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ORIGINAL ARTICLE

Mimetics of the disulfide bridge between the N- and C-terminal cysteines of the KLK3-stimulating peptide B-2

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Abstract Human prostate produces kallikrein-related peptidase 3 (KLK3, also known as prostate specific antigen), which is widely used as a prostate cancer marker. Proteolytically active KLK3 has been shown to inhibit angiogenesis and its expression decreases in poorly differentiated tumors. Thus, it may be possible to control prostate cancer growth with agents that stimulate the proteolytic activity of KLK3. We have earlier developed synthetic peptides, which bind specifically to KLK3 and promote its proteolytic activity. These peptides are cyclic, all containing a disulfide bridge between the N- and C-terminal cysteines. To increase the in vivo stability of the KLK3-stimulating peptide B-2, we made differently cyclized analogues by replacing both terminal cysteines and the disulfide bridge between them. A replacement consisting of γ-amino butyric acid and aspartic acid, where the amino group from the former was linked to the main chain carboxyl group of the latter, was found to be, at high concentrations, more active than the B-2 peptide. Furthermore, as compared to the parent peptide, this analog had an improved stability in plasma and against the enzymatic degradation by KLK3. In addition, the series of analogues also provided valuable information of the structure–activity relationships of the B-2 peptide.

Keywords Synthetic peptide · Stability · Prostate cancer · Kallikrein-related peptidase 3 · KLK3 · Prostate specific antigen · PSA

Abbreviations

Acm	Acetamidomethyl
Ahx	6-Aminohexanoic acid
All	Allyl
Aox	8-Aminooctanoic acid
BHP	Benign hyperplasia
DCM	Dichloromethane
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*′-dimethylformamide
EDT	Ethanedithiol
ESI	Electrospray ionization
Fmoc	Fluorenylmethoxycarbonyl
GABA	γ-Amino butyric acid
HATU	2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HLPC	High-performance liquid chromatography
HOAt	1-Hydroxy-7-azabenzotriazole
HUVEC	Umbilical-vein endothelial cells
KLK2	Kallikrein-related peptidase 2
KLK3	Kallikrein-related peptidase 3
MALDI	Matrix assisted laser desorption ionization

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Mtt	4-Methyltrityl
ODmab	4{*N*-[1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3-methylbutyl]-amino}benzyloxy
PBS	Phosphate buffered saline
PSA	Prostate specific antigen
SPPS	Solid-phase peptide synthesis
*t*BuO	*t*-Butoxy
TFA	Trifluoroacetic acid
TIS	Triisopropyl silane

Introduction

The prostate produces kallikrein-related peptidase 3 (KLK3, also known as prostate specific antigen, PSA), a 28 kDa glycoprotein belonging to the kallikrein family of serine proteases (Yousef and Diamandis 2001). KLK3 is secreted by prostatic glandural epithelium into seminal plasma, where by digesting semenogelins it dissolves the seminal clot formed after ejaculation, and thereby promotes sperm motility (Lilja 1985; Robert et al. 1997). KLK3 is the best currently available cancer marker and it is widely used for detection and monitoring of prostate cancer (Catalona et al. 1991; Stenman et al. 2005). In prostate cancer and some non-malignant conditions, like benign hyperplasia (BHP) and prostatitis, KLK3 leaks into circulation due to altered tissue architecture and thus leads to increased serum concentrations (Stamey et al. 1987; Stenman 1997). Most of the circulating KLK3 is inactivated by protease inhibitors (Christensson et al. 1990; Lilja et al. 1991; Stenman et al. 1991).

Based on in vitro studies, both tumor growth-stimulating and inhibiting properties have been suggested for KLK3 (Borgono and Diamandis 2004; Clements et al. 2004; Williams et al. 2007; Koistinen et al. 2008). Clinical studies have shown that the level of KLK3 is decreased in malignant prostatic epithelium, as compared to normal epithelium, and it is further reduced in poorly differentiated tumors (Abrahamsson et al. 1988; Paju et al. 2007). Furthermore, low tissue concentrations of KLK3 are associated with poor prognosis (Stege et al. 2000) whereas high expression in tumors is associated with low microvessel density (Papadopoulos et al. 2001) suggesting a tumor suppressing role for KLK3. KLK3 has also been shown to exert antiangiogenic properties both in in vitro and in vivo models (Fortier et al. 1999; Fortier et al. 2003). In a cell culture model using umbilical-vein endothelial cells (HUVECs), only enzymatically active isoforms of KLK3 were able to inhibit tube formation, a measure of angiogenic potential of these cells (Mattsson et al. 2008). This study, together with other studies showing that small molecule inhibitors of KLK3 and a KLK3-inhibiting antibody prevents the antiangiogenic activity of KLK3, shows that enzymatic activity of KLK3 is needed for its antiangiogenic activity (Koistinen et al. 2008; Mattsson et al. 2008). Since the active form of KLK3 may suppress tumor growth, it may be possible to control prostate cancer growth by modulating the proteolytic activity of KLK3. Because KLK3 is highly prostate specific, cancer therapy should be possible without serious side effects.

We have used phage-display technology to identify peptides, which specifically bind to KLK3 (Wu et al. 2000). Furthermore, we have shown that synthetic analogues of the peptides promote the proteolytic activity of KLK3 (Pakkala et al. 2004) and are good lead structures for potential therapeutic agents. Strategies to improve in vivo stability and bioavailability of peptides have recently been widely covered in the literature (Adessi and Soto 2002; Sato et al. 2006; Lien and Lowman 2003). These include N- and C-terminal capping, side chain modifications, use of unnatural amino acids and cyclization. In our earlier study, we have shown that most of the amino acid side chains are essential for the promoting activity of KLK3 binding peptide B-2 making the side chain modifications difficult (Pakkala et al. 2004). Furthermore, we have succeeded in increasing the plasma stability of linear KLK2 inhibiting peptide by head-to-tail cyclization (Pakkala et al. 2007). Since the KLK3-stimulating peptides, A-1, B-2 (**1**) and C-4 are cyclic, containing disulfide bridge between the N- and C-terminal cysteines, peptide C-4 containing also a second internal disulfide bridge (Pakkala et al. 2004) our first step in stabilizing the peptides was to use alternative cyclization methods.

Our earlier attempts to perform head-to-tail cyclization of the two most potent peptides B-2 and C-4 have failed with no detectable amounts of cyclic peptides. Commonly cyclization of peptides is highly dependent on the sequence, amino acid composition and the length of the peptide. Several research groups have published successful cyclization of penta- and hexapeptides, but the head-to-tail cyclization of longer sequences is more challenging as recently discussed by Davies (2003). Of the two most active peptides, B-2 (**1**) (Fig. 1) was chosen as the lead peptide, as its conformation is less restricted than that of C-4 (Pakkala et al. 2004). The strategy was to use other types of bridge structures connected by amide bonds suitable for automated solid-phase peptide synthesis (SPPS), and to include other linking units than conventional cysteines. The different bridges were planned to replace the N- and C-terminal cysteines and the disulfide bridge between them, and in some cases also one of the valines on either side of the cysteines. We generated a set of differently cyclized peptides with lysine, ornithine, β-alanine or γ-amino butyric acid (GABA) at the N-terminal site and

aspartic acid at the C-terminal site. The C-terminal aspartic acid was linked to the resin via either the backbone or side chain carboxylic group depending on the synthesized bridge structure. In addition, longer linking units such as 6-aminohexanoic acid (Ahx) and 8-aminooctanoic acid (Aox) were also investigated as replacements. Successfully cyclized structures are shown in Fig. 2.

The stimulation of the KLK3 activity was evaluated for the successfully cyclized peptides. The stability in plasma was evaluated for the peptides which significantly stimulated KLK3 activity. As KLK3 cleaves substrates mainly at the C-terminal side of tyrosine and glutamine (Coombs et al. 1998; Malm et al. 2000) it is feasible that the B-2 peptide also acts as a substrate for KLK3. Therefore, the stability against enzymatic degradation by KLK3 was also studied.

Results and discussion

Synthesis of B-2 variants

Initially, the original B-2 peptide (**1**) was synthesized with C-terminal amide using Rink Amide AM resin, yielding peptide **2**. The synthesis strategies for alternatively cyclized peptides are represented in Fig. 3. In the first set of alternatively cyclized peptides, the disulfide bridge was replaced with a side chain to side chain bridge, using side chain ODmab-protected aspartic acid at the C-terminus and side chain Mtt-protected lysine or ornithine at the N-terminus. The aspartic acid residue was coupled to Rink Amide AM resin via the carboxyl terminus, leaving after ODmab-deprotection the side chain free for cyclization (**10**, **11**, **12**). The length of the modified bridge area (marked with gray background in Fig. 1) was also changed by removing either valine-11 or valine-2 (**11**, **12**). The carboxylic acid group attached to the resin was converted to the corresponding amide using RINK Amide AM resin.

Cyclization of peptides having an N-terminal ornithine was successful, yielding peptides (**3**–**5**), but only when the amino group of the N-terminus of the peptides was acetylated. Cyclization of Fmoc-protected peptides was not successful. Cyclization of the peptides having an N-terminal lysine failed, although the α-amino group was acetylated. Earlier studies with peptide **1** have shown that modification of the N-terminus does not reduce the activity of the peptides (Wu et al. 2004). The peptide with an N-terminal ornithine and C-terminal aspartic acid was also

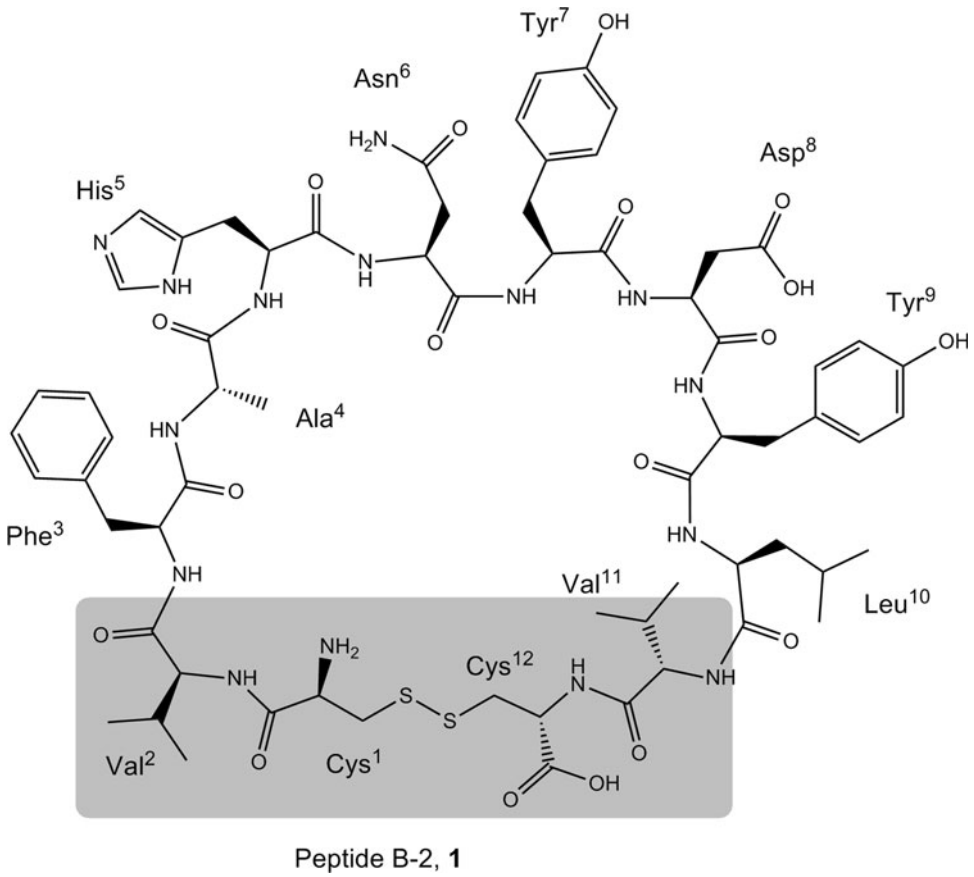


Fig. 1 Structure of peptide B-2. Area for replacement studies is shadowed with *gray*

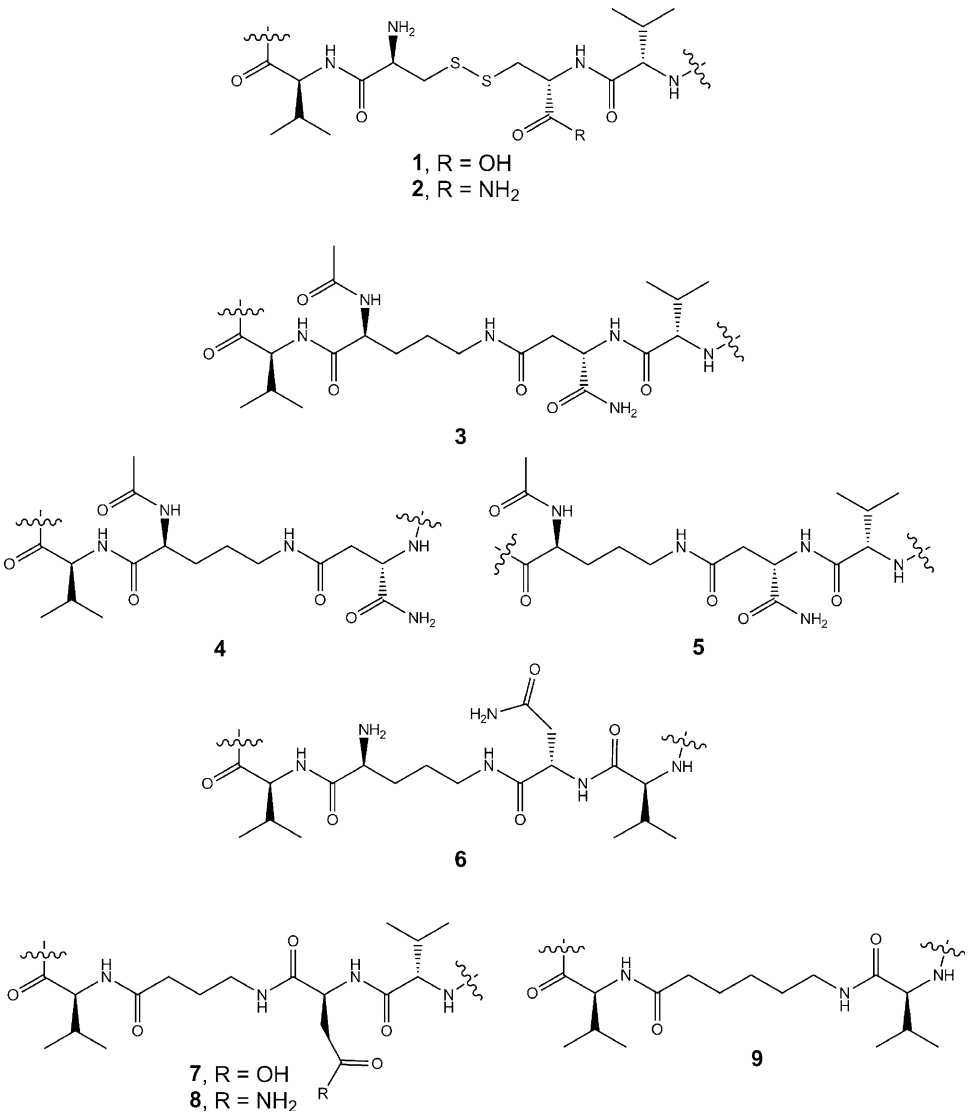


Fig. 2 The structures of the successfully synthesized disulfide bridge replacements

coupled to the resin via the side chain of aspartic acid (**13**), leaving the C-terminal carboxyl group free for cyclization. Cyclization of this peptide, yielding peptide **6**, was successful although the amino group of the N-terminus was not acetylated. In peptide **6** the length of the bridge replacement was reduced by one $–CH_2–$ group as compared to peptide **3**.

Peptides **7** and **8** have a bridge where the amino group of the GABA at the N-terminus is coupled to the main chain carboxylic acid group of aspartic acid at the C-terminus. Peptide **1** contains asparagine at position 6, which provides an additional resin binding site. Peptide **7** was synthesized using C-terminal All-protected aspartic acid, conjugated to Rink Amide resin via the side chain producing asparagine-6 in free peptide (**14**). After completing the synthesis, the peptide was cyclized via the free amino terminus of tyrosine (original position 7) to the deprotected resin-bound aspartic acid. In peptide **8**, aspartic acid at position 12 was conjugated to Rink Amide resin via the side chain and the bridge was formed via GABA (**16**). Peptide **9** was synthesized with the same method as peptide **7** by attaching asparagine-6 to the resin (**15**). The bridge in peptide **9** was made up using Ahx as a linker.

We also tried to synthesize other bridge structures, presented in Fig. 4, but the cyclization of them failed or gave only extremely low yields and purities. These included a cyclization of an N-terminal β-alanine with the side chain of a C-terminal aspartic acid (**17**), where the peptide was attached to the resin from the C-terminal carboxylic acid of the aspartic acid. Another unsuccessful trial (**18**) was the analog of peptide **9** with an Aox linker instead of Ahx. Furthermore, a peptide with a tri-alanine bridge between valines (**19**), using resin-bound asparagine-6 as a cyclization site, was also unsuccessful.

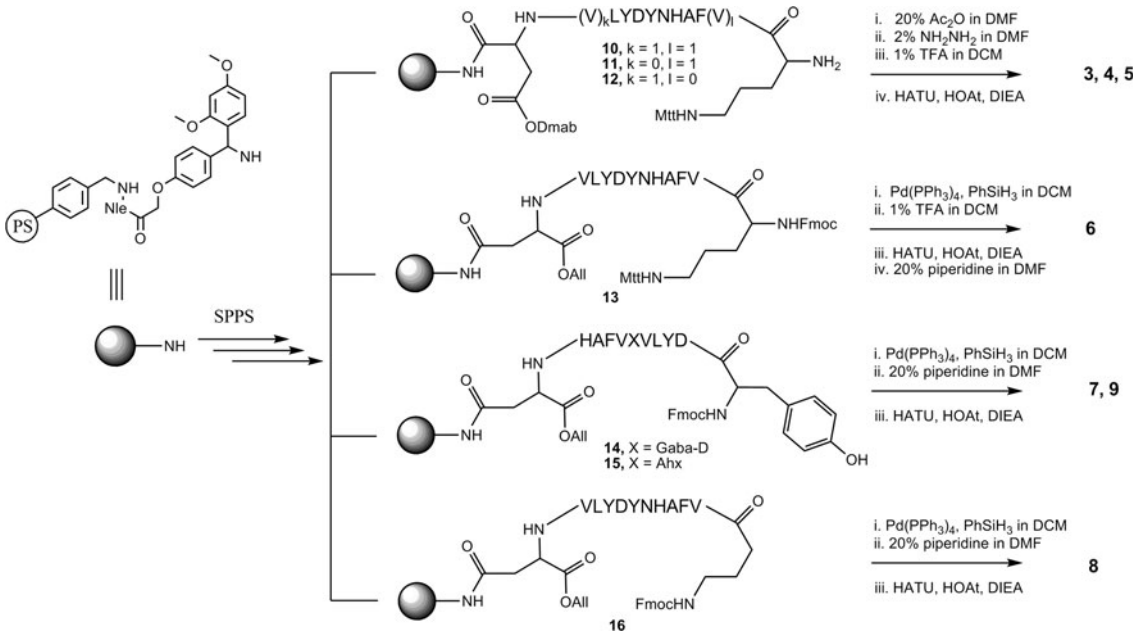
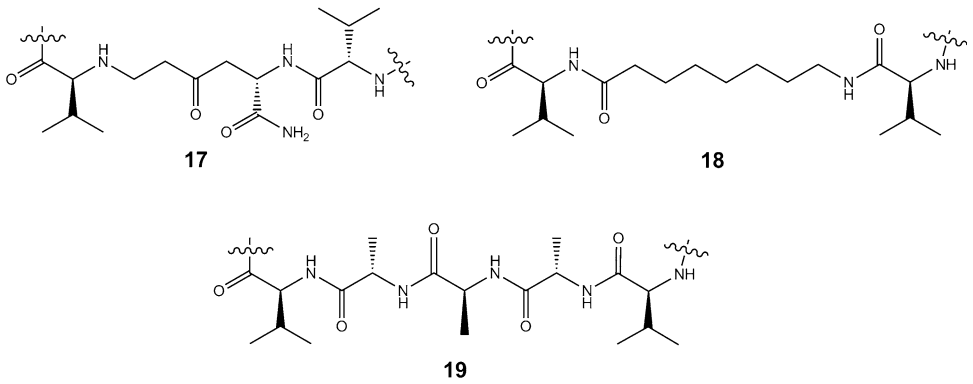


Fig. 3 Representative solid-phase synthesis of peptides **3–9**

Fig. 4 The structures of the unsuccessfully synthesized disulfide bridge replacements



Determination of activity

The ability of the synthesized peptides **1–9** to stimulate KLK3-activity is shown in Fig. 5. Peptides **3–5** did not show any activity against KLK3. These peptides had side chain to side chain cyclizations between an ornithine at the N-terminus and aspartic acid at the C-terminus, and peptides **4** and **5** were shortened with one valine from either side of the bridge. These results suggest that the structure of the bridge is important for activity, especially the length of the bridge. The B-2 peptide (**1**) has 14 atoms counting the atoms in the bridge fragment. In peptide **3** the number of atoms is 16. Peptides **4** and **5**, on the other hand, have only 13 atoms in the bridge and they lack one of the adjacent valine residues. Peptide **6** stimulated KLK3 activity, but less than peptide **1**. Peptide **6** has 15 atoms in the bridge, suggesting again that the length of the bridge is important and the length starts to be closer to the optimal length. In addition, in peptide **6** the configuration of the stereocenter derived from L-aspartic acid is reversed with respect to the main chain in the ring and the carboxylic acid group side chain is one $–CH_2–$ group further apart as compared to the peptide **1**. Peptide **7** was almost as potent as the peptide **1** and peptide **2**. Peptide **7** has the same number of atoms in bridge (14) as the peptide **1**, again emphasizing the importance of the length of the bridge. Furthermore, peptide **7** clearly shows that the amino group at the N-terminus is not needed for the KLK3-stimulating activity. Peptide **8** differs from peptide **7** only in that the carboxylic acid in the side chain in peptide **7** is an amide in peptide **8**. This reduces the activity, which is in agreement with the earlier observation that the free carboxylic acid is preferred over an amide, i.e., peptide **2** is slightly less active than peptide **1**. Interestingly, the relative configuration of the stereocenter next to the carboxylic acid group is not important. Peptide **9** showed no activity, indicating

again the importance of a C-terminal carboxyl group. However, peptide **9** is one atom shorter than peptides **7** and **8**, having only 13 atoms in the bridge, which might also have an effect on the activity.

Concentration dependency of KLK3-stimulation by the peptides

The concentration dependency was studied using the most active peptides, i.e., **1**, **2** and **7**. While these peptides showed a dose-dependent stimulation of KLK3-activity at low concentrations, the original B-2 (**1**) started to show reduced stimulation at higher concentrations (Fig. 6). Our results suggest that peptide **1** could also act as a substrate for KLK3 (see below), especially at high concentrations at which the activation by peptide **2** reaches its maximum. Preliminary molecular modeling has suggested that while the major binding site of peptide **1**, where it is likely to exert its stimulatory effect, is outside of the active center of KLK3, there might be a second binding site at the active site where it could compete with substrate (Henna Härkönen, personal communication). Unlike the peptides **1** and **2**, peptide **7** stimulated KLK3 activity dose dependently at all concentrations studied, exceeding the effect of other peptides. This finding together with a lower degradation rate of peptide **7** by KLK3 or plasma proteases (see below) suggest that the binding of peptide **7** to KLK3 is different from that of peptide **1**.

Stability studies

The original peptide **1** and peptide **7** were cleaved by KLK3 at high concentrations. The cleavage rate of peptide 7 was slower than that of peptide 1. Using a ninefold molar excess of peptides and after 2 h incubation, 59% of peptide **1** and 72% of peptide **7** were intact. After 24 h 5.7% (1) and 34% (7) were found intact, respectively (Fig. 7a). Mass spectrometry analysis indicates that the ring opens between tyrosine-7 and aspartic acid-8 (data not shown). Although the amino acid sequence of peptide **7** is identical to the original peptide, excluding the bridge, it is more stable against KLK3, suggesting different binding behavior.

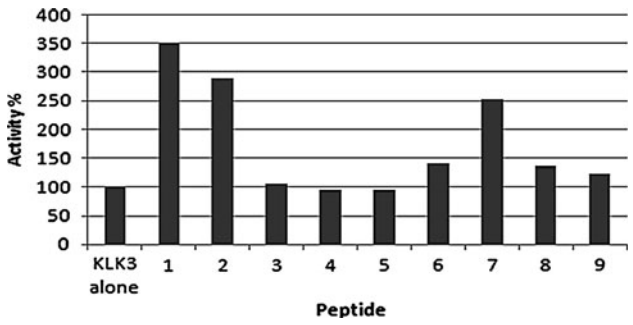


Fig. 5 Effect of the synthetic peptides on KLK3 activity. Stimulation of activity of synthetic peptides as compared to KLK3 without peptides (KLK3 alone is indicated as 100%). Peptide concentration used was 13 μM

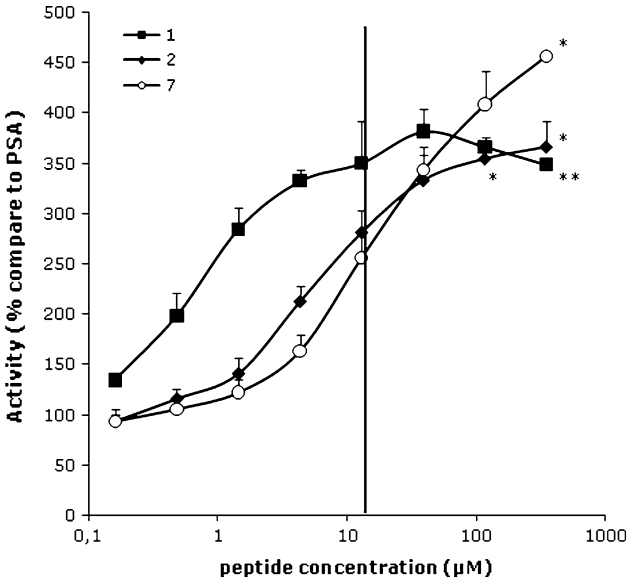


Fig. 6 Concentration dependency of the KLK3 stimulation by peptides **1**, **2** and **7**. Data represent average + SD of three independent experiments, except for $*n = 2$ and $**n = 1$. *Solid line* shows the concentration (13 μM) used for the data shown in Fig. 5

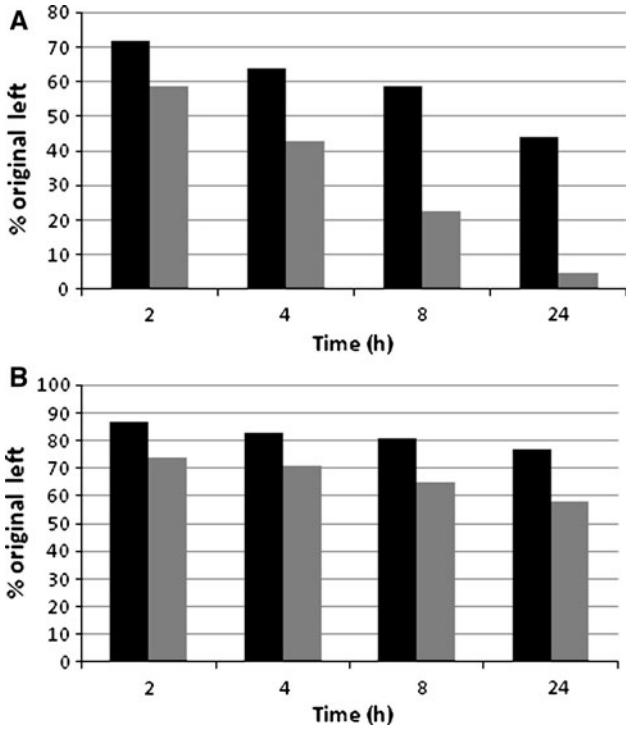


Fig. 7 Stability of peptides 7 (*black columns*) and 1 (*gray columns*) over the time **a** with purified KLK3 and **b** in plasma

The original peptide **1** and peptide **7** were tested for plasma stability. During the first 2 h 26% of peptide **1** and 13% of peptide **7** disappeared, either by degradation or binding to plasma proteins. After 2 h the disappearance rate became slower, finally after 24 h 58% of peptide **1** and 73% of peptide **7** were found intact (Fig. 7b). These results suggest that the replacement enhances the stability of the KLK3-stimulating peptides against plasma proteases or decreases binding to plasma proteins.

Conclusions

Since our earlier studies have shown that tyrosine-7 and aspartic acid-8 are essential for the promoting activity of B-2 peptide (Pakkala et al. 2004), we used the alternative cyclization methods to improve the stability of B-2 peptide instead of side chain modifications. Based on the new analogues of peptide B-2 (**1**), we conclude that the bridge between the N- and C-termini is highly important for the KLK3-stimulating activity. The number of the atoms in the alternative bridge structure was found to be important. A free carboxylic acid group at the C-terminus was also important for the KLK3-stimulating activity, but there was no difference whether the free carboxylic acid was in side chain or the main chain of L-aspartic acid. The free carboxylic acid could be replaced by an amide group, but this replacement reduced the KLK3-stimulating activity. On the other hand, the amino group at the N-terminus could be removed without affecting the KLK3-simulating activity. Peptide **7** with a cysteine based disulfide bridge mimetic consisting of GABA and aspartic acid, where the amino group from the former was linked to the main chain carboxyl group of the latter, is more stable than peptide **1** and, at high concentrations, the activity exceeds that of peptide **1**.

Experimental

Peptide synthesis

The peptides were synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY, USA). All amino acids (GLS Biochem) and other reagents (Aldrich, Fluka, Bachem) were purchased in standard qualities and used without further purification. Rink Amide AM resin (50 mg, loading 0.575 mmol/g), purchased from GLS Biochem, was used as solid-phase with Fmoc strategy and HBTU/DIEA as coupling reagent. The side chain protecting groups used in bridge forming residues were Acm for cysteine, OAll for C-terminus and both *t*-BuO and ODmab for side chain protection of aspartic acid and glutamic acid, Mtt for ornithine and lysine side chain protection. Additional amino acids used for bridge formation were Fmoc-protected β-alanine, GABA, Ahx and Aox. Fmoc group was not removed from peptidyl resins **13–16** after final amino acid coupling. Peptide **1** was obtained from AnaSpec (San Jose, CA, USA) and was >95% pure as detected by HPLC.

Purification and mass spectrometry of peptides

All peptides were cleaved from resin using TFA:TIS:H_2O:EDT (94:1:2.5:2.5) and lyophilized. The lyophilized peptides were purified by HPLC (Shimadzu, Kioto, Japan) on a C_{18} reverse phase column (xTERRA, Waters, Milford, MA, USA) using an CH_3CN gradient (0.1% TFA in H_2O/0–60% CH_3CN gradient for 60 min). The purity and degradation rates were determined by analytical HPLC on a 240 × 1.4 mm C_{18} column (xTERRA, Waters) eluted with 0–90% CH_3CN for 42 min and verified by mass spectrometry on an ABI QSTAR XL hybrid mass spectrometer using MALDI or ESI interface (Applied Biosystems Inc., Foster City, CA, USA) for disulfide bridge containing sequences. Negative mode was used for the detection of head-to-tail, side chain to tail and side chain to side chain cyclic peptides.

Cyclization of the disulfide bridged peptide

Peptide **2** containing cysteines protected with Acm was cyclized by the standard iodination method in solution. Peptide was cleaved from the Rink Amide AM resin yielding C-terminal amide and lyophilized. Lyophilized peptide was purified by HPLC as described above. Fractions containing crude acyclic peptide were pooled, lyophilized and then dissolved in 50% acetic acid (AcOH) in H_2O at a concentration of 2 mg/ml. 1 M HCl (0.1 ml/mg of peptide) and 0.1 M iodine solution in 50% AcOH in H_2O (5 eq/Acm) were added and the resulting solution was stirred vigorously at room temperature for 2 h and the reaction was stopped with 0.1 M sodium thiosulphate. After filtering (0.45 μm) the peptides were purified by HPLC as described above and lyophilized yielding peptide **2**. ESI–MS: (*m*/*z*) 1,443.6 $[M + H]^+$. HPLC: $t_R = 15.6$ (purity > 95%).

Cyclization method for alternatively cyclized peptides

Head-to-tail, side chain to tail and side chain to side chain cyclizations were performed on resin using HATU and HOAt as the coupling agent and DIEA as the base (Fig. 3). The yields were not expected to be higher than a few percent due to the long sequences. Furthermore, bulky tyrosine-7 adjacent to the cyclization sites in case of the

B-2 analogues connected to resin via asparagine 6 (peptides **7** and **9**) expected to cause problems in cyclization. Therefore, the coupling step was performed repeatedly and with different solvent concentrations between DMF and DCM because those solvents swell the resin and peptides differently due to their different polarities. Yields of crude peptides after lyophilization varied between 25 and 35 mg and after purification by HPLC between 1 and 3 mg (2–8%) depending on peptide synthesized. Unsuccessfully cyclized peptides yielded no analytical HPLC detectable amounts of pure peptide.

The Kaiser test was performed for all alternatively cyclized peptides before the cyclization step and all tests were found positive indicating a free amino group (Kaiser et al. 1970). Usually the peptidyl resin was swelled for 30 min with the solvent used in the coupling step, the solvent was removed and the coupling agents, HATU (3 equiv), HOAt (3 equiv) and DIEA (4.5 equiv), were added with a small amount of anhydrous solvent achieving a final volume of 3 ml. The mixture was stirred for 2.5 h at room temperature under argon, the solvent was then removed, and the resin was washed with DMF (3 ml, 3 × 5 min) and with anhydrous DCM (3 ml, 3 × 5 min). The Kaiser test was performed to monitor the success in cyclization. If the Kaiser test results turned out to be positive, the cyclization was repeated. Usually the first cycle was performed in DMF, the second in 25% DCM in DMF and the third in 50% DCM in DMF. Higher concentrations of DCM were discarded due to the low solubility of HATU in DCM. When the Kaiser test was negative, the peptides were cleaved from resin and then lyophilized.

Deprotection and characterization of alternatively cyclized peptides

Peptidyl resins **10**, **11** and **12** with orn–asp bridge as well as corresponding lysine based peptidyl resins (schemes not shown) from automated SPPS were placed in reaction columns and the free N-terminal amino groups were acetylated manually adding 20% Ac_2O in DMF (3 ml) and stirring resulting solution 10 min at RT. Treatments were repeated and resins washed with DMF (3 × 3 ml). Side chain Dmab-protection was removed using 2% hydrazine in DMF (3 ml) agitating 10 min at room temperature. The treatment was repeated twice, followed by washing with 20% DIEA in DMF:H_2O 9:1 (3 ml) to make sure that all traces of Dmab were removed. Resins were washed with DMF (3 × 3 ml) and DCM (3 × 3 ml). N-terminal Mtt was deprotected using 1% TFA in DCM (3 ml) agitating 10 min at room temperature. Treatment was repeated three times and the deprotection was monitored by disappearance of the orange color characteristic for traces of Mtt. Resins were washed with DMF (3 × 3 ml) and DCM (3 × 3 ml) and dried. Peptides were cyclized on resin using HATU/HOAt/DIEA-method as described above. After cleavage peptides were lyophilized yielding peptides **3**, **4** and **5**. Peptide **3**, ESI–MS: (*m*/*z*) 1,493.0 $[M - H]^-$, HPLC: $t_R = 16.0$ (purity > 90%). Peptide **4**, ESI–MS: (*m*/*z*) 1,393.95 $[M - H]^-$, HPLC: $t_R = 16.8$ (purity > 95%). Peptide **5**, ESI–MS: (*m*/*z*) 1,393.90 $[M - H]^-$, HPLC: $t_R = 16.0$ (purity > 85%).

Peptidyl resin **13** from automated SPPS was placed in reaction column and washed with DCM (3 × 3 ml). C-terminal allyl deprotection was performed using a catalytic amount of $Pd(PPh_3)_4$ (0.1 equiv) with $PhSiH_3$ (10 equiv) as a scavenger in anhydrous DCM (3 ml). The mixture was purged with an argon flow and stirred under argon for 1.5 h. The resin was washed with DMF (3 × 3 ml), 0.5% DIEA in DMF (3 × 3 ml), 0.5% sodium diethyldithiocarbamate trihydrate in DMF (3 × 3 ml), DMF (3 × 5 ml) and finally with DCM (3 × 5 ml). The treatment was repeated to drive the deprotection to completion. Deprotection of side chain Mtt was performed as described in previous chapter. Peptidyl resin **13** was additionally deprotected using 2% TFA in DCM (3 ml) to increase the deprotection rates, although this might also lead to cleavage of the peptides from the Rink-resin (Bourel et al. 2000). Peptide was cyclized on resin using HATU/HOAt/DIEA-method as described above. N-terminal Fmoc deprotection was performed after cyclization using 20% piperidine in DMF (3 ml, 2 × 15 min), followed by washing with DMF (3 ml, 3 × 5 min) and DCM (3 ml, 3 × 5 min). After cleavage peptide was lyophilized yielding peptide **6**. ESI–MS: (*m*/*z*) 1,451.0 $[M - H]^-$, HPLC: $t_R = 15.5$ (purity > 95%).

Peptidyl resins **14**, **15** and **16** from automated SPPS were placed in reaction columns and washed with DCM (3 × 3 ml). C-terminal allyl deprotection and N-terminal Fmoc deprotection were performed as described in previous chapter. After deprotection peptides were cyclized on resin using HATU/HOAt/DIEA-method as described above. After cleavage peptides were lyophilized yielding peptides **7**, **8** and **9**. Peptide **7**, ESI–MS: (*m*/*z*) 1,422.5 $[M - H]^-$, HPLC: $t_R = 16.8$ (purity > 95%). Peptide **8**, ESI–MS: (*m*/*z*) 1,421.5 $[M - H]^-$, HPLC: $t_R = 16.4$ (purity > 85%). Peptide **9**, ESI–MS: (*m*/*z*) 1,335.8 $[M - H]^-$, HPLC: $t_R = 18.3$ (purity > 90%).

Effect of the peptides on KLK3 activity

Kallikrein-related peptidase 3 activity was measured by using the chromogenic substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA · HCl) purchased from Chromogenix Instrumentation Laboratory (Milano, Italy). KLK3 (0.3 μM) was incubated for 30 min at room temperature with synthetic

peptides (13 μM) in 0.05 M Tris buffer, pH 7.7, containing 0.154 M NaCl, 8 mM NaN_3 and 0.1% bovine serum albumin. After the addition of the substrate to a final concentration of 0.2 mM, the absorbance at 405 nm was measured at 5 min intervals for 30 min with a Victor 1420 Multilabel fluorometer (Perkin-Elmer-Wallac, Turku, Finland). The concentration dependency of the activity was determined by measuring the activity of KLK3 in the presence of the synthetic peptides in a series of concentration from 0.16 to 351 μM.

Stability tests

Proteolytic cleavage of selected peptides with KLK3 was followed for 24 h. Peptide (0.18 mM) was incubated with KLK3 (0.02 mM in PBS final concentration) in 150 μl volume at +37°C or with PBS as the control. Peptide samples of 35 μl were collected at 2, 4, 8 and 24 h time points and KLK3 was removed from the reaction mixture with a Microcon Centrifucal Filter Device (Microcon YM-10, cut off 10 kDa, Millipore, Bedford, MA, USA) by centrifugation at 15,000 rpm with Eppendorf 5415 D centrifuge (Eppendorf, Hamburg, Germany) for 10 min. The filtrates were analyzed by HPLC as described above.

Plasma stability tests for the selected peptides were performed by incubating the peptides with 250 μl fresh human EDTA plasma, 125 μl PBS and 125 μl peptide solution (0.5 mg/ml in H_2O) for 24 h at +37°C. For controls, the peptides were added to PBS. Peptide samples of 100 μl were collected at 2, 4, 8 and 24 h time points. The peptides were separated from plasma proteins on a Microcon Centrifucal Filter Device and analyzed as described above.

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