Fluorine-18 labeling and simultaneous glycosylation of the model peptide demobesin 1 by the novel prosthetic group, keto-[^18F]FDG.

Dissertation zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Nepal

Bonn, Februar 2014
Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinische Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 07.07.2014
Erscheinungsjahr: 2014
Acknowledgement

I like to acknowledge my mother Mrs. Rajeshwori Malla Thakuri for being the foremost teacher and showing me the right path of moral ethics. I like to remember Prof. Purusottam Basnet and Prof. Natasha Skalko Basnet who motivated me to involve in the field of research. I equally like to remember Prof. Eung-Seok Lee for letting me to start my research career in his group.

I am grateful to PD Dr. rer. nat. Stefan Guhlke and Prof. Dr. med. Hans-Jürgen Biersack for introducing me in the field of nuclear medicine. Equally I am thankful to Dr. rer. nat. Stefan Kürpig for his support from the beginning to the end of my study.

I am thankful to the chemistry department of the University of Bonn for spectroscopic measurements of NMR (Nuclear Magnetic Resonance) and Mass Spectroscopy. I like to appreciate especially Dr. rer. nat. Marianne Engeser for her valuable suggestions and measurements of mass spectrometry.

Similarly, I am thankful to the medical PET centre of our department (Nuclear Medicine, University Hospital of Bonn) for providing me the radiotracer $^{[18\text{F}]}$FDG for my research work.

Last but not the least, I like to acknowledge all my family members in Nepal especially my beloved wife Deepa Thapa Magar for understanding me in my absence.
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1 Introduction

Radioactivity is a naturally occurring process of transforming unstable higher atomic number element into more stable atoms. This activity of the atom was first noticed by Henri Becquerel\(^1\) in 1896 in uranium and is called as radioactivity later by Madam Marie Curie\(^2\). Henri Becquerel, Marie Curie and Pierre Curie jointly shared the Nobel Prize for physics in 1903\(^3\) for their work in the field of radioactivity. Radioactive elements decay by emission of alpha (\(\alpha\)), beta (\(\beta\)) or gamma (\(\gamma\)) rays. These effects can be used in nuclear medicine for therapeutic and diagnostic purposes\(^4,5\).

The phenomenon that some radioactive elements can be produced artificially by the bombardment of alpha particles was first observed by Nobel laureate Frederic and Irene Joliot Curie. Later on neutrons were also used for this purpose by Enrico Fermi\(^6\).

The first applications of radioactivity as radioactive tracers in biological studies were performed by the hungarian physician George Hevesy in 1923 and his continuation in this field was honored with the Nobel Prize in 1943\(^7\).

Another important milestone which boosted nuclear medicine was the invention of the cyclotron by another Nobel laureate Ernest Orlando Lawrence in 1932. Since then many artificial radionuclides were produced and many of these isotopes have been successfully used in medical imaging and therapy\(^8\).
Radionuclides or radioisotopes used in nuclear medicine are referred as radiopharmaceuticals which can be either the radionuclide alone (such as $^{131}$I for therapy of thyroid cancer) or in majority of cases attached to a carrier molecule$^9$. The carrier molecules in nuclear medicine are often targeted to specific organs, receptors, enzymes (or as analogues of specific molecules of diseases or pathways). These carrier molecules are biologically active compounds which may be small molecules, antibodies, antibody fragments, peptides or proteins$^9$. In the near future, with the development of pharmaceutical nanotechnology, list of carrier molecules could be explained by liposomes, nanoparticles, micelles and hydrogels$^{10}$. The carrier molecules like antibodies or proteins with higher molecular weight possess often slow elimination from the body and a slow rate of diffusion. These characteristics might prevent them from being ideal radioligand for imaging or therapy$^{11,12,13}$.

Simply, smaller the size of molecules, more desirable the pharmacokinetic properties such as high target to background ratios in imaging, faster blood clearance and target specific delivery. With the development of synthetic peptide chemistry, peptides as well as radionuclide binding group could be engineered to provide the desired pharmacokinetic properties and efficient binding of desired radionuclide$^{14,15}$. The discovery of new peptide receptors and their function in the biological system combined with the development of synthetic peptide synthesis has made nuclear medicine more successful and effective in diagnostic and therapy compared to other fields of medicine. For instance the receptors of regulatory peptides are overexpressed in some human cancers$^{16}$. Such receptors e.g. somatostatin receptors, vasoactive intestinal peptive (VIP) receptors, cholecystokinin receptors,
bombesin receptors, neurotensin receptors, etc. can be targeted by radiolabeling of specific peptides (natural or analogues) which are specific for these receptors either diagnostic or thereapeutic purposes.

The amount of peptides used for targeting and infact must be very low due to low amount of receptors available. In ideal cases it ranges from micro to picogram levels, hence they do not lead to any physiological, toxicological or immunological response or any side effects.

Radioactive Tracer Studies

![Diagram of radioactive tracer studies]

Figure 1: Multiple approaches to measurements using radiotracers in medicine.
1.1 Nuclear medicine

Nuclear medicine technology is one of the top engineering achievements of the 20\textsuperscript{th} century\textsuperscript{19}. Nuclear medicine is basically known for the use of radiopharmaceuticals for diagnosis and therapy of diseases. It is broadly divided into two classes as:

1.1.1 Nuclear Medicine Therapy (Therapeutic)

1.1.2 Nuclear Medicine Imaging (Diagnostic)

1.1.1 Nuclear medicine therapy

The radionuclides used in radiotherapy are beta (\(\beta\))-emitters, alpha (\(\alpha\))-emitters or auger electrons. Some examples of radiotherapy include\textsuperscript{4}.

i. Brachytherapy (radiation sources implanted into or beside the tumour) for accessible tumours such as those of cervix, prostate, head and neck, breast, bladder, lung and esophagus.

ii. Systemic usually intravenous (i.v). injected radionuclides for selective uptake by tumours. For e.g. \(^{131}\text{I}\) in thyroid cancer, radiolabeled or \(^{111}\text{In}/^{177}\text{Lu}\) labeled somatostatin analogues for neuroendocrine tumours.

iii. Radioimmunotherapy (conjugation of radionuclides to specific antibodies for targeted radiotherapy to tumours containing cells expressing the relevant antigens or receptors.)

The energy of the radionuclides is deposited in the tumour tissue to damage either the cells or the DNA of the diseased cells\textsuperscript{20}.
The commonly used radionuclides in the nuclear medicine therapy are listed in table 1.

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<td>$^{166}$Ho</td>
<td>26.9 h</td>
<td>$^{165}$Ho(n,γ)$^{166}$Ho</td>
<td>β-particle</td>
<td>1853</td>
</tr>
<tr>
<td>$^{188}$Re</td>
<td>17 h</td>
<td>$^{186}$W/$^{188}$Re-generator</td>
<td>β-particle</td>
<td>2110</td>
</tr>
<tr>
<td>$^{89}$Sr</td>
<td>52.7 d</td>
<td>$^{88}$Sr(n,γ)$^{89}$Sr</td>
<td>β-particle</td>
<td>1463</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>14.3 d</td>
<td>$^{32}$P(n, p)$^{32}$P or $^{31}$P(n, γ)$^{32}$P</td>
<td>β-particle</td>
<td>1710</td>
</tr>
<tr>
<td>$^{153}$Sm</td>
<td>46.3 h</td>
<td>$^{152}$Sm(n,γ)$^{153}$Sm</td>
<td>β-particle</td>
<td>810</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>64.1 h</td>
<td>$^{90}$Sr/$^{90}$Y-generator</td>
<td>β-particle</td>
<td>2280</td>
</tr>
<tr>
<td>$^{225}$Ac</td>
<td>10 d</td>
<td>$^{225}$Ra/$^{225}$Ac-generator $^{226}$Ra(p,2n)$^{225}$Ac</td>
<td>4α-particle</td>
<td>~28000</td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>7.2 h</td>
<td>$^{209}$Bi(α,2n)$^{211}$At</td>
<td>α-particle</td>
<td>5870</td>
</tr>
<tr>
<td>$^{213}$Bi</td>
<td>45.6 min</td>
<td>$^{225}$Ac/$^{213}$Bi generator</td>
<td>α-particle</td>
<td>8500</td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>6.7 d</td>
<td>$^{176}$Lu(n,γ)$^{177}$Lu</td>
<td>β-particle</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 1: Commonly used radionuclides in therapy
1.1.2 Nuclear medicine imaging

Images are acquired by detecting the gamma rays emitted by the decay of radioisotopes bound to molecules of known biological properties which are specific to particular sites/receptors and carry the radioisotopes to that particular sites/receptors\textsuperscript{29}. Imaging in nuclear medicine is a noninvasive technology and can visualize the biological process at molecular or cellular levels\textsuperscript{30}. This imaging gives the functional image of the organ/sites which can be used for monitoring of therapies and diagnosis of diseases\textsuperscript{31}. In nuclear medicine, two tomographic methods described below are widely used for imaging.

i. Single photon emission computer tomography (SPECT)

ii. Positron emission tomography (PET)
i. Single photon emission computer tomography (SPECT)

SPECT radionuclides directly emit the desired gamma rays and are generally of relative low energy (100-350 keV). The relatively lower costs of gamma cameras compared to PET had made SPECT imaging more widely available for clinical use than PET scanners. The commonly used SPECT radionuclides are listed in table 2.

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Half-life</th>
<th>Production</th>
<th>Maximum Energy [keV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{123}$I</td>
<td>13.2 h</td>
<td>$^{124}$Xe (p,2n)$^{123}$I</td>
<td>159</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>6 h</td>
<td>$^{99}$Mo/$^{99m}$Tc-generator</td>
<td>140</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>67.9 h</td>
<td>$^{111}$Cd(p,n)$^{111}$In</td>
<td>171/245</td>
</tr>
<tr>
<td>$^{67}$Ga</td>
<td>78.3 h</td>
<td>$^{68}$Zn(p,n)$^{67}$Ga</td>
<td>93/184/300/394</td>
</tr>
<tr>
<td>$^{201}$Tl</td>
<td>73 h</td>
<td>$^{203}$Tl(p,3n)$^{201}$Pb = $^{201}$Tl</td>
<td>137/167</td>
</tr>
</tbody>
</table>

Table 2: Commonly used radionuclides for SPECT imaging.

The most commonly used radionuclide in medical imaging is technetium-99m ($^{99m}$Tc) and reign over 80% of diagnostic radiopharmaceuticals currently in use due to its high-efficiency detection as well as a low radiation exposure to the patients i.e. its pure gamma emission without particle (alpha or beta particle). emission and relative short half life.
ii. Positron emission tomography (PET)

The basic principle of PET is that, the emitted positron from radionuclides combines with a negatron i.e. electron and annihilate in two gamma rays of 511 keV which travels at 180° to each other. These gamma rays pass through the body tissue and can be detected in special camera which consists of lead collimators (which ensure that all detected photons propagated along parallel paths), a crystal scintillator (converts high-energy photon to visible light). The photo-multiplier tubes processing the visible light and associated electronic devices calculates the position of each incident photon from the light distribution in crystal. Higher the energy of the emitted positron, further the travel of a positron before annihilation process and lower its spatial resolution²⁹,³⁹.

Figure 2: Basic principle of PET⁴⁰
Flourine-18 is an ideal radionuclide for PET because of\textsuperscript{30,41}.

i. Low positron energy (635 KeV) compared to other PET radioisotopes, hence low radiation dose to patients and short range in tissue which ultimately leads to highest resolution

ii. High percentage of positron (\(\beta^+\)) emission (97 \%)

iii. Half life of 110 minutes, hence syntheses and imaging procedures can be extended over hours facilitating kinetic studies but still short with respect to radiation burden to the patients.

iv. Small atom size and ability to stable bonds with carbon i.e. mimicking protons and hydroxyl groups.

The commonly used radionuclides used in PET imaging are listed in table 1.

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Half-life</th>
<th>Production</th>
<th>Maximum Energy [keV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{11})C</td>
<td>20.4 mins</td>
<td>(^{14})N(p, (\alpha))(^{11})C</td>
<td>960</td>
</tr>
<tr>
<td>(^{13})N</td>
<td>10 mins</td>
<td>(^{16})O(p, (\alpha))(^{13})N</td>
<td>1190</td>
</tr>
<tr>
<td>(^{15})O</td>
<td>2 mins</td>
<td>(^{14})N(d, n)(^{15})O</td>
<td>1720</td>
</tr>
<tr>
<td>(^{18})F</td>
<td>109 mins</td>
<td>(^{18})O(p, n)(^{18})F</td>
<td>635</td>
</tr>
<tr>
<td>(^{68})Ga</td>
<td>68 mins</td>
<td>(^{68})Ge/(^{68})Ga generator</td>
<td>1920</td>
</tr>
</tbody>
</table>

Table 3: Commonly used radionuclides for PET imaging\textsuperscript{35,42-45}

The functional images of PET and SPECT can be fused with anatomical image of computer tomography (CT) or magnetic resonance imaging (MRI) for precise anatomical localization of radioactivity in the body. Thus hybrid imaging technique (PET/CT; SPECT/CT; PET/MRI and SPECT/MRI) were developed to obtain both anatomical and functional information in a single scanning session\textsuperscript{46-50}. 
1.2 Labeling with radionuclides

Basically there are two types of radionuclides radiometals and radiohalogens. Depending on their nature there are various approaches for radiolabeling\textsuperscript{41,43}.

1.2.1 Labeling using radiometal

Radiometals (Technetium, Lutetium, gallium, rhenium, indium etc) can be bound to a plethora of chelators like DOTA, HEDP, DTPA, etc. The biomolecules are usually previously linked with these chelators and finally labelled with the radiometal called as post conjugate labeling approach. However, pre conjugate labeling where chelator is labeled first and then linked to the biomolecules also exists.

1.2.2 Labeling using radiohalogens

Although radiohalogens are only five in number in the periodic chart of elements, their number of isotopes exceed a hundred.

Radiohalogen nuclides with an atomic mass lower than their stable isotope are primarily produced by bombardment with protons (p,n reactions), deuterons (d,2n reactions) or alpha particles (\(\alpha\),2n reactions) on a target material which is one or two elements left to the halogen group i.e. group 6a and group 5a respectively. Radiohalogens produced by these routes are neutron deficient and decay by emission of photons (from internal conversion), by electron capture (EC) or positron emission.

In contrast, radiohalogens nuclides with an atomic mass higher than the stable isotope are primarily produced by irradiation of the stable halogen with neutrons in a
nuclear reactor and lead to neutron rich radiohalogens that often decay by $\beta^-$ emission.

Radiohalogens are labeled to biomolecules in a similar manner as fluorine-18 which is described in 1.3.

<table>
<thead>
<tr>
<th>Radiohalogen</th>
<th>Half life</th>
<th>Emission</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}\text{F}$</td>
<td>110 min</td>
<td>$\beta^+$</td>
<td>PET Imaging</td>
</tr>
<tr>
<td>$^{122}\text{I}$</td>
<td>3.6 min</td>
<td>$\beta^+$</td>
<td>PET Imaging</td>
</tr>
<tr>
<td>$^{123}\text{I}$</td>
<td>13.2 h</td>
<td>$\gamma$</td>
<td>SPECT Imaging</td>
</tr>
<tr>
<td>$^{124}\text{I}$</td>
<td>4.18 d</td>
<td>$\beta^+$</td>
<td>PET Imaging</td>
</tr>
<tr>
<td>$^{125}\text{I}$</td>
<td>59.4 d</td>
<td>Auger e$^-$</td>
<td>Therapy</td>
</tr>
<tr>
<td>$^{131}\text{I}$</td>
<td>8.02 d</td>
<td>$\beta/\gamma$</td>
<td>Therapy/SPECT Imaging</td>
</tr>
<tr>
<td>$^{75}\text{Br}$</td>
<td>97 min</td>
<td>$\beta^+$</td>
<td>PET Imaging</td>
</tr>
<tr>
<td>$^{76}\text{Br}$</td>
<td>16.2 h</td>
<td>$\beta^+$</td>
<td>PET Imaging</td>
</tr>
<tr>
<td>$^{77}\text{Br}$</td>
<td>57 h</td>
<td>Auger e$^-$</td>
<td>Therapy</td>
</tr>
<tr>
<td>$^{211}\text{At}$</td>
<td>7.21 h</td>
<td>$\alpha$</td>
<td>Therapy</td>
</tr>
</tbody>
</table>

Table 4: Commonly used radiohalogens used in imaging and therapy$^{42,51,53}$. 

1.3 Fluorine-18 labeling

The radiolabeling of fluorine-18 to aliphatic or aromatic organic structures either by electrophilic or nucleophilic substitutions for the development of radiotracer for PET is described below.

1.3.1 Electrophilic substitution labeling with $[^{18}\text{F}]\text{F}_2$

The carrier added or molecular fluorine-18 i.e. $[^{18}\text{F}]\text{F}_2$ is used in this type of reaction. The $^{20}\text{Ne} (d, \alpha)^{18}\text{F}$ nuclear reaction is the most common and well established method for the production of carrier added fluorine-18. The $[^{18}\text{F}]\text{F}_2$ is highly reactive and also involved in oxidation as well as in free radical reactions, hence it is non selective in labeling. Therefore, developing milder and more regioselective agents are desired for electrophilic labeling (e.g. Xe$[^{18}\text{F}]\text{F}_2^{52,53}$, $[^{18}\text{F}]\text{AcOF}^{54}$). Carrier free fluorine-18 diffuses to the target wall and is chemically adsorbed. Hence, stable fluorine-19 is added as carrier to compete with the surface adsorption and recovery of fluorine-18 from the target$^{55,56}$ Thus produced $[^{18}\text{F}]\text{F}_2$ molecule carries one fluorine-18 and one fluorine-19 atom, hence theoretically maximum RCY in this type of reaction of radioactive fluorine-18 is limited upto 50%$^{57}$. The $6-[^{18}\text{F}]\text{fluoro-L-Dopa}$ is one of the routine radiopharmaceutical produced via this type of labeling$^{58}$.

![Scheme 1: Electrophilic radiofluorination](image)

Phe: $R_1 = R_2 = \text{H}$  
Tyr: $R_1 = \text{H}; R_2 = \text{OH}$  
Dopa: $R_1 = R_2 = \text{OH}$
1.3.2 Nucleophilic substitution labeling with $[^{18}\text{F}]$fluoride

The no-carrier-added (n.c.a.) $[^{18}\text{F}]$fluoride ion is used in this type of reactions. The $^{18}\text{O}$ (p, n) $^{18}\text{F}$ nuclear reaction is the common and well established method for the production of n.c.a. $[^{18}\text{F}]$fluoride. In this nuclear reaction heavy water $\text{H}_2\text{O}^{18}$ is irradiated with protons to produce the $[^{18}\text{F}]$fluoride. The expensive heavy water $\text{H}_2\text{O}^{18}$ is recovered commonly by adsorption of $[^{18}\text{F}]$fluoride in an anion exchange resin\textsuperscript{59} for reuse. Fluoride-18 ions are eluted from the cartridge with a small volume of aqueous weak base such as $\text{CO}_3^{2-}$, $\text{HCO}_3^{-}$ or $\text{C}_2\text{O}_4^{2-}$ to avoid protonation of $[^{18}\text{F}]$fluoride ion. The water can be removed by azeotropic evaporation with acetonitrile. $[^{18}\text{F}]$fluoride ions are then solubilised in polar aprotic solvents with the addition of kryptands or large cations (e.g. Cs$^+$, kryptofix 2.22, $\text{Et}_4\text{N}^+$, $\text{tBu}_4\text{N}^+$)$^{40}$. The $[^{18}\text{F}]\text{F}^-$ anion is then called “naked” because of the relative high distance to its positive counterion and as such highly reactive in nucleophilic substitution reactions. The most common example for this kind of nucleophilic substitution reactions is in the synthesis of $[^{18}\text{F}]$FDG (see scheme 2).

\[ \text{OAc} \text{OAc} \text{OTf} \rightarrow \begin{array}{c} \text{OAc} \text{OAc} \text{OH} \\ \text{OH} \text{OH} \text{^{18}F} \text{OH} \end{array} \begin{array}{c} \text{^{18}F} \text{FDG} \\ \text{H}^+ \end{array} \]

\text{Scheme 2: Nucleophilic substitution of fluorine-18$^{115}$}
1.3.3 Fluorine-18 labeling synthons for built up radiosyntheses

Synthons for built up radiosyntheses are also used for $[^{18}\text{F}]$fluoride labeling. It is recently categorized as traditional (radiofluorination on non hypervalent compounds) and more recent approaches (radiofluorination in hypervalent compounds e.g. iodonium, sulfoxides or sulfonium)\textsuperscript{60} (see scheme 3).

Scheme 3 : Approaches of fluorine-18 labeling\textsuperscript{60}
i. Traditional approaches

a. Balz-schiemann and wallach reactions

These approaches are now rarely used due to low radiochemical yield, more isomers and side products. Here, n.c.a. fluorine-18 is directly labeled to the aromatic ring via Balz-Schiemann reactions (e.g. fluorine-18 labeled aromatic amino acids\(^{61,62}\)) or Wallach reactions (e.g. 5-[\(^{18}\)F]fluoro-L-Dopa\(^{63}\), [\(^{18}\)F]haloperidol\(^{64}\)). Both are dediazoniation reactions and diazonium functions were replaced with fluoride in presence of heat/acid. The reaction mechanisms of both reactions is first order nucleophilic substitution reaction type (S\(\text{N}1\) type), where reactive cations are substituted by nucleophilic fluorine-18 (see scheme 3).

b. Aromatic nucleophilic substitution (S\(\text{N}\)Ar)

It is the most widely established method for labeling of fluorine-18 to aromatic ring with high specific activity. In this reaction leaving groups (e. g. NO\(_2\), Cl, Br, I or \(^+\)NMe\(_3\)) are attached to the aromatic ring (in ortho or para position) which are activated with different electron withdrawing groups (EWG) (e. g. NO\(_2\), CN, CHO, COOMe and COOH)\(^{65-67}\) (see scheme 3).

ii. More recent approaches

This includes the radiofluorination to hypervalent compounds of iodonium, sulfoxides or sulfonium salts. Here it is not mandatory for the aromatic ring to carry an EWG and leaving groups. In this reactions both nonactivated and deactivated aryl rings can be labeled with fluorine-18\(^{60,67-69}\) (see scheme 3).
1.3.4 Other fluorine-18 labeling methods

There are many other methods for fluorine-18 labeling as enzymatic fluorine-18 labeling (e.g. 5'-[18F]FDA (5'-fluoro-5'-deoxyadenosine))\(^70\), isotopic fluorine-18 for fluorine-19 exchange at a silicon atom\(^71\), chelation of aluminium fluorine-18 by NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid)\(^72\), microwave enhanced fluorine-18 labeling\(^73\), polymer supported radiofluorination\(^74\) or click chemistry\(^75,76\).

**Scheme 4**: Enzymatic fluorine-18 labeling\(^70\)

**Scheme 5**: Isotopic exchange of fluorine-18 for fluorine-19\(^71\)

**Scheme 6**: Chelation of aluminium fluorine-18 by NOTA\(^72\).
1.4 Fluorine-18 labeling of biomolecules via prosthetic groups

This topic can be broadly divided into two groups as follows:

1.4.1 Fluorine-18 labeling of small monomeric molecules

1.4.2 Fluorine-18 labeling of proteins and peptides

1.4.1 Fluorine-18 labeling of small monomeric molecules

The fluorine-18 labeled aryl fluorides derived from n.c.a. $[^{18}\text{F}]$fluoride were used as labeling precursors for labeling various biomolecules via different chemical reactions such as reductive aminations, carbonyl-olefinations, nucleophilic additions, heteroatom alkylation, palladium mediated C-C and C-N bond formations, etc. The majority of fluorine-18 labeling of small molecules were contributed by $[^{18}\text{F}]$fluorobenzaldehydes, $[^{18}\text{F}]$fluorobenzyl halides and $[^{18}\text{F}]$fluorohalobenzenes whereas $[^{18}\text{F}]$fluorobenzylamines, $[^{18}\text{F}]$fluoroanilines, $[^{18}\text{F}]$fluorophenol and $[^{18}\text{F}]$fluoroacetophenone were utilized to much lower extent\textsuperscript{77}. 


Figure 3: Fluorine-18 labeled aryl fluorides
1.4.2 Fluorine-18 labeling of peptides and proteins

In the case of fluorine-18 labeling of peptides and proteins neither direct n.c.a. added nucleophilic substitution labeling nor direct electrophilic substitution labeling with $[^{18}\text{F}]\text{F}_2$ is possible due to following reasons:

i. In case of direct n.c.a. nucleophilic substitution labeling via $[^{18}\text{F}]\text{F}^-$

The peptides and proteins possess acidic functionalities such as hydroxyls, amides, alcohols, amines or thiols. The n.c.a. nucleophilic substitution reactions for labeling with $[^{18}\text{F}]\text{F}^-$ occur in basic conditions. Hence, the basic conditions of the reactions easily deprotonates the acidic functionalities of peptides and proteins which in turn portonates the fluorine-18 making it unavialble for further reactions. Therefore, n.c.a. nucleophilic substitution reaction for labeling with fluorine-18 is inappropriate.

ii. In case of direct electrophilic substitution labeling with fluorine-18

Electrophilic fluorination is only possible with the carrier-added (c.a.) $[^{18}\text{F}]\text{F}_2$ form. To obtain the molecular fluorine-18 ($[^{18}\text{F}]\text{F}_2$), stable carrier molecule i.e. fluorine-19 is used in the production process which ultimately decreases the specific activity of labeled products. Hence, electrophilic substitution labeling with fluorine-18 is also not suitable for fluorine-18 labeling. In addition, $[^{18}\text{F}]\text{F}_2$ extremely reactive reagents and thus of low regioselectivity.

Hence, both n.c.a nucleophilic and electrophilic substitution for direct labeling are not suitable for peptides and proteins. Hence, most peptides and proteins are labeled through small organic fluorine-18 labeled prosthetic groups. The examples of fluorine-18 prosthetic groups are shown in figure 4.
These fluorine-18 labeled prosthetic groups are then conjugated/coupled to biomolecules either by acylation, amidation, thiol reactive reaction, photochemical conjugation, chemoselective oxime formation, chemoselective hydrazone formation or by enzymatic reactions.
1.5 Bombesin receptors

With the success of somatostatin and its analogues in radiolabeling with different radionuclides for diagnosis and therapy of neuroendocrine tumors in nuclear medicine, there is an increasing interest to investigate for other peptide receptors overexpressed in other tumor diseases\textsuperscript{14,79-81}.

Similar to somatostatin, bombesin is a small neuropeptide which shows high affinity binding for gastrin releasing peptide (GRP) receptors. The GRP receptors are bombesin receptor subtype frequently expressed in tumors (eg. lung, breast, prostate and pancreas)\textsuperscript{82,83}. Hence bombesin and its analogues are considered a promising class of ligands in the development of radiopharmaceuticals for imaging and therapy of GRP expressing tumors.

There are two nomenclature systems prevailing in defining the receptors of bombesin. One system originated from the International Symposium on Bombesin-like Peptides in Health and Disease was held 1987 in Rome, Italy. It names each receptor as BB\textsubscript{x}, where x denotes the order when the receptor was identified. The other system of nomenclature simply names each receptor based on its affinity towards the ligand\textsuperscript{84}. For example GRP-preferring receptor is known as BB\textsubscript{2} or the GRP receptor (GRPR), the NMB-preferring receptor is known as BB\textsubscript{1} or the NMB receptor (NMBR) and the orphan receptor is known as BB\textsubscript{3} or bombesin receptor subtype 3 (BRS-3).
Table 5: Bombesin receptors

<table>
<thead>
<tr>
<th>GRP receptors</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NMBR or BB₁</td>
<td>In rat esophagus, CNS&lt;sup&gt;85,87,89&lt;/sup&gt;</td>
</tr>
<tr>
<td>2. GRPR or BB₂</td>
<td>Cloned from Swiss 3T3 murine embryonal fibroblasts, CNS&lt;sup&gt;85,89&lt;/sup&gt;</td>
</tr>
<tr>
<td>BRS-3 or BB₃ or orphan receptor</td>
<td>Testis and lung carcinoma cells&lt;sup&gt;91&lt;/sup&gt;</td>
</tr>
<tr>
<td>4. BRS-3.5</td>
<td>Avian GRP receptors&lt;sup&gt;94&lt;/sup&gt;</td>
</tr>
<tr>
<td>5. BB₄</td>
<td>Non-mammalian receptors&lt;sup&gt;93&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Functional GRP receptors (GRPR or BB₂) cloned from Swiss 3T3 murine embryonal fibroblasts<sup>86</sup> have high affinity for bombesin whereas NMB receptors (NMBR or BB₁), which were evidenced in rat oesophagus, possess high affinity for neuromedin B<sup>87</sup>. However, the radiolabeled agonist (e.g. <sup>125</sup> I-[Tyr<sup>4</sup>]BN) of bombesin has approximately equal affinities with NMBR or BB₁. Hence, it is difficult to clearly distinguish between the sub-types of bombesin (BN) receptors<sup>88</sup>. The radiolabeled bombesin antagonist i.e. <sup>125</sup> I-[D-Tyr<sup>6</sup>]Bn(6-13) methyl ester, has a >10,000 fold higher affinity towards GRP receptors than towards NMB receptors, thus this antagonist is extremely useful in distinguishing GRP receptors from NMB receptors<sup>90</sup>.

A new BN-like peptide receptor subtype (BRS-3 or BB₃ or orphan receptor) is cloned from the human genome and its gene is located in the human chromosome X. BRS-3 mRNA expression in rat tissues is limited to secondary spermatocytes in rat testes and in human lung carcinoma<sup>91</sup>. It has a higher affinity towards the synthetic
bombesin agonist $^{125}$I-\([\text{D-Phe}^6, \beta\text{Ala}^{11}, \text{Phe}^{13}, \text{Nle}^{14}]\text{bombesin(6-14)}\) compared to other bombesin receptors$^{92}$.

Besides these mammalian receptors another non-mammalian fourth bombesin receptor subtype (BB$_4$) was found in frog brain. It possesses higher affinity for bombesin like peptides e.g. \([\text{Phe}^{13}]\text{bombesin}, [\text{Leu}^{13}]\text{bombesin}\) than GRP$^{93}$.

The new avian receptor **BRS-3.5** has sequence similarities to both mammalian BRS-3 and amphibian BB$_4$ receptor and also possesses high affinity for the synthetic bombesin agonist \([\text{D-Phe}^6, \beta\text{Ala}^{11}, \text{Phe}^{13}, \text{Nle}^{14}]\text{bombesin (6-14)}\). It indicates close evolutionary history between amphibian (frog) BB$_4$, avian (chick) **BRS-3.5** and mammalian **BRS-3$^{94}$**.
1.6 Prostate Cancer (PCa)

The cancer in the prostate gland, the part of male reproductive system located between rectum and bladder which surrounds the urethra, is called PCa\textsuperscript{95}. It usually occurs in older men over 50 years of age\textsuperscript{97}. It is one of the leading causes of cancer death with metastases to adrenal gland, bone, liver and lung\textsuperscript{95}. It is difficult in diagnosis, therapy and prognosis due to its heterogeneity\textsuperscript{95,98}. It is the second most common cancer (after skin cancer) and leading cause of death (after lung cancer) in developed western countries (USA and European countries)\textsuperscript{95,99-101}

It is well known that, in all types of cancer, early diagnosis via imaging technology is important for both the prevention and the cure. Due to its heterogeneity in nature various radiopharmaceuticals for prostate cancer imaging/therapy are developed. In comparison to other strategies for developing radiopharmaceuticals, peptides have been widely investigated with tremendous progress in nuclear medicine\textsuperscript{96}.

![Figure 5: The prostate gland\textsuperscript{95}](image)
1.7 Bombesin (BN)

Amphibian skin is the storage of several biogenic amines and active peptides. Bombesin is one of them which was obtained from the methanol extracts of the skin of two European discoglossid frogs Bombina bombina and Bombina variegata. It was isolated in pure form and recognized as a tetradecapeptide in 1970. Due to the name of the species Bombina, it was named bombesin\textsuperscript{101,102,103}.

Four groups of bioactive peptides have been identified from the amphibian skin. All these peptides have mammalian counterparts except the bombesin like peptides. But the immunoreactivity, immunocytochemistry and radioimmunossay of bombesin or bombesin like peptides (BLPs) were found in endocrine cells which are widely distributed in human gastrointestinal mucosa\textsuperscript{104}, bronchial and bronchiolar epithelium of fetal and neonatal lung\textsuperscript{105} and neuroendocrine tumors\textsuperscript{106,107}. These analyses stand for existence of mammalian counterparts. The gastrin releasing (GRP) heptacosapeptide isolated from porcine non-antral gastric and intestinal tissue, was found to have striking homology in the C-terminal region and showed similar bioactivities\textsuperscript{108,109}. Likewise, bombesin like peptides (BLPs) such as neuromedin-B and neuromedin-C were also isolated from porcine spinal cord with sequence homology to amphibian bombesins\textsuperscript{110,111}.

<table>
<thead>
<tr>
<th>Amino acid sequence of peptide</th>
<th>Name</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH\textsubscript{2}</td>
<td>GRP</td>
<td>1978</td>
</tr>
<tr>
<td>Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH\textsubscript{2}</td>
<td>Neuromedin B</td>
<td>1983</td>
</tr>
<tr>
<td>Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH\textsubscript{2}</td>
<td>Neuromedin C or GRP-10</td>
<td>1984</td>
</tr>
</tbody>
</table>

Table 6: Bombesin and bombesin like peptides
The chromatography of BLI (Bombesin like immunoreactivity) from frog gut tissue \((\textit{Bombina orientalis})\) on sephadex G-50 revealed two peaks of BLI, one similar in size to GRP (fGRP i.e. GRP-29) and another similar in size to amphibian bombesin (GRP-10). Both BLIs were encoded by independent genes. Hence, the mammalian GRP might not be the true mammalian counterpart of amphibian bombesin but there might be yet unidentified mammalian bombesin of similar in size of amphibian bombesin of tetradecapeptides. The GRP of human, porcine, avian, guinea pig and canine possess 27 amino acids while that of frog and rat possess 29 amino acids\textsuperscript{112,113}. Three BLPs from canine intestinal muscle of 27, 23 and 10 (GRP-27, GRP-23 and GRP-10) amino acid residues shows more than one biologically active molecular form of GRP in single species\textsuperscript{116,117}. Hence, the mammalian counterparts of bombesin is still in controversy.

All bombesin like peptides possess C-terminal homology with the C-terminal heptapeptide of bombesin which is the shortest amino acid sequence required for the first appearance of bombesin-like effects. The C-terminal nonapeptide of bombesin is the shortest sequence for maximal bombesin-like effects. Both tryptophan and histidine residues were essential for bombesin-like activity\textsuperscript{88}.

One of the drawbacks of using peptides as carrier molecules is being digested endopeptidases present in plasma. Bombesin is digested at the sites indicated by arrows below

\[
\text{pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH}_2
\]

Many modifications have been studied in the structure of bombesin in order to find more stable carrier molecules targeting radionuclides to the receptors without
compromising the binding affinity. Such examples are the exchange of Met\textsuperscript{14} with norleucine (Nle) and Leu\textsuperscript{13} with cyclohexylalanine (Cha). This resulted in improved stability in human plasma as well as in prostate and breast cancer cells. These modifications did not affect the binding affinity and cell internalization\textsuperscript{116-118}.

The C-terminal amino acid i.e. methionine is only important for initiating biological response but not essential for determining the receptor affinity and is prone to proteolytic cleavage. All bomebesin analogues without methionine i.e. desMet analogues (Bn(1-13)NH\textsubscript{2}) act as antagonists and are commonly referred as demobesin. One of the technetium radiolabelled demobesin derivative ([\textsuperscript{99m}Tc]demobesin 1) has higher tumor uptake for GRPR-expressing tumors compared to other bombesin/demobesin labeled radiopharmaceuticals\textsuperscript{119}. In our study we used the same unmodified peptide of \([\textsuperscript{99m}Tc]demobesin 1\) and referred it with same name demobesin 1 for our convenience as presented below.

DPhe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt

or

[D-Phe\textsuperscript{6}, Leu-NH\textsubscript{13}, des-Met\textsuperscript{14}]bombesin(6-14)

Figure 6 : Demobesin 1
1.8 Glycosylation as related to radiopharmaceuticals

There are various approaches of glycosylation in radiopharmaceutical research due to significantly improved biodistribution and pharmacokinetics characteristics of such modified radiopharmaceuticals. In especially rapid clearance from circulation via the kidneys without increased renal uptake, reduced hepatic uptake and biliary excretion, increased tumor-to-nontumor ratios and minimized effective whole body dose for peptide receptor ligands have been observed\textsuperscript{120}. In addition, it also increased bioavailability\textsuperscript{121,122} due to higher stability of glycopeptides toward metabolic degradation. Also a better contrast between tumor and normal tissues in high resolution PET scans have been reported\textsuperscript{123,124}. Therefore, recently there is an increased interest for glycosylation in the filed of radiolabeled proteins and peptides. Glycosylation in radiopharmaceuticals has been reported with following approaches.

1.8.1 Enzymatic fluorine-18 Glycosylation
1.8.2 Fluorine-18 glycolysation of amino acids (BF\textsubscript{3} mediated/Koenigs-Knorr method)
1.8.3 Maillard Reaction (amodari rearrangement)
1.8.4 Glycosylation via sugar amino acid
1.8.5 S-linked glycosylation
1.8.6 Glycated linker
1.8.7 Chemo-selective Glycosylation
1.8.7.1 Thiol reactive agent
1.8.7.2 Chemoselective oxime formation
1.8.1 Enzymatic $[^{18}\text{F}]$-fluoride glycosylation

The MacDonland synthesis of $[^{18}\text{F}]$FDG-1-phosphate was successfully combined with an enzymatic activation to obtain UDP-$[^{18}\text{F}]$FDG$^{122}$ (see scheme 7). Thus obtained UDP-$[^{18}\text{F}]$FDG, is a substrate of suitable glycosyltransferase enzyme and used for enzymatic transfer of $[^{18}\text{F}]$FDG. Hence, it may be used as a highly selective and mild fluorine-18 labeling method for glycosylated biomolecules$^{125}$.

![Scheme 7: Enzymatic $[^{18}\text{F}]$-Glycosylation.](image)

1.8.2 $[^{18}\text{F}]$-fluoride glycosylation of amino acids (BF$_3$ mediated/Koenigs-Knorr method)

Here, the commonly available intermediate 1,3,4,6-tetra-$O$-acetyl-2-deoxy-2-$[^{18}\text{F}]$fluoroglucopyranose (TA-$[^{18}\text{F}]$FDG) of 2-$[^{18}\text{F}]$fluoro-2-deoxyglucose $[^{18}\text{F}]$FDG is used as prosthetic group. It is coupled to hydroxyl side chain of Fmoc-protected serine and threonine and finally deprotected (see scheme 8)$^{126,127,128}$. This could be adapted for fluorine-18 labeling of bioactive proteins and peptides.
1.8.3 Maillard Reaction (amadori rearrangement)

In this approach glucose molecules are coupled with an amino group of peptides or proteins. The glucose molecules are reacted in excess to achieve high yields. The radiolabeling as shown in figure 6 is radiolabeling performed after the glucose conjugation. Such produced glycosylated products eliminated the hepatobiliary route, showed fast blood clearance, kidneys excretion, significantly decreased activity accumulation in liver and intestines. This work opened a new window in the field of developing peptide receptor binding peptides\textsuperscript{129,130}. 

Scheme 8 : Fluorine-18 labeling by TA-\textsuperscript{[18F]}FDG prosthetic group
Figure 7: Structures of $[^{125}\text{I}]$Gluc-TOC, $[^{125}\text{I}]$Malt-TOC, $[^{125}\text{I}]$Mtr-TOC and $[^{125}\text{I}]$Mtr-TOCA.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$[^{125}\text{I}]$Gluc-TOC</td>
<td>CH$_2$OH</td>
</tr>
<tr>
<td>2</td>
<td>$[^{125}\text{I}]$Malt-TOC</td>
<td>CH$_2$OH</td>
</tr>
<tr>
<td>3</td>
<td>$[^{125}\text{I}]$Mtr-TOC</td>
<td>CH$_2$OH</td>
</tr>
<tr>
<td>4</td>
<td>$[^{125}\text{I}]$Mtr-TOCA</td>
<td>COOH</td>
</tr>
</tbody>
</table>
1.8.4 Glycosylation via sugar amino acid

Sugar molecules (i.e. galactose) can be converted to sugar amino acids (SAA) where carboxylate and amino groups are directly linked to two different carbon atoms of a pyranose ring. The amino group was protected with Fmoc which was later radiolabeled by acylation using $2^{[18}F]$fluoropionate whereas the carboxylate group was used to form an amide linkage with the lysine moiety of peptide (see scheme 9). The introduction of the sugar moiety gave a hydrophilic tracer with predominant renal excretion and suitable metabolic stability in vivo\textsuperscript{131}.

Scheme 9 : Synthesis of $^{[18}F]$Galacto-RGD\textsuperscript{131}.
1.8.5 S-linked glycosylation

Like O-linked glycosylation in Koenigs-Knorr methods or BF$_3$-mediated methods, S-linked glycosylation was possible with reactive thiol groups in Fmoc amino acids or 3-mercaptopropionic acid. This type of glycosylation (see figure 8) as used with the somatostatin analog Tyr$^3$-octreotide as a first $^{18}$F-labeled tracer suitable for routine clinical imaging of sst receptors. The compound was named asCel-S-Dpr($^{18}$FBOA)-TOCA$^{132,133}$.

Figure 8: Structure of Cel-S-Dpr($^{18}$FBOA)-TOCA$^{133}$
1.8.6 Glycated linker

A stabilized BN (7-14) sequence bearing \( (N^\beta\text{His})\text{Ac} \) chelator for radiolabeling of radiometals was also could be modified with amino acid linkers containing a lysine or proparagylglycine could be glycosylated with various methods. The glycated linkers as shown in figure have shown to reduce the abdominal accumulation of radiopharmaceuticals which were unfavourable both for both imaging and therapy. It also improved tumour-to-background ratios of these radiopharmaceuticals\textsuperscript{134,135}.

![Chemical structures]

Figure 9: Glycated linkers in radiopharmaceuticals of radiometals
1.8.7 Chemoselective Glycosylation

i. Thiol reactive reagent

Fluorine-18 mediated by labeling maleimide prosthetic groups carrying oxyamine functions has been labeled by another approach (see scheme 10) i.e. chemoselective reaction with the thiol group of peptides and proteins\(^ {136}\). The maleimide and the oxyamine was used for labeling with \[^{18}\text{F}]\text{FDG}.\]

\[\begin{array}{c}
\text{O} & \text{O} & \text{O} & \text{N} \\
\text{O} & \text{O} & \text{O} & \text{H} \\
\text{H} & \text{C} & \text{H} & \text{N} \\
\text{Cl} & 4 & \text{O} & \text{H} \\
\end{array}\]  \rightarrow  \begin{array}{c}
\text{O} & \text{O} & \text{N} & \text{O} \\
\text{O} & \text{O} & \text{O} & \text{H} \\
\text{H} & \text{C} & \text{H} & \text{N} \\
\text{Cl} & 4 & \text{O} & \text{H} \\
\end{array} + \text{GSH}

\[\begin{array}{c}
\text{O} & \text{O} & \text{O} & \text{N} & \text{O} \\
\text{O} & \text{O} & \text{O} & \text{H} \\
\text{H} & \text{C} & \text{H} & \text{N} \\
\text{Cl} & 4 & \text{O} & \text{H} \\
\end{array}\]

\[^{18}\text{F}]\text{FDG-MHO}\]

phosphate buffer (pH 7.2)

\[^{18}\text{F}]\text{FDG-MHO-GSH}\]

Scheme 10 : Radiolabeling of GSH with \[^{18}\text{F}]\text{FDG-MHO}\] \(^{136}\)
Except fluorine-18 labeling prosthetic groups, 3,4,6-tri-O-acetyl-2-deoxy-2-[^18F]fluoro glucopyranosyl phenylthiosulfonate is also reported for labeling by reacting with site specific thiol group of target peptides\textsuperscript{137}.

![Figure 10](image.png)

**Figure 10**: Fluorine-18 labeling via 3,4,6-tri-O-acetyl-2-deoxy-2-[^18F]fluoro glucopyranosyl phenylthiosulfonate prosthetic group

ii. Chemoselective oxime formation

The carbonyl group can be used for chemoselective coupling to oxyamine modified proteins and peptides with the formation of oximes.

Likewise, 2[^18F]fluoro-2-deoxyglucose (FDG), exists in dynamic equilibrium in aqueous solutions. It isomerizes between cyclic alpha and beta pyranose with the formation of the intermediate acyclic aldehyde form. This intermediate acyclic aldehyde form can be selectively reacted with oxyamine modified proteins and peptides\textsuperscript{138,139} (see scheme 11 and 12).
Various approaches were being used to glycolyse radiopharmaceuticals in nuclear medicine. Some used glucose and its derivatives just to glycolyse the prelabeled radiopharmaceuticals whereas other used radiolabled glucose analogs i.e. $[^{18}F]$FDG or TA-$[^{18}F]$FDG to both glycolyse and radiolabel simultaneously. The idea of glycolysing and radiolabeling at the same time has the advantage of reducing the reaction steps in radiolabeling i.e. with radionuclides of shorter half life. Unfortunately, this approach of using $[^{18}F]$FDG was not ideal due to the requirement of high concentrations of biomolecules to obtain acceptable radiochemical yields. However, if a high radiochemical yield is achieved, it could be a very desirable method to glycolyse and radiolabel biomolecules in a single step. One way to get closer to this ideal case is by modifying the radiolabeled glucose i.e. $[^{18}F]$FDG in order to achieve high radiochemical yields at low biomolecule concentration compared to the reaction performed without its modification. So far there are only few publications regarding modifying $[^{18}F]$FDG in order to increase its reactivity towards direct simultaneous
glycosylation and radiolabeling of biomolecules. Therefore, this thesis describes a new approach by using a new carbonyl modified $[^{18}F]$FDG for the $[^{18}F]$ glycosylation of biomolecules.
2 Aim and Scope

1. $^{18}$F)FDG, a glucose analogue, is well known and established radiopharmaceuticals in nuclear medicine and it is commonly available in every medical PET center. Hence, it is easily available.

2. There are very few examples of $^{18}$F)FDG being used as prosthetic group for fluorine-18 labeling of biomolecules.

3. Chemoselective oxime formation between aminooxy group and carbonyl functionality is successfully being used in radiolabeling of biomolecules.

4. There are various radiopharmaceuticals which have shown improved pharmacokinetics after glycosylation.

Although showing some interesting features $^{18}$F)FDG can not be considered as ideal prosthetic group for fluorine-18 labeling. This may be due to following reasons:

i. The cyclic form (closed pyranose ring form) of $^{18}$F)FDG does not possess the carbonyl functionality whereas the linear form (opened pyranose ring form) of $^{18}$F)FDG does present the carbonyl functionality. Only the carbonyl functionality allows in derivatization with carbonyl reactive functionalities. As the cyclic $^{18}$F)FDG does not present carbonyl functionality it cannot take part in chemoselective reactions with oxyamines. Therefore, it needs high concentrations of biomolecules to obtain acceptable radiochemical yields.

ii. The glucose changes its properties when the prayanose ring is opened. The glycosylation improves biodistribution and pharmacokinetics, increases tumor-to-nontumor ratios, reduces hepatic uptake and biliary excretion, etc. as stated in 1.8. Since only linear $^{18}$F)FDG takes part derivatization the labeled biomolecules


by using unmodified $[^{18}\text{F}]$FDG cannot be described as glycolysed molecules in its classical sense.

Therefore, it will be interesting to modify $^{18}\text{F}]$FDG with a permanent external carbonyl functionality which is always available for derivatization without the pyranose ring of $[^{18}\text{F}]$FDG being opened. Here, the pyranose ring will remain intact and is able to glycolyse the labeled biomolecules in its classical sense. Thereby it will be interesting to see the reactivity of the modified $[^{18}\text{F}]$FDG as compared to $[^{18}\text{F}]$FDG by itself.
3 Result and Discussion

3.1 Synthesis of keto-[\(^{18}\)F]FDG

It is reported that unprotected aldose sugars can be converted to C-glycosidic ketones in an aqueous based knoevenagel reaction using simple β-diketones (pentane-2,4-dione i.e. acetylacetone) at basic conditions with excellent yields retaining the ring integrity of the parent sugar and providing a new carbonyl functionality as well\(^{140,141}\).

It is also reported that 2-deoxy-D-glucose is condensed with acetylacetone to produce its β-anomers\(^{114}\). Hence, we expect that [\(^{18}\)F]FDG could be condensed with acetylacetone in a similar way without losing its ring integrity and without losing glucose properties.

Therefore, we attempted to optimize this reaction (see scheme 13) of [\(^{18}\)F]FDG with acetylacetone at different reactions conditions as explained below.
3.1.1 Synthesis of keto-\(^{18}\)F]FDG using saturated sodium bicarbonate

We tried to modify \(^{18}\)F]FDG at basic conditions of saturated sodium bicarbonate solution (100 µl) with 25 µl of \(^{18}\)F]FDG with different amounts of acetylacetone in acetonitrile solution (25 µl) at 100 °C for 15 minutes in a sealed glass vial.

<table>
<thead>
<tr>
<th>Sat. NaHCO(_3)</th>
<th>Acetylacetone</th>
<th>(^{18})F]FDG</th>
<th>Radiochemical Yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl</td>
<td>20 µl</td>
<td>25 µl</td>
<td>25±2</td>
</tr>
<tr>
<td>100 µl</td>
<td>10 µl</td>
<td>25 µl</td>
<td>35±2</td>
</tr>
<tr>
<td>100 µl</td>
<td>5 µl</td>
<td>25 µl</td>
<td>45±2</td>
</tr>
<tr>
<td>100 µl</td>
<td>2 µl</td>
<td>25 µl</td>
<td>55±2</td>
</tr>
</tbody>
</table>

Table 7: Different approaches of \(^{18}\)F]FDG coupling with different aliquots of acetylacetone in sat. NaHCO\(_3\)

We observed that lower amounts of acetylacetone give better results than higher volumes of acetylacetone.

3.1.2 Synthesis of keto-\(^{18}\)F]FDG using carbonate buffer

Performing the reaction by using saturated sodium bicarbonate solution is not optimal and therefore we changed the reaction conditions to more basic conditions of stable carbonate buffer at different molarities and equimolar ratios of NaHCO\(_3\):Na\(_2\)CO\(_3\) as shown in table 8 keeping the other reaction parameters the same as before with saturated sodium carbonate solution.
<table>
<thead>
<tr>
<th>NaHCO₃:Na₂CO₃ (mmol)</th>
<th>Acetyl Acetone</th>
<th>[¹⁸F]FDG</th>
<th>Radiochemical Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5 µl</td>
<td>25 µl</td>
<td>25±2</td>
</tr>
<tr>
<td>0.25</td>
<td>5 µl</td>
<td>25 µl</td>
<td>35±2</td>
</tr>
<tr>
<td>0.5</td>
<td>5 µl</td>
<td>25 µl</td>
<td>75±2</td>
</tr>
<tr>
<td>0.5</td>
<td>10 µl</td>
<td>25 µl</td>
<td>&gt; 90</td>
</tr>
</tbody>
</table>

Table 8: Different approaches of [¹⁸F]FDG coupling with different ratios of carbonate buffer.

As the yields of keto-[¹⁸F]FDG at equimolar ratio of carbonate buffers (NaHCO₃:Na₂CO₃) with 10 µl and 0.5 mmol concentration was the highest as shown by radio-HPLC, we considered this as optimal reaction condition with respect to the buffer conditions.
3.1.3 Scale up of reaction volume

As useful activities for tracer production require high volumes of $[^{18}\text{F}]$FDG, we increased the reaction volume to 1 ml. We added saline in case of necessity to $[^{18}\text{F}]$FDG solution, directly delivered from medical PET center, in different proportion as shown in table 9 without exceeding the reaction volume beyond 1 ml. In this scheme solid NaHCO$_3$ and Na$_2$CO$_3$ was added in order not to dilute or exceed the reaction volume beyond 1 ml. All reactions conditions shown in table 9 resulted in similar radiochemical yields of $>90\%$.

<table>
<thead>
<tr>
<th>NaHCO$_3$:Na$_2$CO$_3$</th>
<th>Acetyl</th>
<th>$[^{18}\text{F}]$FDG</th>
<th>Saline</th>
<th>Radiochemical Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1:1) 0.5 mmol</td>
<td>10 µl</td>
<td>100 µl</td>
<td>900 µl</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>(1:1) 0.5 mmol</td>
<td>10 µl</td>
<td>200 µl</td>
<td>800 µl</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>(1:1) 0.5 mmol</td>
<td>10 µl</td>
<td>300 µl</td>
<td>700 µl</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>(1:1) 0.5 mmol</td>
<td>10 µl</td>
<td>400 µl</td>
<td>600 µl</td>
<td>$&gt;90$</td>
</tr>
<tr>
<td>(1:1) 0.5 mmol</td>
<td>10 µl</td>
<td>1000 µl</td>
<td>-</td>
<td>$&gt;90$</td>
</tr>
</tbody>
</table>

Table 9: Increasing the $[^{18}\text{F}]$FDG volume in the reaction.

We keep the other reaction parameters the same as before with saturated sodium carbonate solution.

45
3.2 Purification of synthesized keto-[\textsuperscript{18}F]FDG from reaction mixture

In order to make use of keto-[\textsuperscript{18}F]FDG as prosthetic group for modifying biomolecules reactive towards carbonyl groups it is most important to purify the keto-[\textsuperscript{18}F]FDG from the excess of acetylacetone used in the synthesis. The reaction mixture was acidified by 1 M HCl to a pH of 2-3 prior to the purification on the SPE cartridges. Initially, we tried to purify keto-[\textsuperscript{18}F]FDG by C18 solid phase extraction however it proved to be too hydrophilic to be quantitatively to purify on C18 cartridge. Therefore, we choose a more suitable HRP cartridge which quantitatively trapped the synthesized keto-[\textsuperscript{18}F]FDG. Washing with water however did not remove the acetylacetone. Hence, we tried dichloromethane to wash the acetyl acetone completely from the cartridge. Since, dichloromethane did not dissolve our product (i.e. keto-[\textsuperscript{18}F]FDG), we were able to completely wash off the acetylacetone with dichloromethane and were able to elute the purified keto-[\textsuperscript{18}F]FDG with different solvents e.g. acetonitrile, DMF(Dimethylformamide), ethanol or its mixtures with water. We choose 70 % ethanol in water for the elution of keto-[\textsuperscript{18}F]FDG as it eluted the product in low volume and give the opportunity of quick evaporation in case of necessity. The radiochemical yield of keto-[\textsuperscript{18}F]FDG was 70 % after purification whereas the radiochemical purity was >90 %. In summary, due to the inability of water to significantly elute keto-[\textsuperscript{18}F]FDG from a HRP cartridge and the insolubility of keto-[\textsuperscript{18}F]FDG in dichloromethane made this simple and quick purification process possible and very efficient.

We developed a fast and simple keto-[\textsuperscript{18}F]FDG synthesis protocol with a high radiochemical yield and easy purification step. Depending upon further reaction conditions, keto-[\textsuperscript{18}F]FDG can be eluted with solvents of choice such as DMF,
acetonitrile, ethanol or in mixture with water which could be beneficial in fluorine-18 labeling of biomolecules.

Scheme 13: Synthesis and purification of carbonylated $[^{18}\text{F}]$FDG (keto-$[^{18}\text{F}]$FDG) followed by labeling of biomolecules.
3.3 Purification of keto-[\(^{18}\text{F}\)]FDG from keto-glucose

It is known that glucose is a competitive reagent inevitably present in \([^{18}\text{F}]\text{FDG}\) due to the production process of \([^{18}\text{F}]\text{FDG}\)\(^{115,138}\). During the reaction of modifying \([^{18}\text{F}]\text{FDG}\) to keto-[\(^{18}\text{F}\)]FDG, there is equally chances for conversion of glucose to keto-glucose. However, to convert the molecule into C-glycosodies (keto-glucose) it takes a comparatively longer reaction time\(^{140,141}\) than for \([^{18}\text{F}]\text{FDG}\) to keto-[\(^{18}\text{F}\)]FDG. It is unlikely to convert all the glucose present in \([^{18}\text{F}]\text{FDG}\) solution to its keto-glucose form. Anyhow, the competitive formation of keto-glucose still must be taken into consideration. Therefore, we have looked for possibilities to separate keto-glucose from keto-[\(^{18}\text{F}\)]FDG in order to keep the concentration of biomolecules to be labeled as low as possible. In order to avoid a difficult HPLC purification step to separate radiolabeled product from stable byproducts, we investigated different ways as explained in 3.3.1 and 3.3.2 for purification of keto-[\(^{18}\text{F}\)]FDG from keto-glucose.

As most of our studies were performed with small aliquots of \([^{18}\text{F}]\text{FDG}\), glucose was not very prominent. However, in order to simulate larger quantities of \([^{18}\text{F}]\text{FDG}\) solution we explicitly added an amount of 3 mg of keto-glucose considering as an average equivalent of stable glucose present in \([^{18}\text{F}]\text{FDG}\) solution of up to about 110 ml.
3.3.1 HPLC purification of keto-[\(^{18}\)F]FDG from keto-glucose

The retention time to keto-[\(^{18}\)F]FDG in different gradients of acetonitrile (solvent B) in 0.1 % trifluoroacetic acid (TFA) (v/v) in H\(_2\)O (solvent A) at a constant flow of 1 min/ml at 254 nm ultraviolet photometer in radio-HPLC were presented in the table 10.

<table>
<thead>
<tr>
<th>Acetonitrile % (solvent B)</th>
<th>0.1 % TFA in H(_2)O (solvent A)</th>
<th>Retention time of keto-[(^{18})F]FDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-90</td>
<td>100-10</td>
<td>7 min</td>
</tr>
<tr>
<td>0-40</td>
<td>100-60</td>
<td>9 min</td>
</tr>
<tr>
<td>0-10</td>
<td>100-90</td>
<td>13 min</td>
</tr>
<tr>
<td>0-5</td>
<td>100-95</td>
<td>15 min</td>
</tr>
</tbody>
</table>

Table 10 : Retention time of keto-[\(^{18}\)F]FDG in different gradient of radio-HPLC.

The retention time of keto-[\(^{18}\)F]FDG was at 15 min in (0-5 %) acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H\(_2\)O (solvent A) for 30 minutes at a constant flow of 1 ml/min radio-HPLC. Assuming keto-glucose also behave in similar way, we were interested to elute keto-glucose even at slow isocratic gradient of in 0.1 % trifluoroacetic acid (TFA) (v/v) in H\(_2\)O (solvent A) for 30 minutes at a constant flow of 1 ml/min HPLC and aliquots were collected each minute of the gradient. We know that keto-glucose is neither UV-active nor radioactive, hence, it is not possible to visualize it in a HPLC chromatogram. Therefore, the collected aliquots were reacted with an excess of UV active hydroxylamine i.e. O-benzylhydroxylamine at room temperature at pH 5-6. The formation of UV active product was checked by HPLC.

The conditions as described above was eluted as early asd 3-4 minutes, while keto-[\(^{18}\)F]FDG elutes at about 15 minutes gave the isomeric product peak of oxime.
formation made by keto-glucose and O-benzylhydroxylamine, while the other aliquots did not. Hence, we concluded that keto-glucose, when chromatographed in isocratic gradient of 0.1 % TFA water for 30 minutes at a constant flow of 1 ml/m in HPLC, were eluted early at 3-4 minutes.

Figure 11: Excess O-benzylhydroxylamine reacted keto-glucose.

This relative strong difference in retention times (i.e. 3-4 minutes of keto-glucose Vs 15 minutes for keto-[\(^{18}\)F]FDG) Made us to believe observation made us to believe that SPE purification of keto-glucose from keto-[\(^{18}\)F]FDG could also be possible:

- The large difference in retention time of keto-glucose whose retention time was at 3-4 min in isocratic gradient of water and the retention time of keto-[\(^{18}\)F]FDG was at 15 min in slow gradient of HPLC with lower percentage of acetonitrile (0-5 %) in 0.1 % TFA water in radio-HPLC (see table 10).

- Inability of water alone to significantly elute the keto-[\(^{18}\)F]FDG (as described in 3.2 during purification of synthesized keto-[\(^{18}\)F]FDG from its reaction mixture) from HRP a cartridge.
3.3.2 SPE purification of keto-[¹⁸F]FDG from keto-glucose

The HPLC purification of keto-glucose from HRP cartridge as described in 3.3.1, motivated us to try perform a SPE based purification procedure in order to purify keto-glucose from keto-[¹⁸F]FDG. Initially it was studied how much water it would take to elute keto-glucose completely from the HRP cartridge. Again the eluted fractions were reacted with O-benzylationhydroxylamine and observed for oxime formation by HPLC (see 3.3.1) and observe the oxime formation in HPLC. The rinsing/washing volume of water to the cartridge was increased until the oxime product in the chromatogram was not visible anymore. With a single HRP cartridge, 20 ml of water was able to remove ~95 % of the keto-glucose with a loss of 2.5 – 3 % of radioactivity whereas 100 ml of water removed ~98 % keto-glucose with a loss of ~10 % of radioactivity. Alternatively, by using two HRP cartridges and rinsing with a volume as high as 200 ml of water, we were not able to detect the UV active oxime anymore. Thus, we concluded that keto-glucose was nearly quantitatively removed and the loss of activity was still bearable with about 15-20 %.

We also tried to purify keto-glucose from keto-[¹⁸F]FDG by rinsing the HRP cartridge with 1 % acetonitrile in water in order to use a smaller volume of washing solvent. Unfortunately even with 1 % acetonitrile in water, significant amount of keto-[¹⁸F]FDG were eluted from the HRP cartridge.

In summary the findings described above show that a SPE purification of keto-[¹⁸F]FDG from keto-glucose by HRP cartridge (680 mg) is possible by rinsing with sufficient amounts of water without great loss of keto-[¹⁸F]FDG.
3.4 Reference standard for the synthesis of keto-[\textsuperscript{18}F]FDG

In order to confirm the structure of radioactive keto-[\textsuperscript{18}F]FDG, the synthesis of stable keto-[\textsuperscript{19}F]FDG was necessary for analytical purposes. All reactions condition and purification methods were the same as for the synthesis of keto-[\textsuperscript{18}F]FDG. Since stable keto-[\textsuperscript{19}F]FDG is not UV active it was derivatized with a UV active hydroxylamine molecule i.e. O-benzylhydroxylamine and compared the chromatogram with that of keto-[\textsuperscript{18}F]FDG as described in 3.5.2. The chromatograms of products between keto-[\textsuperscript{18}F]FDG and O-benzylhydroxylamine as well as keto-[\textsuperscript{19}F]FDG and O-benzylhydroxylamine showed identical retention time in UV and radio detection. The identity of the reference standard keto-[\textsuperscript{19}F]FDG was confirmed by NMR and MS.

![Stable keto-\textsuperscript{19}FDG (Reference standard)](image)

Figure 12: Mass spectra of stable reference standard i.e. keto-[\textsuperscript{19}F]FDG

![Purified radioactive keto-\textsuperscript{18}FDG in radio-HPLC](image)

Figure 13: Purified radioactive keto-[\textsuperscript{18}F]FDG in radio-HPLC
3.5 Reactivity of keto-$[^{18}\text{F}]$FDG in comparison to $[^{18}\text{F}]$FDG

Both $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG possess the carbonyl functionality in equilibrium and non equilibrium respectively. The pyranose ring of $[^{18}\text{F}]$FDG opens in reaction with oxyamine whereas the pyranose ring of keto-$[^{18}\text{F}]$FDG remains intact in reactions with oxyamines. The close ring integrity of pyranose is considered to possess the glucose functionality which gives desirable pharmacokinetics and biodistribution to radiopharmaceuticals.

We compared the reactivities of $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG by using the UV active oxyamine $O$-benzylhydroxylamine at different concentrations, time, temperature and pH in presence or absence of catalyst aniline (or its derivatives i.e. $p$-anisidine, 4-nitroaniline and $N,N$-dimethylphenylenediamine).

3.5.1 Reactivity of $[^{18}\text{F}]$FDG with $O$-benzylhydroxylamine in the influence of catalyst

In absence of aniline, pH 3-4 is favourable for the oxime formation whereas aniline shows its catalytic effect especially at pH 5-6 as shown in figure 14a and 14b. The aniline derivative, $p$-anisidine (data not shown) also showed a similar pattern of catalytic role. The required temperature for the reaction was decreased from 100 °C to 80 °C at pH 5-6 with the use of catalyst in a time period of 20 minutes.
Figure 14: Reaction of $[^{18}F]$FDG with 5 mg/ml of O-benzylhydroxylamine at different temperatures and at different pH for 20 minutes in (a) absence and (b) presence of 100 mM aniline.
The radiochemical yield of oxime at a concentration of 1.25 mg/ml of O-benzylhydroxylamine increases from 12 % in absence of aniline to 65 % in presence of aniline. The catalytic role of aniline becomes less pronounced for higher concentrations than 5 mg/ml of O-benzylhydroxylamine but still there is an increase in radiochemical yield from 60 % in absence of aniline to 90 % in presence of aniline as shown in figure 15. The aniline derivative, p-anisidine also showed a similar pattern of catalytic role. Hence, the catalytic role of aniline/p-anisidine is prominent at lower concentrations of O-benzylhydroxylamine.

Figure 15 : Comparison of product formation with indicated concentration of O-benzylhydroxylamine at pH 5-6 and 80 °C for 20 minutes with [¹⁸F]FDG with presence/absence of catalyst (100 mM).
3.5.2 Reactivity of keto-\(^{18}\text{F}\)FDG with \(O\)-benzylhydroxylamine in the influence of catalyst

The favorable temperature for the reaction of keto-\(^{18}\text{F}\)FDG with \(O\)-benzylhydroxylamine in presence of aniline is 60 °C as shown in figure 16. This lower optimal temperature of 60 °C (compared to 80 °C for \(^{18}\text{F}\)FDG) also stands for a higher reactivity of keto-\(^{18}\text{F}\)FDG. Keto-\(^{18}\text{F}\)FDG was dramatically more reactive towards oxyamines i.e. \(O\)-benzylhydroxylamine than \(^{18}\text{F}\)FDG as already 0.05 mg/ml of \(O\)-benzylhydroxylamine resulted in a yield of 50 % of oxime at pH 3-4 even without catalyst. The catalytic role of aniline/p-anisidine increases the yield of oxime (product of keto-FDG with \(O\)-benzylhydroxylamine) from 50 % (at pH 3-4) and 30 % (at pH 5-6) to 90 % at both pH values as presented in figure 17.
Figure 16: Reaction of keto-[¹⁸F]FDG with 0.05 mg/ml of O-benzylhydroxylamine in presence of 100 mM aniline at different temperatures for 20 minutes at indicated pH.

Figure 17: 0.05 mg/ml of O-benzylhydroxylamine in absence or presence of 100 mM aniline or p-anisidine at 60 °C for 20 minutes at respective pH with keto-[¹⁸F]FDG.
A 100 mM aniline concentration is appropriate for the reactions at pH 5-6 in both $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG. As higher concentrations of catalyst beyond 100 mM did not improve the reaction and lower concentrations gave lower radiochemical yields. Other aniline derivatives as catalyst were also used at same concentrations. Aniline and $p$-anisidine as nucleophilic catalysts showed improvement in labeling of $\alpha$-effect nitrogens (e.g. in oxyamines or hydrazines) with the carbonyl groups\(^\text{16}\) of $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG. Other aniline derivatives such as 4-nitroaniline and $N,N$-dimethylphenylenediamine did not show catalytic effects, neither with $[^{18}\text{F}]$FDG nor with keto-$[^{18}\text{F}]$FDG.

The reactivity of both $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG were compared with O-benzylhydroxylamine without purifying the glucose from $[^{18}\text{F}]$FDG and keto-glucose from keto-$[^{18}\text{F}]$FDG respectively.

The reactivity of both $[^{18}\text{F}]$FDG and keto-$[^{18}\text{F}]$FDG are high at pH 3-4 in absence of catalyst and at pH 5-6 in presence of catalyst. The $[^{18}\text{F}]$FDG needed 5 mg of O-benzylhydroxylamine to give a similar radiochemical yield (~80 %) as that of 0.05 mg of O-benzylhydroxylamine by keto-$[^{18}\text{F}]$FDG (~80 %) at same reaction conditions.

The highest radiochemical yield (~80 %) for $[^{18}\text{F}]$FDG with 5 mg O-benzylhydroxylamine were obtained at 80 °C, whereas the highest radiochemical yields (~80 %) for keto-$[^{18}\text{F}]$FDG with 0.05 mg O-benzylhydroxylamine was obtained at 60 °C at same pH. On the basis of this data we concluded a higher reactivity of keto-$[^{18}\text{F}]$FDG compared to $[^{18}\text{F}]$FDG.
3.6 Stability test of keto-[^18F]FDG product

3.6.1 *In vitro* stability of oxime

The goal of this work is to use keto-[^18F]FDG, as prosthetic group for fluorine-18 labeling of biomolecules. In this setting, the carbonyl function of keto-[^18F]FDG reacts with oxyamines or hydrazines. We were interested in the stability of keto-[^18F]FDG formed oximes or hydrazones of small model molecules. Hence, we reacted oxyamines (e.g. aminooxy acetic acid, O-benzylhydroxylamine) and hydrazines (e.g. 4-hydrazinobenzoic acid) with keto-[^18F]FDG for the formation of respective oxime and hydrazone products.

![Figure 18: Oxime product of keto-[^18F]FDG with aminooxy acetic acid](image.png)

![Figure 19: Oxime product of keto-[^18F]FDG with O-benzylhydroxylamine](image.png)
Figure 20: Oxime formation of aminooxy acetic acid with keto-[^{18}F]FDG at gradient of 0-90% acetonitrile (solvent B) and 0.1% trifluoroacetic acid (TFA) (v/v) in H$_2$O (solvent A) for 30 minutes at a constant flow of 1 ml/min and wavelength of 254 nm.

Figure 21: Oxime formation of O-benzylhydroxylamine with keto-[^{18}F]FDG at gradient of 0-90% acetonitrile (solvent B) and 0.1% trifluoroacetic acid (TFA) (v/v) in H$_2$O (solvent A) for 30 minutes at a constant flow of 1 ml/min and wavelength of 254 nm.

The products were purified and incubated in human serum at 37 °C for an overall period of four hours. All the products including the hydrazone product of 4-hydrazinobenzoic acid with keto-[^{18}F]FDG remained stable as no peaks except the product peaks of the oximes are visible. This shows that the products are not degraded by enzymes or proteins present in human serum, within the studied time frame of 4 hours.
3.6.2 pH stability

The pH stability of keto-[^18F]FDG and its oxime or hydrazone product were performed at different pH values of pH 2.5, pH 4.7, pH 7 and pH 10.

i. Oxime of aminooxy acetic acid with keto-[^18F]FDG

The oxime product of aminooxy acetic acid was stable in acidic and neutral pH whereas at high basic pH 10 a degradation of about 60% within time period of 3 hours was observed.

ii. Oxime of O-benzylhydroxylamine with keto-[^18F]FDG

The oxime product with O-benzylhydroxylamine was stable in all acidic, neutral and basic pH mentioned above.

iii. Oxime of 4-hydrazinobenzoic acid with keto-[^18F]FDG

The hydrazone product of 4-hydrazinobenzoic acid was stable at neutral and at basic pH whereas in acidic pH the hydrazone was highly sensitive and completely degraded at pH ranges of 1-3 within one hour at room temperature.
3.7 Synthesis of linkers

Linkers are frequently found as bifunctional chelators (BFC) for radiometals to be linked to biomolecules. However, these linkers also are synthesized to react with a certain prosthetic groups at the one end and biomolecules at the other end.

The pharmacokinetic and biodistribution properties of radiopharmaceuticals may depend on these linkers. In most of the radiopharmaceuticals production, the radiolabeling process is performed as the last step to allow for easy purification and high radiochemical yields. Hence we need to modify the biomolecules first with a linker which possess the oxyamine to chemoselectively react with the prosthetic group keto-[\(^{18}\text{F}\)]FDG. In order to protect the oxyamine from being attacked in prior reactions, it is usually protected with boc or other protective groups which are cleaved at the last step before labeling with keto-[\(^{18}\text{F}\)]FDG.

Peptides and proteins are usually modified with linkers by typical peptide coupling reactions where the carboxylic acid moieties of the linkers are first activated by an appropriate peptide coupling reagent. The esters formed by these coupling reagents are often referred to as active esters, which often can be stored for longer time periods and used when needed.

We applied different strategies to synthesize such linkers which possess the oxyamine (or hydrazine) at one end and an activated carboxylic acid at the other side as shown in figure 22. We also synthesized linkers i.e. lysine aminooxyacetyl which introduces an additional positive charge to study the effect of a positive charge in pharmacokinetics and biodistributions of the radiopharmaceuticals of interest.
In especially, the positive charge in the $^{99m}$Tc labeled demobesin 1 claims to be responsible for improved pharmacokinetics and biodistributions as well as the highest tumor uptake in the PC-3 tumor xenografts among bombesin derivatives. As the same peptide was chosen the same peptide as test peptide to this works it was especially interesting to observe the results although there are controversial discussions about the charge in the radiopharmaceuticals i.e. between those labeled by radiometals and radiohalogens. The radiometals with positive charge sometimes showed desired pharmacokinetics and biodistributions behaviour while opposite in case of radiohalogens.
Figure 22: Synthesized linkers with protection and activation.

R₁ = Keto-[18F]FDG
R₂ = peptide/biomolecules
3.7.1 1,3,5,6-tetrafluorophenyl ester of boc protected aminooxy acetic acid (TFP boc-AOA active ester)

We have started to synthesis this active ester with the starting material boc-aminooxy acetic acid. Since it already contains the oxyamine group protected by boc, we only needed to activate the carboxylic acid moiety with an appropriate peptide coupling reagents. We have tried the following reagents for activation. After unsuccessful attempts with 1,1'-carbonylimidazole and Steglich reagent, we observed the combination of DCC (N,N-dicyclohexylcarbodiimide) and 1,3,5,6-tetrafluorophenol as suitable reagents to activate the carboxylic acid of boc-aminooxy acetic acid. The purification was easy by adding ether, in which desired active ester was solubilised and the byproducts that remained insoluble which were later on filtered off. The solvent ether was easily evaporated with stream of argon to obtain the desired active ester.

3.7.2 Hydroxybenzotriazolyl ester of boc protected aminooxybenzoic acid (HOBt boc-PABA active ester)

In this active ester we introduced the boc-protected oxyamine group of N-boc-hydroxylamine to the methyl-4-(bromomethyl)benzoate. Methyl-4-(bromomethyl)benzoate was used for the reaction after the unsuccessful attempt for 4-(bromomethyl)benzoic acid. In this reaction the selection of the base seems to be important. The bases like TEA, DIPEA were leading to additional peaks in HPLC chromatograms, which were not observed in the case of DBU. The completion of the reaction was confirmed by treating the boc-protected product with TFA and shift of the peak in the chromatogram was observed. Once the boc-protected oxyamine group is attached, the benzoate of methyl benzoate was
hydrolyzed using sodium hydroxide solution. The carboxylic acid was finally activated by diBTC (dibenzoyltriazole carbonate).

3.7.3 Hydroxybenzotriazolyl ester of boc protected hydrazinobenzoic acid (HOBt boc-HBA active ester)

The 4-hydrazinobenzoic acid was treated with di-tert-butylcarbonate to protect the hydrazine moiety by a boc group. In the second step, the free carboxylic acid moiety was required activated by a peptide coupling reagent. After some unsuccessful attempts of activation reagents such as DCC and TFP, di(N-succinimidyl)carbonate, di(1-benzotriazolyl) carbonate, 1,1′-carbonyldimidazole, 4-nitrophenol and steglich reagent finally a mixture of DCC with 1-hydroxybenzotriazole lead to the formation of desired active ester of it. The purification was performed by work up with DCM and acidic water. The DCM phase was evaporated to obtain the ester.

3.7.4 1,3,5,6-tetrafluorophenyl ester of boc protected lysine aminooxy amino acid (TFP boc-AOA lysine active ester)

This ester shows a positive charge at physiological pH due to presence of lysine. This linker could be synthesized by addition of lysine to the previously synthesized aminooxy acetyl linker. The boc protected 1,3,5,6-tetrafluorophenyl ester of aminooxy acetic acid could be fused with H-(D)-lysine(boc)-OH. The carboxylic acid moiety of lysine was then activated with peptide coupling reagents. Similar to boc protected aminooxy acetic acid the combination of DCC and TFP was successful in building the ester of TFP after being unsuccessful with other reagents like HOBt (1-hydroxybenzotriazole) and EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide)
combination as well as HBTU (O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium Hexafluorophosphate).

3.8 Reactions of UV-active model amines with oxyamine linkers

Before modifying expensive peptide/biomolecules by the synthesized linkers, we tried to modify some UV active amines with boc-protected aminoxo-acetyl-linkers. The retention times of the amines with the retention times of their corresponding product are listed in figure 23.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Product</th>
<th>Deprotection of boc</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="H-N-CONH2-Fmoc" /></td>
<td><img src="image2.png" alt="Boc-NHCONH2-Fmoc" /></td>
<td><img src="image3.png" alt="H2N-CONH2-Fmoc" /></td>
</tr>
<tr>
<td>H-Lys(Fmoc)-OMe</td>
<td>20 min</td>
<td>21 min</td>
</tr>
<tr>
<td><img src="image4.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image5.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image6.png" alt="H2N-CONH2-Fmoc" /></td>
</tr>
<tr>
<td>Tyramine</td>
<td>6 min</td>
<td>9 min</td>
</tr>
<tr>
<td><img src="image7.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image8.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image9.png" alt="H2N-CONH2-Fmoc" /></td>
</tr>
<tr>
<td>24 min</td>
<td>11 min</td>
<td></td>
</tr>
<tr>
<td><img src="image10.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image11.png" alt="H2N-CONH2-Fmoc" /></td>
<td><img src="image12.png" alt="H2N-CONH2-Fmoc" /></td>
</tr>
<tr>
<td>20 min</td>
<td>13 min</td>
<td></td>
</tr>
</tbody>
</table>
Figure 23: Amines modified by synthesized linkers and their retention times in HPLC at gradient of 0-90 % acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 30 minutes at a constant flow of 1 ml/min and wavelength of 254 nm
3.9 Labeling of peptides
3.9.1 Deprotection of the linkers

The small amines (see figure 23) were successfully modified by aminoxy acetyl or lysine aminooxyacetyl linkers and deprotected by treatment with TFA. After the deprotection step the oxyamine functionality was ready to react with carbonyls as chemoselective oxime formation reaction of carbonyl as described in 3.5 for [\(^{18}\text{F}\)]FDG or keto-[\(^{18}\text{F}\)]FDG with O-benzylhydroxylamine.

3.9.2 Labeling of peptides with keto-[\(^{18}\text{F}\)]FDG

After understanding the behaviour of small amine molecules we were interested in more sophisticated biomolecules for modification and labeling with keto-[\(^{18}\text{F}\)]FDG. We attempted to perform similar modification and labeling reactions with RC160 (vapreotide) as test peptide and bombesin (demobesin 1) as model peptide.
The test peptide RC160(vapreotide) was modified by coupling with TFP boc AOA ester at the lysine residue of peptide. The coupled product of boc protected ester and peptide was purified by C18 SPE and eluted with 90 % ethanol in water. The product was then dried and treated with TFA to cleave the boc group to expose the oxyamine functionality. The oxyamine modified test peptide was reacted with keto-[\(^{18}\text{F}\)]FDG in citrate buffer of pH 2-3 at 100 °C for different reaction times and with or without the presence of catalyst aniline (see table 11 and 12). The resulting structure of the keto-[\(^{18}\text{F}\)]FDG labeled oxyamine modified RC160 is shown in figure 24.

Figure 24: Structure of aminooxy acetylated RC160 labeled by keto-[\(^{18}\text{F}\)]FDG
Table 11: Radiochemical yields for keto-[\(^{18}\)F]FDG labeling of aminooxyacetylated RC160 at various peptide concentrations without the presence of catalyst in citrate buffer of pH 2-3 at 100 °C for given time.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Time (min)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>60±2</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
<td>20±2</td>
</tr>
<tr>
<td>0.5</td>
<td>30</td>
<td>30±2</td>
</tr>
</tbody>
</table>

Table 12: Radiochemical yields for keto-[\(^{18}\)F]FDG labeling of aminooxyacetylated RC160 at various peptide concentrations with the presence of catalyst in citrate buffer of pH 2-3 at 100 °C for given time.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Time (min)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>90±2</td>
</tr>
<tr>
<td>0.5</td>
<td>30</td>
<td>40±2</td>
</tr>
</tbody>
</table>

The reactions were performed without the purification of keto-[\(^{18}\)F]FDG from keto-glucose as described in 3.3.2 because the amount of [\(^{18}\)F]FDG volume was small and significant glucose competition could be ruled out. The radiochemical yields are higher in the presence of aniline as catalyst as shown by an increase of radiochemical yield for 0.5 mg/ml of peptide concentration from 30±2 (absence of catalyst) to 40±2 (presence of catalyst) at 100 °C and 30 minutes. However, the radiochemical yields were strongly increased with longer reaction time and by increasing concentration to 1 mg/ml of peptide. The highest radiochemical yields of up to 90±2 were obtained in the presence of aniline and 1 mg/ml peptide concentration.
3.10 Modification and radiolabeling of the model peptide

3.10.1 Modification of model peptide

As there is no direct labeling of fluorine-18 (see 1.4.2), generally fluorine-18 is labeled to biomolecules via prosthetic groups. The prosthetic groups with fluorine-18 was then labeled with biomolecules via linkers. Here a new prosthetic group for fluorine-18 labeling, keto-[\(^{18}\)F]FDG was labeled to demobesin1 via two different linkers.

i. Aminooxy acetylated linker and

ii. Lysine aminooxyacetylated linker.

The Phe residue of the demobesine 1 was coupled with boc protected active ester of above mentioned linkers. The boc protection of the coupled product was deprotected by the treatment of TFA.

3.10.2 Radiolabeling of modified model peptide with keto-[\(^{18}\)F]FDG

After the deprotection of boc group of modified demobesin 1, the aminooxy funtional group was free to be labeled with the carbonyl (ketone) of prosthetic group keto-[\(^{18}\)F]FDG. For the radiolabeling with demobesin 1, the prosthetic group keto-[\(^{18}\)F]FDG was purified as described in 3.3.2. in order to achieve the highest radiochemical possible. The prosthetic group keto-[\(^{18}\)F]FDG was then labeled to modified demobesin 1 in citrate buffer at pH 2-3 at 60 °C for 30 minutes.

Radiolableling as well as purification was successful as we were able to separate labeled modified demobesin 1 with keto-[\(^{18}\)F]FDG from its unlabeled one by HPLC. This purification increased the specific activity of labeled radiopharmaceuticals which is ideal conditions.
Scheme 14: Synthesis scheme of keto-[18F]FDG labeled demobesin 1.
Figure 25: Keto-[\textsuperscript{18}F]FDG labeled demobesin 1 modified oxyamine by a) aminooxyacetyl linker (represented by solid line) and b) lysine aminooxyacetyl linker (represented by dotted line) in citrate buffer at pH 2-3 at 60 °C for 30 minutes.

The concentrations of the demobesin 1 used in labeling of keto-[\textsuperscript{18}F]FDG were 0.25 mg/ml and 0.125 mg/ml. With the modification of aminooxyacetyl linker, 0.25 mg/ml of demobesin 1 gave radiochemical yield of 50 % whereas 70 % radiochemical yield with modification by lysine aminooxyacetyl linker. Similarly, with 0.125 mg/ml of demobesin 1, modification of aminooxyacetyl linker gave radiochemical yield of 20 % compared to 30 % radiochemical yield in case of lysine aminooxyacetyl.

In comparison of linkers, lysine aminooxyacetyl linker modified demobesin 1 gave higher radiochemical yields with keto-[\textsuperscript{18}F]FDG compared to the aminooxyacetyl linker modified demobesin 1 at same concentrations of peptides as shown in table 10. Hence, lysine aminooxyacetyl linker seems to be more appropriate for higher radiochemical yields.
3.10.3 Reference standard of keto-[\textsuperscript{18}F]FDG labeled model peptide

Keto-glucose was reacted with the modified demobesin1 in same conditions as keto-[\textsuperscript{18}F]FDG. The synthesized product was then purified and analyzed in mass spectroscopy.

3.10.4 HPLC purification of labeled model peptide with keto-[\textsuperscript{18}F]FDG from its unlabeled peptide

In order to increase the specific activity of the labeled radiopharmaceuticals, purification is essential from its unlabeled counterparts. Since, both the labeled and unlabeled demobesin1 showed close retention time in radio-HPLC, SPE is purification is not possible. Hence, we tried to purify them in slow HPLC gradient in order to increase the retention time difference between them and was able to purify the radiolabled demobesin 1 from its unlabled ones (see figure 26 and 27).
Figure 26: Radio (upper) and UV (lower) HPLC chromatogram of keto-[\(^{18}\)F]FDG labeled aminooxy acetylated demobesin 1 at gradient of 20-50 % acetonitrile (solvent B) in 0.1 % trifluoroacetic acid (TFA) (v/v) in H\(_2\)O (solvent A) for 50 minutes at a constant flow of 1 ml/min at UV 280 nm.

Figure 27: Radio (upper) and UV (lower) HPLC chromatogram of keto-[\(^{18}\)F]FDG labeled lysine aminooxy acetylated demobesin 1 at gradient of 20-40 % acetonitrile (solvent B) in 0.1 % trifluoroacetic acid (TFA) (v/v) in H\(_2\)O (solvent A) for 40 minutes at a constant flow of 1 ml/min at UV 280 nm.
## 4 Material and Method

Following are the reagents and solvents used listed in the table 13 with their source and purity.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{18}\text{F}]$FDG</td>
<td>Umbra Medical (Hennef, Germany)</td>
<td></td>
</tr>
<tr>
<td>$[^{19}\text{F}]$FDG</td>
<td>ABX advanced biochemical compounds</td>
<td>&gt;99 %</td>
</tr>
<tr>
<td>4-Nitroaniline</td>
<td>Sigma-Aldrich</td>
<td>≥99 %</td>
</tr>
<tr>
<td>Acetylacetone</td>
<td>Sigma-Aldrich</td>
<td>≥99 %</td>
</tr>
<tr>
<td>$N,N$-Dimethylphenylenediamine</td>
<td>Aldrich</td>
<td>97 %</td>
</tr>
<tr>
<td>$O$-Benzylhydroxylamine</td>
<td>Aldrich</td>
<td>99 %</td>
</tr>
<tr>
<td>Aniline</td>
<td>Aldrich</td>
<td>99.5+ %</td>
</tr>
<tr>
<td>Vapreotide (RC160)</td>
<td>Bachem (Germany)</td>
<td></td>
</tr>
<tr>
<td>Demobesin</td>
<td>American Peptide Company</td>
<td></td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>Merck</td>
<td>≥99 %</td>
</tr>
<tr>
<td>$p$-Anisidine</td>
<td>Sigma-Aldrich</td>
<td>≥99 %</td>
</tr>
<tr>
<td>4-Hydrazinobenzoic acid</td>
<td>Fluka</td>
<td>~97 %</td>
</tr>
<tr>
<td>4-(Bromomethyl)benzoic acid</td>
<td>Sigma-Aldrich</td>
<td>97 %</td>
</tr>
<tr>
<td>(Boc-aminooxy)acetic acid</td>
<td>Aldrich</td>
<td>≥98 %</td>
</tr>
<tr>
<td>2,3,5,6-Tetrafluorophenol</td>
<td>Aldrich</td>
<td>97 %</td>
</tr>
<tr>
<td>Methyl-4-(bromomethyl)benzoate</td>
<td>Aldrich</td>
<td>98 %</td>
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<tr>
<td>$N$-Boc-hydroxylamine</td>
<td>Aldrich</td>
<td>≥98 %</td>
</tr>
<tr>
<td>Di-tert-butyl dicarbonate</td>
<td>Fluka</td>
<td>&gt;97 %</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>Sigma-Aldrich</td>
<td>≥99.5 %</td>
</tr>
<tr>
<td>$N,N$-Dicyclohexylcarbodiimide</td>
<td>Fluka</td>
<td>~99 %</td>
</tr>
<tr>
<td>Reagent</td>
<td>Supplier</td>
<td>Purity</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>----------------------</td>
<td>------------</td>
</tr>
<tr>
<td>N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide</td>
<td>Fluka</td>
<td>≥98 %</td>
</tr>
<tr>
<td>1-Hydroxy-benzotriazole</td>
<td>Fluka</td>
<td>≥98 %</td>
</tr>
<tr>
<td>4-Nitrophenylethylamine HCl</td>
<td>Aldrich</td>
<td>95 %</td>
</tr>
<tr>
<td>2-(2-Napthyl)-ethylamine HCl</td>
<td>Aldrich</td>
<td>97 %</td>
</tr>
<tr>
<td>N,N-Dimethylformamide</td>
<td>Sigma-Aldrich</td>
<td>≥99.5 %</td>
</tr>
<tr>
<td>N-Ethylidiisopropylamine</td>
<td>Merck</td>
<td>≥98 %</td>
</tr>
<tr>
<td>1,8-Diazabicyclo-(5,4,0)undec-7-en</td>
<td>Aldrich</td>
<td>98 %</td>
</tr>
<tr>
<td>N-Fmoc-1,6-hexanediamine</td>
<td>Sigma-Aldrich</td>
<td>≥98 %</td>
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<tr>
<td>Ethanol</td>
<td>Merck</td>
<td>Absolute</td>
</tr>
<tr>
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<td>Merck</td>
<td>≥99.7 %</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>Acors organics</td>
<td>&gt;99 %</td>
</tr>
<tr>
<td>Hexane</td>
<td>Acors organics</td>
<td>&gt;99 %</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Promochem</td>
<td>HPLC grade</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Merck</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>Water</td>
<td>Merck</td>
<td>For chromatography</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>Sigma-Aldrich</td>
<td>99 %</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>Merck</td>
<td>99.9 %</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>Merck</td>
<td>99 %</td>
</tr>
<tr>
<td>Tri-sodium citrate dehydrate</td>
<td>Merck</td>
<td>99 %</td>
</tr>
<tr>
<td>Sodium hydroxide (1N)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Hydrochloric acid (1N)</td>
<td>Merck</td>
<td></td>
</tr>
</tbody>
</table>

Table 13: Reagents and solvents
5 Analytical Method

5.1 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer with the internal standard of tetramethylsilane (TMS). The chemical shifts are calibrated in ppm and coupling constants \( (J) \) in Hertz (Hz) at chemistry department of university of Bonn.

5.2 Mass spectroscopy

Electrospray ionization (ESI) technique was used for the measurement in a micrOTOF Q-flight spectrometer from Bruker Daltonics (Bremen, Germany) in chemistry department of university of Bonn.

5.3 Radio-HPLC

Analytical reversed phase high performance liquid chromatography was performed using LiChrospher 100 RP (18-5 μ, 250 x 4 mm) CS-chromatographie (Langerwehe, Germany) columns, kontron gradienten pump (322), equipped with a rheodyne injector (7125). A UV-detector from Aligent (1100) and a NaI(Tl)-Bohrloch scintillation detector (Raytest) were used for mass and radio detection. Different gradient conditions were used with respect to the varying compounds studied.
5.3.1 Standard radio-HPLC conditions for measurement

A general gradient of 0-90 % acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 30 minutes at a constant flow of 1 ml/min and wavelength of 254 nm was used. In the case of chromatographing the peptide demobesin 1 and its modified or labeled products a wavelength 280 nm was used.

5.3.2 Radio-HPLC conditions for purification purposes

i. Purification of keto-[¹⁸F]FDG from keto-glucose

Isocratic conditions of 0.1 % trifluoroacetic acid (TFA) (v/v) in H₂O for 30 minutes at a constant flow of 1 ml/min at 254 nm ultraviolet photometer was used for purification of keto-[¹⁸F]FDG from keto-glucose.

ii. Purification of keto-[¹⁸F]FDG labeled demobesin 1

A gradient of 20-50 % acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 50 minutes at a constant flow of 1 ml/min and a wavelength of 280 nm for UV detection was used to purify keto-[¹⁸F]FDG labeled aminooxyacteyl linker modified demobesin 1 from its unlabeled peptide demobesin 1.

A gradient of 20-40 % acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 40 minutes at a constant flow of 1 ml/min and a wavelength of 280 nm for UV detection was used to purify keto-[¹⁸F]FDG labeled lysine aminooxyacteyl linker modified demobesin 1 from its unlabeled peptide demobesin 1.
5.4 Conditioning of SPE-cartridges

Throughout the experimental work of this thesis following cartridges were used and conditioning of those cartridges is described as follows:

5.4.1 C18 cartridges

The cartridges were conditioned by rinsing with 5 ml of acetonitrile followed by 5 ml of water.

5.4.2 HRP cartridges

The cartridges were conditioned by rinsing with 5 ml of acetonitrile followed by 5 ml of water.

5.4.3 Silica plus cartridges

Silica plus cartridges were used in micropreparative column chromatography by using an assembly of three silica plus cartridges. The cartridges were rinsed with hexane followed by rinsing with 10 ml solvent used to run in column chromatography i.e. EtOAc:Hexane = 1:3.

5.5 Thin layer chromatography (TLC)

TLC was performed on aluminum sheets of silica gel 60 F_{254} (Merck) to visualize the UV active components by UV light of 254 nm.
6 Experimental

6.1 Synthesis of keto-[^{18}F]FDG

6.1.1 Synthesis of keto-[^{18}F]FDG by using saturated sodium bicarbonate solution

Aliquot of 25µl [^{18}F]FDG solution were dissolved in 100 µl of saturated sodium bicarbonate in presence of different concentration of acetylacetone diluted in a volume of 25 µl acetonitrile. The mixture were then heated at 100 °C for 15 minutes in a closed glass vial. The radiochemical yields of the reaction were measured by radio-HPLC.

6.1.2 Synthesis of keto-[^{18}F]FDG by using carbonate buffer (NaHCO$_3$:Na$_2$CO$_3$)

Aliquots of 25µl [^{18}F]FDG solution were dissolved in 100 µl of different ratios of sodium bicarbonate and sodium carbonate of 0.1 mmol (8.40 mg of NaHCO$_3$ and 10.59 mg of Na$_2$CO$_3$) in presence of 5 µl of acetylacetone. The reactions mixtures then heated at 100 °C for 15 minutes in closed glass vial. The radiochemical yields were measured by radio-HPLC.

6.1.3 Scale up of reaction volume

To different volumes of [^{18}F]FDG, 10 µl of acetylacetone, 0.5 mmol of sodium bicarbonate (42 mg) and sodium carbonate (52.9 mg) were added. The final reaction volume was adjusted to 1 ml by adding saline or water. The reaction mixtures were then heated at 100 °C for 15 minutes. The radiochemical yields were measured by radio-HPLC.
6.2 Purification of keto-$^{18}$FFDG

To the reaction mixture of 1 ml, 1 M HCl (2 ml) was added. The acidic mixture was then diluted with 3 ml of water and passed through a HRP cartridge. The cartridge was then rinsed with 5 ml of water followed by 3 ml of DCM. Keto-$^{18}$FFDG was then eluted with 2 ml of 70 % ethanol in water. The 70 % ethanol in water solvent was evaporated in a stream of argon in case of need to concentrate the radioactivity to a lower volume.

6.3 Purification of keto-$^{18}$FFDG from keto-glucose

Purification of keto-$^{18}$FFDG from keto-glucose was performed in following ways:

6.3.1 HPLC purification of keto-$^{18}$FFDG from keto glucose

Keto-$^{18}$FFDG was chromatographed using various HPLC gradient conditions i.e.

- a) 0-40 % ACN (solvent B) and 0.1 % TFA (v/v) in H$_2$O (solvent A).
- b) 0-10 % ACN (solvent B) and 0.1 % TFA (v/v) in H$_2$O (solvent A).
- c) 0-5 % ACN (solvent B) and 0.1 % TFA (v/v) in H$_2$O (solvent A).

All gradient were performed at a constant flow of 1 ml/min and a wavelength of 254 nm for UV detection.

A volume of 100 µl of 0.004 mmol of keto-glucose dissolved in acetonitrile in water was chromatographed in HPLC. 1 ml fractions of the eluted solvent were collected for 20 minutes. 100 µl samples of each 1 ml fraction were reacted with 100 µl of 5 mg/ml of O-benzylhydroxylamine solutions at a pH of 5-6 in sodium acetate buffer at 60 °C for 15. The fractions containing keto-glucose were indentified by the UV active reaction product of keto-glucose and O-benzylhydroxylamine (see 3.3.1).
6.3.2 SPE purification of keto-[\(^{18}\text{F}\)]FDG from keto-glucose

3 mg of keto-glucose was dissolved in acetonitrile and water, and then it was passed through a HRP cartridge. Two HRP cartridges were rinsed with 200 ml of water. The percentage of remaining keto-glucose was determined after rinsing with 5 ml of DCM and elution with 2 ml of 70 % ethanol in water. 100 μl of final solution was reacted with 100 μl of 5 mg/ml of O-benzylhydroxylamine solution in order to produce the UV active oxime product. The formation of the oxime was checked by radio-HPLC at 220 nm UV detection.

6.4 Synthesis of keto-[\(^{18}\text{F}\)]FDG

The synthesis keto-[\(^{18}\text{F}\)]FDG was performed by dissolving anhydrous sodium carbonate (63.58 mg, 0.6 mmol) and sodium hydrogen carbonate (50.4 mg, 0.6 mmol) in 1 ml of [\(^{18}\text{F}\)]FDG solution (as delivered for patient studies) and subsequent addition of pentane 2,4 dione (10 μl, 0.09 mmol). The mixture was heated at 100 °C for 20 minutes to achieve radiochemical yields of >90 %. The reaction solution was then acidified with 2 ml of 1M HCl (pH 2-3) and purified by passing the solution through a SPE CHROMAFIX ® HR P cartridge (680 mg) and subsequent washing with 5 ml of dichloromethane (DCM). The product was eluted usually by 2 ml of ethanol: water (1:1) and analyzed by radio-HPLC. The product can alternatively be eluted with other solvents such as ethanol, DMF, acetonitrile alone or even with mixtures of those solvents with water which offers a high flexibility in the choice of the solvents.
6.5 Keto-[\textsuperscript{19}F]FDG reference standard

5 mg of 2-fluoro-2-deoxyglucose ([\textsuperscript{19}F]FDG) was processed as described for keto-[\textsuperscript{18}F]FDG and the product was analyzed by NMR. The retention time of keto-[\textsuperscript{18}F]FDG ($t_R = 6$ minutes) using a gradient of 0-90 % acetonitrile (solvent B) and 0.1 % trifluoroacetic acid (TFA) (v/v) in H$_2$O (solvent A) at a constant flow of 1 ml/min at 254 nm corresponds to this standard.

$^1$H NMR (400 MHz, D$_2$O): $\delta$ (ppm) 2.20(s, 3H, CH$_3$), 2.7(dd, $J=9$, 17Hz, 1H, H-3a'), 2.9(dd, $J=2.2$, 17.3 Hz, 1H, H-3b'), 3.34-3.38(m, 2H, H-4,5), 3.55-3.62(m, 2H, H-6a,6b), 3.7(dd, $J=1.75$, 12.4 Hz, 1H, H-3), 3.9(tt, $J=2.6$, 9.2 Hz, 1H, H-1), 4.07(dt, $J=9.1$, 50.2 Hz, 1H, H-2).

$^{13}$C NMR (100 MHz, D$_2$O): $\delta$ (ppm) 32.05 (s, CH$_3$), 47.17 (s, CH$_2'$), 62.6 (s, CH$_2$, C-6), 71.51 (d, $J=9.4$ Hz, C-4), 74.6 (d, $J=23.2$ Hz, C-1), 77.4 (d, $J=17.2$ Hz, C-3), 81.71(s, C-5), 94.3 (d, $J=181.6$ Hz,C-2), 214.6(s, C=O).
6.6 Reactivity of $^{18}$F]FDG and keto-$^{18}$F]FDG with O-benzylhydroxylamine

Different concentrations from 0.031 mmol to 0.0031 mmol of O-benzylhydroxylamine were prepared in acidic to basic buffers with presence or absence of 100 mM aniline (or its derivatives i.e. $p$-anisidine, 4-nitroaniline and $N,N$-dimethylphenylenediamine). To 100 µl buffered O-benzylhydroxylamine (in presence or absence of catalyst), 25 µl of $^{18}$F]FDG solution was added. The mixtures were heated at different temperatures and varying reaction times.

6.6.1 Reactivity of $^{18}$F]FDG with O-benzylhydroxylamine in presence of catalyst

In absence of aniline, pH 3-4 is favourable for the oxime formation whereas aniline shows its catalytic effect especially at pH 5-6 as shown in figure 12a and 12b. The aniline derivative, $p$-anisidine (data not shown) also showed the similar pattern of catalytic role. The temperature for the reaction could be decreased from 100 °C to 80°C for pH 5-6 with the use of catalyst in a time period of 20 minutes.
6.6.2 Reactivity of keto-[\textsuperscript{18}F]FDG with \textit{O}-benzylhydroxylamine in presence of catalyst

The favorable temperature for the reaction of keto-[\textsuperscript{18}F]FDG with \textit{O}-benzylhydroxylamine in presence of aniline is 60 °C (see figure 14). This lower optimal temperature of 60 °C (compared to 80 °C for [\textsuperscript{18}F]FDG) also stands for a higher reactivity of keto-[\textsuperscript{18}F]FDG. keto-[\textsuperscript{18}F]FDG was dramatically more reactive towards oxyamine i.e. \textit{O}-benzylhydroxylamine than [\textsuperscript{18}F]FDG as 0.05 mg/ml of \textit{O}-benzylhydroxylamine was reacted to give 50 % of oxime at pH 3-4 even without catalyst. The catalytic role of aniline/p-anisidine increases the yield of oxime (product of keto-[\textsuperscript{18}F]FDG with \textit{O}-benzylhydroxylamine) from 50 % (pH 3-4) and 30 % (pH 5-6) to 90 % at both pH as presented in figure 15.
6.7 Stability test of keto-[\(^{18}\)F]FDG product

6.7.1 *In vitro* stability of oxime

i. Keto-[\(^{18}\)F]FDG conjugated with aminooxy acetic acid

Boc-aminooxy acetic acid (8 mg, 0.04 mmol) was treated with TFA to cleave the boc group from the aminooxy acetic acid. The completion of the reaction was monitored by HPLC. After completion TFA was evaporated with a stream of argon. The deprotected aminooxy acetic acid was dissolved in 2 ml of keto-[\(^{18}\)F]FDG in ethanol:water (1:1). The pH was adjusted to 2.5 and the solution heated at 100 °C for 15 minutes. The product was purified by using HRP cartridge. After applying the reaction solution to the cartridge, it was washed with 5 ml of water to remove excess of aminooxy acetic acid and eluted with 30 % ethanol in 70 % sodium hydrogen carbonate (0.01 M) solution. The ethanol was evaporated and 25 μl of the product by volume was incubated in 475 μl blood serum at 37 °C. The stability was checked by HPLC at intervals of one hour for an overall period of four hours. After treating the blood serum sample with acetonitrile the supernatant solution was analyzed by HPLC.

ii. Keto-[\(^{18}\)F]FDG conjugated with *O*-benzylhydroxylamine

*O*-benzylhydroxylamine (8 mg, 0.05 mmol) was dissolved in 1 ml of citric buffer of pH 2.5 and mixed with 2 ml of keto-[\(^{18}\)F]FDG eluted from a HRP cartridge by ethanol:water (1:1) and heated at 100 °C for 15 minutes. The oxime product was purified using a C18 Plus SepPak cartridge and washing with 10 ml of 10 % acetonitrile in water. The oxime was eluted with 90 % ethanol. The solvent was evaporated to dryness and the product was redissolved in water. The stability in blood serum was performed as described in 6.7.1.
6.8 Synthesis of linkers

6.8.1 1,3,5,6-tetrafluorophenyl ester of boc protected aminooxy acetic acid (TFP boc-AOA ester) (2)

Boc-aminooxy acetic acid 1 (250 mg, 1.307 mmol) was dissolved in DCM followed by addition of 1,3,5,6-tetrafluorophenol (TFP) (217.16 mg, 1.307 mmol) and DCC (269.81 mg, 1.307 mmol) at room temperature (see scheme 15). The reaction was monitored by HPLC and after completion of the reaction, ether was added. The insoluble byproducts were filtered off. The solvents of the filtrate were evaporated and the ester appeared as a white powder with a yield of 70%.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.51 (s, 9H, Boc), 4.81 (s, 2H, CH$_2$), 7.05 (S, m, Ar), 7.69 (s, 1H, Boc-NH).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 28.15 (CH$_3$, Boc), 72.03 (CH$_2$), 82.73 (Cq, Boc), 103.55, 103.78, 104.01 (Ar), 156.33 (C=O, Boc), 165.62 (C=O).

6.8.2 Hydroxybenzotriazolyl ester of boc protected aminooxybenzoic acid (HOBt boc-PABA ester) (7)

i. Synthesis of methyl-4-(boc-aminooxymethyl)benzoate (5)

Methyl-4-(bromomethyl)benzoate 3 (1 g, 4.365 mmol) was dissolved in 10 ml DCM and stirred with excess N-boc-hydroxylamine 4 (697.48 mg, 5.238 mmol) in presence of diaza-(1,3)-bicyclo-[5.4.0]-undecane (DBU) (930.39 mg, 6.11 mmol) at room temperature for about 24 hours (see scheme 15). The completion of the reaction was checked by HPLC. The DCM phase was washed with acidic water (pH 2-3) in a separatory funnel. The aqueous layer was discarded and DCM was dried with anhydrous sodium sulphate and filtrated DCM was evaporated to give a yield of >90% ester.
ii. 4-(boc-aminoxyymethyl)benzoic acid (6)

Methyl-4-(boc-aminoxyymethyl)benzoate 5 (0.5 g, 1.8 mmol) was hydrolyzed with 1M sodium hydroxide in a mixture of acetonitrile and water (1:1) at room temperature (see scheme 15). The completion of the reaction was checked by HPLC. The workup of the reaction was the same as described in 6.8.2.i. The product was a white solid with a yield of >90%.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.49 (s, 9H, Boc), 4.93 (s, 2H, CH$_2$), 7.48-7.51 (m, 3H, Ar and NH), 8.09 (d, $J=8.4$ Hz, 2H, Ar).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 28.16 (CH$_3$, Boc), 77.73 (CH$_2$), 82.19 (Cq, Boc), 128.67, 129.47, 130.30, 141.60 (Ar), 157.05 (C=O, Boc), 171.16 (COOH).

iii. Esterification of 4-(boc-aminoxyymethyl)benzoic acid (7)

4-(Boc-aminoxyymethyl)benzoic acid 6 (225.50 mg, 0.84 mmol) and (diBTC) (250 mg, 0.84 mmol) were dissolved in DCM in presence of triethylamine (TEA) (0.84 mmol) at room temperature (see scheme 15). The completion of the reaction was checked by HPLC. The workup of the reaction was performed as described in 6.8.2.i. The product was colorless viscous oil with a yield of 90%.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.50 (s, 9H, Boc), 5.01 (s, 2H, CH$_2$), 7.44-7.58 (m, 4H, HOBt, H-4, 5, 6, 7), 7.64 (d, $J=8.7$ Hz, 2H, Ar), 8.11 (dt, 1H, $J=8.5$Hz, oxyamine), 8.26-8.29 (m, 2H, Ar).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 28.16 (CH$_3$, Boc), 77.44 (CH$_2$), 82.23 (Cq, Boc), 108.31, 120.55, 124.50, 124.89, 128.81, 130.88, 143.51, 144.04 (Ar), 156.81 (C=O, Boc), 162.47 (C=O).
6.8.3 Hydroxybenzotriazolyl ester of boc protected hydrazinobenzoic acid (HOBt boc-HBA ester) (10)

i. Synthesis of boc-hydrazinobenzoic acid (9)

4-Hydrazinobenzoic acid 8 (1.3 g, 8.5 mmol) was dissolved with di-tert-butylcarbonate (1.9 g, 9.16 mmol) and triethylamine (TEA) (0.926 g, 9.16 mmol) in 10 ml dimethylformamide (DMF) and was stirred for 24 hours at room temperature (see scheme 15). The completion of the reaction was checked by HPLC. The product was dissolved in diethyl ether and washed with acidic water (pH 2-3) in a separatory funnel. The aqueous layer was discarded and the organic layer was dried with anhydrous sodium sulphate. The product appeared as a pale brown solid after solvent evaporation with a yield of 80 %.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.27 (s, 9H, Boc), 6.17 (s, 1H, NH), 6.59 (s, 1H, NH), 6.8 (d, $J$=8.7 Hz, 2H, Ar), 7.9 (d, $J$=8.7 Hz, 2H, Ar).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 28.16 (CH$_3$, Boc), 81.69 (Cq, Boc), 111.72, 121.58, 128.29, 129.97, 131.89 (Ar), 152.74 (C=O, Boc), 169.83 (COOH).

ii. Esterification of 4-hydrazinobenzoic acid (10)

Boc-hydrazinobenzoic acid 9 (500 mg, 1.98 mmol), N,N-dicyclohexylcarbodiimide (DCC) (449.79 mg, 2.18 mmol) and 1-hydroxybenzotriazole (HOBt) (294.58 mg, 2.18 mmol) were dissolved in 10 ml of dry acetonitrile at room temperature (see scheme 15). The completion of the reaction was checked by HPLC. The workup was performed in C18 cartridge by washing with water, 10 % acetic acid to wash off base and tyramine, 10 % acetonitrile to wash off HOBt. The workup of the reaction was the same as described in 6.8.3.i. The product appeared as a yellow solid after evaporation of solvent with a yield of 70 %.
$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.49 (s, 9H, Boc), 6.45 (s, 1H, NH), 6.63 (s, 1H, NH), 6.91-6.94 (m, 2H, Ar), 7.40-7.54 (m, 3H, HOBt H-4, 5, 6), 8.07 (d, $J$=8.5 Hz, 1H, HOBt H-7), 8.11-8.15 (m, 2H, Ar).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 28.18 (CH$_3$, Boc), 82.11 (Cq, Boc), 108.50, 112.10, 120.38, 124.71, 128.13, 128.22, 132.86, 143.52 (Ar), 154.52 (C=O, Boc), 162.43 (C=O).

6.8.4 1,3,5,6-tetrafluorophenyl ester of boc protected lysine aminooxy amino acid (TFP boc-AOA lysine ester) (13)

i. Synthesis of boc protected AOA lysine acid (12)

H-D-Lys(Boc)-OH (11) (65 mg, 0.26 mmol) was dissolved in 15 ml DMF in presence of DIPEA (50 µl, 0.3 mmol) and TFP AOA ester (100 mg, 0.3 mmol). The mixture was sonicated at room temperature (see scheme 16). The completion of the reaction was checked by HPLC. After the completion of the reaction, the mixture was diluted in a 0.01 N HCl and CH$_2$Cl$_2$. The acidic aqueous layer was discarded and organic layer were evaporated in rotatory evaporator.

ii. Esterification of boc protected AOA lysine acid

The dried boc protected AOA lysine acid (2) (100 mg, 0.17 mmol), TFP (20 µl, 0.17 mmol) and DCC (36.3 mg, 0.17 mmol) were dissolved in CH$_2$Cl$_2$ at RT and reactecd for 12 hours (see scheme 16). The completion of the reaction was checked by HPLC. The product was purified from TFP by three silica plus cartridges with solvent EtOAc:Hex = 1:3, $R_f$ = 0.2.
$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 1.41 (s, Boc, 18H), 1.96 (s, $\gamma$H, Lysine, 2H), 3.11 (b, $\delta$H and $\beta$H, Lysine, 3H), 4.3 (m, OCH$_2$ and $\beta$H, Lysine, 3H) and 4.67 (b, $\varepsilon$H Lysine, 1H), 4.8 (m, $\alpha$H, $\varepsilon$H Lysine and Lysine Boc NH, 2H), 6.97 (m, Aromatic, 1H), 8.01 (s, Boc NH, 1H), 8.81 (b, Lysine NH, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 22.93, 28.41 (CH$_3$, Boc), 28.75, 31.43, 40.47, 52.17, 76.28 (OCH$_2$), 83.62 (Cq, Boc), 103.63, 103.81, 103.99 (Ar), 158.55 (C=O, Boc), 168.43 (C=O, Boc), 170.04 (C=O).
Scheme 15: Synthesis of linkers a) tetrafluorophenyl ester of boc-aminoxy acetic acid and b) benzotriazolyl ester of boc-protected p-oxyaminobenzoic acid c) benzotriazolyl ester of boc-protected 4-hydrizinobenzoic acid.
Scheme 16: Synthesis of Boc protected AOA lysine acid.
6.9 Reaction of UV-active amines with oxyamine linkers

The aminoxyacetic acetyl (from TFP AOA active ester) and aminoxyacetic acetyl lysine (from TFP AOA lysine active ester) linkers were used to modify UV active amines. The boc protected amides were then deprotected for labeling as described below.

6.9.1 H-Lys(Fmoc)-OMe

TFP AOA active ester

TFP AOA active ester (0.007 mmol, 2.5 mg) was dissolved in 100 µl of DMF with addition of N-ethyl-di-isopropylamine (0.007, 1.19 µl) followed by addition of H-Lys(Fmoc)-OMe (0.007 mmol, 2.93 mg). The mixture was left at room temperature for 30 minutes and reaction progress was checked by HPLC. After the completion of reaction, the mixture was diluted with water and passed through a C18 cartridge (Sep Pak Plus). After a rinse with 5 ml of acidic water of pH 2-3 and 5 ml of 10 % acetonitrile in water. The product was eluted with 2 ml of 90 % ethanol in water. The product was dried with a stream of argon and treated with 50 µl of TFA dissolved in 50 µl of DCM for 30 minutes. The deprotection of the boc group was checked by HPLC.
6.9.2 Tyramine

i. TFP AOA active ester

TFP AOA lysine active ester (0.003 mmol, 1 mg) was dissolved in 100 µl of DMF with addition of \(N\)-ethyl-di-isopropylamine (0.003, 0.5 µl) followed by addition of tyramine (0.003 mmol, 0.40 mg). Other reaction procedures were similar as described in 6.9.1.

ii. TFP AOA lysine active ester

TFP AOA lysine active ester (0.0017 mmol, 1 mg) was dissolved in 100 µl of DMF with addition of \(N\)-ethyl-di-isopropylamine (0.0017, 0.29 µl) followed by addition of tyramine (0.0017 mmol, 0.23 mg). Other reaction procedures were similar as described in 6.9.1.

6.9.3 4-nitrophenylenethylamine

i. TFP AOA active ester

TFP AOA lysine active ester (0.003 mmol, 1 mg) was dissolved in 100 µl of DMF with addition of \(N\)-ethyl-di-isopropylamine (0.003, 0.5 µl) followed by addition of tyramine (0.003 mmol, 0.6 mg). Other reaction procedures were similar as described in 6.9.1.

ii. TFP AOA lysine active ester

TFP AOA lysine active ester (0.0017 mmol, 1 mg) was dissolved in 100 µl of DMF with addition of \(N\)-ethyl-di-isopropylamine (0.0017, 0.29 µl) followed by addition of tyramine (0.0017 mmol, 0.34 mg). Other reaction procedures were similar as described in 6.9.1.
6.9.4 *N*-Fmoc-1,6-hexanediamine

**TFP AOA active ester**

TFP AOA lysine active ester (0.003 mmol, 1 mg) was dissolved in 100 µl of DMF with addition of *N*-ethyl-di-isopropylamine (0.002, 0.5 µl) followed by addition of tyramine (0.002 mmol, 1.25 mg). Other reaction procedures were similar as described in 6.9.1.
6.10 Labeling of peptides

6.10.1 RC160 (vapreotide)

RC160 (vapreotide) was modified and labeled as follows:

i. Modifying RC160 (vapreotide)

RC160 (Vapreotide) (2 mg, 1.5 µmol) was modified by reaction with tetrafluorophenyl ester of boc aminooxy acetic acid (0.50 mg, 1.5 µmol) at the lysine residue of the peptide. The active ester and the peptide were dissolved in 200 µl DMF in presence of diisopropylethylamine (DIPEA) (0.25 µl, 1.5 µmol). The completion of the reaction was checked by HPLC. The product was then trapped on a C18 cartridge after diluting the reaction mixture with water. After a rinse with acidic water (pH 2-3), the peptide was eluted with 90 % ethanol in water. Solvents were removed and treated with TFA for removal of the boc group. Finally, TFA was removed by a stream of argon.

ii. Radiolabeling of oxyamine modified RC160 (vapreotide)

The aminooxy modified RC160 (vapreotide) was redissolved in 90 % ethanol in water. Aliquots of different concentrations of peptide maintaining pH 2.5 by citric buffer (100 µl) in the influence of 100 mM aniline were reacted with keto-[\(^{18}\text{F}\)]FDG (25 µl) at 100 °C at various time period.
6.10.2 Labeling of model peptide

Demobesin 1 was modified to its protected oxyamine modified demobesin by two different active ester as described below:

i. TFP AOA ester

Demobesin (2 mg, 2 µmol) was dissolved in 200 µl of DMF with 200 µl of TFP AOA ester (1.4 mg, 4 µmol) in DMF in presence of DIPEA (0.7 µl, 4 µmol) reacted at RT for 2 h. The completion of the reaction was checked by HPLC. The reaction was then purified by C18 SPE after diluting with 5 ml of water. The C18 was washed with 2 ml water, 2 ml of 0.01 N HCl, 2 ml of ether and the product was eluted with 1 ml of 90 % EtOH in water. The solvents were evaporated with a stream of argon.


ii. TFP AOA lysine ester

Demobesin (2 mg, 2 µmol) was dissolved in 200 µl of DMF with 200 µl of TFP AOA Lysine ester (2.3 mg, 4 µmol) in DMF in presence of DIPEA (0.7 µl, 4 µmol) reacted at RT for 4 h. The completion of the reaction was checked by HPLC. The reaction was then purified by C18 SPE after diluting with 5 ml of water. The C18 was washed with 2 ml water, 2 ml of 0.01 N HCl, 2 ml of ether and eluted with 1 ml of 90 % EtOH in water. The solvents were evaporated with stream of argon.

iii. Deprotection of oxyamine modified demobesin of both ester

Protected oxyamine modified demobesin were deprotected from its boc-group by treating with 10 % TFA in CH₂Cl₂ at RT for 30 mins. The completion of the reaction was checked by HPLC.


For deprotection of oxyamine modified demobesin of TFP AOA lysine ester ESI-MS calcd. [M + H⁺] 1185.6; found [M + H⁺] 1185.7, [M + Na⁺ + H⁺] 593.4.

iv. Reference standard for radiolabeled demobesin 1

Cold keto-[¹⁹F]FDG synthesized as 3.2 was reacted with deprotected oxyamine modified demobesin at 60 °C for 30 mins in citrate buffer (pH 2.5) in influence of 100 mM aniline.

For keto-[¹⁹F]FDG labelled demobesin TFP AOA (CUT1):

keto-[¹⁹F]FDG labelled demobesin TFP AOA lysine (CUT2):

v. Radiolabeling of demobesin 1

25 µl keto-[¹⁸F]FDG, 62.5 µl of phosphoric buffer (pH 2.5) and 12.5 µl of oxyamine modified demobesin of both ester were reacted in a glass vial at 60 °C for 30 mins. The completion of the reaction was monitored by HPLC.
vi. Purification of radiolabeled demobesin 1

a. Purification of TFP AOA active ester modified radiolabeled demobesin

A gradient of 20-50% acetonitrile (solvent B) in 0.1% trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 50 minutes at a constant flow of 1 ml/min at 280 nm ultraviolet photometer was used. The unlabeled UV peak appeared at 23 minutes and radiolabeled at 28 minutes as E- and Z-isomers.

b. Purification of TFP Lysine AOA active ester modified radiolabeled demobesin 1

A gradient of 20-40% acetonitrile (solvent B) in 0.1% trifluoroacetic acid (TFA) (v/v) in H₂O (solvent A) for 40 minutes at a constant flow of 1 ml/min at 280 nm ultraviolet photometer was used. The unlabeled UV peak appeared at 25 minutes and radiolabeled at 28 minutes.
7 Summary and Conclusions

With the development of keto-[\(^{18}\text{F}\)]FDG in this work a new powerful prosthetic group for labeling of carbonyl reactive compounds has been added to the tools available for labeling biomolecules with the radionuclide fluorine-18. The main feature that distinguishes this prosthetic group from other existing fluorine-18 labeled carbonyl compounds is the starting material. Whereas most of the existing compounds start with \([^{18}\text{F}]\)-fluoride as primary labeling precursor, keto-[\(^{18}\text{F}\)]FDG starts with 2-[\(^{18}\text{F}\)]fluorodeoxyglucose ([\(^{18}\text{F}\)]FDG) as the radionuclide i.e. fluorine-18 bearing precursor. In terms of availability this circumstance has no restrictions as both \([^{18}\text{F}]\)fluoride as well as \([^{18}\text{F}\)]FDG are readily available to every site that is equipped with a medical cyclotron for the purpose of producing and supplying PET drugs. To the contrary \([^{18}\text{F}\)]FDG is even more widely available as it includes all \([^{18}\text{F}\)]FDG customer sites and \([^{18}\text{F}\)]FDG is the major workhorse of PET. However, in terms of chemical and biochemical properties of the resulting labeled compounds, using \([^{18}\text{F}\)]FDG as precursor leads itself to glycosylated biomolecules of interest. As such keto-[\(^{18}\text{F}\)]FDG might be of use only in selected cases where glycosylation may result in improved biochemical behavior of the labeled compound of interest. In this context it is important to note that the use of keto-[\(^{18}\text{F}\)]FDG, in comparison to \([^{18}\text{F}\)]FDG by itself as glycosylation agent, proved to be far superior with respect to its chemical reactivity. For example a low concentration of only 0.05 mg/ml \(O\)-benzylhydroxylamine when reacted with keto-[\(^{18}\text{F}\)]FDG was able to give the same high radiochemical yield of oxime as compared to a 5 mg/ml \(O\)-benzylhydroxylamine concentration needed for \([^{18}\text{F}\)]FDG to obtain the same high radiochemical yield and this even at lower reaction temperature of 60 °C (in case of keto-[\(^{18}\text{F}\)]FDG) compared to 80 °C (in case of \([^{18}\text{F}\)]FDG). Keto-[\(^{18}\text{F}\)]FDG also offers the additional advantage that it may be purified from accompanying stable keto-glucose by means of a simple
SPE based purification procedure which was also developed in the course of this work and was able to remove the keto-glucose to non detectable levels as measured by HPLC, while purification of \[^{18}F\]FDG from glucose is to our knowledge only possible using much more elaborate HPLC methods. This has important consequences with respect to the apparent specific activity of the final product and also for the absolute amount of biomolecules needed to achieve high radiochemical yields. Another interesting aspect is that keto-\[^{18}F\]FDG modified molecules in contrast to \[^{18}F\]FDG will retain the carbohydrate moiety in its ring closed form while using \[^{18}F\]FDG alone will lead to a ring opened structure.

Using keto-\[^{18}F\]FDG or \[^{18}F\]FDG as carbonyl group bearing prosthetic groups for labeling of biomolecules requires of course a carbonyl reactive moiety on the side of the biomolecule. The targetted group of biomolecules to be \[^{18}F\]-fluoroglycosylated in this work were bioactive peptides i.e. the bombesin analog demobesin 1. As peptides including demobesin 1 do usually not consist of the strongly carbonyl reactive moieties such as hydrazides or oxyamines these have to be attached to the peptides before the actual radiolabeling step can be performed. Therefore a series of bifunctional agents bearing boc-protected oxyamines or hydrazines on one end and an activated ester on the other end and as such acting as “linker” between the peptides amino groups and the carbonyl group of keto-\[^{18}F\]FDG was synthesized and tested first by using simple amines, then the model peptide RC160 and finally the bombesin analog demobesin 1.
Two derivatives were synthesized and radiolabeled. One was obtained by simple aminooxyacetylation while the second derivative was reacted with a special linker introducing not only the aminooxy moiety but also an additional lysine residue in order to add another potential positive charge to the peptide.

Within the group of simple amines they were easily modified by the synthesized linkers and reacted readily to form the expected oximes upon reaction with keto-\([^{18}\text{F}]\)FDG. Within the group of peptides RC160 was chosen as a first test example. RC160 was modified at the lysine group of the peptide by both linker and reacted with keto-\([^{18}\text{F}]\)FDG to a radiochemical yield of 40±2 % at a peptide concentration of 0.5 mg/ml. Demobesin 1 was also modified at D-penylalanine (D-Phe) residue of the peptide by both linker and reacted with keto-\([^{18}\text{F}]\)FDG. The high radiochemical yield of up to 70 % at a peptide concentration as low as 0.25 mg/ml.

In summary, keto-\([^{18}\text{F}]\)FDG proved to be a very useful and highly reactive \([^{18}\text{F}]\)-fluoroglycosylation agent. It can be easily prepared in one step from \([^{18}\text{F}]\)FDG with high radiochemical yields of > 90 % in 15 minutes. If desired additional purification from the keto-glucose analog is possible by using a simple SPE based method. The high radiochemical yields of with the bombesin analog demobesin 1 at a low peptide concentration show the high potential of this method for glycosylation and simultaneous fluorine-18 labeling of complex biomolecules.
## 8 Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>[^{18}F]FDG</td>
<td>2-fluoro-2-deoxyglucose</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computer tomography</td>
</tr>
<tr>
<td>PET</td>
<td>Position emission tomography</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>HEDP</td>
<td>Hydroxyethylidine diphosphate</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>CT</td>
<td>Computer tomography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>5'[^{18}F]FDA</td>
<td>5'-fluoro-5'-deoxyadenosine</td>
</tr>
<tr>
<td>NOTA</td>
<td>1,4,7-triazacyclononane-1,4,7-triacetic acid</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>GRP</td>
<td>Gastrin releasing peptide</td>
</tr>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>NMBR</td>
<td>Neuromedin receptor</td>
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<td>Pca</td>
<td>Prostate cancer</td>
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<tr>
<td>BLPs</td>
<td>Bombesin like peptides</td>
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<tr>
<td>BLI</td>
<td>Bombesin like immunoreactivity</td>
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<td>TA-[^{18}F]FDG</td>
<td>1,3,4,6-tetra-O-acetyl-2[^{18}F]FDG</td>
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<tr>
<td>SAA</td>
<td>Sugar amino acid</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>TFA</td>
<td>Trifluoro Acetic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>BFC</td>
<td>Bifunctional chelators</td>
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<tr>
<td>DCC</td>
<td>N,N-dicyclohexylcarbodiimide</td>
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<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>TFP</td>
<td>1,3,5,6-tetrafluorophenol</td>
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<td>DBU</td>
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<td>S&lt;sub&gt;n&lt;/sub&gt;Ar</td>
<td>Aromatic nucleophilic substitution</td>
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<td>EWG</td>
<td>Electron withdrawing groups</td>
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<td>fGRP</td>
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<td>BN</td>
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<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium Hexafluorophosphate</td>
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10 Abstract


(http://jnumedmtg.snmjournals.org/cgi/content/meeting_abstract/52/1_MeetingAbstracts/1660)

(achieved 1st place in poster presentation in Molecular Targeting Technologies-Radioactive & Nonradioactive Probes Track)