase with increasing peptide size. For peptide macrocycles between 8 and 14

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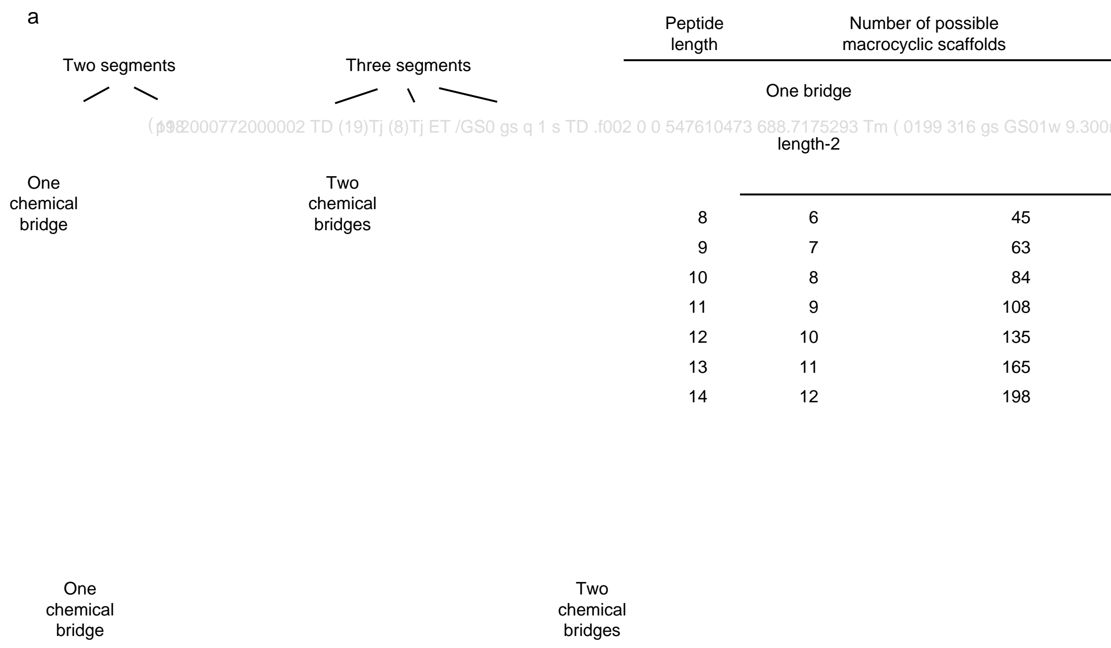

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Peptide length	Number of possible macrocyclic scaffolds	
	One bridge length-2	
8	6	45
9	7	63
10	8	84
11	9	108
12	10	135
13	11	165
14	12	198

amino acids in length (including the four cysteines), a total of 798 different di-bridged bicyclic peptide formats can be obtained versus the 63 that are possible with a mono-bridge connecting three cysteines. The scaffold diversity can be further increased by cyclizing the same peptide library in parallel with many different chemical bridges that can further vary the ring size as well as include functional groups that can participate in binding. Dozens of reagents with two thiol-reactive groups are commercially available[19–21], in direct contrast to the only two commercially available reagents with three symmetrical thiol-reactive groups[18]. The large scaffold diversity obtained by cyclizing peptides with two chemical bridges is illustrated in the animated Supplementary slide show (provided as a PowerPoint presentation and and animated gif). This calculation based on the cyclization of 8- to 14-mer peptides with 10 different bridges yields more than 7,000 different macrocyclic scaffolds. It is likely that some of these formats were already accidentally generated in previous work when cysteine alkylation was utilized to generate monocyclic peptide libraries of the form CX_nC and two additional cysteines appeared in the randomized region[14,23–25]. However, clones with four cysteines had such a low abundance in these libraries that no double-bridged peptides were isolated.

An important goal of our laboratory is the development of small, highly stable peptide macrocycles that can be applied topically or orally. Developing such small and compact ligands can be challenging because they have fewer amino acids that can interact with targets. We speculated that this handicap may be overcome by a molecular shape that is perfectly complementary to a target binding site, and that such molecules could potentially be isolated from macrocyclic libraries with high scaffold diversities. We thus applied the 'double-bridge' cyclization strategy to small-sized peptides and used these to isolate inhibitors of plasma kallikrein. This protein is an important target of the swelling disorder hereditary angioedema (HAE), for which injectable protein-based inhibitors are already in clinical use[26,27] or development[28], but no small-molecule drug is currently available.

Results

A number of reagents containing thiol-reactive groups have been reported to efficiently crosslink or cyclize peptides and proteins via

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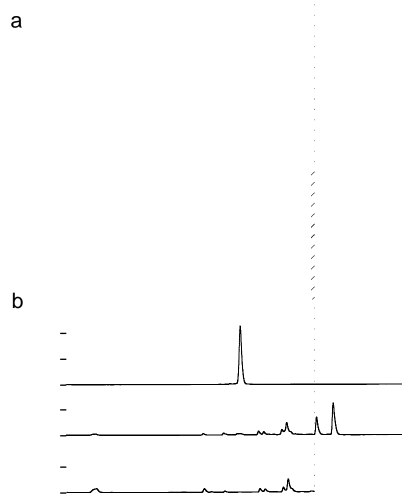

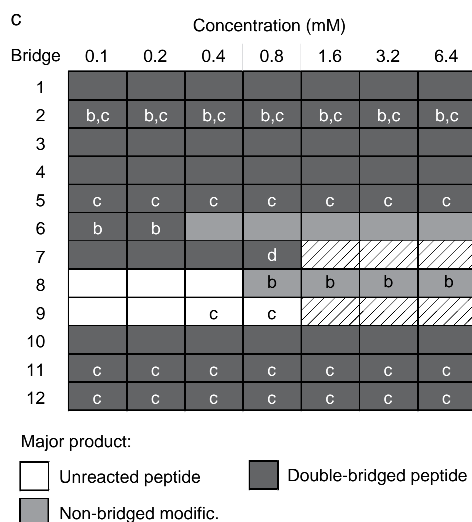

cysteines in aqueous buffer and under mild conditions, including benzylbromides[29,30], allylhalides[31], haloacetamides[32], haloacetones[33], bromopyridazinediones[34] and bromomaleimides[35]. Of those, we chose 12 structurally diverse compounds and tested their ability to quantitatively and selectively link two pairs of cysteines in a model peptide in conditions that are compatible with phage display (ACSRCVECGWCG-NH_2; Fig. 2a). Eight of the 12 reagents produced the double-bridged peptide as the main product in a wide range of concentrations, suggesting that the reactions are robust and would work efficiently with peptides of variable sequences (Fig. 2b,c). The observed side products were mostly peptides with only one bridge and, at higher reagent concentrations, peptides that were modified with more than two linkers such that the cysteines were not bridged. Most of the thiol-reactive compounds did not inactivate filamentous phage or only interfered at concentrations far above those needed to fully cyclize the model peptide (Supplementary Fig. 1).

We applied compounds 1, 3, 4, 7, 10 and 12 to two peptide phage display libraries, each comprising more than 100 million random peptides of the formats XCX_3CX_3CX-phage (library 1) and XCX_4CX_4CX-phage (library 2). All of the peptides of these libraries contain cysteines in three fixed positions (C), and some contain additional cysteines in random positions (X). There was a 20% and 23% chance, respectively, of a peptide containing four cysteines due to the probability of a cysteine occurring in the random NNK-encoded positions. Cyclization of the peptides yielded 54 different backbone formats for each linker (Supplementary Fig. 2), producing 324 different macrocyclic scaffolds in total. The two libraries each cyclized with the six different linkers were individually panned against immobilized plasma kallikrein. High-throughput sequencing of phage isolated after two rounds of selection showed an enrichment for peptides containing four cysteines (Supplementary Table 1), that the fourth cysteine was localized to certain positions depending on the thiol-reactive reagent used (Fig. 3 for library 1; Supplementary Fig. 3 for library 2), and that peptides shared strong consensus sequences (Supplementary Fig. 4). These findings suggested that the library peptides were efficiently cyclized on phage, due to the strong prevalence of four cysteines, and that target-selective peptides were isolated because of the consensus. In phage selections with linkers 3 and 10, certain peptide formats were particularly enriched (Supplementary Fig. 4). For example, most peptides cyclized with linker 3 contained the fourth cysteine in amino-acid position 3. In the selection with linker 10, many of the peptides isolated had the truncated format XCX_2CCX_2CX, which contained 10 instead of 11 amino acids. Such truncated peptides are generated during library cloning through erroneous DNA primers and occur only rarely in the finished library. The strong enrichment of some peptide formats, especially such rare formats, suggested that certain molecular scaffolds are particularly suited for target binding. This finding was the first hint that the high scaffold diversity obtained through the double-bridge strategy was key for the isolation of binders.

We synthesized several peptides and cyclized them by randomly bridging two pairs of cysteines through all three possible combinations, and each isomer was separately isolated

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and tested for binding. We picked the short peptides of 10 or 11 amino acids isolated from library 1 cyclized with linkers 3 and 10 due to our interest in small macrocycles (~1,200 Da). The inhibition constant (K_i) values of the most active isomer for each peptide are indicated in Supplementary Fig. 4. Most of the peptides had activities in the nanomolar range, with some reaching subnanomolar values of K_i. Because the cysteine connectivity of the inhibitors appeared to be important for the activity, the connectivity of two peptides, PK4 and PK6, was deciphered by synthesizing the three isomers using orthogonal cysteine protecting groups Mmt and Dpm (Supplementary Figs. 5 and 6). An efficient procedure including one on-resin cyclization was established that enabled the synthesis of double-bridged peptides in two days. For each peptide, there was one isomer that was much more active than the other isomers. It was determined that for PK4, isomer 3 (Cys1/Cys3, Cys2/Cys4) inhibited the protease with a K_i of 0.7 0.1 nM while the other two isomers showed at least a 200-fold weaker inhibition (Fig. 4a). In PK6, isomer 1 (Cys1/Cys2, Cys3/Cys4) was by far the most active (K_i 3.2 0.5 nM) (Fig. 4a). Not surprisingly, the most active inhibitors of a consensus group had the same cysteine connectivity and thus the same macrocyclic scaffold.

The convergence of peptides to certain consensus groups based on the use of different linkers indicated an important structural and functional role for the chemical bridges. We assessed the role of the two linkers in the double-bridged peptides PK4 (isomer 3) and PK6 (isomer 1) in a structure–activity relationship (SAR) study in which we replaced one linker at a time with a set of diverse linkers (Fig. 4b, Supplementary Figs. 7, 8 and 9), some of which rendered the peptide macrocycles entirely inactive at the highest concentration tested (1 M). This linker swapping experiment showed that both of the two bridges are important. We then wondered if linker substitution could be a strategy for enhancing the macrocycles' inhibitory activities. To test this, we substituted linker 3 in the most active macrocycle PK2 (isomer 3; K_i 0.5 0.1 nM) with *para*-dibromomethyl-benzene linkers 13–19 carrying diverse groups attached to the benzyl ring (Supplementary Fig. 10). Although none of the macrocycles had enhanced activity, we discovered that some of them again had a dramatically reduced activity, showing that even minor structural changes in the linker, such as the addition of small substituents, substantially impact macrocycle activity. This suggested that linkers 13–19, despite the similarity to 3, should all be applied in parallel in future phage selections in order to generate even larger diversities.

Macrocycles PK2, PK4 and PK6 showed high target selectivity in a specificity profile performed with a panel of eight homologous trypsin-like serine proteases (Table 1). Six of the proteases were not inhibited at all. Even coagulation factor XIa (FXIa), sharing the highest sequence identity with plasma kallikrein (69% amino acid homology in the catalytic domain), was only weakly inhibited, and the macrocycles still demonstrated around 1,000-fold selectivity for plasma kallikrein over FXIa. We subsequently investigated the structural determinants of the target specificity for one of the macrocycles, PK2. Specifically, we tested if PK2 could be turned into a FXIa inhibitor by mutating different amino acids (Fig. 4c and Supplementary Fig. 11), but none of the substitutions improved the inhibition of FXIa. Detrimental mutations reduced the inhibition of both proteases to similar extents, indicating that the macrocycle binds both proteases through similar key contacts. Apparently, the backbone formed by a $XCCX_2CX_3CX$ peptide double-bridged by linker 3 fits perfectly to the active site of plasma kallikrein and contributes to the target selectivity.

Short peptides constrained by two linkers promised high proteolytic stability due to the inaccessibility of the peptide backbone to proteases. Indeed, some of the double-bridged peptides, like PK2 (isomer 3), showed an impressive stability in human plasma at 37 °C (Fig. 5a). The two exocyclic amino acids were cleaved by proteases, first the N-terminal serine ($t_{1/2}$ 29 min) and then the C-terminal tyrosine ($t_{1/2}$ 6.9 h), and the double-bridged macrocycle core remained intact after two days. The high stability of PK2 most probably results from interlaced bridging that tightly connects the two macrocycles, reducing their conformational flexibility and/or rendering them less accessible to proteases. This was confirmed by testing a peptide, PK6 (isomer 1), lacking the interlaced configuration due to bridges between Cys1/Cys2 and Cys3/Cys4, which was rapidly degraded ($t_{1/2}$ ~ 5 min; Fig. 5a). For PK2, the loss of the terminal amino acids reduced the activity of the peptide 4-fold (Ser1) and 108-fold (Tyr11), respectively (Fig. 5b). To mitigate this loss, we tested a small set of peptides in which the vulnerable exocyclic residues were replaced by d-amino acids (Fig. 5b). Appending d-Arg at the N terminus and d-Tyr at the C terminus increased the inhibitory activity 1.9- and 9.6-fold, respectively. The resulting macrocycle PK10 had a K_i of 3.6 0.5 nM, compared to 0.5 0.1 nM for the PK2 precursor, but it remained fully intact upon incubation in plasma ($t_{1/2}$

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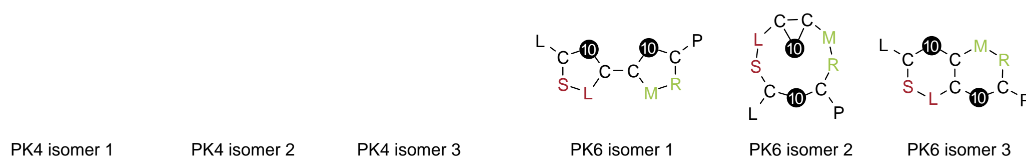

b

small-molecule inhibitors of plasma kallikrein[28,36]. We tested if the double-bridged peptides could block the activation of the intrinsic coagulation pathway in human plasma, which is dependent on the reciprocal activation of plasma kallikrein and factor XII (FXII). Human plasma was incubated with inhibitor, the activation of FXII was triggered by a negatively charged surface (Pathromtin), and the time to coagulation was measured to determine the activated partial thromboplastin time (aPTT). Peptides PK2, PK4, PK6 and PK10 all efficiently inhibited activation of the intrinsic coagulation pathway in human plasma ex vivo (Fig. 5e), and PK10 doubled the aPTT at a concentration of 3 M.

To test if double-bridged peptides could be developed for other protein targets, we performed selections against the cytokine interleukin 17 (IL-17), for which antibody drugs but not small molecules are in clinical use[37]. We designed and cloned a new phage library displaying peptides of the format $XCX_mCX_nCX_oCX$ that all contain four cysteines in fixed positions (library 3, m n o 3–8, 155 formats, library size: 2 10^{10}, Supplementary Fig. 12). Cyclization of library 3 with the same reagents that were applied to libraries 1 and 2 (1, 3, 4, 7, 10, 12) and a new reagent, divinylsulfone (20), yielded a total of 3,255 different macrocyclic backbones, and thus an even larger scaffold diversity than that of

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Table 1 | Target specificity

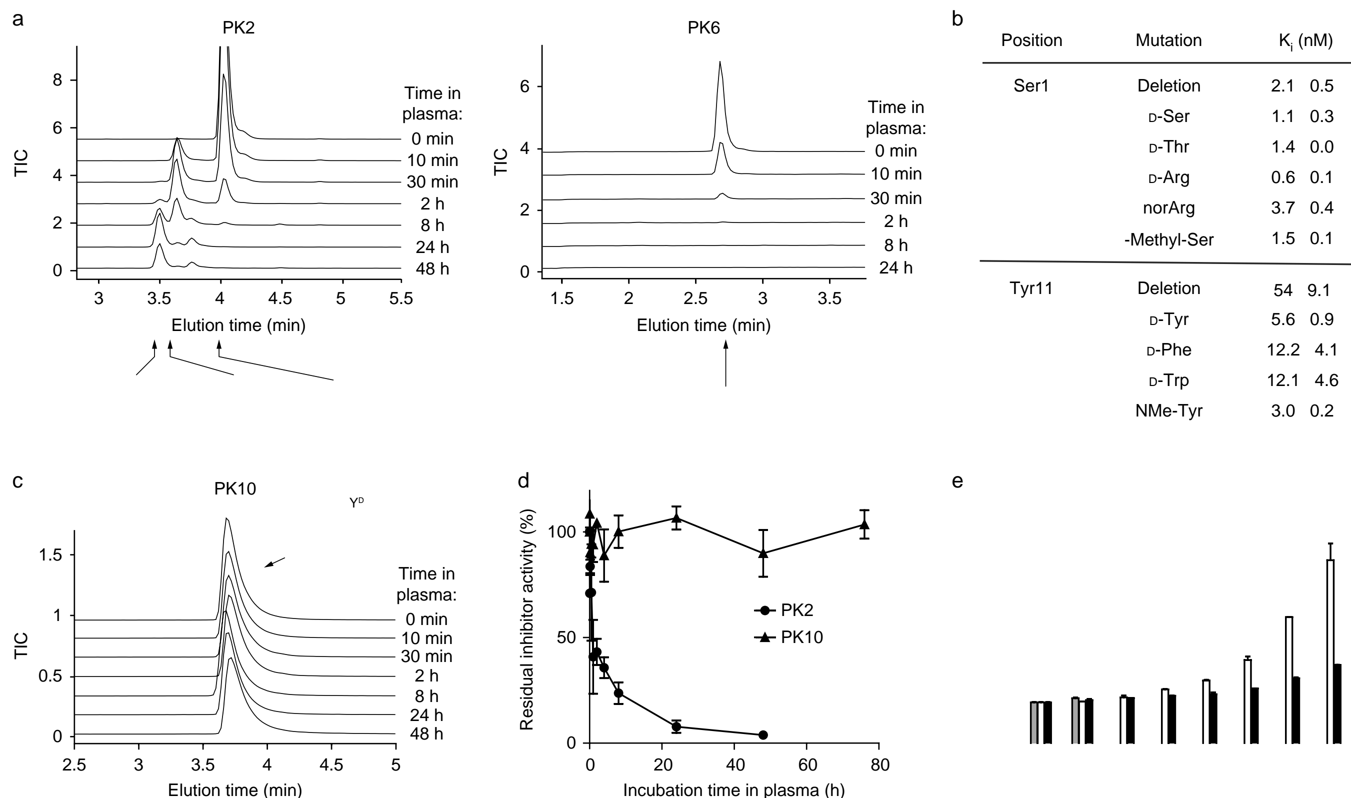
	K_i (nM)			
	PK2	PK4	PK6	PK10
Plasma kallikrein	0.5 0.1	0.7 0.1	3.2 0.5	3.6 0.5
Factor XIa	580 180	1300 500	2700 200	2500 100
Factor XIIa	30,000	30,000	30,000	30,000
Thrombin	30,000	30,000	30,000	30,000
uPA	30,000	30,000	30,000	30,000
tPA	30,000	30,000	30,000	30,000
Plasmin	30,000	30,000	30,000	30,000
Factor Xa	30,000	30,000	30,000	30,000
Factor VIIa	30,000	30,000	30,000	30,000

Inhibition of plasma kallikrein and a panel of structurally homologous or physiologically important paralogous proteases by isolated inhibitors. Average values and standard deviations of at least three measurements are shown.

the libraries described above. After three rounds of affinity selection against IL-17, peptides modified with the two reagents 10 and 20 were strongly enriched over the negative control (no target protein immobilized) and were analysed further. High-throughput DNA sequencing showed that peptides containing four cysteines were highly abundant (90%, Supplementary Table 2) and identified multiple consensus groups (Fig. 6a). Peptides modified with reagent 10 and containing the consensus motif $W^F/_Y{}^D/_E F$ or DYWYGF showed nanomolar binding affinities when the peptides were synthesized as mixtures of the three regioisomers. We synthesized the regioisomers of two of the peptides individually and determined the dissociation constant (K_d) values by fluorescence polarization (Fig. 6a and Supplementary Fig. 13). Like the plasma kallikrein inhibitors, we found that one of the three isomers was far more active than the other two. This was particularly remarkable as two of the isomers, 2 and 3, have a similar backbone and present the consensus motif in a similar manner (Fig. 6b). The best ligand, IL-17-2 isomer 3, bridged at Cys1/Cys3 and Cys2/Cys4, bound IL-17 with a K_d of 36 3 nM (Fig. 6c).

Discussion

We show that macrocyclic peptide libraries with a large structural diversity can be generated by cyclizing peptides with two chemical bridges that each connect a pair of cysteines. This approach yielded libraries comprising many more different macrocyclic scaffolds than any previously developed libraries, which tend to be based on one or, at most, a handful of different scaffolds. High-throughput sequencing of isolated peptides revealed a strong preference for certain peptide formats, clearly showing the importance of the large scaffold diversity. Even though the new strategy generates three macrocyclic isomers that are all encoded by the same phage DNA, we show that multiple products do not impair the phage panning procedure, that the three isomers can be efficiently synthesized with an orthogonal protection strategy, and that the active isomer can



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a

b

IL-17-2 isomer 1

IL-17-2 isomer 2

IL-17-2 isomer 3

easily be identified. Once the active isomer of a consensus group is found, more peptides of the same group can be synthesized directly with the correct cysteine connectivity in order to efficiently screen for the most active sequence.

For both targets used in this study—plasma kallikrein and IL-17—high-affinity double-bridged peptides could be developed. As a direct comparison, similarly sized bicyclic peptides (11 amino acids) that were cyclized by only one chemical linker (connecting three cysteines) were previously developed for plasma kallikrein by screening the same type of library (XCX_3CX_3CX)[38]. The best bicyclic peptide plasma kallikrein inhibitor developed through these pans had a K_i of 5.2 nM and thus a tenfold weaker binding affinity than the di-bridged peptides. It is likely that the affinity improvement can be attributed to the larger scaffold diversity that was generated and sampled with the new approach. A plasma kallikrein inhibitor with a comparable affinity (K_i 0.3 nM) was previously obtained only by screening a library of much longer bicyclic peptides that form a larger binding interface with the target (15 amino acids of the form ACX_5CX_5CA cyclized by one linker)[38]. The development of smaller macrocycles with a molecular weight approaching 1 kDa is of interest for the development of drugs amenable to topical or oral administration. Several orally available peptide macrocycles, including cyclosporin and desmopressin (both 1.2 kDa) have molecular weights in this range, suggesting that phage-selected, double-bridged peptides might be applied as leads for the development of topical or oral drugs.

The SAR study revealed that both of the chemical bridges in the bicyclic peptides are essential for binding, and one bridge is slightly more important in all of the examples studied. Substitution to structurally similar bridges reduced the binding affinity, suggesting that cyclizing peptide phage libraries in parallel with similar reagents such as compounds 13–19 will allow for the generation of even larger macrocycle diversities. More reagents containing two thiol-reactive groups are commercially available, and a nearly endless number of such compounds can be designed and synthesized. A SAR study altering the amino-acid sequence of double-bridged peptide PK2 showed that its high target selectivity was based, at least to some extent, on the specific peptide backbone architecture. None of the numerous amino-acid mutations tested increased the affinity for the homologous protease FXIa. This is in stark contrast to the

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previously developed single-bridged bicyclic peptide inhibitors of plasma kallikrein that also inhibited FXIa when specific amino-acid positions were mutated[38]. This observation is an additional indication that the larger scaffold diversity allowed for the identification of ligands that are perfectly complementary in shape and polarity to the target binding site.

An interesting feature of the ligands with interlaced bridges is the high proteolytic stability. The core structure of PK2 resisted protease degradation when it was incubated for several days in human plasma at 37 °C. This high stability is probably due to the close connection of the two macrocyclic rings, resulting from interlacing bridges Cys1/Cys3 and Cys2/Cys4. This hypothesis is further supported by the low proteolytic stability of the double-bridged PK6 that contains two independent monocycles connected via a flexible linker. The enormous stability of the interlaced peptide macrocycle format might be exploited for the development of oral drugs that must survive the proteolytic pressure in the gastrointestinal tract. This could include either drugs that are absorbed into the bloodstream or that act on targets in the gastrointestinal tract. The feasibility of peptide-based drugs acting in the lumen of the gastrointestinal tract was demonstrated with the recently approved blockbuster drug linaclotide. This 14-amino-acid peptide derived from nature and stabilized by three disulfide bridges is applied orally. The low stability for exocyclic amino acids, such as the Ser1 and Tyr11 in PK2, should not be a limitation as they can be substituted to non-natural building blocks that resist proteolytic cleavage, as demonstrated in this work. Alternatively, exocyclic amino acids may be completely omitted and the loss in affinity compensated by substituting amino acids in the core to unnatural ones that can more efficiently bind the target. The latter strategy would allow a further reduction of the molecular weight of the ligands, and would reduce the size of PK2 to below 1 kDa.

In summary, we have developed an efficient strategy for the generation and screening of macrocyclic peptide libraries comprising an unprecedented scaffold diversity. We show that ligands with a better affinity and target selectivity can be isolated from such libraries despite a rather small molecular mass of the compounds, and that a scaffold format with interlaced macrocyclic rings has a particularly high stability. The combined properties of the generated ligands, including high affinity and selectivity, small size and high proteolytic stability, make them interesting as drug development leads.

Data availability. The principal data supporting the findings of this work are available within the figures and the Supplementary Information. All other data are available from the corresponding author upon request.

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Author contributions
S.S.K., C.V., X.-D.K. and C.H. conceived the strategy, designed experiments, analysed data and wrote the manuscript. S.S.K. established the chemical reactions. C.V. and X.-D.K. performed the phage selections. S.S.K., C.V. and X.-D.K. synthesized, purified and characterized peptides. A.Z. synthesized a linker reagent. K.D. contributed to the writing of the manuscript. S.S.K., C.V. and X.-D.K. contributed equally to this work.

Competing interests
C.H. is a scientific founder of Bicycle Therapeutics. All other authors declare no competing interests.

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Experimental design

1. Sample size

Describe how sample size was determined.

In previous work characterizing peptide-based ligands using the same methods, the standard deviations of inhibitory constants (Ki), dissociation constants (Kd), residual protease activities, plasma half-lives and activated partial thromboplastin times (aPTT) were typically smaller than 20% of the mean values, when three independent measurements were performed. We considered this standard deviation as sufficiently precise and have thus measured the above named parameters three times (or even more often in some cases).

2. Data exclusions

Describe any data exclusions.

No data was excluded.

3. Replication

Describe the measures taken to verify the reproducibility of the experimental findings.

The three first authors had independently applied the new approach to evolve double-bridged peptides by phage display. All of them were able to isolate high-affinity ligands. The measurement of Ki values, Kd values etc. were performed at least three times wherein the replicates were performed in separate experiments.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

None of the experiments required random allocation of samples into groups.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

None of the experiments required blinding.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
[x]	[ ]	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
[x]	[ ]	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
[ ]	[x]	A statement indicating how many times each experiment was replicated
[ ]	[x]	The statistical test(s) used and whether they are one- or two-sided
[x]	[ ]	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
[x]	[ ]	Test values indicating whether an effect is present
[ ]	[x]	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
[ ]	[x]	Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

GraphPad PRISM

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All chemicals are commercially available. All peptides can be obtained from our laboratory or can be ordered from custom peptide synthesis companies for a modest prize.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were applied in this work.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell lines were used.

b. Describe the method of cell line authentication used.

N/A

c. Report whether the cell lines were tested for mycoplasma contamination.

N/A

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	Study did not involve human research participants.