THE SYNTHESIS AND BIOLOGICAL EVALUATION OF INTEGRIN RECEPTOR TARGETING MOLECULES AS POTENTIAL RADIOPHARMACEUTICALS

By

Paul Pellegrini B.Sc., M.Sc. (Hons)

A thesis presented to the University of Western Sydney In partial fulfilment of the requirements for the degree of Doctor of Philosophy

October, 2008

©P. Pellegrini October 2008
To the memory of my loving sister Kelly
ACKNOWLEDGEMENTS

The work presented in this Thesis is the result of a collaborative effort between the University of Western Sydney and the Australian Nuclear Science and Technology Organisation (ANSTO), and I wholeheartedly thank both institutions for making this work possible.

Firstly I would like to show my sincere appreciation to my supervisor Associate Professor Janice Aldrich-Wright for her guidance, not only scientifically but also in professionalism and generosity with her time and knowledge. Her enthusiasm and work ethic is an inspiration, and I thank her for giving me this opportunity.

Dr Ivan Greguric of the Radiopharmaceuticals Research Institute (RRI), ANSTO deserves much praise for his co-supervision of this project and his tireless efforts to make materials and equipment available. His organisation and determination are two factors that have kept the project on track, despite the many setbacks we encountered. Ivan’s knowledge of HPLC is second to none and his guidance was a boon considering the heavy use of HPLC purification this project required.

A great deal of thanks goes to my other co-supervisor Dr Andrew Katsifis, Director of the RRI, for accommodating me with laboratory space, access to equipment and generally making himself available to a pesky student while managing an ever expanding research team. His wealth of knowledge in all facets of pharmaceutical development is quite bewildering, so I am indebted to him for his insight and suggestions.

A great deal of other people played important parts in this multidisciplinary project. In terms of the organic synthesis, Dr Branko Dikic, Dr Christopher Fookes,
and Dr Tien Pham provided much helpful suggestions with the synthetic chemistry and result analyses. Mr Timothy Jackson’s experience in NMR, Mass Spectrometry, and particularly in the brutally unforgiving field of rhenium coordination chemistry helped keep me in good spirits.

In terms of the radiochemistry, Dr Nabil Morcos (formerly of ANSTO), Dr Ahmad Nevissi of the University of Washington, Seattle, Dr Le Van So and Dr Myint Zaw provided a wealth of knowledge with regards to isotope production and separation.

For the biological imaging experiments in this project, I would like to acknowledge Mrs Filomena Mattner for her tireless efforts in setting up the in vitro integrin binding assay, and Miss Paula Berghofer, for her expertise in the SPECT animal imaging experiments. Many thanks also go to Dr Vu Nguyen and Dr Katerina Zavitsanou who assisted in the biological experiments.

I would also like to thank Mitchell Quinlivan, Pamela Sumner, Xiang Liu, Thomas Bourdier and Sergio Cruz for the good natured atmosphere they helped to create.

Finally I would like to thank my family and friends for their love and support during this stressful period of my life, but most importantly I would like to dedicate this thesis to my big sister Kelly, who passed away during the final stages of my writing up period. She was a wonderful inspiration throughout my life, and her continual encouragement and support made this work possible.
STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

................................................

Paul Pellegrini
## TABLE OF CONTENTS

1  **INTRODUCTION** ........................................................................................................... 1

1.1  Integrons and Rattlesnakes ......................................................................................... 2

1.2  What is Cancer? ............................................................................................................. 3

1.3  The Mechanisms of Cancer ......................................................................................... 5

1.4  Angiogenesis ............................................................................................................... 6

1.5  Metastasis .................................................................................................................... 8

1.6  Integron Background .................................................................................................. 10

1.7  Integron Structure ...................................................................................................... 10

1.8  Types of Integrons ...................................................................................................... 11

1.9  The $\alpha_{i}\beta_{j}$ Integrin ........................................................................................... 14

1.10  The $\alpha_{i}\beta_{j}$ Integrin ............................................................................................. 14

1.11  Antagonism and Agonism .......................................................................................... 16

1.12  Peptides vs. Peptide Mimetics .................................................................................. 17

1.13  Integrin Antagonists .................................................................................................. 17

1.14  Synthetic Integrin Antagonists ................................................................................... 20

1.15  Current treatments for cancer (83) ............................................................................ 28

1.16  Radiopharmaceuticals ................................................................................................ 29

1.17  Radionuclides ............................................................................................................ 30

1.18  Diagnostic Radiopharmaceuticals ............................................................................. 33

1.19  Imaging Agents .......................................................................................................... 33

1.20  Positron Emission Tomography (PET) ...................................................................... 34

1.21  Single Photon Emission Computed Tomography (SPECT) ................................... 34

1.22  Technetium-99m Radiopharmaceuticals ................................................................... 35

1.23  Therapeutic Radiopharmaceuticals .......................................................................... 38

1.24  Copper Radiopharmaceuticals ................................................................................... 39

1.25  Lutetium as a Potential Radiopharmaceutical ............................................................. 40

1.26  Aims of This Project ................................................................................................... 42

2  **SYNTHESIS** ................................................................................................................ 45

2.1  **INTRODUCTION** ................................................................................................... 46

2.2  **EXPERIMENTAL** .................................................................................................. 50

2.2.1  General .................................................................................................................... 50

2.2.2  Integrin Antagonist Synthesis (Merck Derivative) ................................................... 51

2.2.2.1  Methyl-4-(2-azidoethoxy) benzote [25] ................................................................ 51

2.2.2.2  Methyl-4-(2-aminoethoxy) benzote [26] .......................................................... 51

2.2.2.3  $(S)$-methyl2-(benzoylcarbonylamino)-3-(4-(2-(pyrimidin-2-ylamino)ethoxy)benzamido)propanoate [27] ................................................................. 52

2.2.2.4  $(S)$-2-Amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid [28] ................................................................. 52

2.2.2.5  $(S)$-Methyl-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoate [29] ................................................................. 55

2.2.3  HYNIC-Integrin Antagonist Conjugate ................................................................... 56

2.2.3.1  $(S)$-tert-Butyl-2-((5-(1-methoxy-1-oxo-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propan-2-ylcarbamoyl)pyridin-2-yl)hydrazinecarb-oxylate [30] ........................................... 56

2.2.3.2  $(S)$-2-(6-hydrazinylnicotinamido)-3-(4-(2-(1,4,5,6-tetrahydro-pyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid [31] ........................................... 57

2.2.4  MAMA-Integrin Antagonist Conjugates and Re Complexes .................................. 58

2.2.4.1  Methyl2-((2-oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetate [32] ........................................................................................................ 58
2.2.4.2 2-((2-Oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetic acid [33]……………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………
2.2.6.5 2-(2-(1-(1H-Benz[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid (Cesium carbonate method) [50] ........................................................................................................ 83
2.2.6.6 Methyl-1-(1H-benz[d]imidazol-2-yl)-4,8,11-trioxo-14-(phenylsulfonamido)-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oic acid [51] .................................................................................................................. 84
2.2.6.7 1-(1H-Benzo[d]imidazol-2-yl)-4,8,11-trioxo-14-(phenylsulfonamido)-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oic acid [52] .................................................................................................................. 85
2.2.6.8 Methyl-1-(1H-benz[d]imidazol-2-yl)-14-(benzyloxycarbonylamino)-4,8,11-trioxo-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oate [53] ........................................................................................................ 87
2.2.6.9 1-(1H-Benzo[d]imidazol-2-yl)-14-(benzyloxycarbonylamino)-4,8,11-trioxo-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oic acid [54] ........................................................................................................ 88
2.2.6.10 Oxorhenium (V) 14-(1H-benzo[d]imidazol-2-yl)-1-carboxoxy-4,7,11-trioxo-1-(phenylsulfonamido)-5,10-bis(sulfidomethyl)-6-amide-3,9,12-triaza tetradecane [23] ........................................................................................................ 89
2.2.6.11 Oxorhenium (V) 18-(1H-benzo[d]imidazol-2-yl)-5-carboxoxy-3,8,11,15-tetraoxo-1-phenyl-9,14-bis(sulfidomethyl)-1)-2-oxa-10-amide-4,7,13,16-tetraaza octadecane [24] .......................................................... 90

2.3 RESULTS AND DISCUSSION .................................................................................. 91
2.3.1 Integrin Antagonist Synthesis ......................................................................... 91
2.3.2 Hydrazinonicotinamide (HNIC) BFC Synthesis and Conjugation .............. 97
2.3.3 MonoAmine MonoAmide Dithiol (MAMA) BFC Synthesis and Conjugation .................................................................................................................. 98
2.3.4 Rhenium MAMA Complex Syntheses ........................................................... 100
2.3.5 2,2',2''2'''-(12-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (p-NCS bz TRITA) BFC Synthesis ........................................................................................................ 104
2.3.6 TRITA-Integrin Antagonist Conjugate [45] and corresponding Cu & Lu Complexes [46, 47] ........................................................................................................ 106
2.3.7 'Integrated Approach’ Ligand Syntheses ....................................................... 108
2.3.8 ‘Integrated Approach’ Rhenium Complexes .................................................. 114

3 RADIOCHEMISTRY OF TARGET MOLECULES .................................................. 117
3.1 INTRODUCTION ................................................................................................. 118
3.2 EXPERIMENTAL .................................................................................................. 122
3.2.1 General ............................................................................................................. 122
3.2.2 Iodine\[^{123}\] Labelling ....................................................................................... 124
3.2.2.1 Radiolabelling Integrin Standard with I\[^{123}\] .............................................. 124
3.2.3 Iodine\[^{125}\] Labelling ....................................................................................... 125
3.2.3.1 Radiolabelling Integrin Standard with I\[^{125}\] .............................................. 125
3.2.4 Technetium-99m Labelling ............................................................................. 126
3.2.4.1 Radiolabelling MAMATr2 Integ Acid [38] with Tc -99m .......................... 126
3.2.4.2 Radiolabelling MAMATr2 Integ Acid [38] with Tc -99m and Tc-99g carrier .................................................................................................................. 127
3.2.4.3 Radiolabelling MAMATr2 Caproic Integ Acid [42] with Tc -99m ............. 128
3.2.4.4 Radiolabelling MAMATr2 Caproic Integ Acid [42] with Tc -99m and Tc99g carrier .................................................................................................................. 129
3.2.4.5 Tc-99m labelling of HYNIC Integ ester [30] with tricine co-ligand (no carrier added) .................................................................................................................. 130
3.2.4.6 Tc-99m +Tc99g carrier added labelling of HYNIC Integ ester with tricine coligand .................................................................................................................. 131
3.2.4.7 Tc-99m labelling of HYNIC Integ ester [30] with EDDA coligand .......... 131
LIST OF TABLES

| Table 1.1 | Selected integrin types and known ligands\(^{19}\) | 13 |
| Table 1.2 | Selected \(\alpha_v\) integrin expression and tissue distribution \(^{33}\) | 15 |
| Table 1.3 | Cyclic RGD containing integrin antagonists | 26 |
| Table 1.4 | Selective small molecule integrin antagonists | 26 |
| Table 3.1 | Characteristics of the radioisotopes used in this project | 121 |
| Table 3.2 | HPLC radiolabelling mobile phase profile #1 | 127 |
| Table 3.3 | HPLC radiolabelling mobile phase profile #2 | 130 |
| Table 3.4 | ICP-MS results for 3 separate Lu\(^{177}\) separations | 155 |
| Table 4.1 | Example of \(\gamma\) counter data for Cu TRITA complex \(^{46}\) | 170 |
| Table 4.2 | IC\(_{50}\) in vitro results for the target molecules | 171 |
| Table 5.1 | Raw scintillation data of tumour to non-tumour ratios of SPECT imaging in rats | 196 |
| Table 5.2 | Increase (percentage) uptake in tumour versus non-tumour tissue in RAT SPECT imaging analysis | 197 |

LIST OF SCHEMES

| Scheme 2.1 | Synthetic route to (S)-methyl-2-amino-3-(4-(2-((1,4,5,6-tetrahyd-ropyrmidin-2-ylamino)ethoxy)benzamido)propanoate | 92 |
| Scheme 2.2 | HYNIC synthetic sequence, as developed by Abrams et. al.\(^{86}\) | 98 |
| Scheme 2.3 | Synthetic Route to MAMAtr2 N’-CH\(_2\)COOCH [33] and MAMAtr2 N’-CH\(_2\)CONH(CH\(_2\))\(_5\)COOH [36] | 99 |
| Scheme 2.4 | The synthetic route to \(\rho\) NCS bz TRITA | 104 |
| Scheme 2.5 | Synthetic route to ‘Integrated approach’ ligands | 108 |
**LIST OF FIGURES**

| Fig 1.1 | Benign and malignant tumours are masses of familiar cells (red) derived from a single mutated parent cell, as opposed to the normal tissue comprised of small groups of differing types of cells (blue and white) | 6 |
| Fig 1.2 | Angiogenesis and tumour expansion from Folkman\(^{(11)}\) | 7 |
| Fig 1.3 | Tumour metastasising from the lung (primary site) to the liver (secondary site). Figure reproduced form Bruce et al\(^{(15)}\) | 9 |
| Fig 1.4 | Stylised representation of an \(\alpha_\beta_3\) integrin protein showing its spanning of the cell membrane and the active site shared by both subunits | 12 |
| Fig 1.5 | Scheme representing the known combinations of selected \(\alpha\) and \(\beta\) subunits | 12 |
| Fig 1.6 | The RGD amino acid sequence | 13 |
| Fig 1.7 | The different effects that antagonists and agonists have upon binding to the \(\alpha_\beta_3\) Integrin receptors | 16 |
| Fig 1.8 | Ribbon diagram of domains 7 -10 of Fibronectin, showing the position and (inset) structural rigidity of the solvent exposed RGD loop | 20 |
| Fig 1.9 | Integrin\(^{®}\), a cyclic peptide antagonist for \(\alpha_{IIb}\beta_3\) | 21 |
| Fig 1.10 | Early \(\alpha_\beta_3\) selective antagonists | 22 |
| Fig 1.11 | Tc-99m Radiotracers in clinical use | 37 |
| Fig 1.12 | The tetraaza DOTA, TRITA and TETA macrocycles | 40 |
| Fig 1.13 | The conjugated and integrated design strategies for metallo-radiopharmaceuticals | 43 |
| Fig 2.1 | The conjugate approach to designing \(\alpha_\beta_3\) integrin selective molecules and radiotracers involves the linking of various bifunctional chelators to \((S)\)-2-amino-3-(4-2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido) propanoic acid | 47 |
| Fig 2.2 | The target molecules synthesised for the 'integrated approach' to \(\alpha_\beta_3\) integrin avid molecules | 48 |
| Fig 2.3 | \(^1\)H spectrum of \((S)\)-2-amino-3-(4-2-(1,4,5,6-tetrahydro-pyrimidin-2-ylamino)ethoxy) benzamido) propanoic acid [28] with integration and peak identifications in D\(_2\)O | 96 |
| Fig 2.4 | Chromatogram displaying the mixture of Re MAMA acid [40] at approx. 11 min and Re MAMA Methyl ester complexes [39] at approx. 13.5 min formed from the acid ligand [59] in mildly acidic conditions with methanol present | 103 |
| Fig 2.5 | Chromatogram of Re MAMA caproic complex [42] and time expansion (inset) of the peak revealing an isomeric mixture(Abs at \(\lambda\) 254 nm) | 103 |
| Fig 2.6 | \(^{13}\)C spectrum expansion of the aliphatic region showing the diastereoisomeric CH signals of [54] at 56 ppm] | 111 |
| Fig 2.7 | HMQC expansion of the aliphatic region of [52] showing correlations | 112 |
| Fig 2.8 | \(^1\)H Spectrum of the aliphatic region showing signals identified from the HMQC of [52] | 112 |
| Fig 2.9 | COSY spectrum of the aliphatic region of the diastereoisomeric acids [52] showing connectivities and \(^1\)H assignments | 113 |
Fig 2.10 Chromatogram of the crude reaction solution of [23] with absorption at 254 nm (red) and 354 nm (black) 114

Fig 2.11 Chromatogram of the crude reaction solution of [24] with absorption at 254 nm (red) and 354 nm (black) showing the ES +ve mass spectral analysis of constituents 115

Fig 2.12 Expansion of the isomeric mixture of complex [23] taken from Fig 2.10 116

Fig 3.1 Route to radio-iodination 140

Fig 3.2 Possible Tc-HYNIC chelation modes adapted from Liu et al \(^{155}\) 143

Fig 3.3 Tc HYNIC interg acid EDDA radio chromatogram 144

Fig 3.4 Tc HYNIC integ acid tricine radio chromatogram 144

Fig 3.5 TcMAMA IntegAcid [87] radio chromatogram 148

Fig 3.6 TcMAMA Integ caprico Acid [88] radio chromatogram 148

Fig 3.7 \(\text{Cu}^{64}\) TETA bz integ radio chromatogram 150

Fig 3.8 Chromatographic separation of Lu\(^{177}\) from Yb\(^{175}\) bulk matrix 155

Fig 3.9 Gamma spectra before (a) and after (b) the separation of Lu\(^{177}\) from the irradiated bulk ytterbium/Yb\(^{175}\) matrix 156

Fig 3.10 Lu\(^{177}\) TETA bz integ radio chromatogram 158

Fig 4.1 Saturation binding curves showing the computer aided plot of the specific binding curve (right) derived from the total binding and the calculated non specific binding curves(left) 165

Fig 4.2 \([^{125}\text{I}]\)-L-775.219, the \(^{125}\text{I}\) labelled \(\alpha_\text{v}\beta_3\) integrin antagonist developed by Merck \(^{86}\) 166

Fig 4.3 Specific binding curve obtained for the Cu TRITA complex \(^{46}\) processed from Kell 6 software (Biosoft\(^\oplus\)) 170

Fig 5.1 Positron - electron, annihilation results in two co-incident gamma rays with energies of 511 keV 178

Fig 5.2 A microSPECT camera with movable gantry and operator console 179

Fig 5.3 Schematic of how scintillation detection works 181

Fig 5.4 Transaxial PET image of a nude mouse bearing an M21 cell based tumour injected with \([^{18}\text{F}]\) Galacto-RGD 181

Fig 5.5 SPECT images of mouse injections at various time points with Tc-99m Hynic Integ Tricine \(^{91}\) and Tc-99m HYNIC Integ EDDA \(^{92}\) radiotracers 187

Fig 5.6 SPECT images of mouse injections at various time points with (1) Tc-99m MAMA Integ and (2) Tc-99m MAMA cap Integ radiotracers 188

Fig 5.7 SPECT images of rat injections at various time points with Tc-99m MAMA Integ radiotracer 191

Fig 5.8 SPECT images of rat injections at various time points with Tc-99m MAMA Cap Integ radiotracer 192

Fig 5.9 SPECT images of rat injections at various time points with Tc-99m HYNIC Integ Tricine radiotracer 193

Fig 5.10 SPECT images of rat injections at various time points with Tc-99m HYNIC Integ EDDA radiotracer 194
Fig 5.11  The percentage increase in Tc-99m uptake in tumours compared to non-tumorous tissue

Fig 6.1  Attachment of the BFC to different parts of the antagonist molecule is worthy of investigation, as well as modification of linker length to reduce steric interactions with the parent molecule and the receptor's binding site
ABBREVIATIONS

1-OS  1-octanesulfonic acid
ANSTO  Australian Nuclear Science &Technology Organisation
ARI  Australian Radioisotopes and Industrials
ARPANSA  Australian Radiation Protection and Nuclear Safety Agency
ATCC  American Type Culture Collection
BFC  bifunctional chelator
B_{max}  maximal binding
BOP  Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Bq  becquerel
©  copyright
CAM  chorioallantoic membrane
CD  circular dichroism
Ci  Curie
corr.  corrected
COSY  2D Correlation Spectroscopy
DEPT  Distortionless Enhancement by Polarization Transfer
DNA  deoxyribonucleic acid
DOTA  1,4,7,10-tetraazacyclododecanetetraacetic acid
DTPA  diethylenetriaminepentaacetic acid
EC  electron capture
ECD  ethyl cysteinate diethylester
ECT  Emission Computed Tomography
EDC  1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDDA  ethylenediaminediacetic acid
EDTMP  ethylenediaminetetramethylene phosphonate
EOB  end of bombardment
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMP</td>
<td>extracellular matrix protein</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration (US)</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HATU</td>
<td>o-(7-Azabenzotriazol-1-yl)-N,N,N',N'-trimethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HDEHP</td>
<td>di-(2-ethylhexyl) phosphoric acid</td>
</tr>
<tr>
<td>α-HIBA</td>
<td>α-hydroxyisobutyric acid</td>
</tr>
<tr>
<td>HIFAR</td>
<td>High Flux Australian Reactor</td>
</tr>
<tr>
<td>HMPA</td>
<td>hexamethylphosphoramide</td>
</tr>
<tr>
<td>HMPAO</td>
<td>hexamethylpropylene amine oxime</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Correlation</td>
</tr>
<tr>
<td>HOBt</td>
<td>hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPGe</td>
<td>high purity germanium</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HYNIC</td>
<td>hydrazinonicotinamide</td>
</tr>
<tr>
<td>IC</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>I.D.</td>
<td>internal diameter</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
</tr>
<tr>
<td>IT</td>
<td>internal transition</td>
</tr>
<tr>
<td>keV</td>
<td>kilo electron volt</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>LD</td>
<td>linear dichroism</td>
</tr>
<tr>
<td>LDV</td>
<td>leucine-aspartic acid-valine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low resolution mass spectrometry</td>
</tr>
<tr>
<td>MadCAM</td>
<td>mucosal addressin cell adhesion molecule</td>
</tr>
<tr>
<td>MAG</td>
<td>mercaptoacetylglycine</td>
</tr>
<tr>
<td>MAMA</td>
<td>monoamine monoamide dithiol</td>
</tr>
<tr>
<td>MDP</td>
<td>methylene diphosphonate</td>
</tr>
<tr>
<td>MeV</td>
<td>mega electron volt</td>
</tr>
<tr>
<td>mIBG</td>
<td>m-iodobenzylguanidine</td>
</tr>
<tr>
<td>MIBI</td>
<td>methoxyisobutyl isonitrile</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>nsb</td>
<td>non-specific binding</td>
</tr>
<tr>
<td>NCA</td>
<td>no carrier added</td>
</tr>
<tr>
<td>NCS</td>
<td>isothiocyanate</td>
</tr>
<tr>
<td>Ng-CAM</td>
<td>neuron-glia cell adhesion molecule</td>
</tr>
<tr>
<td>NMM</td>
<td>N'-methyl morpholine</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OPAL</td>
<td>Open Pool Australian Light-water reactor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDA</td>
<td>photodiode array</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet/endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PEEK</td>
<td>Poly(ether-ether-ketone)</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>Psulf</td>
<td>phenylsulfonyl-</td>
</tr>
<tr>
<td>PIC</td>
<td>paired ion chromatography</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>Py-BOP</td>
<td>benzotriazol-1-yl-oxytrityrrolidinophosphonium-hexafluorophosphate</td>
</tr>
</tbody>
</table>
® registered
rac racemic
Rf relative retention factor
RGD argine-glycine-aspartic acid
SA specific activity
SAR structure-activity relationship
SD standard deviation
SPA Scintillation proximity assay
SPECT single photon emission computed tomography
RNA ribonucleic acid
RT room temperature
TETA 1,4,8,11-tetraazacyclotetradecane \(N,N',N''\)-tetraacetic acid
TFA trifluoroacetic acid
TLC thin layer chromatography
TMS tetramethylsilane
Tr trityl or (triphenylmethyl-)
Tricine \(N\)-tris(hydroxymethyl)methylglycine
Tris tris
TRITA 1,4,7,10-tetraazacyclotridecanetetraacetic acid
uncorr. uncorrected
UWS University of Western Sydney
UV ultra violet
V volt
VCAM vascular cell adhesion molecule
Vis visible
Z benzzyloxycarbonyl (cbz)
ABSTRACT

This thesis reports on the synthesis, characterisation and biological evaluation of a number of metal complexes designed to interact with the $\alpha_\beta_3$ integrin receptor, an important biological target that is heavily involved in angiogenesis, and thus cancer related processes.

Two approaches were used to synthesise the integrin-avid targets. The first was to attach a variety of bifunctional chelators (BFC’s) for the incorporation of different metal centres to a known integrin antagonist, L-748,415, developed by Merck. The BFC’s used were the hydrazinonicotinamide (HYNIC) and monoamine monoamide dithiol (MAMA) systems for coordination to Tc-99m and rhenium of which was used as a characterization surrogate for the unstable Tc core. The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (TRITA) BFC was attached for the inclusion of copper and lutetium. This ‘conjugate’ approach was designed to yield information on how the BFC and the linker length would affect the affinity for the integrin receptor.

The second approach was an ‘integrated’ method where the chelation moiety was integral to the biologically relevant part of the molecule, which in the case of the $\alpha_\beta_3$ integrin receptor, is the arginine-glycine-aspartic acid (RGD) mimicking sequence. Two complexes were created with a modified MAMA derivative placed between a benzimidazole moiety (arginine mimic) and the aspartic acid mimicking terminal carboxylic acid to see how it would affect binding while keeping the molecular weight relatively low.

The molecules were tested in vitro against purified human $\alpha_\beta_3$ integrin receptor protein in a solid phase receptor binding assay to evaluate their inhibition constants against a molecule of known high affinity and selectivity in $[^{125}\text{T}]\text{L-775,219}$, the $[^{125}\text{T}]$ labelled $\alpha_\beta_3$ integrin antagonist. The radiolabelled analogues were also tested in vivo against the A375 human melanoma cell line transplanted into balb/c nude mice as well as Fischer rats implanted with MAT BIII rat mammary adenocarcinoma cells. Animals were imaged on a SPECT camera at various time points and compared to normal tissue to yield tumour/non-tumour uptake ratios.
CHAPTER 1

INTRODUCTION
1. INTRODUCTION

1.1 Integrins and Rattlesnakes

In the cool Arizona twilight, *Crotalus atrox* has emerged from its lair and is surveying the desert scrub landscape.\(^1\) Testing the air with sharp flicks of its sensitive tongue, it is able to pick up on a familiar scent. This Western Diamondback, a rattlesnake common to southern United States and Mexico has not eaten for two weeks, but now has his sights fixed firmly on the squirrel that has unwittingly wandered into his territory. Belonging to the group of new world pit vipers, *Crotalus* and many of his relatives have special organs located between the eyes and the nostrils that can detect perturbations in ambient heat as small as 0.003 °C.\(^1,2\) Using a combination of conventional eyesight as well as this sensitive heat vision, the rattler is locked in on his prey and is gently meandering through the rocky moonscape to get its 2 m long body to within striking distance. After pausing for a brief moment *Crotalus* lunges out at the squirrel and punctures its body with two deep hollow fangs, injecting the mammal with a deadly cocktail of proteins and enzymes. The attack is over in a flash; before the squirrel has had time to react the snake has retreated to a safe distance. A rattlesnake’s fangs are very fragile and it cannot afford to risk breaking them in a struggle with a victim, so the reptile hangs back while the venom goes to work.

The squirrel’s body is rapidly succumbing to the poison. Neurotoxins go to work to immobilise the nervous system, but the major components in pit vipers’ venom; hemotoxic proteins, begin to destroy tissue, including blood cells and skin resulting in the destruction of the circulatory system and internal haemorrhaging.\(^3\) Even before the snake has begun swallowing its victim, the squirrel’s internal organs
are already being digested by these molecules designed to dismantle the cellular structures to ease the snake’s digestion. Amongst the various chemicals invading the squirrel’s body are a class of peptides that inhibit the binding properties of specialised adhesive receptor proteins found on the surface of cells. These adhesive proteins are known as integrins, and the peptides that bind them with high affinity have been termed ‘disintegrins’ as a result of their ability to block the integrin’s function.\(^{(4,5)}\) The affinity and specificity that these peptide toxins have for their target receptors is no mere coincidence, complex evolutionary processes have shaped not only the animal’s physical form but also the design of the molecules it employs. This Darwinian trial and error has led to the creation of disintegrins with such potencies that they can nullify the receptors activity at extremely low nanomolar concentrations.\(^{(5)}\) Having now discovered the importance of integrins and their function, scientists have postulated that by designing synthetic molecules with the ability to bind to integrin receptors, it is possible to create a class of pharmaceuticals that can potentially diagnose and treat various diseases and conditions including cancer.\(^{(6,7)}\)

### 1.2 What is Cancer?

The definition of cancer is not a simple task, because one thing that cancer is not is a single, simple disease. Cancer is a group of many related diseases characterised by abnormal cells dividing in an uncontrolled manner. To illustrate this concept, imagine a person getting badly sunburnt. After a short period the exposed skin will begin to peel. When peeling occurs, the outer layer of the skin breaks away and falls off. This outer layer is replaced by a sophisticated repair mechanism which provides the affected areas with enough new skin cells, but, importantly, not too much. The cells stop growing once the repair has been made. This same system is
applicable when a person gives blood. The bone marrow rapidly replenishes the blood, but only to the level that is required by the body. Both examples illustrate the intelligent control system that regulates the cellular generation process. Unfortunately, these mechanisms can become corrupted resulting in cancer growth. All cancers begin in cells and are caused by mutations. Under normal conditions, cells grow and divide into new cells only when the body requires them. Cells that grow old and die are replaced on this basis, but mutations may occur which can lead to the disruption of this process. New cells are created when the body does not need them and the old cells do not die when they should. These cells form a mass of tissue which is commonly called a tumour or neoplasm. If the tumour does not spread to other regions in the body it is classified as ‘benign’, which are rarely threatening to life. However, if the tumour is spreading to other parts of the body it is termed ‘malignant’ and has the ability to be life threatening.

The mutations in the genetic code of the cells which can lead to cancer can be caused by both internal and external factors. Internal factors include; inherited mutations, hormones, immune conditions and metabolic aberrations. External factors include chemical exposure (including smoking), radiation (light and radioactivity), free radicals as well as biological vectors such as viri. Even relatively non-reactive materials such as silica and asbestos can lead to cancerous activity.

Roughly twenty percent of all cancer victims in developed nations will die as a result of the illness. The incidents of cancer are higher now than they have ever been and this is reflected in the populations increased lifespan and exposure to harmful external stimuli. Cancer has become the second greatest killer in industrialised societies, only heart disease ranks higher.\(^8\) In an effort to counter this, more and more resources are being poured into cancer control, treatment and
Chapter 1

research, more so than practically any other diseases in their entire histories. Thousands of compounds are being tested each year to find effective anti-cancer drugs.

Despite the fantastic inroads researchers and doctors have made into combating cancer, it is in many ways just as mysterious as it was in the days when Hippocrates, the father of western medicine, first diagnosed it over 24 centuries ago.

1.3 The Mechanisms of Cancer

In order to gain an insight into cancer, there is a need to understand its progression from a single cell mutation to a life threatening disease. When a mutation occurs, the genetic integrity of a cell is compromised. More specifically the deoxyribonucleic acid (DNA) structure which codes for the genetic information has changed. By processes of DNA replication, transcription of ribonucleic acid (RNA) from the DNA template and the translation of messenger RNA into protein, the genetic aberrations can be propagated to the cell progeny and manifested in the expression of abnormal gene products. The resulting cancer cell may take on special properties that enable it to replicate faster than normal cells, or even elaborate substances such as ectopic hormones, which can lead to the loss of the cell’s differentiation.\(^{(9)}\) Having acquired metabolic capabilities that are lacking in the parent tissue, the cancer cell divides rapidly until a tumour is formed consisting of the aberrant cells. Early on in the tumour’s formation the cells seem to retain a responsiveness to host control mechanisms such as hormones, but as the cell deviates further from the parent tissue it loses this responsiveness and a stage of independent growth ensues. The resulting tissue loses its mosaic structure as shown in Fig 1.1.
Figure 1.1 Benign and malignant tumours are masses of familiar cells (red) derived from a single mutated parent cell, as opposed to the normal tissue comprised of small groups of differing types of cells (blue and white).

1.4 Angiogenesis

Research into cancer development has revealed that tumours generally develop in stages. The aberrant cells proliferate amongst the normal parent cells until the tumour reaches a certain size and ceases to enlarge. Scientists describe this as a tumour in situ. The reason for the size stabilisation is due to the fact that the number of cells that are being created is counter-balanced by the amounts that are dying. An in situ tumour is generally made up of a few million cells and a tumour can stay in this stage for years in some circumstances. The size limitations imposed on an in situ tumour are on the basis of cell nutrition. The tumour’s cells can rely on the diffusing nutrients and oxygen reaching it if it is within 150 μm of a blood vessel, so this effectively keeps the tumour at a constant size and proximity from the capillary. For a tumour to exceed this size, a new source of nutrition is required, because, as the volume of the tumour increases in three dimensions, the surface area only increases...
by the second power. The surface area dictates the supply of diffusible nutrients and oxygen and also the ability of the cell to expel catabolic breakdown products. This leads to a discrepancy between the demand and supply of nutrients. In order for the continued growth of a tumour beyond this size, new blood vessels are induced to meet the nutrient demand, and within a short time of their formation, the necrotic part of the tumour disappears. Angiogenesis is the term that describes this new capillary growth and it is an integral part of the wound healing process, embryonic development as well as malignant tumour expansion as detailed in Fig 1.2. Angiogenesis is vital to the continued growth of tumours. For example, when a Brown-Pearce tumour is implanted into the anterior chamber of a guinea pig’s eye, near the iris, the tumour remains small, but upon vascularisation will grow to 16,000 times larger over the ensuing two week period. To further illustrate the importance of angiogenesis in tumour development, studies involving chick embryo tumour implants revealed that a stable diameter of 0.93 mm can be achieved in the avascular

**Figure 1.2** Angiogenesis and tumour expansion from Folkman^{(11)}
state, but within 7 days of vascularisation the size was increased to 8 mm, and also leading to the complete disappearance of the necrotic region within 48 h of the capillary formation.\textsuperscript{(12)}

During the abrupt increase in tumour size, and capillary formation, the tumour releases tumour antigens\textsuperscript{(13)} into the blood as well as metastatic cells and this progression is commonly referred to as ‘malignant drift,’ a term denoting the tumour’s adoption of a more malignant behaviour pattern.\textsuperscript{(14)}

1.5 **Metastasis**

Metastasis describes the process in which malignant cancer cells spread from one part of the body (primary site) to another region (secondary site) as shown in Fig 1.3. In fact, it is this potential to spread which makes the cancer malignant. The malignant cells can travel via the bloodstream or lymphatic system through the body and establish themselves in a suitable environment for growth. Generally speaking, the larger a primary tumour is the greater the chance of metastasis, which is the major reason for the importance of early detection and treatment. When a tumour has progressed to the metastatic stage the patient’s chance of survival is greatly reduced, as it is virtually impossible to know the locations of the developing secondary sites and thus treat them accordingly.

It should be noted that metastasis is not necessarily a property that is unique to malignant cells. Similar cell movements correspond to migrations of neuroblasts, melanoblasts and primordial germ cells in embryos. Lymphocytes created in the bone marrow also metastasise through the bloodstream to the lymphoid tissue throughout the body where they perform their immunological purposes. In these two cases the metastasis is a balanced and strictly controlled normal function, but
malignant metastasis is an altered control of a normal process in wide dissemination and the continuing capacity for tumour cell proliferation result in the death of the host.

Figure 1.3  Tumour metastasising from the lung (primary site) to the liver (secondary site). Figure reproduced from Bruce et al.\(^ {15}\)

Metastasis is a rather complex multi-stage process. Before cells can invade and metastasise, they must be able to dislocate from the primary mass. Metastatic cells are able to do this by the degradation of the matrix by proteolytic enzymes. Once freed from the primary site the cells can travel through the body and adhere to a foreign suitable environment. Usually it is a low percentage of cells that survive this journey and go on to form metastases. The success of these cells is dependent on their ‘stickiness’ as they must adhere to the new matrices. This property which is a
prerequisite for metastases is attributed to many surface receptors and the integrin glycoproteins fall into this category.

1.6 Integrin Background

Integrins were discovered primarily as a result of the intense research into the isolation of several cell surface receptors performed in the mid 1980s. Scientists had partially determined some of the amino acid sequences in some of these proteins, and the accumulated knowledge revealed that the receptors belonged to a very big family of structurally similar molecules found on virtually every type of cell in the animal kingdom. The receptors were named ‘integrins’ in 1987 by Hynes in recognition of their importance to the structural integrity of cells and related tissue. Research into integrins increased dramatically, and although much of their structure and function still remain a mystery, scientists have pieced together a respectable knowledge base and are continually demonstrating the importance of the integrin receptor as a pharmaceutical target.

1.7 Integrin Structure

Integrins are a class of heterodimeric transmembrane cell surface receptors that are vital in cell-cell and cell-matrix adhesion processes (Figure 1.4). They are composed of an $\alpha$ and a $\beta$ subunit linked non-covalently. Currently, there are over 26 integrin receptors known consisting of various combinations of at least 19 $\alpha$ and 8 $\beta$ subunits as shown in Fig 1.5. $\alpha$ Subunits usually only associate with certain $\beta$ subunits, whereas $\beta$ subunits tend to be less restricted. All $\alpha$ subunits display high sequence homology and are similarly structured. Their sizes vary from 120 -180 kDa
and possess seven 60 amino acid long tandem repeats, with the C-terminal 4 binding divalent cations. Many α subunits are cleaved during biosynthesis at a site close to the trans-membrane domain. β Subunits are also quite similar to each other, but they tend to be smaller, varying from 90 - 110 kDa. They have a high proportion of cysteine residues concentrated in four repeat domains. The N-terminal domain is disulfide bonded to the N-terminus of the β chain. Importantly, cross-linking investigations have shown that both subunits are involved in ligand recognition. Ligands are primarily extracellular matrix proteins which include intracellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), laminins, collagens and a variety of other proteins (Table 1.1)

1.8 Types of Integrins

To date, the majority of research into the integrin super family has been focused on the αvβ3 and the αIIbβ3. Sharing the β3 sub-unit, these two integrins possess an extremely similar ligand binding site resulting in a variety of ligands that can bind to both receptors. Among many of the integrin heterodimers, the αvβ3 and the αIIbβ3 possess a binding site that has a high selectivity and affinity for ligands containing an arginine-glycine-aspartic acid (RGD) amino acid sequence on the polypeptide chain (Fig 1.6). Ligand binding is affected by the distance between the guanidine and carboxylic acid group as well as the structures surrounding the RGD sequence. Since the ligand recognition site spans both sub-units (Fig 1.9), and they both possess the same β sub-unit, the selectivity of ligands for these similar integrins is dependent on the differing structural character of the α sub-unit.
Figure 1.4  Stylised representation of an $\alpha_\nu \beta_3$ integrin protein showing its spanning of the cell membrane and the active site shared by both subunits.

Figure 1.5  Scheme representing the known combinations of selected $\alpha$ and $\beta$ subunits. Each integrin consists of one $\alpha$ and one $\beta$ subunit linked non-covalently. For example $\alpha_\nu$ has been found to combine with $\beta_1$, $\beta_3$, $\beta_5$, $\beta_6$ and $\beta_8$, but not with $\beta_2$.\(^{(17)}\)
Table 1.1  Selected integrin types and known ligands \(^{(19)}\)

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1\beta_1)</td>
<td>Laminins, collagens I/IV</td>
</tr>
<tr>
<td>(\alpha_2\beta_1)</td>
<td>Collagens, laminins, tenascin</td>
</tr>
<tr>
<td>(\alpha_3\beta_1)</td>
<td>Laminin, epiligrin, collagens, fibronectin, entactin</td>
</tr>
<tr>
<td>(\alpha_4\beta_1)</td>
<td>Fibronectin, VCAM-1</td>
</tr>
<tr>
<td>(\alpha_5\beta_1)</td>
<td>Fibronectin, L1/Ng-CAM, tenascin, invasin</td>
</tr>
<tr>
<td>(\alpha_6\beta_1)</td>
<td>Laminin, merosin, kalinin, invasin</td>
</tr>
<tr>
<td>(\alpha_7\beta_1)</td>
<td>Laminins</td>
</tr>
<tr>
<td>(\alpha_8\beta_1)</td>
<td>Fibronectin, tenascin, vitronectin</td>
</tr>
<tr>
<td>(\alpha_9\beta_1)</td>
<td>Collagen I, laminin, tenascin, VCAM-1</td>
</tr>
<tr>
<td>(\alpha_{11}\beta_1)</td>
<td>Collagen</td>
</tr>
<tr>
<td>(\alpha_{L}\beta_2) (LFA1)</td>
<td>ICAM-1, ICAM-2, ICAM-3</td>
</tr>
<tr>
<td>(\alpha_{M}\beta_2) (Mac1)</td>
<td>Fibrinogen, C3b, ICAM-1, X-factor</td>
</tr>
<tr>
<td>(\alpha_X\beta_2)</td>
<td>Fibrinogen, C3b</td>
</tr>
<tr>
<td>(\alpha_{G}\beta_2)</td>
<td>ICAM-1, VCAM-1</td>
</tr>
<tr>
<td>(\alpha_{IIb}\beta_3)</td>
<td>Fibrinogen, fibronectin, vitronectin, thrombospondin, von Willebrand factor</td>
</tr>
<tr>
<td>(\alpha_{III}\beta_3)</td>
<td>Vitronectin, fibrinogen, fibronectin, osteopontin, thrombospondin, denatured collagens, tenascin, von Willebrand factor, laminin, PECAM-1, L1-CAM</td>
</tr>
<tr>
<td>(\alpha_4\beta_4)</td>
<td>Laminin, kalinin</td>
</tr>
<tr>
<td>(\alpha_4\beta_5)</td>
<td>Vitronectin, osteopontin, fibronectin</td>
</tr>
<tr>
<td>(\alpha_4\beta_6)</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>(\alpha_4\beta_7)</td>
<td>Fibronectin, VCAM-1, MadCAM-1</td>
</tr>
<tr>
<td>(\alpha_{IEL}\beta_7)</td>
<td>E-cadherin,</td>
</tr>
<tr>
<td>(\alpha_4\beta_8)</td>
<td>Fibronectin, vitronectin</td>
</tr>
</tbody>
</table>

It should be noted that not all integrins are receptive to the RGD peptide sequence.

The \(\alpha_4\beta_1\) for example, binds and recognises the leucine-aspartic acid-valine (LDV) sequence within the CS-1 region of fibronectin.

![The RGD amino acid sequence](image)

**Figure 1.6**  The RGD amino acid sequence
1.9 The $\alpha_{\text{IIb}}\beta_3$ Integrin

The $\alpha_{\text{IIb}}\beta_3$ integrin, also known as the fibrinogen receptor or Glycoprotein IIb/IIIa, plays a very important role in wound healing. It is mainly expressed on megakaryocyte derived cells such as platelets as well as melanoma cells.\(^{(20)}\) This integrin binds with varying affinity to a variety of extracellular proteins, which include vitronectin, fibronectin, von Willebrand factor, thrombospondin and of course fibrinogen.\(^{(17)}\) The $\alpha_{\text{IIb}}\beta_3$ integrin is heavily involved in primary and secondary homeostasis. It mediates the cross-linking of platelets at sites of vascular injuries by binding to the collagen filaments in the connective tissue at the rupture crease. A blood clot is then formed by the fibrinogen induced platelet mass to seal the injured tissue.\(^{(21)}\) Defective clot formation occurs in strokes, embolies, deep vein thromboses as well as myocardial infarction, so it is not surprising to see that the $\alpha_{\text{IIb}}\beta_3$ integrin has been targeted for such therapies, including a number involved in clinical trials.\(^{(22-27)}\)

1.10 The $\alpha_v\beta_3$ Integrin

In contrast to the $\alpha_{\text{IIb}}\beta_3$ integrin, the $\alpha_v\beta_3$ or vitronectin receptor is more widely distributed throughout the body (Table 1.2). Like many of the integrins, it can host numerous extracellular matrix proteins (EMPs) which include vitronectin, fibrinogen, fibronectin and osteopontin. The $\alpha_v\beta_3$ integrin is expressed in almost all of the cells originating in the mesenchyme, playing important roles in vascular cell biology.\(^{(7)}\) The $\alpha_v\beta_3$ integrin is implicated in many pathological processes, such as osteoporosis, aberrant angiogenesis (rheumatoid arthritis), tumour development as well as tumour metastasis. The $\alpha_v\beta_3$ integrin is upwardly expressed in many tumour cells such as osteosarcomas, neuroblastomas, various carcinomas including lung,
breast, prostate and bladder, glioblastomas and invasive melanomas.\(^{28-31}\)

Of extreme importance to scientists, it has been found that this particular receptor is linked to tumour-induced angiogenesis. In a study employing the chick chorioallantoic membrane model, inhibition of the \(\alpha_\nu \beta_3\) on the endothelial cells resulted in a drastic reduction of neovascularisation leading to starvation of the tumour.\(^{32}\) The inhibition of the integrin receptor was affected by monoclonal antibodies and peptides that are antagonists for \(\alpha_\nu \beta_3\).

Table 1.2 Selected \(\alpha_\nu\) integrin expression and tissue distribution\(^{33}\)

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Cell/Tissue distribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_\nu \beta_1)</td>
<td>Fibroblasts</td>
<td>(34)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td>(35)</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>Tumour cells</td>
<td></td>
<td>(37)</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>(\alpha_\nu \beta_3)</td>
<td>Endothelial cells</td>
<td>(39)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td>(35)</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td></td>
<td>(40)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td></td>
<td>(41)</td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td>Tumour cells</td>
<td></td>
<td>(43)</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>(\alpha_\nu \beta_5)</td>
<td>Endothelial cells</td>
<td>(44)</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td>(35)</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td></td>
<td>(45)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td></td>
<td>(41)</td>
</tr>
<tr>
<td>Carcinoma cells</td>
<td></td>
<td>(46)</td>
</tr>
<tr>
<td>(\alpha_\nu \beta_6)</td>
<td>Epithelial cells</td>
<td>(45)</td>
</tr>
<tr>
<td>Carcinoma cells</td>
<td></td>
<td>(45)</td>
</tr>
<tr>
<td>(\alpha_\nu \beta_8)</td>
<td>Placenta</td>
<td>(47)</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Ovaries</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Melanoma cells</td>
<td></td>
<td>(48)</td>
</tr>
</tbody>
</table>
1.11 Antagonism and Agonism

In the field of drug development and evaluation, the targeting of a particular receptor by a ligand can have varying effects. The terms antagonist and agonist refer to the positive and negative effects that the ligand binding can have on the receptor function. An antagonist is a ligand that can reduce or totally inhibit a receptor’s ability to perform a task. An agonist, in contrast is a ligand that binds to a receptor to activate a specific response. Agonists are usually the natural ligand for a receptor. In the case of the integrin, the agonist vitronectin binds to the $\alpha_v\beta_3$ receptor by recognition of the RGD sequence. Angiogenesis is the resulting cellular response. If an antagonist binds instead, the receptor recognises the RGD sequence, but binding does not lead to angiogenesis (Fig 1.7). In the case of inhibiting tumour progression through angiogenic pathways, antagonists to the $\alpha_v\beta_3$ integrin have been proposed as therapeutic agents.

![Diagram showing agonist and antagonist binding to $\alpha_v\beta_3$ integrin and resulting angiogenesis versus no angiogenesis.]

**Figure 1.7** The different effects that antagonists and agonists have upon binding to the $\alpha_v\beta_3$ Integrin receptors
1.12 Peptides vs. Peptide Mimetics

An increasingly important phase in the development of pharmaceutical targets is the transition from peptide based lead structures to orally administrable peptide mimetic (peptide mimicking) drugs. Unfortunately, peptides are easily broken down in the body by proteases. Peptide mimetics are designed to have greater stability in vivo, thus giving the agent a longer time to perform a biological task. Another important reason for the move to peptide mimetics is the ability of the scientist to control the polar character and size of the molecule. Rapid excretion of compounds with molecular weights of between 500 and 1000 Da is an inevitable phenomenon either via the kidneys or the liver/bile pathways depending on the hydrophilic/lipophilic profile of the molecule. Scientists usually look to smaller non-peptide targets to overcome these difficulties. Of course, oral administration is not the only possible means of delivering a pharmaceutical. Intravenous, intraperitoneal or subcutaneous injections are options depending on the disease/condition, all the while still considering the polarity of the target environment.

1.13 Integrin Antagonists

There is an ever increasing body of knowledge relating to the inhibitors of integrins functions. The aforementioned disintegrins present in various vipers’ venoms have been shown to be very potent antagonists of the α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{5}β\textsubscript{1} and α\textsubscript{v}β\textsubscript{3} integrins. In fact, echistatin, a commercially available standard employed in binding assays to evaluate and compare potential integrin antagonists is derived from the venom of the Saw-scaled viper Echis carinatus. As previously mentioned,
antagonists of $\alpha_{\text{IIb}}\beta_3$ like echistatin, are efficient at preventing the binding of adhesion proteins such as fibrinogen to the integrin’s recognition site. In doing this it neutralises the integrin’s ability to mediate the formation of fibrous cross links across platelets, thus reducing the possibility of any embolic type activity. Platelet aggregation inhibition and other related thrombolytic functions are the most commonly sought applications for integrin antagonists, but it is by no means the only one. Scientists have found a whole host of ways in which various integrins can be targeted for therapeutic intervention. Inhibitors of $\alpha_v\beta_3$ could potentially be used in the treatment of osteoporosis as well as other bone related illnesses. Osteopontin is a natural ligand to $\alpha_v\beta_3$, and in this case the primary agonist in this process. Since the $\alpha_v\beta_3$ integrin has been shown to be present in osteoclasts (bone resorption cells) but not osteoblasts (bone formation cells), therapeutic intervention involving $\alpha_v\beta_3$ antagonists would limit the bone catabolism and thus increase the bone density.\(^{51,52}\) An equally important intended application of inhibitors of $\alpha_v\beta_3$ is for the diagnosis and treatment of tumours.

Having established the fact that the receptor is highly expressed in the tumours endothelial cells and indeed sites of angiogenesis, there are a number of agents that have been shown to reduce the amount of neovascularisation \textit{in vivo}. In the evaluation of integrin antagonists, researchers have generally compared the binding of the antagonists to both the $\alpha_v\beta_3$ and the $\alpha_{\text{IIb}}\beta_3$ receptors \textit{in vitro}.\(^{53-55}\) This active area of work has detailed the important characteristics of the ligand receptor relationship that discriminate between the two commonly researched integrin subtypes. The vitronectin and fibrinogen receptors both share the $\beta_3$ subunit and as a consequence both recognise the RGD sequence on the binding ligands. Investigations into the binding of ligands to $\alpha_{\text{IIb}}\beta_3$ have proven that the distance between the
relevant pharmacophores (in this case the carboxyl group of the aspartic acid and the guanidine group of the arginine) is critical for activity and specificity. The reasoning behind this discrimination is believed to result from differences in the tertiary structure involved in the integrin region about the RGD recognition site.\(^{(56-58)}\) Most crystal and NMR structures of RGD containing proteins have shown a relatively flexible loop in the case of agents that bind to $\alpha_{\text{IIb}}\beta_3$. NMR studies of the potent $\alpha_{\text{IIb}}\beta_3$ antagonist echistatin for example, have shown to have eight different conformations consisting of a relatively constant globular head but with significant variation about the RGD loop.\(^{(59)}\)

Structural information concerning $\alpha_v\beta_3$ specific ligands has shown some contrasting results. Fibronectin is a protein that can bind various integrins including $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ receptors, although an $\alpha_v\beta_3$ specific site is located on its tenth domain. Crystallographic and NMR data on this tenth domain of fibronectin show a well resolved and rigid solvent exposed RGD loop\(^{(60)}\) as shown in Fig 1.8. Crystal and NMR data from decorsin, another snake venom disintegrin,\(^{(61)}\) the binding domain of tenascin,\(^{(62)}\) as well as $\alpha_v\beta_3$ specific mutant human lysozyme\(^{(63)}\) also support this rigidity about the loop, particularly with a beta turn around the gly-asp moiety. From this structural information, researchers have armed themselves with a relatively clear picture of what is required for the design of agents to bind to, and discriminate between the $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin receptors.
1.14 Synthetic Integrin Antagonists

In light of the importance shed on the integrin’s functions, scientists have made significant inroads into inhibiting their function with synthetic peptides and peptide mimetics. Having established the reasoning for creating peptide mimetics, various research groups have set about testing novel molecules designed to aid in the efficient delivery of such agents to certain target areas.

The first synthetic RGD peptide analogues showed little affinity for the $\alpha_{\text{IIb}}\beta_3$ receptor, and for the reasoning behind this, the researchers had to look no further than the fact that natural ligands such as fibrinogen possessed numerous association modes with the integrin. It was not until the discovery of certain cyclic RGD related peptides that established small molecules as potential integrin inhibitors with affinities in the same order as large peptides like echistatin. Structural modifications
of these molecules improved their activity in vivo by enhancing their affinity, biological half life (stability in vivo) and inhibitory activity in animal thrombosis models. Derivatisation of the unusually highly \( \alpha_{IIb}\beta_3 \) selective disintegrin, barbourin,\(^{(64)} \) led to the novel agent eptifibatide (integrelin\(^{\circledR} \), Fig 1.9), a cyclic heptapeptide, culminating in the development of this and other related drugs into clinical candidates for thrombotic conditions and diseases.\(^{(65)} \) The oral antithrombotic market was a potential market for these agents but unfortunately the cyclic peptides were renowned for poor oral activity. To counter this, the various pharmaceutical companies sought non-peptidic compounds that were more suited to oral administration.

![Diagram of Integrelin\(^{\circledR} \)](image)

**Figure 1.9** Integrelin\(^{\circledR} \), a cyclic peptide antagonist for \( \alpha_{IIb}\beta_3 \)

In the process of evaluating the new \( \alpha_{IIb}\beta_3 \) peptide mimetics it was found that a number of them also displayed varying affinities for the \( \alpha_\ell\beta_3 \) receptor as well. This was not unexpected as it was well established how many natural ligands can bind to both receptors, but for the first time they began focusing on the \( \alpha_\ell\beta_3 \) binding as well. The Merck produced compound L-748,415\(^{(66)} \) as well as the Monsanto-Searle
developed SC-55631 compounds\(^{(67)}\) shown in Fig 1.10 are examples of the molecules that kick-started this break away from the traditional platelet targeting research.

![Chemical structures of L-748,415 and SC-55631](image)

**Figure 1.10** Early \(\alpha_\text{v}\beta_3\) selective antagonists

*In vitro* evaluations of these types of molecules were carried out using inhibition assays against both integrin types. The drugs were incubated with the purified integrin protein and their inhibition was measured as a function of displacement of a known integrin standard. An inhibition constant (\(K_i\)) generally is calculated for the agent against each integrin, which is indicative of the binding affinity. Comparisons of the inhibition constants of a drug for both receptor types elucidated important information on the specificity.

As the pharmaceutical potential of \(\alpha_\text{v}\beta_3\) inhibiting molecules became apparent, researchers began delving into the development of antagonists that preferentially bound to \(\alpha_\text{v}\beta_3\) over \(\alpha_\text{IIb}\beta_3\). The first \(\alpha_\text{v}\beta_3\) antagonists were actually murine monoclonal antibodies of which one, LM609 showed so much promise it eventually was evaluated in Phase I clinical trials for cancer.\(^{(68)}\)

Kessler and co-workers have produced a number of \(\alpha_\text{v}\beta_3\) selective peptides, specifically cyclic systems, and the encouraging results they obtained prompted other groups to adopt similar approaches.\(^{(69-73)}\) Numerous cyclic peptides were produced and evaluated, and from these studies it was found that all of the \(\alpha_\text{v}\beta_3\) selective molecules had a conformational turn about the central glycine. Typically \(\alpha_\text{IIb}\beta_3\)
selective molecules instead possessed an extended central glycine, and the contrasting results helped to confirm the belief that the distance between the cationic guanidinium moiety and the anionic aspartic acid side chain had to be shorter for $\alpha_v\beta_3$ activity. Two cyclic peptides that were extremely similar structurally that had reversed specificities for the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins were the cyclo RGDSPG [7] and XJ-735 [8] as shown in Table 1.2. Three-dimensional analysis of these molecules shows that cyclo RGDSPG is fairly planar with both the arginine and aspartic acid sidechains sitting within the plane. Contrastingly, XJ-735 has the arginine residue sticking out of the plane. This essentially alters the turn from the Type II seen around the $D$-Ala-$(N\text{-Me})$-Arg moiety in cyclo RGDSPG to a Type I turn around the Ala-Arg in XJ-735 turning the molecule into a fairly potent $\alpha_v\beta_3$ inhibitor.\(^{(74)}\)

As the picture of the basic requirements for RGD binding unfolded, researchers became confident that by manipulating the distance between the terminal guanidine and aspartic acid, and the rotational conformation between them, they could give molecules the ability to discriminate between $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptors. The knowledge obtained from the synthetic cyclic peptide work aided strongly in the continuing design and evaluation of peptide mimetics for $\alpha_v\beta_3$.

SmithKline-Beecham were able to convert the potent $\alpha_{IIb}\beta_3$ specific small molecule SB 214857 into a potent $\alpha_v\beta_3$ antagonist by modifying the guanidine mimetic on the 1,4-benzodiazepine based Gly-Asp framework.\(^{(75)}\) The resulting benzimidazole containing SB 223245 extended the notion that the reduced Arg to Asp distance favoured the $\alpha_v\beta_3$ binding site. Interestingly, the Merck produced L-748,415 was created in a similar fashion from highly specific $\alpha_{IIb}\beta_3$ antagonists. Their work involved the modification of piperidine groups of the $\alpha_{IIb}\beta_3$ antagonists to groups including guanidines, alkyl amidine and cyclic guanidines.\(^{(66)}\) It should be
noted that this is a common strategy for $\alpha_\text{v}\beta_3$ binding agent development as not only does it draw on the wealth of knowledge concerning the many $\alpha_{\text{IIb}}\beta_3$ antagonists produced, but it has given the researcher an insight into the discriminatory forces at work in the molecular recognition of these RGD mimetics.

Nicolaou and co-workers evaluated a series of nitroaryl ether based non-peptide mimetics for $\alpha_\text{v}\beta_3$ binding. Their testing included the $\alpha_\text{v}\beta_5$ integrin, also implicated in angiogenesis, as well as the obligatory $\alpha_{\text{IIb}}\beta_3$ receptor. The molecules were designed considering the Merck findings that pointed to the importance of the aryl sulfonamide moiety as an Asp mimetic and the established guanidine mimetic system for Arg. The study produced several small molecules that inhibited all of the integrins studied, but specifically one (Table 1.4) which displayed excellent in vivo inhibition of angiogenesis in the chick chorioallantoic membrane (CAM) angiogenesis assay.\(^{(53)}\)

Non-peptide small molecule integrin antagonists have typically been designed for oral admission, as potential antithrombotic drugs to compete with the likes of aspirin and warfarin. The discovery of $\alpha_{\text{IIb}}\beta_3$ antagonists with oral bioavailabilities of greater than 10% was difficult unless a prodrug strategy was used to mask the ionisable moieties.\(^{(76)}\) Fortunately, considerable progress has been made into the development into $\alpha_\text{v}\beta_3$ analogues, particularly by SmithKline-Beecham. The lead compound SB 223245 (Table 1.4), while a potent antagonist for $\alpha_\text{v}\beta_3$, suffered from poor pharmacokinetics, so efforts were made to improve its biological stability and also improve on its potency. The 1,4-benzodiazepine series was the focus of a follow up study and it revealed that the benzimidazole guanidine mimetic could be exchanged with relatively non-basic aminopyridine based guanidine mimetics to improve the bioavailability. The development of the dibenzocycloheptene analogue
SB 265123 (Table 1.3), with its excellent potency for $\alpha_v\beta_3$ inhibition ($K_i = 4$ nM in a receptor binding assay, and in an $\alpha_v\beta_3$ mediated cell adhesion assay ($IC_{50} = 60$ nM)), was shown to have excellent pharmacokinetics in rats. These properties include a biological half-life of 3-6 h, low plasma clearance ($Cl_p = 3$ mL min$^{-1}$ kg$^{-1}$) as well as an approximately 100% bioavailability, demonstrating that potent $\alpha_v\beta_3$ specific antagonists suitable for oral administration are achievable without having to resort to ester prodrugs.$^{(55)}$

By targeting the $\alpha_v\beta_3$ receptor, researchers have shown that it is possible to produce viable drugs with potent angiogenic properties, as well as other diseases such as osteoporosis, rheumatoid arthritis and restenosis, with a number of these molecules already in various stages of clinical testing.$^{(55)}$
Table 1.3  Cyclic RGD containing integrin antagonists

<table>
<thead>
<tr>
<th>Structure &amp; Code</th>
<th>Binding $K_i$(nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_v\beta_3$</td>
<td>$\alpha_{IIb}\beta_3$</td>
</tr>
<tr>
<td><strong>cyclo-RGDIV [4]</strong></td>
<td>10</td>
<td>42000</td>
</tr>
<tr>
<td>H-Gly-D-Pen(S)-Gly-Arg-Gly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-Ala-Cys(S)-Pro-Ser-Asp</td>
<td>8</td>
<td>110</td>
</tr>
<tr>
<td><strong>cyclo-GpenGRGDSPCA [5]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>75000</td>
</tr>
<tr>
<td><strong>cyclo-RGDSPG [6]</strong></td>
<td>500</td>
<td>8</td>
</tr>
<tr>
<td><strong>XJ-735 [8]</strong></td>
<td>20</td>
<td>18000</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20000</td>
</tr>
<tr>
<td><strong>XK-002 [9]</strong></td>
<td>0.45</td>
<td>200</td>
</tr>
</tbody>
</table>
Table 1.4  Selective small molecule integrin antagonists

<table>
<thead>
<tr>
<th>Structure &amp; Code</th>
<th>Binding IC&lt;sub&gt;50&lt;/sub&gt;(nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α&lt;sub&gt;β3&lt;/sub&gt;</td>
<td>α&lt;sub&gt;IIbβ3&lt;/sub&gt;</td>
</tr>
<tr>
<td>SC-56631 [10]</td>
<td>10</td>
<td>&gt;30000</td>
</tr>
<tr>
<td>SB 223245 [11]</td>
<td>2</td>
<td>30,000</td>
</tr>
<tr>
<td>SB 265123 [12]</td>
<td>4</td>
<td>9000</td>
</tr>
<tr>
<td>Nicolaou et. al [13]</td>
<td>3.9</td>
<td>0.67</td>
</tr>
<tr>
<td>Nicolaou et. al [14]</td>
<td>0.8</td>
<td>24</td>
</tr>
<tr>
<td>Kessler et. al [15]</td>
<td>3</td>
<td>3000</td>
</tr>
</tbody>
</table>
1.15 Current treatments for cancer

In the fight against cancer, there are a number of available options open to patients, dependant upon the tumour’s type, size, location and progression. Successful treatment is dependent upon numerous factors including how early the disease is diagnosed. Unfortunately many tumours are diagnosed too late and metastases may be common before any symptoms are noticed. Therapy may then focus on the palliative treatment of the patient to reduce suffering. Usual treatments include:

Surgical Removal is the primary mode of treatment of solid cancers. It involves the excision of the tumour and generally some of the adjacent tissue. With surgery there is no guarantee of success as the primary tumour may have metastasised.

Chemotherapy involves the use of drugs to kill or suppress tumours. Chemotherapies are ideally cytotoxic drugs, that primarily attack tumour cells, but in reality they possess toxicity to normal cells as well. They are generally designed to be specific for molecules that are more highly regulated in tumours than normal cells, although they can also be designed to take advantage of a tumours’ higher rate of metabolism.

Radiotherapy involves the use of ionising radiation to destroy tumour sites. DNA replication and mitosis are impaired by radiation, resulting in chromatid breaks and abnormal chromosomes and mitoses. The radiosensitivity of tissue is directly related to its mitotic index. By irradiating a rapidly growing tumour (high mitotic index), it is possible to destroy it, while sparing the normal tissue (lower mitotic index). Unfortunately, there are many normal tissues with high mitotic indices (bone marrow and gut mucosa), that reproduce at a rate similar to neoplasms, so radiotherapy does not distinguish between the cancerous and non-cancerous tissue. The radiation is
generally administered as an x-ray beam to the affected areas in a carefully measured dose.

*Bone Marrow Transplantation* involves the transplanting of healthy cells (blood or bone marrow) previously collected from the patient or from a compatible donor. Healthy marrow is infused in the patient’s bloodstream to replace the destroyed bone marrow, where hopefully it can migrate to the cavities in the bone and begin producing normal blood cells.

*Hormone Therapies* work by interfering with the hormone levels in a patient (blocking and supplementing) so it is possible to change the tumour cells life cycle.

*Immunotherapy* promotes the body’s immune system to fight the cancerous activity. Tumours express cancer associated antigens and immunotherapies mediate the regression of established tumours through antibody and cytokine action.

*Nuclear Medicine* involves the administering of radioactive drugs. This approach is a hybrid of the chemo- and radiotherapies, but instead of the patient being exposed to beams of radiation, the radioactivity is ingested or injected into a patient in a form that will target a tumour or cancerous system. Radioactive tracers are unstable versions of bioconjugates that are attracted to cancer sites, either by binding to biomolecules upregulated around the tumour or by mimicking the natural nutrients that the tumorous cells need for growth. This is the fastest growing field in cancer therapy research, as it limits the exposure of the destructive radiation to the tumour site. More details in the nuclear medicine approach are discussed in the following sections.

### 1.16 Radiopharmaceuticals

Nuclear medicine is an extremely valuable emerging field in the diagnosis and treatment of cancer. It involves the elegant approach of taking the radioactivity to
where it is needed, rather than irradiating a large area of a patient. The basis of nuclear medicine therapy is to harness the destructive force of ionising radiation to damage tissue to either kill a tumour or delay its progress, although there are other subtler applications. Radiopharmaceuticals can also be used as diagnostic tools where the radiation is designed to provide a source for imaging or locating a tumour or site of infection, or even normal tissue functioning.

Radiopharmaceuticals can be thought of having two components: a radionuclide and a biologically active pharmaceutical component. The nature of the pharmaceutical component of the molecule will dictate its ability to navigate its way to a certain part of the body or participate in a physiological process. The type of radionuclide component will determine whether the radiopharmaceutical will be used as a diagnostic tool for locating sites of infection or monitoring problematic processes in the body, or as a therapeutic agent for the treatment of diseases.

1.17 Radionuclides

A radionuclide is a radioactive species of an atom characterised by the makeup of its nucleus (the numbers of neutrons and protons) which cause the nucleus to be unstable. Depending on the nuclear makeup and its energy content, the radionuclide can emit radiation to reach a lower energy state, and this process can occur by gamma or x-ray emissions, neutrons, beta particles or alpha particles. Radionuclides can have numerous decay modes with emissions at different energies.

Radioactive decay occurs to stabilise the nucleus of an atom. For the lighter atoms stability is achieved when the ratio of neutrons to protons (N/Z) is one. For elements with the atomic number 20 and upwards, the ratio N/Z must be greater than one because the repulsive force between the increasing number of protons becomes
more prominent, and the presence of neutrons buffers this repulsion. Radionuclides undergo decay to achieve the N/Z ratio of the nearest possible stable nuclide.

The rate of decay of radionuclides is a fundamentally important factor in dealing with radioactive materials. Depending on the nature of the nucleus and the energy emission, the radiation can leave the nucleus at different rates. The rate at which a material decays is expressed in terms of how long it takes for half the radioactivity to disappear, or as it is commonly referred, the half-life (denoted by the Greek symbol \( \lambda \)). The half-life is specific for each different radionuclide. Half-lives of known radionuclides vary from \( 10^9 \) years for very nearly stable isotopes to \( 10^6 \) seconds for highly unstable ones.

There are various decay routes that can lead to emissions and the following is an explanation of the different types of ionising radiation.

**Gamma rays** (denoted by the Greek symbol \( \gamma \)) are high energy photons that are highly penetrating. They begin at energies of 10 keV and continue upwards. Technically, gamma rays and x-rays are identical electromagnetic radiation, but their difference is distinguished by their origin. Gamma rays are produced by nuclear transitions whereas x-rays are created by energy transitions due to accelerating electrons. Gamma rays are ionising radiation but to a lesser extent than alpha and beta particles. The energy of a gamma ray is measured in keV or MeV and this is proportional to the frequency of the radiation. The higher the frequency, the smaller the wavelength and subsequently the more penetrating the gamma ray is. Gamma rays and x-rays have significant effects on biological systems including burns, cancer and genetic mutation.

**Beta particles** (denoted by the Greek symbol \( \beta \)) or beta rays are essentially high speed electrons. They are much less penetrating than gamma rays, as they have significant
mass. They are charged particles, and their ionising potential is only really a danger when ingested into the body due to their weak penetrating power. Beta particles can exist in two forms, the negatively charged beta electron (or negatron $\beta^-$) and a positron ($\beta^+$) which is a positively charged antimatter version of an electron. Unstable nuclei with an excess of neutrons can undergo $\beta^-$ decay where a neutron is converted to a proton, an electron and an electron type antineutrino as follows:

$$ n \rightarrow p + e^- + \nu_e \quad \text{Eqn 1.1} $$

Conversely, unstable nuclei with a deficiency of neutrons can undergo positron ($\beta^+$) decay, where a proton is converted into a neutron, a positron and an electron type neutrino, as follows:

$$ p \rightarrow n + e^+ + \nu_e \quad \text{Eqn 1.2} $$

Alpha particles (denoted by the Greek symbol $\alpha$) are basically the nuclei of the helium atom, consisting of two protons and two neutrons. They are 8000 times the mass of beta particles and are subsequently more ionising. They are very weakly penetrating as a piece of paper or the outer layer of human skin (approx. 40 $\mu$m or a few cells deep) is enough to shield them and thus are only are of any real danger to biological systems if ingested. However, if they are ingested, their mass and strong absorption can cause the most destructive force of all radiation (estimated to be over 100 times greater than that caused by the equivalent amount of other types of radiation). Alpha decay can leave nuclei in an excited state, so it can often be accompanied by a gamma emission to achieve stability.

In the field of nuclear medicine, gamma rays are the most popular option focussed on by clinicians and researchers for imaging and beta particles for therapy.
There are numerous isotopes with differing emission energies and half-lives to suit all sorts of biological applications, whether they are for therapeutic or diagnostic purposes.

1.18 Diagnostic Radiopharmaceuticals

In the field of diagnostic nuclear medicine there are two main streams of applications, in vivo function agents and imaging agents. In vivo agents are designed to monitor the function of a particular organ, based on the absorption, dilution or concentration of measurable radioactivity. No imaging is required in these studies and instead the analyses of the results are based on counting the radioactivity from the organs themselves, the blood or urine samples. Imaging agents are radiopharmaceuticals that provide information on the organs function and morphology (size, shape and location). Imaging agents are designed to localise in the targeted organ within a short amount of time, remain there long enough for the imaging experiment and then be efficiently and rapidly excreted from the body.

1.19 Imaging Agents

Nuclear medicinal imaging involves the detection of an administered radiopharmaceutical, primarily by its gamma ray emissions and processing this into an image relating position to radioactive intensity. There are many commonly used radionuclides suitable for these studies and they include Tc-99m, I$^{123}$, F$^{18}$, O$^{15}$, N$^{13}$, C$^{11}$ and Tl$^{201}$. When these radionuclides are incorporated into the radiopharmaceutical their gamma emissions are detected and processed by Emission Computed Tomography (ECT). This function creates a three-dimensional
distribution map of the radiopharmaceutical quantity which can be analysed for physiological and pathological processes in a non-invasive manner. Positron Emission Tomography (PET)\(^{(84)}\) and Single Photon Emission Computed Tomography (SPECT)\(^{(85)}\) are the two types of ECT commonly employed in imaging clinics and hospitals.

### 1.20 Positron Emission Tomography (PET)

PET imaging requires positron emitting radionuclides such as F\(^{18}\), C\(^{11}\), N\(^{13}\), O\(^{15}\), Ga\(^{68}\) and Rb\(^{82}\). When a positron collides with an electron, two gamma rays are produced in exactly the opposite directions, which are picked up simultaneously by detectors all around the subject. PET agents are usually very biologically similar to their parent biomolecules or drugs as they consist of the biologically important elements.

### 1.21 Single Photon Emission Computed Tomography (SPECT)

SPECT imaging involves the use of radionuclides that emit gamma rays in the range of 70 - 250 keV for maximum efficiency. Common isotopes are the reactor produced Tc-99m and the cyclotron produced I\(^{123}\) and Tl\(^{201}\). SPECT cameras consist of between one and three sodium iodide crystal detectors mounted on a movable gantry linked to a computer for acquisition and analysis. During the analysis the detectors rotate to get a complete picture of the subject.

SPECT suitable isotopes are generally longer lived than their PET equivalents and this is advantageous when multiple steps are required to achieve the desired
agent. Subsequently, the greater half-lives enable the production of more elaborate diagnostic agents. Unlike PET agents, SPECT diagnostics have to incorporate isotopes not usually found in biomolecules like $^{123}$I and Tc-99m, which may require a Bi-Functional Chelator (BFC) for attachment, therefore significantly changing the molecular nature of the agent from the parent molecule. PET is the more sensitive of the techniques partly because of the co-incident nature of the photons, and as a result SPECT agents need to remain in the tissue for longer periods to improve the stochastic quality of the image.

1.22 Technetium-99m Radiopharmaceuticals

Technetium was the first artificially produced element and Tc-99m refers to the ‘metastable’ form of Tc$^{99}$. It is reactor produced and is currently the most commonly used radionuclide used in nuclear medicine. Tc-99m is ideal as a SPECT imaging agent as it has the short half-life of 6.01 h, the gamma energy of 143 keV and is rapid cleared from the body. Technetium is dispensed from a Mo99/Tc99m generator, which can be ‘milked’ for Tc-99m activity, once a day for around two weeks. The technetium is in the reactive pertechnetate form ($\text{TcO}_4^-$) in saline solution. The availability and ideal properties of Tc-99m has generated many technologies to incorporate it into diagnostic imaging agents. Being a metallic element, Tc-99m is linked to the target molecules via coordination bonding to suitable ligand groups, and there are two main types of strategies employed by researchers and clinicians to achieve this. These strategies include direct labelling or by the use of preformed chelates or BFCs to mediate the linking. The direct labelling technique usually involves the use of a reducing agent to convert a number of disulfide linkages to free thiols which bind Tc very efficiently. Imidazole nitrogens
can also be involved in this approach. The advantages of this method is that it is easy and quick to carry out, but the disadvantages are that there is little information about the number of donor atoms or the binding geometries about the central Tc core. This also leads to little control over the stability of the radiotracer and the non-specific binding. This method is good for proteins but not really suitable for smaller peptides as they don’t possess any disulfide bonds, or even if they do they are too critical to the peptides activity to be reduced and coordinated. The second main technique is the preformed chelate approach using BFCs. A number of peptides and small molecules have been linked to Tc-99m using various BFCs, all which impart some of their own character to the target molecule. Various multidentate- and macrocyclic ligands have been created consisting of combinations of amines, amides, imines, thiols and phosphate functional groups designed to ‘wrap up’ the Tc-99m core. The \( \text{N}_2\text{S}_2 \) MonoAmine, MonoAmide dithiol (MAMA) ligand system has been established as an effective BFC for oxotechnetium (Tc=O). Likewise, the hydrazinonicotinamide (HYNIC) system has also become an extremely versatile BFC for Tc-99m although it coordinates via monodentate or bidentate ligation. An advantage of the HYNIC system is that it requires co-ligands to coordinate the unbound sites on the Tc, and by employing various co-ligands such as glucoheptonate, \( \text{N}-\text{tris(hydroxymethyl)methylglycine} \) (tricine) and ethylenediaminediacetic acid (EDDA), the researcher can tune the polarity of the molecule. The polar nature of the molecule can be the difference between success and failure in reaching the desired target.

Tc-99m has radiopharmaceutical applications for proteins, peptides and small molecules designed to target certain biological entities, but there are also small molecule applications as markers and flow agents, as shown in Fig 1.11. These
radiotracers do not actually interact with any specific proteins or receptor but rely on their polar character to get them to the desired part of the body. Tc-99m-hexamethylpropylene amine oxime (Tc-99m-HMPAO) or Ceretech® is one such example of a small molecule radiotracer designed to investigate cerebral blood flow, which is of particular interest to patients who have suffered infarction, strokes and brain tumours. Other important Tc-99m radiotracers include Tc-99m-ECD (Neurolite®), a similar brain perfusion agent, Tc-99m MIBI (Cardiolite®) for heart imaging and the renal function imaging Tc-99m-MAG₃.

Figure 1.11 Tc-99m Radiotracers in clinical use
1.23 **Therapeutic Radiopharmaceuticals**

Therapeutic radiopharmaceuticals are radiolabelled molecules intended to deliver therapeutic doses of ionising radiation to tumours or sites of infection in the body. The attached radionuclide is solely required to provide a cytotoxic environment for the tumour or diseased site. Ideally the administered therapeutic radiopharmaceutical will be highly specific for the target area while delivering minimal or tolerable radiation to the unaffected normal tissue. Of course this specificity can be very hard to achieve due to the many physical and chemical factors of the radiopharmaceutical and indeed the biological environment they are in. Therapeutic radiopharmaceuticals have been in use for over 50 years but in the last two decades discoveries of many immuno-derived molecular carriers and high affinity receptor ligands have enabled more sophisticated and specific delivery techniques. The creation of these types of therapeutics requires the optimisation of the overall molecule to not only reach the target with acceptable specificity with respect to the normal tissue, but to also to clear from the non-target tissue. The residence time of the drug in the target, and the catabolic and metabolic processes that could take place are also factors that must be addressed. There are numerous therapeutic radiopharmaceuticals in clinical trials and commercial availability for various treatments employing a variety of suitable radionuclides and their good *in vivo* characteristics are the result of these optimisations. Some examples of these molecules are $^{131}$I labelled $m$-iodobenzylguanidine (mIBG), which is awaiting approval for clinical use by the US Food and Drug Administration (FDA). Already an approved imaging agent, it has shown excellent activity against neuroendocrine tumours.\(^{92,93}\) $^{131}$I labelled sodium iodide (Na$I^{131}$) has been used extensively to treat hyperthyroidism\(^{94}\) and differentiated thyroid carcinoma.\(^{95}\) Various monoclonal
antibodies (MAbs) are currently being evaluated for specific anti-tumour activity, mostly attached to $^{131}$I, but the development of new BFCs has paved the way for attachment to various metallic radionuclides. One area in which therapeutic radiopharmaceuticals is well established is with the palliative treatment of bone metastases. Bone metastases cause unbearable pain to sufferers so various radiopharmaceuticals have been found to alleviate this pain and improve the life of the cancer patients. Strontium$^{89}$ chloride (Metastron®), P$^{32}$ sodium orthophosphate and Samarium$^{153}$ ethylenediaminetetramethylene phosphonate (Sm$^{153}$-EDTMP or Quadramet®) are all FDA approved for this treatment, although there is considerable research still being conducted to improve the treatments by incorporating different radionuclides with different emission characteristics.$^{(96)}$

### 1.24 Copper Radiopharmaceuticals

Radionuclides of copper lend themselves to both therapeutic and diagnostic applications. Cu$^{64}$ and Cu$^{67}$ have both been used as cytotoxic components of various biologically active molecules in animal and human anti-tumour studies. These molecules include MAbs as well as receptor targeting peptides and small molecule conjugates. Cu$^{64}$ can be produced in a reactor or a cyclotron and its widespread availability make it the preferred Cu radionuclide for applications in which the blood clearance is long. Cu$^{67}$ is not as widely available as it is only produced in usable quantities in spallation reactions in high energy particle accelerators. Cu$^{64}$ also has potential as a diagnostic radiopharmaceutical component along with Cu$^{60}$, Cu$^{64}$ and Cu$^{62}$. All of these radionuclides are positron emitters so they are of great interest to developers of novel PET pharmaceuticals.$^{(97)}$
Several macrocyclic ligand systems have been employed as BFCs to incorporate Cu(II) nuclides in radiodrugs. These include functionalised diethylenetriaminepentaacetic acid (DTPA) derivatives as well as a multitude of tetra-aza macrocycle cyclams. These systems have been sought to provide high stabilities \textit{in vitro} and \textit{in vivo}, as studies had revealed that de-metallation was quite rapid in more accessible complexes. Even still, \textit{in vivo} stability has been improved further by the incorporation of pendant carboxylic acids attached to the cyclic amino groups. These large macrocycles which include the 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), 1,4,8,11-tetraazacyclotetradecanetetraacetic acid (TETA), 1,4,7,10-tetraazacyclotridecanetetraacetic acid (TRITA) and their derivatives have also found use for many larger rare-earth elements (Fig 1.12).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_1_12.png}
\caption{The tetraaza DOTA, TRITA and TETA macrocycles}
\end{figure}

1.25 \textbf{Lutetium as a Potential Radiopharmaceutical}

Lutetium is a lanthanide that was discovered in 1907 by Georges Urbain. It is a relatively air stable silvery white metal and is found, like all rare earths, in minerals mixed with other rare-earths which can be quite problematic when purifying. Lutetium is found primarily in monazite, gadolinite and xenotime (along with the
very physically and chemically similar lanthanide Ytterbium (Yb), and is the most expensive commercially available rare-earth element.

Owing to its favourable decay characteristics, Lu\(^{177}\) is seen as an attractive radionuclide for various therapeutic and diagnostic applications. It has a half-life of 6.7 days and maximum and average \(\beta\) energies of 421 and 133 keV respectively, resulting in short-range transmission in tissue which should be excellent for anti-cancer radiopharmaceuticals. The \(\beta\) decay is accompanied by the emission of the relatively low energy \(\gamma\) radiations at 208.3 keV (11%) and 113 keV (6.5%) which could be suitable for simultaneous imaging. The decay product is Hf\(^{177}\) which is stable.

Lu\(^{177}\) can be produced by the thermal neutron bombardment of Lu\(^{176}\) in natural and Lu\(^{176}\) enriched lutetium oxide (Lu\(_2\)O\(_3\)) targets as follows;

\[
[\text{Lu}^{176}(n,\gamma) \rightarrow \text{Lu}^{177}]
\]

or by the thermal neutron bombardment of Yb\(^{176}\) in the oxide form (Yb\(_2\)O\(_3\)) as follows;

\[
[Yb^{176}(n,\gamma) \rightarrow Yb^{177}(T_{1/2}=1.9h) - \beta^- \rightarrow \text{Lu}^{177}]
\]

The former method is attractive due to the high thermal neutron capture cross section of 2100 barns of the Lu\(^{176}\) which means it can be produced at nuclear reactors in high yield and high specific activities. However, the presence of macroscopic amounts of stable Lu\(^{176}\) can potentially cause problems in labelling biological entities such as MAbs, proteins and small peptides. The latter method overcomes this problem as the ytterbium target material from which the Lu\(^{177}\) forms is chemically different and separable via chromatographic and selective Na(Hg) amalgam cementation methods. As this method has no stable lutetium present (carrier free) a maximal specific activity of 720 GBq/\(\mu\)mol (1.0994 \(\times\) \(10^5\) Ci/g) is achievable.\(^{98}\)
No carrier added (NCA) Lu$^{177}$ has already been incorporated into MAbs, peptides, proteins and small molecules for evaluations as palliative bone therapies,\(^{(99)}\) hypoxia markers, which are designed to detect low oxygen environments, indicative of tumorous and angiogenic processes,\(^{(100,101)}\) as well as receptor avid small molecules.\(^{(102)}\) Being a rather bulky lanthanide rare-earth metal, a lutetium atom can accommodate up to nine sites of ligation. This complex coordination geometry had to be addressed in order to stabilise the metal complexes \textit{in vivo}. Several macrocyclic BFCs have been developed to totally wrap around the metal centre and to protect it from being abstracted. Large macrocyclic systems have been successfully employed and developed into useful BFCs with amenable attachment chemistries to the biologically relevant moieties, and also to the metal coordination. The TETA and TRITA macrocycles are examples of such cage type ligands and their functionalised attachment groups are mainly based on isothiocyanate linking to amino groups on the target molecules.\(^{(103-108)}\) Other strategies include the peptide coupling of N-alkyl acetic acid group to the target amines.\(^{(109-111)}\) Coordination is generally the final step, although there are instances where the coordinated BFC is conjugated to the pharmaceutical component.\(^{(112)}\)

### 1.26 Aims of This Project

Unlike C$^{11}$, N$^{13}$ and O$^{15}$ which are isotopes of atoms that occur naturally in biologically active molecules, and the I$^{123}$ and F$^{18}$ halides which bind covalently, metallic radionuclides like Tc-99m, Cu$^{64}$ and Lu$^{177}$ need special design considerations if they are to be incorporated into radiopharmaceuticals. These metals are not usually associated with biological systems so their metallic characteristics require stabilisation via chelation which may require relatively large multi-heteroatom
systems to hold the metal. To address these problems researchers have employed two different design strategies in the development of metallo-radiopharmaceuticals (Fig 1.14). The first method involves the attachment of a metal containing chelator group to a molecule that has an established affinity for a desired target system. This ‘conjugate design’ has to incorporate the metal moiety in a position where it will hopefully not interfere with the biological interaction. This strategy is very common for proteins and larger peptides, but can present difficulties for the smaller peptides and biomolecules. The ‘integrated design’ method attempts to overcome these problems by incorporating the metal chelator into the biologically relevant moiety. This strategy leads to radio-molecules of similar size and shape while trying to minimise the disruption of the pharmacophore. The ‘integrated design’ method is certainly a more synthetically challenging approach but because the conjugate method risks creating molecules with molecular weights greater than 600, limiting their ability to cross cell membranes and in particular the blood brain barrier.\(^\text{113}\)

![Conjugate Design vs Integrated Design](image)

**Figure 1.13** The conjugated and integrated design strategies for metallo-radiopharmaceuticals
In light of this knowledge the synthetic aim of this project was to create metal complexes using both the ‘integrated’ and ‘conjugated’ design strategies. The ‘integrated’ molecules were based on information regarding the suitable distances between the guanidine and aspartic acid mimetics required for recognition by the $\alpha_\text{v}\beta_3$ receptor, as well as the surrounding groups of the aspartic acid mimetic. The selected chelator is based on the monoamine monoamide dithiol (MAMA) groups for coordination to Tc-99m. For the conjugate design approach, a molecule with an established affinity for $\alpha_\text{v}\beta_3$, Merck’s L-748,415 (Fig 1.10) was chosen as a suitable anchor for the attachment of various BFCs to coordinate the Tc-99m, Cu$^{64}$ and Lu$^{177}$ radionuclides. As Tc-99m has no stable isotope, rhenium will be used a substitute for all characterisation purposes.

The synthesised Re, Cu and Lu metal complexes will be assayed as potential $\alpha_\text{v}\beta_3$ antagonists \textit{in vitro} by displacement of a known high affinity antagonist labelled with I$^{125}$ from well bound purified human $\alpha_\text{v}\beta_3$ receptor protein.$^{(114;115)}$

Selected Tc-99m complexes are to be chosen for \textit{in vivo} SPECT imaging on nude mice bearing tumours of the A375 balb/c human melanoma cell line as well as tumour bearing Fischer rats (F344) arising from implantation with the MAT BIII murine mammary carcinoma cell line.
CHAPTER 2

SYNTHESIS
2 SYNTHESES

2.1 INTRODUCTION

There is a significant body of research already undertaken into the development of $\alpha_v\beta_3$ selective integrin antagonists. Rather than systematically evaluating a series of molecules for their affinity towards the receptor, it was thought that it would be of greater value to choose a moiety of known affinity and selectivity, attach a variety of different bifunctional chelators to the biologically active constituent for the incorporation of different radionuclides. This could broaden the potential of such radiodiagnostic and radiotherapeutic applications. This ‘conjugate approach’ required the synthesis of the chosen integrin antagonist molecule developed by Merck$^{(115)}$ as well as a number of chelating groups to coordinate technetium, copper or lutetium (Fig 2.1).

Oxorhenium(V) complexes were synthesised as structural prototypes for technetium as it has no natural abundance and rhenium is generally considered to be the most chemically similar element available. In fact, rhenium has been used extensively for this reason, although it should be noted that some researchers have been able to characterise Tc-99g complexes.$^{(116)}$ Of course the chemistries of technetium and rhenium are not identical and in some instances it is not possible to synthesise the rhenium equivalent. The HYNIC complexes fit into this category, and in light of this, no attempts were made to synthesise the rhenium complexes of the HYNIC molecules.

The macrocyclic MAMA bifunctional chelator has been used successfully with both Tc-99m and rhenium complexes so two similar target complexes were
synthesised, the oxorhenium(V) \((S)\)-2-(2-((2-mercaptoethyl) (2-mercaptoethylamino)-2-oxoethyl)amino)acetamido)-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino) ethoxy) benzamido) propanoic acid complex, and the oxorhenium(V) \((S)\)-1-mercapto-6-(2-mercaptoethyl)-4,8,15-trioxo-17-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino) ethoxy) benzamido) methyl) -3,6,9,16- tetraazaotadec- an-18-oic acid complex which has a greater linker length between the BFC and the biologically important moiety. This in itself was a minor study to determine the importance of the proximity of two components.

Complexes of copper and lutetium were synthesised using the TRITA bz chelating unit. TRITA and the similar TETA molecules were found to have excellent resistance to liberating larger coordinated metal centres \textit{in vivo}, so TRITA was found to be suitable for both metals and their radioactive analogues produced in Chapter 3.

\[ \text{Figure 2.1} \quad \text{The conjugate approach to designing } \alpha_{v}\beta_{3} \text{ integrin selective molecules and radiotracers involves the linking of various bifunctional chelators to } (S)-2\text{-amino-3-}(4-(2-(1,4,5,6\text{-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido) propanoic acid} \]
Of greater value to this project and also a much riskier proposition in terms of projected receptor affinity, was to attempt to create radiopharmaceuticals based on the ‘integrated’ design methodology. Since this was the first attempt to target the $\alpha_v\beta_3$ integrin, receptor in this manner, (that this researcher is aware of), it was decided to create two target complexes, but with a structural variation adjacent to the aspartic acid moiety of the molecule only. The phenylsulfonyl- and benzyloxycarbonyl-groups were linked via the terminal amino group, a structural feature that has successful enhanced binding selectivity for the $\alpha_v\beta_3$ integrin, receptor. The benzoimidazole group was used at the other end of the molecule as the arginine (R) mimicking moiety, again a feature that has been used to enhance the selectivity.

As much of the molecules are peptidemimetics, a significant amount of peptide coupling technology was used. Various coupling agents were used and much of the chemistry involved the protection and deprotection associated with such synthetic strategies. An extensive amount of literature is available in regards to peptide and peptidemimmetic syntheses, and there are excellent reviews of the various peptide coupling agents including the increasingly popular solid phase
methods.\textsuperscript{(117,118)} This important area of synthetic chemistry utilises agents that promote the condensation of carboxylic acids and amines to form amide linked molecules. To achieve this, these agents work by replacing the carboxylic acid’s hydroxyl group with an efficient leaving group (activated esters for example) that increases the efficiency of the amine – acid reaction. A major hurdle in synthesising peptides and their analogues is the associated racemisation of chiral groups on the molecules. A major driving force behind the development of the variety of peptide coupling agents is the reduction of racemisation of chiral groups by enabling the reactions to take place in gentler environments. Molecules such as hydroxybenzotriazole (HOBt) were originally used to act as the activated ester, often with an activator such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), but improvements in the field led to agents that incorporated both components in the same molecule. Benzotriazole-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP), was developed, as was benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (Py-BOP), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), \textit{o-}(7-azabenztrotiazol-1-yl)-N,N,N',N’-trimethyluronium hexafluorophosphate (HATU) and a significant number of other commercially available agents are the results of such synthetic needs.

The synthesis of the (S)-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid molecule from a different route was adopted to circumvent unsuccessful attempts at the Mitsonobu coupling described in the literature.\textsuperscript{(115)} Despite the reproduction of many of the materials outlined in the literature, it was deemed unnecessary to re-report the syntheses here unless improved upon.
2.2 EXPERIMENTAL

2.2.1 General

All reagents described in this section were used as is from either Sigma-Aldrich, Merck, Maybridge or Lancaster chemical manufacturers. Solvents unless otherwise noted were used as purchased from, Sigma-Aldrich, BDH, Merck, Ajax or Lab-Scan chemicals. For all rhenium coordination reactions involving tetrahydrofuran, the solvent was first eluted through a plug of neutral alumina to remove any peroxides present. Silica gel chromatography was carried out using Merck silica gel 60 (230 - 400 mesh.) Analytical thin layer chromatography was carried out using Merck silica gel plates on polyester with fluorescent dye (254 nm), cut to sizes generally 30 × 70 mm, and visualised by illumination at 254 nm or by exposure in iodine vapour. HPLC purification and analyses were carried out on Waters equipment running Laura Version 1, Millennium and Empower software. A photodiode array detector was employed for all coordination complex purification and analyses. $^1$H NMR and $^{13}$C spectra were recorded on a Bruker AVANCE DPX 400 (400 MHz) spectrometer at 400 MHz and 100 MHz respectively. Deuterated solvents were obtained from Sigma-Aldrich or Cambridge Isotope Laboratories, and were either internally referenced on trimethylsilane or on the residual solvent peak. Shifts were recorded in ppm. The following abbreviations were used to denote the multiplicity of the shifts; s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet and br denoting broadness observed in the peaks. Low resolution Mass Spectroscopy was carried out on a Waters Micromass ZQ quadrupole Mass Spectrometer employing either electrospray positive or negative ionisation in water, acetonitrile or methanol solutions.
2.2.2 Integrin Antagonist Synthesis (Merck Derivative)

2.2.2.1 Methyl-4-(2-azidoethoxy) benzoate [25]

![Chemical Structure]

To a solution of methyl 4-(2-chloroethoxy) benzoate (15.9 g, 74.07 mmol) in N,N'-dimethylformamide (50 mL) was added sodium azide (9.64 g, 148.3 mmol). The mixture was stirred and heated to 140 °C for 2 h. After cooling, water (250 mL) and dichloromethane (250 mL) were added and the product was extracted into the organic layer, washed with water (2 × 100 mL) and dried over magnesium sulfate and evaporated to dryness. The crude solid material was chromatographed on silica gel eluting the azide product in ethyl acetate/petroleum ether (20:80), after drying affording an off-white crystalline solid (13.9 g, 84.8%); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.99 (ArH, d, $J = 8.9$ Hz, 2H), 6.93 (ArH, d, $J = 8.9$ Hz, 2H), 4.19 (CH$_2$, t, $J = 4.9$ Hz, 2H), 3.89 (COOCH$_3$, s, 3H), 3.62 (CH$_2$, t, $J = 4.9$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.53 (ArCOOCH$_3$), 161.77 (ArC), 131.51 (ArCH), 123.14 (ArCCOOCH$_3$), 114.00 (ArCH), 66.90 (CH$_2$O), 51.75 (COOCH$_3$), 49.89 (CH$_2$N$_3$); LRMS (ES+ MS) m/z calc. for C$_{10}$H$_{11}$N$_3$O$_3 = 221.08$, found 244.28 (M+Na)$^+$.

2.2.2.2 Methyl-4-(2-aminoethoxy)benzoate [26]

![Chemical Structure]

Methyl-4-(2-azidoethoxy) benzoate (12.0 g, 61.47 mmol), cobalt chloride hexahydrate (1.29 g, 5.42 mmol) and water (20 mL) were stirred in a 250 mL round
bottom flask at room temperature. To this mixture was added a solution of sodium borohydride (4.10 g, 108.47 mmol) in water (100 mL) dropwise over a 15 min period. The mixture turned black and stirring continued for 3 h, after which it was filtered. The filtrate was extracted with diethyl-ether (3 × 50 mL). The combined organic fractions were dried over sodium sulfate and concentrated under reduced pressure to yield a golden solid (10.3 g, 85.8%); TLC (silica) R$_f$ 0.0 (ethyl acetate/pet. ether(30:70));

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.97 (ArH, d, $J$ = 7.9 Hz, 2H), 6.91 (ArH, d, $J$ = 7.9 Hz, 2H), 4.02 (CH$_2$, t, $J$ = 6.9 Hz, 2H), 3.87 (COOCH$_3$, s, 3H), 3.02 (CH$_2$, br t, 2H), 1.63 (NH$_2$, br s, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.63 (ArCOCOCH$_3$), 162.57 (ArC), 131.42 (ArCH), 122.57 (ArCCOOCH$_3$), 113.94 (ArCH), 70.12 (CH$_2$O), 51.65 (COOCH$_3$), 41.16 (CH$_2$NH$_2$); LRMS (ES+ MS) $m/z$ calc. for C$_{10}$H$_{13}$NO$_3$ = 195.01, found 218.08 (M+Na)$^+$. 

2.2.2.3 (S)-Methyl2-(benzyloxycarbonylamino)-3-(4-(2-(pyrimidin-2-ylamino)ethoxy)benzamido)propanoate [27]

![Chemical Structure Image]

4-(2-(Pyrimidin-2-ylamino) ethoxy) benzoic acid (5.4 g, 20.82 mmol) and (S)-methyl 3-amino-2-(benzyloxycarbonylamino)propanoate (5.29 g, 20.96 μmol) were placed in a 500 mL round bottom flask with N,N'-dimethylformamide (200 mL) under nitrogen. The solution was cooled to 0 °C in an ice bath. Triethylamine (8 mL, 57.52 mmol) was injected into the solution, as was solid benzotriazole-1-yl-oxy-tris-
(dimethylamino)phosphonium hexafluorophosphate (BOP coupling agent) (8.92 g, 20.16 mmol). The solution was stirred at 0 °C for 2 h then at room temperature overnight. The solution was poured into a saturated sodium bicarbonate solution (300 mL) and was extracted into ethyl acetate (2 × 200 mL). The organic phase was washed with brine (100 mL) and was then dried over sodium sulfate and pumped to dryness, using additions of methanol to displace the N,N’-dimethylformamide still present. The crude product was then purified by trituration in ethyl acetate/methanol (95:5), affording a white powder. (6.6 g, 64.2%); \( \text{TLC}_{(\text{silica})} \) \( R_f \) 0.32 (ethyl acetate); 

**\(^1\)H NMR** (400 MHz, \((\text{CD}_3)_2\text{SO}\)) \( \delta \) 8.39 (CONH, t, \( J = 5.6 \) Hz, 1H), 8.29 (ArH, d, \( J = 4.7 \) Hz, 2H), 7.77 (ArH, d, \( J = 8.8 \) Hz, 2H), 7.72 (CONH, d, \( J = 7.7 \) Hz, 1H), 7.39-7.24 (br m, 5H), 7.02 (ArH, d, \( J = 8.8 \) Hz, 2H), 6.59 (ArH, t, \( J = 4.8 \) Hz, 1H), 5.04 (CH\(_2\)O, m, 2H), 4.31 (CH\(_{ABX}\), dd, \( J = 6.2, 13.8 \) Hz, 1H), 4.16 (CH\(_2\)O, t, \( J = 6.0 \) Hz, 2H), 3.66 (CH\(_{ABX}\), dd, \( J = 6.2, 12.0 \) Hz, 1H) 3.54 - 3.63 (CH\(_{ABX}\), CH\(_{ABX}\), COOCH\(_3\), m, 7H); 

**\(^{13}\)C NMR** (100 MHz, \((\text{CD}_3)_2\text{SO}\)) \( \delta \) 171.13 (COOCH\(_3\)), 166.27 (ArCONH), 162.21 (ArC), 160.93 (ArC-O), 158.00 (br ArCH-N) 155.88 (OCONH), 136.77 (ArC), 129.05 (ArCH), 128.34 (ArCH), 127.84 (ArCH), 127.74 (ArCH), 114.01 (ArCH), 110.27 (ArCH), 66.14 (CH\(_2\)O), 65.64 (CH\(_2\)O), 53.85 (CHNH), 51.99 (COOCH\(_3\)), 40.28 (CH\(_2\)NH\(_2\)); 

**LRMS** (ES+ MS) \( m/z \) calc. for \( \text{C}_{25}\text{H}_{27}\text{N}_5\text{O}_6 = 493.51 \), found 516.46 (M+Na\(^+\)).
2.2.2.4 (S)-2-Amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid [28]

(S)-Methyl-2-(benzyloxy carbonylamino)-3-(4-(2-(pyrimidin-2-lamino)ethoxy)benzamido)propanoate (2.0 g, 4.05 mmol) was placed in a 500 mL pressure resistant glass flask with 10% palladium on charcoal (1.0 g). Acetic acid (100 mL) and hydrochloric acid (9 mL) were added and the mixture was degassed by bubbling nitrogen. The vessel was hydrogenated at 50 psi overnight. The mixture was filtered through a plug of celite® and the resulting solution was evaporated to dryness affording a white solid hydrochloride salt (1.33 g, 94.0%): \textbf{TLC} (silica) \text{R}_f 0.35 (chloroform/methanol/aqueous ammonia solution (40:40:20)); \textbf{¹H NMR} (400 MHz, D₂O) \(\delta 7.80 \text{ (ArH, d, } J = 8.8 \text{ Hz, 2H)}, 7.08 \text{ (ArH, d, } J = 8.8 \text{ Hz, 2H)}, 4.24 \text{ (OCH}_2\text{, t, } J = 4.9 \text{ Hz, 2H)}, 4.05 \text{ (CH}_\text{ABX, dd, } J = 3.5, 7.0 \text{ Hz, 1H)}), 3.96 \text{ (CH}_\text{ABX, dd, } J = 14.8, 3.5 \text{ Hz, 1H)}, 3.82 \text{ (CH}_\text{ABX, dd, } J = 14.8, 7.0 \text{ Hz, 1H)}, 3.58 \text{ (NCH}_2\text{, t, } J = 4.9 \text{ Hz, 2H)}, 3.33 \text{ (2 x CH}_2\text{, t, } J = 5.8 \text{ Hz, 4H)}, 1.91 \text{ (CH}_2\text{, qn, } J = 5.9 \text{ Hz, 2H}); \textbf{¹³C NMR} (100 MHz, D₂O) \(\delta 173.23 \text{ (COOH)}, 172.10 \text{ (CONH)}, 162.38 \text{ (ArC-O)}, 154.42 \text{ (N',NH}_\text{CONH}), 130.61 \text{ (ArC)}, 126.79 \text{ (ArCONH)}, 115.84 \text{ (ArC)}, 67.92 \text{ (OCH}_2\text{)}, 56.50 \text{ (CH)}, 41.60 \text{ (CH}_2\text{)}, 41.34 \text{ (CH}_2\text{), 39.52 \text{ (CH}_2\text{)}, 20.72 \text{ (CH}_2\text{); LRMS (ES+ MS) } m/z \text{ calc. for C}_{16}\text{H}_{23}\text{N}_5\text{O}_4 = 349.38, \text{ found 350.48 (M+H)}^+\).
2.2.2.5  (S)-Methyl-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoate [29]

Hydrogen chloride gas was bubbled through 250 mL of anhydrous methanol for 5 min in a 500 mL round bottom flask with constant stirring and cooling in an ice bath. (S)-Methyl-2-((benzyloxycarbonylamino)-3-(4-(2-(pyrimidin-2-lamino)ethoxy)benzamido)propanoate (3.2 g, 9.16 mmol) was added to the solution and was refluxed overnight under nitrogen. After cooling the solution was evaporated to dryness under reduced pressure and pumped to dryness to yield a white crusty dihydrochloride salt (3.84 g, 96.0 %):  

\[ \text{TLC (silica)} \text{ R}_{f} 0.75 \text{ (chloroform/methanol/aqueous ammonia solution (40:40:20))};  \]

\[ \text{^1H NMR (400 MHz, CD}_{3}\text{OD)} \delta \text{ 7.90 (ArH, d, J = 8.9 Hz, 2H), 7.07 (ArH, d, J = 8.9 Hz, 2H), 4.21 (OCH}_{2}\text{, t, J = 5.1 Hz, 2H), 4.15 (CH}_{ABX}\text{, dd, J = 5.0,} \]

\[ \text{5.9 Hz, 1H), 3.89 (CH}_{ABX}\text{, dd, J = 14.3, 5.0 Hz, 1H), 3.85 (COOCH}_{3}\text{, s, 3H), 3.81 (CH}_{ABX}\text{, dd, J = 14.3, 5.9 Hz, 1H), 3.62 (NCH}_{2}\text{, t, J = 5.0 Hz, 2H), 3.39 (2 x CH}_{2}\text{, t, J = 5.8 Hz, 4H), 2.00 (CH}_{2}\text{, qn, J = 5.9 Hz, 2H);}  \]

\[ \text{^13C NMR (100 MHz, CD}_{3}\text{OD)} \delta 171.14 (COOCH}_{3}\text{), 170.66 (CONH), 162.83 (ArC-O), 154.83 (N',NHCNH), 130.57 (ArC), 127.56 (ArCCONH), 115.37 (ArC), 67.68 (OCH}_{2}\text{), 54.82 (CH), 53.57 (COOCH}_{3}\text{), 42.04 (CH}_{2}\text{), 41.53 (CH}_{2}\text{), 39.72 (CH}_{2}\text{), 21.13 (CH}_{2};}  \]

\[ \text{LRMS (ES+ MS)} m/z \text{ calc. for C}_{17}\text{H}_{25}\text{N}_{5}\text{O}_{4} = 363.41, \text{ found 364.56 (M+H)^{+}.}  \]
2.2.3 HYNIC-Integrin Antagonist Conjugate

2.2.3.1 \((S)\text{-}\text{tert-Butyl-2-(5-(1-methoxy-1-oxo-3-(4-(2(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propan-2-ylcarbamoyl)pyridin-2-yl) hydrazinecarboxylate}\) [30]

\[(S)\text{-Methyl-2-amino-3-(4-(2(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoate}\text{ dihydrochloride salt (0.20 g, 0.47 mmol), 6-(2(\text{tert-butoxycarbonyl)hydrazinyl})nicotinic acid (0.12 g, 0.47 mmol), Py-BOP (0.34 g, 0.65 mmol) and diisopropylethylamine (0.5 mL, 2.90 mmol) were combined in a 50 mL round bottom flask with } N,N'\text{-dimethylformamide (5 mL) and dichloromethane (20 mL) at 0 °C with stirring. The reaction was allowed to proceed for 2 h at 0 °C then at room temperature overnight under a N2 atmosphere. The dichloromethane and } N,N'\text{-dimethylformamide were evaporated off under reduced pressure and the residue was suspended in chloroform (50 mL) with a small amount of methanol (5 mL) to aid solubility. The organic phase was washed with water (2 × 50 mL) and saturated sodium bicarbonate solution (2 × 50 mL). The light green organic phase was dried over magnesium sulfate and reduced to dryness at the pump leaving a light brown crusty material (0.2 g crude). The crude material was purified by silica gel chromatography eluting with a solution of methanol/dichloromethane (5:95) yielding} \]
a light brown solid after evaporation (0.21 g, 76.4%): \text{TLC (silica)} Rf 0.50 (20% methanol/80% dichloromethane); \text{\textsuperscript{1}H NMR} (400 MHz, CD$_3$OD) δ 8.58 (ArH, d, J = 2.0 Hz, 1H), 8.03 (ArH, dd, J = 2.0, 8.0 Hz, 1H), 7.80 (ArH, d, J = 8.8 Hz, 2H), 6.97 (ArH, d, J = 8.8 Hz, 2H), 6.70 (ArH, d, J = 8.8 Hz, 1H), 4.14 (CH$_2$O, t, J = 5.0 Hz, 2H), 3.82-3.95 (CH$_2$, m, 2H), 3.75 (COOCH$_3$, s, 3H), 3.56 (CH$_2$N, t, J = 5.0 Hz, 2H), 3.35 (2 x CH$_2$, t, J = 6.0 Hz, 4H), 1.92 (CH$_2$, qn, t, J = 6.0 Hz, 2H), 1.48 (COOC(CH$_3$)$_3$, br s, 9H); \text{\textsuperscript{13}C NMR} (100 MHz, CD$_3$OD) δ 172.18 (COOCH$_3$), 170.42 (CONH), 168.13 (CONH), 163.4 (ArCNHNH), 162.50 (ArC-O), 158.28 (NHNHCOOC(CH$_3$)$_3$), 154.52 (ArC), 149.15 (ArCH), 138.22 (ArCH), 130.25 (ArCH), 127.58 (ArC), 121.29 (ArC), 115.18 (ArCH), 106.60 (ArCH), 81.75 (COCH$_3$)$_3$, 67.42 (CH$_2$O), 54.85 (CHNH), 52.94 (COOCH$_3$), 41.65 (CH$_2$N), 41.32 (CH$_2$NH), 39.52 (2 x CH$_3$NH), 28.45 (C(CH$_3$)$_3$), 20.90 (CH$_2$); \text{LRMS (ES+ MS)} m/z calc. for C$_{28}$H$_{38}$N$_8$O$_7$ = 598.65, found 599.43 (M+H)$^+$. 

2.2.3.2 \text{(S)-2-(6-Hydrazinylnicotinamido)-3-(4-(2-(1,4,5,6-tetrahydro-pyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid [31]}

\begin{center}
\includegraphics[width=0.3\textwidth]{structure.png}
\end{center}

(S)-\textit{tert}-Butyl-2-(5-(1-methoxy-1-oxo-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propan-2-ylcarbamoyl)pyridin-2-yl)hydrazinecarboxylate (48.5 mg, 83.5 μmol) was placed in a 10 mL round bottom flask with dioxane (1 mL)
and a solution of 4 M hydrogen chloride in dioxane (2 mL). The mixture was stirred at room temperature for 2 hr. The mixture was filtered and the resulting crude yellow solid was washed repeatedly with diethyl ether and pumped to dryness (40 mg crude). The material was purified by repeated injections of the crude solution (40 mg/mL methanol) into a HPLC system with a Waters Atlantis 10 μm C18 column (250 mm × 25 mm I.D.) with a mobile phase of acetonitrile/0.1M ammonium acetate solution (17:83) at a flowrate of 20 mL/min and collecting the peak at 12 min. Evaporation of the mobile phase under reduced pressure yielded a white solid (28 mg, 71.3%); LRMS (ES+ MS) m/z calc. for C_{22}H_{28}N_{8}O_{5} = 484.5, found 485.25 (M+H)^{+}.

### 2.2.4 MAMA-Integrin Antagonist Conjugates and Re Complexes

#### 2.2.4.1 Methyl-2-((2-oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetate [32]

To a solution of N-(2-(tritylthio)ethyl)-2-(2-(tritylthio)ethylamino)acetamide (1.0 g, 1.47 mmol) in acetonitrile (100 mL) was added methyl 2-bromoacetate (1.66 g, 10.85 mmol), potassium hydrogen carbonate (0.1 g, 1.0 mmol) and potassium carbonate (0.1 g, 0.72 mmol). The resulting mixture was refluxed at 80 °C overnight under argon atmosphere. The cooled solution was filtered and concentrated under reduced pressure affording a yellow residue. The crude material was chromatographed on a
silica gel column, eluting the product with ethyl acetate/petroleum ether (30:70). The eluate was concentrated in vacuo and pumped dry using high vacuum to afford a golden solid (0.81 g, 73.37%); TLC\textsubscript{(silica)} $R_f = 0.62$ (ethyl acetate/petroleum ether (50:50)); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.69 (CONH, t, $J = 6.4$ Hz, 1H), 7.43-7.48 (ArH, m, 12H), 7.21-7.36 (ArH, m, 18H), 3.65 (COOCH$_3$, s, 3H), 3.24 (CH$_2$, s, 2H), 3.15 (CH$_2$, s, 2H), 3.10 (CH$_2$, q, $J = 6.4$ Hz, 2H), 2.60 (CH$_2$, t, $J = 6.6$ Hz, 2H), 2.43 (CH$_2$, t, $J = 6.4$ Hz, 2H), 2.32 (CH$_2$, t, $J = 6.6$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.08 (COOCH$_3$, CONH), 144.66 (ArC), 144.60 (ArC), 129.50 (ArH), 129.47 (ArH), 127.87 (ArH), 127.85 (ArH), 126.69 (ArH), 126.62 (ArH), 66.83 (S\textsubscript{(Ph)$_3$}), 66.66 (S\textsubscript{(Ph)$_3$}), 58.12 (CH$_2$), 54.78 (CH$_2$), 53.77 (NCH$_2$CH$_2$S), 51.58 (COOCH$_3$), 38.09 (CONHCH$_2$), 31.78 (SCH$_2$), 30.09 (SCH$_2$); LRMS (ES+ MS) m/z calc. for C$_{47}$H$_{46}$N$_2$O$_3$S$_2$ = 750.29, found 751.4 (M+H)$^+$. 

2.2.4.2 2-((2-Oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetic acid \[33\]

![Structural diagram]

To a solution of methyl 2-((2-oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetate (0.81 g, 1.08 mmol) in methanol (7.5 mL), water (3.75 mL) and tetrahydrofuran (7.5 mL) was added lithium hydroxide monohydrate (0.09 g, 2.16 mmol). The solution was stirred mechanically for 3 h, after which the pH was adjusted to 4.0 with dilute hydrochloric acid. The organic solvent was evaporated off in vacuo, and ethyl acetate (20 mL) and water (15 mL) were added to the residue. The product was extracted into the organic fraction, and was subsequently washed
with water (2 x 15 mL) and brine (15 mL), dried over magnesium sulfate, and evaporated to dryness in vacuo affording a white solid material (0.76 g, 96%);

TLC (silica) R_f = 0.17 (methanol/ethyl acetate (10:90)); ¹H NMR (400 MHz, CDCl₃) δ 7.48 (CONH, t, J = 5.9 Hz, 1H), 7.34-7.41 (ArH, m, 12H), 7.13-7.25 (ArH, m, 18H), 3.16 (CH₂, s, 2H), 3.10 (CH₂, s, 2H), 3.01 (CH₂, q, J = 6.4 Hz, 2H), 2.55 (CH₂, t, J = 6.5 Hz, 2H), 2.35 (CH₂, t, J = 6.4 Hz, 2H), 2.25 (CH₂, t, J = 6.5 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.79 (COOH), 171.86 (CONH), 144.78 (ArC), 144.73 (ArC), 129.65 (ArH), 129.62 (ArH), 128.08 (ArH), 128.05 (ArH), 126.89 (ArH), 126.82 (ArH), 66.98 (SC(Ph)₃), 66.88 (SC(Ph)₃), 57.84 (CH₂), 54.61 (CH₂), 53.62 (NCH₂CH₂S), 38.36 (CONHCH₂), 31.82 (SCH₂), 30.37 (SCH₂); LRMS (ES- MS) m/z calc. for C₄₆H₄₄N₂O₃S₂ = 736.28, found 735.5 (M-H)^−.
2.2.4.4 **Methyl-6,10-dioxo-1,1,1-triphenyl-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazahexadecan-17-oate [35]**

![Chemical Structure](attachment:image.png)

2-((2-Oxo-2-(2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetic acid (0.20 g, 0.27 mmol) and methyl-6-aminohexanoate hydrochloride salt (0.05 g, 0.27 mmol) were stirred in anhydrous dichloromethane (10 mL) and N,N'-dimethylformamide (1 mL) in a 25 mL round bottom flask at room temperature. Py-BOP coupling agent (0.155 g, 0.297 mmol) was added as a solid, directly followed by N'-methylmorpholine (0.15 mL, 1.36 mmol). The solution was allowed to stir overnight, after which the dichloromethane was evaporated off under reduced pressure. Ethyl acetate (20 mL) and water (15 mL) was added to the residue, and the product was extracted into the organic phase. The organic layer was washed with more water (2 × 15 mL) and brine (15 mL) and dried over sodium sulfate. The product was pumped dry and then dissolved in the minimum amount of ethyl acetate and chromatographed on a silica gel column eluting the product with ethyl acetate after a stepped gradient of ethyl acetate/petroleum ether (10:90 - 90:10). Evaporation of the collected fractions yielded an off white solid (0.156 g, 66.95 %). \( \text{TLC}_{(\text{silica})} R_f = 0.85 \) (methanol/ethyl acetate (10:90)); \( ^1H \text{NMR} \) (400 MHz, CDCl\(_3\)) \( \delta \) 7.32-7.43 (ArH, m, 12H), 7.16-7.30 (ArH, m, 12H), 7.02 (CONH, t, \( J = 5.8 \) Hz, 1H), 6.80 (CONH, t, \( J = 5.8 \) Hz, 1H), 3.64 (COOCH\(_3\)), 3.12 (CH\(_2\), q, \( J = 6.8 \) Hz, 2H), 2.92-3.02 (CH\(_2\), m, 6H), 2.50 (CH\(_2\), t, \( J = 6.3 \) Hz, 2H), 2.41 (CH\(_2\), t, \( J = 6.3 \) Hz, 2H), 2.31 (CH\(_2\), t, \( J = 6.3 \) Hz, 2H), 2.23 (CH\(_2\), t, \( J = 7.5 \) Hz, 2H), 1.53 (CH\(_2\), qn, \( J = 7.5 \) Hz, 2H), 1.40 (CH\(_2\), qn, \( J = 7.5 \) Hz, 2H), 1.21 (CH\(_2\), m, 2H); \( ^{13}C \text{NMR} \) (100 MHz, CDCl\(_3\)) \( \delta \) 173.91 (COOCH\(_3\)),
Chapter 2

169.61 (CONH), 169.56 (CONH), 144.51 (ArC), 144.42 (ArC), 129.48 (ArH), 129.41 (ArH), 127.99 (ArH), 127.93 (ArH), 126.90 (ArH), 126.78 (ArH), 67.13 (SCPh3), 66.88 (SCPh3), 58.55 (CH2), 57.92 (CH2), 53.92 (CH2), 51.43 (COOCH3), 38.91 (CH2), 37.95 (CH2), 33.831 (CH2), 31.99 (CH2), 30.12 (CH2), 29.14 (CH2), 26.36 (SCH2), 24.40 (SCH2); LRMS (ES+ MS) m/z calc. for C53H57N3O4S2 = 863.38, found 864.45 (M+H)+.

2.2.4.5  6,10-Dioxo-1,1,1-triphenyl-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazaheptadecan-17-oic acid [36]

Methyl-6,10-dioxo-1,1,1-triphenyl-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazaheptadecan-17-oate (0.165 g, 0.191 mmol) and lithium hydroxide monohydrate (79.7 mg, 1.9 mmol) were stirred in a solution of tetrahydrofuran (5 mL), methanol (2.5 mL) and water (5 mL) for 4 h at room temperature. The pH of the resulting solution was adjusted to 4.0 with dilute trifluoroacetic acid (TFA/water (1:10)), and the organic solvent was removed in vacuo. Ethyl acetate (10 mL) and water (5 mL) were added and the product partitioned into the organic phase. Successive washings of the organic layer with water (2 x 50 mL) and brine (5 mL) were performed before drying over sodium sulfate and evaporating under reduced pressure to yield an off-white solid as the TFA salt (0.11g, 59.7%); TLC (silica) Rf = 0.05 (methanol/ethyl acetate modified with ammonia solution (10:90)); 1H NMR (400 MHz, CDCl3) δ 7.33-7.43 (ArH, m, 12H), 7.15-7.30 (ArH, m, 12H), 7.08 (CONH, t, J = 5.8 Hz, 1H), 6.89 (CONH, t, J = 5.8 Hz, 1H), 3.13 (CH2, q, J = 6.8 Hz, 2H), 2.92-3.02 (CH2, m, 6H),
2.50 (CH$_2$, t, $J = 6.3$ Hz, 2H), 2.40 (CH$_2$, t, $J = 6.3$ Hz, 2H), 2.31 (CH$_2$, t, $J = 6.3$ Hz, 2H), 2.24 (CH$_2$, t, $J = 7.5$ Hz, 2H), 1.52 (CH$_2$, qn, $J = 7.5$ Hz, 2H), 1.40 (CH$_2$, qn, $J = 7.5$ Hz, 2H), 1.23 (CH$_2$, m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ; 177.58 (COOH), 169.99 (2 x CONH), 144.47 (ArC), 144.40 (ArC), 129.45 (ArH), 129.39 (ArH), 127.97 (ArH), 127.93 (ArH), 126.88 (ArH), 126.77 (ArH), 67.10 (SC$_3$Ph$_3$), 66.88 (SC$_3$Ph$_3$), 58.38 (CH$_2$), 54.04 (CH$_2$), 53.57 (CH$_2$), 38.93 (CH$_2$), 38.02 (CH$_2$), 33.81 (CH$_2$), 31.90 (CH$_2$), 30.17 (CH$_2$), 28.93 (CH$_2$), 25.92 (SCH$_2$), 24.14 (SCH$_2$); LRMS (ES+ MS) m/z calc. for C$_{52}$H$_{55}$N$_3$O$_4$S$_2$ = 849.36, found 850.26 (M+H)$^+$. 

2.2.4.6 (S)-Methyl 6,10-dioxo-1,1,1-triphenyl-12-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazatridecan-13-oate [37]

(S)-Methyl-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy) benzamido)propanoate (1.0 g, 2.75 mmol) and 2-((2-oxo-2-(2-(tritylthio) ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetic acid (2.05 g, 2.78 mmol) were placed in a flask with N,N'-dimethylformamide (50 mL) and cooled to 0 °C in an ice bath. BOP (1.46 g, 3.30 mmol) was added as a solid, directly followed by triethylamine (1.3 mL, 9.35 mmol) and the resulting solution was stirred for 2 h at 0 °C, after which the ice bath was removed to allow the reaction to proceed at room temperature overnight. A saturated solution of sodium hydrogen carbonate (100 mL)
was added and the reaction mixture was extracted with ethyl acetate (2 × 200 mL). The organic portion was dried over magnesium sulfate and evaporated to dryness under reduced pressure. The crude material was dissolved in the minimum amount of dichloromethane and chromatographed on a silica gel column, eluting the product from a stepped gradient (ethyl acetate/petroleum ether (50:50) to methanol/ethyl acetate (15:85). Evaporation of the eluate yielded a cream solid (1.40 g, 47.03%); 

**TLC** (silica) \( R_f = 0.25 \) (methanol/ethyl acetate (20:80));

\[ ^1H \text{ NMR (400 MHz, CD}_3\text{OD)} \]

\[ \delta = 7.83 (\text{ArCH, d, } J = 8.9 \text{ Hz, 2H}), 7.30-7.40 (\text{ArCH, m, 18H}), 7.15-7.27 (\text{ArCH, m, 12H}), 6.98 (\text{ArCH, d, } J = 8.9 \text{ Hz, 2H}), 4.54 (\text{CH}_2, \text{ t, } J = 5.1 \text{ Hz, 1H}), 4.12 (\text{CH}_2, \text{ t, } J = 5.1 \text{ Hz, 2H}), 3.72 (\text{CH}_2, \text{ s, 2H}), 3.70 (\text{CH}_3, \text{ s, 3H}), 3.56 (\text{CH}_2, \text{ t, } J = 5.1 \text{ Hz, 2H}), 3.32 (2 \times \text{CH}_2, \text{ t, 4H}), 3.21 (2 \times \text{CH}_2, \text{ m, 4H}), 2.88 - 3.18 (2 \times \text{CH}_2, \text{ m, 4H}), 2.23-2.44 (2 \times \text{CH}_2, \text{ m, 4H}), 1.89 (\text{CH}_2, \text{ m, 2H}); \]

\[ ^{13}C \text{ NMR (100 MHz, CD}_3\text{OD)} \]

\[ \delta = 173.11 (\text{C}_\text{OOCH}_3), 172.63 (\text{CONH}), 171.42 (\text{CONH}), 170.42 (\text{CONH}), 162.52 (\text{ArC-O}), 154.70 (\text{N',NHCONH}), 145.99 (\text{ArC}), 130.61 (\text{ArCH}), 130.56 (\text{ArCH}), 130.42 (\text{ArCH}), 128.77, (\text{ArCH}), 128.76 (\text{ArCH}), 127.65 (\text{ArCH}), 127.58 (\text{ArCCONH}), 115.24 (\text{ArCH}), 67.95 (\text{OCH}_2), 67.62 (\text{SC(Ph)_3}), 67.57 (\text{SC(Ph)_3}), 59.56 (\text{CH}_2), 59.13 (\text{CH}_2), 55.71 (\text{CH}_2), 52.86 (\text{COOCH}_3), 41.78 (\text{CH}_2), 41.22 (\text{CH}_2), 39.46 (\text{CH}_2), 39.21 (\text{CH}_2), 32.52 (\text{SCH}_2), 30.35 (\text{SCH}_2), 23.59 (\text{CH}_2), 20.93 (\text{CH}_2); \]

**LRMS (ES+ MS) m/z**

calc. for \( C_{63}H_{67}N_7O_6S_2 = 1080.46 \), found 1081.6 (M+H)^+.
2.2.4.7 (S)-6,10-Dioxo-1,1,1-triphenyl-12-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)met-hyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazatridecan-13-oic acid [38]

To a solution of (S)-methyl-6,10-dioxo-1,1,1-triphenyl-12-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazatridecan-13-oate (0.133 g, 0.106 mmol) in acetonitrile (30 mL) and water (3 mL) was added lithium hydroxide monohydrate (50 mg, 1.19 mmol). The reaction proceeded at room temperature overnight, after which the pH was adjusted to 4.0 with trifluoroacetic acid (1.0M). The resulting mixture was concentrated in vacuo, and ethyl acetate (50 mL) was added to the residue. The organic phase was washed with water (2 × 20 mL) and brine (20 mL). The solution was dried over magnesium sulfate and then evaporated to dryness yielding a white solid (94 mg, 71.6%); TLC (silica) Rf = 0.45 (ethanol/water/ammonia solution (10:1:1)); ^1H NMR (400 MHz, CD$_3$OD); 7.80 (ArCH, d, J = 8.8 Hz, 2H), 7.30-7.40 (ArCH, m, 18H), 7.15-7.27 (ArCH, m, 12H), 6.94 (ArCH, d, J = 8.8 Hz, 2H), 4.50 (CH, t, J = 5.1 Hz, 1H), 4.06-4.12 (CH$_2$, m, 2H), 3.70 (CH$_2$, d, J = 5.5 Hz, 2H), 3.53 (CH$_2$, t, J = 5.1 Hz, 2H), 3.32 (2 x CH$_2$, t, 4H), 2.83 - 3.15 (3 x CH$_2$, m 6H), 2.23-2.44 (3 x CH$_2$, m, 6H), 1.87 (CH$_2$, m, 2H); ^13C NMR (100 MHz, CD$_3$OD) δ 173.25 (COOH), 172.61 (CONH), 171.41 (CONH), 170.39 (CONH), 162.52 (ArC-O), 154.70 (N',NH$_2$NH),
145.97 (ArC), 130.62 (ArCH), 130.56 (ArCH), 130.42 (ArCH), 128.77 (ArCH), 128.76 (ArCH), 127.65 (ArCH), 127.58 (ArC–CONH), 115.21 (ArCH), 67.95 (OCH₂), 67.62 (SC(Ph)₃), 67.57 (SC(Ph)₃), 59.56 (CH₂), 59.13 (CH₂), 55.71 (CH₂), 41.78 (CH₂), 41.22 (CH₂), 39.47 (CH₂), 39.22 (CH₂), 32.52 (SCH₂), 30.34 (SCH₂), 23.59 (CH₂), 20.91 (CH₂); LRMS (ES+ MS) m/z calc. for C₆₂H₆₅N₇O₆S₂ = 1067.44, found 1068.46 (M+H)⁺, (ES-MS) found 1066.40 (M-H)⁻.

2.2.4.8 Oxorhenium(V)(S)-methyl-2-(2-((2-mercaptoethyl)(2-(2-mercaptoethylamino)-2-oxoethyl)amino)acetamido)-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoate complex [39]

(S)-Methyl-6,10-dioxo-1,1,1-triphenyl-12-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazatri-decan-13-oate (58.6 mg, 49.6 µmol) was placed in a flask along with degassed methanol (4 mL) and tetrahydrofuran (2 mL), and heated to reflux under argon with stirring. A solution of tin chloride (25 mg, 131.8 µmol) in 0.1 M hydrochloric acid (200 µL) was injected into the reflux, immediately followed by a solution of sodium perrhenate (27 mg, 100 µmol) in water (100 µL). The golden mixture was refluxed overnight after which the colour changed to a pale pink. The reaction mixture was poured into boiling acetonitrile (50 mL) and the mixture was filtered through a plug of celite°,
with further washings of boiling acetonitrile (3 × 25 mL). The filtrate was evaporated to dryness under reduced pressure and resuspended in a methanol/tetrahydrofuran mixture (1:1, 2 mL) for HPLC purification. Purification was achieved by repeated injections of the crude solution into a HPLC system with an Alltech Econosphere 10 m C18 column (250 mm × 22 mm I.D.) with an isocratic mobile phase of acetonitrile/aqueous 0.1% trifluoroacetic acid solution (40:60) at a flowrate of 20 mL/min. The product peak was collected at 12 min. Evaporation of the mobile phase under reduced pressure yielded a pink glassy film (8.4 mg, 21.2%); \textbf{TLC}_{(silica)} R_f = 0.31 (methanol/ethyl acetate (20:80), modified with ammonia solution); \textbf{^1H NMR} (400 MHz, CD$_3$OD) δ 7.82 (ArCH, d, J = 8.8 Hz, 2H), 7.04 (ArCH, d, J = 8.8 Hz, 2H), 5.23 (CH, br dd, 1H), 4.90 – 2.50 (br m, 25H), 1.96 (CH$_2$, m, 2H); \textbf{^13C NMR} (100 MHz, CD$_3$OD) δ 190.88 (CONH), 171.50 (COOCH$_3$), 167.93 (CONH), 167.74 (CONH), 162.50 (ArC-O), 154.67 (N',NH$\text{C}$NH), 130.25 (ArCH), 127.77 (ArC), 115.18 (ArCH), 69.29 (CH$_2$-O), 67.45 (CH$_2$), 66.78 (CH$_2$), 65.99 (CH$_2$), 63.48 (CH$_2$), 60.47 (CH$_2$), 53.76 (CH$_2$), 52.97 (CH), 41.56 (CH$_2$), 41.26 (CH$_2$), 40.17 (CH$_2$), 39.53 (2 x CH$_2$), 20.94 (CH$_2$); \textbf{LRMS} (ES+ MS) m/z calc. for C$_{25}$H$_{36}$N$_7$O$_7$S$_2$Re = 796.93, found 798.0 (M+H)$^+$; \textbf{UV-Vis} $\lambda_{\text{abs}}$/nm (acetonitrile/H$_2$O) 250.0, 361.8.
2.2.4.9 Oxorhenium(V)(S)-2-(2-(2-mercaptoethyl)(2-(2-mercaptoethylamino)-2-oxoethyl)amino)acetamido)-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid complex [40]

![Chemical Structure](image)

(S)-6,10-Dioxo-1,1,1-triphenyl-12-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazatridecan-13-oic acid dihydrochloride salt (56.6 mg, 49.6 µmol), degassed methanol (4 mL) and tetrahydrofuran (2 mL) were heated to reflux with stirring under argon. A solution of tin chloride (25 mg, 131.8 µmol) in 0.1M hydrochloric acid (200 µL) was injected into the reflux, immediately followed by a solution of sodium perrhenate (27 mg, 100 µmol) in water (100 µL). The golden mixture was refluxed overnight after which the colour changed to a pale pink. The reaction mixture was poured into boiling acetonitrile (50 mL) and the mixture was filtered through a plug of celite®, with further washings of boiling acetonitrile (3 × 25 mL). The filtrate was evaporated to dryness under reduced pressure and resuspended in a methanol/tetrahydrofuran mixture (1:1, 2 mL) for HPLC purification. Purification was achieved by repeated injections of the crude solution into a HPLC system with an Alltech Econosphere 10 m C18 column (250 mm × 22 mm I.D.) employing an isocratic acetonitrile/aqueous 0.1% trifluoroacetic acid solution at a flowrate of 20 mL/min. The product peak eluted at 10 min. Evaporation of the mobile phase under reduced pressure yielded a pink glassy film (4.9 mg, 12.6 %). $^1$H NMR (400 MHz, CD$_3$OD /D$_2$O (95:5)) δ 7.71
(ArCH, d, $J = 8.8$ Hz, 2H), 6.91 (ArCH, d, $J = 8.8$ Hz, 2H), 5.11 (CH, br dd, 1H), 4.50 – 2.80 (br m, 22H), 1.95 (CH$_2$, m, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 191.20 (CONH), 173.25 (COOH), 168.21 (CONH), 167.96 (CONH), 162.66 (ArC-O), 154.84 (N$^\prime$,NH$_2$CNH), 130.49 (ArCH), 128.04 (ArC), 115.41 (ArCH), 69.47 (CH$_2$-O), 67.67 (CH$_2$), 67.26 (CH$_2$), 66.13 (CH$_2$), 63.74 (CH$_2$), 60.70 (CH$_2$), 54.18 (CH), 41.49 (CH$_2$), 40.49 (CH$_2$), 40.31 (CH$_2$), 39.75 (2 x CH$_2$), 21.14 (CH$_2$); LRMS (ES+ MS) $m/z$ calc. for C$_{24}$H$_{34}$N$_7$O$_7$S$_2$Re = 783.15, found 794.0 (M+H)$^+$; UV-Vis $\lambda_{ab}$/nm (acetonitrile/H$_2$O) 250.0, 361.8.

2.2.4.10 (S)-Methyl 6,10,17-trioxo-1,1,1-triphenyl-19-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11,18-tetraazaicosan-20-oate [41]

(S)-Methyl-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoate (75 mg, 0.207 mmol) and 6,10-dioxo-1,1,1-triphenyl-8-(2-(tritylthio)ethyl)-2-thia-5,8,11-triazasaheptadecan-17-oic acid, TFA salt (200 mg, 0.208 mmol) with N,N$^\prime$-dimethylformamide (15 mL) were cooled to 0 °C in an ice bath. Py-BOP coupling agent (130 mg, 0.250 mmol) was added as a solid, directly followed by N$^\prime$-methylmorpholine (0.10 mL, 0.91 mmol) and the resulting solution was stirred for 2 h at 0 °C, after which the ice bath was removed to allow the reaction to proceed at room temperature overnight. The reaction solution was concentrated in
vacuo, and ethyl acetate (50 mL) was added, washed with water (2 × 20 mL), saturated sodium hydrogen carbonate solution (2 × 20 mL) and finally with brine (20 mL), and dried over sodium sulfate. The solution was evaporated to dryness under reduced pressure. The minimum amount of ethyl acetate was added to dissolve the solid and the crude product was chromatographed on a silica gel column eluting with ethyl acetate. Evaporation the eluate and drying the sample yielded a cream solid (152 mg, 61.4%); \textbf{TLC}_{\text{sila}} \text{R}_f = 0.31 (methanol/ethyl acetate (20:80) modified with a few drops of 25% v/v ammonia solution); \textbf{^1H NMR} (400 MHz, CDCl$_3$) $\delta$ 7.74 (ArCH, d, $J$ = 8.3 Hz, 2H), 7.61 (CONH, br s, 1H), 7.13-7.46 (ArCH, 3 × CONH, m, 33H), 6.77 (ArCH, d, $J$ = 8.3 Hz, 2H), 4.63 (CH, br s, 1H), 3.90 - 4.11 (2 × CH, br m, 4H), 3.77 (CH$_2$, br m, 2H), 3.65 (COOCH$_3$, s, 3H) 3.54 (CH$_2$, br m, 2H), 3.18 - 3.40 (4 × CH$_2$, br m, 8H), 2.89-3.07 (2 × CH$_2$, br m, 4H), 2.78 (CH$_2$, br s, 2H), 2.63 (CH$_2$, br m, 2H), 2.32 - 2.46 (2 × CH$_2$, m, 4H), 2.20 1.50 (CH$_2$, br m, 2H), 1.34 (CH$_2$, br m, 2H), 1.18 (CH$_2$, br m, 2H); \textbf{^13C NMR} (100 MHz, CD$_3$OD) $\delta$ 176.12 (COOCH$_3$), 172.29 (CONH), 170.11 (CONH), 162.63 (N',NH$_\text{C'NH}$), 154.77 (Ar-C=O), 146.07 (ArC), 145.81 (ArC), 130.70 (ArCH), 130.40 (ArCH), 129.10 (ArCH), 128.98 (ArCH), 128.06 (ArCH), 127.87 (ArCH), 115.38 (ArCH), 68.42 (OCH$_2$), 67.85 (SC(Ph)$_3$)$_3$, 67.63 (SC(Ph)$_3$)$_3$, 65.10 (CH$_2$), 54.62 (CH), 54.07 (CH$_2$), 53.00 (COOCH$_3$), 47.38 (CH$_2$), 47.36 (CH$_2$), 43.94 (CH$_2$), 41.82 (CH$_2$), 41.51 (CH$_2$), 40.16 (CH$_2$), 39.70 (CH$_2$), 36.59 (CH$_2$), 32.64 (CH$_2$), 29.83 (CH$_2$), 27.37 (CH$_2$), 27.29 (CH$_2$), 26.26 (CH$_2$), 25.01 (CH$_2$), 21.08 (CH$_2$); \textbf{LRMS} (ES+ MS) $m/z$ calc. for C$_{69}$H$_{78}$N$_8$O$_7$S$_2$ = 1194.54, found 1195.37 (M+H)$^+$, 1217.33 (M+Na)$^+$. 
2.2.4.11 (S)-6,10,17-Trioxo-1,1,1-triphenyl-19-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)-ethyl)-2-thia-5,8,11,18-tetraazaicosan-20-oic acid [42]

(S)-Methyl-6,10,17-trioxo-1,1,1-triphenyl-19-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)-ethyl)-2-thia-5,8,11,18-tetraazaicosan-20-oate (50 mg, 41.82 µmol) and lithium hydroxide monohydrate (18 mg, 0.43 mmol) were stirred in a solution of water (3 mL), tetrahydrofuran (3 mL) and methanol (3 mL) for 4.5 h at room temperature. Dilute trifluoroacetic acid was used to adjust the pH of the solution to 4.9, and the organic solvent was removed in vacuo. Ethyl acetate (50 mL) was added and the solution was extracted with water (2 × 20 mL) and brine (15 mL), dried over sodium sulfate and evaporated to dryness yielding an off-white solid as the TFA salt (42 mg, 77.53%).

\[
\text{TLC (silica)} \quad R_f = 0.40 \text{ (ethanol/water/ammonia solution (10:1:1)); } \]

\[
\text{1H NMR (400 MHz, CDCl}_3\text{)} \quad \delta \text{ 7.55 (ArCH, d, } J = 6.8 \text{ Hz, 2H), 7.31-7.38 (ArCH, m, 12H), 7.13-7.27 (ArCH, 2 × CONH, m, 20H), 7.08, (CONH, br s, 1H), 6.95 (CONH, br s, 1H), 6.53 (ArCH, d, } J = 6.8 \text{ Hz, 2H), 4.24 (CH, br m, 1H), 3.84 (CH}_2\text{, br m, 2H), 3.50 (CH}_2\text{, br m, 2H), 3.25 (2 × CH}_2\text{, br m, 4H), 3.07 (CH}_2\text{, br q, } J = 5.6 \text{ Hz, 2H), 2.86-3.03 (4 × CH}_2\text{, m, 8H), 2.48 (CH}_2\text{, t, } J = 5.7 \text{ Hz, 2H), 2.37 (CH}_2\text{, t, } J = 6.3 \text{ Hz, 2H), 2.29 (CH}_2\text{, t, } J = 5.7 \text{ Hz, 2H), 2.08 (CH}_2\text{, 2 × NH, br m, 4H), 1.79 (CH}_2\text{, br qn, 2H), 1.50 (CH}_2\text{, br m, 2H), 1.36 (CH}_2\text{, br m, 2H), 1.19 (CH}_2\text{, br m, 2H); } \]

\[
\text{13C NMR (100 MHz, CD}_3\text{OD)} \quad \delta \text{ 174.42 (COOH), 172.28} \]
(CONH), 170.09 (CONH), 162.63 (Ar-C-O), 154.77 (N'<NH-CNH), 146.08 (ArC), 145.82 (ArC), 130.70 (ArCH), 130.40 (ArCH), 129.11 (ArCH), 128.98 (ArCH), 128.07 (ArCH), 127.87 (ArCH), 115.39 (ArCH), 68.41 (OCH$_2$), 67.84 (SC(Ph)$_3$), 67.62 (SC(Ph)$_3$), 65.10 (CH$_2$), 54.63 (CH), 54.08 (CH$_2$), 47.38 (CH$_2$), 47.36 (CH$_2$), 43.94 (CH$_2$), 41.82 (CH$_2$), 41.51 (CH$_2$), 40.16 (CH$_2$), 39.70 (CH$_2$), 36.59 (CH$_2$), 32.64 (CH$_2$), 29.83 (CH$_2$), 27.37 (CH$_2$), 27.31 (CH$_2$), 26.28 (CH$_2$), 25.02 (CH$_2$), 21.08 (CH$_2$); LRMS (ES+ MS) $m/z$ calc. for C$_{68}$H$_{76}$N$_8$O$_7$S$_2$ = 1180.53, found 1181.24 (M+H)$^+$. 

2.2.4.12 Oxorhenium(V)(S)-1-mercapto-6-(2-mercaptoethyl)-4,8,15-trioxo-17-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-3,6,9,16-tetraazaocotadecan-18-oic acid complex [43]

(S)-6,10,17-Trioxo-1,1,1-triphenyl-19-((4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)methyl)-8-(2-(tritylthio)ethyl)-2-thia-5,8,11,18-tetraaicosan-20-oic acid (20 mg, 16.93 µmol) with methanol (2.5 mL) and tetrahydrofuran (1 mL) were heated to reflux with stirring under argon. A solution of tin chloride (11 mg, 58.01 µmol) in 0.05 M hydrochloric acid (150 µL) was injected into the reflux, immediately followed by a solution of sodium perrhenate (11 mg, 40.26 µmol) in water (18 µL). The golden mixture was refluxed overnight after which the colour changed to pale pink. The reaction mixture was poured into boiling acetonitrile (20
mL) and the mixture was filtered through a plug of celite®, with further washings of boiling acetonitrile (3 × 10 mL). The filtrate was evaporated to dryness under reduced pressure and resuspended in a methanol/tetrahydrofuran mixture (1:1, 1 mL) for HPLC purification. Purification was achieved by repeated injections of the crude solution into a HPLC system with an Alltech Alltima 10 μm C18 column (250 mm × 22 mm I.D.) employing an isocratic acetonitrile/aqueous 0.1M ammonium acetate solution (25:75) with a flowrate of 10 mL/min, collecting the peak eluting between 16 and 19 min. Evaporation of the mobile phase under reduce pressure yielded a pink glassy film (4.8 mg, 31.64%); ¹H NMR (400 MHz, 75% CD₃OD/25% D₂O) δ 7.83 (ArCH, d, J = 8.8 Hz, 2H), 7.09 (ArCH, d, J = 8.8 Hz, 2H), 5.32 (CH₂, br dd, J = 2.7, 17 Hz, 1H), 4.33 - 4.51 (CH₂, br m, 4H), 4.19 (CH₂, br t, J = 4.9 Hz, 2H), 4.10 (CH, br m, 1H), 3.66 - 3.78 (CH₂, br m, 2H), 3.57 (CH₂, br t, 2H), 3.41 - 3.49 (CH₂, br m, 1H), 3.36 (CH₂, br t, J = 5.9 Hz, 4H), 2.98 - 3.20 (CH₂, br m, 4H), 2.25 (CH₂, t, J = 7.4 Hz, 2H), 1.95 (CH₂, qn, J = 5.7 Hz, 2H), 1.81-1.86 (CH₂, m, 2H), 1.66 - 1.77 (CH₂, br m, 1H), 1.55 (CH₂, qn, J = 7.4 Hz, 2H), 1.44 (CH₂, qn, J = 7.4 Hz, 2H), 1.26 (CH₂, br m, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 191.73 (CONH), 191.71 (CONH), 176.47 (COOH), 170.23 (CONH), 167.74 (CONH), 162.29 (ArC-O), 154.54 (N’,NH(CN)H), 130.24 (ArCH), 127.79 (ArC=C=O), 115.46 (ArCH), 69.52 (CH₂O), 67.42 (CH₂), 63.89 (CH₂), 60.51 (CH₂), 56.3 (CH), 47.24 (CH₂), 41.36 (CH₂NH), 40.33 (CH₂), 40.04 (CH₂), 39.55 (2 x CH₂), 36.91 (CH₂), 29.17 (CH₂), 27.20 (CH₂), 27.12 (CH₂), 26.93 (CH₂), 26.12 (CH₂), 20.86 (CH₂); LRMS (ES+ MS) m/z calc. for C₃₀H₄₆N₈O₈S₂Re = 896.24, found 897.33 (M+H)+; UV-Vis λₘₚₑₚₑ/nm (acetonitrile/H₂O) 250.0, 358.9.
2.2.5 TRITA-Integrin Antagonist Conjugates and Lu & Cu Complexes

2.2.5.1 2,2',2'',2'''-(12-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl)tetraacetic acid (p-NCS bz TRITA) [44]

To a solution of 2,2',2'',2'''-(12-(4-aminobenzyl)-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl)tetraacetic acid (70 mg, 0.13 mmol) in water (1 mL) was added a solution of thiophosgene in chloroform (1 M, 0.2 mL). The mixture was vigorously stirred at room temperature for 2 h, after which the aqueous layer was pipetted off and washed with chloroform (2 x 2 mL). The aqueous layer was evaporated to dryness to yield a white solid (72.9 mg, 96.4%); \(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) 7.13 (ArCH, s, 4H), 2.85 - 3.95 (CH\(_2\) br m, 24H), 2.41 - 2.59 (2 x CH\(_2\), CH, m, 3H); \(^{13}\)C NMR (100 MHz, D\(_2\)O) \(\delta\) 172.28 (COOH), 138.95 (ArC), 135.09 (N=C=S), 131.63 (ArCH), 130.37 (ArC), 127.27 (ArCH), 60.57 (CH\(_2\) br), 55.00 (CH\(_2\) br), 54.34 (CH\(_2\) br), 52.64 (CH), 52.01 (CH\(_2\) br), 37.52 (CH\(_2\)), 37.32 (CH\(_2\)), 33.32 (CH\(_2\)); LRMS (ES+ MS) \(m/\)z calc. for C\(_{25}\)H\(_{35}\)N\(_5\)O\(_8\)S = 565.22, found 566.33 (M+H\(^+\)), 589.29 (M+Na\(^+\)).
Chapter 2

2.2.5.2 (S)-2,2’,2”,2’”-(12-(4-(3-(1-Carboxy-2-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)ethyl)thioureido)benzyl)-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl) tetraacetic acid [45]

\[
\begin{align*}
\text{NH} & \quad \text{O} \\
\text{H} & \quad \text{COOH} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{N} \\
\text{H} & \quad \text{O}
\end{align*}
\]

2,2’,2”,2’”-(12-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl) tetraacetic acid (40 mg, 70.72 μmol) and (S)-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid dihydrochloride salt (60 mg, 142 μmol) were placed in flask with a magnetic stir bar. Water (2.5 mL) and diisopropylethylamine (96 μL, 0.55 mmol) were added and the resulting solution was stirred at room temperature for 2 h at which time HPLC monitoring indicated the depletion of the isothiocyanate starting material. The solution was concentrated and purification was achieved by preparative HPLC. Repeated injections of the crude solution into a HPLC system with a Waters μ-bondapak 10 μm C18 column (prep LC100 mm × 25 mm I.D. cartridge) with a 40 min gradient from aqueous 0.1 % trifluoroacetic acid solution to acetonitrile/aqueous 0.1 % trifluoroacetic acid solution (50:50) with a flowrate of 17 mL/min collecting the peak at 9 min. Evaporation of the mobile phase under reduce pressure yielded a colourless glassy solid (45 mg, 69.5 %); \(^1\text{H NMR}\) (400 MHz, D\(_2\)O) δ 7.86 (ArCH, d, \(J = 8.8\) Hz, 2H), 7.39 (ArCH, d, \(J =\)
8.2 Hz, 2H), 7.26 (ArCH, d, J = 8.2 Hz, 2H), 7.04 (ArCH, d, J = 8.8 Hz, 2H), 4.62 (CH, t, J = 5.3 Hz, 1H), 4.18 (CH, t, J = 5.0 Hz, 2H), 3.72 - 4.15 (br overlapped m, 8H), 3.70-3.15 (CH₂, br overlapped m, 20H), 2.95 - 3.15 (2 × CH₂, br m, 4H), 2.79 (CH₂, br m, 1H) 2.71 (CH₂, m, 2H), 1.98 (2 × CH₂, qn, 2H);¹³C NMR (100 MHz, CD₃OD) δ 185.13 (NH,NH'-C=S), 174.39 (COOH), 172.50 (COOH, br), 170.05 (CONH), 162.56 (ArC-O), 154.66 (N',NH-CN), 140.08 (ArC₂-CH₂),133.20 (ArC-NH), 130.43 (ArCH), 130.29 (ArCH), 129.93 (ArCH), 127.75 (ArC), 115.16 (ArCH), 67.43 (CH₂-O), 60.73 (CH₂), 53.00-56.00 (CH₂, CH, br), 41.43 (CH₂), 41.28 (CH₂), 39.51 (CH₂), 37.77 (CH₂), 20.93 (CH₂); LRMS (ES+ MS) m/z calc. for C₄₈H₅₁N₁₀O₁₂S = 914.4, found 897.33.

### 2.2.5.3 Copper 2,2’-(12-(4-(3-((S)-1-carboxy-2-(4-(2-(1,4,5,6-tetrahydro-pyrimidin-2-ylamino)ethoxy)benzamido)ethyI)thioureido)benzyl)-4,10-bis(carboxymethyl)-1,4,7,10-tetraazacyclotridecane-1,7-diyl) diacetate complex [46]

TRITA conjugate [45] (5 mg, 5.46 μmol) was dissolved in a solution of ammonium acetate (0.4 M, 1 mL) with stirring. Copper chloride dihydrate (1.9 mg, 11.15 μmol) was added to the solution and was stirred at room temperature for 2 hr. The solution
immediately turned dark blue but slowly became a dark green. Repeated injections of
the crude solution (7 mg/ mL water) into a HPLC system with a Waters μ-bondapak 10 m C18 column (prep LC 100 mm × 25 mm I.D. cartridge) employing a 40 min gradient from aqueous 0.1% trifluoroacetic acid to acetonitrile/aqueous 0.1% trifluoroacetic acid (50:50) with a flowrate of 17 mL/min, collecting the peak at 13 min. Evaporation of the mobile phase under reduce pressure yielded a blue glassy solid 2.6 mg, 48.9%); LRMS (ES+ MS) m/z calc. for C_{41}H_{56}N_{10}O_{12}SCu = 975.3, found 974.24 (M+H, Cu^{63} isotope), 976.28 (M+H, Cu^{65} isotope); UV-Vis λ_{abs}/nm (acetonitrile/H_{2}O) 255.9, 325.7.

2.2.5.4 Lutetium (/S/-2,2',2'',2''''-(12-(4-(3-(1-carboxy-2-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)ethyl)thioureido)benzyl)-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl)tetaacetate complex [47]

TRITA conjugate [45] (20 mg, 21.86 mol) was dissolved in a solution of ammonium acetate (0.4 M, 2 mL) with stirring. Lutetium nitrate hydrate (20 mg, 55.40 mol) was added to the solution and was heated to 80 °C for 4 h. After cooling to room
temperature, the solution was concentrated to 1 mL for HPLC purification. Repeated injections of the crude solution (40 mg/mL water) into a HPLC system with a Waters μ-bondapak 10 m C18 column (prep LC 100 mm × 25 mm I.D. cartridge) employing a 40 min gradient from aqueous 0.1% trifluoroacetic acid to acetonitrile/aqueous 0.1% trifluoroacetic acid (50:50) with a flowrate of 17 mL/min, collecting the peak at 14 min. Evaporation of the mobile phase under reduce pressure yielded a slightly yellow powder (5.06 mg, 21.3 %) 1H NMR (400 MHz, D2O) δ 7.86 (ArCH, d, J = 8.8 Hz, 2H), 7.41 (ArCH, d, J = 7.8 Hz, 2H), 7.28 (ArCH, d, J = 8.2 Hz, 2H), 7.05 (ArCH, d, J = 8.8 Hz, 2H), 4.63 (CH, m, 1H), 4.22 (CH, m, 2H), 2.05 - 4.12 (br overlapped m series, 35H), 1.99 (2 × CH2, m, 2H); LRMS (ES+ MS) m/z calc. for C41H54N10O12SLu = 1085.3, found 1086.67 (M+H)+; UV-Vis λ_{abs}/nm (acetonitrile/H2O) 257.2.

2.2.6 ‘Integrated Approach’ Antagonist Conjugates and Re Complexes

2.2.6.1 2-(2-(1-Ethoxy-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid [48]  

![Chemical Structure](image)

Ethyl-2-(2-bromoacetamido)-3-(tritylthio)propanoate (3.1 g, 6.05 mmol), 2-amino-3-(tritylthio)propanoic acid (2.75 g, 7.57 mmol) and N,N'-dimethylformamide (100 mL) were stirred together. Triethylamine (2 mL, 14.38 mmol) was added and the solution
was heated at 80 °C overnight with stirring under nitrogen. The solution was then evaporated to an oily consistency under reduced pressure and suspended in ethyl acetate (100 mL). The organic solution was washed with water (3 × 50 mL), brine (2 × 50 mL) and dried over magnesium sulfate. The solution was pumped down to dryness. Repeated additions and evaporations of dichloromethane/diethyl ether (1:1) solutions (50 mL) were performed to help dry the sample and afford a crude golden solid. The crude material was dissolved in the minimum amount of dichloromethane and adsorbed onto the minimum amount of silica gel and pumped dry. The impregnated silica gel was applied to a silica gel column and chromatographed employing a gradient between petroleum ether and methanol/ethyl acetate (30:70) (3.70 g, 77.0%). **TLC** (silica) Rf 0.43 (methanol/ethyl acetate (20:80), modified with triethylamine); **1H NMR** (400 MHz, CDCl₃) δ 7.53 (CONH, d, J = 8.0 Hz, 1H), 7.34 - 7.45 (ArCH, m, 12H), 7.14 - 7.29 (ArCH, m, 18H), 4.36 (CH, m, 1H), 4.06 (COOCH₂CH₃, q, J = 7.2 Hz, 2H), 3.31 (COCH₂NH, d J = 17.2 Hz, 1H), 2.97 (COCH₂NH, d, J = 17.2 Hz, 1H), 2.86 (CH, m, 1H), 2.61 - 2.67 (CH₂S, m, 2H), 2.48 – 2.56 (CH₂S, m, 2H), 1.18 (COOCH₂CH₃, t, J = 7.2 Hz, 3H); **13C NMR** (100 MHz, CDCl₃) δ 175.20 (COOH), 172.20 (COOCH₂CH₃), 171.50 (CONH), 145.62 (ArC), 145.54 (ArC), 130.79 (ArCH), 130.74 (ArCH), 129.27 (ArCH), 129.22 (ArCH), 128.06 (ArCH), 128.05 (ArCH), 68.24 (SCPh₃), 68.23 (SCPh₃), 62.94 (COOCH₂CH₃), 61.80 (CH), 52.90 (CH), 51.20 (COCH₂NH), 35.65 (CH₂S), 34.80 (CH₂S), 15.30 (COOCH₂CH₃); **LRMS (ES+ MS)** m/z calc. for C₄₆H₄₆N₂O₅S₂ = 794.28, found 817.11 (M+Na)⁺.
2.2.6.2 Ethyl 2-(2-(1-(2-(1H-benzo[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoate (HOBt / EDC coupling method) [49]

2-(2-(1-Ethoxy-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid (0.7 g, 0.88 mmol), 2-(1H-benzo[d]imidazol-2-yl)ethanamine hydrochloride (0.21 g, 0.88 mmol), N'-hydroxybenzotriazole monohydrate (HOBt) (0.16 g, 1.15 mmol) and N'-methyl morpholine (0.4 mL, 3.62 mmol) were combined in a 50 mL round bottom flask with N,N'-dimethylformamide (20 mL) at 0°C with stirring. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC coupling agent) (0.22 g, 1.15 mmol) was added quickly and the reaction was allowed to proceed for 2 h at 0 °C then at room temperature overnight under a N₂ atmosphere. The N,N'-dimethylformamide was evaporated to dryness under reduced pressure and the residue was suspended in ethyl acetate (30 mL). The organic phase was washed with water (2 × 30 mL), saturated sodium bicarbonate solution (2 × 30 mL) and finally with brine (30 mL). The golden solution was dried over magnesium sulfate and reduced to dryness at the pump leaving a light brown crusty solid. The crude material was purified by silica gel chromatography employing a gradient form ethyl acetate to methanol/ethyl acetate (5:95) yielding an off-white solid (0.56 g, 67.5%): TLC\text{ (silica)} R\text{ f} 0.7 (methanol/ethyl acetate (20:80) modified with triethylamine); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.19 (benzimidazole NH, s, 1H), 8.24 (CONH, d, $J = 8.4$ Hz, 1H), 8.15 (CONH, t, $J = 5.3$ Hz, 1H), 7.50 (ArCH, d, $J = 6.9$ Hz, 1H), 7.38 (ArCH, d, $J = 7.2$ Hz, 1H), 7.17-7.36 (overlapped ArCH, m, 30H), 7.11 (ArCH, m, 2H), 4.13
(CH, m, 1H), 4.00 (COOCH₃, m, 2H), 3.49 (CH₂, q, J = 4.3 Hz, 2H), 3.07 (CH₂, br d, J = 16.8 Hz, 1H), 2.73 - 2.95 (CH₂, NH m, 5H), 2.53 (CH₂, m, 1H), 2.39 (CH₂, m, 1H), 2.28 (CH₂, br d, J = 6.3 Hz, 2H). 1.07 (COOCH₂CH₃, t, J = 7.1 Hz, 3H); ¹³C NMR (100 MHz, (CD₃)₂SO) δ 173.45 (CONH), 172.40 (CONH), 171.80 (COOCH₂CH₃), 154.76 (NH=N=CH), 145.73 (ArC), 138.9 (ArC=N), 130.77 (ArCH), 130.71 (ArCH), 129.75 (ArCH), 129.670 (ArCH), 128.50 (ArCH), 128.35 (ArCH), 122.80 (ArCH), 113.50 (ArCH), 67.11 (SCH₂), 67.10 (SCH₂), 62.60 (CH), 62.41 (COOCH₂CH₃), 53.35 (CH), 51.14 (COCH₂NH), 39.61 (NHCH₂CH₂), 36.49 (SCH₂), 34.75 (SCH₂), 30.83 (imidazole CCH₂CH₂NH), 15.60 (COOCH₂CH₃); LRMS (ES+ MS) m/z calc. for C₅₇H₅₅N₅O₄S₂ = 937.37, found 938.21 (M+H)⁺, 960.0 (M+Na)⁺.

2.2.6.3 Ethyl-2-(2-(1H-benzo[d]imidazo[2-yl]ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoate (Py-BOP coupling method) [49]

2-(2-(1-Ethoxy-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid (0.7 g, 0.88 mmol), 2-(1H-benzo[d]imidazo[2-yl]ethanamine hydrochloride (0.21 g, 0.88 mmol), benzotriazol-1-yl-oxytris(pyrrolidinophosphonium hexafluorophosphate (Py-BOP) (0.15 g, 1.15 mmol) and N'-methyl morpholine (0.4 mL, 3.62 mmol) were combined with N,N'-dimethylformamide (20 mL) at 0 °C with stirring. The reaction was allowed to proceed for 2 h at 0 °C then at room temperature overnight under a N₂ atmosphere. The N,N'-dimethylformamide was evaporated to dryness under reduced pressure and the residue was suspended in ethyl acetate (30 mL). The organic phase was washed with water (2 × 30 mL), saturated sodium bicarbonate
solution (2 × 30 mL) and finally with brine (30 mL). The golden solution was dried over magnesium sulfate and reduced to dryness at the pump leaving a light brown crusty solid. The crude material was purified by silica gel chromatography employing a gradient form ethyl acetate to methanol/ethyl acetate (5:95) yielding an off-white solid (0.68 g, 82%): Identical properties as for [49] reported in Section 2.2.6.2.

2.2.6.4 2-(2-(1-(2-(1H-Benzo[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid  (Lithium hydroxide method) [50]

Ethyl-2-(2-(1-(2-(1H-benzo[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoate (0.92 g, 0.98 mmol) was placed in a 100 mL round bottom flask with tetrahydrofuran (10 mL), ethanol (5 mL) and water (15 mL). To the stirred solution was added lithium hydroxide monohydrate (0.41 g, 9.77 mmol) and stirring was continued at room temperature for 2 h. The golden solution was then acidified to pH 4.0 with trifluoroacetic acid and the organic solvents were removed in vacuo. To the resulting residue in water was added ethyl acetate (25 mL) and the product was extracted into the organic layer. The ethyl acetate fraction was washed with water (2 × 25 mL) followed by brine (25 mL). The solution was then dried over anhydrous sodium sulfate and pumped to dryness, yielding a tan solid. (0.81 g, 90.8%); \text{TLC(silica)} R_f 0.10 (methanol/ethyl acetate
(20:80) modified with a few drops of ammonia solution; ^1H NMR (400 MHz, (CD$_3$)$_2$SO) δ 8.45 (CONH, t, $J = 5.4$ Hz, 1H), 8.38 (CONH, d, $J = 8.3$ Hz, 1H), 7.53-7.59 (ArCH, m, 2H), 7.18-7.37 (ArCH, overlapped m, 32H), 4.18 (CH, m, 1H), 3.59 (CH$_2$, qn, $J = 6.1$ Hz, 2H), 3.14-3.23 (CH$_2$, m, 2H), 3.06 -3.14 (CH$_2$, m, 2H), 2.96-3.05 (CH$_2$, m, 2H), 2.51-2.58 (CH$_2$, m 2H), 2.38-2.46 (m, 1H), 2.35 (CH$_2$, br d, $J = 7.3$ Hz, 2H), 1.72 (CH$_2$, m, 2H); ^13C NMR (100 MHz, (CD$_3$)$_2$SO) δ 171.23 (COOH), 171.16 (CONH), 168.54 (CONH), 152.26 (NH′N=CCH$_2$), 147.65 (Ar-C-N), 144.03 (ArC), 144.01 (ArC), 128.93 (ArCH), 127.95 (ArCH), 127.93 (ArCH), 126.70 (ArCH), 126.64 (ArCH), 122.70 (ArCH), 114.07 (ArCH), 66.11 (SCPh$_3$), 66.10 (SCPh$_3$), 65.94 (CH), 51.14 (COCH$_2$NH), 48.49 (CH), 36.84 (SC$_2$H), 33.05 (SC$_2$H), 25.83 (imidazole C$_2$H$_2$CH$_2$NH), 25.75; LRMS (ES+ MS) m/z calc. for C$_{55}$H$_{51}$N$_5$O$_4$S$_2$ = 909.34, found 910.0 (M+H)$^+$, (ES- MS) found 908.0 (M-H)$^-$. 

### 2.2.6.5 2-(2-(1-(1H-Benzod)imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid (Cesium carbonate method) [50]

Ethyl-2-(2-(1-(1H-benzo[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoate (2.60 g, 2.77 mmol) was placed in a flask with methanol (100 mL), water (10 mL) and cesium carbonate (1.8 g, 5.522 mmol). The mixture was stirred overnight at room temperature. The resulting solution was acidified to pH 5.0 with dropwise addition of trifluoroacetic acid. The solution was evaporated to dryness under reduced pressure and suspended in ethyl acetate (75 mL). The organic phase was subsequently washed with water (2 × 50 mL) and brine (2 × 50 mL). The solution was then dried over anhydrous sodium...
sulfate and pumped to dryness, yielding a tan solid. (2.52 g, 95.2%): Identical properties as reported for [50] in Section 2.2.6.4.

2.2.6.6 Methyl-1-(1H-benzo[d]imidazol-2-yl)-4,8,11-trioxo-14-(phenylsulfonamido)-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oate [51]

2-(2-(1-(2-(1H-Benzimidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-yl-amino)acetamido)-3-(tritylthio)propanoic acid) (0.70 g, 0.71 mmol) and (S)-methyl 3-amino-2-(phenylsulfonamido)propanoate (0.27 g, 0.91 mmol) were placed in a flask with N,N'-dimethylformamide (35 mL) under nitrogen. The solution was cooled to 0 °C in an ice bath. N'-methylmorpholine (1 mL, 9.05 mmol) was injected into the solution, as was solid Py-BOP (0.56 g, 1.07 mmol). The solution was stirred at 0 °C for 2 h then at room temperature for 48 h. The golden solution was evaporated to dryness under reduced pressure. Ethyl acetate (50 mL) was added to the golden brown material and the organic phase was washed with water (2 × 50 mL), saturated sodium bicarbonate solution (2 × 50 mL) and finally with brine (2 × 50 mL). The ethyl acetate solution was dried over magnesium sulfate and pumped to dryness. The crude product was then purified by silica gel chromatography employing a gradient from ethyl acetate/petroleum ether (50:50) to methanol/ethyl acetate (5:95) to yield an off-white solid diastereoisomeric mixture. (0.48 g, 58.5%): TLC \(_{\text{silia}}\) \(R_f\) 0.80 (methanol/ethyl acetate (20:80) modified with a few drops of triethylamine); \(^1\text{H}\) NMR (400 MHz, CD\(_3\)OD) \(\delta\) 7.79 (ArCH, d, \(J = 7.6\) Hz, 2H), 7.45-7.61 (ArCH,
overlapped m, 5H), 7.14 - 7.38 (ArCH, overlapped m, 32H), 4.21 (CH, t, 1H), 3.98 - 4.14 (CH, m 2H), 3.55 - 3.68 (CH2, m 2H), 3.20 - 3.53 (COOCH3, CH2, overlapped m, 5H), 2.78 - 3.13 (CH2, m, 4H), 2.60 (CH2, m, 2H), 2.46 (CH2, m, 2H); 13C NMR (100 MHz, CD3OD) δ 174.56 (C=OCH3), 173.67 (CONH), 172.45 (CONH), 171.22 (CONH), 153.91 (benzimidazole NH′N=CH2), 145.98 (ArC), 145.96 (ArC), 145.87 (ArC), 141.87 (ArC), 133.79 (ArCH), 133.76 (benzimidazole ArC-N), 130.75 (ArCH), 130.11 (ArCH), 130.10 (ArCH), 129.06 (ArCH), 129.04 (ArCH), 128.15 (ArCH), 128.134 (ArCH), 127.95 (ArCH), 127.88 (ArCH), 123.37 (ArCH), 68.15 (SCPh3), 68.02 (SCPh3), 62.64 (CH), 56.53 (CH), 56.21 (CH), 53.33 (CH), 52.94 (CH2), 52.90 (CH), 51.13 (COOCH3), 48.37 (CH2NH), 42.64 (CH2NH), 38.78 (CH2), 35.76 (CH2), 34.63 (CH2), 29.81 (CH2); LRMS (ES+ MS) m/z calc. for C65H63N7O7S3 = 1149.4, found 1150.15 (M+H)+.

2.2.6.7 1-(1H-Benzo[d]imidazol-2-yl)-4,8,11-trioxo-14-(phenylsulfonamido)-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraaza pentadecan-15-oic acid [52]

To methyl-1-(1H-benzo[d]imidazol-2-yl)-4,8,11-trioxo-14-(phenylsulfonamido)-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oate (0.24 g, 0.21 mmol) and methanol (20 mL) in a flask equipped with a magnetic stir bar, was added water (4 mL) and cesium carbonate (0.14 g, 0.43 mmol). The mixture was stirred at room temperature overnight. Trifluoroacetic acid was used to adjust the pH to 5.0 and the solution was evaporated to dryness under reduced pressure. The solid was suspended
in ethyl acetate (40 mL) and the organic phase was washed with water (2 × 30 mL) and brine (2 × 30 mL) before drying over magnesium sulfate. The golden solution was pumped to dryness to afford a cream solid diastereoisomeric mix. (0.19 g, 80.15%):  

**TLC**<sub>(silica)</sub> R<sub>f</sub> 0.10 (methanol/ethyl acetate (10:90) modified with a few drops of ammonia solution);  

**<sup>1</sup>H NMR** (400 MHz, CD<sub>3</sub>OD) δ 7.84 (ArCH, d, J = 7.6 Hz, 2H), 7.41-7.55 (ArCH, overlapped m, 5H), 7.10-7.38 (ArCH, overlapped m, 32H), 4.18 - 4.28 (2 overlapped diastereoisomeric signals in an approx. 1:2 ratio, CH, m, 1H), 3.78 (CH, q, J = 6.0 Hz, 1H), 3.39-3.70 (2 sets of overlapped CH<sub>2</sub>, m, 4H), 3.20 (CONHCH<sub>3</sub>, t, J = 6.8 Hz, 2H), 2.73 - 2.99 (2 sets of diastereoisomeric CH<sub>AB</sub>, m, J = 63.2 Hz, 2H), 2.58 - 2.73 (CH<sub>2</sub>, split m, 2H), 2.54 - 2.58 (CH, m 1H), 2.33 - 2.53 (CH<sub>2</sub>, split m, 2H);  

**<sup>13</sup>C NMR** (100 MHz, CD<sub>3</sub>OD) δ 174.50 (COOH), 174.19 (CONH), 173.25 (CONH), 171.96 (CONH), 153.16 (benzimidazole NH N=CCH<sub>2</sub>), 145.70 (ArC), 145.68 (ArC), 141.51 (ArC), 141.36 (ArC), 135.22 (ArCH), 135.21 (benzimidazole ArC-N), 133.52 (ArCH), 130.57 (ArCH), 130.48 (ArCH), 129.95 (ArCH), 128.86 (ArCH), 128.84 (ArCH), 128.06 (ArCH), 128.01 (ArCH), 127.75 (ArCH), 127.70 (ArCH), 125.27 (ArCH), 114.93 (ArCH), 67.95 (SCPh<sub>3</sub>), 67.93 (SCPh<sub>3</sub>), 62.26 (CH), 57.15 (CH), 53.06 (CH), 50.76 (COCH<sub>2</sub>NH), 43.34 (CH<sub>2</sub>NH), 43.30 (CH<sub>2</sub>NH), 37.91 (CH<sub>2</sub>NH), 34.79 (CH<sub>2</sub>), 34.33 (CH<sub>2</sub>), 28.25 (CH<sub>2</sub>);  

**LRMS** (ES+ MS) m/z calc. for C<sub>64</sub>H<sub>61</sub>N<sub>7</sub>O<sub>7</sub>S<sub>3</sub> = 1135.38, found 1136.00 (M+H)<sup>+</sup>, (ES- MS) found 1134.0 (M-H)<sup>-</sup>.
2.2.6.8  Methyl-1-(1H-benzo[d]imidazol-2-yl)-14-(benzyloxy carbonylamino)-4,8,11-trioxo-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oate [53]

\[
\begin{array}{c}
\text{N} \quad \text{H} \\
\text{O} \quad \text{N} \\
\text{O} \\
\text{N} \\
\text{S} \\
\text{N} \\
\text{H} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{O} \\
\text{C} \quad \text{O} \\
\text{C} \\
\text{H} \end{array}
\]

2-(2-(1-(2-(1H-Benzo[d]imidazol-2-yl)ethylamino)-1-oxo-3-(tritylthio)propan-2-ylamino)acetamido)-3-(tritylthio)propanoic acid (0.7 g, 0.71 mmol) and (S)-methyl 3-amino-2-(benzyloxy carbonylamino)propanoate (0.23 g, 0.78 mmol) were placed in a flask with N,N'-dimethylformamide (35 mL) under nitrogen. The solution was cooled to 0 °C in an ice bath. N'-methyl morpholine (1 mL, 9.05 mmol) was injected into the solution, as was solid Py-BOP (0.5 g, 0.96 mmol). The solution was stirred at 0 °C for 2 hr then at room temperature for 48 h. The golden solution was evaporated to dryness under reduced pressure. Ethyl acetate (50 mL) was added to the golden brown material and the organic phase was washed with water (2 × 50 mL), saturated sodium bicarbonate solution (2 × 50 mL) and finally with brine (2 × 50 mL). The organic fraction was dried over magnesium sulfate and pumped to dryness. The crude product was then purified by silica gel chromatography employing a gradient from ethyl acetate/petroleum ether (50:50) to methanol/ethyl acetate (5:95) to yield an off-white solid diastereoisomeric mixture (0.35 g, 43.75%): \text{TLC}_{\text{silica}} R_f 0.85 (methanol/ethyl acetate (20:80) modified with a few drops of ammonia solution); \text{^1H NMR} (400 MHz, CDCl_3) \delta 7.45 - 7.52 (ArCH, m, 2H), 7.14 - 7.40 (ArCH, overlapped m, 37H), 4.82 - 5.10 (Ar-CH_2O, m, 2H), 4.30 - 4.40 (CH, m, 1H), 4.22 (CH, t, 1/2H), 4.07 (CH, t, 1/2H), 3.37 - 3.69 (COOCH_3, 2 × CH_2, overlapped m, 7H), 3.06 (CH_2, m, 2H), 2.95 (CH, m, 1H), 2.77 - 2.90 (CH_2, split m, 2H), 2.51 - 2.61
\( (\text{CH}_2, \ t, \ J = 7.8 \ \text{Hz}, \ 2\text{H}) \), 2.38 - 2.50 (\text{CH}_2, \ m, \ 2\text{H}) \); \(^{13}\text{C} \text{NMR} \ (100 \ \text{MHz}, \ \text{CDCl}_3) \ \delta \ 172.57 \ (\text{COOCH}_3), \ 171.81 \ (\text{CONH}), \ 171.43 \ (\text{CONH}), \ 171.35 \ (\text{CONH}), \ 171.15 \ (\text{CONH}), \ 170.95 \ (\text{CONH}), \ 156.42 \ (\text{OCONH}), \ 156.26 \ (\text{OCONH}), \ 152.40 \ (\text{benzimidazole} \ \text{NH} = \text{CCH}_2), \ 144.44 \ (\text{ArC}), \ 136.43 \ (\text{benzimidazole} \ \text{ArC-N}), \ 136.23 \ (\text{ArC}), \ 129.64 \ (\text{ArCH}), \ 128.61 \ (\text{ArCH}), \ 128.14 \ (\text{ArCH}), \ 126.98 \ (\text{ArCH}), \ 122.42 \ (\text{ArCH}), \ 114.73 \ (\text{ArCH}), \ 67.33 \ (\text{SCPh}_3), \ 67.14 \ (\text{ArCH}_2\text{O}), \ 61.86 \ (\text{CH}), \ 61.28 \ (\text{CH}), \ 54.62 \ (\text{CH}), \ 52.80 \ (\text{COOC}_3\text{H}_3), \ 52.24 \ (\text{COCH}_2\text{NH}), \ 50.66 \ (\text{CH}), \ 50.18 \ (\text{CH}_2), \ 41.01 \ (\text{CH}_2\text{NH}), \ 37.93 \ (\text{CH}_2\text{NH}), \ 37.39 \ (\text{CH}_2), \ 33.74 \ (\text{SCH}_2), \ 29.80 \ (\text{CH}_2), \ 29.32 \ (\text{CH}_2), \ 29.00 \ (\text{CH}_2); \ \text{LRMS} \ (\text{ES+ MS}) \ m/z \ \text{calc.} \ \text{for} \ C_{67}H_{65}N_7O_7S_2 = 1143.44, \ \text{found} \ 1143.50 \ (\text{M}+\text{H})^+.

2.2.6.9 \ 1-(1H-\text{Benzo[d]imidazol-2-yl})-14-(\text{benzyloxy carbonylamino})-4,8,11-trioxo-5,10-bis(\text{tritylthiomethyl})-3,6,9,12-tetraazapentadecan-15-oic acid \ [54]

To methyl-1-(1H-benzo[d]imidazol-2-yl)-14-(benzyloxy carbonylamino)-4,8,11-trioxo-5,10-bis(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oate (0.20 g, 0.17 mmol) and methanol (15 mL) in a flask equipped with a magnetic stir bar, was added water (3 mL) and cesium carbonate (0.12 g, 0.36 mmol). The mixture was stirred at room temperature overnight. Trifluoroacetic acid (1.0M) was used to adjust the pH to 5.0 and the solution was evaporated to dryness under reduced pressure. The solid was suspended in ethyl acetate (50 mL) and the organic phase was washed with water
(2 × 50 mL) and brine (2 × 50 mL) before drying over magnesium sulfate. The golden solution was pumped to dryness to afford a cream solid. (0.18 g, 93.0%):

\[ \text{TLC}_{(\text{silica})} \text{ R}_{f} 0.05 \text{ (methanol/ethyl acetate (10:90) modified with a few drops of ammonia solution)}; \]

\[ \text{\textsuperscript{1}H NMR (400 MHz, CD}_{3}\text{OD); } \delta 7.49 - 7.55 (\text{ArCH, m, 2H}), 7.31 - 7.37 (\text{ArCH, m, 6H}), 7.10 - 7.31 (\text{ArCH, overlapped m, 31H}), 4.92 - 5.06 (\text{Ar-CH}_{2}\text{O, m, 2H}), 4.16 - 4.31 (2 \times \text{CH, m, 2H}), 3.46 - 3.72 (2 \times \text{CH}_{2}, \text{m, 4H}), 3.19 (\text{CONHC}_{\text{H}_{2}}, \text{t, } J = 6.5 \text{ Hz, 2H}), 2.75 - 2.98 (\text{CONHCH}_{\text{Ar}}\text{NH, } J = 57.2 \text{ Hz, 2H}), 2.62 - 2.71 (\text{CH}_{2}, \text{m, 2H}), 2.45 - 2.58 (\text{CH}_{2}, \text{m, 2H}), 2.33 - 2.42 (\text{CH}_{2}, \text{split m, 1H}); \]

\[ \text{\textsuperscript{13}C NMR (100 MHz, CD}_{3}\text{OD) } \delta 174.42 (\text{COOH}), 173.44 (\text{CONH}), 172.53 (\text{CONH}), 158.39 (\text{OCONH}), 153.51 (\text{benzimidazole NH'}\text{N}=\text{CCH}_{2}), 145.91 (\text{ArC}), 138.10 (\text{benzimidazole ArC-N}), 136.14 (\text{ArC}), 130.73 (\text{ArCH}), 129.47 (\text{ArCH}), 129.29 (\text{ArCH}), 129.05 (\text{ArCH}), 128.86 (\text{ArCH}), 127.93 (\text{ArCH}), 125.14 (\text{ArCH}), 115.23 (\text{ArCH}), 68.14 (\text{SCH}_{3}), 67.76 (\text{ArCH}_{2}O), 62.45 (\text{CH}), 56.27 (2 \times \text{CH, br d, (diastereoisomers)}), 53.36 (\text{CH}), 50.98 (\text{COCH}_{2}\text{NH}), 42.36 (\text{CH}_{2}\text{NH}), 38.26 (\text{CH}_{2}\text{NH}), 35.03 (\text{CH}_{2}), 34.89 (\text{SCH}_{2}), 28.76 (\text{CH}_{2}); \]

\[ \text{LRMS (ES+ MS) } m/z \text{ calc. for } C_{66}H_{63}N_{7}O_{7}S_{2} = 1129.42, \text{ found 1130.24 (M+H)}^{+}, (\text{ES- MS) } m/z 1128.08 (M-H)}. \]

2.2.6.10 Oxorhenium(V) \text{14-(1H-benzo[d]imidazol-2-yl)-1-carboxy-4,7,11-trioxo-1-(phenylsulfonamido)-5,10-bis(sulfidomethyl)-6-amide-3,9,12-triazatetradecane [23]}

![Oxorhenium(V) structure](image)

1-(1H-benzo[d] imidazol-2-yl)-4,8,11-trioxo-1-(phenylsulfonamido)-5,10-bis (tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oic acid (40 mg, 35 µmol) was placed
in a flask along with degassed methanol (4 mL) and tetrahydrofuran (2 mL) and heated to reflux with stirring under argon. A solution of tin chloride (25 mg, 131.9 µmol) in 0.1 M hydrochloric acid (200 µL) was injected into the reflux, immediately followed by a solution of sodium perrhenate (27 mg, 100 µmol) in water (100 µL). The golden mixture was refluxed overnight after which the colour change to a pale pink. The reaction mixture was poured into boiling acetonitrile (50 mL) and the mixture was filtered through a plug of celite®, with further washings of boiling acetonitrile (3 × 25 mL). The filtrate was evaporated to dryness under reduced pressure and resuspended in a 1:1 methanol/tetrahydrofuran mixture (2 mL) for HPLC purification. Purification was achieved by repeated injections of the crude solution into a HPLC system with a Waters Sunfire Prep C₁₈ OBD 10 m column (150 mm × 30 mm I.D.) with an isocratic mobile phase of acetonitrile/0.1M ammonium bicarbonate (25:75) solution (pH 8.0) with a flowrate of 20 mL/min collecting the peak at 14 - 16 min. Evaporation of the mobile phase under reduced pressure yielded an orange glassy film (2.3 mg, 7.7%); LRMS (ES+ MS) m/z calc. for C₂₆H₂₉N₇O₈S₃Re = 850.08, found 852.14 (M+H)+; UV-Vis λ₅₀0/nm (acetonitrile/H₂O) 272.4, 279.5, 293.1, 304.4, 315.8, 331.2, 345.9.

2.2.6.11 Oxorhenium (V) 18-(1H-benzo[d]imidazol-2-yl)-5-carboxy-3,8,11,15-tetraoxo-1-phenyl-9,14-bis(sulfidomethyl)-2-oxa-10-amide-4,7,13,16-tetraazaocadecane [24]

1-(1H-benzo[d]imidazol-2-yl)-14-(benzyloxycarbonylamino)-4,8,11-trixo-5,10-bis-(tritylthiomethyl)-3,6,9,12-tetraazapentadecan-15-oic acid (20 mg, 17.7 µmol) was
Chapter 2

placed in a flask along with a mixture of degassed methanol/ tetrahydrofuran (7:1, 2 mL) and heated to reflux with stirring under argon. A solution of tin chloride (10 mg, 52.74 µmol) in 0.1M hydrochloric acid (110 µL) was injected into the refluxing solution, immediately followed by a solution of sodium perrhenate (10.6 mg, 38.8 µmol) in water (100 µL). The golden mixture was refluxed for 3 h and then for 48 h at room temperature. The reaction mixture was poured into boiling acetonitrile (50 mL) and the mixture was filtered through a plug of celite®️, with further washings of boiling acetonitrile (3 × 25 mL). The filtrate was evaporated to dryness under reduced pressure and resuspended in a methanol/tetrahydrofuran mixture (1:1, 2 mL) for HPLC purification. Purification was achieved by repeated injections of the crude solution into a HPLC system with an Alltech Alltima 10 μm C18 column (250 mm × 22 mm I.D.) with an isocratic mobile phase of acetonitrile/0.1M ammonium acetate solution (65:35), with a flowrate of 15 mL/min collecting the peak at 14 - 16 min. Evaporation of the mobile phase under reduced pressure yielded an orange powder (1.9 mg, 12.6%). LRMS (ES+ MS) m/z calc. for C_{28}H_{31}N_{7}O_{8}S_{2}Re = 844.12, found 845.99 (M+H)^+; UV-Vis \lambda_{abs}/nm (acetonitrile/H_{2}O) 273.4, 280.7, 336.4.

2.3 RESULTS AND DISCUSSION

2.3.1 Integrin Antagonist Synthesis

In this section an alternative synthetic route to the Merck integrin antagonist molecule was performed, due to an inability to replicate the Mitsonobu coupling reported by Duggan et al. The synthetic route undertaken is outlined in Scheme 2.1. Starting off with commercially available methyl 4-hydroxybenzoate, 1,2-dichloroethane was used to alkylate the molecule to give the corresponding alkyl
Scheme 2.1 Synthetic route to (S)-methyl-2-amino-3-(4-(2-(1,4,5,6-tetrahyd-
ropyrimidin-2-ylamino)ethoxy)benzamido)propanoate [29]
chloride in good yield. This material was subject to azide substitution of the terminal chloride to afford the terminal azide analogue [25]. Reduction of the azide group to the corresponding amine [26] was initially attempted using 10% palladium on charcoal under pressurised hydrogen atmosphere, but these experiments resulted in incomplete transformation. The method of Fringuelli et al was adapted successfully and this involved the chemoselective sodium borohydride reduction catalysed by cobalt(II) chloride in aqueous media.\(^{(119)}\) The reported method called for the use of cetyltrimethylammonium bromide, a cationic surfactant but this was not really necessary as the azide was not hydrophobic. Initially the reaction was attempted with it, but excess frothing resulted in loss of the reaction contents. The reaction worked quite well without the phase transfer catalyst. The mechanism of the reduction is more like a catalytic hydrogenation, as the reaction of cobalt(II) chloride with the borohydride ion, generates the cobalt boride species which acts as a catalyst for borohydride decomposition liberating hydrogen, however, the authors do not exclude the possibility of a cobalt hydride species being responsible. The next step in the synthesis was the alkylation of the amine with 2-bromopyrimidine [57], and this was performed in the same fashion as Duggan et al,\(^{(115)}\) affording a product with the same NMR characteristics and mass spectrum as [58]. Base hydrolysis of this methyl ester yielded the acid [59] as the hydrochloride salt, indicated by the absence of the methyl ester peak in the proton spectrum (3.6 ppm in CD\(_3\)OD).

The construction of the other half of the molecule began with the Hoffman rearrangement of N(\(\alpha\)) Cbz - L- asparagine [60] with iodobenzenediacetate, followed by the acid promoted methyl esterification of the carboxylic acid group [62]. The peptide coupling reaction to form (\(S\))-methyl-2-(benzyloxy-carbonylamino)-3-(4-(2-(pyrimidin-2-ylamino)ethoxy)benzamido)propanoate [27] was performed at 0 °C for
2 h, then at room temperature overnight using the BOP coupling agent. As with all of
the peptide coupling reactions described in this Section, efforts were made to exclude
water as hydrolysis of the coupling agent hampers the reaction. Reactions were
carried out under dry nitrogen and care was taken to minimise the times when solid
coupling agents were added to the reaction. Hexamethylphosphoramide (HMPA), a
by product of the BOP decomposition was difficult to remove chromatographically,
and its high boiling point excluded the possibility of driving it off under high vacuum
with heating.\(^\text{120}\) It was detected by its characteristic doublet at $\delta$ 2.65 ppm (CDCl\(_3\))
in the $^1$H spectrum and only after trituration in ethyl acetate/methanol (95:5) of the
crude product following the extraction, was it found to be an effective method of
HMPA removal. It should also be noted that the HMPA proton peaks appear near
where the protons for $N,N'$-dimethylformamide occur (sharp singlets at $\delta$ 2.88 and $\delta$
2.96 ppm), and this was the source of some confusion initially. The average yield of
this reaction, 64.2% was most likely the result of the trituration step. The $^1$H, $^{13}$C and
mass spectral analysis confirmed the material as the target compound.

The next step involved the catalytic hydrogenation of the material to deprotect
the terminal amine and hydrogenate the pyrimidine ring to its corresponding 1,4,5,6-
tetrahydropyrimidine [28]. This was achieved with 10% palladium on charcoal in a
mixture of acetic acid and hydrochloric acid with hydrogenation at 50 psi. Mass
spectral analysis and $^1$H NMR (Fig 2.3) confirmed the transformation with the
disappearance of the aromatic protons of the benzylxycarbonylamino (Cbz) group as
well as the pyrimidine group. As this molecule was integral component to all of the
‘conjugate approach’ target molecules, Distortionless Enhancement by Polarization
Transfer (DEPT $^{13}$C), 2D Correlation Spectroscopy (COSY) and Heteronuclear
Multiple Quantum Correlation (HMQC) spectra were recorded for the unequivocal
assignment of the shifts observed in the $^1$H and $^{13}$C spectra. The assignment of the protons is shown in Fig 2.3. H1 and H3 appear to be equivalent, indicating the resonance stabilised nature of the tetrahydropyrimidine. Also of note is the ABX system at the C20 and the chiral C21 centre, which is quite clear for this molecule, but is much less apparent in the more complicated analogues derived from this material. Information from the $^{13}$C and DEPT experiment determined the lone CH at 56.50 ppm in D$_2$O. This was also confirmed by the HMQC correlation of $^1$H and $^{13}$C shifts. Mass spectral analysis provided further evidence of the correct structure yielding the expected value of $m/z$ 350.48 (M+1) in electrospray positive conditions.

To protect the terminal carboxylic acid during the following conjugation reactions, conversion to the methyl ester [29] was achieved by refluxing the acid in methanolic hydrochloric acid overnight affording the methyl ester hydrochloride salt in good yield (96.0%). A slight green tint was noticed in the solution and this was most likely due to the nickel dissolution from the syringe needle providing the nitrogen flow. $^1$H NMR confirmed the ester with the appearance of the COOCH$_3$ singlet at $\delta$ 3.85 ppm integrating to approximately 3H in CD$_3$OD. As expected the material was significantly more hydrophobic and thus not soluble in water.
Figure 2.3 $^1$H spectrum of (S)-2-amino-3-(4-(2-(1,4,5,6-tetrahydropyrimidin-2-ylamino)ethoxy)benzamido)propanoic acid [28] with integration and peak identifications in D$_2$O.
2.3.2 Hydrazinonicotinamide (HYNIC) BFC Synthesis and Conjugation

The HYNIC bifunctional chelating unit was constructed as per the method of Abrams et al.\(^{(86)}\) Scheme 2.2 outlines this synthetic route. The Boc group was used to protect the hydrazine moiety, but instead of using the \(N'\)-hydroxysuccinimide group as an activated ester, the carboxylic acid was not modified but rather used as is when coupling to the integrin active molecule using peptide coupling technology. The Boc group was removed (as well as hydrolysis of the methyl ester protector to the acid) using 4.0M HCl\(_\text{aq}\) in dioxane. The ester hydrolysis was not expected, but indicates the presence of a small amount of water in the reaction. Due to the chelating nature of the deprotected hydrazinopyrimidine moiety, faint colours were observed in the purified materials, and this is almost certainly due to coordination to metal impurities. A slight pink colour could have been the result of the iron or nickel coordinated species, which most likely would have had high extinction coefficients (particularly the iron complex) and the colour was visible despite being very low in concentration. Mass spectral analysis confirmed the molecule’s formula weight of 485.25 (M+H) but shed no light on the suspected metal complex impurities. The compound was too unstable for \(^1\text{H} \text{NMR}\) analysis.

No attempt was made to coordinate this molecule to rhenium, due to difficulties reported in the literature.\(^{(116)}\) Tc-99m labelling on the other hand, requires gentler conditions and the Tc-99m complex was successfully synthesised in Chapter 3, utilising different co-ligands to satisfy the coordination requirements of the oxotechnetium core.
2.3.3 MonoAmine MonoAmide Dithiol (MAMA) BFC
Synthesis and Conjugation

The MAMA bifunctional chelating group was constructed using triphenylmethyl (trityl) protection strategies for the sulfur heteroatoms required for rhenium and technetium complexation, as shown in Scheme 2.3. The schemes used were adapted from Katzenellenbogen et al. The use of trityl protection also aided in making the molecules substantially more lipophilic and thus separable on silica gel with petroleum ether/ethyl acetate mixtures, as well as providing a strong chromophore for TLC UV visualisation.

Construction began with the tritylation of cysteamine hydrochloride using triphenylmethanol in trifluoroacetic acid to afford [67]. This step was followed by the N alkylation of the terminal amine with bromoacetyl bromide resulting in [68]. A further alkylation reaction of the bromo-product with a slight excess of cysteamine hydrochloride completed the tetradentate backbone [69]. Methyl bromoacetate was used to alkylate the amine and thus provide an attachment anchor for the biologically active moiety [32]. Base hydrolysis of the resulting methyl ester afforded the acid [33] which was coupled directly to the Merck integrin selective moiety using the BOP.
agent at 0 °C in $N,N'$-dimethylformamide to create the desired methyl ester of the conjugate [37]. A subsequent base hydrolysis of the protected methyl ester group on the newly incorporated integrin specific portion afforded the ligand [38] required for Re and Tc-99m coordination. The synthesis of the caproic acid linked conjugate followed a similar strategy, but with the added coupling of methyl-6-aminohexanoate
hydrochloride salt to the 2-((2-oxo-2-(tritylthio)ethylamino)ethyl)(2-(tritylthio)ethyl)amino)acetic acid tetradentate backbone [33] to create [35] and [36].

2.3.4 Rhenium MAMA Complex Syntheses

Rhenium complexes of the Merck based ligands [37], [38] and [42] were created for characterisation purposes as well as providing analogous information for HPLC separations of the Tc-99m complexes in Chapter 3. In terms of reactivity and coordination structure rhenium is the closest possible metal to technetium which has no natural abundance. As they are not completely similar, Re does not reduce as readily as Tc, which can lead to difficulties in synthesis and stability.

There are a number of strategies used in incorporating Re into complexes, particularly the oxorhenium(V) (Re=O) core which has a net 3+ charge. Macrocyclic and a variety of tridentate and bidentate ligands have been shown to be useful for coordinating oxorhenium, particularly systems with sulphur and amine/amide heteroatoms.\(^{122-124}\) The MAMA system has been used extensively for such coordinations, but due to the easily oxidisable adjacent sulphur atoms, protection of these groups is required to inhibit the bridging reaction (disulfide linkages) between them as is common in cysteine residues in peptides and proteins. The bulky trityl groups present a reasonable methodology for their protection, although the harsh tritylating conditions dictate their use early on in the synthetic procedure, so as to not interfere with other sensitive parts of the molecules, as tritylation is common protective method for alcohols and amines as well.\(^{125}\)

Deprotection of the sulphur atoms was of paramount importance to the rhenium complexation, as the conditions used were conducive to the breakdown of
the peptide mimetic moieties. A variety of methods were trialled, with only one method having any success. Traditional methods generally deprotected the sulphurs in acidic conditions in the presence of an electron acceptor like triethylsilane in neat trifluoroacetic acid to remove the trityl groups. This is followed by the coordination of the isolated tetradentate with a labile oxorhenium species such as triphenylphosphineoxorhenium trichloride in basic conditions (1 M sodium acetate in methanol under a dry nitrogen stream). This method has been used successfully for numerous molecules, but failed for the more delicate materials produced in this chapter. The isolation of the ligand is difficult on account of its hydrophilicity and the relative ease in which the disulfide linking occurs. Other methods incorporate thioanisole to act as an oxidative donor. Another method involved the bubbling of hydrogen sulphide after trityl group cleavage with mercuric acetate, but this strategy was not feasible in our laboratories. The successful method reported here was based on a ‘one pot’ deprotection/coordination step, involving the refluxing of the trityl protected ligand in aqueous alcohol and tetrahydrofuran with the commercially available sodium perrhenate (NaReO$_4$) with stannous chloride as the reducing agent in slightly acidic conditions, under argon. In all of the coordination reactions, the yields were very poor (approx. 12 - 30%). Repeated experiments were attempted to improve the yield but to no avail. $N,N'$-Dimethylformamide was added to improve the solubility but appeared to inhibit the pink colour formation that signified the complex’s presence. Tetrahydrofuran was used successfully for this but had to be peroxide free as the presence of the peroxides could destroy the pink colour immediately indicating that the rhenium was oxidising and thus breaking down the complex. In light of this, the tetrahydrofuran had to be eluted through a plug of neutral alumina to remove peroxide impurities prior to use.
In fact attempts to use tetrahydrofuran as the solvent without any alcohol present were unsuccessful, indicating that the presence of the alcohol was required for the coordination. This was unfortunate as the presence of the alcohol in the mildly acidic refluxing mixture was capable of converting a significant amount of the ligand’s terminal carboxylic acid into the corresponding ester, as shown in the chromatogram in Fig 2.4. HPLC analyses of the Re complexes were carried out on Waters systems equipped with a Photodiode array (PDA) monitoring two wavelengths of 254 and 354 nm. The weak metal-ligand charge transfer characteristics of rhenium MAMA complexes could be discerned from free ligand by a measurable absorbance at 354 nm.

$^1$H NMR analyses of the complexes showed complex splitting patterns of the protons in the macrocycle’s backbone. This observation is in line with previously reported complexes.\(^{(121,131,137)}\) Oxorhenium (and oxotechnetium) can give rise to syn and anti isomerism with respect to the oxygen and groups on the macrocycle. The geometry is a distorted square pyramidal structure. Racemisation of the chiral centre in the Merck derived moiety may also have occurred thus complicating the $^1$H NMR spectrum further. The caproic acid linked conjugate rhenium complex [43] was purified by HPLC and the chromatogram (Fig 2.5) is evidence of some form of isomerism. The peak of the purified metal complex shows two very close overlapped peaks, which would have been extremely difficult to separate in any usable quantity. The $^1$H NMR integration product was as expected for all complexes, and the complex formation is further indicated by the disappearance of the large overlapped multiplets (approx. $\delta$ 7.30 - 7.45 ppm) in the aromatic region arising from the trityl groups, just leaving the two doublets from the aromatic ring in the Merck moiety. Mass spectral analyses of all complexes exhibit the isomeric ratio pattern arising from
the 37.4% and 62.6% isotopic abundances of $^{185}$Re and $^{187}$Re respectively, and UV-Vis electronic absorption spectra display peak and shoulder absorbances towards the visible spectrum indicating coordination of rhenium.$^{(134)}$
2.3.5 2,2′,2″,2‴-(12-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazaclotridecane-1,4,7,10-tetrayl)tetraacetic acid (p-NCS bz TRITA) BFC Synthesis

The synthetic procedure of the p-NCS bz TRITA bifunctional chelator was adapted from literature methods. A variety of these molecules have been created to better hold on to the metal centres in vivo, which is a significant problem for more labile complexes. The TRITA moiety was chosen to fulfil the coordination requirements for both copper and lutetium. The synthetic method is outlined in Scheme 2.4.

Scheme 2.4  The synthetic route to p NCS bz TRITA
The first step of the synthesis involved the alkylation of diethyl malonate [71] with the bromide [70] to form [72] in good yield. This was followed by a condensation reaction with the tetraamine [73] in a large volume of refluxing ethanol over a period of 2 weeks to affect the cyclisation. The resulting diamide [74] was reduced to the corresponding diamine with borane in tetrahydrofuran to form the backbone of the macrocycle [75]. The pendant arms were attached by alkylation of the cyclic amines with 4 equivalents of 2-bromobenzoylacetate, and hydrogenation of this species [76] in the presence of 5% palladium on charcoal afforded the tetra acid aniline [77]. Thiophosgene in chloroform converted the terminal amine to the corresponding isothiocyanate [44], which couples readily with amines in aqueous conditions. This was required, as the Merck derived integrin antagonist acid [28] was only soluble in water.

The difficulties involved in the synthesis of this bifunctional chelator were centred on minimising the materials exposure to metals. These molecules bind avidly to metals, so great care was taken to clean glassware with acid prior to reactions and to purify the materials with HPLC systems equipped with PEEK tubing and pump heads. Of course the catalytic hydrogenation step introduced metal impurities (palladium and platinum) to the sample, but this could not be avoided. This phenomenon was expected as a thorough investigation of the metal and semi-metal impurities was reported in the literature.\(^{(139)}\) Mass spectral and NMR analyses confirmed all of the materials in this synthetic route. Characteristic of the isothiocyanate material was the shielding/deshielding effects on the aromatic system making all 4 aromatic protons equivalent, looking like a large singlet at $\delta$ 7.13 in D$_2$O. Initially the reaction was thought to have failed, because of this proton shifting, but this phenomenon is reported in the literature\(^{(103)}\), and the integration was correct.
The isothiocyanate was found to decompose after long periods in the freezer, so efforts were made to use it quickly.

2.3.6 **TRITA-Integrin antagonist conjugate [45] and corresponding Cu & Lu complexes [46, 47]**

The isothiocyanate group of \( p\)-NCS \(_{\text{ bz TRITA}}\) was reacted with the terminal amine of [28] to form the thiourea conjugate [45] in aqueous conditions at room temperature. The reaction was monitored by HPLC and after 2 h the disappearance of the isothiocyanate peak indicated that the reaction had gone to completion. The material was separated from the excess starting material [28] by HPLC methods, again taking precautions to exclude metal impurities. The material was a glassy coating on the round bottom flask. NMR and mass spectral analyses confirmed the identity of the material, but the mass spectral analyses also showed evidence that the thiourea may be oxidising to the corresponding urea. Upon investigation, it was found that this ‘sulfur extrusion’ type of reaction is possible and has been reported,\(^{(140)}\) but whether this phenomenon is occurring in the actual sample or even under the electrospray conditions is not known.

The copper complex [46] was synthesised by reacting the ligand [45] with copper chloride dihydrate at room temperature in a solution of ammonium acetate over a 2 h period. The HPLC purified samples’ mass spectral analyses showed mass ions corresponding to the complex with both natural Cu isotopic abundances (\( \text{Cu}^{63}, \) 69.2% and \( \text{Cu}^{65}, \) 30.8%). The glassy blue material also possessed absorption maximum at 325.7 nm. The paramagnetism of the copper precluded any NMR analysis. The mass spectral results indicate that two of the macrocycles pendant
carboxylates are deprotonated and thus neutralising the coppers charge to form a neutral complex.

The lutetium complex [47] was synthesised in a similar manner to the copper analogue but with heating to 80 °C in the presence of lutetium nitrate hydrate. Unlike the copper complex, there was no discernible colour change indicating the reaction progress, due to the lanthanides weak metal-ligand charge transfer characteristics. $^1$H NMR of the complex showed the expected integration, although extensive splitting and broad overlapping in the aliphatic region (2.05 - 4.12 ppm) impeded a more comprehensive characterisation. Mass spectral analysis of the lutetium complex yielded an m/z value of 1086.87 (M+1), indicating that each of the macrocycles’ carboxylic acids are deprotonated and coordinated to the lutetium metal centre. This is inline with the established coordination chemistry of lutetium and indeed lanthanides in general. Crystal structure and related structural analyses of such complexes (DOTA, TETA and derivatives support this coordination behaviour, resulting in net anionic charges of complexes where there are 4 available acetate pendant arms.\textsuperscript{(141-147)} As this is the case with [47], it is expected that this complex has a net -1 charge, although no other experiments were conducted to further confirm this. Neutral molecules of these macroyclic sytems have been synthesised by substitution of one of the carboxylate groups with a hydroxyalkyl chain.\textsuperscript{(142)} Lanthanide complexes of this nature are generally 9 coordinate with ligation to the 4 backbone aza nitrogens and 4 carboxylate oxygens arranged in a square antiprismatic arrangement with a water molecule capping the geometry. Diastereoisomerism occurs in these systems, but no further efforts to elucidate this structural information for [47] were undertaken.
2.3.7 ‘Integrated Approach’ Ligand Syntheses

The synthesis of the ‘integrated approach’ ligands began with the tritylation of cysteine’s ethyl ester [78] in trifluoroacetic acid for protective purposes (Scheme 2.5). Alkylation of this species [79] with bromoacetyl bromide gave the bromide [80], which was reacted with trityl cysteine [81] in N,N’-dimethylformamide overnight at 80 °C. Silica gel chromatography of the crude product gave [48] in good yield. $^1$H, $^{13}$C NMR and mass spectrometry confirmed the material which forms the basic framework of the N$_2$S$_2$ Re/Tc-99m chelating tetradentate (Section 2.2.6.1).

Scheme 2.5 Synthetic route to ‘Integrated approach’ ligands
The next step was the attachment of the benzimidazole moiety [82] to [48] and this was achieved by a peptide coupling reaction utilising HOBt and EDC as the coupling and activating agents, to give the product [49] in 67.5% yield after silica gel chromatography (Section 2.2.6.2). $^1$H NMR of the product in (CD$_3$)$_2$SO showed the incorporation of the benzimidazole amine at $\delta$ 12.19 ppm and mass spectrometry confirmed the expected $m/z$ value 938.21 (M+1). In an attempt to improve the yield, the same reaction was attempted using the coupling agent Py-BOP, which has the benefit of being a combined coupling/activating agent (Section 2.2.6.3). A yield of 82% after chromatography was obtained by this method, with the product having identical properties to the one produced in Section 2.2.6.2.

Hydrolysis of the coupled ethyl ester product [49] to the corresponding carboxylic acid [50] was first achieved with lithium hydroxide in a yield of 90.8% (Section 2.2.6.4). Mass Spectral analysis confirmed the transformation, with the acid giving a good signal in ES-MS mode ($m/z$ 908.00 (M-1). $^1$H NMR showed the disappearance of the quartet and triplet associated with the ethyl ester group of the starting material. In an effort to improve the yield of this reaction, and reduce any possible racemisation of the ligand’s chiral centre, an experiment using cesium carbonate was attempted as the hydrolysing agent. This salt has been shown to have a significantly lower racemisation rate than other more typical hydroxide bases in work on peptidic primary esters.$^{(148)}$ The carbonate was used in a 2:1 ratio to the ester, as opposed to the generally higher ratios of lithium hydroxide (10:1) in methanol/water. Overnight stirring at room temperature with the usual work-up gave the product with the same characteristics in a yield of 95.2% (Section 2.2.6.5). No effort was made to quantify any racemisation, although all subsequent hydrolyses employed this method.
To fulfil the aspartic acid mimicking terminal requirements of the target ligands, the acid [50] was coupled to both (S)-methyl-3-amino-2-(phenylsulfonamido)propanoate [83] and (S)-methyl-3-amino-2-(benzyloxy carbonylamino)propanoate [84] to form the ligands [51] and [53] respectively. Both reactions involved coupling with Py-BOP reagent in N,N'-dimethylformamide at 0 °C for 2 h and then at room temperature for 48 h, followed by work up and silica gel chromatography (Sections 2.2.6.6 and 2.2.6.8.). The yields were 58.5% [51] and 43.75% [53] after chromatography, which despite being quite low, are similar to the other yields obtained for the other peptide coupling reactions in this project. Mass spectral analyses gave the expected \( m/z \) values, and \(^1\)H and \(^{13}\)C NMR also corroborated the coupling. Cesium carbonate was again used to hydrolyse the esters in good yields, 80.15% [52] and 93.0% [54] (Sections 2.2.6.7 and 2.2.6.9 respectively).

Although great care was taken to avoid racemisation in these reactions, the \(^{13}\)C NMR results showed sharp doublets of certain signals indicating the presence of diastereoisomers. Both hydrolysed coupling products [52] and [54] exhibited this property, as shown by the peak at 56 ppm in Figure 2.6. The ligands have 3 chiral centres, so technically there is the possibility for \( n^3 \) or 8 isomers, although according to the peak height, there appeared to be two distinct isomers in roughly equal proportions in each cases. The rise of the diastereoisomerism could have resulted from any of the coupling steps or the base hydrolyses, but are most likely the result of epimerisation occurring during the coupling of [50] with [83/84]. No efforts were made to separate them at this stage, unless of course the diastereoisomeric mixtures showed promise in the subsequent biological evaluations. The proton assignment of [52] was aided by the data obtained from the HMQC (Fig 2.7), \(^1\)H NMR (Fig 2.8),
COSY (Fig 2.9), and also DEPT NMR analyses. The spectra were made more complex by the presence of diastereoisomers which were not separated by the HPLC method. The diastereoisomers were assessed as being in a 2:1 ratio by comparison of the peak areas of the CH signal at 4.18 - 4.28 ppm, although this is by no means definitive. This approximated ratio was also observed in the distorted AB quartet of the NHCH\textsubscript{2}CONH moiety (2.73 - 2.99 ppm) atop the intended chelation unit, as seen in the \textsuperscript{1}H spectrum, Fig 2.8. \textsuperscript{1}H - \textsuperscript{13}C correlation is displayed in Fig 2.7, from data obtained in the HMQC spectrum (Fig 2.7). This spectrum was highly useful in determining which signals were accountable from each carbon, as further exemplified in Fig 2.8. Specifically, the AB quartet was determined via the HMQC spectrum (2.73 - 2.99 ppm).

![Figure 2.6 \textsuperscript{13}C spectrum expansion of the aliphatic region showing the diastereoisomeric CH signals of [54] at 56 ppm](image)
Figure 2.7  
HMQC expansion of the aliphatic region of [52] showing correlations

Figure 2.8  
$^1$H Spectrum of the aliphatic region showing signals identified from the HMQC of [52]
The characterisation of the benzyloxycarbonylamino analogue [54] was done with the aid of the various NMR spectra from the similar phenylsulfonyl ligand [52] due to their similar structures.
2.3.8 ‘Integrated Approach’ Rhenium Complexes

The oxorhenium complexes [23] and [24] were extremely difficult to synthesise. The experiments were repeated numerous times in an effort to improve the yields, but values of 7.7% for the phenylsulfonyl ligand [23] and 12.6% for the benzyloxy carbonylamino analogue [24] were the most successful. The one-pot method deprotection/complexation employed for the terminal MAMA complexes in Sections 2.2.4.9, 2.2.4.10 and 2.2.4.12 were deemed the most likely to work for the similarly peptide mimicking molecules. Despite the use of HPLC purified ligands, the reaction conditions gave numerous products, as can be seen in the reaction chromatograms for the phenylsulfonyl complex [23] (Fig 2.10) and the benzyloxy carbonylamino complex [24] (Fig 2.11).

![Chromatogram of the crude reaction solution of [23] with absorption at 254 nm (red) and 354 nm (black) (Gradient elution, Alltech Altima 10µm C18 150 mm x 4.6 mm, 1 mL/min, acetonitrile/0.1 M ammonium acetate (5:95 → 90:10 over 30 min)](image)

The reactions only proceeded in the presence of methanol, which led to the formation of the rhenium complex methyl esters as can be seen in Fig 2.11. The reactions were attempted with mixtures of tetrahydrofuran and water, but without success. The requirement of methanol for such reactions has not been reported, but may be due to
solubilising of the highly lipophilic ligands in the aqueous media. Another possibility is that the alcohol acts as an intermediate in the substitution. Collected fractions from the elution of [24] were analysed by ES+MS as shown in Fig 2.11. This analysis indicates the extent of side products formed during the substitution reaction, some of which could not be determined.

![Figure 2.11](image)

**Figure 2.11** Chromatogram of the crude reaction solution of [24] with detection at 254 nm (red) and 354 nm (black) showing the ES +ve mass spectral analysis of constituents. Gradient elution, Alltech Alltima C18 10µm 150 mm × 4.6 mm, 1 mL/min, acetonitrile/0.1 M ammonium acetate (5:95 → 90:10 over 30 min).

Aside from the low amounts available for analysis, the presence of multiple isomers (Fig 2.10) precluded any sensible $^1$H or $^{13}$C NMR analyses. These isomers which were heavily overlapped in the HPLC analyses also precluded any possible separations. Figure 2.12 is an expanded view of the metal complex [23] elution (from Fig 2.10) showing the difficult to separate isomeric mixture.

Mass spectroscopy results confirmed complexes [23] and [24] with the characteristic rhenium isotope ratio of 5:3 for $^{187}$Re to $^{185}$Re. UV-vis spectrophotometry showed the complexes absorbance maxima and shoulder peaks above 320 nm. The HPLC purified isomeric mixtures were bright orange when solid, but were weakly coloured in aqueous alcoholic solutions. This is indicative of the weak metal to ligand charge transfer properties of the complexes. Interestingly, the
terminal MAMA complexes synthesised earlier [39], [40] and [43] showed a more intense pinkish rose colour in solution.

In summary, this Chapter contains the synthesis and characterisation of the necessary precursors and target materials required for the \textit{in vitro} (Chapter 4) and \textit{in vivo} (Chapter 6) evaluations in an effort to determine their usefulness as $\alpha_\nu \beta_3$ selective integrin antagonists.
CHAPTER 3

RADIOCHEMISTRY
3 RADIOCHEMISTRY OF TARGET MOLECULES

3.1 INTRODUCTION

Nuclear chemistry and radiation physics have become important branches in science and industry. Radioactive sources have found applications in many diverse areas from water monitoring for research and resource management, to fire prevention as in the common household smoke detector. Radiation has become an integral part of medicine with many diagnostic and therapeutic applications. X-rays are used to image broken bones and body functions, as are radioactive bone imaging agents. Radioactive aerosols have even been applied to monitor lung function.\(^{149,150}\)

There are an ever increasing number of therapies involving radiation using electrically produced x-ray beam therapies and sealed sources. This employment of radioactivity to provide cytotoxicity to tumours and sites of infection (therapies) or radiation for gamma imaging (diagnostics) is extended to radiopharmaceuticals that can be administered directly into the body. Monoclonal antibodies (MAbs), proteins, peptides and small biologically active molecules have all been sought to provide possible radio-therapies and diagnostics.

When dealing with radionuclides and radiolabelled compounds, it is important to know the specific activity of the material so as know how much of the material is represented by radioactive nuclei. The specific activity is the measure of the total radioactivity per unit mass, and is often quoted in \(\mu\text{Ci/mg}\) or \(\mu\text{Ci/mmol}\). If the labelled compound contains only the unstable nuclei then it is said to be no carrier added (NCA), where carrier designates the stable isotope of interest present in the material. In many instances carrier added radionuclides are acceptable for various
applications, but when high specific activities are required, NCA isotopes have to be investigated. Whether an isotope can be produced in a NCA state depends on how it was made. If the material that was irradiated in the reactor or cyclotron produced a different isotope of the same element, then the presence of the unconverted starting material would limit its specific activity. If on the other hand the material that is bombarded gets converted to an isotope of a differing element, then it is possible to produce it in a NCA form. Of course the decay scheme also affects this because if the daughter product is the stable isotope of the decaying radioisotope then this will effectively add carrier over time.

Technetium-99m is the most commonly used radioisotope in nuclear medicine, due to availability and ease of use. As stated previously, there are BFCs and direct incorporation strategies that have been used quite extensively to incorporate the metal into a variety of biologically active molecules and indeed biological entities. The fact that it can be dispensed from a Tc-99m/Mo⁹⁹ generator makes it a valuable and convenient source for radiopharmaceuticals. The unit is basically a heavily shielded column which has Mo⁹⁹ bound to alumina. The Mo-99 decays to its short-lived daughter product of Tc-99m which can be eluted off with the introduction of saline solution. The Mo⁹⁹ has a greater decay half-life (2.74 days), and the generator can be ‘milked’ approximately once a day for about a week. All Tc-99m labellings required the milking of the generator prior to experimentation.

Iodine¹²³ and iodine¹²⁵ are isotopes with vastly differing emission characteristics. Iodine¹²³ has a short half life and a highly penetrating gamma emission that has application in imaging of tumours and sites of infection. Iodine¹²⁵ on the other hand has a much longer half life but an extremely weak gamma emission ideal for scintillation counting in radio-assays, as seen in Chapter 4. Other isotopes of
iodine have found applications in nuclear medicine and Iodine$^{131}$, a potent gamma emitter has been used for thyroid treatment.$^{(94,95)}$

Copper$^{64}$ is a positron emitter that has potential for both imaging and therapeutic applications due to its strong gamma and beta emission characteristics.$^{(97)}$ Due to the nature of Cu(II) it has to be wrapped up in a large macrocycle to prevent it from de-metallating in the reactive biological systems.$^{(112)}$

Lutetium$^{177}$ is a new and exciting radiometal that has great potential as a therapeutic agent due to its strong beta emission and weak gamma emission. It has only recently become commercially available, but not in the NCA state. Like copper, lutetium requires suitable macrocyclic ligation to keep the cation attached to the molecule of interest.

This chapter describes the radiolabelling of the target molecules with Tc-99m MAMA moieties and the HYNIC chelator in conjunction with the tricine and ethylenediaminediacetic acid (EDDA) coligands. Experiments were repeated with Tc-99g to confirm the radiotracers molecular weight. The radiometals Cu$^{64}$ and Lu$^{177}$ were incorporated into the intended integrin antagonists with the TRITA tetraaza-tetraacetic acid bifunctional chelator. Lu$^{177}$ was synthesised by irradiation of ytterbium$^{176}$ oxide and separated via chromatographic means. This chapter also describes the labelling of the Merck $\alpha_\text{v}$$\beta_3$ integrin antagonist standard with I$^{123}$ and I$^{125}$ for the stability study and in vitro binding assay described in Chapter 4.
Table 3.1  Characteristics of the radioisotopes used in this project

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Half-Life</th>
<th>Decay Mode</th>
<th>Emission Energies of note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine$^{123}$</td>
<td>13.2 h</td>
<td>Gamma (EC)</td>
<td>159 keV</td>
</tr>
<tr>
<td>Iodine$^{125}$</td>
<td>60.14 days</td>
<td>Gamma (EC)</td>
<td>35.5 keV</td>
</tr>
<tr>
<td>Tc-99m</td>
<td>6.02 h</td>
<td>Gamma (IT)</td>
<td>140.5 keV</td>
</tr>
<tr>
<td>Cu$^{64}$</td>
<td>12.7 h</td>
<td>Beta</td>
<td>653 keV, 578 keV</td>
</tr>
<tr>
<td>Lu$^{177}$</td>
<td>6.73 days</td>
<td>Beta</td>
<td>497 keV, 208 keV / 113 keV</td>
</tr>
</tbody>
</table>

EC = electron capture, IT = internal transition
3.2 **EXPERIMENTAL**

3.2.1 **General**

All radiochemical procedures were carried out in laboratories equipped and designated for radiation handling. Extensive lead shielding in fume hoods and HPLC equipment were employed to minimise the radiation dose. Radiation monitors as well as personal dosimetry badges were operated to indicate the levels of personal exposure. All radioactive waste was labelled and left to decay in the appropriate designated shielded decay areas. Extensive use of ‘double-gloving’ with latex gloves was required when handling radioactive equipment and glassware for heightened protection. Radiochemical reactions requiring heating were generally carried out in pressure resistant screw-capped glassware vials unless otherwise stated. All manipulations were done as carefully as possible in the shortest time to minimise exposure. Autosamplers were used for injecting radioactive materials into HPLCs whenever possible for this reason. All starting materials were HPLC purified to reduce side-reactions and reagents were of the highest purities. Tetrahydrofuran was filtered through a plug of aluminium oxide (activated neutral Brockmann 1) to remove peroxide impurities. Milli-Q deionised water was used for all procedures requiring water. Iodine$^{123}$ was purchased from Australian Radioisotopes and Industrials (ARI) and was in the sodium iodide form in 0.02 M sodium hydroxide (pH 12 - 14). Iodine$^{125}$ was purchased from Amersham Biosciences (General Electric Healthcare) and was in the sodium iodide form in 0.01 M sodium hydroxide (pH 8 - 12). Technetium-99m activity in the pertechnetate form activity was milked from a Mo$^{99}$/Tc-99m generator supplied by ARI. ‘Groundstate’ Tc$^{99}$ in the ammonium pertechnetate form was kindly supplied Dr Christopher Fookes for mass spectral
studies. Copper$^{64}$ was supplied by ARI in the 2+ oxidation state as the chloride. Lutetium$^{177}$ was produced in the HIFAR reactor at the Lucas Heights Science and Technology Centre, ANSTO, NSW. Specific HPLC conditions and columns are stated in each preparation. Iodine$^{123}$ work was carried out on a Berthold system linked to a Pentium® class workstation with Berthold software. Iodine$^{125}$ labelling was performed on a Waters 510 HPLC pump linked to a Pentium® Class workstation with Laura version 1.0 software. Tc-$^{99m}$, Cu$^{64}$ and Lu$^{177}$ labellings were carried out on a Waters 486 pump connected to a Waters 600 controller and Waters 2489 dual channel absorbance detector linked to a Pentium® III class workstation running Waters Empower software (build 1154). All HPLC systems were equipped with sodium iodide crystal gamma radiation detectors, and peak collection was based on this radioactive chromatographic trace. HPLC flow conditions are outlined, however, specialised gradient conditions are shown in Tables 3.2 and 3.3. Gamma spectroscopy was carried out on an Ortec DSPEC Gamma spectrometer, fitted with a High Purity Germanium (HPGe) detector using Gamma vision32 software (ver. 6). Radioactivity is referred to in Becquerels (Bq) and Curies (Ci) and complies with the units most commonly used for each isotope.
3.2.2 Iodine$^{123}$ Labelling

3.2.2.1 Radiolabelling Integrin Standard with I$^{123}$

To stannane (0.1 mg, 0.158 μmol) in a glass vial was added absolute ethanol (200 L). NaI$^{123}$ activity (7.25 mCi, 270 μL) was added and the vessel was slightly agitated. A solution of chloramine-T (1 mg/mL, 100 μL H$_2$O) and hydrochloric acid (0.1 M, 100 L) were added to the vial simultaneously. After further agitation of the vial, the reaction was allowed to proceed for 5 min until quenching with a solution of sodium metabisulfite (100 L of a 50 mg/mL solution) with slight mixing, and finally a solution of sodium bicarbonate (100 L of a 50 mg/mL solution.) HPLC mobile phase (300 L) was added and the entire solution was injected into a HPLC system (Phenomenex Bondclone 10 μ C18, 300 mm × 7.8 mm I.D., acetonitrile, aqueous 0.1% TFA (40:60) with flowrate = 2 mL/min. The I$^{123}$ integrin ester eluted at 19 - 20 min (γ - detection), collected and evaporated to dryness under reduced pressure (4.0 mCi, 55% radiochemical yield). To hydrolyse the ester, acetonitrile (300 L) as well as LiOH.H$_2$O solution (300 L of a 20 mg/mL solution) was added to the ester and was heated to 70 °C on a hotplate for 10 min with a stopper, and a further 5 min with the stopper removed. After cooling, mobile phase (500 L) and TFA (40 L) were added and the entire solution was injected into a HPLC system (Phenomenex Bondclone 10 μ C18, 300 mm × 7.8 mm I.D., acetonitrile, aqueous 0.1% TFA (40:60).
with flowrate = 1.5 mL/min). The $^{125}$I integrin acid eluted at 12 - 13 min ($\gamma$-detection) and collected in a sample vial and frozen until required (3.2 mCi, 80% radiochemical yield uncorr).

### 3.2.3 Iodine$^{125}$ Labelling

#### 3.2.3.1 Radiolabelling Integrin Standard with I$^{125}$

![Chemical Structure](image)

To stannane (0.1 mg, 0.158 µmol) in a glass vial was added absolute ethanol (200 µL). NaI$^{125}$ activity (4 mCi, 40 µL H$_2$O) was added and the vessel was slightly agitated. A solution of chloramine-T (1 mg/mL, 100 µL) and hydrochloric acid (0.1 M, 100 µL) were added to the vial simultaneously. After further agitation of the vial, the reaction was allowed to proceed for 5 min until quenching with a solution of sodium metabisulfite (100 µL of a 50 mg/mL solution) with slight mixing, and finally a solution of sodium bicarbonate (100 µL of a 50 mg/mL solution.) HPLC mobile phase (300 µL) was added and the entire solution was injected into a HPLC system (Phenomenex Bondclone 10 m C18, 300 mm × 7.8 mm I.D., acetonitrile, aqueous 0.1% TFA (40:60) with flowrate = 2 mL/min). The $^{125}$I integrin ester eluted at 19 - 20 min ($\gamma$-detection), was collected and evaporated to dryness under reduced pressure (3.5 mCi, 87.5% radiochemical yield.) To hydrolyse the ester, acetonitrile (300 µL) as well as LiOH.H$_2$O solution (300 µL of a 20 mg/mL solution) was added to the ester and was heated to 70°C on a hotplate for 10 min with a stopper, and a further 5
min with the stopper removed. After cooling, mobile phase (500 μL) and TFA (40 L) were added and the entire solution was injected into a HPLC system (Phenomenex Bondclone 10 μm C18, 300 mm x 7.8 mm I.D., acetonitrile, aqueous 0.1% TFA (40:60) with flowrate = 2 mL/min). The $^{125}$I integrin acid was eluted at t = 12 - 13 min (γ) and collected in a sample vial and frozen until required (2.9 mCi, 83% radiochemical yield uncorr).

3.2.4 Technetium-99m Labelling

3.2.4.1 Radiolabelling MAMATr$_2$ Integ Acid [38] with Tc-99m

Tritylated ligand [38] (0.1 mg, 0.094 μmol) and nitrogen purged hydrochloric acid (0.1 M, 200 μL) were placed in a 4 mL screw-cap vial, with a magnetic stir-bar. A solution of tartaric acid (2 mg, 13.3 μmol) in nitrogen purged hydrochloric acid (0.1 M, 200 μL) was added, along with a solution of Tc-99m activity (sodium pertechnetate in saline, 153 MBq, 200 μL). A solution of stannous chloride (13 μL of a 10 mg/mL of 0.1 M nitrogen purged hydrochloric acid solution) was added, and the resulting mixture was capped and placed in a 70 °C water bath for 1 min with stirring. Tetrahydrofuran (200 μL) was added to the solution and the resulting solution was re-capped and stirred in the water bath for a further 30 min. Upon cooling, acetonitrile (50 μL) was added into the cloudy suspension, and the total volume was injected into
a HPLC system (Waters Bondapak 10 μ C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.2). The peak at 15.2 min (γ - detection) was collected and pumped to dryness, and re-suspended in saline for use in imaging experiments (32.0 MBq, 21% radiochemical yield uncorr).

Table 3.2 HPLC radiolabelling mobile phase profile #1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flowrate (mL/min)</th>
<th>Acetonitrile (vol)</th>
<th>Aqueous (vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.3</td>
<td>2</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.35</td>
<td>4</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>

*All variations in composition and flowrate are linear gradients

3.2.4.2 Radiolabelling MAMATr₂ IntegAcid [38] with Tc-99m and Tc-99g carrier

Tritylated ligand [38] (0.1 mg, 0.094 μmol) and nitrogen purged hydrochloric acid (0.1 M, 200 μL) were placed in a 4 mL screw-cap vial, with a magnetic stir-bar. A solution of tartaric acid (2 mg, 13.3 μmol) in nitrogen purged hydrochloric acid (0.1 M, 200 μL), along with a solution of Tc-99m activity (sodium pertechnetate in saline, 50 MBq, 200 μL) and ammonium pertechnetate solution (50 μL of a 1 mg/mL solution) was pipetted into the solution, quickly followed by a solution of stannous chloride (13 μL of a 10 mg/mL of nitrogen purged 0.1 M hydrochloric acid solution). The resulting mixture was capped and placed in a 70 °C water bath for 1 min with stirring. Tetrahydrofuran (200 μL) was added to the solution and the resulting
solution was re-capped and stirred in the water bath for a further 30 min. Upon cooling, tetrahydrofuran (50 µL) was added into the yellow solution, and the total volume was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.2). The peak at 15.2 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile/water (1:1) for mass spectral analysis. (M.S. \( m/z \) calc. for C_{24}H_{36}N_{7}O_{7}S_{2}Tc (M+H)^{+} = 696.61 found (M+H)^{+} = 696.11)

3.2.4.3 Radiolabelling MAMATr_{2} Caproic IntegAcid [42] with Tc-99m

Tritylated ligand [42] (0.3 mg, 0.254 µmol) and tetrahydrofuran (200 µL) were placed in a 4 mL screw-cap vial, with a magnetic stir-bar. A solution of tartaric acid (2 mg, 13.3 µmol) in nitrogen purged hydrochloric acid (0.1 M, 200 µL) was added, along with a solution of Tc-99m activity (sodium pertechnetate form in saline, 305 MBq, 200 µL). A solution of stannous chloride (13 µL of a 10 mg/mL nitrogen purged 0.1 M hydrochloric acid solution) was added, and the resulting mixture was capped and placed in a 90 °C water bath for 30 min with stirring. Upon cooling, acetonitrile (50 µL) was added into the cloudy suspension, and the total volume was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D
column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.2). The peak at 15.8 min (γ-detection) was collected and pumped to dryness, and re-suspended in saline for use in imaging experiments (38.7 MBq, 12.7% radiochemical yield uncorr).

3.2.4.4 Radiolabelling MAMATr₂ Caproic IntegAcid [42] with Tc-99m and Tc99g carrier

Tritylated ligand [42] (0.3 mg, 0.254 µmol) and tetrahydrofuran (200 µL) were placed in a 4 mL screw-cap vial, with a magnetic stir-bar. A solution of tartaric acid (2 mg, 13.3 µmol) in nitrogen purged hydrochloric acid (0.1 M, 200 µL) was added, along with a solution of Tc-99m activity (sodium pertechnetate form in saline, 50 MBq, 200 µL) and ammonium pertechnetate solution (50 µL of a 1 mg/mL solution in saline). This was quickly followed by a solution of stannous chloride (13 µL of 10 mg/mL of 0.1 M nitrogen purged hydrochloric acid), and the resulting mixture was capped and placed in a 90 °C water bath for 30 min with stirring. Upon cooling, tetrahydrofuran (50 µL) was added into the yellow solution, and the total volume was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.2). The peak at 15.8 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile and water (1:1) for mass spectral analysis. (M.S. m/z calc. for C₃₀H₄₆N₈O₈S₂Tc (M+H)⁺ = 809.77 found (M+H)⁺ = 809.13)
3.2.4.5 Tc-99m labelling of HYNIC Integ ester [30] with tricine co-ligand (no carrier added)

To a 4 mL screw cap vial was added tricine (50 mg, 0.28 mmol) in 500 μL of nitrogen purged succinate buffer (25 mM, pH 5.0) followed by HYNIC Integrin ligand ester [30] (20 μg, 40.12 nmol) in acetonitrile (100 μL). Tc-99m activity (150 MBq) in saline solution (200 μL) was injected, immediately followed by solution of stannous chloride (20 μL of a 10 mg/2 mL solution in 0.1 M hydrochloric acid). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3) collecting the peak (γ - detection) at 13.7 - 14.2 min. (76.5 MBq, 51% radiochemical yield uncorr).

Table 3.3  HPLC radiolabelling mobile phase profile #2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flowrate (mL/min)</th>
<th>Acetonitrile (vol)</th>
<th>Aqueous (vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.10</td>
<td>2</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.30</td>
<td>2</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>0.35</td>
<td>4</td>
<td>5%</td>
<td>95%</td>
</tr>
<tr>
<td>3.00</td>
<td>4</td>
<td>60%</td>
<td>40%</td>
</tr>
</tbody>
</table>

*All variations in composition and flowrate are linear gradients
3.2.4.6 Tc-99m +Tc99g carrier added labelling of HYNIC Integ ester with tricine co-ligand

To a 4 mL screw cap vial was added tricine (50 mg, 0.28 mmol) in nitrogen purged water (500 µL), followed by HYNIC Integrin ligand ester [30] (100 µg, 0.20 µmol) in acetonitrile (100 µL). Radioactive Tc-99m (150 MBq) in saline solution (150 µL) was injected, as was of a solution of ammonium pertechnetate (Tc-99g) (50 µL of a 1 mg/mL in saline.) Immediately following this a solution of stannous chloride (20 µL of 10 mg/2 mL in 0.1 M hydrochloric acid) was added. The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3). The peak at 13.6 - 14.2 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile and water (1:1) for mass spectral analysis (M.S. m/z calc. for C_{29}H_{39}N_{10}O_{10}Tc (M+H)^+ = 772.57 found (M+H)^+ = 772.15)

3.2.4.7 Tc-99m labelling of HYNIC Integ ester [30] with EDDA co-ligand

To a 4 mL screw cap vial was added ethylenediaminediacetic acid (5 mg, 28.4 µmol) in nitrogen purged water (500 µL), followed by HYNIC Integrin ligand ester [30] (20 µg, 40.12 nmol) in acetonitrile (20 µL). Tc-99m activity (pertechnetate form), (150
MBq) in saline solution (200 µL) was injected, immediately followed by 20 µL of a solution of stannous chloride (10 mg/2 mL in 0.1 M hydrochloric acid). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 μ C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3) collecting the peak at 14.4 - 15.1 min (γ - detection), (70.5 MBq, 47% radiochemical yield uncorr).

**3.2.4.8 Tc-99m +Tc99g carrier added labelling of HYNIC Integ ester [30] with EDDA co-ligand**

To a 4 mL screw cap vial was added ethylenediaminediacetic acid (5 mg, 28.4 µmol) in nitrogen purged water (500 µL), followed by HYNIC Integrin ligand ester [30] (100 µg, 0.20 µmol) in acetonitrile (20 µL). Tc-99m activity (pertechnetate form), (150 MBq) in saline solution (150 µL) was injected, as was a solution of ammonium pertechnetate (Tc-99g) in saline (50 µL in 1 mg/mL). This was immediately followed by a solution of stannous chloride (20 µL of 10 mg/2 mL in 0.1 M hydrochloric acid). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 μ C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3). The peak at 14.3 – 15.0 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile and water (1:1) for mass spectral analysis (M.S. m/z calc. for C_{29}H_{38}N_{10}O_{9}Tc (M+H)^+ = 769.57 found (M+H)^+ = 769.16)
3.2.4.9 Tc-99m labelling of HYNIC Integ acid [31] with tricine co-ligand (no carrier added)

To a 4 mL screw cap vial was added tricine (50 mg, 0.28 mmol) in nitrogen purged succinate buffer (500 µL, 25 mM, pH 5.0) followed by HYNIC Integrin ligand acid [31] (20 µg, 40.12 nmol) in acetonitrile (100 µL). Tc-99m activity (150 MBq) in saline solution (200 µL) was injected, immediately followed by of a solution of stannous chloride (20 µL of a 10 mg/2 mL in 0.1 M hydrochloric acid). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm x 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3) collecting the broad peaks at 11.5 - 14.0 min (γ), (76 MBq, 51% radiochemical yield uncorr).

3.2.4.10 Tc-99m +Tc99g carrier added labelling of HYNIC Integ acid [31] with tricine co-ligand

To a 4 mL screw cap vial was added tricine (50 mg, 0.28 mmol) in nitrogen purged water (500 µL), followed by HYNIC Integrin ligand acid [31] (100 µg, 200 nmol) in acetonitrile (100 µL). Tc-99m activity (150 MBq) in saline solution (150 µL) was injected, as was a solution of ammonium pertechnetate (Tc-99g) in saline (50 µL, 1 mg/mL). Immediately following this was of a solution of stannous chloride (20 µL, 10 mg/2 mL in 0.1 M hydrochloric acid). The vial was capped tightly and heated to
70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3). The peak at 11.4 - 14.0 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile and water (1:1) for mass spectral analysis. (M.S. m/z calc. for C_{28}H_{37}N_9O_{10}Tc (M+H)^+ = 758.55 found (M+H)^+ = 758.23)

### 3.2.4.11 Tc-99m labelling of HYNIC Integ acid [31] with EDDA co-ligand

![HYNIC Integ acid](image)

To a 4 mL screw cap vial was added ethylenediaminediacetic acid (5 mg, 28.4 μmol) in nitrogen purged water (500 μL), followed by HYNIC Integrin ligand acid [31] (20 μg, 40.12 nmol) in acetonitrile (20 μL). Tc-99m activity (ammonium pertechnetate form) (190 MBq) in saline solution (200 μL) was injected, immediately followed by of a solution of stannous chloride (20 μL of a 10 mg/2 mL in 0.1 M hydrochloric acid solution). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system ((Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3) collecting the peak at 11.1 - 14.0 min (γ - detection), (100.7 MBq, 53% radiochemical yield uncorr).
3.2.4.12  Tc-99m +Tc99g carrier added labelling of HYNIC Integ acid [31] with EDDA co-ligand

To a 4 mL screw cap vial was added ethylenediaminediacetic acid (5 mg, 28.4 µmol) in nitrogen purged water (500 µL), followed by HYNIC Integrin ligand acid [31] (20 µg, 40.12 nmol) in acetonitrile (20 µL). Tc-99m activity (ammonium pertechnetate form) (150 MBq) in saline solution (150 µL) was injected, as was a solution of ammonium pertechnetate (Tc-99g) in saline (50 µL of a 1 mg/mL solution). This was immediately followed by of a solution of stannous chloride (20 µL of a 10 mg/2 mL in 0.1 M hydrochloric acid solution). The vial was capped tightly and heated to 70 °C for 40 min. After the reaction was allowed to cool to room temperature, the entire solution was injected into a HPLC system (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.3). The peak at 11.0 - 14.0 min (UV and γ detection) was collected and pumped to dryness, left to decay, and re-suspended in a solution of acetonitrile and water (1:1) for mass spectral analysis.  

(M.S. m/z calc. for C_{28}H_{36}N_{10}O_9Tc (M+H)^+ = 754.55 found (M+H)^+ = 755.20)
3.2.5 **Copper\(^{64}\) Labelling**

3.2.5.1 **Cu\(^{64}\) labelling of TRITA Integ acid ligand [45]**

![Cu\(^{64}\) labelling of TRITA Integ acid ligand](image)

TRITA conjugate [45] (0.5 mg, 0.55 \(\mu\)mol) was placed in a 4 mL glass screw cap vial and was dissolved in a solution of ammonium acetate (0.4 M, 300 \(\mu\)L) with stirring. Copper\(^{64}\) chloride (17.5 mCi,) in 0.02 M hydrochloric acid (200 \(\mu\)L) was added and the solution and was warmed to 35 °C for 1 h. After cooling to room temperature, the solution was injected into a HPLC system (Waters \(\mu\)Bondapak 10 \(\mu\)m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01\% TFA) as outlined in Table 3.2.), collecting the peak at 9.0 - 9.5 min (\(\gamma\) - detection), (15.9 mCi, 91\% radiochemical yield uncorr).

3.2.6 **Lutetium\(^{177}\) Synthesis**

3.2.6.1 **Lutetium\(^{177}\) Synthesis and Purification**

A sample of isotopically purified ytterbium\(^{176}\) oxide (11.1 mg, 27.75 \(\mu\)mol) was weighed out into a clean glass ampoule and sealed by flame. The sample was
irradiated in the HIFAR reactor for 7 days at a thermal neutron flux of $5 \times 10^{13}$ n/cm$^2$/sec. After irradiation, the sample was allowed to decay for 24 h. The glass ampoule was cut and transferred to a small beaker in a lead shielded sealed atmosphere glove hood, using hydrochloric acid (6 M) to dissolve the white powder and aid in the transferral. After the sample was fully dissolved in the acid, the beaker was transferred to a lead shielded fume extraction hood equipped with a hot plate stirrer. Hydrogen peroxide solution (0.5 mL, 30%) was added and the suspension was boiled to dryness on the hot plate with a gentle stream of nitrogen blown into it to avoid sputtering. More hydrochloric acid (2 mL, 6 M) and hydrogen peroxide solution (0.5 mL) was added to the residue and boiled off. This was repeated twice more. Water was added in increments of 200 µL and swirled around the beaker to dissolve the residue and transfer the entire solution to a 2 mL eppendorf tube. The solution was then injected into a HPLC system (Waters prep LC, 3 x 100 mm x 25 mm I.D. Novapak cartridge 4 µm C18, using an isocratic mobile phase of 0.1 M octanesulfonic acid sodium salt, α-hydroxyisobutyric acid, 0.25 M, pH 2.25) at a flowrate of 20 mL/min, collecting the radioactive peak between 300 - 325 min (γ – detection). The collected eluate was then acidified to pH <1 with hydrochloric acid (6 M) and passed through a column of Dowex® 50WX-4-200 resin (previously washed with hydrochloric acid (100 mL, 6 M), 100 mL 3 M ammonium chloride solution (3 M, 100 mL) and water (100 mL), all the while monitoring with a hand held Geiger counter to account for radioactivity loss. The Lu$^{3+}$ was eluted with hydrochloric acid (6 M) until the bulk activity was removed from the column. The collected eluate was transferred to a beaker and evaporated to dryness on a hotplate, re-suspended in hydrochloric acid (5 mL, 6 M) and hydrogen peroxide solution (1
mL, 30%) and dried again. The dried residue was taken up in the minimum amount of water and used for labelling experiments.

3.2.7 Lutetium\textsuperscript{177} Labelling

3.2.7.1 Lu\textsuperscript{177} labelling of TRITA Integ acid ligand [45]

[TRITA conjugate][45] (0.5 mg, 0.55 μmol) was placed in a 4 mL glass screw cap vial and was dissolved in a solution of ammonium acetate (0.4 M, 500 μL) with stirring. Lutetium\textsuperscript{177} chloride (3 mCi) in 0.01 M hydrochloric acid (150 μL) was added to the solution and was heated to 70 °C for 1 h. After cooling to room temperature, the solution was injected into a HPLC system (Waters Bondapak 10 μm C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/water (0.01% TFA) as outlined in Table 3.2), collecting the peak at 11.5 - 12.0 min (γ - detection). (2.6 mCi, 87% radiochemical yield uncorr).
3.3 RESULTS AND DISCUSSION

3.3.1 Iodine labelling

Labelling of molecules with iodine radioisotopes is commonly performed by electrophilic iodo-destannylation. These reactions are typically regiospecific provided the reaction time is not too long, which is not a problem considering the time constraints when dealing with short half-life isotopes like I\(^{123}\) and I\(^{131}\). As the radio-iodine is in the iodide state, an oxidising agent is required to provide the electrophilic source of iodine. Chloramine-T and peracetic acid are commonly used for this purpose.\(^{(151)}\) Radio-iodinations like all radiolabelling reactions require highly purified starting materials to minimise side reactions. HPLC is employed to purify the alkylstannane starting materials, and after the reactions are performed they are injected into a HPLC system for purification. Prior to the reactions the HPLC conditions were optimised so as to minimise the time in working up and processing the product. The alkylstannanes are created from chloro or bromo derivatives, although they can be made from iodo molecules as well (Fig 3.1). The latter is not recommended as the minute presence of the cold iodo material can interfere with the labelling and greatly reduce the specific activity of the radiotracers. It should be noted that the cold iodo standard is generally produced to compare the retention times on the HPLC and thus confirm that the molecule is indeed the radio-iodinated derivative. The I\(^{123}\) and I\(^{125}\) labelling of the integrin standard molecule [85, 86] were both performed by converting the trimethylstannane starting material into the corresponding iodo-labelled molecule in the presence of chloramine-T in the acidic conditions. The reaction was performed at room temperature and the chloramine-T was dissolved immediately before it was added to the reaction vessel due to its
instability in aqueous media. After 5 min, the excess iodine was quenched with sodium metabisulfite. The HPLC purified product was then base hydrolysed to afford the carboxylic acid and reintroduced into the HPLC for purification. The iodo-methyl ester was then subsequently hydrolysed using aqueous sodium hydroxide with heating.

![Chemical Structures](image)

**Figure 3.1** Route to radio-iodination

The iodine labelled molecules in this chapter were constructed for the *in vitro* assay described in Chapter 4. The I\(^{123}\) conjugate was used to optimise the HPLC conditions for its efficient purification and also to help determine what buffer or solvent system that it is stable in. After leaving the purified fractions in different solutions overnight at -20 °C and re-injecting on the HPLC, it was determined that the acidic mobile phase of acetonitrile/aqueous 0.1% TFA (40:60) was better than saline and succinate buffer (25 mM, pH 5.0). The information from these experiments was translated to the I\(^{125}\) conjugate which had to be as stable for as long as possible as the experiment was being run over a period of months. The I\(^{125}\) labelled molecule [86] was found to be most stable in the acidic mobile phase compared to saline and succinate buffers as expected, and its radiochemical purity was assessed to reduce by approximately 1% per week at -20 °C.
3.3.2 Technetium-99m labelling

The Tc-99m molecules produced in this chapter incorporated the HYNIC bifunctional chelator as well as the established MAMA tetradentate ligand and its modified derivatives. The HYNIC system has been used quite extensively as the chelating unit for various biological targets including monoclonal antibodies (MAbs), proteins, peptides and small molecules. The success of the HYNIC moiety arises from its relatively simple construction, its ease in which to conjugate it with various targets and the quick and easy way in which it coordinates to technetium. Surprisingly, the HYNIC system produces a stable bond to the Tc atom despite bonding in a monodentate or bidentate fashion. Actually there are a number of hypothesised binding modes in which it coordinates the metal as shown in Fig 3.2. Unfortunately, efforts to coordinate HYNIC to rhenium, the metal that is most similar to the radioactive technetium, have been unsuccessful for these conjugates so a detailed structural analysis of the metal complex is not available. There are examples of crystallised Tc-99g complexes which shed light on the coordination of the Tc-HYNIC system. The Tc- HYNIC chemistry has also achieved great success because it requires co-ligands to bind and stabilise the complex. The employment of co-ligands with different polarities can attenuate the molecule’s overall polarity and thus help it reach its desired destination.

This project used EDDA and tricine as the co-ligands. The syntheses of these molecules were relatively straightforward. The reactions were carried out at 70 °C in a heated water bath in tightly screw-capped HPLC vials. Tc-99m activity in the form of pertechnetate (TcO$_4^-$) in saline was added to the ligand in acetonitrile, followed by stannous chloride as the required reducing agent. A large excess of co-ligand was added immediately after and the reactions were stirred for 40 min. HPLC analyses of
the reactions showed a mixture of two main peaks that may or may not have been made up of two peaks each as shown in Fig 3.3 and 3.4. The broadness of the chromatogram made identification impossible, and this is commonplace for Tc-99m HYNIC chemistry, but repeating the experiments with small amounts of Tc-99g enabled mass spectrometry of the fractions and this showed that they shared the correct molecular weights. The first synthetic strategy employed to create the HYNIC conjugate complexes were via the methyl esters of the integrin antagonist [89, 90]. These reactions were successful but the subsequent attempted base hydrolyses conditions destroyed the complexes which was evident in the mass spectral analyses. In light of this, the ligand was hydrolysed first and the acid derivatives were labelled in the same manner. Expectedly the same peak patterns were observed for the acid complexes [91, 92] but with slightly shorter retention times on the Waters Bondapak semi-prep C-18 column. Of great interest was the observation that there was a degree of interconversion between the Tc-99m HYNIC isomers. Over time it was shown with the tricine complex [91] (Fig 3.4) that the earlier peak (11.5 min) was reducing and the 2nd peak (13.0 min) was increasing after successive injections at longer time points. There were no other significant peaks being observed in the corresponding chromatograms, so it was assumed to be isomeric conversion. This isomeric interconversion could well be the reason why the metal complex’s chromatograms (Fig 3.3 and 3.4) are broad and ill-defined. Another factor that would have certainly contributed to the ambiguity of the Tc-HYNIC molecules was the fact that another monodentate ligand could potentially coordinate to the technetium ion. Variation of the cone voltage on the mass spectrometer yielded some interesting information pertaining to the tricine complexes. The expected (M-H)⁺ values were observed at a cone voltage of 55 V, yet at the gentler conditions of
38 V another peak was observed corresponding to an acetonitrile adduct. This observation indicates a weak ligation of the acetonitrile ligand to Tc, so it is very possible that the abundant acetonitrile (which is present in the labelling reaction, HPLC mobile phase and mass spectral analysis solutions) is rapidly ligating the Tc and disassociating leading to the observed broad peaks. The mass spectra of both the tricine linked methyl ester [89] and acid integrin [91] complexes exhibited this phenomenon.

![Diagram of possible Tc-HYNIC chelation modes](image1)

**Figure 3.2** Possible Tc-HYNIC chelation modes adapted from Liu et al. \(^{(155)}\)
Aside from coordination via HYNIC ligands and direct labelling through thiol and imidazole functional groups on biological conjugates, technetium has commonly
been coordinated to macrocycles containing sulphur and nitrogen. The MAMA bifunctional chelator and many of its derivatives have proven to be a useful and effective means of containing the Tc core in reactive physiological systems. Similar to rhenium, technetium coordinates to this macrocycle in the oxo-form. The technetium coordinates in a square pyramidal fashion with the double bonded oxygen as the apex and the amine, amide and two sulfides as the bases. Coordination of these epimer molecules to technetium, like rhenium, can be syn and anti with respect to the oxo group and any functional groups on the macrocycle backbone. These molecules are neutral with the oxotechnetium core $[\text{Tc}=\text{O}]^{3+}$ charge satisfied by the two sulfides and amine of the macrocycle.

The Tc-99m labelling of the MAMA macrocycles in this chapter proved much more difficult than the HYNIC-integrin antagonist analogues. The experiments were repeated several times with varying degrees of success, as the reactions were not easily reproducible. This may well have been due to the presence of Tc-99m avid impurities in the HPLC purified ligand samples in concentrations too low for the UV analysers to detect. Alternatively, the impurities may lack a chromophore and are not detectable by UV-vis spectroscopy.

As with rhenium chemistry, there are two general approaches when coordinating technetium to MAMA type thiol containing macrocycles. The first method involves cleaving off the protecting group on the highly reactive sulphur atoms, whether they are benzyl or trityl groups. The per technetate is then reacted with the deprotected macrocycle in the presence of stannous chloride as the reducing agent. Deprotection of the two thiols present a problem in itself as the proximal sulphurs can re-oxidise to form disulfide links. To counter this, the coordination should be performed immediately after, or at least using sacrificial reductants such as
mercaptoethanol.\textsuperscript{(136)} To remove the trityl groups from the sulphide requires highly acidic conditions. Traditional methods involve the stirring of the material in neat trifluoroacetic acid in the presence of trimethylsilane as an electron acceptor.\textsuperscript{(136)} After this the mixture is evaporated to dryness, water and petroleum spirits are added and the product partitions into the aqueous layer. Other methods involve cleavage with mercuric acetate followed by bubbling through hydrogen sulphide gas. Having established that these types of de-tritylations destroyed the peptidemimetics, a one-step de-tritylation/coordination method was sought. This method has been used successfully for rhenium and technetium. This method involves heating the tritylated ligand, the pertechnetate, stannous chloride and tartaric acid together in acidic aqueous conditions. Technetium/rhenium coordinations typically work better in slightly basic conditions, but the weak hydrochloric acid is required to promote the cleavage of the sulphur-trityl bond. Conventional heating as well as microwave heating was explored according to the methods detailed by Verbruggen \textit{et al.}\textsuperscript{(136)} For the Tc MAMA-integ complex [87] the radiochemical yield was higher for the conventionally heated reaction, so the decision was made to carry out the rest of the reactions in the heated water bath. Initially the yields were very low, and to improve this, changes to the method were made to see how the order of reactant addition affected the yield. The best results were achieved when the ligand pertechnetate activity was added to the ligand and tartaric acid immediately followed by the stannous chloride. The stannous chloride solution had to be made immediately before the reaction as it oxidised to tin oxide rapidly, which was observable by the solution becoming turbid. Oxidation by air is one of the major factors that work against these reactions, as its presence oxidises the stannous chloride thus reducing its ability to convert the pertechnetate into the oxotechnetium ion. This is why all solutions are
purged with nitrogen prior to the radiolabelling reactions. Tartaric acid weakly coordinates the oxotechnetium so its presence stabilises the oxotechnetium for a long enough period for the trityl-components of the ligand to be removed. The method that this was adapted from, was refined to improve the radiochemical yield and the use of various solvent combinations were explored to achieve a higher yield. In purely aqueous media the dissolution of the fairly non-polar tritylated peptidemimetic ligand was limited to the reactants pulling the insoluble ligand into solution as it was reacted. To counter this, tetrahydrofuran was added to the ligand, as it was miscible with water and dissolved the ligand reasonably well. Alcohols were also considered but their presence promoted the formation of their corresponding ester in the mildly acidic conditions. N,N'-Dimethylformamide was also used but this produced no product at all, as was also the case with dimethylsulfoxide. Dimethylsulfoxide is a known oxidising agent so this may have been the reason its addition caused the reaction to fail. Even though the addition of tetrahydrofuran worked best, the reactions were not always reproducible, and extensive testing of the conditions suggested the purity of the ligand was the major reason. Although all the labelling experiments were carried out with HPLC purified materials, it was found that certain batches worked better than others, while other batches failed to work at all. HPLC purification used UV absorbance for detection, so it is possible that an impurity with no chromophore may have been interfering with the labelling.

Successful labellings of the MAMA [87] and MAMA caproic linked peptidemimetics [88] showed a single major peak at 15.0 min in the chromatograms, as seen in Figs 3.5 and 3.6. This observation indicates that there is either only one epimer forming in the labelling reactions or that both epimers have the same retention. Since this information was echoed with the corresponding rhenium
analogues in Chapter 2, no further investigations were conducted to answer this question. The radiochemical yields were quite low, but understandable on account of the ‘one pot’ technique employed.

**Fig 3.5** TcMAMA Integ Acid [87] (at 15.5 m) radio chromatogram (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient as outlined in Table 3.2).

**Fig 3.6** TcMAMA Integ caproic Acid [88] (at 15.5 min) radio chromatogram (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient as outlined in Table 3.2).
3.3.3 Copper$^{64}$ labelling

The Cu$^{64}$ labelling in this chapter was carried out according to established methods for coordinations of metals to tetraaza-tetra carboxylic acids. The labelling required very similar conditions to that of the stable copper analogue in Chapter 2 [46]. Previous experimental methods reported that Cu(II) complexes of this particular type of macrocycles are neutral. Charged complexes have been achieved when one of the backbone carboxylic acids is converted to an amide by peptide coupling conjugations to the biologically relevant part of the molecule. Coordination of Cu$^{2+}$ has been achieved with DOTA and TETA derivatives as well as the TRITA derivative used in this project. Traditionally chemists have sought simpler tetraaza molecules (with no attached carboxylic acid moieties) to bind copper. Unfortunately it was observed that in physiological conditions it was possible for the complex to be de-metallated. To counter this problem, the addition of acetyl groups was used to further ‘wrap’ the metal up to reduce the observed de-metallation.$^{(112)}$

Despite not having the infrastructure or equipment to image the Cu$^{64}$ conjugate produced in this chapter [93], this work was undertaken to test the technology and feasibility of future Cu$^{64}$ radiopharmaceuticals. Cu$^{64}$ has potential as a therapeutic agent as well as a PET diagnostic so the development of a labelling strategy for this type of molecule could be the first step into the creation of a radiotherapeutic based on these particular integrin antagonists. Eventually this molecule could be tested for its in vivo uptake and tumour destruction. Cu(II) has been attached to bitistatin, a known integrin antagonist, employing the DOTA bifunctional chelator. Stability testing of Cu$^{64}$ DOTA-Bitistatin was carried out in human serum albumin (HSA) at 37 °C showed only 10% breakdown from the original form after 20 h.$^{(157)}$ This high stability was echoed in the mouse plasma
analysis indicating greater than 95% of the Cu\(^{64}\) in the original Cu\(^{64}\) DOTA-Bitistatin form after 1 h.\(^{(157)}\) These findings are in agreement with the stability studies performed on a variety of alkaline earth and transition metal complexes with tetraaza-tetra carboxylic acids.\(^{(158)}\) These studies also compare the calculated stabilities of the similarly shaped DOTA, TETA and TRITA macrocycle backbones (Fig 1.13) with the stability order of DOTA>TRITA>TETA, indicating that a smaller backbone size is preferred by the copper ion.

The Cu\(^{64}\) labelling of the integrin antagonist was performed in a gently warmed waterbath at 35 °C. No colour changes were visible due to the small amount of stable copper present, but the radiochromatogram (Fig 3.8) did show 2 significant peaks, the first at 4.5 min (albeit quite small) and followed by the main peak at 9.0 min. The reason was almost certainly the result of the breakdown of the macrocycle for the earlier peak, which have commonly been observed to lose an N'-acetic acid group. The molecule still avidly binds Cu(II). This problem arises from the extreme difficulties in purifying these macrocyclic systems even with the use of HPLC systems, as discussed in Chapter 2.

![Radiochromatogram](image)

**Fig 3.7** Cu\(^{64}\) TETA bz integ (9-10 min) radio chromatogram (Waters Bondapak 10 m C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient of acetonitrile/aqueous 0.1M ammonium acetate with conditions outlined in Table 3.2).
3.3.4 Lutetium\textsuperscript{177} Synthesis

Lutetium\textsuperscript{177}, a radiolanthanide has been earmarked as a radiopharmaceutical with potential application in diagnostic imaging and therapy on account of its unique emission characteristics. Its 6.73 day half-life enables more extensive chemistries and purifications to be carried out before administering, and its $\beta/\gamma$ dual emission is ideal for combined localised therapies in conjunction with diagnostic imaging. There is an increasing need for Lu\textsuperscript{177}, especially in the NCA state.

NCA Lu\textsuperscript{177} was produced by irradiating isotopically purified ytterbium\textsuperscript{176} oxide in the HIFAR reactor for 7 days. The separation of the formed Lu\textsuperscript{177} from the bulk ytterbium target matrix was the most challenging part due to the extremely similar chemical and physical natures of the two lanthanides. Initial experimentation focussed on the liquid-liquid extraction of Lu\textsuperscript{177} from Yb\textsubscript{2}O\textsubscript{3} using a chelating agent di-(2-ethylhexyl)phosphoric acid (HDEHP) in cyclohexane\textsuperscript{(159)} This particular method relies on the differentiation the ligand has between ytterbium and lutetium at a particular pH. The Lu\textsuperscript{177} complex is reported to partition into the cyclohexane layer. Unfortunately, the complete separation reported by Lebedev et al\textsuperscript{(159)} could not be replicated. Enrichment was achieved but certainly not approaching NCA status. Other methods have been reported that separate these lanthanides by employing selective cementation using sodium amalgams\textsuperscript{(160)} A chromatographic method was seen as an ideal production route as the short half-life did not permit a convoluted separation method. Ion exchange methods using specially modified resins have been created to separate lanthanides, but are not designed for the capacities of bulk matrix material required to produce useful amounts of the radiolanthanides. There is a significant amount of work in the literature dealing with routine lanthanide analysis and these are based on HPLC chromatography using paired ion chromatography
agents to improve the separation. The most popular methods involve the sodium salt of 1-octanesulfonic acid (1-OS) to bind to the C-18 matrix on the HPLC columns (ion pair chromatography). The non-polar octane hydrocarbon chain is attracted to the C-18 modifier leaving the exposed sulfonic acid as a relatively strong cation exchange group. \( \alpha \)-Hydroxyisobutyric acid (\( \alpha \)-HIBA) is also incorporated to chelate the lanthanides present. The lanthanide \( \alpha \)-HIBA complex is retarded to a greater extent than the free hydrated cation, thus enabling a more efficient separation. Understandably, trace amounts of the metals were being separated in this fashion, so an improved method was sought that could handle upwards of 20 mg of the ytterbium oxide starting material. The method of Hashimoto and co-workers\(^{(161)}\) significantly improved this method by building on the established method of using octanesulfonic acid and \( \alpha \)-HIBA at an ideal pH range but utilising high capacity commercially available radial-pak\(^{\circledR}\) technology developed by Waters. Radial-pak\(^{\circledR}\) HPLC involves the uses of plastic cartridges filled with stationary phase, which are compressible to improve the column efficiency. The cartridges are soft enough to be compressed by surrounding liquid, so as to align or order the stationary phase particles. The ordered particles increases the interaction between the stationary and mobile phases and effectively increases the theoretical plates over a column of similar dimensions without external pressurisation. The method is also advantageous as the plastic casing limits the amount of contact the acidic mobile phase has with metal which is an unwanted source of contamination. The method was improved in this project by not only increasing the amounts of ytterbium matrix loaded onto the column but also using custom-made 25 mm ID Radial-pak\(^{\circledR}\) cartridges from Waters to further improve the separation. After the separation, traditional methods were used to free the separated radiolanthanide from the chelating agent, using anion exchange
chromatography followed by ashing with nitric acid and hydrogen peroxide on a hotplate. Final dissolution of the residue in hydrochloric acid and subsequent evaporation afforded the lutetium\textsuperscript{177} trichloride in weak hydrochloric acid solution ready for labelling. The ytterbium oxide starting material used in this experiment was 97.6% isotopically enriched, with 1.93% in the Yb\textsuperscript{174} form. The Yb\textsuperscript{174} present in the sample gets converted to Yb\textsuperscript{175}, a gamma emitter with a half-life of 4.7 days. The presence of this isotope is used as a radiomarker in the HPLC and thus enables the detection of, and separation from the bulk ytterbium matrix.

The Lu\textsuperscript{177} fraction eluted first at 302 min (Fig 3.9) followed by the larger Yb\textsuperscript{175}/Yb fraction. Separation was verging on baseline, so fractionation between the peaks was required to afford the radiochemically pure Lu\textsuperscript{177} to maximise the yield. The radiochemical purity was assessed by gamma spectroscopy and comparing the Lu\textsuperscript{177} photon energies to the ratio of Yb\textsuperscript{175} photon energies. The absence of the Yb\textsuperscript{175} peaks indicated radiochemical purity as shown in Fig 3.10.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine the metal impurities in the sample.

These results showed aluminium and iron to be the major impurities at levels of 2.3 and 12.7 ppm respectively, as shown in Table 3.4. The source of aluminium contamination most likely arose from the use of aluminium foil used to cover and protect beakers, hotplates, and other assorted equipment from radioactive contamination, particularly during the ashing procedure. The iron impurity was most likely to have been introduced from the HPLC itself and the syringe needle that injected the dissolved matrix onto the HPLC system. It should be noted that although the HPLC system used in the radiometal procedures, consisted of PEEK tubing and plastic pump heads, there are parts of the injector loop and system that have exposed
steel and will always be a source of metal contaminants, albeit quite low. There were a number of problems using the Radial-pak® chromatographic method for this radiochemical separation. The first major problem was the back pressure fluctuation during elution. Radial-pak® cartridges have a maximum rated pressure of 1500 psi and above this pressure the plastic casing can rupture. Also for effective usage of the Radial-pak® method, the external liquid pressure has to be greater than the pressure of the mobile phase passing through the column. If the reverse occurs, the mobile phase will leach out into the liquid filled compression chamber and potentially contaminate the system. To avoid this, the HPLC system pressure and the external compression chamber pressure had to be monitored closely and adjusted. Directly after injection of the dissolved ytterbium matrix was the most critical time as the pressure always increases dramatically. By a combination of reducing the initial flowrate and increasing the pressure of the external pressure chamber this was overcome. The reason for the initial pressure build up was most likely to be precipitation of some of the ytterbium matrix when introduced into the mobile phase from the injector loop. The matrix was dissolved in hydrochloric acid (0.1 M) and the sudden mixing with the 1-octanessulfonic acid (0.1 M) / α-HIBA (0.25 M) mobile phase (pH 2.5) may have prompted some to precipitate. The pressure gradually reduced, which could be explained by the re-dissolution of the precipitated material. This precipitation and gradual redissolving of the bulk matrix material would also contribute to the broad tailing of the Yb\(^{175}\) peak. The column required a very long time to separate the extremely similar metal complexes, so a great deal of mobile phase was required. To limit this, mobile phase recycling was employed when the eluent was clear of any radioactivity.
Fig 3.8  Chromatographic separation of Lu$^{177}$ from Yb$^{175}$ bulk matrix (Waters prep LC, 3 x 100 mm x 25 mm I.D. Novapak cartridge 4 µm C18, with mobile phase of 0.1 M octanesulfonic acid sodium salt, 0.25 M α-hydroxyisobutyric acid pH 2.25 at a flow rate of 20 mL/min).

Table 3.4  ICP-MS results for 3 separate Lu$^{177}$ separations

<table>
<thead>
<tr>
<th>Metal</th>
<th>Expt #1 (ppm)</th>
<th>Expt #2 (ppm)</th>
<th>Expt #3 (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu</td>
<td>0.05</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Yb</td>
<td>0.68</td>
<td>0.003</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Er</td>
<td>&lt; 0.002</td>
<td>&lt; 0.002</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Tm</td>
<td>&lt; 0.002</td>
<td>&lt; 0.002</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Al</td>
<td>28.4</td>
<td>74.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Fe</td>
<td>6.6</td>
<td>34.4</td>
<td>12.7</td>
</tr>
</tbody>
</table>
Fig 3.9  Gamma Spectra before (a) and after (b) the separation of Lu$^{177}$ from the irradiated bulk ytterbium/Yb$^{175}$ matrix
3.3.5 Lutetium\textsuperscript{177} labelling

The labelling of the TRITA bz integrin antagonist molecule with Lu\textsuperscript{177} \cite{94} was very similar to that of the analogous Cu\textsuperscript{64} labelling, and indeed the ‘cold’ Lu labelling of the molecule described in Chapter 2 \cite{47}. As is the case with the metal avid tetraaza-tetra carboxylic acid ligands, slight impurities in the ligand and/or the radiometal solution will result in reduced or even no labelling at all. Initially it was found that no labelling could be achieved with slightly impure ligand. A macroscopic amount of ‘cold’ lutetium was required to achieve successful labelling, indicating the presence of an impurity with a higher affinity for lutetium. After painstakingly purifying the ligand \cite{45}, NCA Lu\textsuperscript{177} was successfully incorporated in to the TRITA bz integrin antagonist molecule, resulting in \cite{94}. The reaction differed from the Cu labellings in that 70 °C heat was required to achieve a good yield. The Lu\textsuperscript{177} gamma energies are rather weak in comparison to the other radioisotopes used in the project and the weak signal in the radiochromatogram is a reflection of this (Fig 3.10). Ideally a more sensitive detector would have been used for this labelling experiment like the model used for the HPLC separation of the Lu\textsuperscript{177} from the ytterbium oxide matrix, but this was not available.

This Chapter has demonstrated that ability to label the integrin molecules with various radionuclides using attachment strategies suitable for the differing radiometals used, and from this the Tc-99m radiotracers were evaluated \textit{in vivo} in Chapter 5.
Fig 3.10  Lu$^{177}$ TETA bz integ (at 11 min) radio chromatogram (Waters Bondapak 10 μm C18, 300 mm × 7.8 mm I.D column, employing a programmed gradient between 5% acetonitrile/aqueous 0.1 M ammonium acetate as outlined in Table 3.2).
CHAPTER 4

\textit{IN VITRO} $\alpha_v\beta_3$
INTEGRIN ASSAY
4 IN VITRO $\alpha_v\beta_3$ INTEGRIN ASSAY

4.1 INTRODUCTION

4.1.1 Basic pharmacology

The field of pharmacology is a science that investigates and evaluates drug activity in living systems. The science is well established and arose out of the systematic investigation of the relationship between chemical structure and biological activity in the 1860s. Pharmacology is used to quantify the interactions of drugs with their biological targets and correlate the observed data with mathematical models to help understand the nature of the drug’s effects on the biological targets, whether they be enzymes, ion channels, DNA or one of the myriad of other highly specific protein receptors. It focuses on two major aspects of drug activity, affinity and efficacy. The affinity of a drug for a receptor is a measure of the strength of the interaction between the two entities. The attraction of the molecules is thermodynamic in nature, i.e. enthalpy in regards to changes in heat, as well as entropy regarding the change in disorder. The chemical forces between the drug and the receptor vary in importance in relation to the proximity between the drug and the receptor’s binding surface. Electrostatic interactions, van der Waals forces, hydrogen bonding, and hydrophobic forces dictate the way a drug will occupy the binding site, which is essentially an adopted position of minimum free energy. Of great importance to the pharmacologist is the fact that thermal energy causes the drug to move around and dissociate from the receptor’s binding surface. This dynamic state of associating and dissociation allows other species to bind to the surface and compete for the same binding domain. The
probability that the drug will be at the lowest position of free energy within the
binding site is dependent on the concentration of the drug in the surrounding media
and is the key to measuring the affinity of the drug for the receptor. In fact,
pharmacologists discovered that the pioneering work by Langmuir concerning
adsorption isotherms of molecules binding to metal filaments\(^{(162)}\) in the production of
light, were appropriate to all reversible biological interactions of receptors and their
substrates. By deriving the Langmuir adsorption isotherm, pharmacologists were able
to come up with the following relationship:

\[
p = \frac{[AR]}{[R_i]} = \frac{[A]}{[A] + K_d}
\]

(Equation 4.1)

where \([AR]\) is the amount of the bound receptor ligand complex, and \([R_i]\) is the total
number of receptor domains. The ratio \(p\) is the fraction of maximum binding by a
molar concentration of drug \([A]\) possessing an equilibrium disassociation constant \(K_d\).
\(K_d\) is a measure of the binding between drug and receptor and how easy it is to
separate the two and is essentially a quantifier of affinity. Therefore the lower the \(K_d\)
value, the higher the affinity is. Affinity is the reciprocal of \(K_d\).

Efficacy on the other hand is a measure of the ability of a drug to elicit some
physiological response when it binds to a receptor. This is important for agonists as
they are defined as substrates that promote a biological response as outlined in
Section 1.11. An agonist binding will elicit this response via some sort of
conformational change in the receptor protein, although it is not a static change. The
flexibility of proteins enables a dynamic shifting of the conformational states due to
the heat energy present, but the binding of a certain molecule can bias this dynamic
towards a specific conformation and thus by a biological cascade mechanism cause
some sort of effect or signal.
Whether in fact the molecules are antagonists or agonists is not relevant either as the doses being administered are too small to elicit any response even if they had agonistic properties. So in essence the difference between the development of diagnostic radiotracers and conventional drugs is that only the binding to the target is of any importance due to the extremely low amounts present. Of course diagnostic radiotracers can also give us information such as the density of receptors unlike their non-radioactive counterparts.

4.1.2 Binding assays

Binding assays for receptors have been around for quite some time, and are useful tools for gauging a drug’s affinity for a biological substrate, the amount of a particular receptor present and other binding site related data. This particular in vitro test measures and quantifies the binding of the drug to the receptor, but relies on the ability of the researcher to distinguish the bound from non-bound molecules. The use of radiolabelled drugs was the first method widely employed by pharmacologists. The radioactivity could be measured directly by the observed $\gamma$ or $\beta$ radioactivity or indirectly by scintillation counting. Of course this technology requires the separation of the unbound molecules from the drug-receptor complex, and this can be achieved on the basis of size exclusion of the relatively small drug molecule versus the much larger protein-drug complex (filtration and dialysis.) Centrifugation is another method, as is having the receptor protein bound to a solid phase, enabling the decanting of the supernatant containing the non-bound drug. Scintillation proximity assays (SPAs) employ a bead containing a scintillant material, to which the receptor is bound and thus only the bound radio-ligand is close enough to cause scintillation. Radiolabelled molecules are not the only option open to
pharmacologists in drug-receptor binding studies. Fluorescence and fluorescence polarisation techniques have also been incorporated into binding assays.\(^{(164)}\)

Binding assays can be performed using 3 different approaches; kinetic, equilibrium, and displacement studies. Kinetic studies are measurements of the drug-receptor interactions over a period of time and this data can be used to determine first order rate constants, equilibrium binding constants, reversible binding and allosteric effects. Equilibrium studies involve the monitoring of the binding of a traceable drug to the receptor and by quantifying the maximal number of binding domains and the affinity of the drug for the receptor by applying the Langmuir adsorption isotherm model. Of course for this technique the molecule of interest must be traceable (by radioactivity or other spectroscopies including UV, circular dichroism (CD), linear dichroism (LD), fluorescence and NMR). The displacement assay is similar except it can measure the affinity of a ‘cold’ drug by its interference with the binding of a tracer drug with known binding characteristics for the target receptor.

### 4.1.3 Non specific binding and specific binding

In any binding study there is going to be other sites that can compete with the target receptor domain for the drugs’ occupancy. Whether they are other molecular recognition sites on a purified protein, cell membranes or even the surface media of the glass or plastic vessel where the experiment is taking place, the amount of binding to these must be determined in order to achieve accurate binding data. Non specific binding (nsb) refers to the amount of binding of a drug of interest that is occupying these auxiliary sites other than the receptor’s target domain. Since the aim of the experiment is to relate the concentration of the drug to the amount that is bound to the target receptor binding site (specific binding), the nsb must be calculated and factored
into the data. To determine the non specific binding, a non-tracer standard with known high affinity and selectivity for the target receptor must be available. For traceable ligands, the nsb is linear and non-saturable in the ranges regularly assessed for pharmacological evaluation and is defined in the following relationship;

\[ \text{nsb} = K[A^*] \]  
(Equation 4.2)

where \( K \) is a constant relating the concentration relationship of nsb, and \([A^*]\) is the concentration of the tracer species remaining in solution. Unlike nsb, specific binding is saturable and using the Langmuir isotherm, the following relationship is found;

\[ \text{specific binding} = \frac{[A^*]B_{\text{max}}}{[A^*]+K_d} \]  
(Equation 4.3)

where \( K_d \) is the equilibrium dissociation constant of the receptor-tracer complex. Total binding, the sum of the nsb and specific binding, is given as;

\[ \text{total binding} = \frac{[A^*]B_{\text{max}}}{[A^*]+K_d} + \text{nsb} \]  
(Equation 4.4)

where \( B_{\text{max}} \) is the maximal binding. Appropriate experiments yield both the total binding and the nsb, and by knowing these, the specific binding can be calculated, by simple subtraction or more accurately by fitting the two data sets to Equations 4.2 and 4.4 and solving simultaneously.

### 4.1.4 Equilibrium binding

Equilibrium or saturation binding experiments involve the equilibration of the target receptor with a variety of concentrations of a traceable ligand. These experiments yield the \( K_d \) and \( B_{\text{max}} \) of the tracer for the target receptor. Data for
saturation experiments are processed and visualised in a variety of plot methods, such as Scatchard, Eadie, Eadie-Hofstee depending on the binding characteristics. Logarithmic transformations of plots are regularly used to aid in the visualisation of the binding, although extrapolations can often be inaccurate. Having this data for the tracer would allow for the design of the competition studies that evaluate the non-tracer molecule’s affinities for the receptor. The IC$_{50}$ value is a representation of the binding strength of a ligand for a receptor in a competition study. The IC$_{50}$ specifically refers to the concentration required by the test ligand to displace 50% of the known binder from the receptor sites.

![Saturation binding curves showing the specific binding curve (right) derived from the total binding and the calculated or measured nsb curves (left)](image)

**Figure 4.1** Saturation binding curves showing the specific binding curve (right) derived from the total binding and the calculated or measured nsb curves (left)

### 4.1.5 Integrin *in vitro* binding assay

For the *in vitro* assessment of the synthesised molecules, a method using an I$^{125}$ labelled standard was sought, as that particular isotope has ideal properties for the time consuming assays. Traditionally, researchers had labelled Echistatin (Section 1.13) for $\alpha_i\beta_3$ protein assays, but the development of molecules with improved affinities and selectivities for the integrins have enabled the assaying to be performed with a greater degree of sensitivity and accuracy. The Merck produced [I$^{125}$]L-
775,219 (Fig 4.2) is a superior ligand to Echistatin (IC\textsubscript{50} of 0.1 nM vs Echistatin), and has been used extensively for $\alpha_v\beta_3$ assays for this reasons.\textsuperscript{(114,115)}

\begin{center}
\includegraphics[width=0.5\textwidth]{figure4_2.png}
\end{center}

\textbf{Figure 4.2}  \textsuperscript{[\textsuperscript{125}I]}L-775,219, the \textsuperscript{125}I labelled $\alpha_v\beta_3$ integrin antagonist developed by Merck [86].
4.2 EXPERIMENTAL

4.2.1 General

All radiochemical procedures were carried out in laboratories equipped and designated for radiation handling. Extensive lead shielding in was employed to minimise the radiation dose. Radiation monitors as well as personal dosimetry badges were operated to indicate the levels of personal exposure. All radioactive waste was labelled and left to decay in the appropriate designated shielded decay areas. Extensive use of ‘double-gloving’ with latex gloves was required when handling radioactive equipment and glassware for heightened protection.

All reagents used for buffers and preparations were purchased from Sigma. Buffers were stored in the fridge when not in use. Purified human $\alpha_\nu\beta_3$ integrin receptor protein was purchased from Chemicon and stored at -80 °C when not in use. $[^{125}\text{I}]$L-775,219 [86] (Section 3.2.3.1) was used for the competition assay and stored in a radiological hazard marked freezer. All in vitro testing was performed in triplicate and repeated at least 3 times on separate occasions.

4.2.2 Solid phase Integrin receptor binding competition assay

4.2.2.1 Protein coating of Microtitre plates

Purified human $\alpha_\nu\beta_3$ integrin receptor protein was diluted to 1.5 µg/mL in coating buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM MnCl$_2$). Aliquots (100 µL, 0.15 µg protein) of the solution were added to each of the 96 wells in the microtitre plates and were incubated overnight at 4 °C. The
plates were washed once with buffer (150 µL/well, 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 1% bovine serum albumin) and incubated for 2 h at room temperature. The plates were then rinsed twice with the same buffer prior to the competition assay.

4.2.2.2 Competition study

To each well of the microtitre plate was added [I²³⁵]L-775,219 [86] (adjusted to a specific activity of 500 Ci /mmol (or 1.85×10¹³ Bq/mmol)) to give a final well concentration of 1 × 10⁻⁹ M. Analyte metal complexes [23, 24, 40, 43, 46, and 47] were dissolved in the binding buffer at 9 concentrations ranging from 1 × 10⁻⁵ - 5 × 10⁻¹¹ M, and added to the wells, giving a final volume of 200 µL per well. After incubation at room temperature for 2 h, each well was washed three times with buffer (150 µL/well, 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 1% bovine serum albumin), and the remaining activity was counted on a Wallac γ counter, by dismantling each plate into the individual wells and counting them in kimble tubes. Non-specific binding was determined using 1 × 10⁻⁶ M of the Merck L-775,219 α₃β₃ integrin antagonist.

4.2.2.3 Data analysis

All data obtained for the binding experiments was entered into an IBM-compatible PC workstation running the Kell 6 program suite by Biosoft® created for radioligand binding analysis. Processing and determining the IC₅₀ values were performed using weighted non-linear curve fitting routines (see Appendix, A1).
4.3 RESULTS AND DISCUSSION

Data from the γ counter was converted to decays per minute (dpm) by dividing the counts per minute (cpm) by the efficiency of the counter. The software used this data to create the dose response curves and calculated the IC<sub>50</sub> value. An example of the data obtained from one of the Cu TRITA [46] experiments is shown in Table 4.1. The binding curve for this complex is also shown in Fig 4.3 to illustrate the results.

The results obtained from the in vitro binding assay were quite unexpected in terms of structure and projected affinity for the purified α<sub>ix</sub>β<sub>3</sub> integrin receptor protein (Table 4.2). The two-pronged approach used in this project was to synthesise molecules with a moiety with acknowledged affinity and selectivity for the protein (conjugate approach) as well as the riskier ‘integrated approach.’ The nature of this project was not to systematically construct a series of analogues for a rigorous structure-activity relationship (SAR) investigation. Instead, a small number of analogues were synthesised to see if the incorporation of various bifunctional chelator groups would affect the performance of the known antagonist moiety, and to what extent, as the development of radiotracers is ultimately driving the project. The criteria used in constructing the more synthetically challenging ‘integrated approach’ molecules was the (1) distance between the Arg and Asp mimics, (2) the utilization of the benzimidazole functionality at the Arg mimic terminal and also (3) to determine the effects of the analogous phenylsulfonyl and benzyloxy carbonylamino groups peripheral to the asp mimic terminal. By starting with a totally different framework for this method it was unknown whether a successful target would be achieved.
Table 4.1  Example of γ counter data for Cu TRITA complex [46]

<table>
<thead>
<tr>
<th>Cold drug conc (M)</th>
<th>cpm 1</th>
<th>cpm 2</th>
<th>cpm 3</th>
<th>Average cpm</th>
<th>cpm (decay corr.)</th>
<th>dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56967</td>
<td>54866</td>
<td>53414</td>
<td>55082.3</td>
<td>55648</td>
<td>79497</td>
</tr>
<tr>
<td>$5 \times 10^{-12}$</td>
<td>54662</td>
<td>57582</td>
<td>54338</td>
<td>55527.3</td>
<td>56097</td>
<td>80139</td>
</tr>
<tr>
<td>$5 \times 10^{-11}$</td>
<td>58101</td>
<td>58599</td>
<td>61282</td>
<td>59327.3</td>
<td>59936</td>
<td>85623</td>
</tr>
<tr>
<td>$5 \times 10^{-10}$</td>
<td>52251</td>
<td>51834</td>
<td>56299</td>
<td>53461.3</td>
<td>54010</td>
<td>77157</td>
</tr>
<tr>
<td>$5 \times 10^{-9}$</td>
<td>48520</td>
<td>48843</td>
<td>49762</td>
<td>49041.7</td>
<td>49545</td>
<td>70779</td>
</tr>
<tr>
<td>$5 \times 10^{-8}$</td>
<td>38219</td>
<td>37255</td>
<td>33047</td>
<td>36173.7</td>
<td>36545</td>
<td>52207</td>
</tr>
<tr>
<td>$5 \times 10^{-7}$</td>
<td>15183</td>
<td>15680</td>
<td>16432</td>
<td>15765</td>
<td>15297</td>
<td>22753</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>9403</td>
<td>9443</td>
<td>9853</td>
<td>9566.3</td>
<td>9665</td>
<td>13806</td>
</tr>
<tr>
<td>$5 \times 10^{-6}$</td>
<td>2579</td>
<td>2458</td>
<td>2471</td>
<td>2502.7</td>
<td>2528</td>
<td>3612</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>1440</td>
<td>1455</td>
<td>1455</td>
<td>1450</td>
<td>1465</td>
<td>2093</td>
</tr>
</tbody>
</table>

Figure 4.3  Specific binding curve obtained for the Cu TRITA complex [46] processed from Kell 6 software (Biosoft®)
**Table 4.2  IC<sub>50</sub> in vitro results for the target molecules**

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
<th>( \alpha_\beta_3 \text{ IC}_{50} \text{(nM)} ) &amp; Std Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReMAMA</td>
<td><img src="image" alt="ReMAMA Structure" /></td>
<td>840 ± 200</td>
</tr>
<tr>
<td>ReMAMACap</td>
<td><img src="image" alt="ReMAMACap Structure" /></td>
<td>380 ± 40</td>
</tr>
<tr>
<td>Cu TRITA</td>
<td><img src="image" alt="Cu TRITA Structure" /></td>
<td>110 ± 16</td>
</tr>
<tr>
<td>Lu TRITA</td>
<td><img src="image" alt="Lu TRITA Structure" /></td>
<td>1500 ± 300</td>
</tr>
<tr>
<td>ReNovZ</td>
<td><img src="image" alt="ReNovZ Structure" /></td>
<td>No Inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>at 1 x 10&lt;sup&gt;-5&lt;/sup&gt;M</td>
</tr>
<tr>
<td>RePsulf</td>
<td><img src="image" alt="RePsulf Structure" /></td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>
Since there was no attempt to synthesis a rhenium HYNIC molecule, the conjugated approach molecules tested in vitro were the Re MAMA [40], Re MAMA Cap [43], Cu TRITA [46] and Lu TRITA [47] complexes. The TRITA complexes were expected to be the most similar because it was only the metal centre that was different and any difference in binding would be due to the TRITA complexes’ different size and geometric constraints. The rhenium complexes only differed in the fact that the ReMAMA Cap complex [43] had an aminocaproic acid linker between the MAMA bifunctional chelating unit and the integrin antagonist moiety. The length afforded by this linker was deemed large enough to significantly reduce (but not necessarily inhibit) any steric factors between the bulky Re macrocycle and the binding surface of the receptor. Another reason why the aminocaproic acid group was utilised is because it does not significantly alter the polar character of the molecule, which was certainly confirmed by the similar retention times of the Re MAMA [40] and Re MAMA Cap [43] molecules on reversed phase HPLC (Chapter 2) as well as for the Tc-99m analogues [87, 88] (Chapter 3).

A value of 840 ± 200 nM was obtained for the IC$_{50}$ of the ReMAMA complex [40], which was the experimentally determined concentration required to inhibit 50% of the $^{125}$I labelled Merck standard from binding to the receptor in the radioassay. This is quite poor considering that the phenylsulfonyl parent molecule on which it is based has an IC$_{50}$ value of 0.9 ± 0.7 nM, when performed on wheat germ agglutinin scintillation proximity beads (SPAVs).$^{(115)}$ This result strongly suggests that the addition of the Re macrocycle group drastically reduces the ability of the biologically active moiety to bind to the RGD recognition site on the protein.

The incorporation of the linker in the ReMAMA Cap complex [43] did have a positive effect on the binding of the analogue as it achieved an IC$_{50}$ of 380 ± 40
nM. This data is further evidence that the bifunctional chelator group is inhibiting the binding via some sort of steric interaction, as the linker reduces the proximity between the BFC and the receptor’s recognition site and positively influences the binding.

In terms of the TRITA complexes, there was a rather astonishing difference between the copper and lutetium complexes. The IC$_{50}$ of the CuTRITA complex [46] was found to be 110 ± 16 nM (the lowest in the study), compared to the LuTRITA [47] complexes’ value of 1500 ± 300 nM, approximately an order of magnitude different. The nature of this unexpected result can not be explained easily but it may be the result of charge on the complexes in the buffer systems used in the experiment. The lutetium complex [47] has a net charge of -1 compared to the neutral copper complex [46]. It is known that the molecular recognition is dependent on a variety of factors (Section 4.1.1) and charge is one of them. Another possible reason for the marked difference in binding is the size of the actual complexed TRITA macrocycles. The lanthanoid has a much bigger metal centre and this influences the rather ‘loose’ scaffolding of the TRITA backbone as well as the peripheral acetic acid groups. The larger bulk of the LuTRITA complex could have contributed to steric interference with the binding surface of the integrin receptor. A final possible reason for this unexpected result may be that an un-complexed acetic acid group on the [46] is positively influencing the binding to the protein recognition site. The spatial position of the carboxylic acid may be in a position that is more easily accessed by the aspartic acid selective portion of the RGD binding site, and thus substituting for the terminal carboxylic acid on the Merck derived moiety.

The rhenium complexes developed for the ‘integrated approach’ to design molecules that avidly bind integrins were unsuccessful in terms of specific binding to
the purified $\alpha_v\beta_3$ integrin receptor. There was little observed inhibition up to the maximal tested concentration of $1 \times 10^{-5}$ M for the Re NovZ complex [24] and Re psulf complex [23], indicating that the molecules had no significant attraction to the receptor. Why the phenylsulfonyl derivative is better than the benzyloxycarbonylamino is unknown. Both were chosen as they have shown to be important in binding to RGD sites on other model molecules, although one study comparing des-amide backboned analogues showed an IC$_{50}$ value of 0.3 nM for the phenylsulfonyl molecule compared to the benzyloxycarbonylamino’s 1.8 nM in the SPAV3 assay$^{(165)}$. The observed differences may be the result of steric interactions of these peripheral groups to the acid terminal of the molecules with the protein’s binding surface, or alternatively the varying distortive effects that the phenylsulfonyl and benzyloxycarbonylamino groups impart on the rest of the molecule.

The qualitative comparison of the two ‘integrated approach’ targets is made more difficult because there are diastereoisomers present, not to mention the potential for syn and anti isomerism with respect to the oxorhenium and the groups on the ligand’s backbone. All having the same molecular weights and absorption characteristics, these molecules were detectable by HPLC analysis but inseparable on any preparative scale, so unfortunately the diasterisomeric mixtures were tested in vitro. As the benzyloxycarbonylamino rhenium complex displayed no significant binding, it is fair to assume that none of the isomers are of any relevance to the purified $\alpha_v\beta_3$ integrin receptor. The results obtained for the phenylsulfonyl analogue suggest that at least one of the diastereoisomers could inhibit the binding of the $^{125}$I standard to a degree higher than the observed value which is of course a representation of the whole isomeric mix. Had the binding results for this complex been reasonable it may have been worthwhile to attempt to separate the isomers of the...
phenylsulfonyl complex and further characterise them, which would be a significant body of work in itself, and well beyond the scope of this project.

In summary, this Chapter described the \textit{in vitro} testing of a number of metal complexes comprising of rhenium, copper and lutetium metal centres designed to bind to the $\alpha_v\beta_3$ integrin receptor by testing against well bound purified human $\alpha_v\beta_3$. The copper complex [46] gave the best IC$_{50}$ value (110 ± 16 nM), while the integrated design methodology’s rhenium complexes showed extremely weak or no inhibition at the maximal concentration tested.
CHAPTER 5

IN VIVO ANIMAL IMAGING STUDIES
5 IN VIVO IMAGING ANIMAL STUDIES

5.1 INTRODUCTION

In vivo imaging has become an extremely important technique in diagnostic medicine. Imaging agents include gamma emitters like Tc-99m and $^{123}$I, and positron emitters such as $^{18}$F and $^{64}$Cu. Aside from unstable isotopes, metals such as europium and gadolinium have been employed as Magnetic Resonance Imaging (MRI) agents to monitor bodily functions.\textsuperscript{(166,167)} The ability of a researcher to visualise a drug’s progress in a living biological system adds another dimension to pharmaceutical development. Drug development, thanks to in vivo imaging can be achieved in much less time than previously possible. Tc-99m, as previously stated is the most commonly used radioisotope used in nuclear medicine as its decay properties are ideal for screening and imaging function.

Small animal imaging is the logical first step in evaluating a pharmaceutical in vivo. Mice, rats and hamsters are the main animals used in in vivo imaging experiments. The availability of specific animal models for diseases has allowed their use in a variety of applications and the nude models (immune deficient and as a result, hairless bodied hence ‘nude’) has allowed the implantation of human tumours in the animals without the otherwise normal bodily defences eradicating the tumour. Tumour suppression and destruction is paramount to the oncology researcher, so there is a great deal of literature involving the implantation of tumours into small animals for diagnostic imaging and testing. As well as tumour targeting, small animal imaging has been used quite extensively for targeting receptors in the body and for the basic monitoring of bodily functions and organs. Whatever the application, the radiopharmaceutical can be imaged in the animal using the appropriate camera
apparatus. Gamma scintigraphy and PET scanning are the two main modalities in nuclear diagnostic imaging, and thus require different equipment to detect and subsequently compile the image of detected radioactivity. Both techniques involve the detection of gamma radiation, but in the case of the SPECT agent, the emitted gamma ray is directly detected from the isotope’s decay, whereas the PET agent relies upon the annihilation of the positron with an electron to give rise to two 511 keV photons 180° apart (Fig 5.1). PET scanners are more complex as they contain a circular array of detectors with coincidence circuits designed specifically to detect the 511 keV photons emitted in opposing directions. Because of the increased complexity of the PET scanner, they are significantly more expensive and much less common. The SPECT camera has a similar sodium iodide detector but they are able to detect gamma rays of energies in the range of 100 - 250 keV.

![Diagram of positron-electron annihilation](image)

**Figure 5.1** Positron - electron, annihilation results in two co-incident gamma rays with energies of 511 keV. This radiation can be detected by scintillation cameras in PET imaging.

Energies below 100 keV produce too much scatter, while gamma energies above 250 keV are difficult to collimate so outside this range the image will be of reduced quality. Tc-99m, an ideal SPECT radioisotope has found various uses in imaging of humans for clinical purposes as well as small animal drug discovery. Tc-99m has
been incorporated into effective bone imaging agents as they are deposited in the bones, and bone imaging can provide data which X-ray scanning is incapable of. Tc-99m methylene diphosphonate (MDP) accumulates in areas of high metabolic activity and shows up as a bright spot, correspondingly areas of low metabolic activity show up as a dark spot and this visualisation technique can help diagnose tumours (high metabolic activity) and other associated bone related illnesses. This same principle has enabled SPECT (and PET) to be used for targeting all sorts of functions and sites of infection in clinical and in vivo experimentation with animals using the appropriate imaging equipment (Fig 5.2).

![Fig 5.2 A microSPECT camera with movable gantry and operator console. MicroSPECT refers to systems specifically designed for small animal experimentation and imaging (Gamma Medica).](image)

The SPECT camera’s basic function is to detect the emitted radiation and to analyse the data to produce an image of the origin of the emissions. To do this it requires the movement of the aperture around the subject in order to reconstruct an image of the radioactive target. The camera is usually attached to a movable gantry...
for this reason. The camera takes pictures from various angles and stores the images on a computer. To detect the emissions, the camera consists of a large flat crystal of sodium iodide with thallium doping in a light-sealed housing, as shown in Fig 5.3. The crystal scintillates in response to the incident gamma rays, in a process similar to the photoelectron effect. The gamma ray is absorbed by the crystal and in response an electron is released. The interaction of the electron with the crystal lattice causes a flash of light to be emitted. The fluorescence given off by the crystal is detected by a photomultiplier tube which quantitates the amount of light received. The data from the photomultiplier is processed by the computer to create a two-dimensional image of the spatial distribution of the source of emissions, i.e. the radiotracer in the organ or subject of imaging. The crystal detectors used in gamma cameras require collimation and this is typically done with lead shielding of approximately 2.5 - 7.5 cm thick with thousands of tiny holes. This ensures that any radiation reaching the crystal detector is incident in nature, i.e. exactly from where the camera is pointed. The collimator itself can be a source of blurriness as the perforated lead shielding does not perfectly attenuate the incident photons as there can be some degree of cross-talk between the holes.

Integrin binding radiotracers have been successfully used in imaging small animals implanted with human M21 melanoma cells. The M21 cell line is $\alpha_v\beta_3$ positive and Haubner and co-workers have demonstrated that the positron emitting fluorine-18 labelled RGD containing glycopeptide cyclo(-Arg-Gly-Asp-D-Phe-Lys-(sugar amino acid)-) can image a tumour based on this cell line xenotransplanted in a mouse.\(^1\) Fig 5.4 shows the transaxial PET images of the nude mice bearing the human melanoma xenografts. For comparative purposes, an M21-L $\alpha_v\beta_3$ negative tumour is shown as a control.
Figure 5.3  Schematic of how scintillation detection works. The source (organ, tumour etc) emits gamma rays from the radiotracer present, some of which reach the scintillation crystal via the collimator. The gamma ray excites the crystal, emitting a photon of light, which is detected by the photomultiplier tube array and is converted to an electrical signal. The signal is fed into a computer for position and energy analysis leading to a ‘snapshot’ of the source of emissions.

Figure 5.4  Transaxial PET image of a nude mouse bearing an M21 cell based tumour injected with $[^{18}\text{F} ]$ Galacto-RGD. Figure adapted and reproduced from Haubner et al. (169)
5.2 EXPERIMENTAL

5.2.1 General

Animal ethics was approved and granted by ANSTO’s Animal Care and Ethics Committee (ACEC), protocol 183, approval date 4/7/05 and effective from 1/1/06 to 31/12/06. All radiolabelled molecules used in this chapter were synthesised according to their respective procedures as outlined in Chapter 3. The usual precautions were taken dealing with the radioactive materials, also outlined in Chapter 3. Mice and rats were purchased from The Animal Resource Centre, Western Australia. Animals were cared for in a suitable clean sterile environment with free access to water and Gordons Stock Feed Rodent Breeder Diet in pellet form (Gordons Specialty Stock Feed, Yanderra, NSW, Australia). A375 human melanoma cell lines were purchased from the American Type Culture Collection (ATCC), batch number F-12293, dated 10/01/94. 13762 MAT BIII rat mammary adenocarcinoma cells lines were a gift from the Australian National University. Both cell media contained RPMI-1640 (88%), fetal bovine serum (FBS) (10%), L-glutamine (1% - 200 mM), penicillin/streptomycin (1% - 10,000 units, 10 mg streptomycin. The cultures were stored at -80 °C until required, then thawed out and incubated. Radiopharmaceuticals were administered to animals in sterile saline. Anaesthetisation of the animals was achieved using isoflurane administered via a small modified gas mask attached to an ISOTEC 3/ CIG Midget 3 Anaesthetic Apparatus at a gas flowrate of 200 mL/min. Animal imaging was carried out on Gamma Medica X-SPECT dedicated small animal Gamma Imaging instrument equipped with LumaGEM acquisition software version 5.407lum_10. All mouse and rat studies were performed in triplicate.
5.2.2 Mouse Preparation

Immunocompromised female mice (balb/c nude) were kept in sterile containers and fed a diet Gordons Specialty Stock Feed. Their growth was monitored and at 6 - 10 weeks, they were injected with 100 μL of a solution of A375 human melanoma cells in phosphate buffered saline (PBS) (1 × 10^6 cells/100 μL buffer) into the left flank in a subcutaneous fashion, taking extra care not to inject the cells intra-dermally. The growth of the tumour was monitored and recorded, while ensuring the tumour did not break out externally. After between 24 - 28 days the tumour reached a size of approximately 8 × 12 mm (0.2 - 0.3 g). The animals weighed roughly 20 g, and were ready for imaging studies.

5.2.3 Rat Preparation

Female Fischer F344 rats were kept in sterile containers and fed a diet Gordons Specialty Stock Feed. Their growth was monitored and at 15 - 16 weeks, they were injected with 100 μL of a solution of MAT BIII rat mammary adenocarcinoma cells in PBS buffer (1 × 10^6 cells/100 μL) into the left flank in a subcutaneous fashion, exercising the same care as with the mice. The growth of the tumour was monitored and recorded, while ensuring the tumour did not break out externally. After between 11 and 13 days, the tumours reached a size of approximately 17 × 17 mm (0.8-1.1 g.) The animals which weighed around 180 g were ready for imaging studies.

5.2.4 Animal Injection

The animal was taken from its cage and its tail was suspended in warm water to dilate the blood vessels. Tc-99m radiotracer in sterile saline (0.2 mCi/100 μL for mouse or 0.5 mCi/100 μL for rat) was injected into the animal via the tail vein.
5.2.5 Animal imaging

The animal was placed in the X-Spect dedicated small animal imaging camera and its body aligned with the aid of the focus lasers. For the purposes of ventral imaging (looking at the anterior) the animal was placed on its back with the tumour positioned as far to the right as possible. Manipulation was required to achieve this. Isoflurane/oxygen anaesthetic mixture was administered to the animal via a plastic muzzle loosely fitted over the animal’s head, to maintain anaesthesia during the imaging. The animal was first imaged at 30 minutes, and subsequently at 3, 6 and 24 h time points. After the required experimentation, the animals while still under anaesthesia were sacrificed by cervical dislocation.

5.3 RESULTS AND DISCUSSION

The imaging studies undertaken in this project involved the injection of Tc-99m radiotracers developed in Chapter 3 into immuno-compromised female mice (balb/c nude) bearing tumours based on the A375 human melanoma cells.\(^\text{(170)}\) The fact that the mice had defective immune systems allows the foreign tumour tissue to grow in the body without being rejected or attacked. This cell line was chosen as $\alpha_v\beta_3$ integrins is implicated in melanoma progression. The M21 human melanoma cell line\(^\text{(171,172)}\) was the first choice as it has been shown to express the $\alpha_v\beta_3$, and has successfully been imaged with PET and SPECT radiotracers but was unavailable.\(^\text{(169)}\)

The rat studies on the other hand, involved female Fischer F344 rats (immune systems intact) bearing tumours based on the 13762 MAT BIII rat mammary adenocarcinoma cells.\(^\text{(173)}\) The animals were kept in cages in a suitably sterile environment, with free access to water and pellet type food. Suspension of the
animal’s tails in warm water aided the injection of the radiotracer. The tail vein was a convenient place to inject the animals as it is distant enough from the rest of the organs to not interfere with the imaging if there is any injection spillage, which is always a possibility.

When analysing the \textit{in vivo} imaging results of both the mouse and rat experiments, it should be kept in mind that the visualised radioactivity gives no information on the state of the radiotracer \textit{in vivo}. There is always a possibility that the molecule may have been metabolised to some extent. Whether the Tc-99m ion has dissociated from the bioconjugate, or possibly the Tc-99m bifunctional chelator complex has been cleaved from the bioconjugate is not known. The BFCs, MAMA, and HYNIC were chosen as they have been shown to be relatively stable in physiological systems,\(^{(174)}\) and this coupled with the fact that the peptidemimetics are less likely to be broken down in such systems, should have produced radio-tracers that resist degradation \textit{in vivo}. Unfortunately there is no data to suggest that they are intact without the aid of radiotracer metabolite studies, and unfortunately these scheduled studies could not be fit in. Drug research and development usually employ these metabolite studies only after a radiotracer has shown promise \textit{in vivo}. Whether the molecule was intact or not, accumulation of the radioisotope by the tumour is the important factor.

The mouse studies in this project were not very conclusive in terms of accumulation of the radioactivity in the tumours. Obviously the fact that mice are significantly smaller than rats (20 g versus 180 g) played a part in the diffuse images (Fig 5.5 and 5.6), but it did certainly appear that there was no significant affinity for the Tc-99m radiotracers for the implanted human A375 melanoma cells. It was also quite difficult to recognise the different organs in the compact bodies of the mice. A
great deal of the measured radioactivity was passed through the animal’s gastrointestinal tract and kidneys, and accumulated in the bladder. This data suggests that either the radio-tracer is too polar if in fact it is still intact, or the radio-tracer has been broken down into polar Tc-99m containing metabolites. For the tricine and EDDA HYNIC Tc-99m complexes [91, 92] (Fig 5.5), a great deal of the measured scintillation is already found in the bladder at the 30 - 50 min time frames. At the 3 h time point, there is noticeable accumulation of activity in the stomach and gastrointestinal tract, although the tricine complex [91] seems to be more localised to the stomach. At the 6 h time point there is no great discernible difference from the 3 h time points, still with more scintillation recorded for the stomach in the tricine complex.
Fig 5.5  SPECT images of mouse injections at various time points with Tc-99m Hynic Integ Tricine [91] and Tc-99m Hynic Integ EDDA [92] radiotracers. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot.
Fig 5.6 SPECT images of mouse injections at various time points with Tc-99m MAMA Integ [87] and Tc-99m MAMA cap Integ [88] radiotracers. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot.
At $t = 24\ h$ the residual activity has diffused throughout the animal’s digestive system. There is certainly no discernible activity in the area where the tumour is located on the animal’s hindquarter. For the Tc-9m MAMA Integ [87] and Tc-99m MAMA caproic integ [88] radiotracers (Fig 5.6) the results were no better. These molecules are less polar than the HYNIC complexes so technically they should have persisted in the body longer, if metabolic breakdown is not an issue. Like the HYNIC radiotracers, the MAMA complexes displayed the rapid excretion of the activity through the gastrointestinal tract, kidneys and bladder. Only the 24 h time point showed any significant difference between the radiotracers, with more of the Tc-99m MAMA integ radiotracer [87] still persisting in the gastrointestinal tract. The corresponding Tc-99m MAMA caproic integ radiotracer shows most of the residual radioactivity concentrated in the bladder. Comparisons of the HYNIC and MAMA complexes, although disappointing, did show differences between the two. The HYNIC molecules seemed to better cross the bloodstream into the tissue, whereas the MAMA derivatives were mainly confined to the gastrointestinal tract. It should be reiterated that the A375 is not renowned for its $\alpha_\text{v}\beta_3$ expression as the unavailable ML21 is, but also it should be noted that the molecules tested could be binding to the $\alpha_\text{IIb}\beta_3$ integrin expressed in the platelets, and thus acting as a blood marker. As previously mentioned (Sect 1.8 - 1.10), the binding site is very similar between these two integrins so it is highly possible that the molecules being tested are behaving this way. The lower body clearance particularly exhibited with the HYNIC radiotracers [91] and [92], suggests that they might be displaying this activity. An in vitro binding assay using the $\alpha_\text{IIb}\beta_3$ would provide valuable information into this phenomenon.

The studies carried out in using Fisher rats implanted with rat mammary adenocarcinoma cells were a great deal more informative than the mouse studies.
The significantly larger animals were much more easily manipulated for, and imaged by the SPECT gamma camera. Imaging of the rats was performed by focussing on the lower half of the animal where the tumour location was more discernable from the other organs. A larger dose of radiotracer was required for the rats (0.5 mCi) due to the larger size but the imaging time points were the same, despite the mouse’s higher metabolic rate.
**Fig 5.7** SPECT images of rat injections at various time points with Tc-99m MAMA Integ radiotracer [87]. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot. The rat was rotated to show the tumour at the right most of the image.
Fig 5.8 SPECT images of rat injections at various time points with Tc-99m MAMA Cap Integ radiotracer [88]. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot. The rat was rotated to show the tumour at the right most side of the image.
Fig 5.9 SPECT images of rat injections at various time points with Tc-99m HYNIC Integ Tricine radiotracer [91]. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot. The rat was rotated to show the tumour at the right most of the image.
Fig 5.10  SPECT images of rat injections at various time points with Tc-99m HYNIC Integ EDDA radiotracer [92]. Tumour position is shown on the mouse’s thigh, and scintillation intensity is shown as a colour contour plot. The rat was rotated to show the tumour at the right most of the image.
Quantitative comparisons of the accumulated radioactivity were achieved with the rat studies thanks to the larger and better defined organs and tumour. The raw scintillation data of the tumour to non-tumour ratios is shown in Table 5.1. The processed data in the form of increased percentages of uptake in tumour versus non-tumour tissue is summarised in Table 5.2. Tumour and non-tumour activity was calculated from colour intensity in the imaging software at the designated target areas. Non-tumour tissue data is gathered from the opposite flank of the animal, but other areas were measured when it was difficult to get an accurate reading of the animal’s non-tumored flank. In these cases, lung tissue and skin tissue under the tumour was measured, as noted in Table 5.1.

For the Tc-99m MAMA integ [87] and Tc-99m MAMA caproic integ radiotracers [88] (Figs 5.7 and 5.8), it was shown that these molecules tended to stay more in the gastrointestinal tract in comparison with both HYNIC analogues (Figs 5.9 and 5.10). This correlates with the results seen in the mouse studies. The incorporation of the caproic acid linker in the Tc-99m MAMA caproic integ radiotracer was hoped to reduce the steric interference the BFC moiety would have with the rest of the molecule or indeed the receptor’s recognition site. Upon inspection of the images, it did appear that the caproic linked variant had better resolved the tumour at the 24 h time point but this was not reflected in the measured uptake ratios. At the 24 h time points the averaged increased (percentage) uptake in tumour versus non-tumour tissue was 60.3% for the MAMA molecule and 55.7% for the MAMA caproic linked variant, as shown in Table 5.2.
Table 5.1 Raw Scintillation Data of Tumour to Non-Tumour Ratios of SPECT Imaging in Rats.

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>Animal (ID)</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMA [87] 33</td>
<td></td>
<td>127.68 = 1.87:1</td>
<td>164:184 = 0.89:1</td>
<td>131:90 = 1.46:1</td>
<td>42:22 = 1.91:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164:70 = 0.96:1</td>
<td>131:76 = 1.72:1</td>
<td>42:11 = 3.82:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>73:67 = 1.09:1</td>
<td>116:113 = 1.03:1</td>
<td>64:51 = 1.26:1</td>
<td>7:5 = 1.4:1</td>
</tr>
<tr>
<td></td>
<td>9.05</td>
<td>125:98 = 1.28:1</td>
<td>36:18 = 2:1</td>
<td>52:13 = 4:1</td>
<td>12:8 = 1.5:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125:63 = 1.98:1</td>
<td>36:7 = 5.14:1</td>
<td></td>
<td>12:3 = 4:1</td>
</tr>
<tr>
<td>MAMA cap [88] 2.05</td>
<td></td>
<td>110:79 + 1.39:1</td>
<td>4589:105 = 43.7:1</td>
<td>58:44 = 1.32:1</td>
<td>39:26 = 1.5:1</td>
</tr>
<tr>
<td></td>
<td>3.05</td>
<td>144:79 = 1.82:1</td>
<td>78:49 = 1.59:1</td>
<td>50:46 = 1.09:1</td>
<td>26:12 = 2.17:1</td>
</tr>
<tr>
<td></td>
<td>13.05</td>
<td>41:30 = 1.37:1</td>
<td>43:30 = 1.43:1</td>
<td>51:35 = 1.46:1</td>
<td>7:7 = 1:1</td>
</tr>
<tr>
<td>HYNIC EDDA [92] 4.05</td>
<td></td>
<td>152:144 = 1.06:1</td>
<td>243:168 = 1.45:1</td>
<td>59:57 = 1.04:1</td>
<td>76:55 = 1.38:1</td>
</tr>
<tr>
<td></td>
<td>5.05</td>
<td>208:134 = 1.55:1</td>
<td>292:122 = 2.39:1</td>
<td>192:125 = 1.54:1</td>
<td>74:35 = 2.11:1</td>
</tr>
<tr>
<td>HYNIC Tricine [91] 6.05</td>
<td></td>
<td>186:134 = 1.39:1</td>
<td>183:120 = 1.53:1</td>
<td>183:112 = 1.63:1</td>
<td>41:25 = 1.64:1</td>
</tr>
<tr>
<td></td>
<td>7.05</td>
<td>178:147 = 1.21:1</td>
<td>178:92 = 1.93:1</td>
<td>122:86 = 1.12:1</td>
<td>27:21 = 1.29:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>177:128 = 1.38:1</td>
<td></td>
<td>122:76 = 1.61:1</td>
<td>27:19 = 1.42:1</td>
</tr>
<tr>
<td></td>
<td>10.05</td>
<td>292:233 = 1.25:1</td>
<td>379:370 = 1.02:1</td>
<td>226:141 = 1.60:1</td>
<td>109:50 = 2.18:1</td>
</tr>
</tbody>
</table>

Note: red = tumour to lung, green = tumour to skin under tumour used in place of equivalent tissue (black). Ratios given in bold.
Table 5.2  Increase (Percentage) uptake in Tumour versus non-tumour tissue in RAT SPECT Imaging Analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rat (Image File)</th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMA [87]</td>
<td>33 87</td>
<td>-11</td>
<td>46</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34 9</td>
<td>3</td>
<td>26</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.05 28</td>
<td>2</td>
<td>300*</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>41.3</td>
<td>-2</td>
<td>36</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>Std. dev.</td>
<td>40.7</td>
<td>7.8</td>
<td>14.1</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>MAMAcap [88]</td>
<td>2.05 39</td>
<td>427*</td>
<td>32</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.05 82</td>
<td>59</td>
<td>9</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.05 37</td>
<td>43</td>
<td>46</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>52.7</td>
<td>51</td>
<td>29</td>
<td>55.7</td>
<td></td>
</tr>
<tr>
<td>Std. dev.</td>
<td>25.4</td>
<td>11.3</td>
<td>18.7</td>
<td>58.7</td>
<td></td>
</tr>
<tr>
<td>Hynic EDDA [92]</td>
<td>4.05 6</td>
<td>45</td>
<td>4</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.05 55</td>
<td>139</td>
<td>54</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.05 8</td>
<td>23</td>
<td>122</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>23</td>
<td>69</td>
<td>60</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Std. dev.</td>
<td>27.7</td>
<td>61.6</td>
<td>59.2</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>Hynic-Tricine  [91]</td>
<td>6.05 39</td>
<td>53</td>
<td>55</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.05 25</td>
<td>38</td>
<td>12</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.05 25</td>
<td>2</td>
<td>60</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>29.7</td>
<td>31</td>
<td>42.3</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>Std. dev.</td>
<td>8.1</td>
<td>26.2</td>
<td>26.4</td>
<td>44.8</td>
<td></td>
</tr>
</tbody>
</table>

* = average and SD calculated without this figure

Certainly in the context of this study and the population (n = 3) this small percentage variation can show no significant difference in the MAMA analogues.

In terms of getting radioactivity to the tumour, the HYNIC molecules were the best in the rat studies. There is not a great deal of difference between the tricine [91] and the EDDA analogues [92] although the EDDA variant appears to have resolved
Figure 5.11 The percentage increase in Tc-99m uptake in tumours compared to non-tumorous tissue.

the tumour slightly better as can be seen after comparing Figure 5.9 and 5.10. The tumour is well defined in the HYNIC studies particularly the 3, 6 and 24 h time points for the tricine complex and all of the EDDA complex time points. Cursory inspection of the images also shows the better diffusion of the radioactivity to the non-digestive tract related tissue, i.e. musculature, skin etc. The average increase in uptake in tumorous tissue was higher in the EDDA complex for the 3, 6 and 24 h time points (see Table 5.2 and Fig 5.11 for graphical representation.) After 1 h, the tricine complex was marginally better with a 30% increase versus the EDDA complexes’ 23%. After 3 h the EDDA complex has an averaged percentage increase at 69% compared to the 31% increase for the tricine complex. The 6 and 24 h time points show the same trend with EDDA complex ahead showing an 80% increase at the 24 h
time point. Due to the small population \((n = 3)\) and indeed the significant deviation in the results, there is no proof that the EDDA containing complex is better than the tricine complex in tumour uptake, although this study does suggest that it is slightly better. In terms of clearance of activity from the body, there appears to be a significant difference between the HYNIC molecules and the MAMA analogues. Paralleling the mouse studies, it appears that the radioactivity is persisting in the non GI tract. This slower clearance could be indicative of \(\alpha_{\text{IIb} \beta_3}\) blood binding. Of course the HYNIC molecules were not tested \textit{in vitro} against the purified human \(\alpha_\text{v} \beta_3\) receptor assay in Chapter 4, as testing rhenium HYNIC complexes were not feasible, so whether their very limited uptake is indicative of integrin binding is unknown. In fact, this is true for all of the radiotracers evaluated in the mouse and rat studies. Certainly the limited binding affinity of the MAMA and MAMA caproic integrin molecules shown in Chapter 4 does suggest that the \textit{in vivo} binding would have been low, but whether it was for that reason is unknown. There is certainly a body of evidence to suggest that a molecule with good \textit{in vitro} affinity for a target receptor or substrate, will not necessarily translate to a good \textit{in vivo} binder, and this can be for a whole variety of reasons including metabolic breakdown, polarity related issues, and size constraints. Certainly, these results would be more conclusive if the tumour uptake was significant and comparable to the \textit{in vitro} results in Chapter 4. Another important factor to consider is the fact that the tumours in both the mouse and rat studies are not renowned for their expression of the \(\alpha_\text{v} \beta_3\) receptor in quantities high enough for adequate imaging. The M21 cell line is \(\alpha_\text{v} \beta_3\) positive and would have been a much better option for the immuno-compromised mouse studies but unfortunately this culture was not available for this study.
In summary, this Chapter described the \textit{in vivo} evaluation of the Tc-99m radio-complexes in tumored rats and immuno-deficient mice by imaging on a SPECT scanner at various time points. The results indicated that the tested molecules had levels of tumour uptake too low for their detection and successful imaging, although discrimination was observed between the molecules.
CHAPTER 6

CONCLUSIONS AND FUTURE RECOMMENDATIONS
6 CONCLUSIONS AND FUTURE RECOMMENDATIONS

The aims of this research project were to create molecules that avidly bind to \( \alpha_v\beta_3 \) by conjugated and integrated design methodologies, and assess their pharmaceutical potential via \textit{in vitro} binding assays against the purified receptor and \textit{in vivo} imaging experiments of the radiotracers in tumored animals. The conjugated method involved the attachment of a variety of bifunctional chelators to a derivative of a molecule with good binding properties to the integrin. The chelators were specific for the intended metal/radio metal that was to be coordinated. For rhenium and technetium, monoamine monoamide dithiol moieties were used as well as the highly popular HYNIC chelator. The macrocyclic TRITA chelator was chosen for its suitability to the analogous copper, Cu\textsuperscript{64}, Lu and Lu\textsuperscript{177} chelations. The intended use of these macrocycles was to provide a stable point of attachment so as to minimise any demetallation in the buffered \textit{in vitro} assays and in the physiological conditions in the rat and mouse bodies.

The rhenium complexes synthesised for the integrated approach, were a high risk route to receptor binding targets. These molecules adhered to a very basic set of criteria in the hope that they would display some affinity and selectivity for the \( \alpha_v\beta_3 \) integrin receptor. These criteria were the length between the Arg and Asp mimicking moieties as well as the employment of suitable peripheral groups around the Asp substitute, specifically the phenylsulfonyl- and benzylxycarbonylamino- groups attached to the terminal amine. In terms of synthesis, the integrated approach was quite difficult and was complicated further by the racemisation that occurred in the sequence of peptide coupling reactions to construct the molecules. Chiral separation
was not pursued, so the racemic mixtures were complexed to the rhenium yielding a complex mixture of diastereoisomers coupled to potentially syn and anti oxorhenium centres. Complete separation and characterisation were beyond the scope of this project.

Radiolabelling of the conjugated approach molecules were achieved with Tc-99m, in the case of the MAMA, MAMA-caproic and HYNIC antagonists. The HYNIC molecule required co-ligands to fulfil the coordination requirements of the Tc-99m core so EDDA and tricine were successfully incorporated into the complexes. To demonstrate the potential for PET imaging applications, Cu\textsuperscript{64} was coordinated to the integrin via the tetraazatetraacetic acid TRITA BFC. Lu\textsuperscript{177} was successfully separated from bulk reactor irradiated ytterbium oxide and incorporated in the metal complex via the TRITA BFC as it has shown great potential as a new isotope for simultaneous therapeutic/diagnostic applications.

Of the molecules assayed in the in vitro testing the Cu-TRITA molecule \cite{46} showed the best inhibition of the I\textsuperscript{125} standard \cite{86} with an IC\textsubscript{50} value of 110 ± 16 nM, substantially better than the Lu equivalent \cite{87}, suggesting either a steric interference with the bulkier Lu coordination sphere with the RGD binding site on the receptor or a charge dependency on binding. The incorporation of a caproic acid linker in the Tc-99m MAMA integrin molecules improved the IC\textsubscript{50} over the non-linker analogue, demonstrating the importance of spacers between the BFC and the targeting moiety to radiotracer effectiveness. These successful results have shown that there is great potential in the conjugated approach method and much can be learnt and built upon with future studies focussing on this aspect of radiotracer development.
To complement these results, future work would invariably include an *in vitro* assay of the αIIbβ3 integrin receptor, to test the radiotracer’s selectivity as well as potential for thrombus imaging.

The *in vivo* imaging experiments performed with the immuno-deficient mice were unsuccessful in terms of localised or specific binding to the tumour. The lack of any distinct affinity of the tracers tested coupled with the very compact bodies of the mice made visual discrimination of the tumours impossible.

The results from the rat studies were more informative. Increased uptake of the Tc-99m was observed in all of the complexes tested despite being in the range of 1-1.8 times higher than the non-tumour tissue. Visualisation of the tumours in the SPECT images of the HYNIC molecules was certainly better than for the MAMA molecules, with the EDDA co-ligated species [92] slightly better than the tricine variant [91]. The results showed that none of the molecules could selectively image the tumour *in vivo*. The HYNIC molecules did exhibit a slower clearance from the mouse and rat’s bodies and the αIIbβ3 assay could provide some insight into the reason for this.

In light of the results obtained in this project, certain conclusions can be made regarding the different design approaches to αvβ3 integrin targeting pharmaceuticals. The conjugate method utilising moieties of known affinities and selectivities are a more promising starting point, as the BFCs required for the incorporation of metallic radioisotopes will almost certainly affect the ability of the ligand to dock with the receptor's active site. Of course this depends on the position and proximity of the coordination moiety. As the incorporation of the caproic acid linker did not improve the binding significantly, future efforts could be focussed on the creation of even longer linkers between the RGD active portion and the BFC. This would certainly
provide information on the degree to which it inhibits binding to the receptor. Another possible focus of future work would be to attach the BFC to other parts of the molecule. Attachment of all BFCs were via the terminal amine of the aspartic acid mimic in this project so it would be of great value to assess the *in vitro* binding of molecules with BFCs attached at the Arg mimicking terminal on perhaps from the centre of the molecule (Figure 6.1).

![Figure 6.1](image)

*Figure 6.1* Attachment of the BFC to different parts of the antagonist molecule is worthy of investigation, as well as modification of linker length to reduce steric interactions with the parent molecule and the receptor's binding site.

In regards to the integrated approach rhenium complexes [23, 24], a significant amount of tuning would be required before these molecules showed promise as *in vivo* imaging agents. The fact that the phenylsulfonyl complexes [23] showed a slight inhibition in comparison to the benzyloxycarbonylamino derivatives' [24] total inactivity does demonstrate that this approach has some merit. A more comprehensive study involving molecular modelling of the metal complexes and active comparisons with tried and tested molecules with established αvβ3 integrin
affinities and selectivities, would be required to improve the chance of constructing complexes with suitable in vitro and in vivo characteristics. This approach is important as small molecule probes are being sought for their straightforward and efficient production routes in comparison with monoclonal antibodies and other larger protein/peptide based structures.

As mentioned in Chapter 5, the M21 cell line\textsuperscript{(171,172)} (known to express the $\alpha_v\beta_3$ integrin) was not available for these studies. Human mammary adenocarcinoma cells were used, and in theory, should express the integrin, however, the extent is not known. The results obtained may have been more conclusive if M21 cell based tumours were grown. It would be of great merit to obtain this cell line to see if the radiotracers displayed superior imaging results in the nude mice studies.
REFERENCES
REFERENCES


(9) Hicks, R., *Understanding Cancer*, University of Queensland Press: St. Lucia, Queensland Australia, 1980.


(24) Kleiman, M. D., The American Journal of Cardiology, 1997, 80, 29B-33B.


References


References


References


APPENDIX
Chapter 4

Manual simulation of the IC_{50} calculation from the recorded scintillation data as processed by Kell 6 program suite by Biosoft® created for radioligand binding analysis. From the plotted Specific binding curve (Fig 4.3), the IC_{50} value is measured from the intercept for the 50% of the specific binding (y axis) to the corresponding logarithm of the drug concentration (x axis).

\[
\text{Intercept x value} = \log -6.945 \text{ (concentration)}
\]

Therefore \( x = 113.5 \text{ nM} \) (which approximates to the value that the software obtained (110 ± 16 nM) with its stated weighted non-linear curve fitting routines.)
Chapter 5

Attached Animal Ethics approval (1st page)

<table>
<thead>
<tr>
<th>ANSTO Animal Research Authority AF-2075</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dates this authority remains in force:</td>
</tr>
<tr>
<td>1 Jan 2006 To 31 Dec 2006</td>
</tr>
<tr>
<td>ACEC Title of Protocol</td>
</tr>
<tr>
<td>Evaluation of radionuclide imaging for in vivo imaging of tumour bearing rats and mice using radioisotope analogues of GSAO</td>
</tr>
<tr>
<td>ACEC Protocol No</td>
</tr>
<tr>
<td>183</td>
</tr>
<tr>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Filomena Mattner</td>
</tr>
<tr>
<td>Species/Strain</td>
</tr>
<tr>
<td>C57/Bl6J Mice</td>
</tr>
<tr>
<td>Nude Mice</td>
</tr>
<tr>
<td>F344 Rats</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>75</td>
</tr>
<tr>
<td>08</td>
</tr>
<tr>
<td>Purpose</td>
</tr>
<tr>
<td>Diagnostic Procedures (5)</td>
</tr>
<tr>
<td>Procedure</td>
</tr>
<tr>
<td>Minor Conscious Intervention (3)</td>
</tr>
<tr>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Filomena Mattner</td>
</tr>
<tr>
<td>All persons to whom the authority is issued</td>
</tr>
<tr>
<td>Filomena Mattner, Vu Nguyen, Patrice Ballantine, Paula Bergthof, Kerinne Belbin, Janet Chapman, Emma Millard (in training)</td>
</tr>
</tbody>
</table>

Designated land in or on which the business of animal research is to be carried on

ANSTO, Building 19 Lab y25, Building 21C

The kind of animal research which the authority authorises the persons to carry out

The aim of this project is to evaluate the uptake in tumours of novel GSAO radiolabelled tracers as potential imaging agents. This investigation will include SPECT imaging of the radioisotope analogues in the tumours. From these imaging studies the radiotracers with good uptake in the tumour and good clearance from organs will then be extensively studied in the relevant animal model.

The Animal Ethics Committee (AEC) which has recommended that the authority be issued and under whose supervision the individuals are authorised to carry out animal research

ANSTO Animal Care and Ethics Committee (ACEC)

Name and Number of any Previous related Authorities

ARA 2004/183 and 2005/183 (2)

This authority to conduct the research as outlined above and as the approved ACEC protocol is subject to the conditions below. This authority may be suspended or cancelled at any time or may be surrendered by the Principal Investigator.

1.1 Conditions of Approval Particular to this Authority

- No special conditions.
Attached Animal Ethics approval (2nd page)