Improving the binding affinity of in vitro evolved cyclic peptides by inserting atoms into the macrocycle backbone

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Abstract

Cyclic peptides binding to targets of interest can be generated efficiently with powerful *in vitro* display techniques such as phage display or mRNA display. The cyclic peptide libraries screened with these methods are generated by altering in a combinatorial fashion the amino acid sequence of the peptides, the number of amino acids in the macrocyclic rings, and the cyclization chemistry. A structural element that cannot easily be varied in the cyclic peptides is the backbone that is built of amino acids that each contributes exactly three atoms to the macrocyclic ring structure. Herein, we proposed to affinity mature a phage-selected bicyclic peptide inhibitor of coagulation factor XII (FXII) by screening variants having one or two carbon atoms inserted in different positions of the backbone, and thus tapping into a structural space that was not sampled in the phage display selection. Two mutants with 4.7- and 2.5-fold improved K_is were identified. The better one of the two inhibitors blocked FXII with a K_i of 1.5 ± 0.1 nM and inhibited activation of the intrinsic coagulation pathway with an EC_{2x} of 1.7 μM. The strategy of ring size variation by one or several atoms should be generally applicable for the affinity maturation of *in vitro* evolved cyclic peptides.

Peptide macrocycles have a number of favorable properties that make them an attractive modality for the development of therapeutics. They can bind with high affinity and selectivity to protein targets, their degradation products are non-toxic, they offer different administration options, and they can be chemically synthesized. Another advantage is their fast development; they can be isolated from large combinatorial libraries generated by either chemical[1] or ribosomal synthesis.[2, 3] *In vitro* display techniques such as phage display or mRNA display allow the generation and screening of billions of peptide macrocycles in a short time and for a moderate cost. The chemical and structural diversity of peptide macrocycle libraries is obtained by varying the amino acid sequence, the number of amino acids,[4, 5] and the cyclization chemistry.[2, 6]

For some protein targets, it has been difficult to generate high-affinity peptide macrocycles using *in vitro* display techniques, despite the enormous size and diversity of the libraries that is screened. For therapeutic application, peptide macrocycles typically require a dissociation constant in the low nanomolar or picomolar range. A frequently chosen strategy to affinity mature *in vitro* evolved cyclic peptide ligands is to modify the side chains that make interactions with the target. The modular architecture of peptides, the commercial availability of many unnatural amino acids, and the availability of automated synthesis and purification methods allows efficient preparation of medium-sized libraries of such peptides. A limitation of strategies that rely on amino acid side chain modification can be the low rate of identifying peptides with improved activity. We had recently attempted to improve the binding affinity of the bicyclic peptide FXII618 (herein named bicyclic peptide **1,** Figure 1a),[7] a nanomolar inhibitor of the coagulation factor XII that was isolated by phage display. Following a strategy of substituting amino acid side chains in one position of the bicyclic peptide **1** at a time, we had to synthesize and test more than 50 peptides to find a first peptide variant with a better inhibitory activity (unpublished work).

In this work, we proposed to affinity mature the bicyclic peptide FXII inhibitor **1** by altering the backbone instead of the side chains. Specifically, we proposed to insert one or two carbon atoms in different positions of the macrocyclic rings. Peptides screened by phage display are composed of canonical amino acids that each contributes three atoms to the macrocyclic ring. More precisely, they contain 3*n+m backbone atoms wherein ‘n’ is the number of amino acids and ‘m’ the number of atoms contributed by the cyclization linker. In the phage display screen in which **1** was isolated, cyclic peptides with 3*n+m+1 or 3*n+m+2 backbone atoms were thus not sampled. We speculated that inserting carbon atoms in some sites of the backbone could potentially lead to small changes

in the conformation of the macrocycle, and that this in turn could strengthen existing molecular interactions or allow the formation of new non-covalent contacts of **1** with FXII.

The strategy of inserting atoms into the backbone has been applied to several nature-derived cyclic peptides for studying the structure-activity relationship or enhancing the biological activity. For example Ghadiri and co-workers had inserted single atoms into histone deacetylase (HDAC) inhibitors and measured their cytotoxic activity towards tumor cells and determined their structure.[8] The group of Muir had varied the macrocycle size of so called autoinducing peptides (AIPs) secreted by *Staphylococcus aureus* to study the structure-activity relationship.[9] Hansen and co-workers had extended the ring size of amphipathic cyclic peptides by different number of atoms to investigate the effect on antimicrobial and hemolytic activity.[10] Inserting or deleting single carbon atoms at different positions in macrocyclic rings of peptide ligands has not been used as a systematic approach for improving the binding affinity of *in vitro* evolved cyclic peptides.

A synthetically efficient way of generating cyclic peptides with additional carbon atoms in the rings is by replacing α-amino acids with β-amino acids that contain an additional carbon atom between the amino and carboxyl group (Figure 1b). α-to-β-amino acid substitution has been applied extensively to α-helical peptides, following the observation that β-amino acids can substantially improve their stability.[11-14] In some α-helical peptides, β-amino acids had improved in addition to the stability the binding affinity, as for example in an analogue of parathyroid hormone receptor-1 agonist[15] or an engineered VEGF signaling inhibitor based on the Z-domain.[16] The strategy of α-to-β-amino acid substitution was also applied to a handful of nature-derived cyclic peptides, including the above mentioned HDAC inhibitors and the AIPs.[8, 9] In this work, we chose to apply β-amino acids to efficiently access variants of **1** having a single carbon atom inserted in different positions of the backbone.

In order to identify amino acid positions in which insertion of a carbon atom could potentially improve the binding affinity of bicyclic peptide **1**, we synthesized two series of peptide variants: one having individual amino acids replaced by β-alanine, and the other one having them replaced by glycine (Table 1). Comparison of the peptides containing β-alanine and glycine in a specific position allowed understanding if an additional carbon atom in the specific position enhances binding to FXII, independent of the amino acid side chain. Unmodified bicyclic peptide **1** inhibited

FXII with a K_i of 7 ± 0.4 nM. Substitution of Phe3, Arg4 and Leu5 to β-alanine (**2**, **4**, **6**) or glycine (**3**, **5**, **7**) reduced the inhibitory activity by large factors (> 300-fold). For all three positions, the substitution to β-alanine gave a larger drop in activity than the substitution to glycine, indicating that an additional carbon atom in these three positions of the main chain negatively affects the binding of bicyclic peptide **1**. Substitution of Pro6 to β-alanine (**8**) and glycine (**9**) reduced the affinity around 3- and 15-fold, respectively. The smaller loss in binding affinity found for the β-alanine variant suggested that insertion of one carbon atom in this position enhances the binding to FXII. Amino acid substitution at Arg8 had the opposite effect, giving a larger affinity drop for the peptide with the β-alanine (19- versus 2-fold; **10**, **11**). In the positions Gln9 and Leu10, substitution to β-alanine and glycine reduced the inhibitory activity more than 100-fold (**12**, 1**3**, **14**, **15**). Finally, in position Arg11, replacement of the amino acid with both, β-alanine and glycine reduced the binding by a small factor (3-fold; **16** and **17**).

Based on the results of the β-alanine/glycine screen, we considered Pro6 and Arg11 as the most promising sites for α-to-β-amino acid substitutions. β-amino acids can have side chains at either the alpha (C2) carbon or the beta (C3) carbon and are denoted β^2- and β^3-residues, respectively. Given that these carbon atoms can have R or S configuration, four diastereoisomeric β-amino acids exist for any given side chain. We first substituted Pro6 with a range of cyclic β-amino acids (Table 2). All these substitutions reduced the inhibitory activity 80-fold or more (**18**-**22**). It is likely that the cyclic β-amino acids imposed conformational constraints onto **1**, hindering efficient binding to FXII. We subsequently synthesized peptides in which Pro6 was substituted with acyclic β-amino acids having methyl groups linked to either the carbon 3, carbon 2 or the amino group (**23**-**27**; Table 2). We reasoned that the methyl groups could potentially replace interactions that were formed by the proline ring. Three of these peptides had double-digit inhibitory constants (**23**, **26**, **27**), but they all had weaker K_is than the peptide with β-alanine in this position. The second position that was identified to tolerate the β-alanine substitution reasonably well was Arg11. We replaced this amino acid with the β^3-amino acid structurally resembling best L-Arg (S configuration in C3; (S)-β^3-homoarginine) (**28**). This substitution yielded a peptide with a 4.7-fold improved K_i (we named the bicyclic peptide FXII700; 1.5 ± 0.1 nM; Table 2). Given that the two peptides **16** and **17**, having β-alanine and glycine in position Arg11, had a comparable affinity, it is likely that the affinity improvement achieved with the (S)-β^3-homoarginine substitution resulted from interactions of the arginine side chain with FXII that are apparently better for the β^3-amino acid than for L-Arg.

Another efficient strategy for inserting carbon atoms into the macrocyclic rings of bicyclic peptide **1** is by substituting the cysteines with homocysteine or 5-mercapto-norvaline, having one and two additional carbons in the side chains, respectively (Figure 1b). We synthesized six variants of **1**, each having one of the three cysteines replaced by either of the two cysteine analogs (Table 3). Replacement of the Cys12 with homocysteine and 5-mercapto-norvaline improved the binding 2.5- and 1.2-fold, respectively (K_i of **31** = 2.8 ± 0.5 nM, K_i of **34** = 5.8 ± 1.2 nM). Replacement of the other two cysteines conserved the binding affinity in the case of homocysteine (bicyclic peptides **29** and **30**) and slightly reduced the binding for 5-mercapto-norvaline (bicyclic peptides **32** and **33**). We named the bicyclic peptide **31** having the best K_i FXII701. Having found that cysteine analogues with longer side chains can improve the affinity, we tried also the conformationally constrained cyclic amino acid 4-mercapto-proline, having either 2*S*,4*S* or 2*S*,4*R* configuration (Table 3). In these two amino acids, the sulfur and carbon α atoms are spaced by two carbon atoms, as in homocysteine. Replacement of the Cys12 with 4-mercapto-proline (2*S*,4*S*) or (2*S*,4*R*) improved the binding 2.8- and 1.7-fold, respectively (K_i of **37** = 2.5 ± 0.4 nM, K_i of **40** = 4 ± 1.1 nM). Replacement of Cys7 with 4-mercapto-proline improved the binding for one of the diastereomers (2*S*,4*R*; K_i of **39** = 5.8 ± 1 nM) but not for the other one (2*S*,4*S*; K_i of **36** = 1228 nM). In position Cys2, replacement with both 4-mercapto-proline diastereomers reduced the binding more than 1000-fold (**35**, **38**). We subsequently combined two amino acid substitutions that improved the inhibitory activity of **1** by large factors, Arg11 to (S)-β^3-homoarginine and Cys12 to homocysteine. The resulting peptide (**41**) had a K_i of 2.7 ± 0.32 nM, showing that the affinity enhancement of the two modifications is not additive. This finding was not surprising given the close proximity of Arg11 and Cys12. Insertion of a carbon atom in one of the two positions presumably allows a small conformational change in this region of the macrocycle resulting in stronger molecular interactions of an amino acid side chain, such as the one of Arg11 with FXIIa.

We finally tested if the affinity-matured bicyclic peptides **28** and **31** were blocking better intrinsic coagulation pathway activation than the lead peptide **1**. All three peptides dose-dependently prolonged the intrinsic coagulation time (aPTT) of human plasma wherein **28** and **31** were superior to **1** (Figure 2a). The inhibitor with the highest affinity, bicyclic peptide **28** showed the best prolongation with an EC_{2x} of 1.7 μM as compared to peptide **1** which has an EC_{2x} of 3.1 μM. As the lead peptide **1**, the affinity-matured inhibitors did not affect the extrinsic coagulation (PT), showing that the high target selectivity was conserved (Figure 2b).

In summary, we have proposed to improve the binding affinity of *in vitro* evolved cyclic peptide ligands by varying the size of the macrocyclic rings by one or two carbon atoms. We reasoned that this chemical space is not sampled by screening genetically encoded cyclic peptide libraries and could potentially offer a rich source for slightly improved ligands. Indeed, synthesis and screening of only 40 peptide variants of the bicyclic peptide FXII inhibitor **1** yielded several inhibitors with substantially improved K_is.

Materials and methods

Materials

Fmoc-L-α-amino acids, O-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBt), and Rink Amide AM resin were purchased from GL Biochem. Fmoc-β-amino acids were purchased from Chemimpex, Polypeptide and TCI. Fmoc cysteine derivatives were purchased from Polypeptide.

Peptide synthesis

Peptides were synthesized on an Advanced ChemTech 348-Ω peptide synthesizer (Aapptec) by solid phase peptide synthesis using standard Fmoc procedures. Rink Amide AM resin was used as solid support (0.03 mmol scale) and DMF as solvent. Each amino acid was coupled twice (4 eq, 0.2 M in DMF) using HBTU/HOBt (4 eq, 0.45 M in DMF) and DIPEA (6 eq, 0.5 M in DMF) (RT, 30 min, 400 rpm).The resin was washed four times with DMF after the coupling reaction. The N-terminal Fmoc protecting group was removed with 20% (v/v) piperidine in DMF (RT, 2x5 min, 400 rpm). The resin was washed five times with DMF after deprotection.

Peptide cleavage from the resin

The side chains were deprotected and the peptide cleaved from the Rink Amide AM resin by incubation with 5 mL cleavage solution (90% v/v TFA, 2.5% v/v 1,2-ethanedithiol, 2.5% w/v phenol, 2.5% v/v thioanisole, 2.5% v/v H_2O) for 2 h with shaking. The resin was removed by vacuum filtration, and the peptides were precipitated with ice-cold diethyl ether (50 mL), incubated for 30 min at -20 °C, and pelleted by centrifugation for 5 min at 2700 g. The diethyl ether was discarded, and the precipitate was then washed twice with diethyl ether. The remaining solvent was evaporated at RT.

Peptide cyclization with TATA:

The crude peptide (typically ~ 50 mg) was dissolved in 6 mL 33% MeCN and 67% H_2O (resulting in a concentration of ~ 3.5 mM). 1,3,5-triacryloyl-1,3,5-triazinane (TATA) (10 mM, 1.2 eq) in MeCN was added to the dissolved peptide. The reaction was started by the addition of degassed aqueous NH_4HCO_3 buffer (60 mM, pH = 8.0) until a final peptide concentration of 1 mM (1 h at 30 °C in water bath). The peptide was lyophilized.

Peptide purification by reversed-phase HPLC

Modified peptide powder was dissolved in 1 mL DMSO, 2 mL MeCN containing 0.1% TFA, and 7 mL H_2O containing 0.1% TFA, and purified on a preparative C18 column (Vydac C18 TP1022 250, 22 mm, 10 mm) using a linear gradient of solvent B (MeCN 0.1% v/v TFA) over solvent A (H_2O, 0.1% v/v TFA) (13 min, 15–28% MeCN, flow rate: 20 $mLmin^{-1}$). Fractions containing the desired peptide product were identified by ESI-MS analysis and lyophilized.

Protease Inhibition assays

The inhibitory activity of the synthesized bicyclic peptides was determined by incubation with the protease and quantification of the residual activity at various peptide concentrations using a fluorogenic substrate. For each peptide, 2 mM stock solutions were prepared in H_2O. Residual enzymatic activities were measured in aqueous buffer containing 10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% (v/v) Triton X-100 and 0.1 % (w/v) BSA (pH 7.4) in a final volume of 150 μl. The final concentration of human β-FXIIa (HFXIIAB; Molecular Innovations) was 0.5 or 1 nM. Dilutions of peptides ranging from 10,000 to 0.1 nM, were prepared with buffer. The final concentration of the fluorogenic substrate Boc-Q-G-R-AMC (Bachem) was 50 μM. The fluorescence was measured using an Infinite M200 Pro plate reader (excitation at 368 nm, emission at 467 nm; Tecan). The fluorescence was recorded for 60 min with a measurement every minute at 25 °C. Sigmoidal curves were fitted to the data using the following dose response equation wherein x = peptide concentration, y = % activity of reaction without peptide, A_1 = 100%, A_2 = 0%, p = 1. IC_{50} values were derived from the fitted curve using the GraphPad Prism 5 software.

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(LOG_x 0 - x)p}}$$

The inhibitory constant K_i was 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. The K_M for Boc-Gln-Gly-Arg-AMC has been determined to be 256 ± 42 μM (mean ± SD, n = 4).

Coagulation assays

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 is monitored. The time until the ball stops moving is 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).

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Tables

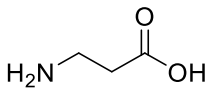
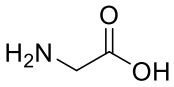
position	β-alanine 		Glycine 	
	peptide	K_i (nM)	peptide	K_i (nM)
Phe3	2	> 10000 (65%)	3	2289
Arg4	4	> 10000 (83%)	5	6513
Leu5	6	> 10000 (75%)	7	> 10000 (51%)
Pro6	8	25 ± 1	9	109 ± 21
Arg8	10	132 ± 65	11	14 ± 0.6
Gln9	12	963 ± 36	13	2000
Leu10	14	1673	15	1119
Arg11	16	22 ± 0.7	17	23 ± 4

Table 1. β-alanine and glycine screen. Standard deviations were determined for peptides with K_is below 1 μM and are indicated. For peptides with K_is above 10 μM, the remaining protease activity at a concentration of 10 μM is indicated in brackets.

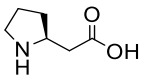
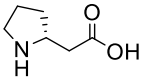
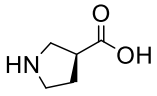
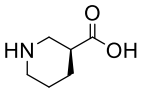
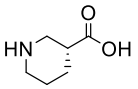
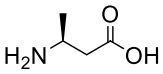
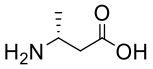
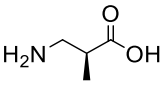
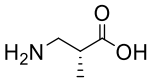
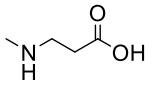
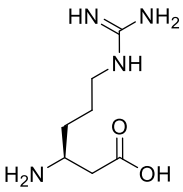
position	peptide	β-amino acid		K_i (nM)
		Name	structure	
Pro6	18	(S)-β^3-homoproline		1810
Pro6	19	(R)-β^3-homoproline		> 10000 (89%)
Pro6	20	(S)-β^2-proline		6783
Pro6	21	(S)-β^2-homoproline		> 10000 (68%)
Pro6	22	(R)-β^2-homoproline		578 ± 132
Pro6	23	(S)-β^3-homoalanine		82 ± 10
Pro6	24	(R)-β^3-homoalanine		4628
Pro6	25	(S)-β^2-homoalanine		232 ± 7
Pro6	26	(R)-β^2-homoalanine		35 ± 1
Pro6	27	N-Me-β-alanine		74 ± 8
Arg11	28	(S)-β^3-homoarginine		1.5 ± 0.1

Table 2. Substitution of Pro6 and Arg11 by β-amino acids. Standard deviations were determined for peptides with K_is below 1 μM and are indicated. For peptides with K_is above 10 μM, the remaining protease activity at a concentration of 10 μM is indicated in brackets.

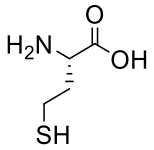
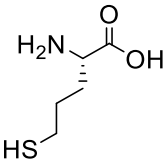
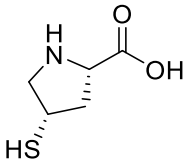
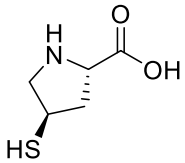
position	homocysteine 		5-mercapto-norvaline 		(2*S*,4*S*) 4-mercapto-proline 		(2*S*,4*R*) 4-mercapto-proline 	
	peptide	K_i (nM)	peptide	K_i (nM)	peptide	K_i (nM)	peptide	K_i (nM)
Cys2	29	6 ± 0.6	32	12 ± 0.3	35	> 10000 (68%)	38	> 10000 (62%)
Cys7	30	7 ± 1	33	33 ± 5	36	1228	39	5.8 ± 1
Cys12	31	2.8 ± 0.5	34	5.8 ± 1.2	37	2.5 ± 0.4	40	4 ± 1.1

Table 3. Substitution of cysteines with homocysteine, 5-mercapto-norvaline and 4-mercapto-proline. Standard deviations were determined for peptides with K_is below 1 μM and are indicated. For peptides with K_is above 10 μM, the remaining protease activity at a concentration of 10 μM is indicated in brackets.

Figures

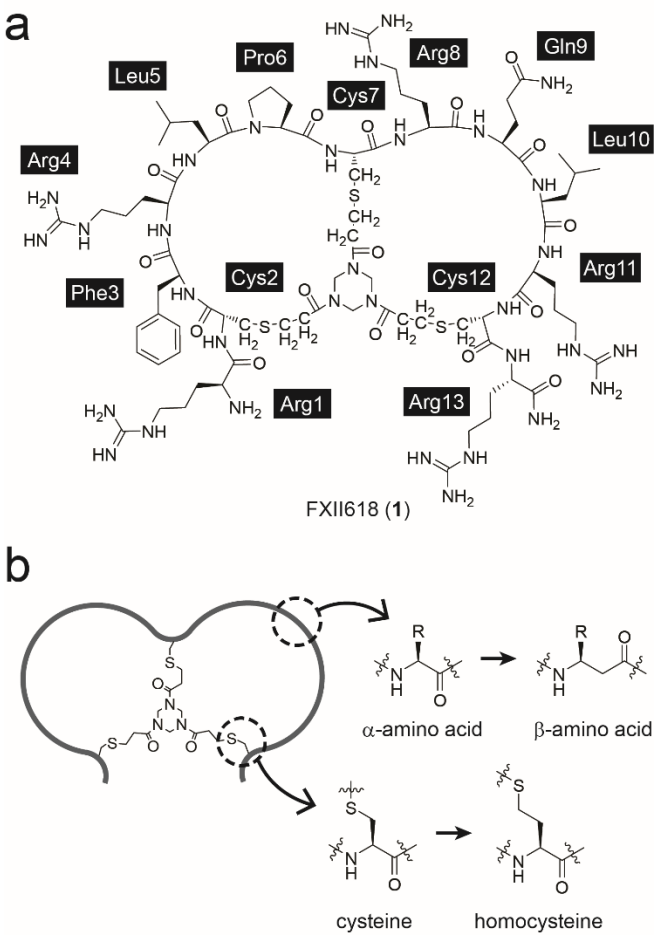


Figure 1. (a) Chemical structure of FXII618 (**1**). (b) Affinity maturation strategy. Carbon atoms are inserted into the peptide backbone by replacing α-amino acids with β-amino acids, or by replacing cysteines connected to the cyclization linker with cysteine homologues having longer side chains.

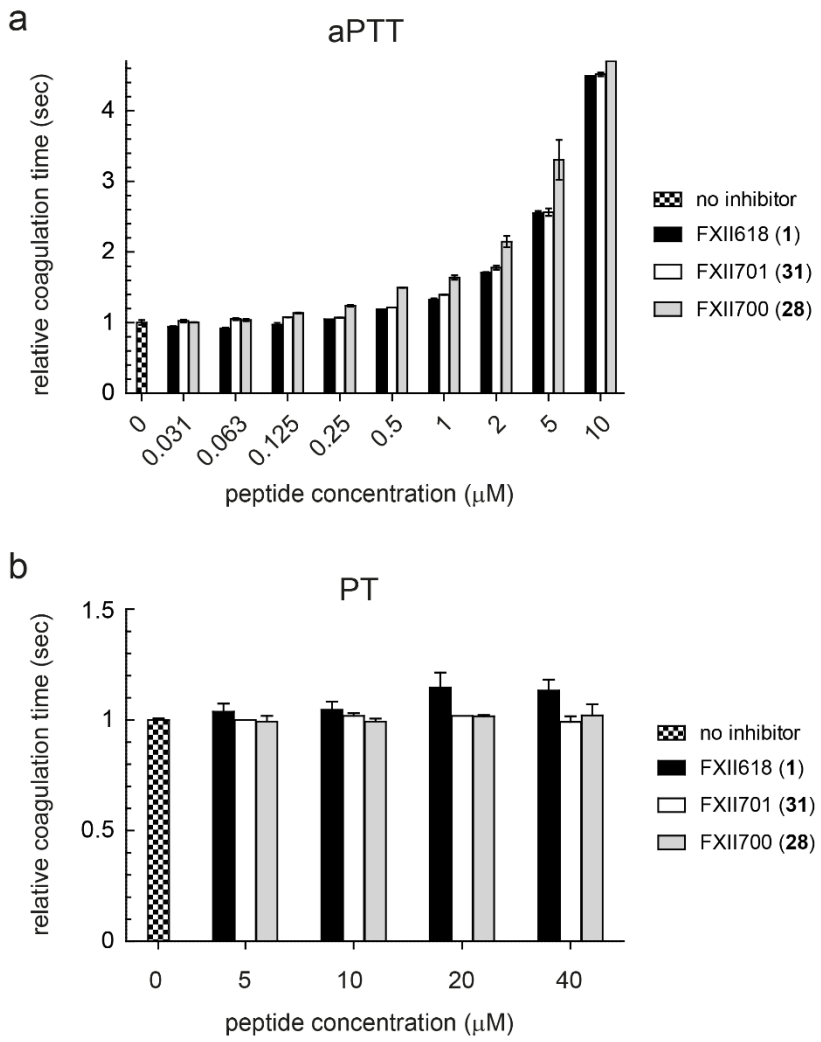


Figure 2. Coagulation parameters aPTT (a) and PT (b) for bicyclic peptides **1** (FXII618), **28** (FXII700) and **31** (FXII701).

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