

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

SYNTHESIS OF CATIONIC ANTIBACTERIAL PEPTIDES



M.Sc. THESIS

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Department of Chemistry

Chemistry Programme

AUGUST 2018

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ABBREVIATIONS

ACN	: Acetonitrile
AIDS	: Acquired Immune Deficiency Syndrome
Ala (A)	: Alanine
AMP	: Antimicrobial Peptides
Arg (R)	: Arginine
Asp (D)	: Aspartic Acid
CPP	: Cell Penetrating Peptides
CT	: Computed Tomography
CTLA-4	: Cytotoxic T-Lymphocyte–Associated Antigen 4
CTLs	: cytotoxic T lymphocytes
DCM	: Dichloromethane
DIPEA	: Diisopropylethylamine
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic acid
DOTA	: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
FDA	: Food and Drug Administration
FDG	: 2-deoxy-2-(¹⁸ F)fluoro-D-glucose
Fmoc	: 9H-fluorene-9-ylmethoxycarbonyl
Glu	: Glutamic Acid
HBTU	: O-(Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Ile (I)	: Isoleucine
Leu (L)	: Leucine
LS-MS	: Liquid Chromatography–Mass Spectrometry
Lys (K)	: Lysine
MALDI-TOF	: Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry
MBHA	: 4-Methylbenzhydrylamine resin
MRI	: Magnetic Resonance Imaging
NEt₃	: Triethylamine
NMR	: Nuclear Magnetic Resonance Spectroscopy

NSCLC	: Non-Small Cell Lung Cancer
PD₁	: Programmed Death-1
PD-L₁	: Programmed Death-1 Ligand-1
PD-L₂	: Programmed Death-1 Ligand-2
PET	: Positron Emission Tomography
PET-CT	: Positron Emission Tomography - Computed Tomography
Phe (F)	: Phenylalanine
PPI	: Protein Protein Interactions
RAM	: Rink Amide MBHA resin
RP-HPLC	: Reversed Phase High Performance Liquid Chromatography
RT	: Room Temperature
SPECT	: Single Photon Emission Computed Tomography
t-Boc	: tert-butoxycarbonyl
TFA	: Trifluoroacetic Acid
TIPS	: Triisopropylsilane
Tyr (Y)	: Tyrosine
UIPAC	: International Union of Pure and Applied Chemistry
VS	: Virtual Screening

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SYNTHESIS OF CATIONIC ANTIBACTERIAL PEPTIDES

SUMMARY

When a tissue is damaged, inflammations, biological response of the body to the injury, is maintained to repair the area. Tissues are regenerated by a chemical network system and then inflammation is removed after repair completed. However, when tissue cells continue to proliferate in an inflammation rich environment, tumor cells evolve from inflammatory cells. As the relationship between inflammation and tumor progression becoming clear, it is crucial to distinguish inflammation from infections by means of early diagnosis of cancer. For this matter, cationic antibacterial peptides can be useful as they accumulate at infection but not inflammations.

Surgery, radiotherapy and chemotherapy are the main treatments of cancer. In the early stage of cancer, surgery is the standard application used for either therapy or diagnosis. Besides the constantly improving treatment strategies, new diagnostic agents are needed to be explored. In case of early detection and diagnosis, it is important to utilize proteins that are formed around tumor microenvironment as biomarkers. Specific proteins and its ligands are chosen as they are the basis of the tumor microenvironment formation that consequently provides tumor generation and development. The recent technology named molecular imaging can be used in monitoring these molecular imaging agents.

The aim of this thesis is to develop new imaging agents for the detection of infections and imaging of tumors at the cellular and molecular levels. Thus, labeled peptides and peptidomimetics that are able to bind with high affinity and selectivity to bacteria membranes and to biomarkers expressed by immune and stromal cells of tumors respectively were designed and produced. In the scope of this study, molecules were designed with pharmacophore approach. More than one antibacterial peptide sequences were synthesized and then Rhodamine B, which is an antibacterial imaging agent was added. The aim of this project is to use these dye-labeled peptides as molecular contrast agents. As a result of the experiments, antibacterial peptide dye fixation was successfully carried out and the analysis proved that the result was correct. Additionally, organic molecules as fragments of various peptidomimetic structures were synthesized, they were purified and characterized.

After laboratory studies are completed biomarker affinity and stability analyses will be performed. According to the obtained results from these studies, modifications and alterations will be applied to improve performance. Once the biologically active compounds have been identified, they will be conjugated with DOTA-chelates radio-labeling with Ga⁶⁸ for PET-CT imaging.



KATYONİK ANTİBAKTERİYEL PEPTİTLERİN SENTEZİ

ÖZET

Vücudtaki bir doku hasar gördüğünde, hasara karşı vücudun biyolojik müdahalesi olan inflamasyonlar zarar görmüş bölgeyi düzeltmek için oluşturulur. Dokular kimyasal bağlantılar tarafından rejenarasyona uğrarlar ve ardından düzeltme tamamlandıktan sonra inflamasyonlar kaldırılır. Doku hücreleri inflamasyonca zengin çevrede çoğalmaya devam ettiğinde, inflamasyon hücreleri tümör hücrelerine dönüşür. Tümör gelişimi ve inflamasyon arasındaki bu ilişki daha belirgin hale geldiğinden, inflamasyonu enfeksiyondan ayırt etmek kanserin erken teşhisinde çok önemlidir. Bu bağlamda, inflamasyonlar yerine sadece enfeksiyonlarda biriken katyonik antibakteriyel peptitler kullanışlı olabilirler.

Kanserin erken evrelerinde tedavi veya tanı için kullanılan standart uygulama ise cerrahi müdahaledir. Tedavi yöntemlerinin sürekli gelişmesine rağmen sınırlı sayıda teşhis ajanı mevcuttur ve yeni ajanların geliştirilmesine ihtiyaç vardır. Erken tanı ve teşhiste kullanılabilecek yöntemlerden biri tümör mikroçevresinin etrafında oluşan proteinlerin biyobelirteçler olarak belirlenmesidir. Tümör oluşumu ve gelişimi sağlayan bu spesifik proteinler ve ligandları, tümör mikroçevresini oluşturdukları için biyobelirteç olarak seçilmektedirler. Günümüzdeki en son gelişmiş yöntem olan moleküler görüntüleme teknolojisi, bu biyobelirteçlere bağlanacak moleküler görüntüleme ajanlarının görüntülenmesinde kullanılabilmektedir.

Bu tezin amacı, konvansiyonel PET ajanlarına alternatif olarak kullanılmak üzere tümör görüntülemesi için protein tabanlı peptidomimetiklerin ve enfeksiyon odaklarının hücresel ve moleküler düzeyde ayırt edilmesinde kullanılmak üzere peptitlerin geliştirilmesidir. Bu sebeple, tümör mikroçevresindeki immün ve stromal hücrelerinde bulunan biyobelirteçlere ve enfeksiyon hücrelerine yüksek bağlanma eğilimi ve seçicilikle bağlanabilen radyonüklidler ile işaretlenmiş peptidomimetikler ve peptitler tasarlanmış ve üretilmiştir.

Öncelikli olarak sentezlenecek olan katyonik antibakteriyel peptit sekansları tasarlanmıştır. Bu peptitlerin toplam pozitif yük ve amfipatik özellik olmak üzere iki ana yapısal karakteristiği vardır. Katyonik yük pozitif yüklü amino asitlerle sağlanmaktadır. Bu sebeple peptit sekanlarına toplam yükü pozitif olan arjinin ve lisin aminoasitleri eklenmiştir. Antibakteriyel peptitlerin diğer anahtar özelliği ise, peptitlerin lipid yapılarına hızlı ve kolay bağlanmasını sağlayan amfipatik gruplardır. Pozitif yüklü peptitler ve negatif yüklü mikrobiyal hücre yüzeyleri arasındaki ilk etkileşim elektrostatik çekim kuvvetidir. Hayvan hücre zarlarının aksine, mikrobiyal hücre zarlarının yüzeyleri negatif yüklüdür. Bu sebeple antibakteriyel peptitler hayvan hücresinden bakteriyeye bağlanmayı tercih ederler.

Mevcut farmasötikler yeterince hücreye özel olmadıklarından enfeksiyon ve inflamasyonları ayırt edememektedir. İnflamasyon belirtileri ve semptomları belirgin olmayan, çeşitlilik gösteren ve hatta belirlenemeyen özelliklere sahip olduğundan, ayırt edici ajanlar olarak antibakteriyel peptitler kullanılabilir. Antibakteriyel peptitler memeli hücreleri yerine tercihen bakterilere bağlanma özelliklerine sahiptir ve dokulara kolayca nüfuz edebilmektedirler. Birçok hastalığın tedavisinde erken teşhis önemli yer tuttuğundan katyonik antibakteriyel peptitler ayırt edici ajanlar ve görüntüleme ajanları olarak umut veren adaylardır.

Bu bilgiler doğrultusunda tez kasamında yapılan çalışmalarla, tasarlanan birden fazla antibakteriyel peptit sekansı sentezlenmiş ve ardından antibakteriyel görüntüleme ajanı olan Rodamin B boyası eklenmiştir. Bu boya, amino asitlerin birbirlerine bağlanma mekanizmasıyla aynı yöntem kullanılarak peptit sekansına eklenmiştir. Yapılan deneyler sonucunda antibakteriyel peptit boya eşleşmesi başarıyla gerçekleştirilmiş ve peptitler RP-HPLC ile saflaştırılmıştır. Ardından analiz sonuçların doğruluğu kütle spektrometresiyle kanıtlanmıştır. Rodamin B'nin floresan özelliği sayesinde izlenebilir hale getiren peptitler, ileriki çalışmalarda kararlılık, bağlama eğilimi ve stabilite analizleri için kullanılacaktır.

Katyonik antibakteriyel peptit çalışmalarına ek olarak, tümör hücrelerine bağlanmak üzere organik tabanlı peptidomimetikler üzerinde çalışılmıştır. Peptidomimetik yapısının çeşitli parçaları olan organik moleküller tasarlanmış, sentezlenmiş, saflaştırılmış ve karakterize edilmiştir. Bu çalışmalar boyunca üretilen peptidomimetikler farmakofor yaklaşımı ile tasarlanmıştır. Tasarlama prosesi boyunca, ligand-protein bağlanma etkileşimini engellemeyecek optimal kısımlar her bir farklı molekül için belirlenmiştir. Tasarlanan bu moleküller arasından sentezlenebilir olmayanlar elenmiştir. Molekülün ana gövdesine karar verildikten sonra, farmakofor yaklaşımla molekül yapısı geliştirilmeye devam edilmiştir. Ana gövde tasarım kolaylığı ve sentez metodu sebebiyle üç parçaya bölünmüştür. Her bir parça çeşitli gruplarla türevlendirilerek molekül tasarımına eklenmiştir. Tez çalışmaları kapsamında gerekli parçaların tasarımları, sentezleri ve saflaştırmaları optimize edilmiştir. Sentezlenen parçalar gerekli teknikler kullanılarak saflaştırılmıştır. Ardından $^1\text{H-NMR}$ kullanılarak karakterizasyonları gerçekleştirilmiş ve sentezlerin doğruluğu onaylanmıştır.

Bu çalışmalar tamamlandıktan sonra, bireysel parçalar tüm bir peptidomimetik elde etmek üzere birleştirilecektir. Öncelikli olarak floresan boya ile işaretlenen bu peptitlerin ortogonal şekilde türevlendirilerek çeşitlendirilmesi amaçlanmaktadır.

Peptidomimetik parçaları üzerinde ortogonal koruma gerçekleştirilerek DOTA-şelatlarının gerekli etkileşimleri engellemeyecek şekilde bağlanması tasarlanmıştır. Radyofarmasötik alanda PET-BT cihazları için en yaygın olarak kullanılan aktif izotop Ga^{68} 'dir. DOTA şelati Ga^{68} 'e uygun oyuk çapı sebebiyle seçilmiştir ve peptidomimetiklerin elverişli bölgelerine eklenecektir.

Laboratuvar çalışmaları tamamlandıktan sonra biyobelirteçlere bağlanma eğilimi ve stabilite analizleri yapılacaktır. Bu çalışmalardan elde edilen sonuçlara göre, yüksek bağlanma eğilimi ve seçicilik performanslarını geliştirmek için çeşitli modifikasyonlar ve değişiklikler uygulanacaktır. Peptitlerin kararlılığını arttırmak üzere L ve D konformasyonları arasındaki stabilite farklılıklarından yararlanılacaktır. Peptidomimetiklerde ise sanal tarama yöntemi kullanılarak olası aktif moleküller belirlenecektir.

Pozitron emisyon tomografisi-bilgisayarlı tomografi (PET-BT), günümüzde çoğunlukla kullanılan en gelişmiş teknolojilerdendir. PET-BT sadece anatomik bilgi değil aynı zamanda doku ve organların metabolik ve fonksiyonel durumu hakkında da bilgi vererek onkolojik görüntüleme için kullanılmaktadır. Bu sebeple, biyolojik olarak aktif olduğu tespit edilen bileşikler tanımlandıktan sonra bu bileşikler PET-BT cihazlarında görüntüleme amacıyla kullanılmak üzere Ga⁶⁸ ve DOTA-şelatları ile radyolojik olarak işaretleneceklerdir.





1. INTRODUCTION

Various failure or mutations in DNA is the root cause of all diseases. These variations may be natal (genetic defects) or may be gained during life (mutation). Occasional mistakes during performing DNA replication or protein synthesis, DNA damaging occurs. Cellular mechanisms of the body try to repair both gene-caused and wound-associated damaging. When a tissue is damaged, body tries to repair injured areas by initiating an maintaining a response which can be called as inflammation. Inflammation is removed after repair completed. However, when tissue cells continue to proliferate in an inflammation rich environment, tumor cells evolve from inflammatory cells. As the relationship between inflammation and tumor progression becoming clear, it is crucial to distinguish inflammation from infections by means of early diagnosis of cancer.

Some peptide structures that are effective in the cell have been discovered to enter the cell membrane effectively and comfortably. As a result of some new research, protein families with positive charged amino acid members can easily pass through cell membranes (natural or synthetic) (Okarvi, 2004). Due to Lysine and Arginine have (+) charge in the side chains, which provide them to have net positive charge, peptide sequences that have K or R in their structure have been found linked to the cell membrane easily. Additionally, specific proteins that are formed around tumor microenvironment, is used as biomarkers. Biomarkers are like molecular plates that indicate the physiological state of a cell at a particular time, and can be active genes, genetic material, related proteins, hormones, antigens, enzymes and other organic chemicals produced by the cell. Biomarkers are sensitive, selective, synchronous, rapid, easy to diagnose and able to be monitored continuously. Because of these advantages, cancer studies focused on identifying biomarkers.

In this study, probe carried peptides to distinguish and image the focus of inflammation as well as organic molecules to image the tumor cells synthesized and develop as diagnostic imaging agents to identify and localized the defected cells and tissues.



2. MOLECULAR IMAGING

Diseases are biological processes and molecular imaging visualizes the environment of these processes in living subjects in cellular extent. It differs from 'classical' diagnostic imaging with means of identifying the molecular abnormalities which have caused the diseases instead of the effects of these disease processes. Revealing molecular pathways and understanding the cellular molecular mechanism of many diseases to obtain an appropriate signal and to localize that signal as accurately as possible where the primary aim of the molecular imaging.

Today there are many important goals wanted to achieve in biomedical research which are; monitor multiple molecular alterations at the same time, optimize drug and gene therapy, image drug effects at the cellular level, evaluate disease development at the molecular level detection of physiological alterations of disease when it is still at a curable stage, adjusting treatment management in real time and ability to real-time the disease drug development process (Weissleder, *Molecular Imaging in Cancer*, 2006); (Massoud & Gambhir, 2003).

Molecular imaging characterizes specific disease processes in different patients so that it personalizes the treatment. In addition, it allows the unique approach to management of disease conditions. Risk evaluation, stratification, and follow-up of patients can be performed during disease progression and treatment management (Maisey, 2005). Unrevealing of molecular and physiological information that obtained about each patient by imaging methods demonstrates the patient-specific and disease-specific principle of molecular imaging.

One of the most important usage area of molecular imaging is in oncology. The tumor extent and location is stated by characterization of the tumor properties (Maisey, 2005). Not only tumor location and extent is indicated but also the activity of specific biological processes of those tumors that is the basis of tumor behavior and response to therapy are imagined (Weissleder, *Molecular Imaging in Cancer*, 2006).

2.1 Molecular Imaging Targets and Agents

Molecular imaging devices use molecular probes as a source of image contrast to reveal biochemical processes. Molecular probe, which is known as tracer, is a molecule provides imaging signal for target areas by injected to the patient (Gambhir, 2002). Tracers consist of an affinity and signaling component. Affinity component interacts with the target when signaling component is used for imaging (Massoud & Gambhir, 2003).

Molecular probes can be divided into two categories either as radiolabeled or activatable probes. If a probe produces a signal when interacting with the target, it is called as activatable probes. In contrast to activatable probes, radiolabeled probes produce continuous signal through the decay of the radioisotope (Massoud & Gambhir, 2003). Such probes may be small molecules or affinity ligands with higher molecular weight like antibodies and recombinant proteins (Weissleder & Mahmood, Molecular Imaging, 2001). Figure 2.1 illustrates molecular probe and its potential targets *in vivo*.

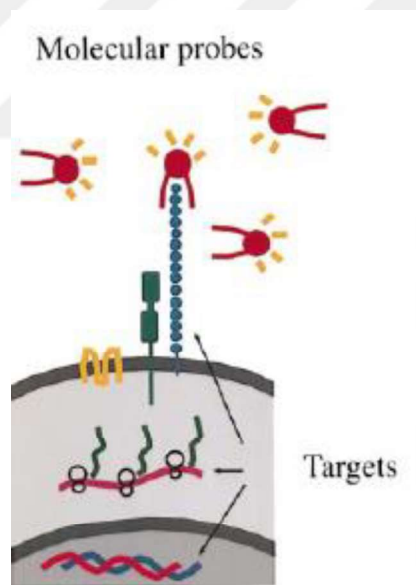


Figure 2.1: Molecular imaging path *in vivo*.

Most of the target molecules of current drugs are receptors (45%), rest are enzymes (30%) and others (25%) (Weissleder & Mahmood, Molecular Imaging, 2001). There are nearly 500 molecular targets utilized in biomedicine estimated number of the potential targets are 5000-10000 (Weissleder & Mahmood, Molecular Imaging, 2001).

Even though there are thousands of possible targets, designing and development of efficient imaging agents are more difficult processes. Potential probes must be biocompatible, stable, target specific and have enough target abundance to release amplified signal (Massoud & Gambhir, 2003); (Jaffer & Weissleder, 2005).

2.2 Imaging Pathways and Strategies

To develop more effective and convenient imaging agent, it is crucial to understand the basis of imaging principle. Molecular mechanisms of the disease process are the key for molecular imaging. Angiogenesis and apoptosis are the specific molecular regulation pathways that lead the current imaging techniques.

Angiogenesis is transforming and remodeling of a basic vascular network to a complex system (Weissleder & Mahmood, Molecular Imaging, 2001). Under normal physiological conditions, angiogenesis occurs during embryonic development, wound healing and the female reproductive cycle (Weissleder & Mahmood, Molecular Imaging, 2001). However, this balance changes with up/down regulations of angiogenesis inhibitors which are the reasons of many diseases including cancer, immunological and cardiovascular diseases (Weissleder & Mahmood, Molecular Imaging, 2001). One of the specific approaches of imaging is to visualize this vascular growth by targeting imaging probes presented on tumor surface (Weissleder & Mahmood, Molecular Imaging, 2001). This method allows detecting the earliest stage while it images the molecule needed to be treated (Weissleder & Mahmood, Molecular Imaging, 2001).

Apoptosis is a controlled progress of the programmed cell that used for organ development and elimination of redundant cells (Lowe & Lin, 2000). Apoptosis takes place by recognition of some cellular signals which result in activation of proteases called caspase (Lowe & Lin, 2000). Regulation of apoptosis is used in treatments of various disease for intense autoimmune disease, AIDS and cancers (Lowe & Lin, 2000). Imaging the intracellular caspase activity or imposed cells are another approaches that are presented by imaging techniques (Weissleder & Mahmood, Molecular Imaging, 2001).

Another major component of imaging pathways is signal amplification. Higher signal levels are provided by the frequency of interaction between target and probe which

lead to higher sensitivity (Massoud & Gambhir, 2003). Due to the adequate target number imaging of proteins or protein function are convenient and feasible. It is also significant to select a target that can produce sufficient signal amplification for imaging.

Many different imaging systems developed to detect the molecular probes or interaction with molecules. Nuclear (radionuclide) imaging, optical imaging, magnetic resonance imaging (MRI), computed tomography (CT) and ultrasound are the current molecular imaging systems used today (Massoud & Gambhir, 2003).

2.3 Nuclear Imaging Technique: PET/CT

Nuclear imaging is using a probe which labeled with specific radiopharmaceuticals (Weissleder & Mahmood, Molecular Imaging, 2001). The nuclear imaging system includes single photon emission computed tomography (SPECT) and positron emission tomography (PET) which are the most sensitive molecular imaging techniques (Ametamey, Honer, & Schubiger, 2008).

PET has a special advantage of being quantitative however, it needs a cyclotron to produce its probes and patient has to exposure to radiation during PET scanning (Jaffer & Weissleder, 2005). Positron emission tomography (PET) images and measures the biochemical process *in vivo* by using positron-emitting radioisotope that bound to molecular probe (Massoud & Gambhir, 2003); (Phelps, 2000).

When ^{15}O , ^{13}N , ^{11}C and ^{18}F are generally used, ^{14}O , ^{64}Cu , ^{62}Cu , ^{76}Br , ^{68}Ga , ^{124}I and ^{92}Rb as well as some isotopes of Zn, P and Fe are used as positron emitters (Massoud & Gambhir, 2003); (Phelps, 2000). Figure 2.2 shows the structure of some chelator used for radionuclide labeling (McQuade, Deborah W., & Michael J., 2005).

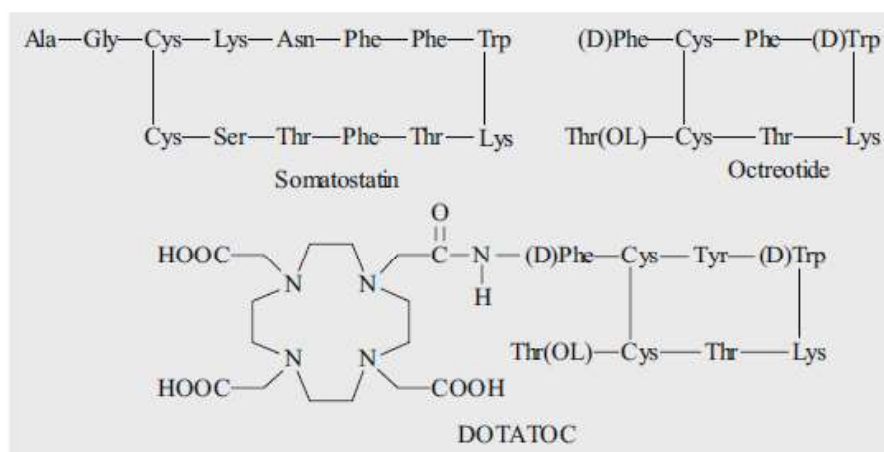


Figure 2.2: Structures of radionuclide chelates.

Positron emitter labeled molecules generate two γ -rays through its nucleus in which PET records from within the subject (Massoud & Gambhir, 2003). Currently, PET and SPECT have the most developed clinical agents when compared to other systems. The major tracer of PET is 2-deoxy-2- (^{18}F) fluoro-D-glucose (FDG) which is used for diagnosis, staging and monitoring the cancer cells. In addition to current tracers, developed tracers must meet the primary requirements such as high binding affinity to the specific target, metabolic stability and proper pharmacokinetics like sufficient lipophilicity in order to cross cell membranes (Phelps, 2000); (Ametamey, Honer, & Schubiger, 2008).

Firstly, the tracer is injected into the subject, then PET can measure the labeled tissue concentration over time that results in the image of process rate (Phelps, 2000). Due to the short half-lives of positron-emitting isotopes, the introduction of isotope into the subject must be performed rapidly. In Figure 2.3, a PET imaging study of the human brain is shown. Glucose metabolism is used to create a map of the brain during daily activities (Phelps, 2000).

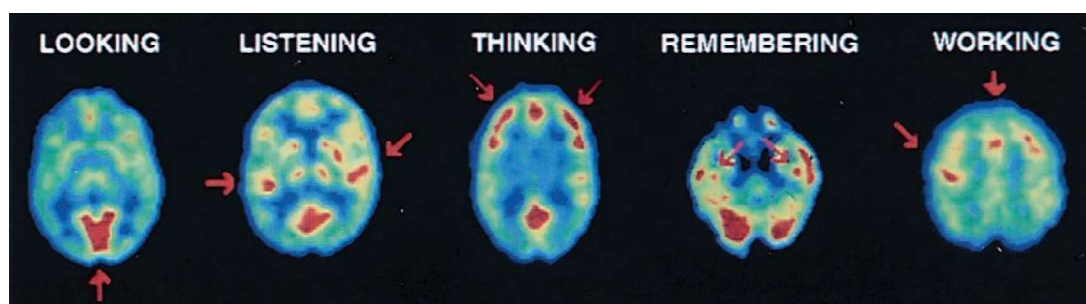


Figure 2.3: PET studies the with the response of human brain when performing different tasks.

PET scans are shown in color which reflects the isotope location and PET sensitivity is liberated from the location depth of the tracers (Massoud & Gambhir, 2003).

PET is one of the rare current technology approved by the FDA which is used in three important clinical areas (Weissleder, Molecular Imaging in Cancer, 2006). For cancer diagnosis and management, PET scans are used in the clinical practice of cardiology to measure myocardial perfusion (by using ^{13}N) and myocardial viability (by using ^{18}F). For neurology, presurgical workup of epilepsy patients, management of brain tumors and identification of neoplastic syndrome tumors (Maisey, 2005). Additionally, PET is used specifically to monitor the early stage of diseases and duration of therapy *in vivo* (Gambhir, 2002).

Although the PET resolution is relatively high [nearly $(6-8)^3 \text{ mm}^3$] the precise cell number that can be imaged with PET depends on the tracer level amount in the tissue (Gambhir, 2002). PET scan measures tissue radioactivity in the definite unit which is Bq/mL (Ametamey, Honer, & Schubiger, 2008).

PET has a specific advantage of being developed for the whole body imaging that provides an examination of all organ systems in a single study (Phelps, 2000). For instance, PET demonstrates its accuracy with 8 to 43% ratio for living and colorectal cancer detection compared to other workups (Phelps, 2000). Moreover, PET is an effective technique used for individualized evaluating of data during disease management. Studies show that more than 25% of patients treatment is validated crucially with PET. Decision change of nonsmall cell lung cancer surgery, staging of lymphoma and labeling the high-risk coronary artery disease are some examples of many (Maisey, 2005).

Although PET is one of the most sensitive methods and has the ability to specific determination, it yields limited anatomic information (Antoch, et al., 2003). With the development of combined PET-CT systems, functional and morphologic data can be accurately generated. CT system facilitates accurately assessed tumor size and adjacent structures (Antoch, et al., 2003). Additionally, PET-CT systems decrease the examination time by up to 30% (less than 10 minutes for all body) (Gambhir, 2002). In figure 2.4, an application of PET-CT can be seen which is used to image the non-small cell lung cancer and prostate cancer.

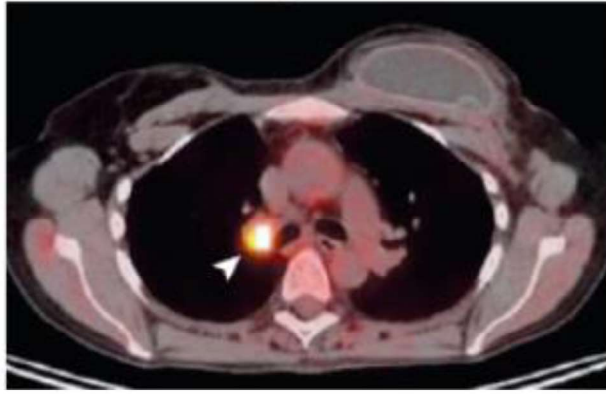


Figure 2.4: PET-CT imaging of lung cancer.

Research demonstrates the dual modality ability to provide system have more accurate and 40% additional information with respect to separate PET and CT scans (Jaffer & Weissleder, 2005). It is estimated that PET will be replaced entirely by PET-CT systems in near future (Gambhir, 2002).

As a result, molecular imaging techniques contribute a better understanding of molecular pathways of disease that lead to the development of new drugs and imaging agents. These tools provide early diagnosis efficient treatments and better disease progress management which led to improved patient care.



3. IMAGING AGENTS

There is an increasing need for diagnosing disease, imaging disease process, following the treatment, personalized information of physiology and biochemistry. Molecular imaging is the major element for this evaluation associated with a contribution in drug discovery and development.

In the successful imaging agent, target binding molecule and signaling part must not dissociate. Also, imaging agent must clarify from the non-target part of the body like blood and lymphatic serum (Gambhir, 2002). Small molecules like enzyme substrates and receptor ligands or ligands with higher molecular weight like antibodies and proteins can be used as molecular imaging probes (Massoud & Gambhir, 2003).

Imaging probes can label two categories which are radionuclide probes and activatable probes as shown in Figure 3.1 (Massoud & Gambhir, 2003).

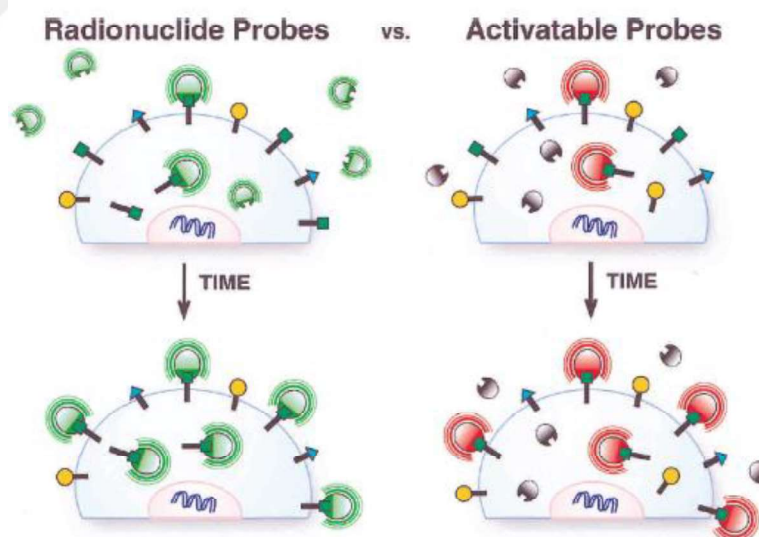


Figure 3.1: Type of molecular imaging probes.

For radionuclide probe, interaction with their target to produce a signal is not necessary in contrast to activatable probe. Radionuclide probe generates signal before and after the coupling with the target when the activatable probe generates signal only with interaction time (Massoud & Gambhir, 2003).

Target of molecular imaging probes which is called biomarkers can be group according to their purpose of use. Type 0 biomarkers are used for imaging of the progression of the disease, Type I biomarkers are used for drug mechanism and Type II biomarkers are used for surrogation of endpoints (Agdeppa & Spilker, 2009).

Target interacted probes can determine the presence, quantity, and location. However, probes do not have to be functional for modifying and treatment of diseases. Nonfunctional probes can have many markers of disease progression like structural proteins membrane lipids, extracellular peptides, and proteins (Agdeppa & Spilker, 2009). Chemical probes are given to subject in very low concentrations to detect ligand-target interaction without causing a pharmacological effect (Agdeppa & Spilker, 2009).

For imaging agent performance *in vivo*, the amount of accessible target for high signal-to-noise ratio is important (Agdeppa & Spilker, 2009). The main contributors of imaging agent performance are pharmacokinetic behavior and signal production so that chemical synthesis and preclinical studies are iterative for diagnostics (Agdeppa & Spilker, 2009). Major pharmacokinetic behaviors that are important for a specific imaging probe are able to reach its target at the adequate amount and to remain there for enough time to detection. Additionally, it is also important to overcome delivery barriers (Massoud & Gambhir, 2003).

Protein-protein interaction (PPI) is the origin of designing molecular imaging agents. When cellular proteins coupled to regulate cellular functions, its called as PPI (Akram, et al., 2014). The cellular functions are the key processes to decipher disease pathways as well as modeling tracers for these diseases. PPIs are changed by metabolic diseases specifically in cancer. Pathway of PPI mechanism can be imitated to develop chemical probe-biomarker binding process for modeling this tracer.

One of the main usage areas of tracers is to image tumors that have low growth rate. As discussed at section 4, Tumor Microenvironment, there are many cell-surface receptors in tumor cells which could be probable biomarkers. However, a new chemical probe must develop and radiolabeled for each targeted biomarkers.

In the scope of this thesis, peptides as small ligands and peptidomimetics as radionuclide labeled molecules is chosen as tracers to target specific receptors for PET imaging.

3.1 Antibacterial Peptides for Delivery of Contrast Agent

Peptides are protein chains that consist of linked amino acid monomers by peptide (amide) bonds. Peptides can be used for cell-targeting or cell-penetrating in the concept of delivery of tracers. Small peptides are used to provide effective internalization and selective binding to bind cell-surface proteins (biomarkers) with high selectivity and high affinity. Peptides are natural ligands to significant cell-surface receptors (Akram, et al., 2014).

Antibacterial peptides exhibit antibacterial properties by interacting with the bacterial cell membrane or the metabolism of the bacterium. Altering the antibacterial effect is possible by changing the structure of the amino acids and the structure of the peptides. Some protein structures that are effective in the cell have been discovered to enter the cell membrane effectively and comfortably. This new construct is now called CPP (cell penetrating peptides). As a result of some new research, protein families with positive amino acid sequence can easily pass through cell membranes (natural or synthetic) (Okarvi, 2004). Antibacterial AMP (Antimicrobial Peptides) are short peptide chains with L-configuration. Lysine and Arginine, which have (+) charge in the side chains, allow CPP and AMP to have net positive charge.

Although these peptides differ in size, structure, amino acid sequence, biological activity, they are usually helical in the form of common ancestral secondary structures (Henriques, Melo, & M.Castanho, 2006). In order to attack the bacterial membrane, antibacterial peptides require some amphipathic (both hydrophobic and hydrophilic) properties. Throughout it is thought that the antibacterial activities of AMPs are determined by hydrophobic amino acids and cationic residues. The peptides form an electrostatic attraction with the negatively charged bacterial surface through the cationic sites present and enter the lipid layer of the membrane layer through hydrophobic sites. As a result of this mechanism, the structures of the phospholipids in the bacterium and the charges are among the criteria that influence the mechanism of action of the antibacterial peptides. Antibacterial peptides have been found to bind strongly to phospholipids, the most basic structure of the cell membrane. The observation that these peptides bind more strongly to negatively charged liposomes than uncharged liposomes suggests that being positively charged is required for antibacterial activity.

The most general strategy to develop a cell-targeting peptide is to use phage display. Basically, phage display is expression and purification of a particular biomarker then using as a substrate for scanning peptide library for high-affinity ligands (Juliano, Alam, Dixit, & Kang, 2009). Probe-carrier peptides which generated with phage display or modeling are generally small chains with 6-12 amino acids. After peptide is designed, it is coupled with a radioactive element to deliver radionuclide directly to targeted cells (Akram, et al., 2014).

3.2 Peptidomimetics as Radiolabeled Probes

While peptides have many advantages as an imaging agent, they need to be chemically and biologically stable *in vivo*. Their natural structure is sensitive to peptidase which causes to break the amide bonds in blood and some tissues (Jong, et al., 2008). In order to overcome this hindrance peptidomimetics are developed and used in the living systems. Peptidomimetics are small organic molecules which mimic peptides or peptide segments of proteins. Small structural changes like the execution of peptide bonds or the using variations of natural amino acids can have significant effects on the target-binding capacity as well as the stability toward to enzymes and immune system (Akram, et al., 2014). Moreover, peptidomimetics have advantages over peptide-based structures such as high transportation through membranes, slow excretion and high target specificity due to their restricted structures (Akram, et al., 2014). Peptidomimetics gain these advantages thanks to their chemically modified structures which includes amino acid mimics, backbone amid bond changes and the addition of hydrophilic/hydrophobic residues (Akram, et al., 2014). When their structures can be various, interaction spots with relevant target protein remains the same in their 3-D conformation. That is to say, peptidomimetics target specific PPIs to achieve purposed cellular binding pathways.

There are three types of peptidomimetics called as types I, type II and type III which are categorized by their chemical modifications. Type I is conformationally restricted short-peptides which replicate α -helix structures of local parts of PPIs. Type II has a resemblance to the amino acid backbone of peptides that replicates secondary structure. Type III mimetics are lack of the main peptide backbone structure but only have necessary functional groups for binding interactions. They are highly modified structures (Akram, et al., 2014).

4. MECHANISM OF ACTION OF PEPTIDES AND PEPTIDOMIMETICS

4.1 Peptide Structure and Antibacterial Activity

Cationic antibacterial peptides are small, positively charged molecules which have broad antibacterial activity. These peptides have two main structural characteristics overall positive charge and amphipathic property. The cationic charge is gained by the positively charged amino acids and hydrophobic residues. The other key feature of antibacterial peptides is the amphipathic conformation. Amphipathic groups lead to bind of a peptide to the lipid structure fast and easy.

The first association between the peptides and bacterial membrane is the electrostatic interactions. The initial step of these interactions occurs between cationic (positively charged) peptide and negatively charged cell surface. Therefore the main requirement leading to desired association is electrostatic attraction. Also, an increase in charge accompanies with the decrease in hydrophobicity. While microbial membranes are negatively charged on their surface, mammalian cells have headgroups. Thus antibacterial peptides prefer to bind bacteria over mammalian cells. (Lupettti et al.,2003)

Some research showed that arginine-rich peptides have the ability to translocate the membrane components, therefore they can be acting as delivery agents. Additional studies were conducted with the fluorescent labeled peptides in order to develop antibacterial properties and the structures. The antibacterial activity of peptides could also be improved with the further modeling studies. One of the study fields of these labeled peptides is the detection and distinction of infection. Although physical examination, laboratory tests and studies were conducted, it is difficult to identify the inflammation. Biopsies and microbiological culture tests must be performed to identify the pathogens. Moreover, inflammation signs and symptoms are generally non-typical subtle and even non-existing. Yet, the early detection of an inflammation is crucial for treatment of many diseases. For this matter, labeled peptides can be used as antibacterial peptides accumulate at infection sites but not inflammations. Current

radiopharmaceuticals are unable to distinguish inflamed and infected areas as they are not adequately cell-specific. Due to their favorable binding characteristics to bacteria over mammalian cells and easy penetration into tissues, antibacterial peptides are promising candidates for discriminating and monitoring agents (Powers & Hancock, 2003). One main requirement of peptide labeling is the binding sites should not prevent or affect the binding mechanism of the peptide to the mechanism (Lupetti et al., 2003).

4.1 Peptidomimetics on Tumor Tissue

Immune-based therapies have gain importance as cancer cells demonstrate tumor-specific and tumor-selective antigens (Suzanne L., Charles G., & Drew M., 2012). Although there are many factors promotes cancer cells such as living habits, dysfunction of the immune system, environmental factors, genetic mutation; development and progression of the tumor is related to the specific tumor-immune microenvironment (He, Hu, Hu, & Li, 2015). In the treatment of tumor specimens which associated with tumor regression, tumor physiology, tumor microenvironment, and how their expression is regulated in that microenvironment must be fully comprehended. (Ribas, 2012)

Tumor microenvironment has a significant role in cancer progression due to the intercellular signaling between tumor cells and their microenvironment. Tumor cell can continue to live and proliferate in a metastatic environment based on its ability to increase the antigenic outcome of its microenvironment (Watnick, 2017). Tumor cells obtain the ability to escape the immune system and progress potential vital disease.

As shown in Figure 4.1, tumor tissue forms a new vasculature system in and around its surface to extend the diffusion limit of oxygen (Watnick, 2017). Besides, cancer cells adapt various pathways to regulate negative signals through cell surface and inhibit T-cell activation.

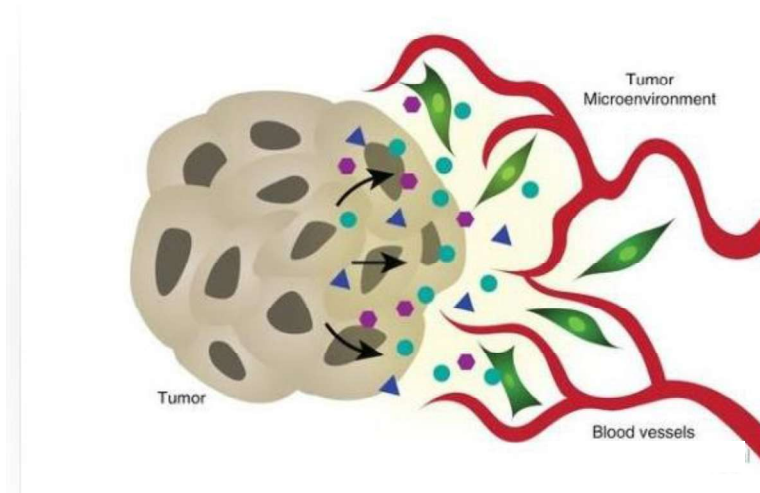


Figure 4.1: The interaction of the tumor and its microenvironment.

Tumor growth becomes independent in which tumor progression initiates by the invasion of stromal cells by the tumor. Cancer cell hides from the immune system by disguising of immune cells checkpoints which are induced on T cell activation (Ribas, 2012). Especially in multiple areas tumorigenesis and subsequent outgrowth of tumors are highly related to pathways of tumor stromal cell signaling (Watnick, 2017). Infiltrating cells are comprised of immune/inflammatory cell that contains B and T cells, neutrophils, mast cells, dendritic cells and macrophages (Watnick, 2017). The T cells identify cancer cell then originates cytotoxic T lymphocytes (CTLs) and eliminate cancer cells. Under normal conditions, so-called 'immune checkpoints' have in balance that generates a proper intensity of T cell as a response.

Latest studies focus on reducing this inhibition by local manipulation of immune-checkpoint molecules in the tumor microenvironment (Taube, et al., 2014). Immune checkpoints on molecules are concentrate on cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), Programmed Death-1 (PD₁) and its ligands PD-L₁ and PD-L₂, (He, Hu, Hu, & Li, 2015). The PD-1 receptor is an inhibitory T cell receptor and CTLA-4 is a coinhibitory receptor that regulates T cells (Ribas, 2012).

Figure 4.2 describes how these receptors blocking signaling in tumor immunotherapy. T cells recognize antigens on the cancer cell surface with T cell receptors (TCR). CTLA-4 initiates negative regulation signaling on T cells in case of PD-1 receptor, negative regulation on T cells is established within tissues and tumor microenvironment during ligation with PD-L₁ and PD-L₂ (Ribas, 2012).

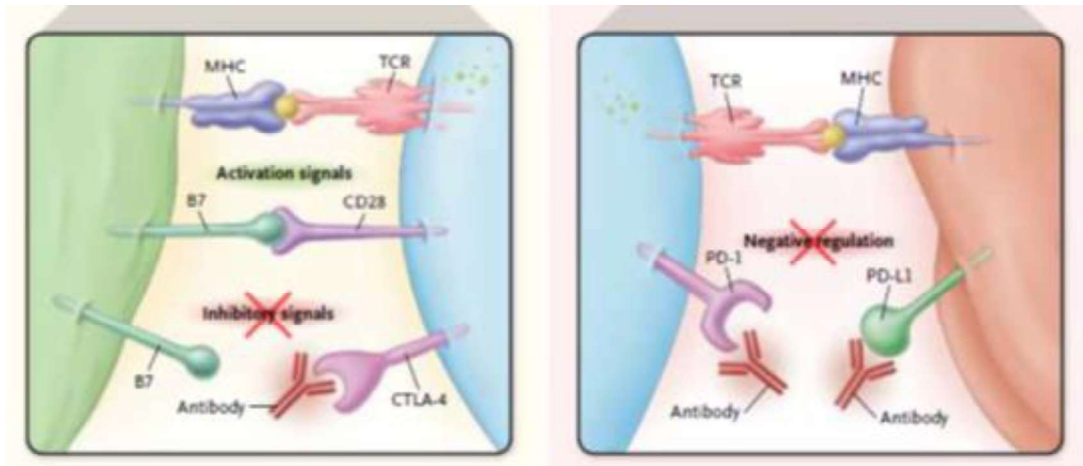


Figure 4.2: Blockade the signaling pathway of PD-1 and CTLA-4 in the tumor environment.

Contrary to CTLA-4, the main role of PDL-1 is to restrain the T cell activity (Suzanne L., Charles G., & Drew M., 2012). Regulation pathway of T cells demonstrates that PD-1 inhibition has less side effect and better antitumor activity than CTLA-4 inhibition (Ribas, 2012).

As explained in Figure 4.2, PD-1 and CTLA-4 express different way of regulating T cell immunity. Due to their ability to limit the activity of T cells at the time of on inflammatory response. PD-1 antigens provide the protection of normal tissues from collateral destruction (Ribas, 2012). In other words, immense distribution of this molecule provides a more general role in protecting tissues from inflammation (He, Hu, Hu, & Li, 2015). Various types of cancers contain PD-L₁ molecules especially non-small cell lung cancer (NSCLC).

One of the methods that can enhance the antitumor immune response is reversing the tumor microenvironment by blocking of PD-1 / PD-L₁ pathway. A new wide space for antitumor activity in immunotherapy can be provided by blocking PD-1 or PD-L₁ by antibodies. PD-1 and PD-L₁ antibodies have little toxicity a good safety and tolerated method (He, Hu, Hu, & Li, 2015).

The most interesting results from clinical trials demonstrate that PD-L₁ blockage pathway for advanced tumors is more effective than utilizing a therapeutic method that PD-1 region (He, Hu, Hu, & Li, 2015). Therefore, in order to suggest more feasible therapeutic options, varying approaches for combining PD-L₁ pathway inhibitors have been studied recent years. In this pathway, less toxicity can be achieved by modulating

the immune response selectively in the tumor microenvironment by using PD-L₁ as a therapeutic and diagnostic target.

In specific patterns PD-L₁ expression at tumor surface is associated with tumor types like melanoma, kidney and lung cancer. That information provides research area for identifying tumor types that respond the PD-L₁ (Taube, et al., 2014). Hence, understanding the implementing tumor selectivity characteristics of PD-1/PD-L₁ antibodies is leading data for this research on the basis of tumor markers as the local tumor microenvironment is the key of molecular marking. In order to generate specified therapies, inducing a long-lasting tumor response is vital. Inhibition of PD-L₁ meets this requirement to keep a durable tumor response (Suzanne L., et al., 2012). With the discovery of immune regulation mechanism, the PD-L₁ pathway is utilized in the studies for cancer diagnosis. In addition to that, continuous research on biomarkers which combined with the PD-1/PD-L₁ antigen detection has shown significant progress. With the current studies in our laboratories that aiming to synthesize and develop of highly-selective identifiers are still performed.



5. MOLECULAR MODELING

Molecular modeling is using theoretical models to reveal and comprehend structures, interactions and properties of molecules that cannot be obtained empirically (Bordessa, 2009). Recent advances in computational technology have been using to design potential affinity ligands. Using of molecular modeling provides the evaluation of alternatives and feasibility of these ligands.

5.1 Pharmacophore

Pharmacophore is an approach that has been used to develop drug and imaging agents, *de novo* design and virtual screening (Yang, 2010). According to IUPAC, "pharmacophore is an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response" (Wermuth, Ganellin, Lindberg, & Mitscher, 1998). The basis of pharmacophore is to recognize the biological target by interaction of corresponded parts on the target which are shared with designed molecules (Wermuth, Ganellin, Lindberg, & Mitscher, 1998). General features typically used are hydrogen-bond acceptors, hydrogen bond donors, positively and negatively charged groups, hydrophobic and hydrophilic features donor-acceptor positions, (Leach, Gillet, Lewis, & Taylor, 2010).

To build a pharmacophore model; ligand-based manner or structure-based manner can be utilized. Common chemical properties of multiple ligands which are necessary for their bioactivity are extracted to establish a ligand-based pharmacophore modeling (Yang, 2010). This strategy is used when there are no particular macromolecular target (Yang, 2010). In contrast to ligand-based model, structure-based pharmacophore modeling is based on the 3-D structure of the target or ligand directly (Yang, 2010). Principal of structure-based pharmacophore model involves probing the interaction points between ligand and target, then assembling these features to the designed molecule (Yang, 2010).

The main advantage of pharmacophore is using the generated model for pharmacophore-based virtual screening (VS) (Yang, 2010). Virtual screening is 3-D chemical database that one can search potential ligands among the thousands of known molecules (Yang, 2010). Alternative structures are chosen according to their common or scaffold moiety and their framework from databases consist of both ‘real’ and ‘virtual’ compounds to exhibit the same biological activity (Leach, Gillet, Lewis, & Taylor, 2010).

Along with virtual screening, another approach of pharmacophore model that have been used is *de novo* design of ligands (Yang, 2010). In contrast to virtual screening, original candidate molecules is formed based on the necessities of a given model (Yang, 2010).

5.2 Methodology for Design Peptides and Peptidomimetics

The most simple way to design biologically active 3-D peptide analogs is mimicing the specific interactions between the ligand and proteins (Leach, Gillet, Lewis, & Taylor, 2010). These specific interactions are called as hot-spots which contain the key binding elements to provide fitting into the active site of ligands. Figure 5.1 shows the designing steps of biologically active peptidomimetic (Akram, et al., 2014).

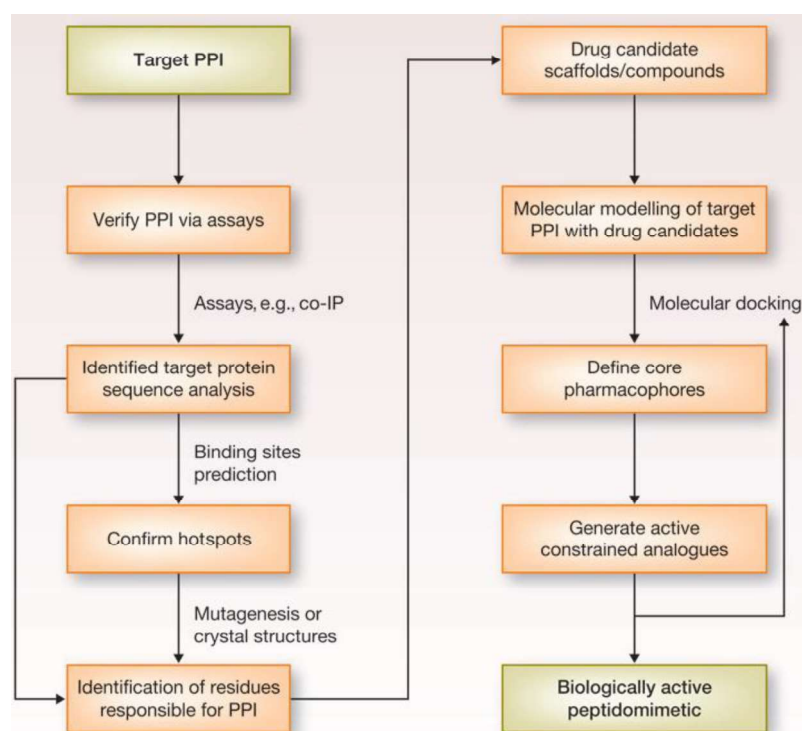


Figure 5.1: Schematic diagram of development of peptidomimetic pathway steps.

In order to design peptides, one must consider conformational limitations where the intramolecular interactions of the protein could be lost (Ruzza, 2012). The most straightforward way to overcome this problem is cyclization of peptide sequence which increase the *in vivo* stability by connecting the C-end with the N-terminus (Ruzza, 2012). Another way to maintain stability is to introduce the alkyl group at the side chain which provide to overcome membrane by enhancing the lipophilicity of the peptide (Ruzza, 2012). Many cancer types express peptide receptors characteristic *in vivo*. According to these findings, peptide-probes are labeled with radionuclides for imaging of tumors (Ruzza, 2012). Generally labeled peptide-probes are developed by isolation of natural peptides or structure-based *de novo* design (Ruzza, 2012). Both peptides and peptidomimetics show great potential as imaging agents either rational and empirical aspects.



6. PURPOSE OF THE THESIS AND FUTURE AIMS

Disease results from molecular failure of regulated functions of normal cells. Inflammation are maintained as biological response of the body to the injury. After tissues are regenerated, inflammation is removed from damaged area. However, when tissue cells continue to proliferate in an inflammation rich environment, tumor cells evolve from inflammatory cells. Cancer is one of these diseases that occurs due to tissue cells proliferation. Although the development of new therapeutic drugs, there has been limited studies about imaging probes which can help early diagnose therefore improves the recovery rate and process. New features gained by immune cells and stromal cells located in the tumor microenvironment can be identified by biomarker *in vivo* applications. In this thesis it is aimed to synthesize the that can bind to negatively charged bacteria membranes to identify and monitor the focus of infections. Furthermore, peptidomimetics were designed and synthesized to bind specific biomarkers of immunological and stromal cells in tumor microenvironment with high selectivity and affinity.

In this thesis, imaging of cancer cells with organic molecules (peptidomimetics) and imaging of focus of inflammation with probe carried peptides are aimed to be achieved by considering four main elements; (i) molecular target relevant to disease of interest, (ii) affinity of ligand that will bind to the target (iii) appropriate molecular imaging system and (iv) synthesize of molecular imaging agent to detect desired target. By this means PET-CT, pharmaceutical sciences and biochemistry come together to produce new molecules that can image biological function in living subject.

Peptides and various fragments of peptidomimetics were synthesized, they were purified and characterized. After laboratory studies are completed biomarker affinity and stability analyses will be performed. According to obtained results from these studies, modifications and alterations will be applied to improve performance. Once biologically active compounds has been identified, they will be conjugated with DOTA-chelates radio-labeling with Ga⁶⁸ for PET-CT imaging.



7. EXPERIMENTAL PART

7.1 Peptide Synthesis

Peptide synthesis pathway consist of removal of fmoc group of the resin, coupling of aminoacides respectively, removal of F-moc group, coupling of dye and cleavage of peptide from resin. These steps are the same for every peptide sequence except the amount of aminoacid weights that used for coupling reactions. Table 7.1 provides individual aminoacid weights to synthesize each sequence of peptides.

Table 7.1: Aminoacid Weights Used for Coupling Reactions.

Aminoacid Code		Aminoacid Name	Weight in mg
Lys	K	Lysine	110 mg
Leu	L	Leucine	82.2 mg
Arg	R	Arginine	152 mg
Ile	I	Isoleucine	82.8 mg

According the molecular docking results following peptide sequences were synthesized. All aminoacids that were used in peptide chains below have the L conformation.

- Rhodamine B-KLKLK
- Rhodamine B-RLKLR
- Rhodamine B-RIRIKIRI

7.1.2 Synthesis of KLKLK sequence with Fmoc-chemistry

96.6 mg (0.087 mmol, 0.44 meq/g) rink amide MBHA resin (RAM) is weighed in reaction vessel. The resin is swelled in DMF (2x) for 20 minutes. Then, the solvent is drained.

7.1.2.1 Removal of Fmoc group of the resin

The Fmoc protecting group is removed by treating the pre-swollen resin with 20% piperidine solution (4.2 mL piperidine, 16.8 mL DMF) in DMF for 10 minutes (2x10 mL). Then, the solution is drained and the resin is excessively washed with DMF.

7.1.2.2 Coupling of lysine (K)

The Fmoc-protected amino acid K (110 mg, 5.5 eq.) dissolved in HBTU (80.8 mg, 5 eq.) and DMF (1.5 mL) solution, followed by DIPEA (85.2 μ L). After addition of DIPEA, solution is mixed and added to the resin in 30 seconds. Mixture is applied to the resin and allowed to stand for 1 hour under constant agitation. Then, the solution is filtered off and the resin is washed excessively with DMF and DCM. The Fmoc group is removed by treating the resin with 20% piperidine in DMF solution (1.7 mL) (2x10 minutes). The resin is subsequently washed with DMF to remove excess piperidine and other by-products.

7.1.2.3 Coupling of leucine (L)

The Fmoc-protected amino acid L (82.8 mg, 5.5 eq.) dissolved in HBTU (80.8 mg, 5 eq.) and DMF (1.5 mL) solution, followed by DIPEA (85.2 μ L). After addition of DIPEA, solution is mixed and added to the resin in 30 seconds maximum. Mixture is allowed to stand for 1 hour under constant agitation. Then, the solution is filtered off and the resin is washed excessively with DMF and DCM. The Fmoc group is removed by treating the resin with 20% piperidine in DMF solution (1.7 mL) (2x10 minutes). The resin is subsequently washed with DMF to remove excess piperidine and other by-products.

7.1.2.4 Removal of the last Fmoc group

The Fmoc group is removed by treating the resin with 20% piperidine in DMF solution (1.7 mL) (2x10 minutes). The resin is subsequently washed with DMF to remove excess piperidine and other by-products. Upon deprotection of last Fmoc group, resin is washed with DMF (4 x 2 mL) (to remove excess piperidine and other by-products.) and DCM (4 x 2 mL) (to remove DMF from milieu, which otherwise interferes with the cleavage step). Then, the resin was dried under suction.

7.1.2.5 Coupling of dye

Rhodamin B (112.2 mg, 5.5 eq.) dissolved in HBTU (80.8 mg, 5 eq.) and DMF (1.5 mL) solution, followed by DIPEA (85.2 μ L). After addition of DIPEA, solution is mixed and added to the resin. Mixture is allowed to stand for 1 hour under constant agitation. Then, the solution is filtered off and the resin is washed excessively with DMF and DCM. The Fmoc group is removed by treating the resin with 20% piperidine in DMF solution (1.7 ml) (2x10 minutes). The resin is subsequently washed with DMF to remove excess piperidine and other by-products.

7.1.2.6 Cleavage of peptide

98% TFA, %1 DCM and %1 TIPS (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS) solution which is known as cleavage cocktail is employed to cleave the peptide sequence from resin. The cocktail (2 x 2.5 mL) added to the resin and waited for 1 hour. Then, the solution is collected and TFA was removed under reduced pressure. The crude material was submitted to LC-MS for detailed analysis.

7.1.3 RLKLR synthesis

This peptide sequence is synthesized based on the procedure depicted previously. The amounts of amino acids and the other reagents are summarized below in Table 7.2.

Table 7.2: Synthesis of RLKLR+Rhodamine B Sequence with Fmoc-chemistry.

Reagent	Amount	Reagent	Amount
Resin	96.6 mg	Leucine (L)	82.2 mg
HBTU	80.8 mg	DIPEA	85.2 μ L
DMF	2x1.5 mL	DMF-piperidine	2x1.7 mL
Arginine (R)	152 mg	Rhodamine B	112.2 mg
Lysine (K)	110 mg		

After the coupling of Rhodamine dye, dye conjugated peptide is cleaved from resin with TFA:DCM:TIPS (v/v/v 98:1:1). 1.1 mL of this cocktail (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS) is added to resin and the resin is allowed to stand for one hour (the step is repeated twice). After volatile materials are removed under reduced pressure,

the crude material is purified with HPLC and the product is submitted to LC-MS for characterization.

7.1.4 Synthesis of RIRIKIRI sequence with Fmoc-chemistry

This peptide sequence is synthesized based on the procedure depicted previously. The amounts of amino acids and the other reagents are summarized below in Table 7.3

Table 7.3: Synthesis of RIRIKIRI+Rhodamine B Sequence with Fmoc-chemistry.

Reagent	Amount	Reagent	Amount
Resin	96.6 mg	Isoleucine (I)	82.8 mg
HBTU	80.8 mg	DIPEA	85.2 μ L
DMF	2x1.5 mL	DMF-piperidine	2x1.7 mL
Arginine(R)	152 mg	Rhodamine B	112.2 mg
Lysine(K)	110 mg		

After the coupling of Rhodamine dye, dye conjugated peptide is cleaved from resin with TFA:DCM:TIPS (v/v/v 98:1:1). 1.1 mL of this cocktail (1078 μ L TFA, 11 μ L DCM, 11 μ L TIPS) is added to resin and the resin is allowed to stand for one hour (the step is repeated twice). After volatile materials are removed under reduced pressure, the crude material is purified with HPLC and the product is analyzed by LC-MS for characterization.

7.2 Peptidomimetics as Organic Molecules

7.2.1 Synthesis of tert-butyl 2-chloroethylcarbamate (2)

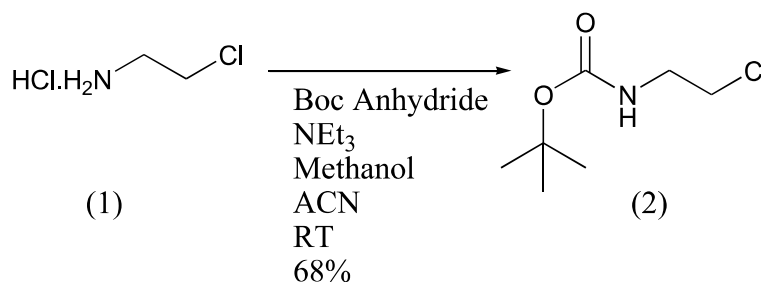


Figure 7.1: Synthesis of tert-butyl 2-chloroethylcarbamate (2).

Tert-butoxycarbonyl anhydride (3.38 g, 15.60 mmol), and trimethylamine (4.35 mL, 31.20 mmol) are dissolved in a mixture of methanol:acetonitrile (50:50, 30 mL), and the reaction is stirred at room temperature for 15 minutes. Then, 2-chloroethylamine hydrochloride (1) (1.81 g, 15.60 mmol) is dissolved in a mixture of methanol/acetonitrile (1:1, 15 mL) and is added to reaction mixture over 1.5 hours. Reaction is completed after this addition. The solvents are evaporated under reduced pressure. The residue is redissolved in EtOAc (40 mL), and washed with distilled water (2x30 mL). Organic phase is dried over sodium sulfate and concentrated under reduced pressure to afford the title compound as a viscous liquid (Baell, et al., 2004). Yield: 68 %. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ ppm: 1.43 (s, 9H), 3.39 (t, 2H, $J = 6.5$ Hz), 3.72 (t, 2H, $J = 6.5$ Hz), 4.95 (s, 1H).

7.2.2. Synthesis of tert-butyl 3-bromopropyl-carbamate (4)

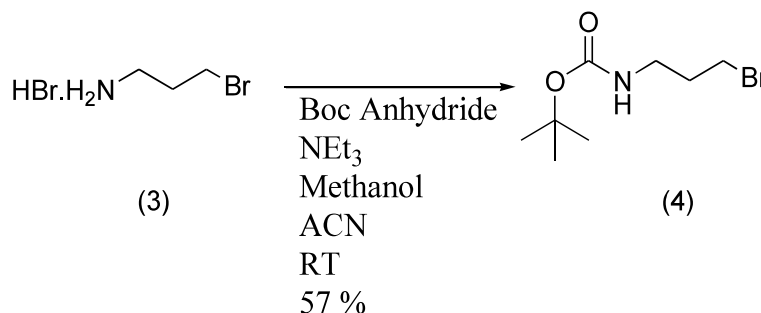


Figure 7.2: Synthesis of tert-butyl 3-bromopropyl-carbamate (4).

Tert-butoxycarbonyl anhydride (3.38 g, 15.60 mmol), and trimethylamine (4.35 mL, 31.20 mmol) are dissolved in a mixture of methanol/acetonitrile (1:1, 30 mL), and the reaction is stirred for 15 minutes at room temperature. 3-Bromopropylamine

hydrobromide (3) (3.42 g, 15.60 mmol) dissolved in a mixture of methanol: acetonitrile (50:50, 15 mL) is added to reaction mixture over 1.5 hours. Reaction is completed after this addition. The solvents are evaporated under reduced pressure. The residue is redissolved in EtOAc (40 mL) and washed with water (2x30 mL). Organic phase is dried over sodium sulfate, and is concentrated under reduced pressure to afford the title compound as a viscous liquid (Baell, et al., 2004). Yield: 57 %. ^1H -NMR (500 MHz, CDCl_3) δ ppm: 1.45 (s, 9H), 2.06 (q, 2H, , J = 6.5 Hz), 3.28 (q, 2H, J = 6.5 Hz), 3.46 (t, 2H, J = 6.5 Hz), 4.66 (s, 1H)

7.2.3 Synthesis of tert-butyl 2-(2-(ethoxycarbonyl)phenoxy)ethyl-carbamate (5)

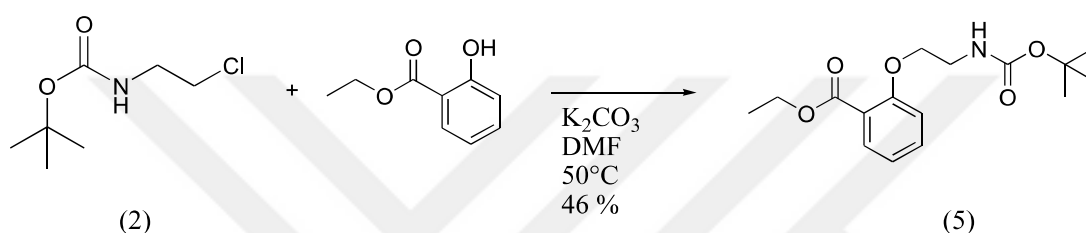


Figure 7.3: Synthesis of tert-butyl 2-(2-(ethoxycarbonyl)phenoxy)ethylcarbamate (5).

Ethyl salicylate (1.52 mL, 10.34 mmol), K_2CO_3 (5.73 g, 41.46 mmol), and tert-butyl 2-chloroethylcarbamate (2) (1.84 g, 10.23 mmol) are stirred in DMF (30 mL) at 50°C for 24 h. Subsequently, the reaction is cooled down to room temperature and is washed with a mixture of EtOAc (40 mL), and 1M HCl (40 mL). Organic phase is washed with saturated NaHCO_3 solution (2x40 mL), and brine solution (2x40 mL). Then, it is dried over sodium sulfate, filtered and concentrated. The residue is purified by flash column chromatography (eluent: ethyl acetate/ hexanes [40:60, v/v]) to give (5) (2 g, 46%) as yellow liquid (Baell, et al., 2004).

7.2.4 Synthesis of tert-butyl 3-(2-(ethoxycarbonyl)phenoxy)propylcarbamate (6)

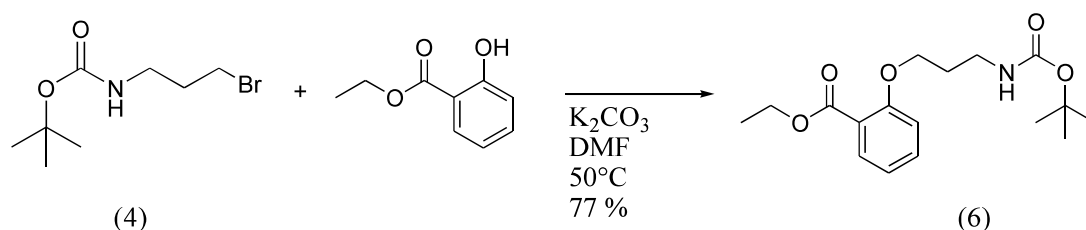


Figure 7.4: Synthesis of tert-butyl 3-(2-(ethoxycarbonyl)phenoxy)propylcarbamate (6).

Ethyl salicylate (0.88 mL, 5.98 mmol), K_2CO_3 (3.31 g, 23.98 mmol) and tert-butyl 3-bromopropylcarbamate (4) (2.10 g, 5.92 mmol) are stirred in DMF (20 mL) at 50 °C for 24 h. Subsequently, the reaction is cooled down to room temperature and washed with a mixture of EtOAc (20 mL) and 1M HCl (20 mL). Organic phase is washed with saturated $NaHCO_3$ solution (2x20 mL) and brine solution (2x20 mL). Then, it is dried over sodium sulfate, filtered and concentrated. The compound is obtained as viscous oil (Baell, et al., 2004). Yield: 77%. ^1H -NMR (500 MHz, $CDCl_3$) δ ppm: 1.39 (t, 3H, $J = 7$ Hz), 1.45 (s, 9H), 2.05 (q, 2H, $J = 6.5$ Hz), 3.41 (q, 2H, $J = 6.5$ Hz), 4.13 (t, 2H, $J = 6.5$ Hz), 4.38 (q, 2H, $J = 7$ Hz), 5.96 (s, 1H), 6.96 - 7.01 (m, 2H), 7.47 (t, 1H, $J = 8$ Hz), 7.95 (d, 1H, $J = 8$ Hz)

7.2.5 Synthesis of 2-[2-(Boc-amino)ethoxy]benzoic acid (7)

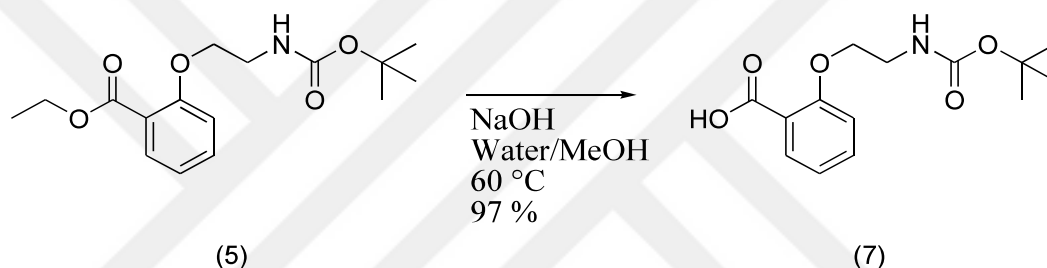


Figure 7.5: Synthesis of 2-[2-(Boc-amino)ethoxy]benzoic acid (7).

Ethyl ester (5) (1.94 g, 6.56 mmol) and NaOH (0.79 g, 19.67 mmol) are solved in mixture of water/methanol (25/75, 25 ml) and refluxed for 6 hours. The solvents are removed under reduced pressure to give a white solid which is then redissolved in ether (15 mL). After mixture is extracted with 2M NaOH (3x15 mL) aqueous layer was washed with ether (15 mL) then acidified with 3M HCl to pH=1 on ice bath. This acidic solution is extracted with ether (3x15 mL). Organic phases are combined, washed with brine (2x15 mL), dried with sodium sulfate. After filtration and removal of the solvent, the title compound was obtained as viscous oil (Baell, et al., 2004). (7). Yield: 97%. ^1H -NMR (500 MHz, $CDCl_3$) δ ppm: 1.43 (s, 9H), 3.65 (t, 2H, $J = 7$ Hz), 4.32 (t, 2H, $J = 7$ Hz), 5.03 (s, 1H), 7.05 (m, 1H, $J = 8$ Hz), 7.15 (m, 1H, $J = 8$ Hz), 7.58 (m, 1H, $J = 8$ Hz), 8.18 (dd, 1H, $J = 8$ Hz).

7.2.6 Synthesis of tert-butyl 2-(4-formylphenoxy)ethyl-carbamate (8)

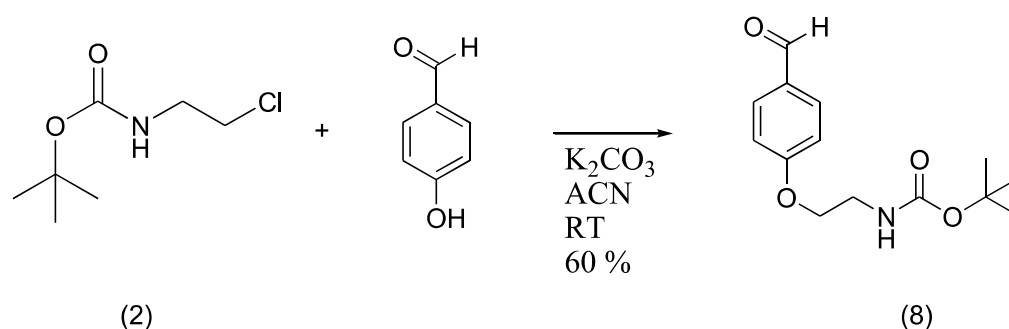


Figure 7.6: Synthesis of tert-butyl 2-(4-formylphenoxy)ethyl-carbamate (8).

To a solution of tert-butyl 2-chloroethylcarbamate (2), (1 g, 5.59 mmol) and 4-hydroxybenzaldehyde (0.75 g, 5.59 mmol) in ACN (15 mL) K_2CO_3 (1.71 g, 12.41 mmol) is added, and the reaction is stirred at room temperature for 15 hours. Then, inorganic salts are filtered and the solvent is evaporated under reduced pressure. The residue is resolved with ethyl acetate (2x25 mL) then extracted water (25 mL) and triturated with 2M NaOH (2x15 mL). After drying with sodium sulfate, EtOAc was evaporated to give (8) as a yellow liquid (Baell, et al., 2004). Yield: 60%. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ ppm: 1.43 (s, 9H), 3.58 (t, 2H, $J = 7$ Hz), 4.1 (t, 2H, $J = 7$ Hz), 4.98 (s, 1H), 6.93 (d, 2H, $J = 8$ Hz), 8.03 (d, 2H, $J = 8$ Hz)

7.2.7 Synthesis of tert-butyl 2-(4-(methoxycarbonyl)phenoxy)ethylcarbamate (9)

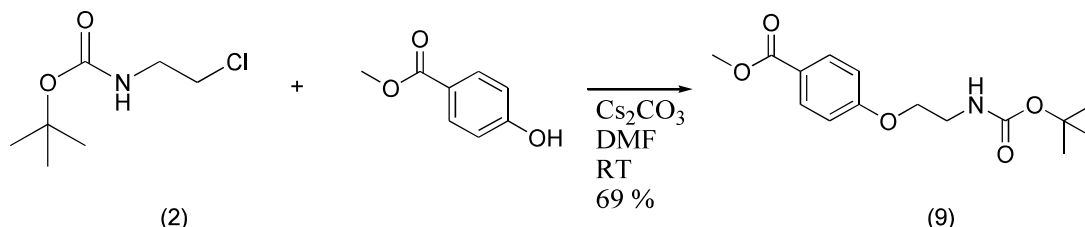


Figure 7.7: Synthesis of tert-butyl 2-(4-(methoxycarbonyl)phenoxy)ethylcarbamate (9).

To a solution of *N*-Boc 2-chloro ethylamine (2), (1.137 g, 6.44 mmol) in DMF (20 mL) Cs_2CO_3 is added then solution is stirred. Methyl 4-hydroxybenzoate is added the reaction flask and the reaction mixture is stirred at room temperature for 48 hours. Inorganic salts are removed with filtration and the solvent is evaporated under reduced pressure. The residue is extracted with ethyl acetate (3x30 mL) and water (30 mL). After drying with sodium sulfate the EtOAc was evaporated. The residue is purified with column chromatography (eluent: ethyl acetate/ hexanes [2:1, v/v]. Yield: 69%.

7.2.8 Synthesis of 6-(*p*-toloxy)benzo[d]thiazol-2-amine (11)

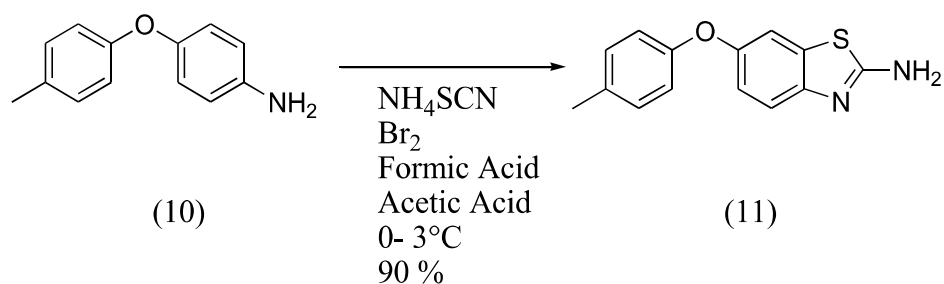


Figure 7.8: Synthesis of 6-(*p*-toloxy)benzo[d]thiazol-2-amine (11).

To a solution of 4-(*p*-toloxy)benzenamine (10) (0.5 g, 2.51 mmol) in a mixture of formic acid/acetic acid (20:80) (72 mL), NH_4SCN (0.69 g, 3.62 mmol) is added and the reaction mixture is cooled to approximately $0-3^\circ\text{C}$ under inert atmosphere. With the exclusion of light from the reaction mixture, bromine (0.182 g, 2.81 mmol) dissolved in acetic acid (18.2 mL) is added dropwise over 1 hour. After the light shield is removed, the mixture is allowed to warm to room temperature overnight. Sodium hydroxide pellets and ice were added with stirring until $\text{pH} = 11$ is attained. Then, the reaction mixture is extracted with EtOAc (3x145 mL). The organic layer is filtered through celite to remove polythiocyanogen $(\text{SCN})_n$. The organic layer is washed with water (2x110 mL), and saturated NaHCO_3 (2x70 mL), respectively. Then it is dried Na_2SO_4 , filtered and concentrated to give (11) as yellow solid (Baell, et al., 2004).

7.2.9 Synthesis of 6-(4-chlorophenoxy)benzo[d]thiazol-2-amine (13)

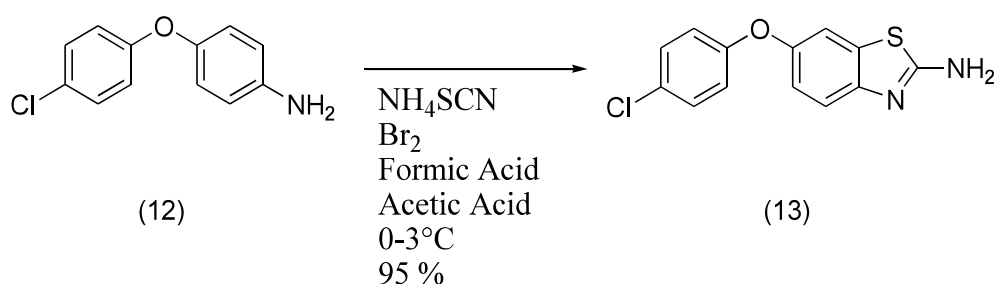


Figure 7.9: Synthesis of 6-(4-chlorophenoxy)benzo[d]thiazol-2-amine (13).

To a solution of aniline (12) (0.551 g, 2.51 mmol) in a mixture of formic acid/acetic acid (20:80) (72 mL), NH_4SCN (0.69 g, 3.62 mmol) is added and the reaction mixture is cooled approximately to $0-3^\circ\text{C}$ under inert atmosphere. With the exclusion of light from the reaction mixture, bromine (0.182 g, 2.81 mmol) dissolved in 18.2 mL acetic acid is added dropwise over 1 hour. After the light shield is removed, the mixture is

allowed to warm to room temperature overnight. Until pH = 11 is attained, sodium hydroxide pellets and ice were added with stirring. The reaction mixture is extracted with EtOAc (3x145 mL). The organic layer is filtered through celite to remove polythiocyanogen. The organic layer is washed with water (2x110 mL) and saturated NaHCO₃ (2x70 mL), respectively. Then, it is dried with sodium sulfate, filtered and the solvent is concentrated to give (11) as yellow solid (Baell, et al., 2004). Yield: 95%. (Baell, et al., 2004). ¹H-NMR (500 MHz, CDCl₃) δ ppm: 5.18 (s, 2H), 6.9 (d, 2H, *J*=7 Hz), 7.02 (m, 1H), 7.28 (dd, 3H, *J* = 8 Hz), 7.49 (d, 1H, *J* = 8 Hz).

7.2.10 Synthesis of 4-[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-propoxy] benzaldehyde (15)

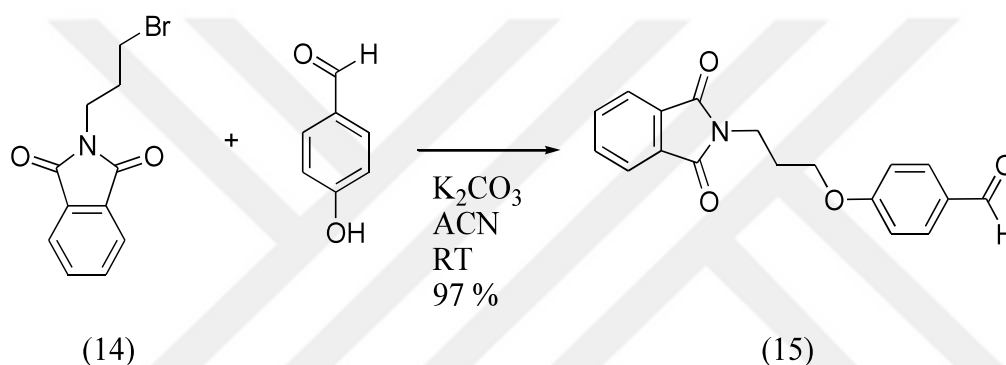


Figure 7.10: Synthesis of 4-[3-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-propoxy] benzaldehyde (15).

To a solution of 3-bromo-*N*-propylphthalimide (14) (1.5 g, 5.59 mmol) and 4-hydroxybenzaldehyde (0.75 g, 6.15 mmol) in acetonitrile (15 mL), K₂CO₃ (1.71 g, 12.41 mmol) is added and the reaction is stirred at room temperature for 15 h. Upon completion, the reaction mixture is poured into iced water (100 mL). The resulting white precipitate is filtered out, triturated with 2M NaOH (2x15 mL), and water (15 mL), respectively. The title compound (15) is obtained as white needles (Baell, et al., 2004). Yield: 97%. ¹H-NMR (500 MHz, CDCl₃) δ ppm: 3.92 (t, 2H, *J* = 7 Hz), 4.13 (t, 2H, *J* = 7 Hz), 6.88 (d, 2H, *J* = 7 Hz), 7.71 (m, 2H), 7.78 (m, 2H), 7.85 (m, 2H), 9.87 (s, 1H).

7.2.11 Synthesis of tert-butyl (2-hydroxyethyl)-carbamate (17)

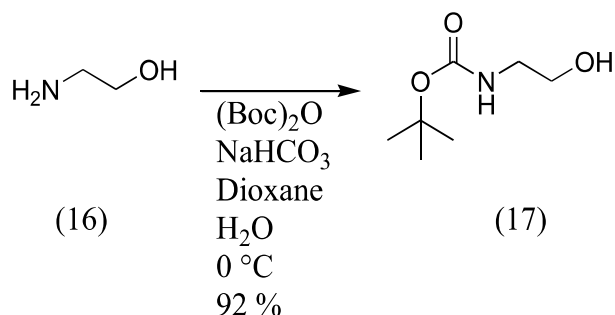


Figure 7.11: Synthesis of tert-Butyl (2-hydroxyethyl)-carbamate (17).

To a solution of ethanolamine (0.5 mL, 8.30 mmol) in dioxane (10 mL) and H_2O (5 mL), saturated NaHCO_3 solution (5 mL) is added in room temperature. The reaction mixture is cooled to 0°C , and is treated dropwise with $(\text{Boc})_2\text{O}$ (2 g, 9.19 mmol). Upon the completion of addition, the reaction is allowed to warm to room temperature and is further stirred for 1 hour. Then, the mixture is diluted with water (10 mL) and extracted with ethyl acetate (2x20 mL). Organic phases are combined, dried over sodium sulfate and concentrated under reduced pressure. Tert-Butyl (2-hydroxyethyl) carbamate (2) is obtained as yellow oily liquid. Yield: 92%. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ ppm: 6.65 (t, 1H), 4.55 (t, 2H, $J = 5$ Hz), 3.34 (q, 2H, $J = 5$ Hz), 2.97 (q, 2H, $J = 5$ Hz), 1.36 (s, 9H).

7.2.12 Synthesis of 2-[Boc-amino]ethyl-mesylate (18)

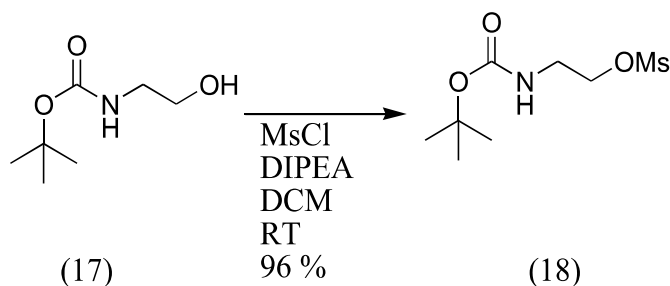


Figure 7.12: Synthesis of 2-(Boc-amino)ethyl-mesylate (18).

Tert-butyl (2-hydroxyethyl) carbamate (1.343 g, 8.33 mmol) and diisopropylethylamine (1.07 mL, 8.33 mmol) is dissolved in DCM (15 mL) under N_2 and cooled with ice. Afterwards, methane sulfonyl chloride (645 μL , 8.33 mmol) is added over 15 minutes and the reaction mixture is stirred at room temperature for 4 hours. The reaction mixture is diluted with DCM and washed with cold water (2x20 mL). Organic phase is dried over sodium sulfate, filtered, and concentrated under

reduced pressure to afford the title compound as a viscous oil. Yield: 96 %. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ ppm: 4.93 (bs, 1H), 4.29 (t, 2H, $J = 5$ Hz), 3.48 (q, 2H, $J = 5$ Hz), 3.04 (s, 3H), 1.45 (s, 9H).

7.2.13 Synthesis of tert-butyl 2-(4-formylphenoxy)ethyl-carbamate (8)

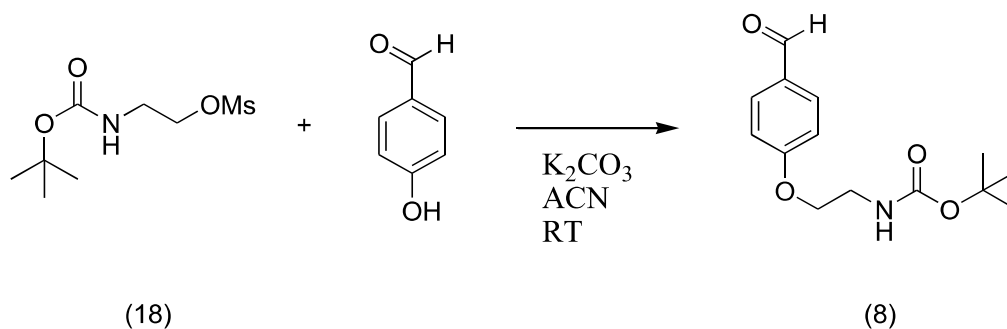


Figure 7.13: Synthesis of tert-butyl 2-(4-formylphenoxy)ethyl-carbamate (8).

To a solution of tert-butyl 2-chloroethylcarbamate (18), (1.56 g, 6.5 mmol) and 4-hydroxybenzaldehyde (0.87 g, 7.15 mmol) in acetonitrile (20 mL), K_2CO_3 (1.99 g, 14.43 mmol) is added, and the reaction is stirred at room temperature for 15 hours. Then, inorganic salts are removed by filtration, and the solvent is evaporated under reduced pressure. The residue is extracted with ethyl acetate (2x35 mL) and water (35 mL), triturated with 2M NaOH (2x25 mL). According to TLC, the yield of the reaction is poor (Baell, et al., 2004).

8. RESULTS AND DISCUSSIONS

In the scope of this project, developing and testing functionally new imaging agents for the imaging of tumors microenvironments and the inflammations at the cellular and molecular level for conventional PET agents are aimed. Therefore, peptide sequences and peptidomimetics which will be labeled with fluorescent dye and radionuclides, respectively were designed, synthesized, purified and characterized. Peptidomimetics which are able to bind with high affinity and selectivity will be utilized to bind biomarkers expressed by immune and stromal cells of tumor microenvironment. In addition, peptides will be used for imaging of inflammation in body.

8.1 Antibacterial Peptide Design for Imaging of Inflammation

In conjunction with the overall purpose of our work, we have been intrigued in the synthesis of antibacterial peptides to monitor inflammation in body. Antibacterial AMP (Antimicrobial Peptides) are short peptide chains which have α -helical structure with L-configuration. They have exhibit antibacterial properties by interacting with the bacterial cell membrane or the metabolism of the bacterium. Altering the antibacterial effect is possible by changing the structure of the amino acids and the structure of the peptides.

In order to attack the bacterial membrane, antibacterial peptides require some amphipathic (both hydrophobic and hydrophilic) properties as shown in Figure 8.1. Throughout it is thought that the antibacterial activities of AMPs are determined by hydrophobic amino acids and cationic residues. The peptides form an electrostatic attraction with the negatively charged bacterial surface through the cationic sites present and enter the lipid layer of the membrane layer through hydrophobic sites. As a result of this mechanism, the structures of the phospholipids in the bacterium and the charges are among the criteria that influence the mechanism of action of the antibacterial peptides. Antibacterial peptides have been found to bind strongly to phospholipids, the most basic structure of the cell membrane. The observation that

these peptides bind more strongly to negatively charged liposomes than uncharged liposomes suggests that being positively charged is required for antibacterial activity.

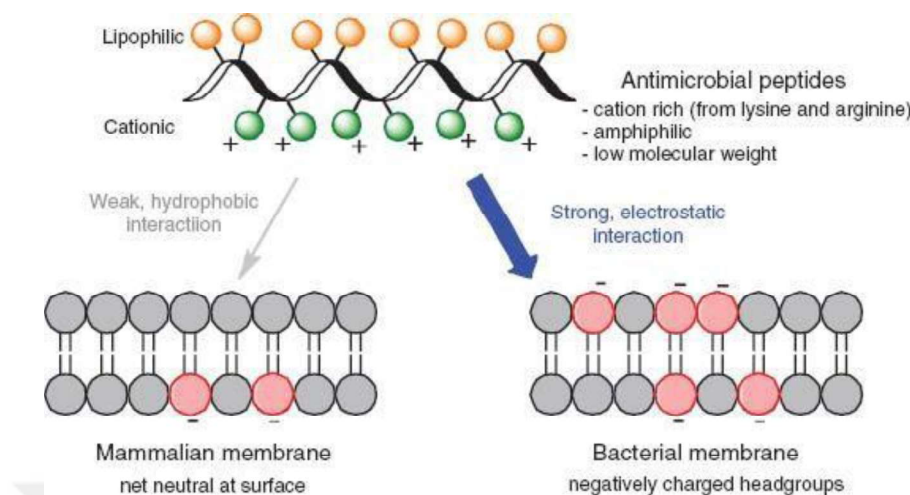


Figure 8.1: Antibacterial peptide structure.

Various peptides were modeled based on their positive/negative charge distribution and positions, hydrophobic/hydrophilic amino acid distribution and positions. The structural characteristics of these sequences is that their central parts interact with to the negatively charged cell membrane. In order to create an electrostatic interaction between the peptide and the cell membrane, positively charged amino acids as in the Figure 8.2, such as Lysine (K) and Arginine (R) will be substituted in the peptide sequence.

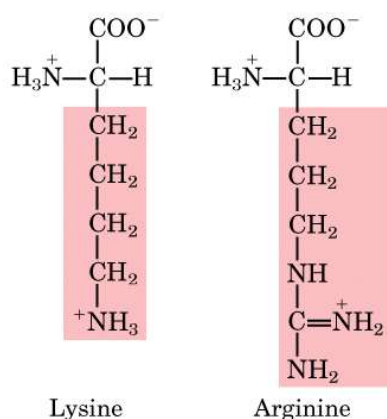


Figure 8.2: Positively charged side chains of K and R amino acids.

According to these criteria following peptide sequences are chosen;

- RLKLR
- RIRIKIRI

- KLKLLK

8.1.1 Solid-phase peptide synthesis

These peptides are synthesized by solid phase peptide synthesis on rink amide resin according to literature. In each cycle of peptide synthesis, the carboxylic acid of amino acid is first activated with HBTU/DIPEA and then reacted with free amine on the resin. Subsequently, Fmoc group on the resin is cleaved in the presence of piperidine so that the next amino acid can be added. Rhodamine B is coupled with the peptide on the resin as the same procedure. The synthesized peptide is cleaved from the resin with a mixture of TFA/water/TIPS and crude material is purified by RP-HPLC with ACN/TFA gradient system. The final product is characterized by MALDI-TOF. Figure 8.3 shows a schematic flowchart of peptide synthesis.

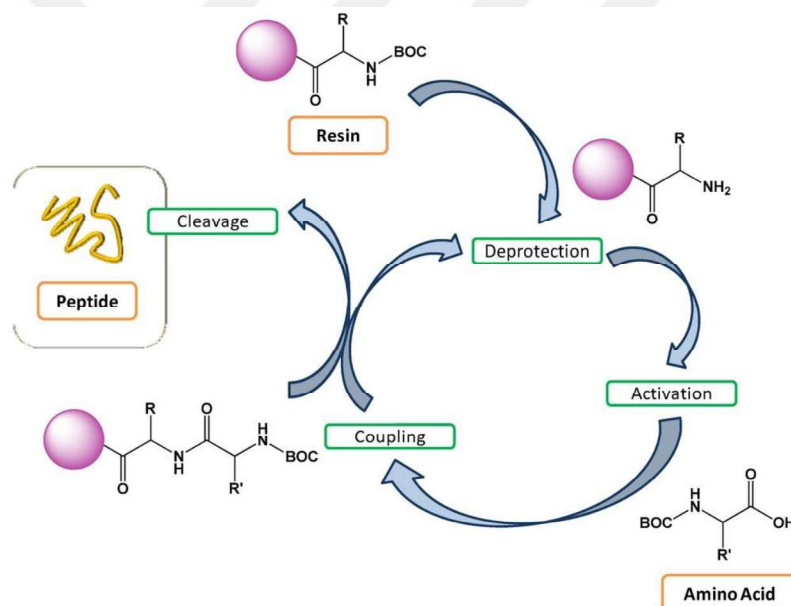


Figure 8.3: Peptide synthesis pathway.

Peptides must traditionally be prepared through so-called orthogonal protection, allowing a strict control over the reactivity of amine residues both side chain and N-terminal. The most widely employed protecting groups on rink amide resins are *tert*-butoyloxycarbonyl (*t*-Boc,) and 9H-fluorene-9-ylmethoxycarbonyl (Fmoc). In comparison to *t*-Boc, Fmoc is cleaved under milder reaction conditions (i.g., the treatment of piperidine in DMF) and hence, the latter is a better choice. Although Fmoc group can be removed by primary and tertiary amines, using cyclic secondary amine is more effective because of their nucleophilicity (Omar, et al., 2016). As shown in the

Figure 8.4, a specific protection group (for this example *t*-Boc) is used for protection of all side chains while the other one (for this example Fmoc) is used for protection of amine groups at the N-terminal of each amino acid. According to this methodology, two protecting groups that are cleaved under different conditions are utilized to protect amine groups. Hence, based on the cleaving conditions, one can control which functional groups become reactive, thereby to minimize the likelihood of side-reaction and by-products.

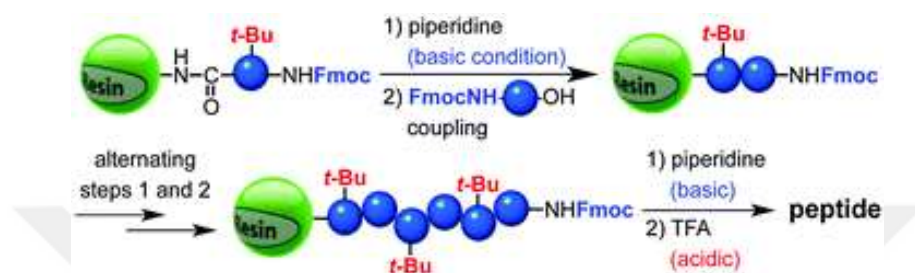


Figure 8.4: Orthogonal protection for peptide synthesis.

As discussed in the sections above, these molecules are designated to bind bacteria cells with high affinity. After their binding and stability analyses are performed, specified molecules will be labeled with radionuclides. In order to perform these analyses, 3 peptides are labeled with Rhodamine-B dye to be able to follow peptides in their target affinity analyses. The purpose of coupling the dye with peptides is to be able to monitor the peptide bioactivity in fluorescence microscopy. By means of the carboxylic acid group in the Rhodamine B structure, it can be easily coupled with amines as the same method used in peptide synthesis. The structure of Rhodamine is shown in Figure 8.5.

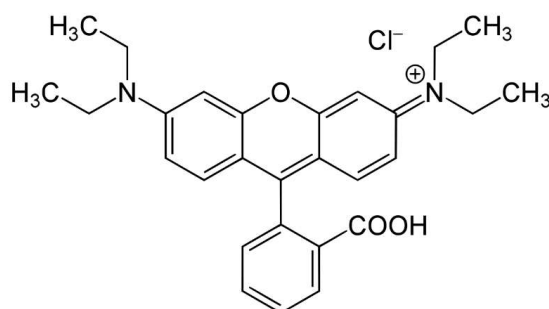


Figure 8.5: Chemical Structure of Rhodamine B.

8.1.2 Rink amid resin for peptide synthesis

The resin employed in solid-phase peptide synthesis is Rink Amide (RAM) resin as it provides preferable structural characteristics to peptides. Amide functional group on C - terminal is especially preferred because they tend to be more stable under in vivo conditions, due to their resistance against serum proteases enzymes. The role of these amides groups is to mask C-terminal, which serves as a recognition site for the protein-cleaving enzymes. In addition, its contribution to peptide stability, amide group at the C-terminal also makes easier to the peptide chains forms α -helix formation at the 3D structure. Figure 8.6 shows the difference between the structure of natural peptide and *de novo* synthesized peptide with rink amide resin.

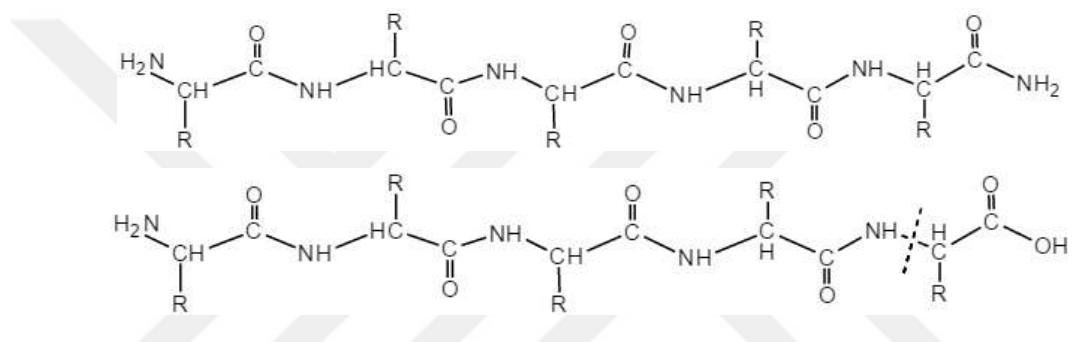


Figure 8.6: The natural structure of peptide chain and peptide synthesized with RAM resin.

8.1.3 Peptide synthesis and labelling the peptides with Rhodamine B

HPLC and mass spectroscopy chromatogram of KLKLLK + Rhodamine B sequence are given below in Figure 8.7 and Figure 8.8 respectively. Additionally, 2-D chromatogram results from the synthesized peptide in Figure 8.9.

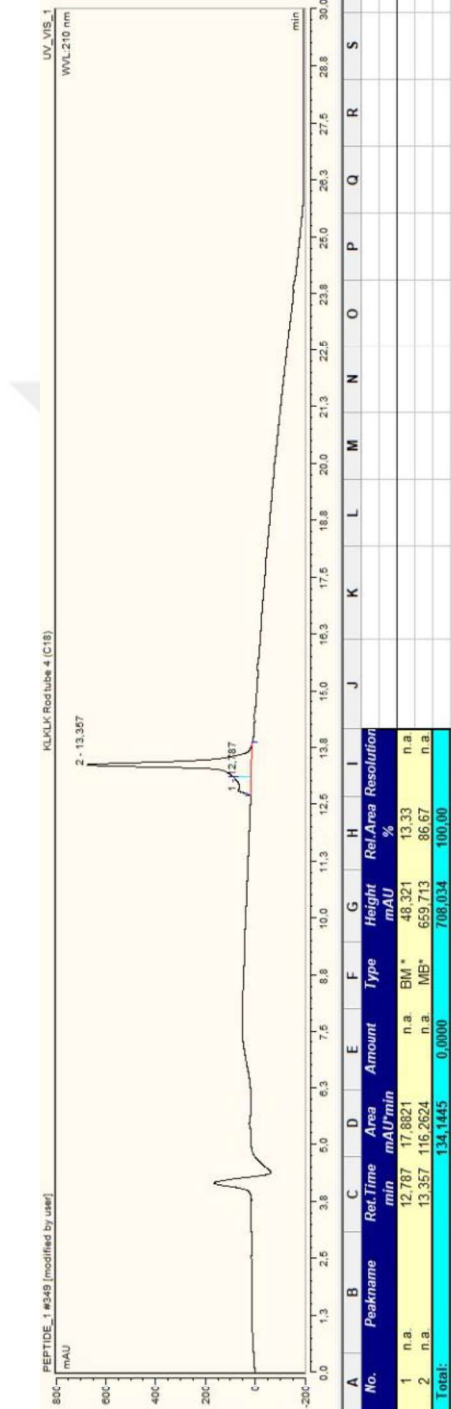


Figure 8.7: HPLC chromatogram of purified KLK1K + Rhodamine B.

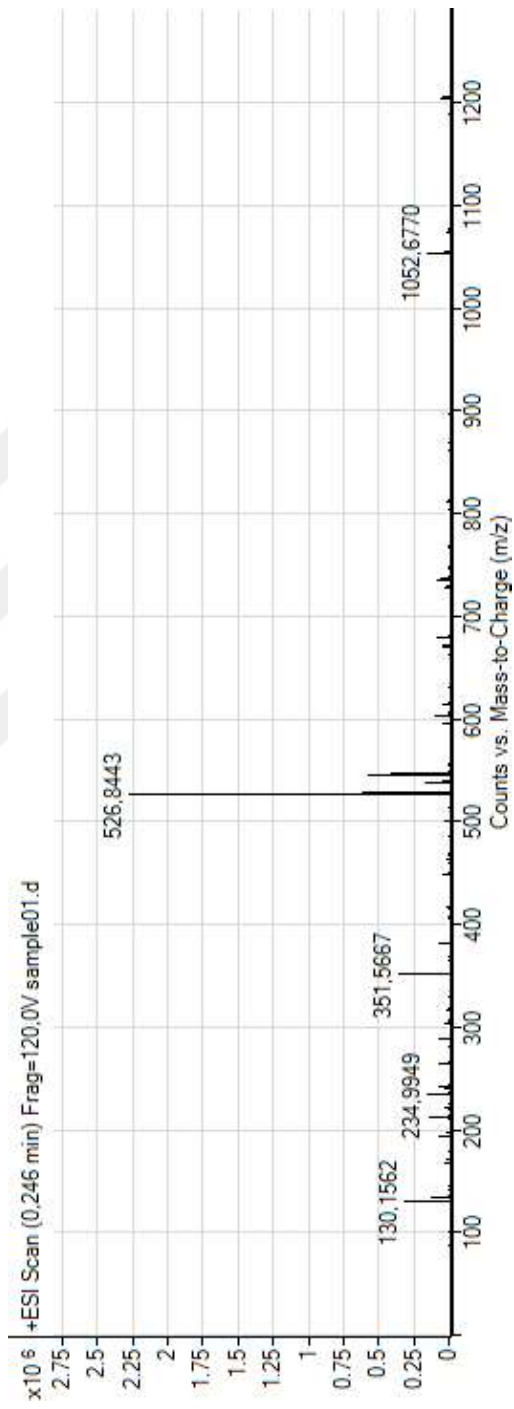


Figure 8.8: Mass chromatogram of KLK1K + Rhodamine B.

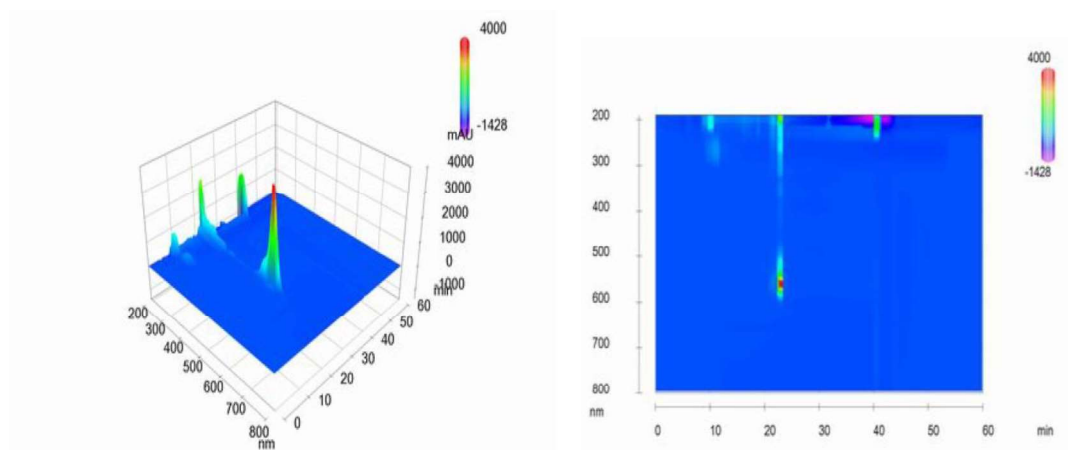


Figure 8.9: Pre-purified KLKLLK+Rhodamine B Sequence at 10 mg/ml concentration.

According to these results, the mass chromatogram shows that the peptide + dye sequence is at the expected weight, as a result of which we have concluded we had successfully synthesized the peptide. Molecular mass of KLKLLK sequence with C-term amide and Rhodamine B is 627.8 g/mol and 479 g/mol, respectively. Total mass of peptide sequence is the sum of these masses with elimination of water and chloride anion which is equal to 1053.3 g/mol. Because of the two charges in the structure, m/z ratio will be 526.6 which is seen as in the mass chromatogram. The signal obtained between retention time of 12 minutes in the first chromatogram indicates that targeted KLKLLK+Rhodamine B sequence has a purity of 86,67 % in light of integration of the corresponding signal. The observed signal at 500-600 nm on the chromatogram indicates the dye was coupled with the peptide sequence successfully.

HPLC and mass spectroscopy chromatogram of RLKLR + Rhodamine B sequence are given below in Figure 8.10 and Figure 8.11 respectively.

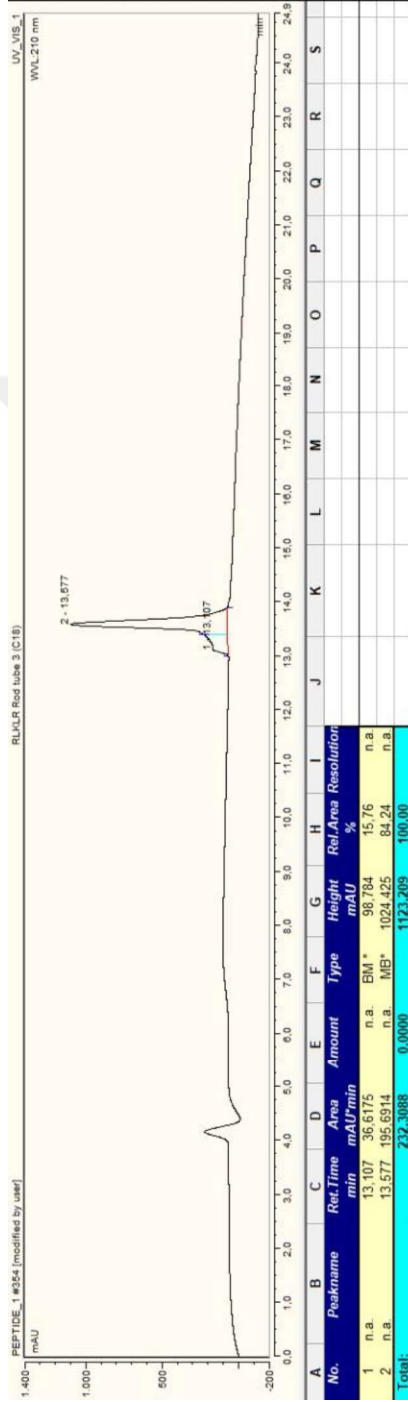


Figure 8.10: HPLC chromatogram of purified RLKLR + Rhodamine B.

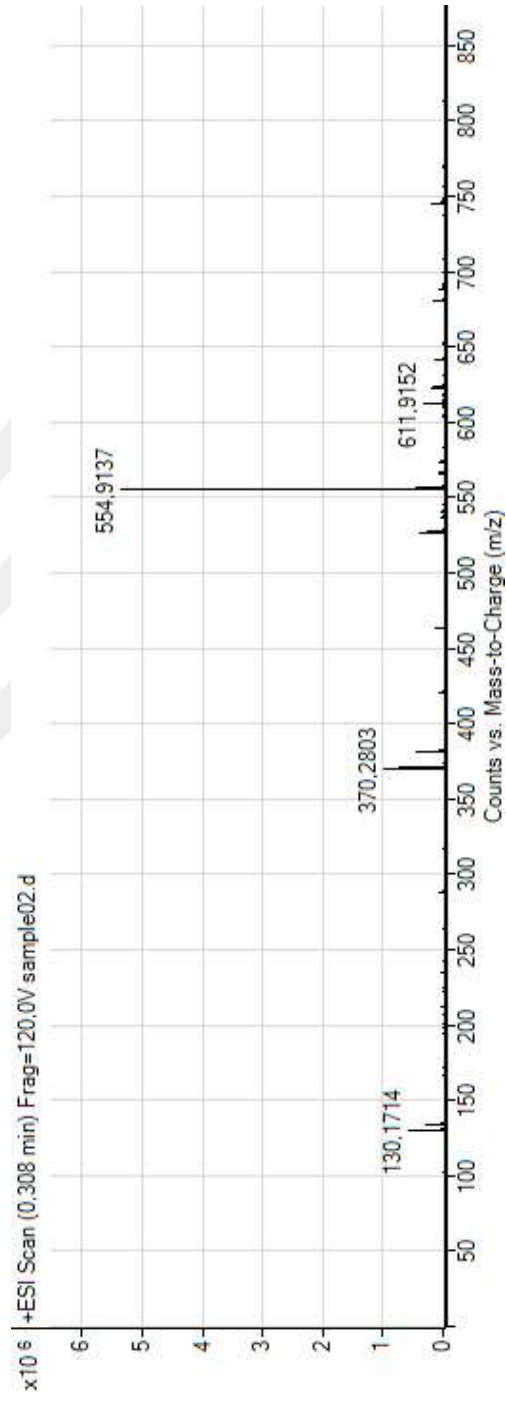


Figure 8.11: Mass chromatogram of RLKLR + Rhodamine B.

According to these results, the mass chromatogram shows that the peptide + dye sequence is at the expected weight, as a result of which we have concluded we had successfully synthesized the peptide. Molecular mass of RLKLR sequence with C-term amide and Rhodamine B is 683.8 g/mol and 479 g/mol, respectively. Total mass of peptide sequence is the sum of these masses with elimination of water and chloride anion which is equal to 1108.4 g/mol. Because of the two charges in the structure, m/z ratio will be 554.2 which is seen as in the mass chromatogram. The signal obtained between retention time of 13 minutes in the first chromatogram indicates that targeted RLKLR + Rhodamine B sequence has a purity of 84,24 % in light of integration of the corresponding signal.

HPLC and mass spectroscopy chromatogram of RIRIKIRI + Rhodamine B sequence are given below in Figure 8.12 and Figure 8.13 respectively.

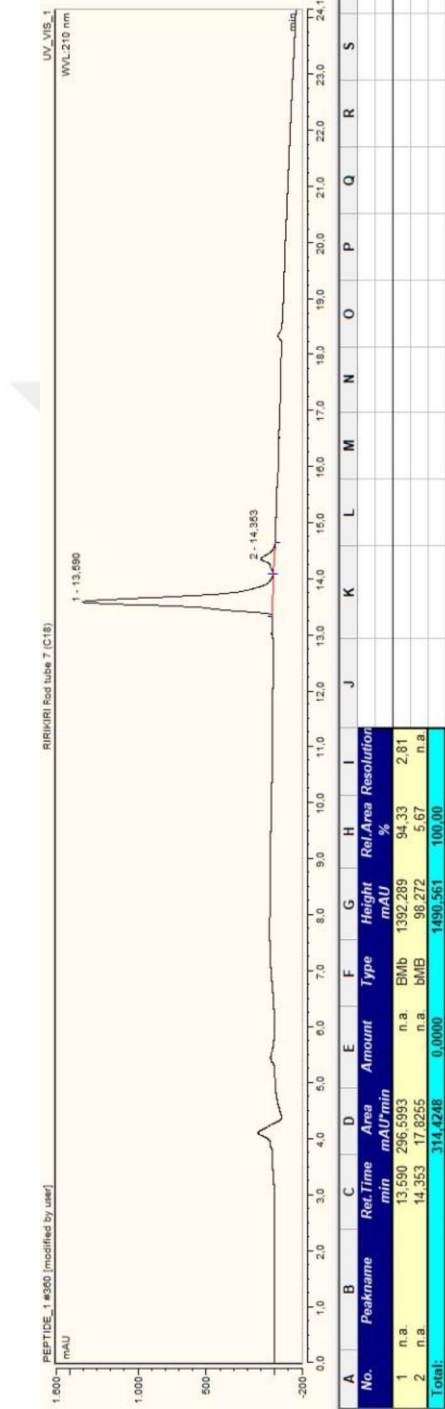


Figure 8.12: HPLC chromatogram of purified RIRIKRI + Rhodamine B.

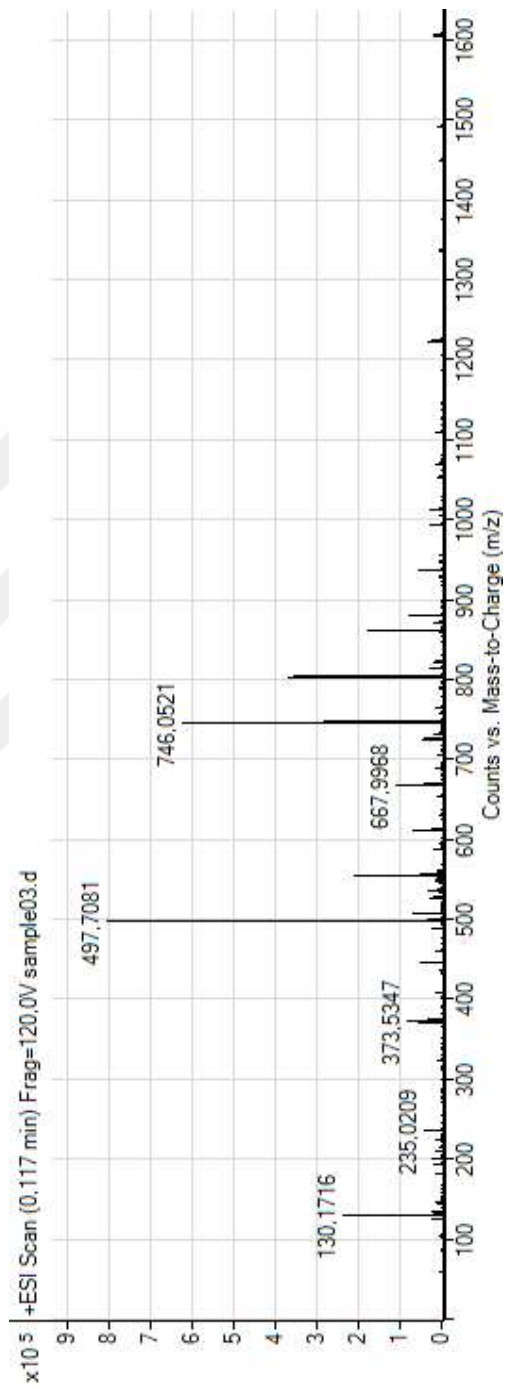


Figure 8.13: Mass chromatogram of RIRIKRI + Rhodamine B.

According to these results, the mass chromatogram shows that the peptide + dye sequence is at the expected weight, as a result of which we have concluded we had successfully synthesized the peptide. Molecular mass of RIRIKIRI sequence with C-term amide and Rhodamine B is 1066.3 g/mol and 479 g/mol, respectively. Total mass of peptide sequence is the sum of these masses with elimination of water and chloride anion which is equal to 1491.8 g/mol. Because of the two charges in the structure, m/z ratio will be 745.9 which is seen as in the mass chromatogram. The signal obtained between retention time of 13 minutes in the first chromatogram indicates that targeted RIRIKIRI+Rhodamine B sequence has a purity of 94,33 % in light of integration of the corresponding signal.

8.2 Peptidomimetic Design for Imaging of Tumor Cells

Firstly, using our rational design algorithm, our project team has conducted a preliminary study to introduce a 'targeting molecule' (ligand) for the PD-L₁ biomarker, which is among the most promising target molecules for cancer immunotherapy today. With the help of bioinformatics databases and scientific literature, ligands that are able to bind with high affinity and specificity to the PD-L₁ protein were designed with a rational approach and *in silico* techniques. For the designing of this ligands, pharmacophore approach was used for each potential candidate. According to these findings, precursor of peptidomimetics were designed, synthesized, purified and characterized as well as peptides.

Initially, the docking studies were performed to identify the appropriate compounds that will be examined in virtual scans. During the designing process, the optimal parts for binding of the radiolabel/DOTA (not to interfere with ligand-protein binding interaction) were also determined for every molecule as discussed following sections. Among the designed molecules, synthesizable molecules were selected and synthesized. Selection criteria include high scores according to docking studies, ligand-protein interaction and ligand-protein compatibility. It is seen that the peptidomimetic structure inspired by ω -conotoxin GVIA compound is suitable as PD-L₁ ligand.

According to modeling studies, the main chemical structure of peptidomimetic is identified as shown in the Figure 8.14. A Type III peptidomimetic was comprised of three parts based on the interactions between amino acids and its fragments. For

instance, Glu and Asp are highly hydrophilic and negatively charged amino acids in neutral pH. Positively charged amide group that mimic to side chain of Lys was chosen for that specific fragment of molecule.

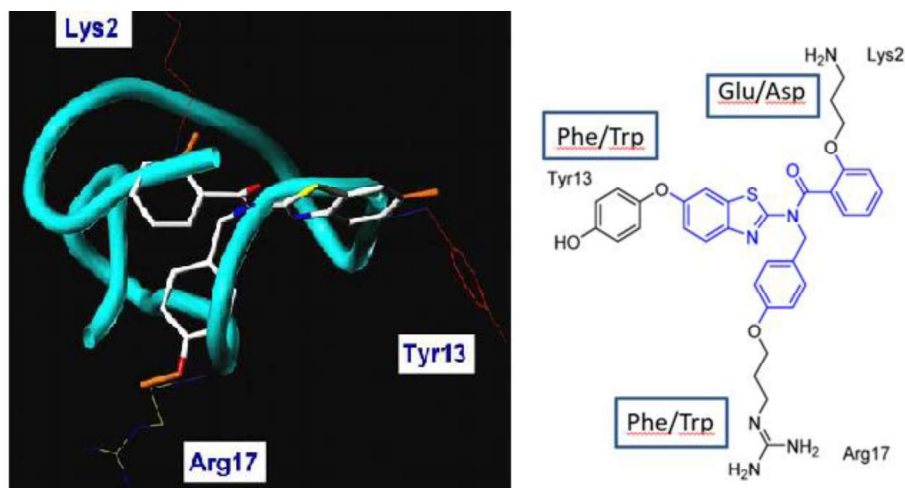


Figure 8.14: First approach for peptidomimetic structure.

Figure 8.15 shows an example of virtual screening studies. Peptidomimetic formed with chosen fragments is the subject of this VS study. There is shape conformity between selected molecule and the protein binding site. The p-ethyl phenoxy group (R1) of the compound is located in the active region of the hydrophobic amino acids (Ile116, Ala121, Tyr56 (B), Ile116 (B)). The primary aliphatic amine group (R3) bound to the 1,2-disubstituted phenyl group of molecule formed a hydrogen bond with the backbone of the amino acid Phe19. The benzothiazole ring has hydrophobic interactions with Tyr123 (B) and Tyr56 amino acids. In addition, the primary aliphatic amine group (R2) found in the alkyloxy chain linked to the 1,4-disubstituted phenyl ring has formed two hydrogen bonds with the backbone of the amino acid Arg125 (B) and the amino acid Asp26 (B). The interaction area of the PD-L₁ protein is shown in dark grey colour and the molecule structure is shown as green colored. Hydrogen bonds are shown with red dashed lines.

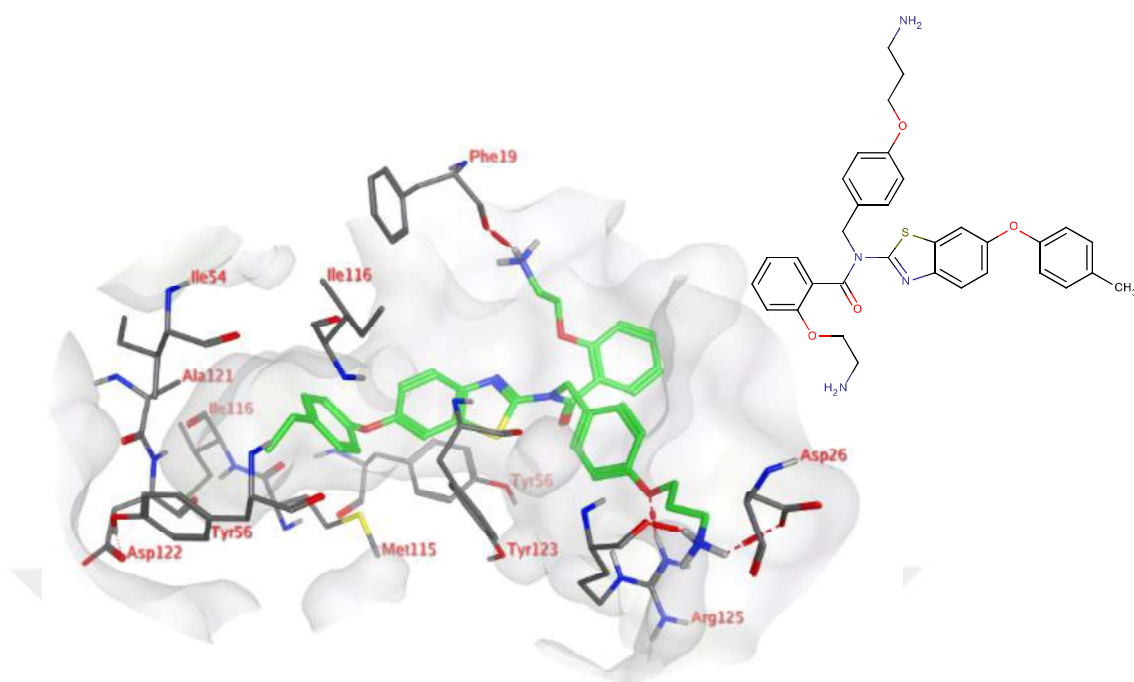


Figure 8.15: VS study example of selected peptidomimetic and target protein.

8.2.1 Derivatization of identified peptidomimetic

After the main body of the molecule was determined, it has been continued to develop according to pharmacophore approach. The main structure was fragmented into three parts (R_1 , R_2 , and R_3) and different segments with various groups were introduced to the design. As a part of my thesis, the synthesis of necessary precursors were optimized. After completion of the studies, individual parts will be combined to acquire the intact peptidomimetics. After R_1 and R_2 coupled with reductive amination, R_3 is coupled with formed structure with peptide (amide) bond. Figure 8.16 describes the R_1 , R_2 , and R_3 as well as their derivatizations.

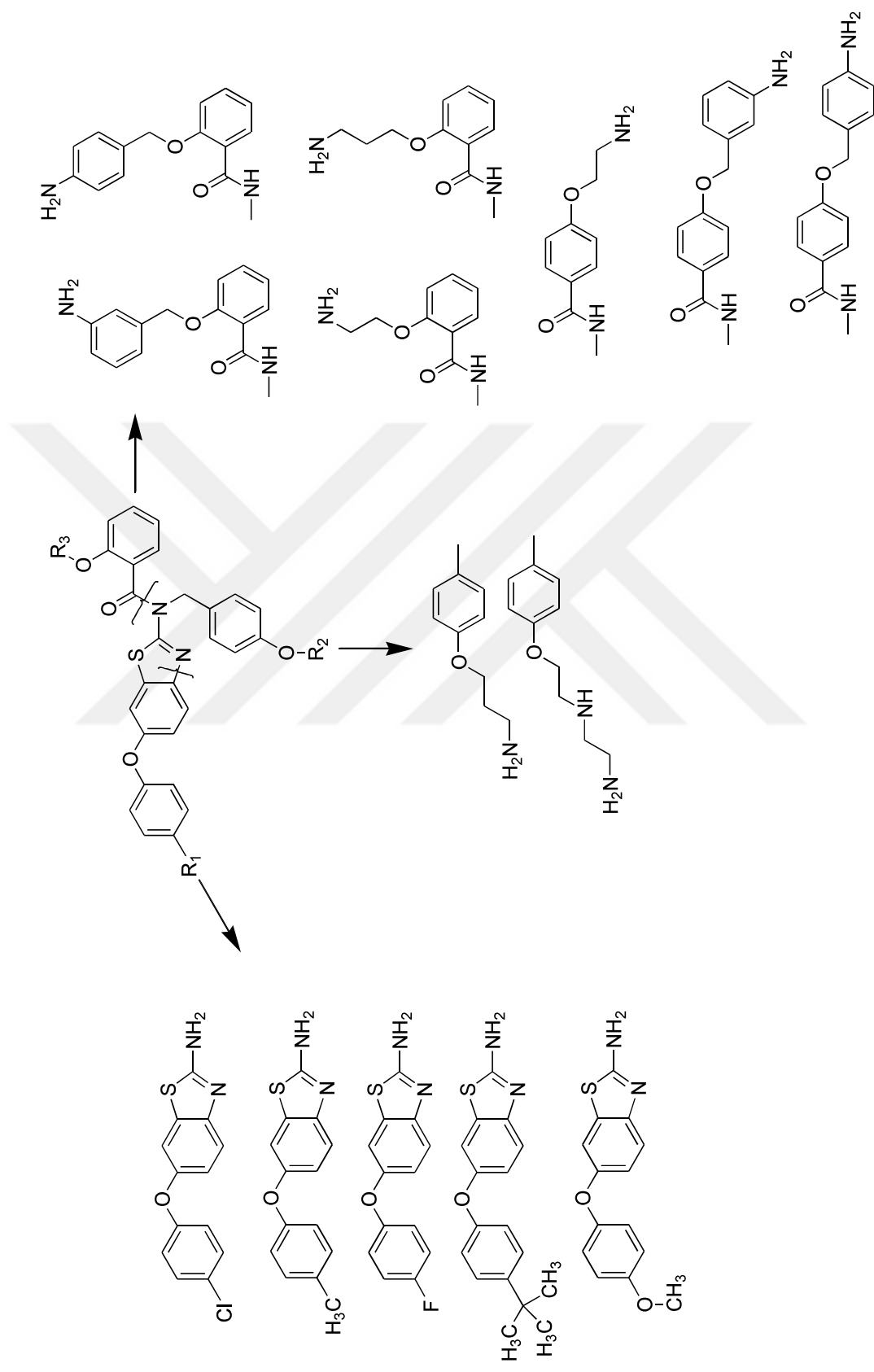


Figure 8.16: Molecule diversification strategy.

After completion of the studies, individual parts will be combined to acquire the intact peptidomimetics. After R_1 and R_2 coupled with reductive amination, R_3 is coupled with formed structure with peptide (amide) bond. Figure 8.17 describes the R_1 , R_2 , and R_3 as well as their derivatizations.

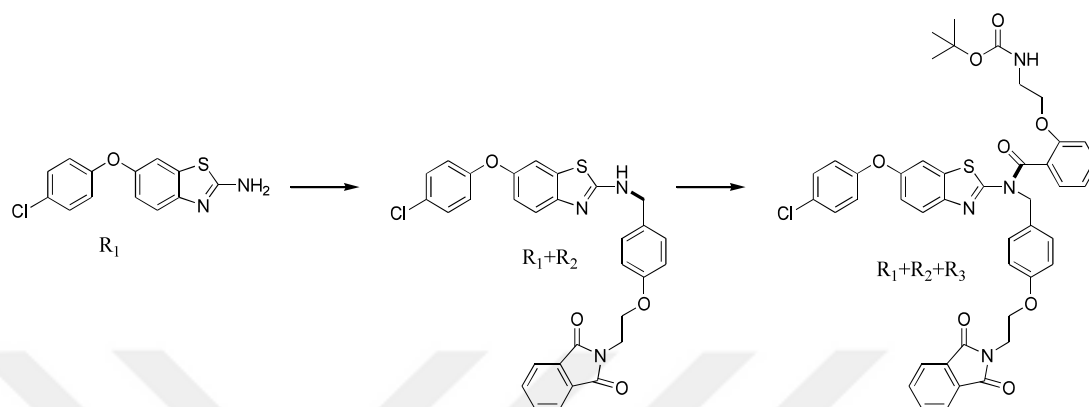


Figure 8.17: Coupling of R_1 , R_2 , and R_3 fragments.

8.2.2 Orthogonal protection for DOTA labeling

As can be seen in the Figure 8.15, there are two molecules harbors two moieties wherein DOTA group could be conjugated. In order to distinguish amine groups synthetically, orthogonal protection is required to conjugate DOTA moiety, later on. As discussed at section above, two protecting groups that are cleaved under different conditions are utilized to provide selective binding to amine groups. Therefore amine groups of R_2 and R_3 segments are synthesized both phthalimide and t-Boc protection groups. While t-Boc is cleaved by TFA which is not affect to Phth protecting group, phthalamide is cleaved by hydrazine hydrate. Figure 8.18 shows an example of an orthogonally protected peptidomimetic. Desired amide group can be deprotected for binding of DOTA.

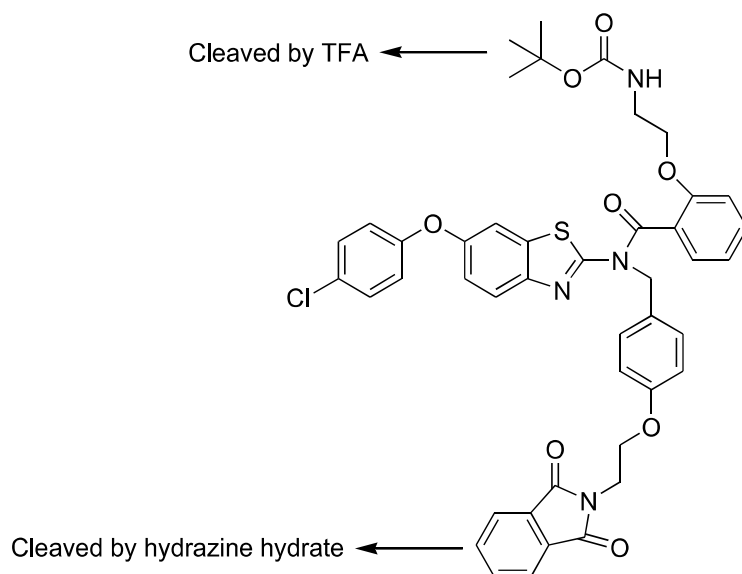


Figure 8.18: Design of orthogonal protection.

8.2.3 Excluding of propylamine derivatives

In the beginning, propylamine contained segments were discussed for R₂ and R₃ parts of peptidomimetic as shown in Figure 8.19. However, after further docking studies it is shown that 2 carbons were preferred instead of 3 carbons because of the minimum free spin and better interaction. Therefore, propylamine is replaced with ethylamine bridge.

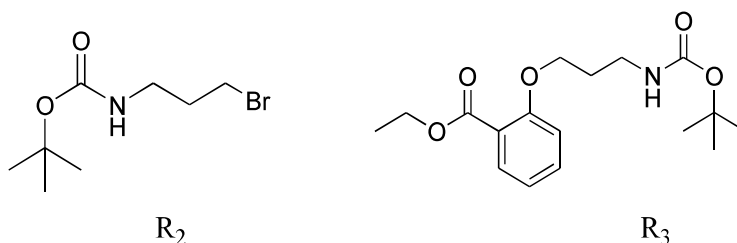


Figure 8.19: Excluded segments from the model.

8.2.4 Mesylchloride chemistry

In order to synthesize the tert-butyl 2-(4-formylphenoxy)ethylcarbamate which is shown in Figure 8.20 as (8), our first attempt was using Cl chemistry for this reaction. Product were afforded with 60% yield. Based on our previous experiences we have turned our attention to obtain better yields to MsCl, giving that MsCl is a better electrophile than Cl. Mesylation of 2-(Boc-amino) ethanol went smoothly, affording 2-(Boc-amino) ethyl mesylate (18) was synthesized in high yield under N₂ atmosphere at a room temperature.

To fulfill the synthesis, 2-(Boc-amino) ethyl mesylate is coupled with 4-hydroxybenzaldehyde in the presence of K_2CO_3 . However, our NMR results indicated no product formation with K_2CO_3 in ACN. As the synthesis tert-butyl 2-(4-formylphenoxy)ethyl-carbamate (8) is hard to achieve, we focused on the 4-[3-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-propoxy] benzaldehyde (15) synthesis.

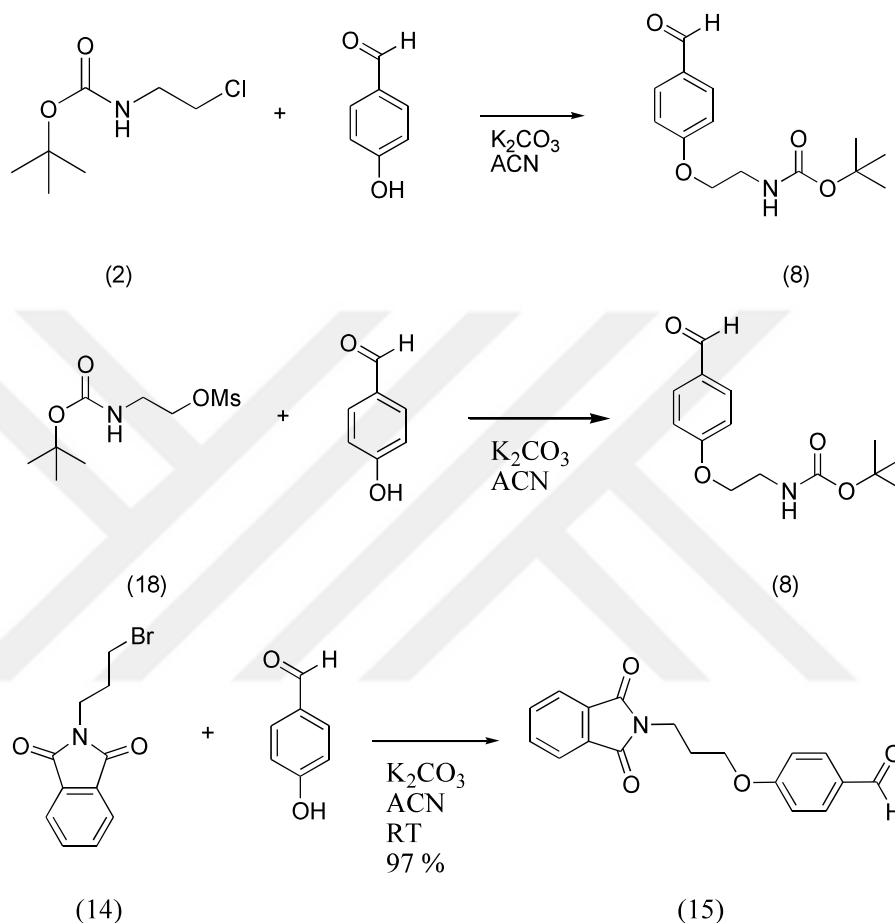


Figure 8.20: Two synthesis steps for molecule (8).



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APPENDICES

¹H-NMR spectra

Figure B1 : ¹H-NMR spectrum of tert-butyl 2-chloroethylcarbamate (2).

Figure B2 : ¹H-NMR spectrum of tert-butyl 3-bromopropylcarbamate (4).

Figure B3 : ¹H-NMR spectrum of tert-butyl 3-

(2(ethoxycarbonyl)phenoxy)propylcarbamate (6).

Figure B4 : ¹H-NMR spectrum of 2-[2-(Boc-amino)ethyloxy]benzoic acid (7).

Figure B5: ¹H-NMR spectrum of tert-butyl 2-(4-formylphenoxy)ethylcarbamate (8).

Figure B6 : ¹H-NMR spectrum of 6-(4-chlorophenoxy)benzo[d]thiazol-2-amine (13).

Figure B7: ¹H-NMR spectrum of 4-[3-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)propoxy]benzaldehyde (15).

Figure B8 : ¹H-NMR spectrum of tert-Butyl (2-hydroxyethyl) carbamate (17).

Figure B9: ¹H-NMR spectrum of 2-(Boc-amino) ethyl mesylate (18).



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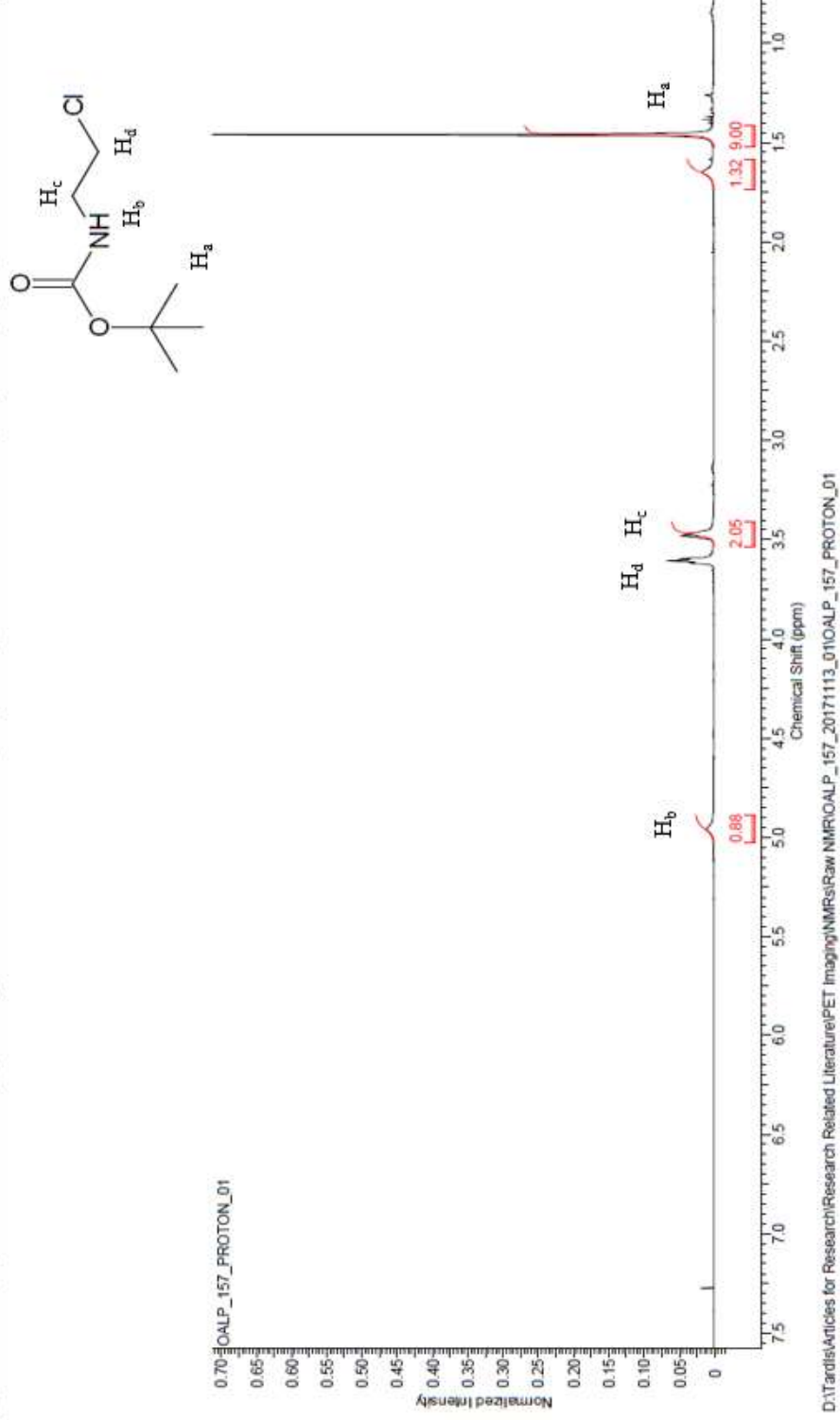


Figure B1 : ¹H-NMR spectrum of tert-butyl 2-chloroethylcarbamate (2).

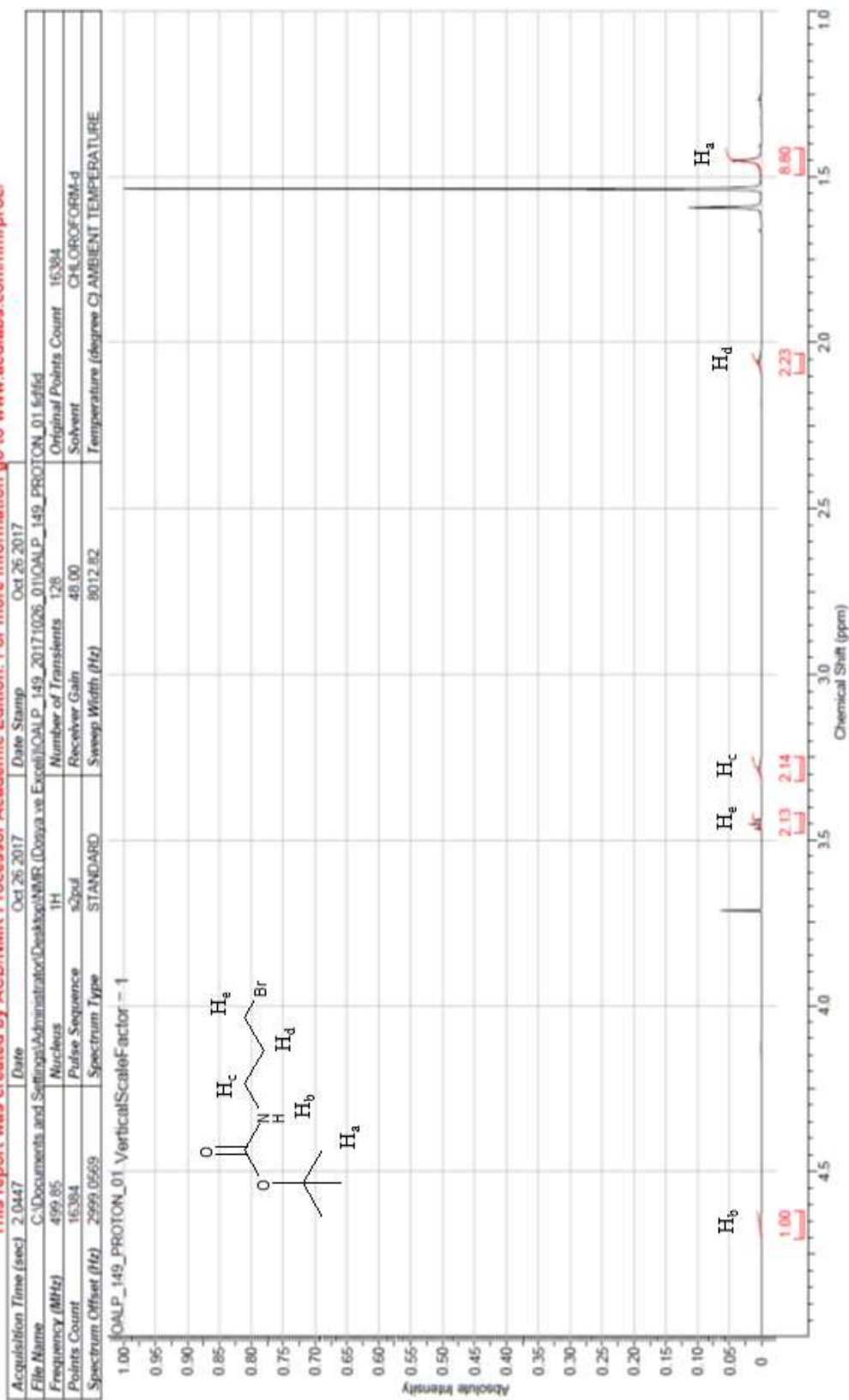


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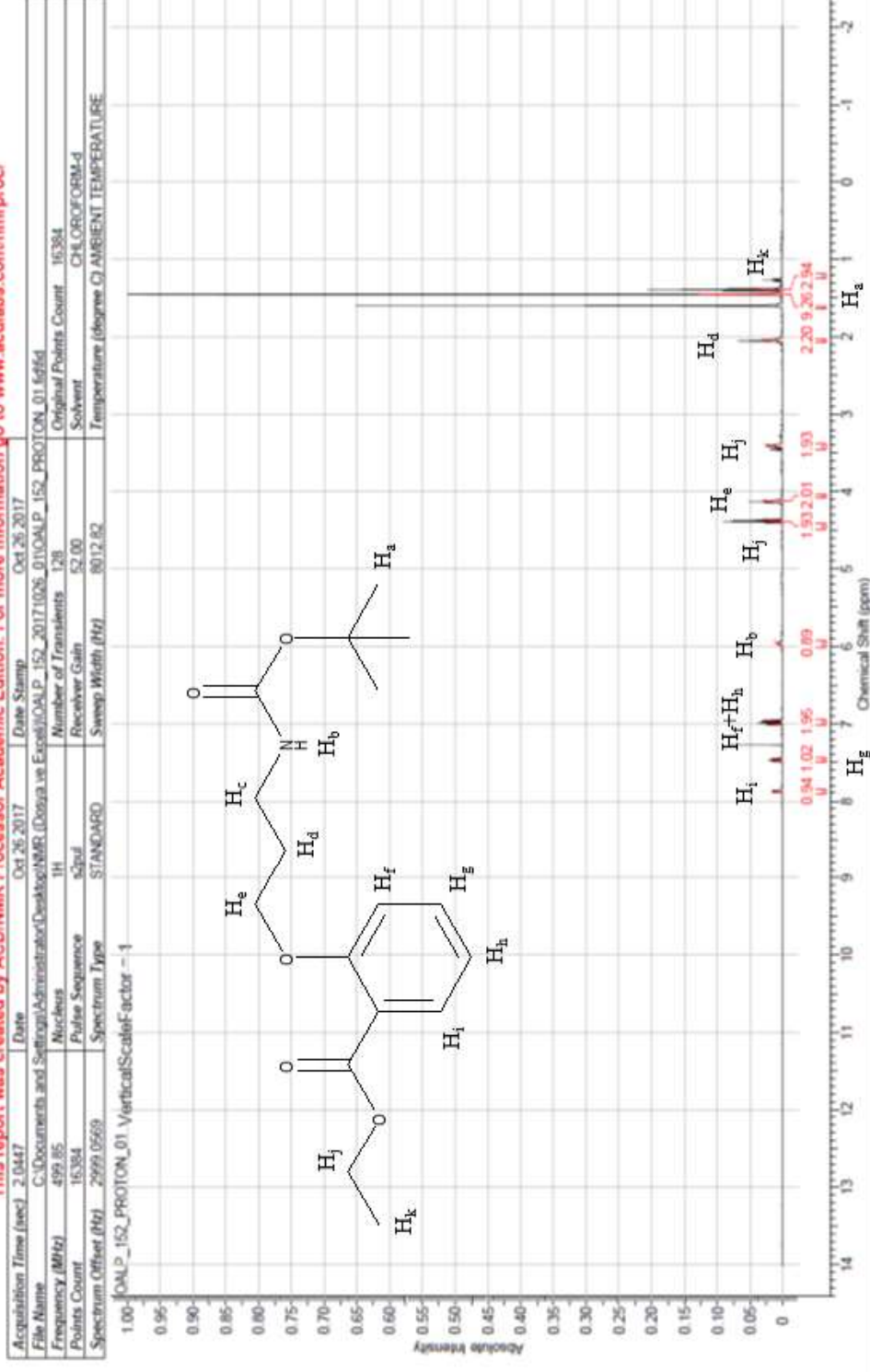


Figure B3 : ¹H-NMR spectrum of tert-butyl 3-(2-(ethoxycarbonyl)phenoxy)propylcarbamate (6).

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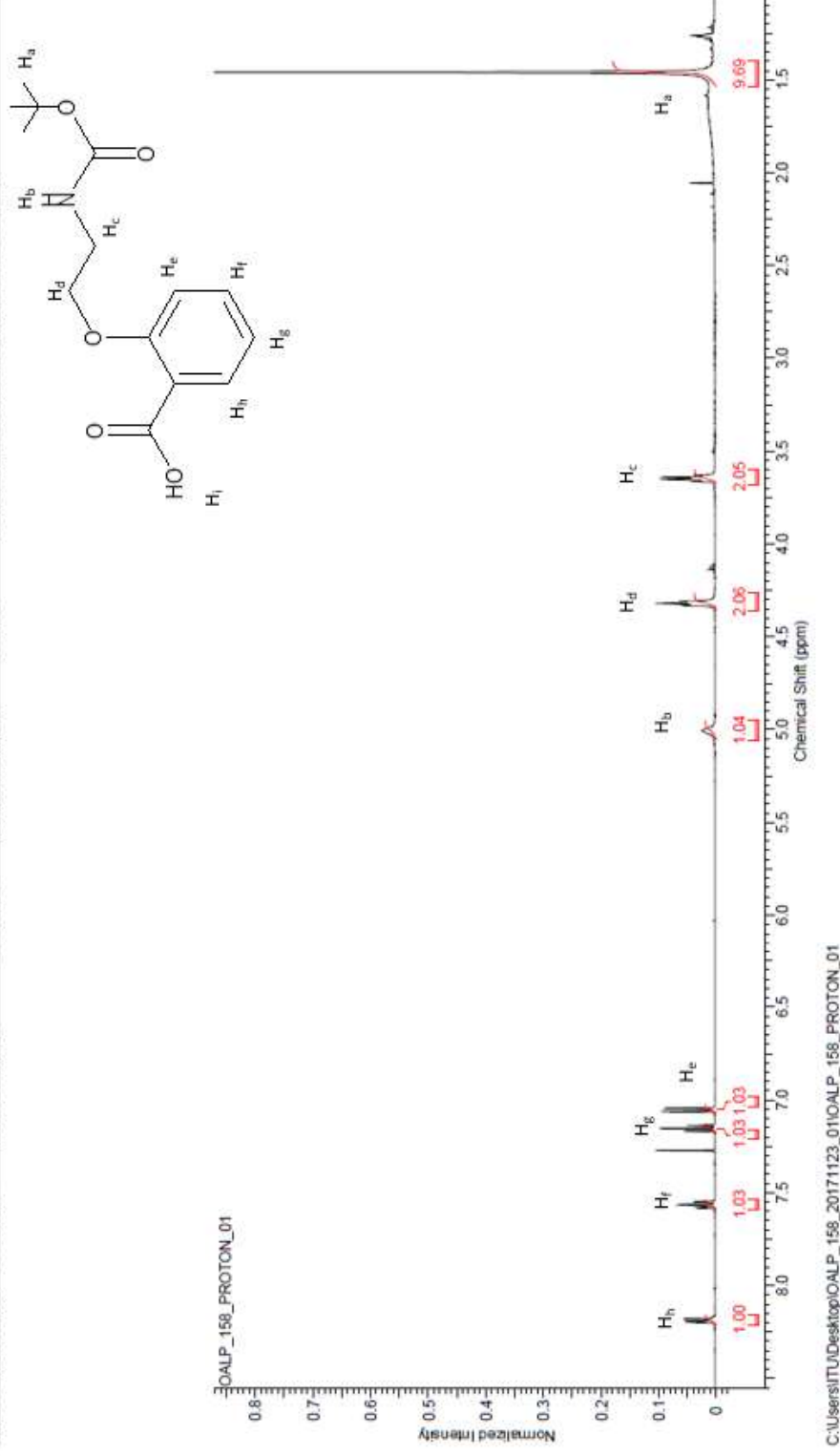


Figure B4 : ¹H-NMR spectrum of 2-[2-(Boc-amino)ethoxy]benzoic acid (7).

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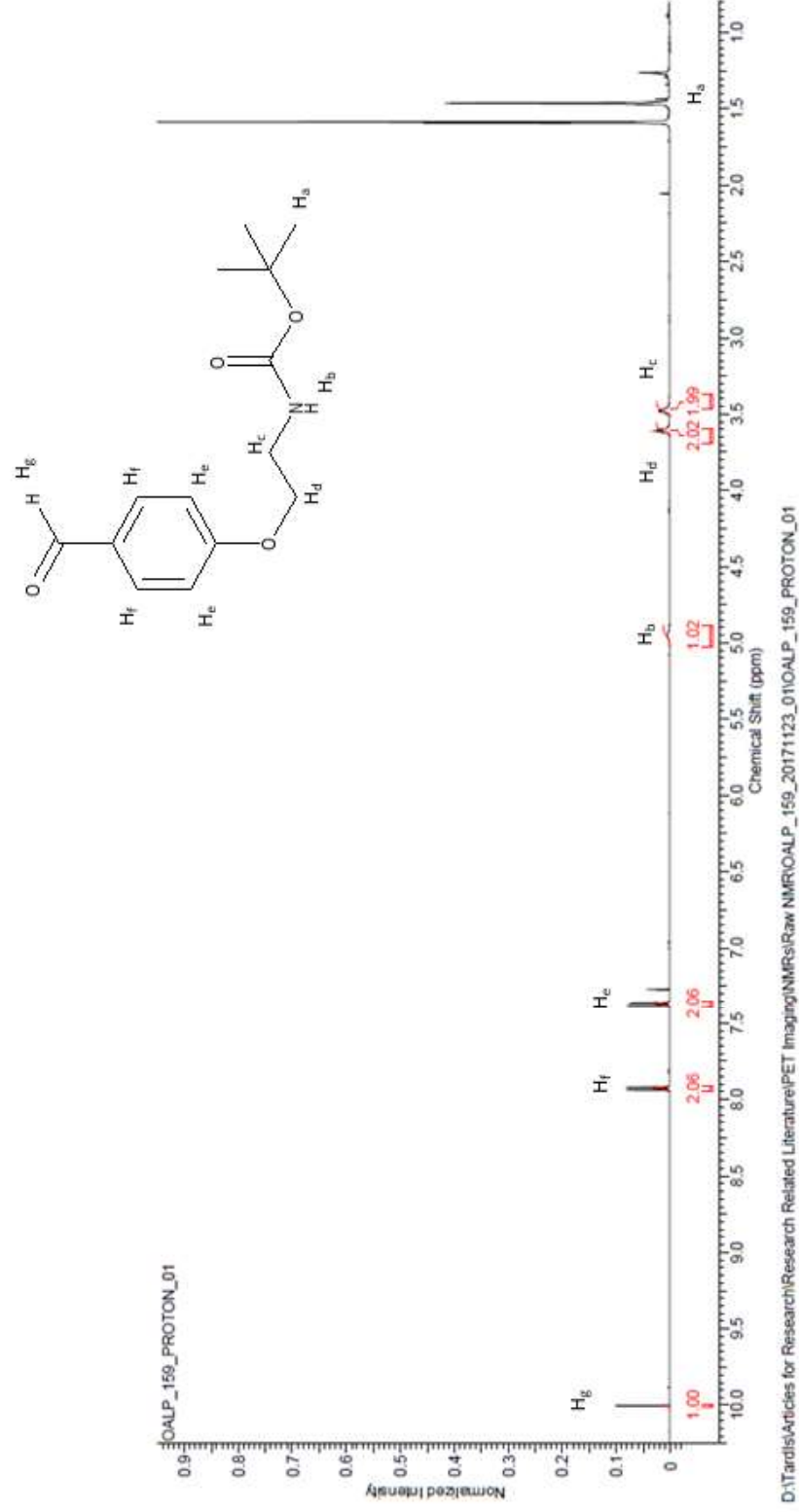
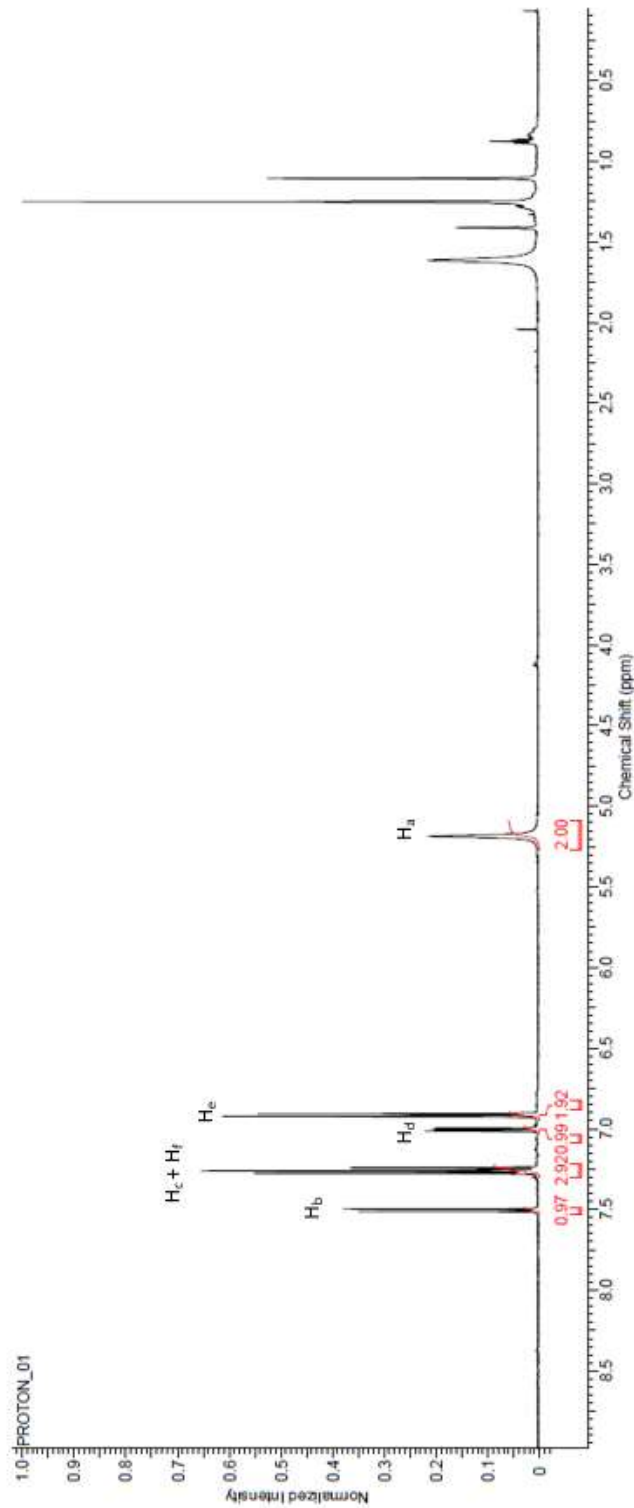
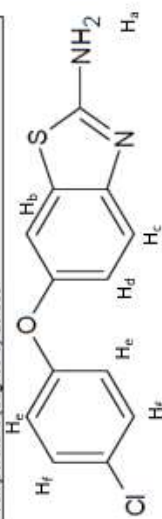


Figure B5: ¹H-NMR spectrum of tert-butyl 2-(4-formylphenoxy)ethylcarbamate (8).

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Points Count	32768	Pulse Sequence	s2pul	Receiver Gain	44.00	Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	3598.1741	Spectrum Type	STANDARD	Sweep Width (Hz)	9615.38	Temperature (degree C)	25.000



D:\Tardis\Articles for Research\Research Related Literature\PET Imaging\NMRs\Raw NMR\Cl halka kapama_20180108_01.fid

Figure B6 : ¹H-NMR spectrum of 6-(4-chlorophenoxy)benzo[d]thiazol-2-amine (13).

Acquisition Time (sec)	2.0447	Date	Feb. 7 2018	Date Stamp	Feb. 7 2018
File Name	D:\Tardis\Articles for Research\Research Related Literature\PET Imaging\NMRs\Raw NMR\OALP_162_20180207_01\OALP_162_PROTON_01.fid				
Frequency (MHz)	499.85	Nucleus	¹ H	Number of Transients	128
Points Count	16384	Pulse Sequence	s2pul	Receiver Gain	50.00
Spectrum Offset (Hz)	2999.0569	Spectrum Type	STANDARD	Sweep Width (Hz)	8012.82
				Solvent	CHLOROFORM-d
				Temperature (degree C)	25.000

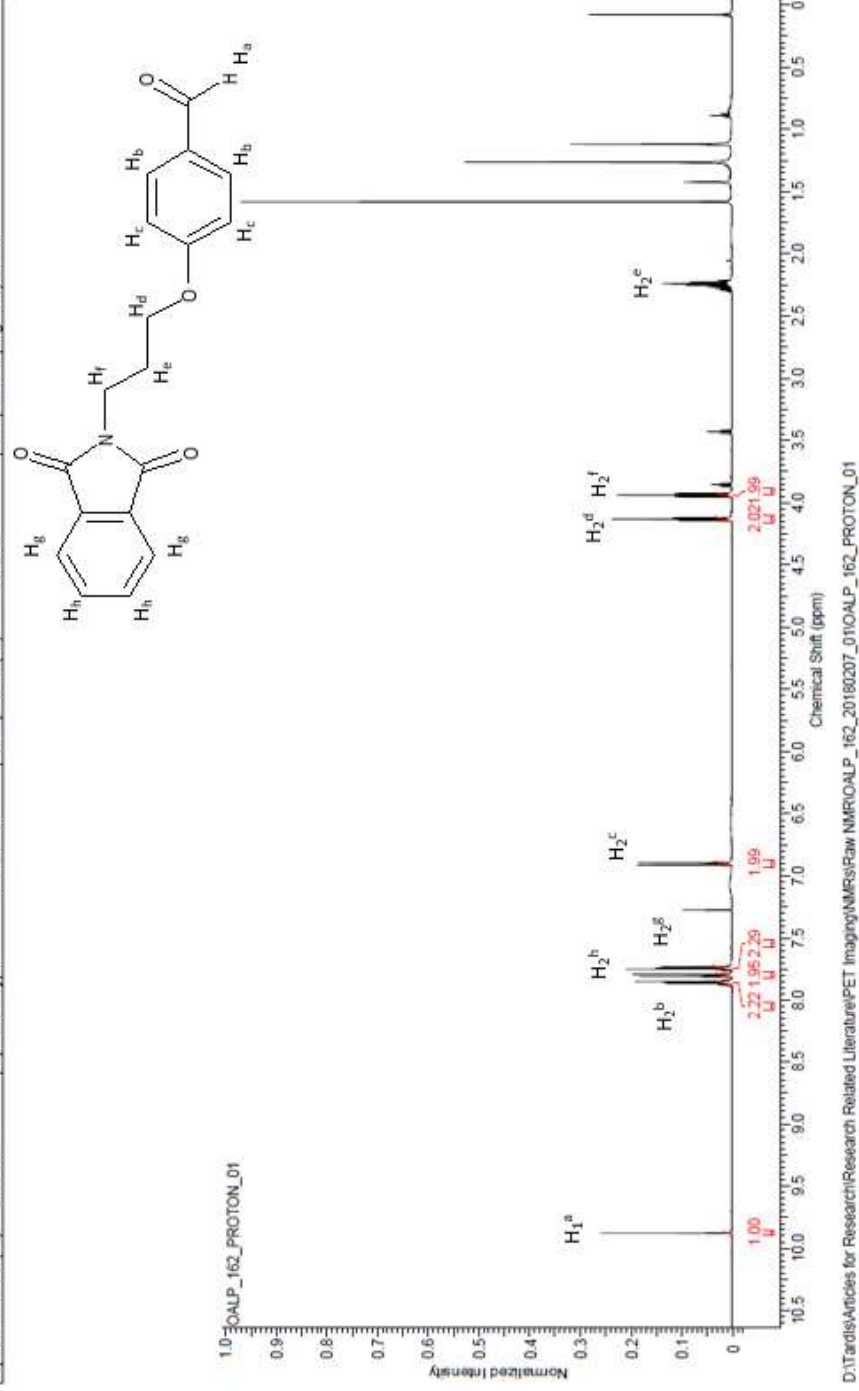
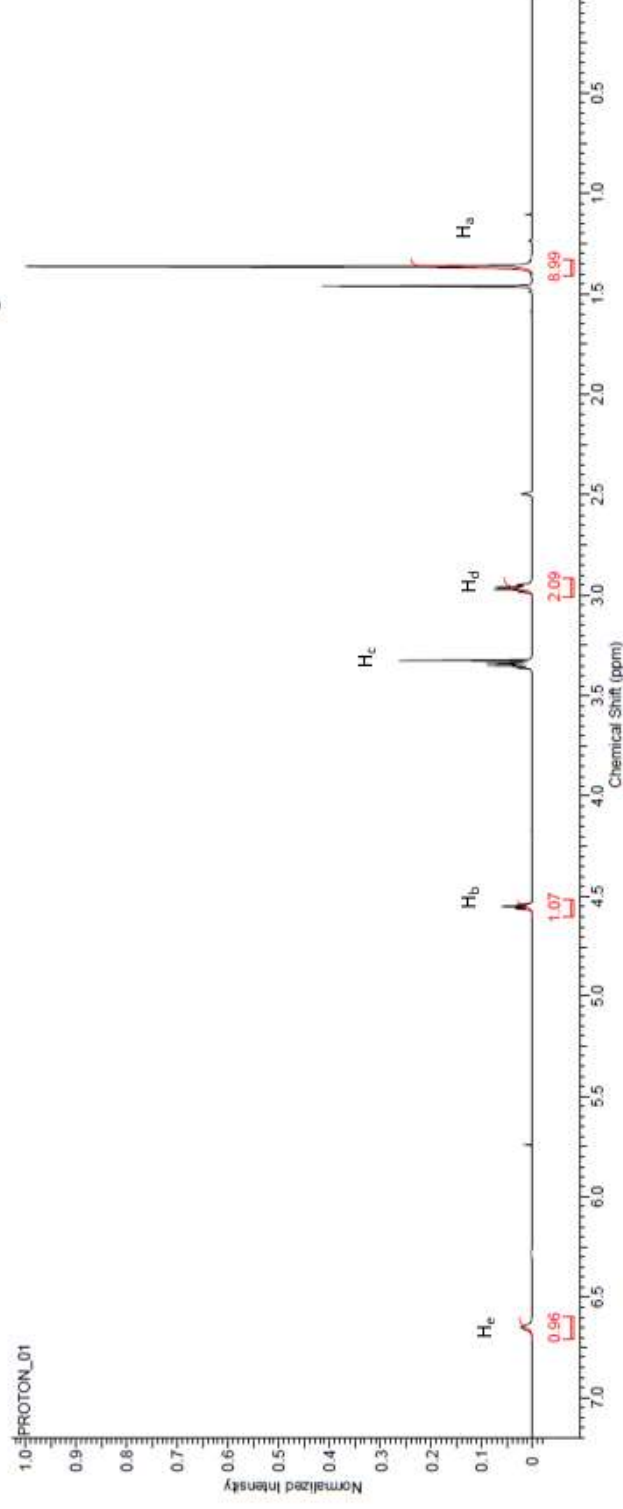
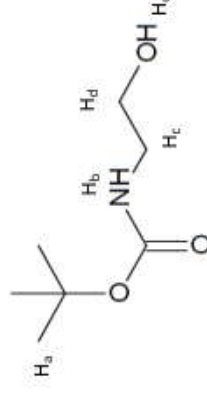


Figure B7 : ¹H-NMR spectrum of 4-[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propoxy] benzaldehyde (15).

Acquisition Time (sec)	2.0447	Date	Nov 24 2015	File Name	D:\Results\NMR\N-Boc ethanolamine\PROTON_01.fid
Frequency (MHz)	499.85	Nucleus	¹ H	Original Points Count	16384
Pulse Sequence	s2pul	Receiver Gain	32.00	Points Count	16384
Sweep Width (Hz)	8012.82	Temperature (degree C)	25.000	Spectrum Type	STANDARD



D:\Results\NMR\N-Boc ethanolamine\PROTON_01

Figure B8 : ¹H-NMR spectrum of tert-Butyl (2-hydroxyethyl) carbamate (17).

Acquisition Time (sec)	2.0447	Date	Jan 25 2016	Date Stamp	Jan 25 2016
File Name	C:\Users\user\Desktop\OALP_131_20160125_01\OALP_131_PROTON_01.fid.tif				
Nucleus	¹ H	Number of Transients	128	Original Points Count	16384
Pulse Sequence	s2pul	Receiver Gain	38.00	Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2999.0569	Spectrum Type	STANDARD	Sweep Width (Hz)	8012.82
				Temperature (degree C)	25.000

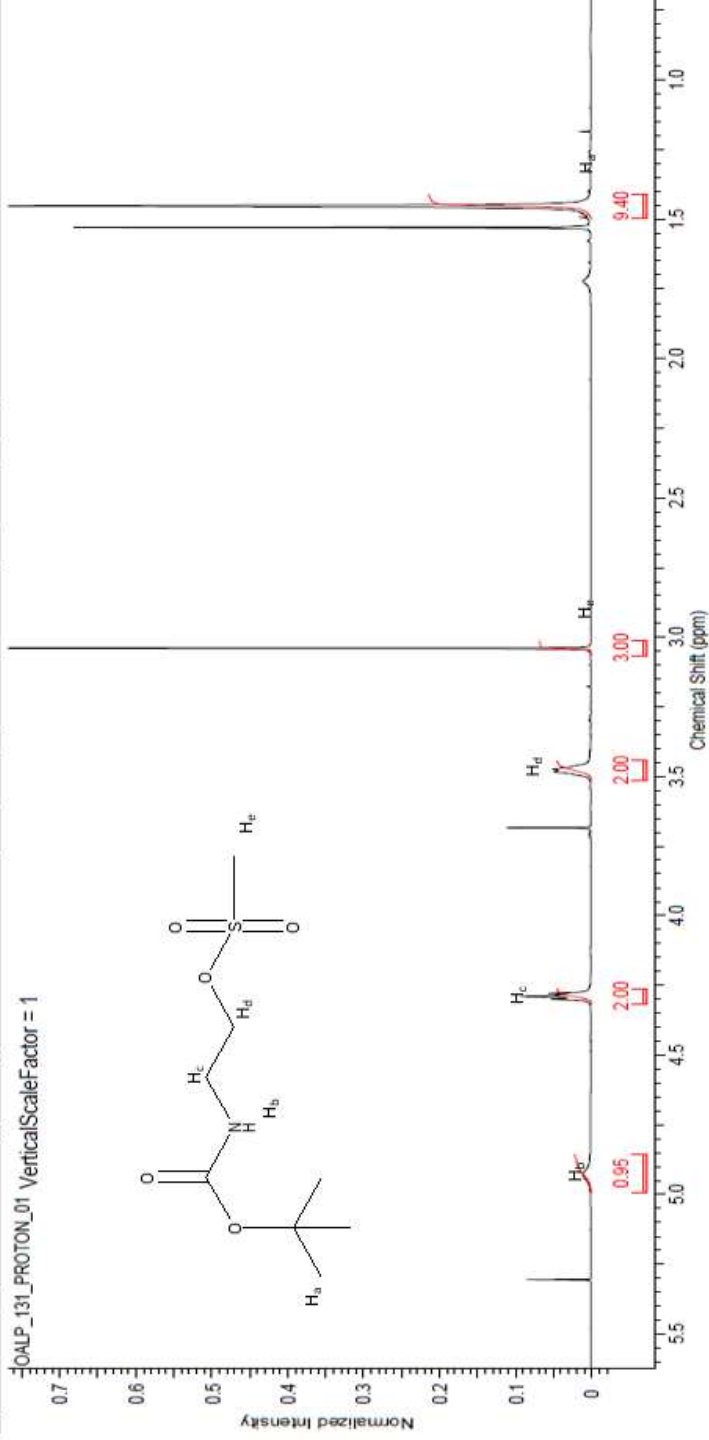


Figure B9 : ¹H-NMR spectrum of 2-(Boc-amino) ethyl mesylate (18).



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OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- Alptürk, O., Yesilbas, Z.S., Sarıoglu, G., Karaçaylı, A., Saylam, A., Özçubukcu, S. 2018. Optimization of synthetic route to PNA-T-OH monomers. *J. Turk. Chem. Soc., Sect. A: Chem.*, 5(2): 457-468.