Peptide macrocycle inhibitor of coagulation factor XII with subnanomolar affinity and high target selectivity

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Abstract

Factor XII (FXII) is a plasma protease that has emerged in recent years as a potential target to treat or prevent pathological thrombosis, to inhibit contact activation in extracorporeal circulation, and to treat the swelling disorder hereditary angioedema. While several protein based inhibitors with high affinity for activated FXII (FXIIa) were developed, the generation of small molecule inhibitors has been challenging. In this work, we have generated a potent and selective FXIIa inhibitor by optimizing a peptide macrocycle that was recently evolved by phage display (K_i = 0.84 ± 0.03 nM). A fluorine atom introduced in the *para*-position of phenylalanine enhanced the binding affinity as much as 10-fold. Furthermore, we improved the proteolytic stability by substituting the N-terminal arginine by norarginine. The resulting inhibitor combines high inhibitory affinity and selectivity with a good stability in plasma (K_i = 1.63 ± 0.18 nM, >27,000-fold selectivity, $t_{1/2}$ plasma = 16 ± 4 h). The inhibitor efficiently blocked activation of the intrinsic coagulation pathway in human blood *ex vivo*.

Introduction

FXII, originally called Hageman factor, is a serine protease that plays an important role in the kallikrein-kinin system and the intrinsic coagulation pathway.[1] It is expressed as an 80 kDa zymogen, primarily by hepatocytes, and circulates in plasma at a concentration of around 0.4 μM. FXII is converted into its activated form, FXIIa, by proteolytic cleavage between the heavy chain and the enzymatic light chain. This process is initiated when blood is exposed to negatively charged surfaces or compounds. FXIIa has the capacity to cleave factor XI, initiating the intrinsic coagulation pathway, and plasma kallikrein, activating the kallikrein-kinin system.[1] Activated plasma kallikrein can reciprocally activate FXII, leading to rapid activation of the contact system. FXIIa can also trigger the complement and fibrinolytic systems. Human and animals deficient in FXII do not show abnormal bleeding from injury sites and have a normal hemostatic capacity. The physiological role of FXII and its benefit for individuals are not completely understood but are subject of active research.[2, 3]

While the beneficial function of FXII for the body is currently not fully understood, more is known about its harmful role. FXII activity causes or contributes to a number of medical conditions leading to coagulation and/or inflammation.[4] The protease has received much attention in recent years due to its role in thrombosis. Mice deficient in FXII have a much reduced risk of thrombus formation and are protected from experimental ischemic stroke and pulmonary embolism.[5, 6] This observation and the limited role of FXII in hemostasis raised the prospect that inhibiting FXIIa could offer an antithrombotic therapy with low bleeding risk. The role of FXII in thrombosis has been confirmed in various experimental animal models, including rat, rabbit and baboon.[4] Several *in vivo* activators of the plasma contact system were reported, including platelet and bacterial polyphosphates, extracellular RNA and DNA, amyloid β peptide and misfolded proteins.[1] FXII also constitutes a constant threat in medical procedures that depend on extracorporeal circulation such as cardiopulmonary bypass surgery, extracorporeal membrane oxygenation (ECMO), or hemodialysis.[7, 8] In these procedures, contact with the materials of the medical devices, such as tubing and membranes, can

activate FXII and trigger the coagulation and inflammation pathways. FXII can also be activated by contact with catheters which can lead to catheter occlusion and thrombosis.[9] In hereditary angioedema (HAE), a life-threatening inherited swelling disease, FXIIa activates plasma kallikrein that in turn cleaves high-molecular-weight kininogen to release the vasoactive hormone bradykinin.[10] HAE type I and II result from a deficiency (type I) or malfunction (type II) of C1 esterase inhibitor, which is the main inhibitor of plasma kallikrein and FXII. In type III HAE, mutations in FXII cause recurrent edema attacks.[11]

The important role of FXII in multiple diseases prompted academic laboratories and pharmaceutical companies to develop inhibitors of the protease.[4] A range of natural proteins that inhibit FXIIa with high affinity were identified and exploited to block FXIIa in experimental animal models.[4, 12, 13] Monoclonal antibodies either preventing activation of FXII or inhibiting the activated FXIIa were developed.[4] The humanized antibody 3F7 inhibited arterial and venous thrombosis in mouse and rabbit, and prevented coagulation in extracorporeal circulation in rabbits.[7] The antibody 15H8 blocked thrombosis in mice and baboon.[14] The development of small molecule FXIIa inhibitors has been challenging. The high structural similarity of FXII to numerous homologous trypsin-like serine proteases makes it difficult to generate selective inhibitors.[15, 16] Our laboratory has recently developed selective FXIIa inhibitors based on peptide macrocycles that have a relatively small size (< 2000 Da) and that are accessible to chemical synthesis.[17, 18] The best inhibitor reported is the bicyclic peptide **1** (FXII618; Figure 1a), inhibiting FXIIa with a K_i of 8.1 ± 0.7 nM and has a more than 2,000-fold selectivity over physiologically relevant homologous proteases. The peptide macrocycle inhibited the intrinsic coagulation pathway with an EC_{2x} for the activated partial thromboplastin time (aPTT) of around 3 μM, without affecting the prothrombin time (PT) at high concentrations (< 20% increase at 40 μM). We demonstrated that the inhibitor can be applied as a reagent for the inhibition of contact activation in diagnostic coagulation assays.[17]

For therapeutic application, a synthetic FXIIa inhibitor must block the protease with high affinity. In this work, we aimed at developing high affinity FXIIa inhibitors by improving the binding affinity of the above described bicyclic peptide **1**. In the phage display selections that had been performed to isolate peptide **1**, all of the bicyclic peptides that were screened contained exclusively natural amino acids. Herein, we proposed to synthesize and screen variants of peptide **1** containing unnatural amino acids. In addition, we aimed at improving the stability of peptide **1** to prevent proteolytic degradation when the peptide is applied *in vivo*.

Results and Discussion

Improving the inhibitory activity

Based on sequence similarities of peptides isolated in the phage selections against FXIIa,[17] the four amino acids of the first ring (Phe3, Arg4, Leu5, Pro6) and the middle two amino acids of the second ring of bicyclic peptide **1** (Gln9, Leu10) appeared to be most important for the binding (Figure 1a). In a first attempt to improve the inhibitory activity of peptide **1**, we chose to replace the amino acids in these positions with natural amino acids having similar side chains. Suitable amino acid substitutions were chosen using the two scoring matrices BLOSUM62 and PAM250 that indicate the evolutionary substitution probability of an amino acid by another one (Table 1). To allow precise quantification of the synthesized peptides by absorption spectrometry, a tryptophan residue was appended to the C-terminus of all peptides. Peptide **1** with a C-terminal Trp, named peptide **2,** had a K_i of 10.2 ± 0.5 nM versus 8.1 ± 0.7 nM of the original peptide and thus a comparable activity. A total of 26 bicyclic peptides, each having one amino acid substituted in one of the six positions, were synthesized. While none of the peptides had an improved K_i (Table 1), valuable structure-activity relationship (SAR) data was obtained. In the position of Phe3, replacement of phenylalanine with tryptophan conserved the activity (peptide **3**; K_i = 12 ± 2). All other amino acid substitutions in this positon reduced the activity more than 2-fold (tyrosine, leucine, proline; peptides **4**-**6**) or more than 10-fold (alanine, valine, isoleucine; peptides **7**-**9**). In position Arg4, only the substitution to lysine was tested as this residue should have a positive charge to form an electrostatic interaction with Asp189 (trypsin numbering) at the bottom of the S1 binding pocket of FXIIa. Lysine in this position reduced the inhibitory activity 10-fold (peptide **10**; K_i = 111 ± 8). In the position of Leu5, all substitutions, even those with amino acids closely resembling leucine such as valine (peptide **11**) or isoleucine (peptide **12**), reduced the affinity more than 50-fold. Peptides containing methionine (peptide **13**) or alanine (peptide **14**) in this position did not inhibit FXIIa at the highest concentration tested (3 μM). In position Pro6, two of the amino acid replacements reduced the activity less than 2-fold, namely alanine (peptide **15**) and leucine (peptide **18**). In the second macrocyclic ring, all of the substitutions reduced the

activity of peptide **2** substantially. In positions Gln9 and Leu10, the smallest losses were seen for substitutions of aspartate (peptide **21**, 54-fold) and isoleucine (peptide **26**, 8-fold), respectively.

In a second attempt to improve the activity of peptide **1**, we chose to replace the amino acids with unnatural residues that are structurally similar to the natural ones (Table 2). In position Phe3, unnatural amino acids were tested resembling phenylalanine (peptides **29-32**) and tryptophan (peptides **33-37**), as peptides with these two natural amino acids were the most active (peptides **2** and **3**). Two of the phenylalanine analogues reduced the binding affinity around 30-fold (peptides **29** and **30**) and two of them did not inhibit FXIIa at the highest concentration tested (1 μM; peptides **31** and **32**). Two out of the five peptides with tryptophan analogues had slightly improved activity: peptide **33** containing 2-naphthylalanine had a 1.7-fold improved inhibitory activity (K_i = 6.0 ± 0.6) and peptide **35** containing 3-benzothienylalanine showed a 1.5-fold improvement (K_i = 6.8 ± 1.8). In position Arg4, the amino acid was replaced with one lysine analogue (homolysine; peptide **38**) and three arginine analogues (homoarginine, norarginine and 4-guanidinophenylalanine; peptides **39-41**). All of these substitutions reduced the binding affinity of the peptide 5-fold or more. In the two positions Leu5 and Leu9, several aliphatic amino acids structurally resembling leucine were tested. All of the substitutions in position Leu5 greatly reduced the binding affinity wherein the smallest drop in affinity was found for norvaline (22-fold; peptide **42**). In position Leu10, all the five unnatural amino acids reduced the affinity by less than 8-fold.

Many of the 48 peptide variants described above had substantially reduced activities, despite the fact that only one amino acid was replaced at a time and that most of the inserted amino acids were structurally similar to the substituted ones. In a third attempt to improve the inhibitory activity of peptide **1**, we aimed for amino acid substitutions that would result in only small structural changes in the side chains. We chose to modify position Phe3 as this was the only site in which two substitutions with unnatural amino acids had yielded slightly improved inhibitors (Table 3). The addition of a methyl group to the phenyl ring of Phe3 in

ortho- (peptide **51**) and *para*-position (peptide **53**) decreased the activity around 4-fold. In contrast, a methyl group in *meta*-position increased the affinity 2.7-fold (peptide **52**; K_i = 3.7 ± 0.2). This result indicated that some space around the side chain of phenylalanine is available but that the space is limited. We subsequently modified the phenyl ring of Phe3 by introducing heteroatoms or by halogenation, both modifications that conserved the size and shape of the amino acid side chain. Replacement of a carbon atom with nitrogen in the phenyl ring in *meta*-position improved the activity around 3-fold (peptide **54**; K_i = 3.0 ± 0.1). The same substitution in *para*-position reduced the inhibitory activity around 6-fold (peptide **55**). Substitution of hydrogen atoms on the phenyl ring with halogen atoms altered the activity dramatically in both directions. Fluorine in *ortho*-position reduced the activity around 2.5-fold (peptide **56**). In contrast, fluorine in *meta*- and *para*-position enhanced the activity 2- and 11-fold, respectively (peptide **57**: K_i = 4.5 ± 0.4; peptide **58**: K_i = 0.89 ± 0.15). Bicyclic peptide **58** was the first synthetic FXIIa inhibitor with a subnanomolar inhibitory constant. We subsequently tested more electronegative atoms or groups in *para*-position. Iodine in this position reduced the activity 9-fold (peptide **59**) and a nitro group improved the binding affinity 6-fold (peptide **60**; K_i = 1.64 ± 0.19). Finally, the peptide with the best inhibitory activity, peptide **58** containing a 4-fluorophenyl substitution in position Phe3, was synthesized without the C-terminal tryptophan that was introduced at the beginning to enable precise determination of the concentration based on absorption. The resulting peptide **61** (also termed FXII800), differing from peptide **1** only by the fluorine atom in phenylalanine, had a K_i of 0.84 ± 0.03 nM and thus displayed an approximately 10-fold improvement in inhibitory activity (Table 4).

Structural modeling and interactions of 4-fluoro-phenylalanine

The large affinity improvement achieved by only replacing a hydrogen atom with fluorine in Phe3 raised the question about the underlying molecular basis. In protein-ligand interactions, fluorine can strengthen binding affinities through direct interactions with proteins (e.g. polar interactions, hydrophobic effects) or by influencing other groups of the ligand that interact with the protein (e.g. change of pK_a and thus

protonation state, change of electron distribution in an aromatic ring).[19, 20] We analyzed the environment of Phe3 in a structural model of the peptide **61** bound to FXIIa (Figure 1b). The structure was obtained by simply replacing the hydrogen atom in the *para* position of Phe3 with fluorine in the previously developed model for the complex peptide **1**:FXIIa (PDB file 'FXIIa-61_model' in Supporting Information).[17] The first macrocyclic ring of the bicyclic peptide containing the FRLP motif fits perfectly into the active site of FXIIa in terms of shape and polarity. The side chain of 4-fluoro-phenylalanine is buried in an aromatic pocket that is formed by two surface loops of FXIIa. The fluorine atom is in close proximity to positively polarized C=O and H-C_α groups and can potentially form polar interactions. A possible indirect effect of fluorine could be an alteration of the electron distribution in the aromatic ring which may enhance π-π stacking interactions between the 4-fluoro-phenylalanine and facing aromatic amino acids His393 and Tyr439.

Improving the proteolytic stability

The stability of the FXIIa inhibitors can be assessed by incubating the bicyclic peptides in blood plasma and by subsequently measuring the remaining inhibitory activity in an assay with FXIIa and a fluorogenic substrate. Prior to this measurement, the plasma/bicyclic peptide samples were heat-inactivated to prevent cleavage of the fluorogenic substrate by other blood proteases or inhibition of FXIIa by blood-derived inhibitors. The assay quantifies not only the activity of intact bicyclic peptide but also the activity of potential metabolic products. Bicyclic peptide **1** was found to be inactivated by proteolysis when incubated in human plasma at 37 °C for extended time periods ($t_{1/2}$ = 4 ± 2 h). While a half-life of four hours is sufficient to block contact activation *in vitro* in coagulation assays, as demonstrated in our previous work,[17] it may not be long enough for clinical applications in which FXIIa presumably needs to be inhibited for longer time periods. Mass spectrometric analysis of peptide **1** incubated in human plasma showed that the first proteolytic modification is removal of a 156 Da fragment corresponding to the mass of arginine. Derivatives of bicyclic peptide **1** lacking either the N- or C-terminal arginine had K_i values of 330 ± 30 nM (peptide **62**) and 9.3 ± 0.6 nM (peptide **63**), respectively (Table 4). This result suggested that plasma

proteases clip off the arginine at the N-terminus. To improve the stability, we searched for amino acid substitutions in position Arg1 that i) do not reduce significantly the binding affinity, and ii) prevent proteolytic cleavage of the first amino acid. Bicyclic peptides containing substitutions in position Arg1 were synthesized without the C-terminal tryptophan residue (Table 5). Natural amino acids such as isoleucine, valine and alanine in place of Arg1 all reduced the affinity > 5-fold (peptides **64**-**66**). Replacement with lysine conserved the binding affinity (peptide **67**; 9.0 ± 0.9 nM), suggesting that a positive charge in the side chain is important. D-arginine reduced the binding affinity 3-fold (peptide **68**; K_i = 34.4 ± 0.7 nM). Based on this structure-activity data, we tested a number of unnatural amino acids carrying positively charged side chains (peptides **69**-**72**). These peptides had a comparable or slightly reduced affinity, with K_i value between 6.5 and 15 nM.

Next, we tested the stability of the bicyclic peptides **69**-**72**, using the assay described above (Table 5). Plasma proteases were heat-inactivated by incubating the samples at 65 °C for 20 minutes. The heat-treatment did not affect the activity of the bicyclic peptides. Two of the four peptides had an improved stability compared to peptide **1** (Figure 2a). The longest half-life, 22 ± 3 h and thus a 5.5-fold improved stability compared to peptide **1,** was found for peptide **71** which has a norarginine residue at the N-terminus. Finally, we combined the norarginine substitution with the amino acid replacement that most improved the inhibitory activity of peptide **1** (4-fluoro-phenylalanine in position 3 of the peptide). The resulting peptide **73**, also termed FXII801, had a K_i of 1.63 ± 0.18 nM and a plasma half-life of 16 ± 4 h.

Target selectivity

The target selectivity of peptide **73** was assessed by measuring the inhibition of structurally related or functionally important proteases and compared to the specificity profile of peptide **1** (Figure 2b).[17] Eight of the ten proteases were inhibited by less than 20% at the highest inhibitor concentration tested (40 μM). Only

two of the paralogous proteases were inhibited more than 50% by peptide **73** at the tested concentrations, namely trypsin (K_i = 1.30 ± 0.01 μM) and plasma kallikrein (K_i = 45.1 ± 1.5 μM). The same two proteases were also inhibited by the peptides **1** and **61** at similar extents. These results showed that the affinity towards FXIIa could be improved while the activity towards other proteases remained essentially unchanged. The new FXIIa inhibitor peptide **73** exhibits a good selectivity of 800-fold over trypsin (a protease that is not present in the blood) and at least a 27,000-fold selectivity over all of the other tested plasma proteases.

Inhibition of the intrinsic coagulation pathway

The ability to selectively suppress the intrinsic coagulation pathway was assessed in *in vitro* coagulation assays. aPTT and PT measure the time to coagulation upon initiation of the intrinsic and extrinsic coagulation pathway, respectively. Coagulation via the intrinsic pathway was triggered by addition of silicon dioxide particles, plant phospholipids and calcium. Coagulation via the extrinsic pathway was triggered with recombinant human tissue factor, synthetic phospholipids and calcium. The tests were performed in human plasma in the presence of different concentrations of FXIIa inhibitor. Selective FXIIa inhibition is expected to increase aPTT but not PT. The coagulation tests were carried out using peptide **61**, the most potent inhibitor, peptide **73**, the most stable variant, and the previously developed FXIIa-inhibitor peptide **1**, for comparison. The presence of a peptide inhibitor prolonged aPTT in a concentration dependent manner (Figure 3a), whereas PT remained almost unaffected up to a concentration of 40 μM for all peptides tested (Figure 3b). The optimized peptides **61** and **73** inhibited the intrinsic coagulation pathway more efficiently than peptide **1**. The potency of an intrinsic coagulation inhibitor can be expressed as the concentration required to double aPTT (EC_{2X}). Peptides **61** and **73** showed EC_{2X}s of 1.6 μM and 2.0 μM, respectively, while peptide **1** had an EC_{2X} of 2.9 μM.

Conclusions

The binding affinity and proteolytic stability of a bicyclic peptide inhibitor of FXIIa were improved substantially by synthesizing and screening a library of around 70 variants of an existing peptide macrocycle. These variants were created by substituting structurally similar natural and unnatural amino acid analogues for various macrocycle residues. It was discovered that the inhibitory activity of the bicyclic peptide could be most improved with conservative mutations, such as replacing the atoms in the amino acid side chain with halogens or heteroatoms, which resulted in only small structural changes. The best affinity improvement, on the order of 10-fold, was achieved by replacing a hydrogen with a fluorine atom in para position of a phenylalanine side chain. The blood plasma half-life of the bicyclic peptide was improved around 5-fold by replacing the N-terminal arginine with an unnatural arginine analogue.

This work is the first study in which the activity and stability of a phage-selected bicyclic peptide was improved by systematically synthesizing and testing a large number of peptide macrocycle variants containing unnatural amino acids. The result shows that synthesizing several dozen bicyclic peptide variants is sufficient to identify a handful of substitutions that significantly improve the affinity or stability. Given that small structural changes most improved the inhibitory activity, it is recommended that future optimization of phage-selected bicyclic peptides should be performed by synthesizing variants with structurally conservative mutations.

The inhibition constants of the developed bicyclic peptides are several orders of magnitude better than any small molecule FXIIa inhibitor reported thus far. The FXIIa inhibitors effectively prolong aPTT while leaving PT unchanged. With their favorable binding properties and high stability, the peptide macrocycles will be valuable tools to study the biology of FXII and could be exploited as lead structures for the development of a therapeutic agent.

Experimental Section

Bicyclic peptide synthesis:

Peptides were synthesized in house by standard solid-phase peptide synthesis using Fmoc-protected amino acids and Rink Amide AM resin on a 0.03 mmol scale. Amino acids were coupled twice at 4-fold molar excess using HBTU (4 eq) and HOBt (4 eq) as coupling reagents and DIPEA (6 eq). Coupling of unnatural amino acids was performed manually by adding 2 eq of Fmoc-protected amino acid, 2 eq of HATU and 4 eq of DIPEA. All peptides synthesized have a free N-terminus and an amidated C-terminus. Peptides were cleaved from the resin under reducing conditions (90% TFA, 2.5% H_2O, 2,5% thioanisol, 2,5% phenol, 2.5% 1.2-ethanedithiol). Crude peptide at a concentration of 1 mM was reacted with 1.5 mM 1,3,5-triacryloyl-1,3,5-triazinane (TATA) in 70% aqueous buffer (50 mM NH_4HCO_3, pH 8.0) and 30% acetonitrile for 1 hour at 30 °C followed by lyophilization. The cyclized peptides were dissolved in 1 ml MeCN and 9 ml H_2O (containing 0.1% TFA), purified by reversed-phase chromatography on a preparative C18 column (Vydac C18 TP1022, 250 x 22 mm, 10 μm) using a linear gradient of solvent B (MeCN, 0.1% v/v TFA) over solvent A (H_2O, 0.1 % v/v TFA) (gradient: 15-28% MeCN in 19 min, flow rate 20 ml min^{-1}). Pure bicyclic peptides were lyophilized and dissolved in water. The purity of all peptides was assessed by analytical HPLC (UV detection at 220 nm) using a C18 column (Agilent Zorbax 300SB, 4.6 mm x 250 mm, 5 μm, flow: 1 ml min^{-1}, gradient: 0-50% solvent B over solvent A in 15 min) and accepted if > 95% calculated by integration of the area under the curve. The correct mass of all peptides was verified by ESI-MS using a Shimadzu LCMS-2020 with a detector voltage of 1.05 kV. The analytical data of all bicyclic peptides is shown in Figure S1 and S2 and Table S1.

Concentration determination of peptide stock solutions

The concentration of peptide was determined by absorption spectrometry at 280 nm or by weighing the lyophilized peptide before dissolving in water, or both. For determining the concentration of peptides by absorption spectrometry, theoretical extinction coefficients (ε280) were calculated based on the presence of

tryptophan ($\varepsilon = 5500\ M^{-1}cm^{-1}$) and tyrosine ($\varepsilon = 1490\ M^{-1}cm^{-1}$). For determining the concentration of peptides by weighing, it was assumed that one trifluoroacetic acid molecule is bound per positive charge of the peptide. The concentration of peptides that contain unnatural amino acids absorbing UV light at 280 nm with unknown ε280, or that lack tryptophan or tyrosine, was determined by weighing.

Protease inhibition assays

The inhibitory activity of bicyclic peptides was determined by incubation with proteases and quantification of the residual activity with a fluorogenic substrate. Residual enzymatic activities were measured in buffer containing 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% w/v BSA, 0.01% v/v Triton-X100, and 1% v/v DMSO in a volume of 150 µL. In a first series of experiments, 2.5 nM of enzyme (human β-FXIIa, HFXIIAB; Molecular Innovations) and 50 µM of the fluorogenic substrate Z-Gly-Gly-Arg-AMC were used. For measuring the activity of peptides with single-digit nanomolar or subnanomolar affinity, enzyme was used at a concentration of 0.5 nM in combination with the more sensitive substrate Boc-Gln-Gly-Arg-AMC (50 µM). Dilutions of peptides ranging from 0.01 nM to 4 µM were prepared depending on the binding affinity. Peptides were incubated together with the enzyme for 10 min before triggering the reaction by addition of substrate. Fluorescence intensity was measured with an Infinite M200Pro fluorescence plate reader (excitation at 368 nm, emission at 467 nm; Tecan) for a period of 30 min with a read every minute. The reactions were performed at 25 °C. Sigmoidal curves were fitted to the data using the following dose response equation wherein x = peptide concentration, y = % protease activity, p = Hill slope. IC_{50} values were derived from the fitted curve.

$$y = \frac{100}{1 + 10^{(\log IC50 - x)p}}$$

The bicyclic peptides inhibit most likely, as the parent peptide **1**, FXII in a competitive manner. The inhibitory constants (K_i) were thus calculated according to the equation of Cheng and Prusoff $K_i = IC_{50}/(1+([S]_0/K_m)$ wherein IC_{50} is the functional strength of the inhibitor, $[S]_0$ is the total substrate concentration, and K_M is the Michaelis-Menten constant. K_M for the two substrates Z-Gly-Gly-Arg-AMC and Boc-Gln-Gly-Arg-AMC were determined to be 180 ± 30 and 260 ± 40 μM, respectively (mean ± SD, n = 3-4).

For testing the specificity of the bicyclic peptides, the following final concentrations of human serine proteases were used: tPA (Molecular Innovations) 7.5 nM, uPA (Molecular Innovations) 1.5 nM, factor XIa (Innovative Research) 6 nM, plasma kallikrein (Innovative Research) 0.25 nM, thrombin (Molecular Innovations) 1 nM, plasmin (Molecular Innovations) 2.5 nM, trypsin (Molecular Innovations) 0.05 nM, factor VIIa (Haematologic Technologies Inc.) 50 nM and factor Xa (Haematologic Technologies Inc.) 6 nM. Dilutions of peptide were prepared ranging from 0.04 to 40 μM. The following fluorogenic substrates were used at a final concentration of 50 μM: Z-Phe-Arg-AMC (Bachem) for plasma kallikrein, Boc-Phe-Ser-Arg-AMC (Bachem) for factor XIa, Z-Gly-Gly-Arg-AMC (Bachem) for tPA, uPA, thrombin, and trypsin, H-D-Val-Leu-Lys-AMC (Bachem) for plasmin, and D-Phe-Pro-Arg-ANSNH-C_4H_9 (Haematologic Technologies Inc.) for FVIIa and FXa. The residual activity of FXIIa (raw data) for the most important bicyclic peptides **1**, **61** and **73** are shown in Figure S2.

Structural model and structure analysis

A structural model of the peptide **61**-FXIIa complex was obtained by modifying a model of bicyclic peptide **1** bound to FXIIa that was previously developed by homology modeling and molecular dynamics simulation as described in Baeriswyl, V. *et al.*[17] The hydrogen atom in the *para* position of Phe3 was substituted with fluorine using the 'Build' function of Pymol. The resulting model for peptide **61** bound to FXIIa is provided in the Supporting Information (PDB file FXIIa-61_model).

Plasma stability assays

Peptide (2 µl of 2 mM in H_2O) was added to 398 µl human plasma (Innovative Research) to obtain a final peptide concentration of 10 µM. The mixture was incubated in a water bath at 37 °C. At different time points (0, 0.5, 1 , 2 , 4 , 8 , 12, 24 and 48 hr), samples of 30 µl were removed, diluted to 200 µl with aqueous buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) and incubated for 20 min at 65 °C to inactivate plasma proteases. Control experiments showed that the bicyclic peptide remains functional during the protease-inactivation step. The peptide/plasma samples were stored at -20 °C until the residual inhibitory activity of the peptides was measured in a FXIIa inhibition assay. For the activity assay, the peptide/plasma samples were centrifuged for 5 min at 16,000 g and the supernatant was collected. Two-fold dilutions of the peptide/plasma samples were prepared (peptide concentration ranges from 0.5 nM to 0.5 µM) and the residual activity of 0.5 nM FXIIa with 50 µM Boc-Gln-Gly-Arg-AMC substrate was measured. IC_{50} values were derived from the fitted curve using the equation indicated above. Residual inhibition in % was calculated using the equation $IC_{50,0h}/IC_{50,xh}$*100 wherein $IC_{50,0h}$ is the functional strength of the inhibitor at time point 0 and $IC_{50,xh}$ the functional strength of inhibitor after one of the different plasma incubation periods mentioned above.

aPTT and PT coagulant activity measurements

Coagulation times (aPTT and PT) were determined in human plasma using a STAGO STart4 Coagulation analyzer (Diagnostica). Human single donor plasma was used (Innovative Research). For the extrinsic coagulation, 50 µl of plasma was placed in the incubating chamber of the instrument for 2 min at 37 °C. After incubation, 100 µl of Innovin (recombinant human tissue factor, synthetic phospholipids, and calcium in stabilized HEPES buffer system; Dade Behring/Siemens) was added using the pipette connected to the instrument. Upon addition of this reagent the electromagnetically induced movement of a steel ball in the

plasma was monitored. The time until the ball stops moving was recorded as coagulation time. For the intrinsic coagulation, 100 µl of plasma was incubated with 100 µl of Pathromtin* SL (silicon dioxide particles, plant phospholipids in HEPES buffer system, Siemens) for 2 min at 37 °C. Subsequently, the coagulation was triggered by addition of 100 µl $CaCl_2$ solution (25 mM, Siemens). Statistical analysis was performed using the Student's t-test showing two level of significance (*: $p < 0.05$ and **: $p < 0.01$).

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xyz.

Analytical data of bicyclic peptides (HPLC chromatograms, MS spectra)

Raw data of FXII inhibition assay

Mass table of bicyclic peptides

Structure model for bicyclic peptide **61** bound to FXIIa (PDB file)

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Abbreviations

aPTT, activated partial thromboplastin time; EC_{2x}, concentration at which coagulation time is doubled; ECMO, extracorporeal membrane oxygenation; HEA, hereditary angioedema; PT, prothrombin time

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Tables

peptide	position	substitution	K_i (nM)
2	-	-	10.2 ± 0.5
3	Phe3	Trp	12 ± 2
4		Tyr	32.2 ± 1.6
5		Leu	28 ± 5
6		Pro	36.9 ± 1.2
7		Ala	132 ± 19
8		Val	160 ± 20
9		Ile	310 ± 15
10	Arg4	Lys	111 ± 8
11	Leu5	Val	570 ± 60
12		Ile	890 ± 190
13		Met	> 3000
14		Ala	> 3000
15	Pro6	Ala	19.6 ± 0.4
16		Ser	29.4 ± 1.2
17		Val	47 ± 3
18		Leu	13.0 ± 1.3
19		Asn	104 ± 4
20		Asp	120 ± 30
21	Gln9	Asp	550 ± 30
22		Asn	1000 ± 70
23		Glu	> 3000
24		His	> 3000
25	Leu10	Val	326 ± 12
26		Ile	78 ± 3
27		Met	143 ± 5
28		Ala	1040 ± 90

Table 1. Natural amino acid substitutions in bicyclic peptide **2**. Standard deviations of at least three measurements are indicated.

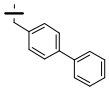
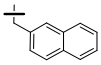
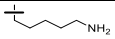
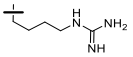
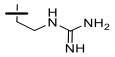
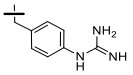
peptide	position	substitution	K_i (nM)
29	Phe3		290 ± 4
30			366 ± 5
31			> 1000
32			> 1000
33			6.0 ± 0.6
34			10 ± 2
35			6.8 ± 1.8
36			94 ± 10
37			114 ± 10
38	Arg4		51 ± 18
39			71 ± 18
40			156 ± 16
41			380 ± 40
42	Leu5		220 ± 80
43			2700 ± 900
44			> 3000
45			> 3000
46	Leu10		72 ± 5
47			29.4 ± 1.2
48			17.7 ± 0.4
49			30.6 ± 1.2
50			38 ± 2

Table 2. Unnatural amino acid substitutions in bicyclic peptide **2**. Standard deviations of at least three measurements are indicated.

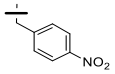
peptide	position	substitution	K_i (nM)
51	Phe3		40 ± 2
52			3.7 ± 0.2
53			39 ± 6
54			3.0 ± 0.1
55			57 ± 7
56			26.8 ± 0.5
57			4.5 ± 0.4
58			0.89 ± 0.15
59			92 ± 13
60			1.64 ± 0.19

Table 3. Substitutions of Phe3 in bicyclic peptide **2**. Standard deviations of at least three measurements are indicated.

peptide	sequence	K_i (nM)
61	RCF$^{4\text{-}F}$RLPCRQLRCR	0.84 ± 0.03
62	CFRLPCRQLRCR	330 ± 30
63	RCFRLPCRQLRC	9.3 ± 0.6

Table 4. Removal of the C-terminal tryptophan from bicyclic peptide **58** yielded the bicyclic peptide **61**. Removal of the N- or C-terminal arginine from bicyclic peptide **1** yielded the bicyclic peptides **62** and **63**, respectively. Standard deviations of at least three measurements are indicated.

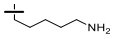
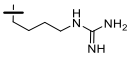
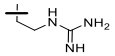
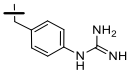
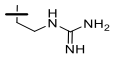
peptide	position	substitution	K_i (nM)	$t_{1/2}$ (hrs)
64	Arg1	Ile	83 ± 3	
65		Val	99 ± 17	
66		Ala	59.2 ± 1.5	
67		Lys	9.0 ± 0.9	
68		D-Arg	34.4 ± 0.7	
69			6.5 ± 0.3	2.0 ± 0.7
70			8.8 ± 1.4	5 ± 3
71			14 ± 2	22 ± 3
72			15 ± 3	13 ± 2
73	Arg1 Phe3	 4-F-Phe	1.63 ± 0.18	16 ± 4

Table 5. Substitution of Arg1 in bicyclic peptide **1**. The stability in human citrated plasma is indicated as half-life, $t_{1/2}$. Standard deviations of at least three measurements are indicated.

Figures

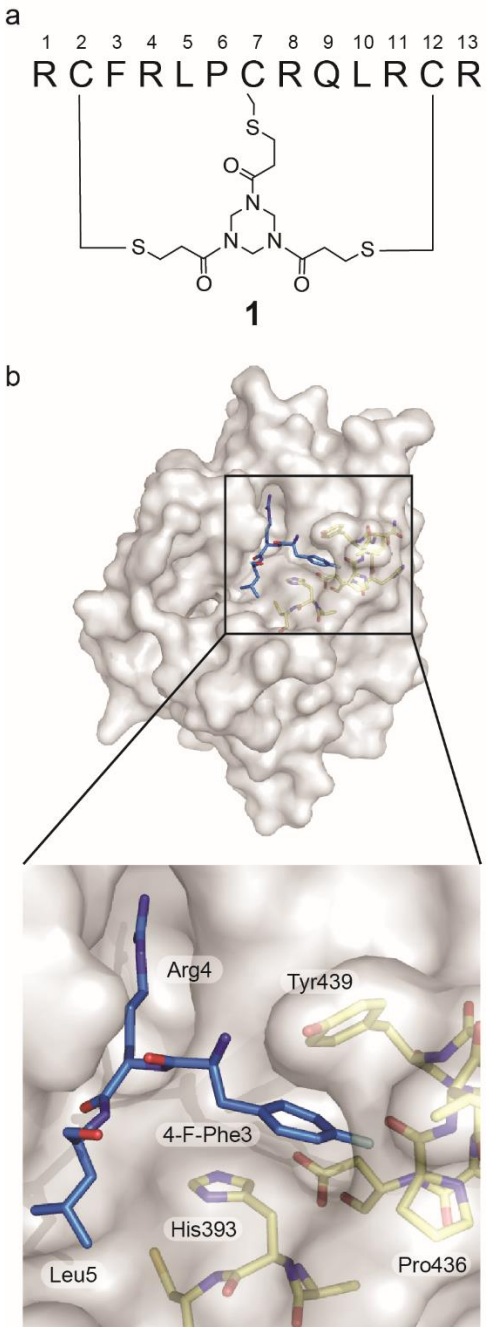


Figure 1. (a) Schematic representation of bicyclic peptide **1**. (b) Structural model for the three key amino acids 4-fluoro-phenylalanine (4-F-Phe3), Arg4 and Leu5 of bicyclic peptide **61** bound to FXIIa. Amino acids of two surface-exposed loops of FXIIa, surrounding 4-fluoro-phenylalanine are shown in yellow.

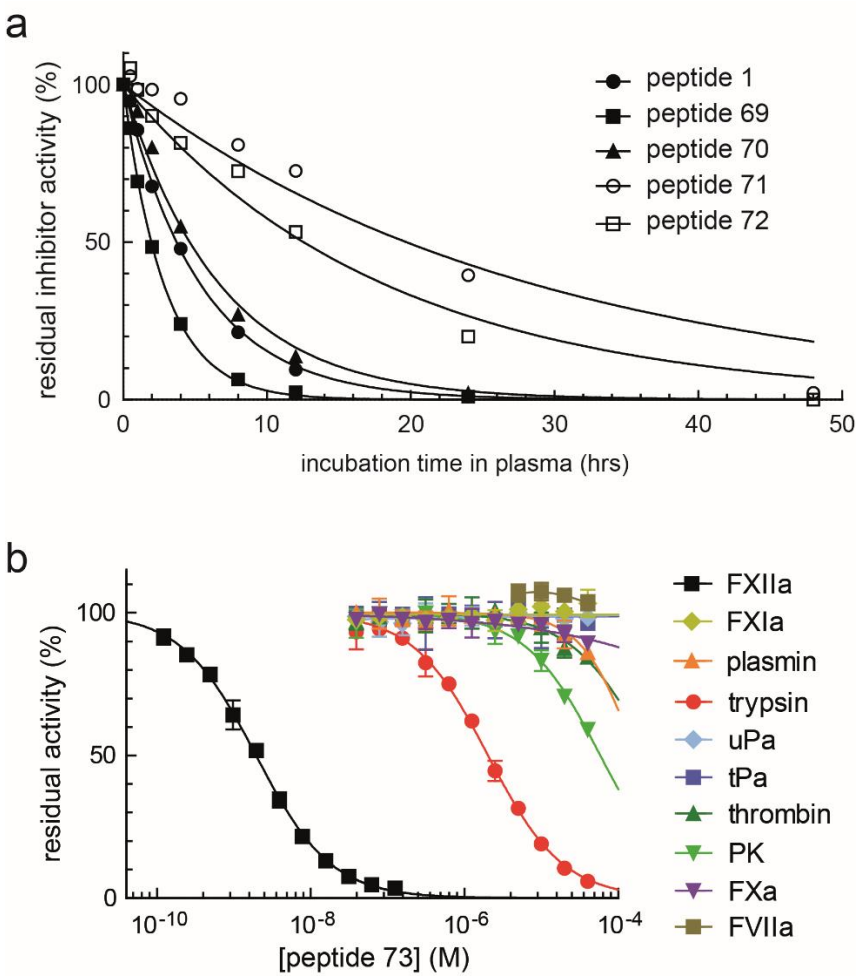


Figure 2. (a) Stability of bicyclic peptides in human plasma. The apparent IC_{50} was determined after incubating the peptides in human plasma at 37 °C for the indicated time periods. Average values of three measurements are indicated. (b) Specificity of bicyclic peptide **73**. Average values of at least three measurements. Standard deviations are indicated.

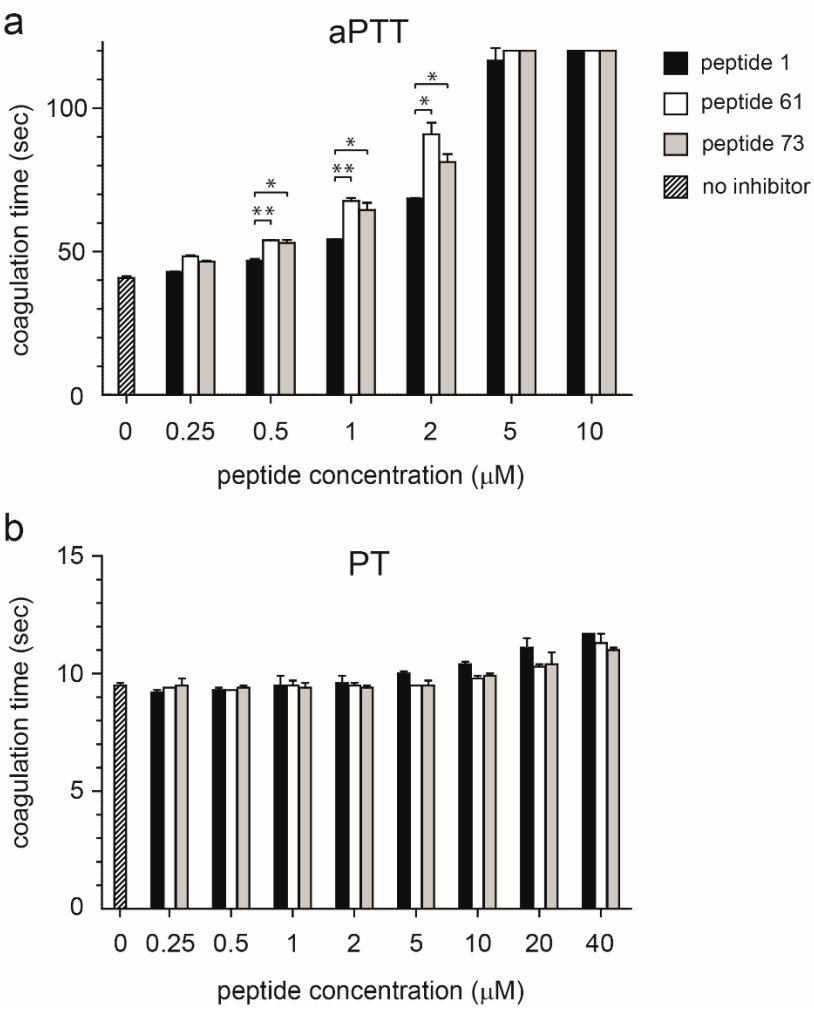


Figure 3. Coagulation parameters aPTT (a) and PT (b) for bicyclic peptides **1**, **61** and **73**. Standard deviations of aPTT, PT are calculated based on three measurements (*: $p < 0.05$; **: $p < 0.01$).

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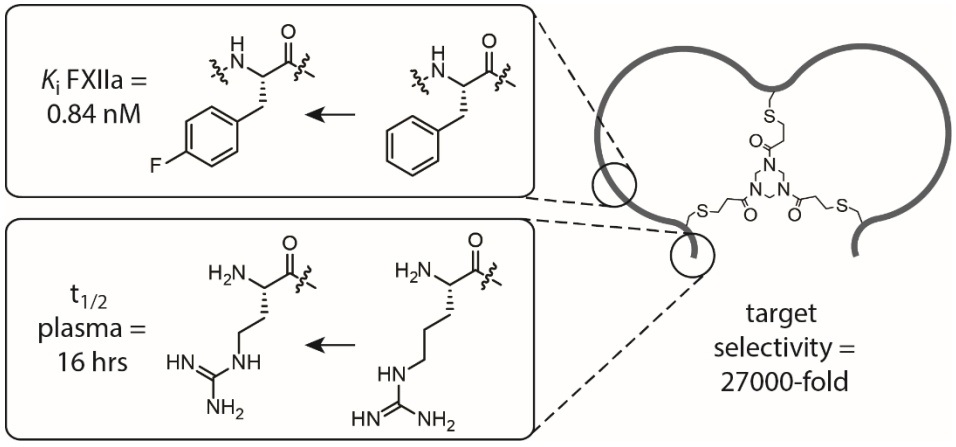

Supporting Information

Peptide macrocycle inhibitor of coagulation factor XII with subnanomolar affinity and high target selectivity

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Supporting Figures

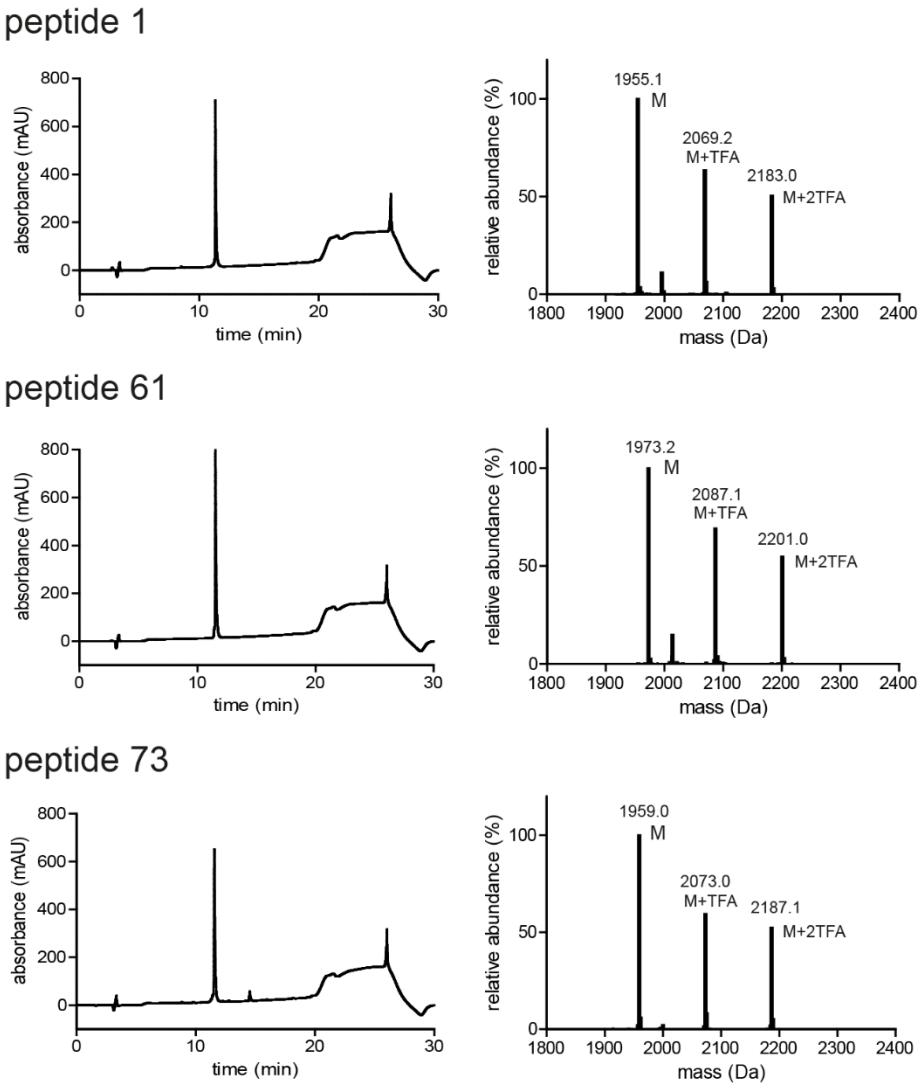


Figure S1. Analysis of bicyclic peptide **1**, **61** and **73** by reversed phase chromatography (left) and mass spectrometry (right). Absorption in HPLC was measured at 220 nm. Integration of the area under curve indicated purities > 95% for all three peptides characterized. Masses were measured by ESI-MS. Calculated masses differed by less than 0.5 Da compared to the expected masses for all peptides analyzed. TFA adducts were observed for all peptides (+114 Da).

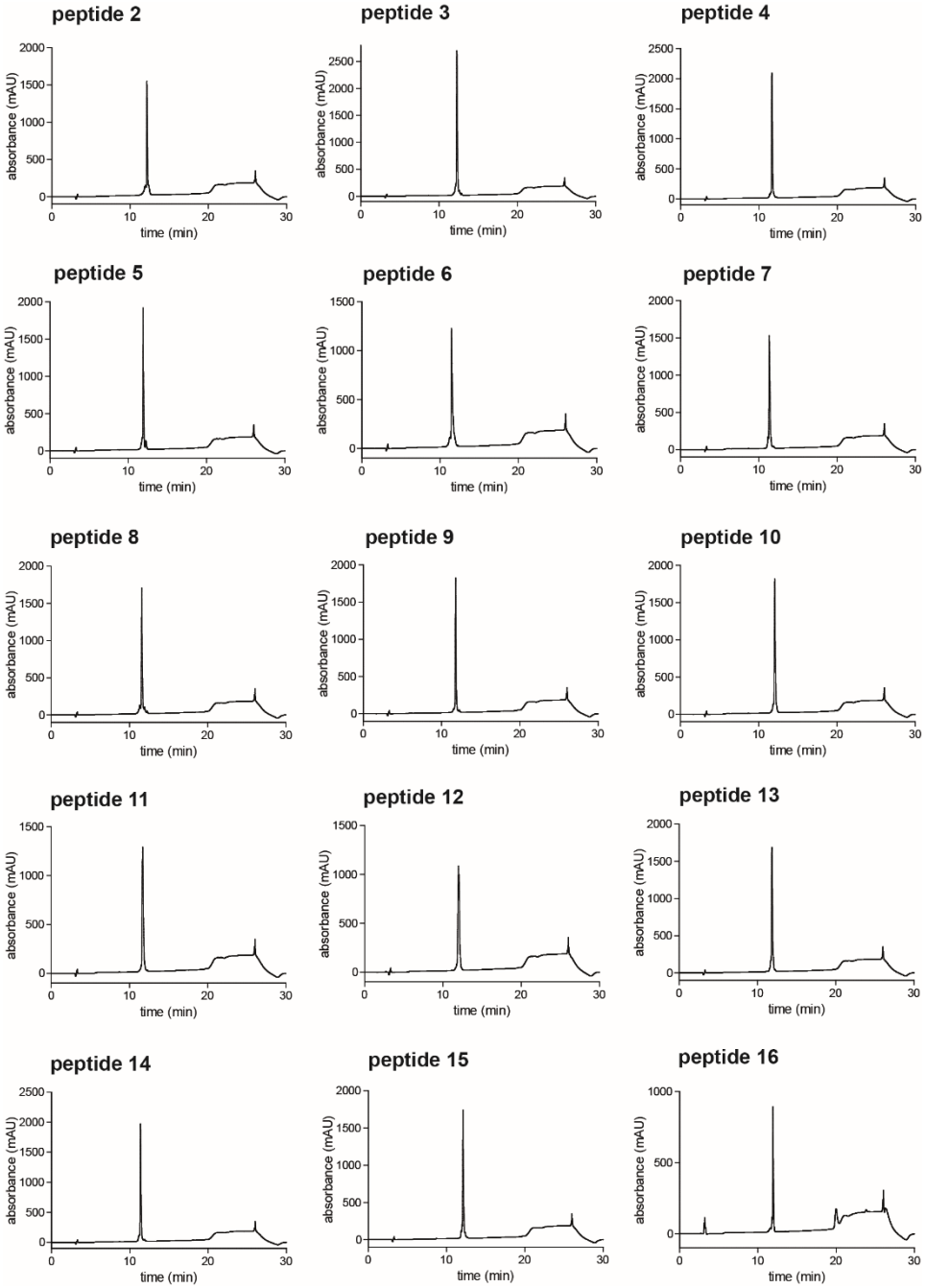
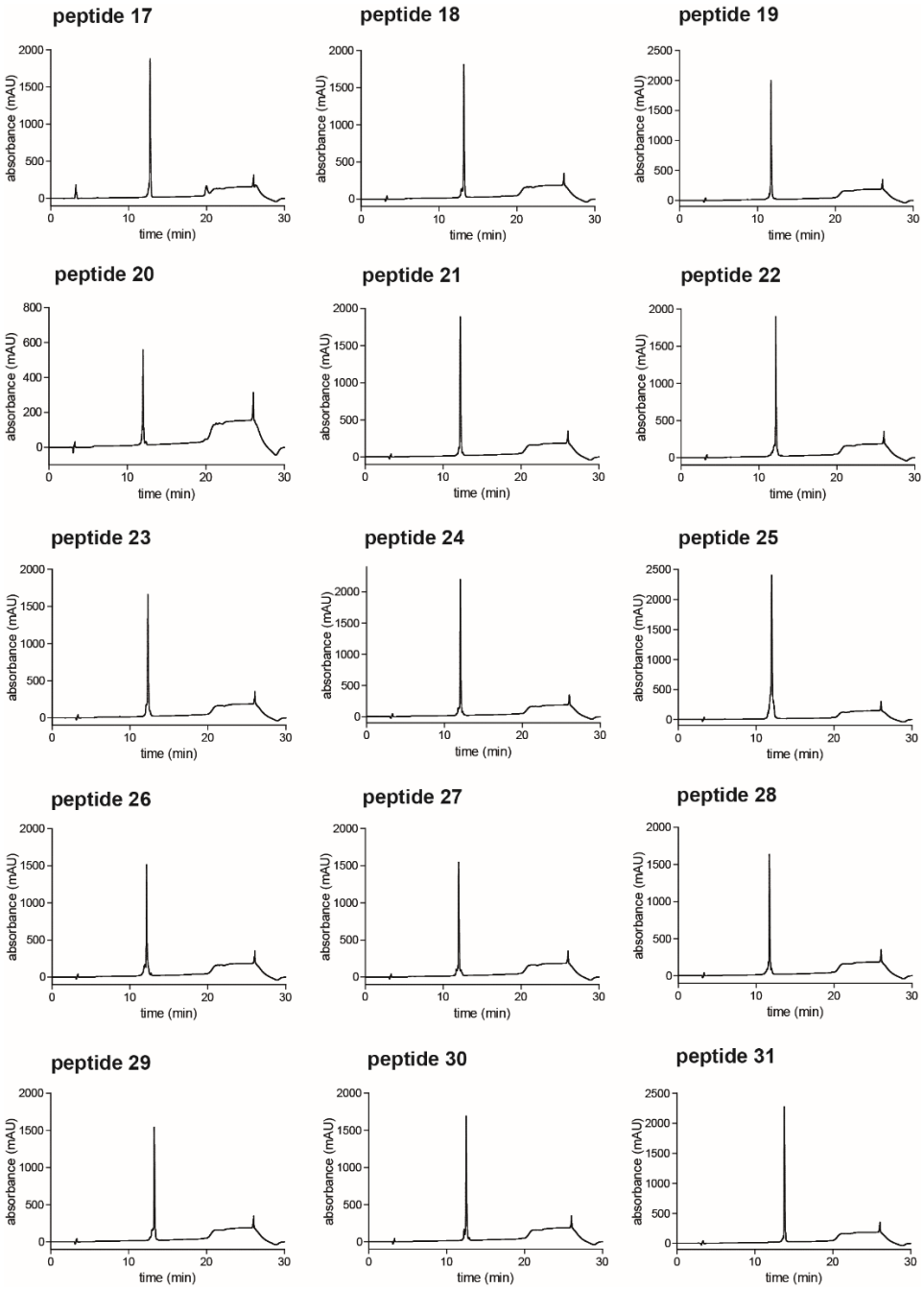


Figure S2. Analysis of bicyclic peptides by reversed phase chromatography. Absorption in HPLC was measured at 220 nm.



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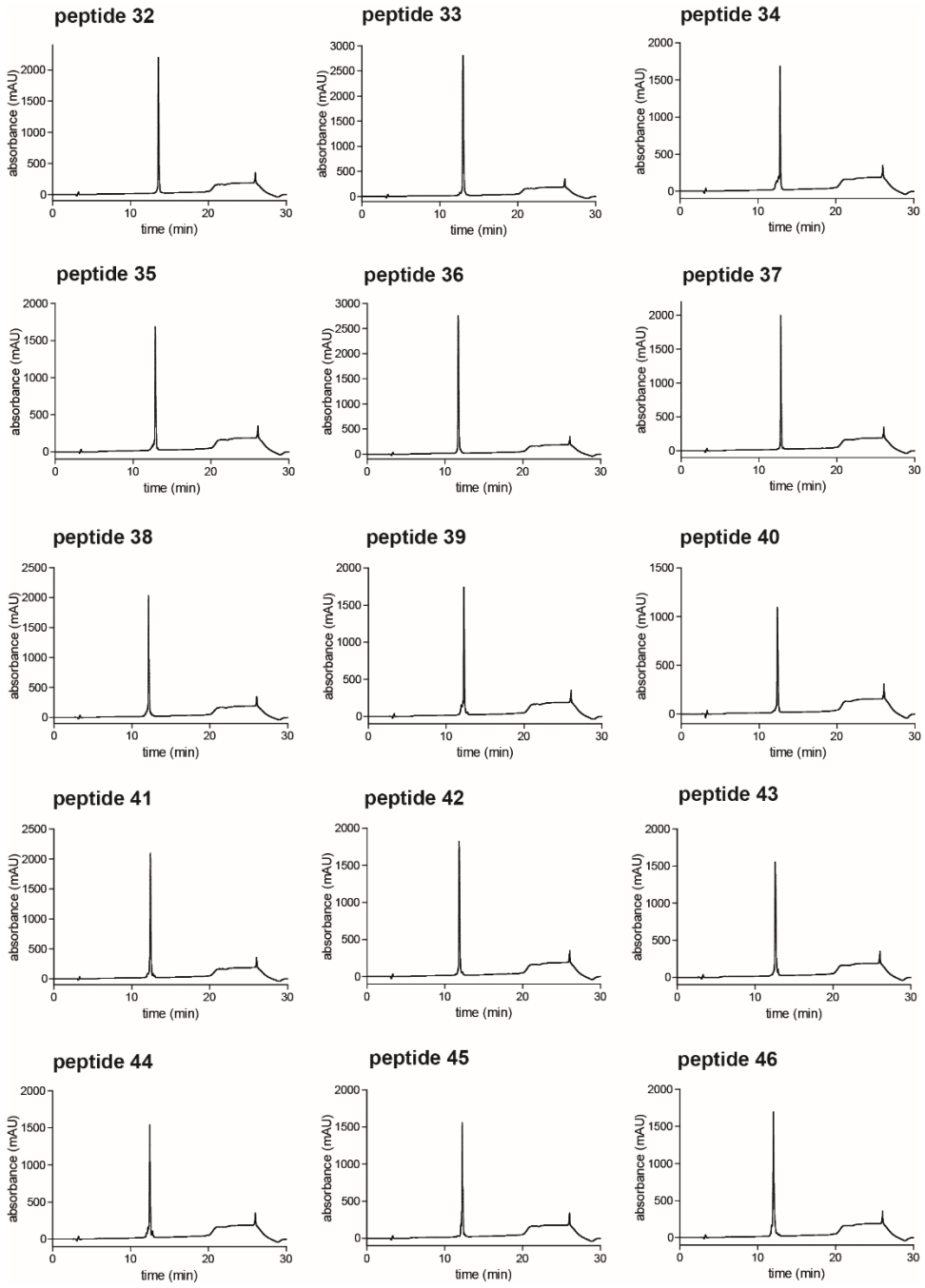

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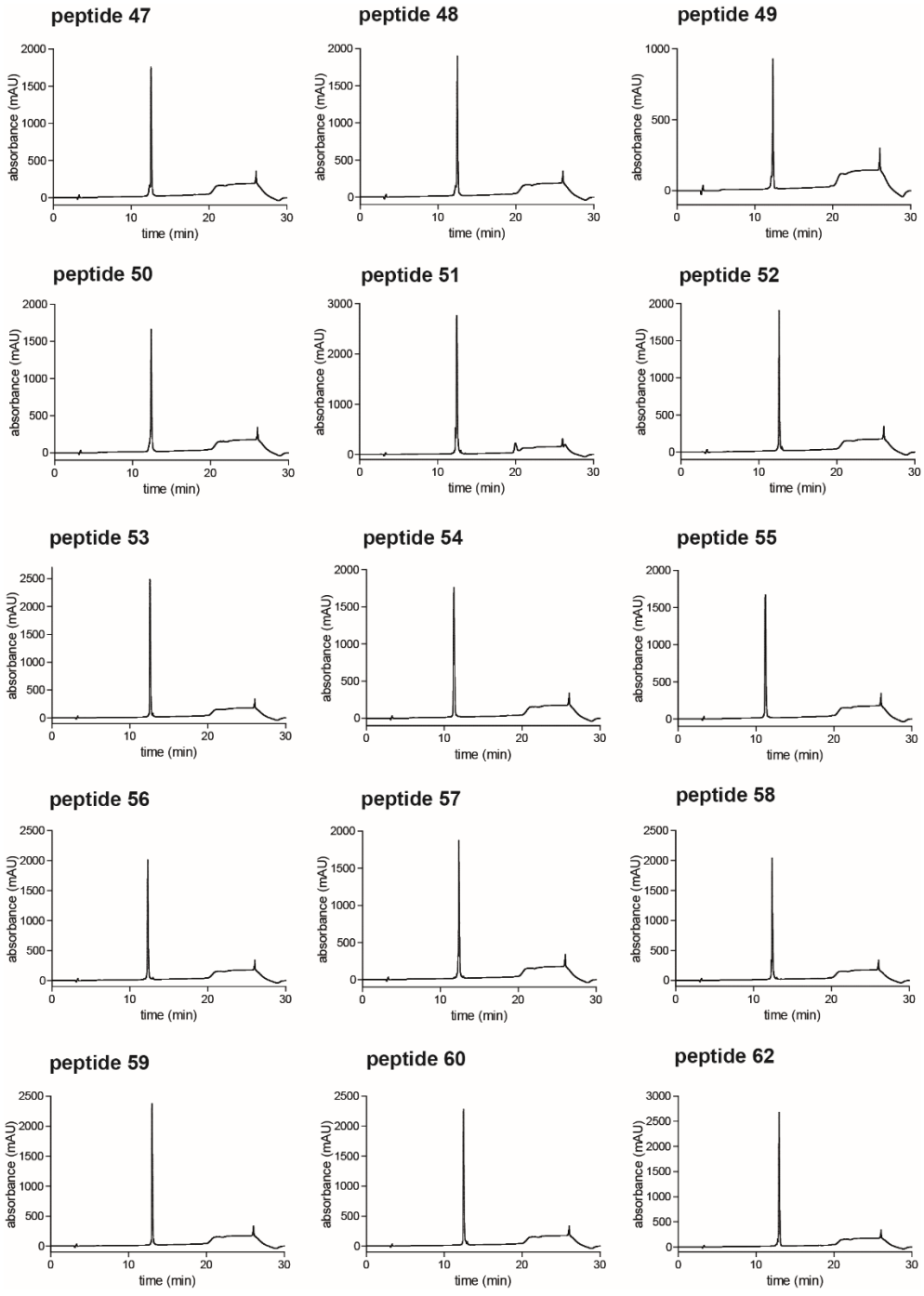

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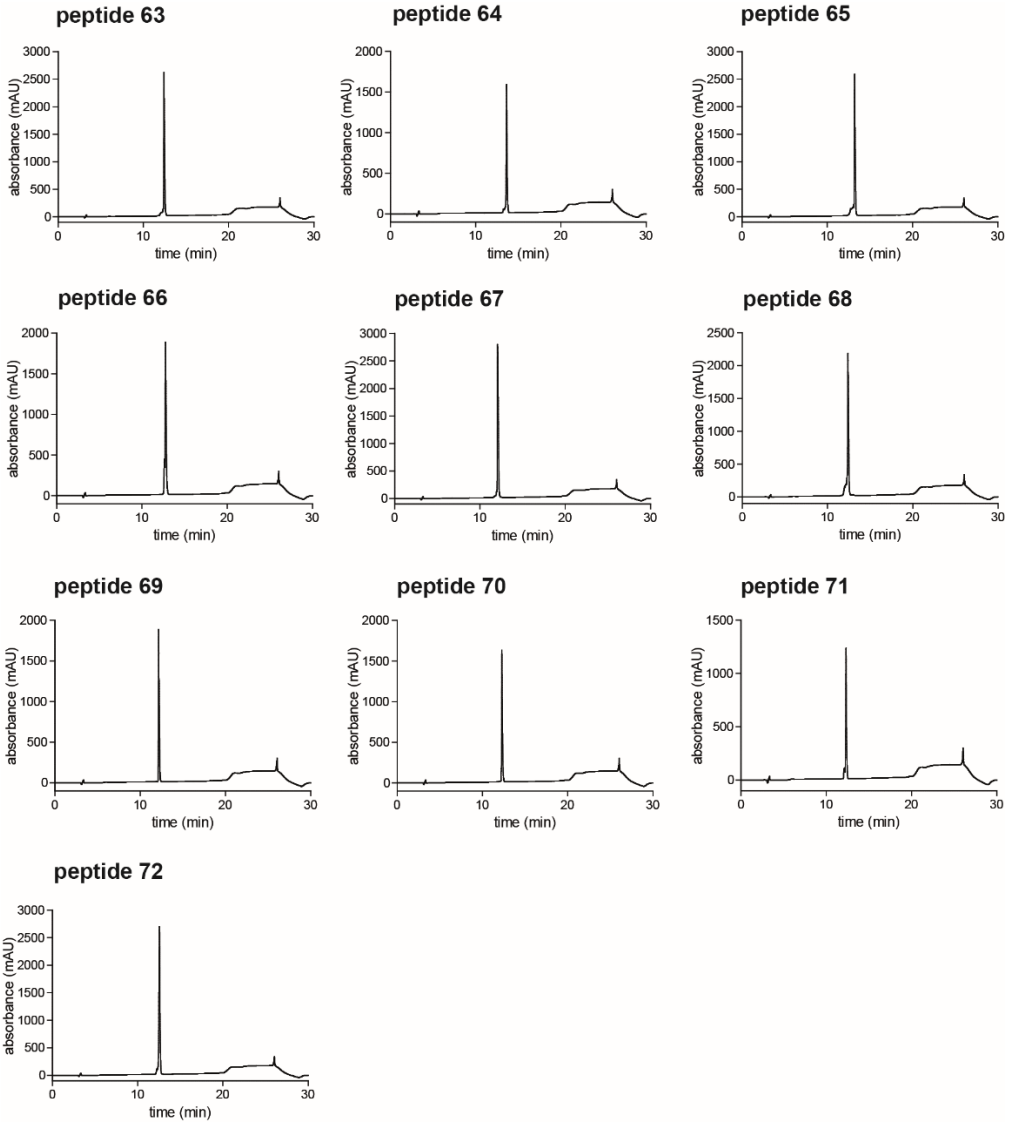

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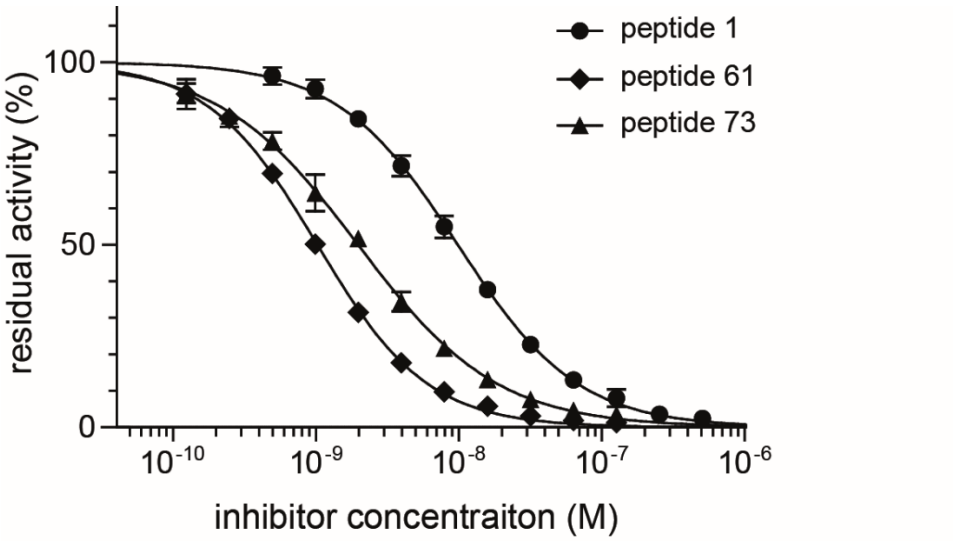

Figure S3. Inhibition of FXIIa by bicyclic peptides **1**, **61** and **73**. Hill slopes are 1.04, 1.13 and 0.89, respectively.

Supporting Table

peptide	observed $[M+4H]^{4+}$	observed $[M+3H]^{3+}$	calculated MW	theoretical MW
1	489.8	652.8	1955.1	1955.4
61	494.3	658.7	1973.2	1973.4
73	490.7	654.1	1959.0	1959.4
2	536.4	714.7	2141.4	2141.6
3	546.2	727.7	2180.4	2180.7
4	540.4	720.1	2157.5	2157.6
5	527.8	703.5	2107.3	2107.6
6	523.8	698.2	2091.4	2091.6
7	517.4	689.4	2065.3	2065.5
8	524.4	698.8	2093.3	2093.6
9	527.8	703.5	2107.4	2107.6
10	529.3	705.5	2113.2	2113.6
11	532.9	710.2	2127.5	2127.6
12	536.4	714.9	2141.6	2141.8
13	540.9	720.9	2159.5	2159.7
14	525.8	700.8	2099.2	2099.5
15	529.8	706.2	2115.4	2115.6
16	533.8	711.5	2131.2	2131.6
17	536.9	715.5	2143.6	2143.6
18	540.4	720.2	2157.6	2157.7
19	540.7	720.5	2158.5	2158.6
20	540.9	720.8	2159.5	2159.6
21	533.2	710.5	2128.6	2128.6
22	532.9	710.1	2127.4	2127.6
23	536.6	715.2	2142.4	2142.6
24	538.7	717.9	2150.6	2150.6

peptide	observed $[M+4H]^{4+}$	observed $[M+3H]^{3+}$	calculated MW	theoretical MW
25	532.9	710.1	2127.4	2127.6
26	536.4	714.9	2141.5	2141.6
27	540.9	720.8	2159.5	2159.7
28	525.8	700.8	2099.2	2099.5
29	541.4	721.5	2161.3	2161.6
30	539.9	719.5	2155.6	2155.6
31	550.4	733.6	2197.5	2197.7
32	555.5	740.3	2217.8	2217.7
33	548.9	731.6	2191.5	2191.5
34	548.9	731.5	2191.4	2191.5
35	550.4	733.3	2197.3	2197.6
36	550.2	733	2196.3	2196.5
37	542.9	723.5	2167.6	2167.5
38	532.9	710.2	2127.6	2127.6
39	539.9	719.6	2155.5	2155.6
40	532.9	710.2	2127.6	2127.6
41	548.5	730.9	2189.8	2189.5
42	532.9	710.2	2127.5	2127.6
43	540.0	719.5	2155.7	2155.6
44	539.9	719.5	2155.6	2155.6
45	536.4	714.8	2141.4	2141.6
46	532.8	710.0	2127.1	2127.6
47	540.0	719.6	2155.7	2155.6
48	539.9	719.5	2155.6	2155.6
49	536.5	714.9	2141.7	2141.7
50	539.9	719.5	2155.6	2155.6
51	539.9	719.5	2155.6	2155.7
52	540.0	719.5	2155.7	2155.7
53	539.9	719.5	2155.6	2155.7

peptide	observed $[M+4H]^{4+}$	observed $[M+3H]^{3+}$	calculated MW	theoretical MW
54	536.6	715.2	2142.5	2143.2
55	536.7	715.1	2142.6	2143.2
56	540.9	720.8	2159.5	2159.6
57	540.9	720.9	2159.6	2159.6
58	540.9	720.8	2159.5	2159.6
59	567.9	756.8	2267.5	2267.5
60	547.7	729.8	2186.5	2186.6
62	463.3	617.5	1849.2	1849.3
63	463.3	617.5	1849.2	1849.3
64	491.5	655.1	1962.2	1962.5
65	488.0	650.5	1948.2	1948.4
66	481.1	641.1	1920.3	1920.4
67	495.4	660.1	1977.4	1977.5
68	502.4	669.4	2005.3	2005.5
69	498.9	664.8	1991.5	1991.5
70	505.7	674.1	2019.0	2019.5
71	498.9	664.8	1991.3	1991.5
72	514.4	685.5	2053.5	2053.5

Table S1. Experimentally determined and theoretical molecular weights of all bicyclic peptides synthesized. The experimentally determined molecular weight was calculated based on the mass peaks $(M+4H)^{4+}$ and $(M+3H)^{3+}$.