ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Replacement of the Disulfide Bridge in a KLK3-Stimulating Peptide Using Orthogonally Protected Building Blocks

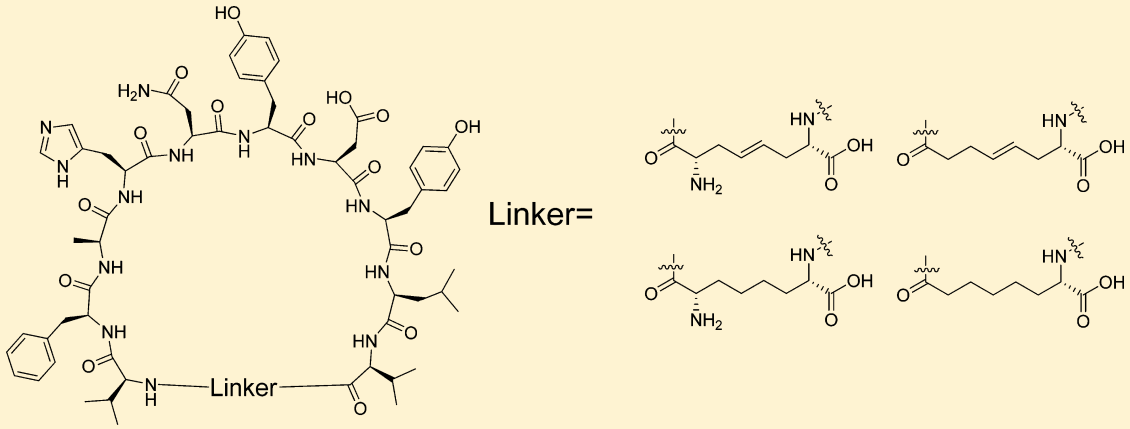
Kristian Meinander,*[,†] Miikka Pakkala,[‡,∥] Janne Weisell,[‡] Ulf-Håkan Stenman,[§] Hannu Koistinen,[§] Ale Närvänen,[‡] and Erik A. A. Wallén[†]

[†]Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, P.O. Box 56, FIN-00014 Helsinki, Finland

[‡]School of Pharmacy, University of Eastern Finland, P.O. Box 1627, FIN-70211 Kuopio, Finland

[§]Department of Clinical Chemistry, Haartman Institute, University of Helsinki and Helsinki University Central Hospital, Haartmaninkatu 8, FIN-00290 Helsinki, Finland

Supporting Information



ABSTRACT: Peptide "B-2", which is one of the most potent kallikrein-related peptidase 3 (KLK3)-stimulating compounds, consists of 12 amino acids and is cyclized by a disulfide bridge between the N- and C-terminal cysteines. Orthogonally protected building blocks were used in the peptide synthesis to introduce a disulfide bridge mimetic consisting of four carbon atoms. The resulting pseudopeptides with alkane and *E*-alkene linkers doubled the proteolytic activity of KLK3 at a concentration of 14 μM. They were almost as potent as the parent "B-2" peptide, which gives a 3.6-fold increase in the proteolytic activity of KLK3 at the same concentration.

KEYWORDS: *Disulfide bridge mimetic, human kallikrein-related peptidase 3, prostate specific antigen, proteolytic activity, pseudopeptide, peptide synthesis*

Kallikrein-related peptidase 3 [KLK3, also known as prostate specific antigen (PSA)] is currently the most widely used serum marker for prostate cancer.[1−3] KLK3 has been shown to inhibit angiogenesis.[4−6] This anti-angiogenic activity is associated with the proteolytic activity of KLK3 and can be enhanced by peptides that stimulate KLK3.[5,6] Furthermore, a high level of KLK3 expression in tumors is associated with a low microvessel density, and the level of expression of KLK3 decreases in poorly differentiated tumors, suggesting a tumor suppressing role for KLK3.[7−9]

Peptide **1a** (B-2) and its equipotent amide analogue, **1b**, stimulate the proteolytic activity of KLK3.[10] Peptide **1a** along with two other peptides with similar biological activity was identified using phage-display technology.[10,11] The two most potent peptides are **1a**/**1b** and C-4 (structure not shown).[10] Although previously published results indicate that peptide C-4 has a stronger stimulating effect on KLK3 with small peptide substrates, **1a** has proven to be more potent with larger protein substrates and to have a higher affinity for KLK3 (J. M. Mattson and H. Koistinen, unpublished results). These peptides represent the most potent KLK3-stimulating compounds, and the search for low-molecular weight compounds with the same biological activity has so far been unsuccessful, although one low-molecular weight compound possessing both a very weak stimulating effect on its own and a weakly enhancing effect on the stimulation by **1a** has been identified.[12]

In an earlier study of **1a**,[13] it was shown that most of the amino acid side chains are essential for the KLK3 stimulating activity. To improve the *in vivo* stability of the KLK3-stimulating peptides, alternatives to the disulfide bridge for cyclizing the peptide have been studied.[14] These alternatives are

Received: October 18, 2013
Accepted: December 15, 2013
Published: December 16, 2013

based on cyclizing the peptide in a head-to-tail fashion using an amide bond. The only potent replacement for the terminal disulfide bridge consisted of γ-aminobutyric acid at the N-terminus and aspartic acid at the C-terminus connected via an amide bond (peptide **2**). The analogue had improved stability in plasma compared to that of **1a**.[14] The same study also showed that the terminal NH_2 group could be removed from the peptide without a significant loss of biological activity.

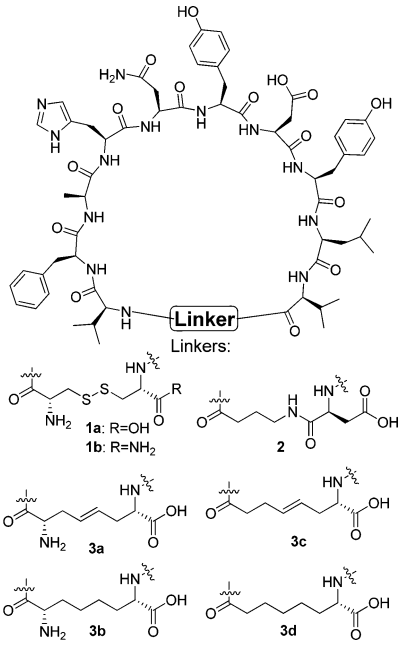


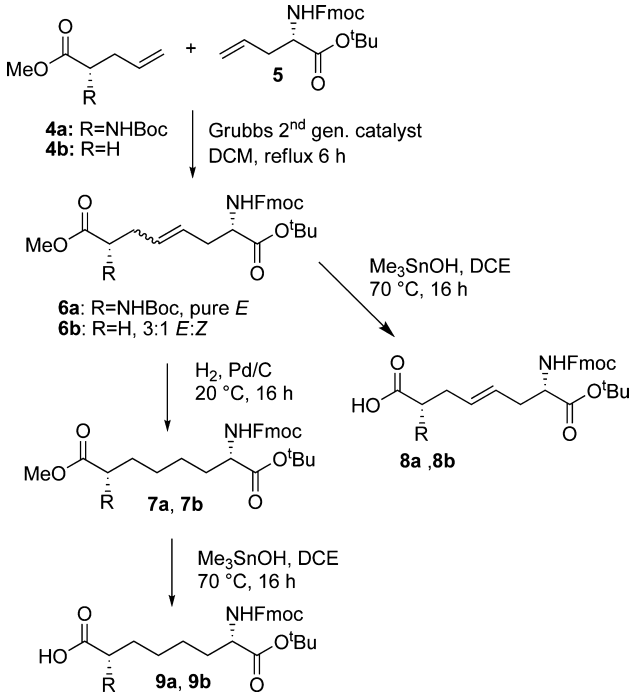
Figure 1. Structures of synthesized pseudopeptides and the linkers used.

We were interested in replacing the disulfide bridge in peptide **1a** with some of the orthogonally protected disulfide bridge mimetics based on hydrocarbon linkers that we had developed previously,[15] to afford pseudopeptides **3a** and **3b**. As the terminal NH_2 group does not seem to be critical for biological activity, analogues **3c** and **3d** were also included in the series. Replacing the disulfide bridge with a hydrocarbon linker has been reported to considerably increase the *in vivo* stability and bioavailability of peptides.[16,17] A similar alkene linker has already been shown to be a successful disulfide bridge replacement in the other KLK3-stimulating peptide, C-4.[18] However, in the C-4 peptide, the alkene linker was introduced after the peptide synthesis using a ring-closing metathesis reaction that gave an inseparable mixture of the *E*- and *Z*-alkenes.

The synthetic route to pseudopeptides **3a** and **3b** started with the synthesis of the mimetics containing orthogonal protecting groups compatible with the Fmoc protocol for solid phase peptide synthesis (SPPS). The synthesis of building blocks **8a** and **9a** needed for synthesis of pseudopeptides **3a** and **3b**, respectively, is presented in Scheme 1 and is based on our previously published procedure.[15]

Mimetics **8b** and **9b** lacking the terminal NH_2 group were synthesized in a manner analogous to that of **8a** and **9a** (Scheme 1). However, the obtained cross metathesis product (**6b**) was in this case an inseparable 3:1 mixture of the *E*- and *Z*-isomers (as determined by ^1H and ^{13}C NMR spectroscopy, assignment of peaks using gCOSY, gHSQC, and gHMBC

Scheme 1. Synthesis of Building Blocks 8a, 8b, 9a, and 9b



spectra). This was further confirmed by the reduction of the double bond in **6b** that yielded only one product (**7b**).

Interestingly, the hydrolysis of the methyl ester in **6b** was found to enrich the *E*-form of building block **8b**, yielding the *E*-isomer in pure form. The cause for this difference in reactivity between the two isomers is unclear, but the observation is confirmed by NMR spectroscopy of both the isolated product and unreacted starting material.

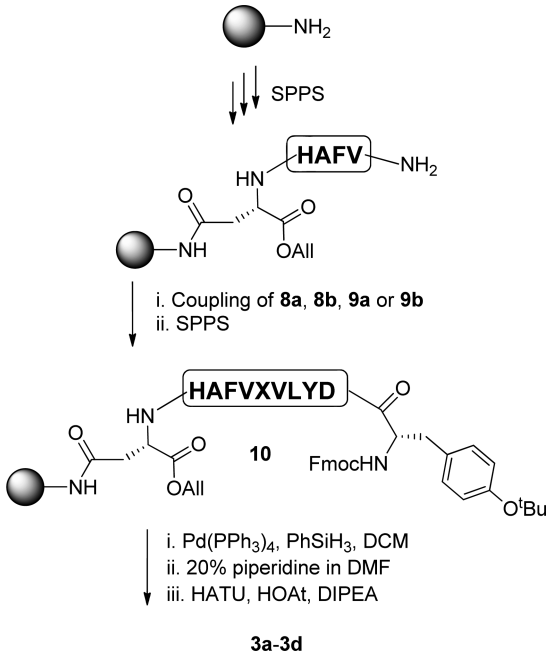
The synthesized mimetics **8a**, **8b**, **9a**, and **9b** were introduced into the peptide following a previously published protocol.[14] The peptide was attached to the resin (Rink Amide AM) from the side chain of Asn6, followed by coupling of amino acids and mimetics using Fmoc chemistry, and then finally cyclizing the pseudopeptide between residues Asn6 and Tyr7 (Scheme 2). Longer reaction times (4 h) were used for coupling the orthogonally protected building blocks to ensure a high yield in these steps. The yield-limiting step in the syntheses of the pseudopeptides was expected to be the head-to-tail cyclization. The main cause of this is the length of the peptide and the bulky tyrosine at the terminal site of the cyclization point that has previously resulted in low yields.[14] One common impurity according to MS studies of pseudopeptides **3a** and **3b** was a linear peptide resulting from incomplete cleavage of the allylic ester used for protecting the main chain carboxylic acid of the aspartic acid, thus preventing the head-to-tail cyclization.

To verify the stereochemistry of the double bond in the purified pseudopeptide **3c**, we also analyzed it by NMR using a method we have utilized in a previous study.[18] This showed that the peptide exclusively contained the *E*-isomer. After purification of the pseudopeptides to >90% purity, the yields of peptides were 0.6−5%.

The effect of the pseudopeptides on the proteolytic activity of KLK3 was determined in an assay using a chromogenic chymotrypsin substrate.[13,14,19] The ability of pseudopeptides **3a**−**3d** to stimulate the activity of KLK3 is summarized in Table 1. All four pseudopeptides stimulated the activity of KLK3; however, the effects were weaker than that of **1b**, and

dx.doi.org/10.1021/ml400419g | ACS Med. Chem. Lett. 2014, 5, 162−165

Scheme 2. Synthesis of Pseudopeptides 3a−3d[a]



[a]The X in intermediate **10** refers to a bridge mimetic corresponding to building block **8a**, **8b**, **9a**, or **9b**.

Table 1. Stimulation of KLK3 Activity by the Synthesized Pseudopeptides at 14 μM (N = 2)

compound	activity ± standard error (%)
KLK3 alone	100
1b	360 ± 5.4
3a	160 ± 8.1
3b	250 ± 10
3c	160 ± 0.5
3d	170 ± 15

the activity of the best one (**3b**) was equal to that of the previously published analogue with a linker consisting of aspartic acid and γ-aminobutyric acid as the bridge mimetic (peptide **2**, 250% stimulation compared to that with KLK3 alone).[14] Pseudopeptides **3a** and **3b** containing the disulfide bridge mimetics increased the enzymatic activity to 160 and 250%, respectively, of that of KLK3 alone. Similarly, pseudopeptides **3c** and **3d** stimulated KLK3 activity to 160 and 170%, respectively.

In this work, we have shown that it is possible to replace the disulfide bridge in KLK3-stimulating peptide **1a** with a hydrocarbon linker without a significant loss of biological activity. Interestingly, the four-carbon linkers containing an alkane or *E*-alkene seem to be able to mimic the disulfide bridge well despite the carbon atoms in the bridge being smaller in size than the corresponding sulfur atoms in the natural disulfide bridge. Furthermore, we have also confirmed that the terminal NH_2 group next to the disulfide bridge is not critical for the biological activity of the pseudopeptides, although removing it slightly decreases the stimulating effect of the pseudopeptide. Even though the stimulating effect of the synthesized pseudopeptides is weaker than that of the parent peptide, they still significantly stimulate KLK3. In the pseudopeptides both with and without the terminal NH_2 group, the fully saturated linker seems to result in a KLK3 stimulator slightly more potent than the *E*-alkene linker. This can possibly be attributed to the higher flexibility of the saturated linker that allows the peptide to adopt a conformation closer to that of the biologically active conformation of the parent peptide.

In this work, we have presented the replacement of a terminal disulfide bridge using an orthogonally protected building block. Together with the pseudopeptides of the KLK3-stimulating peptide C-4, which we have published previously,[18] we have now successfully introduced similar hydrocarbon-based disulfide bridge replacements in two structurally different KLK3-stimulating pseudopeptides. Notably, pseudopeptides **3a−3d** have been synthesized employing a synthetic strategy different from that used for pseudopeptides based on C-4. We thereby conclude that the presented disulfide bridge mimetics and the synthetic strategies for introducing them could also be applied to other biologically relevant peptides.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for the synthesis and purification of peptides **3a−3d** and compounds **6a** and **7−9** and information about the biological assay. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +358-9-191 59172. Fax: +358-9-191 59556. E-mail: kristian.meinander@helsinki.fi.

Present Address

[‖]M.P.: Nesteoil Corp., P.O. Box 310. FIN-06101 Porvoo, Finland.

Funding

This work was supported by the Academy of Finland (Grants 126969, 135937, and 140929), the Graduate School of Organic Chemistry and Chemical Biology, Research Funds of the University of Helsinki, the Helsinki University Central Hospital, the Finnish Cancer Foundation, the Sigrid Juselius Foundation, Finska Läkaresällskapet, and the Magnus Ehrnrooth foundation.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully thank Prof. Jouko Vepsäläinen and Dr. Pasi Soininen for help with the NMR analysis of peptide **3c**, Ms. Marianne Niemelä for the biological testing, and Ms. Tiina Ahonen for help with the peptide synthesis.

■ ABBREVIATIONS

KLK3, human kallikrein-related peptidase 3; DCE, 1,2-dichloroethane; HOAT, 1-hydroxy-7-azabenzotriazole; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; TBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *N*-*tert*-butoxycarbonyl; tBu, tertiary butyl; SPPS, solid phase peptide synthesis

REFERENCES

(1) Catalona, W. J.; Smith, D. S.; Ratliff, T. L.; Dodds, K. M.; Coplen, D. E.; Yuan, J. J. J.; Petros, J. A.; Andriole, G. L. Measurement of Prostate-Specific Antigen in Serum as a Screening Test for Prostate Cancer. *N. Engl. J. Med.* **1991**, *324*, 1156−1161.

(2) Stenman, U.; Abrahamsson, P.; Aus, G.; Lilja, H.; Bangma, C.; Hamdy, F. C.; Boccon-Gibod, L.; Ekman, P. Prognostic value of serum markers for prostate cancer. *Scand. J. Urol. Nephrol.* **2005**, *39*, 64−81.

(3) Lilja, H.; Ulmert, D.; Vickers, A. J. Prostate-specific antigen and prostate cancer: Prediction, detection and monitoring. *Nat. Rev. Cancer* **2008**, *8*, 268−278.

(4) Fortier, A. H.; Nelson, B. J.; Grella, D. K.; Holaday, J. W. Antiangiogenic Activity of Prostate-Specific Antigen. *J. Natl. Cancer Inst.* **1999**, *91*, 1635−1640.

(5) Mattsson, J. M.; Valmu, L.; Laakkonen, P.; Stenman, U.; Koistinen, H. Structural characterization and anti-angiogenic properties of prostate-specific antigen isoforms in seminal fluid. *Prostate* **2008**, *68*, 945−954.

(6) Mattsson, J. M.; Närvänen, A.; Stenman, U.; Koistinen, H. Peptides binding to prostate-specific antigen enhance its antiangiogenic activity. *Prostate* **2012**, *72*, 1588−1594.

(7) Abrahamsson, P.; Lilja, H.; Falkmer, S.; Wadströ, L. B. Immunohistochemical distribution of the three predominant secretory proteins in the parenchyma of hyperplastic and neoplastic prostate glands. *Prostate* **1988**, *12*, 39−46.

(8) Papadopoulos, I.; Sivridis, E.; Giatromanolaki, A.; Koukourakis, M. I. Tumor Angiogenesis Is Associated with MUC1 Overexpression and Loss of Prostate-specific Antigen Expression in Prostate Cancer. *Clin. Cancer Res.* **2001**, *7*, 1533−1538.

(9) Paju, A.; Hotakainen, K.; Cao, Y.; Laurila, T.; Gadaleanu, V.; Hemminki, A.; Stenman, U.; Bjartell, A. Increased Expression of Tumor-Associated Trypsin Inhibitor, TATI, in Prostate Cancer and in Androgen-Independent 22Rv1 Cells. *Eur. Urol.* **2007**, *52*, 1670−1681.

(10) Wu, P.; Leinonen, J.; Koivunen, E.; Lankinen, H.; Stenman, U. Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. *Eur. J. Biochem.* **2000**, *267*, 6212−6220.

(11) Koistinen, H.; Närvänen, A.; Pakkala, M.; Hekim, C.; Mattsson, J. M.; Zhu, L.; Laakkonen, P.; Stenman, U. Development of peptides specifically modulating the activity of KLK2 and KLK3. *Biol. Chem.* **2008**, *389*, 633−642.

(12) Härkönen, H. H.; Mattsson, J. M.; Määttä, J. A. E.; Stenman, U.; Koistinen, H.; Matero, S.; Windshügel, B.; Poso, A.; Lahtela-Kakkonen, M. The Discovery of Compounds That Stimulate the Activity of Kallikrein-Related Peptidase 3 (KLK3). *ChemMedChem* **2011**, *6*, 2170−2178.

(13) Pakkala, M.; Jylhäsalmi, A.; Wu, P.; Leinonen, J.; Stenman, U.; Santa, H.; Vepsäläinen, J.; Peräkylä, M.; Närvänen, A. Conformational and biochemical analysis of the cyclic peptides which modulate serine protease activity. *J. Pept. Sci.* **2004**, *10*, 439−447.

(14) Pakkala, M.; Weisell, J.; Hekim, C.; Vepsäläinen, J.; Wallen, E. A. A.; Stenman, U.; Koistinen, H.; Närvänen, A. Mimetics of the disulfide bridge between the N- and C-terminal cysteines of the KLK3-stimulating peptide B-2. *Amino Acids* **2010**, *39*, 233−242.

(15) Tadd, A. C.; Meinander, K.; Luthman, K.; Wallén, E. A. A. Synthesis of Orthogonally Protected Disulfide Bridge Mimetics. *J. Org. Chem.* **2011**, *76*, 673−675.

(16) Veber, D. F.; Strachan, R. G.; Bergstrand, S. J.; Holly, F. W.; Homnick, C. F.; Hirschmann, R.; Torchiana, M. L.; Saperstein, R. Nonreducible cyclic analogues of somatostatin. *J. Am. Chem. Soc.* **1976**, *98*, 2367−2369.

(17) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. Synthesis of Oxytocin Analogues with Replacement of Sulfur by Carbon Gives Potent Antagonists with Increased Stability. *J. Org. Chem.* **2005**, *70*, 7799−7809.

(18) Meinander, K.; Weisell, J.; Pakkala, M.; Tadd, A. C.; Hekim, C.; Kallionpää, R.; Widell, K.; Stenman, U.; Koistinen, H.; Närvänen, A.; Vepsäläinen, J.; Luthman, K.; Wallén, E. A. A. Pseudopeptides with a centrally positioned alkene-based disulphide bridge mimetic stimulate kallikrein-related peptidase 3 activity. *Med. Chem. Commun.* **2013**, 549−553.

(19) Wu, P.; Stenman, U.; Pakkala, M.; Närvänen, A.; Leinonen, J. Separation of enzymatically active and inactive prostate-specific antigen (PSA) by peptide affinity chromatography. *Prostate* **2004**, *58*, 345−353.

NOTE ADDED AFTER ASAP PUBLICATION

This paper published ASAP on December 19, 2013. Scheme 1 was replaced and the revised version was reposted on January 8, 2014.