SYNTHESIS AND CYCLIZATION OF MODIFIED PROSTATE-SPECIFIC ANTIGEN ACTIVATING PEPTIDES VIA SOLID-PHASE PEPTIDE SYNTHESIS METHOD

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DATE: 18-01-2012
Abstract

Peptides have been used, in pharmaceutical research in diverse areas (neurology, endocrinology and haematology) due to their important role in many biologically relevant processes. For the therapeutic use, they can be modified chemically to increase their enzymatic stability, specificity, and bioavailability. Prostate specific antigen (PSA) also known as kallikrein-related peptidase 3 (KLK3) is a potential target (biomarker) for cancer therapy due to its suggested antiangiogenetic properties. Discovering new lead molecules is one of the great challenges in drug research. Cyclic synthetic peptides B2 (CVFAHNYDYLVC) and C4 (CVAYCIEHHCWTC) have been shown to increase the proteolytic activity of KLK3 and therefore, the development of newer modified B2 and C4 peptides for in vivo use is at high priority. B2 and C4 analogues (B2-W3 and C4_JE01-04) have been suggested to increase the proteolytic activity of KLK3, and previous research has shown that peptide prodrugs based on KLK3 substrates have therapeutic effect in vivo. The B2 and C4 analogues were synthesized from Fmoc-Cys(Acm)-Wang resin using solid-phase peptide synthesis method with double coupling. The Kaiser or ninhydrin test was performed to confirm that complete coupling has occurred on all free amines on the resin. The completely synthesized peptide was cleaved from the resin and side chain protecting groups removed using a cleavage cocktail. The lyophilized peptides were cyclized using the iodination and/or iodination with subsequent ether precipitation method and the purity and molecular weight of the peptide was verified with analytical HPLC and mass spectrometry, respectively.
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<tbody>
<tr>
<td>AA</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Acm</td>
<td>Acetamidomethyl</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>ACT</td>
<td>α1-anti-chymotrypsin</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Alloc</td>
<td>Allyloxy carbonyl</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>Asp</td>
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</tr>
<tr>
<td>Boc</td>
<td>t-Butoxymethyl</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>EDT</td>
<td>Ethanedithiol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>Eq.</td>
<td>Equivalent</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>HBTU</td>
<td>2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>hK2</td>
<td>Human kallikrein 2</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>KLK3</td>
<td>Kallikrein-related peptidase 3</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
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<tr>
<td>Mtr</td>
<td>4-Methoxy-2,3,6-trimethyl-benzenesulfonyl</td>
</tr>
<tr>
<td>Meb</td>
<td>p-Methylbenzyl</td>
</tr>
<tr>
<td>Mob</td>
<td>p-Methoxybenzyl</td>
</tr>
<tr>
<td>Mtt</td>
<td>4-Methyltrityl</td>
</tr>
<tr>
<td>ODmab</td>
<td>4(N-[1-(4,4-Dimethyl-2,6-dioxo-cyclohexyllidene)-3-methylbutyl]-amino)benzyloxy</td>
</tr>
<tr>
<td>Orn</td>
<td>Ornithine</td>
</tr>
<tr>
<td>OtBu</td>
<td>t-Butoxy</td>
</tr>
<tr>
<td>Pbf</td>
<td>2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl</td>
</tr>
<tr>
<td>Pmc</td>
<td>2,2,5,7,8-Pentamethylchromane-6-sulfonyl</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>Symbol</td>
<td>Name</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Phacm</td>
<td>Phenylacetamidomethyl</td>
</tr>
<tr>
<td>Pic</td>
<td>4-Picolyl</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid-phase peptide synthesis</td>
</tr>
<tr>
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<td>Serine</td>
</tr>
<tr>
<td>S^Bu</td>
<td>tert-Butylmercapto</td>
</tr>
<tr>
<td>tBu</td>
<td>Tert-butyl</td>
</tr>
<tr>
<td>Tmob</td>
<td>2,4,6-Trimethoxybenzyl</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropyl silane</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Trt</td>
<td>Trityl</td>
</tr>
<tr>
<td>t_R</td>
<td>Retention time</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>Xan</td>
<td>Xanthyl</td>
</tr>
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</table>
1. Introduction

Prostate cancer or adenocarcinoma of the prostate (Pca) initiates in the prostate gland and becomes severe once it metastasizes to other parts of the body, i.e., bones and lymph nodes. Common symptoms are difficulty in urinating (dysuria), blood in the urine or semen (hematuria), problems during intercourse (erectile dysfunction), and may cause bone pain or tenderness, etc., and some other symptoms that can augment during the later phase of the disease. Previous studies have shown that the lowest level of prostate cancer is registered in Japanese men living in Japan, vegetarians, and very high level in America (resulting in the death of ~25,000–29,900 American men annually). The development of the prostate cancer has been attributed to factors including genetics and diet. The presence of the prostate cancer may be indicated by symptoms, physical examination (rectal palpation), biopsy, or measuring the prostate specific antigen (PSA) levels in serum. PSA, commonly known as human tissue kallikrein 3 (KLK3) is a serine protease produced by prostate gland and its function is to keep the semen in its liquid form and allows sperm to swim freely. PSA has also been suggested to stimulate tumor growth by cleaving the insulin-like growth factor binding protein-3 (IGFBP) responsible for the release of active IGF-I, which stimulates cell growth. On the contrary, Frontier et al. has reported the anti-angiogenic properties of KLK3, which may indicate tumor suppressing properties of KLK3. KLK3 is secreted in the lumen (figure 1.) as an inactive proenzyme (proPSA) and its concentration ranges from 0.5 – 2.0 mg/mL.
Figure 1. PSA biosynthesis in a normal and cancer infected prostate epithelium cell. The prostate gland is surrounded by a consecutive layer of basal and basement membrane. proPSA produced by the secretory epithelium is deposited into the lumen where it is cleaved by hK2 to produce active PSA. A fragment of the active PSA bound to protease inhibitor (ACT) in the serum and the other fraction is converted to inactive PSA via proteolysis and goes into the serum as free PSA. A characteristic feature of a Pca cell are losses of large fragments of basal cells and disruption of basement membrane resulting to a decrease in production of proPSA and thus active PSA leading to an increase in bound PSA and proPSA in the serum.\textsuperscript{11-12}

ProPSA is a 28 kDa protein consisting of 244 amino acids and becomes activated and displays chymotrypsin-like enzymatic activity when 7 amino acids are cleaved by KLK2 from the N-terminus.\textsuperscript{13} The significant physiological role of KLK3 is to cleave the gel-forming proteins (seminogelin I and seminogelin II) in the semen leading to liquefaction of semen impediments after ejaculation.\textsuperscript{14} KLK3 has also been identified in trace amounts in other tissues and biological fluids i.e. breast and saliva but its biological roles in these tissues is unclear.\textsuperscript{15}

In the serum a majority of KLK3 is in the complex with α1-anti-chymotrypsin (ACT)/serpin A3, with minor amounts adhering to α2-macroglobulin or α1-antitrypsin/serpin A1 and the remaining exists as unbound inactive enzyme (free PSA).\textsuperscript{16} Only free PSA and PSA-ACT (total PSA) are detectable in immunoassays. During the development of Pca tumor PSA is released to the blood circulation and serum concentration of PSA is widely used in the diagnosis of the Pca. The accuracy of the test is interfered by benign prostate hyperplasia. The ratio between free and total PSA are frequently found to be lower in prostate cancer than in benign hyperplasia.\textsuperscript{17} The expression of KLK3 is regulated by androgen due to the presence of androgen response
elements in its gene promoter.\textsuperscript{18} The presence of high levels of enzymatically active PSA only within the extracellular fluid of prostate cancer tumors suggests that a PSA inhibitor based method could be used for imaging and therapy for metastatic prostate cancer sites.\textsuperscript{19} On the contrary it has been shown that metastatic breast cancer with high levels of KLK3 has good prognosis and it has been suggested that further modulation of KLK3 proteolytic activity may inhibit the growth of tumor.\textsuperscript{20} In a cancer infected prostatic epithelium cell, the expression of KLK3 is lower than in normal prostate tissue, and even more reduced in violent tumors indicating poor diagnosis. KLK3 has been suggested to exhibit both cancer promoting and cancer suppressing activities in the regulation of cancer growth even though this has not been extensively examined. Higher levels of KLK3 in tumor tissue are linked with low microvessel density.\textsuperscript{54, 76-79}

Another important serine protease secreted by the prostate gland is KLK2 which exhibit 78\% amino acid identity with KLK3 and has trypsin-like activity.\textsuperscript{21,22} KLK2 is tissue specific i.e. high levels have been detected only in prostate. KLK2 have been associated with increase of prostate cancer growth and metastasis hence, predict the presence of prostate cancer at biopsy.\textsuperscript{23-27} The roles of kallikreins are interconnected though the expression and regulation of different kallikreins in prostate cancer has not been compared.\textsuperscript{28}

1.1. The overall 3D structure of kallikrein-related peptidase 3

The 3D structure of prostate kallikrein reveals that the S1 pocket of chymotrypsin-like proteases consists of the catalytic triads His57, Ser195 and Asp102 (figure 2) located near the heart of the enzyme (active site) and function in catalysis. Ser195 binds to the substrate polypeptide to the side of surrounding residues i.e. phenylalanine, tryptophan, or tyrosine residues closer to the C-terminus, hence keeping it in place while the His57 and Asp102 then hydrolyze the bond.
Figure 2. The overall 3D structure of prostate kallikrein isolated from stallion seminal plasma with five disulfide bridges (green) and the locations of the mercury and zinc atom-binding sites.

The amino acid residue at the bottom of the S1 pocket exerts the chymotrypsin-like specificity for the P1 position and in most kallikrein the residue has been identified as negatively charged aspartic acid i.e. a variety of kallikrein exhibit trypsin-like specificity. In PSA the residue at the bottom is serine (Ser189) which is similar to that found in chymotrypsin as such they share some substrate specificity in common. Zn$^{2+}$ and Hg$^{2+}$ are potential inhibitors of PSA activity and binds to peptides having sequences matching Zn$^{2+}$ binding sites on the zinc finger proteins. Zn$^{2+}$ has been also shown to mediate high binding affinity between trypsin and its small molecule size inhibitor. Furthermore, Zn$^{2+}$ is said to stabilize the 3-D structure of the peptides just like it interacts with the zinc finger proteins.$^{30,31}$

**1.2. Development of KLK3-binding peptides using phage display**

Phage display method is a technique used to find zinc-finger proteins that will bind to a specific DNA target sequence using bacteriophages. The bacteria uses specifically the Gene 3 on the phage to produce a protein which assembles on one end of the phage hence, by inserting a variety of same zinc-finger protein into Gene 3 of the phage a library of phages each expressing a different zinc-finger protein can be produced.$^{32-35}$ Phages modified from the original purpose has further used to characterized any linear and cyclic
peptide binding specifically to the target molecule. Phage display has been used to discover peptides that fine-tune the enzyme activity of proteases and such peptides are reported to stop tumor growth in vivo.\textsuperscript{34,36-37} The synthetic cyclic peptides B-2 and C-4 were identified using phage display peptide libraries and have been reported to increase the serine protease activity of PSA.\textsuperscript{38,39} These peptides with different amino acid sequence were identified to bind to the same region of active site making them potential lead compounds to target PSA. The prerequisites for binding was studied by alanine replacement.\textsuperscript{40} Recent development of phage display has led to the discovery of small-molecule mimicking peptides i.e. biotin and sugar mimics. Streptavidin have a high affinity for biotin (a B-complex vitamin) and the non-covalent interaction between streptavidin and biotin is considered one of the strongest in nature. As a result of the resistance to organic solvents, denaturants the streptavidin-biotin complex is used in many applications e.g. molecular biology and bionanotechnology. Another protein avidin (cut off from egg white) share about 30% sequence identity with streptavidin also have very high affinity for biotin but not commonly used because it is easily glycosylated. The use of combinatorial methods like phage display strategies developed by Devlin et al.\textsuperscript{74} have provided flexible means to discover peptide-based biotin functional mimics.\textsuperscript{75}

1.3. Peptide modification strategies

There is often challenges’ to use peptides as drugs because they generally have poor in vivo stability, poor pharmacokinetics, and poor bioavailability, but this does not mean that they play no role in medicinal chemistry. They serve as highly important lead compounds for the design of novel drugs e.g. goserelin is a peptide drug used against breast and prostate cancers.

1.4. Advantages and Disadvantages of peptides as drugs

Peptides are versatile and have tremendous therapeutic properties due to their chemical and biological diversity. Furthermore, they exhibit high specificity, affinity and molecular recognition.
1.4.1. Disadvantages of peptides as drugs

Some of the main disadvantages of peptides as drugs are described below:

1. Undergo rapid degradation by peptidases in the gastrointestinal tract and in serum.
2. Due to their relative high molecular weight and lack of specific transport systems, it is difficult to cross the plasma membrane or blood brain barrier.
3. It undergoes rapid excretion via the liver or kidneys without reaching the target. Excretion may be also beneficial, since excess of the drug should be neutralized.
4. Result to undesired side effects due to interaction with multiple receptors besides the target attributed to its inherent flexibility.

1.5. Peptidomimetics and pseudopeptides

As a result of the shortcomings of peptides as potential drug candidates, it is necessary to structurally modify the peptides to improve their therapeutic efficacy and conformational stability. Peptidomimetics are structures, which improve the bioavailability of the polypeptides without losing its function. Peptidomimetics are designed using unnatural amino acids, dipeptides or conformational restraints to mimic the bioactive conformation of the peptides and thus retain the ability to interact with the biological target with the same biological effect as original polypeptide. Peptidomimetic approaches are used for the design and synthesis of peptide analogues with improved pharmacological properties known as pseudopeptides or peptide bond surrogates by replacing one or more bonds with an isostere. Scheme 1. represents some examples of peptidic bond transformation to peptidomimetics containing the amide surrogates i.e. isosteric with the natural peptidic amide bond.

Peptidomimetic structures are used to optimize the specificity, oral bioavailability, decrease the side effects and increase the half-life through minimizing enzymatic...
degradation and optimizing the potency and selectivity.\textsuperscript{42} Several methods have been developed in the discovery, development and optimization of pseudopeptides:

1. Amide bond replacements - replacement of peptide bonds by non-peptide analogs e.g. carbamate, urea, hydrazines, lactams etc.
2. Incorporation of non-peptide moieties – insertion of various bi-functional, non-peptidic molecules within the peptide sequence.
3. Peptide small molecule conjugates – small molecules attachment to side chains, and use of N-alkylated and non-natural amino acid derivatives
4. Backbone cyclization – ring formation using proprietary building units, leads to cyclic peptides in which the amino acid side - chains are unaltered.

Scheme 1. Common amide bond isosteres.
1.5.1 Peptidomimetics in drug design

A lead molecule is a chemical compound that has biological activity and whose chemical structure is used as a starting point for chemical modifications to enhance potency, selectivity or pharmacokinetic properties. The structure activity relationship studies (SARs) are the starting point for the modulation of the peptide sequence with biological activity taking into consideration the binding affinities of the peptide derivatives to the target.\textsuperscript{41, 43-47} To improve the bioavailability i.e. enzyme stability, improved half life e.t.c. conformational restrictions are used.\textsuperscript{41, 48} Cyclization is achieved by local and global constraints,\textsuperscript{43} N-methylation,\textsuperscript{49} isosteric replacement,\textsuperscript{50} or by secondary replacement.\textsuperscript{51}

1.6. Cyclic peptides and cyclization strategies

Cyclic peptides are a class of compounds with improved biological activities and ranges from antibiotics to immunosuppressant drugs and toxins. They are much more resistant to enzyme degradation than non-cyclic peptide chain and are able to bind drug targets in vivo. These cyclic peptides can be modified using several approaches e.g. N- and C-terminal capping, D-amino acids, side chain modifications, unusual amino acids, N-alkylation of amide nitrogen and cyclization hence, enhance peptide stability.\textsuperscript{52}

Most natural peptides are linear and consist of a sequence of amino acids linked together by an amide bond. They have N-terminus with the amino group at one end and a C-terminus with a carboxyl group at the other end. A cyclic polypeptide chain is formed by linking the N and C terminals together. Aside their biological activity, they act as carriers to convey drugs into the body e.g. Sophisen is a good carrier for Dichlofenac sodium by passive diffusion through the cornea.\textsuperscript{53}
1.6.1. Peptide cyclization advantages and methods

Cyclization can be used to mimic natural peptide structures or to synthesize more stable peptide structures, resulting in enhanced conformational stability when compared to their natural counterparts. A variety of peptide cyclization methods have been developed:

1. Amide condensation (amide bond formation, lactam formation)
2. Thiol oxidation (disulfide bridge formation)
3. On resin cyclization
4. Solution phase cyclization

The two most commonly used methods of peptide cyclization will be described below.

1.7. Amide condensation

In this method an intramolecular amide bond is formed between an amino group (NH\textsubscript{2}) and a carboxyl group (COOH). There are four possibilities for amide condensation to take place:

1. Between N-terminal to C-terminal
2. Between N-terminal to internal COOH
3. Between C-terminal to internal NH\textsubscript{2}
4. Between NH\textsubscript{2} to internal COOH

1.7.1. Thiol oxidation (Disulfide bridge formation)

In this method a disulfide bridge is formed between two thiol (SH) groups from the side chain of cysteine or cysteine analogues resulting to intermolecular (two peptide molecules are linked via the disulfide bridge forming homodimers or heterodimers) or intramolecular (cyclization within one peptide molecule) oxidation. Protecting groups are used to prevent undesired linkage. The disulfide bridge is formed between two cysteines, head-to-tail (i.e. backbone) cyclization between the N- and C-terminus, between side chains, between the terminus and side chain, or between unnatural amino acids or
synthetic building blocks. Head-to-tail cyclization has reduced the risk of peptide degradation i.e. enhanced the stability of peptides against proteases by eliminating the N- and C-termini.\(^5^4\)

### 1.8. Side-chain protecting groups

About 50% of all amino acids encountered in SPPS have side-chains containing reactive functional groups. During peptide synthesis masking of these reactive groups is beneficial due to the severe conditions used to achieve the desired sequence and also to achieve the highest possible recovery of the product. Protecting groups that can be easily removed with TFA are usually used because they can be easily cleaved from the peptide or support during the deprotection phase. However, a variety of protecting groups can be used and thus will require different conditions for their removal (table 1).

<table>
<thead>
<tr>
<th>Side-chain functionality of AA</th>
<th>Protecting groups</th>
<th>Cleavage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>Mtr, Pmc, Pbf</td>
<td>90-95% v/v TFA, 4-6 h TFA-anisole(9:1), 1 h</td>
</tr>
<tr>
<td>Asp/Glu</td>
<td>(\text{O}^3\text{Bu})</td>
<td>90% v/v TFA, 30 min</td>
</tr>
<tr>
<td></td>
<td>(\text{OAll})</td>
<td>(\text{Pd(Ph}_3\text{P})_4)-AcOH-NMM</td>
</tr>
<tr>
<td></td>
<td>(\text{ODmab})</td>
<td>2% (\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}) in DMF, 5-10 min</td>
</tr>
<tr>
<td>Asn/Gln</td>
<td>Trt</td>
<td>90% v/v TFA, 30-60 min</td>
</tr>
<tr>
<td></td>
<td>Tmob</td>
<td>90% v/v TFA, 1h</td>
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<tr>
<td></td>
<td>Mtt</td>
<td>95% v/v TFA, 30 min</td>
</tr>
<tr>
<td>Cys</td>
<td>Acm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trt</td>
<td>90% v/v TFA, 30 min</td>
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### Table 1.55 Common side-chain protecting groups used in Fmoc/tBu SPPS

<table>
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<tr>
<th>Side-chain functionality of AA</th>
<th>Protecting groups</th>
<th>Cleavage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>S⁻Bu</td>
<td>Tmob</td>
<td>5% TFA-3% TES in DCM</td>
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<tr>
<td>Mmt</td>
<td>0.5-1% TFA in DCM-TES(95:5), 30 min</td>
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</tr>
<tr>
<td>His</td>
<td>Trt</td>
<td>50% TFA in DCM, 30 min</td>
</tr>
<tr>
<td>Boc</td>
<td>90% v/v TFA, 30 min</td>
<td></td>
</tr>
<tr>
<td>Lys/Orn</td>
<td>Boc</td>
<td>90% v/v TFA, 30 min</td>
</tr>
<tr>
<td>Alloc</td>
<td>Pd(Ph₃P)₄(0.1 eq.)-PhSiH₃(24 eq.) in DCM, 10 min</td>
<td></td>
</tr>
<tr>
<td>Mtt</td>
<td>1% TFA in DCM, 30 min AcOH-TFE-DCM(1:2:7), 1 h</td>
<td></td>
</tr>
<tr>
<td>Ser/Thr/Tyr</td>
<td>S⁻Bu</td>
<td>90% v/v TFA, 30 min</td>
</tr>
<tr>
<td>Trt</td>
<td>1-5% TFA in DCM, 2-5 min</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>Boc</td>
<td>i) 90% TFA, 1 h; ii) 1% aq. TFA, 1-2 h</td>
</tr>
</tbody>
</table>

1.9. Cysteine protection

The presence of an atom of sulphur in cysteine and/or methionine makes them special amino acid residues in peptide chemistry. Handling of cysteine is challenging because some targets require Cys with its side-chain in the free thiol form, meanwhile an even number of Cys residues is required for other targets to ease the formation of
disulphide connection between each pair. The Cys protecting groups that are frequently used in Fmoc/tBu strategy are listed (table 2) together with the conditions in which they are stable and possible methods of removal. Different reaction conditions can yield the free thiol, thiolate metal salt (mercaptide), or disulphide. Peptides with four or more cysteine residues present a statistical obstacle, since they can, in principle give rise to three or more different intramolecular isomers. This obstacle has been addressed by using highly optimized conditions to produce a major regioisomer with the naturally occurring pairing.

<table>
<thead>
<tr>
<th>Lability</th>
<th>Protecting group</th>
<th>Stability</th>
<th>Removal condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate acid</strong></td>
<td>Xan, Tmob, Mmt, Trt</td>
<td>Base, Nucleophiles</td>
<td>Dilute TFA/scavengers, $I_2$, $Tl(III)$, $Hg(II)$, $Ag(I)$, RSCl</td>
</tr>
<tr>
<td><strong>Concentrated acid</strong></td>
<td>Mob, Mob, tBu</td>
<td>TFA, Base, RSCl, $I_2$, $Ag(I)$, HF(0°C)</td>
<td>HF(0°C), $Tl(III)$, Ph(SO)Ph$-\text{CH}_3\text{SiCl}_3$, Hg(II), NpsCl</td>
</tr>
<tr>
<td><strong>Metal ions</strong></td>
<td>Acm, Phacm</td>
<td>HF(0°C), TFA, Base, HF</td>
<td>Hg(II), $Ag(I)$, $I_2$, $Tl(III)$, RSCl, Ph(SO)ph$-\text{CH}_3\text{SiCl}_3$, penicillin amidohydrolase</td>
</tr>
<tr>
<td><strong>Reducing agents</strong></td>
<td>S-tBu, Pic</td>
<td>TFA, HF (partial), Base, RSCl, HBr$-\text{AcOH}$</td>
<td>RSH, Bu$_3$P, other reducing agents, Electrolytic reduction, Zn-AcOH</td>
</tr>
</tbody>
</table>

Table 2. Cysteine protecting groups compatible with Fmoc/tBu SPPS
1.10. Overview of Preparative and Analytical HPLC

In high-performance liquid chromatography, analyte molecules are injected into a column filled with particles capable of retaining them (stationary phase), and they are eluted out of the column with solvents (mobile phase) so that the least strongly retained analyte is eluted first into detector. Different additives are added to the mobile phase to optimize the separation and also for pH adjustment e.g., formic and acetic acids, trifluoroacetic acid (TFA), ammonium acetate, or phosphate buffers. HPLC is commonly used in a wide range of applications, including drug discovery and purification for the pharmaceutical and biotechnology industries, environmental analysis, forensics, petrochemical analysis, food, cosmetics and vitamins. Different columns are used with respect to the sample to be analyzed i.e. reversed-phase (for molecules that posses some degree of hydrophobic character e.g. peptides and can be separated with excellent recovery and resolution), normal-phase, hydrophilic interaction liquid chromatography (HILIC), ion-exchange, ion-exclusion, size-exclusion, and affinity stationary phases for small- and large-molecule separations. For proper performance of a HPLC column the following properties have to be taken into account i.e. particle shape, pore size and pore volume, particle size and particle size distribution, surface properties and metal impurities. For peptide separation C18 columns are recommended as the first choice because of their high capacity.

Preparative chromatography is considered to a large extent to be a purification process using liquid chromatography. Preparative chromatography could be done in linear or non-linear mode i.e. in the former the equilibrium concentrations of the component in the stationary and mobile phases are proportional and band shapes and retention times are independent of the amount of each component in the injected sample. The peak height is directly proportional to the amount and composition of the sample. In the latter i.e. non-linear chromatography the concentration of a component in the stationary phase at equilibrium is not proportional to its concentration in the mobile phase as such the band shape, retention time and peak maximum depend on the amount and composition of the sample. In preparative chromatography larger amounts of sample are injected with the
main task to recover as much purified product as possible in each run. In analytical chromatography, smaller amounts of samples are often used (depending on the detection method) in order to identify and quantify the concentrations of the components in a mixture. The two chromatographic processes can be distinguished not by the size of sample or equipment but exclusively by the task of the separation process (table 3).

<table>
<thead>
<tr>
<th>Analytical HPLC</th>
<th>Preparative HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample goes from detector into waste</td>
<td>Samples goes from detector into fraction collector</td>
</tr>
<tr>
<td>Main goal is for quantification and/or identification of compounds</td>
<td>Main goal is for isolation and/or purification of compounds</td>
</tr>
<tr>
<td>Sample can be processed, handled and modified to generate required information, including degradation, labeling or changing the nature of the compound under investigation</td>
<td>The sample has to be recovered in exact condition before undergoing the separation i.e. no degrading elution conditions</td>
</tr>
</tbody>
</table>

Table 3. Overview of the differences between analytical and preparative HPLC

### 1.11. Mass spectrometry

Mass spectrometry (MS) is an analytical tool used for measuring the mass-to-charge ratio (m/z) of charged particles. It is also used to determine the chemical structures of molecules e.g. peptides and other chemical compounds. Based on the ionization technique used, the mass spectrum may show a large number of peaks from fragment ions, that provide the structural data of the analyzed compound, or it may contain a peak solely for the molecular ion, providing information of the molecule or about the elemental composition of the molecule. An ionization source creates charged ions within the gas phase and a mass analyzer separates ions according to their mass to charge ratio value. The ions produced are as a result of loss or gain of one or more electrons, making them negatively or positively charged. The two primary methods for ionization of whole
proteins are Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). In a typical MS procedure:

1. A sample is loaded onto the MS instrument and undergoes vaporization
2. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions)
3. Directing the ions into an electric and/or magnetic field
4. Computation of the mass-to-charge ratio of the particles based on the details of motion of the ions as they transit through electromagnetic fields, and
5. Detection of the ions, by a quantitative method and the ion signal is processed into a mass spectra.

A mass spectrometer consists of three basic components:

1. An ion source, which can convert gas phase sample molecules into ions
2. A mass analyzer, which sorts the ions by their masses by applying electromagnetic fields
3. A detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present
2. Project tasks

The use of peptides in pharmaceutical research is increasing due to the important role in many biological processes\(^6^2\) i.e. in the fields of neurology, hematology and endocrinology. Only few peptide drugs are available in the market for the treatment of disease because of their inherent enzymatic instability of unmodified peptides and their relatively poor bioavailability i.e. limits their potential as drug candidates. Contrary to their shortcomings, they are less toxic, more soluble but more complicated to synthesize than small organic molecules.\(^6^3\) Recent advances in genomics and proteomics, have led to the discovery of numerous protein and peptide drugs of therapeutic potential and a great number of peptides in various phases of clinical trials.\(^6^4\) About 720 peptide drugs and drug candidates were reported in 2004, of which 1% are registered, 5% are already in the market, 38% are in clinical trials and 56% in advanced preclinical phases.\(^6^5\) The discovery of a large number of naturally occurring peptides with potent and biological activities during the past 35 years has boosted their importance.\(^6^6\) Peptide ligand act as agonists or antagonists at cell-surface receptors or acceptors that activate cell function and animal behavior e.g. learning, memory, response to stress, pain, addiction, sexual behavior and many others. The synthesis and development of peptide molecules that can target the receptors or acceptors that activate these biological processes is of high priority.\(^6^7\)

The B-2 peptide containing phenylalanine, tyrosine, valine (in second position) and the C-4 peptides have been shown to bound specifically to KLK3, stimulate its proteolytic activity and is potential therapeutic peptide for PCa.\(^6^8,4^0,5^4\)

The main goal of this study was to synthesize modified PSA activating peptides B-2 and C-4 (Table 4) using Fmoc-based solid-phase peptide synthesis (SPPS) method under standard conditions. B-2 (CVFAHNYDYLVC) is a peptide sequence consisting of 12 amino acids and undergoes structural modification by replacement from F3 to W3 to form the peptide sequence B2-W3 (CVWAHNYDYLVC). C-4 (CVAYCIEHHCWTC) is a 13 amino acid peptide containing cysteines at fixed positions (1, 5, 10 and 13) and undergoes substitution point mutations to form the analogues C4_JE01-C4_JE04 as shown below. Cyclization was
performed through disulfide bridging between two cysteins in the N- and C-terminals (Figure 3.).

<table>
<thead>
<tr>
<th>Peptide ID</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>CVFAHNHYDYLVC</td>
</tr>
<tr>
<td>C4</td>
<td>CVAYCIEHHCWTC</td>
</tr>
<tr>
<td>B2-W3</td>
<td>CVWAHNHYDYLVC</td>
</tr>
<tr>
<td>C4_JE01</td>
<td>CVAYCIAHACWTC</td>
</tr>
<tr>
<td>C4_JE02</td>
<td>CVAYCAEHACWTC</td>
</tr>
<tr>
<td>C4_JE03</td>
<td>CVAYCAAHACWTC</td>
</tr>
<tr>
<td>C4_JE04</td>
<td>CVAYCVEHHCWTC</td>
</tr>
</tbody>
</table>

Table 4. Structurally modified B-2 and C-4 peptide analogues from B2 and C4.

After cleavage from the solid support and purification by high-performance liquid chromatography (HPLC), their structures were confirmed by mass spectrometry and the purity was determined by analytical reversed-phase HPLC.

a) CVFAHNHYDYLVC
   S——S

b) CVAYCIEHHCWTC
   S——S

Figure 3. The original B-2 (a) and C-4 (b) cyclic peptides with formation of Disulfide Bridge via two cysteins.

The structures of the four modified peptides used in this study are shown below (figure 4).

Figure 4. Structures of successfully synthesized peptides 1-4 with corresponding ID B2-W3, C4_JE01, C4_JE02 and C4_JE03 respectively.
3. Results and discussion

The first protocol for the synthesis of B-2 analogue, B2-W3 failed due to incomplete coupling of the amino acid residues as shown in the mass spectra. The second strategy was slightly modified from the first protocol to increase the coupling efficiency of the added amino acid. In the first protocol, a solution of activated Fmoc amino acid in 1 ml DMF was added to the resin and stirred for 40 min at RT for coupling and the second protocol, the activated Fmoc amino acid (1 ml) was divided into two (i.e. ½ ml solution in DMF) and added at intervals of 30 min with vigorous stirring for coupling to occur (double coupling). B2-W3 and C-4 analogues were synthesized using the second strategy. The successfully synthesized peptides were cleaved from the resin and lyophilized under high vacuum. The yields of the crude peptide obtained are shown in table 5 below.

<table>
<thead>
<tr>
<th>Peptide ID</th>
<th>Peptide Sequence</th>
<th>Yields (crude peptide after lyophilization) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2-W3</td>
<td>CVWAHYDLVC</td>
<td>67.7</td>
</tr>
<tr>
<td>C4_JE01</td>
<td>CVAYCIALWC</td>
<td>25.2</td>
</tr>
<tr>
<td>C4_JE02</td>
<td>CVAYCAHACWTC</td>
<td>26.5</td>
</tr>
<tr>
<td>C4_JE03</td>
<td>CVAYCAHACWTC</td>
<td>21.9</td>
</tr>
<tr>
<td>C4_JE04</td>
<td>CVAYCVEHWC</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table 5. Yields of crude peptide after cleavage from resin and lyophilization.

The low yield of C4_JE04 was as a result of the resins broken into powder by the magnetic stirrer. As a result of this it was difficult for the peptide to filter through the PD-10 column easily.

Detectibility of the peptides in UV detector depends on the different amino acid residues in the peptide sequence. Since our peptides consists of both non-aromatic and aromatic residues, the UV light detection was set at 220 nm and 280 nm, respectively which offer selective detection of the amino acid residues and amide bonds at these wavelengths. The
results of Kaiser Test of B2-W3 and C-4 analogue peptides are shown in Table 6 below. The Kaiser test was not performed after each deprotection due to the limited amount of resin.

**Table 6 - Results of Kaiser Test after the coupling of the respective amino acids as indicated with their respective subscripts (i.e. AA2 indicate after addition of amino acid residue 2).**

<table>
<thead>
<tr>
<th>Kaiser test</th>
<th>Resin</th>
<th>AA2</th>
<th>AA3</th>
<th>AA6</th>
<th>AA9</th>
<th>AA12/AA13</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-/-</td>
</tr>
<tr>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

<sup>a</sup> After coupling of amino acid residue 2,3,6,9, 12 and 13 respectively. <sup>b</sup> Kaiser test before deprotection with 20% piperidine to give a yellow colour (negative test). <sup>c</sup> Kaiser test after deprotection to give a dark blue colour (positive test).

The first cyclization was unsuccessful for the C4 analogues as indicated by the mass spectra i.e. there was incomplete cyclization as such all the Acm protecting groups were still attached to the crude peptide hence no disulfide bond was formed. We made a recombination of all the fractions with similar masses for the four peptides and we obtained yields as shown in table 7 below.

**Table 7. Yield of C4 analogues with Acm protecting groups attached.**

<table>
<thead>
<tr>
<th>Peptide ID</th>
<th>C4_JE01 (mg)</th>
<th>C4_JE02 (mg)</th>
<th>C4_JE03 (mg)</th>
<th>C4_JE04 (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>3</td>
<td>5.1</td>
<td>5.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Each of these peptides except C4_JE04 (cyclized using second method) was subjected to a second cyclization phase with same procedure as described above. The peptide recovery was very low (-0.1-0.7 mg) and the MS showed more of the oxidization products [M+32] and the potassium adduct [M+38] hence suppressing the signal intensity of the molecular ion [M+1]. The potassium ion could have been generated from the solvents or from the glassware used. The oxidation product is suggested to have emerged from the autoxidation of the diethyl ether used for washing the peptide after iodine cyclization. From the analytical HPLC these oxidation product seems difficult to isolate because it is merged with the peptide signal i.e. have same retention time as the peptide being analyzed.
We decided to continue with the second cyclization method with the remaining crude peptide (C4_JE01-C4_JE04) i.e. iodine cyclization with subsequent ether precipitation as described in the experimental section. After the cyclization process was completed, to the crude peptide solution was added into ice-cold ether and subsequent freezing and centrifugation afforded a light yellow or colourless crude peptide solid (> 7 mg except for C4_JE04). The peptides were purified by preparative HPLC, purity checked with analytical HPLC and finally characterized by mass spectrometry. However, in the case of C4_JE04 instead of obtaining a precipitate of peptide, two solution layers were obtained consisting of an upper ether layer and a lower aqueous layer containing the peptide. The biphasic solution was cooled overnight in freezer after which the lower layer peptide solution froze allowing the upper ether layer to be decanted carefully. After thawing, the aqueous layer was then characterized as described above.

Since the C4 point mutated peptides have four cysteines, there is a possibility of disulphide bond connection different from the structures in figure 10 above. i.e. pairing can occur between Cys 1-9 and Cys 4-13 and/or Cys 1-4 and Cys 9-13 (figure 5). The correct pattern can be determined using other experimental techniques e.g., mass spectrometry with respect to the fragments that will be observed from the mass spectra.

![Figure 5. Other possible disulphide bond connection between the Cysteine residues.](image)

### 3.1. Purification of peptides

The lyophilized crude peptide was analyzed with analytical HPLC to check the impurity profile and to determine if purification was required before iodine-cyclization. Both preparative and analytical HPLC were performed using Xterra™ RP C18 5 µm 4.6 x 250 mm column which afforded better separation than the previous column used for the first cyclization method i.e. Phenomenex Jupiter C18 RP 250 x 10.00 mm, 10 micron for
preparative and Xterra waters C18 240 x 1.4 mm column for analytical HPLC. The mass spectra indicated mass of peptide with the Acm protecting groups and we decided to go on with the cyclization process before purification. The retention time of the analyzed peptides were within the expected range for the B2 and C4 peptide analogues (9-17 minute) with respect to the column used. The retention time varies due to slight differences in the peptide sequence and also due to external factors e.g., temperature, ratio/composition of solvent used, flow rate of mobile phase and the strength of analyte interaction with the stationary phase).

The analytical HPLC chromatogram of B2-W3 (appendix 5) and C4_analogues (appendix 6-8) with respect to their retention time and % purity are shown in table 8 below. The HPLC chromatogram of C4_JE01 (appendix 5) showed an extra peak at retention time 18,333 originating from the background as observed from the blank run. The analytical chromatogram of C4_JE02 (appendix 6) showed three peaks close together thus, indicating the peptide is still impure and requires further purification since the purity is expected to be > 90% for the biological testing.

3.2. Mass spectrometry of peptides

The peptide recovery was higher than the previous method (0.5-2.5 mg) and the MS showed more of the potassium adduct [M+38] as the most abundant ion and some trace of the sodium adduct [M+23] hence, suppressing the signal intensity of the molecular ion [M+1] (table 6). The ionization was performed in the positive ion mode mass spectra from m/z 400-2000 with a time-of-flight MS instrument to determine the elemental composition of the peptides with high resolution and accuracy. The ions observed in the MS may be quasimolecular ions created by the addition of a proton (a hydrogen and denoted [M+H]⁺, or of another cation such as sodium ion, [M+Na]⁺, or the removal of a proton [M-H]⁻. Multiply-charged ions such as [M+nH]ⁿ⁺ (where n>1) are also often observed especially for large macromolecules.

The mass spectrum (appendix 1) indicates a base peak at m/z 742.3683 assuming the peptide ions are most abundant in the 2+ charge state and molecular ion peak at m/z
1483.6731, which corresponds to the molecular mass of the protonated \([\text{M+H}]^+\) form of the cyclic B2-W3 peptide i.e., \([\text{C}_{68}\text{H}_{90}\text{N}_{16}\text{O}_{18}\text{S}_2]^+\).

**Table 8-** Experimental data of B2-W3 and C-4 analogues

<table>
<thead>
<tr>
<th>Comp. ID</th>
<th>Calculated molecular mass (g)</th>
<th>Measured molecular mass (g)</th>
<th>Observed ions (m/z)</th>
<th>% purity</th>
<th>Retention Time((t_R)), min ((\lambda = 280 \text{ nm}))</th>
<th>Disulfide pairing</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2-W3</td>
<td>1482.61</td>
<td>1483.7224</td>
<td>742.3269,</td>
<td>&gt; 90</td>
<td>10.200(^c)</td>
<td>Cys 1-12</td>
</tr>
<tr>
<td>C4_JE01</td>
<td>1437.72</td>
<td>1438.4053</td>
<td>719.7604, 738.7366, 1476.4617, 1460.2901</td>
<td>&gt; 90</td>
<td>9.583(^c)</td>
<td>Cys 1-13, Cys 4-9</td>
</tr>
<tr>
<td>C4_JE02</td>
<td>1453.44</td>
<td>1454.3824</td>
<td>738.7301, 746.7157, 1492.3209, 1477.4341</td>
<td>&gt;50</td>
<td>14.975(^d)</td>
<td>Cys 1-13, Cys 4-9</td>
</tr>
<tr>
<td>C4_JE03</td>
<td>1395.64</td>
<td>1396.3341</td>
<td>717.7145, 1418.4253, 1433.4255, 1417.4190</td>
<td>&gt; 90</td>
<td>15.458(^d)</td>
<td>Cys 1-13, Cys 4-9</td>
</tr>
</tbody>
</table>

\(^a\) Calculated using HighChem Mass Frontier software. \(^b\) Mass of purified peptide obtained. \(^c\) eluted using Jupiter C18 4.6x150mm column. \(^d\) eluted using Xterra\(^\text{TM}\) RP C18 5 \(\mu\)m 4.6 x 250 mm column

For the C4 analogue peptides with two disulfide bridges, the ions monitored were the protonated molecules \([\text{M+H}]^+\), the alkali metal cation adducts i.e. the sodium adduct \([\text{M+Na}]^+\) and potassium adduct \([\text{M+K}]^+\). C4_JE01 (appendix 2) showed the expected molecular ion peak at m/z 1438.4983, a very intense base peak at m/z 738.7404 corresponding to the 2+ charge adduct of potassium and a weak signal of m/z 1460.4642 indicating the presence of sodium adduct. Secondly, C4_JE02 (appendix 3) we observed a weak precursor ion with m/z 1454.4526 and very intense 2+ charge adduct of potassium at m/z 746.7157 and a trace of 2+ charge at m/z 738.7301 corresponding adduct of sodium. Finally, for C4_JE03 (appendix 4) we observe the expected parent ion at m/z 1396.4579, a very intense base peak at m/z 717.7187 corresponding to the potassium
adduct and a weak sodium adduct ion signal with m/z 1418.4253 respectively. The C4_JE04 did not show any expected ion thus, results are not reported in this study. The presence of the disulfide bridge(s) increases the structural stability and can also improve biological activity\textsuperscript{69-70} of these peptides.

4. Conclusion and future aspects

In this study we have successfully used solid-phase peptide synthesis method to synthesis and cyclized the B2 and C4 modified peptide B2-W3 and C4_JE01-C4_JE03 respectively. Analytical reversed-phase HPLC was used for product isolation (purification) and mass spectrometry with ESI source in positive ion mode for peptide mass identification. ESI is considered a soft ionization technique because it typically produces only a few fragment ion and an intensive protonated molecule (deprotonated in negative ion mode), or in many cases adducts with alkali metal cations. The Na/K adducts observed are due to small amounts in the MS solvents, or MALDI matrix or even picked up from glass sample vials. The cation adducts are suggested to be suppressed using a basic HPLC buffer, an acidic ion exchange resin or performing the MS in the negative ion mode e.g., running C4_JE01 (calculated molecular mass, 1437) showed an intense parent ion at m/z 1436.9340 and a weak signal at m/z 1472.9066 (M+36) which can be attributed to addition of two extra water molecules. The cation adducts were not observed in the negative ion mode. Furthermore, the presence of the cation adducts do not have any effect on the purity of the peptide as determined by the analytical chromatograms giving rise to very sharp single peak as expected. The retention time of the peptide varies with respect to the column used for the analysis. The C4_JE04 can be re-synthesized and taking into consideration the magnetic stirrer used for the synthesis so as not to break the resin into powder as previously observed. Both cyclization strategies proved efficient but the higher yield obtained from the ether precipitation method makes it to be more efficient over the normal iodine cyclization method. Depending on the \textit{in vitro} biological assay evaluation result, we can draw conclusions whether the modified peptide binds specifically to KLK3 and increases its proteolytic activity as suggested. This will act as a
lead compound (starting point) for further development of peptidomimetic analogues of B2-W3 and C4 peptides to target prostate cancer.

In summary, we have shown that the double coupling SPPS and ether precipitation method are more efficient and reliable approach for the synthesis and cyclization of B2 and C4 analogue peptides with high purity.

5. Experimental section

5.1. General considerations

All solvents and reagents were purchased from Sigma Aldrich and/or Fluka and all Fmoc amino acids/Wang resin from GLS Biochem and used without further purification. The reaction was performed in the empty PD-10 column. DMF was used for washing off impurities from other solvents and 20% piperidine in DMF was used as deprotection reagent i.e. removal of Fmoc Amino-Terminal protecting group. The “Kaiser Test” was used to confirm that each coupling step goes to completion and all the Fmoc protecting groups are removed. The “Kaiser Test” is a colorimetric test for the presence of amino groups and based on the reaction of ninhydrin with amino groups to form a dark blue adduct. The test consists of three test solutions: Ninhydrin (5% w/v) in ethanol (solution A), phenol (4:1 w/v) in ethanol (solution B) and potassium cyanide (2%, v/v, of a 1 mmole/liter aqueous solution) in pyridine (solution C). The test is carried out by adding one drop of solutions A, B and C to the test sample in a small glass tube and then heating the tube for 2 minutes in a water bath (in beaker) at 100°C. The incomplete coupling develops a blue color of the resin (positive Kaiser Test) while coupling to completion will afford a yellow coloration (negative Kaiser Test). The Kaiser test is based on the color of the resin and not the colour of the solution because some amino acid residues can give unusual color change from red to blue e.g. Asn, Cys, Ser, Thr. HBTU and DIPEA were used as activation reagents (to activate the carboxylic acid by esterifying it) prior to coupling. The peptide was cleaved from the resin with the cleavage cocktail made from a combination of trifluoroacetic acid, ethanedithiol, Tri-isopropylsilane and water. The cleavage cocktail
remove also the amino acid protecting groups. The peptide was precipitated and washed with diethyl ether and centrifuged using Hettich Zentrifugen Rotina 46 R. The lyophilized peptide was purified by HPLC (Shimadzu, Kioto, Japan) on a C$_{18}$ reversed phase column (250 x 10.00 mm, 10 micron) (Phenomenex Jupiter) or Xterra™ RP C18 5 µm 4.6x250 mm using an CH$_3$CN gradient (0.1% TFA in H$_2$O/0-90% CH$_3$CN gradient for 60 min.). The purity were determined by analytical HPLC on a 240 x 1.4 mm C$_{18}$ column (Xterra, Waters) and/or Xterra™ RP C18 5 µm 4.6x250 mm eluted with 0-90% CH$_3$CN and fractions verified by mass spectrometry on an ABI QSTAR XL hybrid mass spectrometry using ESI interface (Applied Biosystems Inc., Foster City, CA, USA) for disulfide bridge formation.

5.2. Peptide synthesis

Peptides can be obtained by partial hydrolysis of proteins or by peptide synthesis. There are two methods for peptide synthesis:

1. Solution or liquid phase peptide synthesis (SPS)
2. Solid phase peptide synthesis (SPPS)

5.3. Solution phase peptide synthesis

Solution phase synthesis (SPS) is a classical approach for peptide synthesis and it is still useful in large-scale production of bioactive peptides in industry. However, it is time consuming, purification needed after each steps and coupling is less than 90% convention. I will not go into details of SPS since our target is to use SPPS to build our peptides under investigation.

5.4. Solid phase peptide synthesis

Solid phase synthesis SPPS has replaced the SPS due to its high efficiency and purification, head-to-tail cyclization of peptide with minimal risk of intermolecular reactions i.e. dimerization and oligomerization even under high concentration. In SPPS amino acids are assembled on solid support from the C-terminal to the N-terminal. The α-
carboxyl group is attached via an acid-labile linker to a solid support, resin (figure 6). The resins are typically made from polystyrene. The amino terminal end of the amino acid is protected e.g. by a base-labile Fmoc or acid-labile Boc protecting groups while the side chains are protected to block the reactivity of that group (can be easily removed using mild reaction conditions) by acid-labile groups such as tBu. After the first amino acid is conjugated onto the resin, the Fmoc group is removed using piperidine (deprotection). A Kaiser test is then performed to confirm that all of the Fmoc protecting groups are removed. The next Fmoc amino acid is then attached to the growing peptide by activation of its carboxyl group (coupling). A kaiser test is then performed to confirm that complete coupling has occurred on all free amines on the resin. Synthesis then proceed via a cycle of

1. Deprotection of Fmoc amino terminus groups
2. Coupling of the next amino acid until the peptide is completely synthesized.
3. The completely synthesized peptide is cleaved from the resin and side chain protecting groups are removed using TFA (cleavage) or other cleavage cocktail depending on the types of protecting groups present.

Figure 6. General scheme for SPPS.
5.4.1. General procedure for solid-phase Peptide synthesis

Peptides were synthesized on Fmoc-Cys (Acm)-Wang resin (1 eq.) in a PD-10 column. All couplings (double coupling method was used to ensure total coupling of the added amino acid) were performed in DMF and reagent solutions were added manually. Fmoc amino acids with the following protecting groups were used: acetamidomethyl (Acm) for Cysteins, tert-butyl (tBu) for tyrosine, tert-butyl ester (OtBu) for aspartic acids, tert-butoxycarbonyl (Boc) for tryptophan, trityl (Trt) for asparagine and histidine. Fmoc amino acids (4.9 eq.) were activated with HBTU (5 eq.) and DIPEA (10 eq.). All coupling reactions (after 30 min x 2 stirring at r.t.) were monitored using the Kaiser Test as described earlier for each amino acid. The Fmoc protecting group was removed by 20% piperidine in DMF (2 ml x 3) for 5 minutes intervals and washing with DMF (2 ml) for 5 minutes after each deprotection step to ensure complete removal of piperidine with vigorous stirring. After final Fmoc deprotection, the resin was washed with DMF several times and dried under vacuum. Cleavage of the peptide from the resin and deprotection of acid labile protecting groups was accomplished by treatment with TFA/EDT/TIS/H₂O (95:3:1:1, 2 ml) for 3 hrs. at room temperature (RT). The resin was filtered off and washed with acetic acid, and the filtrate (crude peptide) was precipitated into cold diethyl ether (-20°C, 30 ml) for 20 minutes. The precipitate was centrifuged (8 min, 2800 rpm +4°C) and decants the ether away from the flask carefully. The peptide pellet was washed with 7 ml of diethyl ether and centrifuged as described above and decant the ether away from the flask. The crude peptide was lyophilized from a mixture of water and 50% acetic acid.
6. Cyclization of the disulfide bridged peptide

We have used two strategies to accomplish the cyclization of these B2-W3 and C4 analogue peptides. i.e. iodine cyclization and the iodine/ether precipitation method.

6.1. Iodine cyclization (B2-W3)

The disulfide bond formation (cyclization) was formed via iodine oxidation method which is suitable for peptides with Acm protecting groups. This simultaneously removes the sulfhydryl protecting groups and disulfide bond formation between two cysteines. The lyophilized peptide (15 mg) was dissolved in H₂O (0.75 ml), 100% AcOH (0.75 ml), 1M HCl (0.3 ml) and 0.1M I₂ in 50% AcOH (1.8 ml). The above mixture was stirred vigorously at RT. under a N₂ atmosphere for 2 hrs to avoid oxidation of the Cys residues. The reaction was stopped with addition of 1M Na₂S₂O₃ dropwise until the solution becomes colourless i.e. solution changes from yellow to colourless. The peptide was filtered prior to analytical HPLC purification using a 0.45 µM filter and the formation of disulfide bond characterized with mass spectrometry after product isolation from different fractions using preparative HPLC as described above. The peptide was further repurified using the same column as described above since the purity for biological testing is expected to be > 90%.

6.2. Simultaneous deprotection/oxidation of S-Acm with Iodine and subsequent ether precipitation (C4_JE01-C4_JE03)

The crude peptide (10 mg) was dissolved in glacial acetic acid (10 ml) and to this solution were added 12.5 ml of 20 mM iodine/acetic acid and 5.0 ml of 60 mM HCl. The reaction mixture is purged by bubbling through N₂, the tube is sealed and covered with aluminium foil, and stirring is carried out for 120 min at 25°C. The reaction was stopped by the addition of ice-cooled diethyl ether 50 ml (9 eq.). The resulting mixture was kept in the freezer overnight and then centrifuged (4100 rpm for 10 min, +4°C). The ether solution was carefully decanted and the yellow precipitate was washed with 10 ml of diethyl ether
and further centrifuged as above and the ether decanted from the flask. The crude peptide (yield > 7 mg) was lyophilized from a mixture of water and 50% acetic acid and the resulting yellow or colourless solid was purified using preparative HPLC and purity checked with analytical HPLC. The peptides were characterized by mass spectrometry.
7. Acknowledgments

I extend my sincere appreciation and thanks to the following people for their continuous supervision, support, guidance and assistance during my project work: Ale Närvänen (supervisor), Miikka Pakkala, and Janne Weisell (co-supervisors). Their assistance was so vital for the success of this work.

I will like to thank my HOD Tapani Pakkanen for giving me the opportunity to participate in this research program and for the financial support throughout the program.

Special thanks to my program coordinators Mari Heiskanen (Joensuu campus) and Antti Poso/Jukka Leppanen (Kuopio campus) for their continuous assistance.

My gratitude to my medicinal chemistry mates in the research chemist program.

I like to extend my heartfelt thanks to my beloved family Maureen L. Mbake (wife), daughter and son (Angele Misori Okia and Ryan Tristan Okia) and others for their love and being there when I needed them most.

I extend my deepest gratitude to my dad David M. Ekabe, Chief Elias M. Mbake, Mrs Julie Mbake for their love and support.

Finally I dedicate this work to my late mum Angela Misori Ekabe whom I lost when I needed her most in my life. May her soul rest in perfect peace.
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9. Appendices

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Appendix 6: Mass spectra of C4_JE01.

Appendix 7: Mass spectra of C4_JE02.

Appendix 8: Mass spectra of C4_JE03.

Appendix 2: HPLC chromatogram of C4_JE01.
Appendix 3: HPLC chromatogram of C4_JE02.

Appendix 4: HPLC chromatogram of C4_JE03

Appendix 6: Mass spectra of C4_JE01.
Appendix 7: Mass spectra of C4_JE02.

Appendix 8: Mass spectra of C4_JE03.