



Development of Molecular Probes Responsive to Matrix Metalloproteinases

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Abstract

Matrix metalloproteinases (MMPs) have been identified as biomarkers for cancer, offering prognostic potential. However, they lack non-invasive detection. This thesis highlights the work carried out on the design, synthesis and development of ^{19}F MRI probes for the molecular imaging of MMP activity.

The design of these MMP-activated imaging agents was based on the paramagnetic effects of gadolinium(III) on the NMR/MRI signal of a CF_3 group in close proximity to the metal cation. 1st generation probes were prepared, incorporating MMP-2 selective peptide substrates SPAYYTA or SLAYYTA as a linker between gadolinium(III)-DOTA and Asp[(trifluoromethoxy)benzylamide]-OH. Attempts to monitor MMP-2 mediated peptide hydrolysis via HPLC and fluorescent assays proved problematic. The use of ^{68}Ga and its complexation to DOTA provided a highly sensitive assay for the assessment of the peptide compounds. Using this method, assay conditions were optimised to observe on average 20% peptide cleavage over 2 hours with MMP-2, with selectivity observed against MMP-9 and MMP-14. The original gadolinium analogues of these compounds were then analysed by ^{19}F NMR spectroscopy, successfully demonstrating a change in signal upon incubation with MMP-2.

Development of the probes was then carried out to improve their clinical potential. Two pathways were explored; increasing compound reactivity through the introduction of spacer groups and increasing ^{19}F sensitivity via increased local concentrations of fluorine, carried out utilising activated cell penetrating peptides (ACPPs). The latter method proved more successful, displaying similar reactivity and selectivity to 1st

generation compounds. These ACPD probes are still in the early stages of development, with scope for future investigation.

The transferability of 1st generation MMP-2-activated probes was also investigated via the production of a compound of analogous design, incorporating an MMP-14 selective substrate RIGFLR. Testing and development revealed this compound required incorporation of a two amino acid spacer group (SG) in order to demonstrate partial MMP-14 selectivity.

Author's Declaration

The work described in this thesis was carried out within the Comprehensive Cancer Imaging Centre in the Department of Surgery and Cancer, and the Department of Chemistry at Imperial College London between October 2009 and June 2013. This was done under the supervision of Professor Eric Aboagye and Professor Ramon Vilar. All of the work is my own, except where stated otherwise, and has not been previously submitted for a degree at this or any other university.

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Abbreviations

ACPP – Activated cell penetrating peptide

APMA – 4-Aminophenylmercuric acetate

CPP – Cell penetrating peptide

DCM – Dichloromethane

DIPEA – N,N-Diisopropylethylamine

DMF – Dimethylformamide

DMSO – Dimethylsulphoxide

DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

DTPA – Diethylenetriaminepentaacetic acid

ECM – Extracellular matrix

EGFR – epidermal growth factor receptor

ESI – Electrospray ionisation

Fmoc – 9-fluoromethoxycarbonyl

FRET – Förster resonance energy transfer

HARP – Heparin affinity regulatory peptide

HATU – 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid
hexafluorophosphate

HBTU – O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate

HPLC – High performance liquid chromatography

MALDI – Matrix-assisted laser desorption/ionisation

MMP – Matrix metalloproteinase

MMPI – Matrix metalloproteinase inhibitor

MRI – Magnetic resonance imaging

MS – Mass spectrometry

NIRF – Near infrared fluorescence

NMM – *N*-methylpyrrolidone

NMP – *N*-methylmorpholine

NMR – Nuclear magnetic resonance

PET – Positron emission tomography

PRE – Paramagnetic relaxation enhancement

SEM – Scanning electron microscopy

SNR – Signal to noise ratio

SPE – Solid phase extraction

SPECT- Single photon emission computed tomography

TEM – Transmission electron microscopy

TFA – Trifluoroacetic acid

UV – Ultraviolet

VEGF – Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Cancer

Cancer research UK states that in the UK alone around 325,000 people were diagnosed with cancer in 2010 and worldwide 7.6 million people died from the disease in 2008.¹

² Undoubtedly improvements have been made year on year to increase survival rates, but in order to ‘fight’ a disease such as cancer greater understanding of the processes by which a cell becomes cancerous and spreads throughout the body is necessary. Such investigation could not only deliver valuable information when looking for new treatments for the disease but could also be utilised for early cancer detection and diagnostics to improve patient treatment.

Cancer is a disease which centres on mutations of the genome and is characterised by uncontrolled growth and spread of abnormal cells leading to fatality.^{3, 4} If we look in more detail we discover ‘cancer’ is in fact an umbrella term used to describe not one disease but many diseases, generating difficulties in understanding and treatment. Over the last few decades extensive research has revealed cumulative layers of complexity to the disease and in order to simplify such a diverse subject it is important to understand its underlying principles; these can be described by a series of markers. Six of hallmarks of cancer were originally highlighted by Hanahan and Weinberg in 2000; evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential and sustained angiogenesis.^{3, 4}

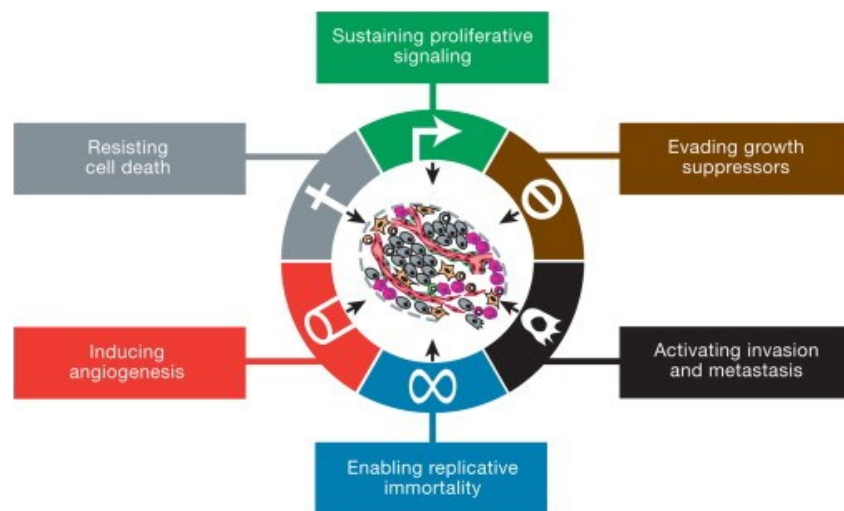


Figure 1- The six original hallmarks of cancer describing cellular changes which lead to malignant growth. Figure taken from Hanahan and Weinberg's original paper 'The Hallmarks of Cancer'.^{3,4}

A further 4 markers were later added to this list in 2011; avoiding immune destruction, tumour promoting inflammation, genome instability and mutation and deregulating cellular energetics.

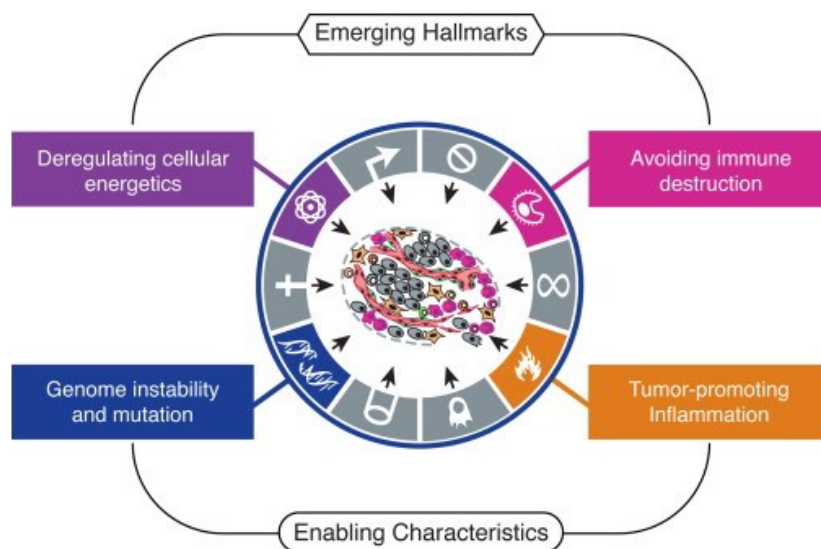


Figure 2- This picture describes four categories that were later added to the original six hallmarks of cancer. These were categorised in to two which were emerging hallmarks and two which are enabling characteristics necessary for the development of the original six hallmarks. Figure taken from Hanahan and Weinberg 'Hallmarks of Cancer: The next generation'.^{3,4}

These hallmarks of cancer are widely accepted to apply to numerous different types of cancer cell, and understanding these hallmarks provides good foundation for research into the disease.

Of the hallmarks originally identified by Hanahan and Weinberg in 2000, the acquired ability for tumour invasion and metastasis was highlighted as “the last great frontier of exploratory cancer research”.³ Within this hallmark one of the key areas of discussion was matrix degrading proteases. The major group of these proteases, called Matrix metalloproteinases (MMPs) have been found to be involved not only in invasion and metastasis, but also disease pathways such as angiogenesis.⁵ Additionally, these enzymes have also been implicated in a number of other processes tied to different hallmark capabilities.³ As such MMPs have been described as promising biomarkers for cancer and research has been carried out scrutinising their roles and their potential use within cancer treatment. Understanding these valuable biomarkers within cancer enables us to further our knowledge of the disease and the hallmarks the action of these biomarkers falls within. The use of techniques such as molecular imaging to investigate important biomarkers and processes within cancer has the potential to provide greater understanding of the disease via non-invasive methods. Additionally, this could produce a system of early tumour detection with diagnostic properties which could improve patient monitoring and treatment.⁶

1.2 *Matrix Metalloproteinases*

MMPs are a set of zinc dependent endopeptidases that are widely acknowledged to collectively have the capability of degrading all components of the extracellular matrix and basement membranes. Breakdown of the extracellular matrix (ECM) is crucial for

the body's normal function for processes such as embryonic development, morphogenesis, reproduction, and tissue remodelling and resorption.^{7, 8} More recently new functions of MMPs have been uncovered extending beyond simple degradative enzymes, for example in regulation of signalling molecules.^{5, 7-10}

1.2.1 Structure and activity - classes of MMP

The MMP family is comprised of around 24 distinct gene encoding members with related structures. Basic MMP domain structure consists of a signal peptide which directs cell secretion and a conserved catalytic domain which contains a zinc binding motif.^{10, 11} The catalytic domain also incorporates a pro-peptide which coordinates to the catalytic site maintaining latency until activation.

While all members of the MMP family contain similar primary, secondary and tertiary structures likely relating to a common ancestor, evolution and distinction of each individual member has resulted in structural and functional diversity.¹² Different MMPs maintain different roles within the body, for example collagenases are responsible for initial cleavage of the tough triple helical fibres in collagens, whose degradation products can then be further divided up by gelatinases.¹³ Structural differences (i.e. the inclusion of differing domains and modules) accommodate this substrate specificity and define the various functions of MMPs. MMPs can be organised into subgroups based on their structural design as shown below in Figure 3.¹⁰

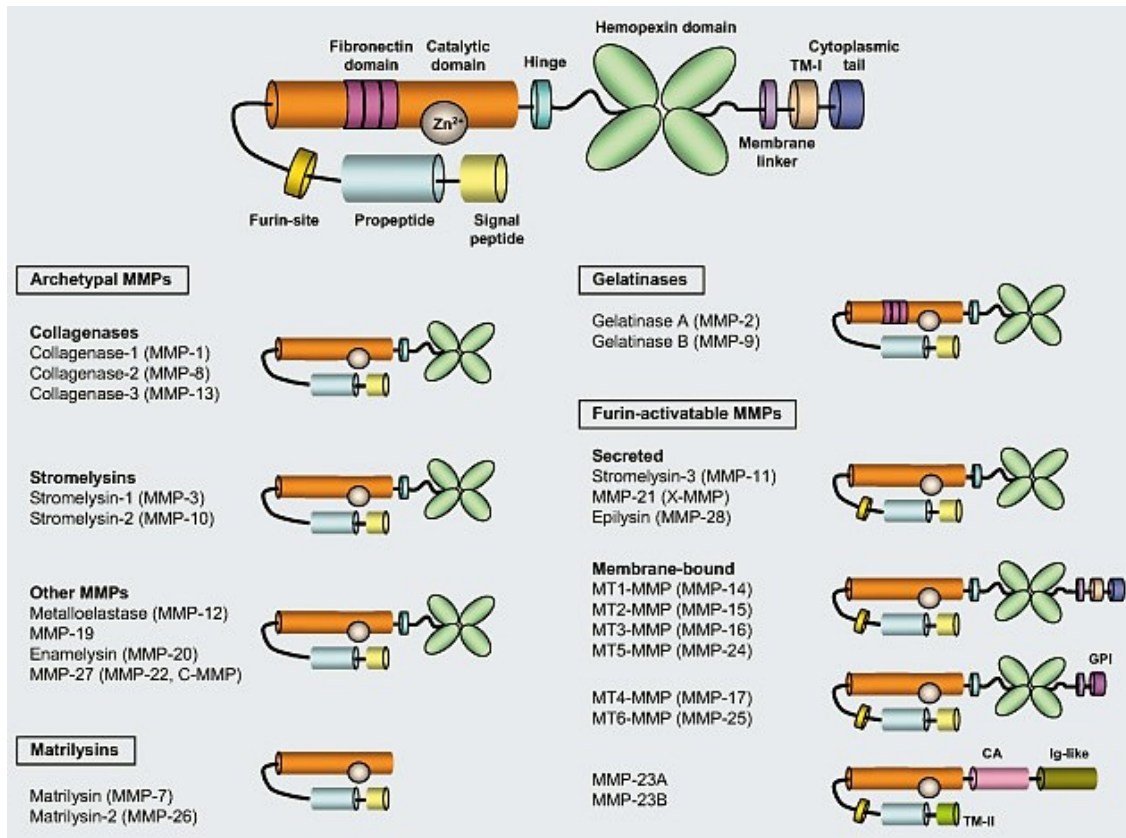


Figure 3- Schematic description of Human MMPs structure and their structural classification based on domain organisation. Figure taken from Folgueras *et al.*¹⁰

MMPs in the Matrilysins subgroup are made up from the minimal domain structure as previously described, with MMP-7 being the smallest in size.⁷ Collagenases and Stromelysins as well as a number of other secreted MMPs follow an archetypal structural design, which is made up of the signal peptide and catalytic domain present in the minimal design, along with a C-terminal hemopexin domain which plays a role in substrate specificity and interactions with endogenous inhibitors.^{10, 12} Gelatinases structure is closely related to that of the archetypal MMP group though with addition of a fibronectin domain which provides a collagen binding domain.^{10, 12} There is also a subgroup of furin-activateable MMPs which contain basic residues in-between the catalytic domain and pro-peptide which may be cleaved by furin like serine proteases allowing activation of the catalytic site, these include some MMPs which are not secreted but membrane bound.^{10, 12} Membrane bound MMPs are localised on the cell

surface and contain an additional 20 amino acid transmembrane domain and a small cytoplasmic domain. It is important to remember that structural variances between MMPs occurs not only through the presence of certain domains and modules as described schematically in Figure 3, but also at a finer level of structural organisation with differences in the amino acid motifs within these.¹²

Substrate specificity of different MMPs can occur through a number of structural elements, most widely recognised is the binding of the substrate to the active site cleft and the specificity induced by subsite pockets that define the active site.^{12, 14} These subsites differ between MMPs to accommodate different peptide substrate backbones. Often highlighted as most important of these subsite interactions and described as a key molecular determinant of substrate specificity is the S₁' specificity pocket.^{12, 14} This relates to the interaction of the side chain of the amino acid residue immediately after the scissile bond (P₁'). However, recently other subsite interactions along both the primed and unprimed sides of the active site have been found to be important and impart selectivity between potential substrates.¹⁵⁻¹⁸ In addition to the active site, other important interactions at exosites (on discrete substrate binding domains or functional modules) have been found to contribute to substrate binding and specificity.¹² These additional interactions utilised by macromolecular substrates are located away from and not influenced by primary specificity subsites, they can be involved in tethering the enzyme and substrate or can help to prepare substrates for enzymatic reaction.

Proteolysis of substrates via MMPs occur catalytically at a Zn²⁺ ion which is coordinated by three histidine residues in the common binding motif HExxHxxGxxH in the active site.^{8, 12, 14} The accepted mechanism of catalytic activity (analogous to bacterial thermolysin collagenase) is via polarised catalytic water bound to zinc and stabilised by a glutamic acid residue. Upon coordination, the water molecule becomes

a better nucleophile and reacts on the carbonyl of the substrates scissile bond forming a tetrahedral intermediate; proton transfer results in the cleavage of the substrate which is then released from the catalytic site.^{12, 19-21} A schematic of the catalytic cycle is shown in Figure 4; this example incorporates second associated water into the mechanism which has been observed in a number of X-ray crystallographic studies.²¹

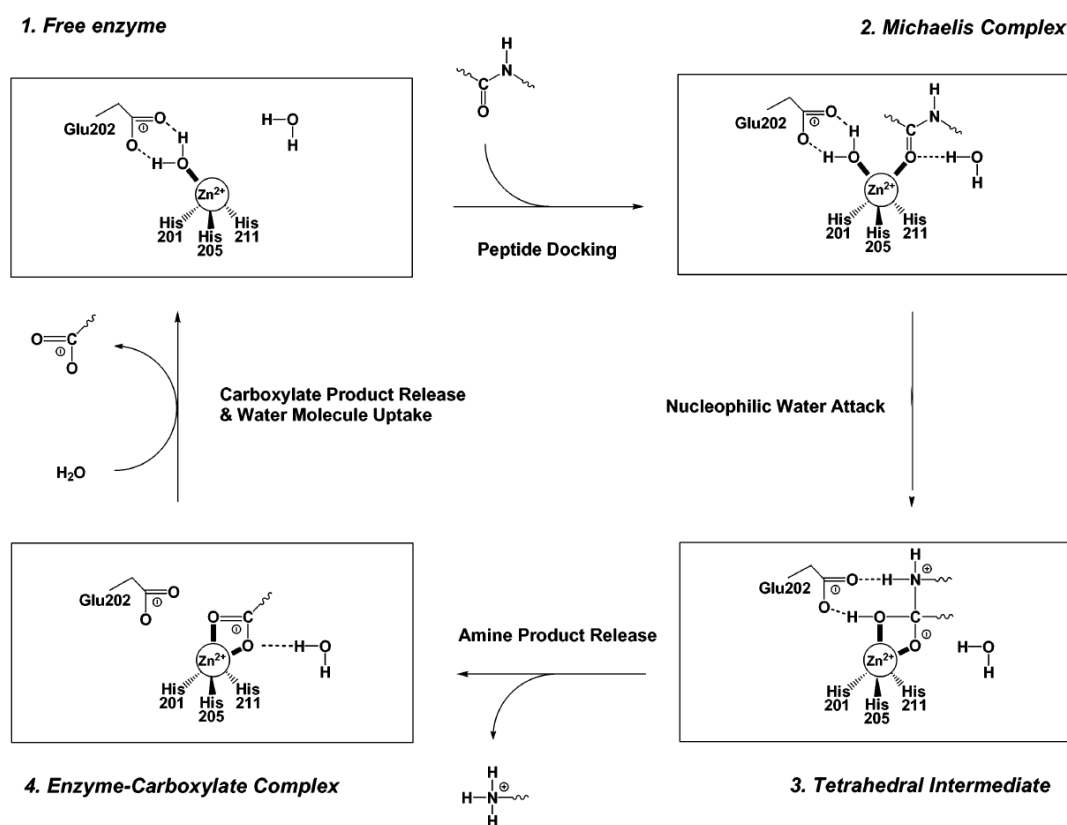


Figure 4- Catalytic cycle of substrate hydrolysis by MMPs; reaction occurs around the catalytic Zn²⁺ ion, a bound water molecule act as a nucleophile attacking the carbonyl of the substrates scissile bond forming a tetrahedral intermediate, subsequent proton transfer results in substrate cleavage and products are released from the catalytic site. Figure taken from Pelmeshnikov and Siegbahn.²¹

1.2.2 Relevance to disease

The activity of MMPs within the body is generally under tight control. These proteinases are only expressed in small amounts *in vivo* and their transcription is

regulated by cytokines, hormones, growth factors and cellular transformation.¹³ The overexpression of MMPs has been associated with a variety of diseases including inflammatory diseases like rheumatoid arthritis and osteoarthritis, cardiovascular diseases such as atherosclerosis, and significantly cancer. The contribution of MMPs within cancer has been confirmed with transgenic and knockout mice models where the absence of certain MMPs resulted in a decrease in the growth of tumours and the spread of the disease.^{7, 22} Examples include studies on MMP-2 null mice which demonstrated around a 50% decrease in angiogenesis induction, melanoma growth and lung carcinoma metastasis; as well as investigations into MMP-9 deficient mice which showed reduced formation of melanoma metastasis.^{13, 23}

Due to the ability of MMPs to breakdown virtually all ECM components their importance in facilitating the spread of the disease has long been acknowledged.^{5, 7, 23, 24} Degradation of the ECM allows for tumour cell invasion of surrounding tissues which is important in processes such as tumour growth, angiogenesis and metastasis. Angiogenesis is an important process for tumour progression; it describes the formation of new blood vessels from pre-existing ones and is imperative for tumour growth, whilst metastasis describes the ability of the cancer cells to break away from the initial tumour site and go on to form secondary tumours resulting in an increase in patient mortality. It is therefore unsurprising that high levels of MMPs have been detected in malignant tissues and the serum of patients in the later stages of the disease.^{5, 13}

More recently studies into MMPs have revealed that their function in cancer is not solely breaking down the ECM; their over expression is thought to be important for the activation of growth factors, the suppression of cell apoptosis as well as destruction of the chemokine gradients developed by host immune response.^{5, 10, 22} Figure 5 taken

from a review by Roy *et al* pictorially demonstrates the 4 main roles MMPs have in cancer.⁵ This includes the breakdown or proteolysis of the ECM which has been described above, but also highlights the importance of three other areas: the liberation of angiogenic factors, the regulation of cell adhesion and migration, and the processing of growth factors and cytokines.

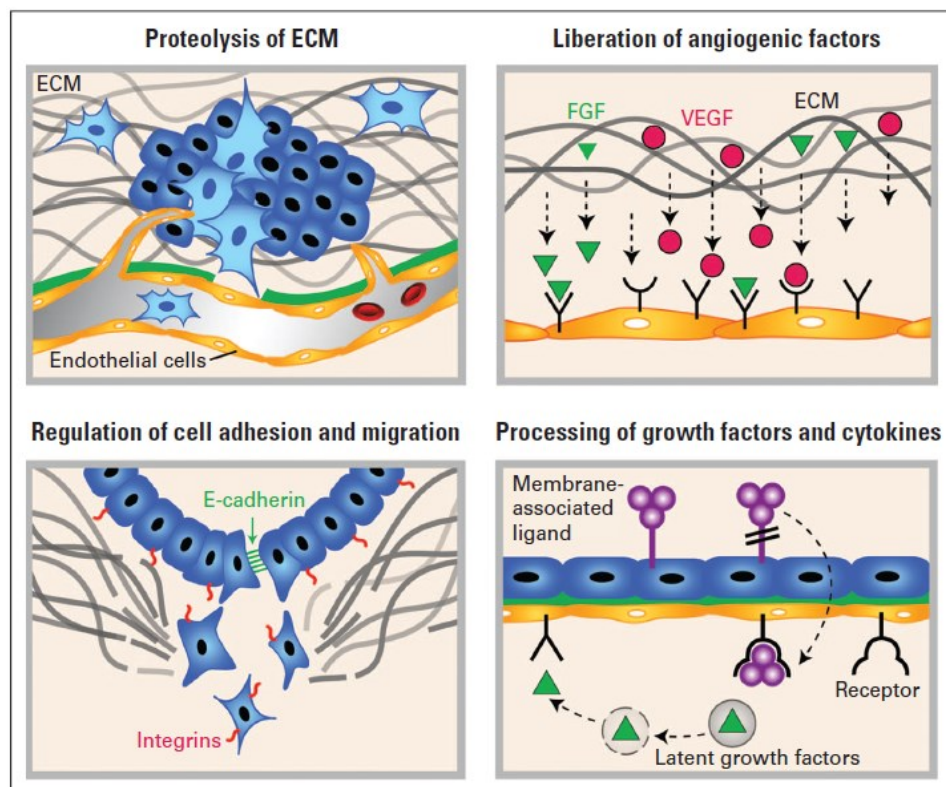


Figure 5 – The various roles of MMPs have been found to play in cancer progression (clockwise); breakdown of the ECM facilitating angiogenesis and metastasis, cleavage of ECM substrates to release various angiogenic factors, shedding of growth factors from the cell surface and the modulation of tumour and stromal cell interaction via processes such as the cleavage of E-cadherin. Figure taken from Roy *et al.*⁵

Angiogenesis as described above is an important process for tumour progression; as shown in Figure 5 MMPs are involved in angiogenesis, not only through the breakdown of the ECM necessary for remodelling of tissue during angiogenesis, but it has been established that they also liberate angiogenic factors. MMPs are known to

release the proangiogenic vascular endothelial growth factor (VEGF) via proteolytic release from bound inhibitory complexes such as the MMP-2 cleavage of HARP (Heparin affin regulatory peptide).²² In addition to this MMPs have been implicated in a process known as the angiogenic switch. The angiogenic switch describes a discrete step in tumour development which occurs in the very earliest stages of tumour growth and progression.²⁵ MMP-2 and MMP-9 have both been linked to the induction of the angiogenic switch: MMP-2 was found to play a crucial role in the development of the angiogenic phenotype in a model of tumour progression replicating the switch, whilst MMP-9 has been found to be a regulator of the switch in a pancreatic tumour model.^{26,}

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Furthermore MMPs have been shown to play important roles in signalling. Many ECM degradation products demonstrate certain biological properties which trigger a variety of cellular signals. For instance, as shown in Figure 5 the breakdown of E-cadherin by MMPs in the ECM causes the release of soluble E-cadherin which can disrupt intercellular interactions resulting in a loss of cell adhesion allowing migration to increase.⁵ MMPs are also known to modulate the bioavailability of growth factors necessary for tumour growth and angiogenesis, as well as regulating aspects of immunity and inflammation via cytokine signalling. For example MMP-2, -9 and -14 have been implicated in the release epidermal growth factor receptor (EGFR) ligands resulting in upregulation of signalling via the EGFR pathway and which leads to uncontrolled proliferation, migration and survival of cancer cells.²⁸

In addition to the well-known pro-cancer effects of MMPs, it is now acknowledged that certain MMPs can provide an anti-cancer effect.^{10, 23} For instance MMPs have been shown to cleave substrates plasminogen and collagen XVIII into angiostatin and endostatin respectively which have demonstrated potential as angiogenesis inhibitors.

Therefore, over expression of MMPs in the incidence of cancer may not only be due to their necessity for the progression of the disease but rather a response triggered by the abnormal cell activity.

Figure 6 provides a pictorial summary demonstrating a few of the important routes of progression in cancer and highlighting the MMPs known to be involved in these processes.

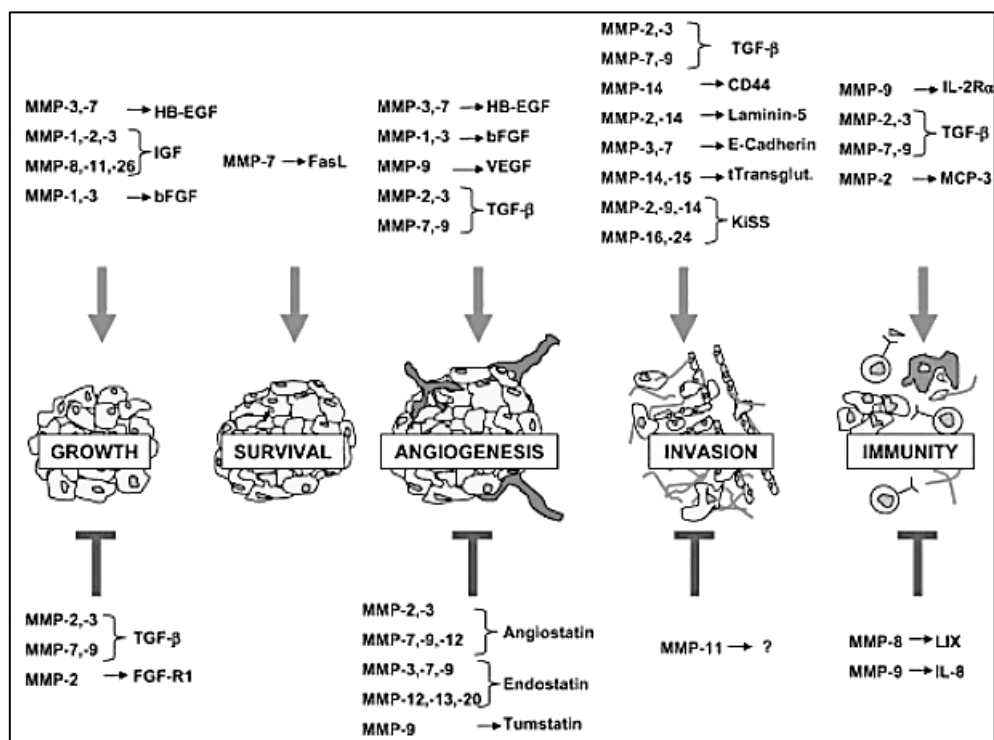


Figure 6 – Schematic describing some of the roles different MMPs have to play in different processes in cancer. Arrows highlight MMPs involved in cancer progression at these stages whilst the red non-arrows indicate anti-cancer activity in those stages. Figure taken from Folgueras *et al.*¹⁰

While MMPs have shown involvement in numerous processes facilitating the progression of cancer it is difficult to fit their activity into just one of the hallmarks of cancer (as defined by Hanahan and Weinberg). It is this unique mix of roles, their activity, and involvement in a variety of different disease pathways that make them interesting targets for furthering our understanding of cancer. Additionally, MMPs represent a potential early marker for cancer whose detection could pinpoint certain

downstream effects, making them desirable molecular targets for diagnostics and drug treatments.

1.2.3 MMPs as targets for inhibition

As well-known contributors to cancer progression MMPs have been the subject of considerable interest in investigations into the treatment of the disease through the inhibition of these proteases.^{10, 11, 23} A number of MMP inhibitors (MMPIs) have reached the clinical trial phase of drug development many based around peptidomimetic or small compounds which target the catalytic zinc(II) centre within the MMP active site. While small animal studies on such compounds provided positive results, these have not translated into increased survival rates in patients during clinical trials. The reasons for this failure are thought to come down to a number of factors.^{10, 11, 23} Firstly, dosing of patients was not optimal; adverse musculoskeletal effects of many MMPIs during clinical trials limited the maximum tolerated dose of these early MMPIs, limiting drug efficacy and the search for optimum dose analysis. Secondly, the clinical trials in question were conducted with patients in the later stages of the disease, past the beginning of metastatic spread when MMP inhibition could be most helpful. Finally, the MMPIs in question were non-specific, targeting a broad spectrum of MMPs, or demonstrating only limited selectivity. It is now believed that this broad spectrum recognition would not only block MMPs involved in processes such as metastasis and angiogenesis, but similarly the anti-cancer action that some of the proteases provide. In addition to this, another issue raised in clinical trials involving MMPIs was the difficulty monitoring the effect of drug treatment, this was on the basis that MMPIs are cytostatic rather than cytotoxic so conventional drug monitoring

systems (i.e. tumour size) were not applicable to MMPIs preventing accurate evaluation of efficacy.

In order to advance MMP inhibition a number of areas have been highlighted to facilitate improvement. A greater understanding of the individual roles of MMPs within cancer is needed, along with the identification of targets and anti-targets.²³ More specific MMPIs will need to be produced which target only those MMPs which negatively impact on health. Some MMPs pose the issue that they have different roles at different stages of cancer progression (they may provide anti-cancer action at one point but pro-cancer action at another making it difficult to define them as targets or anti-targets), however a greater understanding of these MMPs and their actions could help with understanding when MMPIs may be most useful to patients.²³ Furthering the knowledge of MMP activity *in vivo* will be essential in the design of more effective inhibitors. Additionally, the need for better monitoring systems of these cytostatic drugs has been emphasised, the ability to non-invasively detect the action of these proteases is necessary for complete evaluation of MMPIs. Both of these areas emphasised in the development of MMPIs could be aided with the use of molecular imaging, this could provide insight into the functions of MMPs in cancer and lead to effective MMP-targeting therapies.^{10, 11}

1.2.4 MMPs as targets for imaging

Expression of MMPs has been shown to be amplified in virtually every type of human cancer making them valuable biomarkers for the disease as well as desirable targets for molecular imaging.^{7, 29} Molecular imaging of MMPs offers the potential for not only detection of cancer at an earlier stage (as discussed earlier MMPs 2 and 9 are known to

play a role in the angiogenic switch which occurs at the earliest stages of tumour progression), but could similarly contribute to patient monitoring during treatment and aftercare. For example Ranuncolo *et al* conducted a study in which it was seen that for patients who had previously had breast cancer a gradual increase in the plasma activity of MMP-9 was observed 1-8 months before the return of the cancer could be clinically diagnosed.³⁰ There have been numerous studies which have highlighted the potential of MMPs as prognostic markers within a number of cancers, and there are examples of varying expression in different histotypes of individual cancers, highlighting the diagnostic potential of these enzymes.^{13, 31, 32} These examples all demonstrate the potential of MMPs as a useful clinical tools as we head towards an age of personalised cancer medicine and therapy.

Despite the potential of MMPs as biomarkers, complete understanding of these enzymes has still not been reached, and there is room for furthering our understanding of these proteases. Greater appreciation of MMPs and their function, through non-invasive monitoring via molecular imaging, would further our understanding of cancer and the hallmarks that MMPs fall within, this will in turn increase their diagnostic potential. This includes the production of more specific and appropriate MMPIs (via the identification of targets and anti-targets), as well as providing an understanding of when MMPIs would be most useful to patients. The use of imaging modalities would make it easier to evaluate the activity of MMPIs *in vivo* and may offer help when trying to prescribe an effective dose of an inhibitor.

In addition to their over expression in cancer and their potential applications as biomarkers, there are other factors which make MMPs ideal molecular targets for imaging. As enzymes which are secreted and activated in the extracellular matrix, any imaging probe designed would not require the ability to transfer into the cellular

environment, increasing synthetic ease and potentially allowing for more design creativity.²⁹ MMPs importantly also provide catalytic activity which allows for potential signal amplification through the use of an imaging probe which is activated by the catalytic site of the protease, as opposed to a target- receptor type interaction which is intrinsically on a 1:1 ratio.²⁹

Of the MMPs known to be up-regulated in the incidence of cancer those which could be considered most interesting to image are MMP-2 and MMP-9, as they are both associated with the angiogenic switch and have shown high levels of expression in a wide variety of cancers. Both of these MMPs have been widely evaluated in terms of their roles and overexpression in cancer and have been investigated with regards to defining a number of selective substrates. Additionally, it would be interesting to investigate MMP-14 as it is a membrane type MMP and is known to turn pro-MMP-2 into its active form. Thus, its overexpression would be expected before or alongside that of MMP-2. All three of these members of the MMP family have also been implicated as having prognostic potential.^{5, 28}

1.3 Molecular imaging and state of the art MMP imaging

Molecular imaging has been broadly defined as ‘the *in vivo* characterisation and measurement of biological processes at the cellular and molecular level’.³³ It aims to probe molecular abnormalities and changes that are the basis of disease rather than the end effects of these molecular alterations (which is the domain of classical diagnostic imaging). The goal within cancer imaging is to identify tumour specific biomarkers and utilise molecular imaging to facilitate the early detection of cancer and enable the personalisation of cancer therapy. Imaging of highlighted biomarkers such as MMPs

has the potential to provide valuable information on molecular alterations occurring within cancer and offer important advances for the diagnosis and treatment of cancer; including the provision of information regarding a patient's prognosis and a contribution to effective aftercare.²⁹ Molecular imaging can be carried out by any number of the imaging modalities available and a variety of different modalities have recently been investigated for the targeted molecular imaging of MMPs. These include optical imaging, positron emission tomography (PET), single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI).

1.3.1 Optical imaging

Of the imaging modalities which have been investigated for targeted molecular imaging of MMPs, optical imaging techniques represent the group which has been most prolific. Optical imaging is a modality which encompasses a variety techniques based on the use of light to interrogate cellular or molecular functions within the living body, or animal and plant tissues.³⁴ Optical probes which are designed for the clinical environment generally provide detection via the accumulation or activation of a near-infrared fluorescent (NIRF) species at a specific target site which on excitation will emit photons. Fluorescent agents can be designed to target different receptors within the body, or as is often the case in MMP imaging can be designed to be activated in the presence of a certain enzyme, protease or catalyst.

The use of optical imaging to visualise MMP activity within the body was first demonstrated in a study by Bremer *et al.*³⁵ In this investigation an imaging probe was developed which contained a near-infrared fluorochrome linked via an MMP-2

cleavable peptide to a non-immunogenic polymer backbone which had been designed to be an efficient delivery vehicle Figure 7.

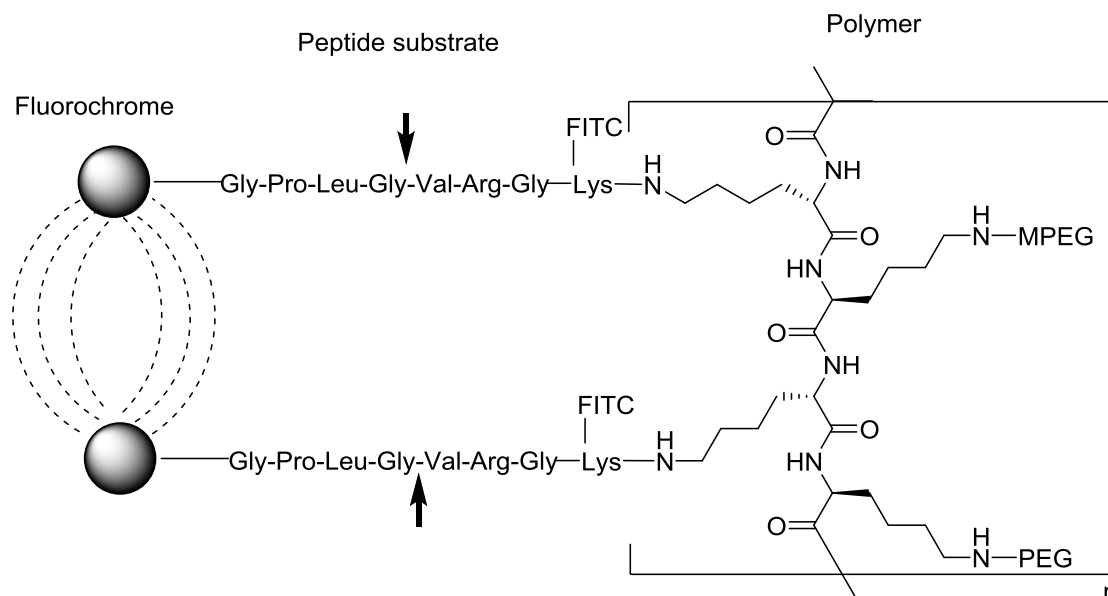


Figure 7 - Schematic showing the design of MMP-2-activated fluorescent probe from Bremer et al, with fluorescent compounds attached to a polymer backbone via MMP-2 cleavable peptide substrates.

Internal fluorescence quenching occurs (represented by orange dotted lines) prior to enzymatic cleavage.³⁵

The proximity of the fluorophores to one another resulted in quenching of the fluorescent signal which could be broken by enzymatic reaction. This probe was tested *in vitro* with MMP-2, against a scrambled control peptide with no specificity for MMP-2 cleavage. This comparison showed that the probe with the MMP-2 cleavable peptide had a significant increase in fluorescence (up to 850%) when treated with active purified MMP-2, whereas the control compound exhibited very little change in fluorescence. This demonstrated the potential of the probe for imaging the activity of MMP-2. Further analysis was carried out on the probe to explore its selectivity with regards to other MMPs which would be found *in vivo*. The probe was tested against a variety of MMPs: MMP-1, -2, -7, -8 and -9, and it was seen that although fluorescent

signal could be observed in these experiments, the increase was not as high as with MMP-2.

In vivo investigations were subsequently carried out on the probe to evaluate its potential to image MMP-2 expression. Two models were set up one using a HT1080 fibrocarcinoma model which is reported to produce high levels of MMP-2 and a BT20 tumour model which was chosen because of its relative lack of MMP-2. The MMP sensitive probe and the control probe were injected into the HT1080 and BT20 tumour bearing animals. Imaging results showed that for the HT1080 tumour model there was a significant difference in fluorescence between the mice injected with the MMP-2 probe and the control probe, it was also observed that for the BT20 tumour model there was a much lower fluorescence signal than seen in the HT1080 model.

Bremer *et al* went on to investigate the use of the probe for analysing the abilities of MMP inhibitors both *in vitro* and *in vivo*. In a number of *in vitro* investigations it was seen that fluorescence signals could be completely blocked with the use of certain MMP inhibitors such as phenanthroline. *In vivo* the study was focused on prinomastat, again using the HT1080 tumour model. Tumours were allowed to grow to 2-3mm before the mice were treated twice daily with either prinomastat or a control. Mice treated with prinomastat produced a significantly smaller MMP-2 NIRF signal when compared to mice bearing untreated tumours. This work illustrated the potential use for an MMP imaging probe in analysis of drugs as well as validating MMP specific activation. This probe demonstrated the first example of MMP imaging and highlighted the potential of such probes for the detection of MMPs *in vivo* and for their use in drug treatment response analysis. However, initial MMP optical probe designs such as this lacked the ability to quantitatively assess and determine specific MMP activity. These problems have been overcome to some extent in later work where

techniques using sensor and reference probes provide the potential of quantitative measures of proteolytic activity

An example of this comes from the work by McIntyre *et al* in which a probe was designed based on a dendrimeric scaffold which has a fluorogenic substrate built on to it and which could be selectively cleaved by MMP-7.³⁶ In addition to this the probe contained a non-cleavable internal reference fluorophore, allowing the probe to be detected even when MMP-7 cleavage had not taken place.

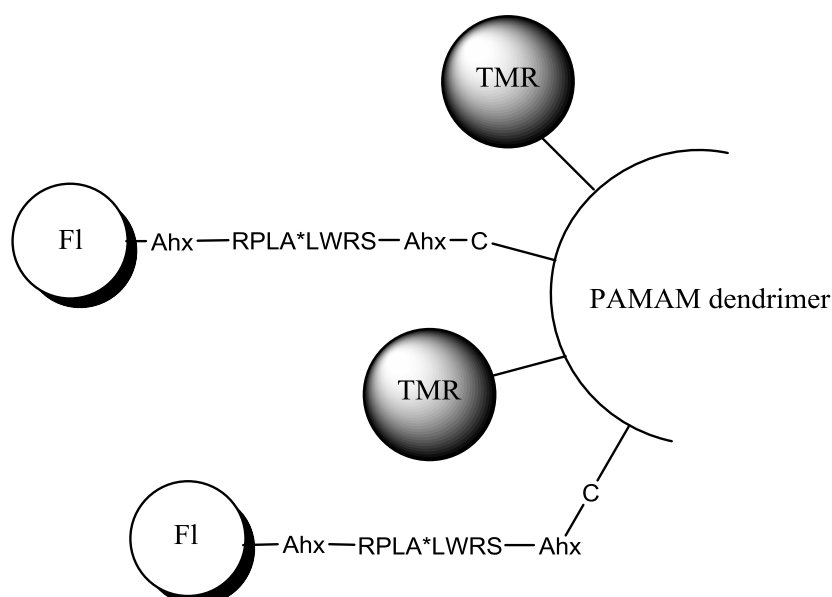


Figure 8 – Diagram describing the MMP-activated optical imaging probe (fluorescein, Fl attached to poly amido amino dendrimer via MMP cleavable peptide) with non cleavable internal reference

(tetramethylrhodamine, TMR) from McIntyre *et al*.³⁶

This system allows for simultaneous imaging of biodistribution and MMP activity, providing a different response between MMP-7 expressing tumours and tumours with no MMP-7. Additionally, this probe demonstrated selectivity for MMP-7 over MMP-2.

Another issue in using optical probes based around this ‘activatable’ concept for imaging MMPs is the probable complexity involved when attempting to image cancers

which lack membrane bound MMPs. The fact that fluorophores may not remain localised at the site of tumours once MMP cleavage has taken place could be problematic. A strategy was devised by Tsien *et al* which attempted to overcome these concerns by designing fluorescent probes which on cleavage by MMP-2 or MMP-9 have the ability to penetrate or translocate into the cytosol of mammalian cells Figure 9.³⁷ This process allows for the build-up of fluorescence in cells in the area of MMP activity. In experiments with HT1080 cells significant uptake of activated cell penetrating peptide (ACPP) was observed compared to a scrambled peptide control.

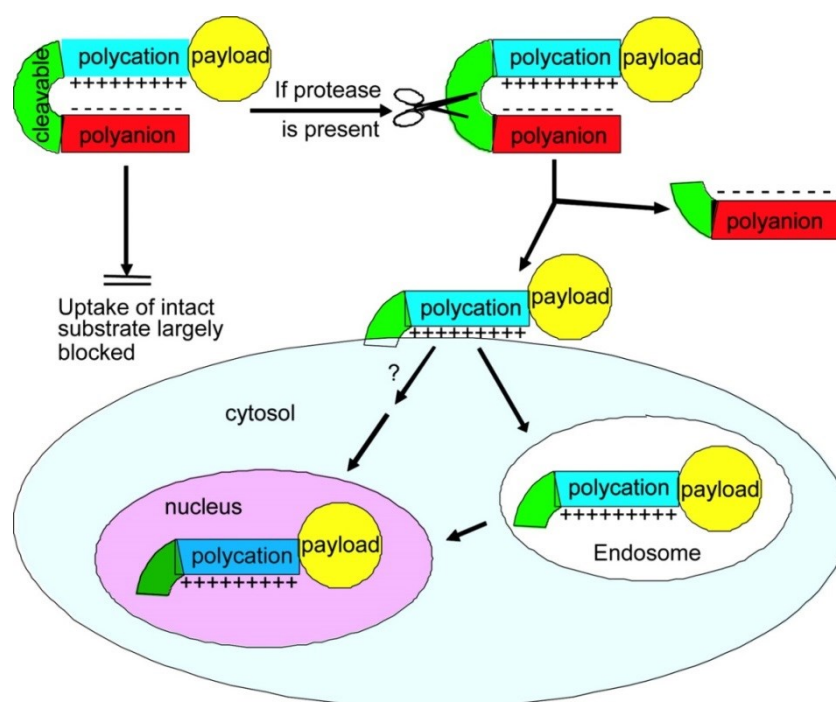


Figure 9- Schematic describing the mode of action of MMP activated cell penetrating probes, showing initial blocking of cell penetration by polyanionic residue, linker cleavage and subsequent cellular uptake of the polycationic residue, which delivers the payload into the cells. Figure taken from Tsien *et al.*³⁷

However such probes lack an activated signal component relying on an activated targeting mechanism which could potentially result in high overall background signal due to no initial quenching of fluorescence.

Despite the high sensitivity of optical imaging probes often only modest changes in fluorescence signals between quenched and unquenched states are seen. Recent studies have addressed this with the use of gold nanoparticles which act as almost perfect quenchers of fluorescence and therefore provide a much larger change in signal between the quenched and un-quenched states.³⁸ Lee *et al* describes the use of gold nanoparticles functionalised with a Cy5.5 dye via an MMP cleavable peptide to measure the presence of MMPs. In the initial state fluorescence is highly quenched by the gold nanoparticle core and additional quenching takes place due to the proximity of dye molecules to one another at the particle surface, this multi-quenched state ensures pronounced NIRF recovery on MMP activity.

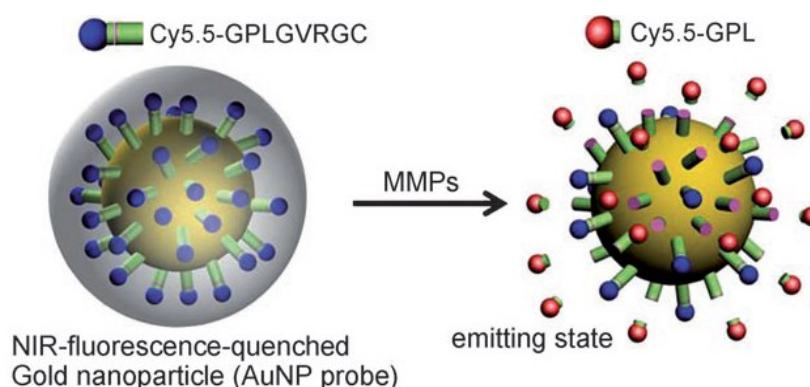


Figure 10 – Figure taken from Lee *et al* showing the mode of action of MMP sensitive gold nanoparticle for activated optical imaging of MMP-2.³⁸

These nanoparticles were shown *in vitro* to produce strong NIRF signals when tested against a number of MMPs. This was compared to a probe synthesised with a scrambled MMP-2 substrate linker with whom no NIRF signals were recovered. It was observed that in the presence of an MMP-2 inhibitor the NIRF signals exhibited by the gold nanoparticle probe were strongly disrupted. *In vivo* investigations were carried out using mice infected with SCC7 tumour cell carcinomas which are known to express MMP-2. It was seen that in normal nude mice NIRF signal was comparable to

the background tissue signal, whereas in mice bearing the SCC7 tumours the gold nanoparticle probes produced a high intensity NIRF signal allowing visualisation of the tumour site. Furthermore, SCC7 tumour bearing mice were tested to ascertain the effects an MMP-2 inhibitor would have on the fluorescence of the gold nanoparticle probe, as with *in vitro* experiments the inhibitor significantly decreased the NIRF signal. *In vivo* results can be visualised in Figure 11.

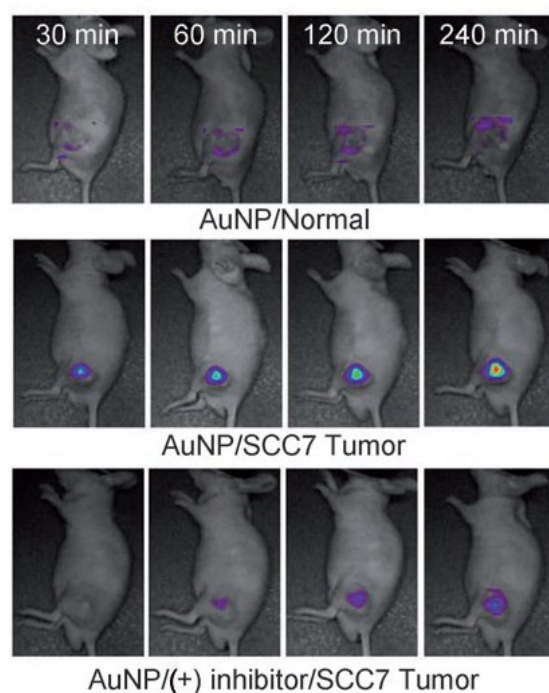


Figure 11- Images of normal and SCC7 tumour bearing mice after injection with the MMP activated gold nanoparticle and with or without an MMP inhibitor present, figure from Lee *et al.*³⁸

While these probes demonstrated the benefits of using ‘perfect’ quenching to increase fluorescence signal changes in optical imaging, these probes lack the quantification capabilities of those imaging probes which present a pair of fluorophores. Additionally, these probes despite being designed as MMP-2 probes show activity with a number of MMPs tested.

The examples shown here represent a sample of the large number of MMP activated optical probes, they demonstrate the potential and progression of the optical imaging

of MMPs. However, despite all the positive examples, optical imaging comes with the inherent problem of limited depth penetration through tissue.³⁴ While not a significant issue in mouse models this would be problematic for *in vivo* applications in humans and transfer of successful probes in to the clinical environment. Near infra-red fluorescence imaging has a limited penetration of light of less than 10 mm.³⁵ It is this low depth penetration that is the main factor which prevents optical imaging from being used effectively *in vivo*. Ultimately, in spite of the sensitivity of optical imaging and its ability to successfully image the activity of MMPs, the inability to penetrate deep within the tissue of a patient will be the limiting factor of optical imaging techniques for non-invasive clinical applications such as the imaging of cancer. Nevertheless, the wide range of probes developed for optical imaging of MMPs elucidate properties that are desirable when designing future MMP imaging probes; ‘activatable’ or ‘on-off’ systems based on MMP activity, ability for quantification (internal reference) and the issue of compound retention at the activation site with these extracellular enzymes.

1.3.2 Positron emission tomography (PET) and Single photon emission computed tomography (SPECT)

PET is a very sensitive and quantitative molecular imaging modality which is highly utilised in drug discovery and has been expanding into a useful tool in the treatment of a number of diseases. It involves the labelling of a tracer compound with a radioactive isotope such as ^{11}C or ^{18}F which emits positrons. An emitted positron will travel no more than a few millimetres before collision with a nearby electron results in annihilation, emitting two 511 eV γ -ray photons in opposite directions to one another.³⁹

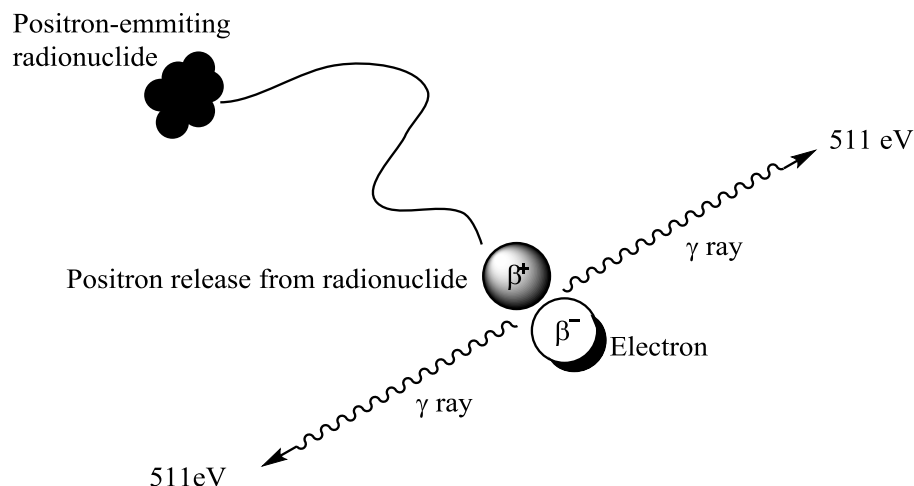


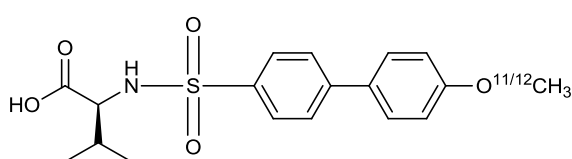
Figure 12- Schematic description of the positron decay process and the release of collinear γ rays.

The emitted photons travel in a straight line and can be detected by collinearly aligned detectors to localise the source of the collision. Detectors are generally arranged in a ring around the target allowing radioactivity measurement from a large number of angles. Reconstruction algorithms are then used on collected data to make allowances for attenuation and scattering of the γ rays. Labelled compounds are designed to interact with a specific target or mechanism within the body or organism and often potential drugs are labelled to monitor their action. PET therefore allows for the tracking of labelled compounds within the body to provide molecular imaging. The most noteworthy example of a PET radiotracer for imaging cancer comes from [^{18}F]-2-deoxy-2-fluoro-D-glucose otherwise known as FDG, which is a glucose analogue and is widely used for the clinical diagnosis of cancers.

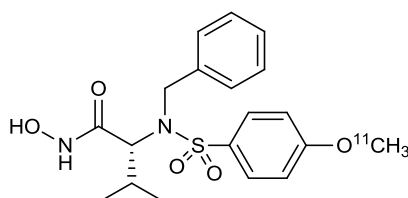
SPECT is another imaging technique based on the radiolabelling of compounds, however in this case gamma radiation is utilised.²⁹ The signal produced by these probes can be directly measured on simple gamma camera which when used at multiple angles can be computed to give a 3D tomographic reconstruction. While SPECT imaging benefits from isotopes with longer half-lives and can be used to

monitor relatively slow kinetic processes, it suffers from lower sensitivity and imaging quality than PET imaging.⁴¹

While there have been attempts to utilise MMPs natural inhibitors (TIMPs) for imaging of MMPs (for example the use of ^{111}In labelling of an N-TIMP-2 DTPA conjugate with the potential for SPECT imaging of MMPs) the majority of PET and SPECT studies into the imaging of MMPs have involved the labelling of small molecule or peptidomimetic MMP inhibitors.^{29, 42} Given the failures of MMPIs in the clinic it is unsurprising that many of the studies into the labelling of MMPIs have not provided successful imaging. This is in contrast to *in vitro* data on labelled MMPIs and is thought to be due to large amounts of non-specific binding found with these radiolabeled compounds. For example Zheng *et al* have conducted a number of investigations where potent MMPIs such as [^{11}C] MSMA and [^{11}C] CGS25966 have been radiolabeled, in these cases both compounds have exhibited high amounts of non-specific binding when targeting breast cancers in mouse models preventing efficient imaging of MMP activity.⁴³



[^{11}C]MSMA1



[^{11}C]CGS 25996

Figure 13 - Structures of two labelled radiotracer MMP inhibitor compounds investigated in Zheng *et al*.⁴³

Additionally, the labelling of MMPIs such as Marimastat (via [^{18}F]marimastat-aryltrifluoroborate) which reached phase III clinical trials demonstrated similar results with high levels of non-specific binding. It is likely these MMPI labelling examples suffered from high levels of non-pathogenic uptake due to the non-selective nature of the MMPIs labelled.⁴⁴

Furumoto and co-workers have provided an example where radiolabelling of a specifically designed MMPI has resulted in good tumour uptake in comparison to other tissues and with development into a prodrug has demonstrated improved biodistribution.⁴⁵ In this study a tracer was designed based on an MMP-2 inhibitor labelled with an ^{18}F as (seen in Figure 14) called (2R)-2-[4-(6-[^{18}F]Fluorohex-1-ynyl)-benzenesulfonylamino]-3-methylbutyric acid, or otherwise referred to as [^{18}F]SAV03.

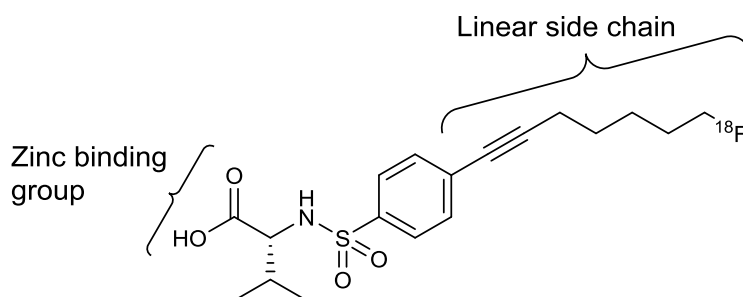


Figure 14 - Radiotracer designed by Furumoto *et al* for MMP-2 inhibition and imaging incorporating a zinc binding group and a linear side chain for S_1 subsite interaction.⁴⁵

This compound was designed to contain features making it highly selective for MMP-2 inhibition. Figure 14 highlights the important domains of the molecule, namely the carboxylic acid group which will bind to the active-site zinc ion in the catalytic domain of MMP-2 and the linear side chain which specifically interacts with the channel like S_1 subsite of MMP-2. As well as the compound shown above experiments were also conducted on a potential prodrug for the above inhibitor [^{18}F]SAV03M in which the carboxylic acid group was replaced with a methyl ester.

Biodistribution studies were carried out for both radiolabelled compounds using Ehrlich tumour bearing mice as tumour models (which have the potential to produce MMP-2 during tumour growth). On investigation of [^{18}F]SAV03 *in vivo*, it was found that the tumour model demonstrated tumour-specific uptake of the radioactive compound, with a high tumour to muscle ratio (8.4) at 120 minutes after injection. The highest uptake of radioactivity was seen in the liver and small intestine which was attributed to first pass effects by Furumoto *et al.*⁴⁵ The first pass effect describes the early metabolism of the compound which would result in reduced availability of the tracer around the other organs of the mouse. It was considered likely that it was the carboxylic acid group of [^{18}F]SAV03 which was the main factor generating this first pass effect.

In order to overcome the problematic metabolism of the MMP targeting tracer before it reached its target site, the use of the aforementioned compound [^{18}F]SAV03M as a potential prodrug was investigated in order to observe its biodistribution. Preliminary assays ascertained that SAV03M displayed similar inhibition qualities as SAV03 and [^{18}F]SAV03M *in vivo* exhibited a reduced uptake of the radiotracer in the liver, with radioactivity approximately halved. Due to this uptake in the blood, heart, lung, spleen and muscle showed a comparative increase 30 minutes after injection. It was found that after 120 minutes the tumour uptake of the radiotracer had a 2.4 fold increase. Metabolites of [^{18}F]SAV03M in plasma and tumour tissue after 30 minutes post injection were analysed with radio-TLC and revealed that [^{18}F]SAV03M was metabolised *in vivo* into the parent tracer, weakening the first pass effect. Further investigation into [^{18}F]SAV03M using whole body autoradiography supported the conclusion that [^{18}F]SAV03M provided superior availability *in vivo* compared to

[^{18}F]SAV03, and that tumour specific uptake of the prodrug was observed in relation to other organs and tissues.

This investigation by Furumoto *et al* demonstrates one of the better examples of small molecule MMPI radiolabelling, however the relationship of tumour uptake and MMP-2 expression has not yet been fully elucidated.⁴⁵ The tumour model chosen may not be the best for assessment of MMP-2 activity. Additionally, from what is known about MMP selectivity, the design of this radiotracer is unlikely to be MMP-2 specific displaying many features of broad spectrum recognition. MMP specificity would need to be assessed further to confirm no issues relating to non-pathogenic uptake which has been a problem in many other radiolabelled MMPIs.

A recent example of MMP imaging shows a move away from small molecule radiolabelling in an attempt to improve selective uptake using a $^{99\text{m}}\text{Tc}$ labelled aptamer with the potential for SPECT imaging of MMP-9.⁴⁶ The term aptamer refers to a macromolecular drug made up of oligonucleotides or peptides selected to bind to a specific target via iterative cycles of selection and amplification. In the study by Da Rocha Gomes *et al*, a suitable MMP-9 aptamer is identified, minimal domain structure for selectivity determined and adaptations for use in biological media made before conjugation to a ligand and $^{99\text{m}}\text{Tc}$.

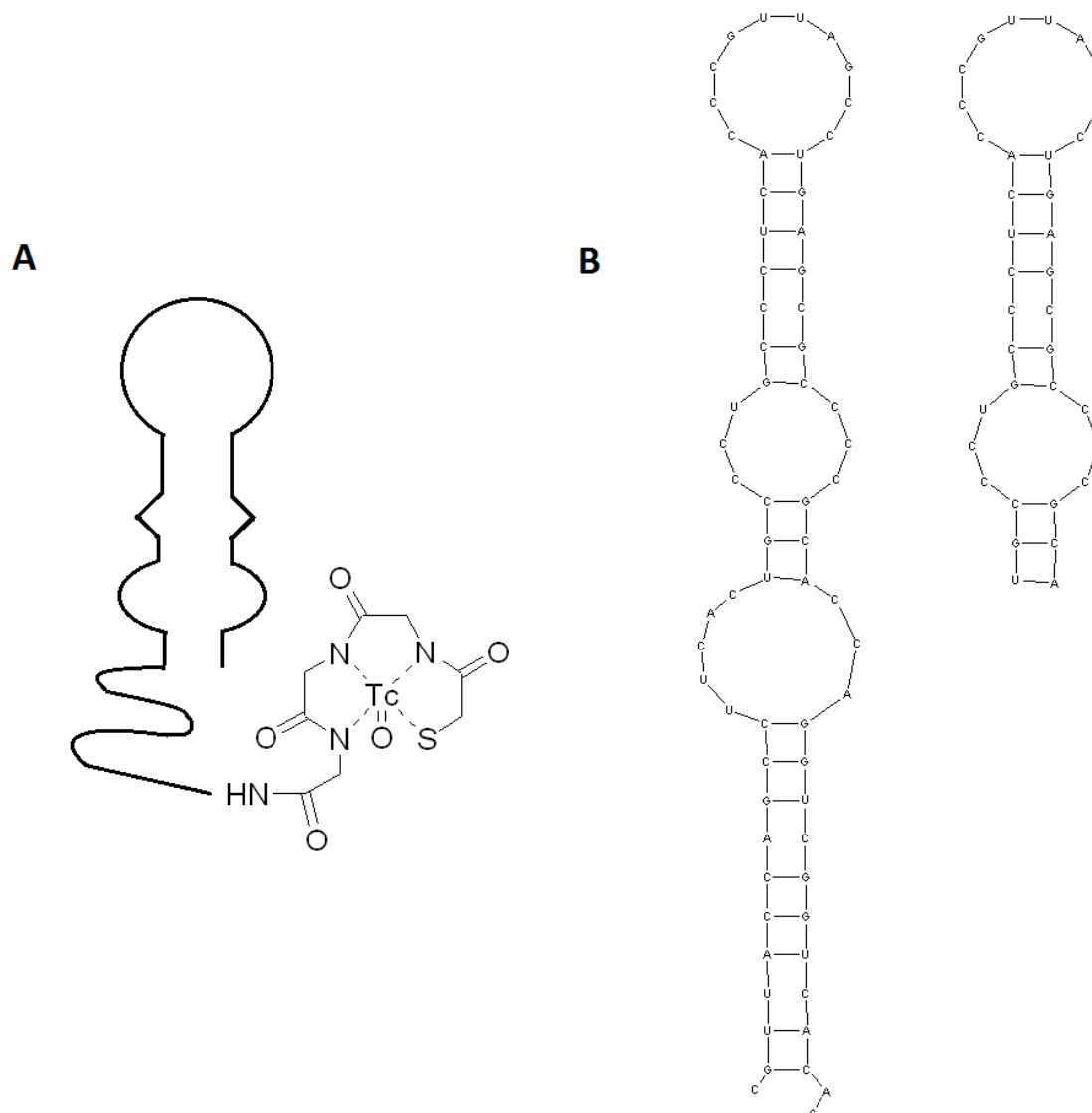


Figure 15- A: basic structure of radiolabelled aptamer SPECT tracer designed to target MMP-9. B: originally identified aptamer and minimal structure required for MMP-9 specificity.⁴⁶

MMP-9 binding remained intact on functionalization and radiolabelling and selectivity over MMP-2 and MMP-7 was demonstrated with minimal aptamer domain. This probe demonstrated immunostaining of MMP-9 expressing glioblastomas versus a control sequence and ^{99m}Tc-ligand alone. Despite high specificities and positive early results imaging studies are yet to be carried out on this compound so *in vivo* characteristics and nonspecific binding remain unknown.

The use of PET or SPECT for imaging of MMPs has great potential for the examination of MMP inhibitors and their effectiveness *in vivo*, as well as this imaging of good inhibitors would provide useful biomarker detection and could be used for molecular imaging. However, due to the lack of high specificity MMP inhibitors PET and SPECT imaging will remain difficult until more selective compounds have been found. As described previously current radiolabelled MMPIs have suffered from large amounts of non-specific binding. Despite the work by Furumoto *et al*, definitive proof of a small molecule PET tracer's ability to be effective indicators of proteolytic activity has yet to be demonstrated and further work is needed in this area. Though PET has many positives such as its high sensitivity and the early use in human subjects, it does not utilise one of the major advantages of enzymatic imaging, the potential for the amplification of signal and the use of an 'on/off' imaging system which can help to increase the signal-to-noise ratio. In addition to this, there is the disadvantage that many PET tracers are synthetically difficult to produce; many inhibitors may have lengthy synthetic routes which must be shortened in order to be effective for the radioactive technique. Issues relating to the lack of an MMP-activated component and signal amplification are paralleled for SPECT imaging, which also suffers from lower sensitivity and image quality than PET imaging.

1.3.3 Magnetic Resonance Imaging (MRI)

MRI is a widely available technique which is utilised in many hospitals and clinics around the world enabling excellent soft tissue visualisation. Relying on the same principles as nuclear magnetic resonance (NMR), MRI allows for the visualisation of soft tissues throughout the body via a process of magnetisation, excitation and relaxation of the protons present. While excited protons process around the axis of

magnetisation, measurement is carried out by RF coils and processed into an image using the Fourier transform.

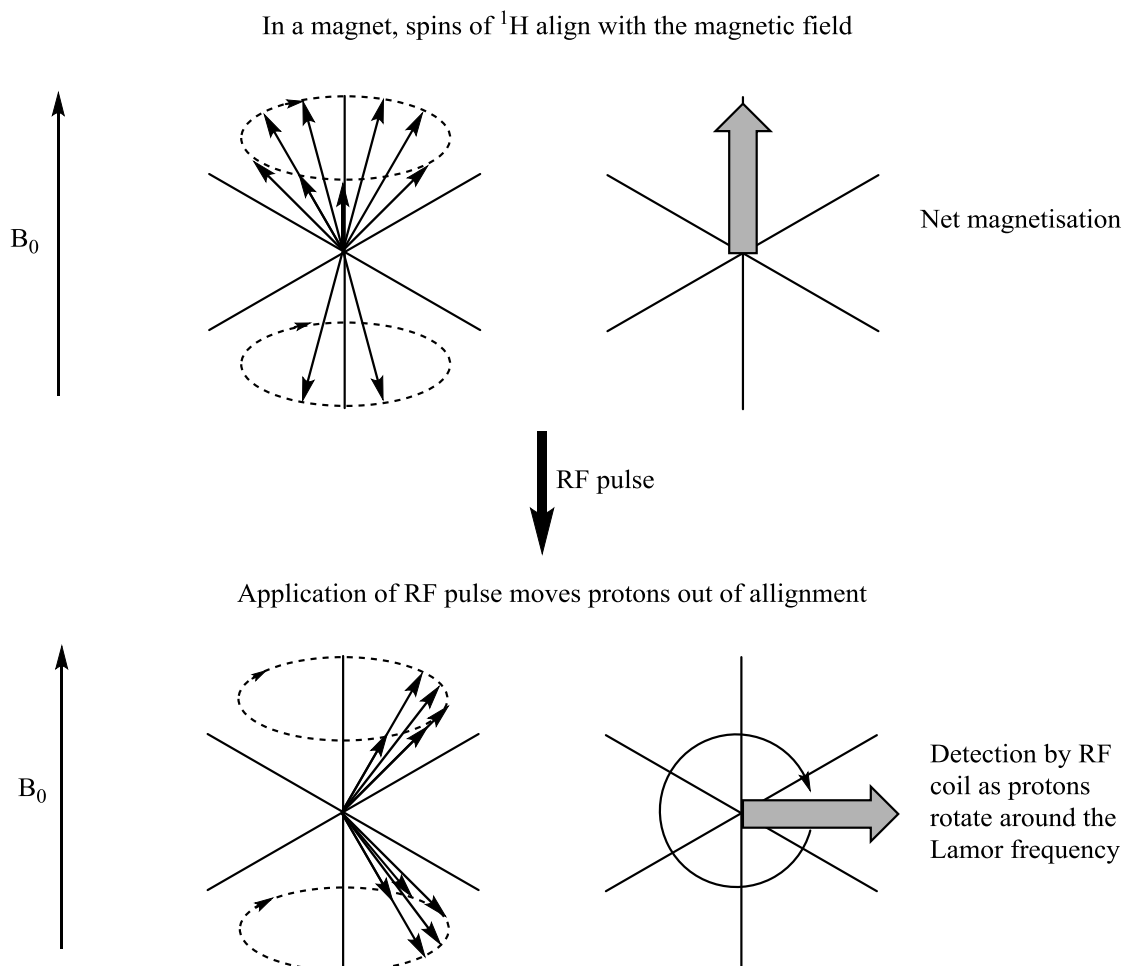


Figure 16- Visual description of signal production within an MRI; spin magnetisation and excitation of spins via an RF pulse.

For more in-depth explanation into the principles of MR imaging the reader is referred to books on the area.^{47, 48} Although MRI is highly recognised as a technique for structural imaging, its use in molecular imaging studies has yet to become widespread. Contrast agents based on paramagnetic gadolinium(III) complexes are already in clinical use worldwide to broadly increase image contrast in certain tissues of the subject. By applying a targeting or activatable system onto such contrast agents provides the potential for molecular imaging using MRI.

There are fewer examples in literature of the use of MRI contrast agents for the selective targeting of MMPs compared to techniques such as optical imaging or PET. Lepage *et al* began investigations into MMP activated MRI by developing a contrast agent based around a gadolinium(III)-DOTA which experienced a change in solvation on contact with MMP-7, these were later developed to be MMP-2 active.⁴⁹⁻⁵¹ These MMP-2 activated probes centre on a selectively cleaved peptide (SPAY↓YTAA, arrow represents cleavage point), functionalised at the N-terminus with a hydrophobic alkyl chain which attached to the DOTA ligand of the Gd complex, this peptide was then linked to a polyethylene glycol (PEG) at the C-terminus (Figure 17). PEG is a water soluble polymer which has low toxicity in the body; it is the attachment of this PEG group which determines the solubility of the contrast agent designed by Lepage *et al*.⁴⁹ The loss of PEG in the cleavage reaction facilitated by MMP-2 causes the loss of solubility of the contrast agent resulting in localisation in the area in which the MMP was situated i.e. the tumour site.

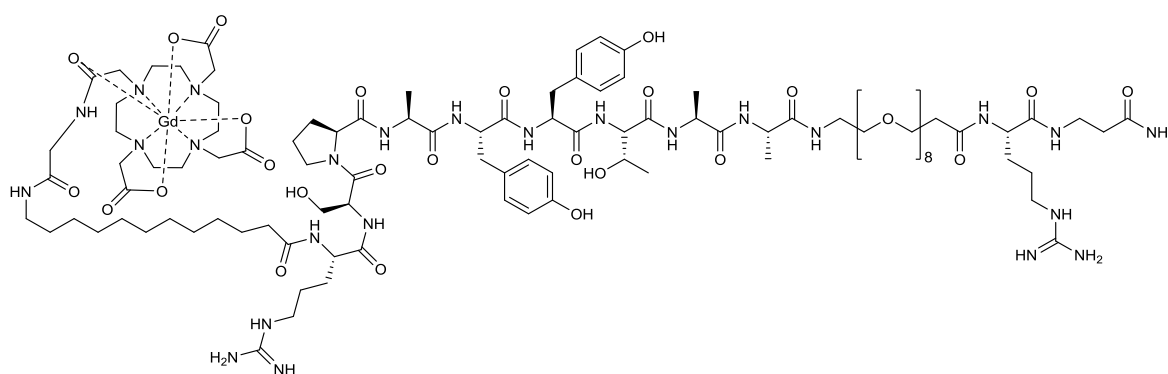


Figure 17- The MMP-2-activated solubility switch contrast agent as described in Lepage *et al*. Design is based around an MMP-2 specific substrate, functionalised with a soluble PEG chain which is removed on protease activity, decreasing the solubility of the gadolinium-DOTA.⁴⁹

The compound shown in Figure 17 was tested both *in vitro* and *in vivo* against a similar compound with a scrambled peptide chain as a control. *In vivo* experiments

were carried out using testing on animal models with two tumours; one from MC7-L1-WT cells which over express MMP-2 and MC7-L1-KD cells which have reduced MMP-2 activity. A control experiment using Gd-DTPA revealed that both tumour varieties had similar kinetics and therefore comparable vascular permeability. MRI experiments with both the MMP-2 cleavable contrast agent and the control were conducted on the animal models. It was found that the MMP-2 cleavable contrast agent in the MC7-L1-WT tumour the relative ΔR_1 value reached a plateau between 5 and 10 minutes before getting to a maximum at 40 minutes, whereas in the MC7-L1-KD tumour only a continuous decrease was seen. With the scrambled control compound it was observed that an early maximum in ΔR_1 was followed by a continual decrease in both tumour models. These results suggested that the cleavage of the peptide in the MMP activatable contrast agent by MMP-2 resulted in an accumulation of contrast agent at the site of cleavage allowing for imaging of MMP activity *in vivo* using the solubility switch method.

Another similar example of a contrast agent for the molecular imaging of MMPs using MRI comes from Schellenberger *et al* in which functionalised iron oxide nanoparticles were used as MMP-9 activateable MRI contrast agents.⁵² The nanoparticles designed in this study were based on very small (hydrodynamic diameter of 7.7 ± 2.1 nm) electrostatically stabilized and citrate coated nanoparticles. To add MMP sensing capabilities to these very small iron oxide nanoparticles (VSOPs) they were functionalised via a positive arginine rich coupling domain with a peptide containing an MMP-9 cleavable domain. The other side of the cleavage domain of the peptide was bound to PEG. These functionalised compounds were defined as MMP-9 activatable protease specific iron oxide particles (PSOPs) and their design can be seen in Figure 18.

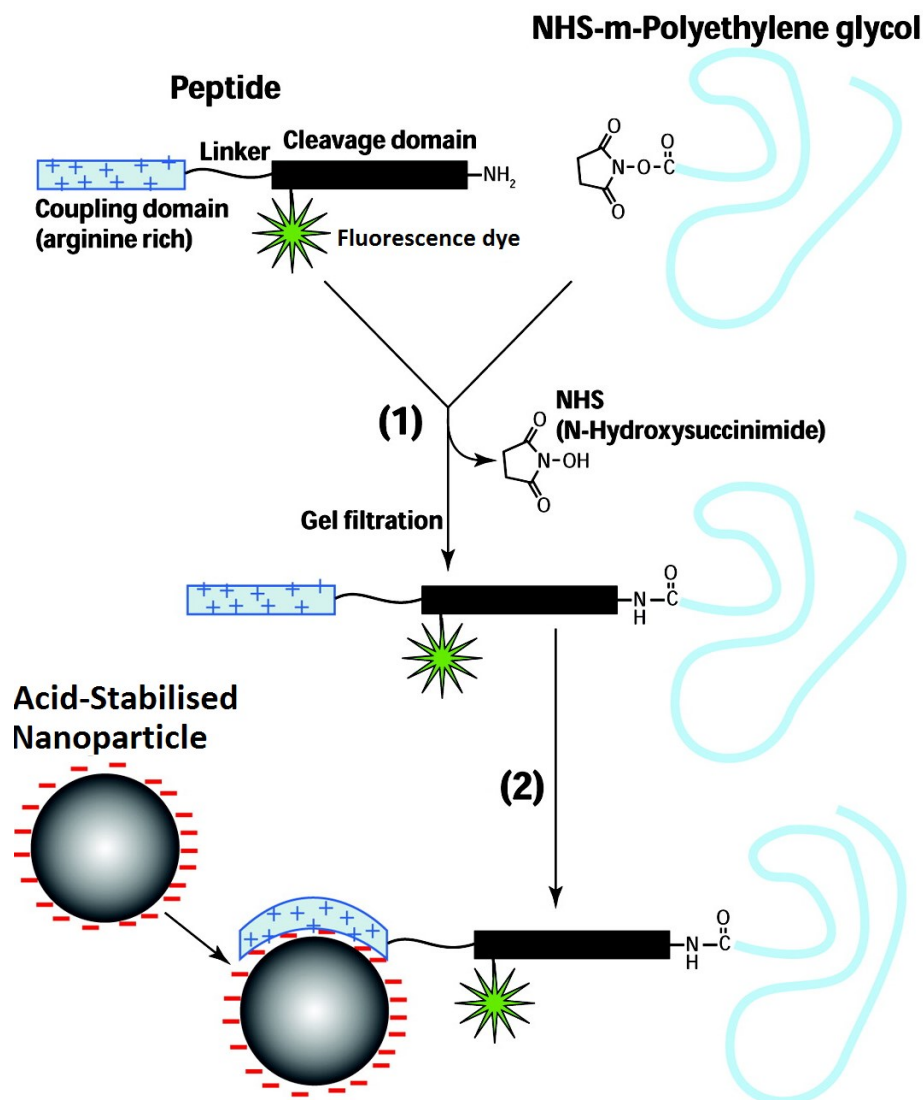


Figure 18 – Figure taken from Schellenberger *et al* showing the synthetic route to an MMP-9 activatable nanoparticle.⁵²

On cleavage of the peptide the PSOPs will lose the sterically stabilising mPEG shell. The particles will then aggregate driven by magnetic and electrostatic interactions caused by the superparamagnetic properties of the iron oxide core and the positively and negatively charged areas of the nanoparticle surface. This aggregation will cause an increase in relaxivity due to clustering of the nanoparticles and accumulation of the particles in the area which they were cleaved. This process can be visualised in Figure 19.

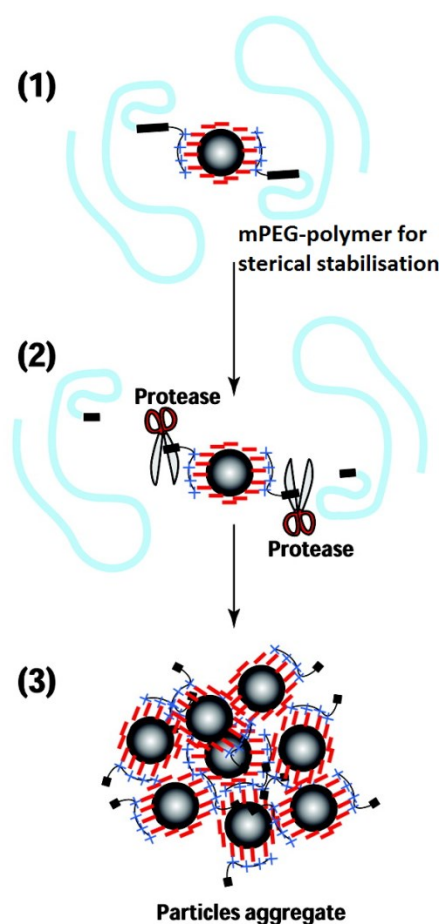


Figure 19- Figure taken from Schellenberger *et al* demonstrating the effect of MMP-9 activity on the nanoparticles. Protease releases the nanoparticle from the bound polymer and the charged particles now aggregate altering the relaxation time and increasing the contrast⁵²

Initial tests were carried out to determine if a change in hydrodynamic diameter was seen upon incubation of the MMP-9-PSOPs with MMP-9; it appeared from these tests that incubation with MMP-9 caused significant aggregation after only a few minutes, with particles growing to the micrometer range. A further test was carried out using the same conditions but with the additional presence of an MMP-9 inhibitor, here it was seen that the size of the nanoparticles remained unchanged over a 2 hour period. MRI experiments were then carried out on the MMP-9-PSOPs using different concentrations of the nanoparticles (300 nM, 150 nM, 75 nM and 38 nM) and 1.3 U of MMP-9, with T^{2*} weighted images being taken every 15 seconds for 50 minutes.

Signal intensities were seen to decrease depending on concentration and reach a minimum at approximately 15 minutes for 75 nM and around 27 minutes for 300 nM, with differences attributed to the time it took for the MMP to cleave the different amounts of peptide substrates.

It was found that there were some limitations to *in vitro* experiments carried out in this study. Firstly, it was seen that size measurements of the nanoparticles were only valid up until the point at which precipitation occurred and the particles dropped out of the zetasizer measurement field. Furthermore, this effect was observed in MR experiments with the decrease in T^{2*} contrast reducing at a certain point, which was likely to have been caused by similar precipitation of aggregates. Moreover, there is an issue in the fact that aggregation of the nanoparticles results in fewer water molecules feeling the influence of the magnetic field therefore decreasing the contrast that occurs.

No *in vivo* experiments were reported in the paper by Schellenberger *et al*, but it is stated in their paper that the precipitation which is an issue for the *in vitro* experiments is an intended and useful function when it comes to *in vivo* experiments.⁵² The hypothesis behind this is that the aggregated particles should accumulate in the area at which they were cleaved allowing for effective imaging of the site of MMP action. Although accumulation on aggregation of the nanoparticles is expected by the authors and would help to provide greater contrast, this theory has yet to be proven in an *in vivo* study. Despite the problems which were discovered in the *in vitro* testing of these PSOPs, they did still exhibited effective change in the MRI signal on treatment with MMP-9, and there is the potential that *in vivo* testing will alleviate some of the issues seen *in vitro*. Schellenberger *et al* provide an interesting example of MMP imaging using MRI contrast agents and highlight the opportunity to use this imaging technique in innovative ways to allow molecular imaging.

These two studies demonstrate the possibility of utilising contrast agents which alter relaxivity, along with the ability of MMPs to cleave specific peptides for creating a system in which MMPs can be visualised *in vitro* and *in vivo* using MRI. There are many positive points in using MR for the imaging of MMPs; for example it is a well-established clinical technique and if utilised for the detection of MMPs could provide important structural information combined with the detection of biomarker activity. However, as with the other imaging techniques discussed there are issues with using MRI for molecular imaging, for example its inherent sensitivity problems and the potential issues in acquiring quantitative data. There are on the other hand possibilities for overcoming these issues emerging through the use of new and innovative contrast agents.

1.3.4 Multimodal imaging

The ability to combine imaging techniques has long been a desirable concept, allowing for the amalgamation of the favourable characteristics each individual modality provides, whilst helping to reduce individual modality deficit. Already used in the clinic multimodal PET/CT imaging has becoming a highly valuable diagnostic tool. Combining imaging modalities within molecular imaging has the potential to provide detailed quantitative analysis of biological processes. Of particular interest has been the combination of PET/MR due to the high sensitivity of PET imaging and the unmatched soft tissue detail of MRI.⁵³

Within the area of MMP imaging there have been a number of examples where multimodality probes have been investigated. One of the earlier examples of this comes from Tsien *et al* in a continuation of their work on activated cell penetrating

optical probes described above.⁵⁴ Designs that incorporated ACPs onto a dendrimeric polyamidoamine (PAMAM) nanoparticle which could then be linked to fluorescent dye (Cy5.5) and gadolinium-DOTA complexes were developed. It had previously found that these large macromolecular dendrimers contributed to favourable improvements with the ACPs (i.e. longer tumour circulation time and less background uptake) and additionally afforded the incorporation of multiple imaging modalities.

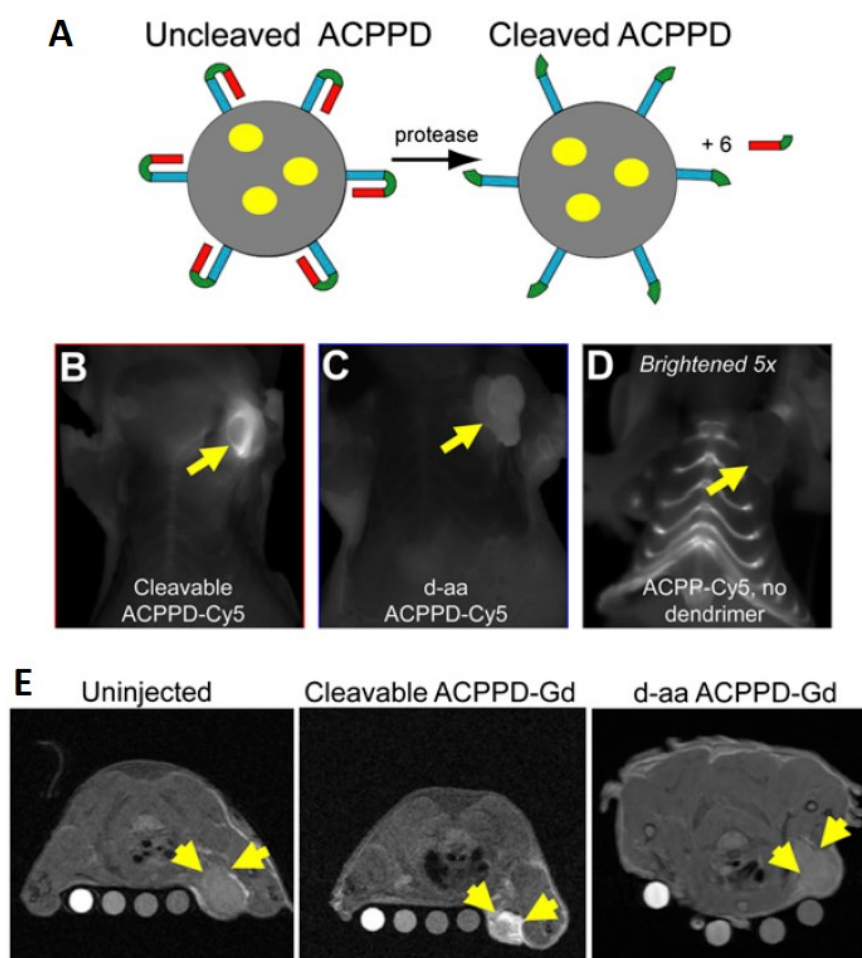


Figure 20 – Diagrams and images from Tsien *et al* A: Schematic of the dual fluorescence and gadolinium(III) nanoparticle functionalised with an activated cell penetrating moiety. B-D: *in vivo* fluorescence imaging of PyMT breast cancer model. E: MRI of nanoparticle uptake in HT-1080 model.⁵⁴

This probe enabled activated uptake of both fluorescence in a PyMT breast cancer model and of gadolinium in a HT-1080 model. This can be seen in Figure 20 where uptake in cleavable peptide can be compared to a D-amino acid control compound (and with the fluorescence example with the non dendrimeric fluorescent probes). Tumour uptake compared to background (muscle and blood) was good, however high uptake was seen in the liver and kidneys of the animals. These probes were taken forward and used for the guided removal of tumours in mice providing a postoperative imaging method for detection of any remaining pathogenic tissue, which could then be excised further. This study successfully demonstrates the ability to combine imaging methods in MMP imaging and for such probes to be utilised for guided surgery. However, these probes do not contain any element of an 'Off/On switching' system which could be utilised and the ability of probes to supply quantitative data is not discussed.

Another recent example looking at the combination of MRI and optical imaging modalities comes from Park *et al.*⁵⁵ This was designed with the aim of imaging membrane bound MT1-MMP (MMP-14) and its protease activity simultaneously. A probe was designed incorporating an MT1-MMP activated fluorogenic peptide (containing a fluorescence dye and quenching group) and an iron oxide magnetic nanocrystal. The proposed mode of action can be seen in Figure 21 involving the localisation of imaging probes to the surface of cells for MT1-MMP cleavage resulting in increase in fluorescence signal and localisation of nanoparticle in cells via endocytosis.

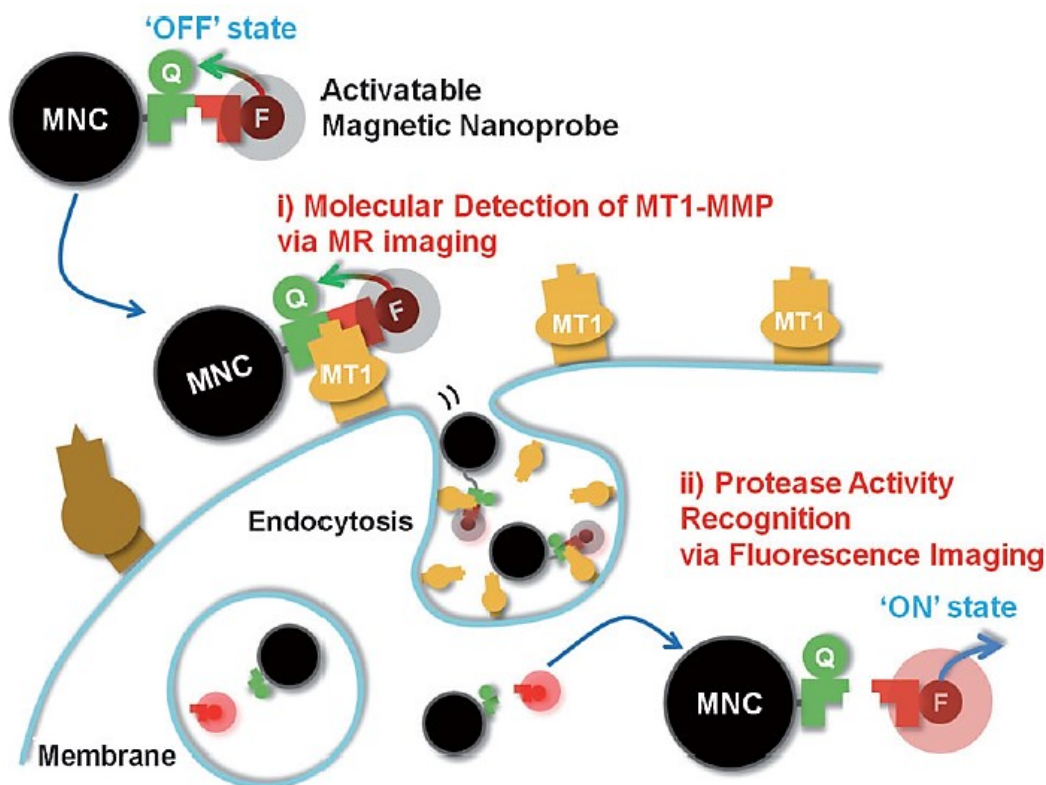


Figure 21 - Schematic taken from Park *et al* describing the proposed mode of action of dual MRI and optical probes for MT1-MMP (MMP-14) imaging; based on the localisation of probe on the cell surface via MMP- substrate binding, followed by activation of the fluorescent probe via protease activity, and incorporation of the contrast agent into the cell through endocytosis.⁵⁵

These probes demonstrated significant fluorescence change on incubation with MT1-MMP compared with scrambled control and selectivity was seen when compared to MMP-3 and MMP-7 incubation. MR signal change was also exhibited *in vitro* on treatment of HT180 cells which are MT1-MMP active. Specific uptake of the compound was shown via a loss of signal change on treatment with a broad spectrum MMP inhibitor. Subsequent *in vivo* studies gave positive results, although different timescales were needed to visualise peak fluorescence and MR signals. Further studies with these compounds would be necessary to determine selectivity of uptake with regards to tumours which express other membrane bound MMPs and to assess the potential for extracellular activation by other soluble MMPs such as MMP-2 and

MMP-9 (often present at similar points to MT1-MMP). In addition to this, probes produced visible background signal in both MR and fluorescent experiments which could be problematic.

As well as the combination of MRI and optical imaging techniques there have been examples exploring the amalgamation of optical imaging with different modalities, for instance Huang *et al* investigated the combination of optical imaging and PET.⁵⁶ In this example an MMP activated optical imaging probe based around an MMP cleavable peptide is functionalised with an additional ^{64}Cu -DOTA group (Figure 22). This group would allow for more accurate information about the compounds biodistribution and potentially allow for quantification of signal.

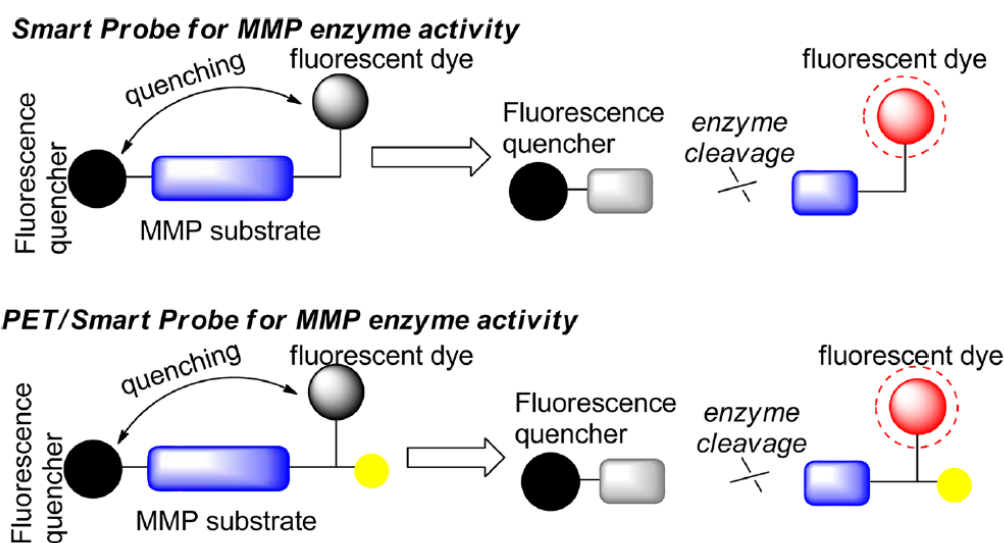


Figure 22 - Schematic taken from Huang *et al* describing the design of dual optical and PET imaging probe, top schematic describing an MMP-activated optical smart probe and below the same design incorporating a PET component in yellow which acts as an internal reference.⁵⁶

The dual fluorescence/PET probe demonstrated breakdown and increase of fluorescence in *in vitro* studies with MMP-13 and proof of concept *in vivo* imaging was carried out. This involved the subcutaneous injection of MMP-13 (at a constant concentration) and the imaging probe (at different concentrations). This demonstrated

the ability to utilise PET to discern the difference in signal due to higher imaging probe concentration rather than increased MMP expression and activity. While this is an interesting example of the combination of PET and optical imaging, the PET element of this imaging probe acts as an internal standard (much like the fluorescent pairs described earlier) rather than a molecular imaging probe in its own right, which could perhaps be a more interesting combination of modalities. Additionally, this probe suffered from a number of issues relating to nonspecific peptide degradation, poor probe retention time and pharmacokinetics which need further development.

These multimodal imaging examples represent one future direction of molecular imaging, moving towards combined systems which reduce the weaknesses of individual modalities alone. However, as these examples show there are difficulties in mixing imaging modalities in to compound design. The complexity of multimodality imaging is further emphasised when exploring issues relating to the equipment required for such analysis (i.e. PET/MRI machines), and combined imaging technologies are not readily available in the clinic. Additionally, the costs and justification of these techniques are still being established. Looking at the examples described above for MMPs, the use of optical imaging in all the examples reflects the large number of investigations carried out using this modality for these proteases. Nevertheless, optical imaging is still difficult to transfer into the clinic even in these combined modality agents.

1.3.5 The future of MMP imaging

While the examples above represent large steps forward in the molecular imaging of proteolytic events a single MMP imaging probe has yet to fulfil the full potential of

MMP imaging (i.e. use in the clinical environment). This can be related to the failure to transfer successful optical imaging probes into a more clinically useful imaging modality. Modalities such as PET and SPECT are more difficult to develop into activated ‘on/off’ switching probes and therefore lack the ability to utilise the amplification benefits of proteolytic activity. One might argue that efficient targeting of radiolabelled tracers would result in sensitive enough probes that amplification is not necessary; however, issues with efficient MMP targeting mute such opinions. There has been little exploration into the use of MRI for the activated imaging of enzymatic reactions. While these systems remain more difficult to design than optical probes there is scope for innovative development. MRI probes would provide contrast agents easily transferable to the clinical environment, ideal for longitudinal studies which have the potential to provide both structural and molecular information. MR imaging therefore represents the most interesting area for investigations into single modality MMP imaging. Ultimately, a probe which enables multimodality imaging has the potential to provide an all-round approach to MMP imaging combining sensitive imaging with specific on-off design and easy transfer to the clinic. To enable this, successful single modality investigations will need to be carried out and optimised.

Apart from the issues of clinical applicability another feature which is highlighted in the above MMP imaging examples is the specificity of the probes. Despite being targeted towards a specific MMP, many imaging probes have in reality been broad spectrum. Much like with MMP inhibitors this represents an area for improvement within MMP imaging. Such broad spectrum probes are more likely to suffer from high background signals related to non-pathogenic cleavage and have potentially reduced value as prognostic markers. This lack of selectivity reduces the ability to use these

imaging probes to further our knowledge on the roles of MMPs within cancer and for the development of specific MMP inhibitors.

1.4 *MRI and the use of hetero-nuclei*

Introduced earlier, MRI is recognised as one of the most important diagnostic tools available in the clinic today. It is a non-invasive technique which is radiation free, offers excellent spatial resolution and good soft tissue contrast without any issues related to depth penetration. It is also ideal for longitudinal studies as it is low risk to patients. The ability to utilise MRI for molecular imaging of biomarkers such as MMPs would be indispensable as it would allow for an easy combination of structural and molecular imaging of patients, using an imaging technique already available in many hospitals worldwide.⁶ Nevertheless, MRI does have problems associated with its low sensitivity, which with molecular imaging becomes increasingly problematic, due to the signal arising from background water. This results in low signal to noise ratios and a difficulty in discriminating between normal background and targeted molecular events.

New possibilities for overcoming the issues associated with MRI are continually emerging, with many employing new and innovative contrast agents to achieve this. For example the sensitivity of MRI contrast agents may be improved through the use of polymeric constructs to increase the local delivery of the contrast agent. Increases in relaxivity may also be achieved through an increased size and a longer rotational correlation time all of which have been investigated to improve the MRI technique. Increasingly more innovative techniques are being investigated such as CEST or PARACEST, demonstrating a move away from the conventional imaging of bound

water to altering magnetisation in bulk water, or the use of alternative nuclei such as ^{19}F , ^{31}P and even ^{13}C , which can be visualised with the use of hyperpolarisation.⁵⁷⁻⁵⁹ Many of these techniques not only allow for increased signal to noise ratios and sensitivity, but can also provide quantitative data and allow for contrast agents which can be “switched on” *in vivo* allowing for separate imaging of structure and the molecular target. These emerging MRI techniques represent exciting areas of development for molecular imaging.

An area of particular interest for this investigation is the aforementioned employment of a hetero-nuclear approach to MRI. One of the most promising of hetero-nuclides is ^{19}F due to its sensitivity being comparable to ^1H and the lack of fluorine background signal within the body.⁵⁹ The ability to image ^{19}F within the body alongside a regular ^1H image could provide both structural information and specific information on fluorine position; this could provide solutions to a number of the issues connected to MRI.

1.4.1 Fluorine MRI

^{19}F is a 100% naturally abundant spin $\frac{1}{2}$ nucleus with a high gyromagnetic ratio of 40.05 T similar to that of ^1H (allowing for the use of existing NMR/MRI equipment) and a comparable sensitivity to ^1H of 83%.⁵⁹ Perhaps most importantly, whilst present in the body endogenous fluorine produces a negligible ^{19}F MRI signal due to non-detectable physiological concentrations of fluorine, with the only high concentrations in the body existing in solid salts such as the bone matrix and the teeth.⁵⁹⁻⁶¹ In these circumstances the fluorine is solid and immobilized producing an extremely shortened T_2 (spin-spin or transverse) relaxation time resulting in a signal which is beyond the

detection limits of NMR/MRI detection in most biological systems. Thus, any exogenous fluorine containing agent or compound can be detected without interference from background signals resulting in a very good signal to noise ratio. It is the similar sensitivity to ^1H and this lack of background signal within biological systems that makes fluorine very attractive for molecular imaging. However, only relatively low concentrations of fluorinated probes may be introduced into a biological system because of issues with toxicity; this can prove problematic as in order to achieve detectable signals and decent images a sufficient amount of fluorine must be present. This means that fluorine MRI can suffer from similar problems with sensitivity as ^1H .

In addition to inconsequential background noise fluorine has a number of other attractive qualities. For example ^{19}F chemical shifts are incredibly sensitive to even small perturbations in the chemical microenvironment and ^{19}F has a large range of chemical shifts (greater than 300 ppm) meaning that even small changes in the state of a fluorinated probe (i.e. metabolite formation) can often easily be differentiated.⁵⁹ However, unlike ^1H and ^{13}C , the ^{19}F chemical shift can appear to be arbitrary with very little predictability (though some attempts have been made to rationalise the values). Additionally one of the major advantages of using ^{19}F MR imaging is the potential to quantify amounts of the exogenous probe in an area of interest, for example a number of protocols for detecting and quantifying cardiovascular abnormalities using fluorine can be found in literature.^{62, 63}

1.4.2 Fluorine MR Imaging probes

A large number of fluorine imaging probes have already been designed and tested, in fact early ^{19}F MRI systems were suggested in the 1970s around the time first human

^1H MRI was taking place. At this time early ^{19}F MR images were taken using NaF and perfluorotributylamine (PFTA) and represent some of the earliest examples of contrast agents for MRI.⁶¹ Contemporary examples of fluorinated probes can be separated into 2 basic categories: exogenous agents which are detected and quantified directly by ^{19}F , and those where the fluorinated contrast agents can be used for indirect detection of molecular changes or environmental factors.⁶¹

Directly detected agents include compounds which act as passive reporter molecules, such as fluorinated gases or liquid perfluorocarbons (PFCs) which can be utilised for functional ^{19}F imaging of the lungs.^{61, 64} Importantly, another area of direct fluorine detection encompasses the investigation of fluorine containing drugs monitoring their activity and metabolism. One of the best examples of this comes from a number of studies investigating the anticancer drug 5-Fluorouracil or 5-FU, which is one of the most popular drugs for chemotherapy.^{59, 65} For this drug a number of ^{19}F NMR and MRS studies have been carried out in order to determine the metabolism of the drug within the body and determine its efficacy and toxicity. These studies have shown that other than the desired mode of action of the drug a competing catabolic pathway converts 5-FU into FBAL (α -fluoro β -alanine) and various other molecules which offer little toxicity. It has also been reported that the 5-FU pharmacokinetics are pH sensitive and fluorine NMR studies have shown that the chemical shift of the metabolites of 5-FU exhibit sensitivity to pH. In addition to studies of 5-FU itself, ^{19}F NMR and MRS have been used to investigate a number of minimally toxic pro-drugs (necessary due to the high dose limiting toxicity of 5-FU).

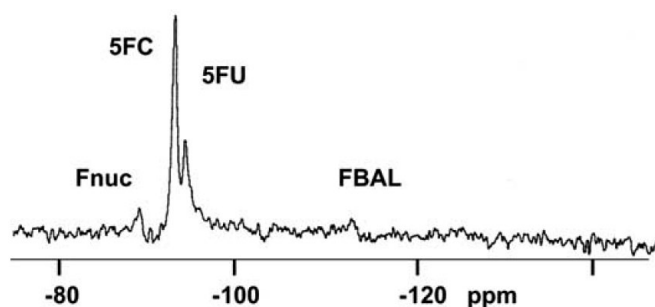


Figure 23 - Example taken from reference 57 of a successful study in to a 5-FU pro-drug 5-fluorocytosine (5-FC), this converted to 5-FU via Cytosine deaminase (CD), conversion can be monitored through a change chemical shift of approximately 1.5ppm.⁵⁹

All of these investigations into 5-FU its pro-drugs and metabolites have demonstrated the potential of the fluorine nuclei for imaging in drug discovery and development, as well as a means to monitor and optimise pro-drug administration. Unfortunately some of the studies using fluorine MRI suffered from issues due to the low sensitivity of the MR technique which must be addressed.

Fluorinated contrast agents designed for indirect detection include compounds which are susceptible to changes in pH, ion concentration (such as Na^+ , Mg^{2+} or Ca^{2+}), oxygen concentration or the presence of specific enzymes or proteins.⁶⁶⁻⁶⁹ For example, the use of on-off self-assembling nanoprobe for the detection of proteins (using human carbonic anhydrase I or hCAI for proof of principal).⁷⁰ These probes were designed with a fluorine-containing group, a ligand of interest (in this case targeting hCAI) and the capability to assemble into nanoaggregates. These nanoaggregates result in attenuated fluorine signal due to broadening. On contact with the protein of interest ligand binding would result in disassembly of the aggregated monomers and an increase in signal (mode of action shown in Figure 24).

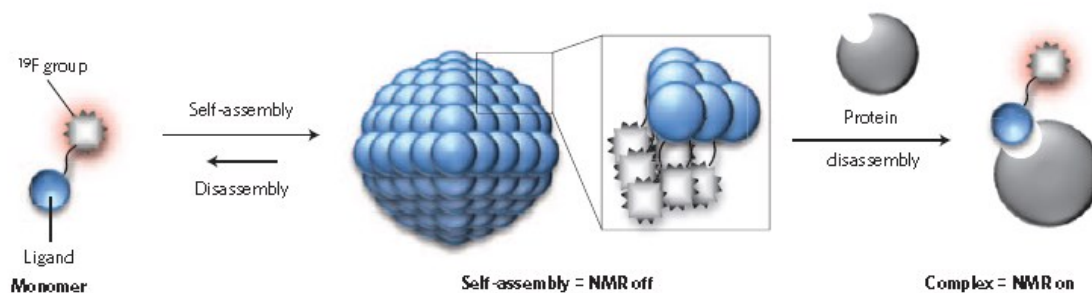


Figure 24 - Schematic from Takaoaka *et al*, describing the mode of action of self-assembling fluorinated nanoparticles and their protein mediated disassembly.⁷⁰

^{19}F NMR of self-assembled aggregates demonstrated no signal, however on addition of hCAI a signal at -62.6ppm was observed, confirming the potential of these compounds.

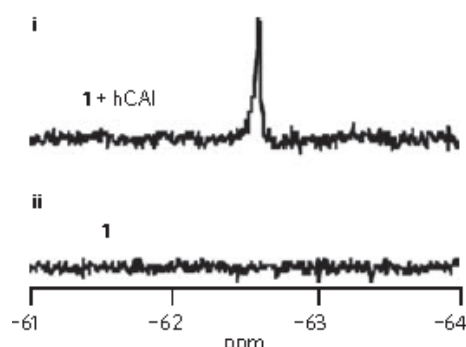


Figure 25 – Picture taken from reference 63 Change in ^{19}F NMR signal from assembled nanoparticle (bottom) to disassembled fluorinated compounds (top) on contact with hCAI.⁷⁰

Presence of nanoaggregates and their disassembly was further proven by TEM, SEM and UV spectrum. These systems were then successfully transferred to interrogate presence of trypsin and avadin.

It has been found that with well thought out compound design it can be possible to monitor 2 variables with only one ^{19}F contrast agent.⁷¹ One such example of this is in the detection of β -galactosidase whereby a substrate compound can be employed which will change its fluorine chemical shift on metabolism by the β -galactosidase enzyme; the product of this enzymatic reaction can then also detect local pH due to

chemical shift variations of the ^{19}F signal.⁷¹ 4-fluoro-2-nitrophenyl- β -D-galactopyranoside or PFONPG acts as an efficient substrate for β -galactosidase and upon cleavage of the glycosidic bond a chemical shift change of $>3.6\text{ppm}$ is seen. PFONPG itself shows no pH sensitivity but the product of this cleavage reaction aglycone or PFONP has a pH sensitive chemical shift range of about 9ppm, and importantly there is no overlap between the chemical shift of the substrate and the product.

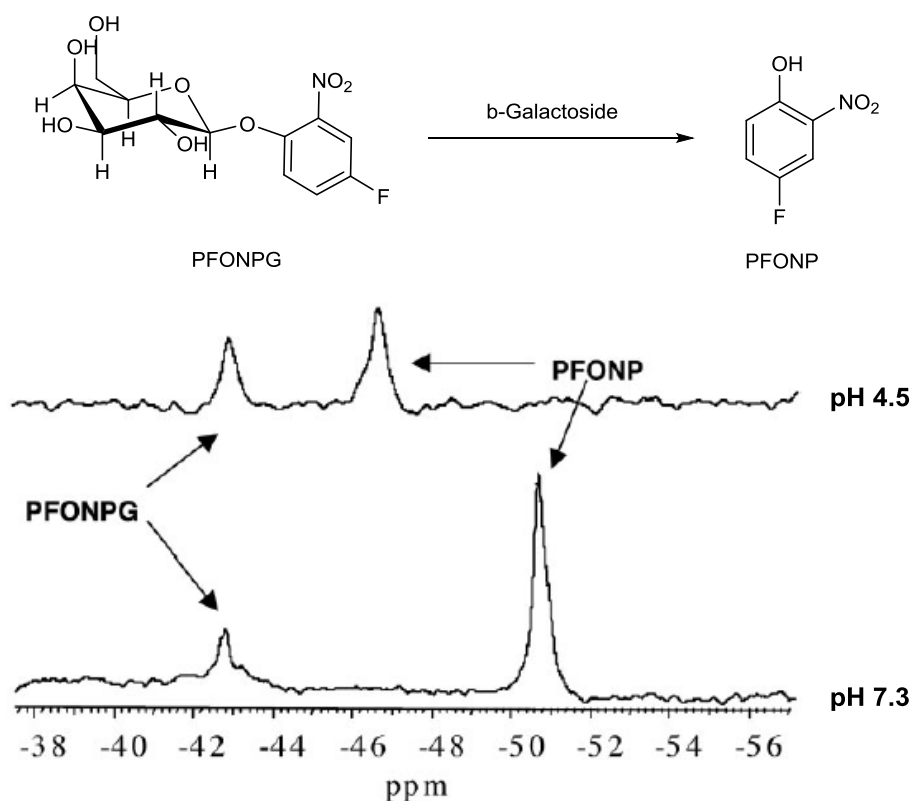


Figure 26 – Scheme showing the reaction of PFONPG with β -galactoside and the ^{19}F NMR spectrum from reference 68 showing the change in chemical shift differences between parent compound PFONPG and enzymatic product PFONP and the pH dependant shift of PFONP.⁷¹

This example demonstrates nicely the potential for a number of parameters to be simultaneously detected via the sensitive chemical shift changes of the fluorine nucleus.

1.4.3 ^{19}F MRI: Applications to enzymatic imaging

A large amount of work has been carried out which describes the development of various fluorine MRI contrast agents, but those examples which are most interesting for this investigation are those related to activated enzyme imaging. An example previously described in this report focused on work to detect chemical shift changes on enzymatic reaction, whilst helpful this approach is dependent on the magnitude of chemical shift changes.⁷¹ The ability to manipulate the signal strength of fluorine NMR/MRI through enzymatic reaction would provide a more easily applicable technique for activated imaging of enzymes such as MMPs.

Examples from Mizukami *et al* demonstrate such a system for caspase imaging, whereby only a low fluorine signal can be observed in the substrate compound prior to enzymatic reaction and this signal is then greatly increased in the product of the reaction.^{67, 68} Such an effect is achieved through the utilisation of paramagnetic relaxation enhancement (PRE) which describes the ability of paramagnetic metal ions (Fe^{3+} , Gd^{3+}) or paramagnetic molecules (O_2) to shorten the T_2 of fluorine. In particular Gd^{3+} has a very strong relaxivity due to its large electron spin quantum number so can exert a very strong paramagnetic effect on ^{19}F resulting in an extremely shortened T_2 which will attenuate the fluorine MRI signal. The basic design principle in these studies was that an intramolecular paramagnetic effect on the T_2 of the ^{19}F signal could be modulated by protease activity Figure 27.

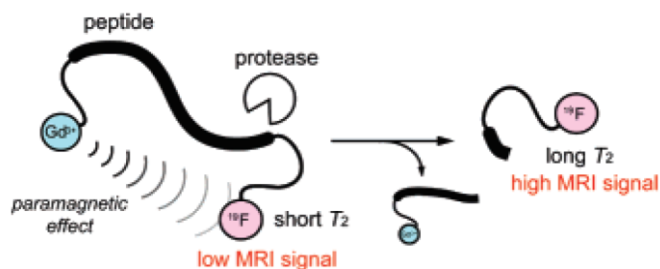


Figure 27 - Demonstration of PRE for protease imaging: Paramagnetic centre is attached to a fluorinated group via a protease substrate, prior to cleavage PRE effect is shortening the T_2 of the fluorine resulting in low signal intensity, on cleavage paramagnetic centre no longer has an effect and the T_2 is lengthened increasing fluorine signal. Figure from Mizukami *et al.*⁶⁷

One of the compounds designed can be seen in Figure 28 and shows a Gadolinium chelate attached to a fluorine containing group via a peptide sequence which is a caspase -3 substrate sequence.

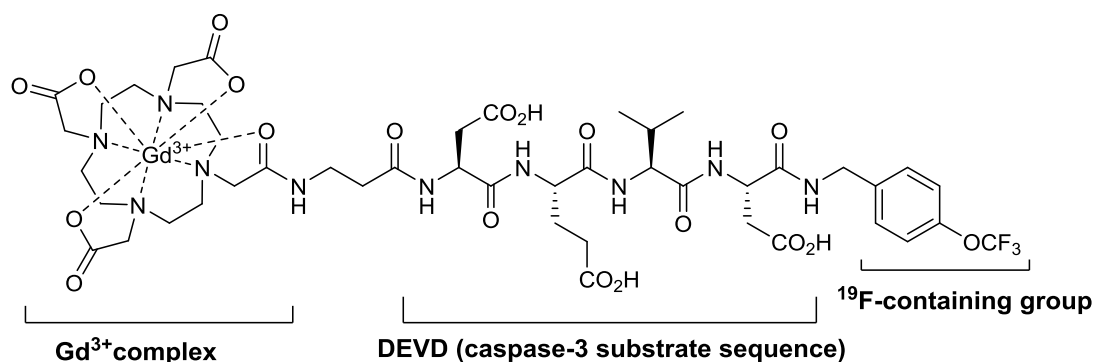


Figure 28 – Structure of Gd-DOTA-DVED-Tfb, a fluorine MRI contrast agent for the imaging of caspase 3 via PRE, gadolinium-DOTA is in close proximity to CF_3 containing group via a peptide substrate of caspase 3.⁶⁷

In this compound the ^{19}F signal will be low due to the PRE effects of the Gd^{3+} complex. On incubation with caspase-3, the peptide should be cleaved and the fluorine containing group released, resulting in an increase in signal as the paramagnetic effects of the gadolinium can no longer be felt by ^{19}F (increasing the T_2). Testing on this

compound with caspase-3 using NMR and MRI phantoms demonstrated the ability of this technique to visualise enzyme activity *in vitro*:

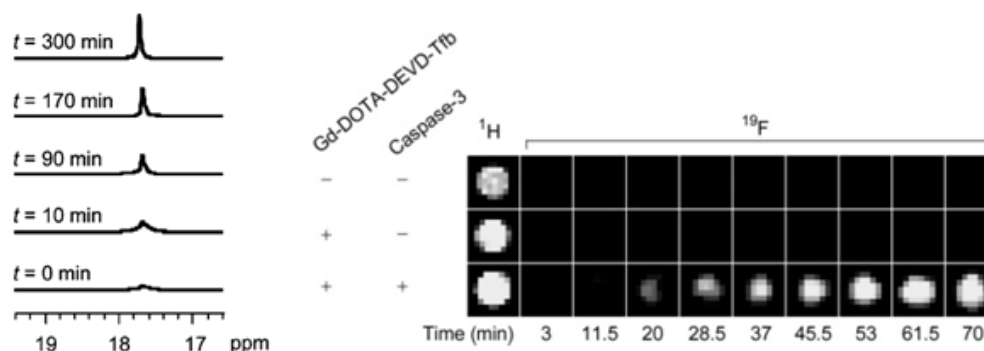


Figure 29 – Results from Mizukami *et al* of the enzymatic testing on Gd-DOTA-DVED-Tfb using NMR and MRI, showing increases in signal intensity of both ^{19}F NMR and MRI on enzymatic incubation.⁶⁷

A further study investigated the incorporation of a fluorescence molecule into a similar system and again provided successful imaging results demonstrating both an increase in ^{19}F signal and fluorescence on enzymatic reaction.⁶⁸ This principle for designing enzyme activated contrast agents using the ^{19}F nuclei and PRE appears to have great potential and could be easily applied to a variety of proteases such as MMPs offering a similar off-on system that is usually used by optical imaging probes.

1.4.4 Improving the ^{19}F signal

Whilst fluorine MRI has a great deal of potential in molecular imaging one of its major issues is that despite similar sensitivity to ^1H , hydrogen represents almost two thirds of the body's nuclei. Therefore, in order to produce images of similar quality to those of ^1H MRI the ^{19}F signal needs to be increased on the molecule and concentrated within the tissue (at the desired location). Detection limits of MRS studies in whole body scanners at 1.5T suggest a $30\text{ }\mu\text{mol/g}$ wet weight in a volume of about 33 mL with a 6 minute acquisition time.⁶¹ Other reports have suggested that lower amounts may be

used along with stronger magnets and longer acquisition times.⁶¹ Therefore in order for fluorine MRI to be successfully implemented in molecular imaging techniques through which sensitivity issues may be resolved will need to be explored.

Methods to increase the fluorine signal can be as simple as increasing the number of fluorines present on the contrast agent in question. Compounds such as PFCs for example can provide high numbers of fluorines on one molecule or the use of fluorinated nanoparticles can be employed.^{61, 72} There are however issues with how such a large concentration of fluorine on a molecule will affect solubility, biodistribution and toxicity. Another simple method of improving fluorine imaging (and the contrast to noise ratio) is to increase the applied magnetic field strength, though at present this is not really viable for clinical translations.

Another possibility is the use of lanthanide metals to increase the signal intensity of a smaller group of fluorines. Whilst previously we discussed the use of gadoliniums paramagnetic relaxation enhancement to increase the T_2 signal to the point of broadening and suppressing the ^{19}F signal, it has been seen that other lanthanide metals have an optimum point where increase of relaxivity has a positive effect on the signal. Work by Parker *et al* describes how a balance can be achieved between the paramagnetic relaxation of the T_1 (longitudinal relaxation) to enhance the fluorine signal and the detrimental effects of broadened spectral lines associated with the fast relaxation of the T_2 (transverse relaxation).^{60, 69, 73} It has been found that careful positioning of a CF_3 reported within $7\text{\AA}$ of a paramagnetic centre, a lanthanide (III) ion such as Tb^{3+} , Dy^{3+} , Ho^{3+} , Er^{3+} or Tm^{3+} will enhance the rate of ^{19}F relaxation and increase signal intensity. In addition to this, pseudocontact shift (PCS) induced by the lanthanide ion will amplify the fluorine chemical shift sensitivity resulting in chemical shift differences between compounds being much greater and more easily

distinguished from one another. There is a great deal of potential in the use of lanthanides to improve the sensitivity of fluorine MRI and this is well highlighted in the work by Parker *et al.*

Another way in which fluorine sensitivity may be increased is via an increased local concentration of the fluorinated contrast agent. This could be carried out via nanoparticle delivery to a specific site or by designing the compound to “stick” at the point of interaction (i.e. enzymatic cleavage) to allow a build-up of the fluorinated contrast agent.^{62, 63} One example of this comes from the use of PFC nanoparticles functionalised for targeted imaging of fibrin using a fibrin antibody and resulted in high delivery of fluorine to a target site.⁶² A method such as this would increase retention times (allowing longer acquisition) and local concentrations up to levels that would allow for more effective imaging.

Sensitivity will be a very important factor for the progression of ^{19}F MRI imaging and any probes designed for ^{19}F detection will need to acknowledge this and develop to improve the technique. As described above there is a large amount of potential for increasing the sensitivity of ^{19}F MRI with a number of promising methods that may be employed in imaging probe design to varying degrees of success.

1.5 *Aims and objectives*

The main aim of this work was to design and synthesise a molecular imaging probe for the detection of MMPs which in the future could develop into a clinically applicable imaging agent. This imaging agent would allow for further investigation into MMPs and their roles within cancer, and have the potential to act as a tool in the diagnosis and treatment of the disease. The issues highlighted in literature within MMP imaging

focus around lack of selectivity of imaging agents, and probes which are ill designed for the clinical environment. It was determined the best route to solve these issues was via the exploration of MRI, a modality which has been neglected within MMP imaging. While the solubility switch approach as described in literature for MMP imaging has shown some positive results, high background signals from water will limit the sensitivity of these contrast agents *in vivo*. Therefore, the exploitation of alternative nuclides, namely ^{19}F , was deemed an innovative route for compound design, with the added potential to provide MMP-activateable imaging. Additionally, it was decided imaging probes would be designed to be MMP specific, beginning with a focus on MMP-2 due to prolific reference to this protease in literature. Thus, this work focused on the following objectives: design, synthesis and testing of a potential ^{19}F MR imaging probe targeting MMP-2 activity, development of this probe to improve its potential for *in vivo* imaging, and finally testing the transferability of imaging probe design to other MMPs.

Chapter 2

Design and synthesis of an MMP-2 - activated imaging agent

2.1 *Introduction*

MMP-2 has been widely discussed in relation to disease due to its overexpression in a large number of cancers. It has long been highlighted as a potentially important biomarker and is widely acknowledged to be involved in metastasis and facilitating the spread of the cancer throughout the body.^{5, 13} Involvement of MMP-2 occurs not only via mechanisms of ECM breakdown but also through altering cell adhesion and promoting cell migration via processes such as the cleavage of Laminin-5 and also mediating the release of growth factors for example VEGF.^{22, 74} Along with MMP-9 it has been implicated in the angiogenic switch which occurs at early stages of tumour progression and involves the vascularisation of the tumour environment.¹⁰ MMP-2 null mice have been shown to have reduced vascularisation compared to wild type controls, while MMP-2/MMP-9 doubly deficient mice have revealed the synergistic effect these proteases have in angiogenesis.^{10, 23} Additionally MMP-2 expression in malignant tumour cells has also been linked to patient prognosis in a number of cancers for example prostate cancer and levels of expression have been found to vary in different histotypes of ovarian cancer, indicating its potential as a diagnostic tool.^{31, 75 32}

The attraction of being able to image MMPs individually comes from the need to understand their individual roles in cancer and what these mean for patients. While

MMP-2 and MMP-9 appear to be highly structurally related and are involved in similar processes such as angiogenesis, there are indicators that they do in fact have highly distinct roles.^{16, 28} The choice of focusing on MMP-2 over MMP-9 comes from numerous examples of its prognostic potential, its known activation on tumour and stromal cell surface by MMP-14, and its high concentration on leading edges of invading tumours (which suggests potential as a localised marker of early tumour progression).^{28, 31, 32, 74} Furthermore, MMP-2 has also been implicated in an MMP-9 activation pathway alluding to activation before other downstream effects.^{28, 74, 76}

As previously discussed MMPs have been imaged using a wide variety of methods, the most successful of these being optical imaging. MMP-2 itself has been widely targeted but in most examples this has either been carried out alongside MMP-9 (the other gelatinase of the MMP family) due to the similarity of their peptide substrates and their highly related structure, or been investigated without looking into selectivity. One of the first examples of MMP imaging was an optical imaging probe designed by Bremer *et al* aimed towards MMP-2 activation.³⁵ This probe was the first demonstration of the potential of MMP imaging as a diagnostic or developmental tool (see Chapter 1). Despite the success of these probes not only as markers of MMP activity *in vitro* and *in vivo*, but also demonstrating an ability to monitor tumour response to treatment, the probes did not display complete specificity for MMP-2 (demonstrating breakdown with a number of other MMPs) and lacked the ability to quantitatively assess MMP activity. Other examples of MMP-2 imaging include targeting alongside MMP-9 in probes such as the aforementioned ACPs as designed by Tsien *et al* and further investigated by a number of other groups including Grull *et al*; while these demonstrated a promising strategy for MMP-2/-9 *in vivo* imaging, problems arose

from unspecific cleavage occurring in blood which could be attributed to the non-specific nature of the probes.^{37, 77}

The features which impart substrate recognition and distinction for MMPs are not fully understood; generally it is thought MMPs impart much of their selectivity through differences in domains and exosites, however these only apply to macromolecule recognition, thus for small molecule and substrate recognition differences in the catalytic cleft need to be examined.^{12, 14} For all of the MMP family structural features of the catalytic clefts are generally similar, containing a zinc(II) ion and glutamic acid residues involved in catalysis. Additionally, MMPs also display several recognition pockets either side of the catalytic zinc ion most significantly a deep S1' pocket which generally favours large hydrophobic residues;^{12, 14} this has been used as the site of binding for the majority of pharmaceutical MMP inhibitors and substrates targeting them to a broad spectrum of MMPs.^{10, 12, 14}

In 2002 Chen *et al* looked to investigate MMP-2 selectivity within the catalytic cleft and studied a large number of peptides highlighting those which were selective substrates.¹⁶ Features of these selective peptides were analysed to determine how selectivity arose. From this investigation the P₂ amino acid position of substrates was highlighted as important (with recognition profile P₃P₂P₁↓P₁'P₂'P₃' where the arrow denotes point of cleavage) with its interaction with the S₂ subsite contributing to MMP-2/-9 selectivity. The S₂ pocket is remarkably similar for both enzymes apart from a glutamic acid (Glu⁴¹²) in MMP-2 which is replaced by an aspartic acid (Asp⁴¹⁰) in MMP-9, it is suggested from modelling studies that glutamic acid extends further into the S₂ pocket and can form a hydrogen bond with the backbone of selective substrates.¹⁵ This bond influences the turnover number (K_{cat}) of selective substrates signifying an involvement in positioning of substrate for optimal catalysis.¹⁵ Mutations

of MMP-2 at this glutamic acid to an aspartic acid produced detrimental effects on the rate of hydrolysis of selective MMP-2 substrates due to a decrease in the K_{cat} underlining the importance of this interaction.¹⁵ However, interestingly these MMP-2 mutations had significantly less effect on substrates which show both MMP-2 and MMP-9 activity indicating alternate interactions are important for broad spectrum peptide recognition and catalysis.¹⁵ It is important to note that that whilst providing a substantial contribution the interaction at P₂/S₂ is not solely responsible for MMP-2 selectivity, as shown by the incomplete conversion to MMP-9 activity on glutamic acid mutation.

Another important consideration comes from research which suggests the catalytic cleft generally favours substrates which deviate from linearity produced by a proline at the P₃ position; however it has been found that inclusion of this amino acid at P₃ can remove selectivity and alter recognition mechanisms from a selective to a broad spectrum mode.^{16, 17} This is demonstrated by the fact many broad spectrum MMP substrates contain a proline at P₃ while many of those which show high selectivity for MMP-2 do not.

A number of MMP-2 selective peptide substrates are reported in the aforementioned Chen *et al* paper and could be incorporated into a selective MMP-2 imaging agent. One example which has taken one of these selective substrates and looked at MMP-2 specific imaging comes from Lepage *et al*.⁴⁹ This work was outlined in Chapter 1 and describes an MRI contrast agent based on a solubility switch design utilising the SPAYYTA motif discovered by Chen and co-workers.^{16, 49} This substrate is functionalised at both the N and C-terminus and demonstrates positive results *in vitro* and *in vivo* for an MMP-2 activated change in compound solubility, however this paper does not go into detail on selectivity of this compound. Our initial choice of

substrate was based on the work by Chen *et al* into MMP-2 selective peptides, utilising both the SPAYYTA motif exploited by Lepage *et al* and another similar motif SLAYYTA; further details are given in section 2.2.2.

2.2 *Results and discussion*

2.2.1 Aims and objectives

The aim of this work was to begin the development of an MRI probe suitable for the detection of MMP-2 *in vivo*, focusing on creating a highly MMP-specific probe which features an activatable component, utilising the enzymatic amplification of these proteases. This was carried out by concentrating on the following objectives:

1. Design and synthesis of an MMP-2 activated MRI imaging probe, focused on the potential of ¹⁹F MRI.
2. Development of an efficient method of testing enzymatic cleavage of peptides.
3. Testing of probes for their ¹⁹F MRI potential: proof of principle that paramagnetic relaxation enhancement can be applied to the activated fluorine imaging of MMP-2.

2.2.2 Design of MMP-2 activated MRI probe

Initial design for an MMP-2-activated fluorine probe was based on the concept described in Mizukami *et al* which was designed for the activated imaging of a caspase-3 enzyme.^{67, 68} As discussed previously, this was based on the paramagnetic relaxation enhancement (PRE) on fluorine by gadolinium.⁷⁸ The design as seen in Figure 30 incorporates a MMP-2 specific peptide substrate SPAY↓YTA or

SLAY↓YTA, which is functionalised at the C terminus by a fluorinated aspartic acid group (D-Tfb) and at the N-terminus by a DOTA ligand that coordinates gadolinium(III).

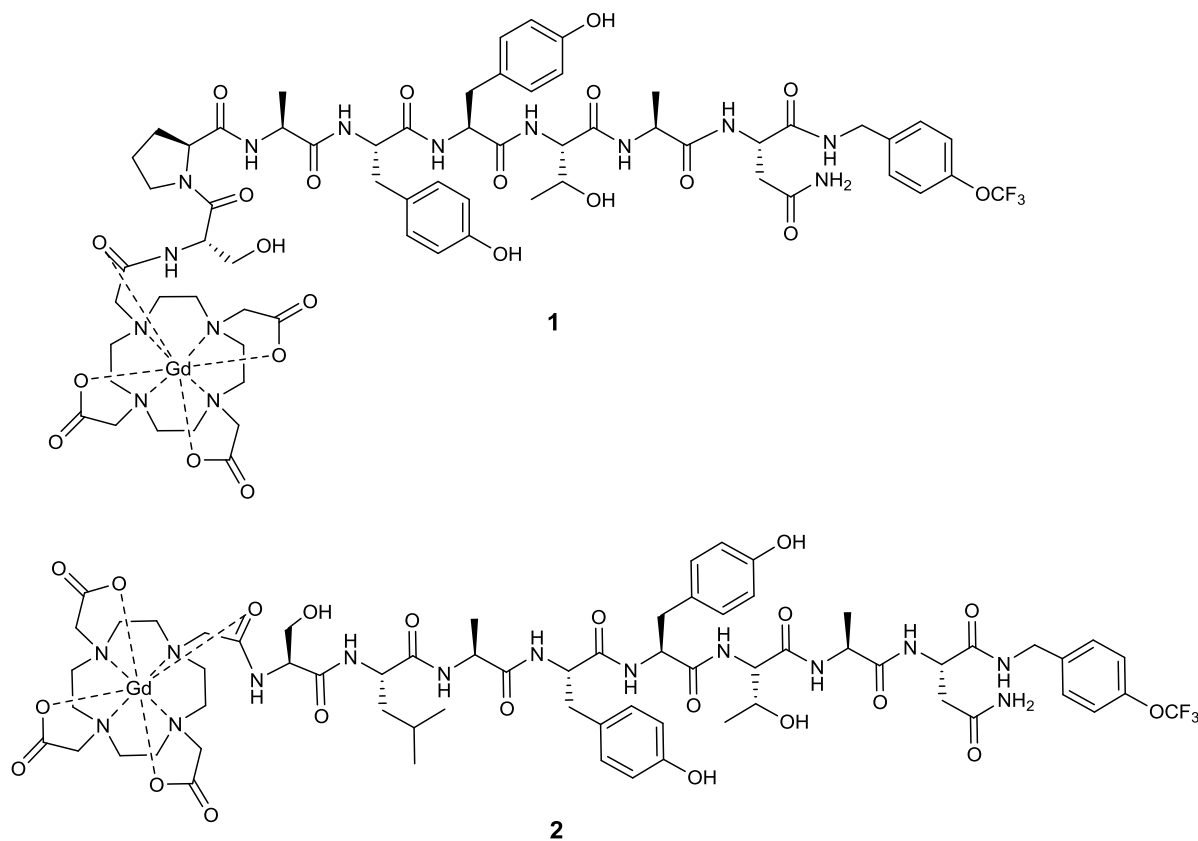
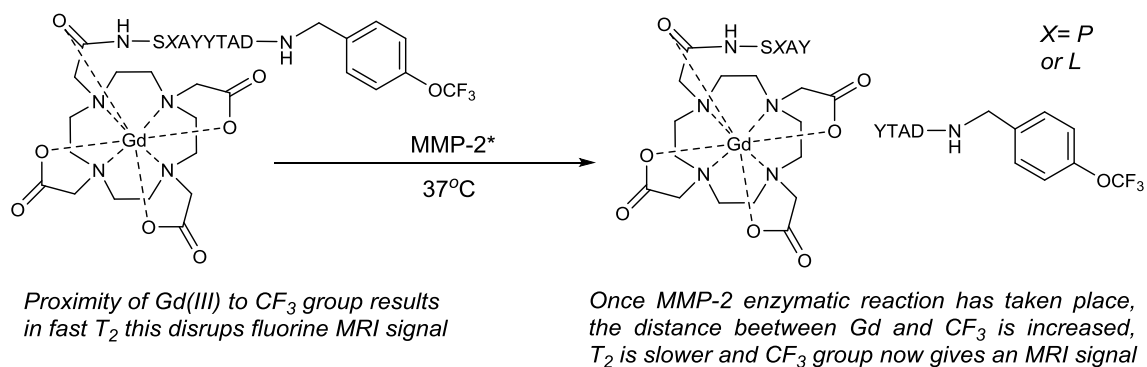


Figure 30-Selective MMP-2-activated ^{19}F MRI probe designs.

It is proposed that for these compounds the gadolinium is close enough to the CF_3 group to have a paramagnetic effect on the fluorine atoms shortening their T_2 relaxation time, which causes a broadening of their signal, attenuating the MRI. In the presence of MMP-2 the peptide will be cleaved between the tyrosine residues and the paramagnetic relaxation enhancement effect would no longer take place, this increases the T_2 relaxation time resulting in a higher intensity of the ^{19}F signal. This proposed mode of action is schematically shown in Figure 31.

Figure 31 - Proposed mode of action for MMP-2 activated ^{19}F MRI probes.

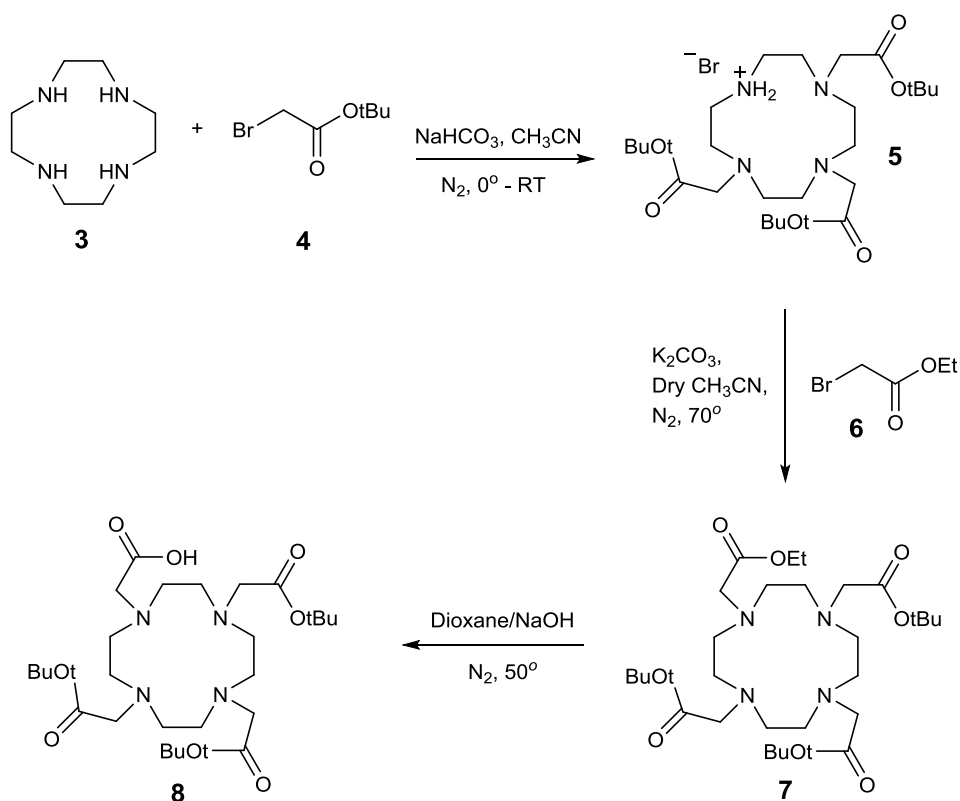
It is important to note that while caspase-3 is an exopeptidase requiring a short 3 amino acid residue for substrate recognition, MMP-2 is an endopeptidase requiring a longer peptide chain. Since the paramagnetic effect of lanthanides is a distance-dependent effect, the increased distance between Gd(III) and CF_3 in this design could decrease the PRE and thus the attenuation of the fluorine signal might not be as prominent as in the caspase-3 example. While it would be possible to synthesise a peptide chain which was functionalised with gadolinium and fluorine closer together along the substrate, this was more likely to have a detrimental effect on substrate recognition. Thus, this first compound was designed to test the feasibility of translating the paramagnetic relaxation enhancement effect of gadolinium(III) on fluorine to MMP imaging given the increased peptide length.

As previously mentioned the choice of peptide substrates in this initial design was based on the work by Chen et al; these two peptide substrates have shown increased MMP-2 specificity over MMP-9.¹⁶ Peptide substrate SPAYYTA was specifically chosen due to its use by Lepage *et al* in an MRI solubility switching probe demonstrating literature precedence that functionalisation retains MMP-2 activity.⁴⁹ While SLAYYTA was chosen as this one amino acid change demonstrated increased

MMP-2 selectivity (demonstrating decreased hydrolysis with active MMP-9), despite a lower MMP-2 reactivity ($K_{\text{cat}}/K_{\text{m}}$) when investigated by Chen *et al.*¹⁶

2.2.3 DOTA ligand synthesis

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid (**7**) was synthesised from cyclen via intermediates **5** and **7** using a slightly modified literature protocol as shown in Scheme 1.⁷⁹⁻⁸¹



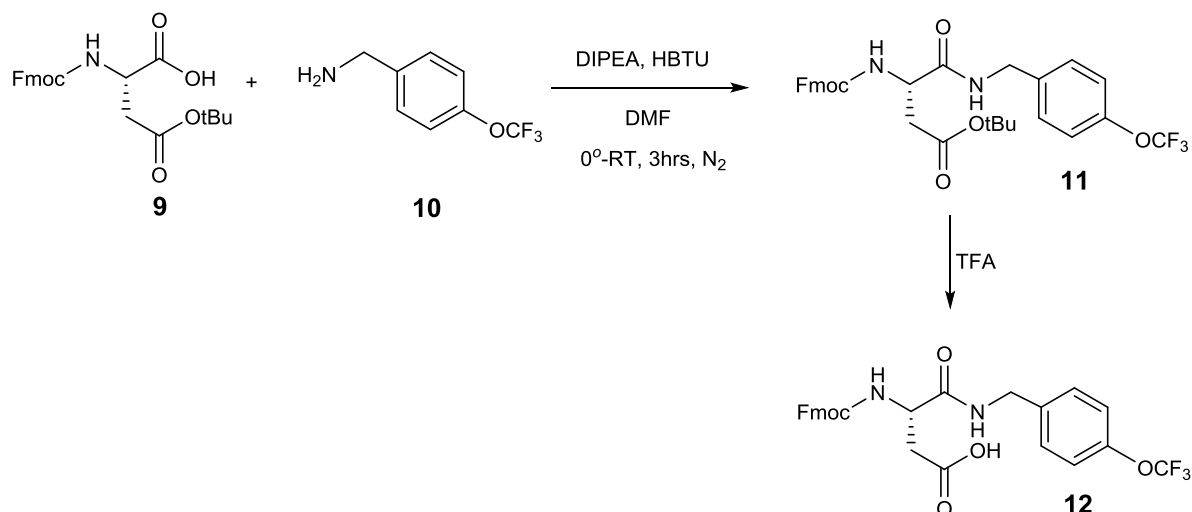
Scheme 1- Synthesis of triprotected DOTA ligand compound **8**.

The initial step involved the dropwise addition of *tert*-butyl bromo acetate (**4**) to a cyclen (**3**) and NaHCO_3 mixture, synthesising intermediate **5**, a 1,4,7-Tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane hydrobromide salt. Synthesis was confirmed by ^1H NMR spectroscopy and after purification by recrystallation, a yield of 48% was obtained.⁶⁷ This reaction could be successfully scaled up to 5 g

without significant change in yield. The next step in this reaction scheme involved a 24 hour reflux of **5** with K_2CO_3 and ethyl bromoacetate (**6**) in dry acetonitrile. Presence of compound **7** was confirmed by 1H NMR spectroscopy. After purification via column chromatography 74% isolated yield of **7** was recorded for smaller scale reactions, while increasing the scale saw this drop to around 50%. Triprotected DOTA ligand **8** could then be synthesised, using a literature protocol, from **7** via selective deprotection of the ethyl ester group in dioxane and 0.4 M NaOH at 50 °C.⁸⁰ Yields were around 60% after the 4 hour reaction time stated in the literature, but could be increased to 80% in 6 hours. Loss of ethyl ester protons in the 1H NMR spectrum confirmed compound synthesis along with ESI(+)-MS which demonstrated a $[M+H]^+$ peak at 573 a.m.u. Yields from the synthesis of **7** and **8** were lower than those reported in literature likely due to the larger scale of the reactions reported here.^{79, 80}

2.2.4 Functionalised amino acid synthesis

The functionalised aspartic acid group Fmoc-L-Asp[(trifluoromethoxy)benzylamide]-OH (**12**) was synthesised via intermediate Fmoc-L-Asp[(trifluoromethoxy)benzylamide]-OtBu (**11**) as shown in Scheme 2 following an adaptation of literature procedures.⁶⁷

Scheme 2- Synthesis of Fmoc-L-Asp[(Trifluoromethoxy)benzylamide]-OH (**12**).

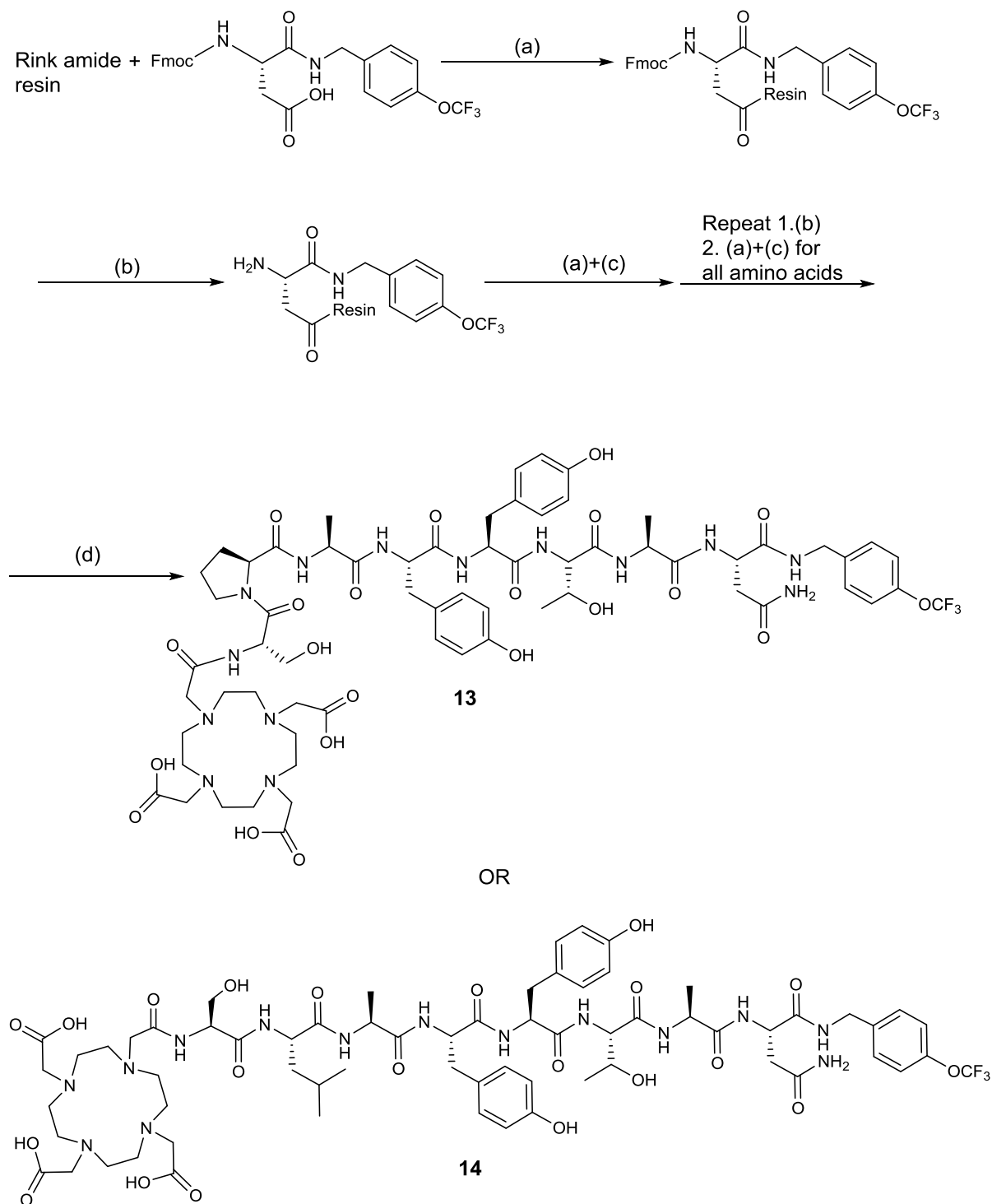
Fmoc-L-Asp[(trifluoromethoxy)benzylamide]-OtBu was synthesised from Fmoc protected aspartic acid (**9**) via a peptide coupling reaction with 4-(trifluoromethoxy)benzylamine (**10**) using HBTU and DIPEA as peptide coupling agents. After washing a crude yield of 90% was obtained and did not need further purification prior to use. Synthesis was confirmed by ^1H NMR spectroscopy and ESI(+)-MS which showed an $[\text{M}+\text{H}]^+$ peak at 585 a.m.u. In order to produce an amino acid ready for peptide synthesis the *t*Bu group was removed from compound **11** using TFA. Synthesis was confirmed by loss of *tert*-butyl protons on the ^1H NMR spectrum, and after purification a yield of 58% was obtained. This compound showed a sharp ^{19}F NMR peak at -59.6 ppm. Overall yield of Fmoc-L-Asp[(trifluoromethoxy)benzylamide]-OH (**12**) was consistent with literature values.⁶⁷

2.2.5 Peptide synthesis

Peptide ligands DOTA-SPAYYTAD-Tfb (**13**) and DOTA-SLAYYTAD-Tfb (**14**) were synthesised via the combination of the triprotected DOTA ligand (**8**) and fluorinated

amino acid Fmoc-L-Asp-(Tfb)-OH (**12**) on to a peptide substrate via manual Fmoc solid phase peptide synthesis on a Rink amide resin. The procedure is outlined in Scheme 3 and involved fmoc deprotection steps followed by amino acid coupling reactions, using HBTU and DIPEA as coupling reagents. Coupling reactions of 30 minutes were carried out twice, this process was repeated for each sequential amino acid. The method was developed to include alternative coupling protocol for Fmoc-L-Asp-(Tfb)-OH (**12**) onto the resin and of the triprotected DOTA (**8**) onto the peptide chain; this was carried out using HATU as the peptide coupling agent.

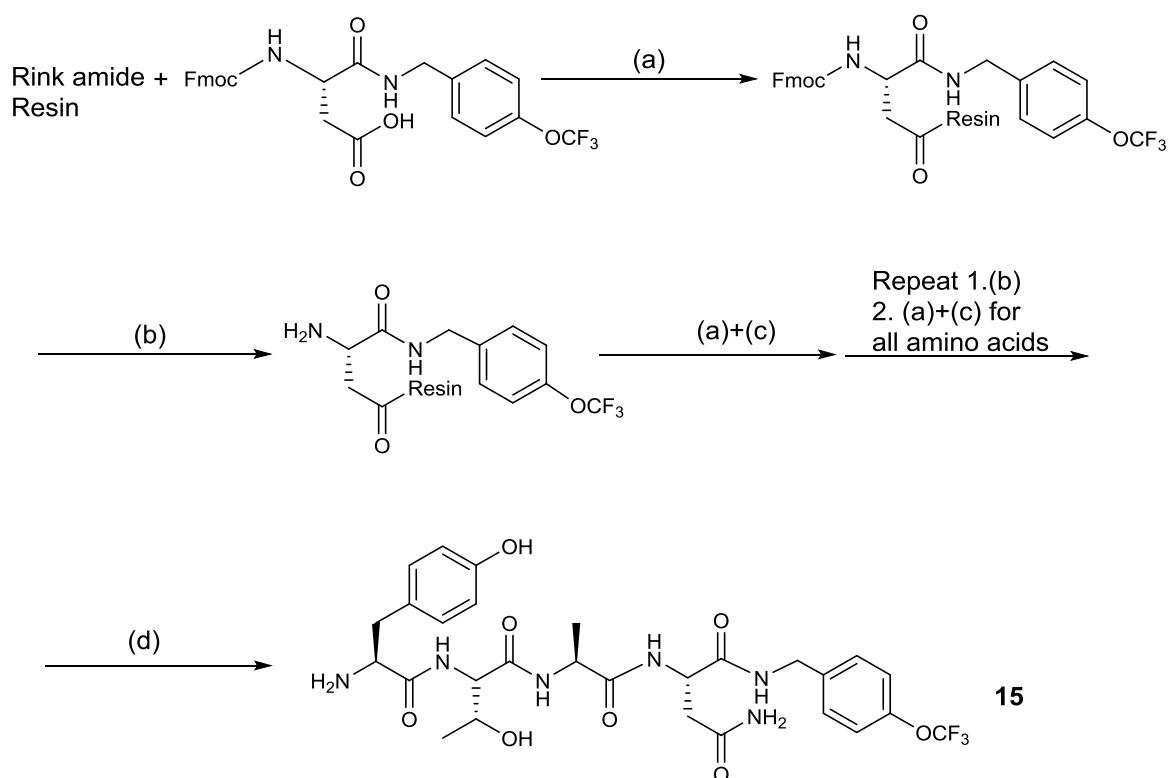
After the peptide was cleaved from the resin, purification was carried out using reversed phase prep-HPLC in Water/Acetonitrile 0.1% FA. The corresponding peptides were then isolated via lyophilisation in a yield of 59% for DOTA-SPAYYTAD-Tfb (**13**) and 40% for DOTA-SLAYYTAD-Tfb (**14**) which is reasonable for solid phase peptide synthesis. Peptide synthesis was confirmed by ESI(+)-MS with $[M+H]^+$ peak at 1446.6 a.m.u. and 1461.5 a.m.u. respectively. ^{19}F NMR spectroscopy showed a sharp peak centred at -59 ppm for both peptides consistent with what was expected for this CF_3 group.



(a) HBTU, DIPEA (b) DMF/Piperidine (c) Fmoc-Ala-OH, Fmoc-Thr(^tBu)-OH, Fmoc-Tyr(^tBu)-OH, Fmoc-Tyr(^tBu)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH/ Fmoc-Leu- OH, Fmoc-Ser(^tBu)-OH, 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid
(d) TFA, TIS, H₂O

Scheme 3- Standard Fmoc solid phase peptide synthesis of compounds **13** (DOTA-SPAYYTAD-Tfb)
and **14** (DOTA-SLAYYTAD-Tfb).

In addition to the complete peptide substrates, the expected cleavage product of the MMP-2 enzymatic reaction of compounds **1** and **2**, namely YTAD-Tfb (**15**), was also synthesised via manual Fmoc solid phase synthesis (Scheme 4). Synthesis was confirmed by ESI(+)-MS with a $[M+H]^+$ peak at 642.3 a.m.u., yields for this peptide were 41% and ^{19}F NMR spectroscopy showed a sharp peak at -59.9 ppm.



(a) HBTU, DIPEA (b) DMF/Piperidine (c) Fmoc-Ala-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH
(d) TFA, TIS, H₂O

Scheme 4- Synthesis of YTAD-Tfb (**15**) expected product of enzymatic cleavage of compounds **1** and **2**

gadolinium and $[M+H]^+$ peaks present at 1600.5 a.m.u. for **1** and 1616.5 a.m.u. for **2**.

An example of the isotopic pattern is shown for **1** in Figure 32.

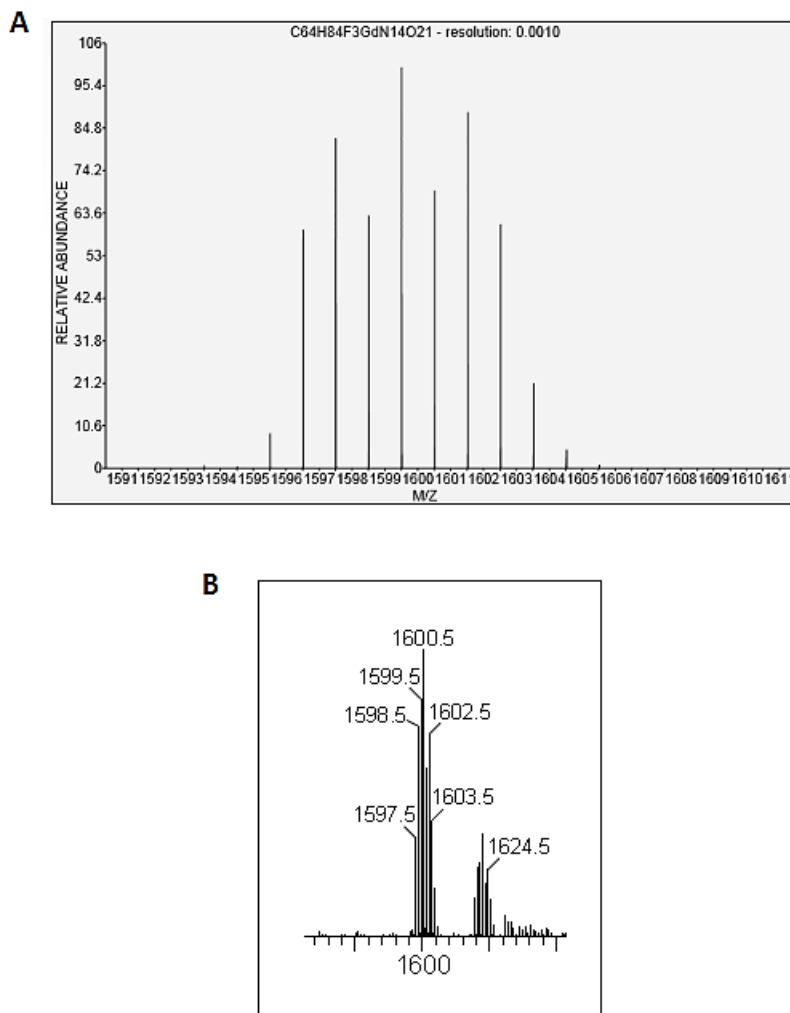


Figure 32- A: calculated molecular ion peaks for compound **1**.⁸² B: experimental ESI⁺ m/z of compound **1** demonstrating a mixture of molecular ion and $[M+H]^+$ peaks at 1599.5 and 1600.5 a.m.u. respectively and $[M+Na]^+$ peaks, showing the isotopic pattern of gadolinium.

The ¹⁹F NMR spectrum showed a peak in the same region as the free peptide ligand but it appeared to be significantly broadened indicating that the complexation of gadolinium(III) was having an effect on the CF₃ group despite a longer than optimal separation. Comparison between the ¹⁹F NMR spectra of the free peptide ligands and the corresponding Gd complexes can be seen in Figure 33 and Figure 34.

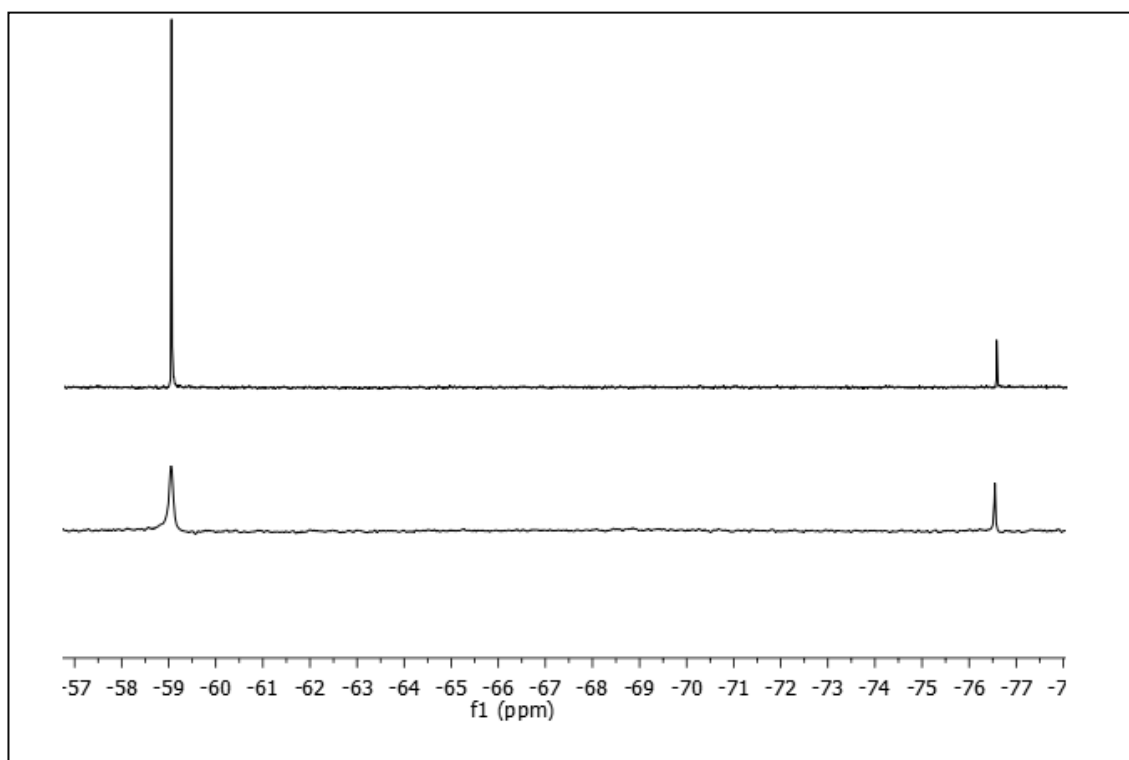


Figure 33- Comparison of ^{19}F NMR spectra for DOTA-SPAYYTAD-Tfb (top) and Gd-DOTA-SPAYYTAD-Tfb (bottom).

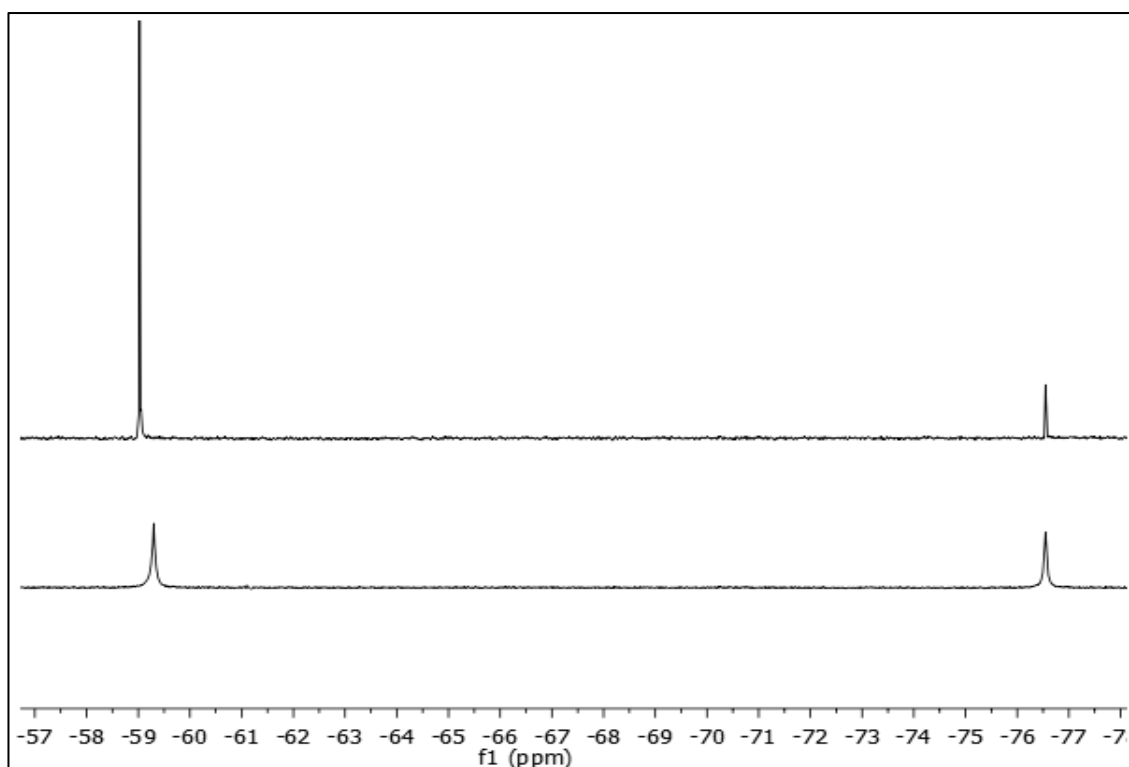


Figure 34- Comparison of ^{19}F NMR spectra for DOTA-SLAYYTAD-Tfb (top) and Gd-DOTA-SLAYYTAD-Tfb (bottom).

This observable difference in the ^{19}F NMR spectra on complexation to Gd^{III} provided an initial proof of principle for the probe design. This result indicated that the gadolinium(III) centre was having a strong PRE effect resulting in visible signal broadening of the OCF_3 group and alteration of the ^{19}F NMR spectrum. This result was consistent with complexation as described by Mizukami *et al* and provides evidence of the potential of these probes for MMP-2 activated ^{19}F MR imaging.^{67, 68}

2.2.7 T_1 and T_2 measurements

While visual comparisons of the ^{19}F NMR spectra between gadolinium-complexed and ligand compounds clearly illustrated some changes, T_1 and T_2 measurements were carried out to quantify this. The visual data should correspond to decreased T_1 and T_2 relaxation times as a result of complexation and proximity to gadolinium to the CF_3 group. In addition to free ligands **13** and **14**, and complexes **1** and **2**, the expected product of enzymatic reaction **15** was also investigated in order to predict the outcome of enzymatic cleavage. These can be seen in Table 1.

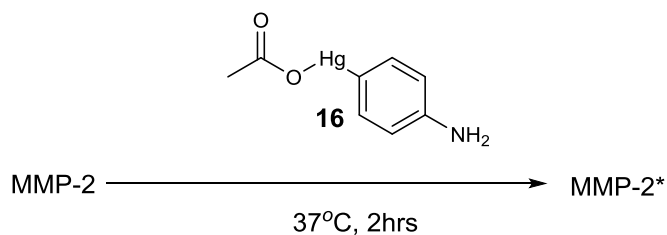
Table 1- Relaxation times for peptide ligands **13** and **14**, complexes **1** and **2**, and peptide **15**. 400MHz, 298K, standard error $\pm 0.5\text{ms}$.

Compound	T_1 - longitudinal relaxation time (seconds)	T_2 – Transverse relaxation time (seconds)
DOTA-SPAYYTAD-Tfb (13)	1.514	1.044
Gd-DOTA-SPAYYTAD-Tfb (1)	0.051	0.009
DOTA-SLAYYTAD-Tfb (14)	1.518	1.061
Gd-DOTA-SLAYYTAD-Tfb (2)	0.065	0.011
YTAD-Tfb (15)	1.577	1.181

T_1 and T_2 results demonstrated a significant decrease in relaxation times in the presence of gadolinium (complex **1** and **2**), when compared to both uncomplexed ligands (**13** and **14**) and expected cleavage product **15**. Free ligands and expected cleavage product show comparable relaxation times. The shortening of the T_2 explains the broadening of the fluorine signal observed in the gadolinium complex's ^{19}F NMR spectrum. These results clearly validate that the gadolinium(III) centre is exerting a PRE effect on the fluorine atoms, which results in significant changes in relaxation times and should correspond to signal attenuation. Results also indicated that theoretically a change can be expected in fluorine signal on enzymatic reaction of complex **1** or **2** with MMP-2. These were important results confirming the potential of compounds **1** and **2** to act as MMP-2 activated imaging agents.

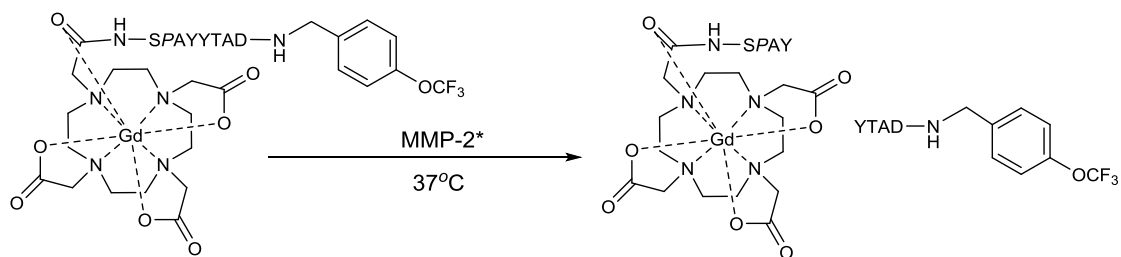
2.2.8 MMP-2 Enzymatic reaction assay

In order to determine the reactivity of the newly synthesised peptide complexes **1** and **2** with MMP-2, enzymatic assays were carried out. This was necessary in order to confirm peptide cleavage was taking place in these new compounds and to develop an appropriate assay for investigating effects of MMP-2 cleavage by ^{19}F NMR spectroscopy. Initially focus was placed on DOTA-SPAYYTAD-Tfb and HPLC was chosen as the method of detection due to simple acquisition and literature precedence.^{35, 37} The assay method involved the activation of recombinant human MMP-2 with APMA (**16**) at 37 °C for 2 hours as shown in Scheme 6 (activation was confirmed with analysis with a fluorescent control compound Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (**17**) purchased from R&D systems).



Scheme 6- MMP-2 activation procedure from latent to active form

Activated MMP-2 was then incubated with peptide complex **1** at time points 5, 30 and 60 minutes; the enzymatic reaction was quenched with 1,10 phenanthroline and the resulting reaction mixture injected into the HPLC (analytical C18 column, UV at 210 and 254 nm).

Scheme 7- expected enzymatic reaction of **1** with MMP-2* resulting in the production of **15**.

It was expected that if enzymatic reaction was taking place appearance of a new peak would be present in the HPLC trace and its retention time should correspond to that previously determined for peptide **15**, the expected cleavage product. However, due to the large excesses of phenanthroline and APMA used along with the low UV absorbance of Gd-DOTA-SPAYYTAD-Tfb (**1**) there was great difficulty in visualising any change in the peptide complex peak or appearance of the expected cleavage product peak (whose retention time had previously been determined) and results were inadequate to draw any conclusions.

By increasing the concentration of the peptide complex (fourfold), and adaptation of HPLC to an isocratic method (60% H₂O with 0.1% FA, 40% Acetonitrile with 0.1% FA), slightly improved visualisation of the peptide complex peak was obtained.

Additionally, two further time points were added to the reaction (90 and 120 minutes), however still no cleavage product peak was seen after substrate incubation with the active enzyme. Additionally, little change was seen in the relative percentage area of peptide complex **1** – results can be seen in Figure 35.

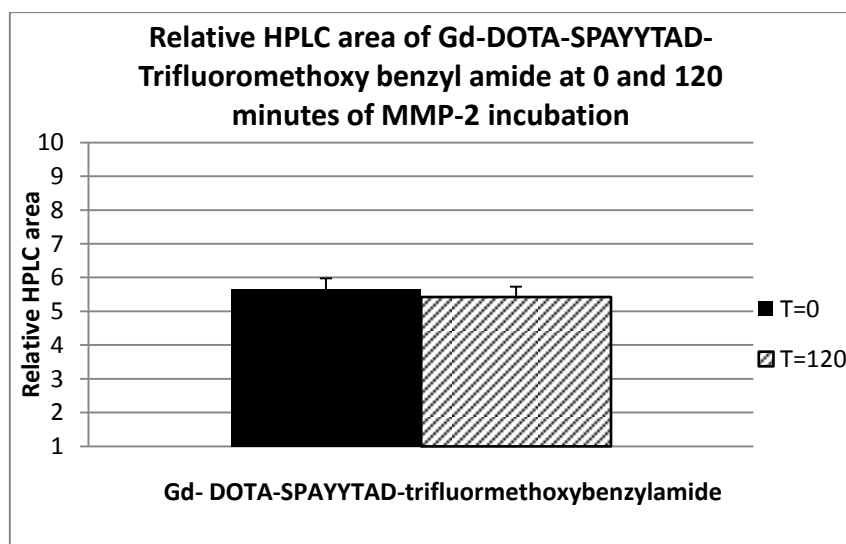
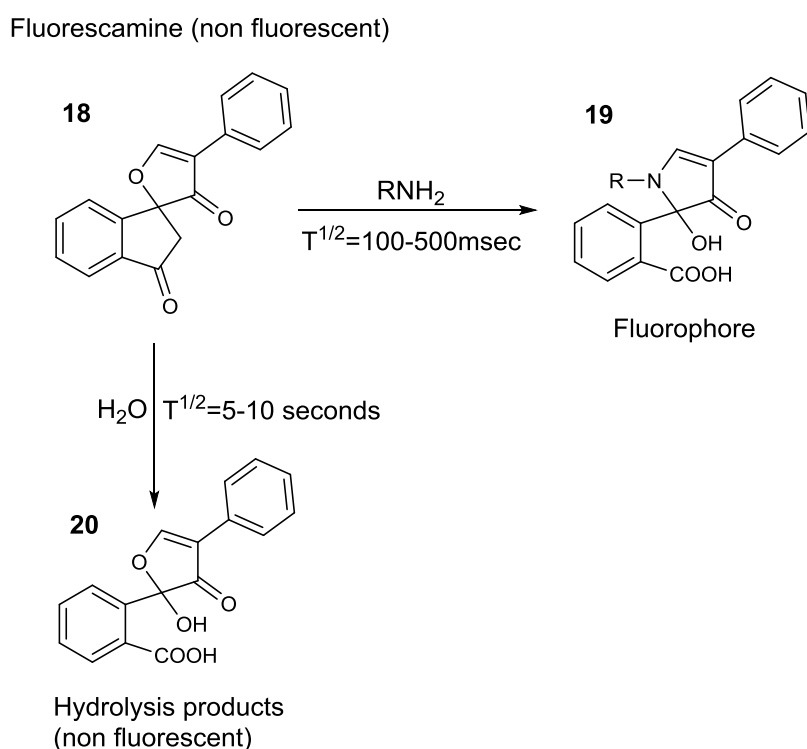


Figure 35- Graph showing the relative HPLC area of Gd-DOTA-SPAYYTAD-Tfb (**1**) before and after enzymatic reaction (n=3, error bars showing standard deviation).

Whilst we had some concerns about the reliability of this assay, the results suggested that no enzymatic reaction had taken place with compound **1**; it was thought this could be due to the bulky Gd-DOTA interfering with enzyme recognition. We hypothesised that the serine-proline motif within the peptide could be forming a turn in the peptide substrate which was forcing the DOTA complex towards the cleavage site of the peptide.^{17, 83, 84} Theoretically if this was the case Gd-DOTA-SLAYYTAD-Tfb (**2**) should be a more successful MMP-2 probe, due to the substitution of proline and thus potentially more linear character. In order to explore this, alternative assay methods were deemed necessary due to the low sensitivity of the HPLC analysis method (due to high background signals and low UV absorbance of **1** and **2**) combined with other

problems, namely the large amounts of MMP-2 and peptide needed under these conditions.

Fluorescamine (**18**) has been previously reported to be a useful compound for determining peptide/protein cleavage due to its reaction with primary amines to form fluorescent compounds (Scheme 8).^{16, 49, 85, 86}



Scheme 8- Describing the reaction of fluorescamine with amines or with water.

Its use in fluorescent analysis of enzymatic cleavage assays is well documented in literature (including examples with MMPs) and was easily applicable for use in analysis of compounds **1** and **2** as neither contains primary amine groups prior to enzymatic reaction. Assay conditions remained similar to those previously described bar change in assay buffer (from tris to tricine, to remove unwanted primary amine) which should not alter the assay and a reduction in the quantities of MMP-2 and peptide complex used. These were now in line with those prescribed for the control peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (**17**) (20 μM final concentration of

peptide substrate and 2.8 nM final concentration of MMP-2). Enzymatic reactions were stopped with phenanthroline and 200 μ l of 5 mM fluorescamine solution in acetone plus assay buffer were added. The mixture was left for 5 minutes before analysis on a 96 well fluorescent plate reader at excitation 390 nm and emission 475 nm. Additionally reaction mixtures were analysed via fluorescent HPLC.

Due to the improved ease of analysis and lower quantities of MMP-2 needed, a number of compounds could be analysed simultaneously using this method. Thus in addition to complexes **1** and **2**, basic peptide substrates SPAYYTAD (**21**) and SLAYYTAD (**22**) were synthesised and tested along with monofunctionalised SPAYYTAD (SPAYYTAD-Tfb (**23**) and Gd-DOTA-SPAYYTAD (**24**)) (Figure 36), in order to fully investigate changes in cleavage on functionalisation.

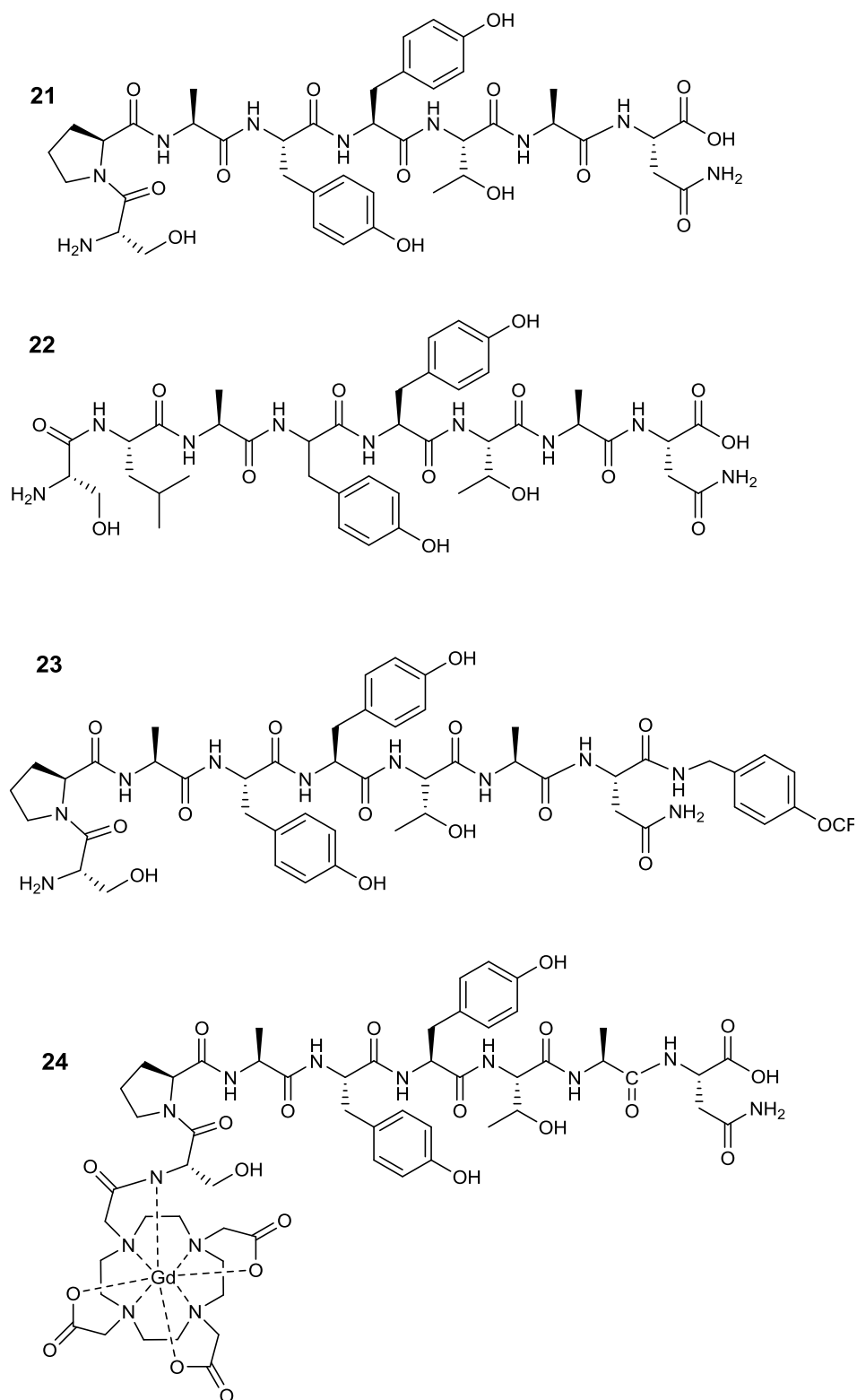


Figure 36- Compounds SPAYYTAD (**21**), SLAYYTAD (**22**), SPAYYTAD-Tfb (**23**) and Gd-DOTA-SPAYYTAD (**24**).

Methods of synthesis and purification followed those described previously, with exclusion of the DOTA ligand and/or the fluorinated amino acid from the peptide

sequence where required. Yields for each peptide compound and complex can be seen in Table.

Table 2- Yields for peptides **21**, **22** and **23**, and complex **24**

Compound	Yield
SPAYYTAD (21)	31%
SLAYYTAD (22)	28%
SPAYYTAD-Tfb (23)	21%
Gd-DOTA-SPAYYTAD (24)	23%

Fluorescamine analysis of compounds **1**, **2**, and the newly synthesised group of related peptide substrates (**21-24**) after MMP-2 enzymatic reactions provided the results shown in Figure 37.

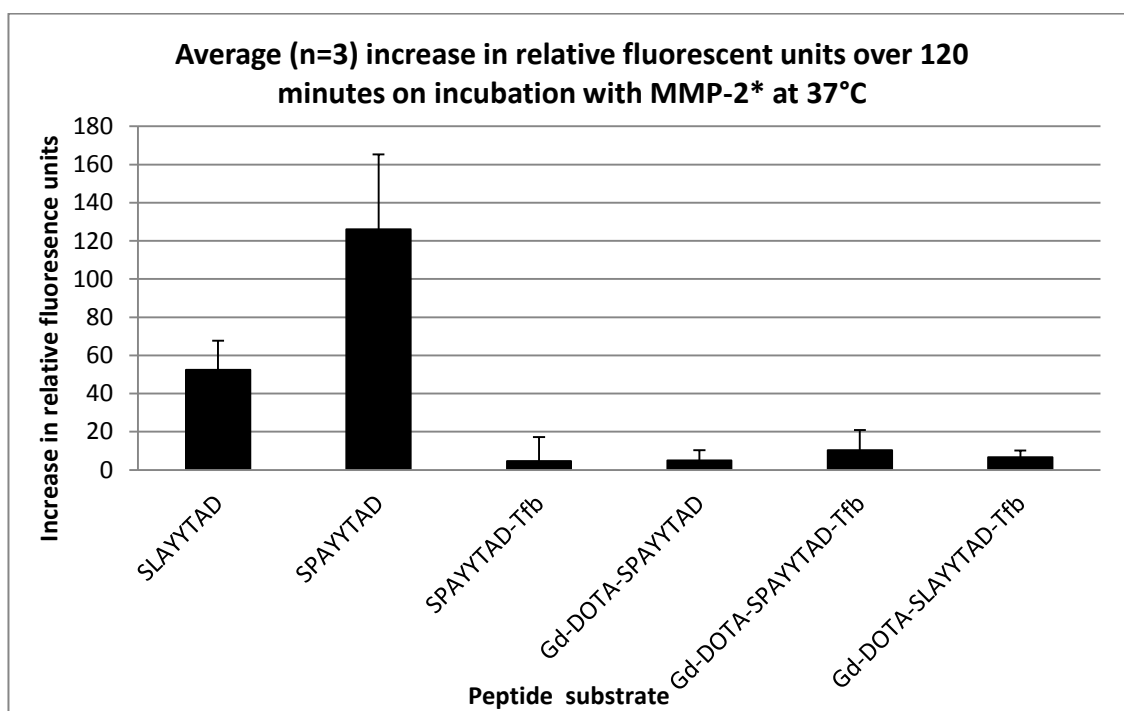


Figure 37- Graph showing the average (n=3, error bars showing standard deviation) increase in relative fluorescence units over 120 minutes.

This data demonstrated that only unfunctionalised peptides showed any notable change in fluorescence (which would indicate an increase in primary amines resulting from

peptide cleavage). This suggests that functionalization is hindering MMP-2 activity on the peptide compounds. However, on closer analysis of data (Figure 38), changes in fluorescence over two hours are still relatively low for SLAYYTAD and SPAYYTAD when compared to high background signals caused by the presence of primary amine groups.

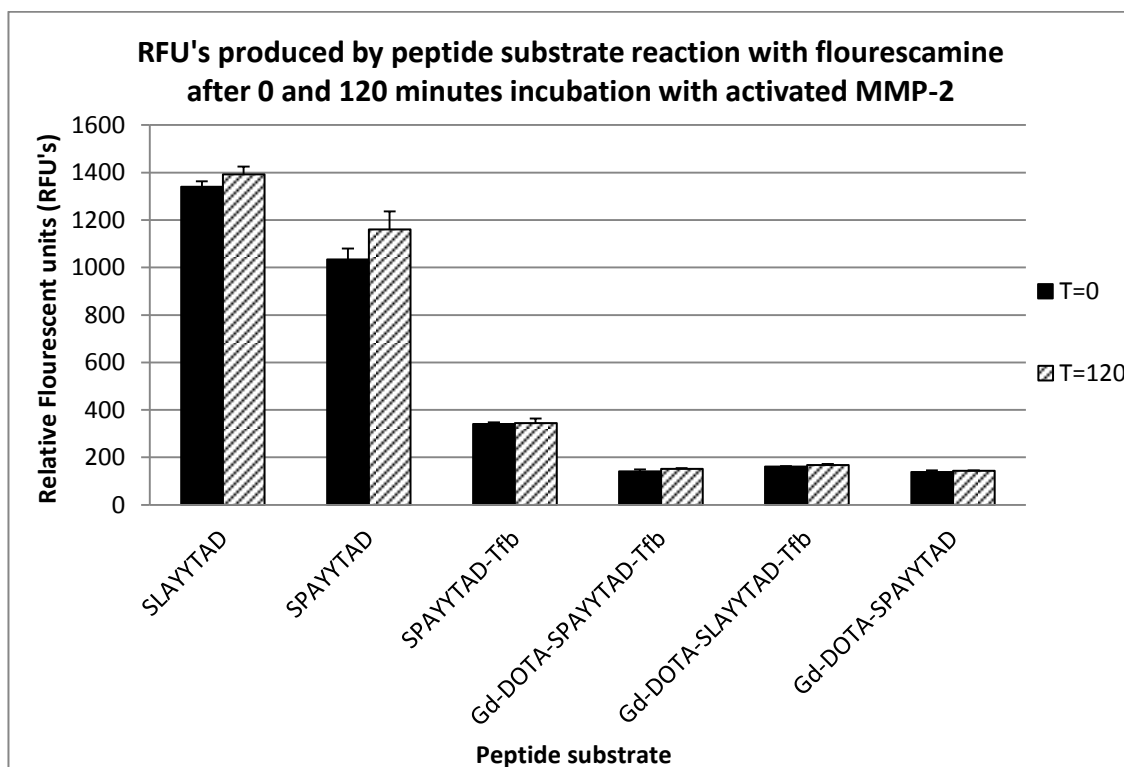


Figure 38- Graph comparing T=0 fluorescence with T=120 for each peptide substrate.

On analysis of fluorescent HPLC data it was determined that the assay buffer solution and activated MMP-2 (after reaction with APMA (15)) were reacting with fluorescamine and producing fluorescent signals which were contributing to background (Figure 39).

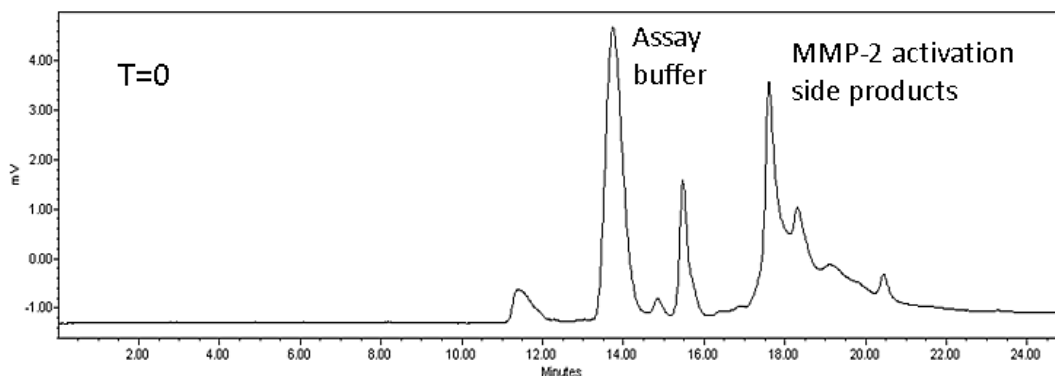


Figure 39- Fluorescence HPLC trace of a sample at 0 minutes incubation, containing no peptide, showing the background signal produced by assay buffer and MMP-2 activation products.

The background signal in this assay reduced the sensitivity of detection, making it difficult to identify small amounts of peptide cleavage impeding comprehensive assessment of the peptide compounds. While further adaptations to this assay could have been explored, it was decided that sensitivity would most probably continue to adversely affect the efficacy of the results. Therefore, an alternative method of monitoring enzymatic reaction was needed, focusing on finding a highly sensitive system with simple analysis methods.

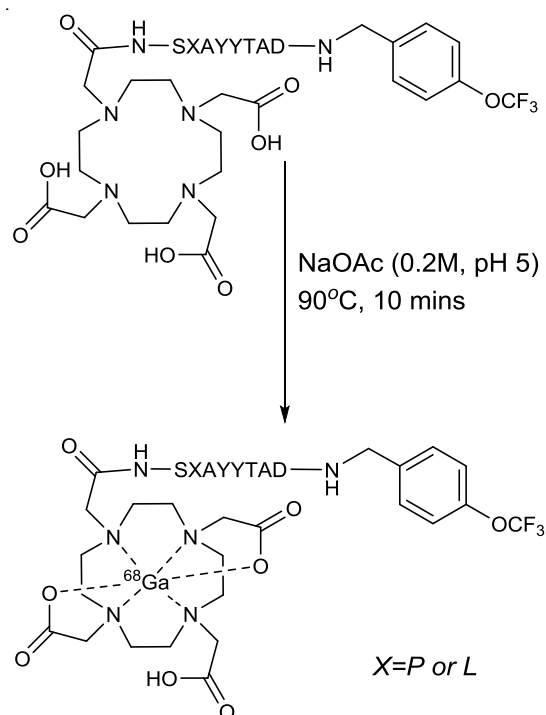
2.2.9 Gallium-68 and MMP-2 Enzymatic reaction assay

In order to develop a more sensitive method of monitoring the enzymatic reaction of MMP-2, the use of ^{68}Ga was proposed as a substitute for gadolinium in the peptide compounds. This PET isotope would allow for the use of sensitive radio-HPLC techniques for the monitoring of enzymatic reaction, alleviating the problem of low UV signal in previous HPLC analysis and removing any issues of background signal associated with fluorescent detection.

Gallium-68 is a radiometal whose use in PET imaging has gained momentum in recent years. This can be attributed to the fact that ^{68}Ga can be produced with a desktop

generator from germanium-68 without the need for a cyclotron, enabling those without accelerators to carry out PET examinations. In addition to this, ^{68}Ga possesses a number of qualities necessary for a useful PET isotope including a $\tau_{1/2}$ of 67.6 minutes, the majority of its emission by positrons (89%) and its easy incorporation into a number of existing ligands including DOTA.^{87, 88}

DOTA is arguably not the best available chelator of ^{68}Ga , with lower stability than other available chelators (such as NOTA and its derivatives). However, it forms highly stable complexes with kinetic inertness under physiological conditions and its use is well documented in literature with a large number of examples of PET imaging agents utilising this ligand.⁸⁸ Reactions of $^{68}\text{Ga(III)}$ with DOTA are simple involving high temperature reactions with reaction times of between 10-15 minutes at acidic pH, and yields are typically high. Reactions have been carried out with DOTA conjugated to a wide variety of peptide substrates most notably octreotide ($^{68}\text{GaDOTATOC}$) and Octreotate ($^{68}\text{GaDOTATATE}$) which have gone on to be useful neuroendocrine tumour imaging agents.^{89, 90} $^{68}\text{Ga(III)}$ does coordinate to DOTA differently to gadolinium(III) in a hexadentate rather than oxadentate manner, leaving two free carboxylic acid/amide arms.⁸⁷ Labelling of peptide ligands **13** and **14** was carried out in NaOAc buffer (pH5, 0.2 M) at 90 °C for 10 minutes (Scheme 9) after which time reactions were analysed with radio-HPLC (C18 column, water/acetonitrile 0.1%FA) which demonstrated high conversions of greater than 90%.

Scheme 9- Reaction scheme for the ^{68}Ga labelling of peptide ligands.

These reactions were shown to be highly reproducible for both peptide ligands, with average decay corrected yields (as calculated from radio-HPLC trace) of ^{68}Ga -DOTA-SPAYYTAD-Tfb (**25**) and ^{68}Ga -DOTA-SLAYYTAD-Tfb (**26**) being around 98%. Investigations into alternative conditions such as lower temperatures or alternative pH (4/6) demonstrated varying decreases in yields. It was vital that glassware was well cleaned (soaked overnight in a 1% nitric acid solution, washed with water and acetone and then oven dried) prior to use in labelling reactions, failure to do so resulted in significant loss of yield. After the syntheses, the corresponding peptides could be easily purified from any remaining free ^{68}Ga and concentrated using a TC₁₈light SPE cartridge, with the majority of activity collected in approximately 200 μL of ethanol (demonstrating easy formulation into an appropriate injectable volume).

High yielding and reproducible radiolabelling of peptides afforded the use of **25** and **26** for enzymatic assay analysis. Once purified radioactive material was obtained in 200 μL of ethanol this was diluted with 300 μL of assay buffer. 100 μL of the labelled

peptide solution was then mixed with 100 μL of MMP-2 (2.8 nM) and heated in a water bath at 37 $^{\circ}\text{C}$ until removal and injection into HPLC at time points 30, 60 and 90 minutes. Observation of peptide hydrolysis into enzymatic cleavage products by radio-HPLC for compound **26** was very small (around 8% after 90 minutes) Figure 40, while for **25** it was non-existent, indicating that little to no cleavage was taking place.

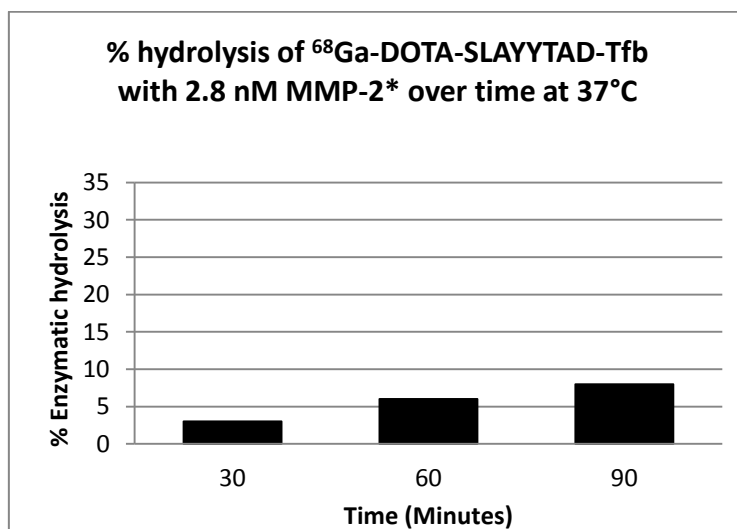
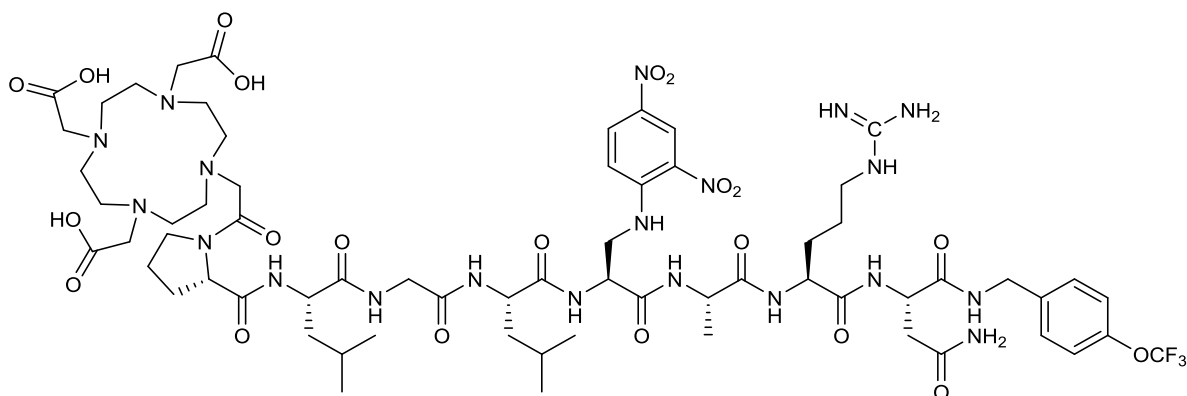


Figure 40- Graph showing % of enzymatic hydrolysis of peptide ^{68}Ga -DOTA-SLAYYTAD-Tfb over time with activated MMP-2 (n=1).

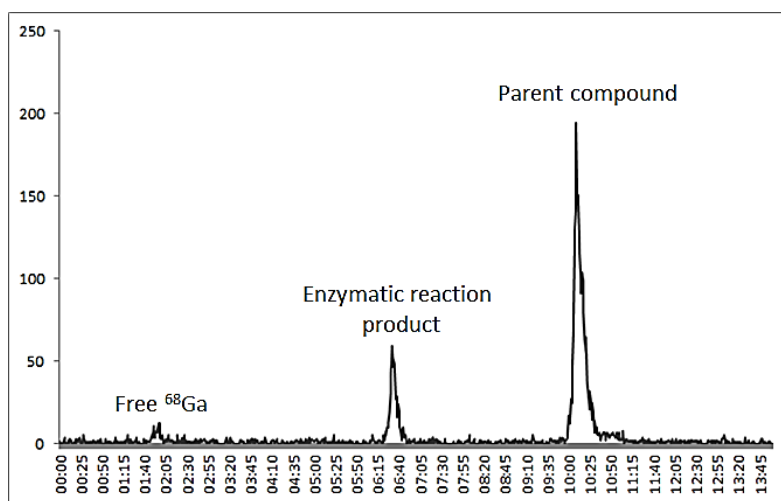
It was postulated that functionalisation of peptide substrates had reduced their reactivity with MMP-2 and that addition of bulky groups were blocking interactions of MMP-2 along the peptide substrate. The result supported the theory that compound **26** which is potentially more linear in character was affected less by functionalization as interaction of bulky DOTA ligand would be less prominent. While compound **25** which contains a serine-proline motif known to form a 'kinked' shaped substrate is likely to be suffering from blocked interactions caused by the bulky DOTA ligand and its proximity to the unprimed side of the catalytic cleft.^{15, 17} However, literature provided examples of functionalisation with large species without loss of activity for a wide variety of broad spectrum MMP substrates, suggesting that this was a problem associated only with selective compounds potentially due to increased specificity

(arising from multiple interactions along the catalytic cleft). It is known that MMP-2 specific peptides substrates show additional interactions than those of the large S₁' pocket of most broad spectrum peptides, it is thought that these interactions can lead to differences in docking and catalytic reactions.¹⁷ For broad spectrum substrates, the reaction is driven by fast docking and by a strong binding affinity generally to the S₁' pocket or the active site, while modelling experiments have shown that specificity appears to be driven by speed of catalytic reaction and interactions at subsites (such as the S₂) which allow the peptide substrate to be held in a favourable orientation for enzymatic reaction.¹⁵ This difference could explain the absence, or decrease of enzymatic proteolysis if S₂ subsite interaction is more substantially affected by functionalisation than that of the S₁' pocket.

In addition to this, it became apparent that the fluorescent control compound **17** (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, which had demonstrated enzymatic cleavage throughout, and on whose prescribed method the enzymatic assay was based) used much lower MMP concentrations for enzymatic reactions than many examples of MMP-2 specific peptides.^{16, 49} It was decided that to fully assess compounds **25** and **26** using this ⁶⁸Ga enzymatic assay, higher MMP-2 concentrations would have to be explored. In order to do this efficiently and to probe the differences between functionalised broad spectrum and specific peptide substrates, an adapted version of the positive control compound was synthesised (DOTA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-Asp-Tfb (**27**); see Figure 41). This was done using the same Fmoc peptide synthesis methods described previously by simply substituting amino acids resulting in a yield of 55%. This compound would provide a broad spectrum basis on which to explore the conditions of the assay.

Figure 41- Positive control peptide DOTA-PLGL-Dpa-ARD-Tfb **27**.

DOTA- Pro-Leu-Gly-Leu-Dpa-Ala-Arg-Asp-Tfb (**27**) could be easily labelled with ^{68}Ga following the protocol already developed for peptide ligands **13** and **14** with conversions above 90% achieved (n=4). Purification and formulation were also carried out as described previously using a TC₁₈ light SPE cartridge. ^{68}Ga -DOTA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-Asp-Tfb (compound **28**) could then be incubated with MMP-2 at a concentration of 2.8 nM for 30, 60 and 90 minutes as previously described and additionally at 120 minutes. On analysis by radio-HPLC, the enzymatic cleavage of the labelled peptide was observed with the following yields: 12% at 90 min and of 19% after 120 min (Figure 42).

Figure 42- Radio HPLC trace of ^{68}Ga -DOTA-PLGL-Dnp-ARD-Tfb after 120 minutes incubation with MMP-2*.

Analysis of **28** incubated at 37 °C solely in assay buffer (MMP-2 free) over the same time scale showed no enzymatic reaction. These results demonstrated that this broad spectrum “control” compound was being broken down by MMP-2, although complete cleavage was not seen at this MMP-2 concentration over a two hour time scale. It was expected that this substrate would show more significant enzymatic hydrolysis than that observed, based on reactions with the related fluorescent control (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂). The next step was to investigate increased MMP-2 (final) concentrations 11 nM and 22 nM. Results of all of these assays are summarised in Figure 43.

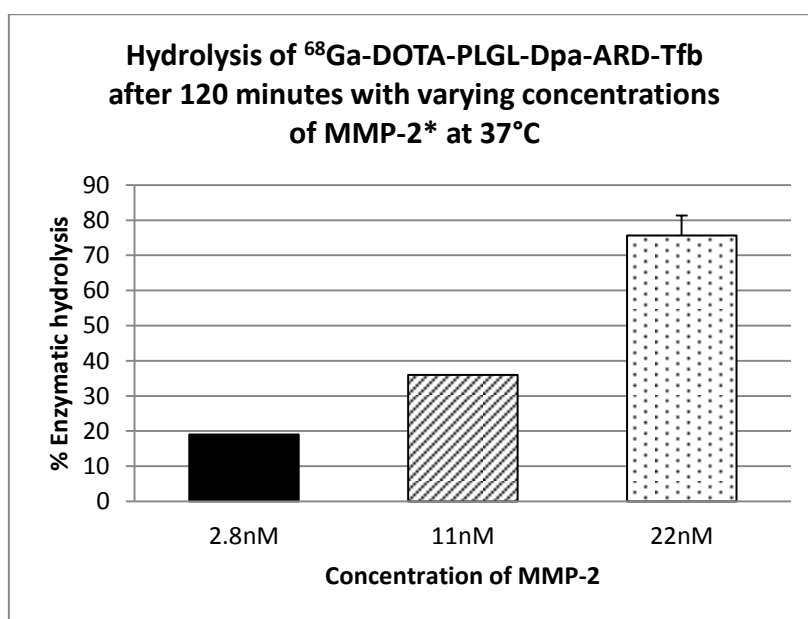


Figure 43- Comparison of enzymatic reaction product over 120 minutes at varying MMP-2* concentrations.

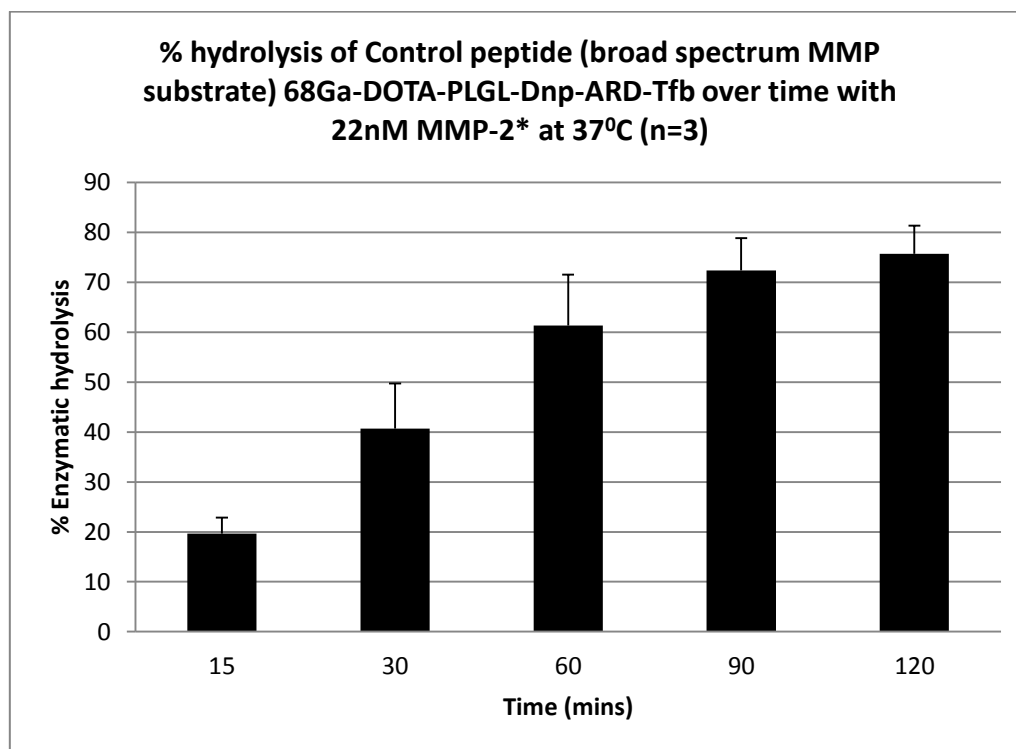


Figure 44- Graph showing the % of enzymatic hydrolysis of peptide ^{68}Ga -DOTA-PLGL-Dnp-ARD-Tfb over time on incubation with 22nM MMP-2* (n=3, error bars show standard deviation).

The plot in Figure 43 shows that the highest concentration tested (22 nM) resulted in an average of 75% hydrolysis over 2 h; this was greatly increased from the original concentration of 2.8 nM. It was decided that this would be a suitable concentration to re-test compounds **25** and **26**, in order to achieve the most useful results using the ^{68}Ga assay whilst still maintaining low MMP-2 concentrations. Increasing MMP concentrations too greatly was undesirable due to increased MMP consumption and deviation from biologically applicable concentrations.

Radiolabelled compounds **25** and **26** were retested using optimised assay conditions and analysis carried out with radio-HPLC as before. Results (shown in Figure 45) demonstrate increased hydrolysis of compounds over a 120 minute time frame.

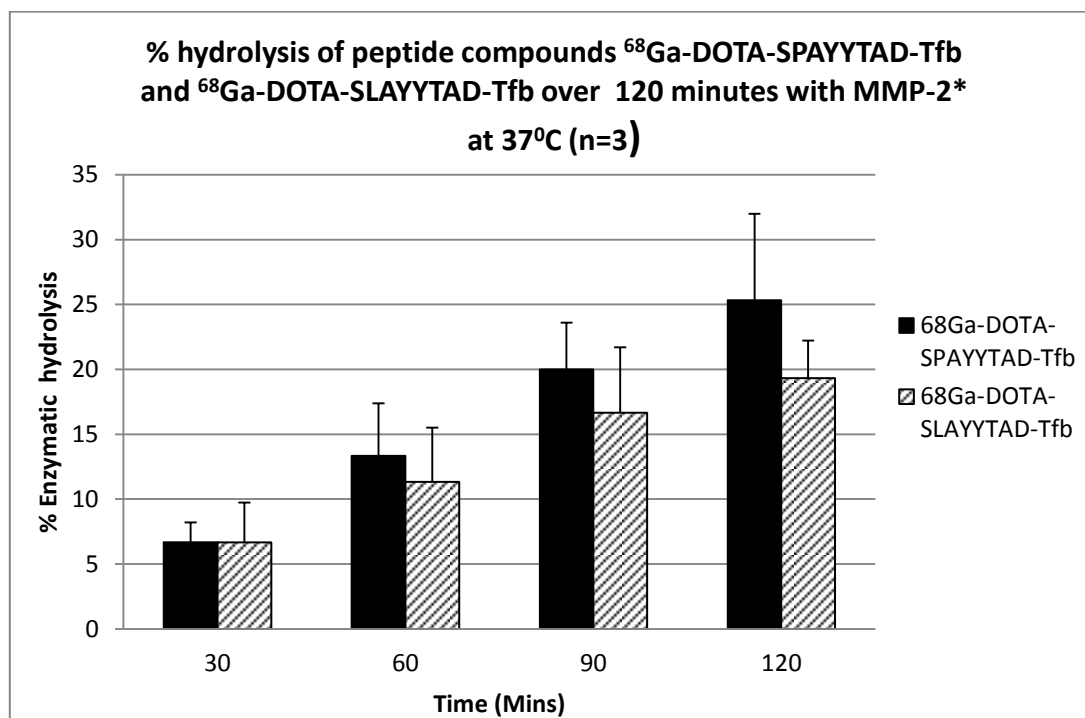


Figure 45- Graph showing the % of enzymatic hydrolysis of labelled peptides **25** and **26** on enzymatic reaction with MMP-2* (n=3, error bars show standard deviation).

Stability of compounds **25** and **26** was confirmed by incubation in assay buffer (MMP free) at 37 °C for 120 minutes, which demonstrated no enzymatic reaction. Compounds **25** and **26** exhibited average enzymatic conversions (n=3) of 25% and 19% respectively, when incubated with MMP-2. Compound ^{68}Ga -DOTA-SPAYYTAD-Tfb (**25**) demonstrated slightly larger hydrolysis than ^{68}Ga -DOTA-SLAYYTAD-Tfb (**26**) as would be expected from literature data on unfuctionalised peptide substrates.¹⁶ These results demonstrated that MMP-2 enzyme recognition could be achieved with the peptide compounds synthesised. However, compared to the broad spectrum “control” peptide, compound hydrolysis was significantly lower. This could be due to the discussed differences in the route to catalytic reaction. It should also be noted that compared to similar compounds in literature, reactivity with MMP-2 appears reduced, likely due to a combination of peptide functionalisation and differing assay conditions.^{16, 49} Enzymatic hydrolysis of compounds **25** and **26** in the ^{68}Ga assay

timescale while lower than that of the broad spectrum peptide, still demonstrate considerable reactivity with MMP-2. If this could be extended to a longer timescale, with the use of ^{19}F MRI (in which no background signal should be seen), we could expect greater peptide hydrolysis and potentially a significant change in fluorine signal. However, *in vivo* this will be limited by the clearance times of these compounds, which are likely to be relatively fast due to lack of any targeting moieties. The main advantage of these compounds in their current formulation is likely to be found in their selectivity; despite the lower reactivity with MMP-2 it was expected that these peptide compounds **25** and **26** would show selectivity over other MMPs within the family, most importantly MMP-9.

2.2.10 Selectivity studies – MMP-9 and MMP-14 ^{68}Ga enzymatic reaction assays

MMP-9 has a highly related structure to MMP-2; many MMP-2 substrates are also substrates for MMP-9 and many imaging agents previously reported target both enzymes together. Nevertheless, as discussed in previous sections, these two enzymes have been found to have distinct roles within cancer. It was therefore of great interest to determine the specificity of MMP-9 against compounds **25** and **26**. Additionally MMP-14 was investigated as it is known to be involved with the cell surface activation of pro-MMP-2; it would be expected for it to be expressed at similar points or places within cancer progression.^{8, 28} In order to analyse the selectivity of **25** and **26** for MMP-2 over MMP-9 and MMP-14 assays were carried out with these enzymes, results can be seen in Figure 46 and Figure 47.

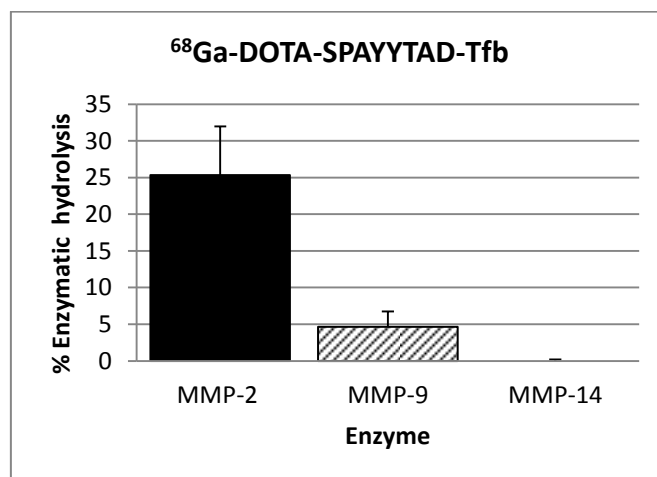


Figure 46- Graph showing the % of enzymatic hydrolysis of ^{68}Ga -DOTA-SPAYYTAD-Tfb after two hours with MMP-2, MMP-9 or MMP-14 (n=3, error bars show standard deviation).

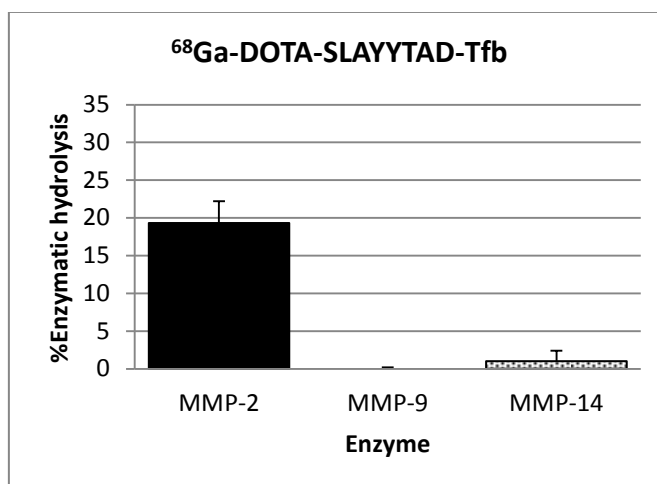


Figure 47- Graph showing the % of enzymatic hydrolysis of ^{68}Ga -DOTA-SLAYYTAD-Tfb after two hours with MMP-2, MMP-9 and MMP-14 (n=3, error bars show standard deviation).

MMP-9 was activated using similar conditions to those used for MMP-2, however required longer activation times of 24 hours. After activation, the enzyme was diluted to 22 nM and its activity was confirmed with the fluorescent control peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (**17**). The assay was carried out using the same protocol employed for MMP-2 with the radiolabeled substrates **25** and **26**. For ^{68}Ga -DOTA-SLAYYTAD-Tfb (**25**) no hydrolysis was observed with MMP-9 over a 120 min time frame while for ^{68}Ga -DOTA-SPAYYTAD-Tfb (**26**) average hydrolysis of 4.7% was observed over 120 min. These results represent significant selectivity for

MMP-2 over MMP-9 for compounds **25** and **26** demonstrating their potential to provide specific imaging for this enzyme. Additionally the behaviour of these compounds reflects what could be expected from literature, as unfuctionalised peptide SLAYYTA was shown to have improved selectivity over SPAYYTA with regards to MMP-9. The slightly decreased selectivity of SPAYYTA and compound **25** can be related back to the concept already introduced in this chapter of the serine-proline turn and of proline at P₃ contributing to a 'kinked' peptide conformation, often favoured by broad spectrum MMP substrates. It should be highlighted that while 4.7% does not represent significant enzymatic activity, it could cause issues in longer timeframe experiments.

MMP-14 enzyme required alternative activation conditions involving 1 hour incubation at 37 °C with trypsin before addition of ASEBF for 15 minutes at room temperature to quench the enzymatic activation reaction. The enzyme could then be diluted to 22 nM ready for the ⁶⁸Ga enzymatic reaction assay which was carried out as described above. The activity was confirmed with the fluorescent control peptide Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (**17**). ⁶⁸Ga-DOTA-SPAYYTAD-Tfb (**25**) showed no hydrolysis over 120 minutes, while with ⁶⁸Ga-DOTA-SLAYYTAD-Tfb (**26**) an average of 1% hydrolysis of the parent compound was observed. These peptides both demonstrated high levels of selectivity for MMP-2 over MMP-14 providing more support for their potential as MMP-2 selective imaging agents.

If these results are compared to the selectivity of broad spectrum control peptide ⁶⁸Ga-DOTA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-Asp-Tfb (**28**) against MMP-9 and MMP-14 (Figure 48), the vast difference between broad spectrum MMP peptides and specific substrates becomes apparent. The broad spectrum compound shows similar activity with each of the enzymes, demonstrating around 80% average hydrolysis with all 3

enzymes tested. This was as expected from this broad spectrum substrate whose parent compound is known to be active with a number of the MMP enzyme family.

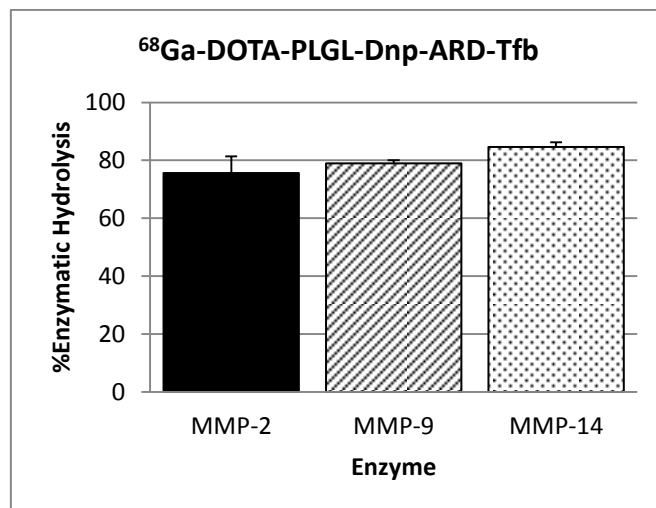


Figure 48- Graph showing the % hydrolysis of broad spectrum peptide ⁶⁸Ga-PLGL-Dnp-ARD-Tfb over 2 hours with MMP-2, MMP-9 or MMP-14 (n=3, error bars show standard deviation).

This data demonstrates that while Compounds **25** and **26** are less active than the broad spectrum compound **28** they provide significant improvements on selectivity which could allow for the novel investigation of the individual MMP-2 enzyme. Additionally, although compounds **25** and **26** demonstrate lower reactivity with MMP-2 than expected, selectivity of the compounds reflects what has been previously reported, showing that addition of DOTA and CF₃ to the peptide substrate have not affected MMP-2 specificity. DOTA-SLAYYTAD-Tfb in particular could provide a useful MMP-2 specific imaging agent due to its significant selectivity over MMP-9 (and good selectivity over MMP-14), this could be used to probe MMP-2's individual role in cancer in more detail.

2.2.11 ^{19}F NMR spectroscopy enzymatic assays

Using ^{68}Ga provided a highly sensitive technique which allowed for the optimisation of MMP-2 assay conditions and allowed the confirmation that peptide modifications had not resulted in loss of substrate activity. However, in order to test compound viability as ^{19}F MRI agents the MMP-2 assay was carried out using the original gadolinium(III) analogues compounds **1** and **2** and using ^{19}F NMR spectroscopy to monitor peptide cleavage. For these experiments, procedures remained the same as with radiolabelled assay experiments but with the peptide and MMP-2 concentrations scaled up in order to provide a significant fluorine signal. Initially, Gd-DOTA-SPAYYTAD-Tfb (**1**) was tested incubating 100 μM of the peptide with 45 nM of MMP-2; the reaction was stopped with phenanthroline (at time points 5, 30, 60, 120 minutes and 24 hours) and ^{19}F NMR spectroscopy carried out. The final concentration of peptide for ^{19}F NMR spectroscopy was 50 μM . Results are summarised in Figure 49.

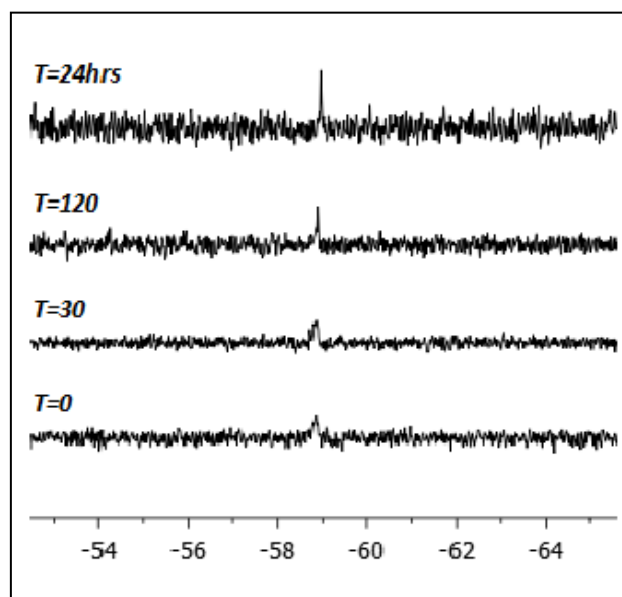


Figure 49- ^{19}F NMR spectra of $100\mu\text{M}$ Gd-DOTA-SPAYYTAD-Tfb (**1**) incubation with 45nM MMP-2* over 24 hours ($50\mu\text{M}$ final peptide concentration in D_2O /Tricine buffer, recorded on 400MHz NMR instrument at 298K).

From Figure 49 we can see that the ^{19}F NMR signal changes over time with the incubation of MMP-2, demonstrating peak sharpening and a slight chemical shift. This indicates that MMP-2 is successfully cleaving the peptide compound and this is subsequently having an effect on the ^{19}F NMR signal. Unfortunately, the signal produced from the peptide was very small due to the low concentration of the sample and we were unable to quantify this with T_1 and T_2 data.

In order to provide a more suitable sample for analysis, peptide concentrations were increased further to $500\mu\text{M}$ during enzymatic incubation with 90 nM MMP-2 (increase in MMP-2 was not equivalent), which would be diluted down to a final concentration of $250\mu\text{M}$ for the ^{19}F NMR spectroscopic experiment after the reaction was quenched with phenanthroline. These conditions were used to investigate both Gd-DOTA-SPAYYTAD-Tfb (**1**) and Gd-DOTA-SLAYYTAD-Tfb (**2**) (it is important to note that while **1** went into solution readily, **2** was more difficult to dissolve at this higher concentration).

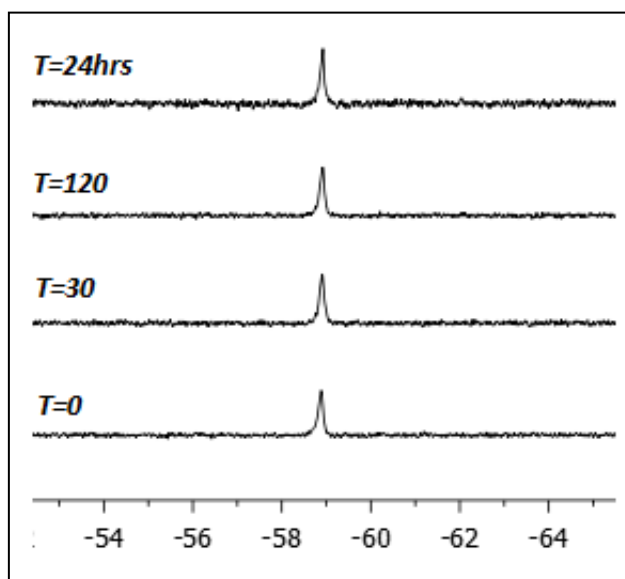


Figure 50- ^{19}F NMR spectra of 500 μM Gd-DOTA-SPAYYTAD-Tfb (1) on incubation with 90 nM MMP-2* over 24 hours (250 μM final peptide concentration in D_2O /Tricine buffer, recorded on 400 MHz NMR instrument at 298K).

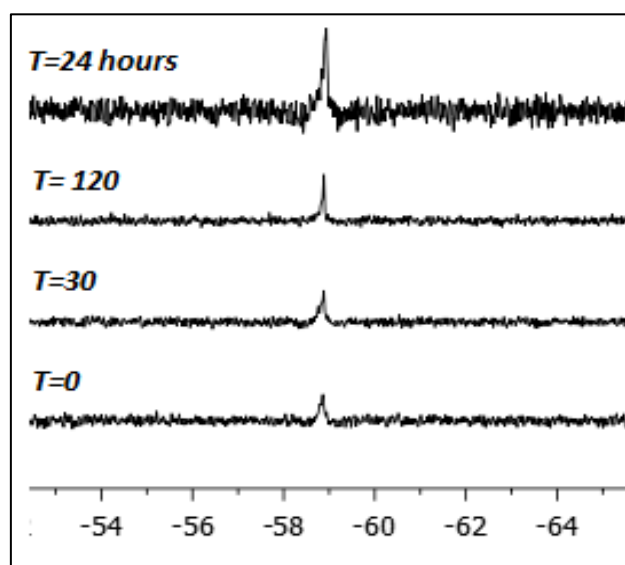


Figure 51- ^{19}F NMR spectra of 500 μM Gd-DOTA-SLAYYTAD-Tfb (2) on incubation with 90 nM MMP-2* over 24 hours (250 μM final peptide concentration in D_2O /Tricine buffer, recorded on 400 MHz NMR instrument at 298K).

These experiments gave mixed results. Figure 50 depicts the ^{19}F NMR spectroscopic data for the cleavage experiment using Gd-DOTA-SPAYYTAD-Tfb (1) which shows no marked change in signal intensity or chemical shift, while in Figure 51 (Gd-DOTA-

SLAYYTAD-Tfb) (**2**) signal changes are evident along with a slight chemical shift indicating peptide hydrolysis. For compound **1** in Figure 50 this lack of observable change in signal can be explained by the increased concentration of peptide sample. This result could be produced by the incomplete hydrolysis of the peptide sample which is causing the signal to be blocked by unreacted **1** which is now at a high enough concentration to conceal the signal from fragment **15**. This incomplete hydrolysis was later confirmed by LCMS. For Gd-DOTA-SLAYYTAD-Tfb (**2**), although change in ^{19}F signal is observed, Figure 51 demonstrates both a sharp signal peak and a broad hump in the 24 hour time point suggesting that enzymatic reaction is incomplete and compound **2** remains in the sample. In this case unreacted **2** is not fully blocking signal from enzymatic reaction product as is suspected for **1** in Figure 50. The likely explanation for this dissimilarity between the two peptide compounds is the reduced solubility of compound **2**. Neither of these NMR signals provided sufficient SNR for T_1 or T_2 quantification.

To optimise these ^{19}F NMR spectroscopic studies and investigate the problems that increased peptide concentration presented, a further study was carried out with Gd-DOTA-SPAYYTAD-Tfb (**1**) increasing the MMP-2 concentration (in line with that of the peptide). Thus for 100 μl of the 1 mM peptide sample 100 μl of 500 nM MMP-2 was added and incubated for 24 hrs (final concentrations in assay 500 μM and 250 nM respectively).

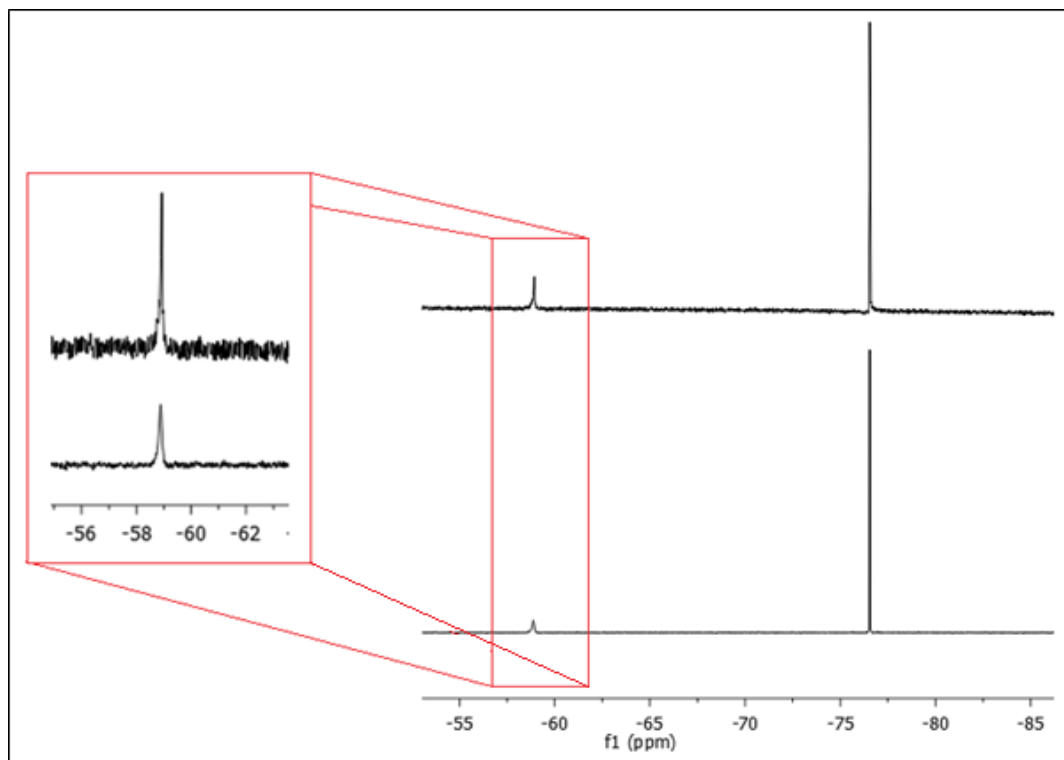


Figure 52- ^{19}F NMR spectra of Gd-DOTA-SPAYYTAD-Tfb on incubation with 250 nM MMP-2* over 24 hours (250 μM final peptide concentration in D_2O /Tricine buffer, 400 MHz NMR instrument at 298K, peak at -77ppm corresponds to TFA standard).

Under these new conditions a clear change in the ^{19}F NMR spectroscopic signal was observed indicating peptide cleavage of substrate **1**. This confirms that in previous experiments (Figure 50) MMP-2 cleavage had not gone to completion. Unfortunately, while this was a positive result demonstrating the potential of this compound to detect MMP-2 activity with change in ^{19}F NMR spectroscopic signal, the intensity was still insufficient to obtain meaningful T_1 and T_2 results due to the signal to noise ratio. Without the T_1 and T_2 values it is difficult to quantitatively analyse this data, but from visual inspection and comparison of these ^{19}F NMR spectroscopic with the results obtained with the free ligand, it does not appear full recovery of ^{19}F NMR signal has taken place. This incomplete signal recovery is similar to that demonstrated by Mizukami *et al* and is likely due to the presence and close proximity of gadolinium

within the NMR sample even after enzymatic cleavage of the compound, this could potentially be a result of the increased concentration.⁶⁷ This is an issue that would need to be resolved before *in vivo* experiments, ensuring complete separation of the two enzymatic reaction products.

2.2.12 MRI Characterisation

As Compounds **1** and **2** had shown promise in ¹⁹F NMR spectroscopy, basic MRI characterisation was attempted at 4.7 T. ¹⁹F MR spectra were acquired in a pulse-and-acquire experiment with the following parameters: TR = 800 ms, receiver bandwidth of 5 KHz, receiver gain = 58, N_A = 50 or 512 (T_A = 40 sec and 6 min respectively).

Figure 53 shows the MRS of DOTA-SPAYYTAD-Tfb (**13**) at 2 mM with N_A = 512 which shows a sharp peak, while the spectra of Gd-DOTA-SPAYYTAD-Tfb (**1**) (Figure 54) on the other hand demonstrates a decrease and a broadening of the signal at the same concentration with the same number of averages. At N_A = 50 while a signal was present for peptide ligand **13**, no signal for complex **1** was visible at all. These results are consistent with the ¹⁹F NMR spectroscopic data and continued to support the proof of principle of these compounds as potential ¹⁹F imaging agents for MMP-2.

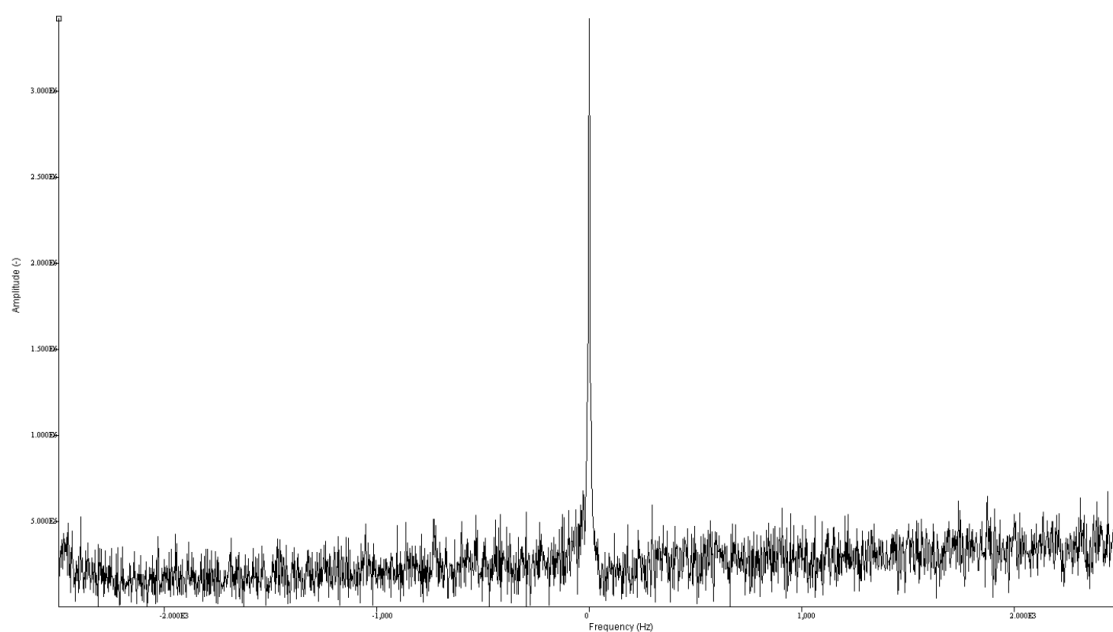


Figure 53- ^{19}F MRS of DOTA-SPAYYTAD-Tfb (**12**) 2mM, $N_A=512$ at 4.7T at 298K.

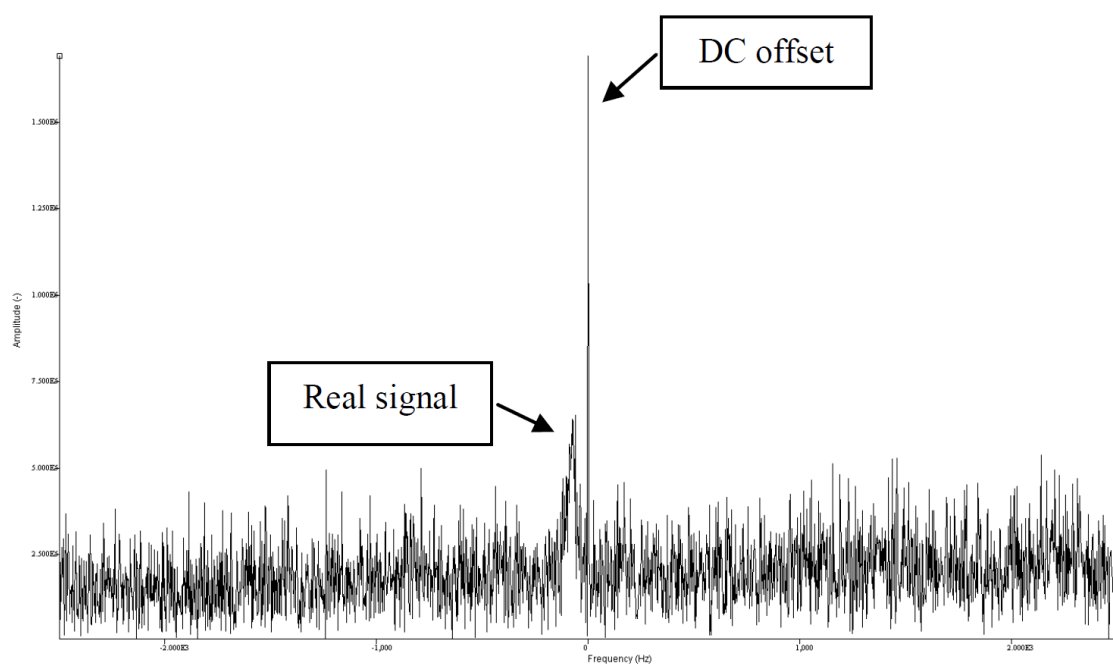


Figure 54- ^{19}F MRS spectra Gd-DOTA-SPAYYTAD-Tfb (**1**) 2mM, $N_A=512$ at 4.7T at 298K.

As well as the signal in Figure 54 a sharper peak is present due to a DC offset artefact.

These results support the already discussed ^{19}F NMR spectroscopic data.

For DOTA-SLAYYTAD-Tfb (**14**) due to solubility issues a concentration of 1 mM was used, again with 512 averages, a sharp peak is seen in the MR spectra as would be expected for this compound.

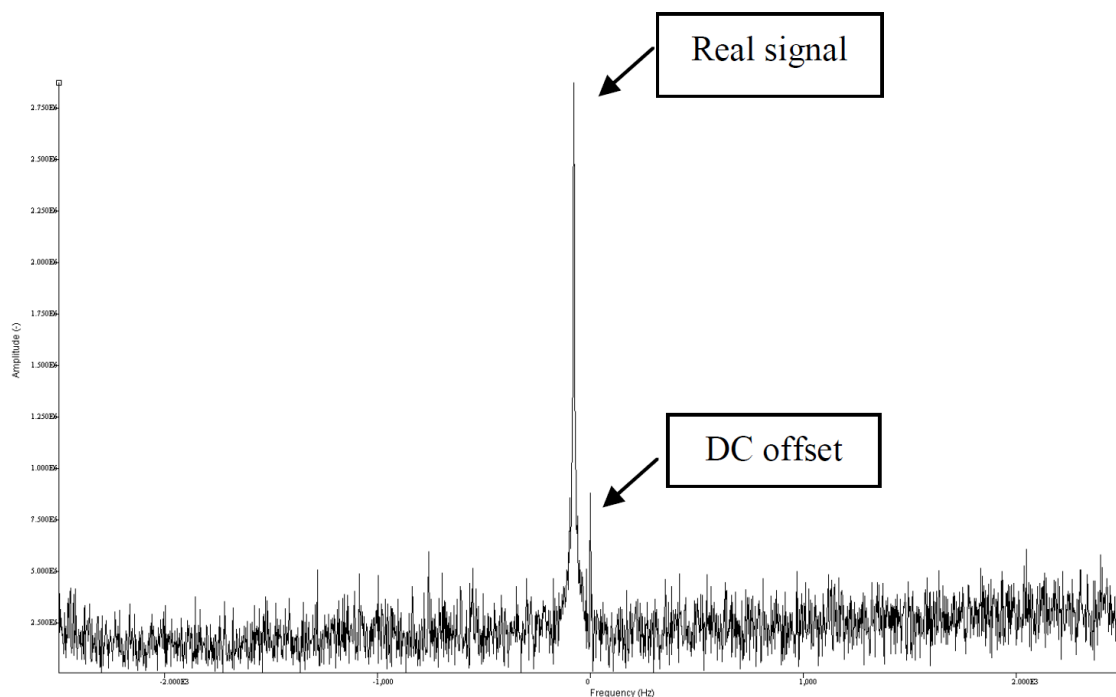


Figure 55- ^{19}F MRS of DOTA-SLAYYTAD-Tfb (**14**) 1mM, $N_A=512$ at 4.7T at 298K.

Unfortunately due to low solubility and time constraints with the MRI equipment no spectra were obtained for Gd-DOTA-SLAYYTAD-Tfb (**2**), it would be expected for this compound to produce a spectrum similar to that in Figure 54 for complex **1** (based on the ^{19}F NMR spectrum and T_1 and T_2 analysis).

Expected enzymatic cleavage product YTAD-Tfb (**15**) was also investigated (Figure 56) this again showed a sharp peak consistent with those seen for free ligand compounds **13** and **14** and in agreement with ^{19}F NMR and T_1 and T_2 data.

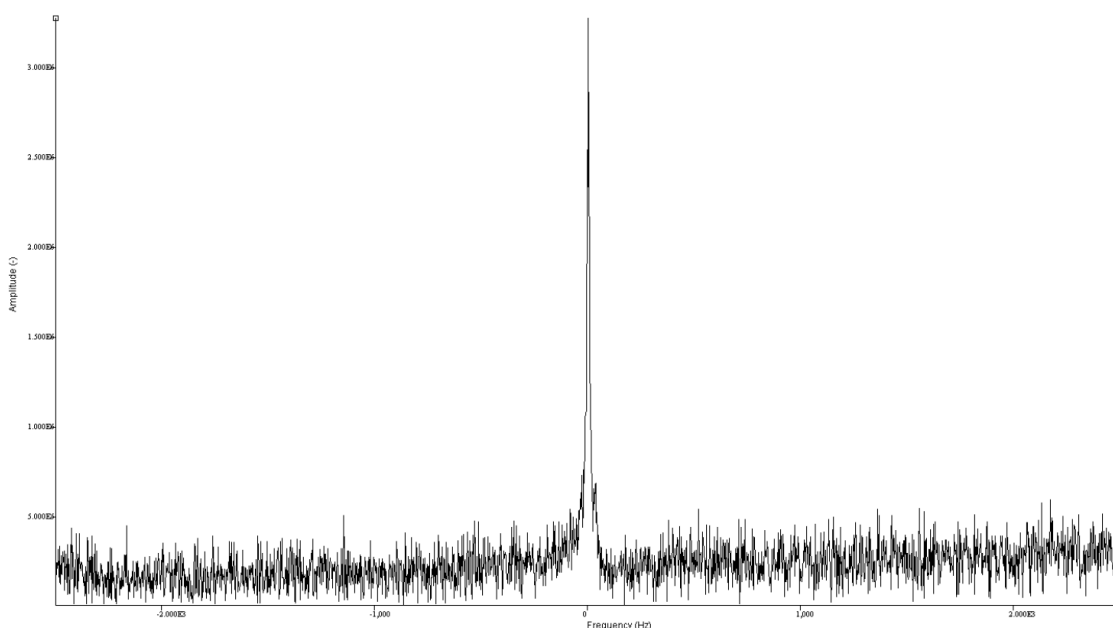


Figure 56- ^{19}F MRS of YTAD-Tfb (**15**) 2mM, $N_A=512$ at 4.7T at 298K.

The results shown here support the ^{19}F NMR spectroscopic data on these compounds and should translate into a lack of signal in the fluorine MRI spectrum for gadolinium complexes. Unfortunately, further characterisation through T_1 and T_2 as well as the production MR images could not be carried out due limited time on MRI equipment combined with problems with sample solubility.

2.3 Conclusions

Compounds Gd-DOTA-SPAYYTAD-Tfb (**1**) and Gd-DOTA-SLAYYTAD-Tfb (**2**) were designed as MMP-2 specific ^{19}F MRI probes; synthesis was carried out successfully, and their potential as MMP-2-activated ^{19}F imaging probes was investigated. Despite initial difficulties, reactivity of peptide compounds with MMP-2 and selectivity over MMP-9 and MMP-14 has been confirmed through the investigation of ^{68}Ga analogues (compounds **25** and **26**) which provided a highly sensitive assay method. This method also afforded reduced peptide and MMP-2

consumption during the project. Despite the good selectivity of **25** and **26**, reactivity was lower than expected and desired. It was hypothesised that this was due to introduction of functionalisation which was corrupting subsite interactions. ^{19}F spectroscopy (NMR and MRI) provided some good results demonstrating changes in the fluorine signal on complexation and during incubation with MMP-2. These were backed up by T_1 and T_2 data obtained using ^{19}F NMR. However, problems with the low solubility of these compounds along with the low sensitivity of the fluorine group proved problematic when attempting to get more in depth MR data and images during the limited timeframe available on the equipment. While Compounds **1** and **2** currently show a lot of potential, improvements would be paramount to their usefulness as MMP-2-activated MRI contrast agents. The major developmental areas of these compounds could be focused around two key areas which are looked at in more detail in Chapter 3; improving the reactivity of compounds with MMP-2 and improving sensitivity. In addition to these, another important factor for consideration in future work will be ensuring the compound does not migrate from the point of enzymatic reaction; this will be vital for a successful *in vivo* probe.

Chapter 3

Development of MMP-2 - activated imaging agent

3.1 *Introduction*

In Chapter 2 compounds **1** and **2** were introduced, both demonstrating potential as MMP-2-activated ^{19}F MR imaging agents. However, a number of issues with these compounds were revealed; including the low MMP-2 reactivity (when compared to broad spectrum compounds and literature examples), and the ^{19}F sensitivity, highlighting the need for further development. These two issues represent the main areas where improvements could be made to these compounds to increase their clinical potential. The development of compounds **1** and **2** forms the basis of the following Chapter.

3.2 *Results and discussion*

3.2.1 *Aims and objectives*

The aim of the research discussed in this chapter was to advance compounds **1** and **2**, building on their potential as ^{19}F MR imaging agents of MMP-2 activity, and finding the best route to developing them into viable molecular probes for clinical imaging. The objectives of this work were to:

1. Design and synthesise ‘2nd generation compounds’ with the potential to improve MMP-2 reactivity and/or ^{19}F sensitivity.

2. Analyse these '2nd generation compounds' via T₁ and T₂ interrogations, ⁶⁸Ga labelling and MMP assays, to determine the advantages and limitations of each.

3.2.2 Increasing reactivity - Introduction of spacer groups

One of the issues highlighted with peptides **1** and **2** (via radiolabelled analogues **25** and **26**) was their low MMP-2 reactivity compared with the functionalised broad spectrum peptide (**28**). In addition to this, comparison of compounds **25** and **26** to unfunctionalised 'parent' substrates revealed lower MMP-2 reactivity, with parent substrates producing near complete cleavage during two hour incubation with MMP-2.¹⁶ While this could be explained to some extent by differences in assay conditions (for example the form and amount of MMP-2 enzyme used), our hypothesis was that functionalisation had a detrimental effect on substrate reactivity. This effect was less apparent on broad spectrum compound **28**, likely a result of the different recognition profiles between broad spectrum and specific substrates, with the presence of multiple subsite interactions in specific peptides equating to greater difficulties in functionalisation.¹⁵⁻¹⁷

In order to improve reactivity of peptides **1** and **2**, the introduction of spacer groups was proposed. This was based on the assumption that functionalisation of the peptide chain was affecting its recognition at the S₂ subsite which is known to be involved in MMP-2/ -9 specificity. For MMP-2 specific peptides, it is known that the P₂ position has a role in activity thought to be related to positioning the substrate for successful hydrolysis within the catalytic site. It has been demonstrated that altering the P₂ in peptide substrates can affect MMP-2 recognition, and can lead to dramatically reduced hydrolysis.^{15, 16} The addition of a large DOTA group near this subsite may be causing

distortion in this S₂ interaction, and it is likely that this functionalisation at the N-terminus is causing low reactivity.⁸⁴ Whilst the C-terminus of the compounds **1** and **2** has also been functionalised, this group is much smaller and not adjacent to any amino acids which have shown a specific recognition interaction. Additionally, this group is likely to be hydrophobic in character which is in keeping with the favourable characteristics of this primed side of the catalytic cleft.^{12, 14, 91, 92}

When designing and developing compounds for molecular imaging the use of pharmacokinetic modifiers (i.e. hydrocarbon chains or PEG groups) to distance the biologically active site from bulky chelators is well known. There is evidence to suggest that the inclusion of such spacer groups into compounds **1** and **2** would improve reactivity. Lepage *et al* demonstrates that functionalisation of SPAYYTA with the gadolinium DOTA is possible without great effect on reactivity; in this case a small carbon chain is present between the ligand and the peptide.^{49, 50} We can infer from this that the spacer group in this compound (which was introduced for reasons un-related to reactivity) provided an ideal platform for retaining the high activity of unfunctionalised parent substrate. Additionally, other reports on specific peptide substrates have found similar problems with bulky substituents. Park *et al* described showed that loss of peptide activity with MMP-14 was observed upon functionalisation with bulky substituents.⁵⁵ However, this could be reversed on addition of a 3 amino acid spacer group.

We proposed the introduction of the following spacer groups into compounds **1** and **2**; firstly a 6-aminohexyonyl group which is a small carbon chain similar to that described by Lepage *et al*; secondly a small PEG₃ linker chosen due to PEG's documented use as a pharmacokinetic modifier; finally a 3 amino acid spacer group (Serine-Glycine-Glutamic acid) which was chosen from the extended version of the

peptide substrate described in Chen *et al.*^{16, 49, 50} These spacers groups were straightforward to incorporate into the already existing synthesis method. They were introduced into both peptide compounds **13** and **14** (DOTA-SPAYYTAD-Tfb and DOTA-SLAYYTAD-Tfb) between the amino acid chain and the bulky gadolinium DOTA species (Figure 57).

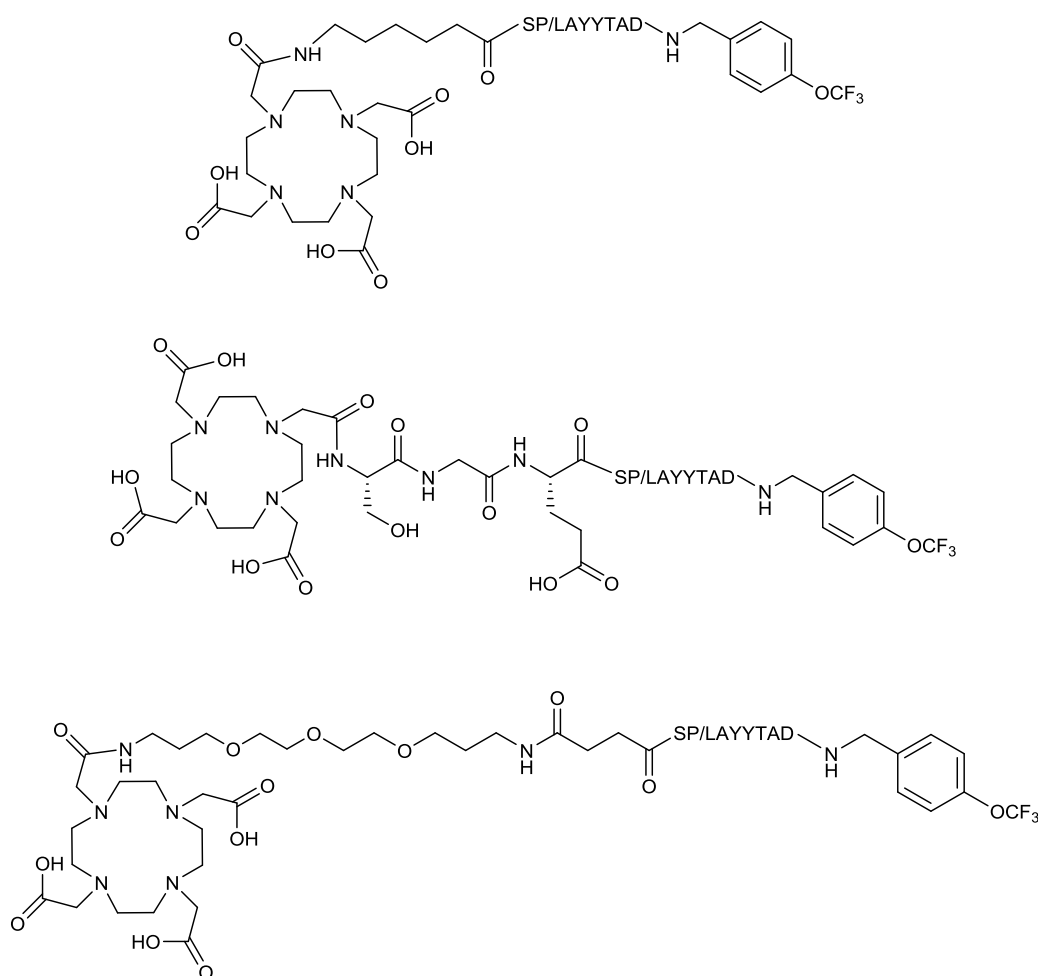


Figure 57- Diagram outlining the spacer groups which will be introduced to both peptide substrates.

These peptides were synthesised following analogous procedures to those described in Chapter 2. This involved the synthesis of triprotected DOTA ligand (**8**) and of Fmoc-Asp-[(trifluoromethoxy)benzyl amide]-OH (**12**), which were then combined into a peptide substrate via Fmoc solid phase synthesis procedures. This included three additional coupling steps for the SGE spacer group under normal conditions (HBTU

and DIPEA), or an additional coupling step for spacer groups Ahx and PEG₃ using an alternative coupling reagent HATU. Coupling of the DOTA ligand and removal from the solid resin could then be carried out as previously described. Successful synthesis of these peptides was confirmed by mass spectrometry, with yields for peptides shown in Table 3.

Table 3- Yields of spacer group compounds after solid phase peptide synthesis and HPLC purification

Peptide	Yield
<i>DOTA-Ahx-SPAYYTAD-Tfb (29)</i>	32%
<i>DOTA-Ahx-SLAYYTAD-Tfb (30)</i>	27%
<i>DOTA-SGESPAYYTAD-Tfb (31)</i>	42%
<i>DOTA-SGESPAYYTAD-Tfb (32)</i>	26%
<i>DOTA-PEG₃-SPAYYTAD-Tfb (33)</i>	30%
<i>DOTA-PEG₃-SLAYYTAD-Tfb(34)</i>	28%

Consequently, these ligands could be complexed to gadolinium(III) (GdCl₃.6H₂O in H₂O adjusted to pH7 at RT overnight) using identical methods to those outlined in Chapter 2. Mass spectrometry was used to confirm complex formation, demonstrating the expected isotopic patterns for gadolinium. Yields for complexation reactions are shown in Table 4.

Table 4- Yields for spacer group compound complexation to gadolinium (III)

Complex	Yield
<i>Gd-DOTA-Ahx-SPAYYTAD-Tfb (35)</i>	69%
<i>Gd-DOTA-Ahx-SLAYYTAD-Tfb (36)</i>	78%
<i>Gd-DOTA-SGESPAYYTAD-Tfb (37)</i>	52%
<i>Gd-DOTA-SGESLAYYTAD-Tfb (38)</i>	79%
<i>Gd-DOTA-PEG₃-SPAYYTAD-Tfb (39)</i>	62%
<i>Gd-DOTA-PEG₃-SLAYYTAD-Tfb (40)</i>	85%

T_1 and T_2 ^{19}F NMR experiments were carried out on both peptide ligands (**29-34**) and complexes (**35-40**) in order to determine the effects from gadolinium(III) (Table 5).

Table 5- ^{19}F NMR T_1 and T_2 results for all spacer group compounds, free ligands and gadolinium(III) complexes. 400MHz, 298K, standard error $\pm 0.5\text{ms}$.

Peptide	T_1 – longitudinal relaxation time (seconds)	T_2 – Transverse relaxation time (seconds)
DOTA-Ahx-SPAYYTAD-Tfb (29)	1.519	1.123
Gd-DOTA-Ahx-SPAYYTAD-Tfb (35)	0.045	0.008
DOTA-SGESPAYYTAD-Tfb (30)	1.523	1.031
Gd-DOTA-SGESPAYYTAD-Tfb (36)	0.077	0.011
DOTA-PEG-SPAYYTAD-Tfb (31)	1.542	1.193
Gd-DOTA-PEG-SPAYYTAD-Tfb (37)	0.058	0.012
DOTA-Ahx-SLAYYTAD-Tfb (32)	1.536	1.041
Gd-DOTA-Ahx-SLAYYTAD-Tfb (38)	0.050	0.013
DOTA-SGESLAYYTAD-Tfb (33)	1.458	1.031
Gd-DOTA-SGESLAYYTAD-Tfb (39)	0.083	0.013
DOTA-PEG-SLAYYTAD-Tfb (34)	1.482	1.282
Gd-DOTA-PEG-SLAYYTAD-Tfb (40)	0.050	0.012

This data shows that gadolinium(III) complexation resulted in a decrease of T_1 and T_2 relaxation times when compared to metal-free peptides, confirming the presence of a paramagnetic effect on the fluorine atoms in compounds **35-40**. Relaxation times are comparable to those described in Chapter 2, indicating that introduction of the spacers has not had a detrimental effect on PRE, and the mode of action has not been affected. Moreover, results in Table 5 can be compared to compound **15**, the expected cleavage product of enzymatic reaction (see Chapter 2), as this has not changed; this shows comparable relaxation times to the free ligands described (**29-34**) in Table 5. Overall

results in Table 5 provide proof of principle that theoretically, on peptide cleavage, a change in ^{19}F MR signal can be expected.

Free ligands **29-34** were then labelled with ^{68}Ga using the protocol outlined in Chapter 2 (10 minutes, 90 °C, NaOAc buffer, pH 5). Decay corrected radiochemical yields are shown in Table 6.

Table 6- Radiochemical yields of the gallium labelling of peptide ligands **27-32**. ^a decay-corrected using radio-HPLC, n=3.

Complex	Radiochemical Yield ^a
^{68}Ga -DOTA-Ahx-SPAYYTAD-Tfb (41)	95.7±2.5%
^{68}Ga -DOTA-Ahx-SLAYYTAD-Tfb (42)	97.3±0.5%
^{68}Ga -DOTA-SGESPAYYTAD-Tfb (43)	95.0±4.6%
^{68}Ga -DOTA-SGESPAYYTAD-Tfb (44)	96.0±3.0%
^{68}Ga -DOTA-PEG ₃ -SPAYYTAD-Tfb ^{68}Ga - (45)	95.3±3.5%
^{68}Ga -DOTA-PEG ₃ -SLAYYTAD-Tfb ^{68}Ga - (46)	92.0±2.0%

Purification and formulation was carried out on the solid phase as previously described, and radiolabelled compounds were isolated in 200 µl of ethanol for biological testing.

Gallium labelling allowed for analysis of peptide substrates reactivity with MMP-2. The assay was carried out using the protocol outlined in Chapter 2; employing APMA to activate pro-MMP-2 which could then be incubated at 22 nM with ^{68}Ga labelled peptides at 37 °C for 30, 60, 90 or 120 minutes before radio HPLC analysis. Results for all spacer group peptides can be seen in Figure 58.

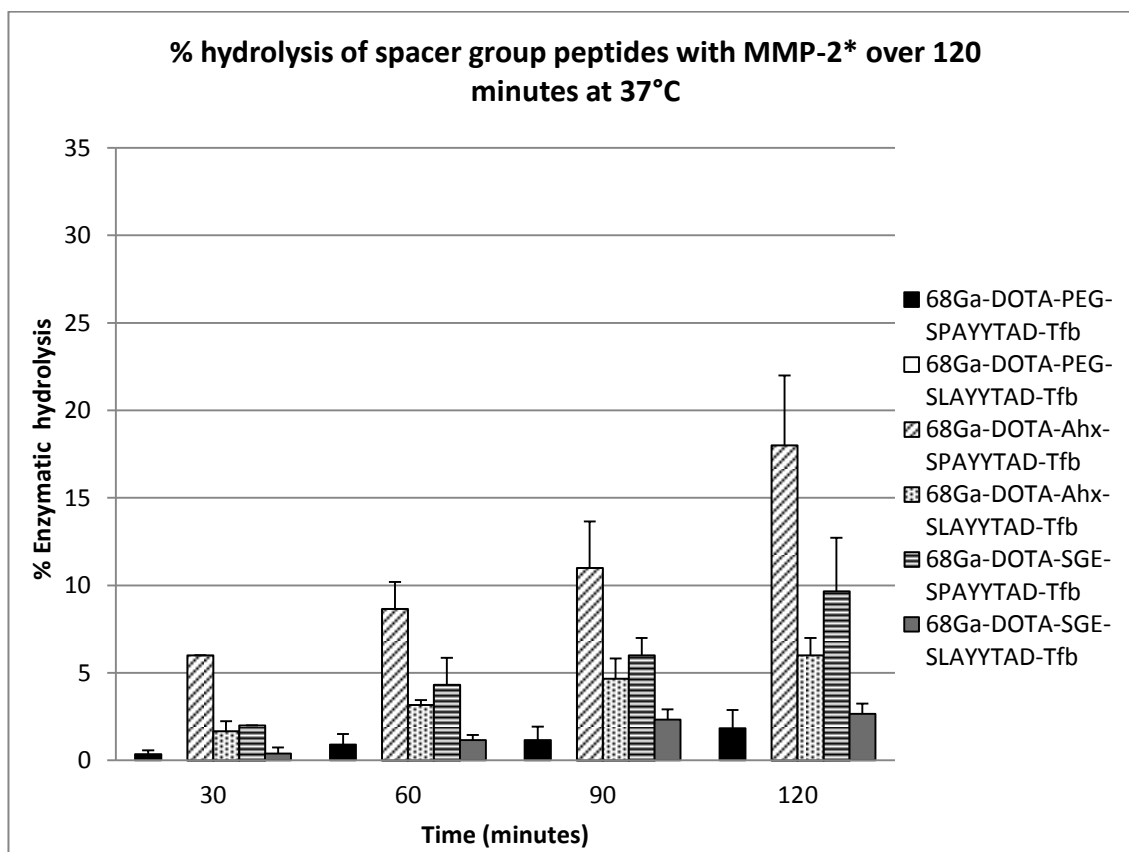


Figure 58 – Graph showing the % of enzymatic hydrolysis found on incubation of radiolabelled compounds **41-46** with MMP-2* after 120 minutes of reaction at 37° C (n=3, error bars show standard deviation).

Apart from ^{68}Ga -DOTA-PEG₃-SLAYYTAD-Tfb (**46**), all the radiolabelled peptides demonstrated enzymatic conversion with MMP-2. However, only ^{68}Ga -DOTA-Ahx-SPAYYTAD-Tfb (**41**) showed similar enzymatic cleavage to compounds **25** and **26**; the other labelled compounds showed significantly decreased reactivity. Interestingly, peptides with the SLAYYTA core peptide substrate were more greatly affected by the introduction of a spacer group than those with the proline containing motif (SPAYYTA). This general loss of reactivity is likely to be due to the increased size of the peptide substrates. The results show that, as the spacer group becomes larger, the enzymatic conversion of the peptide decreases, with compounds containing the PEG₃ spacer showing the lowest levels of enzymatic cleavage. Spacer groups appear to be

affecting the interactions of the peptide substrate with the catalytic cleft. The difference between the proline and leucine containing peptides could be explained by the different shapes of the compounds, with SLAYYTA being more linear in character. Literature states that all MMPs favour deviations from linearity generally arising from a proline at the P₃ position, so additional broad spectrum character of the SPAYYTA could be contributing to its increased recognition over SLAYYTA on additional functionalisation.¹⁴⁻¹⁷ It could be hypothesised that the compounds containing the SPAYYTA motif are likely to demonstrate an element of secondary structure due to the presence of the Ser-Pro turn, which is helping retain recognition in these larger substrates.⁸³

As ⁶⁸Ga-DOTA-Ahx-SPAYYTAD-Tfb (**41**) was the only peptide which showed similar reactivity to first generation compounds **25** and **26**, this was further analysed with selectivity studies versus MMP-9 and MMP-14. MMP activation and assays were carried out as described in Chapter 2; the results are summarised in Figure 59.

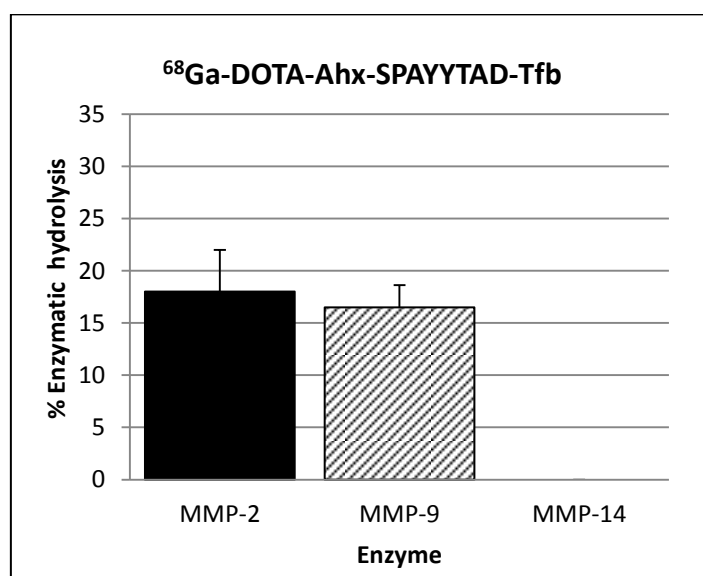


Figure 59- Graph showing the enzymatic hydrolysis of **41** after 2 hours incubation with activated MMP-2, MMP-9 or MMP-14 (MMP-2 and MMP-14 n=3, MMP-9 n=2, error bars show standard deviation).

No hydrolysis was observed with MMP-14 using compound **41** as substrate, while MMP-9 demonstrated activity, as was expected from the unfunctionalised 'parent' peptide. However, this was greater than that exhibited with compound **25** (an average of $16.5 \pm 2\%$ versus $4.7 \pm 2\%$) indicating that introduction of the Ahx spacer group had resulted in a loss of selectivity. This could be explained by the introduction of an alternative mode of recognition within the catalytic site that has been triggered by the addition of the Ahx carbon chain. Loss of selective character has been described by Kridel *et al* who demonstrated that a selective substrate could be made broad spectrum by a simple amino acid substitution away from the primary selective interaction.¹⁷ Molecular modelling of this event showed two forms of recognition in which contact points for specific interactions could be altered by changing a proximate amino acid. It is also well documented that proline at P₃, as is present in SPAYYTA, is generally associated with broad spectrum recognition, due to its induction of a 'kinked' conformation, favoured by majority of the MMP family. However, Chen *et al* demonstrated that this peptide retained selectivity for MMP-2 despite this proline amino acid at P₃.^{15, 16} Therefore, it could be inferred that the SPAYYTA substrate demonstrates both selective and broad spectrum recognition at different subsites along the peptide chain. If this is the case, it is possible that, while the spacer group may not be affecting broad spectrum interactions (for example the S₁' binding site), it could be altering specific ones occurring along the peptide substrate (for example at the S₂ subsite), changing the mode of recognition. This broad spectrum recognition of the SPAYYTA peptide would explain why it is less detrimentally affected by spacer group addition than the more selective SLAYYTA substrate. It is interesting to note that in the investigation by Lepage *et al* which uses the DOTA functionalised SPAYYTA

motif with a similar spacer group to Ahx, no investigations into compound selectivity were reported.⁴⁹

Unfortunately the introduction of spacer groups into the 1st generation probes did not result in any improvement on the reactivity of peptides **25** and **26**. This suggested that alternative methods of compound development should be in the forefront of future work.

3.2.3 Increasing sensitivity- Cell penetration

Increasing the activity of peptide substrates would help increase the signal changes of the fluorine over a short period of time, however perhaps a more important area of improvement for compounds **1** and **2** would be increasing their sensitivity. The NMR and MR experiments described in Chapter 2 demonstrated a number of issues with the intensity of the ¹⁹F signal at low concentrations of **1** and **2**. This would ultimately make these imaging probes difficult to use in a clinical environment. In Chapter 1, a number of methods for increasing ¹⁹F sensitivity were outlined, the easiest of which is to increase the fluorine content of the compound.^{60, 63, 64, 73} However, while additional fluorine atoms would provide a small increase in sensitivity, there are possible effects on biocompatibility (reduced solubility and increased toxicity). Additionally, the difficulty of dealing with highly fluorinated agents could add synthetic complexity to compound synthesis.

Advanced designs for increasing sensitivity discussed in Chapter 1 included the use of other lanthanides (which have shown an ability to increase the fluorine signal) or targeted delivery to increase fluorine concentrations.^{60, 63, 64, 73} Whilst investigations into dual lanthanide/gadolinium probes could provide some interesting results, this

would be difficult to efficiently incorporate into our current ^{19}F imaging system. On the other hand, increasing local concentrations of fluorine at the point of MMP expression could be achieved via a number of methods; such as nanoparticle delivery or addition of a targeting moiety. This area of design modification could also increase compound clearance times, allowing for longer experimental timescales.

The incorporation of a ‘targeting’ moiety into the fluorinated side of the peptide complex has the potential to provide local build-up of ^{19}F at the site of enzymatic cleavage, prevent migration of the contrast agent and increase retention times. While traditional targeting methods could be employed, such as the addition of a moiety to target receptors like $\alpha_v\beta_3$ integrins (which are also overexpressed in cancer with links to MMP-2), a more viable area of interest was introducing a cell penetrating component.²⁸ As MMPs are expressed extracellularly, cell penetration would allow for localisation of ^{19}F within cells, providing the potential for signal build up and increased local concentration.

The majority of cell penetrating motifs are based on peptides, the most prolific of which include those based on the HIV Tat sequence, polyarginine residues and penetratin.^{93, 94} Whilst these have the ability to enable cell penetration of compounds, they are non-discriminant, and will enter into cells at any point in the biological system which, if incorporated into compounds **1** and **2**, could be before the extracellular enzymatic reaction. However, as discussed in Chapter 1, Tsien *et al* developed a set of activated cell penetrating peptide (ACPP) sequences which do not enter cells initially but respond to enzymatic reaction to release a cell penetrating component.³⁷ Such systems have been utilised for MMP imaging via activated localisation of a ‘payload’ such as fluorescent dye, gadolinium (for ^1H MRI) or radioactive species.^{37, 54, 77} These sequences consist of a poly-arginine residue acting as a cell penetrating peptide,

followed by an MMP cleavable peptide substrate (in Tsien's work, a MMP-2/-9 substrate sequence), and then by a poly-glutamic acid residue. The C-terminus of this peptide (poly-cationic arginine residue) is attached to the payload that needs to be delivered into the cells. In the initial state, the poly-cationic cell penetrating component of these compounds is balanced by the poly-anionic residue and compounds will not enter into cells. Once enzymatic reaction takes place, the two charged residues are no longer in contact and cell penetration can take place. These activated cell penetration systems have demonstrated positive *in vitro* and *in vivo* results with increased tumour uptake of ACPs, and have shown potential for incorporation into nanoparticles and multimodal agents. There are now example emerging from Xia *et al* of modified ACP compounds being developed for activated drug delivery, that are demonstrating positive preliminary results.⁹⁵ However, some *in vivo* studies using doubly radiolabelled ¹⁷⁷Lu and ¹²⁵I broad spectrum (MMP-2/-9) ACP probes show aspecific peptide cleavage is occurring, potentially in the vasculature.⁷⁷ This suggests increased tumour uptake of these broad spectrum probes may only be an artefact of enhanced permeability and retention, combined with high tumour vascularisation.⁷⁷

These probes represent an interesting area for investigation into increasing local concentrations of fluorine; incorporation of ACP motifs into the MMP-2 specifically cleaved imaging agent design could provide a probe which is specifically activated and simultaneously enters the cell. These probes would have the additional advantage of ensuring complete separation of the CF₃ group from the gadolinium-DOTA which should improve ¹⁹F signal recovery on enzymatic cleavage (an issue in compounds **1** and **2** during *in vitro* investigations). Figure 60 shows the proposed basic structure for the incorporation of ACPs into MMP activated ¹⁹F imaging probes.

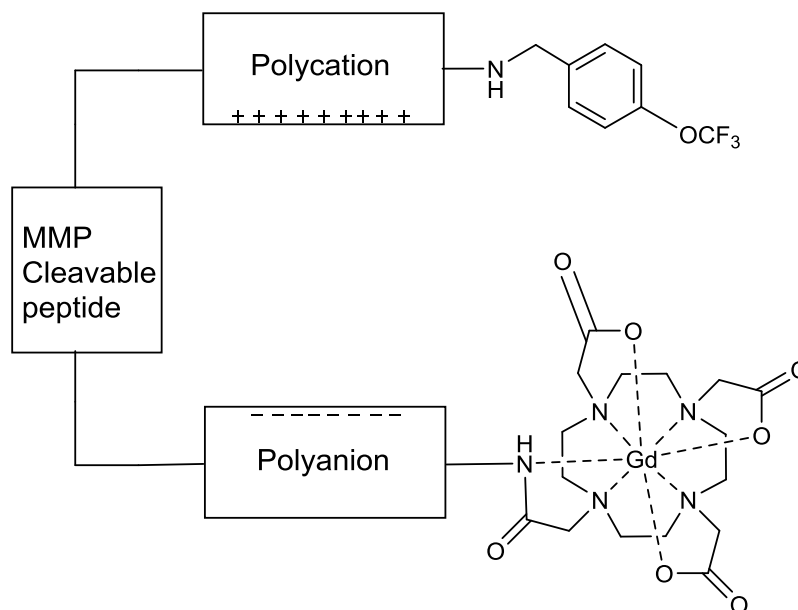


Figure 60- Proposed basic struction of ACPP comppunds for activated ^{19}F MRI.

This structure involves the functionalization of the cell penetrating polycationic arginine residue with CF_3 reporter group and the polyanionic glutamic acid residue with Gd-DOTA.

One issue with such cell penetrating systems is the increase in peptide length. Many cell penetrating sequences are in the region of 9-15 amino acid residues long; in the case of the ACPPs (such as that described by Tsien and co-workers) there are around 24 amino acid residues and two 6-aminohexonyl spacer groups.³⁷ These increased lengths could mean that if N-terminus and C-terminus functionalization took place as with 1st generation imaging probes and spacer group peptides, gadolinium-DOTA species and fluorine containing groups would be an increased distance apart, which could significantly affect the paramagnetic relaxation interaction. This could potentially remove or reduce the change in ^{19}F signal on MMP activation. Importantly, in the case of ACPPs, it has been shown (through NOE effects and short range couplings) that the peptide forms into a hairpin conformation (Figure 61).³⁷

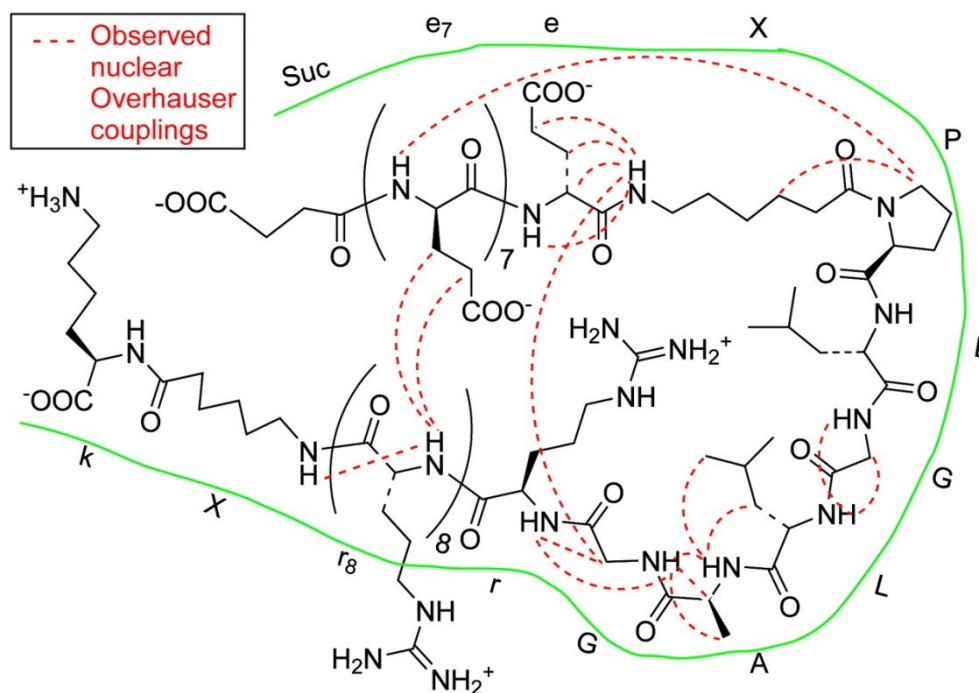


Figure 61- Pictorial representation of nuclear overhauser effects which demonstrate the hairpin structure of ACPPs, figure taken from Tsien *et al.*³⁷

This secondary peptide structure could be enough to force the two end groups into close proximity, despite the increased peptide length, thus retaining the PRE effect. Although compounds could be functionalised at any point along the peptide substrate, moving functionalised groups away from the active site of enzymatic cleavage is desirable. Therefore, as shown in Figure 60 the bulky DOTA group and the functionalised amino acid group are now removed from the MMP active peptide substrate, but will be held close together via interactions along the peptide backbone. The proposed mode of action of our ACPP probe is shown in Figure 62.

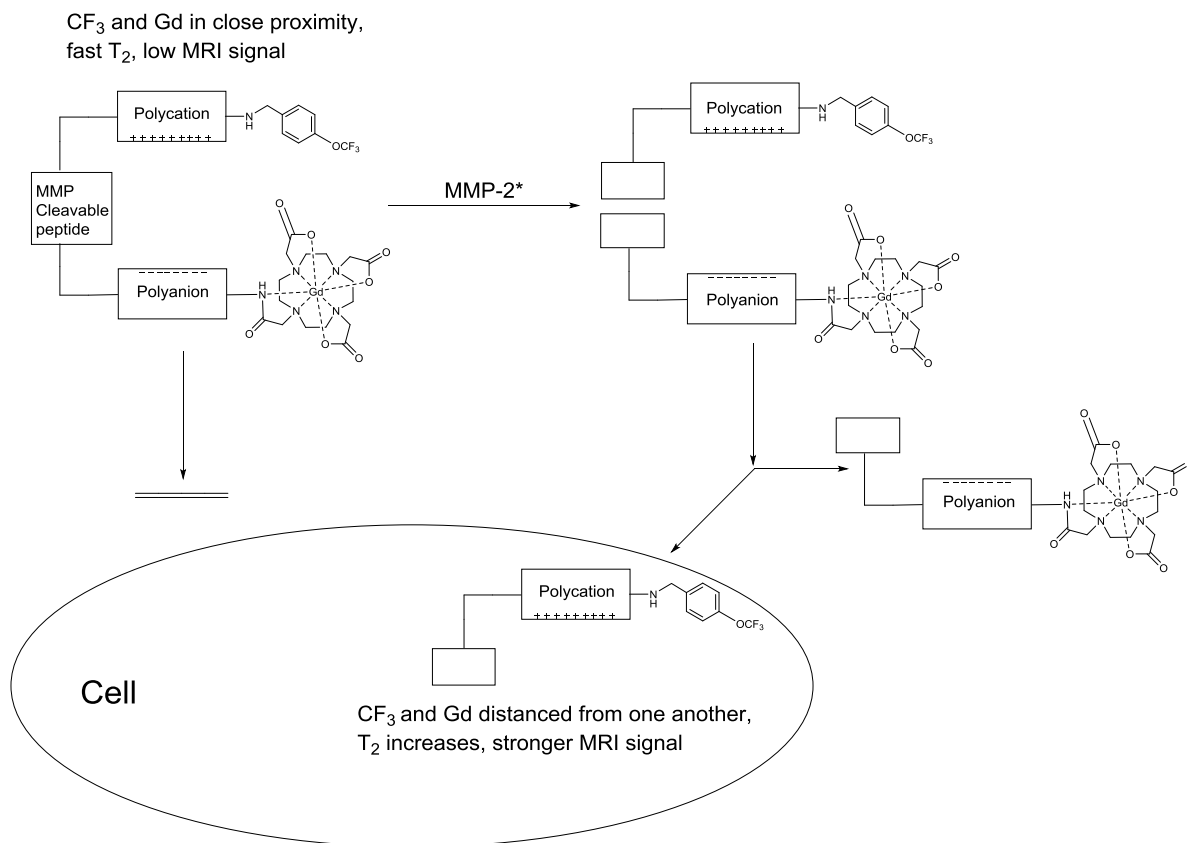


Figure 62- Proposed mode of action for MMP activated ACPP compounds for activated ¹⁹F MRI.

It was decided that this compound design would be applied to two MMP cleavable peptide substrates (Figure 63); firstly, the widely reported ACPP sequence (with MMP-2/-9 cleavable motif PLGLAG) originally described by Tsien *et al*, as this is the structure which has demonstrated the hairpin conformation experimentally, and secondly, one of the MMP-2 selective peptide sequences utilised for 1st generation imaging probes, namely SPAYYTA. This sequence was chosen over the slightly more selective SLAYYTA due to its retention of activity on extension with spacer groups (as described previously) and due to the presence of a proline amino acid, which is also present in the broad spectrum substrate and known to cause ‘kinks’ in peptide shape. This proline amino acid may be involved in promoting hairpin conformations.

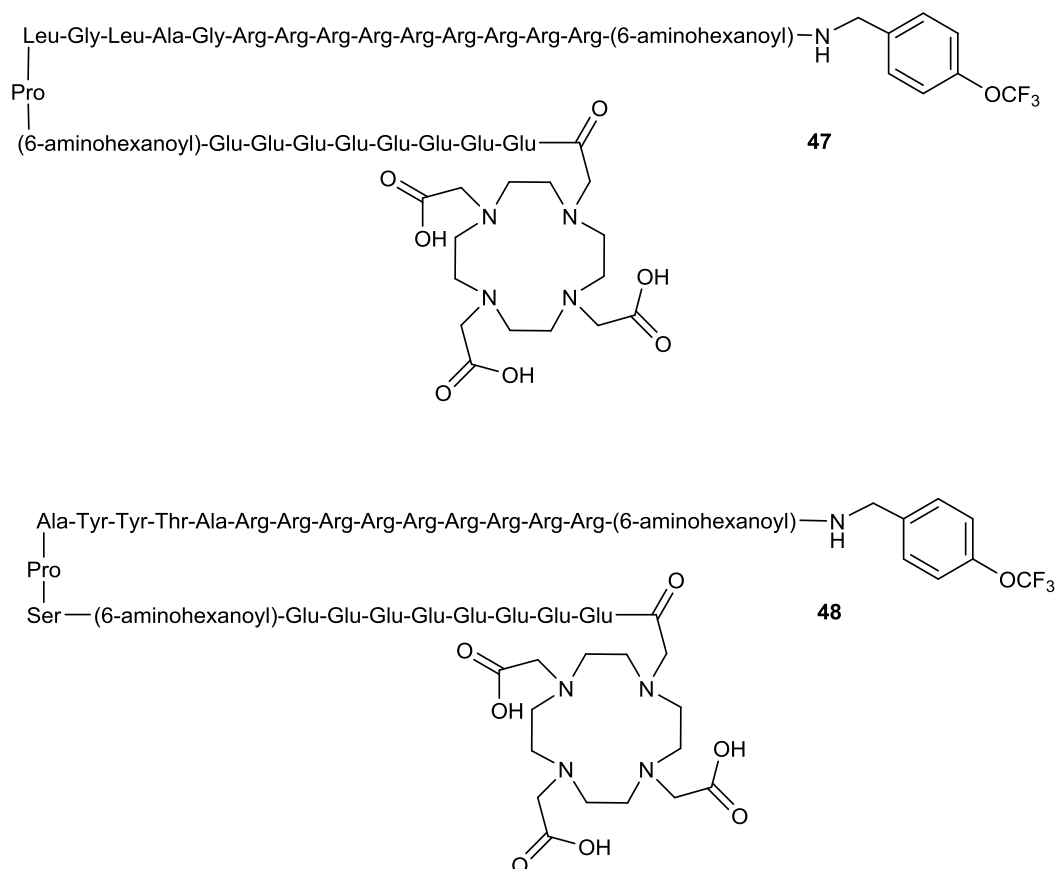


Figure 63- Diagram showing designs for compound **47** and **48**. **47** is based on a reported ACPP design, with **48** incorporating an MMP-2 specific substrate, both for activated cellular uptake and activation of ^{19}F .

Subsequently, it was deemed interesting to attempt to synthesise an ACPP compound which allowed ^{68}Ga delivery, this is similar to that described in Grull *et al*, which uses the same basic structure as Tsien *et al* and involves incorporation of the DOTA ligand on the cell penetrating (poly-cationic) C-terminus of the peptide.⁷⁷ This was to be carried out with the SPAYYTA substrate and could be labelled with ^{68}Ga (using the simple and high yielding labelling methods described for other DOTA peptides) to provide selective MMP-2 activated cell penetration of the radionuclide. In addition to providing easy analysis of cell penetration via use of radiodetector, if successful, there is potential for this to be incorporated along with a ^{19}F MRI probe onto a nanoparticle surface. The proposed modified peptide is shown in Figure 64.

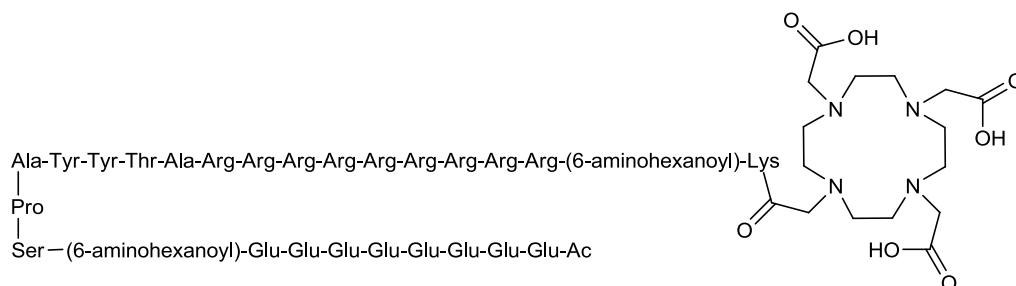


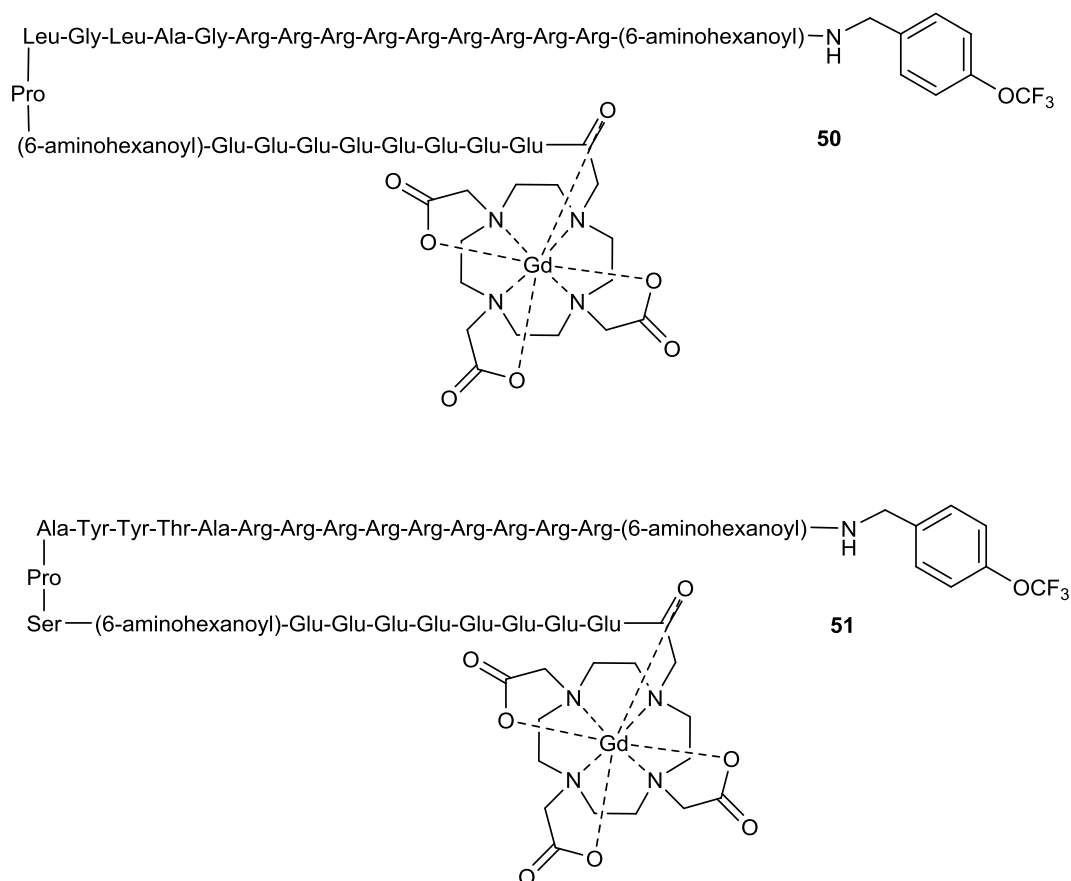
Figure 64 – Proposed design of ACPP for MMP-2 selective uptake of ^{68}Ga , compound **49**.

It is interesting to note that this design is very similar to that of Grull *et al* bar the use of ^{68}Ga instead of ^{177}Lu and the MMP-2 specific peptide substrate. Whilst Grull demonstrates cellular uptake of ACPP (pre-incubated with activated MMPs), similar to that of a CPP, *in vivo* studies indicated high background uptake, relating on further investigation to aspecific cleavage.⁷⁷ It was hoped that the use of a specific peptide substrate would eliminate much of this problem resulting in a more efficient radiotracer. For all peptide substrates poly-anionic and poly-cationic residues were synthesised using D-amino acids to prevent non-specific peptide breakdown within the body.

As before, synthesis of triprotected DOTA ligand (**8**) and of Fmoc-Asp-[(trifluoromethoxy)benzyl amide]-OH (**12**) were carried out using aforementioned methods. ACPP substrates could then be synthesised up to the final glutamic acid residue on a peptide synthesiser. Once peptide synthesis was complete up to this final residue with Fmoc deprotection, the peptides were removed from the synthesiser for manual steps. For the synthesis of peptides **47** and **48**, DOTA ligand was coupled manually using HATU and DIPEA in a 2 hour procedure which was carried out twice. For peptide **49**, acetylation took place at the N-terminus, before the Mtt protecting group was selectively removed (1.8% v/v TFA in DCM with 2% v/v triisopropylsilane as a scavenger in 12 × 3min washes). At this point, coupling with 1.5 equivalents of

DOTA succinimidyl ester with DIPEA took place over 24 hours. All peptides could then be cleaved from the rink amide resin, deprotected and isolated as previously described, using an extended 6 hour deprotection time ensuring removal of Pfb protecting groups. Before HPLC purification, all peptides were analysed with mass spectrometry, which revealed that while **47** and **48** had been synthesised, **49** had not. This was likely to be an issue with Mtt deprotection or reaction with DOTA succinimidyl ester and unfortunately due to time constraints this synthesis could not be repeated. Compounds **47** and **48** were purified using prep-HPLC to give yields 30% and 38% respectively. Analytical HPLC demonstrated broad peaks, thought to be due to multiply charged species.

Subsequently, these peptide ligands were complexed to gadolinium(III) (GdCl_3 in H_2O adjusted to pH7 at RT overnight) and purified by prep HPLC to give complexes **50** and **51**, with yields of 77% and 66% respectively.

Figure 65- Gadolinium (III) complexes of ACPD peptides **50** and **51**.

^{19}F NMR spectroscopic analysis on peptide ligands **47** and **48**, and their corresponding complexes **50** and **51** was carried out focusing on T_1 and T_2 (see Table 7).

Table 7 - ^{19}F NMR T_1 and T_2 results for ACPD compounds, free ligands and gadolinium(III) complexes.

D amino acids represented in lower case (e and r). 400MHz, 298K, standard error $\pm 0.5\text{ms}$.

Compound	T_1 – Longitudinal relaxation time (seconds)	T_2 - Transverse relaxation time (seconds)
DOTA-(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (47)	1.405	0.879
Gd-DOTA-(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (50)	0.307	0.055
DOTA-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-D-Tfb (48)	1.389	0.827
Gd-DOTA-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-D-Tfb (51)	0.312	0.041

These results demonstrate a change in T_1 and T_2 relaxation times upon complexation, indicating PRE effect is taking place. The similarity in relaxation times between both peptides (PLGLAG and SPAYYTA) suggests that similar conformations and distance between gadolinium(III) and CF_3 are present in both peptides. However, the change in relaxation times is not as great as for first generation compounds **1** and **2**, or spacer group peptides (**35-40**), indicating a weaker paramagnetic effect. This suggests that the increased distance has an effect on paramagnetic interactions, and that through-space interactions are not strong enough to counteract this change. The ^{19}F signal in the MRI spectrum for these complexes may not be completely attenuated at this T_2 length, meaning that, upon cleavage, change in signal intensity would be less significant than for 1st generation compounds. This could be problematic, resulting in ^{19}F background signals and reduced signal to noise ratios and will require further investigation. This suggests that the hairpin conformation is not enough to bring these two groups into close proximity for gadolinium to have as pronounced a paramagnetic relaxation enhancement effect as was first hoped. Nevertheless, comparison of the ^{19}F NMR spectroscopic data (shown in Figure 66 and Figure 67) did show changes in the ^{19}F signal on complexation with broadening of the peaks and loss of signal intensity, indicating a paramagnetic interaction. However, this could be due to the dissolved gadolinium (III) complex affecting bulk magnetic susceptibility.

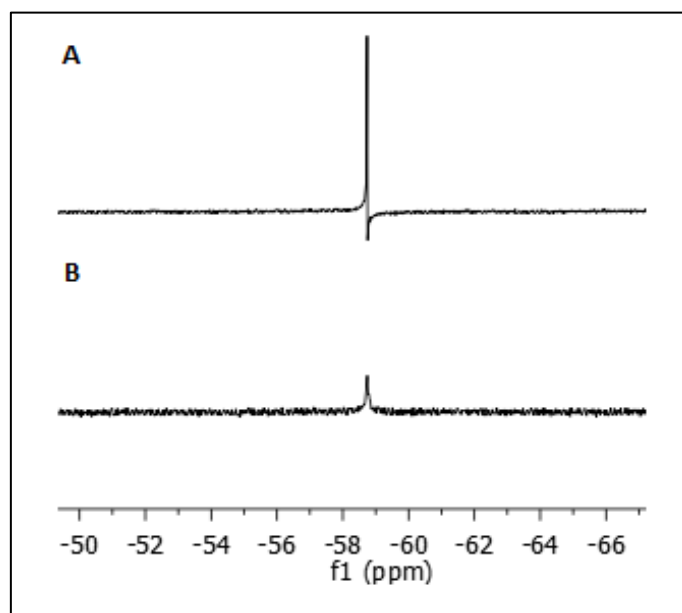


Figure 66- Comparison of ^{19}F NMR signals of compounds **47** (A) and **50** (B), demonstrating the paramagnetic relaxation effect.

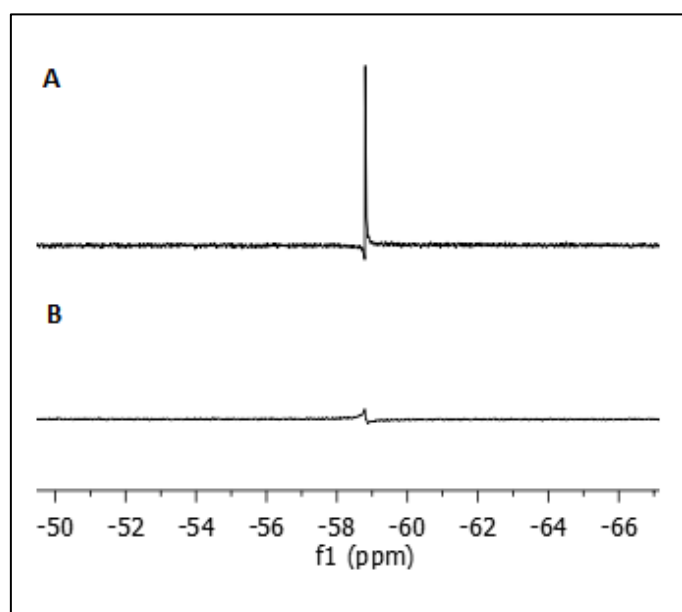


Figure 67- Comparison of ^{19}F NMR signals of compounds **48** (A) and **51** (B), demonstrating the paramagnetic relaxation effect.

Subsequent analysis of these ACPD compounds was carried out via radiolabelling of Peptides **47** and **48**, and analysis of MMP activity and selectivity. This could be carried out as previously described (90°C, NaOAc pH5 0.2 M, 10 minutes), purification was

carried out on C18 light SPE cartridges, using acidified ethanol to obtain the majority of the labelled compound in 200 μ l.

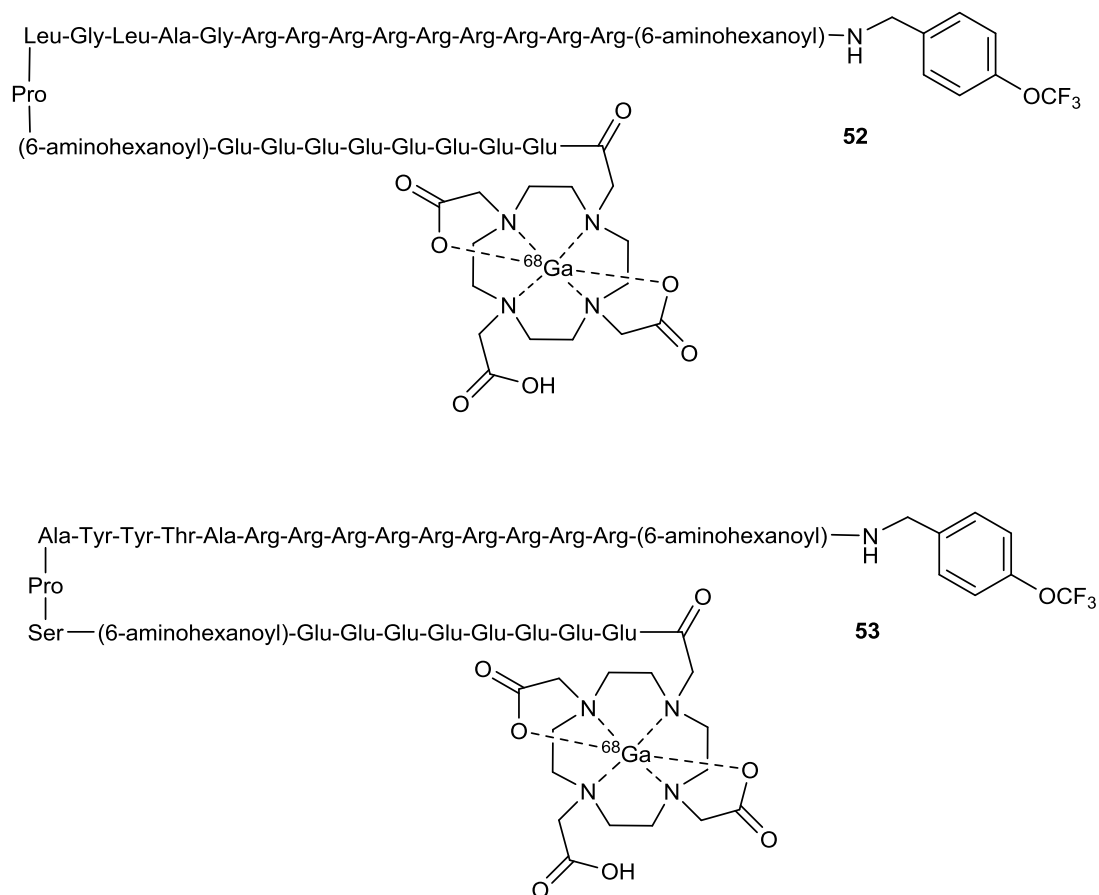


Figure 68- ^{68}Ga labelled peptides **52** and **53**.

Radiochemical yields for **52** and **53** were $95.7 \pm 2.5\%$ and $92.7 \pm 5.5\%$ respectively ($n=3$, decay corrected as calculated by radio HPLC).

^{68}Ga labelling allowed for MMP-2 and MMP-9 assays to take place as previously described incubating the radiolabelled compounds with 22 nM of enzyme to determine reactivity and selectivity of ACPPs. Analysis of the enzymatic reaction took place using radio-HPLC methods, though alternative HPLC gradients were needed for ACPPs to separate out the highly charged species (gradient: water with 0.1% TFA and 10-40% acetonitrile with 0.1% TFA over 20 minutes). The incubation of ^{68}Ga -DOTA-

(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (**52**) with MMP-2 over 2 hours resulted in an average of 61% conversion to hydrolysis products as shown in Figure 69.

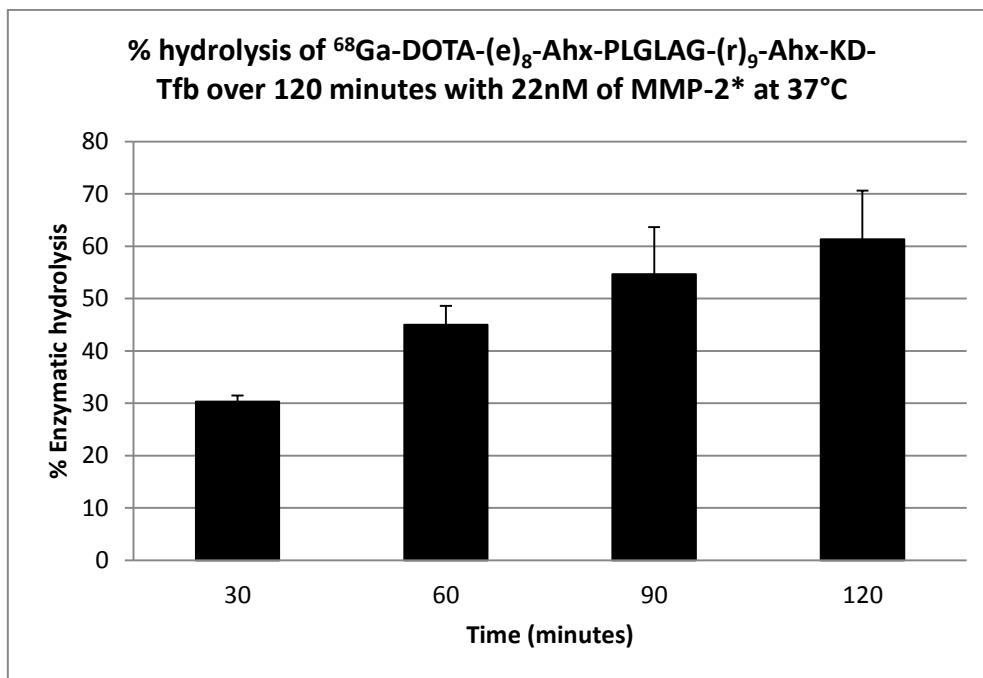


Figure 69- Graph showing the enzymatic hydrolysis of **52** during a 120 minute incubation with MMP-2* at 37 °C (n=3, error bars show standard deviation).

This peptide shows substantial conversion to enzymatic product over two hours, but this is slightly less than would be expected for this peptide substrate from literature comparison.^{37, 77} This could simply be an artefact of slightly differing assay methods, enzyme concentrations or MMP-2 used. Nevertheless, this compound shows good MMP-2 activity as we expected for this broad spectrum substrate. As anticipated, analysis of selectivity revealed **50** also had high MMP-9 activity as can be seen in Figure 70.

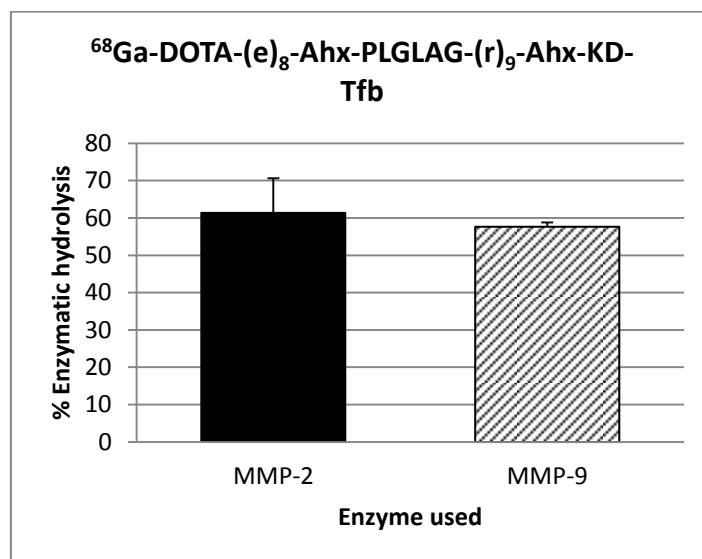


Figure 70- Variation of the percentage of enzymatic hydrolysis of **52** over a 120 minute incubation with MMP-2 or MMP-9 at 37 °C (n=3, error bars show standard deviation).

This shows that over a two hour period, almost equivalent conversion takes place with MMP-9 (average of 58%) demonstrating the broad spectrum nature of this substrate. The lack of selectivity was expected for this peptide and had been previously shown for similar compounds (which contain the same PLGLAG substrate).^{37, 77} While MMP14 analysis did not take place, this peptide substrate has previously shown activity towards this enzyme in other studies of imaging probes incorporating this substrate.⁷⁷

⁶⁸Ga-DOTA-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-D-Tfb (**53**) incorporated the MMP-2 selective substrate previously chosen from Chen *et al* for 1st generation selective MMP-2 imaging probes. As expected, activity with MMP-2 was lower than that of the broad spectrum substrate compound **52**. These results can be seen in Figure 71.

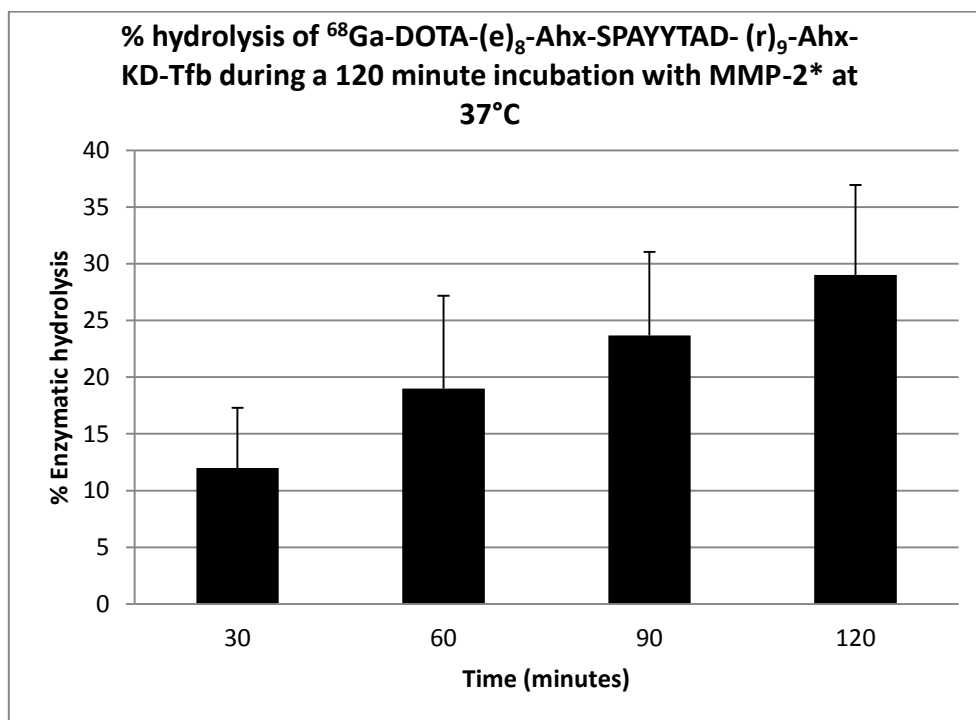


Figure 71- Graph showing the enzymatic hydrolysis of compound **53** during a 120 minute incubation with MMP-2* at 37 °C (n=3, error bars show standard deviation).

^{68}Ga -DOTA-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-D-Tfb (**53**) demonstrated an average of 29% conversion over 2 hours with MMP-2 at 37 °C. This was consistent with results from compounds **25** and **26** (1st generation probes), indicating that incorporation into this extended peptide system had not affected reactivity with MMP-2. Selectivity of the compound was then tested against MMP-9 (Figure 72).

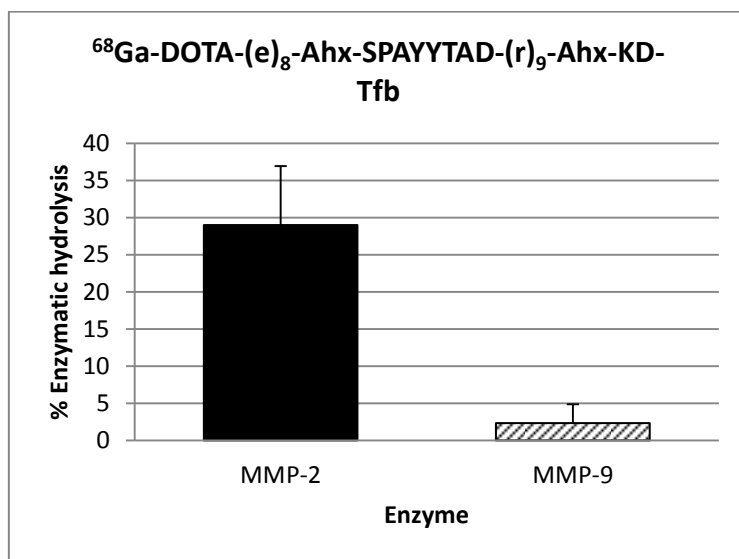


Figure 72- Variation of the percentage of enzymatic hydrolysis of **53** over a 120 minute incubation with MMP-2 or MMP-9 at 37 °C (n=3, error bars show standard deviation).

Only small amounts of peptide conversion were seen with MMP-9 (average of 2%). This was comparable to the observations with the 1st generation imaging probes, indicating the retention of substrate selectivity despite incorporation into this large ACP system. This is a positive result indicating that large ACP constructs can be combined with selective substrates. This compound should enable MMP-2 selective cellular uptake of the CF₃ containing group allowing for localisation and build-up of this species. MMP-14 assay was not carried out, but no significant enzymatic activity would be expected as shown with compound **25**. This result demonstrated the potential of selective peptide substrates to be incorporated into ACPs.

Both ACP substrates demonstrated activity on incubation with MMP-2 with $^{68}\text{Ga-DOTA-(e)}_8\text{-Ahx-SPAYYTAD-(r)}_9\text{-Ahx-K-D-Tfb}$ providing selectivity over MMP-9. Additionally, both compounds showed a change in T₁ and T₂ on complexation but this was not as significant as the effect seen with 1st generation probes **1** and **2**. However, while the paramagnetic effect of gadolinium on the fluorine in these peptide compounds was not as significant as initially expected, the real potential in these

probes lies in the combined effect of activated imaging and cell penetration. The cell penetrating potential of these compounds has been described previously in literature but would need to be confirmed for both peptide substrates with further experiments. While a preliminary experiment was carried out, cell penetration was not observed. This was likely due to issues of detection arising from the use of ^{19}F NMR. It was apparent this needed further optimisation via a fluorinated cell penetrating control compound; this would ideally be done with the pre-synthesised CPP component of compounds **50** and **51**.

Nevertheless, these ACPD probes could have the potential to provide local build-up of fluorine, eliminating the issue of migration of probes around the biological system after activation, along with increased retention times and complete separation from cleaved gadolinium. These factors would mark a vast improvement on 1st generation probes. In the meantime substrates could be adapted to move DOTA ligand and fluorine containing group closer in proximity.

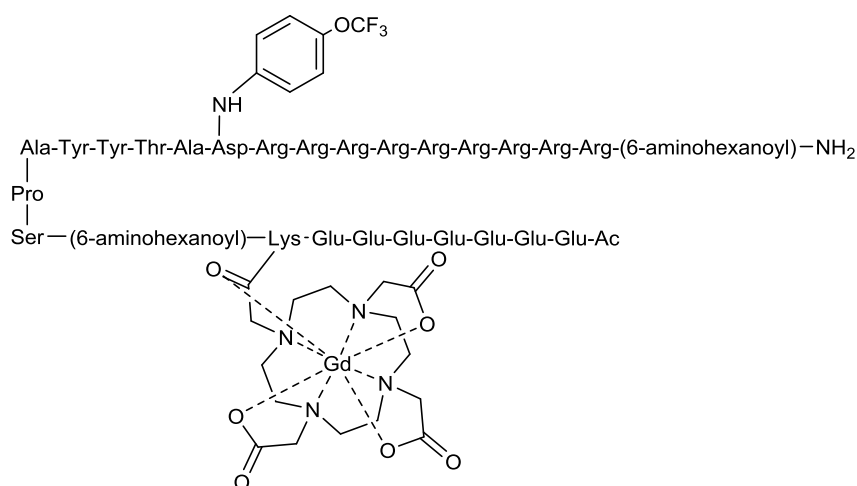


Figure 73- Proposed change to ACPD compound design to increase paramagnetic relaxation between the Gd-DOTA and the CF_3 group.

This would help to increase the T_2 relaxation of the CF_3 group in the intact complex and attenuate any ^{19}F MR background signal. Additionally while the reversed peptide

ligand Ac-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-DOTA (**49**) was not synthesised successfully, probes such as this have the potential to be combined with activated MRI designs into multimodal imaging agents around nanoparticles cores.

3.3 *Conclusions and future work*

In order to develop compounds **1** and **2** into viable clinical imaging probes, two areas were investigated: increasing the reactivity of probes with MMP-2, which was carried out via the incorporation of spacer groups, and increasing the sensitivity of the probes, which was carried out by employing a cell penetrating peptide to allow build-up of local fluorine concentration. It was hoped this second method would also improve other design issues with **1** and **2**, in that it would prevent movement of extracellular probes away from point of activation (MMP-2 activity). Additionally, the cell penetration component is likely to increase compound retention times *in vivo*.

Synthesis of a group of spacer group peptides for both **13** and **14** was carried out successfully, peptide compounds (**29-34**) could be complexed to gadolinium(III) or labelled with ⁶⁸Ga using methods described in Chapter 2. Gadolinium analogues (**35-40**) demonstrated similar relaxation times to **1** and **2**, showing the paramagnetic effects were not disrupted by introduction of spacer groups. However, MMP-2 assays carried out on ⁶⁸Ga analogues (**41-46**) showed a general decrease in reactivity contrary to expectations. This was hypothesised to be a result of increased size of the compounds and a lack of secondary structure. Testing the selectivity of compound **41** (DOTA-Ahx-SPAYYTAD-Tfb), the only peptide to demonstrate similar reactivity to **25** and **26**, revealed a loss of selectivity resulting from the introduction of this spacer group. It was thought that the spacer group was causing a change in the compounds mode of

recognition by altering previously specific interactions. Ultimately, none of the spacer group peptides (**41-46**) offered any potential improvement as ^{19}F imaging probes and alternate modes of compound enhancement were investigated.

Activated cell penetrating peptides ligands **47** and **48** were synthesised successfully and could be easily complexed to gadolinium(III) (**50** and **51**) or labelled with ^{68}Ga (**52** and **53**). While it was hoped that the assumed secondary 'hairpin' structure of ACPs would accommodate strong paramagnetic relaxation effects on complexation of gadolinium(III), T_1 and T_2 studies on compounds **47**, **48**, **50** and **51** showed a decreased effect compared with **1,2** and spacer group peptides (**35-40**). This was likely a result of the increased length of the peptides and distance between the gadolinium-DOTA and the OCF_3 group, which was not recompensed by through space interactions. This could potentially result in more difficulties observing signal change and would give reduced signal to noise ratios. Nevertheless, ^{19}F NMR spectroscopic analysis still showed a visual change in ^{19}F signal on complexation. MMP-2 and -9 assay investigations with ^{68}Ga analogues of both ACP compounds (**52** and **53**) demonstrated that both compounds showed activity with MMP-2 (**52** greater than **53** as expected) but that **53** showed superior selectivity over MMP-9. This was as expected with **52** derived from a broad spectrum peptide substrate. Unfortunately, cell penetration experiments could not be repeated or optimised due to time constraints, but ACP **50** and **51**'s potential for this has been validated by a number of other groups.^{37, 54, 77, 95} These results highlight a valuable route for vast improvements in compounds **1** and **2**, and warrant further in depth investigation *in vitro* and *in vivo*.

Future work which would need to be carried out in order to validate the prospects of **50** and **51** as ^{19}F MRI agents for activated MMP imaging, include: cell penetration experiments, MRS and MRI investigations, and further enzymatic experiments.

Looking further into the future, the successful synthesis of **49** could provide a MMP-2 selective ^{68}Ga labelled ACPP for PET experiments and possibly warrant the combination of techniques. Furthermore, the use of nanoparticle technology could accommodate the delivery of a large number of these ACPPs and have been shown to improve distribution in tumour environments, highlighting another prospective area of investigation.^{54, 95}

Chapter 4

Design and synthesis of an MMP-14 - activated imaging agent

4.1 *Introduction*

MMP-14 (also referred to as MT1-MMP) is another member of the MMP family which has been highlighted as a potential target for molecular imaging due to its strong association to a number of cancers (breast, cervical, colon, bladder, pancreatic etc.).^{5, 28, 96} It is a membrane bound protease meaning that in addition to the general MMP structure (of a conserved catalytic domain, propeptide, signal peptide and a hemopexin domain), it contains a C-terminal transmembrane domain (to anchor the enzyme on the cell surface) and a number of basic residues between the propeptide and catalytic domain (which can be cleaved by furin-like serine proteases) for intracellular activation.^{10, 17, 28}

MMP-14 is found on both tumour and stromal cells in a wide variety of cancers and has been considered as an early biomarker for invasive cancers.⁹⁷ One of the dominant roles MMP-14 is known to play in cancer is its activation of MMP-2; this is carried out on the cell surface by forming a trimolecular complex with TIMP-2 or through $\alpha_v\beta_3$ integrin promoted pathways.^{8, 28} As we have already discussed MMP-2 is then involved in a number of processes including ECM breakdown and release of growth factors. It is therefore unsurprising that increased expression of MMP-14 and MMP-2 has been linked to poor patient prognosis in a number of cancers (i.e. advanced

neuroblastoma, small cell lung cancer, ovarian cancer, bladder cancer, tongue and squamous cell carcinoma, head and neck carcinoma).²⁸ Its early expression before the downstream effects of MMP-2 activity offers the potential for an early diagnosis imaging agent. In addition to this, MMP-14 has been shown to have an individual role to play in the disease, not only in its contribution to breakdown of the ECM in tumour invasion and metastasis, but also in cell adhesion, cell signalling and angiogenesis.²² For example the α_v chain of $\alpha_v\beta_3$ integrin (known to be involved in tumour angiogenesis, invasion and metastasis) can be processed by MMP-14 into a functional form.²⁸ It is also thought that while many MMPs are involved in ECM breakdown MMP-14 is the predominant enzyme involved in cell migration through its activity towards cell adhesion molecules.^{22, 28, 96} Furthermore, it is interesting to note that studies have demonstrated that MMP-2 overexpression in the absence of MMP-14 doesn't promote tumour development in nude mice, whilst MMP-14 alone can induce tumour growth.²⁸ These roles of MMP-14 within cancer along with evidence of its prognostic potential make this protease an obvious choice of biomarker for both molecular imaging and inhibition and a good choice for testing the transferability of activated ^{19}F MRI probes described in Chapter 2.

MT1-MMP has a number of advantages over other MMPs (such as MMP-2/-9) as a target for molecular imaging; most significantly that it is membrane bound thus ensuring activation occurs in or around the tumour site. Release of soluble MMPs into the vasculature (such as MMP-2 and 9) has in some imaging examples been thought to have caused signals away from tumour environment resulting in a high background signal. The imaging of membrane bound rather than soluble proteases may circumvent this issue and enable better targeting of tumour and surrounding tissues.⁹⁷

As with MMP-2 features which impart specific substrate recognition are not fully understood for MMP-14. Like other members of the MMP family main points of recognition within the catalytic cleft focus on the zinc ion and associated glutamic acid group, as well as the S_{1'} subsite. However, as with MMP-2 alternative subsite interactions have also been investigated and give insight into specific substrate recognition. Kridel *et al* conducted a study investigating specific substrates for MMP-14; this revealed that an arginine residue at P₄ had a favourable interaction with an S₄ subsite on the enzyme.¹⁷ It is thought that this S₄ subsite in MMP-14 (not utilised for recognition by many MMPs) favours long side-chains capable of hydrophobic interactions. Although unlikely to be the only mode of selective recognition for the MMP-14 catalytic cleft, it was shown to have a substantial effect through studies which demonstrated loss of MMP-14 activity on replacing P₄ arginine with alanine. Another important subsite interaction highlighted in this study came from investigation of the S₃ subsite and the effect of proline in this position, which only occurred in non-selective substrates. Substitution of proline into previously selective peptides at the P₃ position resulted in an increase in MMP-9 activity and a loss of selectivity. On further investigation it was determined from molecular modelling studies that this proline group caused a change in the shape of the peptide from a more linear to a pronounced kinked conformation. This change in shape appeared to change the mode of recognition, once proline was introduced at P₃, arginine was no longer interacting with the enzyme at P₄, proline was now involved with interactions at this point of the enzyme. A number of the selective MMP-14 substrates highlighted in this paper by Kridel *et al* could be incorporated into a selective imaging agent; the information on substrate recognition is also valuable in the design and development of these probes.¹⁷

There are only few examples of MMP-14 specific imaging probes described in literature. This includes the multimodal (MR and optical) probe reported by Park *et al* which demonstrated activated fluorescence on contact with MMP-14 and cellular uptake of an iron oxide MRI active nanoparticle (see Chapter 1 for more details).⁵⁵ One of the earliest attempts at MMP-14 specific imaging described by Watkins *et al* comes from a SPECT imaging probe based on MMP-14 activated cell penetration similar to the design introduced by Tsien *et al*.^{37, 97} This probe was designed using a specific MMP-14 peptide substrate chosen from a study by Kridel *et al*, charged residues (polyanionic or polycationic) were incorporated on either side of the peptide and ^{99m}Tc and ligand was attached to the polycationic (cell penetrating) residue (Figure 74).¹⁷

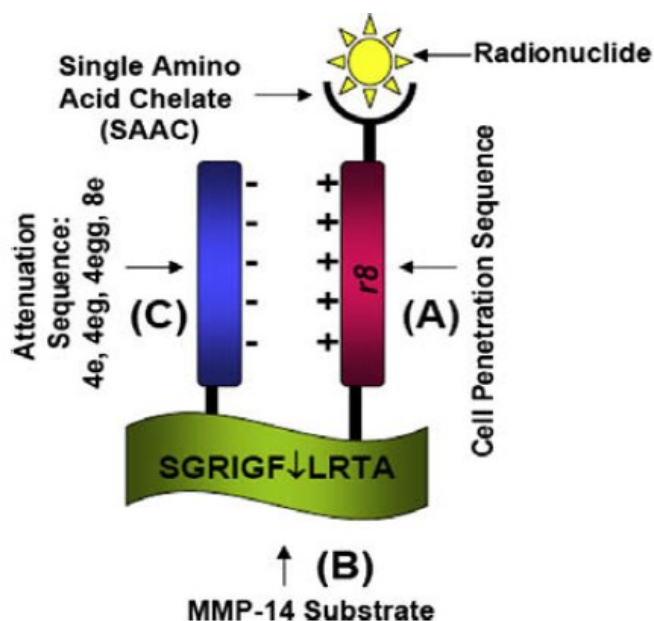


Figure 74- Activated cell penetrating MMP-14 SPECT probe designed by Watkins *et al*. Figure taken from reference.⁹⁷

These probes (particularly the 4eg version) showed good cellular uptake on cells transfected with MMP-14, with subsequent MMP inhibition resulting in decreased uptake, indicating compound specificity. However, non-specific cellular uptake on

non-transfected ‘negative control’ cells also took place in *in vitro* studies indicating a level of development is still needed for these activated cell penetrating probes to tailor them to MMP-14.

A more recent example of MMP-14 imaging includes an optical probe developed by Zhu *et al*; based around a peptide substrate (GRIGFLRTAKGG) selected for MMP-14 reactivity and selectivity, this was functionalised at the C-terminus by NIR dye (Cy5.5) and at the N-terminus with a NIR quencher group (BHQ-3).⁹⁸ This probe exhibited an increase in fluorescence *in vitro* on incubation with MMP-14 which could be translated into *in vivo* imaging of MMP-14 expressing tumours over 24 hours.

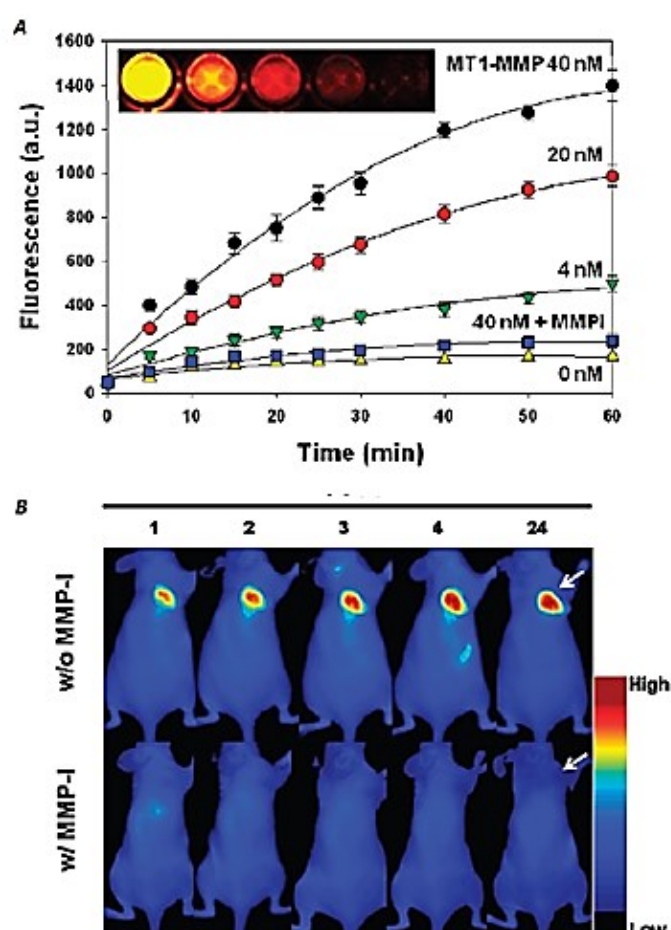


Figure 75- A: *in vitro* fluorescence on incubation with varying concentrations of MMP-14 (with or without MMPI) over 60 minutes. B: *in vivo* fluorescence on mice models with MMP-14 expressing tumours (MDA-MB-435) over 24 hours. Figure taken from Zhu *et al*.⁹⁸

Imaging demonstrated activation of probes within 1 hour and high fluorescent signals in the tumour were retained for up to 24 hours. Biodistribution experiments showed high tumour fluorescence (activation) and appear to confirm specific MMP-14 activation. However, while selectivity of the peptide substrate had been previously tested against soluble MMPs (i.e. MMP-2 and MMP-9) screening against other membrane bound enzymes had not taken place. Testing against MT2-MMP and MT3-MMP revealed similar breakdown with all three membrane type enzymes, but also demonstrated retention of selectivity over MMP-2 and MMP-9 on functionalization.

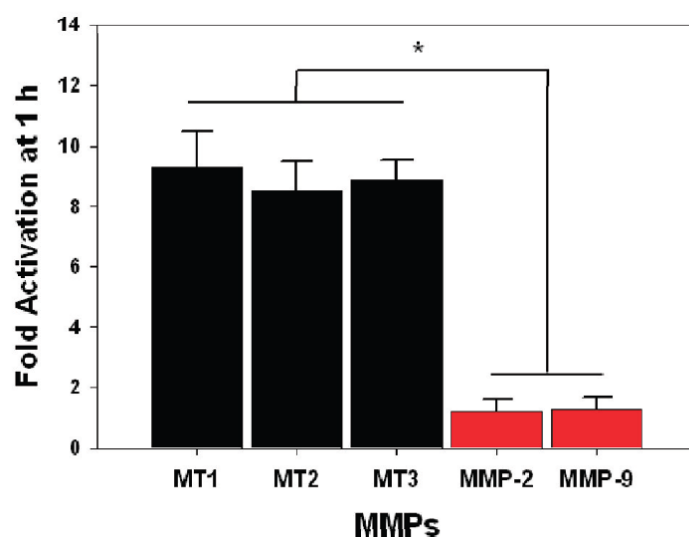


Figure 76- Taken from Zhu *et al*, showing activation of MMP-14 selective imaging probe on incubation with membrane bound and soluble MMPs for 60 minutes at 37 °C.⁹⁸

The compound described by Zhu *et al* represents a novel probe targeted solely at membrane-bound proteases which demonstrated good *in vivo* characteristics and allowed for optical imaging of MT1-MMP tumours in mice. However, the use of the optical imaging modality in this probe does not allow for easy translation into a clinical environment where such imaging agents could be very useful tools. Additionally, lack of selectivity between MT-MMPs using this compound could be

problematic, with the possibility of unspecific enzymatic reaction occurring *in vivo*, akin to that described for some MMP-2 /-9 selective probes.

Imaging of membrane bound MMPs had been less widely investigated than extracellular soluble proteases, despite the advantages these enzymes could impart as both imaging agents and targets for MMPIs. Of the few examples of MMP-14 imaging, none have been developed into a clinically useful imaging probe, analogous to the wider MMP family (suffering from similar issues i.e. non clinical modalities and possible broad spectrum character), leaving room for development in this area.

4.2 *Results and discussion*

4.2.1 Aims and objectives

The aim of this work was to design an MMP-14 activatable ^{19}F MRI probe based on in the work described in Chapter 2. This would test the feasibility of transfer of these imaging probes across to different MMP substrates and assess the difficulty of doing so. The following specific objectives were outlined:

1. Synthesise a MMP-14 specific compound based on compounds **1** and **2**, starting with a substrate with high MMP-14 reactivity and good specificity.
2. ^{68}Ga labelling of peptide ligands to evaluate the reactivity and selectivity of the probe. This will then be used as a guide for peptide development, working towards a highly reactive and selective probe.
3. Once a highly reactive and selective Ga-based probe has been found, the gadolinium(III) analogue will be synthesised and used as a 1st generation ^{19}F

NMR probes for MMP14 activity. This probe could subsequently be developed for *in vivo* applications.

4.2.2 Design of MMP-14 activated imaging probe

Probe design was based on peptides described in Chapter 2 of this thesis, which showed potential for activated ^{19}F MR imaging of MMPs. This was aimed at testing the transferability of the basic concept of these MMP-activated ^{19}F MRI probes to specific substrates of alternative MMPs. The initial design which can be seen in Figure 77, incorporates an MMP-14 specific substrate RIGF↓LR into a compound which is functionalised at the C-terminus by an aspartic acid containing a CF_3 group, and at the N-terminus by a DOTA ligand.

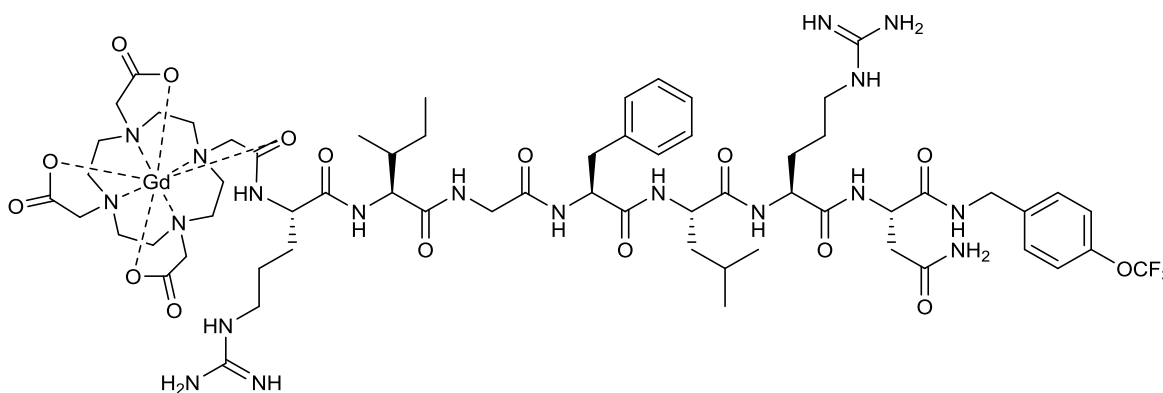


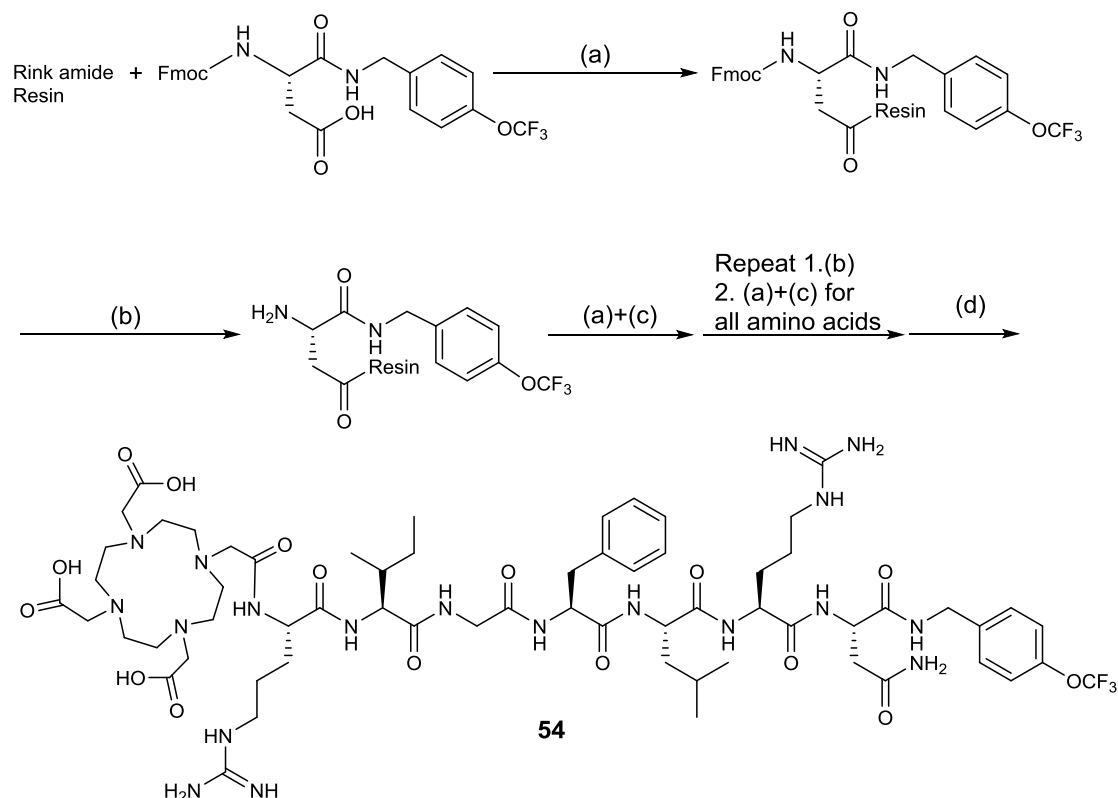
Figure 77- Design for an MMP-14 activate ^{19}F MRI probe

The peptide substrate for this initial design was chosen based on the work by Kridel *et al*; this small peptide sequence was part of a group of peptides which were identified using a phage substrate display as selective for MMP-14.¹⁷ This particular substrate showed very high reactivity with MMP-14 accompanied with good selectivity over MMP-2 and MMP-9. Furthermore, it was thought to display specific interactions with the enzyme other than the favourable S_4 interaction with arginine; studies investigating

the effects of alanine substitution at P₄ showed a much smaller decrease in reactivity than other peptides investigated, suggesting other MMP-14 promoting interactions along the peptide.¹⁷ It was hoped this high reactivity with MMP-14 would result in retention of activity on functionalization of the peptide substrate which has been problematic in other probes. Additionally this peptide substrate has been identified and utilised in other previously reported imaging probes.^{97, 98} It is important to note that this initial design was based on the core peptide substrate initially identified by Kridel *et al* as MMP-14 selective.¹⁷ Although they go on to expand this into a longer sequence with subsequent amino acids on both ends of the peptide chain, it was hoped this core structure would be enough for specific recognition. The work described in Chapter 3 on spacer group peptides suggested that the core recognition profile was most successfully functionalised without loss of selectivity and activity.

4.2.3 Compound synthesis and testing

Tri-protected DOTA Ligand (**8**) and Fmoc-Asp-[(trifluoromethoxy)benzylamide]-OH (**12**) were synthesised as described in Chapter 2. The peptide ligand could then be assembled using Fmoc solid phase peptide synthesis on a rink amide resin (Scheme 10).



(a) HBTU, DIPEA (b) DMF/Piperidine (c) Fmoc-Arg(Pfb)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Arg(Pfb)-OH, 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid (d) TFA, TIS, H₂O

Scheme 10 - Reaction scheme showing Fmoc solid phase peptide synthesis of DOTA-RIGFLRD-Tfb

Once cleaved from the resin peptide **54** could be purified by preparative HPLC, resulting in a yield of 17%. Synthesis of **54** was confirmed by mass spectrometry and a molecular ion peak at 1434 a.m.u. The ¹⁹F NMR spectrum of **54** showed a sharp peak at -58.9 ppm, comparable to both compound **12** and other peptides investigated in this report.

Before attempting complexation of ligand to gadolinium(III), **54** was tested for its MMP-14 reactivity and selectively by labelling the ligand with ⁶⁸Ga and carrying out appropriate radio-HPLC monitored assays, using the same protocol as described in Chapters 2 and 3. ⁶⁸Ga labelling (0.2 M NaOAc buffer, pH 5, 90 °C, 10 minutes) gave a decay corrected yield of 99±0.5% (n=3, as determined by radio-HPLC). Purification

of radiolabelled compound from any unreacted ^{68}Ga was carried out on the solid phase, simultaneously concentrating the compound in 200 μl of ethanol. Labelled compound (Figure 78) could then be diluted with assay buffer and undergo MMP-14, MMP-2 and MMP-9 assays.

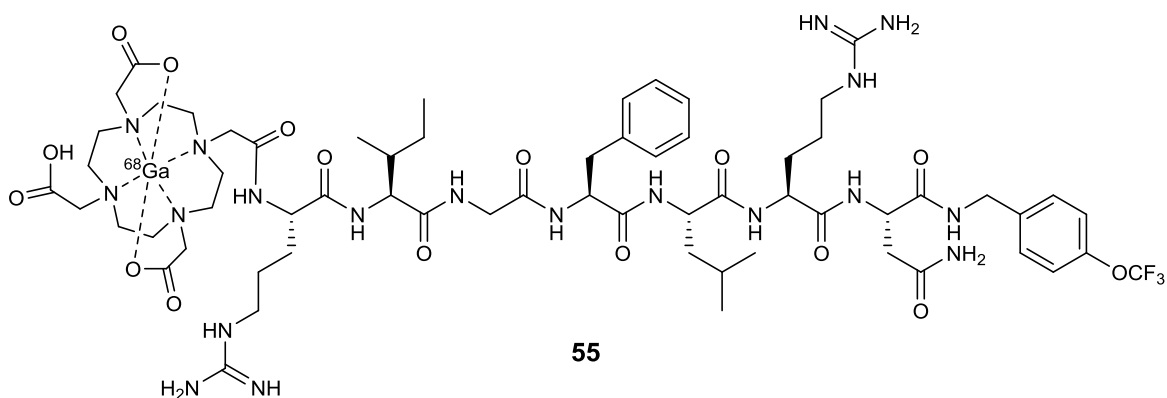


Figure 78- Gallium labelled ligand: ^{68}Ga -DOTA-RIGFLRD-Tfb

Assays were carried out as previously described incubating 22 nM of enzyme with the labelled peptide at 37 °C for 2 hours and monitoring peptide hydrolysis by radio-HPLC. Results from reaction of labelled compound **55** with MMP-14 are summarised in Figure 79.

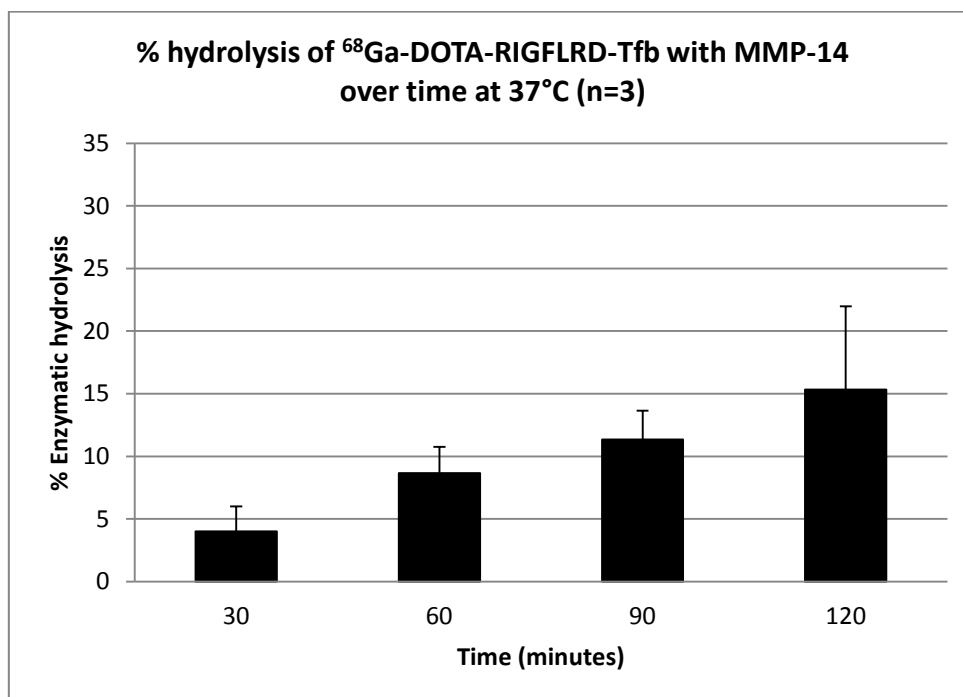


Figure 79- Graph showing the percentage hydrolysis of ^{68}Ga -DOTA-RIGFLRD-Tfb with MMP-14 at 30 minute time points over 120 minutes (n=3, error bars show standard deviation).

This shows that active MMP-14 was reacting with compound **55** but hydrolysis over two hours was only around 15%. This was lower than would be expected of this peptide from literature and was likely due to the functionalisation of the specific peptide substrate. Primarily it was thought that the P_4 interaction of arginine with the S_4 subsite (as discussed previously, an important interaction for specificity) has been partially blocked by addition of DOTA. This idea is supported by the data produced on compound selectivity against MMP-2 and MMP-9 (Figure 80).

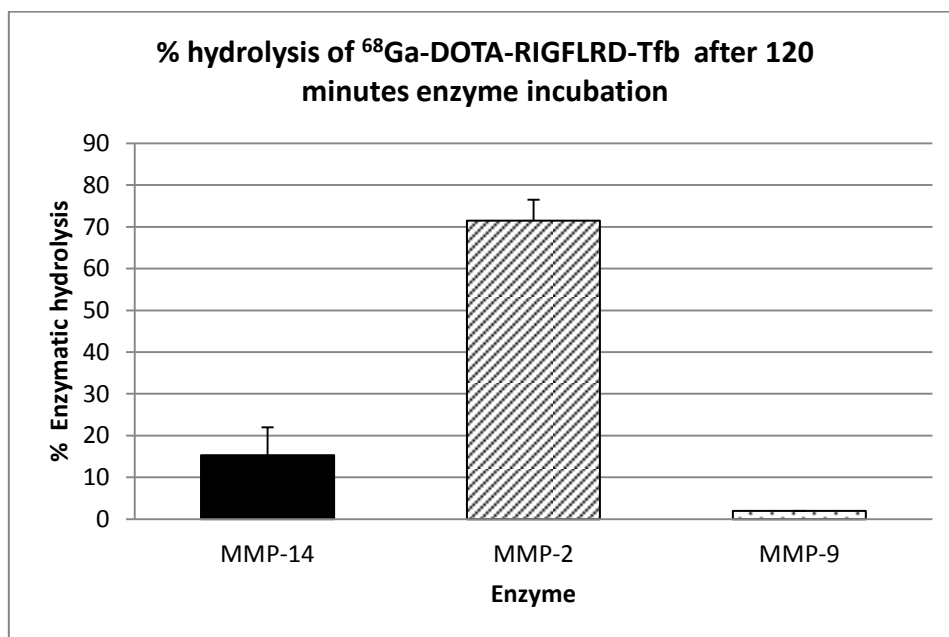
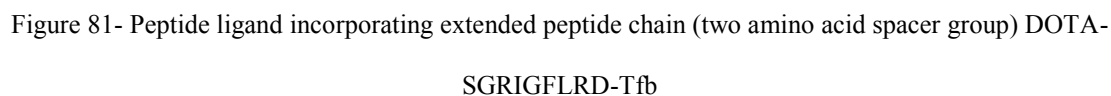


Figure 80- Graph showing the percentage hydrolysis of ^{68}Ga -DOTA-RIGFLRD-Tfb after 120 minutes incubation with MMP-14, -2 or -9 (n=3, error bars show standard deviation).

This data showed that incubation with MMP-2 resulted in greater hydrolysis than MMP-14 over 120 minutes incubation with equivalent amounts of activated enzyme. This is in direct contradiction to what was expected of this peptide substrate from literature on the parent peptide, which exhibited significant preference for MMP-14.¹⁷ Therefore, we can theorise that the arginine residue at P₄ (which is said to provide an important interaction in MMP-14 selectivity) is not contributing to the recognition of this compound. Rather interactions along the rest of the peptide chain must be taking precedence potentially altering the mode of recognition (as described by Kridel and co-workers) to a broad spectrum recognition conformation.¹⁷ It is interesting to note that compound **55** now demonstrates significant MMP-2 reactivity and selectivity which could deliver an alternative imaging construct to compounds **1** and **2** (as described in Chapter 2). However, on development of such probes the movement of DOTA away from arginine at P₄ would likely change reactivity and selectivity profile.



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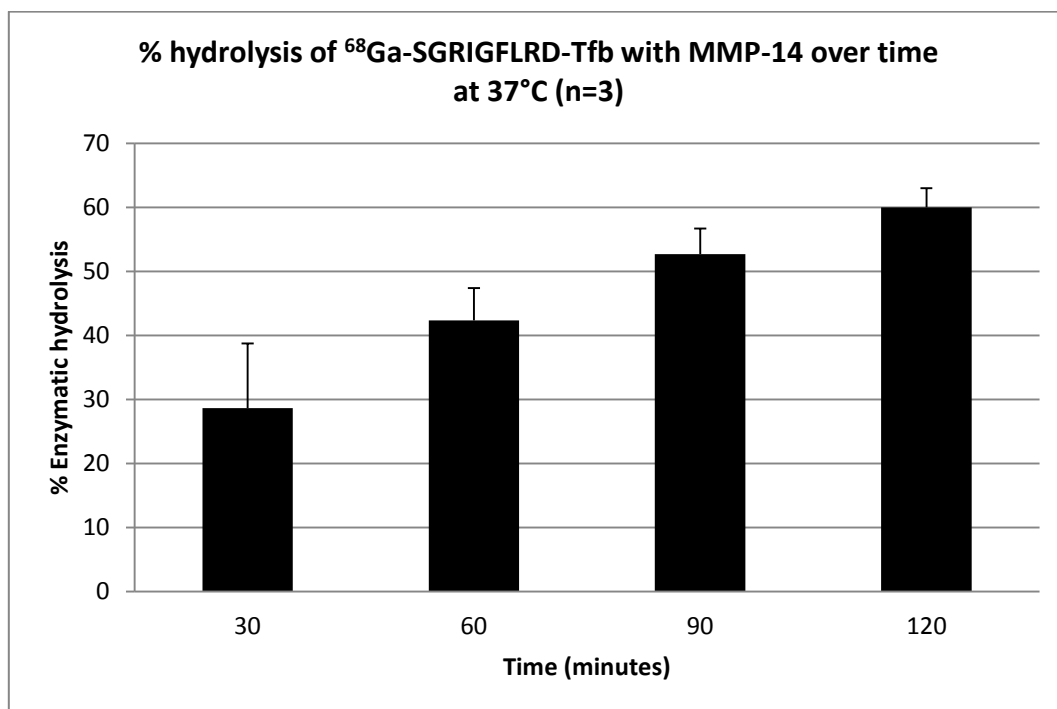


Figure 82- Graph showing the percentage hydrolysis of ^{68}Ga -DOTA-SGRIGFLRD-Tfb after 120 minutes incubation with MMP-14, -2 or -9 (n=3, error bars show standard deviation).

This shows greatly increased MMP-14 hydrolysis (fourfold) compared to compound **55**, with average hydrolysis over 120 minutes of 60%. This was significantly higher and closer to results described in literature, supporting the hypothesis that P₄ arginine interaction was blocked by DOTA and that introduction of a longer spacer between the two reinstated selective interactions. On investigating reaction of the labelled peptide compound with MMP-2 results were also different when compared to compound **55** (Figure 83).

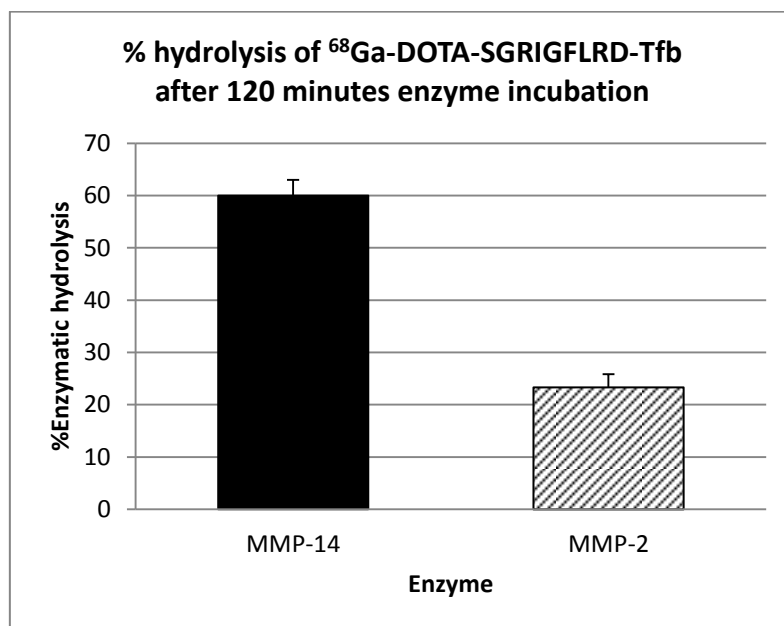


Figure 83 Graph showing the percentage hydrolysis of ^{68}Ga -DOTA-SGRIGFLRD-Tfb after 120 minutes incubation with MMP-14 or MMP-2 (n=3, error bars show standard deviation).

MMP-2 activity is still present but it has been reduced by more than half, showing greatly decreased enzyme recognition. This result is much closer to what we would expect from literature on this peptide substrate (in its unfunctionalised form) and supports the idea that in compound **55** proximity of DOTA ligand to the P₄ arginine was blocking specific S₄ subsite recognition. This change in selectivity adds weight to the hypothesis explored in Smith *et al* of alternate modes of substrate recognition for selective and broad spectrum peptides. The introduction of a small spacer group has in this example returned compound reactivity towards that of the unfunctionalised substrate demonstrating the detrimental effects large groups can have on specific peptides. Substrate recognition derives from many interactions along the peptide chain and this data supports the theory that small alterations can change the dominance of these interactions.

This improved peptide substrate (**57**) showed greatly increased MMP-14 activity but still incomplete selectivity against MMP-2 which would probably remain problematic

for *in vivo* interrogations. Unfortunately due to time constraints MMP-9 selectivity could not be tested and gadolinium analogues were not synthesised. The next step for this compound would be to complex it to gadolinium, analyse its effect on the ^{19}F NMR signal and carry out the MMP-14 and MMP-2 assays whilst monitoring the fluorine signal. Similar behaviour to the compounds described in Chapter 2 would be expected but this information would help give an idea on the usefulness of this compound and how to develop it further.

4.3 *Summary and future work*

The peptide ligand from the initial design for an MMP-14 activated fluorine MRI probe DOTA-RIGFLRD-Tfb (**54**) was synthesised with Fmoc solid phase peptide synthesis in a reasonably good yield. Labelling of this ligand with ^{68}Ga was carried out using previous methods highly successfully; however this probe (**55**) showed low MMP-14 reactivity and little selectivity over MMP-2 and -9. It was hypothesised that this was due to the proximity of the DOTA ligand to the P₄ arginine and thus compound DOTA-SGRIGFLRD-Tfb (**56**) was synthesised and labelled with ^{68}Ga to test this hypothesis. This radiolabelled compound (**57**) demonstrated significantly better MMP-14 activity and improved selectivity (with decreased MMP-2 activity); although not completely selective this was now in agreement with literature data for unfunctionalised peptide substrate. These results suggested that P₄ had been an absent interaction in the initial peptide compound, but had been restored on inclusion of two amino acid spacer groups. This observation supported the experimental and molecular modelling results from Kridel *et al*, which suggested there could be alternative modes of recognition depending on P₄ interaction.¹⁷

Ligand DOTA-SGRIGFLRD-Tfb showed potential as an imaging agent with large hydrolysis on incubation with MMP-14, however due to time constraints gadolinium analogues could not be synthesised or analysed. Future work would involve the gadolinium complexation of this ligand (Figure 84) and analysis of its potential as a fluorine MRI agent.

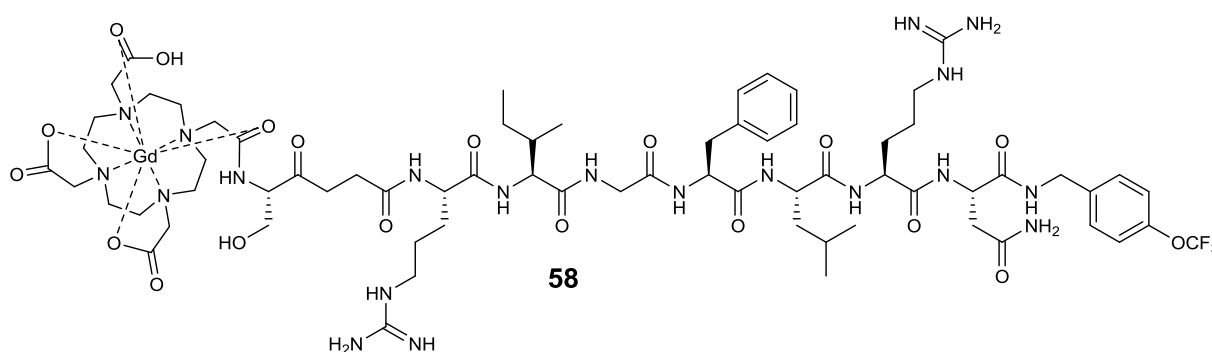


Figure 84- Gadolinium (II) complex of ligand DOTA-SGRIGFLRD-Tfb

This could then lead on to development of this peptide (**58**), looking at incorporating an activating cell penetrating component as described in Chapter 3.

In its current form compound **58** does not offer sufficient selectivity to be MMP-14 specific and further work would need to be conducted, investigating and developing this. It is important to note the peptide substrate chosen for compounds **54** and **56** was not the most selective substrate highlighted by Kridel and co-workers. Alternative substrates could provide a system with improved selectivity but compound reactivity would be likely to suffer; ultimately a balance will have to be found between reactivity and selectivity.

The results described in this chapter demonstrate to the ability to synthesise similar compounds to those described in Chapter 2, targeted to different MMPs. Further development of these could lead to similar 1st generation MMP-14 activated ¹⁹F imaging probes. It also highlights the importance of understanding the unique

recognition interactions of each catalytic cleft and incorporating these into compound design and choice of peptide substrate. Indicating that while the design of these enzymatically activated agents can be easily transferred between different peptide substrates, in order to retain specificity (rather than create another broad spectrum imaging agent) each compound will require a level of unique design considerations.

Chapter 5

Experimental

5.1 General considerations

Reaction conditions: Reagents were bought from commercial sources and were used as received unless stated otherwise. Where anhydrous conditions were used, reactions were carried out using Schlenk line techniques under a nitrogen atmosphere.

Solvents: Milli-Q water was used for HPLC and biological applications. HPLC-grade methanol was bought from VWR. HPLC-grade acetonitrile and FA, along with deuterated solvents for use in NMR spectroscopy were bought from Sigma-Aldrich. Acetonitrile was purchased from Sigma Aldrich and was dried over potassium carbonate (5% w/v, dried in oven) for 24 hours, which was followed by stirring over 3Å molecular sieve powder for 24 hours (5% w/v, activated by heating under vacuum) under nitrogen. Alternatively anhydrous acetonitrile was purchased from Sigma Aldrich and used as received under nitrogen. Anhydrous DMF was purchased from Sigma Aldrich and used as received under nitrogen. All other solvents were purchased from Sigma Aldrich and used without further purification unless otherwise stated.

¹H NMR spectra: Were recorded at 400 MHz on a Bruker AV 400 instrument at 298K. Chemical shifts (δ_{H}) are given in parts per million (ppm) and referenced to any residual solvent peaks (¹H: CDCl₃ δ 7.26; CD₃OD δ 3.34; D₆-DMSO δ 2.54), coupling constants are in Hz.

^{19}F NMR spectra, T_1 and T_2 values: Recorded on Bruker DRX-400 MHz, at 400MHz and 298K, T_1 and T_2 experiments carried out by Pete Haycock. Samples dissolved in DMSO (1mM) for relaxation time measurements. T_1 measurements were carried out using T_1 weighted inversion recovery (tlir), T_2 measurements were carried out using Carr Purcell Meiboom Gill (CPMG) pulse sequence. For comparison studies samples were dissolved in water/ D_2O or DMSO- d_6 and TFA was used as a standard. Chemical shift are reported relative to trichlorofluoromethane.

Mass Spectra (m/z): MALDI analysis was carried out on a Micromass MALDI-ToF using a 337 nm nitrogen laser, Operates in reflectron (under 7KDa) or linear (over 7KDa) modes. ESI and Elemental composition was carried out with ES-ToF, Waters LCT Premier/Acquity uPLC. Capillary Voltage: 2000, Cone Voltage: 30, Desolvation Temp: 350 degrees, Source Temp 120 degrees, desolvation gas 400 L/hr, Cone gas 10 L/hr. Only major peaks reported, all gadolinium complexes reported for ^{158}Gd .

Reversed phase HPLC: Analysis and purification of peptides was carried out using a Waters HPLC system comprised of a 600 pump and 2487 UV detector using a Luna 5u C18 column; 5 μM (particle size); 100Å (pore diameter); 30 mm inner diameter and 75 mm length. This system was also used for fluorescent HPLC with the addition of a Waters 470 scanning fluorescence detector, using a Waters u Bondapak; C18; 10 μM (particle size); 125Å (pore diameter); 7.8 mm inner diameter and 300 mm length column. Analysis of biological assays and semi-prep could also be carried out on Agilent technologies 1200 series system which comprised a G1379B vacuum degasser, and a G1312A Bin pump. The column used was a Waters u Bondapak; C18; 10 μM (particle size); 125Å (pore diameter); 7.8 mm inner diameter and 300 mm length. For radiochemical studies analysis was carried out on HP1100 series system comprising of a G1322A degasser, GA1312A bin pump, G1514A UV detector and a Flowcount BFC

200 radiodetector. The column used was a Gemini 5u; C18; 5 μ M; 110A (pore diameter); 4.6 mm inner diameter and 150 mm length.

LCMS: Analysis and purification were carried out on a Waters HPLC system using a Waters 2767 autosampler for samples injection and collection, a Waters 515 HPLC pump for delivery of the mobile phase to the source, a Waters 3100 mass spectrometer with ESI and Waters 2998 Photodiode Array (detection at 200-600 nm). The columns used were XBridge C18 columns (Waters, 4.6 mm D $\times$ 100 mm L analytical, 19 mm D $\times$ 100 mmL preparative). Both preparative and analytical analysis using 15 or 18 minute run times in water with 5-98%, 30-98% methanol or 50-98% or methanol.

MRS: Scanning was carried out by Dr Ioannis Lavdas at Hammersmith Hospital in the Medical Research Council (MRC) biological imaging Centre, 3rd floor cyclotron building. MRS was performed in a 4.7T horizontal bore Direct Drive Varian MRI system (Palo Alto, CA). A 20-mm diameter surface coil was used in transmit/receive (T/R) mode. Samples were in 200 μ l of methanol (1mM or 2mM) in 250 μ l eppendorf tubes and placed in the middle of the coil loop. Pulse-and-acquire experiment: $TR=800$ ms, receiver bandwidth= 5 kHz, $N_A=50$ or 512 ($T_A=40$ sec and 6 min respectively).

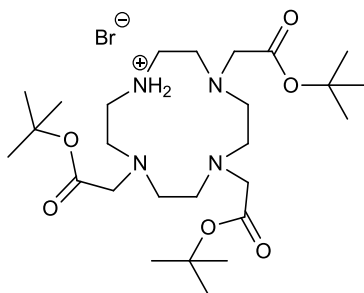
Fluorescent plate readers: Fluorescamine studies were carried out on a Tecan Infinite 200 series, multiple wavelength fluorescent plate reader. Positive control (compound **17**) investigations were carried out on a Perkin Elmer Victor ³ plate reader fitted with filters for optical excitation 340/70 and emission 405/10.

Gallium generator: ⁶⁸Ga was synthesized using a desktop generator supplied by Ekhert & Ziegler containing a ⁶⁸Ge source. Elution of ⁶⁸Ga was carried out using 0.1 M HCL producing activity in approximately 2 ml.

5.2 *Synthetic procedures*

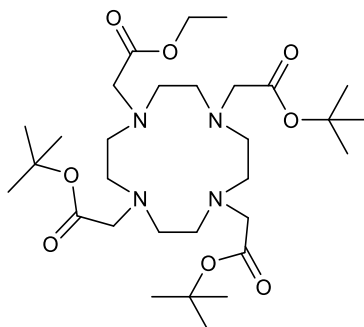
5.2.1 Ligand synthesis

Preparation of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane hydrobromide (**5**)



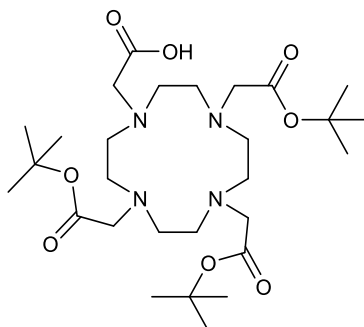
This compound was prepared by small modifications of a previously reported procedure.⁸¹ 1,4,7,10-tetraazacyclododecane (1 g, 5.80 mmol, 1.0 eq) and NaHCO₃ (2.44 g, 29.0 mmol, 5.0 eq) were stirred in CH₃CN (40 ml) at 0°C under N₂. *Tert*-butylbromoacetate (2.6 mL, 17.4 mmol, 3.0 eq) was added dropwise over a time period of 30min at 0°C. The reaction mixture was allowed to reach RT and stirred under N₂ for 24 hrs. Inorganic solids were removed by filtration and the filtrate was evaporated under reduced pressure leaving a cream solid residue. Recrystallization from toluene afforded 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane hydrobromide salt as a white solid (1.57 g, 2.64 mmol, yield 48 %). ¹H NMR (CDCl₃,): δ 1.47 ppm (s, 27H, OC(CH₃)₃), 2.85-3.02 ppm (m, 12H, NCH₂CH₂N), 3.09-3.18 ppm (m, 4H, H₂N⁺CH₂CH₂N), 3.32 ppm (s, 2H, NCH₂COOH), 3.42 ppm (s, 4H, NCH₂COOH); MS (ESI⁺) m/z: 515.4 [M+H]⁺; MP: 180-181°C (lit 178-180°C).

Preparation of 1-(ethoxycarbonylmethyl)-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (7)



This compound was prepared by small modifications of a previously reported procedure:^{79, 80} tris(*tert*butoxy carbonyl methyl)-1,4,7,10-tetraazacyclododecane hydrobromide salt (1.49 g, 2.50 mmol, 1.0 eq) and K₂CO₃ (690 mg, 5.0 mmol, 2.0 eq) was dissolved in anhydrous CH₃CN (40 mL) under N₂, the mixture heated to 80°C for 30 min before ethyl bromoacetate (0.27 ml, 2.5 mmol, 1.0 eq) was added and the mixture was refluxed for 24h under N₂. After this time TLC analysis of the reaction mixture appeared to show only one visible product under UV. After removing any excess of K₂CO₃ by filtration, acetonitrile was evaporated under reduced pressure. The resulting dark- yellow oil was analysed via TLC now showing 3 compounds on the TLC plate, the oil purified by flash column chromatography on silica gel (dichloromethane:methanol 95:4) to give 1-(ethylacetate)-4,7,10-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane as a light yellow solid (1.11g, 1.85 mmol, 74% yield). ¹H NMR (CDCl₃): δ 1.28 ppm (t, 3H, ³J=7 Hz, ROCH₂CH₃), 1.46 ppm (s, 27H, OC(CH₃)₃), 2.05-3.60 ppm (broad m, 24H, RCH₂N), 4.18 ppm (broad s, 2H, OCH₂CH₃); MS (ESI⁺) m/z: 601.4 [M+H]⁺, 623.4 [M+Na]⁺; MP: Decomposition at 139-140°C (lit decomposition ≥140°C).

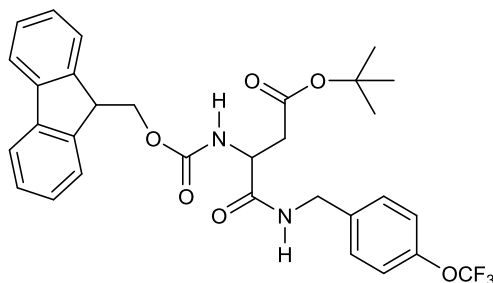
Preparation of 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid (8)



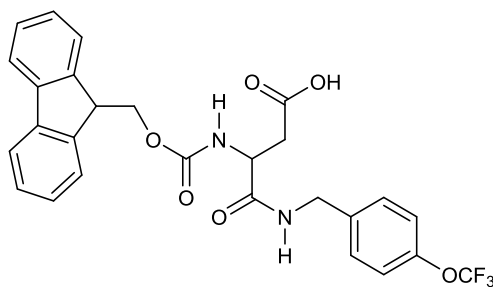
This compound was prepared by small modifications of a previously reported procedure.^{79, 80} 1-(Ethyl acetate)-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (917 mg, 1.52 mmols, 1.0 eq) was dissolved in (15 mL) of dioxane and (5 ml) 0.4M NaOH solution (ratio of 3:1 (v:v)). This solution was stirred for 6h under N₂ at 50°C. Dioxane was then removed under vacuum and water (20 mL) was added. The mixture was extracted with CH₂Cl₂ (3 × 30 mL). Organic phases were combined and further washed with water and brine. The organic solution was dried using Na₂SO₄ and the solvent was removed to afford a very pale yellow/cream solid (695 mg, 1.22 mmol, yield 80%). ¹H NMR (CDCl₃): δ 1.47 ppm (s, 27H, OC(CH₃)₃), 2.0-3.70 ppm (broad m, 24H, RCH₂N); MS (ESI⁺) m/z: 573 [M+H]⁺, 595 [M+Na]⁺; HRMS (elemental composition) [M+Na]⁺: Mass calculated for C₂₈H₅₂N₄O₈Na 595.3683, found 595.3689.

5.3 *Functionalised Amino acid synthesis*

Fmoc-Asp[(trifluoromethoxy)benzylamide]-OtBu (11)



This compound was prepared by modification of a previously reported procedure:⁶⁷ Fmoc-Asp-OtBu-OH (430 mg, 1.0 mmol, 1.0 eq.), HBTU (379 mg, 1.0 mmol, 1.0 eq) and DIPEA (0.26 ml, 1.26 mmol, 1.2 eq) were dissolved in dry DMF (20 mL) at 0 °C under N₂, then (trifluoromethoxy)benzylamine (200mg, 1.05 mmol, 1.0 eq.) was added at 0 °C. The mixture was stirred at 0 °C for 2 h under N₂, then warmed to RT and stirred for 1 h. The solvent was removed under vacuum, and the crude product was diluted with ethyl acetate and washed with 4% NaHCO₃ aq., 10% citric acid, and brine. The organic layer was dried over MgSO₄ and removed under vacuum to give crude Fmoc-L-Asp-[(trifluoromethoxy)benzylamide] as a beige solid (552 mg, 0.94 mmol, yield 90%). ¹H NMR (CDCl₃): δ 1.46 ppm (s, 9H, ROCCH₃), 2.60 ppm (dd, 1H, ³J=6 Hz, ²J=-17 Hz, R₃*CCH₂COOR), 3.01 ppm (dd, 1H, ³J=4 Hz, ²J=-17 Hz, R₃*CCH₂COOR), 4.21 ppm (t, 1H, ³J=6 Hz, R₂CHCH₂R), 4.46 ppm (m, 4H, CH₂), 4.58 ppm (broad s, 1H, *CHR₃), 5.96 ppm (broad d, 1H, ³J=8.0 Hz, NH), 6.81 ppm (broad s, 1H, NH), 7.17-7.79 ppm (m, 12H, aromatic protons); ¹⁹F NMR (CDCl₃): δ -59.5 ppm (s, ROCF₃); MS(ESI⁺)m/z: 585 [M+H]⁺, 607 [M+Na]⁺.

Fmoc-Asp[(trifluoromethoxy)benzylamide]-OH (12)

This compound was prepared by small modifications of a previously reported procedure.⁶⁷ Fmoc-Asp[*p*-(trifluoromethoxy)benzylamide]-*O**t*Bu **1** (552 mg, 0.94 mmol, 1.0 eq) was dissolved in TFA (15 mL) and stirred at RT for 2 h. TFA was then removed under vacuum to yield the crude product. Recrystallization from ethyl acetate and *n*-hexane (8:1) afforded compound **2** (289 mg, 0.55 mmol, yield 58%). ¹H NMR (DMSO): δ 2.55 ppm (dd, 2H, ³J= 9 Hz, ²J=−15 Hz, R₃*CCH₂COOR), 2.72 ppm (dd, 2H, ³J=5 Hz, ²J=−16 Hz, R₃*CCH₂COOR), 4.27 ppm (m, 5H, 2 CH₂, R₂CHCH₂R), 4.41 ppm (m, 1H, *CHR₃), 7.25-7.92 ppm (m, 12H, aromatic protons); ¹⁹F NMR (DMSO): δ -59.6 ppm (s, ROCF₃); MS (ESI⁺) *m/z*: 529.2 [M+H]⁺, 551.1 [M+Na]⁺; HRMS (elemental composition) [M+H]⁺: Mass calculated for C₂₇H₂₄N₂O₆F₃ 529.1586, found 529.1577.

5.4 Peptide synthesis

Depending on the length of the peptide substrate, functionalised peptide ligands could be prepared manually or using a solid phase peptide synthesiser. Both methods used standard Fmoc solid phase peptide synthesis techniques.

Standard protocol 1 – Manual Fmoc solid phase peptide synthesis

Rink amide resin (40 μ mol, 1.0 eq or 20 μ mol, 1.0 eq) was prepared for synthesis by being swollen with 2ml of DMF for 30 minutes before being rinsed with DMF/CH₂Cl₂/DMF. The resin was then Fmoc deprotected by shaking resin bound compound with a 2 ml mixture of DMF/piperidine (80:20) for 5 minutes carried out 3 times for each deprotection. The resin was then washed with DMF/CH₂Cl₂/DMF ready for the first coupling reaction. Coupling reactions were carried out using 3 separate protocols depending on the amino acid/compound:

Coupling A: Used for standard Fmoc protected amino acids. Amino acid (5.0 eq) and DIPEA (10 eq) in DMF were mixed together before adding HBTU coupling reagent (4.9 eq) in DMF for 5 minutes preactivation, this was then added to the resin for 30 minutes. This process was repeated (to ensure efficient couplings) with a DMF/CH₂Cl₂/DMF wash between and after couplings.

Coupling B: Used for unusual amino acids or other compounds which can be introduced into the peptide chain; Fmoc-Asp[(trifluoromethoxy) benzyl amide]-OH, 6-(Fmoc-amino)hexanoic acid and *N*-Fmoc-*N*'-succinyl-4,7,10-trioxa-1,13-tridecanediamine. Amino acid (2.5 eq) and DIPEA (5 eq) in DMF were mixed together before adding HATU coupling reagent (2.5 eq) in DMF for 5 minutes preactivation, this was then added with the resin for 30 minutes. Process was repeated (to ensure efficient couplings) with a DMF/CH₂Cl₂/DMF wash between and after couplings.

Coupling C: Used only for 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid (1.5 eq) which was mixed with DIPEA (3 eq) in DMF before addition of HATU (1.5 eq in DMF) for 5 minutes pre activation, this mixture was then added to the resin for 60 minutes. This process was repeated (to

ensure efficient couplings) with a DMF/CH₂Cl₂/DMF wash between and after couplings.

Between each amino acid coupling step Fmoc deprotection took place, as previously described. After all the amino acids/ compounds had been sequentially coupled together, the resin was washed with DMF/DCM/MeOH/diethylether and left to dry overnight under vacuum. Peptides were then cleaved from the resin by shaking with 95% TFA, 2.5% water and 2.5% triisopropylsilane for 3 hours. Cold diethyl ether (15 ml) was added to the TFA peptide mixture forming a solid peptide which was collected as a pellet via centrifugation, this pellet was washed with cold diethyl ether (15 ml) , after a second centrifugation solvent was drained from the pellet and it was left to dry under vacuum overnight. Peptide could then be dissolved ready for purification via prep HPLC on Waters HPLC with Luna C18 column.

Standard protocol 2 – Fmoc solid phase peptide synthesis on a peptide synthesiser

Peptide synthesiser: Intavis AG ResPep SL instrument

Amino acids were dissolved in *N*-methylpyrrolidone (NMP) and loaded on to the peptide synthesiser along with HBTU, HATU, *N*-methylemorpholine (NMM), Capping solution (5% acetic anhydride in DMF), DCM and DMF/piperidine (80/20). Rink amide resin was loaded into wells in a 20 µM scale. Two types of cycle were used for the synthesis:

Cycle A: Used for standard Fmoc amino acids and 6-(Fmoc-amino)hexanoic acid.

- Fmoc deprotection (initially of resin, then of each Fmoc-amino acid) with 20% piperidine in DMF (400 µL) for 10 min x 3
- Wash with DMF (400 µL) x 8

- Coupling of subsequent amino acid with HBTU in NMP (0.5 M, 170 μ L), NMM in NMP (4M, 52 μ L), NMP (5 μ L), Fmoc-amino acid in NMP (0.5 M, 175 μ L) for 35 min x 2
- Capping with 5% acetic anhydride in DMF (400 μ L) for 5 min
- Wash with DMF (600 μ L) x 2 and DMF (500 μ L) x 5

Cycle B: Used for D-amino acids and Fmoc-Asp[(trifluoromethoxy) benzyl amide]-OH.

- Fmoc deprotection (initially of resin, then of each Fmoc-amino acid) with 20% piperidine in DMF (400 μ L) for 10 min x 3
- Wash with DMF (400 μ L) x 8
- Coupling of subsequent amino acid with HATU in NMP (0.25 M, 170 μ L), NMM in NMP (4M, 52 μ L), NMP (5 μ L), Fmoc-amino acid in NMP (0.25 M, 175 μ L) for 40 min x 2
- Capping with 5% acetic anhydride in DMF (400 μ L) for 5 min
- Wash with DMF (600 μ L) x 2 and DMF (500 μ L) x 5

After all cycles were complete final Fmoc deprotection and washing takes place (as described in cycles A and B), peptides were then removed from the peptide synthesiser for manual steps and subsequent cleavage from the resin.

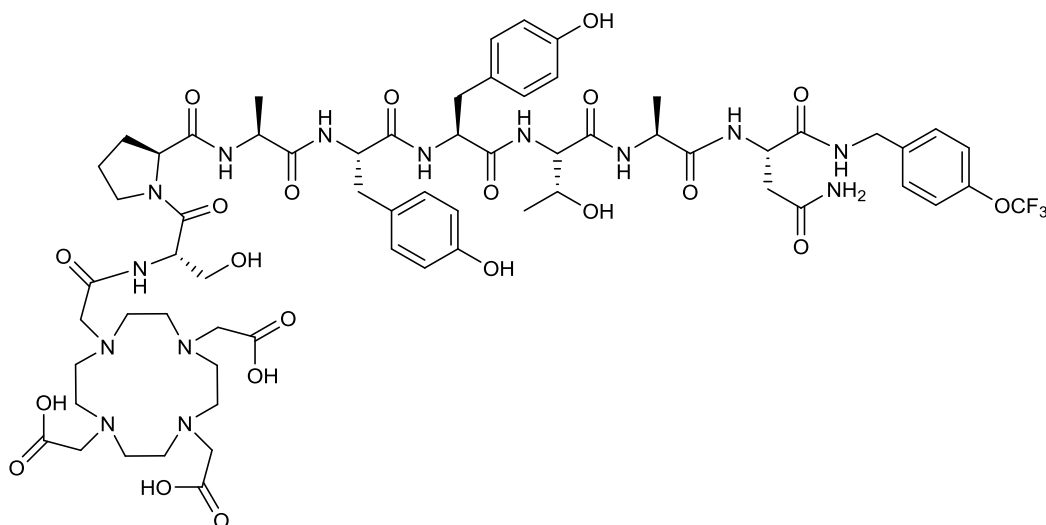
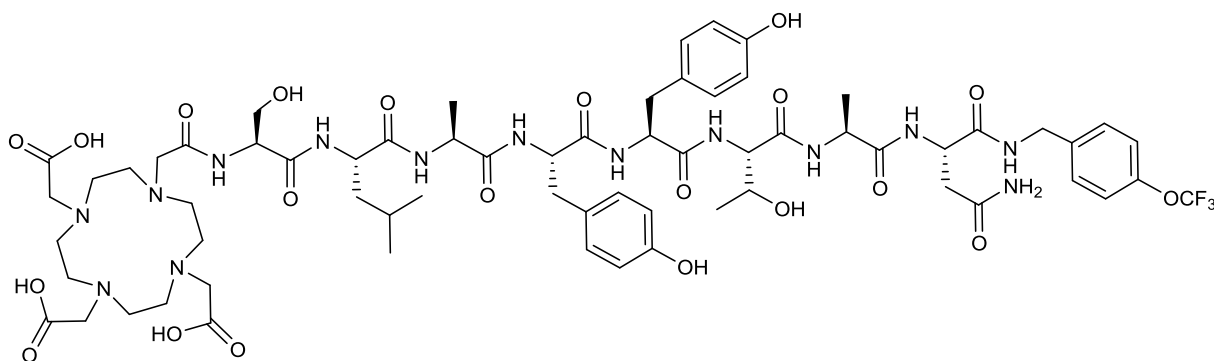


Figure 85 - HPLC trace of purified **13** at 254 nm.

DOTA-SLAYYTAD-(trifluoromethoxy)benzylamide synthesis (14)

DOTA-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.7 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 23.5 mg, 40% yield). ^{19}F NMR (Methanol- d): δ -59.1ppm (s, ROCF_3); MS(ESI^+) m/z : 731.2 and 731.7 $[\text{M}+2\text{H}]^{2+}/2$, 1461.5 $[\text{M}+\text{H}]^+$; MS (MALDI $^+$) m/z : 1461.7 $[\text{M}+\text{H}]^+$, 1483.6 $[\text{M}+\text{Na}]^+$.

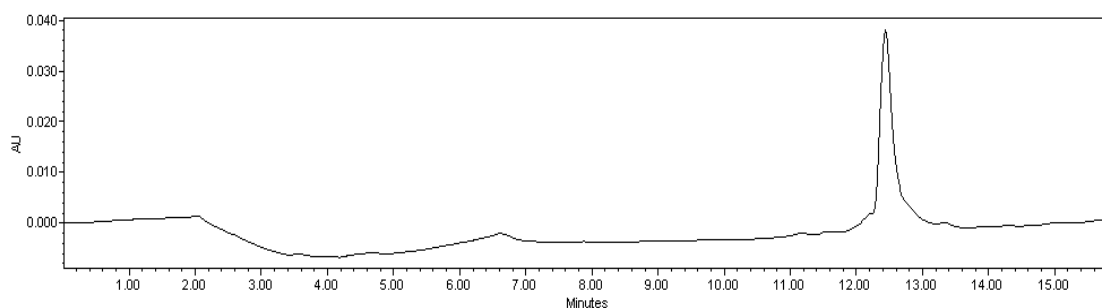
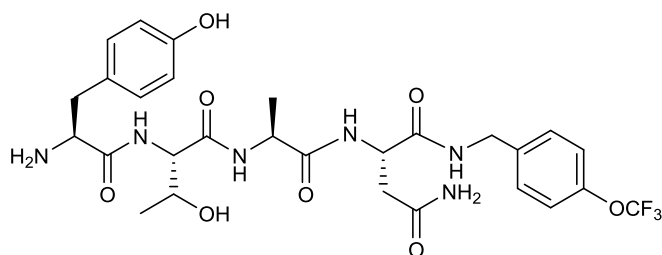


Figure 86 - HPLC trace of purified 14 at 254 nm.

YTAD-(trifluoromethoxy)benzylamide synthesis (15)

Compound **15** was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 20 minutes, peak at 11.35 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 90\%$ purity by HPLC, 10.5 mg, 41 % yield). ^{19}F NMR (Methanol- d): δ -59.9ppm (s, ROCF_3). MS(ESI^+) m/z : 641.3 $[\text{M}+\text{H}]^+$ 663.2 $[\text{M}+\text{Na}]^+$. MS (MALDI $^+$) m/z : 641.5 $[\text{M}+\text{H}]^+$.

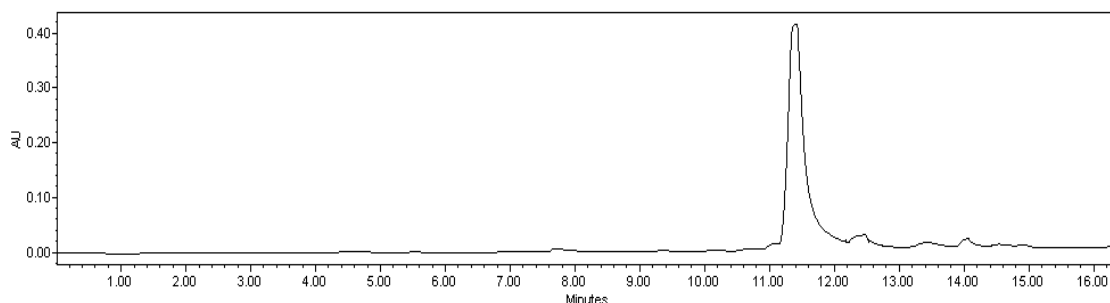
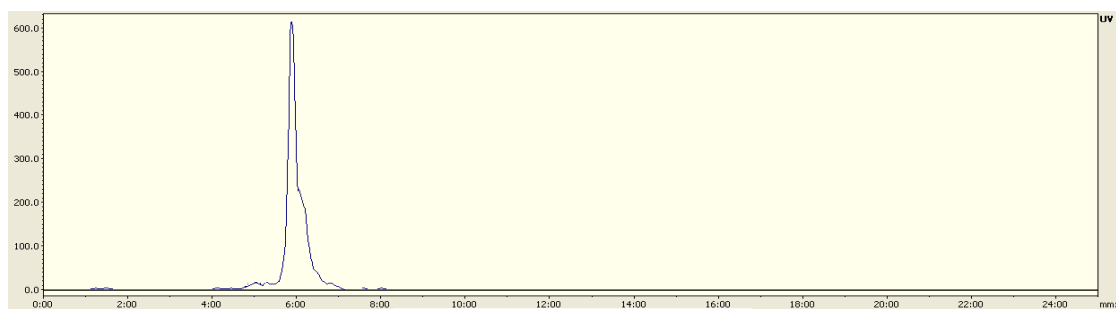
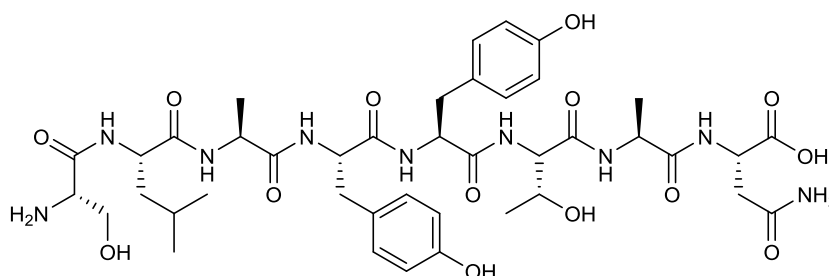
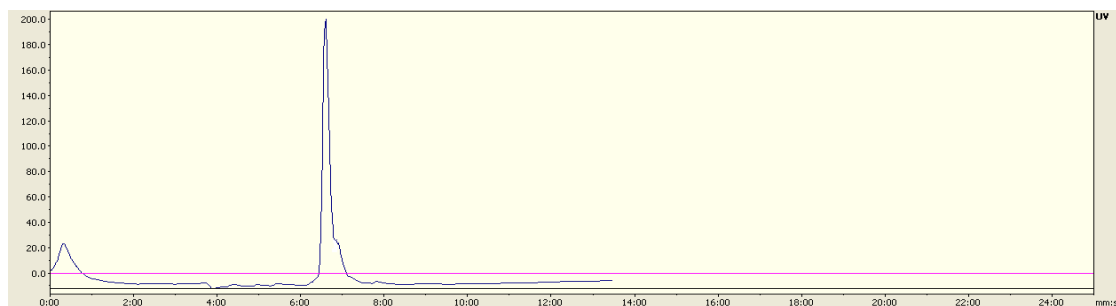


Figure 87 - HPLC trace of purified **15** at 254 nm.

Figure 88 - HPLC trace of purified **21** at 254 nm.**SLAYYTAD (22)**

SLAYYTAD was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification was carried out using prep LCMS (water 0.1% FA with 30-98% methanol 0.1% FA over 15 minutes), centrifugal evaporation white solid, yield 28% (5 mgs, $\geq 95\%$ purity by HPLC). MS(ESI⁺)m/z: 902.7[M]⁺, 903.7 [M+H]⁺, 926.10 [M+Na]⁺.

Figure 89 - HPLC trace of purified **22** at 254 nm.

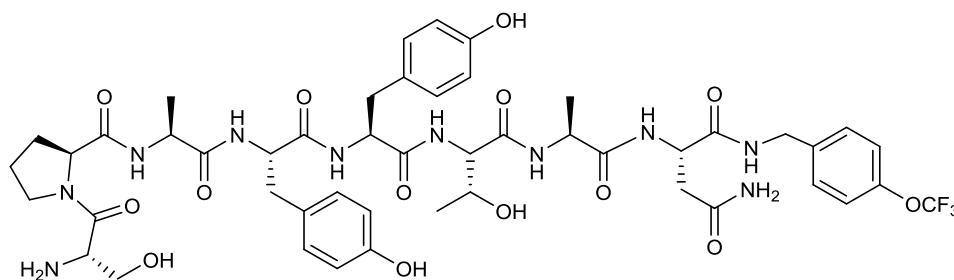
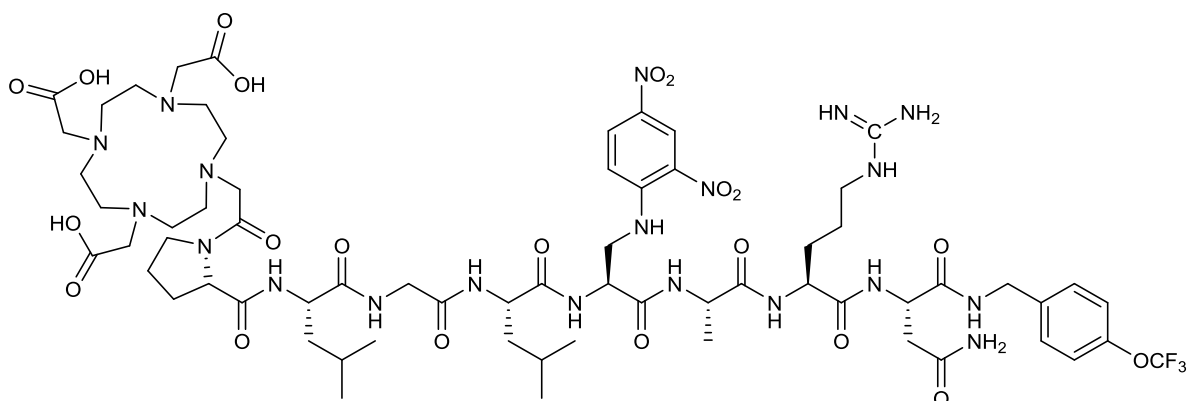


Figure 90 - HPLC trace of purified **23** at 254 nm.

DOTA-PLGL-Dpa-ARD-Tfb (27)

DOTA-PLGL-Dpa-ARD-Tfb was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 μ M scale. Purification was carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, peak at 8.3 Minutes, UV at 210 nm and 254 nm) lyophilisation yielded a bright yellow solid ($\geq 95\%$ purity by HPLC, 34 mg, , 55% yield). ^{19}F NMR (DMSO- d_6): δ -59.4ppm; MS (ESI $^+$) m/z: 1589.7 [M+K] $^+$; MS (MALDI $^+$) m/z: 1551.8 [M+H] $^+$.

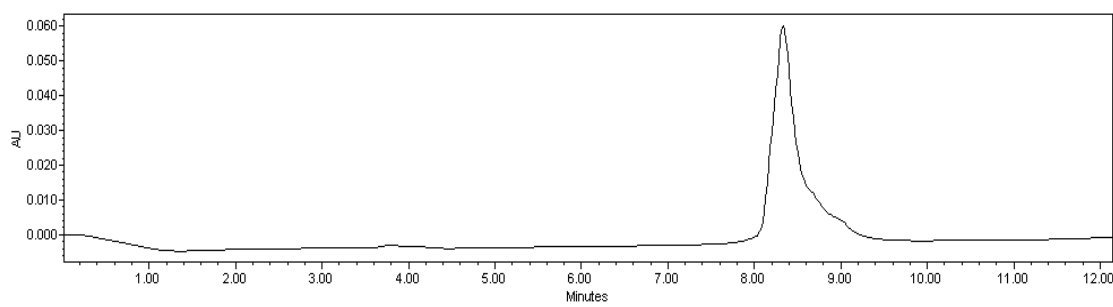
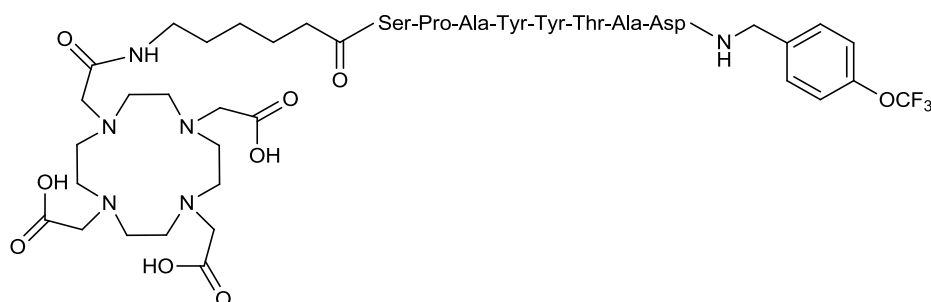


Figure 91 - HPLC trace of purified **27** at 254 nm.

DOTA-Ahx-SPAYYTAD-Tfb (29)

DOTA-Ahx-SPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.3 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 90\%$ purity by HPLC, 10 mg, 32% yield). ^{19}F NMR (DMSO- d_6): δ -59.6 ppm (s, ROCF_3); MS (MALDI $^+$) m/z : 1558.9 $[\text{M}]^+$, 1559.9 $[\text{M}+\text{H}]^+$, 1597.9 $[\text{M}+\text{K}]^+$.

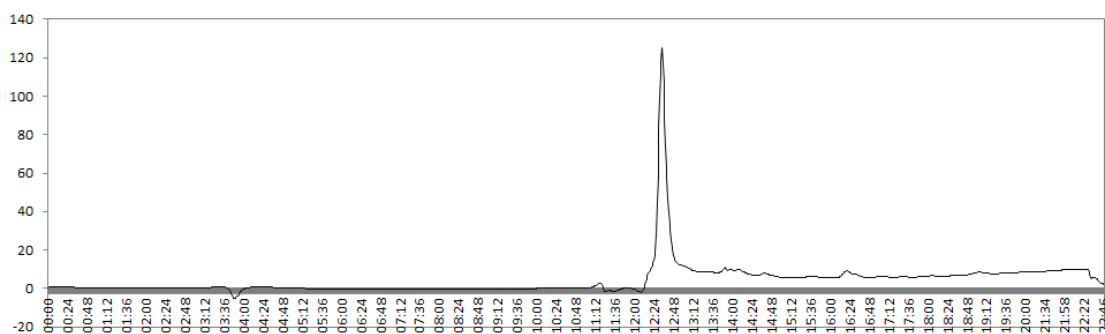
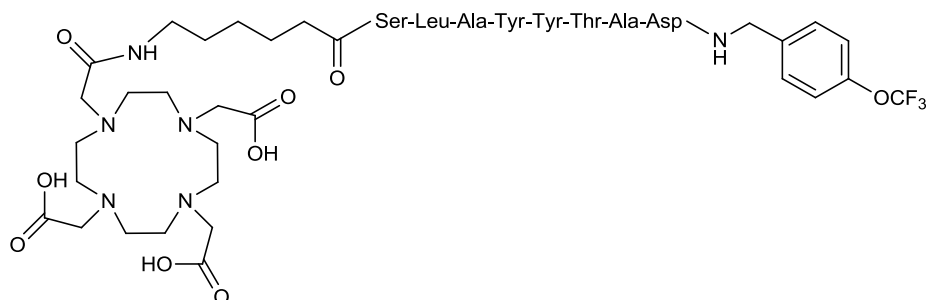


Figure 92 - HPLC trace of purified **29** at 254 nm.

DOTA-Ahx-SLAYYTAD-Tfb (30)

DOTA-Ahx-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 13.1 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 90\%$ purity by HPLC, 8.5 mg, 27% yield). ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROCF_3); MS (MALDI $^+$) m/z : 1575.3 $[\text{M}]^+$, 1576.3 $[\text{M}+\text{H}]^+$.

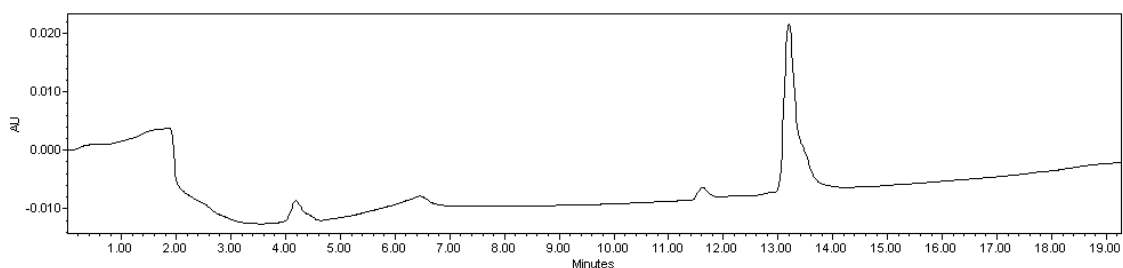
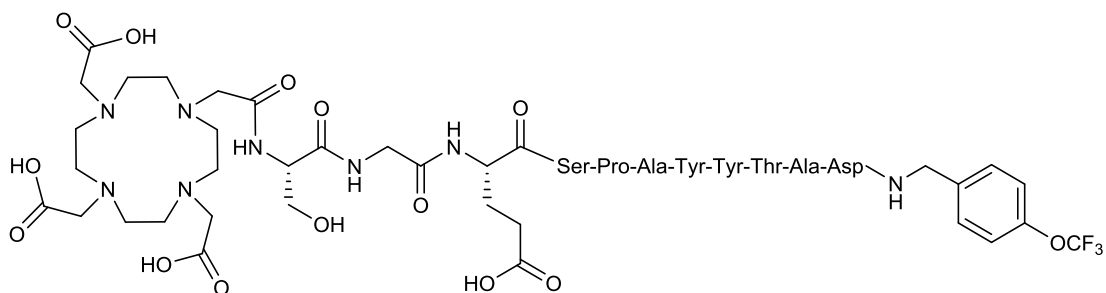


Figure 93 - HPLC trace of purified **30** at 254 nm.

DOTA-SGESPAYYTAD-Tfb (31)

DOTA-SGESPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, UV monitored at 210 nm and 254 nm, peak at 12.6 minutes) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 14.5 mg, 42% yield). ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROCF_3); MS (MALDI^+) m/z : 1719.2 $[\text{M}]^+$, 1758.2 $[\text{M}+\text{K}]^+$.

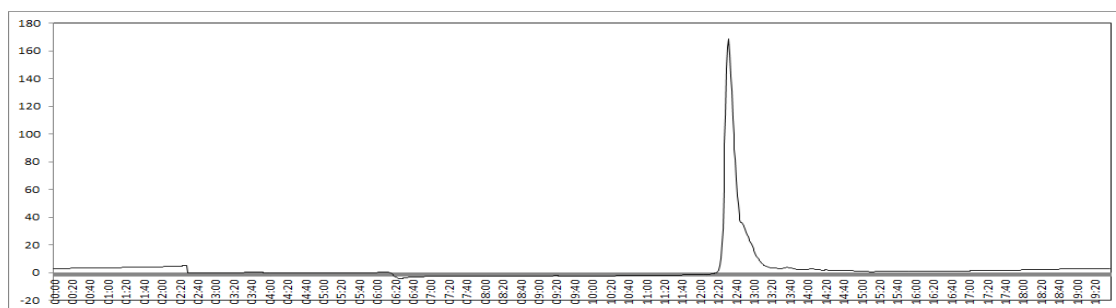
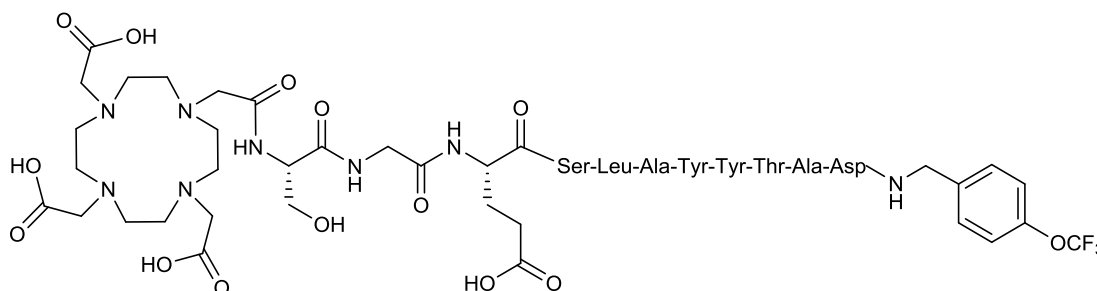


Figure 94 - HPLC trace of purified **31** at 254 nm.

DOTA-SGESLAYYTAD-Tfb (**32**)



DOTA-SGESLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.3 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 9 mg, 26%

yield). ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROCF_3); MS (MALDI^+) m/z : 1735.1 $[\text{M}]^+$, 1736.1 $[\text{M}+\text{H}]^+$.

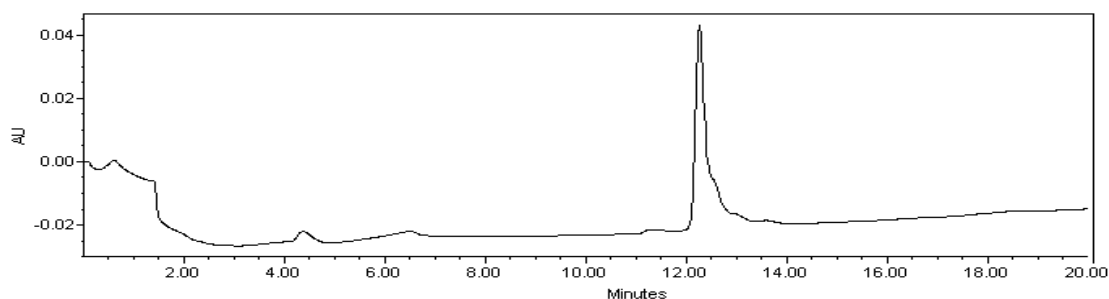
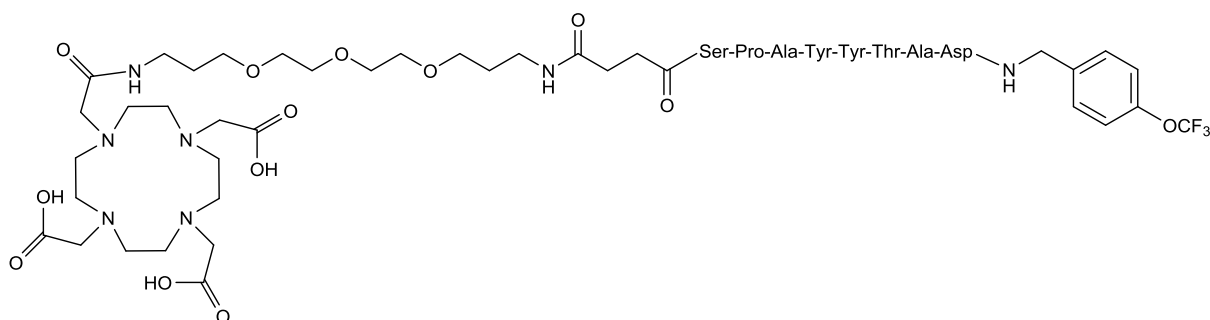
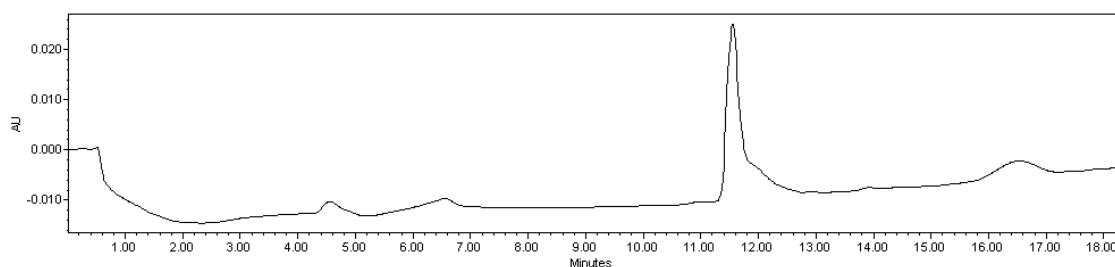
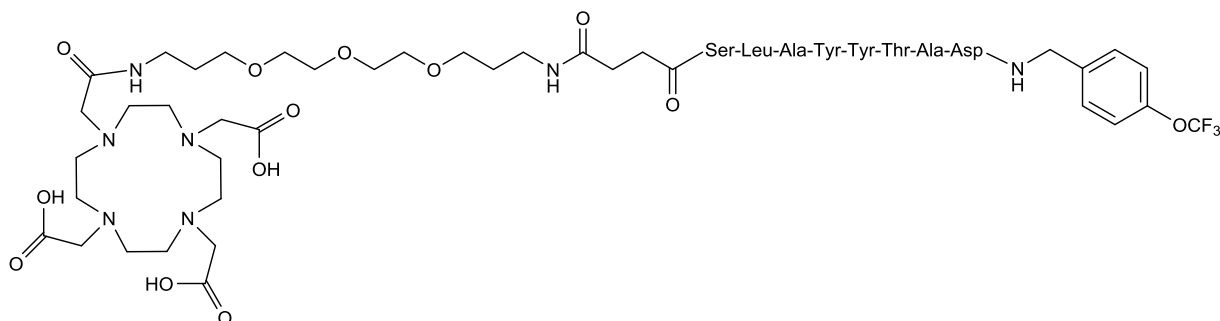


Figure 95 - HPLC trace of purified **32** at 254 nm.

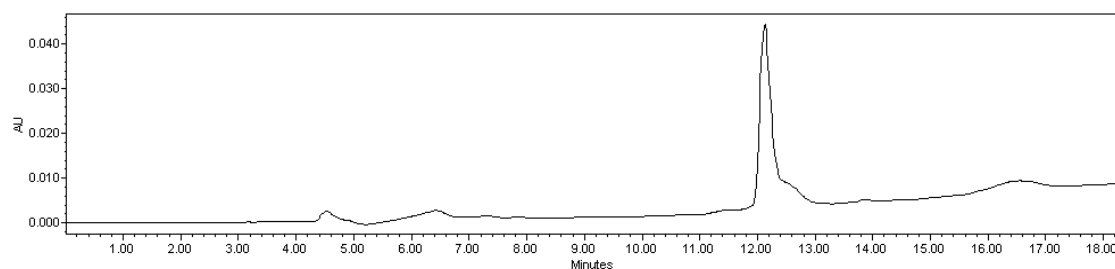
DOTA-PEG₃-SPAYYTAD-Tfb (**33**)

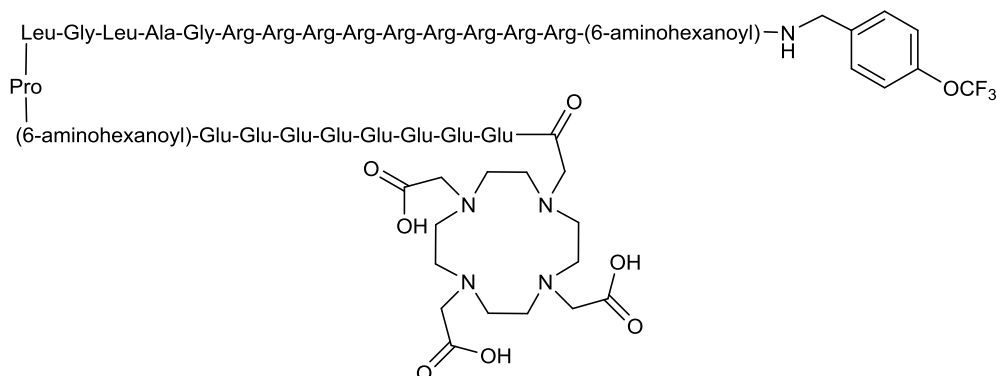


DOTA-PEG₃-SPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 11.6 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 10.5 mg, 30% yield). ^{19}F NMR (DMSO-d_6): δ -59.1 ppm (s, ROCF_3); MS (MALDI^+) m/z : 1749.1 $[\text{M}]^+$, 1750.1 $[\text{M}+\text{H}]^+$.

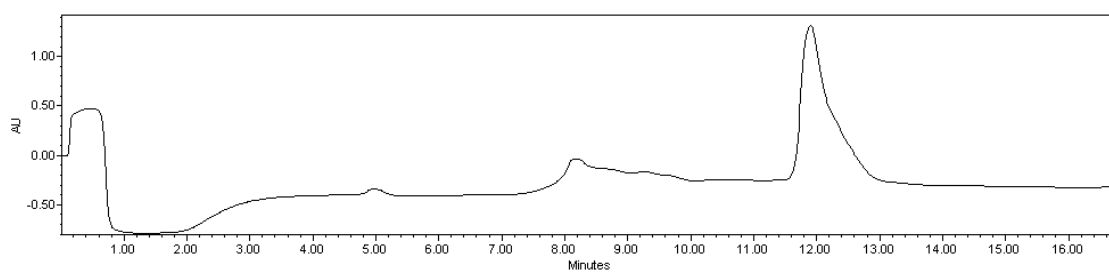
Figure 96 - HPLC trace of purified **33** at 254 nm**DOTA-PEG₃-SLAYYTAD-Tfb (34)**

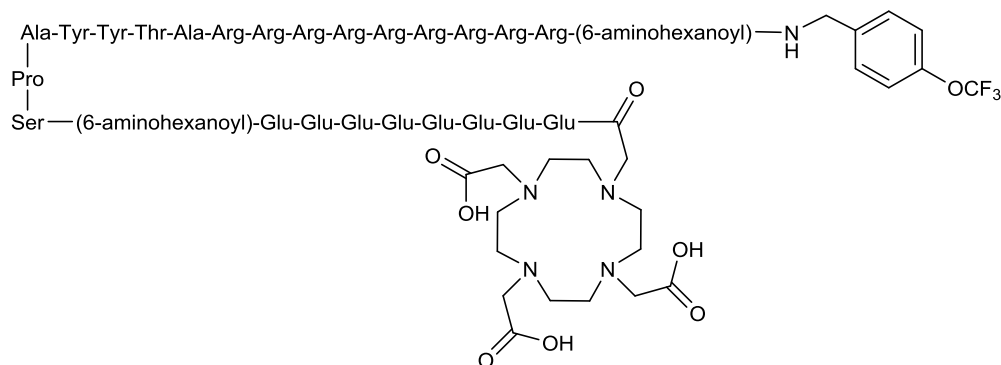
DOTA-PEG₃-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.1 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 10 mg, 28% yield). ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROCF_3); MS (MALDI^+) m/z : 1764.0 $[\text{M}+\text{H}]^+$, 1802.0 $[\text{M}+\text{K}]^+$.

Figure 97 - HPLC trace of purified **34** at 254 nm.

DOTA-(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (47)

47 was synthesised using standard protocol 2 (peptide synthesiser) up to final glutamic acid coupling and deprotection. Triprotected DOTA was coupled on manually using the steps described for protocol 1. Deprotection and cleavage from the resin used the same cleavage mix (95 %TFA, 2.5 % water and 2.5 % triisopropylsilane) but reaction was carried out for 6 hours. Compound was isolated as described in protocol 1. **47** was purified using Prep HPLC; Water with 0.1% TFA and 0-90% Acetonitrile with 0.1% TFA over 1-21 minutes, UV monitored at 210 nm and 254 nm, broad peak at 11.9 minutes. Lyophilisation yielded **47** as a white solid ($\geq 95\%$ purity by HPLC, 24 mg, 30% yield). ^{19}F NMR (DMSO- d_6): δ -59.6 ppm (broadened s, ROCF_3); MS (ESI $^+$) m/z : 666.62 $[\text{M}+6\text{H}]^{6+}/6$, 799.8 $[\text{M}+5\text{H}]^{5+}/5$, 999.4 $[\text{M}+4\text{H}]^{4+}/4$, 3993.0 $[\text{M}+\text{H}]^+$, 4032.0 $[\text{M}+\text{K}]^+$; MS (ESI $^-$) m/z : 1330.2 $[\text{M}-3\text{H}]^{3-}/3$; MS (MALDI $^+$) m/z : 3993.9 $[\text{M}+\text{H}]^+$.

Figure 98 - HPLC trace of purified **47** at 210 nm.



48 was synthesised using standard protocol 2 (peptide synthesiser) up to final glutamic acid coupling and deprotection. Triprotected DOTA was coupled on manually using the steps described for protocol 1. Deprotection and cleavage from the resin used the same cleavage mix (95 %TFA, 2.5 % water and 2.5 % triisopropylsilane) but reaction was carried out for 6 hours. Compound was isolated as described in protocol 1. **48** was purified using Prep HPLC; Water with 0.1% TFA and 0-90% Acetonitrile with 0.1% TFA over 1-21 minutes, UV monitored at 210 nm and 254 nm, broad peak at 11.5 minutes. Lyophilisation yielded **48** as a white solid ($\geq 90\%$ purity by HPLC, 32 mg, 38% yield). ^{19}F NMR (DMSO- d_6): δ -59.6 ppm (broadened s, ROCF_3); MS (ESI^+) m/z: 707.4 $[\text{M}+6\text{H}]^{6+}/6$, 848.5 $[\text{M}+5\text{H}]^{5+}/5$, 1060.1 $[\text{M}+4\text{H}]^{4+}/4$, 4237.0 $[\text{M}+\text{H}]^+$; MS (ESI^-) m/z: 1411.8 $[\text{M}-3\text{H}]^{3-}/3$; MS (MALDI $^+$) m/z: 4237.0 $[\text{M}+\text{H}]^+$.

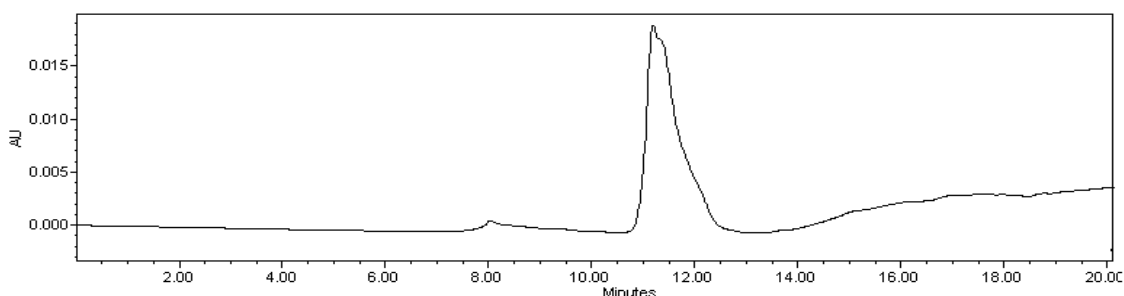
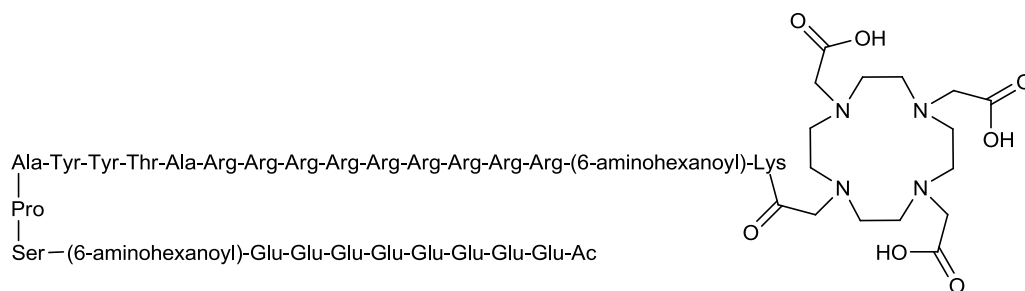
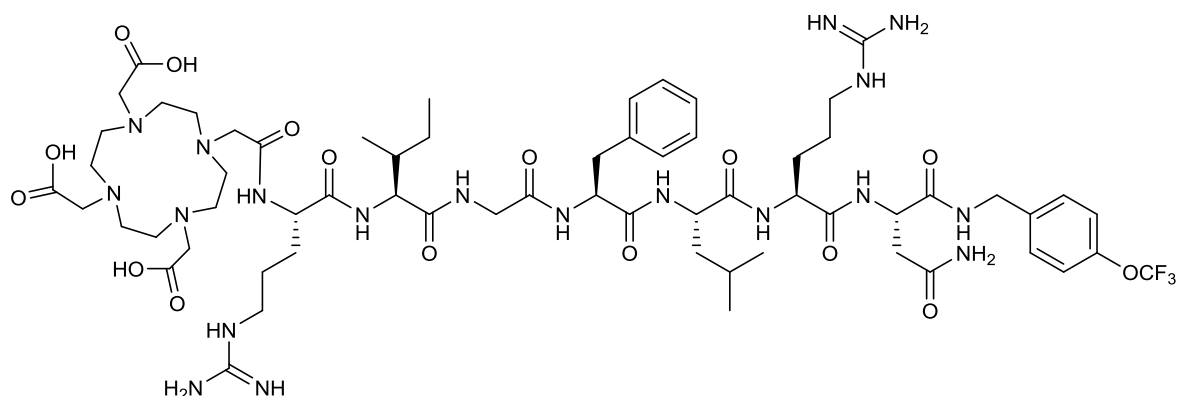


Figure 99 - HPLC trace of purified **48** at 254 nm.

Attempted synthesis of Ac-(e)₈-Ahx-SPAYYTAD-(r)₉-Ahx-K-DOTA (49)

49 was synthesised using standard protocol 2 (peptide synthesiser) from lysine to final glutamic acid residues. At this point resin was removed from the synthesiser for manual steps. N-terminus was protected via acetylation using a capping mixture of 10% acetic anhydride v/v with 20% DIPEA v/v in DMF for 1 hour, before Mtt protecting group was selectively removed using 1.8% v/v TFA in DCM with 2% v/v triisopropylsilane as a scavenger, washes were carried out for 3min at a time with 12 washes needed until yellow colour disappeared. DOTA succinimidyl ester (1.5 eq) and DIPEA (3 eq) were then mixed with the resin overnight before resin was washed and dried overnight. Deprotection and cleavage from the resin used the same cleavage mix as protocol 1 (95 %TFA, 2.5 % water and 2.5 % triisopropylsilane) but reaction was carried out for 6 hours. Compound was isolated as described in protocol 1. Crude Mass spec analysis showed no peak corresponding to compound **49**, major peak in MS (ESI⁺) m/z: 1200.7 [M-3H]³⁺/3, corresponding to peptide without DOTA.

DOTA-RIGFLRD-Tfb (54)

DOTA-RIGFLRD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 11.2 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid ($\geq 95\%$ purity by HPLC, 5 mg, 17% yield). ^{19}F NMR (DMSO- d_6): δ -58.9 ppm (s, ROCF_3); MS (MALDI $^+$) m/z : 1435.0 $[\text{M}]^+$, 1473.0 $[\text{M}+\text{K}]^+$.

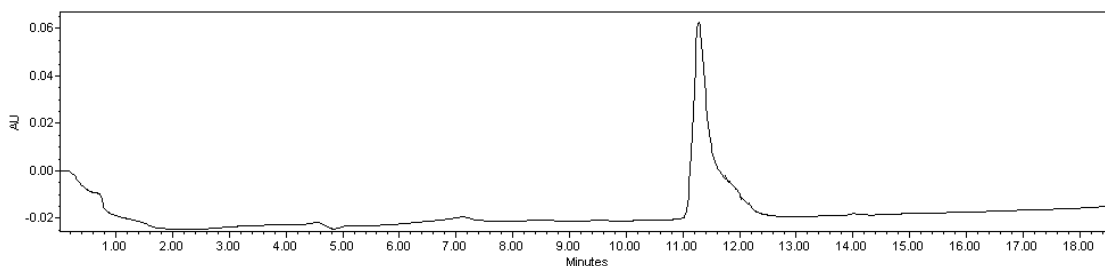
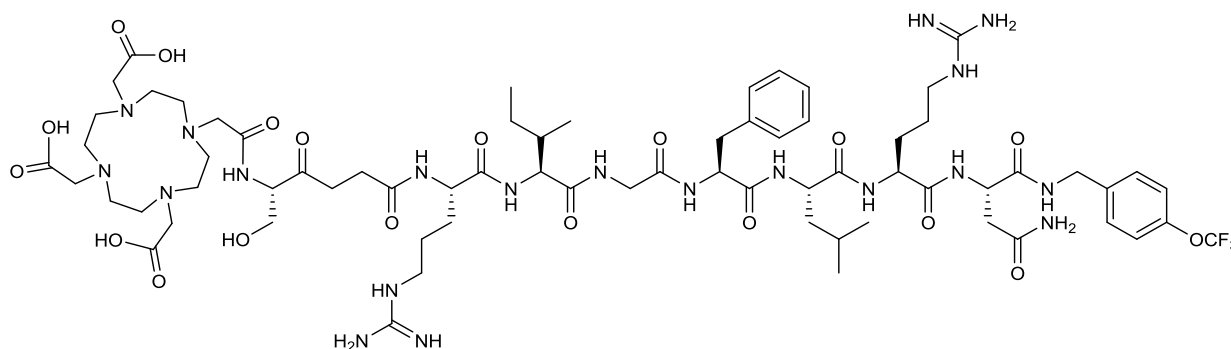


Figure 100 - HPLC trace of purified **54** at 254 nm.



DOTA-SGRIGFLRD-(trifluoromethoxy)benzylamide was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 μ M scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, peak at 12.0 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a fluffy white solid ($\geq 90\%$ purity by HPLC, 10 mg, 31% yield). ^{19}F NMR (DMSO- d_6): δ -58.9 ppm (s, ROCF_3); MS (MALDI $^+$) m/z : 1579.9 $[\text{M}+\text{H}]^+$, 1580.9 $[\text{M}+2\text{H}]^{2+}$.

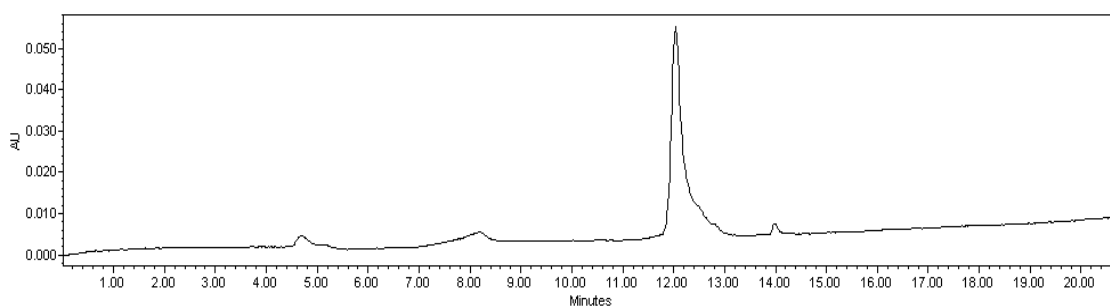
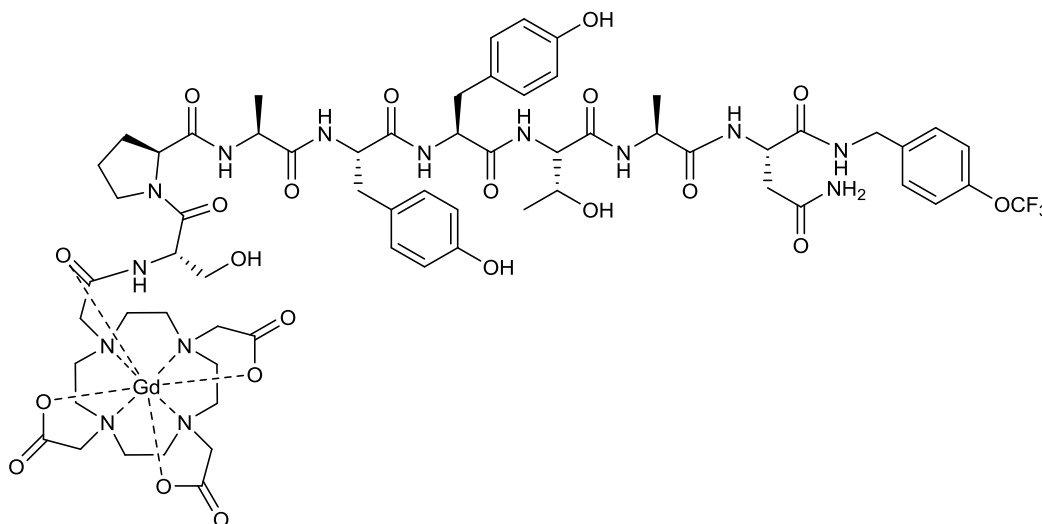


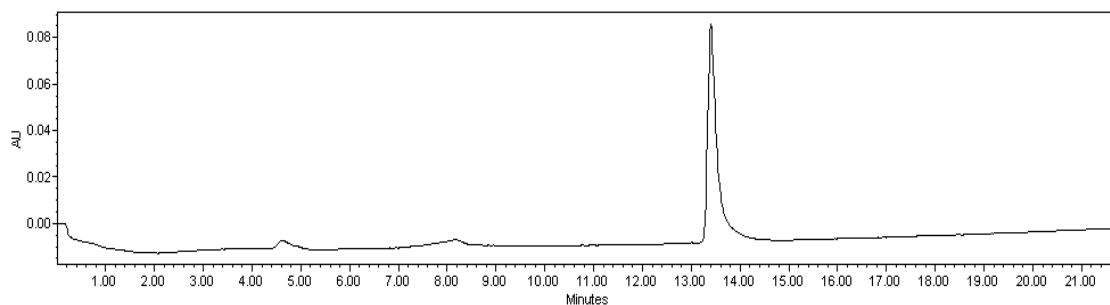
Figure 101 - HPLC trace of purified **56** at 254nm.

5.5 Gadolinium (III) complexes

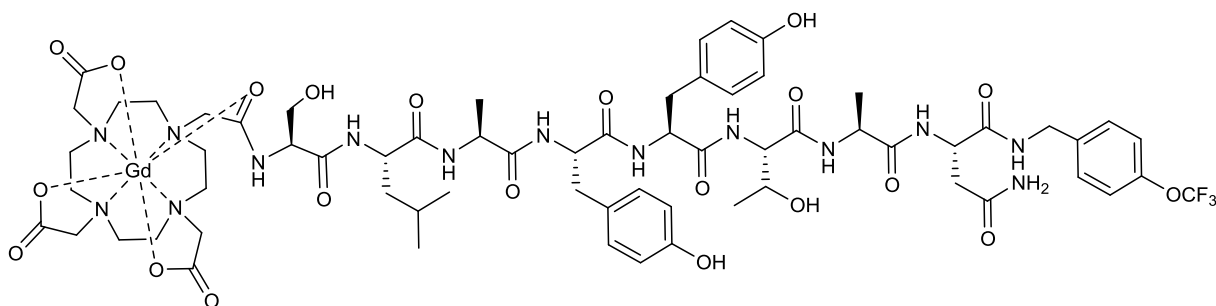
Gd-DOTA-SPAYYTAD-(trifluoromethoxy)benzylamide synthesis (1)



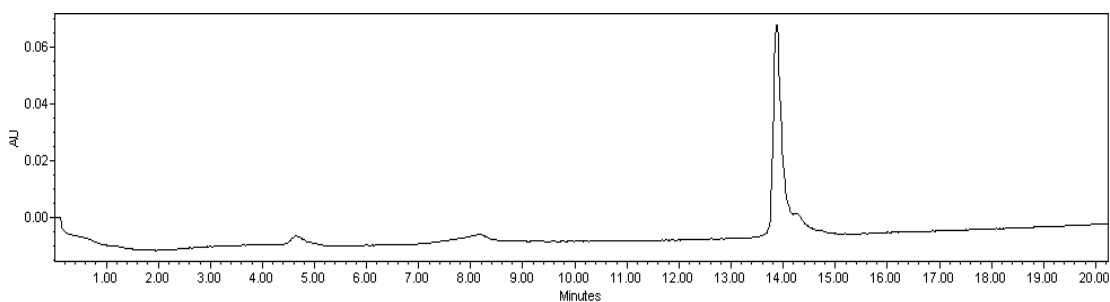
DOTA-SPAYYTAD- (trifluoromethoxy)benzylamide (10 mgs, 6.9 μmol s, 1.0 eq) was partially dissolved in 4 mls of MilliQ water then an excess of $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ was added as 2 ml of an 6 mM solution in MilliQ water (4.5 mgs, 12 μmol s, 1.75 eq), pH adjusted with NaOH to between 6-7 (checking with pH indicator paper) and the mixture was left stirring at room temperature. After 1 hour the pH of the mixture was checked again using indicator paper to ensure pH had not changed and then the reaction was left stirring overnight. The mixture was then purified using reversed phase Prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, peak at 13.4 Minutes, 210 nm and 254 nm) to give pure Gd-DOTA-SPAYYTAD-(trifluoromethoxy)benzylamide as a fluffy white solid ($\geq 98\%$ purity by HPLC, 9.6 mgs, 87% yield). ^{19}F NMR (DMSO-d_6): δ -59.2 ppm (broadened s, ROCF_3); MS (ESI^+) m/z: 800.7 $[\text{M}+2\text{H}]^{2+}/2$, 1599.5 $[\text{M}]^+$. MS (MALDI^+) m/z: 1601.0 $[\text{M}+\text{H}]^+$, 1622.8 $[\text{M}+\text{Na}]^+$.

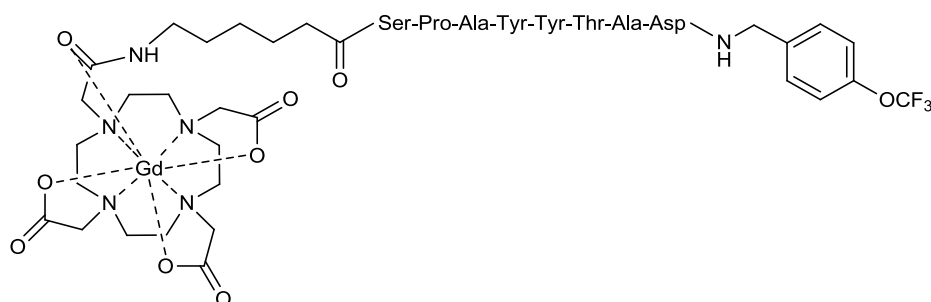
Figure 102- HPLC trace of purified **1**, 254 nm

Gd-DOTA-SLAYYTAD-(trifluoromethoxy)benzamide synthesis (2)

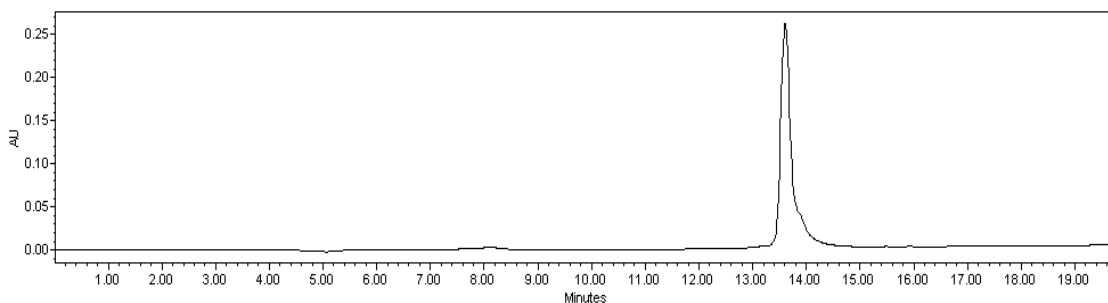
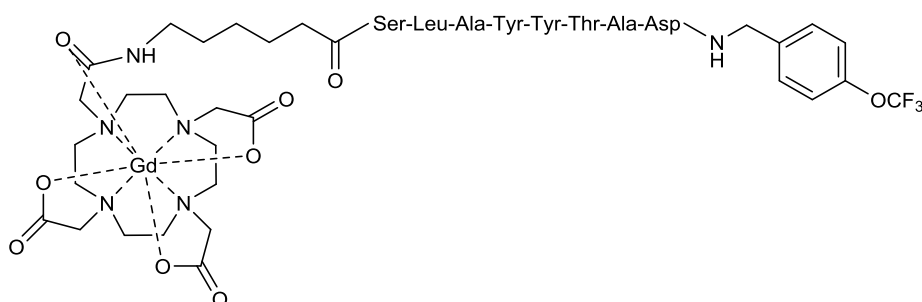


Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-SLAYYTAD-Tfb (8 mgs, 5.5 μ mol, 1eq) yield 70% (6.2 mgs, $\geq 90\%$ purity by HPLC Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes peak at 13.8 minutes UV at 210 nm and 254 nm) ^{19}F NMR (DMSO- d_6): δ -59.6 ppm (broadened s, ROCF_3). MS(ESI^+)m/z: 808.8 $[\text{M}+2\text{H}]^{2+}/2$, 1616.5 $[\text{M}]^+$, 1638.5 $[\text{M}+\text{Na}]^+$. MS (MALDI $^+$) m/z: 1615.9 $[\text{M}]^+$.

Figure 103 - HPLC trace of purified **2**, 254nm.

Gd-DOTA-Ahx-SPAYYTAD-Tfb (35)

Prepared as described for compound **1** (Gd-DOTA-Ahx-SPAYYTAD-Tfb), starting material DOTA-Ahx-SPAYYTAD-Tfb (2.5 mgs, 1.6 μ mol, 1 eq) to afford **35** as a white solid in a yield of 69% (1.9 mgs, $\geq 98\%$ purity by HPLC Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, UV monitored at 210 nm and 254 nm, peak at 13.6 minutes). ^{19}F NMR (DMSO-d_6): δ -59.9 ppm (broadened s, ROCF_3); MS (MALDI $^+$) m/z : 1714.0 $[\text{M}+\text{H}]^+$, 1736.0 $[\text{M}+\text{Na}]^+$.

Figure 105 - HPLC trace of purified **35**, 254 nm.**Gd-DOTA-Ahx-SLAYYTAD-Tfb (36)**

Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-Ahx-SLAYYTAD-Tfb (3.5 mgs, 2.2 μ mol, 1 eq) to afford **36** as a white solid in a yield of 78% (3 mgs, $\geq 98\%$ purity by HPLC Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210 nm and 254 nm, peak at 14.1 minutes). ^{19}F NMR (DMSO- d_6): δ -59.3 ppm (broadened s, ROCF_3); MS (MALDI $^+$) m/z : 1730.0 $[\text{M}]^+$.

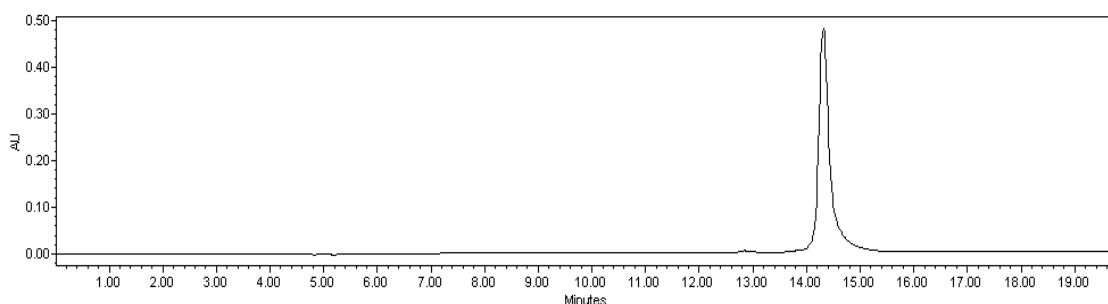
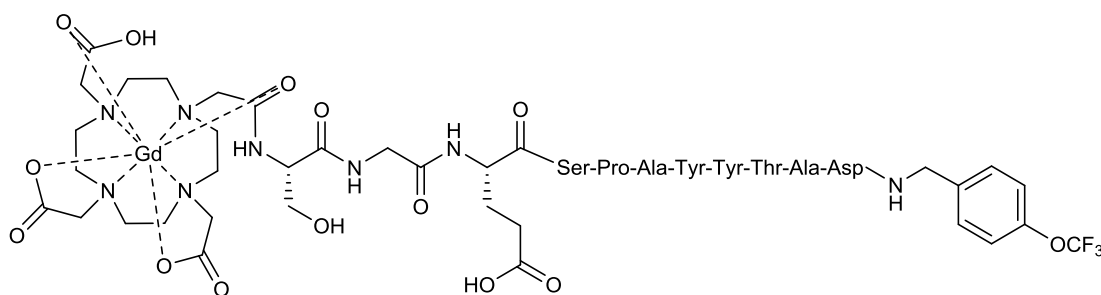
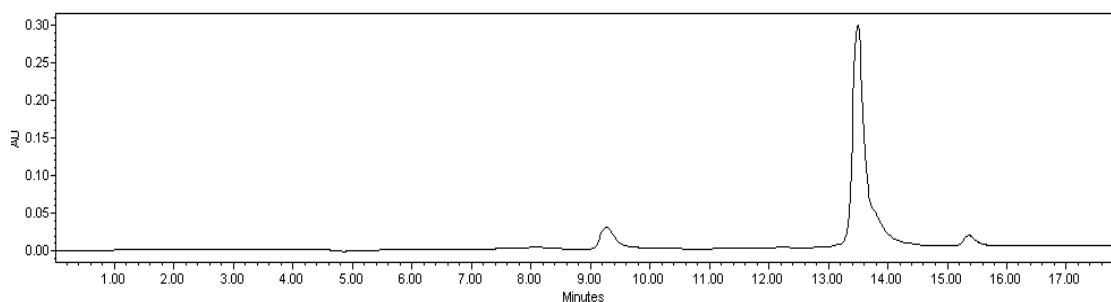
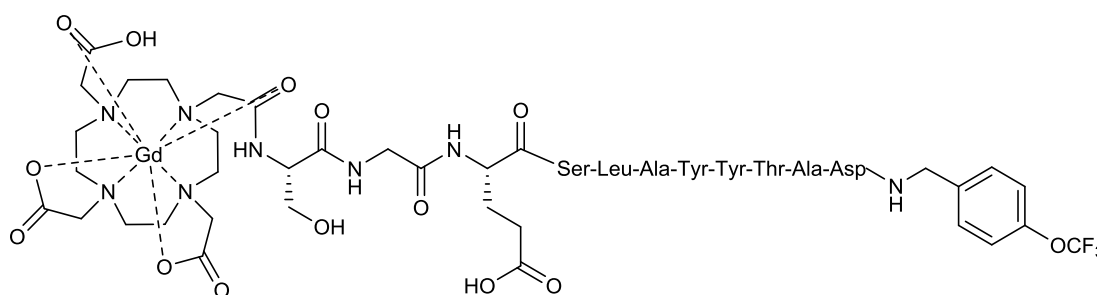


Figure 106 - HPLC trace of purified **36**, 254 nm.

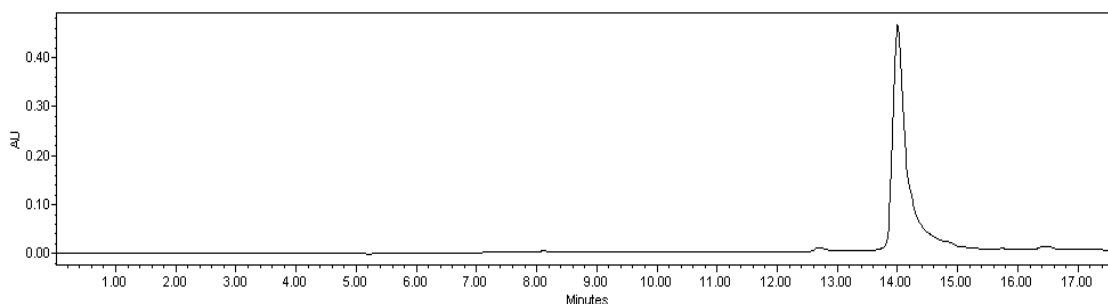
Gd-DOTA-SGESPAYYTAD-Tfb (**37**)

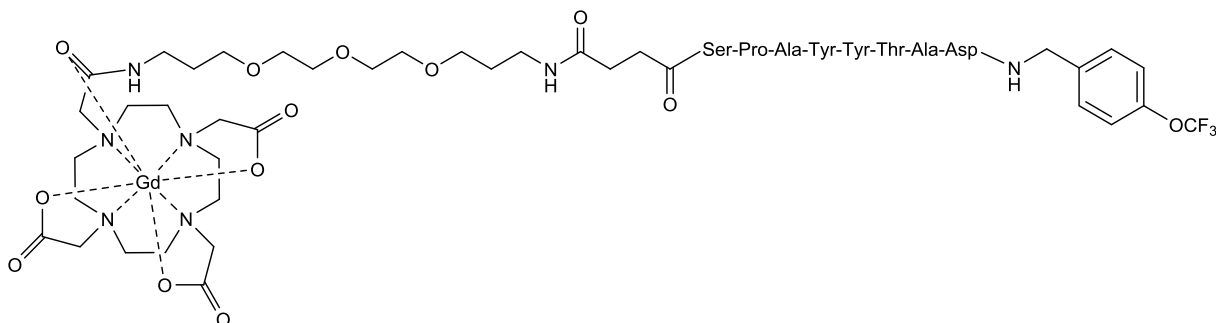


Prepared as described for compound **1** (Gd-DOTA-SGESPAYYTAD-Tfb), starting material DOTA-SGESPAYYTAD-Tfb (3.5 mgs, 2 μ mol, 1 eq) to afford **37** as a white solid in a yield of 52% (2 mgs, $\geq 90\%$ purity by HPLC: Water (0.1% FA) and 0-90% Acetonitrile (0.1% FA) over 1-21 minutes, UV monitored at 210 nm and 254 nm, peak at 13.5 minutes). ^{19}F NMR (DMSO- d_6): δ -59.5 ppm (broadened s, ROCF_3); MS (MALDI $^+$) m/z : 1875.0 $[\text{M}]^+$, 1913.8 $[\text{M}+\text{K}]^+$.

Figure 107 - HPLC trace of purified **37**, 254 nm.**Gd-DOTA-SGESLAYYTAD-Tfb (38)**

Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-SGESPAYYTAD-Tfb (3.5mgs, 2 μ mols, 1eq) to afford **38** as a white solid in a yield of 79% (3mgs, $\geq$ 95% purity by HPLC Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 14.0 minutes). ^{19}F NMR (DMSO- d_6): δ -59.5ppm (broadened s, ROCF_3); MS (MALDI $^+$) m/z: 1889.2 $[\text{M}]^+$, 1912.2 $[\text{M}+\text{Na}]^+$.

Figure 108 - HPLC trace of purified **38**, 254nM

Gd-DOTA-PEG₃-SPAYYTAD-Tfb (39)

Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-PEG₃-SPAYYTAD-Tfb (2.5mgs, 1.4 μ mol, 1eq) to afford **39** as a white solid of in a yield of 62% (1.7mgs, $\geq$ 98% purity by HPLC Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 13.5 minutes). ¹⁹F NMR (DMSO-d₆): δ -59.6ppm (broadened s, ROCF₃); MS (MALDI⁺) m/z: 1904.2 [M]⁺.

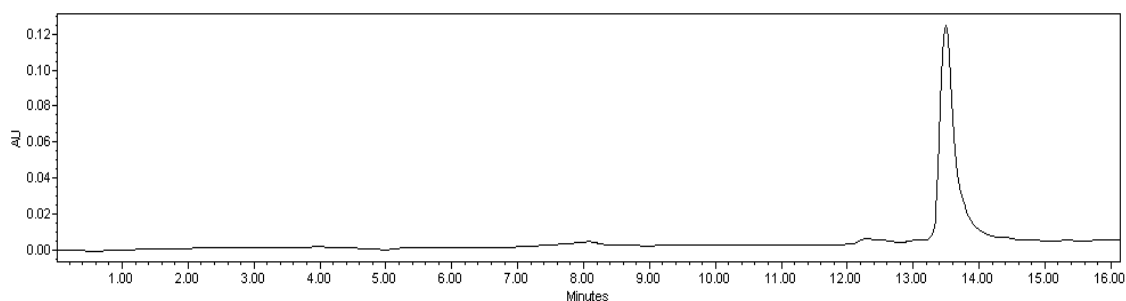
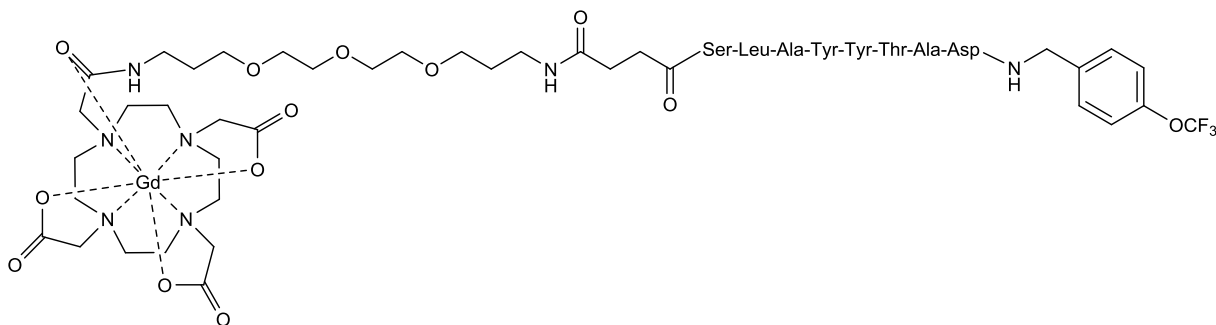


Figure 109 - HPLC trace of purified **39**, 254nm

Gd-DOTA-PEG₃-SLAYYTAD-Tfb (40)

Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-PEG₃-SLAYYTAD-Tfb (3mgs, 1.7 μ mol, 1eq) to afford **40** as a white solid in a yield of 85% (2.8mgs, $\geq$ 98% purity by HPLC, Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 14.2 minutes). ^{19}F NMR (DMSO- d_6): δ -59.6ppm (broadened s, ROCF_3); MS (MALDI $^+$) m/z: 1918.2 $[\text{M}]^+$, 1941.1 $[\text{M}+\text{Na}]^+$.

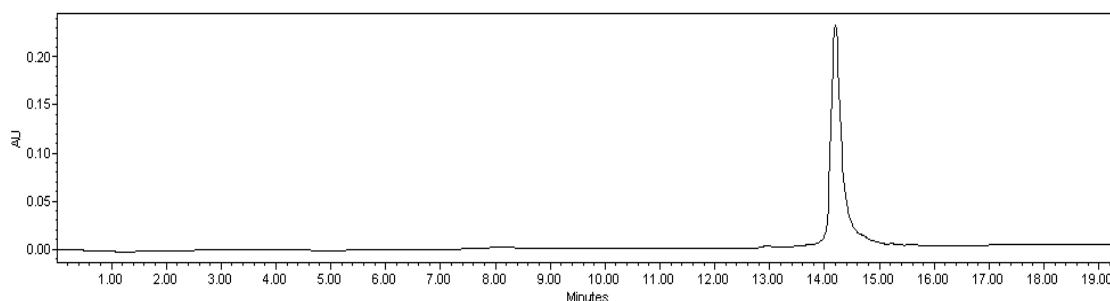
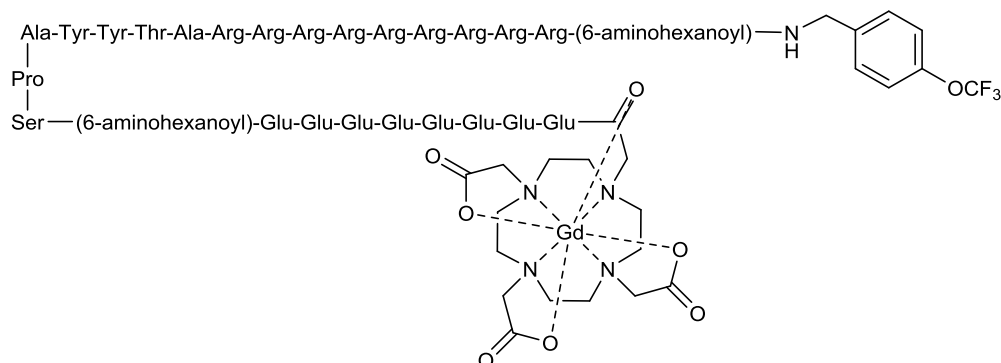


Figure 110 - HPLC trace of purified **40**, 254 nm.



Prepared as described for compound **1** (Gd-DOTA-SPAYYTAD-Tfb), starting material DOTA-(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (20 mgs, 4.8 μmols, 1 eq) to afford **51** as a white solid in a yield of 66% (13.7 mgs, ≥95% purity by HPLC Water with 0.1% TFA and 0-90% Acetonitrile with 0.1% TFA over 1-21 minutes, UV monitored at 210 nm and 254 nm, broad peak at 11.8 minutes). ¹⁹F NMR (DMSO-d₆): δ -59.6 ppm (broadened s, ROCF₃); MS (ESI⁺) m/z: 733.3[M+6H]⁶⁺/6, 879.7[M+5H]⁵⁺/5, 1099.5[M+4H]⁴⁺/4. MS (ESI⁻) m/z: 1462.96[M-3H]³⁻/3.

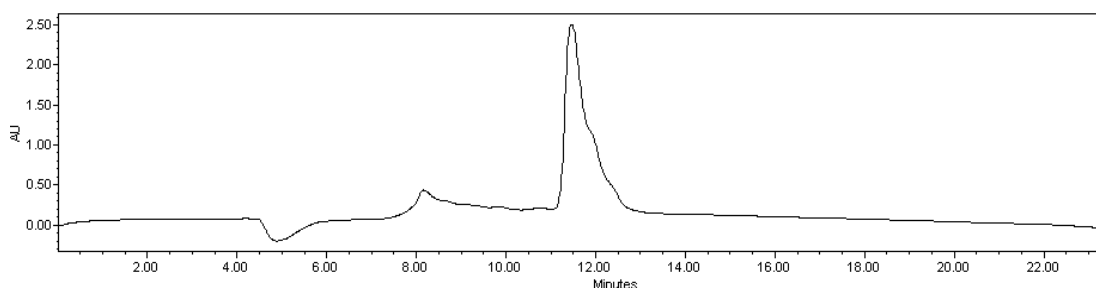


Figure 112 - HPLC trace of purified **51**, 210 nm.

5.6 Radiochemistry

General protocol: peptide ligand dissolved to 700 μ M in DMSO, 50 μ l of peptide ligand solution was mixed with 1 ml of NaOAc buffer (pH 5, 0.2 M) in a Wheaton

vial. ^{68}Ga was eluted from the generator in 2 ml of 0.1M HCL and 1 ml of this was added to the peptide solution. Reaction mixture was heated at 90°C for 10 minutes and then reaction mixture was removed from the heat. Radio-HPLC was carried out to determine radiochemical yields of compound versus free gallium (which elutes with the solvent front at 2 minutes). Remaining gallium could be removed from sample via solid phase extraction, this was carried out on a TC18 light cartridge (prepared by flushing with 5 mls of ethanol and the 10 mls of water), sample was loaded onto cartridge in reaction mixture with any free ^{68}Ga passing straight through, sample was then washed with water and could be removed from the cartridge with 0.8 ml of ethanol (acidified with HCL for compounds **52** and **53**) collected in 100 μl aliquots, the majority of the radiolabelled sample obtained between aliquots 2 and 3. Analytical radio-HPLC was carried out again to confirm purification. If being used for assay conditions samples 2 and 3 were combined and diluted with 300 ml of MMP-2 assay buffer (50 mM Tricine, 10 mM CaCl_2 , 150 mM NaCl, 0.5% Brij-35; pH 7.5).

Radio-HPLC conditions: Gradient, water (0.1% FA) with 0 - 90% acetonitrile (0.1%FA) over 20 minutes. For Activated cell penetrating compound **47** and **48**, two alternate HPLC conditions were used; water (0.1% TFA) 0-90% acetonitrile (0.1% TFA) over 20 minutes or water (0.1% TFA) 10-40% acetonitrile (0.1% FA) over 20 minutes. All HPLCs were carried out using C18 Gemini column, as described in section 5.1.

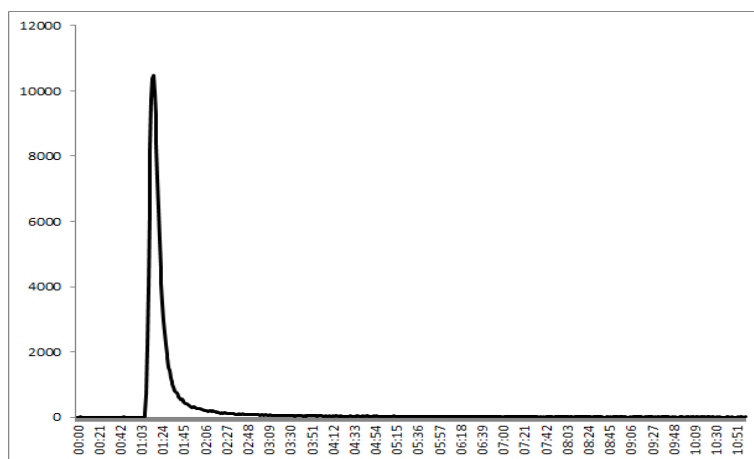
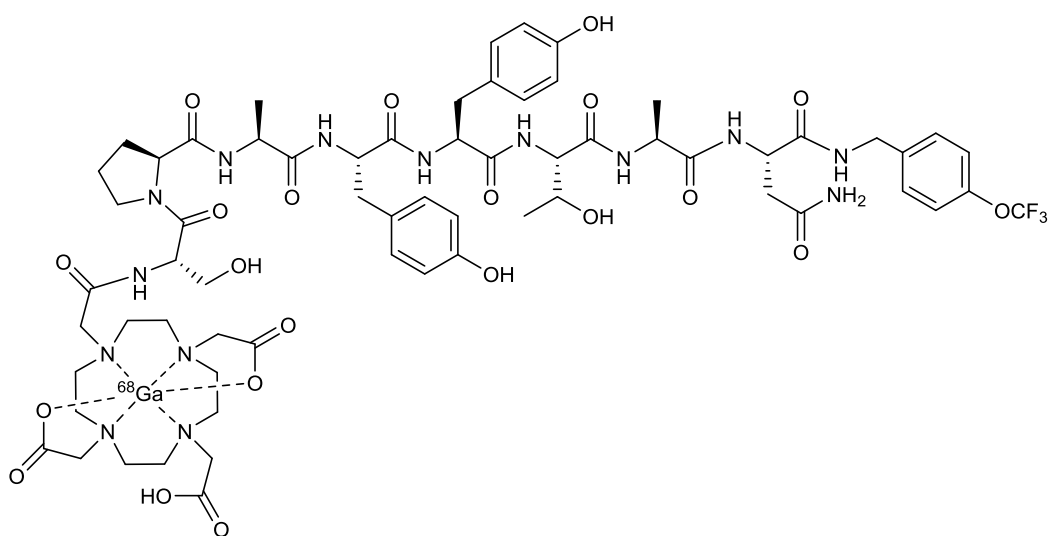
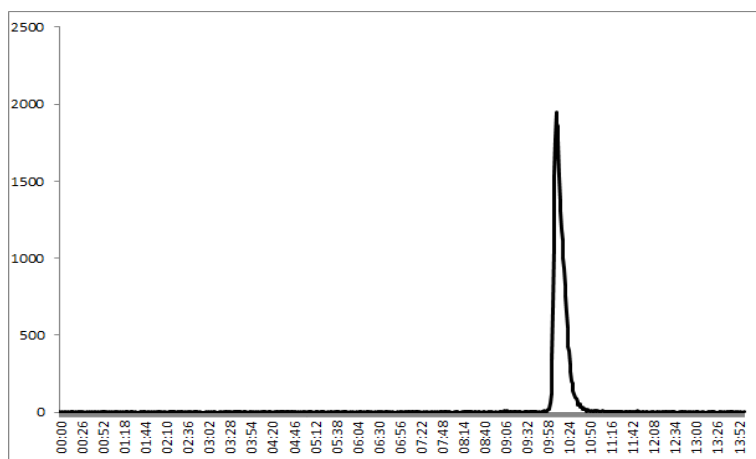
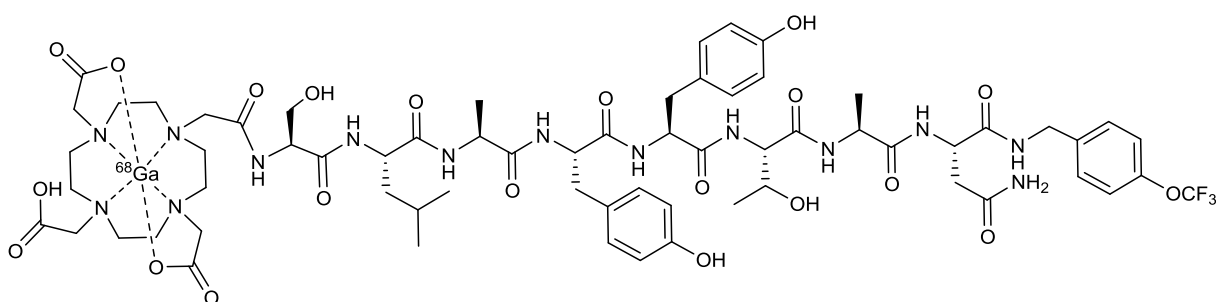


Figure 113- radio HPLC trace of free unreacted ^{68}Ga eluted from the generator.

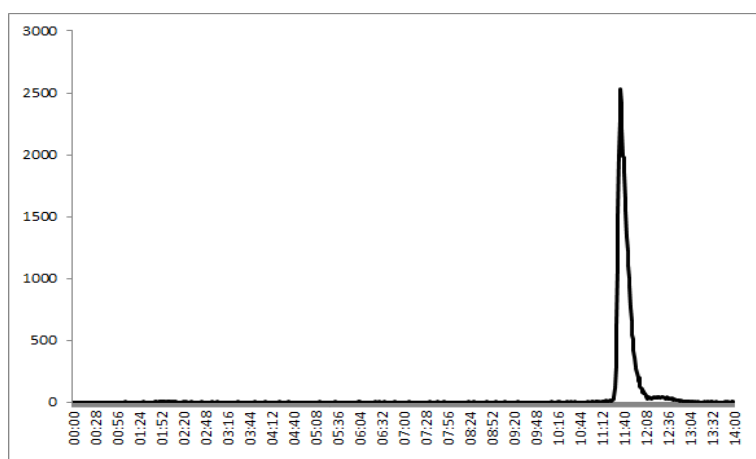
^{68}Ga -DOTA-SPAYYTAD-Tfb (25)

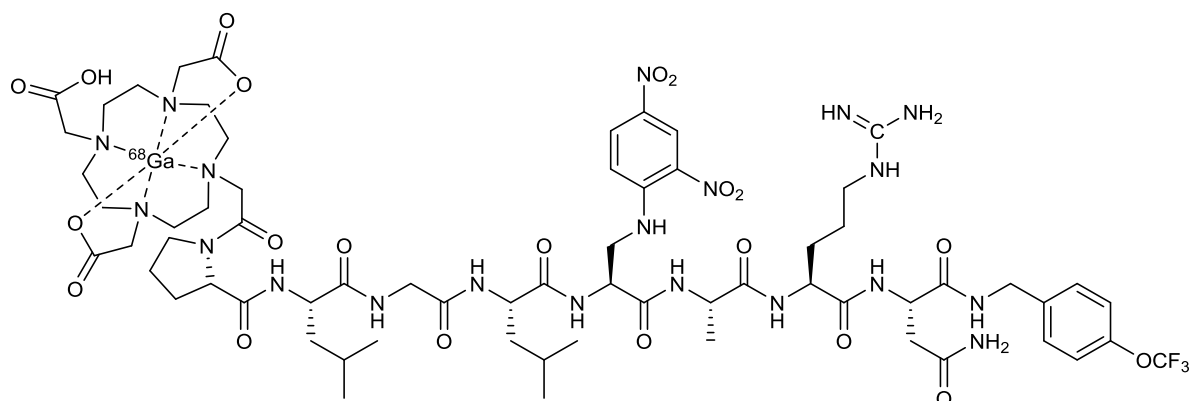


Decay corrected yield: $98.0 \pm 0.8\%$ (n=4)

Figure 114- radio HPLC trace of synthesis of **25**, before purification.**⁶⁸Ga-DOTA-SLAYYTAD-Tfb (26)**

Decay corrected yield: 97.0±1.4% yield (n=4)

Figure 115- radio HPLC trace of synthesis of **26**, before purification

^{68}Ga -DOTA-PLGL-Dnp-ARD-Tfb (28)

Decay corrected yield: $98.0 \pm 1.0\%$ (n=3)

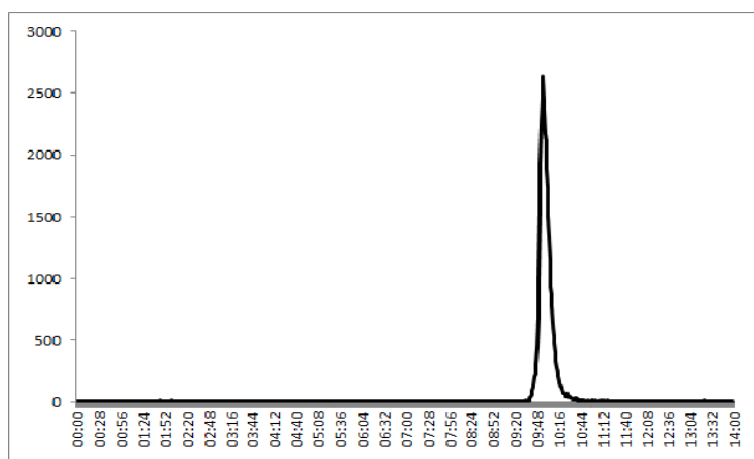
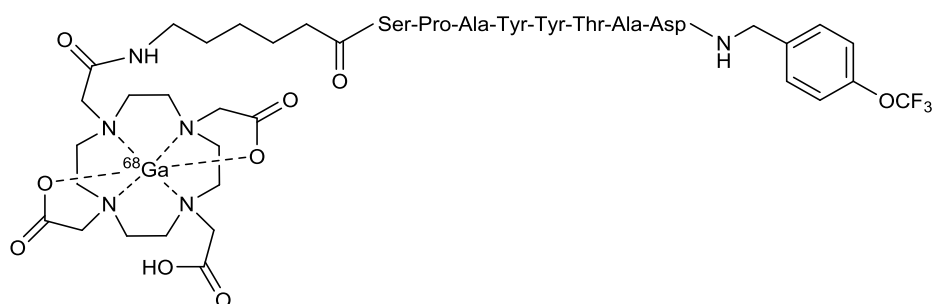
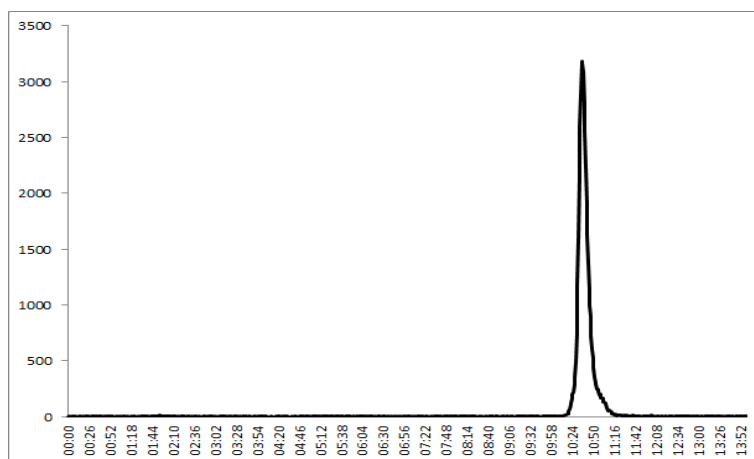
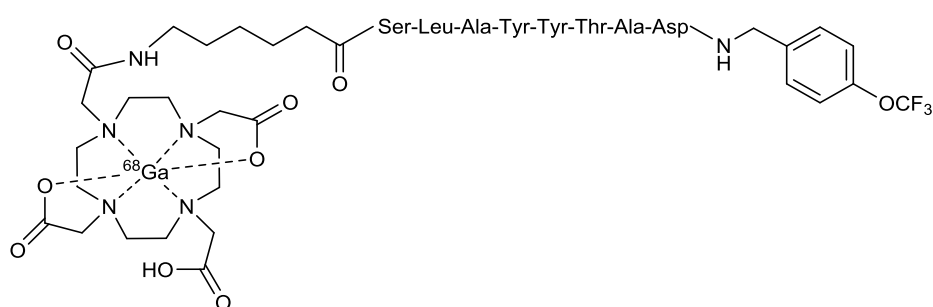


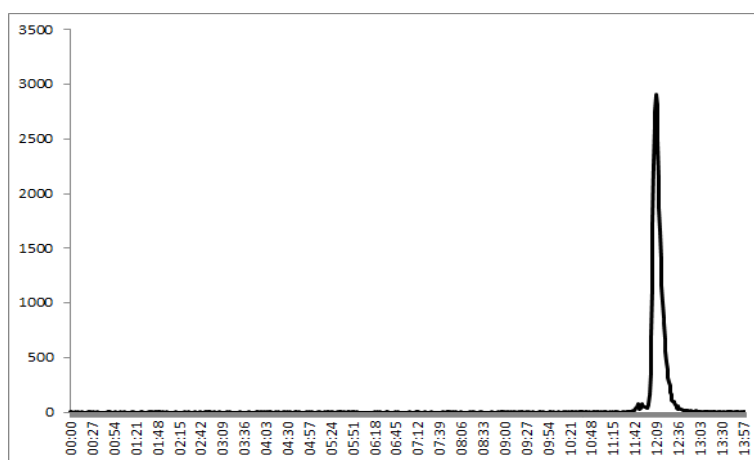
Figure 116- radio HPLC trace of synthesis of **28**, before purification

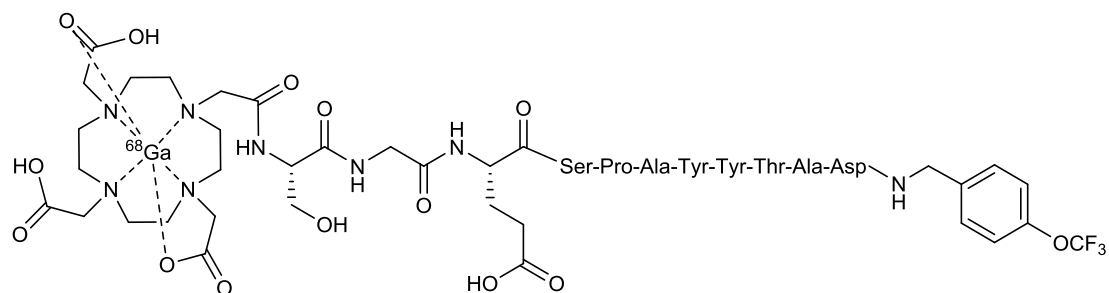
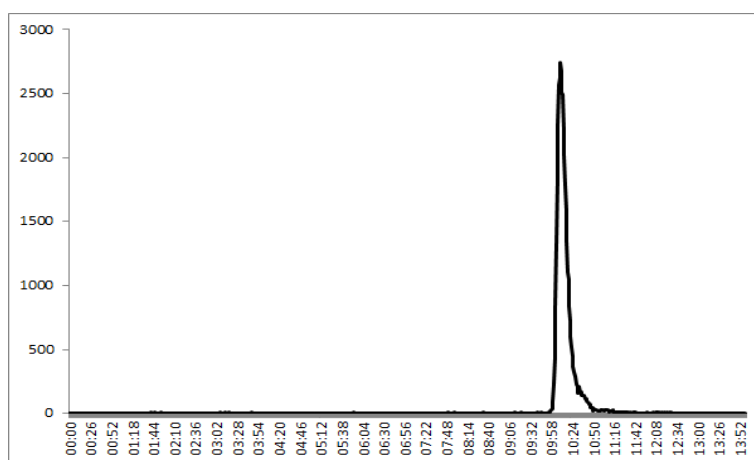
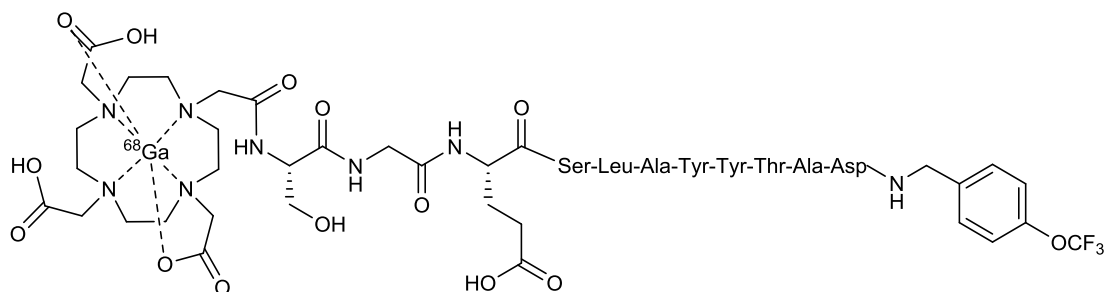
 ^{68}Ga -DOTA-Ahx-SPAYYTAD-Tfb (41)

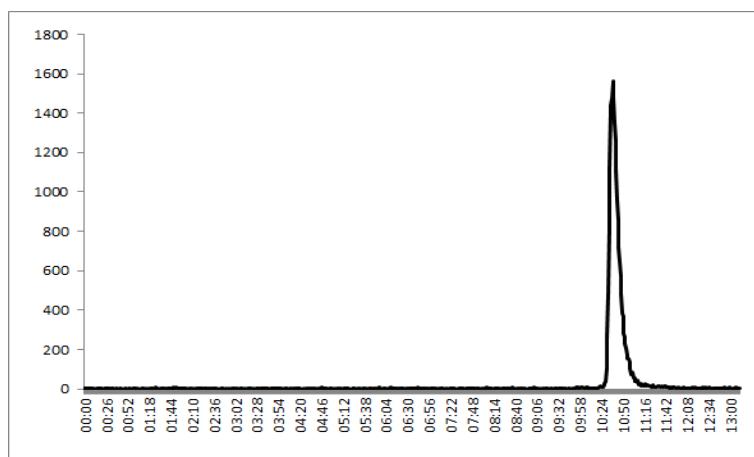
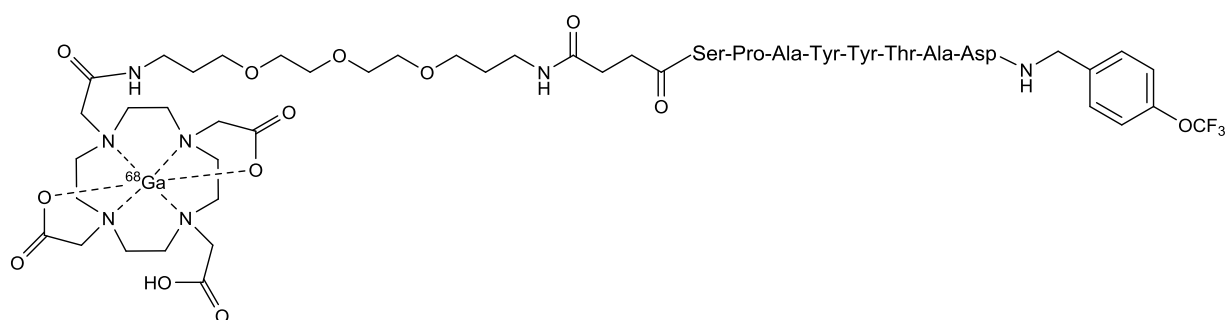
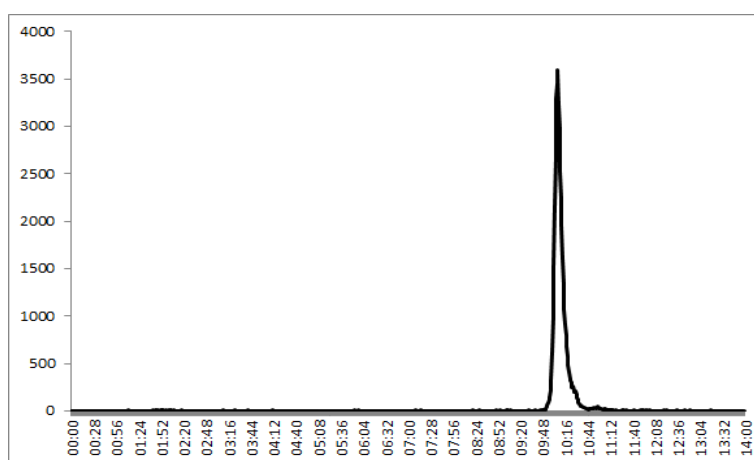
Decay corrected yield: $95.7 \pm 2.5\%$ (n=3)

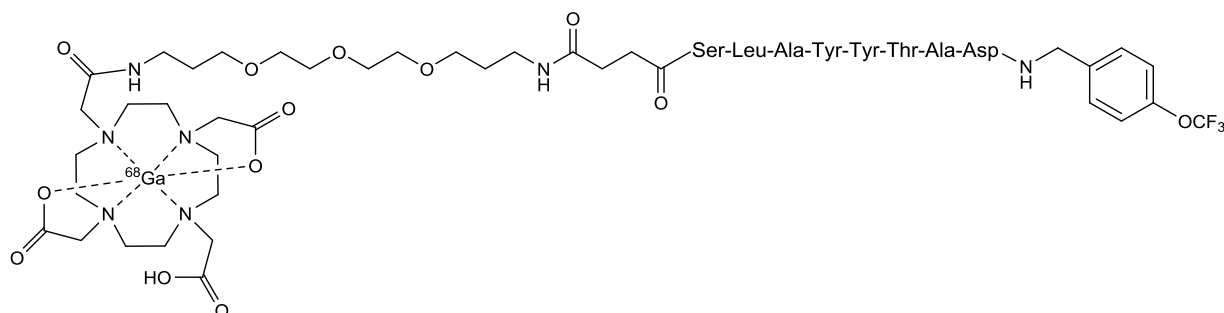
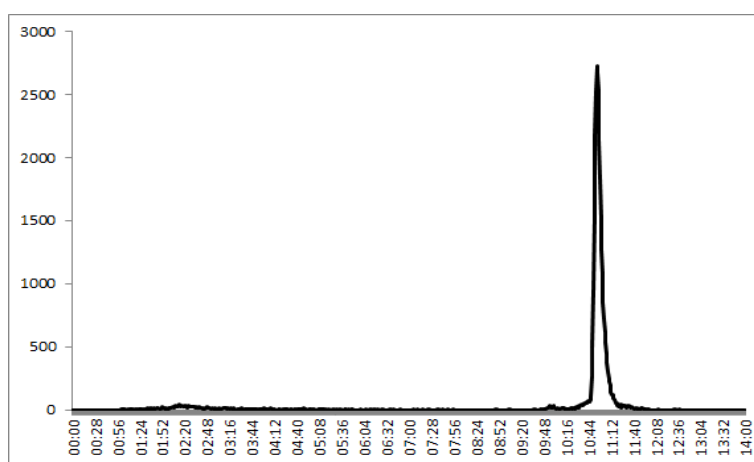
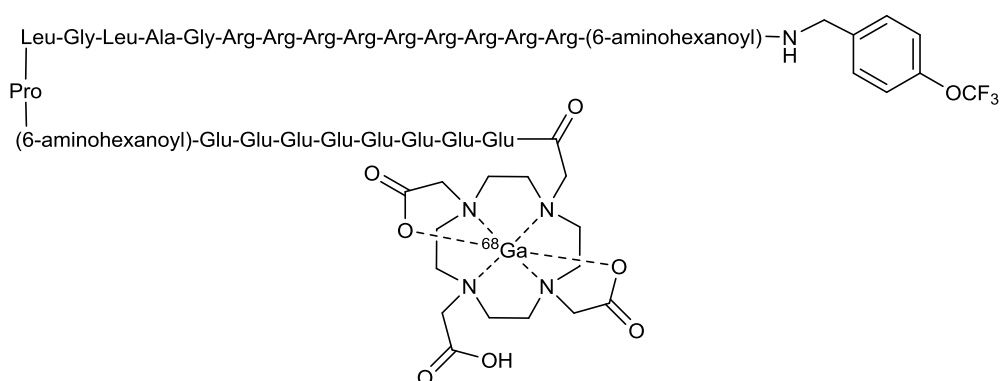
Figure 117- radio HPLC trace of synthesis of **41**, before purification**⁶⁸Ga-DOTA-Ahx-SLAYYTAD-Tfb (42)**

Decay corrected yield: 97.3±0.5% (n=3)

Figure 118 radio HPLC trace of synthesis of **42**, before purification

^{68}Ga -DOTA-SGESPAYYTAD-Tfb (43)Decay corrected yield: $95.0 \pm 4.6\%$ (n=3)Figure 119- radio HPLC trace of synthesis of **43**, before purification **^{68}Ga -DOTA-SGESLAYYTAD-Tfb (44)**Decay corrected yield: $96.0 \pm 3.0\%$ (n=3)

Figure 120 - radio HPLC trace of synthesis of **44**, before purification **^{68}Ga -DOTA-PEG₃-SPAYYTAD-Tfb (45)**Decay corrected yield: $95.3 \pm 3.5\%$ (n=3)Figure 121- radio HPLC trace of synthesis of **45**, before purification

^{68}Ga -DOTA-PEG₃-SLAYYTAD-Tfb (46)Decay corrected yield: $92.0 \pm 2.0\%$ (n=3)Figure 122 - radio HPLC trace of synthesis of **46**, before purification **^{68}Ga -DOTA-(e)₈-Ahx-PLGLAG-(r)₉-Ahx-K-D-Tfb (52)**Decay corrected yield: $95.7 \pm 2.5\%$ (n=3)

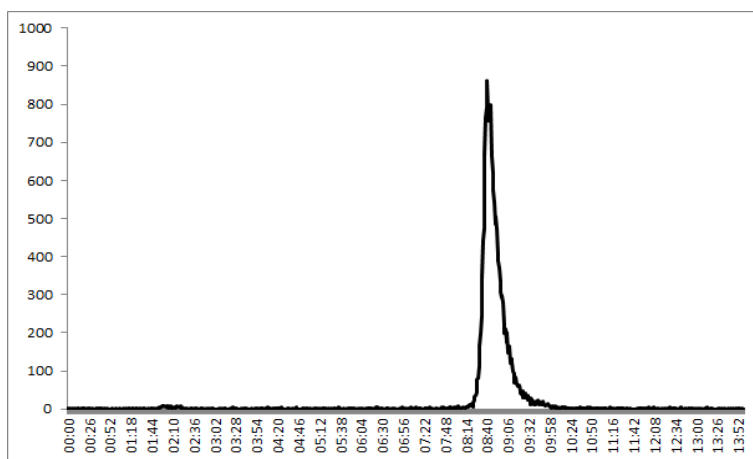


Figure 123- radio HPLC trace of synthesis of **53**, before purification

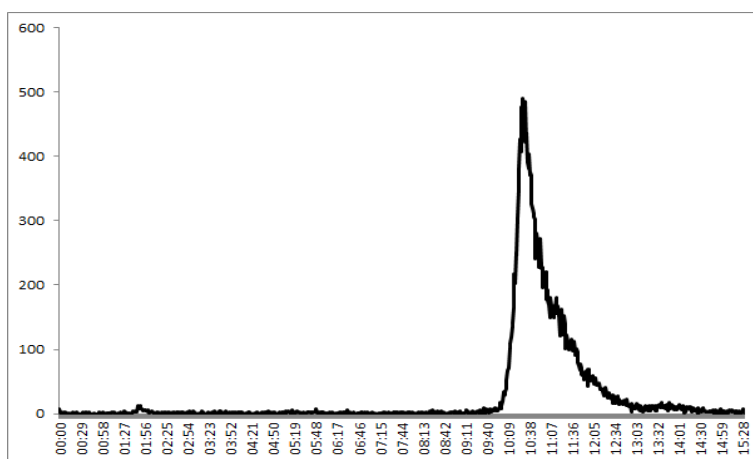
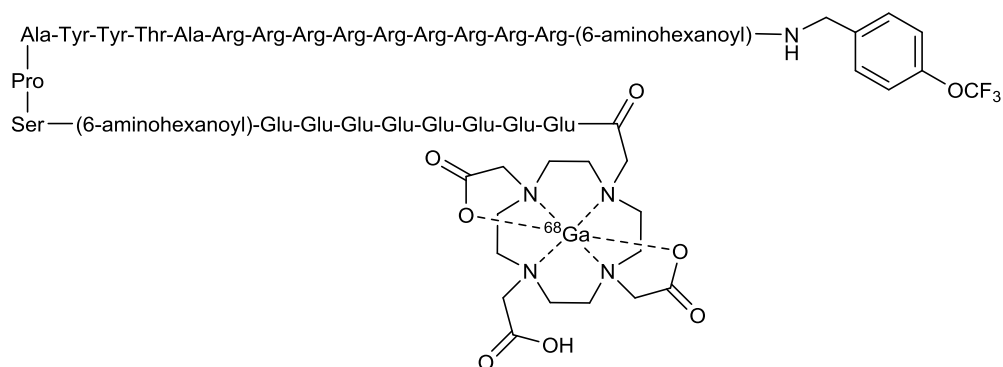


Figure 124- radio HPLC trace of synthesis of **52**, before purification HPLC 10-40% acetonitrile (0.1% TFA)

^{68}Ga -DOTA-(e)₈-Ahx-SPAYYTA-(r)₉-Ahx-K-D-Tfb (53)

Decay corrected yield: $92.7 \pm 5.5\%$ (n=3)

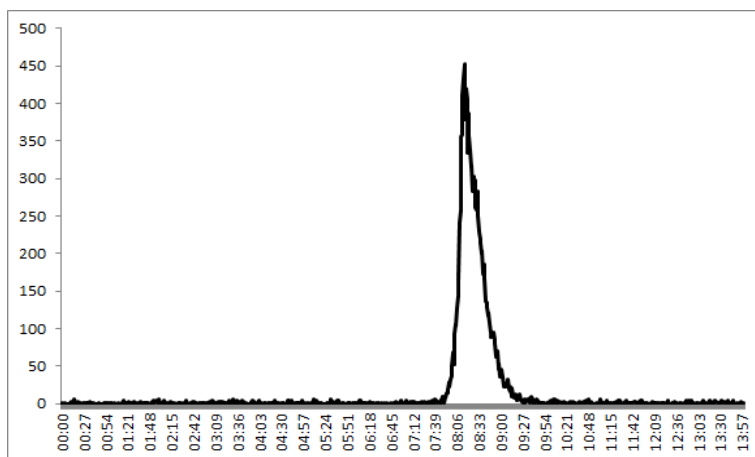


Figure 125- radio HPLC trace of synthesis of **53**, before purification

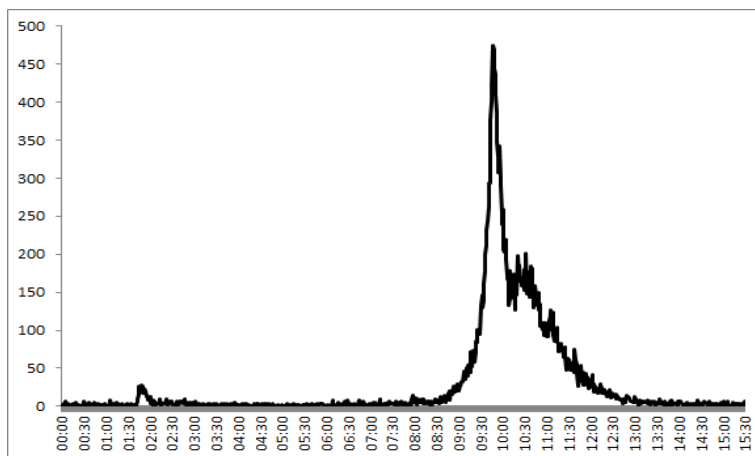


Figure 126 - radio HPLC trace of synthesis of **53**, before purification HPLC 10-40% acetonitrile (0.1% TFA)

Decay corrected yield: $98.5 \pm 0.6\%$ (n=3)

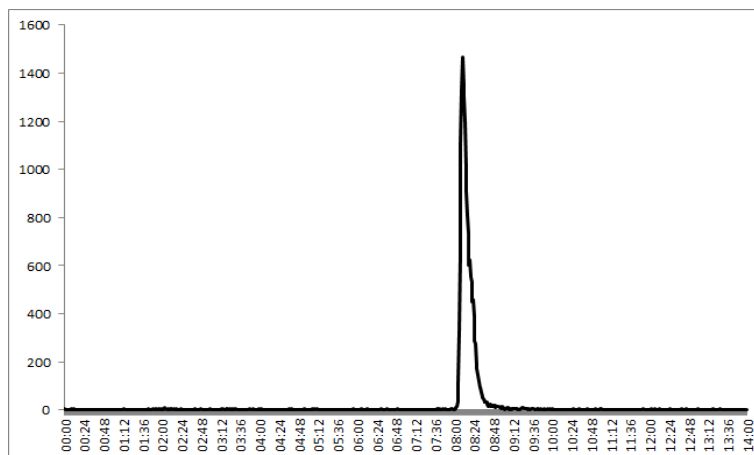


Figure 128 - radio HPLC trace of synthesis of **57**, before purification

5.7 *Enzyme activity assays*

MMP-2 assay positive control compound.

Assay buffer: 50 mM Tris, 10 mM CaCl_2 , 150 mM NaCl, 0.5% Brij-35; pH 7.

Control compound: Mca-Pro-Leu-Gly-Leu-Dnp-Ala-Arg- NH_2 (Mca: (7-methoxycoumarin)acetyl, Dpa: N-3-(2, 4-dinitrophenyl)-L-2, 3-diaminopropionyl).

Fluorescent readings at 320 ex and 405 em.

MMP-2 activation: MMP-2 dissolved to 694 nM in assay buffer and 2.5 mM APMA (final concentration) was added (from stock solution 100 mM in DMSO) and incubation at 37°C took place for 2 hours.

MMP-2 assay: Activated MMP-2 diluted in assay buffer (to 400ng/ml or 5.6nM), 100 μl of MMP-2 added (final concentration 2.8 nM) to 100 μl of fluorescent peptide (final

concentration of 20 μM or 40 μM) in a 96 well plate. Incubation carried out at 37°C with fluorescent signals monitored at 0, 5, 15, 30, 45, 60, 75, 90, 105 and 120 minutes.

Results: As seen in graph below at both concentrations of control compound show fourfold increase in fluorescence over 1 hour of incubation after which time increase in fluorescence begins to plateau indicating majority of cleavage had taken place during this time.

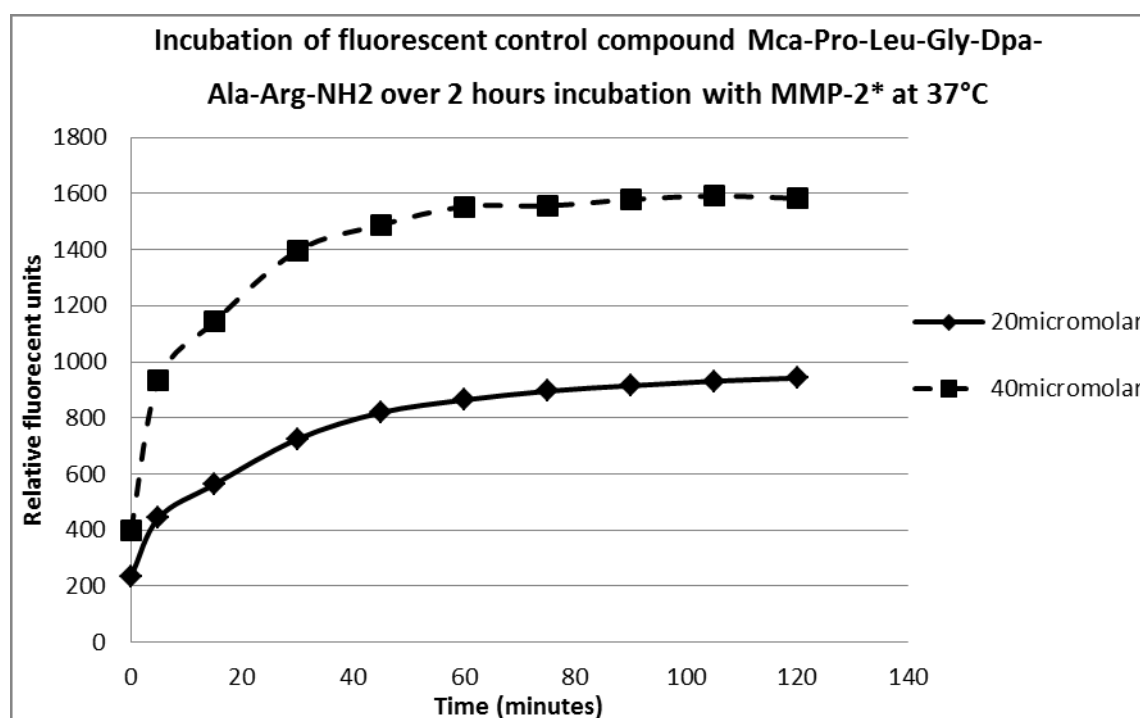


Figure 129- Graph showing the relative fluorescent units over time on incubation of fluorescent control compound Mca- Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ with MMP-2

MMP-2 assay HPLC analysis

Assay buffer: 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.5% Brij-35; pH 7.5

Peptides tested: Gd-DOTA-SPAYYTAD-Tfb

MMP-2 activation: MMP-2 dissolved to 200 nM in assay buffer and 1 mM APMA (final concentration) was added (from stock solution 100 mM in DMSO) and

incubation at 37°C took place for 2 hours. MMP-2 activity was confirmed by incubation with control peptide as described above (fluorescent compound Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ purchased from R&D systems, incubation for 1 hour resulted in a 4 fold increase in fluorescence).

Method A: Peptide was dissolved in assay buffer 20mM stock solution. 100µl of MMP-2 (100 nM) and 100 µL peptide compound (10 mM) were mixed by a short vortex followed by incubation at 37°C for time points 0, 5, 30 and 60 minutes. After incubation 100 µl of phenanthroline (10 mM) was added to reaction mix to quench enzymatic reaction. Analysis was carried out on agilent HPLC (Waters u Bondapak column), gradient in water (0.1% FA) with 0-90% acetonitrile (0.1% FA) over 20 minutes. APMA peak was seen at 3.5, Phenanthroline peak at 9.4 and peptide peak at 13.1, no enzymatic reaction products were visualised, gradient resulted in sloping baseline making results difficult to analyse.

Method B (assay optimisation): Carried out in triplicate. Peptide was dissolved in assay buffer to 80 mM stock solution. 100 µl of MMP-2 (100 nM) and 100 µL peptide compound (40 mM) were mixed together with a short vortex followed by incubation at 37 °C for time points 0, 5, 30 60, 90 and 120 minutes. After incubation 100 µl of phenanthroline (10 mM final concentration) was added to reaction mix to quench enzymatic reaction. Analysis was carried out on Agilent HPLC system (Waters u Bondapak column) with an isocratic method of water (0.1% FA) 75%, acetonitrile (0.1%FA) 25% over 20 minutes. APMA and phenanthroline peak co-eluted at 2 minutes and peptide peak was seen at 6.5 minutes. Expected product (YTAD-Tfb **15**) was tested on the system and peak expected at 4 minutes this was not observed in any of the HPLC traces.

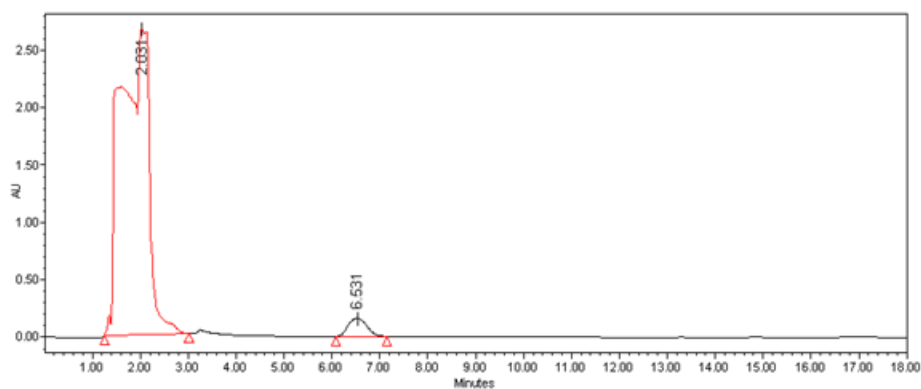


Figure 130- HPLC trace of enzymatic assay at T=0

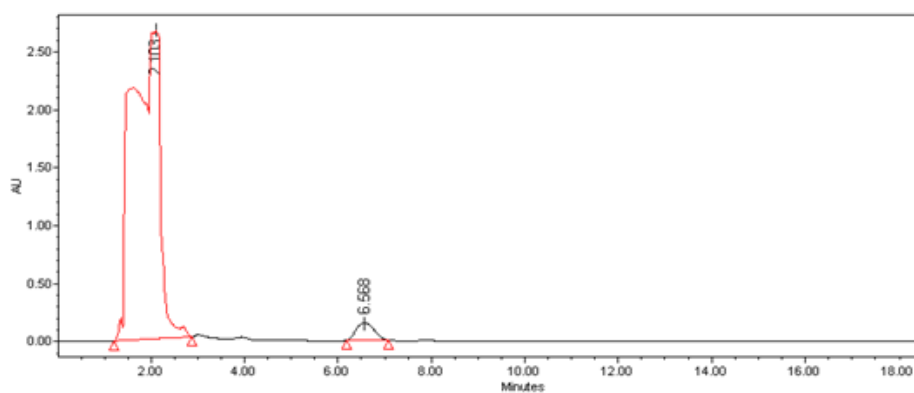


Figure 131- HPLC trace of enzymatic assay at T=120

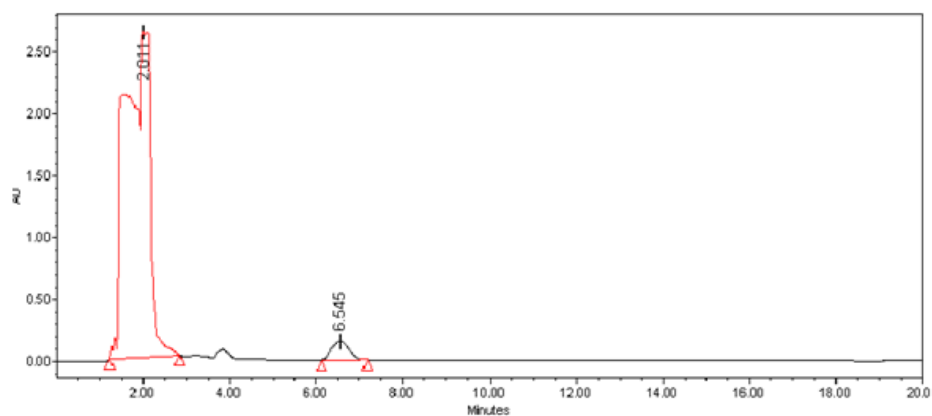


Figure 132- HPLC trace of enzymatic reaction assay of compound **1** (40 μ M) spiked with expected cleavage product **15** (visible between 3.5-4 minutes).

MMP-2 assay fluorescent analysis

Assay buffer: 50 mM Tricine, 10 mM CaCl₂, 150 mM NaCl, 0.5% Brij-35; pH 7.5

MMP-2 activation: MMP-2 dissolved to 694 nM in assay buffer and 2.5 mM APMA (final concentration) was added (from stock solution 100 mM in DMSO) and incubation at 37°C took place for 2 hours. MMP-2 activity was confirmed by incubation with control peptide (fluorescent compound Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ purchased from R&D systems, incubation for 1 hour resulted in 4 fold increase in fluorescence). MMP-2 then was diluted to 5.6 nM ready for MMP assay.

Method: Carried out in triplicate for each peptide. Peptide dissolved in assay buffer 40 mM stock solution. Reaction was carried out in 96 well plates (Corning black). 100 µl of MMP-2 (final concentration, 2.8 nM) and 100 µL peptide compound (final concentration, 20 mM) short vortex followed by incubation at 37°C for time points 0, 5, 30, 60, 90 and 120 minutes. After incubation 50 µl of phenanthroline (10 mM final concentration) was added to reaction mix to quench enzymatic reaction (for Time 0, phenanthroline was added prior to peptide to prevent any reaction) and then 5 mM (final concentration) fluorescamine (in acetone) was added to each well. Fluorescence signals were recorded on a fluorescent plate reader and 1 sample from each triplicate was analysed using Waters HPLC, gradient in water (0.1% FA) with 0-90% acetonitrile (0.1%FA) over 20 minutes. Fluorescence trace read at 390 ex and 475 em. HPLC traces were difficult to analyse due to background signals from assay buffer and MMP-2 activation blocking site of expected cleavage product in most samples. No appearance of significant new peaks was observed in any of the HPLC traces.

MMP assays ⁶⁸Ga labelling- radioHPLC analysis

Assay buffer: 50 mM Tricine, 10 mM CaCl₂, 150 mM NaCl, 0.5% Brij-35; pH 7.5

Radiolabelled peptides: Synthesised and purified as described above into approximately 200 µl of ethanol. This would be diluted by 300 µl of assay buffer.

Method A: MMP-2 activation was carried out as described previously for fluorescence assay. Reaction was carried out in Wheaton vial, 100 µl of MMP-2 (2.8nM final concentration) and 100 µL radiolabelled peptide compound added(15-40 µci), incubation at 37°C for time points 0, 30, 60 and 90 minutes. After incubation peptide was injected into radio-HPLC to monitor formation of new radioactive compounds.

Method Optimisation: using compound ⁶⁸Ga-DOTA-PLGL-Dnp-ARD-Tfb (**28**) testing varying concentrations of MMP-2 (11 nM and 22 nM final concentrations) following the same conditions as described previously. Additional time point of 120 minutes was added. Assay was optimised to result in near complete hydrolysis of this control compound.

Method B (standard protocol following optimisation): MMP-2 activation was carried out as described previously for fluorescence assay; enzyme was diluted to 44 nM. Reaction was carried out in wheaton vial, 100 µl of MMP-2 (22 nM final concentration) and 100 µL radiolabelled peptide compound added(15-40 µci), incubation at 37°C for time points 0, 30, 60, 90 and 120 minutes. After incubation peptide was injected into radio-HPLC, monitoring formation of new radioactive compounds. Simultaneously stability studies were carried out on all peptides, incubating radiolabelled peptide (15-40 µci) in assay buffer without enzyme for 2.5 hours at 37 °C followed by incubation into radio-HPLC.

MMP-9 and MMP-14 assays were also carried out using Method B. Enzyme activation was carried out as follows. MMP-9 was activated using the aforementioned method for MMP-2 but with a longer incubation time of 24 hours. MMP 14 assays required the addition of ZnCl 5 μ M to the assay buffer solution. MMP-14 activation was carried out with MMP-14 dissolved in assay buffer to 166.7 nM (final concentration) and 3.9 nM (final concentration) of trypsin was added, incubation took place at 37 °C for 1 hour. After this time AEBSF 1 mM (final concentration) was added to the mixture and it was left at room temperature for 15 minutes. This could then be diluted to 22 nM ready for assay.

¹⁹F NMR enzymatic assays

Assay protocol: MMP-2 activation was carried out as described previously. Enzymatic reaction was carried out by incubating 100 μ l of MMP-2 and 100 μ l of peptide at 37 °C for 0, 5, 30, 60, 120 minutes and 24 hours, after which time enzymatic reaction was stopped with 200 μ l of a phenanthroline solution (20 mM in buffer and D₂O 2:1). Reaction mixture was then submitted for ¹⁹F NMR with the peptide compound now at a 50 μ M concentration. ¹⁹F peak was seen to get sharper over the enzymatic reaction.

For Gd-DOTA-SPAYYTAD-Tfb (**1**) concentrations used were as follows: peptide 100 μ M and enzyme 45 nM with a final peptide concentration of 50 μ M for NMR, peptide 500 μ M and enzyme 90 nM with a final peptide concentration of 250 μ M for NMR, peptide 500 μ M and enzyme 500 nM with a final peptide concentration of 250 μ M for NMR.

For Gd-DOTA-SLAYYTAD-Tfb (**2**) concentration used was peptide 500 μ M and enzyme 90 nM with a final peptide concentration of 250 μ M for NMR.

Formation of compound **15** on enzymatic reaction was confirmed by LCMS analysis which found small peaks corresponding to **1** and **15** on analysis of high concentration samples.

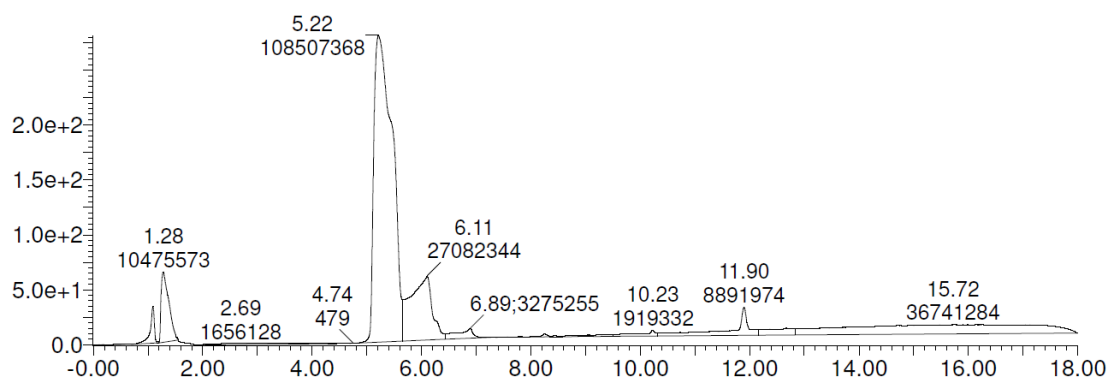


Figure 133- LCMS UV trace of enzymatic assay sample from NMR analysis (500 μ M peptide with 90 nM MMP-2 after 24 hours), peaks at 10.23 corresponding to compound **15**, and peak at 11.90 to compound **1** as confirmed by Mass spectroscopy. (Large peaks around 5.22 correspond to APMA and phenanthroline).

Preliminary study - cell penetration

MMP-2 activation was carried out as previously described for fluorescence assays; enzyme was diluted to 300 nM in assay buffer. 100 μ l of MMP-2 (final concentration 150 nM) was incubated with 100 μ l of compounds **50** and **51** (final concentration 550 μ M) at 37 $^{\circ}$ C for 5 hours. Incubation mixture was then added to plated A431 cells for 1 hour at 37 $^{\circ}$ C. After this, cells were lysed, 400 μ l of this solution was taken, 100 μ l of D₂O was added along with a TFA internal standard (50 μ M final concentration) and samples submitted for 19 F NMR. Control groups included compounds **50** and **51** incubated with cells at the same concentration without pre-incubation with MMP-2, and cell lysates, which were also analysed via 19 F NMR.

Chapter 6

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