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Design, Synthesis, Photophysical and Biological Evaluation of Novel 1,8-Naphthalimide Derivatives as DNA Binders and Potential Anti-Cancer Agents

By

Emma B. Veale

August 2007

University of Dublin
Trinity College

Based on research carried out under the direction of
Prof. Thorfinnur Gunnlaugsson

A thesis submitted to the School of Chemistry, University of Dublin, Trinity College in fulfillment of the requirements for the degree of Doctor of Philosophy.
Declaration

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Emma B. Veale
Summary

This thesis, titled 'Design, Synthesis, Photophysical and Biological Evaluation of Novel 1,8-Naphthalimide Derivatives As DNA Binders and Potential Anti-Cancer Agents' is divided into seven chapters. The first chapter is an introduction to the discovery and the progression of 1,8-naphthalimides as anticancer agents. This research represents an important contribution to the design of potent therapeutic agents, some of which have entered clinical trial. For example, Quinamed® developed by GhemGenex Pharmaceutica, has been shown to be active in Gleevec resistant patients with prostate cancer. This Chapter also deals with the utility of DNA cleaving proteins for achieving greater sequence specificity in DNA binding, and on the natural occurrence of thiazole and oxazole containing cytotoxic cyclic peptides. The final section of Chapter 1, reviews the properties and the applications of synthetic Tröger’s base derivatives, which in the past have been used as receptors, as well as chiral solvating agents. Despite the many highly desirable properties of the Tröger’s base, there only exist a few reports on its incorporation into known DNA-binding motifs. These examples are also reviewed in this Chapter. Chapter 2 describes the synthesis and the characterisation of aldehyde, alkene and thiazole modified 1,8-naphthalimide derivatives containing α-amino acids. The first section of this Chapter deals with the synthesis of four α-amino-1,8-naphthalimide aldehyde derivatives, which are utilised as precursors for a transformation to α,β-unsaturated esters. The second section of this Chapter deals with the synthesis of a family of thiazole modified 1,8-naphthalimides containing α-amino acids. This is achieved using a convergent approach whereby thaizole containing amino acids are synthesised as heterocyclic building blocks and then subsequently incorporated into the skeleton of the 1,8-naphthalimide derivatives containing α-amino acids. Solid-state analysis and epimerisation studies using 1H NMR of the thiazolidine intermediates are also presented. Chapter 3 discusses the design, synthesis and photophysical evaluation of four Bis-1,8-naphthalimide containing Tröger’s base derivatives. These molecules are designed as potential C2-symmetric DNA-binding molecules and they have tertiary amino functionalities at the 'terminus' of their side chains, which render them water-soluble while providing favourable electrostatic interactions with the negatively charged backbone of DNA. In this Chapter, a determination of their pKa values and the existence of an ICT excited state will be presented and also show how such information will aid in the analysis of their DNA binding affinities using various
spectroscopic techniques. Chapter 4 discusses the DNA-binding affinities of the Bis-1,8-naphthalimide containing Tröger’s base derivatives studied in aqueous solution with calf-thymus DNA (ct-DNA) and the homopolymers, poly(dG-dC) and poly(dA-dT), using various spectroscopic methods including UV/Vis, fluorescence, T\textsubscript{m} (melting temperature) and circular dichromism. These molecules exhibit large enhancements in their DNA-binding ability and become bound to ct-DNA with an impressive P/D (Phosphate to Dye) ratio of ca. 1. Such strong binding to ct-DNA is maintained even in competitive media (50 mM and 160 mM NaCl) and is also found to be irreversible regardless of the ionic strength. Their binding affinity for ct-DNA, poly(dA-dT) and poly(dG-dC) will also be compared to that of their 4-amino-1,8-naphthalimide precursors, determined by fitting of data using ‘intrinsic’ methods and ethidium bromide displacement assays. The latter method gives outstanding binding constants in the range of $10^{-6}$-$10^{-7}$ M\textsuperscript{-1} for the Bis-1,8-naphthalimide containing Tröger’s base derivatives. The final section of this Chapter will present the results from the T\textsubscript{m} and CD studies, which are carried out to help in further understanding the nature by which these molecules bind to DNA. In Chapter 5, the cytotoxic activity of the alkene, thiazole and Tröger’s base modified 1,8-naphthalimide derivatives in the HL-60 promyelocytic cell line and in the more resistant erythroleukemia K562 cell line is discussed. Mr. Daniel Omar Frimannsson in the Department of Hematology, St. James’ Hospital, Dublin, carried out these investigations and will present these findings in a PhD thesis to be submitted early 2008. However, in the interest of the reader of this thesis, it is important that the cytotoxicity of these molecules is shown to emphasise the importance of their further development. Both the aldehyde and alkene modified 1,8-naphthalimide derivatives show good cytotoxicity, with IC\textsubscript{50} values in the range of 1.9 – 12.0 \textmu M, while the cytotoxic activity of the 1,8-naphthalimide containing Tröger’s base derivatives correlate well with their strong DNA binding affinity. One of these molecules also exhibits the ability to enter the cell, localise in the nucleus and induce apoptosis, which makes it an ideal candidate for further development as a potential anticancer agent. Chapter 6 is the experimental section where the procedures and analytical data for the molecules in the Chapters 2-3 are presented. Chapter 7 gives the references cited.
For My Family
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I wish to sincerely thank my supervisor Prof. Thorri Gunnlaugsson for his support and guidance and for providing me with this wonderful opportunity. The day I entered the lab of the TG Group, I knew I had made the right decision. Thanks for all your optimism, encouragement and motivation over the last a few years. It’s been an amazing experience! Thank you so very much.

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Thank you all.
Abbreviations Most Commonly Used

- br s: Broad singlet
- CD: Circular Dichroism
- CML: Chromic Myeloid Leukaemia
- d: Doublet
- dd: Double doublet
- δ: Chemical shift
- DNA: Deoxyribonucleic acid
- EDCI: 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide Hydrochloride
- Eq.: Equivalents
- HMBC: Heteronuclear Multiple Bond Correlation Spectrum
- HMQC: Heteronuclear Multiple Quantum Coherence Spectrum
- HOBT: N-Hydroxybenzotriazole
- HPLC: High Performance Liquid Chromatography
- Hz: Hertz
- Ir: Intensity of fluorescence
- IR: Infra red
- J: Coupling constant
- m: Multiplet
- m.p.: Melting point
- MTT: [3-[4,5-Dimethylthiazol-2-yl]-2,3-diphenyl] tetrazolium bromide
- m/z: Mass charge ratio
- NMR: Nuclear magnetic resonance
- Pd/C: Palladium on carbon catalyst
- Phe: Phenylalanine
- ppm: Parts per million
- q: Quartet
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<td>Total Correlation Spectroscopy</td>
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Chapter 1: Introduction

1.1 Introduction

In the past few decades there has been considerable interest in the development of small-molecules as deoxyribonucleic acid (DNA) binders because of their potential therapeutic use for targeting cancer and genetic related diseases. In particular, research into the use of the 1,8-naphthalimide structure as a potential anticancer agent has resulted in the formation of several such derivatives entering into clinical trials. However, many of these have failed these trials due to issues such as high toxicity. A major shortcoming of many current DNA-directed anticancer drugs is the lack of selective targeting of (specific regions within) the DNA structure. In this Thesis, two approaches have been undertaken to enhance the selectivity and potency of 1,8-naphthalimide based DNA targeting drugs. The first approach was to incorporate into its structure, peptide moieties functionalised with recognition elements found in many naturally occurring cyclic peptides. This approach was inspired by the fact that many oxazole- and thiazole- containing cyclic peptides from marine organisms (fungi and algae) constitute a growing class of naturally occurring cytotoxic substances. The second approach was to modify the naphthalimide chromophore by the introduction of the Tröger’s base structural unit as a means of reinforcing its DNA binding capacity.

In this Chapter the topic of naturally occurring cyclic peptides as cytotoxic agents will be dealt with, followed by a review on the properties and the applications of synthetic Tröger’s base derivatives. The development of the 1,8-naphthalimide structure as an anticancer agent and the utility of DNA cleaving proteins for achieving sequence specificity has already been extensively reviewed within the Gunnlaugsson group in the PhD Theses of Phelan, Hussy and Gillespie. Nevertheless, a small discussion is still necessary and consequently, this Chapter will begin with a brief overview on these topics; a short discussion on the relationship between cancer and the cell cycle followed by DNA as a promising target for anticancer drug design.

1.2 Cancer and the Cell Cycle

Cancer is a disease of the cell. It occurs because of failures in the mechanisms that regulate cell growth and division, of which proteins play a major role. Advances in
anticancer treatments have required an in-depth understanding of how such processes are disrupted in cancer cells.

The life cycle of the cell, depicted in a conventional manner in Figure 1.1, involves four phases, and begins when a cell emerges from the resting phase known as $G_0$. The cell then enters the S phase (for DNA Synthesis) from the gap phase called $G_1$, wherein the cell is actively metabolising but not dividing. In the S phase, the DNA of the cell replicates, duplicating the genome and producing two copies of each chromosome. After a resting period in the gap phase $G_2$ the cell enters the M (Mitosis) phase. Here the chromosomes separate in the nucleus and the division of the cytoplasm occurs. After the cell life cycle has culminated in mitosis, the cell is either signalled to enter the $G_0$ resting phase or begin the cycle again.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{The four phases of a mammalian cell cycle.}
\end{figure}

The entire cell division process can be regulated in two ways. The first is by a series of protein-phosphorylations, which activates (or inhibits) the function of a protein and passes the cell into the different phases of its life cycle. Advancement into the S phase is helped by the production of Cyclin D, Cyclin E and their active Cyclin-Cdk complexes, with the latter being the main regulation control element (Figure 1.1). Upon complex formation, the Cdk can act as a kinase and catalyse protein phosphorylation, thereby, helping to turn target proteins functionality "on" or "off". The second method of regulation is by a set of checkpoints in $G_1$ and $G_2$, which control the entry of the cell into the S and M phases, respectively. If the DNA becomes damaged during the cell cycle, the checkpoints slow the progression of the cell so as to allow time for the DNA to be repaired. Otherwise, if the DNA is beyond repair, the cell can respond in apoptosis; a process that involves programmed suicide of the affected cell.

The relationship between cancer and the cell cycle is that cancer cells never enter the $G_0$ phase and continually progress through the replicating cycle. In general, a cancerous cell can progress through all of the cell cycle stages, (Figure 1.1), with a lack of response to
growth inhibitors and an increase in metabolic transport capabilities. This situation arises due to a mutation of the protein-encoding genes (called proto-oncogene) giving rise to oncogenes and inactive tumor suppresser genes. These genetic alterations are known as "gain-of-function" and "loss-of-function" mutations, respectively. It is the proto-oncogenes that produce the protein products that normally enhance cell division or inhibit normal cell death, while tumor suppresser genes make proteins that inhibit cell growth and prevent tumor formation. Therefore, a loss of this regulation can lead to cancer.

The most common mutation leading to cancer is in the gene that produces p53. In fact, it is mutated in over 50 % of all human cancers. Upon the binding of p53 to DNA, the transcription of a protein called p21 is activated. This protein blocks the activity of the Cdk allowing the cell to repair the DNA before it is replicated. However, if the DNA cannot be repaired, p53 triggers the cell to undergo apoptosis. Therefore, the efficient functioning of p53 in the cell cycle may deter cancer development and cancer cells with such a function respond to chemotherapy by endorsing their own death.

1.2.1 Chemotherapy As a Treatment of Cancer

The treatment of cancer using chemotherapy involves the use of cytotoxic compounds to selectively target rapidly proliferating cancer cells in preference to the body’s own cells. Anticancer drugs generally exert their activity by inhibiting any one of the many processes that take place during the cell cycle (c.f Figure 1.1). In particular, many of the more successful anticancer drugs target specific active kinases. These drugs tend to be less toxic because they specifically kill cancer cells while sparing their normal counterparts. Two such drugs, Gleevec® (Imatinib mesylate) and Desatinib (BMS-354825), developed for the treatment of chronic myeloid leukaemia (CML), inhibit cancer cell growth by targeting the tyrosine kinase inhibitor and ultimately cause the cancer cell to undergo apoptosis.

![Figure 1.2: Structure of Gleevec® (Imatinib mesylate) and Desatinib (BMS-354825).]
Chapter I: Introduction

Leukaemia is a type of cancer in which the bone marrow produces an excessive number of abnormal (leukaemic) white blood cells. These abnormal cells suppress the production of normal white blood cells, which act to protect the body against infection. The targeted kinase, that 1 and 2 inhibit, is the protein produced by a DNA translocation ("Philadelphia chromosome") that appears central to the CML disease process. Moreover, 2 has been shown to be active against many of the mutations known to cause resistance of CML patients to the therapy of 1.

The development of targeting therapeutics, such as 1 and 2, contrasts dramatically with the way in which chemotherapy was discovered; as an unsuspected side effect of mustard-gas weapons on people suffering from cancer of the lymph nodes. This became the basis of variations of chemotherapy that kill both healthy and damaged cells. Today chemotherapy is more sophisticated and accurate in targeting specific proteins or process within the cell cycle. Nevertheless, rational drug design is a challenging exercise because cancer is a group of more than 200 diseases, in most cases their etiology is unknown and it is particularly difficult to design selective agents against transformed cells.

1.3 Targeting DNA

DNA as the carrier of genetic information is a major target for drug interaction because of the ability to interfere with transcription (gene expression and protein synthesis) and DNA replication. Anticancer drugs that attack DNA use this epigenetic information to target their preferred DNA sequences.

The vast majority of naturally occurring DNA is in the B-form (Figure 1.3), consisting of two polynucleotide strands wound about a common axis with a right handed twist. A molecule of DNA consists of two strands, composed of a large number of chemical compounds, called nucleotides, linked together to form a chain. These chains are arranged like a

---

* Copyright © 2004 Kothavale, M.; Markwort, R.; Sandhu, P. The University of Birmingham.
ladder that has been twisted into the shape of a winding staircase, called a double helix. Each nucleotide consists of three units: a sugar molecule called deoxyribose, a phosphate group, and one of four different nitrogen-containing bases. The four bases are adenine (abbreviated A), guanine (G), thymine (T), and cytosine (C). Each turn of the helix contains 10 base pairs and the distance between base pairs is 3.4 Å.

The deoxyribose molecule occupies the centre position in the nucleotide, flanked by a phosphate group on one side and a base on the other. The phosphate group of each nucleotide is also linked to the deoxyribose of the adjacent nucleotide in the chain. These linked deoxyribose-phosphate subunits form the parallel side rails of the ladder. The bases face inward toward each other, forming the rungs of the ladder. The sequence of bases along each strand provides all the genetic information needed to carry out the cells activities. The double helix leaves two edges on each base exposed; one edge in the minor groove and the other in the major groove (Figure 1.4). The major and minor grooves differ significantly in electrostatic potential, hydrogen bonding characteristics, steric effects and hydration.

Figure 1.4: The major and minor grooves within B-DNA. (R = deoxyribosyl linkage to DNA polymer)

Having briefly reviewing the structure of DNA, it is openly apparent that DNA has many features that make it amenable as a potential drug target, such as:

- The hydrophobicity, the presence of heteroatoms and the level of unsaturation of the four nucleobases, together with the specificity of hydrogen bonding and π-stacking interactions between them, make them chemically accessible and ideal for DNA specific drug-target interactions.
Chapter 1: Introduction

- The particular topology of the DNA helix and the uniqueness of any long nucleobase sequence should provide further potential DNA specific drug-target interactions.

Conversely, DNA is a complex drug design target. In humans, a unique DNA sequence comprises a span of about 15-16 bases, which can only be read from the exterior of the helical structure. Two bases can be read from the minor groove and four from the major groove. A base specific targeting molecule must therefore be relatively large. Most DNA interactive drugs are however, small molecules. This is generally the case because the pharmaceutical industry prefers producing small molecular weight drugs for solubility reasons. Thus, the design of a DNA interactive small molecule with high specificity is quite challenging. Furthermore, the location of DNA in the cell makes it a more difficult target than enzymes or receptors as a drug must traverse two membranes, the cell membrane and the nuclear membrane, in order to interact with DNA.

Drug-DNA interactions may be classified as either: (i) covalent binding, or (ii) noncovalent binding. Covalent binding involves alkylation or coordination of the drug to DNA. There are three types of noncovalent binding mechanisms relevant to DNA-drug interactions (i) intercalation, (ii) groove binding and (ii) electrostatic interactions.

Electrostatic interactions occur between the negatively charged phosphate backbone and positively charged amino groups, or any (+)-charged species. Intercalation occurs when planar, heteroaromatic molecules stack between the base pairs, thereby distorting the DNA backbone conformation and preventing association with the DNA topoisomerase enzymes that initiate DNA replication. Doxorubicin, and Ethidium bromide, are examples of drugs, with planar aromatic regions, which can intercalate between the base pairs of DNA.
Doxorubicin also has a groove-binding domain and is one of the most potent and clinically useful agents of the anthracycline antibiotic family currently used in cancer chemotherapy. Groove binding occurs in either the minor or major grooves of DNA. Drugs bind in the grooves through hydrogen bond donor or acceptor sites and cause little distortion of the DNA backbone. Distamycin, and Netropsin, are examples of natural products, which are comprised of aromatic rings joined by linkages such that they have an overall curvature that matches that of the minor groove. They also have hydrogen-bond donors on the inside edge and they have one or more positive charge at physiological pH. The combination of these features has given rise to preferential binding of these molecules at A-T sequences in the minor groove.

New classes of synthetic polyamides have evolved from that can bind to a range of DNA sequences with high affinity and specificity. These molecules contain hairpin pyrrole-imidazole (Py/Im) amino acids and can permeate living cells, enter the nucleus and downregulate endogenous gene expression. Dervan et al., developed pairing rules for these polyamides so that the sequence specificity of predetermined DNA sequences can be controlled. For instance polyamide 7, which contains an unsymmetrical combination of
three rings—Py, Im and Hp, was found to specifically recognize each of the four Watson-Crick base pairs: Im/Py and Hp/Py were specific for G.C and T.A sequences, respectively.\textsuperscript{27} The advancement in this area of research has been vast; further pairing rules have been established, binding models structurally verified, and biological applications for these polyamides have been discovered.\textsuperscript{28}

Despite these advances, DNA intercalating agents are still some of the most common anticancer drugs used in clinical therapy of human tumours.\textsuperscript{29} Among this category of DNA binders known to have antitumour activity are the 1,8-naphthalimides,\textsuperscript{30} as previously mentioned.

1.4 Antitumor Mono-naphthalimides- Historical background

In 1973, Bräna \textit{et al.}\textsuperscript{31} initiated the development of novel anticancer agents based on the 1,8-naphthalimide structure, which were known from the dyestuff industry.\textsuperscript{32} The lineage of this compound can be traced back to the (i) cytotoxic aristolochic acid, which contains a \(\beta\)-nitronaphthalene moiety, (ii) glutarimide rings of cycloheximide and CG-603, and (iii) the basic side chain of tilorone (Figure 1.5 (a))\textsuperscript{30}

![Figure 1.5: (a) Cytotoxic compounds considered in the design of naphthalimides. (b) The best monosubstituted mononaphthalimides.](image-url)
With this in mind, Brâna et al.\textsuperscript{33} synthesised a large series of imide derivatives of 1,8-naphthalic acid by varying the side chain and the naphthalimide ring substituent. Waring et al.\textsuperscript{34} showed that compounds of this type bind to DNA, increase the length of sonicated DNA and cause unwinding of closed circular superhelical DNA, characteristics typical of intercalating agents.\textsuperscript{15}

Brâna et al.\textsuperscript{30} found the following influential in achieving the best antitumour activity (Figure 1.1 (b)): (a) a basic terminal group in the side chain must be present, (b) the terminal nitrogen of the side chain must be separated from the naphthalic ring nitrogen by two methylene units, (c) the terminal nitrogen atom should not have many attached substituents, and (d) substitution should occur at position 3 in the naphthalic ring. The latter was confirmed by Wilson et al.\textsuperscript{35d} who synthesised and tested the DNA binding ability of a series of naphthalimides with a 3-(dimethylamino)propyl group on the imide nitrogen. The 3-nitro analogue had a greater binding affinity for DNA compared to the 4-nitro derivative. A rationale for this is steric effects where the nitro group in position 4 rotates significantly out of the monoimide plane, leading to perturbed stacking in the intercalated complex.

Two of the most active naphthalimides of the Brâna series were Amonafide, 8 and Mitonafide, 9.\textsuperscript{30} Clinical evaluation showed that both had high activity against human tumour cell lines; 8 exhibiting an IC\textsubscript{50} value, (inhibitory concentration of a drug needed to inhibit viral replication by 50 %), of 0.47 μM and 9 an IC\textsubscript{50} value of 8.80 μM against HeLa cell lines in culture.\textsuperscript{35a,b} In fact, the dihydrochloride salt of 8 developed by ChemGenex Pharmaceuticals (under the generic name of Quinamed ®) has successfully entered into phase II clinical trials for prostate cancer (CaP).\textsuperscript{35c}

Their mechanism of action involves binding to double-stranded DNA by intercalation and hindering the religation step of Topoisomerase II action.\textsuperscript{36} Mitonafide 9 has been shown to inhibit DNA synthesis and induce strand breaks in Chinese hamster ovary cells.\textsuperscript{37}
Andersson and Beran have observed that amonafide produces DNA single and double strand breaks.\textsuperscript{38} It also produces DNA-protein cross-links in human myeloid leukaemia cells through a \textit{Topoisomerase II}-mediated reaction.\textsuperscript{39} Nevertheless, they have different mechanism of actions. The electron withdrawing nitro group in Mitonafide favours the formation of a charge-transfer complex with the DNA bases. On the other hand, the electron donor amino group in Amonafide tends to form hydrogen bonds with the sugar phosphate chain leading to a stabilization of the DNA-drug complex.\textsuperscript{40}

Many approaches have been undertaken to improve the activity of the mononaphthalimides. Bräna \textit{et al.}\textsuperscript{40} designed a new series of mononaphthalimides containing both the nitro and amino group ring substituents. These disubstituted naphthalimides actually showed higher cytotoxicity than monosubstituted mitonafide and amonafide. Zee-Cheng and Cheng\textsuperscript{41} prepared several dinitro and diamine derivatives of mitonafide, which possessed, both \textit{in vitro} and \textit{in vivo}, prominent antileukemia and antimelanoma activity, while Remers \textit{et al.}\textsuperscript{42} substituted an anthracene for the naphthalimide unit to reinforce its DNA binding capacity. The resulting drug, Azonafide 10 exhibited an increase in cellular cytotoxic potency over amonafide\textsuperscript{43}, presumably because of the higher affinity of azonafide for DNA than of Amonafide.\textsuperscript{44} These promising results then lead to the synthesis and testing of phenanthrene and azaphenanthrene analogues of azonafide, 11 - 13.\textsuperscript{45} They proved to be less potent than azonafide against tumour cells indicating that the linear anthracene chromophore is preferred.
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So far, the main efforts have been directed at increasing the binding capacity to DNA. However, no attempt has been made to link the naphthalimides with known minor groove-binding agents. In light of this, Gupta et al.\textsuperscript{46} reported on the synthesis, DNA sequence specificity and biological evaluation of DNA-directed alkylating agents \textbf{14 (a)-(c)} comprising of naphthalimide, nitrogen mustard and lexitropsin moieties. The biological properties of these compounds were tested against KB human nasopharangeal tumour cells and, interestingly, the compound lacking the lexitropsin moiety was the most active.

![Chemical structure](image)

To further enhance the potency of their mononaphthalimides, Br\={a}na \textit{et al.} made three key studies. A series of mononaphthalimides in which an imidazole ring was fused to the naphthalene unit, were developed.\textsuperscript{47} However, dimerization of the chromophore resulted in no significant improvement in antitumour activity. This discovery prompted Br\={a}na \textit{et al.}\textsuperscript{48} to explore the DNA binding capability of Amonafide derivatives bearing a \(\pi\)-deficient pyrazine ring fused to the naphthalene moiety. The pyrazonaphthalimide \textbf{15} showed values of IC\(_{50}\) very similar to the values found for Amonifide in the same experimental conditions, and exhibited typical intercalation capability.\textsuperscript{49} It was shown also to inhibit DNA relaxation by \textit{Topoisomerase I} and \textit{Topoisomerase II}. The most recent contribution from Br\={a}na \textit{et al.}\textsuperscript{50} involved the design and synthesis of Amonafide related monointercalators, all of which were capable of forming stable DNA complexes. In this case, the naphthalimide unit was fused to a furan or thiophene ring, which increased the planar surface of the chromophore and enhanced its stacking properties. The planar chromophore of the most active furonaphthalimide,
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MCI3334, 16, intercalates between the GC base pairs and the appended cationic side chain is directed toward the major groove (Figure. 1.6). This molecule was also shown not to promote DNA cleavage by Topoisomerase II, in contrast to the related monomer, aminoflide.\(^{52}\)

![Intercalating Domain and Major Groove Binding Domain](image)

**Figure 1.6:** Schematic representation of the binding of 16 to DNA.

These latest attempts have allowed Brana et al., to propose that the electronic nature of the fused heterocycle has no influence over the DNA binding process of either the latter naphthalimides or the imidazo- or the pyrazinonaohthalimide analogues. The fusion of a heterocycle to the naphthalene moiety has reinforced the stacking properties, but this is apparently at the expense of Topoisomerase II inhibition. The incorporation of two naphthalimide units into the same molecule has been carried out, resulting in higher binding affinity and increased anti-tumor activity.\(^{30}\) The progress of this work has been thoroughly reviewed by Phelan, Hussey and Gillespie,\(^3\) and so will not be discussed in this Chapter. However, a few examples will be discussed in Chapter 3.

1.5 Sequence Specific DNA Cleaving Proteins

A specific binding site is recognized in terms of specific sequences of base pairs. The only mechanism that can discriminate individual base pairs in double stranded DNA is complementary hydrogen bonding through the major or minor groove.\(^{53,54}\) Most proteins exhibit binding specificity through interaction with the major groove of DNA.\(^{15}\)

Some DNA-interactive drugs initially bind to DNA but then, under certain conditions, react in such a way as to cause strand cleavage.\(^{15}\) DNA cleavage usually occurs by radical generation followed by oxygen dependent cleavage of the ribose phosphate backbone.\(^{15}\)

In 1988, Iverson et al.\(^{55}\) reported on the design and synthesis of a sequence specific DNA cleaving metalloprotein consisting wholly of naturally occurring \(\alpha\)-amino acids. A
new 55-residue protein with two structural domains was afforded and its DNA cleaving capabilities analysed.\textsuperscript{55a} The two structural domains had distinct functions, sequence specific recognition, and cleavage of double helical DNA. The formation of this new protein, GGH (Hin 139-190) 17, was achieved by the attachment of the copper binding domain, the tripeptide H-Gly-Gly-His-OH, to the amino terminus of the DNA binding domain of Hin recombinase (residues 139-190). The DNA binding location of the new protein was found to be at the four Hin sites, each 13 base pairs in length. However, GGH only exhibited strong DNA cleavage by oxidative degradation at one of the Hin half sites. Nevertheless, the Gly-Gly-His ligand system was shown to be chemically activated in the presence of Cu(II) or Ni(II) to produce oxidizing equivalents capable of effecting DNA or protein cleavage.\textsuperscript{55,56} However, a limitation of this metal binding tripeptide GGH, is that its incorporation is restricted to the amino terminus of polypeptide structures.

As a consequence, Long et al.\textsuperscript{57} created a more versatile peptide-based system, which preserved the metal binding, electronic and catalytic properties of GGH. The strategy involved the synthetic redesign of the latter into two model tripeptides 18 and 19 each containing a carboxy-terminal and interior Cu(II)-binding domain. In essence, this simple modification left the interior $\alpha$-amino group of ornithine free to participate in Cu(II) complexation while allowing the incorporation of its ($\delta$)-amino group into any sterically-permissable location along the polypeptide chain.
1.6 Peptide-Based DNA Intercalators

The design and synthesis of novel sequence-specific DNA-binding agents has received considerable interest. Much research has focused on enhancing selectivity and binding affinity by combining the effects of two different types of binding motifs on the same molecule.

Iverson et al. developed polyintercalating molecules comprising naphthalene diimides linked by peptide chains. The compounds fully intercalate upon binding to DNA and exhibit a preference for GC sequences. To achieve the optimum length and flexibility required to span two base pairs, the peptide linkers comprise four amino acids. Simply changing the sequence of the peptide linker segment could alter the sequence specificity of the molecules. In fact, Plaumbo et al. showed that the amino acid composition of certain chiral peptidyl-anthraquinones play a key role in DNA-binding, sequence specificity and cytotoxicity.

Dixon et al. improved the DNA cleavage abilities of the metal chelating Gly-Gly-His motifs by the attachment of the known threading intercalator, naphthalene diimide. In the presence of nickel and Oxone, the Ni(II).GGH motif appended to a naphthalene diimide intercalator was approximately 100-fold more efficient at inducing single-stranded cleavage than Ni(II).GGH-CONH₂ itself. The side chains provide the necessary eight rotatable bonds between the diimide nucleus and the Ni(II).GGH moiety allowing the Ni center to reach the expected site of attack, the H-4' in the minor groove. The addition of a second Ni(II).GGH did not improve the cleavage efficiency of the mono-Gly-Gly-His-NDI motif.

In an effort to design practically useful DNA-cleaving amino acids, Saito et al. incorporated the water-soluble L-Lysine into a 1,8-napthalimide moiety. The resulting compounds, 21-23, exhibited high DNA cleavage efficiency and selectivity upon photoirradiation at 320-380 nm. In fact, the presence of a nitro group in the napthalimide ring, changed the selectivity of DNA cleavage from the 5' side of guanine-guanine (-GG-) sequences to a thymine (T)-specific cleavage. Furthermore, the 4-nitro derivative, 23
cleaved at both 5-GG-3 and T sites to an equal extent. Thus, simply changing the aromatic substitution pattern of the naphthalimide can alter the sequence selectivity of the DNA-cleavage.

In summary, extensive research has shown that 1,8-naphthalimides are a class of lead compounds with high anti-tumour activity. It has also been shown that there is a great potential for peptide-based ligands to achieve sequence specific DNA binding. Hence, the pursuit of enhanced selectivity and stronger binding affinity may well be fulfilled, by combining the intercalating chromophore of naphthalimide with sequence specific peptidyl side chains.

1.7 Recent Advances by Gunnlaugsson et al.

Gunnlaugsson et al., are involved in the design and incorporation of nucleic acid recognition moieties into the naphthalimide structure. To date several hundred mono- and di-peptide-based 1,8-naphthalimide conjugates have been synthesised, using solution phase peptide chemistry. The basic structure comprises either an amino, nitro, chloro or bromo substituted naphthalimide chromophore connected to a carboxylic anchor functionalised with a variety of \( \alpha \)-amino acid/esters such as glycine, \( L \)-alanine, leucine, phenylalanine (Figure 1.7) and \( L \)-lysine (Figure 1.8).

![Diagram of naphthalimide structures](image)

**Figure 1.7:** Examples of some of the mono-and di-peptide based naphthalimide derivatives.
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Figure 1.8: Examples of some of the L-lysine containing mono-and di-peptide based naphthalimide derivatives.

All of the peptide-based naphthalimide series synthesised to date have been tested in HL-60 cell lines (a CML cell line) and a selection of them in the more resistant K562 and PC-3 cell lines. These studies were carried out in collaboration with Daniel Ómar Frímannsson, Dr. Tony McElligott and Prof. Mark Lawlor, Department of Haematology, St. James Hospital, Dublin. From these results, Gunnlaugsson et al., in a manner similar to Brâna et al., have established their own building rules. On comparing the activities of peptide-based naphthalimides, the following have been found influential in achieving the best antitumor activity: (a) a large side chain amino acid should be present, i.e. leucine and phenylalanine are more active than glycine and alanine derivatives, (b) in the dipeptides, leucine should be positioned as the first amino acid followed by alanine, when reading from the 1,8-naphthalimide terminus, (c) (S)-α-amino acids should be used rather that the (R)-enantiomers, and (d) substitution by a nitro group in the 4-position of the naphthalimide unit should occur.

The latter is in contradiction to the 3-position rule established by Brâna et al. Nevertheless, some of the 4-nitro substituted compounds show only a ten-fold decrease in activity compared to the reference compound 8. In fact, the L-phenylalanine methyl ester 4-nitro derivatives showed an IC$_{50}$ value of 1.80 μM while 8 showed an IC$_{50}$ value of 0.23 μM. Furthermore, there is a lack of activity seen for either the 3- or 4-amino derivatives. For instance, the L-leucine methyl ester 4-nitro derivative showed an IC$_{50}$ value of 2.20 μM, while, the value for its amino derivative was 22.0 μM.

Of the lysine-based 1,8-naphthalimide derivatives, (Figure 1.8), the most active molecule was the Boc-protected 4-nitro derivative (IC$_{50}$ = 2.0 μM), while the Cbz-lysine
tert-butyl ester 4-amino derivative was the least active (IC$_{50}$ > 100 μM). Furthermore, the di-peptide based 1,8-naphthalimide consisting of either leucine and Boc-lysine amino acids (IC$_{50}$ = 1.4 μM) or phenylalanine and lysine amino acids (IC$_{50}$ > 50 μM) were found to be the most and least potent, respectively. Removal of the lysine-protecting group to yield a family of charged dipeptides also resulted in an approximate 10-fold decrease in activity in comparison to the protected derivatives. These results were surprising as it was expected that the presence of a positive charge might enhance their ability to bind DNA by interacting electrostatically with the anionic phosphate group of the helix. An inability to enter into the cell and cross the hydrophobic membrane was given as a rationale for these results.

The ability of the latter charged molecules to bind DNA was evaluated using UV/Vis, fluorescence, circular dichroism (CD), and melting temperature experiments ($T_m$). The measurements were conducted using 24 – 25, in aqueous buffered solution, and the binding constants determined from the absorbance data were 8.6 x 10$^3$ M$^{-1}$ and 2.1 x 10$^4$ M$^{-1}$, respectively. A slight increase in the melting temperature of ct-DNA (68 °C → 71 °C) was also observed, indicating some stabilisation of the DNA helix by these molecules towards thermal denaturation. The CD studies showed that these molecules could also enhance the chirality of ct-DNA, further supporting an efficient binding interaction, possibly through an intercalation binding mechanism.

A large range of peptide-based bis-naphthalimides has also been developed. The first family synthesised by Phelan and Blais$^3$ (Figure 1.9), involved the linkage of two 1,8-naphthalimide chromophores using either 1,3-propane or spermidine to give the “Side on Side” derivatives, while the “Top to Tail” derivatives were obtained by linkage via an amine linker from the 4-position of one chromophore to the imide position of the other.
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Figure 1.9: Examples of some of the (a) "Side on Side" and (b) "Top to Tail" bis-naphthalimide derivatives.

The general trend in cytotoxicity for these molecules followed that of the mono-naphthalimide series, with the 4-nitro derivatives being more potent than the 3-amino analogues. Nevertheless, the activity of the “Side on Side” bis-naphthalimides was much less in comparison to their parent molecules. Replacing 1,3-propane for the longer spermidine linker also resulted in a further reduction in their cytotoxicity. With regards to the “Top to Tail” bis-naphthalimides, the greatest activity was seen for the 4-amino substituted derivatives with leucine methyl ester as the amino acid. With an IC\textsubscript{50} value of 1.2 µM, this molecule was the most active compound of the peptide-based 1,8-naphthalimide series.

The second family of bis-naphthalimides (synthesized by Hussey)\textsuperscript{3} consisted of “Head to Head”, “Tail to Tail” and “Head to Tail” L-lysine based derivatives. Figure 1.10 shows examples of the most and least potent of these molecules along with their corresponding IC\textsubscript{50} values (HL-60 cell line). Similar trends were observed, as previously discussed, with the 4-nitro derivatives being more active than the corresponding 3-amino or 4-nitro derivatives. This provided further evidence that the introduction of an amino group at the 4-position, instead of a nitro group, leads to diminished activity and lowers IC\textsubscript{50} values. The DNA-binding interactions of these molecules were not evaluated because they were not water-soluble, a problem observed for many of the compounds discussed above.
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The final family consisted of a number of symmetrical "Head to Head" and "Tail to Tail" naphthalene diimide linked bis-naphthalimides. These molecules were synthesized by Gillespie,\textsuperscript{3c} and the most active of these in the HL-60 cell lines are shown in Figure 1.11.

On comparing the activity of these molecules, the following have been found influential in achieving the best antitumour activity: (a) "head-to-head" naphthalene linked
bis-naphthalimides are better than the "tail-to-tail" derivatives (b) in the dipeptides derivatives, phenylalanine should be positioned as the first amino acid followed by alanine when reading from the 1,8-naphthalimide terminus.

The Cbz-lysine-alanine functionalised "Tail to Tail" derivative, which contained an amino-hexyl linker chain, showed an IC$_{50}$ of 1.51 $\mu$M, whereas the propyl analogue showed no cytotoxic activity during the incubation period of 24 h. Nevertheless, removal of the Cbz protecting group from this molecule resulted in an enhancement in cytotoxicity (IC$_{50}$ = 8.14 $\mu$M). This was attributed to a reduction in the size of the molecule allowing it to be more easily transported across the cell membrane. Overall, these results indicated that the length of the linker chain played a role in potency, with the general trend being; hexyl $>$ pentyl $>$ propyl. These trends were similar to the binding constants ($K_b$) determined from the UV/Vis DNA-binding experiments. Here, the Cbz-lysine-alanine derivatives showed $K_b$ values ranging between $10^4 \rightarrow 10^5$ M$^{-1}$, where the hexyl analogue exhibited the largest value, $K_b = 4.0 \times 10^5$ M$^{-1}$.

All of the peptide-based naphthalimide, bis-naphthalimide and the naphthalimide conjugates, along with exhibiting $\mu$M IC$_{50}$ values, have also been shown to inhibit cell cycle arrest and induce apoptosis in HL-60, K562 and the PC-3 cell lines. Furthermore, fluorescence imaging and co-staining studies, (the results from which will be discussed in the Thesis of Mr. Frömmansson), have shown that some of the naphthalimide conjugates can penetrate the nucleus of HL-60 cells.

Building on work already carried out in the Gunnlaugsson group, the aim of one of the projects discussed within this Thesis was to investigate whether simple modifications of the peptide moiety, would endow improved biological activity. As previously mentioned, one such modification will involve the incorporation of thiazole units found in many naturally occurring cytotoxic cyclic peptides.

1.8 Thiazole Containing Cyclic Peptides.

Two classes of thiazole containing cyclic peptides which exhibit cytotoxic activity, are the lissoclinamides and the patellamides/ulithiacyclamides.$^{64}$ In particular, lissoclinolides 4, 29, and 7, 30, have been shown to be active against MRC5CV1 and T24 cells lines with IC$_{50}$ values of 0.8 $\mu$g/mL and 0.04 $\mu$g/mL, respectively.$^{65}$ These natural products, isolated from the truncate L. patella, which is an ascidian belonging to the mammalian phylum Chordata, are composed of the same sequence of amino acids, but they
differ in the oxidation state of the two sulfur-containing rings, shown in blue and red, respectively, and in the absolute stereochemistry of the amino acids.

Although it is known that the oxazoline and thiazole rings play an important role in the biological activity of these cyclic peptides, their mode of action has not been fully determined to date. However, it has been indicated that these peptides have potential for metal ion chelation and transport in vivo.\(^6\) With this in mind, Pattenden et al.,\(^7\) reported the total synthesis of 29, whereby the thiazoline ring was produced simultaneously with an oxazoline ring in a "one-pot" double cyclodehydration sequence from the cyclopeptide precursor 31 (Figure 1.12). Following this, and with the aim of investigating the capacity of these cyclic peptides to bind metal ions, two 18- and 24-membered ring analogues of 29 were synthesized using cyclooligomerisation/macrolactamisation reactions of thiazole amino acid monomers.\(^8\)

Figure 1.12: Retrosynthetic analysis of 29.
Also isolated from *L. patella* were the octapeptide tawicyclamides A, 34, and B, 35. These molecules differ from 29 and 30 as they lack an oxazoline ring, to the absence of which was attributed their weak cytotoxicity against human colon tumor cell lines (IC$_{50}$ ~ 30 µg/mL). The cyclic peptides that also exhibited cytotoxic activity are Bistramides A, 36, and B, 37. These cyclic peptides were isolated from the ascidian named *Lissoclinum aestrum*, found in the Great Barrier Reef, Australia. These molecules contain oxazoline and thiazoline rings, however, 37 also contains a thiazole amino acid. Both 36 and 37 showed IC$_{50}$ values between 50 and 100 µg/mL against the human MRC5CV1 fibroblasts and T24 bladder carcinoma cells.

The antitumor and the anti-drug resistance properties of members of this family of cyclic peptides warranted the synthetic preparation of natural products related to 36-37. Meyers *et al.*, reported the total synthesis of Bistramide C, 38, and D, 39, which were isolated from a Phillippine collection of *Lissoclinum bistratum*. In particular, 39 is more cytotoxic than the other cyclic hexapeptides and has been found to induce depressant...
effects in mice when administered by intracerebral injection.\textsuperscript{72(b)} The synthetic strategy towards the formation of 38-39 involved the assembly of enantiomerically pure oxazole, thiazole and oxazoline segments derived from amino acids followed by a final macrocyclisation step (Figure 1.13). Many methods exist for the synthesis of amino acid-derived thiazoles, and these will be discussed in more detail in Chapter 2.

![Retrosynthetic analysis of 38 and 39.](image1)

More recently, Kelly \textit{et al.}\textsuperscript{74} reported the synthesis of Bistramide E, 43, and J, 44, which exhibit moderate cytotoxic activity against a human colon tumor cell line. The synthesis was also achieved by forming the thiazole containing dipeptides 45 in a step-wise manner, followed by a final macrocyclisation step (Figure 1.14).

![Retrosynthetic analysis of 43 and 44.](image2)
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As previously mentioned, the anti-drug resistance property exhibited by a number of naturally occurring thiazole containing cyclic peptides, has spurred efforts towards their total synthesis. In particular, Dendroamide A, 46, isolated from a blue-green alga exhibits multiple drug resistance (MDR) in tumor cells that over express the transport protein, P-glycoprotein (Pgp).\(^7\) Pgp functions as a plasma membrane localized pump mechanism that can transport anticancer drugs out of the cell.\(^6\) Therefore, it acts as an important target for cancer therapy and great interest has been focused on the development of effective inhibitors of Pgp-mediated MDR. For instance, the total synthesis of 46 has been achieved by Pattenden \textit{et al.},\(^7\) using peptide cyclooligomerisation procedures, while Smith \textit{et al.},\(^7\) and Kelly \textit{et al.},\(^7\) achieved the synthesis of 46 in a highly convergent fashion from appropriately protected thiazole and oxazole amino acids.

Another family of closely related cyclic peptides isolated from \textit{L. patella}, which have been shown to reduce multi drug resistance \textit{in vitro}, is the patellamides B, 47, and C, 48.\(^7\) In 2001, Jaspars \textit{et al.},\(^7\) studied their metal binding selectivity and it was shown that 48 exhibited extreme selectivity for Cu\(^{2+}\) over Zn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) and Hg\(^{2+}\), indicating that copper is the biologically relevant metal for these cyclic peptides. The significance of this result is that the metal cation complexation by cyclopeptides \textit{in vivo} may facilitate the transport of such cations across the hydrophobic cell membrane, as previously mentioned.

So far discussions have concentrated on families of thiazole and oxazole containing cyclic peptides isolated from marine ascidians. However, many other naturally occurring cyclopeptides have been isolated from microorganisms. For instance, Kanoh and Paco \textit{et al.},\(^8\) recently isolated a new thiazole and oxazole containing cyclic depsipeptide, named mechercharmycin A 49, from \textit{Thermoactinomyces sp. YM3-251}, and from actinomycete strain ES7-008, respectively. This molecule exhibited significant cytotoxicity for A549 cells (human lung cancer) and Jurket cells (human leukaemia) with IC\(_{50}\) values of 40 nM and 46 nM, respectively.
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A structurally related polyoxazole-thiazole-based cyclopeptide, 50 isolated from *Streptomyces nobilis*, has been shown to inhibit the growth of human cervical cancer HeLa Se cells in a dose-dependent manner with an IC_{50} value of 14 nM. Furthermore, the biological activity of 50 is similar to that of Telomestatin, 51, which is a potent telomerase inhibitor and shows promising activity as an anticancer agent. In light of this, Pattenden and Deeley, recently reported the total synthesis of 50 and confirmed that its structure comprised a continuum of five azoles linked via a glycine-valine-isoleucine tripeptide tether.

In summary, many thiazole and/or oxazole-containing cyclic peptides have been recently isolated from marine ascidians and microorganisms. Their activities, including cytotoxicity, multiple drug resistance pump inhibition, as well as their metal binding, and transport properties have led to much synthetic interest. The next section of this introduction will deal with the second theme of this Thesis, the modification of the 1,8-naphthalimide structure using Tröger’s base.

1.9 Tröger’s Base

Tröger’s Base, 52, is a chiral cleft-containing molecule whose chirality with a C_{2} axis of symmetry is provided by two bridgehead sterogenic nitrogen atoms. Tröger’s base, 52, was created when Julius Tröger in 1887 reacted p-toluidine with formaldehyde in the presence of HCl. Half a century later, Spielman established the correct structure of 52, while Wilcox reported its X-ray structure showing that the aromatic rings are orientated approximately at right angles to each other. The dihedral
angle was approximately 104°, thus creating a twist within the molecule. Dihedral angles ranging from 82° to 104° have since been reported for a number of simple dibenzo analogues indicating that the dihedral angle is dependent upon the nature of the substituents on the ring. The geometry of 52, with the aromatic rings almost perpendicular to each other, has played a considerable role in the field of supramolecular chemistry, specifically in molecular recognition. Bag and Král et al. have independently reviewed this area of research. In this section, the properties of 52 and its use for the design of novel DNA binding agents will be discussed.

1.9.1 Chirality of Tröger’s Base

The majority of reported applications of Tröger’s base analogues explore only their geometry and deal with racemates. Consequently, the intended utility of enantiomerically pure Tröger’s base derivatives is limited, and purely hampered by the fact that the resolution of these molecules is difficult to achieve. The resolution of 52 is known to be challenging due to rapid pyramidal inversion of the diamine and such racemization is acid catalysed (Scheme 1). The mechanism for the acid-induced racemization was initially investigated by Prelog and Wieland and was proposed to proceed via the intermediary of the iminium ion 53. On the other hand, spectral studies involving X-ray crystallography, NMR and UV/Vis spectroscopy carried out by Greenberg et al., did not detect such a species in dilute acid solution. The racemization process was thus suggested to involve rapid proton exchange between the two-bridgehead nitrogens. However, the formation of an iminium ion was detected in more concentrated acid giving rise to the conclusion that in dilute acid the iminium ion may have simply been present in undetectable amounts.

Scheme 1: Proposed mechanism for the acid-catalyzed racemization of 52.
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The ease with which 52 can undergo acid-catalyzed enantiomerization, is in fact an important aspect of its chemistry because such racemization is a necessary condition for complete asymmetric transformation into a single enantiomer. This was exploited by Wilen et al.,\textsuperscript{89b} by transforming racemic 52 into (+)-52 in 93\% yield using (-)-1,1′-binaphthalene-2,2′-diyd hydrogen phosphate. Nonetheless, the preparation of both enantiomers is of current importance.

1.8.3 Resolution of Tröger’s Base

In general, the resolution of 52 by the formation of diastereoisomeric salts with enantiomerically pure acids has been considered unfeasible. However, a number of instances of resolution through diastereomeric salt formation with strong chiral acids have been reported.\textsuperscript{80b, 91, 92} The first successful chromatographic resolution of 52 was achieved in 1944 on a D-lactose chiral column.\textsuperscript{88a} Since then, 52 has become a popular standard probe for studying the efficiency of newly designed chiral stationary phases. Sergeyev et al.,\textsuperscript{93} recently demonstrated the successful enantioseparation of a number of Tröger’s base analogues by HPLC on commercially available celluose derived chiracel OJ and brush-type whelk O1 chiral stationary phases.

Owing to the fact that the resolution of 52 is achievable but extremely challenging, an attractive option in avoiding the aforementioned racemization mechanism would be to enhance its configurational stability. Lenev et al.,\textsuperscript{94} recently reported the preparation of configurationally stable, bis-methylated methano and ethano-Tröger’s base derivatives, 54 and 55, respectively. Further racemization kinetic studies carried out on the bis-orthomethyl substituted Tröger’s bases 56 and 57 showed that the enantiomerization barriers of these molecules in acidic media were raised relative to 52. It was believed that the presence of large groups in the ortho-position gave rise to strain in the transition state of 53, which created a rate-deceleration effect by preventing possible rotation about the C4-N5 bond to close the methylene bridge on the opposite side.

![Chemical structures](image-url)
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1.9.3 Applications of Tröger’s Base Analogues

Many researchers have incorporated the cleft structure of 52 into the skeleton of their various supramolecular receptors. For instance, Wilcox, Wilen, Crossley and Hansson among others have utilised 52 for the construction of molecular torsion balances, water-soluble cyclophanes 58, chiral solvating agents and hydrogen bonding receptors 59-60. Tröger’s base 58 was used to bind aminopyridine while 59 was found to bind to small dicarboxylic acids.

The cleft structure of 52 has also been incorporated into macrocycles for the detection of cyclohexanols and various cations. Many other structural analogues have also been used to bind biotin, urea and adenine. In particular, Crossley et al. designed a bisporphyrin Tröger’s base analogue which was found to bind enantioselectivity to lysine benzyl esters and histidine esters. In this way, the design of 61 took advantage of the chirality of Tröger’s base to result in the differential binding of enantiomers.
1.8.5 Tröger’s Base Analogues as Targets of DNA

Despite the fact that the geometry of the Tröger’s base could give a helical shape to a molecule that may possibly be the same as the helicity of DNA, there are only a very small number of reports within the literature on the construction of such DNA-binding motifs. Yashima et al.\textsuperscript{101} pioneered this work by studying the chiral recognition of a racemic bis-(1,10-phenanthroline) containing Tröger’s base by DNA. Circular dichromism (CD) measurements showed that racemic 62 interacted with DNA to a higher degree than the parent 5-amino-1,10-phenthroline and a copper (I) complex of 62 caused strand scission by conversion of closed circular pUC18 plasmid to open circular DNA.\textsuperscript{101}

![Image of molecule 62]

In the same manner, Demeunynck et al.\textsuperscript{102, 103} developed a symmetric acridine containing Tröger’s base 63, which gave encouraging results for the enantioselective recognition of DNA. The binding of 63 to DNA was evaluated using UV/Vis and circular dichroism studies, which also indicated that such interaction might be accompanied by protonation of the two-acridine moieties.\textsuperscript{102} Successful resolution of 63 was then achieved and the (-)-(7R,17R) enantiomer was primarily shown to bind preferentially to calf-thymus DNA (ct-DNA) by liquid-liquid partition studies of (±)-63. In fact such binding was later shown to be both enantio- and sequence-specific.\textsuperscript{103}

![Image of molecule 63]

\( T_m \) measurements, complementary biochemical assays using DNA Topoisomerase inhibition and DNase I footprinting experiments were also conducted. Results indicated that the (-)-isomer of 63 recognised certain DNA sequences containing both A.T and G.C
base pairs while the (+)-isomer showed no sequence selectivity. The mode of binding of 63, was suggested to involve interaction of the acridine ligand within the minor groove of the double helix, due to a lack of interference with the methylation of N7 guanidine residues of DNA. Typically, acridine derivatives are strong intercalators, however, on this occasion, results from both electric linear dichromism (ELD) and topoisomerase inhibition measurements indicated that the acridine rings of 63 were not both intercalated into DNA. Accordingly, molecular modelling studies of the interaction of the (R,R) and (S,S) isomers of 63 with short oligonucleotides was carried out. Two binding modes were analysed, as a means of resolving the above binding discrepancy, where one of the acridine ring is intercalated between adjacent base pairs and the other acridine rings is set in the minor or major groove (Figure 3.3). Unfortunately the elucidation of the exact mode of binding of 63 with DNA was not achieved because the symmetric structure of this molecule complicated the analysis of the DNA interaction process.

To circumvent this problem, Bailly et al. prepared the asymmetric racemic acridine-phenthroline Tröger's base 64 and studied its interaction with DNA using complementary biochemical and biophysical methods.

Figure 1.15: Acridine Tröger's base 63 binding with DNA: (a) through intercalation with minor groove interaction and (b) in the minor groove.

To circumvent this problem, Bailly et al. prepared the asymmetric racemic acridine-phenthroline Tröger's base 64 and studied its interaction with DNA using complementary biochemical and biophysical methods.
Spectroscopic methods using UV/Vis, and T_m experiments, showed that racemic 64 interacted with DNA, and both CD and electric linear dichromism (ELD) experiments indicated a bimodal binding process, implicating intercalation of an acridine ring coupled with groove binding of the phenanthroline moiety. Such measurements were possible because the acridine and the phenanthroline chromophores have different spectroscopic properties. Additionally, at high drug concentration, 64 inhibited the relaxation of supercoiled plasmid DNA by topoisomerase I, providing further evidence for an intercalating binding process. The sequence selectivity of 64 was also investigated by DNase I footprinting using a \(^{32}\)P-radiolabeled DNA restriction fragment of 117 base pairs.\(^{105}\) The results obtained were quite interesting as the footprint showed that the sequence selectivity of 64 was similar to that of the phenanthroline analog 62, rather than that of the parent acridine 63. From this, the triplet sequences 5'-GTC-5'-GAC were suggested as providing an optimal binding site for Tröger's base 64.

In recent work by Král et al.,\(^{106}\) a series of distamycin analogues 65-68 possessing Tröger's base were prepared, as enantio-selective bidentate minor groove binders. As previously mentioned, Distamycin A is a naturally occurring antibiotic agent that can reversibly bind to the minor groove of DNA and exhibits a strong preference for A.T rich sequences.\(^{23}\) Conversely, DNA binding studies using ethidium bromide displacement experiments showed that for 67 and 68, the presence of the Tröger's base unit caused an enhanced affinity to G.C sequences. Furthermore, such displacement assays showed that the enantiomer (4R,9R) of 67 had an enhanced affinity for DNA and exhibited higher discrimination for poly(dA-dT)\(^2\).\(^{106}\)

In summary, the work of Demeunynck, Bailly and Král et al. has shown that Tröger's base is a rational structural unit for the incorporation into DNA-targeting agents for achieving enhanced binding affinity and enantioselective recognition of the nucleic acid
structure. However, as stated above only a few such examples have been developed to date. Hence, the second theme of this Thesis will deal with the incorporation of such structures into the 1,8-naphthalimide unit.

1.10 Conclusion

The interaction of small molecules with nucleic acids has been the subject of intense interest, and an understanding of such interactions has been pivotal to the design and development of novel chemotherapeutic agents. In this Chapter, the discovery and the progression of the mono-1,8-naphthalimides as potential anticancer agents has been discussed. This research represents an important contribution to the design of potent therapeutic agents, some of which have entered clinical trials. For example, Quinamed® developed by GhemGenex Pharmaceutica, has been shown to be active in Gleevec resistant patients with prostate cancer.\textsuperscript{11}

The utility of DNA cleaving proteins for achieving greater sequence specificity was also discussed. The results have shown that the use of aromatic-peptide conjugates is of particular interest, where the peptide acts as a DNA recognition site and the aromatic unit as an intercalator, and/or groove binder. With this in mind, Gunnlaugsson et al., have developed a large family of peptide-based 1,8-naphthalimide derivatives, many of which have exhibited potent cytotoxic activity in leukaemia cell lines.

This chapter has also focused on the natural occurrence of thiazole and oxazole containing cyclic peptides from marine ascidians and microorganisms. Their potent cytotoxic activity and proven ability to reverse multi-drug resistance has spurred much effort in achieving their total synthesis. Moreover, these results have suggested that there exists the potential of achieving potent biological activity by incorporating such thiazole amino acids with other known anticancer drugs.

The final section of this Chapter, dealt with the Tröger’s base, which in the past, has been used as receptors, as well as chiral solvating agents. More recently, the idea emerged that these chiral cleft-like molecules may represent useful probes of nucleic acid structures. Despite the many highly desirable properties of Tröger’s base, there only exist a few reports on its incorporation into known DNA-binding motifs and these examples were reviewed. Nevertheless, the results from these investigations has provided valuable information about the manner in which these molecules bind to DNA and thus have
highlighted the utility of the cleft structure in the design of DNA targeting agents. Consequently, there exists a window of opportunity to exploit this research.

From this conclusion, two main areas have been reviewed (Thiazole amino acids and Tröger’s bases). These structures had not previously been incorporated into the 1,8-naphthalimide building block. The objective of this PhD work was to explore such targets, and investigate their anticancer properties using various methods. This work is outlined in the following section.

1.11 Work Described Within this Thesis

Building on work already carried out within the Gunnlaugsson group, the aim of the work discussed in Chapters 2 and 5, respectively, is to investigate whether simple modifications of the peptide moiety will endow improved biological activity. This will be achieved by the construction of a family of functionalised peptide-based naphthalimide derivatives. A series of naphthalimide based optically active α-amino aldehydes, such as 69, will be synthesised as synthons for further synthetic modification. This will involve a chemical transformation with the aim of developing α,β-unsaturated esters and thiazole amino acids, such as 70 and 71, respectively. The cytotoxic activity of these molecules will then be investigated and compared to the peptide-based naphthalimide derivatives developed in the Gunnlaugsson group.
Chapter 1: Introduction

The second objective of the work discussed in Chapters 3 and 4, respectively, is to reinforce the DNA binding capacity of the 1,8-naphthalimide, by modifying its chromophore. This will be achieved by the incorporation of the Tröger’s base unit into the skeleton of the naphthalimide structure for the development of potential novel $C_2$-symmetric DNA binding agents, such as \textbf{151-154}. Following the synthesis of these target molecules, their photophysical properties in organic solvents, in water and as a function of pH, will be investigated using UV/Vis and fluorescence spectroscopy.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\end{figure}

In Chapter 4, the DNA-binding affinities of \textbf{151 - 154} in aqueous solution will be evaluated. These evaluations will be carried out with ct-DNA and the homopolymers, poly(dG-dC) and poly(dA-dT), using UV/Vis and fluorescence spectroscopy and ethidium bromide displacement assays. $T_m$ experiments and CD studies will also be conducted to further help in understanding the nature of their DNA-binding interactions.

In Chapter 5, the cytotoxic activity of the functionalised peptide-based naphthalimide derivatives and those containing Tröger’s base will be evaluated. Their ability to induce apoptosis in cancer cells and inhibit \textit{topoisomerase} action will also be investigated. Mr. Daniel Ömar Frímannsson, at the Department of Haematology, St. James Hospital, carried out this research and will present the final data in his own PhD thesis, which is to be submitted in early 2008. Nevertheless, some highlights of these results will be included in this Thesis.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

2.1 Introduction

Aldehydes are very versatile functional groups that are widely used in organic synthesis. Basic transformations that can be carried out on these include Diels-Alder cycloaddition, aldol condensation and Wittig reactions. The condensation reaction of an α-amino aldehyde with the amino moiety of cysteine derivatives gives rise to the formation of thiazolidine derivatives. Oxidation of these products then leads to the corresponding thiazole derivatives. Therefore, an aldehyde functionality acts as an excellent starting point for the further development of functionalised α-amino acid-based naphthalimide derivatives, which is the main theme of this Chapter.

The first section of this Chapter discusses the development of alkene modified 1,8-naphthalimide derivatives from optically active α-amino aldehydes. Both Phelan and Hussey made many attempts at achieving the synthesis of these molecules using the Wittig reaction from the corresponding aldehyde, but with limited success, due to the general unreactivity of the α-amino aldehyde moiety. This was most likely due to steric constraints inflicted by the naphthalimide moiety. Consequently, for this work a different synthetic approach was devised. As mentioned in Chapter 1, Gunnlaugsson et al. have shown that a nitro group in the 4-position of the naphthalimide unit contributes to a good cytotoxic activity. As a result, the herein research focuses on using 4-nitro substituted α-amino-(1,8-naphthalimide) aldehyde as the starting material. The synthesis involves the use of (S)-α-amino acids, alanine, leucine and phenylalanine, and also the R-enantiomer of phenylalanine, all of which were then transformed into an α-amino aldehyde. The reasons for the predominant use of (S)-α-amino acids being, in nature, proteins, which exhibit site-specific DNA interactions, are comprised almost exclusively of (S)-α-amino acids.

The second section of this Chapter deals with the synthesis of thiazole modified 1,8-naphthalimides containing α-amino acids. As a means of achieving this, two synthetic approaches were considered. The first approach involved the synthesis of the thiazole directly from the α-amino-(1,8-naphthalimide) aldehyde precursor. The second and preferred approach would be to develop the target molecules in a convergent fashion where thiazole-containing amino acids were prepared and thereafter incorporated into the naphthalimide skeleton.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

2.2 Synthesis of α-Amino-(1,8-naphthalimide) Aldehyde Precursors

A four-step synthesis for the α-amino-(1,8-naphthalimide) aldehyde was developed originally by Phelan\(^3\) and further modified by Hussey\(^3\). The first two steps in the reaction sequence is shown in Scheme 2.1, and involves a condensation reaction (the mechanism of which can be found in the Thesis of Phelan, Hussey and Gillespie, respectively),\(^3\) between the commercially available 4-nitro-1,8-naphthalic anhydride and the hydrochloride salt of the relevant α-amino as its tert-butyl ester derivative. The reaction was carried out refluxing the two components in anhydrous toluene overnight in the presence of 2 equivalents of triethylamine. As the naphthalic anhydrides themselves are sensitive to water, molecular sieves were included in the reaction mixture to remove any water produced. Following this procedure, the α-amino acid-based naphthalimides 72-75 were synthesised and their corresponding yields are listed in Table 2.1. All four were purified by a recrystallisation from MeOH. The next step in the reaction sequence involved ester hydrolysis using neat TFA, which gave 76-79 in high yields as shown in Table 2.1, without the need for further purification.

![Scheme 2.1](image)

**Scheme 2.1**: Preparation of α-amino-(1,8-naphthalimide) derivatives.

<table>
<thead>
<tr>
<th>Cp</th>
<th>R</th>
<th>Yield</th>
<th>Cpd</th>
<th>R</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>CH(_2)CH(CH(_3))(_2) (S)</td>
<td>76 %</td>
<td>76</td>
<td>CH(_2)CH(CH(_3))(_2) (S)</td>
<td>99 %</td>
</tr>
<tr>
<td>73</td>
<td>CH(_2)(C(_6)H(_5)) (S)</td>
<td>84 %</td>
<td>77</td>
<td>CH(_2)(C(_6)H(_5)) (S)</td>
<td>97 %</td>
</tr>
<tr>
<td>74</td>
<td>CH(_2)(C(_6)H(_5)) (R)</td>
<td>61 %</td>
<td>78</td>
<td>CH(_2)(C(_6)H(_5)) (R)</td>
<td>98 %</td>
</tr>
<tr>
<td>75</td>
<td>CH(_3) (S)</td>
<td>66 %</td>
<td>79</td>
<td>CH(_3) (S)</td>
<td>99 %</td>
</tr>
</tbody>
</table>

**Table 2.1**: Synthesis of naphthalimide derivatives using above Scheme 2.1.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

As discussed in Chapter 1, a large number of α-amino acid-based 1,8-naphthalimide derivatives were synthesised by both Phelan and Hussey\textsuperscript{3a,b}, and it was found that all of these molecules retained their chirality. The retention of chirality was investigated using optical rotation experiments, europium \textsuperscript{1}H NMR shift experiments, chiral HPLC measurements and crystal structure analysis. Although 72-79 had been previously synthesised within the Gunnlaugsson group, they were still analysed by \textsuperscript{1}H and \textsuperscript{13}C NMR, mass spectroscopy and by IR analysis to determine their purity. The \textsuperscript{1}H NMR spectrum (CDCl\textsubscript{3}, 400 MHz) of 72 is shown in Figure 2.1. The aromatic region between 8.00 and 8.75 ppm is composed of four doublets and one triplet, all of which integrate for one proton. The α-proton appears as a double-doublet at 5.68 ppm, giving rise to an ABX system. The methylene unit next to the chiral centre, with two non-equivalent protons, appears as two double-double-doublets at 2.08 and 2.21 ppm, respectively. However, this signal appears as a multiplet, due to overlapping of the peaks. The two sets of methylene protons integrate for two protons each, and appear as doublets at 0.94 and 1.02 ppm, while the adjacent CH yields a complex multiplet at 1.55 ppm and integrates for one proton. The protons of the tert-butyl ester-protecting group show as a sharp singlet at 1.45 ppm.

![Figure 2.1: The \textsuperscript{1}H NMR spectrum (CDCl\textsubscript{3}, 400 MHz) of 72.](image)

The next step in the reaction sequence involved the treatment of 76-79 with HO\textsubscript{B}t and EDCI.HCl in dry THF and subsequent peptide coupling of aminoaldehyde diethyl acetal in a one-pot reaction (Scheme 2.2). Each of the acetal derivatives 80 - 83, listed in Table 2.2, was purified by column chromatography (neutral silica, CH\textsubscript{2}Cl\textsubscript{2}/MeOH-NH\textsubscript{3},


37
12:2) and $^1$H NMR (CDCl$_3$, 400 MHz) spectra were consistent with their structures. The synthetic route was completed on removal of the acetal-protecting group using TFA in CH$_2$Cl$_2$ giving the desired aldehyde derivatives 84-87 which were obtained in high yields as listed in Table 2.2.

Scheme 2.2: Preparation of α-amino-(1,8-naphthalimide) acetal and aldehyde derivatives.

Table 2.2: Synthesis of naphthalimide derivatives using above Scheme 2.2.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>R</th>
<th>Yield</th>
<th>Cpd</th>
<th>R</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>CH$_2$CH(CH$_3$)$_2$ (S)</td>
<td>76 %</td>
<td>84</td>
<td>CH$_2$CH(CH$_3$)$_2$ (S)</td>
<td>90 %</td>
</tr>
<tr>
<td>81</td>
<td>CH$_2$(C$_6$H$_5$) (S')</td>
<td>51 %</td>
<td>85</td>
<td>CH$_2$(C$_6$H$_5$) (S)</td>
<td>94 %</td>
</tr>
<tr>
<td>82</td>
<td>CH$_2$(C$_6$H$_5$) (R)</td>
<td>60 %</td>
<td>86</td>
<td>CH$_2$(C$_6$H$_5$) (R)</td>
<td>96 %</td>
</tr>
<tr>
<td>83</td>
<td>CH$_3$ (S')</td>
<td>64 %</td>
<td>87</td>
<td>CH$_3$ (S')</td>
<td>99 %</td>
</tr>
</tbody>
</table>

The $^1$H NMR spectra (CDCl$_3$, 400 MHz) of 81 and 87 are shown in Figure 2.2 and 2.3, respectively. The aromatic region of both spectra includes four doublets and one triplet between 7.9 and 8.8 ppm, while 81 (Figure 2.2) also shows a multiplet between 7.08 and 7.23 ppm. The amide proton of both 81 and 87 appears as a broad triplet at 6.10 ppm and 6.83, respectively, while the α-proton appears as a doublet of doublets at 6.03 ppm and 5.80, respectively. With regards to 81 (Figure 2.2), both the methine proton and the two sets of methyl protons of the acetal moiety appear as triplets at 4.53, 1.22 and 1.09 ppm, respectively. The three sets of methylene protons from the aminoaldehyde diethylacetel part of the molecule appear as two multiplets due to the effect of the chiral centre, making each of them chemically un-equivalent. The protons labelled d and e, show as two double-quartets for each, while those labeled b appear as two double-double-doublets from
coupling with each other, the NH proton and the vicinal methine proton. A 2D TOSCY was used to assign these resonances. $^1$H NMR (CDCl$_3$, 400 MHz) analysis confirmed the formation of 87 by the presence of a singlet at 9.74 and 4.43 ppm for the aldehyde resonance and the adjacent methylene protons, respectively (Figure 2.2). However, purification of 87 was not completely successful, as there are a few impurities still present as shown in the $^1$H NMR spectrum.

**Figure 2.2:** The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 81

**Figure 2.3:** The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 87
2.3 Synthesis of α-Amino-(1,8-naphthalimide) α,β-Unsaturated Esters

2.3.1 Introduction

Compounds 88 and 89 were chosen as synthetic targets for the current study because there are a plethora of reactions available for the functionalisation of alkenes, leading to diverse organic structures.\(^{107}\) One reaction in particular is epoxidation. The utility of epoxide functionality is that they can form anti-diols by acid-catalysed hydration and are also susceptible to ring opening by nucleophiles to give the corresponding alcohols or diols.\(^{107}\)

Many well-documented olefination reactions exist,\(^{109}\) the Wittig reaction being the most desirable as it provides a means of positioning the double bond at the desired location within a given structure.\(^{110}\) As previously mentioned, both Phelan and Hussey have made numerous attempts to achieve the synthesis of α-amino-(1,8-naphthalimide) α,β-unsaturated esters using this reaction method, but with limited success.\(^{3a,b}\) In an effort to synthesise 88 and 89, it was deemed necessary to take a different approach to that taken by Phelan and Hussey. A powerful method for the stereoselective preparation of α,β-unsaturated esters is the Horner-Wadsworth-Emmons (HWE) reaction of aldehydes.\(^{111}\) It was chosen because it demonstrates synthetic and practical advantages over the traditional Wittig reaction. For instance, phosphonate-stabilized carbanions are more nucleophilic and thus more basic than the corresponding phosphonium ylides and the dialkylphosphate salt by-product is readily removed by aqueous extraction. In using the Wittig reaction, a problem encountered by Phelan was the general unreactivity of the reagents employed, while Hussey experienced difficulties in successfully removing the triphenylphosphine oxide by-product.

The major product of olefinations with phosphonate carbanions is usually the (E)-isomer,\(^{109}\) and the mechanism for this reaction is shown in Figure 2.4. The phosphoryl-stabilized carbanion attacks the carbonyl in a stepwise manner, to give oxyanion intermediate \(1_{E,Z}\), which then decomposes via a transient four-centred intermediate, \(2_{E,Z}\), to yield olefin.\(^{109}\) Carbanion-stabilizing group (W) at the phosphorous-substituted carbon is necessary for elimination to occur. It is the stereoselectivity in the initial carbon-carbon bond-forming step combined with perhaps the reversibility of the intermediates that the
stereochemistry of the HWE reaction is determined. Like the analogous Wittig reagents, stabilized-phosphonates tend to give trans \((E)\) olefins if the substituents on the phosphorus are simple alkoxy groups, and if \(\text{Li}^+\) or \(\text{Mg}^{2+}\) counterions are used.

![Figure 2.4: Mechanism of the HWE reaction for an aldehyde \((R'\text{CHO})\) condensation.](image_url)

Normally, strong bases such as butyllithium (n-BuLi) or NaH are required to generate the phosphonate carbanion.\(^{113}\) Once generated, the nature of the metal ion influences the nucleophilicity of that carbanion. Addition of a metal ligand to the reaction mixture also influences the relative ease with which the \(\alpha\)-proton is removed by the base.\(^{114}\) A variation of this method was used to prepare the target \(88\) and \(89\) and is discussed in the following sections. All synthetic attempts are collected in Table 2.3 in Section 2.3.1.

2.3.2 HWE Reaction using Magnesium Salts: (Reactions A, B and C)\(^{114}\)

In the presence of Lewis acids, phosphonate carbanions can be generated by the use of tertiary amine bases.\(^{114}\) Rathke and Nowak studied the HWE reaction in the presence of magnesium or lithium halides using the weaker but much less expensive base triethylamine,\(^{112}\) and found that using such reagents in the HWE reaction gave yields of unsaturated esters comparable to those reported for other, stronger bases.\(^{109}\)
As shown in Table 2.3, the first attempt at this reaction involved stirring 84 in dry THF with the ylide prepared from treating triethyl phosphonate with MgBr₂ (1.2 eq.) and freshly distilled triethylamine (1.1 eq.). Analysis of the reaction mixture showed it consisted mainly of unreacted starting material. However, the presence of 88 was evident in the NMR spectrum (CDCl₃, 400 MHz). Consequently, 84 was again treated with the ylide prepared as above using the stronger base DIPEA (2.5 eq.). Despite these modifications, TLC analysis showed that no reaction had occurred and starting material was recovered. The reaction was then repeated in acetonitrile and the ylide was prepared using DBU (1 eq.) as a base. This particular reaction was difficult to follow by TLC analysis (various eluting systems were tried), however, ¹H NMR yielded the same result as before. It is possible that using a mild organic base is not adequate enough for this transformation.

### Table 2.3: Summary of procedures investigated for the synthesis of 88, 89 and 90. The aldehyde 86 is the R-enantiomer of 85 and the olefin 90 is the R-enantiomer of 88.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Aldehyde</th>
<th>Reagent</th>
<th>Time</th>
<th>Solvent</th>
<th>Temp.</th>
<th>Olefin</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>84</td>
<td>MgBr₂, NEt₃</td>
<td>4 days</td>
<td>THF</td>
<td>45 °C</td>
<td>88</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>84</td>
<td>MgBr₂, DIPEA</td>
<td>48 h</td>
<td>THF</td>
<td>70 °C</td>
<td>88</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>84</td>
<td>MgBr₂, DBU</td>
<td>8 days</td>
<td>MeCN</td>
<td>r.t.</td>
<td>88</td>
<td>0 %</td>
</tr>
<tr>
<td>D</td>
<td>84</td>
<td>LiOH</td>
<td>4 days</td>
<td>THF</td>
<td>r.t.</td>
<td>88</td>
<td>0 %</td>
</tr>
<tr>
<td>E</td>
<td>84</td>
<td>BuLi</td>
<td>1 h</td>
<td>THF</td>
<td>-78 °C</td>
<td>88</td>
<td>40 %</td>
</tr>
<tr>
<td>F</td>
<td>85</td>
<td>BuLi</td>
<td>2 h</td>
<td>THF</td>
<td>-78 °C</td>
<td>89</td>
<td>53 %</td>
</tr>
<tr>
<td>G</td>
<td>86</td>
<td>BuLi</td>
<td>2 h</td>
<td>THF</td>
<td>-78 °C</td>
<td>90</td>
<td>55 %</td>
</tr>
</tbody>
</table>

2.3.3 HWE Reaction using LiOH: (Reaction D, Table 2.3)

Bonadies and Cardilli showed that aldehydes could undergo efficient E-stereoselective HWE olefination by generation of phosphonate carbanion with LiOH, a reagent that includes both the required basic property and the presence of the lithium.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

cation.\textsuperscript{115} Compound 84 was treated with a slight excess of phosphonate carbanion (1.1 eq) generated by the reaction of triethyl phosphonate with LiOH.H₂O (1.1 eq.) in dry THF. According to \textsuperscript{1}H NMR no reaction occurred and only the starting material was recovered. The rationale for the last two attempts stem from our interest in using bases weak enough as not to affect the chirality of the molecules and also in using bases that are accessible to industry.

2.3.3 HWE Reaction using n-BuLi: (Reactions E, F and G, Table 2.3)

The compounds 84 85 and 86 were cooled in THF to -78 °C and reacted with the ylide being prepared from treating triethyl phosphonate with a 1.02 eq. of n-BuLi. After two hours stirring, water was added to the reaction mixture and the THF was removed under reduced pressure. The resulting residue was then extracted with CH₂Cl₂ and washed with water. According to \textsuperscript{1}H NMR analysis, this reaction procedure resulted in the successful formation of 88, 89 and 90. Unfortunately, Hussey\textsuperscript{3b} previously reported that the purification of these compounds by column chromatography was not effective. Because of this, various different methods of purification were investigated and are summarised in Table 2.4. The most efficient method was found to be a precipitation of 88-90 (five times) from a mixture of ethyl acetate/hexane. However, this gave the molecules in low yield as detailed in Table 2.3. Therefore, the purification of these compounds will have to be further investigated.

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Purification method</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trituration in diethyl ether</td>
<td>Unsuccessful</td>
</tr>
<tr>
<td>2</td>
<td>CHCl₃/hexane ppt</td>
<td>Unsuccessful</td>
</tr>
<tr>
<td>3</td>
<td>CHCl₃/diethyl ether ppt</td>
<td>Unsuccessful</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl acetate/hexane ppt</td>
<td>Improved \textsuperscript{1}H NMR</td>
</tr>
<tr>
<td>5</td>
<td>Recrystallisation from MeOH</td>
<td>Unsuccessful</td>
</tr>
</tbody>
</table>

Table 2.4: Methods investigated for the purification of 88, 89 and 90.

The molecules 88-90 were fully characterised by \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, IR, ES mass spectroscopy and elemental analysis. The \textsuperscript{1}H NMR spectrum (CDCl₃, 400 MHz) of 88, shown in Figure 2.5, confirmed its identity by the presence of a doublet of triplets at 6.87 ppm pertaining to the β-proton on the double bond. The α-proton on the double bond
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

appeared as a doublet at 6.0 ppm and the two protons of the ester methylene group as a quartet at 4.20 ppm. The methylene unit adjacent to the olefin bond yielded a multiplet at 4.10 ppm while the ethyl ester group as a triplet at 1.30 ppm. The spectra also clearly showed the trans geometry of 88 with a characteristic olefinic proton-coupling constant of 15.9 Hz. The $^{13}$C NMR, DEPT 135 and HMQC spectra showed the two alkenic α-C and β-C carbon signals appearing at 143.5 and 122.0 ppm, respectively. The effect of the electron withdrawing carboxyl group on the electron density of the alkenic carbon atoms was also observed. In a conjugated unsaturated system like this, the π-bond of the CC double bond will be polarised by the presence of the electron-withdrawing group so that β-C will have a slightly greater electron density than α-C (Figure 2.6). Furthermore, the $^{13}$C NMR signal at 165.6 ppm assigned to the ester carbonyl and an [M + Na]$^+$ m/z peak at 490 confirmed formation of the desired target 88.

![Figure 2.6](image)

Figure 2.6: Schematic representation of the trans geometry of 88.

Figure 2.5: The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 88.

The formation of 89 and 90 was verified by circular dichroism (CD) spectroscopy. The CD spectroscopy measures differences in the absorption of left-handed circularly polarized light versus right-handed polarized light, which arises due to structural asymmetry. Chiral or asymmetric molecules produce a CD spectrum because they absorb left and right handed circularly polarised light to different extents and thus are considered
to be "optically active". This can be clearly seen in the CD spectra shown in Figure 2.6 of the $S$ and $R$ enantiomers 89 and 90, respectively. This is an induced CD effect from amino acid to chromophore.

![Figure 2.6: The CD spectra of the $S$ (red line) and $R$ (blue line) enantiomers, 89 and 90 in CH$_2$Cl$_2$.](image)

In summary, four $\alpha$-amino (1,8-naphthalimide) aldehyde derivatives 84-87 were generated as synthons for further modification. This involved the successful transformation to the targets $\alpha$,$\beta$-unsaturated ester derivatives 88-90, which was the principal concept of this research. The transformation was achieved through the use of the HWE reaction. The discovery of the most suitable variation of this reaction method was successful, however, the purification of the molecules was difficult and consequently they were obtained in poor yields. This is a reason why 88-90 were not reduced to their corresponding 4-amino derivatives. Regarding the fact that the $\alpha$,$\beta$-unsaturated esters can act as a building block towards other functionalities such as epoxides, optimisation of their synthesis may require further investigations.

The cytotoxic activity of 80-90 was evaluated in the HL60 cell line, the results of which will be discussed in Chapter 5. Attempts were also made at evaluating the DNA binding affinity of these molecules using UV/Vis and fluorescence spectroscopy. The utility of such techniques for a determination of potential DNA binding interactions will be discussed in more detail in Chapter 4. However, all of the molecules were insoluble in aqueous solution and were also found to precipitate out of a 2% DMSO and 98 % phosphate buffer solution. The next section deals with the synthesis of a phthaloyl protected $\alpha$-amino aldehyde containing L-Leucine. This molecule was synthesised so that its cytotoxic activity could be analysed and compared with that of its naphthalimide analogue 84.
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2.4 Synthesis of the Phthaloyl Protected \( \alpha \)-Amino Aldehyde 93

The first step in the reaction sequence shown in Figure 2.3 was to protect the amino group of \( \text{L-leucine} \). There are a large number of complementary protecting groups available and the choice of one over the other depends largely on the conditions, which are to follow in the reaction sequence. Phthaloyl (Phth) was chosen as a protecting group because it is stable towards acidic and basic conditions. However, it can be removed upon treatment with a solution of hydrazine in ethanol.\(^{116}\)

![Chemical structure of amino aldehyde 93 from \( \alpha \)-amino acid 91.]

\[
\text{NH}_2 \quad \text{OH} \quad + \quad \text{HN} \quad \text{NEt}_3 \text{Toluene} \quad \text{EDCI, HOBt} \quad \text{NEt}_3 \text{, THF, r.t}
\]

\[
\text{91}
\]

\[
\text{92} \quad \text{TFA, CHCl}_3 \quad \text{r.t} \quad \text{93}
\]

**Scheme 2.3**: Synthesis of amino aldehyde 93 from \( \alpha \)-amino acid 91.

\( N \)-Phthaloyl protection was achieved by treatment of \( \text{L-Leucine} \) in toluene with phthalic anhydride and triethylamine under reflux, using a Dean-Stark apparatus to remove the water generated. This gave 91 as a white crystalline solid in 69 % yield after a recrystallisation from hexane. This compound was characterised by the usual methods and its \( ^1 \text{H} \) NMR spectrum (CDCl\(_3\), 400 MHz) is shown in Figure 2.7. The aromatic region is composed of two sets of multiplets pertaining to the protons of the phthalimide moiety. The \( \alpha \)-proton appears as a double doublet at 5.02 ppm, while the adjacent methylene protons show as a multiplet between 2.37 and 1.95 ppm. The upfield region of the spectrum consists of a multiplet at 1.51 ppm for the methine proton and two doublets at 0.95 ppm and 0.93 ppm for the two sets of methyl protons, respectively.
Figure 2.7: The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 91.

Treatment of 91 with HOBt and EDCI.HCl in dry THF and subsequent coupling in a one-pot reaction to aminoaldehyde diethyl acetal gave 92 in 60 % yield, following precipitation from a mixture of CH$_2$Cl$_2$/hexane. It gave satisfactory spectral analysis and ESMS data with an [M + Na]$^+$ peak at 399. The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) had similar characteristics to that seen for 91 (Figure 2.8), with the addition of a broad singlet at 6.35 ppm for the amide proton and also four additional signals pertaining to the aminoaldehyde diethyl acetal part of the molecule. These included a multiplet between 3.67 and 3.34 ppm for the methylene protons, a triplet at 4.50 ppm for the methine proton and two triplets at 1.19 and 1.15 ppm for the two sets of methyl protons. Not surprisingly, this molecule exhibited poor cytotoxic activity, when tested in the HL-60 cell line (Chapter 5).

The next step in the reaction sequence involved removing the acetal-protecting group from 92. This was achieved using TFA in CH$_2$Cl$_2$ and gave 93 as brown oil in 91 % yield. $^1$H NMR (CDCl$_3$, 400 MHz) confirmed the identity of 93 by the absence of the signals pertaining to the aminoaldehyde diethyl acetal unit and by the presence of the aldehyde peak at 9.96 ppm and a singlet at 4.28 ppm for the adjacent methylene protons. Furthermore, the $^{13}$C NMR signal at 193.9 ppm assigned to the aldehyde carbon and an [M + Na]$^+$ m/z peak at 279 confirmed its formation. In a similar manner to 92, this molecule also exhibited poor cytotoxicity in the KL-60 cell line (Chapter 5).

As previously mentioned, the naphthalimide moiety may be inhibiting the reactivity of the aldehyde functionality of 84 and 85, thus making the transformation to the $\alpha,\beta$-
unsaturated ester difficult. Consequently it may be advantageous to determine if this is the case because further modification at the α-amino aldehyde terminus was essential for the development of functionalised amino acid-based naphthalimide conjugates. One method of investigation could involve the synthesis of an α-amino-α,β-unsaturated ester, such as 94, in the absence of the naphthalimide moiety. Ease of reaction would then confirm the latter. This may be achieved by using 93 or by preparing a Cbz-protected analogue of 93 and carrying out the transformation to the ester using the HWE reaction, previously described in Section 2.3.3. Prior to carrying out the reaction the phthaloyl protecting group can be removed because it has a similar structure to the 1,8-naphthalimide. However, due to the fact that the reactivity between them is very different, the HWE reaction could also be attempted with the phthaloyl group present. To date this investigation has not been carried out because both 88 and 90 were not as cytotoxic towards the HL-60 cell line as expected (Chapter 5). Nevertheless, the discussed strategy may act as a convenient method for the preparation of 88-90 for further functionalisation.

Scheme 2.4: Synthesis of 88 and 89 from α-amino aldehyde 93.

Overall, the ultimate goal of this research, has been to utilise the α-amino-(1,8-naphthalimide) aldehyde as a stepping stone for the preparation of many other functionalised naphthalimide derivatives. However, due to the unreactivity of the α-amino aldehyde moiety when attached to the 1,8-naphthalimide structure, (which has been proven by both Phelan and Hussey), this research was not continued any further.

The next section of this Chapter will discuss the development of thiazole modified 1,8-naphthalimides containing α-amino acids. As mentioned in Chapter 1, our interest in this area stems from the fact that many of the naturally occurring cyclic peptides, which exhibit high cytotoxicity, contain thiazole amino acids. Even though the proposed systems will be acyclic, the thiazole units will be flanked on either side with amino acids and the
presence of the intercalating chromophore of naphthalimide may further aid in biological activity.

2.5 Synthesis of Thiazole Based-Naphthalimide Containing α-Amino Acids

In terms of a synthetic strategy, two pathways were considered to achieve the synthesis of thiazole based-1,8-naphthalimides containing α-amino acids, such as 95 and 96 (Scheme 2.5). As previously mentioned, the first approach was to utilise 84 and 85 and condense them with L-cysteine methyl ester. Oxidation of the thiazolidine intermediate would then generate the desired thiazole derivative. However, two problems existed with this route: (i) the experienced unreactivity of the aldehyde moiety as previously discussed and (ii) a possible loss of chirality at the chiral centre of the neighbouring amino acid. The latter was a problem experienced by Hussey for the attempted synthesis of 95. A preferred synthetic pathway would involve a convergent approach, utilising orthogonally protected thiazole units such as 97. This approach would, thus, allow each thiazole unit to act as a heterocyclic building block, which can be pieced together to give a wide variety of thiazole based naphthalimide derivatives.

Scheme 2.5: Synthetic strategy for the preparation of 95 and 96, respectively.

A number of methods for the synthesis of amino acid-derived thiazoles are known and include: (i) condensation of an amino acid-derived thioamide with ethyl
bromopyruvate (a modification of the Hantzsch reaction),\(^{117}\) (ii) condensation of \(\alpha\)-amino aldehydes with \(L\)-cysteine esters,\(^{118}\) (iii) condensation between cysteine esters and \(N\)-protected imino esters,\(^{119}\) and (iv) cyclodehydration of \(\beta\)-hydroxythioamides using Burgess reagent.\(^{120}\) However, thiazolidines synthesised by the last three procedures are readily converted into thiazoles by oxidation. Consequently, this second reaction method was chosen for the preparation of the thiazole derivatives, 95 and 96, and the manner in which this was achieved will be discussed in the following section.

### 2.5.1 Synthesis of the Thiazolidine Intermediates 100 and 101

For the synthesis of the orthogonally protected thiazole 97, a phthaloyl protected aminoaldehyde diethylacetal, 98, was chosen as starting material. Phthaloyl was the protecting group of choice for the same reasons as given earlier, *i.e.* it is stable to both acidic and basic conditions. The two-step synthesis of the thiazolidine intermediates 100 and 101 is shown in Scheme 2.6. The first step involved an acetal hydrolysis of 98 using a mixture of TFA and chloroform which gave the corresponding aldehyde derivative, 99 in quantitative yield as a white solid without the need for further purification. \(^1\)H NMR \((CDCl_3, 400\) MHz) analysis confirmed its successful formation by the presence of a singlet at 9.67 ppm for the aldehyde resonance and second singlet at 4.58 ppm for the adjacent methylene group. It was also fully characterised by \(^13\)C NMR, IR, mass spectroscopy and elemental analysis.

![Scheme 2.6: Synthesis of the thiazolidine intermediates 100 and 101.](image)

Subsequent cyclocondensation reactions using the hydrochloride salts of \(L\)-Cysteine methyl ester and its corresponding free acid gave the thiazolidines 100 and 101 in 75% and
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76% yields, respectively, after a recrystallisation from methanol. The general procedure involved treating 98 in ethanol with a solution of either L-Cysteine or L-Cysteine methyl ester in water, KHCO$_3$ was then added and the mixture was stirred at room temperature for 5 hours, during which time the product gradually precipitated. The mechanism for the formation of 100-101 is shown in Scheme 2.7. The first step involves a nucleophilic attack by the amino moiety of L-Cysteine methyl ester on the carbonyl carbon of the aldehyde to give a dipolar tetrahedral intermediate. Proton transfer followed by a loss of water and a second nucleophilic attack by the thiol group on the electron deficient iminium ion gives the thiazolidine product.$^{116}$

![Scheme 2.7: Mechanism for thiazolidine formation.$^{116}$](image)

2.5.1.1 Characterisation of 100 and 101

The $^1$H NMR spectra of both 100 and 101 exhibited two ABX systems and also showed that they were obtained as single diastereoisomers. The $^1$H NMR spectrum (CDCl$_3$, 600 MHz) of 100 and its Cosy spectrum are shown in Figure 2.8 and 2.9, respectively. The geminal pair of protons, $H_a$ and $H_a'$, are diastereotopic and their signals appear as two double doublets at 4.15 and 4.05 ppm, respectively. Each proton is coupled to each other with a large coupling constant of 14.5 Hz. They are also coupled to $H_z$, which appears as a double doublet at 4.86 ppm. The second geminal pair of protons, $H_b$ and $H_b'$ appear as a double doublet and a triplet at 3.33 and 2.92 ppm, respectively. Each proton is coupled to each other as well as $H_x$, which shows as a triplet at 3.82 ppm. The NH proton appears as a broad singlet at 2.61 ppm while the protons of the methyl ester appear as a sharp singlet at 3.80 ppm.
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Figure 2.8: $^1H$ NMR spectrum (CDCl$_3$, 600 MHz) of 100.

Figure 2.9 Cosy spectrum (CDCl$_3$, 600 MHz) of 100, (Aromatic region not shown).

Although not shown, the $^1H$ NMR spectrum of 101 only differed from that of 100 by the absence of the singlet, pertaining to the protons of the methyl ester group. Both molecules were also fully characterised by the usual methods and ESMS gave [M + Na]$^+$ m/z peaks at 329 and 292 for 100 and 101, respectively.
2.5.1.2 Solid State Analysis of 100

Single off-white crystals of 100 were grown by the slow evaporation of CH₂Cl₂ overnight from a suspension of the compound in MeOH. They were suitable for X-ray diffraction analysis, which was carried out by Dr. T. McCabe in the School of Chemistry, Trinity College Dublin, and the resulting crystal structure of 100 is shown in Figure 2.10. Assuming that the reaction was stereospecific at the C-2 position, the stereochemistry of 100 was assigned as (2R, 4R). Selected bond lengths and angles are also summarised in Table 2.5, which were obtained from an analysis of the CIF files using Mercury 1.1©.

![Figure 2.10: Crystal structure of 100, grey represents carbon atoms, red oxygen atoms, blue nitrogen atoms and white hydrogen atoms](image)

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 C14</td>
<td>1.453 (3)</td>
<td>N1 C14 C9</td>
<td>110.2 (2)</td>
</tr>
<tr>
<td>C14 C9</td>
<td>1.543 (3)</td>
<td>N2 C10 C12</td>
<td>113.07 (18)</td>
</tr>
<tr>
<td>C9 S1</td>
<td>1.836 (2)</td>
<td>N2 C9 S1</td>
<td>110.57 (16)</td>
</tr>
<tr>
<td>S1 C11</td>
<td>1.805 (2)</td>
<td>N2 C10 C11</td>
<td>109.94 (18)</td>
</tr>
<tr>
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<td>1.445 (3)</td>
<td>O3 C12 C10</td>
<td>110.60 (19)</td>
</tr>
<tr>
<td>N2 C10</td>
<td>1.473 (3)</td>
<td>C10 C11 S1</td>
<td>106.74 (15)</td>
</tr>
<tr>
<td>C10 C11</td>
<td>1.539 (3)</td>
<td>C14 C9 S1</td>
<td>110.57 (16)</td>
</tr>
<tr>
<td>C10 C12</td>
<td>1.533 (3)</td>
<td>N2 C9 C14</td>
<td>112.21 (18)</td>
</tr>
<tr>
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<td>1.204 (3)</td>
<td>O4 C12 C10</td>
<td>124.3 (2)</td>
</tr>
<tr>
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<td>1.325 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O3 C13</td>
<td>1.453 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: Selected bond lengths and bond angles for 100.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

The view along the crystallographic c axis of the packed structure of 100 is shown in Figure 2.11. Upon structure analysis, intermolecular hydrogen bonding interactions were observed between the sulfur atom (S1) of the thiazolidine ring and one of the amide oxygens (O1). There are also non-bonding contacts between the NH proton, the sulfur atom (S1) and the amide carbonyl oxygen (O1).

![Figure 2.11: View along the crystallographic c axis of 100.](image)

Following the crystal structure analysis, a number of NMR experiments were also carried out on a sample of 100 in DMSO-d6. These included $^1$H, $^{13}$C, $^{15}$N, Cosy, HMQC, HMBC, NOE, TOSCY and Decoupling experiments, which were carried out by Dr. John O'Brien in the School of Chemistry, Trinity College Dublin. The measurements were conducted using a Bruker Spectrospin DPX-600 instrument, and each spectrum was recorded immediately after sample preparation (the reasons for this will be discussed in Section 2.8).

The $^1$H NMR spectrum ((CD$_3$)$_2$SO, 600 MHz) and the NOE of 100 is shown in Figure 2.12. As can be seen the spectrum is somewhat different to that recorded in CDCl$_3$ (Figure 2.10). The most important feature is that the signal pertaining to the NH proton appeared as a triplet at 3.39 ppm (located using $^{15}$N NMR), because of its coupling to both adjacent chiral protons, Hx and Hz. This was evident by the fact that the NOE experiment, in which the signal at 3.99 ppm was irradiated, showed a direct link between it and the signal for Hx and Hz. Interestingly, the results also showed a link with the signal pertaining to the methylene protons of the thiazolidine ring, Hb,Hb'. Although not shown, this was further evidenced by the results obtained from the long range coupling experiment.
Figure 2.12: $^1$H NMR spectrum ((CD$_3$)$_2$SO, 600 MHz) of 100 (aromatic region not shown), with NOE as insert.

Figure 2.12 also shows the chiral proton, H$_z$, appearing as a multiplet at 4.82 ppm because of its coupling with both the NH proton and the adjacent methylene protons, H$_a$, H$_a'$. This was confirmed upon irradiating the H$_z$ proton and observing the collapse of the signals for the above protons (Figure 2.13). Irradiation of the NH proton also caused the collapse of the H$_x$ proton and the H$_z$ proton, which, in addition to the long range coupling experiment, further supports that discussed previously.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

In a similar manner to the $^1$H NMR spectrum of 100 recorded in CDCl$_3$ (Figure 2.8), the magnetically non-equivalent methylene protons, Ha and Ha', show as two double doublets, but they appeared more upfield at 3.94 and 3.88 ppm, respectively. The latter signal is overlapping with the signal for the Hx proton, which can be observed as a multiplet integrating for two protons. In a similar manner to the Ha and Ha' protons, the second geminal protons, Hb and Hb', appear as two well defined double doublets at 3.17 and 2.93 ppm.

Further NOE analysis of the structure of 100 involved irradiating the protons of the methyl ester group, and upon doing so; it was found that there was a link between it and the aromatic protons. Even though the signals have not been included in the spectra, shown in Figure 2.12, they appear as two multiplets at 7.90 and 7.86 ppm, respectively. This splitting arises because they are coupled together in an AA'BB' system because the two pairs of nuclei are not magnetically equivalent. When the crystal structure of 100 was analysed again, a through space interaction was observed between the carbonyl oxygen and one of the CH of the aromatic ring (Figure 2.14). While this is a solid-state phenomenon, it is likely that a contribution from such an interaction also exists in solution, which further supports the above effect.

2.5.2 Synthesis of the Thiazole 102

The thiazolidine, 100, was oxidised to the corresponding thiazole derivative, 102, (Scheme 2.8), in 63 % yield by employing activated MnO$_2$ and carrying out the reaction in the presence of pyridine in dry CH$_2$Cl$_2$ at refluxing temperature.
The discovery of the most suitable condition for this reaction was challenging and many modifications were carried out as summarised in Table 2.6. The use of CH$_2$Cl$_2$ as solvent rather than THF, and carrying out the reaction in the presence of pyridine (1.13 eq.) resulted in a decrease in the reaction time by 48 hours (7 days $\rightarrow$ 5 days). In doing so, 102 was obtained without the need for further purification. However, when THF was used as solvent it was always necessary to recrystallise the crude product from ethanol, thus lowering the overall isolated yield of 100. Furthermore, when the reaction was carried out in the absence of pyridine the crude product was a mixture of unreacted starting material and product, which were separated by flash column chromatography (silica, CH$_2$Cl$_2$/MeOH, 99:1). From this, perhaps, it could be suggested that pyridine plays a role in controlling the reactivity of active MnO$_2$, thereby improving its selectivity.

### Table 2.6. The Synthesis of 102.

<table>
<thead>
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<th>Attempt</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>MnO$_2$ (eq.)</td>
</tr>
<tr>
<td>Solvent</td>
</tr>
<tr>
<td>Pyridine (eq.)</td>
</tr>
<tr>
<td>Rxn Time</td>
</tr>
<tr>
<td>Yield (%)</td>
</tr>
</tbody>
</table>

Compound 102 was fully characterised by $^1$H NMR, $^{13}$C NMR, IR, ESMS and HRMS. The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 102 is shown in Figure 2.15, and as expected is significantly simpler than that of 100 (Figure 2.10). The aromatic region is composed of a singlet at 8.15 ppm pertaining to that of the methine proton of the thiazole ring and two sets of doublet of doublets for the benzene ring protons. The methylene
protons of the amide appear as a singlet at 5.25 ppm, while the methyl ester protons appears as a sharp singlet at 3.95 ppm. This compound was further analysed by using X-ray crystallography.

Figure 2.15: $^1$H NMR spectrum (CDCl$_3$, 400 MHz.) of 102.

2.5.2.1 Solid State Analysis of 102

Crystals of 102 were obtained by the slow evaporation of the compound from a solution containing MeOH and CH$_2$Cl$_2$ (3:1 mixture) and were suitable for X-ray crystallography diffraction analysis. The crystal structure obtained by Dr. T. McCabe, in the School of Chemistry, Trinity College Dublin, is shown in Figure 2.16. Selected bond lengths and bond angles are given in Table 2.7.

Figure 2.16: Crystal structure of 102, grey represents carbon atoms, red oxygen atoms, blue nitrogen atoms and white hydrogen atoms
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing \(\alpha\)-Amino Acids

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
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<td>1.449 (2)</td>
<td>N1 C9 C10</td>
<td>113.51 (15)</td>
</tr>
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<td>N2 C10 C9</td>
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<td>1.736 (2)</td>
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<td>1.297 (2)</td>
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<td>1.379 (2)</td>
<td>C10 C11 S1</td>
<td>106.74 (15)</td>
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</table>

Table 2.7: Selected bond lengths and bond angles for 102.

The packing diagram of 102 is shown in Figure 2.17 viewing along the crystallographic a axis. There were no measurable \(\pi - \pi\) stacking interactions observed. However, intermolecular hydrogen bonding interactions were observed between the sulfur atom and one of the imide oxygens. Further analysis showed short contacts between the carbonyl oxygen (O3) and the one of the para hydrogens of the aromatic ring.

Figure 2.17: View along the crystallographic a axis of 102.

2.5.3 Removal of the Protecting Groups of 102

The next two steps in the synthesis towards the development of the thiazole modified naphthalimides, involved removing both the methyl ester and phthaloyl protecting groups to give 103 and 104, respectively (Scheme 2.9). Cleavage of the methyl ester group to give 103 was firstly attempted using a base catalysed hydrolysis reaction. This involved using either LiOH or NaOH as a base, and carrying out the reactions as summarised in Table 2.8.
Scheme 2.9: Deprotection of 102 to give 103 and 104, respectively.

<table>
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<tr>
<th>Attempt</th>
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<th>Solvent</th>
<th>Temp.</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
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<td>MeOH/H₂O (1:1)</td>
<td>Reflux</td>
<td>24 hr</td>
<td>0 %</td>
</tr>
<tr>
<td>B</td>
<td>LiOH.H₂O (1.1 eq.)</td>
<td>MeOH/H₂O (1:1)</td>
<td>Reflux</td>
<td>4 days</td>
<td>0 %</td>
</tr>
<tr>
<td>C</td>
<td>NaOH (10 eq.)</td>
<td>THF/H₂O (9:1)</td>
<td>r.t.</td>
<td>2 days</td>
<td>0 %</td>
</tr>
<tr>
<td>D</td>
<td>HCl</td>
<td>Acetone/H₂O (7:1)</td>
<td>Reflux</td>
<td>2 days</td>
<td>55 %</td>
</tr>
</tbody>
</table>

Table 2.8: Synthesis of 103.

According to \(^1\)H NMR ((CD\(_3\))\(_2\)SO, 400 MHz), none of the reactions occurred and starting material was recovered. Consequently, the cleavage was attempted by an acid catalysed hydrolysis using HCl. As soon as the reaction had come to completion, any precipitate, which had formed, was collected by suction filtration, and washed with a small amount of acetone. The filtrate was then removed under reduced pressure and the remaining residue was dissolved in KHCO\(_3\) (1.2 M). The solution was then filtered and brought to pH 1 by the slow addition of conc. HCl. Ethanol was subsequently added and heated to dissolve the precipitate. The product 103 crystallised out of solution and was obtained as fine colourless needles in 55 % yield, without the need for further purification. ESMS data confirmed the molecular formulae of 103 by giving \(m/z = 311\) for [M + Na]\(^+\), which was also verified by accurate mass determination. \(^1\)H NMR ((CD\(_3\))\(_2\)SO, 400 MHz) and DEPT showed the absence of a singlet for the methyl ester protons and the resonance for the methyl carbon, respectively.

For the cleavage of the phthaloyl-protecting group a number of hydrazinolysis reactions were attempted. The reaction of hydrazine with phthalimide or its \(N\)-substituted
derivatives is used in the synthesis of primary amines, and this procedure is known as the Ing-Mankse reaction.\textsuperscript{121} In 2003, McMurry \textit{et al.},\textsuperscript{122} confirmed and revised the structures of the isolated intermediates of this reaction. It was found that 105 reacts with hydrazine at room temperature to afford 106, while at higher temperature or in the presence of acid, this intermediate cyclises to give 107.

\[ R = \text{C}_6\text{H}_5\text{OCH}_2\text{CHOHCH}_2, \text{C}_6\text{H}_5\text{CH}_2 \]

\textbf{Scheme 2.10: Intermediates 106 and 107 in the Ing-Manske reaction.}

As summarised in Table 2.9, the formation of 104 by hydrazonysis was quite difficult to achieve successfully in high yields. It was established that if the reaction was carried out at reflux the thiazole ring broke down and if too small amount of hydrazine monohydrate (less than 2 eq.) was used a mixture of products were obtained. These included 102, 104 and the phthalhydrazide by-product, all of which were very difficult to separate, either by classical acid-base extraction work-up or by using flash column chromatography on silica.

<table>
<thead>
<tr>
<th>Attempt</th>
<th>N$_2$H$_4$H$_2$O</th>
<th>Solvent</th>
<th>Temp.</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>Dioxane</td>
<td>r.t</td>
<td>Unable to isolate 104</td>
</tr>
<tr>
<td>B</td>
<td>1.1</td>
<td>EtOH</td>
<td>r.t</td>
<td>Unable to isolate 104</td>
</tr>
<tr>
<td>C</td>
<td>1.1</td>
<td>MeOH</td>
<td>r.t</td>
<td>Unable to isolate 104</td>
</tr>
<tr>
<td>D</td>
<td>1.2</td>
<td>EtOH</td>
<td>Reflux</td>
<td>Decomposition of 104</td>
</tr>
<tr>
<td>E</td>
<td>1.2</td>
<td>Dioxane</td>
<td>Reflux</td>
<td>Decomposition of 104</td>
</tr>
<tr>
<td>F</td>
<td>1.5</td>
<td>EtOH</td>
<td>r.t</td>
<td>Unable to isolate 104</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>EtOH</td>
<td>r.t</td>
<td>104 obtained in 53 % yield</td>
</tr>
</tbody>
</table>

\textbf{Table 2.9. Synthesis of 104.}
The hydrazinolysis was eventually achieved by the addition of hydrazine monohydrate (2 eq.) to a boiling suspension of 102 in ethanol (Attempt G, Table 2.7). Upon solvation, the reaction was stirred at room temperature for 4 h and the appearance of a white voluminous precipitate was produced indicating the formation of the phthalhydrazide side-product. This precipitate was collected by filtration and characterized by $^1$H NMR, $^{13}$C NMR ($(CD_3)_2$SO, 400 MHz) and ESMS, all of which identified it as the correct product. The filtrate was then concentrated in vacuo and the resulting residue was re-dissolved in EtOH. Any remaining precipitate was collected by suction filtration and washed with a little EtOH. The filtrate and washings were then evaporated again under reduced pressure. This procedure was repeated until an oil-like residue was obtained. The residue was then dissolved in H$_2$O and extracted three times with CHCl$_3$. The combined CHCl$_3$ layers were washed three times with HCl (0.1 M) and the combined aqueous phases were extracted two times with CH$_2$Cl$_2$. The aqueous phase was then brought to pH 12 by the addition of NaOH (0.1 M) and the product was extracted three times with CHCl$_3$. This gave 104 in 53% yield. According to $^1$H NMR (CDCl$_3$, 400 MHz), the cleavage of the phthaloyl-protecting group was evident by the absence of the multiplet signals for the aromatic protons and the ESMS gave $m/z = 325$ for [M+Na$^+$].

The major problem with the above reaction method was that low yields of 104 were obtained (Attempt G, Table 2.7) after such intensive purification. As previously discussed, purification of this molecule was lengthy and not that trivial either. As a means of recovering additional 104, the precipitate formed during the reaction, was heated in a mixture of HCl (2 M) and ethanol at 50°C for 10 minutes. The reaction mixture was then allowed to cool to room temperature and the resulting precipitate was collected by suction filtration. The filtrate was evaporated to dryness and $^1$H NMR analysis ($(CD_3)_2$SO, 400 MHz) showed the presence of only the aromatic peaks for the phthalaldehyde side-product. The $^1$H NMR spectrum ($(CD_3)_2$SO, 400 MHz) of the filtrate, however, still showed the presence of some quantity of the phthalaldehyde side-product. Consequently, the filtrate was evaporated to dryness and the resulting solid was initially precipitated from a mixture of MeOH and diethyl ether and then further purified by a recrystallisation from ethanol. According to $^1$H NMR and $^{13}$C NMR analysis ($(CD_3)_2$SO, 400 MHz) (Figure 2.18), the desired 104 was obtained, however, the methyl ester group had also been hydrolysed under these acidic conditions to give the amino acid 108. As for the intermediates discussed above, we were also able to analyse compound 104 by X-ray crystallography.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing \( \alpha \)-Amino Acids

![Figure 2.18: (A) \(^1\)H NMR and (B) \(^{13}\)C NMR ((\(\text{CD}_{3}\))\(_2\)SO, 400 MHz) spectra of 108.](image)

### 2.5.3.1 Solid State Analysis of Thiazole 104

A recrystallisation of 104 from EtOH resulted in the formation of fine single off-white crystals, which were suitable for by X-ray crystallographic diffraction analysis. The crystal structure of 104 is shown in Figure 2.19 and selected bond lengths and angles are given in Table 2.10.

![Figure 2.19: Crystal structure of 104, grey represents carbon atoms, red oxygen atoms, blue nitrogen atoms and white hydrogen atoms.](image)
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazoie Modified
1,8-Naphthalimide Containing α-Amino Acids

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 C1</td>
<td>1.448 (3)</td>
<td>N1 C1 C2</td>
<td>110.5 (2)</td>
</tr>
<tr>
<td>C1 C2</td>
<td>1.503 (4)</td>
<td>C1 C2 N2</td>
<td>125.6 (2)</td>
</tr>
<tr>
<td>C2 S1</td>
<td>1.737 (3)</td>
<td>C1 C2 S1</td>
<td>119.06 (19)</td>
</tr>
<tr>
<td>C2 N2</td>
<td>1.295 (2)</td>
<td>N2 C4 C5</td>
<td>121.8 (2)</td>
</tr>
<tr>
<td>S1 C3</td>
<td>1.707 (3)</td>
<td>C3 C4 C5</td>
<td>122.2 (2)</td>
</tr>
<tr>
<td>N2 C4</td>
<td>1.383 (3)</td>
<td>C4 C5 O2</td>
<td>112.9 (2)</td>
</tr>
<tr>
<td>C3 C4</td>
<td>1.364 (4)</td>
<td>C4 C5 O1</td>
<td>123.0 (2)</td>
</tr>
</tbody>
</table>

Table 2.10: Selected bond lengths and bond angles for 104.

The view along the crystallographic b* axis in the packing diagram of 104 is shown in Figure 2.20. Upon analysis of this packing, no intermolecular hydrogen bonding interactions were observed. However, there are non-bonding contacts between the NH proton and the carbonyl oxygen atom (O1), and the oxygen atom (O2) and the methine proton (H3) of the thiazoie ring.

![Figure 2.20: View along the crystallographic b* axis of 104.](image)

In summary, the synthesis of 101 - 104 was tough because each reaction method required efficient modification. Although, the oxidation reaction to give 103 was successfully optimised, the length of the reaction was still very long (5 days) and the maximum the reaction could be scaled up to was approximately 10 g. Cleavage of the phthaloyl-protecting group from 103 to give 104 was also synthetically taxing and the hydrazinolysis reaction required lengthy analysis and numerous attempts. Furthermore, the required purification methods resulted in low yields of 104 being obtained. Consequently,
this problem would affect the preparation of a wide variety of thiazole-α-amino based naphthalimide derivatives. Nevertheless, the development of the synthetic strategy undertaken was necessary because Hussey had tried to synthesise the target molecules directly from an naphthalimide containing α-amino aldehyde but was unsuccessful in doing so. It was found that upon oxidation of the thiazolidine moiety, the chiral centre of the adjacent amino acid epimerised resulting in the formation of a pair of diasterisomers, which could not be separated by flash silica column chromatography.

As previously mentioned, there exist other methods for the preparation of thiazole containing α-amino acids. Consequently, the preparation of thiazole 109, was attempted using the synthetic methods devised by Pattenden et al. However, the synthesis of this molecule was unsuccessful and was abandoned, because significant effort had been devoted to the synthesis of 104. For the above reasons, we decided on an alternative target where the amine was directly attached to the heterocycle, the synthesis of which could be achieved in a single step giving 110. The next section deals with this synthesis. However, as will be discussed in Section 2.5.7, this molecule was very unreactive and difficult to further functionalise. Therefore, we eventually used 104 for incorporation into the 1,8-naphthalimide containing α-amino acid structure.

2.5.4 Synthesis of the Thiazole 110 via a Modified Hantzsch Procedure

The thiazole 110 was synthesized by treating thiourea with the commercially available ethyl 2-chloro-methyl acetoacetate in EtOH at 78 °C. The reaction mixture was allowed to cool to room temperature and the precipitate was then collected by suction filtration. This gave 110 in 72 % yield as a white solid (Scheme 2.11) with both its 1H NMR (CDCl₃, 400 MHz) and 13C NMR (CDCl₃, 100 MHz) spectra being consistent with its presented structure.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

2.5.5 Synthesis of the Thiazole-α-Amino Acid Based-1,8-Naphthalimides, 118 – 121, Using Peptide Coupling Reactions

Initially, and as a means of investigating the reactivity of thiazole amino acid 103 using simple peptide coupling reactions, a Boc protected glycine, 111, and the Fmoc protected L-leucine, 112, were reacted with 104 using EDCI and HOBt as the coupling reagents (Scheme 2.12). The reactions were carried out in dry THF at room temperature in the presence of Et$_3$N. Upon completion, the THF was evaporated in vacuo and the remaining residue was dissolved in CH$_2$Cl$_2$. The organic phase was then washed twice with sat. NaHCO$_3$, water and finally with brine. This gave 113 and 114 in 77 % and 78 % yields, respectively, after recrystallisation from EtOH.

Scheme 2.12: Synthesis of 113 and 114.

Both 113 and 114 were fully characterized by $^1$H NMR, $^{13}$C NMR, ESMS, IR and either elemental analysis or high-resolution mass analysis. The ESMS gave m/z = 352 and 530 for [M+Na]$^+$, respectively, which were also verified by accurate mass determination. The $^1$H NMR (CDCl$_3$, 400 MHz) spectra of 113 was much simpler in comparison to that of 114, because there are no sterogenic centres present in this molecule. Confirmation of their formation was also made by the presence of a singlet and a triplet at 5.5 ppm and 3.8 ppm pertaining to the amide proton for 113 and 114, respectively.

For the synthesis of 118 – 122 (Scheme 2.13), it was decided that the amino acid-based naphthalimides 76, 77, 115, 116 and 117 would be reacted with 103 using the same
peptide coupling reagents used for the formation of 113 and 114. However, the Cbz-protected L-lysine 4-nitro naphthalimide, 117, and the 3-nitro naphthalimides 115 and 116 needed to be firstly prepared. This was carried out using the same reaction methods as for the two-step synthesis of 76 and 77, previously described in Section 2.2. This gave 115, 116 and 117 in 99 %, 98 % and 99 % yields, respectively, with ¹H NMR (CDCl₃, 400 MHz) being consistent with their structures. Since all of these molecules have been previously synthesised either by Phelan or Hussey, they were only further characterised by ¹³C NMR, IR and ESMS. Following this, the desired compounds 118 - 121 were synthesised, purified and obtained in yields as summarised in Table 2.11. The synthesis of 122 was difficult to achieve, despite being tried a number of times.

**Scheme 2.13 The synthesis of 118 – 122.**

<table>
<thead>
<tr>
<th>Cpd.</th>
<th>Purification Method</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>118</td>
<td>Recrystallisation from MeOH</td>
<td>74 %</td>
</tr>
<tr>
<td>119</td>
<td>Recrystallisation from MeOH</td>
<td>77 %</td>
</tr>
<tr>
<td>120</td>
<td>Column chromatography on silica (ethyl acetate/hexane 4:1)</td>
<td>73 %</td>
</tr>
<tr>
<td>121</td>
<td>Column chromatography on silica (1 → 3% MeOH-NH₃/CH₂Cl₂)</td>
<td>70 %</td>
</tr>
<tr>
<td>122</td>
<td>Column chromatography on silica (1 → 3% MeOH-NH₃/CH₂Cl₂)</td>
<td>0 %</td>
</tr>
</tbody>
</table>

**Table 2.11: Synthesis and method of purification of 118 – 122.**
The first two attempts for the synthesis of 122 involved carrying out the same coupling reactions used for the preparation of 118 - 121. According to $^1$H and $^{13}$C NMR analysis (CDCl$_3$, 400 MHz), both reactions resulted in a mixture of products, consisting of 104, 117, 122 and unknown impurities which were not possible to separate by flash column chromatography on neutral silica (1 → 3 % MeOH-NH$_3$/CH$_2$Cl$_2$). The third attempt was made using the acid chloride of 117, but this reaction was also unsuccessful. As a result, attempts at the synthesis of 122 were not continued any further.

2.5.5.1 Characterisation of 118 – 121

The compounds 118 – 121 were all fully characterised by $^1$H and $^{13}$C NMR, IR and HRMS. The $^1$H NMR spectrum of 119 is shown in Figure 2.21, demonstrating that the aromatic region is composed of three doublets and one triplet for the naphthalimide protons, one singlet for the methine proton of the thiazole ring and a multiplet for the aromatic ring of L-phenylalanine. The amide proton appeared as a triplet at 7.41 ppm, while the $\alpha$-proton resonance as a doublet of doublets at 6.02 ppm. The methylene group adjacent to the amide appears as a multiplet between 4.66 and 4.77 ppm. The methyl ester group shows as a sharp singlet at 3.85 ppm, while the methylene protons adjacent to the sterogenic centre appear as two doublet of doublets at 3.72 and 3.55 ppm, respectively. The $^1$H NMR spectrum of 118 was similar to that for 119, but differed by having signals pertaining to the L-Leucine side chain, which were previously described for compound 72 shown in Figure 2.1.
Figure 2.22 shows the $^1$H NMR spectra of 120, with the nitro group in the 3-position. The aromatic region of the spectrum differs to that seen for 119 (Figure 2.21), by showing three singlets, two doublets and one triplet for the protons of the 3-nitro-substituted naphthlaimide chromophore. However, the signals for the $\alpha$-proton, the methyl protons, the amide proton and the adjacent methylene protons are similar to that described for 119. Having successfully synthesised these thiazole derivatives, the next step was to reduce the 4-nitro compounds to give the corresponding 4-amino derivatives 123 and 124. This will be dealt with in next section.

**Scheme 2.14:** Synthesis of 123 and 124.
Both 123 and 124 were synthesized with the aim of investigating their cytotoxic activity and comparing them with the rest of the biological tests carried out on the compounds made herein. These results will be discussed in Chapter 5. As mentioned in Chapter 1, the 4-amino substituted 1,8-naphthalimides containing α-amino acids were found to be much less active than their 4-nitro derivatives. Nevertheless, it would be important to ascertain the cytotoxic activity of the 4-amino derivatives, because their mode of action could change with the presence of the thiazole moiety. Furthermore, they have the advantage of being highly fluorescent due to the internal charge transfer nature of the molecule (this will be discussed in greater detail in Chapter 3). This property can aid in determining the mechanism by which these molecules operate in cells.

Both 123 and 124 were fully characterized by the usual methods. The $^1$H NMR spectrum ($\text{(CD}_3\text{)}_2\text{SO, 400 MHz}$) of 123 is shown in Figure 2.23, where the reduction of the nitro group was confirmed by both the presence of the singlet at 7.54 ppm for the NH$_2$ protons, and the upfield shift of the H3 aromatic proton at 6.87 ppm. Having successfully synthesised 123 and 124, the next course of action was to utilise the thiazole 110 in the preparation of another series of thiazole based naphthalimide derivatised α-amino acid. The efforts directed towards achieving this will be discussed in the next section.

Figure 2.23: $^1$H NMR spectrum ($\text{(CD}_3\text{)}_2\text{SO, 400 MHz}$) of 123.
2.5.7: The Attempted Synthesis of Thiazole-α-Amino Acid Based-1,8-Napthalimides using Thiazole 110

Compound 76 was chosen as the starting material for the introduction of 110 into the napthalimide structure through an amide bond to give the desired molecule 124 (Scheme 2.15). For this work, three different coupling reagents were employed and the results from these reactions are summarised in Table 2.12.

Scheme 2.15: Synthesis of 124.

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Coupling Reagent</th>
<th>Solvent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>EDCI, HOBt</td>
<td>CH₂Cl₂</td>
<td>No reaction</td>
</tr>
<tr>
<td>B</td>
<td>EDCI, HOBt</td>
<td>THF</td>
<td>No reaction</td>
</tr>
<tr>
<td>C</td>
<td>DCC/HOBt</td>
<td>CH₂Cl₂</td>
<td>No reaction</td>
</tr>
<tr>
<td>D</td>
<td>DCC/HOBt</td>
<td>THF</td>
<td>No reaction</td>
</tr>
<tr>
<td>E</td>
<td>PyBOP</td>
<td>CH₂Cl₂</td>
<td>No reaction</td>
</tr>
<tr>
<td>F</td>
<td>PyBOP</td>
<td>THF</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

Table 2.12: The synthesis of 124. (All reactions were conducted at RT and both of the solvents were freshly distilled before use).

Unfortunately, the synthesis of 124 was unsuccessful regardless of the coupling reagents employed or the solvent used. Each of the reactions were carried out at room temperature the general work-up involved removing the solvent under reduced pressure and dissolving the resulting residue in CH₂Cl₂. The organic phase was then washed with sat. NaHCO₃, water and then finally with brine.

The coupling reagents, EDCI and HOBt were chosen, as they had already been utilised for the successful synthesis of 118 - 121. However, on this occasion they were unsuccessful in giving rise to the formation of 124, with only the starting material being
recovered. The next two attempts were carried out using DCC and HOBT. The problem associated with using this particular method is the difficulty associated with removing the dicyclohexylurea (DCU) side-product from the reaction mixture. In general, DCU is insoluble in both CH$_2$Cl$_2$ and THF, and therefore should precipitate from the reaction mixture. Nevertheless, when attempting to form 124, it was found that some of the DCU dissolved in the THF and then proceeded to precipitate at various times during work-up. Strangely, according to $^1$H NMR, neither of these reactions seemed to have occurred and only the starting material was recovered. The final attempt of achieving the synthesis of 124, involved using the phosphonium salt coupling reagent, PyBop, but this method again proved unsuccessful. The reason for the unsuccessful formation of 124 was probably due to the unreactivity of 109 as it is weakly nucleophilic. Consequently, due to time constraints, this work was not continued any further.

Having established that 109 could not be used any further, work was devoted to removing the methyl ester protection group of 118 – 121 and 123 – 124, as this would allow for a second thiazole amino acid unit to be attached at the newly liberated carboxylate terminus, giving rise to the formation of polyamine naphthalimide derivatives, such as 125. This work could then be even further extended by "simply" coupling the free acid to an $\alpha$-amino ester (to give 126), an $\alpha$-amino alcohol (to give 127), or a variety of basic units such as hydrazine and dimethylidiamine ethylene.

![Scheme 2.16: Target polyamine derivatives 125 – 127.](image-url)
Both Phelan and Hussey had investigated the hydrolysis of methyl and ethyl esters of naphthalimide based peptides. However, both were unable to remove the methyl ester group from such naphthalimide derivatives, and therefore it was anticipated that this proposed work would not be trivial. However, as the presence of the thiazole moiety may, in fact, enhance the cleavage of the methyl ester-protecting group, it was decided to investigate the hydrolysis of the ester. This work will now be discussed.

2.5.8: Removal of the Methyl Ester-Protecting Group from 118 and 123

Initial studies for achieving the hydrolysis of the methyl esters were carried out on 118 (Scheme 2.17). As previously discussed in Section 2.5.3, the removal of a methyl ester-protecting group can be carried out either using acid-catalysed hydrolysis or by saponification. Unfortunately, when the base-catalysed hydrolysis of the methyl ester of 118 was attempted using NaOH (10 eq.) in MeOH, either at room or by stirring at reflux, the reaction was unsuccessful and only the starting material was isolated (Table 2.13).

![Scheme 2.17: Attempted synthesis of 128.](image)

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Reagent</th>
<th>Solvent</th>
<th>Temp.</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NaOH (10 eq.)</td>
<td>MeOH/H₂O (1:1)</td>
<td>RT or Reflux</td>
<td>No reaction</td>
</tr>
<tr>
<td>B</td>
<td>HCl</td>
<td>Acetone/H₂O (7:1)</td>
<td>Reflux</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

Table 2.13: Reaction conditions used for the synthesis of 128.

As previously mentioned, Phelan had also carried out hydrolysis on a number of α-amino acid based naphthalimide methyl esters. These attempts involved using either NaOH (1 eq. or 10 eq.) or LiOH (1 eq.) and stirring the reactions at reflux in MeOH. Unfortunately, none of these procedures were successful in yielding the free acid.
Consequently, the second attempt at synthesising 128 was carried out using acid-catalysed hydrolysis, using the same procedures as used for the formation of the phthaloyl-protected thiazole 103. This involved stirring 118 in acetone at reflux in the presence of conc. HCl. However, this method again proved unsuccessful. In the light of these results, attempts at removing the methyl ester-protecting group from 118 were abandoned, and not investigated for the 3- or 4-nitro substituted derivatives 119 - 121.

The inability to remove the methyl ester is a major disadvantage towards the further functionalisation of 118 – 121. Consequently, an attempt was made at obtaining the free acid of the corresponding 4-amino derivative 124 to give 129. The hydrolysis was carried out using NaOH (10 eq.) as a base and stirring the reaction in a solution mixture of THF and water (9:1) at room temperature (Scheme 2.18).

Following completion of the reaction, a small amount of water was added to dissolve any remaining precipitate. The aqueous layer was then washed three times with ethyl acetate, and acidified to pH 4 by the addition of 10 % citric acid. Following this, the product was extracted with ethyl acetate and the organic extracts were combined and washed with water and then finally with brine. Unlike that seen for 119 – 121, this method gave the corresponding free acid 129 as a bright yellow solid in 61 % yield before further purification. Unfortunately, the $^1$H NMR ((CD$_3$)$_2$SO, 400 MHz) spectrum of this molecule showed the presence of a minor (unidentifiable) impurity, which could not be removed by either a recrystallisation using MeOH or by using flash column chromatography on neutral silica (CH$_2$Cl$_2$/MeOH-NH$_2$ 2 %). The cleavage of the methyl ester was, however, confirmed using ESMS giving $m/z$ = 523 for [M+Na]$^+$. The presence of the small amount of impurity in the $^1$H NMR spectrum resulted in this molecule not being submitted for cytotoxic analysis.
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing \( \alpha \)-Amino Acids

An alternative design strategy for achieving the formation of the polyamide derivatives was considered. This involved treating 103 with 104 in a peptide coupling reaction to give the corresponding di-peptide 130 (Scheme 2.19). Cleavage of the phthaloyl protecting group from this molecule, followed by a second coupling reaction to the naphthalimides 76 - 78, could then lead to the desired target polyamine conjugates, such as 125 shown in Scheme 2.8. It was anticipated that the formation of 130 should be easily achieved, given that 104 had already been successfully coupled with the free acid of 76, 77, 115 and 116 (Section 2.5.5).

\[
\begin{align*}
\text{Scheme 2.19: The formation of 130 using 103 and 104.}
\end{align*}
\]

2.5.9 Synthesis of the Thiazole Containing Peptide 130

The synthesis of 130 was readily achieved by treating 103 with PyBop (1.2 eq) and DIPEA in dry THF at 0 °C and then further reacted with 104 at room temperature. This gave 130 as a creamy white solid in 87 % yield, following a recrystallisation from EtOH. The compound was characterized by the usual methods, with \( ^1H \) NMR being consistent with its structure. Unfortunately, this reaction could only conducted on a small scale due to the limited supply of 103 and 104. Nevertheless, the ease with which 130 can be synthesized, coupled with the cleavage of the phthaloyl protecting group of 102, will aid in the development of the desired polyamine thiazole containing naphthalimides. This work is currently being carried out.

Up to this point, six novel thiazole-based naphthalimide containing \( \alpha \)-amino acids have been synthesised in a six-step synthesis. These molecules were prepared in a convergent fashion from the appropriately protected thiazole unit 102, which was synthesized by the oxidation of the corresponding thiazolidine intermediate 100. Both of the protecting groups of 102, \( i.e \) the methyl ester and the phthaloyl group, were successfully cleaved, which was an essential step towards the development of the target molecules. However, the discovery of the most suitable conditions to conduct these
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids

The synthesis of aldehyde, alkene and thiazole modified 1,8-naphthalimide containing α-amino acids was challenging and in some cases only achieved in low yields. Most importantly, the successful synthesis of 118 - 121 without any racemization was a major breakthrough for the development of these compounds within our laboratory. All of the target compounds synthesised, including the intermediates 100 - 104 have been analysed for their cytotoxic activity in leukaemia cell lines by Daniel Ömar Frimannsson, at the Department of Haematology, St. James Hospital. These results will be highlighted in Chapter 5, but the results from this chapter will be discussed in more detail in Mr. Frimannsson's PhD thesis.

As previously mentioned, the above strategy for the synthesis of the target thiazole containing naphthalimide derivatives was developed because of the experienced unreactivity of the α-amino aldehyde functionality. It has been speculated (by both Phelan and Hussey)\(^1\) that the naphthalimide moiety may inhibit the reactivity of the aldehyde moiety due to steric constraints and also by possible electronic effects. Hence, it was decided to determine if this was definitely the case by attempting to synthesise a naphthalimide-based aldehyde, and reacting it with L-Cysteine to form the corresponding thiazolidine intermediate 133. A lack of reactivity would then fortify the rationale in the synthetic route undertaken for the development of the target molecules discussed within this Chapter.

2.6 Synthesis of Naphthalimide Derivatives Functionalised with a Thiazole Containing Amino Acid

The four-step synthesis of the thiazolidine-based naphthalimide derivative 133 is shown in Scheme 2.20.

![Scheme 2.20: Attempted synthesis of 133.](image-url)
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified 1,8-Naphthalimide Containing \( \alpha \)-Amino Acids

The first step of the reaction sequence involved the condensation reaction between 4-nitro-1,8-naphthalic anhydride and aminoaldehyde diethyl acetal, which gave 131 in 95% yield after recrystallisation from MeOH. This compound was fully characterised by \( ^1 \)H and \( ^{13} \)C NMR, IR, and HRMS. The \( ^1 \)H NMR spectrum (CDCl\(_3\), 400 MHz) of 131 is shown in Figure 2.24 and shows the expected aromatic signals, each of which integrate to one proton. The CH of the aminoaldehyde diethylacetel moiety appears as a triplet at 5.04 ppm, while the adjacent methylene protons resonate as a doublet at 4.40 ppm. The methylene protons appear as two sets of quartets between 3.54 and 3.82 ppm and the protons of the methyl groups appear as doublets at 1.16 ppm.

![Figure 2.24: \( ^1 \)H NMR spectrum (CDCl\(_3\), 400 MHz) of 131.](image)

Subsequent acetal hydrolysis using neat TFA gave the desired aldehyde derivative 132 as a white solid in 89% yield. According to \( ^1 \)H NMR analysis ((CD\(_3\))\(_2\)SO, 400 MHz), the successful formation of 132 was verified by the presence of a singlet at 9.60 ppm for the aldehyde proton and the by the absence of the signals of the acetal proton, as previously described (Figure 2.23). To prepare the corresponding thiazolidine intermediate 133, the aldehyde 132 was treated with L-Cysteine using the same conditions employed for the synthesis of 100 (Section 2.5.1). However, this attempt was unsuccessful by giving rise to an undistinguishable \( ^1 \)H NMR spectrum (CDCl\(_3\), 400 MHz) confirming the unreactivity of the aldehyde moiety for further functionalisation, at least in this reaction, while also supporting the synthetic strategy taken for the work discussed within this chapter.

As an alternative means of generating 134 and 135, the thiazole 104 was simply condensed with both 3 and 4-nitro-1,8-naphthalic anhydride in a single step (Scheme 2.21). The reaction was carried using the same procedure as discussed for 72–75 (Section 2.2),

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however, without the use of NEt₃. This gave 134 and 135 in 71 % and 88 % yield, respectively, following a recrystallisation from MeOH.

Scheme 2.21: Synthesis of 134 and 135.

The ¹H NMR spectrum of 135, shown in Figure 2.25, is quite simple, with the aromatic region showing four doublets and a triplet for the naphthalimide protons, and a singlet at 8.15 ppm for the thiazole proton. The methylene protons and the methyl protons show as sharp singlets at 5.76 and 3.94 ppm, respectively.

Figure 2.25: ¹H NMR spectrum (CDCl₃, 400 MHz.) of 135.

Unfortunately when the cytotoxic activity of all the thiazole modified 1,8-naphthalimide containing α-amino acids discussed within this chapter were evaluated, the results were not as good as anticipated (see details in Chapter 5). Consequently, the development of these molecules was not continued any further. The main reason being that at this stage very promising results had been obtained on the DNA binding capability of the 1,8-naphthalimide containing Tröger’s bases developed within this PhD programme (these are discussed in Chapter 3 and 4). Nevertheless, as shown in Chapter 5, the cytotoxic
activity of 118 – 121 and 123 - 124 were still greater than some of the more simple \( \alpha \)-amino acid containing naphthalimides, previously synthesised within the Gunnlaugsson group. Consequently, there currently exists an interest in the further development of these molecules, which will be carried out by another member of the group. In the meantime we decided on further utilising the thiazolidine intermediate 100 by attempting a series of \( N \)-alkylation reactions. The following section deals with this.

### 2.7 \( N \)-Alkylation of the Thiazolidine Intermediate 100

For the \( N \)-alkylation of thiazolidine 100, a number of reactions were conducted by varying the base and the reaction conditions (Scheme 2.30). The two first attempts were carried out using NEt\(_3\) as a base and benzylchloride as the alkylating agent. The general procedure involved treating 100 with NEt\(_3\) (1 eq.) for one hour at room temperature, after which benzylchloride (1 eq.) was added and the reaction mixture was stirred for 24 hours at reflux. The first reaction was carried out in CH\(_2\)Cl\(_2\) (Attempt A) while the second was carried out in CH\(_3\)CN (Attempt B).

![Scheme 2.22: Attempted \( N \)-alkylation of 100 to give 136.](image)

According to \(^1\)H NMR analysis both reactions had been unsuccessful in giving the desired alkylated product. Instead both spectra showed the presence of a mixture of diastereoisomers of 100. The \(^1\)H NMR spectrum (CDCl\(_3\), 400 MHz) from Attempt A is shown in Figure 2.26. Upon comparing this spectrum with that of 100 (Figure 2.10, Section 2.5.1.1) the peaks for one of the diastereoisomers were found to belong to that of the \((2R,4R)\) diasterisomer. As previously discussed in Section 2.5.1.2, solid-state analysis of 100 allowed its absolute stereochemistry to be assigned. The peaks for the ABX system of the other diastereoisomer, 138, are highlighted in red boxes in Figure 2.20 and according to the integration of the peaks, which appear at 5.2 and 4.9 ppm, respectively, the ratio of the two diastereoisomers is approximately 95:5.
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Figure 2.26: \(^1H\) NMR spectrum (CDCl\(_3\), 400 MHz) of 100 and its other diastereisomer.

Unfortunately the stereochemistry of the second diastereoisomer cannot be assigned. Nevertheless, it has been reported in the literature that thiazolidine rings are susceptible to epimerisation and it has also been suggested that this process may occur at the C-2 chiral centre of the thiazolidine ring.\(^{125}\) Therefore, it could be speculated that the stereochemistry of the other diastereisomer is \((2S, 4R)\). Nevertheless, a solid-state analysis of this molecule could only verify such a statement.

An attempt at separating the diastereoisomers using column chromatography was not carried out simply because the ratio between them was too large (\(i.e.\) one of the compounds was only present in a small amount). Alternatively, as a means of increasing the ratio of the other unknown diastereoisomer, the mixture was treated with NEt\(_3\) in CH\(_2\)Cl\(_2\) overnight at room temperature. According to \(^1H\) NMR, this procedure gave rise to a 50:50 mixture of the diasterisomers. Following this, an attempt was made to separate these products by column chromatography on flash silica (ethyl acetate/CH\(_2\)Cl\(_2\)/hexane 3:2:3). This method of purification was successful, and the \(^1H\) NMR spectra (CDCl\(_3\), 400 MHz) of the 50:50 mixture, 100 and 138 are shown in Figure 2.27a, 2.27b and 2.27c, respectively. The resonances for 138 were assigned by means of both a COSY and NOE experiments. As can be seen in Figure 2.26c, the geminal pair of protons, \(Ha\) and \(Ha'\), appear as two double doublets at 3.95 and 3.75 ppm, respectively, while the adjacent \(\alpha\)-proton, \(Hz\), appears as a double doublet at 5.50 ppm. The second geminal pair of protons, \(Hb\) and \(Hb'\) appear as a double doublet and a triplet at 3.43 and 2.92 ppm, respectively. Each proton is coupled to each other as well as \(Hx\), which shows as a triplet at 4.21 ppm. As expected, two clear
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ABX systems can be observed, but the signals are well shifted with respect to the signals for the ABX system of 100, shown in Figure 2.27b.

![ABX systems](image)

Figure 2.27: $^1H$ NMR spectrum (CDCl$_3$, 400 MHz) of (A) the 50:50 mixture of diasterisomers, (B) 100 and (C) 137. (The aromatic region is not included).

As a means of determining the stereochemistry of 138, an attempt was made at growing crystals of the compound by slow evaporation from a solution mixture containing CH$_2$Cl$_2$ and MeOH. This resulted in the formation of off-white single crystals, which were fortunately suitable for X-ray crystallography diffraction analysis, which was carried out by Dr. T. McCabe in the School of Chemistry, Trinity College Dublin.

2.7.1 Solid State Analysis of 138

The crystal structure of 138 is shown in Figure 2.28 and selected bond lengths and angles are summarised in Table 2.14. The relative stereochemistry of 138 was assigned as (2S, 4R), confirming that the epimerisation of 100 must have occurred at the C-2 position of the thiazolidine ring. To the best of our knowledge, the use of X-ray crystallography to prove such epimerisation has not been achieved to date.
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Figure 2.28: Crystal structure of 138, grey represents carbon atoms, red oxygen atoms, blue nitrogen atoms and white hydrogen atoms

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N4 C9</td>
<td>1.452 (2)</td>
<td>N4 C9 C10</td>
<td>110.75 (13)</td>
</tr>
<tr>
<td>C9 C10</td>
<td>1.532 (2)</td>
<td>C9 C10 S2</td>
<td>108.26 (11)</td>
</tr>
<tr>
<td>C10 S2</td>
<td>1.836 (17)</td>
<td>C9 C10 N3</td>
<td>112.00 (14)</td>
</tr>
<tr>
<td>S2 C11</td>
<td>1.804 (18)</td>
<td>N3 C12 C13</td>
<td>110.68 (13)</td>
</tr>
<tr>
<td>C11 C12</td>
<td>1.535 (2)</td>
<td>C11 C12 C13</td>
<td>109.08 (13)</td>
</tr>
<tr>
<td>C12 N3</td>
<td>1.456 (2)</td>
<td>C12 C13 O4</td>
<td>109.99 (15)</td>
</tr>
<tr>
<td>N3 C10</td>
<td>1.445 (2)</td>
<td>O3 C13 O4</td>
<td>125.25 (16)</td>
</tr>
<tr>
<td>C12 C13</td>
<td>1.514 (2)</td>
<td>C13 O4 C28</td>
<td>115.79 (13)</td>
</tr>
<tr>
<td>C13 O3</td>
<td>1.203 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C13 O4</td>
<td>1.328 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O4 C28</td>
<td>1.450 (2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.14: Selected bond lengths and bond angles for 138.

The packing diagram of 138 is shown in Figure 2.29 viewed along the crystallographic b* axis. Upon analysing this packing, intermolecular hydrogen bonding interactions were observed between the sulphur atom (S2) of the thiazolidine ring and one of the amide oxygens (O1). The length between this contact was determined to be 3.04 Å. There were also non-bonding contacts between the nitrogen atom (N4), the Oxygen (O3) and between the oxygen atom (O2) and an aromatic proton.
Having determined the absolute stereochemistry of 138 as being (2S, 4R) and that it was indeed the C-2 sterogenic centre of 100 which had epimerised, the mechanism shown in Scheme 2.23 was considered. As shown, the mechanism would involve the deprotonation of the acidic NH proton causing the thiazolidine ring to open. This gives rise to Schiff base intermediate, which then undergoes a ring closure, ultimately changing the stereochemistry at C-2 chiral centre. In fact, the base-catalysed isomerization of thiazolidines through a Schiff base intermediate has been reported within the literature,\textsuperscript{125} which further support the suggested mechanism shown in Scheme 2.31. However, to the best of our knowledge, the deprotonation by a weak base such as NEt\textsubscript{3} has not been reported within the literature.

**Scheme 2.23:** Possible mechanism for the C-2 epimerisation of 100 via an schiff base intermediate to give 138.
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At this stage the alkylation of 100 had not been successfully achieved. Consequently, three more attempts were carried out using K$_2$CO$_3$ as a base. The reaction conditions used and the outcome of each reaction attempt are summarised in Table 2.15 (C – E).

![100](image1) → Base, RX → ![136](image2) 137

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Base</th>
<th>RX</th>
<th>Solvent</th>
<th>Conditions</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Et$_3$N</td>
<td>C$_6$H$_5$CH$_2$Cl</td>
<td>CH$_2$Cl$_2$</td>
<td>Reflux 24 h</td>
<td>R = H. R,R,S,R 95:5</td>
</tr>
<tr>
<td>B</td>
<td>Et$_3$N</td>
<td>C$_6$H$_5$CH$_2$Cl</td>
<td>CH$_3$CN</td>
<td>Reflux 21 h</td>
<td>R = H. R,R,S,R 95:5</td>
</tr>
<tr>
<td>C</td>
<td>K$_2$CO$_3$</td>
<td>C$_6$H$_5$CH$_2$Cl</td>
<td>CH$_3$CN</td>
<td>Reflux 18 h</td>
<td>R = H. R,R,S,R 50:50</td>
</tr>
<tr>
<td>D</td>
<td>K$_2$CO$_3$</td>
<td>C$_6$H$_5$CH$_2$Cl</td>
<td>CH$_3$CN</td>
<td>Reflux 18 h, KI</td>
<td>R = CH$_2$C$_6$H$_5$</td>
</tr>
<tr>
<td>E</td>
<td>K$_2$CO$_3$</td>
<td>CH$_3$I</td>
<td>CH$_3$CN</td>
<td>Reflux 2 days</td>
<td>R = CH$_3$. S,R 45 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = H. R,R 55 %</td>
</tr>
</tbody>
</table>

Table 2.15: Attempted synthesis of 136 and 137

When thiazolidine 100 was treated with K$_2$CO$_3$ (1 eq.) in acetonitrile and subsequently reacted with benzylchloride (1 eq.), the alkylation reaction was again unsuccessful, giving rise to a 50:50 mixture of 100 and 138. The next attempt was carried out in the presence of potassium iodide and also by increasing the amount of K$_2$CO$_3$ and benzylchloride to 1.5 and 1.2 equivalents, respectively. This reaction resulted in the successful formation of 136, which was obtained as an off-white solid in 69 % yield, following purification by column chromatography on neutral flash silica (ethyl acetate/hexane 1:1 and 1 % NEt$_3$). According to $^1$H NMR analysis (CDCl$_3$, 400 MHz), 136 had been formed as a single diastereoisomer (Figure 2.30). However, the ability to determine the stereochemistry of this molecule comparing it with either 100 or 136, was not possible as the signals for the ABX system had shifted due to the presence of the benzyl group. With this in mind, an attempt was made at growing crystals of 136 by slow
evaporation from a solution mixture containing CH$_2$Cl$_2$ and MeOH. Unfortunately, the resulting off-white crystals were unsuitable for crystallographic diffraction analysis.

Figure 2.30: $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 136.

Compound 136 was further characterised by $^{13}$C NMR, IR, ESMS and HRMS analysis. ESMS analysis gave m/z = 419 for [M+Na], while in the $^{13}$C NMR spectrum (CDCl$_3$, 400 MHz), the three methylene carbon resonances appeared at 61.8, 42.8 and 33.4 ppm, respectively, while the seven resonances for the carbons of the aromatic rings appeared between 137.8 and 122.6 ppm.

With the above successes, the alkylation reaction was again repeated but using CH$_3$I as the alkylating agent instead of benzylchloride. This gave rise to the formation of a 55:45 mixture of the successfully alkylated (2R, 4R) diastereoisomer, 137, and the (2S,4R) diastereoisomer 138. The successful formation of 137 was confirmed by $^1$H NMR analysis as well as by ESMS analysis, which gave m/z = 434 and 329 for [M+Na] for 137 and 138, respectively. Rather than trying to separate these products, the mixture was oxidised using MnO$_2$ following the procedure used for the preparation of the thiazole 102 (Section 2.5.2). In fact, this resulted in the formation of 102, with $^1$H and $^{13}$C NMR (CDCl$_3$, 400 MHz) being consistent with its structure.
In summary, the alkylation of 100 with benzylchloride was achieved using \( \text{K}_2\text{CO}_3 \) as a base in the presence of KI. This method gave rise to the formation of desired product 136 as a single diastereoisomer. When the reaction was carried out using NEt\(_3\) or in the absence of KI, the alkylation of 100 was unsuccessful and a mixture of diastereoisomers was obtained. The absolute stereochemistry of these molecules was established using X-ray crystallography as being \((2R, 4R)\) for 100 and \((2S, 4R)\) for 138. From this, it was established that the epimerisation process was occurring at the C-2 centre of the thiazolidine ring.

As an extension to the above finding, we decided to investigate the rate of epimerisation in a range of solvents of different polarities by \(^1\text{H}\) NMR. Our interest for conducting such a study stems from the fact that the formation of the thiazolidine ring, by the reaction of an aldehyde with cysteine, has been implicated in many biochemical processes and also constitutes the first step in the synthesis of penicillin and biotin.\(^{126}\)

### 2.8 Epimerisation Studies of the Thiazolidine 100

To investigate the rate of epimerisation, the \(^1\text{H}\) NMR spectra of 100 was recorded at 300 K immediately after dissolving the compound in the appropriate solvent until the process reached equilibrium. As shown in Figure 2.21 (Section 2.7), the apparent difference between the chemical shifts of the C-2 protons of the \((2R, 4R)\) and the \((2S, 4R)\) diasterisomers would allow the process to be easily followed. The solvents used in these studies were CDCl\(_3\), \((\text{CD}_3)_2\text{SO}\), \((\text{CD}_3)_2\text{CO}\) and \(\text{CD}_3\text{CO}_2\text{D}\). The epimerisation of 100 was also followed using CDCl\(_3\) in the presence of a small amount of \((\text{C}_2\text{H}_5\text{O})\text{BF}_3\), to determine whether or not the process was acid-catalysed. As previously mentioned, the crystals of 100 were grown by the slow evaporation of \(\text{CH}_2\text{Cl}_2\) from a saturated solution of the compound in MeOH. Consequently, it was decided to also follow the process in CDCl\(_3\) and MeOD (1:3) as well.

The stack plot of the \(^1\text{H}\) NMR spectra (CDCl\(_3\), 600 mHz) of 100 after recording the sample over five days is shown in Figure 2.31. The changes in the resonance for the Hz proton at 4.87 ppm were followed. After 6 hours, the signal for the Hz proton of the \((2S, 4R)\) diasterisomer was observed at 5.14 ppm. Upon integrating both signals, it was determined that there was approximately a 4:1 ratio of the \((2R,4R)/(2S,4R)\) diasterisomers present in solution. This process, while being slow, reaches equilibrium after 5 days with a \((2R, 4R)/(2S, 4R)\) ratio of 1:2.
When the above experiment was repeated in the presence of a small amount of (C\textsubscript{2}H\textsubscript{5}O)BF\textsubscript{3}, the epimerisation of 100 also came to equilibrium after 5 days. However, unlike that seen above, after 3 hours there was 3:1 ratio of (2R, 4R) to (2S, 4R) and upon reaching equilibrium there was a (1R, 4R)/(2S, 4R) ratio of 1:4 present in solution. This would indicate that the process is acid-catalyzed. Further evidence for this was given by the fact that the epimerisation process in (CD\textsubscript{3})\textsubscript{2}CO did not occur. Even though the polarities of CDCl\textsubscript{3} and (CD\textsubscript{3})\textsubscript{2}CO are quite similar, CDCl\textsubscript{3} is slightly acidic. With this in mind, the epimerisation of 100 was followed in C\textsubscript{2}D\textsubscript{4}O\textsubscript{2} (Figure 2.32), and was found to reach equilibrium after 1.5 days with a (R,R)/(S,R) ratio of 1:1 and remained unchanged even after 4 days.
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When the spectra were recorded in \((CD_3)_2SO\), (Figure 2.33), the process came to equilibrium after 6 days with a \((2R,4R)/(2S,4R)\) ratio of 1:2. Typical first order kinetics were observed for this process and the rate constant was determined to be \(3 \times 10^{-6} \text{ S}^{-1}\). However, it took nearly three days for a 50:50 mixture of the diasterisomers to be present in solution, indicating that the epimerisation of 100 occurs at a slower rate in a more polar solvent.

![Figure 2.33: Stack plot of the \(^1H\) NMR spectra ((CD$_3$)$_2$SO, 600 MHz) of 100.](image)

The final study undertaken involved following the epimerisation of 100 in a mixture of CDCl$_3$ and MeOD (1:3). After 12 hours, formation of the \((2S,4R)\) diasterisomer was not observed. Interestingly, this is the same timeframe in which the crystals of 100 were formed, as discussed in Section 2.5.1.2. Moreover, it was only after eight days that the signal pertaining to the Hx proton of the \((2S,4R)\) diasterisomer was observed and an integration of each of the signals for the protons of the C-2 center gave a \((2R,4R)/(2S,4R)\) ratio of 6:1. This indicated that the presence of a protic solvent significantly reduces the rate of epimerisation, maybe through hydrogen bonding interactions with the NH of 100.

As previously mentioned, the rate of epimerisation in \((CD_3)_2SO\) followed a first order kinetics giving a rate constant of \(3 \times 10^{-6} \text{ S}^{-1}\). Strangely, it was found that the epimerisation of 100 in all of the other solvents did not obey first order or second order kinetics, and consequently a rate constant could not be determined. Nevertheless, it can be summarised that the epimerisation of 100 was found to be acid-catalyzed. In acidic solutions the mechanism may involve isomerisation via a sulfonium ion, as shown in Scheme 2.24. According to Pesek et al.,\(^{125a}\) the stabilization of the ring structure with respect to open-chain intermediates occurs in moderately acidic solutions, where the
monoprotonated form are predominant. However, isomerisation through a sulphonium ion requires the presence of excess of a strong acid to give a fully protonated system.

\[
\begin{align*}
\text{(2R,4R)} & \quad \leftrightarrow \\
\text{(2S,4R)} & 
\end{align*}
\]

**Scheme 2.24:** Possible mechanism for the C-2 epimerisation via a sulfonium ion intermediate.

In summary, the epimerisation of 100 has been studied in a range of solvents by varying their acidity and polarity. The process has been shown to be acid-catalyzed by a dramatic increase in the formation of the (2S,4R) diasterisomer when monitored in C\textsubscript{2}D\textsubscript{4}O\textsubscript{2}. The epimerisation of 100 was, however, nonexistent in (CD\textsubscript{3})\textsubscript{2}CO, while it did occur in (CD\textsubscript{3})\textsubscript{2}SO, but to a much slower extent in comparison to the same in CDC\textsubscript{13}.

So far this Chapter has dealt with the synthesis of aldehyde, alkene and thiazole modified 1,8-naphthalimides containing α-amino acids. The synthesis of these molecules was challenging due to the general unreactivity of the α-amino aldehyde moiety. For this reason a different synthetic route was undertaken for the preparation of the thiazole modified naphthalimides. This was achieved in a convergent fashion, whereby the thiazolidine containing amino acids were prepared and then subsequently incorporated into the naphthalimide structure. All the target molecules synthesized and discussed within this Chapter have been tested for their anticancer activity in the leukemia cell lines HL-60 and K562, the results of which will be discussed in Chapter 5, as well as being the theme of Mr. Daniel Frimannson’s PhD thesis, as previously mentioned.

As mentioned in Chapter 1, the second approach taken for enhancing the biological activity of the 1,8-naphthalimide was to modify its structure by the introduction of the Tröger’s base structural unit. This was also carried out as a means of reinforcing its DNA binding capacity. However, before embarking on this work, we became interested in developing thiocyclic fused naphthalimide containing α-amino acids, the results of which will be dealt with in the following section.
2.9 Thiocyclic-Fused Naphthalimide Derivatives

Recently Qian et al.\textsuperscript{127} reported the synthesis and photobiological activity of two novel thiocyclic-fused naphthalimide hydroperoxides. In their report they showed that a thiocyclic-fused ring on the naphthalimide moiety contributed significantly to its binding affinity with DNA. From these results, the aim was to synthesise the equivalent peptide-based naphthalimides 144 and 145.

2.6 Synthesis of the Thiocyclic-Fused Naphthalic Anhydrides 142 and 143

Qian et al.\textsuperscript{127a} has recently synthesised the reagents 142 and 143 from 4-bromo-3-nitro-1,8-naphthalic anhydride through a Pschorr cyclisation, as shown in Scheme 2.25.

Following published procedures,\textsuperscript{127a} 139 was synthesised via an electrophilic aromatic substitution reaction between the commercially available 4-bromo-1,8-naphthalic anhydride and the nitronium ion formed from nitric and sulphuric acids. Precipitation from
water followed by a recrystallisation from acetic anhydride gave 139 as a beige solid in 74 % yield and was characterised by $^1$H, $^{13}$C NMR (CDCl$_3$, 400 MHz) and ESMS.

The nitro compound 139 was then treated with thiophenol in dry ethanol. After stirring at reflux for five hours, the reaction mixture was allowed cool to room temperature and the resulting precipitate was collected by suction filtration. This gave 140 as a bright yellow solid in 97 % without the need for purification. Subsequent reduction of the nitro group was achieved by treating 140 with a mixture of stannous chloride and concentrated hydrochloric acid. After warming to 40 °C, the temperature rose spontaneously to 85 °C and was maintained at 85 °C for one hour. After cooling the reaction mixture, the resulting precipitate was collected by suction filtration. This gave 141 as an olive green solid in 88 % yield. $^1$H NMR analysis (CDCl$_3$, 400 MHz) confirmed the formation of 141 by the presence of a singlet peak at 5.08 ppm for the amino group and ESMS gave m/z = 321 for [M + H]$^+$. The synthesis of the isomers 142 and 143 was attempted via a Pschorr cyclisation reaction. This reaction has been widely used in the preparation of polycyclic compounds.$^{46,57}$ The amine 141 was treated at 0 - 5 °C with nitrous acid, which was prepared by the treatment of sodium nitrite in acetic acid with sulfuric acid. The progress of the reaction was accompanied by a dramatic colour change from green to dark red. The diazonium salt coupling was then carried out by treating the reaction mixture with a boiling solution of CuSO$_4$ in water and acetic acid. The reaction mixture was stirred at reflux for 30 minutes, after which the precipitated product was collected by suction filtration. Both the TLC (ethyl acetate/hexane 2:1) and the $^1$H NMR ((CD$_3$)$_2$SO, 400 MHz) analysis showed the presence of a mixture of products. Purification was attempted by preparatory plate chromatography on silica (using CH$_2$Cl$_2$ as eluent), where each time the preparatory plate was ‘developed’ three times. According to the $^1$H NMR (CDCl$_3$, 400 MHz) analysis, this method of purification had been unsuccessful. However, it was discovered some time later that the exact purification method for separating these isomers had been excluded from the experimental section of the publication made by Qian et al.$^{127a}$ After a communication with the authors, it was found that the purification method involved at least six separations by preparatory plate chromatography on silica. This would consequently dramatically reduce the yield of the desired molecules and for this reason the synthesis of 142 and 143 was not continued any further.
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2.10 Conclusion

The first section of this Chapter dealt with the synthesis of four α-amino-(1,8-naphthalimide) aldehyde derivatives \(^{84 - 87}\) as synthons for further modification. This involved the successful transformation to the target α-amino-(1,8-naphthalimide) αβ-unsaturated ester derivatives \(^{88 - 90}\) which was the principal concept of this research. The transformation was achieved through the use of the HWE reaction. The discovery of the most suitable variation of this reaction method was challenging, because the α-amino aldehyde moieties were found to be unreactive, possibly due to steric and electronic effects inflicted by the naphthalimide moiety. The cytotoxic activity of these target molecules has been determined in the leukaemia cell line HL-60, the results of which will be discussed in Chapter 5.

The second section of this chapter dealt with the synthesis of the thiazole modified 1,8-naphthalimides containing α-amino acids \(^{118 - 124}\). Due to the experienced unreactivity of the α-amino aldehyde moiety, the synthesis of the target molecules was attempted in a convergent fashion whereby thiazole containing amino acids were synthesised and subsequently incorporated into the naphthalimide skeletons. Using this approach, two phthaloyl based thiazolidine containing amino acids \(^{100}\) and \(^{101}\) and the corresponding thiazole \(^{102}\) were synthesised, after efficient synthetic optimisation of reaction conditions. Following this, the methyl ester and the phthaloyl-protecting group of \(^{102}\) were removed to give the corresponding the free acid \(^{103}\) and the free amine \(^{104}\), respectively. Both deprotection steps required efficient synthetic modification and the thiazole ring was found to be sensitive to the conditions under which the hydrazinolysis reaction was conducted. Single crystals of \(^{100}\), \(^{102}\) and \(^{104}\) were successfully grown by the slow evaporation of CH\(_2\)Cl\(_2\) from a saturated solution of the compound in MeOH, all of which were suitable for X-ray crystallography diffraction analysis. Furthermore, the crystal structure of \(^{100}\) allowed for its absolute stereochemistry to be assigned as \((2R, 4R)\).

Having successfully synthesised the thiazole containing amino acid \(^{104}\), four thiazole amino acid-based 4-nitro naphthalimides \(^{118 - 121}\), and their corresponding amino derivatives \(^{123 - 124}\) were synthesised in high yields. All of these target molecules have been analysed for their cytotoxic activity in both the HL-60 and in the more resistant K-562 cell line. These results will be highlighted in Chapter 5.

Efforts were also directed towards developing 1,8-naphthalimide derivatives wherein the thiazole containing amino acid was directly incorporated into its structure. In an effort
Chapter 2: Synthesis of Aldehyde, Alkene and Thiazole Modified
1,8-Naphthalimide Containing α-Amino Acids

to achieve this, two synthetic routes were adopted. Within route one, the 1,8-naphthalimide containing aldehyde 132 and subsequently reacted with L-Cysteine methyl ester. However, this proved to be unsuccessful. Within route two, the desired naphthalimide containing thiazoles 134 and 135 were obtained by a simple condensation reaction between 3- or 4-nitro 1,8-naphthalic anhydride and thiazole 104.

A series of attempts were made at N-alkylating the thiazolidine intermediate 100, the results of which were discussed in Section 2.7. The synthesis was achieved by using K$_2$CO$_3$ as a base in the presence of KI. This gave the alkylated product as a single diastereoisomer. All other attempts resulted in the formation of a mixture of the (2R, 4R) and the (2S, 4R) diastereoisomers of 100. The absolute stereochemistry of the (2S, 4R) diasterisomer was also determined by X-ray crystallographic analysis. Following on from these results, the rate of epimerisation of 100 was monitored in a range of solvents by $^1$H NMR. It was found that the rate of epimerisation increased in the presence of acid whereas it decreased in polar solvents. From these results we can conclude that when L-cysteine methyl ester is reacted with a phthaloyl protected aldehyde a single diasteroisomer, as the kinetic product, is obtained in the solid state. However, over time, selective inversion occurs to give a mixture of C-2 epimeric thiazolidines. The final section of this chapter dealt with the synthesis of thiocyclic-fused naphthalimide derivatives. Unfortunately this proved unsuccessful because the isomers 142 and 143 were difficult to separate.

Having achieved the synthesis of the thiazole modified naphthlamide containing α-amino acids; the next course of action was to develop the naphthalimide containing Troger’s base derivatives. As mentioned in Chapter 1, the purpose of this work was to enhance the DNA binding affinity of the naphthalimide structure. The results from this work will be discussed in Chapter 3 and 4, respectively.

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Chapter 3: Design and Synthesis of Bis-1,8-Naphthalimides Containing the Tröger’s Base as Novel C$_2$-Symmetric DNA-Binding Molecules

3.1 Introduction

Many chemotherapeutic agents currently in clinical trials, are small molecules which exert their biological activity by interacting with DNA. As mentioned in Chapter 1, these binding modes include π-stacking interactions associated with intercalation of a planar aromatic group between the base pairs, hydrogen-bonding and van der Waals interactions of functionalities bound along the grooves of the DNA helix, and the electrostatic binding of a cation with the phosphate group of DNA. Such modes of binding have been extensively studied in the hope of learning new design principles for targeting DNA with an enhanced affinity. Moreover, investigations directed toward the design of site- and conformation-specific reagents provide rationales for new drug design, as well as a means of developing novel sensitive chemical probes for targeting nucleic acid structure.

Within the last decade, several research groups, as discussed in Chapter 1, have focused their attention on combining the effects of two binding motifs, in the same molecule to develop new targeting agents with enhanced selectivity, stronger binding affinity, or that act as a chemical probe or reporter molecules. In particular, the development of the bis-1,8-naphthalimide series, as efficient binders of DNA, has significantly contributed to this field of research. These molecules can, in principle, bind to DNA through intercalation, bis-intercalation, groove binding, and electrostatic interactions, depending on the nature of the chromophore and the spacer group present in them. The aim of this chapter is to incorporate the Tröger’s base motif into such a 1,8-naphthalimide skeleton as a means of developing novel bis-1,8-naphthalimide molecules as stronger DNA binders. Mr. Frimannsson in the Department of Hematology, St. James’ Hospital, Dublin, would carry out an evaluation of their biological activity in cancer cells.

Various applications of the Tröger’s base motif in the area of molecular recognition were discussed in Chapter 1 and in particular how its concave (V-shaped) structure has been utilised in various supramolecular structures, such as “torsion balances”, water-soluble cyclophanes, chiral solvating agents and hydrogen and metal-ligand-bonding receptors. Despite the many highly desirable properties of Tröger’s base, there only exist a few reports on its incorporation into known DNA-binding motifs but the results from these investigations have provided valuable information about the manner in which
these molecules bind to DNA, thus highlighting the utility of the cleft structure in the design of DNA targeting agents. This research was discussed in Chapter 1. Consequently, there exists a window of opportunity to exploit this current research, by developing several DNA targeting naphthalimide functionalised Tröger’s bases, which is the objective of the work discussed herein and Chapter 4.

The incorporation of two naphthalimide units into a single compound has not yet been discussed within this thesis and this chapter begins with a short discussion of such compounds. This will be followed by the objectives of our design where two naphthalimide units and the Tröger’s base structural motif are employed in the same molecule, the synthesis of these targets and finally by the evaluation of their various photophysical properties in both organic solvents, in water and as a function of pH. The interaction of these new molecules with DNA will be discussed in the Chapter 4.

3.2 Bis-1,8-naphthalimides

The development of the bis-naphthalimide series, as a means of enhancing both binding and anti-tumour activities, has already been thoroughly discussed in the theses of Phelan and Hussey. Consequently, this section will concentrate on those molecules that are the more cytotoxic of the bis-naphthalimide series. The general structure of these molecules involves two naphthalimide moieties joined together in a ‘head to head’ or ‘head to tail’ fashion using alkylamine linkers while maintaining the structural features of the more successful monoimides. Several lines of evidence indicate that they intercalate into the major groove of the DNA double helix. The lead drugs currently in clinical trials are Elinafide 1 (LU 79553), Bisnafide 2 (DMP 840), Bisfuronaphthalimides, 3 (MCI3335) and 4 (BisBF1). While all of these molecules demonstrate high-level antitumor activity they differ significantly in their mode of action.

Elanifide 146, developed by Brána et al., interacts with DNA through bisintercalation and binds selectivity to alternating purine-pyrimidine motifs. This is particularly the case for those containing GpT (ApC) and TpG (CpA) steps, but only weakly inhibits topoisomerase II. Bisnafide 147, developed by Chen et al., binds strongly with DNA through monointercalation and strongly inhibits topoisomerase II. The precise DNA binding site and mechanism of cytotoxic action of these bisnaphthalimides have not been determined, although from these results, it would appear that the ability of these drugs to bis-intercalate seems to effect topoisomerase II inhibition.
Elinafide and Bisnafide differ in the structure of the linker between the two-imide functions and this feature gives rise to their different modes of binding. The aminoalkyl linker chain of Elinafide separates the two chromophores by ca. 12.3 Å and this is sufficient for bis-intercalative binding. The spacer group in Bisnafide is, however, shorter and insufficient to span two base pairs, and consequently gives rise to mono-intercalation, with the second chromophore lying outside the DNA helix. Generally, bis-intercalative binding occurs in a molecule when the linker chain length is more than 10.2 Å, which enables the DNA interacting chromophores to occlude two base pairs between them.

The Bis-furonaphthalimide 148 binds to DNA with a marked preference for GC sites, but it does not promote DNA cleavage by Topoisomerase I or II. Bis-naphthalimide 149 interacts with DNA through bis-intercalation and it is more potent than parent compound 146. A lack of topoisomerase inhibition was attributed to the presence of the fused furan
Chapter 3: Design and Synthesis of Bis-1,8-Naphthalimides Containing the Tröger’s Base as Novel C₂-Symmetric DNA-Binding Molecules

ring, which gave rise to the bis-tetracyclic structures, 148 and 149, having stronger tendencies to self-associate. In general, all of these bis-naphthalimide drugs have higher cytotoxic activities, stronger binding affinity and selectivity than their parent mononaphthalimide compounds.

The peptide-based bis-naphthalimides and those incorporating naphthalene diimides, investigated by Gunnlaugsson et al., as mentioned in Chapter 1, have also shown significant enhancement in DNA-binding affinity and cytotoxic activity within leukaemia cell lines. In particular, some of the ‘tail to tail’ bis-naphthalimides synthesised by Blais, such as 150, which is shown in Figure 3.1, have exhibited promising cytotoxic activity (IC₅₀ in the range of 2.7-4.7 µM with HL60 cell line). These molecules were constructed by linking the chromophores together at the 4 positions using various polyamines and the imide part of the naphthalimide was attached to either leucine methyl ester or phenylalanine methyl ester. These molecules are, unlike those bis-naphthalimides discussed previously, highly fluorescent in the visible region. Thus, combining the latter with the activities of 146 - 149 it is clear that the formation of dimeric molecules using the 1,8-naphthalimide structure is worthwhile and DNA is obviously an ideal bioreceptor for such derivatives.

![Figure 3.1: The general structure of a 'tail to tail' bis-naphthalimide.](image)

3.3 Design of Bis-1,8-Naphthalimides Containing the Tröger’s Base

As discussed in Chapter 1, Tröger’s base 52 (Figure 3.2) has been widely utilised within supramolecular chemistry due its ability to bind guests and to allow for functional groups to be oriented in well-defined spatial arrangement. An even more exciting feature of Tröger’s base is that its chiral, cleft-like structure with a dihedral angle between two
Chapter 3: Design and Synthesis of Bis-1,8-Naphthalimides Containing the Tröger’s Base as Novel C$_2$-Symmetric DNA-Binding Molecules

aromatic rings of ca 90°, that can give rise to a helical shape and thus may result in the enantiospecific recognition of molecules within the major or minor grooves of the DNA structure.$^{94a,104}$ With this in mind, the aim of the work discussed in this chapter was to develop novel $C_2$-symmetric molecules, which could potentially act as $C_2$-chiral DNA binding agents, by incorporating the Tröger’s base motif into the skeleton of the 1,8-naphthalimide structure. Even though the methano-1,5-diazocine unit (shown in red in Figure 3.2) between the two naphthalimides will not be long enough for bis-intercalation, the introduction of a V-shaped cavity may lead to a different binding mechanism, which we aim to investigate. The results from this investigation will be detailed in Chapter 4.

![Figure 3.2: (a) Structure and (b) optimised geometry$^{165}$ of Tröger’s base 52.](image)

As mentioned in Chapter 1, the majority of reported applications of Tröger’s base analogues, within supramolecular chemistry, explore only its geometry and deal with racemates. Consequently, the intended utility of enantiomerically pure Tröger’s base has been limited and hampered by the fact that these enantiomers are difficult to resolve. While this is of critical importance in drug discovery, we decided to prepare the first example of the bis-1,8-naphthalimide containing the Tröger’s base as a racemic mixture and investigate their interactions with DNA, prior to their resolution. The reason being, that it was necessary to first establish if the design principle would give rise to enhanced DNA binding affinity and also that the resolution of the Tröger’s bases may not be trivial.

When designing the bis-1,8-naphthalimide containing Tröger’s base derivatives, it was decided, that they should ideally be water-soluble and positively charged at physiological pH, for achieving better interaction with DNA. In fact, of the mononaphthalimides, prepared by Bräna et al,$^{30}$ it was found that if the side chain contained a basic terminal group, in the form of a tertiary amine, which was separated from the naphthalimide ring nitrogen by two methylene units, then the best anticancer activity was achieved. It was, therefore, decided to have similar hydrophilic cationic functionalities, in
Chapter 3: Design and Synthesis of Bis-1,8-Naphthalimides Containing the Tröger’s Base as Novel C$_2$-Symmetric DNA-Binding Molecules

the form of two dialkylaminoethyl sidechains, attached to the naphthalimide structure. The side chains could then differ in their dialkyl substituents, so that their effect on the strength of DNA binding could be evaluated. Another aspect which was considered for the design, was the aforementioned fact that, the methano-1,5-diazocine unit is chiral and hence formed racemic. It was therefore, thought best to have the naphthalimide unit achiral, so that the complications of diastereoisomer formation could be avoided. These design features resulted in target molecules 151, 152, 153, and model compound 154.

![Diagram](image)

In using such a design strategy, these potential DNA-binding agents, are expected to be relatively rigid chiral molecules in which the two-naphthalimide moieties should be held in a well-defined position. The dihedral angle between the planes of the two aromatic rings of many of the examined Tröger’s base analogues generally lies in a range between $82^\circ$ – $104^\circ$. In terms of an interaction with DNA, the naphthalimide chromophore is well-known to bind by intercalation. Nevertheless, due to the V-shaped geometry, this is an unlikely binding mode for our targets. However, it is more likely that the binding may occur in either the major or minor grooves of the DNA helix, as the V-shape in conjunction with the di-cationic functionality, in the form of a tertiary amine, may interact electrostatically with the DNA phosphate anionic backbone. We anticipate, however, that as 154 lacks these amino moieties, it will not interact electrostatically and thus it was designed as a model compound.

Ultimately, the proposed Tröger’s base derivatives should ideally be prepared as single enantiomers, and moreover they should not easily racemize in the presence of DNA. We anticipate, however, that the resolved structures should not racemize. As mentioned in Chapter 1, 52 is susceptible to racemization under acidic conditions, however, this will depend on ease of protonating the nitrogens of the methano-1,5-diazocine unit. The p$\text{Ka}$ of
Troger's base 52, monoprotonated salt, has been determined to be 3.2 in 50 % aqueous alcohol.\textsuperscript{141} Therefore, in our design, at physiological pH, it would be expected that the tertiary amine of the side chain would be protonated and not the bridgehead nitrogens of the methano-1,5-diazocine unit. Moreover, due to the presence of the internal charge transfer (ICT) excited state of the naphthalimide chromophore, which the amines of the methano-1,5-diazocine unit participate in, their $pK_a$ may be reduced even further which should inhibit racemization completely. The ICT excited state of the bis-naphthalimide containing Troger's base 152 was investigated and will be discussed in Section 3.7. In addition to the above, and as mentioned in Chapter 1, Lenev \textit{et al.}\textsuperscript{94b} have recently proposed that enhancing the configurational stability of the Troger's base is an attractive option in avoiding the aforementioned racemization mechanism. Therefore, given the fact that both the ortho- and meta- positions of the naphthalimide are substituted, configurational inversion which aids the racemization process, could be prevented or slowed down.

This chapter will now deal with the synthesis of the target naphthalimid es 151, 152, 153 and 154 as racemic mixtures, and this will then be followed with a discussion on their photophysical properties. It seems necessary, however, to discuss the general synthesis of the original Trogers' base 52 before preceding any further, as the formation of the methano-1,5-diazocine unit often gives rise to problematic side reactions that can occur, and which are generally influenced by the structure of the starting aromatic amine.

### 3.4 General synthesis of the Troger's base 52

The mechanism for the formation of Troger's base 52, discovered by Wagner \textit{et al.}\textsuperscript{142} is due to an electrophilic aromatic substitution reaction. The reaction goes \textit{via} a number of intermediates shown in Scheme 3.1. Recently, Coelho \textit{et al.}\textsuperscript{143} identified and characterised these key intermediates using direct infusion electrospray ionisation mass and tandem mass spectrometric experiments (ESI-MS/(MS)).

The first step of the reaction sequence, as shown in Scheme 3.1, is a condensation reaction, which involves nucleophilic attack of the aromatic amine 155 on formaldehyde to form an imine intermediate, which upon protonation, and nucleophilic attack by a second molecule of aromatic amine, gives rise to methylene-bis-amine 156.
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Scheme 3.1: Reaction scheme of the steps towards formation of the Tröger's base 52.

The next step involves conversion of the methylene-bis-amine 156 to aminobenzylarylamine 157, through an acid-induced rearrangement, which is shown in Scheme 3.2. Wagner reported that this reaction is not an intramolecular rearrangement or isomerization. Rather, it involves a proton-induced cleavage of methylene-bis-amine 156 forming carbonium ions which couple together, followed by loss of a proton.

Scheme 3.2: Conversion of methylene-bis-amine 156 to aminobenzylamine 157.
Chapter 3: Design and Synthesis of Bis-1,8-Naphthalimides Containing the Tröger’s Base as Novel C$_2$-Symmetric DNA-Binding Molecules

The aminobenzylarylamine 157, then subsequently condenses with excess formaldehyde to firstly form tetrahydroquinazoline 158 (Scheme 3.3), which then undergoes a further condensation to form the 1-hydroxymethyl tetrahydroquinazoline 159. An elimination of water then gives 52.

![Scheme 3.3: Conversion of aminobenzylamine 157 to tetrahydroquinazoline 158.](image)

During the formation of the Tröger’s base 52, two side reactions are liable to occur: \[144\] (i) irreversible conversion of aminobenzylarylamine 157 to dihydroacridine 162 via diaminophenylmethane 160, shown in Scheme 3.4 and (ii) dehydrogenation of tetrahydroquinazoline 158 to dihydroquinazoline 163, shown in Scheme 3.5. The formation of dihydroacridine 162, shown in Scheme 3.4, involves an elimination of ammonia from diaminophenylmethane 161 and a subsequent internal ring closure. In general, this reaction is very slow and partial because the ortho-hydrogen of the liberated aromatic amine is not readily displaced. However, if the para-hydrogen of the starting aromatic amine is available, this coupling will occur leading to a polymerised product. Therefore, to ensure successful formation of the methano-1,5-diazocine ring, the para-position of the starting aromatic amine should be blocked.
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Scheme 3.4: Conversion of aminobenzylarylamine 157 to dihydroacridine 162.

Conversion of the tetrahydroquinazoline 158 to dihydroquinazoline 163, is shown in Scheme 3.5, and is more likely to occur if the (starting) aromatic amine contains electron-withdrawing substituents attached at the para-position. Such a situation will render the amine weakly nucleophilic and consequently unable to condense with formaldehyde to form the hydroxymethyl tetrahydroquinazoline and ultimately the desired Tröger’s base. In fact, its elucidation explained the presence of methylated amines among the products formed, upon reaction of formaldehyde with p-substituted amines in the presence of acid.\(^\text{145}\)

Scheme 3.5: Conversion of tetrahydroquinazoline 158 to dihydroquinazoline 163.
In general, for the formation of Tröger's base derivatives, the starting aromatic amine can be fully substituted except for the ortho-position where the desired cyclisation is to occur, noticeably, the nature of the substituent playing a role in controlling the regiochemistry\(^{146-150}\) and yield\(^{87a}\) of the desired Tröger's base through electronic and steric effects. The most common method for the preparation of Tröger's base derivatives involves treating an aromatic amine with formaldehyde in the presence of an acid.\(^{87a}\) The variation in the reaction method is quite large with the possibility of using a formaldehyde equivalent such as paraformaldehyde, hexamethylenetetramine (HMT),\(^{95c,151}\) dimethoxymethane\(^{152}\) or even DMSO\(^{153}\) and varying the acid by either using acetic acid, HCl or trifluoroacetic acid (TFA). The use of more than one of these methods is possible, while also varying the reaction conditions, such as changing temperature and length of reaction time, etc., with only the yields of the reaction product differing.\(^{154}\)

### 3.5 Synthesis of Bis-1,8-naphthalimides Containing the Tröger's Base

The 1,8-naphthalimide containing Tröger's base derivatives 151, 152, 153 and 154 were prepared as racemates in three steps, starting from the commercially available 4-nitro-1,8-naphthalic anhydride. This involved firstly making the N-substituted 4-nitro- and 4-amino-1,8-naphthalimide precursors using standard literature procedures,\(^{41}\) like those used and discussed in Chapter 2. During the progress of this work, alkyl based Tröger's naphthalimide derivatives were reported by Deprez et al.\(^{155}\) However, these were only studied for their photophysical properties in organic solvents.

#### 3.5.1 Synthesis of the 1,8-naphthalimide Precursors 168 - 171

By treating 4-nitro-1,8-naphthalic anhydride with the relevant alkylamine and refluxing the two components in anhydrous toluene for 24 hours, four 4-nitro-1,8-naphthalimides 164, 165, 166 and 167 were synthesised. They are listed in the table in Scheme 3.6, with their corresponding yields. In general, the work-up procedure involved a simple wash with saturated NaHCO\(_3\), and this was found to be as effective as conducting an acid-base extraction of the crude residue. Each of the 4-nitro-naphthalimides were then purified by a recrystallisation from ethanol. Subsequent reductions of the 4-nitro-1,8-naphthalimides by catalytic hydrogenation with 10 % Pd/C, at 3 atm H\(_2\), in methanol or ethyl acetate, gave the corresponding amino derivatives 168, 169, 170 and 171 in good
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yields (Scheme 3.6), after filtration through a celite plug under reduced pressure and without the need for any further purification.

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{O}_2\text{N} & + \quad \text{H}_2\text{N}-\text{R} \quad \xrightarrow{\text{Toluene}} \quad \text{120 °C} \\
\text{164 - 167} & \quad \text{Pd/H}_2 \\
\text{MeOH or Ethyl acetate} & \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>% Yield</th>
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<tbody>
<tr>
<td>164</td>
<td>N(CH$_3$)$_2$</td>
<td>91</td>
</tr>
<tr>
<td>165</td>
<td>N(CH$_2$CH$_2$)$_2$NCH$_3$</td>
<td>77</td>
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<td>166</td>
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<td>88</td>
</tr>
<tr>
<td>168</td>
<td>N(CH$_3$)$_2$</td>
<td>98</td>
</tr>
<tr>
<td>169</td>
<td>N(CH$_2$CH$_2$)$_2$NCH$_3$</td>
<td>98</td>
</tr>
<tr>
<td>170</td>
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<td>87</td>
</tr>
<tr>
<td>171</td>
<td>CH$_3$</td>
<td>99</td>
</tr>
</tbody>
</table>

Scheme 3.6: Synthesis of 4-nitro- and 4-amino-1,8-naphthalimide derivatives.

The 4-nitro- and 4-amino-1,8-naphthalimides, 164 and 168, were prepared by Bräna et al., and, in fact they are the analogues of Mitonafide and Amonafide, respectively. Both 168 and 171 are also known compounds within the literature. Nonetheless, all of the 4-nitro- and 4-amino-1,8-naphthalimides synthesised 164 - 171, were completely analysed by standard methods to gain a full understanding of the nature of such compounds. More importantly, such analysis was conducted as a means to verify the purity of 168 - 171, which were intended to be used as model compounds for the DNA binding analysis of the target Tröger's base analogues 151 - 154. All the compounds gave satisfactory CHN and ESMS data with spectroscopic data being consistent with their structures.
3.5.2. Synthesis of the Tröger’s Base Analogue 151 - 154

Towards formation of the bis-1,8-naphthalimide containing Tröger’s base derivatives, the classical method developed by Tröger was initially explored (Scheme 3.7).\(^{87(a)}\) This method proved successful and involved treating the 4-amino-1,8-naphthalimide precursor with a mixture of formaldehyde and HCl in ethanol (Method 1). Thereafter, attempts were made at optimising this reaction condition, by varying the temperature and length of reaction time. Furthermore and as previously mentioned, modifying the reaction method by simply varying the type of acid used can have an effect on the overall yield of the reaction, and such an investigation was also undertaken (Method 2).

<table>
<thead>
<tr>
<th>Method</th>
<th>Reaction condition</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>CH(_2)O, HCl, EtOH, 60 °C</td>
</tr>
<tr>
<td>2</td>
<td>CH(_2)O, TFA, RT</td>
</tr>
</tbody>
</table>

Scheme 3.7: Reaction protocol for the synthesis of C\(_2\)-symmetric bis-1,8-naphthalimides containing the Tröger’s base 151 - 154.

Formation of the Tröger’s base naphthalimide 151 was initially attempted by treating 4-amino-1,8-naphthalimide 168 with an ice-cold mixture of 7 equivalents of formaldehyde and concentrated HCl in ethanol and stirring the reaction mixture at 60°C (Method 1). Unfortunately, it was not possible to monitor the reaction by TLC (various eluting systems were tried), and so consequently after 4 hours stirring and upon the formation of a yellow and sparingly soluble precipitate, a small portion of the reaction mixture was collected and
cooled to room temperature. The precipitate was then extracted into CDCl$_3$ after dissolving it in D$_2$O and basifying to ca. pH 12 by the addition of saturated NaHCO$_3$. According to $^1$H NMR (CDCl$_3$, 400 MHz), the reaction had not proceeded efficiently and a mixture of both the desired product 151 and 4-amino-1,8-naphthalimide 168 were obtained. Consequently, the reaction mixture was left stirring for a further 20 hours. It was then cooled to room temperature and the precipitate was collected by suction filtration, dissolved in water and basified to pH 12 by the slow addition of 10 % aqueous ammonia. The product was then extracted with CH$_2$Cl$_2$ and the combined organic extracts were washed with brine and purification of 151 was successfully achieved on a short plug of flash silica (CH$_2$Cl$_2$/MeOH-NH$_3$, 95:5). This method of purification was chosen as a means of investigating the compound’s stability towards silica gel, upon which naphthalimides are sometimes known to decompose.

Tröger’s base 151 was fully characterised by $^1$H and $^{13}$C NMR, ESMS, IR and elemental analysis. The $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 151 is shown in Figure 3.3 and confirms its identity by the presence of a well-separated doublet of doublets between 5.14 and 4.34 ppm, pertaining to the methylene protons of the diazocine ring, while also clearly reflecting the $C_2$ plane of symmetry of the molecule. The aromatic region (ca. 7.5 – 9.0 ppm) is composed of 4 signals, which includes two doublets, a singlet and a triplet, all of which integrated for two protons. Such integration for the naphthalimide chromophore confirms the symmetric nature of 151.

The methylene protons of the dimethylaminoethyl amino side chain appear as two triplets at 4.25 and 2.56 ppm, respectively, each integrating for two protons and the two N-methyl groups appear as a sharp singlet at 2.30 ppm integrating for 12 protons. All of the signal resonances were assigned by $^1$H-$^1$H-COSY and HMQC experiments. The $^{13}$C NMR spectrum (CDCl$_3$, 100 MHz) also showed the symmetric nature of the molecule by having two more signals, pertaining to the carbons of the diazocine ring, than that of 4-aminonaphthalimide 168. The ESMS showed a peak at $m/z = 603$ (M+H)$^+$ and IR spectral data were consistent with the assigned structure.
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Figure 3.3: The $^1$H NMR spectrum (CDCl₃, 400 MHz) of 151.

Unfortunately, upon a reaction scale-up using the same procedure as before, the $^1$H and $^{13}$C NMR (CDCl₃, 400 MHz) spectral analysis indicated the presence of a mixture containing the desired Tröger’s base 151 and an unidentifiable product. In fact, the presence of two such products was visibly evident in the precipitate, which contained a mixture of red and yellow coloured solids. The $^1$H NMR spectrum (CDCl₃, 400MHz), confirmed that the yellow solid was the desired Tröger’s base 151. The structure of the other molecule, following several recrystallisation attempts, was not further investigated due to a complex $^1$H NMR spectrum. Ultimately, the desired Tröger’s base product 151 had to be isolated using flash column chromatography on neutral silica (CH₂Cl₂: MeOH-NH₃, 95:5) and in doing so was obtained in 23 % yield. As a result of these poor yields, the reaction was repeated using the same conditions as before but was left stirring for 72 hours. This resulted in the successful formation of the bis-1,8-naphthalimide containing Tröger’s base 151 in an improved 31 % yield after a recrystallisation from EtOH and the formation of other products or the recovery of 168 was not observed.

As previously mentioned, attempts were made at enhancing the yield of the reaction by changing the type of acid used. The first attempt involved treating the 4-amino-1,8-
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naphthalimide 168 with 1.5 equivalents of formaldehyde in neat TFA over 12 hours at room temperature (Method 2). Upon completion, the reaction mixture was neutralised and further basified to pH 12 by the slow drop wise addition of NaOH (6M) and the product was extracted with CH₂Cl₂. This resulted in the successful formation of 151 in a greatly improved yield of 61 %, following a recrystallisation from ethanol. An advantage of this particular method was that the extraction workup was far less tedious than previously experienced. Unfortunately, varying the reaction method by replacing HCl with the much milder acetic acid proved unsuccessful with a complete recovery of starting material 168, indicating that the pH of the solution was possibly not acidic enough to perhaps form the imine intermediate of the reaction sequence (Scheme 3.1).

Formation of the 1,8-naphthalimide containing Tröger’s base derivative 152 was also initially attempted using Method 1 (i.e by treating 169 with formaldehyde in the presence of HCl in an ethanol solution). However, on this occasion and as a means of investigating the utility of varying the reaction conditions, 14 equivalents (rather than 7 equivalents) of formaldehyde were used. As before, the progress of the reaction was monitored by ¹H NMR (CDCl₃, 400MHz), however, after 48 hours the methano-1,5-diazocine ring had only partially formed. The reaction was left stirring for a further 72 hours and upon base extraction the desired bis-1,8-naphthalimide containing Tröger’s base 152 was obtained in 67 % yield. Unfortunately, ¹H NMR (CDCl₃, 400 MHz) analysis showed the presence of a small amount of impurity, and as such, the crude product was subjected to purification by column chromatography on neutral alumina (CH₂Cl₂: MeOH, 98:2). This method of purification was chosen because the previous attempt at purifying 151 using flash chromatography on silica, even though successful, was very difficult and troublesome. Nevertheless, chromatography on neutral alumina was completely ineffective due to an unidentifiable contamination of 152, according to ¹H NMR (CDCl₃, 400 MHz). As a means of ascertaining the sensitivity of these molecules towards alumina, a purified sample of the bis-1,8-naphthalimide containing Tröger’s base 151 was stirred with neutral alumina in a mixture of CH₂Cl₂ and methanol and as expected similar contamination occurred. Owing to such a lack of success, Method 2 was then employed for the formation of 152, which involved treating the 1,8-naphthalimide 169 with 1.5 equivalents of formaldehyde in neat TFA at room temperature for 12 hours. This method, again, proved successful and ¹H NMR (CDCl₃, 400MHz) confirmed the formation of desired bis-1,8-naphthalimide containing Tröger’s base 152 without the presence of any side-products or starting material.
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Upon usual work-up and a recrystallisation from ethanol, 152 was obtained in 50% yield and fully characterised using standard techniques. The ESMS showed a peak at \( m/z = 713 \) corresponding to \((\text{M+H})^+\) and satisfactory CHN data confirmed the molecular formula of the product. The \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra (CDCl\(_3, 400\) MHz) also reflected the \(C_2\) plane of symmetry of the molecule. The \(^1\text{H}\) NMR spectrum was similar to that of 151 (Figure 3.3) while also showing two broad singlets at 2.43 and 2.39 ppm, respectively each integrating for eight protons, pertaining to the protons of the pyrazine ring and the \(N\)-methyl group was characterised by the presence of a sharp singlet at 2.23 ppm integrating for six protons. These signals were assigned using a H-H cosy experiment. The \(^{13}\text{C}\) NMR spectra of 152, shown in Figure 3.4, is composed of the same number of signals as found in the \(^{13}\text{C}\) NMR spectrum of its precursor, 4-amino-1,8-naphthalimide 169, with the addition of two signals for the methylene carbons of the diazocine ring which come into resonance at 56.6 and 36.9. These signals were assigned with the aid of a 2-D HMQC experiment.

![Figure 3.4: The \(^{13}\text{C}\) NMR spectrum (CDCl\(_3, 100\) MHz) of 152 exhibiting \(C_2\) symmetry.](image)

With the latter success, Method 2 was also employed for the formation of bis-1,8-naphthalimides containing Tröger’s base derivatives 153 and 154. Formation of 153 was achieved in 49% yield, by treating 4-aminonaphthalimide 170 with formaldehyde in neat TFA over 12 hours and was obtained after a simple recrystallisation from ethanol.
Similarly, 154 was prepared from 4-amino-1,8-naphthalimide 171 and was obtained in 60% yield after a recrystallisation from butan-1-ol. To check the generality of Method 2, 171 was also treated with formaldehyde in ethanol in the presence of HCl (Method 1) but 154 was obtained in a lower yield of 39% after a recrystallisation from butan-1-ol.

Both 153 and 154 were characterised using standard techniques and Figure 3.5 shows the $^1$H NMR spectra (CDCl$_3$, 400 MHz) of 153. As can be seen, the two sets of methylene protons of the dialkylaminoethyl side chains appear as two broad singlets at 4.31 and 2.71 ppm, each of which integrate for four protons, and the methylene protons of the morpholine ring appear as two broad singlets at 3.66 and 2.62 ppm, both integrating for eight protons. The appearance of the $^1$H NMR spectrum (CDCl$_3$, 400 MHz) of 154 only differed in the most downfield aliphatic region, which was composed of a triplet at 0.97 ppm integrating for six protons, pertaining to the methyl group of the dialkylaminoethyl side chain and the two sets of methylene protons appeared as a triplet and as a double doublet of doublets at 4.09 ppm 1.71 ppm, respectively, both of which integrated for four protons. The $^{13}$C NMR (CDCl$_3$, 400 MHz) also confirmed the $C_2$-symmetric nature of these molecules and IR and mass spectral results and CHN determination were consistent with their assigned structures.

![Figure 3.5: The $^1$H NMR spectra (CDCl$_3$, 400 MHz) of 153.](image)
In summary, **Method 1** used for the formation of the desired 1,8-naphthalimide containing Tröger's base derivatives was unsuccessful, leading in some cases to intractable mixtures. Otherwise, upon varying the reaction conditions poor yields were obtained. A modification of the reaction procedure, **Method 2**, worked well for the preparation of all the bis-1,8-naphthalimide containing Tröger's bases, 151, 152, 153 and 154 which were obtained in reasonably moderate yields, as listed in Table 3.1. Furthermore, confirmation that each of these derivatives actually formed as a racemic mixture was achieved using CD spectroscopy by the absence of a signal, due to equal handedness.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield % Method 1</th>
<th>Yield % Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>151</td>
<td>N(CH₃)₂</td>
<td>31</td>
<td>61</td>
</tr>
<tr>
<td>152</td>
<td>N(CH₂CH₂)₂NCH₃</td>
<td>67 crude</td>
<td>50</td>
</tr>
<tr>
<td>153</td>
<td>N(CH₂CH₂)₂O</td>
<td>-</td>
<td>49</td>
</tr>
<tr>
<td>154</td>
<td>CH₃</td>
<td>39</td>
<td>60</td>
</tr>
</tbody>
</table>

**Table 3.1:** *Synthesis of 1,8-naphthalimide containing Tröger’s base derivatives*

The moderate yields obtained are probably due to the cross conjugated nature of the 4-amino-1,8-naphthalimide derivatives 168, 169, 170 and 171 which reduces the nucleophilicity of the amino group considerably, due to the partial positive charge on the nitrogen atom (Figure 3.6). As a means of improving yields, optimisation of the reaction conditions could be attempted by using formaldehyde equivalents such as hexamethylenetetraamine and dimethoxymethane as discussed in section 3.6 and by varying the conditions of such reactions. However, this has not been done yet. Furthermore, methods of resolving these derivatives has not been attempted, but could possibly be achieved using chiral acids or even possibly HPLC, for reasons as explained in Section 3.3.
3.6 Spectroscopic Evaluation of 152 in Solvents of Varying Polarity

1,8-Naphthalimide derivatives are very sensitive to their media and their electronic emission and absorption spectra are frequently affected by the nature of the solvent and temperature. The reason is that the ICT excited state of, for example, the 4-amino-1,8-naphthalimide 172 gives rise to a large excited state dipole moment (Figure 3.6) which can vary during electronic transitions depending on the solvents polarity and its hydrogen-bond donor (HBD) or acceptor (HBA) capacity.\(^\text{157}\) The excited state dipole moment arises, as previously mentioned, due to the effective 'push-pull' character of the chromophore due to the electron donating amine and electron accepting imide.

![Figure 3.6: The effect of ICT upon the excited state of 172.](image)

In various investigations carried out on N-substituted 1,8-naphthalimides, the fluorescent emission wavelength and quantum yield were found to be dependent on the solvent's polarity and its HBD capability.\(^\text{157}\) The bis-1,8-naphthalimide containing Tröger's base derivatives may possess such a property, however, it is anticipated that it will not be completely the same. Thus, as a means of investigating whether the bis-1,8-naphthalimide containing Tröger's base derivatives actually exhibit an ICT excited state, and to what extent, the electronic absorption and emission spectra of 152 were recorded in protic and aprotic solvents of varying polarity.

The absorption and emission spectra for 152 recorded in a range of solvents are shown in Figure 3.7 and 3.8, respectively and relevant spectral data is summarized later in Table 3.2. All measurements were carried out using an optical density (OD) of 0.15 at the excitation wavelength, which was chosen as the \(\lambda_{\text{max}}\) observed in the absorption spectrum of 152. The solvents used in the study were hexane, dichloromethane (CH\(_2\)Cl\(_2\)), acetone, methanol (MeOH), dimethylformamide (DMF), acetonitrile (MeCN) and water (H\(_2\)O).
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The absorption spectra of 152, (Figure 3.7), when recorded in methanol, showed a broad band between 280 and 450 nm, with $\lambda_{\text{max}}$ at 385 nm ($\varepsilon_{152} = 11,452 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$) and a structural band at 230 nm. In general, the absorption spectrum was not significantly affected by the polarity and or the hydrogen-bonding ability of the medium. However, the ICT nature of the fluorophore is evident from the fact that the absorption is fairly broad and intense with the molar extinction coefficient lying between 11,500 and 20,000 mol$^{-1}$ cm$^{-1}$ and that the peak position is slightly affected by the polarity of the medium (Table 3.2). A change from hexane to methanol typically resulted in a 15 nm bathochromic shift of the absorption maximum. This is unlike that usually seen for 4-amino-naphthalimides, which, in general, show a much greater shift. Nevertheless, such a shift in the absorbance spectrum to longer wavelengths indicates that during the $\pi-\pi^*$ transition, the polar solvent is more strongly hydrogen-bonded, therefore causing a decrease in the energy gap between the ground state (S$_0$) and the excited state (S$_1$). The bathochromic shift observed for 152 with increasing solvent polarity is therefore characteristic of an ICT band.$^{157}$

![Figure 3.7](image-url)

**Figure 3.7: Solvent effects on the absorption properties of 169 and 152.**

Figure 3.7 also shows the absorption spectra of the 4-amino-1,8-naphthalimide 169, which was recorded for comparison purposes. When recorded in MeOH, a broad structurless band centered at 433 nm ($\varepsilon_{169} = 10,474 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$) was observed and is well characterized as being due to an ICT excited state of the fluorophore. Upon a decrease in the polarity and HBD ability of the medium, the ICT absorption band exhibited a hypsochromic shift of 27 nm. By comparing the changes of the absorbance shift in $\lambda_{\text{max}}$ of
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152 with that of 169 allows for a further characterization of the long wavelength band of 152 as that of the ICT. Furthermore, the smaller shift in the absorbance spectrum of 152 may be due to its inherent geometry, derived from the V-shaped structure of the methano-1,5-diazocine ring, which perhaps effects the ability of the amine to donate electrons into the naphthalimide ring.

The influence of solvent polarity on the photophysical behavior of 152 and 169 were also studied using fluorescence emission and excitation spectroscopy, using the same solvents as for their absorbance studies. Here, 152 was excited at $\lambda_{\text{max}}$ 385 nm and 169 at $\lambda_{\text{max}}$ 433 nm and Figure 3.8 shows their overlaid emission spectra both of which exhibit similar trends.

![Figure 3.8: Solvent effects on the emission properties of 152 (A) and 169 (B).](image)

When excited in CH$_2$Cl$_2$ the spectra for 169 and 152 exhibited maxima at 497 nm and 489 nm, respectively. Both bands are not particularly symmetrical, but tail to longer wavelengths. This supports the characterisation of the excitation bands of 152 as having contributions from ICT transitions. Upon increasing solvent polarity, from hexane to methanol, the fluorescence maximum of 152 exhibited a bathochromic shift of 85 nm and this effect was more pronounced than that of the shift in its absorption maximum. In fact, the magnitude of the spectral shift was 5 times greater than in the absorption spectrum (Table 3.2). This is typical of solvent stabilization of more polar excited states and the greater magnitude of the shift compared supports the characterization of the emission as ICT.\(^{157}\) Likewise, the emission spectra of 169 also shown in Figure 3.8, exhibited a clear
bathochromic shift and a decrease in the emission intensity upon an increase in the polarity and HBD ability of the solvent. On comparison with 152, the latter result gives further evidence that this charge separating process is occurring, but to a far lesser extent in the bis-1,8-naphthalimide containing Tröger’s base derivatives.

Additional evidence for the presence of an ICT excited state in 152 is that its fluorescence quantum efficiencies ($\Phi_F$), as shown in Table 3.2, are low and were also found to decrease on changing the solvent from CH$_2$Cl$_2$ to methanol. This effect has been previously reported and is in agreement with the low fluorescent quantum efficiencies of $N$-substituted 1,8-naphthalimide derivatives.\textsuperscript{158,159} It must be noted, however, that the quantum yield of 152 when recorded in hexane was very low, and this is attributed to insolubility of 152 in that solvent.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{max}$ Abs. (nm)</th>
<th>$\lambda_{max}$ Flu. (nm)</th>
<th>log $\epsilon_{max}$ M$^{-1}$ cm$^{-1}$</th>
<th>$\Phi_{Flu}$ (±10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>370</td>
<td>434</td>
<td>-</td>
<td>0.0063</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>385</td>
<td>500</td>
<td>4.30</td>
<td>0.1803</td>
</tr>
<tr>
<td>Acetone</td>
<td>383</td>
<td>519</td>
<td>4.09</td>
<td>0.0363</td>
</tr>
<tr>
<td>MeOH</td>
<td>385</td>
<td>517</td>
<td>4.10</td>
<td>0.0114</td>
</tr>
<tr>
<td>DMF</td>
<td>387</td>
<td>523</td>
<td>4.14</td>
<td>0.0232</td>
</tr>
<tr>
<td>MeCN</td>
<td>384</td>
<td>518</td>
<td>4.23</td>
<td>0.0222</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>381</td>
<td>505</td>
<td>4.06</td>
<td>0.0122</td>
</tr>
</tbody>
</table>

Table 3.2: UV/Visible and emission spectral data for 152 recorded in a range of solvents.

The fluorescence quantum yields of the systems were measured using quinine sulphate ($\Phi_F = 0.542$ in 0.1N H$_2$SO$_4$) as the primary reference standard.\textsuperscript{160} The fluorescence quantum yield is an essential source of information that gives the probability of the excited state being deactivated by fluorescence rather than by another, non-radiative mechanism. Two comparative methods were employed by: (A) using optically dilute solutions\textsuperscript{161} and (B) acquiring data at a number of different concentrations.\textsuperscript{162} For method A, dilute solutions with OD of 0.03 at the excitation wavelength 368 nm (which corresponded to a concentration in the given micromolar range) were used so that corrections for self-absorption and of incident and emitted light on the emission intensities, were not required. In method B, a number of solutions ranging from OD of 0.10 to 0.02
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were excited at 368 nm and linearity across the concentration range was maintained. For both measurements, optically matched solutions of the test sample and reference were used and excited at the same wavelength (368 nm) using an excitation slit of 5 nm and an emission slit of 10 nm and the data was collected between 375 - 800 nm. Therefore, solutions of the standard and test samples with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons.

For method A and B, $\Phi$ were calculated by comparing the integrated areas underneath the emission band of the spectra using Eq. 3.1 and 3.2, respectively.

\[
\Phi_s = \Phi_r \cdot \frac{A_s}{A_r} \cdot \frac{F_s}{F_r} \cdot \frac{(\eta_s)^2}{(\eta_r)^2} \quad (3.1)
\]

\[
\Phi_s = \Phi_r \cdot \frac{m_s}{m_r} \cdot \frac{(\eta_s)^2}{(\eta_r)^2} \quad (3.2)
\]

where, $s$, $r$, $A$, $F$ and $\eta$ refer to the test sample, reference standard, absorbance, integrated area and the solvent refraction index, respectively.

The integrated areas were calculated using Origin programme (version 7.5). For method B, $\Phi$ were measured by, firstly plotting the integrated fluorescence area vs. absorbance, independently for the standard and the test samples. A straight line should be obtained and the gradients, $m$, are proportional to the quantum yield of the different samples. The quantum yield was then calculated according to Eq 3.2. In both methods, all fluorescence spectra were corrected for the instrumental response and each measurement was repeated twice. Calculated quantum yields from both methods were compared and error values are approximately 10%.

All of the results obtained for 152, as summarized in Table 3.2, are also in accordance with the results reported by Deprez et al. on the photophysical properties of a number of $N$-alkyl-1,8-naphthalimides with Tröger's base derivatives in a range of solvents. The electronic absorption spectra of the Tröger's bases were relatively insensitive to solvent, whereas the fluorescence emission intensity of the dyes was medium dependent and a decrease in quantum yield with increasing solvent polarity was generally observed.

The knowledge that 152 exhibits an ICT excited state, along with being able to recognize the position of the characteristic absorption and emission bands of 152 and 169, will aid in examining the interactions between such compounds with DNA, or homopolymers. As previously stated, Chapter 4 will deal with evaluating the affinity of 151 -153
for DNA using such techniques. The final section of this chapter deals with the sensitivity of these molecules towards pH and a determination of the pKa value of the dialkylamino functionality in the side chains in 151 - 153.

### 3.7 Photophysical Studies of 151 - 153

As previously mentioned, the dialkylamino moiety of the side chains of the bis-1,8-naphthalimides containing Tröger's bases should ideally be protonated at physiological pH so that electrostatic interaction with the DNA helix could be optimised. As a means of ascertaining this, the spectroscopic properties of all the Tröger's bases derivatives 151, 152 and 153 were investigated as a function of pH in water, using 100mM NaCl as buffer to maintain a constant ionic strength. However, 154 was not included in the study, because it does not have a protonable amine at the 'terminus' of its side chains and is not soluble in aqueous solution. The 4-amino-1,8-naphthalimide 168 was also investigated for comparison purposes. All of the molecules were studied with an OD of 0.1 – 0.15 and their relevant spectral parameters are collected in Table 3.3.

<table>
<thead>
<tr>
<th>Property</th>
<th>151</th>
<th>152</th>
<th>153</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (acid), nm</td>
<td>380</td>
<td>381</td>
<td>381</td>
<td>433</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ (base), nm</td>
<td>384</td>
<td>385</td>
<td>391</td>
<td>433</td>
</tr>
<tr>
<td>% $H_{\text{Abs}}$ [a] (acid-base)</td>
<td>70</td>
<td>40</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>log $\varepsilon$ (pH = 7.0), M$^{-1}$ cm$^{-1}$</td>
<td>4.11</td>
<td>4.06</td>
<td>4.01</td>
<td>3.99</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ (acid), nm</td>
<td>500</td>
<td>505</td>
<td>534</td>
<td>550</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ (base), nm</td>
<td>500</td>
<td>517</td>
<td>540</td>
<td>547</td>
</tr>
<tr>
<td>% $H_{\text{Abs}}$ [a] (acid-base)</td>
<td>10</td>
<td>30</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>$\Phi_{\text{Flu}}$ (pH = 7.0), nm</td>
<td>0.032</td>
<td>0.012</td>
<td>0.0045</td>
<td>0</td>
</tr>
<tr>
<td>pKa (±0.1)</td>
<td>7.7</td>
<td>-</td>
<td>6.22</td>
<td>-</td>
</tr>
<tr>
<td>pKa*(±0.1)</td>
<td>8.7</td>
<td>8.2</td>
<td>6.03</td>
<td>8.7</td>
</tr>
</tbody>
</table>

**Table 3.3:** Optical and protonation properties (in ground (pKa) and excited state (pKa*)) of 168, 151, 152 and 153. [a] % $H$, is the percentage hypochromism of the absorbance and emission spectra upon increase in pH [b] $\Phi_{\text{Flu}}$ was not determined [c] absorbance changes are too small to permit reliable determination of pKa value.
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Their relevant pKa values were calculated from both the changes in the absorbance or the emission spectra (where possible) by fitting the data accordingly using equations 3.3 and 3.4.

\[
\text{pK}_a(S_0) = \text{pH} - \log\left(\frac{\text{Abs}_{A\text{H}} - \text{Abs}_A}{\text{Abs}_A - \text{Abs}_{A^-}}\right) \quad (3.3)
\]

\[
\text{pK}_a(S_1) = \text{pH} - \log\left(\frac{\text{If}_{A\text{H}} - \text{If}_A}{\text{If}_A - \text{If}_{A^-}}\right) \quad (3.4)
\]

where, \(\text{Abs}_{A\text{H}}\) and \(\text{Abs}_{A^-}\) are the absorbance values in acidic and basic solution and \(\text{Abs}_A\) is the absorbance value at each pH value within the changes of the pH-A/A_o plot. \(\text{If}_{A\text{H}}\) and \(\text{If}_{A^-}\) are the emission intensities in acidic and basic solution and \(\text{If}_A\) is the emission intensity at each pH value within the changes of the pH-I/I_0 plot.

The effect of pH on the ground states of 168 and 151 were firstly examined and their overlaid absorbance spectra are shown in Figure 3.9. When recorded in dilute acid solution using HCl, both gave rise to broad absorbance bands with \(\lambda_{\text{max}}\) at 433 nm (\(\epsilon_{168} = 9,782\, \text{mol}^{-1}\, \text{dm}^3\, \text{cm}^{-1}\)) and 380 nm (\(\epsilon_{151} = 12,893\, \text{mol}^{-1}\, \text{dm}^3\, \text{cm}^{-1}\)), for 168 and 151, respectively. Upon titration with base, using dilute NaOH, the absorption spectrum of 151 showed a decrease in absorbance of ca. 70% whereas the ICT band of 168, in comparison showed no significant change. Such changes were found to be fully reversible.

**Figure 3.9:** The absorption spectra of (A) 151 (11μM) and (B) 168 (16μM), upon a gradual increase in pH.
A plot of their absorbance versus pH is shown in Figure 3.10 and their relevant spectral parameters are collected in Table 3.4. As expected, the emission of 168 is relatively independent of pH as the protonation of the 4-amino moiety is difficult due to the ICT. Furthermore, the extensive delocalisation of the negative charge over the carboximide group, which arises due to the ICT excited state character of the fluorophore, reduces any interaction with the amine of the sidechain to a very low level.\textsuperscript{163,164} Conversely, a sigmoidal curve was obtained for 151, however this curve is not ideal. Normally, the midpoint of the curve should occur between two log units, but as shown in Figure 3.10, this is not the case. Nevertheless, the changes between pH 5 and 9 were used to obtain a pKa value of 7.7 (± 0.1) for 151. Perhaps, one of the reason for this is the tendency of the tertiary amine of the aminoethyl sidechain to donate a proton into the ring is more likely to be favourable, due to the potential attenuation of the electron-releasing character of the nitrogen in the 4-position. The latter effect may arise, as mentioned in Section 3.6, because the 4-amino group may be some what twisted due to the V-shaped structure of the methano-1,5-diazocine ring. The origin of the charge repulsion in 168 is, therefore not as strong as in 151 due to a weaker push-pull ICT fluorophore. As also mentioned in Section 3.6, 152 was found to exhibit an ICT excited state but such a charge separation process seems to occurs to a lesser extent than in the classical 4-amino-1,8-naphthalimides. However, the reason for the changes observed in the absorbance spectrum of 151 with increasing pH effect maybe much more complicated than the above explanation and therefore further studies should be carried out.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure310.png}
\caption{The normalised changes in absorbance of 168 (○) at 433 nm and of 151 (●) at 381 nm with respect to pH.}
\end{figure}
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The changes in the fluorescence emission spectra of 168 and 151 were also studied as a function of pH and their relevant spectra are shown in Figure 3.11. When Tröger’s base 151 was excited in acidic solution at 368 nm, a broad band between 375 and 675 nm, with \( \lambda_{\text{em}} \text{ max at 500 nm} \), was observed which subsequently decreased by 10 % upon titration with base. Whereas, the ICT emission band of the aminonaphthalimide 168, observed between 475 and 675 nm in acidic solution, with \( \lambda_{\text{em}} \text{ max at 550 nm} \), increased by 10 % upon increasing the pH. Both of these changes were found to be fully reversible.

Figure 3.11: The emission spectra of (A) 168 (16 μM, excitation at 433 nm) and (B) 151 (11 μM, excitation at 368 nm), upon a gradual increase in pH.

According to the corresponding \( \frac{I_f}{I_0} \)-pH profile for 168 and 151 (Figure 3.12) changes occurred within the same pH range, (7.6–10), giving rise to a pKₐ of 8.7 (± 0.1) for both.

Figure 3.12: The normalised changes in emission of 168 (●) at 550 nm and 151 (●) at 502 nm with respect to pH.
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The very slight fluorescence enhancement observed for 168 upon deprotonation may be attributed to the loss of the hydrogen bond, which may have formed between the imide carbonyl oxygen and the protonated amine of the side chain.\(^{163}\) Whereas, the reason for the observed fluorescence quenching of 151 upon deprotonation could be, again, due to the fact that the effect of its ICT excited state is weak. Therefore, because of the less active ICT excited state of Tröger’s base 151, an electron transfer from the amine of the side chain may be allowed and upon deprotonation of the dimethylamino functionality, the fluorescence is quenched. Again, further studies would be required to clarify this argument.

The absorbance and emission spectra of 152 and 153 were also examined as a function of pH. When recorded in acidic solution, both gave rise to similar absorbance spectra, which exhibited a broad band centred at 381 nm (\(\epsilon_{152} = 11,452 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}\) and \(\epsilon_{153} = 10,220 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}\)) and a sharp band at 232 nm, like that of 151. Upon titration with base the absorption spectrum of 152 was found to decrease by 40 \% whereas the absorbance band of 153 decreased by 80 \%. These changes were found to be fully reversible. With regard to Tröger’s base 153, a plot of the changes in the absorbance with respect to pH (Figure 3.13) showed a sigmoidal step occurring between pH 4-7, which has a pK\(\text{a}\) of 6.22 (± 0.1). Unfortunately, the changes in the absorbance spectrum of 152 were not large enough to allow for an accurate calculation of a pK\(\text{a}\) value.

![Figure 3.13: Plot of the changes at 381 nm for 153 as a function of pH.](image)

When the emission spectra of 152 and 153 (Figure 3.14) were recorded in acidic solution, similar results were observed as discussed above. Upon excitation at 420 nm in acidic solution, broad emission bands between 450 and 750 nm were observed, with \(\lambda_{em}\) max of 505 nm and 534 for 152 and 153, respectively. Upon titration with base, the
emission spectra of 152 exhibited a decrease of 30% along with a bathochromic shift of 12 nm, whereas, the emission spectra of 153 decreased by 15% and its $\lambda_{\text{em max}}$ red-shifted slightly by 6 nm. Again these changes were fully reversible. As shown in Figure 3.15, the $I_f/I_0$ profile for 152 showed a sigmoidal step between pH 6 and 10, giving a pKa of 8.2 (±0.1). Whereas, the $I_f/I_0$ profile for 153, exhibited changes between pH 4 and 7 from which a pKa of 6.03 (±0.1) was determined. As expected the morpholine nitrogen is more acidic due to the electron withdrawing effect of the oxygen.

![Figure 3.14](image-url)

**Figure 3.14:** The emission spectra of (a) 152 (9 μM) and (b) 153 (15 μM), upon a gradual increase in pH. Excitation at 420 nm.

![Figure 3.15](image-url)

**Figure 3.15:** The normalised changes in emission of 152 (▲) at 505nm and 153 (▲) at 534nm with respect to pH.

The determination of these pKa values were an essential means of ascertaining whether the side chain of the relevant bis-1,8-naphthalimide containing Tröger’s base...
derivatives would be protonated at physiological pH 7.4, so that binding with DNA would be enhanced by electrostatic interaction with the phosphate anionic backbone of the DNA helix. As expected, Tröger's base 153 having a pK\textsubscript{a} of ca. 6, will not be protonated at physiological pH. This will, however, allow us to use 153 rather than 154 as a model for determining whether the discussed binding occurs because 154 was found to be highly insoluble in most solvents including water. Apart from pK\textsubscript{a} determinations, this photophysical study has also further indicated that these molecules have a weaker ICT excited state character in comparison to the corresponding 4-aminonaphthalimides precursors.

### 3.8 Conclusion

In this chapter four bis-1,8-naphthalimide containing Tröger's base derivatives 151, 152, 153 and 154 were successfully prepared as racemic mixtures, in reasonable yields after efficient optimisation of reaction conditions. These molecules were designed as potential C\textsubscript{2}-symmetric DNA-binding molecules and they simply differ from each other in the dialkyl substituent of their dialkylaminoethyl sidechains.

Their photophysical characteristics were determined in a range of solvents of varying polarity and hydrogen-bonding capability and compared to their 4-amino-1,8-naphthalimide precursors, using both ground state and excited state spectroscopy. However, 154, because it lacks a protonatable group at each end of its terminus, was not water-soluble and hence, was not analysed. It was found that by the incorporation of the Tröger’s base moiety into the skeleton of the 4-amino-1,8-naphthalimides, the ICT excited state character was withheld. However, such a charge separation occurs to a lesser extent as compared to their 4-amino-1,8-naphthalimide precursors. The weak ICT excited state character was given as a reason for the changes observed in their absorbance and emission spectra upon a change in pH value. Nevertheless, further studies should be carried out to obtain a greater understanding for such an effect. The spectral changes obtained, even though not ideal, allowed for a determination of the pK\textsubscript{a} values of the alkylamine substituent of their side chains. It was found that bis-1,8-naphthalimide containing Tröger’s bases, 151 and 152 having pK\textsubscript{a} values of 8.7 and 8.2, respectively, will indeed be protonated at physiological pH. However, 153 with a pK\textsubscript{a} value of 6.0 will not be expected to give rise to significant electrostatic interactions with DNA. It was therefore decided, due
to a lack of water solubility of 154, that 153 could be used as a model compound for analysing potential electrostatic binding with DNA.

Determination of their pKa values and the existence of an ICT excited state was an important study and such information will aid in the analysis of their DNA binding affinities using various spectroscopic techniques, such as, UV/Vis, fluorescence, Tm experiments and CD. This study is discussed in Chapter 4.
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-Naphthalimides Containing the Tröger’s Base

4.1 Introduction

In Chapter 3, bis-1,8-naphthalimide derivatives 151, 152, 153 and 154, incorporating the Tröger’s base structural motif (Figure 4.1) were designed and synthesised as potential $C_2$-symmetric DNA targeting molecules. The Tröger’s base (methano-1,5-diazocine) was chosen because of its chiral, cleft-like structure, which gives rise to a helical shape and thus may result in the enantiospecific recognition of these molecules within the major or minor groves of the DNA structure. However, 151 – 154 were synthesised as racemic mixtures. They have tertiary amino functionalities at the ‘terminus’ of their side chains, which render them water-soluble while providing favourable electrostatic interactions with the negatively charged backbone of DNA. In Chapter 3, the photophysical properties of these molecules in organic solvents and in water and as a function of pH, were investigated. In this chapter their ability to bind to DNA in buffered aqueous solutions will be explored.

![Figure 4.1: Structures of the bis-1,8-naphthalimide containing Tröger’s base derivatives 151 – 154 designed and synthesised in Chapter 3.](image)

4.2 Physical Evaluations of Drug-DNA Interactions

Generally, high-resolution techniques such as X-ray diffraction are utilised for obtaining accurate information on the mode of DNA binding by small molecules. However, in the absence of such techniques, the mode of binding is typically carried out using solution studies. As discussed in Chapter 3, the photophysical properties of 152 were highly sensitive to a variation in solvent polarity and pH. Therefore, it was anticipated that
the photophysical properties of 151 – 154 could also be affected as a result of binding to DNA.

When a small molecule as an organic chromophore binds to double stranded DNA, any of the following three noncovalent binding interactions are possible:15 (i) intercalation between the base pairs, (ii) groove-binding in the major or minor grooves and (iii) electrostatic binding to the anionic phosphate groups. In order to maximize these interactions a planar structure is an important feature needed for efficient intercalation, while size and curvature generally aids groove-binding interactions.19,166,167

The ability to deduce the binding mode of a new DNA targeting agent typically rests on the fundamental structural differences resulting from intercalation and groove binding.168 Small molecules, which bind in the grooves of DNA through hydrogen bonding interactions, cause little distortion to the DNA backbone. In contrast, intercalation, in which a planar, aromatic ligand moiety is inserted between the base pairs, significantly distorts the DNA backbone conformation giving rise to a lengthening, stiffening, and unwinding of the double stranded helix.19,130,166

Spectroscopic techniques such as UV/vis and fluorescence, even though they do not normally distinguish intercalation from groove binding, can be used as a preliminary study for reliably detecting an interaction with DNA.168 Dichroism (circular and linear) and fluorescence energy transfer methods, are more sensitive to structural change and are commonly employed for more effectively distinguishing intercalation from groove binding.168,169 This is possible, as such techniques can evaluate the orientation of a ligand chromophore relative to the DNA helix and also its proximity to the DNA bases. When the binding of a drug involves an intercalation mechanism, the planar chromophore of the ligand is orientated roughly perpendicular to the DNA helix axis while also being in close contact with the DNA base pairs.15 In addition, intercalation normally causes an increase in the viscosity of DNA and a decrease in the sedimentation coefficient as well as a stabilisation of the DNA helix towards thermal denaturation. Accordingly relative viscosity studies170 and Tm studies (using absorbance spectroscopy) can also be used to determine a mode of binding.

The rationale used in the design of the 1,8-naphthalimide containing Tröger’s bases, discussed in Chapter 3, is shown schematically in Figure 4.2. It was anticipated that the V-shaped methano-1,5-diazocine ring would position the two chromophores into either the major or minor grooves of the DNA. At the same time, the di-cationic terminus of the side
chains would be able to interact electrostatically with the anionic phosphate backbone. While the minor groove may act as a better receptor in terms of its size, flexibility, electrostatic potential and water bonding properties, many 1,8-naphthalimide derivatives are well-characterized intercalating agents. Consequently, a potential binding interaction may involve intercalation of one of the naphthalimide chromophores while the other acts as a groove-binding domain. For instance, and as discussed in Chapter 1, Bailly et al. reported that an asymmetric acridine and phenanthroline containing Tröger’s base bound to DNA by intercalation of the acridine ring coupled with groove binding of the phenanthroline moiety.

Figure 4.2: Rationale in the design of 151 as DNA binding agent, and schematic diagram of methods of binding to DNA helix.*

As a means of evaluating the DNA-binding affinity of 151, 152 and 153, the following methods were chosen. Firstly, UV-vis and fluorescence titrations were used to determine whether there was any interaction between these molecules and DNA. Thermal denaturation studies were then carried out, followed by evaluation by circular dichroism. In order to estimate whether the compounds showed any sequence selectivity, the above measurements were also carried out using the homopolymers, poly (dA-dT) and poly (dG-dC).

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Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger’s Base

4.3 Ground State and Excited State Investigations

As shown in Chapter 3, the naphthalimide chromophores of 151, 152 and 153 absorb in the 350 – 500 nm region of the electromagnetic spectrum. The nucleobases of DNA absorb at ca. 250-260 nm. Therefore, any changes in the absorbance spectra of these molecules upon interacting with DNA can be easily followed. Such changes could involve a decrease in the absorption intensity (hypochroism), and a shift in the wavelength of maximum absorbance (bathochromic or hypsochromic shifts). Changes in the emission properties of the Tröger’s base derivatives, such as an enhancement or quenching of fluorescence and a shift of the emission band to longer (red shift) or shorter wavelengths (blue shift) would also be indicative of DNA-binding interactions.

The ground state and the excited state investigations of these molecules with ct-DNA and the homopolymers poly(dA-dT) and poly(dG-dC) were carried out in aqueous 10 mM phosphate buffer (1 M KH$_2$PO$_4$ and K$_2$HPO$_4$) solutions at pH 7.4. At this pH, the dialkylamino functionality of the side chains of 151 and 152 were protonated (Chapter 3), whilst the side chain of DNA is largely deprotonated. Hence, at this pH, electrostatic interactions should be maximized between the two. Tröger’s base 153, however, has a pKa value of ca. 6.2 and therefore measurements were carried out at both pH 7.4 and 6.0, to investigate whether the cationic side chain was important in the DNA-binding event. Furthermore, 154 which was primarily designed as a model compound was not included in the studies, because, it lacks the protonatable group which renders it not water-soluble.

4.3.1 Methods for the Determination of Binding Constants

A determination of the intrinsic binding constant $K_b$, for the interaction of compounds 168 - 153 with DNA, would allow for the evaluation and the comparison of their binding affinities under the same specific experimental conditions.

In general, $K_b$, can be described as:

$$K_b = \frac{L_b}{L_f S_f} \quad (4.1)$$

where, $L_b$ is the concentration of bound ligand, $L_f$ is the concentration of free ligand, and $S_f$ is the free site concentration, which is usually taken to be the DNA base concentration divided by the number of DNA bases required for the ligand binding site.
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger’s Base

The binding constants for all compounds tested were determined using both the absorbance and the luminescence spectral data. As it is constructive to compare binding parameters determined by different methods, three binding models were utilised: (i) The Kumar model\(^{171}\) (ii) The Bard model\(^{172}\) and (iii) The McGhee/von Hippel model.\(^{173}\) These will be discussed below. The absorbance spectral data was fitted to all three models whereas the luminescence data could only be fitted to the last of these models. All titration measurements were repeated three times and each of the data sets were fitted to the above models. The \(K_b\) values presented in this thesis are the average of these measurements.

The binding constant described by Kumar et al.\(^{171}\) was determined using equation 4.2:

\[
\frac{[\text{DNA}]}{(\epsilon_a-\epsilon_f)} = \frac{[\text{DNA}]}{(\epsilon_b-\epsilon_f)} + \frac{1}{K_b}\left(\epsilon_b-\epsilon_f\right) \tag{4.2}
\]

where, \(\epsilon_f\) corresponds to the extinction coefficient for the free compound, \(\epsilon_b\) corresponds to the compound when bound to DNA, recorded at the wavelength of maximum absorbance \((\lambda_{\text{max}})\) of the free compound and \([\text{DNA}]\) is the DNA concentration in base pairs. By plotting \(1/\text{[DNA]}\) against the absorbance at \(\lambda_{\text{max}}\) and dividing the y-intercept by the concentration of the compound in the absence of DNA, gives \(\epsilon_b\). The apparent extinction coefficient, \(\epsilon_A\) was calculated according to the Beer-Lambert law. The absorbance data was then fitted graphically using the above equation, with the ratio of the slope to the y-intercept giving, \(K_b\).

Utilising the Bard model, which is valid for the assumption of non-cooperative, non-specific binding to DNA that has only one type of discreet binding site, \(K_b\) was determined from equations 4.3 and 4.4: \(^{172}\)

\[
\frac{(\epsilon_a-\epsilon_f)}{(\epsilon_b-\epsilon_f)} = \frac{b - (b^2 - 2K_b^2C_t[\text{DNA}]/n)^{1/2}}{2KC_t} \tag{4.3}
\]

\[
b = 1 + K_bC_t + K_b[\text{DNA}]/2n \tag{4.4}
\]

where, \(\epsilon_a\), \(\epsilon_f\) and \(\epsilon_b\) correspond to the apparent extinction coefficient, the extinction coefficient for the free compound, and the extinction coefficient for the compound in the fully bound form, respectively. \(C_t\) is the total compound concentration, \([\text{DNA}]\) is the DNA
concentration in base pairs, and $n$ is the number of nucleotides occupied by the bound molecule. It can be assumed that when further addition of DNA does not change the absorbance, the compound is fully bound and $\epsilon_b$ can be calculated from the Beer-Lambert law. $K_b$ was then determined by fitting a plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ versus $[\text{DNA}]$ to equation 4.3, using the non-linear regression analysis programme, Sigmaplot (Version 10.0).

According to the McGhee and von Hippel model, $K_b$ was determined using Scatchard plots. Using the luminescence data, the concentration of the bound compound $C_b$ and free compound $C_f$, at any given concentration, were firstly determined from equations 4.5 and 4.6:

$$C_b = \frac{I_f - I}{I_f - I_b} \cdot C \quad (4.5)$$

$$C_f = C - C_b \quad (4.6)$$

Here, $I_f$ and $I_b$ are the fluorescence intensities of the free and bound compound, $I$ is the fluorescence response of the mixture of free and bound compound and $C$ is the total compound concentration. The absorbance data was treated in a similar manner. Scatchard analysis was then carried out according to the method of McGhee and von Hippel described in equation 4.7, where, $r = C_b/[\text{DNA}]$, ([DNA] is the concentration of DNA in base pairs). The analysis was done using the plot of $r/C_f$ versus $r$ and the curve was fitted to equation 4.7, using Sigmaplot 10.0.

$$\frac{r}{C_f} = K_b(1-nr) \left( \frac{1-nr}{1-(n-1)r} \right)^{n-1} \quad (4.7)$$

### 4.3.2 Binding Affinity of the Tröger’s Bases 151-153 for ct-DNA

Following the procedure used by Kelly et al., for the study of naphthalimide and diimide derivatives, the ground state and the excited state investigations were conducted by monitoring the changes in the absorbance and emission spectra of 151, 152 and 153 (8.7 $\mu$M), respectively, at pH 7.4 (10 mM phosphate buffer) upon successive additions of 2, 5, 10 or 20 $\mu$L aliquots of ct-DNA. In the following discussion, the results will be quoted using both the concentration of ct-DNA (1.6 mM) in base pairs (bp), and a “P/D”...
(nucleotide Phosphate to Dye) ratio. The ratios of the ct-DNA concentration to compound were calculated upon each addition of ct-DNA. A general procedure for the preparation of stock solutions of each sample and ct-DNA is given Chapter 6. The purity of ct-DNA was confirmed prior to these measurements by obtaining a ratio of about 1.8 at absorbance of 260 to that of 280 nm. This indicated that the ct-DNA was sufficiently free of protein. As stated before, all of the DNA-binding measurements were repeated three times using three different stock solutions.

4.3.2.1 Ground-State Studies

Compound 151 was first to be evaluated. Its absorbance spectrum, shown in Figure 4.3, was substantially affected by the successive addition of ct-DNA. These changes can be decomposed into two steps. The initial addition of ct-DNA (0 → 21 µM) resulted in a significant hypochromism of 33% for the λ_{max} = 381 nm, with an accompanying bathochromic shift of 11 nm. A clear isosbestic point was also observed at 415 nm at a P/D of 0.18, which indicates the presence of two spectroscopically distinct free and DNA bound chromophores.

![Figure 4.3](image)

**Figure 4.3:** (A) The normalised absorption spectra of 151 (8.7 µM) in 10 mM phosphate buffer (pH 7.4) with increasing concentration of ct-DNA (0 → 245 µM). (B) Plot of \((\epsilon_a - \epsilon_d)/(\epsilon_b - \epsilon_d)\) versus DNA (M, bp) (○) using data between P/D = 0 → 2.4 and the best fit of the data (—) using the Bard equations 4.4 and 4.3 as described in Section 4.3.1. There is slight error present on graph, nevertheless the error from mathematical calculation were small.
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The significant decrease in the absorption and the concomitant red shift indicates that 151 interacts quite strongly with the DNA base pairs and that these findings provide the first indication of the formation of ground-state complexes between 151 and the double-stranded ct-DNA. However, upon a further addition of ct-DNA within the 23 - 172 μM concentration range, the absorbance spectrum of 151, exhibited a small enhancement of 18 % at λ_{abs} 392 nm. Thereafter, no changes were observed. This is perhaps indicative of a second mode of binding.

The changes in the absorbance at 381 nm were plotted with respect to P/D, (Figure 4.4), showed evidence of a biphasic-binding phenomenon for 151. Here, the initial addition of ct-DNA resulted in a hypochromism of 33 %, which reached a plateau at a P/D of 1.3. Subsequent additions resulted in a minor reversal of this effect, which ceased to change at a P/D of 23. The initial changes were also plotted against the binding parameter, β, which is the fraction of change in the absorbance at the λ_{max}. As shown in the insert in Figure 4.4, the hypochromic effect in the absorption spectrum of 151 reached a plateau at P/D = 1.3, which ceased to change until a P/D = 2.3.

![Figure 4.4: Plot of the changes in the absorption spectra of 151 (■) and 152 (○) at 381 nm in the presence of ct-DNA. Insert: The binding curve for the changes in absorbance at 381 nm from P/D 0 → 2.5.](image)

The overall changes in the absorbance spectra of 152 (Appendix 1) at low ct-DNA concentration were identical to that of 151; exhibiting a bathochromic shift of 11 nm and the appearance of an isosbestic point at 415 nm. When such changes were plotted at 381 nm, a similar binding isotherm was obtained as shown in Figure 4.4, with the
absorption maximum exhibiting a hypochromism of 36 % between P/D 0 – 1.3 and, thereafter, subsequently enhancing by 25 %, reaching a plateau at a P/D = 20. Overall, the changes in the absorption spectra of 151 and 152 were fully reproducible.

In the literature, an analogous variation of the absorption spectra was encountered during the binding of the pyropheophorbide-spermidine (H$_2$PP-sp) conjugate 172 to ct-DNA in phosphate buffer (pH 7). In this respect, the biphasic-binding phenomenon was attributed to a "template directed effect" as shown above. The absorbance and the fluorescence initially decreased, as a result of DNA-induced aggregation of 172, causing them to stack on the DNA backbone, and was then found to subsequently increase due to a destacking event, allowing a redistribution of the fluorophore over the length of the DNA polyanion. Since 172 actually forms aggregates in phosphate buffered solution (pH = 7), the DNA-directed interaction between the H$_2$PP-sp molecules, therefore, simply enhances the aggregation of the dye.

There are several factors, which affect the strength of molecular aggregation, such as the structure of the molecule, the type of solvent, the temperature, or the presence of electrolytes. Molecules are more likely to aggregate in water due to its high dielectric constant, which reduces the repulsive force between the molecules in the aggregate. Furthermore, the addition of an electrolyte will increase the dielectric constant of a solvent, thereby facilitating aggregation. With regards to 151 and 152, aggregation in phosphate buffered solution is unlikely, owing to the fact that their absorption and emission spectra were found to be concentration independent, with the absorbance at $\lambda_{\text{max}}$ 381 nm obeying the Beer Lambert law up to a concentration of 1.0 X 10$^{-4}$ M. Furthermore, as shown in Chapter 3 (Section 3.6), the spectral characteristics of 152 are identical in 10 mM phosphate buffer solution to that in MeOH. Moreover, upon the addition of 200 mM NaCl
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to 151 in 10 mM phosphate buffer, the absorbance spectra, as shown in Figure 4.5, exhibited only an 8 % hypochromic effect with no shifts in $\lambda_{\text{max}}$. Additionally, the luminescence intensity of 151 did not change upon increasing ionic strength, nor were there any spectral shifts observed in the excitation spectra. Such behaviour suggests that no aggregation is occurring in the phosphate buffer.

Figure 4.5: Changes in the absorption spectrum of 151 (20 $\mu$M) in 10 mM phosphate buffer (—) upon the addition of 2 M NaCl (—) or 10 mM phosphate buffer (—). Insert: The Stern-Volmer plot demonstrating the lack of fluorescence quenching of 151 by NaCl.

Even though the addition of electrolytes generally quench the fluorescence and decrease the absorbance of an aggregated dye,\(^{178-180}\) the small decrease observed for 151 can be simply attributed to dilution effects.\(^*\) This was evidenced by the fact that both the absorbance and emission bands of 151 decreased to the same extent with the addition of 10 mM phosphate buffer in the absence of NaCl, (also shown in Figure 4.5). Hence, since the absorbance and emission of 151 and 152 are independent of the salt effect, further supports the lack of aggregation in aqueous solution for these molecules.

With this in mind, it could be proposed that at low ct-DNA concentrations both 151 and 152 undergo a DNA-induced aggregation, which causes them to bind along the DNA backbone, possibly through electrostatic interactions. At higher DNA concentration, the molecules reorganise along the polyanion possibly inserting into the grooves of the helix. This is inline with the situation where at low DNA concentrations, molecules tend to crowd around the polyanion whereas at high DNA concentrations, the many sites available for either intercalation or for groove-binding cause most of the drug to be bound in those forms, with the remainder of the molecules free in solution.\(^{181}\) On the other hand, the

\(^*\) Dilution effects were taken into consideration for the DNA binding studies.
possibility of the enantiomers of 151 stacking together at low DNA concentration or even both of them interacting with the DNA helix maybe a reason for the observed biphasic curve shown in Figure 4.4.

The DNA-binding affinity of 153 for ct-DNA was determined at both pH 7.4 and pH 6.0. Troger's base 153, having a pK\(a\) of 6.2, was not expected to be protonated at physiological pH and consequently it was anticipated that this study, might give an insight as to whether or not the protonated side chains were a necessary feature for achieving stronger DNA-binding. The affinity of 153 for ct-DNA was evaluated at pH 6.0 using a coadylate buffer (0.01 M). The stability of ct-DNA at this pH was confirmed before each titration, using CD spectroscopy, which showed that it exhibited the classic features of B-form DNA (Figure 4.4, Section 4.6).

The absorption spectra of 153 (8.7 \(\mu\)M) in 10 mM phosphate buffer (pH 7.4) and in 10 mM coadylate buffer (pH 6.0) upon successive addition of ct-DNA are shown in Figure 4.6a and 4.6b, respectively.

![Figure 4.6: UV/Vis absorption spectra of 153 (8.7 \(\mu\)M) in 10 mM phosphate buffer at (a) pH 7.4 and (b) pH 6.0, with increasing concentration of ct-DNA (1.6 mM).](image)

Upon increasing concentration of ct-DNA both spectra exhibited an initial decrease in the absorbance followed by a small increase. Such changes, however, were not accompanied with a bathochromic shift or the appearance of an isosbestic point as observed for 151 and 152. When 153 was recorded in 10 mM phosphate buffer at pH 7.4, it exhibited an absorbance maximum at 391 nm, which was found to decrease by 32 % upon addition of ct-DNA, within the 0 – 42 \(\mu\)M concentration range. This was followed by a subsequent increase of 10 % at larger ct-DNA concentrations (92 – 192 \(\mu\)M). In comparison, the
absorbance spectrum of 16 when recorded in the coadcylate buffer (pH 6.0) exhibited a smaller hypochromic effect of 24 % at 385 nm after initial additions of ct-DNA (0 – 20 µM). This was followed by a subsequent hyperchromic effect of 16 % upon further additions of ct-DNA (60 – 157 µM).

According to a plot of the changes in absorbance for 153, at pH 7.4 and pH 6.0, with respect to the increasing concentration of ct-DNA, (Figure 4.7), a biphasic mode of binding was observed, that is again similar to that seen previously for 151 and 152.

Figure 4.7: Plot of the changes in the absorption spectra of 153 at pH 7.4 at 391 nm (●) and pH 6.0 (●) at 385 nm in the presence of ct-DNA. Inset: The binding curve for the changes in absorbance from P/D 0 – 7.5.

However, in comparison to that of 151 and 152 the changes observed for 153 occur at higher ct-DNA concentrations. At pH 7.4, saturation behaviour of the initial spectral changes took place at a P/D = 5 and the subsequent small increase in absorbance only occurred after a P/D of 12; reaching a plateau at a P/D of 25. In comparison, at pH 6.0, the decrease in the absorbance reached a plateau at a P/D = 2.4, and the subsequent increase occurred between P/D of 6 - 22. This suggests that the binding interaction of 153 with ct-DNA at pH 7.4 is not as strong in comparison to that at pH 6.0 when 153 was fully protonated.

The changes observed in the absorbance spectra of 151, 152 and 153, as previously discussed, are summarised in Table 4.1. The red shifts, the presence of an isosbestic point and much greater hypochromicity in the absorbance spectra of 151 and 152, suggest that they bind more strongly to ct-DNA in comparison to 153.
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Table 4.1: Photophysical properties of 151 – 153 (10 mM phosphate buffer, pH 7.4) in the presence of ct-DNA. * Troger’s base 153 (coadcylate buffer, pH 6.0). [a] $\lambda_{\text{max F}}$ is the wavelength of maximum absorbance of the free compound (P/D = 0). [b] $\lambda_{\text{max B}}$ is the wavelength of maximum absorbance of the compound bound to ct-DNA (P/D = 1.3 – 5). [c] $\Delta \lambda$ is the bathochromic shift observed in $\lambda_{\text{max}}$ from the free to the bound compound. [d] $\epsilon_f$ is the extinction coefficient of the free compound. [e] $\epsilon_b$ is the extinction coefficient of compound bound to ct-DNA at $\lambda_{\text{max}}$ Abs. By plotting $1/[\text{DNA}]$ versus $\lambda_{\text{max}}$ and dividing the y-intercept by [Dye (P/D = 0)] gives $\epsilon_b$. [f] % Hypo is the percentage hypochromicity at $\lambda_{\text{max}}$ Abs (P/D = 0). [g] IP is the isosbestic point for titration of the compound with ct-DNA. [h] Bound P/D values are calculated in base pairs and correlate to the phosphate to dye ratio at which the decrease in the absorbance spectra reach an initial plateau. [i] % Hyper is the percentage hyperchromicity at $\lambda_{\text{max}}$ Abs that occurs after the plateau of the initial hypochromic effect. [j] Bound P/D at which the subsequent increase in the absorbance spectra reach a plateau. Errors: $\lambda_{\text{max}} \pm 0.2$ nm, $\epsilon \pm 100$.

4.3.2.1.1 Binding Constants Determined from the Absorbance Data

The binding constants, $K_b$, for 151, 152 and 153 were determined by analysing the initial changes in their absorbance spectra based on the Kumar model and on the site-exclusion model of Bard et al, from which the binding site size, $n$, were also obtained. A description of these binding models was previously discussed in Section 4.3.1. In all cases the spectral data from each repeated titration was fitted to both models and the average $K_b$ value was taken as the accurate binding constant. A representative Bard plot for 153 is shown as insert in Figure 4.3. Other relevant binding plots for 151, 152 and 153 are shown.
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in Appendix 1. Unfortunately, it was not possible to generate non-linear Scatchard binding isotherms from these spectral changes. Nevertheless, the obtained binding constant, $K_b$, for each molecule is summarised in Table 4.2. These results show that there is very good agreement between the two binding models, which clearly show the same binding trends.

The binding constants determined for 151, 152 and 153 are large, being in the range of $10^6$ M$^{-1}$. This binding to DNA is larger than most commonly used systems; 9-(3-aminopropyl)anthracene has a binding constant of $1.4 \times 10^4$ M$^{-1}$ (determined using the Kumar equation)$^{171}$ while the $K_b$ for $\text{[Ru(NH}_3)_4dppz]}^{2+}$ is $1.24 \times 10^5$ M$^{-1}$ (determined using the Bard equation).$^{182}$ The binding constants for Methylene blue, Azure B and Thionine, which were determined by Kelly et al.,$^{183}$ using the McGhee and von Hippel model, are also complimentary to that for 151-153.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bard Model</th>
<th>Kumar Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_b$ (x $10^6$ M$^{-1}$)</td>
<td>$n^{[a]}$</td>
</tr>
<tr>
<td>151</td>
<td>7.40 (± 0.2)</td>
<td>0.34 (± 0.015)</td>
</tr>
<tr>
<td>152</td>
<td>4.24 (± 0.3)</td>
<td>0.29 (± 0.003)</td>
</tr>
<tr>
<td>153</td>
<td>0.79 (± 0.2)</td>
<td>0.52 (± 0.07)</td>
</tr>
<tr>
<td>153*</td>
<td>2.26 (± 0.09)</td>
<td>0.64 (± 0.016)</td>
</tr>
</tbody>
</table>

Table 4.2: Binding constants determined for 151 – 153 (8.7 μM) bound to ct-DNA in 10 mM phosphate buffer, pH 7.4. *Tröger’s base 153 in coacrylate buffer, pH 6.0. The data was obtained from the changes in the absorbance spectra upon addition of ct-DNA and are the average of three experiments. [a] Number of nucleotides occluded by a bound ligand.

As shown in Table 4.2, 151 and 152 gave larger $K_b$ values in comparison to 153 at pH 7.4. However, when 153 is protonated at pH 6.0, the binding constant obtained does not significantly differ from that of 151 and 152. Therefore, the ability of Tröger’s bases 151 and 152 to interact electrostatically with the negatively charged backbone of DNA at physiological pH may account for the enhancement of $K_b$. Interestingly, binding site sizes less than unity were obtained for all the compounds, which are not in accordance with the neighbour exclusion principle.$^{176}$ This would suggest that the binding interaction of the Tröger’s bases are possibly occurring in the grooves of the DNA helix and not by intercalation between the base pairs. Consequently, investigations were undertaken to
elucidate the possible binding model for these compounds. The results from these studies will be discussed in Section 4.5 and 4.6.

4.3.2.2 Excited-State Studies

The fluorescence emission spectra of 151 and 152 (8.7 μM) in 10 mM phosphate buffer (pH 7.4), along with their corresponding excitation spectra are shown in Figure 4.8a and 4.8b, respectively. The titrations were performed by excitation at approximate isosbestic point, ~415 nm, so that the absorbance of sample was independent of DNA concentration. Upon the successive addition of ct-DNA (0 – 245 μM), the emission spectrum of 151 dramatically decreased by ca. 90 % and exhibited a concomitant bathochromic shift of 34 nm, from 505 nm to 534 nm. In comparison, the emission spectra of 152, exhibited a hypochromic effect of ca 75 % (at λ_{maxEm} of 510 nm), accompanied by a bathochromic shift of 19 nm. In both cases, the two-step phenomenon as previously seen in the absorbance studies was absent; with the fluorescence being significantly quenched at large ct-DNA concentrations. This fluorescence quenching can possibly be attributed to an efficient photoinduced electron transfer (PET) from the DNA bases to the excited state of the naphthalimide chromophore.

![Figure 4.8: The overlaid excitation (λ_{Em} 505 nm) and emission spectra (λ_{Ex} 415 nm) of 151 (A) and 152 (B) in 10 mM phosphate buffer (pH 7.4) upon the addition of increasing concentration of ct-DNA (0 – 245 μM).](image)

As shown in Figure 4.8, the excitation spectra of 151 and 152 in the absence of DNA, exhibit three bands of maximum intensity at 302, 368 and 425 nm. When 151 was excited
at these wavelengths (the resulting emission spectra are shown in Appendix 1), dramatic hypochromic effects of 90% was observed coupled with red-shifts of 34 nm, on binding to ct-DNA. When excited at 368 nm, the emission spectra showed, however, a smaller red shift of 7 nm. Interestingly, the emission spectrum of 152, when excited at 368 nm, also exhibited a hypochromic effect of 90% at λ_{max}Em 510 nm, but with an accompanying bathochromic shift of 19 nm. Excited at 425 nm, the resulting emission spectrum of 152 showed a decrease of 75% in the fluorescence intensity with a concomitant red shift of 19 nm on binding to ct-DNA.

The emission spectra of 153 (8.7 μM) when excited at 415 nm in 10 mM phosphate buffer (pH 7.4) and in 10 mM coadycylate buffer (pH 6.0) are shown in Figure 4.9a and 4.9b, respectively, along with their corresponding excitation spectra (λEx = 535 nm).

Figure 4.9: The overlaid excitation (λEm 535 nm) and emission spectra (λEx 415 nm) of 153 (A) in 10 mM phosphate buffer (pH 7.4) and (B) in 10 mM coadycylate buffer (pH 6.0) upon the addition of increasing concentration of ct-DNA (0 – 245 μM).

Upon an increasing concentration of ct-DNA (0 – 245 μM), the fluorescence intensity of the emission and the excitation spectra at pH 7.4 was quenched by 60% whereas at pH 6.3, the emission and excitation spectra exhibited a larger hypochromism of 70%. In comparison to 151 and 152, such changes were not accompanied by a bathochromic shift at the wavelength of λ_{max}. On the other hand, the emission band of the excitation spectra, centered at 415 nm, exhibited a significant bathochromic shift of 36 nm, as a function of increasing concentrations of ct-DNA at both pH values, along with the formation of isoexcitation points at ca. 473 nm. Such changes were not observed in the excitation
spectra of either 151 nor 152 (Figure 4.8) and neither do they reflect the changes observed in the absorbance spectra of 16 in the presence of ct-DNA (Figure 4.6).

Plots of I/I₀ at 415 nm vs. P/D are shown in Figure 4.10 and demonstrate the dramatic reduction in the emission intensity of both 151 and 152 in comparison to that observed for 153, on successive addition of ct-DNA. This corresponds to an approximately 9-fold and 7-fold decrease in the luminescence intensity of 151 and 152, respectively, when bound to ct-DNA. In comparison, 153 showed a more modest 5-fold decrease at pH 7.4. Nevertheless, when 153 was protonated at pH 6.3, a 6-fold decrease in luminescence intensity occurred upon binding to ct-DNA. Interestingly, the decrease in the luminescence intensity for 151 and 153 reached a plateau at ca. P/D of 20 and 15, respectively, whereas the saturation effect for 152 occurred at a much lower P/D = 8.

Figure 4.10: Plot of the changes in the emission spectra (λex 415 nm) of 151 (○), 152 (■) and 153 (▲) in 10 mM phosphate buffer (pH 7.4) and 153 (▲) in 10 mM coadecylate buffer (pH 6.3) with respect to increasing concentration of ct-DNA.

An attempt was made to evaluate the efficiency of fluorescence quenching by ct-DNA using the Stern-Volmer equation, 4.8,\(^\text{184}\)

\[
\frac{I_0}{I} = (1 + K_{SV}[Q])
\]

(4.8)

where, I₀ and I are the fluorescence intensities in the absence and presence of DNA, respectively, K_{SV} is the Stern-Volmer quenching constant, and [Q] is the ct-DNA concentration. It must be noted, however, that Stern-Volmer analysis is usually used for studying diffusional process. Hence, it is probably not an appropriate method of analysing a
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DNA binding event. Consequently, when the aforementioned titration data were used to construct a plot of $\frac{I_0}{I}$ vs. [DNA], the resulting Stern-Volmer plots, shown in Figure 4.11, were non-linear. This indicated that the fluorescence quenching of 151-153 was not purely dynamic, (i.e., quenching solely by collisional deactivation of the excited state). However, this deviation from linearity is difficult to explain.

Figure 4.11: Stern-Volmer plot of 151 (●), 152 (■) and 153 (▲) in 10 mM phosphate buffer (pH 7.4) and 153 (▲) in 10 mM coadcylate buffer (pH 6.0) with respect to increasing concentration of ct-DNA.

Firstly, the upward curvature in the above Stern-Volmer plot may be due to a static component in the quenching process. In theory, the static quenching of fluorescence is dependent on the formation of a nonfluorescent complex between the fluorophore and the quencher prior to excitation. When the fluorescence quenching of a tetrakis (4-N-methylpyridyl)porphine by guanine (which is the most reducing of the nucleotide bases) was evaluated by Larsen et al., the resulting Stern-Volmer plot was upward curving. It was thought that such a plot was consistent with an emissive complex formation. However, this may not be the case for 151-153. For instance, the observation of a static quenching component in the quenching data does not prove that the formation of a nonfluorescent complex is the reason for the deviations away from linearity. The apparent static quenching may be due to the presence of the quencher molecule being within a certain distance (also known as "sphere of action") of the fluorophore at the time of excitation.

Another possibility for the deviation away from linearity could be that some of the fluorophores are less accessible than others to dynamic quenching. For instance, it was reported by Eftink et al. that the dynamic quenching of LADH (Liver Alcohol
Dehydrogenase: which is a dimer with two tryptophan residues per identical monomer) intrinsic protein fluorescence by iodide, resulted in an upward curving Stern-Volmer plot. The rationale given was that there were two types of tryptophanyl residues in LADH, one readily accessible to iodine and one that was not. Therefore, the nonlinear Stern-Volmer plot for 151-153 could be due to one of the naphthalimide chromophores residing on the DNA surface and therefore being accessible to quenching and the other being groove bound and inaccessible to quenching. Again, this may also not be the case for 151-153. Overall, the fluorescence quenching efficiency of 151-153 by ct-DNA is not a simple process. Nevertheless, it has not been the purpose of this discussion to make a definite statement on the reason for the deviation from the Stern-Volmer equation.

4.3.2.2.1 Binding Constants Determined from the Emission Data

The fluorescence-titration data obtained from the changes in the emission spectra upon excitation at the isosbestic point (415 nm) were further used to derive Scatchard plots, which were analyzed according to the model of McGhee and von Hippel. As described in Section 4.3.1, this would give information about $K_b$, and the number of base pairs, $n$, covered by each ligand. For comparative purposes, the fluorescence data obtained from the changes in the emission spectra upon excitation at 302 nm, 368 m, and 425 nm for both 151 and 152 were also analyzed using this method. Binding isotherms for the interaction of 151, 152 and 153 and ct-DNA are shown in Appendix 1 and the fits of this are listed in Table 4.3.

Tröger’s bases 151 and 152, have an affinity to ct-DNA, which was approximately 14 times larger than 153 at physiological pH, presumably because of the binding contribution of the protonated amino side chain. The binding constant determined for 153 at pH 6.0 was, however, only 2 times smaller than 151 at pH 7.4. All of the binding constants were smaller than those obtained from the absorption titrations, yet, they follow the same trend and as such lend credibility to these measurements as well as to the binding models utilized. Interestingly the $n$ values were greater than unity, which is in contrast to that obtained using the bard model. In general, for a binding interaction, regardless of the mode of interaction, $n$ values should be greater than unity. Therefore this may suggest that the $n$ values (shown in Table 4.2, Section 4.3.2.1) determined using the Bard model are inaccurate, and indeed that the Bard Model may be too simplistic.
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Table 4.3: Photophysical properties of 151 – 153 (10 mM phosphate buffer, pH 7.4) in the presence of ct-DNA. λ<sub>ex</sub> 415 nm. * Tröger’s base 153 (coacetylate buffer, pH 6.0). [a] λ<sub>maxF</sub> is the wavelength of maximum emission of the free compound (P/D = 0), excited at 415 nm. [b] λ<sub>maxB</sub> is the wavelength of maximum emission of the compound bound to DNA. [c] % Hypo is the percentage hypochromicity at λ<sub>max</sub> (P/D = 0). [d] B<sub>shift</sub> is the bathochromic shift observed in λ<sub>max</sub> on going from the free to the bound compound. [e] K<sub>b</sub> is the intrinsic binding constant obtained by the methods of McGhee and von Hippel. [f] n the number of base pairs per binding site.

<table>
<thead>
<tr>
<th>Property</th>
<th>151</th>
<th>152</th>
<th>153 (pH 7.4)</th>
<th>153* (pH 6.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a]&lt;sub&gt;λ&lt;/sub&gt;maxF (nm)</td>
<td>505</td>
<td>509</td>
<td>538</td>
<td>540</td>
</tr>
<tr>
<td>[b]&lt;sub&gt;λ&lt;/sub&gt;maxB (nm)</td>
<td>535</td>
<td>530</td>
<td>535</td>
<td>538</td>
</tr>
<tr>
<td>[c]% Hypo</td>
<td>90</td>
<td>75</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>[d]B&lt;sub&gt;shift&lt;/sub&gt; (nm)</td>
<td>30</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[e]K&lt;sub&gt;b&lt;/sub&gt; (x 10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.75 (± 0.04)</td>
<td>2.56 (± 0.09)</td>
<td>0.18 (± 0.012)</td>
<td>0.80 (± 0.08)</td>
</tr>
<tr>
<td>[f]n</td>
<td>1.70 (± 0.03)</td>
<td>1.51 (± 0.04)</td>
<td>0.48 (± 0.007)</td>
<td>1.48 (± 0.01)</td>
</tr>
</tbody>
</table>

4.3.3 Electrostatic Effects on the Binding of 151 - 152 with ct-DNA

In general, an increase in ionic strength causes the DNA helix to shrink due to a reduction in the phosphate-phosphate bond repulsion. Therefore, within a high ionic environment; the strength at which a drug binds to DNA is expected to decrease. These electrostatic effects were investigated, by monitoring the changes in the luminescence intensity of 151 and 152 when fully bound to ct-DNA, upon the addition of a NaCl solution (2 M). Further investigations involved preparing stock solutions of 151 and 152 (8.7 μM) in 10 mM phosphate buffer containing either 50 mM NaCl or 160 mM NaCl. The UV/Vis and fluorescence titrations with ct-DNA were then performed in the same manner as those previously discussed in Section 4.3.2.

The emission spectra of 151 and 152 upon excitation at 415 nm, in the absence and in the presence of ct-DNA, are shown in Figure 4.12a and 4.12b, respectively. These show that the addition of an increasing concentration of NaCl (0 → 250 mM) to a mixture of 151 and ct-DNA (P/D = 34), does not give rise to an significant increase in the luminescence intensity. This indicated that 151 was strongly bound to ct-DNA. When 152 was examined in the same manner, a more pronounced increase in the emission intensity was observed. Nevertheless, the luminescence intensity of 152/ct-DNA (P/D = 34), at high ionic strength
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(250 mM NaCl), was still less than that compared to that observed for free 152 (P/D = 0). Therefore, the results signify that the binding processes were irreversible

![Emission spectra](image)

**Figure 4.12:** The emission spectra of (A) 151 and (B) 152 bound to ct-DNA (P/D = 34) in 10 mM phosphate buffer, upon an increasing concentration of NaCl (0 – 0.25 M).

4.3.3.1 DNA-Binding Affinity of 151 in competitive Media

The changes in the absorption spectra for the titration of 151 with ct-DNA, in 50 mM and 160 mM NaCl, are shown respectively, in Figure 4.13a and 4.13b.

![Absorption spectra](image)

**Figure 4.13:** The UV/Vis absorption spectra of 151 (8.7 μM) in (A) 50 mM NaCl and (B) 160 mM NaCl, upon titration with ct-DNA (0 – 245 μM).
As shown in Figure 4.13, the spectrum of 151, at low ionic strength (50 mM NaCl), was affected in an analogous manner to that observed in the absence of NaCl (Figure 4.3, Section 4.3.2.1). These changes are summarised in Table 4.4. The degree of hypochromism was approximately 34 % for the $\lambda_{\text{max}} = 381$ nm, while the concomitant bathochromic shift of 11 nm. An isosbestic point was also observed at 410 nm. Further additions of $ct$-DNA lead to additional 13 % hyperchromism. At higher ionic strength (160 mM NaCl), the absorption spectrum of 151, shown in Figure 4.13b, was less affected upon the addition of $ct$-DNA. Here, a hypochromism of 26 %, coupled with a bathochromic shift of 3 nm were observed up to a P/D of 10. Further additions of $ct$-DNA only induced a negligible effect.

<table>
<thead>
<tr>
<th>Property</th>
<th>151 (50 mM NaCl)</th>
<th>151 (160 mM NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max,F}}$ (Abs/nm)</td>
<td>381</td>
<td>381</td>
</tr>
<tr>
<td>$\lambda_{\text{max,B}}$ (Abs/nm)</td>
<td>392</td>
<td>384</td>
</tr>
<tr>
<td>$B_{\text{shift}}$ (nm)</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>$\epsilon_F$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>12,893</td>
<td>12,893</td>
</tr>
<tr>
<td>$\epsilon_B$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>7,972</td>
<td>9,426</td>
</tr>
<tr>
<td>% Hypo</td>
<td>33</td>
<td>26</td>
</tr>
<tr>
<td>IP (nm)</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>Bound P/D</td>
<td>1.5 $\rightarrow$ 2.4</td>
<td>10</td>
</tr>
<tr>
<td>% Hyper</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Bound P/D</td>
<td>20</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 4.4: Photophysical properties of 151 in competitive media in the presence of $ct$-DNA. [a–j] are defined in Table 4.1. Errors: $\lambda_{\text{max}} \pm 0.2$ nm, $\epsilon \pm 100$.

A plot of $A/A_0$ vs. P/D, comparing these absorbance changes are shown in Figure 4.14. The results show that within the environment of low ionic strength, 151 experienced the biphasic-binding interaction with $ct$-DNA, in a manner previously discussed in Section 4.3.2.1. However, a single binding interaction predominated when these titrations were carried out at higher ionic strength. This would imply that electrostatic interactions no longer play a prominent role in the binding of 151 to $ct$-DNA under these conditions.
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Figure 4.14: Plot of the changes in the absorption spectra of 151 at 381 nm in the presence of ct-DNA. (●) 0 mM NaCl (▲) 50 mM NaCl and (■) 160 mM NaCl: Insert: The binding curve for the changes in absorbance at 381 nm.

The values of $K_b$ determined from the absorbance data are shown in Table 4.5. Representative binding plots are shown in Appendix 2. From these results it is clear that the binding of 151 to ct-DNA is quite sensitive to the ionic strength of the solution. According to the Bard model, the $K_b$ value for 151 at high ionic strength is approximately five times less than that at low ionic strength. Furthermore, the $K_b$ value for 151 in the absence of NaCl was determined as $7 \times 10^5$ M$^{-1}$ (Section 4.3.2.1), which is an order of magnitude greater than the $K_b$ value for 151 in 160 mM NaCl.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Kumar $K_b$ (x 10$^6$ M$^{-1}$)</th>
<th>Bard $K_b$ (x 10$^6$ M$^{-1}$)</th>
<th>McGhee and von Hippel $K_b$ (x 10$^6$ M$^{-1}$)</th>
<th>$n$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2.0</td>
<td>3.0 (± 0.3)</td>
<td>0.27 (± 0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>0.63</td>
<td>0.61 (± 0.3)</td>
<td>0.69 (± 0.17)</td>
<td>0.17 (± 0.03)</td>
<td>[a]</td>
</tr>
</tbody>
</table>

Table 4.5: Binding constants determined for 151 bound to ct-DNA in competitive media. [a] Data did not give good fitting values.

The emission spectra for the titration of 151 with ct-DNA, upon excitation at 410 nm in 50 mM and 160 mM NaCl, are shown respectively, in Figure 4.15a and 4.15b. In both
cases, the successive addition of ct-DNA, caused a 86 % quenching (at $\lambda_{\text{max,em}} = 510$ nm), with a concomitant bathochromic shift of 28 nm (Table 4.6).

![Emission spectra](image)

**Figure 4.15:** The overlaid emission spectra ($\lambda_{\text{ex}} 410$ nm) of 151 (8.7 $\mu$M, 10 mM phosphate buffer, pH 7.4) in (A) 50 mM NaCl and (B) 160 mM NaCl, upon titration with ct-DNA.

![Plot](image)

**Figure 4.16:** Plot of the changes in the emission spectra ($\lambda_{\text{ex}} 415$ nm) of 151 in the presence of ct-DNA, (●) 0 mM NaCl (■) 50 mM NaCl and (▲) 160 mM NaCl.

According to a plot of $I/I_0$ vs. P/D (Figure 4.16), the luminescence intensity of 151 upon binding to ct-DNA decreased more gradually at high ionic strength (160 mM NaCl) then it did at low ionic strength (0/50 mM NaCl). These results showed that the binding of 151 with ct-DNA was affected in a solution of higher ionic strength. Again, this implies that electrostatic interactions play a role in the binding of 151 with ct-DNA.
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Table 4.6: Photophysical properties of 151 in the presence of ct-DNA (λ_{ex} 410 nm). \([a \text{−} f]\) are defined in Table 4.3. Errors: ± 0.2 nm, \(R^2 = 0.99\)

\[
\begin{array}{lll}
\text{Property} & 151 (50 \text{ mM NaCl}) & 151 (160 \text{ mM NaCl}) \\
[\text{[a]}] \lambda_{\max, F} (\text{nm}) & 505 & 505 \\
[\text{[b]}] \lambda_{\max, B} (\text{nm}) & 535 & 531 \\
[\text{[c]}] \% \text{Hypo} & 87 & 85 \\
[\text{[d]}] B_{\text{shift}} (\text{nm}) & 30 & 27 \\
[\text{[e]}] K_b (x 10^6 \text{ M}^{-1}) & 1.03 (± 0.05) & 0.72 (± 0.03) \\
[\text{[f]}] n & 1.68 (± 0.03) & 1.50 (± 0.03) \\
\end{array}
\]

The values of \(K_b\) determined from the emission changes are shown in Table 4.6. Representative Scatchard plots are shown in Appendix 3. In the absence of salt, \(K_b\) for 151 was determined as \(1.75 \times 10^6 \text{ M}^{-1}\) (Table 4.3, Section 4.3.2.2). This value does not differ greatly from those shown in Table 4.6, and demonstrate that the binding of 151 is not significantly affected by the increase in the ionic strength of the solution. However, this is in contradiction to the results determined from the absorption spectra.

4.3.3.2 DNA-Binding Affinity of 152 in Competitive Media

The absorption spectra for the titration of 152 with ct-DNA, in the presence of 50 mM and 160 mM NaCl solutions, are shown in Figure 4.17a and 4.17b, respectively. Upon binding to ct-DNA at low ionic strength, the changes observed in the absorption spectra of 152 were analogous to those in the absence of added salt (Appendix 2). However, the magnitude of the shifts was dramatically smaller. The degree of hypochromism was approximately 29 %, while the red shift was of 5 nm and the degree of hyperchromism was ca. 13 % (Table 4.7). At higher ionic strength (160 mM NaCl), the degree of hypochromism was 13 %, and the bathochromic shift of 3 nm was observed. The changes in the spectra reached a plateau at a P/D ratio of ca. 6, and thereafter, further additions of ct-DNA induced only a negligible effect. Interestingly, the formation of a clear isosbestic point was not observed in the absorption spectra during these titrations.


**Figure 4.17:** The changes in the absorption spectra of 152 (8.7 μM, 10 mM phosphate buffer, pH 7.4) in (A) 50 mM NaCl and (B) 160 mM NaCl, upon increasing concentration of ct-DNA (0 – 245 μM).

<table>
<thead>
<tr>
<th>Property</th>
<th>152 (50 mM NaCl)</th>
<th>152 (160 mM NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a]$\lambda_{\text{maxA}}$ (Abs/nm)</td>
<td>381</td>
<td>381</td>
</tr>
<tr>
<td>[b]$\lambda_{\text{maxB}}$ (Abs/nm)</td>
<td>386</td>
<td>384</td>
</tr>
<tr>
<td>[c]$B_{\text{shift}}$ (nm)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>[d]$\epsilon_F$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>11,452</td>
<td>11,452</td>
</tr>
<tr>
<td>[e]$\epsilon_B$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>7,806</td>
<td>8,686</td>
</tr>
<tr>
<td>[f]% Hypo</td>
<td>29</td>
<td>13</td>
</tr>
<tr>
<td>[g]IP (nm)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>[h]Bound P/D</td>
<td>2.4 → 3.5</td>
<td>6</td>
</tr>
<tr>
<td>[i]% Hyper</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>[j]Bound P/D</td>
<td>20</td>
<td>—</td>
</tr>
</tbody>
</table>

**Table 4.7:** Photophysical properties of 152 in the presence of ct-DNA. *No IP. [a – j] are defined in Table 4.1. Errors: $\lambda_{\text{max}} \pm 0.2$ nm, $\epsilon \pm 100$.

The plots of A/A$_0$ vs. P/D for these titrations are given in Figure 4.18. The biphasic-binding observed in the absence of salt was withheld at low ionic strength. However, the changes in absorbance were less dramatic. In a similar manner to that obtained for 151, the binding for 152 at higher ionic strength was again characteristic of a single binding mode.
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Figure 4.18: Plot of the changes observed in the absorption spectra of 152 at 381 nm in the presence of ct-DNA. (**) 0 mM NaCl (**) 50 mM NaCl and (**) 160 mM NaCl: Insert: The binding curve for the changes in absorbance at 381 nm.

The \( K_b \) values for the interaction of 152 with ct-DNA at increased ionic strength are shown in Table 4.8. The representative binding plots of the absorbance data are shown in Appendix 2. The \( K_b \) determined for 152 in the absence of salt was \( 4.24 \times 10^6 \) M\(^{-1} \) for the Bard Model and \( 5.0 \times 10^6 \) M\(^{-1} \) using the Kumar model (Table 4.2, Section 4.3.2.1). Therefore, at higher ionic strength (160 mM NaCl), the \( K_b \) for 152 has decreased by an order of magnitude.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Kumar ( \times 10^6 ) M(^{-1} )</th>
<th>Bard ( \times 10^6 ) M(^{-1} )</th>
<th>McGhee and von Hippel ( \times 10^6 ) M(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>3.4 (± 0.07)</td>
<td>2.7 (± 0.07)</td>
<td>- [a]</td>
</tr>
<tr>
<td>160</td>
<td>0.63 (± 0.1)</td>
<td>0.5 (± 0.1)</td>
<td>0.52 (± 0.12)</td>
</tr>
</tbody>
</table>

Table 4.8: Binding constants determined for 152 bound to ct-DNA in competitive media. [a] Data did not give good fitting values.

The emission spectra for the titration of 152 with ct-DNA, upon excitation at 415 nm in 50 mM and 160 mM NaCl, are shown in Figure 4.19a and 4.19b, respectively. In each case, both hypochromism and red shifts (at \( \lambda_{\text{Em}} = 510 \) nm) were observed. The degree of
hypochromism was approximately 72% at low ionic strength and ca. 66% at higher ionic strength. The bathochromic shifts were about 20 nm for both.

Figure 4.19: The overlaid emission spectra ($\lambda_{ex}$ 415 nm) of 152 (8.7 µM, 10 mM phosphate buffer, pH 7.4) in (A) 50 mM NaCl and (B) 160 mM NaCl, upon increasing concentration of ct-DNA (0 – 245 µM).

The plot of the changes in emission spectra in the presence of ct-DNA at different ionic strength is shown in Figure 4.20. The decrease was more gradual at higher ionic strength and reached a plateau at a P/D of about 23. In comparison, the decrease in the emission at lower ionic strength reached a plateau at a P/D = 8.

Figure 4.20: Plot of the changes in the emission spectra ($\lambda_{ex}$ 415 nm) of 152 in the presence of ct-DNA, (■) 0 mM NaCl (■) 50 mM NaCl and (■) 160 mM.
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger's Base

As before, the emission data was used to determine $K_b$. The results are shown in Table 4.9. The binding constants determined for 152 at increased ionic strength were dramatically smaller than the $K_b$ ($2.56 \times 10^6$ M$^{-1}$) determined for 152 in the absence of salt. In contrast to 151, the emission data showed that the binding of 152 to ct-DNA was highly sensitive to changes in the ionic strength.

<table>
<thead>
<tr>
<th>Property</th>
<th>152 (50 mM NaCl)</th>
<th>152 (160 mM NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{maxF}$ (nm)</td>
<td>510</td>
<td>510</td>
</tr>
<tr>
<td>$\lambda_{maxB}$ (nm)</td>
<td>533</td>
<td>530</td>
</tr>
<tr>
<td>$%$ Hypo</td>
<td>72</td>
<td>66</td>
</tr>
<tr>
<td>$B_{shift}$ (nm)</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>$K_b$ (x $10^6$ M$^{-1}$)</td>
<td>0.96 ($\pm$ 0.03)</td>
<td>0.1 ($\pm$ 0.02)</td>
</tr>
<tr>
<td>$n$</td>
<td>1.70 ($\pm$ 0.03)</td>
<td>1.58 ($\pm$ 0.04)</td>
</tr>
</tbody>
</table>

Table 4.9: Photophysical properties of 152 in the presence of ct-DNA ($\lambda_{ex} = 415$ nm). [$a$ – $f$] are defined in Table 4.3. Errors: $\lambda_{max} \pm 0.2$ nm, $R^2 = 0.99$.

4.3.4 Summary

The results from the ground state and the excited state investigations carried out in this Chapter have shown that 151, 152 and 153 exhibit a significant binding interaction with ct-DNA. The absorbance data showed that this binding interaction possibly involves two modes of binding. However, the changes observed in the emission spectra of 151, 152 and 153 supports only a single mode of binding. It was also shown that 151 and 152 have a greater affinity for ct-DNA than 153, at pH 7.4. The fact that 153 (as its di-cation) had a greater affinity for ct-DNA at pH 6.0 than at pH 7.4 indicated that the cationic side chain was crucial for achieving a DNA binding interaction. As expected, the binding efficiency of 151 and 152 to ct-DNA was reduced at higher ionic strength. Nevertheless, the $K_b$ values, which were determined under these conditions, were large, being in the $10^5$ M$^{-1}$ range. These results indicated that electrostatic interactions play a role in the binding of these molecules to ct-DNA. In the following section, results on the sequence specificity of 151 and 152 are discussed.
4.3.5 Binding Affinity of 151 - 152 for Polynucleotides

In order to investigate the sequence specificity of 151 and 152, their binding interaction with the two polynucleotides, poly(dA-dT), and poly(dG-dC), was evaluated. The measurements were conducted in a similar manner to that with ct-DNA (Section 4.3.2). The changes in the resulting absorption spectra were also analogous to those observed in the case of ct-DNA, i.e., hypochromism and a red shift were seen in the absorption band, followed by an increase in the absorption band at higher P/D ratios. The appearance of an isosbestic point was also observed during these titrations. Changes in the emission spectra involved an overall red shift in the fluorescence where an initial quenching followed by an emission enhancement with increasing P/D ratios were observed. However, these changes were dependent on the polynucleotides used.

4.3.5.1 Ground State and Excited State Investigations of 151

The resulting absorption spectra for the titration of 151 with poly(dG-dC) and poly(dA-dT) are shown in Figure 4.21a and 4.21b, respectively. For both, 36% hypochromism and red shift of about 10 nm was observed. These changes suggest that 151 interacted strongly with these polynucleotides. The appearance of an isosbestic point was also observed at ca. 415 nm, signifying the presence two distinct species in solution, bound and free chromophore.

![Figure 4.21: (A) The absorption spectra of 151 (8.2 μM) upon the successive addition of poly(dG-dC) (0.5 mM). (B) The absorption spectra of 151 (10 μM) upon the successive addition of poly(dA-dT) (0.75 mM)](image)
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Figure 4.22: Plot of the changes in the absorption spectra of 151 at 381 nm in the presence of poly(dG-dC) (■) and poly(dA-dT) (●). Insert: The binding curve for the changes in absorbance at 381 nm.

As shown for the plot of A/A₀ vs. P/D, (Figure 4.22), the subsequent increase in absorbance as a function of polynucleotide concentration was, however, different. Here, the degree of hyperchromism for poly(dG-dC) was approximately 12 %, while for poly(dA-dT) it was approximately 22 %.

<table>
<thead>
<tr>
<th>Property</th>
<th>Poly(dG-dC)</th>
<th>Poly(dA-dT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a] λₐₘₐₓ(F) (Abs/nm)</td>
<td>381</td>
<td>381</td>
</tr>
<tr>
<td>[b] λₐₘₐₓ(B) (Abs/nm)</td>
<td>389</td>
<td>391</td>
</tr>
<tr>
<td>[c] B_shift (nm)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>[d] εₐ (M⁻¹ dm³ cm⁻¹)</td>
<td>12,893</td>
<td>12,893</td>
</tr>
<tr>
<td>[e] ε₉ (M⁻¹ dm³ cm⁻¹)</td>
<td>7,643</td>
<td>7,515</td>
</tr>
<tr>
<td>[% Hypo]</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>[f] IP (nm)</td>
<td>417</td>
<td>410</td>
</tr>
<tr>
<td>[g] Bound P/D</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>[% Hyper]</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>[h] Bound P/D</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Kₙ(x 10⁶ M⁻¹) (Bard Model)</td>
<td>2.42 (± 0.2)</td>
<td>2.83 (± 0.2)</td>
</tr>
<tr>
<td>n</td>
<td>0.19 (± 0.018)</td>
<td>0.22 (± 0.014)</td>
</tr>
</tbody>
</table>

Table 4.10: Photophysical properties of 151 in the presence of DNA. [a – j] are defined in Table 4.1. Errors: λₐₘₐₓ ± 0.2 nm, ε ± 100, R² = 0.99.
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The binding constants for the interaction of 151 with poly(dG-dC) and poly(dA-dT) were similar, as shown in Figure 4.10, suggesting that 151 does not bind to DNA in a sequence specific manner. The very low binding site size of ca. 0.2 base pairs is not in agreement with the intercalation of 151 into the double strand of the polynucleotides and suggests that groove-binding is perhaps the major mode of binding.

The emission spectra for the titration of 151 with poly(dG-dC) and poly(dA-dT) are shown in Figure 4.23a and 4.23b, respectively. Upon the successive addition of poly(dG-dC) (0 → 100 \(\mu\)M), the luminescence intensity of 151 was quenched by 87 % and the emission band centred at 515 nm, exhibited a bathochromic shift of ca. 25 nm. The corresponding excitation spectrum (\(\lambda_{em} 510\) nm) and the emission spectra, upon excitation at 302 nm, 368 nm and 425 nm, are shown in Appendix 3. Similar emission quenching and red shifts were observed upon binding to poly(dG-dC) for these excitation wavelengths.

Figure 4.23: (A) The emission spectra of 151 (8.2 \(\mu\)M, \(\lambda_{ex} 415\) nm) upon the successive addition of poly(dG-dC) (0.5 mM). (B) The emission spectra of 151 (10 \(\mu\)M, \(\lambda_{ex} 415\) nm) upon the successive addition of poly(dA-dT) (0.75 mM).

The binding of 151 with poly(dA-dT) showed quite a different behaviour. Here the changes in the emission spectra shown in Figure 4.23b were decomposed into two steps. Initial addition of poly(dA-dT) (0 → 26 \(\mu\)M) resulted in a hypochromism of approximately 58 % with a concomitant bathochromic shift of about 23 nm. Further additions (28 → 87 \(\mu\)M) gave rise to an enhancement in the emission intensity accompanied by a hypsochromic shift at \(\lambda_{max} 538\) nm. This hyperchromism was approximately 30 %, while the blue shift was about 23 nm (Table 4.6).
According to the plot of $I/I_0$ vs. P/D (Figure 4.24), the decrease in the emission of 151, upon interaction with poly(dG-dC), reached a plateau at a P/D ratio of ca. 14. Whereas, upon interaction with poly(dA-dT), the emission decreased until a P/D of about 3 and thereafter increased, levelling off at a P/D of about 13. These changes (which were found to be fully reproducible) indicated that two processes were occurring on binding to poly(dA-dT) the first of them probably involving the external binding of the molecule to the DNA surface (electrostatic interactions), while the second interaction could involve the binding of 151 in the grooves of the DNA.

**Figure 4.24:** Plot of the changes in the emission spectra of 151 in the presence of poly(dG-dC) ( ■ ) ($\lambda_{ex}$ 417 nm) and poly(dA-dT) ( ● ) ($\lambda_{ex}$ 415 nm).

<table>
<thead>
<tr>
<th>Property</th>
<th>Poly(dG-dC) ($\lambda_{ex}$ 417 nm)</th>
<th>Poly(dA-dT) ($\lambda_{ex}$ 410 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[a]<em>{\lambda</em>{max}}$ F (nm)</td>
<td>515</td>
<td>515</td>
</tr>
<tr>
<td>$[b]<em>{\lambda</em>{max}}$ B (nm)</td>
<td>540</td>
<td>538</td>
</tr>
<tr>
<td>$[c]_{%}$ Hypo</td>
<td>87</td>
<td>58</td>
</tr>
<tr>
<td>$[d]<em>{B</em>{shift}}$ (nm)</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>$[e]_{K_b}$ ($x 10^6$ M$^{-1}$)</td>
<td>1.33 (± 0.03)</td>
<td>—*</td>
</tr>
<tr>
<td>$[f]_n$</td>
<td>0.96 (± 0.02)</td>
<td>—*</td>
</tr>
<tr>
<td>$[g]_{%}$ Hyper</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>$[h]<em>{H</em>{shift}}$ (nm)</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 4.11:** Photophysical properties of 151 in the presence of polynucleotide. $[a - h]$ are defined in Table 4.3. $[g]$ % Hyper is the percentage hyperchromicity at $\lambda_{max}$ $[h]$ $H_{shift}$ is the hypochromic shift observed in $\lambda_{max}$. * Poor fitting of data. Errors: $\lambda_{max} \pm 0.2$ nm, $R^2 = 0.99$
Unfortunately, a binding constant for the interaction of 151 with poly(dA-dT), could not be determined accurately from the emission data. However, a $K_b$ value of $1.33 \times 10^6$ M$^{-1}$ with a binding site size (n) of 0.96 was determined for the binding of 151 with poly(dG-dC) (Table 4.11). Therefore, the sequence specificity of 151 was not successfully determined. According to the ground state results, it exhibited equal selectivity of AT and GC rich sequences.

4.3.5.2 Ground State and Excited State Investigations of 152

The absorption spectra for the titration of 152 with poly(dG-dC) and poly(dA-dT) are shown in Appendix 3 and the results are summarised in Table 4.7. The degree of hypochromism observed upon the successive addition of poly(dG-dC) was approximately 43 % while it was ca. 36 % for poly(dA-dT). These changes reached a plateau at a P/D of about 2. The plot of $A/A_0$ vs. P/D is given in Figure 4.25, as well as the binding curve for the initial changes. At higher concentrations of poly(dA-dT), the degree of hyperchromism was 20 % whereas there was no substantial increase in the absorbance with the further addition of poly(dG-dC). Therefore, according to these ground state results, 152 only exhibited biphasic binding with poly(dA-dT).

![Figure 4.25: Plot of the changes in the absorption spectra of 152 at 381 nm in the presence of poly(dG-dC) (●) and poly(dA-dT) (♦). Insert: The binding curve for the changes in absorbance at 381 nm.](image)

Unfortunately, fitting of the initial absorbance data to the Bard model gave rise to the same binding constant for the interaction of 152 with each of the polynucleotides. The $K_b$
values are shown in Table 4.12 and the Bard plots are given in Appendix 3. This suggested that 152, in the same manner as 151, did not show any sequence selectivity at low polynucleotide concentration. Attempts were made to determine a $K_b$ for 151 and 152 from the subsequent increase in absorbance. However, unacceptable errors of fitting were obtained on each occasion.

<table>
<thead>
<tr>
<th>Property</th>
<th>Poly(dG-dC)</th>
<th>Poly(dA-dT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^a]$$\lambda_{maxF}$ (Abs/nm)</td>
<td>381</td>
<td>381</td>
</tr>
<tr>
<td>$[^b]$$\lambda_{maxB}$ (Abs/nm)</td>
<td>389</td>
<td>390</td>
</tr>
<tr>
<td>$[^c]$$\Delta$$\lambda_{shift}$ (nm)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>$[^d]$$\epsilon_F$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>11,452</td>
<td>11,452</td>
</tr>
<tr>
<td>$[^e]$$\epsilon_B$ (M$^{-1}$ dm$^3$ cm$^{-1}$)</td>
<td>5,953</td>
<td>6,725</td>
</tr>
<tr>
<td>$[^f]$$%$ Hypo</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td>$[^g]$$%$ IP (nm)</td>
<td>No IP</td>
<td>415</td>
</tr>
<tr>
<td>$[^h]$Bound P/D</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>$[^i]$$%$ Hyper</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>$[^j]$Bound P/D</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>$K_b$ (x $10^6$ M$^{-1}$) (Bard Model)</td>
<td>2.62 ($\pm$ 0.2)</td>
<td>2.5 ($\pm$ 0.3)</td>
</tr>
<tr>
<td>$n$</td>
<td>0.22 ($\pm$ 0.013)</td>
<td>0.20 ($\pm$ 0.01)</td>
</tr>
</tbody>
</table>

Table 4.12: Photophysical properties of 152 in the presence of DNA. [$a$ – $j$] are defined in Table 4.1. Errors: $\lambda_{max} \pm 0.2$ nm, $\epsilon \pm 100$, $R^2 = 0.99$.

The type of changes observed in the emission spectra of 152 on binding to poly(dA-dT) and poly(dG-dC) were similar to those previously discussed for 151. The relevant spectra are shown in Appendix 3 and a plot of $I/I_0$ at $\lambda_{max} = 415$ nm vs. P/D is given in Figure 4.26. The graph shows that 152, also binds with poly(dA-dT) in a biphasic manner and by a single mode with poly(dG-dC). Furthermore, it would appear that the binding of 152 occurred at a lower polynucleotide concentration in comparison to that of 151.
Figure 4.26: Plot of the changes in the emission spectra of 152 in the presence of poly(dG-dC) (△) and poly(dA-dT) (■) (λ_ex 415 nm).

The overall changes which were observed in the emission spectra of 152 upon binding to poly(dA-dT) and poly(dG-dC) are summarised in Table 4.13. The fitting of the emission data to the method of McGhee and von Hippel gave a $K_b$ value of $3.5 \times 10^6$ M$^{-1}$ for the binding of 152 to poly(dG-dC). This value is nearly a three-times greater binding constant than the $K_b$ observed for 151, indicating that 152 has a significant greater affinity for poly(dG-dC) than 151. Again $K_b$ could not be determined for the interaction with poly(dA-dT). Therefore, no conclusions could be made on the sequence specificity of 152.

<table>
<thead>
<tr>
<th>Property</th>
<th>Poly(dG-dC)</th>
<th>Poly(dA-dT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a] $\lambda_{max}$ (nm)</td>
<td>510</td>
<td>510</td>
</tr>
<tr>
<td>[b] $\lambda_{max}$ (nm)</td>
<td>538</td>
<td>530</td>
</tr>
<tr>
<td>[c] % Hypo</td>
<td>87</td>
<td>48</td>
</tr>
<tr>
<td>[d] $B_{shift}$ (nm)</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>[e] $K_b$ (x 10$^6$ M$^{-1}$)</td>
<td>3.51 (± 0.07)</td>
<td>—*</td>
</tr>
<tr>
<td>[f] $n$</td>
<td>0.99 (± 0.01)</td>
<td>—*</td>
</tr>
<tr>
<td>[g] % Hyper</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>[h] $H_{shift}$ (nm)</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4.13: Photophysical properties of 152 in the presence of polynucleotides (λ_ex 415 nm). [a-f] and [g-h] are defined in Table 4.3 and 4.11, respectively. * Poor fitting of data. Errors: $\lambda_{max} \pm 0.2$ nm, $R^2 = 0.99$
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As a means of monitoring the binding preference of 152 with either AT or GC rich sequences, a competitive binding assay were also carried out. This involved adding fixed aliquots of poly(dA-dT) to a solution mixture of 152/poly(dG-dC) at a P/D of about 2 and monitoring any reverse changes in fluorescence emission. The results from this titration are shown in Figure 4.27. As before the emission of 152 bound to poly(dG-dC) was significantly quenched. However, the subsequent addition of poly(dA-dT), resulted in the progressive restoring of the luminescence intensity up to a P/D = 16. At this stage, fixed aliquots of poly(dG-dC) were added. However, the emission was seen to only slightly decrease, indicating that 152 was more tightly bound to poly(dA-dT) than poly(dG-dC). However, these are not conclusive results regarding sequence specificity.

![Figure 4.27: Plot of the emission changes of 152, bound to poly(dG-dC) (▲) with respect to increasing concentration of poly(dA-dT) (●) and bound to poly(dA-dT) (■) with respect to increasing concentration of poly(dG-dC) (●).](image)

4.3.5.3 Summary

The determination of the sequence specificity of 151 and 152 was examined using UV/Vis and fluorescence spectroscopy in the same manner as that for ct-DNA. According to the ground state results, both molecules exhibited equal selectivity for AT and GC rich sequences. Unfortunately, the emission data could not be used to make any further comparisons because the $K_b$ value for the binding of both 151 and 152 with poly(dA-dT) could not be determined. Nevertheless, the changes in the absorbance spectra showed that the binding of 151 and 152 to AT rich sequences was biphasic. Furthermore, the emission data indicated that both 151 and 152 interacted differently with each of the polynucleotides.
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Further investigations on the sequence specificity of 151 and 152 were carried out using ethidium bromide displacement assays and thermal denaturation experiments. The results from these studies are discussed in Section 4.3.5 and 4.5.1. However, the next section deals with the DNA-binding affinity of 168 and 169. For comparisons purposes, it was important to carry out these investigations, as this would allow the nature of the binding interactions of 151 and 152 to be further understood.

4.3.6 DNA-Binding Affinity of 168 and 169

The ground state and excited state studies of 168 and 169 in the presence of ct-DNA were carried out using the exact same conditions as for the absorption and fluorescence experiments with 151 and 152, discussed in Section 4.3.2. As previously mentioned, it was anticipated that this study would allow for a further understanding of the nature of the binding interaction of the Tröger’s base derivatives. Furthermore, the DNA binding interaction of naphthalimide 168 has been extensively studied by Wilson et al.\textsuperscript{179} and the results have shown that 168 binds to DNA by intercalation.

![Figure 4.28: Structures of the 4-amino-1,8-naphthalimide precursors 168 and 169.](image)

4.3.6.1 Ground State Studies

The absorption spectrum of 168 (8.7 μM) in 10 mM phosphate buffer (pH 7.4) is shown in Figure 4.29. Upon increasing concentration of ct-DNA (0 – 245 μM) the absorption band centred at 433 nm was hypochromially shifted by ca. 42 %, along with a bathochromic shift of 11 nm. The appearance of an unclear “isosbestic point” ranging between 471 nm and 475 nm was also observed. Nevertheless, the significant decrease and the red shift in absorbance, indicates that 168 has significant binding affinity for ct-DNA.
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Figure 4.29: (A) The absorption spectra of 168 (8.7 μM) in 10 mM phosphate buffer (pH 7.4) with increasing concentration of ct-DNA (0 – 245 μM). (B) Plot of \((\epsilon_a - \epsilon_b)/\epsilon_b\) vs. DNA (M⁻¹, bp) (●) using data between P/D = 0 – 10 and the best fit of the data (—) using the Bard equation.

A plot of A/A₀ vs. P/D for the changes observed at 433 nm shown in Figure 4.30 further illustrates the apparent reduction in the absorbance band of 168, until saturation occurred and a plateau region was reached. The changes in the absorbance spectrum of the Tröger’s base 151, obtained in Section 4.3.2.1, are also plotted for comparison.

Figure 4.30: Plot of the changes in the absorption spectra of 168 (●) at 433 nm and 151 (■) at 381 nm in the presence of ct-DNA. Insert: The binding curve for the changes in absorbance at 433 nm for 168 from P/D 0 → 8 and at 381 nm for 151 from P/D 0 → 2.5.
As can been seen in Figure 4.30, the biphasic DNA binding exhibited by 151, is not evident for 168. Such a result is quite interesting and clearly highlights the difference in the ground state interactions of 168 vs. 151, upon binding to DNA. Similar decreases in the absorption spectra have been observed for unsubstituted\textsuperscript{176,189} and 3- and 4-amino substituted 1,8-naphthalimides.\textsuperscript{179,190} Therefore, the presence of the Tröger’s base moiety is obviously influencing the manner in which the naphthalimide chromophore interacts with DNA. Plotting the changes in the absorbance spectra of 168 against the $\beta$ factor, allowed for further comparisons to be made with the Tröger’s base 151. The resulting binding curves are shown in the insert of Figure 4.30. As can been seen, 168 is binding to ct-DNA at a P/D of 6, whereas the initial binding interaction exhibited by 151 occurs at a much lower P/D of 1.3. This clearly demonstrates the advantage in the design of 151 over 168.

In a similar manner to 168, the absorption spectra of 169 (8.7 $\mu$M, 10 mM phosphate buffer, pH 7.4) shown in Figure 4.31, exhibited a hypochromic effect of 36 \% at $\lambda_{\text{max}}$ 433 nm upon binding to ct-DNA. Further additions of ct-DNA induced a negligible effect in the absorption spectra. The bathochromic shift of 7 nm, (ranging from 433 nm to 440 nm) and the appearance of an (unclear) isosbestic point at 473 nm for 169 was also observed.

**Figure 4.31:** (A) The normalised UV/Vis absorption spectra of 169 (8.7 $\mu$M) in 10 mM phosphate buffer (pH 7.4) with increasing concentration of ct-DNA (0 – 245 $\mu$M). (B) Plot of $(\epsilon_a - \epsilon_b)/(\epsilon_b - \epsilon)$ versus DNA (M$^{-1}$, bp) (●) using data between P/D = 0 – 10 and the best fit of the data (—) using the Bard equation.
The plot of $A/A_0$ vs. P/D, (Figure 4.32) further shows the significant difference in their binding interaction with ct-DNA. The absorbance of both 169 and 152 decreased to almost the same extent. However, by plotting these changes against the $\beta$ factor, it can be seen that 169 binds to ct-DNA with a P/D of 13 whereas 152 binds at a P/D of 1.3. Again demonstrating the advantage of the Tröger’s base unit in these DNA targeting molecules. The overall changes in the absorbance spectra of 168 and 169 are summarized in Table 4.14.

![Figure 4.32: Plot of the changes in the absorption spectra of 169 (•) at 433 nm and 152 (○) at 381 nm in the presence of ct-DNA. Insert: The binding curve for the changes in the absorbance at 433 nm for 11 from P/D 0 – 16 and at 381 nm for 15 from P/D 0 – 2.5.]

<table>
<thead>
<tr>
<th>Property</th>
<th>168</th>
<th>169</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{a}]\lambda_{\text{maxF}}$ (nm)</td>
<td>433</td>
<td>433</td>
</tr>
<tr>
<td>$[\text{b}]\lambda_{\text{maxB}}$ (nm)</td>
<td>444</td>
<td>440</td>
</tr>
<tr>
<td>$[\text{c}]\beta_{\text{shift}}$ (nm)</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>$[\text{d}]\varepsilon_F$ (M⁻¹ dm³ cm⁻¹)</td>
<td>9782</td>
<td>10474</td>
</tr>
<tr>
<td>$[\text{e}]\varepsilon_B$ (M⁻¹ dm³ cm⁻¹)</td>
<td>5182</td>
<td>6139</td>
</tr>
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<td>$[\text{f}]%\text{ Hypo}$</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>$[\text{g}]IP$ (nm)</td>
<td>473</td>
<td>473</td>
</tr>
<tr>
<td>$[\text{h}]\text{Bound P/D}$</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 4.14: Photophysical properties of 168 and 169 (10 mM phosphate buffer, pH 7.4) in the presence of ct-DNA. $[\text{a–h}]$ are defined in Table 4.1. Errors: $\lambda_{\text{max}} \pm 0.2$ nm, $\varepsilon \pm 100$. 

166
4.3.6.1.1 Binding Constants Determined From the Absorbance Data

Quantitative analysis of the absorbance changes, up to P/D values of 8 and 16 for 168 and 169, respectively, permitted determination of $K_b$ based on both the Kumar model and on the Bard, and the McGhee and von Hippel models. Representative Bard plots for 168 and 169 are shown in the inset of Figure 4.29 and 4.31, respectively. Binding isotherms in the form of the Scatchard plots, and the Kumar plots, were generated using the extinction coefficients listed in Table 4.14. The best fits of the data from each of these binding isotherms, (shown in Appendix 4), gave the respective $K_b$ values and binding site sizes, all of which are summarised in Table 4.15.

<table>
<thead>
<tr>
<th></th>
<th>Kumar $K_b$ ($10^6$ M$^{-1}$)</th>
<th>Bard $K_b$ ($10^6$ M$^{-1}$)</th>
<th>n</th>
<th>McGhee/ von Hippel $K_b$ ($10^6$ M$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>1.0</td>
<td>2.8 ($\pm$ 0.05)</td>
<td>2.15 ($\pm$ 0.03)</td>
<td>2.1 ($\pm$ 0.05)</td>
<td>2.3 ($\pm$ 0.03)</td>
</tr>
<tr>
<td>169</td>
<td>0.6</td>
<td>1.44 ($\pm$ 0.4)</td>
<td>2.65 ($\pm$ 0.13)</td>
<td>1.96 ($\pm$ 0.016)</td>
<td>2.14 ($\pm$ 0.01)</td>
</tr>
</tbody>
</table>

Table 4.15: Binding constants determined for 168 and 169 (8.7 μM) bound to ct-DNA in 10 mM phosphate buffer, pH 7.4. Error: $R^2 = 0.99$

The binding constants for 168 and 169 were large being in the range of $10^6$ M$^{-1}$. This was expected because molecules which contain the 1,8-naphthalimide structure have been well characterised as a strong DNA binding agents. Furthermore, the binding site size determined for 168 using the method of McGhee and von Hippel is in line with that reported by Wilson et al. Such a result, not only confirms the quality of the absorbance-titration data for 168 and 169, but also the accuracy in the manipulation of this data for a determination of their respective binding constants. Nevertheless, the binding constants for 168 and 169 in comparison to 151 and 152 (Table 4.2, Section 4.3.2.1) were 7-times smaller, according to the Kumar model, and 3-times smaller according to the Bard model. Thus the Tröger’s base moiety has enhanced the DNA-binding affinity for 151 and 152. Furthermore, the occurrence of the less than unity binding site sizes ($n = 0.3 - 0.6$) for 151 and 152 (Table 4.2, Section 4.3.2.1) are characteristic of DNA groove binding, involving hydrophobic interaction. A binding site size of ca. 2 would have indicated that the binding was occurring by an intercalation mechanism, which would appear to be the case for 168-169.
In essence, the binding constants and neighbour exclusion parameters of 168 and 169 in comparison to that seen for 151 and 152, indicate a direct dependence between the structural changes and the resulting binding capability on incorporation of the Tröger's base moiety into the skeleton of the naphthalimide structure. Nevertheless, the binding constants are not that much larger in comparison to those determined for 168 and 169, which demonstrates that 151 and 152 are strong binders of DNA.

4.3.6.2 Excited State Studies

The emission spectra of 168 (8.7 μM, 10 mM phosphate buffer, pH 7.4) in the absence and presence of ct-DNA (P/D = 0 - 34) upon excitation at 433 nm and at the approximate isosbestic point, 473 nm, are shown in Figure 4.33. The corresponding excitation spectrum following the emission at 548 nm is also included in Figure 4.33.

![Figure 4.33](image)

**Figure 4.33:** The excitation spectrum (λ<sub>Em</sub> at 548 nm) (A) and emission spectra (λ<sub>Ex</sub> at 433 nm and λ<sub>Ex</sub> at 473 nm) (B) of 168 (8.7 μM) in 10 mM phosphate buffer (pH 7.4) with the addition of increasing concentration of ct-DNA (0 – 245 μM). The emission spectra λ<sub>Ex</sub> at 473 nm is normalized for clarity.

Upon the titration with ct-DNA (0 – 245 μM), the emission spectra of 168 when excited at 433 nm was found to decrease by 44 % (at λ<sub>em</sub> = 548 nm) with a concomittant hypsochromic shift of ca. 12 nm. The emission spectra excited at the isosbestic point 473 nm, however, increased by 15 % (at λ<sub>em</sub> = 530 nm) and showed the appearance of an
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isoemissive point at 550 nm upon binding to ct-DNA. These latter changes were also accompanied by a 10 nm hypsochromic shift.

Plots of \( \frac{I}{I_0} \) vs. P/D (Figure 4.34) show the changes observed in the emission intensity of 168 in comparison to the Tröger's base 151, as a function of ct-DNA concentration. The luminescence intensity of 168 showed a small enhancement followed by quenching when excited at the isosbestic point 473 nm, whereas for 151, a more significant 9-fold reduction in emission was observed. Furthermore, the luminescence intensity of 168 when excited at 433 nm, in comparison to 151, showed only a 4-fold reduction in the luminescence intensity upon binding to ct-DNA.

![Figure 4.34: Plot of the changes in the emission spectra of 151 (○) \( \lambda_{ex} \) 415 nm and 168 (♦) \( \lambda_{ex} \) 433 nm, (▲, ▲) \( \lambda_{ex} \) 473 nm in 10 mM phosphate buffer (pH 7.4) with respect to increasing concentration of ct-DNA.](image)

The emission spectra of 169 (8.7 µM, 10 mM phosphate buffer, pH 7.4) in the absence and presence of ct-DNA (P/D = 0 - 34) are shown in Figure 4.35a and 4.35b, respectively. When 169 was excited at 433 nm and upon the titration with ct-DNA, the resulting emission spectra, exhibited the appearance of an isoemissive point at 548 nm, accompanied by a hypsochromic shift of 12 nm. The spectrum was also found to increase by 27 % at 530 nm and decrease by 16 % at 568 nm, respectively, changes which were not observed in the emission spectra of 168 (\( \lambda_{Em} \) at 433 nm), upon binding to ct-DNA (Figure 4.33). When 169 was excited at 361 nm, the changes observed were a hypochromism of 36 % accompanied by a hypsochromic shift of 17 nm (Figure 4.35b). Whereas, significant hyperchromism of 45 % with a concomitant hypsochromic shift of 16 nm was observed in the emission spectrum when 169 was excited at the isosbestic point 473 nm.
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Figure 4.35: The emission spectra of 169 (8.7 μM) in 10 mM phosphate buffer (pH 7.4) upon titration with ct-DNA (0 → 245 μM). (A) \( \lambda_{ex} \) 433 nm, (B) \( \lambda_{ex} \) 473 nm and 361 nm. The emission spectra \( \lambda_{ex} \) = 473 nm is normalized for clarity.

The plot of \( I/I_0 \) vs. P/D for the changes observed in the above emission spectra are shown in Figure 4.36, along with the changes which were observed in the emission spectra of 152. In comparison to the 7-fold decrease in the emission intensity of 152, a 5-fold increase in intensity (\( \lambda_{ex} \) = 473 nm) was seen for 169 upon binding with ct-DNA. These results show that 152 and 169 were affected in a different manner upon binding with DNA.

Figure 4.36: Plot of the changes in the fluorescence spectra of 152 (■) \( \lambda_{ex} \) 415 nm and 169 (♦) \( \lambda_{ex} \) 361 nm (▲, △) \( \lambda_{ex} \) 433 nm and (♦) \( \lambda_{ex} \) 473 nm in 10 mM phosphate buffer (pH 7.4) with respect to increasing concentration of ct-DNA.
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The efficiency of the fluorescence quenching for 168 and 169 by ct-DNA were also investigated. As shown in Figure 4.37, the resulting Stern-Volmer plots were linear, indicating dynamic quenching. This is contrast to the complicated quenching process for 151 and 152, which was evidenced by upward curving Stern-Volmer plots. These results further highlight the significant difference in the excited state interactions between these molecules with DNA.

\[
y = 11573x + 1.0104 \\
R^2 = 0.9959
\]

\[
y = 4482.6x + 1.0133 \\
R^2 = 0.9917
\]

\[
y = 2938.4x + 1.0039 \\
R^2 = 0.9943
\]

\[
y = 1618.6x + 1.0049 \\
R^2 = 0.9909
\]

Figure 4.37: Stern-Volmer plot of 168 (■) λex 433 nm, (▲) λex 473 nm and 169 (▲) λex 361 nm, (▲) λex 433 nm in 10 mM phosphate buffer (pH 7.4) with respect to ct-DNA concentration.

4.3.6.2.1 Binding Constants Determined from the Emission Data

The overall changes in the emission spectra of 168 and 169 are summarised in Table 4.16. The results indicated that both interact strongly with ct-DNA. However, such changes are significantly different in comparison to those observed in the emission spectra of 151 and 152, as summarised in Table 4.3 (Section 4.3.2.2). Quantitative analysis of the emission data for 168 and 169, permitted determination of $K_b$ and $n$, in a similar manner to 151 and 152, based on the McGhee and von Hippel model. Representative Scatchard plots are shown in Appendix 4, while the average best fits of such data plots (from a number of repeated titrations) are included in Table 4.16.
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### Table 4.16: Photophysical properties of 168 and 169 (10 mM phosphate buffer, pH 7.4) in the presence of ct-DNA. [a-g] are defined in Table 4.3. [h] poor fitting of data. Errors: $\lambda_{\text{max}} \pm 0.2$ nm, $R^2 = 0.99$

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$</th>
<th>$\lambda_{\text{max}}$</th>
<th>%Hypo</th>
<th>%Hyper</th>
<th>H$_{\text{shift}}$</th>
<th>$K_b$</th>
<th>n</th>
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<tr>
<td></td>
<td>(nm)</td>
<td>(nm)</td>
<td></td>
<td></td>
<td>(nm)</td>
<td>(x $10^6$ M$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>168</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{\text{ex}}$ 433 nm</td>
<td>548</td>
<td>536</td>
<td>44</td>
<td>0</td>
<td>12</td>
<td>0.98 (± 0.03)</td>
<td>2.70 (± 0.03)</td>
</tr>
<tr>
<td>$\lambda_{\text{ex}}$ 473 nm</td>
<td>548</td>
<td>535</td>
<td>11</td>
<td>15</td>
<td>10</td>
<td>[h]</td>
<td>[h]</td>
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<tr>
<td>169</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{\text{ex}}$ 433 nm</td>
<td>548</td>
<td>533</td>
<td>16</td>
<td>27</td>
<td>15</td>
<td>[h]</td>
<td>[h]</td>
</tr>
<tr>
<td>$\lambda_{\text{ex}}$ 473 nm</td>
<td>548</td>
<td>534</td>
<td>0</td>
<td>45</td>
<td>16</td>
<td>0.23 (± 0.04)</td>
<td>2.91 (± 0.02)</td>
</tr>
<tr>
<td>$\lambda_{\text{ex}}$ 361 nm</td>
<td>548</td>
<td>531</td>
<td>36</td>
<td>0</td>
<td>17</td>
<td>0.20 (± 0.02)</td>
<td>2.14 (± 0.01)</td>
</tr>
</tbody>
</table>

In comparison to 151 and 152 (Table 4.3, Section 4.3.2.2), the binding constants obtained (from the emission data) for 168 and 169, are significantly smaller, being in the range of 10$^5$ M$^{-1}$. In fact, 168 has a binding constant nearly two times as less than that observed for 151, while 152 exhibits nearly a 10-fold higher value than precursor 169. Furthermore, n values for 168 and 169 are in the range generally found for organic intercalators. The binding site sizes for 151 and 152, are nearly 2-times smaller, leading to the assumption that they are binding perhaps by another mechanism. The difference in the Stern-Volmer plot provides further evidence for this, where the emission of 168 and 169 was dynamically quenched. Overall, these results are comparable to those obtained from the absorbance data discussed in Section 4.3.5.1.1.

### 4.3.7 Summary

The ground state and excited state studies of 168 and 169 with ct-DNA were conducted so that comparisons could be made with the DNA-binding interactions of 151 and 152. According to the absorbance data, both 168 and 169 exhibited a single mode of binding with ct-DNA. In contrast, the absorbance data for both 151 and 152 suggested a biphasic binding interaction. Therefore, this indicates that the presence of the Tröger's base unit leads to significant changes in the manner in which the naphthalimide chromophore binds to DNA. When the excited state behaviour of 168 and 169 were examined (by
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exciting at the isosbestic point), the fluorescence emission intensity enhanced in the presence of ct-DNA. However, when the experiments were conducted by exciting at the wavelength of maximum absorbance, the emission intensity of both 168 and 169 was quenched. When this emission data was evaluated using the Stern-Volmer equation, linear plots were obtained consistent with dynamic quenching. However, as discussed in Section 4.3.2.2, both 151 and 152 exhibit complicated quenching processes that gave upward curving Stern-Volmer plots. The variation in the binding interactions between 168-169 and 151-152 can obviously be accounted by their structural differences.

The binding constants for 168 and 169 were determined in the same manner as for 151 and 152. The $K_b$ values obtained were still large being in the range of $10^5$-$10^6$ M$^{-1}$, which was expected because the 1,8-naphthalimide structure is well known to bind strongly with DNA. In comparison, 151 and 152 produced larger $K_b$ values, indicating that the rationale in the design of these molecules was well founded. Furthermore, binding site sizes ($n$) consistent with intercalation were determined for 168 and 169. Regarding both 151 and 152, $n$ values less than unity were determined, which would suggest that they do not bind in the same manner as 168 and 169. Overall, these results have indicated that the introduction of the Tröger’s base moiety into the skeleton of the naphthalimide structure has significantly affected the binding interaction of the naphthalimide chromophore with the DNA helix and has ultimately resulted in an enhancement in the binding affinity of 151 and 152. The next section will further discuss and compare the binding affinities of these molecules, which were determined from displacement assays using ethidium bromide.

4.4 Displacement Assays using Ethidium Bromide

The DNA binding affinity and sequence specificity of 168 - 152 was further examined by carrying out ethidium bromide displacement assays. This method was first introduced by LePecq and Paoletti$^{191}$ and is commonly used to evaluate the DNA binding efficiency of both intercalative and non-intercalative drugs.$^{192}$ As mentioned in Chapter 1, ethidium bromide, intercalates between the base pairs and interacts electrostatically with the anionic phosphate groups on the DNA surface. When bound, ethidium bromide is strongly emissive. Upon displacement by a competitive DNA binding molecule, the changes in the emission of ethidium bromide can be monitored and employed to obtain the $C_{50}$ value, which is defined as the concentration of the compound leading to a 50 % reduction in the fluorescence intensity of ethidium bromide bound to DNA.
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The fluorescence titrations were performed using 168 – 152 in 10 mM phosphate buffer (pH 7.4) according to the procedures developed by Cain et al. and Boger et al. The fluorescence of ethidium bromide was firstly measured (λmax = 546 nm) and normalized to 0 % relative fluorescence. An appropriate amount of ct-DNA, poly(dA-dT) or poly(dG-dC) was then added so that the ratio of ethidium bromide to DNA (base pairs) was 2:1. At this ratio it could be presumed that all intercalation sites were occupied. The fluorescence was measured again after an incubation period of 20 minutes and normalized to 100 % relative fluorescence. The changes in the emission spectra of ethidium bromide bound to DNA were then monitored upon the successive additions of 2 μL aliquots of compounds 168 - 152. The titrations were continued until the decrease in emission reached saturation and remained constant.

The emission spectrum for the titration of ethidium bromide bound to ct-DNA with 151 is shown in Figure 4.38. All other spectra results for the titrations of ethidium bromide bound to ct-DNA, poly(dA-dT) and poly(dG-dC) upon displacement by compounds 151-153 are given in Appendix 5. As shown in Figure 4.40, ethidium bromide is weakly emissive when free in solution and strongly emissive when bound to ct-DNA. In general, the addition of 168–152 caused the emission intensity of ethidium bromide/ct-DNA at λmax = 600 nm to decrease dramatically. This indicated that they were all capable of substituting ethidium bromide from DNA.

![Figure 4.38: The emission spectra of ethidium bromide (EB) when free and bound to ct-DNA (1.25 μM: 2.5 μM) in 10 mM phosphate buffer upon the titration of 151.](image-url)
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Analysis of the emission data from each titration allowed for a determination of $C_{50}$ values, as discussed above. The plots of the relative decrease in intensity (IF$_{rel}$/%) of ethidium bromide/ct-DNA against the concentration of added 168 – 152 are given in Figure 4.39 and the relevant $C_{50}$ values are listed in Table 4.17.

![Figure 4.39: The fluorescence decrease of ethidium bromide induced by the competitive binding of 168 (■), 169 (▲), 171 (●), 151 (▲) and 152 (■) to ct-DNA.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$C_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ct-DNA</td>
</tr>
<tr>
<td>151</td>
<td>0.38</td>
</tr>
<tr>
<td>152</td>
<td>0.625</td>
</tr>
<tr>
<td>168</td>
<td>10</td>
</tr>
<tr>
<td>169</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 4.17: $C_{50}$ values from an ethidium bromide displacement assay (10mM phosphate buffer, pH 7.4). Results are an average of three titrations. Error: ± 10 %

It is clear from these results that the Tröger’s bases 151 and 152 and their precursors 168 and 169 are able to displace ethidium bromide effectively from bound ct-DNA, which gives rise to a quenching of the emission of ethidium bromide. The displacement capability is in the order of 151 > 152 > 168 > 169. Molecule 171, is the 4-amino-1,8-naphthalimide which lacks the tertiary amine of the terminus and is the precursor to the Tröger’s base 154. Interestingly, this compound did not displace ethidium bromide. Therefore, it can be
concluded that the displacement by the remaining molecules must involve a strong contribution from electrostatic interactions, rather than by intercalation.

It must also be noted that the titration was conducted with 171 dissolved in 100 % DMSO and ethidium bromide bound to ct-DNA in phosphate buffer. According to the method developed by Boger et al., the ethidium bromide displacement assays were conducted by titrating the competitive DNA binding molecule dissolved in DMSO. As a means of ascertaining if the same conditions could be used, the above titrations were all repeated with 168, 169, 151 and 152 dissolved in DMSO. They were then added to a mixture of ethidium bromide and ct-DNA in the phosphate buffer and the decrease in the emission was monitored. On comparing the results from these latter measurements with those in 100 % phosphate buffer, no noticeable difference was observed. Therefore, the lack of displacement ability displayed by 171 can be taken as an accurate measurement of the inability of this compound to bind to DNA.

The sequence specificity of 168 - 152 was also evaluated in phosphate buffer using poly(dA-dT) and poly(dG-dC). The plot of IFrel/% vs. concentration of compound is shown in Figure 4.40 and relevant C_{50} values were summarised in Table 4.17. According to these results, both 151 and 152 displace ethidium bromide from poly(dG-dC) and poly(dA-dT) at nearly the same C_{50} values. On the other hand, 168 and 169 exhibit sequence specificity by displacing ethidium bromide from poly(dA-dT) at C_{50} values that were two-times lower than poly(dG-dC).

![Figure 4.40: The fluorescence decrease of ethidium bromide induced by the competitive binding of 168 (●), 169 (○), 151 (▲) and 152 (●) to poly(dG-dC) and 168 (●), 169 (○), 151 (▲) and 152 (▲) to poly(dA-dT).](image-url)
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4.4.1 Binding Constants

The C50 values shown in Table 4.17 were further used to determine the DNA-binding strength of 168-152. The apparent binding constant $K_{\text{app}}$ was calculated according to a competitive model described by Boger et al.$^{194}$ using equation 4.9:

$$K_{\text{app}} = K_{\text{EB}}[\text{EB}]/[\text{Agent}]_{50\%\text{FI}} \quad (4.9)$$

where, [Agent] is the concentration of compound (168-152) at 50% reduction in the emission of ethidium bromide, [EB] is the ethidium bromide concentration and $K_{\text{EB}}$ is the binding constant for ethidium bromide. These values are summarised in Table 4.18.

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>$K_{\text{EB}}$ ($\times 10^6$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ct-DNA</td>
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</tr>
<tr>
<td>Poly(dA-dT)</td>
<td>0.65</td>
</tr>
<tr>
<td>Poly(dG-dC)</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 4.18: Ethidium Bromide Binding Constants$^{195}$

The resulting $K_{\text{app}}$ for 168 - 152 are shown in Table 4.19. The $K_{\text{app}}$ value for Distamycin A is included for comparison purposes.$^{194}$ Distamycin A binds to AT-rich sequences in the minor groove.$^{196}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{\text{app}}$ ($\times 10^7$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ct-DNA</td>
</tr>
<tr>
<td>151</td>
<td>3.5</td>
</tr>
<tr>
<td>152</td>
<td>2.4</td>
</tr>
<tr>
<td>168</td>
<td>0.15</td>
</tr>
<tr>
<td>169</td>
<td>0.054</td>
</tr>
<tr>
<td>Distamycin A$^{31}$</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.19: Binding constants for 168 – 152 determined using displacement assays with ethidium bromide. $K_{\text{app}}$ for Distamycin A is included for comparison.
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger’s Base

The results show that, 151 and 152 have a greater affinity towards ct-DNA than 168 and 169, respectively. In fact the $K_{\text{app}}$ values for 151 and 152 are significantly larger, showing again that the design of these molecules was well founded. Interestingly, 151 and 152 have larger $K_{\text{app}}$ values for poly(dG-dC) than for poly(dA-dT). In contrast, the $C_{50}$ values, shown in Table 4.17, were the same. A reason for the difference in $K_{\text{app}}$ values may be due to the fact that $K_{EB}$ with poly(dA-dT) is much smaller than that for poly(dG-dC) (Table 4.18). This would obviously effect an accurate calculation of $K_{\text{app}}$ with the polynucleotides.

4.4.2 Summary

The results from the ethidium bromide displacement assays with ct-DNA were in good agreement with the result from the ground and excited state investigations. As previously discussed in Section 4.3, the trends in the $K_b$ values with ct-DNA determined from both the absorbance and emission data were 151 >152 >168 >169. As shown in Table 4.18, the values determined from the ethidium bromide displacement assays followed the same trend, which highlights the accuracy in such measurements coupled with the greater DNA-binding affinity of both 151 and 152. Unfortunately, the sequence specificity of 151 and 152 was not accurately determined for the reasons discussed above. However, the results showed that both 151 and 152 exhibit a significantly stronger binding interaction with the polynucleotides in comparison to 168 and 169.

The results from a series of thermal denaturation experiments and CD studies will be discussed in the next Section. Thermal denaturation experiments were carried out as a means to further compare the nature of the binding interactions of 168-169 with that of 151-152, while the CD studies were conducted in an attempt to determine the mode of binding of 151-152 with ct-DNA.

4.5 Thermal Denaturation Experiments

A thermal denaturation experiment determines the stability of the DNA-structure. When double-stranded DNA is denatured, (i.e., the individual strands separate), there is a corresponding increase in the absorbance at $\lambda_{\text{max}} = 260$ nm. The temperature at which the strands of DNA are half denatured is taken as the melting temperature, $T_m$, which is dependent on the base sequence of the DNA as well as the solvent, but is independent of DNA concentration. A typical melting curve for ct-DNA in aqueous solvent (pH 7.4) is
shown in Figure 4.41a. The $T_m$ value (69 °C) for ct-DNA is the midpoint of the transition curve and can be determined from the maximum of a first-derivative plot of absorbance vs. temperature (Figure 4.41b). When a small molecule binds to DNA, it either stabilises, or destabilises, the double helix and hence modulates this number. As shown in Figure 4.41b, any stabilisation is reflected in a higher $T_m$ value, while a lower temperature indicates that the bound molecule destabilises the structure.

**Figure 4.41:** (a) The melting curve of ct-DNA (150 μM) in 10 mM phosphate buffer, showing the changes in the absorbance at $\lambda_{max}$ as a function of temperature. (b) The first derivative plot of the melting curve showing the stabilisation of ct-DNA by a typical DNA binding molecule.

The thermal denaturation studies of ct-DNA (150 μM) alone and in the presence of 168-152 were conducted in 10 mM phosphate buffer (pH 7.4). For these measurements, a P/D ratio of 10 was used because 151 and 152 bind to ct-DNA at P/D ratios lower than this value (Section 4.3.2). Before heating all solutions were degassed. The absorbance at $\lambda_{max}$ 260 nm was then monitored as the temperature was gradually ramped from 30 → 90 °C (1°C per minute). The resulting melting curves and the $T_m$ values determined from the corresponding first derivative plots are shown in Figure 4.42.

In general, all of the molecules stabilised the ct-DNA structure with similar increases in $T_m$. However, the $T_m$ values for 151 and 152 did not correlate with the midpoint of the transition curves, as the melting of ct-DNA had not finished at 90 °C. The temperature of
the sample could not be increased further due to evaporation/boiling of the buffer. Therefore, it would appear that 151 and 152 had stabilised the ct-DNA structure with a much greater increase in $T_m$ compared to the values determined by extrapolation of the absorbance data. These results were fully reproducible and pre-incubation of the molecules with ct-DNA for 12 hours did not change these results.

![Graph](image_url)

**Figure 4.42.** (A) Thermal denaturation curves of ct-DNA (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence (■) and in the presence of 151 (●), 152 (●), 168 (□) and 169 (▲) at a P/D of 10. (B) The $T_m$ values from first derivatives plots of the melting curve.

As a means of further investigating the sequence specificity of 151 and 152, the thermal denaturation studies were repeated, using poly(dA-dT) and poly(dG-dC). Upon an increasing temperature, the absorbance was monitored at 254 nm and at 262 nm for poly(dG-dC) and poly(dA-dT), respectively. Whilst poly(dG-dC) did not melt below 95 °C, poly(dA-dT) had a $T_m$ of 51 °C. Therefore, the melting of poly(dG-dC) in the presence of 151 or 152 was not examined. On the other hand, a substantial increase in the melting temperature of poly(dA-dT) occurred in the presence of both 151 and 152. The results are shown in Figure 4.43. In a similar manner to ct-DNA, the melting did not stop before 90 °C. Therefore, the values determined from the first derivative plot of the absorbance are in fact higher than 71 °C. This would consequently give a 20 °C change in the melting temperature for poly(dA-dT), suggesting a significant stabilization of its structure upon the binding of 151 and 152. However, upon closer examination of the $T_m$ curve, it appears that there are two phases present. This is a phenomenon associated with drug redistribution, and
thus correlates well with the results obtained from the fluorescence emission studies (Section 4.3.5).

![Graph showing thermal denaturation curves of poly(dA-dT) (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence (■) and in the presence of 151 (■) and 152 (▲) at a P/D = 10.](image)

<table>
<thead>
<tr>
<th></th>
<th>T_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dA-dT)</td>
<td>51 °C</td>
</tr>
<tr>
<td>151</td>
<td>&gt;71 °C</td>
</tr>
<tr>
<td>152</td>
<td>&gt;71 °C</td>
</tr>
</tbody>
</table>

**Figure 4.43.** (A) Thermal denaturation curves of poly(dA-dT) (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence (■) and in the presence of 151 (■) and 152 (▲) at a P/D = 10. (B) The T_m values determined from first derivatives plots of the melting curve.

In summary, 151 and 152 were both found to stabilise ct-DNA and poly(dA-dT) using thermal denaturation studies. The increase in T_m for poly(dA-dT) was very large, but unfortunately could not be compared to that for poly(dG-dC). Overall, these results support that the trend observed from the excited state results discussed previously with the trends in T_m being similar to both the K_b and the C_50 results, discussed in Section 4.3 and 4.4.

### 4.6 Circular Dichroism Studies

The CD studies were conducted to evaluate the potential of 151 and 152 to effect conformational changes in the double helical structure of ct-DNA. In general, CD is more sensitive to the conformation of a molecule than absorption. This is because a CD spectrum has both sign and magnitude at each wavelength. In addition, CD depends on the magnitude of the electron displacement as well as on the shape of the path taken by the electron density during an electronic transition. The shape of the path is generally in the form of a helical motion, which may be due to either a chiral molecular structure, or to perturbations induced by an asymmetric environment.

The CD of DNA is dependent on the stacking geometry of the bases. In isolation, the bases and the phosphates are achiral but when joined together with the sugar moiety
they exhibit an induced chirality. Therefore, the CD spectrum detects the induced transitions of the bases as a result of their coupling with the backbone transitions. In general, the chiral ribose-phosphate backbone has no important transitions (as far down as 180 nm), whereas, the transitions of the purine bases (adenine and guanine) have a negative signal, while the pyrimidines (cytosine and thymine) have a positive signal.\(^\text{198}\) A typical CD spectrum of ct-DNA (\(\lambda_{\text{max}}\) 260 nm) in aqueous buffered solution is given in Figure 4.44. It exhibits a positive absorption band at 275 nm, due to base stacking, and a negative band at 234 nm due to the right-handed helicity of B-DNA. It also displays a weak negative band around 210 nm and a positive band signal at 200 nm. These bands as well as those on either side of 260 nm are known as exciton bands.\(^\text{198}\)

\[\text{Wavelength (nm)}\]

![Figure 4.44: CD spectrum of ct-DNA (150 \(\mu\)M, OD = 1) in 10 mM phosphate buffer.]

In general, two experimental methods can be conducted to determine a mode of binding using CD. Firstly, intercalation can be distinguished from groove binding by measuring the CD spectra of an achiral molecule and monitoring any induced chirality upon binding to DNA. The signal and the magnitude of the induced CD (ICD) band can be used to infer the orientation of the chromophore relative to the base pair.\(^\text{199}\) For instance, if the long axis of the intercalator is orientated parallel to the base pair long axis, a negative ICD is observed while a positive ICD would indicate a perpendicular orientation. Groove-bound molecules usually exhibit a much greater ICD signal intensity in comparison with compounds that are intercalated within the helix.\(^\text{200}\) The second method involves monitoring the conformational changes in the double helical structure of DNA in the presence of either an achiral or chiral molecule. Because 151 and 152 are racemic mixtures (with equal amounts of mirror images) the latter method was carried out.
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger’s Base

The conformational changes were studied by keeping the concentration of ct-DNA constant at 150 μM (OD = 1, at 260 nm) while varying the concentration of compound from 1.5 μM to 30 μM. To avoid dilution affects, a range of solutions containing ct-DNA and the compound in 10 mM phosphate buffer were individually prepared at P/D ratios of 100, 75, 50, 30, 20, 10 and 5. The CD spectra were recorded immediately thereafter. Allowing the mixtures to equilibrate overnight had no effect on the results. Furthermore, the background CD spectrum of the buffer was initially recorded and then subtracted from all the following CD spectra.

The resulting CD spectra of ct-DNA in the presence of 151 are shown in Figure 4.45. The negative absorption peak showed an increase in the molar ellipticity of ct-DNA from -7.8 to -3.2. The changes in ellipticity are related to the conformational changes observed from the CD. Therefore the increase in the negative absorption band reflects the distortion in the helical nature of B-DNA. The intensity of the positive band also increased with a slight red-shift. This showed that 151 induced a pronounced dipole interaction between the adjacent stacked bases. Nevertheless, the shape of the spectra remained the same suggesting a single mode of binding. Furthermore, no induced CD signals were observed in the absorption region of 151 (λmax 381 nm). This could be expected if the binding mode of both enantiomers of the racemic mixture were identical. Consequently, an accurate mode of binding could not be determined from these results.

![Figure 4.45: CD spectra of ct-DNA (150 μM) in 10 mM phosphate buffer in the absence and presence of 151 at varying P/D ratios.](image-url)
Tröger’s base \textbf{152} caused similar changes in the CD spectra of \textit{ct}-DNA and did not give rise to an induced CD signal. The spectra are shown in Appendix 5. The changes were indicative of a binding interaction but unfortunately they also did not give any definite information on the mode of binding.

The effect of \textbf{168} on the CD spectrum of \textit{ct}-DNA is shown in Figure 4.46. Increasing the concentration of \textbf{168} resulted in the positive band increasing by more than that of \textbf{151}. However, the increase observed in the negative band was smaller, suggesting that \textbf{168} affects the helical structure of DNA to a lesser degree. The CD spectra of \textit{ct}-DNA in the presence of \textbf{169} are shown in Appendix 5. The changes observed were the same as that for \textbf{168}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cd_spectra.png}
\caption{CD spectra of \textit{ct}-DNA (150 \textmu M) in 10 mM phosphate buffer in the absence and presence of \textbf{168} at varying P/D ratios.}
\end{figure}

In summary, the mode of binding of \textbf{151} and \textbf{152} with \textit{ct}-DNA and their respective binding constants ($K_b$) could not be determined using CD spectroscopy. Nevertheless, the results showed that, upon binding to \textit{ct}-DNA, the chirality of its helical structure was enhanced. This provided further evidence that \textbf{151} and \textbf{152} exhibited an efficient binding interaction with DNA. Moreover, it would appear that such binding interactions were greater in comparison to \textbf{168} and \textbf{169}. However, the concave structure of these molecules may lead to an assumption that the possible mode of binding would be that of groove...
Chapter 4: Physical Evaluation of the DNA-Binding Affinity of Bis-1,8-naphthalimides Containing the Tröger's Base

binding and not by intercalation. Ideally, 151 and 152 should be resolved into single enantiomers to allow for a better determination of their mode of binding.

4.7 Conclusion

In this chapter, the DNA-binding affinities of 151-153 in aqueous solution has been discussed and compared with that of their precursors 168 and 169. The evaluations were carried out using ct-DNA and the homopolymers, poly(dG-dC) and poly(dA-dT), using UV/Vis and fluorescence spectroscopy. Ethidium bromide displacement assays, T_m experiments and CD studies were also conducted to further aid in understanding the nature of their DNA-binding interactions.

The ground state and the excited state interactions of 151 and 152 with ct-DNA were firstly evaluated and addition of ct-DNA to a 10 mM phosphate buffered solution of the 151 and 152 at pH 7.4, lead to distinct and impressive changes in the absorption spectrum of the naphthalimide, with concurrent formation of a single isobestic point. Analysis of the absorbance data indicated that both molecules were fully bound to ct-DNA at very low P/D ratios (ca. 1.3) and the binding seemed to be occurring by two possible modes of interaction. It was proposed that the first mode of binding might be due to simple electrostatic interactions between the positively charged amino groups of the side chains of 151 and 152 with the negatively charged phosphate backbone of DNA. The second mode may, however, involve binding within the grooves of the helix. The DNA-binding interaction of 153, which was determined to have a pK_a of 6.2, was also investigated at both pH 7.4 and pH 6.0. Analysis of the binding data again indicated a biphasic binding interaction similar to that seen for 151 and 152. Nevertheless, the extent of the changes observed in the absorbance spectra at both pH values was less than that seen for 151 and 152, respectively. These results indicated that the binding interaction of 153 with ct-DNA at pH 7.4 was not as strong in comparison to that at pH 6.0, presumably due to a lack of electrostatic interactions with the phosphate backbone.

Upon binding to ct-DNA, the luminescence intensity of 151-153 was substantially quenched, and bathochromic shifts at the lambdamax were also observed for all three. Analysis of the emission data showed that these molecules (when protonated) became bound to ct-DNA at very low P/D ratios (ca. ~ 2). When 153 was examined at pH 7.4, its binding was not as strong. These results again indicated that a protonated amine in the side chains was crucial to achieve the strong binding interactions with DNA. The efficiency of the
fluorescence quenching by ct-DNA was also evaluated using the Stern-Volmer equation. However, the resulting Stern-Volmer plot was upward curving, which indicates that the process was not purely dynamic in nature. A number of possible explanations for this observation were proposed. The first involved a static component in the quenching process due to either the formation of a non-fluorescent complex between 151-153 and DNA or a sphere of action effect prior to excitation. Another possibility considered was that some of the DNA-bound fluorophores of 151-153 were less accessible than others to dynamic quenching. Nonetheless, it was difficult to draw a definite conclusion for the reasons for this deviation from the Stern-Volmer equation.

The DNA binding constants \((K_b)\) of 151-153 were determined by fitting both the absorbance and the emission data to the Kumar, the Bard and the McGhee and von Hippel models, respectively. The binding constants obtained from each of these models agreed well and the average values for all molecules were in the \(10^6 \text{ M}^{-1}\) range. Furthermore, these investigations established a general trend in binding ability, which was in the trend of 151 > 152 > 153. As discussed above, molecule 153 exhibited the lowest \(K_b\) value, which was attributed to a lack of electrostatic binding with DNA at pH 7.4. It was also found that 151-153 had binding site size values were less than unity, which suggested that the binding interaction of these molecules was possibly occurring in the grooves of the DNA helix and not by intercalation between the base pairs.

The DNA binding interactions of 151 and 152 with ct-DNA were also analysed in competitive media using 50 mM and 160 mM NaCl as constant ionic strength and using absorption and fluorescence spectroscopy. In general, the strong binding observed in the absence of NaCl was maintained under low ionic strength conditions. It was also found to be irreversible regardless of the ionic strength. However, the \(K_b\) values determined under higher ionic strength conditions were on all occasions smaller. This indicated that both 151 and 152 were sensitive to increasing ionic strength and that electrostatic interactions must play a role in the binding of these molecules with DNA. When the absorbance data was further analysed additional evidence for this became apparent as it was found that both 151 and 152 experienced biphasic-binding interaction with ct-DNA under low ionic strength conditions, whereas a single binding interaction predominated when the titrations were carried out at higher ionic strength.

The sequence specificity of 151 and 152 was also investigated using the polynucleotides poly(dA-dT) and poly(dG-dC). The changes in the resulting absorption
spectra were analogous to those observed in the case of \( ct \)-DNA. Analysis of the absorbance data indicated that neither 151 or 152 exhibited any sequence selectivity because similar \( K_b \) values (ca. 2.5 \( \times 10^6 \) M\(^{-1} \)) were determined with both of the polynucleotides. On the other hand, the changes observed in the emission spectra suggested that 151 and 152 interacted differently with the polynucleotides. Upon titration with poly(dG-dC), the emission intensity of both molecules was substantially quenched. Whereas, it was found that a binding interaction with poly(dA-dT), caused the luminescence intensity of both molecules to decrease and subsequently increase at higher poly(dA-dT) concentrations. Unfortunately, a \( K_b \) value could not be determined from such emission data. Nevertheless, both molecules displayed large \( K_b \) values (\( \times 10^6 \) M\(^{-1} \)) for their binding with poly(dG-dC). As a means of monitoring the binding preference of 152 with either AT or GC rich sequences, a competitive binding assay was also carried out using fluorescence spectroscopy. The results suggested that 152 had a binding preference for poly(dA-dT) over poly(dG-dC). However, these were still not conclusive results regarding sequence specificity.

The ground state and excited state studies of 168 and 169 with \( ct \)-DNA were conducted so that comparisons could be made with the DNA-binding interactions of 151 and 152. According to the absorbance data, both 168 and 169 exhibited a single mode of binding with \( ct \)-DNA. When the emission data was evaluated using the Stern-Volmer equation, linear plots were obtained consistent with dynamic quenching. These results showed that the presence of the Tröger’s base moiety had significantly affected the binding interaction of the naphthalimide chromophore with the DNA helix. The reason being that both 151 and 152 exhibited biphasic binding with \( ct \)-DNA and the fluorescence quenching observed upon binding appeared not to be purely dynamic. The \( K_b \) values determined for 168 and 169 were quite large, being in the range of \( 10^5-10^6 \) M\(^{-1} \), (which was expected because the 1,8-naphthalimide structure is well known to bind strongly with DNA). Nevertheless they were smaller in comparison to that determined for either 151 or 152. These results indicated that enhanced binding affinity has been achieved by the incorporation of the Tröger’s base unit into 168 and 169.

The DNA binding affinity and sequence specificity of 168 - 152 was further examined by carrying out ethidium bromide displacement assays. The results were in good agreement with those from the ground and excited state investigations by exhibiting similar trends in DNA binding affinity; 151 > 152 > 168 > 169. Tröger’s bases 151 and 152
appeared to exhibit a binding preference for AT sequences. However, these results were regarded as non-conclusive.

A mode of binding for 151 and 152 was investigated by carrying out T\textsubscript{m} experiments and CD studies. The T\textsubscript{m} studies showed that 151 and 152 efficiently stabilised both ct-DNA and poly(dA-dT) from thermal denaturation. The increase in T\textsubscript{m} was far greater for the Tröger's base derivatives than that observed for 168 and 169. The results also displayed the same trends as observed in the ground state and excited state results. The large increases in melting temperature would be somewhat indicative of binding by an intercalation mechanism. However, a groove-binding interaction may also result in similar T\textsubscript{m} increases.

The results from the CD studies showed that both 151 and 152 enhanced the chirality of ct-DNA (B-form) upon binding. However, neither 151 nor 152 produced an induced CD spectrum, possibly due to the fact that these molecules were used as racemic mixtures. Consequently a mode of binding could not be determined, but it could be predicted that these molecules bind in the grooves of the DNA structure. Overall, these results are speculative, and molecular modelling, crystal structures or NMR could help provide more structural information.

Overall, the DNA binding interactions of 151-153 indicated that the rationale of the design of these molecules, discussed in Chapter 3, was well founded. They exhibited a greater affinity for ct-DNA over the 4-amino-1,8-naphthalimides 168-169, of which 168 has within the literature been shown to be an efficient binder of DNA. These results are thus highly encouraging and indicate that the development of such novel water-soluble molecules will hopefully represent a major milestone in the utility of the 1,8-naphthalimide structure as an anticancer agent.
Chapter 5: Biological Examination of Alkene, Thiazole and Tröger's Base Modified 1,8-Naphthalimide Derivatives

5.1 Introduction

As discussed in Chapter 1, several hundred peptide-based naphthalimide derivatives have been developed within the Gunnlaugsson group, many of which have exhibited promising cytotoxic activity in the leukemic HL-60 and K562 cell lines. The focus of the work carried out and discussed in this Thesis has been to enhance the biological activity of these molecules by the incorporation of (i) alkene and thiazole containing amino acids and (ii) the Tröger's base structural unit. The incorporation of the thiazole moiety was inspired by the fact that many of the naturally occurring thiazole containing cyclic peptides exhibit strong cytotoxic activity. The development of the Tröger's based naphthalimide derivatives was carried out because the chiral concave structure of the Tröger's base unit may give rise to enantiospecific binding of such naphthalimide analogues in the minor or major grooves of DNA. Consequently, it was anticipated that this would lead to a good cytotoxic activity. The synthesis of the alkene and the thiazole modified 1,8-naphthalimides was discussed in Chapter 2, while the synthesis and the DNA binding interactions of the Tröger's base based 1,8-naphthalimides was dealt with in Chapter 3 and Chapter 4, respectively.

In this chapter, the cytotoxic activity of the above target molecules in the HL-60 promyelocytic cell line and in the more resistant erythroleukemia K562 cell line are discussed. Dr. Tony McElligott in the Department of Hematology, St. James' Hospital, Dublin, carried out the biological testing of the alkene modified 1,8-naphthalimide derivatives, while the testing of the thiazole and the Tröger's base modified 1,8-naphthalimides was carried out by Mr. Frimannsson. The biological activity of the Tröger's based naphthalimide derivatives was then further evaluated by Mr. Frimannsson using confocal microscopy, cell cycle analysis and topoisomerase I and II inhibition studies. These results will be discussed in the PhD Thesis of Mr. Frimannsson, which is to be submitted in 2008. However, in the interest of the reader of this Thesis, it is important that the cytotoxicity of these molecules is highlighted to emphasise the importance of their further development, and to support our original objectives.

For this Chapter, each of the target molecules was split into three groups, with the aldehyde and the alkene modified 1,8-naphthalimide derivatives forming Group 1.
Group 2 consists of the thiazole modified 1,8-naphthalimide derivatives, while Group 3 consists of the Tröger's base analogues. The cytotoxicity of each molecule will be quoted as an IC\(_{50}\) value, which is the concentration of drug that causes 50 % growth inhibition of living cells, and can be determined by means of an MTT assay. The activity of each molecule in the HL-60 cell line will be compared to the IC\(_{50}\) values of the reference compounds 8, 9, 164 and 168, shown in Table 5.1.\(^a\) For tests in the K562 cell line, a IC\(_{50}\) of 1.56 \(\mu\)M for 8 will be used as a reference value. Furthermore, all results were compared with the cytotoxicity of the peptide-based 1,8-naphthalimides previously developed within the Gunnlaugsson group.\(^3\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>H</td>
<td>NH(_2)</td>
<td>3.00</td>
</tr>
<tr>
<td>9</td>
<td>H</td>
<td>NO(_2)</td>
<td>0.23</td>
</tr>
<tr>
<td>164</td>
<td>NO(_2)</td>
<td>H</td>
<td>4.48</td>
</tr>
<tr>
<td>168</td>
<td>NH(_2)</td>
<td>H</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Table 5.1: IC\(_{50}\) values obtained for reference compounds in HL 60 cells, after incubation for 24 h.

At this point, it must be emphasised that the purpose of this Chapter is to simply illustrate the biological activity of the target molecules developed within this Thesis. It is not the intention to discuss or to explain the theory or nature behind any of the biological results, as these will be dealt with in the PhD thesis of Mr. Frimannsson, as previously mentioned. Thus, this Chapter will be concise and will begin by showing the IC\(_{50}\) values for the molecules within Group 1. This will be then followed by the results from Group 2 and 3, respectively.

5.2 The Cytotoxic Activity of the Alkene Modified 1,8-Naphthalimide Containing \(\alpha\)-Amino Acids – Group 1

All the compounds developed in this Thesis, and discussed below, were found, if active, to induce apoptosis of the cell cultures investigated over 24 or 48 hours. The IC\(_{50}\) values of the acetal, aldehyde and alkene functionalised \(\alpha\)-amino acid-based naphthalimide conjugates, evaluated in the HL-60 cell line, are shown in Table 5.2.
Table 5.2: IC\textsubscript{50} values for compounds 80 – 89 after incubation for 24 h in HL-60 cells. Errors will be presented in Frimansson Thesis.

<table>
<thead>
<tr>
<th>Cpd.</th>
<th>R</th>
<th>IC\textsubscript{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>CH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2} (S)</td>
<td>2.2</td>
</tr>
<tr>
<td>81</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (S)</td>
<td>2.0</td>
</tr>
<tr>
<td>82</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (R)</td>
<td>2.1</td>
</tr>
<tr>
<td>83</td>
<td>CH\textsubscript{3} (S)</td>
<td>12.00</td>
</tr>
<tr>
<td>84</td>
<td>CH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2} (S)</td>
<td>2.4</td>
</tr>
<tr>
<td>85</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (S)</td>
<td>2.8</td>
</tr>
<tr>
<td>86</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (R)</td>
<td>2.6</td>
</tr>
<tr>
<td>87</td>
<td>CH\textsubscript{3} (S)</td>
<td>11.0</td>
</tr>
<tr>
<td>88</td>
<td>CH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2} (S)</td>
<td>1.4</td>
</tr>
<tr>
<td>89</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (S)</td>
<td>1.9</td>
</tr>
<tr>
<td>90</td>
<td>CH\textsubscript{2}(C\textsubscript{6}H\textsubscript{5}) (R)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

From these results, discrimination between the above molecules can be observed. The alkene 88 was the most potent by exhibiting a IC\textsubscript{50} value of 1.4 \mu M, while the corresponding aldehyde 84 and acetal 80 derivatives were less active, exhibiting values of 2.4 \mu M and 2.2 \mu M, respectively. In fact, all of the alkene derivatives 88 - 90 are more cytotoxic than their corresponding acetal 84 and aldehyde 80 derivatives. However, these are still excellent results in comparison to a lot of the other compounds in the family of peptide-based 1,8-naphthalimide derivatives, developed within the Gunnlaugsson group.

An interesting modulation of the cytotoxic parameters was further observed by changing the amino acid in the side chain of the acetal, aldehyde and alkene derivatives, respectively. For instance, the phenylalanine acetal derivative 81 and the alanine acetal derivative 83 gave IC\textsubscript{50} values of 2.0 \mu M and 12.0 \mu M, respectively, clearly showing that the phenylalanine amino acid is more active than the alanine amino acid. Perhaps it is \pi-stacking interactions between the aromatic ring of phenylalanine and the base pairs of DNA, which contribute to its greater cytotoxicity, or perhaps this molecule inhibits the cell growth and induces apoptosis in a different manner. These results are in-line with initial results obtained by Phelan,\textsuperscript{3a} which showed that in general, phenylalanine and leucine
derivatives are more effective than alanine derivatives in inducing apoptosis in leukaemia cell lines. Overall, the reasons behind the high activity of 88 - 90 are not obvious. Further functionalisation of these derivatives and testing of the resulting compounds will lead to a more concise evaluation. Nevertheless, 80 - 90 have shown an improvement in cytotoxic activity over the more simple α-amino acid based naphthalimides previously developed within the Gunnlaugsson Group. For instance, Phelan reported a IC_{50} value of 3.0 μM, for a corresponding leucine tert-butyl ester naphthalimide derivative. Even though this is a good result, it still shows that the development of the above molecules has been worthwhile.

As mentioned in Chapter 2, the phthaloyl protected α-amino acid 91, the acetal 92 and the aldehyde 93 derivatives were synthesised so that their cytotoxic activity could be tested and compared with 76, 80 and 84. The resulting IC_{50} values are shown in Figure 5.1 and as can be seen, their cytotoxicity is considerably reduced by the absence of the naphthalimide moiety. This result merits the further development of 88 - 90 as potential anticancer agents. The next section will discuss the cytotoxicity of the thiazole modified 1,8-naphthalimide containing α-amino acids.

![Figure 5.1: IC_{50} values for compounds 91 – 93 after incubation for 24 h in HL-60 cells.](image)

5.3 The Cytotoxic Activity of the Thiazole Modified 1,8-Naphthalimide Containing α-Amino Acids – Group 2

The IC_{50} values of the 4-nitro and 4-amino-1,8-naphthalimide based thiazole derivatives evaluated in the K562 cell line are shown in Table 5.3. The most active of the series was 118 and the least active was 124, which showed no activity during the incubation period (24 hours). This result is in-line with the trends seen by both Phelan and Hussey, where the 4-nitro substituted naphthalimides are generally the most active. As discussed in Chapter 2, the synthesis of the above molecules was challenging and the
achievement of which was a major breakthrough. Consequently, the poor cytotoxic activity of 119, 123 and 124 was disappointing.

<table>
<thead>
<tr>
<th>Cpd.</th>
<th>R</th>
<th>R₁</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>118</td>
<td>CH₂CH(CH₃)₂</td>
<td>NO₂</td>
<td>6.43</td>
</tr>
<tr>
<td>119</td>
<td>CH₂C₆H₅</td>
<td>NO₂</td>
<td>23.46</td>
</tr>
<tr>
<td>123</td>
<td>CH₂CH(CH₃)₂</td>
<td>NH₂</td>
<td>&gt;100</td>
</tr>
<tr>
<td>124</td>
<td>CH₂C₆H₅</td>
<td>NH₂</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

**Table 5.3:** IC₅₀ values for compounds 118 – 124 after incubation for 48 h in K562 cells. Errors will be presented in Frimansson Thesis.

Nevertheless, the cytotoxicity of 118 is still quite promising as its IC₅₀ value is only four times higher than that of the reference compound 8 (Section 5.1). Furthermore, the naphthalimide derivative 134, which has a thiazole amino acid directly incorporated into its structure, exhibited a higher IC₅₀ value (10.5 µM) when tested in the HL-60 cell line, suggesting that the presence of the leucine amino acid and the thiazole ring in compound 118 must contribute to a good cytotoxic activity. Further evidence for this, was provided by the phthaloyl protected thiazolidine 100 and thiazole 102 containing amino acids (Chapter 2) failing to exhibit any cytotoxicity when tested in the HL-60 cell line.

The 3-nitro derivatives 120 and 121, which were also synthesised and discussed in Chapter 2, are currently undergoing further biological testing. Since it has been reported by both Phelan and Hussey that the 4-nitro-1,8-naphthalimide containing α-amino acids are more active that the corresponding 3-nitro analogues,³ it could be expected that 120 and 121 may not exhibit an enhancement in cytotoxicity. Nevertheless, the biological testing of these molecules is still essential as the above trends may change by the presence of the thiazole unit. In fact, this has already been shown by the leucine derivative 118 being more active than the phenylalanine derivative 119, a result that is not in-line with the trends reported by Phelan.³

Overall compound 118 has been the most active of the thiazole analogues tested, however, the cytotoxicity of the other derivatives 119 - 124 were very poor. It must be
Chapter 5 Biological Examination of Alkene, Thiazole and Tröger’s base Modified 1,8-Naphthalimide Derivatives

noted, however, that the quoted IC$_{50}$ values are for their activity in the more resistant K562 cell line. With this in mind, these molecules may exhibit better activity in the less resistant HL-60 cell line, and such investigations are currently being carried out.

The next group of molecules to undergo cytotoxic analysis were the 1,8-naphthalimide containing Tröger’s base derivatives. Prior to such analysis, it had been established, using UV/Vis and fluorescence spectroscopy, Tm experiments and CD studies, that these molecules bind strongly with ct-DNA, poly(dA-dT) and poly(dG-dC). All results from this work are discussed in Chapter 4. Consequently, it was anticipated that they would also show promising activity in cancer cells. The results will be detailed in the next section.

5.4 The Cytotoxic Activity of the 1,8-Naphthalimide Containing Tröger’s Base Derivatives – Group 3

The cytotoxic activity of the Tröger’s base derivatives 151 – 153 and their 4-amino precursors 168 – 170, tested in both the HL-60 and the K562 cell line, are shown in Table 5.4.

![Chemical structures](image)

<table>
<thead>
<tr>
<th>Cpd.</th>
<th>R</th>
<th>IC$_{50}$ HL-60</th>
<th>IC$_{50}$ K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>151</td>
<td>N(CH$_3$)$_2$</td>
<td>4.02 µM</td>
<td>3.82 µM</td>
</tr>
<tr>
<td>152</td>
<td>N(CH$_2$CH$_2$)$_2$NCH$_3$</td>
<td>4.44 µM</td>
<td>17.0 µM</td>
</tr>
<tr>
<td>153</td>
<td>N(CH$_2$CH$_2$)$_2$O</td>
<td>—*</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>168</td>
<td>N(CH$_3$)$_2$</td>
<td>0.94</td>
<td>—*</td>
</tr>
<tr>
<td>169</td>
<td>N(CH$_2$CH$_2$)$_2$NCH$_3$</td>
<td>72.5</td>
<td>28.1</td>
</tr>
<tr>
<td>170</td>
<td>N(CH$_2$CH$_2$)$_2$O</td>
<td>—*</td>
<td>28.7</td>
</tr>
</tbody>
</table>

Table 5.4: IC$_{50}$ values for the Tröger’s bases 151 – 153 and the 4-amino derivatives 168 – 170 after incubation for 24 h in HL-60 and for 48 h in K562 cells. * Compound still under investigation. Errors will be presented in Frimannsson Thesis.
Upon comparing the Troger's base derivatives, the cytotoxic activity of 151 and 152 in the HL-60 cell line are very similar. This result is not surprising as the DNA binding affinity of these molecules, determined with ct-DNA in aqueous solution at pH 7.4, did not differ greatly, with 151 and 152 exhibiting $K_b$ values of $7.4 \times 10^6 \text{ M}^{-1}$ and $4.3 \times 10^6 \text{ M}^{-1}$, respectively. However, the difference between their IC$_{50}$ values in the K562 cell line is more pronounced. Perhaps the mechanism of cell death inflicted by these molecules differs between the HL-60 and K562 cell lines. Possible reasons for such a discrepancy will be discussed in more detail in the PhD Thesis of Mr. Frimannsson.

The morpholine derivative 153, showed very poor cytotoxic activity in the K562 cell line and consequently was not tested in the HL-60 cell line. This result again was somewhat expected, because the DNA binding ability of this molecule was less than that of 151 and 152, by exhibiting a $K_b$ value of $0.79 \times 10^6 \text{ M}^{-1}$, when analysed with ct-DNA at pH 7.4. The possible reason for the reduced binding affinity was the lack of potential electrostatic interactions between it and the anionic phosphate backbone of the DNA helix. As shown in Chapter 3, 153 was determined to have a p$K_a$ value of 6.0, and thus was not protonated at pH 7.4. On the other hand, it could be assumed that a protonated side chain would inhibit the entry of a molecule into the cell, thus reducing its cytotoxicity. However, molecules 151 and 152, having p$K_a$ values of 8.7 and 8.2, respectively, both showed good cytotoxic activity. With this in mind, further studies using confocal microscopy were carried out by Mr. Frimannsson to determine the rate of uptake in cells of 151.

As shown in Table 5.4, the cytotoxic activity of the 4-amino precursors, 168 – 170, were also evaluated for comparison purposes. As was expected, the reference compound 168 is strongly cytotoxic, exhibiting an IC$_{50}$ value of 0.94 $\mu$M. However, the precursor 169 showed a reduced cytotoxic activity in comparison to the Troger's base 152. This result, in combination with the enhanced DNA binding ability of 151 and 152 (as discussed in Chapter 4), has indicated that the design of these molecules was well founded. Having established that Troger's base 151 is the most toxic in the K562 cell line, the biological activity of this molecule was further studied. As this is the sole work of Mr. Frimannsson, these results will not be shown in this Chapter. Nevertheless, the exciting results obtained from the confocal fluorescent imaging of 151 needs to be mentioned, as these results really emphasise the potential of this molecule as an anticancer agent. These measurements showed that 151 was quickly taken up into the cells, localised within the nucleus and induced apoptosis. This molecule emits in the green ($\lambda_{\text{fluor}} \sim 500 \text{ nm}$) as shown
in Figure 5.2, wherein the apoptotic cell is highlighted in the red circle. These are very encouraging results for the further development of this molecule as an anticancer agent.

![Figure 5.2: A fluorescent picture of 151 in HL-60 cells showing localisation of the drug in the nucleus: (A) phase (B) propidium iodide (C) 151 (10 μM) (D) overlay of (B) and (C).](image)

**5.5: Conclusion**

In this Chapter, the cytotoxic activity of the alkene, thiazole and Tröger’s base modified 1,8-naphthalimide derivatives were discussed. The most potent was the alkene derivative 88, which showed an IC$_{50}$ value of 1.4 μM in the HL-60 cell line. Nevertheless, all target molecules showed good cytotoxicity, with IC$_{50}$ values in the range of 1.9 – 12.0 μM. Unfortunately, the cytotoxic activity of the thiazole based 1,8-naphthalimide derivatives was poor. However, the most active of this series was the leucine analogue, which showed an IC$_{50}$ value of 6.43 μM in the more resistant K562 cell line.

The cytotoxic activity of the 1,8-naphthalimide containing Tröger’s base derivatives correlated well with their strong DNA binding affinity. Upon comparing the cytotoxicity of these derivatives, the Tröger’s base analogue 151 was shown to be the most potent. Consequently, this molecule has undergone further biological studies to gain a better understanding of its cytotoxic activity. These results were not discussed in this Chapter as
they will be discussed in the Thesis of Mr. Frimannsson. Overall, the results detailed within this Chapter have shown that the development of these molecules as potential anticancer agents was worthwhile.
Chapter 6: Experimental

6.1 General Experimental Details

All chemicals were obtained from Sigma-Aldrich, Fluka, or Lancaster and unless specified, were used without further purification. Deuterated solvents for NMR use were purchased from Apollo Ltd. Dry solvents were prepared using standard procedures, according to Vogel, with distillation prior to each use. Chromatographic columns were run using Silica gel 60 (230-400 mesh ASTM). Solvents for synthesis purposes were used at GPR grade unless otherwise stated. Analytical TLC was performed using Merck Kieselgel 60 F254 silica gel plates or Polygram Alox N oxide plates. Visualisation was by UV light (254 nm). NMR spectra were recorded using a Bruker Advance III spectrometer, operating at 400 MHz and 600 MHz for $^1$H-NMR and 100 MHz for $^{13}$C-NMR. Shifts are referenced relative to the internal solvent signals. NMR data were processed using Bruker Win-NMR 5.0 software. Electrospray mass spectra were recorded on a Mass Lynx NT V 3.4 on a Waters 600 controller connected to a 996 photodiode array detector with HPLC-grade methanol, water or acetonitrile as carrier solvents. Accurate molecular weights were determined by a peak-matching method, using leucine enkephaline (H-Tyr-Gly-Gly-Phe-Leu-OH) as the standard internal reference ($m/z = 556.2771$); all accurate mass were calculated to $\leq 5$ ppm. Samples were prepared as solutions in MeOH, EtOH or CH$_3$CN. Melting points were determined using an Electrothermal IA9000 digital melting point apparatus. Infrared spectra were recorded on a Mattson Genesis II FTIR spectrometer equipped with a Gateway 2000 4DX2-66 workstation and on a Perkin Elmer Spectrum One FT-IR Spectrometer equipped with Universal ATR sampling accessory. Oils were analysed using NaCl plates; solid samples were dispersed in KBr and recorded as clear pressed discs or as neat samples. Elemental analysis was carried out at the Microanalysis Laboratory, School of Chemistry and Chemical Biology, University College Dublin. X-ray diffraction studies were carried out by Dr. T. McCabe, School of Chemistry, Trinity College Dublin, Dublin.

6.2 UV/Vis Measurements

UV-visible absorption spectra and optical density were recorded by means of a Varian CARY 50 spectrophotometer. Solutions were measured in 3 cm (10 mm x 10 mm) cuvettes. The wavelength range was 200-550 nm with a scan rate of 600 nm min$^{-1}$. Water
used in DNA related work was triply distilled, autoclaved and filtered (Millipore, HV, 0.45 μm). Phosphate buffer: two 1 M stock solutions of K$_2$HPO$_4$ and KH$_2$PO$_4$ (using 10 mL volumetric flasks) were made up with water (triply distilled, autoclaved and filtered), portions of each solution were diluted together to achieve 10 mM phosphate buffer of pH 7. Baseline correction measurements were used for all spectra. All solutions were prepared freshly prior to measurement.

The ct-DNA, and the homopolymers, poly(dA-dT) and poly(dG-dC), were obtained from Sigma Aldrich as their sodium salts. They were stored at −20 °C to prevent bacterial growth. The concentrations of ct-DNA and the homopolymers were accurately determined by quantification by UV-Vis analysis. Detection for the presence of contaminant proteins in ct-DNA was performed through UV-Vis analysis where the absorption ratio $A_{260 nm}/A_{280 nm}$ must be greater than 1.8 for protein-free DNA, and the homopolymers were checked for purity using CD spectroscopy. The DNA concentration per nucleotide was determined spectrophotometrically using the molar extinction coefficient, 6600 M$^{-1}$cm$^{-1}$ at 260 nm for ct-DNA and 6000 M$^{-1}$ cm$^{-1}$ at 262 nm for poly(dA-dT) and 8400 M$^{-1}$ cm$^{-1}$ at 254 nm for poly(dG-dC).

6.3 Fluorescence Measurements

Fluorescence and luminescence measurements were made with a Varian Carey Eclipse Fluorimeter equipped with a 1.0 cm path length quartz cell. The solvents used were of HPLC grade. The concentrations of the compounds under investigation were the same as those used for the UV-visible absorption measurements, as detailed in Chapter 3 and Chapter 4.

6.4 UV/Vis Temperature-Based Measurements

For the $T_m$ studies, semi-micro UV-Vis cuvettes were used (path length of 1 cm and window width of 4 mm, from Starna). The temperature-based measurements were obtained using a Cary Temperature controller in conjunction with the Varian Cary 300 UV-Vis spectrometer. The temperature was ramped from 30-90 °C at a rate of 0.5 °C/min. All solutions were thoroughly degassed before the experiment.
6.5 CD Measurements

CD spectra were recorded in CH$_2$Cl$_2$ or in 10 mM phosphate buffer (for the DNA titrations) on a Jasco J-810-150S spectropolarimeter. All CD spectra are represented as mdeg vs. wavelength (nm). The baseline of the solvent was taken and removed from all spectra shown. CD spectra were recorded at a concentration corresponding to an optical density of approximately 1.0. One O.D. unit is the amount of oligonucleotide which, when dissolved in water (1 mL), results in an absorbance reading of 1.0 at 260 nm in a 1 cm quartz cuvette (O.D. = optical density).

6.6 General Experimental Procedures for Chapter 2

6.6.1 Procedure 1: Condensation Reaction With $\alpha$-Amino Esters$^3$

To a mixture of the relevant $\alpha$-amino ester hydrochloride salt (1.4 eq.) and 4-nitro-1,8-naphthalic anhydride (1 eq.) in anhydrous toluene, was added triethylamine (2 eq.). The reaction mixture was refluxed for 24 h. The solution was then filtered while hot through celite. Subsequently, the solvent was evaporated under reduced pressure. The resulting residue was dissolved in CH$_2$Cl$_2$ and washed twice with 0.1 M HCl and once with water. The organic layer was dried over MgSO$_4$, filtered and evaporated to dryness. Purification was carried out, if necessary, by recrystallisation.

6.6.2 Procedure 2: Hydrolysis of $t$-Butyl-1,8-Naphthalimide Esters$^3$

A solution of the tert-butyl ester in trifluoroacetic acid (TFA) was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and co-evaporated several times with CH$_2$Cl$_2$ to remove all traces of TFA. The resulting product was dried further under high vacuum. Further purification was not necessary.

6.6.3 Procedure 3: Peptide Bond Formation via coupling reaction$^3$

To a solution of the 1,8-naphthalimide containing $\alpha$-amino acid (1 eq.) in either dry THF or CH$_2$Cl$_2$ was added HOBT (1.05 eq.) and aminoaldehyde diethylacetal (1 eq.) or the aminomethylthiazolecarboxylate 104 (1 eq.). The stirring solution was cooled in an ice-bath for 15 min. EDCI.HCl (1.1 eq.) and triethylamine (1.1 eq.) were then added and the reaction mixture was left stirring at room temperature for 48 h. The solvent was evaporated under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ The organic layer was washed twice with sat. NaHCO$_3$, once with H$_2$O and then once with brine. The organic
layer was dried over MgSO$_4$, filtered and evaporated to dryness. Purification was carried out by either recrystallisation or by column chromatography using flash silica, depending on the individual compound.

6.6.4 Procedure 4: Acetal Hydrolysis$^3$

A solution of the relevant acetal in a mixture of dry TFA and dry CHCl$_2$ (1:1) was stirred at 0 $^\circ$C for 90 min and for a further 4 h at room temperature. The solvent was evaporated under reduced pressure and co-evaporated several times with CH$_2$Cl$_2$ to remove all traces of TFA. Further purification was not necessary.

6.6.5 Procedure 5: Horner-Wadsworth-Emmons Reaction

Triethyl phosphonoacetate (1.0 eq.) in freshly dried THF was cooled to 0 $^\circ$C, under a dry argon atmosphere and n-BuLi (1.6 M solution in hexanes) was added dropwise and the resulting solution stirred for 10 min. The flask was then further cooled to $-78$ $^\circ$C and the mono-peptide 4-nitro-1,8-naphthlaimide aldehyde in dry THF was added in one portion and the mixture stirred for 2 h at $-78$ $^\circ$C. Water (10 ml) was added and the THF removed in vacuo. The residue was extracted into CH$_2$Cl$_2$ (2 $\times$ 30 ml), washed with brine (1 $\times$ 20 ml), dried over Na$_2$SO$_4$ and the solvent removed to leave a crude product, which was precipitated (5 times), from ethyl acetate/hexane.

6.6.6 Procedure 6: Reduction Reaction via Hydrogenation

The relevant 4-nitro-1,8-naphthalimide (1 eq) in MeOH was hydrogenated at 3 atm pressure, in the pressure of 10 % Pd/C catalyst (0.2 eq) for 24 h. The reaction mixture was filtered through celite and washed with MeOH. The filtrate and washings were evaporated under reduced pressure.

$N$-[(IS)-tert-Butoxycarbonyl-3-methylbutyl]-4-nitro-1,8-naphthalimide (72)$^{3a}$

72 was synthesised by refluxing $S$-leucine tert-butyl ester hydrochloride (3.2 g, 14.39 mmol, 1.4 eq) with 4-nitro-1,8-naphthalic anhydride (2.5 g, 10.28 mmol, 1 eq.) and Et$_3$N (2.08 g, 2.84 ml, 2 eq.) according to Procedure 1 in anhydrous toluene (120 ml) to yield the product as a light yellow solid (3.23 g, 76 %) after recrystallisation from MeOH. m.p. 125 - 127 $^\circ$C (ref.,$^{3a}$ 125 - 127 $^\circ$C); $\delta_H$ (400 MHz, CDCl$_3$), 8.86 (1H, d, $J = 8.5$ Hz).
Hz, Ar-H7), 8.75 (1H, d, J = 7.0 Hz, Ar-H5), 8.71 (1H, d, J = 8.0 Hz, Ar-H2), 8.42 (1H, d, J = 8.0 Hz, Ar-H3), 8.01 (1H, t, J = 8.0 Hz, Ar-H6), 5.67 (1H, dd, J = 9.0, 5.0 Hz, CH), 2.23 - 2.05 (2H, m, CHCH2CH), 1.55 (1H, m, CH(CH3)2), 1.43 (9H, s, (CH3)3), 1.01 (3H, d, J = 6.5 Hz, CH3), 0.96 (3H, d, J = 7.0 Hz, CH3); δc (100 MHz, CDCl3), 168.2, 162.0, 161.2, 150.0, 132.4, 129.6, 129.5, 129.1, 128.5, 126.0, 123.4, 123.0, 121.2, 80.5, 53.2, 35.3, 28.2, 25.2, 22.4, 20.6; m/z: 413 (M + H)+.

**N-[(1S)-tert-Butoxycarbonyl-2-ethylphenyl]-4-nitro-1,8-naphthalimide (73)**

73 was synthesised by refluxing S-phenylalanine tert-butyl ester hydrochloride (2.23 g, 8.64 mmol, 1.4 eq.), with 4-nitro-1,8-naphthalic anhydride (1.5 g, 6.17 mmol, 1 eq.) and Et3N (1.3 g, 1.8 ml, 2 eq.) according to Procedure 1 in toluene (100 ml) to yield the product as a yellow solid (2.3 g, 84%). No further purification was necessary. m.p. 137 - 139 °C (ref.,3b 137 - 139 °C); δH (400 MHz, CDCl3), 8.83 (1H, d, J = 8.5 Hz, Ar-H7), 8.67 (1H, d, J = 7.5 Hz, Ar-H5), 8.61 (1H, d, J = 8.0 Hz, Ar-H2), 8.38 (1H, d, J = 8.0 Hz, Ar-H3), 7.98 (1H, t, J = 7.5 Hz, Ar-H6), 7.20 - 7.11 (5H, m, C6H5), 5.94 (1H, dd, J = 10.5, 5.5 Hz, CH), 3.68 (2H, m, β-CH2), 1.47 (9H, s, (CH3)3); δc (100 MHz, CDCl3), 167.6, 162.3, 161.5, 149.2, 136.9, 132.2, 129.5, 129.1, 128.5, 127.9, 127.8, 126.1, 126.0, 124.8, 123.4 123.2, 122.0, 82.0, 55.1, 34.4, 27.5; m/z: 469 (M + Na)+.

**N-[(1R)-tert-Butoxycarbonyl-2-ethylphenyl]-4-nitro-1,8-naphthalimide (74)**

74 was synthesised by refluxing R-phenylalanine tert-butyl ester hydrochloride (2.23 g, 8.64 mmol, 1.4 eq.) with 4-nitro-1,8-naphthalic anhydride (1.5 g, 6.17 mmol, 1 eq.) and Et3N (1.3 g, 1.8 ml, 2 eq.) according to Procedure 1 in toluene (100 ml) to yield the product as a caramel yellow solid (1.65 g, 64%). No further purification was necessary. m.p. 137 - 139 °C; HRMS: 469.1386 ([M + Na]+. C25H22N2O6Na requires 469.1376); vmax (KBr)/cm−1 3066, 3028, 2978, 1738, 1530, 1455, 1346, 1275; 1H, 13C and m/z as seen above for its enantiomer, N-[(1S)-tert-Butoxycarbonyl-2-ethylphenyl]-4-nitro-1,8-naphthalimide, 73.
Chapter 6: Experimental

\[ \text{N-[(IS)-tert-Butoxycarbonyl-ethyl]-4-nitro-1,8-naphthalimide (75)} ^{3a} \]

75 was synthesised by refluxing \(R\)-alanine tert-butyl ester hydrochloride (1.57 g, 8.64 mmol, 1.4 eq.) with 4-nitro-1,8-naphthalic anhydride (1.5 g, 6.17 mmol, 1 eq.) and triethylamine (1.20 g, 1.7 ml, 2 eq.) according to Procedure 1 in toluene (100 ml) to yield the product as a pale brown solid (1.5 g, 66%). No further purification was necessary. m.p. 161 - 163 °C (ref.,\(^{3a}\) 161 - 163 °C); \(\delta_H\) (400 MHz, CDCl\(_3\)), 8.78 (1H, d, \(J = 8.5\) Hz, Ar-H7), 8.76 (1H, d, \(J = 7.5\) Hz, Ar-H5), 8.72 (1H, d, \(J = 8.0\) Hz, Ar-H2), 8.43 (1H, d, \(J = 8.0\) Hz, Ar-H3), 8.03 (1H, dd, \(J = 8.5, 7.0\) Hz, Ar-H6), 5.66 (1H, q, \(J = 7.0\) Hz, CH), 1.68 (3H, d, \(J = 7.0\) Hz, CH\(_3\)), 1.46 (9H, s, (CH\(_3\))\(_3\)); \(\delta_c\) (100 MHz, CDCl\(_3\)), 168.3, 162.2, 161.5, 149.5, 134.9, 129.3, 129.1, 129.0, 128.5, 126.3, 123.8, 123.4 122.2, 52.0, 22.0, 15.1; \(m/z\): 763 (2M + Na)^+.

\[ \text{N-[(IS)-Carboxy-3-methylbutyl]-4-nitro-1,8-naphthalimide (76)} ^{3a} \]

76 was prepared using 72 (1.35 g, 3.26 mmol) and TFA (6 ml) according to Procedure 2, to yield the product as a sticky yellow solid (1.10 g, 99%). m.p 139 - 141 °C (ref.,\(^{3a}\) 138 - 140 °C); \(\delta_H\) (400 MHz, CDCl\(_3\)), 8.87 (1H, d, \(J = 8.5\) Hz, Ar-H7), 8.76 (1H, d, \(J = 7.5\) Hz, Ar-H5), 8.72 (1H, d, \(J = 8.0\) Hz, Ar-H2), 8.43 (1H, d, \(J = 8.0\) Hz, Ar-H3), 8.03 (1H, dd, \(J = 9.0, 5.0\) Hz, CH\(_2\)), 2.27 - 2.08 (2H, m, CH\(_2\)CH\(_2\)), 1.55 (1H, m, CH(CH\(_3\))\(_2\)), 1.01 (3H, d, \(J = 6.5\) Hz, CH\(_3\)), 0.94 (3H, d, \(J = 6.5\) Hz, CH\(_3\)); \(\delta_c\) (100 MHz, CDCl\(_3\)), 174.5, 162.1, 161.2, 150.5, 132.7, 129.7, 129.2, 129.1, 128.5, 126.0, 123.6, 123.0, 121.8, 52.2, 35.6, 23.8, 22.4, 20.6; \(m/z\): 357 (M + H)^+.

\[ \text{N-[(IS)-Carboxy-2-ethylphenyl]-4-nitro-1,8-naphthalimide (77)} ^{3a} \]

77 was prepared using 73 (0.9 g, 2.02 mmol), TFA (4 ml) and DCM (4 ml) according to Procedure 2, to yield the product as a yellow solid (0.78 g, 97%). m.p 132 - 134 °C (ref.,\(^{3a}\) 131 - 133 °C); \(\delta_H\) (400 MHz, CDCl\(_3\)), 8.83 (1H, d, \(J = 8.5\) Hz, Ar-H7), 8.67 (1H, d, \(J = 7.5\) Hz, Ar-H5), 8.62 (1H, d, \(J = 8.0\) Hz, Ar-H2), 8.38 (1H, d, \(J = 8.0\) Hz, Ar-H3), 7.98 (1H, t, \(J = 7.5\) Hz, Ar-H6), 7.19 - 7.09 (5H, m, C\(_6\)H\(_5\)), 6.09 (1H, dd, \(J = 10.5, 5.5\) Hz, CH), 3.70 (1H, m, \(J = 14.0, 5.5\) Hz, \(\beta\)-CH\(_2\)); \(\delta_c\) (100 MHz, CDCl\(_3\)), 172.6, 162.3, 161.5, 149.2,
136.9, 132.7, 129.4, 129.2, 128.9, 128.7, 127.8, 127.1, 126.0, 124.8, 123.4 123.2, 122.0, 121.5, 55.1, 34.4; m/z: 413 (M + Na)^+

**N-[(IR)-Carboxy-2-ethylphenyl]-4-nitro-1,8-naphthalimide (78)**

78 was prepared using 74 (1.65 g, 3.69 mmol), TFA (6 ml) and DCM (6 ml) according to Procedure 2, to yield the product as a yellow solid (1.42 g, 98%). m.p. 132 - 134 °C (ref., 3a 132 - 134 °C); HRMS: 413.0745 ([M + Na]^+); C_{21}H_{14}N_{2}O_{6}Na requires 413.0750; ^1H, ^13C and m/z as seen above for its enantiomer, N-[(IS)-Carboxy-2-ethylphenyl]-4-nitro-1,8-naphthalimide, 77.

**N-[(IS)-Carboxyl-ethyl]-4-nitro-1,8-naphthalimide (79)**

79 was prepared using 75 (1.41 g, 3.79 mmol), and TFA (6 ml) according to Procedure 2, to yield the product as a creamy/brown solid (1.19 g, 99%). m.p. 196 - 198 °C (ref., 3a 196 - 198 °C); δ_H (400 MHz, d_{6}-DMSO), 8.78 (1H, d, J = 8.5 Hz, Ar-H7), 8.68 (2H, m, Ar-H2, Ar-H3), 8.76 (1H, d, J = 7.5 Hz, Ar-H5), 8.10 (1H, dd, J = 7.5, 8.5 Hz, Ar-H6), 5.57 (1H, q, J = 5.0 Hz, CH), 1.63 (3H, d, J = 5.0 Hz, CH3); δ_C (100 MHz, d_{6}-DMSO), 171.3, 162.2, 161.5, 149.5, 132.9, 129.9, 129.8, 129.0, 128.5, 126.3, 124.8, 123.0 122.5, 50.0, 15.7; m/z: 315 (M + Na)^+

**N-[(IS)-(2,2-Diethoxy-ethyl)carboxamido-3-methylbutyl]-4-nitro-1,8-naphthalimide (80)**

80 was synthesised using 76 (1.24 g, 3.48 mmol, 1 eq.), aminoaldehyde diethyl acetal (0.46 g, 0.5 ml, 3.48 mmol, 1 eq.), HOBt (0.63 g, 4.69 mmol, 1.35 eq.), EDCI.HCl (0.73g, 3.83 mmol, 1.1 eq.) and Et$_3$N (0.38 g, 0.55 ml, 3.83 mmol, 1.1 eq.) according to Procedure 3 and was isolated as a yellow solid (1.24 g, 76 %). No further purification was necessary. m.p. 105 - 107 °C (ref., 3b 106 - 108 °C); δ_H (400 MHz, CDCl$_3$), 8.876 (1H, d, J = 8.5 Hz, Ar-H7), 8.74 (1H, d, J = 7.0 Hz, Ar-H5), 8.70 (1H, d, J = 7.5 Hz, Ar-H2), 8.43 (1H, d, J = 8.0 Hz, Ar-H3), 8.01 (1H, t, J = 8.0 Hz, Ar-H6), 6.10 (1H, br t, NH), 5.75 (1H, dd, J = 9.5, 5.0 Hz, CHCH$_2$),
4.55 (1H, t, J = 5.5 Hz, CH(OCH₂CH₃)₂), 3.75 - 3.62 (2H, m, bCH₂CH₃), 3.71 - 3.53 (2H, m, aCH₂CH₃), 3.49 - 3.42 (2H, m, CH₂NH), 2.34 - 1.98 (2H, m, CHCH₂CH), 1.55 (1H, m, CH(CH₃)₂), 1.24 (3H, t, J = 7.0 Hz, bCH₂CH₂), 1.13 (3H, J = 7.0 Hz, aCH₂CH₂), 1.01 (3H, d, J = 6.5 Hz, CH₃), 0.96 (3H, d, J = 7.0 Hz, CH₃); δₑ (100 MHz, CDCl₃), 195.4, 172.0, 168.9, 150.2, 132.5, 131.8, 129.9, 129.8, 129.4, 129.3, 123.7, 122.5, 101.5, 63.0, 62.8, 55.4, 42.0, 37.5, 25.7, 23.6, 21.5, 14.9, 14.6; m/z: 965 (2M + Na)⁺.

**N-[(IS)-(2,2-Diethoxy-ethyl)carboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide (81)**

81 was synthesised using 77 (0.81 g, 2.08 mmol, 1 eq.), aminoaldehyde diethyl acetal (0.27 g, 0.30 ml, 2.08 mmol, 1 eq.), HOBt (0.38 g, 2.81 mmol, 1.35 eq.), EDCl.HCl (0.44 g, 2.29 mmol, 1.1 eq.) and Et₃N (0.23 g, 0.33 ml, 2.29 mmol, 1.1 eq.) according to Procedure 3. After purification by column chromatography on flash silica (CH₂Cl₂/MeOH, 12:2) the product was obtained as a golden yellow solid (0.54 g, 51 %). m.p. 107 - 109 °C (ref. 3b 108 - 110 °C); δₑ (400 MHz, CDCl₃), 8.84 (1H, d, J = 8.5 Hz, Ar-H7), 8.67 (1H, d, J = 6.5 Hz, Ar-H5), 8.62 (1H, d, J = 8.0 Hz, Ar-H2), 8.39 (1H, d, J = 8.0 Hz, Ar-H3), 7.97 (1H, t, J = 7.5 Hz, Ar-H6), 7.22 - 7.08 (5H, m, C₆H₅), 6.11 (1H, br t, NH), 6.02 (1H, dd, J = 9.0, 6.5 Hz, CHCH₂(C₆H₅)), 4.55 (1H, t, J = 5.5 Hz, CH(OCH₂CH₃)₂), 3.74 - 3.61 (2H, m, bCH₂CH₃), 3.70 - 3.52 (2H, m, aCH₂CH₃), 3.49 - 3.42 (2H, m, CH₂NH), 3.75 - 3.43 (2H, m, CH₂(C₆H₅)), 1.22 (3H, t, J = 7.0 Hz, bCH₂CH₂), 1.08 (3H, t, J = 7.0 Hz, aCH₂CH₂); δₑ (100 MHz, CDCl₃), 168.1, 162.6, 161.8, 149.2, 136.3, 132.3, 129.7, 129.5, 129.1, 128.6, 128.5, 128.2, 126.5, 125.9, 123.4, 123.1, 122.0, 100.2, 62.7, 62.6, 55.5, 41.2, 34.2, 14.8; m/z: 528 (M + Na)⁺.
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*N-[(1R)-(2,2-Diethoxy-ethyl)carboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide (82)*

82 was synthesised using 78 (1.45 g, 3.714 mmol, 1 eq.), aminoaldehyde diethyl acetal (0.49 g, 0.54 ml, 3.714 mmol, 1 eq.), HOBt (0.68 g, 5.014 mmol, 1.35 eq.), EDCI.HCl (0.78g, 4.085 mmol, 1.1 eq.) and Et3N (0.42 g, 0.59 ml, 2.29 mmol, 1.1 eq.) according to Procedure 3. After purification by column chromatography on flash silica (CH2Cl2/MeOH, 12:2) 82 was obtained as a golden yellow solid (1.13 g, 60 %). m.p. 107 - 109 °C; HRMS: 528.17 ([M + Na]⁺. C27H27N3O7Na requires 528.18); νmax (KBr)/cm⁻¹ 3332, 3073, 2953, 2360, 1671, 1584, 1530, 1455, 1426, 1416, 1371, 1343, 1239, 1060; ¹H, ¹³C and m/z as seen above for its enantiomer, *N-[(1S)-(2-diethoxy-ethylcarboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide, 81.*

**N-[(1S)-(2,2-Diethoxy-ethyl)carboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide (83)**

83 was synthesised using 79 (1.18 g, 3.755 mmol, 1 eq.), aminoaldehyde diethyl acetal (0.49 g, 0.55 ml, 3.755 mmol, 1 eq.), HOBt (0.69 g, 5.07 mmol, 1.35 eq.), EDCI.HCl (0.79g, 4.13 mmol, 1.1 eq.) and Et3N (0.34 g, 0.49 ml, 4.13 mmol, 1.1 eq.) according to Procedure 3. After purification by column chromatography on flash silica (CH2Cl2/MeOH, 12:2) 83 was obtained as a yellow solid (1.025 g, 64 %). m.p. 99 - 101 °C; HRMS: 452.1427 ([M + Na]⁺. C21H23N3O7Na requires 452.1434); δH (400 MHz, CDC13), 8.84 (1H, d, J = 8.5 Hz, Ar-H7), 8.72 (1H, d, J = 7.5 Hz, Ar-H5), 8.68 (1H, d, J = 8.0 Hz, Ar-H2), 8.40 (1H, d, J = 8.0 Hz, Ar-H3), 7.99 (1H, t, J = 7.5 Hz, Ar-H6), 6.13 (1H, br t, NH), 5.72 (1H, dd, J = 14.0, 7.0 Hz, CHCH₂), 4.56 (1H, t, J = 5.5 Hz, CH(OCH₂CH₃)₂), 3.75 - 3.62 (2H, m, bCH₂CH₃), 3.71 - 3.53 (2H, m, aCH₂CH₃), 3.49 - 3.42 (2H, m, CH₂NH), 1.74 (3H, d, J = 7.0 Hz, NCHCH₃), 1.23 (3H, t, J = 7.0 Hz, bCH₂CH₃), 1.15 (3H, t, J = 7.0 Hz, aCH₂CH₃); δc (100 MHz, CDC13), 169.28, 162.1, 149.64, 132.74, 130.11, 129.95, 129.56, 129.17, 126.83, 123.90, 123.60, 122.85, 100.78, 63.07, 50.57, 42.34, 15.38, 15.29, 14.51; m/z: 452 (M + Na)⁺; νmax (KBr)/cm⁻¹ 3372, 3075, 2976, 1710, 1670, 1426, 1191, 870.

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\[ N-\{(IS)-(2-Oxo-ethyl)carboxamido-3-methylbutyl\}-4-nitro-1,8-naphthalimide (84) \]  

84 was prepared using 76 (0.79 g, 1.67 mmol), TFA (10 ml) and CHCl\(_3\) (15ml) according to Procedure 4 to yield the product as a yellow solid (0.60 g, 90 %). m.p. 160 - 162 °C (ref.,\(^{3b}\) 160 - 162 °C); \(\delta_H\) (400 MHz, CDCl\(_3\)), 9.72 (1H, s, CHO), 8.87 (1H, d, \(J = 8.5\) Hz, Ar-H7), 8.74 (1H, d, \(J = 7.5\) Hz, Ar-H5), 8.70 (1H, d, \(J = 8.0\) Hz, Ar-H2), 8.42 (1H, d, \(J = 8.0\) Hz, Ar-H3), 8.01 (1H, t, \(J = 8.5\) Hz, Ar-H6), 6.99 (1H, br s, NH), 5.84 (1H, dd, \(J = 10.0, 5.0\) Hz, CHCH\(_2\)), 4.34 (2H, s, NHCH\(_2\)CHO), 2.39 - 1.98 (2H, m, CHCH\(_2\)CH), 1.54 (1H, m, \(CH(CH_3)_2\)), 1.01 (3H, d, \(J = 6.5\) Hz, CH\(_3\)), 0.96 (3H, d, \(J = 6.5\) Hz, CH\(_3\)); \(\delta_c\) (100 MHz, CDCl\(_3\)), 195.69, 170.11, 162.93, 162.14, 149.22, 132.49, 129.93, 129.51, 129.23, 128.72, 126.02, 123.45, 123.11, 122.01, 52.99, 50.10, 36.93, 25.01, 22.84, 21.46; \(m/z\): 420 (M + Na). 

\[ N-\{(IS)-(2-Oxo-ethyl)carboxamido-2-ethylphenyl\}-4-nitro-1,8-naphthalimide (85) \]  

85 was prepared using 81 (0.44g, 0.87 mmol), TFA (10 ml) and CHCl\(_3\) (15 ml) according to Procedure 4 to yield the product as a yellow solid (0.35 g, 94 %). m.p. 146 - 148 °C (ref.,\(^{3b}\) 146 - 148 °C); \(\delta_H\) (400 MHz, CDCl\(_3\)), 9.68 (1H, s, CHO), 8.84 (1H, d, \(J = 8.5\) Hz, Ar-H7), 8.66 (1H, d, \(J = 7.0\) Hz, Ar-H5), 8.62 (1H, d, \(J = 8.0\) Hz, Ar-H2), 8.42 (1H, d, \(J = 8.0\) Hz, Ar-H3), 7.98 (1H, t, \(J = 7.5\) Hz, Ar-H6), 7.25 - 7.13 (5H, m, C\(_6\)H\(_5\)), 6.77 (1H, br s, NH), 6.10 (1H, dd, \(J = 9.0, 6.5\) Hz, NCHCH\(_2\)), 4.33 (2H, d, \(J = 4.8\) Hz, NHCH\(_2\)CHO), 3.73 (1H, m, \(\beta\)-CH\(_2\)); \(\delta_c\) (100 MHz, CDCl\(_3\)), 195.8, 168.3, 162.9, 161.5, 149.1, 136.5, 132.7, 129.4, 129.2, 128.8, 128.4, 128.3, 128.1, 126.6, 125.4, 123.2, 123.0, 121.6, 58.3, 50.0, 34.4 \(m/z\): 432 (M + H).
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N-[(IR)-(2-Oxo-ethyl)carboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide (86)\(^{3b}\)

86 was prepared using 82 (1.03 g, 2.04 mmol), TFA (10 ml) and CHCl\(_3\) (15 ml) according to Procedure 4 to yield the product as a dark yellow solid (0.85 g, 96 %). m.p 146 - 148 °C; HRMS: 432.1199 ([M + Na]\(^+\). C\(_{23}\)H\(_{18}\)N\(_3\)O\(_6\)Na requires 432.1196); \(^1\)H, \(^{13}\)C and m/z as seen above for its enantiomer N-[(IS)-(2-Oxo-ethyl)carboxamido-2-ethylphenyl]-4-nitro-1,8-naphthalimide, 85.

N-[(IS)-(2-Oxo-ethyl)carboxamido-2-ethyl]-4-nitro-1,8-naphthalimide (87)

87 was prepared using 83 (200 mg, 0.46 mmol), TFA (5 ml) and CHCl\(_3\) (10 ml) according to Procedure 4 to yield the product as a beige solid (0.16 g, 99 %). m.p 159 - 160 °C; HRMS: 378.3159 ([M + Na]\(^+\). C\(_{17}\)H\(_{13}\)N\(_3\)O\(_6\)Na requires 378.3156); \(^1\)H (400 MHz, CDCl\(_3\)), 9.74 (1H, s, CHO), 8.85 (1H, d, J = 8.5 Hz, Ar-H7), 8.75 (1H, d, J = 8.0 Hz, Ar-H5), 8.72 (1H, d, J = 8.0 Hz, Ar-H2), 8.43 (1H, d, J = 8.0 Hz, Ar-H3), 8.01(1H, d, J = 8.0 Hz, Ar-H6), 6.83 (1H, br s, NH), 5.81 (1H, dd, J = 8.0, 6.5 Hz, NCH\(_3\)), 4.43 (2H, s, NCH\(_2\)CHO), 1.81 (1H, d, J = 5.0, CH\(_3\)); \(^13\)C (100 MHz, CDCl\(_3\)), 195.0, 168.1, 161.5, 160.1, 145.01, 132.49, 129.93, 129.51, 128.72, 52.99, 53.10, 36.93, 25.01, 22.84, m/z: 378 (M + Na\(^+\). v\(_{\text{max}}\) (KBr)/cm\(^{-1}\) 3310, 3005, 2900, 2560, 2670, 1450.

N-[(IS)-(but-2-enoic acid ethyl ester)carboxamido-3-methylbutyl]-4-nitro-1,8-naphthalimide (S,E) (88)

88 was synthesised by treating 84 (0.3 g, 0.755 mmol, 1 eq.) in THF (10 ml) with the ylide prepared by treating triethyl phosphonacetate (0.17 g, 0.15 ml, 0.755 mmol, 1 eq.), in THF (20 ml) with n-BuLi (0.33 g, 0.48 ml, 0.77 mmol, 1.02 eq.) according to Procedure 5 and was isolated as a shiny brown solid (0.12 g, 40 %). m.p 154 - 156 °C; HRMS: 490.1603 ([M + Na]\(^+\). C\(_{24}\)H\(_{22}\)N\(_3\)O\(_7\)Na requires 490.1590); \(^1\)H (400 MHz, CDCl\(_3\)), 8.85 (1H, d, J = 8.5 Hz, Ar-H7), 8.75 (1H, d, J = 7.0 Hz, Ar-H5), 8.71 (1H, d, J = 8.0 Hz, Ar-H2), 8.42 (1H, d, J = 8.0 Hz, Ar-H3), 8.01 (1H, t, J = 8.5 Hz, Ar-H6), 6.87 (1H,
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dt, J = 16.0, 5.0 Hz, CH$_2$CH$^b$CH), 6.18 (1H, br s, NH), 6.0 (1H, d, J = 16.0 Hz, CH$^b$CH$^f$), 5.78 (1H, dd, J = 10.5, 5.0 Hz, NCHCH$_2$), 4.20 (2H, q, J = 6.8 Hz, CH$_2$$^d$CH$_3$), 4.11 (2H, m, NHCH$_2$$^a$), 2.37 - 1.97 (2H, m, CHCH$_2$CH), 1.40 (1H, m, CH(CH$_3$)$_2$), 1.30 (3H, t, J = 7.0 Hz, CH$_2$CH$_3$$^d$), 1.01 (3H, d, J = 6.5 Hz, CHCH$_3$), 0.95 (3H, d, J = 6.5 Hz, CHCH$_3$); $\delta_c$ (100 MHz, CDCl$_3$), 169.0, 165.6, 162.9, 162.2, 149.3, 143.1, 132.5, 129.9, 129.5, 129.1, 128.8, 126.2, 123.5, 123.2, 122.2, 121.6, 60.1, 53.3, 40.1, 37.0, 25.2, 22.9, 21.5, 13.8; m/z: 490 (M + Na)$^+$; $\nu$ max (KBr)/cm$^{-1}$ 3327, 3076, 2958, 2360, 1670, 1410, 975.

$N$-[($I$S)-($\text{but-2-enoic~acid~ethyl~ester}$)carboxamido-3-ethylphenyl]-4-nitro-1,8-naphthalimide ($S,E$) (89)

![Structure of compound 89](image)

89 was synthesised by treating 85 (0.25 g, 0.579 mmol, 1 eq.) in THF (10 ml) with the ylide prepared by treating triethyl phosphonacetate (0.14 g, 0.12 ml, 0.579 mmol, 1 eq.), in THF (20 ml) with n-BuLi (0.25 g, 0.36 ml, 0.770 mmol, 1.02 eq.) according to Procedure 5 and was isolated as a light brown solid (0.13 g, 53 %). m.p 166 - 168 °C (ref.,$^{45b}$ 166 - 168 °C); HRMS: 524.5113 ([M + Na]$^+$). C$_{27}$H$_{32}$N$_3$O$_7$Na requires 524.1112; $\delta_H$ (400 MHz, CDCl$_3$), 8.78 (1H, d, J = 8.5 Hz, Ar-H7), 8.65 (1H, d, J = 7.0 Hz, Ar-H5), 8.59 (1H, d, J = 8.0 Hz, Ar-H2), 8.34 (1H, d, J = 8.0 Hz, Ar-H3), 7.94 (1H, t, J = 7.5 Hz, Ar-H6), 7.23 - 7.12 (5H, m, C$_6$H$_5$), 6.82 (1H, dt, J = 15.5, 5.0 Hz, CH$_2$CH$^b$CH), 6.20 (1H, br t, NH), 6.04 (1H, dd, J = 9.5, 6.5 Hz, NCHCH$_2$), 5.90 (1H, d, J = 16.0 Hz, CH$^b$CH$^f$), 4.16 (2H, q, J = 6.8 Hz, CH$_2$$^d$CH$_3$), 4.07 (2H, m, NHCH$_2$$^a$), 3.70 (1H, dd, J = 14.0, 6.5, Hz, CH$^b$CH$^f$), 3.57 (1H, dd, J = 14.0, 9.5 Hz, CH$^f$CH$^b$), 1.28 (3H, t, J = 7.0 Hz, CH$_2$CH$_3$$^d$); $\delta_c$ (100 MHz, CDCl$_3$), 168.2, 165.4, 162.7, 161.9, 149.2, 143.1, 136.1, 132.4, 129.8, 129.5, 129.2, 128.6, 128.5, 128.4, 128.3, 126.7, 125.9, 123.4, 123.0, 121.9, 121.4, 60.1, 55.5, 39.9, 34.1, 13.8; m/z: 524 (M + Na)$^+$. $\nu$ max (KBr)/cm$^{-1}$ 3362, 3084, 1711, 1626, 1410, 1031, 974.
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**N-[(IR)-(but-2-enoic acid ethyl ester)carboxamido-3-ethylphenyl]-4-nitro-1,8-naphthalimide (S,E) (90)**

90 was synthesised by treating 87 (0.30 g, 0.69 mmol, 1 eq.) in THF (10 ml) with the ylide prepared by treating triethyl phosphonacetate (0.16 g, 0.14 ml, 0.579 mmol, 1 eq.), in THF (20 ml) with n-BuLi (0.30 g, 0.44 ml, 0.77 mmol, 1.02 eq.) according to Procedure 5 and was isolated as a light brown solid (0.19 g, 55 %). m.p 166 - 168 °C; HRMS: 524.1437 ([M + Na]^+). C_{27}H_{23}N_{3}O_{7}Na requires 524.1434; \( \nu_{\text{max}} \) (KBr)/cm\(^{-1}\) 3386, 3082, 1703, 1430, 1173, 845; \(^1\)H, and \(^{13}\)C and \(m/\zeta\) as seen above for its enantiomer, \(N-[(S)-\text{but-2-enoic acid ethyl ester)carboxamido-3-ethylphenyl]-4-nitro-1,8-naphthalimide (S,E), 89.\)

**N-Phthaloyl-S-leucine (91)^202**

91 was synthesised by suspending S-Leucine (5.0 g, 38.12 mmol, 1 eq.) in toluene (125 ml) to which phthalic anhydride (5.93 g, 40.02 mmol, 1.05 eq.) and Et\(_3\)N (4.2 g, 6 ml, 42.0 mmol, 1.1 eq.) were added. The mixture was refluxed overnight, during which time the water evolved from the reaction was separated and collected by means of a Dean-Stark apparatus. The solution was then evaporated to dryness. The residue was dissolved in CH\(_2\)Cl\(_2\), washed with water, HCl (2 M) and brine. The organic phase was dried over Na\(_2\)SO\(_4\) and the solvent removed to leave the crude product as a white crystalline solid (6.87 g, 69 %), after recrystallisation from CH\(_2\)Cl\(_2\)/hexane. m.p. 117 - 118 °C; HRMS: 284.0896 ([M + Na]^+). C\(_{14}\)H\(_{15}\)NO\(_4\)Na requires 284.0899; \( \delta_{\text{H}} \) (400 MHz, CDCl\(_3\)), 7.89 - 7.81 (2H, m, Ar-H2, Ar-H5), 7.77 - 7.75 (2H, m, Ar-H3, Ar-H4), 5.02 (1H, dd, \( J = 11.5, 4.5 \) Hz, CH\(_2\)CHNPhth), 2.37 - 1.95 (2H, m, CH\(_2\)CH\(_2\)), 1.51 (1H, m, CH\(_3\)(CH)CH\(_2\)), 0.95 (3H, d, \( J = 6.4 \) Hz, CH\(_3\)(CH)CH\(_2\)); \( \delta_{\text{C}} \) (100 MHz, CDCl\(_3\)), 175.3, 167.3, 133.8, 131.2, 123.1, 49.9, 36.5, 24.6, 22.6, 20.5; \(m/\zeta\): 284 (M + Na)^+.
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2-(1,3-Dioxo-1,3-dihydro-isoinodol-2-yl)-4-methyl-pentanoic acid (2,2-diethoxy-ethyl)-amide (92)

92 was synthesised using 91 (1.20 g, 4.59 mmol, 1 eq.), aminoaldehyde diethyl acetal (0.61 g, 0.67 ml, 4.59 mmol, 1 eq.), HOBT (0.84 g, 6.19 mmol, 1.35 eq.), EDCI.HCl (0.97 g, 5.04 mmol, 1.1 eq.) and Et3N (0.51 g, 0.73 ml, 3.83 mmol, 1.1 eq.) according to Procedure 3 and was isolated as a creamy yellow semi-solid (1.03 g, 60 %). No further purification was necessary. m.p 104-106 °C; HRMS: 399.1887 ([M + Na]+; C29H28N2O5Na requires 399.1896); δH (400 MHz, CDCl3), 7.89 - 7.87 (2H, m, Ar-H2, Ar-H5), 7.78 - 7.76 (2H, m, Ar-H3, Ar-H4), 6.35 (1H, br s, NH), 4.90 (1H, dd, J = 11.5, 5.0 Hz, CH2CH2NPhth), 4.5 (1H, t, J = 5.5 Hz, CH(OCH2CH3)2), 3.67 - 3.50 (2H, m, CH2CH2), 3.54 - 3.46 (2H, m, aCH2CH3), 3.44 - 3.34 (2H, m, NHC=CH), 2.37 - 1.85 (2H, m, CHCH2CH), 1.19 (3H, t, J = 7.0 Hz, bCH2), 0.96 (3H, t, J = 7.0 Hz, CH3); δc (100 MHz, CDCl3), 168.9, 167.6, 133.8, 131.1, 123.0, 100.1, 62.5, 52.4, 41.7, 36.8, 24.7, 22.6, 20.7, 14.8; m/z: 399 (M + Na)+; νmax (NuJol)/cm⁻¹ 1461, 1376, 439.

2-(1,3-Dioxo-1,3-dihydro-isoinodol-2-yl)-4-methyl-pentanoic acid (2,2-oxy-ethyl)-amide (93)

93 was prepared using 92 (0.50 g, 1.33 mmol), TFA (5 ml) and dry CHCl3 (10ml) according to Procedure 4 to yield 93 as a caramel oil (0.45 g, 91 %). m.p 142 - 148 °C; HRMS: 325.3321 ([M + Na]+; C18H18N2O4Na requires 325.3311); δH (400 MHz, CDCl3), 9.66 (1H, s, CHO), 7.91 - 7.87 (2H, m, Ar-H2, Ar-H5), 7.80 - 7.76 (2H, m, Ar-H3, Ar-H4), 5.04 (1H, br s, NH), 4.90 (1H, dd, J = 11.5, 5.0 Hz, CH2CH2NPhth), 4.28 (2H, s, NHCH2CHO), 2.45 - 1.84 (2H, m, CHCH2CH), 1.50 (1H, m, CH(CH3)2), 0.96 (3H, t, J = 7.0 Hz, CH3), 0.95 (3H, t, J = 7.0 Hz, CH3); δc (100 MHz, CDCl3), 193.9, 172.2, 169.4, 167.7, 133.7, 131.3, 123.3, 50.0, 45.5, 36.6, 22.7, 20.5; m/z: 325 (M + Na)+; νmax (KBr)/cm⁻¹ 3375, 2960, 2360, 1775, 1535, 1386, 1172, 960.
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*N*-Phthaloyl-methanal (99)

99 was prepared by stirring Phthalimidoacetaldelyde diethylacetal (26.0 g, 98 mmol) in a mixture of TFA (175 mL) and CHCl₃ (350 mL) in an ice/water bath, under an argon atmosphere, for 1 h. The reaction was stirred at room temperature for a further 5 h. The solvent was removed in *vacuo* and co-evaporated with CH₂Cl₂ several times, to remove the remaining traces of TFA. This yielded 99 as an off-white solid (18.78 g, 100 %). No purification was necessary. m.p 102 – 103 °C; Found: C, 63.23; H, 3.02; N, 6.58 %. C₁₀H₇NO₃ requires C, 63.49; H, 3.73; N, 7.40 %; δ₁H (400 MHz, CDCl₃), 9.67 (1H, s, CHO), 7.90 & 7.78 (4H, AA’BB’ system, Phth), 4.58 (2H, s, CH₂); δc (100 MHz, CDCl₃), 193.2 (CH), 167.1, 133.9, 131.4, 123.2, 46.9; m/z: 190 (M + H)⁺; νmax (KBr)/cm⁻¹ 3100, 2965, 2850.

*N*-Phthaloyl-methyl-2(7R)-thiazolidine-4(R)-carboxylate (100)

100 was prepared by adding a solution of L-cysteine methyl ester hydrochloride (32.47, 189.0 mmol, 2 eq) in H₂O (250 mL) to the aldehyde 99 (18.0 g, 94.6 mmol, 1 eq) suspended in EtOH (500 mL). KHCO₃ (18.9 g, 189.0 mmol, 2 eq) was then added and the resulting mixture was stirred under argon at r.t for 5 h with thiazolidine 100 gradually precipitating from the reaction medium. The reaction was allowed to sit overnight at 0 °C. The crude product 2 (27.0 g, 93 % yield) was collected by suction filtration and washed several times with EtOH. Recrystallisation from MeOH yielded 100 as a white crystalline fibrous solid (22.7 g, 79 %). m.p 81 – 82 °C; Found: C, 54.08; H, 4.23; N, 8.79 %. C₁₄H₁₄N₂O₄S requires C, 54.89; H, 4.61; N, 9.14 %; HRMS: 329.0562 ([M + Na]⁺. C₁₄H₁₄N₂O₄NaS requires 329.0572); δ₁H (400 MHz, CDCl₃), 7.87 & 7.74 (4H, AA’BB’ system, Phth), 4.86 (1H, dd, J = 8.5 & 4.0 Hz, Hz), 4.14 (1H, dd, J = 14.5, 4.0 Hz, Ha), 4.06 (1H, dd, J = 14.0, 8.5 Hz, Ha’), 3.82 (1H, dd, J = 9.5, 7.6 Hz, Hx), 3.80 (3H, s, OCH₃), 3.33 (1H, dd, J = 1.0, 6.5 Hz, Hb), 2.92 (1H, dd, J = 10.0, 8.0 Hz, Hb’), 2.53 (1H, br s, NH); δc (100 MHz, CDCl₃), 170.9 (CO), 168.1 (CO), 134.1 (CH), 131.7 (CH), 123.5 (CH), 68.2 (CH), 65.5 (CH), 52.5 (CH₂), 41.1 (CH₂), 38.2 (CH₂); m/z: 329 (M + Na)⁺. νmax (KBr)/cm⁻¹ 3500, 3180, 2951, 2850, 1603.
**N-Phthaloyl-methyl-2(R)-thiazolidine-4(R)-carboxylic acid (101)**

101 was prepared by adding a solution of L-cysteine hydrochloride (7.32 g, 46 mmol, 2 eq) in H₂O (100 mL) to the aldehyde 99 (4.42 g, 94.6 mmol, 1 eq) suspended in EtOH (200 mL). KHCO₃ (4.6 g, 46 mmol, 2 eq) was then added and the resulting mixture was stirred under argon at r.t for 5 h with thiazolidine 101 gradually precipitating from the reaction medium. The reaction was allowed to sit overnight at 0 °C. The white precipitate was collected by suction filtration and washed several times with EtOH to give 101 as a white solid (5.09 g, 76 % yield). m.p 67 – 68 °C; Found: C, 53.21; H, 3.88; N, 8.25 %. C₁₃H₁₂N₂O₄S requires C, 53.42; H, 4.14; N, 9.58 %. δ_H (400 MHz, CD₃SO), 7.90 & 7.64 (4H, AA'B'B' system, Phth), 4.77 (1H, dd, J = 6.6 & 5.0 Hz, Hz), 4.10 (1H, dd, J = 14.5, 5.0 Hz, Ha), 3.89 (1H, dd, J = 14.5, 7.0 Hz, Ha'), 3.77 (1H, dd, J = 8.5, 7.0 Hz, Hx), 3.20 (1H, dd, J = 10.0, 7.0 Hz, Hb), 2.85 (1H, t, J = 9.5 Hz, Hb'); δ_c (100 MHz, CDCl₃), 170.9 (C =O), 167.0 (CO), 135.6 (CH), 134.2 (CH), 126.0 (CH), 67.5 (CH), 64.1 (CH), 50.1 (CH₂), 38.0 (CH₂); m/z: 315 (M + Na)^+. υ_max (KBr)/cm⁻¹ 3500, 3180, 2901, 1603, 1410.

**N-Phthaloyl-methyl-thiazole-4-carboxylate (102)**

102 was prepared by stirring 100 (22.50 g, 73.9 mmol, 1 eq), activated MnO₂ (224.9 g, 2580 mmol, 35 eq) and pyridine (6.60 g, 6.7 mL, 83.5 mmol, 1.13 eq) at reflux in dry CH₂Cl₂ under an argon atmosphere for 5 days. The reaction mixture was filtered hot through celite, washing several times with CH₂Cl₂. The filtrate and washings were evaporated to dryness and the white residue was re-dissolved in CH₂Cl₂ and washed three times with HCl (0.1 M) and once with H₂O. The organic layer was dried over MgSO₄, filtered and evaporated to dryness to give the product as a white solid (13.39 g, 63 % yield) after recrystallisation from MeOH. m.p 164 – 164 °C; Found: C, 54.99; H, 2.76; N, 8.68 %. C₁₄H₁₀N₂O₄S requires C, 55.62; H, 3.33; N, 9.27 %. HRMS: 325.0265 ([M + Na]^+). C₁₄H₁₀N₂O₄NaS requires 325.0259); δ_H (400 MHz, CDCl₃), 8.15 (1H, s, CH), 7.93 & 7.77 (4H, AA'B'B' system, Phth), 5.25 (2H, s, CH₂), 3.95 (3H, s, OCH₃); δ_c (100 MHz, CDCl₃), 166.7 (C = O), 165.6 (C = O), 161.1 (C), 146.3 (C), 134.4 (CH), 131.3 (C), 128.3 (CH), 123.7 (CH), 52.0 (CH₃), 38.7 (CH₂); m/z: 325 (M + Na)^+. υ_max (KBr)/cm⁻¹ 3260, 3150, 2701, 2650, 1589.
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*N-Phthaloyl-methyl-thiazole-4-carboxylic acid (103)*

103 was prepared by adding 102 (1.68 g, 5.5 mmol, 1 eq) to a mixture of acetone (28 mL), H₂O (17 mL) and concentrated HCl (8.5 mL). The suspension was stirred at reflux for 48 h. The reaction mixture was allowed to cool and any precipitate present was collected by suction filtration and washed with a little acetone. The filtrate and washings were evaporated under reduced pressure. The residue was dissolved in K₂CO₃ (1.2 M). The solution was filtered and brought to pH 1 by adding concentrated HCl. A small amount of EtOH was added and the mixture was gradually heated to dissolve the precipitate. On cooling the product separated and was collected by suction filtration, washed with a little EtOH and dried to yield 103 as fine colourless needles (0.87 g, 55 %). m.p 102 – 103 °C; HRMS: 311.0114 ([M + Na]⁺. C₁₃H₈N₂O₄NaS requires 311.0102); δH (400 MHz, (CD₃)₂SO), 8.42 (1H, s, CH), 7.95 & 7.90 (4H, AA’BB’ system, Phth), 5.12 (2H, s, CH₂); δc (100 MHz, CDCl₃), 16.1 (C = O), 165.3 (C = O), 161.3 (C), 146.6 (C), 134.8 (CH), 131.4 (C), 12.6 (CH), 123.5 (CH), 39.2 (CH₂); m/z: 311 (M + Na)⁺. v_max (KBr)/cm⁻¹: 3260, 3150, 2650, 1589, 1360.

**2-Aminomethyl-thiazole-4-carboxylate (104)**

104 was prepared by adding hydrazine monohydrate (3.9 g, 3.8 mL, 79.4 mmol, 2 eq) to a suspension of 102 (12 g, 39.7 mmol, 1 eq) in boiling EtOH (180 mL). The resulting solution was stirred at r.t for 4 h. A white precipitate was produced as the reaction progressed, indicating the formation of phthaylhydrazide side-product. The reaction was allowed sit at r.t overnight. The voluminous white precipitated phthaylhydrazide (6.24 g, 97 % yield) was collected by suction filtration and washed with a little EtOH. (¹H, ¹³C NMR and MS confirmed the structure to be C₈H₆O₂). The filtrate and washings were evaporated under reduced pressure. The white residue was re-dissolved in EtOH and any remaining precipitate was collected by suction filtration and washed with a little EtOH. The filtrate and washings were evaporated under reduced pressure. This procedure was repeated until an oil-like residue was obtained. The residue was then dissolved in H₂O and extracted three times with CHCl₂. The combined chloroform layers were washed three times with HCl (0.1 M) and the combined aqueous phases were extracted two times with CH₂Cl₂. The aqueous phase was brought to pH 12 by adding
NaOH (0.1 M) and extracted three times with CHCl₂. The solvent was removed in vacuo to give 104 as an off-white solid (3.6 g, 53 % yield). m.p 162 - 163 °C; HRMS: 195.0125 ([M + Na]⁺. C₆H₈N₂O₂NaS requires 195.0229); δ_H (400 MHz, CDCl₃), 8.13 (1H, s, CH), 4.22 (2H, s, CH₂), 3.94 (3H, s, OCH₃); δ_c (100 MHz, CDCl₃), 175.4 (CO), 161.5 (C), 146.3 (C), 128.3 (CH), 51.9 (CH₃), 43.52 (CH₂); m/z: 195 (M + Na)⁺. ν_max (KBr)/cm⁻¹ 2957, 2843, 2810, 1650.

**Ethyl 2-amino-4-methyl thiazole-5-carboxylate (110)**

110 was prepared by adding thiourea (2.3 g, 30.4 mmol, 1 eq.) to a solution of ethyl 2-chloroacetoacetate (5.0 g, 4.2 mL, 1 eq.) in EtOH. The mixture was stirred at reflux (78 °C) for 4 h. The reaction was allowed cool to r.t and the white precipitate was collected by suction filtration and washed with a little EtOH which yielded 110 as a white solid (4.20 g, 75 %). m.p 176 - 180 °C (ref.; 176 - 180 °C); δ_H (400 MHz, CDCl₃), 4.22 (2H, q, J = 7.0 Hz, CH₂), 2.45 (3H, s, CCH₃), 2.07 (2H, s, NH₂), 1.25 (3H, t, J = 7.0 Hz, CH₃); δ_c (100 MHz, CDCl₃), 168.7, 160.7, 148.9, 107.3, 61.1, 30.7, 14.2; m/z: 187 (M + H)⁺.

**N-[tert-Butoxycarbonylamino]-methyl-2-carboxyethoxy-4-thiazolidine (113)**

113 was synthesised using Boc-Glycine (102 mg, 0.58 mmol, 1 eq.), 104 (100 mg, 0.58 mmol, 1 eq.), HOBt (105 mg, 0.60 mmol, 1.35 eq.), EDCI.HCl (122 mg, 0.64 mmol, 1.1 eq.) and Et₃N (64 mg, 0.1 ml, 0.64 mmol, 1.1 eq.) according to Procedure 3 using THF (20 mL) as solvent and was yielded as a off-white hydroscopic solid (0.1 g, 77 %). m.p. 154 - 157 °C; HRMS: 352.1117 ([M + Na]⁺. C₃H₁₉N₃O₅NaS requires 533.1107); δ_H (400 MHz, CDCl₃), 8.13 (1H, s, CH), 7.48 (1H, br s, NHCH₂), 5.38 (1H, br s, NH), 4.78 (2H, d, J = 6.0 Hz, CONHCH₂), 3.94 (3H, s, OCH₃), 3.88 (2H, d, J = 6.0, CH₂), 1.42 (9H, s, CH₃ x 3); δ_c (100 MHz, CDCl₃), 169.6 (CO), 167.9 (CO), 161.2 (C), 145.8 (C), 128.0 (CH), 52.0 (CH₃), 43.7 (CH₂), 40.3 (CH₂), 28.3 (CH₃); m/z: 532 (M + Na)⁺. ν_max (KBr)/cm⁻¹ 3298, 3200, 2960, 2850, 2890, 2810.
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N-[9-Fluorenlymethoxycarbonylamino]-methyl-2-carboxyethoxy-4-thiaazolidine (114) was synthesised using Fmoc-L-Leucine (205 mg, 0.58 mmol, 1 eq.), HOBT (105 mg, 0.60 mmol, 1.35 eq.), EDCI.HCl (122 mg, 0.64 mmol, 1.1 eq.) and Et₃N (64 mg, 0.4 ml, 0.64 mmol, 1.1 eq.) according to Procedure 3 using THF (20 mL) as solvent and was yielded as an off-white fluffy solid (226 mg, 78 %). m.p. 185 - 187 °C; HRMS: 530.2531 ([M + Na]^+). C₂₇H₂₉N₃O₇Na requires 530.2532; δ_H (400 MHz, CDCl₃), 8.06 (1H, s, SCH), 7.78 (2H, d, J = 8.0 Hz, Ar-H), 7.67 (2H, d, J = 7.5 Hz, Ar-H), 7.42 (2H, d, J = 8.0 Hz, Ar-H), 7.28 (2H, d, J = 7.5 Hz Ar-H), 7.30 (1H, br t, J = 6.0 Hz, NH), 5.36 (1H, br s, NH), 5.75 (1H, dd, J = 10.0, 5.0 Hz, CHCH₂), 4.62 (2H, m, NHCH₂), 4.46 (2H, s, OCH₂), 3.81 (3H, s, OCH₃), 3.23 - 2.17 (2H, m, β-CH₂), 1.63 - 1.51 (1H, m, CH(CH₃)₂), 0.99 (3H, d, J = 6.5 Hz, CH₃); δ_C (100 MHz, CDCl₃), 168.3, 163.2, 145.8, 143.1, 140.8, 128.3, 127.3, 126.5, 120.5, 119.3, 66.5, 60.1, 52.0, 45.3, 44.4, 40.6, 24.2, 22.5, 21.3; m/z: 530 (M + Na)^+; ν_max (KBr)/cm⁻¹ 3200, 3156, 2956, 2850, 2810, 1623, 1632.

N-[2S-[1-(Thiazole-4-carboxylic acid methyl ester)methyl]carboxamido-3-methylbutyl]-4-nitro-1,8-naphthalimide (118) was synthesised using 76 (206 mg, 0.58 mmol, 1 eq.), HOBT (80 mg, 0.60 mmol, 1.05 eq.), EDCI.HCl (122 mg, 0.64 mmol, 1.1 eq.) and Et₃N (64 mg, 0.1 ml, 0.64 mmol, 1.1 eq.) according to Procedure 3 and was yielded as a yellow solid (220 mg, 74 %) after recrystallisation with MeOH. m.p. 129 - 130 °C; Found: C, 56.34; H, 4.21; N, 10.87 %. C₂₄H₂₂N₄O₇S requires C, 56.46; H, 4.34; N, 10.97 %; HRMS: 533.1117 ([M + Na]^+). C₂₄H₂₂N₄O₇Na requires 533.1107; δ_H (400 MHz, CDCl₃), 8.81 (1H, d, J = 8.0 Hz, Ar-H7), 8.69 (1H, d, J = 7.0 Hz, Ar-H5), 8.63 (1H, d, J = 8.0 Hz, Ar-H2), 8.38 (1H, d, J = 8.0 Hz, Ar-H3), 8.02 (1H, s, SCH), 7.97 (1H, dd, J = 8.5, 7.0 Hz, Ar-H6), 7.31 (1H, br t, J = 6.0 Hz, NH), 5.77 (1H, dd, J = 10.0, 5.0 Hz, CHCH₂), 4.75 (2H, m, NHCH₂), 3.83 (3H, s, OCH₃), 2.30 - 2.07 (2H, m, β-CH₂), 1.50 - 1.46 (1H, m, CH(CH₃)₂), 0.97 (3H, d, J = 6.5 Hz, CH₃); δ_C (100 MHz, CDCl₃), 145.8, 143.1, 140.8, 128.3, 127.3, 126.5, 120.5, 119.3, 66.5, 60.1, 52.0, 45.3, 44.4, 40.6, 24.2, 22.5, 21.3; m/z: 530 (M + Na)^+; ν_max (KBr)/cm⁻¹ 3200, 3156, 2956, 2850, 2810, 1623, 1632.
$^{13}$C NMR (CDCl$_3$), 169.4 (CO), 168.0 (CO), 162.0 (CO), 161.0 (C), 149.2 (C), 145.4 (CH), 132.4 (CH), 129.8 (CH), 129.5 (CH), 129.1 (CH), 128.6 (CH), 128.1 (C), 126.1 (C), 123.4 (CH), 123.0 (C), 122.1 (C), 53.1 (CH), 51.9 (CH3), 40.6 (CH2), 36.8 (CH2), 25.0 (CH), 22.8 (CH3), 21.5 (CH3); $m/z$: 533 (M + Na)$^+$. $\nu_{\text{max}}$ (KBr)/cm$^{-1}$ 3317, 3073, 1711, 1667, 1598, 1536, 1420, 1330, 1244, 1104.

$N$-[2S-[1-(Thiazole-4-carboxylic acid methyl ester)methyl]carboxamido-ethylphenyl]-4-nitro-1,8-naphthalimide (119)

119 was synthesised using 77 (1.13 g, 2.9 mmol, 1 eq.), 104 (500 mg, 2.9 mmol, 1 eq.), HOBt (410 mg, 3.04 mmol, 1.05 eq.), EDCI.HCl (612 mg, 0.64 mmol, 1.1 eq.) and Et$_3$N (322 mg, 0.46 ml, 3.19 mmol, 1.1 eq.) according to Procedure 3 and the crude product was isolated as a dark brown solid (1.46 g, 93 % yield). Recrystallisation with MeOH yielded 119 pure (1.20 g, 77 %). m.p. 121 - 123 °C; Found: C, 59.23; H, 3.52; N, 9.84 %. C$_{27}$H$_{20}$N$_4$O$_7$S requires C, 59.55; H, 3.70; N, 10.29 %; HRMS: 567.0932 ([M + Na]$^+$. C$_{27}$H$_{20}$N$_4$O$_7$NaS requires 567.0950); $\delta_{\text{H}}$ (400 MHz, CDCl$_3$), 8.76 (1H, d, $J = 8.5$ Hz, Ar-H7), 8.57 (1H, d, $J = 7.5$ Hz, Ar-H5), 8.53 (1H, d, $J = 7.5$ Hz, Ar-H2), 8.32 (1H, d, $J = 8.0$ Hz, Ar-H3), 8.00 (1H, s, SCH), 7.92 (1H, t, $J = 7.5$ Hz, Ar-H6), 7.41 (1H, br t, $J = 5.5$ Hz, NH), 7.15 - 7.04 (5H, m, C$_6$H$_5$), 6.02 (1H, dd, $J = 10.0$, 6.5 Hz, $\alpha$-H), 4.75 (2H, m, NHCH$_2$), 3.82 (3H, s, OCH$_3$), 3.73 - 3.51 (2H, m, $\beta$-CH$_2$); $\delta_{\text{C}}$ (100 MHz, CDCl$_3$), 168.7 (CO), 168.0 (CO), 162.5 (CO), 161.8 (C), 161.0 (C), 149.2 (C), 145.4 (C), 136.2 (CH), 132.2 (CH), 129.6 (CH), 129.5 (CH), 129.1 (CH), 128.5 (CH) 128.1 (CH), 126.5 (CH), 125.8 (C), 123.4 (CH), 122.9 (C), 121.9 (C), 55.3 (CH), 51.9 (CH3), 50.39 (C), 40.6 (CH$_2$), 34.0 (CH$_2$); $m/z$: 567 (M + Na)$^+$. $\nu_{\text{max}}$ (KBr)/cm$^{-1}$ 3455, 3365, 1711, 1667, 1579, 1536, 1420, 1372, 1244.
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\[ N\text{-}\{2S\text{-}[1\text{-}(Thiazole-4-carboxylic acid methyl ester)methyl]carboxamido-3-methylbutyl\}-3\text{-}nitro-1,8\text{-}naphthalimide (120) \]

120 was synthesised using 115 (258 mg, 0.72 mmol, 1 eq.), 104 (125 mg, 0.72 mmol, 1 eq.), HOBT (102 mg, 0.75 mmol, 1.05 eq.), EDCI.HCl (143 mg, 0.75 mmol, 1.1 eq.) and Et\(_3\)N (77 mg, 0.11 ml, 0.64 mmol, 1.1 eq.) according to Procedure 3 and was yielded as a brown solid (315 mg, 73 %). Column chromatography on flash silica (50 mg: ethyl acetate/hexane 4:1) yielded 120 pure (36.7 mg, 73 % yield). m.p. 128 - 130 °C; Found: C, 56.21; H, 3.86; N, 10.37 %. C\(_{24}\)H\(_{22}\)N\(_4\)O\(_7\)S requires C, 56.46; H, 4.34; N, 10.97 %. HRMS: 533.1116 ([M + Na]\(^+\). C\(_{24}\)H\(_{22}\)N\(_4\)O\(_7\)NaS requires 533.1107); \(\delta\)\(_H\) (400 MHz, CDCl\(_3\)), 9.21 (1H, s, Ar-H2), 9.13 (1H, s, Ar-H4), 8.71 (1H, d, \(J = 7.0\) Hz, Ar-H7), 8.45 (1H, d, \(J = 8.0\) Hz, Ar-H5), 8.00 (1H, s, SCH), 7.94 (1H, t, \(J = 8.0\) Hz, Ar-H6), 7.33 (1H, br t, \(J = 6.0\) Hz, NH), 5.78 (1H, dd, \(J = 10.0, 5.0\) Hz, CH\(_{CH_2}\)), 4.77 - 4.66 (2H, m, NHCH\(_2\)), 3.82 (3H, s, OCH\(_3\)), 2.31 - 2.07 (2H, m, \(\beta\text{-CH}_{2}\)), 1.49 (1H, m, CH(CH\(_3\))\(_2\)), 0.96 (3H, d, \(J = 6.5\) Hz, CH\(_3\)), 0.90 (3H, d, \(J = 6.5\) Hz, CH\(_3\)); \(\delta\)\(_C\) (100 MHz, CDCl\(_3\)), 169.4 (CO), 162.6 (C O), 162.0 (CO), 161.0 (C), 145.8 (C), 145.3 (C), 135.4 (CH), 134.4 (CH), 130.4 (C), 129.7 (C), 128.7 (CH), 128.7 (CH), 128.2 (CH), 124.0 (CH), 123.8 (C), 122.3 (C), 53.1 (CH\(_3\)), 51.9 (CH), 40.6 (CH\(_2\)), 36.8 (CH\(_2\)), 25.0 (CH), 22.8 (CH\(_3\)), 21.4 (CH\(_3\)); \(m/z\): 533 (M + Na\(^+\), 511 (M + Na\(^+\)), 534 (M+H+Na\(^+\)); \(v_{\text{max}}\) (KBr)/cm\(^{-1}\) 3207, 3153, 1751, 1597, 1548, 1501, 1410, 1330, 1214, 1004.

\[ N\text{-}\{2S\text{-}[1\text{-}(Thiazole-4-carboxylic acid methyl ester)methyl]carboxamido-ethylphenyl\}-3\text{-}nitro-1,8\text{-}naphthalimide (121) \]

121 was synthesised using 116 (160 mg, 0.41 mmol, 1 eq.), 104 (70 mg, 0.41 mmol, 1 eq.), HOBT (58 mg, 0.43 mmol, 1.05 eq.), EDCI.HCl (86 mg, 0.45 mmol, 1.1 eq.) and Et\(_3\)N (45 mg, 0.064 ml, 0.45 mmol, 1.1 eq.) according to Procedure 3 and the crude product was isolated as a dark brown solid (181 mg, 81 % yield). Column chromatography on neutral flash silica (gradient elution using 1 % MeOH-NH\(_3\)/CH\(_2\)Cl\(_2\) followed by 3 % MeOH-NH\(_3\)/CH\(_2\)Cl\(_2\)) yielded 121 pure (175 mg, 70 %). m.p.
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120 - 121 °C; Found: C, 59.05; H, 3.62; N, 10.14 %.

C$_{27}$H$_{20}$N$_4$O$_7$S requires C, 59.55; H, 3.70; N, 10.29 %; HRMS: 567.0955 ([M + Na]$^+$)

C$_{27}$H$_{20}$N$_4$O$_7$S requires 567.0950; δ$_H$ (400 MHz, CDCl$_3$), 9.15 (1H, s, Ar-H2), 9.10 (1H, s, Ar-H4), 8.64 (1H, d, J = 7.0 Hz, Ar-H7), 8.40 (1H, d, J = 8.0 Hz, Ar-H5), 8.05 (1H, s, SCH), 7.90 (1H, t, J = 7.5 Hz, Ar-H6), 7.19 - 7.06 (5H, m, C$_6$H$_5$), 6.06 (1H, dd, J = 9.5, 6.5 Hz, ø-H), 4.75 (2H, d, J = 6.0 Hz, NHCH$_2$), 3.85 (3H, s, OCH$_3$), 3.75 - 3.70 (1H, dd, J = 14.0, 6.0 Hz, β-CH$_2$), 3.59 - 3.53 (1H, dd, J = 9.5, 10.0 Hz, β-CH$_2$); δ$_C$ (100 MHz, CDCl$_3$), 168.6 (CO), 167.95 (CO), 162.3 (CO), 161.8 (CO), 161.0 (C), 145.5 (C), 136.1 (C), 135.4 (CH), 134.3 (CH), 130.4 (C), 129.5 (C), 128.7 (CH), 128.6 (CH) 128.5 (CH), 128.2 (CH), 128.1 (CH), 126.4 (CH), 123.9 (CH), 123.5 (CH), 122.0 (CH), 55.3 (CH), 51.9 (CH$_3$), 40.6 (CH$_2$), 33.9 (CH$_2$); m/z: 567 (M + Na)$^+$. $v_{max}$ (KBr)/cm$^{-1}$ 3275, 3102, 1641, 1603, 1544, 1340, 1252, 1164.

$N$-[2S-[1-(Thiazole-4-carboxylic acid methyl ester)methyl] carboxamido-3-methylbutyl]-4-amino-1,8-naphthalimide (123)

123 was synthesised using 118 (220 mg, 0.43 mmol, 1 eq.) according to Procedure 6 and was yielded as a orange solid (150 mg, 75 %) after recrystallisation with Ethyl acetate. m.p. 115 - 117 °C; Found: C, 59.54; H, 4.86; N, 4.97 %.

C$_{14}$H$_{10}$N$_2$O$_4$S requires C, 59.99; H, 5.03; N, 11.66 %; HRMS: 503.1366 ([M + Na]$^+$)

C$_{24}$H$_{24}$N$_4$O$_4$SNaS requires 503.1365; δ$_H$ (400 MHz, CDCl$_3$), 8.77 (1H, br t, J = 6.0 Hz, NH), 8.64 (1H, d, J = 8.5 Hz, Ar-H7), 8.43 (1H, s, SCH), 8.41 (1H, d, J = 8.0 Hz, Ar-H5), 8.19 (1H, d, J = 8.0 Hz, Ar-H2), 7.66 (1H, t, J = 7.5 Hz, Ar-H6), 7.31 (1H, br s, NH$_2$), 6.86 (1H, d, J = 8.5 Hz, Ar-H3), 5.56 (1H, dd, J = 9.5, 5.0 Hz, ø-H), 4.52 (1H, dd, J = 16.5, 6.0 Hz, NHCH$_2$), 4.45 (1H, dd, J = 16.0, 6.0 Hz, NHCH$_2$), 3.80 (3H, s, OCH$_3$), 2.13 - 1.99 (2H, m, β-CH$_2$), 1.43 - 1.37 (1H, m, CH(CH$_3$)$_2$), 0.87 (3H, d, J = 6.5 Hz, CH$_3$), 0.82 (3H, d, J = 7.0 Hz, CH$_3$); δ$_C$ (100 MHz, CDCl$_3$), 169.4 (CO), 168.0 (CO), 162.0 (CO), 161.0 (C), 149.2 (C), 145.4 (CH), 132.4 (CH), 129.8 (CH), 129.5 (CH), 129.1 (CH), 128.6 (CH), 128.1 (C), 126.1 (C), 123.4 (CH), 123.0 (C), 122.1 (C), 53.1 (CH), 51.9 (CH$_3$), 40.6 (CH$_2$), 36.8 (CH$_2$), 25.0 (CH), 22.8 (CH$_3$), 21.5 (CH$_3$); m/z: 503 (M + Na)$^+$. $v_{max}$ (KBr)/cm$^{-1}$ 3338, 3073, 1740, 1700, 1669, 1625, 1450, 1346, 799.
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124 was synthesised using 119 (500 mg, 0.92 mmol, 1 eq.) according to Procedure 6 and was yielded as an orange solid (211 mg, 60 %) following a recrystallisation with Ethyl acetate. m.p. 105 - 106 °C; Found: C, 62.99; H, 4.30; N, 10.75 %. C_{27}H_{22}N_{4}O_{5}S requires C, 63.02; H, 4.31; N, 10.89 %; HRMS: 533.1117 ([M + Na]^+). C_{24}H_{22}N_{4}O_{7}NaS requires 533.1107; δ_{H} (400 MHz, CDCl_{3}), 8.81 (1H, d, J = 8.0 Hz, Ar-H7), 8.69 (1H, d, J = 7.0 Hz, Ar-H5), 8.63 (1H, d, J = 8.0 Hz, Ar-H2), 8.38 (1H, d, J = 8.0 Hz, Ar-H3), 8.02 (1H, s, SCH), 7.97 (1H, dd, J = 8.5, 7.0 Hz, Ar-H6), 7.31 (1H, br t, J = 6.0 Hz, NH), 5.77 (1H, dd, J = 10.0, 5.0 Hz, CHCH_{2}), 4.75 (2H, m, NHCH_{2}), 3.83 (3H, s, OCH_{3}), 2.30 - 2.07 (2H, m, β-CH_{2}), 1.50 - 1.46 (1H, m, CH(CH_{3})_{2}), 0.97 (3H, d, J = 6.5 Hz, CH_{3}), 0.91 (3H, d, J = 6.5 Hz, CH_{3}); δ_{C} (100 MHz, CDCl_{3}), 169.4 (CO), 168.0 (CO), 162.0 (CO), 161.0 (C), 149.2 (C), 145.4 (CH), 132.4 (CH), 129.8 (CH), 129.5 (CH), 129.1 (CH), 128.6 (CH), 128.1 (C), 126.1 (C), 123.4 (CH), 123.0 (C), 122.1 (C), 53.1 (C), 51.9 (CH_{3}), 40.6 (CH_{2}), 36.8 (CH_{2}), 25.0 (CH), 22.8 (CH_{3}), 21.5 (CH_{3}); m/z: 533 (M + Na)^+. v_{max} (KBr/cm): 3455, 3365, 3250, 2927, 1783, 1685, 1638, 1579, 1372, 776.

N-[2,2-Diethoxy-ethyl]-4-nitro-1,8-naphthalimide (131)

131 was prepared by stirring aminoaldehyde diethyl acetal (2.30 g, 2.50 mL, 17.22 mmol, 1.4 eq), 4-Nitro-1,8-naphthalide anhydride (3 g, 12.3 mmol, 1 eq) and NEt_{3} (2.5 g, 3.5 mL, 2 eq) at reflux (120 °C) in anhydrous toluene (300 mL) with molecular sieves, under argon, for 48 h. The reaction was filtered hot through celite washing several times with toluene. The filtrate and washings were evaporated under reduced pressure. The residue was dissolved in CH_{2}Cl_{2} and washed once with HCl (0.1 M), once with H_{2}O and once with brine. The organic layer was dried over MgSO_{4}, filtered and evaporated under reduced pressure to give the crude product as a brown solid (4.21 g, 95 % yield). Recrystallisation from MeOH yielded 131 pure (3.3 g, 78 %). m.p. 99 - 101 °C; HRMS: 381.1054 ([M + Na]^+). C_{18}H_{18}N_{2}O_{6}Na requires 381.1063; δ_{H} (400 MHz, CDCl_{3}), 8.84 (1H, d, J = 9.0 Hz, Ar-H7), 8.74 (1H, d, J = 7.0 Hz, Ar-H5), 8.70 (1H, d, J = 8.0 Hz, Ar-H2), 8.41 (1H, d, J = 8.0 Hz,
Ar-H3), 8.00 (1H, t, J = 9.0 Hz, Ar-H6), 5.02 (1H, t, J = 6.0 Hz, CH), 4.40 (2H, d, J = 5.5 Hz, NCH3), 3.83 - 3.75 (2H, m, CH2CH3), 3.61 - 3.51 (2H, m, CH2CH3), 1.16 (6H, t, J = 7.0, CH3); δc (100 MHz, CDCl3), 162.8 (CO), 162.0 (CO), 149.1 (C), 132.1 (CH), 129.5 (CH), 128.9 (CH), 128.6 (C), 126.4 (C), 123.5 (CH), 123.2 (C), 122.4 (C), 98.2 (CH), 61.5 (CH2 x 2), 41.5 (CH2), 14.8 (CH3); m/z: 381 (M + Na)+. νmax (KBr)/cm−1 3180, 2960, 2889, 1600, 1570.

N-[2-Oxo-ethyl]-4-nitro-1,8-naphthalimide (132)

132 was synthesised by stirring 12 (3.1 g, 8.6 mmol) in a mixture of TFA (60 mL) and CHCl3 (120 mL) in an ice/water bath, under an argon atmosphere, for 1 h. The reaction was stirred at room temperature for a further 5 h. The solvent was removed in vacuo and co-evaporated with CH2Cl2 several times, to remove the remaining traces of TFA to give 132 as an off-white solid (2.3 g, 89 % yield). m.p. 115-116 °C; HRMS: 285.2551 ([M + Na]+. C14H9N2O4 requires 285.2451); δH (400 MHz, CD3)2SO), 9.60 (1H, s, CHO), 8.76 (1H, d, J = 8.0 Hz, Ar-H7), 8.66 (2H, br t, J = 8.0 Hz, Ar-H5, Ar-H2), 8.58 (1H, d, J = 8.0 Hz, Ar-H3), 8.13 (1H, dd, J = 8.5, 7.5 Hz, Ar-H6), 4.97 (2H, s, CH2); δc (100 MHz, CDCl3), 196.2, 168.2, 163.0, 149.1, 133.5, 130.1, 129.5, 129.2, 128.4, 126.0, 123.6, 123.2, 122.01, 36.7; m/z: 285 (M + H)+. νmax (KBr)/cm−1 3180, 2900, 2889, 1600, 1570.

N-[Thiazole-4-carboxylic acid methyl ester)methyl]-4-nitro-1,8-naphthalimide (134)

134 was synthesised by stirring 104 (100 mg, 0.58 mmol, 1.4 eq), 4-Nitro-1,8-naphthalide anhydride (100 mg, 0.41, 1 eq) and NEt3 (80 mg, 0.12 mL, 2 eq) at reflux (120 °C) in anhydrous toluene (15 mL) with molecular sieves, under argon, for 48 h. The reaction was filtered hot through celite washing several times with toluene. The filtrate and washings were evaporated under reduced pressure. The residue was dissolved in CH2Cl2 and washed once with HCl (0.1 M), once with H2O and once with brine. The organic layer was dried over MgSO4, filtered and evaporated under reduced pressure to give the crude product 134 as a brown solid (156 mg, 95 % yield). A recrystallisation from MeOH yielded 134 pure (116 mg, 71 %). m.p. 125 - 126 °C; HRMS: 420.0253 ([M + Na]+. C18H11N3O6NaS requires 420.0266); δH (400 MHz, CDCl3), 8.84 (1H, d, J = 8.5 Hz, Ar-
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H7), 8.77 (1H, d, J = 7.0 Hz, Ar-H5), 8.73 (1H, d, J = 8.0 Hz, Ar-H2), 8.41 (1H, d, J = 8.0 Hz, Ar-H3), 8.15 (1H, s, CH), 8.02 (1H, t, J = 8.0 Hz, Ar-H6), 5.75 (2H, s, CH2), 3.92 (3H, s, CH3); δ2 (100 MHz, CDCl3), 165.3 (C = O), 162.5 (C = O), 161.2 (C), 149.5 (C), 146.2 (C), 132.6 (CH), 130.1 (CH), 129.6 (CH), 129.5 (CH), 128.7 (CH), 127.9 (C), 125.8 (C), 123.5 (CH), 123.3 (C), 121.9 (C), 52.0 (CH3), 41.3 (CH2); m/z: 420 (M + Na)+. νmax (KBr)/cm⁻¹: 3400, 2954, 2810, 1640, 1578, 1545.

N-[Thiazole-4-carboxylic acid methyl ester)methyl]-3-nitro-1,8-naphthalimide (135)

135 was synthesised by stirring 104 (100 mg, 0.58 mmol, 1.4 eq), 3-Nitro-1,8-naphthalide anhydride (100 mg, 0.41, 1 eq) and NEt3 (80 mg, 0.12 mL, 2 eq) at reflux (120 °C) in anhydrous toluene (15 mL) with molecular sieves, under argon, for 48 h. The reaction was filtered hot through celite washing several times with toluene. The filtrate and washings were evaporated under reduced pressure. The residue was dissolved in CH2Cl2 and washed once with HCl (0.1 M), once with H2O and once with brine. The organic layer was dried over MgSO4, filtered and evaporated under reduced pressure to give the crude product 135 as a brown solid (148 mg, 88 % yield). A recrystallisation from MeOH yielded 135 pure (116 mg, 71 %). m.p. 126 - 127 °C; HRMS: 420.0253 ([M + Na]+). C18H11N3O6NaS requires 420.0266); δH (400 MHz, CDCl3), 8.84 (1H, d, J = 8.5 Hz, Ar-H7), 8.77 (1H, d, J = 7.0 Hz, Ar-H5), 8.73 (1H, d, J = 8.0 Hz, Ar-H2), 8.41 (1H, d, J = 8.0 Hz, Ar-H3), 8.15 (1H, s, CH), 8.02 (1H, t, J = 8.0 Hz, Ar-H6), 5.75 (2H, s, CH2), 3.92 (3H, s, CH3); δ2 (100 MHz, CDCl3), 165.3 (CO), 162.5 (CO), 161.2 (C), 149.5 (C), 146.2 (C), 132.6 (CH), 130.1 (CH), 129.6 (CH), 129.5 (CH), 128.7 (CH), 127.9 (C), 125.8 (C), 123.5 (CH), 123.3 (C), 121.9 (C), 52.0 (CH3), 41.3 (CH2); m/z: 420 (M + Na)+. νmax (KBr)/cm⁻¹: 3374, 2904, 2765, 1520, 1508, 1500.
3-Nitro-4-bromo-1,8-naphthalic anhydride (139)\textsuperscript{127b}

139 was synthesised by gradually treating 4-bromo-1,8-naphthalic anhydride (3 g, 10.8 mmol) in sulphuric acid (20 ml) at 15 - 20 °C with a mixture of nitric acid (0.5 ml) and sulphuric acid (10 ml) and the whole heated for 1 h at 100 °C. 139 was obtained as a beige solid (2.5 g, 74 %) after precipitation from water and recrystallisation from acetic anhydride. m.p 229 - 230 °C (ref.\textsuperscript{127b} 230 - 232 °C); \(\delta_H\) (400 MHz, \text{d}_6-DMSO), 8.92 (1H, s, Ar-H7), 8.82 (1H, d, \(J = 9\) Hz, Ar-H3), 8.74 (1H, d, \(J = 7.5\) Hz, Ar-H1), 8.18 (1H, t, \(J = 8.5\) Hz, Ar-H2); \(\delta_C\) (100 MHz, CDCl\textsubscript{3}), 161.8, 160.9, 152.7, 134.8, 134.5, 132.8, 130.7, 130.3, 129.5, 128.3, 126.9, 125.4; \textit{m/z}: 322 (M + H)^+.

3-Nitro-4-phenylthio-1,8-naphthalic anhydride (140)\textsuperscript{127b}

140 was synthesised by treating 139 (2.0 g, 6.2 mmol, 1 eq.) with thiophenol (1.03 g, 0.96 ml, 9.3 mmol, 1.5 eq.) in dry EtOH (20 ml) for 5 h. The liquor was reduced in volume and filtered, washed with some EtOH and dried to give 140 as a yellow solid (2.10 g, 97 %). No further purification was necessary. m.p 177 - 178 °C (ref.\textsuperscript{127b} 178 - 179 °C); \(\delta_H\) (400 MHz, \text{d}_6-DMSO), 8.96 (1H, d, \(J = 8.5\) Hz, Ar-H3), 8.80 (1H, s, Ar-H7), 8.73 (1H, d, \(J = 7.0\) Hz, Ar-H1), 7.92 (1H, t, \(J = 8.5\) Hz, Ar-H2), 7.28 - 7.21 (5H, m, C\textsubscript{6}H\textsubscript{5}); \(\delta_C\) (100 MHz, CDCl\textsubscript{3}), 158.6, 158.0, 151.7, 135.8, 135.3, 135.0, 133.2, 132.4, 130.5, 129.8, 129.4, 127.9, 126.9, 126.7, 126.1; \textit{m/z}: 351 (M + H)^+.

3-Amino-4-phenylthio-1,8-naphthalic anhydride (141)\textsuperscript{127b}

141 was synthesised by treating 140 (1.8g, 5.18 mmol, 1 eq.) with a mixture of stannous chloride (5.84 g, 26.0 mmol, 5 eq.) and concentrated hydrochloric acid (8 ml). After warming to 40 °C, the temperature rose spontaneously to 85 °C and was maintained at 85 °C for 1h. The suspension was cooled and filtered to give 141 as an olive green solid (1.4 g, 88 %). No further purification was necessary. m.p 224 - 225 °C (ref.\textsuperscript{127b} 224 - 225 °C); \(\delta_H\) (400 MHz, \text{d}_6-DMSO), 8.43 (1H, d, \(J = 8.0\) Hz, Ar-H3), 8.17 (1H, s, Ar-H7), 8.09 (1H, d, \(J = 6.5\) Hz, Ar-H1), 7.71 (1H, t, \(J = 8.5\) Hz, Ar-H2), 7.25 - 7.01 (5H, m, C\textsubscript{6}H\textsubscript{5}), 4.73 (2H, s, NH\textsubscript{2}); \(\delta_C\)
(100 MHz, CDCl₃), 160.6, 160.5, 150.8, 135.3, 130.3, 129.3, 128.7, 126.9, 126.4, 125.8, 123.4, 121.2, 119.4, 108.8; m/z: 321 (M + H)^+.

6.7 General Experimental Procedures for Chapter 3

6.7.1 Procedure 1. Formation of the 4-Nitro-1,8-Naphthalimide Derivatives 164 - 167

4-Nitro-1,8-naphthalic anhydride (1 eq.) was placed into a round bottomed flask along with molecular sieves. The flask was sealed and evacuated. Anhydrous toluene then was added via cannula. The appropriate dialkylaminoethylamine (1.4 eq.) was subsequently added via syringe. The reaction mixture was stirred at reflux (120 °C) for 48 hours under an argon atmosphere. After completion of the reaction the solution mixture was immediately filtered while hot through celite, washing several times with toluene. The filtrate and washings were removed under reduced pressure and the residue dissolved in CH₂Cl₂. The organic solution was washed twice with sat. NaHCO₃, twice with H₂O and once with brine. The organic layer was dried over MgSO₄ and evaporated under reduced pressure to dryness. The crude product was then purified by recrystallisation using either EtOH or MeOH and dried under vacuum.

6.7.2 Procedure 2. Formation of the 4-Amino-1,8-Naphthalimide Derivatives 168 - 171

The reduction reaction of the relevant 4-nitro-1,8-naphthalimide (1 eq) in MeOH was carried out using a Parr hydrogen shaker apparatus at 3 atm pressure, in the presence of 10 % Pd/C catalyst (0.2 eq.) until no more hydrogen gas was consumed. The reaction mixture was filtered through celite, washing with MeOH. The filtrate and washings were evaporated under reduced pressure and further dried under vacuum.

6.7.3 Procedure 3: Formation of the Bis-1,8-Naphthalimide Containing Tröger's Base Derivatives 151 – 154, Method A:

A solution of conc. aqueous HCL (5 eq.) and paraformaldehyde (7.2 eq., 37 % w/v), in the minimum amount of EtOH, were cooled in an ice-bath for 2 hours. The relevant 4-amino-1,8-naphthalimide (1 eq.) was added and the resulting mixture was stirred at 60°C for 3 days under an argon atmosphere. The mixture was then cooled to room temperature and the precipitate was collected by suction filtration and air-dried. The collected precipitate was dissolved in the minimum amount of H₂O and the pH was brought to
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pH 9/12 (depending on pKₐ value of the molecule) by the slow addition of 10% aqueous ammonia. The aqueous solution was extracted three times with CH₂Cl₂ and the combined organic extracts were washed twice with brine. The organic layer was dried over MgSO₄ and the solvent was removed under reduced pressure. The product was purified by recrystallisation or by column chromatography on flash silica (using CH₂Cl₂/MeOH-NH₃, 95:5 as eluant).

**Method B:**

A mixture of the relevant 4-amino-1,8-naphthalimide (1 eq.) and paraformaldehyde (1.5eq.) in neat TFA were stirred at room temperature for 12 hours under an argon atmosphere. The reaction was then brought to pH 9/12 (depending on pKₐ value of the molecule) by the slow addition of aqueous NaOH (6 M). The aqueous solution was extracted three times with CH₂Cl₂. The organic extracts were combined and the solvent was removed under reduced pressure. The crude product was purified by recrystallisation and dried under vacuum.

**N-[1-Dimethylamino-ethyl]-4-nitro-1,8-naphthalimide (164)**

164 was synthesised by reacting N,N-Dimethylethylenediamine (1.50 g, 1.86 mL, 17.3 mmol, 1.4 eq.) with 4-nitro-1,8-naphthalic anhydride (3.0 g, 12 mmol, 1 eq.) and Et₃N (2.5 g, 3.56 mL, 24.6 mmol, 2 eq.) in anhydrous toluene (200 ml), according to Procedure 1, to yield the product as a shiny brown solid (2.83 g, 91 %) after a recrystallisation from EtOH. m.p. 106 - 108 °C (ref., 156 106 - 109 °C); HRMS: 314.1141 ([M + H]⁺). C₁₆H₁₆N₉O₄ requires 314.1129; δ₁H (400 MHz, CDCl₃), 8.80 (1H, d, J = 8.5 Hz, Ar-H7), 8.71 (1H, d, J = 7.0 Hz, Ar-H5), 8.67 (1H, d, J = 8.0 Hz, Ar-H2), 8.39 (1H, d, J = 8.0 Hz, Ar-H3), 7.97 (1H, dd, J = 8.0 & 7.5 Hz, Ar-H6), 4.33 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₃)₂), 2.67 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₃)₂), 2.35 (6H, s, N(CH₃)₂); δc (100 MHz, CDCl₃), 162.9 (CO), 162.0 (CO), 149.0 (C), 132.0 (CH), 129.4 (CH), 129.3 (CH), 128.8 (CH), 128.6 (C), 126.4 (C), 123.4 (CH), 123.1 (C), 122.5 (C), 56.4 (CH₂), 45.3 (CH₃), 38.0 (CH₂); m/z: 314 (M + H)⁺.
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N-[1-Dimethylamino-ethyl]-4-amino-1,8-naphthalimide (168)

165 was synthesised using 164 (1.80 g, 5.7 mmol, 1 eq.) according to Procedure 2 and was yielded as an orange solid (1.57 g, 98%). m.p. 184 - 185 °C (ref. 156 184 - 185 °C); HRMS: 284.1399 ([M + H]^+). C_{18}H_{18}N_{3}O_{2} requires 284.1388; δ_{H} (400 MHz, (CD_{3})_{2}SO), 8.60 (1H, d, J = 8.5 Hz, Ar-H7), 8.41 (1H, d, J = 7.0 Hz, Ar-H2), 8.18 (1H, d, J = 8.0 Hz, Ar-H5), 7.64 (1H, dd, J = 7.5 & 8.5 Hz, Ar-H6), 7.46 (2H, br. s, NH_{2}), 6.83 (1H, d, J = 8.5 Hz, Ar-H3), 4.11 (2H, t, J = 7.0 Hz, NCH_{2}CH_{2}N(CH_{3})_{2}), 2.80 (6H, s, N(CH_{3})_{2}); δ_{C} (100 MHz, CDCl_{3}), 163.7 (CO), 162.8 (CO), 152.7 (C), 133.9 (CH), 131.0 (CH), 129.9 (C), 129.3 (CH), 123.9 (CH), 121.7 (C), 119.3 (C), 108.1 (CH), 107.4 (C), 56.6 (CH_{2}), 45.3 (CH_{3}), 37.0 (CH_{2}); m/z: 284 (M + H)^{+};

Bis-{N-[1-Dimethylamino-ethyl]}-9,18-methano-1,8-naphthalimido[1,5]-diazocine (151)

151 was synthesised by reacting 168 (1.30 g, 4.58 mmol, 1 eq.) with paraformaldehyde (1.083 g, 1 mL, 33.0 mmol, 7.2 eq.) and conc. HCl (0.84 g, 0.7 mL, 22.9 mmol, 5 eq.) in EtOH (45 mL), according to Procedure 3, Method A, to yield the product as an orange solid (850 mg, 31%) after a recrystallisation from EtOH. 151 was also synthesised by reacting 168 (200 mg, 0.71 mmol, 1 eq.) with paraformaldehyde (32.5 mg, 0.03 mL, 1.1 mmol, 1.5 eq.) in TFA (3.5 mL), according to Procedure 3, Method B, to yield the product as an orange solid (260 mg, 61%) after a recrystallisation from EtOH. m.p. 231 - 233 °C; Found: C, 69.53; H, 5.62; N, 13.56 %. C_{35}H_{34}N_{6}O_{4} requires C, 69.75; H, 5.69; N, 13.94 %; HRMS: 603.2740 ([M + H]^+). C_{35}H_{34}N_{6}O_{4} requires 603.2720; δ_{H} (400 MHz, CDCl_{3}), 8.69 (2H, d, J = 8.0 Hz, Ar-H7, Ar-H7'), 8.60 (2H, d, J = 7.5 Hz, Ar-H5, Ar-H5'), 8.07 (2H, s, Ar-H2, Ar-H2'), 7.87 (2H, t, J = 8.0 Hz, Ar-H6, Ar-H6'), 5.12 (2H, d, J = 17.0 Hz, Ar-CH_{2}N), 4.67 (2H, s, NCH_{2}N), 4.59 (2H, d, J = 17.0 Hz, Ar-CH_{2}N), 4.29 (4H, t, J = 6.5 Hz, NCH_{2}CH_{2}N(CH_{3})_{2}), 2.64 (4H, t, J = 6.5 Hz, NCH_{2}CH_{2}N(CH_{3})_{2}), 2.30 (12H, s, N(CH_{3})_{2}); δ_{C} (100 MHz, CDCl_{3}), 163.7 (CO), 163.1 (CO), 148.6 (C), 130.5 (CH), 130.0 (CH), 128.3 (CH), 127.8 (C), 126.8
(CH), 126.7 (C), 124.7 (C), 122.6 (C), 118.4 (C), 66.6 (CH₂), 56.6 (CH₂), 56.4 (CH₂), 45.2 (CH₃), 37.5(CH₂); m/z: 603 (M + H)⁺; v max (neat sample)/cm⁻¹ 2955, 2859, 2819, 2769, 1652, 1595, 787.

**N-[1-Methyl-pyrrazino-ethyl]-4-nitro-1,8-naphthalimide (165)**

165 was synthesised by reacting 1-(2-aminoethyl)-4-methylpiperazine (2.47 g, 2.58 mL, 17.2 mmol, 1.4 eq.) with 4-nitro-1,8-naphthalic anhydride (3.0 g, 12.3 mmol, 1 eq.) and Et₃N (2.5 g, 3.56 mL, 24.6 mmol, 2 eq.) in anhydrous toluene (200 ml), according to Procedure 1, to yield the product as a brown solid (3.50 g, 77 %) after a recrystallisation from MeOH. m.p. 109 - 111 °C; HRMS: 369.1554 ([M + H]⁺, C₁₉H₂₁N₄O₄ requires 369.1563); δ (400 MHz, CDCl₃), 8.85 (1H, d, J = 9.0 Hz, Ar-H7), 8.74 (1H, d, J = 7.5 Hz, Ar-H5), 8.70 (1H, d, J = 8.0 Hz, Ar-H2), 8.42 (1H, d, J = 8.0 Hz, Ar-H3), 8.00 (1H, t, J = 8.0 Hz, Ar-H6), 4.36 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 2.73 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 2.65 (4H, br. s, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 2.44 (4H, br. s, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 2.28 (3H, s, NCH₂CH₂N(CH₂CH₂)₂NCH₃); δ (100 MHz, CDCl₃), 162.7 (CO), 161.9 (CO), 149.0 (C), 131.8 (CH), 129.4 (CH), 129.2 (CH), 128.7 (CH), 128.5 (C), 126.4 (C), 123.4 (CH), 123.1 (C), 122.4 (C), 54.9 (CH₂), 54.63 (CH₂), 52.7 (CH₂), 45.52 (CH₃), 45.5 (CH₃), 37.3 (CH₂); m/z: 369 (M + H)⁺; v max (neat sample)/cm⁻¹ 3078, 2928, 2793, 2757, 1655, 1522, 1339, 824, 761.

**N-[1-Methyl-pyrrazino-ethyl]-4-aminio-1,8-naphthalimide (169)**

169 was synthesised using 165 (1.0 g, 2.7 mmol, 1 eq.) according to Procedure 2 and was yielded as an orange solid (900 mg, 98 %). m.p. 182 - 183 °C; HRMS: 361.1829 ([M + Na]⁺, C₁₉H₂₁N₄O₂Na requires 361.1821); δ (400 MHz, (CD₃)SO), 8.62 (1H, d, J = 8.5 Hz, Ar-H7), 8.41 (1H, d, J = 7.0 Hz, Ar-H2), 8.18 (1H, d, J = 8.5 Hz, Ar-H5), 7.65 (1H, t, J = 8.0 Hz, Ar-H6), 7.46 (2H, br s, NH₂), 6.84 (1H, d, J = 8.5 Hz, Ar-H3), 4.12 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 3.25 (2H, t, J = 7.0 Hz, NCH₂CH₂N(CH₂CH₂)₂NCH₃), 2.45 (4H, br s, NCH₂CH₂N(CH₂CH₂)₂NCH₃); δ (100 MHz,
Bis-{N-[1-Methyl-pyrazino-ethyl]}-9,18,-methano-1,8-naphthalimido[\(\beta_i\)]\[1,5\]-diazocine (152)

152 was synthesised by reacting 169 (200 mg, 0.059 mmol, 1 eq.) with paraformaldehyde (21.6 mg, 0.02 mL, 0.086 mmol, 1.5 eq.) in TFA (3 mL), according to Procedure 3, Method B, to yield the product as a yellow solid (210 mg, 50%) after a recrystallisation from EtOH.

m.p. 225 - 227 °C; Found: C, 68.69; H, 6.10; N, 15.71 %. C\(_{41}\)H\(_{44}\)N\(_8\)O\(_4\) requires C, 69.08; H, 6.22; N, 15.72 %; HRMS: 713.3580 ([M + H]\(^{+}\)). C\(_{41}\)H\(_{45}\)N\(_8\)O\(_4\) requires 713.3564; \(\delta\)H (400 MHz, CDCl\(_3\)), 8.62 (2H, d, \(J = 8.5\) Hz, Ar-H\(_7\), Ar-H\(_7'\)), 8.51 (2H, d, \(J = 7.0\) Hz, Ar-H5, Ar-H5'), 8.02 (2H, s, Ar-H2, Ar-H2'), 7.81 (2H, t, \(J = 8.0\) Hz, Ar-H6, Ar-H6'), 5.10 (2H, d, \(J = 17.0\) Hz, Ar-CH\(_2\)N), 4.65 (2H, s, NCH\(_2\)N), 4.53 (2H, d, \(J = 17.0\) Hz, Ar-CH\(_2\)N), 4.23 (4H, t, \(J = 6.5\) Hz, NCH\(_2\)CH\(_3\)N(CH\(_2\))\(_4\)CH\(_3\)), 2.60 (4H, t, \(J = 6.5\) Hz, NCH\(_2\)CH\(_2\)N(CH\(_2\))\(_4\)CH\(_3\)), 2.43 (8H, br s, NCH\(_2\)CH\(_2\)N(CH\(_2\))\(_4\)CH\(_3\)), 2.39 (8H, br s, NCH\(_2\)CH\(_2\)N(CH\(_2\))\(_4\)CH\(_3\)), 2.23 (6H, s, NCH\(_3\)); \(\delta\)C (100 MHz, CDCl\(_3\)), 163.5 (CO), 162.9 (CO), 148.5 (C), 130.4 (CH), 129.8 (CH), 128.2 (CH), 127.7 (C), 126.7 (C), 126.6 (CH), 124.7 (C), 122.5 (C), 118.3 (C), 66.5 (CH\(_2\)), 56.5 (CH\(_2\)), 55.1 (CH\(_2\)), 54.5 (CH\(_2\)), 52.6(CH\(_2\)), 45.4 (CH\(_3\)), 36.8 (CH\(_2\)); m/z: 713 (M + H)\(^{+}\); \(\nu\)max (neat sample)/cm\(^{-1}\) 2936, 2795, 1650, 1595, 783.
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\[ N-[1-\text{Morpholino-ethyl}-4-\text{amino-1,8-naphthalimide (170)} \]

166 was synthesised by reacting 4-(2-aminoethyl)-morpholine (1.60 g, 1.62 mL, 12.3 mmol, 1 eq.) with 4-nitro-1,8-naphthalic anhydride (3.0 g, 12.3 mmol, 1 eq.) in toluene (30 ml), according to Procedure 1, to yield the product as a brown solid (3.17 g, 74 %) after a recrystallisation from EtOH. m.p. 115 - 117 °C; HRMS: \[ m/z \] 355.3663 ([M + H]^+. \( \text{C}_{18}\text{H}_{18}\text{N}_{3}\text{O}_{5} \) requires 355.3563); \( \delta \) (400 MHz, CDCl\(_3\)), 8.84 (1H, d, \( J = 8.5 \text{ Hz, Ar-H7} \)), 8.73 (1H, d, \( J = 7.5 \text{ Hz, Ar-H5} \)), 8.69 (1H, d, \( J = 8.0 \text{ Hz, Ar-H2} \)), 8.41 (1H, d, \( J = 8.0 \text{ Hz, Ar-H3} \)), 8.00 (1H, t, \( J = 4.5 \text{ Hz, NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 3.69 (4H, t, \( J = 4.5 \text{ Hz, NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 2.75 (2H, t, \( J = 7.0 \text{ Hz, NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 2.63 (4H, br. s, \( \text{NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)); \( \delta_c \) (100 MHz, CDCl\(_3\)), 162.9 (CO), 162.0 (CO), 149.1 (C), 132.0 (CH), 129.5 (CH), 129.4 (CH), 128.9 (CH), 128.4 (C), 126.4 (C), 123.5 (CH), 123.2 (C), 122.5 (C), 66.4 (CH\(_2\)), 55.5 (CH\(_2\)), 53.3 (CH\(_2\)), 36.9 (CH\(_2\)); \( m/z: 355 \text{ (M + H)}^+; \nu_{\text{max}} \text{ (neat sample)}/\text{cm}^{-1} 3078, 2950, 2855, 1656, 1523, 1339, 1117, 839, 787.

170 was synthesised using 166 (1.0 g, 2.7 mmol, 1 eq.) according to Procedure 2 and was yielded as a yellow solid (800 mg, 87 %). m.p. 175 - 177 °C; HRMS: \[ m/z \] 348.3829 ([M + Na]^+. \( \text{C}_{18}\text{H}_{19}\text{N}_{3}\text{O}_{3}\text{Na} \) requires 348.3828); \( \delta \) (400 MHz, (CD\(_3\))\(_2\)SO), 8.60 (1H, d, \( J = 8.0 \text{ Hz, Ar-H7} \)), 8.41 (1H, d, \( J = 7.0 \text{ Hz, Ar-H2} \)), 8.18 (1H, d, \( J = 8.5 \text{ Hz, Ar-H5} \)), 7.64 (1H, t, \( J = 8.0 \text{ Hz, Ar-H6} \)), 7.46 (2H, br s, NH\(_2\)), 6.83 (1H, d, \( J = 8.5 \text{ Hz, Ar-H3} \)), 4.13 (2H, t, \( J = 7.0 \text{ Hz, NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 3.54 (4H, br s, \( \text{NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 2.54 (2H, br s, \( \text{NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)), 2.45 (4H, br s, \( \text{NCH}_2\text{CH}_2\text{N(CH}_2\text{CH}_2\text{)}_2\text{O} \)); \( \delta_c \) (100 MHz, (CD\(_3\))\(_2\)SO), 163.7 (CO), 162.8 (CO), 152.7 (C), 133.9 (CH), 131.0 (CH), 129.6 (C), 129.3 (CH), 123.9 (CH), 121.7 (C), 119.3 (C), 108.1 (CH), 107.4 (C), 66.2 (CH\(_2\)), 55.8 (CH\(_2\)), 53.4 (CH\(_2\)), 36.4 (CH\(_2\)); \( m/z: 348 \text{ (M + H)}^+; \nu_{\text{max}} \text{ (neat sample)}/\text{cm}^{-1} 3253, 2964, 2919, 2861, 2813, 1638, 1572, 863, 774.

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Bis-{N-[1-Morpholino-ethyl]}-9,18-methano-1,8-naphthalimido[β,φ][1,5]-diazocine

(153)

153 was synthesised by reacting 170 (200 mg, 0.060 mmol, 1 eq.) with paraformaldehyde (27.0 mg, 0.025 mL, 0.092 mmol, 1.5 eq.) in TFA (3 mL), according to Procedure 3, Method B, to yield the product as a yellow solid (205 mg, 49 %) after a recrystallisation from EtOH. m.p. 231 - 232 °C; Found: C, 67.92; H, 5.26; N, 12.02 %. C_{39}H_{38}N_{6}O_{6} requires C, 68.21; H, 5.58; N, 12.24 %; HRMS: 687.7180 ([M + H]^+). C_{39}H_{39}N_{6}O_{6} requires 687.7164; δH (400 MHz, CDCl3), 8.70 (2H, d, J = 8.5 Hz, Ar-H7, Ar-H7'), 8.60 (2H, d, J = 7.0 Hz, Ar-H5, Ar-H5'), 8.10 (2H, s, Ar-H2, Ar-H2'), 7.88 (2H, t, J = 8.0 Hz, Ar-H6, Ar-H6'), 5.16 (2H, d, J = 19.5 Hz, Ar-CH2N), 4.69 (2H, s, NCH2N), 4.60 (2H, d, J = 17.0 Hz, Ar-CH2N), 4.30 (4H, br s, NCH2CH2N(CH2CH2)2O), 3.66 (8H, br s, NCH2CH2N(CH2CH2)2O), 2.71 (4H, br s, NCH2CH2N(CH2CH2)2O); δc (100 MHz, CDCl3), 163.7 (CO), 163.1 (CO), 148.7 (C), 130.5 (CH), 130.0 (CH), 128.3 (CH), 127.8 (C), 126.8 (CH), 126.7 (C), 124.8 (C), 122.6 (C), 118.3 (C), 66.5 (CH2), 66.2 (CH2), 56.6 (CH2), 55.5 (CH2), 53.1 (CH2); m/z: 686 (M + H)^+; νmax (neat sample)/cm⁻¹ 2955, 2863, 2754, 1592, 1112, 783.

N-[1-Propyl]-4-nitro-1,8-naphthalimide (167)

167 was synthesised by reacting propylamine (1.02 g, 1.42 mL, 17.2 mmol, 1.4 eq.) with 4-nitro-1,8-naphthalic anhydride (3.0 g, 12.3 mmol, 1 eq.) and Et3N (2.5 g, 3.56 mL, 24.6 mmol, 2 eq.) in toluene (200 ml), according to Procedure 1, to yield the product as a brown solid (3.0 g, 88 %) after a recrystallisation from EtOH. m.p. 98 - 101 °C; HRMS: 285.1141 ([M + H]^+). C_{15}H_{13}N_{2}O_{4} requires 285.1129; δH (400 MHz, CDCl3), 8.84 (1H, d, J = 9.0 Hz, Ar-H7), 8.74 (1H, d, J = 7.0 Hz, Ar-H5), 8.70 (1H, d, J = 8.0 Hz, Ar-H2), 8.41 (1H, d, J = 8.0 Hz, Ar-H3), 8.00 (1H, t, J = 7.5 Hz, Ar-H6), 4.16 (2H, t, J = 7.5 Hz, NCH2CH2CH3), 1.80 (2H, ddd, J = 7.5 Hz, NCH2CH2CH3), 1.04 (3H, t, J = 7.0 Hz, NCH2CH2CH3); δc (100 MHz, CDCl3), 162.8
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CO, 162.0 (CO), 149.0 (C), 131.9 (CH), 129.4 (CH), 129.3 (CH), 128.7 (CH), 128.5 (C),
126.5 (C), 123.4 (CH), 123.1 (C), 122.5 (C), 41.8 (CH$_2$), 20.85 (CH$_3$), 11.03 (CH$_3$);
m/z: 285 (M + H)$^+$; $\nu_{max}$ (neat sample)/cm$^{-1}$ 3078, 2959, 2875, 1657, 1522, 1342, 827, 759.

$N$-[1-Propyl]-4-nitro-1,8-naphthalimide (171)

171 was synthesised using 167 (700 mg, 2.4 mmol, 1 eq.) according to Procedure 2 and was yielded as a bright orange solid (605 mg, 99%). m.p. 105 - 107 °C; HRMS: 277.0956 ([M + Na]$^+$. C$_{15}$H$_{14}$N$_2$O$_2$ requires 277.0953); $\delta_H$ (400 MHz, (CD$_3$)$_2$SO), 8.56 (1H, d, J = 7.5 Hz, Ar-H7), 8.40 (1H, d, J = 6.5 Hz, Ar-H2), 8.16 (1H, d, J = 8.5 Hz, Ar-H5), 7.62 (1H, t, J = 8.5 Hz, Ar-H6), 7.39 (2H, br. s, NH$_2$), 6.83 (1H, d, J = 8.5 Hz, Ar-H3), 3.93 (2H, t, J = 7.0 Hz, NCH$_2$CH$_2$CH$_3$), 1.58 (2H, ddd, J = 7.0 Hz, NCH$_2$CH$_2$CH$_3$), 0.87 (3H, t, J = 7.5 Hz, NCH$_2$CH$_2$CH$_3$); $\delta_C$ (100 MHz, (CD$_3$)$_2$SO), 163.8 (CO), 163.0 (CO), 152.6 (C), 133.9 (CH), 131.1 (CH), 129.5 (CH), 129.2 (CH), 124.0 (C), 121.6 (C), 119.2 (CH), 108.2 (C), 107.4 (CH), 78.9 (CH$_2$), 20.9 (CH$_3$), 11.3 (CH$_2$); m/z: 277 (M + Na)$^+$; $\nu_{max}$ (neat sample)/cm$^{-1}$ 3352, 2958, 2873, 1672, 1632, 1570, 815, 753.

Bis-[N-[1-Propyl]-9,18-methano-1,8-naphthalimido[6,5][1,5]-diazocine (154)

154 was synthesised by reacting 171 (500 mg, 1.9 mmol, 1 eq.) with paraformaldehyde (410 mg, 0.38 mL, 13.6 mmol, 7.2 eq.) and conc. HCl (358 mg, 0.29 mL, 9.8 mmol, 5 eq.) in ethanol (20 mL), according to Procedure 3, Method A, to yield the product as a yellow solid (420 mg, 39%) after a recrystallisation from butan-1-ol. 154 was also synthesised by reacting 171 (200 mg, 0.78 mmol, 1 eq.) with paraformaldehyde (23.6 mg, 0.02 mL, 0.78 mmol, 1.5 eq.) in TFA (3.5 mL), according to Procedure 3, Method B, to yield the product as an orange solid (251 mg, 60%) after a recrystallisation from butan-1-ol. m.p. 210 - 211 °C; HRMS: 545.6340 ([M + H]$^+$. C$_{33}$H$_{29}$NaO$_4$ requires 545.6720); $\delta_H$ (400 MHz, CDCl$_3$), 8.70 (2H, d, J = 8.5 Hz, Ar-H7, Ar-H7'), 8.61 (2H, d, J = 7.5 Hz, Ar-H5, Ar-H5'), 8.12 (2H, s, Ar-H2, Ar-H2'), 7.88 (2H, t, J = 8.0 Hz, Ar-H6, Ar-H6'), 5.15 (2H, d, J = 17.0 Hz, Ar-CH$_2$N), 4.70 (2H, s, NCH$_2$N), 4.61 (2H, d, J = 17.0 Hz, Ar-CH$_2$N),
4.09 (4H, t, J = 7.5 Hz, NCH₂CH₂CH₃), 1.70 (4H, ddd, J = 7.0 Hz, NCH₂CH₂CH₃), 0.96 (6H, t, NCH₂CH₂CH₃); δ (100 MHz, CDCl₃), 163.7 (CO), 163.1 (CO), 148.5 (C), 130.5 (CH), 129.9 (CH), 128.2 (CH), 127.8 (CH), 126.8 (C), 126.6 (C), 124.8 (C), 122.7 (C), 118.5 (C), 66.6 (CH₂), 56.6 (CH₂), 41.3 (CH₂), 20.87 (CH₂), 10.9 (CH₃); m/z: 545 (M + H)⁺; νmax (neat sample)/cm⁻¹ 2954, 2873, 1653, 1589, 785.
Chapter 7: References


[93] Sergeyev, S.; Diederich, F. Chirality 2006, 18, 707.


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Appendix 1

Figure A1.1: (A) The UV/visible absorption spectra of 152 (8.7 mM) in 10 mM phosphate buffer (pH 7.4) with increasing concentration of ct-DNA (0 - 245 μM). (B) Plot of $(\epsilon_a - \epsilon_f)/((\epsilon_b - \epsilon_f)$ versus DNA (M, bp) (●) using data between P/D = 0 → 2.4 and the best fit of the data (—) using the Bard equation 4.3 and 4.4 (Chapter 4, Section 4.3.1).

Figure A1.2: The Plot of $(\epsilon_a - \epsilon_f)/((\epsilon_b - \epsilon_f)$ versus DNA (M, bp) (●) and the best fit of the data (—) using the Bard equations 4.3 and 4.4 (Chapter 4, Section 4.3.1) for 153 (A) at pH 7.4 using absorbance data between P/D = 0 → 12 and (B) at pH 6.3 using absorbance data between P/D 0 → 7.
Figure A1.3: Plot of DNA (M/L) versus DNA/($\epsilon_{T}-\epsilon_{0}$) (•) and best fit of the absorbance data (−) for (A) 151 and (B) 152, in 10 mM phosphate buffer (pH 7.4) according to the Kumar equation 4.2 (Section 4.3.1, Chapter 4)

Figure A1.4: Plot of DNA (M/L) versus DNA/($\epsilon_{T}-\epsilon_{0}$) (•) and best fit of the absorbance data (−) for 153 in (A) 10 mM phosphate buffer (pH 7.4) and (B) 10 mM coadcyate buffer (pH 6) according to the Kumar equation 4.2 (Section 4.3.1, Chapter 4)
Figure A1.5: The overlaid emission spectra (A) $\lambda_{ex} 302$ nm (B) $\lambda_{ex} 368$ nm of 151 (8.7 $\mu$M) in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of ct-DNA (0 – 245 $\mu$M).

Figure A1.6: (A) The overlaid emission spectrum ($\lambda_{em} 425$ nm) and (B) excitation spectrum ($\lambda_{em} 505$ nm) of 151 (8.7 $\mu$M) in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of ct-DNA (0 – 245 $\mu$M)
Figure A1.7: The overlaid emission spectra (A) $\lambda_{ex}$ 368 nm (B) $\lambda_{ex}$ 425 nm of 152 (8.7 $\mu$M) in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of ct-DNA (0–245 $\mu$M).

Figure A1.8: (A) The overlaid excitation spectra of 152 ($\lambda_{em}$ 510 nm) in 10 mM phosphate buffer (pH 7.4) with increasing concentration of ct-DNA (0–245 $\mu$M) (B) The plot of the changes in the emission spectra of 151 and 152 with respect to increasing concentrations of ct-DNA.
Figure A1.9: Scatchard plot (●) and best fit of the emission data (−) for (A) 151 and (B) 152, in 10 mM phosphate buffer, according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).

Figure A1.10: Scatchard plot (●) and best fit of the emission data (−) for 153 in (A) 10 mM phosphate buffer (pH 7.4) and (B) 10 mM coadecylate buffer (pH 6.3), according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Appendix 2

Figure A2.1: Plot of $(\varepsilon_a - \varepsilon_d)/(\varepsilon_b - \varepsilon_d)$ versus DNA (M, bp) (●) and the best fit of the data (—) for 151 in 10 mM phosphate buffer (pH 7.4) with (A) 50 mM NaCl and (B) 160 mM NaCl, according to the Bard equations 4.3 and 4.4 (Chapter 4, Section 4.3.1).

Figure A2.2: Plot of DNA (M/L) versus DNA/(ε_a-ε_d) (●) and best fit of the absorbance data (—) for 151 (8.7 µM) in 10 mM phosphate buffer with (A) 50 mM NaCl and (B) 160 mM NaCl, according to the Kumar equation 4.2 (Section 4.3.1, Chapter 4)
Figure A2.3: Plot of $(\epsilon_a - \epsilon_f)/((\epsilon_b - \epsilon_f)$ versus DNA $(M, bp)$ (●) and the best fit of the data (—) for 152 in 10 mM phosphate buffer (pH 7.4) with (A) 50 mM NaCl and (B) 160 mM NaCl, according to the Bard equations 4.3 and 4.4 (Chapter 4, Section 4.3.1).

Figure A2.4: Plot of DNA $(M/L)$ versus DNA/$(\epsilon_a-\epsilon_f)$ (●) and best fit of the absorbance data (—) for 152 (8.7 μM) in 10 mM phosphate buffer with (A) 50 mM NaCl and (B) 160 mM NaCl, according to the Kumar equation 4.2 (Section 4.3.1, Chapter 4)
**Figure A2.5:** Scatchard plot (●) and best fit of the absorbance data (−) for (A) 151 and (B) 152, in 10 mM phosphate buffer with 160 mM NaCl according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).

**Figure A2.6:** Scatchard plot (●) and best fit of the emission data (−) for 151 (A) in 10 mM phosphate buffer and 50 mM NaCl and (B) in 10 mM phosphate buffer and 160 mM NaCl, according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Figure A2.7: Scatchard plot (●) and best fit of the emission data (—) for 152 (A) in 10 mM phosphate buffer and 50 mM NaCl and (B) in 10 mM phosphate buffer and 160 mM NaCl, according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Figure A3.1: (A) The excitation spectra ($\lambda_{\text{em}}$ 510 nm) and (B) the emission spectra ($\lambda_{\text{ex}}$ 302 nm) of 151 in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of poly(dG-dC)

Figure A3.2: The emission spectra of 151 in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of poly(dG-dC), (C) ($\lambda_{\text{ex}}$ 368 nm) (D) ($\lambda_{\text{ex}}$ 425 nm)
Figure A3.3: The Plot of \((\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)\) versus DNA (M, bp) (●) and the best fit of the absorbance data (—) using the Bard equations 4.3 and 4.4 (Chapter 4, Section 4.3.1) for 151 (A) with poly(dA-dT) (B) with poly(dG-dC)

\[K_b = 2.8 \times 10^6 \text{ M}^{-1}\]
\[n = 0.22\]

\[K_b = 2.4 \times 10^6 \text{ M}^{-1}\]
\[n = 0.20\]

Figure A3.4: Scatchard plot (●) and best fit of the emission data, \(\lambda_{Ex} 417 \text{ nm}\) (—) for 151 and poly(dG-dC), according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Figure A3.5: (A) The excitation spectra ($\lambda_{em}$ 510 nm) and (B) the emission spectra ($\lambda_{ex}$ 302 nm) of 152 in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of poly(dA-dT).

Figure A3.6: The emission spectra of 152 in 10 mM phosphate buffer (pH 7.4) upon increasing concentration of poly(dA-dT), (C) ($\lambda_{ex}$ 368 nm) (D) ($\lambda_{ex}$ 425 nm)
Figure A3.7: The absorbance spectra of 152 (5.2μM) upon the titration with (A) poly(dG-dC) and with (B) poly(dA-dT).

Figure A3.8: The Plot of $(ε_a - ε_b)/(ε_b - ε_d)$ versus DNA (M, bp) (●) and the best fit of the absorbance data (—) using the Bard equations 4.3 and 4.4 (Chapter 4, Section 4.3.1) for 152 (A) with poly(dA-dT) (B) with poly(dG-dC)
Figure A3.9: The emission spectra of 152 (5.2 μM) in 10 mM phosphate buffer (pH 7.4) upon titration with (A) poly(dG-dC) and (B) poly(dA-dT).

Figure A3.10: Scatchard plot (●) and best fit of the emission data, λ<sub>e</sub> 415 nm (—) for 152 and poly(dG-dC), according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Appendix 4

Figure A4.1: Scatchard plot (●) and best fit of the emission data (−) for (A) 168 and (B) 169, in 10 mM phosphate buffer, according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).

Figure A4.2: Plot of DNA (M/L) versus DNA/(ε_a-ε_j) (●) and best fit of the absorbance data (−) for (A) 168 (8.7 μM) and (B) 169 (8.7 μM) in 10 mM phosphate buffer (pH 7.4) according to the Kumar equation 4.2 (Section 4.3.1, Chapter 4).
Figure A4.3: Scatchard plot (●) and best fit of the emission data (—) for (A) 168 ($\lambda_{ex}$ 433 nm) and 169 (B) $\lambda_{ex}$ 473 nm (C) $\lambda_{ex}$ 361 nm, according to the neighboring-site exclusion model of McGhee and von Hippel, equation 4.7 (Section 4.3.1, Chapter 4).
Appendix 5

Figure A5.1: The emission spectra of ethidium bromide, free and bound to poly(dG-dC) (1.5 \mu M : 3 \mu M) in 10 mM phosphate buffer, upon the titration of 151

Figure A5.2: The emission spectra of ethidium bromide, free and bound to poly(dA-dT) (1.4 \mu M : 2.65 \mu M) in 10 mM phosphate buffer, upon the titration of 151
Figure A5.3: The emission spectra of ethidium bromide, free and bound to ct-DNA (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 152

Figure A5.4: The emission spectra of ethidium bromide, free and bound to poly(dG-dC) (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 152
Figure A5.5: The emission spectra of ethidium bromide, free and bound to poly(dA-dT) (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 152

Figure A5.6: The emission spectra of ethidium bromide, free and bound to ct-DNA (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 168
Figure A5.7: The emission spectra of ethidium bromide, free and bound to poly(dG-dC) (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 168

Figure A5.8: The emission spectra of ethidium bromide, free and bound to poly(dA-dT) (1.4 μM: 2.75 μM) in 10 mM phosphate buffer, upon the titration of 168
Figure A5.9: The emission spectra of ethidium bromide, free and bound to ct-DNA (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 169

Figure A5.10: The emission spectra of ethidium bromide, free and bound to poly(dG-dC) (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 169
Figure A5.11: The emission spectra of ethidium bromide, free and bound to poly(dA-dT) (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 169

Figure A5.12: The emission spectra of ethidium bromide, free and bound to ct-DNA (1.5 μM: 3.0 μM) in 10 mM phosphate buffer, upon the titration of 170
Figure A5.13  CD spectra of ct-DNA (150 μM) in 10 mM phosphate buffer in the absence and the presence of 152 at varying P/D ratios.

Figure A5.14  CD spectra of ct-DNA (150 μM) in 10 mM phosphate buffer in the absence and the presence of 169 at varying P/D ratios.