Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.
Targeting DNA: Synthesis, Biophysical and Biochemical Studies of New Aminoalkyl Derivatives of Diphenyl Guanidines and Acridine

By

Caitriona McKeever B. A. (Mod.)

A thesis presented to the University of Dublin for the degree of Doctor of Philosophy

Under the supervision of Prof. Isabel Rozas

School of Chemistry
Trinity College Dublin  August 2012
Declaration

This work comprises a doctoral thesis submitted for the consideration of Trinity College Dublin.

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with due acknowledgement and reference given to the work of others, where appropriate.

I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.
Acknowledgements

I would like to express my immense gratitude to all the people who helped and inspired me throughout my Ph.D. This thesis would not have been possible without you.

First and foremost, I offer my sincerest thanks to my supervisor Prof. Isabel Rozas for her guidance and terrific support, expertise and advice during my research. Thank you for always believing in me. One simply could not wish for a better or friendlier supervisor.

I would like to express gratitude to Prof. Reto Brun and Marcel Kaiser from the Swiss Tropical and Public Health Institute (Swiss TPH) who were responsible for carrying out the cytotoxicity assays in this thesis.

A special thanks goes to all past and present members of the Rozas group. Thank you all for always being there for me when I needed you. I am deeply grateful to the postdoctoral fellow Dr. Alessandra Cordeiro Machado for her invaluable assistance in the laboratory and for her optimistic nature. To Dr. Padraic Nagle, thank you for all your help, advice and friendship both inside and outside of the laboratory. Thanks also go to Dr. Fernando Blanco for his encouragement and friendship. Thanks to Dr. Daniel H. O’ Donovan, Dr. Amila Kahanavić and Dr. Aoife Flood, for all your help, support, advice, encouragement in the laboratory and for all the fun times we had together. Thanks for always being such great friends. Thanks to Brendan, Elena, Julian and Paddy for all the laughs, great nights out, chats and coffees and for making every day in the laboratory great craic. I would not have been able to do this without you all. I wish you all the best in the rest of your PhDs.

I would like to thank the head of school, Prof. David Grayson for his help with administrative matters and constant support. Thanks to Dr. John O’Brien and Dr. Manuel Ruether for running my NMRs and for answering all my questions. Thanks to Manuel for all the help with the various UV, CD and LD machines. Thanks also go to Dr. Martin Feeney, Dr. J. Bernard Jean-Denis and Dr. Dilip Rai for running the mass spectra. I would like to thank all of the technicians in the Chemistry department for their help while at Trinity College.
I would also like to thank Prof. Thorri Gunnlaugsson and all the members of his group for their help and advice in the laboratory. In particular, I would like to thank Swagata and Samantha for their invaluable assistance and great chats.

I would like to show my appreciation to all of my friends outside of the laboratory - especially Moira, Sarah, Aoife, Kim, Claire and James. To Moira and Sarah whom I known for years and have always been there for me. To Aoife thanks for being a truly great friend and for making the years at college a great experience. To James, Kim and Claire, my fellow classmates, thank you for your constant support and encouragement. Thanks for all the great nights out and for helping to take my mind off chemistry.

To Darragh thanks you for always being there for me. Thanks for putting up with me and for being good to me when times were hard and stressful. Thank you for all your support, encouragement, help and love.

I would like to thank all of my family who supported me throughout my Ph.D.

Thanks to my brother Tommy for all your endless support and love. Thanks for all the encouragement, laughs and for being such a great brother.

Thanks to my parents, Eileen McKeever and Tom McKeon whom I am also forever indebted. To my Dad who unfortunately is no longer with us, thanks for all your encouraging words, love and support throughout my life. You always believed in me and were always so proud of me. I miss you and will always love you.

To my Mum, you are an inspiration to me with all that you have achieved and have been through in your life. Thank you from the bottom of my heart for all your help, support, guidance, advice and love and for always being there for me. I love you more than words can say.

-Caitriona McKeever
I would like to dedicate this work to my parents Tom McKeon and Eileen McKeever
Abstract

Research on the design and synthesis of DNA targeting molecules is widespread. There are a number of diseases which are treated using DNA-targeting agents. DNA ligands have been shown to exhibit anticancer, antiprotozoal, antitrypanosomal, antiviral and antibacterial activity. Many of these drugs display promising results and a number are in clinical use against a variety of protozoal infections (Trypanosomiasis and malaria) and cancers. There are a number of minor groove binding/intercalator/alkylating or dual binding agents that display toxic side effects due to a lack of sequence specificity. Further, optimisation of DNA targeting agents is crucial so as new and improved agents can be discovered.

Compounds that target the minor groove have a clear potential for treating protozoal infections and cancer by disrupting the biochemistry of a cell at fundamental levels by inhibiting the actions of DNA-dependent enzymes or by direct inhibition of transcription. Many types of drugs are recognised for interacting with DNA through intercalation which involves insertion of a flat polyaromatic chromophore between consecutive base pairs of the DNA double-helix. Dual agents that act as intercalator/minor groove binders show an increased affinity and activity for DNA. The aim of this project is to synthesise a new family of minor groove binders, a new family of intercalators and from these a new family of dual (minor groove binding/intercalating) DNA targeting agents. The minor groove binders possess similar structural characteristics required for strong binding; crescent shape, dications and hydrogen bond donating or accepting groups. The synthesis of the aminoalkyl derivatives of guanidine di-aromatic minor groove binders is displayed in Chapter 3. The synthesis of the alkylcarboxylic acid derivatives of acridine which consists of an acridine core linked to an aliphatic carboxylic acid chain is described in Chapter 4. In Chapter 5, the various synthetic routes explored in the attempted synthesis of the alkyl linked minor groove binder-intercalator dual binders are presented.

In Chapter 6 the binding affinity and mode of binding of the molecules is evaluated by a number of biophysical assays. In Chapter 7 the cytotoxic effect of the minor groove binders against Trypanosomiasis, malaria and L6 (skeletal muscle myoblastoma) cell lines is studied.
Abbreviations

A: Adenine
Å: Armstrong
A.a: Amino acid
AcOH: Acetic Acid
Ac$_2$O: Acetic Anhydride
AIDS: Acquired immunodeficiency syndrome
Ar: Argon
atm: Atmospheres
app: apparent
Boc: $\text{tert}$-Butoxycarbonyl
Boc$_2$O: di-$\text{tert}$-butyldicarbonate
BOP: Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate
Bp: Base Pair
br: Broad
C: Cytosine
Cbz: Carboxybenzyl
Cbz-Cl: Benzyl Chloroformate
CD: Circular Dichroism
CDKs: Cyclin-dependent Kinases
CHN: Microanalysis
CLL: Chronic lymphocytic leukemia
CIC: N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and N-cyclohexyl-N'-isopropylcarbodiimide
d: Doublet
DACA: N-[2-(dimethylamino)ethyl]acridine-4-carboxamide
DAPI: 4',6-diamidino-2-phenylindole
DCC: N,N'-Dicyclohexylcarbodiimide
DCM: Dichloromethane
DCU: N,N'-dicyclohexylurea
DDD: Drew-Dickerson-dodecamer
dd: Double doublet
DIC: Diisopropylcarbodiimide
DIEA: N,N-Diisopropylethylamine
DMAP: 4-Dimethylaminopyridine
DMF: N,N-dimethylformamide
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic Acid
DNase: Deoxyribonuclease
DSC: Differential scanning calorimetry
dt: Double triplet
ERK: Extracellular-signal-regulated kinases
EDCI: 3-(ethyliminomethyleneamino)-N,N-dimethyl-propan-1-amine
Et₂O: Diethyl Ether
ES⁺: Electrospray
EtOH: Ethanol
EtOAc: Ethyl Acetate
FPIX: Ferriprotoporphyrin IX
Fmoc-Cl: Fluorenylmethyloxycarbonyl chloride
Fmoc: 9-fluoromethyloxy carbonyl
g: Grams
GS-X: Glutathione S-conjugate export pump
G: Guanine
GPR: General purpose reagent
h: Hours
HATU: 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium
HBTU: O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
HAT: Human African trypanosomiasis
HBs: Hydrogen Bonds
HEPES: ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV: Human immunodeficiency virus
HL-60: Human Caucasian promyelocytic leukemia cells
HOAt: 1-Hydroxy-7-azabenzotriazole
HOBt: Hydroxybenzotriazole
HOMO: Highest occupied molecular orbital
HMPA: Hexamethylphosphoramide  
HPLC: High-pressure Liquid Chromatography  
HRMS: High-Resolution Mass Spectrometry  
Hz: Hertz  
ICD: Induced Circular Dichroism  
IHF: Integration host factor  
ILD: Induced Linear Dichroism  
IPA: Isopropyl Alcohol  
IR: Infrared  
In Vacuo: Under Vacuum  
ITC: Isothermal Titration Calorimetry  
J: Coupling Constants  
LD: Linear Dichroism  
LUMO: Lowest unoccupied molecular orbital  
M: Molar  
m: Multiplet  
MD: Molecular Dynamics  
MeCN: Acetonitrile  
MeOH: Methanol  
MIR: Multiple isomorphous replacement  
mol: Mole  
ml: Millilitre  
mp: Melting point  
NC: Nitracrine  
NETGA: Netropsin-acridine conjugate  
NetAMsa: Netropsin-Amsacrine conjugate  
NHL: Non-Hodgkin’s lymphoma  
NMR: Nuclear Magnetic Resonance  
NPP: New permeability pathways  
NTD: Neglected tropical diseases  
PCNA: Proliferating cell nuclear antigen  
P/D: Phosphate: Drug  
PCP: Pneumocystis jiroveci carinii pneumonia  
P. falciparum: Plasmodium falciparum  
PRC: Phenothiazinium redox cyclers
PDT: Photodynamic therapy
Ph: Phenyl
PyBOP: (Benzotriazol-1-yl oxy) tripyrrolidinophosphonium hexafluorophosphate
q: Quartet
q: Quaternary
rt: Room temperature
RNA: Ribonucleic Acid
RU: Response Units
s: Singlet
st: Salmon testes
SAR: Structure–Activity Relationship
SIR: single isomorphous replacement
SPR: Biosensor-surface Plasmon Resonance
Spp: Species
TBTU: O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumtetrafluoroborate
t: Triplet
T: Thymine
T. b. r: Trypanosoma brucei rhodesiense
TBT: TATA-box binding protein
TEA: Triethylamine
THF: Tetrahydrofuran
TFA: Trifluoroacetic acid
TLC: Thin Layer Chromatography
T_m: Thermal Melt
TMU: Tetramethylurea
TMUCI: Chlorotetramethyluronium chloride
TL: Tegumentary leishmaniasis
Topos: Topoisomerases
TRK: Tropomyosin-receptor-kinase
UV: Ultraviolet
VL: Visceral leishmaniasis
WHO: World Health Organisation
Table of Contents

1. Introduction ........................................................................................................... 2
  1.1. DNA: Structure and Function .............................................................................. 2
  1.1.1. The Structure of DNA ....................................................................................... 2
  1.1.2. DNA Polymorphs: A-, B- and Z-DNA .............................................................. 5
  1.1.3. DNA function .................................................................................................. 9
  1.2. DNA Targeting Agents ...................................................................................... 9
  1.2.1. Alkylating Agents .......................................................................................... 10
  1.2.2. Intercalating Agents ...................................................................................... 11
  1.2.3. Major and Minor Groove Binding Agents ...................................................... 18
  1.2.4. Dual DNA Targeting Agents ........................................................................ 28
  1.3. DNA as a therapeutic target .............................................................................. 33
  1.3.1. Tropical Diseases .......................................................................................... 34
  1.3.2. Cancer ............................................................................................................ 41
    1.3.2.1. Oncogenesis .............................................................................................. 43
    1.3.2.2. The Cell Cycle and Apoptosis ..................................................................... 43
  1.4. Biophysical experiments to evaluate ligand DNA affinity ................................... 44
  1.4.1. DNA thermal denaturation ........................................................................... 45
  1.4.2. Circular and Linear Dichroism (CD and LD) ................................................... 45
  1.4.3. Surface Plasmon Resonance (SPR) ................................................................ 46
  1.5. Previous work that constitutes the basis of this research .................................... 46
  1.6. References ....................................................................................................... 47

2. Objectives ............................................................................................................. 55
  2.1. Objectives .......................................................................................................... 56
  2.2. References ......................................................................................................... 60

  3.1. Introduction ....................................................................................................... 62
3.2. Synthesis of the Aminoalkyl Derivatives of Guanidine Di-aromatic Minor Groove Binders ....................................................................................................................67

3.2.1. Synthesis of the Guanidylating Agent ...........................................................67

3.2.2. Synthesis of the Mono-Guanidine Derivatives ............................................69

3.2.3. Synthesis of the Boc-protected amino acids .............................................73

3.2.4. Synthesis of the Boc-protected aminoalkyl mono-guanidine conjugates ......77

3.2.5. Synthesis of the dihydrochloride salts of the aminoalkyl mono-guanidine conjugates ............................................................................................................87

3.3. Conclusions ........................................................................................................96

3.4. References ..........................................................................................................97

4. Synthesis of Alkylcarboxylic Acid Derivatives of Acridine as Intercalators 100

4.1. Introduction ........................................................................................................101

4.2. Synthesis of Alkylcarboxylic Acid Derivatives of Acridine...............................106

4.2.1. Synthesis of amino acid methyl esters .......................................................106

4.2.2. Synthesis of the methyl (acridine-9-carboxamido)alkylcarboxylates ..........108

4.2.3. Synthesis of (acridine-9-carboxamido)alkylcarboxylic acids .................113

4.3. Conclusions ........................................................................................................116

4.4. References ..........................................................................................................117

5. Attempted Synthesis of Minor Groove Binder-Intercalator Dual DNA Targeting Agents 118

5.1. Introduction ........................................................................................................119

5.2. Attempted synthesis involving orthogonal protection using Cbz-protected amino acids and Boc-protected mono-guanidines ...........................................123

5.2.1. Synthesis of the Cbz-protected amino acids .............................................124

5.2.2. Synthesis of the Boc-protected mono-guanidine Cbz-amino acid conjugates 125

5.2.3. Attempted deprotection of the Boc-protected mono-guanidine Cbz-amino acid conjugates ......................................................................................................126

5.3. Attempted synthesis involving orthogonal protection using Fmoc-protected amino acids and Boc-protected mono-guanidines ..............................................127
5.3.1. Synthesis of the Fmoc-protected amino acids ..................................................128
5.3.2. Synthesis of the Boc-protected mono-guanidine Fmoc-amino acid conjugates .................................................................................................................................129
5.3.3. Deprotection of the Boc-protected mono-guanidine Fmoc-amino acid conjugates .................................................................................................................................129
5.4. Attempted coupling reaction using 9-chloroacridine ..........................................130
5.5. Attempted coupling reaction using acridine-9-carboxylic acid..........................131
5.6. Attempted coupling reactions using methyl (acridine-9-carboxamido)alkyl carboxylate and Boc protected mono-guanidines .......................................................133
5.7. Attempted coupling reactions using ethyl (acridine-9-carboxamido)alkyl carboxylate and Boc protected mono-guanidines .......................................................133
5.8. Attempted coupling reactions using (acridine-9-carboxamido)alkylcarboxylic acids and Boc-protected mono-guanidines ..........................................................136
5.9. Conclusions .........................................................................................................146
5.10. References ..........................................................................................................147


6.1. Introduction ........................................................................................................149
6.2. Evaluation of the protonation state of the compounds prepared .....................150
6.2.1. Determination of pK_a......................................................................................151
6.3. Studying the dependence of increasing the ionic strength on the binding affinity 154
6.4. DNA Thermal Denaturation Assays ................................................................156
6.4.1. DNA thermal denaturation assays of the aminoalkyl di-aromatic mono-guanidine derivatives ..........................................................159
6.4.2. DNA thermal denaturation assays of the alkylcarboxylic acid derivatives of acridine ..........................................................................................................................167
6.5. Circular Dichroism Studies ...............................................................................169
6.5.1. Circular Dichroism studies of the aminoalkyl di-aromatic mono-guanidines 170
6.5.2. Circular Dichroism studies of the alkylcarboxylic acid derivatives of acridine ..........................................................................................................................175
6.6. Electric Flow Linear Dichroism ........................................................................176
6.6.1. Linear Dichroism studies of the aminoalkyl di-aromatic mono-guanidines... 177
6.6.2. Linear Dichroism studies of the alkylcarboxylic acid derivatives of acridine 178
6.7. Ultraviolet intercalator displacement assays ...................................................... 179
6.7.1. UV intercalator studies of the aminoalkyl di-aromatic mono-guanidines .... 180
6.8. Conclusions ........................................................................................................ 182
6.9. References ......................................................................................................... 184

7. Biological Evaluation 186
7.1. Introduction ....................................................................................................... 187
7.2. Trypanosoma brucei rhodesiense STIB 900 cell line ............................................. 190
7.3. Plasmodium falciparum NF54 cell line ................................................................. 190
7.4. The skeletal myoblasts L6 cell line ..................................................................... 191
7.5. Results obtained with the T. b. rhodesiense STIB 900 cell line ...................... 191
7.6. Correlation between Binding Affinity and Cytotoxicity towards Trypanosoma brucei rhodesiense STIB 900 cells ................................................................. 193
7.7. Cytotoxicity Experiments of the Plasmodium falciparum NF54 cell line .......... 195
7.8. Correlation between Binding Affinity and Cytotoxicity towards Plasmodium falciparum NF54 cell line ................................................................. 196
7.9. Correlation between antiparasitic activity of previously synthesised bis-(2-aminoimidazolines) and bis-guanidines and the current aminoalkyl di-aromatic mono-guanidines .......................................................................................................................... 197
7.10. Conclusions ..................................................................................................... 199
7.11. References ....................................................................................................... 200

8. Conclusions and Future Work 202
8.1. Conclusions ....................................................................................................... 203
8.1.1. Synthesis ..................................................................................................... 203
8.1.2. Biophysical Experiments .......................................................................... 206
8.1.3. Biochemistry .............................................................................................. 207
8.2. Future Work ..................................................................................................... 209
8.2.1. Synthesis ..................................................................................................... 209
8.2.2. Biophysical Experiments ................................................................. 212
8.2.3. Biochemistry ..................................................................................... 212
8.3. References ............................................................................................. 214

9. Experimental Section ............................................................................. 216

9.1. Chemistry ............................................................................................... 217
  9.1.1. General procedures for the preparation of compounds ...................... 217
  9.1.2. Preparation and characterisation of compounds .................................. 219

9.2. Biophysical Experiments ...................................................................... 276
  9.2.1. DNA and Buffers ............................................................................... 276
  9.2.2. DNA Binding Assays ........................................................................ 276
  9.2.3. Determination of the pKₐ .................................................................. 276
  9.2.4. UV Spectroscopy: The effect of increasing the ionic strength ............. 277
  9.2.5. Circular Dichroism Spectroscopy ...................................................... 277
  9.2.6. Linear Dichroism Spectroscopy ........................................................ 277
  9.2.7. UV Spectroscopy: Determination of DNA binding affinity ............... 278

9.3. Biochemical Experiments ..................................................................... 278
  9.3.1. In Vitro Activity against T. brucei rhodesiense STIB 900 ...................... 278
  9.3.2. In Vitro Activity against P. falciparum NF54 ..................................... 278
  9.3.3. In Vitro cytotoxicity with L6 cells .................................................... 279

9.4. References ............................................................................................. 279

Appendix i

General procedures for the preparation of compounds .................................. ii
Preparation and characterisation of compounds .............................................. iii
Chapter 1

Introduction
1.1. DNA: Structure and Function

The focus of this project is the preparation and study of molecules that bind to deoxyribonucleic acid (DNA). These types of compounds are very important as many anticancer, antiprotozoal, antiviral and antibacterial treatments include compounds that bind to and/or modify DNA. This binding occurs through various interactions such as π-π stacking, hydrophobic forces, hydrogen bonds (HBs) or ionic interactions.1,2 There are numerous modes of DNA binding; these include alkylating, intercalating and groove binding.

1.1.1. The Structure of DNA

One must understand the structure of DNA in order to appreciate the DNA-ligand interactions that are involved in DNA binding. This nucleic acid contains the genetic information that is responsible for the development and functioning of all known living organisms. The structure of DNA was first described by James Watson and Francis Crick and consists of two helical chains each coiled round the same axis. Each helix consists of repeating units known as nucleotides. Nucleotides are composed of nucleosides which are structures consisting of a base covalently bound to a five-carbon sugar (either ribose in ribonucleic acid -RNA- or 2'-deoxyribose in DNA). When the nucleoside is attached to a phosphate group it forms the structure known as a nucleotide (Figure 1.1).

![Figure 1.1.- Structure of a generic nucleotide](image)

The information in DNA is stored as a code consisting of four chemical bases: adenine (A), guanine (G), cytosine (C) and thymine (T). These four bases can be categorised by their heterocyclic structure; adenine and guanine are purines and cytosine and thymine are pyrimidines. The two chains are connected together by these purine and pyrimidine bases...
and these are joined in pairs by HBs (Figure 1.2). It is the sequence of these four bases along the backbone that encodes the information required for building and maintaining all living organisms.\(^4\)

Figure 1.2.- Structure of the four DNA bases showing hydrogen bonding between them (purine bases: G-guanine and A-adenine; pyrimidine bases: C-cytosine and T-thymine)

The sugar rings in nucleotides possess important structural features. First of all the deoxyribose sugar ring is composed of five atoms and they do not all lie in one plane, since at least one atom must be out of plane. This non-planarity is known as puckering. Puckering can be described as a simple qualitative description of the conformation of the sugar relating to the atoms deviating from ring coplanarity. In ring puckers the C2' atom can be out of plane on the same side as the base (C2' \textit{endo}). The structure of this pucker gives phosphate groups up to \(~7\) Å apart. The ring pucker can also either have the C3' atom out of plane (C3' \textit{endo}). In this conformation the phosphate groups are \(~5.8-6\) Å apart.\(^5,6\)

Figure 1.3.- DNA Sugar Puckers, C2' \textit{Endo} and C3' \textit{Endo}\(^6\)

The bond that links a deoxyribose sugar to a base is known as the glycosidic bond and connects C1'-N9 in the purines and C1'-N1 in pyrimidines. The covalent glycosidic bond
can exist in two conformations: syn and anti. The anti conformation is the most preferable conformation because it is lower in energy; however, an exception to this rule is seen in guanosine-containing nucleotides. These nucleotides have a small preference for the syn conformation due to the favourable electrostatic interactions between the exocyclic N2 amino group of guanine and the 5' phosphate atom. The anti conformation has the N1, C2 face of purines and the C2, N3 face of pyrimidines pointing away from the sugar so that the hydrogen atoms attached to C8 of the purines and C6 of the pyrimidines, lie over the sugar ring. Therefore, the hydrogen bonding groups of the bases are facing away from the sugar. In the syn conformation the opposite occurs, the hydrogen bonding groups are now directed towards the sugars. (Figure 1.4).^5

![Figure 1.4.-Guanine base in the anti conformation (a) and in the syn conformation (b)](Image)

The asymmetric ends of DNA strands are known as the 5' and 3' ends, with the 5' end having a terminal phosphate group and the 3' end a terminal hydroxyl group. The two helices are related by a dyad of bases perpendicular to the fibre axis. As the negative phosphate groups are on the outside, monovalent and divalent cations have easy access to them and the binding of these cations leads to the stabilisation of DNA. The open structure of DNA has a high water content known as the "spine of hydration". At lower water content the bases tilt so that the structure becomes more compact. The chains follow right-handed helices, but owing to the dyad, the sequences of the atoms in the two chains run in opposite directions and are therefore antiparallel with respect to each other (Figure 1.5). The distribution of electrostatic potential along a DNA sequence is also dependent upon the base pair sequence, with a run of A:T base pairs having the greatest negative potential and a G:C run having the greatest positive potential.
1.1.2. DNA Polymorphs: A-, B- and Z-DNA

The right-handed double-helical Watson and Crick model for the B-form of DNA is the best known form of this macromolecule. Several other forms, referred to as polymorphs, exist and it is apparent that the DNA molecule can assume different structures depending on the base sequence and its environment. There are many different polymorphs of DNA, with A-DNA, B-DNA and Z-DNA being the most commonly observed structures (Figure 1.6).8

The different structural features of A-, B- and Z-DNA are displayed in the Table 1.1. The key difference between the forms is that A-DNA and B-DNA are both right-handed, uniform double-helical structures, while Z-DNA is a left-handed double helix with a dinucleotide repeat and the backbone follows a zig-zag path. A-DNA occurs under conditions of low humidity while Z-DNA exists in the alternating sequence poly(dC-
dG)_{poly}(dC-dG) and is believed to be the structure formed in solutions of high salt concentration (>2.5 M NaCl).\textsuperscript{5,8} This stabilises the helix by reducing the electrostatic repulsions between the intrastrand phosphate groups since they are held closer together in Z-DNA than A- and B-DNA.\textsuperscript{5}

Table 1.1.- Differences between A-DNA, B- DNA and Z-DNA\textsuperscript{5,8,10}

<table>
<thead>
<tr>
<th></th>
<th>A-DNA</th>
<th>B-DNA</th>
<th>Z-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conditions</strong></td>
<td>Reduced humidity</td>
<td>High levels of hydration</td>
<td>Each purine is rotated about the glycosidic bond</td>
</tr>
<tr>
<td><strong>Helix Sense</strong></td>
<td>right-handed</td>
<td>right-handed</td>
<td>left-handed</td>
</tr>
<tr>
<td><strong>Repeating</strong></td>
<td>1 base pair (bp)</td>
<td>1 bp</td>
<td>2 bp</td>
</tr>
<tr>
<td><strong>Rotation/ bp</strong></td>
<td>32.7°</td>
<td>35.9°</td>
<td>60°/2</td>
</tr>
<tr>
<td><strong>N (number of base pairs per turn)</strong></td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td><strong>Inclination of bp to axis</strong></td>
<td>+19°</td>
<td>-1.2°</td>
<td>-9°</td>
</tr>
<tr>
<td><strong>Rise/bp along axis</strong></td>
<td>2.3 Å</td>
<td>3.32 Å</td>
<td>3.8 Å</td>
</tr>
<tr>
<td><strong>Z (distance between each base pair)</strong></td>
<td>2.55 Å</td>
<td>3.46 Å</td>
<td>7.43 Å</td>
</tr>
<tr>
<td><strong>Mean propeller twist</strong></td>
<td>+18°</td>
<td>+16°</td>
<td>0°</td>
</tr>
<tr>
<td><strong>Glycosyl angle</strong></td>
<td><em>Anti</em></td>
<td><em>Anti</em></td>
<td>C: <em>anti</em></td>
</tr>
<tr>
<td></td>
<td>G: <em>syn</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Major and Minor Grooves</strong></td>
<td>Major groove: width: 2.12Å depth: 13.0Å Minor groove: width: 11.1Å depth: 2.6Å</td>
<td>Major groove: width: 11.6Å depth: 8.5Å Minor groove: width: 6.0Å depth: 8.2Å</td>
<td>Deep minor groove, no discernible major groove</td>
</tr>
<tr>
<td><strong>Sugar Pucker</strong></td>
<td>C3*Endo</td>
<td>C2*Endo</td>
<td>C2<em>Endo at dC C3</em>Endo at dG</td>
</tr>
<tr>
<td><strong>Helix Diameter</strong></td>
<td>23 Å</td>
<td>19 Å</td>
<td>18 Å</td>
</tr>
</tbody>
</table>
In Table 1.1 it is shown how the sugar pucker is C3'-endo in the A-DNA meaning that the C3' atom is out of plane. In this conformation the sugar phosphate backbone is shorter and therefore produces a double helix in which the base pairs are slightly displaced from the centre of the helix leading to a flatter and wider helix. The C2'-endo conformation is favoured in B-DNA with C2'endo out of plane and on the same side as the base. The sugar pucker can be C2'-endo for the cytosine base or C3'-endo for the guanine base in the Z-DNA. The glycosidic bond is in an anti conformation in A-DNA and B-DNA and can be in syn (guanine) or anti (cytosine) conformation in Z-DNA.\textsuperscript{5,8} The anti conformation is extremely important as it determines the polymorph of DNA that is present.\textsuperscript{5}

In B-DNA there is a nucleotide on each chain every 3.4 Å and an angle of 36° between adjacent nucleotides in the same chain, so that the structure repeats after 10 nucleotides on each chain. The distance of each phosphorus atom from the fibre axis is 10 Å. The sugar-phosphate backbone spirals around the outer surface of DNA and between these backbones are two different sized grooves, designated the major and minor grooves (Figure 1.7). The width of the minor groove in B-DNA differs significantly from that of the major groove but they are almost equal in depth. It is now known that groove width varies with sequence. The major groove consists mostly of G:C base pairs and the minor groove is rich in A:T base pairs. In particular, continuous runs of A:T base pairs tend to have narrow minor grooves. This is probably due to their ability to accommodate the spine of hydration. Alternatively, runs of sequence with a high proportion of G:C base pairs tend to have widened minor groove widths. The grooves form as a consequence of HBs between complementary bases of DNA leading to sugar groups to stick out at 120° angles from each other instead of 180°. They run parallel with the phosphate backbone and the edges of the bases and the sugar-phosphate backbone form the floor and the walls of the grooves respectively.\textsuperscript{1,11}

Figure 1.7.- The structure of the DNA double helix showing the major and minor grooves\textsuperscript{12}
In 1979 the first single crystal structure of more than a complete turn of right-handed B-DNA was analysed. This example of B-DNA was composed of the self-complementary dodecanucleotide d(CGCGAATTTCGCG)\textsubscript{2} known as the Drew-Dickerson-dodecamer (DDD). The crystal structure was obtained using multiple isomorphous replacement (MIR) methods. MIR involves introducing heavy atoms into a DNA or protein structure. Discovery of the crystal structure of the Drew-Dickerson sequence was of great importance due to the fact that it showed an antiparallel right-handed B-DNA double helix which was proof of the accuracy of the Watson-Crick model for the DNA structure. This crystallographic analysis displayed many sequence-dependent structural features including:

- In the 5' AATT region a narrow minor groove of 3.2 Å was discovered, compared to 6.0 Å in average canonical B-DNA. The major groove was extremely wide with a width of 12.7 Å.

- A well-ordered and regular network of water molecules was found within the minor groove, the 'spine of hydration', which bridges adenine N3 and thymine O2 atoms in adjacent base-pairs. These outer water molecule layers are linked by a second layer. It is particularly ordered in the AATT centre, and is disrupted at the CGCG ends, due to the presence of the N2 amino groups on guanine residues. The presence of this well-ordered network of water molecules has been proven by NMR (nuclear magnetic resonance) studies, high resolution crystallographic studies and molecular dynamics simulations studies.

Many A-DNA octamers have been crystallised, such as d(GGGGCCCC)\textsuperscript{5,18} and d(GGGTACCC),\textsuperscript{5,19} showing that the minor groove has a width of 15 Å in d(GGGGCCCC) and 9.7-9.8 Å in d(GGGTACCC).

The first oligonucleotide duplex single-crystal structure to be solved by MIR was actually a left-handed helix. Unlike A- and B-DNA the dG and dC residues of the backbone have very distinct conformations giving rise to a zig-zag (Z-DNA) arrangement of phosphate groups. Crystal structures have been analysed from sequences such as d(CGCGCGCGC)\textsuperscript{20} and d(CGCGCGCGC)\textsuperscript{21} showing the flexibility of Z-DNA. It has been proven that Z-DNA can have A:T base pairs indicating that the left-handed helix remains undamaged when a G:C base pair is replaced with an A:T base pair. Nevertheless, A:T sequences greatly destabilise the Z-form DNA because they are unable to support the groove hydration that is essential to maintain the structure of Z-DNA. This polymorph also prefers
the G:C base pair because of the ability of guanosine nucleotides to adopt the syn glycosidic conformation instead of the anti conformation.\textsuperscript{22,23}

There are many different polymorphs of DNA\textsuperscript{8} but this research focuses on the binding of molecules to B-DNA, therefore the remainder of this report will concentrate on B-DNA.

1.1.3. DNA function

DNA has two main functions:

- Transcription: Information is retrieved from the DNA by ribonucleic acid, RNA, and used to synthesise proteins in the body. Proteins are involved in all body processes and have many roles. e.g. hormones, enzymes, carriers, structural proteins, receptors, regulators etc.
- Replication: DNA is responsible for its own regeneration, i.e., DNA self replicates
- The majority of the human genome (80.4\%) participates in biochemical RNA- and/or chromatin-associated events in different types of cell. Examples include different types of RNA which cover 62\% of the genome and regions involved in histone modifications consist of 56.1\% of the genome.\textsuperscript{23}

Transcription and replication are vital for cell survival and proliferation and for maintaining the correct functioning of all body processes. DNA starts transcribing or replicating only when it receives a signal which is usually given by the binding of a regulatory protein to a particular region of the DNA. Therefore, if the binding specificity and strength of this regulatory protein is mimicked by a small molecule, then DNA functioning can be modified, inhibited or activated. Activation would produce more of the regulatory protein or could lead to DNA replication depending on which site the small molecule targets. Inhibition would stop protein synthesis or replication and could cause cell death. Consequently, a small molecule can act as a drug to cure or control a disease, when activation or inhibition of DNA function is required. Although both activation and inhibition are possible, DNA is generally targeted in an inhibitory mode in order to destroy cells as seen in antitumour or antibacterial treatments.\textsuperscript{11}

1.2. DNA Targeting Agents

There are several types of chemotherapeutic agents that have diverse targets within the cancer cell: antimetabolites, antitumour antibiotics, topoisomerase inhibitors, mitotic
inhibitors and many more. In particular, those chemotherapeutic agents that target different loci on DNA of cancer cells are of interest for the present work. These drugs can be divided into three categories: alkylating agents, intercalators and minor groove binders.

1.2.1. Alkylating Agents

Interestingly, it was due to chemical warfare during World War I that the modern era of cancer chemotherapy began. Sulphur mustards are cytotoxic and vesicant chemical warfare agents with the ability to form large blisters on the exposed skin and in the lungs. They were first used in chemical warfare and, in contrast, it was the most stable derivatives, the nitrogen mustards, that were developed for cancer chemotherapy and the first alkylating agents used medically. Some examples of nitrogen mustards developed as treatments include cyclophosphamide (1) and bendamustine (2) (Figure 1.8). Bendamustine has recently re-emerged for the treatment of indolent non-Hodgkin’s lymphoma (NHL), chronic lymphocytic leukemia (CLL) and multiple myeloma. Alkylating agents such as nitrogen mustards interact non-specifically with DNA by transferring an alkyl group to the heterocyclic bases of DNA and one of the primary sites of alkylation is the N7 on guanine. These agents are highly electrophilic and can react with nucleophiles to form covalent bonds.

![Figure 1.8.- Structures of cyclophosphamide (1) and bendamustine (2)](image)

Another example of an alkylating agent is cisplatin (3) wherein the platinum atom binds covalently to the N7 position of purine bases such as guanine. Drugs with two alkylating groups can react with a guanine on each DNA strand and crosslink the chains (interstrand) or they can link two guanines on the same side chain forming 1,2 or 1,3-intrastrand crosslinks, blocking access to enzymes required for normal functioning. Cisplatin is an intrastrand DNA crosslinker and can be used in the treatment of testicular, ovarian and head and neck cancer. Some other examples of Pt based alkylating agents include carboplatin (4) and oxaliplatin (5) (Figure 1.9). The chloride, cyclobutane-1,1-
dicarboxylate and oxalate ions give good aqueous solubility and greater stability to these Pt complexes and in the process of binding to DNA, they act as leaving groups.\textsuperscript{27}

\[
\begin{array}{c}
\text{H}_3\text{N}^-\text{Pt}^+\text{Cl}^- \\
\text{H}_3\text{N}^-\text{Pt}^+\text{Cl}^- \\
\end{array}
\]

3

\[
\begin{array}{c}
\text{H}_3\text{N}^-\text{Pt}^+\text{O}^-\text{C}_\text{O} \\
\text{H}_3\text{N}^-\text{Pt}^+\text{O}^-\text{C}_\text{O} \\
\end{array}
\]

4

\[
\begin{array}{c}
\text{H}_2\text{N}^-\text{Pt}^+\text{O}^-\text{C}_\text{O} \\
\text{H}_2\text{N}^-\text{Pt}^+\text{O}^-\text{C}_\text{O} \\
\end{array}
\]

5

Figure 1.9.- Chemical structures of three well known alkylating agents: cisplatin (3), carboplatin (4) and oxaliplatin (5)\textsuperscript{26}

Nitrosoureas are another type of alkylating agent that contains nitroso (NO) groups and a urea. Some examples include carmustine (6) and lomustine (7) (Figure 1.10). They are lipophilic and thus can cross the blood-brain barrier easily, making them valuable in the treatment of brain tumours such as glioblastoma multiforme which is the most common and most aggressive malignant primary brain tumour in humans.\textsuperscript{28}

\[
\begin{array}{c}
\text{Cl}^-\text{N}^-\text{N}^-\text{C}_\text{O}^-\text{N}^-\text{N}^-\text{Cl}^- \\
\text{Cl}^-\text{N}^-\text{N}^-\text{C}_\text{O}^-\text{N}^-\text{N}^-\text{Cl}^- \\
\end{array}
\]

6

\[
\begin{array}{c}
\text{Cl}^-\text{N}^-\text{N}^-\text{C}_\text{O}^-\text{N}^-\text{N}^-\text{Cl}^- \\
\text{Cl}^-\text{N}^-\text{N}^-\text{C}_\text{O}^-\text{N}^-\text{N}^-\text{Cl}^- \\
\end{array}
\]

7

Figure 1.10.- Structures of carmustine (6) and lomustine (7)

1.2.2. Intercalating Agents

Intercalation is another method by which molecules can interact with DNA. Many types of drugs such as daunomycin hydrochloride (8) and acridine (9) (Figure 1.11) are known to interact with DNA through intercalation which involves insertion of a flat polyaromatic chromophore between consecutive base pairs of the DNA double-helix. This insertion subsequently leads to the lengthening and unwinding of the helix. The intercalator forms non-covalent bonds (\(\pi-\pi\) or stacking interactions) between its aromatic system and the two
base pairs perpendicular to the axis and causes the distortion of the polysaccharide backbone causing potential deletions in the coding region.

![Figure 1.11.- Structures of known intercalators: daunomycin hydrochloride (8) and acridine (9)](image)

Intercalators can be either cationic or neutral. A cationic intercalator induces an electrostatic force of attraction between the DNA macromolecule and itself. This is followed by the insertion of the planar moiety in between the DNA base pairs due to hydrophobic and π-π interactions. From computational studies, it has been shown that one of the most important driving forces for the intercalation of a neutral intercalator such as 10 (Figure 1.12) is the interaction between the highest occupied molecular orbital (HOMO) of DNA and the lowest unoccupied molecular orbital (LUMO) of intercalators.

![Figure 1.12.- An example of a neutral intercalator (10)](image)

The mode of binding of intercalators is related to their anticancer activity and the strength of intercalation is relevant to the drug’s potency. Also, the kinetics of binding is decidedly important due to the fact that average residence time of intercalation is associated with biological activity. It is important to note that upon intercalation, the drug leads to distortion on the angle of the phosphate groups. The unwinding of the double strand leads to a lengthening of the helix by approximately 3.4 Å, causing a conformational change of some sugar moieties involved.
S. Lerman was the first scientist to propose intercalation. He reported the occurrence of a noncovalent interaction between acridine and DNA, suggesting an intercalative mode. To understand this intercalation process, the so-called “neighbour exclusion principle” was developed. The nearest neighbour-exclusion principle proposed by Crothers is one of the most basic rules for intercalative binding of planar drugs to DNA and it states that intercalators can, at most, only bind at alternate base pair sites on DNA. Therefore, there is only ever a maximum of one intercalator between every second possible binding site. In the first instance, before addition of any ligand, all base pair spaces have an equal potential to be intercalation sites for a non-specific compound. However, when an intercalator binds to a particular site, the neighbour exclusion principle states that binding of additional ligand molecules at sites immediately adjacent is not possible. This has been proven experimentally in fiber diffraction studies on nucleic acid fibers bound to metallointercalative agents, in single-crystal studies on complexes of dinucleoside monophosphate and an intercalator, and in solution studies on binding of ethidium bromide to oligonucleotides of ribose and deoxyribose sugars.

Another example of an intercalator is doxorubicin (11) and in Figure 1.13 two doxorubicin molecules intercalating in between base pairs according the nearest neighbour exclusion principle are shown.

It is not only the binding strength of a DNA intercalator that leads to its biological activity. The cytotoxicity of intercalators is dependent on both their ability to interact with DNA and their ability to poison topoisomerase (Topos) enzymes that are directly involved in DNA recognition. Topos can be poisoned in the necessary steps of cellular growth when DNA replication is active; in the S phase of the cell cycle, in which the topology of DNA
plays a significant role and in the M phase of the cell cycle, where these enzymes are involved in arranging the chromatin.

The structure of DNA before, during, and after replication is vital for the process of cell division. In this sense, Topos manage the topology of DNA by controlling three parameters: linking number, twist and writhe. Hence, topological problems of the double helix can be overcome by Topos' binding to either single-stranded or double-stranded DNA, cutting the phosphate backbone and allowing the DNA to be untangled or unwound. Afterwards, the DNA backbone is resealed again. Topos enzymes can be divided into two main families: Topoisomerase I enzymes cleave only one strand of the DNA even though both strands are involved in the interaction with the enzyme. This family can be further classified as either type IA subfamily if the protein link is to a 5' phosphate or type IB subfamily if the protein is bound to a 3' phosphate. Topoisomerase II enzymes cleave both strands of the DNA double helix. Another family, Topoisomerase III enzymes, has also been described but their mechanism of action is still unknown. A fourth family, known as the gyrases, have been found in bacteria.35

A DNA intercalator displays cytotoxicity when it poisons the Topos enzymes by stabilising the ternary complex DNA–intercalator–topoisomerase in such a way that enzymatic processes cannot continue forward or backward. Once the enzyme–DNA complexes are poisoned by intercalators, the ternary complex is recognised by the cell as a damaged portion, leading to a sequence of events, one of which involves the tumour suppressor protein p53, which induces apoptosis (Figure 1.14).

![Figure 1.14.- DNA intercalator and the mechanism of its cytotoxicity.](image)

Topos are found in all eukaryotic cells; therefore, both healthy and infected cells can be affected during treatment with intercalators. Since tumour cells replicate more frequently than normal cells, it will be the tumour cells that will be mostly affected by these drugs.36
As it will be presented in the Objectives (chapter 2), the molecules aimed in this thesis contain an acridine core as the intercalator moiety. There are three main families of intercalators that contain the acridine system: the 9-anilinoacridines, the acridinecarboxamides and the nitroacridines.

An example of a 9-anilinoacridine is asulacrine (12) (Figure 1.15), which acts by inhibiting topoisomerase II enzymes resulting in DNA protein cross-links and DNA breaks. Asulacrine has been shown to have therapeutic effects on non-small cell lung and breast cancer, but not in colorectal and gastric cancer. Studies have been carried out on combination therapies of asulacrine and cisplatin. These studies carried out in mice with both intraperitoneally and intravenously implanted P388 leukemia and also with advanced stage sub-cutaneously implanted LC-12 squamous cell carcinoma, showed that combinations of asulacrine and cisplatin or carboplatin were considerably more effective than treatment with just asulacrine. The activity of the combination therapy was 50-fold larger than that obtained with asulacrine alone suggesting that such combinations may have clinical potential.

Figure 1.15.- Chemical structure of asulacrine (12)

The second family of acridines, 9-aminoacridine-4-carboxamides, were first described in 1984, as a new group of DNA-intercalating agents with potent cytotoxicity and in vivo antileukemic activity. Structure-activity studies showed that their cytotoxicity was related to the kinetics of dissociation of the drugs from DNA. These drugs remained bound to DNA for longer time therefore resulting in stronger activity. Crystal structure studies of complexes of 9-aminoacridine-4-carboxamides with hexanucleotides showed the acridine chromophore intercalated parallel to the base pairs and the 4-carboxamide side chain inserted into the major groove. An example of an aminoacridine-4-carboxamide is N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) (13). Research carried out by Antonini et al. showed that, in comparison to DACA, N-4-(ω-aminoalkyl)-1-[(ω-
aminoalkyl]amino]-4-acridinecarboxamides possess potent cytotoxic activity and relevant DNA-binding properties. The introduction of a second side chain to the chromophore leads, in a number of cases, to an increase of \textit{in vitro} activity and DNA-binding ability. These derivatives are a new potential anticancer lead possessing useful broad-spectrum cytotoxic activity in the 4-7 nM range (Figure 1.16).

![Figure 1.16.- Structure of DACA (13) and N-4-(ω-aminoalkyl)-1-[((ω-(aminoalkyl))amino]-4 acridinecarboxamides derivatives](image1)

Nitroacridines are the third and final family of intercalating acridines that will be discussed in this Introduction. These intercalators are substituted at the 1-position with a nitro group which is essential for their activity. Nitracrine (NC, 14), a 1-nitro-9-aminoalkyl derivative of acridine (Figure 1.17) displays potent cytotoxic effects which are a result of its metabolic activation, followed by covalent binding to DNA.

![Figure 1.17.- Chemical structure of nitracrine (14)](image2)

Another interesting class of intercalator agents are the \textit{bis}-intercalators, which are compounds that intercalate twice. Waring et al.\textsuperscript{42} reported the DNA-binding characteristics of the first known \textit{bis}-intercalating natural product, echinomycin (15).
This bifunctional molecule consists of a cyclic octapeptide minor groove binding region and two linked chromophores capable of simultaneous intercalation. The minor groove binding bicyclic depsipeptide backbone forms HBs to DNA bases while the aromatic rings bind to DNA via intercalation.\(^{43}\) The compound DMP-840 (16, Figure 1.19) has excellent activity in human tumour cells. These studies led to the development of the DACA chromophore (acridine-4-carboxamide) bis-intercalators (17, Figure 1.19). Structure-activity relationship (SAR) studies of substituted bis-(acridine-4-carboxamides) linked by a mono-cationic chain revealed that small substituents at the 5-position gave the most cytotoxic compounds with IC\(_{50}\) values as low as 2 nM against Lewis lung carcinoma cells. These compounds have been shown to inhibit topoisomerase I enzymes rather than topoisomerase II enzymes.\(^{2}\)
Due to the highly conjugated ring system in acridines and in many other intercalators these compounds possess a high fluorescence. Therefore, they can be used as dyes and photosensitzers (photodynamic therapy -PDT-). Acridine orange (18, Figure 1.20) is used as a dye in cell cycle determination. This compound stains human cells in green and other organisms in bright orange, hence, it can be used for detection with a fluorescence microscope for example in the examination of smears for cancer cells. Methylene blue (19, Figure 1.20) is an intercalator that has been experimentally investigated for use in PDT. Treatment of cancer by PDT requires photosensitzing agents which absorb light to be excited to an energetic triplet state capable of transferring its energy efficiently to ground state triplet molecular oxygen to form excited singlet oxygen. Recent research proposes that compounds such as methylene blue that contain redox active 3,7-diaminophenothiazinium pharmacophore (phenothiazinium redox cycler-PRC compounds) selectively target melanoma and other cancer cells with induction of apoptosis by expression of NAD(P)H. It has been shown that photosensitized oxidation of plant auxin (indole-3-acetic acid) by phenothiazinium dyes like methylene blue, and red light offers the potential to kill oxic and hypoxic tumour cells using low light doses.

![Figure 1.20.- Structure of acridine orange (18) and methylene blue (19)](image)

### 1.2.3. Major and Minor Groove Binding Agents

Another class of chemotherapeutic drugs that act by interacting with DNA is that of the major and minor groove binders. As mentioned before (section 1.1.2.), in DNA the sugar-phosphate backbone spirals around the outer surface of DNA and between these backbones are two different sized grooves known as the major and minor grooves. The major groove is the larger one measuring 11.6 Å wide and 8.5 Å deep. It consists mainly of G:C base pairs having the greatest positive potential in comparison with the negative electrostatic potential seen in the minor groove which is rich in A:T base pairs. Both grooves are surrounded by physiologically essential cations for example Na⁺, K⁺, Ca²⁺ and Mg²⁺. The G:C rich sequences localise cations in the major groove and the A:T regions favourably concentrate them in the minor groove. Water molecules are also present within both grooves; these molecules form water-mediated interactions (hydrogen bonded structures)
and support the organisation of cations.\textsuperscript{47} The dynamics of these water molecules contrasts greatly between the two grooves. It has been shown by NMR and simulation studies that the water dynamics in the major groove are much faster than those in the minor groove. The water molecules in the minor groove have residence times exceeding one nanosecond whereas the water in the major groove is highly mobile, with residence times shorter than 500 picoseconds. This observation confirms that the water molecules in the minor groove have an important structural role in the duplex architecture.\textsuperscript{48}

The DNA major and minor grooves are the interaction site for many replicative and repairing enzymes and transcription control proteins. Groove binding molecules target the DNA duplex aiming to interfere with the normal helical structure. In doing so, they compete with endogenous cellular components such as nucleic acid-binding proteins therefore disrupting typical processes like transcription and replication which maintain the homeostasis within the cell. Binding to either groove requires the molecules to have different and specific characteristics and this binding induces different physiological effects depending on which groove is targeted. Proteins generally bind to the major groove while the majority of small molecules interact with the minor groove. Although some small drugs such as methyl green (20, Figure 1.21) have been shown to bind to the major groove,\textsuperscript{49} proteins bind more often to the major groove due to their size and also because of the large number of hydrogen bonding interactions that can be formed. Very specific recognition and control processes such as accurate gene expression occur within the major groove.\textsuperscript{50,51}

![Chemical Structure of methyl green (20)](image)

These protein-DNA interactions in the major groove do not lead to major alterations to the helical structure. In contrast, proteins binding in the minor groove induce significant changes. For example the TATA-box binding protein (TBT) binds to the minor groove and gives rise to a dramatic DNA deformation.\textsuperscript{52}
Considering that, as mentioned, the minor groove is an interaction site for a number of enzymes and transcription control proteins, compounds that target the minor groove have a clear therapeutic potential because they will inhibit transcription directly or block the actions of DNA-dependent enzymes. These compounds have been shown to exhibit antiprotozoal, antiviral, antibacterial and anticancer activity. Both, natural products and synthetic molecules, greatly contrasting in structure, have been shown to interact with the DNA minor groove displaying various modes of action and mechanisms. Hence, to understand the reasons why DNA minor groove binders specifically target the minor groove and to comprehend the nature of the interactions formed, it is essential to be aware of the characteristics of this groove.

The surface of the minor groove is convex, which complements the concave structure of a typical minor groove binder. Minor groove binding agents usually have long and planar structures allowing them to adopt a crescent shape that fits into the minor groove, forming close contacts in the deep, narrow space formed between the two strands of DNA. The shape of the minor groove is vital for the recognition of enzymes and for the normal functioning of cellular processes. An example of this is the integration host factor (IHF) protein which is a dimer of closely related chains that seem to function in genetic recombination as well as in translational and transcriptional control. The DNA minor groove must undergo enormous changes in its width and the ability of DNA to support these changes is a fundamental factor in the selection of the specific targets by IHF. Upon binding to the minor groove, IHF causes the DNA to be bent by 160°. The intercalating prolines of IHF give short stretches of very wide minor grooves (>10 Å) located approximately one helical turn apart at the top corners of the bent DNA. These are in turn evenly interspersed with three distinct areas of narrowed minor groove. These are separated by one helical turn (10 base pairs), and therefore are located at the centre and sides of the inside of the DNA bend. Thus, the minor groove incorporating the three characteristics alternates between narrow and wide every half helical turn, and the three sites having the narrowest minor grooves are positioned to contact the top and sides of the protein. This arrangement accommodates many direct and water-mediated electrostatic interactions between the IHF protein and the narrow minor grooves.

As previously mentioned minor groove binders normally form HBs with the DNA base pairs within the groove. Hydrogen bonding to base edges in the groove offers the ability to discriminate between A:T and G:C base pairs because the different numbers of HB donors
and acceptors. Within the minor groove, A:T base pairs prefer HB donation from the minor groove binders due to the ability of the N3 and O2 of adenine and thymine respectively to accept HB.\textsuperscript{55} There are more HB donors and acceptors on the major groove edge of each base pair than on the minor groove edge. Therefore, within the major groove there are more opportunities for discriminating between different base pairs using HBs. In the minor groove, the key difference between base pairs is that G:C base pairs contain an exocyclic amino group that protrudes into the groove. This amino group makes the steric and electronic environment of the minor groove at G:C base pairs extremely different from that at A:T base pairs.\textsuperscript{56} This exocyclic amino group blocks close contacts between the minor groove and the molecule (Figure 1.22).\textsuperscript{55}

![Figure 1.22.- HB donors (D) and acceptors (A) within the major and minor groove available for ligand-DNA interactions\textsuperscript{57}](image)

A number of different types of interactions occur within the minor groove. Analysis of crystal structures of 60 protein–DNA and 14 drug–DNA complexes by Morávek et al.\textsuperscript{58} showed that the base pair atoms that bind to protein or drug residues are purine N3, pyrimidine O2, guanine N2 and deoxyribose O4'. Interactions with N3 and O2 are the most common ones because they are more polar than contacts to O4'. The surfaces of the minor groove walls are more hydrophobic, due to the backbone hydrogen atoms.\textsuperscript{55} Because of this, hydrophobic interactions can occur within the groove. These interactions act as secondary to the preferable interactions of N3 and O2. Many protein contacts are facilitated by water, in order to increase the DNA effective surface. Water mediated contacts are less commonly observed in DNA-drug complexes.

As previously indicated the minor groove has the greatest negative electrostatic potential in comparison to the rest of the DNA duplex. Extensive research has been carried out on the molecular electrostatic potential of the sugar-phosphate backbone of the B-DNA helix, the
geometric factors controlling the negative electrostatic potential within the groove, the overall shape of the minor groove and the differences in electrostatic potential values between the minor and major groove and the phosphate backbone. It seems that the electrostatic potential is altered depending on the position of the base pairs within the grooves relative to the double helix. The cations Na⁺, K⁺, Ca²⁺ and Mg²⁺ shield the negatively charged phosphates and this also has an effect on the negative electrostatic potential of the groove demonstrating the importance of the ions surrounding DNA. These cations determine whether electrophilic or nucleophilic molecules bind to the minor groove. The arrangement of the base pairs within the minor groove is very important and research within in this area has led to determining the preference of base pairs for electrostatic interactions with ligands.

Bearing in mind the features of the minor groove, classical ligands designed to bind to it comprise of certain features that make them an extremely important group of DNA binding molecules. Among the characteristics that minor groove binders show is that they have linked rather than fused aromatic and/or heterocyclic rings; therefore, they have high flexibility that allows for a better fit into the groove. The minor groove has a strong negative potential which complements with the cations usually present in minor groove binders. These DNA/minor groove binder complexes are usually stabilised by HBs, van der Waals contacts, and/or ionic interactions, and if the molecule is positively charged, this is typically accompanied by the release of a large number of water molecules known as spine of hydration by means of hydrophobic interactions, thereby leading to an increase in entropy. Distamycin (21) and netropsin (22) were amongst the first molecules found to bind to the minor groove (Figure 1.23) and many other drugs that target the minor groove have been discovered since.

Netropsin is an example of the ‘ideal’ minor groove binder because it possesses several of the essential structural features required: it is not planar and it is twisted in a screw sense that fits into the B-DNA minor groove. Netropsin binds to the 5’AATT region of the groove, with the walls of the groove in close contact with the pyrrole and amide groups. This interaction is driven by the highly enthalpic displacement of the spine of hydration. The crystal structure of a netropsin complex with the dodecanucleotide duplex sequence d(CGCGAATTCGCG)₂ provided detailed experimental information of the interactions established with the A:T base pairs explaining how netropsin is selective for these A:T base pairs. Netropsin binds to the minor groove according to the isohelicity concept which
states that heterocyclic molecules targeting the minor groove require a shape that complements the convex surface of the minor groove. A number of HBs stabilise netropsin within the minor groove. Each amide group has its nitrogen atom pointed inwards, and forms three centred HBs with the O2 of thymine. The terminal amidinium groups are also involved in hydrogen bonding to the bases.\textsuperscript{65}

Distamycin extracted from Streptomyces distamyces is another example of a molecule that strongly binds to the minor groove. It comprises of three N-methylpyrrole amino acids and is structurally similar to netropsin. Studies of the crystal structure of distamycin bound to the sequence d(CGCAAATTTGCG)\textsubscript{2} shows that it differs from netropsin by the existence of only one cationic guanidinium group. Binding of distamycin and netropsin to DNA is similar but not identical. These differences are due to the additional pyrrole amide group in distamycin and to the fact that the terminal residues are different. Distamycin covers five of the six A:T base pairs in the minor groove, whereas netropsin covers four A:T base pairs.\textsuperscript{55,68}

The difference in structural features contributes to the difference in binding of these molecules to the minor groove. Studies using 2D NMR (NOESY) showed that at low concentrations of the drug, distamycin mainly displays a 1:1 binding mode to the d(CGCAAATTTGCG)\textsubscript{2} sequence; in contrast, at higher drug concentrations, a 2:1 binding mode was favoured.\textsuperscript{69} The minor groove width in the 2:1 complex is twice that observed in 1:1 complex. The width of the groove expands from 3.4 to 6.8 Å showing that the minor
groove is extremely flexible particularly when a molecule is bound to it. Figure 1.24 shows the 2:1 complex of distamycin bound to d(CGCAATTTGCG)_2 by covalent hydrogen bonds to the O2 of thymine and the N3 of adenine.

Figure 1.24.- Distamycin (21)-DNA 2:1 complex showing detailed hydrogen bonding to bases in both strands of a five base pair A:T sequence.

Following the research carried out on the original crystal structure of a netropsin–DNA complex it was noted that alteration of netropsin to give an appropriately-placed acceptor group would lead to recognition of the minor groove exocyclic N2 atom of guanine, this would result in active recognition of a G:C base pair. Recognition of G:C base pair is achieved by replacement of the pyrrole ring in netropsin or distamycin with an N-
methylimidazole ring. This led to the discovery of lexitropsins, a novel class of minor groove binding molecules in which one of more pyrrole rings have been replaced with other heterocyclic moieties such as imidazole (23) or thiazole (24), yielding ligands with an increased selectivity for G:C base pairs at their binding sites (Figure 1.25).\textsuperscript{1,51,71}

![Figure 1.25.: Structures of lexitropsins with imidazole (23) and thiazole (24) rings](image)

Another type of minor groove binders is that of the diarylamidines. These molecules consist of two phenyl rings separated by a linker of various lengths and composition. The molecules are concave in shape, positively charged and are stabilised by HBs, ionic interactions and van der Waals forces. Some examples include pentamidine (25), berenil (26) and 4',6-diamidino-2-phenylindole (DAPI, 27) (Figure 1.26). Berenil is an aromatic diaminidine which can be used in the treatment of trypanosomiasis in animals. Compounds such as berenil and furamidine (28, Figure 1.27) inhibit kinetoplasts which are disk-shaped masses of circular DNA inside a large mitochondrion that contains the mitochondrial DNA of the parasite.

Crystal structure studies of berenil with the dodecanucleotide d(CGCGAATTCGCG) showed that it is bound at the 5'-AAT-3' region. At one end of the drug the amidinium group forms HBs with N3 of the adenine base complementary to the thymine of the AAT. At the other end the second amidinium group does not make direct contact with the DNA; instead, a water molecule serves as a bridge for a HB between them.\textsuperscript{72,1}
Pentamidine is another diarylamidine that binds to the minor groove. It has been used to treat a number of diseases including trypanosomiasis, leishmaniasis, pneumocystis carinii pneumonia (PCP) and malaria.\textsuperscript{73,74,75}

Pentamidine has been shown to bind to duplex DNA, with a preference for A:T sequences. Footprinting and X-ray crystallographic studies have shown that pentamidine binds to four A:T base pairs and does not bind in the presence of a G:C base pair within the site. The two aromatic rings of the bound pentamidine molecule are twisted by 35° with respect to each other, resulting in the rings being aligned to be parallel to the walls of the minor groove, and therefore complementing the curvature of the groove.\textsuperscript{76,77}

Furamidine is the most important of all of the diarylamidines. It is a diphenylfuran diamidine and is the leading compound in the group of antitrypanosomiasis, antimicrobial and antiparasitic agents.\textsuperscript{73,78,74,79,75,80,81} Furamidine has two phenyl rings, connected by a furan, and basic amidine ends which are cationic by nature. The concave structure of the furamidine molecules complements the convex minor groove. Van der Waals contacts and HB interactions occur between furamidine and the DNA bases. An X-ray structure of the furamidine-d(CGCGAATTGC)\textsubscript{2} complex shows that the molecule spans four base pairs within the minor groove, specifically AATT sequences. Interactions with A:T base pairs along the floor of the groove and hydrogen bonding with the amidine groups stabilise the complex.\textsuperscript{82}
A number of analogues of furamidine have been synthesised including selenophen and thiophene analogues where the furan oxygen is replaced with a Se or a S atom.\textsuperscript{83,74} Analogues incorporating benzimidazole ring systems have also been synthesised.\textsuperscript{84}

Minor groove binding agents normally adopt a crescent shape that fits into the minor groove, though recently it has been found that linear compounds can also bind to the minor groove.\textsuperscript{85} It has been shown by Wilson et al. that linear compounds display a higher binding affinity when compared with the equivalent crescent shaped compounds. X-ray crystallographic analysis of DB921 (29) complexed with AATT demonstrates that the structural change in DB921 induced by the fit reduces the twist of the biphenyl to complement the groove therefore placing the functional groups in position to interact with bases at the floor of the groove. The phenylamidine of DB921 forms indirect contacts with the bases through a bound water molecule.\textsuperscript{84,86,87} Binding is assisted by highly structured water molecules that can support groove binders in a number of ways with a bridging water molecule being a prime example.\textsuperscript{88}

![Chemical structure of DB921 (29)](image)

Another example of a linear diamidine that binds to the minor groove is CGP-40215A (30) which has been found to have excellent antitrypanosomal activity. Research was carried out on this compound using DNase I footprinting, biosensor-surface plasmon resonance (SPR), X-ray crystallography, and molecular dynamics (MD) simulations in order to show that CGP-40215A strongly binds to the DNA minor groove. It was quite surprising that this molecule displayed strong binding to the minor groove as it was expected that it would poorly bind due to its lack of curvature. Thus, to understand the interaction of CPG-40215A and the minor groove, this ligand was crystallised with the d(CGCGAATTCGCG)\textsubscript{2} duplex. As a result, it was found that it binds to the AATT sequence with the two NH groups of the linker pointed into the groove to form direct HBs with the O2 groups of three thymines. As a consequence of the linear structure of CGP-40215A, both amidines cannot form direct HBs with the bases. The crystal structure showed that one amidine forms a HB with the O2 of thymine-8 by an indirect, water-
mediated HBs to the adenine that is complementary to thymine-8 as well as to cytosine-9. The NH group of the linker forms split HBs with the interstrand O2 of thymine-7 and thymine-19, meanwhile the amidine at the other end of this molecule points away from the groove. Hence, two water molecules form a hydrogen bonding bridge between this amidine group and the base pairs. The binding strength of CGP-40215A has been shown to be approximately six times larger than that of the crescent shaped minor groove binder berenil.\(^8\) Another example of a minor groove binder that strongly binds to the minor groove via a water molecule is DB185 (31, Figure 1.29).\(^8\)

![Linear minor groove binders CGP 40215A (30) and DB185 (31)](image)

Figure 1.29.- Linear minor groove binders CGP 40215A (30) and DB185 (31)

### 1.2.4. Dual DNA Targeting Agents

There are a small number of drugs which simultaneously bind to DNA by two different modes by combining two different DNA targeting functionalities. These drugs are known as dual binders and, for example, can be composed of minor groove binder-intercalator agents, minor groove binder-alkylating agents or major groove binder-intercalator agents.

In order for a dual binder to form strong interactions it must combine the qualities that make the individual moieties efficient DNA targeting agents. Therefore, the intercalating moiety must consist of a cationic or neutral planar polyaromatic chromophore that inserts between the base pairs of the DNA double helix. This consequently leads to the lengthening and unwinding of the helix. The intercalator should form non-covalent bonds (\(\pi-\pi\) or stacking interactions) between its aromatic system and the two base pairs perpendicular to the axis causing the distortion of the polysaccharide backbone. The minor groove binder moiety should be sequence-specific enough to bind to particular A:T rich sequences within the minor groove. As mentioned before, minor groove binding agents generally have long and flat structures allowing them to form close contacts in the deep, narrow space formed between the two strands of DNA. Minor groove binder functionalities should be positively charged to complement the negative potential of the groove and help the release of the spine of hydration. Finally, these moieties must be able
to form HBs, van der Waals contacts, and/or ionic interactions to stabilise the drug-DNA complex. If the dual binder contains an alkylating functionality it must be capable of transferring an alkyl group to DNA at the primary site of alkylation which is the N7 on guanine. Additionally, it would have to be highly electrophilic to be able to react with nucleophiles to form covalent bonds and strongly alkylate with the binding site.

Another characteristic that is essential for efficient dual binding is that the spacer between the two binding moieties must be of optimal length and composition. The length is important to allow both functionalities to simultaneously and synergistically bind to DNA. The composition of the linker is also important because it must not cause unfavourable interactions therefore leading to a decrease in binding affinity. It must form favourable interactions such as HBs, ionic interactions or van der Waal forces to stabilise the molecule at the various binding sites. Also the linker must have the correct degree of flexibility or rigidity allowing the two binding moieties to fit into their respective binding sites. Possessing all these features is what makes a successful dual binder.

Examples of dual agents are molecules that intercalate and bind to the minor groove simultaneously. These dual binders aim to improve the affinity and cytotoxic activity of the molecule towards DNA. Studies show that in a netropsin-acridine conjugate (NETGA) (32, Figure 1.30) the minor groove binding component of netropsin and the intercalation of the acridine can successfully occur simultaneously and also that this dual binding process induces a local distortion of the DNA helix near the intercalation site. That means that NETGA acts synergistically showing an increase in activity and affinity for DNA with respect to the single interaction moieties. Dual binders such as these are known as combilexins and they are expected to result in an improved antitumor activity.90 They are prepared by connecting sequence specific molecules to an intercalator. Accordingly, the sequence specific part of the molecule will act as a hook with the minor groove binding moiety serving as a director for specific sequences while the intercalator inserts in between the base pairs, thus exerting its activity in certain areas of the DNA double helix. When compared with the minor groove binding analogues dual binders showed an increase in binding affinity due to the increased binding site size that was found to be 5-6 base pairs long. Importantly, the presence of the acridine ring, in the NETGA and similar compounds did not affect the A:T sequence selectivity.91,92
Distel(1+) (33) and distel(2+) (34) (Figure 1.31) are other interesting examples of combilexins. Distel(1+) is capable of bidentate binding to DNA. This reaction is mainly driven by the charged ellipticine intercalator moiety of the hybrid. The ellipticine chromophore has noticeably strengthened the affinity of the ligand for DNA, but this result has unfortunately also caused a reduction in DNA sequence selectivity. Computational studies showed that the addition of a positive charge on the distamycin terminal group would favour binding to A:T sequences, thus improving the sequence selectivity. The hypothesis proved to be correct. Certainly, the substitution of the terminal formamido group of distel(1+) by an aminopropionamido group charged at neutral pH, resulting in distel(2+), was the precise way to proceed to transform a nonspecific derivative into a highly selective A:T-specific DNA binder. In contrast to distel(1+), the interaction of distel(2+) with DNA seems to be driven equally by both the distamycin and the ellipticine residues. Of the two distamycin-ellipticine hybrids, distel(1+) and distel(2+), only the mono-cationic hybrid distel(1+) showed to be a topoisomerase inhibitor like aforementioned intercalators. Its di-cationic analogue distel(2+) produced virtually no effect on both topoisomerase I and II, despite its superior DNA binding properties. The poisoning of topoisomerase I by distel(1+) enhances the cytotoxic effect since P388CPT5 cells, which are resistant to camptothecin (a powerful topoisomerase I inhibitor), display a notable cross-resistance to distel(1+).71,93
An example of a trifunctional molecule (35, Figure 1.32), which is composed of a bis-pyrrole skeleton for minor groove DNA binding linked to two different intercalating chromophores, anilinoacridine and ethidium, has been described. The binding mode and affinity of this compound to DNA were studied using various biophysical techniques including absorption and fluorescence spectroscopy as well as circular and linear dichroism.

Further dual action molecules have also been synthesised containing an intercalator functionality linked to an alkylating moiety. The cytotoxic complex molecule known as PT-ACRAMTU (36, Figure 1.33) is a dual platinating/intercalating DNA binder. In the DNA duplex, PT-ACRAMTU targets guanine (80%) and adenine (20%) bases. The DNA-drug complex is formed by replacement of the chloro leaving group in PT-ACRAMTU with the endocyclic nitrogen N7 of guanine, which is located in the major groove of the DNA. Upon binding, PT-ACRAMTU forms a mono-nucleoside adduct, dGuo* (Figure 1.33) and single crystals of this adduct, which were suitable for X-ray analysis, led to the determination of its structure. The unusual type of self-association observed in this
structure provides a strong rationale for the proposed covalent-intercalative DNA binding mode of PT-ACRAMTU. The crystal structure of dGuo* shows that the flexibly linked acridine unit can adopt a geometry that results in almost perfect coplanarity with the platinated guanine base. On the basis of the geometry seen in the crystal, intercalation of acridine into the DNA base stack perpendicular to the helical axis and parallel to the platinated base pair seems to the most likely binding mode.  

Nitrogen mustards can be attached to intercalators or sequence selective molecules in order to form another class of dual binders. Nitrogen mustards interact non-specifically with DNA by transferring an alkyl group to DNA at the primary site of alkylation which is the N7 on guanine. However, when an intercalator is linked to a nitrogen mustard the selectivity moves from the N7 of guanine to the adenine N3. Research carried out on 9-aminoacridine nitrogen mustards demonstrated that binding to DNA occurs by intercalation between base pairs as it was expected. Moreover, it was found that once the molecule is intercalating between the base pairs of DNA, the tethered nitrogen mustard moiety can react with the DNA at neighbouring nucleophilic sites, depending on their steric accessibility which depends upon the local DNA sequence. A two-step reaction sequence of non-covalent binding (intercalation) followed by alkylation is consistent with 10-100-fold lower concentrations of the acridine-nitrogen mustards.
Alkylator-groove binder dual agents display higher selectivity in comparison to intercalators. Other examples of a nitrogen mustard-minor groove binder are MGB1 and MGB2 developed by Ferguson et al. The results obtained with these molecules showed that targeting DNA with nitrogen mustards attached to a DNA minor groove binder such as the bis-benzimidazole Hoechst 33258 makes it possible to direct DNA alkylation to more specific stretches of DNA.

Moreover, to increase the DNA binding affinity of a bis-arginyl porphyrin which has displayed favourable binding to the major groove of the d(GGCGCC)_2 sequence, Perée-Fauvet et al. synthesised bis- and tris-intercalating derivatives where one or both arginyl arms are connected through a flexible chain to an acridine ring. Molecular modelling of their complexes with a G:C rich sequence showed that when the porphyrin was intercalated into the central d(CpG)_2 site with both arginyl side chains bonded to the guanines flanking the intercalation site, the acridine rings could intercalate immediately upstream from the central d(CpG)_2. A significant preference for major groove binding over minor groove binding was found. These compounds proved to be efficient major groove-intercalator dual agents.

1.3. DNA as a therapeutic target

DNA is a key target for drugs aiming to interfere with normal cellular processes because of its involvement with how genes are expressed and how proteins are created. Furthermore, the replication process for DNA can be affected, which is important for cell growth as well as for the cell's ability to successfully divide. The discovery of the sequence of the human genome has helped in the development of DNA targeting molecules which are gene-selective and are uniquely able to down-regulate the expression of a single abnormally expressed or mutant gene. There are several ways in which drugs can target DNA. One particular way is by controlling special enzymes and factors involved in transcription. In order to do this, the drugs target proteins that bind to DNA. Another mode of DNA targeting involves molecules that bind to the double helix parts of DNA interfering with the interactions between DNA and proteins. There are many diseases in which DNA is targeted in order to find a cure. DNA ligands have been shown to exhibit anticancer, antiprotozoal, antitrypanosomal, antiviral and antibacterial activity. We will concentrate in how DNA targeting can be useful in treating tropical diseases and cancer.
1.3.1. Tropical Diseases

Tropical diseases, generally referred to as neglected tropical diseases (NTDs), are medically diverse and all are strongly associated with poverty. Some causes of the spread of these diseases include lack of access to safe water and sanitation, filthy environments, and abundant insects and other vectors which contribute to efficient transmission of infection. Most of them are ancient diseases that have plagued humanity for thousands of years. Once widely prevalent, the occurrence of many of these diseases gradually diminished from large parts of the world as societies developed and living conditions and hygiene improved. In 2010 the World Health Organisation (WHO) reported that over one billion people are infected with one of the 14 diseases categorised within the class of NTDs. These diseases are the most common infections in the 2.7 billion people living on less than $2 a day. They affect those often marginalised and forgotten by governments. NTDs can all cause severe disability or death, and are a major economic burden on endemic countries from Africa, Asia, and the Americas. In 2002 over 120,000 people across Africa, America, South East Asia, the Western Pacific and the Eastern Mediterranean died as a result of tropical diseases. Human exploration of tropical rainforests and deforestation has led to a recent increase in tropical diseases. A study showed that malarial mosquitoes prefer to inhabit recently cleared forests. A rise in immigration, international air travel and other tourism to tropical regions has also led to an increased incidence of such conditions.

These are widespread diseases that are unique to tropical and subtropical regions and they are less common in temperate climates because of occurrence of a cold season, which controls the insect population by forcing hibernation. Insects such as mosquitoes and flies are by far the most common disease carriers. These insects may infect a human or an animal with a parasite, bacterium or virus. Generally, the disease is transmitted by an insect bite, which causes transmission of the infectious agent through subcutaneous blood exchange. Some examples include leishmaniasis, Chagas disease and African trypanosomiasis. Next, some other example of tropical diseases and their effects are presented. Onchocerciasis and trachoma cause blindness. Leprosy and lymphatic filariasis lead to disfigurement of the body. Buruli ulcer maims, especially when limbs have to be amputated to save a life. Human African trypanosomiasis (sleeping sickness) severely debilitates before it kills, and mortality approaches 100% in untreated cases. Leishmaniasis, leaves deep and permanent scars or entirely destroys the mucous
membranes of the nose, mouth and throat. In its most severe form, it infects the internal organs and is rapidly fatal if left untreated. Chronic Chagas disease can cause life-threatening heart and digestive system disorders.103

Human African trypanosomiasis (HAT), also known as sleeping sickness is a parasitic disease transmitted by the bite of the Glossina insect, generally known as the tsetse fly (Figure 1.34).104

Figure 1.34.- Tsetse fly105

HAT, which is present in sub-Saharan Africa and affects between 50,000 and 70,000 people,106 is a life-threatening disease caused by bloodstream infections with parasitic protozoans of the subspecies Trypanosoma brucei (T. b.) rhodesiense or T. b. gambiense.71 After the bite of the infected fly, the parasite multiplies in the lymph and the blood, leading to headaches, fever, weakness, sweating, joint pain, and stiffness. Signs of infection are not always seen immediately. With time the parasite crosses the blood-brain barrier entering the central nervous system. This results in various neurological changes which include psychiatric disorders, seizures, coma and eventually death. T. b. rhodesiense results in acute infections, lasting from a few weeks to several months whereas in T. b. gambiense infections the disease is chronic, generally lasting several years without any major signs or symptoms.107 Two well-known examples of DNA binders that combat HAT are the minor groove binders furamidine and pentamidine whose structures are shown in Figure 1.26 and 1.27. While pentamidine is effective in the treatment of the tropical disease, furamidine is a key aromatic diamidine that is active against Trypanosoma species in vitro and DB289 (37) is an orally active analogue of furamidine.72 A few years ago, Rozas and co-workers show that bis-guanidine and bis-2-aminoimidazoline diphenyl derivatives such as compound 38 (Figure 1.35), display potent antitrypanosomal activity in vitro and in vivo against T. b. rhodesiense.106
These studies suggested that compounds with 2-aminoimidazoline cations had higher selectivity for the parasites and similar activities with respect to their guanidine counterparts. Also, a correlation between antitrypanosomal activity and DNA binding affinity was observed, suggesting a possible mechanism of action for these compounds by targeting DNA.

An X-ray structure was obtained for the complex of furamidine with the duplex oligomer d(CGCGAATTCGCG)\textsubscript{2} as shown in Figure 1.36.\textsuperscript{78} This X-ray structure displays the snug fit of this compound within the AATT minor groove along with the HBs to A:T base pairs at the floor of such a minor groove. This result proves that this dication binds to the minor groove suggesting further biological action. Thus, the DNA-furamidine complex could be responsible for the inhibition of the microbial topoisomerase enzyme leading to antitrypanosomal activity.\textsuperscript{1} In addition, the X-ray crystal structure of a bis-2-aminoimidazoline derivative (compound 38 in Figure 1.35) has been published (see Figure 1.36) showing very similar binding mode to that of furamidine.\textsuperscript{108} Drugs available for HAT are outdated and can cause major side effects. Recently, drug resistance has emerged causing an increase in treatment failures.\textsuperscript{106}

Figure 1.35.- Structures of DB289 (37) and 4,4'-\textit{bis}(4,5-dihydro-1\textit{H}-2-imidazolylamino)diphenylamine (38).

Figure 1.36.- X-Ray structure of the complex between (a) furamidine and the DNA dodecamer d(CGCGAATTCGCG)\textsubscript{2}\textsuperscript{78} and (b) 4,4'-\textit{bis}(2-aminoimidazoline)diphenylamine within a self-complementary oligonucleotide, 5'-d(CTTAATTCGAATTAAG)\textsuperscript{108}
Chagas disease also known as American trypanosomiasis, is a chronic infection caused by *Trypanosoma cruzi*, a single-cell flagellate that is transmitted to animals and humans via the bite of the triatomine insect vector. The disease affects about eight million people in Latin America. In the past thirty years, the control and management of Chagas disease has improved substantially. Large-scale vector control programmes and screening of blood donors have reduced disease incidence and prevalence. Although more effective trypanocidal drugs are needed, treatment with benznidazole (or nifurtimox) is reasonably safe and effective, and is now recommended for a widened range of patients. Unfortunately, these drugs have many toxic side effects and, therefore, new drugs with fewer side-effects and increased trypanocidal activity are urgently needed.\(^{109}\)

Another tropical disease that is targeted using DNA binders is Leishmaniasis. This disease is caused by protozoan parasites that come from the genus *Leishmania* and is transmitted by the bite of certain species of sand fly. The two general forms of the disease, which are caused by several species of Leishmania, are visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL). Leishmaniasis affects approximately 12 million people worldwide; the annual incidence of new cases is approximately 2 million, and 350 million people are at risk for infection. There are 500,000 new cases of VL per year around the world, 90% of these occur in just five countries (India, Bangladesh, Brazil, Nepal and Sudan). VL infects the internal organs, if left untreated it is usually fatal within two years. TL forms affect 1,500,000 people globally, and though TL is not a lethal disease, disfigurement, disability, and social and psychological stigma are all severe consequences of TL.\(^{110}\) *Pneumocystis carinii pneumonia* (PCP) caused by the yeast-like fungus *Pneumocystis jirovecii* is a common disease in developing countries. It is a form of pneumonia widespread among people with weak immune systems, for example severely malnourished children, and especially people living with HIV (Human immunodeficiency virus)/AIDS (acquired immunodeficiency syndrome). Symptoms of PCP include fever, non-productive cough, shortness of breath, weight loss and night sweats. The fungus can invade other visceral organs, such as the liver, spleen and kidney, but only in a minority of cases. Both of these diseases have been treated using pentamidine over the past 70 years;\(^{74}\) unfortunately, pentamidine is ineffective when given orally and it can cause severe toxicity. Many toxic side effects have been reported for pentamidine, these include abscesses at the site of injection, abnormal liver function, hypotension, nephrotoxicity, cardiotoxicity and hypoglycemia sometimes leading to insulin-dependent diabetes mellitus.
The aim of the research carried out by Wilson et al.\textsuperscript{74} during the last ten years has been to design an orally effective replacement for pentamidine. This long and flat structure is able to adopt a crescent shape which forms close contacts in the deep, narrow space between the two strands of DNA. The surface of the minor groove is convex, which complements the concave structure of a typical minor groove binder such as pentamidine. Analysis of these features, suggested that the diamidines designed by Wilson et al.\textsuperscript{74} should bind to DNA. Initial studies with diamidines indicated that they bind to DNA but not by intercalation. These diamidines showed binding to A:T sequences of at least four consecutive A:T base pairs in the binding site. Compounds that bound well to A:T sequences possess an appropriate curvature to complement the convex shape and helical twist of the DNA minor groove and hence they are usually minor groove binders. Moreover, the amidines provide HB donating NH groups to interact with HB acceptors, N3 of adenine and O2 of thymine at the floor of the minor groove in AT sequences.

The compounds designed by Rahmathullah et al.\textsuperscript{111} and Boykin et al.\textsuperscript{79} had excellent activity against eukaryotic parasites that cause diseases such as sleeping sickness and leishmaniasis affecting millions of people each year. The most active compounds strongly bind to the minor groove of DNA and in particular to A:T sequences. The compounds enter parasite cells rapidly and appear first in the kinetoplast and, after some time, they are also seen in the cell nucleus. The kinetoplast degrades and disappears from the mitochondria of treated cells and at this instant, the compounds begin to be observed in other areas of the cell. Cells normally die in 24-48 hours after treatment with active compounds. These compounds display selective targeting to long A:T sequences and cause changes in kinetoplast DNA minicircles that lead to a synergistic destruction of the catenated kinetoplast DNA network and cell death.

Malaria is generally not considered a neglected tropical disease since it receives more treatment and funding than other tropical diseases. It is referred to as one of the "big three" diseases in South America, Africa and South Asia. Malaria is caused by a parasite that is spread amongst humans by the bite of infected \textit{Anopheles} mosquitoes. After infection, the parasites known as sporozoites travel through the bloodstream to the liver, here they mature and release another form of parasite called merozoites. These parasites then enter the bloodstream and infect red blood cells. Here, they multiply causing the red blood cells to break open within 48 to 72 hours, infecting more red blood cells.
Symptoms usually first arise ten days to four weeks after infection, though they can appear as early as eight days or as long as a year after infection. The symptoms occur in cycles of 48 to 72 hours. Symptoms include high fevers, shaking, chills, flu-like symptoms, and anaemia.

Malaria can also be transmitted from a mother to her unborn baby (congenitally) and by blood transfusions. The Centre for Disease Control and Prevention estimates that there are 300-500 million cases of malaria each year, causing more than one million deaths. It is a major disease threat for tourists in warm climates. Some mosquitoes in certain parts of the world carry malaria that has developed resistance to insect repellent. Additionally, the parasites have developed resistance to some antibiotics. These conditions have led to difficulty in controlling both the rate of infection and spread of this disease. *Plasmodium falciparum* (*P. falciparum*) malaria, affects more red blood cells than the other four types of malaria and is much more serious. It can be fatal within a few hours of the first symptoms.\(^{113}\)

Wilson et al.\(^{74}\) described prodrug approaches to provide oral bioavailability for the dication class of diamidines. The furamidine prodrug DB289 has shown low toxicity in human trials in African, Asian, Caucasian and Hispanic populations. This compound that is the amidoxime prodrug of furamidine has shown activity against tropical diseases since it is
Chapter 1

Introduction

Orally active and metabolically converted to furamidine. DB289 underwent phase IIb human clinical trials for treatment of early-stage HAT, PCP, and malaria. Studies carried out by Yeramian et al. showed that DB289 is a promising new antimalarial compound that could become an important component of new antimalarial combinations.

Of the analogues synthesised by Rahmathullah et al. and Boykin et al. the compounds that gave the best results were DB596 (39) and DB539 (40). These compounds gave equivalent oral efficacy to that of DB289 in an immunosuppressed rat model for PCP.

In a study to determine if these compounds could be made orally effective, the alkyl carbamates of two of the more potent N'-alkylfuramidines (DB181, 41 and DB244, 42) were examined. In the systems studied it was found that the 2,2,2-trichloroethyl carbamate of both the N-iso-propyl (DB485, 43) and the N-cyclopentyl (DB517, 44) furamidine derivatives were quite effective on oral dosing in an immunosuppressed rat model for PCP. Therefore, carbamates of amidines and N-alkylamidines in oral doses can act as effective prodrugs. We now know that produgs of diamidines can be synthesised to allow effective oral delivery of the active dication.

Yeramian et al. stated that diamidines may have both novel transport pathways and novel targets. These compounds seem to be transported into P. falciparum–infected erythrocytes via a parasite-induced permeability pathway in the host cell membrane. It is thought that intracellular targets of diamidines include mitochondrial respiration.
haemoglobin degradation\textsuperscript{75} and DNA replication.\textsuperscript{80,81} Lanteri et al.\textsuperscript{83} proposed that furamidine prevents mitochondrial function. Yeast cells relying upon mitochondrial metabolism for energy production are particularly sensitive towards furamidine which targets the mitochondria of living yeast cells leading to collapse of the mitochondrial membrane potential. Additionally, treatment with furamidine results in immediate inhibition of oxidative phosphorylation and subsequent inhibition of respiration.

Another mechanism of action that has been suggested for diamidines is by hemoglobin degradation.\textsuperscript{75} Studies carried out by Stead et al.\textsuperscript{75} showed that pentamidine is impermeable to normal uninfected erythrocytes but enters malaria infected erythrocytes rapidly. Penetration of the infected erythrocytes occurs via a parasite specific pore with properties similar to those of the new permeability pathways (NPP) initiated by the presence of the parasite on the surface of the infected erythrocyte. Following penetration through the NPP, the concentration of pentamidine is largely driven by its binding to ferrirprotoporphyrin IX (FPIX) generated during the digestion of hemoglobin. Malaria parasites generally crystallise toxic FPIX into nontoxic hemozoin (malarial pigment). Diamidines inhibit the formation of hemozoin from FPIX by interacting directly with FPIX. It is this interaction that is predominantly responsible for the antimalarial activity of the diamidines.

### 1.3.2. Cancer

DNA targeting molecules are mainly used in the treatment of cancer. Cancer is one of the leading causes of death worldwide and accounted for 7.6 million deaths (approximately 13\% of all deaths) in 2008.\textsuperscript{115} There are currently 24.6 million people living with cancer worldwide and this is expected to increase with an estimated 11 million deaths by 2030.\textsuperscript{116,117} In Ireland, in 2005, cancer killed approximately 8,000 people with 3,500 of these being under the age of 70.\textsuperscript{118} These facts prove that we must do everything in our power to combat this family of diseases. Research is helping to identify the causes of cancer and is pointing in the direction to improved methods of diagnosis and treatment.

Cancer is a class of diseases in which a group of cells display uncontrolled growth and proliferation. Cancer cells do not respond to the body's control mechanisms, they divide excessively and invade other tissues. Cancer can be caused by many factors but, ultimately, its outcome is that cells divide and grow in the absence of growth signals, or they are able to overcome the resistance placed by antigrowth signals. This enables them to out-grow the constraints of their normal cellular compartment. Normally, under these conditions of
deregulated division and growth, cells experience many stresses signalling apoptosis, which usually protects us from cancer; therefore, it is even more important that tumours must also gain mechanisms that resist these apoptotic signals in order to succeed.\textsuperscript{119}

There are many different types of cancer that can affect almost every organ of the human body. According to the American Cancer Society the most common cancers include bladder cancer, lung cancer, breast cancer, melanoma, colon and rectal cancer, non-Hodgkin lymphoma, endometrial cancer, pancreatic cancer, kidney (renal cell) cancer, prostate cancer, leukemia and thyroid cancer. The most common type of cancer is prostate cancer, with more than 240,000 new cases expected in the United States in 2011. Figure 1.40 shows the estimated number of new cases of different types of cancers and the number of estimated deaths due to these cancers in the USA in 2011.\textsuperscript{120}

\begin{center}
\includegraphics[width=\textwidth]{cancer_cases Deaths.png}
\end{center}

Figure 1.40.- New cases of cancer and cancer deaths for 2011.\textsuperscript{120}

Every case of cancer is unique, with its own set of genetic changes and growth properties. Some cancers develop quickly while others can take years to become life-threatening. The differences between cases of cancer, even of the same organ, are the main reason why it is so difficult to treat. In spite of these differences, all cancers share some common features which are the basis for many cancer treatments and research efforts. The main feature that all cancers possess is the uncontrolled growth and proliferation of abnormal cells that can be triggered by a number of risk factors. Generally, the three main factors that can lead to cancer are: environmental factors (smoking may cause cancer of the lungs), genetic factors
(this is the case in breast and prostate cancer), and viral infections (human papillomavirus can result in cervical cancer).\textsuperscript{121}

Cancer is preventable in many cases. Learning what causes cancer and what the risk factors are is the first step towards cancer prevention. Unhealthy lifestyles such as smoking tobacco, poor diet, excessive alcohol consumption and physical inactivity are having a major influence on public health. More than 50\% of cancers are believed to be attributable to lifestyle factors. In the US, smoking is estimated to account for approximately 30\% of all cancers, obesity for 15\%, poor diet for at least 10\%, inactivity for 5\%, and alcohol intake for 4\%.\textsuperscript{122} Another risk factor that causes cancer is overexposure to Ultraviolet (UV) radiation which causes early aging of the skin and can lead to skin cancers such as melanoma.\textsuperscript{123} Both breast and prostate cancer are hereditary. Breast cancer is the main cause of death from cancer in women worldwide.\textsuperscript{124} Men with a family history of prostate cancer have twice the risk of developing prostate cancer.\textsuperscript{125,126}

\subsection*{1.3.2.1. Oncogenesis}

Oncogenesis is defined as the transformation of a normal cell into a cancerous cell. It is described as a number of changes on cellular and genetic level that cause a cell to undergo uncontrolled cell division, consequently forming a malignant tumor. It has been proposed that there are six vital alterations within cell physiology that lead together to malignant growth. These alterations show the difference between a healthy normal cell and a cancerous cell and include, self-sufficiency in growth signals, insensitivity to antigrowth signals (both leading to uncontrolled growth of the cancer cells), evasion of apoptosis, limitless replicative potential (immortality), continuous angiogenesis so as the tumour can grow beyond the limitations of passive nutrient diffusion, ability to invade other tissues and finally metastasis. Cancerous cells also lose the ability to repair genetic errors, resulting in an increased mutation rate thus initiating all the other changes listed above. These mutations produce oncogenes and tumor suppressor genes.\textsuperscript{127}

\subsection*{1.3.2.2. The Cell Cycle and Apoptosis}

The cell cycle describes the life of all cells in the human body. The cell cycle leads to cell duplication. It involves the complete and error-free replication of the whole genome, as well as the accurate segregation of sister chromatids to the daughter cells. The cell cycle is made up of four different phases, G\textsubscript{1} phase, S phase, G\textsubscript{2} and finally the M phase in which
mitosis occurs. DNA replication occurs during the S phase and chromosome segregation (process of nuclear division) occurs during M phase. In somatic (human body) cells, the S and M phases are segregated from gap phases known as $G_1$ which occurs before DNA replication and $G_2$ which occurs before mitosis. In the $G_1$ phase the cell is preparing for DNA synthesis and this is followed by $G_2$ during which the cell prepares for mitosis.

Cells in the $G_1$ phase can, before starting DNA replication, enter a resting state known as $G_0$ phase. Cells in $G_0$ phase make up for the major part of the non-growing, non-proliferating cells in the human body.\(^{128,129,130}\) Transition between the cell cycle phases occurs in a controlled manner and is regulated by different cellular proteins; these are a family of threonine/serine protein kinases known as cyclin-dependent kinases (CDKs). When these proteins are activated downstream processes are induced by phosphorylating selected proteins.\(^{130}\)

A set of checkpoints also monitors the completion of critical events and delays progression to the next phase of the cell cycle if necessary. Checkpoint control is not essential, they sense errors in critical events for example DNA replication and chromosome segregation. When checkpoints are activated, for example by underreplicated or damaged DNA, signals are relayed to the cell cycle-progression machinery causing a delay in cycle progression, until the danger of mutation has been averted.\(^{131}\)

Apoptosis is the process of programmed cell death that can occur in a multicellular organism. Each day, approximately 50 to 70 billion cells die in the average adult due to apoptosis. Apoptosis is necessary to make room for the billions of new cells produced daily. Programmed cell death is a complicated network of cell death blockers and inducers and therefore must be controlled in order to achieve homeostasis.\(^{132}\)

1.4. **Biophysical experiments to evaluate ligand DNA affinity**

The study of DNA minor/major groove binding, intercalating, alkylating and/or dual binding agents involves diverse fields of science, including biophysical and biochemical experiments. Some examples of biophysical experiments that allow evaluating DNA binding of small molecules include DNA thermal denaturation assays, circular dichroism (CD), linear dichroism (LD) and surface plasmon resonance (SPR) which are carried out to establish where and how well a ligand binds into DNA.
1.4.1. DNA thermal denaturation

This is an optical technique used to measure the binding affinity of a molecule to DNA. It provides a rapid, qualitative method for ranking compounds according to their binding affinity. A solution of DNA is prepared and the absorbance is measured as DNA is heated. This energy input breaks the intermolecular forces that hold the double helix together resulting in two single strands. This is known as melting of the DNA double helix and occurs at a precise temperature for different types of DNA. Minor groove binders/intercalators/dual binders that attach to the minor groove or intercalate will displace water molecules within the minor groove and therefore leading to an increase in the stability of the DNA-drug molecule complex. Therefore, more energy in the form of heat is necessary to lead to denaturation. This process is entropically favourable and is measured by monitoring the change in temperature ($\Delta T_m$) and is detected by a plot of absorbance vs. melting temperature.\(^{82}\)

1.4.2. Circular and Linear Dichroism (CD and LD)

CD is an optical technique that involves measuring the binding strength and mode of binding of molecules to DNA. It measures the differential absorption of left- and right-handed circularly polarised light within the framework of the chiral sugar–phosphate backbone. Stereospecific coupling of degenerate or near-degenerate electronic transitions between neighbouring base chromophores produces a CD signal. Typically, DNA binding ligands are achiral and, therefore, are optically inactive. Nevertheless, upon interaction with DNA, a ligand can acquire an induced CD (ICD) signal through the coupling of electronic transition moments of the ligand and the DNA bases. The ICD signal within the absorption bands of the achiral ligand indicates ligand–DNA interaction. Intercalators generally display small negative ICD signals, <10 M\(^{-1}\) cm\(^{-1}\) at the maximum of the ICD signal. Minor groove binders result in larger positive ICD signals. Data obtained from a CD spectrum of CD vs. wavelength is used to calculate binding constants and determine stoichiometry.\(^{133}\)

LD is the differential absorption of parallel and perpendicular linearly polarised light. The latter is chosen in the absorption band of DNA or of the ligand bound to DNA. A positive induced LD (ILD) signal indicates minor groove binding and a negative ILD signal indicates intercalation. This technique is used to estimate the mode of binding to DNA and
is particularly useful when analysing hybrid molecules due to the fact that simultaneous minor groove binding and intercalation can be displayed.\textsuperscript{134}

\subsection*{1.4.3. Surface Plasmon Resonance (SPR)}

SPR is also a valuable technique that provides kinetic and thermodynamic information and is a useful comparison to the previously mentioned experiments.\textsuperscript{135} In an SPR experiment, the corresponding oligonucleotide is immobilised onto a sensor chip, normally made of gold. The technique is based on the fact that light will reflect differently over this chip when it has immobilised DNA than when it has DNA bound to a molecule. Steady-state binding analyses are carried out with multiple injections of different compound concentrations over the immobilised DNA surface. Solutions of known ligand concentration are injected through the flow cells until a constant steady-state response is obtained. This is then replaced by buffer flow, resulting in dissociation of the complex. The response from the blank cell is subtracted from the response in each cell containing DNA to give a signal (response units (RU)) that is directly proportional to the amount of bound molecule. The predicted maximum response per bound molecule in the steady-state region (RU\textsubscript{max}) is determined from the DNA molecular weight, the amount of DNA on the flow cell, the compound molecular weight, and the refractive index gradient ratio of the molecule and DNA. Registering the changes in the RU with time, a sensorgram can be produced.\textsuperscript{136} SPR provides the real-time kinetics of a complex association and dissociation to be determined directly and can be used for reactions that have low to zero binding enthalpy. It also provides kinetic and thermodynamic information and has a number of advantages. It requires very small amounts of sample and can be used for determining a wide range of binding constants.\textsuperscript{137}

\section*{1.5. Previous work that constitutes the basis of this research}

Recent studies in the Rozas group have shown that different series of di-aromatic symmetric and asymmetric guanidinium/2-aminoimidazolinium molecules (Figure 1.41) structurally related to Furamidine\textsuperscript{138} strongly bind to the minor groove of DNA. Previous studies have shown that both guanidinium and 2-aminoimidazolium cations exhibit similarities geometrically and electronically.\textsuperscript{139} These molecules possess linked aromatic systems, cationic ends and HB acceptors and/or donors. The central linker of the diphenyl system was varied (X=CH\textsubscript{2}, CH\textsubscript{2}CH\textsubscript{2}, O, CO, NH, S, NHCONH, CONH, piperazine and fluorene).\textsuperscript{63,140}
Introduction

Chapter

The synthesis of isouroniums and hydroxyguanidinium (Figure 1.42) derivatives was also carried out and then, by using various biophysical techniques, some of these diphenyl dicationst possessing variable central bridges, have shown to strongly bind to the DNA minor groove. These studies have shown very encouraging results.\(^{63,138,141}\)

Thus in this work a new family of dual minor groove binder/intercalator agents were designed (Figure 1.43) combining a known DNA intercalator –acridine and a minor groove binders connected by an alkyl linker,

1.6. References

Introduction


48


34. http://en.wikipedia.org/wiki/File:Doxorubicin%E2%80%93DNA_complex_1D12.png


57. http://employees.csbsju.edu/hjakubowski/classes/ch331/bind/olbindtransciption.html


100. www.doctorswithoutborders.org/publications/article.cfm?id=4276&cat=ideas-opinions#fnref-1
103. First WHO report on neglected tropical diseases: working to overcome the global impact of neglected tropical diseases, 2010.

52
120. Cancer Facts and Figures 2011, American Cancer Society
124. E. Washbrook, Women’s Health Medicine, 2006, 3, 8.
127. D. Hanahan and R.A. Weinberg, Cell, 200, 100, 57.

Chapter 2

Objectives
2.1. **Objectives**

The three main objectives in this research are as follows:

1) **Synthesis of minor groove binding/intercalating dual agents and, hence, the corresponding preparation of new single minor groove binders and single intercalators:**

The synthesis of a new family of dual action agents combining a known DNA intercalator (acridine) and minor groove binders connected by different alkyl linkers will result in the identification of the optimal linker length between the minor groove binder moiety and the intercalator moiety. To be able to assess the effect of the alkyl linker on the potential activity of these dual agents, each independent DNA targeting moiety (minor groove binder and intercalator) attached to the alkyl linker will be prepared separately. Then, the evaluation of these three different families will allow us to evaluate possible synergistic interactions.

Firstly, we will synthesise the minor groove binder family which consists of aminoalkyl conjugates of *mono*-guanidine derivatives previously prepared by us.¹ These molecules involve the minor groove binder moiety being coupled with amino acids by means of an amide functionality, with varying minor groove binder central linkers and varying lengths of the amino acid chain (Figure 2.1).

![Figure 2.1- Minor groove binders previously prepared in Rozas' group and the amino acid conjugates of the *mono*-guanidine derivatives](#)
This preparation will follow the synthetic scheme of the *mono*-guanidylolation of the starting diamine previously developed by Rozas et al.,\(^2\) which will then be followed by a coupling reaction to connect the aminoalkyl chain with the minor groove binding moiety. Since these steps involved Boc protected groups, the last step will consist of the deprotection of the molecules.

The synthesis and analysis of this family will allow us to determine the influence of the attached alkyl chain on the minor groove binder DNA interaction. The results of this will be compared with that of the isolated minor groove binder which affinity has been previously determined.\(^1\)

Once the syntheses of the aminoalkyl conjugates of the *mono*-guanidine derivatives are complete, the preparation of the alkylcarboxylic acid intercalator derivatives will be carried out (Figure 2.2) incorporating a well known intercalator, acridine. This will allow us to determine the influence of the alkyl chain and the amide linker on the DNA intercalation.

![Figure 2.2.- Amino acid-intercalator derivatives](image)

Finally, syntheses of the dual action molecules composed of the minor groove binding and the intercalator moieties linked by means of an alkyl chain will be carried out (Figure 2.3). Evaluation of these compounds will allow to determine the difference in affinity and activity between the *mono*-functional and the bi-functional molecules and to consider possible synergies.

![Figure 2.3.- Minor groove binding/intercalating dual agents](image)
Objectives

Once all the molecules are prepared their DNA affinity and their antiparasitic activity will be analysed by means of biophysical and biochemical assays.

2) Evaluation of the DNA affinity of the mono- and bi-functional molecules prepared using different physicochemical techniques:

The second objective of this research is to carry out biophysical experiments to assess the DNA binding affinity and mode of binding of the molecules prepared. Strength of affinity and selectivity along with the mode of the interaction (intercalation vs. minor groove binding, or both) will be determined. Thermal denaturation experiments, which provide a rapid, qualitative method for ranking compounds according to their binding affinity, will be initially carried out to select the best DNA binding agents. These experiments will be carried out using salmon testes DNA to measure their binding affinity for mixed sequences. This will be followed by thermal denaturation assays with poly(dA-dT)$_2$ and poly(dA)$\cdot$poly(dT) DNA to investigate if the molecules show selectivity for AT sequences.

Thermal denaturation assays give limited information on binding to DNA. Therefore, it is necessary to carry out further experiments to gain more insight into, for example, the strength or mode of binding of the molecules to DNA. Accordingly, the protonation states (pK$_a$) of the minor groove binder molecules will be calculated to show if the molecules are mono- or di-cationic at physiological pH. If the minor groove binder molecules prove to be di-cationic they should interact more favourably within the minor groove. Intercalators can be either cationic or neutral, though acridine derivatives are in generally neutral at physiological pH. Hence, the pK$_a$ of the intercalators will be also measured. This study will be followed by reverse salt titration experiments with salmon testes DNA to confirm that the minor groove binder molecules are di-cationic within the minor groove. Another application for this technique is to distinguish between binding modes (intercalation vs. minor groove binding) since the minor groove binder can be easily displaced from the groove upon increasing ionic strength whereas the intercalator is not so easily displaced because it would be inserted in between the base pairs and would not be affected by the increasing salt concentration as much. Moreover, to compare the binding affinities and determine the binding mode of these compounds to DNA, circular dichroism (CD) experiments will be carried out. Additionally, Linear dichroism (LD) will be used to confirm the mode of binding of these molecules. This technique is used to estimate the
mode of binding to DNA and is particularly useful when analysing dual action molecules due to the fact that simultaneous minor groove binding and intercalation can be displayed.³

Lastly, Ultraviolet intercalator displacement experiments will be performed to determine the strength of binding, binding constants and mode of binding.

Once all the biophysical techniques are performed on the mono-functional DNA binders (minor groove binders and intercalators), the final aim of this project is to carry out physicochemical measurements to evaluate the DNA binding affinity and mode of the dual action molecules. Similar methodology will be follow, i.e. thermal denaturation assays will be carried out with salmon testes DNA and these experiments will be repeated in the presence of AT sequences for the best molecules. Considering that these tests provide good DNA binding results, further physicochemical measurements, such as the ones mentioned above, will be used to further analyse the binding affinity and mode of these compounds in detail.

3) **Biochemical assays to determine the cytotoxic activity of our compounds in Human African trypanosomiasis (sleeping sickness) and Plasmodium falciparum (malaria) cells:**

In collaboration with the Swiss Tropical and Public Health Institute, biochemical assays will be carried out on those compounds that display good DNA binding affinities, to measure their cytotoxic activity. This will deepen our understanding of the possible effects resulting from the DNA interactions of these molecules within the minor groove. Their cytotoxic effect will be evaluated in the line of *Trypanosoma brucei rhodesiense* STIB 900 and *Plasmodium falciparum* NF54 cells. Cytotoxic effects of the minor groove binders will also be assessed in L6 cells (rat skeletal muscle myoblast). The corresponding IC₅₀ values will be calculated for all compounds in the three cell lines. These biochemical assays will be performed not only to measure the cytotoxicity of our compounds, but also to compare the DNA binding affinity with the cytotoxicity results and see whether a correlation can be made between both sets of data. If such a correlation exists, we could conclude that the cytotoxicity was related to the DNA binding affinity.
2.2. References


Chapter 3

Synthesis of Aminoalkyl Derivatives of Di-Aromatic Guanidine Minor Groove Binders
3.1. Introduction

The minor groove of double helical B-DNA is a target site of great interest for developing new drugs due to the fact that it is the site of non-covalent high sequence specific interactions for a large quantity of small molecules. Minor groove binders have been shown to exhibit several novel biological activities including antiprotozoal, antiviral, and antibacterial properties. Additionally, some of these ligands have displayed antitumor activity leading to the discovery of many anticancer drugs. Some examples of minor groove binders include antibiotics of natural origin as well as synthetic and semisynthetic compounds, varying in molecular structure and mechanism of action.

Pentamidine, furamidine and a number of other diarylamidines known to be minor groove binders have been shown to be active against tropical diseases such as trypanosomiasis, leishmania, Pneumocystis carinii pneumonia and malaria. Lown et al. designed a number of minor groove binders displaying anticancer activity. They synthesised a series of new bis-(pseudo)benzimidazoles in which one of the units could be a benzimidazole, a pyridoimidazole or a imidazoquinine system connected to a piperazinyl functional group while the other unit is a benzimidazole containing different leaving groups (Figure 3.1). These bis-benzimidazoles showed to be actively cytotoxic against many human cancer cell lines in particular in renal, central nervous system, colon, melanoma, and breast cancer.

![Figure 3.1.- Bis-benzimidazoles synthesised by Lown et al.](image)

Even though a number of minor groove binders have been developed displaying antiprotozoal or anticancer activity, unfortunately, a number of them also display a high level of toxicity. Pentamidine is ineffective when given orally and therefore must be given by injection. Side effects such as abscesses at the site of injection, abnormal liver
functioning, hypotension, nephrotoxicity, cardiotoxicity and hypoglycemia can occur as a result of taking this drug.\textsuperscript{10}

Netropsin and distamycin are examples of model minor groove binders displaying all the essential structural features required for binding to the minor groove. They are ideal for studying the physical characteristics necessary for targeting the minor groove, such as their concave shape to complement the convex groove and specific hydrogen bonding donor and acceptors. Research on netropsin and distamycin has led to the development of new and improved drugs. Hybrid molecules such as the uramustine-distamycin derivatives (45) (Figure 3.2) designed by Baraldi et al.\textsuperscript{11} have shown significantly enhanced antitumor activity relative to that of the parent compounds distamycin A and uramustine.

A number of studies have been carried out to get a better understanding of the mode of action of minor groove binders. The crystal structure of the complex formed between the dodecanucleotide d(CGCGAATTCCGCG)\textsubscript{2} and the drug pentamidine has been determined.\textsuperscript{12} Analysis of the structure shows the drug to be bound in the 5'-AATT minor groove region of the duplex, with the amidinium groups hydrogen bonded to adenine N3 atoms in an interstrand manner. The pentamidine molecule adopts an extended conformation, and the immediate binding site spans four base pairs. This crystal structure proved that these types of compounds bind to the minor groove and also provided information about the structural features required to achieve binding. Another crystal structure of a trypanocidal 4,4'-\textit{bis}-(imidazolinylamino)diphenylamine bound to the oligonucleotide sequence 5'd(CTTAATTCGAATTAAG) was determined\textsuperscript{13} and showed that although 4,4'-\textit{bis}-(imidazolinylamino)diphenylamine is expected to be highly twisted in its energy minimized state,\textsuperscript{14} it adopts a more planar crescent shape when bound in the
minor groove of the DNA. Interactions of the molecule with 5'-AATT comprise of bifurcated HBs resulting in selectivity for this site and favourable van der Waals interactions in a slightly widened minor groove. Therefore, an induced fit results from conformational changes in both the small molecule and DNA.

The X-ray crystal structures of two N-alkylamidine furans bound to a specific DNA sequence were also obtained, further proving minor groove binding. This research allowed for the determination of ideal functionalities for binding and thus large families of furamidine derivatives to be synthesised. A number of studies have analysed these physical features of minor groove binders such as the importance of the position and nature of the cationic groups and the role that water molecules play in the binding of more linear derivatives.

To design new minor groove binders, it is important to understand why furamidine and its derivatives strongly bind to the minor groove. Thus, it is essential to identify the physical characteristics that make a certain compound an efficient minor groove binder and to compare these features with those of our target compounds. Minor groove binders generally have long and flat structures, allowing them to adopt a crescent shape forming close interactions in the deep, narrow space between the DNA strands. The curvature of the minor groove is thought to be essential for enzyme recognition and for the correct functioning DNA-associated processes. In general, minor groove binders have unfused aromatic and/or heterocyclic systems; resulting in a high degree of flexibility that gives a snug fit in the groove. The surface of the minor groove is convex, which complements the concave structure of a typical minor groove binder. In addition, the minor groove has a strong negative potential which complements the cations usually present in minor groove binders. These structures are generally stabilised by HBs with the N3 of adenine and the O2 of thymine, as well as hydrophobic and ionic interactions. The interaction of the cationic terminal groups of typical minor groove binders with the negative potential of the groove causes a release of a large number of water molecules (spine of hydration) thereby leading to an increase in entropy.

The structure of twin molecules such as furamidine is extremely important for minor groove binding. The symmetry and the existence of the two cationic groups are vital as the mono-cations have shown to cause a decrease in binding affinity. It has also been proven
that the two aromatic rings are most effective as phenyl ring and in a \textit{para-para} substituent arrangement.\textsuperscript{17}

We have reported in the past the preparation of symmetric and asymmetric di-aromatic guanidinium/2-aminoimidazolinium derivatives.\textsuperscript{17,18} These molecules possess similar characteristics to furamidine such as a crescent shape, two cationic ends, HB donor groups, two phenyl rings, and a central linker containing polar atoms. Replacing the amidine group in furamidine with guanidine should lead to higher binding affinity, due to the extra NH HB donor and a larger flexibility. Guanidine and 2-aminoimidazoline cations are similar in terms of geometrical parameters and in electronic level properties.\textsuperscript{19} These two types of cations are suitable and commonly present in minor groove binding agents. Netropsin has a guanidine moiety and derivatives of netropsin containing 2-aminoimidazoline cations have been synthesised.\textsuperscript{20,21}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{furanamide.png}
\caption{Furamidine, pentamidine and symmetric (bis-guanidine, bis-2-aminoimidazoline) and asymmetric (guanidine/2-aminoimidazole) derivatives previously prepared in the Rozas group.}
\end{figure}

\textit{Mono}-cationic derivatives and symmetric/asymmetric dications containing guanidinium and/or 2-aminoimidazolinium were synthesised by Rozas and co-workers\textsuperscript{22,18} to examine the importance of symmetry within the molecule, and the importance of electrostatic interactions between the molecule and the minor groove. The central linker was varied where $X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH}, \text{S}, \text{NHCONH}, \text{CONH}, \text{piperazine}, \text{CO}, \text{NHCONH}$ to study the effect of electron donating or electron withdrawing groups. This led to the investigation into the optimal geometry and electronic properties of the compounds. HB formation was also studied using different $X$ linkers. Once the compounds were prepared, biophysical studies were carried out to determine their binding affinity and mode towards DNA. Techniques such as CD, LD and SPR were used to determine the exact mode of
binding, whilst ITC was used to determine the thermodynamics of this binding process. These results show that these compounds exhibit strong DNA binding affinity that correlates with the expected 1:1 binding ratio.\textsuperscript{23,24,14}

The synthesis of isouronium and hydroxyguanidinium derivatives with varying X linkers has also been carried out previously in our laboratory.\textsuperscript{25,26} The isouronium derivatives replace the NH (HB donor) of guanidine with an O (HB acceptor) group and the hydroxyguanidinium derivatives contain a hydroxyl group which can act simultaneously as a HB acceptor and an extra HB donor. An additional contact was therefore introduced with the intention of improving DNA binding.

![Chemical structures](image)

Figure 3.4.- Isouronium and hydroxyguanidinium derivatives prepared by Rozas and co-workers\textsuperscript{25}

Taking this into account and based on the binding activity of our previously prepared minor groove binders we intend to combine the aliphatic linker of pentamidine and the diaromatic structure of our minor groove binders by attaching aminoalkyl chains to one of the aromatic rings. The central linker group will be varied where X= CH\textsubscript{2}, CH\textsubscript{2}CH\textsubscript{2}, O, CO, and NH. Also the length of the aminoalkyl chain will be varied with three, four, seven, ten and 11 methylene groups. This will allow us to determine the influence of the aliphatic chain on the minor groove binding. Thus, the synthesis carried out in order to achieve these goals and the subsequent results obtained are described next.

![Chemical structures](image)

Figure 3.5.- General structure of the compounds prepared in the present study
3.2. Synthesis of the Aminoalkyl Derivatives of Guanidine Di-aromatic Minor Groove Binders

The preparation of the target aminoalkyl derivatives of guanidine di-aromatic minor groove binders involves first, the preparation of the guanidylating agent, then syntheses of the mono-guanidylated di-aromatic systems, as previously prepared by us,\(^{24}\) followed by the Boc-protection of the amino terminal group of various aminoalkylcarboxylic acids of different lengths. Coupling of both moieties is then carried out by means of an amide functionality. Finally, deprotection of the Boc protected aminoalkyl derivatives of guanidine di-aromatic minor groove binders is carried out to give the final dihydrochloride salts. This synthesis is described in detail in Scheme 3.1.

Scheme 3.1

3.2.1. Synthesis of the Guanidylating Agent

Considering that the target molecules contain a guanidine moiety, the synthesis of all the proposed molecules must involve the introduction of this particular functionality. Hence, the first step in the preparation of the target compounds was the synthesis of such guanidylating agent. Bearing in mind the low nucleophilicity of the aromatic amines used in the synthesis of the mono-guanidines, the first step was the activation of the corresponding commercial guanidylating agent. Kim and Qian\(^ {27} \) proposed that the reactivity of thiourea
46. Scheme 3.2) as a guanidylating agent is increased by the introduction of Boc (tert-butoxycarbonyl) protecting groups to give the \( N,N'-\text{bis-(tert-butoxycarbonyl)} \) thiourea (47).

The Boc protecting groups remove the nucleophilicity from each of the amino groups whilst simultaneously increasing the electrophilic character of the central carbon, thus activating it towards attack. The Boc protecting group was chosen because the synthesis of 47 is simple and the carbamate protecting group is stable in the presence of bases and nucleophiles and can also be easily removed. Thus, the Boc protection reaction of thiourea was achieved by reacting thiourea with di-tert-butyldicarbonate (Boc\(_2\)O) in dry tetrahydrofuran (THF) in the presence of sodium hydride. This reaction was stirred overnight under argon initially at 0 °C when Boc\(_2\)O was added, and then brought up to 20 °C. Upon completion of the reaction it was quenched using saturated NaHCO\(_3\) and subsequently worked-up. Purification by washing with hexane afforded a white solid with a final yield of 44%. This low yield could be a result of solidification of the reaction overnight which leads to a slower reaction.

\[
\begin{array}{c}
\text{H}_2\text{N} \quad \text{NaH, Boc}_2\text{O, THF} \\
\text{S} \quad 0^\circ\text{C-rt, 20h} \\
\text{NH}_2 \quad \text{BocHN} \\
\end{array}
\]

Scheme 3.2

The mechanism of this reaction involves both of the amino groups on the thiourea being irreversibly deprotonated by NaH expelling H\(_2\) gas. The highly basic and reactive thiourea salt attacks di-tert-butyldicarbonate to yield the relevant mono-tert-butoxycarbonyl-protected thione, CO\(_2\), and sodium tert-butoxide. As there is a further thioamide group on the mono-Boc-protected thione, the two steps previously mentioned are repeated, resulting in the formation of 47.\(^{25,26}\)

The synthesis of a guanidylating agent with a different protecting group, the Fmoc derivative \( N,N'-\text{bis-(fluorenylmethyloxycarbonyl)} \) thiourea (48), was also attempted. Thus, to a solution of thiourea in 10% aqueous acetic anhydride (AcOH) Fmoc chloride dissolved in 1,4-dioxane was added (Scheme 3.3). The mixture was stirred overnight at room temperature. Unfortunately, this reaction did not go to completion and therefore this method was abandoned.
Finally, the alternative carboxybenzyl (Cbz) protected guanidylating agent \( N,N'-\text{bis-} \) (carboxybenzyl)thiourea (49) was also synthesised. This reaction was initially carried out similarly to that with Fmoc and also failed; for that reason, a second method of synthesis was used involving the same procedure followed for the Boc derivatives (Scheme 3.4). Purification of the product afforded a white solid with a yield of 28%. This molecule was synthesised as a precursor to a different method of synthesis of the final dual binding agents that will be discussed in Chapter 5. The experimental for compound 49 is described in the appendix.

3.2.2. Synthesis of the Mono-Guanidine Derivatives

Once the guanidylating agent was prepared by Boc protection as described above, the next step was to use it in the mono-guanidylation of the aromatic diamines (50a-e). This reaction can be carried out by two methods. The first one involves mono-Boc protection of one the aromatic amino groups, followed by guanidylation of the remaining amino group. Three equivalents of the aromatic diamine are reacted with one equivalent of Boc to avoid Boc protection of both amino groups. Then, the mono-Boc diamine obtained is reacted with the guanidylating agent 47 in a 1:1 ratio, using mercury (II) chloride to give the mono-Boc protected guanidines (51a-e). The second method involves the introduction of the guanidine moiety by reacting the Boc-protected thiourea derivative 47 with the corresponding commercial aromatic diamines directly in the presence of excess triethylamine (TEA) in dichloromethane (DCM) or dimethylformamide (DMF) depending on the polarity of the starting diamine. This reaction is carried out with a 3:1 ratio of amine:guanidylating agent in order to minimise the formation of the bis-guanidine
derivatives. As mercury (II) chloride (HgCl₂) was used, a characteristic black precipitate (mercury (II) sulphide, HgS) was normally formed. This was considered a sign of a complete reaction as displayed in Scheme 3.5. The reaction conditions were quite mild and involved stirring, initially at 0 °C and then overnight at room temperature under argon. Progress and completion of the reaction was monitored by thin layer chromatography (TLC) accordingly. Once the reaction was completed, a work-up involving dilution with ethyl acetate (EtOAc) followed, and then the removal of the HgS by filtering the reaction mixture through a pad of celite was performed. Next, the crude product was washed with water and brine, dried over anhydrous Na₂SO₄ and concentrated under vacuum to give an oil that was purified by silica gel chromatography. This led to the preparation of compounds 51a-e (Scheme 3.5); compounds 51a, 51b and 51c are all white solids while 51d is a pale yellow solid and 51e is a purple solid.

\[
\text{Scheme 3.5}
\]

Previous work within our group shows that the second method of those described before is more efficient and results in higher yield overall because it involves only one step instead of the two steps required in the first method. Additionally, the second amino group needs to be deprotected for the coupling reaction in the next step. Therefore, the second strategy was the one followed.

A commercially available derivative of 47, 1,3-\textit{bis}(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (52) was found to work in a similar manner and, therefore, was used as the guanidylating agent in the subsequent reactions.

\[
\text{Figure 3.6.- Structure of 1,3-\textit{bis}(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (52)}
\]
Kim and Qian\textsuperscript{27} suggested that this reaction mechanism proceeds through a carbodiimide intermediate formed by desulphurisation of the substituted thiourea. The carbodiimide then undergoes nucleophilic attack by the amine to generate the product guanidine. The mechanism of this reaction involves coordination of the sulphur of 47 to the thiophilic mercury of the HgCl\textsubscript{2} catalyst. This results in the formation of a new bond and the subsequent loss of one of the chlorine atoms producing a highly electrophilic carbon atom. Triethylamine (TEA) then deprotonates one of the N atoms and the other one then donates its lone pair of electrons to the central carbon, resulting in the formation of mercury (II) sulphide as well as the loss of the remaining chlorine. Finally, deprotonation by TEA leads to the formation of a carbodiimide species which is believed to be the actual guanidylating reagent (Scheme 3.6).

![Scheme 3.6](image)

Carbodiimides are well recognised for their reaction with amines to form guanidines.\textsuperscript{28} Thus, it is possible that guanidine derivatives are formed via the carbodiimide mechanism. Carbodiimides are highly unstable species; the central carbon is unusually electrophilic and therefore is susceptible to be attacked by any available nucleophiles. Because of this, the carbodiimide could be formed \textit{in situ} in the synthesis of the \textit{mono}-guanidylated diamines; thus, the nucleophilic aromatic NH\textsubscript{2} of di-aromatic diamines 50a-e would attack the carbodiimide intermediate at the electrophilic central carbon, resulting in the cleavage of the double bond and the migration of an electron pair onto one of the nitrogen atoms. Finally, a proton transfer could happen resulting in the formation the \textit{mono}-guanidylated di-aromatic derivatives (Scheme 3.7, 51a-e).
Aminoalkyl Guanidine Minor Groove Binders

Chapter 3

Kim and Qian,\textsuperscript{27} unfortunately, were unable to isolate the bis-Boc carbodiimide intermediate; although, they obtained spectra \textit{in situ} which proves the existence of a mercury complex of bis-Boc carbodiimide. The authors were unsuccessful in determining the exact structure of the intermediate.

The five resulting compounds 51a-e were obtained in moderate to good yields ranging between 34 and 73\%. These compounds were chosen based on the DNA thermal denaturation results previously obtained where the most interesting binding was achieved for compounds with the linkers X= CH\textsubscript{2}, CH\textsubscript{2}CH\textsubscript{2}, O, NH, and CO. These compounds have been previously reported by us and, therefore, analysis of the $^1$H NMR and melting points of these compounds proved the successful preparation the Boc-protected mono-guanidines.

Table 3.1.- Yields obtained for the Boc protected mono-guanidines (51a-e).

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>51a</td>
<td>CH\textsubscript{2}</td>
<td>59</td>
</tr>
<tr>
<td>51b</td>
<td>CH\textsubscript{2}CH\textsubscript{2}</td>
<td>57</td>
</tr>
<tr>
<td>51c</td>
<td>O</td>
<td>73</td>
</tr>
<tr>
<td>51d</td>
<td>CO</td>
<td>42</td>
</tr>
<tr>
<td>51e</td>
<td>NH</td>
<td>34</td>
</tr>
</tbody>
</table>
3.2.3. Synthesis of the Boc-protected amino acids

The next step in the synthesis of the target compounds was the Boc protection of the amino terminal of various amino acids. Different amino acids with three, four, seven, ten and 11 methylene groups [4-aminobutanoic (53), 5-aminopentanoic (54), 8-aminooctanoic (55), 11-aminoundecanoic (56) and 12-aminododecanoic (57) acids] were considered. These amino acids were chosen due to the fact that they resemble the aliphatic linker seen in pentamidine and to find the optimal length of the aliphatic chain. Considering this and the binding affinity of our previously prepared minor groove binders, coupling of an aminoalkyl carboxylic acid with di-aromatic minor groove binders will be carried out by attaching the carboxylic group of the corresponding amino acid to the amino group of the mono-guanidine derivatives.

First of all, the amino acid must be protected so that the amino group of the mono-guanidine attacks at the terminal carboxyl leading to a successful coupling reaction and avoiding polymerisation. A number of test reactions, based on different research found in the literature, were carried out to find the best reaction conditions for Boc-protection of the amino acids. The Boc protecting group was chosen because its stability under basic conditions, which are utilised in the coupling reaction in the next step; additionally, the use of the Boc protecting group is advantageous because it is also used in the synthesis of the Boc-protected mono-guanidines and, thus, all three Boc groups can be removed simultaneously to give the final dihydrochloride salts.

The first method of Boc protection tested involved dissolving amino acid 54 in methanol (MeOH) in the presence of TEA. This reaction was left to stir at room temperature for 30 minutes. Di-tert-butyl dicarbonate was added to the reaction mixture and this was left to stir overnight at room temperature (Scheme 3.8). Work up involved addition of ethyl acetate to the mixture, which was extracted with water and the combined organic phases were washed with brine, dried over Na₂SO₄ and concentrated under vacuum. Unfortunately, this reaction did not go to completion and, therefore, gave a mixture of starting material and product which was difficult to purify. This reaction was attempted with other amino acids such as 53 and 55 giving similar results, hence, this method was abandoned and other routes were investigated.
The next set of conditions involved the reaction using Boc₂O, TEA and dioxane as a solvent at 0 °C overnight. A solution of di-tert-butyl dicarbonate in dioxane was added dropwise to a solution of amino acid 55 in dioxane in the presence of TEA at 0 °C (Scheme 3.9). The reaction was allowed to stand overnight. The crude product was concentrated under reduced pressure, 1 M HCl was added and then EtOAc. The organic layer was washed with water, dried over Na₂SO₄ and concentrated under vacuum. The compound was obtained as an impure oil and considering that these impurities interfered in the next reaction, purification was attempted even though leading to minimal recovery of the final compound.

The third set of conditions tested involved the use of Boc₂O, TEA and THF/H₂O as a solvent at room temperature for 20 hours and it was not useful for the protection of most amino acids due to solubility difficulties regarding most of the starting materials. Only amino acid 56 was soluble under these conditions. Thus, TEA was added to a stirred solution of 56 in a mixture of THF/H₂O (1:1). After ten minutes di-tert-butyl dicarbonate was added and the reaction mixture left to stir for 20 hours (Scheme 3.10). The solution was reduced in volume, dissolved in DCM and washed with 1 M HCl. The organic layer was dried over Na₂SO₄, filtered and the filtrate reduced in volume to obtain a colourless powder that was recrystallised from hexane to afford the product in a yield of 40%.
Due to the low yield and solubility problems, a final test reaction was attempted involving the use of MeOH at 60 °C for 24 hours in the presence of TEA. Since the previous reaction using MeOH (Scheme 3.8) did not fully go to completion it was thought that refluxing at 60 °C it could increase the possibility of the reaction to go to completion. After work-up (concentration of the product and the residue redissolved in EtOAc), the mixture was washed with 0.25 M HCl, dried, filtered and concentrated under vacuum to give a white solid which was purified by recrystallisation over hexane. Recrystallisation was chosen as a method of purification because it has given higher yields in previous test reactions (Scheme 3.11).

Scheme 3.11

In Table 3.2 the conditions utilised within the various test reactions for the Boc-protection of the different amino acids (53-57) are summarised.

Table 3.2.- Conditions tested for Boc-protections of amino acids (53-57).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Boc$_2$O</th>
<th>Temperature</th>
<th>Base</th>
<th>Solvent</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>53, 54, 55 (1 eq)</td>
<td>1 eq</td>
<td>Rt</td>
<td>TEA (1.4 eq)</td>
<td>MeOH</td>
<td>-</td>
</tr>
<tr>
<td>55 (1 eq)</td>
<td>1.5 eq</td>
<td>0 °C</td>
<td>TEA (10 eq)</td>
<td>Dioxane</td>
<td>-</td>
</tr>
<tr>
<td>56 (2.2 eq)</td>
<td>1 eq</td>
<td>Rt</td>
<td>TEA (1 eq)</td>
<td>THF/H$_2$O</td>
<td>40</td>
</tr>
<tr>
<td>57 (1 eq)</td>
<td>1 eq</td>
<td>60 °C</td>
<td>TEA (1.2 eq)</td>
<td>MeOH</td>
<td>57</td>
</tr>
<tr>
<td>55 (1 eq)</td>
<td>1 eq</td>
<td>60 °C</td>
<td>TEA (1.2 eq)</td>
<td>MeOH</td>
<td>60</td>
</tr>
</tbody>
</table>
This last reaction was carried out on a small scale for 12-aminododecanoic and 8-aminooctanoic acids giving satisfactory yields; hence, the reaction was upscaled and repeated for the remaining amino acids in the series. The five resulting compounds (58a-e) gave high yields ranging between 57 and 100% (Table 3.3). As these compounds have been previously prepared they were characterised only by $^1$H NMR and melting points.

Table 3.3.- Yields obtained in the preparation of the Boc protected amino acids (58a-e).

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>N</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>58a</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>58b</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>58c</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>58d</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>58e</td>
<td>11</td>
<td>57</td>
</tr>
</tbody>
</table>

The Cbz-protected amino acids were also synthesised to be used later on in a coupling reaction with the Boc-protected mono-guanidines to form orthogonally protected conjugates for the preparation of dual agents (this synthesis is discussed in detail in Chapter 5 of this thesis). The amino group of amino acids 53, 54 and 55 was protected with Cbz by the method described in Scheme 3.12. A mixture of the corresponding amino acid in a 2 M aqueous sodium hydroxide solution was cooled in an ice bath to 0 °C. Under vigorous stirring benzyl chloroformate and a 2 M aqueous sodium hydroxide solution were simultaneously added within two minutes. The mixture was stirred for 20 minutes at room temperature and extracted with diethyl ether. The aqueous layer was separated and acidified with concentrated HCl to a pH of 2. The resulting emulsion was extracted with ethyl acetate. The organic phases were combined, washed with brine, and dried with Na$_2$SO$_4$. Concentration in vacuo gave white needles, which were dried under high vacuum.
The three resulting compounds 58f-h gave yields ranging between 42-51%. The experimental for these three compounds can be found in the appendix section. As these compounds have been previously prepared they were only characterised by $^1$H NMR and melting points.

### 3.2.4. Synthesis of the Boc-protected aminoalkyl mono-guanidine conjugates

Having synthesised the two moieties required to form the Boc-protected aminoalkyl mono-guanidine conjugates (60a-e, 61a-e, 62a-e, 63a-e, 64a-e), the next step was to carry out the corresponding coupling reaction. To join the Boc-protected amino acids with the di-aromatic mono-guanidines, different reactions were explored. The first method attempted involved the formation of the acid chloride of the amino acid, which was then reacted with the mono-guanidine in acetonitrile (MeCN) in the presence of TEA (Scheme 3.13).

The conversion of the starting Boc-protected amino acid to its acid chloride involved dissolving the Boc derivative in DMF, stirring it for ten minutes, then, adding an excess of thionyl chloride (SOCl$_2$) under inert atmosphere and allowing the reaction to reflux for 5 hours. Following this, the mixture was cooled and excess SOCl$_2$ was mostly removed by distillation with traces being eliminated under vacuum. A bright yellow oil (acid chloride
of 4-aminobutyric acid or 5-aminovaleric acid) was obtained which was used in the next step of the reaction without further purification or characterisation.

\[
\text{BocHN} \quad \xrightarrow{\text{SOCl}_2 (10.0 \text{ eq.})} \quad \text{BocHN} \quad \xrightarrow{\text{DMF reflux, } 80^\circ \text{C, } 5 \text{ h}} \quad \text{Cl} \\
\text{n=3 (58a), 4 (58b)} \quad \xrightarrow{\text{DMF reflux, } 80^\circ \text{C, } 5 \text{ h}} \quad \text{n=3 (59a), 4 (59b)}
\]

Scheme 3.14

The acid chloride produced is then reacted with the Boc-protected mono-guanidine in MeCN and in the presence of TEA. This reaction can also be performed by dissolving the Boc-protected mono-guanidine in DMF along with a catalytic amount of 4-dimethylaminopyridine (DMAP) at 0 °C under inert atmosphere. In this case, the acid chloride is dissolved in DMF and added drop-wise to the amine solution leaving the reaction stirring for 20 hours on ice. Work up involves dissolving in EtOAc, washing with 2 M HCl solution, saturated NaHCO\(_3\) solution and finally brine. Drying with MgSO\(_4\) and concentrating under vacuum should result in the formation of the required product.

The reaction mechanism for the synthesis described (Scheme 3.14) to form the acid chloride is noteworthy. First of all, the conversion of the starting carboxylic acid to its acid chloride is a derivative of the Vilsmeier-Haack reaction where SOCl\(_2\) is used instead of POC\(_1\(_3\). The reaction of an N,N-disubstituted formamide, such as DMF or N-methyl formanilide, with acid chlorides, such as phosphoryl chloride (POCl\(_3\)) or phosgene, leads to the formation of adducts. These complexes which are usually referred to as the Vilsmeier-Haack reagent have many important applications in synthetic organic chemistry especially in the formulation of electron rich aromatic compounds or alkenes. The Vilsmeier–Haack reagent which is formed \textit{in situ} is a chloroiminium salt and it is a weak electrophile; therefore, the reaction works better with electron-rich carbocycles and heterocycles.\(^{33}\) The Vilsmeier-Haack reaction encompasses the use of DMF and POCl\(_3\) to form a carbon electrophile without the need for a strong acid or Lewis acid and is an alternative to the Friedel-Crafts acylation.\(^{34}\)

The first step of mechanism is the reaction between the catalytic DMF and the highly electrophilic sulphur atom of SOCl\(_2\). This nucleophilic substitution of chlorine encourages the migration of electrons producing a highly electrophilic chloroiminium cation. Reaction of the chlorine anion with the C=N bond leads to the formation of the tetrahedral
intermediate, with SO₂ and HCl as gaseous side-products. This reactive intermediate is unstable and, therefore, reacts rapidly with the nucleophilic carboxylic acid of the amino acid. The carboxylic acid loses a proton and the electrons move to the nitrogen atom thus neutralising the positive charge upon it, the new intermediate breaks down with the loss of another chlorine and the formation of another intermediate species. The chloride then interacts with the carbonyl carbon giving the required acid chloride and DMF as a side product. The mechanism of this reaction is displayed in Scheme 3.15.

Once the acid chloride is successfully synthesised, it is then reacted with the corresponding amine in MeCN and in the presence of TEA. As stated earlier, this reaction can also be carried out in the presence of a catalytic amount of DMAP in DMF. DMAP is chosen as an appropriate acylation catalyst because the amino group supports its nucleophilic nature making it highly reactive; thus, only a small quantity of DMAP is required. The mechanism (Scheme 3.16) for this reaction starts with the migration of the lone pair of electrons from the NMe₂ to form the double bond. Then the electrons of aryl ring migrate to N of the pyridine which then attacks the carbonyl carbon which forms an intermediate. Subsequently, the C-Cl bond is cleaved to give the intermediate N-acyl pyridinium. The amine then attacks the carbonyl carbon followed by rearrangement of the electrons resulting in the formation of the amide-linked product and the regeneration of DMAP. This regeneration of the catalyst allows for recycling of the catalyst which can be used in further reactions.
Unfortunately, this reaction was unsuccessful due to the occurrence of polymerisation. It may also have not worked, considering that our reaction may have come in contact with moisture in the atmosphere, the thionyl chloride could have decomposed.

Due the unsuccessful formation of the Boc-protected aminoalkyl mono-guanidine conjugates following the method above described, it was necessary to find an alternative coupling. A number of coupling reagents were studied and both DCC (N,N'-dicyclohexylcarbodiimide) and TBTU (O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate) were considered useful. DCC is a carbodiimide whose primary use is to couple amino acids during automated peptide synthesis. It is commonly used to prepare amides, esters and acid anhydrides from carboxylic acids. This reagent can also be used to convert an aldehyde into an oximes and dehydration of the latter into nitrile, which can be useful in organic synthesis. Carbodiimides contain two weakly basic nitrogen atoms, which are adequate to trigger a reaction with an acid to generate an O-acylisourea. The O-acylisourea of a peptide is one of the most reactive species and, therefore, rapidly undergoes aminolysis in the presence of an amine to produce the peptide. Within a few minutes, a large amount of N,N'-dicyclohexylurea (DCU) precipitates out of any reaction using DCC and traces of DCU are difficult to remove (even after passage through a chromatographic column), since it is soluble only in trifluoroacetic acid (TFA). Alternatives to DCC have been developed such as
diisopropylcarbodiimide (DIC), \( N\)-ethyl-\( N'\)-(3-dimethylaminopropyl)carbodiimide and \( N\)-cyclohexyl-\( N'\)-isopropylcarbodiimide (CIC) which are all relatively soluble in DCM.\(^{36}\)

The general reaction mechanism of DCC involves the abstraction of the proton of the carboxylic acid resulting in the formation of the carboxylate anion and the positively charged carbodiimidinium. Then, the carboxylate attacks at the central carbon of the carbodiimidinium causing neutralisation of the positive charge forming an intermediate ester. The amine then reacts with this ester at the carbonyl carbon and movement of the electrons forms the amide and the by-product DCU (Scheme 3.17). The ester is activated because substitution with any nucleophile such as an amine expels this very stable urea as a leaving group.

![Scheme 3.17](image)

As previously mentioned coupling reactions using DCC generate the side-product DCU which is difficult to remove. DCC is also a potent allergy inducer so sensitised individuals can develop a nasty rash and swollen throat when exposed to it. Because of these two reasons the use of DCC was rejected. Alternatives such as DIC and EDC were not available in the lab. The hydrate of hydroxybenzotriazole (HOBt) can be used to eliminate the production of DCU but was not useful under the conditions of coupling considered since an inert atmosphere was required.

Taking all this into account, TBTU was proposed as a coupling reagent to join the Boc-protected amino acids to the Boc-protected mono-guanidines. TBTU is a aminium salt coupling reagent which has been used in the past as an activating agent of carboxylic acids for the preparation of 1,2,4, oxadiazoles\(^{37}\), amides and phenylhydrazides\(^{38}\) and esters. The tetrafluoroborate or hexafluorophosphate anion seen in TBTU and \(O\)-benzotriazole-
Aminoalkyl Guanidine Minor Groove Binders

Chapter 3

*N,N,N′,N′*-tetramethyluroniumhexafluoro phosphate (HBTU) is generally used as the non-nucleophilic counterion in aminium reagents. A comparison study between HBTU and TBTU showed that the counterion had no significant influence on the coupling rate or racemisation.\(^{39}\) The general mechanism for the synthesis of amides using TBTU is described in Scheme 3.18 below.

\[
\begin{align*}
\text{DIEA (N,N-diisopropylethylamine) deprotonates the carboxylic acid to give the carboxylate which, then, attacks the positively charged aminium salt. Migration of the electrons from the dimethyl nitrogen forms the O-benzotriazole; then, the negatively charged oxygen attacks at the carbonyl carbon of the ester cleaving it. This leads to the formation of the ester derivative of benzotriazole which is next attacked by the amine at the carbonyl carbon to give the required amide and 1-benzotriazolol as a side product which is easily removed by purification with silica gel chromatography. The conditions that were first attempted involved TBTU, TEA, the Boc-protected *mono*-guanidine and the Boc-protected amino acid being dissolved in DMF at room temperature under argon (Scheme 3.19.).}^{37}
\end{align*}
\]

The Boc-protected amino acid was dissolved in DMF and TEA was added to this. The Boc-protected *mono*-guanidine was added and the reaction left to stir until it dissolved. At 0 °C, TBTU was dissolved in DMF and added to the reaction mixture. The mixture was left to stir for one hour at 0 °C, brought up to room temperature and left for an additional three hours. The work up involved the reaction mixture being poured over
EtOAc and extracted with water dried with Na$_2$SO$_4$ and concentrated under vacuum. This reaction was attempted with a number of different Boc-protected mono-guanidines (51a and 51b) and one of the Boc-protected amino acids (58a). This coupling reaction gave impure products in low yields which were difficult to purify; it did not go to completion and required longer reaction times. Moreover, to fully deprotonate the amine, more equivalents of base were required. Lastly, other solvents were considered due the high boiling point of DMF. Therefore, different conditions were explored for this reaction.

Scheme 3.19

The method which gave the best results involved preparing a solution of the corresponding Boc-protected amino acid (1.2 equivalents) in MeCN and treating it with 3.8 equivalents of DIEA. Then, the di-aromatic mono-guanidine (1 equivalent) and TBTU (1.2 equivalents) were added to the reaction mixture under inert atmosphere. The mixture was stirred at room temperature for 18 hours and the reaction was monitored by TLC. Upon completion, the reaction was partitioned between brine and EtOAc, the organic layer washed with 0.1 M HCl and 5% NaHCO$_3$, dried over anhydrous Na$_2$SO$_4$, filtered and concentrated under vacuum. Purification by flash chromatography with silica gel eluting with hexane/EtOAc (2:1) yielded the required products 60a-e, 61a-e, 62a-e, 63a-e and 64a-e in moderate to good yields ranging between 22-98%. The 25 compounds thus obtained were fully characterised by means of $^1$H NMR, $^{13}$C NMR, HRMS, IR and mp.

Scheme 3.20
In the $^1$H NMR spectrum of compound 60e the protons for the Boc are observed at 1.42 and 1.51 ppm. The signal at 1.42 integrates for 18 protons showing that the two Boc groups on the guanidine are so closely related that they show as a single signal. Two triplets and a multiplet are observed for the aliphatic CH$_2$ protons. Four doublets are observed in the aromatic region corresponding to the protons of the phenyl rings. Five NH proton signals are observed; two that correspond to the guanidine NH protons at 10.09 and 11.64 ppm, one at 8.77 ppm that correlates with the amide proton, another at 6.06 ppm that relates to the NH linker proton between the two phenyl rings and finally one at 5.11 ppm (Figure 3.7).

The $^{13}$C NMR of this compound 60e shows the eight signals that correspond to the aromatic carbons of the compound. It also shows the three Boc–CH$_3$ signals at 27.9, 28.0 and 28.2 ppm respectively and the quaternary carbons of the Boc groups which are seen as
three signals between 79.1 and 83.4 ppm. The five other quaternary carbons (CO and the CN) are also observed (Figure 3.8).

The CH COSY and long range COSY showed that the NH proton at 6.06 ppm is in close proximity to the C atoms in the aromatic region, therefore indicating that this signal corresponds to the NH linker. The peaks at 10.09 and 11.64 ppm were determined to be the guanidine NH protons as they existed in the \(^1\)H NMR spectra of the mono-guanidine compounds. The signal at 8.77 ppm which correlates to the amide proton showed a relation to both the aliphatic carbons of the aliphatic amino acid alkyl chain as well as the carbons of the aryl ring in the CH COSY. It is also located close to the peak at 171.0 ppm which correlates to the CO carbon of the amide bond (Figure 3.9).
In Table 3.4 the yields obtained for all the Boc-protected aminoalkyl mono-guanidine conjugates are presented.
Table 3.4.- Yields obtained for the Boc-protected aminoalkyl *mono*-guanidine conjugates

![Aminoalkyl Guanidine Minor Groove Binders](image)

\[ n=3, X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH} (60a-e) \]
\[ n=4, X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH} (61a-e) \]
\[ n=7, X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH} (62a-e) \]
\[ n=10, X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH} (63a-e) \]
\[ n=11, X= \text{CH}_2, \text{CH}_2\text{CH}_2, \text{O}, \text{CO}, \text{NH} (64a-e) \]

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>n</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>60a</td>
<td>CH_2</td>
<td>3</td>
<td>86</td>
</tr>
<tr>
<td>60b</td>
<td>CH_2CH_2</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>60c</td>
<td>O</td>
<td>3</td>
<td>79</td>
</tr>
<tr>
<td>60d</td>
<td>CO</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>60e</td>
<td>NH</td>
<td>3</td>
<td>68</td>
</tr>
<tr>
<td>61a</td>
<td>CH_2</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>61b</td>
<td>CH_2CH_2</td>
<td>4</td>
<td>92</td>
</tr>
<tr>
<td>61c</td>
<td>O</td>
<td>4</td>
<td>68</td>
</tr>
<tr>
<td>61d</td>
<td>CO</td>
<td>4</td>
<td>81</td>
</tr>
<tr>
<td>61e</td>
<td>NH</td>
<td>4</td>
<td>78</td>
</tr>
<tr>
<td>62a</td>
<td>CH_2</td>
<td>7</td>
<td>88</td>
</tr>
<tr>
<td>62b</td>
<td>CH_2CH_2</td>
<td>7</td>
<td>68</td>
</tr>
<tr>
<td>62c</td>
<td>O</td>
<td>7</td>
<td>22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>n</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>62d</td>
<td>CO</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>62e</td>
<td>NH</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td>63a</td>
<td>CH_2</td>
<td>10</td>
<td>49</td>
</tr>
<tr>
<td>63b</td>
<td>CH_2CH_2</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>63c</td>
<td>O</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>63d</td>
<td>CO</td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td>63e</td>
<td>NH</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>64a</td>
<td>CH_2</td>
<td>11</td>
<td>74</td>
</tr>
<tr>
<td>64b</td>
<td>CH_2CH_2</td>
<td>11</td>
<td>72</td>
</tr>
<tr>
<td>64c</td>
<td>O</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>64d</td>
<td>CO</td>
<td>11</td>
<td>56</td>
</tr>
<tr>
<td>64e</td>
<td>NH</td>
<td>11</td>
<td>54</td>
</tr>
</tbody>
</table>

Having synthesised all the derivatives of the Boc-protected aminoalkyl *mono*-guanidine conjugates the next step was the Boc-deprotection of these compounds and generation of the final hydrochloride salts.

### 3.2.5. Synthesis of the dihydrochloride salts of the aminoalkyl *mono*-guanidine conjugates

The *tert*-butoxycarbonyl group is still one of the most popular amino protecting groups because it is stable in the presence of most nucleophiles and bases. Hence, an orthogonal protection strategy using for example a base-labile protection group such as Fmoc is
possible. Cleavage of the Boc group occurs under anhydrous acidic conditions with the production of tert-butyl cations. Scavengers such as thiophenol may prevent nucleophilic substrates from being alkylated. Generally, acids such as formic acid, trifluoroacetic acid, bromohydric acid, hydrochloric acid and Lewis acids can be used.\textsuperscript{41} As well, Mohapatra et al.\textsuperscript{42} discovered a simple, mild, and efficient protocol for the deprotection of the Boc group from Boc-protected carbamates, amides and nitrogen atoms present in the aromatic ring under basic conditions. This involved using a Cs₂CO₃ and imidazole system at 70 °C. Research has been carried out into milder conditions for Boc deprotection. For example, Wang et al. found that boiling water could efficiently catalyse the deprotection of N-Boc groups, thus meeting all of the requirements for a green chemical reaction.\textsuperscript{43}

The most common method used for Boc group removal is the use of trifluoroacetic acid either neat or in DCM solution. The standard reaction followed in our laboratory involves treatment with 50:50 TFA/DCM at room temperature for 3-5 hours followed by solvent removal under vacuum.\textsuperscript{24} This method has always given good results even with derivatives containing the CONH moiety.\textsuperscript{18} Thus, following this approach, the corresponding trifluoroacetate salts were generated and then treated with Amberlite chloride anion exchange resin overnight. The formation of the dihydrochloride salts was expected, but, unfortunately, the elimination of water from the resin treatment led to fragmentation of the molecules by the hydrolysis of the amide bond. This resulted in very low yields of the final dihydrochloride salts.

The mechanism of this reaction involves protonation of the carbonyl group by the TFA; the electrons then migrate so that the positively charged carbonyl is quenched. This is followed by the movement of the lone pair of electrons from the O to reform the carbonyl bond and CO₂ is expelled to form the deprotected amine (Scheme 3.22).
Considering the peptide bond hydrolysis observed, another deprotection was attempted using 1.25 M HCl/MeOH.\textsuperscript{44} This involved dissolving the Boc-protected derivative in 30 equivalents of 1.25 M HCl/MeOH and left to stir at room temperature overnight (Scheme 3.23). Work-up involved concentration of the product under vacuum. This unfortunately also resulted in the hydrolysis of the amide bond.

Deprotection was also attempted by bubbling 2.5 M HCl with the protected amines (60a and 60b) dissolved in MeOH overnight at room temperature (Scheme 3.24). The reaction was worked up by concentration of the compound under vacuum to give the product. This reaction also led to fragmentation at the amide bond. From these unsuccessful reactions it was determined that aminoalkyl mono-guanidine conjugates cannot be deprotected using HCl/MeOH or TFA/DCM because upon protonation they can be attacked by H\textsubscript{2}O which will hydrolyse the amide bond.

The next conditions for Boc-deprotection consisted of using 1 M HCl/ether (Scheme 3.25). This reaction involved the corresponding Boc-protected precursors being dissolved in a minimal amount of diethyl ether and then treated with six equivalents of 1 M HCl/ether.
until the reaction was complete as monitored by TLC. The solvent was eliminated under vacuum to generate the hydrochloride salt. Purification was carried out using reverse phase column chromatography eluting with MeCN/H$_2$O. This reaction led to the formation of the hydrochloride salts of some of the Boc-protected derivatives but, unluckily, led to the fragmentation of others. Compounds 65a, 65b, 66b and 67d were obtained using this method in yields of 64-79%.

\[ \text{Scheme 3.25} \]

Due to the fact that this reaction also led to degradation of some of the derivatives, further research was carried out leading to improved deprotection methods. The first of these used a more dilute concentration of TFA:DCM (1:100) which was successful and gave the hydrochloride salts upon treatment with Amberlite anion resin overnight (Scheme 3.26). This reaction was carried out on a number of the Boc-protected derivatives giving yields between 39-89%.

\[ \text{Scheme 3.26} \]

Deprotection using TFA:DCM mixtures involved two steps for the formation of the hydrochloride salt; therefore, a more efficient way to optimise the reactions using HCl to cleave the Boc group was sought. The determinant step on the overall yields is the final Boc-deprotection and for that reason different deprotection conditions were explored. From all of these the most successful results were obtained with 4 M HCl/dioxane in IPA (isopropylalcohol)/DCM leading to the direct formation of the hydrochloride salts (Scheme 3.27). This involved the Boc-protected derivatives being dissolved in 4 M HCl/dioxane under argon, the mixture was then diluted with IPA/DCM (1:1) to make up a 0.2 M HCl
solution and stirred for four hours. The HCl/dioxane was then removed under vacuum. The residue was redissolved in H₂O and washed with DCM. The H₂O layer was concentrated under vacuum. Purification was carried out using reverse phase column chromatography eluting with MeCN/H₂O resulting in yields of 28-99%.

The total overall yields obtained for all the salts prepared are presented in Table 3.5. The best results were obtained for those compounds with n= 10 alkyl chains. Regarding the linkers in the di-aromatic moiety, the worst yields were obtained for those compounds with X= NH and the best for those with X= CH₂ and CH₂CH₂ (except for compound 66b). The 24 compounds obtained in this way were fully characterised and thus HPLC (High Pressure Liquid Chromatography), ¹H NMR, ¹³C NMR, HRMS, IR and mp were obtained for each of them.
Table 3.5.- Overall yields obtained for the dihydrochloride salts of the aminoalkyl mono-guanidine conjugates.

IR absorbencies corresponding to the Boc carbonyl groups are no longer present with the major peaks for the amino group at approximately 3200–3400 cm\(^{-1}\). Analysis of \(^1\)H and \(^13\)C NMR of the dihydrochloride salts of the aminoalkyl carboxylic acid conjugates of the mono-guanidine derivatives show that Boc-methyl group signals have disappeared with the signals for the methylene chain and the phenyl remaining. These NMR spectra were recorded in D\(_2\)O therefore the NH proton peaks are not visible.

For example, compound 67e, shown in Figure 3.10, displayed a singlet corresponding to six of the methylene chain protons as well as two triplets and a multiplet corresponding to
the remaining CH$_2$ protons of the aminoalkyl chain. All aromatic peaks were observed between 7.01 and 7.21 ppm.

![NMR spectra of compound 67e](image)

Figure 3.10.- $^1$H NMR spectrum of compound 67e

The $^{13}$C NMR spectra (Figure 3.13) of these compounds consists of eight signals in the aromatic region, four that correspond to the CH carbons of the phenyl rings and four quaternary carbons as seen in the Boc-protected derivatives. Due to deprotection there are now only two other quaternary carbons corresponding to the CN of the guanidine and the CONH of the amide bond. The three Boc-CH$_3$ peaks, which were generally found between 78 and 83 ppm respectively, have now disappeared. The methyl signals of the Boc in the aliphatic region are also not present therefore only the signals for CH$_2$ carbons of the aminoalkyl chain remain.

As an interesting point of comparison, the Boc-protected derivatives of the molecules where X= NH gave broad aromatic signals as seen in Figure 3.11. In the $^1$H NMR spectra
of these species, restricted rotation of the phenyl rings results in an extremely short relaxation time for the aromatic signals. This gives rise to aromatic protons appearing as broad, amorphous signals such as the broad doublet at 6.85 ppm. Aromatic signals in the $^{13}$C NMR spectra (example in Figure 3.12) are not as intense as seen in other derivatives. Deprotection of the Boc-protected derivatives reduces the steric congestion experienced by the aromatic rings. The relaxation times return to ordinary levels and the aromatic NMR signals of the deprotected hydrochloride salts of the aminoalkyl carboxylic acid conjugates of the mono-guanidine derivatives are correspondingly sharpened (Figure 3.10).

Figure 3.11.- $^1$H NMR spectrum of compound 62e
Figure 3.12. $^{13}$C NMR spectrum of compound 62e
In addition to the above analysis, these compounds were further characterised by HPLC showing all of them one peak with a purity percentage of 95% or more.

3.3. Conclusions

In this chapter, the preparation of a new series of hydrochloride salts of the aminoalkyl mono-guanidinium di-aromatic conjugates is presented. The rationale for preparing these compounds was to explore the effect of the aminoalkyl chain on the DNA binding activity of the minor groove binding moiety. Five different X groups connecting the di-aromatic moiety have been explored (CH₂, CH₂CH₂, O, CO, NH) and different lengths of the amino acid system considered (three –n=3-, four –n=4-, seven –n=7-, ten –n=10- and eleven –n=11- methylene groups).
Firstly, the di-aromatic mono-guanidines were prepared. This synthesis involves firstly, guanidylation, as was previously used in the synthesis of bis-guanidines and bis-2-aminoimidazolines in which the functional group was introduced by employing Boc-protected thiourea as an activated guanidylating agent in the presence of HgCl₂ and TEA. Mono-guanidylation of the starting diamine with the central linkers being CH₂, CH₂CH₂, O, CO and NH was obtained. Yields achieved for the five mono-guanidines (51a-e) were good ranging between 34 and 73%. Then, the different aminoalkyl carboxylic acids were Boc protected to facilitate the reaction with the NH₂ group of the mono-Boc protected guanidines. Finally, condensation between both moieties yielded the Boc-protected aminoalkyl conjugates of the mono-guanidine derivatives (60-64a-e). Yields achieved were ranged between 22-98%.

Finally, Boc-deprotection was optimised since the usual treatment with TFA and later treatment with ionic interchange resin resulted in the hydrolysis of the amide bond. This problem was not foreseen since previously this methodology had been successful with derivatives containing the CONH moiety. Removal of the Boc-groups using 4 M HCl/dioxane in IPA/DCM successfully produced the corresponding hydrochloride salts. In this way a total of 24 (65-69a-e) molecules were prepared.

3.4. References


Chapter 4

Synthesis of Alkylcarboxylic Acid Derivatives of Acridine as Intercalators
4.1. Introduction

As mentioned in the first Chapter, there are several ways in which compounds can target DNA; for example, DNA-binding agents such as intercalators can affect the replication process of DNA, which is important for cell growth as well as for its ability to successfully divide. Many types of drugs such as daunomycin and acridine are known to interact with DNA through intercalation which is essentially the insertion of a flat polyaromatic chromophore between consecutive base pairs of the double-helix, thus interfering with normal cellular processes.

Intercalation is an extremely important process, particularly in relation to anticancer agents. As previously stated, it consists in the insertion of a planar aromatic system between DNA base pairs with concomitant unwinding and lengthening of the DNA helix.\(^1\) The insertion of an intercalator between adjacent base pairs causes substantial change in DNA structure such as lengthening, stiffening and unwinding of the DNA helix. As a result of intercalation, DNA loses its regular double helical structure of the DNA backbone. This has been proven by fiber diffraction patterns which show a loss of resolution in their inner layer lines to give a spacing greater than 10.2 Å. The sugar-phosphate torsional angles become altered in order to accommodate the aromatic compound, separating the base pairs, leading to lengthening of the double helix and a decrease in the helix diameter (i.e. unwinding) at the intercalation site.\(^2\) Since S. Lerman discovered intercalation in 1961, thousands of organic and inorganic compounds have been developed as potential anticancer drugs and diagnostic agents.

There are a number of metallo-derivatives such as rhodium and ruthenium octahedral complexes that act as intercalators. Friedman et al. showed that metal complexes with dppz (dipyrido[3,2-a:2',3'-c]phenazine) groups such as [Ru(phen)_2dppz]^2+ and [Ru(bipy)_2dppz]^2+ (70, Figure 4.1.) bind to DNA via intercalation with binding constants greater than 10^6 M\(^{-1}\).\(^3\) Octahedral intercalators incorporating the dpq (dipyrido-[3,2-a:2',3'-c](6,7,8,9-tetrahydro)phenazin)) ligand instead have also been shown to readily intercalate with DNA, but with more base sequence selectivity than dppz.\(^4\) Unfortunately, octahedral metallo-intercalators have not displayed much potential in overcoming drug resistance, but they may have other uses in cancer treatment. Compounds [Ru(phen)_2dppz]^2+ and [Ru(bipy)_2dppz]^2+ (70) can act as “molecular light switches” for DNA. While the metallo-
intcalators show significant solvatochromic luminescence in organic solvents, they display low photoluminescence in aqueous solution, but they show bright luminescence upon intercalation with DNA. A correlation was subsequently established between the extent of protection from the aqueous solvent and the luminescence characteristics. Thus, octahedral metallo-intercalators, which are sequence selective, could be used in the development of diagnostic agents, for example in an in vitro test they fluoresce strongly in the presence of cells with DNA markers for cancers (e.g. mutant p53 genes).

![Metallo-intercalator](image)

Figure 4.1: Metallo-intercalator [Ru(bipy)$_2$dpdz]$^{2+}$ (70) that can be used as a molecular switch

Jennette et al. showed that intercalation of square-planar transition metal complexes can occur. This was proven with [Pt(terpy)S(CH$_2$)$_2$OH)]$^+$ ([Pt(terpy)(HET)])$^+$, 71, Figure 4.2.) This metallo-intercalator was shown to insert into double stranded DNA by different techniques such as UV-vis, viscosity, CD, fluorescence displacement and unwinding experiments.

By using different substituted intercalating ligands (in particular those containing methylated phenanthrolines) and the use of chiral ancillary ligands, it is now possible to improve the cytotoxicity of Pt intercalators; such metallo-intercalators have been shown to be active at much lower concentrations than cisplatin, and to be able to overcome cisplatin resistance in several cancer cell lines. A SAR study was carried out on such compounds and based on the results obtained, a lead compound, [Pt(5,6-Me$_2$-phen)(S,S-dach)]$^{2+}$ (72, Figure 4.2.) was found, showing to be 12 times more cytotoxic that cisplatin. This compound shows cytotoxicity in a number of cancer cell lines such as L1210 (murine leukemia), A-427 (human lung cancer), RT-112 and RT-4 (human bladder cancer), KYSR-70 (human oesophagus cancer), SISO (human cervical cancer), MCF-7 (human breast
cancer), and 2008 (human ovarian carcinoma) and also shows activity in cell lines with acquired and intrinsic resistance to chemotherapy.²

The intercalators synthesised in this thesis are organic intercalators, thus, from here on we will focus on organic intercalators and their many uses. There are a number of different organic intercalators used for medicinal purposes, which chemical structures are all based on six different intercalator frameworks: anthracenes (73), acridines, anthraquinones (74), phenanthridines (75), phenanthrolines (76) and ellipticines (77) (see Figure 4.3).

Daunorubicin and doxorubicin are organic intercalators containing an anthraquinone core and are currently used in the treatment of different human cancers. On the one hand, doxorubicin, which was previously discussed in the first Chapter, is used to treat many different human cancers including acute lymphoblastic leukemia and breast carcinoma.
Daunorubicin, on the other hand, is only used in the treatment of non-lymphocytic leukemia in adults and acute lymphocytic leukemia in children and adults.

The structure of the intercalators studied in this project incorporates an acridine core. The biological activity, in particular the anticancer activity, of acridines is mainly attributed to the planar chromophore they contain. Reasons for using acridine as a standard intercalator include their ready synthesis, biological stability, and their ability to efficiently bind to DNA leading to disruption of DNA cellular function. They have mainly been explored as chemotherapeutic, antibacterial and antiprotozoal agents because of the ability of the acridine chromophore to intercalate DNA and inhibit topoisomerase and telomerase enzymes, as previously discussed in detail in the Introduction section. As mentioned, 9-anilinoacridines, 9-aminoacridine-4-carboxamides and nitroacridines target topoisomerases; but, in general, intercalators also target telomeres. Telomeres comprise of a tandem of DNA mono-stranded TTAGGG repeats that cap and protect the ends of chromosomes. Normal human cells possess a fixed replicative potential which is controlled by telomere shortening occurring at each cell division. This telomere shortening can be inhibited by telomerase, which is expressed in almost all human cancers but is inactive in most normal cells. Therefore, telomeres and/or telomerase are important targets for the design of new anticancer agents that might have minimal side effects. Telomeres can adopt a four-stranded conformation know as a G-quadruplex. Numerous small molecules have been developed to inhibit telomeres via stabilisation of these G-quadruplex, hence inhibiting telomerase action. These include acridine-based derivatives in particular tri-substituted acridines. Neidle et al. synthesised and studied the effects of tri-substituted acridines, including 3,6,9-tri-substituted derivatives (78, Figure 4.4), which showed EC₅₀ values of less than 0.2 μM.

Figure 4.4.- 3,6,9-Trisubstituted acridines (78) synthesised by Neidle et al.

Despite the large number of different intercalators that have been developed, their clinical uses are limited by their major side-effects as well as acquired resistance to the drugs. For
example, these compounds can lead to heart damage, and thus, patients can experience heart failure during the course of treatment and for up to two years after the treatment is finished. This cardiotoxicity is also accumulative; the risk of heart failure increases with every treatment and for patients with a cumulative dose of approximately 400 to 550 mg/m$^2$ their chance of heart failure rises by as much as 20%. Because of these side effects and drug’s resistance, researchers are recently examining ways to increase the localisation of organic intercalators in cancer cells and studying their interactions with DNA in great detail.\textsuperscript{9} Recently, new Doxorubicin derivatives have been shown to overcome multi-drug resistance and have been reported of being two to 36 times more active than doxorubicin in the advanced neuroblastoma cell lines tested.\textsuperscript{10} Due to the toxic side effects of current intercalators, it is necessary to find novel compounds showing a decrease in the severity of side effects caused.

Taking all this into account and exploiting the strong intercalating ability of acridine, we have designed new acridine derivatives by attaching an aliphatic linker to acridine-9-carboxylic acid (80) to explore the influence that such an alkylcarboxylic acid chain could have on the intercalation activity (Figure 4.5). The effect of the alkylcarboxylic acid chain on intercalation can be compared with the aminoalkyl chain effect on the minor groove binders which syntheses was previously discussed in Chapter 3. Thus, in this chapter we present the synthesis of a series of alkylcarboxylic acid derivatives of 80 as intercalators. The final aim of this work is to develop dual agents combining different DNA targeting moieties joined together by an aliphatic chain and the study of these acridine derivatives will allow us to determine the contribution of the intercalator to the efficacy of the overall dual compound.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure45.png}
\caption{General structure of the acridine derivatives prepared}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure46.png}
\caption{Figure 4.6.- General structure of the acridine derivatives prepared}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure47.png}
\caption{Figure 4.7.- General structure of the acridine derivatives prepared}
\end{figure}
4.2. **Synthesis of Alkylcarboxylic Acid Derivatives of Acridine**

The preparation of the alkylcarboxylic acid derivatives of 80 firstly involves the syntheses of the amino acid methyl esters, the coupling of these molecules with 80 to yield the corresponding methyl esters of the (acridine-9-carboxamido)alkylcarboxylates (82a-e) and, finally, the hydrolysis of the methyl esters to give the final (acridine-9-carboxamido)alkylcarboxylic acids (83a-e) as shown in Scheme 4.1.

![Scheme 4.1](image)

**4.2.1. Synthesis of amino acid methyl esters**

Different aminoalkylcarboxylic acids with three, four, seven, ten and eleven methylene groups (53, 54, 55, 56 and 57) were considered. The amino acid must be protected to avoid the carboxylic group interacting with its own amino group causing polymerisation. Esterification of the carboxylic group allows for successful coupling between the free amino group and the carboxylic group of 80. Methyl esterification can be carried out by a number of different methods and three of them were studied in this work searching for the best conditions. The first method attempted involves dropwise addition of three equivalents of thionyl chloride to a stirred solution of the amino acid in ten equivalents of methanol at
0 °C (Scheme 4.2). The resulting solution is then heated to reflux overnight. The solvent and excess of thionyl chloride are then removed under reduced pressure.

\[
\begin{align*}
\text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OH} & \xrightarrow{\text{SOCl}_2, \text{MeOH, reflux,overnight}} \text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OMe} \\
n= 3 \ (53), 4 \ (54), 7 \ (55), 10 \ (56), 11 \ (57) \quad n= 3 \ (79a), 4 \ (79b), 7 \ (79c), 10 \ (79d), 11 \ (79e)
\end{align*}
\]

Scheme 4.2

This method was abandoned because SOCl\(_2\) was difficult to remove leading to impure products. Methyl esterification can also be carried out by adding H\(_2\)SO\(_4\) to the amino acid in methanol. Thus, H\(_2\)SO\(_4\) is added dropwise until all of the amino acid is dissolved. The reaction mixture is then refluxed at 70 °C overnight and the product is then concentrated under vacuum (Scheme 4.3). This method, on a small scale, was more successful than the previous one; however, when the scale of this reaction was increased it became difficult to remove the H\(_2\)SO\(_4\) leading to impure oils as products.

\[
\begin{align*}
\text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OH} & \xrightarrow{\text{H}_2\text{SO}_4, \text{MeOH, reflux,overnight}} \text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OMe} \\
n= 3 \ (53), 4 \ (54), 7 \ (55), 10 \ (56), 11 \ (57) \quad n= 3 \ (79a), 4 \ (79b), 7 \ (79c), 10 \ (79d), 11 \ (79e)
\end{align*}
\]

Scheme 4.3

Finally, methylation was carried out in MeOH in the presence of HCl/MeOH yielding the corresponding compounds 79a-e (Scheme 4.4). This reaction involves the amino acid being dissolved in MeOH and then HCl/MeOH is added. The reaction mixture is stirred at room temperature overnight, and then concentrated under vacuum to give the required product.

\[
\begin{align*}
\text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OH} & \xrightarrow{\text{HCl/MeOH, MeOH, reflux,overnight}} \text{H}_2\text{N} \left( \text{\textbullet} \right)_n \text{O} \left( \text{\textbullet} \right) \text{OMe} \\
n= 3 \ (53), 4 \ (54), 7 \ (55), 10 \ (56), 11 \ (57) \quad n= 3 \ (79a), 4 \ (79b), 7 \ (79c), 10 \ (79d), 11 \ (79e)
\end{align*}
\]

Scheme 4.4
This reaction was the most successful of the three. It was possible to increase the scale and still obtain pure products in good yields. Thus, the five compounds required were obtained in yields ranging between 66 and 95% as presented in Table 4.1. As these compounds have been previously described in the literature, only $^1$H NMR and melting points were recorded for each compound.

Table 4.1.- Yields obtained in the preparation of the methyl esters of the amino acids

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>n</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>79a</td>
<td>3</td>
<td>83</td>
</tr>
<tr>
<td>79b</td>
<td>4</td>
<td>87</td>
</tr>
<tr>
<td>79c</td>
<td>7</td>
<td>66</td>
</tr>
<tr>
<td>79d</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>79e</td>
<td>11</td>
<td>95</td>
</tr>
</tbody>
</table>

Once all of the methyl esters of the aminoalkyl acids were prepared the next step in the synthesis was to couple them with acridine 9-carboxylic acid.

4.2.2. Synthesis of the methyl (acridine-9-carboxamido)alkylcarboxylates

To connect the methyl esters of the amino acids with 80 to give the corresponding coupled derivatives, different reactions were explored. Thus, we tried to use the corresponding acid chloride (as tested in Chapter 3 for different derivatives) of compound 80 with the amino acid methyl ester in MeCN in the presence of TEA; and also, we tested different coupling reagents such as TBTU or benzotriazole-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP).

The formation of the acid chloride was successful along with its coupling to the amine to form the required peptide linked products. In this reaction, a catalytic amount of DMF was added to a solution of 80 in excess of SOCl₂ and was refluxed for 5 hours. Removal of the SOCl₂ yielded the required acid chloride (81) as a yellow solid (Scheme 4.5).
The yellow solid was used without further purification and dissolved in MeCN with TEA under argon. A solution of the corresponding amino acid methyl ester (79a-e) in MeCN was added to the 81 solution at 0 °C for one hour and at room temperature for three hours. The crude product was filtered and washed with EtOAc. Recrystallisation using MeOH followed by purification by chromatography on silica gel using EtOAc:Hexane (2:1) as an eluent gave the required compounds (Scheme 4.6).

The mechanism of the peptide bond formation involves the amino group of the amino acid methyl ester attacking at the carbonyl group of the acridine acid chloride, then the electrons of the double bond migrate to give a negatively charged oxygen. The Cl atom is then expelled in the reformation of the carbonyl group and TEA then deprotonates the positively charge peptide bond to give the stable amide product.

Other coupling reagents were also investigated to see if higher yields could be obtained. The coupling reaction can be carried out using TBTU or BOP as a coupling reagent. This type of reaction involves the amino acid methyl ester and 80 being dissolved in CH$_2$Cl$_2$ at room temperature under argon, the mixture is then stirred and cooled to 0 °C. Four equivalents of DIEA are then slowly added, followed by the addition 1.5 equivalents of the coupling reagent (TBTU or BOP) as shown in Scheme 4.7. The reaction is stirred overnight at 20 °C and work-up involves EtOAc being added to the reaction mixture and the organic layer is successively washed with a 1 M HCl solution, a 20% NaHCO$_3$ solution and brine. The organic layer is dried over MgSO$_4$ and concentrated under vacuum. The
crude residue obtained was purified by chromatography on a silica gel column using a gradient mixture of hexane:EtOAc eluent (5:1-3:1-1:1-EtOAc) affording the final compound.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{C} & \quad \text{Me} \\
\text{79a-e} & \quad \text{O}_{\text{20}^\circ C, \text{overnight}} \\
\text{BOP or TBTU} (1.5 \text{ eq.}), \text{DIEA} (4 \text{ eq.}), \text{CH}_2\text{Cl}_2, \\
 & \quad \text{n= 3 (82a), 4 (82b), 7 (82c), 10 (82d), 11 (82e)}
\end{align*}
\]

Scheme 4.7

This reaction was carried out using TBTU and BOP for compound 82e and it was found that BOP gave a higher yield of 60% in comparison with 48%. Therefore, it was decided that the rest of the alkylcarboxylic acid acridine derivatives would be synthesised using BOP.

BOP is a phosphonium salt coupling reagent that contains the racemisation suppressant HOBt, therefore leading to minimal racemisation when compared with carbodiimide coupling reagents. The mechanism of a coupling reaction involving BOP is different to that of TBTU which was previously described in Chapter 3. An advantage of phosphonium salts over aminium/uronium salts such as TBTU is that the phosphonium does not react with the amino function of the incoming moiety and thus the phosphonium does not terminate the peptide chain. This is relevant in fragment coupling and cyclisations when both reactants are in equimolar quantities and an excess of the coupling agent reacts with the amino component.\(^{11}\) It involves deprotonation of 80 by the non-nucleophilic base DIEA to give the carboxylate anion which then interacts with the positively charged phosphorous of BOP due to the strong affinity of oxygen and phosphorous. Simultaneously, the cleavage of the O-P bond in BOP occurs, and the benzotriazole anion can then attack the intermediate ester, eliminating hexamethylphosphoramide (HMPA, PO(NMe\(_2\))\(_3\)). Formation of an activated ester occurs, which then reacts with the amine group of the amino acid methyl ester resulting in the corresponding methyl (acridine-9-carboxamido)alkylcarboxylate and benzotriazol-1-ol (Scheme 4.8).
Following this approach we were able to prepare in moderate to good yields (46-71%, Table 4.2) compounds 82a-e which were fully characterised, thus $^1$H NMR, $^{13}$C NMR, HRMS, IR and mp were obtained for each compound.

Table 4.2.- Yields obtained for the preparation of methyl (acridine-9-carboxamido) alkylcarboxylate

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>N</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>82a</td>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td>82b</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>82c</td>
<td>7</td>
<td>46</td>
</tr>
<tr>
<td>82d</td>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>82e</td>
<td>11</td>
<td>60</td>
</tr>
</tbody>
</table>
The $^1$H NMR of these compounds, for example in the case of compound 82c (Figure 4.6), shows four aromatic signals between 7.48 and 8.11 ppm corresponding to the aromatic protons of the acridine core. Two multiplets, a triplet and in this particular derivative where $n=7$, another multiplet corresponding to the CH$_2$ protons of the alkyl chain are observed within the aliphatic region of the spectrum. Finally, a quartet is recorded at 3.72 ppm which corresponds to the methyl protons (Figure 4.6).

![Figure 4.6. $^1$H-NMR spectrum of compound 82c](image)

The $^{13}$C NMR spectrum of these compounds (for example 82c as shown in Figure 4.7) consists of seven signals in the aromatic region, four between 125.2 and 130.5 ppm corresponding to the eight tertiary carbons of the acridine ring, and three signals seen at 122.1, 141.4 and 148.2 ppm respectively corresponding to its five quaternary carbons. There are two other quaternary carbons corresponding to the carbonyl groups of the amide and the ester at 166.9 and 174.2 ppm, respectively. There are seven aliphatic C signals
between 24.8 and 40.2 ppm along with a peak at 51.5 ppm corresponding to the carbon of the methyl group (Figure 4.7).

Figure 4.7.- $^{13}$C NMR spectrum of compound 82c

4.2.3. Synthesis of (acridine-9-carboxamido)alkylcarboxylic acids

Finally, hydrolysis of the methyl esters was carried out to form the corresponding alkyl carboxylic acids (83a-e). Both acid and base catalysed hydrolyses were attempted and saponification using KOH followed by acidification using HCl proved to give the best results. The methyl esters were dissolved in THF and 1M KOH was added, the reaction was left stirring overnight, then, the pH of the solution was adjusted from 9 to 3 and the reaction mixture was concentrated in vacuo in order to form the corresponding acids 83a-e in yields between 28-89% (Scheme 4.9).
This involves the nucleophilic hydroxide attacks at the electrophilic carbonyl of the ester, breaking the double bond and creating a tetrahedral intermediate. The intermediate collapses, reforming the C=O cleaving the methyl ester group and leading to the carboxylic acid. The methoxy functions as a base deprotonating the carboxylic acid and the carboxylate can then form the potassium salt. Acidic work up finally results in formation of the carboxylic acids.

The total overall yields, ranging between 9-50% for all the acids prepared, are presented in Table 4.3. The best yields were obtained for those compounds with alkyl chains of eleven methylene groups (n= 11). The determinant step on the overall yields is the coupling reaction and for that reason different coupling reactions have been explored.

Table 4.3.- Overall yields obtained for the (acridine-9-carboxamido)alkylcarboxylic acids

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>n</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>83a</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>83b</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>83c</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>83d</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>83e</td>
<td>11</td>
<td>50</td>
</tr>
</tbody>
</table>
All final compounds were fully characterised by $^1$H NMR, $^{13}$C NMR, HRMS, IR and mp. Analysis of $^1$H and $^{13}$C NMR of compounds 83a-e show that the methyl protons and carbon signals have disappeared and been replaced with a carboxylic acid proton and carbon signal proving that the ester has been hydrolysed to form the acid. For example, compound 83e shows four aliphatic peaks, two multiplets, a triplet and a quartet corresponding to the protons of the alkyl chain. It is clear that the methyl protons previously seen at approximately 3.75 ppm have now disappeared. In the aromatic region, the two doublets and two apparent triplets remain but the amide NH proton has shifted from 6.63 to 9.02 ppm also proving that hydrolysis has occurred (Figure 4.8).

The $^{13}$C NMR spectra of these compounds have a similar pattern to that of the methyl esters except that the signal corresponding for the methyl carbon has disappeared. For example, in the spectrum of compound 83a the methyl ester signal at 51.9 ppm no longer exists as shown in Figure 4.9. Also the quaternary carbon that corresponds to the carbonyl
of the ester group has shifted from 173.6 to 176.1 ppm, in agreement now with the C of a carboxylic acid proving once again that hydrolysis has occurred.

4.3. Conclusions

The preparation of a new series of alkylcarboxylic acid derivatives of 80 has been presented in this chapter. These compounds were prepared in order to explore the effect of the alkylcarboxylic chain on the DNA binding activity of the intercalator moiety. Five different amino acids with chains of different lengths have been explored (n= 3, 4, 7, 10, 11 methylene groups). First, the methyl esters of the different amino acids were prepared to facilitate the reaction of the free NH$_2$ group with 80. Yields obtained for compounds 79a-e were between 66 and 95%. The amino acid methyl esters (79a-e) were then coupled with 80 using BOP as a coupling reagent to form the five methyl (acridine-9-carboxamido) alkylcarboxylates (82a-e) in yields of 46-71%. Finally, base catalysed hydrolysis was carried out using KOH to yield the corresponding (acridine-9-carboxyamido)alkylcarboxylic acids (83a-e). A total of five new derivatives were prepared with total yields ranging between 9 and 50%.
4.4. References


Chapter 5

Attempted Synthesis of Minor Groove Binder-Intercalator Dual DNA Targeting Agents
5.1. **Introduction**

By combining the essential characteristics of the individual DNA targeting agents such as intercalators, minor/major groove binders or alkylators into one molecule an increase in activity and affinity towards DNA is expected. The different moieties would bind to DNA simultaneously leading to a synergistic effect. As previously mentioned in Chapter 1, a small number of dual DNA targeting agents exist. In some cases, these dual binders have shown improved anticancer and antiprotozoal activity when compared with their mono-functional counterparts.

NetAmsa (84, Figure 5.1) is a good example of a dual binder that acts as a potent antitumour drug. NetAmsa is a combilexin, which consists of a minor groove binder linked to an intercalator. This is derived from a covalent coupling of the minor groove binder netropsin and the intercalator amsacrine. NetAmsa has displayed enhanced sequence specificity when compared with mono-intercalators.\(^1\) Interestingly, this molecule shows three modes of binding with duplex DNA: (i) sequence-specific recognition of the DNA minor groove via the netropsin moiety; (ii) DNA intercalation of the acridine chromophore, and (iii) threading of the methane sulfonanilino group into the major groove.\(^2\)

Based on the structure of NetAmsa, Pindur et al.\(^3\) synthesised a series of new combilexins with different heterocycles (such as indole or carbazole) or a nitro group as the left side building block. The cytotoxicity of these compounds was tested on three cell lines NCI-
H640 (lung carcinoma), MCF-7 (breast carcinoma) and SF-268 (glioma). Some of these combilexins limited cell growth to 32% or less and, therefore, further testing was carried out on such derivatives. The potency of these compounds was measured by determining the GI$_{50}$ which is defined as the concentration at which growth is inhibited in a 50%. Unfortunately, none of these compounds showed GI$_{50}$ values that justify further biological testing. This was confirmed by DNA thermal denaturation and ethidium bromide displacement assays, both experiments indicating weak binding of the compounds to DNA.

Then, the authors proceeded to synthesise a number of $N,N$-dimethylaminopropylamide based compounds and their cytotoxicity was measured in the same cell lines. Of the two derivatives containing anthraquinone moieties, which gave the most promising results, one compound displayed strong binding to DNA while another inhibited topoisomerase I at 50 μM. These compounds were considered promising candidates for further study.\(^4\)

Further, a series of oligopyrrole carboxamides was synthesised as combilexins containing naphthalimide, acridone, anthraquinone or acridine moieties. Two of the combilexins containing acridone moieties showed good selectivity for melanoma and non-small cell lung cancer culture, whereas the iminostilbene derivative was particularly active against breast cancer cell lines.\(^5\) Recently, a family of anthrapyrazole–netropsin hybrid molecules (85, Figure 5.2) designed to both intercalate and bind in the DNA minor groove were synthesised and tested for their ability to inhibit cell growth. The compounds that had the strongest affinity for DNA and the strongest cell growth inhibitory activity had two $N$-methylpyrrole groups and positively charged (dimethylamino)alkyl side chains. These compounds displayed submicromolar cytotoxicity towards K562 human leukemia cells.\(^6\)

![Figure 5.2.- Anthrapyrazole-netropsin hybrid molecules (85)](image)

These dual binders have shown some promising preliminary results but have yet to show significant levels of cytotoxicity or inhibition of topoisomerases. Therefore, further
investigation into new dual binders is required. Taking this into account we attempt to synthesise dual binders by combining the compounds previously synthesised in Chapter 3 (minor groove binders) and Chapter 4 (intercalators). These dual binders will consist of the aminoalkyl minor groove binders linked to an acridine core. The general structure of such compounds varying in different central linkers (X) and different chain lengths is described in Figure 5.3.

\[
\text{Figure 5.3.- Proposed dual minor groove binder/intercalator agents}
\]

The synthesis of all these dual binders involves a coupling reaction to join the two individual DNA binding moieties. For example, the trifunctional DNA ligand R-132 (Figure 5.4) was synthesised by coupling the netropsin moiety with acridine and, then, coupled with the ethidium system to give the final compound. The coupling reagent 3-(ethyliminomethyleneamino)-N,N-dimethylpropan-1-amine (EDCI) was used in both cases. EDCI is a water soluble carbodiimide, usually obtained as a hydrochloride, which can be used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds.

\[
\text{Figure 5.4.- R-132 trifunctional combilexin}
\]

Compound R-132 stabilises the topoisomerase-II/DNA covalent complexes and displays potent cytotoxic activities due to the strong binding of the netropsin moiety to DNA minor groove and intercalation of the ethidium and acridine units. Its binding to DNA was investigated by thermal denaturation assays and SPR, which resulted in high \( \Delta T_m \) (23 °C) and binding constant of \( K_{eq} 4.81 \times 10^6 \). Circular Dichroism (CD) spectra displayed both a
negative and positive induced CD signal representative of minor groove binding and intercalation. Linear Dichroism (LD) spectra showed negative signals at the 440 and 530 nm corresponding to the anilinoacridine and ethidium absorption bands, respectively. In contrast, positive signals were observed in the netropsin absorption band at 310 nm. This confirms the intercalation of both the ethidium and acridine moieties and the minor groove binding of the netropsin system. Compound R-132 easily enters and accumulates in cell nuclei, as proven by confocal microscopy and, thus, provides a novel lead compound for the design of gene-targeted anticancer agents. 

Other examples of coupling reagents used in the synthesis of dual targeting DNA agents include DCC, TBTU and BOP, which have all been previously mentioned in Chapters 3 and 4. For example, a series of acridine-triazene comblexins were synthesised using DCC as a coupling reagent in order to combine the acridine moiety with the 4-(2',4',6-trimethylbenzenesulfonamido)benzoic acid which is then converted to the triazene. These acridine-triazene comblexins were studied using thermal denaturation assays and fluorescence experiments. The results of these biophysical tests indicate that the hybrid agents have a stronger affinity for DNA in comparison to that of the acridine or triazene components alone. These compounds display AT-sequence selectivity due to the difunctionalised 1,3-diaryltriazene residues, despite weak GC preferential behaviour associated with the acridine chromophore. In vitro cytotoxicity tests were carried out using L1210 mouse leukemia and A2780 human colon cancer cell lines. The results obtained showed that the comblexins were 10 to 40-fold more potent than the acridine or triazene subunits and have activities that favourably compare with those of other reported synthetic comblexins.

There are a number of different methods of coupling a carboxylic acid with an amine to form an amide bond. Coupling involves attack by the amino group of one residue at the carbon atom of the carboxyl-containing component that has been activated by the introduction of an electron-withdrawing group. The latter immediately undergoes aminolysis to give the amide. Alternatively, it can react with a second nucleophile (from the reactants or added for the purpose to give the more stable active ester or symmetrical anhydride) whose aminolysis then generates the amide.

Many different types of coupling reagents exist that activate the carboxylic acid. Some of these include chlorinating reagents such as SOCl₂, phosphonium salts such as BOP and
Minor Groove Binder-Intercalator Dual Binders

Chapter 5

(benzotriazol-1-yloxy)trippyrrolidinophosphonium hexafluorophosphate (Py-BOP), aminium salts (HBTU), 2-(1H-7-azabenotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU), TBTU, ethyl chloroformate which forms a mixed anhydride which is then attacked by the amine and the Yamaguchi reagent which forms an ester which is attacked by the amine.  

The attempted syntheses of the proposed dual binders (Figure 5.3) involved many different synthetic routes and different coupling reactions. Thus, the syntheses carried out and the subsequent results obtained are described in detail next. The experimental of all the compounds synthesised in this chapter are described in the appendix.

5.2. Attempted synthesis involving orthogonal protection using Cbz-protected amino acids and Boc-protected mono-guanidines

The first method involved the use of orthogonally protected Cbz (carboxybenzyl) amino acids and Boc-protected mono-guanidines which subsequently would be coupled with each other. Then, the Cbz group would be removed, the free amino group coupled with the acridine moiety and finally removal of the Boc-protecting groups should yield the required dual binders (Scheme 5.1). The first step in this synthetic route requires the Cbz-protection of the amino acids.
5.2.1. **Synthesis of the Cbz-protected amino acids**

The amino group of 53, 54 and 55 was protected with Cbz by the method described in Scheme 5.2. A mixture of the corresponding amino acid in a 2 M aqueous sodium hydroxide solution was cooled in an ice bath to 0 °C. Under vigorous stirring benzyl chloroformate and a 2 M aqueous sodium hydroxide solution were simultaneously added within two minutes. The mixture was stirred for 20 minutes at room temperature and extracted with diethyl ether. The aqueous layer was separated and acidified with concentrated hydrochloric acid to a pH of 2. The resulting emulsion was extracted with ethyl acetate. The organic layers were combined, washed with brine, and dried with \( \text{Na}_2\text{SO}_4 \). Concentration under vacuum gave white needles, which were dried under vacuum.
The three resulting compounds 58f-h were obtained in yields ranging between 42-51%. As these compounds have been previously prepared, only \(^1\)H NMR and melting points were obtained for each compound.

### 5.2.2. Synthesis of the Boc-protected mono-guanidine Cbz-amino acid conjugates

The next step in the synthesis is the coupling of the Cbz-protected amino acid with the mono-guanidylated di-aromatic diamine. This reaction was carried out in the same manner as when the Boc-protected amino acid was coupled with the Boc-protected mono-guanidines since this synthesis gave good results. First, the reaction was attempted with 58f and 51a as a test reaction. The Cbz-protected amino acid, the Boc-protected mono-guanidine, TBTU and DIEA were dissolved in acetonitrile at room temperature under inert atmosphere. This reaction involved 1.2 equivalents of the corresponding Cbz-protected amino acid in acetonitrile and treatment with 3.8 equivalents of DIEA. Then, the mono-guanidine (1 equivalent) and TBTU (1.2 equivalents) were added to the reaction mixture under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours (Scheme 5.3). Work-up of this reaction involved partition between brine and EtOAc. The organic layer was washed with 0.1 M HCl and 5% NaHCO₃, dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. Purification by flash chromatography with silica gel eluting with hexane/EtOAc (2:1) yielded 86 in 84% yield which was fully characterised by \(^1\)H NMR, \(^{13}\)C NMR, HRMS, IR and mp.

![Scheme 5.3](image)
5.2.3. Attempted deprotection of the Boc-protected mono-guanidine Cbz-amino acid conjugates

Once 86 was synthesised, the Cbz protecting group had to be removed selectively so that the acridine moiety could couple with the correct amino group. Cbz protecting groups are removed by hydrogenolysis or reaction with HBr. Boc-protecting groups are acid labile but are stable under hydrogenolysis. Therefore, the Boc-groups should remain intact whilst the Cbz-group is removed by a reaction with Pd/C in a particular solvent under hydrogen atmosphere. A number of different conditions were investigated for this reaction (Table 5.1) but all proved unsuccessful.

The general procedure consists in mixing the N-Cbz-protected amino acid tert-butyl ester and 10% Pd/C which was stirred for a certain number of hours in different solvents under hydrogen atmosphere as shown in Table 5.1. The reaction was monitored by TLC until the starting material was consumed. The catalyst is then filtered off through a celite pad which is washed with ethyl acetate. The filtrate and washings were combined and evaporated expecting to give the tert-butyl ester compound as an oil, which would be dried under high vacuum.

Even though a range of reaction conditions were attempted the Boc-protected mono-guanidine amino acid conjugate could not be isolated. In the first reaction with 10% Pd/C and 3 atm of hydrogen pressure in THF for 6 hours, the compound decomposed. In the second reaction, the Cbz group was not fully removed and the deprotected amine could not be isolated from the protected amine. The third reaction was too harsh due to the pressure used and, therefore, led to decomposition of the compound. It was considered that a shorter reaction time would not be so harsh and, hence, might lead to successful deprotection. Unfortunately, the conditions were not strong enough and purification failed. Finally, the reaction was left on overnight but this time was not sufficient for full deprotection. After several attempts this general synthetic route was abandoned.
Table 5.1.-Hydrogenolysis of Boc-protected mono-guanidine-Cbz-amino acid conjugate

<table>
<thead>
<tr>
<th>Pd/C (%)</th>
<th>Atm. Pressure (atm)</th>
<th>Time (h)</th>
<th>Solvent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3</td>
<td>6</td>
<td>THF</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>6</td>
<td>MeOH</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>6</td>
<td>MeOH</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>3</td>
<td>MeOH</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>Overnight</td>
<td>MeOH</td>
<td>0</td>
</tr>
</tbody>
</table>

5.3. **Attempted synthesis involving orthogonal protection using Fmoc-protected amino acids and Boc-protected mono-guanidines**

The next route that was investigated involved the protection of the amino acids with the fluorenylmethyloxycarbonyl (Fmoc) protecting group (Scheme 5.4). This was then followed by a coupling reaction with the Boc-protected mono-guanidines to give Boc-protected mono-guanidine Fmoc-amino acid conjugates. The next step that was attempted involved deprotection of the Fmoc-protected amines. Then, this would be followed by coupling with acridine and subsequent Boc-deprotection to give the hydrochloride salts.
5.3.1. Synthesis of the Fmoc-protected amino acids

The amino group of 54 was protected with Fmoc by the method described in Scheme 5.5. Thus, 54 was dissolved in 10% sodium carbonate aqueous solution. Next, dioxane was added and the mixture was stirred in an ice-water bath; an equivalent of 9-fluorenylmethylchlorocarbonate was then added, stirring was continued for 3.5 hours at 0 °C and, additionally, for 6.5 hours at room temperature. The resulting reaction mixture was poured into water and extracted with four portions of diethyl ether. The aqueous phase was cooled in an iced water bath and acidified to pH 3.0 with concentrated hydrochloric acid under vigorous stirring; this was followed by extraction with EtOAc. The organic layers were combined, dried over MgSO₄, and concentrated under vacuum to give a white foam. Recrystallisation from CH₃CN gave 87 in a 95% yield. As this compound was previously synthesised it was only characterised by melting point and ¹H NMR.
5.3.2. Synthesis of the Boc-protected mono-guanidine Fmoc-amino acid conjugates

As mentioned, the next step in the synthesis of dual agents should consist in the coupling of the Fmoc-protected amino acid with the mono-guanidylated diamine. This reaction was first attempted with compound 87 and the mono-guanidines 51d and 51e as these derivatives gave good results in the biophysical experiments. The reaction, as shown in Scheme 5.6, involved the Fmoc-protected amino acid, mono-guanidine and HOBT being dissolved in dry THF; next, the mixture was cooled to 0 °C, and EDCI was added. The mechanism of this reaction is similar to that of the DCC coupling described in Chapter 3. The reaction mixture was stirred at room temperature overnight, then quenched with water and extracted with EtOAc. The combined organic layers were washed with a saturated aqueous solution of NaHCO₃ and brine, dried over anhydrous Na₂SO₄ and concentrated under vacuum. The crude product was filtered over a pad of basic alumina to remove the amino acid in excess and then purified by flash column chromatography using hexane/ethyl acetate (2:1) as eluent to give a yield of 48% for 88d (X=CO) and 41% for 88e (X=NH). These compounds were fully characterised by ¹H NMR, ¹³C NMR, HRMS, IR and mp.

5.3.3. Deprotection of the Boc-protected mono-guanidine Fmoc-amino acid conjugates

As with the Cbz-derivatives the Fmoc group must be selectively removed for the coupling of the Boc-protected mono-guanidine amino acid conjugate with acridine-9-carboxylic acid. The Fmoc protecting groups are base labile whereas Boc-groups are acid labile, therefore, Fmoc should be selectively removed in the presence of a strong base such as
piperidine. Thus, piperidine (15 equivalents) was added to $88d$ or $88e$ in diethyl ether at 0 °C, stirred for 1 hour and then at room temperature for ten minutes (Scheme 5.7). The product was then extracted with 2 M HCl into the aqueous layer and, after separation and neutralisation with aqueous NaOH, re-extracted with diethyl ether. The combined ether layers were washed with brine, dried over Na$_2$SO$_4$ and concentrated. The crude mixture was purified by column chromatography on silica, eluting with hexane/ethyl acetate. However, upon purification the product that was isolated had both the Fmoc and Boc-protecting groups removed. The removal of the Boc-groups must have occurred due to the acidic work-up. As a result of this, other synthetic routes towards the dual binders were investigated.

![Scheme 5.7](image)

**5.4. Attempted coupling reaction using 9-chloroacridine**

Other coupling reactions were explored in an effort to synthesise the dual binders. Firstly, the dihydrochloride salts of the mono-guanidine amino acid conjugates from Chapter 3 and 9-chloroacridine were reacted together in the presence of TEA for 24 hours at room temperature. The basis of this reaction was that the aliphatic amino group is more reactive than the guanidine functionality and therefore should preferentially attack 9-chloroacridine. The experimental procedure involved a solution of 9-chloroacridine in DMF being treated with TEA and compound 65a. The reaction mixture was stirred at room temperature for 24 hours and partitioned between H$_2$O and EtOAc. The organic layer was washed with 0.1 M HCl and concentrated under vacuum. A reverse phase column was used to purify the required compound; yet, the mixture proved difficult to separate since the components were of similar polarity and regrettably, the required dual binder could not be isolated (Scheme 5.8).
5.5. Attempted coupling reaction using acridine-9-carboxylic acid

Reactions were also carried out using acridine-9-carboxylic acid, the dihydrochloride salts of the mono-guanidine amino acid conjugates and various coupling reagents. The first of these involved acridine-9-carboxylic acid being converted to the acid chloride and then, as a test reaction, this acid chloride would be reacted with compound 65a. Thus, the acridine derivative was dissolved in an excess of SOCl₂ with a catalytic amount of DMF under inert atmosphere and the reaction was allowed to reflux for 5 hours. Following this, the mixture was cooled and excess SOCl₂ was removed by distillation with traces being eliminated under vacuum. This afforded the acid chloride of acridine-9-carboxylic acid as a bright yellow oil which was used in the next step of the reaction without further purification. The acid chloride produced was then reacted at room temperature overnight with 65a in DMF in the presence of TEA. Work up involved dissolving in EtOAc and washing with 2 M HCl solution, saturated NaHCO₃ solution and finally brine. Drying with MgSO₄ and concentrating under vacuum should have resulted in the formation of the required product; however, this synthetic route failed since, as previously mentioned in Chapter 3, polymerisation or decomposition of SOCl₂ could have occurred.

Since successful coupling reactions had been achieved in Chapters 3 and 4 in the presence of TBTU and BOP it was thought that these reagents would be useful in the coupling of acridine-9-carboxylic acid and 65a. Initially, the reaction was attempted with TBTU and thus, a solution of acridine-9-carboxylic acid in DMF was treated with DIEA, 65a and TBTU under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours and partitioned between water and EtOAc. The organic layer was washed with 0.1 M HCl and concentrated under vacuum. Purification of the mixture obtained was attempted
but failed due to the close polarity of the different compounds. The $^1$H NMR spectrum of this mixture after the column chromatography showed no aromatic signals and, therefore, decomposition must have occurred on the column. The reaction was also carried out for 67b but again proved to be problematic.

In addition, the reaction was attempted using BOP as a coupling reagent. These reactions were monitored by TLC over a 48 hour period but all of the starting material was not consumed and the reaction did not go to completion. Purification and isolation of the different fractions of the mixture was again unsuccessful. The different conditions for the attempted reactions of acridine-9-carboxylic acid and the dihydrochloride salts of the mono-guanidine amino acid derivatives are summarised in Table 5.2.

Table 5.2.- Coupling of acridine-9-carboxylic acid and the dihydrochloride salts 65a or 67b

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Dihydrochloride Salt</th>
<th>Acridine Moiety</th>
<th>Coupling Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X= CH$_2$, n=3 (65a)</td>
<td>9-chloroacridine</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>X= CH$_2$, n=3 (65a)</td>
<td>acridine-9-carboxylic acid</td>
<td>SOCl$_2$</td>
</tr>
<tr>
<td>3</td>
<td>X= CH$_2$, n=3 (65a)</td>
<td>acridine-9-carboxylic acid</td>
<td>TBTU</td>
</tr>
<tr>
<td>4</td>
<td>X= CH$_2$CH$_2$, n=7 (67b)</td>
<td>acridine-9-carboxylic acid</td>
<td>TBTU</td>
</tr>
<tr>
<td>5</td>
<td>X= CH$_2$, n=3 (65a)</td>
<td>acridine-9-carboxylic acid</td>
<td>BOP</td>
</tr>
</tbody>
</table>

![Diagram of the reaction](image-url)
5.6. **Attempted coupling reactions using methyl (acridine-9-carboxamido)alkyl carboxylate and Boc protected mono-guanidines**

According to the literature, esters such as methyl and ethyl esters can be coupled with amines to form amides. Such a reaction would be very useful in the synthesis of the proposed dual agents. A solution of $82e$ in MeCN was treated with DIEA, $51a$ and TBTU under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours and partitioned between water and EtOAc. The organic layer was washed with 0.1 M HCl and concentrated under vacuum (Scheme 5.9). This reaction was monitored by TLC and low resolution mass spectrometry which showed no sign of reaction occurring even after 18 hours. Therefore, the synthesis was ceased at this point.

![Scheme 5.9](image)

5.7. **Attempted coupling reactions using ethyl (acridine-9-carboxamido)alkyl carboxylate and Boc protected mono-guanidines**

In the literature, ethyl esters have also been shown to react with amines to form peptide bonds. In order to discover if this reaction would be successful in the synthesis of the dual binders the ethyl (acridine-9-carboxamido)alkylcarboxylates must be first prepared.

The synthesis of the ethyl (acridine-9-carboxamido)alkylcarboxylates is very similar to that of the methyl esters described in Chapter 4, using ethanol instead of methanol. Thus, the amino acids are converted to the corresponding ethyl esters and next, a coupling reaction between these ethyl esters and acridine-9-carboxylic acid in the presence of BOP is undertaken to form the corresponding alkylcarboxylate acridine derivatives. Next, and to form the dual binders, another coupling reaction should be carried out joining the intercalator and minor groove binder moieties and finally removal of the Boc groups would yield the hydrochloride salts of the dual binders (Scheme 5.10).
The reaction to prepare the ethyl ester was carried out on the five different amino acids (53-57). The resulting compounds 89a-e gave yields ranging between 19 and 98% (Table 5.3). As these compounds have been previously prepared, only $^1$H NMR and melting points were obtained for each compound.

Table 5.3.- Yields obtained for the amino-acetic acid ethyl esters

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>N</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>89a</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>89b</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>89c</td>
<td>7</td>
<td>97</td>
</tr>
<tr>
<td>89d</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>89e</td>
<td>11</td>
<td>19</td>
</tr>
</tbody>
</table>

The next step was to couple the ethyl ester of the amino acids with acridine-9-carboxylic acid. This reaction is similar to those for the coupling of the methyl esters and acridine-9-carboxylic acid previously discussed in Chapter 4. At room temperature and under argon,
ethyl 4-aminobutanoate and acridine-9-carboxylic acid in equimolar concentration in anhydrous CH$_2$Cl$_2$ were stirred and cooled to 0 °C. Four equivalents of DIEA were slowly added, followed by the addition of one and a half equivalents of the coupling reagent BOP. The reaction was stirred overnight at 20 °C. Usual work-up followed by purification by silica gel column chromatography using a gradient mixture of hexane:EtOAc eluent (5:1-3:1-1:1-EtOAc) afforded the final compound. Thus, five compounds 90a-e (Scheme 5.11) were prepared in good yields (49-68%) which were fully characterised by $^1$H NMR, $^{13}$C NMR, HRMS, IR and mp. The yields obtained for this reaction are summarised in Table 5.4.

![Scheme 5.11](image)

Table 5.4.-Yields obtained for the ethyl (acridine-9-carboxamido)alkylcarboxylates

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>N</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>90a</td>
<td>3</td>
<td>67</td>
</tr>
<tr>
<td>90b</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td>90c</td>
<td>7</td>
<td>49</td>
</tr>
<tr>
<td>90d</td>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td>90e</td>
<td>11</td>
<td>68</td>
</tr>
</tbody>
</table>

Next, the synthesis of the dual binders involving the coupling of the 90b with 51b was attempted. The coupling reaction was performed in the same manner as for the reaction with the methyl esters in section 5.6. Similar reactivity was observed for the ethyl esters...
and thus the reaction did not go to completion and the product could not be isolated (Scheme 5.12).

![Scheme 5.12](image)

5.8. Attempted coupling reactions using (acridine-9-carboxamido)alkylcarboxylic acids and Boc-protected mono-guanidines

The synthesis of the dual binders was then approached by a different synthetic method involving the use of the (acridine-9-carboxamido)alkylcarboxylic acids and the Boc-protected mono-guanidines synthesised in Chapters 4 and 3, respectively. This would then be followed by the Boc-deprotection to give the final dihydrochloride salts of the dual binders (Scheme 5.13).

![Scheme 5.13](image)

The first method involved converting the (acridine-9-carboxamido)alkylcarboxylic acid to the corresponding acid chloride using SOCl₂ and reacting this with the Boc-protected mono-guanidine. Initially, a test reaction was carried out on a small scale, a catalytic amount of DMF was added to a solution of 83b in excess of SOCl₂ and was refluxed for 5 hours. Removal of the SOCl₂ using toluene as a co-solvent under vacuum yielded the required compound as a yellow solid. Then, compound 51a was dissolved in dry DMF
along with a catalytic amount of DMAP and was kept at 0 °C under argon. The acid chloride was then dissolved in dry DMF and added drop-wise via syringe to the amine solution under argon and this mixture was stirred overnight at 0 °C (Scheme 5.14). Then, EtOAc was added resulting in a precipitate that was removed by filtration and the organic layer washed with 2 M HCl solution and saturated NaHCO₃. Next, it was washed with brine, dried with anhydrous Na₂SO₄ and concentrated under vacuum. The crude obtained was purified by silica gel column chromatography using a chloroform:methanol:ammonia (8:20:3) as eluent mixture. This reaction was successful and the required product (91) along with the two starting materials was observed by mass spectrometry confirming the mass peak for 91. The reaction was then carried out using three equivalents of compound 51a instead of one. However, this scaled up reaction did not result in a significantly higher yield and, hence, the product could not be properly characterised or brought forward for the next step.

Scheme 5.14

Next, the reaction using BOP as a coupling reagent was examined. Thus, (acridine-9-carboxamido)alkylcarboxylic acid and the mono-guanidine derivative (a number of different derivatives with different alkyl chain lengths and central linkers were used) in anhydrous CH₂Cl₂ were mixed at room temperature under argon. The mixture was stirred and cooled to 0 °C and DIEA was slowly added, followed by the addition of the coupling reagent BOP dissolved in anhydrous CH₂Cl₂. The reaction was stirred for 24 hours at 20 °C, EtOAc was added and the organic layer was successively washed with a 1 M HCl solution, a 20% NaHCO₃ solution and brine. The organic layer was dried over MgSO₄ and concentrated in vacuo. The crude residue was purified by chromatography on a silica gel column using hexane:EtOAc as eluent [(5:1), (3:1), (1:1) successively]. Analysis of the fractions was carried out by mass spectrometry but, unfortunately, none of them showed the mass of the required product or any definitive side products (Scheme 5.15).
The reaction was also carried out under the same conditions but using TBTU as a coupling reagent to couple 83a with 51a (Scheme 5.15); again no reaction occurred.

Based on the positive results obtained earlier when coupling the Fmoc-protected amino acid with the mono-guanidine and on literature examples,\(^4\) EDCI was thought to be a good candidate for the coupling of 83a and 51b. Thus, the starting materials, HOBt-hydrate and TEA were dissolved in dry DCM at 0 °C followed by addition of EDCI hydrochloride. This reaction was left stirring at room temperature overnight (Scheme 5.16).

The mechanism of this reaction is similar to the mechanism described for DCC in Chapter 3. It involves the lone pair of the N atom reacting with the hydrogen of the carboxylic acid resulting in the formation of the carboxylate anion and the positively charged carbodiimide. The carboxylate then attacks at the central carbon of the carbodiimide and the electrons migrate causing neutralisation of the positive charge forming an intermediate ester. Next, HOBt attacks the carbonyl carbon and the 3-(ethyliminomethyleneamino)-N,N-dimethylpropan-1-amine moiety is cleaved to form the HOBt-derivative. The amine then reacts with this ester at the carbonyl carbon and movement of the electrons forms the amide. This reaction was unsuccessful and only starting material was observed. This may have been due to the presence of the water molecule in the HOBt-hydrate. This reaction requires dry conditions in order for this reaction to proceed correctly.
The next coupling reagent explored was HATU (92) which is a tetramethylammonium salt. The preparation of this commercially available reagent is achieved by transformation of tetramethylurea (TMU) into the corresponding chlorouronium salt (chlorotetramethyluronium chloride, TMUCI), by treatment with COCl₂ in toluene followed by exchange with NH₄PF₆ or KPF₆ and then reaction of TMUCI with 1-hydroxy-7-azabenzotriazole (HOAt) (Scheme 5.17).

![Scheme 5.17](image)

The mechanism of this reaction is similar to that of TBTU described in Chapter 3. First, DIEA deprotonates the carboxylic acid to give the carboxylate which then attacks the positively charged aminium salt. The electrons then move to form the O'-azabenzotriazole which attacks the carbonyl carbon of ester and cleaves it. This leads to the formation of the ester derivative of azabenzotriazole which is then attacked by the amine at the carbonyl carbon to give the required amide and 1-azabenzotriazolol as a side product. Purification by column chromatography would eliminate this side product.

The reaction is carried out by the same method as when TBTU was used. A solution of 83a in DMF was treated with DIEA, 51b and HATU under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours (Scheme 5.18). Usual work up and purification by flash chromatography with silica gel eluting with hexane/EtOAc (2:1) yielded 93.
This reaction was initially carried out on a small scale and was successful. Compound 93 was observed by high resolution mass spectrometry but was not isolated. On the basis of this result, the scale was increased by an order of ten. Regrettably, the reaction failed on a larger scale.

This reaction was also attempted using HBTU for the coupling of 51e with 83d or 83e (Scheme 5.19). It has the same mechanism and is carried out by the same procedure as described above. The reaction was also carried out on a small scale for both derivatives but failed once again when upscaled.

The Yamaguchi esterification can be used in the synthesis of highly functionalised esters and amides. Thus, the Yamaguchi reagent (2,4,6-trichlorobenzoyl chloride) reacts with the carboxylic acid to form a mixed anhydride; the volatiles are removed and the reaction of the anhydride with an alcohol or amine in the presence of a stoichiometric amount of DMAP results in the required ester or amide.

The mechanism of this reaction proceeds by addition of the Yamaguchi reagent to the carboxylic acid chloride forming the mixed anhydride. Then, if DMAP is used in the reaction as a catalyst, it regioselectively reacts at the less hindered carbonyl carbon, since
DMAP is a stronger nucleophile than the alcohol or amine. The newly formed intermediate is less hindered, the acyl group is still polarized and DMAP is a good leaving group, all of which enable a fast reaction with the alcohol or amine (Scheme 5.20)

\[
\text{Scheme 5.20}
\]

In the experimental procedure, the Yamaguchi reagent is added to a mixture of 83b and TEA in DMF and the reaction mixture was stirred for 20 minutes at room temperature. This was monitored by TLC until the starting material was consumed. After the removal of triethylamine hydrochloride by filtration, the filtrate was evaporated and the residue was redissolved in DCM. To this solution a mixture of 51a and DMAP in DCM was added and the resulting solution was stirred overnight at room temperature (Scheme 5.21). The reaction mixture was diluted with ether, washed successively with 3% aqueous HCl, water and aqueous NaHCO₃, then dried with MgSO₄ and concentrated under vacuum. This reaction was carried out on a small scale and mass spectrometry was used to analyse the results of the reaction. Unfortunately, the required product was not observed, and only the peak corresponding to the molecular weight of the mono-guanidine could be identified.
The intermediates in that reaction did not seem sufficiently reactive and therefore another coupling reagent was considered. (Benzotriazol-1-yl oxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) is a derivative of BOP where the dimethylamine is replaced by pyrrolidine. It has been used to avoid the formation of the carcinogenic side product hexamethylphosphoramide (HMPA) which is a by-product of the BOP coupling reaction.

PyBOP works by the same mechanism as BOP, as it was described in Chapter 4. In a test reaction, 83b was dissolved in DMF; then, PyBOP and TEA were successively added followed by addition of 51a and the mixture left stirring at room temperature. The reaction was monitored by TLC until the starting material had been consumed (around 3 hours). Next, the solvent was removed, the mixture was purified by column chromatography using Hexane:EtOAc (2:1) and then fractions were examined using mass spectrometry and $^{1}$H NMR. The product was not identified maybe because the reactants were not reactive enough for the reaction to proceed (Scheme 5.22).

Ethyl chloroformate is used as a coupling reagent in peptide synthesis. The procedure involves separate preparation of a mixed anhydride by addition of ethyl chloroformate to the $N$-alkoxycarbonylamino acid anion that is generated by deprotonation of the corresponding acid by a tertiary amine, such as $N$-methylmorpholine or TEA. The amine then attacks the mixed anhydride and the amide is formed along with ethanol and CO$_2$ (Scheme 5.23).
This reaction was attempted on a number of different derivatives with different alkyl chain lengths in the acridine moiety and several central linkers in the mono-guanidine one. The procedure used consisted of utilising three equivalents of the (acridine-9-carboxamido)alkylcarboxylic acid dissolved in dry DCM at 0 °C, followed by addition of three equivalents of both TEA and ethyl chloroformate. The reaction mixture was stirred for 30 minutes under N₂ atmosphere and evaporated to dryness under reduced pressure. The resulting solid was re-dissolved in dry DCM at 0 °C followed by the addition of a solution of one equivalent of the corresponding mono-guanidine in dry DCM and excess TEA. The reaction mixture was stirred for 1 hour at 0 °C and overnight at room temperature under N₂ atmosphere. Then, it was washed with 5% NaHCO₃ and water. The organic phase was dried over Na₂SO₄ and the solvent evaporated under reduced pressure. The resulting solid was purified by silica gel chromatography using hexane:EtOAc (2:1) as eluent. This reaction was initially carried out on a small scale and optimisation was attempted. Thus, three equivalents of the amine and the one equivalent of the acid were used and the base 4-methylmorpholine was also tested. The effect of time and/or temperature change was also explored.
Unfortunately, this optimisation did not lead to an improvement in the results of the reaction even though all attempts were successful detecting the [M+H] molecular ion in the mass spectra. The reaction with 51c and 83b gave the best result producing a sufficient quantity of the product for full characterisation (Figure 5.5 and 5.6, and Scheme 5.24).

![Scheme 5.24](image)

Figure 5.5.-\textsuperscript{1}H NMR spectrum of compound 96
The $^1$H NMR spectrum (Figure 5.5) shows the aromatic signals for the 16 aromatic protons. Also, it displays two triplets and a multiplet for the aliphatic carbons of the aminoalkyl chain. The $^{13}$C NMR spectrum (Figure 5.6) shows all aromatic and quaternary carbons of the two aromatic structures. It also displays the quaternary carbons for the Boc groups. In addition, it shows four signals between 22.4 and 39.2 ppm which correspond to the methylene chain carbons.

Although accurate characterisation was obtained, there was not enough product synthesised to bring forward to the next step of deprotection. The reaction scale was increased using 83d by an order of three but disappointingly the yield did not substantially increase and there was still not enough material for deprotection. As a result of time constraints and minimal starting materials available, optimisation of this reaction was ceased at this point even though this particular set of conditions and reaction gave the best results of all the coupling reactions attempted and shown in this Chapter.
Chapter 5

5.9. Conclusions

The final aim of this thesis was to prepare dual binders composed of an intercalator moiety attached to a minor groove binder. Thus, in this chapter the various synthetic routes towards the preparation of dual binders consisting of an acridine moiety and a diphenyl mono-guanidine moiety linked by an alkyl chain were presented. The length of the alkyl chain between the two moieties was varied as well as the central linker of the minor groove binder. If the dual binders had been successfully synthesised one would have been able to compare the activity of the dual binders with that of the mono-functional compounds described in Chapters 3 and 4 to determine if synergistic effects could be observed.

Five different linkers connecting the di-aromatic moiety of the minor groove binder were explored (X = CH₂, CH₂CH₂, O, CO, NH) and different lengths of the alkyl chain tested (three, four, seven, ten and eleven methylene groups). Using the mono-guanidines and the (acridine-9-carboxamido)alkylcarboxylic acids prepared as described in previous Chapters, various coupling reactions and conditions were investigated.

Initially, routes involving orthogonal protection of the amino group of the aminoalkyl minor groove binders were attempted. The Cbz-protected aminoalkyl acids and Boc-protected guanidines were coupled to form the Boc-protected mono-guanidine Cbz-amino acid conjugates. However, the Cbz-group could not be removed and, thus, coupling of the conjugates and the acridine moiety could not be carried out. This synthetic route was also attempted using Fmoc as a protecting group. Disappointingly, this also proved unsuccessful.

Coupling of 9-chloroacridine or acridine-9-carboxylic acid using various coupling reagent such as SOCl₂, TBTU or BOP with the dihydrochloride salts of the aminoalkyl minor groove binders failed. Methyl and ethyl (acridine-9-carboxamido)alkylcarboxylates were synthesised and the coupling reactions of these with the mono-guanidines using TBTU were attempted but proved ineffective.

The coupling of (acridine-9-carboxamido)alkylcarboxylic acids and Boc-protected mono-guanidines was attempted using different coupling reagents such as chlorinating agents (SOCl₂), phosphonium salts (BOP and PyBOP), aminium salts (HBTU, HATU and TBTU), the Yamaguchi reagent which forms an ester which is attacked by the amine and, finally, ethyl chloroformate which forms a mixed anhydride which is then attacked by the
amine. The reactions using SOCl₂, HATU, HBTU and ethyl chloroformate were all successful showing the [M+H] molecular ion of the required product in their mass spectra. The reaction involving ethyl chloroformate gave the best results yielding enough product for full accurate characterisation. Unfortunately, due to limited time and minimal starting materials available, further optimisation was not possible. Further improvements of this reaction conditions could lead to successful coupling of the two moieties in sufficient yields. Upon synthesis of the Boc-protected dual binder, deprotection would then be carried out giving the dihydrochloride salt of the required dual binders.

5.10. References

Chapter 6

Biophysical Evaluation
6.1. Introduction

Once the syntheses of the DNA targeting agents previously discussed in Chapters 3 to 5 were completed, biophysical evaluation was undertaken to determine their affinity towards DNA and deduce their mode of binding. There are a number of physicochemical experiments which can be carried out to determine DNA interactions and binding constants of minor groove binders, intercalators and minor groove binder-intercalator dual agents.

It is extremely important to determine whether or not these molecules bind to DNA because, then, it will be possible to investigate their potential as anticancer, antiprotozoal, antiviral and/or antibacterial agents. As previously mentioned in Chapter 1, these diseases cause millions of deaths every year. If a molecule strongly binds to the DNA minor groove it may have the potential to block important enzymes and transcription control proteins that are essential for the growth of cancerous cells, bacteria, protozoan and viruses. If a molecule strongly intercalates, it can lead to lengthening and unwinding of the helix causing potential deletions in the coding region, which is related to their anticancer activity. Additionally, an intercalator exhibits cytotoxicity by means of poisoning the topoisomerase enzymes via stabilisation of the ternary complex (DNA–intercalator–topoisomerase).

Biophysical assays have contributed significantly to the determination of the strength and mode of binding of minor groove binders and intercalators. In this research, a number of optical physicochemical techniques including thermal denaturation assays, circular dichroism (CD), linear dichroism (LD) and reverse salt titration studies have been carried out to characterise the binding of these molecules to DNA. As well, evaluation of the pK_a by a non-optical method was performed. In this chapter, the basic theory behind each technique will be described followed by the explanation and analysis of the experiments carried out to determine DNA binding affinity and mode of binding of our compounds.

Firstly, evaluation of the protonation state by measuring the pK_a of our compounds is discussed. The minor groove has a strong negative potential, there, the bases are located inside the double helix with the negative phosphate groups on the outside. Therefore, mono- and di-cations have easy access to the groove and the binding of these cations leads to the stabilisation of DNA. Thus, UV spectroscopy can be used to determine whether a
molecule is mono- or di-cationic at physiological pH. Evaluation of pKₐ values was carried out to determine if the guanidine and/or aliphatic amine groups of our molecules were protonated at physiological pH. Thus, the pKₐ of the aminoalkyl derivatives of mono-guanidine di-aromatic minor groove binders will indicate if the compounds are positively charged and, therefore, have the ability to form electrostatic interactions within the negatively charged minor groove of DNA.

In addition, reverse salt titrations, which were used in this research, are helpful to prove that the minor groove binders are di-cationic within the minor groove. A reverse salt titration involves increasing the ionic strength of the DNA-ligand solution by addition of NaCl. Accordingly, the competition for the binding sites between the ligand and the salt cation increases, leading to displacement of the ligand. This is detected in the UV spectra when a decrease in the DNA-ligand absorption band is observed. This technique can also be used to determine binding mode.

Sequence selectivity has also been determined by carrying out different DNA binding experiments (thermal denaturation, CD and LD) on oligonucleotides containing specific base sequences such as poly(dA-dT)₂ which consists of a run of alternating adenine-thymine bases. The results obtained from these experiments are then compared with DNA binding experiments that were carried out in the presence of wild type salmon testes-DNA made up of 32% G:C base pairs.

6.2. Evaluation of the protonation state of the compounds prepared

When designing any new potential drug, it is crucial to determine their corresponding pKₐ in aqueous environments at physiological pH since their protonation state and basicity will be critical in the ligand–receptor interaction and it will play an essential role in their pharmacokinetic profile. For example, if a compound is in its protonated form, it will be probably soluble at physiological pH and, therefore, easily transported into cells. For example, it is postulated that compounds such as pentamidine gain entry into the cell via the human organic cation transporter 1 (hOCT1). As the environment of the DNA minor groove is considerably more electron rich than its surroundings,² the pKₐ value allows us to determine whether the molecules are protonated in the minor groove.
Before the pKₐ values were experimentally determined, some estimates of the corresponding values for the functional groups present in the compounds prepared were found in the literature.³ It has been previously determined in the Rozas group that the guanidine groups of the bis-guanidine di-aromatic derivatives, which are highly basic, show pKₐ values in the range of 9.4-10.8.⁴ Subsequently, it was expected that the guanidine groups of the compounds presented in this thesis would be protonated at physiological pH in the DNA minor groove. Secondly, the pKₐ values of amino acids such as 5-aminopentanoic acid and gamma-aminobutyric acid are 10.76 and 10.55, respectively. Taking this into consideration the aliphatic amine of the aminoalkyl chains was expected also to be protonated at physiological pH in the DNA minor groove. Thirdly, previous studies in Rozas group have shown that in those molecules containing an NH linker, this amine has a pKₐ of 2.9.⁴ Hence, the molecules prepared as minor groove binders in the present research are expected to be di-cationic at physiological pH.

As the compounds here studied are asymmetric, we expect to observe two pKₐ values, one for each functional group, as displayed in Figure 6.1.

![Protonatable sites of the hydrochloride salts of the aminoalkyl mono-guanidine di-aromatic derivatives.](image)

**Figure 6.1.-** Protonatable sites of the hydrochloride salts of the aminoalkyl mono-guanidine di-aromatic derivatives.

### 6.2.1. Determination of pKₐ

The pKₐ values of the compounds in this research were experimentally determined with a method based on the Henderson-Hasselbalch equation (6.1)

$$\text{pH} = \text{pK}_a + \log \left[\text{A}^+\right]/[\text{AH}]$$  (6.1)
According to this equation, when the concentration of the protonated acid and its conjugated base is the same, the logarithmic term is cancelled and then the pK$_a$ will be equal to the experimentally measured pH. Thus, if a 0.08 mmol solution of the compound is made up (pH = 7) and 4 mL of a 0.01 M NaOH standard solution is added, the pK$_a$ values of the first protonation of the hydrochloride salt can be obtained.

For compound 66e (Figure 6.2), the pH on addition of the 4 mL of 0.01 M NaOH was 11.33. Therefore, the pK$_a$ of the first protonation is 11.33. The pK$_a$ of the second protonation was determined by adding eight additional mL of the 0.01 M NaOH standard solution and measuring the pH afterwards which was 11.69. Therefore, the pK$_a$ of the second protonation is 11.69. To determine the pK$_a$ values corresponding to the third protonation (compound 66e has a X = NH linker) the pH of solutions containing 0.08 mmol of 66e and 4 mL of a 0.01 M HCl standard solution was measured as 2.60. Therefore, the pK$_a$ of the third protonation in this particular molecule was found to be 2.60.

The results obtained can be rationalised as follows. Firstly, three pK$_a$ values were measureable indicating that deprotonation occurs at different pH for all of the functional groups. The difference in the pK$_a$ values found for the first and second protonation is very small and, hence, it is difficult to distinguish which pK$_a$ is associated with the aliphatic amine and which is associated with the guanidine (both pK$_a$ values estimated to be around 9.4-10.76).2 Secondly, the pK$_a$ values obtained are in agreement with the literature basicity values of di-aromatic bis-guanidines and 5-aminopentanoic acid (10.8 and 10.76) respectively.2 Considering that the pK$_a$ of the NH linker in 66e was determined to be 2.60, this secondary amine would not be protonated at physiological pH. From these results it can be concluded that both the guanidine and the aliphatic amine would be protonated at physiological pH therefore making these molecules di-cationic within the minor groove of DNA.

Figure 6.2.- Experimental pK$_a$ values obtained for the guanidine, aliphatic amine and secondary amine functionalities in compound 66e.
Basicity can also be determined using UV spectroscopy. This method involves titration of the molecule with aliquots of NaOH (0.1 M) and if an increase in absorbance is observed this indicates deprotonation of the guanidine and/or aliphatic amine. Then, by plotting pH against normalised absorbance, the area in which spectral changes occur can be found. Consequently, the pKₐ for the guanidine and aliphatic amine could be evaluated from these plots by using the Henderson-Hasselbalch equation. The pKₐ of the X=NH linker could be determined by UV by adding aliquots of 0.1 M HCl and recording the change in absorption. A second plot of pH vs. normalised absorbance would show the area in which spectral changes occur and accordingly, the pKₐ for the NH linker can be evaluated from these plots. This method has been carried out in previous studies by the Rozas group, however, in our case the change in absorbance was not significant enough and therefore, accurate results could not be obtained.

The pKₐ values of the alkylcarboxylic acid acridine derivatives were also calculated. Acridine has a pKₐ of 5.6 and, hence, a large proportion of the acridine molecule is neutral at physiological pH. Substitution of acridine with different functional groups can alter its basicity. Thus, the pKₐ values of the alkylcarboxylic acid acridines prepared in this research were measured to determine the effect that the alkylcarboxylic chain could have on the basicity of the acridine moiety. The Henderson-Hasselbalch equation was used as before and, thus, a 0.08 mmol solution of 83a (Figure 6.3) was prepared and the pH measured. This solution had a pH of 6.92. Next, 4 mL of a 0.01 M HCl solution were added to the acridine solution and the pH was measured again giving a value of 5.33 (pKₐ= 5.33 in the N of the acridine moiety). The expected pKₐ of an aliphatic carboxylic acid is 4.23 and, hence, this should be deprotonated at physiological pH; thus, an additional 8 mL of 0.01 M HCl was added to our compound solution resulting in a pH of 2.69 (pKₐ of the carboxylic acid of 83a = 2.69). Therefore, the carboxylic acid would be deprotonated at physiological pH.

![Figure 6.3.- Basicity (pKₐ values) of the acridine butyric acid derivative 83a](image-url)
6.3. Studying the dependence of increasing the ionic strength on the binding affinity

A reverse salt titration was carried out on compound **66e** to prove that the molecule was dicationic at physiological pH. Reverse salt titrations involve increasing the ionic strength of the DNA-ligand solution by addition of NaCl. Consequently, the competition for the binding sites between the ligand and the cation from the salt increases and the bound ligand is displaced upon increasing salt concentration. This is detected in the UV spectra when a decrease in the DNA-ligand absorption band is observed. This technique can also be used to determine binding mode (intercalation vs. minor groove binding) since a minor groove binder can be easily displaced from the groove upon increasing ionic strength whereas an intercalator is not easily displaced because it is inserted in between the base pairs and would not be affected by the salt concentration increment. Figure 6.4 shows that upon raising the ionic strength by increasing the salt concentration, spectral alterations were observed for the UV spectrum of compound **66e** with salmon testes DNA. This indicates that significant quantities of the ligand were displaced from the DNA helix confirming that this molecule binds to the minor groove.
Figure 6.4.- UV-vis absorption spectrum for the reverse salt titration of compound 66e (top), the dependence of the binding constant on the salt concentrations is shown on the graph (bottom).

From the UV-vis absorption spectrum (Figure 6.4, top), the dependence of the binding of 66e on the salt concentration was investigated by plotting logK vs. log[Na+] and the slope of the graph obtained (SK) is calculated according to equation (6.2) where Z is the charge on the ligand and ψ is the charge on the phosphate:  

\[
SK = \frac{\delta \log K}{\delta \log [Na^+]} = -Z \psi \quad (6.2)
\]

\[
Z = -SK/\psi \quad (6.3)
\]
The slope of the graph (SK) was found to be -1.74 and if the equation (6.2) is rearranged to give equation (6.3) then Z is calculated to be 2. Therefore, the number of cations is two confirming that the molecule is di-cationic at physiological pH.

A reverse salt titration was also carried out on the alkylcarboxylic acid acridine 83a to understand the effect that increasing ionic strength has on the binding of this molecule to DNA. As mentioned before, since intercalators are inserted in between the base pairs they should not be easily displaced by NaCl. In Figure 6.5 it is shown that upon increasing the salt concentration, the absorbance of the peak at 360 nm in the UV spectrum of 83a bound to salmon testes DNA did not decrease as much as with the minor groove binders. This indicates that a small number of ionic interactions are established in the binding of this molecule, which is neutral at the N atom of acridine when binding, and shows that it binds to DNA via intercalation.

Figure 6.5.- UV-vis absorption spectrum for the reverse salt titration experiment of compound 83a and salmon sperm DNA.

6.4. DNA Thermal Denaturation Assays

The bases in the double helix of DNA are arranged in stacked helical configuration. Thus, they are shielded from the light by the external phosphate groups and can only absorb a minimal quantity of light. If the temperature of a solution containing a sample of a helical nucleic acid such as DNA is increased, this energy input breaks the hydrogen bonds that hold the double helix together resulting in two single strands. This process is known as thermal denaturation. As a result of this an increase in light absorbance occurs due to the
fact that free bases are not shielded by phosphates anymore and therefore are able to rotate and absorb light freely. This increased absorbance of light by the DNA bases can be correlated with the gradual hyperchromic shift seen in a UV spectrum (Figure 6.6).

Unwinding occurs initially in areas rich in A:T base pairs, which are bound in a weaker manner than G:C due to the smallest number of intermolecular hydrogen bonds. Thus, strands of DNA sequences rich in G:C do not unwind until higher temperatures are reached. Each region melts in a narrow temperature range and each melting creates two phase boundaries of melted and helical forms. In a melting curve, the increase in UV absorbance is measured as the temperature increases tracking the unwinding and denaturation of DNA. The melting point \( (T_m) \) is the midpoint of this increase in a population of DNA molecules correlating to the temperature at which half the DNA is unwound (see Figure 6.6).

Initially, the DNA thermal denaturation of the oligonucleotide alone is carried out, followed by the thermal denaturation of the oligonucleotide bound to a ligand. The melting points \( (T_m) \) of these two experiments are compared and the difference in such thermal melting temperatures \( (\Delta T_m) \) is determined by subtracting the \( T_m \) of oligonucleotide alone from that of the ligand bound to the oligonucleotide. This value can be used as a measurement of the affinity of the ligand towards DNA.

An increase in the melting temperature occurs when a minor groove binder binds into the minor groove. This leads to considerable stabilisation of the double helical structure as a result of the formation of van der Waals contacts and HBs with the DNA base pairs, which
would be expected to contribute to the enthalpy of interaction. Upon binding to the minor groove, the molecule displaces a large number of water molecules (from the spin of hydration) and cations (which are stabilising the double helix) resulting in a favourable entropic increase. Therefore, the process of binding of a molecule to DNA is thermodynamically favourable because DNA-minor groove binder complexes are both enthalpically and entropically stable. Consequently, a higher input of energy is required to induce thermal denaturation of the complex resulting in the increased Tm value.10

The melting temperature of DNA is dependent on its specific sequence. The observed melting temperature for wild type salmon testes DNA is 68 °C, while poly(dA-dT)$_2$, which is the AT specific oligonucleotide used in this work, has quite a low melting temperature of 47 °C. The lower melting temperature of this oligonucleotide is due to the lack of G:C base pairs which form stronger HB interactions than the A:T base pairs.11

Melting temperatures can be detected using NMR or CD;12 however, UV absorbance is the most popular method used due to its simplicity, sensitivity and reproducibility. This technique is advantageous because spectrophotometers are inexpensive, only small amounts of drug and nucleic acid are required and no spectral signal from the drug is necessary. Additionally, a Tm value can provide an estimate for the binding constant of a drug. Unfortunately, there are some disadvantages associated with thermal denaturation assays; for example, ligands might have to be stable at temperatures as high as 90 °C, they must be soluble in optically transparent buffers, binding interactions of ligands are compared at the melting temperature not at a standard temperature of 25 °C or at physiological temperature 37 °C, lastly Tm measurements do not provide specific information regarding the structure of the nucleic acid-ligand complex or about the kinetics of the nucleic acid-ligand interaction.13 For these reasons, thermal denaturation assays are generally carried out as preliminary experiments for ranking compounds according to their binding affinity. They are usually followed by other biophysical techniques which give more in depth information of the binding affinity and mode of the DNA binder.

As mentioned, thermal denaturation experiments are generally carried out by using a UV-vis spectrophotometer and recording absorbance at 260 nm ($\lambda_{max}$ of DNA) as a function of temperature; but it can also be determined by using a fluorescence emission signal or the intensity of an NMR signal. This biophysical technique has a number of different
applications other than analysing quantitatively drug–nucleic acid interactions. Thermal
denaturation is particularly beneficial when characterising molecules with extremely high
binding affinity to DNA. An example of this is given by Chaires et al. who used thermal
denaturation along with other biophysical techniques to characterise the interaction of the
new bis-intercalating anthracycline antibiotic WP631 with DNA. Upon binding of this bis-
intercalator to herring sperm DNA a high $\Delta T_m$ of 26.3 was obtained.\textsuperscript{14} Thermal
denaturation can also be utilised to determine the stability of RNA with RNA binders as
well as to determine whether a compound is DNA sequence selective.

6.4.1. DNA thermal denaturation assays of the aminoalkyl di-aromatic mono-
guanidine derivatives

DNA thermal denaturation experiments were carried out on all the aminoalkyl di-aromatic
mono-guanidine systems. This technique provides information on the general binding
strength and shows the effect that the nature and length of the aminoalkyl chain have on
the binding of the minor groove binder moiety. Salmon testes DNA (wild type, sequence
unspecific oligonucleotide) was used for these experiments. Salmon testes DNA has 32%
G:C content, therefore, the minor groove will be average in width, and would possibly
accommodate large flexible molecules. Firstly, the melting temperature of DNA was
determined by preparing a solution of known concentration of unspecific wild type salmon
testes DNA (150 $\mu$M, giving an absorbance of 1 au at 260 nm), which was heated in a
temperature range of 30-90 °C simultaneously measuring the absorbance. In the range of
65-70 °C, an increase in absorbance was observed and a characteristic melting temperature
curve was obtained. When the DNA is heated to this temperature, enough energy is given
to split the double helix into two single strands which absorb more strongly than the double
helix. The first derivative of the absorbance function is then calculated to give the thermal
melting temperature result ($T_m$) which, in the case of salmon testes DNA was found to be
68 °C.

The next step was to determine the binding strength of the aminoalkyl di-aromatic mono-
guanidine derivatives. The thermal denaturation experiments were repeated by adding the
specific compound to be tested (at 15 $\mu$M) to a solution of DNA (150 $\mu$M) to achieve a
final phosphate:drug (P/D) ratio of 10. The experiment was then performed, whilst
employing the same conditions, as described for the DNA alone. If binding occurs, an
increase in the $T_m$ value should be observed due to the extra stabilisation provided to the
double helix. Hence, for each DNA/aminoalkyl di-aromatic mono-guanidine complex, a characteristic plot was obtained and the corresponding T_m value calculated. The ΔT_m was obtained by deducting the melting temperature of salmon testes DNA from that of the DNA/aminoalkyl di-aromatic mono-guanidine complex. Duplicates were obtained to confirm reproducibility.

The binding of each molecule to the DNA resulted in an increase in melting temperature in comparison to 68 °C obtained for the melting temperature of DNA alone. Results obtained for the -CH_2CH_2, n=3 (65b) -O, n=4 (66c) and -O, n=7 (67c) derivatives are shown in Figure 6.7. Shifts to higher temperatures than that of DNA can be observed proving the binding of these compounds to DNA.

![Figure 6.7. Plot showing the DNA (—) thermal denaturation results of compounds 65b [X=CH_2CH_2, n=3(—)], 66c [X=O, n=4(—)] and 67c [X=O, n=7(—)] with salmon testes DNA](image)

The results obtained for the thermal denaturation assays performed on aminoalkyl di-aromatic mono-guanidines are displayed in Table 6.1. It has to be mentioned that when studying the non alkylated di-aromatic guanidines the best results were achieved for the symmetric bis-guanidinium derivatives (compounds 97-e^{15} in Table 6.1); however, together with the values obtained for these bis-guanidinium derivatives and for a better structural comparison with the present conjugates, the ΔT_m values obtained for the mono-guanidiniums (98a-e^{16}) are also presented in Table 6.1. This will show the effect of the
alkylamino chain on the affinity of the di-aromatic mono-guanidines. It will also indicate the optimal length for these alkylamino chains in terms of DNA affinity.

Table 6.1.-Thermal denaturation experiment results\(^a\) of the aminoalkyl di-aromatic mono-guanidine conjugates (65a-e to 69a-e), the results for the bis- and mono-guanidinium unconjugated minor groove binders (97a-e and 98a-e) are presented for the sake of comparison.

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>n</th>
<th>(\Delta T_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65a</td>
<td>CH(_2)</td>
<td>3.</td>
<td>1.0 ±0.01</td>
</tr>
<tr>
<td>65b</td>
<td>CH(_2)CH(_2)</td>
<td>3</td>
<td>5.0 ±0.82</td>
</tr>
<tr>
<td>65c</td>
<td>O</td>
<td>3</td>
<td>1.0 ±0.82</td>
</tr>
<tr>
<td>65d</td>
<td>CO</td>
<td>3</td>
<td>5.0 ± 0.01</td>
</tr>
<tr>
<td>65e</td>
<td>NH</td>
<td>3</td>
<td>1.0 ±0.01</td>
</tr>
<tr>
<td>66a</td>
<td>CH(_2)</td>
<td>4</td>
<td>4.0 ±1.0</td>
</tr>
<tr>
<td>66b</td>
<td>CH(_2)CH(_2)</td>
<td>4</td>
<td>5.0 ±0.58</td>
</tr>
<tr>
<td>66c</td>
<td>O</td>
<td>4</td>
<td>6.0 ±0.41</td>
</tr>
<tr>
<td>66d</td>
<td>CO</td>
<td>4</td>
<td>5.0 ±1.0</td>
</tr>
<tr>
<td>66e</td>
<td>NH</td>
<td>4</td>
<td>6.0 ± 0.01</td>
</tr>
<tr>
<td>67a</td>
<td>CH(_2)</td>
<td>7</td>
<td>0 ±0.01</td>
</tr>
<tr>
<td>67b</td>
<td>CH(_2)CH(_2)</td>
<td>7</td>
<td>2.0 ±0.01</td>
</tr>
<tr>
<td>67c</td>
<td>O</td>
<td>7</td>
<td>2.0 ±0.82</td>
</tr>
<tr>
<td>67e</td>
<td>NH</td>
<td>7</td>
<td>2.0 ±0.01</td>
</tr>
</tbody>
</table>
In Table 6.1 it can be observed that the introduction of an aminoalkyl chain, and, in particular short chains, results in increased $T_m$. In general, most of the aminoalkyl di-aromatic mono-guanidine conjugates showed moderate increased binding to DNA with $\Delta T_m$ between 2 and 6. Looking at the nature of the X linker, -NH- linked derivatives gave the best results in general, but some of the -O-, -CO- and -CH$_2$CH$_2$- linked compounds (65b, 65d, 66c, 66e and 67c) displayed the strongest binding. The most significant results ($\Delta T_m = 6$) were achieved for compounds with -O- and -NH- linkers and aminoalkyl chains of four methylene groups ($n=4$, compounds 66c and 66e). This result could suggest that the

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>68a</td>
<td>CH$_2$</td>
<td>10</td>
<td>0 ±0.01</td>
</tr>
<tr>
<td>68b</td>
<td>CH$_2$CH$_2$</td>
<td>10</td>
<td>1.0 ±0.82</td>
</tr>
<tr>
<td>68c</td>
<td>O</td>
<td>10</td>
<td>2.0 ±0.01</td>
</tr>
<tr>
<td>68d</td>
<td>CO</td>
<td>10</td>
<td>2.0 ±0.89</td>
</tr>
<tr>
<td>68e</td>
<td>NH</td>
<td>10</td>
<td>3.0 ±0.01</td>
</tr>
<tr>
<td>69a</td>
<td>CH$_2$</td>
<td>11</td>
<td>0 ±0.01</td>
</tr>
<tr>
<td>69b</td>
<td>CH$_2$CH$_2$</td>
<td>11</td>
<td>1.0 ±0.41</td>
</tr>
<tr>
<td>69c</td>
<td>O</td>
<td>11</td>
<td>2.0 ±0.01</td>
</tr>
<tr>
<td>69d</td>
<td>CO</td>
<td>11</td>
<td>2.0 ±0.01</td>
</tr>
<tr>
<td>69e</td>
<td>NH</td>
<td>11</td>
<td>5.0 ±0.01</td>
</tr>
<tr>
<td>97a$^{15}$</td>
<td>CH$_2$</td>
<td>-</td>
<td>8.0 ±0.01</td>
</tr>
<tr>
<td>97d$^{15}$</td>
<td>CO</td>
<td>-</td>
<td>4.0 ±0.01</td>
</tr>
<tr>
<td>97e$^{15}$</td>
<td>NH</td>
<td>-</td>
<td>8.0 ±0.01</td>
</tr>
<tr>
<td>98a$^{16}$</td>
<td>CH$_2$</td>
<td>-</td>
<td>3.0 ±0.01</td>
</tr>
<tr>
<td>98b$^{16}$</td>
<td>CH$_2$CH$_2$</td>
<td>-</td>
<td>0 ±0.01</td>
</tr>
<tr>
<td>98c$^{16}$</td>
<td>O</td>
<td>-</td>
<td>2.0 ±0.01</td>
</tr>
<tr>
<td>98d$^{16}$</td>
<td>CO</td>
<td>-</td>
<td>0 ±0.01</td>
</tr>
<tr>
<td>98e$^{16}$</td>
<td>NH</td>
<td>-</td>
<td>2.0 ±0.01</td>
</tr>
</tbody>
</table>

*Melting temperature of salmon testes DNA in phosphate buffer (10 mM) is 68 °C.*
strong HB acceptor nature of the –O- group (compound 66c) and the strong HB donor nature of the –NH- group (compound 66e) could be responsible for the good binding. Furthermore, compound 69e (n=11) shows a ΔTm = 5 probably due to the nature of the –NH- linker. However, the corresponding di-aromatic mono-guanidines did not show strong binding to DNA indicating that the aminoalkyl chain is playing a favourable role in the interaction with DNA.

There are differences between the di-aromatic mono-guanidines and their aminoalkyl conjugates that may lead to increments in the affinity for DNA. First, the total length of the aminoalkyl di-aromatic mono-guanidines is larger than the corresponding di-aromatic mono-guanidines, which could lead to a better fitting into the minor groove occupying more space and displacing more of the water molecules within. Second, the aminoalkyl di-aromatic mono-guanidines are di-cationic at physiological pH, whereas the di-aromatic mono-guanidines are mono-cations and it is known that di-cationic molecules favour the interactions in the negatively charged environment of the minor groove. Third, the aminoalkyl chain may better direct the charged ammonium group to establish strong HBs, hydrophobic forces and ionic interactions with the nucleic bases in the minor groove of DNA.

Regarding the aminoalkyl chain length, those compounds with four methylene groups (n= 4, compounds 66a-e) gave the best results and elongating the chain seemed to interfere with the binding except for those compounds with a NH group as a linker (68e, n=10 and 69e, n=11). Compounds 66b, 66c and 66d showed differences in the ΔTm of 5, 4 and 5 °C, respectively, when compared to the corresponding mono-guanidines (98b-d). This could indicate that very short alkyl chains cannot contribute with extra interactions to DNA in order to improve the binding, whereas alkyl chains longer than four CH2 groups are too long or too lipophilic to achieve an optimal interaction. Compounds 65b (X= CH2CH2) and 65d (X= CO) showed the strongest binding for the series where n= 3. This difference can be attributed to the extra CH2 group in the linker in the case of compound 65b, thereby giving the molecule an extra degree of freedom. As well, when comparing the ΔTm, it has to be considered that the distance between the cations in 65b is larger than that of 65a. In 65d the HB acceptor CO linker could lead to stronger interactions with the minor groove.
Therefore, two effects should be considered for future derivatives. On the one hand, even though the presence of the aminoalkyl chain does not increase the binding in all the cases, those between four and seven methylene groups give the best results and should be incorporated in future derivatives. On the other hand, considering that the bis-guanidinium minor groove binders produced a better DNA interaction than the aminoalkyl conjugates, probably because the extra HB contacts that the second guanidinium cation provides, the introduction of a second guanidinium functionality between the minor groove binder system and the aminoalkyl chain should be considered.

Once the experiments with salmon testes DNA were completed, the sequence selectivity of the aminoalkyl di-aromatic mono-guanidines was investigated by carrying out thermal denaturation experiments of some of the derivatives in poly(dA-dT)\textsubscript{2} DNA. This oligonucleotide is completely made up of A:T base pairs. Due to the lack of G:C base pairs the minor groove is consequently narrower than the minor groove of natural salmon testes DNA. Molecules that have a better fit in the minor groove of poly(dA-dT)\textsubscript{2} than in that of salmon testes DNA should display an increased binding affinity. Also the exocyclic amino group from the G:C pairs in salmon testes DNA could prevent a good fit of the molecules in the minor groove. In order for a molecule to bind into the narrower minor groove, they should be relatively planar with a small dihedral angle between the two aromatic rings. Derivatives 66\textsubscript{d} and 66\textsubscript{e} (X=CO and NH, n=4) and 69\textsubscript{e} (X=NH, n=11) were chosen for this study with the A:T oligonucleotide because they showed the strongest binding from the salmon testes assays. In addition, 66\textsubscript{d} and 66\textsubscript{e} were selected to correspond with the asymmetric guanidine/2-aminoimidazoline derivatives that were previously studied in detail using various biophysical techniques giving good results, and derivative 69\textsubscript{e} was chosen to show the effect of a long aminoalkyl chain on binding to the narrower minor groove of poly(dA-dT)\textsubscript{2}.

These experiments were carried out as previously described for the salmon testes DNA thermal denaturation experiments. They were performed using a P/D ratio of 10 but due to fact that poly(dA-dT)\textsubscript{2} is expensive, lower concentrations of 50 \(\mu\)M for DNA and 5 \(\mu\)M for the ligand were used. The results obtained are displayed in Table 6.2.
Table 6.2.-Thermal denaturation experiment results$^a$ of the aminoalkyl di-aromatic mono-guanidine conjugates (66d, 66e and 69e) with poly(dA-dT)$_2$

\[
\text{HCl H}_2\text{N}\left(\begin{array}{c}
\text{O} \\
n
\end{array}\right)\text{NH}_2 \\
\text{NH}
\]

\[n=4, X= \text{CO, NH (66d,e)}
\]
\[n=11, X= \text{NH (69e)}
\]

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>n</th>
<th>$\Delta T_m\text{(ST)}$</th>
<th>$\Delta T_m\text{(AT)}$</th>
<th>Rel. $T_m$</th>
<th>Selectivity $\Delta T_m\text{(AT)}/\Delta T_m\text{(ST)}$</th>
<th>Sequence Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>66d</td>
<td>CO</td>
<td>4</td>
<td>5.0±1.0</td>
<td>7.4±0.01</td>
<td>2.4</td>
<td>1.48</td>
<td>AT</td>
</tr>
<tr>
<td>66e</td>
<td>NH</td>
<td>4</td>
<td>6.0±0.01</td>
<td>6.2±0.01</td>
<td>0.2</td>
<td>1.03</td>
<td>None</td>
</tr>
<tr>
<td>69e</td>
<td>NH</td>
<td>11</td>
<td>5.0±0.01</td>
<td>6.6±0.01</td>
<td>1.6</td>
<td>1.32</td>
<td>AT</td>
</tr>
</tbody>
</table>

$^a$Melting temperature for poly(dA-dT)$_2$ was found to be 47 °C

These results indicate that both 66d and 69e show sequence selectivity for AT sequences. Compound 66e has a very small preference for AT sequence over mixed natural DNA sequences.

Relative $T_m$ values can be obtained from equation (6.4) by defining the difference of thermal melting temperature between poly(dA-dT)$_2$ and salmon testes DNA as:

\[
\text{Rel. } T_m = \Delta T_m\text{(AT)} - \Delta T_m\text{(ST)}
\]  

(6.4)

All compounds show an increase in the $\Delta T_m$ in the order of 66e < 69e < 66d with relative $T_m$ (Rel. $T_m$) from 0.2 to 2.4. The CO linker of 66d allows for a better fit within the minor groove than the NH linker in 66e. Thus, 66d displays a greater affinity for poly(dA-dT)$_2$ than 66e. The longer aminoalkyl chain in 69e allows for a better fit and stronger binding affinity to the A:T oligonucleotide than 66e which binds to poly(dA-dT)$_2$ and salmon testes DNA with very similar affinity. The extra methylene groups in 69e could increase the binding affinity by forming extra van der Waals contacts with the DNA base pairs, or by latching around the DNA minor groove pocket.

Next, thermal melting experiments were carried out with poly(dA)*poly(dT) in the presence of the same compounds following similar methodology. This polynucleotide
differs from poly(dA-dT)₂ because it has non-alternating AT bases. The results obtained are shown in Table 6.3.

Table 6.3.-Thermal denaturation experiment results* of the aminoalkyl di-aromatic mono-guanidine conjugates (66d, 66e and 69e) with poly(dA)•poly(dT)

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>X</th>
<th>N</th>
<th>ΔT&lt;sub&gt;m(A&lt;/sub&gt;T) (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>66d</td>
<td>CO</td>
<td>4</td>
<td>6.0 ±0.01</td>
</tr>
<tr>
<td>66e</td>
<td>NH</td>
<td>4</td>
<td>1.4 ±0.01</td>
</tr>
<tr>
<td>69e</td>
<td>NH</td>
<td>11</td>
<td>2.0 ±0.01</td>
</tr>
</tbody>
</table>

*Melting temperature for poly(dA)•poly(dT) was found to be 60 °C

These assays confirmed that 66d is selective for AT sequences but 66e and 69e did not display a significant increase in T<sub>m</sub> and, therefore, must not have achieved an optimal interaction with the poly(dA)•poly(dT) sequence.

In conclusion, the affinity of these compounds towards salmon testes, poly(dA-dT)₂ and poly(dA)•poly(dT) DNA indicate that the aminoalkyl di-aromatic mono-guanidines 65b, 65d, 66b, 66c, 66e, 68e and 69e have the strongest affinity for salmon testes DNA while 66d and 69e have the strongest affinity for the narrower minor groove of poly(dA-dT)₂. These experiments show that in all cases the incorporation of the aminoalkyl chain results in significant increments in the melting temperature of the DNA complexes.

Further, to investigate the role of electrostatic interaction in the binding process, the binding of 66e and 69e with poly(dA-dT)₂ was explored via thermal denaturation assays in the presence of 25 mM NaCl (Table 6.4). An overall decrease in the magnitude of binding affinity was observed with increasing ionic strength of the solution, indicating significant contribution of electrostatic interaction in the ligand binding process.
Table 6.4.- Salt dependent thermal denaturation experiment results\(^a\) of the aminoalkyl di-aromatic mono-guanidines 66e and 69e with poly(dA-dT)\(_2^\text{\textsuperscript{a}HCl}}\) and poly(dA-dT)\(_2^\text{\textsuperscript{a}NH}_2\) 

\[ n=4, \text{X= NH (66e)} \]
\[ n=11, \text{X= NH (69e)} \]

\[ 66e \quad \text{NH} \quad 4 \quad 0.2 \]
\[ 69e \quad \text{NH} \quad 11 \quad -1.8 \]

\(^a\)Melting temperature for poly(dA-dT)\(_2\) in 10 mM phosphate buffer with 25 mM NaCl was found to be 57.4 °C

6.4.2. DNA thermal denaturation assays of the alkylcarboxylic acid derivatives of acridine

In order to determine the affinity of the alkylcarboxylic acid derivatives of acridine-9-carboxylic acid intercalators towards DNA, different thermal denaturation experiments were performed. A solution of known concentration of unspecific wild type salmon testes DNA (150 µM) was used and the change in the thermal melting temperature (\(\Delta T_m\)) was monitored when adding the molecule to be tested at a known concentration (15 µM).

The results obtained for these thermal denaturation assays are displayed in Table 6.5. Additionally, and for comparison purposes, the binding affinity of the starting material (acridine-9-carboxylic acid), the known intercalator proflavin (99, Figure 6.8) and the acridine p-toluidine derivative (100\(^5\), Figure 6.8) were also measured.

![Figure 6.8.- Structure of proflavin (99) and the p-toluidine acridine derivative (100)](image)

This will show the effect of the alkylcarboxylic acid chain on the DNA affinity of acridine derivatives. It will also show the optimal length for these chains in terms of DNA affinity.
Table 6.5. - Thermal denaturation results\(^a\) of the alkylcarboxylic acid acridine derivatives

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>(n)</th>
<th>(\Delta T_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proflavin (99)</td>
<td>-</td>
<td>3.4±0.01</td>
</tr>
<tr>
<td>9-Acridine carboxylic acid</td>
<td>-</td>
<td>4.0±0.01</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>2.0±0.01</td>
</tr>
<tr>
<td>83a</td>
<td>3</td>
<td>5.0±0.01</td>
</tr>
<tr>
<td>83b</td>
<td>4</td>
<td>2.0±0.01</td>
</tr>
<tr>
<td>83c</td>
<td>7</td>
<td>0±0.01</td>
</tr>
<tr>
<td>83d</td>
<td>10</td>
<td>2.0±0.41</td>
</tr>
<tr>
<td>83e</td>
<td>11</td>
<td>1.0±0.01</td>
</tr>
</tbody>
</table>

\(^a\)Melting temperature of salmon testes DNA in phosphate buffer (10 mM) is 69 °C.

It can be observed in Table 6.5 that the introduction of an alkylcarboxylic acid chain, and in particular short chains, can result in increased binding affinity to DNA, for example, in the case of compound 83a. Thus, stabilisation of salmon testes DNA in the presence of 83a (P/D = 10) was indicated by a 5 °C increase of the DNA melting temperature of the oligonucleotide in 10 mM buffer solution. Even though intercalators cause mainly destabilisation by leading to lengthening and unwinding of the helix, some of them may also cause a small degree of stabilisation. In general, the rest of the compounds (83b-e) showed either no change or a decrease in affinity in comparison to proflavin. Compounds 83b and 83d show a decrease in binding affinity when compared with proflavin and acridine-9-carboxylic acid but display the same binding affinity as the \(p\)-toluidine derivative 100.

Regarding the alkylcarboxylic acid chain length, compound 83a with an alkyl chain \(n=3\) gave the best results and elongating the chain seemed to interfere with the binding leading to no change or a decrease in binding affinity. This could indicate that chains with very
short alkyl chains cannot contribute with extra interactions with DNA to improve the binding, whereas alkyl chains longer than three methylene groups are too long or too lipophilic to achieve an optimal interaction. The alkylcarboxylic acid chains would also induce hydrophobic interactions as well as extra van der Waals contacts which may be favourable for binding. Another possible reason for the low binding affinity could be the rigid amide bond attached to the acridine-9 position that does not allow the acridine moiety to fully insert in between the DNA base pairs.

Compound 83a has been proven by LD and CD (see further sections) to act as an intercalator but it also stabilises DNA by a small degree as indicated by the positive ΔT_m. The other derivatives such as 83b and 83e display less stabilisation and are also proven to be efficient intercalators by subsequent biophysical methods. The stabilisation may be a result of the aliphatic chain.

6.5. Circular Dichroism Studies

Circular Dichroism (CD) is a spectroscopic technique that can be used to measure the binding strength and mode of binding of molecules to DNA. It is based on the difference between absorption of left- and right-handed circularly polarised light by a chiral molecule.18 DNA bases are achiral, but become chiral when placed within the framework of the chiral sugar–phosphate backbone. CD measures the differential absorption of left- and right-handed circularly polarised light within the framework of the chiral sugar–phosphate backbone. Ligand–DNA interactions can be analysed by virtue of the interpretation of induced ligand CD signals resulting from the coupling of electronic transition moments of the ligand and DNA bases within the asymmetric DNA environment. DNA binding ligands such as minor groove binders or intercalator are generally achiral molecules and, as a result, are optically inactive. When a molecule binds to DNA, an induced CD (ICD) signal is acquired through the coupling of electronic transition moments of the ligand and the DNA bases. The ICD signal within the absorption bands of the achiral ligand indicates ligand–DNA interaction. Intercalators generally display small ICD signals<10 M⁻¹ cm⁻¹ at the maximum of the ICD signal. Minor groove binders result in larger positive ICD signals. Calculations suggest that the ICD signal of a groove binder is one-two orders of magnitude larger than that of an intercalator, although, experimentally the difference can be smaller.19 Data obtained from a CD spectrum (mdeg -
millidegrees) vs. wavelength (nm) can be used to calculate binding constants and determine stoichiometry.\textsuperscript{20}

CD has a number of advantages over other techniques such as NMR and X-ray crystallography because it only requires a small amount of sample and measurements can be carried out in solution. This biophysical technique is not limited by the molecular weight or size of a molecule and can be performed easily and rapidly. CD can be used to study chiral molecules, as each enantiomer will interact with different amounts of right-handed and left-handed light thus resulting in a CD spectrum. Each enantiomer should result in different CD spectra. If a molecule is achiral, no spectrum will be observed because the molecule will interact with the same amount of right- and left-handed light.

The CD spectrum of B-DNA is composed of a positive signal at 275 nm and a negative one at 245 nm, passing zero at 258 nm. CD is regularly used to monitor conformational changes of DNA and interactions of ligands with DNA. In addition, to the changes due to achiral drugs binding, spectral changes can be observed indicating structural modifications occurring to the double helix. The stronger the binding affinity of the molecule, the more structural changes will occur to the DNA and as a result a large induced CD signal is register. Hence, the CD spectrum obtained can be used not only to evaluate the binding mode but also to calculate the binding affinity of the molecules.

In this work, CD measurements were performed by titrating the ligands into a DNA solution and observing the changes in the spectra. CD measurements were carried out on the aminoalkyl di-aromatic mono-guanidines 66d, 66e and 69e in salmon testes and poly(dA-dT)\textsubscript{2} DNA using P/D ratios of 0, 10 and 2. CD was also used to analyse the interactions between the alkylcarboxylic acid acridines 83a, 83b and 83e in salmon testes DNA.

### 6.5.1. Circular Dichroism studies of the aminoalkyl di-aromatic mono-guanidines

As previously mentioned, minor groove binders, due to their close proximity to chiral sugar molecules, characteristically exhibit strong positive ICD signals. The aminoalkyl di-aromatic mono-guanidines are achiral with no inherent CD signals; however, when bound to DNA they may show an induced signal. Thus, CD measurements were performed by
increasing the compound to DNA P/D ratio from 0 to 2 over 2 additions using first, salmon testes DNA to observe the effect of the compounds on a wild mixed sequence of DNA.

A CD titration was carried out on compound 66d with salmon testes DNA. Firstly, the CD spectrum of a solution of known concentration (150 μM) of salmon testes DNA was measured. Next, the molecule was added to the solution at a P/D ratio of 10 (15 μM 66d added) and the changes in the CD spectra were recorded. Finally, the P/D ratio was increased to 2 by a further addition of 60 μM of the compound to give 75 μM of the compound in solution. The spectral changes due to the binding can be observed in Figure 6.9.

![CD titration spectra obtained for 66d produced upon addition to DNA in phosphate buffer](image)

A positive induced circular dichroism (ICD) peak is observed at 320 nm. The increase of the signal upon addition of the molecule is characteristic of compounds that bind to the minor groove instead of intercalating between the DNA base pairs. From the spectra it can be observed that the aminoalkyl di-aromatic monoguanidine is binding to the chiral environment of the DNA helix.

Upon binding of the drug, the structure of the DNA backbone is altered. Hence, as the compound concentration is increased and binding occurs, the changes in the ICD signal with the changes in the DNA environment (i.e. changes in the DNA spectrum at 275 nm)
can be compared. In the case of compound $66d$ and salmon testes DNA, the ellipticity of the positive band at 275 nm decreased, while that of the negative band at 240 nm increased. These changes are consistent with the alteration of the helical structure of B-DNA in the presence of $66d$. Furthermore, there is no evidence of a bathochromic shift, indicating that there is only one mode of binding. Correspondingly, the magnitude of the ICD peak at 320 nm is consistent with minor groove binding since a large ICD signal is typical of minor groove binding whereas a small one is characteristic of intercalation due to the lack of interaction with the chiral sugars. CD titrations were also carried out in the presence of $66e$ and $69e$ under the same conditions used for $66d$. The changes in the CD spectra as a result of addition of the molecules can be observed in Figure 6.10 and Figure 6.11.

![Figure 6.10.- CD titration of compound 66e with salmon testes DNA](image)
In the CD spectra of 66e a large ICD signal is observed at 320 nm which is indicative of minor groove binding as seen before in 66d. As the concentration of the ligand is increased, spectral changes indicating structural changes occurring in DNA is observed. As the P/D ratio of 66e is increased, a bathochromic shift is observed in the DNA spectrum from 275 nm to 285 nm indicating that the structure of the DNA backbone is altered upon binding.

The CD spectra of 69e display a much smaller induced CD signal than that of 66d and 66e. Although the ICD signal at 327 nm is smaller than in previous compounds it is still a magnitude greater than that of the DNA alone and, therefore, is characteristic of a minor groove binder. As aliquots of the ligand were added to the DNA solution, spectral changes were observed indicating DNA structural changes. Upon binding, a decrease in the signal at 275 nm is observed indicating DNA binding. The changes in the ICD signal are similar to that of the DNA environment (changes in the DNA spectrum at 275 nm and 245 nm), showing that the binding of the drug changes the DNA backbone structure to the same degree.

CD titrations were also carried out using poly(dA-dT)\textsubscript{2} and the same protocol as described for salmon testes DNA. The CD titration of 66d (Figure 6.12) in the presence of poly(dA-dT)\textsubscript{2} showed a large positive ICD signal at 320 nm as with salmon testes DNA, which is indicative of minor groove binding. In contrast to the CD spectra with salmon testes DNA,
the changes in the ICD peak are of a greater magnitude than in the DNA environment (changes in DNA spectrum at 275 nm). Therefore, the binding of the drug changes the DNA structure, but it does not induce a large structural change to the DNA backbone. Additionally, there is no evidence of a bathochromic shift which indicates that there is only one form of binding occurring.

Figure 6.12.- CD titration spectra of 66d in the presence of poly(dA-dT)$_2$

The CD spectra of 66e and 69e with poly(dA-dT)$_2$ showed similar result as seen for 66d. Compound 66e shows a positive ICD signal at 320 nm indicating that binds to the minor groove of DNA. As the P/D ratio was increased, a decrease in the signal at 270 nm was seen proving DNA binding. The changes in the ICD signal compared to those in the DNA environment (changes in the DNA spectrum at 275 nm) show that the binding of the drug changes the DNA structure but not the DNA backbone to the same extent. In the case of 69e a positive ICD signal at 327 nm indicative of minor groove binding is observed. Minimal spectral changes are displayed at 275 nm indicating that the binding does not cause alterations to the DNA backbone. There is no bathochromic shift which signifies that only one species is formed in solution and that there is only one binding mode associated to this compound. The CD spectra of these compounds bound to poly(dA-dT)$_2$ are displayed in Figure 6.13 and Figure 6.14.
6.5.2. Circular Dichroism studies of the alkylcarboxylic acid derivatives of acridine

CD studies were also performed on three of the alkylcarboxylic acid acridines. The CD spectra of 83a, 83b and 83e in the presence of salmon testes DNA were recorded and the spectra for 83a, which is the strongest intercalator, is displayed in Figure 6.15. The three acridines showed similar results; thus, the ellipticity of the positive band at 275 nm increased, while that of the negative band at 240 nm decreased slightly in all of them. These changes are consistent with the alteration of the helical structure of B-DNA in the presence the intercalators. A large ICD signal would be expected if the molecules were
bound to the minor groove of DNA. However, no ICD signal was observed for the bound molecules demonstrating that they bind to DNA via intercalation.

![Figure 6.15.- CD titrations of the alkylcarboxylic acid intercalator 83a with salmon testes DNA](image)

**6.6. Electric Flow Linear Dichroism**

Linear Dichroism (LD) spectroscopy, which is progressively being found to be a very powerful method for evaluating the binding of small molecules to nucleic acids, was used to examine the mode of binding of the aminoalkyl di-aromatic mono-guanidines 66d, 66e and 69e to salmon testes DNA. LD is defined as the differential absorption of parallel (A<sub>parallel</sub>) and perpendicular (A<sub>perpendicular</sub>) linearly polarised light according to equation (6.5).

\[
LD = A_{\text{parallel}} - A_{\text{perpendicular}} = \frac{3}{2}S(3\cos^2\alpha - 1) \tag{6.5}
\]

Where S is the orientation factor (S = 1 if the DNA is aligned whereas S = 0 if the DNA is not aligned), and \( \alpha \) is the angle that denotes the orientation of the transition moment that is responsible for the absorption of light.

The DNA was flow-oriented in a couette cell at 2000 rpm. The LD spectrum of salmon testes DNA alone (378.8 \( \mu \)M) shows a negative LD signal around 260 nm characteristic of B-DNA, which arises from the orientation of the transition dipole of the base pairs approximately perpendicular to the helical axis of DNA. When a molecule intercalates
between the base pairs, it would be parallel with respect to the base pairs, therefore, the angle \( \alpha \) would be equal to 90°. If \( \alpha = 90° \) is inserted into the equation above, a negative LD signal is obtained. Upon binding to the minor groove of DNA, \( \alpha \) would be equal to 45° and then the equation would result in a positive induced LD signal. Thus, LD experiments are very useful of the binding mode of the ligand.

6.6.1. Linear Dichroism studies of the aminoalkyl di-aromatic mono-guanidines

The orientation axis used in the present study was that of salmon testes DNA in order to probe the mode of binding. Thus, the LD spectra for 66d, 66e and 69e were monitored at increasing compound to DNA ratio (Figure 6.16, 6.17 and 6.18)

![Figure 6.16.- LD spectra for salmon testes DNA with 66d](image_url)
The LD spectra for salmon testes DNA titrated with the three compounds in 10 μM phosphate buffer at 25 °C were recorded. Titrations were carried out at a 378.8 μM concentration of salmon testes DNA working with a P/D ratio of 0, 2 and 10; varying the P/D ratio from 10 to 2 over 2 additions. In these experiments, the DNA and compound concentration was kept constant to avoid dilution effects. The reason for this is that we are dealing with very small quantities (80 μL). Consequently, each solution was prepared individually and the corresponding spectra were recorded.

All three compounds showed a notable positive induced LD signal. There was an increase in the absorbance at 312 nm (66d) and 300 nm (66e and 69e) where the DNA does not absorb. Positive induced signals are indicative of minor groove binding and, hence, these LD results with salmon testes DNA show that the aminoalkyl di-aromatic mono-guanidines 66d, 66e and 69e bind in the minor groove.

6.6.2. Linear Dichroism studies of the alkylcarboxylic acid derivatives of acridine

The LD spectrum of 83a was also monitored at increasing compound to DNA ratio (Figure 6.19). The LD spectra for salmon testes DNA titrated with 83a in 10 μM phosphate buffer at 25 °C were recorded. Titrations were carried out with a 300 μM concentration of salmon testes DNA working with a P/D ratio of 0, 0.5, 1 and 2. As before, the DNA and compound
concentration was kept constant to avoid dilution effects. The LD measurement was carried in the same manner as before for the aminoalkyl di-aromatic mono-guanidines. In the LD spectrum of salmon testes DNA in the presence of compound 83a a new negative induced LD signal appears at 359 nm where DNA does not absorb. Negative induced signals are characteristic of intercalation and therefore this negatively induced LD signal at 359 nm proves that 83a intercalates between the base pairs and that this intercalating molecule is parallel with respect to the base pairs giving an $\alpha$ angle of 90°.

6.7. Ultraviolet intercalator displacement assays

As stated earlier UV studies have a number of uses such as to determine the ultraviolet properties of the molecules, to establish the DNA binding properties of small molecules and proteins, sequence selectivity, binding constants and mode of binding. Ultraviolet intercalator displacement assays can be used for establishing DNA binding affinity. The advantages associated with this technique are that it is non-destructive, technically non-demanding, it is not limited to small molecule assessments and has been used with a variety of ligands, including proteins and triplex-forming oligonucleotides.

This experiment involves the addition of aliquots of a ligand to a sample of DNA and ethidium bromide (a well known intercalator) in the appropriate buffer while recording its UV spectra. If the molecule binds to DNA therefore displacing the bound ethidium bromide spectral changes are observed. Generally, a hypochromic shift in the ligand’s absorbance spectra will occur which represents a decrease in the concentration of the free molecule. Also a decrease in absorbance spectra of the prebound ethidium bromide will
occur. A new absorbance band is generally observed at a different wavelength representing the formation of the DNA-ligand complex. With individual sequences, quantitative titration of a compound against a DNA sequence prebound with ethidium can be performed to give reliable binding constants. Binding constants are determined by plotting the change in absorbance against the equivalents of compound. Binding constants are calculated by using a Scatchard plot of the of $\Delta A/[\text{free agent}]$ versus $\Delta A$, giving a straight line plot where the slope provides the binding constant ($K_a$).

**6.7.1. UV intercalator studies of the aminoalkyl di-aromatic mono-guanidines**

Aliquots of a 0.5 mM 66d solution were added to the poly(dA-dT)$_2$ DNA (2 µM) and ethidium bromide (1 µM) solution, and a small hypochromic shift was observed representing the disappearance of the free 66d molecule. A decrease in the absorbance of the ethidium bromide-DNA complex was observed. We could observe a DNA-molecule species band forming at $\lambda_{\text{max}} = 320$ nm, proving the increasing concentrations of the DNA–ligand complex due to the strong binding of this molecule to DNA. Binding constants are determined by plotting the change in absorbance against the equivalents of compound (Figure 6.20).

![Figure 6.20.- UV intercalator displacement assay of 66d. Inset: Plot of absorbance vs. equivalents.](image)

A solution of the compound 66e of known concentration (0.5 mM) was prepared and aliquots of this were added to a solution of poly(dA-dT)$_2$ DNA (2 µM) and ethidium
bromide (1 μM) until saturation occurred and the corresponding absorption spectrum was recorded. Results are presented in Figure 6.21 showing a small hypochromic shift was representing the disappearance of the free 66e molecule. A decrease in the absorbance of the ethidium bromide-DNA complex was also observed. We observed an increase in the absorbance spectra for the ligand-DNa complex $\lambda_{\text{max}} = 320$ nm. This shows that by increasing the concentration of the ligand, the formation of the DNA-ligand complex results while the ethidium bromide-DNA complex at 490 nm and 280 nm decreases. Binding constants are determined by plotting the change in absorbance against the equivalents of compound (Figure 6.21).

![Figure 6.21.- UV intercalator displacement assay of 66d. Inset: Plot of absorbance vs. equivalents.](image)

From these experiments it was possible to calculate the corresponding binding constants of 66d and 66e to poly(dA-dT)$_2$ oligonucleotide and the results are presented in Table 6.6, confirming that 66d binds more strongly to poly(dA-dT)$_2$ than 66e. These results are in agreement with the thermal denaturation results obtained. These compounds bind moderately to DNA and their binding constants are in the range of $10^4$. These results can be compared with the binding constants of asymmetric di-aromatic guanidinium/2-aminoimidazolinium derivatives in poly(dA-dT)$_2$ DNA (Figure 6.22).
Figure 6.22.- Asymmetric di-aromatic guanidinium/2-aminoimidazolinium derivatives

Table 6.6.- Binding constants calculated from scatchard plot analysis for compounds 66d, 66e, 101 and 102 with poly (dA-dT)₂ DNA

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Binding Constant (K) (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(dA-dT)₂</td>
<td></td>
</tr>
<tr>
<td>66d</td>
<td>1.5 x 10⁴</td>
</tr>
<tr>
<td>66e</td>
<td>1.3 x 10⁴</td>
</tr>
<tr>
<td>101</td>
<td>0.6 x 10⁵</td>
</tr>
<tr>
<td>102</td>
<td>2.5 x 10⁵</td>
</tr>
</tbody>
</table>

6.8. Conclusions

In this chapter the physicochemical techniques used to study the molecules synthesised in Chapters 3 and 4 are presented. Biophysical assays were carried out for the binding of the molecules to DNA [salmon testes and poly(dA-dT)₂]. The experiments performed and presented in this chapter are based on mainly optical techniques that depend on the use of the UV-vis spectrometer, CD and LD.

The protonation state (pKₐ) of the molecules at physiological pH was first of all determined. It was found that in the aminoalkyl di-aromatic mono-guanidines, both the guanidine and the aliphatic amine would be protonated at physiological pH. The NH linker in compound 66e was found to have a low pKₐ and therefore would not be protonated at physiological pH. Thus, these molecules are di-cationic within the minor groove of DNA. The pKₐ of the alkylcarboxylic acid acridines was also measured and found to be 5.33 and 2.69, therefore the alkylcarboxylic acid acridine derivatives are neutral at the N atom of the acridine core at physiological pH.

Then, a reverse salt titration was performed to confirm that the aminoalkyl di-aromatic mono-guanidines were di-cationic upon binding to the DNA minor groove. These experiments can be used to determine the mode of binding of the molecules. Increasing the ionic strength by increasing the salt concentration led to spectral alterations in the UV spectrum of compound 66e with salmon testes DNA. This indicates that significant
quantities of the ligand were displaced from the DNA helix by NaCl proving that this molecule acts as a minor groove binder within the DNA double helix. The alkylcarboxylic acridine 83a showed minimal spectral changes upon increasing salt concentration demonstrating that it was not displaced much by NaCl and therefore must bind to DNA via intercalation.

Next, the DNA binding affinity to salmon testes DNA of the molecules synthesised in Chapters 3 and 4 were investigated by using DNA thermal denaturation experiments. Compounds 65b, 65d, 66b-e, 68e and 69e showed the strongest affinity. The incorporation of the aminoalkyl chain resulted in significant increments in the melting temperature of DNA when complexed with the di-aromatic mono-guanidines.

Using also thermal denaturation techniques, the sequence selectivity of some of the dicationic molecules was investigated. It was observed that 66d and 69e displayed selectivity for AT sequences, while 66e stabilised poly(dA-dT)₂ to the same extent that it stabilised salmon testes DNA. The DNA binding affinity of the molecules to poly(dA)•poly(dT) was also investigated. Compound 66d showed a ΔTₘ of 6 °C while 66e and 69e, which both have NH central linker, did not show favourable interaction with the A:T DNA displaying low ΔTₘ values. The CO central linker of 66d must form stronger interactions with poly(dA)•poly(dT) DNA.

Thermal denaturation assays were also carried in the presence of 25 mM NaCl to examine the role of electrostatic interaction in the binding of the 66e and 69e in the minor groove of poly(dA-dT)₂. A decrease in binding affinity was recorded with increasing ionic strength of the solution, showing that electrostatic interactions contribute greatly to the binding of these molecules to the minor groove of DNA.

Thermal denaturation assays were also carried out on the alkylcarboxylic acid acridines. Compound 83a showed the strongest affinity. The incorporation of short (n=3) alkylcarboxylic acid chains resulted in an increase in the melting temperature of the DNA, but decreased when a long chain was incorporated. Other derivatives showed less stabilisation of the DNA sequence and therefore displayed low ΔTₘ values as a result of destabilisation which would be in agreement with the results obtained from the CD an LD studies showing that all these molecules act as intercalators.
Circular dichroism experiments were performed to determine strength and mode of binding to salmon testes and poly(dA-dT)$_2$. Large positive ICD signals were observed upon binding of the molecules 66d, 66e and 69e confirming that they are minor groove binders. The CD spectra of alkylcarboxylic acid acridine derivatives 83a, 83b and 83e with salmon testes DNA displayed no induced signals, therefore proving that they bind to DNA via intercalation.

Linear dichroism was also carried out to confirm the mode of binding to DNA. In this technique, a positive induced signal is indicative of minor groove binding whereas a negative induced signal would be indicative of intercalation. From the experiments of the aminoalkyl di-aromatic mono-guanidines, we observed positive induced signals confirming that 66d, 66e and 69e bind to the minor groove of DNA. In contrast, a clearly negative induced LD signal was observed for compound 83a proving that this compound binds to DNA via intercalation.

Overall, a great deal of information has been acquired from these physiochemical techniques helping us to determine the binding affinity and mode of binding of the molecules synthesised in this research.

6.9 References

Chapter 7

Biological Evaluation
7.1. Introduction

Once all of the biophysical assays were carried out to characterise the binding of the molecules synthesised in chapter 3 the next step was to assess the antiprotozoal activity and cytotoxicity of these compounds. An essential part of the drug design process involves the evaluation of the compounds on biological models of a specific disease with the objective to improve their activity.\(^1\) Eight of the aminoalkyl di-aromatic mono-guanidines prepared (compounds 65b, 66b-e, 67c, 68e and 69e, Figure 7.1) were tested \textit{in vitro} against \textit{Trypanosoma brucei rhodesiense} (T.b.r) STIB 900 and \textit{Plasmodium falciparum} (P. falciparum) NF54 which are related to African trypanosomiasis and malaria respectively.

From these evaluations we can gain an insight into the essential structural features for a compound to exhibit significant antiprotozoal activity and this will help us to design new potential antiprotozoal agents.

By comparing the thermal denaturation results with the IC\(_{50}\) values obtained we would be able to determine whether the binding affinity to the minor groove of DNA may be the mechanism by which their antiprotozoal activity is exerted. In general only those compounds that displayed the largest DNA binding affinity were subjected to biochemical testing. Compounds 67c and 68e, which contain alkyl chains of different length, were also chosen so that the effect of these different alkyl chains could be examined. The antiprotozoal activity and cytotoxicity of these compounds was evaluated by cellular growth inhibition assays and were determined by Marcel Kaiser at Prof. Reto Brun’s laboratory in the Swiss Tropical and Public Health Institute (Basel, Switzerland).

As stated in Chapter 1 human African trypanosomiasis and malaria are very serious diseases that cause millions of deaths ever year. Drugs available for HAT are outdated and exhibit unacceptable side effects such as hypotension, nephrotoxicity, cardiotoxicity and hypoglycaemia. Recently, there has also been an increase in treatment failures due to
emergence of drug resistance. Regarding the therapeutic agents used to treat malaria, the
effect of some drugs has recently diminished due to the appearance of drug resistant strains
of *Plasmodium* species. Thus, drugs such as chloroquine, mefloquine, halofantrine and
artemisinin are now virtually ineffective. Consequently, the World Health Organisation
now recommends the use of antimalarial drug combinations (e.g., artesunate/mefloquine,
artesunate/amodiaquine) in order to stop the development of resistant strains and, hence,
the syntheses of new safe and efficient antiprotozoal agents to treat HAT and malaria are
of great significance in international health. 

The *in vitro* assay against *T. b. rhodesiense* STIB 900 is based on the evaluation of cell
proliferation. It involves the use of the alamarBlue® assay which is non-radioactive,
reliable, sensitive, reproducible, water soluble and non-toxic with respect to the cells. It
does not require cell lysis, washing or extraction procedures and is a simple one step assay.
AlamarBlue® viability tests were carried out in order to measure the effect of the
compounds on STIB 900 cell proliferation. This test uses a cell viability dye, resazurin (7-
hydroxy-3H-phenoxazin-3-one 10-oxide) which is a blue non-fluorescent dye. When
subjected to the reducing power of the proliferating cells, this dye is reduced to the red and
highly fluorescent resorufin (Figure 7.2). The amount of fluorescence is proportional to the
number of living cells; therefore, it is a good indication of the effect of the compound on
cell proliferation. 

![Figure 7.2.-Reaction involved in the AlamarBlue® assay](image)

AlamarBlue® can be used in large-scale *in vitro* screening of many different biological
systems. In addition, to the *in vitro* assays of African trypanosomes, a type of primary cell
lines derived from rat skeletal myoblasts, the L6 cells, have been used for the analysis of
cell proliferation.

The procedure followed for the alamarBlue® assay consists first of preparing serial drug
dilutions covering a range of concentrations in a 96-well plate. Next, *T. b. rhodesiense*
STIB 900 was added to each well and the plates incubated at 37 °C for 70 hours. Then,
alarmBlue® was added to each well and incubated for a further 2–4 hours; during this time, reduction of resazurin to resorufin occurs and the conversion and extent of the resulting fluorescence, is dependent on compound concentration and activity. Next, the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wave length of 536 nm and an emission wave length of 588 nm. Finally, results were analysed by plotting obtained fluorescence values against compound concentration; the IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves. The same procedure was carried out when examining the effect of the compounds on L6 cells except that they were only incubated with alarmBlue® for 2 hours. An example of a 96-well plate analysed using alarmBlue® is shown in Figure 7.3.

![Figure 7.3. A 96-well plate containing cell line and compound incubated with alarmBlue®](image)

The *in vitro* activity against erythrocytic stages of *P. Falciparum* was determined using a ^3^H-hypoxanthine incorporation assay. This is a sensitive method that measures the incorporation of ^3^H-hypoxanthine into parasitic DNA as a measure of parasitic replication in red blood cells. Using the uptake of ^3^H-hypoxanthine to measure antimalarial activity *in vitro* involves dissolving the compounds in dimethylsulfoxide (DMSO) and adding them to parasite cultures which were incubated without hypoxanthine. Then, serial drug dilutions covering a range of concentrations were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C, and after 48 hours ^3^H-hypoxanthine was added to all of the wells. Further incubation was carried out for 24 hours. The plates were then harvested with a Betaplate™ cell harvester and the red blood cells transferred onto a glass fibre filter and then washed with distilled water. The dried filters were inserted into a plastic foil with scintillation fluid, and counted in a Betaplate™ liquid scintillation counter. The corresponding IC_{50} values were calculated as it was done for *T. b. rhodesiense* activity.
7.2. *Trypanosoma brucei rhodesiense* STIB 900 cell line

*T. b. rhodesiense* (Figure 7.4) causes fast onset of acute trypanosomiasis in humans. It is most common in southern and eastern Africa in countries such as Uganda, Tanzania and Malawi, where game animals and livestock are thought to be the main hosts. The *T. b. rhodesiense* STIB 900 cell line was isolated in 1982 from a male patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions. This cell line is known to be sensitive to all currently used drugs and it has been extensively used throughout the literature. Different minor groove binders have been tested for antitrypanosomal activity on this cell line.\[2,7,8\]

![Figure 7.4.- Trypomastigotes of *T. b. rhodesiense* in blood](image)

7.3. *Plasmodium falciparum* NF54 cell line

The *P. falciparum* NF54 cell line (Schiphol Airport, The Netherlands,\[10\] Figure 7.5.) is susceptible to chloroquine and was used in this research to analyse the toxicity of the compounds prepared towards malaria. Chloroquine was the most common antimalarial drug for decades, unfortunately, a number of chloroquine resistant strains of *Plasmodium* species have emerged rendering chloroquine practically ineffective. Resistance to another common drug, pyrimethamine, is also widespread. Mutations in the malarial gene for dihydrofolatereductase may reduce the effectiveness of pyrimethamine.
7.4. The skeletal myoblasts L6 cell line

Cytotoxicity of the aminoalkyl di-aromatic mono-guanidines was measured in skeletal myoblasts L6 cells in order to determine the selectivity of the compounds for *T. b. rhodesiense* STIB 900 and *P. falciparum* NF54. The L6 cells are a primary cell line derived from rat skeletal myoblasts; this myogenic line was isolated originally by Yaffe from primary cultures of rat thigh muscle preserved for the first two passages in the presence of methylcholanthrene. The L6 cells fuse in culture to form multinucleated myotubes and striated fibers (Figure 7.6).

![Figure 7.6.- L6 cells (mouse muscle cell line)](image)

The results of the *in vitro* assays using *T. b. rhodesiense* STIB 900 and *P. falciparum* NF54 will be described in detail separately and their cytotoxicity towards L6 cells will be incorporated into these explanations.

7.5. Results obtained with the *T. b. rhodesiense* STIB 900 cell line

The experiments performed with the STIB 900 cell line would allow us to determine the importance of the cationic moieties, which differ in basicity and hydrogen bonding ability, and the number of cations within the molecule. Comparison with compounds previously synthesised within our group would allow us to identify the most favourable cation. The relevance of the central linker groups, varying in polarity, HB ability and geometry, could
also be evaluated. The importance of the length of the aliphatic chain and therefore the length and flexibility of the molecule could be assessed as well. Lastly, a correlation, if it exists, could be drawn between DNA binding affinities of the different molecules and their antiprotozoal activity.

AlamarBlue® viability assays using the *T. b. rhodesiense* STIB 900 cell line were carried out with the aminoalkyl di-aromatic *mono*-guanidines shown in Figure 7.1. Melarsoprol, which is a very active drug with an IC\textsubscript{50} value of 0.0055 \(\mu\text{M}\), was used as a control in these experiments. The effect of increasing the concentration of the aminoalkyl di-aromatic *mono*-guanidines (from 100 to 0.002 \(\mu\text{g} \text{ mL}^{-1}\)) upon the growth of *T. b. rhodesiense* STIB 900 cell lines was recorded and the calculated IC\textsubscript{50} values (concentration required to inhibit 50\% of the cellular growth) are displayed in Table 7.1.

Table 7.1.- *In vitro* antitrypanosomal activity of the aminoalkyl di-aromatic *mono*-guanidines

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} ((\mu\text{M}))</th>
<th>Selectivity Index (SI)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T.\textit{b.r}^\text{a})</td>
<td>L6 cells</td>
</tr>
<tr>
<td>65b (X=CH\textsubscript{2}CH\textsubscript{2}, n=3)</td>
<td>13.3</td>
<td>133</td>
</tr>
<tr>
<td>66b (X=CH\textsubscript{2}CH\textsubscript{2}, n=4)</td>
<td>20.2</td>
<td>109</td>
</tr>
<tr>
<td>66c (X=O, n=4)</td>
<td>13.1</td>
<td>33.5</td>
</tr>
<tr>
<td>66d (X=CO, n=4)</td>
<td>121</td>
<td>140</td>
</tr>
<tr>
<td>66e (X=NH, n=4)</td>
<td>57.7</td>
<td>223</td>
</tr>
<tr>
<td>67c (X=O, n=7)</td>
<td>31.9</td>
<td>136</td>
</tr>
<tr>
<td>68e (X=NH, n=10)</td>
<td>85.4</td>
<td>157</td>
</tr>
<tr>
<td>69e (X=NH, n=11)</td>
<td>4.06</td>
<td>11.9</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>0.0055</td>
<td>-</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>-</td>
<td>0.0145</td>
</tr>
</tbody>
</table>

\textsuperscript{a} *T. brucei rhodesiense* STIB 900 strain.

\textsuperscript{b} Selectivity index= (IC\textsubscript{50} L6 cells)/(IC\textsubscript{50} \(T.\textit{b. rhodesiense}\))
From Table 7.1 we can see that all of the aminoalkyl di-aromatic mono-guanidines display IC$_{50}$ values against *T. b. rhodesiense* in the micromolar range. It can be observed that in general upon lengthening the aliphatic chain activity decreases. Compound 69e (X= NH, n=11) is an exception to this because it displays the greatest activity of the series despite having a long aliphatic chain. This could be due to the central NH linker which has a good hydrogen bonding ability. Unfortunately, this compound is very cytotoxic towards L6 cells and is, therefore, very unselective towards *T. b. rhodesiense* cell lines. The compounds with CH$_2$CH$_2$ as a linker (65b and 66b) display good activity with IC$_{50}$ of 13.3 and 20.2 μM respectively. These compounds are selective towards the parasite, since they show IC$_{50}$ values of 133 and 109 μM towards L6 cells. The derivative containing the electron withdrawing CO linker (compound 66d) showed the lowest activity. The IC$_{50}$ values obtained for derivatives containing electron donating linkers such as NH (69e) and CH$_2$CH$_2$ (65b and 66b) are in the low μM range. Compound 68e (X=NH, n=10)) displayed a moderate-poor IC$_{50}$ value of 85.4 μM, and in comparison to the other derivatives containing the NH linker, this decrease in activity could be due to the length of the aliphatic chain. Compound 66e (X=NH, n=4)) displayed a moderate IC$_{50}$ value of 57.7 μM with very good selectivity for *T. b. rhodesiense* cell lines. It displays a high IC$_{50}$ of 223 μM against L6 cells. Compounds with an O linker (66c and 67c) display moderate to low *in vitro* activity.

The selectivity index (SI) shows how safe these compounds are. Thus, compounds 65b and 66b are the safest with the largest SI values. Since these compounds have low IC$_{50}$ values and high SI values they have a good potential as antitrypanosomal agents.

### 7.6. Correlation between Binding Affinity and Cytotoxicity towards *Trypanosoma brucei rhodesiense* STIB 900 cells

It is important to determine if there is any correlation between the antitrypanosomal activity of the aminoalkyl di-aromatic mono-guanidines towards *T. b. rhodesiense* and their minor groove binding ability. Theoretically, the compounds that have the strongest affinity for the minor groove should inhibit DNA replication most effectively and, hence, elicit the strongest antitrypanosomal activity. Thus, results obtained for the thermal denaturation and antitrypanosomal activity are shown in Table 7.2.
Table 7.2.- Correlation between $IC_{50}$ values of aminoalkyl di-aromatic mono-guanidines in *T. b. rhodesiense* STIB 900 cells and DNA binding affinity ($\Delta T_m$) using salmon-testes$^b$ and poly(dA-dT)$_2$ DNA$^c$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$IC_{50}$ (pM)</th>
<th>$\Delta T_m$ s.t. DNA (°C)$^b$</th>
<th>$\Delta T_m$ A:T (°C)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>65b (X=CH$_2$CH$_2$, n=3)</td>
<td>13.3</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>66b (X=CH$_2$CH$_2$, n=4)</td>
<td>20.2</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>66c (X=O, n=4)</td>
<td>13.1</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>66d (X=CO, n=4)</td>
<td>121</td>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>66e (X=NH, n=4)</td>
<td>57.7</td>
<td>6</td>
<td>6.2</td>
</tr>
<tr>
<td>67c (X=O, n=7)</td>
<td>31.9</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>68e (X=NH, n=10)</td>
<td>85.4</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>69e (X=NH, n=11)</td>
<td>4.06</td>
<td>5</td>
<td>6.6</td>
</tr>
</tbody>
</table>

$^a$ *T. b. rhodesiense* STIB 900 strain. Control: melarsoprol, $IC_{50}= 0.0055 \mu M$

$^b$ Using salmon-testes DNA ($T_m= 68°C$); obtained in this work

$^c$ Using poly(dA-dT)$_2$ DNA ($T_m= 47°C$); obtained in this work

Thus, no direct correlation was found between antiparasitic activity ($IC_{50}$) and binding affinity ($\Delta T_m$). Compound 66e, which has one of the strongest binding affinities, has shown to display low antiparasomal activity while compound 66c which has the same $\Delta T_m$, displayed a much lower $IC_{50}$ value. However, compounds 65b, 66b and 69e showed very good $IC_{50}$ values and had shown to strongly bind to salmon testes DNA. Compound 66d which had a $\Delta T_m$ of 5 showed low activity with an $IC_{50}$ of 121 pM which is the worst of the set. Compounds 67c and 68e showed the lowest DNA binding affinity and quite low activity, in particular 68e with an $IC_{50}$ of 85.4 pM.

A correlation plot of the $\Delta T_m$ and log (1/$IC_{50}$) was sought as well, however, little or no correlation between DNA binding affinity and cytotoxicity was found.

Next, the correlation between antiparasomal activity and binding affinity towards poly(dA-dT)$_2$ was analysed; again no correlation was found. Compound 66d, which showed the highest $\Delta T_m$ had the lowest activity with and $IC_{50}$ value of 121 pM, while
compounds 66e and 69e which had smaller ΔT_m values, displayed much greater activity with IC_{50} values of 57.7 and 4.06 μM respectively.

7.7. **Cytotoxicity Experiments of the *Plasmodium falciparum* NF54 cell line**

A ^3^H-hypoxanthine incorporation assay was used to measure the *in vitro* activity of aminoalkyl di-aromatic mono-guanidines against the *P. falciparum* NF54 cell line. This method involves measuring the incorporation of ^3^H-hypoxanthine into parasitic DNA as a measure of its replication in red blood cells. The aminoalkyl di-aromatic mono-guanidines showed very high activity with IC_{50} values in the nano- and micromolar range. The results obtained from the antiplasmodial experiments against *P. falciparum* NF54 on these compounds are displayed in Table 7.3.

**Table 7.3.-In vitro antiplasmodial activity of the aminoalkyl di-aromatic mono-guanidines**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. f. NF54</em></td>
<td><em>L6 cells</em></td>
</tr>
<tr>
<td>65b (X=CH₂CH₂, n=3)</td>
<td>1.14</td>
<td>133</td>
</tr>
<tr>
<td>66b (X=CH₂CH₂, n=4)</td>
<td>1.06</td>
<td>109</td>
</tr>
<tr>
<td>66c (X=O, n=4)</td>
<td>0.106</td>
<td>33.5</td>
</tr>
<tr>
<td>66d (X=CO, n=4)</td>
<td>2.55</td>
<td>140</td>
</tr>
<tr>
<td>66e (X=NH, n=4)</td>
<td>1.46</td>
<td>223</td>
</tr>
<tr>
<td>67e (X=O, n=7)</td>
<td>0.149</td>
<td>136</td>
</tr>
<tr>
<td>68e (X=NH, n=10)</td>
<td>3.79</td>
<td>157</td>
</tr>
<tr>
<td>69e (X=NH, n=11)</td>
<td>2.71</td>
<td>11.9</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.0039</td>
<td>-</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>-</td>
<td>0.0145</td>
</tr>
</tbody>
</table>

*P. falciparum* NF54 strain. Control: chloroquine, IC_{50} = 0.0039 μM

*Selectivity index* = (IC_{50} L6 cells)/(IC_{50} *P. falciparum*).

It can be observed that, in general, upon lengthening the aliphatic chain, larger IC_{50} values are obtained. Compounds 66c and 67c, (both with X=O) which have four and seven
methylene groups respectively, displayed the greatest activity of the series within the nanomolar range. These compounds showed very good and selective in vitro activity against *P. falciparum* with IC$_{50}$ values of 0.106 and 0.149 µM against *P. falciparum* NF54. Compound 67c is particularly non-toxic towards L6 cells with an IC$_{50}$ value of 136 µM against these cells. Due to these very good results, these derivatives could be candidates for in vivo testing. This high activity could be due to the central O linker, a good hydrogen bond acceptor, which is common to two molecules. The rest of the derivatives displayed high in vitro activity against *P. falciparum* showing very low µM IC$_{50}$ values. The compounds with CH$_2$CH$_2$ central linkers (65b and 66b) display IC$_{50}$ values of 1.14 and 1.06 µM, respectively. These compounds are more selective towards the *P. falciparum* than *T. b. rhodesiense* and are also quite non-toxic towards L6 cells exhibiting IC$_{50}$ values of 133 and 109 µM. The compounds 68e, 69e (both with X=NH) and 66d (X=CO) showed the lowest activity with IC$_{50}$ values of 3.79, 2.71 and 2.55 µM. The decrease in activity of 68e and 69e (n=10 and 11) could be due to the long aliphatic chains, which may not be favourable for antimalarial activity. In the case of compound 66e, the electron withdrawing CO linker may be the cause of the decrease in activity. Finally, compound 66e (X=NH, n=4) displayed a low IC$_{50}$ value of 1.46 µM; this increase in activity compared to the other derivatives containing the NH linker, could be due to the shorter aliphatic chain.

All compounds showed greater selectivity towards the *P. falciparum* NF54 cell line over the *T. b. rhodesiense* STIB 900 and the high selectivity indices of many of these compounds show how safe they are. Compounds 66c and 67c are the safest with the largest SI values. Since these compounds have nanomolar IC$_{50}$ values and very high SI values they have good potential as antiplasmodial agents.

### 7.8. Correlation between Binding Affinity and Cytotoxicity towards *Plasmodium falciparum* NF54 cell line

It is essential to draw comparisons between the antiplasmodial activity of the aminoalkyl di-aromatic mono-guanidines and their DNA binding ability. The same principle applies as before where, the compounds that have the strongest affinity for the minor groove should inhibit DNA replication most effectively and elicit the strongest antiplasmodial activity. Analysis of the thermal denaturation experiments results and the IC$_{50}$ values obtained (Table 7.4) can provide some evidence for this hypothesis.
Table 7.4.- Correlation between IC_{50} values of aminoalkyl di-aromatic mono-guanidines in *P. falciparum* NF54 cells and DNA binding affinity (ΔT_m) using salmon-testes and poly(dA-dT)₂ DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50}(μM)</th>
<th>ΔT_m s.t.DNA (°C)</th>
<th>ΔT_m A:T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65b (X=CH₂CH₂, n=3)</td>
<td>1.14</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>66b (X=CH₂CH₂, n=4)</td>
<td>1.06</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>66c (X=O, n=4)</td>
<td>0.106</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>66d (X=CO, n=4)</td>
<td>2.55</td>
<td>5</td>
<td>7.4</td>
</tr>
<tr>
<td>66e (X=NH, n=4)</td>
<td>1.46</td>
<td>6</td>
<td>6.2</td>
</tr>
<tr>
<td>67c (X=O, n=7)</td>
<td>0.149</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>68e (X=NH, n=10)</td>
<td>3.79</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>69e (X=NH, n=11)</td>
<td>2.71</td>
<td>5</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*P. falciparum* NF54 strain. Control: chloroquine, IC_{50} = 0.0039 μM
Using salmon testes DNA (T_m = 68°C); obtained in this work
Using poly(dA-dT)₂ DNA (T_m = 47°C); obtained in this work

When exploring the *P. falciparum* activity, a certain linear relation (ΔT_m = -0.646×(IC_{50}) + 6.183; R² = 0.648) was found when removing the data for compound 67c, which is one of the compounds with the very good activity results but a very poor DNA binding according to the ΔT_m value obtained. This seems to indicate that this compound exerts its antimalarial activity by a different mechanism of action.

7.9. Correlation between antiparasitic activity of previously synthesised *bis*(2-aminoimidazolines) and *bis*-guanidines and the current aminoalkyl di-aromatic mono-guanidines

A series of guanidine and 2-aminoimidazole analogues previously synthesised within our laboratory (Figure 7.7) had been assayed *in vitro* against *T. b. rhodesiense* STIB 900 and *P. falciparum* K1 by our collaborators at the Swiss Tropical and Public Health Institute (Basel, Switzerland). The di-cationic diphenyl compounds displayed the largest activities with IC_{50} values against *T. b. rhodesiense* and *P. falciparum* in the nanomolar range.
The results for the in vitro testing against *T. b. rhodesiense* showed that it was essential to have a di-cationic structure in order to obtain IC$_{50}$ values within the nanomolar range. The aminoalkyl di-aromatic mono-guanidines synthesised in this work are di-cationic but only showed IC$_{50}$ values within the micromolar range. This could be due to the nature of the cations (aminium-guanidinium vs. bis-guanidinium like) or to the aminoalkyl chain which causes the cations to be positioned further apart from each other depending on the length of the aminoalkyl chain.

When the *bis*-guanidines and *bis*-2-aminoimidazolines derivatives have an electron withdrawing group at the X linker between the aromatic moieties, a loss of activity resulted. This was observed also for compound 66d (X= CO) which has the highest IC$_{50}$ (i.e. the lowest activity). Electron donating groups such as NH and CH$_2$CH$_2$ at the linker of the symmetric di-cations resulted in compounds with low micromolar IC$_{50}$ values. This same trend in activity can be observed for the aminoalkyl di-aromatic *mono*-guanidines where 65b, 66b (both with X= CH$_2$CH$_2$) and 69e (X= NH) gave low IC$_{50}$ values probably due to electron donating effect of their central linkers.

Usually, the di-cationic *bis*-guanidine and *bis*-2-aminoimidazoline analogues displayed lower IC$_{50}$ values than the aminoalkyl di-aromatic *mono*-guanidines (i.e. better activity). Exceptions are the *bis*-guanidine, *bis*-2-aminoimidazoline and Boc-protected *bis*-2-aminoimidazoline with sulfonyl linkers which exhibited worse antitrypanosomal activity than some of the aminoalkyl derivatives synthesised in this research. The aminoalkyl di-aromatic *mono*-guanidines (65b, 66b-e, 67c, 68e and 69e) showed similar activity to the
Biological Evaluation

Chapter 7

diphenyl mono-cationic guanidines and 2-aminimidazolines and much greater activity when compared with the phenyl mono-cationic compounds.

The same trends were observed for in vitro activity against *P. falciparum* with bis-guanidinium bis-2-aminimidazolium compounds showing the best activity. Electron withdrawing linkers and removal of a cation led to a reduction in activity as before. The IC$_{50}$ values were a lot lower for *P. falciparum* than for *T. b. rhodesiense* in both the bis-guanidine like derivatives and aminoalkyl di-aromatic mono-guanidines. The aminoalkyl di-aromatic mono-guanidines in this project exhibited nanomolar to low micromolar IC$_{50}$ values for *P. falciparum* as did the bis-guanidiniums and bis-2-aminimidazoliums. The aminoalkyl derivatives showed much better activity than both the diphenyl and phenyl mono-cationic compounds. Those guanidines and 2-aminimidazolines with the best activity were tested further in vivo; unfortunately, they were not active enough in the animal model.$^2$ Of the aminoalkyl di-aromatic mono-guanidines compounds 66c and 67c (X=O, n=4 and 7, respectively) displayed the best activity (nanomolar) and will be interesting candidates for in vivo testing.

7.10. Conclusions

The antiparasitic activity and cytotoxicity of eight of the aminoalkyl di-aromatic mono-guanidines prepared, with different central linkers and aminoalkyl chain lengths was evaluated on three different cell lines. The in vitro activity against *Trypanosoma brucei rhodesiense* STIB 900 and *Plasmodium falciparum* NF54 cell lines was measured and, to assess how safe these compounds were towards other cells, the cytotoxicity against L6 skeletal cells was also determined. The alamarBlue® viability assay was employed for both the *T. b. rhodesiense* and L6 cell lines as a suitable method of accurately monitoring the reducing environment of the cells and thereby allowing viability and proliferation to be assessed. To measure the in vitro activity of the compounds against *P. falciparum* NF54 a $^3$H-hypoxanthine incorporation assay was used. This assay involves evaluating the absorption of $^3$H-hypoxanthine into parasitic DNA as a measure of parasitic replication in red blood cells. From all these assays, IC$_{50}$ values were calculated and, in addition, these IC$_{50}$ values were compared with the Δ$T_m$ values calculated from the thermal denaturation experiments; however, no correlation was found. Also, the required structural characteristics for targeting each cell line were identified.
For *T. b. rhodesiense*, the IC\textsubscript{50} values obtained for the aminoalkyl di-aromatic mono-guanidines (65b, 66b-e, 67c, 68e and 69e) were in the micromolar range varying from 4.06 to 121 \mu M. Compounds 65b, 66b and 69e showed the best activity; these compounds contained electron donating groups at the linker between the phenyl rings. Compound 66c also displayed good *in vitro* activity with a low IC\textsubscript{50} value of 13.1 \mu M. A relation between the antitrypanosomal activity and DNA binding affinity of the molecules was searched; however, plots of the IC\textsubscript{50} values against the thermal denaturation results showed poor correlation. This could be due to the antitrypanosomal activity being related to other cellular mechanisms. A certain relation was found between the antimalarial activity and the DNA affinity when removing the data of compound 66c. This could be due to the antiplasmodial activity being produced by a different cellular mechanism.

The aminoalkyl di-aromatic mono-guanidines proved to be more potent and selective towards *P. falciparum* NF54, exhibiting low micromolar to nanomolar IC\textsubscript{50} values. These ranged between 0.106 and 3.79 \mu M. Compounds 66c and 67c displayed excellent *in vitro* activity against *P. falciparum* with low nanomolar IC\textsubscript{50} values. Both these compounds showed very good and selective activity with selectivity indices of 315 and 913 and IC\textsubscript{50} values of 0.106 and 0.149 \mu M, respectively. In view of these promising results, the aminoalkyl di-aromatic mono-guanidines could be good candidates for *in vivo* assays and more investigation as antiprotozoal agents should follow.

7.11. References


9. [http://158.83.1.40/Buckelew/Trypanosoma%20brucei%20rhodesiense](http://158.83.1.40/Buckelew/Trypanosoma%20brucei%20rhodesiense) section.htm


Chapter 8

Conclusions and Future Work
8.1. Conclusions

The work carried out in this project has resulted in a number of objectives achieved in the areas of synthesis, biophysical chemistry and biochemistry. Based on the results obtained, a number of conclusions can be drawn.

8.1.1. Synthesis

A) The successful synthesis of novel hydrochloride salts of several aminoalkyl di-aromatic mono-guanidinium conjugates was achieved. Initially, the two precursor molecules, the Boc-protected mono-guanidines and the Boc-protected amino acids were prepared. First, based on the synthetic methodology previously developed by Rozas' group\(^1\) (based on the methodology originally described by Kim et al.\(^2\)), mono-guanidylation of the starting di-aromatic diamines with central linkers being CH\(_2\), CH\(_2\)CH\(_2\), O, CO and NH was achieved. These syntheses involve the introduction of the Boc-protected guanidine by employing Boc-protected thiourea as the guanidylating agent in the presence of HgCl\(_2\) and TEA. Secondly, amino acids of various chain lengths (n= 3, 4, 7, 10 and 11) were Boc protected in MeOH in the presence of TEA.\(^3\)

This was followed by a coupling reaction of the two precursors to give a total of 25 derivatives of the Boc-protected aminoalkyl di-aromatic mono-guanidine conjugates (60-64a-e). The coupling reagent TBTU was used in the presence of DIEA\(^4\) to obtain the conjugates in moderate to high yields which varied with the length of the alkyl chain and with the central linker of the mono-guanidines. Then, after optimisation of the method to prevent hydrolysis of the amide bond, Boc-deprotection was carried out using 4 M HCl/Dioxane in IPA/DCM to give the hydrochloride salts of the mentioned conjugates. This successfully produced a novel family of 24 aminoalkyl di-aromatic mono-guanidinium dihydrochloride salts (65-69a-e) in satisfactory yields.

B) Following the successful preparation of these minor groove binders, the design and synthesis of an intercalator family was explored. Thus, a new series of alkylcarboxylic acid derivatives of acridine were synthesised by firstly forming the methyl ester of the same amino acids used in the preparation of the mono-guanidine conjugates by reacting them with HCl/MeOH in MeOH. Next, different coupling reactions were explored to connect the
methyl aminoalkylcarboxylates with acridine-9-carboxylic acid. The coupling reagent BOP was used to form five of these acridine conjugates in moderate to high yields. These derivatives underwent base catalysed hydrolysis using KOH yielding the corresponding five (acridine-9-carboxyamido)alkylcarboxylic acids (83a-e) in reasonable yields.

C) The next aim was to synthesise minor groove binder-intercalator dual agents also known as combilexins. With this in mind, various methods were explored to couple the Boc protected mono-guanidines or the aminoalkyl mono-guanidinium di-aromatic conjugates with the (acridine-9-carboxyamido)alkylcarboxylic acids or acridine-9-carboxylic acid.

Firstly, methods involving orthogonal protection using Cbz, Fmoc and Boc protecting groups were explored. The amine of the amino acids was protected with either Cbz of Fmoc and then coupled with the Boc-protected mono-guanidines. Removal of the Cbz/Fmoc group was then attempted to allow the molecule to be coupled with acridine-9-carboxylic acid to yield the protected dual binder. However, attempts to remove the Cbz or Fmoc protecting groups failed even after several optimisations of the reactions were attempted. Thus, other methods of forming the dual binders were explored.

Coupling of 9-chloroacridine or acridine-9-carboxylic acid with the dihydrochloride salts of the aminoalkyl di-aromatic mono-guanidines using various coupling reagents such as SOCl₂, BOP and TBTU proved unsuccessful.

Based on the fact that only one amino group of the Boc-protected mono-guanidines was free to react with the (acridine-9-carboxyamido)alkylcarboxylic acids, it was hoped that coupling reactions involving these two moieties would lead to the successful synthesis of the dual binders. Coupling of the methyl (acridine-9-carboxyamido)alkylcarboxylates and the Boc-protected di-aromatic mono-guanidines was attempted on the basis of literature examples, but, unfortunately, failed because the methyl ester was not sufficiently reactive. This reaction was also attempted using the ethyl esters because it was thought that they could be cleaved upon attack of the amine to form the dual binders. Initially, the ethyl esters of the amino acids were prepared using SOCl₂ in EtOH. These esters were obtained in high yields and used in a coupling reaction with acridine-9-carboxylic acid to form the corresponding ethyl (acridine-9-carboxyamido)alkylcarboxylates in yields ranging 49-
69%. Next, the coupling reaction with the Boc-protected di-aromatic mono-guanidines was attempted using TBTU as a coupling reagent in the presence of DIEA. Regrettably, this also proved insufficiently reactive and so the reaction did not go to completion.

A number of coupling reactions were explored for the connection of the di-aromatic mono-guanidines with the (acridine-9-carboxyamido)alkylcarboxylic acids to form the required dual binders. The reaction of SOCl₂ with the acids to form the acid chlorides which could be reacted with the di-aromatic mono-guanidines to form the Boc protected dual binders was investigated. However, it was successful only on a small scale for a test reaction but failed when upscaled, therefore, this synthetic route was abandoned. Next, the coupling of the di-aromatic mono-guanidines and the (acridine-9-carboxyamido)alkylcarboxylic acids was tried with BOP and TBTU but both reactions were unsuccessful. The coupling reagent EDCI was also tested in the presence of HOBT but led to a similar result as with BOP and TBTU. The Yamaguchi esterification was next investigated which involves the reaction of 2,4,6-trichlorobenzoyl chloride with the carboxylic acid to form a mixed anhydride, which should then be reactive enough to couple with the amine of the di-aromatic mono-guanidine. Unfortunately, the mass spectrum of this reaction showed only the mass of the mono-guanidine. The pyrrolidine derivative of BOP, PyBOP, was then used as a coupling reagent but similar result where achieved as with BOP.

Derivatives of TBTU proved more successful; thus, both HATU and HBTU were successful in coupling the (acridine-9-carboxyamido)alkylcarboxylic acid with the di-aromatic mono-guanidine, but, regrettably, failed when the reactions were upscaled.

Ethyl chloroformate gave the most positive results. It was used to react with the (acridine-9-carboxyamido)alkylcarboxylic acid to form the mixed anhydride which then was reacted immediately with the di-aromatic mono-guanidine to form the dual binder. This reaction was optimised and upscaled but insufficient yields were still obtained and, thus, an unsatisfactory quantity of the Boc-protected dual binder was achieved. As a result it was not possible to perform Boc-deprotection and due to time constraints the synthesis of the dual binders was ceased at this point. Although a sufficient quantity of the dual binders was not obtained the reaction did proceed and, with further optimisation in the future, could prove successful in yielding the required dual binders.
8.1.2. Biophysical Experiments

After the syntheses of the aminoalkyl di-aromatic mono-guanidinium conjugates and the (acridine-9-carboxyamido)alkylcarboxylic acid family were successfully completed, the next step consisted of the evaluation of their affinity and binding mode to DNA. Hence, a number of biophysical experiments were carried out.

A) To assess the protonation state of the molecules at physiological pH, the pKₐ values of one of the aminoalkyl di-aromatic mono-guanidine conjugates (66e) and one of the (acridine-9-carboxyamido)alkylcarboxylic acids (83a) were determined by using the Henderson-Hasselbalch equation titration method. It was found that the aminoalkyl di-aromatic mono-guanidine conjugates were protonated at both the guanidine and aliphatic amine at physiological pH. Compound 66e displayed pKₐ values of 11.33 and 11.69 for the amine and guanidine, while the central NH linker had a pKₐ value of 2.60, showing that these compounds are di-cationic at physiological pH. The pKₐ of the amino group of the (acridine-9-carboxyamido)alkylcarboxylic acid 83a was 5.33 and that of the carboxylic acid was found to be 2.68, showing that the amino group is neutral and the carboxylic group is anionic at physiological pH.

B) A reverse salt titration was performed to confirm that the aminoalkyl di-aromatic mono-guanidine conjugates are di-cationic upon DNA binding. In addition, this technique can help to determine the mode of binding of these molecules to DNA. Therefore, the ionic strength of the solution was augmented by increasing the salt concentration which resulted in spectral alterations in the UV spectrum of compound 66e with salmon testes DNA. This result shows that significant quantities of the ligand were displaced from the DNA helix proving that this molecule acts as a minor groove binder. In contrast, the alkylcarboxylic acid acridine derivative 83a showed minimal spectral changes indicating that it is bound to DNA via intercalation.

C) The DNA binding affinity of the molecules prepared was investigated using thermal denaturation assays. The aminoalkyl di-aromatic mono-guanidine conjugates showed ΔT_m ranging from 0-6 °C, with compounds 65b, 65d, 66b-e, 68e and 69e showing the strongest affinity for salmon testes DNA. The incorporation of the aminoalkyl chain and HB donor or acceptor central linkers resulted in significant increments in the melting temperature of
the DNA when complexed with these modified minor groove binders.\textsuperscript{12} Sequence selectivity of the molecules was investigated by carrying out thermal denaturation assays using poly(dA-dT)\textsubscript{2}. Thus, compound 66d proved to be the most selective for the narrower minor groove of this DNA sequence. A decrease in binding affinity was observed with increasing ionic strength, indicating that electrostatic interactions contribute greatly to the binding of these molecules into the minor groove of DNA.

Thermal denaturation experiments with the acridine alkylcarboxylic acid derivatives in salmon testes DNA were performed. The shorter alkyl chains resulted in an increase in the melting temperature of the DNA while the longer ones resulted in a decrease.

D) Circular dichroism experiments were used to determine mode of binding to salmon testes and poly(dA-dT)\textsubscript{2}. Large positive induced CD signals were observed upon binding of the aminoalkyl di-aromatic mono-guanidine conjugates confirming that they act as DNA minor groove binders, while the CD spectra of the acridine alkylcarboxylic acid derivatives with salmon testes DNA displayed no induced signals confirming that they are intercalators.

Linear dichroism was used to confirm the binding mode of these compounds. The spectra of the aminoalkyl di-aromatic mono-guanidine displayed positive induced signals proving that they bind into the minor groove of DNA. A negative induced LD signal was observed for the acridine derivative 83a confirming the CD results and that these compounds are intercalators.

8.1.3. Biochemistry

Once confirmed the binding to DNA of the aminoalkyl di-aromatic mono-guanidines, biochemical assays were performed to determine the antiparasitic activity of eight of these compounds (65b, 66b-e, 67c, 68e and 69e). The eight derivatives that were chosen varied in the central linker of the di-aromatic mono-guanidine moiety and in the alkyl chain length. The \textit{in vitro} activity against \textit{Trypanosoma brucei rhodesiense} STIB 900 (human African trypanosomiasis) and \textit{Plasmodium falciparum} NF54 (malaria) cell lines was measured. The cytotoxicity towards L6 (skeletal myoblast) cells was also assessed to determine the selectivity of the compounds versus the other two cell lines.\textsuperscript{12}
A) The alamarBlue® viability assay was used to calculate the activity of the compounds towards the *T. b. rhodesiense* cell line. This assay is a good method of measuring the cytotoxic effects on cell viability and proliferation. All the compounds except one were active (IC$_{50} < 100$ μM) displaying IC$_{50}$ values ranging from 4.06-85.4 μM. Compound 66d displayed the lowest activity with an IC$_{50}$ value of 121 μM. This low activity could be due to the electron withdrawing effect of the CO linker. Derivatives 65b, 66b and 69e showed the best cytotoxicity in this STIB 900 cell line and all of them contained electron donating groups as linkers. A trend was observed for the *in vitro* activity of these compounds, the longer the alkyl chain the lower the activity. Compounds with shorter alkyl chains showed the best activity with IC$_{50}$ values of 13.3, 20.2 and 4.06 μM. Compounds with longer chains such as 68e, showed lower activity (IC$_{50} = 85.4$ μM). Compound 69e was an anomaly in this trend, being the compound with the longest alkyl chain and the best activity. The relation between the thermal denaturation ΔT$_{m}$ values and the IC$_{50}$ ones was explored, but poor correlation was found. Therefore, minor groove binding cannot be confirmed as the only mode of action of this group of compounds and their activity may be related to other cellular mechanisms.

B) The *in vitro* activity of the same eight compounds was evaluated in the *P. falciparum* NF54 cell line using a ³H-hypoxanthine incorporation assay which involves measuring the absorption of ³H-hypoxanthine into parasitic DNA as an indication of parasitic replication in red blood cells. These compounds displayed very good activity, with IC$_{50}$ values in the nano- and micromolar range. The compounds showed selectivity for the *P. falciparum* NF54 cell line over the *T. b. r.* STIB 900. The molecules proved to be much more potent towards malaria with IC$_{50}$ values ranging between 0.106 and 3.79 μM. A similar trend was observed for the activity and length of the alkyl chain, lengthening the chain led to poorer activity and higher IC$_{50}$ values. Compounds with O atoms as central linkers (66c and 67c) showed the best activity with excellent IC$_{50}$ values of 0.106 and 0.149 μM. These derivatives are very selective towards *P. falciparum* NF54 with SI (selectivity index) values of 315 and 913. These nanomolar IC$_{50}$ and high SI values show that these compounds would be excellent candidates for *in vivo* testing. The rest of the derivatives displayed a high level of cytotoxicity with low micromolar IC$_{50}$ values. As before, although some of the derivatives that bind strongly to the minor groove of DNA were found to be active in this cell line, a direct correlation between DNA-binding affinity and cytotoxicity could not be found. In light of the encouraging results obtained for these
compounds, further investigation as potential antimalarial agents would prove very interesting.

C) To see how safe these eight compounds are, their cytotoxicity against L6 cells (skeletal myoblasts) was measured. Overall the compounds displayed selectivity towards *T. b. rhodesiense* and *P. falciparum* cell lines. High SI values of *T. b. rhodesiense* and L6 cells showed compounds 65b and 66b to be the most selective of the eight compounds. The eight compounds were more selective towards *P. falciparum* exhibiting very high SI values.

D) A direct structure-activity relationship (SAR) between the biophysical and biochemical results could not be established for these eight compounds. Although some of the stronger minor groove binders showed good IC$_{50}$ values, there were derivatives that did not have good ΔT$_{m}$ values and were quite potent. In particular, compound 67c was very active against *P. falciparum* and had a low change in DNA melting temperature of 2 °C. As previously mentioned, this questions the expected mode of action.$^{15}$ Therefore, further research should be carried out to determine the exact targets and modes of action of some of the compounds.

Nonetheless, the results obtained are promising and, therefore, the aminoalkyl-minor groove binders deserve more investigation as antiprotozoal (particularly as antimalarials) agents and DNA minor groove binders.

8.2. Future Work

The results obtained and presented in this thesis regarding synthesis, biophysical tests and biochemistry were very promising and interesting. This research led to a number of ideas for possible future work which are described next.

8.2.1. Synthesis

Synthetically, with regards to the new minor groove binders it would be interesting to synthesise the *bis*-guanidine derivatives with the aminoalkyl chain connected to one of the guanidine groups. This would increase the number of cations in the molecules and,
therefore, encourage uptake and nuclear accumulation as well as being the driving force for binding of the molecules to the DNA minor groove.

Previous studies in our research group have shown that bis-2-aminoimidazoline derivatives are more active when binding to DNA than the bis-guanidine ones; therefore, it may also be useful to substitute one of the guanidinium groups by a 2-aminoimidazolinium cation.\textsuperscript{16}

In addition, in the molecules synthesised in this project, the guanidine cation could be exchanged for other cations such as hydroxyguanidinium or isouronium. All of these cations have been previously studied\textsuperscript{17} in our group and some have shown to bind to DNA nicely and therefore incorporating them into the aminoalkyl minor groove binders could lead to an improved binding and hence activity.

One of the conclusions of the biophysical assays was that the longer alkyl chains of some compounds may be too flexible to optimally bind within the minor groove. Therefore, the synthesis of more rigid linkers containing double bonds or aromatic rings could produce compounds with a more favourable fit into the minor groove.

Complexation of the minor groove binders to metals could also be carried out. Research on attaching Pt to minor groove binders is currently on-going within our group. This would draw the molecules more strongly towards the groove and the Pt would alkylate DNA bases. Biophysical and cytotoxicity assays on these compounds would be interesting.

Symmetrical and asymmetrical di-aromatic guanidinium/2-aminoimidazolinium compounds have been synthesised with an amide central linker and have shown strong binding to DNA;\textsuperscript{18} thus, the synthesis of the corresponding aminoalkyl minor groove binder as described in this thesis with an amide linker should be investigated.

Other modifications could consist of: to include a guanidine as a central linker since it could lead to more points of interaction between the molecule and the minor groove; the phenyl rings could be changed to pyrroles to mimic the structure of netropsin and distamycin; thiophene rings may also prove interesting.
Throughout the synthesis of the alkylcarboxylic acid intercalators, a number of ideas for future work emerged. Further acridine conjugates could be prepared if a guanidine or an amide was added at the end of the alkyl chain. This would lead to further interactions and thus, on top of the DNA intercalation of the acridine core, the alkylguanidine could bind in the minor groove.

From the thermal denaturation assays performed in this thesis, it was observed that upon lengthening the alkyl chain the intercalation worsened; thus, it would be interesting to study the effect of incorporating more rigid linkers in the alkyl chains of these acridine derivatives.

Other intercalator cores such as anthracene or phenanthridines instead of acridine should be considered to see if these molecules would intercalate more efficiently and later on would facilitate the preparation of the corresponding dual agents.

Since the preparation of the dual binders was not successfully completed there are still numerous synthetic routes towards dual binders that could be investigated. Hence, further optimisation of the coupling reaction between the mono-guanidines and (acridine-9-carboxyamido)alkylcarboxylic acid using ethyl chloroformate should be carried out. Other coupling reagents such as DCC and 1,1'-carbonyldiimidazole (CDI) could also be investigated.

Considering that the carboxylic acid in the 9 position of the acridine does not seem to be very reactive, it may be useful to reduce it to the alcohol which could then be converted to the chloride using SOCl₂. Various coupling reactions could then be attempted in the hope of forming dual binders. The corresponding acridine conjugates thus prepared would yield more flexible linkers that will enable the acridine moiety to intercalate more efficiently and exert its cytotoxicity.

If a second guanidinium is incorporated into the minor groove binder derivatives then when attached to the intercalator moiety would result in dual binders containing three cations which would prove interesting to study with regards to biophysical chemistry and biochemistry.
8.2.2. **Biophysical Experiments**

Further biophysical experiments could be carried out on both the aminoalkyl minor groove binders and the alkylcarboxylic acid intercalators. For example, biophysical assays could be carried out using GC sequences such as poly(dG-dC)_2 DNA to observe whether or not the compounds intercalate or bind to the minor groove in GC sequences.

Also more physicochemical experiments could be carried out on the alkylcarboxylic acid intercalators such as thermal denaturation, CD and LD with AT sequences. Fluorescence assays should be carried out also to obtain binding constants.

Isothermal Calorimetry experiments (ITC) could be carried out for all the aminoalkyl minor groove binders. This method is based on the difference in heat supplied between the control and the experiment and allows the determination of the thermodynamics of binding such as the changes in the enthalpy, entropy and Gibbs free energy. Positive entropy denotes that more disorder was introduced to the system due to disruption of the hydration spine as a consequence of binding, a negative change represents formation of more hydrogen bonds between the molecule and the DNA bases.19

DNase I footprinting could be attempted on the compounds. This technique is used to determine if a compound is sequence-selective and is based on an enzymatic method that uses the enzyme DNase I for differentiating the sites of reversible binding of DNA-targeting drugs.20

Finally, Surface Plasmon Resonance (SPR) could be carried out on all the compounds which is a technique used to measure binding affinities and kinetics towards different DNA sequences (hairpins) and corresponding binding constants can be calculated.21

8.2.3. **Biochemistry**

The biochemical studies throughout this work have allowed to achieve many important conclusions and to obtain very exciting results. One of the most remarkable is the nanomolar activity of the aminoalkyl minor groove binders 66c and 67c against *P. falciparum* NF54 cell lines. Statistics show that 1,238,000 people died from malaria in 2010, around 90% of malaria-related deaths occurring in sub-Saharan Africa, and about
60% of these are children younger than five years old. *P. falciparum* is responsible for the most severe form of malaria and causes the vast majority of deaths associated to the disease. However, therapeutic agents for malaria are impaired by the appearance of drug resistant strains of *Plasmodium* species. Therefore, chloroquine, which was the most common antimalarial drug for decades, is now basically ineffective and development of resistance towards other drugs such as mefloquine, halofantrine, or artemisinin is beginning to occur. As a result of this there is a demand for new safe and efficient antiprotozoal treatment strategies. Taking this into account, minor groove binding agents, such as 66c and 67c, prepared in this research could lead to new therapies to treat diseases such as malaria and human African trypanosomiasis.

As stated within this project, compounds 66c and 67c would be interesting candidates for *in vivo* antimalarial studies. The *in vivo* tests for malaria involve groups of three female National Medical Research Institute (NMRI) mice being intravenously infected with *P. berghei*. Compounds would then be added and then parasitemia will be determined on day 4 post infection (24 h after last treatment) by fluorescence-activated cell sorting (FACS) analysis. The survival time in days is also recorded up to 30 days after infection and a compound will be considered curative if the animal survives to day 30 after infection with no detectable parasites.

The *in vitro* assays should be carried out on the remainder of the aminoalkyl minor groove binder series. If other molecules in the series exhibit nanomolar activity in *T. b. rhodesiense* or *P. falciparum* these should be followed by *in vivo* cytotoxicity assays.

Previous research in our group has shown that minor groove binders are effective at killing some cancer cell lines and, in some cases, inducing apoptosis. It would be interesting to observe the effect of the aminoalkyl minor groove binders synthesised in this project on Human leukemia (HL-60), MCF-7 (breast cancer) and Kelly neuroblastoma cell lines. Both *in vitro* cytotoxicity assays and more in depth analysis including FACS and apoptosis evaluation could be evaluated by agarose gel electrophoresis and western blot analysis. Gel electrophoresis is a technique that separates components such as proteins on the basis of physical characteristics and could therefore be used for measuring DNA fragmentation of nuclear extracts or for probing specific proteins associated with apoptotic pathways.
Western blotting involves transferring the specific protein from the gel to a nitrocellulose membrane via specific protein-binding antibodies.

Cytotoxicity experiments need to be carried out on the alkylcarboxylic acid intercalators, and, if synthesised, in the dual binders to see if they exhibit cytotoxicity against cancer and protozoal cell lines. Such future work may lead to interesting results and producing potential new anticancer or antiprotozoal agents.

8.3. References

Conclusions and Future Work

Chapter 9

Experimental Section
9.1. Chemistry

All commercial chemicals were obtained from Sigma-Aldrich or Fluka and were used without further purification. Deuterated solvents for NMR use were purchased from Apollo. Dry solvents were prepared using standard procedures, according to Vogel, with distillation prior to use. Chromatographic columns were run using Silica gel 60 (230-400 mesh ASTM). Chromatographic columns were also run using a Biotage SP4 flash purification system with Biotage SNAP silica cartridges. Solvents for synthetic purposes were used at general purpose reagent (GPR) grade. Analytical TLC was performed using Merck Kieselgel 60 F$_{254}$ silica gel plates. Visualisation was by UV light (254 nm). NMR spectra were recorded in a Bruker DPX-400 Avance spectrometer, operating at 400.13 MHz and 600.1 MHz for $^1$H-NMR and 100.6 MHz and 150.9 MHz for $^{13}$C-NMR. Shifts are referenced to the internal solvent signals. NMR data were processed using Bruker Win-NMR 5.0 and Bruker TOPSPIN software. HRMS spectra were measured on a Micromass LCT electrospray TOF instrument with a WATERS 2690 autosampler with methanol as carrier solvent. Melting points were determined using a Stuart Scientific Melting Point SMP1 apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR Spectrometer equipped with a Universal ATR sampling accessory. HPLC purity analysis was carried out using a Varian ProStar system equipped with a Varian Prostar 335 diode array detector and a manual injector (20 µL). UV detection was performed at 245 nm and peak purity was confirmed using a purity channel. The stationary phase consisted of an ACE 5 C18-AR column (150 x 4.6 mm), and the mobile phase used the following gradient system, eluting at 1.0 cm$^3$/min: aqueous formate buffer (30 mM, pH 3.0) for ten minutes, linear ramp to 85% methanol buffered with the same system over 25 minutes, hold at 85% buffered methanol for ten minutes.

9.1.1. General procedures for the preparation of compounds

Method A: General method for the preparation of Boc-protected di-aromatic mono-guanidines

To a cooled solution (0 °C) of the starting di-aromatic diamine (15 mmol), 1,3-bis-(tert-butoxycarbonyl)-2-methyl-2-pseudothiourea (5 mmol) and TEA (15 mmol) in DCM or DMF (5 cm$^3$) depending on the polarity of the starting material, HgCl$_2$ (5.5 mmol) was added. This mixture was left stirring for 1 hour at 0 °C and a further 23 hours at room
temperature. The resulting mixture was diluted with EtOAc and filtered through a pad of celite in order to remove the mercury sulphide precipitate formed. The filter cake was rinsed with EtOAc. The resulting organic phase was washed with brine (2×30 cm³), dried over Na₂SO₄, and concentrated under vacuum. Purification by flash chromatography with silica gel, eluting with the appropriate hexane/EtOAc mixture yielded the required product.

Method B: General method for the preparation of Boc-protected amino acids
The corresponding amino acid (4.65 mmol) was suspended in MeOH (15 cm³) with TEA (0.78 cm³, 5.58 mmol) and dissolved di-tert-butyldicarbonate (1.01 g, 4.65 mmol) and heated under reflux at 60 °C for 24 hours. The solvent was eliminated under vacuum and the residue was redissolved in EtOAc. The mixture was washed with 0.25 M HCl (2×100 cm³), dried and filtered. Evaporation of the solvent afforded the Boc-protected amino acids as white solids which were then purified by recrystallisation from hexane.

Method C: General method for the preparation of Boc-protected aminoalkyl di-aromatic mono-guanidine conjugates
A solution of the corresponding Boc-protected amino acid (1.2 mmol) in MeCN (10 cm³) was treated with DIEA (3.8 mmol), the di-aromatic mono-guanidine (1 mmol) and TBTU (1.2 mmol) under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours and partitioned between brine (4 cm³) and EtOAc (10 cm³). The organic layer was washed with 0.1 M HCl (2×5 cm³) and 5% NaHCO₃ (2×5 cm³), dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. Purification by flash chromatography with silica gel eluting with hexane/EtOAc (2:1) yielded the required product.

Method D: General method for Boc deprotection and preparation of hydrochloride salts
The Boc-protected amino acid conjugate of the mono-guanidine derivative (1 mmol) was dissolved in 4 M HCl/dioxane (4.5 cm³) under argon. The mixture was made up to 0.2 M solution with IPA/DCM (1:1) (0.5 cm³) and stirred 3-4 hours. The HCl/dioxane mixture of solvents was then removed under vacuum. The residue was dissolved in H₂O and washed with DCM (3×5 cm³). Concentration of the aqueous layer followed by reverse phase column chromatography eluting with H₂O yielded the required product.
Method E: General method for the preparation of amino acid methyl esters

The amino acid was dissolved in MeOH (5 cm$^3$) and HCl/MeOH (2 cm$^3$) was added. The reaction mixture was stirred at room temperature overnight. The compound was concentrated under vacuum to afford the required product.

Method F: General method for the preparation of methyl (acridine-9-carboxyamido)alkylcarboxylate

In a 50 cm$^3$ round bottomed flask were placed, at room temperature under argon, the corresponding amino acid methyl ester (1 mmol) and acridine-9-carboxylic acid (1 mmol) in anhydrous CH$_2$Cl$_2$ (10 cm$^3$). The mixture was stirred and cooled to 0 °C. DIEA (4 mmol) was slowly added, followed by the addition of the coupling reagent BOP (1.5 mmol) dissolved in anhydrous CH$_2$Cl$_2$. The reaction was stirred overnight at 20 °C under an inert atmosphere. EtOAc was added to the reaction mixture and the organic layer was successively washed with a 1 M HCl solution, a 20% NaHCO$_3$ solution and brine. The organic layer was dried over MgSO$_4$ and concentrated under vacuum. Purification by flash chromatography with silica gel eluting with hexane/EtOAc (5:1)-(3:1)-(1:1) yielded the required product.

Method G: General method for the preparation of (acridine-9-carboxyamido)alkylcarboxylic acids

The ester was dissolved in THF (4 cm$^3$) and 1 M KOH (2 cm$^3$) was added to the mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum yielding the required product.

9.1.2. Preparation and characterisation of compounds

$N,N'$-bis-(tert-Butoxycarbonyl)thiourea (47)$_1$

A solution of thiourea (2.499 g, 32.88 mmol) in dry THF (450 cm$^3$) was prepared and cooled to 0 °C under nitrogen. To this NaH (5.89 g, 245.5 mmol, 60% in mineral oil) was added. The ice bath was removed after five minutes and the mixture stirred for ten more minutes at room temperature. The mixture was then cooled to 0 °C, di-tert-butyl dicarbonate (15.23 g, 69.87 mmol) was added neat. After 30 minutes, the ice-bath was
removed and the reaction was left to stir overnight. The reaction was then quenched by the careful addition of NaHCO₃ saturated solution. It was then added to deionised water (300 cm³). The aqueous layer was extracted using EtOAc and the combined organic phases were washed with brine. The solution was dried using MgSO₄ and concentrated under vacuum, washing with hexane afforded 47 as a white solid (4.01 g, 44%) Mp: 123-125 °C (literature: 127-129 °C).²

$$\delta_H (400 \text{ MHz, CDCl}_3): 1.55 \text{ (s, 18H, } 2(\text{CH}_3)_3)$$²

4-Amino-4'-[1,3-di(tert-butoxycarbonyl)guanidino]diphenylmethane (51a)³

Following Method A and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 51a was obtained as a white solid (1.30 g, 59%). Mp: 108-110 °C (literature: 106-108 °C).³

$$\delta_H (400 \text{ MHz, CDCl}_3): 1.52 \text{ (s, 9H, } (\text{CH}_3)_3), 1.55 \text{ (s, 9H, } (\text{CH}_3)_3), 3.86 \text{ (s, 2H, H-5), 6.65 (d, 2H, J 8.0, H-2/H-2'), 6.99 (d, 2H, J 8.0, H-3/H-3'), 7.15 (d, 2H, J 8.0, H-7/H-7'), 7.52 (d, 2H, J 8.0, H-8/H-8') 10.27 \text{ (broad s, 1H, NH), 11.65 \text{ (broad s, 1H, NH)}}$$³

4-Amino-4'-[1,3-di(tert-butoxycarbonyl)guanidino]diphenylethylene (51b)³

Following Method A and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 51b was obtained as a white solid (1.31 g, 57%). Mp: decomposes over 246 °C (literature: decomposes over 245 °C).³

$$\delta_H (400 \text{ MHz, CDCl}_3): 1.52 \text{ (s, 9H, } (\text{CH}_3)_3), 1.56 \text{ (s, 9H, } (\text{CH}_3)_3), 2.35 \text{ (broad s, 2H, NH}_2), 2.77-2.87 \text{ (m, 4H, H-5 and H-6), 6.66 (d, 2H, J 8.0, H-2/H-2'), 6.99 (d, 2H, J 8.0, H-3/H-3'), 7.15 (d, 2H, J 8.0, H-8/H-8'), 7.51 (d, 2H, J 8.0, H-9/H-9'), 10.30 \text{ (broad s, 1H, NH), 11.64 \text{ (broad s, 1H, NH)}}$$³
Experimental Section

Chapter 9

4-Amino-4'-[1,3-di(tert-butoxycarbonyl)guanidino]diphenyl ether (51c)\(^3\)

Following Method A and after purification by flash chromatography with silica gel eluting with hexane/EtOAc (3:2), 51c was obtained as a white solid (1.62 g, 73%). Mp: decomposes over 255 °C (literature: decomposes over 260 °C).\(^3\)

\[\delta_H (400 \text{ MHz, CDCl}_3): 1.53, (s, 9H, (\text{CH}_3)_3), 1.56 (s, 9H, (\text{CH}_3)_3), 3.60 \text{ (broad s, 2H, NH}_2), 6.65 \text{ (d, 2H, J 8.0, H-2/H-2')}, 6.99 \text{ (d, 2H, J 8.0, H-3/H-3')}, 7.15 \text{ (d, 2H, J 8.0, H-6/H-6')}, 7.52 \text{ (d, 2H, J 8.0, H-7/H-7')}, 10.28 \text{ (broad s, 1H, NH)}, 11.67 \text{ (broad s, 1H, NH)}\]

4-Amino-4'-[1,3-di(tert-butoxycarbonyl)guanidino]benzophenone (51d)\(^3\)

Following Method A and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (3:2), 51d was obtained as a pale yellow solid (970.4 mg, 42%). Mp: decomposes over 205 °C (literature: decomposes over 205 °C).\(^3\)

\[\delta_H (400 \text{ MHz, CDCl}_3): 1.55, (s, 9H, (\text{CH}_3)_3), 1.57 (s, 9H, (\text{CH}_3)_3), 4.15 \text{ (broad s, 2H, NH}_2), 6.71 \text{ (d, 2H, J 8.5, H-2/H-2')}, 7.72-7.77 \text{ (m, 6H, H-3/H-3', H-7/H-7' and H-8/H-8')}, 10.59 \text{ (broad s, 1H, NH)}, 11.67 \text{ (broad s, 1H, NH)}\]

4-Amino-4'-[1,3-di(tert-butoxycarbonyl)guanidino]diphenylamine (51e)\(^3\)

Following Method A and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (3:1), 51e was obtained as a purple solid (755.5 mg, 34%). Mp: decomposes over 230 °C (literature: decomposes over 230 °C).\(^3\)
Experimental Section

4-(tert-Butoxycarbonylamino)butyric acid (58a)

Following Method B 58a was obtained as a white solid (717.7 mg, 76%). Mp: 56-58 °C (literature: 52-54 °C).

4-(tert-Butoxycarbonylamino)pentanoic acid (58b)

Following Method B 58b was obtained as a white solid (727.1 mg, 72%). Mp: 43-44 °C (literature: 44.5 °C).

8-(tert-Butoxycarbonylamino)octanoic acid (58c)

Following Method B 58c was obtained as a white solid (728.5 mg, 60%). Mp: 56-59 °C (literature: 56-57 °C).
11-(tert-Butoxycarbonylamino)undecanoic acid (58d)\textsuperscript{4}

Following method B 58d was obtained as a white solid (1.4 g, 100%). Mp: 64-66 °C (literature: 68 °C).\textsuperscript{10}

δ\textsubscript{H} (400 MHz, CDCl\textsubscript{3}): 1.29 (broad s, 12H, H-3-H-8), 1.47 (broad s, 11H, (CH\textsubscript{3})\textsubscript{3} and H-9), 1.63-1.67 (m, 2H, H-2), 2.37 (t, 2H, J 7.5, H-10) 3.12 (t, 2H, J 7.5, H-1), 4.55 (broad s, 1H, NH)\textsuperscript{10}

12-(tert-Butoxycarbonylamino)dodecanoic acid (58e)\textsuperscript{4}

Following Method B 58e was obtained as a white solid (830.4 mg, 57%). Mp: 72-74 °C (literature: 76-79 °C).\textsuperscript{11}

δ\textsubscript{H} (400 MHz, CDCl\textsubscript{3}): 1.30 (broad s, 14H, H-3-H-9), 1.47 (broad s, 11H, (CH\textsubscript{3})\textsubscript{3} and H-10), 1.62-1.69 (m, 2H, H-2), 2.37 (t, 2H, J 7.0, H-11), 3.12 (t, 2H, J 7.0, H-1), 4.55 (broad s, 1H, NH)\textsuperscript{4}

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino butanamido]diphenylmethylen (60a)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 60a was obtained as a yellow solid (537 mg, 86%). Mp: 72-74 °C.

δ\textsubscript{H} (400 MHz, CDCl\textsubscript{3}): 1.48 (s, 9H, (CH\textsubscript{3})\textsubscript{3}), 1.51 (s, 9H, (CH\textsubscript{3})\textsubscript{3}), 1.55 (s, 9H, (CH\textsubscript{3})\textsubscript{3}), 1.85-1.90 (m, 2H, H-2), 2.39 (t, 2H, J 4.0, H-3), 3.27 (t, 2H, J 5.5, H-1), 3.93 (s, 2H, H-9), 4.81 (s, 1H, NHCO), 7.15 (d, 4H, J 8.0, H-6/H-6’ and H-12//H-12’), 7.51 (d, 2H, J 8.0, H-7/H-7’), 7.55 (d, 2H, J 8.0, H-11/H-11’), 8.65 (s, 1H, CONH-Ar), 10.33 (broad s, 1H, NH), 11.67 (s, 1H, NH)
Experimental Section

\[ \delta_c (100 \text{ MHz, CDCl}_3): 26.7 \text{ (CH}_2, \text{ C-2}), 27.6 \text{ (CH}_3\text{_3}), 27.7 \text{ (CH}_3\text{_3}), 27.9 \text{ (CH}_3\text{_3}), 30.5 \text{ (CH}_2, \text{ C-3}), 38.9 \text{ (CH}_2, \text{ C-9}), 40.3 \text{ (CH}_2, \text{ C-1}), 79.1 \text{ (q, C(CH}_3\text{_3)}), 79.3 \text{ (q, C(CH}_3\text{_3)}), 83.2 \text{ (q, C(CH}_3\text{_3)}), 119.4 \text{ (CH, Ar., C-6/C-6’), 122.0 \text{ (CH, Ar., C-12/C-12’), 128.8 \text{ (CH, Ar., C-7/C-7’), 128.9 \text{ (CH, Ar., C-11/C-11’), 134.2 \text{ (q, Ar., C-8), 136.1 \text{ (q, Ar., C-10), 136.1 \text{ (q, Ar., C-5), 137.4 \text{ (q, Ar., C-13), 152.8 \text{ (q, CO) 153.1 \text{ (q, CO), 156.7 \text{ (q, CO), 163.1 \text{ (q, CN), 173.2 \text{ (q, CONH, C-4)}}\]}

\[ \nu_{\text{max}} \text{ (film/cm}^2\text{: 2978 (NH), 1723, 1684, 1633, 1601 (CO, CN), 1512, 1408 (C-C), 1367 (CH}_2, 1340, 1306 (C-N), 1229, 1119, 1056, 1028 (C-N) 986, 858, 819, 768 (N-H, C-H)\]}

HRMS (m/z -ES): Found: 626.3559 (M\text{+} + H. \text{ C}_{33}\text{H}_{48}\text{N}_5\text{O}_7 \text{ Requires: 626.3554).}\]

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino butanamido]diphenylethylene (60b)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 60b was obtained as a yellow oil (597.3 mg, 93%).

\[ \delta_h (400 \text{ MHz, CDCl}_3): 1.42 \text{ (s, 9H, (CH}_3\text{_3)}), 1.46 \text{ (s, 9H, (CH}_3\text{_3)}), 1.51 \text{ (s, 9H, (CH}_3\text{_3)}), 1.77-1.83 \text{ (m, 2H, H-2), 2.30 \text{ (t, 2H, J 6.0, H-3), 2.80 \text{ (broad s, 4H, H-9 and H-10) 3.15 \text{ (t, 2H, J 6.0, H-1), 5.16 \text{ (s, 1H, NHCO), 7.06 \text{ (d, 4H, J 8.0, H-6/H-6’ and H-12/12’), 7.41-7.48 \text{ (m, 4H, H-7/H-7’ and H-13/H-13’), 8.91 \text{ (s, 1H, CONH-Ar), 10.24 \text{ (s, 1H, NH), 11.65 \text{ (broad s, 1H, NH)}}\]}

\[ \delta_c (100 \text{ MHz, CDCl}_3): 26.2 \text{ (CH}_2, \text{ C-2), 27.6 \text{ (CH}_3\text{_3)}, 27.7 \text{ (CH}_3\text{_3)}, 27.9 \text{ (CH}_3\text{_3)}, 34.0 \text{ (CH}_2, \text{ C-3), 36.8 \text{ (CH}_2, \text{ C-9), 36.9 \text{ (CH}_2, \text{ C-10), 39.0 \text{ (CH}_2, \text{ C-1), 78.8 \text{ (q, C(CH}_3\text{_3)}), 79.1 \text{ (q, C(CH}_3\text{_3)}), 83.1 \text{ (q, C(CH}_3\text{_3)}), 119.3 \text{ (CH, Ar., C-6/C-6’), 122.0 \text{ (CH, Ar., C-12/C-12’), 128.2 \text{ (CH, Ar., C-12/C-12’), 128.4 \text{ (CH, Ar., C-7/C-7’), 133.9 \text{ (q, Ar., C-8), 136.1 \text{ (q, Ar., C-11), 136.6 \text{ (q, Ar., C-5), 138.0 \text{ (q, Ar., C-14), 152.8 \text{ (q, CO), 153.3 \text{ (q, CO), 156.4 \text{ (q, CO), 163.0 \text{ (q, CN), 170.8 (q, CONH, C-4)}}\]}

224
Experimental Section

\( \nu_{\text{max}} \) (film)/cm\(^{-1} \): 3650, 2972, 2884 (NH) 1739, 1713, 1638, 1600 (CO, CN) 1514, 1463 (C-C), 1372 (C-N) 1299, 1241, 1153, 1091, 1051, (C-N) 954, 815, 782, 749, 656 (N-H, C-H)

HRMS (m/z -ES): Found: 640.3701 (M\(^+\) + H \( \text{C}_{34}\text{H}_{50}\text{N}_{5}\text{O}_{7} \) Requires: 640.3710).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino butanamido]diphenyl ether (60c)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 60c was obtained as a yellow oil (496.8 mg, 79%).

\( \delta_{\text{H}} \) (400 MHz, CDCl\(_3\)): 1.48 (s, 9H, (CH\(_3\))\(_3\)), 1.50 (s, 9H, (CH\(_3\))\(_3\)), 1.56 (s, 9H, (CH\(_3\))\(_3\)), 1.85-1.90 (m, 2H, H-2), 2.35 (t, 2H, J 6.5, H-3), 3.25 (t, 2H, J 2.0, H-1), 4.89 (s, 1H, NH, NHCO), 6.91 (d, 2H, J 8.5, H-6/H-6'), 6.99 (d, 2H, J 8.5, H-7/H-7'), 7.51 (d, 2H, J 8.5, H-10/H-10'), 7.61 (d, 2H, J 8.5, H-11/H-11'), 8.90 (s, 1H, NH, CONH-Ar), 10.28 (broad s, 1H, NH), 11.67 (s, 1H, NH)

\( \delta_{\text{C}} \) (100 MHz, CDCl\(_3\)): 26.7 (CH\(_2\), C-2), 27.2 ((CH\(_3\))\(_3\)), 27.6 ((CH\(_3\))\(_3\)), 27.7 ((CH\(_3\))\(_3\)), 31.1 (CH\(_2\), C-3), 38.9 (CH\(_2\), C-1), 79.3 (q, C(CH\(_3\))\(_3\)), 79.3 (q, C(CH\(_3\))\(_3\)), 83.3 (q, C(CH\(_3\))\(_3\)), 118.0 (CH, Ar., C-6/C-6'), 119.2 (CH, Ar., C-11/C-11'), 120.8 (CH, Ar., C-10/C-10'), 123.7 (CH, Ar., C-7/C-7') 131.1 (q, Ar., C-8), 133.9 (q, Ar., C-9), 152.4 (q, Ar., C-5), 152.9 (q, Ar., C-12), 153.4 (q, CO), 154.4 (q, CO), 156.7 (q, CO), 163.0 (q, CN), 170.6 (q, CONH, C-4)

\( \nu_{\text{max}} \) (film)/cm\(^{-1} \): 3285, 2979 (NH) 1715, 1685, 1671, 1633 (CO, CN) 1501 (C-C) 1458 (CH\(_2\)), 1406 (C-C) 1364 (CH\(_2\)) 1302, 1273 (C-N) 1220, 1112, 1093, 1056, (C-N) 874, 854, 834, 805, 760, 728, (N-H, C-H)

HRMS (m/z -ES): Found: 628.3342 (M\(^+\) + H \( \text{C}_{32}\text{H}_{46}\text{N}_{5}\text{O}_{8} \) Requires: 628.3346).
4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino butanamido]benzophenone (60d)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 60d was obtained as a yellow oil (383.6 mg, 60%).

$\delta$H (400 MHz, CDCl$_3$): 1.47 (s, 9H, (CH$_3$)$_3$), 1.53 (s, 9H, (CH$_3$)$_3$), 1.56 (s, 9H, (CH$_3$)$_3$), 1.86-1.90 (m, 2H, H-2), 2.42 (t, 2H, J 6.5, H-3), 3.26 (t, 2H, J 6.0, H-1), 4.97 (broad s, 1H, NHCO), 7.76-7.81 (m, 8H, H-6/H-6’,H-7/H-7’, H-11/H-11’ and H-12/H-12’), 9.50 (s, 1H, CONH-Ar), 10.61 (broad s, 1H, NH), 11.65 (broad s, 1H, NH)

$\delta$C (100 MHz, CDCl$_3$): 26.9 (CH$_2$, C-2), 27.6 ((CH$_3$)$_3$), 27.7 ((CH$_3$)$_3$), 27.9 ((CH$_3$)$_3$), 34.2 (CH$_2$, C-3), 38.7 (CH$_2$, C-1), 79.5 (q, C(CH$_3$)$_3$), 79.7 (q, C(CH$_3$)$_3$), 83.7 (q, C(CH$_3$)$_3$), 118.2 (CH, Ar., C-6/-C-6’), 120.7 (CH, Ar., C-12/C-12’), 130.7 (CH, Ar., C-11/C-11’), 131.0 (CH, Ar., C-7/C-7’), 132.3 (q, Ar., C-8), 133.2 (q, Ar., C-10), 140.1 (q, Ar., C-5), 142.0 (q, Ar., C-13), 152.9 (q, CO), 155.7 (q, CO), 157.0 (q, CO), 162.8 (q, CN), 171.3 (q, CONH, C-4), 194.3 (q, PhCOPh, C-9)

$\nu$ max (film)/cm$^{-1}$: 2979, 2925 (NH) 1739, 1637 (CO, CN) 1408 (C-C) 1370 (CH$_2$) 1308, 1238 (C-N) 1150, 1122, 1099, 1046 (C-N) 847, 769, 693 (N-H, C-H)

HRMS (m/z -ES): Found: 640.3350 (M$^+$ + H. C$_{33}$H$_{46}$N$_5$O$_8$ Requires: 640.3346).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino butanamido]diphenylamine (60e)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 60e was obtained as a purple solid (423.7 mg, 68%). Mp: 58-60 °C.
δ_\text{H} (400 MHz, CDCl₃): 1.42 (s, 18H, ((CH₃)₃)), 1.51 (s, 9H, (CH₃)₃), 1.78-1.82 (m, 2H, H-2), 2.28 (t, 2H, J 5.5, H-3), 3.16 (t, 2H, J 5.5, H-1), 5.11 (s, 1H, NHCO), 6.06 (s, 1H, ArNHAr), 6.82 (d, 2H, J 8.5, H-6/H-6'), 6.91 (d, 2H, J 8.5, H-7/H-7'), 7.28 (d, 2H, J 8.5, H-10/H-10'), 7.40 (d, 2H, J 8.5, H-11/H-11'), 8.77 (s, 1H, CONH-Ar), 10.09 (s, 1H, NH), 11.64 (broad s, 1H, NH).

δ_\text{C} (150 MHz, CDCl₃): 26.3 (CH₂ C-2), 27.9 (C(CH₃)₃), 28.0 (C(CH₃)₃), 28.2 (C(CH₃)₃), 34.2 (CH₂, C-3), 39.5 (CH₂, C-1), 79.1 (q, C(CH₃)₃), 79.4 (q, C(CH₃)₃), 83.4 (q, C(CH₃)₃), 116.8 (CH, Ar., C-6/C-6'), 118.7 (CH, Ar., C-11/ C-11'), 121.1 (CH, Ar., C-10/C-10'), 124.3 (CH, Ar., C-7/C-7'), 128.5 (q, Ar., C-8), 132.0 (q, Ar., C-9), 138.9 (q, Ar., C-5), 141.4 (q, Ar., C-12), 153.1 (q, CO), 154.0 (q, CO), 156.6 (q, CO), 163.6 (q, CN), 171.0 (q, CONH, C-4).

ν_max (film)/cm⁻¹: 3300, 2928 (NH) 1715, 1605, (CO, CN) 1452, (C-C) 1300 (C-N) 1227, 1148, 1098, 1056, 1027 (C-N) 818, 776, 699 (N-H, C-H).


4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino pentanamido]diphenylmethene (61a)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 61a was obtained as a yellow solid (630.3 mg, 98%). Mp: 92-94 °C.

δ_\text{H} (400 MHz, CDCl₃): 1.45 (s, 9H, (CH₃)₃), 1.50 (s, 9H, (CH₃)₃), 1.55 (s, 9H, (CH₃)₃), 1.72-1.78 (m, 4H, H-2 and H-3), 2.39 (t, 2H, J 7.0, H-4), 3.19 (t, 2H, J 6.5, H-1), 3.91 (s, 2H, H-10), 4.70 (s, 1H, NHCO), 7.13 (d, 4H, J 8.5, H-7/H-7’ and H-13/H-13’), 7.47 (d, 2H, J 8.5, H-8/H-8’), 7.51 (d, 2H, J 8.5, H-12/H-12’), 7.62 (s, 1H, CONH-Ar), 10.28 (s, 1H, NH), 11.65 (s, 1H, NH).
Experimental Section

**Chapter 9**

\[ \delta_C \text{ (100 MHz, CDCl}_3\text{): 22.1 (CH}_2\text{, C-3), 27.6 ((CH}_3\text{)_3), 27.7 ((CH}_3\text{)_3), 28.0 ((CH}_3\text{)_3), 29.0 (CH}_2\text{, C-2), 30.5 (CH}_2\text{, C-4), 38.8 (CH}_2\text{, C-10), 40.3 (CH}_2\text{, C-1), 78.8 (q, C(CH}_3\text{)_3), 79.2 (q, C(CH}_3\text{)_3), 83.3 (q, C(CH}_3\text{)_3), 119.4 (CH, Ar., C-7/C-7'), 119.5 (CH, Ar., C-13/C-13'), 128.9 (CH, Ar., C-12/C-12'), 129.0 (CH, Ar., C-8/C-8'), 135.7 (q, Ar., C-9), 136.4 (q, Ar., C-11), 137.3 (q, Ar., C-6), 148.5 (q, Ar., C-14), 151.0 (q, CO), 152.8 (q, CO), 153.1 (q, CO), 169.5 (q, CN), 172.9 (q, CONH, C-5) \]

\[ \nu_{\text{max}} \text{ (film)/cm}^{-1}: 3284, 2977, 2931, (\text{NH}) 1711, 1690, 1663, 1633, 1604 (\text{CO, CN}) 1562, 1512, 1477 (\text{C-C}) 1457 (\text{CH}_2) 1411 (\text{C-C}) 1367, (\text{CH}_2) 1301, 1271, (\text{C-N}) 1233, 1148, 1122, 1113, 1097, 1055, 1027 (\text{C-N}) 852, 830, 808, 785, 761, 728, 700 (\text{N-H, C-H}) \]

HRMS \( m/z \text{ -ES): Found: 640.3709 (M}^+ + \text{H. C}_{34}\text{H}_{50}\text{N}_{5}\text{O}_{7} \text{ Requires: 640.3710).} \]

**4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino pentanamido]diphenylethylene (61b)**

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 61b was obtained as a yellow oil (605.5 mg, 92%).

\[ \delta_H \text{ (400 MHz, CDCl}_3\text{): 1.47 (s, 9H, (CH}_3\text{)_3), 1.53 (s, 9H, (CH}_3\text{)_3), 1.56 (s, 9H, (CH}_3\text{)_3), 1.60 (broad s, 4H, H-2 and H-3), 2.41 (t, 2H, J 5.0, H-4), 2.87 (broad s, 4H, H-10 and H-11), 3.21 (t, 2H, J 4.0, H-1), 4.67 (s, 1H, NH), 7.13 (d, 4H, J 8.0, H-7/H-7' and H-13/H-13'), 7.44 (s, 1H, CONH-Ar), 7.46 (d, 2H, J 8.0, H-8/H-8') 7.52 (d, 2H, J 8.0, H-14/H-14'), 10.29 (s, 1H, NH), 11.66 (s, 1H, NH) \]

\[ \delta_C \text{ (100 MHz, CDCl}_3\text{): 22.1 (CH}_2\text{, C-3), 27.6 ((CH}_3\text{)_3), 27.7 ((CH}_3\text{)_3), 28.0 ((CH}_3\text{)_3), 29.1 (CH}_2\text{, C-2), 30.5 (CH}_2\text{, C-4), 36.8 (CH}_2\text{, C-10), 36.9 (CH}_2\text{, C-11), 39.1 (CH}_2\text{, C-1), 78.8 (q, C(CH}_3\text{)_3), 79.1 (q, C(CH}_3\text{)_3), 83.2 (q, C(CH}_3\text{)_3), 119.3 (CH, Ar., C-7/C-7'), 121.8 (CH, Ar., C-14/C-14'), 126.4 (CH, Ar., C-13/C-13'), 128.4 (CH, Ar., C-8/C-8'), 134.1 (q, Ar., C-9), 135.6 (q, Ar., C-12), 137.0 (q, Ar., C-6), 137.8 (q, Ar., C-15), 152.9 (q, CO), 153.1 (q, CO), 155.9 (q, CO), 163.1 (q, CN), 170.8 (q, CONH,C-5) \]
Experimental Section

\[ \nu_{\text{max}} \text{(film)/cm}^{-1}: \ 3265, \ 3024, \ 2916, \ 2562, \ (\text{NH}) \ 1660, \ 1634, \ (\text{CO, CN}) \ 1597 \ (\text{C-C}), \ 1408 \ (\text{C-C}) \ 1302, \ 1265 \ (\text{C-N}) \ 1194, \ 1102 \ (\text{C-N}) \ 827, \ 733, \ 716 \ (\text{N-H, C-H}) \]

HRMS \( m/z \ -\text{ES} \): Found: 654.3870 (M⁺ + H. C_{35}H_{52}N_{5}O_{7} \text{ Requires: 654.3867)\]

4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino pentanamido]diphenylether (61c)

\[
\text{Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 61c was obtained as a yellow oil (438.5 mg, 68%).}
\]

\[ \delta_{\text{H}} \ (400 \text{ MHz, CDCl}_3): \ 1.46 \ (s, 9\text{H}, (\text{CH}_3)_3), \ 1.53 \ (s, 9\text{H}, (\text{CH}_3)_3), \ 1.56 \ (s, 9\text{H}, (\text{CH}_3)_3), \ 1.61 \ (m, 2\text{H}, \text{H-2}) \ 1.75-1.79 \ (m, \text{2H}, \text{H-3}), \ 2.42 \ (t, \text{2H}, J \ 7.5, \text{H-4}), \ 3.22 \ (t, \text{2H}, J \ 6.5, \text{H-1}), \ 4.69 \ (\text{broad s, 1H, NHCO},) \ 6.94-6.99 \ (m, \text{4H, H-7/H-7' and H-11/H-11'}), \ 7.53 \ (d, \text{4H, J 9.0, H-8/H-8' and H-12/H-12'}), \ 7.55 \ (s, \text{1H, CONH-Ar}), \ 10.33 \ (\text{broad s, 1H, NH}), \ 11.69 \ (\text{broad s, 1H, NH})
\]

\[ \delta_{\text{C}} \ (100 \text{ MHz, CDCl}_3): \ 22.1 \ (\text{CH}_2, \text{C-3}), \ 27.6 \ ((\text{CH}_3)_3), \ 27.7 \ ((\text{CH}_3)_3), \ 27.9 \ ((\text{CH}_3)_3), \ 30.4 \ (\text{CH}_2, \text{C-2}), \ 36.1 \ (\text{CH}_2, \text{C-4}), \ 39.1 \ (\text{CH}_2, \text{C-1}), \ 78.9 \ (q, \text{C(CH}_3)_3), \ 79.2 \ (q, \text{C(CH}_3)_3), \ 83.3 \ (q, \text{C(CH}_3)_3), \ 115.7 \ (\text{CH, Ar., C-7/C-7'}), \ 117.3 \ (\text{CH, Ar., C-12/C-12'}), \ 118.5 \ (\text{CH, Ar., C-11/C-11'}), \ 120.1 \ (\text{CH, Ar., C-8/C-8'}), \ 130.6 \ (q, \text{Ar, C-9}), \ 132.2 \ (q, \text{Ar, C-10}), \ 133.1 \ (q, \text{Ar., C-6}), \ 142.1 \ (q, \text{Ar., C-13}), \ 148.6 \ (q, \text{CO}), \ 154.7 \ (q, \text{CO}), \ 156.0 \ (q, \text{CO}), \ 162.0 \ (q, \text{CN}), \ 170.8 \ (q, \text{CONH, C-5})
\]

\[ \nu_{\text{max}} \text{(film)/cm}^{-1}: \ 3366, \ 2984, \ (\text{NH}) \ 1737, \ 1683, \ 1659, \ (\text{CO, CN}) \ 1447 \ (\text{C-C}) \ 1372 \ (\text{CH}_2) \ 1302 \ (\text{C-N}) \ 1234, \ 1166, \ 1098, \ 1043 \ (\text{C-N}) \ 847, \ 783 \ (\text{N-H, C-H}) \]

HRMS \( m/z \ -\text{ES} \): Found: 642.3508 (M⁺ + H. C_{33}H_{48}N_{5}O_{8} \text{ Requires: 642.3503}).
4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino pentanamido]benzophenone (61d)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 61d was obtained as a yellow solid (531.8 mg, 81%). Mp: 66-68 °C.

δH (400 MHz, CDCl3): 1.41 (s, 9H, (CH3)3), 1.49 (s, 9H, (CH3)3), 1.53 (s, 9H, (CH3)3), 1.70-1.76 (m, 4H, H-2 and H-3), 2.40 (t, 2H, J 8.0, H-4), 3.13 (t, 2H, J 6.5, H-1), 4.84 (s, 1H, NHCO), 7.69-7.76 (m, 8H, H-7/H-7’, H-8/H-8’, H-12/H-12’, H-13/H-13’), 8.60 (s, 1H, CONH-Ar), 10.59 (s, 1H, NH), 11.64 (s, 1H, NH)

δC (100 MHz, CDCl3): 22.0 (CH2, C-3), 27.7 ((CH3)3), 28.0 ((CH3)3), 29.1 ((CH3)3), 36.3 (CH2, C-2), 38.2 (CH2, C-4), 39.0 (CH2, C-1), 78.8 (q, C(CH3)3), 79.8 (q, C(CH3)3), 83.8 (q, C(CH3)3), 118.3 (CH, Ar., C-7/C-7’), 120.7 (CH, Ar., C-13/C-13’), 130.7 (CH, Ar., C-12/C-12’), 131.0 (CH, Ar., C-8/C-8’), 132.3 (q, Ar., C-9), 133.2 (q, Ar., C-11), 140.1 (q, A., C-6), 141.9 (q, Ar., C-14), 152.8 (q, CO), 153.0 (q, CO), 156.0 (q, CO), 162.7 (q, CN), 171.5 (q, CONH, C-5), 194.3 (q, PhCOPh, C-10)

υmax (film)/cm⁻¹: 2993, (NH) 1713, 1623, (CO, CN) 1589 (C-C) 1403 (C-C) 1364 (CH2) 1298, 1279, (C-N) 1228, 1142, 1117, 1093, 1054, 1026, (C-N) 842, 803, 767, 681 (N-H, C-H)

4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino] pentanamido]diphenylamine (61e)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 61e was obtained as a purple solid (498.2 mg, 78%). Mp: 93-95 °C.

$\delta_H$ (400 MHz, CDCl$_3$): 1.45 (s, 9H, (CH$_3$)$_3$), 1.49 (s, 9H, (CH$_3$)$_3$), 1.55 (s, 9H, (CH$_3$)$_3$), 1.67-1.77 (m, 4H, H-2 and H-3), 2.36 (t, 2H, J 7.3, H-4), 3.17 (t, 2H, J 7.0, H-1), 4.64 (s, 1H, NHCO), 4.74 (s, 1H, ArNHAr), 6.93 (d, 2H, J 9.0, H-7/H-7’), 6.99 (d, 2H, J 9.0, H-11/H-11’), 7.38-7.43 (m, 4H, H-8/H-8’ and H-12/H-12’), 7.78 (s, 1H, CONH-Ar), 10.18 (s, 1H, NH), 11.66 (s, 1H, NH)

$\delta_C$ (150 MHz, CDCl$_3$): 21.7 (CH$_2$, C-3), 28.4 ((CH$_3$)$_3$), 28.4 ((CH$_3$)$_3$), 29.4 ((CH$_3$)$_3$), 29.5 (CH$_2$, C-2), 34.7 (CH$_2$, C-4), 38.6 (CH$_2$, C-1), 79.1 (q, C(CH$_3$)$_3$), 79.4 (q, C(CH$_3$)$_3$), 80.3 (q, C(CH$_3$)$_3$), 115.6 (CH, Ar., C-7/C-7’), 116.1 (CH, Ar., C-12/C-12’), 120.6 (CH, Ar., C-11/C-11’), 124.9 (CH, Ar., C-8/C-8’), 126.9 (q, Ar., C-9), 128.5 (q, Ar., C-10), 128.8 (q, Ar., C-6), 132.6 (q, Ar., C-13), 133.1 (q, CO), 143.5 (q, CO), 156.0 (q, CO), 156.1 (q, CN), 169.7 (q, CONH, C-5)

$\nu_{max}$ (film)/cm$^{-1}$: 3300, 2975, 2932, (NH) 1714, 1688, 1604, (CO, CN) 1454, 1407 (C-C), 1365 (CH$_2$) 1300 (C-N), 1098, 1056, 1028 (C-N) 818, 775 (N-H, C-H)

HRMS (m/z -ES): Found: 641.3664 (M$^+$ + H. C$_{33}$H$_{49}$N$_6$O$_7$ Requires: 641.3663).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino] octanamido]diphenylmethylene (62a)
Following Method C, a solution of 8-(tert butoxycarbonylamino)octanoic acid (129.6 mg, 0.50 mmol) in MeCN (10 cm³) was treated with DIEA (0.28 cm³, 1.6 mmol), 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'[4-(tert-butoxycarbonyl)amino]butanamido]diphenylethylene (184.9 mg, 0.42 mmol) and TBTU (134.9 mg, 0.50 mmol) under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours. Usual work-up followed by silica gel chromatography, eluting with hexane/EtOAc (2:1) afforded 62a as a yellow oil (162.7 mg, 88%).

δ_H (400 MHz, CDCl₃): 1.36 (broad s, 6H, 3CH₂, H-3–H-5), 1.46 (s, 9H, (CH₃)₃), 1.51 (s, 9H, (CH₃)₃), 1.55 (s, 9H, (CH₃)₃), 1.67-1.73 (m, 4H, H-2 and H-6), 2.34 (t, 2H, J 7.5, H-7), 3.13 (t, 2H, J 6.5, H-1), 3.91 (s, 2H, H-13), 4.55 (s, 1H, NHCO), 7.13 (d, 4H, J 8.5, H-10/H-10' and H-16/H-16'), 7.35 (s, 1H, CONH-Ar), 7.47 (d, 2H, J 8.5, H-11/H-11'), 7.51 (d, 2H, J 8.5, H-15/H-15'), 10.28 (broad s, 1H, NH), 11.65 (broad s, 1H, NH)

δ_C (100 MHz, CDCl₃): 25.0 (CH₂, C-6), 26.0 (CH₂, C-3), 27.6 ((CH₃)₃, 27.7 ((CH₃)₃), 28.0 ((CH₃)₃), 28.3 (CH₂, C-4) 28.5 (CH₂, C-5), 29.5 (CH₂, C-2), 37.1 (CH₂, C-7), 40.0 (CH₂, C-1), 40.3 (CH₂, C-13), 78.6 (q, C(CH₃)₃), 79.1 (q, C(CH₃)₃), 83.2 (q, C(CH₃)₃), 119.4 (CH, Ar., C-10/C-10'), 122.0 (CH, Ar., C-16/C-16'), 128.8 (CH, Ar., C-15/C-15'), 129.0 (CH, Ar., C-11/C-11'), 134.3 (q, Ar., C-12), 135.7 (q, Ar., C-14), 136.4 (q, Ar., C-9), 137.2 (q, Ar., C-17), 152.8 (q, CO), 153.1 (q, CO), 155.9 (q, CO), 162.8 (q, CN), 170.8 (q, CONH, C-8)

v_max (film)/cm⁻¹: 2981, 2934, (NH) 1737, 1639, 1603, (CO, CN) 1409 (C-C) 1371 (CH₂) 1302, (C-N) 1235, 1151, 1098, 1044 (C-N) 847, 810, 780 (N-H, C-H)

HRMS (m/z -ES): Found: 682.4173 (M⁺ + H. C₃⁷H₅₆N₅O₇ Requires: 682.4180).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'[4-(tert-butoxycarbonyl)amino]octanamido]diphenylethylene (62b)

Following Method C, a solution of 8-(tert butoxycarbonylamino)octanoic acid (105.9 mg, 0.40 mmol) in MeCN (10 cm³) was treated with DIEA (0.23 cm³, 1.6 mmol), 4-[2,3-
di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)aminobutanamido]diphenyl
ethylene (154.7 mg, 0.34 mmol) and TBTU (128.4 mg, 0.40 mmol) under inert
atmosphere. The reaction mixture was stirred at room temperature for 18 hours. Usual
work-up followed by silica gel chromatography, eluting with hexane/EtOAc (2:1) afforded
62b as a yellow solid (105.7 mg, 68%). Mp: 68-70 °C.

δH (400 MHz, CDCl3): 1.36 (broad s, 6H, H-3—H-5), 1.46 (s, 9H, (CH3)3), 1.51 (s, 9H,
(CH3)3), 1.57 (s, 9H, (CH3)3), 1.73-1.75 (m, 4H, H-2 and H-6), 2.35 (t, 2H, J 5.0, H-7),
2.83 (s, 4H, H-13 and H-14), 3.13 (t, 2H, J 6.5, H-1), 4.55 (s, 1H, NHCO), 7.12 (d, 2H, J
5.3, H-10/H-10'), 7.15 (d, 2H, J 5.3, H-11/H-11'), 7.34 (s, 1H, CONH-Ar), 7.45 (d, 2H, J
5.3, H-16/H-16'), 7.49 (d, 2H, J 5.3, H-17/H-17'), 10.41 (s, 1H, NH), 11.63 (s, 1H, NH)

δC (100 MHz, CDCl3): 25.3 (CH2, C-6), 26.4 (CH2, C-3), 28.1 ((CH3)3), 28.4 ((CH3)3),
28.4 ((CH3)3), 28.8 (CH2, C-4), 28.9 (CH2, C-5), 29.0 (CH2, C-2), 30.8 (CH2, C-7), 37.2
(CH2, C-13), 37.3 (CH2, C-14), 40.4 (CH2, C-1), 79.0 (q, C(CH3)3), 81.9 (q, C(CH3)3), 83.5
(q, C(CH3)3), 117.8 (CH, Ar., C-10/C-10'), 119.8 (CH, Ar., C-17/C-17'), 122.6 (CH, Ar.,
C-16/C-16'), 128.9 (CH, Ar., C-11/C-11'), 130.0 (q, Ar., C-12), 133.3 (q, Ar., C-15), 133.8
(q, Ar., C-9), 137.3 (q, Ar., C-18), 153.4 (q, CO), 156.0 (q, CO), 158.1 (q, CO), 163.5 (q,
CN), 171.2 (q, CONH, C-8)

νmax (film)/cm⁻¹: 3291, 2975, 2929, 2857, (NH) 1716, 1627, 1602 (CO, CN) 1454, 1407
(C-C) 1366 (CH2) 1336, 1301, (C-N) 1235, 1147, 1105, 1056, 1027 (C-N) 813, 774 (N-H,
C-H)


4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino
octanamido]diphenylether (62c)

Following Method C and after purification by flash chromatography with silica gel, eluting
with hexane/EtOAc (2:1), 62c was obtained as a yellow oil (148.4 mg, 22%).
Experimental Section

\[ \delta_H (400 \text{ MHz, CDCl}_3): 1.16-1.24 \text{ (m, 6H, } H-3-H-5), 1.41 \text{ (s, 9H, } (\text{CH}_3)_3), 1.50 \text{ (s, 9H, } (\text{CH}_3)_3), 1.57 \text{ (s, 9H, } (\text{CH}_3)_3), 1.59-1.69 \text{ (m, 4H, } H-2 \text{ and } H-6), 2.18 \text{ (t, 2H, J 7.2, } H-7), 3.04 \text{ (t, 2H, J 4.5, } H-1), 4.81 \text{ (s, 1H, NHCO), 6.75 \text{ (d, 2H, J 8.0, } H-10/H-10'), 6.88 \text{ (d, 2H, J 8.0, } H-11/H-11'), 7.38 \text{ (d, 2H, J 8.0, } H-14/H-14'), 7.54 \text{ (d, 2H, J 8.0, } H-15/H-15'), 8.69 \text{ (s, 1H, CONH-Ar), 10.20 \text{ (s, 1H, NH), 11.67 \text{ (s, 1H, NH)}} \]

\[ \delta_C (100 \text{ MHz, CDCl}_3): 26.1 \text{ (CH}_2, C-6), 26.4 \text{ (CH}_2, C-3), 27.5 ((\text{CH}_3)_3), 27.6 ((\text{CH}_3)_3), 27.9 ((\text{CH}_3)_3), 28.5 \text{ (CH}_2, C-4) 28.7 \text{ (CH}_2, C-5), 34.1 \text{ (CH}_2, C-2), 36.6 \text{ (CH}_2, C-7), 40.0 \text{ (CH}_2, C-1), 78.4 \text{ (q, C(\text{CH}_3)_3), 79.3 \text{ (q, C(\text{CH}_3)_3), 83.3 \text{ (q, C(\text{CH}_3)_3), 117.5 \text{ (CH, Ar., C-10/C-10'), 119.2 \text{ (CH, Ar., C-15/C-15'), 120.8 \text{ (CH, Ar., C-14/C-14'), 124.0 \text{ (CH, Ar., C-11/C-11'), 130.5 \text{ (q, Ar., C-12), 134.3 \text{ (q, Ar., C-13), 151.7 \text{ (q, Ar., C-9), 152.8 \text{ (q, Ar., C-16), 153.7 \text{ (q, CO), 154.8 \text{ (q, CO), 155.7 \text{ (q, CO), 162.9 \text{ (q, CN), 171.3 (q, CONH, C-8)}}}} \]

\[ \nu_{\text{max}} \text{ (film)/cm}^{-1}: 2980, 2934, \text{(NH) } 1737, 1639, \text{(CO, CN) } 1499, 1407 \text{ (C-C) } 1370 \text{ (CH}_2 \text{) } 1305, \text{(C-N) } 1236, 1151, 1113, 1044 \text{ (C-N) } 875, 846, 779 \text{ (N-H, C-H)}} \]


4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino]octanamido]benzophenone (62d)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 62d was obtained as a yellow solid (684.1 mg, 93%). Mp: 42-44 °C.

\[ \delta_H (400 \text{ MHz, CDCl}_3): 1.27-1.34 \text{ (m, 6H, } H-3-H-5), 1.47 \text{ (s, 9H, } (\text{CH}_3)_3), 1.55 \text{ (s, 9H, } (\text{CH}_3)_3), 1.58 \text{ (s, 9H, } (\text{CH}_3)_3), 1.69-1.74 \text{ (m, 4H, } H-2 \text{ and } H-6), 2.38 \text{ (t, 2H, J 7.0, } H-7), 3.12 \text{ (t, 2H, J 5.0, } H-1), 4.63 \text{ (s, 1H, NHCO), 7.76-7.81 \text{ (m, 8H, H-10/H-10', H-11/H-11', H-15/H-15' and H-16/H-16'), 8.26 \text{ (s, 1H, CONH-Ar), 10.64 \text{ (s, 1H, NH), 11.68 \text{ (s, 1H, NH)}}} \]

234
Experimental Section

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino octanamido]diphenylamine (62e)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 62e was obtained as a black solid (254.0 mg, 37%). Mp: 54-56 °C.

δH (600 MHz, CDCl3): 1.27-1.33 (m, 6H, H-3—H-5), 1.45 (s, 9H, (CH3)3), 1.52 (s, 9H, (CH3)3), 1.55 (s, 9H, (CH3)3), 1.70 (broad s, 2H, H-2), 1.86-1.89 (m, 2H, H-6), 2.29 (t, 2H, J 4.0, H-7), 3.10 (broad s, 2H, H-1), 4.60 (s, 1H, NHCO), 5.83 (s, 1H, ArNHAr), 6.96 (app d, 4H, H-10/H-10’ and H-15/H-15’), 7.36 (d, 2H, J 5.4, H-11/H-11’), 7.41 (d, 2H, J 5.4, H-14/H-14’), 7.77 (s, 1H, CONH-Ar), 10.15 (s, 1H, NH), 11.66 (s, 1H, NH)

δC (150 MHz, CDCl3): 26.4 (CH2, C-6), 26.7 (CH2, C-3), 27.9 ((CH3)3), 28.0 ((CH3)3), 28.3 ((CH3)3), 28.8 (CH2, C-4), 28.9 (CH2, C-5), 29.8 (CH2, C-2), 37.2 (CH2, C-7), 40.3 (CH2, C-1) 78.9 (q, C(CH3)3), 79.4 (q, C(CH3)3), 83.4 (q, C(CH3)3), 117.1 (CH, Ar., C-10/C-10’), 118.6 (CH, Ar., C-15/C-15’), 121.2 (CH, Ar., C-14/C-14’), 124.1 (CH, Ar., C-
4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino undecanamido]diphenylmethylene (63a)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 63a was obtained as a white solid (354.3 mg, 49%). Mp: 62-65 °C.

δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 1.27 (broad s, 12H, H-3-H-8), 1.45 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>), 1.50 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>), 1.54 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>), 1.69-1.72 (m, 4H, H-2 and H-9), 2.33 (t, 2H, J 5.0, H-10), 3.10 (t, 2H, J 6.4, H-1), 3.90 (s, 2H, H-16), 4.57 (s, 1H, NHCO), 7.11 (d, 4H, J 5.5, H-13/H-13’ and H-19/H-19’), 7.46 (d, 2H, J 5.5, H-14/H-14’), 7.49 (d, 2H, J 5.5, H-18/H-18’), 7.56 (s, 1H, CONH-Ar), 10.26 (s, 1H, NH), 11.64 (s, 1H, NH)

δ<sub>C</sub> (150 MHz, CDCl<sub>3</sub>): 25.5 (CH<sub>2</sub>, C-9), 26.6 (CH<sub>2</sub>, C-3), 27.9 ((CH<sub>3</sub>)<sub>3</sub>), 28.0 ((CH<sub>3</sub>)<sub>3</sub>), 28.3 ((CH<sub>3</sub>)<sub>3</sub>), 29.1 (CH<sub>2</sub>, C-8), 29.1 (CH<sub>2</sub>, C-7), 29.1 (CH<sub>2</sub>, C-4), 29.2 (CH<sub>2</sub>, C-5) 29.3 (CH<sub>2</sub>, C-6), 29.9 (CH<sub>2</sub>, C-2), 30.7 (CH<sub>2</sub>, C-10), 38.4 (CH<sub>2</sub>, C-16), 40.6 (CH<sub>2</sub>, C-1), 78.8 (q, C(CH<sub>3</sub>)<sub>3</sub>), 79.4 (q, C(CH<sub>3</sub>)<sub>3</sub>), 83.5 (q, C(CH<sub>3</sub>)<sub>3</sub>), 119.7 (CH, Ar., C-13/C-13’), 122.3 (CH, Ar., C-19/C-19’), 129.1 (CH, Ar., C-18/C-18’), 129.2 (CH, Ar., C-14/C-14’), 134.6 (q, Ar., C-15), 136.2 (q, Ar., C-17), 136.5 (q, Ar., C-12), 137.6 (q, Ar., C-20), 153.2 (q, CO), 153.4 (q, CO), 155.9 (q, CO), 163.3 (q, CN), 171.0 (q, CONH, C-11)

ν<sub>max</sub> (film)/cm<sup>-1</sup>: 3352, 2979, 2923, 2848, (NH) 1719, 1678, 1666 (CO, CN), 1600 (C-C) 1410 (C-C) 1364 (CH<sub>2</sub>) 1319, 1279 (C-N) 1229, 1152, 1105, 1057, 1028 (C-N) 859, 806, 780, 767 (N-H, C-H)

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino undecanamido]diphenylethylene (63b)

Following Method C, a solution of 11-(tert-butoxycarbonylamino)undecanoic acid (79.8 mg, 0.26 mmol) in MeCN (10 cm³) was treated with DIEA (0.15 cm³, 0.84 mmol), 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)aminobutanamido]diphenylethylene (100 mg, 0.22 mmol) and TBTU (85 mg, 0.26 mmol) under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours. Usual work-up followed by silica gel chromatography, eluting with hexane/EtOAc (2:1) afforded 63b as a white solid (109 mg, 67%). Mp: 75-77 °C.

δH (400 MHz, CDCl₃): 1.25-1.29 (m, 12H, H-3–H-8), 1.45 (s, 9H, (CH₃)₃), 1.50 (s, 9H, (CH₃)₃), 1.54 (s, 9H, (CH₃)₃), 1.71 (m, 4H, H-2 and H-9), 2.33 (t, 2H, J 7.5, H-10), 2.84 (s, 4H, H-16 and H-17), 3.11 (t, 2H, J 6.5, H-1), 4.58 (s, 1H, NHCO), 7.11 (d, 4H, J 8.0, H-13/H-13' and H-20/H-20'), 7.45 (d, 2H, J 8.0, H-14/H-14') 7.49 (d, 2H, J 8.0, H-19/H-19'), 7.52 (s, 1H, CONH-Ar), 10.29 (s, 1H, NH), 11.67 (s, 1H, NH)

δC (100 MHz, CDCl₃): 25.2 (CH₂, C-9), 26.3 (CH₂, C-3), 27.6 ((CH₃)₃), 27.7 ((CH₃)₃), 28.0 ((CH₃)₃), 28.7 (CH₂, C-8), 28.8 (CH₂, C-7), 28.8 (CH₂, C-4), 28.9 (CH₂, C-5), 29.0 (CH₂, C-6), 29.6 (CH₂, C-2), 36.8 (CH₂, C-10), 36.9 (CH₂, C-16), 37.2 (CH₂, C-17), 40.1 (CH₂, C-1), 78.5 (q, C(CH₃)₃), 79.2 (q, C(CH₃)₃), 83.2 (q, C(CH₃)₃), 119.3 (CH, Ar., C-13/C-13'), 121.8 (CH, Ar., C-20/C-20'), 128.4 (CH, Ar., C-19/C-19'), 128.5 (CH, Ar., C-14/C-14'), 134.1 (q, Ar., C-15), 135.6 (q, Ar., C-18), 136.9 (q, Ar., C-12), 137.8 (q, Ar., C-21), 152.8 (q, CO), 153.1 (q, CO), 155.6 (q, CO), 163.8 (q, CN), 170.7 (q, CONH, C-11)

νmax (film)/cm⁻¹: 3350, 2982, 2922, 2853 (NH) 1739, 1677, 1640, (CO, CN) 1410, (C-C) 1366 (CH₂) 1335, 1296, (C-N) 1235, 1151, 1108, 1045 (C-N) 867, 831, 778, 721, 692 (N-H, C-H)

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino undecanamido]diphenylether (63c)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 63c was obtained as a white solid (625.2 mg, 86%). Mp: 74-76 °C.

δ₁H (400 MHz, CDCl₃): 1.26-1.30 (m, 12H, H-3–H-8), 1.46 (s, 9H, (CH₃)₃), 1.50 (s, 9H, (CH₃)₃), 1.56 (s, 9H, (CH₃)₃), 1.64-1.75 (m, 4H, H-2 and H-9), 2.35 (t, 2H, J 7.5, H-10), 3.13 (t, 2H, J 6.0, H-1), 4.54 (s, 1H, NHCO), 6.96 (d, 2H, J 9.0, H-13/H-13’), 6.99 (d, 4H, J 9.0, H-18/H-18’), 7.35 (s, 1H, CONH-Ar), 7.51 (m, 4H, H-14/H-14’ and H-17/H-17’), 10.36 (s, 1H, NH), 11.70 (s, 1H, NH)

δ₁C (100 MHz, CDCl₃): 25.2 (CH₂, C-9), 26.3 (CH₂, C-3), 27.6 ((CH₃)₃), 27.7 ((CH₃)₃), 28.0 ((CH₃)₃), 28.7 (CH₂, C-8), 28.8 (CH₂, C-7), 28.8 (CH₂, C-4), 28.9 (CH₂, C-5), 29.6 (CH₂, C-6), 30.5 (CH₂, C-2), 37.2 (CH₂, C-10), 40.2 (CH₂, C-1), 78.4 (q, C(CH₃)₃), 80.2 (q, C(CH₃)₃), 84.7 (q, C(CH₃)₃), 118.4 (CH, Ar., C-13/C-13’), 119.0 (CH, Ar., C-18/C-18’), 121.0 (CH, Ar., C-17/C-17’), 123.6 (CH, Ar., C-14/C-14’), 131.3 (q, Ar., C-15), 138.0 (q, Ar., C-16), 148.0 (q, Ar., C-12), 152.8 (q, Ar., C-19), 153.2 (q, CO), 156.2 (q, CO), 159.5 (q, CO), 164.6 (q, CN), 170.9 (q, CONH, C-11)

νmax (film)/cm⁻¹: 3296, 2980, 2929 (NH) 1715, 1608 (CO, CN) 1498, 1406 (C-C) 1366 (CH₂) 1335, 1304 (C-N) 1216, 1147, 1110, 1056, (C-N) 875, 829, 764 (N-H, C-H)

HRMS (m/z -ES): Found: 726.4449 (M⁺ + H. C₃₉H₆₉N₅O₈ Requires: 726.4442).
Experimental Section

Chapter 9

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino undecanamido]diphenylbenzophenone (63d)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 63d was obtained as a yellow solid (417.5 mg, 56%). Mp: 72-74 °C.

δH (400 MHz, CDCl3): 1.31 (broad s, 12H, H-3–H-8), 1.47 (s, 9H, (CH3)3), 1.54 (s, 9H, (CH3)3), 1.58 (s, 9H, (CH3)3), 1.63 (broad s, 4H, H-2 and H-9), 2.42 (t, 2H, J 7.5, H-10), 3.14 (t, 2H, J 6.5, H-1), 4.54 (s, 1H, NHCO), 7.50 (s, 1H, CONH-Ar), 7.69 (d, 2H, J 8.0, H-13/H-13′), 7.77-7.83 (m, 6H, H-14/H-14, H-18/H-18’ and H-19/H-19”), 10.69 (s, 1H, NH), 11.71 (s, 1H, NH)

δC (100 MHz, CDCl3): 26.8 (CH2, C-9), 27.5 (CH2, C-3), 27.6 ((CH3)3), 28.0 ((CH3)3), 28.8 ((CH3)3), 28.9 (CH2, C-8), 29.2 (CH2, C-4), 29.3 (CH2, C-5), 29.9 (CH2, C-6), 30.4 (CH2, C-7), 31.5 (CH2, C-2), 35.8 (CH2, C-10), 40.2 (CH2, C-1), 79.4 (q, C(CH3)3), 81.7 (q, C(CH3)3), 113.4 (CH, Ar., C-13/C-13’), 120.7 (CH, Ar., C-19/C-19’), 130.4 (CH, Ar., C-18/ C-18’), 132.4 (CH, Ar., C-14/C-14’), 135.6 (q, Ar., C-15), 141.4 (q, Ar., C-17), 144.5 (q, Ar., C-12), 148.1 (q, Ar., C-20), 152.8 (q, CO), 156.5 (q, CO), 160.0 (q, CO), 165.0 (q, CN), 171.5 (q, CONH, C-11), 193.9 (q, PhCOPh, C-16)

νmax (film)/cm−1: 3325, 2980, 2923, 2852, (NH) 1719, 1679, 1636, (CO, CN) 1594, (C-C) 1408 (C-C) 1367 (CH2) 1336, 1301, 1277 (C-N) 1233, 1149, 1121, 1100, 1056 (C-N) 807, 769, 684 (N-H, C-H)


4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino undecanamido]diphenylamine (63e)
Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), \(63e\) was obtained as a black solid (482.8 mg, 67\%). Mp: 77-80 °C.

\[\delta_h (400 \text{ MHz, CDCl}_3): 1.17-1.21 (\text{m}, 12\text{H}, \text{H-3--H-8}), 1.37 (\text{s}, 18\text{H}, (\text{CH}_3)_3), 1.48 (\text{s}, 9\text{H}, (\text{CH}_3)_3), 1.60 (\text{broad s}, 4\text{H}, \text{H-2 and H-9}), 2.20 (\text{broad s}, 2\text{H}, \text{H-10}), 3.03 (\text{t}, 2\text{H}, J 7.9, \text{H-1}), 4.76 (\text{s}, 1\text{H}, \text{NHCO}), 6.10 (\text{s}, 1\text{H}, \text{ArNHAr}), 6.84 (\text{m}, 4\text{H}, \text{H-13/H-13' and H-18/H-18'}) 7.21 (\text{m}, 2\text{H}, \text{H-14/H-14'}), 7.35 (\text{d}, 2\text{H}, J 8.0, \text{H-17/H-17'}), 8.30 (\text{s}, 1\text{H}, \text{CONH-Ar}), 10.05 (\text{s}, 1\text{H}, \text{NH}), 11.63 (\text{s}, 1\text{H}, \text{NH})\]

\[\delta_c (150 \text{ MHz, CDCl}_3): 25.1 (\text{CH}_2, \text{C-9}), 25.5 (\text{CH}_2, \text{C-3}), 26.6 ((\text{CH}_3)_3), 27.9 ((\text{CH}_3)_3), 28.0 ((\text{CH}_3)_3), 28.3 (\text{CH}_2, \text{C-7}), 29.0 (\text{CH}_2, \text{C-8}), 29.2 (\text{CH}_2, \text{C-4}), 29.9 (\text{CH}_2, \text{C-5}), 31.4 (\text{CH}_2, \text{C-6}), 34.5 (\text{CH}_2, \text{C-2}), 37.4 (\text{CH}_2, \text{C-10}), 40.5 (\text{CH}_2, \text{C-1}), 78.8 (\text{q}, \text{C(CH}_3)_3), 79.5 (\text{q}, \text{C(CH}_3)_3), 83.5 (\text{q}, \text{C(CH}_3)_3), 117.2 (\text{CH}, \text{Ar}, \text{C-13/C-13'}), 118.7 (\text{CH}, \text{Ar}, \text{C-18/C-18'}), 121.2 (\text{CH}, \text{Ar}, \text{C-17/C-17'}), 124.1 (\text{CH}, \text{Ar}, \text{C-14/C-14'}), 129.0 (\text{q}, \text{Ar}, \text{C-15}), 131.7 (\text{q}, \text{Ar}, \text{C-16}), 139.0 (\text{q}, \text{Ar}, \text{C-12}), 141.0 (\text{q}, \text{Ar}, \text{C-19}), 153.3 (\text{q}, \text{CO}), 154.0 (\text{q}, \text{CO}), 155.9 (\text{q}, \text{CO}), 163.5 (\text{q}, \text{CN}), 171.2 (\text{q}, \text{CONH}, \text{C-12})\]

\[\nu_{\text{max}} (\text{film}) / \text{cm}^{-1}: 3341, 2925, (\text{NH}) 1626, 1595 (\text{CO, CN}) 1393 (\text{C-C}) 1304, 1285, (\text{C-N}) 1243, 1141, 1122, 1059, (\text{C-N}) 827, 766, 721, 678 (\text{N-H, C-N})\]

HRMS (m/z -ES): Found: 725.4606 (M⁺ + H. C_{39}H_{61}N_{6}O_{7}). Requires: 725.4602.

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino dodecanamido]diphenylmethylene (64a)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), \(64a\) was obtained as a white solid (545.4 mg, 74\%). Mp: 45-47 °C.

\[\delta_h (400 \text{ MHz, CDCl}_3): 1.28 (\text{broad s}, 14\text{H}, \text{H-3--H-9}), 1.44 (\text{s}, 9\text{H}, (\text{CH}_3)_3), 1.49 (\text{s}, 9\text{H}, (\text{CH}_3)_3), 1.54 (\text{s}, 9\text{H}, (\text{CH}_3)_3), 1.63-1.69 (\text{m}, 4\text{H}, \text{H-2 and H-10}), 2.30 (\text{t}, 2\text{H}, J 7.5, \text{H-11}), 3.11 (\text{t}, 2\text{H}, J 6.0, \text{H-1}), 3.89 (\text{s}, 2\text{H}, \text{H-17}), 4.60 (\text{s}, 1\text{H}, \text{NHCO}), 7.09 (\text{d}, 4\text{H}, J 8.0, \text{H-}}
Experimental Section

14/H-14' and H-20/H-20'), 7.47 (d, 4H, J 8.0, H-15/H-15' and H-19/H-19'), 7.73 (s, 1H, CONH-Ar) 10.27 (s, 1H, NH), 11.67 (s, 1H, NH)

$\delta_C$ (100 MHz, CDCl$_3$): 25.6 (CH$_2$, C-10), 28.1 ((CH$_3$)$_3$), 28.2 ((CH$_3$)$_3$), 28.5 ((CH$_3$)$_3$), 29.2 (CH$_2$, C-3), 29.3 ((CH$_3$)$_3$), 29.4 (CH$_2$, C-9), 29.4 (CH$_2$, C-8), 30.0 (CH$_2$, C-4), 31.0 (CH$_2$, C-5), 31.0 (CH$_2$, C-6), 31.0 (CH$_2$, C-7), 35.4 (CH$_2$, C-2), 37.8 (CH$_2$, C-11), 38.7 (CH$_2$, C-17), 40.7 (CH$_2$, C-1), 78.8 (q, C(CH$_3$)$_3$), 79.4 (q, C(CH$_3$)$_3$), 83.4 (q, C(CH$_3$)$_3$), 119.9 (CH, Ar., C-14/C-14'), 122.4 (CH, Ar., C-20/C-20'), 129.3 (CH, Ar., C-19/C-19'), 129.4 (CH, Ar., C-15/C-15'), 134.6 (q, Ar., C-16), 136.2 (q, Ar., C-18), 137.7 (q, Ar., C-13), 143.0 (q, Ar., C-21), 153.3 (q, CO), 156.0 (q, CO), 159.8 (q, CO), 163.6 (q, CN), 171.4 (q, CONH, C-12)

$\nu$$_{\text{max}}$ (film)/cm$^{-1}$: 3338, 2978, 2919, 2849, (NH) 1720, 1678 (CO, CN) 1410 (C-C) 1364 (CH$_2$) 1327, 1302, 1278 (C-N) 1230, 1155, 1106, 1057, 1028 (C-N) 858, 807, 766, 720 (N-H, C-H)

HRMS (m/z -ES): Found: 738.4797 (M$^+$ + H. C$_{41}$H$_{64}$N$_5$O$_7$ Requires: 738.4806).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino dodecanamido]diphenylethylene (64b)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 64b was obtained as a white solid (543 mg, 72%). Mp: 56-58°C.

$\delta_H$ (400 MHz, CDCl$_3$): 1.26-1.29 (m, 14H, H-3-H-9), 1.45 (s, 9H, (CH$_3$)$_3$), 1.49 (s, 9H, (CH$_3$)$_3$), 1.54 (s, 9H, (CH$_3$)$_3$), 1.70-1.74 (m, 4H, H-2 and H-10), 2.34 (t, 2H, J 7.5, H-11), 2.85 (s, 4H, H-17 and H-18), 3.11 (t, 2H, J 6.5, H-1), 4.56 (s, 1H, NHCO), 7.12 (d, 4H, J 8.5, H-14/H-14' and H-21/H-21') 7.45 (d, 4H, J 8.5, H-15/H-15' and H-20/H-20'), 7.48 (s, 1H, CONH-Ar), 10.35 (s, 1H, NH), 11.69 (s, 1H, NH)
Experimental Section

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 64c was obtained as a pale yellow solid (332.6 mg, 45%). Mp: 92-95 °C.

δH (400 MHz, CDCl3): 1.29 (broad s, 14H, H-3–H-9), 1.46 (s, 9H, (CH3)3), 1.50 (s, 9H, (CH3)3), 1.56 (s, 9H, (CH3)3), 1.70-1.73 (m, 4H, H-2 and H-10), 2.33 (t, 2H, J 7.5, H-11), 3.13 (t, 2H, J 6.5, H-1), 4.54 (s, 1H, NHCO), 6.92 (d, 2H, J 9.0, H-14/H-14’), 6.98 (d, 2H, J 9.0, H-15/H-15’), 7.44 (s, 1H, CONH-Ar), 7.53 (d, 4H, J 9.0, H-18/H-18’ and H-19/H-19’), 10.30 (s, 1H, NH), 11.68 (s, 1H, NH)

δC (100 MHz, CDCl3): 25.2 (CH2, C-10), 26.3 (CH2, C-3), 27.6 ((CH3)3), 27.7 ((CH3)3), 28.0 ((CH3)3), 28.8 (CH2, C-9), 28.8 (CH2, C-8), 28.9 (CH2, C-4), 28.9 (CH2, C-5), 29.0 (CH2, C-6), 29.6 (CH2, C-7), 36.8 (CH2, C-2), 37.2 (CH2, C-11), 40.2 (CH2, C-1), 78.4 (q, C(CH3)3), 79.4 (q, C(CH3)3), 83.4 (q, C(CH3)3), 118.2 (CH, Ar., C-14/C-14’), 119.1 (CH, Ar., C-14/C-14’), 122.3 (CH, Ar., C-21/C-21’), 126.9 (CH, Ar., C-20/C-20’), 128.9 (CH, Ar., C-15/15’), 134.6 (q, Ar., C-16), 136.1 (q, Ar., C-19), 137.4 (q, Ar., C-13), 138.3 (q, Ar., C-22), 153.3 (q, CO), 153.6 (q, CO), 156.0 (q, CO), 163.5 (q, CN), 171.5 (q, CONH, C-12)

νmax (film)/cm⁻¹: 3301, 2920, 2851, (NH) 1719, 1678, (CO, CN) 1409 (C-C) 1365 (CH2) 1334, 1300 (C-N) 1151, 1105, 1057, 1028 (C-N) 809, 765, 720 (N-H, C-H)

HRMS (m/z -ES): Found: 752.4954 (M⁺ + H. C42H66N5O7 Requires: 752.4962).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino dodecanamido]diphenylether (64c)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 64c was obtained as a pale yellow solid (332.6 mg, 45%). Mp: 92-95 °C.

δH (400 MHz, CDCl3): 1.29 (broad s, 14H, H-3–H-9), 1.46 (s, 9H, (CH3)3), 1.50 (s, 9H, (CH3)3), 1.56 (s, 9H, (CH3)3), 1.70-1.73 (m, 4H, H-2 and H-10), 2.33 (t, 2H, J 7.5, H-11), 3.13 (t, 2H, J 6.5, H-1), 4.54 (s, 1H, NHCO), 6.92 (d, 2H, J 9.0, H-14/H-14’), 6.98 (d, 2H, J 9.0, H-15/H-15’), 7.44 (s, 1H, CONH-Ar), 7.53 (d, 4H, J 9.0, H-18/H-18’ and H-19/H-19’), 10.30 (s, 1H, NH), 11.68 (s, 1H, NH)

δC (100 MHz, CDCl3): 25.7 (CH2, C-10), 26.8 (CH2, C-3), 28.1 ((CH3)3), 28.2 ((CH3)3), 28.4 ((CH3)3), 29.2 (CH2, C-9), 29.3 (CH2, C-8), 29.3 (CH2, C-4), 29.4 (CH2, C-5), 29.5 (CH2, C-6), 30.9 (CH2, C-7), 37.3 (CH2, C-2), 37.7 (CH2, C-11), 38.6 (2CH2, C-17 and C-18), 40.7 (CH2, C-1), 79.6 (q, C(CH3)3), 83.6 (q, C(CH3)3), 119.8 (CH, Ar., C-14/C-14’), 122.3 (CH, Ar., C-21/C-21’), 126.9 (CH, Ar., C-20/C-20’), 128.9 (CH, Ar., C-15/15’), 134.6 (q, Ar., C-16), 136.1 (q, Ar., C-19), 137.4 (q, Ar., C-13), 138.3 (q, Ar., C-22), 153.3 (q, CO), 153.6 (q, CO), 156.0 (q, CO), 163.5 (q, CN), 171.5 (q, CONH, C-12)
Experimental Section

Ar., C-19/C-19'), 120.9 (CH, Ar., C-18/C-18'), 123.6 (CH, Ar., C-15/C-15'), 131.3 (q, Ar., C-16), 133.3 (q, Ar., C-17), 147.4 (q, Ar., C-13), 152.7 (q, Ar., C-20), 153.3 (q, CO), 155.6 (q, CO), 157.1 (q, CO), 162.9 (q, CN), 170.9 (q, CONH, C-12)

ν max (film)/cm⁻¹: 3332, 2918, 2850, (NH) 1718, 1676 (CO, CN) 1501, 1408, (C-C) 1365 (CH₂) 1328, 1303, 1286 (C-N) 1219, 1155, 1106, 1057, 1029 (C-N) 877, 857, 823, 810, 764, 720 (N-H, C-N)


4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxycarbonyl)amino dodecanamido]diphenylbenzophenone (64d)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 64d was obtained as a yellow solid (421 mg, 56%). Mp: decomposes at 174-176 °C.

δ H (400 MHz, CDCl₃): 1.25 (broad s, 14H, H-3–H-9), 1.43 (s, 9H, (CH₃)₃), 1.52 (s, 9H, (CH₃)₃), 1.56 (s, 9H, (CH₃)₃), 1.69 (broad s ,4H, H-2 and H-10), 2.36 (t, 2H, J 8.0, H-11), 3.08 (t, 2H, J 8.0, H-1), 4.64 (s, 1H, NHCO), 7.71-7.76 (m, 8H, H-13/H-13', H-14/H-14', H-15/H-15' and H-19/H-19'), 8.53 (s, 1H, NH), 10.66 (s, 1H, NH), 11.69 (s, 1H, NH)

δ C (100 MHz, CDCl₃): 25.3 (CH₂, C-10), 26.5 (CH₂, C-3), 27.9 ((CH₃)₃), 28.2 ((CH₃)₃), 29.0 ((CH₃)₃), 29.0 (CH₂, C-9), 29.1 (CH₂, C-8), 29.1 (CH₂, C-4), 29.2 (CH₂, C-5), 29.3 (CH₂, C-6), 29.3 (CH₂, C-7), 29.8 (CH₂, C-2), 37.5 (CH₂, C-11), 40.4 (CH₂, C-1), 78.7 (q, C(CH₃)₃), 81.5 (q, C(CH₃)₃), 84.0 (q, C(CH₃)₃), 113.4 (CH, Ar., C-14/C-14'), 118.5 (CH, Ar., C-20/C-20'), 120.9 (q, Ar., C-16), 127.3 (CH, Ar., C-19/C-19'), 130.6 (CH, Ar., C-15/C-15'), 131.0 (q, Ar., C-18), 132.6 (q, Ar., C-13), 142.2 (q, Ar., C-21), 150.8 (q, CO), 153.2 (q, CO), 155.9 (q, CO), 166.5 (q, CN), 170.9 (q, CONH, C-12), 194.4 (q, PhCOPh, C-17)
Experimental Section

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3460, 3363, 3242, 2983 (NH) 1722, 1687, (CO, CN) 1583 (C-C) 1498 (C-C) 1463, (CH$_2$) 1440, 1407 (C-C) 1366 (CH$_2$) 1309, 1280, (C-N) 1230, 1171, 1146, 1119, 1098, 1058 (C-N) 841, 824, 803, 766, 746, 724, 696, 681 (N-H, C-H)

HRMS ($m/z$ -ES): Found: 752.4606 (M$^+$ + H. $C_{41}H_{62}N_5O_8$ Requires:752.4598).

4-[2,3-Di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxycarbonyl)amino dodecanamido]diphenylamine (64e)

Following Method C and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 64e was obtained as a black solid (398.2 mg, 54%). Mp: 84-86 °C.

$\delta_H$ (600 MHz, CDCl$_3$): 1.24 (broad s, 14H, H-3—H-9), 1.43 (s, 9H, (CH$_3$)$_3$), 1.45 (s, 9H, (CH$_3$)$_3$), 1.54 (s, 9H, (CH$_3$)$_3$), 1.64—1.70 (m, 2H, H-2 and H-10), 2.26 (t, 2H, J 4.0, H-11), 3.09 (t, 2H, J 4.0, H-1), 4.61 (s, 1H, NHCO), 5.90 (s, 1H, ArNHAr), 6.83 (d, 2H, J 5.5, H-14/H-14’), 6.91 (d, 2H, J 5.5, H-15/H-15’), 7.30 (d, 2H, J 5.8, H-18/H-18’), 7.39 (d, 2H, J 5.8, H-19/H-19’), 7.93 (s, 1H, NH), 10.11 (s, 1H, NH), 11.66 (s, 1H, NH)

$\delta_C$ (150 MHz, CDCl$_3$): 25.5 (CH$_2$, C-10), 26.6 (CH$_2$, C-3), 27.9 ((CH$_3$)$_3$), 28.0 ((CH$_3$)$_3$), 28.3 ((CH$_3$)$_3$), 29.1 (CH$_2$, C-9), 29.2 (CH$_2$, C-8), 29.3 (CH$_2$, C-4), 29.3 (CH$_2$, C-5), 29.9 (CH$_2$, C-6), 31.0 (CH$_2$, C-7), 31.4 (CH$_2$, C-2), 37.3 (CH$_2$, C-11), 40.5 (CH$_2$, C-1), 78.8 (q, C(CH$_3$)$_3$), 79.4 (q, C(CH$_3$)$_3$), 83.4 (q, C(CH$_3$)$_3$), 116.9 (CH, Ar., C-14/C-14’), 118.7 (CH, Ar., C-19/C-19’), 121.1 (CH, Ar., C-18/C-18’), 124.2 (CH, Ar., C-15/C-15’), 128.6 (q, Ar., C-16), 132.0 (q, Ar., C-17), 138.9 (q, Ar., C-13), 141.3 (q, Ar., C-20), 153.2 (q, CO), 154.0 (q, CO), 155.9 (q, CO), 163.4 (q, CN), 171.0 (q, CONH, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 2927 (NH) 1721, 1629 (CO, CN) 1593 (CH$_2$) 1408 (C-C) 1368 (CH$_2$) 1305, 1285 (C-N) 1236, 1147, 1058 (C-N) 827, 765, 678 (N-H, C-H)

HRMS ($m/z$ -ES): Found: 739.4765 (M$^+$ + H. $C_{40}H_{63}N_6O_7$ Requires: 739.4758).

244
Dihydrochloride salt of N-(4-(4'-guanidinobenzyl)phenyl)-4-aminobutanamide (65a)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminobutanamido]diphenylmethylene (359.9 mg, 0.58 mmol) was dissolved in 4 M HCl/dioxane (2.61 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.29 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 65a as a yellow solid (87 mg, 46%). Mp: 58-60 °C.

δ$_h$ (400 MHz, D$_2$O): 1.87-1.94 (m, 2H, H-2), 2.41 (t, 2H, J 7.0, H-3), 2.96 (t, 2H, J 7.0, H-1), 3.82 (s, 2H, H-9), 7.07 (d, 2H, J 7.5, H-6/H-6'), 7.14 (d, 2H, J 7.5, H-12/H-12'), 7.18-7.24 (m, 4H, H-7/H-7' and H-11/H-11')

δ$_c$ (100 MHz, D$_2$O): 22.3 (CH$_2$, C-2), 32.5 (CH$_2$, C-3), 38.3 (CH$_2$, C-1), 39.6 (CH$_2$, C-9), 121.5 (CH, Ar., C-6/C-6'), 125.6 (CH, Ar., C-12/C-12'), 128.8 (CH, Ar., C-7/C-7'), 129.6 (CH, Ar., C-11/C-11'), 131.5 (q, Ar., C-8), 134.4 (q, Ar., C-10), 138.0 (q, Ar., C-5), 141.1 (q, Ar., C-13), 155.8 (q, CN), 172.9 (q, CONH, C-4)

ν$_{max}$ (film)/cm$^{-1}$: 2874, 2585, 2011 (NH) 1728, 1655, (CO, CN) 1601 (C-C) 1410, 1439, 1410 (C-C) 1353 (CH$_2$) 1333, 1305, 1258 (C-N) 1203, 1181, 1148, 1112, 1093, 1020 (C-N) 861, 811, 773, 758 (N-H, C-H)


Purity by HPLC: 95.4% (t$_R$ = 22.21)

Dihydrochloride salt of N-(4-(4'-guanidinophenylethyl)phenyl)-4-aminobutanamide (65b)
Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminobutanamido]diphenylethylene (271 mg, 0.42 mmol) was dissolved in 4 M HCl/dioxane (1.89 cm^3) under argon. The mixture was diluted with IPA/DCM (1:1) (0.21 cm^3) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 65b was obtained as a yellow oil (55 mg, 39%).

δ$_\text{H}$ (400 MHz, D$_2$O): 1.87-1.94 (m, 2H, H-2), 2.42 (t, 2H, J 8.0, H-3), 2.84 (m, 4H, H-9 and H-10), 2.95 (t, 2H, J 8.0, H-1), 7.07 (d, 2H, J 7.0, H-6/H-6'), 7.11 (d, 2H, J 8.0, H-13/H-13'), 7.16-7.20 (m, 4H, H-12/H-12' and H-7/H-7')

δ$_\text{C}$ (100 MHz, D$_2$O): 30.1 (CH$_2$, C-2), 32.9 (CH$_2$, C-3), 35.8 (CH$_2$, C-9), 35.9 (CH$_2$, C-10), 38.7 (CH$_2$, C-1), 121.8 (CH, Ar., C-6/C-6'), 125.8 (CH, Ar., C-13/C-13'), 129.2 (CH, Ar., C-12/C-12'), 129.9 (CH, Ar., C-7/C-7'), 131.6 (q, Ar., C-8), 134.4 (q, Ar., C-11), 138.9 (q, Ar., C-5), 141.7 (q, Ar., C-14), 156.3 (q, CN), 173.3 (q, CONH, C-4)

ν$_{\text{max}}$ (film)/cm$^{-1}$: 3283, 3026, 2846, 2583 (NH) 1659, 1636 (CO, CN) 1597 (C-C) 1407 (C-C) 1353 (CH$_2$) 1302, 1247 (C-N) 1195, 1105, 1053 (C-N) 894, 827, 734 (N-H, C-H)

HRMS (m/z -ES): Found: 340.2140 (M$^+$ + H. C$_{19}$H$_{26}$N$_5$O Requires: 340.2137).

Purity by HPLC: 95.4% (t$_R$= 23.20)

**Dihydrochloride salt of N-(4-(4'-guanidinophenyl)phenylether)-4-aminobutanamide (65c)**

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminobutanamido]diphenylether (347 mg, 0.55 mmol) was dissolved in 4 M HCl/dioxane (2.48 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.28 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 65c as a brown solid (153.5 mg, 85%). Mp: 98-100 °C.
δ_H (400 MHz, D_2O): 1.89-1.97 (m, 2H, H-2), 2.45 (t, 2H, J 7.5, H-3), 2.98 (t, 2H, J 7.5, H-1), 7.98-7.01 (m, 4H, H-6/H-6' and H-11/H-11'), 7.21 (d, 2H, J 8.5, H-7/H-7'), 7.33 (d, 2H, J 8.5, H-10/H-10')

δ_C (100 MHz, D_2O): 22.7 (CH_2, C-2), 32.8 (CH_2, C-3), 38.7 (CH_2, C-1), 119.4 (CH, Ar., C-6/C-6'), 119.7 (CH, Ar., C-11/C-11'), 123.8 (CH, Ar., C-10/C-10'), 124.6 (CH, Ar., C-7/C-7'), 128.0 (q, Ar., C-8), 129.1 (q, Ar., C-9), 132.6 (q, Ar., C-5), 153.5 (q, Ar., C-12), 156.5 (q, CN), 173.5 (q, CONH, C-4)

ν_{max} (film)/cm^{-1}: 3307, 2927, 2862, 2538 (NH) 1676, 1643 (CO, CN) 1494, 1452 (C-C) 1303, 1318 (C-N) 1192, 1174, 1050 (C-N) 825, 758 (N-H, C-H)

HRMS (m/z - ES): Found: 328.1760 (M^+ + H. C_{17}H_{22}N_{5}O_{2} Requires: 328.1774).

Purity by HPLC: 95.2% (t_R= 21.85)

Dihydrochloride salt of N-(4-(4'-guanidinophenyl)phenylmethanone)-4-aminobutanamide (65d)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminobutanamido]diphenylbenzophenone (102.3 mg, 0.1599 mmol) was dissolved in 4 M HCl/dioxane (0.71 cm^3) under argon. The mixture was diluted with IPA/DCM (1:1) (0.08 cm^3) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H_2O afforded 65d as a yellow solid (53.5 mg, 99%). Mp: 138-140 °C.

δ_H (400 MHz, D_2O): 1.46-1.52 (m, 2H, H-2), 2.25 (t, 2H, J 7.5, H-3), 2.85 (t, 2H, J 7.5, H-1), 7.33-7.38 (m, 4H, H-6/H-6' and H-7/H-7'), 7.74-7.79 (m, 4H, H-11/H-11' and H-12/H-12')
\( \delta \)C (100 MHz, D\textsubscript{2}O): 29.7 (CH\textsubscript{2}, C-2), 30.0 (CH\textsubscript{2}, C-3), 38.3 (CH\textsubscript{2}, C-1), 119.7 (CH, Ar., C-6/C-6'), 123.7 (CH\textsubscript{2}, Ar., C-12/C-12'), 129.8 (q, Ar., C-8), 131.3 (CH, Ar., C-11/C-11'), 131.4 (CH, Ar., C-7/C-7'), 131.8 (q, Ar., C-10), 132.1 (q, Ar., C-5), 138.5 (q, Ar., C-13), 155.4 (q, CN), 176.5 (q, CONH, C-4), 197.3 (q, PhCOPh, C-9)

\( \nu \)\textsubscript{max} (film)/cm\textsuperscript{-1}: 3316, 3129, 2585 (NH) 1674, 1643, (CO, CN) 1453, 1408 (C-C) 1318, 1290, 1261 (C-N) 1192, 1175, 1151, 1019 (C-N) 852, 834, 757 (N-H, C-H)

HRMS (m/z -ES): Found: 340.1781 (M\textsuperscript{+} + H. C\textsubscript{18}H\textsubscript{22}N\textsubscript{5}O\textsubscript{2} Requires: 340.1774).

Purity by HPLC: 98.4% (t\textsubscript{R}= 19.68)

**Dihydrochloride salt ofN-(4-(4'-guanidinophenyl)phenylamine)-4-aminobutanamide (65e)**

Following Method D and after purification by reverse phase silica gel chromatography, eluting with H\textsubscript{2}O afforded 65e as a black solid (134.1 mg, 41%). Mp: 38-40 °C.

\( \delta \)H (600 MHz, D\textsubscript{2}O): 1.80-1.84 (m, 2H, H-2), 2.39 (t, 2H, J 7.5, H-3), 2.92 (t, 2H, J 7.5, H-1), 7.11 (app d, 6H, J 6.0, H-7/H-7', H-10/H-10', H-6/H-6'), 7.20 (d, 2H, J 8.0, H-11/H-11')

\( \delta \)C (150 MHz, D\textsubscript{2}O): 21.9 (CH\textsubscript{2}, C-2), 30.5 (CH\textsubscript{2}, C-3), 38.6 (CH\textsubscript{2}, C-1), 118.7 (CH, Ar., C-6/C-6'), 119.2 (CH, Ar., C-11/C-11'), 122.6 (CH, Ar., C-10/C-10'), 124.0 (CH, Ar., C-7/C-7'), 127.3 (q, Ar., C-8), 127.6 (q, Ar., C-9), 142.1 (q, Ar., C-5), 143.3 (q, Ar., C-12), 156.5 (q, CN), 176.8 (q, CONH, C-4)

\( \nu \)\textsubscript{max} (film)/cm\textsuperscript{-1}: 3671, 3648, 3263, 2928, 2862, 2542 (NH) 1728, 1659 (CO, CN) 1498, 1466, 1452, 1404 (C-C) 1236, 1224, 1150, 1098, 1076, 1051 (C-N) 877, 825 (N-H, C-H)

HRMS (m/z -ES): Found: 327.1940 (M\textsuperscript{+} + H. C\textsubscript{17}H\textsubscript{23}N\textsubscript{6}O Requires: 327.1933).
Purity by HPLC: 95.1% (t<sub>R</sub> = 8.19)

Dihydrochloride salt of \( N-(4-(4'-\text{guanidinophenyl})\text{phenylmethylene})-4-\text{aminopentanamide} \) (66a)

Following Method D, the \( 4-[2,3-\text{di(}t\text{-butoxycarbonyl})\text{guanidino}]4'-[4-(t\text{-butoxy carbonyl})\text{aminopentanamido}]\text{diphenylmethylene} \) (203 mg, 0.31 mmol) was dissolved in 4 M HCl/dioxane (1.40 cm<sup>3</sup>) under argon. The mixture was diluted with IPA/DCM (1:1) (0.16 cm<sup>3</sup>) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H<sub>2</sub>O afforded 66a as a yellow solid (92.7 mg 88%). Mp: 135-137 °C

\[ \begin{align*}
\delta_H (400 \text{ MHz, } D_2O): & \ 1.62 \ (\text{broad s, } 4\text{H, H-2 and H-3}), \ 2.35 \ (t, \ 2\text{H, J 6.5, H-4}), \ 2.91 \ (t, \ 2\text{H, J 7.0, H-1}), \ 3.89 \ (s, \ 2\text{H, H-10}), \ 7.13 \ (d, \ 2\text{H, J 8.0, H-7/H-7'}), \ 7.19 \ (d, \ 2\text{H, J 8.0, H-8/H-8'}), \\
& \ 7.22-7.26 \ (m, \ 4\text{H, H-12/H-12'} \text{ and H-13/H-13'})
\end{align*} \]

\[ \begin{align*}
\delta_C (100 \text{ MHz, } D_2O): & \ 22.0 \ (\text{CH}_2, \ C-3), \ 26.1 \ (\text{CH}_2, \ C-2), \ 35.5 \ (\text{CH}_2, \ C-4), \ 39.1 \ (\text{CH}_2, \ C-1), \\
& \ 40.1 \ (\text{CH}_2, \ C-10), \ 122.4 \ (\text{CH, Ar}, \ C-7/C-7'), \ 126.3 \ (\text{CH, Ar}, \ C-13/C-13'), \ 129.4 \ (\text{CH, Ar}, \ C-12/C-12'), \ 130.2 \ (\text{CH, Ar}, \ C-8/C-8'), \ 132.1 \ (\text{q, Ar}, \ C-9), \ 134.9 \ (\text{q, Ar}, \ C-11), \ 138.7 \ (\text{q, Ar}, \ C-6), \\
& \ 141.7 \ (\text{q, Ar}, \ C-14), \ 156.4 \ (\text{q, CN}), \ 174.9 \ (\text{q, CONH, C-5})
\end{align*} \]

\[ \begin{align*}
\nu_{\text{max}} \ (\text{film})/\text{cm}^{-1}: & \ 2963, \ (\text{NH}) \ 1649, \ 1623 \ (\text{CO, CN}) \ 1600, \ (\text{CH}_2) \ 1467 \ (\text{C-H}) \ 1411 \ (\text{C-C}) \\
& \ 1306, \ 1250 \ (\text{C-N}) \ 1204, \ 1110 \ (\text{C-N}) \ 860, \ 817, \ 781, \ 739 \ (\text{N-H, C-N})
\end{align*} \]

HRMS \( m/z \ -\text{ES} \): Found: 340.2146 (M<sup>+</sup> + H. \ C<sub>19</sub>H<sub>26</sub>N<sub>5</sub>O Requires: 340.2137).

Purity by HPLC: 98.7% (t<sub>R</sub> = 22.60)
Dihydrochloride salt of \(N-(4-(4'\text{-guanidinophenylethyl})\text{phenyl})-4\text{-aminopentanamide} (66b)\)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminopentanamido]diphenylethylene (602 mg, 0.92 mmol) was dissolved in 4 M HCl/dioxane (4.14 cm\(^3\)) under argon. The mixture was diluted with IPA/DCM (1:1) (0.46 cm\(^3\)) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H\(_2\)O afforded \(66b\) as a yellow solid (26.9 mg, 8\%). Mp: 76-78 °C.

\(\delta_H\) (400 MHz, D\(_2\)O): 1.62 (broad s, 4H, H-2 and H-3), 2.34 (t, 2H, J 6.5, H-4), 2.85 (s, 4H, H-10 and H-11), 2.91 (t, 2H, J 5.0, H-1), 7.08 (d, 2H, J 8.0, H-7/H-7'), 7.12 (d, 2H, J 8.0, H-8/H-8'), 7.19 (d, 4H, J 8.0, H-13/H-13' and H-14/H-14')

\(\delta_C\) (100 MHz, D\(_2\)O): 26.0 (CH\(_2\), C-2), 35.4 (CH\(_2\), C-3), 35.8 (CH\(_2\), C-4), 35.9 (2CH\(_2\), C-10 and C-11), 39.0 (CH\(_2\), C-1), 122.1 (CH, Ar., C-7/C-7'), 125.8 (CH, Ar., C-14/C-14'), 129.1 (CH, Ar., C-13/C-13'), 130.0 (CH, Ar.,C-8/C-8'), 131.7 (q, Ar., C-9), 134.5 (q, Ar., C-12), 139.0 (q, Ar., C-6), 141.8 (q, Ar., C-15), 156.4 (q, CN), 174.7 (q, CONH, C-5)

\(\nu_{\text{max}}\) (film)/cm\(^{-1}\): 3283, 2870, 2582 (NH) 1660, 1636 (CO, CN) 1597 (C-C) 1407 (C-C) 1302, 1265 (C-N) 1193, 1106 (C-N) 892, 821, 715 (N-H, C-H)

HRMS (m/z -ES): Found: 354.2294 (M\(^+\) + H. C\(_{20}\)H\(_{27}\)N\(_5\)O Requires: 354.2292).

Purity by HPLC: 98.1% (\(t_R= 23.59\))

Dihydrochloride salt of \(N-(4-(4'\text{-guanidinophenylethyl})\text{phenylether})-4\text{-aminopentanamide} (66c)\)
Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminopentanamido]diphenylether (309.2 mg, 0.48 mmol) was dissolved in 4 M HCl/dioxane (2.16 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.24 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 66c as a brown solid (69.7 mg, 43%) Mp: 52-55 °C.

$\delta_H$ (400 MHz, D$_2$O): 1.63 (broad s, 4H, H-2 and H-3), 2.37 (t, 2H, J 6.5, H-4), 2.92 (t, 2H, J 8.0, H-1), 6.98-7.01 (m, 4H, H-7/H-7' and H-8/H-8'), 7.21 (d, 2H, J 8.0, H-11/H-11'), 7.32 (d, 2H, J 8.0, H-12/H-12')

$\delta_C$ (100 MHz, D$_2$O): 22.0 (CH$_2$, C-3), 26.1 (CH$_2$, C-2), 35.4 (CH$_2$, C-4), 39.0 (CH$_2$, C-1), 119.4 (CH, Ar., C-7/C-7'), 119.6 (CH, Ar., C-12/C-12'), 123.5 (CH, Ar., C-11/C-11'), 127.8 (CH, Ar., C-8/C-8'), 129.0 (q, Ar., C-9), 132.8 (q, Ar., C-10), 153.1 (q, Ar., C-6), 156.3 (q, Ar., C-13), 156.4 (q, CN) 174.5 (q, CONH, C-5)

$\nu_{max}$ (film)/cm$^{-1}$: 2859 (NH) 1637, 1603 (CO, CN) 1499, 1409 (C-C) 1286 (C-N) 1243, 1167, 1107 (C-N) 886, 826, 756, 702 (N-H, C-H)

HRMS (m/z -ES): Found: 342.1924 (M$^+$ + H. C$_{18}$H$_{24}$N$_5$O$_2$ Requires: 342.1930).

Purity by HPLC: 95.4% ($t_R$ = 21.84)

Dihydrochloride salt of $N$-(4-(4'-guanidinophenyl)phenylmethanone)-4-aminopentanamide (66d)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminobutanamido]diphenylbenzophenone (178.3 mg, 0.273 mmol) was dissolved in 4 M HCl/dioxane (1.23 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.136 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel
Experimental Section

chromatography, eluting with H₂O afforded 66d as a yellow solid (42.5 mg, 44%). Mp: 40-42 °C.

δH (400 MHz, D₂O): 1.59 (broad s, 4H, H-2 and H-3), 2.34 (t, 2H, J 8.0, H-4), 2.91 (t, 2H, J 8.0, H-1), 7.20 (d, 2H, J 8.0, H-7/H-7'), 7.39 (d, 2H, J 8.0, H-8/H-8'), 7.47 (d, 2H, J 8.0, H-12/H-12'), 7.52 (d, 2H, J 8.0, H-13/H-13')

δC (100 MHz, D₂O): 21.4 (CH₂, C-3), 25.4 (CH₂, C-2), 35.8 (CH₂, C-4), 38.3 (CH₂, C-1), 119.6 (CH, Ar., C-7/C-7'), 122.5 (CH, Ar., C-13/C-13'), 131.2 (CH, Ar., C-12/C-12'), 131.3 (CH, Ar., C-8/C-8'), 134.5 (q, Ar., C-9), 136.9 (q, Ar., C-11), 138.6 (q, Ar., C-6), 141.6 (q, Ar., C-14), 155.3 (q, CN), 174.3 (q, CONH, C-5), 196.9 (q, PhCOPh, C-10)

νmax (film)/cm⁻¹: 3310, 3097 (NH) 1674, 1639 (CO, CN) 1592 (C-C) 1407 (C-C) 1311, 1282, 1257 (C-N) 1174, 1151, 1116 (C-N) 852, 835, 758, 767, 679 (N-H, C-H)


Purity by HPLC: 95.5% (tR= 21.33)

Dihydrochloride salt ofN-(4-(4'-guanidinophenyl)phenylamine)-4-aminopentanamid (66e)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminopentanamido]diphenylamine (498.2 mg, 0.78 mmol) was dissolved in 4 M HCl/dioxane (3.51 cm³) under argon. The mixture was diluted with IPA/DCM (1:1) (0.39 cm³) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H₂O afforded 66e as a black solid (10.7 mg, 4%). Mp: 75-77 °C.
\(\delta_H\) (400 MHz, D\(_2\)O): 1.64 (broad s, 4H, H-2 and H-3), 2.39 (t, 2H, J 4.5, H-4), 2.97 (t, 2H, J 4.5, H-1), 7.17-7.22 (m, 6H, H-7/H-7', H-8/H-8' and H-11/H-11'), 7.28 (d, 2H, J 5.6, H-12/H-12')

\(\delta_C\) (100 MHz, D\(_2\)O): 20.9 (CH\(_2\), C-3), 26.0 (CH\(_2\), C-2), 33.1 (CH\(_2\), C-4), 39.0 (CH\(_2\), C-1), 118.4 (CH, Ar., C-7/C-7'), 118.9 (CH, Ar., C-12/C-12'), 124.0 (CH, Ar., C-11/C-11'), 126.9 (CH, Ar., C-8/C-8'), 127.3 (q, Ar., C-9), 127.9 (q, Ar., C-10), 142.6 (q, Ar., C-6), 143.7 (q, Ar., C-13), 155.6 (q, CN), 178.6 (q, CONH, C-5)

\(v_{\text{max}}\) (film)/cm\(^{-1}\): 2958 (NH) 1666, 1606 (CO, CN) 1511 (C-C) 1304 (C-N) 1110 (C-N) 821 (C-H)

HRMS \((m/z\ \text{-ES})\): Found: 341.2095 (M\(^+\) + H. C\(_{18}\)H\(_{25}\)N\(_{6}\)O Requires: 341.2090).

Purity by HPLC: 97.6% \((t_R= 18.73)\)

**Dihydrochloride salt of \(N\)-(4-(4'-guanidinophenyl)phenylmethylene)-4-amino\noctanamide (67a)**

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxy carbonyl)aminoocatanamido]diphenylmethylene (339.1 mg, 0.497 mmol) was dissolved in 4 M HCl/dioxane (2.14 cm\(^3\)) under argon. The mixture was diluted with IPA/DCM (1:1) (0.24 cm\(^3\)) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H\(_2\)O afforded 67a as a yellow oil (183.3 mg, 96%).

\(\delta_H\) (400 MHz, D\(_2\)O): 1.05 (broad s, 6H, H-3—H-5), 1.36 (broad s, 4H, H-2 and H-6), 2.07 (t, 2H, J 7.0, H-7), 2.71 (t, 2H, J 7.0, H-1), 3.50 (s, 2H, H-13), 6.82 (d, 4H, J 7.5, H-10/H-10' and H-16/H-16'), 6.90 (d, 2H, J 7.5, H-11/H-11'), 7.10 (d, 2H, J 7.5, H-15/H-15')

\(\delta_C\) (100 MHz, D\(_2\)O): 25.2 (CH\(_2\), C-6), 25.4 (CH\(_2\), C-3), 26.6 (CH\(_2\), C-4), 27.9 (CH\(_2\), C-5), 28.0 (CH\(_2\), C-7), 36.4 (CH\(_2\), C-2), 39.4 (CH\(_2\), C-13), 40.0 (CH\(_2\), C-1), 121.2 (CH, Ar., C-10/C-10'), 125.5 (CH, Ar., C-16/C-16'), 129.1 (CH, Ar., C-15/C-15'), 130.0 (CH, Ar., C-
Experimental Section

11/C-11\(^{1}\), 131.8 (q, Ar., C-12), 135.3 (q, Ar., C-14), 137.6 (q, Ar., C-9), 141.1 (q, Ar., C-17), 155.9 (q, CN), 175.1 (q, CONH, C-8)

\(\nu_{\text{max}} \text{ (film)/cm}^{-1}\): 3330, 2925, 2852, 2587 (NH) 1726, 1668, 1625, (CO, CN) 1411 (C-C)
1309 (C-N) 1247, 1214, 1184, 1145, 1094, 1021 (C-N) 815, 761, 722 (N-H, C-H)

HRMS (\(m/z\) -ES): Found: 382.2601 (M\(^+\) + H. \(C_{22}H_{32}N_{5}O\) Requires: 382.2607)

Purity by HPLC: 95.7\% (\(t_R=23.24\))

**Dihydrochloride salt of \(N-(4-(4'-\text{guanidinophenyl}) \text{ phenylethylene})-4-\text{amino octanamide (67b)}\)**

Following Method D, 4-[2,3-di(\text{tert-butoxycarbonyl})guanidino]-4'-[4-(\text{tert-butoxy carbonyl})aminoocetanamido]\text{diphenylethylene (86.3 mg, 0.12 mmol) was dissolved in 4 M HCl/dioxane (0.54 cm}^3\) under argon. The mixture was diluted with IPA/DCM (1:1) (0.06 cm}^3\) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H\(_2\)O afforded 67b as a yellow solid (45.9 mg, 97\%). Mp: 143-145 °C.

\(\delta_H\) (400 MHz, \(D_2\)O): 1.18 (broad s, 6H, H-3–H-5), 1.47 (broad s, 4H, H-2 and H-6), 2.19 (t, 2H, J 7.0, H-7), 2.63 (broad s, 4H, H-13 and H-14), 2.82 (t, 2H, J 7.5, H-1), 6.90-6.94 (m, 4H, H-10/H-10' and H-17/H-17') 7.00 (d, 2H, J 7.5, H-11/H-11'), 7.17 (d, 2H, J 8.0, H-16/H-16')

\(\delta_C\) (100 MHz, \(D_2\)O): 25.3 (CH\(_2\), C-6), 25.6 (CH\(_2\), C-3), 26.5 (CH\(_2\), C-4), 28.3 (CH\(_2\), C-5), 28.7 (CH\(_2\), C-7), 28.9 (CH\(_2\), C-2), 33.4 (CH\(_2\), C-13) 36.4 (CH\(_2\), C-14), 39.0 (CH\(_2\), C-1), 118.7 (CH, Ar., C-10/C-10'), 119.1 (CH, Ar., C-17/C-17'), 121.3 (CH, Ar., C-16/C-16'), 126.5 (CH, Ar., C-11/C-11'), 128.3 (q, Ar., C-12), 133.9 (q, Ar., C-15), 151.6 (q, Ar., C-9), 155.5 (q, Ar., C-18), 156.0 (q, CN), 173.3 (q, CONH, C-8)
Experimental Section

\( \nu_{\text{max}} \) (film)/cm\(^{-1}\): 3369, 2728, 2100 (NH) 1659, 1619 (CO, CN) 1468, 1410 (C-C) 1244, 1021 (C-N) 819 (C-H)

HRMS \( m/z \) -ES): Found: 396.2768 (M\(^+\) + H. \( \text{C}_{23}\text{H}_{34}\text{N}_{5}\text{O} \) Requires: 396.2763).

Purity by HPLC: 98.4% (\( t_R = 28.12 \))

Dihydrochloride salt of \( N-(4-(4'-\text{guanidinophenyl})\text{phenylether})-4\)-amino-octanamide \( (67c) \)

Following Method D, 4-[2,3-di(rerr-butoxycarbonyl)guanidino]-4'-[4-(rerr-butoxy carbonyl)aminobutanamido]diphenylethylene (271 mg, 0.42 mmol) was dissolved in 4 M HCl/dioxane (1.89 cm\(^3\)) under argon. The mixture was diluted with IPA/DCM (1:1) (0.21 cm\(^3\)) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H\(_2\)O afforded \( 67c \) a brown solid (158.9 mg, 53%). Mp: 50-52 °C.

\( \delta \)\(_H\) (400 MHz, D\(_2\)O): 1.26 (broad s, 6H, H-3—H-5), 1.55 (m, 4H, H-2 and H-6), 2.30 (t, 2H, J 7.0, H-7), 2.86 (t, 2H, J 7.5, H-1), 6.98-7.02 (m, 4H, H-10/H-10' and H-15/H-15'), 7.21 (d, 2H, J 8.5, H-11/H-11') 7.30 (d, 2H, J 8.5, H-14/H-14')

\( \delta \)\(_C\) (100 MHz, D\(_2\)O): 25.2 (CH\(_2\), C-6), 26.5 (CH\(_2\), C-3), 27.5 (CH\(_2\), C-4), 27.7 (CH\(_2\), C-5), 27.8 (CH\(_2\), C-7), 36.1 (CH\(_2\), C-2), 39.3 (CH\(_2\), C-1), 119.4 (CH, Ar., C-10/C-10'), 119.6 (CH, Ar., C-15/C-15'), 124.0 (CH, Ar., C-14/C-14'), 125.3 (CH, Ar., C-11/C-11'), 128.0 (q, Ar., C-12), 129.1 (q, Ar., C-13), 132.7 (q, Ar., C-9), 153.4 (q, Ar., C-16), 156.5 (q, CN), 175.9 (q, CONH, C-8)

\( \nu_{\text{max}} \) (film)/cm\(^{-1}\): 3262, 2929, 2853, 2031 (NH) 1727, 1659, 1621 (CO, CN) 1585 (C-C) 1499, 1467, 1404 (C-C) 1296 (C-N) 1236, 1224, 1151, 1098 (C-N) 881, 851, 827 (N-H, C-H)
HRMS ($m/z$ -ES): Found: 384.2409 (M$^+$ + H. C$_{21}$H$_{30}$N$_5$O$_2$ Requires: 384.2400).

Purity by HPLC: 98.8% (t$_R$ = 24.07)

**Dihydrochloride salt of N-(4-(4'-guanidinophenyl)phenylamine)-4-aminocatanamide (67e)**

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminoctanamido]diphenylamine (80.5 mg, 0.12 mmol) was dissolved in 4 M HCl/dioxane (0.54 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.06 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 67e a black solid (5.6 mg, 12%). Mp: 52-54 °C.

$\delta$$_H$ (400 MHz, D$_2$O): 1.25 (broad s, 6H, H-3-H-5), 1.52-1.56 (m, 4H, H-2 and H-6), 2.28 (t, 2H, J 8.0, H-7), 2.85 (t, 2H, J 8.0, H-1), 7.01-7.05 (m, 4H, H-10/H-10' and H-15/H-15'), 7.09 (d, 2H, J 8.5, H-11/H-11'), 7.21 (d, 4H, J 8.5, H-14/H-14')

$\delta$$_C$ (150 MHz, D$_2$O): 25.0 (CH$_2$, C-6), 25.2 (CH$_2$, C-3), 26.5 (CH$_2$, C-4), 27.6 (CH$_2$, C-5), 30.1 (CH$_2$, C-2), 36.1 (CH$_2$, C-7), 39.3 (CH$_2$, C-1), 117.8 (CH, Ar., C-10/C-10'), 119.2 (CH, Ar., C-15/C-15'), 123.8 (CH, Ar., C-14/C-14'), 126.1 (CH, Ar., C-11/C-11'), 127.8 (q, Ar., C-12), 130.5 (q, Ar., C-13), 140.2 (q, Ar., C-9), 143.7 (q, Ar., C-16), 156.7 (q, CN), 176.0 (q, CONH, C-8)

$\nu$$_{max}$ (film)/cm$^{-1}$: 3315, 2931 (NH) 1726, 1667 (CO, CN) 1412 (C-C) 1318 (C-N) 1227, 1153, 1095, 1057 (C-N) 817, 695 (N-H, C-H)


Purity by HPLC: 95.1% (t$_R$ = 22.48)
Dihydrochloride salt of $N'$-(4-(4'-guanidinophenyl)phenylmethylene)-4-amino undecanamide (68a)

Following Method D, the 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminoundecanamido]diphenylmethylene (452.1 mg, 0.624 mmol) was dissolved in 4 M HCl/dioxane (2.81 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.31 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 68a as a white solid (152.5 mg, 58%). Mp: 80-82 °C.

$\delta$$_H$ (400 MHz, D$_2$O): 1.17 (broad s, 12H, H-3–H-8), 1.46-1.56 (m, 4H, H-2 and H-9), 2.27 (t, 2H, J 8.0, H-10), 2.83 (t, 2H, J 8.0, H-1), 3.88 (s, 2H, H-16), 7.13 (d, 2H, J 8.5, H-13/H-13’), 7.18 (d, 2H, J 8.5, H-14/H-14’), 7.21-7.25 (m, 4H, H-18/H-18’ and H-19/H-19’)

$\delta$$_C$ (100 MHz, D$_2$O): 25.1 (CH$_2$, C-9), 25.3 (CH$_2$, C-3), 26.5 (CH$_2$, C-8), 27.9 (CH$_2$, C-7), 28.0 (CH$_2$, C-4), 28.1 (CH$_2$, C-5), 28.2 (CH$_2$, C-6), 28.2 (CH$_2$, C-10), 36.3 (CH$_2$, C-2), 39.4 (CH$_2$, C-16), 40.0 (CH$_2$, C-1), 122.3 (CH, Ar., C-13/C-13’), 126.2 (CH, Ar., C-19/C-19’), 129.2 (CH, Ar., C-18/C-18’), 130.0 (CH, Ar., C-14/C-14’), 132.0 (q, Ar., C-15), 134.9 (q, Ar., C-17), 138.5 (q, Ar., C-12), 141.6 (q, Ar., C-20), 156.3 (q, CN), 176.1 (q, CONH, C-11)

$\nu$$_{max}$ (film)/cm$^{-1}$: 3360, 2924 (NH) 2472, 1637 (CN, CO) 1582 (C-C) 1441 (C-C) 1206 (C-N) 1099 (C-N)

HRMS (m/z -ES): Found: 424.3073 (M$^+$ + H. C$_{25}$H$_{38}$N$_5$O Requires: 424.3076).

Purity by HPLC: 95.5% (t$_R$=27.69)
Dihydrochloride salt of \( N-(4-(4'\text{-guanidinophenyI})\text{phenylethylene})-4\text{-amino undecanamide (68b)} \)

Following Method D, 4-[2,3-di(\text{\textit{tert}-butoxycarbonyl})guanidino]-4'-[4-(\text{\textit{tert}-butoxy carbonyl})aminoundecanamido]diphenylethylene (416.6 mg, 0.564 mmol) was dissolved in 4 M HCl/dioxane (2.54 cm\(^3\)) under argon. The mixture was diluted with IPA/DCM (1:1) (0.28 cm\(^3\)) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H\(_2\)O afforded 68b as a yellow solid (222.3 mg, 90%). Mp: 128-130 °C.

\[ \delta_H (400 \text{ MHz, } D_2O): 1.17 (\text{ broad s, 12H, H-}3\text{-H-8}), 1.51-1.55 (\text{ m, 4H, H-2 and H-9}), 2.26 (\text{ t, 2H, J 8.0, H-10}), 2.85 (\text{ broad s, 6H, H-1, H-16 and H-17}), 7.07 (\text{ d, 2H, J 8.0, H-13/H-13'}), 7.11 (\text{ d, 2H, J 8.0, H-14/H-14'}), 7.18 (\text{ d, 4H, J 7.5, H-19/H-19'} and H-20/H-20') \]

\[ \delta_C (100 \text{ MHz, } D_2O): 25.3 (\text{CH}_2, \text{C-9}), 25.5 (\text{CH}_2, \text{C-3}), 26.6 (\text{CH}_2, \text{C-8}), 28.0 (\text{CH}_2, \text{C-7}), 28.1 (\text{CH}_2, \text{C-4}), 28.2 (\text{CH}_2, \text{C-5}), 28.3 (\text{CH}_2, \text{C-6}), 28.4 (\text{CH}_2, \text{C-10}), 35.9 (\text{CH}_3, \text{C-2}), 36.0 (\text{CH}_2, \text{C-16}), 36.4 (\text{CH}_2, \text{C-17}), 39.5 (\text{CH}_2, \text{C-1}), 122.1 (\text{CH, Ar.}, \text{C-13/C-13'}), 125.9 (\text{CH, Ar.}, \text{C-20/C-20'}), 129.2 (\text{CH, Ar.}, \text{C-19/C-19'}), 130.1 (\text{CH, Ar.}, \text{C-14/C-14'}), 131.8 (\text{q, Ar.}, \text{C-15}), 134.6 (\text{q, Ar.}, \text{C-18}), 138.9 (\text{q, Ar.}, \text{C-12}), 141.8 (\text{q, Ar.}, \text{C-21}), 160.4 (\text{q, CN}), 176.2 (\text{q, CONH, C-11}) \]

\[ \nu_{\text{max}} (\text{film})/\text{cm}^-1: 3299, 2920, 2850 (\text{NH}) 2464, 1651 (\text{CN, CO}) 1592 (\text{C-C}) 1470, 1409 (\text{C-C}) 1300, 1252 (\text{C-N}) 1184, 1093, 1021 (\text{C-N}) 966, 819 (\text{N-H, C-H}) \]

HRMS (m/z -ES): Found: 438.3226 (M\(^+\) + H. C\(_{26}\)H\(_{40}\)N\(_5\)O Requires: 438.3233)

Purity by HPLC: 99.0% (t\(_R\) = 27.83)
Dihydrochloride salt of \(N-(4-(4'-\text{guanidinophenyl})\text{phenylether})-4\text{-amino undecanamide (68c)}\)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminoundecamido]diphenylether (100 mg, 0.14 mmol) was dissolved in 4 M HCl/dioxane (0.63 cm\(^3\)) under argon. The mixture was diluted with IPA/DCM (1:1) (0.07 cm\(^3\)) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with \(\text{H}_2\text{O}\) afforded \(68c\) a yellow solid (59.5 mg, 99%). Mp: 89-92 °C.

\(\delta_H\) (400 MHz, D\(_2\)O): 1.18 (broad s, 12H, H-3-H-8), 1.51-1.56 (m, 4H, H-2 and H-9), 2.29 (t, 2H, J 8.0, H-10), 2.84 (t, 2H, J 7.5, H-1), 6.96-7.00 (m, 4H, H-13/H-13' and H-14/H-14’) 7.20 (d, 2H, J 8.5, H-17/H-17’), 7.30 (d, 2H, J 8.0, H-18/H-18’)

\(\delta_C\) (100 MHz, D\(_2\)O): 25.6 (CH\(_2\), C-9), 25.8 (CH\(_2\), C-3), 26.8 (CH\(_2\), C-8), 28.5 (CH\(_2\), C-7), 28.6 (CH\(_2\), C-4), 28.8 (2CH\(_2\), C-5 and C-6), 28.8 (CH\(_2\), C-10), 36.7 (CH\(_2\), C-2), 39.5 (CH\(_2\), C-1), 119.2 (CH, Ar., C-13/C-13’), 119.6 (CH, Ar., C-18/C-18’), 122.6 (CH, Ar., C-17/C-17’), 127.6 (CH, Ar., C-14/C-14’), 129.0 (q, Ar., C-15), 133.7 (q, Ar., C-16) 152.6 (q, Ar., C-12) 156.3 (q, Ar., C-19) 156.5 (q, CN), 174.7 (q, CONH, C-11)

\(\nu_{max}\) (film)/cm\(^{-1}\): 3327, 2922 (NH) 2164, 1643 (CN, CO) 1501, 1439, 1403 (C-C) 1235, 1165, 1100 (C-N) 870, 723 (N-H, C-H)

HRMS (m/z -ES): Found: 426.2860 (M\(^+\) + H. C\(_{24}\)H\(_{36}\)N\(_5\)O\(_2\) Requires: 426.2869)

Purity by HPLC: 97.8% (t\(_R\) = 27.47)

Dihydrochloride salt of \(N-(4-(4'-\text{guanidinophenyl})\text{phenylmethanone})-4\text{-amino undecanamide (68d)}\)
Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxy carbonyl)aminoundecanamido]diphenylbenzophenone (720.8 mg, 0.98 mmol) was dissolved in 4 M HCl/dioxane (4.41 cm³) under argon. The mixture was diluted with IPA/DCM (1:1) (0.49 cm³) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H₂O afforded 68d as an orange solid (339.5 mg, 79%). Mp: 71-73 °C.

δ₁ (400 MHz, D₂O): 1.15 (broad s, 12H, H-3-H-8), 1.48-1.53 (m, 4H, H-2 and H-9), 2.30 (t, 2H, J 8.0, H-10), 2.83 (t, 2H, J 8.0, H-1), 7.32 (d, 2H, J 8.0, H-13/H-13’), 7.49 (d, 2H, J 8.5, H-18/H-18’), 7.64 (d, 2H, J 8.5, H-19/H-19’), 7.68 (d, 2H, J 8.0, H-14/H-14’)

δC (100 MHz, D₂O): 25.0 (CH₂, C-9), 25.4 (CH₂, C-3), 26.5 (CH₂, C-8), 27.9 (CH₂,C-7), 28.0 (CH₂, C-4), 28.2 (CH₂, C-5), 28.3 (CH₂, C-6), 28.3 (CH₂, C-2), 36.6 (CH₂, C-10), 39.4 (CH₂, C-1), 119.9 (CH, Ar., C-13/C-13’), 123.9 (CH, Ar., C-19/C-19’), 131.7 (CH, Ar., C-18/C-18’), 131.7 (CH, Ar., C-14/C-14’), 132.1 (q, Ar., C-15), 135.0 (q, Ar., C-17), 138.9 (q, Ar., C-12), 142.1 (q, Ar., C-20), 155.7 (q, CN), 176.1 (q, CONH, C-11), 197.9 (q, PhCOPh, C-16)

νₘₐₓ (film)/cm⁻¹: 3027, 2849, 2917 (NH) 1724, 1644 (CO, CN) 1598 (C-C) 1469, 1406 (C-C) 1312, 1284, 1257 (C-N) 1225, 1175, 1150, 1100 (C-N) 835, 758 (N-H, C-H)


Purity by HPLC: 95.1% (tᵣ= 26.89)

Dihydrochloride salt of N-(4-(4’-guanidinophenyl)phenylamino)-4-amino undecanamide (68e)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(tert-butoxy carbonyl)aminoundecanamido]diphenylamine (482.8 mg, 0.67 mmol) was dissolved in 4 M HCl/dioxane (3 cm³) under argon. The mixture was diluted with IPA/DCM (1:1) (0.35 cm³) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H₂O afforded 68e as an orange solid (339.5 mg, 79%). Mp: 71-73 °C.

δ₁ (400 MHz, D₂O): 1.15 (broad s, 12H, H-3-H-8), 1.48-1.53 (m, 4H, H-2 and H-9), 2.30 (t, 2H, J 8.0, H-10), 2.83 (t, 2H, J 8.0, H-1), 7.32 (d, 2H, J 8.0, H-13/H-13’), 7.49 (d, 2H, J 8.5, H-18/H-18’), 7.64 (d, 2H, J 8.5, H-19/H-19’), 7.68 (d, 2H, J 8.0, H-14/H-14’)

δC (100 MHz, D₂O): 25.0 (CH₂, C-9), 25.4 (CH₂, C-3), 26.5 (CH₂, C-8), 27.9 (CH₂,C-7), 28.0 (CH₂, C-4), 28.2 (CH₂, C-5), 28.3 (CH₂, C-6), 28.3 (CH₂, C-2), 36.6 (CH₂, C-10), 39.4 (CH₂, C-1), 119.9 (CH, Ar., C-13/C-13’), 123.9 (CH, Ar., C-19/C-19’), 131.7 (CH, Ar., C-18/C-18’), 131.7 (CH, Ar., C-14/C-14’), 132.1 (q, Ar., C-15), 135.0 (q, Ar., C-17), 138.9 (q, Ar., C-12), 142.1 (q, Ar., C-20), 155.7 (q, CN), 176.1 (q, CONH, C-11), 197.9 (q, PhCOPh, C-16)

νₘₐₓ (film)/cm⁻¹: 3027, 2849, 2917 (NH) 1724, 1644 (CO, CN) 1598 (C-C) 1469, 1406 (C-C) 1312, 1284, 1257 (C-N) 1225, 1175, 1150, 1100 (C-N) 835, 758 (N-H, C-H)


Purity by HPLC: 95.1% (tᵣ= 26.89)
cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded $68e$ as a black oil (81.6 mg, 29%).

$\delta_H$ (600 MHz, D$_2$O): 1.25 (broad s, 12H, H-3–H-8), 1.55-1.66 (m, 4H, H-2 and H-9), 2.35 (t, 2H, J 6.4, H-10), 2.91 (t, 2H, J 8.0, H-1), 7.11 (app t, 4H, H-13/H-13' and H-18/H-18'), 7.17 (d, 2H, J 5.7, H-14/H-14'), 7.28 (d, 2H, J 5.7, H-17/H-17')

$\delta_C$ (150 MHz, D$_2$O): 25.3 (CH$_2$, C-9), 26.5 (CH$_2$, C-3), 27.9 (CH$_2$, C-8), 28.0 (CH$_2$, C-7), 28.1 (CH$_2$, C-4), 28.2 (CH$_2$, C-5), 28.2 (CH$_2$, C-6), 34.2 (CH$_2$, C-2), 36.2 (CH$_2$, C-10), 39.4 (CH$_2$, C-1), 117.8 (CH, Ar., C-13/C-13'), 119.2 (CH, Ar., C-18/C-18'), 123.7 (CH, Ar., C-17/C-17'), 126.1 (q, Ar., C-15), 127.7 (CH, Ar., C-14/C-14'), 130.6 (q, Ar., C-16), 140.1 (q, Ar., C-12), 143.7 (q, Ar., C-19), 156.6 (q, CN), 176.0 (q, CONH, C-11)

$\nu_{max}$ (film)/cm$^{-1}$: 2927, 2533, 2160, 2030 (NH), 1977, 1625 (CO, CN), 1497 (C-C), 1225 (C-O), 1201, 1049 (C-N), 824, 773 (N-H, C-H)

HRMS (m/z -ES): Found: 425.3033 (M$^+$ + H. C$_{24}$H$_{37}$N$_6$O Requires: 425.3029).

Purity by HPLC: 98.2% ($t_R= 25.77$)

Dihydrochloride salt of N-(4-(4'-guanidinophenyl)phenylmethylene)-4-aminododecanamide (69a)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminododecanamido]diphenylmethylene (694.7 mg, 0.94 mmol) was dissolved in 4 M HCl/dioxane (4.23 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.47 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded $69a$ as a white solid (224.6 mg, 55%). Mp: 114-116 °C.

$\delta_H$ (400 MHz, D$_2$O): 1.14 (broad s, 14H, H-3–H-9), 1.47-1.55 (m, 4H, H-2 and H-10), 2.26 (t, 2H, J 7.0, H-11), 2.82 (t, 2H, J 7.0, H-1), 3.87 (s, 2H, H-17), 7.12 (d, 2H, J 8.0, H-
Experimental Section

14/H-14'), 7.17 (d, 2H, J 8.5, H-19/H-19'), 7.21-7.25 (m, 4H, H-15/H-15' and H-20/H-20')

δ_C (150 MHz, D_2O): 25.1 (CH_2, C-10), 25.4 (CH_2, C-3), 26.5 (CH_2, C-9), 28.0 (CH_2, C-8), 28.0 (CH_2, C-4), 28.1 (CH_2, C-5), 28.2 (CH_2, C-6) 28.3 (CH_2, C-7), 28.3 (CH_2, C-11), 36.3 (CH_2, C-2), 39.4 (CH_2, C-17), 40.0 (CH_2, C-1) 122.2 (CH, Ar., C-14/C-14), 126.2 (CH, Ar., C-20/C-20'), 129.2 (CH, Ar., C-19/C-19'), 130.0 (CH, Ar., C-15/C-15'), 132.0 (q, Ar., C-16), 134.9 (q, Ar., C-18), 138.4 (q, CH., C-13), 141.6 (q, Ar., C-21), 156.3 (q, CN), 176.1 (q, CONH, C-12)

ν_max (film)/cm⁻¹: 3291, 3144, 2996, 2918, 2849 (NH) 1683, 1656, (CO, CN) 1594 (C-C) 1469, 1408 (C-C) 1305, 1250 (C-N) 1208, 1181, 1104, 1038 (C-N) 856, 815, 772, 719 (N-H, C-H)

HRMS (m/z -ES): Found: 438.3241(M⁺ + H. C_{26}H_{40}N_{5}O Requires: 438.3233).

Purity by HPLC: 96.2% (t_R= 28.68)

Dihydrochloride salt of \(N-(4-(4'-guanidinophenyl)phenylethylene)-4-aminododecanamide\) (69b)

Following Method D, \(4-[2,3-di(\text{t}ert-\text{butoxycarbonyl})\text{guanidino}]-4'-[4-(\text{t}ert-\text{butoxy carbonyl})\text{aminododecanamido}]\text{diphenylethylene}\) (447 mg, 0.59 mmol) was dissolved in 4 M HCl/dioxane (2.65 cm³) under argon. The mixture was diluted with IPA/DCM (1:1) (0.30 cm³) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H_2O afforded \(69b\) as a white solid (265.4 mg, 99%) Mp: 116-117 °C.

δ_H (400 MHz, D_2O): 1.15 (broad s, 14H, H-3—H-9), 1.50-1.54 (m, 4H, H-2 and H-10), 2.26 (t, 2H, J 7.0, H-11), 2.84 (broad s, 6H, H-1, H-17 and H-18), 7.05-7.10 (m, 4H, H-14/H-14' and H-21/H-21), 7.17 (d, 4H, J 7.0, H-15/H-15' and H-20/H-20')
Experimental Section

$\delta_C$ (100 MHz, D$_2$O): 25.4 (CH$_2$, C-10), 25.6 (CH$_2$, C-3), 26.8 (CH$_2$, C-9), 28.2 (CH$_2$, C-8), 28.3 (CH$_2$, C-4), 28.5 (3CH$_2$, C-5, C-6 and C-7), 30.2 (CH$_2$, C-11), 35.9 (CH$_2$, C-2), 36.1 (CH$_2$, C-17), 36.4 (CH$_2$, C-18), 39.7 (CH$_2$, C-1), 122.0 (CH, Ar., C-14/C-14'), 125.9 (CH, Ar., C-21/C-21'), 129.2 (CH, Ar., C-20/C-20'), 130.1 (CH, Ar., C-15/C-15'), 131.9 (q, Ar., C-16), 134.8 (q, Ar., C-19), 138.8 (q, CH, C-13), 141.8 (q, Ar., C-22), 156.5 (q, CN), 176.1 (q, CONH, C-12)

$\nu_{max}$ (film)/cm$^{-1}$: 3476, 2921 (NH) 2499, 1655 (CN, CO) 1470, 1408 (C-C) 1204, (C-N) 865, 816, 793 (N-H, C-H)

HRMS (m/z -ES): Found: 452.3384 (M$^+$ + H, C$_{27}$H$_{42}$N$_5$O Requires: 452.3389).

Purity by HPLC: 96.7% ($t_R =$ 28.93)

Dihydrochloride salt of $N$-(4-(4'-guanidinophenyl)phenylether)-4-amino dodecanamide (69c)

Following Method D, 4-[2,3-di(D-tert-butoxycarbonyl)guanidino]-4'-[4-(D-tert-butoxy carbonyl)aminododecanamido]diphenylether (304.9 mg, 0.41 mmol) was dissolved in 4 M HCl/dioxane (1.85 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.2 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 69c as a yellow solid (64.2 mg, 36%). Mp: 78-80 °C.

$\delta_H$ (400 MHz, D$_2$O): 1.14 (broad s, 14H, H-3—H-9), 1.48-1.54 (m, 4H, H-2 and H-10), 2.27 (t, 2H, J 8.0, H-11), 2.83 (t, 2H, J 8.0, H-1), 6.94-6.98 (m, 4H, H-14/H-14' and H-19/H-19'), 7.19 (d, 2H, J 9.0, H-15/H-15'), 7.30 (d, 2H, J 8.5, H-18/H-18')

$\delta_C$ (150 MHz, D$_2$O): 25.2 (CH$_2$, C-10), 25.4 (CH$_2$, C-3), 26.5 (CH$_2$, C-9), 28.0 (CH$_2$, C-8), 28.2 (CH$_2$, C-4), 28.3 (3CH$_2$, C-5, C-6 and C-7), 28.4 (CH$_2$, C-11), 36.2 (CH$_2$, C-2), 39.4 (CH$_2$, C-1) 119.4 (CH, Ar., C-14/C-14'), 119.7 (CH, Ar., C-19/C-19'), 123.8 (CH, Ar., C-
Experimental Section

18/C-18'), 128.0 (CH, Ar., C-15/C-15'), 129.1 (q, Ar., C-16), 132.8 (q, Ar., C-17), 153.3 (q, Ar., C-13), 156.5 (q, Ar., C-20), 156.5 (q, CN), 176.0 (q, CONH, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3296, 2917 (NH) 2850, 1649 (CN, CO) 1467, 1408 (C-C) 1232, 1167, 1102 (C-N) 963, 826 (N-H, C-H)

HRMS (m/z -ES): Found: 440.3032 (M$^+ + H$). C$_{25}$H$_{38}$N$_5$O$_2$ Requires: 440.3026.

Purity by HPLC: 97.5% ($t_R=27.91$).

Dihydrochloride salt of N-(4-(4'-guanidinophenyl)phenylmethanone)-4-amino dodecanamide (69d)

Following Method D, 4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminododecanamido]diphenylbenzophenone (303.1 mg, 0.4 mmol) was dissolved in 4 M HCl/dioxane (1.8 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (0.2 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 69d as a yellow solid (46.4 mg, 26%). Mp: 60-62 °C.

$\delta_H$ (400 MHz, D$_2$O): 1.20 (broad s, 14H, H-3--H-9), 1.55-1.62 (m, 4H, H-2 and H-10), 2.38 (t, 2H, J 4.9, H-11), 2.90 (t, 2H, J 4.9, H-1), 7.40 (d, 2H, J 5.8, H-14/H-14'), 7.58 (d, 2H, J 5.8, H-15/H-15'), 7.73 (d, 2H, J 5.8, H-19/H-19'), 7.77 (d, 2H, J 5.8, H-20/H-20')

$\delta_C$ (100 MHz, D$_2$O): 25.0 (CH$_2$, C-10), 25.4 (CH$_2$, C-3), 26.5 (CH$_2$, C-9), 28.0 (CH$_2$, C-8), 28.0 (CH$_2$, C-4), 28.2 (CH$_2$, C-5), 28.3 (CH$_2$, C-6), 28.4 (CH$_2$, C-7), 28.4 (CH$_2$, C-11), 36.6 (CH$_2$, C-2), 39.4 (CH$_2$, C-1), 120.0 (CH, Ar., C-14/C-14'), 123.9 (CH, Ar., C-20/C-20'), 131.7 (CH, Ar., C-19/C-19'), 131.7 (CH, Ar., C-15/C-15'), 132.2 (q, Ar., C-16), 135.1 (q, Ar., C-18), 138.9 (q, CH, C-13), 142.1 (q, Ar., C-21), 155.7 (q, CN), 176.2 (q, CONH, C-12), 198.0 (q, PhCOPh, C-17)
Experimental Section

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3374, 2916, 2850 (NH) 2478, 1649 (CN, CO) 1601 (C-C) 1443 (C-C) 1315 (C-N) 1209 (C-N) 934, 766 (N-H, C-H)

HRMS ($m/z$ -ES): Found: 452.3032 (M$^+$ + H. C$_{26}$H$_{38}$N$_{5}$O$_{2}$ Requires: 452.3026).

Purity by HPLC: 95.3% ($t_R$ = 28.04)

**Dihydrochloride salt of $N$-(4-((4'-guanidinophenyl)phenylamine)-4-amino dodecanamide (69e)**

Following Method D, 4-[[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(tert-butoxy carbonyl)aminododecanoicamido]diphenylamine (162.7 mg, 0.22 mmol) was dissolved in 4 M HCl/dioxane (0.99 cm$^3$) under argon. The mixture was diluted with IPA/DCM (1:1) (1.11 cm$^3$) and stirred for 3-4 hours. Usual work-up followed by reverse phase silica gel chromatography, eluting with H$_2$O afforded 69e as a black oil (29.3 mg, 10%).

$\delta H$ (400 MHz, CDCl$_3$): 1.22 (broad s, 14H, H-3–H-9), 1.52-1.63 (m, 4H, H-2 and H-10), 2.33 (t, 2H, J 8.0, H-11), 2.89 (t, 2H, J 8.0, H-1), 7.11 (m, 4H, H-14/H-14' and H-19/H-19'), 7.16 (d, 2H, J 8.8, H-15/H-15'), 7.28 (d, 2H, J 8.8, H-18/H-18')

$\delta C$ (150 MHz, CDCl$_3$): 25.3 (CH$_2$, C-10), 25.5 (CH$_2$, C-3), 26.7 (CH$_2$, C-9), 28.1 (CH$_2$, C-8), 28.1 (CH$_2$, C-4), 28.2 (CH$_2$, C-5), 28.3 (CH$_2$, C-6), 28.4 (CH$_2$, C-7), 28.5 (CH$_2$, C-2), 36.3 (CH$_2$, C-11), 39.5 (CH$_2$, C-1), 117.9 (CH, Ar., C-14/C-14'), 119.3 (CH, Ar., C-19/C-19'), 123.8 (CH, Ar., C-18/C-18'), 126.2 (CH, Ar., C-15/C-15'), 127.9 (q, Ar., C-16), 130.7 (q, Ar., C-17), 140.3 (q, Ar., C-13), 143.9 (q, Ar., C-20), 156.7 (q, CN), 176.2 (q, CONH, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3128, 3017, 2971 (NH), 1739, 1665 (CN, CO), 1649, 1606 (N-H), 1587 (C-C), 1487, 1435, 1422, (C-C) 1366 (C-H), 1296, 1279 (C-N), 1230, 1217, 1149, 1138, 1110, 1092, 1053, 1032 (C-O) (C-N), 1009 (C-O), 949, 920, 908, 891, 870, 810, 762, 740, 671 (N-H, C-H)
Experimental Section


Purity by HPLC: 97.1% (tᵣ = 27.24)

4-Aminobutyric acid methyl ester (79a)

Following Method E, 79a was obtained as a white solid (283 mg, 83%). Mp: 73-75 °C (literature: 73-75 °C).

δ_H (400 MHz, CDCl₃): 1.77-1.84 (m, 2H, CH₂, H-2), 2.44 (t, 2H, J 8.0, H-3) 2.75-2.83 (m, 2H, H-1), 3.41 (s, 3H, CH₃), 7.99 (br s, 2H, NH₂)

HRMS (m/z -ES): Found: 118.0869 (M⁺ + H. C₅H₁₂NO₂ Requires: 118.0868).

5-Aminopentanoic acid methyl ester (79b)

Following Method E, 79b was obtained as a white solid (292 mg, 87%). Mp: 120-122 °C (no literature value available).

δ_H (400 MHz, DMSO): 1.54-1.58 (m, 4H, H-2 and H-3), 2.34 (t, 2H, J 6.5, H-4), 2.76 (t, 2H, J 6.5, H-1), 3.38 (s, 3H, CH₃), 8.04 (s, 2H, NH₂)


8-Aminoocctanoic acid methyl ester (79c)

Following Method E, 79c was obtained as a white solid (229 mg, 66%). Mp: 129 °C (literature: 129 °C).
Experimental Section

11-Aminoundecanoic acid methyl ester (79d)

Following Method E, 79d was obtained as a white solid (145 mg, 67%). Mp: 60-65 °C (no literature value available).

δ\textsubscript{H} (400 MHz, CDCl\textsubscript{3}): 1.21 (broad s, 12H, H-3–H-8), 1.45-1.54 (m, 4H, H-2 and H-9), 2.26 (t, 2H, J 7.4, H-10), 2.86 (t, 2H, J 7.4, H-1), 3.56 (s, 3H, CH\textsubscript{3})

HRMS (m/z -ES): Found: 216.1974 (M\textsuperscript{+} H. C\textsubscript{12}H\textsubscript{26}N\textsubscript{2}O\textsubscript{2} Requires: 216.1964).

12-Aminododecanoic acid methyl ester (79e)

Following Method E, 79e was obtained as a white solid (217 mg, 95%) Mp: 30-32 °C (no literature value available).

δ\textsubscript{H} (400 MHz, DMSO): 1.09 (broad s, 14H, H-3–H-9), 1.36-1.41 (m, 4H, H-2 and H-10), 2.13 (t, J 7.3, 2H, H-11), 2.66 (t, 2H, J 6.5, H-1), 3.43 (s, 3H, CH\textsubscript{3}), 7.49 (s, 2H, NH\textsubscript{2})

HRMS (m/z -ES): Found: 230.2113 (M\textsuperscript{+} H. C\textsubscript{13}H\textsubscript{28}N\textsubscript{2}O\textsubscript{2} Requires: 230.2120).

4-(Acridine-9-carboxyamido)butyric acid methyl ester (82a)
Following Method F and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 82a was obtained as a yellow solid (209 mg, 65%). Mp: 55–57 °C.

\[ \delta_H (400 \text{ MHz}, \text{CDCl}_3): 2.17 (q, 2H, J 7.1, H-10), 2.57 (t, 2H, J 7.1, H-9), 3.68 (s, 3H, CH\_3), 3.81 (q, 2H, J 7.1, H-11), 6.55 (br s, 1H, NH), 7.58 (dt, 2H, J 1.3, 6.5, H-4/H-4'), 7.81 (dt, 2H, J 1.3, 6.5, H-3/H-3'), 8.01 (d, 2H, J 8.8, H-2/H-2'), 8.21 (d, 2H, J 8.8, H-5/H-5') \]

\[ \delta_C (100 \text{ MHz}, \text{CDCl}_3): 24.7 (\text{CH}_2, C-10), 31.6 (\text{CH}_2, C-11), 39.7 (\text{CH}_2, C-9), 51.9 (\text{CH}_3), 122.2 (\text{q, Ar., C-6/C-6}'), 125.1 (\text{CH, Ar., C-5/C-5}'), 126.9 (\text{CH, Ar., C-3/C-3}'), 129.7 (\text{CH, Ar., C-4/C-4}'), 130.5 (\text{CH, Ar., C-2/C-2}'), 140.9 (\text{q, Ar., C-7}), 148.6 (\text{q, Ar., C-1/C-1}'), 167.2 (\text{q, CONH, C-8}), 173.6 (\text{q, COOMe, C-12}) \]

\[ \nu_{\text{max}} \text{ (film)/cm}^{-1}: 3428, 2927 \text{ (NH)}, 1729, 1629, 1084 \text{ (COO)} \]

HRMS (m/z -ES): Found: 323.1393 (M\(^+\) +H. C\(_{19}\)H\(_{19}\)N\(_2\)O\(_3\) Requires: 323.1396).

5-(Acridine-9-carboxyamido)pentanoic acid methyl ester (82b)

Following Method F and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 82b was obtained as a yellow solid (168 mg, 50%). Mp: 65-67 °C.

\[ \delta_H (400 \text{ MHz}, \text{CDCl}_3): 1.85 (q, 4H, H-10 and H-11), 2.46-2.50 (m, 2H, H-9), 3.73 (s, 3H, CH\_3), 3.71-3.78 (m, 2H, H-12), 6.73 (s, 1H, NH), 7.50 (dt, 2H, J 1.5, 7.5, H-4/H-4'), 7.71 (dt, 2H, J 1.5, 7.5, H-3/H-3'), 8.01 (d, 2H, J 8.8, H-2/H-2'), 8.10 (d, 2H, J 8.8, H-5/H-5'). \]

\[ \delta_C (100 \text{ MHz}, \text{CDCl}_3): 22.2 (\text{CH}_2, C-11), 29.1 (\text{CH}_2, C-10), 33.4 (\text{CH}_2, C-9), 39.9 (\text{CH}_2, C-12), 51.7 (\text{CH}_3), 122.2 (\text{q, Ar., C-6/C-6}'), 125.4 (\text{CH, Ar., C-5/C-5}'), 127.2 (\text{CH, Ar., C-3/C-3}'), 128.3 (\text{CH, Ar., C-4/C-4}'), 131.4 (\text{CH, Ar., C-2/C-2}'), 142.6 (\text{q, Ar., C-7}), 147.4 (\text{q, Ar., C-1/C-1}'), 166.7 (\text{q, CONH, C-8}), 173.8 (\text{q, COOMe, C-13}) \]
Experimental Section

\[ \nu_{\text{max}} \text{ (film)} / \text{cm}^{-1}: 3428, 2928 \text{ (NH)}, 1738, 1627, 1166 \text{ (COO)} \]

HRMS \((m/z - \text{ES})\): Found: 337.1553 \((\text{M}^+ + \text{H})\). \(\text{C}_{20}\text{H}_{21}\text{N}_{2}\text{O}_{3}\) Requires: 337.1552.

\[ \text{8-(Acridine-9-carboxyamido)octanoic acid methyl ester (82c)} \]

Following Method F and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), \(82c\) was obtained as a yellow solid (528.9 mg, 46%).

Mp: 65-67 °C.

\[ \delta_H \text{ (400 MHz, CDCl}_3\text{: )} \]
\[ 1.36-1.57 \text{ (m, 6H, H-11-H-13), 1.61-1.71 \text{ (m, 2H, H-10), 1.77-1.84} \text{ (m, 2H, H-14), 2.34 \text{ (t, 2H, J 8.0, H-9), 3.67 \text{ (s, 3H, CH}_3\text{), 3.72 \text{ (q, 2H, J 8.0, H-15), 6.79} \text{ (s, 1H, NH), 7.48 \text{ (dt, 2H, J 1.0, 6.5, H-4/H-4'), 7.72 \text{ (dt, 2H, J 1.0, 6.5, H-3/H-3'), 7.95 \text{ (d, 2H, J 8.8, H-2/H-2'), 8.10 \text{ (d, 2H, J 8.8, H-5/H-5')}}} \text{) \}} \text{) \}} \]

\[ \delta_C \text{ (100 MHz, CDCl}_3\text{: )} \]
\[ 24.8 \text{ (CH}_2\text{, C-14), 26.9 \text{ (CH}_2\text{, C-11), 28.9 \text{ (CH}_2\text{, C-12), 29.0 \text{ (CH}_2\text{, C-13), 29.6 \text{ (CH}_2\text{, C-10), 34.0 \text{ (CH}_2\text{, C-9), 40.2 \text{ (CH}_2\text{, C-15), 51.5 \text{ (q, CH}_3\text{), 122.1 \text{ (q, Ar., C-6/C-6'), 125.3 \text{ (CH, Ar., C-5/C-5'), 126.8 \text{ (CH, Ar., C-3/C-3'), 129.2 \text{ (CH, Ar., C-4/C-4'), 130.5 \text{ (CH, Ar., C-2/C-2'), 141.4 \text{ (q, Ar., C-7), 148.2 \text{ (q, Ar., C-1/C-1'), 166.9 \text{ (q, CONH, C-8), 174.2 \text{ (q, COOME, C-16) \}} \text{) \}} \text{) \}} \text{) \}} \text{) \}} \]

\[ \nu_{\text{max}} \text{ (film)} / \text{cm}^{-1}: 3215, 3017 \text{ (NH), 2971, 2943, 2861 (C-H), 1737 (C-O), 1664, 1637 (N-H), 1366 (C-H), 1230, 1217, 1193, 1171, 1140, 1093, 1053, 1030 (N-H), 1009, 982, 907, 844, 796, 751, 724 (N-H) \]

HRMS \((m/z - \text{ES})\): Found: 379.2020 \((\text{M}^+ + \text{H})\). \(\text{C}_{23}\text{H}_{27}\text{N}_{2}\text{O}_{3}\) Requires: 379.2022.
11-(Acridine-9-carboxyamido)undecanoic acid methyl ester (82d)

Following Method F and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 82d was obtained as a yellow solid (300 mg, 71%). Mp: 58-60 °C.

$\delta$H (400 MHz, CDCl$_3$): 1.31-1.58 (m, 12H, H-11-H-16), 1.60-1.79 (m, 2H, H-10), 1.81-1.85 (m, 2H, H-17), 2.28 (t, 2H, J 7.5, H-9), 3.63 (s, 3H, CH$_3$), 3.73 (q, 2H, J 6.7, H-18), 6.77 (s, 1H, NH), 7.45 (dt, 2H, J 1.2, 8.1, H-4/H-4'), 7.70 (dt, 2H, J 1.2, 8.1, H-3/H-3'), 7.94 (d, 2H, J 8.8, H-2/H-2'), 8.08 (d, 2H, J 8.8, H-5/H-5')

$\delta$C (100 MHz, CDCl$_3$): 24.9 (CH$_2$, C-17), 27.1 (CH$_2$, C-11), 29.1 (CH$_2$, C-12), 29.2 (CH$_2$, C-13), 29.3 (CH$_2$, C-10), 29.3 (CH$_2$, C-14), 29.5 (CH$_2$, C-15), 29.7 (CH$_2$, C-16), 34.1 (CH$_2$, C-9), 40.3 (CH$_2$, C-18), 51.5 (CH$_3$), 122.1 (q, Ar., C-6/C-6'), 125.2 (CH, Ar., C-5/C-5'), 126.7 (CH, Ar., C-3/C-3'), 129.3 (CH, Ar., C-4/C-4'), 130.4 (CH, Ar., C-2/C-2'), 141.3 (q, Ar., C-7), 148.3 (q, Ar., C-1/C-1'), 166.9 (q, CONH, C-8), 174.4 (q, COOMe, C-19)

$\nu$$_{max}$ (film)/cm$^{-1}$: 2982, 2920 (NH), 1604, 1031 (COO)

HRMS (m/z -ES): Found: 421.2484 (M$^+$ + H. C$_{26}$H$_{33}$N$_2$O$_3$ Requires: 421.2491).

12-(Acridine-9-carboxyamido)dodecanoic acid methyl ester (82e)

Following Method F and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 82e was obtained as a yellow solid (260 mg, 60%). Mp: 62-64 °C.
\[ \delta_H (400 \text{ MHz, CDCl}_3): 1.33-1.52 (m, 14H, H-11-H-17), 1.63 (m, 2H, H-10), 1.80-1.84 (m, 2H, H-18), 2.31 (t, 2H, J 7.5, H-9), 3.67 (s, 3H, CH\text{}_3), 3.75 (q, 2H, J 7.1, H-19), 6.63 (s, 1H, NH), 7.54 (dt, 2H, J 1.2, 7.9, H-4/H-4'), 7.75 (dt, 2H, J 1.2, 7.9, H-3/H-3'), 8.01 (d, 2H, J 8.8, H-2/H-2'), 8.15 (d, 2H, J 8.8, H-5/H-5') \]

\[ \delta_C (100 \text{ MHz, CDCl}_3): 24.9 (\text{CH}_2, C-18), 27.1 (\text{CH}_2, C-11), 29.1 (\text{CH}_2, C-12), 29.2 (\text{CH}_2, C-13), 29.3 (\text{CH}_2, C-10), 29.4 (\text{CH}_2, C-14), 29.5 (\text{CH}_2, C-15), 29.5 (\text{CH}_2, C-16), 29.7 (\text{CH}_2, C-17), 34.1 (\text{CH}_2, C-9), 40.3 (\text{CH}_2, C-19), 51.5 (\text{CH}_3), 122.2 (q, \text{Ar.}, C-6/C-6'), 125.3 (\text{CH, Ar.}, C-5/C-5'), 126.8 (\text{CH, Ar.}, C-3/C-3'), 129.3 (\text{CH, Ar.}, C-4/C-4'), 130.6 (\text{CH, Ar.}, C-2/C-2'), 142.1 (q, \text{Ar.}, C-7), 148.3 (q, \text{Ar.}, C-1/C-1'), 166.9 (q, \text{CONH}, C-8), 174.4 (q, \text{COOMe}, C-20) \]

\[ \nu_{\text{max}} (\text{film})/\text{cm}^{-1}: 3382, 3197, 2917, 2852 (\text{NH}), 1737, 1633, 1116 (\text{COO}) \]

HRMS (m/z -ES): Found: 435.2662 (M\textsuperscript{+}+H. \text{C}_{27}\text{H}_{35}\text{N}_2\text{O}_3 \text{Requires: 435.2648}).

**4-(Acridine-9-carboxyamido)butyric acid (83a)**

![Diagram of 4-(Acridine-9-carboxyamido)butyric acid](image)

Following Method G, methyl 4-(acridine-9-carboxyamido)butanoate (577.2 mg, 1.89 mmol) was dissolved in THF (4 cm\textsuperscript{3}) and 1 M KOH (2 cm\textsuperscript{3}) was added to mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum, 83a was obtained as a yellow solid (547.9 mg, 89%). Mp: 81-83 °C.

\[ \delta_H (400 \text{ MHz, } \text{D}_2\text{O}): 1.93 (q, 2H, H-10), 2.28 (t, 2H, J 7.6, H-9), 3.56 (t, 2H, J 7.0, H-11), 7.53 (app t, 2H, H-4/H-4'), 7.73 (app t, 2H, H-3/H-3'), 7.84 (d, 2H, J 8.7, H-2/H-2'), 7.89 (d, 2H, J 8.8, H-5/H-5'), 8.36 (s, 1H, NH) \]

\[ \delta_C (100 \text{ MHz, } \text{D}_2\text{O}): 25.1 (\text{CH}_2, C-10), 30.7 (\text{CH}_2, C-11), 35.6 (\text{CH}_2, C-9), 122.0 (q, \text{Ar.}, C-6/C-6'), 125.6 (\text{CH, Ar.}, C-5/C-5'), 126.9 (\text{CH, Ar.}, C-3/C-3'), 129.1 (\text{CH, Ar.}, C-4/C-4) \]
Experimental Section

Chapter 9

4'), 131.1 (CH, Ar., C-2/C-2'), 143.1 (q, Ar., C-7), 148.6 (q, Ar., C-1/C-1'), 166.7 (q, CONH, C-8), 176.1 (q, COOH, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3249 (N-H), 3017, 2971 (COOH), 1739 (CO), 1607, 1549, 1487, 1421, 1407 (C-C), 1366 (C-H), 1293, 1271, 1231 (C-N), 1217, 1165, 1138, 1110, 1092, 1045, 1030 (C-N), 1010 (C-O), 844, 779, 763, 741 (C-H), 667 (N-H).

HRMS (m/z -ES): Found: 307.1083 (M$^+$ - H. C$_{18}$H$_{15}$N$_2$O$_3$ Requires: 307.1083).

Purity by HPLC: 97.0% ($t_R$ = 25.51)

5-(Acridine-9-carboxyamido)pentanoic acid (83b)

Following Method G, methyl 5-(acridine-9-carboxyamido)pentanoate (871.2 mg, 2.71 mmol) was dissolved in THF (4 cm$^3$) and 1 M KOH (2 cm$^3$) was added to mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum, 83b was obtained as a white solid (594.9 mg, 68%). Mp: 137-139 °C.

$\delta_H$ (400 MHz, DMSO): 1.15-1.22 (m, 4H, H-10 and H-11), 1.64 (t, 2H, J 6.0, H-9), 3.48 (t, 2H, J 6.0, H-12), 7.68 (app t, 2H, H-4/H-4'), 7.88 (app t, 2H, H-3/H-3'), 8.01 (d, 2H, J 8.8, H-2/H-2'), 8.20 (d, 2H, J 8.8, H-5/H-5'), 9.17 (t, J 5.5, 1H, NH)

$\delta_C$ (100 MHz, DMSO): 23.8 (CH$_2$, C-11), 29.3 (CH$_2$, C-10), 30.7 (CH$_2$, C-9), 37.5 (CH$_2$, C-12), 121.7 (q, Ar., C-6/C-6'), 125.6 (CH, Ar., C-5/C-5'), 126.7 (CH, Ar., C-3/C-3'), 129.2 (CH, Ar., C-4/C-4'), 130.6 (CH, Ar., C-2/C-2'), 142.6 (q, Ar., C-7), 148.1 (q, Ar., C-1/C-1'), 165.8 (q, CONH, C-8), 178.0 (q, COOH, C-13)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3220 (N-H), 3016, 2970 (COOH), 1740 (C=O), 1607, 1574, 1549, 1529, 1486, 1421, 1403 (C-C), 1366 (C-H), 1333, 1295, 1258 (C-N), 1232, 1217, 1192, 1165, 1139, 1110, 1053, 1030, 1010 (C-O), 908, 890, 850, 810, 797, 761, 742 (C-H), 670 (N-H).
HRMS (m/z -ES): Found: 323.1407 (M⁺ + H. C₁₉H₁₈N₂O₃ Requires: 323.1396).

Purity by HPLC: 96.8% (t_R = 26.49)

4-(Acridine-9-carboxyamido)octanoic acid (83c)

Following Method G, methyl 8-(acridine-9-carboxyamido)octanoate (1.94 g, 5.13 mmol) was dissolved in THF (4 cm³) and 1 M KOH (2 cm³) was added to mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum, 83c was obtained as a yellow oil (528.9 mg, 28%).

δ_H (400 MHz, DMSO): 1.43-1.58 (m, 6H, H-11-H-13), 1.60-1.64 (m, 2H, H-10), 1.66-1.73 (m, 2H, H-14), 2.34 (t, 2H, J 8.0, H-9), 3.52 (t, 2H, J 10.0, H-15), 7.71 (app t, 2H, H-4/H-4'), 7.90 (app t, 2H, H-3/H-3'), 8.00 (d, 2H, J 8.9, H-2/H-2'), 8.22 (d, 2H, J 8.6, H-5/H-5'), 9.05 (s, 1H, NH)

δ_C (100 MHz, DMSO): 24.6 (CH₂, C-14), 26.5 (CH₂, C-11), 28.5 (CH₂, C-12), 28.7 (CH₂, C-13), 28.9 (CH₂, C-10), 30.7 (CH₂, C-9), 33.7 (CH₂, C-15), 121.8 (q, Ar., C-6/C-6'), 125.5 (CH, Ar., C-5/C-5'), 126.8 (CH, Ar., C-3/C-3'), 129.3 (CH, Ar., C-4/C-4'), 130.6 (CH, Ar., C-2/C-2'), 142.6 (q, Ar., C-7), 148.3 (q, Ar., C-1/C-1'), 165.8 (q, CONH, C-8), 174.5 (q, COOH, C-16)

v_max (film)/cm⁻¹: 3246 (N-H), 2951 (COOH), 1739 (C=O), 1611, 1574, 1530, 1486, 1403 (C-C), 1366 (C-H), 1332, 1294, 1273, 1258 (C-N), 1232, 1217, 1192, 1165, 1139, 1110, 1093, 1053, 1031, 1010 (C-O), 909, 890, 867, 797, 779, 762, 741 (C-H), 663 (N-H).


Purity by HPLC: 95.0% (t_R = 30.41)
11-(Acridine-9-carboxyamido)undecanoic acid (83d)

Following Method G, methyl 11-(acridine-9-carboxyamido)undecanoate (298.2 mg, 0.71 mmol) was dissolved in THF (4 cm³) and 1 M KOH (2 cm³) was added to mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum, 83d was obtained as a white solid (195.5 mg, 67%) Mp: 158-160 °C.

δ_H (400 MHz, CDCl_3): 1.19 (broad s, 12H, H-11-H-16), 1.40 (m, 4H, H-10 and H-17), 1.79 (t, 2H, J 8.0, H-9), 3.51 (d, 2H, J 6.3, H-18), 7.68 (app t, 2H, H-4/H-4'), 7.89 (app t, 2H, H-3/H-3'), 7.99 (d, 2H, J 8.5, H-2/H-2'), 8.21 (d, 2H, J 8.6, H-5/H-5'), 9.08 (s, 1H, NH)

δ_C (100 MHz, CDCl_3): 21.4 (CH_2, C-17), 26.0 (CH_2, C-11), 26.6 (CH_2, C-12), 26.7 (CH_2, C-13), 28.7 (CH_2, C-10), 29.0 (CH_2, C-14), 29.1 (CH_2, C-13), 29.2 (CH_2, C-15), 29.6 (CH_2, C-9), 34.6 (CH_2, C-18), 121.7 (q, Ar., C-6/C-6'), 125.7 (CH, Ar., C-5/C-5'), 126.9 (CH, Ar., C-3/C-3'), 129.3 (CH, Ar., C-4/C-4'), 131.1 (CH, Ar., C-2/C-2'), 142.4 (q, Ar., C-7), 148.2 (q, Ar., C-1/C-1'), 166.0 (q, CONH, C-8), 173.5 (q, COOH, C-19)

v_max (film)/cm⁻¹: 3221 (N-H), 2951 (COOH), 1740 (C=O), 1552, 1487, 1401 (C-C), 1366 (C-H), 1295, 1279, 1257 (C-N), 1233, 1217, 1190, 1165, 1139, 1110, 1092, 1053, 1030, 1008 (C-O), 908, 890, 859, 828, 810, 797, 779, 762, 741 (C-H), 700, 664 (N-H).

HRMS (m/z -ES): 407.2335 Found: (M⁺ + H. C_{25}H_{31}N_{2}O_{3} Requires: 407.2335).

Purity by HPLC: 95.5% (t_R= 33.57)
12-(Acridine-9-carboxyamido)dodecanoic acid (83e)

Following Method G, methyl 12-(acridine-9-carboxyamido)dodecanoate (489.8 mg, 1.16 mmol) was dissolved in THF (4 cm³) and 1 M KOH (2 cm³) was added to mixture. This reaction was left stirring overnight. The pH of the solution was adjusted from 9 to 3 using a solution of 1 M HCl and the reaction mixture was concentrated under vacuum, 83e was obtained as red solid (425 mg, 87%). Mp: 72-74 °C

δH (400 MHz, CDCl₃): 1.26 (broad s, 14H, H-11–H-17), 1.40-1.49 (m, 2H, H-10), 1.64 (m, 2H, H-18), 2.19 (t, 2H, J 7.0, H-9), 3.51 (q, 2H, J 7.1, H-19), 7.67 (app t, 2H, H-4/H-4’), 7.89 (app t, 2H, H-3/ H-3’), 8.00 (d, 2H, J 8.6, H-2/H-2’), 8.21 (d, 2H, J 9.0, H-5/H-5’), 9.03 (t, 1H, J 5.0, NH)

δC (100 MHz, CDCl₃): 21.0 (CH₂, C-18), 25.9 (CH₂, C-11), 29.0 (CH₂, C-12), 29.0 (CH₂, C-13), 29.1 (CH₂, C-10), 29.2 (CH₂, C-14), 29.2 (CH₂, C-15), 29.5 (CH₂, C-16), 29.6 (CH₂, C-17), 32.7 (CH₂, C-9), 36.8 (CH₂, C-19), 125.7 (q, Ar., C-6/C-6’), 126.8 (CH, Ar., C-5/C-5’), 128.3 (CH, Ar., C-3/C-3’), 129.3 (CH, Ar., C-4/C-4’), 130.7 (CH, Ar., C-2/C-2’), 143.1 (q, Ar., C-7), 148.4 (q, Ar., C-1/C-1’), 165.6 (q, CONH, C-8), 173.6 (q, COOH, C-20)

νmax (film)/cm⁻¹: 3242 (N-H), 2923 (COOH), 1740 (C=O), 1550, 1486, 1405 (C-C), 1366 (C-H), 1332, 1296, 1276, 1257 (C-N), 1233, 1217, 1191, 1165, 1139, 1109, 1092, 1053, 1030, 1010 (C-O), 908, 890, 867, 809, 796, 779, 761 (C-H), 666 (N-H).

HRMS (m/z -ES): Found: 421.2498 (M⁺ + H. C₂₆H₃₃N₂O₃ Requires: 421.2491).

Purity by HPLC: 95.3% (tR= 2.12)
9.2. Biophysical Experiments

9.2.1. DNA and Buffers

Salmon testes, poly(dA-dT)$_2$ and poly(dA).poly(dT) DNA were purchased from Sigma Aldrich. Phosphate buffer solutions contained 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ adjusted to pH 7 and were prepared using Millipore water.

9.2.2. DNA Binding Assays

Thermal melting experiments were conducted with a Varian Cary 300 Bio spectrophotometer equipped with a 6x6 multicell temperature-controlled block. Temperatures were monitored with a thermistor inserted into a 1 cm quartz cuvette containing the same volume of water as in the sample cells. Absorbance changes at 260 nm were monitored from a range of 30 °C to 90 °C with a heating rate of 1 °C/minute and a data collection rate of five points per °C. The salmon testes DNA was purchased from Sigma Aldrich (extinction coefficient $\varepsilon_{260}=6600$ cm$^{-1}$M$^{-1}$ base). A quartz cell with a 1 cm path length was filled with a 1 cm solution of DNA polymer or DNA-compound complex. For thermal denaturation using salmon testes DNA, the DNA polymer (150 µM base) and the compound solution (15 µM) were prepared in a phosphate buffer [0.01 M Na$_2$HPO$_4$/NaH$_2$PO$_4$], adjusted to pH 7) so that a compound to DNA base ratio of 0.1 was obtained. The thermal melting temperatures of the duplex or duplex-compound complex obtained from the first derivative of the melting curves are reported.

For thermal denaturation assays using poly (dA-dT)$_2$ (extinction coefficient $\varepsilon_{260}=6600$ cm$^{-1}$M$^{-1}$ base) and poly(dA).poly(dT) DNA (extinction coefficient $\varepsilon_{260}=6000$ cm$^{-1}$M$^{-1}$ base), the DNA polymer (50 µM base) and the compound solution (5 µM) were prepared in a phosphate buffer [0.01 M Na$_2$HPO$_4$/NaH$_2$PO$_4$], adjusted to pH 7) so that a compound to DNA base ratio of 0.1 was obtained. The thermal melting temperatures of the duplex or duplex-compound complex obtained from the first derivative of the melting curves are reported.

9.2.3. Determination of the pK$_a$

The pK$_a$ value of the first protonation of the hydrochloride salt was determined by measuring the pH of solutions containing 0.08 mmol of the salt and 4 mL of a 0.01 M NaOH standard solution. To obtain the pK$_a$ values of the second protonation, eight
additional mL of the NaOH standard solution was added and the pH was measured afterwards.

To determine the pKₐ values of the third protonation (NH linker) the pH of solutions containing 0.08 mmol of the salt and 4 mL of a 0.01 M HCl standard solution was measured.

To determine the pKₐ value of the N atom of the alkylcarboxylic acid acridine derivatives 4 mL of a 0.01 M HCl solution were added to the acridine solution and the pH was measured. An additional 8 mL of 0.01 M HCl was added to our compound solution to determine the pKₐ of the carboxylic acid.

All the measurements were carried out in an Orion 420A pH-meter with an epoxy body, semi-micro tip combination pH electrode (910600). A pH 7.00 standard solution with disodium hydrogen phosphate/potassium dihydrogen phosphate was used to calibrate the apparatus.

9.2.4. UV Spectroscopy: The effect of increasing the ionic strength

A solution DNA-complex where 70% of the compound was initially bound was prepared in phosphate buffer (10 mM) at pH 7. Aliquots of a salt solution (3 M NaCl) were titrated until the salt concentration reached 0.2 M. From a plot of log(Na⁺) against log(K), the slope SK (=Zψ) was determined.

9.2.5. Circular Dichroism Spectroscopy

CD spectra were collected with a JASCO J–800 spectrometer at different ratios of compound to DNA [salmon testes and poly(dA-dT)₂ we used] at 25 °C in phosphate buffer. Titrations were carried out by adding the compound (P/D ratio 0 to 2 over 2 additions) in a DNA solution (150 µM) in a 1 cm quartz cuvette and scanned over a desired wavelength range.

9.2.6. Linear Dichroism Spectroscopy

LD spectra were collected with a JASCO J–810 spectrometer at different ratios of compound to DNA at 25 °C in phosphate buffer. Each flow LD spectrum was acquired from 200 nm to 400 nm and reflects the average of two scans. The couette flow orientation
was used for sample orientation for all LD studies. The DNA solutions were 378.8 \mu\text{M}
with a compound to DNA (phosphate) ratio of 2 to 10.

**9.2.7. UV Spectroscopy: Determination of DNA binding affinity**

For the evaluation of the DNA binding affinity stock solutions of the DNA (2 \mu\text{M}) and
ethidium bromide (1 \mu\text{M}) were made up that contained 10 \text{mM} sodium phosphate buffer
(pH 7) were used. The compound at increasing ratios was then titrated into the solution and
the corresponding absorption spectra were recorded under the same conditions. All
concentrations were determined using the appropriate extinction coefficients.

**9.3. Biochemical Experiments**

**9.3.1. In Vitro Activity against \textit{T. brucei rhodesiense} STIB 900**

This stock was isolated in 1982 from a human patient in Tanzania and after several mouse
passages cloned and adapted to axenic culture conditions.\textsuperscript{15} Minimum Essential Medium
(50 \mu\text{L}) supplemented with 25 \text{mM} HEPES, 1 g/L additional glucose, 1\% MEM
non-
essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate and 15\%
heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial
drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 \mu\text{g} \text{mL}^{\text{-1}}
were prepared. Then 4x10^{11} bloodstream forms of \textit{T. b. rhodesiense} STIB 900 in 50 \mu\text{L} was
added to each well and the plate incubated at 37 °C under a 5\% \text{CO}_2 atmosphere for 70
hours. 10 \mu\text{L} alamarBlue (resazurin, 12.5 mg in 100 \text{ml} double-distilled water) was then
added to each well and incubation continued for a further 2–4 hours.\textsuperscript{16} Then the plates were
read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices
Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an
emission wave length of 588 nm. The IC\textsubscript{50} values were calculated by linear regression\textsuperscript{17}
from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices
Cooperation, Sunnyvale, CA, USA).

**9.3.2. In Vitro Activity against \textit{P. falciparum} NF54**

\textit{In vitro} activity against erythrocytic stages of \textit{P. falciparum} was determined using a \textsuperscript{3}H-
hypoxanthine incorporation assay, using the drug sensitive NF54 strain (Schiphol Airport,
The Netherlands) and the standard drug chloroquine (Sigma C6628). Compounds were
dissolved in DMSO at 10 mg \text{mL}^{\text{-1}} and added to parasite cultures incubated in RPMI 1640
medium without hypoxanthine, supplemented with HEPES (5.94 g/l), NaHCO\textsubscript{3} (2.1 g L\textsuperscript{-1}), neomycin (100 U mL\textsuperscript{-1}), Albumax\textsuperscript{R} (5 g L\textsuperscript{-1}) and washed human red cells A+ at 2.5% haematocrit (0.3% parasitaemia). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 μg mL\textsuperscript{-1} were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C; 4% CO\textsubscript{2}, 3% O\textsubscript{2}, 93% N\textsubscript{2}. After 48 hours 50 μL of \textsuperscript{3}H-hypoxanthine (=0.5 μCi) was added to each well of the plate. The plates were incubated for a further 24 hours under the same conditions. The plates were then harvested with a Betaplate\textsuperscript{TM} cell harvester (Wallac, Zurich, Switzerland), and the red blood cells transferred onto a glass fibre filter then washed with distilled water. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid, and counted in a Betaplate\textsuperscript{TM} liquid scintillation counter (Wallac, Zurich, Switzerland). IC\textsubscript{50} values were calculated from sigmoidal inhibition curves by linear regression\textsuperscript{17} using Microsoft Excel.

### 9.3.3. \textit{In Vitro} cytotoxicity with L6 cells

Assays were performed in 96-well microtiter plates, each well containing 100 μL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4000 L6 cells (a primary cell line derived from rat skeletal myoblasts).\textsuperscript{18,19} Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 μg mL\textsuperscript{-1} were prepared. After 70 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 μL of alamarBlue was then added to each well and the plates incubated for another 2 hours. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC\textsubscript{50} values were calculated by linear regression\textsuperscript{17} from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA). Podophyllotoxin was the reference drug used.

### 9.4. References

Experimental Section


12. K. Liu, J. Qie. Y. Liang, X. Zhao, CN 1467220 A 2004 CAPLUS


Appendix
General procedures for the preparation of compounds

Method H: General method for the preparation of Cbz-protection of amino acids
A mixture of the corresponding amino acid (4.85 mmol) in a 2 M aqueous sodium hydroxide solution (17 cm$^3$) was cooled in an ice bath to 0 °C. Under vigorous stirring benzyl chloroformate (5.45 mmol) and a 2 M aqueous sodium hydroxide solution (19 cm$^3$) were simultaneously added within two minutes. The mixture was stirred for 20 minutes at room temperature and extracted with diethyl ether (4×40 cm$^3$). The aqueous layer was separated and acidified with concentrated hydrochloric acid to a pH of 2. The resulting emulsion was extracted with ethyl acetate (3×30 cm$^3$). The organic phases were combined, washed with brine, and dried with sodium sulphate. Concentration under vacuum afforded the Cbz-protected amino acids as white needles, which were dried under high vacuum.

Method I: General method for the preparation of Fmoc-protected mono-guanidine amino acid conjugates
The Boc-protected mono-guanidine (0.5 mmol), Fluorenylmethyloxycarbonyl -5-aminopentanoic acid (0.75 mmol), and HOBt (0.75 mmol) were dissolved in dry THF (8 cm$^3$). The mixture was cooled to 0 °C, and EDCl (0.75 mmol) was added. The reaction mixture was stirred at room temperature overnight, then quenched with water (10 cm$^3$) and extracted with ethyl acetate (3×20 cm$^3$). The combined organic layer was washed with a saturated aqueous solution of NaHCO$_3$ (20 cm$^3$) and brine (20 cm$^3$), dried over anhydrous Na$_2$SO$_4$, and concentrated. The crude product was filtered over a pad of basic alumina to remove the amino acid in excess and then purified by flash column chromatography with silica gel eluting with hexane/EtOAc (2:1) yielded the required product.

Method J: General method for the preparation of Amino-acetic acid ethyl esters
Thionyl chloride (25.5 mmol) was added dropwise to a stirred solution of amino acid (8.5 mmol) and ethanol (85 mmol) at 0 °C. The resulting solution was then heated to reflux in ethanol (0.4 cm$^3$/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure.
Method K: General method for the preparation of ethyl (acridine-9-carboxyamido)alkylcarboxylate

In a 100 cm$^3$ round-bottomed flask were placed, at room temperature under argon, amino-acetic acid ethyl ester (5 mmol) and acridine-9-carboxylic acid (5 mmol) in anhydrous CH$_2$Cl$_2$ (50 mL). The mixture was placed under stirring and cooled to 0 °C. DIEA (20 mmol) was slowly added, followed by the addition of the coupling reagent (BOP) (6.5 mmol). The reaction was stirred overnight at 20 °C. EtOAc was added to the reaction mixture and the organic layer was successively washed with a 1 M HCl solution, a 20% NaHCO$_3$ solution and brine. The organic layer was dried over MgSO$_4$ and concentrated in vacuo. The crude residue was purified by chromatography on a silica gel column eluting with hexane/EtOAc (5:1)-(3:1)-(1:1) yielded the required product.

Method L: General method for the preparation of Boc-protected dual binders

(Acridine-9-carboxyamido) alkylcarboxylic acid (0.3 mmol) was dissolved in dry DMF at 0 °C followed by addition of triethylamine (0.36 mmol) and ethyl chloroformate (0.36 mmol). The reaction mixture was stirred under N$_2$ atmosphere for at 30 °C until reaction was completed. An aqueous work up was carried out to remove the DMF. The resulting solid was redissolved in dry DCM at 0 °C followed by the addition of a solution of the Boc-protected mono-guanidine (0.1 mmol) in dry DCM and triethylamine (0.36 mmol). The reaction mixture was stirred for 1 hour at 0 °C and overnight at room temperature under N$_2$ atmosphere. The reaction mixture was washed with a solution of 5% NaHCO$_3$ (m/v) (3×20 cm$^3$) and water (3×20 cm$^3$). The organic phase was dried over Na$_2$SO$_4$ and the solvent evaporated under reduced pressure. The resulting solid was purified by silica gel chromatography eluting with hexane/EtOAc (2:1) yielded the required product.

Preparation and characterisation of compounds

$N,N$-bis(carboxybenzyl)thiourea (49)$^1$

A solution of thiourea (100 mg, 1.315 mmol) in dry THF (10 cm$^3$) was prepared and cooled under nitrogen. To this NaH (69.4 mg, 2.89 mmol, 60% in mineral oil) was added. The ice bath was removed after five minutes and the mixture stirred for ten more minutes at room temperature. The mixture was then cooled to 0 °C, CbzCl (1009 mg, 5.91 mmol)
was added neat. After 30 minutes, the ice-bath was removed and the reaction was left to stir overnight. The reaction was then quenched by the careful addition of NaHCO₃ saturated solution. It was then added to deionised water (100 cm³). The aqueous layer was extracted using EtOAc and the combined organic phases were washed with brine. The solution was dried using MgSO₄ and concentrated under vacuum; washing with hexane afforded 49 as a white solid (124.9 mg, 28%) Mp: 122-124 °C (no literature value available).

δ_H (400 MHz, CDCl₃): 5.23 (d, 4H, J 10.5, CH₂-Cbz), 7.35-7.45 (m, 10H, Cbz)


4-(Fluorenlymethyloxycarbonyl)pentanoic acid (87)¹

5-Aminovaleric acid (300 mg, 2.56 mmol) was dissolved in 6.5 cm³ of 10% sodium carbonate aqueous solution. Dioxane (4 cm³) was added and the mixture was stirred in an ice-water bath. A small portion of 9-fluorenylmethyl chlorocarbonate (666 mg, 2.56 mmol) was then added and stirring was continued for 3.5 hours at 0 °C and additionally for 6.5 hours at room temperature. The resulting reaction mixture was poured into 80 cm³ of water and extracted with diethyl ether (4×25 cm³). The aqueous phase was cooled in an iced water bath and acidified to pH 3.0 with concentrated hydrochloric acid under vigorous stirring followed by extraction with EtOAc (3×50 cm³). The organic layers were combined, dried over MgSO₄, and concentrated under vacuum to give a white foam. Recrystallisation from CH₃CN gave 87 as a white solid (824 mg, 95%). Mp: 132-135 °C (literature: 135-136 °C).²

δ_H (400 MHz, ((CD₃)₂CO)): 1.55-1.64 (m, 4H, H-2 and H-3), 2.32 (t, J 7.8, 2H, H-4), 3.19 (t, J 6.6, 2H, H-1), 4.22 (t, J 7.0, 1H, CH-Fmoc), 4.34 (d, J 7.0, 2H, CH₂-Fmoc), 6.53 (s, 1H, Ar, Fmoc), 7.31-7.88 (m, 8H, Ar, Fmoc)
Appendix

4-(Benzyloxycarbonylamino)butanoic acid (58f)³

Following Method H, 58f was obtained as a white solid (586.2 mg, 51%). Mp: 74-76 °C (literature: 67-68 °C).⁴

δ_H (400 MHz, DMSO): 1.83-1.89 (m, 2H, H-2), 2.40 (t, 2H, J 7.1, H-1), 3.24-3.28 (q, 2H, H-3), 5.09 (broad s, 2H, CH₂-Cbz), 7.32-7.38 (m, 5H, Cbz)

4-(Benzyloxycarbonylamino)pentanoic acid (58g)⁴

Following Method H, 58g was obtained as a white solid (520.3 mg, 42%). Mp: 94-96 °C (literature: 105 °C).⁴

δ_H (400 MHz, CDCl₃): 1.57-1.73 (m, 4H, H-2 and H-3), 2.42 (t, 2H, J 7.0, H-1), 3.23-3.28 (q, 2H, H-4), 5.12 (broad s, 2H, CH₂-Cbz), 7.33-7.39 (m, 5H, Cbz)

8-(Benzyloxycarbonylamino)octanoic acid (58h)⁴

Following Method H, 58h was obtained as a white solid (738.7 mg, 51%). Mp: 44-46 °C (literature: 63-64 °C).⁵

δ_H (400 MHz, CDCl₃): 1.35 (s, 6H, H-3—H-5), 1.50-1.65 (m, 4H, H-2 and H-6), 2.37 (t, 2H, J 7.5, H-7), 3.18-3.24 (q, 2H, H-1), 5.11 (broad s, 2H, CH₂-Cbz), 7.34-7.39 (m, 5H, Cbz)
Appendix

4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(carboxybenzyl)aminopentanamido]benzophenone (86)

A solution of 4-(Benzylloxycarbonylamino)butanoic acid (1.2 mmol) in MeCN (10 cm$^3$) was treated with DIEA (3.8 mmol), the 4-amino-4’-[1,3-di(tert-butoxycarbonyl)guanidino]diphenylmethane (1 mmol) and TBTU (1.2 mmol) under inert atmosphere. The reaction mixture was stirred at room temperature for 18 hours and partitioned between brine (4 cm$^3$) and EtOAc (10 cm$^3$). The organic layer was washed with 0.1 M HCl (2x5 cm$^3$) and 5% NaHCO$_3$ (2x5 cm$^3$), dried over anhydrous Na$_2$SO$_4$, filtered and concentrated under vacuum. Purification by flash chromatography with silica gel eluting with hexane/EtOAc (2:1), 86 was obtained as a white solid (554.3 mg, 84%). Mp: 130-132 °C.

$\delta_H$ (400 MHz, CDCl$_3$): 1.55 (d, 18H, (CH$_3$)$_3$), 1.93 (t, 2H, J 6.3, H-2), 2.39 (t, 2H, J 6.5, H-3), 3.34 (t, 2H, J 6.5, H-1), 3.92 (s, 2H, H-9), 5.12 (s, 2H, CH$_2$-Cbz), 7.12-7.15 (m, 4H, H-6/H-6’, and H-7/H-7’), 7.37 (s, 5H, Ar-H, Cbz), 7.52 (d, 4H, J 8.0, H-11/H-11’ and H-12/H-12’)

$\delta_C$ (100 MHz, CDCl$_3$): 27.2 (CH$_2$, C-2), 27.6 ((CH$_3$)$_3$), 33.7 (CH$_2$, C-3), 38.2 (CH$_2$, C-9), 39.5 (CH$_2$, C-1), 66.5 (CH$_2$, Cbz), 78.6 (q, C(CH$_3$)$_3$), 78.8 (q, C(CH$_3$)$_3$), 81.5 (q, C(CH$_3$)$_3$), 119.5 (CH, Ar., C-12/C-12’), 121.9 (CH, Ar., C-6/C-6’), 125.4 (CH, Ar., C-7/C-7’), 127.7 (CH, Ar., C-11/C-11’), 128.1 (Cbz, Ar), 128.8 (Cbz, Ar), 129.0 (Cbz, Ar), 134.3 (q, Ar., C-10), 136.4 (q, Ar., C-5), 137.4 (q, Cbz-Ar) 138.4 (q, Ar., C-8), 143.6 (q, Ar., C-13), 153.1 (q, CO), 156.7 (q, CO), 160.3 (q, CO), 163.2 (q, CN), 171.2 (q, CONH, C-4)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3322 (NH), 1723, 1705, 1671, 1633, 1602 (CO, CN), 1588, 1409, 1368 (C-C), 1338 (CH$_2$), 1317, 1304, 1229, 1153 (C-N), 1119, 1095, 1056, 1026, (C-N) 857, 807, 781, 757, 734, 694 (N-H, C-H)

HRMS ($m/z$ -ES): Found: 660.3397 (M$^+$ + H. C$_{36}$H$_{46}$N$_5$O$_7$ Requires: 660.3397).
4-[2,3-di(tert-butoxycarbonyl)guanidino]-4’-[4-(Fluorenethylmethoxycarbonyl)aminopentanamido]benzophenone (88d)

Following method I and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 88d was obtained as a yellow oil (186.2 mg, 48%).

$$\delta_H (400 \text{ MHz, CDCl}_3): 1.53 (s, 9H, (CH_3)_3), 1.58 (s, 9H, (CH_3)_3), 1.73-1.86 (m, 4H, H-2 and H-3), 2.42 (t, 2H, J 8.0, H-4), 3.24 (t, 2H, J 8.0, H-1), 4.21 (t, 1H, J 4.0, CH-Fmoc), 4.44 (d, 2H, J 8.0, O-CH$_2$-Fmoc), 5.12 (s, 1H, NHCO), 7.32 (d, 2H, J 8.0, H-8/H-8’), 7.39 (d, 2H, J 8.0, H-12/H-12’), 7.41(d, 2H, J 8.0, H-7/H-7’), 7.59-7.70 (m, Ar, 12H, H-13/H-13’ and Fmoc), 8.39 (s, 1H, CONH-Ar), 10.64 (s, 1H, NH), 11.68 (s, 1H, NH)

$$\delta_C (150 \text{ MHz, CDCl}_3): 22.6 (CH$_2$, C-3), 27.7 ((CH$_3$)$_3$), 29.0 (CH$_2$, C-2), 31.5 (CH$_2$, C-4), 36.6 (CH$_2$, C-1), 39.9 (CH, Fmoc), 66.3 (CH$_2$, Fmoc), 80.1 (q, C(CH$_3$)$_3$), 83.8 (q, C(CH$_3$)$_3$), 118.2 (CH, Ar., C-13/C-13’), 119.7 (CH, Ar., C-7/C-7’), 121.1 (q, Ar., C-11), 124.8 (CH, Ar., C-8/C-8’), 126.6 (CH, Ar., C-12/C-12’), 127.3 (q, Ar., C-9), 130.7 (CH, Ar., Fmoc), 131.0 (CH, Ar., Fmoc), 132.3 (CH, Ar., Fmoc), 133.3 (CH, Ar., Fmoc), 140.0 (q, Ar., C-6), 140.9 (q, Ar, C-14), 141.9 (q, Ar, Fmoc), 143.4 (q, Ar, Fmoc), 152.8 (q, CO), 153.0 (q, CO), 156.6 (q, CO), 162.8 (q, CN), 171.4 (q, CONH, C-5), 194.2 (q, PhCOPh, C-10)

$\nu_{max}$ (film)/cm$^{-1}$: 2984, (NH) 1729, 1673, 1506, (CO, CN) 1370, (C-C), 1355 (CH$_2$) 1263, 1153 (C-N), 1099 (C-N) 905, 841, 726 (N-H, C-H)

HRMS (m/z -ES): Found: 798.3486 (M$^+$ + Na.C$_{44}$H$_{49}$N$_5$O$_9$Na Requires: 798.3479).
4-[2,3-di(tert-butoxycarbonyl)guanidino]-4'-[4-(Fluorenylmethyloxycarbonyl)aminopentanamido]diphenylamine (88e)

Following method I and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 88e was obtained as a black oil (155 mg, 41%).

δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 1.30 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>), 1.39 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>), 1.46-1.58 (m, 4H, H-2 and H-3), 2.16 (t, 2H, J 8.0, H-4), 3.05 (t, 2H, J 8.0, H-1), 4.03 (t, 1H, J 6.0, CH-Fmoc), 4.21 (d, 2H, J 7.0, O-CH<sub>2</sub>-Fmoc), 5.62 (s, 1H, NHCO), 6.21 (s, 1H, ArNHR), 6.74 (d, 2H, J 8.0, H-8/H-8'), 7.13 (d, 2H, J 8.0, H-11/H-11'), 7.18-7.28 (m, 4H, H-7/H-7' and H-12/H-12'), 7.44 (d, 2H, J 8.0, Ar, Fmoc), 7.57 (d, 2H, J 8.0, Ar, Fmoc), 7.59 (d, 2H, J 8.0, Ar, Fmoc), 8.33 (s, 1H, CONH-Ar), 9.99 (s, 1H, NH), 11.59 (s, 1H, NH)

δ<sub>C</sub> (100 MHz, CDCl<sub>3</sub>): 22.3 (CH<sub>2</sub>, C-3), 27.7 ((CH<sub>3</sub>)<sub>3</sub>), 28.4 (CH<sub>2</sub>, C-2), 35.6 (CH<sub>2</sub>, C-4), 39.8 (CH<sub>2</sub>, C-1), 46.6 (CH, Fmoc), 66.2 (CH<sub>2</sub>, Fmoc), 79.6 (q, CH(CH<sub>3</sub>)<sub>3</sub>), 83.3 (q, CH(CH<sub>3</sub>)<sub>3</sub>), 117.6 (CH, Ar., C-12/C-12'), 118.4 (CH, Ar., C-7/C-7'), 119.8 (CH, Ar., C-8/ C-8'), 121.2 (CH, Ar., C-11/C-11'), 124.1 (CH, Ar., Fmoc), 125.0 (CH, Ar., Fmoc), 126.6 (CH, Ar., Fmoc), 127.2 (CH, Ar., Fmoc), 129.6 (q, Ar., C-6), 131.3 (q, Ar., C-10), 139.2 (q, Ar., C-13), 140.2 (q, Ar., C-9), 141.0 (q, Fmoc), 143.5 (q, Fmoc), 153.0 (q, CO), 153.2 (q, CO), 156.3 (q, CO), 163.8 (q, CN), 170.5 (q, CONH, C-5).

ν<sub>max</sub> (film)/cm<sup>-1</sup>: 3220, 2920, 2932, (NH) 1733, 1636, 1564, (CO, CN) 1458, 1398 (C-C), 1367 (CH<sub>2</sub>) 1290 (C-N), 1225, 1200, 1150 (C-N) 898, 843, 820, 782, 718, 749, 699 (N-H, C-H)

HRMS (m/z -ES): Found: 761.3464 (M<sup>+</sup> + H.C<sub>43</sub>H<sub>49</sub>N<sub>6</sub>O<sub>7</sub> Requires: 761.3663).
Appendix

4-Aminobutyric acid ethyl ester (89a)

Following Method J, thionyl chloride (0.21 cm$^3$, 29.1 mmol) was added dropwise to a stirred solution of amino acid (1000 mg, 9.7 mmol) and ethanol (90 cm$^3$, 97 mmol) at 0 °C. The resulting solution was then heated to reflux in ethanol (0.4 cm$^3$/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure, 90a was obtained as a white solid (1201.4 mg, 97%). Mp: 87-90 °C (no literature value available).

δ$_H$ (400 MHz, DMSO): 1.33 (s, 3H, CH$_3$), 1.77-1.84 (m, 2H, H-2), 2.32-2.44 (m, 2H, H-3) 2.79 (t, 2H, J 8.0, H-1), 3.86-4.09 (m, 2H, CH$_2$), 8.08 (broad s, 2H, NH$_2$)

5-Aminopentanoic acid ethyl ester (89b)

Following Method J, 89b was obtained as a white solid (1179 mg, 96%). Mp: 63-66 °C (no literature value available)

δ$_H$ (400 MHz, DMSO): 1.20 (s, 3H, CH$_3$), 1.54-1.58 (m, 4H, H-2 and H-3) 2.33 (t, 2H, J 6.7, H-4), 2.77 (t, 2H, J 5.5, H-1), 4.03-4.08 (m, 2H, CH$_2$), 7.90 (s, 2H, NH$_2$)

8-Aminoocitoanoic acid ethyl ester (89c)

Following Method J, thionyl chloride (0.14 cm$^3$, 18.85 mmol) was added dropwise to a stirred solution of amino acid (1001.9 mg, 6.29 mmol) and ethanol (63 cm$^3$, 63 mmol) at 0 °C. The resulting solution was then heated to reflux in ethanol (0.4 cm$^3$/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure, 89c was obtained as a white solid (1140 mg, 97%). Mp: 70-72 °C (no literature value available).

δ$_H$ (400 MHz, DMSO): 1.28 (t, 3H, J 7.0, CH$_3$), 1.36-1.43 (m, 6H, H-3—H-5), 1.63 (t, 2H, J 7.0, H-2) 1.79 (t, 2H, J 7.0, H-6), 2.30 (t, 2H, J 7.5, H-7), 3.01 (t, 2H, J 7.5, H-1), 4.15 (q, 2H, CH$_2$), 8.29 (s, 2H, NH$_2$)
11-Aminoundecanoic acid ethyl ester (89d)

Following Method J, thionyl chloride (0.11 cm$^3$, 14.92 mmol) was added dropwise to a stirred solution of amino acid (1000 mg, 4.97 mmol) and ethanol (49.7 cm$^3$, 50 mmol) at 0 °C. The resulting solution was then heated to reflux in ethanol (0.4 cm$^3$/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure, 89d was obtained as a white solid (524 mg, 46%). Mp: 63-65 °C (literature: 65 °C).$^7$

$\delta_H$ (400 MHz, DMSO): 1.24 (t, 3H, CH$_3$), 1.24 (broad s, 12H, H-3-H-8), 1.50-1.56 (m, 4H, H-2 and H-9), 2.26 (t, 2H, J 8.0, H-10), 2.72 (t, 2H, J 7.6, H-1), 4.00-4.06 (m, 2H, CH$_2$), 8.09 (s, 2H, NH$_2$)$^8$

12-Aminododecanoic acid ethyl ester (89e)

Following Method J, thionyl chloride (0.10 cm$^3$, 13.92 mmol) was added dropwise to a stirred solution of amino acid (1000 mg, 4.64 mmol) and ethanol (45 cm$^3$, 46.4 mmol) at 0 °C. The resulting solution was then heated to reflux in ethanol (0.4 cm$^3$/mmol) overnight. The solvent and thionyl chloride in excess were then removed under reduced pressure, 89e was obtained as a white solid (275 mg, 19.2%). Mp: 54-56 °C (literature: 56-58 °C).$^8$

$\delta_H$(400 MHz, DMSO): 1.15-1.19 (q, 3H, CH$_3$), 1.25 (broad s, 14H, H-3-H-9), 1.52 (broad s, 4H, H-2 and H-10), 2.27 (t, 2H, J 7.5, H-11), 2.74 (t, 2H, J 7.5, H-1), 4.02-4.07 (m, 2H, CH$_2$), 7.93 (s, 2H, NH$_2$)$^9$

4-(Acridine-9-carboxyamido)butyric acid ethyl ester (90a)

x
Following Method K and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 90a was obtained as a yellow solid (1125 mg, 67%). Mp: 68-70 °C.

$\delta_H$ (400 MHz, CDCl$_3$): 1.05-1.12 (m, 3H, CH$_3$), 1.86 (t, 2H, J 8.0, H-10), 2.28 (t, 2H, J 8.0, H-9), 3.41 (t, 2H, J 6.5, H-11), 3.89-3.96 (m, 2H, CH$_2$), 7.08 (app t, 2H, H-4/H-4'), 7.34 (app t, 2H, H-3/H-3'), 7.46 (d, 2H, J 8.5, H-2/H-2'), 7.66 (d, 2H, J 9.0, H-5/H-5'), 7.94 (s, 1H, NH)

$\delta_C$ (100 MHz, CDCl$_3$): 13.6 (CH$_3$), 24.1 (CH$_2$, C-10), 31.1 (CH$_2$, C-11), 38.7 (CH$_2$, C-9), 60.0 (CH$_2$), 120.9 (q, Ar., C-6/C-6'), 124.4 (CH, Ar., C-5/C-5'), 125.8 (CH, Ar., C-3/C-3'), 128.2 (CH, Ar., C-4/C-4'), 129.6 (CH, Ar., C-2/C-2'), 140.3 (q, Ar., C-7), 147.0 (q, Ar., C-1/C-1'), 166.4 (CONH, C-8), 172.5 (COOEt, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3228, 2987 (NH), 1729, 1644, 1080 (COO)

HRMS (m/z -ES): Found: 335.1402 (M$^+$ - H.C$_{20}$H$_{19}$N$_2$O$_3$ Requires: 335.1396).

5-(Acridine-9-carboxyamido)pentanoic acid ethyl ester (90b)

Following Method K and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 90b was obtained as a yellow solid (1021 mg, 58%). Mp: 170-172 °C.

$\delta_H$ (400 MHz, CDCl$_3$): 1.28 (t, 3H, J 7.0, CH$_3$), 1.86 (broad s, 4H, H-10 and H-11), 2.45 (t, 2H, J 6.5, H-9), 3.76 (t, 2H, J 6.0, H-12), 4.13-4.18 (m, 2H, CH$_2$), 6.30 (s, 1H, NH), 7.55 (app t, 2H, H-4/H-4'), 7.75 (app t, 2H, H-3/H-3'), 8.03 (d, 2H, J 8.5, H-2/H-2'), 8.15 (d, 2H, J 9.0, H-45/H-55')

$\delta_C$ (100 MHz, CDCl$_3$): 13.8 (CH$_3$), 21.8 (CH$_2$, C-10) , 28.7 (CH$_2$, C-11), 33.3(CH$_2$, C-9), 39.4 (CH$_2$, C-9), 60.0 (CH$_2$), 121.7 (q, Ar., C-6/C-6'), 124.9 (CH, Ar., C-5/C-5'), 126.5
(CH, Ar., C-3/C-3’), 128.3 (CH, Ar., C-4/C-4’), 130.4 (CH, Ar., C-2/C-2’), 141.4 (q, Ar., C-7), 147.3 (q, Ar., C-1/C-1’), 166.4 (CONH, C-8), 173.0 (COOEt, C-12)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3440, 2930 (NH), 1730, 1628, 1158 (COO)

HRMS (m/z -ES): Found: 349.1559 (M$^+$ - H.C$_{21}$H$_{21}$N$_2$O$_3$ Requires: 349.1552).

8-(Acridine-9-carboxyamido)octanoic acid ethyl ester (90c)

Following Method K and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (5:1)-(3:1)-(1:1), 90c was obtained as a yellow solid (955 mg, 49%). Mp: 78-80 °C.

$\delta$H (400 MHz, CDCl$_3$): 1.25-1.28 (m, 3H, CH$_3$), 1.40-1.53 (m, 6H, H-11-H-13), 1.64-1.83 (m, 4H, H-10 and H-14), 2.33 (t, 2H, J 7.5, H-9), 3.72-3.77 (m, 2H, H-15), 4.10-4.15 (m, 2H, CH$_2$), 6.53 (s, 1H, NH), 7.57 (app t, 2H, H-4/H-4’), 7.78 (app t, 2H, H-3/H-3’), 8.05 (d, 2H, J 8.5, H-2/H-2’), 8.19 (d, 2H, J 8.5, H-5/H-5’)

$\delta$C (100 MHz, CDCl$_3$): 14.7 (CH$_3$), 24.8 (CH$_2$, C-14), 26.9 (CH$_2$, C-11), 28.9 (CH$_2$, C-12), 29.0 (CH$_2$, C-13), 29.6 (CH$_2$, C-10), 34.2 (CH$_2$, C-9), 40.4 (CH$_2$, C-15), 60.3 (CH$_2$), 122.2 (q, Ar., C-6/C-6’), 125.3 (CH, Ar., C-5/C-5’), 126.9 (CH, Ar., C-3/C-3’), 129.0 (CH, Ar., C-4/C-4’), 130.8 (CH, Ar., C-2/C-2’), 141.9 (q, Ar., C-7), 147.9 (q, Ar., C-1/C-1’), 166.6 (q, CONH, C-8), 173.8 (q, COOEt, C-16)

$\nu_{\text{max}}$ (film)/cm$^{-1}$: 3400, 2933 (NH), 1733, 1632 (COO)

HRMS (m/z -ES): Found: 393.2168 (M$^+$ + H.C$_{24}$H$_{29}$N$_2$O$_3$ Requires: 393.2178).
Appendix

4-Amino-4’-[1,3-di(tert-butoxycarbonyl)guanidino]diphenyl ether 5-(Acridine-9-carboxamido)pentanoate (96)

Following Method L and after purification by flash chromatography with silica gel, eluting with hexane/EtOAc (2:1), 96 was obtained as a yellow solid.

δ_H (400 MHz, CDCl_3): 1.56 (s, 9H, (CH_3)_3), 1.60 (s, 9H, (CH_3)_3), 1.91-2.03 (m, 4H, H-10 and H-11), 2.57 (t, 2H, J 8.0, H-12), 3.82 (t, 2H, J 6.5, H-9), 6.95 (d, 4H, J 8.5, H-20/H-20’ and H-19/H-19’), 7.02 (s, 1H, CONH-Ar), 7.48 (d, 2H, J 9.0, H-16/H-16’), 7.54-7.61 (m, 4H, H-15/H-15’ and H-4/H-4’), 7.83 (app t, 2H, J 7.0, H-3/H-3’), 8.09 (d, 2H, J 7.5, H-5/H-5’), 8.29 (d, 2H, J 9.5, H-2/H-2’), 10.29 (broad s, 1H, NH), 11.66 (broad s, 1H, NH)

δ_C (100 MHz, CDCl_3): 22.5 (CH_2, C-11), 29.5 ((CH_3)_3), 29.6 ((CH_3)_3), 31.7 (CH_2, C-10), 36.3 (CH_2, C-12), 39.2 (CH_2, C-9), 79.5 (q, C(CH_3)_3), 83.7 (q, C(CH_3)_3)), 118.7 (CH, Ar., C-20/C-20’), 119.2 (CH, Ar., C-15/C-15’), 121.3 (CH, Ar., C-19/C-19’), 122.2 (CH, Ar., C-16/C-16’), 123.7 (CH, Ar., C-5/C-5’), 124.9 (q, Ar., C-6/C-6’), 126.9 (CH, Ar., C-3/C-3’), 130.0 (CH, Ar., C-4/C-4’), 131.2 (CH, Ar., C-2/C-2’), 132.9 (q, Ar., C-18), 135.8 (q, Ar., C-17), 137.8 (q, Ar., C-21), 147.3 (q, Ar., C-1/C-1’), 148.5 (q, Ar.,C-14), 153.2 (q, CO), 154.7 (q, CO), 156.6 (q, CO), 163.4 (q, CN), 167.4 (q, CONH, C-8), 170.7 (q, CONH, C-13)

HRMS (m/z -ES): Found: 747.3537 (M^+ + H. C_{42}H_{47}N_6O_7 Requires: 747.3506).

References


