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Development of a Gallium-Containing One-Bead One-Compound Peptide Library for the Discovery of New Molecular Imaging Probes

Fernanda C. Bononi
The University of Western Ontario

Supervisor
Leonard G Luyt
The University of Western Ontario

Graduate Program in Chemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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**Development of a Gallium-Containing One-Bead
One-Compound Peptide Library for the Discovery
of New Molecular Imaging Probes**

(Thesis format: Monograph)

by

Fernanda C Bononi

Graduate Program in Chemistry (Molecular Imaging)

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
Western University
London, Ontario, Canada

Abstract

An eight amino acid one-bead one-compound (OBOC) peptide library, containing a gallium-DOTA complex, was developed in an attempt to overcome common issues associated with the later addition of radionuclides to peptide chains of imaging probes. The further addition of a radionuclide often changes the binding properties of a peptide, as it adds bulk, and possibly charges. MALDI (matrix-assisted laser desorption ionization) tandem mass spectrometry was determined to be the method of choice in order to deconvolute gallium-containing peptide sequences. The library obtained was screened against the breast cancer cell lines MDA-MB-231 and MCF-7. Positive beads were isolated and sequences were determined before ligand validation, which included synthesis of peptides and further testing for binding affinity and specificity. Furthermore, positive peptides obtained through screening were developed into potential imaging agents by radiolabelling with ^{68}Ga .

Keywords: Molecular Imaging, OBOC library, gallium, MALDI, DOTA, peptide fragmentation

Co-Authorship Statement

Chapter 2: All work in this chapter was completed by the author with the exception of MALDI tandem mass spectrometry data acquisition. This work was done by Ms. Kristina Jurcic of the UWO MALDI MS Facility under the supervision of Dr. Ken Yeung, Associate Professor at the University of Western Ontario jointly appointed to the Departments of Chemistry and Biochemistry.

Chapter 3: All work in this chapter was completed by the author with the exception of MALDI tandem mass spectrometry data acquisition, which was done by Ms. Kristina Jurcic of the UWO MALDI MS Facility.

Chapter 4: All work in this chapter was completed by the author with the exception of MALDI tandem mass spectrometry data acquisition, which was done by Ms. Kristina Jurcic of the UWO MALDI MS Facility.

“It always seems impossible until it's done.”

— *Nelson Mandela*

“If I have seen further, it is by standing on the shoulders of giants.”

- *Isaac Newton*

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List of Abbreviations

ANP	2-amino-3-(2-nitrophenyl)propanoic acid
Boc	<i>tert</i> -butoxycarbonyl
BOP	benzotriazol-1-yloxy)tris(dimethylamino)prospanium hexafluorophosphate
CE-MRI	contrast enhanced magnetic resonance imaging
CHCA	<i>trans</i> - α -cyano-4-hydroxycinnamic acid
CID	collision-induced dissociation
COPAS	complex object parametric analyzer and sorter
CT	x-ray computed tomography
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DM	diabetes mellitus
DMF	dimethylformamide
DIPEA	<i>N,N</i> -diisopropylethylamine
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
FDG	2-fluoro-2-deoxy-D-glucose
Fmoc	fluorenylmethyloxycarbonyl
HBTU	O-(Benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HCTU	O-(6-chlorobenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry

hSSTr2	human somatostatin receptor subtype 2
mAb	monoclonal antibodies
MALDI	matrix assisted laser desorption/ionization
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MRI	magnetic resonance imaging
MS	mass spectrometry
MS/MS	tandem mass spectrometry
Mtt	4-methyltrityl
NETs	neuroendocrine tumours
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
OBOC	one-bead one-compound
PED	partial Edman degradation
PED-MS	partial Edman degradation mass spectrometry
PEG	polyethylene glycol
PET	positron emission tomography
PITC	phenylisocyanate
PTH	phenylthiohydantoin
RCY	radiochemical yield
RES	reticuloendothelial system
RP-HPLC	reverse-phase high-performance liquid chromatography
SPECT	single photon emission computed tomography
SPPS	solid-phase peptide synthesis
SST	somatostatin
SUV	standardized uptake values
TBME	<i>tert</i> -butyl methyl ether
tBu	<i>tert</i> -butyl
TFA	trifluoroacetic acid
TOF	time-of-flight
TIS	triisopropylsilane
TOF/TOF	tandem time-of-flight

UV

ultraviolet

Chapter 1: Introduction

1.1 Molecular Imaging

Molecular imaging can be defined as the real-time non-invasive visualization, characterization and evaluation of specific biological processes at the cellular and sub-cellular level in an organism.^{1,2} By combining different areas of expertise, such as cell and molecular biology, chemistry, physics, mathematics, bioinformatics and medicine, we can obtain data that helps the understanding of the physiology and mechanisms of disease.^{1,2,3} Since molecular imaging looks for more specific molecular events than simple differentiation between diseased and healthy tissues, it has the potential to provide insight not only in early detection of diseases, but also in their physiology, progression and response to therapy.² Besides their recent advent, molecular imaging modalities are showing promising results in early detection, differentiation and therapy response for a variety of diseases, including cancer.

There are a number of different molecular imaging modalities, which are often combined to provide detailed information about the presence, the development and the prognosis of a disease.⁴ Some examples are MRI (magnetic resonance imaging), PET (positron emission tomography) and SPECT (single photon emission computed tomography). While MRI relies only on energy-tissue interactions using magnetism to produce images, PET and SPECT require the administration of agents or reporting probes containing a radionuclide.⁴

Table 1.1 Characteristics of imaging modalities PET, SPECT and MRI.⁵

Imaging Modality	Spatial Resolution	Sensitivity (mole L⁻¹)	Amount of molecular probe used
MRI	25-100 μm	10^{-3} to 10^{-5}	Micrograms to Milligrams
PET	1-2 mm	10^{-11} to 10^{-12}	Nanograms
SPECT	1-2 mm	10^{-10} to 10^{-11}	Nanograms

PET and SPECT have been broadly used for the past few decades for diagnosis and therapeutic evaluation of diseases as they offer the sensitivity required to monitor drug distribution, pharmacokinetics and pharmacodynamics.⁴ While both techniques rely on the use of radionuclides and the detection of emitted radiation, differences exist in the way that the radiation is detected. SPECT imaging detects gamma ray radiation emitted directly from radionuclides, and the image formed is a result of the radiation data collected by a gamma camera. The most commonly used radioisotope, ^{99m}Tc, is a well known gamma emitter; it has ideal nuclear properties and can be easily obtained from commercial generators.⁶ Its 6 h half-life allows for its preparation, hospital distribution, administration, accumulation time in the target tissue, and image collection, while reducing patient exposure to large doses of radiation.⁶

PET, on the other hand relies on positron emitting radionuclides like ¹⁵O, ¹³N, ¹⁸F, and, more recently, ⁶⁸Ga, ¹²⁴I, ⁶⁴Cu. As the radioisotope undergoes decay, it emits a positron that travels a short distance before interacting with an electron, thereby resulting in an annihilation event. The encounter produces a pair of 511 keV gamma photons that

travel opposite to one another (180°). Both photons are detected and the information obtained is used to create an image via mathematical reconstruction procedures of “coincidences” registered by the photon detectors.⁷ Isotopes used in PET imaging can be either cyclotron-produced, such as ^{18}F , or generator-produced, such as ^{68}Ga . Although both techniques offer limitless depth of penetration, PET offers advantages over SPECT, including improved sensitivity, and the ability to detect and record a higher percentage of emitted events. These advantages are due to the absence of a collimator and the use of the coincidence-detection method. This improvement in sensitivity can lead to better image resolution, improved temporal resolution, and the possibility of performing shorter scans.⁸

Because of the difference in radionuclides used in PET and SPECT imaging, the discovery of suitable radiopharmaceutical probes is required for their use in the successful diagnosis and treatment of a specific disease. Despite the aforementioned advantages in using PET over SPECT, SPECT agents are often capable of providing more specific targeting, making SPECT the preferred modality for a variety of applications within biological organisms.⁸ As a result, the application of radionuclide-based imaging techniques for disease diagnosis or treatment will depend on factors such as the discovery of suitable probes, the equipment available and the availability of radionuclides.

1.2 PET and SPECT Imaging Agents

Some of the characteristics that are important for molecular imaging agents are high selectivity for the target or process of interest, a good ratio of specific over non-specific binding, a good safety profile, good *in vivo* stability, time and cost-effective synthesis, and signal amplification.³

Imaging agents can be of two different classes: targeted and non-targeted. Non-targeted agents rely on general chemical properties, such as size, charge and lipophilicity, to determine their movement within the body.⁶ On the other hand, targeted agents utilize a targeting entity to drive the agent to the target of interest.

¹⁸FDG (2-[¹⁸F]-fluoro-2-deoxy-D-glucose) (**Figure 1.1**) is the most well known targeted agent currently in use. It is a glucose analog known for causing significant inhibition of glycolysis in ascites tumour cells grown *in vitro*.⁹ This probe is taken up by high-glucose-using cells, which are capable of internalizing it, but cannot undergo glycolysis, resulting in the probe being trapped in the intracellular environment as 2-[¹⁸F]-fluoro-2-deoxy-D-glucose-6-phosphate (FDG-6-P).¹⁰

The tracer is able to measure the status of a malignant lesion; however, it only distinguishes between metabolically active cells and less active cells. Tumour tissue is nonhomogeneous; clusters of normal cells exist simultaneously with malignant or premalignant cells. As a consequence, the use of FDG does not reflect the real metabolic status of the malignancy. In addition, macrophages also demonstrate FDG uptake, as their activity increases in response to inflammation or after tumor therapy, thereby producing misleading imaging results.^{10,11}

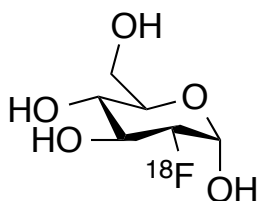


Figure 1.1 ^{18}F FDG (2- ^{18}F]-fluoro-2-deoxy-D-glucose), a widely used analogue of glucose for imaging tumours due to high sugar uptake.

Targeted imaging agents are composed of an imaging entity, and a targeting entity, which is responsible for the biodistribution of the agent throughout the body. These two portions may be separated by a linker, which is used to add distance between the two entities, resulting in a better receptor-ligand binding (**Figure 1.2**).⁶

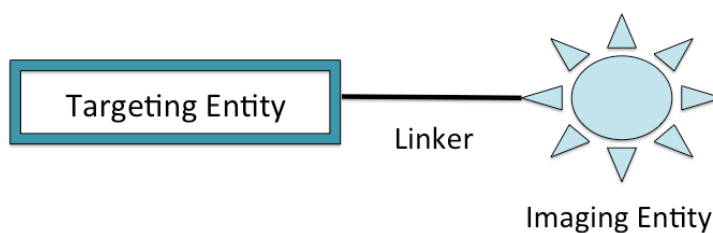


Figure 1.2 The general scheme of a targeted imaging agent: a targeting entity, responsible for driving the agent to the site of action and interacting with the receptor; an imaging entity, responsible for emitting detectable radiation; and a linker, which is sometimes present to reduce interference between the two portions of the imaging agent.

The radionuclide used as the imaging entity must have a half-life suitable for emitting enough detectable radiation within a reasonable time frame without exposing the patient to unsafe doses of radiation. In addition, its half-life must be long enough that it

can be synthesized, incorporated into a probe, transported, and injected into the body without significant decay. A variety of radionuclides are currently being employed in the different imaging modalities; however, applications with ^{68}Ga are becoming more popular due to the availability of the radionuclide and its outstanding properties.

Different radionuclides are attached to targeting entities differently: ^{18}F and ^{11}C , for example, can be integrated directly into the structure of an imaging agent, while ^{64}Cu , $^{99\text{m}}\text{Tc}$ and ^{68}Ga require the presence of a chelator to incorporate the radionuclide into the imaging agent.

The targeting entity is the vehicle that directs the imaging entity to the target or tissue of interest.⁷ It must have the ability to move through the body rapidly, avoid normal tissue uptake, and only interact with the target of interest, which results in improved performance and reduces any possible side effects of radiation exposure to the patient. Additionally, it should not produce an immune response, and must also be readily cleared from the body after imaging, ultimately leading to minimal patient exposure to radiation.

The targeting entity is the variable part of targeted imaging agents, and can be modified in order to increase affinity or specificity to the target of interest. Thus, the development of new molecular imaging agents is dependent on the discovery and further development of new targeting entities.

Table 1.2 Radionuclides used for SPECT imaging.⁵

Nuclide	Half-life (h)	Type of emission	Principal photon emission energies (MeV)
¹²³ I	13.2	Electron capture	0.16
^{99m} Tc	6	Isomeric transition	0.14
¹¹¹ In	67.9	Electron capture	0.17/0.25
⁶⁷ Ga	78.3	Electron capture	0.09/0.19/0.30
²⁰¹ Tl	73.1	Electron capture	0.17

Table 1.3 Radionuclides used for PET imaging.⁵

Nuclide	Half-life (min)	Type of emission	Maximum energy (MeV)
¹¹ C	20.3	β^+	0.97
¹³ N	10	β^+	1.20
¹⁵ O	2	β^+	1.74
¹⁸ F	110	β^+	0.64
⁶⁴ Cu	76.2	β^+ /electron capture	0.66
⁶⁸ Ga	68.1	β^+ /electron capture	1.90
⁷⁶ Br	972	β^+ /electron capture	4.00
¹²⁴ I	60192	β^+ /electron capture	2.14

1.3 ^{68}Ga as an Imaging Entity

^{68}Ga , a generator-produced radionuclide, has a half-life of approximately 68 minutes, which makes it possible for it to be synthesized, incorporated into an imaging agent, and applied for posterior injection and detection of the radiation emitted using PET imaging.

^{68}Ga is becoming a PET radioisotope of interest for reasons related to its convenient half-life of 68 minutes, economical viability and its accessibility from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator.¹² This radionuclide decays to the stable daughter isotope, ^{68}Zn , by positron emission (89%) and electron capture (11%) with a maximum positron energy of 1899 keV.^{13,14}

Within the past few years, the number of references to ^{68}Ga -labeled analogues for PET imaging has increased substantially,^{15,16} and recent studies show that peptides labeled with ^{68}Ga are able to provide better images than analogues labeled with other radionuclides, such as ^{111}In .^{17,18} To date, ^{68}Ga has been used to image a variety of ailments, including tumours¹⁷, bone metastasis¹⁹, and infections.^{20,21} A bifunctional chelator, which contains a portion for attachment to a molecule or peptide and a portion responsible for chelating gallium, is attached to a targeting entity in order for the radionuclide to be incorporated into the imaging agent's structure.

A $^{68}\text{Ge}/^{68}\text{Ga}$ generator is composed of a column containing a stationary phase on which ^{68}Ge , the parent isotope, is absorbed. A solution of EDTA (ethylenediaminetetraacetate) or HCl, in the newer generators, is used to elute the

radionuclide. The shelf life of such a generator is one to two years, and it can be used up to three times a day.⁷

The chelation chemistry of ⁶⁸Ga is well understood, as its prevalent state in aqueous solution is +3, and at higher pH, it has the propensity to demetallate from its complexes, forming insoluble Ga(OH)₃ in the absence of stabilizing ligands, and at physiological pH, forms a gallate anion, Ga(OH)₄⁻.^{6,22} Gallium is classified as a hard Lewis acid due to its high charge density and small ionic radius, and, therefore, it binds strongly to ligands that are hard Lewis bases, such as nitrogen and oxygen atoms.²³ Its small ionic radius (0.62 Å) allows it to be six-coordinate in a distorted octahedral geometry²⁴, and complexes that have spare coordination sites are more sensitive to hydrolysis.⁶

As mentioned previously, polydentate ligands with hard donor groups are the most common class of chelators for ⁶⁸Ga. Common chelators include DTPA (diethylenetriaminepentaacetic acid), NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid); the number of labeled molecules using these chelators has increased over the past few years (**Figure 1.3**).

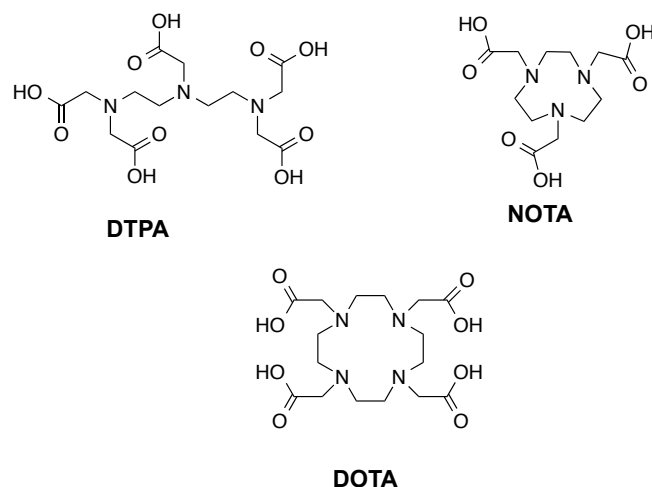


Figure 1.3 Schematic representation of bifunctional chelators DTPA, NOTA and DOTA.

1.4 Peptides as Targeting Entities

There are a variety of compounds that can be used as targeting entities: small molecules, peptides, monoclonal antibodies (mAb) or mAb fragments.⁷

Small molecules have a lower molecular weight when compared to antibodies and peptides, which allows them to move through the body and access major targets more easily, as well as increases their resistance to enzymatic degradation. The affinity of a targeted imaging agent for a biological target of interest should be unchanged or very close to that of the targeting entity alone after radiometal labeling; however, this is rarely the case when small molecules (MW<1000) are incorporated into the structure of an imaging agent, as the imaging entity corresponds to a considerable part of the probe size and weight.

Antibodies have the advantage of having extremely high affinity and specificity to targets and also being very stable to degradation. Monoclonal antibodies, such as trastuzumab, have been approved for clinical use²⁵; however, their large molecular weights (usually over 100 kDa) prevent them from being able to cross biological barriers and can lead to problems related to immunogenicity²⁶, which is the result of their being easily recognized by the immune system, taken up by the reticuloendothelial system (RES)²⁷, the latter of which can also cause a number of undesired side effects.²⁸

Peptides present a number of important advantages over other molecules. Their favourable pharmacokinetics²⁹, high receptor binding affinity and selectivity³⁰, and the lack of side effects³¹ make peptides an attractive class of molecules for the design of new imaging probes.

In addition to promising biological characteristics, the chemistry of peptides is very compelling. The process of synthesizing peptides is straightforward, radiolabeling is easily performed, especially when bifunctional chelators are utilized, as they possess a well-established chelation chemistry and can be easily incorporated into the peptide structure.³² Peptides are stable to the chemical modifications that are associated with the addition of radionuclides, including elevated experimental temperatures and the use of organic solvents. In addition, peptides can be easily modified to avoid rapid enzymatic degradation³⁰ through the use of natural and unnatural amino acids, and D-amino acids, all of which can be used to alter the chain length and properties.

Peptide-based imaging agents were introduced into the clinic more than two decades ago.^{33,34} Specifically, the somatostatin (SST) analog ¹¹¹In-DTPA⁰-octreotide

(^{111}In -OctreoScan or ^{111}In -pentetrotide) is an agent that effectively images SST receptor-positive lesions such as neuroendocrine tumours (NETs) and small cell lung cancer (**Figure 1.4**).^{35,36,37}

Following the advent of ^{111}In -DTPA⁰-octreotide, peptide imaging agents were further developed to include analogs of neurotensin³⁸, exendin³⁹ and new analogs for SST receptors, such as DOTATOC, which binds to the human somatostatin receptor subtype 2 (hSSTr2), which has been shown to be highly expressed in various human tumours.⁴⁰

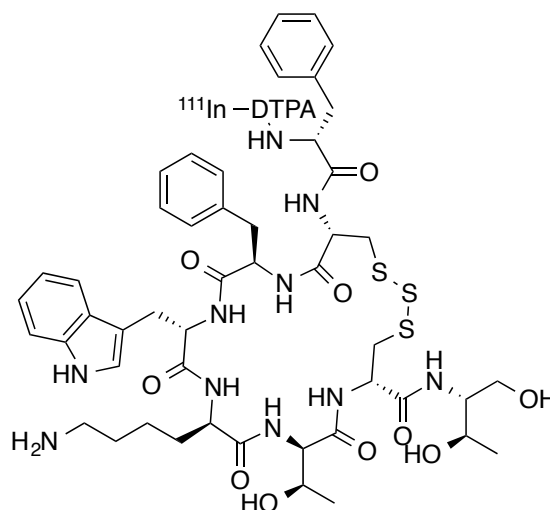


Figure 1.4 ^{111}In -DTPA⁰-octreotide. A peptide based imaging agent that acts on the somatostatin receptor. The probe contains a D-Phe and D-Trp, is cyclized via a disulfide bond between the two cysteine residues and contains ^{111}In -DTPA (diethylenetriaminepentacetic acid) as the imaging entity.

DOTATOC was initially developed for labeling with ^{90}Y or ^{177}Lu , and was used for targeted radiotherapy⁴⁰; however, it has more recently been studied as a potential PET agent when chelated with ^{68}Ga (**Figure 1.5**) because it shows promising results in the diagnosis of NETs when compared to conventional detection methods.^{17,41}

The advantages in the synthesis of peptides and the ease of incorporating them into an imaging entity has resulted in their becoming important molecules in the development of novel molecular imaging agents.

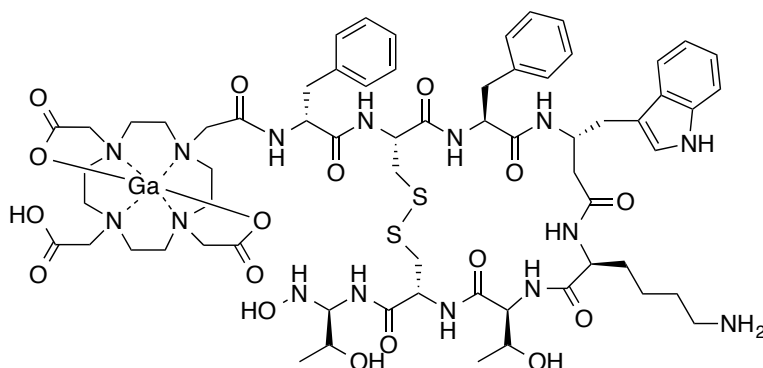


Figure 1.5 ^{68}Ga -DOTATOC, a somatostatin analog that is showing promising results in the diagnosis of neuroendocrine tumours (NETs).

1.5 Discovery and Development of Peptide-based Imaging Agents

The strategy for design and synthesis of new imaging probes is similar to the process of generating drug-like molecules. Probes are expected to show high affinity and selectivity for the target receptor or organ of interest. As a result, small amounts of the radioactive probe are required to generate precise and well-defined images of the receptor.⁵

The discovery of new peptide-based imaging probes is accomplished by two different methods: a rational approach, in which standard peptide chemistry is carried out to determine the regions critical for receptor binding affinity of an endogenous peptide that is known to interact with the desired target, and a combinatorial approach, in which a

large number of compounds are screened against a particular target to determine which ligands show the most promising results.

Rational design is a broadly used method for drug discovery, and more recently, has been applied for the discovery of imaging agents. The first step in rational design is to identify a molecular target of relevance for a particular disease, and subsequently to identify a lead peptide, which can be of natural or synthetic origin. Endogenous peptides are prone to quick degradation, and, therefore, are normally not useful imaging agents. However, endogenous peptides can be modified into more stable agents while maintaining high selectivity and binding affinity by the incorporation of D-amino acids or unnatural amino acids. Furthermore, more hydrophilic and lipophilic amino acids, as well as charged amino acids can be added to change the properties of the peptide.

Despite the advantages of rational design, it is limited by the fact that it depends on information available on the target of interest and the endogenous ligand, which are not always readily available. General methods to obtain such information can be time consuming; for example, crystallization leads to limited or no information on certain targets. Because of these limitations, other design methods have been developed and are now currently in use.

Peptide libraries generally overcome the limitations of rational design, as information about the target of interest or endogenous ligand is not required. With peptide libraries, a large number of peptide sequences are simultaneously tested against a cell line or receptor for binding affinity. The sequences showing high affinity to the target can then be identified, and further modifications can lead to the development of new

imaging agents. This approach is less time consuming and more economical than rational design, as a large number of potential imaging agents can be discovered and developed simultaneously. The use of automated synthesizers and the development of more efficient ways of screening such compounds have popularized this approach in the past few decades.

The first big advancement in the development of peptide libraries was in the early 1990s when Furka *et al.*⁴² presented a method for the simultaneous synthesis of a mixture of 27 tetrapeptides and that of 180 pentapeptides. This new synthetic method laid the foundation for the development of some types of peptide libraries available today.

There currently exist a variety of methods for developing random peptide libraries, and subsequently obtaining the sequences of peptides that show affinity for a particular target of interest. For example, the SPOT-synthesis technique and light directed-chemical synthesis are methods in which the sequence of the peptides is known based on physical location. On the other hand, phage display and one-bead one-compound (OBOC) libraries are completely random, requiring the use of other techniques to determine the sequences of the positive peptides or “hits” obtained through screening against a target of interest.

SPOT libraries were first presented in 1990 by Frank *et al.*⁴³; this type of library is designed to synthesize hundred or thousands of peptides arranged on a planar cellulose support. The chemical reaction occurs on little spots, corresponding to where droplets of the reagents are added. The peptide is subsequently attached to the cellulose support by a method that is similar to standard solid phase peptide synthesis. The surface holds a large

number of spots, or a large number of peptide sequences, which can be identified by their location on the support. The size of the spot reflects the concentration of the peptide (Figure 1.6).⁴⁴

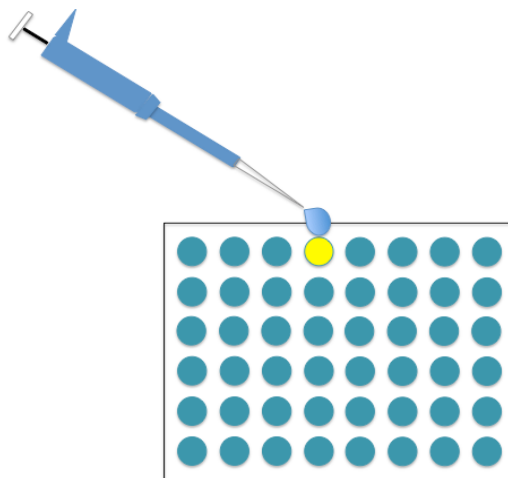


Figure 1.6 A cartoon representing the principle of SPOT libraries. Adapted from Frank *et al.* (2002).⁴⁴

Light-directed parallel chemical synthesis combines tools from solid phase peptide chemistry, photolabile protective groups, and photolithography for the synthesis of a peptide library.⁴⁵ The precision of the methodology allows for the synthesis of thousands of compounds to be retained in highly compact matrices. The immobilized compounds are incubated with the molecular binding agent of interest. Negative and positive binding events are then recorded, and sequences are determined based on their location.

The major limitation of these two methods is the limited number of peptide sequences that can be screened at any given time. Even when the processes are automated, the two methods described result in a maximum of an one hundred thousand peptide sequences.⁴⁴ For drug or imaging agent discovery, by screening a larger number

of compounds, it increases the possibility of obtaining positive results. As a result, there is a need for new methods for designing libraries, in which random sequences are used, requiring further experiments to determine the sequences of the positive peptides obtained by screening.

Of all of the types of combinatorial peptide libraries, phage display and one-bead one-compound (OBOC) libraries are two techniques that provide the possibility of screening a larger number of peptide sequences, potentially numbering in the millions.

Phage display libraries utilize standard recombinant DNA technology to engineer phage for specific peptide sequences with DNA coding, and combinations of different codon sequences are then cloned into phage libraries. After infection of *E. coli* cells with phage, the peptide sequences are synthesized as a coat protein for the phage.⁴⁶ To screen these libraries, the receptor is bound to a solid support, and the mixture containing all phages is passed over the immobilized receptor. Phages containing peptide sequences that show affinity to the receptor are retained on the surface while the unbound ones are washed away. The addition of a solution that removes the receptor-peptide bonds results in the collection of the positive phages in an “enriched” solution, which contains only phages that show some affinity to the receptor in question. The positive phages are still able to infect fresh bacterial host cells, leading to an amplified eluate, which is used for other rounds of affinity selection. After the final screening, the positive phages in the eluate are propagated and characterized individually. The positive sequences are then determined by ascertaining the corresponding coding sequence in the viral DNA.⁴⁶

A phage display library has the potential to display billions of peptide sequences, and subsequent DNA sequencing identifies bioactive peptides. This type of library is accessible commercially, and peptide sequences can be readily obtained. However, despite their ability to contain unnatural amino acids^{47,48}, phage display libraries are limited by their inability to contain non-peptide structures, as sequencing methods cannot account for these structures. OBOC libraries, on the other hand, are not biological in nature, which permits them to easily incorporate D-amino acids, unnatural amino acids, as well as non-peptide structures. Even though OBOC libraries are usually smaller than phage display libraries, they allow thousands to millions of compounds to be synthesized in a short period of time.⁴⁹

1.6 One-Bead One-Compound Peptide Libraries

Described in 1991, the one-bead one-compound (OBOC) combinatorial method allows for the synthesis and screening of thousands of compounds against a variety of targets in a short period of time using on-bead binding assays.⁴⁹ Using TentaGel as a solid support, the first OBOC study described a library synthesis using the “split-mix” methodology (**Figure 1.7**), which consists of having a specific number of peptide vessels corresponding to the number of amino acids to be used in the random sequences, and subsequently mixing and splitting the contents of the vessels again at the end of each coupling cycle. This allows for the creation of a library of compounds, where each individual 90 μm bead displays up to 10^{13} copies of one chemical entity.^{49,50}

OBOC libraries benefit from being able to contain unnatural amino acids, D-amino acids, and non-peptide structures, and are therefore, suitable for drug or imaging agent development, as peptides containing unnatural amino acids are generally more resistant to proteolysis.⁵¹

While initially developed to screen libraries consisting of short linear peptide sequences, this methodology has more recently been used to develop cyclic peptides⁵², peptidomimetic and small molecule libraries.⁵³ In addition, it has been successfully used in many high-throughput screening assays to identify protein kinase and protease substrates and inhibitors⁵⁴⁻⁵⁷, G-coupled protein receptor inhibitors⁵³, ligands against mRNA precursors⁵⁸, and integrin-specific peptides against breast cancer cells⁵¹, among others.

The main steps in OBOC methodology are synthesis, screening, and sequence deconvolution of peptide beads. Subsequent ligand validation is the key step that follows sequence determination of positive “hits”, as it leads to the synthesis and characterization of the discovered ligands prior to further biological evaluation for target affinity.

Although revolutionary in its concept, improvements to the synthesis and screening of such libraries have been made throughout the years, resulting in different design approaches being incorporated according to the needs of particular studies.

In order to make it possible to screen the same beads against different targets, the concept of “rainbow beads” was developed, which allows for the possibility of screening different libraries simultaneously by encoding each library with a different colour.⁵⁹ A similar concept was explored when a method to immobilize beads in a gel support was

reported. It allowed for the screening of an entire library sequentially many times with a series of distinct probes.^{60,61}

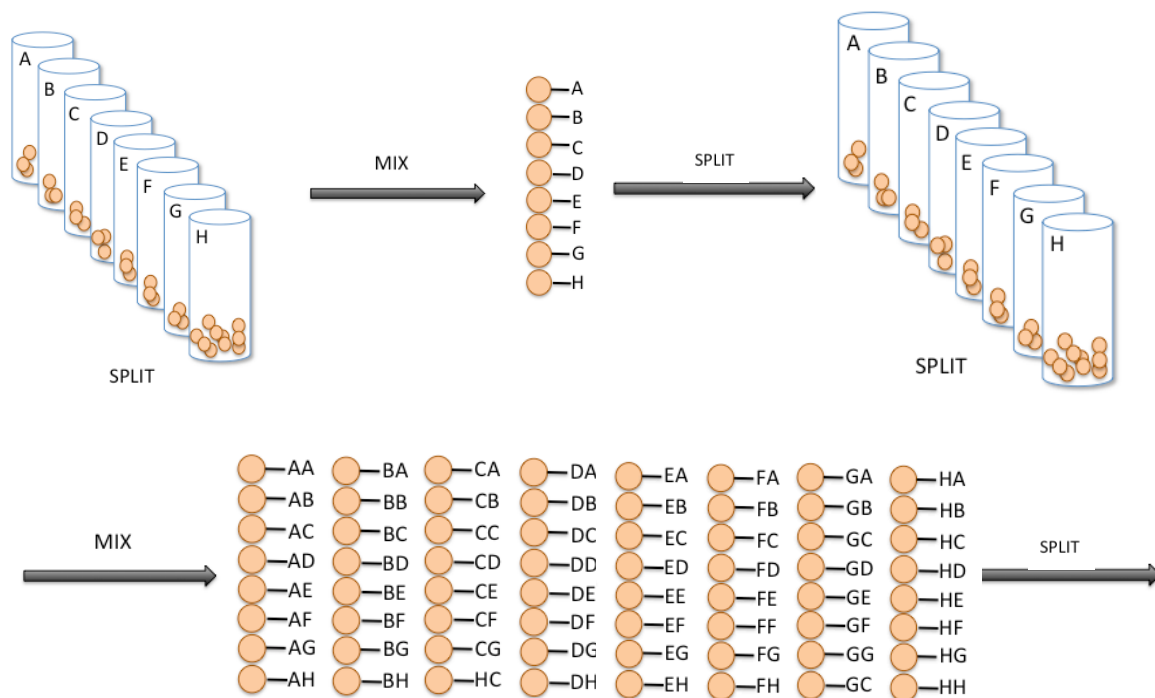


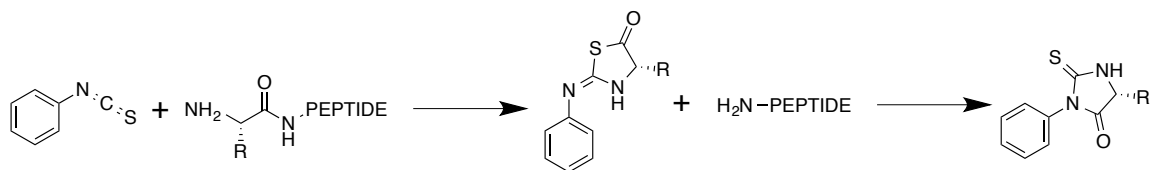
Figure 1.7 The synthesis of OBOC peptide libraries, where A-H are amino acids and using two “split-mix” steps. The number of possible peptide sequences will increase exponentially in each step following $N = aa^x$, where N is the number of sequences, aa is the number of amino acids in a peptide, and x is the number of amino acids available for each step.

The screening process, which involves the selection of positive beads for further identification, was initially performed manually using a confocal microscope and a pipette. Recent studies attempted to automatize the screening process by using cells that express a fluorescent protein and a Complex Object Parametric Analyzer and Sorter (COPAS) platform, which sorts beads based on the fluorescence of the cells. More recently, Cho *et al.*²⁷ reported the development of a screening method that combined

fluorescence and magnetic field for the purpose of bead separation. Separation procedures with two different steps may decrease the number of false positives normally encountered with beads sorting through the COPAS platform.

1.7 Sequence Deconvolution and Identification of Peptide Sequences

The first synthesized OBOC library contained 19 natural amino acids, excluding cysteine due to the potential of disulfide bond formation, and the sequences of positive beads were determined by automated Edman degradation⁴⁹, which has been the method of choice since the advent of OBOC libraries. In this method, each amino acid is converted to its phenylthiohydantoin (PTH) derivative, from N-terminus to C-terminus. The formed PTH amino acids are identified based on their retention times by reversed-phase high-performance liquid chromatography (RP-HPLC) and compared with reference standards for all 20 natural amino acids.⁶²



Scheme 1.1 A schematic of Edman degradation using phenylisothiocyanate to form a phenylthiohydantoin derivative of the N-terminus amino acid residue. R represents the side chains of amino acids.

Liu *et al.*⁶² reported the use of Edman microsequencing to identify unnatural amino acids within a peptide structure. They were able to trace the elution profile of 19

out of 35 unnatural amino acids, which can be readily distinguished from the 20 natural amino acids on a modified elution gradient.

Although useful for peptide sequencing, this technique is limited when used for applications related to non-peptide structures and N-capped peptides. The reaction with phenylisothiocyanate does not occur, and it is not possible to identify these structures. In addition, the high cost per sample and time-consuming procedure make it challenging to sequence multiple samples.⁶³

Matrix-assisted laser desorption ionization (MALDI) tandem mass spectrometry for peptide deconvolution was first described by Youngquist *et al.*⁶⁴. It used capped deletions in order to encode a combinatorial library and simplify the sequencing process. This method not only revealed a rapid method for sequencing, but it also allowed a direct assessment of the quality of the synthetic library in question, as side-reaction products, incomplete products, and deletion peptides could be observed.⁶⁴

Few studies had since been developed to show the usefulness of this methodology until Amadei *et al.*⁶³ established a fast, economical, reliable, and reproducible method for structure determination of small peptides without the need for further complex experiments. This study used only the MALDI tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) instrument to photocleave, desorb, ionize and fragment ‘on-bead’ peptides. A related report by Semmler *et al.* also describes the use of a photocleavable library and sequencing using MALDI.⁶⁵ In this report, the peptide sequence is determined by a two-step process; the peptide is first cleaved off of the resin, and is then analyzed by MALDI tandem mass spectrometry. Thus, the focus of recent

studies is to optimize the process of removing the peptide from resin and subsequently submitting the sample for analysis, in order to make it a high-throughput methodology.

1.8 [Ga]DOTA Peptide Libraries

The discovery of peptide-based targeted imaging agents is often difficult due to the required addition of a radionuclide to a peptide. When either rational design or random library approach is used, the addition of an imaging entity, consisting of a radionuclide, a chelator, and a linker, results in modifications to the binding properties of the original peptide, as it adds bulk and possible charges. This is even the case with the discovery of agents that present high affinity for a particular target. For small peptides (<2000 Da), for example, the imaging entity corresponds to a significant portion of the mass and size of an imaging agent, suggesting that the changes to the binding properties are more perceptible; however, studies have shown that even larger peptides, where the imaging entity mass and size corresponds to a smaller portion of the probe, can be influenced by the addition of the imaging entity.

After the addition of the imaging entity, the peptide structure is modified in order to create a compound with more affinity and specificity to the target of choice. This step is often an inefficient process, particularly when peptide sequences are discovered using a random library approach such as OBOC, where information of how the peptide binds to its target, its conformation, and bond distance are not available.

Recent advances in the synthesis and screening of OBOC libraries, and the ability to incorporate non-peptide structures, suggest that the addition of an imaging entity to a peptide library prior to screening has the potential to overcome the current limitations of OBOC, as it will account for changes in binding affinity before the screening. Thus, only those sequences that show affinity with the radionuclide present will be further analyzed and characterized.

As mentioned previously, ^{68}Ga is becoming a relevant isotope for diagnostic purposes. The incorporation of ^{68}Ga is achieved with bifunctional chelators; the most widely used functional chelator for ^{68}Ga is DOTA.⁶⁶

More recently, DOTA labeled with ^{68}Ga has been added to a variety of peptides to be used against a variety of targets. For example, Octreotide, a metabolic stable SST derivative, was conjugated with DOTA and labeled with ^{68}Ga for imaging of beta cell mass in diabetes mellitus (DM) model rats showing pancreatic accumulation, which was found to be significantly reduced in normal animals and animals that had excess amounts of unlabeled Octreotide administered previously⁶⁷.

The design of a [Ga]DOTA peptide library will lead to a more efficient discovery and development of new peptide-based PET imaging tracers. Preliminary work is required to determine the feasibility of such a library, and to determine if synthesis, screening and sequence deconvolution can be performed when the bifunctional chelator and metal are present.

1.9 Thesis Rationale

Gallium is becoming an isotope of relevance for PET oncological imaging, which fits our purpose for the synthesis of imaging-entity containing libraries. Although little is known about library screening of metal-containing libraries, the stability of the [Ga]DOTA complex in physiological conditions is already known to be satisfactory. Moreover, to date, there has been no report on either successful on-resin gallium coordination or the use of MALDI tandem mass spectrometry to determine the sequences of peptides containing gallium.

Furthermore, the first step in exploring the utility of a [⁶⁸Ga]DOTA peptide library involves the synthesis of a test peptide, which is coupled to DOTA, coordinated with ^{69/71}Ga and is then analyzed using MALDI tandem mass spectrometry, in order to determine how the presence of gallium, and its typical isotropic signature, will interfere with the fragmentation profile of peptides.

Due to the limitations associated with Edman degradation and other types of sequencing methods, MALDI tandem mass spectrometry will be evaluated as a method of identifying peptide sequences that contain the [Ga]DOTA complex as it has the potential to be a more efficient way of obtaining the sequences of bioactive peptides.

Lastly, with a reliable method to identify unknown peptide sequences, and a stable peptide library coordinated with gallium, the library will be tested against useful targets in order to validate the method. For this purpose, the [Ga]DOTA peptide library is screened against MDA-MB-231 and MCF-7 cells as a way of identifying those peptides

that bind specifically to one cell line, thereby allowing for the potential detection and monitoring of breast cancer.

Chapter 2: Synthesis, coordination and sequence deconvolution of a [Ga]DOTA-octapeptide

2.1 Introduction

Due to the properties of DOTA as a stable and well characterized chelator and gallium as a great imaging entity, the [Ga]DOTA complex is the imaging entity of choice for the development of an OBOC library. Furthermore, the patterns of fragmentation of [Ga]DOTA-containing peptides are analyzed utilizing MALDI tandem mass spectrometry, which is not only able to identify peptide sequences, but also show the presence of gallium and account for non-peptide structures.

2.1.1 Peptide Synthesis

Peptide synthesis consists in the coupling of amino acids protected with orthogonal protecting groups to a solid support. It was developed in an attempt to optimize the synthesis of long chain polypeptides, when solubility and purification of peptides become more challenging as the number of residues increases.⁶⁸

The general concept involved in solid phase peptide synthesis is the attachment of the first amino acid of the peptide chain to a solid polymer (resin) by a covalent bond. The subsequent amino acids are added in a stepwise manner until the desired sequence is obtained. The sequence is then removed from the support.

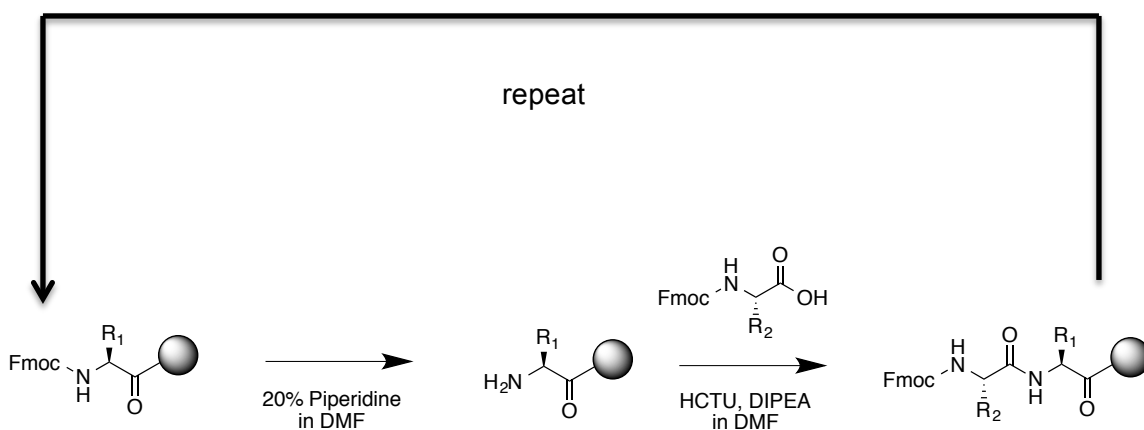
Having the peptide attached to an insoluble support allows for the proper washing and filtering of reagents and byproducts, leading to more pure intermediates without the use of time consuming techniques like recrystallization or chromatography.

The support used for solid phase peptide synthesis has to be insoluble in all solvents used during the process, so that filtration can be easily performed. It contains an insoluble polystyrene cross-linked backbone where polyethylene glycol (PEG) groups are attached. It also has to contain functional groups, within the PEG groups, where the first protected amino acid can be linked by a covalent bond. The functional group present will depend on the type of resin used and the existence of methods to remove the peptide from the support after synthesis.

The most common protecting group for the backbone amines is fluorenylmethyloxycarbonyl (Fmoc), a base-labile group that can be removed with the use of a piperidine solution in dimethylformamide (DMF). Side chains, on the other hand, are protected with acid-labile protecting groups. That way, selective deprotection of terminal amines and coupling of subsequent amino acids can be achieved. There are a variety of acid-labile protecting groups, with *tert*-butoxycarbonyl (Boc) being the most commonly used amine protective groups and *tert*-butyl (tBu) esters for carboxylic acids.

The process of adding amino acids one by one to a solid support follows a procedure deprotection followed by coupling of amino acids. The resin contains a free amine that will react with the free carboxylate of an Fmoc-amino acid to form an amide bond. Since the carboxylate is the only portion of the amino acid not protected, there will be only one possible reaction.

The coupling reaction between amines, carboxylates and amide bonds is known to be slow, which leads to the need of coupling agents (**Scheme 2.1**). The first coupling reagents to be used were carbodiimide derivatives such as *N,N'*-dicyclohexylcarbodiimide (DCC) and *N,N'*-diisopropylcarbodiimide (DIC). These compounds react with the carboxyl group of amino acids to form a highly reactive O-acylisourea intermediate, which is quickly displaced by a nucleophilic attack from the deprotected amine at the N-terminus of the growing sequence. However, besides the possibility of amino acid racemization, these reagents are also known to produce a reactive intermediate and a number of side reactions. Agents that required the addition of an activating base to mediate the coupling were then developed, such as (benzotriazol-1-yloxy)tris(dimethylamino) prospanium hexafluorophosphate (BOP), O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and O-(6-chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU). The activating base most commonly used is diisopropylethylamine (DIPEA).



Scheme 2.1 A diagram showing the steps of peptide synthesis: Fmoc deprotection using a 20% piperidine solution in DMF, and amino acid coupling using HCTU and DIPEA. This cycle is repeated until the length of the peptide chain is reached.

The side chain protecting groups, which are acid labile, are removed by the addition of acids such as trifluoroacetic acid (TFA). The solution (cocktail) used to remove acid-labile groups contains a high percentage of acid and a small percentage of scavenging agents such as water, phenol and triisopropylsilane (TIS). The scavenging agents are responsible for removing the electrophiles produced from the cleavage of protecting groups by reacting with them. That way, reactions of these groups with the peptide chain are avoided.

2.1.2 Ga Labeling using DOTA as a Chelator

Due to its small cationic radius, Ga(III) is often six-coordinate in a distorted octahedral fashion.²⁴ As a result, polydentate ligands with hard donor groups are the first choice for gallium labeled biomolecules. DOTA, a ligand based on polyaza-macrocycles, when in a Ga-DOTA complex, shows high thermodynamic stability, which makes it a good fit for clinical practice.⁶ In [Ga]DOTA-D-Phe-NH₂, Ga(III) is coordinated by a N₄O₂ donor set while one carboxylic acid is deprotonated and does not coordinate to the metal. The remaining carboxylic group is conjugated with the amino acid via an amide bond.⁶⁹

DOTA-tris(tert-butyl ester) is a popular ligand for attaching DOTA to a peptide on solid support as it can be used to derivatize the N-terminus of a peptide chain attached to the solid support or a lysine side chain.⁷⁰

2.1.3 OBOC Sequencing Methods

Although conventional Edman degradation has been widely used for sequence determination of OBOC peptide libraries, due to its intrinsic limitations, new coding methods that enable the identification of unnatural and N-termini blocked elements have been developed. One of these strategies requires the addition of a “recognition arm”, a branched linker that is synthesized in parallel with an arm that encodes the recognition polymer’s sequence. The identity of this recognition polymer is determined by sequencing the coding arm.^{71,72} Although great in theory, the addition of a coding polymer adds extra steps to the library synthesis process and may also interfere with the screening against the target protein.

In order to deal with these limitations, a variety of other sequencing methods have been proposed, including the addition of coding molecules to the interior of beads, while keeping the test compound on the exterior, which decreases or eliminates the interference of the coding molecule with the binding of the testing compound to the target.⁷³ In this method, the synthetic step of both coding and testing compounds are combined, eliminating the extra steps present within the first strategy. The most efficient and reliable method for the modification of the bead surface consists on the use of a biphasic solvent environment, causing the surface of TentaGel beads to be exposed to organic solvent containing a derivatized reagent, such as Fmoc-Osu, while the interior of the bead remains in water, without any reagents.⁷³

The limitations of the former procedure led to a search for faster methods like the “ladder-sequencing” method.⁷⁴ Here, the N-terminus of each bead-bound peptide was

partially degraded, generating a series of peptide “ladders”, which were later analyzed by mass spectrometry. The sequences can be determined by the mass difference between adjacent peaks, as it will account for the molecular weight of one amino acid.⁷⁵ A similar method developed by Youngquist *et al.*⁷⁶ utilizes the same concept, but by capping the N-terminus portion of the peptides after each coupling cycle during the library synthesis, it can be also applied to small molecules and peptidomimetic libraries, a significant limitation of the previous method, which could only be applied to standard peptide libraries.

Partial Edman degradation and mass spectrometry (PED-MS) is similar to the ladder-sequencing method, where an OBOC bead will contain a series of capped peptide sequences. The advantage here is that all capping is performed after library screening, reducing the interference with the binding of the testing peptide to its target. In this method, a resin-bound peptide is treated with a mixture of phenylisocyanate (PITC) and a small amount of a capping agent. Both the PITC and capping agent compete for reacting with the N-terminal amine of the peptide. After addition of the reagents, 90-95% of the peptide undergo cleavage, while 5-10% react with the capping agent in each step. Repetition of the PED reaction for as many cycles converts the original peptide into a series of sequence-related truncation products.^{77,78} By analyzing the peptide mixture using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), the sequence of the original peptide is obtained (**Figure 2.1**). The reliability of this method has been tested by obtaining the sequence of peptides from an OBOC library using Fmoc-OSu as a capping agent.⁷⁹ The major limitation of this method, however, is the

requirement that peptides have to contain free N-terminal amines, which is not ideal for libraries containing non-peptide structures or that went through N-terminal acetylation.

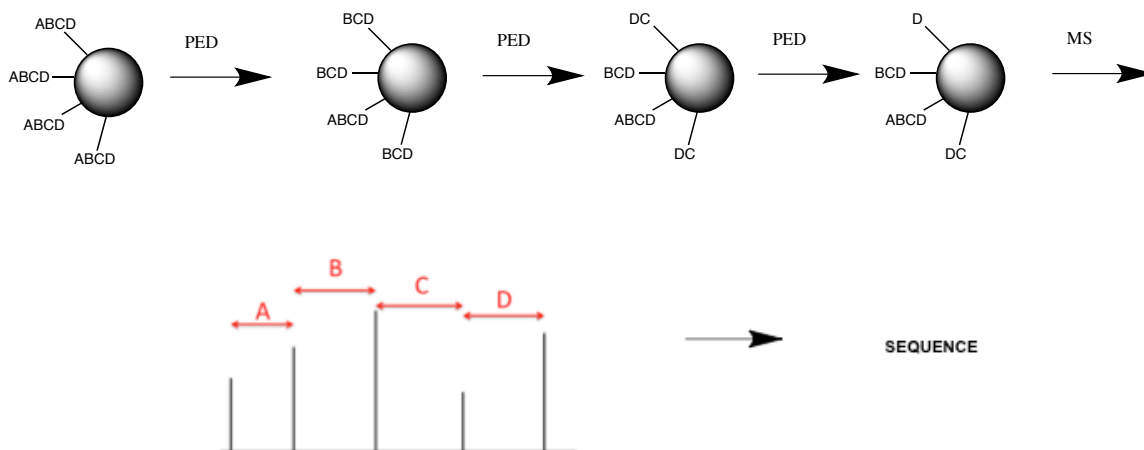


Figure 2.1 The procedure for peptide sequencing by the use of ladder synthesis and partial Edman degradation and mass spectrometry.

Tandem mass spectrometry (MS/MS) is an important technique that has proven to be valuable for sequence deconvolution of combinatorial libraries. It employs two stages of mass analysis, which makes it able to selectively examine the fragmentation of a particular ion or a mixture of ions. Here, a peptide is ionized, the ions formed are identified and separated based on their m/z ratio. The precursor ion is chosen based on the mass range of choice for further fragmentation, where information on the sequence of a peptide is obtained. The most commonly used methods for the soft ionization of peptides are ESI (electrospray ionization) and MALDI (matrix-assisted laser desorption/ionization).

The use of MALDI tandem mass spectrometry for the sequence determination of peptide libraries have been recently reported by different research groups^{80,81}, and is becoming the sequencing method of choice, as it is a fast, reliable and economic method that is also able to identify non-peptide structures.

In order to simplify the spectrum and help discern specific fragment series, some groups reported on the use of chemical derivatization, which consists of the addition of chemical labels, creating a mass shift or fragmentation pattern that helps to identify and differentiate fragment series. Examples of this technique are the introduction of amidine labels, creating a mass shift that is useful for protein identification⁸², and the introduction of halogenated mass-tags, either on topologically segregated bifunctional beads along with the bioactive sequence itself⁸³, or directly to the N-terminus of a peptide⁸⁴, facilitating the differentiation between different elements due to the unique isotope patterns associated with the halogen.

The use of MALDI for sequence determination of a resin-bound molecule has been reported, where an ultraviolet (UV) MALDI laser was used to cleave the molecule off resin and to promote its gas phase ionization for subsequent analysis, making a one step process.⁸⁵ The same results were achieved when using a 355-nm Nd:YAG laser in a MALDI-TOF/TOF (tandem time-of-flight) instrument, where peptide fragments were identified with a mass accuracy of 0.1 Da of expected values.⁶³

Besides the variety of sequencing methods for OBOC libraries that are currently available, with the addition of a chelator and a metal, there are a few limitations to most of these methods, as the N-terminus of the peptides is no longer a free amine, and also

due to the presence of non-peptide structures. Overall, tandem mass spectrometry, using MALDI as the ionization method turned out to be the best fit for sequence investigation of a [Ga]DOTA-containing OBOC peptide library.

2.1.4 Tandem Mass Spectrometry

When a peptide is submitted to fragmentation, the bond breakage will mainly occur through the lowest energy pathways, which in the case of a peptide is through the amide bond. This leads to the two most common ions in tandem mass spectrometry: the *b* and *y* ions (**Figure 2.2**). Other fragments that may be found are *a*, *c*, *x*, *z*. In *a*, *b* and *c* fragments, the charge is retained by the amino terminal fragment, while for *x*, *y* and *z*, the charge is retained by the carboxy-terminal fragment (**Figure 2.3**).⁸⁶

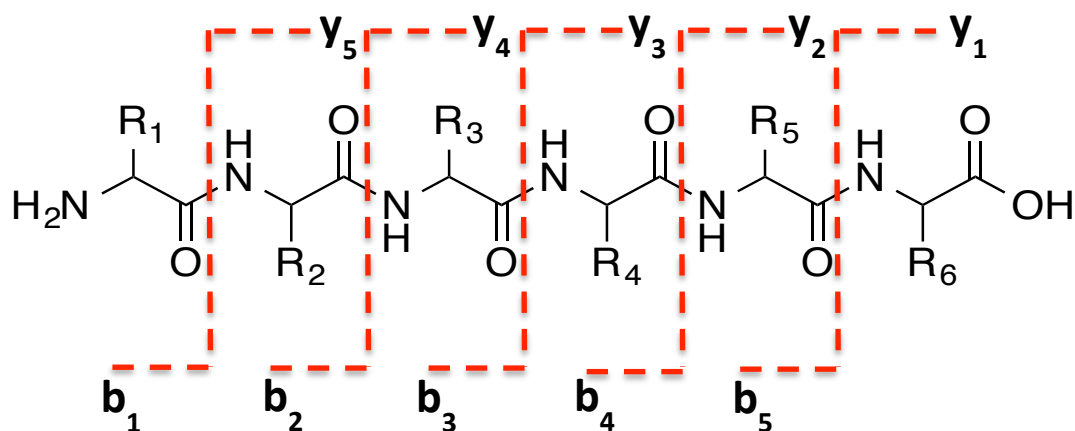


Figure 2.2 A general peptide structure showing the bonds that are broken to form *b* and *y* ions, the most common ions visualized in tandem mass spectrometry.

The most important characteristic of peptide fragmentation is that the patterns of fragmentation will depend entirely on the sequence of the peptide, specifically, it will depend on the side chains of the amino acids involved. During the initial ionization, basic residues normally accept protons from the matrix, but upon excitation, using collision-induced dissociation (CID) for example, the protons that are added to a peptide can migrate to various protonation sites before fragmentation. This means that one predominant structure will be present for peptide ions where the added proton is sequestered, and differently protonated forms will be present for easily fragmented species. This theory is known as the mobile proton theory, and is the most complete explanation of how peptide fragmentation occurs in tandem mass spectrometry.⁸⁷

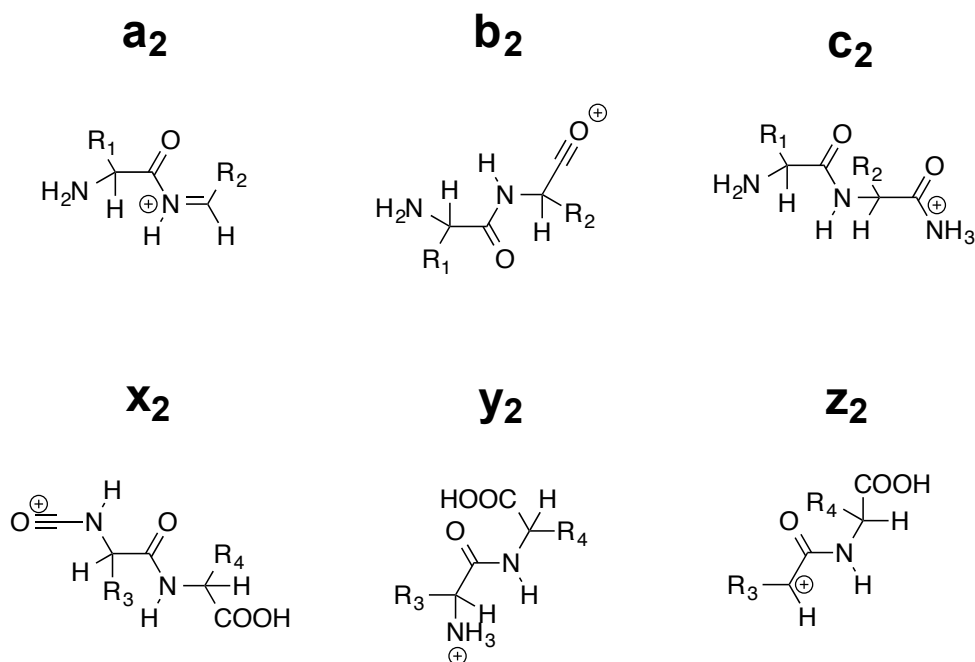


Figure 2.3 Fragments in tandem mass spectrometry. In *a*, *b* and *c* fragments the charge is retained on the N-terminus of the peptide, while in *x*, *y* and *z* the charge is retained on the C-terminus.

In tandem mass spectrometry, the difficulty lies in the fact that information in a spectra is not always complete. The presence of intervening peaks or absence of peaks due to poor fragmentation will interfere on the ability to determine the sequence of peptides. In other words, proper fragmentation by mass spectrometry will solely depend on the ability of the peptides to produce a well defined series of peptide fragments, where the difference between two neighbouring peaks produced correspond to an individual amino acid.

Although still an issue, with the use of databases, it is still possible to obtain enough information to match the data obtained from mass spectrometry uniquely to a peptide present in a database, based on the profiles of observed and expected ions. But when adding certain modifications to peptides, most of the time, information obtained from spectra cannot be matched with existent databases, as some modifications are not accounted for. However, the presence of certain isotopic labels in the N-terminus of peptide sequences can enhance the quality of the spectra obtained as it adds an isotopic signature to *b* ions, which can be easily differentiated, and sequences can be more easily determined.⁸⁴

In a similar fashion, the addition of the [Ga]DOTA entity to the N-terminus of peptide sequences is expected to produce similar fragmentation profile: an isotopic signature that will enable differentiation of *b* fragments, facilitating sequence determination. It is expected that MALDI tandem mass spectrometry is a reliable method for the deconvolution of such peptide sequences.

In addition to the difficulties in fully predicting the fragmentation of a peptide, the amino acids present, the three-dimensional conformation of peptides, and the presence of a chelator and metal complex will also interfere with the mass spectrometry data obtained. It can affect proton movement within the peptide, and possibly direct to a few favourable fragmentation pathways. As such, the peculiarities of the addition of DOTA and gallium to peptide fragmentation should be addressed in order to establish MALDI tandem mass spectrometry as the method of choice for sequence deconvolution of gallium-containing peptide libraries.

2.2 Results and Discussion

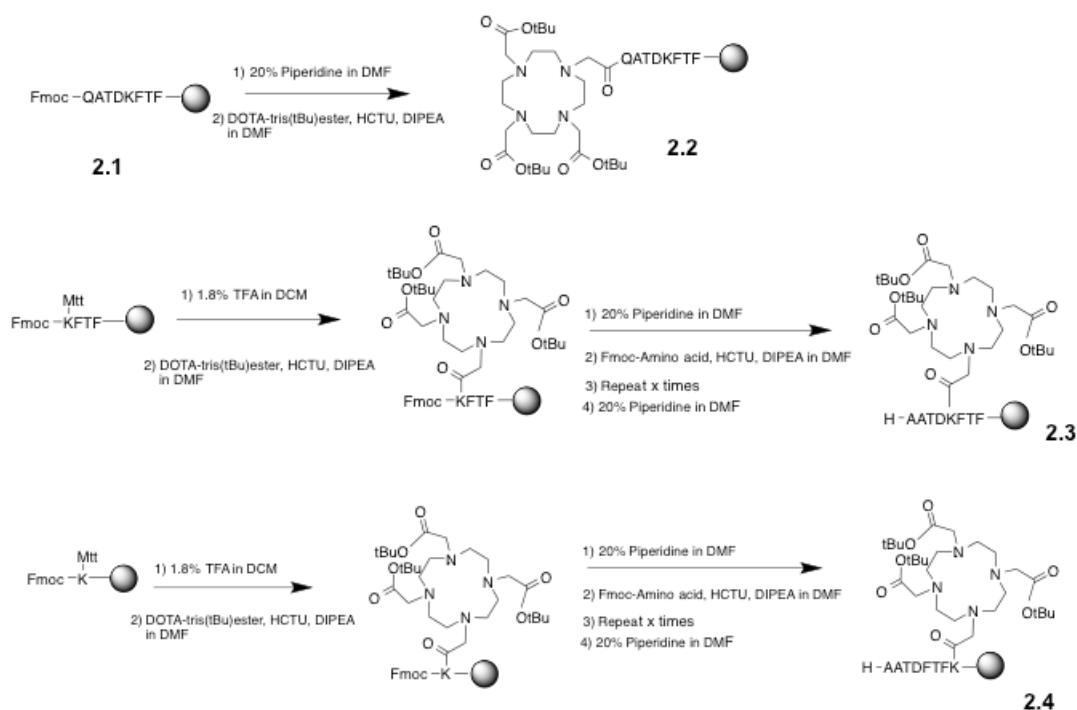
This chapter will outline the analysis of the fragmentation pattern of a test peptide containing the [Ga]DOTA motif at the N-terminus. The synthesis of a [Ga]DOTA-octapeptide will first be discussed, followed by the analysis of MALDI TOF/TOF tandem mass spectrometry data with remarks being made on how to overcome possible limitations and adapt this methodology to be used in the sequence deconvolution of [Ga]DOTA-containing libraries.

2.2.1 Synthesis of [Ga]DOTA Peptides

The first step for the synthesis of a [Ga]DOTA-containing OBOC peptide library is to analyze the method of choice for sequence deconvolution. There are no reports on the typical fragmentation of gallium-containing peptides using MALDI tandem mass

spectrometry, and as such, in order to establish this method for this particular study, peptides containing a [Ga]DOTA complex were synthesized on Rink Amide resin using standard Fmoc peptide synthesis protocols. Since non-radioactive gallium labeling and further analysis of the peptides was performed off resin, an acid-labile resin is chosen as the solid support for this synthesis.

The sequences of the three peptides synthesized were based on a sequence that has previously been reported to show good fragmentation patterns when utilizing MALDI TOF/TOF mass spectrometry (QATDKFTF-NH₂).⁸⁸ Compound **2.1** became the standard peptide sequence for such testing in our lab. The first peptide synthesized, compound **2.2** contains the [Ga]DOTA complex on the N-terminus of the peptide sequence, in order to understand the effects of the presence of gallium and the bifunctional chelator to the fragmentation of a peptide, as a preliminary analysis. After this first analysis, two other peptides were synthesized, with small changes to the peptide sequence in order to accommodate the [Ga]DOTA complex and also avoid issues with cyclization that might be present when adding such a chelator and having an amino acid with a free amine on its side chain. Compound **2.3** contains the complex in the fourth amino acid of its sequence, while **2.4** contains it in the C-terminus of the peptide, on a lysine side chain (**Scheme 2.2**).



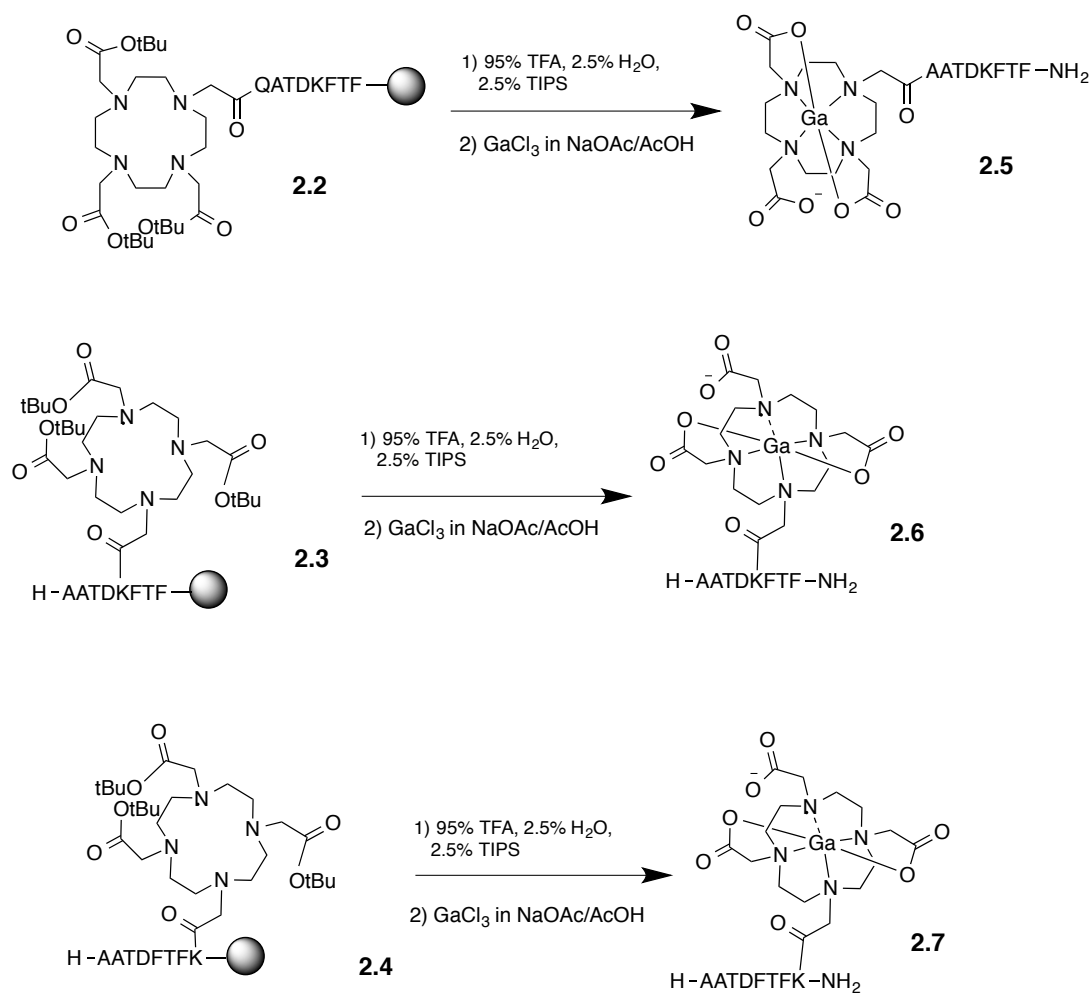
Scheme 2.2 Synthesis of [Ga]DOTA peptides. Sequences on **2.3** and **2.4** are variations from **2.1**. In **2.2**, the chelator (DOTA) is added after a last Fmoc deprotection in a standard solid phase peptide synthesis procedure. In **2.3** and **2.4**, the chelator is added before the peptide sequence is completed, after selective Mtt deprotection using 1.8% TFA in DCM.

The major goal when changing the position of the [Ga]DOTA complex was to study the possibility of designing a library where the position of the chelator with the metal could also be randomized, like the position of regular amino acids.

For **2.2**, the Fmoc group on the N-terminus of the peptide was removed using standard conditions. The bifunctional chelator (DOTA) is then coupled to the N-terminus utilizing standard amino acid coupling using HCTU and DIPEA in DMF. For compounds **2.3** and **2.4**, the use of an orthogonal group is necessary in order to selectively deprotect

the side chain of a lysine, where the chelator will be coupled, leaving the Fmoc group on the terminal amine of the lysine. In order for this to happen, instead of using regular Fmoc-Lys(Boc)-OH, for these two peptides, Fmoc-Lys(Mtt)-OH was used. When synthesizing compound **2.3**, after the coupling of lysine, the Fmoc group is kept and the 4-methyltrityl (Mtt) group from the side chain of lysine is removed selectively using a solution of 1.8% TFA in dichloromethane (DCM), 9 times for 3 minutes until the solution colour comes from yellow to clear. The same approach is used on **2.4**, where lysine is the first amino acid to be added. The chelator is then coupled using the same reagents as for **2.2**. After the coupling of the chelator, the addition of the remaining amino acids is carried on using standard procedures.

For simplicity the gallium coordination of these compounds is conducted off resin after purification. To remove the peptides from resin and also have side chain groups deprotected, a solution of 95% TFA : 2.5% H₂O : 2.5% TIS, known as Reagent K, is added to the resin for a period of three hours. This results in a solution containing a peptide with a C-terminal amide. These compounds were purified by HPLC (high-performance liquid chromatography) and their identity confirmed by mass spectrometry. Gallium coordination is performed using gallium chloride (GaCl₃) in an acetate buffer solution (NaOAc/Hac, pH 4), since under basic conditions, gallium will form insoluble gallate ions [Ga(OH)₄]⁻.⁸⁹ The procedure for non-radioactive gallium coordination is well known and straightforward, where the coordinated compound can be easily separated from the non-labeled. The processes of resin removal and side chain deprotection as well as the non-radioactive gallium labeling for all three peptides are shown in **Scheme 2.3**.



Scheme 2.3 Side chain deprotection, removal from resin and gallium coordination for **2.2**, **2.3** and **2.4**.

2.2.2 MALDI Tandem Mass Spectrometry

As previously mentioned, MALDI tandem mass spectrometry use on [Ga]DOTA peptides has never been reported, leading to the necessity to perform initial studies on purified peptides in order to investigate the viability of this method as well as observe how the addition of this metal-chelator complex will affect the fragmentation profile for peptides.

The peptides were dissolved in water and mixed with an equal volume of a solution of *trans*- α -cyano-4-hydroxycinnamic acid (CHCA), which is one of the most commonly used matrices for short peptides on MALDI tandem mass spectrometry. The resulting solution was spotted on a MALDI target plate and the contents allowed to slowly evaporate. The matrix then absorbs light from a laser, which results in ablation and the transfer of a proton to the peptide, leading to detection. Results obtained show the expected mass for all peptides and a very characteristic gallium 69/71 isotopic signature (**Figure 2.4**). The masses of the peptides are then selected for further fragmentation.

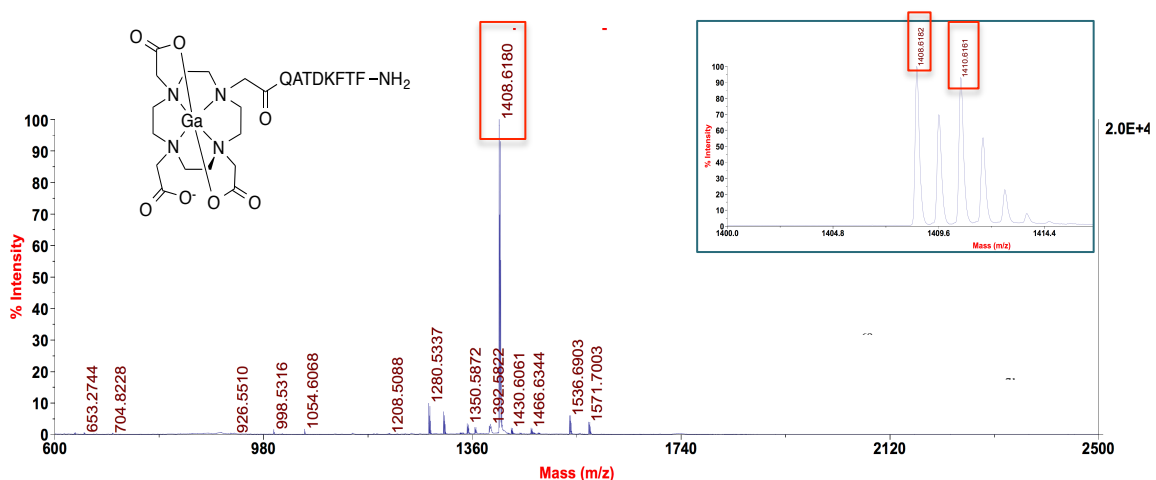


Figure 2.4 MALDI tandem mass spectrometry for compound 2.5 showing the desired mass of the product. The zoomed frame shows the typical gallium isotopic signature, where the 69 isotope is relatively more abundant (60%) than the 71 isotope (40%).

All compounds showed strong signals from MALDI tandem mass spectrometry, and when peaks are selected for further fragmentation, these gallium-containing peptides give a well-defined, clear fragmentation pattern that can be used to obtain the amino acid

sequences, where *b* series fragments are more abundant. However, compound **2.5** shows a higher number of distinguishable fragments (**Figure 2.5**). It is possible to identify clear fragments for the *b* series and *y* series on **2.5**, while for **2.6** and **2.7**, fragmentation does not provide well-defined peaks for *b* and *y* series.

Table 2.1 Fragment assignment from *b* and *y* series for compound 2.5. $[M+H]^+$ *m/z* = 1408.5475 (gallium-69 peak).

Series	Label	Fragment	Peak (m/z)	Difference between peaks	Amino Acid Assignment
<i>b</i> <i>series</i>	<i>b</i> ₀	[Ga]DOTA	-----	-----	-----
	<i>b</i> ₁	[Ga]DOTA-Q	581.17	-----	Glutamine
	<i>b</i> ₂	[Ga]DOTA-QA	652.21	71.04	Alanine
	<i>b</i> ₃	[Ga]DOTA-QAT	753.25	101.04	Threonine
	<i>b</i> ₄	[Ga]DOTA-QATD	868.27	115.02	Aspartic Acid
	<i>b</i> ₅	[Ga]DOTA-QATDK	996.34	128.07	Lysine
	<i>b</i> ₆	[Ga]DOTA-QATDKF	1143.39	147.05	Phenylalanine
	<i>b</i> ₇	[Ga]DOTA-QATDKFT	1244.45	101.06	Threonine
	<i>b</i> ₈	[Ga]DOTA-QATDKFTF	1391.50	147.05	Phenylalanine
<i>y</i> <i>series</i>	<i>y</i> ₁	-----	-----	-----	-----
	<i>y</i> ₂	-----	-----	-----	-----
	<i>y</i> ₃	FTF	413.12	-----	-----
	<i>y</i> ₄	KFTF	541.33	128.21	Lysine
	<i>y</i> ₅	DKFTF	-----	-----	-----
	<i>y</i> ₆	TDKFTF	757.40	-----	Threonine
	<i>y</i> ₇	ATDKFTF	828.44	71.00	Alanine
	<i>y</i> ₈	[Ga]DOTA-QATDKFTF	-----	-----	Glutamine

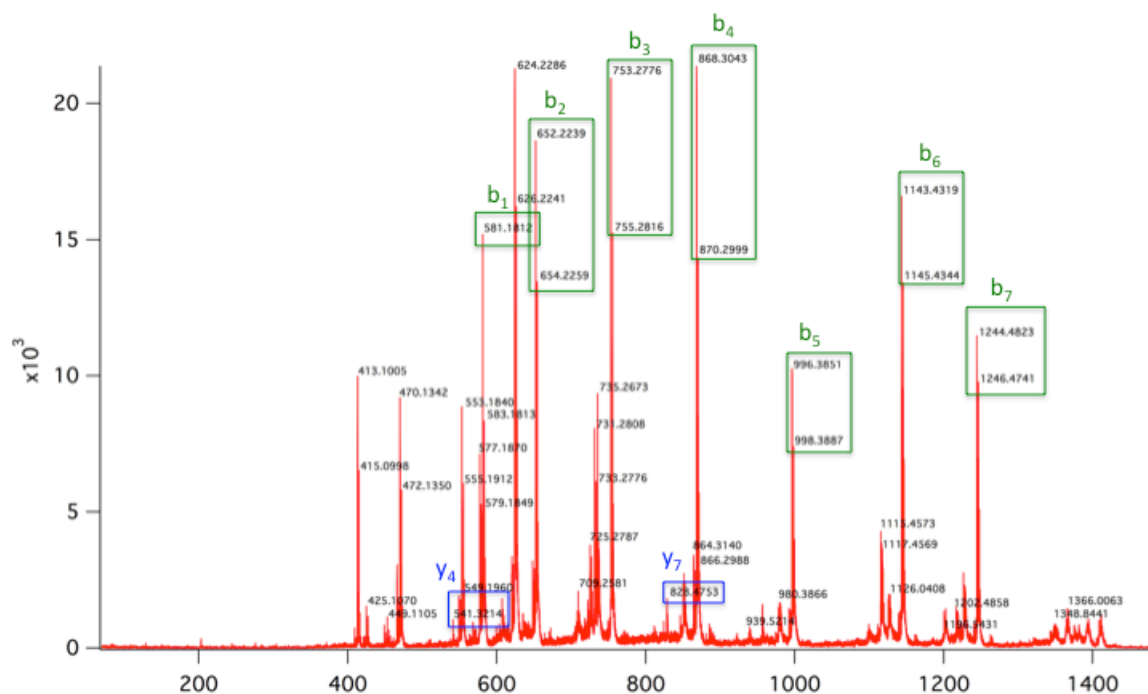


Figure 2.5 MALDI tandem mass spectrum for compound 2.5. The *b* series fragments can be easily identified.

For **2.6** and **2.7**, there is a prevalence of gallium-containing ions, meaning not all *y* and *b* fragments are easily identified (**Figures 2.6 and 2.7**). For **2.6**, it is only possible to identify *b* fragments for higher masses, where the isotopic signature can be visualized, making it a challenge to predict the first few amino acids of the peptide sequence. For **2.7**, it is even harder to assign the correct peaks and only *y*₁ and *y*₇ can be easily visualized. Even though these particular sequences can be determined, the absence of peaks and difficulties in assigning the correct corresponding mass for a single peak can add an extra challenge when attempting to determine peptide sequences containing the [Ga]DOTA complex in randomized positions.

Table 2.2 Fragment assignment from *b* and *y* series for compound **2.6**. $[M+H]^+$ m/z = 1351.5503 (gallium-69 peak).

Series	Label	Fragment	Peak (m/z)	Difference between peaks	Amino Acid Assignment
<i>b</i> series	b_1	H-A	-----	-----	-----
	b_2	H-AA	-----	-----	-----
	b_3	H-AAT	-----	-----	-----
	b_4	H-AATD	-----	-----	-----
	b_5	H-AATDK([Ga]DOTA)	939.2092	-----	-----
	b_6	H-AATDK([Ga]DOTA)F	1086.2496	147.0404	Phenylalanine
	b_7	H-AATDK([Ga]DOTA)FT	1187.2853	101.0357	Threonine
	b_8	H-AATDK([Ga]DOTA)FTF	1334.2766	146.9913	Phenylalanine
<i>y</i> series	y_1	F	-----	-----	-----
	y_2	TF	-----	-----	-----
	y_3	FTF	-----	-----	-----
	y_4	K([Ga]DOTA)FTF	995.2423*	-----	-----
	y_5	DK([Ga]DOTA)FTF	1108.2667	115.0244	Aspartic Acid
	y_6	TDK([Ga]DOTA)FTF	1209.2937	101.027	Threonine
	y_7	ATDK([Ga]DOTA)FTF	-----	-----	-----
	y_8	AATDK([Ga]DOTA)FTF	-----	-----	-----

* Corresponds to the gallium-71 peak.

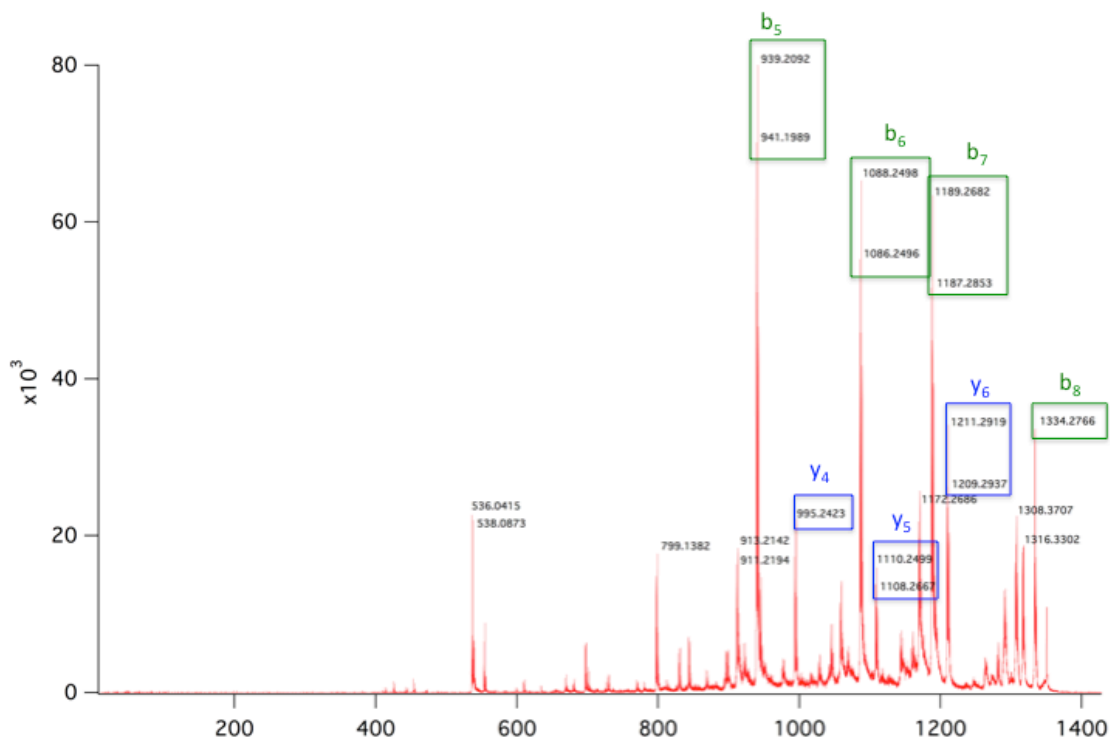


Figure 2.6 MALDI tandem mass spectrum for compound **2.6**. The b ions can be easily identified when containing the [Ga]DOTA complex, however, lower mass fragments are not easily identified.

For a matter of comparison, a fourth peptide was synthesized, containing a [Ga]DOTA motif on the N-terminus, but substituting the glutamine residue by an alanine (**2.8**) (**Figure 2.8**). By performing such change, it is possible to establish a closer comparison between products **2.6**, **2.7** and **2.8** on their fragmentation profiles. The fragmentation profile of **2.9** is shown on **Figure 2.9**.

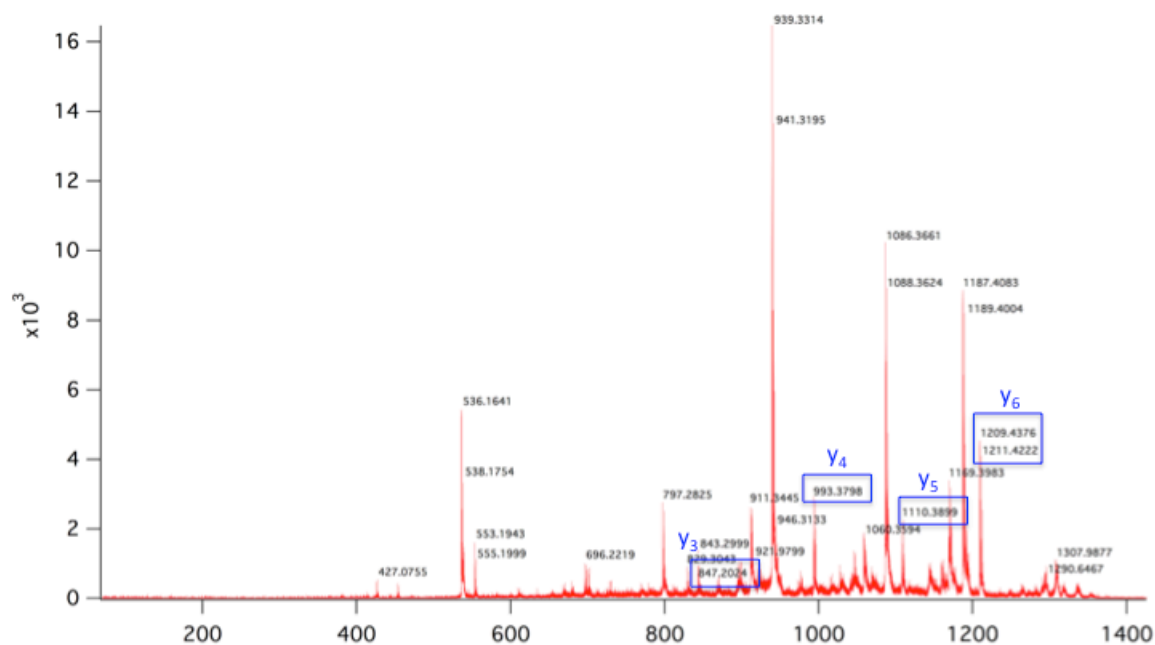


Figure 2.7 MALDI tandem mass spectrum for compound **2.7**. The *b* ions cannot be easily identified. Ions containing the [Ga]DOTA motif can be better identified; however, it is challenging to assign fragments for this sequence.

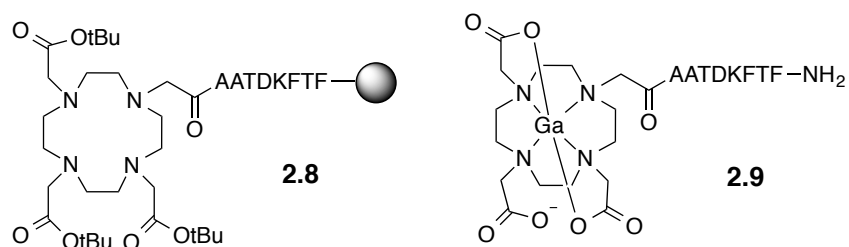


Figure 2.8 Structure of compounds **2.8** and **2.9**, which are a variations of compound **2.2** and **2.5**, and shows a structure that is similar to **2.6**, being the position of the [Ga]DOTA surrogate the only major difference.

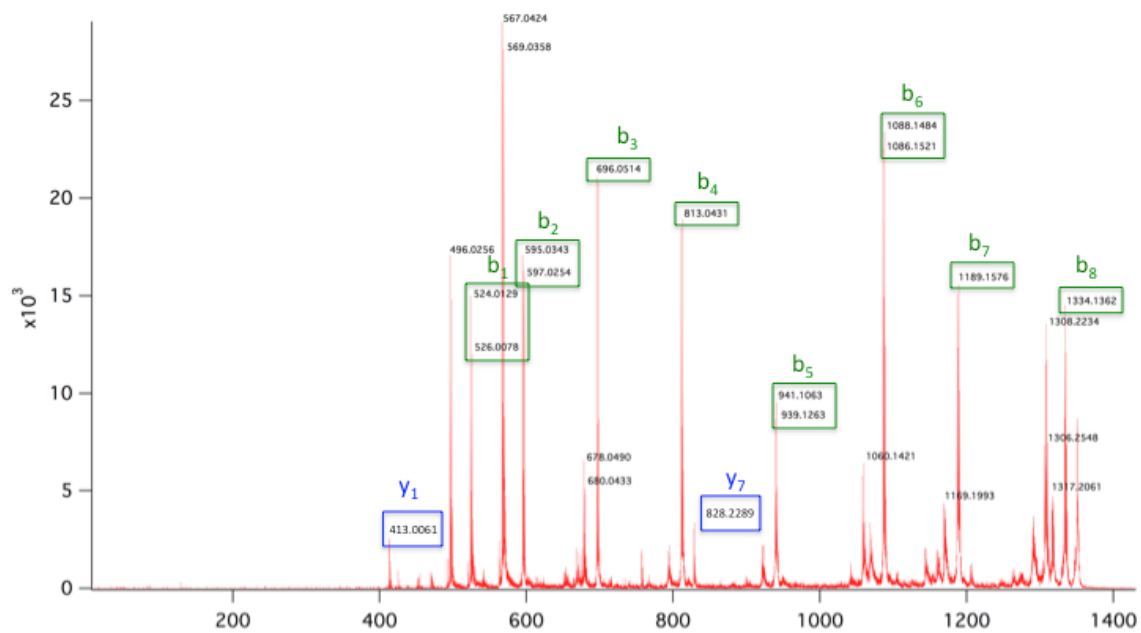


Figure 2.9 MALDI tandem mass spectrum for compound **2.9**. The *b* series fragments can be easily identified.

Table 2.3 Fragment assignment from *b* and *y* series for compound **2.9**. $[M+H]^+$ m/z = 1351.1435

Series	Label	Fragment	Peak (m/z)	Difference between peaks	Amino Acid Assignment
<i>b</i> <i>series</i>	b_0	[Ga]DOTA	-----	-----	-----
	b_1	[Ga]DOTA-A	524.01	-----	Alanine
	b_2	[Ga]DOTA-AA	595.03	71.04	Alanine
	b_3	[Ga]DOTA-AAT	696.05	101.04	Threonine
	b_4	[Ga]DOTA-AATD	813.04	115.02	Aspartic Acid
	b_5	[Ga]DOTA-AATDK	939.13	128.07	Lysine
	b_6	[Ga]DOTA-AATDKF	1086.16	147.05	Phenylalanine
	b_7	[Ga]DOTA-AATDKFT	1187.16	101.06	Threonine
	b_8	[Ga]DOTA-AATDKFTF	1334.14	147.05	Phenylalanine

<i>y</i> <i>series</i>	y_1	-----	-----	-----	-----
	y_2	-----	-----	-----	-----
	y_3	FTF	413.12	-----	-----
	y_4	KFTF	-----	-----	-----
	y_5	DKFTF	-----	-----	-----
	y_6	TDKFTF	-----	-----	-----
	y_7	ATDKFTF	828.23	-----	-----
	y_8	[Ga]DOTA-QATDKFTF	-----	-----	-----

2.3 Conclusions

The results obtained during the investigation of the fragmentation patterns of gallium-containing peptides gives useful information regarding the effects of the addition of a chelator and a metal to different positions on a peptide chain. These results have shown the first sequencing of [Ga]DOTA-containing peptides, showing the gallium isotope signature, which assists with the determination of *b* versus *y* ions, it also provides

starting point for further research on the identification of peptide sequences obtained from a [Ga]DOTA-containing OBOC peptide library for the development of imaging agents.

Similar peptide sequences were used for this experiment, with the only change being in the position of the [Ga]DOTA motif. Compounds containing the complex in the N-terminus were successfully sequenced, while the sequencing of peptides containing the complex in different positions within the peptide chain was challenging due to the absence of a few fragment peaks. This would raise a concern in the synthesis of a library containing the [Ga]DOTA complex in a random position within the peptide sequence.

The results shown proved that MALDI TOF/TOF is a reliable method for the sequence determination of gallium-containing OBOC peptide libraries.

2.4 Experimental Methods

2.4.1 General Materials and Procedures

All chemicals and solvents were purchased from commercially available sources and used without further purification. Fmoc amino acids, coupling reagents and Rink Amide MHBA (4-(2',4'-dimethoxyphenyl-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin resin were purchased from Peptides International (Louisville, KY) or Aapptec (Louisville, KY).

Solvents were purchased from Fisher Scientific and Sigma-Aldrich. Analytical HPLC was performed using a Waters Sunfire C18 column 4.6 x 150 mm, 5 μ m, while

semi-preparative HPLC was performed utilizing a Waters Sunfire Prep C18 OBD column 19 x 150 mm, 5 μ m. The gradient system consists of acetonitrile + 0.1% TFA (Solvent A) and water + 0.1% TFA (Solvent B). Ultraviolet absorbance measurements were obtained utilizing a Waters 2998 Photodiode Array Detector and Mass spectra were obtained via electrospray ionization utilizing a Micromass Quatro Micro LCT mass spectrometer.

MALDI mass spectrometry experiments were done utilizing an Applied Biosystems 4700 PDS, equipped with a Nd: YAG laser tuned to a wavelength of 355 nm, with a 200 Hz laser rate operating in reflectron mode and tandem time-of-flight ion detectors in series. The instrument was calibrated with a mixture of angiotensin I (m/z 1296.685), Glu-1-Fibrino-peptide B (m/z 1570.677) to a mass accuracy of \pm 0.5 Da.

2.4.2 General Peptide Synthesis

Standard Fmoc solid phase peptide synthesis (SPPS) procedures were utilized to obtain the desired peptides containing all side chain protecting groups. Rink amide MHBA NH₂ resin (0.1 mmol, 0.52meq/g) containing a Fmoc protecting group was used. The resin was swelled using DCM washes, and the Fmoc group was removed using a 20% piperidine in DMF solution (2x, 5 min and 20 min, respectively).

The deprotection solution was removed from resin using a series of DMF and DCM washes. Fmoc protected amino acids (0.3 mmol, 3 eq) were dissolved in approximately 0.5 mL DMF, along with HCTU (0.3 mmol, 3 eq) and DIPEA (0.6 mmol, 6 eq). For the peptides described above, the amino acids used were: Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ala-OH and

Fmoc-Gln(Trt)-OH. The solution of coupling reagents plus activated amino acid was then combined with the resin and agitated for one hour. The resin was then washed with DMF and DCM multiple times. This process of coupling and deprotection was then repeated until the desired sequence of the peptides was obtained. Kaiser test⁹⁰ was performed to confirm the presence of free primary amino groups after deprotection and the absence after coupling of amino acids. For each peptide sequence, the deprotection step preceding the addition of the chelator DOTA was performed in different steps of the synthesis, using different reagents, which will be specified below.

(DOTA)QATDKFTF-NH₂ (2.2)

After a last Fmoc deprotection, the chelator DOTA, in its tBu-protected form (tris-tBu-DOTA) was added manually by the use of solid phase peptide synthesis techniques (4 eq, 0.4 mmol), using HCTU (4 eq, 0.4 mmol) and DIPEA (8 eq, 0.8 mmol) as coupling reagents.

H-AATDK(DOTA)FTF-NH₂ (2.3)

Peptide synthesis is performed as described on 2.4.2 until the coupling of the second phenylalanine. For the next coupling Fmoc-Lys(Mtt)-OH is used instead of Fmoc-Lys(Boc)-OH. Before removing the Fmoc group from the lysine, a Mtt deprotection is performed using a 1.8% TFA solution in DCM (9x, 3 minutes). The chelator DOTA, in its tBu-protected form (tris-tBu-DOTA) was added manually using standard solid phase peptide synthesis techniques (4 eq, 0.4 mmol), using HCTU (4 eq, 0.4 mmol) and DIPEA (8 eq, 0.8 mmol) as coupling reagents. After coupling the chelator, the Fmoc group from lysine was removed, and the remaining of the peptide was built up to completion.

H-AATDFTFK(DOTA) (2.4)

Following a very similar procedure to compound **2.3**, here, Fmoc-Lys(Mtt)-OH is used. After the first coupling step, before removing the Fmoc group from the lysine, a Mtt deprotection is performed using a 1.8% TFA solution in DCM (9x, 3 minutes), until change of colour from clear to yellow was no longer observed. The chelator DOTA, in its tBu-protected form (tris-tBu-DOTA) was added manually using standard solid phase peptide synthesis techniques (4 eq, 0.4 mmol), using HCTU (4 eq, 0.4 mmol) and DIPEA (8 eq, 0.8 mmol) as coupling reagents. After coupling the chelator, the Fmoc group from lysine was removed, and the remaining of the peptide was built up to completion.

(DOTA)AATDKFTF-NH₂ (2.8)

After a last Fmoc deprotection, the chelator DOTA, in its tBu-protected form (tris-tBu-DOTA) was added manually by the use of solid phase peptide synthesis techniques (4 eq, 0.4 mmol), using HCTU (4 eq, 0.4 mmol) and DIPEA (8 eq, 0.8 mmol) as coupling reagents.

2.4.3 Peptide Deprotection, Cleavage and Purification

After the chelator coupling, the peptide was mixed with 5 ml of a solution of 95% TFA, 2.5% H₂O and 2.5% TIS for six hours to remove the peptide from resin, as well as remove side-chain protecting groups. The resulting solution was cooled on ice and cold tert-butyl methyl ether (TBME) (20 mL) was added to the solution until a white

precipitate formed. The solution was then centrifuged at 0°C at 3000 rpm for 10 minutes. The liquid was decanted and the resulting pellet was mixed with another 20 mL of cold TBME and subsequently centrifuged again under identical conditions to obtain the crude peptide. Following the removal of the supernatant, the peptide pellets were dissolved in water, frozen at -78°C and lyophilized. Small samples of the resin were cleaved using the full deprotection procedure in order to monitor reaction completion.

The crude peptides were dissolved in water, purified by HPLC (15 min linear gradient 15% solvent A in solvent B to 70%) and identified by mass spectrometry, giving white powders after lyophilization.

(DOTA)QATDKFTF-NH₂ (2.2)

Purity: >95% [M+H]⁺ *m/z* calc: 1342.66 obs: 1342.94.

H-AATDK(DOTA)FTF-NH₂ (2.3)

Purity: >99% [M+H]⁺ *m/z* calc: 1285.42 obs: 1285.93

H-AATDFTFK(DOTA) (2.4)

Purity: >99% [M+H]⁺ *m/z* calc: 1285.42 obs: 1285.99

(DOTA)AATDKFTF-NH₂ (2.8)

Purity: >99% [M+H]⁺ *m/z* calc: 1286.42 obs: 1286.01

2.4.4 Cold Ga Coordination Procedures

The procedures here stated correspond to compounds **2.5**, **2.6**, **2.7** and **2.9**.

10 mg of each DOTA-peptide, dissolved in 0.1 M pH 4 NaOAc/HOAc buffer were added to a round bottom flask containing 5 mg of GaCl₃. The mixture was stirred at room temperature for 30 minutes, then heated to 70° C and stirred for another 30 minutes. The resulting mixture was cooled prior to purification using a light C18 RP Sep-Pak© cartridge. The reaction mixture was passed through the Sep-Pak©, and the cartridge was washed with 10 mL of water in order to remove unreacted GaCl₃. Ethanol is then used to wash out the labeled product. The EtOH was then removed *in vacuo*.

[Ga]DOTA-QATDKFTF-NH₂ (**2.5**)

Purity: >98% MS (MALDI) [M+H]⁺ *m/z* calc: 1408.56 obs: 1408.63 (Gallium-71)

H-AATDK([Ga]DOTA)FTF-NH₂ (**2.6**)

Purity: 90% MS (MALDI) [M+H]⁺ *m/z* calc: 1353.13 obs: 1353.55 (Gallium-71)

H-AATDFTFK([Ga]DOTA)-NH₂ (**2.7**)

Purity: >90% MS (MALDI) [M+H]⁺ *m/z* calc: 1353.54 obs: 1353.89 (Gallium-71)

[Ga]DOTA-AATDKFTF-NH₂ (**2.9**)

Purity: >90% MS (MALDI) [M+H]⁺ *m/z* calc: 1353.13 obs: 1353.52 (Gallium-71)

2.4.5 MALDI Tandem Mass Spectrometry

Purified peptides were dissolved in water to a final concentration of approximately 0.1 mg/mL. A portion of the peptide solutions (3 μ L) and an equal portion of CHCA (matrix) solution (3 μ L) were mixed for a few minutes before spotting. CHCA is a mixture of 50:50 acetonitrile/water with 0.1% TFA (v/v) at a concentration of 5 mg/mL. A portion of the peptide/matrix mixture (70 μ L) was spotted onto a MALDI target plate in duplicate for further analysis. The spotted mixture is allowed to evaporate, resulting in the co-crystallization of the matrix with the peptide. Samples were then analysed using MALDI tandem mass spectrometry. The spectra obtained show gallium-69/71 typical isotopic distributions, being the 69 isotope 60% abundant in nature, while the 71 isotope corresponds to 40%. The gallium-69 peak was chosen for fragmentation via CID with argon gas. Tandem mass spectra were obtained and peaks assigned by manual peptide analysis.

Chapter 3: Synthesis, Coordination and Sequence

Deconvolution of a [Ga]DOTA-Octapeptide on TentaGel Resin

3.1 Introduction

Since the advent of OBOC, TentaGel resin is the solid support of choice, as it is stable under acidic and basic conditions required for the removal of protecting groups of peptides. In TentaGel resin, polyethylene glycol (PEG) units of approximately 3 kDa are cografted onto a low-cross-linked-polystyrene.⁹¹ This structure gives TentaGel the ability of swelling in both organic solvents and water, which makes it suitable for cell studies, where the peptide sequences to be tested should be on resin to allow for screening and better visualization. Furthermore, it also allows for the projection of attached groups into solution rather than anchored closer to the polymer backbone.⁹¹

Libraries are synthesized using a split-mix procedure, which will lead to different peptide sequences with the same length, where one individual 90 μm bead contains approximately 10^{13} copies of only one peptide sequence.^{49,50} Here, a single bead should be able to provide strong enough signal for MALDI tandem mass spectrometry, meaning that a single bead should have enough material to be detected by the mass spectrometer and for further fragmentation. Considering that the sensitivity of the MALDI time-of-flight (TOF) instrument is in the order of pmol, and the amount of material available from a single TentaGel bead (0.29 meq) is in the range of 10^{-10} mol, one bead provides enough peptide material for fragmentation.⁹² However, since most cleavage protocols are not able to totally remove a peptide off resin, and the presence of bulk from the chelator and

gallium complex may interfere with the number of sites available to a reacting molecule, test sequences were built on TentaGel using a cleavable linker in order to simulate the sequence deconvolution from a single bead.

3.2 Results and Discussion

3.2.1 Choice of a Suitable Linker

In order to obtain the sequences of positive peptides from an OBOC library using MALDI tandem mass spectrometry, the peptide has to be successfully cleaved off resin after screening without damaging its structure or producing significant byproducts that might interfere with its fragmentation and sequence deconvolution. For this to happen, a stable linker is coupled to the resin before the peptide sequence is built.

When choosing a suitable linker, some important characteristics should be considered, such as the stability of the linker on resin under synthesis and screening conditions, how efficient is the process of peptide removal from resin, and the presence of other cleavage byproducts that can interfere with fragmentation and consequent sequence deconvolution. Two linkers often used in solid phase peptide synthesis (SPPS) were evaluated. Both linkers were added to TentaGel resin, and a peptide sequence was built thereafter (QATDKFTF). The two linkers used were 2-amino-3-(2-nitrophenyl)propanoic acid (ANP), linker, which was previously used in our lab⁶³, and a methionine linker, that is commonly used when light sensitivity becomes an issue.⁹³

The two peptides were cleaved off-resin and analyzed by MALDI tandem mass spectrometry for sequence determination, which showed good fragmentation results with

both linkers (**Figure 3.1**). However, when using the methionine linker, a large number of impurities or byproducts are present, due to its chemical cleavage mechanism, leading to the presence of unwanted fragments that can interfere with the sequence analysis. In addition to that, during the chemical reaction that removes the bead from resin, there is the formation of a homoserine lactone on the C-terminus of the peptide, which might change the peptide fragmentation profile, and make the sequencing process more challenging.

The ANP linker, on the other hand, leaves an amide on the C-terminus of the peptide, which removes the extra challenge of accounting for an extra mass on some fragments. Furthermore, it provides clean spectra, as its light-dependent cleavage mechanism does not produce any significant byproduct.

Due to its easy manipulation, simple cleavage protocol and the fact that it provided clearer results when compared to the methionine linker, the ANP linker became the linker of choice for the design of the [Ga]DOTA OBOC peptide library. The coupling step to add the ANP linker to resin is shown on **Scheme 3.1**. Although the mechanism for ANP off-resin cleavage is not completely understood, it has been proposed to relate to the electronic excitation of 2-nitrobenzyl compounds, via a 1-5 proton shift, which quickly generates their *aci*-nitro tautomers (**Figure 3.2**).^{94,95}

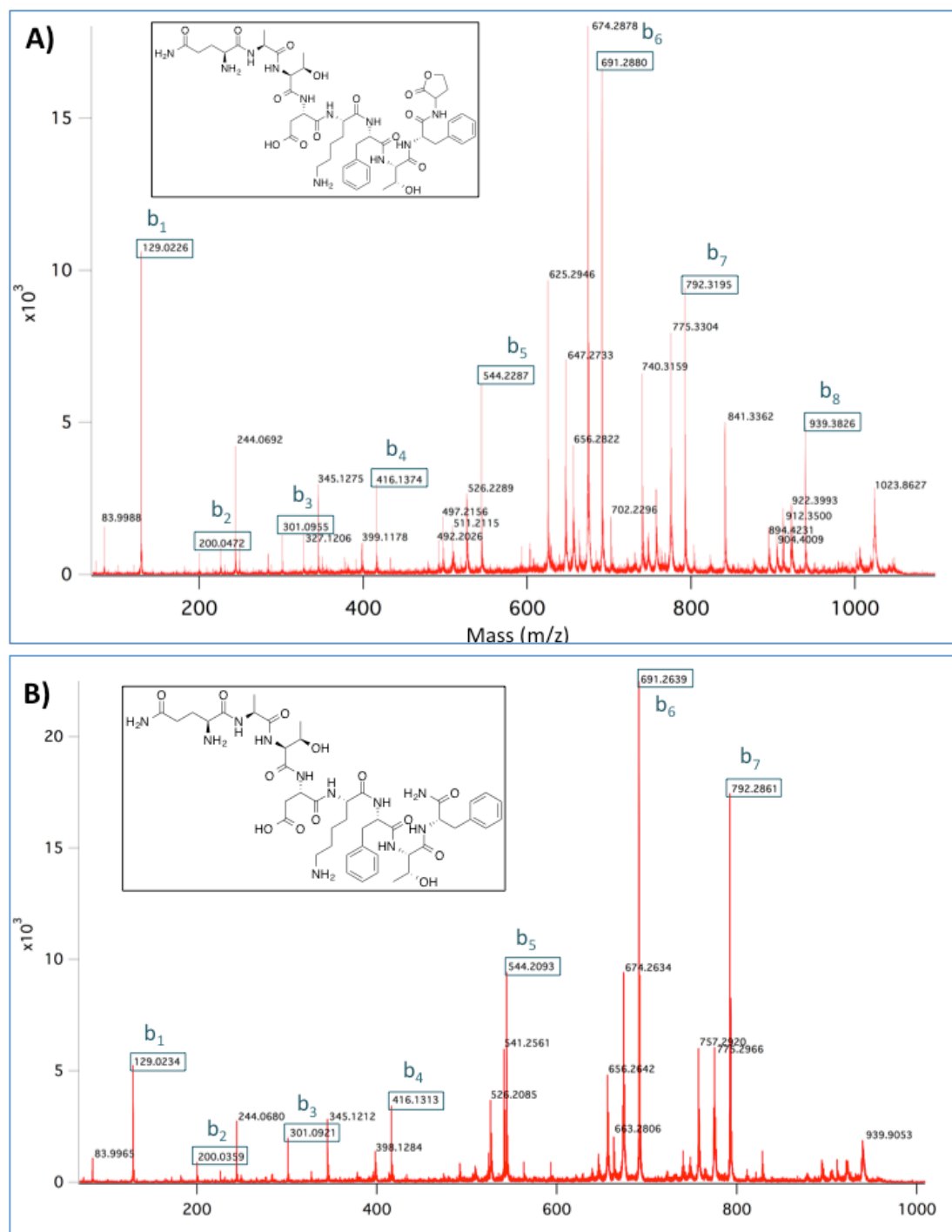
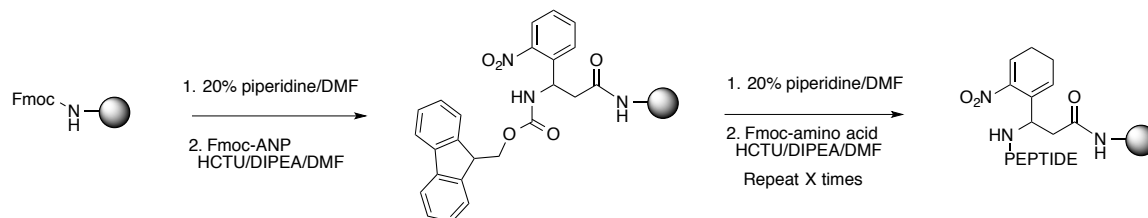


Figure 3.1 a) MS/MS spectrum for QATDKFTF-NH₂ obtained from a chemical cleavage using the methionine linker. After cleavage, there is the formation of a homoserine lactone in the C-terminus of the peptide. b) MS/MS spectrum for QATDKFTF-NH₂ obtained using the photocleavable linker ANP. This linker gives a clear spectrum as it does not contain traces of chemicals.



Scheme 3.1 Coupling of ANP linker to TentaGel resin prior to the addition of Fmoc protected amino acids.

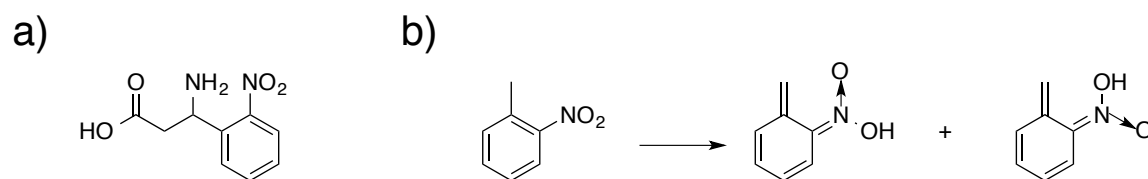


Figure 3.2 a) Structure of ANP linker b) formation of *aci*-nitro tautomers for 2-nitrobenzyl compounds, suggested to be the first step on the cleavage mechanism of ANP linker.

3.2.2 Peptide Synthesis and Gallium Labeling on Resin

The peptide synthesis on TentaGel is performed in a similar fashion to the synthesis on Rink Amide resin, with the only difference being the addition of the ANP linker prior to building up the peptide sequence. Reagent K is used to remove the amino acids side chains, but in the case of TentaGel, it does not remove the peptide off-resin. TentaGel contains a free amine, which forms an amide bond once amino acids are attached. Therefore, the regular acidic cleavage conditions used to remove side-chain protecting groups are not able to remove the peptide from resin.

For this experiment, the gallium labeling procedure was performed on resin, in an attempt to simulate the library synthesis. As the peptide synthesis is conducted in organic solvents, the TentaGel beads do not mix appropriately with the aqueous buffer solution containing gallium chloride, which might cause incomplete on-resin coordination. Therefore, an elaborate washing procedure that will eliminate as much traces of organic solvent as possible is a fundamental step before gallium coordination on resin.

The initial attempts for coordinating gallium to DOTA on TentaGel resin were not successful. With the addition of a greater excess of GaCl_3 , an optimized series of organic solvent and water washes and a longer time for the reaction, complete coupling was achieved and confirmed using MALDI tandem mass spectrometry (**Figure 3.3**).

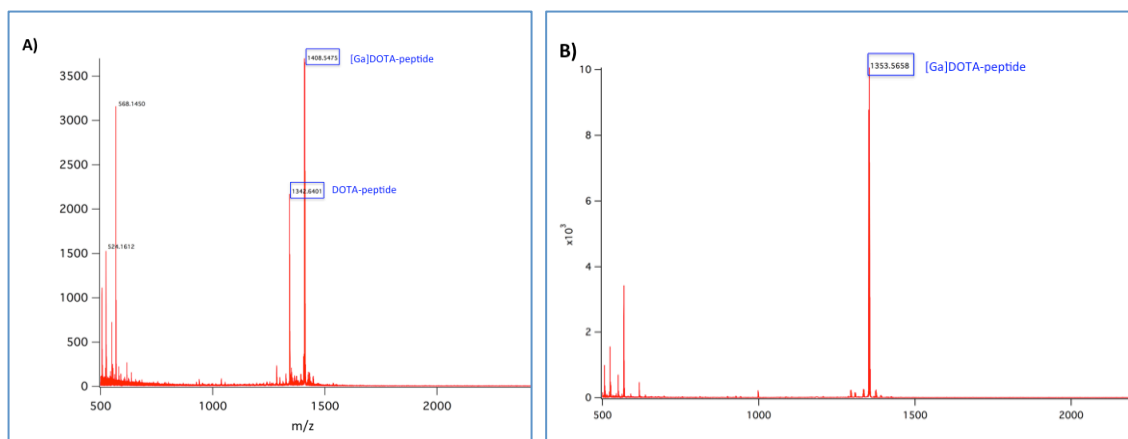


Figure 3.3 a) MS spectrum for $[\text{Ga}]\text{DOTA-QATDKFTF-NH}_2$ showing incomplete gallium coordination on resin, as it shows two major peaks corresponding to the DOTA-peptide with and without gallium. b) MS spectrum for $[\text{Ga}]\text{DOTA-AATDKFTF-NH}_2$ showing complete gallium coordination on resin after increasing reaction time and the concentration of GaCl_3 (from 5 to 10 equivalents).

3.2.3 Sequence Deconvolution

For sequence deconvolution, the positive beads are placed in wells on a 96-well plate with 40 μ L of water. Peptide beads were then exposed to a 365 nm light source for a period of 4 hours to cleave peptides from resin.

MALDI tandem mass spectrometry results showed not only that it is possible to obtain enough material to be detected by the mass spectrometer and also to produce visible fragments, but that is also possible to perform cold gallium labeling on TentaGel resin, by using an excess of gallium chloride in NaOAc/AcOH buffer (pH =5), in a procedure similar to the regular peptide cold gallium coordination described in the previous chapter, as already shown on **Figure 3.3**. Proof that a single bead provides enough material for sequence determination is given on **Figure 3.4**, where the fragment assignments for the test peptide can be observed, predominantly *b* ions.

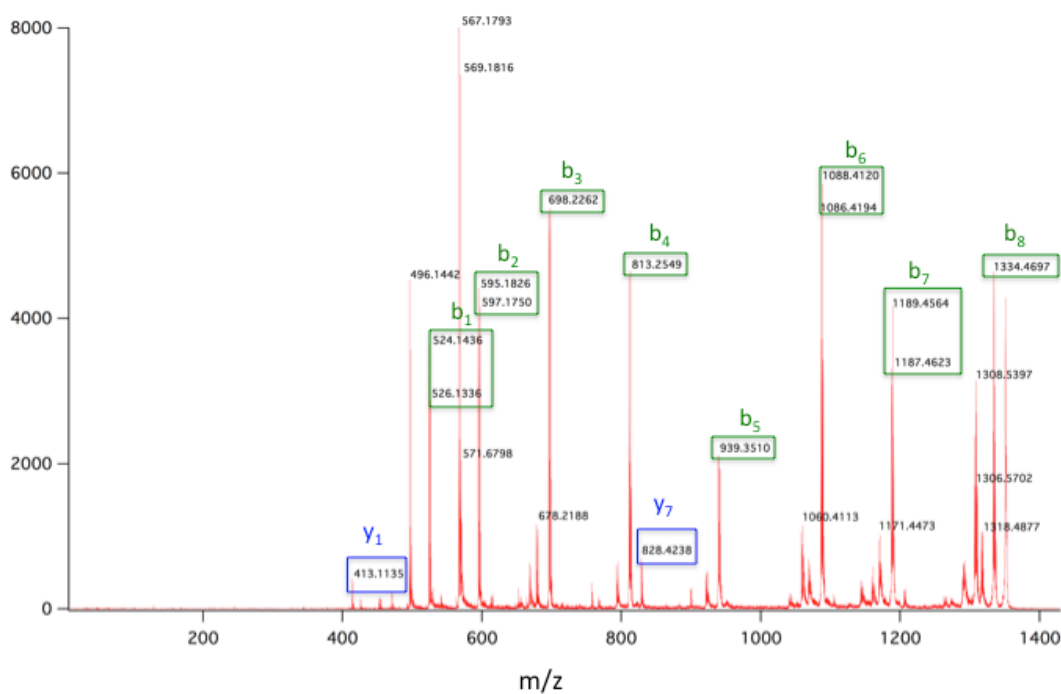


Figure 3.4 MS/MS spectrum for peptide [Ga]DOTA-AATDKFTF-NH₂ obtained from a single bead. Here, b ions can be easily identified and the peptide sequence can be readily obtained.

3.3 Conclusions

The screening of OBOC libraries is performed on resin, while for the deconvolution of positive sequences using MALDI tandem mass spectrometry, the peptide must be off resin. For this to happen, it is necessary to place a stable linker on resin prior to building the peptide sequence. Of the two linkers tested, the ANP linker showed better results, as it provides a cleaner spectra when compared to the methionine linker. The reason for this difference may be due to the presence of chemical reagents in the methionine linker samples that are analyzed by MALDI tandem mass spectrometry, due to its cleavage protocol.

Using ANP as a linker, $^{69/71}\text{Ga}$ was successfully coordinated on resin after optimization of the amount of reagent used and the reaction time. Furthermore, peptide sequences can be determined for this gallium-peptide using only a single bead for analysis. The signal obtained is strong enough to easily identify correspondent peaks leading to amino acid assignments, with *b* ions more common than *y* ions for the sequence used.

Considering the combination of positive results acquired for the gallium-containing model peptide, we were confident to proceed to the synthesis, screening and sequence deconvolution of a gallium-containing peptide library.

3.4 Experimental Methods

Chemicals and solvents were purchased and used without further purification. Resin, Fmoc amino acids and coupling agents were purchased from Peptides International (Louisville, KY) or Aapptec (Louisville, KY), unless stated otherwise. Solvents were purchased from Fisher Scientific and Sigma Aldrich.

The automated peptide synthesis was performed using a Biotage® Syro Wave™ Peptide Synthesizer. MALDI tandem mass spectrometry experiments were performed using an Applied Biosystems 4700 PDS, equipped with an Nd: YAG laser tuned to a wavelength of 355 nm. The instrument was calibrated using a mixture of angiotensin I (m/z 1296.685), Glu-1-Fibrino-peptide B (m/z 1570.677) to a mass accuracy of ± 0.5 Da.

3.4.1 Peptide Synthesis using different linkers

Standard Fmoc solid phase peptide synthesis was performed to obtain the peptides. Tentagel S NH₂ resin (0.1mmol, 0.29 mmol/g) containing a Fmoc protecting group was utilized. The resin was swelled with DCM washes. A solution of 20% piperidine was used to remove the Fmoc group (1x 5min, and 1x 15 minutes, respectively) followed by DMF and DCM washes. Either Fmoc-ANP linker or Fmoc-methionine (0.3 mmol, 3 eq) were dissolved in approximately 5 mL DMF along with HCTU (0.3 mmol, 3 eq) and DIPEA (0.6 mmol, 6 eq). The aminoacids coupled after the linker were: Fmoc-Phe-OH, Fmoc-Thr (tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ala-OH and Fmoc-Gln(Trt)-OH. A final Fmoc deprotection and side chain deprotection were performed before following the cleavage protocols. The cleavage cocktail used for side chain deprotection consisted of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5% H₂O.

3.4.2 Chemical Cleavage Protocol

Peptide beads were isolated using a microscope and placed in a 96-well plate with conical bottom with 40-50 μ L of water. The liquid was allowed to evaporate overnight before 20-30 μ L of a 0.25 M CNBr in 70% aqueous formic acid solution was added to each well and left for 12 hours at room temperature. The wells were then washed 3 times with 30 μ L of water. The contents of the wells were allowed to evaporate in between washes. The final product was kept in 40-50 μ L of water prior to MALDI analysis.

3.4.3 Photocleavage Protocol

Peptide beads were isolated using a fluorescent microscope and placed individually on wells on a 96-well plate with approximately 40-50 μL of water. The peptide beads were then exposed to a 365 nm light source for a period of 4 hours, replenishing the volume of water to 40-50 μL frequently, in order to prevent the wells containing the beads from drying, which could lead to peptide degradation.

3.4.4 MALDI Tandem Mass Spectrometry Analysis

After following the different cleavage protocols, 2 μL of each peptide solution obtained was mixed with 2 μL of α -Cyano-4-hydroxycinnamic acid (CHCA) matrix for 5 minutes. An aliquot of 0.75 μL of this mixture was spotted onto a MALDI target plate in duplicate for analysis. The solvent was allowed to evaporate, leading to a co-crystallization of the matrix and peptide. The samples were then analyzed utilizing MALDI tandem mass spectrometry.

3.4.5 Peptide Synthesis on TentaGel

Peptide synthesis was performed using the automated peptide synthesizer, on TentaGel S NH_2 resin. The sequences built were the same described on section 2.4.2.

The first step, coupling the Fmoc-ANP linker was performed manually. The resin was swelled in DCM, Fmoc deprotection was performed and the linker was dissolved in DMF and added to the resin along with HCTU (4 eq, 0.4 mmol) and DIPEA, (8 eq, 0.8

mmol). The mixture was then stirred for one hour, and the solution was removed from the vessels by DMF and DCM washes. The resin containing the ANP linker was then transferred to an automated peptide synthesizer vessel and the peptide synthesis was carried on, following the same procedures stated on 2.4.2.

After proceeding with automated synthesis, the chelator coupling was also performed manually, after a final Fmoc deprotection.

Samples were analyzed by MALDI tandem mass spectrometry to check for the presence of the desired peptide (mass).

3.4.6 Peptide Side Chain Deprotection

Side chain deprotection was performed using a cocktail of 95% TFA, 2.5% water and 2.5% TIS, and the washing after 4 hours was performed as follow: 1) 3x DMF, 2) 2x MeOH, 3) 2x 5%DIPEA in DMF, 4) 3x DMF, 5) 3x MeOH, 6) 3x DCM, 7) 3x DMF, 8) 3 x 50% DMF in water, 9) 5 x water. The peptides are then kept in water solution prior to gallium coordination and MALDI analysis.

3.4.7 ^{69/71}Ga Coordination on TentaGel Resin

TentaGel resin (10 mg) containing the peptide sequence of interest, was added to a small round bottom flask containing initially 5, and later on 10 mg of GaCl₃ dissolved in 0.1 M pH 4 NaOAc/HOAc buffer. The mixture was stirred for 45 minutes at room temperature and another 45 minutes at 75° C. The resulting mixture was cooled to room temperature, added to a small peptide vessel, and washed with water multiple times. The

peptide beads were kept in water for further studies using MALDI tandem mass spectrometry.

3.4.8 MALDI Analysis of a Peptide From a Single TentaGel Bead

Beads were isolated using an Olympus IX70 inverted fluorescent microscope and placed individually on wells on a 96-well plate with around 50 μL of water and irradiated with UV light for a period of four hours.

An aliquot of the peptide solution (2 to 4 μL) is mixed with an equal volume of CHCA matrix, and a portion (0.75 μL) was spotted onto a MALDI target in duplicate for mass spectrometry analysis. The solvent on the mixture is then allowed to evaporate. After that, samples were analyzed using MALDI tandem mass spectrometry, showing a typical gallium isotopic signature (69 and 71 isotopes). Tandem mass spectra were obtained and peaks assigned by manual *de novo* peptide analysis.

Chapter 4: Synthesis, Screening and Sequence Deconvolution of a Gallium-containing OBOC Peptide Library

4.1 Introduction

4.1.1 Breast Cancer

Breast cancer is one of the most common cancers affecting Canadian women each year. In 2013, the Canadian Cancer Society estimated that 23,800 Canadian women will be diagnosed with breast cancer and approximately 5,000 would die from it.⁹⁶

The current standard diagnostic tools for tumour detection are mammography and ultrasound, which have been able to detect tumours at a relatively early stage.⁹⁷ Over the years, the low sensitivity and specificity of these techniques have proven inefficient in patients with dense breast tissue, breast implants or postsurgical abnormalities^{98,99}, and therefore, the need for the development of non-invasive diagnostic tools is of great interest. These tools will not only provide a better diagnosis for breast cancer but may also be able to provide information on the physiology of a tumour, disease progression and therapy response.

Many efforts have been made to improve imaging detection in order to better discriminate between benign and malignant tumours, as well as to differentiate them based on their pathology. One of the modalities recently studied for such matter is contrast enhanced magnetic resonance imaging (CE-MRI), which provides high sensitivity (over 90%), but still a low to moderate specificity for lesion characterization

(72%)¹⁰⁰⁻¹⁰², which still makes it a challenge to differentiate between cancer and benign lesions, or aggressive versus non-aggressive tumours.¹⁰³

In PET imaging, ¹⁸F-FDG has been used to assess response to therapy or disease progression. Quantitative PET-CT is becoming a valuable tool for the assessment of individual response to therapy, as it accounts for changes in tumour size and metabolism. By calculating the changes in tumour standardized uptake values (SUVs), it is possible to evaluate a patient's response to therapy and the disease progression. However, with the use of ¹⁸F-FDG and the available tools from PET-CT imaging, there is still significant interference from different error sources, compromising the ability to detect SUV changes, and even though it has become a useful tool for management of disease progression, especially regarding metastasis, it is still not reliable on the differentiation of some aggressive versus non-aggressive tumours, which is still a challenge when diagnosing breast cancer.¹⁰⁴

Although recent efforts were made in the field of non-invasive methods for breast cancer detection, mammography is still the more efficient test for the detection of breast tumours, even though it may still not discriminate tumours correctly.

Relying on anatomic density and distortions, and byproducts of a cancer mammogram is far from ideal, leading to a need for more specific tests for the early efficient detection of aggressive breast tumours, which would not only increase the chances of survival, but also help improve therapy accordingly. The design of gallium-containing peptide libraries aims to discover bioactive peptide sequences that can be

developed into PET imaging agents to be used as a non-invasive method for the early detection and determination of cancer aggressiveness of breast tumours.

4.1.2 OBOC Library Screening

One-bead one-compound combinatorial (OBOC) libraries have been successfully used to identify ligands for a large number of biological targets, using a variety of screening methods.^{50,61,105} In 1991, when the OBOC library concept was first reported, the library screening consisted in having acceptor molecules coupled to an alkaline phosphatase enzyme and added in soluble form to the peptide bead library. The positive beads were easily identified due to the intensive staining.⁴⁹

A method for the discovery of anti-cancer drugs was developed using beads containing 2 orthogonal linkers that release the bead-bound compound at different pH. Here, cells mixed with beads were plated in soft agarose containing cell culture medium. A portion of the peptide is released from the beads at neutral pH, and the cytotoxic effect is observed as a clear ring of tumour cell lysis. Positive beads are easily selected and the remaining of the compound is cleaved off resin for structural analysis.¹⁰⁶

Although very efficient, the use of enzyme-linked colorimetric assays have some limitations, especially regarding the presence of false positive interactions, usually corresponding to peptide beads binding to other screening agents such as secondary antibodies, streptavidin, and reporting enzymes. In order to overcome these limitations, a dual colour screening method was developed.¹⁰⁷ This is a two-stage method that aims to rapidly identify compounds that bind only to a specific protein and monitor the intensity

of this interaction. The first stage consists in colorimetrically labeling nonspecific interactions with background proteins, and the second stage consists in colorimetrically labeling the beads with the target protein. For these two steps, beads are immobilized in agarose and scanned on flat-bed transparency scanner after the first and second stages. By using an image subtraction technique, the resulting image highlights beads that specifically bind the desired target protein.^{107,108}

With the advent of the automated bead sorter, the complex parametric analyzer and sorter (COPAS), which sorts positive beads based on size and fluorescence, studies where the target protein is conjugated to fluorescent dyes instead of enzymes were developed. These studies improved the efficiency of the screening of libraries by scanning an even larger number of compounds in a short period of time.^{81,109}

Although the described approaches are feasible for a variety of targets, during the purification and derivatization process prior to screening, these proteins can adopt an altered conformation that changes their function and interaction with bioactive peptides. In order to preserve the protein's conformation and enable proper display of biologically relevant epitopes¹¹⁰, the presence of the whole plasma membrane and binding partners are often used. Therefore, binding assays that utilize cells are still preferred as a primary method of library screening, and have been successfully utilized to discover ligands against a variety of human cancer cell lines such as breast cancer⁵¹ and Jurkat T-leukemia.¹¹¹

4.2 Results and Discussion

4.2.1 OBOC Library Synthesis

As previously described, it is possible to determine the sequence of a peptide from a single peptide bead when using the ANP linker. This allowed for the synthesis of a [Ga]DOTA-containing peptide library for screening against different cancer cell lines using standard solid phase peptide synthesis approaches. For the synthesis of this library, all natural amino acids were used, except cysteine and methionine. Cysteine has the ability to form disulfide bonds between peptide chains, thus adding some challenges during the sequencing process. On the other hand, methionine is known to be unstable to UV cleavage conditions and is also predisposed to oxidation.

When constructing an eight amino acid library using eighteen natural amino acids, it would result in approximately 10^{10} possible sequences. However, this would lead to the use of kilograms of resin, which becomes impractical. As a proof-of-concept, 1 g of resin beads were used in the design of this library, which allowed for the synthesis of approximately 1.5 million individual peptide sequences.

The library synthesis followed a “split-mix” procedure. After manual Fmoc deprotection and coupling of Fmoc-ANP-OH to 1 g of resin, the resin was divided equally among 18 different wells on the peptide synthesizer, each corresponding to a different natural amino acid. Beads from each well were recombined after deprotection and coupling step, mixed thoroughly and divided again among the eighteen wells. This procedure was repeated until the desired length of the library was reached. This method

guaranteed that only one amino acid was coupled to each well in every step, allowed for sequence randomization and provided peptides of the same length.

After eight coupling cycles, the resin was again recombined, and the coupling of DOTA, as well as gallium coordination, were performed manually. For DOTA, 4 equivalents of the chelator on its tri-*t*Bu protected form were added to the resin beads, together with 4 equivalents of HCTU and 8 equivalents of DIPEA.

Side chain deprotection is achieved by using 8 mL of a TFA/H₂O/TIPS mixture prior to gallium coordination, which is performed by using excess of GaCl₃ in NaOAc/HOAc pH 4 buffer, in a procedure already described on chapter 3. The extra purification step, using a Sep Pak® cartridge was not necessary here, since the resin beads are retained in the peptide vessel when washing out unreacted gallium.

The purity of the library is assessed through MALDI tandem mass spectrometry, prior to library screening. A total of 50 beads were selected from the library using a fluorescence microscope and were irradiated with UV light for four hours with approximately 40 μL of water. The resultant mixtures were co-crystallized with CHCA and the MALDI tandem spectra were obtained and sequenced. Results showed the presence of only one peptide on each single bead, all containing eight amino acid sequences (**Figure 4.1**).

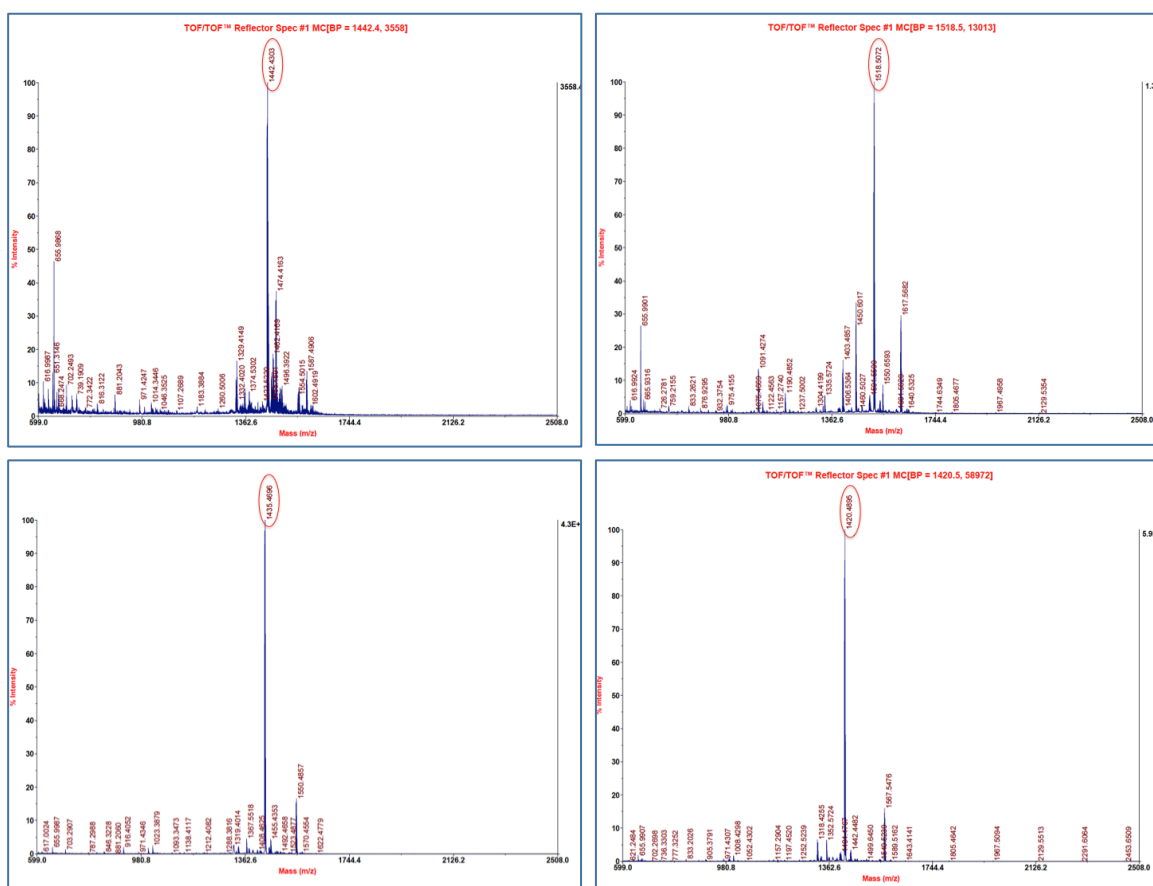


Figure 4.1 Samples from the [Ga]DOTA OBOC library showing the presence of only one peptide on a single bead.

4.2.2 Library Screening

For the screening of the designed library, we were looking to find peptides that would bind specifically to a more aggressive breast cancer cell line, MDA-MB-231, by screening the obtained library against those cells and later on performing a negative versus positive screening using MCF-7 cells, as both cells have a different receptor expression. MDA-MB- 231 cells expressing the tdTomato protein and MCF-7 cells expressing zSgreen protein, both stably transfected, were kindly provided by Dr. Hon

Sing Leong and Dr. Ann Chambers (Departments of Oncology, Medical Biophysics and Pathology, University of Western Ontario). These cells were incubated with the peptide library and observed under a fluorescence microscope one hour after incubation. The desired outcome was to observe MDA-MB-231 cells attached to a number of beads, which means those beads contain a bioactive sequence (**Figure 4.2**).

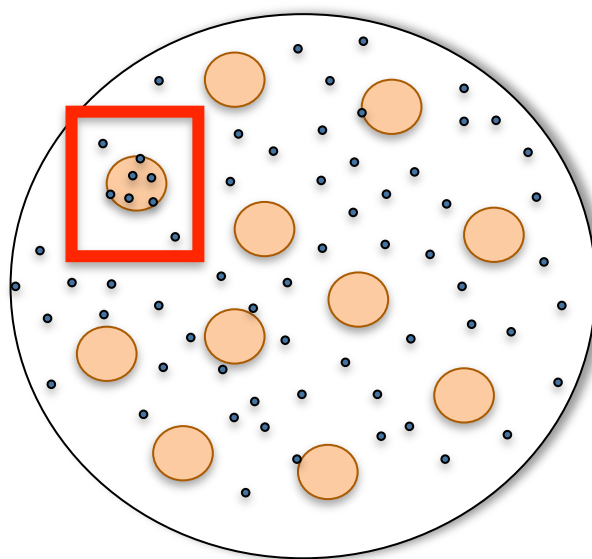


Figure 4.2 Schematic of a microscope view of beads and cells showing a positive bead surrounded by cells.

The selection of positive beads was performed manually after using a reversible chemical cross-linking method^{112,113}, where the cells and beads were incubated with 4% formaldehyde before analysis. After the incubation, a 2M glycine solution was added to the wells to quench cross-linking and allow for the screening process. This procedure of cross-linking is known to show no interference with MALDI tandem mass spectrometry analysis.¹¹⁴ Crosslinking can be easily reverted by washing the beads extensively with 95% ethanol. Selected beads were analyzed by MALDI tandem mass spectrometry. Of

50 beads selected, 33 sequences were fully determined, giving a success rate of 66% (Figure 4.3).

The presence of multiple positive hits, showing the high affinity of this library also raised concerns regarding the specificity of such peptides (Figure 4.4). For that reason, an extra step of screening was made necessary, where positive sequences were resynthesized and screened in parallel with two different cell lines (MDA-MB-231 versus MCF-7), in a positive versus negative screening.

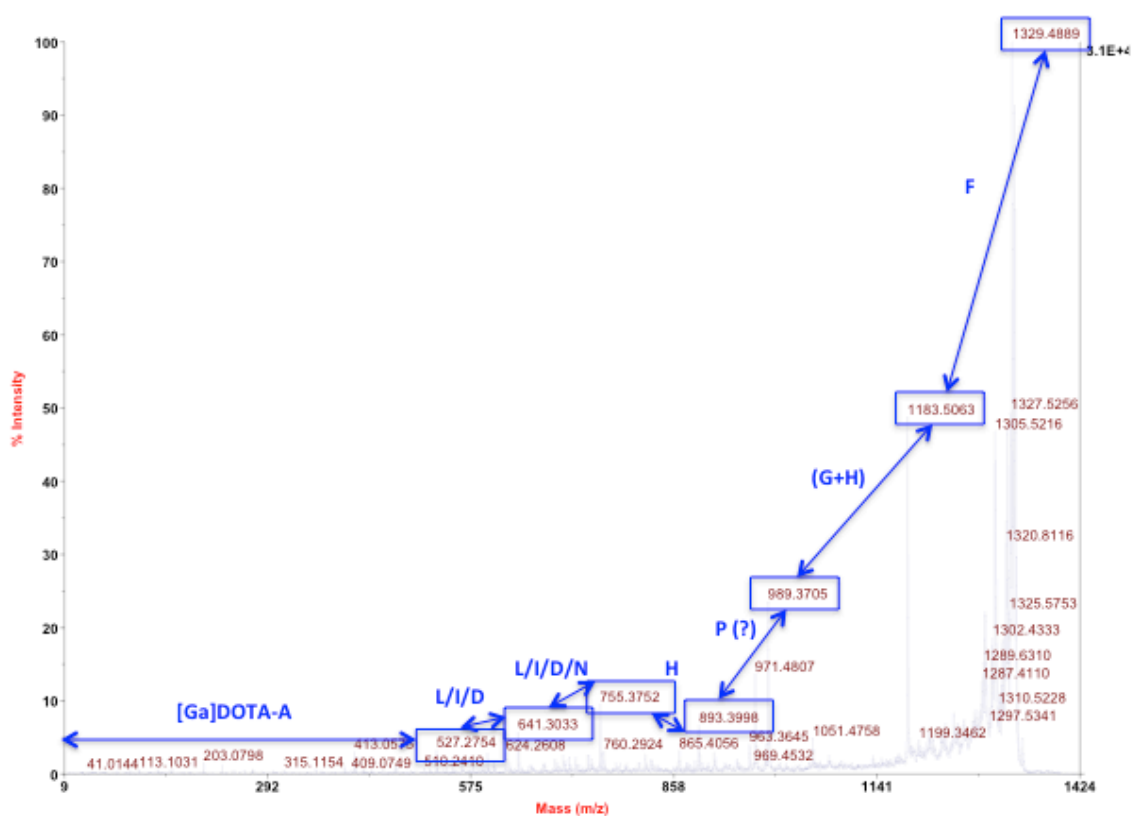


Figure 4.3 Fragment assignments for one of the positive sequences obtained through screening of the OBOC library. Due to the presence of more than one possibility for a certain position, small libraries containing variations of positive peptides were built.

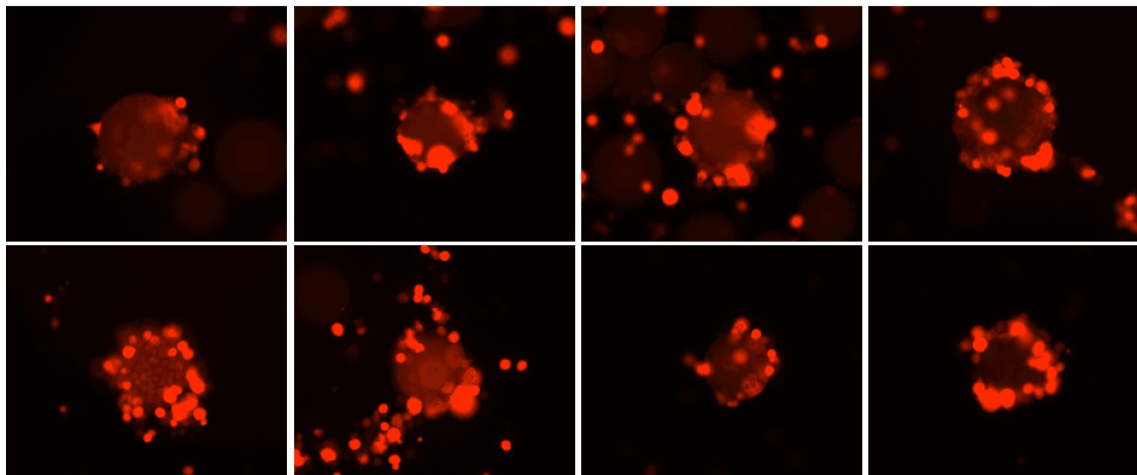


Figure 4.4 Positive beads covered with MDA-MB-231 cells in suspension after screening and before cross-linking.

4.2.3 Positive Sequences Validation and Negative Screening

Of all positive sequences obtained through library screening and subsequent MALDI tandem mass spectrometry, a total of 33 peptides were synthesized, including sequences determined by MALDI as well as a few sequence variations for peptides that did not provide a clear spectra of fragmentation or included amino acids with similar molecular weight such as lysine and glutamine or leucine and isoleucine. For those 33 peptides, a second cell binding experiment was performed, where the peptides were incubated with both MDA-MB 231 and MCF-7 cells in parallel, in order to investigate their specificity to a more aggressive cell line in comparison to a less aggressive cell line. In order to perform this experiment, MCF-7 cells transfected with zSgreen protein were used (**Figure 4.5**).

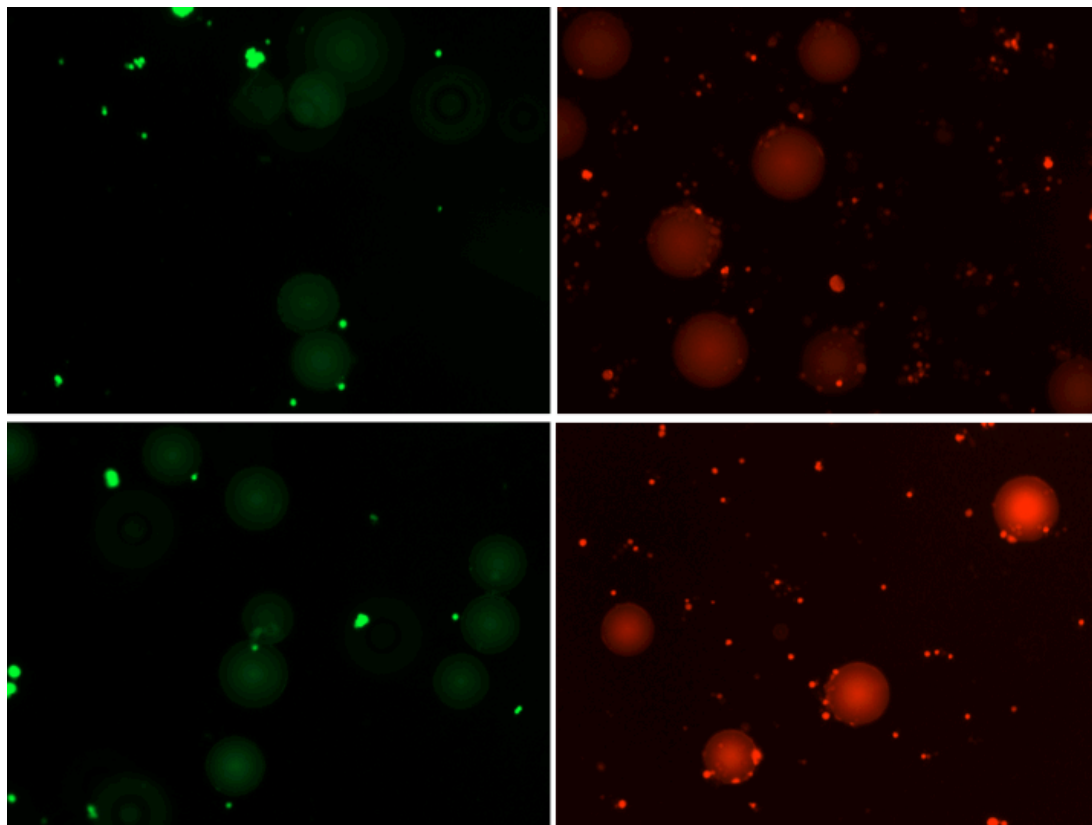


Figure 4.5 Positive versus negative screening for peptides **4.8** (top) and **4.9** (bottom). MCF-7 cells are shown in green (left), and MDA-MB-231 cells in red (right).

As expected, most peptides showed remarkable binding with both cells lines, showing no specificity to specific breast cancer markers. Of 33 peptides analyzed, five peptides showed a distinction in binding when comparing MDA-MB-231 and MCF-7 cells. Out of these five peptides there was no obvious similarities on the peptide sequences to allow for the study their structure activity relationship, which is due to the small percentage of possible sequences that were screened and also the limited size of the library (**Figure 4.6**).

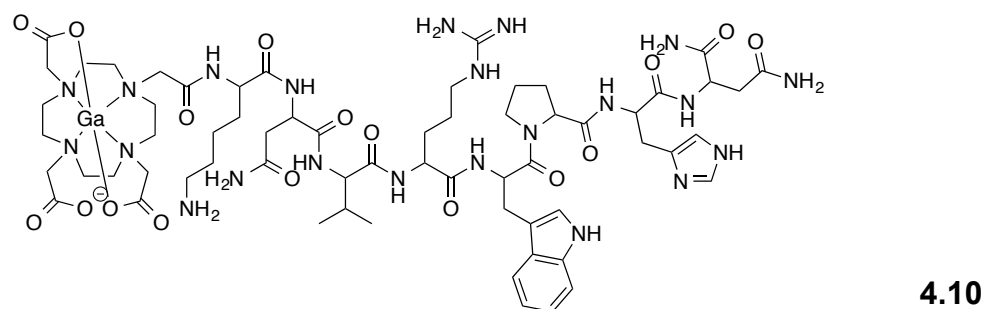
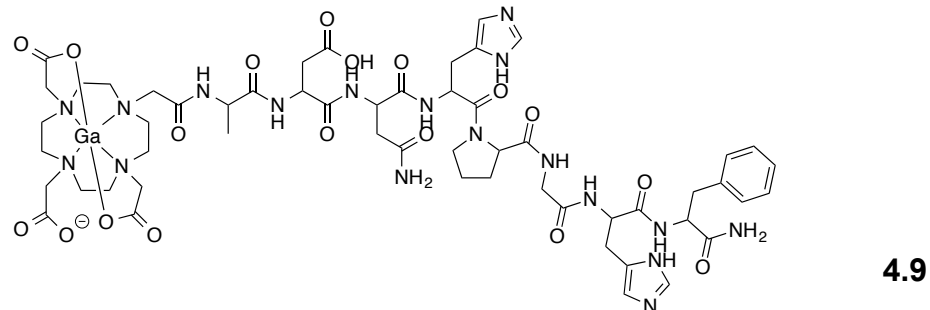
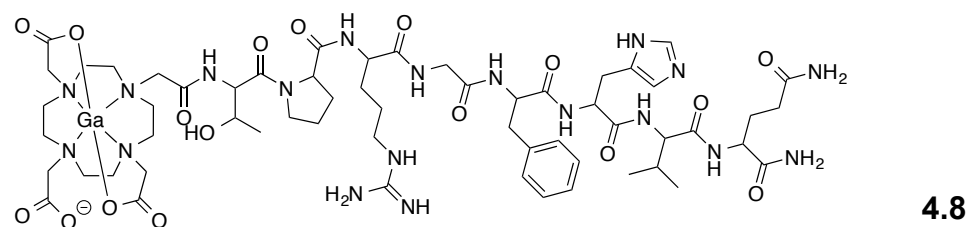
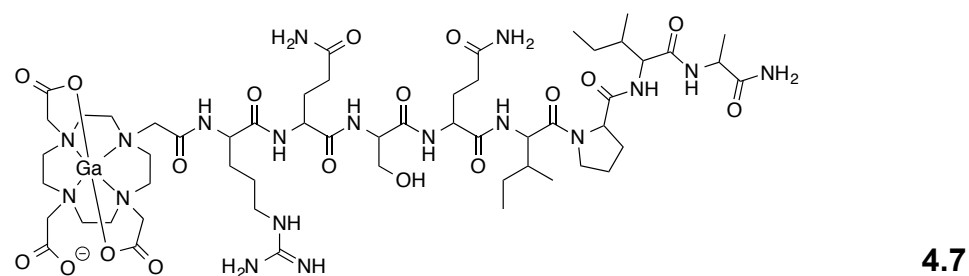
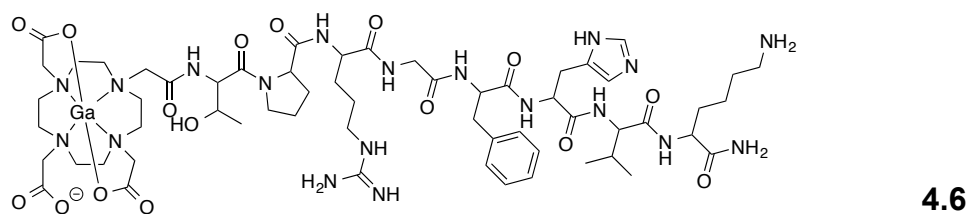


Figure 4.6 Five gallium-peptides that showed specificity to a more aggressive breast cancer cell line.

The five peptides confirmed by this screening were synthesized for further evaluation and development into potential imaging agents.

4.2.4 Synthesis and Characterization of Positive Peptides

Positive peptides, after validation, were resynthesized in order to be developed into imaging agents. Using standard solid phase peptide synthesis and purification procedures, five DOTA-containing peptides were prepared and purified for radiolabeling using ^{68}Ga . As it requires a longer time of reaction, the coupling step for DOTA was performed manually after a last Fmoc deprotection. Reagent K was used to remove the peptide off resin and also remove side chain protecting groups, allowing for later radionuclide labeling.

The purified peptides were characterized using HPLC and mass spectrometry. High-resolution mass spectrometry (HRMS) spectra were obtained using electrospray ionization (ESI), fragmentation was confirmed by the use of MALDI tandem mass spectrometry and compared with spectra obtained from library screening in order to confirm the peptide sequences. Since the concentration of pure peptides is normally higher than what is obtained from a single bead, the signal obtained from the spectrometer is also higher than for the library beads, meaning that fragments that were not previously observed can be clearly seen, and differences between some sequence variations can be clearly spotted.

In order to fully characterize these compounds, coordination with non-radioactive gallium ($^{69/71}\text{Ga}$) was performed for all five peptides. **Table 4.1** shows the results of the [M+H] (high-resolution mass spectrometry) and purity of the desired compounds before and after $^{69/71}\text{Ga}$ coordination (**Figure 4.7**). Compounds **4.1** to **4.5** correspond to the non-gallium coordinated version of peptides **4.6** to **4.10**, respectively.

Table 4.1 Analysis of DOTA-peptides and [$^{69/71}\text{Ga}$]DOTA-peptides.

Compound	Peptide	Calculated m/z [M+2H] ²⁺	Observed m/z [M+2H] ²⁺	Purity*
4.1	DOTA-TPRGFHVK-NH ₂	663.8675	663.8667	>99%
4.2	DOTA-RQSQIPIA-NH ₂	649.3575	649.3419	>99%
4.3	DOTA-TPRGFHVK-NH ₂	663.8498	663.8340	>99%
4.4	DOTA-ADNHPGHF-NH ₂	640.2948	640.2773	>99%
4.5	DOTA-KNVRWPHN-NH ₂	718.3818	718.3800	>99%
4.6	[$^{69/71}\text{Ga}$]DOTA-TPRGFHVK-NH ₂	696.8190	696.8086	>99%
4.7	[$^{69/71}\text{Ga}$]DOTA-RQSQIPIA-NH ₂	682.3163	682.3066	>90%
4.8	[$^{69/71}\text{Ga}$]DOTA-TPRGFHVQ-NH ₂	696.8008	696.7919	>95%
4.9	[$^{69/71}\text{Ga}$]DOTA-ADNHPGHF-NH ₂	673.2459	673.2443	>99%
4.10	[$^{69/71}\text{Ga}$]DOTA-KNVRWPHN-NH ₂	751.3328	751.3275	>95%

* Purity was determined by integration of the area under the curve

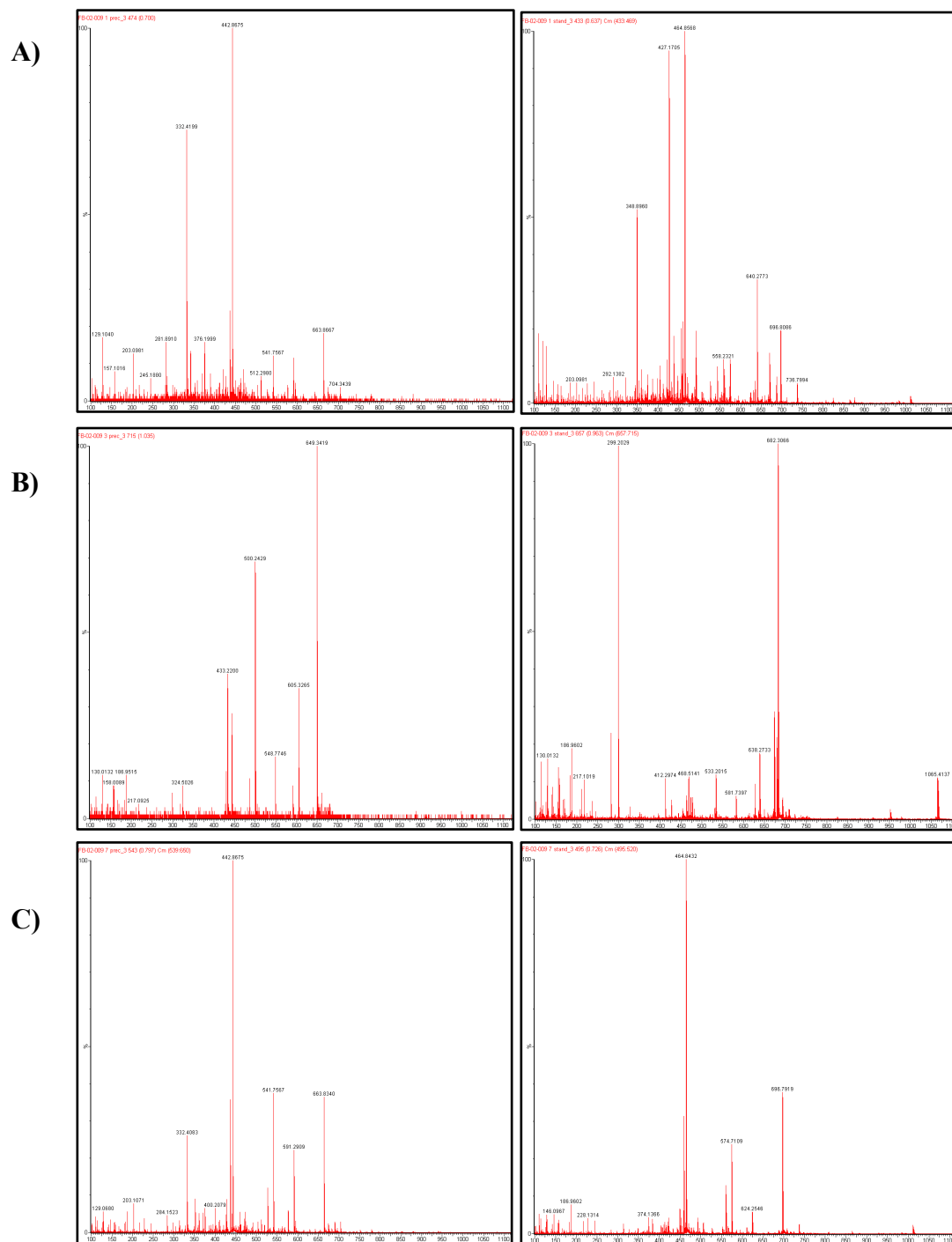


Figure 4.7 A) MS spectra for compounds **4.1** (left) and **4.6** (right). B) MS spectra for compounds **4.2** (left) and **4.7** (right). C) MS spectra for compounds **4.3** (left) and **4.8** (right).

4.2.5 ^{68}Ga Radiolabeling

Radiolabeling of the five peptides was performed in HEPES buffer by heating the reaction vial using an Eckert and Ziegler $^{68}\text{Ge}/^{68}\text{Ga}$ generator, connected to a series of synthesis modules, controlled remotely by the user via a process computer (**Figure 4.8**). The interface of the program used for the radiolabeling of peptides is shown on **Figure 4.9**.

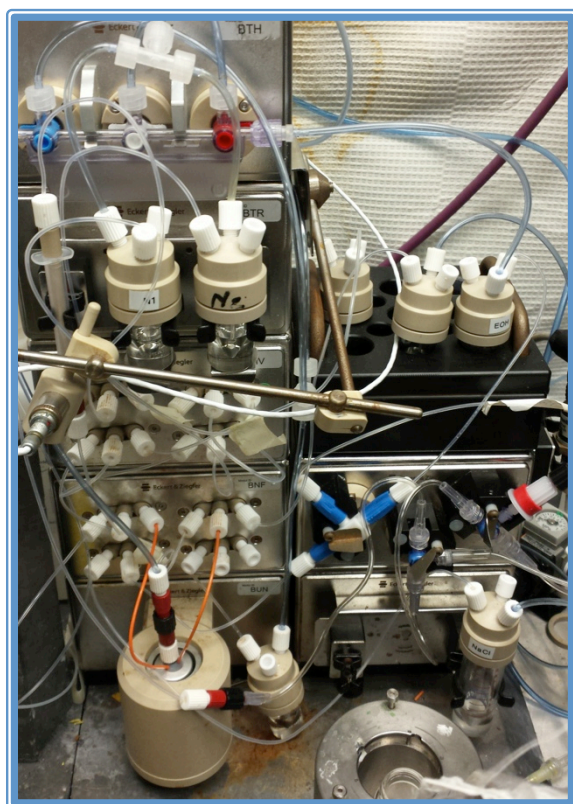


Figure 4.8 Eckert and Ziegler modular lab automated synthesis unit used for ^{68}Ga labeling.

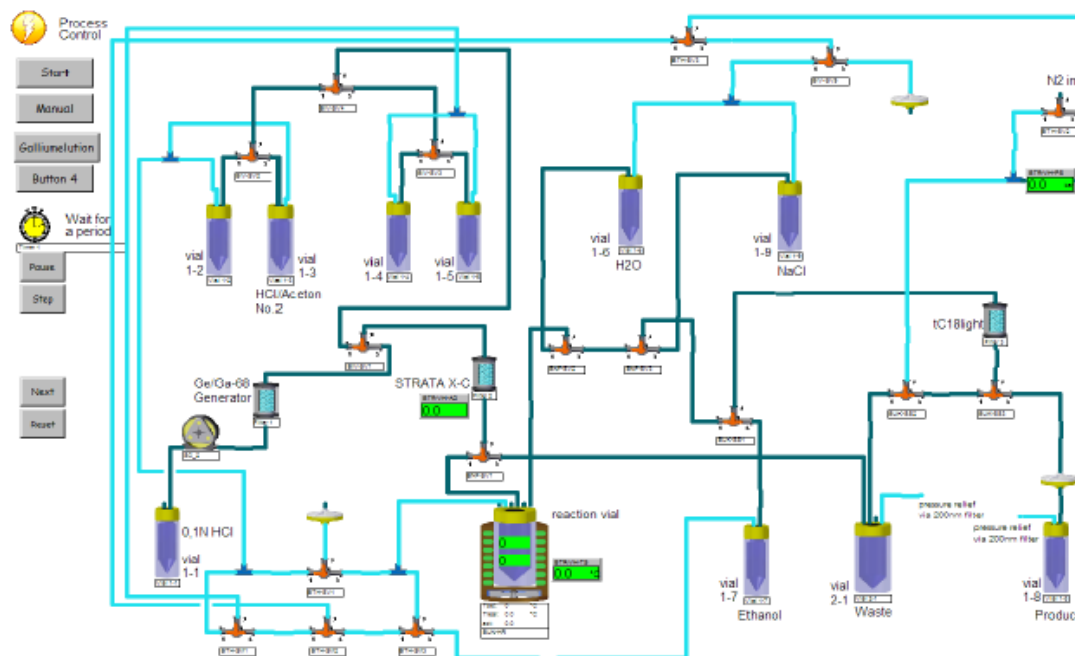


Figure 4.9 Scheme of the program used for peptide radiolabeling (Modular Lab).

^{68}Ga is eluted from the generator via a squeeze pump and approximately 3 mL of 0.1 N HCl (vial 1-1). The eluate, containing ^{68}Ga and other impurities, travels to a strong cation exchange column, which provides an initial purification step. From this column, ^{68}Ga is eluted using 0.5 mL of a 0.05 N HCl in 98% acetone solution (N_2 solution, vial 1-3), and then carried to the reaction vial, which contains the peptide dissolved in HEPES buffer. After completion, the mixture is eluted through a C18 light Sep Pak for further purification. An aliquot of 2 mL of water is passed through the cartridge initially, to remove any free ^{68}Ga . After this wash, the final product is eluted using EtOH into a clean glass vessel. An aliquot of the final product is then diluted with H_2O and acetonitrile for HPLC analysis.

Peptides **4.4** and **4.5** were successfully radiolabeled with ^{68}Ga , initially using 100 μg of the precursor, with radiochemical yields (RCY) of 76% (**4.9***) and 67% (**4.10***) (decay-corrected) respectively, and radiopurity greater than 99%. Confirmation of peptide identity was done by co-injection of the radiolabeled peptide and the cold-labeled compound on HPLC. An HPLC comparison of radiolabeled, cold-labeled and precursors for all five peptides is shown on **Figures 4.10, 4.11** and **4.12**, which show the similar retention times of radiolabeled compounds when compared to the $^{69/71}\text{Ga}$ -peptides and precursor samples.

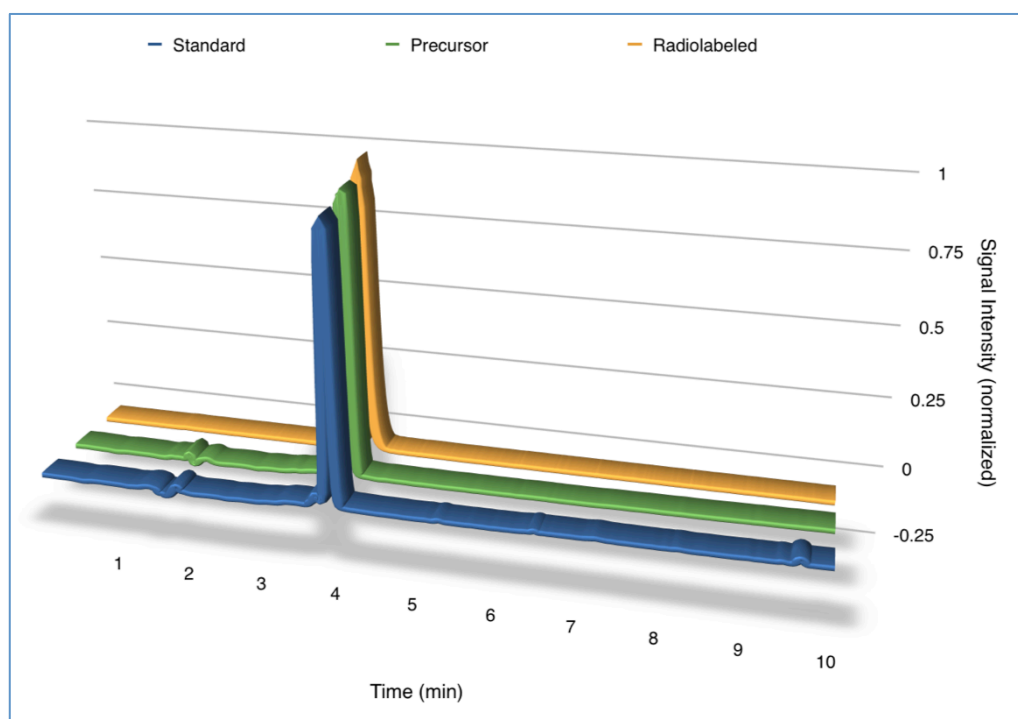


Figure 4.10 Stacked HPLC traces for peptide **4.6*** showing the traces for the precursor (**4.1**) (green), cold gallium-labeled standard (**4.6**) (blue) and radiolabeled (**4.6***) (yellow) products.

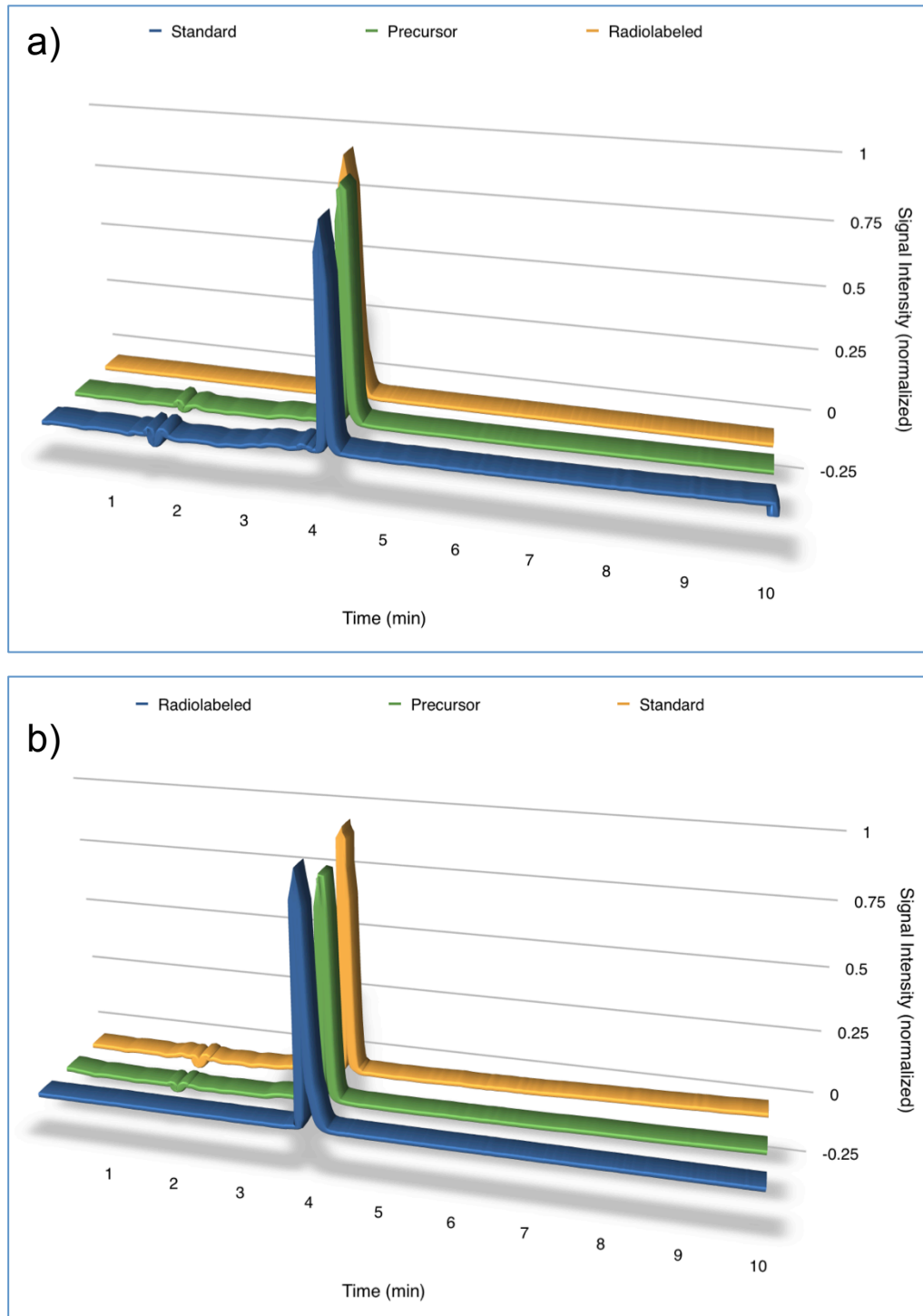


Figure 4.11 Stacked HPLC traces for peptides **4.7*** (a) and **4.8*** (b) showing the traces for the precursor (**4.2**, **4.3**) (green), cold gallium-labeled standard (**4.7**, **4.8**) (blue) and radiolabeled (**4.7***, **4.8***) (yellow) products.

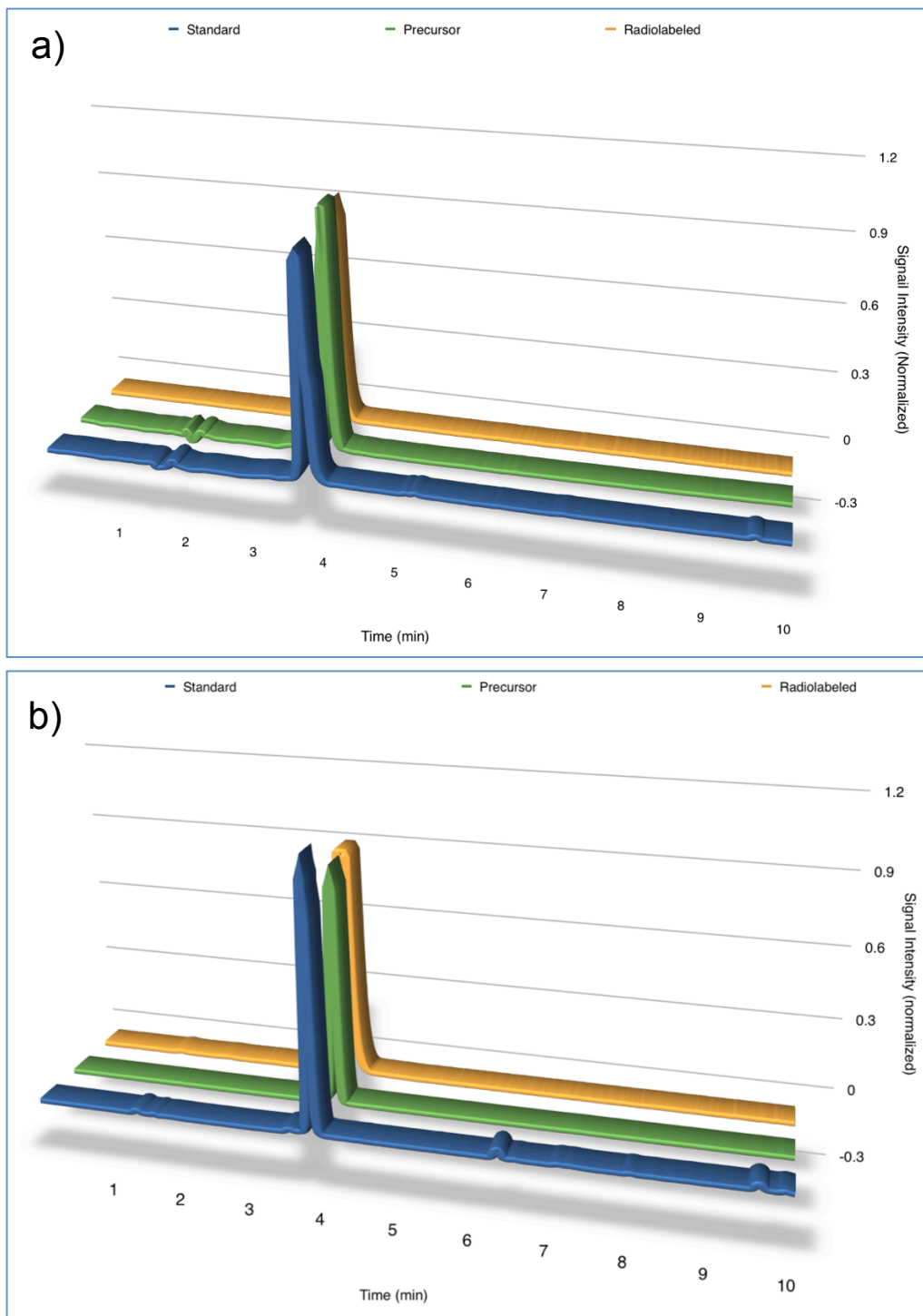


Figure 4.12 Stacked HPLC traces for peptides **4.9*** (a) and **4.10*** (b) showing the traces for the precursor (**4.4**, **4.5**) (green), cold gallium-labeled standard (**4.9**, **4.10**) (blue) and radiolabeled (**4.9***, **4.10***) (yellow) products.

The specific activity when using 100 μg of precursor was determined to be between 1.3-2.0 GBq/ μmol , which is lower than preferred for a targeted imaging agent. In order to improve these values, while preserving radiochemical yields for these peptides, a smaller amount of the precursor was used for radiolabeling (10 μg). Results obtained showed excellent radiopurity (>99%), with high radiochemical yields and improved effective specific activity (**Table 4.2**).

Table 4.2 Radiochemical yield, radiopurity and specific activity for the five lead candidates.

Compound Structure	⁶⁸ Ga-DOTA-TPRGFHVK-NH ₂	⁶⁸ Ga-DOTA-RQSQIPIA-NH ₂	⁶⁸ Ga-DOTA-TPRGFHVQ-NH ₂	⁶⁸ Ga-DOTA-ADNHPGHF-NH ₂	⁶⁸ Ga-DOTA-KNVRWPHN-NH ₂
Compound Number	4.6*	4.7*	4.8*	4.9*	4.10*
Precursor amount (μg)	10 μg	10 μg	10 μg	10 μg	10 μg
Specific Activity (GBq/ μmol)	13.19	12.14	5.77	6.19	17.95
RCY (%) decay corrected (d.c.)	82.5%	84.7%	36.1%	31.6%	86%
Radiopurity (%)	>99%	>99%	>99%	>99%	>99%

4.3 Conclusions

A gallium-containing OBOC octapeptide library was synthesized containing the chelator and metal motif on the N-terminus of the peptides. Screening of this library against MDA-MB-231 cells resulted in the identification of 50 positive hits, from which 33 sequences were obtained, leading to a sixty-six percent success rate. This satisfactory number once again shows that MALDI tandem mass spectrometry can be successfully used to determine peptide sequences that contain the [Ga]DOTA complex.

In a search for peptide sequences that would specifically bind to a more aggressive cell line (MDA-MB-231), peptides containing the sequences discovered by MALDI tandem mass spectrometry. This “positive versus negative” screening led to 5 peptides that showed a better binding affinity to a more aggressive cell line, instead of MCF-7 cells. These peptides were then chosen to be developed into imaging agents, which includes further characterization, radiolabeling and *in vivo* animal PET imaging. All five peptides were successfully characterized and radiolabeled using ^{68}Ga , however, it would be ideal to repeat the labeling for compounds such as **4.8*** and **4.9***, in order to obtain better radiochemical yields.

Experiments are now being conducted for small animal PET imaging using tumour-bearing mice (MDA-MB-231 cells), looking for specific tumour targeting.

4.4 Experimental Methods

All standard Fmoc protected amino acids and coupling agents were obtained from Peptides International. Fmoc-Rink amide MBHA resin (4-(2',4'-dimethoxyphenyl-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamidonorleucyl-4-methyl benzhydrylamine resin) was obtained from Nova Biochem. RP-C18 Sep-Pak© cartridges were obtained from Waters. ^{68}Ge - ^{68}Ga generator was obtained from Eckert and Ziegler.

4.4.1 OBOC Library Synthesis

The OBOC library was synthesized using the Biotage® Syro Wave™ Peptide Synthesizer. For that, eighteen natural amino acids, excluding cysteine and methionine, were used. Fmoc-ANP-OH (4 eq.) was manually coupled to Tentagel S NH_2 resin (loading 0.29 mmol/g) using standard Fmoc peptide synthesis procedures. The resin was kept in the dark during the synthesis process due to the nature of the ANP linker. In the automated synthesizer, the Fmoc group was removed using a 20% piperidine in DMF solution (800 μL /well), twice. To every well, one different amino acid (3 eq.), HCTU (3 eq.) and DIPEA (6 eq.) in DMF were added. After each coupling step, the resin was rinsed with DMF and DCM multiple times and recombined in a peptide vessel. After mixing it thoroughly, the resin was again divided among the seventeen vessels and placed back on the synthesizer for another round of Fmoc deprotection and coupling. The process of deprotection and coupling was then repeated until the library reached its size of eight amino acids. The resin was recombined, a last Fmoc deprotection was performed and the coupling step for the chelator was performed manually, using DOTA-

tris(tBu)ester (4 eq.), HCTU (4 eq.) and DIPEA (4eq), under shaking, for a period of 3 hours.

Gallium coordination was performed by adding 1 g of GaCl₃ in a round bottom flask containing the library in approximately 150 mL of NaOAc/HOA buffer (pH = 4). The mixture was left for stir at room temperature for 45 minutes and another 45 minutes at 75°C. The library was kept dry under -80°C until the day of usage. Small samples were solubilized in PBS and left at room temperature prior to library screening.

4.4.2 Cell Growth

MDA-MB-231 cells stably transfected with tdTomato protein and MCF-7 cells stably transfected with zSGreen protein were kindly provided by Dr. Hon Sing Leong and Dr. Ann Chambers.

MDA-MB-231 cells expressing tdTomato protein were grown in Alpha modification of Eagle's medium (AMEM) media supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 1% sodium pyruvate.

MCF-7 cells expressing zSGreen protein were grown in Dulbecco's modified Eagle's medium (DMEM) low glucose media, supplemented with 10% FBS.

4.4.3 Library Screening

MDA-MB-231 cells (approximately 500,000 cells) were added to multiple wells on 6-well plates with approximately 3 mL of AMEM serum-free cell culture media. To these wells, approximately 15,000 library beads, solubilized in 30 μ L of PBS were added.

The plates were protected from light and submitted to gentle shaking in an incubator, on 37° C, 85 rpm, for one hour. After one hour, cells and beads were imaged using the Olympus IX70 inverted fluorescent microscope prior to fixation. Fixing of the cells was performed using a 4% formaldehyde solution for 15 minutes. The cells were then washed with PBS twice before observation on a fluorescence microscope for binding confirmation.

For sequence determination later on, the beads were treated rigorously with ethanol to remove any bound cells, and then washed several times with water.

4.4.4 MALDI Tandem Mass Spectrometry

After following the cleavage protocol (UV light, 4 hours), 2 μ L of each peptide solution obtained, in water, was mixed with 2 μ L of trans- α -cyano-4-hydroxycinnamic acid (CHCA) matrix for a few minutes. 0.75 μ L of this mixture was spotted onto a MALDI target plate in duplicate for analysis. The solvent was allowed to evaporate, leading to a co-crystallization of the matrix and peptide. The samples were then analyzed utilizing MALDI tandem mass spectrometry.

4.4.5 Negative Screening

MCF-7 cells (approximately 500,000) were added to multiple wells on 6-well plates with approximately 3 mL of serum-free low glucose DMEM cell culture media. To each well, different peptide beads were added, to a total of 33 wells. The same procedure was followed for MDA-MB-231 cells, on serum-free AMEM media. The plates were protected from light and submitted to gentle shaking in an incubator on 37° C, 85 rpm, for one hour.

After incubation, the wells corresponding to each peptide sequence were observed in parallel in order to compare the binding of a more aggressive cell line versus a less aggressive one. For peptides that specifically bind to the cell line of interest, pictures were taken for a visual comparison.

4.4.6 General Peptide Synthesis Procedures

The procedure described corresponds to the synthesis of peptides **4.1**, **4.2**, **4.3**, **4.4** and **4.5**.

Standard Fmoc solid phase peptide synthesis (SPPS) procedures were utilized to obtain the desired peptides containing all side chain protecting groups. Rink amide MHBA NH₂ resin (0.1 mmol, 0.52meq/g) containing a Fmoc protecting group was swelled using DCM washes, and the Fmoc group was removed using a 20% piperidine in DMF solution (2x, 5 min and 20 min, respectively). The deprotection solution was removed from resin using a series of DMF and DCM washes. Fmoc protected amino

acids (0.3 mmol, 3 eq) were dissolved in approximately 0.5 mL DMF, along with HCTU (0.3 mmol, 3 eq) and DIPEA (0.6 mmol, 6 eq). For the peptides described above, the amino acids used were: Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ala-OH and Fmoc-Gln(Trt)-OH. The solution of coupling reagents plus activated amino acid was then combined with the resin and agitated for one hour. The resin was washed with DMF and DCM multiple times. This process of coupling and deprotection was then repeated until the desired sequence of the peptides was obtained. Kaiser test⁹⁰ was performed to confirm the presence of free primary amino groups after deprotection and the absence after coupling of amino acids. After a last Fmoc deprotection, the chelator DOTA, in its tBu-protected form (tris-tBu-DOTA) was added manually by the use of solid phase peptide synthesis techniques (4 eq, 0.4 mmol), using HCTU (4 eq, 0.4 mmol) and DIPEA (8 eq, 0.8 mmol) as coupling reagents.

4.4.7 Peptide Deprotection, Cleavage and Purification

After the chelator coupling, peptides were mixed with 5 ml of a solution of 95% TFA, 2.5% H₂O and 2.5% TIPS for six hours to remove the peptide from resin as well as remove side-chain protecting groups. The resulting solution was cooled on ice and cold TBME (20 mL) was added to the solution until a white precipitate formed. The peptide solutions were then centrifuged at 0°C at 3000 rpm for 10 minutes. The liquid was decanted and the resulting pellet was mixed with cold TBME (20 mL) and subsequently

centrifuged again under identical conditions to obtain the crude peptide. Following the removal of the supernatant, the peptide pellets were dissolved in water, frozen at -78°C and lyophilized. Small samples of the resin were cleaved using the full deprotection procedure in order to monitor reaction completion.

The crude peptides were dissolved in water, purified by HPLC (15 minutes, linear gradient 15% solvent A in solvent B to 70%) and identified by mass spectrometry, giving white powders after lyophilization. High-resolution mass spectrometry spectra were obtained using ESI-MS (Water Micromass Quattro MicroTM API). Refer to **Table 4.1** for mass spectrometry data.

4.4.8 ^{69/71}Ga Coordination Procedures

The procedures here stated correspond to compounds **4.6**, **4.7**, **4.8**, **4.9** and **4.10**.

10 mg of each DOTA-peptide (**4.1**, **4.2**, **4.3**, **4.4**, **4.5**), dissolved in 0.1 M pH 4 NaOAc/HOAc buffer were added to a round bottom flask containing 5 mg of GaCl₃. The mixture was stirred at room temperature for 30 minutes before heating to 70° C for another 30 minutes. The resulting mixture was purified using a light C18 RP Sep-Pak[©] cartridge. The reaction mixture was passed through the Sep-Pak[©], and the cartridge was washed with 10 mL of water in order to remove unreacted GaCl₃. Ethanol is then used to wash out the labeled product and subsequently removed *in vacuo*. The peptide products are isolated as white powders and characterized using HPLC and mass spectrometry. Refer to **Table 4.1** for mass spectrometry data.

4.4.9 ⁶⁸Ga Radiolabeling

The procedure for radiolabeling was the same for all peptides in this chapter (4.6*, 4.7*, 4.8*, 4.9* and 4.10*).

10 µg of the DOTA-peptide (4.1, 4.2, 4.3, 4.4, 4.5) solution (1mg/mL in pH 3.5 HEPES buffer) was added to a clean glass microwave vessel. To this aliquot, another 700 µL of the buffer was added in order to obtain a final solution with concentration in the order of µM. To this vessel, 10 mCi of ⁶⁸GaCl₃ was added, eluted from a ⁶⁸Ge/⁶⁸Ga generator using 3 mL of a 0.1 M HCl solution. Purification and isolation of the final product were achieved using a pre-activated light C18 RP Sep-Pak© cartridge, prior to the transfer to a clean microwave vessel (using a 0.05 N HCl in Acetone solution as eluent). The purity of the products was analyzed using analytical RP-HPLC (Sunfire™ RP-C18 column 4.6 x 150 mm, 5µm) coupled to a gamma detector. The system employed a Waters 1525 Binary HPLC pump, Waters 2487 dual λ absorbance detector, Waters In-Line degasser and Breeze Software (version 3.30).

Chapter 5: Conclusions

The advent of molecular imaging led to an increasing search for new potential biological targets and the study of new and optimized ways of incorporating radionuclides into imaging agents. Furthermore, it also resulted in more detailed studies on radioisotopes, in order to improve its production, accessibility, radiochemical yields and specific activity. For this matter, more recently, ^{68}Ga is been explored as a PET imaging agent, as it has a very suitable half-life, can be easily obtained from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator and its chelation chemistry is well understood, especially when using bifunctional chelators such as DOTA and NOTA.

In the present research, we are able to offer enough proof that OBOC combinatorial libraries containing the [Ga]DOTA surrogate prior to screening are able to provide positive hits that can be further developed into imaging agents. The library designed showed great stability under synthesis and screening conditions and positive hits obtained can be easily developed into potential imaging agents.

The aim of this research was to prove the ability of not only synthesizing such a library, but also being able to screen for a biological target and obtain the positively interacting peptide sequences using MALDI tandem mass spectrometry. After confirmation of the ability of sequencing [Ga]DOTA-containing peptides, a breast cancer cell line was chosen for the screening of the library.

Although one of the goals for this study was to be able to randomize the position of the radionuclide with the chelator, due to the changes in the fragmentation pattern for

the peptides, it would increase the challenge of determining the sequences of positive peptides. Therefore, a library containing the imaging entity on the N-terminus was synthesized. In order to look at the different possibilities for the position of such an entity, further studies are needed in order to understand the fragmentation of peptides containing the [Ga]DOTA complex in different positions within peptide chain and how those changes would affect the peptide conformation on the bead and consequently cell binding.

The OBOC library synthesized contains between 1 to 2 million unique peptide sequences, which contained all of the natural amino acids, excluding cysteine and methionine, and was screened against MDA-MB-231 cells, an aggressive breast cancer cell line. For this study, a target was not yet identified, as the search was for positive peptides that would only bind to the cell line of interest. To confirm specificity, positive sequences obtained, and its variants, were resynthesized and screened against MCF-7 cells in parallel with MDA-MB-231 cells, and peptides that specifically bound to MDA-MB-231 cells were selected for further characterization. Since there is no previous knowledge on the target and also the five peptides that show a positive interaction have different sequences, it is difficult to comment on structure-activity relationship.

One of the main advantages of OBOC libraries is the ability of incorporating unnatural amino acids^{115,116} and non-peptide structures, such as chelators and radionuclides, and when using MALDI tandem mass spectrometry as the sequence determination method, those structures can be easily accounted for. It is known that unnatural amino acids or non-peptides structures show a better *in vivo* stability, and further studies on sequences obtained by the screening of this library include the

substitution of natural amino acids by unnatural amino acids or non-peptide structures in order to search for better specificity and stability.

The fact that the library already contains the imaging entity prior to screening represents an advantage, as it might overcome the present problems with the development of new imaging probes. The imaging entity corresponds to a very significant part of the agent, and it will most likely affect the characteristics of a targeting entity, changing its binding affinity and specificity.

In conclusion, a novel gallium-containing OBOC peptide library was synthesized utilizing standard solid phase peptide synthesis approaches. MALDI tandem mass spectrometry was proved to be a useful technique for the identification of positive peptide sequences obtained through screening of this library with a breast cancer cell line, MDA-MB-231.

A study was previously reported in our lab using rhenium-containing libraries⁸⁸, and by showing the same approach using a different radionuclide, we believe that this technique can be applied to other radionuclide surrogates, with proper investigation of its fragmentation patterns and chelate stability under synthesis and screening conditions. This approach for the development of new imaging probes has the potential to make the process of discovering new agents faster and more economically feasible.

References

1. Ottobriani, L., Ciana, P., Biserni, A., Lucignani, G., Maggi, A. Molecular imaging: a new way to study molecular processes in vivo. *Mol Cell End* **246**, 69-75 (2006).
2. Massoud, T.F., Gambhir, S.S. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev* **17(5)**, 545-580 (2003).
3. James, M.L., Gambhir, S.S. A molecular imaging primer: modalities, imaging agents and applications. *Physiol Rev* **92**, 897-965 (2012).
4. Rudin, M., Weissleder, R. Molecular imaging in drug discovery and development. *Nat Rev Drug Disc* **2**, 123-131 (2003).
5. Pimlott, S.L., Sutherland, A. Molecular tracers for the PET and SPECT imaging of disease. *Chem Soc Rev* **40**, 149-162 (2011).
6. Bartholoma, M.D., Louie, A.S., Valliant, J.F., Zubieta, J. Technetium and Gallium derived radiopharmaceuticals: comparing and contrasting the chemistry of two important radiometals for the molecular imaging era. *Chem Rev* **110**, 2903-2920 (2010).
7. Bhattacharyya, S., Dixit, M. Metallic radionuclides in the development of diagnostic and therapeutic radiopharmaceuticals. *Daltons Trans* **40**, 6112-6128 (2011).
8. Rahmim, A., Zaidi, H. PET versus SPECT: strenghts, limitations and challenges. *Nuc Med Comm* **29**, 193-207 (2008).
9. Coe, E.L. Inhibition of glycolysis in ascites tumor cells preincubated with 2-deoxy-D-glucose. *Biochim Biophys Acta* **264**, 319-327 (1972).
10. Pauwels, E.K.J., Ribeiro, M.J., Stoot, J.H.M.B., McReady, V.R., Bourguignon, M., Mazière, B. FDG accumulation and tumor biology. *Nuc Med Biol* **25**, 317-322 (1998).
11. Kubota, R., Yamada, S., Kubota, K., Ishiwata, K., Tamahashi, N., Ido, T. Intramural distribution of fluorine-18-fluorodeoxyglucose in vivo: High accumulation in macrophages and granulation tissue studies by microautoradiography. *J Nucl Med* **33**, 1972-1980 (1992).
12. Ehrhardt, G.J., Welch, M.J.. A new germanium-63/gallium-68 & 1978;19:925-9, g.J.N.M. A new germanium-68/gallium-68 generator. *J Nucl Med* **19**, 925-929 (1978).

13. Fani, M., Andre, J.P., Maecke, H.R. ^{68}Ga -PET: a powerful generator-based alternative to cyclotron-based PET radiopharmaceuticals. *Contrast Media Mol Imag* **3**, 67-77 (2008).
14. Breeman, W.A.P., de Blois, E., Chan, H.S., Koninjenberg, M., Kwekkeboom, D.J., Krenning, E.P. ^{68}Ga -labeled DOTA-peptides and ^{68}Ga -labeled radiopharmaceuticals for positron emission tomography: current status of research, clinical applications, and future perspectives. *Semin Nucl Med* **41**, 314-321 (2011).
15. Klivényi, G., Schuhmacher, J., Patzelt, E., Hauser, H., Matys, R., Moock, M., Regiert, T., Maier-Borst, W.. Gallium-68 chelate imaging of human colon carcinoma xenografts pretargeted with bispecific anti-CD44V6/anti-gallium chelate antibodies. *J Nucl Med* **39**, 1769-1776 (1998).
16. Eisenwiener, K.P., Prata, M.I., Buschmann, I., Zhang, H.W., Santos, A.C., Wenger, S., Reubi, J.C., Mäcke, H.R. NODAGATOC, a new chelator-coupled somatostatin analogue labeled with [$^{67}/^{68}\text{Ga}$] and [^{111}In] for SPECT, PET, and targeted therapeutic applications of somatostatin receptor (hsst2) expressing tumors. *Bioconjug Chem* **13**, 530-541 (2002).
17. Gabriel, M., Decristoforo, C., Kendler, D., Dobrozemsky, G., Heute, D., Uprimny, C., Kovacs, P., Von Guggenberg, E., Bale, R., Virgolini, I.J. ^{68}Ga -DOTA-Tyr3-octreotide PET in neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and CT. *J Nucl Med* **48**, 508-518 (2007).
18. Buchmann, I., Henze, M., Engelbrecht, S., Eisenhut, M., Runz, A., Schafer, M., et al. . Comparison of ^{68}Ga -DOTATOC PET and ^{111}In -DTPAOC (Octreoscan) SPECT in patients with neuroendocrine tumours. . *Eur J Nucl Med Mol Imaging* **34**, 1617–1626 (2007).
19. Fellner, M., Biesalski, B., Bausbacher, N., Kubicek, V., Hermann, P., Rösch, F., Thews, O. (^{68}Ga)-BPAMD: PET-imaging of bone metastases with a generator based positron emitter. *Nucl Med Biol* **39**, 993-999 (2012).
20. Ebenahn, T., Zeevaart, J.R., Venter, J.D., Govender, T., Kruger, G.H., Jarvis, N.V., Sathekge, M.M. Preclinical evaluation of ^{68}Ga -labeled 1,4,7-triazacyclononane-1,4,7-triacetic acid-ubiquicidin as a radioligand for PET infection imaging. *J Nucl Med* **55**, 308-314 (2014).
21. Ebenhan, T., Chadwick, N., Sathekge, M.M., Govender, P., Govender, T., Kruger, H.G., Marjanovic-Painter, B., Zeevaart, J.R. Peptide synthesis, characterization and ^{68}Ga -radiolabeling of NOTA-conjugated ubiquicidin fragments for prospective infection imaging with PET/CT. *Nucl Med Biol* **41**, 390-400 (2014).
22. Wadas, T.J., Wong, E.H., Weisman, G.R., Anderson, C.J. Coordinating radiometals of copper, gallium, indium, yttrium and zirconium for PET and SPECT imaging of disease. *Chem Rev* **110**, 2858-2902 (2010).

23. Hancock, R.D., Martell, A.E. Ligand design for selective complexation of metal ions in aqueous solution. *Chem Rev* **89**, 1875-1914 (1989).
24. Bandoli, G., Dolmella, A., Tisato, F., Prochia, M., Refosco, F. Mononuclear six-coordinated Ga(III) complexes: a comprehensive survey. *Coord Chem Rev* **253**, 56-77 (2009).
25. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C., Dantis, L., Sklarin, N.T., Seidman, A.D., Hudis, C.A., Moore, J., Rosen, P.P., Twadell, T., Henderson, I.C., Norton, L., . Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. *Semin Oncol* **26**, 78-83 (1999).
26. Tabrizi, M.A., Roskos, L.K. Preclinical and clinical safety of monoclonal antibodies. *Drug Discov Today* **12**, 540-547 (2007).
27. Cho, C.F., Amadei, G.A., Breadner, D., Luyt, L.G., Lewis, J.D. Discovery of novel integrin ligands from combinatorial libraries using a multiplex "beads on a bead" approach. *Nano Lett* **12**, 5957-5965 (2012).
28. Wu, A.M. Antibodies and antimatter: the resurgence of immuno-PET. *J Nucl Med* **50(1)**, 2-5 (2009).
29. Tweedle, M.F. Peptide-targeted diagnostics and radiotherapeutics. *Acc Chem Res* **42**, 958-968 (2009).
30. Okarvi, S.M. Peptide-based radiopharmaceuticals: future tools for diagnostic imaging of cancers and other diseases. *Med Res Rev* **24**, 357-397 (2004).
31. Lee, S., Xie, J., Chen, X. Peptides and peptide hormones for molecular imaging and disease diagnosis. *Chem Rev* **110**(2010).
32. Fani, M., Maecke, H.R., Okarvi, S.M. Radiolabeled peptides: valuable tools for the detection and treatment of cancer. *Theranostics* **2**, 481-501 (2012).
33. Krenning, E.P., Bakker, W.H., Breeman, W.A., Koper, J.W., Kooij, P.P., Ausema, L., et al. Localisation of endocrine-related tumours with radioiodinated analogue of somatostatin. *Lancet* **1**, 242-244 (1989).
34. Otte, A., Mueller-Brand, J., Dellas, S., Nitzsche, E.U., Herrman, R., Maecke, H.R. Yttrium-90 labelled somatostatin-analogue for cancer treatment. *Lancet* **351**, 417-418 (1998).
35. Rufini, V., Calcagni, M.L., Baum, R.P. Imaging of neuroendocrine tumors. *Semin Nucl Med* **36**, 228-247 (2006).
36. Ambrosini, V., Fani, M., Fanti, S., Forrer, F., Maecke, H.R. Radiopeptide imaging and therapy in Europe. *J Nucl Med* **52**, 42S-55S (2011).

37. Kaltsas, G.A., Papadoglas, D., Makras, P., Grossman, A.B. Treatment of advanced neuroendocrine tumours with radiolabelled somatostatin analogues. *Endocrin Relat Cancer* **12**, 683-699 (2005).
38. Buchegger, F., Bonvin, F., Kosinski, M., Schaffland, A.O., Prior, J., Reubi, J.C., et al. Radiolabeled neurotensin analog, ^{99m}Tc-NT-XI, evaluated in ductal pancreatic adenocarcinoma patients. *J Nucl Med* **44**, 1649-1654 (2003).
39. Wild, D., Behe, M., Wicki, A., Storch, D., Waser, B., Gotthardt, M., et al. [Lys40(Ahx-DTPA-111In)NH₂]exendin-4, a very promising ligand for glucagon-like peptide-1 (GLP-1) receptor targeting. *J Nucl Med* **47**, 2025-2033 (2006).
40. Zhang, H., Moroz, M.A., Serganova, I., Ku, T., Huang, R., Vider, J., Maecke, H.R., Larson, S.M., Blasberg, R., Smith-Jones, P.M. Imaging expression of the human somatostatin receptor subtype-2 reporter gene with ⁶⁸Ga-DOTATOC. *J Nucl Med* **52**, 123-131 (2011).
41. Oberauer, G.M., Dobrozemsky, G., et al. ⁶⁸Ga-DOTA-Tyr³-octreotide PET for assessing response to somatostatin-receptor mediated radionuclide therapy. *J Nucl Med* **50**, 1427-1434 (2009).
42. Furka, A., Sebestyén, F., Asgedom, M., Dibó, G. General method for rapid synthesis of multicomponent peptide mixtures. *Int J Pept Protein Res* **37**, 487-493 (1991).
43. Frank, R., Güler, S., Krause, S., Lindenmaier, W. Facile and rapid 'spot synthesis' of large numbers of peptides on membrane sheets. in *21st European Peptide Symposium* 151 (ESCOM, Barcelona, Spain, 1991).
44. Frank, R. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *J Immunol Methods* **267**, 13-26 (2002).
45. Fodor, S.P., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T., Solas, D. Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**, 767-773 (1991).
46. Smith, G.P., Petrenko, V.A. Phage Display. *Chem Rev* **97**, 391-410 (1997).
47. Liu, C.C., Mack, A.V., Tsao, M.L., Mills, J.H., Lee, H.S., Choe, H., Farzan, M., Schultz, P.G., Smider, V.V. Protein evolution with an expanded genetic code. *Proc Natl Acad Sci* **105**, 17688-17693 (2008).
48. Liu, C.C.M., A.V., Brustad, E.M., Mills, J.H., Groff, D., Smider, V.V., Schultz, P.G. Evolution of proteins with genetically encoded "chemical warheads". *J Am Chem Soc* **131**, 9616-9617 (2009).

49. Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M., Knapp, R.J. A new type of synthetic peptide library for identifying ligand-binding activity. *Letters to Nature* **354**, 82-84 (1991).
50. Lam, K.S., Lebl, M., Krchnák, V. The "one-bead-one-compound" combinatorial library method. *Chem Rev* **97**, 411-448 (1997).
51. Yao, N., Xiao, W., Wang, X., Marik, J., Park, S.H., Takada, Y., Lam, K.S. Discovery of targeting ligands for breast cancer cells using the one-bead one-compound combinatorial method. *J Med Chem* **52**, 126-133 (2009).
52. Aina, O.H., Marik, J., Liu, R., Lau, D.H., Lam, K.S. Identification of novel targeting peptides for human ovarian cancer cells using "one-bead one-compound" combinatorial libraries. *Mol Cancer Ther* **4**, 806-813 (2005).
53. Appell, K.C., Chung, T.D.Y., Solly, K.J., Chelsky, D. Biological characterization of neurokinin antagonists discovered through screening of a combinatorial library. *J Biomol Screen* **3**, 19-27 (1998).
54. Mc Bride, J.D., Freeman, N., Domingo, G.J., Leatherbarrow, R.J. Selection of chymotrypsin inhibitors from a conformationally-constrained combinatorial peptide library. *J Mol Biol* **259**, 819-827 (1996).
55. Abato, P., Conroy, J.L., Seto, C.T. . Combinatorial library of serine and cysteine protease inhibitors that interact with both the S and S' binding sites. *J Med Chem* **42**, 4001-4009 (1999).
56. Lam, K.S., Liu, R., Miyamoto, S., Lehman, L., Tuscano, J.M. Applications of one-bead one-compound combinatorial libraries and chemical microarrays in signal transduction research. *Acc Chem Res* **36**, 370-377 (2003).
57. St. Hilaire, P.M., Alves, L.C., Herrera, F., Renil, M., Sanderson, S.J., Mottram, J.C., Coombs, G.H., Juliano, M.A., Juliano, L., Arevalo, J., Meldal, M. Solid-phase library synthesis, screening, and selection of tight-binding reduced peptide bond inhibitors of a recombinant *Leishmania mexicana* cysteine protease B. *J Med Chem* **45**, 1971-1982 (2002).
58. Chirayil, S., Chirayil, R., Luebke, J. Discovering ligand for a microRNA precursor with peptoid microarrays. *Nucleic Acids Research* **37**, 5486-5497 (2009).
59. Luo, J., Zhang, H., Xiao, W., Kumaresan, P.R., Shi, C., Pan, C., Aina, O.H., Lam, K.S. Rainbow beads: a color coding method to facilitate high-throughput screening and optimization of one-bead one-compound combinatorial libraries. *J Comb Chem* **10**, 599-604 (2008).

60. Xiao*, W., Bononi*, F.C., Townsed, J., Liu, R., Lam, K.S., Immobilized OBOC combinatorial bead array to facilitate multiplicative screening. *Comb Chem High Throughput Screen* **16**, 441-448 (2013).
61. Townsend, J.B., Shaheen, F., Liu, R., Lam, K.S. Jeffamine derivatized TentaGel beads and poly(dimethylsiloxane) microbead cassettes for ultrahigh-throughput in situ releasable solution-phase cell-based screening of one-bead-one compound combinatorial small molecule libraries. *J Comb Chem* **12**, 700-712 (2010).
62. Liu, R., Lam, K.S. Automatic Edman microsequencing of peptides containing multiple unnatural amino acids. *Anal Biochem* **295**, 9-16 (2001).
63. Amadei, G.A., Cho, C-F., Lewis, J.D., Luyt, L.G. A fast, reproducible and low-cost method for sequence deconvolution of 'on-bead' peptides via 'on-target' maldi-TOF/TOF mass spectrometry. *J Mass Spec* **45**, 241-251 (2009).
64. Youngquist, R.S., Fuentes, G.R., Lacey, M.P., Keough, T., Baillie, T.A. Matrix-assisted laser desorption ionization for rapid determination of the sequences of biologically active peptides isolated from support-bound combinatorial peptide libraries. *Rapid Comm Mass Spec* **8**, 77-81 (2005).
65. Semmler, A., Weber, R., Przybylski, P., Wittmann, V. De novo sequencing of peptides on single resin beads by MALDI-FTICR tandem mass spectrometry. *J Am Chem Soc Mass Spectrom* **21**, 215-219 (2010).
66. Ebenhan, T., Chadwick, N., Sathekge, M.M., Govender, P., Govender, T., Kruger, H.G., Marjanovic-Painter, B., Zeevaart, J.R. Peptide synthesis, characterization and (68)Ga-radiolabeling of NOTA-conjugated ubiquitin fragments for prospective infection imaging with PET/CT. *Nucl Med Biol* **41(5)**, 390-400 (2014).
67. Sako, T., Hasegawa, K., Nishimura, M., Kanayama, Y., Wada, Y., Hayashinaka, E., Cui, Y., Kataoka, Y., Senda, M., Watanabe, Y. Positron emission tomography study on pancreatic somatostatin receptors in normal and diabetic rats with 68Ga-DOTA-octreotide: a potential PET tracer for beta cell mass measurement. *Biochem Biophys Res Commun* **442**, 79-84 (2013).
68. Merrifield, R.B.J.A.C.S., 8, 255-73. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **8**, 255-273 (1963).
69. Heppeler, A., Froidevaux, S., Macke, H.R., Jermann, E., Behe, M., Powell, P., Hennig, M. Radiometal-labelled macrocyclic chelator-derivatised somatostatin analogue with superb tumour-targeting properties and potential for receptor-mediated internal radiotherapy. *Chem Eur J* **5(7)**, 1974-1981 (1999).
70. De Leon-Rodriguez, L.M., Kovacs, Z. The synthesis and chelation chemistry of DOTA-peptide conjugates. *Bioconjugate Chem* **19**, 391-402 (2008).

71. Needels, M.C., Jones, D.G., Tate, E.H., Heinkel, G.L., Kochersperger, L.M., Dower, W.J., Barrett, R.W., Gallop, M.A. Generation and screening of an oligonucleotide-encoded synthetic peptide library. *Proc Natl Acad Sci* **90**, 10700-10704 (1993).
72. Nikolaiev, V., Stierandova, A., Krchnak, V., Seligmann, B., Lam, K.S., Salmon, S.E, Lebi, M. Peptide-encoding for structure determination of nonsequenceable polymers within libraries synthesized and tested on solid-phase supports. *Peptide Res* **6**, 161-170 (1993).
73. Liu, R., Marik, J., Lam, K.S. A novel peptide-based encoding system for "one-bead one-compound"peptidomimetic and small molecule combinatorial libraries. *J Am Chem Soc* **124**, 7678-7680 (2002).
74. Chait, B.T., Wang, R., Beavis, R.C., Kent, S.B.H. Protein ladder sequencing. *Science* **262**, 89-92 (1993).
75. Wang, X., Zhang, J., Song, A., Lebrilla, C.B., Lam, K.S. Encoding method for OBOC small molecule libraries using a biphasic approach for ladder-synthesis of coding tags. *J Am Chem Soc* **126**, 5740-5749 (2004).
76. Youngquist, R.S., Fuentes, G.R., Lacey, M.P., Keough, T. Generation and screening of combinatorial peptide libraries designed for rapid sequencing by mass spectrometry. *J Am Chem Soc* **117**, 3900-3906 (1995).
77. Wang, P., Arabaci, G., Pei, D. Rapid Sequencing of Library-Derived Peptides by Partial Edman Degradation and Mass Spectrometry. *J Comb Chem* **3**, 251-254 (2001).
78. Sweeney, M.C., Pei, D. An Improved Method for Rapid Sequencing of Support-Bound Peptides by Partial Edman Degradation and Mass Spectrometry. *J Comb Chem* **5**, 218-222 (2003).
79. Thakkar, A., Wavreille, A.S., Pei, D. Traceless capping agent for peptide sequencing by partial edman degradation and mass spectrometry. *Anal Chem* **78**, 5935-5939 (2006).
80. Jee, J.E., Ang, Y.L., Cha, J., And, M.W., Ling, J., Lim, J., Lee, S.S. Combinatorial bead-based Peptide libraries improved for rapid and robust screenings. *Comb Chem High Throughput Screen* **17**, 520-530 (2014).
81. Marani, N.M., Martínez Ceron, M.C., Giudicessi, S.L., de Oliveira, E., Côté, S., Erra-Balsells, R., Albericio, F., Cascone, O., Camperi, S.A. Screening of one-bead-one-peptide combinatorial library using red fluorescent dyes. Presence of positive and false positive beads. *J Comb Chem* **11**, 146-150 (2009).
82. Beardsley, R.L., Reilly, J.P. Fragmentation of amidinated peptide ions. *J Am Soc Mass Spectrom* **15**, 158-167 (2004).

83. Hwang, S.H., Lehman, A., Cong, X., Olmstead, M.M., Lam, K.S., Lebrilla, C.B., Kurth, M.J. OBOC small-molecule combinatorial library encoded by halogenated mass-tags. *Org Lett* **6**, 3829-3832 (2004).
84. Kim, J.-S., Song, J.-S., Kim, Y., Park, S.B., Kim, H.-J. De novo analysis of protein N-terminal sequence utilizing MALDI signal enhancing derivatization with Br signature. *Anal Bioanal Chem* **402**, 1911-1919 (2012).
85. Fitzgerald, M.C., Harris, K., Shevlin, C.G., Siuzdak, G. Direct characterization of solid phase resin-bound molecules by mass spectrometry. *Bioorg Med Chem Lett* **6**, 979-982 (1996).
86. Steen, H., Mann, M. The ABC's (and XYZ's) of peptide sequencing. *Nat Rev Mol Cell Biol* **5**, 699-711 (2004).
87. Paizs, B., Suhai, S. fragmentation pathways of protonated peptides. *Mass Spec Rev* **24**, 508-548 (2005).
88. Cruickshank, D.R. University of Western Ontario (2012).
89. Kubíček, V., Havlíčková, J., Kotek, J., Tircsó, G., Hermann, P., Tóth, E., Lukes, I. Gallium(III) complexes of DOTA and DOTA-monoamide: kinetic and thermodynamic studies. *Inorg Chem* **49(23)**, 10960-10969 (2010).
90. Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal Biochem* **34**, 595-598 (1970).
91. Quarell, R., Claridge, D.W., Weaver, G.W., Lowe, G. Structure and properties of TentaGel resin beads: implications for combinatorial library chemistry. *Mol Div* **1**, 223-232 (1996).
92. LLC, A. Practical synthesis guide to solid phase peptide chemistry.
93. Hancock, W.S., Marshall, G.R. Letter: Cyanogen bromide as a cleavage procedure in solid phase peptide synthesis. *J Am Chem Soc* **97**, 7488-7489 (1975).
94. Pelliccioli, A.P., Wirz, J. Photoremovable protecting groups: reaction mechanisms and applications. *Photochem Photobiol Sci* **1**, 441-458 (2002).
95. Gaplovsky, M., Il'ichev, Y.V., Kamdzhilov, Y., Kombarova, S.V., Mac, M., Schwörer, M.A., Wirz, J. . Photochemical reaction mechanisms of 2-nitrobenzyl compounds: 2-Nitrobenzyl alcohols form 2-nitroso hydrates by dual proton transfer. *Photochem Photobiol Sci* **4**, 33-42 (2004).
96. Canadian Cancer Society. Breast Cancer Statistics. www.cancer.ca. Accessed in June, 10, 2014.

97. Zhu, J., Zheng, Z., Wang, J., Sun, J., Wang, P., Cheng, X., Fu, L., Zhang, L., Wang, Z., Li, Z.. Different miRNA expression profiles between human breast cancer tumors and serum. *Front Genet* **5**, 1-7 (2014).
98. Leibman, A.J., Kruse, B. Breast cancer: mammographic and sonographic findings after augmentation mammoplasty. *Radiology* **174**, 195-198 (1990).
99. Jackson, V.P., Hendrick, R.E., Feig, S.A., Kopans, D.B. Imaging of the radiographically dense breast. *Radiology* **188**, 297-301 (1993).
100. Boetes, C., Mus, R.D., Holland, R., Barentsz, J.O., Strijk, S.P., Wobbes, T., Hendriks, J.H., Ruys, S.H. Breast tumors: comparative accuracy of MR imaging relative to mammography and US for demonstrating extent. *Radiology* **197**, 743-747 (1995).
101. Mumtaz, H., Hall-Craggs, M.A., Davidson, T., Walmsley, K., Thurell, W., Kissin, M.W., Taylor, I. Staging of symptomatic primary breast cancer with MR imaging. *Am J Roentgenol* **169**, 417-424 (1997).
102. Orel, S.G., Schnall, M.D. MR imaging of the breast for the detection, diagnosis and staging of breast cancer. *Radiology* **220**, 13-30 (2001).
103. Menezes, G.L.G., Knuttel, F.M., van den Bosch, M.A.A.J. Magnetic resonance imaging in breast cancer: a literature review and future perspectives. *World J Clin Oncol* **5**, 61-70 (2014).
104. Alunni-Fabbroni, M., Müller, V., Fehm, T., Janni, W., Rak, B. Monitoring in metastatic breast cancer: is imaging outdated in the era of circulating tumor cells? *Breat Care* **9**, 16-21 (2014).
105. Lam, K.S. Enzyme-linked colorimetric screening of a one-bead one-compound combinatorial library. *Methods Mol Biol* **87**, 7-12 (1998).
106. Salmon, S.E., Liu-Stevens, R.H., Zhao, Y., Lebl, M., Krnchňák, V., Wertman, K., Sepetov, N., Lam, K.S. High-volume cellular screening for anticancer agents with combinatorial chemical libraries: a new methodology. *Mol Divers* **2**, 57-63 (1996).
107. Lehman, A., Gholamin, S., Hahn, M., Lam, K.S. Image subtraction approach to screening one-bead-one-compound combinatorial libraries with complex protein mixtures. *J Comb Chem* **8**, 562-570 (2006).
108. Miyamoto, S., Liu, R., Hung, S., Wang, X., Lam, K.S. Screening of a one-bead-one compound combinatorial library for β -actin identifies molecules active toward Ramos B-lymphoma cells. *Anal Biochem* **374**, 112-120 (2008).
109. Cha, J., Lim, J., Zheng, Y., Tan, S., Ang, Y.L., Oon, J., Ang, M.W., Ling, J., Bode, M., Lee, S.S. Process automation toward ultra-high-throughput screening

- of combinatorial one-bead-one-compound (OBOC) peptide libraries. *J Lab Autom* **17**, 186-200 (2012).
110. Cho, C.F., Behnam Azad, B., Luyt, L.G., Lewis, J.D. High-throughput screening of one-bead-one-compound peptide libraries using intact cells. *Comb Sci* **15**, 393-400 (2013).
 111. Peng, L., Liu, R., Marik, J., Wang, X., Takada, Y., Lam, K.S. Combinatorial chemistry identifies high-affinity peptidomimetics against $\alpha 4\beta 1$ integrin for in vivo tumor imaging. *Nat Chem Biol* **2**, 381-389 (2006).
 112. Cha, J.L., J.; Zheng, Y.; Tan, S.; Ang, Y. L.; Oon, J.; Ang, M. W.; Ling, J.; Bode, M.; Lee, S. S. Process Automation toward UltraHigh-Throughput Screening of Combinatorial One-Bead-One Compound (OBOC) Peptide Libraries. *J Lab Autom* **17**, 186-200 (2012).
 113. Marani, M.M., Ceron, M.C.M., Giudicessi, S.L., Oliveira, E., Côté, S., Erra-Balsells, R., Albericio, F., Cascone, O., Camperi, S.A. Screening of one-bead-one-peptide combinatorial library using red fluorescent dyes. Presence of positive and false positive beads. *J Comb Chem* **11**, 146-150 (2009).
 114. Cho, C.F., Behnam, Azad. B., Luyt, L.G., Lewis, J.D. High-Throughput Screening of One-Bead-One-Compound Peptide Libraries Using Intact Cells. *ACS Comb Sci* **15**, 393-400 (2013).
 115. Göksel, H., Wasserberg, D., Möcklinghoff, S., Araujo, B.V., Brunsveld, L. An on-bead assay for the identification of non-natural peptides targeting the androgen receptor-cofactor interaction. *Bioorg Med Chem* **19**, 306-311 (2011).
 116. Teng, P., Zhang, X., Wu, H., Qiao, Q., Sebt, S.M., Cai, J. Identification of novel inhibitors that disrupt STAT3-DNA interaction from a γ -AApeptide OBOC combinatorial library. *Chem Commun*, Ahead of print (2014).

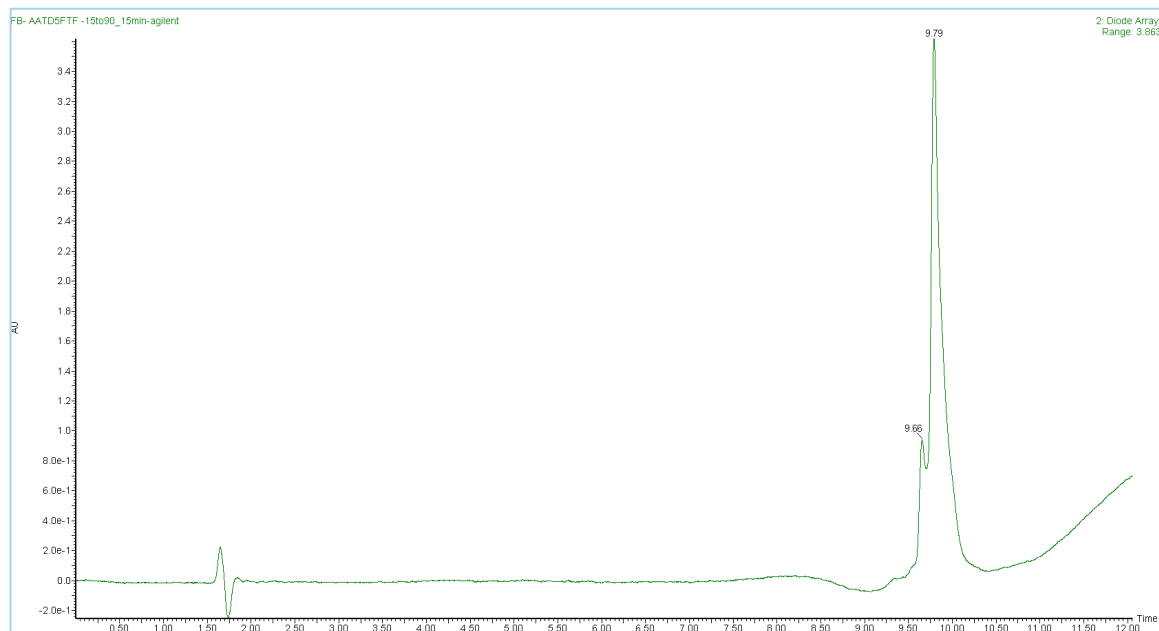
APPENDIX I – CHAPTER 2

HPLC TRACES

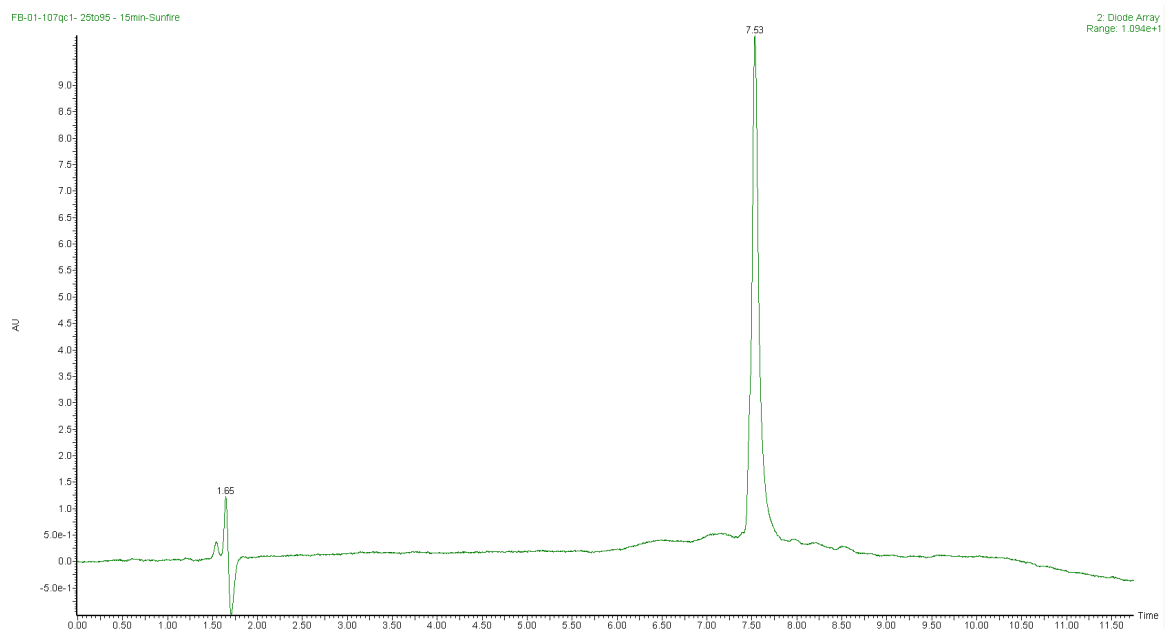
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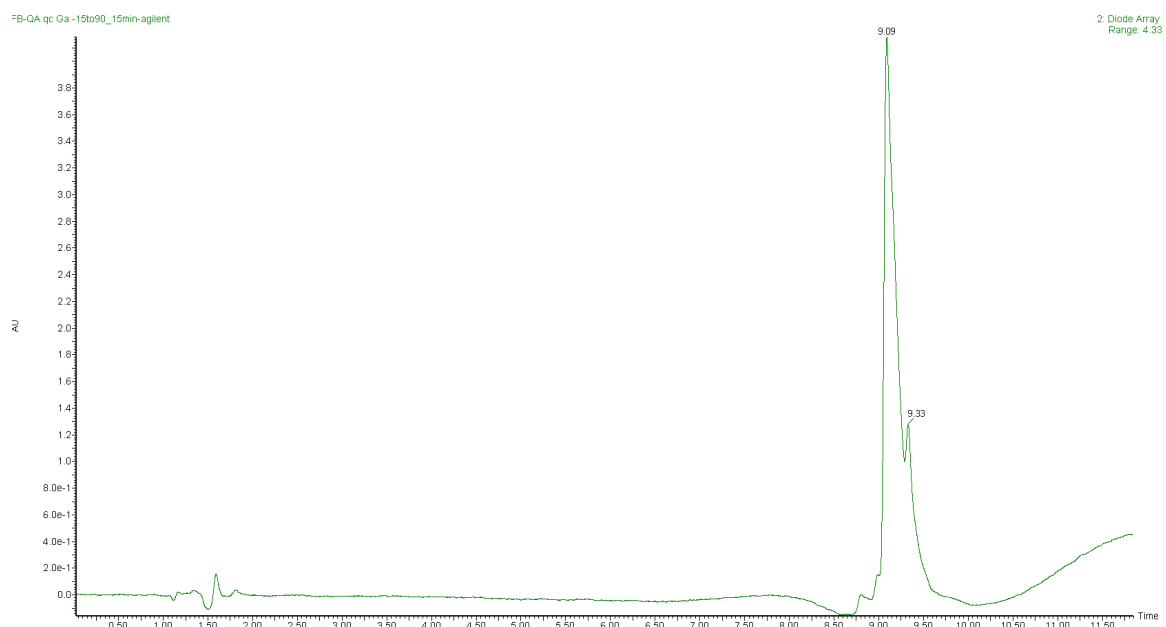
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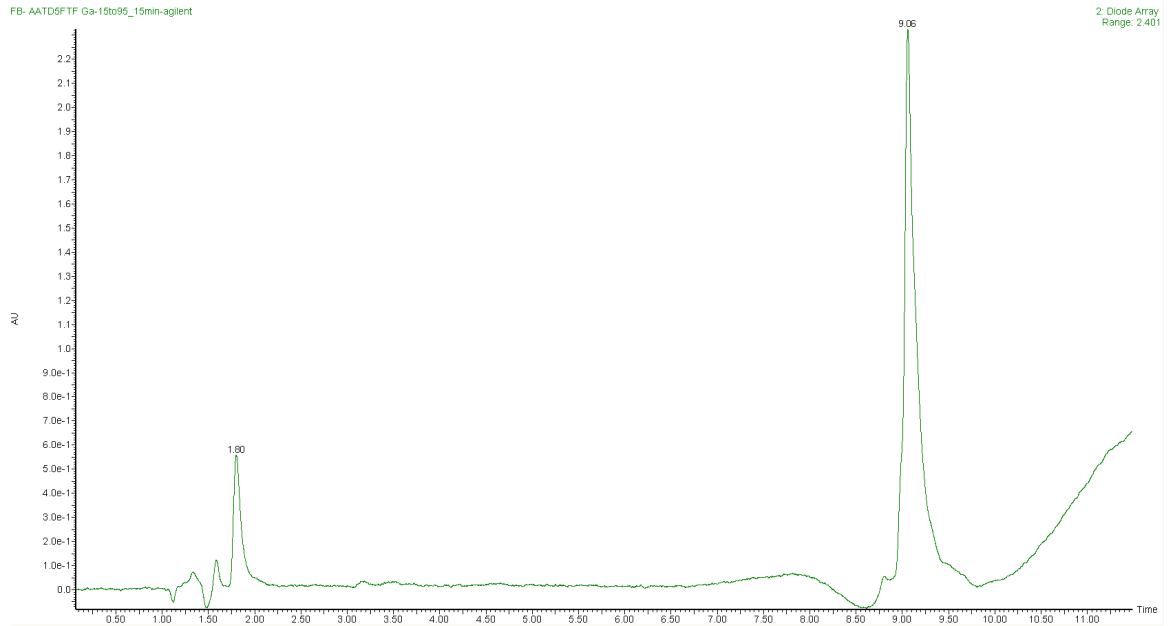
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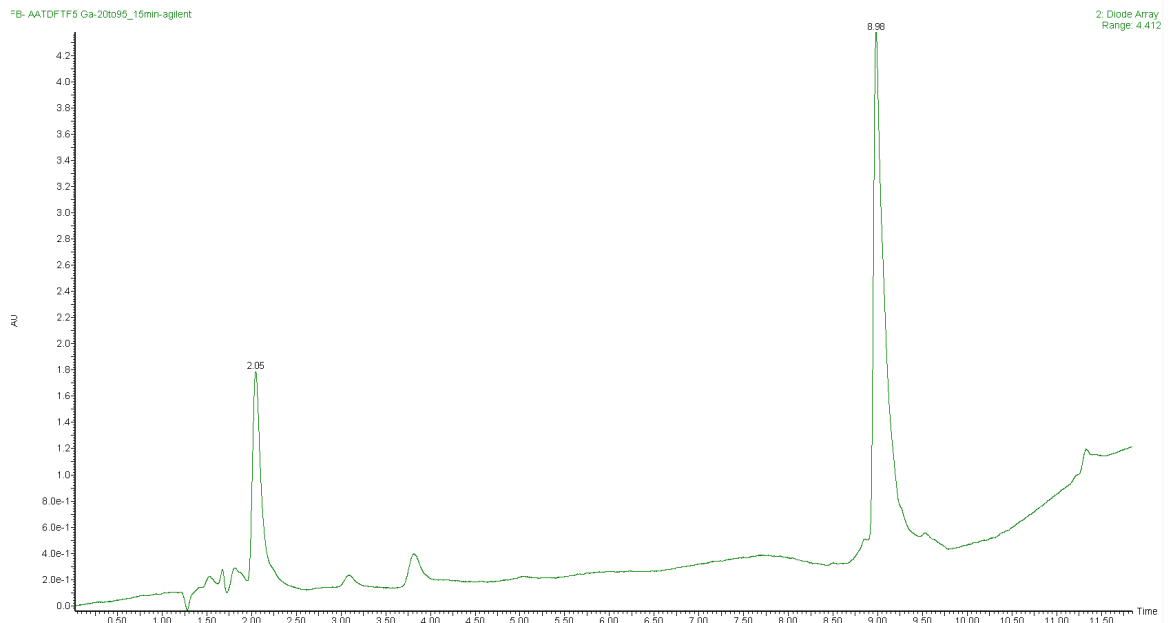
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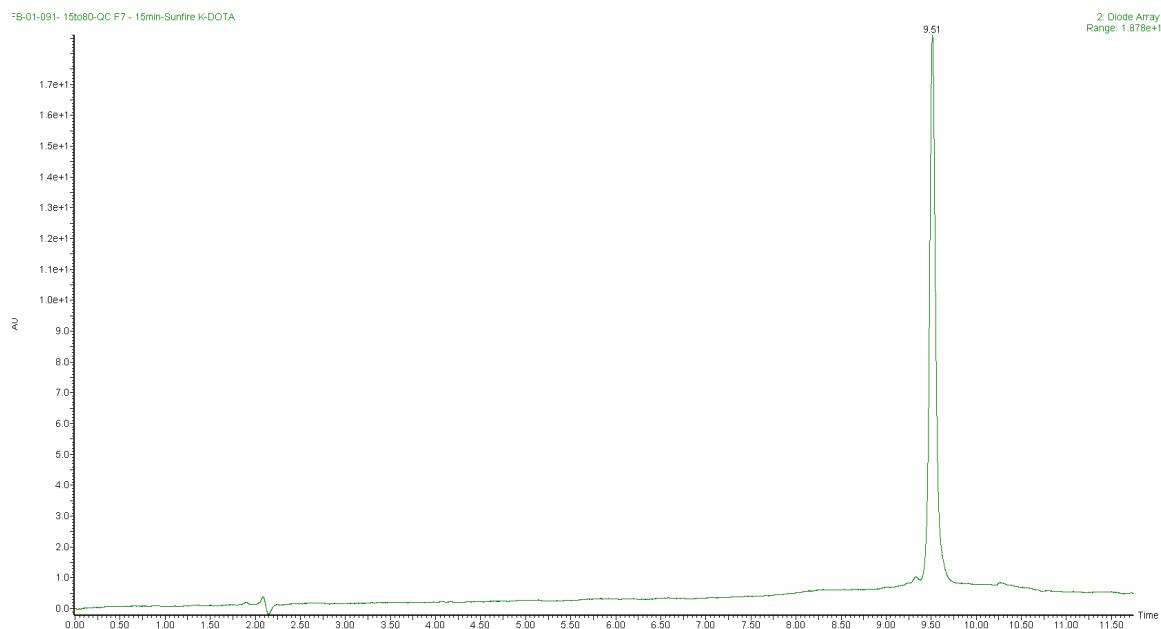
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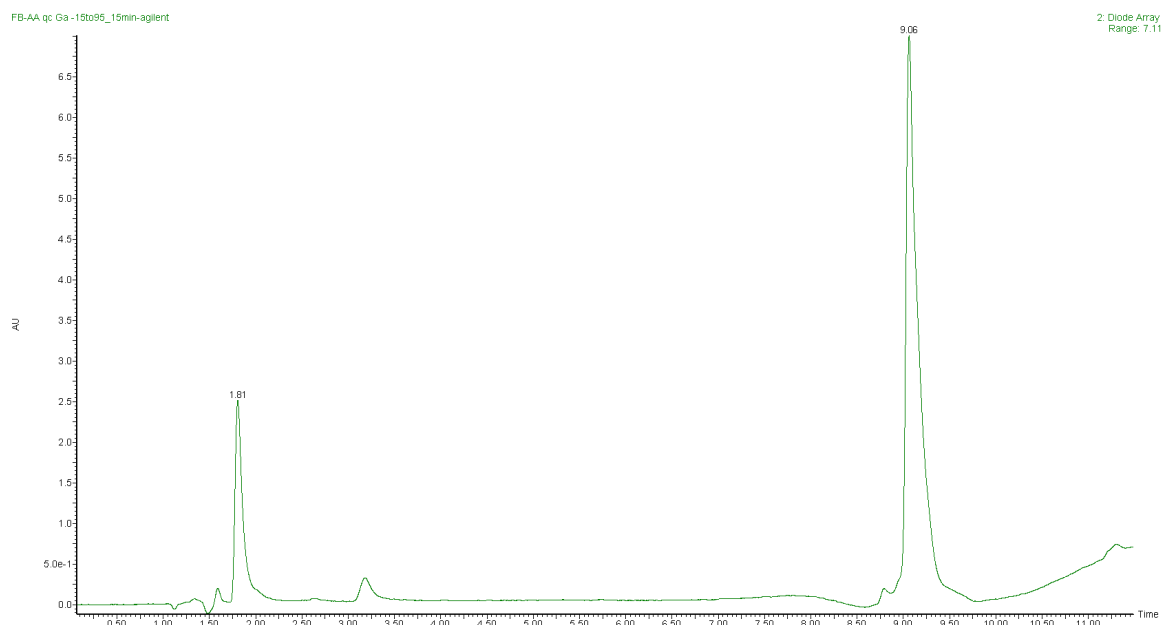
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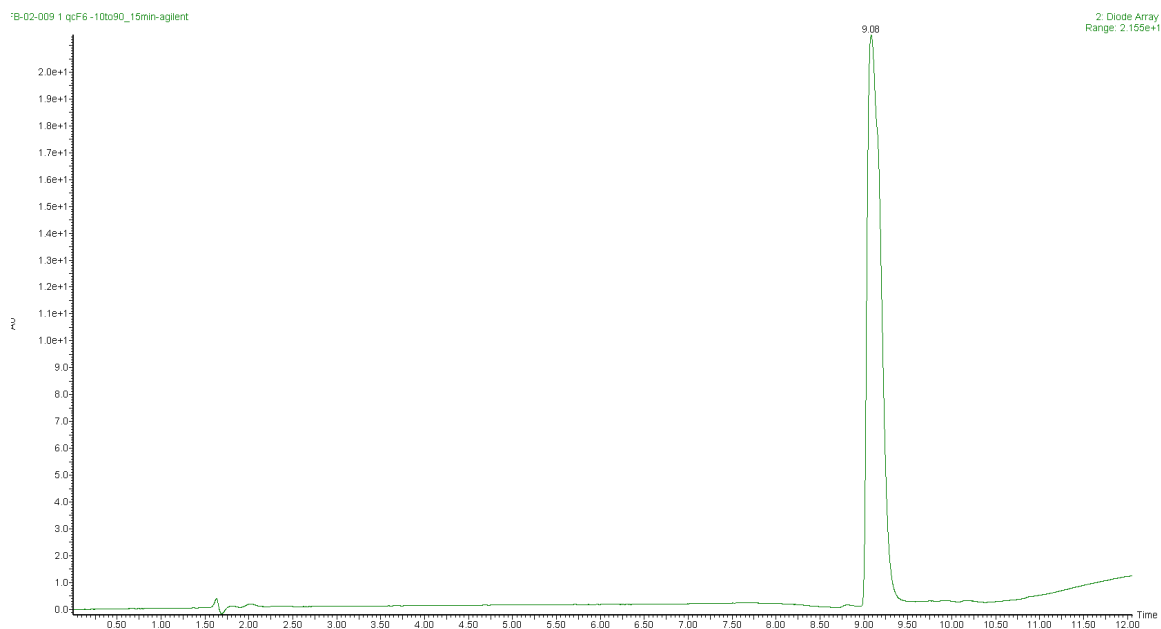
HPLC trace for 2.9



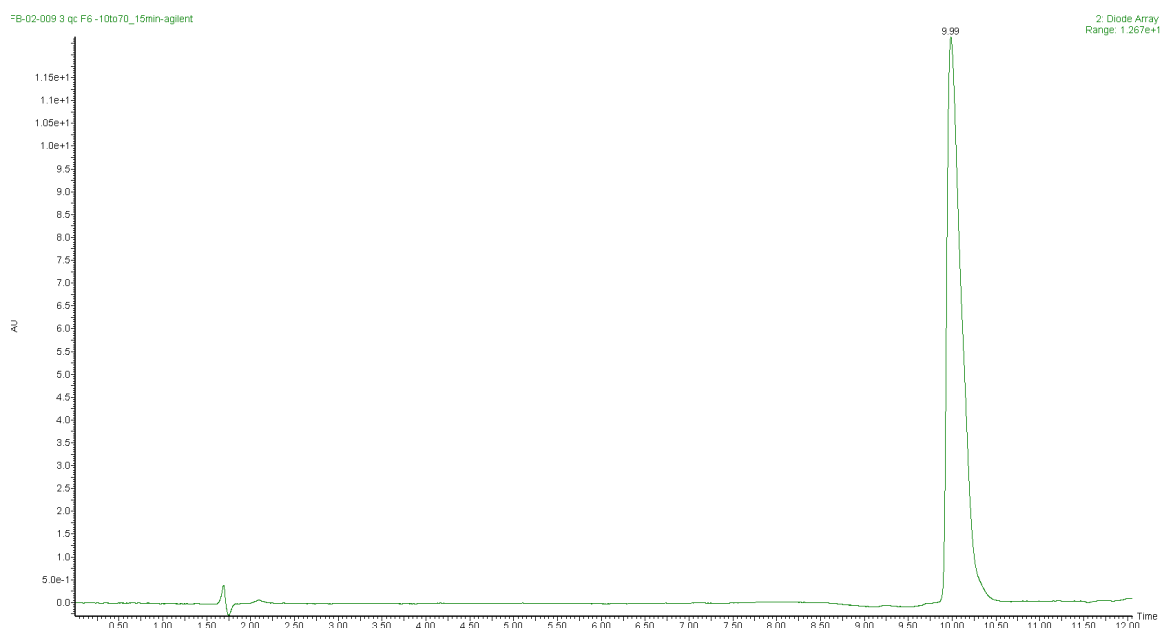
APPENDIX II – CHAPTER 4

HPLC TRACES

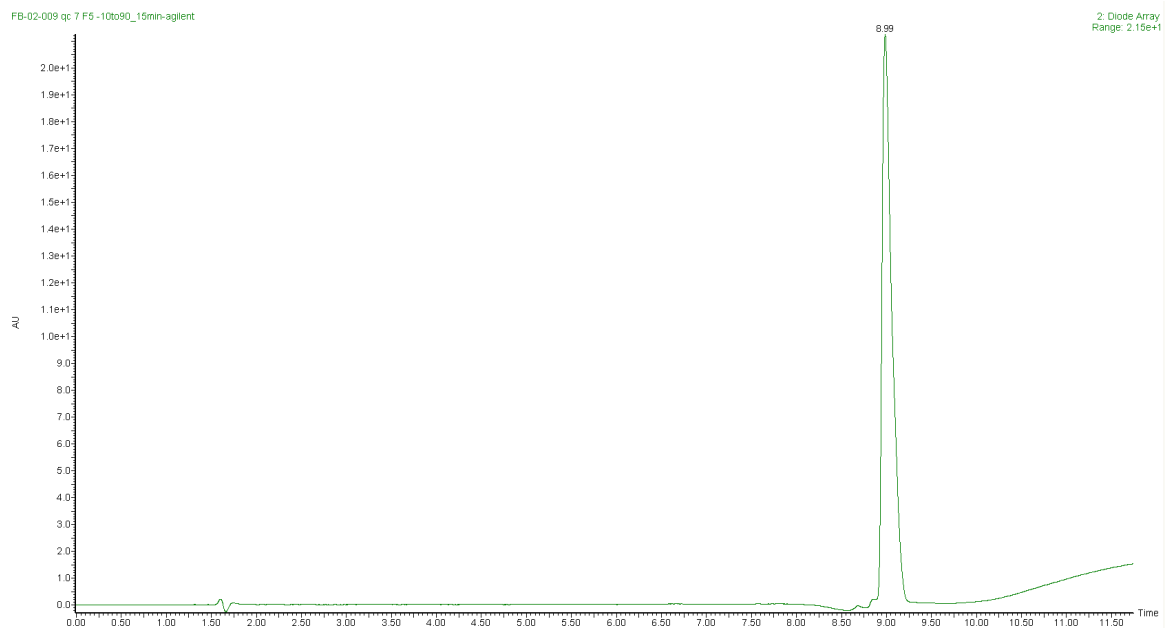
HPLC trace for 4.1



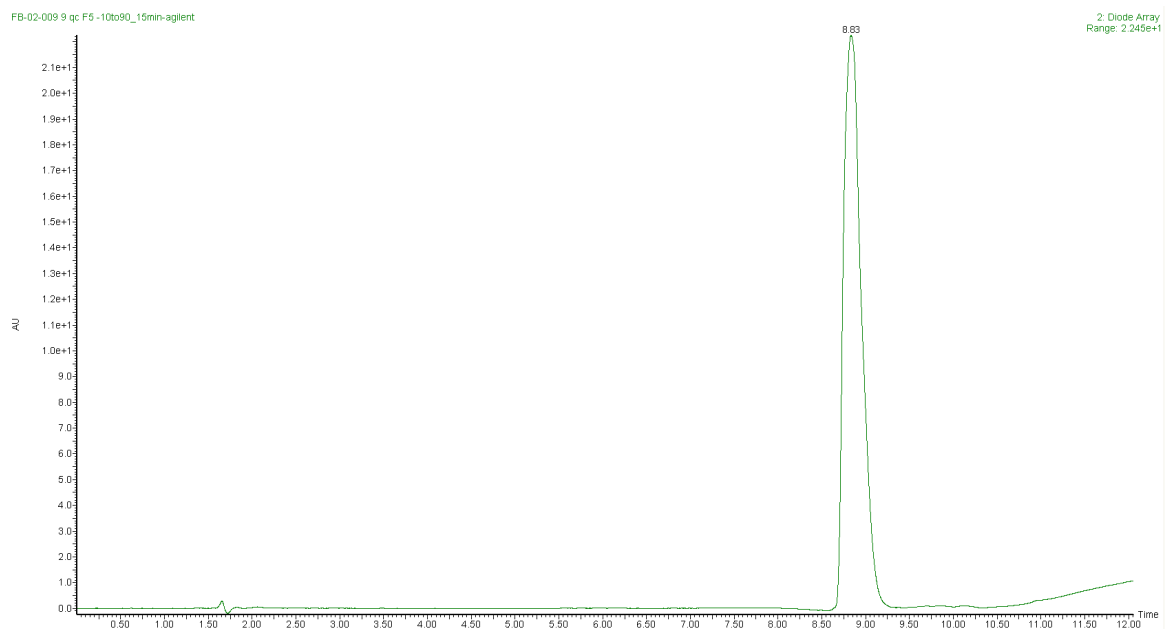
HPLC trace for 4.2



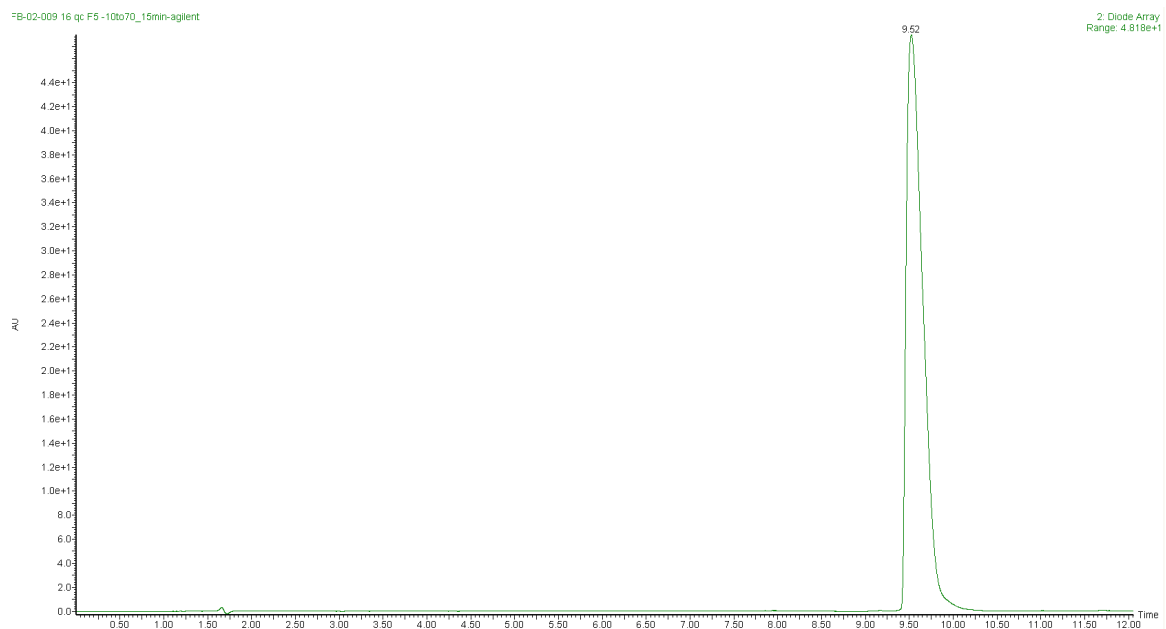
HPLC trace for 4.3



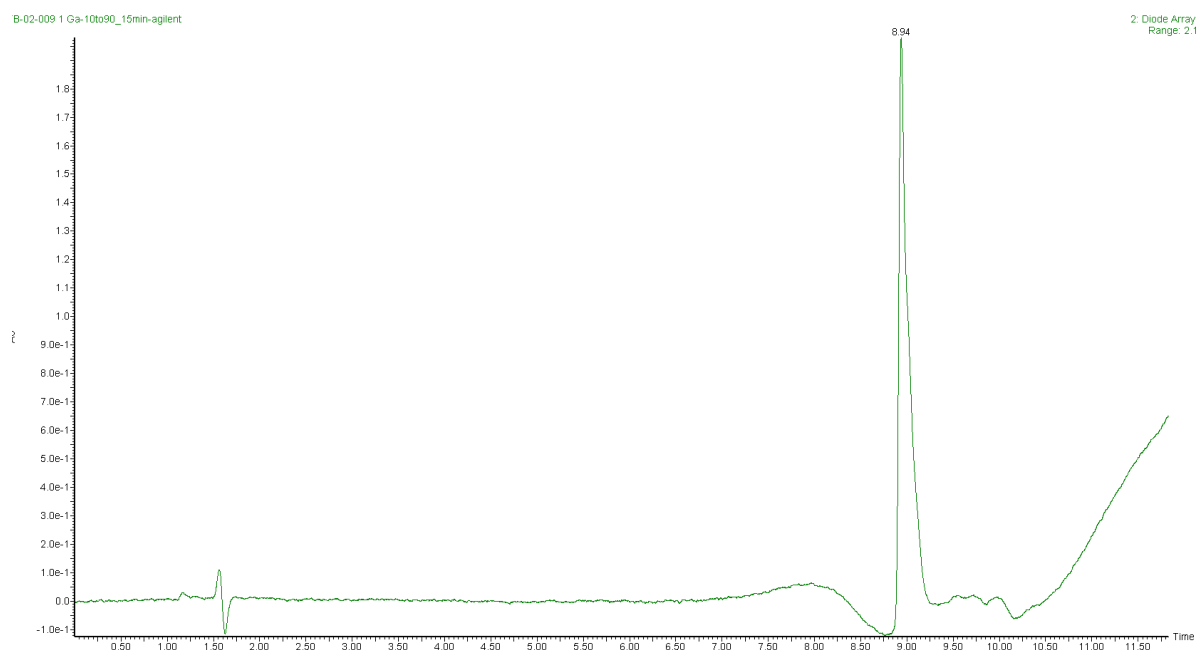
HPLC trace for 4.4



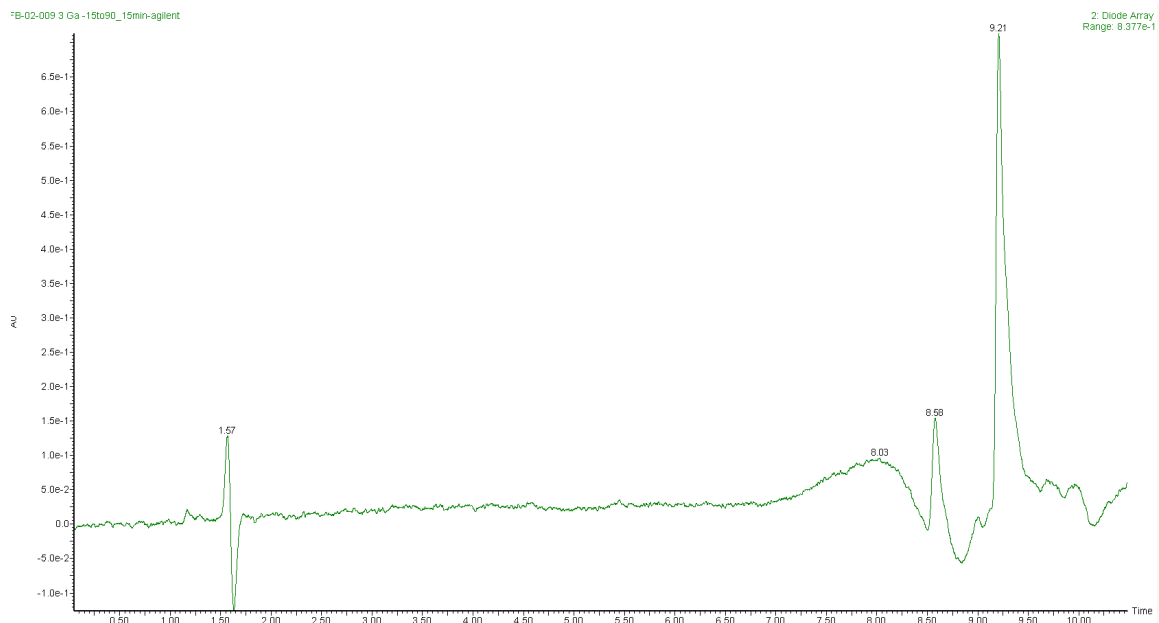
HPLC trace for 4.5



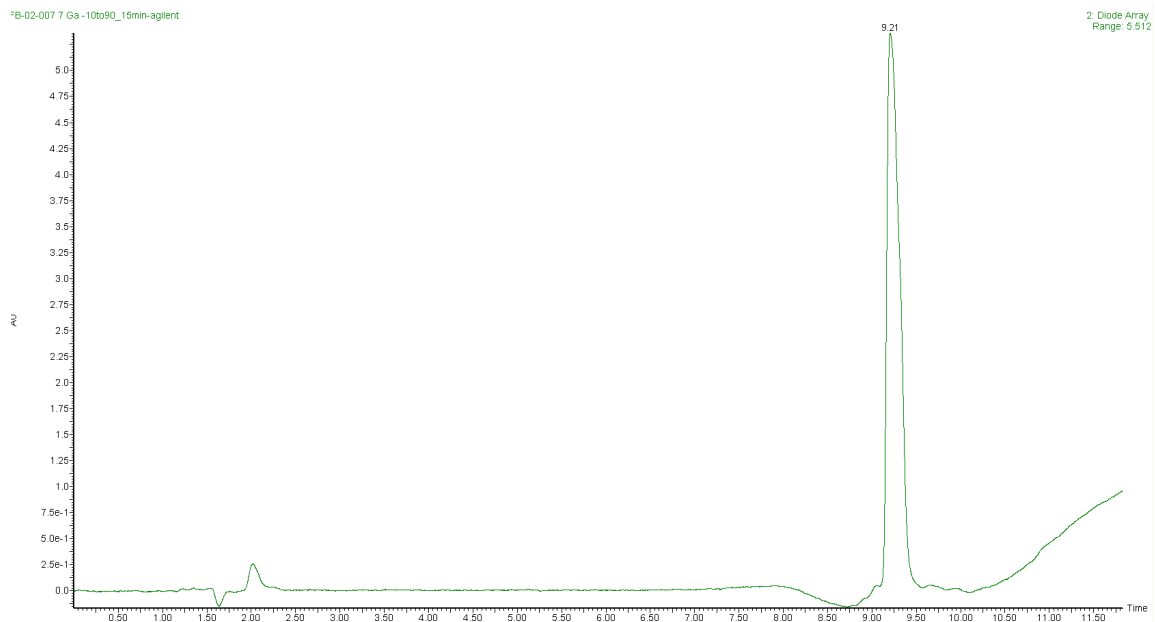
HPLC trace for 4.6



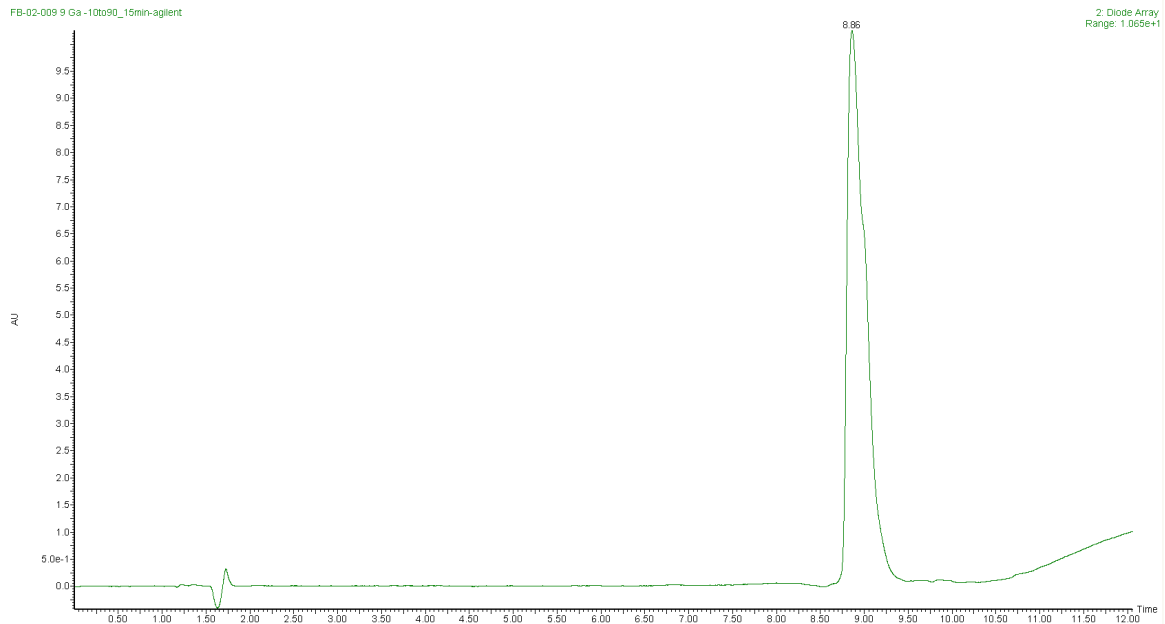
HPLC trace for 4.7



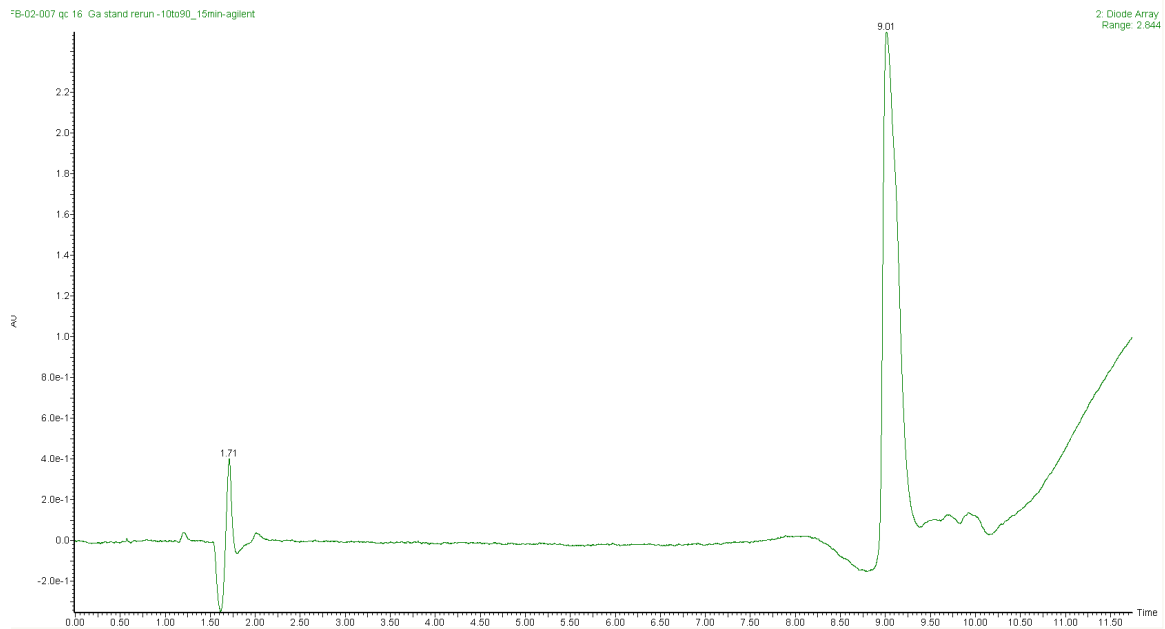
HPLC trace for 4.8



HPLC trace for 4.9

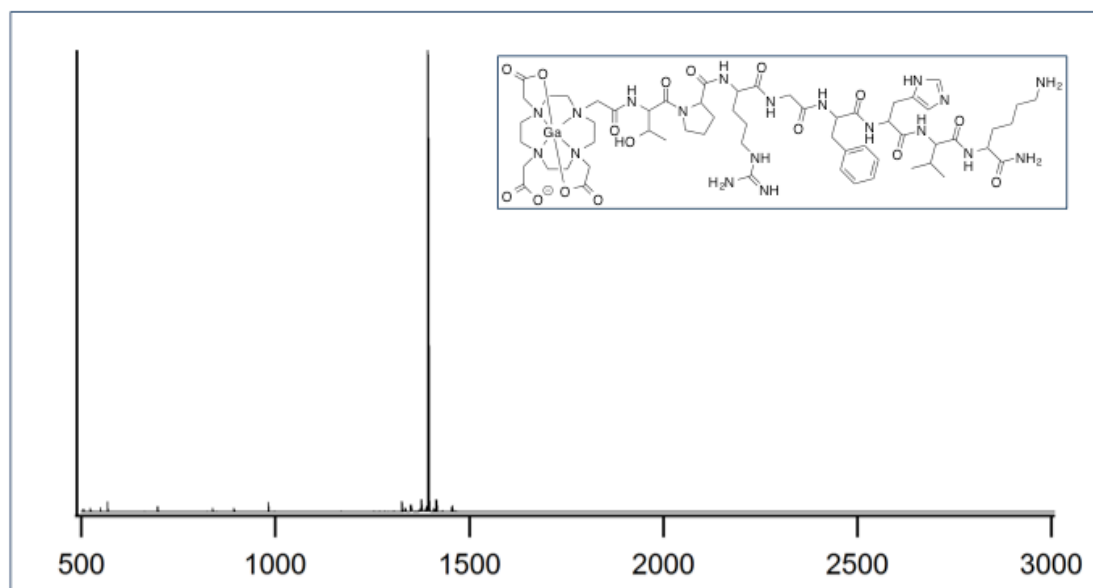


HPLC trace for 4.10

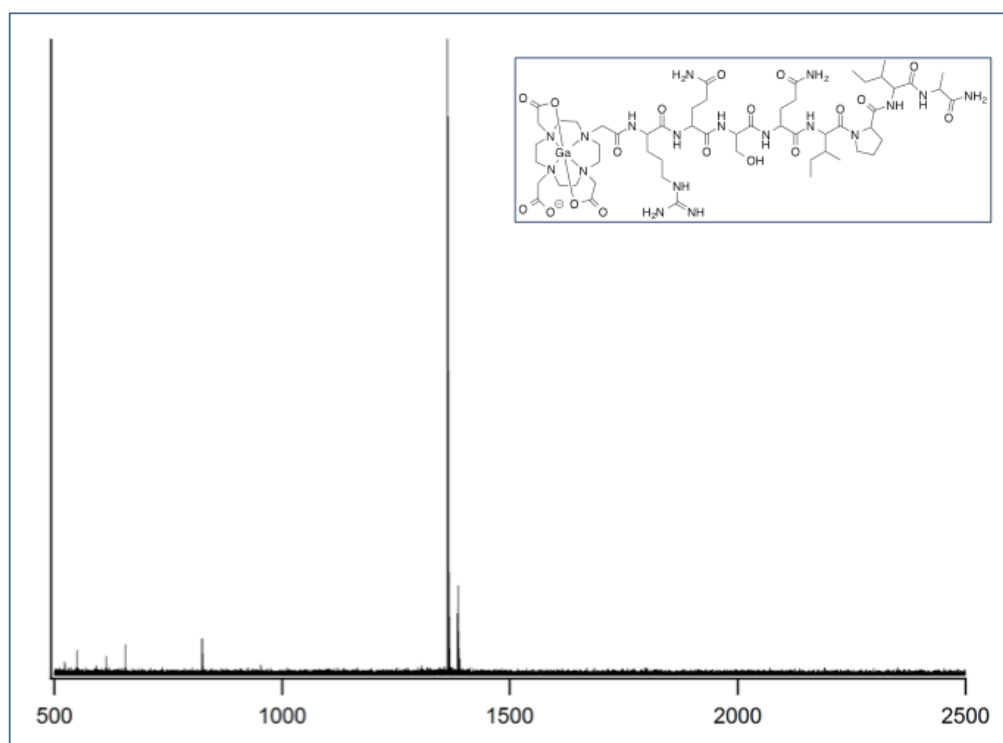


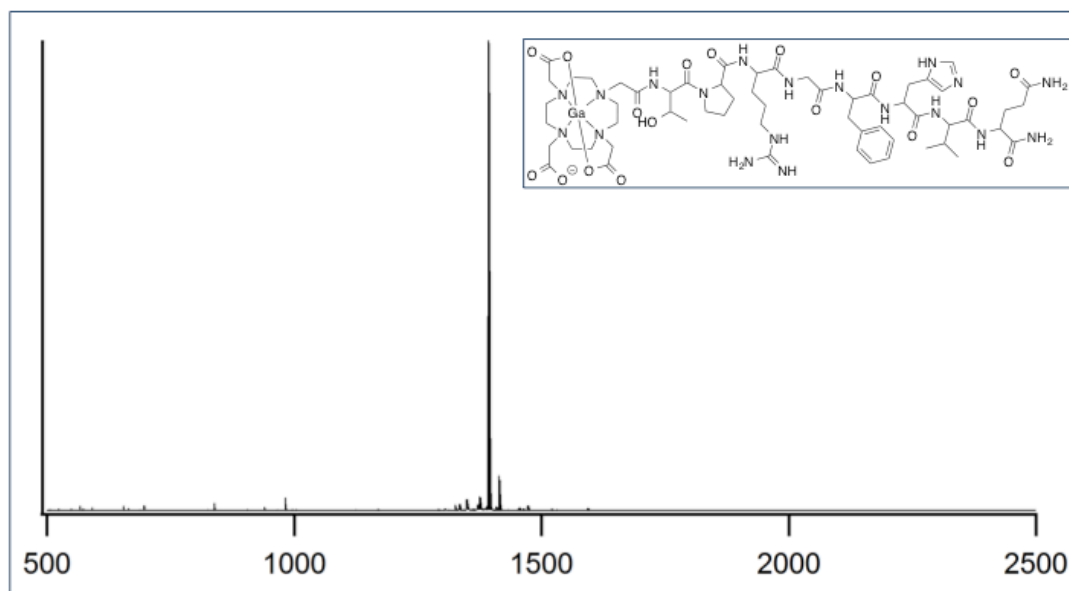
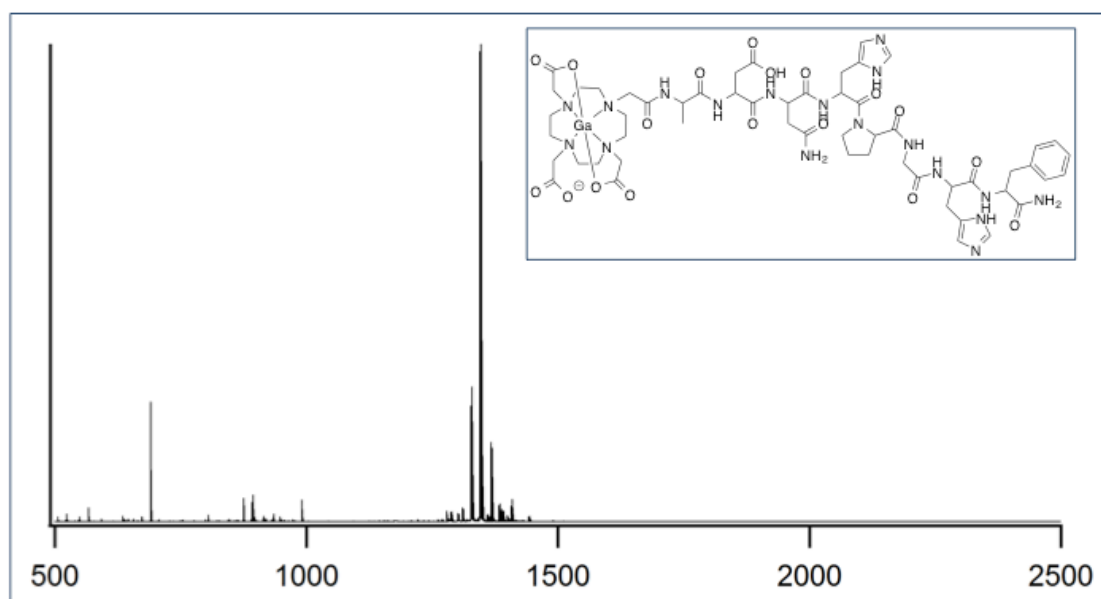
MALDI TANDEM MASS SPECTROMETRY

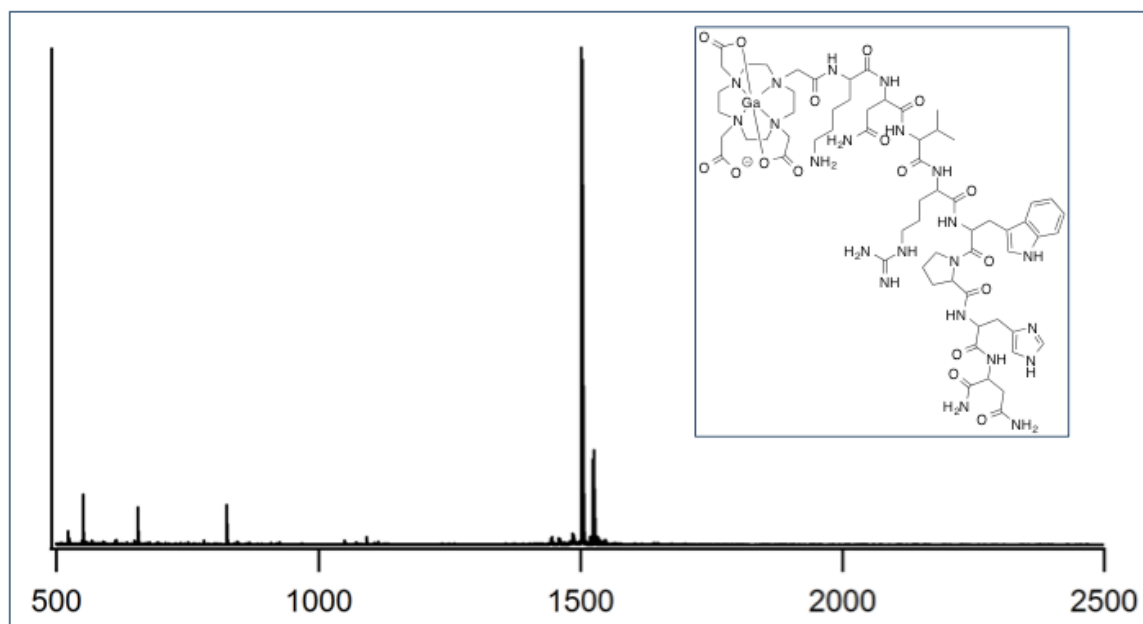
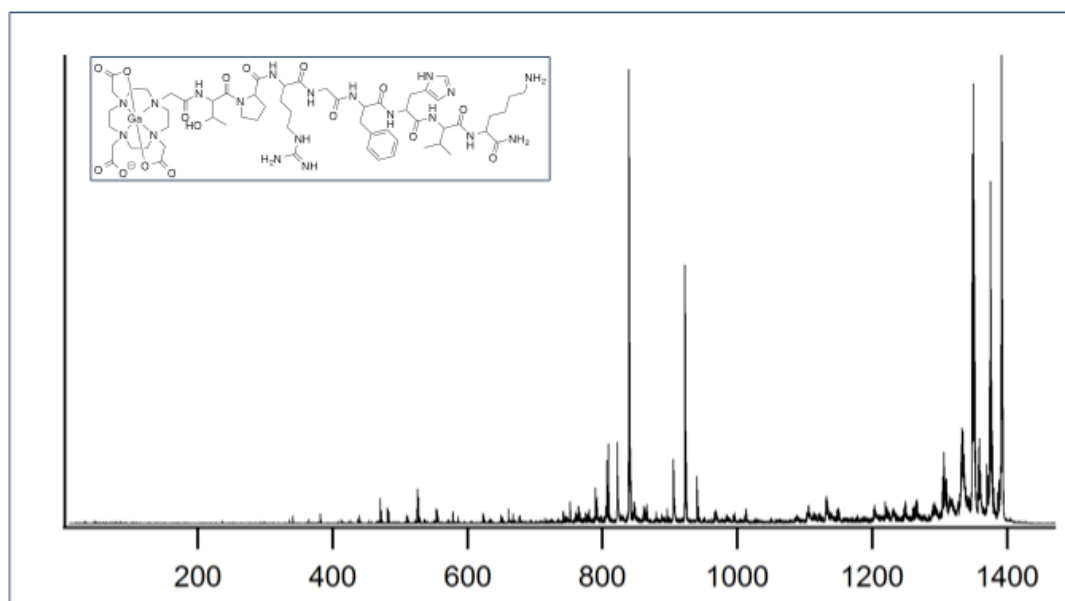
MS Spectrum for **4.6**



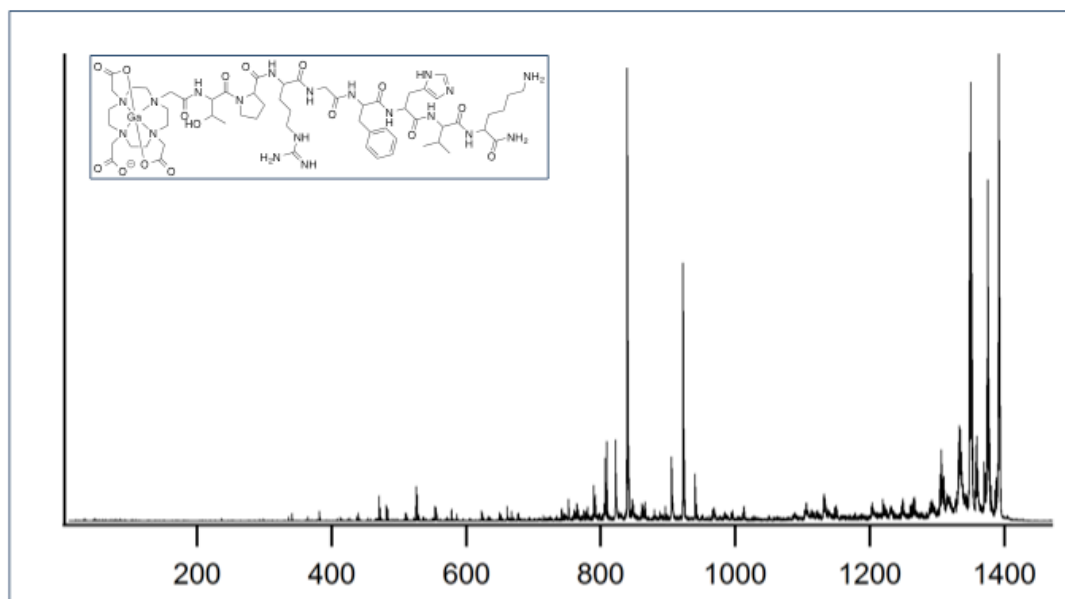
MS Spectrum for **4.7**



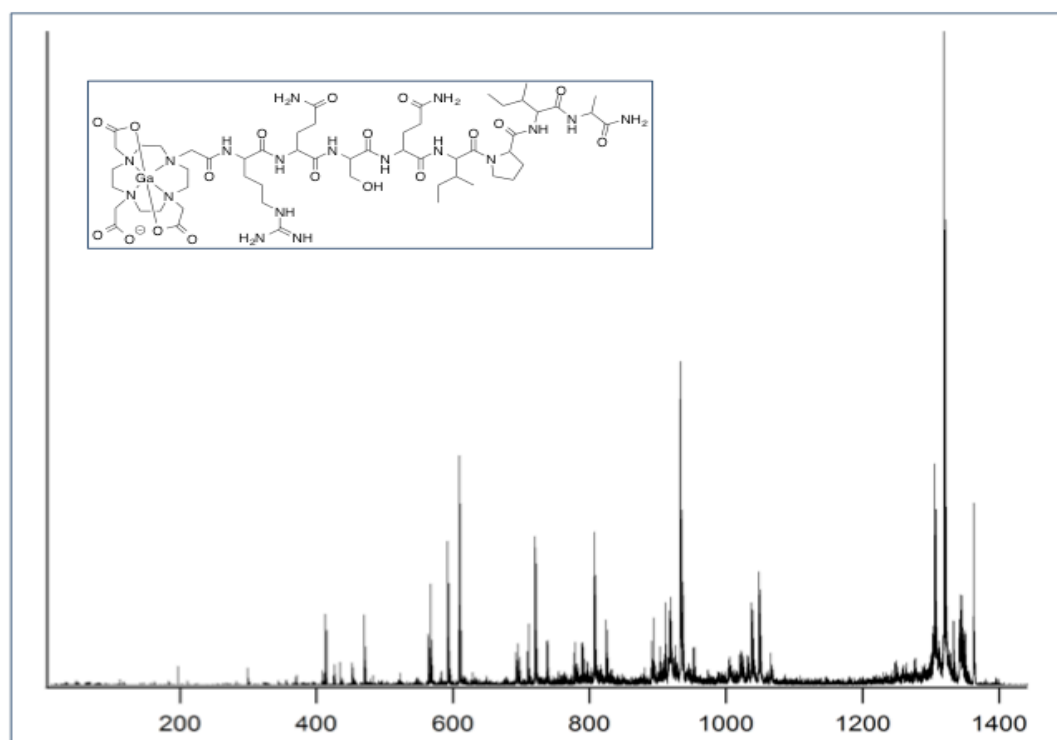
MS Spectrum for **4.8**MS Spectrum for **4.9**

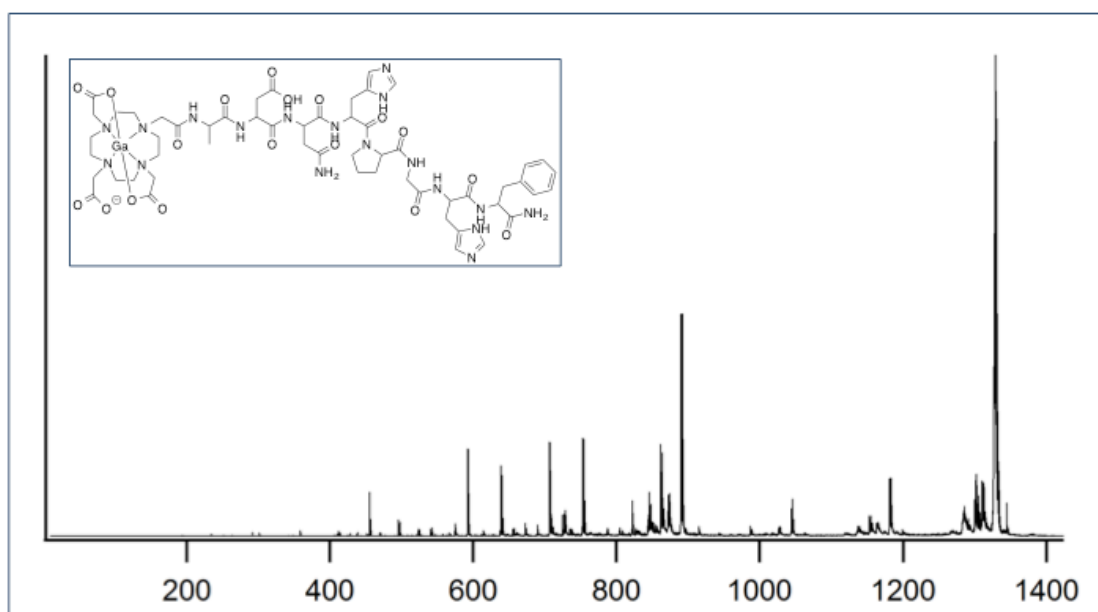
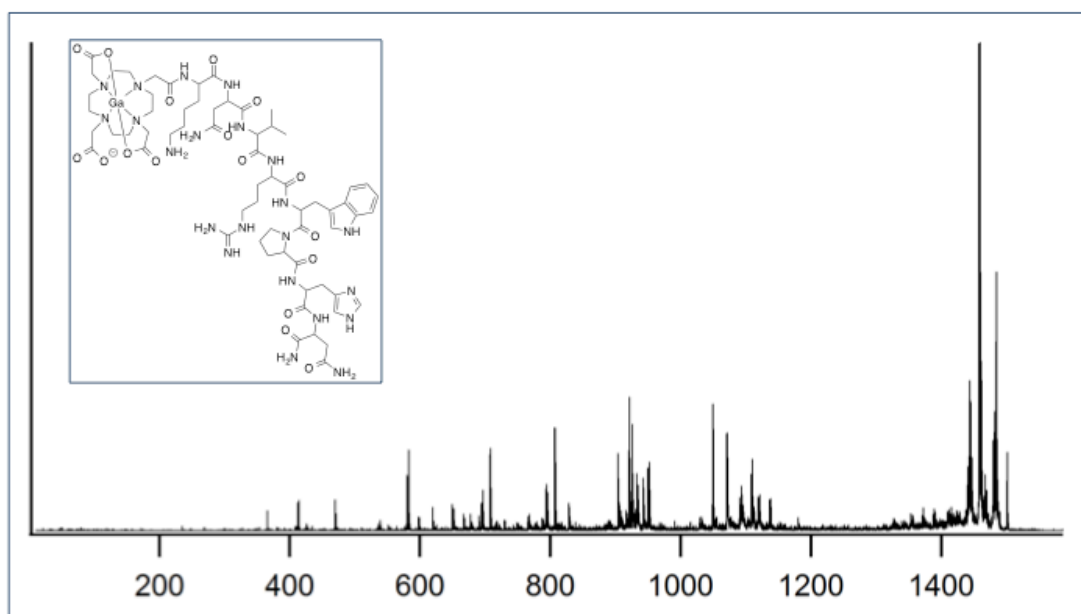
MS Spectrum for **4.10**MSMS Spectrum for **4.6**

MSMS Spectrum for 4.7



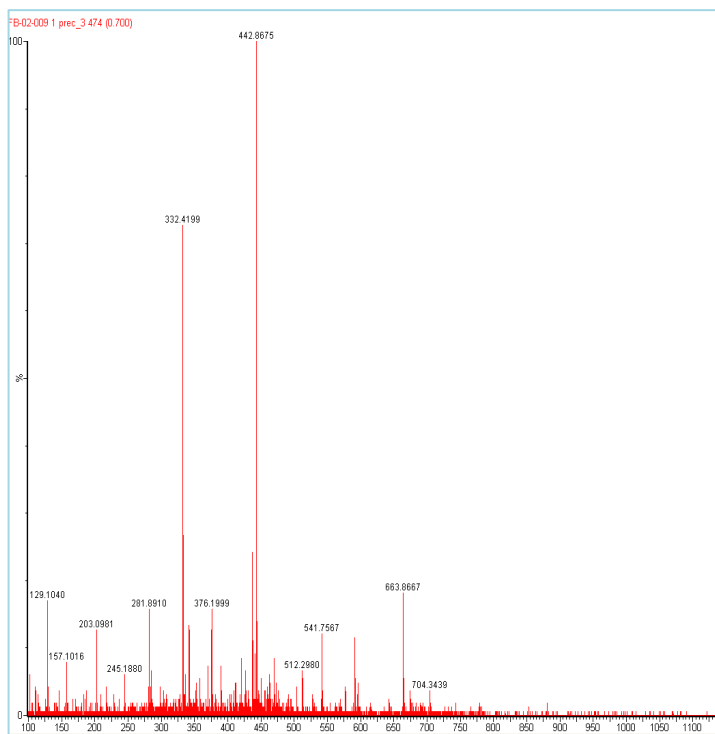
MSMS Spectrum for 4.8



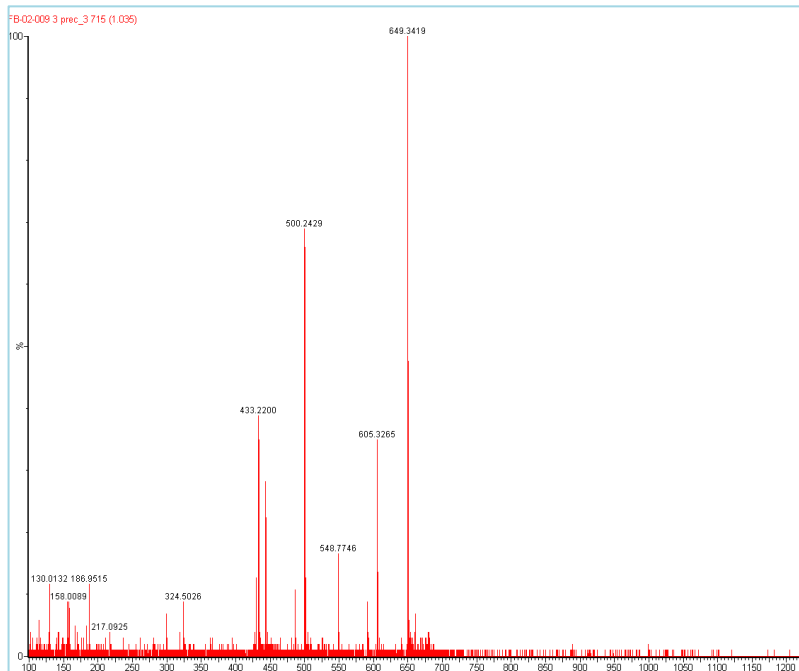
MSMS Spectrum for **4.9**MSMS Spectrum for **4.10**

HIGH-RESOLUTION MASS SPECTROMETRY

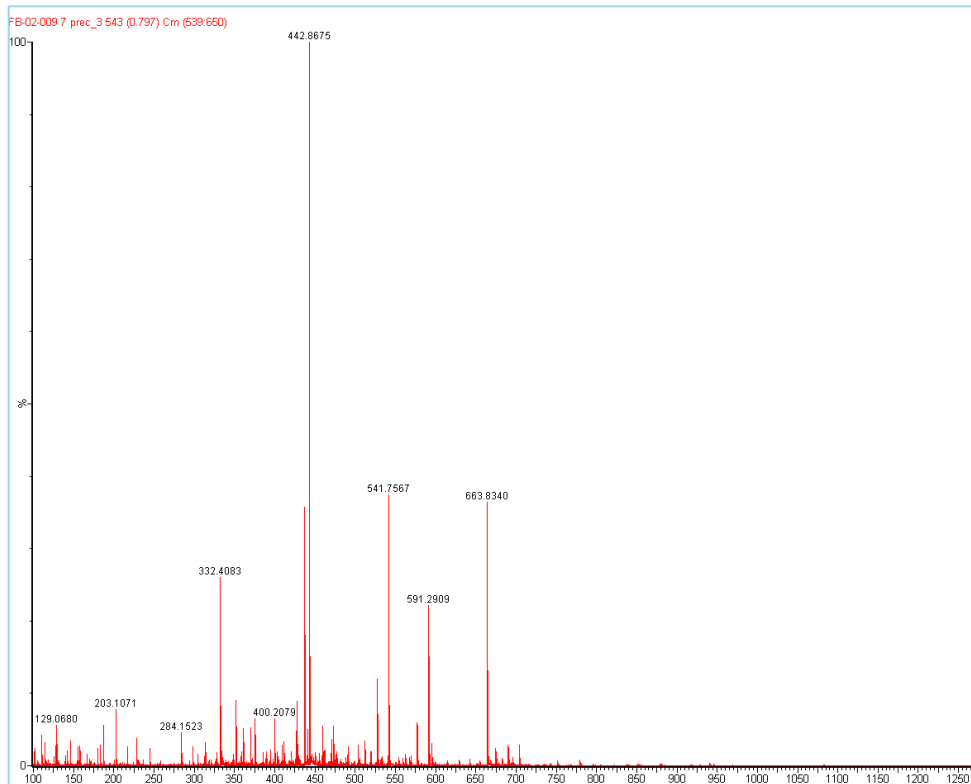
Compound 4.1



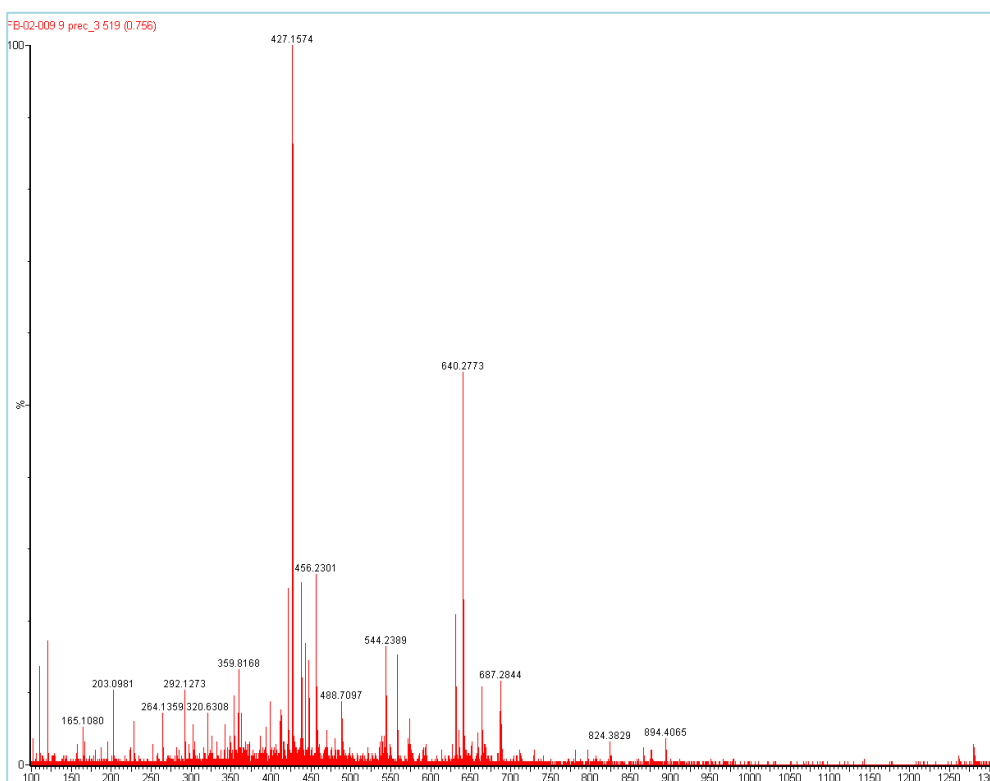
Compound 4.2



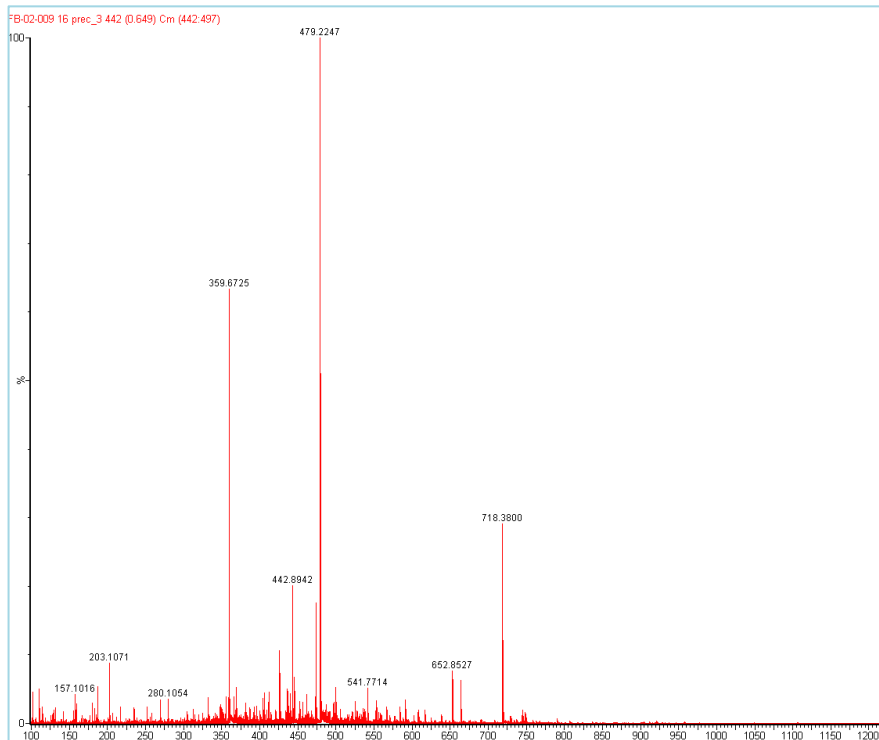
Compound 4.3



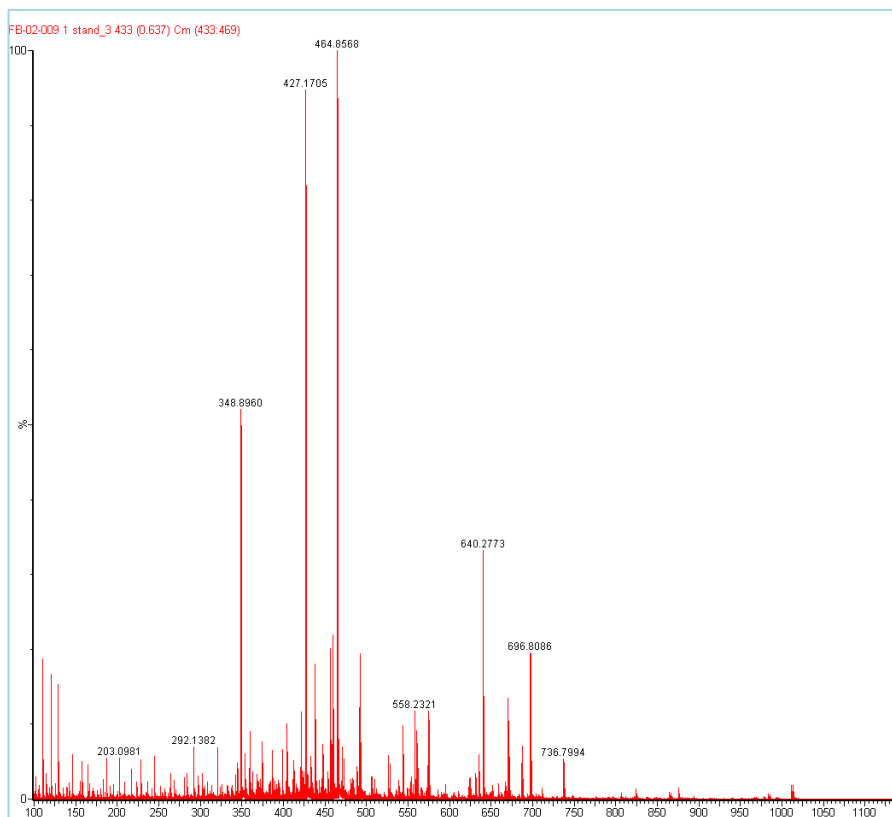
Compound 4.4



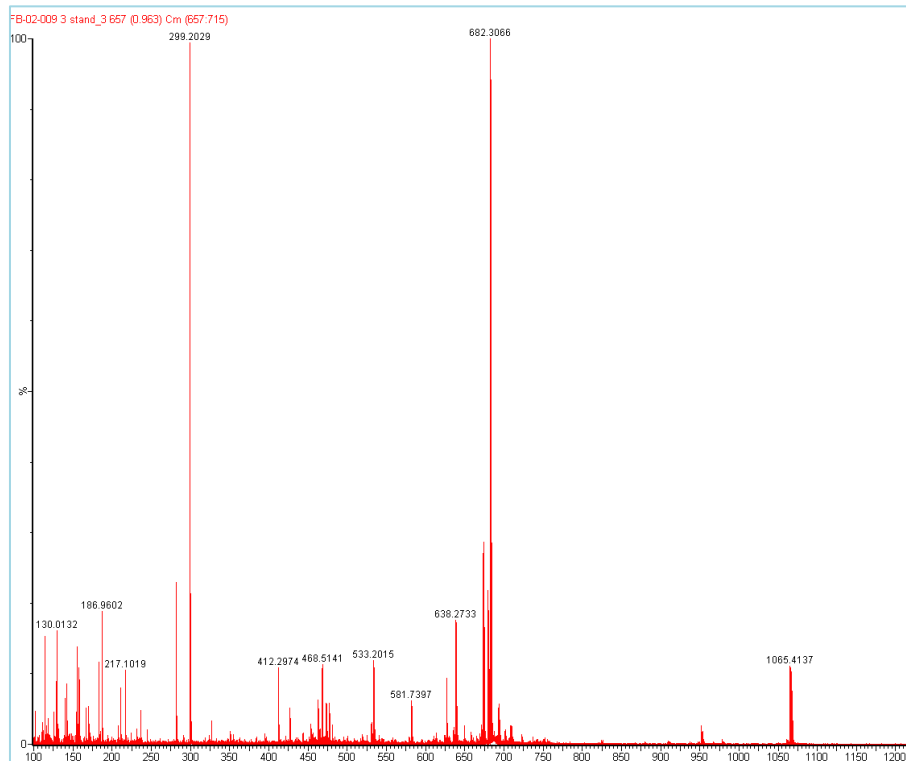
Compound 4.5



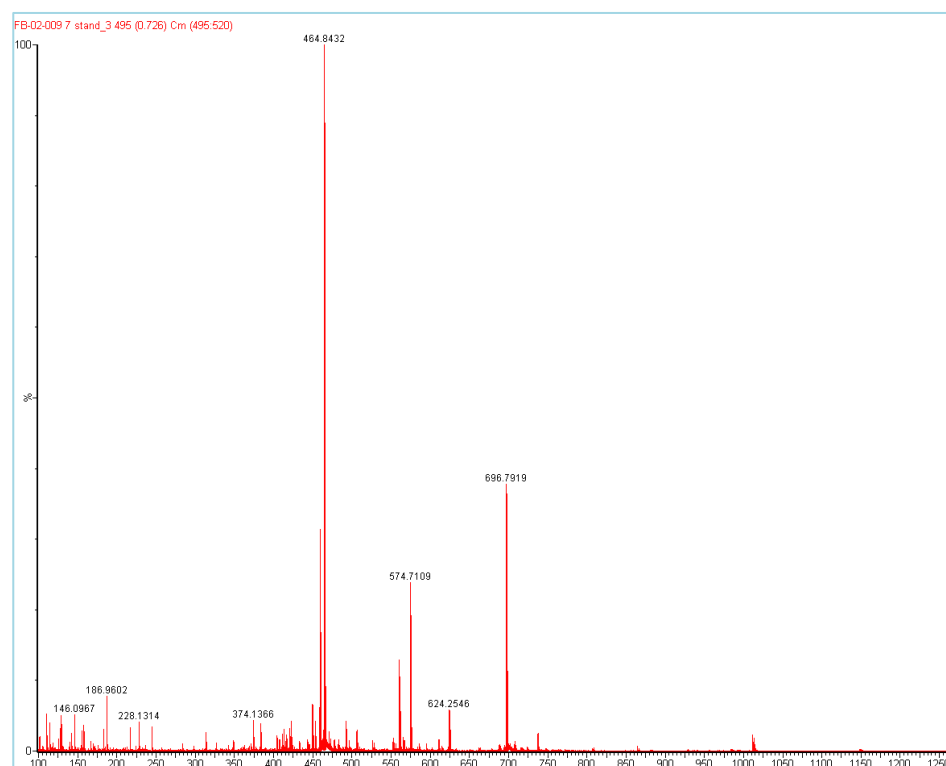
Compound 4.6

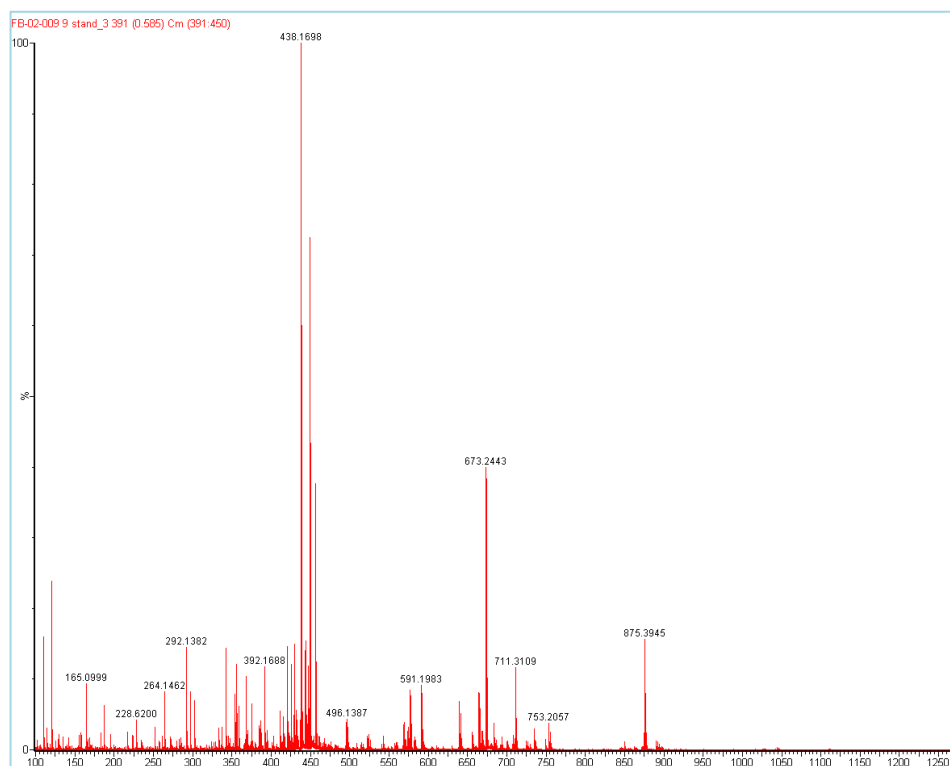
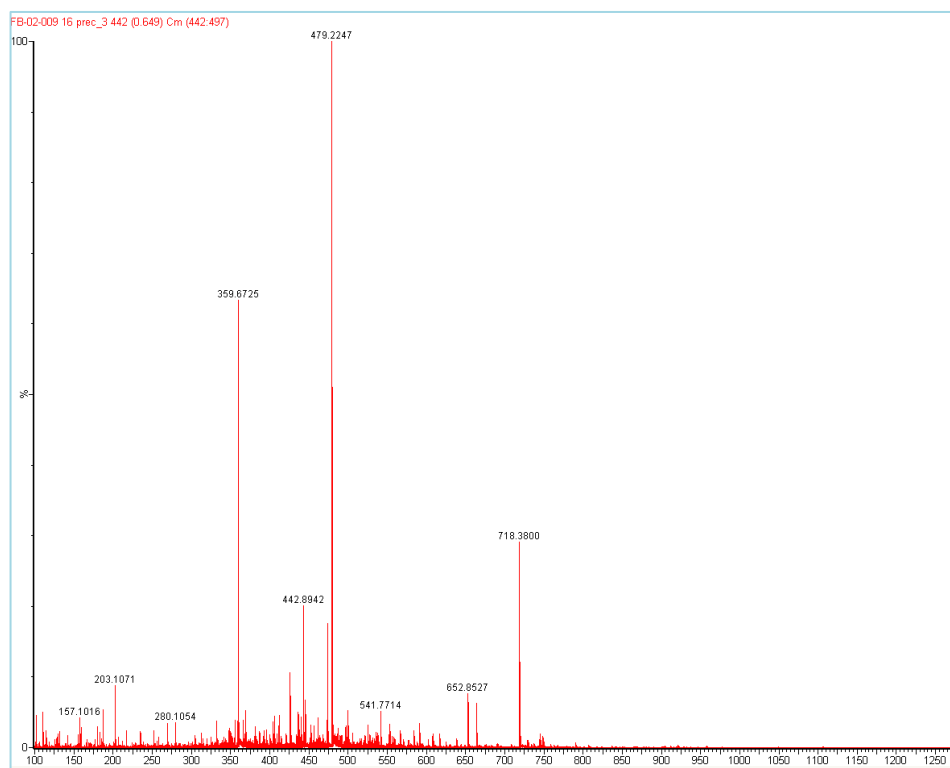


Compound 4.7



Compound 4.8

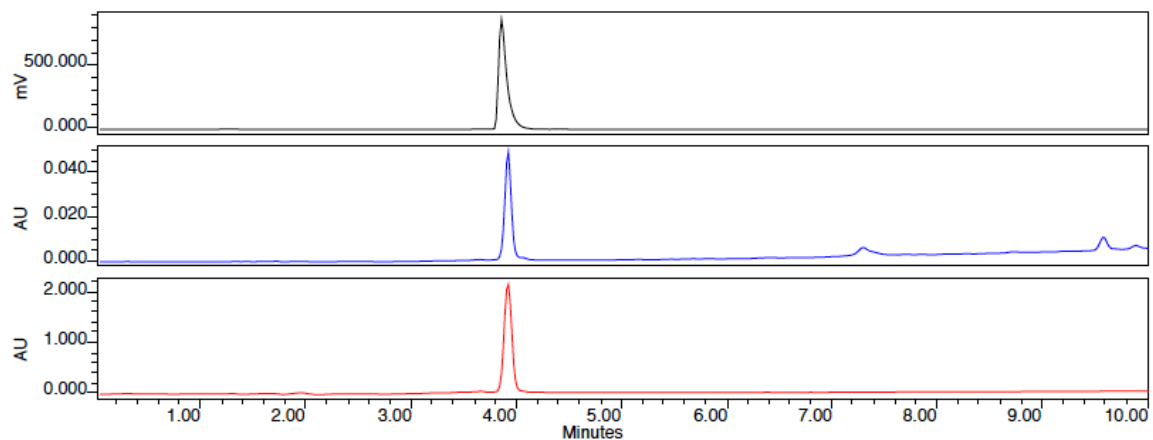


Compound 4.9**Compound 4.10**

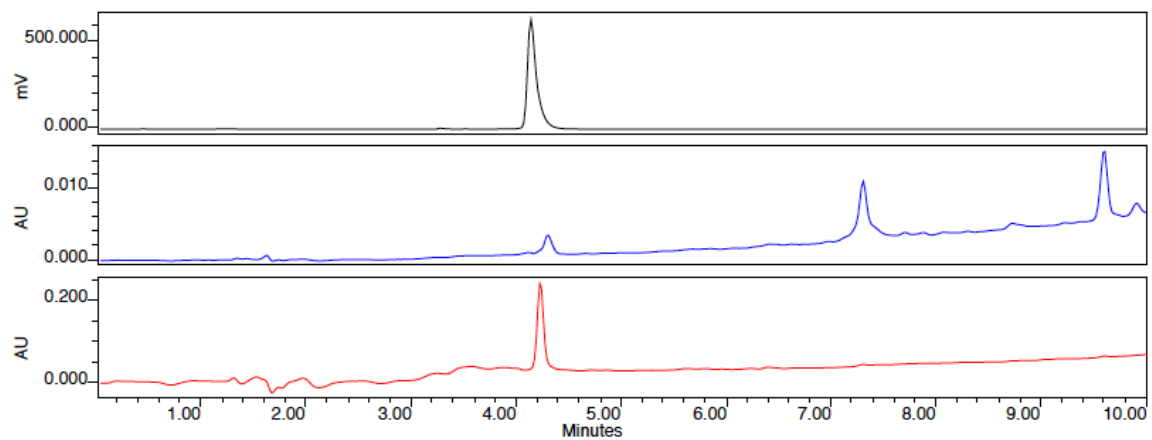
^{68}Ga RADIOLABELING

Black: satin; Blue: UV 254 nm; Red: UV 220 nm.

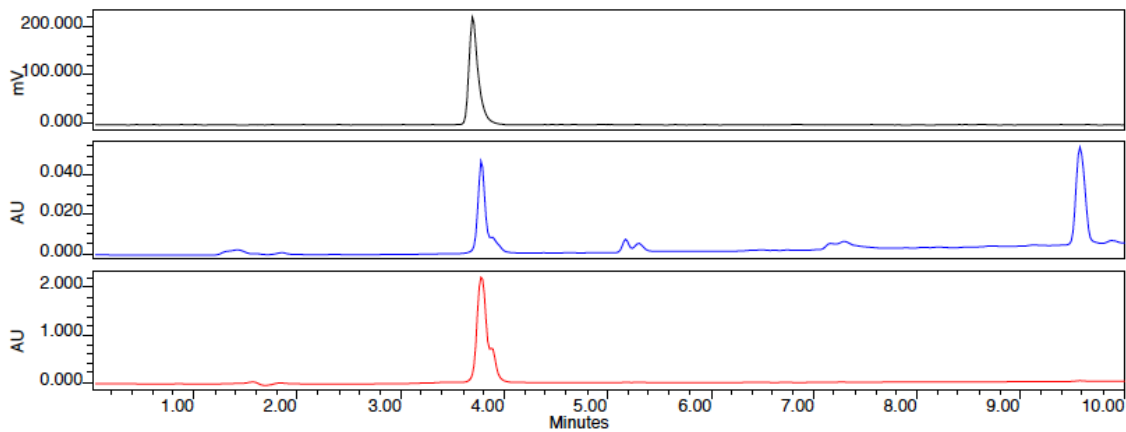
Co-injection of radiolabeled compound **4.6*** with its cold labeled form **4.6**.



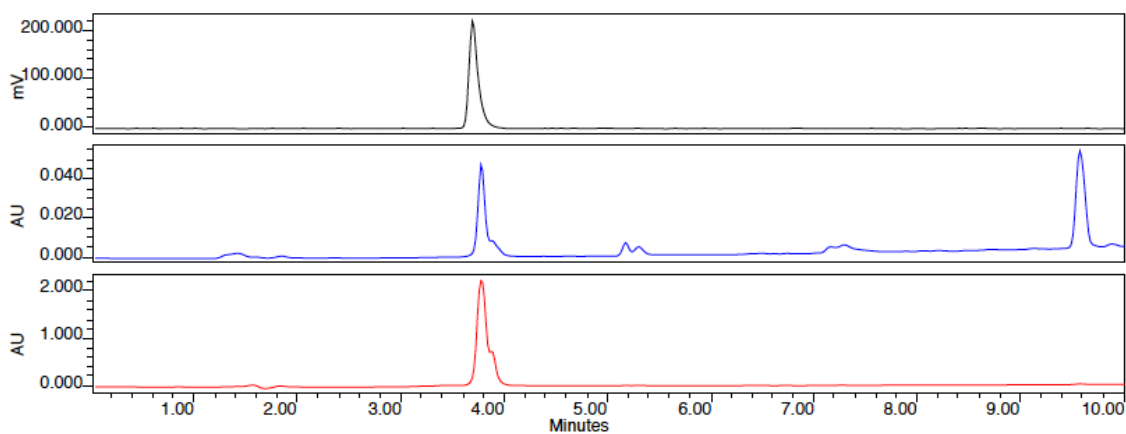
Co-injection of radiolabeled compound **4.7*** with its cold labeled form **4.7**



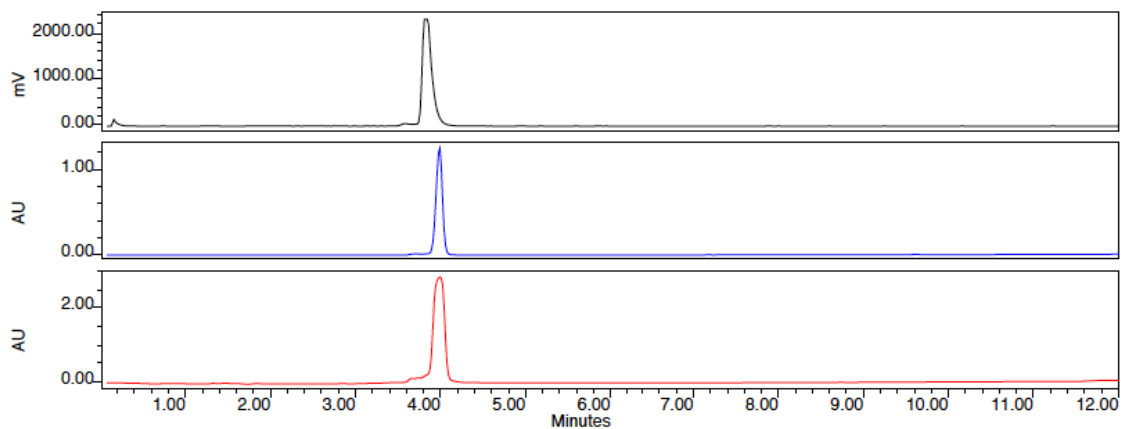
Co-injection of radiolabeled compound **4.8*** with its cold labeled form **4.8**



Co-injection of radiolabeled compound **4.9*** with its cold labeled form **4.9**



Co-injection of radiolabeled compound **4.10*** with its cold labeled form **4.10**



Curriculum Vitae

Fernanda Cristina Bononi

Education

2012 – 2014 - Western University.

- Master of Science (M.Sc.) degree in Chemistry/Molecular Imaging

2005 – 2009 - Universidade de São Paulo, Brazil.

- Bachelor of Science, Pharmacy and Biochemistry.
- Thesis: A comparative study between Mikania glomerata and Mikania laevigata with emphasis on coumarin and kaurenoic acid concentrations and the bronchodilator effects on rat and guinea pig trachea.

Research Experience

03/2011 – 06/2012 - University of California, Davis.

- Junior Specialist. Combinatorial chemistry, drug design. Dr. Kit S. Lam. Department of Biochemistry and Molecular Medicine.

01/2011 – 03/2011 – University of California, Davis.

- Extended Training. Design, chemical synthesis, molecular modification and characterization. Dr. Heike Wulff. Department of Pharmacology.

03/2007 – 07/2009 – Universidade de São Paulo

- Extracurricular Training Internship. Chromatographic analysis of plant composition, respiratory pharmacology, cardiovascular pharmacology, natural product chemistry. Dr. Ana Maria de Oliveira and Dr. Fernando Batista da Costa. Department of Physics and Chemistry.

Publications

Journals

- Xiao*, W., Bononi*, F.C., Townsend, J., Li, Y., Liu, R., Lam, K.S. (2013). Immobilized OBOC combinatorial bead array to facilitate multiplicative screening. *Comb Chem High Throughput Screen*, 16, 441-448 (2013).
- Silva, C. H. T. P., Silva, V. B., Resende, J., Rodrigues, P. F., Bononi, F. C., Benevenuto, C. G., Taft, C. A. Computer-aided drug design and ADMET predictions for identification and evaluation of novel potential farnesyltransferase inhibitors in cancer therapy. *J Mol Graph Model*, 28, 513-523 (2010).

Chapters

- Bononi, F.C., Luyt, L.G. (2014). Synthesis and cell-based screening of one-bead one-compound peptide libraries. *Methods in Molecular Biology*. Submitted.

Other Publications

- Liu, R., Xiao, W.; Tseng, H.; Liu, Y. ; Sanchez, E. ; Mazloom, A. ; Lin, J. ; Bononi, F. C. ; Lam, K. S. (2011). Optimization of glioblastoma-targeting peptide ligand LXY1 using one-bead one-compound focused library approach.

Proceedings of the 22nd American Peptide Symposium, American Peptide Society. P. 388-389.

Abstracts

- Bononi, F.C., Luyt, L.G. Development of gallium-containing OBOC peptide libraries for the discovery of new molecular imaging probes". *97th Canadian Chemistry Society Conference*, Vancouver, BC, Canada. [Oral Presentation].
- Simpson, E.J., Gobbo, P., Bononi, F., Workentin, M.S., Luyt, L.G. Bombesin-functionalized water-soluble gold nanoparticles for prostate cancer imaging. *97th Canadian Chemistry Society Conference*, Vancouver, BC, Canada.
- Bononi, F.C., Luyt, L.G. (2013). Development of a gallium-containing OBOC peptide library for use in molecular imaging. Sao Paulo Advanced School on Bioorganic Chemistry (ESPCA), Araraquara, SP, Brazil, June 30th , July 5th.
- Liu, R., Xiao, W., Bononi, F.C., Sanchez, E., Liu, Y., Mazloom, A., Tseng, H., Lam, K.S. (2012). Development of a highly potent and non-invasive optical imaging agent for glioblastoma. 103rd Annual Meeting of American Association for Cancer Research (AACR), Chicago, IL, March 31st-April 4th.
- Liu, R., Xiao, W., Bononi, F.C., Sanchez, E., Liu, Y., Mazloom, A., Tseng, H., Lam, K.S. (2011). Optimization and SAR study of Glioblastoma-targeting Peptide Ligand LXY1. 16th Annual Cancer Research Symposium, UC Davis Cancer Center, Sacramento, CA, October, 11th.
- Bononi, F.C., Oliveira, A.M. (2009). A comparative study between *Mikania glomerata* and *Mikania laevigata* with emphasis on coumarin and kaurenoic acid concentrations and the bronchodilator effects on rat and guinea pig trachea. 17^o Simpósio Internacional de Iniciação Científica (SIICUSP), Ribeirão Preto, São Paulo, Brazil.

Teaching Experience

09/2012 – 05/2014 – Western University

- Organic Chemistry for Life Sciences (2213a).
- Organic Chemistry of Biological Molecules (2223b).

Honours and Awards

06/2014 – Oral Presentation Award – *97th Canadian Chemistry Society Conference*,
Vancouver, BC, Canada

09/2012 – 09/2014 – Western Graduate Research Scholarship – Chemistry

06/2007 – 06/2008 – PIBIc Fellowship for Brazilian Undergraduate Students