Synthesis of Potent Ligands for Targeting Prostate Specific Membrane Antigen Receptor

A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree

of

Master of Science

by

Jyotsana Tyagi

DISCIPLINE OF CHEMISTRY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Synthesis of potent ligands for targeting prostate specific membrane antigen receptor** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF CHEMISTRY, INDIAN INSTITUTE OF TECHNOLOGY INDORE**, is an authentic record of my own work carried out during the time period from July 2014 to June 2016 under the supervision of Dr. Chelvam Venkatesh, Assistant Professor, and Indian Institute of Technology Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree in this or any other institute.

Jyotsana Tyagi

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Dr. Chelvam Venkatesh

Jyotsana Tyagi has successfully given her M.Sc. Oral Examination held on **5th July 2016**.

Signature of Supervisor of MSc thesis
Date:

Convener, DPGC
Date:

Signature of PSPC Member
Date:

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Date:

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DEDICATION

This thesis is dedicated to my family

ABSTRACT

Carcinoma of prostate is one of the most common malignancy among men in the United States of America and it is the second most cause of cancer related death worldwide after lung cancer. The method of diagnosis involvesmeasurement of PSA (prostate specific antigen) level inman's blood.Increased level of PSA in blood doesn't always indicate the presence of prostate cancer as it also increases duringdisorders like BPH (benign prostatic hyperplasia), which is enlargement of prostate gland and prostatitis, which is inflammation in prostate gland. There is a constant ambiguity in determining the presence of disease with the help of PSA level examination. Thus it is important to discover an efficient biomarker for the early diagnosis of prostate cancer. Prostate specific membrane antigen (PSMA) is a useful biomarker fortargeting and imaging purposes. Its levelincreases to several foldsduring prostate cancer and also it catalyzes the hydrolysis of N-acetylaspartyl acetate (NAAG), a neurotransmitter to provide N-acetyl aspartate and glutamate, which is another neurotransmitter. Increased level of glutamate causes neuro disorders and sometimes death. Several ligands have beendesignedby making use of the interactions shown by NAAG with the PSMA protein.

In this work, we have synthesized PSMA selective diagnostic agent **25**through solid phase synthesisso that it can be utilized for targeting and imaging of prostate cancer cells. Computational studyshowsthat the small molecule inhibitor **2**, which is used as a targeting ligand in the conjugate **25**, has better binding affinityto PSMA protein as compared to other known ligands.

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NOMENCLATURE

nM	nanomolar
k-Da	kilodalton
Å	angstrom
mL	milliliter
g	gram
mol%	mole percent

ACRONYMS

ACS	American Cancer Society
PCa	Prostate Cancer
U.S	United States
PSMA	Prostate Specific Membrane Antigen
PSA	Prostate Specific Antigen
GCPII	Glutamate Carboxypeptidase II
NAAG	N-Acetylaspartyl glutamate
NAA	N-Acetyl aspartate
CD	Cytoplasmic Domain
ED	Epithelial Domain
TM	Transmembrane
FLNa	Filamin A
Glu	Glutamic acid
Asp	Aspartic acid
Arg	Arginine
Ser	Serine
Ile	Isoleucine
Tyr	Tyrosine
Gly	Glycine
Lys	Lysine
His	Histidine

Zn	Zinc
DMF	Dimethyl formamide
DCM	Dichloromethane
TIPS	Triisopropylsilyl ether
Fmoc	Fluorenylmethyloxycarbonyl chloride
EDT	1,2-Ethane dithiol
Na_2SO_4	Sodium sulphate
Me_3SnOH	Trimethyl tin hydroxide
1,2-DCE	1, 2-Dichloro ethane
NaOH	Sodium hydroxide
DMSO	Dimethyl sulfoxide
DIPEA	N,N-Diisopropylethylamine
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxidohexafluorophosphate
K_2CO_3	Potassium carbonate
DMAP	4-Dimethylaminopyridine
DCC	*N,N'*-Dicyclohexylcarbodiimide
PTSA	*para*-Toluene sulphonic acid
Cs_2CO_3	Cesium carbonate
SPPS	Solid Phase Peptide Synthesis
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography

H-Ala-2-ClTrt	H-Alanine 2-chlorotrityl

Chapter 1

Introduction

1.1 General introduction

In spite of new developments in treatment strategies, prostate cancer is still the second leading cause of cancer-associated mortality among men, behind lung cancer, in the United States of America [1-4, 9]. The American Cancer Society (ACS) projects more than 27,000 deaths from prostate cancer in 2015 [2]. One man in seven is diagnosed with prostate cancer (PCa) during his lifetime in the U.S.A. Cancer cell surface proteins have been used extensively for diagnosis, staging, and therapeutic monitoring. The well-known diagnosis method is based on the triad of digital rectal examination, blood prostate specific antigen (PSA) measurement, and ultrasound-guided prostate biopsy [5]. Despite the successful adoption of these diagnostic modalities for detecting PCa, these are still limited, leaving most early malignancies undiagnosed and sites of metastasis in advanced disease undetected, for which complementary or alternative diagnostic options are needed [6, 7]. For instance, the introduction of PSA screening decreased mortality by 4% between 1994 and 2006[8], but the use of PSA as a diagnostic serum marker still presents several drawbacks [7]. It is known that the concentration of this protein in the bloodstream increases during the development of cancer but also secreted as a result of benign prostatic hyperplasia, prostatitis, or other traumas to prostate cells [2, 10]. Therefore, this method suffers from low specificity and consequent over diagnosis and overtreatment [8].Therefore, reliable biomarkers are strongly recommended so that patients receive appropriate treatment earlier in the course of their

disease[5].Prostate-specific membrane antigen (PSMA), unlike PSA, is a useful target biomarker for imaging and radiotherapy of prostate cancer. PSMA, which is also known as glutamate carboxypeptidase II (GCPII), or N-acetyl-aspartyl-glutamate peptidase I, NAAG peptidase, is an integral binuclear zinc peptidase membrane protein predominantly localized in the epithelial cells of the prostate gland[5,11,12].The extracellular portion of GCPII folds into three distinct domains: the protease domain (domain I, residues 57–116 and 352–590), the apical domain (domain II, residues 117–351), and the C-terminal domain (residues 591–750) (Figure 1). Amino-acid residues from all three domains are involved in substrate recognition. Although the asymmetric unit of the crystal contains a monomer of GCPII, a homodimer is formed through crystallographic two-fold symmetry [5]. It shows carboxypeptidase activity by hydrolyzing NAAG into N-acetyl-L-aspartate (NAA) and L-glutamate and also acts as folate hydrolase in the membrane brush border of small intestine [11].

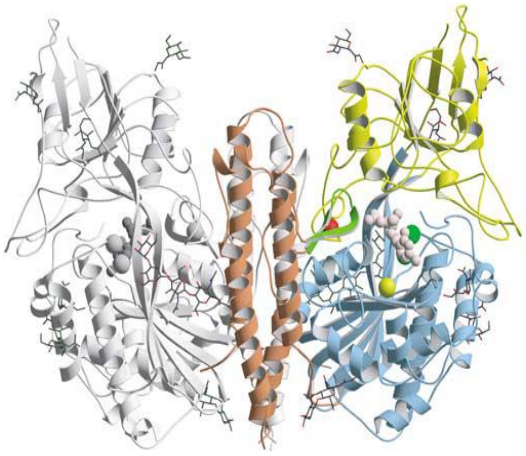


Figure 1. Structure of glutamate carboxypeptidase II

Figure 1 shows one subunit is shown in grey, while the other is colored according to association into these domains. There are three

domains which have been distinguished by showing in three different colors as Domain I, is light blue; Domain II, yellow; Domain III, brown. The dark green spheres shown here are the two Zn^{2+} ions, a red sphere represents the Ca^{2+} ion near the monomer –monomer interface, whereas a yellow sphere represents Cl^- ion.

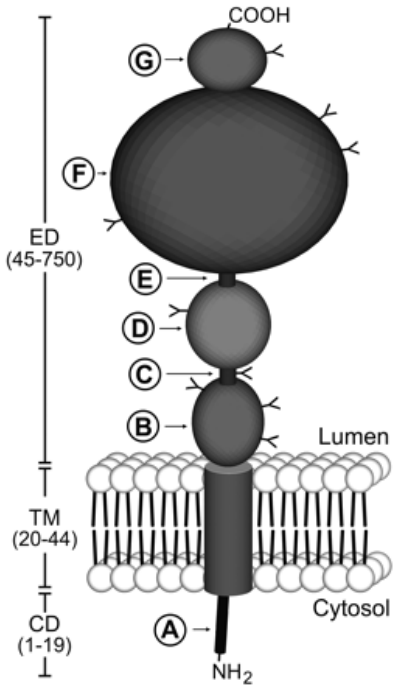


Figure 2. Schematic illustration of prostate specific membrane antigen

Figure 2 shows the representation of PSMA and it is clear from the figure that PSMA is a type II transmembrane protein which is highly glycosylated with nine predicted *N*-glycosylation sites shown with the alphabet Y. There are two domains with unknown function in an endoplasmic domain (ED). There are two domains in ED called B, D (domain 1) and C, E(domain 2) B has span amino acid residues (44–150)and D has (151–274), proline- and glycine-rich regions that span amino acid residues (145–172) and (249–273), respectively are C and E . There are two more domains in ED of unknown function, a catalytic domain which spans amino acid residues (274–587) F, and a final domain

which also of unknown function (amino acids 587–750) to which a helical dimerization domain (amino acids 601–750) is localized G [12].

The low expression of PSMA in normal prostate epithelial cell increases several fold in high-grade prostate cancers, metastatic and androgen-insensitive prostate carcinoma [13].The PSMA concentration increases from 0.25 nM to approximately 3.5 nM in prostate cancer patients' biological fluids, including urine. PSMA, a 750-residue, 90-kDa glycoprotein, is over-expressed on the surface of tumor cells as a non-covalent homodimer in primary (>94.3%) and metastatic (>57.7%) prostate cancers [14]. Elevated PSMA levels also connect with the aggressiveness of tumor growth. Moreover, PSA is a secretory protein thus metastases of prostate cancer cannot be diagnosed by the PSA test [2, 14].On the other hand, metastases can be diagnosed by PSMA imaging as PSMA is a membrane anchored protein.

PSMA is also expressed in the neovasculature of most other solid tumors but not in the vasculature of healthy tissues. These features have made it emerge as one of the most attractive biomarkers in the diagnosis, detection, and treatment of PCa [14, 15]. Due to the above given facts, there are efforts to synthesize a variety of low molecular weight inhibitors that selectively target PSMA.

1.2 Biological function of PSMA

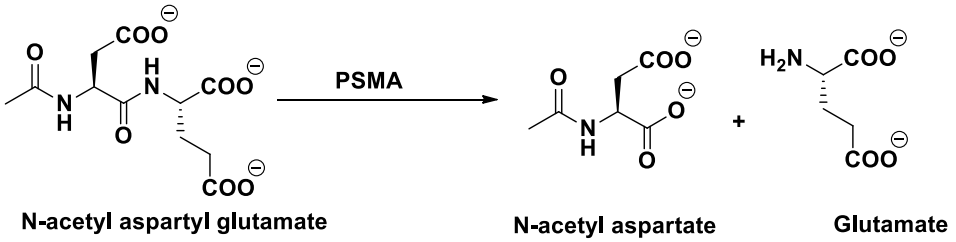


Figure 3. Hydrolysis of N-acetylaspartyl glutamate by PSMA

NAAG (N-acetylaspartyl glutamate) is one of the three predominant neurotransmitter found in central nervous system and when it

is catalytically hydrolyzed, it gives another neurotransmitter called glutamate [11].Glutamate is a common and abundant excitatory neurotransmitter in the central nervous system; however, if there is too much glutamate transmission, this can kill or damage neurons and as a consequence neurological disorders take place. Therefore the balance, which NAAG peptidase contributes to, is quite important. Increased level of PSMA or GCPII has been shown to increase the concentration of glutamate in the extracellular space. GCPII cleaves NAAG into NAA and glutamate. Researchers have been able to show that selective GCPII inhibitors are able to decrease the brain's levels of glutamate and even provide protection from apoptosis or degradation of brain neurons in many animal models of stroke, amyotrophic lateral sclerosis, and neuropathic pain.

1.3Binding Site of PSMA

In the catalytic mechanism discussed here for GCPII, Glu424 acts as catalytic base.

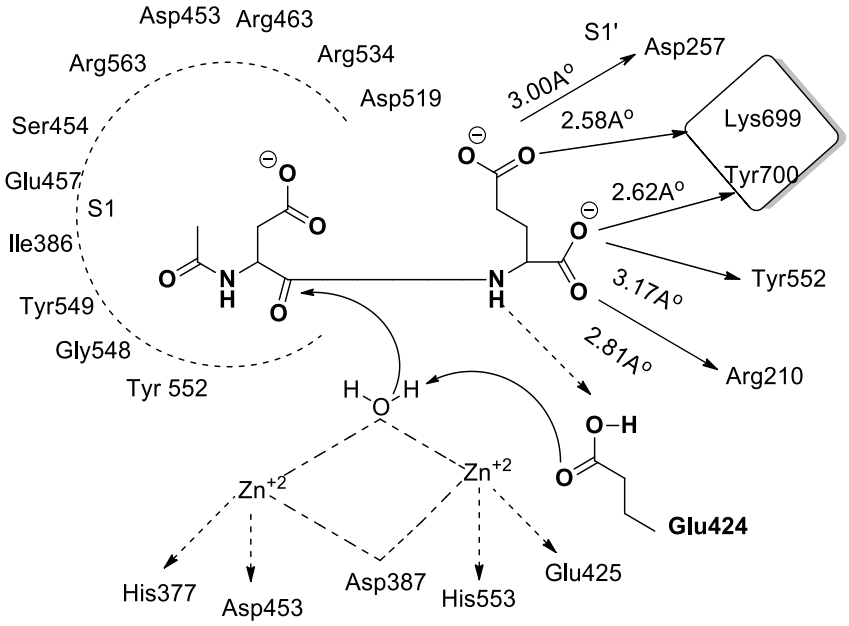


Figure 4.PSMA catalytic site, indicating NAAG substrate as bound to the enzyme

An approximate 20Å deep funnel leads from the surface of GCPII to the active site that contains two zinc ions. The binding of NAAG in the cavity of PSMA is shown in figure 4.

The aspartyl residue of substrate does not interact with S1 pocket (left) whereas glutamate residue interacts with S1' pocket significantly. Lys699 and Tyr700 are called 'glutamate sensor' amino acid residues and are shown inbox.Glu424 acts as catalyst of GCPII for the hydrolysis of NAAG substrate. In the glutamate complex, that is, in the free state of the catalytic center, one of its carboxylate oxygens is hydrogen-bonded (2.56Å) to the water molecule bridging the two zinc ions, whereas the other interact atom interacts with the free amino group of the bound glutamate, the product of the cleavage reaction. The first step of catalytic reaction is the activation of the central nucleophile, HO–H, with the help of a base, Glu424. There is a formation of hydroxyl ion which acts as a nucleophile to attack the peptide bond of the substrate at the carbonyl group of the aspartyl residue of NAAG. Proton gets transported by Glu424 to the amino group of leaving product, glutamate [11].

Chapter 2

Review of Past Work

2.1 Structure of PSMA scaffold

Based on the NAAG interaction in the binding pockets of PSMA three kinds of PSMA scaffold have been reported [9] which can inhibit the hydrolysis of NAAG. These molecules are named as (a) glutamate−urea heterodimers **1**,(b) glutamate containing phosphoramidates **2** and (c) 2-(phosphinylmethyl)pentanedioic acid **3** containing a similar binding pentanedioic acid motif shown in box (Figure 5).

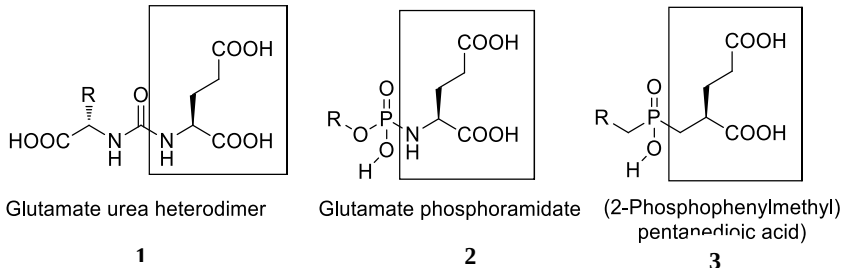


Figure 5. Structures of the PSMA scaffolds: **1** (glutamate−urea heterodimer), **2** (glutamate phosphoramidate), and **3** (2-(phosphinylmethyl)pentanedioic acid)

2.2 Objective of proposal

Keeping in mind the interaction of S1' pocket of PSMA and oxygen atoms of carboxylic acid group of glutamate moiety of NAAG and on the basis of reported ligands which have been shown above, we develop new small molecule ligands, by mimicking NAAG, that have higher affinity for PSMA. Herein, we are presenting our research work in which we have synthesized a new small molecule inhibitor having glutamate moiety as it was in NAAG and replaced the N-acetylaspartyl acetate group with protected tyrosine group which is expected to enhance

affinity towards PSMA to several folds as predicted by computational studies.

Chapter 3

Experimental Section

3.1 General information and methods

H-Ala-2-ClTrt resin, amino acids and coupling agents, reagents and solvents used in solid phase peptide synthesis (SPPS) as well as in chemical synthesis were purchased from Iris Biotech GmbH, Sigma Aldrich, Merck and Spectrochem. Dry solvents were prepared by using drying agents and following usual methods. All peptide synthesis was performed manually by using peptide vessels (Chemglass) and standard peptide coupling procedures. ^{1}H and ^{13}C NMR data were recorded by using Bruker AV 400MHz NMR spectrometer with TMS as internal reference. $CDCl_3$ was used for the NMR solvent. All mass data were recorded on Bruker micro TOF-Q II by positive mode and negative mode electrospray ionization method. Reactions were monitored by TLC and all compounds were purified by column chromatography using 100-200 or 320-400 mesh silica-gel. Distilled hexane and distilled ethyl acetate were used as eluents in column chromatography.

3.1.1 Resin swelling

All resins used in solid phase peptide synthesis were swelled initially with 5 mL DCM for 30 minutes by bubbling nitrogen and after draining DCM, the resin is swelled once again with 5 mL DMF thrice for 15 minutes each.

3.1.2 Kaiser test

Few resin beads were taken in a test-tube and 2 drops each of ninhydrin, phenol and 0.1% potassium cyanide solution were added to the test-tube and heated for 2 minutes at 110 °C through sea sand kept in a beaker. The presence of free amine group is confirmed by the appearance

of blue colored resin bead in the test tube. The test is performed after coupling each amino acid by the aforementioned procedure.

3.1.3 Fmoc deprotection

The Fmoc protected amino group in the growing peptide chain is deprotected using 20% piperidine in DMF (7 mL) by bubbling nitrogen for 10 minutes through the swelled resin beads in the peptide synthesizer vessel. The procedure is repeated thrice to ensure complete deprotection of Fmoc protecting group from the amino acid of growing chain.

3.1.4 Resin cleavage

A mixture of 9.5 mL TFA, 0.25 mL TIPS, and 0.25 mL $H_2O$was prepared and 5 mL of this cocktail solution was added to resin beads and nitrogen was bubbled through the solution for 30 minutes. The cleaved peptide solution was collected from the peptide vessel in a round bottom flask. The residual peptide in the resin was mixed with cocktail solution twice and the same procedure was repeated as above. The remaining cleaved peptide solution collected together in a round bottom flask was evaporated using 15 mL centrifuge tube by rotary evaporator.

3.2 Synthesis of*(S)-Di-tert-butyl-2-(3-((S)-1-tert-butoxy-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)ureido)pentanedioate*(11)

Triphosgene **7** (0.1 g, 0.33 mmol) was dissolved in 5 mL dry DCM and the solution was stirred at −50 °C under inert atmosphere. Then bis(*tert*-butyl)-L-glutamate.HCl **8** (0.293 g, 1 mmol) was dissolved in 2mL dry DCM and the solution was added to the triphosgene solution and after that triethylamine (0.459 mL,3.3 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred for 1.5 h at –50 °C and stirred for another 1.5 h at room temperature for the generation of isocyanate intermediate **9**. Thereafter, a solution of L-tyrosine *tert*-butyl ester **10** (0.237 g, 1 mmol) and triethylamine (0.1 mL, 0.66 mmol) was

added to the reaction mixture. The progress of the reaction was monitored through TLC using ethyl acetate and hexane (1:3) mixture. The reaction mixture was stirred overnight at room temperature. After completion of the reaction, the reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate and washed with water. The organic layer dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude reaction mixture was purified by column chromatography using 100-200 mesh silica gel using ethyl acetate and hexane. The purified compound was obtained as colorless viscous liquid which gradually solidified to white powder. Yield:90% (0.469 g).^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.99 (d, 2H, *J* = 8.0 Hz), 6.71 (d, 2H, *J* = 8.0 Hz), 5.20 (d, 1H, -NH), 5.08 (d, 1H, -NH), 4.56 (dd, 1H, CH), 4.33 (dd, 1H, CH), 2.96 (d, 2H), 2.30(m, 2H), 2.05 (s, 1H) 1.78 (m, 2H), 1.45 (s, 9H),1.42 (s, 18H);^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.58, 172.49, 171.72, 156.91, 155.37, 130.62, 127.54, 115.51, 82.28, 82.09, 80.72 54.66, 52.95, 37.70, 31.75, 31.63, 28.28, 28.01; $C_{27}H_{42}N_2O_8$(522)ESI–MS (positive mode):m/z 545.4$[M+ Na]^+$.

3.3 Synthesis of 3-(4-(*(S)-3-(tert-butoxy)-2-(3-((S)-1, 5-di-tert-butoxy-1, 5-dioxopentane-2-yl) ureido)-3-oxopropyl) phenoxy) propionic acid* (12)

A mixture of **11** (0.050 g, 0.095 mmol), 1N NaOH and DMSO was taken in a culture tube. The reaction mixture was stirred for 10 minutes. To this mixture, the solution of bromopropionic acid in DMSO was added and the resulting mixture was stirred at room temperature for 1h and monitored by TLC. At the end of the reaction, the starting material was found unreacted in the mixture. Reaction failed.

3.4 Synthesis of esters of bromopropionic acid

Method 1

3.4.1 Methyl ester formation of bromopropionic acid

Bromopropionic acid (1 g, 6.53 mmol) was dissolved in 5 mL dry DMF and K_2CO_3 (2.7 g, 19.61 mmol) was added to the solution. The reaction mixture was stirred for 1h at room temperature. After an hour, methyl iodide (0.44mL, 7.18mmol) was added to the reaction mixture and stirred overnight at room temperature. Reaction mixture was quenched with ethyl acetate and washed with brine solution. The organic layer was dried over anhydrous Na_2SO_4 and filtered. It was then concentrated under reduced pressure by rotary evaporator, purified by column chromatography using 100-200 mesh silica gel and ethyl acetate and hexane mixture as eluent. The product isolated was not the desired bromo -methylpropionate.

Method 2

3.4.2 Benzyl ester formation of bromopropionic acid

Reaction of bromopropionic acid with benzyl alcohol was carried out with different reagents and at different conditions for the formation of corresponding ester. However none of the reaction conditions yielded the required product.

Table1. Reagents and conditions for preparation of bromo benzylpropionate

Reagents	Amount required	Reaction conditions
1. DIPEA	0.68 mL, 3.98 mmol	90 minutes
2. HATU	0.596 g, 1.568 mmol	Room temperature
3. DMF	4 mL	

1. DCC 2. DMAP 3. DMF	0.80 g, 3.92 mmol 10 mol% 5 mL	Overnight Room temperature
1. PTSA 2. Toluene	0.124 g, 0.653 mmol 5mL	Overnight 140 °C

3.5 Synthesis of 13

Protection of phenolic –OH of 11 with benzyl 3-bromo propionate:

Compound **11** (0.107 g, 0.206 mmol) and Cs_2CO_3 were suspended in 5 mL dry DMF and stirred for 20 minutes. Thereafter benzyl 3-bromo propionate (0.050 g, 0.206 mmol) was added to the reaction mixture and stirred overnight at room temperature. The reaction was monitored by TLC only to recover the starting material**11**.

3.6 Synthesis of*(S)-Di-tert-butyl-2-(3-((S)-1-tert-butoxy-3-(4-(2-methoxy-2-oxoethoxy)phenyl)-1-oxopropan-2-yl)ureido)pentanedioate* (15)

Compound **11** (0.317 g, 0.6073 mmol) and Cs_2CO_3 (0.593 g, 1.821 mmol) were suspended in freshly distilled dry DMF (5 mL) and the resulting mixture was stirred for 30 minutes at room temperature. Methyl bromo acetate (0.223 mL, 2.43 mmol) was added to the reaction mixture and the reaction was monitored by TLC using ethyl acetate and hexane (1:3) as eluent. The reaction was continued until all the starting material was consumed and quenched immediately to avoid side product formation by adding ethyl acetate and brine solution. The organic layer was extracted and dried over anhydrous Na_2SO_4. It was then filtered, concentrated under reduced pressure using rotary evaporator. The crude reaction mixture was purified through column chromatography using 100-200 mesh silica gel

and ethyl acetate-hexane mixture as eluent. The pure compound was obtained as colorless viscous liquid which solidified on standing. Yield: 65% (0.360 g). ^{1}H NMR ($CDCl_3$, 400 MHz) δ:7.10 (d, 2H, *J* = 8.0 Hz), 6.82 (d, 2H, *J* = 8.0 Hz), 4.95 (d, 1H, -NH), 4.86 (d, 1H, -NH), 4.60 (s, 2H, CH_2), 4.57 (dd, 1H, CH), 4.33 (dd,1H,CH),3.6(s,3H), 3.02 (m, 2H), 2.32-2.27 (m,4H), 2.07(m, 1H), 1.46 (s, 9H); 1.42 (s,9H), 1.39 (s,9H);^{13}C NMR ($CDCl_3$, 100 MHz) δ:171.39, 171.02, 170.33, 168.43, 155.71, 155.40, 129.76, 128.74, 113.48, 81.03, 81.00, 79.50, 64.38, 53.49, 52.00,51.21, 36.80, 30.55, 28.67, 27.30, 27.04, 26.95;$C_{30}H_{46}N_2O_{10}$(594),ESI–MS (positive mode):m/z 617$[M + Na]^+$.

3.7 Synthesis of *2-(4-((S)-3-(tert-butoxy)-2-(3-((S)-1, 5-di-tert-butoxy-1, 5-dioxopentan-2-yl)ureido)-3oxopropyl)phenyl) acetic acid*(16)

A mixture of **15** (0.235 g, 0.395 mmol), Me_3SnOH (0.215 g, 1.19 mmol) and 5 mL dry 1, 2-dichlorethane was placed in a preheated oil bath, the temperature of which was maintained at 80 °C. The reaction mixture was monitored by TLC and quenched after 2h. After 2h, the reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate and washed with 0.1N $KHSO_4$ solution. The organic layer was extracted, dried over anhydrous Na_2SO_4, filtered and then concentrated under reduced pressure. The crude residue was purified through column chromatography using 100-200 mesh silica gel and mixture of ethyl acetate and hexane as eluent. The pure compound was obtained as colorless viscous liquid. Yield: 75.5%; (0.170 g); ^{1}H NMR ($CDCl_3$, 400 MHz) δ:7.08 (d, 2H, *J* = 8.0 Hz), 6.8 (d, 2H, *J* = 8.0 Hz), 5.4 (d, 1H, -NH), 5.3 (d, 1H, -NH), 5.2 (s, 2H, CH_2), 4.57 (dd, 1H, CH), 4.36 (dd,1H,CH), 3.00 (d, 2H), 2.34–2.20 (m,4H), 1.85 (m, 1H), 1.46 (s, 27H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ:177.40, 171.58, 170.68, 156.14, 155.62, 138.23,

129.78, 127.09, 113.04, 81.29, 81.16, 79.68, 53.56, 52.00, 48.68, 36.58, 30.90, 28.67, 28.34, 26.97;$C_{29}H_{44}N_2O_{10}$(580),ESI–MS (positive mode):m/z 603$[M + Na]^+$

3.8 Solid phase synthesis procedure

H-Ala-2-ClTrt resin (0.175 g) was swelled in DCM and DMF as mentioned in 3.1.1 of experimental section. Then *N*-Fmoc-Lys-(Tfa)-OH (0.0833 g, 0.179 mmol), PyBOP (0.0933 g, 0.179 mmol) and DIPEA (0.125 mL, 0.718 mmol) in 0.5 mL DMF was added to peptide vessel and the coupling reaction was continued overnight. The resin was washed with DMF (5 mL x 3) followed by the washing with isopropanol (3mL x 3). Completion of reaction was confirmed by performing Kaiser test according to section 3.1.2. Then a solution of 20% piperidine in DMF (3 mL) was added to the peptide vessel to cleave the NHFmoc protecting group according to the section 3.1.3. Resins were washed with DMF (3 mL x 3) and isopropanol (3 mL x 3). The formation of free amine was confirmed by the Kaiser test. After swelling the resin in DMF, next amino acid Fmoc-8-aminocaprylic acid (0.0684 g, 0.179 mmol), PyBOP (0.0933 g, 0. 0.179 mmol) and DIPEA (0.125 mL, 0.718 mmol) in 0.5 mL DMF was added twice to the resin and same steps were followed as described above. A series of amino acids including of Fmoc-(*L*)-Phe-OH (0.0695 g, 0.179mmol), 8-aminocaprylic acid (0.0684 g, 0.179 mmol), compound **16** (0.0624 g, 0.108 mmol)was added following the same procedure as above. Trifuoroacetyl protecting group in lysine amino acid residue of the peptide chain is cleaved by using 2M aqueous piperidine overnight. The formation of free amine was confirmed by the Kaiser test. After swelling the resin in 3mL DMF, a solution of rhodamine B (0.0516 g, 0.108 mmol), PyBOP (0.0933 g, 0.179 mmol) and (0.125 mL, 0.718 mmol) DIPEA in 3 mL DMF was added to the resin and the reaction was continued overnight.

The resin was washed with DMF (5 mL x 3) followed by isopropanol (3 mL x 3) and the completion of reaction was confirmed by the Kaiser Test. The resins were dried by passing nitrogen gas through peptide vessel for 30 minutes and cleaved using the cocktail solution mentioned in section 3.1.4. The crude mixture was concentrated under reduced pressure using rotary evaporator and ice cold ether was added to precipitate the required PSMA targeting agent **25** as pink colored solid.

Chapter 4

Results and Discussion

In search of new inhibitors for PSMA protein, we begin our journey by carrying out molecular docking studies of newly designed inhibitor **2**, in comparison to known ligands, the structure of which is shown in Chart 1.

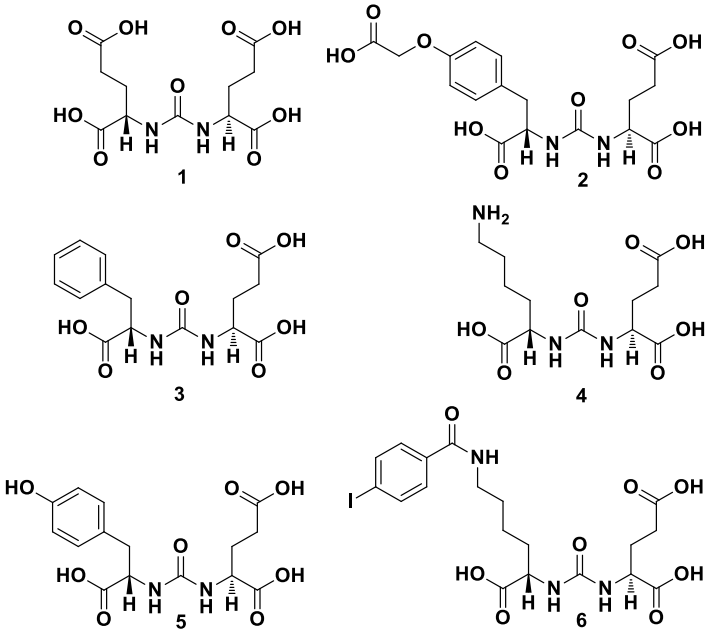


Chart 1. Structure of PSMA Inhibitors

The inhibitor **2** consists of three carboxylic acid units and a hydrophobic phenyl ring connected to acetic acid side chain that would not only enhance H-bonding interaction with PSMA but also acts as a handle for conjugation of drugs, biologics or diagnostic agents through peptidic spacer. The inhibitor **2** affinity will be further enhanced as soon a peptidic spacer with two phenyl alanine residues are introduced that can interact with the hydrophobic pocket present in the 20 Å tunnel of the PSMA protein.

Computational docking studies of DUPA**1**, new PSMA inhibitor **2**, Phe-CONH-Glu **3**, Lys-CONH-Glu **4**, Tyr-CONH-Glu **5** and 3D7H-inhibitor **6** in the active site of PSMA protein were performed to find out the number of favorable interactions. The dock score values and different kinds of interaction shown by the inhibitors in the active site of PSMA were determined (Tables 2 and3).

Table 2. Dock Scores and binding energy values of Urea based inhibitors in GCPII docking study

	(-)CDOCKER ENERGY	BINDING ENERGY
DUPA **1**	116.742	-765.062
Inhibitor **2**	113.868	-724.682
Phe-CONH-Glu **3**	103.988	-561.688
Lys-CONH-Glu **4**	103.634	-427.293
Tyr-CONH-Glu **5**	89.371	-571.817
3D7H-inhibitor **6**	102.834	-620.232

It was observed that all these ligands (Chart 1) when docked to the active site of PSMA protein exhibit common aligned conformations (Figure 6). However, the dock score value of PSMA inhibitor **2** was found to be the highest amongst other ligands studied. The docking interaction poses of the above mentioned ligands are depicted in figure 6. From the dock score and binding energy value (Table 2) it is found that DUPA**1** and inhibitor **2** have higher docking score values whereas ligands **3** to **6** exhibit moderate score.

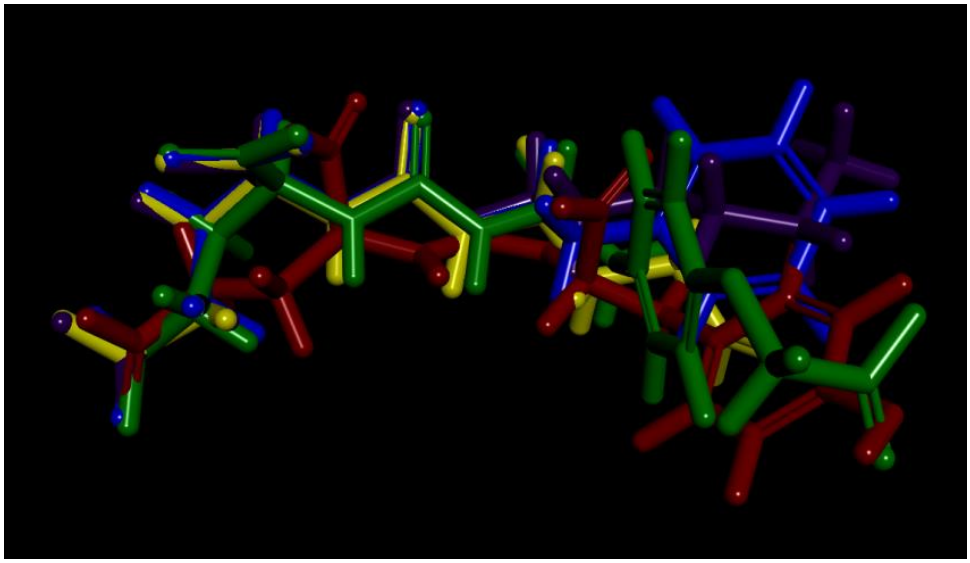


Figure 6. Superimposed pose of all urea based ligands

A study of various interactions shown by these ligands was also carried out (Table 3). Interactions of ligands with the active site of PSMA are denoted by different alphabets. E represents electrostatic, V stands for vander waals interactions and M for metallic interactions. Y denotes interaction of water molecule at the active site of PSMA. Inhibitor **2** shows 19 electrostatic interactions when compared to DUPA **1** and Lys-CONH-Glu **4** which shows only 16 interactions. Also presence of urea moiety enables interaction with zinc atoms at the PSMA binding site.

Table 3. Amino acid interactions of urea based inhibitors in comparison with co-crystallized ligand **6**

Amino Acids	DUPA **1**	Inhibitor **2**	Phe-CONH-Glu **3**	Lys-CONH-Glu **4**	Tyr-CONH-Glu **5**	3D7H-inhibitor **6**
Phe546		E			E	
Arg534	E	E	V	E	E	E
Asp453	E	E	E	E	E	E

Gly548		E	V	V	V	
Ser547		E				
His553	E	E	E	E	E	V
Asp387	E	E	E	E	E	
Tyr552	E	E	E	E	E	E
Glu424	E	E	E	E	E	E
Leu428	V	E	E	V	V	
Gly427	E	E	E	E	V	
Glu425	E	E	E	E	E	E
Lys699	E	E	E	E	E	E
Asn257	E	E	E	E	E	E
Tyr700	E	E	E	E	E	E
Arg210	E	E	E	E	E	E
Gly518	E	E	E	E	E	E
Arg536	E	E	E	E	E	E
Asn519	E	E	E	E	E	E
Lys207		V				
Arg463	E	V	V	E	V	
Phe209	V	V	V	V	V	
HOH1037		Y				Y
HOH1025	Y	Y	Y	Y	Y	Y
HOH926	Y	Y	Y	Y	Y	Y
HOH928	Y	Y	Y	Y	Y	Y
HOH901	Y	Y	Y	Y	Y	Y
HOH942	Y	Y	Y	Y	Y	Y
Zn817	**M**	**M**			**M**	**M**
Zn818	**M**	**M**	**M**	**M**	**M**	**M**

Glu457	E		V	E	V	
Ser454	E			E		
Tyr549			V	E		
Trp381			V			

E- Electrostatic interaction, V - Van der Waals Interaction, M- Metallic interactions, Y- Interaction with water molecules

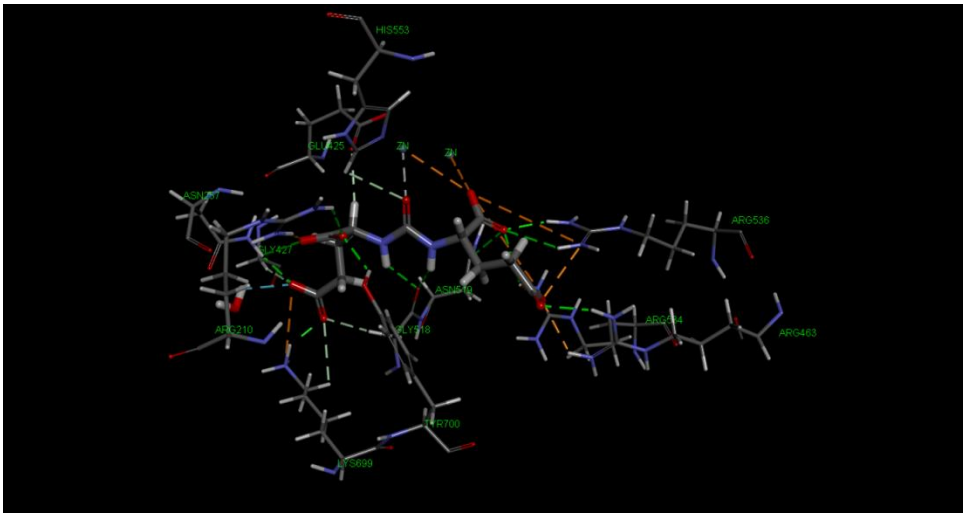


Figure 7. DUPA **1**, docking interaction pose of urea based derivative

Also, with the help of interactions listed in table 3, it was found that all the reported amino acid interactions of co-crystallized ligand**6** were also shown by PSMA targeting ligand **2**. Further some additional interactions with amino acids such as PHE546, GLY548, SER547 and LYS207were also shown by PSMA targeting ligand **2**. The introduction of two carbon unit in **5** to give **2** makes the molecule to show better binding affinity than**5.**The docking pose interactions of various ligands like DUPA **1**, inhibitor **2,** Phe-CONH-Glu**3,** Lys-CONH-Glu **4** and Tyr-CONH-Glu **5** were depicted in figures 7-11. From these studies it can be predicted that biological activity of PSMA targeting molecule **2** would be better or at

least similar to DUPA **1** and Lys-CONH-Glu **4** ligands which are known as potent PSMA protein inhibitors.

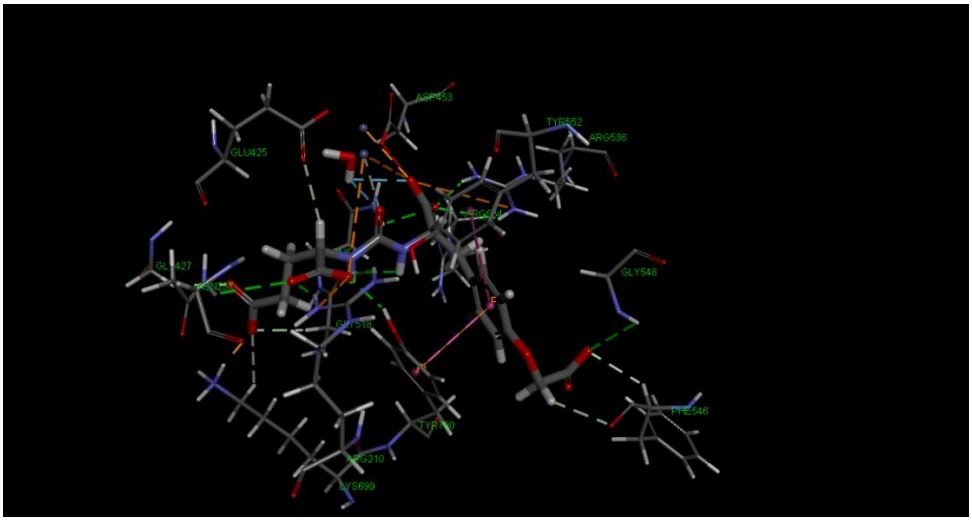


Figure 8. Inhibitor **2**, docking interaction pose of urea based derivative

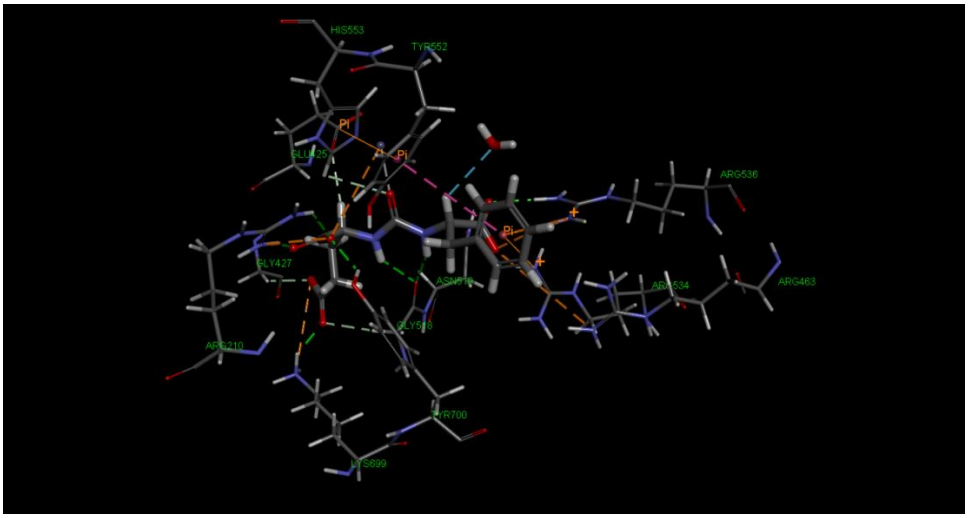


Figure 9. Phe-CONH-Glu **3**, docking interaction pose of urea based derivative

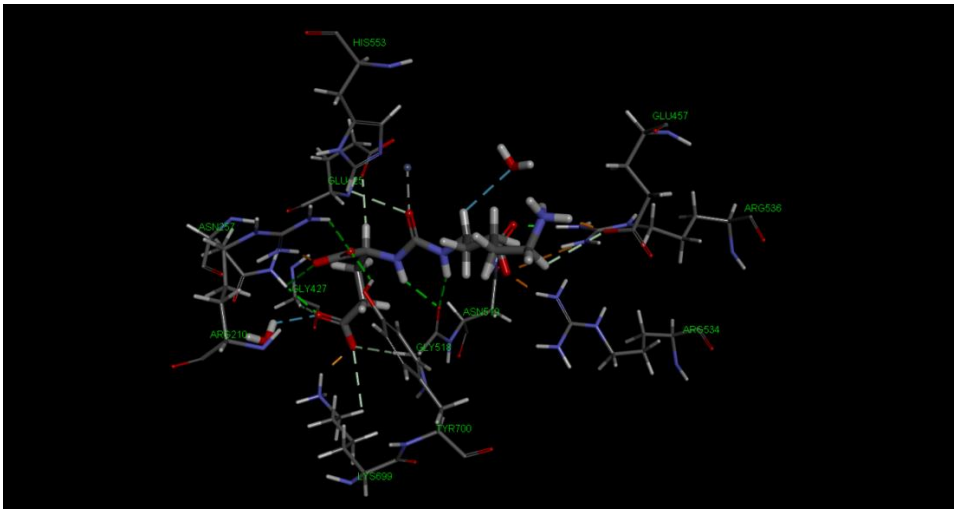


Figure10. Lys-CONH-Glu**4**,docking interaction pose of urea based derivative

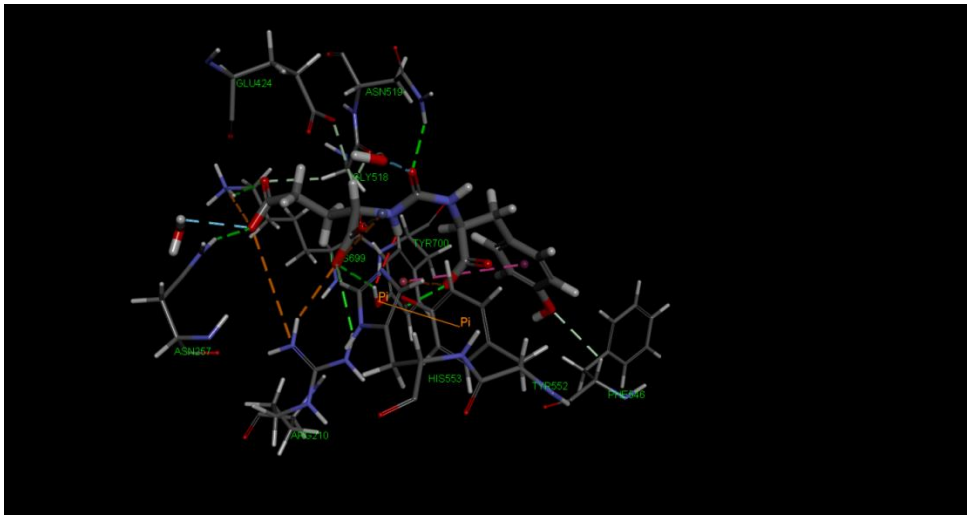
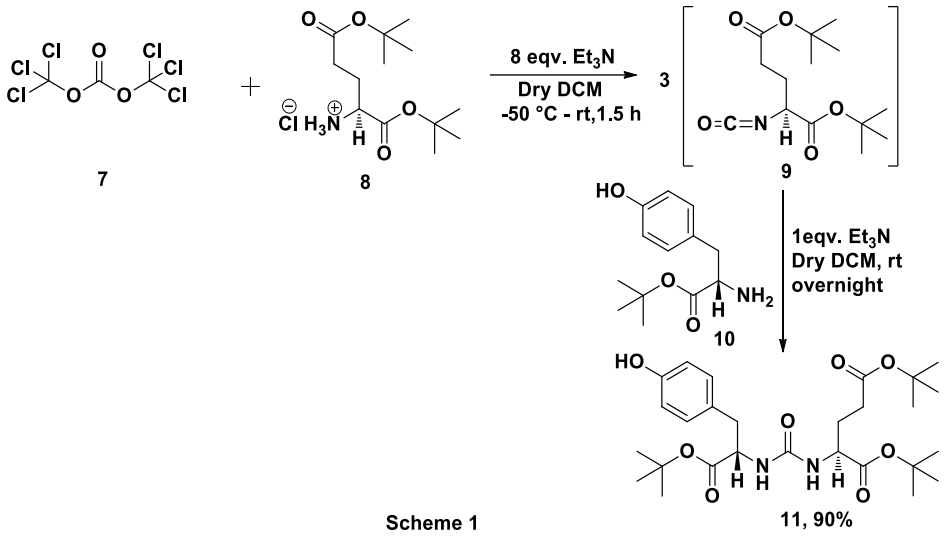


Figure11. Tyr-CONH-Glu **5**, docking interaction pose of urea based derivative

After the completion of computational studies, we next proceeded for the synthesis of PSMA conjugate **25**.The prostate specific membrane antigen (PSMA) targeting fluorescent conjugate **25** required for PCa analysis is synthesized as enumerated in Schemes 1−7. The first step of the synthesis of **16** begins with the solution phase synthesis of PSMA

targeting precursor **11** as shown in Scheme 1. Brieflytriphosgene**7** was reacted with bis(*tert*-butyl)-L-glutamate **8** at low temperature in presence of triethylamine as base to form isocyanate intermediate**9** which is not stable for isolation. Therefore intermediate **9** was utilized in one pot by reacting with the unprotected phenolic amino acid **10** to give the corresponding urea product **11**in high yield of 90%. Free phenolic hydroxyl present in L-tyrosine *tert*-butyl ester **10** doesn't compete with reaction of isocyanate **9** to give carbamate. However, the amino group present in **10** reacts exclusively with isocyanate **9** to give t-butyl protected urea precursor **11** in excellent yield**.**



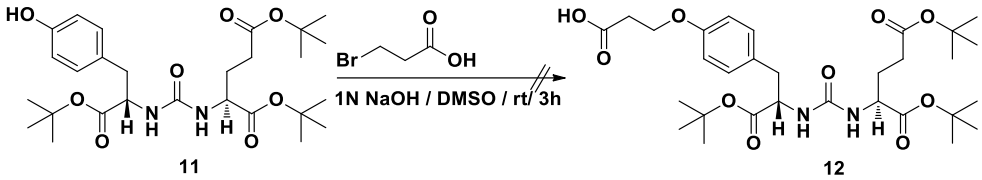
7 8 9 10 11, 90%

Scheme 1

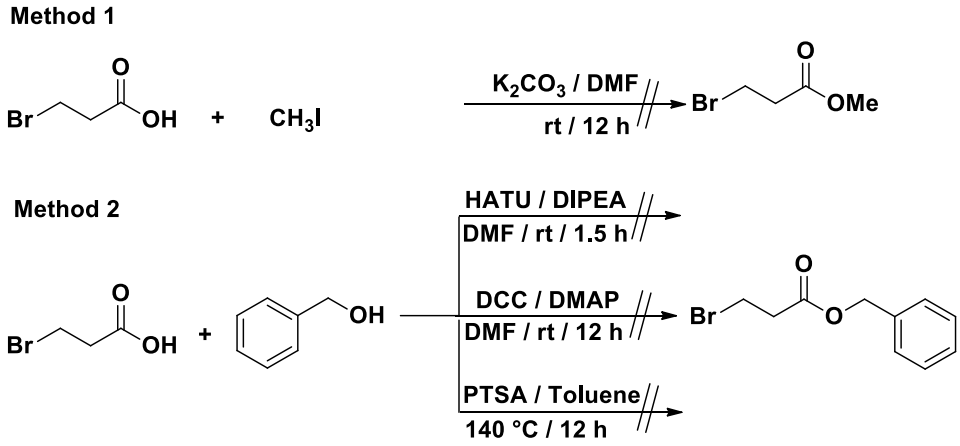
To incorporate urea precursor **11** in the later part of solid phase synthesis, it is necessary to introduce carboxylic acid functionality through free phenolic hydroxy group present in **11** that can undergo amide coupling with the free amino group of the growing peptide chain. However our attempt to introduce the side chain in **11** as described in Scheme 2 to give **12** was not successful.

Hence, few test reactions were carried out to protect carboxylic acid of bromopropionic acid as either methyl or benzyl ester as shown in Scheme 3. Protection of free carboxylic acid group to methyl ester using

methyl iodide and potassium carbonate did not yield the expected methyl ester of bromo propionic acid. Also reactions using coupling agents of alcohol and acid such as HATU, DCC did not result in the formation of corresponding benzylester of bromo propionic acid that can be later used to protect phenolic hydroxy group of **11**. Even mild acid catalyzed (PTSA) ester formation reaction from benzyl alcohol and bromopropionic acid failed to produce expected 3-bromo benzylpropionate.

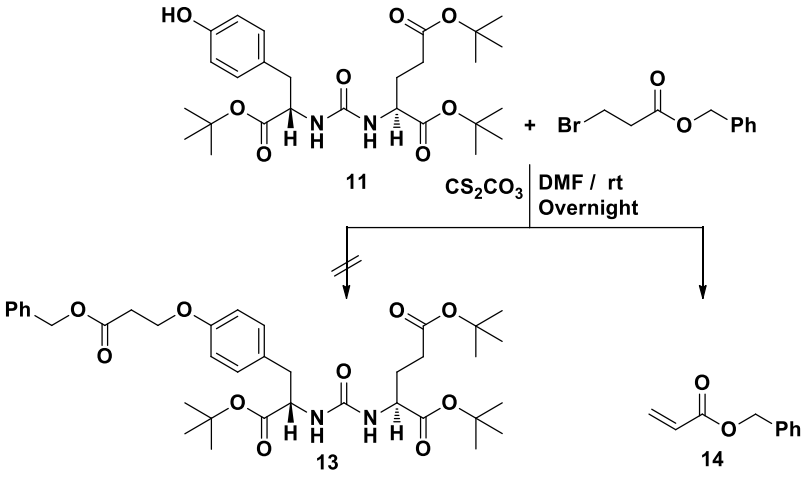


Scheme 2



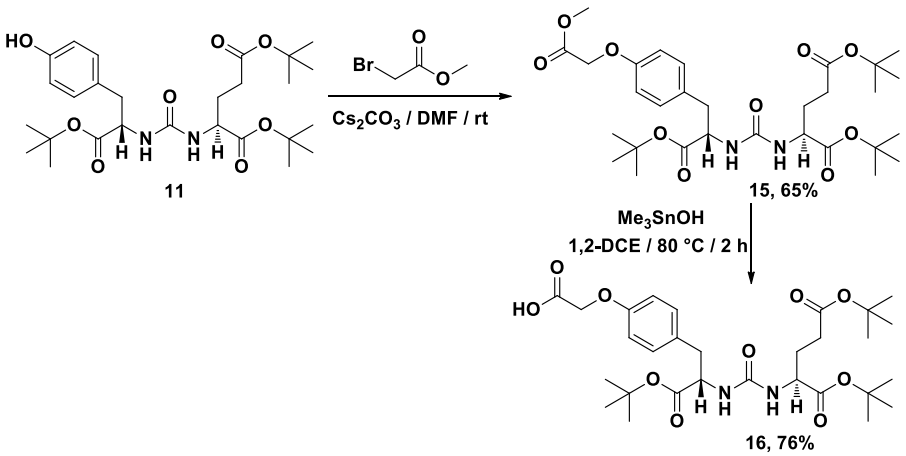
Scheme 3

Finally the precursor **11** was reacted with commercially available benzyl 3-bromo propionate (Scheme 4) in presence of Cs_2CO_3 as base and DMF as solvent.



Scheme 4

The expected product **13** was not obtained during this reaction and instead benzyl 3-bromo propionate, used as protecting agent, underwent 1, 2-elimination of HBr to yield **14** as confirmed by ^{1}H NMR. To avoid elimination reaction, a new strategy was planned in which an ester with one carbon unit less, i.e., methyl bromoacetate, would be introducedas side chain so that there will be no chance of 1, 2-elimination reaction during base catalyzed protection of phenolic hydroxy group in **11**(Scheme 5).

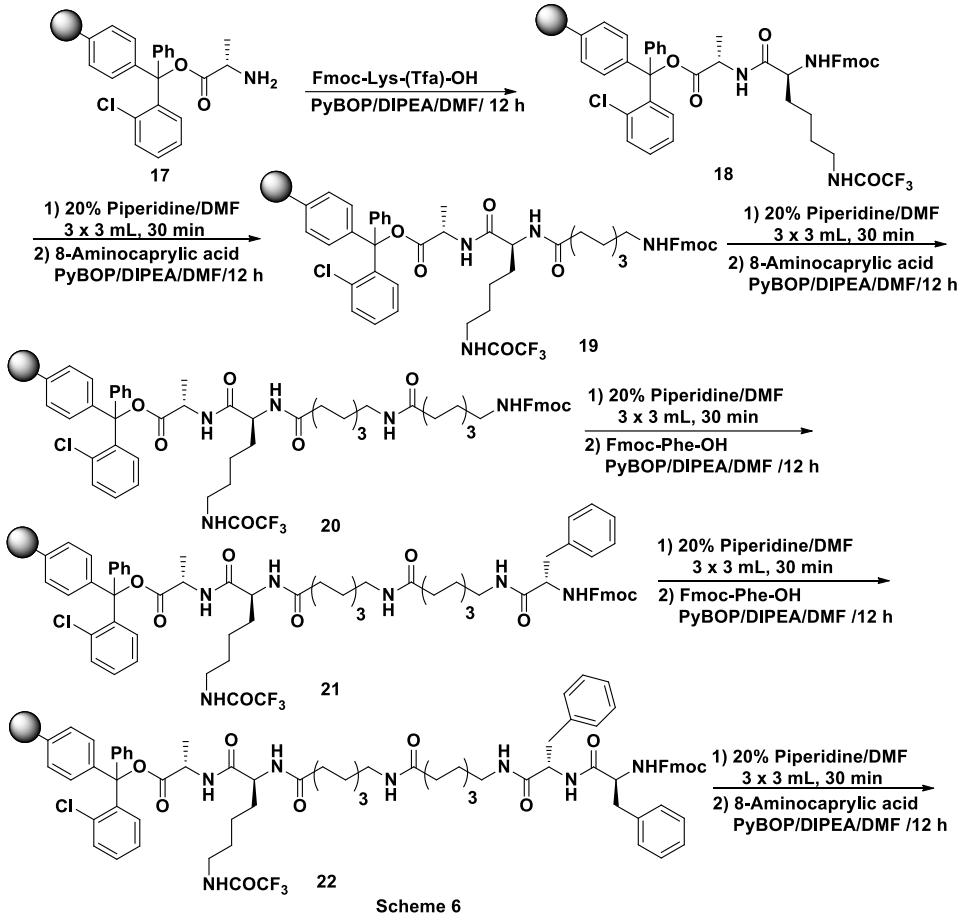


Scheme 5

As predicted, **11** reacted with α-bromomethyl acetate in presence of Cs_2CO_3 in DMF at room temperature to yield the product **15** in moderate yield (Scheme 5). The product**15** was characterized by ^{1}H, $^{13}CNMR$ and ESI-MS techniques. The final step for regenerating carboxylic acid group from **15**, required for solid state phase synthesis, is carried out by hydrolysis of methylester **15** using trimethyltin hydroxide as saponificating agent. The methyl ester of **15** was successfully hydrolyzed in good yield (76%) to provide the required PSMA ligand precursor **16** with free carboxylic acid side chain. With the ligand precursor **16** in hand, we next proceeded for the synthesis of final PSMA targeting fluorescent conjugate **25** that can utilized for diagnosis of PCa cells.

An elaborate solid state phase synthesis for the construction of required PSMA fluorescent conjugate **25** is described in Schemes 6 and 7. We begin our solid state phase synthesis of **25** from commercially available 2-chlorotrityl resin **17** pre-attached to alanine amino acid via carboxylic acid functional group (Scheme 6). The free amino group present in **17** is coupled with Fmoc-Lys(Tfa)-OH using PyBOP as amide coupling agent to provide dipeptide chain **18** with trifluoroacetyl protected ε-amino group of lysine. It is important to mention that the α-amino group of lysine is protected as NHFmoc which is labile to 20% piperidine in DMF and readily available for constructing next amide bond in the growing dipeptide chain. Whereas the ε-amino group of lysine protected as trifluoroacetyl group is stable under NHFmoc cleavage condition and it readily undergoes deprotection in 2M aqueous piperidine. The selection of α- and ε-amine protecting groups in lysine amino acid, which are labile under different basic conditions, is crucial to strategize the synthesis of attaching fluorescent agent to ε-amino group of lysine in the final step of the preparation of conjugate **25**. The NHFmoc amino group in the growing dipeptide chain **18** is deprotected using a solution of 20% piperidine in

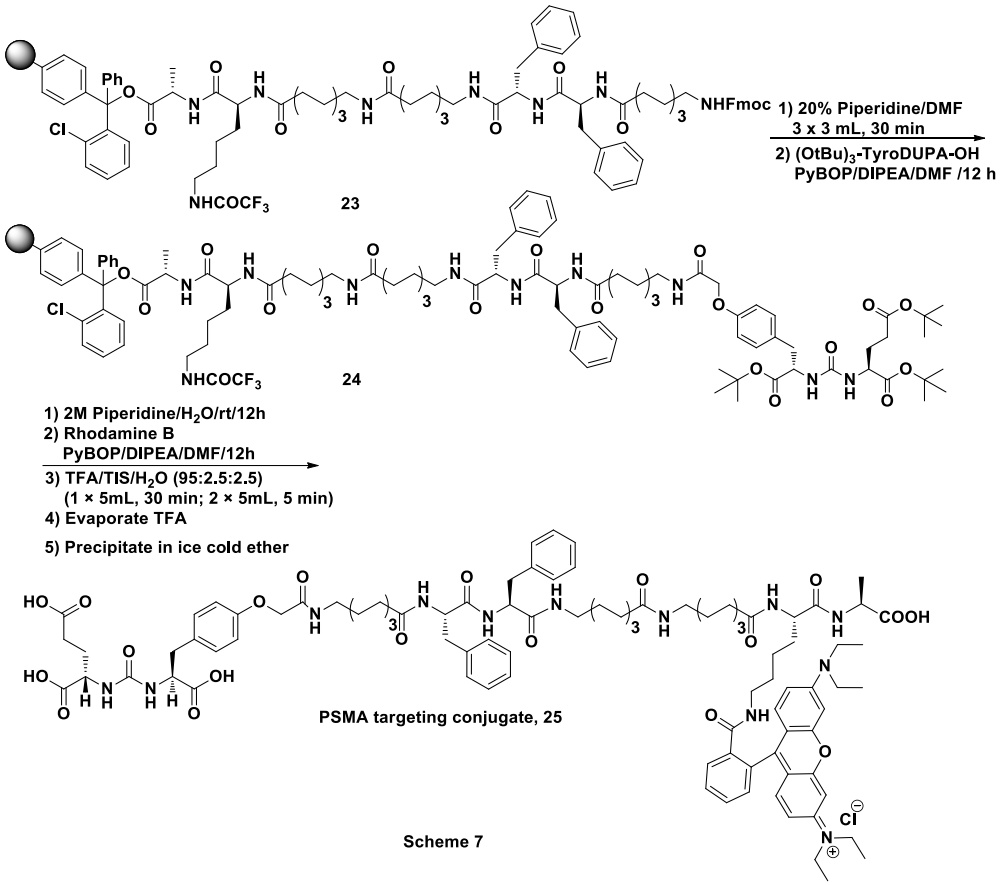
DMF. The Fmoc free amino group in **18** is now coupled with Fmoc-8-aminocaprylic acid using standard amide coupling reagents to provide growing tripeptide chain **19**.



Scheme 6

Fmoc-8-aminocaprylic acid is once again added to the tripeptide chain **19** to provide NHFmoc protected tetrapeptide chain **20**. Addition of two molecules of 8-aminocaprylic acid to the peptide chain provides a 16-carbon atoms alkyl thread and ensures that the cargo of fluorescent agent is safely distanced away from the hydrophobic pocket along 20Å deep tunnel present in the PSMA protein. The tetrapeptide chain **20** is next attached to two molecules of phenyl alanine amino acids to give hexapeptide chain **22** which provides necessary hydrophobic interaction with the binding pocket present in 20Å funnel of PSMA protein.

The amino group present in NHFmoc protected hexapeptide chain **22** is further attached via amide coupling to NHFmocprotected-8-aminocaprylic acid to afford heptapetide chain **23**which is awaiting for final amide coupling with the *t*-butyl protected tyrosine carboxylic acid PSMA inhibitor **16**(Scheme 7).



Scheme 7

This was successfully achieved to provide the required trifluoroacetyl amino protected lysine polypeptide chain **24** and confirmed by observation of negative Kaiser test. Having constructed the masked polypeptide chain **24**, the end game is to deprotect the trifluoroacetylaminoprotected lysine aminoacid under aqueous piperidine conditions mentioned in Scheme 7 to expose free amino group from lysine that can be tagged with fluorescent agent, Rhodamine B. The trifluoroacetyl protected amino group in lysine amino acid of polypeptide

chain **24** is deprotected successfully using 2M aqueous piperidine and the fluorescent agent, Rhodamine B is coupled to lysine amino using PyBOP as coupling agent to give *t*-butyl protected precursor of final conjugate **25**.

The PSMA targeting rhodamine B conjugate **25** is released from the chlorotrityl resin and simultaneously all the carboxylic acid protecting *tert*-butyl groups of amino acids are cleaved with the help of cleaving cocktail TFA:TIS:H_2O (95:2.5:2.5). Excess trifluoroacetic acid is evaporated under reduced pressure using rotary evaporator and the turbid pink viscous liquid is precipitated by addition of ice cold ether. The pink colored precipitate is washed thrice with ice cold ether, centrifuged and dried under inert atmosphere to provide the final PSMA targeting Rhodamine B conjugate **25** that can be used for targeting prostate cancer cells *in vitro*.

Chapter 5

Conclusions

In this work, we are reporting the successful synthesis of a PSMA targeting fluorescent conjugate **25** which has finally been prepared by attaching a *tert*-butyl protected form of inhibitor **2** to the grown chain of peptide through solid state phase synthesis. Inhibitor **2** shows more number of interactions and better binding affinity with the active site of PSMA protein in comparison to inhibitors **1** and **3-6**. Therefore, inhibitor **2** can be considered effective to target PSMA protein for the diagnosis of prostate cancer. It can be further utilized as imaging agent to detect the early prostate malignancy.

Chapter 6

Appendix

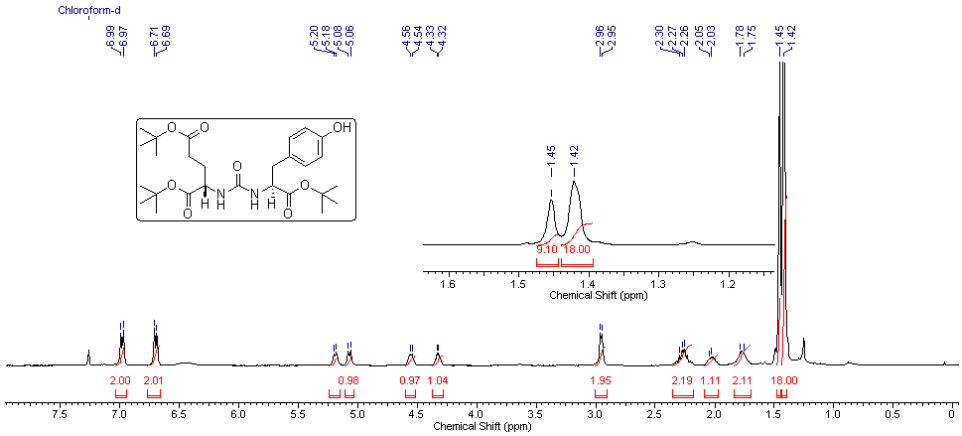


Figure 12. ^{1}H NMR spectrum of **11** in $CDCl_3$

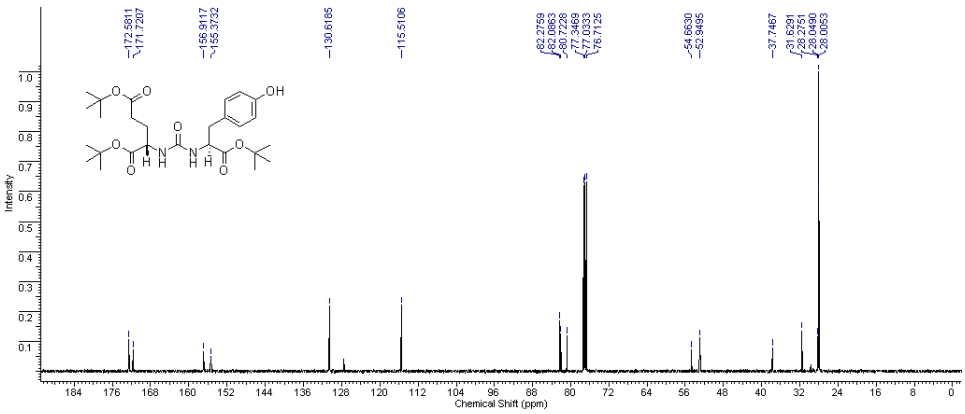


Figure 13. ^{13}C NMR spectrum of **11** in $CDCl_3$

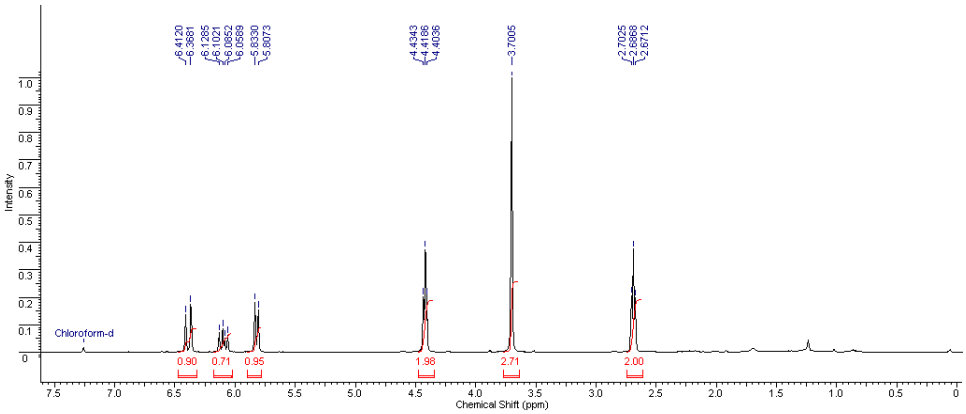


Figure 14. ^{1}H NMR spectrum of **14** in $CDCl_3$

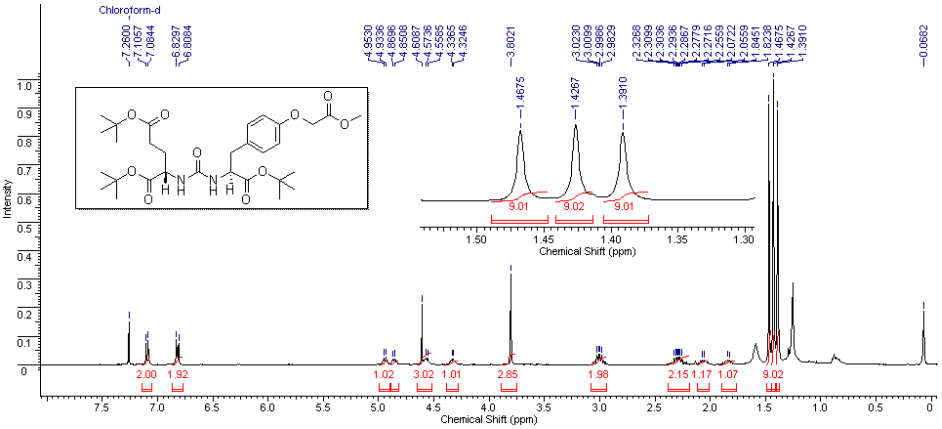


Figure 15. ^{1}H NMR spectrum of **15** in $CDCl_3$

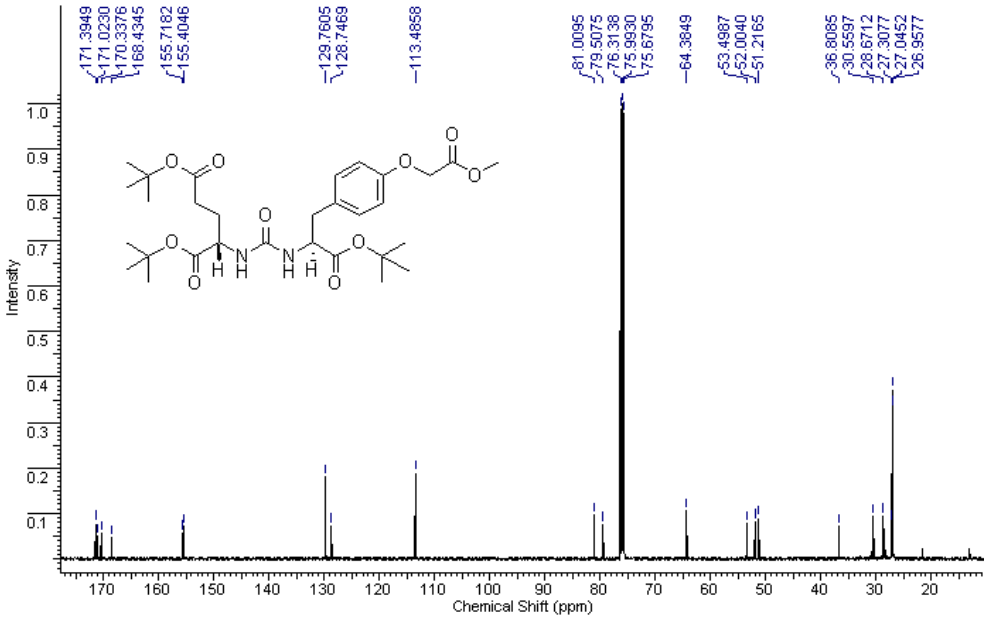


Figure 16. ^{13}C NMR spectrum of **15** in $CDCl_3$

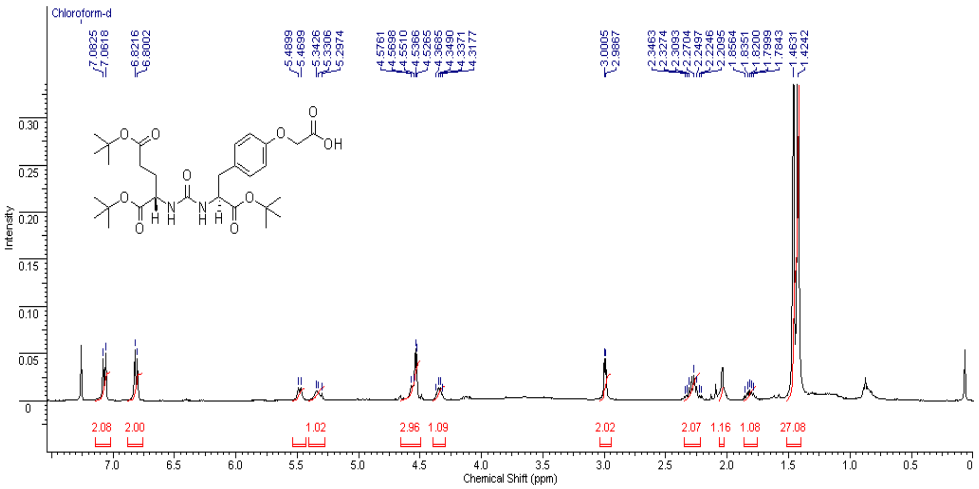


Figure 17. ^{1}H NMR spectrum of **16** in $CDCl_3$

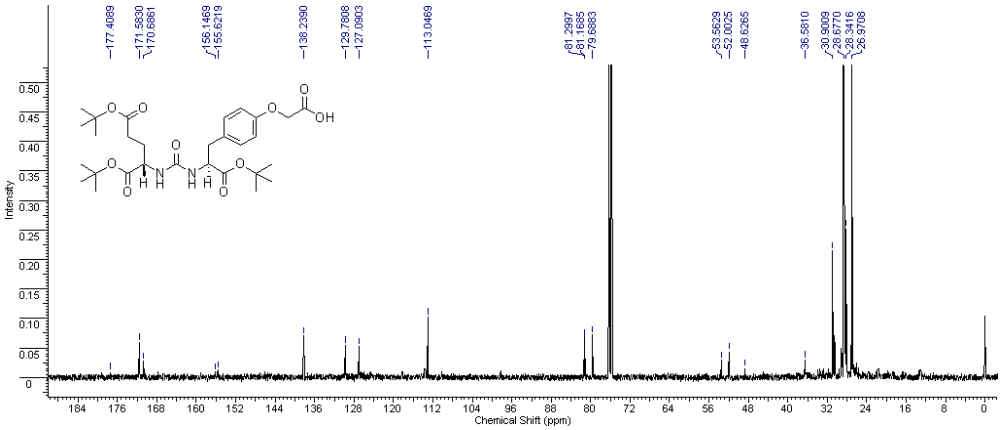


Figure 18. ^{13}C NMR spectrum of **16** in $CDCl_3$

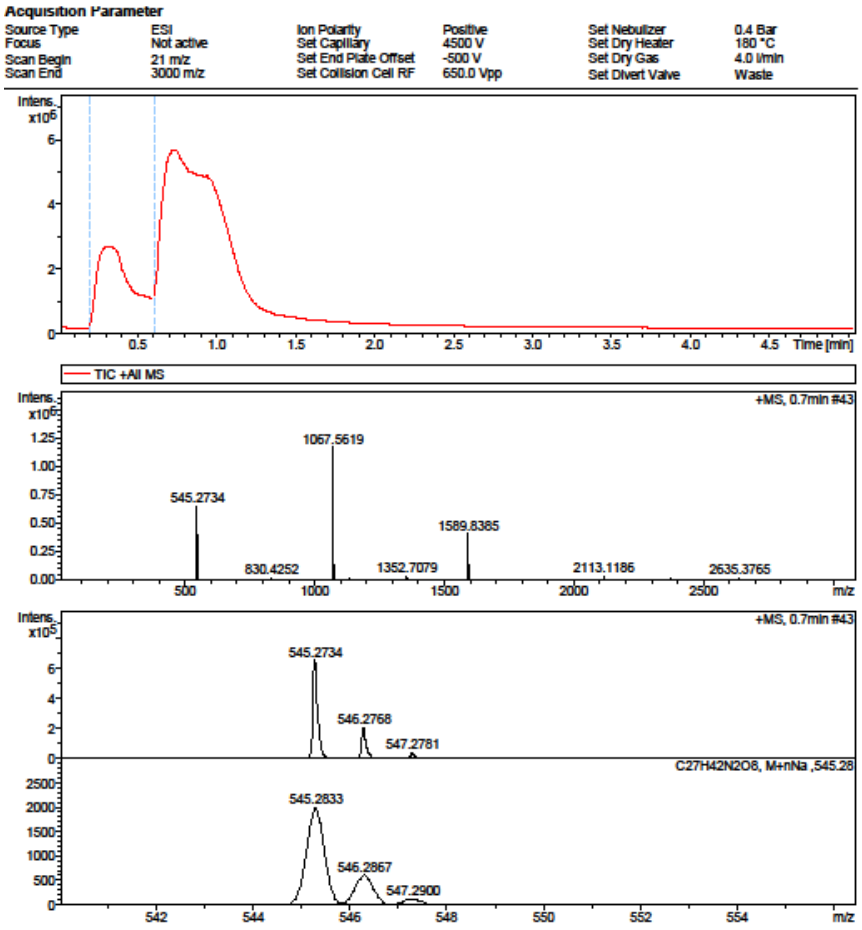


Figure 19. HRMS spectrum of **11**in CH_3CN

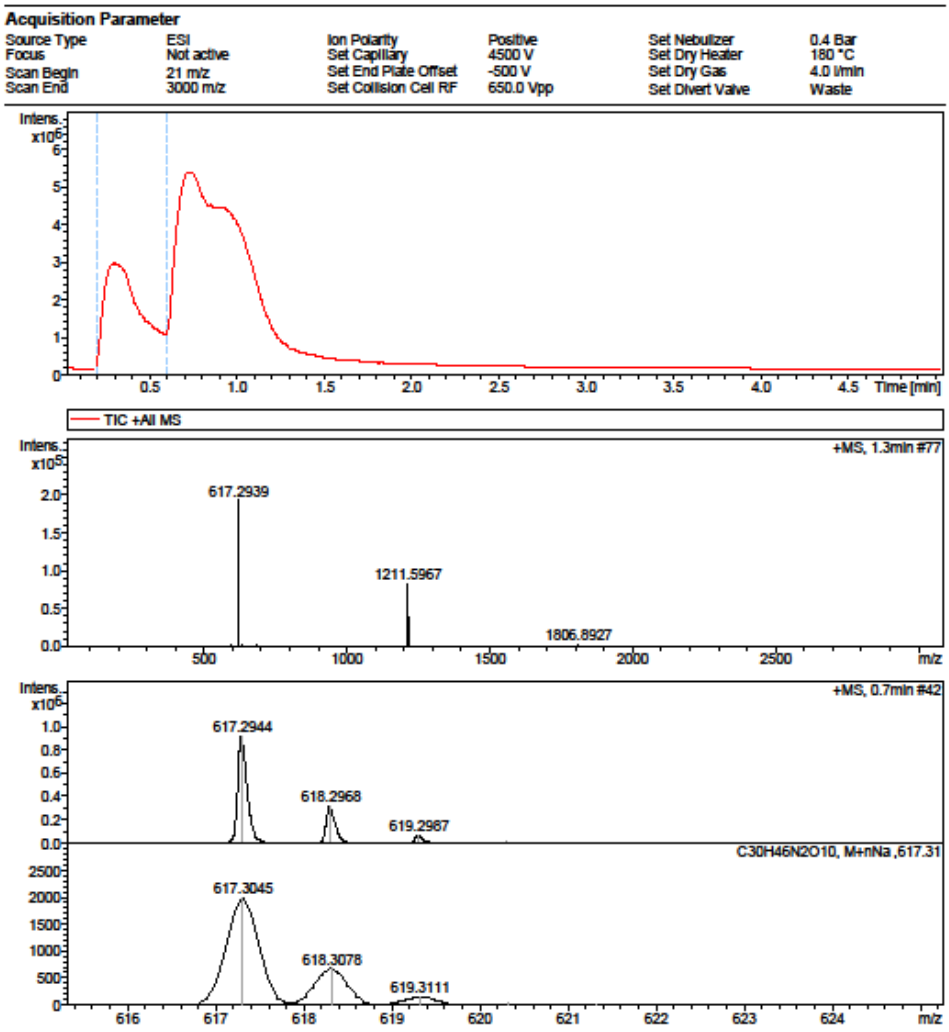


Figure 20. HRMS spectrum of **15** in CH_3CN

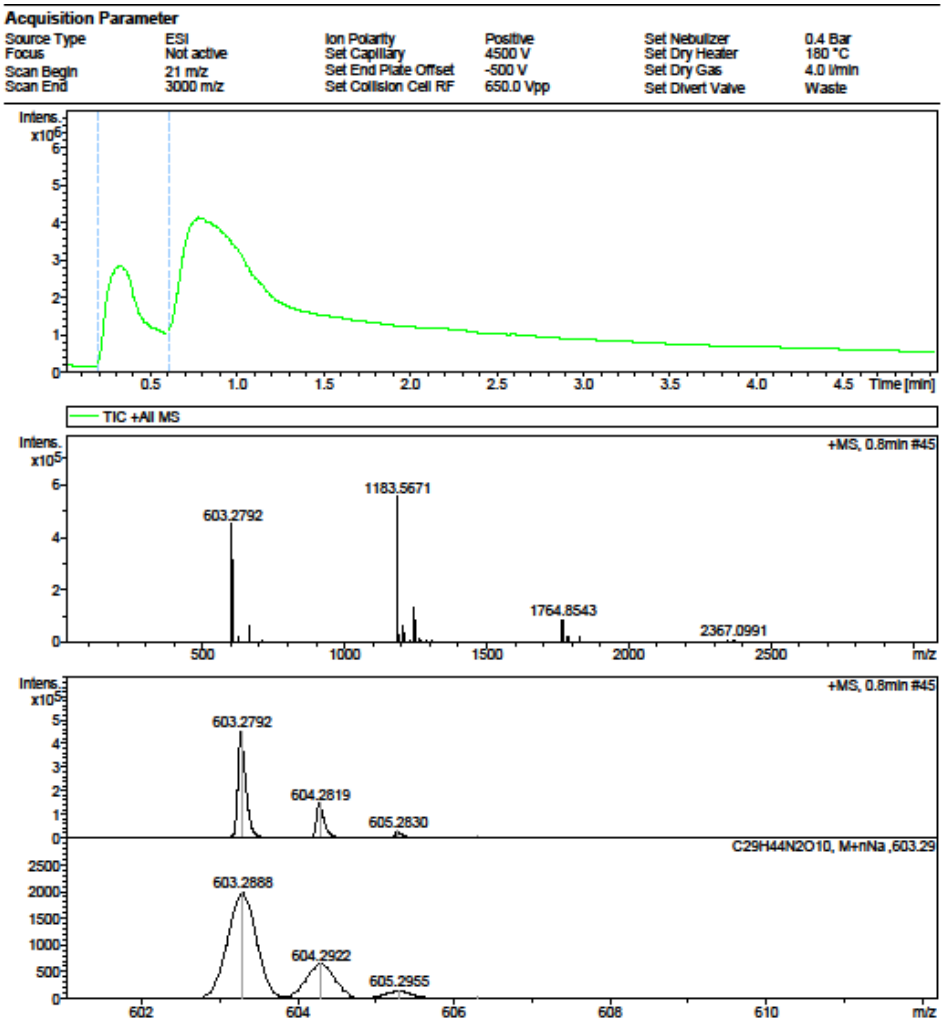


Figure 21. HRMS spectrum of **16** in CH_3CN

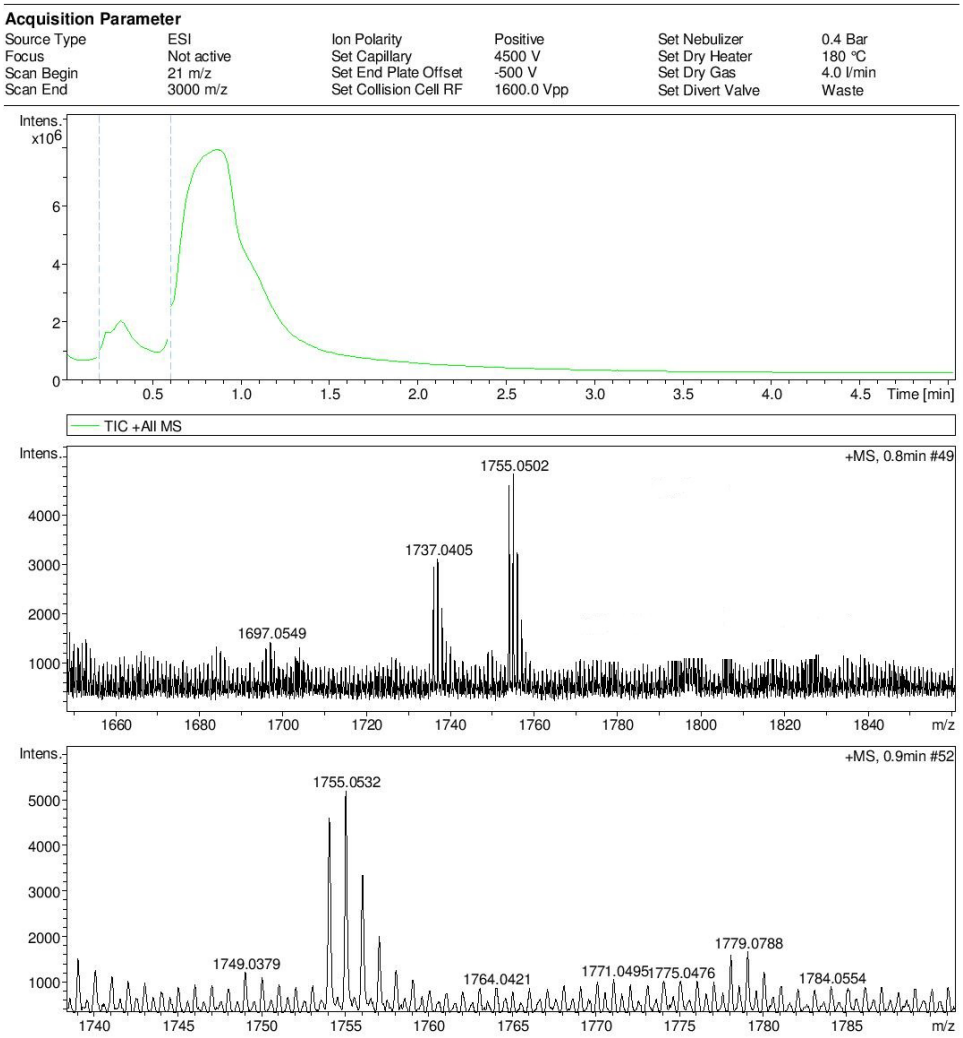


Figure 22. HRMS of compound **25** in 1:1 CH_3CN:H_2O

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