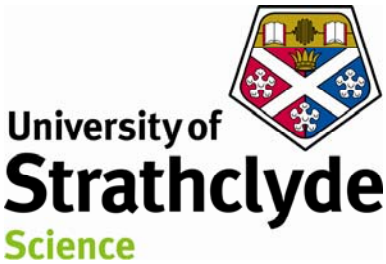
Structure and Binding Affinity in DNA Minor Groove Binders

By

Gavin Donoghue



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Contents

Abbreviations

A	Adenine
Ac	Acetyl
Bi	benzimidazole
Boc	Butyloxycarbonyl
B.P.	Boiling point
C	Cytosine
CE	Capillary electrophoresis
CPI	Cyclopropylpyrrolylindole
Ct	Chlorothiophene
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DMF	Dimethylformamide
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic acid
Et	Ethyl
FSCE	Free solution capillary eletrophoresis
G	Guanine
HBTU	O-Benzotriazole-*N,N,N',N'*-tetramethyl uronium hexafluoro-phosphate
HOX	Homeobox
HPCE	High performance capillary eletrophoresis
Hz	Hydroxybenzamidazole
I	Inosine
Im	Imidazole
Ip	Imidazopyridine
Irp	Isopropyl
I.R.	Infra red
ITC	Isothermal titration calorimetry
MCPBA	*m*-Chloroperbenzoic acid
Me	Methyl
MGB.	Minor groove binder
MIC	Minimum inhibitory concentration

M.P.	Melting point
NMM	N-methylmorpholine
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
O/N	Overnight
PDB	Pyrrolobenzodiazepines
Py	*N*-Methylpyrrole
r.t.	Room temperature
SecBut	Secondary butyl
Spec.	Spectroscopy
T	Thymine
TALE	Three amino acid loop extension
TEA	Triethylamine
Tet.	Tetrazole
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC.	Thin layer chromatography
TMR	Tetramethyrhodamine
TO	Thiazole orange
Ubx	ultrabithorax
UV	Ultra violet
YO	Oxazole yellow

NMR data

s = SINGLET
d = DOUBLET
t = TRIPLET
q = QUARTET
Q = QUINTET
S = SEPTET
o = OCTET
m = MULTIPLET

1. Abstract

Minor groove binders are small molecules that bind selectively to the minor groove of DNA. Many synthetic MGBs are analogues of the natural products netropsin and distamycin; these belong to the polyamide class of MGBs. These compounds consist of two and three methylpyrrole monomers linked by amides, with a head and tail group also linked by amides. They bind selectively to AT base pairs in DNA and have potential antiviral, antibiotic, and anti oncolytic properties, however they are also toxic. Much work has been carried out to create analogues of these compounds with similar or improved activity with reduced toxicity. Analogues of these compounds typically consist of a tail group linked to a sequence of heterocycles via amide bonds ending with a larger head group than formyl.

This project is concerned with developing analogues of Distamycin and netropsin with increased lipophilicity. The aim is to synthesise molecules with improved ability to cross membranes thus improving bioavailability. This primarily involves the synthesis of MGBs with alternative linking groups to amide, namely alkene and diazo. Using linking groups with varying polarity will allow us to test the hypothesis that hydrophobic binding is a major driving force in DNA binding. Using diazo and alkene linkages also opens up interesting applications in terms of the potential colour and fluorescent nature of the resulting molecules.

The second aim of this project is to purposely design a series of molecules that will allow us to explore the link between DNA binding and biological activity. Although it is known that certain groups such as branched alkyl chains produce good biological activity there is still a lot to be learned about how and indeed if these groups actually interact with DNA.

The synthetic routes employed to make the various diazo and alkene analogues of existing compound are described. Also the discovery of several active lead compounds plus the synthesis of analogues of these leads designed to probe the effects of various linking groups and head group substituents on antibacterial activity and DNA binding are described. This was done by testing all compounds for biological activity and by investigating the DNA binding of selected compounds

by physical methods. Various analytical techniques were employed to do this including NMR, thermal denaturation, ITC, capillary electrophoresis and DNA footprinting. A good correlation between antibacterial activity and DNA binding was found. In general it was found that compounds with amide, alkene and diazo bonds all bound to DNA to a certain extent however it would appear that overall alkene linkages produce better results both in terms of antibacterial activity and evidence of DNA binding.

2. Introduction

2.1 DNA: Structure and Function

In order to describe a series of molecules whose primary function is to bind to DNA (deoxyribonucleic acid) it is necessary to give a description, in particular the features of it that facilitate its interaction with the minor groove binders, as well as an overview of its function in the body.

DNA is the material in organisms that allows genetic information to be passed on from parents to their offspring. DNA molecules vary in length depending on their use however in organisms they are generally long and complex and are made up of thousands of genes. Genes are areas of DNA that are responsible for expressing certain characteristics in organisms. DNA essentially is responsible for almost every activity that occurs in a cell. This control is not direct however and is achieved via the ability of DNA to provide the information to produce specific proteins, which are the tools used by the body for most biological functions. Again DNA is not directly responsible for protein synthesis; genes in the DNA direct synthesis of molecules of messenger RNA (mRNA, ribonucleic acid), which is complementary to the DNA, using an enzyme called RNA polymerase in a process called transcription. This mRNA will now contain the information needed to synthesise one or more peptide strands. This information is stored as a specific series of bases. These bases are split into groups of three known as codons, since there are four possible bases in RNA this means there are 64 different codons. These codons encode the twenty standard amino acids meaning most amino acids have more than one codon. The mRNA then interacts with the ribosomes in the cell which decode the mRNA and pair it up with complementary transfer RNA (tRNA) that carries the appropriate amino acid. The amino acid sequence is then built up until the intended peptide strand is completed.[1] Given that the production of proteins is vital to many major functions in the body it is easy to see why DNA is so important. It follows that any potential level of control we could assert over the functions of DNA could be hugely beneficial in therapeutic medicinal terms. This thesis deals with synthetic

molecules with the ability to interact with DNA, the details of which will be discussed further in section 2.3.

Both DNA and RNA are classed as nucleic acids and have the same general structure; they are polymers that are made up of repeating units called nucleotides. The nucleotides are themselves made up of three parts: pentose sugar, phosphate group and a third molecule called a base. This is shown schematically in figure 1.

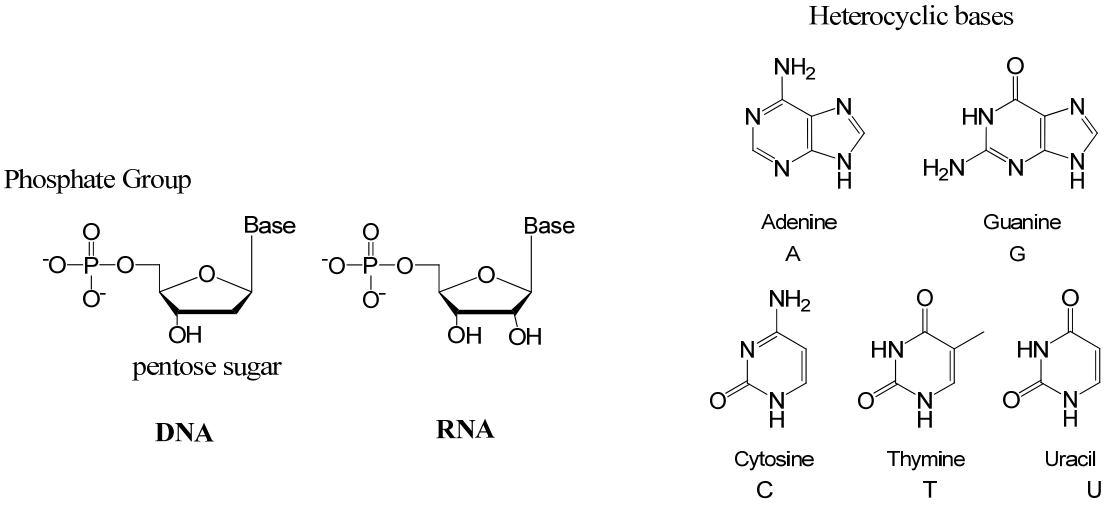


Figure 1

Structural components of nucleic acids

There are five common bases that can occur in nucleic acids and they fall into two categories; purines and pyrimidines. The purines are adenine (A) and guanine (G), the pyrimidines are cytosine (C), thymine (T) and uracil (U). Adenine, cytosine and guanine are found in both DNA and RNA however uracil is specific to RNA and thymine is specific to DNA (figure 1)

The other main structural difference between DNA and RNA is that the 2-position of the pentose sugar in RNA has a hydroxyl group attached whereas in DNA the position is unsubstituted.

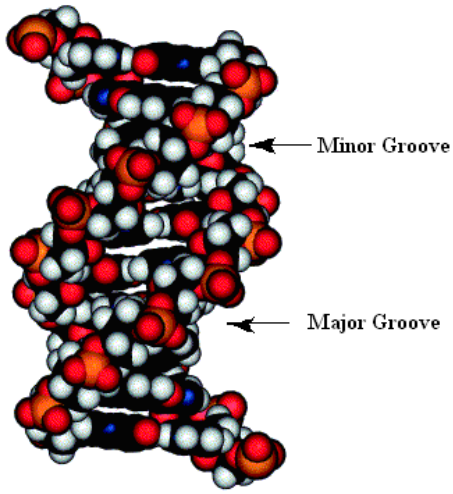


Figure 2

The Watson-Crick model of DNA, (reproduced with permission, see appendix 1a)

In 1953, James Watson and Francis Crick proposed a model for the three dimensional structure of DNA. In this model the DNA molecule consists of two polynucleotide strands bound together spiralling around an axis in a double-helical arrangement.[2] The sugar-phosphate backbones of the two strands point towards the outside of the helix while the bases point inwards. In fact it is the interactions of these bases which allow the DNA strands to bind together in the helix. [3] Hydrogen bonds are formed between the bases on each strand however the structure of the bases is such that hydrogen bonding will only occur between specific bases. One complete turn is 34 Å and encompasses ten base pairs. The dimensions of the major groove are 11.6 Å x 8.2 Å (width x depth) and the minor groove's dimensions are 6.0 Å x 8.2 Å (width x depth), and the helix has an overall diameter of 20 Å (see figures 2 and 3).

Cytosine will only bind to guanine and vice-versa; a similar relationship occurs between adenine and thymine (or uracil in the case of RNA). It is these sequences of bases that allow DNA to code for specific proteins as sequences of three bases (called codons) correspond to specific amino acids which are the building blocks for production of proteins. It is this ability of DNA to influence cellular functions that makes it an important target for drug molecules. The pairings of the bases are demonstrated in fig. 3. The specific pairings of A, T, G and C are based on the number of hydrogen bonds formed between them. We can see adenine forming two hydrogen bonds with thymine while cytosine forms three bonds with guanine.

Effective hydrogen bonds cannot be formed between other pairings, due to clashes of lone pairs of electrons or protons destabilising binding.

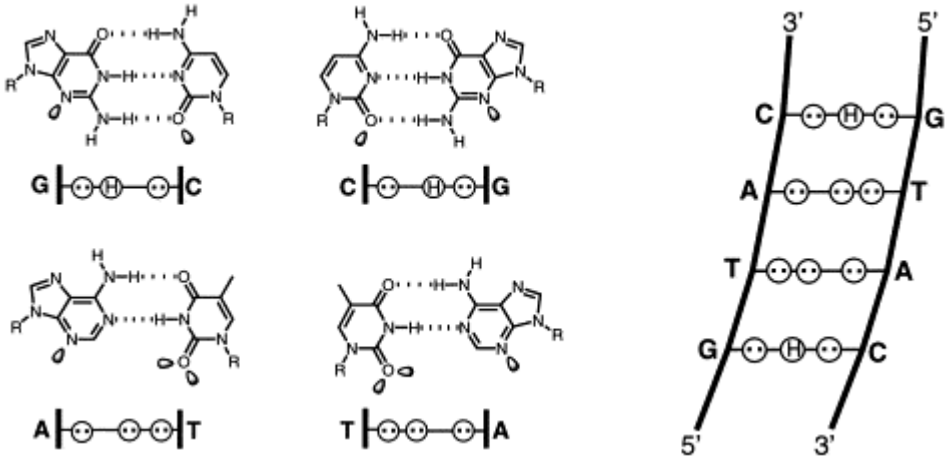


Figure 3

Hydrogen bonds formed in base pairing, (reproduced with permission, see appendix 1a). Circles with dots represent lone pairs of N(3) of purines and O(2) of pyrimidines, and circles containing an H represent the 2-amino group of guanine.

2.2 Chemical Synthesis of DNA [4, 5 ,6]

It became clear that if a method existed to effectively synthesise DNA then a more thorough analysis of it could be made, for example in order to define the genetic code. For this reason there was a substantial amount of research carried out on this subject and there have been several methods used. However the method in figure 4 represents one of the more common methods used for DNA synthesis.[4]

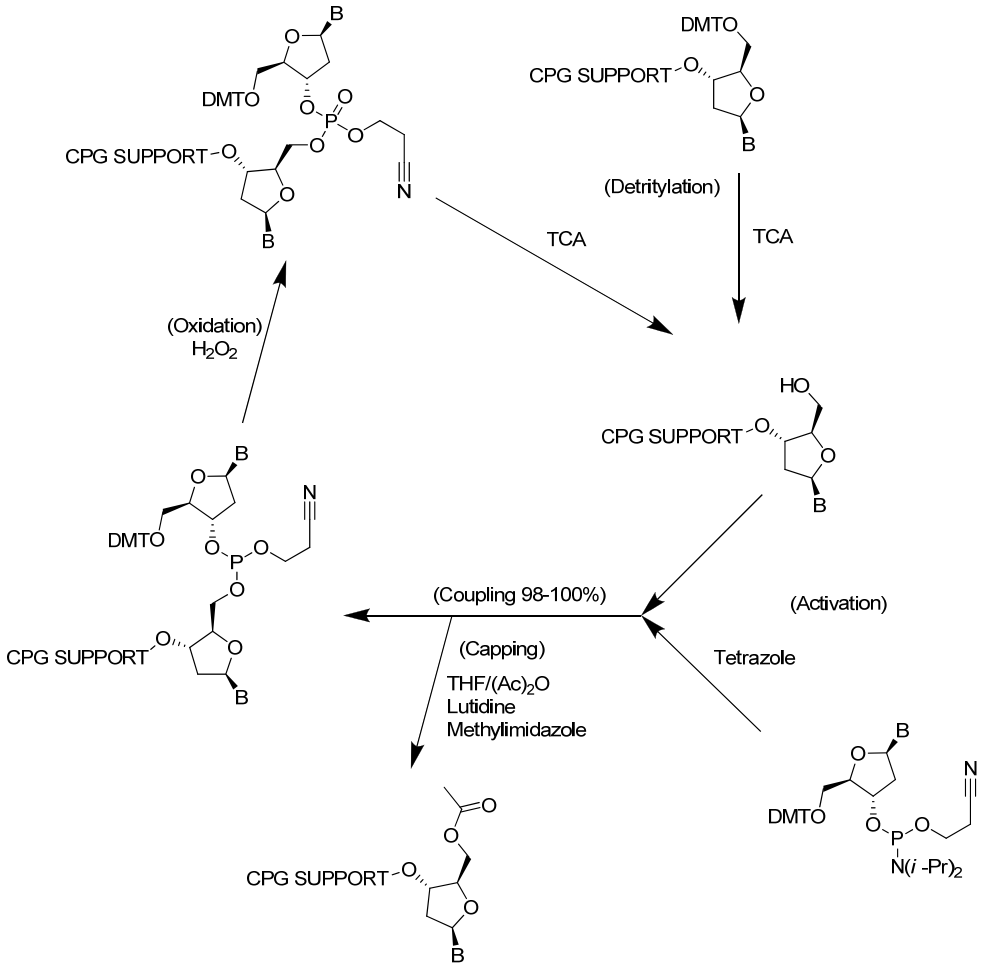


Figure 4

Schematic for the synthesis of DNA

The DNA synthesis cycle begins with the 3'-hydroxyl group of a nucleoside attached to a solid polymer support through a long spacer arm. This allows excess reagents to be used and removed by filtration and eliminates the need for purification steps between base additions.

The first step in the synthesis involves the removal of the dimethoxytrityl (DMT) group with trichloroacetic acid (TCA); this frees the 5'-hydroxyl group for the coupling reaction. The chain is lengthened by stepwise addition of phosphoramidite derivatives of nucleotides which have been activated by the addition of tetrazole. Tetrazole protonates the nitrogen of the phosphoramidite, making the phosphorus susceptible to nucleophilic attack. The resulting intermediate is very reactive therefore the coupling step is complete in less than 30 seconds. To prevent reaction at the 5'-OH group of the phosphoramidite this position is blocked by a DMT group.

The capping step terminates any chains that did not undergo coupling. Any unreacted chains will have a free 5'-OH which can be terminated or capped by acetylation and removed from the synthetic cycle. This is achieved with acetic anhydride and 1-methylimidazole. The DMT group of the successful coupling step protects the 5'-OH end from being capped, this minimizes the length of impurities and therefore gives easier HPLC purification. The internucleotide linkage is then converted from the less stable phosphite to the phosphotriester in the oxidation step. Iodine is used as the oxidizing agent and water as the oxygen donor. After oxidation, the DMT group is removed with TCA and the cycle is repeated until chain elongation is complete. Cleavage from the solid support is then carried out with concentrated ammonium hydroxide. Ammonia treatment also removes the cyanoethyl phosphate protecting groups. The crude DNA in ammonium hydroxide solution is then heated at 65 ˚C for 1 hour to remove the protecting groups on the exocyclic amines of the base. This product is then purified by reversed-phase HPLC.

2.3 Minor Groove Binders

Minor groove binders (MGBs) are molecules that bind to DNA in the minor groove. The minor groove of the title refers to the structure of DNA, which is helical in nature and is made up of two strands bound together. DNA exists in several conformations, however in the B conformation (which is the most common, therefore the most relevant in terms of drug discovery) the gaps between the strands are referred to as major and minor grooves. [7] The gaps alternate between major and minor grooves throughout the whole molecule with the major groove (as the name suggests) being the larger of the two. This is shown in figure 5.

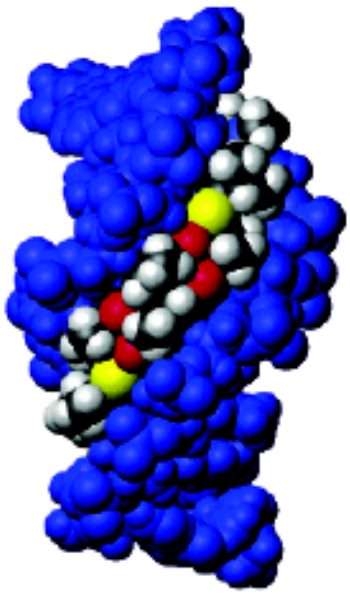


Figure 5

Diagram of two MGBs bound to a DNA strand Key: Blue – DNA. Red, yellow, black and white- MGB (reproduced with permission, see appendix 1b)

Minor groove binders are capable of binding selectively to specific regions of DNA. This is mainly due to the nature of the binding of the two strands in DNA; each strand of DNA is made up of repeating units called nucleotides; nucleotides are made up of a sugar and a phosphate molecule and a structure called a base. Just as base pairing provides for selective binding between DNA strands, MGBs are also able to 'read' sequence information from the base pairs exposed in the minor groove. The binding of MGBs to DNA and resulting base selectivity is due to a combination of hydrogen bonds, hydrophobic interactions and ionic binding. The

ability of the MGB to hydrogen-bond makes it capable of binding to bases, which in turn makes specific sequence recognition possible. The ability to bind to these bases also comes from the fact that the bases are exposed on the floor of the minor groove allowing the minor groove binders access to them. This is shown schematically in figure 3, there are however several other classes of MGB. This will be discussed in greater detail later (section 2.6). [8]

The ability of MGBs to recognise base sequences has many potential applications and several have been researched intensely.[9] The first and probably most useful application is as an anti-tumour agent, in other words, an anti-cancer drug. Cancer is basically the production in the body of a large collection of cells known as a tumour; tumour cells are essentially malfunctioning by replicating themselves to an excessive degree. Cell self-replication begins with the DNA of the cell being replicated in order to produce another, identical, cell. This process is complex and has several steps; one of the first steps is the unravelling of the DNA into two separate strands. By using a minor-groove binder attached to the molecule of DNA, the process of unravelling would be disrupted and thus the growth of the tumour would be halted. [9]

There are also several other applications of MGBs, one of which is the use of them as a dye or a fluorescent marker. By attaching a dye or fluorescent molecule (or by making the MGB itself inherently fluorescent) specific cells containing a certain sequence of bases could be identified. Identification of cancerous cells is a possible application of this feature. [9]

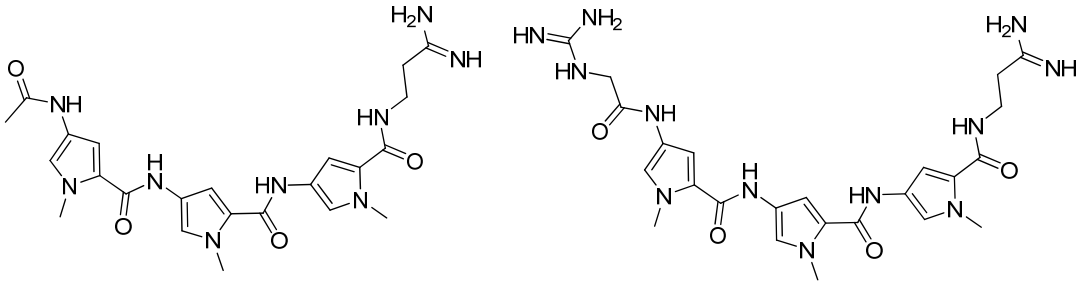
The sequence of bases in a molecule of DNA determines the function of the molecule. Long sequences of bases are called genes and are responsible for the production of various characteristics in the body. This occurs by the use of DNA sequences to synthesise protein molecules. By using MGBs to bind to certain areas of these genes it should be possible to have a degree of control over the expression/repression of these genes. Control over gene expression would have various applications however would likely require sequence-specificity for longer sequences of bases than is currently possible. The importance of controlling genes has increased because the human genome, *i.e.* the sequence of bases in the DNA of

all humans, has largely been mapped and various genes and their applications have been identified.[10] Our knowledge in this area is constantly improving, and along with regular improvements to the specificity and overall binding capability of MGBs, will prove to be an important factor in the development of minor-groove binders.

2.4 Polyamide Minor Groove Binders

When designing a molecule with the goal of using it to bind to a specific region in the body (for example a specific receptor in a cell) the starting point is often examination of natural products. Looking at natural products that bind securely to the site you are examining can often give valuable insights into the nature of the binding capabilities at that particular site and also provide a starting point from which to design synthetic molecules. Natural products themselves may be of no practical use due to reasons of high toxicity or low selectivity, thus the goal in designing drugs is commonly to produce similar molecules that overcome these obstacles. Minor groove binders are no exception and consequently most of the major groups of MGB's can be classed according to the natural product from which they are derived.

There are a number of classes of MGBs. One of the largest and most widely researched is derived from the natural products distamycin (**1**) and netropsin (**2**)(figure 6).[11]



1 Distamycin

2 Netropsin

Figure 6

Structures of netropsin and distamycin

While these compounds are known to have antiviral and antibacterial properties there are various reasons for developing alternatives, such as their toxicity for example. Compounds derived from these natural products have subtle changes made and are known as analogues; however the majority of the new compounds retain the central core characteristics of the originals. An example of this is the feature of five-membered heterocyclic rings bound together with amide linkages present in distamycin and netropsin, which has been used extensively and is known

to produce favourable interactions with the minor groove of DNA. The bond angles produced by the carbon and nitrogen atoms in the amide bonds of these molecules also lead to an inherent curvature in polyamide MGBs. This curvature is significant as it allows the molecule to follow the natural curve of the minor groove of DNA allowing for closer interaction. In fact this curvature is a feature present to some extent in all MGBs; this will be discussed in greater depth in section 2.6. Another significant feature of the amide linkages is that they provide potential sites for hydrogen bonding both as an acceptor and a donor. This is important as hydrogen bonding is a key factor in the binding of MGBs to DNA.

There are various other features of distamycin and netropsin that have been exploited in order to produce effective binding with DNA. For example, netropsin binds with the minor groove in a 1:1 ratio. Distamycin on the other hand has been shown to bind with DNA in a 2:1 ratio, in other words with two molecules bound in the minor groove.[12] This finding had several implications for the design of MGBs. Firstly it implied a certain amount of conformational flexibility in the DNA molecule. The binding of one molecule could be tolerated with a fairly small amount of disruption in the minor groove. The binding of two molecules however would necessitate a large change in the conformation of DNA causing a significant deviation in the conformation from the usual B-form of DNA. Secondly the ability to bind two molecules in the minor groove could have implications for the selectivity of the compounds. The size of netropsin and distamycin, and thus their analogues, has a limitation in that they are capable of binding to only 4-5 bases. Two molecules bound in the minor groove instead of one means that longer sequences could in principle be read. This could be achieved by having two analogues bound together in a head to tail fashion using a linker group; such molecules would then bind side by side in the minor groove. These particular types of molecules are known as hairpin molecules and there are various examples in the literature where this particular feature has been used to great effect. One example can be found in the research group of P.B Dervan. The diagram below shows an example of one of the 'hairpin' molecules designed by this group (**3**, fig. 7) [12] and this will be discussed in more detail later.

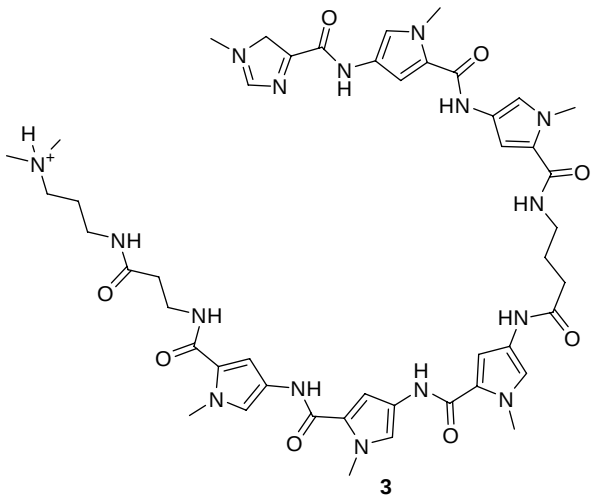


Figure 7

Example of Hairpin MGB

There are also several other features associated with netropsin and distamycin analogues, some of which are unfavourable, and attempts have been made to produce practical solutions to these problems. One of the main problems was in the molecules' ability to bind to specific DNA sequences. Netropsin and distamycin have an affinity for base sequences that are rich in the bases thymine and adenine.[8] There were concerns that molecules that could not bind effectively to sequences including the bases cytosine and guanine would be limited in their capability as antiviral and antibacterial agents. This issue was tackled by Dervan's research group as described in the next section.

2.5 Binding rules

The understanding of the specific H-bonding interactions made by the ligands in the minor groove was instrumental in determining how to create sequence-specific MGBs. An excellent example of this is in the work carried out by a number of parties, notably Dervan, in determining a set of binding rules for minor groove binders.[8] An important breakthrough in this was made prior to any knowledge regarding the ability of two molecules to bind side by side in the minor groove. It was concerned with the acquisition of a detailed X-ray structure of a molecule of netropsin bound in the minor groove of DNA in a paper by Dickerson.[13] From this the hydrogen-bonds made by the ligand could be determined. Figure 8 shows a schematic representation of these H-bonds. This represents the NH's of the amide bond in the minor groove binder making H-bonds with the N-2 of adenine and the O-2 of thymine.

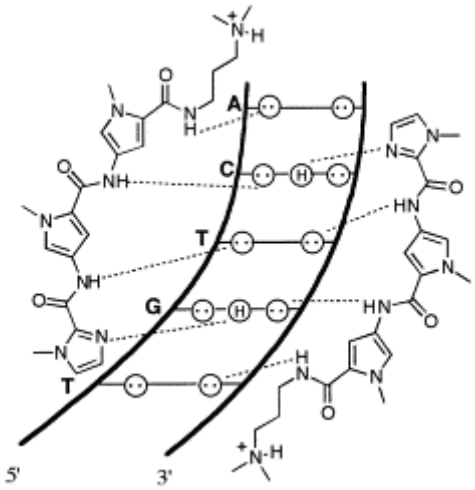


Figure 8

An imidazole containing MGB bound in 2:1 fashion in the minor groove of DNA [11]
(reproduced with permission, see appendix 1a)

Dickerson and Lown concluded that replacing one of the pyrrole rings with an imidazole ring may produce tolerance for the G:C base pair, thus expanding the sequences of bases that could be read.[14] Research into this theory by Wade[15] produced some surprising results regarding sequence specificity that were rationalised in a paper published at the time by Wemmer.[16] This study used NMR to confirm that distamycin could bind to DNA in a stoichiometry of 2:1 rather than

just 1:1 as was previously assumed, thus having two MGBs bound in an antiparallel fashion. It was concluded that the imidazole-containing distamycin analogue produced by Dervan was also binding 2:1 in an antiparallel fashion as shown in figure 8.

It was deduced from this model that it was the pairing of rings side by side that caused that specificity in the reading of sequences. A pairing of imidazole/pyrrole would recognise a G:C pair and a pyrrole/imidazole would recognise a C:G pair meanwhile neither one would recognise an A:T or a T:A sequence.[17] This is due to the fact that the N of imidazole would be able to form a hydrogen bond with the exocyclic amine of guanine. An imidazole on the other side of a G:C base pairing would be unable to form this optimum hydrogen bond thus imidazole will favour G over C. The pairing of pyrrole/pyrrole would recognise an A:T or a T:A pair but would not distinguish between the two. From this information a number of studies was made into improving the affinity of these molecules by creating hairpin structures using aliphatic tethers. Solid-phase synthesis allowed a large number of compounds to be tested in a relatively short space of time.[18] MGB **3** is an example of these hairpin molecules.

This work was later completed when a heterocycle sequence capable of distinguishing A:T from T:A was discovered by the same group. The heterocyclic ring hydroxypyrrole (HP) was used in order to create the unsymmetrical pair of HP/Pyrrole.[19] Compound **4** is an example of the use of the HP group in an MGB (highlighted in red, figure 9).

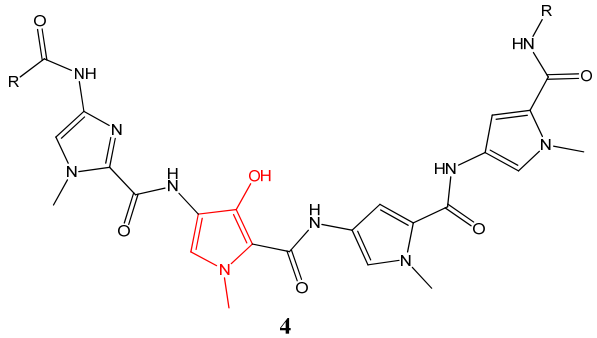


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Figure 9

Example of HP containing MGB

It was thought that introducing a substituent on the corner of a pyrrole ring would produce a steric clash with the base adenine that would be tolerated by thymine.

Thus the pairing of HP/pyrrole would favour the base pairing of T:A while the pairing of pyrrole/HP would favour the A:T base pair. Thus a set of ring pairings was deduced that would distinguish between all four possible base pairings

Since the discovery of these binding rules for sequence specificity several groups, most notably Dervan's, have used them to create huge number of MGBs with a wide range of structures and applications. For example, it has been previously mentioned that MGBs with sequence specificity would have particular usefulness in the area of gene regulation with the mapping of the human genome completed. One example of this is in the work geared towards the inhibition of the NF-κB transcription factor. [20] NF-κB is a transcription factor which is activated via a complex series of biological pathways and is capable of producing significant responses in gene expression through the binding to specific regions of DNA[21]. NF-κB in its inactive form is bound in the cytoplasm of cells by inhibitory IκB; it's activation is brought about primarily by the phosphorylation of IκB. This complex is then eventually broken down into the active form of NF-κB. This complex then permeates the nucleus and binds to specific regions of DNA corresponding to particular genes[22]. This transcription factor is important clinically as it is thought to be a key step in several virus modes of action including HIV. It has also been linked to inflammatory diseases such as arthritis and asthma [19, 20]. For these reasons it would be beneficial to be able to identify the genes this factor is targeting, determine the sequence of DNA associated with it and design a MGB, using the binding rules discussed previously, that would target this sequence. Thus the MGB would effectively act as a competitive inhibitor for this transcription factor.

Dervan designed a MGB that would bind selectively to portions of the binding site of NF-κB, 5'-GGGACTTTCC-3'. This resulted in the synthesis of several compounds with varying specificity. These were effectively split into two groups, those that bound to the sequence 5'-GGACT-3' and those that bound to the sequence 5'-ACTTTCC-3'. This was due to the fact that a molecule that would cover the entire binding site would be of an unreasonable size. It was found that MGBs that bound to the 5'-GGACT-3' site were NF-κB inhibitors while MGBs binding to the site 5'-ACTTTCC-3' were not. An example of the former (**5**) is shown in figure 10. The run of imidazole-imidazole-pyrrole-pyrrole (highlighted in

blue) was the area of the molecule designed to target the sequence 5'-GGAC- 3' while the run of pyrrole-spacer-pyrrole-imidazole (highlighted in red) was designed to target the complementary sequence of 5'-CCTG-3'.

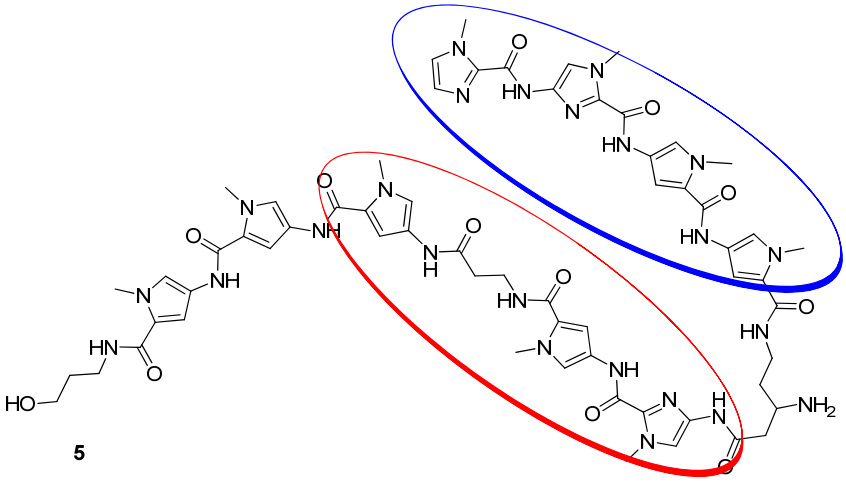

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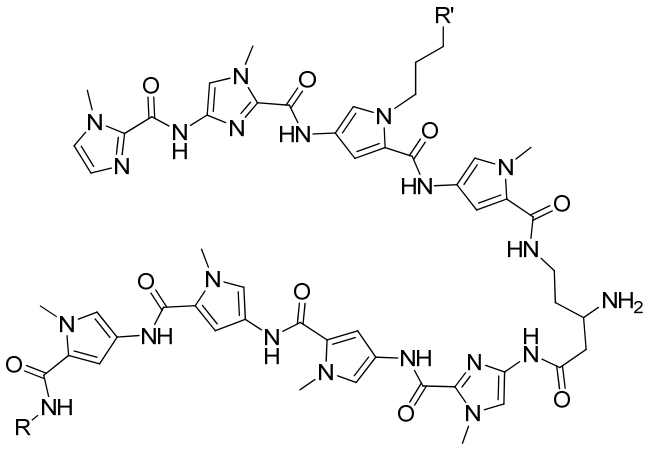
Figure 10

Example of NF-κB inhibitors

The use of hairpin linked MGBs has been expanded within the Dervan group to include various functions for example the area of MGBs as artificial regulators has been recently explored with some success [25]. A good example of this is in the mimicking of HOX proteins.

Hox (Homeobox) proteins, which include the protein Ultrabithorax (ubx), are part of a family of transcription regulators that all contain a DNA binding section that is almost identical across a wide range of organisms[26]. These proteins are of particular interest as they are thought to play a vital role in the patterning of developing embryos [27]. One example where this has been drastically highlighted is in the case of *Drosophila melanogaster* where mutations in the ubx gene resulted in the transformation of Halteres or balancing organs into a set of wings[2829]. Taken on their own, Hox proteins provide generally poor affinity and sequence specificity for DNA, however it is thought that these proteins often interact with TALE (Three Amino Acid Loop Extension) and thus bind to DNA as a dimer. This dimer appears to show much greater affinity to DNA with significant sequence specificity [30]. This interaction is thought to occur via a short peptide sequence of varying length[31] which is a feature of all Hox proteins. Recent crystal studies have shown that ubx

interacts with the drosophile TALE protein extradenticle (exd) through such a peptide link.[32] Dervan's work was centred on synthesising a polyamide MGB with a short peptide side chain that would mimic the action of ubx by interacting with exd to form a DNA binding complex. This led to the synthesis of the following compounds.



6a (R' = H)
6b (R' = Ac-Phe-Tyr-Pro-Trp-Met-Lys-Gly-)
6c (R' = Ac-Phe-Tyr-Pro-Ala-Ala-Lys-Gly-)

Figure 11

MGBs designed to target exd

Tests carried out using electrophoretic mobility shift assays (EMSAs) showed that while complexes **6a** and **6c** (figure 11) were not successful in associating with exd, complex **6b** improves the affinity of exd for its binding site by a factor of ~200.[25] Furthermore, in quantitative DNase I footprinting assays **6b** showed significant affinity for the binding site of exd, 5'-TGGTCA-3'.[33]

2.6 Examples of Variety in MGBs

2.6.1 Duocarmycins and CC-1065 analogues

Other classes of MGB's based on natural products include the complex heterocyclic compounds based upon duocarmycins and CC-1065 (**7**, figure 12). [34, 35, 36] There are several key features in these compounds such as the presence of spirocyclopropylquinone, the shape of which is complementary to the helix of DNA. The cyclopropane ring is also pivotal to their biological activity as it is capable of becoming an alkylating agent with the ability to form covalent bonds with DNA.

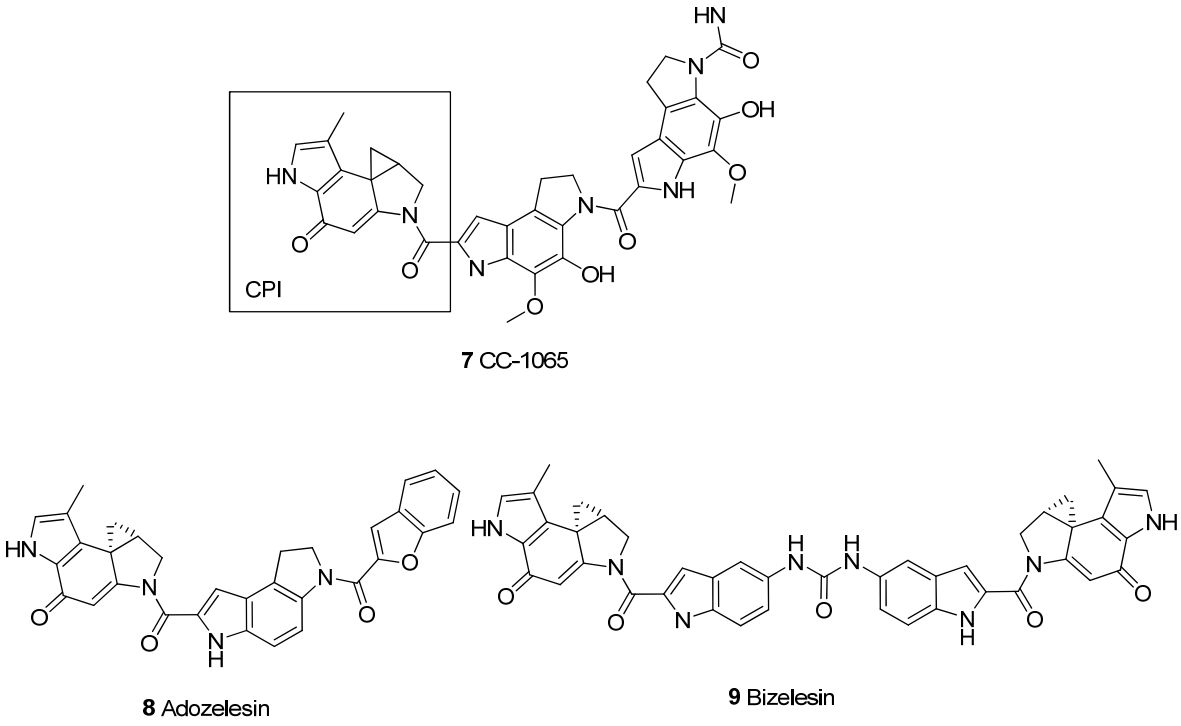


Figure 12

CC – 1065 plus analogues

Again the natural products that are the precursors of this series are unavailable for clinical tests for reasons of toxicity; however the spirocyclopropylquinone functionality means that there is a wide range of variation possible. As before analogues have been produced in large part exploring the different groups that could be used to add to the cyclopropylpyrrolylindole (CPI) moiety which is the main core of the molecule as shown in compound **7**. Examples of this include the

analogues bizelesin (**8**) and adozelesin (**9**) which are molecules that have been taken forward for clinical trials (Figure 12).[34, 36]

A common technique in production of novel minor groove binders is the inclusion in the molecule of functional groups that are known to be biologically active. For example, the polyamide MGBs based on netropsin/distamycin analogues have in the past been extended to include biologically active moieties to improve biological activity. For example nitrogen mustards are known to bind covalently to DNA (by alkylation, similar to CC-1065), they are however toxic. This is partially due to their lack of specificity; incorporating such a group into the structure of an MGB had been shown to improve specificity. One such example of this is tallimustane (figure 13) which has an ID_{50} of 0.5 μg/ml against L1210 cells.[37] Another example of this is brostacillin (fig. 13) which incorporates the DNA alkylating group bromoacrylamide. This has been shown to be active against several tumour cell lines.[38] Similarly the reactive core of CC-1065 and duocarmycins (i.e the CPI functionality) has been included in several classes of MGBs as an extension to improve activity. [11]

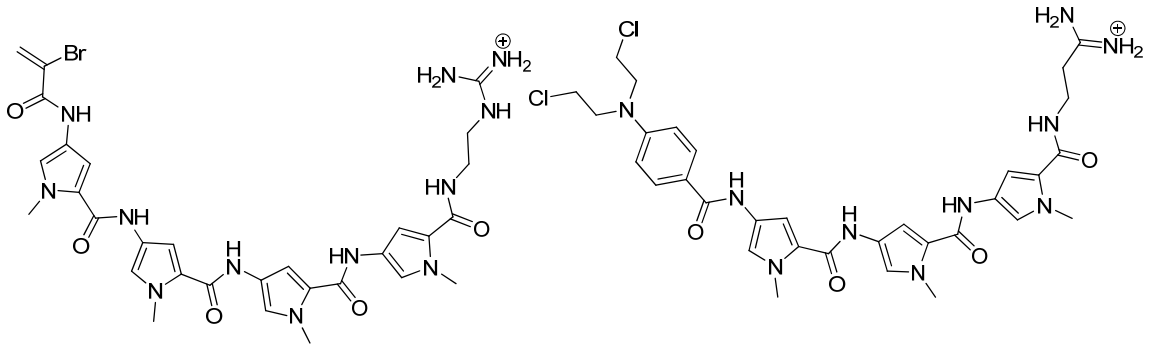


Figure 13

Structures of Brostacillin and Tallimustane

2.6.2 Pyrrolobenzodiazepines

One of the other significant groups of MGBs based on analogues of heterocyclic natural products is the pyrrolobenzodiazepines (PDBs) which are based on the naturally occurring antibiotic anthramycin (**10**, figure 14). As before, key features of the original molecule are retained in the analogues while variation is introduced in various ways to overcome toxicity problems. In the case of PDBs, variation is found by the introduction of different substituents on the benzene ring and by varying the unsaturation in the 5-membered ring. A feature present in all compounds of this type is a functional group, carbonyl for example, that is capable of hydrogen bonding to an amino group from a guanosine base.[8] An example of this is compound **11** which demonstrates both the variation possible on the benzene ring substituents and the varying level of saturation in the five membered ring.[39] This compound was found to be biologically active as antitumour antibiotics at 10 nM levels.

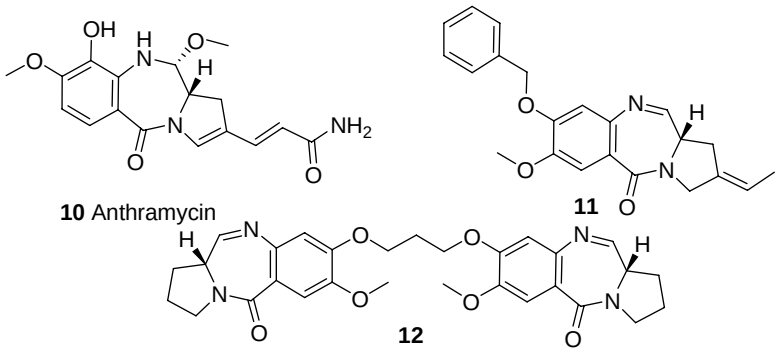


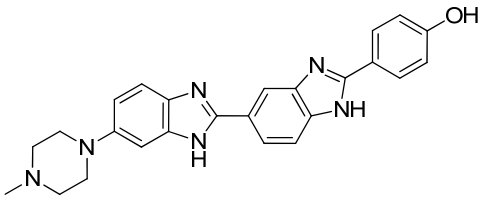
Figure 14

Anthramycin plus analogues

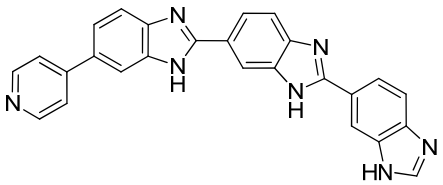
Other areas of interest in this particular class include the synthesis of dimers based on analogues of anthramycin that are bound together by an ether linkage.[40] These molecules are not only more active than their respective monomers but also show greater selectivity for base sequences; this is not entirely surprising given their increased size. This is demonstrated in compound **12** which was shown to be 1000-fold more active than its corresponding monomer. As in the monomers, there is wide scope for introducing variety in the molecules via substituents on the benzene rings of the compounds. Various functional groups have been used in this process including cyano, methoxy and carbonyl.

2.6.3 Hoechst-33258 analogues.

Hoechst-33258 (**13)** was found to bind strongly to the minor groove of DNA, which prompted further research based into benzimidazoles as starting points for design of minor groove binders.[41]



13 Hoechst-33258



14 Example of a Hoechst-33258 analogue

Figure 15

Hoescht – 33258 and example of analogue

This research largely centred around keeping the middle two benzimidazole groups intact and experimenting with different substituents at either end of the molecule. For example the group at Rutgers State University replaced the terminal tertiary amine group with various functional groups such as pyridyl, benzyl and various substituted phenyl groups. Compound **14** is an example of this (figure 15). [42] This class of compound was shown to be a topoisomerase 1 inhibitor with IC_{50} values showing an improvement of 1000 fold over Hoechst compounds.[43] The binding mode of such compounds, observed by studies of their crystal structures, has shown interaction with the minor groove via a series of hydrogen bonds over a binding site of 4-5 base pairs with affinity for repeating A:T sequences.[44] This binding is analogous with the binding mode of Hoechst-33258 itself.[7] As well as the significant biological activity of Hoechst analogues it is important to note that they can also function as fluorescent dyes; this will be discussed further in section 2.10.

2.7 Polyamide MGBs with increased lipophilicity

The examples given previously for different classes of minor groove binders show the variation available in this one group of compounds. One particularly noticeable feature that can be taken from observations of these different classes is the huge differences in structure between them. There are some structural similarities, for example all of these compounds possess an inherent curvature that allows them to match the curve of the minor groove. Another common feature is the ability of these compounds to form hydrogen bonds which as discussed is an important feature of binding to the minor groove. The general structures of these compounds, however, appear to be completely different. These differences in structure come from the fact that they are derived from different natural products. However, it is unusual for a binding site to tolerate such a diverse array of molecular structures.

One reason for this range of structures is the range of flexibility available within the minor groove of DNA. It appears to be able to accommodate a wide selection of sizes of molecule while the resulting disruption to the typical B-conformation of the DNA seems minimal. Essentially the disruption is a widening of the minor groove that has little effect on the overall conformation of the DNA. One outcome of this is that it is unlikely that a drug will be found that has exactly the correct conformation to bind to the minor groove with maximum efficiency, being in the optimal position to produce all interactions with the appropriate residues. It is for this reason that several research groups have based a large part of their study on producing molecules that would be favourable for drug use in ways other than simply their binding capability. One fairly obvious way in which drugs can be improved is in their bioavailability.

The ability of a drug to reach the area in the body at which they are designed to carry out their function depends on factors such as molecular weight, charge and polarity. For this reason there have been various studies into producing bioavailable MGBs, one of which has been carried out by Professor Suckling's group at Strathclyde University and forms the basis for this research project. [8, 45]

Most of the published work on minor groove binders has centred on creating molecules that produce favourable binding to the floor of the minor groove; in particular hydrogen bonding has been focused on.[12] To this end there have been various attempts to create large molecules that bind strongly to the minor groove with base sequence specificity. In the category of polyamide MGBs, one of the most common and successful strategies has been to create dimers of the analogues of natural products (i.e. distamycin and netropsin) and this refers to the hairpin linked molecules mentioned earlier in the report (see compound **3** for example, page 12).[12] While hairpin molecules fit the criteria for reading sequences of appropriate length and show strong binding, there are several concerns over them. The problems are concerned largely with the bioavailability of these compounds. The molecules themselves are very large and this is often a problem when it comes to molecules being able to cross the appropriate membranes in order to reach their target. The size of the molecules is also reflected in the number of H-bond donors/acceptors, the large number of which will also hinder the ability of the molecule to pass through membranes. This is a hypothesis based on Lipinski's rule of 5 which is a series of guidelines to aid in the design of effective drugs with good bioavailability. It states that an orally active drug should have no more than one violation of the following criteria: Not more than 5 hydrogen bond donors; not more than 10 hydrogen bond acceptors; a molecular weight under 500 daltons and finally a logP of less than 5. Despite these molecules obvious violation of the rules of five there have been several hairpin MGBs that have shown significant biological activity. This would suggest that they are able to reach their intended target, however if the bioavailability of them could be improved this could raise their activity even further. Furthermore, making MGBs more compliant with the rules of five could for example increase their potential for use as oral drugs.

A general property related to the ability of a molecule to pass through biological membranes is its lipophilicity. The principal component of biological membranes is phospholipids therefore one of the aims of the Suckling group has been to improve the lipophilicity of minor groove binders. To do this the issue of too many hydrogen bond donors/acceptors needed to be addressed. This was not the only reason to decrease the number of H-bonding sites however. Experimental data have suggested that the main driving force of molecules binding to the minor groove may not, as

originally thought, be the formation of H-bonds. The data suggested that the main driving force may be hydrophobic, in other words the energy benefit from exclusion of structured water molecules from the spine of DNA upon close binding of a receptor molecule.[46]

Taking as an example the work carried out by the research group at Strathclyde University, I will give a review of the work carried out in producing minor groove binders that incorporate all the features listed above. In summary, the aim of the group was to create a series of novel minor groove binders and the design of these molecules focused on several key factors. As in all polyamide MGBs, the template for design was the natural products distamycin and netropsin, particularly distamycin. The size of the molecules would be kept low, comparable to the size of distamycin, and an emphasis would be placed on creating favourable hydrophobic interactions rather than H-bonding interactions.

These factors were taken into account in the design of the molecules by the setting of several criteria for the molecules. One of these goals concerned the surface of the minor groove; this surface has varying positive and negative charges throughout, areas with potential for hydrogen bond donors and acceptors as well as large hydrophobic patches. In order to design an efficient MGB it would be necessary to match the properties of the molecule as well as possible with the areas of the minor groove surface it is likely to interact with.

The issue of lipophilicity would be approached by attempting to raise the logP of the molecules in an effort to aid binding and penetration through membranes. Improving the hydrophobic interactions in the molecule would be achieved by using larger side-chains in the molecules. These strategies will be discussed in greater depth in the next section.

2.8 Lipophilic distamycin analogues.

The research group at Strathclyde has synthesised various distamycin analogues taking into account the factors previously discussed regarding bioavailability etc. All of these compounds had a general structure similar to distamycin as follows:

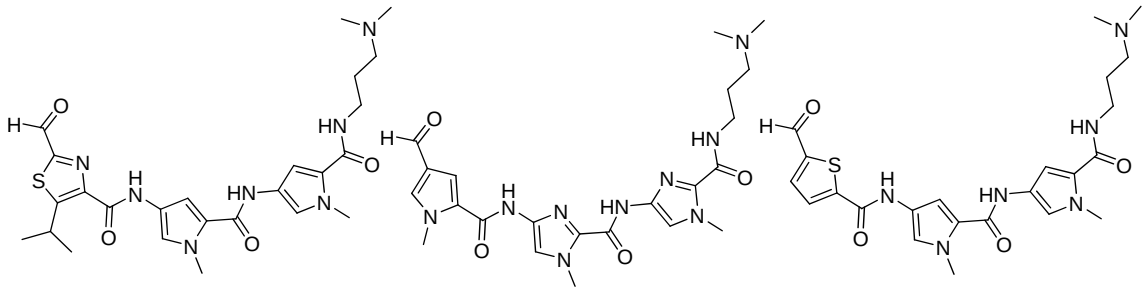
Head group- Ring 1-Ring 2-Ring 3- Tail group

The heterocyclic ring systems in distamycin are exclusively methyl-substituted pyrrole rings. However in order to create diversity and to experiment with binding capability and lipophilicity, a number of other heterocyclic rings were incorporated into the molecules. Diversity was also created by altering the head and tail groups of distamycin from the typical amidine function at the tail group and the formyl group at the head of the molecule. Thus a list of rings to be used as monomers was compiled along with a selection of head and tail groups such that various combinations of these different groups could be achieved via a relatively simple synthetic process.[7]

Various different rings were experimented with including *N*-alkylpyrroles, thiazoles, thiophenes, imidazoles and oxazoles for a variety of reasons. For example, it was felt that the pyrrole rings in distamycin that are substituted with methyl group do not take full advantage of the available hydrophobic interactions on the edge of the minor groove; therefore larger *N*-alkyl groups were investigated. These groups were chosen to represent a wide range of sizes and shapes in order to evaluate the potential for binding to the backbone of the DNA molecule. For example cycloalkyl, isopropyl and 3-methylbutyl were used to represent a fairly wide range of steric bulk. Further discussion of distamycin analogues containing such substituents from the Suckling group along with examples can be found in section 2.12.2.

As previously mentioned a number of compounds were synthesised that incorporated a thiazole ring (**15**) in place of an imidazole ring (**16,** see figure 16). This is due to the fact that thiazoles are substantially more lipophilic than imidazoles (due mainly to the presence of an S atom) but structurally the two

compounds are analogous to one another. Thus a study could be carried out to assess the importance of lipophilicity in the compounds. Thiazoles were also tested because, similar to imidazole rings, they permit binding to GC base pairs (see earlier section on binding rules). Similarly, a number of thiophene containing compounds were synthesised, which, like the thiazoles, incorporate a sulfur atom in the ring but are structurally similar to pyrrole (**17**). Finally in order to complete the comparisons in the molecules for lipophilicity, a small number of imidazoles and oxazoles were made. Again these molecules provide a structural comparison with pyrrole but these compounds would be more lipophilic than pyrrole. As with the pyrrole substituents, a variety of alkyl substituents were experimented with in the thiazole, thiophene, oxazole and imidazole rings.[47]



15 **16** **17**

Figure 16

Examples of variation of heterocycles

The variation in head and tail groups was also mainly concerned with altering the size and shape of the molecule as well as conformational rigidity. One noticeable general feature with respect to this was the removal of the amidine function in the tail group of distamycin, which was replaced with various branched alkylamines in an effort to produce different physiochemical properties. This change in properties would come from the fact that at physiological pH, amidine groups are permanently protonated while tertiary amines are approximately 1% unprotonated under the same conditions. This property would give an advantage in terms of ability to cross membranes.[45] Examples of distamycin analogues with variations at the tail group are shown in figure 17. Typically a dimethylamino group was used; however there are several examples of compounds containing morpholine and pyrrolidine (compounds **18**, **19** and **20**, figure 17).

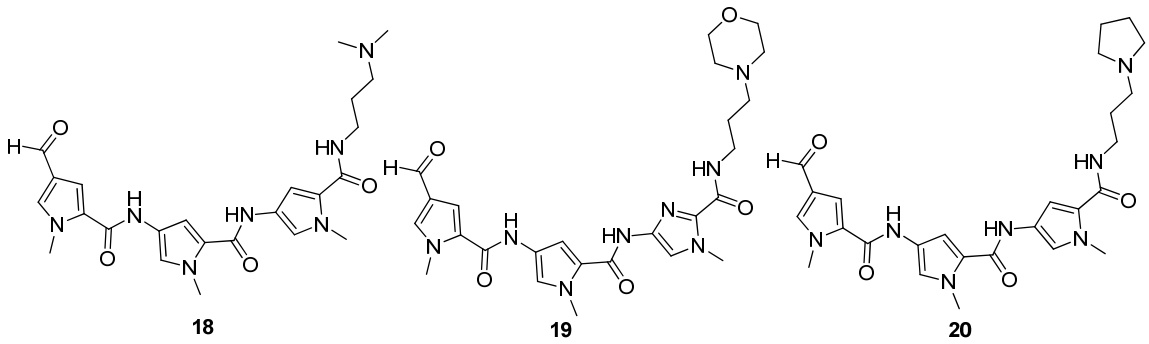


Figure 17

Examples of tail group variation

Similarly there was considerable variation in the use of head groups, ranging from the standard formyl found in distamycin to acetyl (**21**), cinnamoyl (**22**) and dimethylbutyl (**23**) (figure 18).

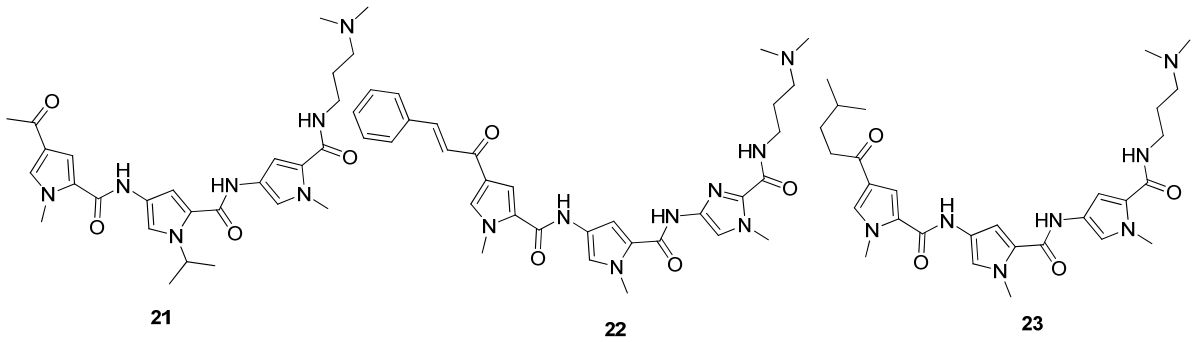
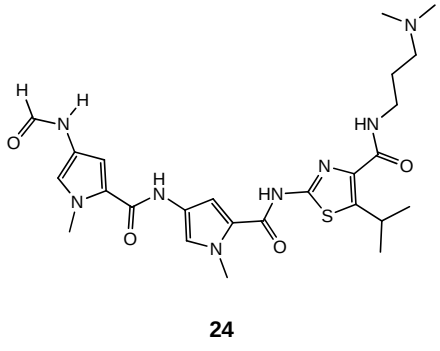


Figure 18

Examples of head group variation

Evaluation of the binding in the synthesised molecules highlighted several general trends mostly in terms of the effect that the different head and tail groups have on the binding ratio of the molecule in the minor groove. There was however one exceptional compound, **24**, which highlighted several important features and directly relates to the work carried out in this research project; compound **24** has become known as thiazotropsin A.



Although it has been previously mentioned that various ring systems were produced, most compounds produced contained only pyrrole rings and as such had affinity for A/T rich sequences (see binding rules section). Compound **24** had noteworthy binding capabilities in that it had affinity for sequences containing guanine bases. Pyrrole rings produce a steric clash with guanine; however thiazole rings have much higher tolerance for this base. Consequently while almost all the other compounds synthesised had affinity for A/T sequences it was found that **24** had very high affinity for the sequence 5'-ACTAGT-3'.[46]

The stoichiometry of these molecules was also of particular interest. Alteration of ratio of ligand to DNA with respect to changes in the head and tail groups has already been mentioned. In a series of distamycin analogues designed at Strathclyde, it was thought that the introduction of bulkier side-chains to improve hydrophobicity might inhibit the side-by-side binding of two molecules by steric repulsion. These molecules had the general structure shown in figure 19 with various bulky alkyl substituents present in the R^1, R^2 and R^3 positions instead of the methyl groups present in distamycin. The side chains used were ethyl, isopropyl, isopentyl, cyclopentyl and cyclopropyl (see figure 19).

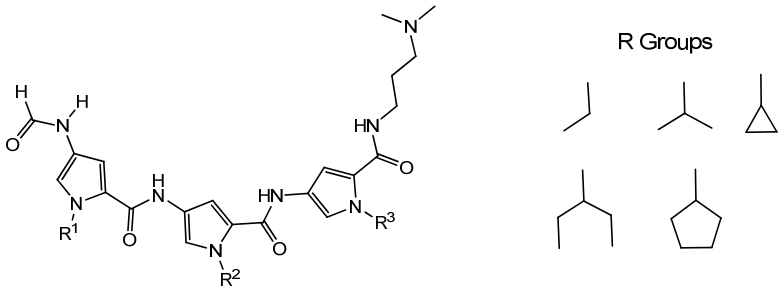
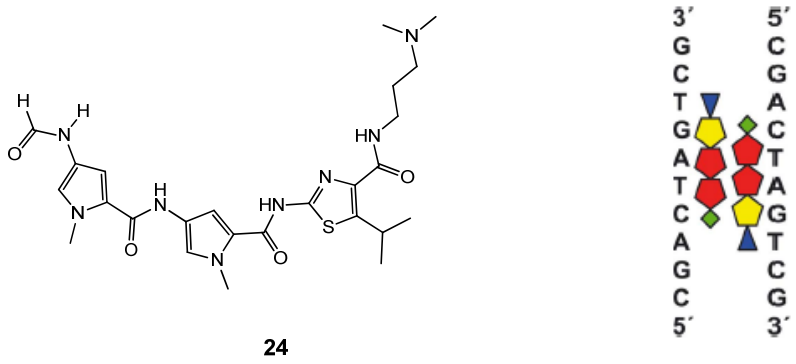


Figure 19

(General structure of distamycin analogues)

This would result in only one molecule bound in the active site resulting in a 1:1 binding ratio. It was found that the introduction of any of these alkyl groups to the R^2 position did not perturb the 1:2 (DNA:ligand). However when two bulky groups were added (isopropyl, at the R^1 and R^3 positions) the binding ratio changed to 1:1. Introduction of isopropyl groups at all three positions showed only very weak binding to DNA while using a bulkier group at this position (isopentyl) resulted in

no binding at all. The idea of using bulkier alkyl side chains was explored further in a later series of compounds; this will be discussed in section 2.12. Binding ratios seemed to be affected mostly by head/tail group variation with the use of any alkyl group larger than the typical formyl head group of distamycin resulting in a shift to 1:1 binding. Similarly the binding mode of the thiazole containing compound **24** was found to be 1:2 indicating the bulky isopropyl group was not affecting side by side binding, however the exact conformation of binding was not initially clear. This was determined by ^{1}H NMR studies that showed symmetrical binding between the ligand and the DNA molecule. Given that the ligand itself is unsymmetrical this could only occur if the two ligand molecules were bound antiparallel head to tail. This head to tail binding could occur in one of two ways, either end to end binding or side by side binding.[45] The side-by side binding model was later confirmed by NMR studies. A schematic of this binding mode is shown in figure 20.



24

Figure 20:

Schematic of two molecules bound in a staggered geometry in antiparallel fashion (reproduced with permission, see appendix 1b) [47]

Key: Green, formyl head; red, N-methyl pyrrole; yellow, thiazole; blue, DMAP tail

As previously mentioned, one of the reasons for the design of this polyamide MGB (**24**) was to increase the potential for hydrophobic interactions, thus disrupting the organised water molecules. Close observation of the molecule **24** bound in the minor groove using NMR to study NOEs showed close contact between the isopropyl group and the hydrogens of the backbone of the DNA.[46] Similar interactions were apparent between the methyl groups of the pyrrole groups and the

backbone. Generally speaking using larger groups would lead to enhanced hydrophobic interactions, leading to greater solvent exclusion. According to the theory that MGB binding is an entropically driven (rather than enthalpically driven) process this would promote tighter binding in the active site.[46]

Another important feature of the binding of compound **24** is concerned with the 2:1 binding mode and is to do with the binding of the two ligands in relation to each other. In the 2:1 binding mode there can be one of two conformations adopted; a staggered geometry or an overlapped geometry. In both of these geometries there is an amide bond in one ligand hanging over the heterocyclic rings. The difference is that in the staggered conformation the first amide bond of one molecule hangs over the thiazole ring of the other molecule whereas in the overlapped geometry the amide bond hangs over the tail group of the adjacent molecule. NMR studies confirmed that compound **24** adopts this staggered formation as shown schematically in figure 20.[47] This has one very important implication in that it allows the reading frame of the ligand to be increased by one. The reading frame is the number of base-pairs in the minor groove that the molecule binds to and therefore determines the number of bases that can be read at any one time by that molecule. With one of these molecules the number of bases expected to be read would be five, however with two of them next to each other in a staggered way it would allow six bases to be read. This is significant as it allows the reading frame of the MGB to be increased without enlarging the molecule and paying the corresponding bioavailability penalty. It also has the added bonus of increasing the distance between the charged tails of the group compared to an overlapped geometry thus reducing electrostatic repulsion. [13]

Figure 21 shows two molecules of compound **24** bound side by side in a DNA strand in an anti-parallel manner and this demonstrates the close fit involved in this type of binding.

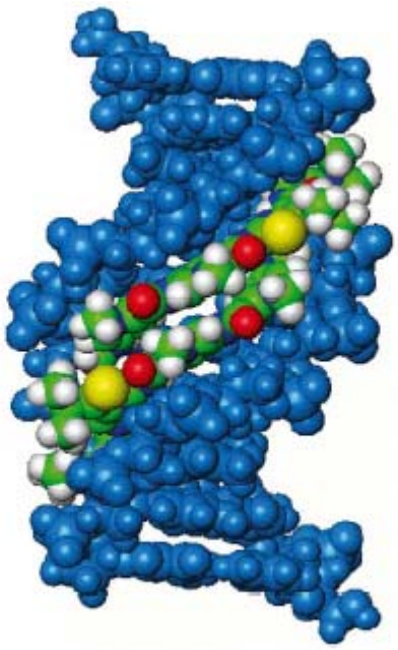


Figure 21

Two molecules of compound **24** bound side by side in the minor groove of DNA (reproduced with permission, see appendix 1b)

So far we have considered the effects of alterations to the type of heterocycle, and to the type of alkyl substitution on the rings. It has been shown that alterations to the alkyl group can affect the binding mode and lead to an extended reading frame. It has also been shown that changing the head and tail group of MGBs can affect their binding modes. Now we will look at the links between the different heterocycles and consider whether changes to them could affect DNA binding.

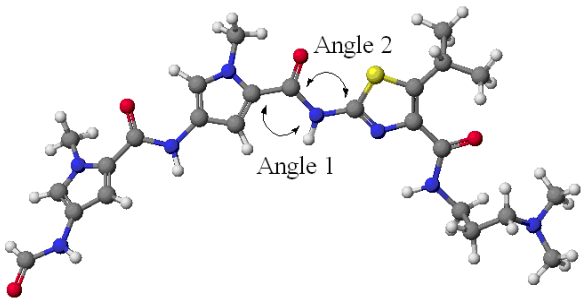
2.9 Amide Isosteres: alkene and diazo linkages.

The aim of this project is to further the investigation carried out at Strathclyde University with regards to synthesising minor groove binders that have increased lipophilicity and promote hydrophobic bonding rather than hydrogen bonding. The starting point in this project comes from the discovery of the thiazole containing compound **24**. This compound not only has a thiazole but also incorporates the bulky isopropyl group to promote hydrophobicity. One of the aims of this project is to synthesise similar molecules that replace one of the hydrogen bonding amide linkages with appropriate isosteres. Synthesis and subsequent biological testing of molecules of this type would provide important information regarding the importance of hydrophobic interactions in minor-groove binders compared with hydrogen-bonding capability. The hypothesis is that the penalty paid in terms of binding for losing hydrogen-bonding sites will be made up for by the hydrophobic interactions produced.

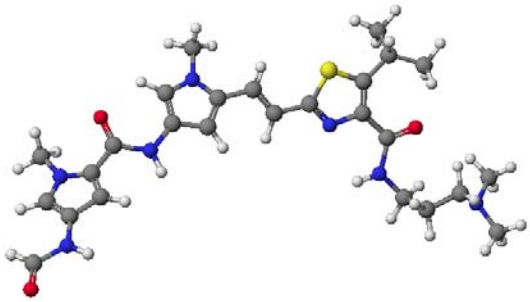
One of the most likely replacements for the amide function is an alkene bond. This has similar geometry to an amide link and would cause little disruption to the shape of the molecule as a whole. Alkene linkages represent a non-polar alternative to amide links; thus any potential for hydrogen bonding in this area of the molecule would be removed. This would be important in terms of investigating hydrogen bonding compared with potential hydrophobic interactions. Another possibility for an isostere would be a diazo linkage; again this molecule has similar geometry to an amide link and would likely be a reasonable replacement. In addition, the diazo bond would be an interesting alternative to the amide and alkene bonds, offering different possibilities in terms of hydrogen bonding compared with the amide bond. The amide bond contains a dipole while diazo and alkene links do not. Also given the fact that increasing lipophilicity has been an important feature in MGB design at Strathclyde, the differences in logP would be significant. A simple comparison of benzanilide, stilbene, and azobenzene using calculated logP gives values of 2.65, 4.83, and 3.85 respectively. As expected, the alkene is the most lipophilic with the diazo link between the alkene and the amide. An MGB with a diazo link would therefore offer further insight into the importance of hydrogen bonding in MGBs and the importance of increasing lipophilicity. In addition to potential binding

advantages of the diazo linkage the compounds produced would almost certainly be coloured; diazo compounds are generally used as dyes due to an extended conjugated π-electron system that allows them to absorb in the visible region of the electromagnetic spectrum. There are several advantages of having a minor groove binder that is a dye; for example a dye would be much easier to track in the body or even in a cell if, for example, a better understanding of exactly where in the body a drug would act. In addition, if a minor groove binder were able to specifically target base sequences corresponding to cancerous cells and was also a dye, this would allow easier identification of the areas in the body affected by the cancer. Similarly, use of an alkene linkage could produce fluorescent compounds that could also be used as markers and potential diagnostic agents.

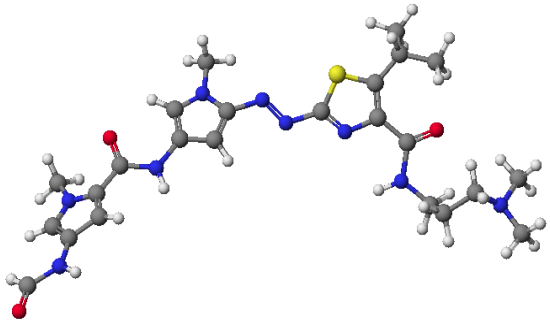
Figure 22 shows compound **24** compared with the same molecule with the amide function closest to the thiazole ring replaced with, firstly an alkene bond and secondly a diazo linkage.



Original Thiazole molecule **24** (amide linkage)



Thiazole molecule (alkene link)



Thiazole molecule (diazo linkage)

Figure 22

(Compound **24** with amide, alkene and diazo linkages)

Comparison of these three molecules shows that the change in the geometry of the molecule upon changing the amide link is minimal. In both the case of the alkene isostere and the diazo isostere a slight shortening of the bond is observed as expected (see table 1).

Bond	Bond angle 1 (^{o})	Bond angle 2(^{o})	Bond length (Å
Amide	114.8	119.7	1.367
Alkene	120.1	119.9	1.337
Diazo	107.4	107.4	1.248

Table 1

Bond angles for alkene, amide and diazo linkages. See fig. 22 for reference to bond angle 1 and 2.

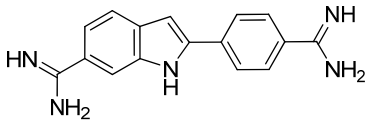
However the bond angles produced in relation to the pyrrole and thiazole rings are very similar. In fact superimposition of these molecules on the original amide molecule shows no significant change at all. Therefore taking into account the fact that the original amide compound provided a good fit in the minor groove (see diagram of **24** in minor groove, figure 21) it is reasonable to assume that either of these isosteres would be appropriate for use in a minor groove binder.

In order to investigate this theory the research group at Strathclyde University has begun to synthesise various minor groove binders that incorporate alkene and diazo linkages. These compounds have been designed to represent a range of structural diversity while at the same time providing an interesting comparison with similar compounds containing amide linkages. The spectroscopic properties of these compounds have been a very important feature, especially in terms of colour and fluorescence. These properties have yet to be fully investigated but it is likely that they will become important soon. The next section will describe in more detail why coloured and fluorescent compounds are useful in drug design.

2.10 Coloured and Fluorescent compounds as Minor Groove Binders

There are various applications for coloured compounds or fluorescent compounds in medicinal chemistry. They are frequently used as dyes for biological markers, to identify chromosomes for example.[48] Their spectroscopic properties may also be exploited, a compound designed to bind to a specific biological target could have a change in fluorescence upon binding. It therefore is a logical step to attempt to produce minor groove binders with interesting spectroscopic properties. It would be extremely useful to be able to identify sequences of DNA with fluorescent or coloured markers especially if the marker was specific to certain sequences of DNA. One obvious way to achieve this would be to simply attach a fluorescent molecule to one end of a simple DNA intercalator. It has been demonstrated that it is possible to combine two different moieties in an MGB that have different functions and still produce a useful molecule as demonstrated in section 2.6.1. Although this is possible it would be more useful to design an MGB that has these spectroscopic properties built in to the structure.

There have been several examples of these types of compounds in the literature some of which are unsymmetrical cyanine dyes related to the Hoechst 33258 (**13**) basic design.



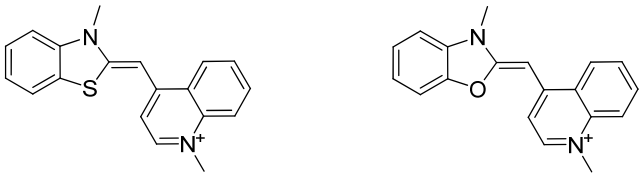
25. DAPI

Figure 23

Structure of DAPI

Compounds such as Hoechst-33258 (**13**) [49] and DAPI (**25**, figure 23) [50] have previously been used for their spectroscopic properties. Both show increased fluorescence upon binding to DNA.[51] This increase is significant and allows for their use as fluorescent markers in various contexts, for example the tethering of the Hoechst fluorophore to DNA triplexes has been used to provide insight into the stabilisation of such structures by DNA intercalators. [52]

There are examples in the literature of dyes that have much more appealing spectroscopic properties. Cyanine dyes such as picogreen, YO (**27**) and TO (**26**) show increases in fluorescence upon DNA binding of up to 1000-fold compared to around 20 fold for DAPI and Hoechst (figure 24).[53] These dyes also have various applications; for example picogreen has been used to investigate the differences of binding to single-stranded and double-stranded DNA.[54] The drawback for these dyes is that they show little or no specificity for any sequence of DNA and this limits their usefulness to a degree. In fact these compounds do not bind in the minor groove of DNA; instead they intercalate thus extending the DNA helix. They have also been known to bind to single stranded DNA.

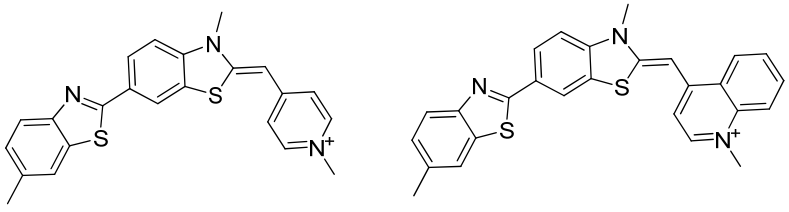


26 TO **27** YO

Figure 24

Structures of TO and YO

There have been several attempts to produce minor groove binders with properties similar to those of the dyes mentioned above. One example of this was carried out by Karlsson *et al.* who attempted to combine the properties of the cyanine dyes with minor-groove binding capabilities of Hoechst compounds. The result of this was the synthesis of several unsymmetrical cyanine dyes such as BEBO (**28**) [55] and BETO (**29**) (see figure 25).[56] These molecules extended the basic structure of the dyes with a further benzothiazole unit. These compounds have some obvious structural similarities to Hoechst compounds; also the curvature of the compounds would lend themselves to binding in the minor-groove.



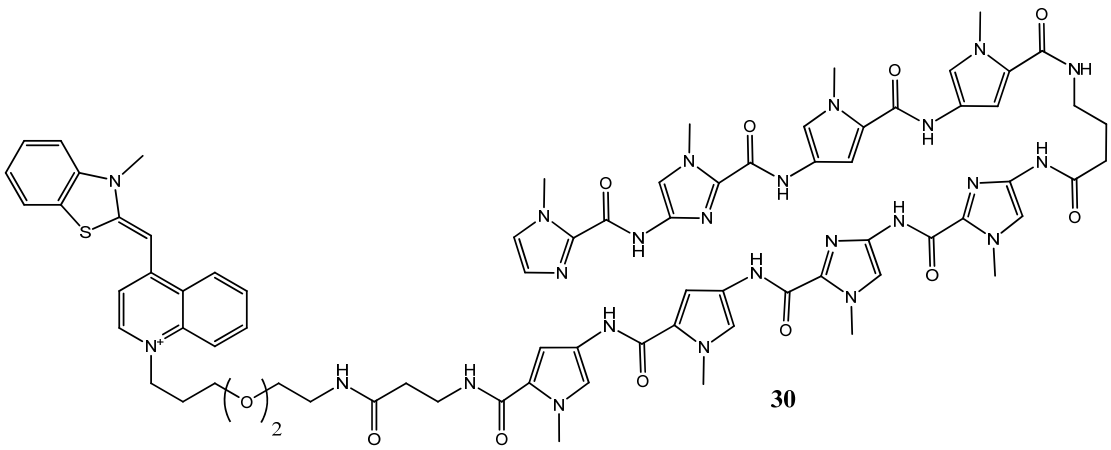
28 BEBO **29** BETO

Figure 25

Structures of BEBO and BETO

These examples show that there has been some research into the area of fluorescent DNA binding molecules; however there is a lot of room for expansion in this area, particularly in minor groove binding molecules.

More recently there has been extensive work carried out in this field by Dervan's group at Caltech. Much of this work has been involved with the tethering of fluorescent moieties to polyamide MGB structures. This work has been mainly geared towards the detection of double-stranded DNA in solutions. Previously pyrrole-imidazole containing MGBs have been conjugated with known fluorophores for example thiazole orange (TO, see compound **30**, fig. 26) and tetramethyl rhodamine (TMR)[57-59]. It was discovered that in the case of TMR conjugates fluorescence in these compounds was quenched when covalently bound to the pyrrole of the tethering MGB. It was subsequently discovered that this fluorescence was restored upon binding to double-stranded DNA. Other compounds incorporating TO for example showed a marked increase in fluorescence upon binding to double-stranded DNA. The advantages with respect to detection of DNA in either case are obvious.



30

Figure 26

Example of MGB tethered to TO

This idea was then expanded to the design of MGBs that incorporate a fluorophore directly into the structure of the molecule to the extent that it would contribute to the sequence specificity of the molecule. Compound **31** (figure 27) is an example of one of these molecules containing chlorothiophene-benzimidazole (Ct-Bi), pyrrole-

imidazopyridine (Py-Ip) and imidazole-hydroxybenzimidazole (Im-Hz) recognition modules and was shown by DNA footprinting studies to have sequence specificity for the biologically significant DNA sequence 5'ATACGT- 3'[60-62].

A library of double-stranded DNA hairpins containing six base-pair matches along with various mismatch sites was then used to assess the emission properties of the molecule. It was found that a marked increase in fluorescence was observed in the molecule following addition of DNA containing its match site. The addition of DNA containing single base-pair mismatches caused a moderate lowering of fluorescence activity while the addition of DNA with multiple base pair mismatches caused a significantly larger decrease in activity.[63]

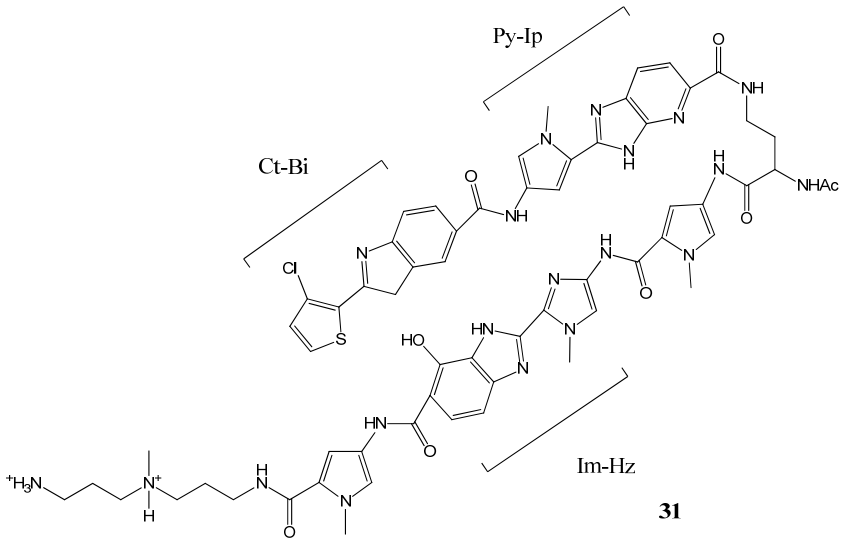


Figure 27

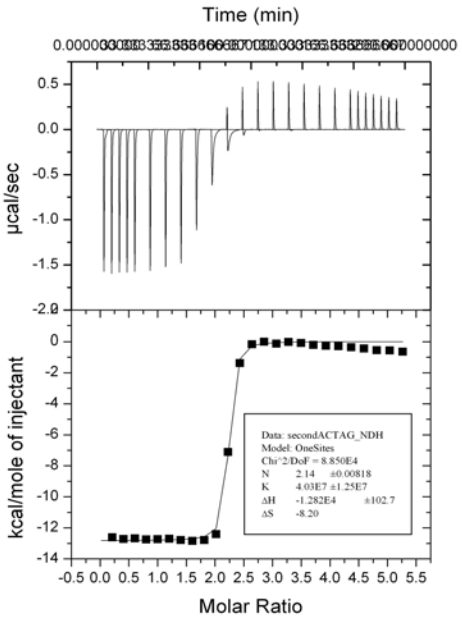
Example of MGB incorporating fluorophore

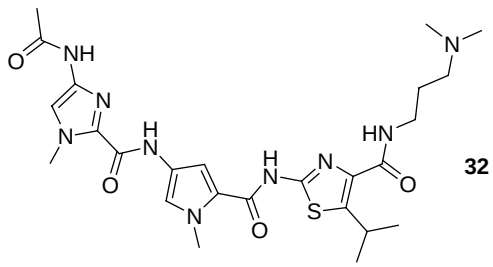
2.11 Methods used for MGB Analysis

A large part of this project involves an investigation into the binding properties of MGBs. This section deals with the various analytical techniques that are available to carry out such an investigation.

2.11.1 ITC (Isothermal titration calorimetry) [68]

Isothermal titration calorimetry is a method by which the Gibbs free energy change (ΔG) produced when a ligand binds to a particular binding site is measured. This is achieved by the titration of a syringe containing a ligand (in this case an MGB) into a sample cell containing DNA via a series of injections. When this binding occurs there is an associated exothermic or endothermic response, the resulting temperature change is monitored and kept at zero (relative the reference cell, containing buffer) by increasing or decreasing the feedback power to the sample cell. Ultimately a graph is produced similar to the one in figure 28.





32

Figure 28

Example of ITC curve
This diagram shows MGB **32** being titrated against a DNA oligomer.

The area under each peak in this graph is equal to the heat released/absorbed for that particular injection; this integrated heat is then plotted against the molar ratio of DNA in the sample cell thus giving the binding isotherm for the injection. The data is then fit as shown in the graph (figure 28) based on the number of binding sites, in this case one. From here the ITC software can carry out several calculations to give the binding ratio, binding constant (K), enthalpy change (ΔH) and entropy change (ΔS). From this the Gibbs free energy (ΔG) can be calculated from the following relationship: $\mathbf{\Delta G = \Delta H - T\Delta S}$

2.11.2 UV melt experiments [64]

UV melt experiments are used to determine the strength of binding of a particular minor groove binder to DNA. This is measured by the stabilising effect on a sequence of the MGB on double-stranded DNA. When two complementary strands of oligonucleotide are placed in solution under standard conditions (room temperature in a buffered solution) they will interact to form a double-stranded duplex according to the Watson-Crick model of a DNA molecule. If this solution is raised past a certain temperature (dependent on concentration and base sequence for example), the hydrogen bonds between the base pairs will break and the two strands of oligonucleotide will separate. As the temperature is raised in a solution of double stranded DNA a process called denaturation takes place. This is a process by which the hydrogen bonds holding the DNA strands together break as the thermal energy becomes too great for them to remain intact and the strands begin to separate. Eventually if the temperature is raised high enough the strands will separate completely. The mid-point of this denaturation process is called the melt temperature or T_m of the DNA. This T_m can be determined by exploiting the difference in UV absorption between single-stranded and double-stranded DNA. The UV absorbance of single-stranded DNA is much higher than that of double-stranded DNA. Placing the two strands of DNA in solution in a cell of a UV spectrometer and raising the temperature in the spectrometer should provide a UV spectrum that shows a pronounced increase in absorbance of approximately 15% at the UV melt temperature. From this spectrum a precise temperature can be derived that represents the mid point at which the two DNA strands separated. The mid-point of this transition is the melting temperature of the oligomer. Taking a 1^{st} derivative of this spectrum provides a graph showing a peak which corresponds to this mid-point thus an accurate melting point can be obtained (see figure 29 for example of spectrum).

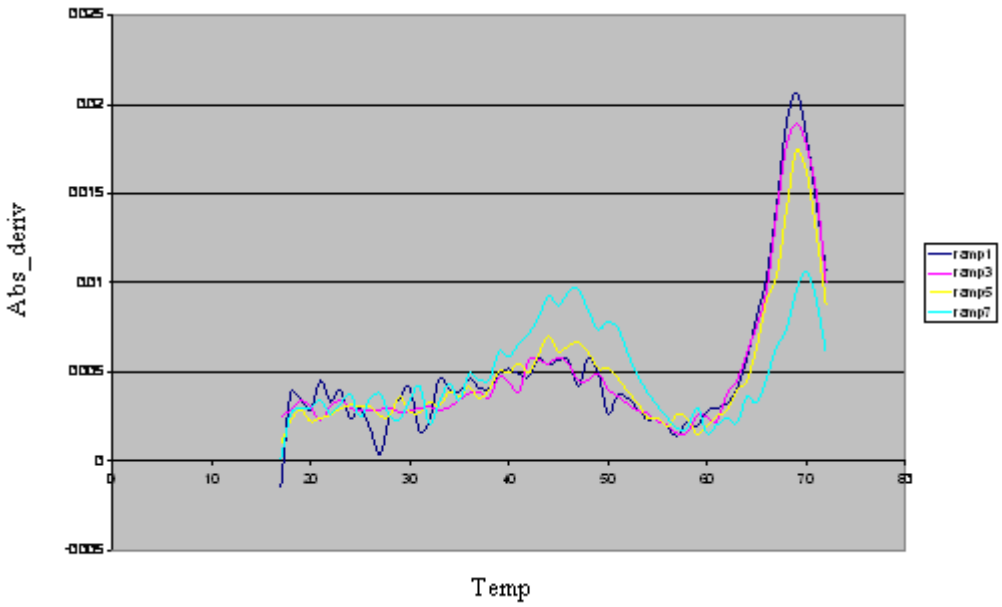
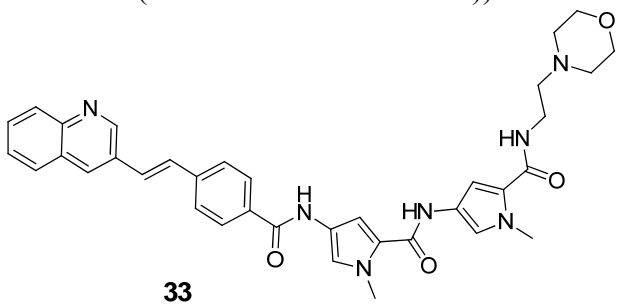


Figure 29

Example of 1st derivative of UV melt trace.
(This is a trace of MGB **33** bound to the DNA sequence 5'- GCGATATATGCG- 3'
(see section 3.5 for details))



33

If a molecule that binds to DNA is placed in solution with the two strands of oligonucleotide then binding will occur as soon as the DNA duplex is formed. DNA binding molecules have a stabilising effect on double stranded DNA, thus having the molecule bound will mean that more energy will be required to separate the two strands. Therefore the UV-melt temperature of a DNA molecule bound to an MGB for example will be higher than that of unbound DNA. By comparing the rise in UV-melt temperature with that of a standard intercalator of known binding strength, a measure of the binding strength of the molecule can be determined. The same property of double-strand stabilisation is shown when minor groove binders associate with a target oligonucleotide.

2.11.3 Capillary Electrophoresis

The term electrophoresis refers to the migration of a charged species in an electrolyte solution upon the application of a potential gradient to that solution. The rate of migration of a particular species is dependent on a number of factors including charge, shape and size. Thus this technique can be used to separate different components in a mixture depending on their different characteristics. The charged species in the electrolyte solution will assume a constant rate of migration towards the cathode (cations) or towards the anode (anions). The rate of migration of a species becomes a constant when the attractive force exerted by the electrode is balanced by the frictional forces resulting from the movement of the species through the solution. The friction is a direct result of the factors mentioned earlier (size, charge, shape of species) and the net result of all these forces is characterised by the term electrophoretic mobility (μ).[65]

High performance capillary electrophoresis (HPCE), also known as free solution capillary electrophoresis (FSCE), involves the process of electrophoresis taking place in a narrow bore fused silica capillary linked to an on-line detector. Different components of a mixture that have been inserted into the capillary will migrate along it depending on the potential gradient. The different components will then pass through the detector producing a profile similar to chromatographic peaks (see figure 30 for an example).[65]

The relevance of this technique to minor groove binders is that it can be used to study ligand-DNA interactions.[66] In this paper, FSCE was used to determine the sequence selectivity of simple DNA intercalators and MGBs by titration of the compounds with two DNA sequences. In order to do this a method of CE was developed that was capable of separating two equally sized oligonucleotides based solely on their base sequence. This was significant as previously separation of oligonucleotides had only been achieved based on their differing lengths.[67] In this experiment each of the DNA binding molecules were placed in solution with two DNA duplexes (oligonucleotides) each twelve bases in length. One comprised an A and T-containing sequence and the other comprised a G and C sequence.

FSCE was then carried out on each of these solutions and the resulting profiles analysed. A profile containing peaks corresponding to each of the AT and GC sequences would indicate that no binding had occurred. A profile that showed that the peak corresponding to the GC sequence had disappeared to be replaced with a new peak would indicate that the binder had formed a complex with that sequence indicating preferential binding for GC sites over AT sites. The identity of the new peak would then be confirmed showing that it was in fact a complex formed between the binder and the GC duplex. This is a powerful analytical technique in the field of MGBs as it allows the possibility to estimate binding strength, binding ratios and sequence specificity. Figure 30 shows an example of a CE trace.

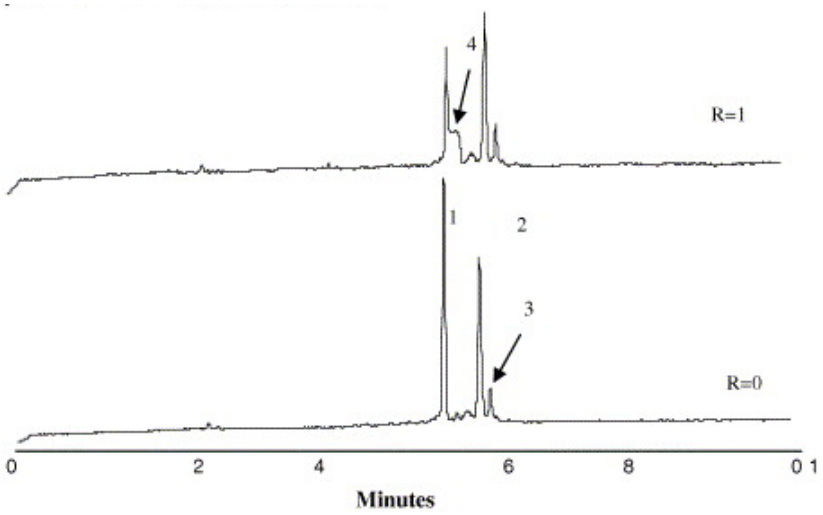


Figure 30

Electropherogram showing titration of compound **24** (see page 29) against a mixture of double stranded AT and GC dodecamers (5'-AAATTATATTAT/5'-ATAATATAATTT and 5'-GGGCCGCGCCGC/5'-GCGGCGCGGCCC). R = **24** :oligomer ratio. 1 = free AT dodecamer, 2 = free GC dodecamer, 3 = unhybridized fragment, 4 = AT dodecamer–**24** complex. Y-axis is absorbance (260nm)[67]

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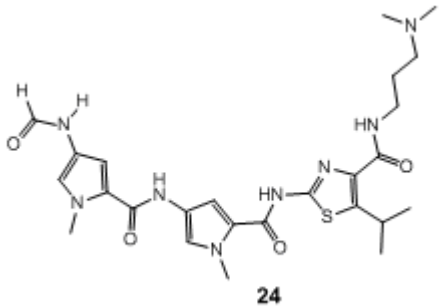


24

2.11.4 Potential uses for coloured/ fluorescent minor groove binders

One of the main advantages of synthesising a series of compounds that are fluorescent (alkene compounds) or quenchers (coloured compounds: diazo linkages) is the ways in which their physical properties can be exploited. The paper by Hampshire *et al*. [69] provides a template for the types of measurements that could be taken with such compounds. In this paper the DNA sequence recognition of thiazotropsin B (**32**, page 40) was tested using a variety of techniques; those of interest here are the tests involving fluorescence measurements using a technique first described by Darby *et al*. [70]

Thiazotropsin B (**32**) is analogous to thiazotropsin A, the major difference being that the first pyrrole unit of the molecule is replaced with an imidazole. As previously mentioned, thiazotropsin A has an affinity for the DNA sequence ACTAGT and it was thought that due to its different structure thiazotropsin B would have an affinity for a slightly different sequence. One of the proposed sequences or binding sites for this molecule was ACGCGT. In order to test this proposal, a strand of DNA incorporating the sequence ACTAGT was synthesised with the fluorescent molecule fluorescein attached at the 5' end. The complementary strand was then synthesised with a molecule of the dye methyl red at the 3' position. When the two strands were placed in solution the two strands form a DNA duplex. This causes the fluorescein and the methyl red to come into close proximity. The methyl red (which is a quencher) quenches the fluorescence of the fluorescein. Heating this solution to a certain temperature then causes the duplex to melt thus separating the fluorescent molecule from the quencher with a consequent rise in fluorescence. Measuring the point at which the rise in fluorescence occurs will give the melt temperature (T_m) of the DNA duplex. The same process was carried out using a DNA strand incorporating the ACGCGT sequence and its complementary strand. If the same experiment was carried out with the addition of a DNA intercalator, the stability of the duplex would rise due to the binding of the intercalator with the duplex irrespective of sequence. Therefore in order to test the binding strength of thiazotropsin A and B with each of these sequences each molecule was added separately to a solution of each sequence and the rise in (T_m) observed in each case. It was found that, as expected from previous

results [71] thiazotropsin A (**24**) caused a large increase of 19°C in the T_m of the sequence ACTAGT and had little effect on the T_m of the sequence ACGCGT with a Tm increase of 3.1°C. In the case of thiazotropsin B the reverse was found to be true with a large increase of 12°C observed in the T_m of ACGCGT and a much smaller T_m increase (4°C) for ACTAGT thus proving that the binding sites of the two molecules are significantly different (see figure 31).

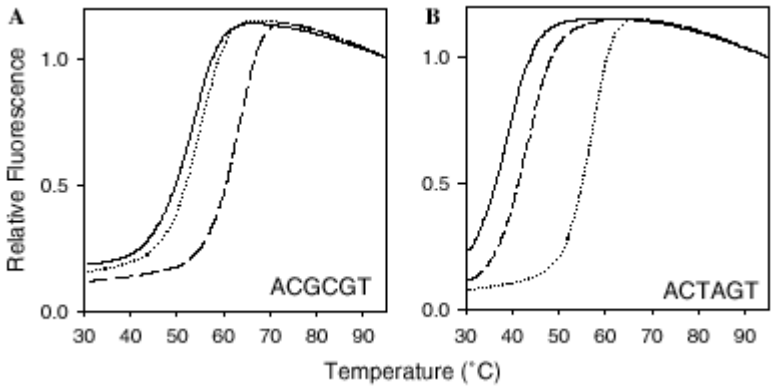


Figure 31

(Fluorescence melting curves of sequences ACGCGT and ACTAGT with binding ligands. Filled line, no added ligand; dotted line, thiazotropsin A (**24**) added; dashed line thiazotropsin B (**32**) added)[71]

The significance of this experiment for the synthesis of inherently fluorescent or coloured MGB is in the way that the interactions between these types of molecules can be used for analytical purposes. Here fluorescent/quenching markers were attached to each end of a DNA duplex; however if the DNA intercalator itself were fluorescent or a quencher this would provide some interesting possibilities to explore. For example if a fluorescent marker was attached to a DNA duplex and an MGB was added that was a quencher, then a drop in fluorescence would occur upon binding of the MGB. The opposite would be true for a fluorescent MGB and a quenching marker on the duplex. Also if, for example, the sequence specificity of an MGB was to be tested, a much longer DNA sequence could be used with two or more potential binding sites, a fluorescent/quenching marker could be placed near

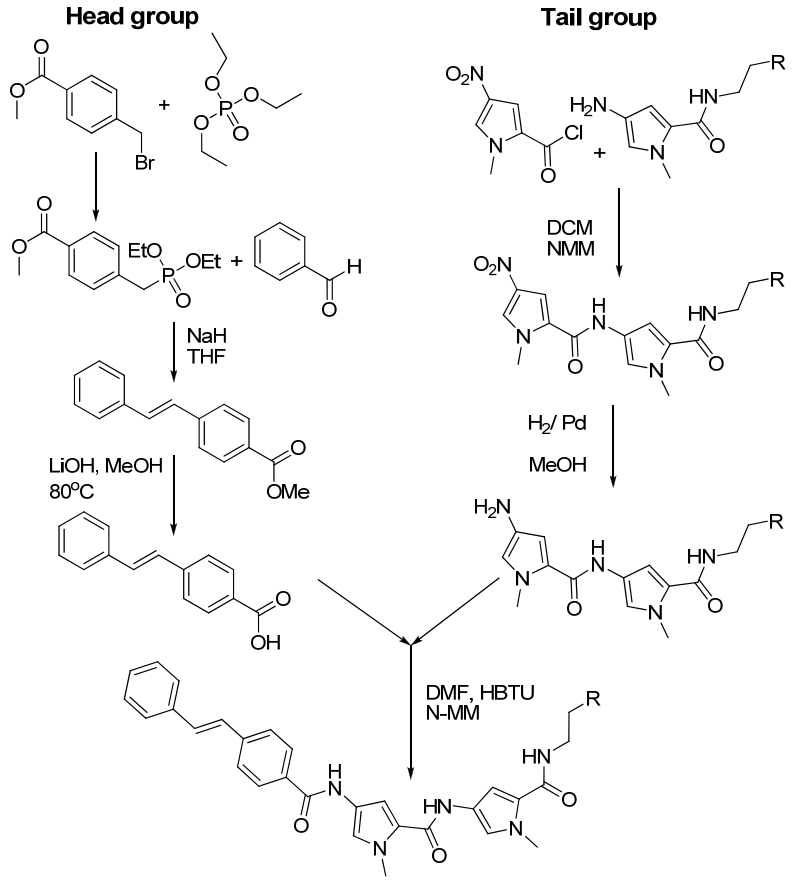
to one of the sites. Addition of a fluorescent/quenching MGB and the subsequent drop in fluorescence would provide an indication as to which site the MGB had greater affinity for.

At the time of writing these are simply potential uses and have not been tested; however the example of Hampshire *et al*.[69] shows that there are practical applications in the field of DNA binding for coloured or fluorescent compounds.

2.12 Review of alkene containing MGBs

2.12.1 General synthesis of alkene containing MGBs

Scheme 1 shows the general synthetic route followed to produce minor groove binders with alkene containing head groups. While the synthetic details will not be discussed in great depth here this will serve as a template for the standard methods used in MGB synthesis, these details will be discussed in more detail in section 3.2. The alkene containing head group is synthesised using Horner-Wadsworth-Emmons chemistry between a phosphonate ester and an aldehyde. The ester group of this compound is then hydrolysed to a carboxylic acid. The tail group is synthesised via standard amide coupling chemistry, the nitro group of this compound is then hydrogenated to the corresponding amine. This amine is then used directly in the coupling reaction with the carboxylic acid of the head group. This final coupling is carried out using HBTU with *N*-methylmorpholine in DMF.

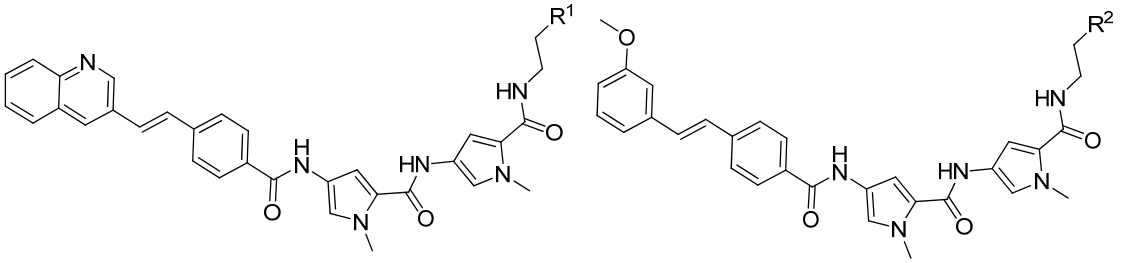


Scheme 1

General scheme for the synthesis of alkene containing MGBs

2.12.2 Characteristics of lead compounds

The upcoming sections of this thesis are in part concerned with MGB compounds containing the stilbene motif. It would therefore be appropriate to give a brief review of some of the structural features present and to briefly describe some of the key compounds in this series in terms of both biological activity and structural significance. Coincidentally, some of the first compounds in this series have been found to have among the most promising antibacterial activity in the entire series. Two compounds of particular significance have been compounds **33** and **35** (figure 32), both of which have shown excellent activity.[72] They are also of structural significance in light of later work carried out in terms of producing analogues to these compounds. This will be discussed in greater detail in later sections.

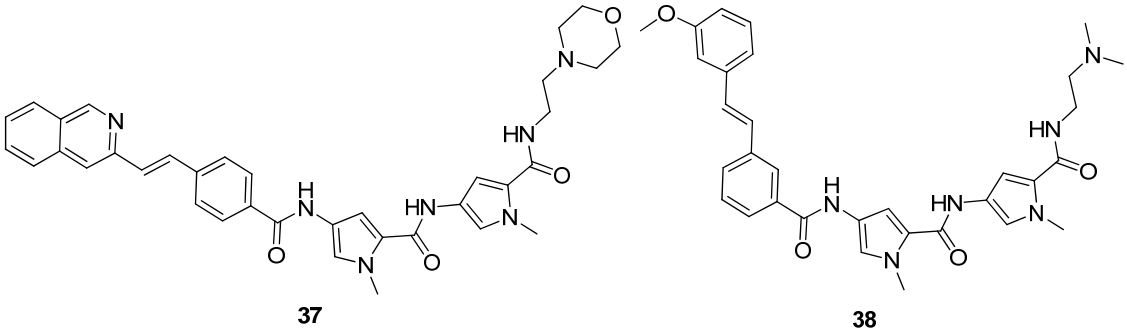


33 (R^1 = morpholino), **34** (R^1 = dimethylamino) **35** (R^2 = morpholino), **36** (R^2 = dimethylamino)

Figure 32

Structures of lead compounds

Also significant is the fact that there have been tail group variations on these compounds producing compounds **34** and **36** (figure 32)**.** The discovery of these lead candidates led to further synthesis to produce similar compounds such as **37** and **38** (figure 33)**.**



37 **38**

Figure 33

Analogues of lead compounds

Both of these are based somewhat on the lead compounds shown earlier, compound **33** being just one of several compounds that expanded on the idea of having a quinoline ring system as head group. Many of these were designed to probe the effects of having the nitrogen in different positions on the ring (for example the 2 position as in compound **37**). Significantly a similar compound (**39,** figure 34) was synthesised to test whether or not the presence of the nitrogen in the head group was required at all. Interestingly, this compound showed no antibacterial activity or binding to DNA suggesting that the presence of the nitrogen atom is vital to binding. This will be discussed in greater depth in section 3.5.

Several compounds were synthesised to include a thiazole ring in the structure. This tactic was successful in one of the previous compounds in the Suckling group, thiazotropsin A (**24**), therefore it was logical to try to replicate this success in this series. This led to the synthesis of several compounds; an example is compound **40** (figure 34).

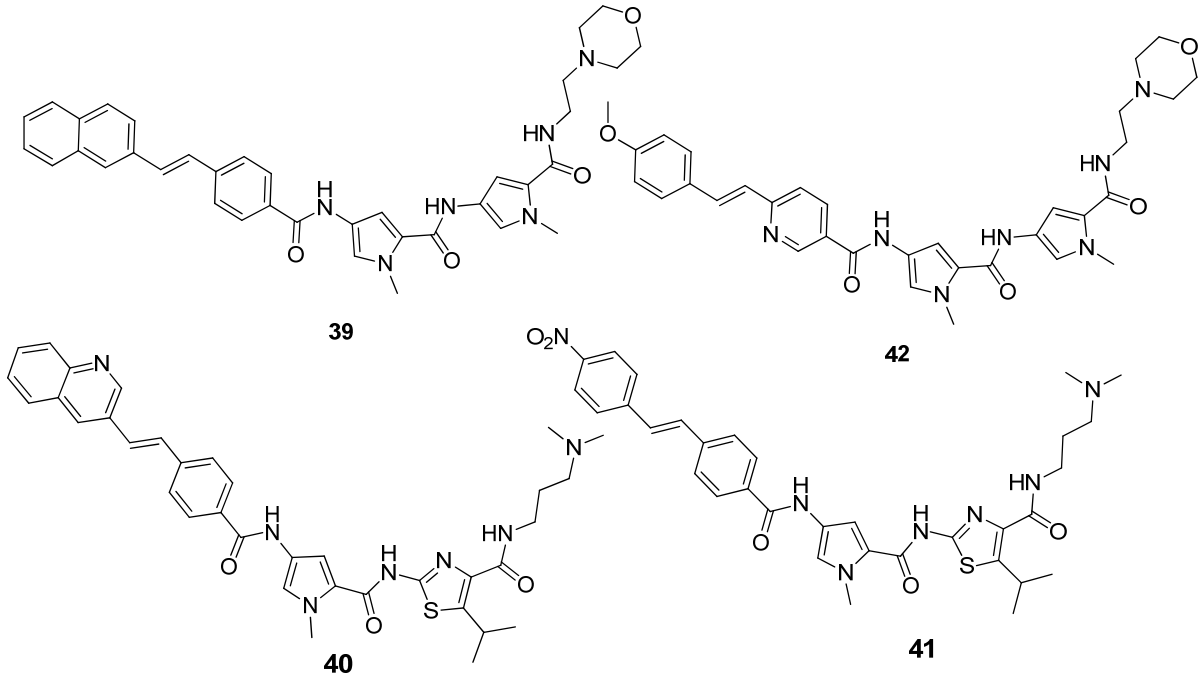


Figure 34

Structures of compounds **39**, **40**, **41** and **42**

These compounds incorporate elements of both thiazotropsin A and lead compound **33**. Compound **41** (figure 34) shows another example of use of the thiazole group and also an example of variation of substituents on the terminal benzene ring.

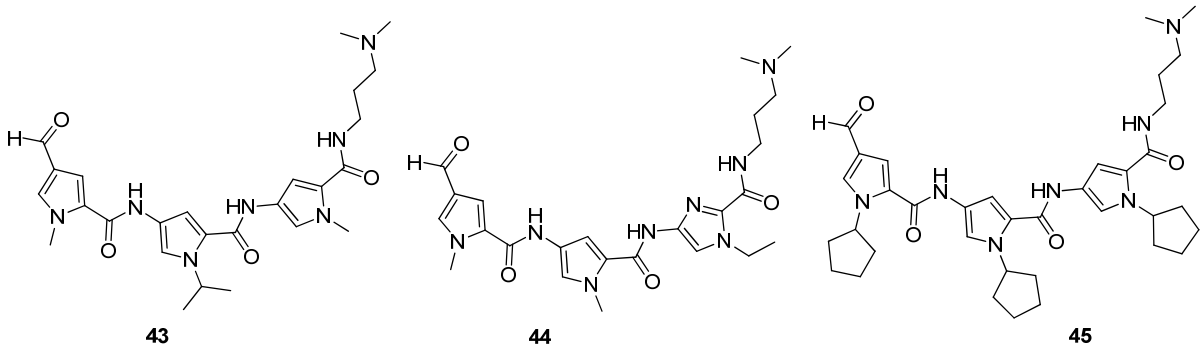
One of the other significant features of these compounds is the presence of nitrogen atoms in either of the aromatic rings of the head group. Several compounds were synthesised to explore whether this would be beneficial and in fact many of the quinoline compounds made experimented with the placement of the nitrogen in the ring. For example compound **33** has the nitrogen placed in the 3 position of the ring while compound **37** has it placed in the 2 position. Another example of this is in the use of pyridine rings in the stilbene section of the molecule. Several molecules were synthesised incorporating this feature however the only one that was significant in terms of biological activity was compound **42** (figure 34).[72] Several SAR studies were also carried out on this molecule. This compound was identified as one of the most promising lead candidates from this series for antibacterial activity

2.12.3 Alkyl group variation

As mentioned previously, there are several ways in which we could potentially improve the binding of MGBs to DNA. At Strathclyde one of the key methods is increasing the lipophilicity of the molecules. This can be achieved in a variety of ways however thus far the focus has been on reducing the polarity of MGBs. This has led to several structural features such as the inclusion of alkene and diazo links.

One of the other possible methods is to increase the size of the alkyl chains in the molecule. This would also increase the lipophilicity of the molecule thus increasing the possibility of membrane permeability while at the same time allowing for the exploitation of potential hydrophobic interactions in the minor groove (as discussed in section 2.8).

There are several further areas of the molecule that could be used to explore the effects of larger alkyl groups, for example increasing the length of the alkyl group. This area has been explored to a certain extent leading to the discovery of the ethyl group linking the first heterocycle to the basic tail group (in most cases morpholine). Another area that could be explored is the inclusion of alkyl groups of varying sizes on the terminal aromatic ring of the head group. Although several substituents have been experimented with at this position this is an area that has yet to be full explored. Perhaps the most obvious choice when looking to vary the alkyl group size is the substituents on the first and second heterocyles of the MGB. Typically the heterocyles used in these positions are pyrrole rings and generally speaking the groups attached at the nitrogen position are methyl. There are several examples in the Suckling group library where larger groups have been used. The examples shown in compounds **43**, **44** and **45** (figure 35) are some of the examples of compounds that have been synthesised with alkyl groups other than methyl. *Iso*-propyl, cyclopentyl and ethyl represent a fairly wide range of steric bulk, however in most cases direct comparisons cannot be made due to the lack of analogous compounds. Furthermore there has been no detailed DNA binding studies carried out on them and, as previously mentioned, the equivalent alkene containing compounds have not been made.[45]



43 44 45

Figure 35

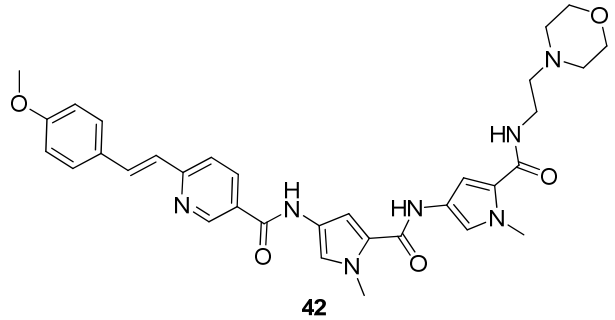
Examples of MGBs with alkyl group variation

The same problem exists with these alkyl-substituted compounds as with the compounds containing stilbene-type groups. They lack analogous compounds that allow us to make a direct comparison between larger and smaller alkyl groups and crucially there are no examples of compounds containing both the stilbene-type head groups that have been proven to be active and to bind strongly to DNA and compounds containing branched alkyl. Again in order to make a meaningful study of the effects of these groups a series of compounds would have to be synthesised in a more organised fashion.[45]

In order to conduct such a study a suitable lead compound (or compounds) would have to be chosen to provide a starting point or a 'control' for the experiment. A sensible starting point was to look at the pool of compounds containing alkene links under the umbrella of compounds with the stilbene motif. This would allow a study on how combining the loss of hydrogen bonding groups (by replacing amide bonds with alkene) with the increased lipophilicity from increasing alkyl bulk affects binding and activity. Initially two compounds, **33** and **42**, were chosen as templates mainly based on the excellent biological activity shown by both of them. Another reason to choose these two was that they had both shown strong evidence of binding to DNA previously; this will be discussed in more detail later (section 3.5).

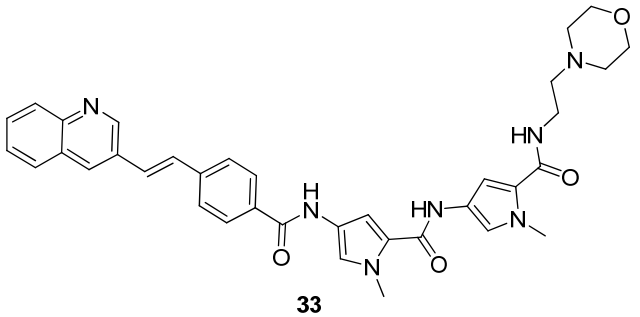
MGB **42**, (also known as AIK 20/25-1) was first prepared by Dr. Khalaf using Horner-Wadsworth-Emmons alkene chemistry (described previously in section 2.12.1) to synthesise the head group with the rest of the molecule being synthesised using standard procedures. This compound was submitted for biological testing and was shown to have excellent antibacterial activity in vitro (see section 3.4) and was

one of the strongest candidates put forward from this group of compounds.[72] Binding of this compound to DNA was also confirmed using thermal denaturation experiments.



42

The second compound **33**, (also known as AIK 19/56-2) was also prepared by Dr. Khalaf using Horner-Wadsworth-Emmons chemistry. This compound also showed excellent antibacterial activity both in vitro and in vivo and has in fact been taken the farthest in terms of pharmaceutical tests than any other compound in the series. Binding of this compound to DNA was also confirmed using thermal denaturation and capillary electrophoresis experiments (see section 3.5).



33

Previous sections have outlined the range of chemical structures that have featured in the Strathclyde MGB programme. As well as giving us a chance to explore the effects of increased lipophilicity in MGBs these compounds also provided a chance to explore the effects of making small changes in structure in broader terms. As explained previously the binding site of DNA in the case of MGBs is not rigid and is very flexible, hence the tolerance for a wide variety of molecular structures. It is therefore unlikely that an ideal fit would be found since there are no pockets to be filled and specific binding interactions take into account many factors such as hydrophobic interactions and hydrogen bonding. However this does not mean that simple SAR experiments cannot be carried out. By taking a starting compound such as **33** or **42** and making small changes to the structure we can determine the effects (if any) to be found in activity upon making these adjustments. In other words we

can determine the feasibility of fine tuning active compounds to improve activity and also make a start at determining what changes would be beneficial and which would be detrimental. In addition, by combining biological assays on these compounds with experiments to determine relative binding strength we can learn more about the connection between biological activity and binding to DNA. Since there is still little known about mechanism of action of active compounds this will further expand our knowledge in this area.

When looking to expand on the idea of using varying sizes of alkyl groups the sensible option would be to propose a selection of groups that cover a wide range of steric bulk. However in reality this would be quite a large undertaking in terms of the synthesis involved. As has been the case with previous MGBs, much of the chemistry is relatively straightforward however there are still several steps involved which are both time consuming and typically low yielding. This synthesis will be described in more detail in section 3.2.

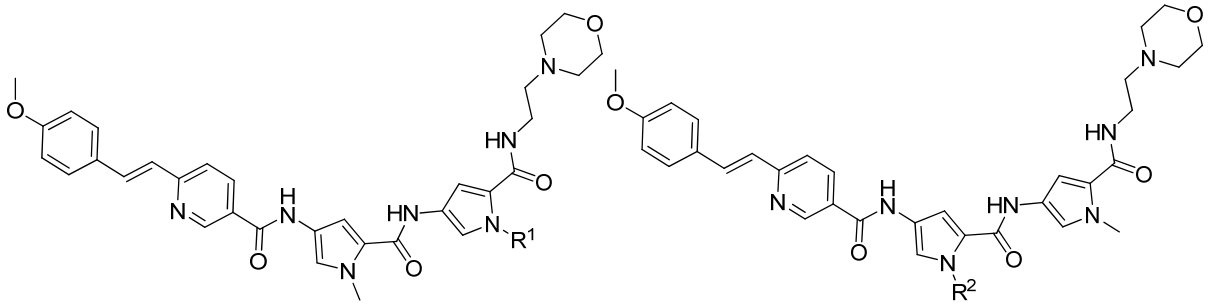
In addition to this any groups chosen to be tested would have to be placed on both the first and the second heterocycle, effectively doubling the number of compounds to be synthesised. It was therefore decided that in addition to the existing methyl group, isopropyl and secondary butyl groups would also be used (figure 36).

Figure 36

Pyrrole monomers with (1) Isopropyl and (2) Secondary butyl groups

Use of these three groups would represent increasing steric bulk going from methyl to isopropyl and finally secondary butyl. The placing of these groups in both the first and second heterocycle is a further attempt to probe the sensitivity of DNA binding to small incremental changes in structure. To this end, two series of compounds were synthesised by Fraser Scott, the first of which was based on the lead compound **42** described earlier.

The first two compounds (**46** and **47**) had both the isopropyl (**46**) and secondary butyl (**47**, racemic mixture of isomers) groups respectively attached to the first heterocycle as shown in figure 37.



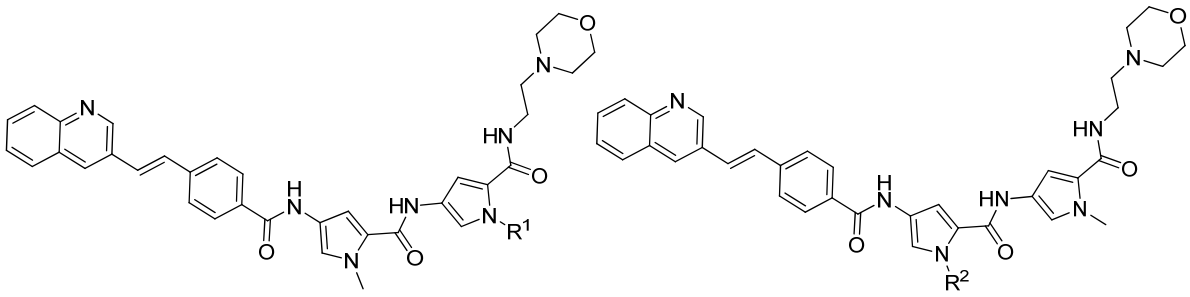
46 (R^1 = isopropyl), **47** (R^1 = secondary butyl) **48** (R^2 = isopropyl), **49** (R^2 = secondary butyl)

Figure 37

Analogues of lead compounds

In the second pair of compounds both the isopropyl (**48**) and secondary butyl (**49**, racemic mixture of isomers) groups respectively are attached to the second heterocycle (figure 37).

Similarly in the next series, based on compound **33**, the first two compounds, **50** and **51**, had both the isopropyl and secondary butyl groups respectively attached to the first heterocycle as shown in figure 38.



50 (R^1 = isopropyl), **51** (R^1 = secondary butyl) **52** (R^2 = isopropyl), **53** (R^2 = secondary butyl)

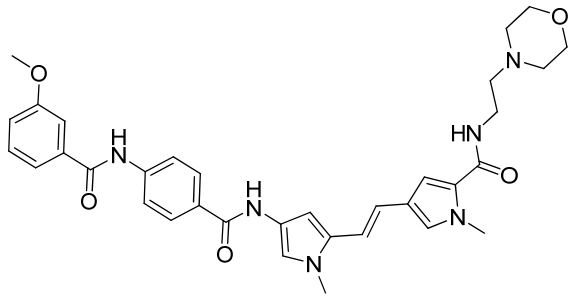
Figure 38

Analogues of lead compounds

In the second pair of compounds both the isopropyl (**52**) and secondary butyl (**53**) groups respectively are attached to the second heterocycle (figure 38)

2.12.4 MGBs with internal alkene links and alternative tail groups[73]

It has been previously noted in the introduction and in section 2.7 that much of the work carried out in the Suckling group is concerned with increasing the lipophilicity of minor groove binders. A large portion of this work has involved reducing the polarity of the MGBs by replacing polar groups such as amide bonds with non-polar groups such as alkene. This has manifested itself in the inclusion of the stilbene motif as the head group in our MGBs as shown in compounds **33** and **35**. In terms of comparing the effectiveness of polar groups versus non-polar groups this presented a problem in that the stilbene containing compounds had no existing analogous polar compounds. This problem is addressed in a later section of this thesis (section 3.3), however there were other areas in the molecule at which polar groups could be replaced with non-polar moieties. The amide bonds that linked first and second heterocycle could be replaced with alkene links thus creating so-called internal alkene compounds. This would pose more of a synthetic challenge than in previous alkene containing compounds due to the fact that the bond would be formed between two pyrrole rings. The method development of this synthesis was carried out by Dr. David Breen allowing the synthesis of several examples of compounds of this type. An example of this type of internal alkene is compound **54**.[73]

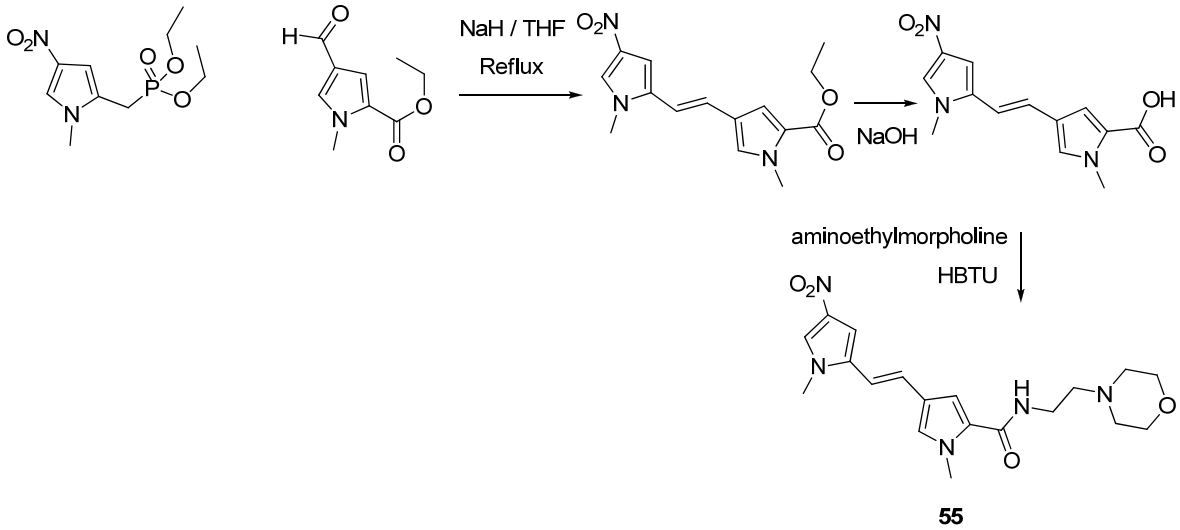


54

Here the amide bond linking the two pyrroles has been replaced with an alkene bond; comparing this molecule with an analogous amide containing MGB will not only give us more information about the importance of increasing lipophilicity in general but also tell us more about specifically which areas of the molecule are forming hydrogen bonds. Obviously amide bonds are hydrogen bonding groups so replacing one with a non-hydrogen bonding group should give us some information

as to the importance of hydrogen bonding in that particular area. This would be done by comparison of binding properties of this compound with an amide containing analogue (see section 3.1.3).

The synthesis of the head group is the same for both compounds however the tail group (**55**, scheme 2) in compound **54** requires a different synthetic approach as described in the following synthetic route. It is important to note that the following approach is a summary of the methods used and was the end result of a lengthy investigation into the method development for this approach (scheme 2).[73]



Scheme 2

Synthesis of tail group **55**

Coupling of monomers was carried out using Horner-Wadsworth-Emmons chemistry to give the dimer shown. The ester group was then hydrolysed using sodium hydroxide to give the carboxylic acid. Attachment of the aminoethylmorpholine tail group was then carried out using simple HBTU coupling to give completed tail group. The rest of the molecule was then synthesised using standard methods, the only other noteworthy point was that reduction of the nitro group to the amine was carried out using sodium borohydride in the presence of palladium on charcoal and that some reduction of the alkene link was observed.

Breen also investigated the possible benefits of replacing the penultimate aromatic ring with a heterocycle. This led to the synthesis of several compounds with

differing degrees of activity. One such compound is **56** with a thiazole ring replacing the benzene ring; this compound (**56,** figure, 39) is also analogous to compound **33** (page 50).

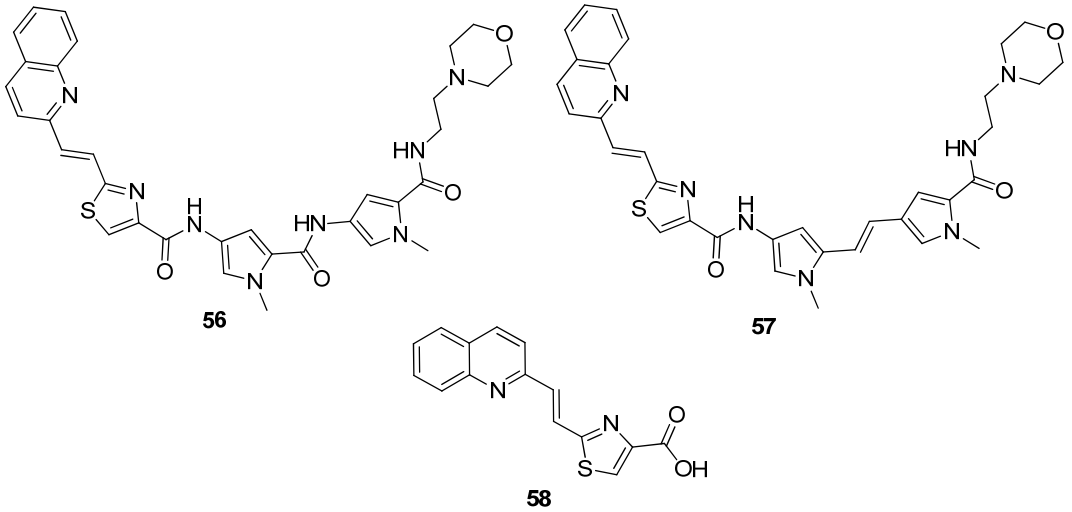


Figure 39

Structures of analogous compounds **56** and **57** and head group **58**

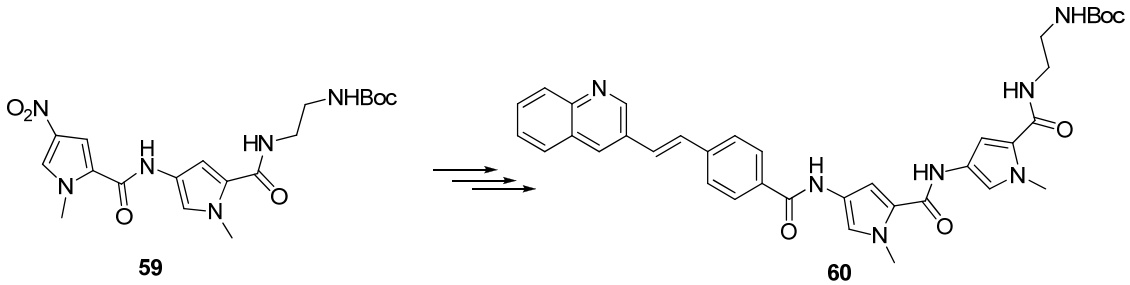
The synthesis of this molecule involved making the head group **58** using the Horner-Wadsworth-Emmons reaction described previously Figure 39.[73]

Coupling of this dimer with the usual ethyl morpholine tail group dimer using standard methods afforded the final product **56**. An analogue to this compound (**57**) containing an alkene bond between the pyrrole heterocycles was also synthesised using the same head group which was coupled with the alkene containing tail group described previously (figure 39). Interestingly compound **56** showed significant antibacterial activity while compound **57** did not. This gives us valuable insight into the importance of the internal double bond in **56**. This will be discussed in greater depth in section 3.5.

Tail group variations

The final group of compounds of interest in this section contain novel tail groups. Generally speaking most of the compounds mentioned in this report will have basic tail groups and will therefore be protonated at biological pH. It was of interest to synthesise some examples of compounds with neutral tails which would have

potential to form H-bonds (but not be protonated) in an area of these molecules that have yet to be exploited in this type of bonding. The synthesis of these compounds was relatively straightforward and involved the synthesis of the dimer **59**.



Scheme 3

Synthesis of MGB **60**

This dimer was then coupled to a quinoline containing head group using standard methods to give compound **60** (scheme 3). Boc deprotection and coupling with the appropriate tail groups was then carried out in situ. This produced Breen's final compounds; of these only the nitroalkene **61** would be neutral and the guanidine tail group MGB **62** will be essentially permanently positively charged at physiological pH (figure 40).

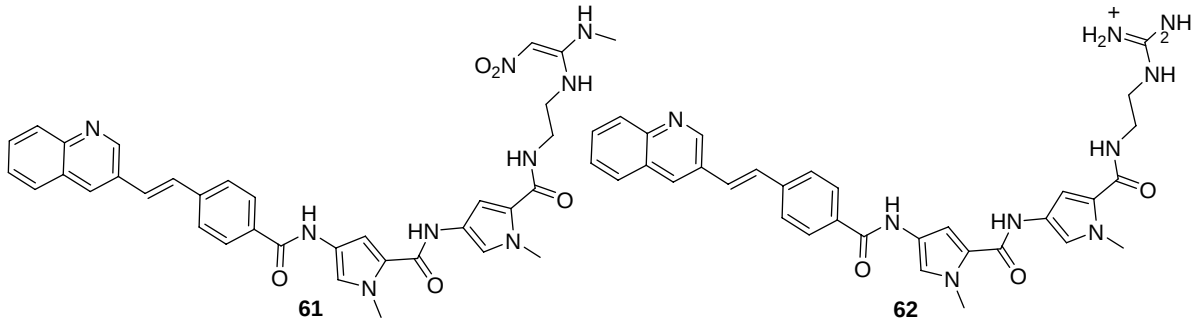


Figure 40

MGBs with varied tail groups

So far a wide range of minor groove binders has been discussed covering many structural categories. This has yielded several active lead compounds however little of the investigation into these compounds had been planned out and organised with a view to identifying specific structural features that promote activity. Taking a more systematic approach to the investigation would involve the design and synthesis of compounds specifically designed to probe the various structural features of MGBs. It would also involve using a wide range of analytical techniques to investigate the properties of these compounds with respect to their DNA binding. This will be discussed in the next section.

3. Results and Discussion

3.1 Project Aims

The research described below is divided into two main sections: the first is concerned with the synthesis of MGBs containing diazo links and an investigation of their properties and the second is concerned with comparative binding studies on MGBs with wider structural variation including *N*-alkyl substituents, alkene links, and tail groups.

3.1.1 MGBs with azo linkages

As explained previously, the concept of MGBs with azo linkages ties in with the general philosophy of MGB design at Strathclyde University. The diazo bond when compared to the amide bond would provide greater lipophilicity for the molecule as a whole thus providing potentially better transport in the body to the target, i.e. the DNA. The diazo bond is an isostere of the peptide bond. The geometry, as discussed earlier, is very similar however it has different properties in terms of polarity and hydrogen bonding capabilities (see section 2.9). Because of this it makes sense to explore a range of compounds that could be made by utilising it, therefore one of the first aims of this project was to investigate these possibilities. The possibilities would be limited by the relatively few methods of synthesising a diazo bond. Probably the most reliable method is to use diazonium salts to create the bonds via electrophilic aromatic substitution.[74] This method has several limitations in that the formation of the diazonium salts normally requires the presence of a stable amine, something that is not always possible when using heterocycles (as would be the case in the majority of the compounds attempted). Also electrophilic aromatic substitution would only be possible at sites strongly activated towards this, thus again limiting the number of sites where a diazo bond could be formed.[75] The other possibility in synthesising diazo bonds would be to carry out a condensation reaction between a nitroso group and an aromatic amine. This reaction is not particularly reliable and much of the literature on the subject is based on condensations between very simple aromatic compounds;[76] this would

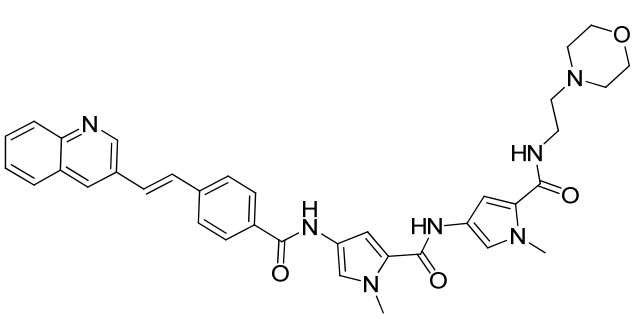
also limit the number of compounds that could be reliably synthesised. Overcoming these and other synthetic challenges to create a range of compounds with diazo linkages was the first aim in this section.

The second aim relates to minor groove binder synthesis in the context of the entire research group at Strathclyde University. It has already been mentioned that the use of diazo linkages fits in with the overall strategy of lipophilic MGBs, this strategy has recently included the synthesis of a range of alkene containing compounds. In order to provide a complete analysis of the differences between polar amide linkages and non-polar alkene or diazo linkages it has been necessary to synthesise compounds that are direct analogues of one another. To prove that changing from amide to alkene or diazo has a positive impact on binding in a particular MGB it must be compared with another MGB that is a direct amide analogue of it. Similarly in order to compare diazo and alkene links analogous compounds containing each of the linkages must be synthesised. Again the synthetic limitations of diazo linkages would mean that the extent to which this can be done would be limited. Hence overcoming these limitations to provide analogues of amide and alkene compounds was another aim of this project, as was the analytical comparison of the finished product. This means that portions of this project were carried out in collaboration with other members of Prof. Suckling's research group.

3.1.2 Comparison of linking groups in active Minor Groove Binders

As mentioned previously there has been considerable research in the Strathclyde group into synthesising MGBs that contain alkene bonds. A significant amount of this research has been directed towards making compounds that have a head group similar in structure to a substituted stilbene molecule. The reason for using this stilbene motif was that it moved the general structure of the MGBs towards being more hydrophobic with alkene linkages being used instead of amide. An added advantage was the ease with which the synthesis could be carried out; it was theorised that using simple substituted benzene molecules head groups could be readily synthesised using Wittig type chemistry.

Using this approach, many examples of alkene containing compounds were synthesised with variation in the substitution in the terminal benzene moiety and also with the introduction of various hetero atoms in either of the benzene groups.[72] Of these compounds several were found to be reasonably active however a few showed exceptional antibacterial activity and have at the time of writing been taken forward to the animal testing phase where numerous experiments have been carried out. Notable examples of this are compounds **33** and **35**. Obviously using this synthetic methodology has proven successful in producing a wide variety of compounds with several promising hits emerging, however it gives us very little insight into the structure activity relationships of these molecules.



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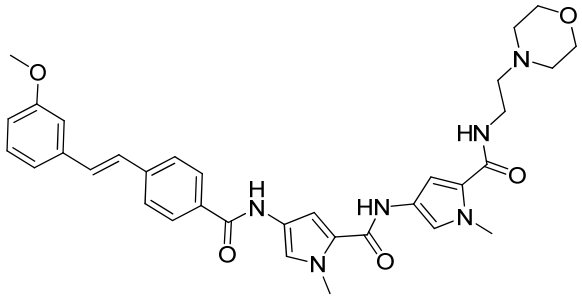


35

It has already been mentioned that SAR in these types of molecules is difficult due to the huge amount of variation in molecules that will bind to the minor groove indicating the tolerance of this binding site to many moieties. Thus far several structural features have been identifies that are now known to promote antibacterial activity. There has, however been limited information gathered with respect to the binding of these compounds to DNA. In order to gather information in this area it would be necessary to plan a series of compounds with analogous structures and to analyse them as fully as possible with respect to biological activity and DNA binding. This way specific features that promote DNA binding could easily be identified and we can begin to analyse the links between binding strength and activity.

As mentioned, in order to test the theory that increased lipophilicity promotes binding it would be necessary to compare two compounds that are analogous in every way except for the presence of a hydrogen bonding group in one that was absent in the other. It was therefore decided to take some of the more active compounds from the stilbene range and to synthesise analogues which replace the

alkene group with an amide group and observe the change (if any) in activity. It was also decided to synthesise analogues of these compounds which replace the alkene linking group with a diazo group which would have very similar geometry to the alkene and have the ability to form similar hydrophobic interactions with the minor groove. This would serve to test whether any activity gained or lost was due to the loss of a hydrogen bonding group or due to some specific interaction of the alkene group.

3.1.3 Selection of Compounds

The first compound selected was AIK 19/56-2 (**33**), synthesised by Dr. Khalaf. The structure is a slight variation on the stilbene motif with the introduction of a quinoline group as the terminal building block. This is an interesting feature as the nitrogen in the quinoline has the potential to act as a hydrogen boding acceptor. It could be speculated that the position of this acceptor may improve binding; however more detailed experiments would be necessary to prove this. This compound showed excellent antibacterial activity in vivo; it was therefore decided to synthesise the analogous diazo and amide compounds **63** and **64** (figure 41).

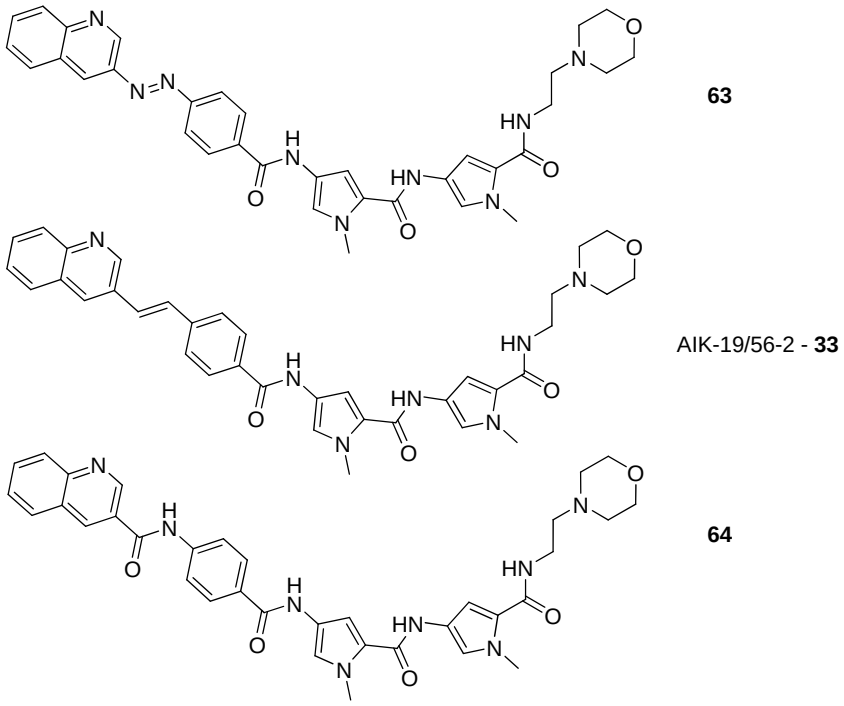


Figure 41:
MGB **35** plus analogues

The compound AIK 19/56-1 (**35**), also synthesised by Dr. Khalaf was another of the stilbene series that showed exceptional activity in vitro. Again the diazo (**65**) and amide (**66**) equivalents would be synthesised to complete the set as shown in figure 42.

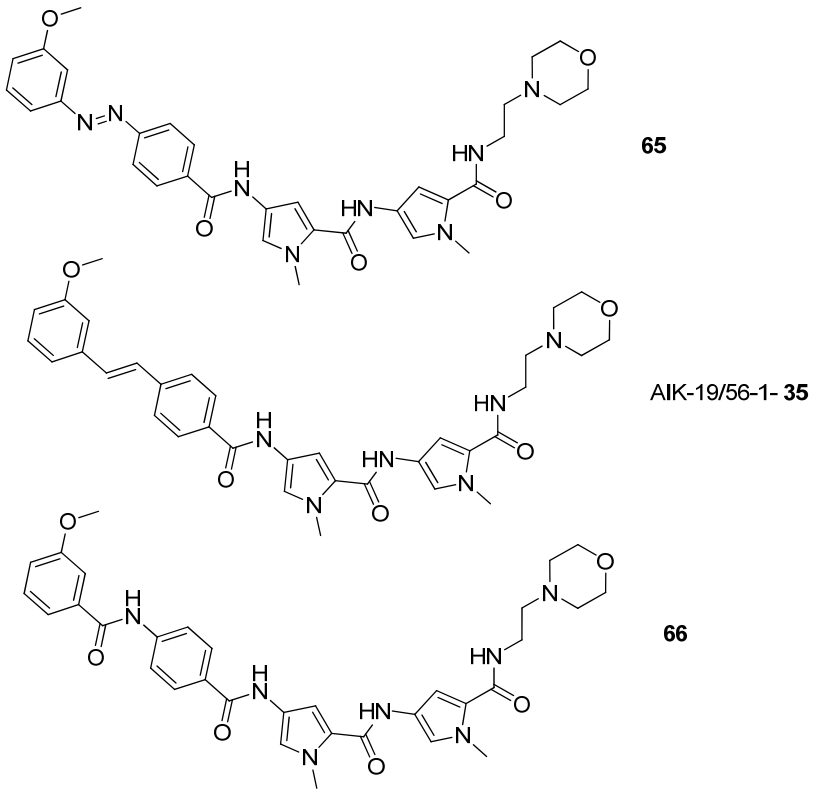


Figure 42:
MGB **35** plus analogues

The final set of compounds chosen was based upon the compound GD/B1/65 (**67**); this compound had a diazo link in place of an alkene link but showed only moderate antibacterial activity. It was decided to synthesise the alkene containing equivalent in order to observe any improvement in activity (compound **68**), again the amide equivalent (**69**) would be synthesised to complete the set of three. The completed set is shown in figure 43.

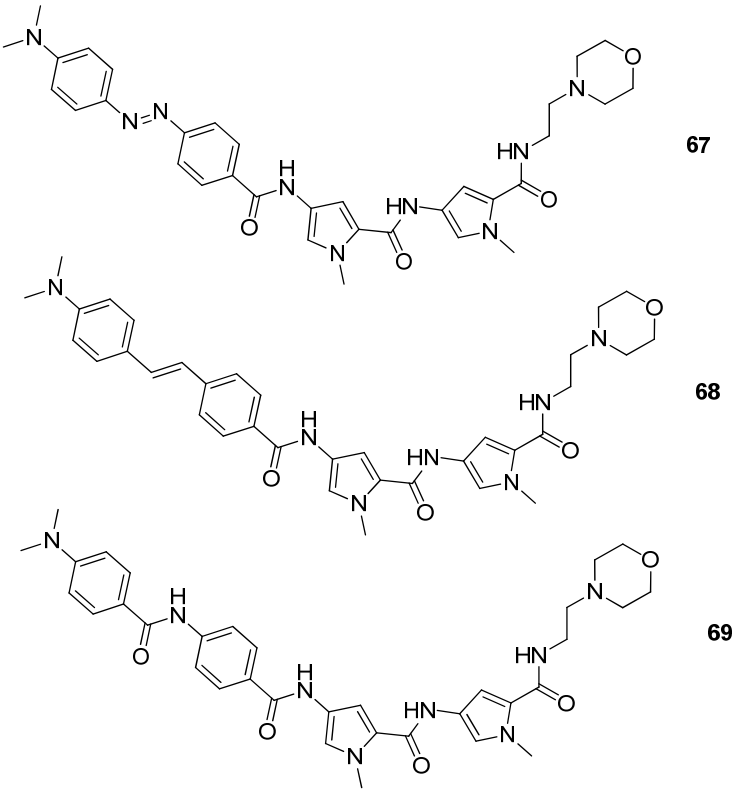


Figure 43:

MGB **67** plus analogues

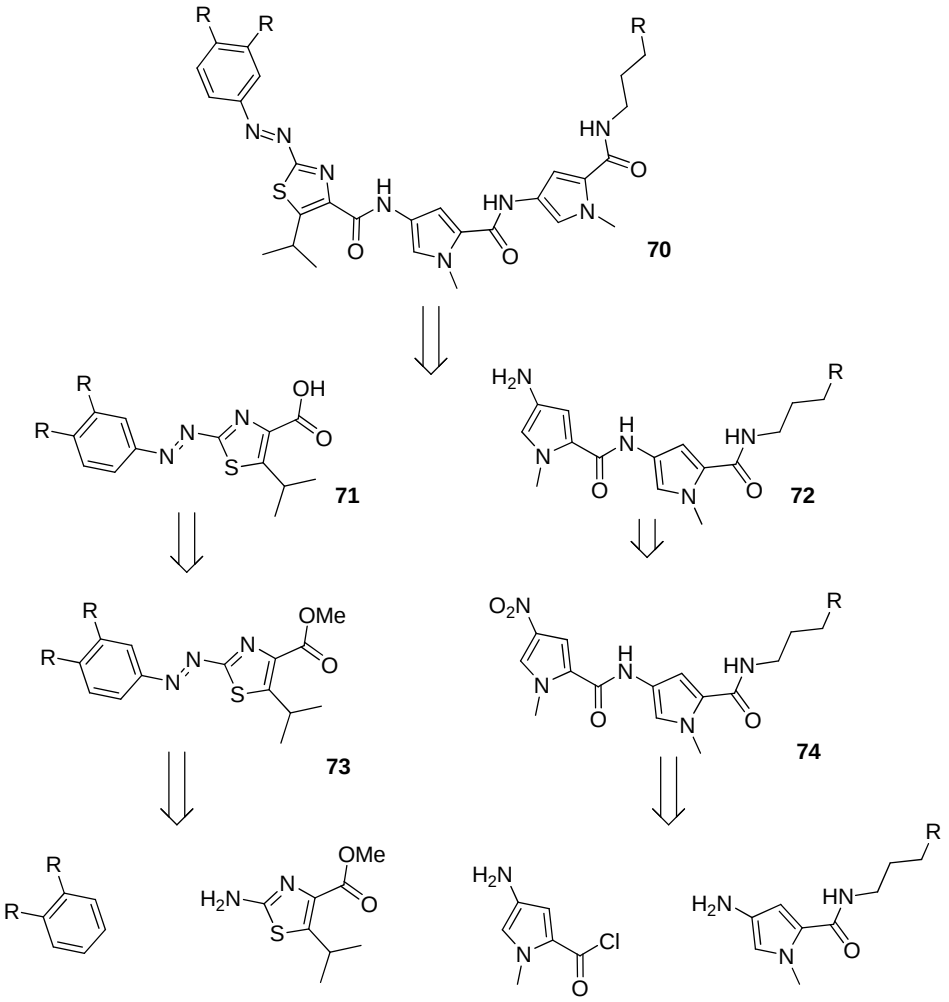
3.2 Compounds Containing the Diazo Linkage

3.2.1 Thiazole-containing diazo compounds

The choice of compounds that could be synthesised with diazo links is limited thus synthetic accessibility influenced the choice of the initial target molecule (a general structure of which is shown, **70**, scheme 4). Not only does the molecule contain a diazo link but it also contains a thiazole group, which has been a feature in some of the Strathclyde group's most potent compounds including thiazotropsin A. The rest of the molecule takes the standard pyrrole-pyrrole- and dimethylaminopropyl group seen in many distamycin analogues.

The decision to use an aromatic head group was also one that was based on synthetic strategy; in order for electrophilic aromatic substitution to occur to give the diazo link at the correct position, the R group must be strongly electron donating. Previous work on using electrophilic aromatic substitution to create diazo bonds has shown that groups such as hydroxy or dimethylamino in the para position to the site of substitution are sufficiently electron donating.[77] Not many other groups are suitable for this purpose but there remains some degree of variation in the ring bearing the amino substituent from which the diazo link is formed which would be useful in synthesising a variety of compounds.

The retrosynthetic analysis for MGB **70** is shown in scheme 4.



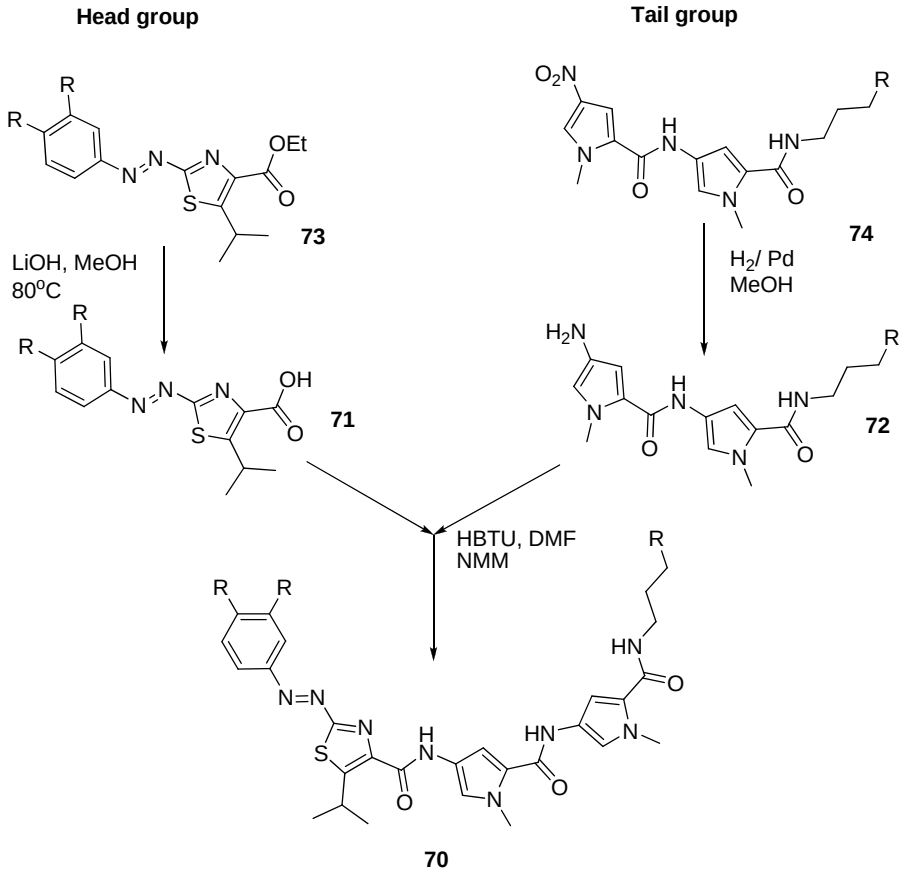
Scheme 4

Proposed synthetic route for thiazole containing MGBs

As is often the case in the making of MGBs, a convergent synthesis involving two dimers is necessary. In this case, the pyrrole-pyrrole-DMA dimer (tail group, **72**) would be synthesised using a relatively simple synthesis and the diazo containing dimer (head group, **71**) would be synthesised using a diazonium salt reaction.

General route for synthesis of MGBs

The general route for the synthesis of MGBs is shown in scheme 5. As can be seen from the retrosynthesis shown in scheme 4 it involves the 2+2 coupling of two dimers, in this case **71** and **72**. The ester group contained in the head group is hydrolysed to its corresponding carboxylic acid using lithium hydroxide. The nitro group contained in the tail group is hydrogenated to its corresponding amine using H_2 and palladium catalyst. This amine is then used immediately in the coupling step with the carboxylic acid to avoid decomposition of the amine. The coupling step is carried out using HBTU in DMF.



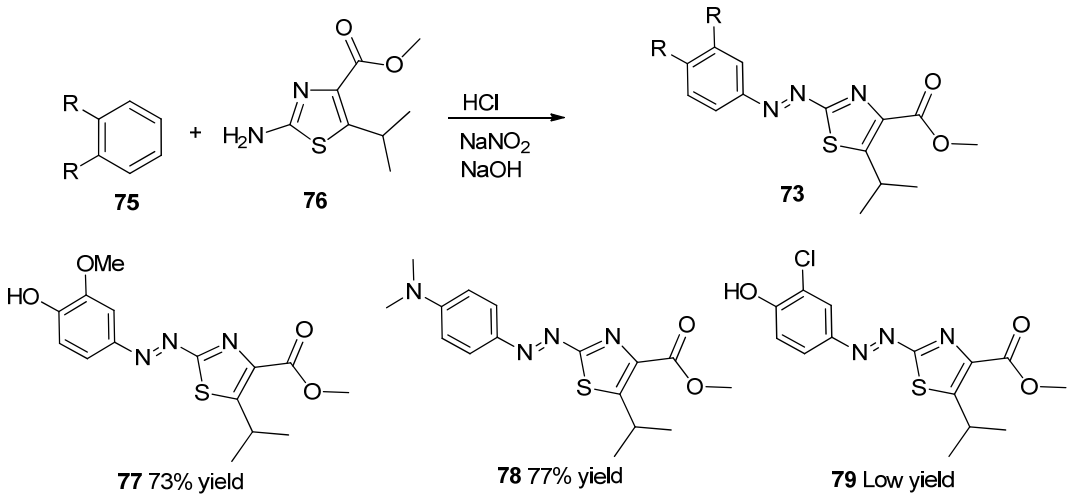
Scheme 5

General synthesis for thiazole containing MGBs

This diagram describes the synthesis for thiazole containing compounds; however the general idea of a 2+2 coupling between a carboxlylic acid group and an amine can be applied to the synthesis of all the MGBs described in this thesis.

Synthesis of Head groups

The general synthetic route for the synthesis of the diazo dimer is shown in scheme 6 along with dimers of this type that have been synthesised so far.

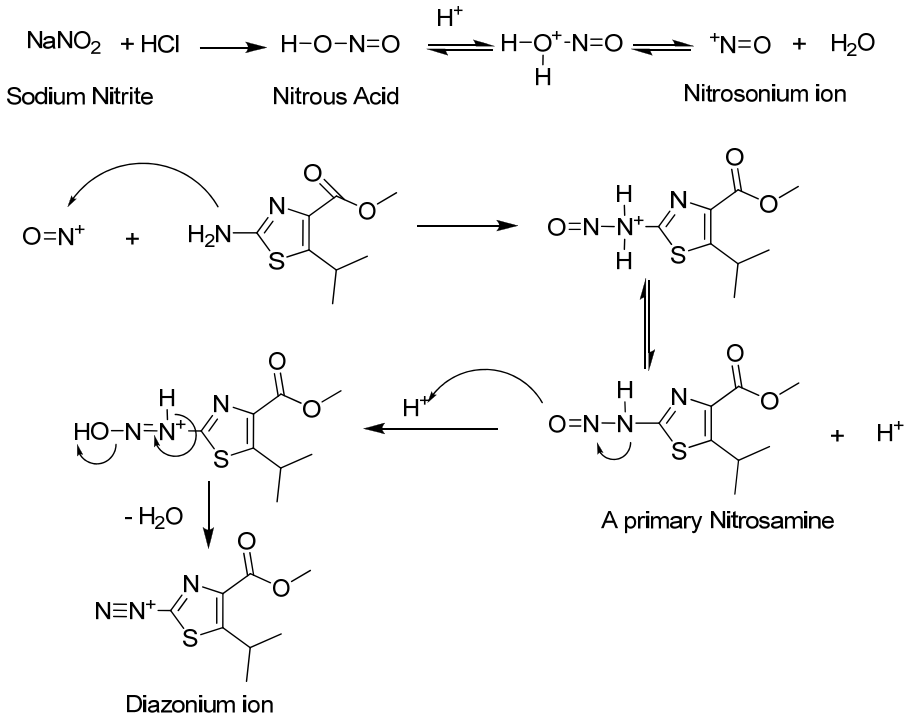


Scheme 6

Synthetic route for diazo containing thiazole dimer molecules

Mechanism of diazo coupling [78]

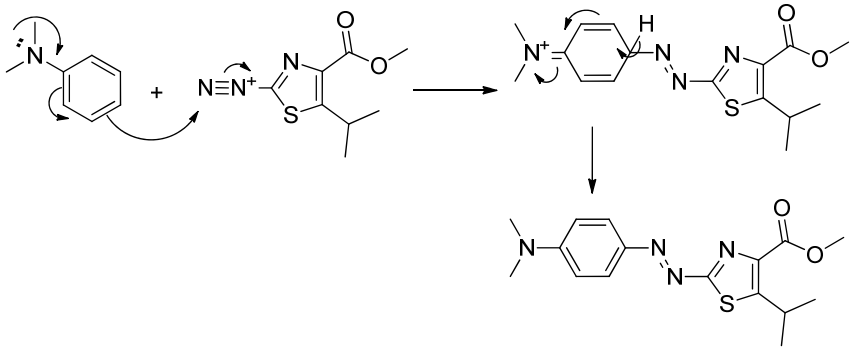
Formation of a diazonium ion requires the presence of nitrous acid, however this substance is unstable at room temperature so, generally speaking, in these types of reactions the nitrous acid is formed in situ from sodium nitrite and a strong acid (in this case conc. HCl) at low temperature. At about $0^{o}C$ the nitrous acid solution is relatively stable. Protonation of nitrous acid followed by loss of water produces a nitrosonium ion which is the species that reacts with the amine. Nucleophilic attack of the amine (in this case the thiazole compound) on the nitrosonium ion followed by loss of a proton produces a primary nitrosamine as shown in scheme 7. Protonation of the oxygen in this nitrosamine followed by loss of water then produces the diazonium ion.



Scheme 7

Formation of diazonium ion

The diazonium ion will exist in solution as the chloride salt, hence the term diazonium salt. This diazotisation can only occur in primary amines. The diazonium salt is fairly stable and can be kept at low temperatures for several hours. They are useful intermediates in organic synthesis as they can be replaced by various nucleophiles. However in this case the diazonium salt is used almost immediately in an electrophilic aromatic substitution reaction as shown in scheme 8.



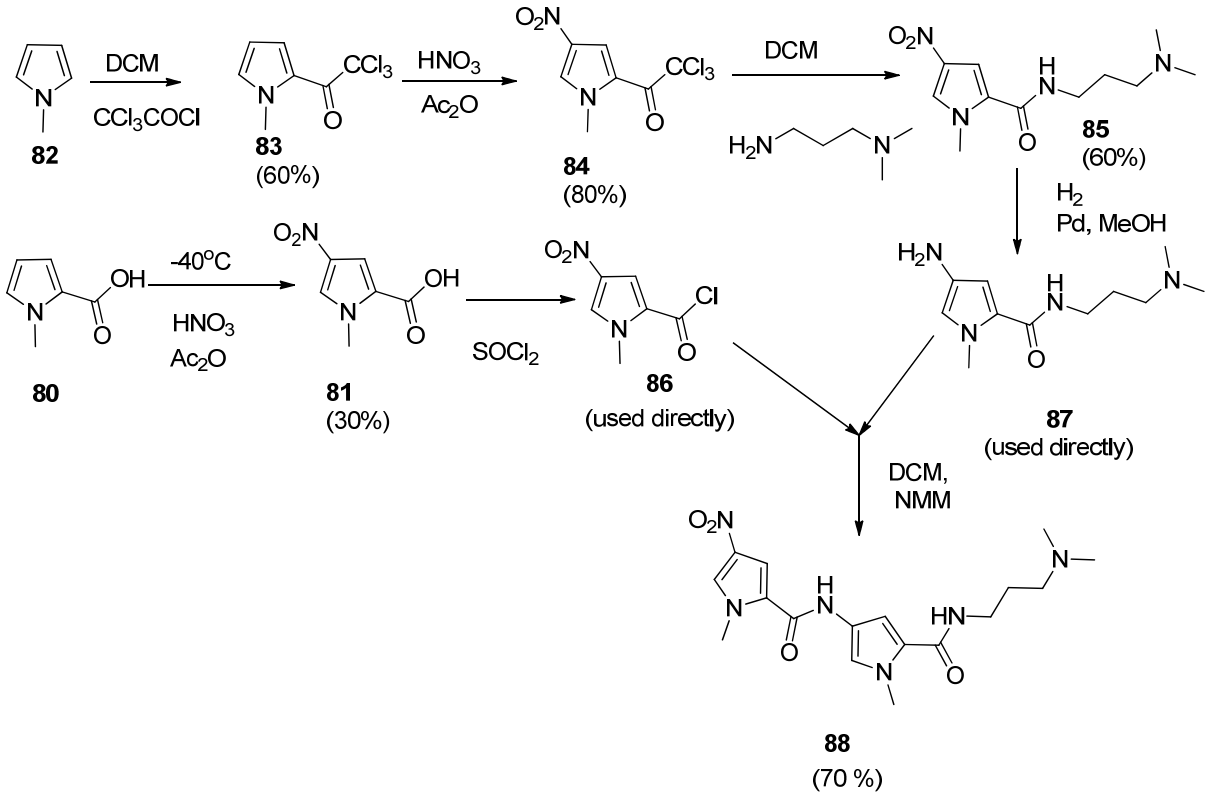
Scheme 8:

Diazonium salt in electrophilic aromatic substitution reaction

Synthesis of Tail groups

The second dimer consists of two pyrrole groups linked with an amide bond with the tail generally being a nitrogen containing group. Several different tail groups will be used in this thesis, the first of these is compound **88**. The synthetic route for this compound is shown in full in scheme 9. This can be taken as a general route for the synthesis of tail groups.

Synthesis of this dimer was carried out using standard procedures[16] (see scheme 9). Two monomers were synthesised, the first of these (**81**) was made by nitrating pyrrole 2-carboxylic acid **80**; this reaction was carried out at –40°C (30% yield) according to the procedure developed by Barton et al [77].



Scheme 9:

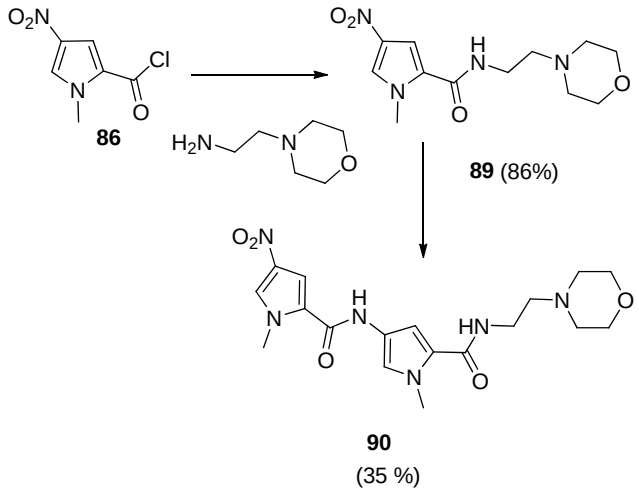
Synthesis of compound **88**

In the second monomer the trichloroacetyl pyrrole **83** was made by reaction of *N*-

methylpyrrole **82** with trichloroacetyl chloride at room temperature (60% yield). This was then nitrated in a similar fashion to the pyrrole carboxylic acid (**80**) to give the corresponding nitro compound **84** (80% yield) which was then coupled with *N,N*-dimethylpropane-1,3-diamine in DCM at room temperature to give the monomer **85** (60% yield). In order to carry out the coupling reaction between the two monomers it was necessary to first convert the acid **81** into the acid chloride **86**, using thionyl chloride under reflux.

The pyrrole nitro-compound **85** was then hydrogenated over a palladium catalyst to give the corresponding amine **87**. The Suckling group has shown from previous work that amines of this type are unstable and are oxidised readily upon contact with air; [75] therefore when carrying out reactions involving them it is necessary to use the compound immediately after synthesis. The unstable nature of the acid chloride meant that this compound was also used immediately after synthesis. Coupling was carried out in DCM with NMM to give the dimer **88** (70%, scheme 9).

A similar dimer was produced with a morpholino ethyl group to replace the dimethylamino group. The preparation was very similar except that the monomer **89** was prepared from the acid chloride **86** rather than the trichloroacetyl pyrrole, **84**. The acid chloride was prepared as described in scheme 9 and used immediately due to its unstable nature.



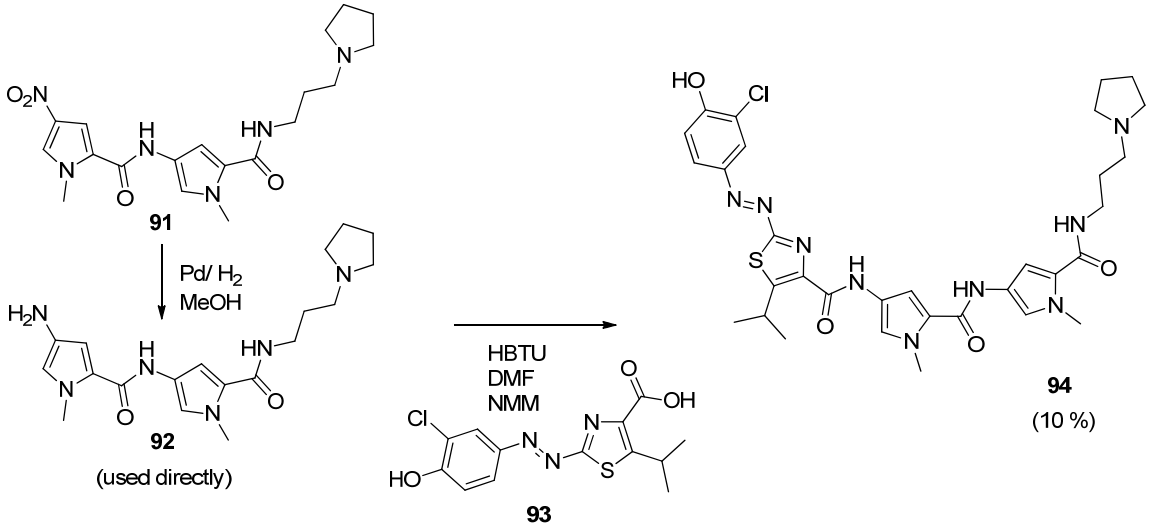
Scheme 10:

Synthetic route for **90**

Coupling of **89** with the acid **81** to give the dimer **90** was then carried out as described previously, with conversion of the carboxylic acid and nitro groups to acid chloride and amine respectively and immediate reaction (scheme 10).

Synthesis of MGBs

As mentioned previously, this project was started during my final year project. During this time synthesis of the three dimers was attempted using a modified version of the method of Barton et al. [77] This gave poor results in all three cases; however dimer **79** was isolated in very small amounts. It was decided to couple **79** with the dimer **91** since this was readily available at the time (scheme 11).



Scheme 11

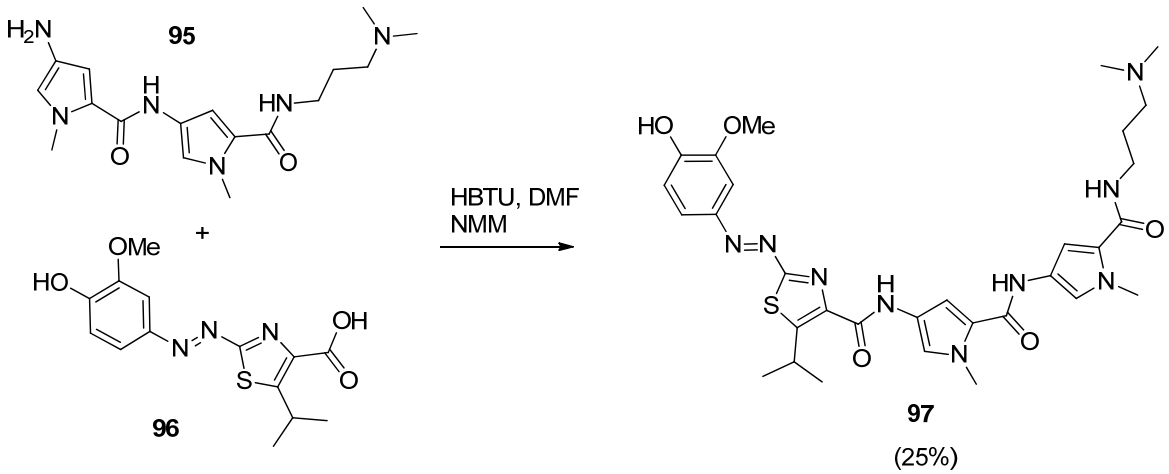
Synthesis of MGB **94**

For the coupling reaction it was necessary to reduce the nitro group of **91** to the corresponding amino group (**92**); this was carried out using palladium catalyst with hydrogen gas and the amine was used directly in the next step without analysis. The ester of the diazo compound **79** was hydrolysed using lithium hydroxide in methanol to give the carboxylic acid **93**. The acid produced was not fully characterised due to lack of material however some analytical data was obtained. These analytical data, while not conclusive, provided enough evidence that hydrolysis had taken place to proceed to the coupling step. The coupling step was then carried out using HBTU and *N*-methylmorpholine in DMF. Purification by HPLC was then carried out directly on this reaction mixture with gradient elution of

90% water in MeCN to 10% water in MeCN. Identification of the product peak was carried out using LCMS and collection of the appropriate fractions gave the completed minor groove binder **94**. Although mass spectral data suggested this compound had been formed it could not be fully analysed by NMR.

The synthesis of similar compounds was continued when this project was started. Further investigation into the reaction conditions was needed due to poor results using the previous procedure. Thus a procedure developed by Dimmock et al.[79] was found and using this method, dimers **77** and **78** (page 72) were synthesised in 70-80% yields. It was decided to couple these compounds with a different dimer from the one used previously. Literature precedent shows that the pyrrole-pyrrole-dimethylamino tail group generally gives good biological activity in MGBs therefore it was decided that this dimer (**88**) should be used.[46]

Another aspect of the coupling that was changed was the method used to hydrolyse the ester of the diazo dimers. It was thought that it may not be necessary to convert the carboxylic acid salt to the free acid and that using it directly as the lithium salt that is formed in the hydrolysis reaction would not affect the overall coupling reaction. To test this, the ethyl ester of dimer **77** was hydrolysed using lithium hydroxide in aqueous methanol; the solvent was then removed in a freeze dryer to give the lithium salt of the acid.



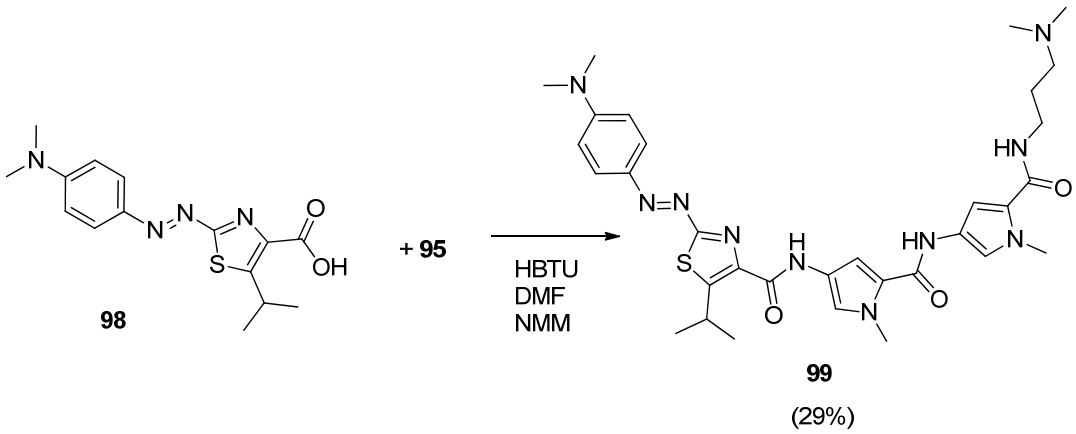
Scheme 12:

Synthesis of MGB **97**

Coupling was then carried out as before with the amine **95,** obtained from hydrogenation of **88,** using HBTU and NMM. HPLC was then carried out on this mixture however no product could be identified from this. It was therefore decided that this would not be a viable route and that the free acid should be obtained prior to coupling. To this end the lithium salt was converted to the free acid **96** and coupling was repeated with the amine **95**. In this case HPLC showed that the coupling had successfully produced the MGB tetramer **97** in 25% yield (scheme 12).

Ester **78** was then similarly hydrolysed to the carboxylic acid **98** using lithium hydroxide, the dimer **88** was hydrogenated to the amine **95** as before and coupling was carried out on these two dimers using HBTU (scheme 13).

As before HPLC was carried out directly on the reaction mixture as in the previous two reactions to give the completed minor groove binder **99** in 29% yield after freeze drying.



Scheme 13

Synthesis of MGB **99**

3.2.2 Amide isostere of diazo compounds

Producing minor groove binders with diazo linkages is interesting as a class of compounds but in order to determine if there is any advantage of this class over the standard amine linkages it would be necessary to produce diazo and amide compounds that are direct analogues of each other. To this end it was decided to attempt to synthesise amide equivalents of one or more of the three diazo intermediates already produced. Diazo compound **78** was chosen and its amide equivalent, **100** (figure 44), was identified as the target molecule; the amide **100** would then be hydrolysed and coupled with the same dimer molecule as its diazo equivalent.

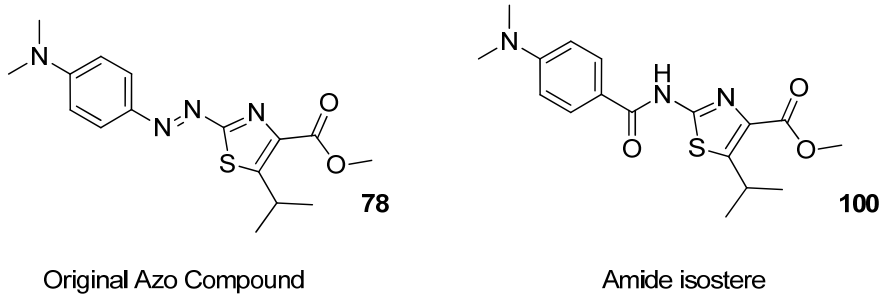
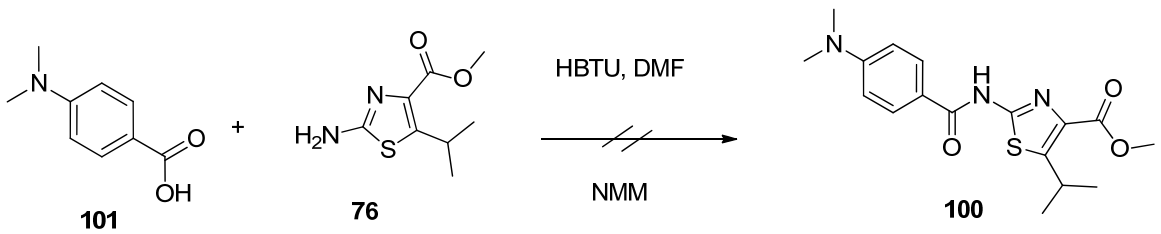


Figure 44

Comparison of azo and amide isosteres

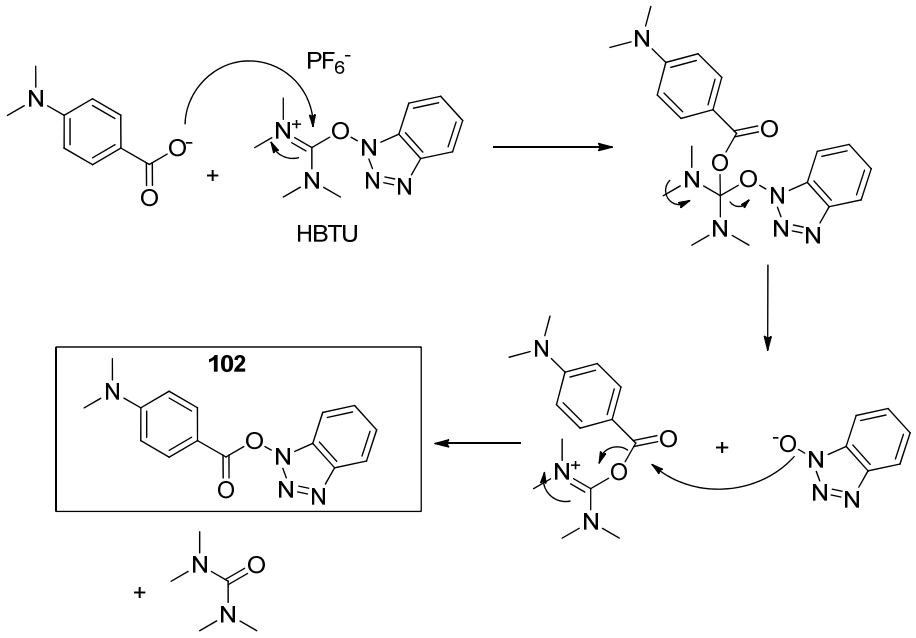
Initial attempts to synthesise compound **100** involved conversion of the carboxylic acid **101** into an acid chloride followed by coupling with the amine **76** in the presence of *N*-methylmorpholine (scheme 14). The reaction was followed by TLC, however after 24 hours there was no evidence that any coupling reaction had taken place. The same coupling reaction was then carried out using the carboxylic acid and the amine using standard HBTU coupling conditions.



Scheme 14:

Reaction scheme for amide isostere

Aqueous work up was followed by TLC analysis which appeared to show formation of a new product which was isolated by flash column chromatography and analysed. ^{1}H NMR and mass spectral analysis did not correspond to the expected product but unfortunately an exact structure could not be determined from these data. One possible explanation is the formation of an intermediate formed from reaction between the acid and HBTU as shown in scheme 15. [80]



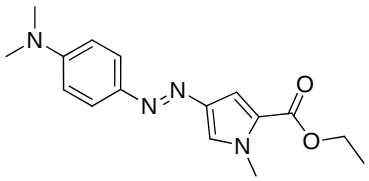
Scheme 15:

Mechanism of HBTU coupling to produce active ester

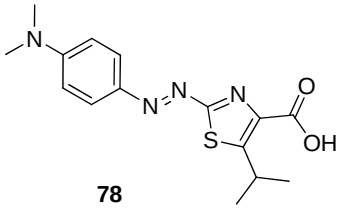
This product **102** would normally be the active intermediate in the amide coupling reaction; this would then be attacked by the amine to form the amide bond. If, however, the amine is not a strong nucleophile and does not attack the intermediate you may observe a stable intermediate. As mentioned, structure **102** could not be confirmed for the isolated product by NMR. However the presence of unreacted amine in the TLC along with the almost complete disappearance of the carboxylic acid would suggest this as a strong possibility. This would suggest that the thiazole amine **76** is a weak nucleophile and is therefore unsuitable for these types of amide couplings.

3.2.3 Synthesis of Pyrrole-Containing Diazo MGBs

Thiazoles were chosen as one of the components of lipophilic MGBs but it was important to have a good comparator for the thiazole in diazo MGBs and *N*-methylpyrrole was chosen. This would also mean that we could look at MGBs that have an affinity for AT sequences (pyrrole) and GC sequences (thiazole, see section 2.8). Therefore the dimer **103** (figure 45) was identified as an intermediate target molecule. Again, upon successful synthesis of this compound it would be coupled with the same dimer (**88**) as before. This head group is analogous to compound **78**.



103



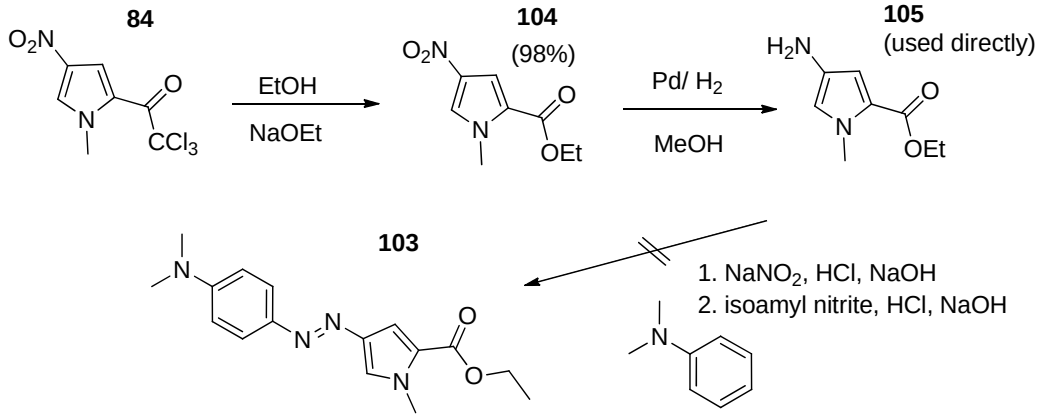
78

Figure 45

Head group **103** with analogous, thiazole containing head group **78**

First synthetic route for diazo-MGBs containing pyrroles

The first proposed synthetic route for the production of a diazopyrrole dimer (**103**) involved using the same procedure as for the thiazole equivalent namely making the diazonium salt of the amine and coupling this with *N,N*-dimethylaniline in an electrophilic aromatic substitution reaction (see scheme 16). Making the diazonium salt requires the presence of a stable amine; it was thought that this may present a problem as amines formed at the required position on pyrroles are unstable. This has been demonstrated in the amide coupling reactions described previously where the amine formed from the hydrogenation reactions (compound **95**) is observed to decompose when not used immediately.



Scheme 16:

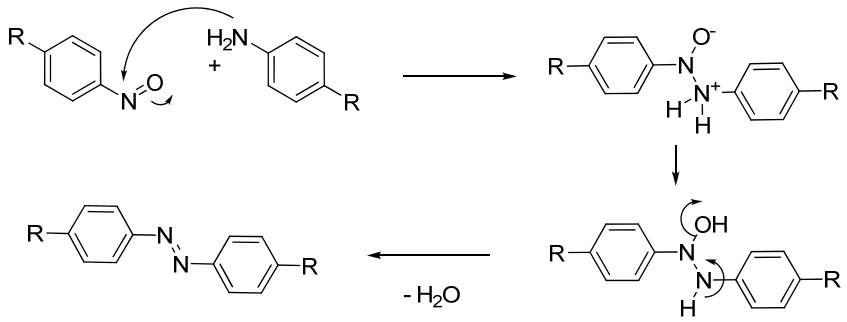
Reaction scheme for synthesis of pyrrole dimer **103**

Despite the possible difficulty this reaction was attempted. The amine **105** was formed by hydrogenation of the nitro group in **104** and immediately used in the diazotisation step and then shortly afterwards the coupling was attempted with *N,N*-dimethylaniline. This reaction was unsuccessful and no amine starting material was recovered from the reaction mixture suggesting decomposition had occurred. Several variations on this procedure were attempted to try to improve it; for example it has been demonstrated that the addition of HCl to the reaction mixture when carrying out hydrogenation would cause the hydrochloride salt of the amine to form as soon as it was made. [81] The more stable amine salt might be more suited as a starting material for the preparation of the diazonium salt. However, this appeared to have no effect on the reaction. Several substitutes for the sodium nitrite reagent were tried including isoamyl nitrite; however these also had no effect on the reaction. It was therefore decided that a new synthetic route should be devised that was more suitable for these compounds.

Second synthetic route for diazo-MGBs containing pyrroles

The second method of producing a diazo linkage is to carry out a condensation between an amine and a nitroso compound. There is literature precedent for this however it is concerned with condensation between two very simple aromatic compounds. Therefore there were some doubts as to whether or not this would be a viable route in this case.[76] The mechanism of this reaction involves nucleophilic

attack of the amine on the nitroso followed by loss of water to give the diazo bond (scheme 17).



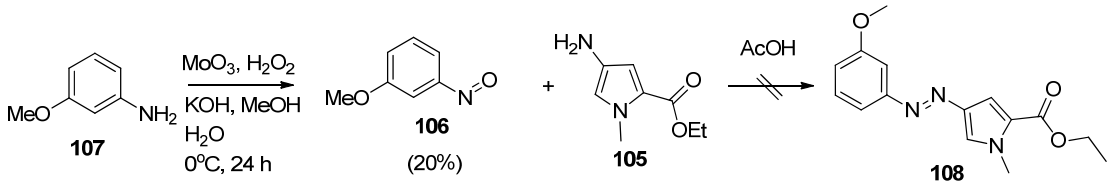
Scheme 17:

Mechanism of condensation reaction

The first step in this route was to find a procedure to oxidise an amino to a nitroso group. This was necessary because there are very few nitroso compounds commercially available to buy. The literature precedent for these types of conversion is limited to very simple aromatic compounds. [76] For this reason 3-methoxynitrosobenzene (**106**) was chosen to be the head group for the pyrrole dimer.

Although this head group does not exactly match the head group for the thiazole dimer it contains an important feature, the *m*-methoxy substituent, which is thought to be a group that leads to strong binding in DNA and good biological activity (see section 3.4). In the case of the preparation of the thiazole compounds, the *p*-hydroxy group was there to facilitate electrophilic aromatic substitution in the molecule. Oxidation was therefore attempted on *m*-anisidine (**107**) as shown in scheme 19 according to the procedure by Defoin.[76]

This reaction was low yielding with difficult purification. Also the product appeared to show signs of decomposition after being stored for several days. However the product was obtained in sufficient quantity to attempt a condensation reaction with a pyrrole monomer. As before the pyrrole nitro compound **104** was hydrogenated to give the corresponding amine **105**, and this was used immediately in the condensation reaction with the nitroso compound in the presence of acetic acid [82] (scheme 18)

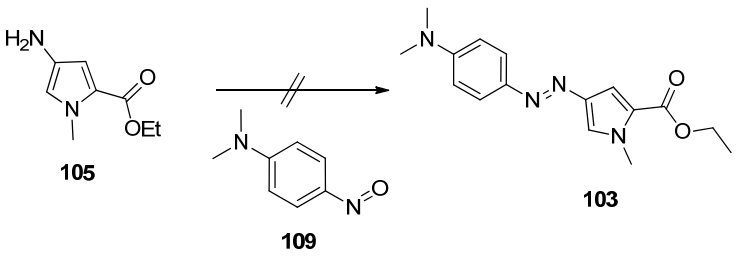


Scheme 18:

Attempted condensation of amine and nitroso

This reaction produced a dark brown solution that could not be purified to identify any products. There was however quite a large range of differing reaction conditions in the literature [83, 84] for these types of reactions therefore it was decided to carry out some test reactions. These were carried out using a different nitroso compound, 4-dimethylaminonitrosobenzene (**109**), which is commercially available and also leads to being a direct analogue of one of the previous thiazole containing MGBs (**99**).

The following reaction was carried out under a range of reaction conditions, for example in neat acetic acid at room temperature and at reflux; with acetic acid/ ethanol mixtures in varying ratios, also at room temperature and reflux. However none of these conditions produced any identifiable products. Scheme 19 shows an example of the reactions attempted.

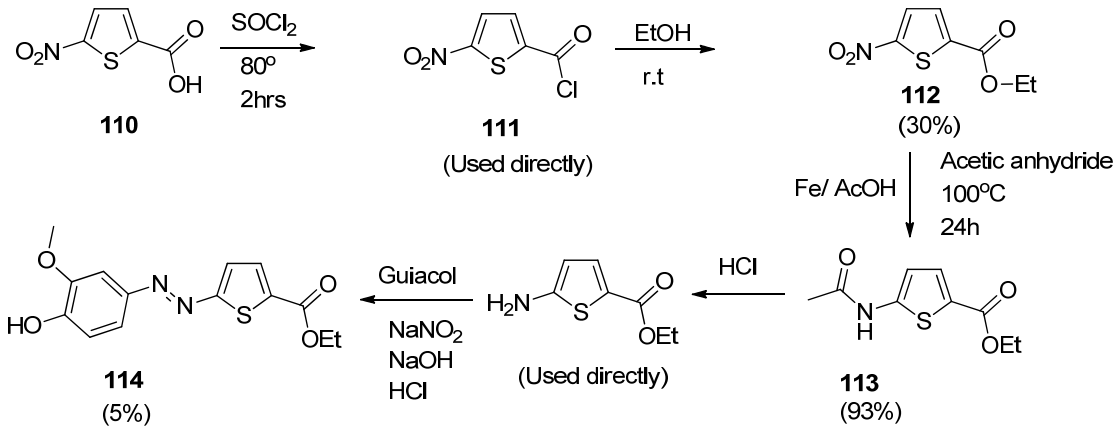


Scheme 19:

Attempted condensation of amine and nitroso groups

Protection of the amine in situ

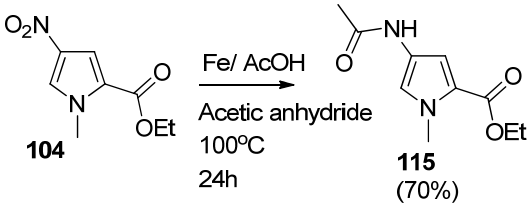
Investigations were carried out into possible alternative methods for hydrogenation of the nitro group to the amine with a possible view to synthesising the amine and carrying out the diazotisation in situ. During this time investigations were being carried out to determine the feasibility of introducing a thiophene ring into MGBs. This did not yield any products, however a paper was found written by Beers *et al*. [74] in which a nitro-thiophene compound was hydrogenated using acetic acid with an iron catalyst and protected with acetic anhydride in situ. This reaction was carried out to verify the result using the method shown in scheme 20 and the resulting acetamide **113** was found to be stable over a period of several days; also the yield of the reaction was excellent. Also shown is the synthesis of the dimer molecule **114** which was completed in very low yield.



Scheme 20

Synthesis of compound **114**

The reaction was then repeated with the pyrrole monomer, the same result was observed producing amide **115** in slightly lower but still acceptable yield (scheme 21).

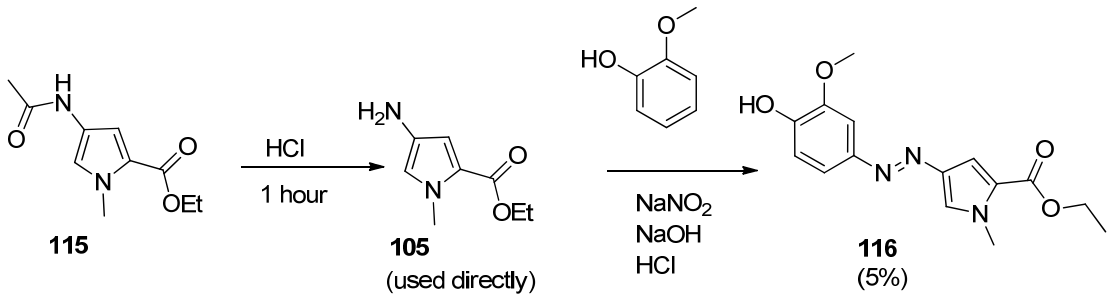


Scheme 21

Synthesis of compounds **115**

This result was important for several reasons. Firstly it showed that the aminopyrrole ester intermediate leading to **115** does form and is at least stable enough to react with acetic anhydride upon production. Secondly, it showed that the amine could survive long enough to react even under strongly acidic conditions (this was important given the acidic conditions needed for diazotisation of the amine). This was highlighted by the excellent yield suggesting little or no amine decomposition had occurred. Finally it highlighted the idea that the amine of this pyrrole **104** could be protected and that this protected amine could possibly be de-protected *in situ* to undergo a successful diazotisation reaction, thus providing a new strategy for the synthesis of pyrrole dimer compounds.

While the acetamide formed in the reduction reactions did provide useful information about both the thiophene and the pyrrole monomers there was some doubt as to whether or not it would be useful as an intermediate for the diazotisation process. The acetamide group is not particularly acid labile as a protecting group therefore it would possibly require harsh acidic conditions to remove it. Although the reaction of the amine in acetic acid showed that the amine was seemingly stable in acidic conditions the amine would have only been present in solution for a very short space of time. The amine resulting from the reduction would have reacted almost instantly with the acetic anhydride in solution whereas in the case of deprotection, such a short exposure to acidic conditions would be more difficult to achieve. Because of this, deprotection was attempted under reasonably mild conditions with dilute HCl at room temperature for 1 hour. This solution was then cooled to 0^{o}C and the diazotisation reaction was carried out followed by coupling with guaiacol. This reaction did produce the expected material **116** (scheme 22). Unfortunately the yield in this reaction was extremely low (~5%), scale-up of the reaction was attempted with no success. It appeared that either a small amount of deprotection had occurred or the acidic conditions had decomposed the bulk of the amine upon deprotection. Either one of these possible results would have suggested that the use of the acetamide protecting group was not appropriate for this type of reaction.



Scheme 22

Synthesis of compound **116**

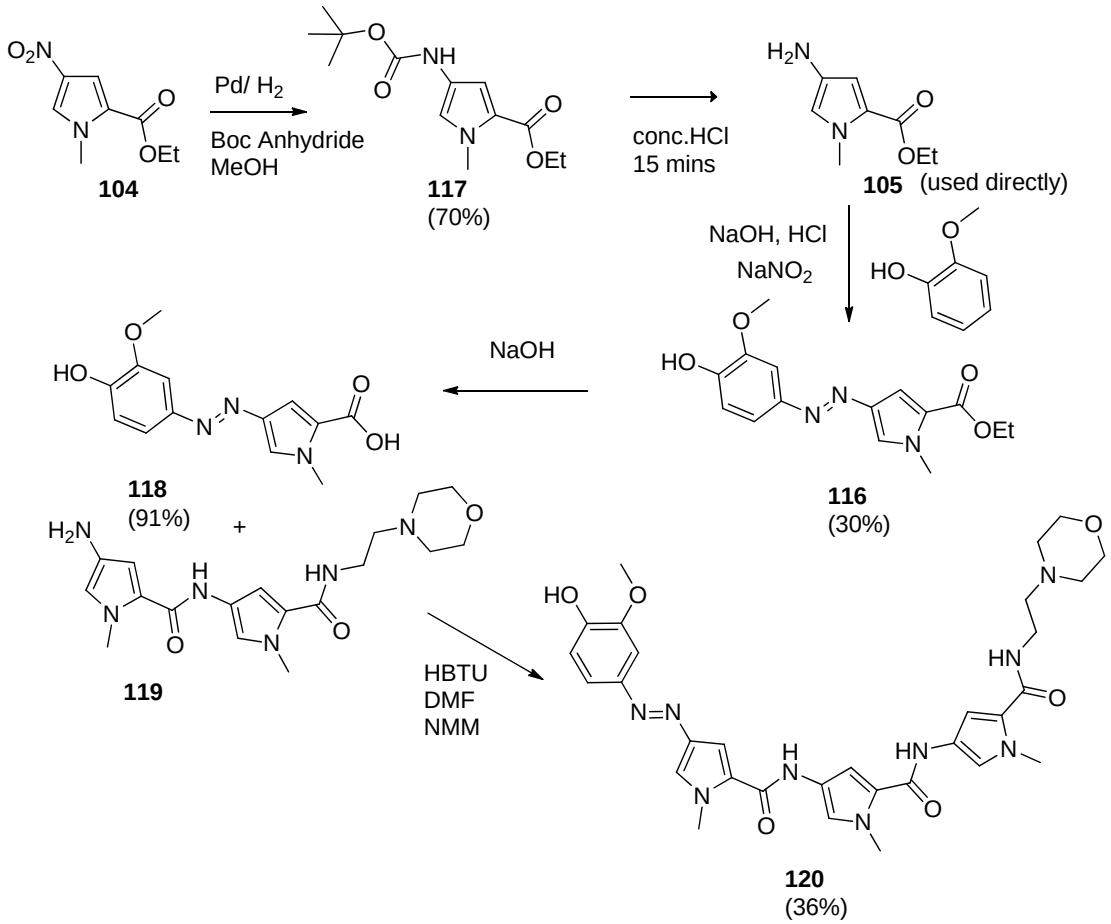
A possible alternative to the acetamide protecting group was to use a Boc protecting group. This group is acid-labile; therefore deprotection is more likely under mild acidic conditions. There is also literature precedent for reduction of nitro groups in heterocyclic compounds, particularly pyrrole, with Boc deprotection *in situ*.[85] The method used to Boc-protect the amine of the pyrrole was to use the standard, palladium-catalysed hydrogenation of nitropyrrole **104.** This would simply involve the addition of Boc-anhydride to the reaction mixture followed by purification by flash column chromatography to remove excess Boc anhydride.

This reaction was successful using the nitropyrrole **104** with the pure product **117** being formed in 70% yield (scheme 23). This was unsurprising due to the literature precedent for compounds of this type.[85] This compound appeared to be stable and therefore several test reactions were carried out to determine appropriate conditions for removing the Boc group and carrying out a diazotisation reaction.

In the next reaction only HCl was used to deprotect the amine followed by diazotisation and coupling; in this case the product was formed in 30% yield. This yield was reasonable compared to previous coupling reactions, however scale-up of the reaction gave drastically decreased yields. The product **116** was however formed in high enough quantities to proceed to the next step, which was hydrolysis of the ester group to give the corresponding carboxylic acid **118** in 91% yield (scheme 23).

With the carboxylic acid in hand it was now possible to attempt an amide coupling reaction between this dimer and the morpholino tail group **119** shown in scheme 23. This dimer possesses a morpholino tail group as opposed to the dimethylamino group used in previous MGBs. This was chosen due to the fact that it is the predominating tail group used in the alkene MGBs synthesised so far because the best antibacterial activity is found in compounds with the morpholinoethyl tail group.

As before, the pyrrole-pyrrole dimer had to have its nitro group reduced to an amine group (**119**); once this had been carried out the dimer was used immediately in the coupling step with the carboxylic acid dimer **118**. HPLC was then carried out directly on the reaction mixture to give the product **120** in 36 % yield (scheme 23).



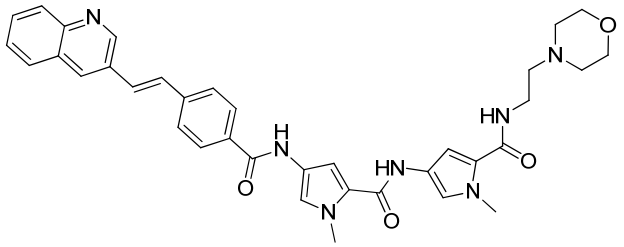
Scheme 23:

Reaction Scheme for pyrrole containing MGB **120**

3.3 Synthesis of lead compound analogues

3.3.1 Synthesis of compound **33** and analogues

As mentioned previously compound **33** (scheme 24) was synthesised by Dr. Khalaf however a brief description of the synthesis has been added to demonstrate the synthetic method for compounds of this type.

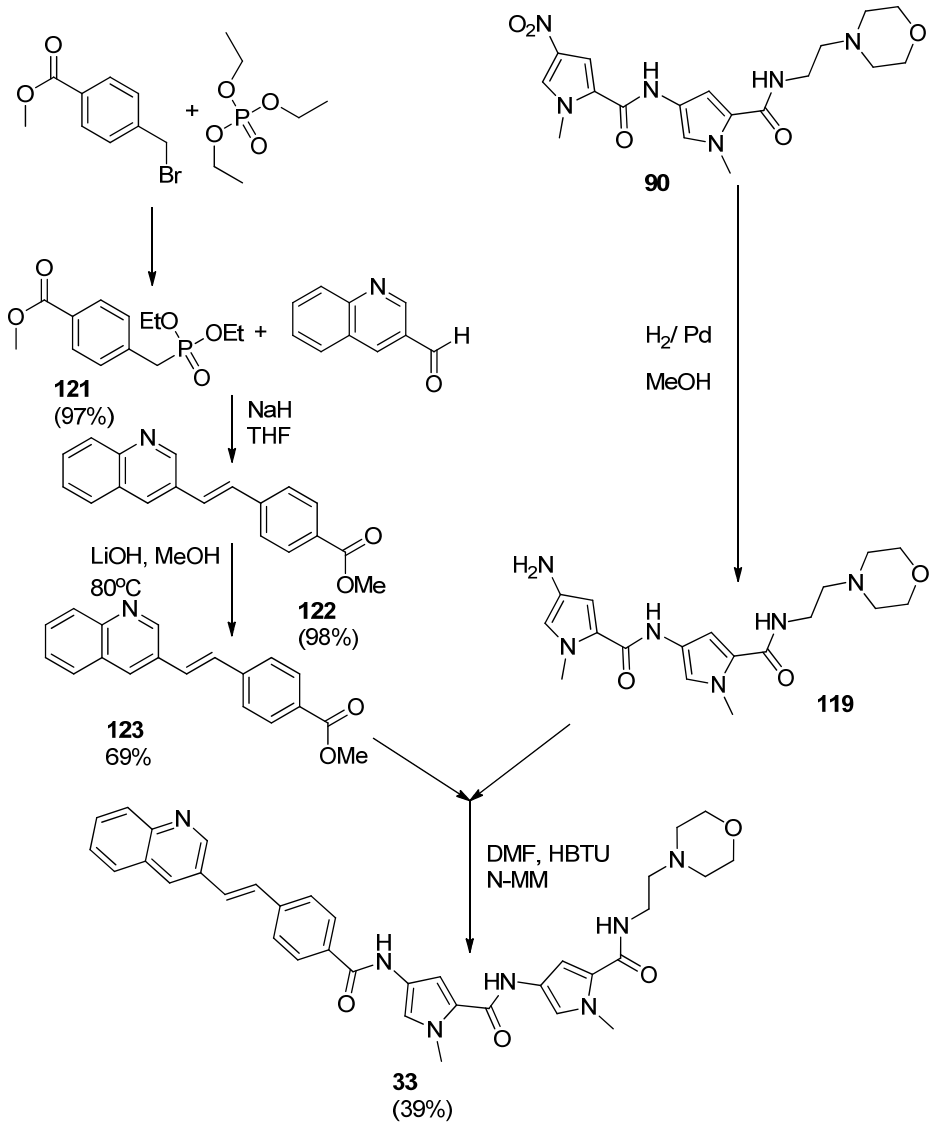


33

As in previous MGBs the compound was prepared via a convergent synthesis involving two dimers, the preparation of the tail end of the molecule is identical to previous preparations. The head section of the MGB was synthesised using the Horner-Wadsworth-Emmons method.

The Horner-Wadsworth-Emmons method involves the reaction of a phosphonate with an aldehyde. The aldehyde in this case would be quinoline-3-carbaldehyde however the phosphonate had to be synthesised from methyl 4-(bromomethyl)benzoate as shown in scheme 24. This was carried out by refluxing the starting material with an excess of triethyl phosphite; removal of the volatile triethyl phosphite then gave the desired product **121** in quantitative yield.

This phosphonate was then reacted with quinoline-3-carbaldehyde in THF with sodium hydride to give the desired product **122** shown in scheme 24.



Scheme 24:

Synthesis of MGB **33** (Carried out by Dr. Khalaf)

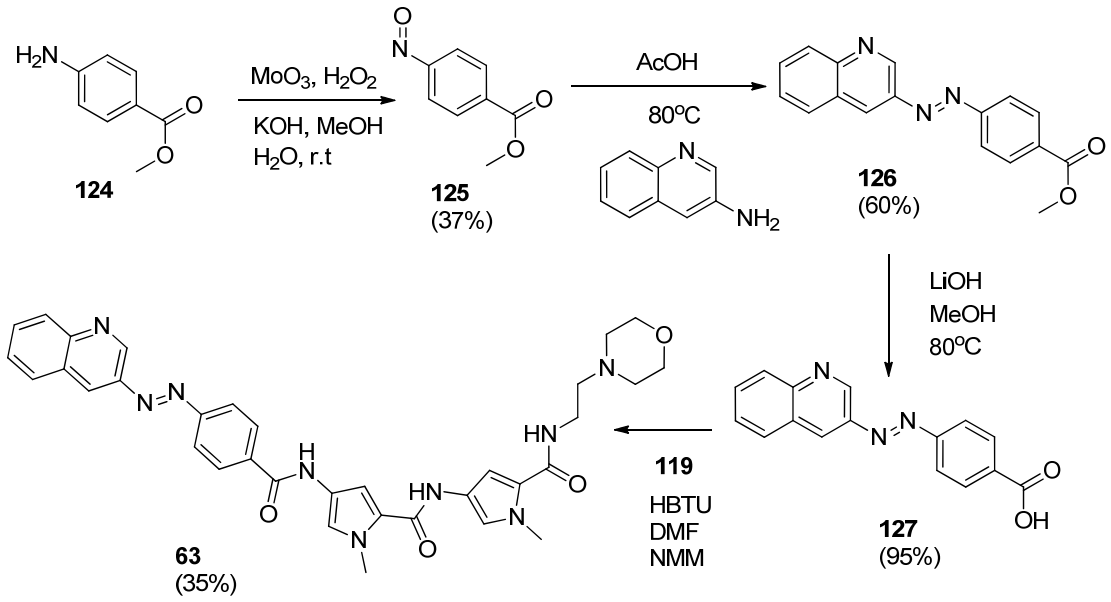
The methyl ester group was then hydrolysed to the corresponding carboxylic acid **123**.

With the carboxylic acid of the dimer in hand it was now possible to attempt the coupling reaction with the morpholino based dimer. The nitro group of the dimer **90** was hydrogenated over palladium catalyst to give the corresponding amine **117**. This amine was then coupled with the carboxylic acid dimer **121** in an HBTU coupling reaction. HPLC was then carried out directly on the reaction mixture to give the desired product **33** in 39% yield (scheme 24).

The next target compound was the diazo MGB (**63**), which is the diazo analogue of the antibacterial lead compound **33.** This would, as before, mean synthesising the dimer molecule **127** shown in scheme 25. This synthesis was carried out by the author.

It was quickly decided that a molecule of this type could not be synthesised from a diazonium salt reaction as it does not possesses the strongly electron donating group necessary for electrophilic aromatic substitution on either side of the molecule. It was therefore decided to attempt the alternative diazo synthesis, a condensation reaction between an amine and a nitroso group. Earlier attempts at these reactions had proved unsuccessful; however it was thought that a condensation reaction between two relatively simple aromatic compounds such as the components of **127** might work.[76]

It was first necessary to convert the amine, ethyl 4-aminobenzoate **124** to its corresponding nitroso compound **125** as shown in scheme 25. This reaction was carried out according to literature precedent[76] giving the nitroso compound **125** in similar yields to those reported. The condensation reaction was carried out between the nitroso compound (**125)** and 3-aminoquinoline using a modified version of the procedure used by Ogata [86] to give the diazo compound **126** in 60 % yield. This was a pleasing result given the previous lack of success in these reactions.



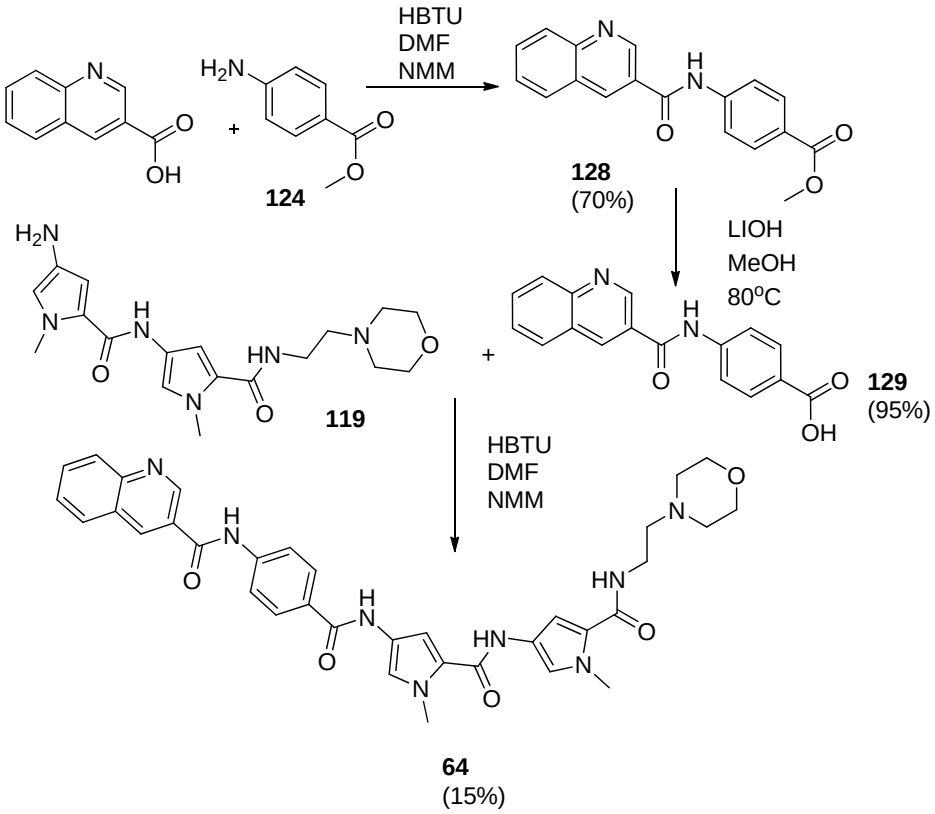
Scheme 25

Synthesis of MBG **63**

The methyl ester of the dimer **126** was then hydrolysed to give the corresponding carboxylic acid **127** in 95% yield.

With the carboxylic acid **127** in hand it was now possible to attempt the coupling reaction with the morpholino based dimer used previously. As before the nitro group of the dimer **90** was hydrogenated over a palladium catalyst to give the corresponding amine **119**. This amine was then coupled with the carboxylic acid dimer **127** in an HBTU coupling reaction. HPLC was then carried out directly on the reaction mixture to give the desired product **63** in 35% yield (scheme 25).

The final target in this series is the amide analogue of AIK 19/56-2, **33**, which, perhaps surprisingly, had not been prepared before. This required synthesising the amide dimer **128** (Scheme 26) which involved a simple amide coupling reaction between commercially available building blocks, quinoline-3-carboxylic acid and methyl 4-aminobenzoate as shown in scheme 26.



Scheme 26

Synthesis of MGB **64**

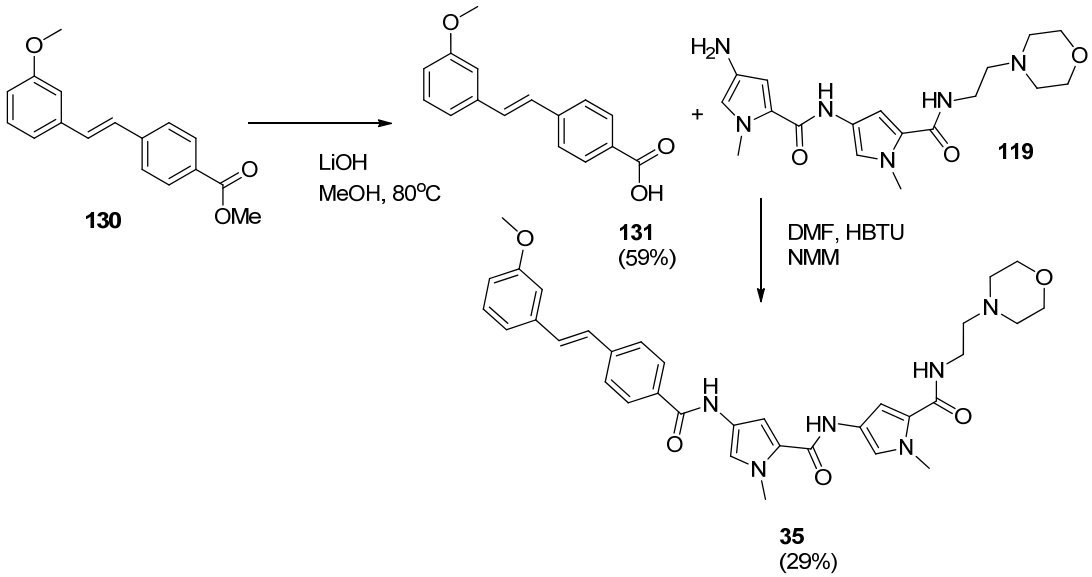
The methyl ester of the dimer was then hydrolysed to give the corresponding carboxylic acid **129** in 95% yield. The completion of the synthesis of **64** used methods previously outlined for other MGBs containing the pyrrole dimer **119** in 15 % yield (scheme 26).

3.3.2 Synthesis of compound **35** and analogues

As mentioned previously compound **35** was synthesised by Dr. Khalaf however a brief description of the synthesis has been added to demonstrate the synthetic method for compounds of this type.

As in previous MGBs the compound would be prepared via a convergent synthesis involving two dimer, the preparation of the tail end of the molecule is identical to previous preparations. The head section of the MGB would be synthesised using the Horner-Wadsworth-Emmons method.

The phosphonate **121** was prepared as described previously, this was then reacted with commercially available 3-methoxybenzaldehyde in THF with sodium hydride to give the desired product **130** shown in scheme 27.



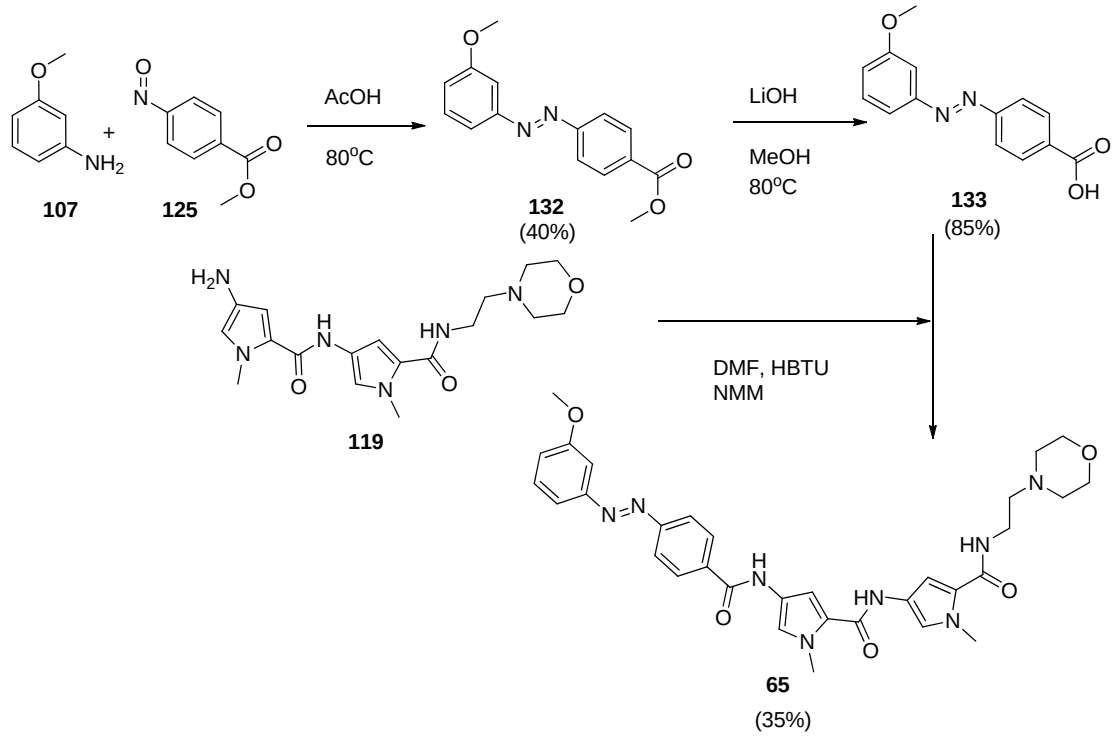
Scheme 27

Synthesis of MGB **35** (carried out by Dr. Khalaf)

The methyl ester group was then hydrolysed to the corresponding carboxylic acid **131**. With the carboxylic acid of the dimer in hand it was now possible to attempt the coupling reaction with the morpholino based dimer used previously. As before the nitro group of the dimer **90** was hydrogenated over a palladium catalyst to give the corresponding amine **119**. This amine was then coupled with the carboxylic acid dimer **131** in an HBTU coupling reaction. HPLC was then carried out directly on the reaction mixture to give the desired product **35** in 50% yield (scheme 27).

The following molecule with the alkene group replaced by an diazo group (**65**) is shown in scheme 28.

As before, this would involve the synthesis of the following dimer **132**. Similar to the diazo analogue for AIK 19/56-2, this molecule could not be synthesised by the diazonium salt reaction described earlier in the report. This is again due to the lack of a strongly electron donating group in the correct position to facilitate electrophilic aromatic substitution on either of the benzene rings.



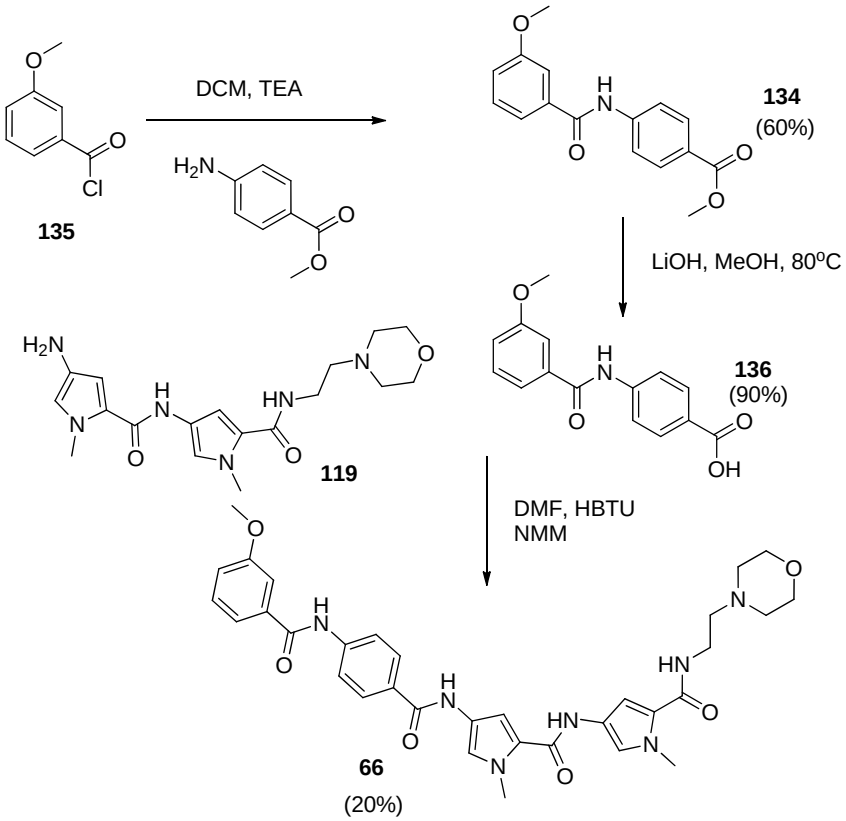
Scheme 28:

Synthesis of MGB **65**

This meant that a condensation reaction would have to be carried out between commercially available *m*-anisidine and the nitroso compound **125** described previously. The reaction was therefore carried out in the presence of AcOH to give, after purification by column chromatography, the product in 40% yield. The methyl ester of the dimer was then hydrolysed to give the corresponding carboxylic acid **133** in 85% yield.

Coupling of the carboxylic acid **133** of the dimer and the morpholino tail group **119** was then carried out according to standard procedures. HPLC was then carried out directly on the reaction mixture to give the desired product **65** in 35% yield (scheme 28).

The following molecule **66** with the alkene group replaced by an amide group is shown (scheme 29). As before, this would involve the synthesis of the dimer **134**. This could have been achieved as in the previous amide head group with a simple HBTU coupling however it was felt that the compound could be more efficiently synthesised using an acid chloride (**135**) and carrying out a coupling reaction with the appropriate amine as follows (scheme 29); this method gave the desired product **134** in 60% yield.



Scheme 29:

Reaction scheme for MGB **66**

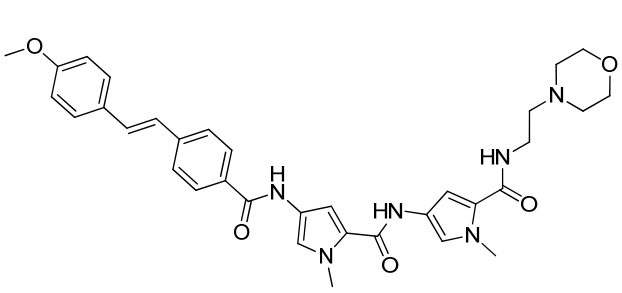
The methyl ester of the dimer was then hydrolysed to give the corresponding carboxylic acid **136** in 90% yield.

Coupling was then carried out as described previously using the carboxylic acid **136** and the amine **119** (obtained by reduction of **90**) using HBTU. HPLC was then carried out directly on the reaction mixture to give the desired product **66** in 20% yield (scheme 29).

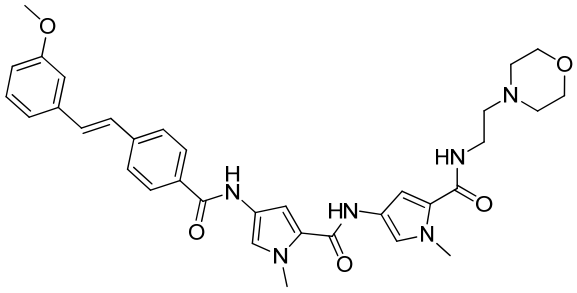
3.3.3 *Para*- Methoxy analogues of compound **35**

Given that the MGB **35** had shown excellent activity in vitro it was decided that some further insight was needed as to the source of this activity. Since other stilbene compounds with different substituents on the terminal benzene ring had shown either weak activity or no activity at all it was clear that the methoxy group was very important. In order to test whether the activity in these molecules was sensitive to the position of the methoxy group it was decided to synthesise a series of compound with the substituent in the para position instead of the meta as before.

The synthesis of the alkene MGB **137** was identical to that described for compound **35** except that 3-methoxybenzaldehyde was replaced with 4-methoxybenzaldehyde in the alkene coupling step. Although mass spectral data suggested the product had formed the NMR data was not conclusive.

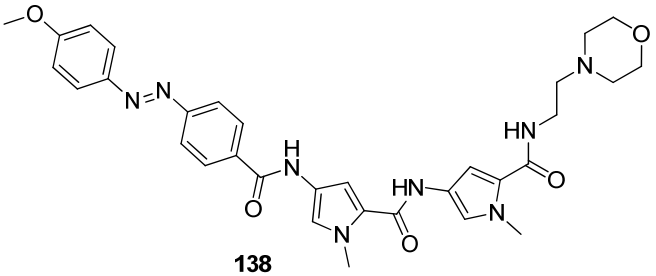


137



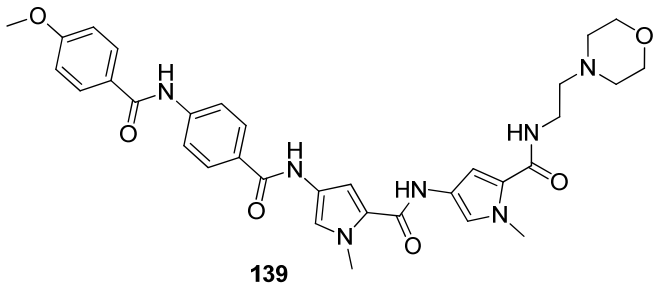
35

Similarly the synthesis of the diazo compound **138** was identical to the method used for compound **65** except for the use of *p*-anisidine as apposed to *m*-anisidine in the diazo coupling step; **138** was obtained in 40% yield.



138

Finally the amide analogue **139** was synthesised using the same method as for compound **66** except for the use of 4-methoxybenzoyl chloride instead of 3-methoxybenzoyl chloride; **139** was obtained in 25% yield.



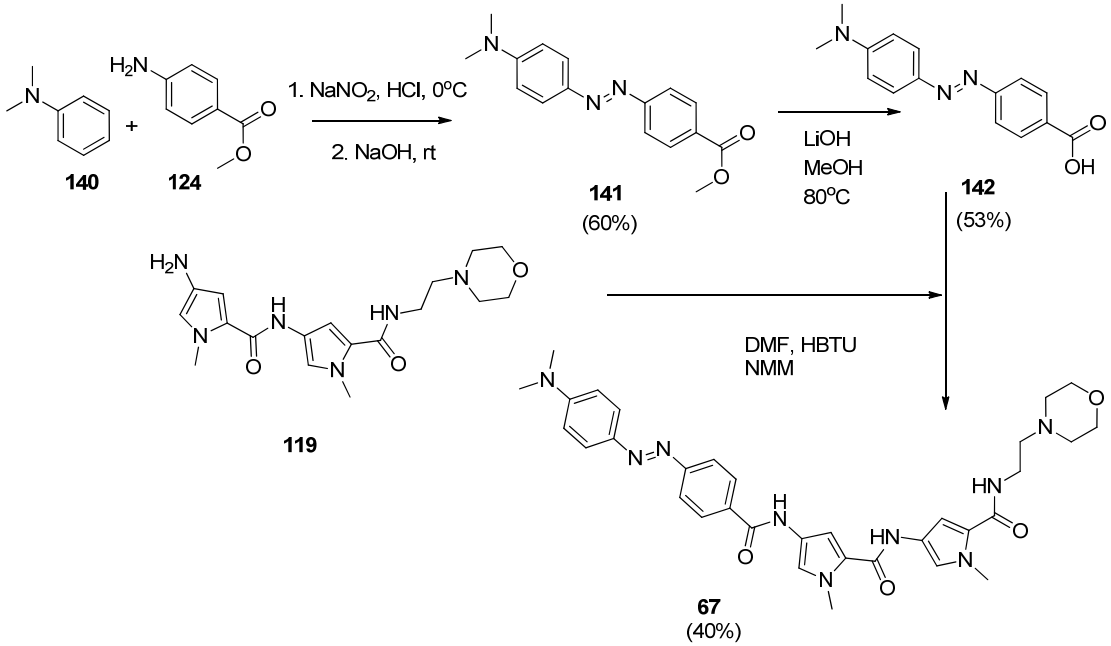
139

3.3.4 Synthesis of Dimethylaminophenyl MGBs

The first molecule synthesised in this series is shown in scheme 30, the structure was not a direct analogue of any stilbene type MGBs that had been synthesised at the time. Instead the structure was chosen due to the relatively simple synthesis involved in making it with a view to synthesising the subsequent alkene and amide analogues afterwards.

The first target in this series was to produce the dimer **141** shown in scheme 30. This dimer does not directly relate to any of the existing alkene compounds as none of them possess a head group substituted with a dimethylamino group. It does however have the same general shape of the alkene compounds.

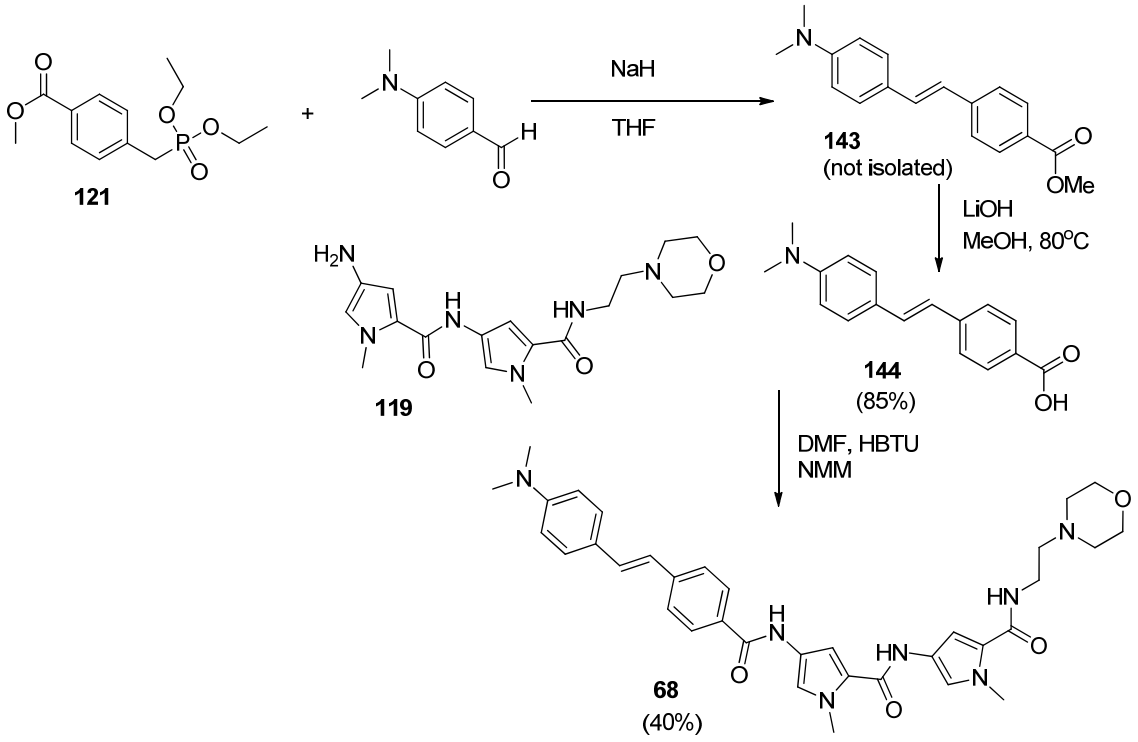
The synthesis of this dimer would require a diazotisation reaction on the amine group of methyl 4-aminobenzoate followed by electrophilic aromatic substitution of this diazonium salt on *N,N'*-dimethylaniline. Both of these starting materials are commercially available which meant the synthesis would be relatively straightforward (Scheme 30).



Scheme 30:
Preparation of MGB **67**

This reaction was carried out and the product **141** was obtained in 60% yield. As before the methyl ester of the dimer had to be converted to the carboxylic acid **142**, this was carried out using lithium hydroxide and the acid was obtained in 53% yield. This was now ready to be used in the coupling step. Coupling with the tail group **90** was carried out as described previously with the nitro group reduced to the amine (**119**) which was used immediately in the coupling step. HPLC was then carried out directly on the reaction mixture to give the desired product **67** in 40% yield (scheme 30).

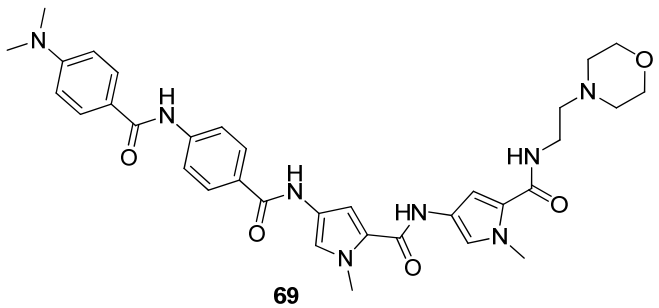
The alkene analogue of this compound (**68**) was then prepared according to the method set out by Dr. Khalaf for compounds of this type. As before, the head section of the MGB was synthesised using the Horner-Wadsworth-Emmons method (scheme 31). The phosphonate **121** was prepared as described previously, this was then reacted with commercially available 4-(dimethylamino)benzaldehyde in THF with sodium hydride. The product of this reaction was found to be a mixture of the product **143** and the corresponding carboxylic acid **144**, it was therefore decided to take this material forward to the next step to convert all of the product to compound **144**.



Scheme 31:
Synthesis of MGB **68**

Again the carboxylic acid (**144**) was used in an HBTU coupling reaction with the amine **119**. HPLC was then carried out directly on the reaction mixture to give the desired product **68** in 40% yield as in scheme 31.

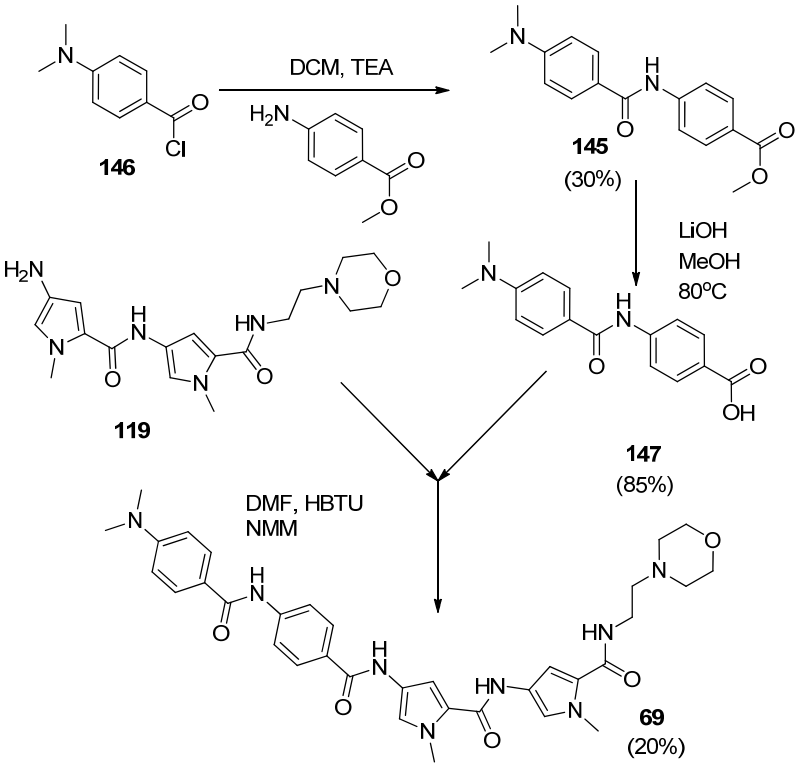
The following molecule has the alkene group of **68** replaced by an amide group (compound **69)**.



69

As before, this would involve the synthesis of the dimer (**145,** scheme 33). This was prepared similarly to the 3-methoxy compound using an acid chloride (**146**) and carrying out a coupling reaction with the appropriate amine to give the product in 30% yield as in scheme 32.

The methyl ester of the dimer **145** was then hydrolysed to give the corresponding carboxylic acid **147** in 85% yield. The remainder of the synthesis was carried out similarly to previous MGBs as shown in scheme 32. HPLC was then carried out directly on the reaction mixture to give the desired product **69** in 20% yield.
Figure 46 shows a representative HPLC trace of an MGB.



Scheme 32:

Synthesis of compound **69**

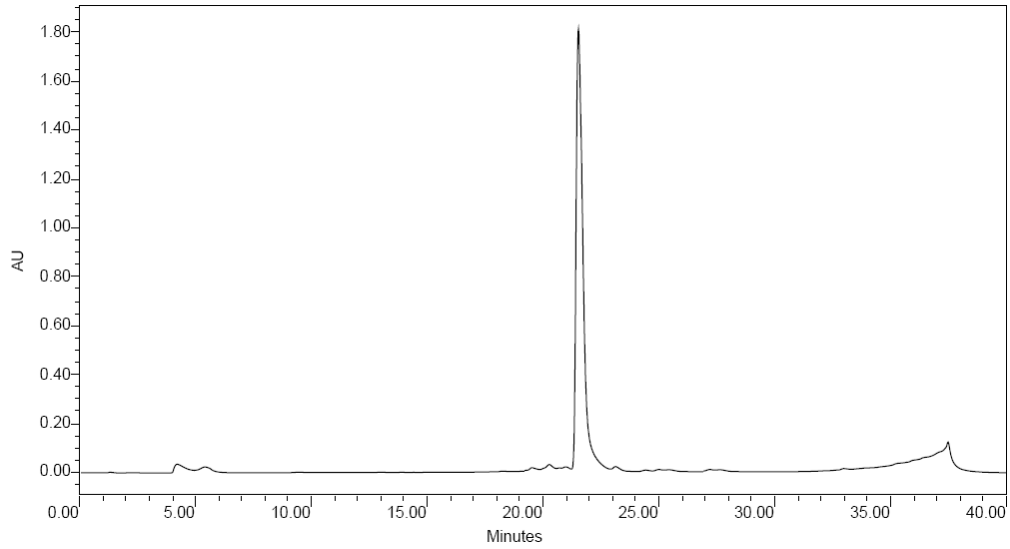


Figure 46

HPLC trace of compound **63**. Peak at 21.5 mins corresponds to MGB **63.**

3.4 Biological assay results.

The MGBs successfully synthesised where submitted for biological testing. These compounds were submitted to Allen Drummond's lab at Strathclyde University. The minimum inhibitory concentration (MIC) of compound was tested against three bacteria namely two strains of *Staphylococcus aureus*, 1 and 2, and *Staphylococcus faecalis*. Each compound was also tested against the fungi *Aspergillus niger* and *Candida albicans*. However very little activity was recorded. These results will be summarized and discussed briefly with more detailed discussion to follow in a later section. Minimum inhibitory concentration is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

Bacteria/ Fungi	*Control drug*	*MIC*
S. aureus and S. faecalis	Amoxicilin	0.49μM (0.19μg/mL)
C. albicans and A. niger	Fluconazole	326μM (100μg/mL)
M. .fortuitum	Streptomycin	5.36μM (3.12μg/mL)

Table 2

The MICs of the control drugs for each of the bacteria and fungi tested. All data are minimum inhibitory concentrations, given as μg mL^{-1} and μM.

Antibacterial activity of the lead head group alkene containing MGBs

Cmpd	*S. aur 1 (μM)*	*S. aur 2 (μM)*	*S. faec (μM)*	*Structure (Head*– 1 – 2 – 3 – tail)
33	0.16(0.12)	0.32(0.25)	33.5(25)	3-quin = p-ph – PyrMe – PyrMe - MorphE
35	2.7(2.0)	11.0(8.0)	69(50)	m-MeO= p-ph – PyrMe – PyrMe - MorphE
39	34(25)	68(50)	136(100)	Naphth=p-ph – PyrMe – PyrMe - MorphE
42	8.6(6.25)	17.2(12.5)	8.6(6.25)	2-pyridine=p-ph–PyrMe – PyrMe - MorphE

Table 3

Biological data for lead compunds. All data are minimum inhibitory concentrations, given as μg mL^{-1} and μM

Table 3 shows a list of the biological activities for all the compounds synthesised by Dr. Khalaf that have been previously mentioned in this thesis (shown in figure 47). It is important to note that where possible these compounds have been designed to have only one variation from molecule to molecule. For example, all of these compounds have an ethyl morpholine tail group and virtually all contain the phenyl-pyrrole-pyrrole section in the centre of the molecule. The first of these compounds is **33** which was shown to be extremely potent against *Staphylococcus aureus* 1 with a MIC of 0.16μM with similar activity against *Staphylococcus aureus 2*. An interesting comparison can be made between this compound and **39**. This compound is identical to **33** except the terminal aromatic group is naphthalene instead of quinoline. It has been previously mentioned that there have been some studies into the placement of a nitrogen atom in these head groups. **39** serves as an example of the effect of removing the nitrogen atom completely and interestingly shows little or no activity suggesting the nitrogen atom in this case is vital to binding.

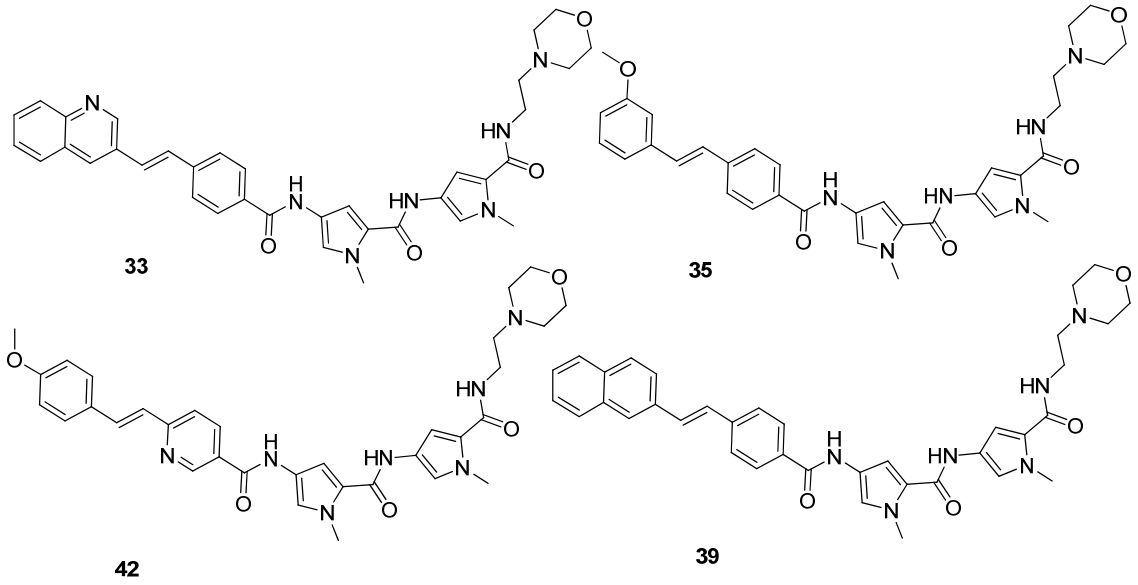


Figure 47

Structures of alkene containing MGBs

Antibacterial activity of alkene containing MGbs

Cmpd	*S.aur 1 (μM)*	*S. aur 2 (μM)*	*S. faec (μM)*	*Structure (Head–* 1 – 2 – 3 – tail)
54	>100	>100	100	3-OMe-ph - p-ph – PyrMe = PyrMe - MorphE
56	3.12	6.25	25	2-quin=Thia – PyrMe – PyrMe - MorphE
57	>100	>100	100	2-quin=Thia - PyrMe=PyrMe - MorphE
61	1.0-3.9	1.9	500	3-Quin=p-ph – PyrMe – PyrMe - nitro alkene
62	7.8	15.6	500	3-Quin=p-ph – PyrMe – PyrMe - guanidine

Table 4

Biolocal data for alkene containing MGBs. All data are minimum inhibitory concentrations, given as μM

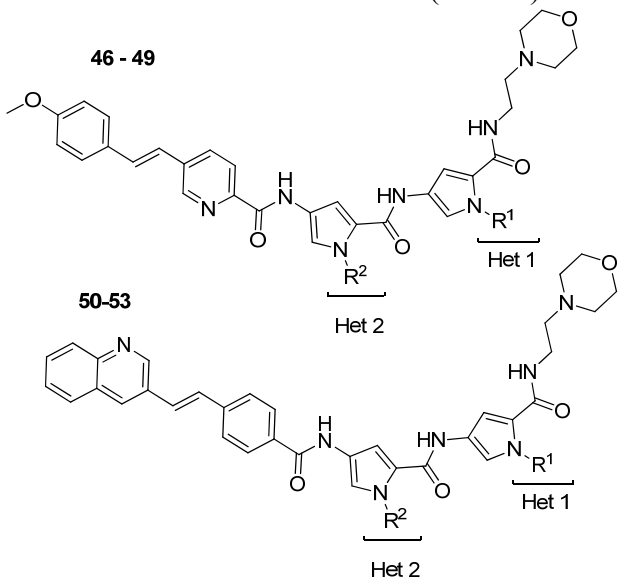
Of the compounds in this report synthesised by Dr. Breen there are several that have interesting activity (Table 4). For example a comparison of compound **56** with compound **57** (see page 60) shows that the former has significant activity against *Staphylococcus aureus* 1 while compound **57** has no notable biological activity whatsoever (within the limits of these assays). The only difference between these compounds is in the presence of an alkene link between the two pyrrole rings in **57** that is replaced with an amide bond in **56**. Combining this result with the binding studies carried out in this report will provide an interesting study in the link between biological activity and DNA binding. Comparing compound **54** with **66** described earlier in the report would provide a similar comparison. Finally the biological activity noted in compounds **61** and **62** (see page 61) are noteworthy due to the novel nature of the tail groups involved. Again further studies carried out in this report will give more insight into the nature of this activity in terms of DNA binding.

Biological activity of compounds investigating Alkyl group variation

Cmpd	*E.coli*	*S.aur 1 (μM)*	*S. aur 2 (μM)*	*M.aurum (μM)*	*Structure (Head–* 1 – 2 – 3 – tail)
	% of control	*% of control*			
46	103.2	8.5	4	8	4-OMe=pyridyl – PyrMe – PyriPr - MorphE
47	115.6	2.8	16	16	4-OMe=pyridyl – PyrMe – Pyr*sec*-but - Morph
48	98.6	6.7	16	8	4-OMe=pyridyl – PyriPr – PyrMe - MorphE
49	100.9	3.2	8	31	4-OMe=pyridyl – Pyr*sec*-but – PyrMe - Morph
50	94.7	0.8	<1	2	3-Quin=p-ph – PyrMe – PyriPr - MorphE
51	74.2	0.3	<1	2	3-Quin=p-ph – PyrMe – Pyr*sec*-but - MorphE
52	80.9	2.3	<1	2	3-Quin=p-ph – PyriPr – PyrMe - MorphE
53	69.0	2.9	<1	2	3-Quin=p-ph – Pyr*sec*-but – PyrMe - MorphE

Table 5

Biological data for MGBs with varied alkyl groups. All data are minimum inhibitory concentrations, given as μM. In addition to the bacteria typically used compounds 46 – 53 were also tested against *Mycobacterium aurum* (*M. aurum*) and *Escherichia coli* (*E. Coli*)



Compound	R1	R2
46	Ipr	Me
47	SecBut	Me
48	Me	Ipr
49	Me	SecBut
50	Ipr	Me
51	SecBut	Me
52	Me	Ipr
53	Me	SecBut

Figure 48

Structures of compounds **46 - 53**

As previously mentioned these compounds were designed to provide a comparison with the lead compounds **33** and **42**. Compounds **46-49** (figure 48) all relate to **42** and all of them show good biological activity (table 5). This is unsurprising since they are closely related to an active lead compound; however their purpose was to ascertain whether the differences in them lead to differences in behaviour. Looking at the range of results within the series it is clear there are differences however they are reasonably small and in tests such as these could be considered negligible

therefore it is difficult to draw any conclusion. It is for this reason that binding studies were carried out on these compounds, the results of which can be found in section 3.5.5. Compounds **50-53** (figure 48) all relate to compound **33** (page 104) and again clearly the activities are comparable. The same problem however exists as in the previous series, therefore binding studies were also carried out on this series.

Lead compound analogues containing diazo or amide linkages

Cmpd	*S.aur 1(μM)*	*S. aur 2 (μM)*	*S. faec (μM)*	*A. nig (μm)*	*C. alb (μm)*	*Structure (Head–* 1 – 2 – 3 – tail)
94	>126(>100)	>126(>100)	126(100)	126(100)	63(50)	m-Cl-p-OHN=N – PyrMe – PyrMe – PyrMe - Morph
63	>134(>100)	>134(>100)	134(100)	67(50)	34(25)	3-Quin N=N - p-ph – PyrMe – PyrMe - MorphE
120	17(12.5)	34(25)	8.4(6.25)	67(50)	134(100)	GuaiacolN=N PyrMe – PyrMe – PyrMe - MorphE

Table 6

Biological data for lead compound analogues. All data are minimum inhibitory concentrations, given as μg mL^{-1} and μM

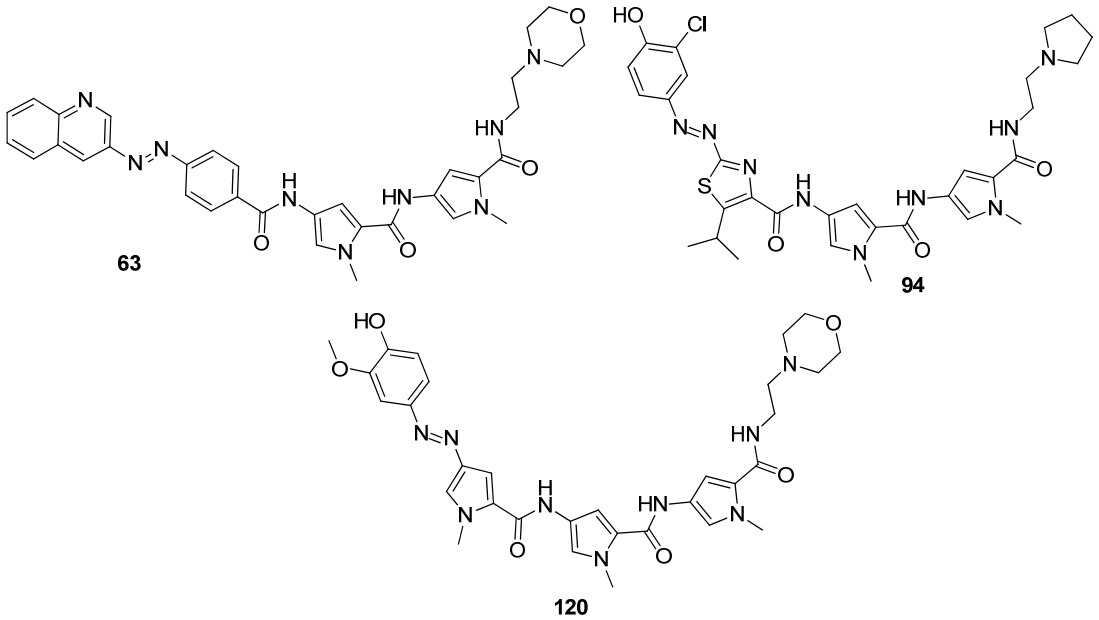


Figure 49

Lead compound analogues containing diazo linkages.

The series of compounds containing diazo linkages and thiazole groups (**94**, **97** and **99**) showed no significant antibacterial activity (see table 6). Compound **94** (figure

49) however showed some antifungal activity against *C. albicans*; a MIC of 35μM (25μg/mL) was recorded, a four fold increase in activity over the control drug fluconazole. Likewise compound **120** (figure 49) containing a pyrrole group showed little antibacterial or antifungal activity in biological assays; low antibacterial activity against *S. aureus* and *S. faecalis* was recorded however no improvement on the control amoxicilin was observed. Low antibacterial activity against *M. fortuitum* was also shown. However no improvement on the control, streptomycin, was found.

Analogues of lead compounds containing diazo or amide linkages showed little or no biological activity.

Compound **63** (figure 49, analogue of lead compound **33**) showed weak antifungal activity against *Aspergillus niger* corresponding to a twofold increase in activity compared with the control, fluconazole. Activity against *C. albicans* was also observed corresponding to a fourfold increase in activity compared with fluconazole**.**

Compounds **63**, **64**, **65**, **66**, **67**, **68**, **69**, **137**, **138**, **139** and **151** showed no significant antibacterial or antifungal activity.

Some important conclusions can be drawn from these data. Comparison of lead compound **33** which showed excellent in vivo activity with diazo and amide analogues **63** and **64** which showed no activity would imply that the alkene link is vital for activity. This is supported by comparison of lead compound **35** with its diazo and amide analogues **65** and **66** which also showed no activity. Having identified the alkene as a key feature for antibacterial activity, the question arises whether this activity correlates with binding to DNA. Binding studies were carried out on these compounds to learn more about the links between biological activity and binding to DNA as well as relative binding strengths of analogous compounds.

3.5 Determination of Binding strength to DNA

3.5.1 Binding studies of lead compounds

As previously mentioned all of the final compounds synthesised were submitted for biological testing; this made sense as one of the main purposes behind making these analogous compounds was to observe the effect of changing linking groups on activity. Therefore comparing the results of diazo, amide and alkene linked compounds should give us at least an idea of their relative importance in terms of biological activity. However since we know very little about the mode of action of the active compounds we cannot say for sure that the increases/decreases in activity are due to their strength of binding to DNA. It is possible that activity in compounds such as 19/56-1 (**35**) is due to some other aspect of their structure that has as yet been undetermined. This makes it difficult to design similar molecules with potentially greater binding.

If we knew the relative importance of different functional groups, especially the amide, alkene, and diazo links, for DNA binding then it would be easier to start to make sensible decisions about what changes in the molecule could result in more effective minor groove binders.

Therefore the decision was made to run several experiments with the groups of molecules described in order to assess their binding strength. One of the simplest experiments was to carry out T_m (melt temperature) experiments on them in order to show the increase in stability of a DNA oligomer when bound to these compounds by observing changes in melt temperature. This would give a good indication of binding strength. [64]

Another method that could be used was capillary electrophoresis (CE); this has been explained in section 2.11.2 and would give a clear indication of whether of not the compounds are binding and to the relative ratios of binding and also, to some degree, the strength of binding.[65]

Finally ITC (isothermal titration calorimetry) could be used to measure changes in heat upon binding of these compounds to DNA which would give a very good indication of relative binding strength leading directly to thermodynamic parameters.[68]

All of the above methods are based on binding of these compounds to one specific DNA sequence; this sequence would have to be chosen to be one likely to bind to the compounds under test, as the preferred sequence for these compounds is as yet unknown.

For this reason DNA footprinting tests are also being carried out by our collaborator, Professor Keith Fox (University of Southampton). However this can be a lengthy procedure therefore UV melt, ITC and CE experiments were carried out using an AT-containing DNA oligomer typical of those to which this class of MGB is known to bind. Previously several tests had been carried out on MGBs involving CE and ITC, these are outlined below as they were helpful in the selection of an appropriate DNA oligomer.

ITC Experiments

As described in section 2.1.3 ITC (isothermal titration calorimetry) is a method by which a syringe containing an MGB is titrated into a cell containing DNA. The temperature change (exothermic or endothermic) upon titration is measured. The temperature difference between the sample and reference cells (ΔT) is kept at zero by increasing or decreasing the feedback power to the sample cell. This produces a graph such as the one shown in figure 50.

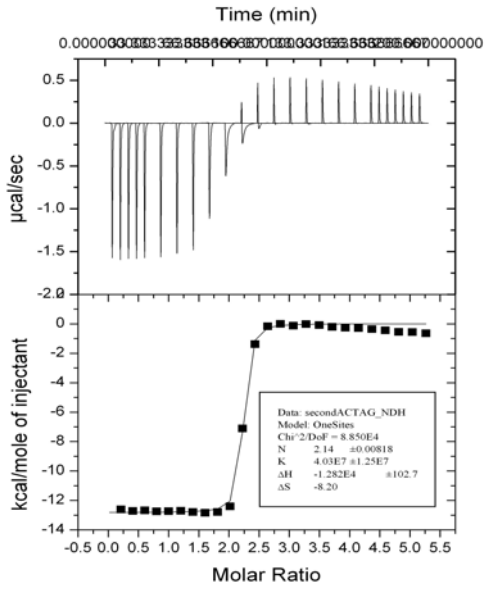
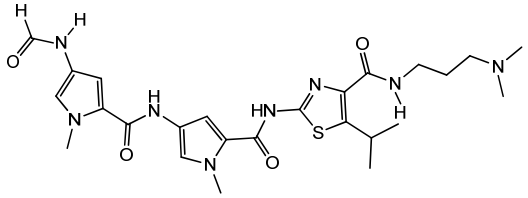


Figure 50

Output of ITC injection of compound **24**

The calculated area underneath each peak is equal to heat released for that injection. Integrated heat is plotted against molar ratio of ligand to DNA in the cell, producing binding isotherm for the interaction. Data are fit based on number of binding sites, in this case one.

Thiazatropsin A (**24**) was titrated against five self complimentary DNA oligomer sequences: (Work carried out by Hasan Alniss).



24

1. 5'-GCGACTAGTCGC-3'

2. 5'-GCGGCTAGCCGC-3'

3. 5'-GCGCCTAGICGC-3'

4. 5'-GCGTCTAGACGC-3'

5. 5'-GCGCCTAGGCGC-3'

The results of ΔG obtained from the five experiments are shown in table 7.

DNA Sequence	ΔG (kcal.mol-1)
1	-10.37
2	-9.49
3	-10.6
4	-10.15
5	-8.49

Table 7

ΔG values obtained for binding of **24** with oligomers 1-5

The results from these series of experiments are significant in terms of choosing a suitable oligomer sequence for binding experiments. As previously mentioned it is impossible to determine the optimum binding site for each of the MGBs in this report without detailed DNA footprinting studies. However Thiazatropsin A is one of the few compounds in the library that has been tested in this manner thus the optimum binding site for this compound is known, 5'-ACTAGT-3'. This sequence was incorporated in oligomer 1 of this experiment. The other four sequences were designed to show a reasonably wide range of alternative sequences for example 2, 3 and 5 have predominantly GC sequences with only a two base AT sequence at the centre. Sequence 4 has the binding site 5' – TCTAGA - 3' which is very similar to the preferred binding site of compound **24** with the A and T at either end swapped. Interestingly, oligomer 3 also incorporates I (inosine, see fig 51), this structure is analogous to G, this provides an interesting comparison with oligomer 5 which had the same sequence except that I is replaced with G.

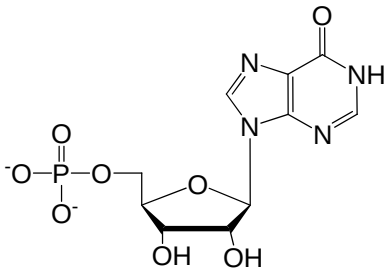


Figure 51

Structure of inosine

Unsurprisingly the data show binding of thiazatropsin A to oligomer 1. However binding is also shown in all four of the alternative sequences. There is a range of ΔG across the five sequences but they are all reasonably similar. This demonstrates that if an MGB is shown to bind to DNA at all, although it may have an optimum binding site, it will likely bind to a fairly wide range of generic sequences also. This would mean that hopefully choosing a fairly standard generic sequence for the binding studies would show binding in the majority of compounds that are actually capable of binding.

CE studies

In order to understand more clearly the sequence specificity of compounds containing the stilbene type head group, several CE tests were carried out on compound **33** (page 43, test carried out in Prof. Simon MacKay's laboratories). Again a selection of DNA oligomers of varying size and sequence (table 8) were used.

Code	Sequence 5'-3'	Bases	Normalised K_a ratio
IJ1	TGCAATTAACGT	12	0.76
IJ2	GTGCATATCGTG	12	0.03
IJ3	TGCATATATAGT	12	1
IJ4	GGTGCATATACGTGGT	16	0.19
IJ5	GGTGCAAATTTCGTGG	16	0.5
IJ6	CAGGTGCATATATCGTGGAC	20	0.86

Table 8

This table shows only DNA sequence in the 5'-3' direction, all of these oligomers were used alongside a complementary sequence to create a DNA double helix.

The oligomers were picked to have a variety of lengths (IJ1 – 12 bases to IJ6 – 20 bases) as well as varying length of AT runs at the centre of the sequence (IJ2 – 4 bases, IJ5 6 bases). The AT runs at the centre of the oligo would likely act as the binding site for the MGB as these type of compounds would be expected to bind to AT sequences. The oligomers were also chosen to have a variety of alternating AT

bases and blocks of two or three A or T bases. These were chosen by Prof. Mackay. This time CE was used and a series of competition experiments was set up. This involved adding **33** in increasing ratios to a mixture of these oligomers and observing the peaks formed. By determining which peak corresponded to which MGB:oligo complex and comparing their size, the relative binding constants of the MGB to each oligo could be determined. The results are summarised in table 8. It is difficult to draw any direct conclusions from this data as the MGB clearly binds to all six sequences, albeit with varying degrees of strength. One thing all of these sequences have in common is a run of AT bases at the centre of the sequence. In the case of the oligomers with the two strongest K_a values, IJ3 and IJ6, the sequence is alternating AT bases lasting seven and six bases respectively. Conversely, the oligomers with the two weakest K_a values, 1J2 and IJ4, also have a series of six and five alternating AT bases respectively. IJ1 and IJ5 have higher K_a values and contain the sequences AATTAA and AAATTT respectively at their centre. Based on this information it was decided to use a sequence with a central run of six AT bases in the binding experiments. The data is slightly ambiguous as the MGB does not show a clear preference for alternating AT bases over blocks of two or three A or T however the two highest K_a values are for oligomers with alternating AT. Most of these oligomers also have GC bases on either side of the AT runs. It is therefore reasonable to conclude that the oligomer, 5'-GCGATATATGCG-3', selected to carry out the UV melt and CE studies in this thesis will likely show binding to these types of compounds. Similar to IJ3 it contains an alternating AT run at the centre as well as G and C bases on either side of this. The AT run at the centre would act as the binding site for the MGB and the presence of GC bases on either side of this would not only mirror the above sequences but also increase the overall T_m of the oligomer, this would make it easier to carry out experiments with it.

3.5.2 UV Melt Experiments summary

Analogues of lead compounds

Oligo sequence: 5'-GCGATATATGCG-3' plus complement.

As explained in the previous section, this oligomer was chosen as a compromise of the types of sequence that these MGBs bind to. It is, however, important to note that this sequence does not represent the optimum binding site for any of these compounds. Therefore if a compound does not show binding to this sequence we cannot conclude that it does not bind to DNA as it may show binding for another sequence. Due to time constraints it was decided to use only one oligomer however the use of alternative sequences may prove to be an interesting subject for further study.

Concentration of oligomer and MGB: $6x10^{-6}$ M

NaCl conc: 50 mM

All experiments were carried out in 10 mM PBS buffer at pH 7.4.

The results obtained from the UV melt experiments carried out are summarised in table 9.

Compound	Equiv	Heating/ Cooling	Ist T_m (^{o}C)	2nd T_m (^{o}C)	Increase in Tm (^{o}C)
Unbound oligo	-	Heating	48	-	0
		Cooling	46	-	0
33	4	Heating	66	-	18
		Cooling	65	-	19
	2	Heating	46	68	20
		Cooling	43	67	21
64	4	Heating	46	65	17
		Cooling	43	63	17
63	4	Heating	48	65	17
		Cooling	43	63	17
35	4	Heating	46	-	0
		Cooling	46	-	0
66	4	Heating	60	-	12
		Cooling	58	-	12
	2	Heating	58	-	10
		Cooling	56	-	10
65	4	Heating	49	-	1
		Cooling	45	-	0
68	4	Heating	50	-	2
		Cooling	45	-	0
69	4	Heating	48	66	18
		Cooling	45	64	18
67	4	Heating	56	-	8
		Cooling	52	-	6

Table 9

T_m values obtained for lead compounds and lead compound analogues

Errors

It has been discussed previously that a T_m increase in the region of ~10^{o}C clearly represents a significant stabilisation of the DNA double helix. However an important issue is determining the extent to which small differences in Tm become significant. In other words to what extent can differences in T_m be attributed to experimental error? Literature precedent has shown that the accuracy of using thermal denaturation experiments can be relatively inaccurate in terms of determining the absolute melting temperature of DNA with errors estimated in the region of ±1^{o}C. [87] However in terms of repeatability within experiments carried out using the same conditions the errors have been shown to be approximately ± 0.1^{o}C. Therefore assuming that there is a standard or control T_m taken in the experiment to

compare results to the temperature changes can sensibly be measured to one decimal place. This also means that compounds that differ in T_ms to the extent of one or two degrees can be said to have a significant difference.

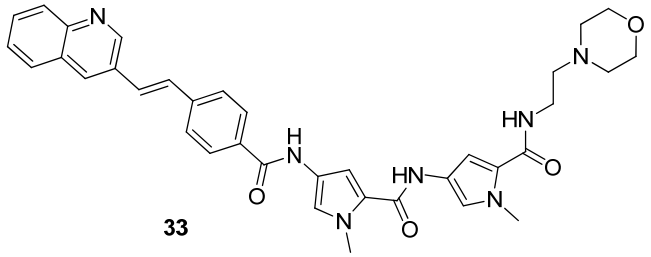
Details of results

Oligo(no MGB)

The first experiment carried out was to determine the T_m of the DNA oligomer with no added MGBs, a T_m of 48°C was observed (details of the exact conditions used can be found in the experimental section).

To begin with when adding MGB to the oligomer, an excess of MGB was used, this was to ensure that the oligomer was saturated as the binding ratio of MGB to oligo was unknown.

Compound **33**



33

Adding four equivalents of AIK 19/56-2 (**33**) to the oligomer and repeating the experiment resulted in a heating curve that showed a T_m of 66°C. This was obviously a large increase (18°C) compared with unbound oligo and indicates strong binding. The experiment was then repeated using two equivalents of MGB; the heating curve in this case appeared to show two T_ms. One appeared at 46°C and was very small and one appeared at 68°C which was much larger. It would appear that the peak at 43°C corresponds to the Tm of unbound oligo and the peak at 68°C corresponds to oligo bound to MGB. This is unusual; the presence of the peak representing unbound oligo was unexpected. It is clear that the MGB binds to oligo in a ratio higher than 2:1 since this ratio results in unsaturated oligo however it

would be expected that even in this case the MGB would bind 1:1 with all available oligo first before binding 2:1 and 3:1 etc. This would not appear to be the case and would possibly indicate that there is some interaction of molecules of MGB to prearrange themselves prior to binding. This cannot be confirmed from this experiment alone however.

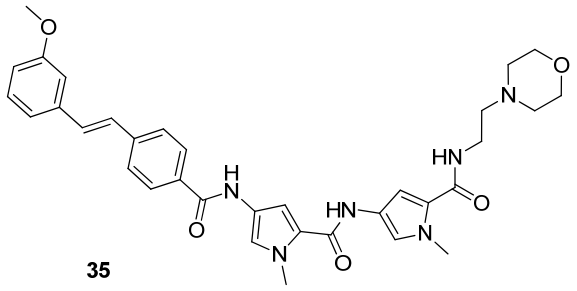
Compound **64**, analogue to **33** with amide linkage

Adding four equivalents of **64** to the oligomer and repeating the experiment resulted in a heating curve that showed two T_ms. One appeared at 46^{o}C and was very small and one appeared at 65^{o}C which was much larger. Again it would appear that the peak at 46^{o}C corresponds to the T_m of unbound oligo and the peak at 65^{o}C corresponds to oligo bound to MGB. However in this case the oligo seemed to be unsaturated even at four equivalents; further experiments would be necessary to find the saturation point of this molecule, however the presence of a peak at 65^{o}C would indicate binding is taking place.

Compound **63**, analogue to **33** with diazo linkage

Adding four equivalents of **63** to the oligomer and repeating the experiment resulted in a heating curve again showing two T_ms. One appeared at 48^{o}C and was very large and was very small and one appeared at 65^{o}C which was much smaller. Again it would appear that the peak at 48^{o}C corresponds to the T_m of unbound oligo and the peak at 65^{o}C corresponds to oligo bound to MGB. Again the oligo seems unsaturated at this number of equivalents and the fact that peak corresponding to bound oligo is much smaller than that for unbound oligo may suggest weaker binding.

Compound **35**



35

Adding four equivalents of AIK 19/56-1 (**35**) to the oligomer and repeating the experiment resulted in a heating curve that showed a T_m of 46°C. This is a small increase in T_m compared with unbound oligo and may even be attributed to experimental error. Either way it would suggest that with this particular DNA sequence the DNA binding is weaker than for **33** or at least shows that any binding that does occur does not significantly increase the stability of the DNA helix.

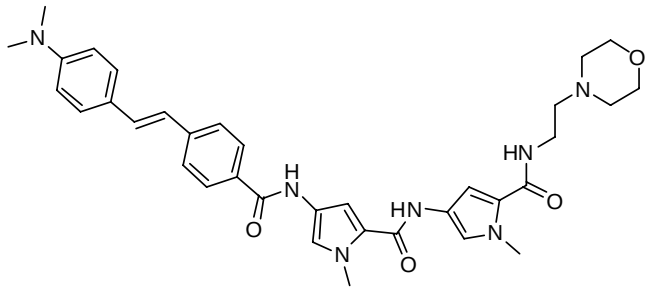
Compound **66**, analogue to **35** with amide linkage

Adding four equivalents of **66** to the oligomer and repeating the experiment resulted in a heating curve that showed a T_m of 60°C, this is a significant increase in T_m (12°C) and would indicate strong binding. The lack of any peak corresponding to the unbound oligo would also suggest the oligo is saturated at this point. The experiment was therefore repeated using two equivalents of MGB, the heating curve in this case appeared to show a T_m of 58°C and again no peak for unbound oligo which would suggest that the oligo is also saturated at 2 equivalents.[73]

Compound **65**, analogue to **35** with diazo linkage

Adding four equivalents of **65** to the oligomer and repeating the experiment resulted in a heating curve that showed a Tm of 49°C. This is a small increase in T_m compared with unbound oligo and may even be attributed to experimental error. The data would suggest weak binding or no binding of this compound to the oligomer sequence.

Compound **68**



68

Adding four equivalents of **68** to the oligomer and repeating the experiment resulted in a heating curve that showed a T_m of 50°C. This is a small increase in T_m compared with unbound oligo and may again be attributed to experimental error.

Compound **69**, analogue to **68** with amide linkage

Adding four equivalents of **69** to the oligomer and repeating the experiment again resulted in a heating curve with two T_ms. One appeared at 48°C and was very small and one appeared at 66°C which was much larger. From this a similar conclusion to the results from **64** can be drawn with strong binding indicated, however the oligomer may not be saturated.

Compound **67**, analogue to **68** with diazo linkage

Adding four equivalents of **67** to the oligomer and repeating the experiment resulted in a heating curve that showed a T_m of 56°C. This is a relatively small increase in Tm (8°C) it is however still a significant increase indicating a degree of binding. Future experiments will be carried out to determine the binding ratio of this compound to DNA.

Overview

Compound **33** and analogues:

While the diazo, alkene and amide analogues of this compound all showed some degree of binding, the strength of the binding and the point at which saturation occurs appear to differ.

Compound **35** and analogues:

Both the alkene and diazo analogues seem to show no binding to the oligo, or at least no stabilisation of the helix occurs upon binding. This is surprising since 19/56-1 has shown significant binding in past NMR studies (see section 3.5.4).[73] The amide analogue however shows significant binding to the DNA.

Compound **68** and analogues:

The alkene analogue in this series appears to show no binding to the oligo, the amide and diazo analogues do show some binding although the amide binding seems to be significantly stronger than the diazo.

Overall it is difficult to draw many conclusions from the data above. The only trends seem to be that in all cases the amide analogues show some degree of binding. It is, however, clear that the strength of binding differs by a large amount upon changing the linking group from alkene to diazo or amide.

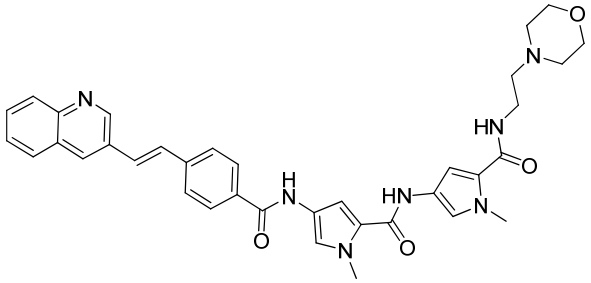
3.5.3 Capillary electrophoresis results

Oligo sequence: 5'-GCGATATATGCG-3' plus complement.
Concentration of oligomer and MGB: $2x10^{-5}$ M
NaCl conc: 20 mM

As mentioned previously another method for measuring the binding of our compounds to DNA is capillary electrophoresis (CE). CE is generally not used to assess the strength of binding since no definite figures can be produced from the data. This method is generally used to determine two things. Firstly, whether or not the compound is actually binding to DNA, this is determined by the appearance of a new peak corresponding to the DNA-MGB complex. Secondly, the ratio of binding which is determined by the incremental addition of equivalents of MGB until saturation is achieved (characterised by the disappearance of the peak corresponding to unbound DNA). However analysis of CE data from analogous compounds should give us more information about their behaviour when they interact with DNA. As is the case in several of the compounds, the saturation of the DNA does not always occur even when the equivalents of the MGB reaches four or five. This is unusual since it would be expected for these compounds to bind in a 2:1 fashion. There are several possibilities to explain this, the first of which is the issue of solubility. It has been discussed previously that the very fact that these compounds are designed to bind side by side has led to some problems in the form of aggregation of the molecules in solution. This had led in some cases to the precipitation of the compounds when they are dissolved in water. For this reason it is difficult to rely on the concentrations of the prepared solutions used for the CE experiments as they may be lower than expected due to this. The second possible explanation is that of relative binding strength. As previously noted it is difficult to determine binding strength of these compounds from CE but some degree of relative binding strength may be theorised based on these results. When the MGB binds to DNA it is likely there is an equilibrium set up between bound and unbound DNA. Depending on the strength of binding the equilibrium may be biased towards unbound DNA or bound DNA. If the binding is weak then it may be that we see a peak corresponding to unbound DNA beyond 2 equivalents even if the binding ratio is 2:1.

Compound **33** and analogues

Table 10 summarises the results from the CE experiments for these compounds and puts them alongside the corresponding T_m data obtained previously. This will be discussed in detail in this section, the table is there for easy reference to the results.



33

Compou	Linkage	Equiv	1st T_m	2nd T_m	T_m increase	CE results
Oligo	-	-	48	-	-	-
33	alkene	4	66	-	18	New peak
		2	46	68	20	-
64	Amide	4	46	65	17	No new peak
63	diazo	4	48	65	17	New peak

Table 10

T_m values and CE results for compound **33** plus analogues

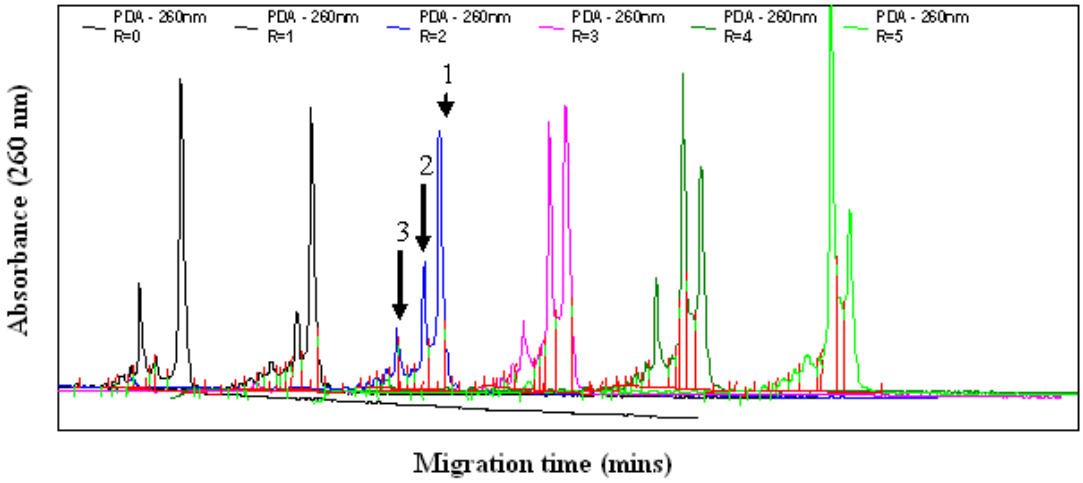


Figure 52

CE trace of compound **33.** 1= oligomer peak, 2= oligomer:MGB complex, 3=unknown peak. R = MGB:oligomer ratio

This CE trace of compound **33** (fig. 52) shows clearly the unbound oligomer peak (1) being slowly reduced as the peak for the DNA:MGB complex (2) grows. As the

diagram shows, the free oligomer peak is still present up to 5 equivalents of the MGB. As discussed previously this could possibly be due to the lack of solubility in the compound. There is a third peak in this diagram (3) which has not as yet been identified however may be single stranded oligomer.

Compound **64** - The CE trace of compound **64** shows no apparent binding with the DNA oligomer.

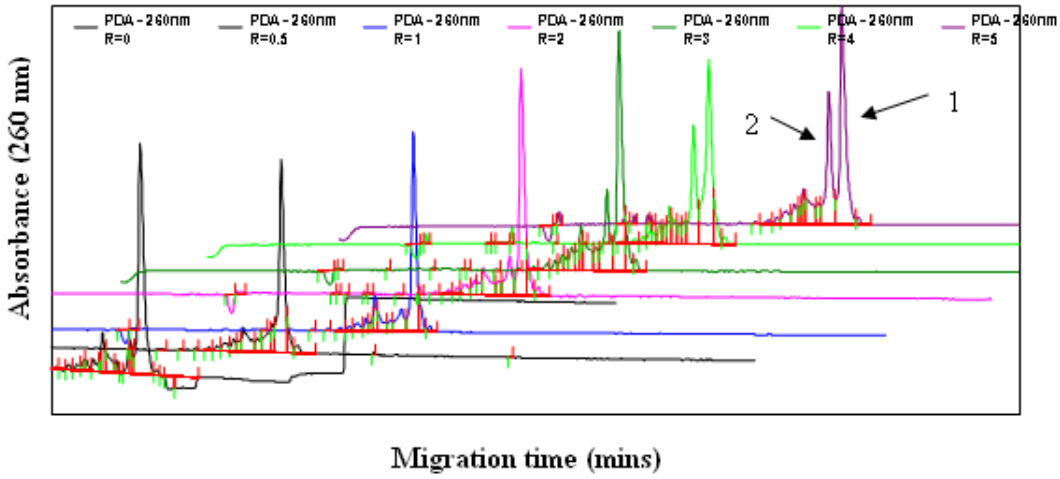


Figure 53

CE trace of compound **63.** 1= oligomer peak, 2= oligomer:MGB complex. R = MGB:oligomer ratio

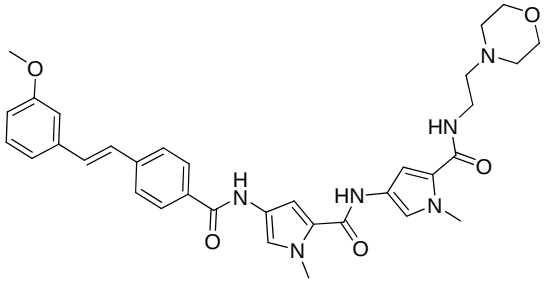
This CE trace of **63** (fig. 53) shows the unbound oligomer peak (1) being slightly reduced as the peak for the DNA:MGB complex (2) grows. As the diagram shows, the peak for the DNA:MGB complex does not appear until 2 equivalents of MGB have been added and even up to 5 equivalents the free oligomer peak is still larger than the complex peak. This may be indicative of weaker binding to the oligomer.

Discussion and comparison with T_m results

In the case of **33** there is a large increase in Tm alongside evidence from the CE results that there is a new peak formed corresponding to the DNA:MGB complex, this would suggest that there is binding to DNA taking place. In the case of the amide analogue **64** there is a T_m increase observed however it is important to note that the data from this compound also showed a peak present with a Tm corresponding to unbound oligomer and no extra peak in CE. This would suggest

that perhaps binding in this case is not as strong as in **33**. The fact that the CE data does not show evidence of binding to DNA would support this. Finally the diazo analogue **63** shows a Tm increase similar to the amide analogue **64** and also shows the presence of a peak corresponding to unbound oligomer. In this case the CE data does show evidence of binding however the peak for oligo:MGB complex is considerably smaller than in **33**, again suggesting weaker binding.

Compound **35** and analogues



35

Again table 11 summarises the CE and UV melt results for these compounds and serves as a reference for the more detailed discussion to follow.

Compound	linkage	Equiv	1st T_m	2nd T_m	T_m increase	CE results
35	Alkene	4	46	-	-	New peak
66	Amide	4	60	-	12	No new peak
		2	58	-	10	
65	diazo	4	49	-	1	New peak

Table 11

T_m values and CE results for compound **35** plus analogues

In the case of compound **35**, because the peaks for unbound oligo (1) and oligo:ligand complex (2) are of a similar retention time in this case it is difficult to single out peak 2 (see figure 54) . However at two equivalents of the MGB we start to see the peak clearly emerging. The peak continues to grow alongside the shrinking of peak one until five equivalents where the free oligomer peak has almost completely disappeared. This would indicate strong binding.

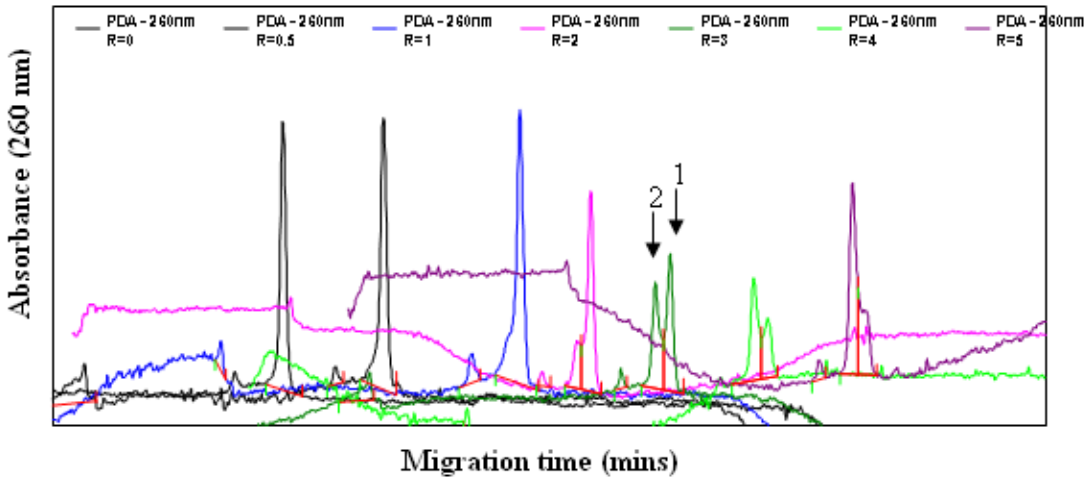


Figure 54

CE trace of compound **35.** 1= oligomer peak, 2= oligomer:MGB complex. R = MGB:oligomer ratio.

Compound **66** - This diagram of Compound **66** shows no apparent binding with the DNA oligomer.

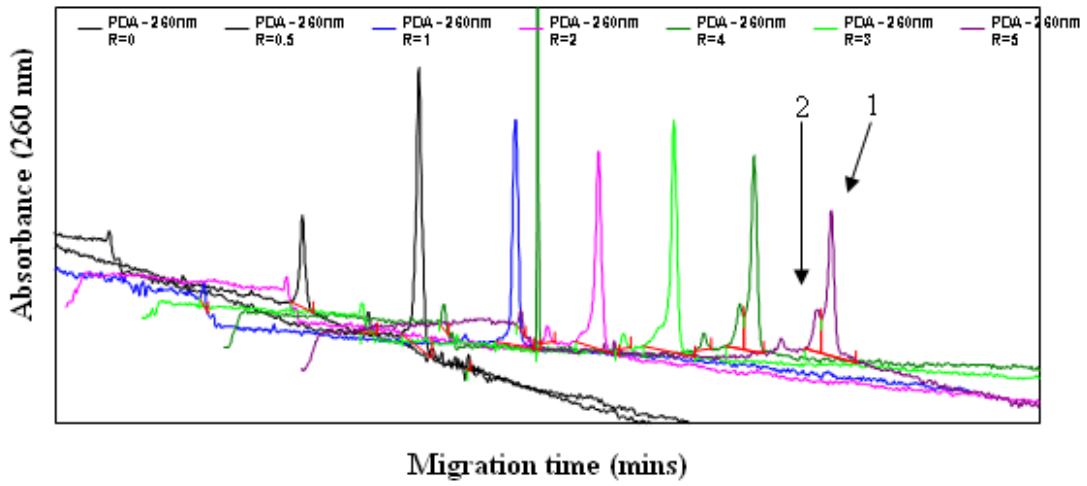


Figure 55

CE trace of Compound **65.** 1= oligomer peak, 2= oligomer:MGB complex. R = MGB:oligomer ratio.

In the case of compound **65**, similar to **35** peaks 1 and 2 are of a similar retention time however at four equivalents of MGB peak 2 can be seen more clearly (see figure 55). However at five equivalents of MGB the peak corresponding to unbound oligo is still the dominant peak suggesting that binding is weaker in this case.

Comparison with T_m UV data

The result for compound **35** is surprising; the CE data would suggest that binding is taking place and that the oligo is close to being saturated at five equivalents of MGB. However the T_m UV data shows no increase in melt temperature over the free oligomer. Given the CE data and the excellent biological activity of the compound a reasonably large Tm increase would be expected. It is important to note however that while an increase in Tm is a good indicator of binding, an absence of such an increase does not necessarily mean that no binding is taking place. The amide analogue **66** shows T_m increase of 12^oC which is a significant increase while the CE data shows no new peak corresponding to the oligo:MGB complex. Again this may be due to the fact that binding is too weak in this case. There is also the possibility that the peaks may be overlapping. The example of compound **35** shows that the two peaks can be very close in retention time and it is possible that this is masking the peak for the complex. The diazo analogue **65** is also similar to **35**; the Tm data shows no significant increase in T_m however the CE data shows evidence of binding, albeit weaker than in the alkene analogue, this could also be due to the reasons explained earlier.

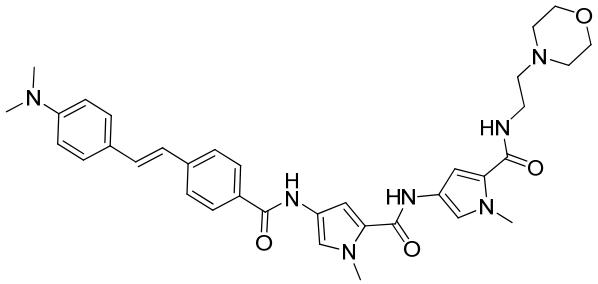
Compound **68** and analogues

Table 12 summarises the CE and UV melt experiments for compound **68** and its analogues.

Compound	Linkage	Equiv	1st T_m	2nd T_m	T_m increase	CE results
68	Alkene	4	50	-	2	No New peak
69	Amide	4	48	66	18	New peak
67	diazo	4	56	-	8	New peak

Table 12

Tm values and CE results for compound **68** plus analogues



68

Compound **68** - The CE trace of compound **68** shows no apparent binding with the DNA oligomer.

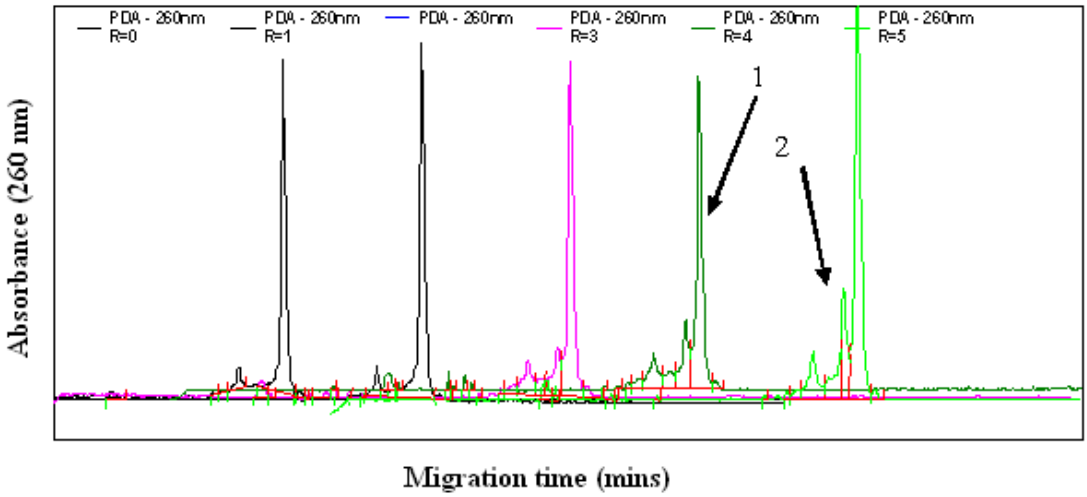


Figure 56

CE trace of Compound **69.** 1= oligomer peak, 2= oligomer:MGB complex. R = MGB:oligomer ratio.

This CE trace of compound **69** (figure 56) shows the unbound oligomer peak (1) being slightly reduced as the peak for the DNA:MGB complex (2) grows. As the diagram shows, the peak for the DNA:MGB complex does not appear until four equivalents of MGB have been added and the peak is very small compared to the unbound oligo peak.

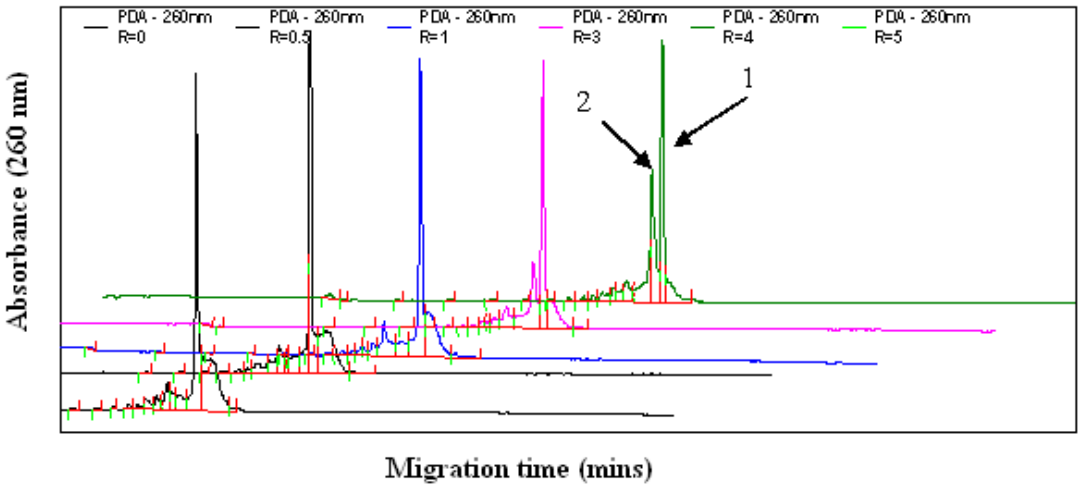


Figure 57

CE trace for Compound **67.** 1= oligomer peak, 2= oligomer:MGB complex R = MGB:oligomer ratio.

This CE trace of compound **67** (figure 57) shows the peak for oligo:MGB complex appear at three equivalents. It is fairly small but by four equivalents it has grown considerably indicating binding is taking place.

Comparison with T_m UV data

Compound **68** shows no new peak for the oligo:MGB complex in the CE data, this along with the fact that the UV data shows no significant increase in T_m would suggest that no binding is taking place. The amide equivalent **69** shows an increase in T_m of 18^{o}C however the data also shows that there is still a significant amount of unbound oligo. Likewise the CE data shows the emergence of a small peak corresponding to the DNA:MGB complex however there is still a large amount of unbound oligomer present. Finally the diazo equivalent **67** shows both an increase in T_m and the emergence of a new peak corresponding to the complex. The T_m increase is relatively small however (8^{o}C) and again the peak for the complex is still small compared to the unbound oligo peak indicating weaker binding.

3.5.4 NMR Data for Compound **35** [73]

There have been several detailed NMR binding studies carried out on a selection of the MGBs mentioned in this report. The most significant was carried out on AIK 19/56-1 (**35**).[73] A detailed analysis was made of this compound in solution with the same oligomer used previously in the binding studies (figure 58). This work was carried out by Dr. John Parkinson.

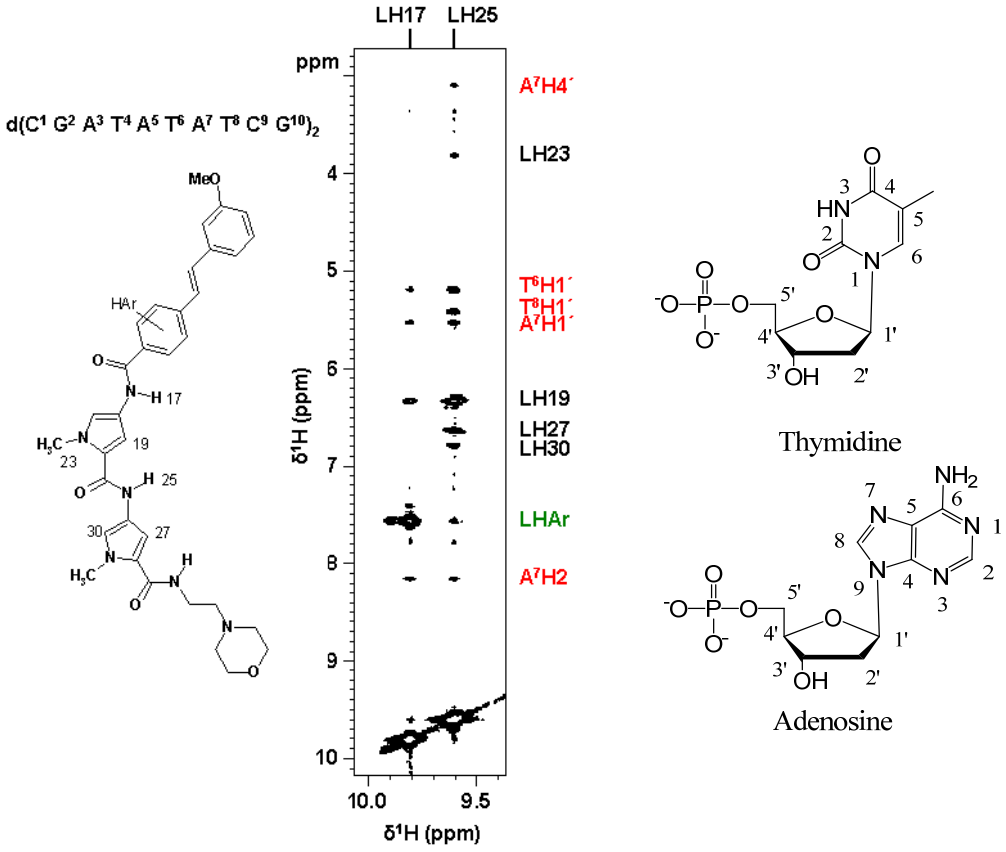


Figure 58

NOEs for the 2:1 complex formed between **35** and the oligonucleotide shown plus numbering of adenosine and thymidine.

The lead compound **35** was studied bound 2:1 to a similar recognition sequence as used in previous experiments. This was however a shorter sequence more suited to NMR experiments; the self-complementary oligonucleotide, $CGA^3TAT^6A^7T^8CG$. These studies confirmed the binding of compound **35** in this sequence and also showed significant NOEs between ligand N*H* of the inter-pyrrole peptide bond (replaced in **54** by an alkene) and DNA hydrogens T^6H1', A^7H4', A^7H1', A^7H2 and T^8H1' (see fig. 58 for numbering of nuclsosides). The results also establish that this

NH bond is oriented towards the concave edge of the minor groove making it available for hydrogen bonding. The available data and bond distances would suggest that there is significant H-bonding in this area of the minor groove binder. This is a significant result as it establishes the binding of this molecule and emphasises the importance of H-bonding in this linkage as established by comparison of compounds **54** and **66** (see section 3.5.6).

The results clearly show the MGB bound in an antiparallel 2:1 fashion in the minor groove of the DNA oligomer. This clearly demonstrates not only that these molecules are definitely binding to the DNA but also that the binding mode is what was predicted previously. It also shows important interactions of the oligomer with the head group of the MGB as well as with several of the internal amide linkages. It also serves to demonstrate some of the shortcomings of the thermal denaturation experiments. While an increased T_m in the oligomer almost certainly corresponds to binding to the DNA it is also true that if there is no T_m increase observed this does not necessarily correspond to a lack of binding. This is very clearly illustrated in the case of compound **35** which shows no apparent binding according to UV measurements however gives very convincing evidence from both NMR and CE experiments. This highlights the notion that ideally all of these compounds should be analysed fully by NMR as this would undoubtedly give us greater insight into their binding capabilities. Unfortunately due to the huge amount of work involved in this and the expertise required this is currently not a realistic goal.

It should be noted that the concentration of MGB as well as the salt concentration varies across the different experiments described in this thesis. Where possible this was kept constant or very similar however due to the practical limitations of each technique and the fact that the experiments were not all carried out by the same scientists there is considerable variation. The concentrations used are as follows:
UV melt (MGB conc: $6x10^{-6}$ M; Salt conc: 50mM); CE (MGB conc: $2x10^{-5}$ M; Salt conc: 20mM); ITC (MGB conc: $1.5x10^{-5}$; Salt conc: 0.2M); NMR (MGB conc: 4.34mM)

The differences in ligand and oligomer concentration in solution will undoubtedly have an effect on the binding of the MGB to the oligomer, with a low MGB

concentration possibly being detrimental to binding. The varying salt concentration may also affect the ability of an MGB to bind to the minor groove. It is therefore unsurprising that the results from each technique do not always agree. Compound **35** is a good example of this, with the CE and NMR data clearly indicating binding however no T_m increase is observed in the UV melt experiments. For this reason it is problematic to make direct comparisons between the techniques and it is important to assess the results from each technique individually.

3.5.5 The effect of N-substitution on binding to DNA

Oligo sequence 5'-GCGATATATGCG-3' plus complement.

Concentration of oligomer and MGB: $6x10^{-6}$ M. NaCl conc: 50 mM
All experiments were carried out in 10 mM PBS buffer at pH 7.4.

The results obtained from the UV melt experiments carried out are shown in table 13.

Compound	R1	R2	equiv	T_m (^oC)	increase
33	Me	Me		66	18
50	Ipr	Me	2	59	11
51	SecBut	Me	2	47	0
52	Me	Ipr	2	47, 65	17
53	Me	SecBut	2	47, 63	15

Table 13

T_m values obtained for compound **33** plus analogues

Figure 59 shows the 1st derivative of the UV melt trace obtained for compound **33**.

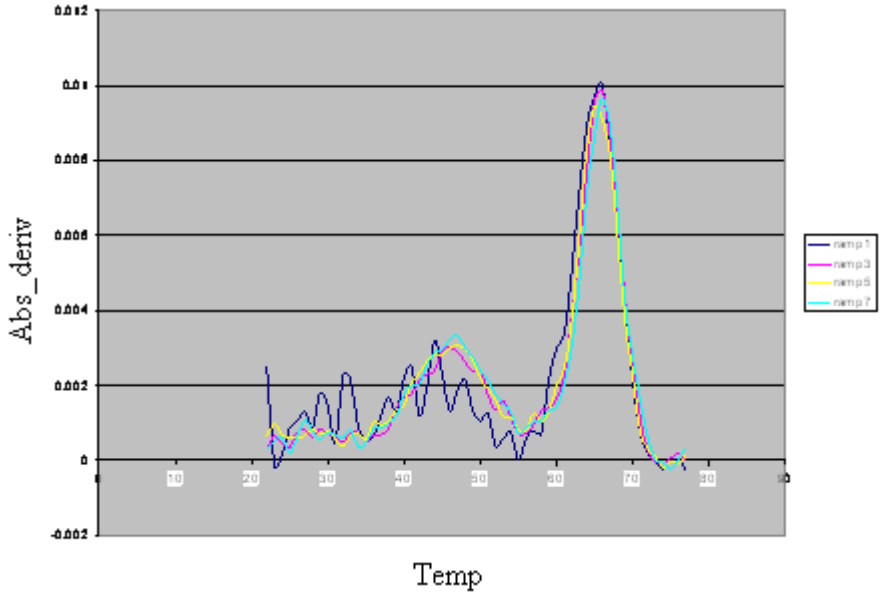


Figure 59

1st derivative of UV melt graph for compound **33**

Analogues of compound **33**

As mentioned previously the first group of compounds in this series is based on the lead compound **33**. In order to simplify descriptions the relevant heterocycles have been labelled Het 1 and Het 2 as shown in figure 60. Also shown are the two groups, isopropyl and secondary butyl that were used in the subsequent analogues of this compound.

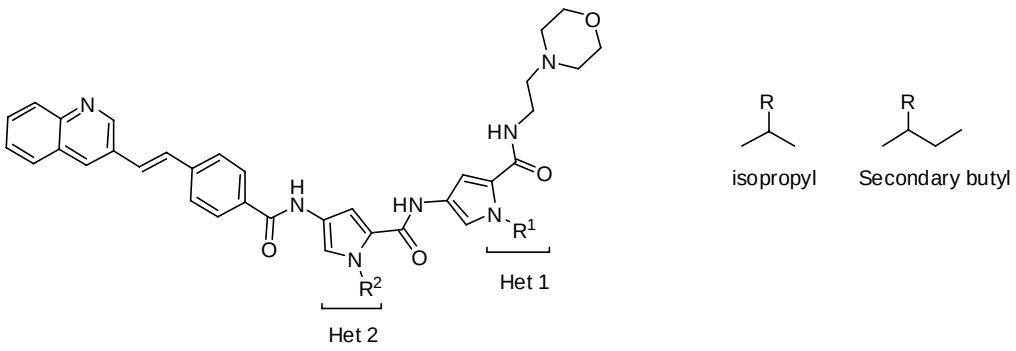


Figure 60

Structure showing R groups used in compound **33** analogues.

Heterocycle 1

The two compounds with changes made to Het 1 were **50** and **51** which changed the methyl group to isopropyl and secondary butyl respectively. **50** showed a T_m increase of 11^oC over the free oligonucleotide. This clearly demonstrates an increase in stability of the duplex; however the lead compound **33** shows a Tm increase of 18^oC. This would imply that in this case increasing the steric bulk in the region of Het 1 is not favourable. **51** showed no T_m increase at all over the standard, this would appear to follow the pattern of increasing steric bulk being detrimental to binding.

Heterocycle 2

The two compounds with changes made to Het 2 were **52** and **53** which changed the methyl group to isopropyl and secondary butyl respectively. **52** showed a Tm increase of 17^oC over the standard. While this is lower than the lead compound it is

still a significant result; previously the use of an isopropyl group (**50**) caused a very pronounced decrease in T_m (9^oC) while in this case the difference is very small (1^oC). While this change in steric bulk did not result in increased binding it would certainly imply that this area of the molecule is more tolerant to alterations in structure. This is backed up somewhat by **53** which showed a T_m increase of 15^oC. Again this is lower than the lead compound but it does fit into the pattern of a large increase in steric bulk being detrimental to binding. However the fact that the T_m is only lower by 3^oC therefore making it stronger than the secondary butyl group in Het 1 emphasises the notion that increased steric bulk is more easily tolerated in this second heterocycle.

Analogues of compound **42**

The second group of compounds in this series is based on AIK 20/25-1 (**42**), again the relevant heterocycles are noted as Het 1 and Het 2 in figure 61. Tm values are summarised in table 14.

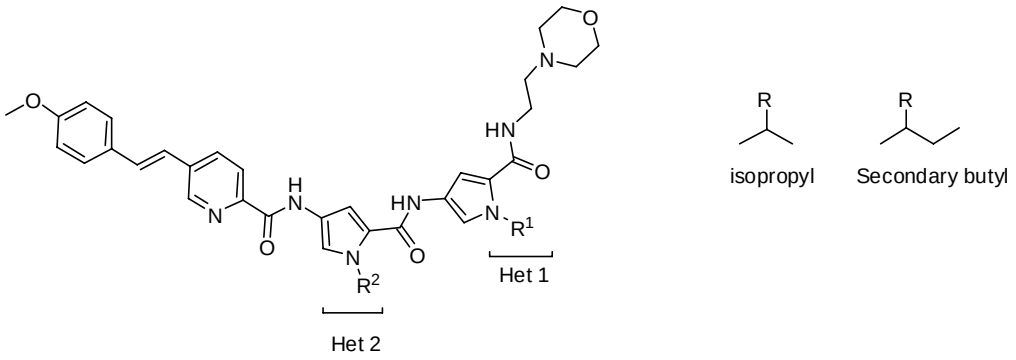


Figure 61

Structure showing R groups used in compound **42** analogues

Compound	R1	R2	Equiv	T_m	Tm increase
42	Me	Me	2	64.0	16
46	Ipr	Me	2	64.0	16
47	SecBut	Me	2	61.1	13
49	Me	SecBut	2	63.9	16
48	Me	Ipr	2	67.1	19

Table 14

T_m values obtained for compound 42 plus analogues

Heterocycle 1

In the case of this series the lead compound AIK 20/25-1 (**42**) showed a T_m increase of 16°C (see figure 62).

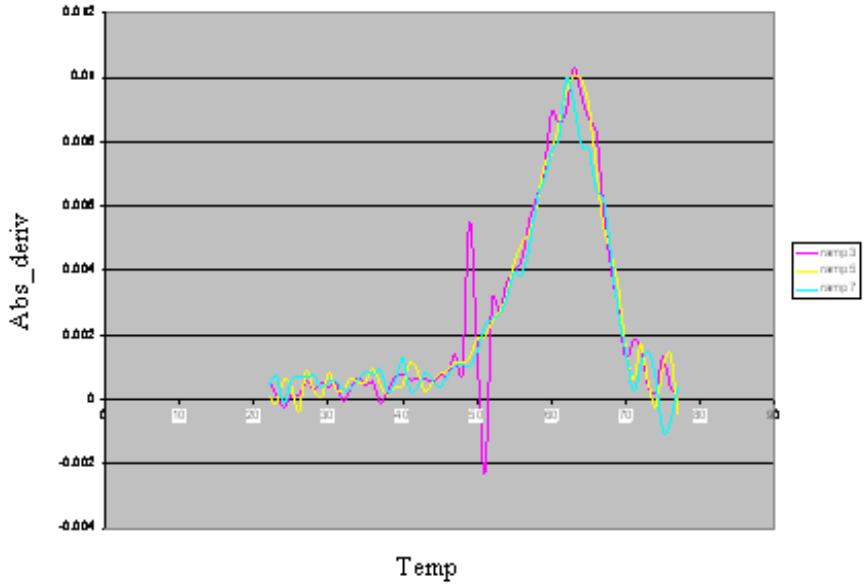


Figure 62

1st derivative of UV melt graph for compound **42**

In this case compounds **46** and **47** changed the methyl group to isopropyl and secondary butyl respectively. **46** showed a T_m increase of 16°C over the standard, this is exactly the same as the lead compound. This shows that in the case of this compound changes can be made to the steric bulk of Het 1 with no detrimental effect on binding. **47** shows an increase of 13°C, 3°C less than the lead compound. However this fits into the pattern that increasing steric bulk past a certain point is generally a hindrance to binding.

Heterocycle 2

The two compounds with changes made to Het 2 were **48** and **49** which changed the methyl group to isopropyl and secondary butyl respectively. **48** showed a T_m increase of 19°C, this is a very significant result as it is the first compound in either of these series to show an improvement in binding over the lead compound. This is further proof that this second heterocycle is tolerant to increases in steric bulk; in fact in this case it is advantageous. In **49** when the steric bulk is increased to

secondary butyl a T_m increase of 16°C is observed, this is also significant as this is on a par with the binding observed in the lead compound. Therefore even a relatively large steric bulk such as secondary butyl in this position does not cause a reduction in binding strength.

Comparison with biological activity

Comparing the results obtained from UV melt data shows some obvious similarities. In most cases the T_m increase shown by the analogues of **33** or **42** are at least comparable with the lead compounds. Likewise the biological activity of the analogues is on a par with that of the lead compounds (see table 9). However measuring biological activity is not as precise as measuring T_m therefore it is difficult to draw any conclusions or discern any patterns from the data. The most that can be said from this data is that alterations of the steric bulk certainly do not kill the biological activity of these molecules however it is not realistic to attempt to compare activity within a series.

Overview

Although examination of the biological data did not yield many observations other than those stated above, the thermal denaturation data provides a lot of information. In the analogues of **33** increasing steric bulk from methyl to iso-propyl on the first heterocycle resulted in a reduction of binding strength. A further increase of bulk to secondary butyl resulted in no binding. If the aim of this experiment was to determine if increasing steric bulk was beneficial to binding this would seem to imply that it is not, however there are other factors to consider. For example it is likely that these molecules will bind in a 2:1 fashion therefore two molecules will bind side by side in the minor groove. It may be the case that increasing steric bulk at this point in the molecules disrupts the side by side binding, it may also be the case that there is little opportunity for hydrophobic bonding in the area of the minor groove where this group would sit. It is for these two reasons that the second heterocycle was also experimented with. Here it was shown that increasing steric bulk to an isopropyl group resulted in a loss of binding of only 1°C. This would

imply that increasing of steric bulk in this area is certainly more tolerated if not favourable. Again increasing steric bulk to a secondary butyl group results in further reduction of binding.

In the analogues of **42** we can observe that using an isopropyl group on the first heterocycle provides the same binding strength as the lead compound. This result alone would imply that this molecule would be more tolerant to changes in steric bulk. Once again, however, increasing steric bulk to a secondary butyl group results in a loss of binding corresponding to a $3^{o}C$ loss in T_m over the lead.

From all of this information we can make several conclusions, firstly that increasing steric bulk on heterocycle 2 (see page 134) would appear to more tolerated than in the heterocycle 1 (see page 134). As shown in **48** this can actually lead to an increase in binding however it would appear that there is an optimum size of group since any attempts to use the larger secondary butyl group resulted in a loss of binding compared to the isopropyl analogue. This is likely due to disruption in the side by side binding of the two molecules in the minor groove, however in order to confirm this a wider range of groups would be needed. Increasing steric bulk on the first heterocycle would not appear to be beneficial or at least not as beneficial as in the second heterocycle. The final observation it is important to make is between the two sets of compounds. Although both follow the same general pattern it is important to note that no increase in binding over the lead was observed in the analogues to compound **33** while the analogues to compound **42** showed a significant increase in one case. This shows the large differences that can be observed in binding upon changing a head group therefore, as is typical in these compounds, it is difficult to make general statements as what is beneficial in one compound may not be for another and vice-versa. There are some trends that can be observed however which will hopefully be of considerable benefit to future attempts at lead optimisation.

It should also be noted that the shape of the derivative of the melting curves can give us some information about ΔH, the energy required to separate the two strands of DNA. The melting temperature of a duplex gives us information about its stability, this will take in factors such as ΔH and the energy of the oligomer: MGB

complex and the separated strands of DNA however the peak shape gives us specific information about the ΔH value. A sharp transition from double stranded to single stranded DNA will result in a derivative that has a sharp peak whereas a slow transition will result in a broader peak. Relatively, a sharp peak represents a higher ΔH value than a broad peak therefore we can say that the energy required to separate the strands of DNA in the case of a sharp transition is higher than that of a broad transition. This value could not be quantified in the case of the melting curves obtained here. However using the melting curve derivative of compound **33** as an example (see fig 59, page 133) we can see that the peak corresponding to unbound oligomer (47^{o}C) is far more broad than the peak corresponding to the oligomer: MGB complex (66^{o}C). This would indicate that the energy required to separate the strands is higher in the case of the oligomer: MGB complex than in the case of the unbound oligomer, this is consistent with the theory that the binding of MGBs to the oligomer stabilises the DNA duplex.

3.5.6 DNA binding properties of additional analogues of existing compounds

Oligo sequence: 5'-GCGATATATGCG-3' plus complement
Concentration of oligomer and MGB: $6x10^{-6}$ M, NaCl conc: 50 mM
All experiments were carried out in 10 mM PBS buffer at pH 7.4.

In addition to the series of compounds that have been made to serve as comparisons to the lead compounds or other compounds in the Suckling group there have been several other 'one shot' compounds that have been synthesised. These compounds also serve as comparisons; however they do not fit in easily with previous series shown therefore the binding study results of them will be described here. Results obtained from UV melt data are summarised in table 15.

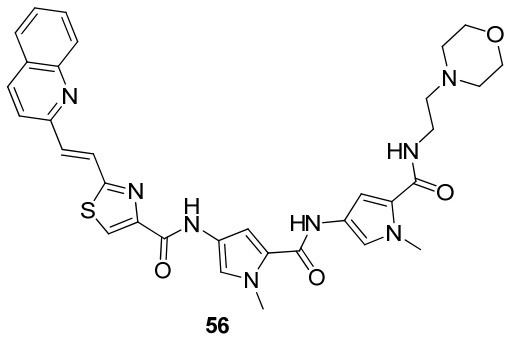
Compound	Equiv	T_m	T_m increase
61	2	54.1	16
62	2	49.1, 77.0	29
54	2	48	0
66	2	60	12
56	2	48	0
57	2	48	0
39	2	48	0
33	2	67	19

Table 15

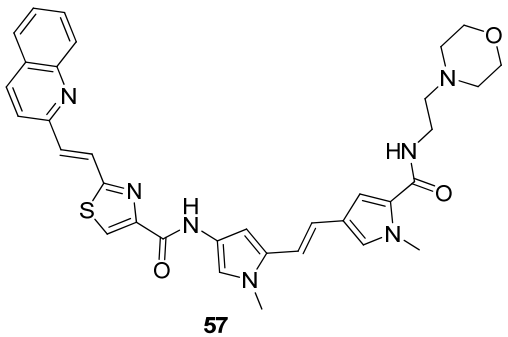
T_m values obtained for alkene containing MGBs

The importance of the internal amide linkage to DNA binding

Compounds **56** and **57**



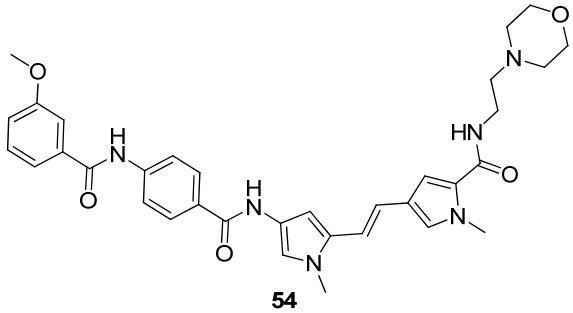
56



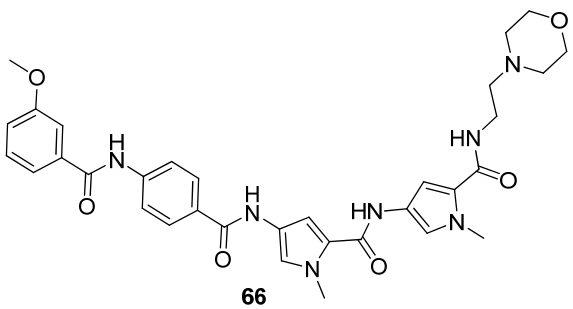
57

Biological results showed that **56** has significant antibacterial activity while **57** had no such activity. The only structural difference between these compounds is an amide bond between Het1 and Het2 in **56** which is replaced by an alkene link in **57**. This would suggest the presence of an H-bonding group is vital to activity in this molecule. It was of interest to compare the binding strengths of these two compounds to see if these results could be confirmed via binding strength. Surprisingly neither compound was shown to bind to DNA using UV melt experiments; however this negative result illustrates an important point and is therefore appropriate to mention. It has been mentioned previously that MGBs are DNA sequence specific and therefore any compound we have made that binds to DNA will likely have an optimum binding site. Furthermore it has also been shown that certain groups have a preference for certain bases or sequences of bases. The DNA oligomer sequence used in these experiments was picked to include a binding site rich in A and T since most MGBs will bind to a sequence of AT bases if they are going to bind at all. However it is perfectly reasonable that a compound may bind to DNA and in fact in this case show excellent biological activity and still not bind to this particular sequence, or at least not strongly enough to show a change in T_m. These compounds in particular contain a thiazole group which, as has been mentioned shows a preference for GC sequences. A possible next step as regards testing of these molecules may be to explore their binding strength with other DNA oligomers, possible containing GC in the binding site. It is however an important point that the chosen oligomer sequence represents a compromise for what most of the MGBs will likely bind to and is not an optimum binding site for any of them. This could be determined via DNA footprinting studies as discussed previously and this data will hopefully become available at a later date.

Compounds **54** and **66**



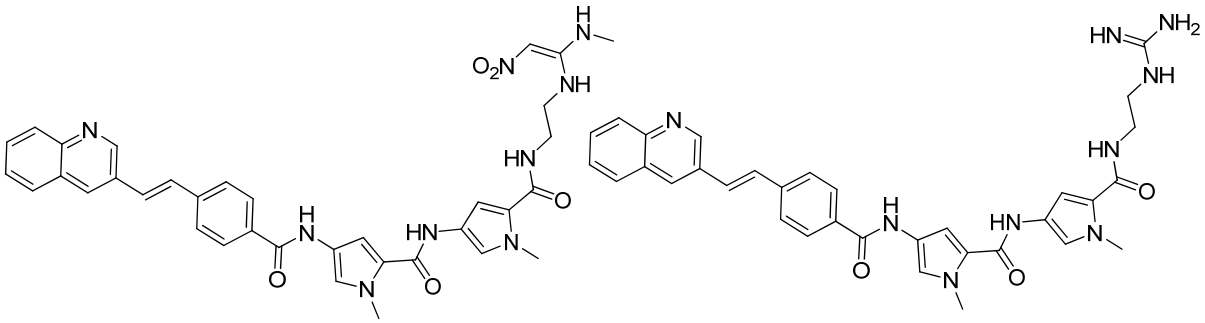
54



66

Unfortunately the comparison of binding strength between **56** and **57** did not yield any meaningful results. A similar comparison can be made between the compounds noted above. Compound **54** is an analogue of **66** with the internal amide link between Het1 and Het2 replaced with an alkene link. Again testing the binding strength of the two compounds should give us some information regarding the importance of H-bonding groups in this region. Unfortunately both compounds were biologically inactive therefore we cannot derive any conclusions from that data. Testing the T_m increase of the standard oligomer showed an increase of 12°C for **66** whereas **54** showed no T_m increase over the free oligomer (up to 4 equivalents of MGB).[73] Although a T_m increase of 12°C is smaller compared to some of the other compounds mentioned it is still significant. This result demonstrates that while the potential for hydrophobic interactions in the middle section of the MGB can be exploited it is clear that there are also H-bonds being formed here that are not only beneficial to binding but in this case are actually vital.

Comparing biological activity with DNA binding



61

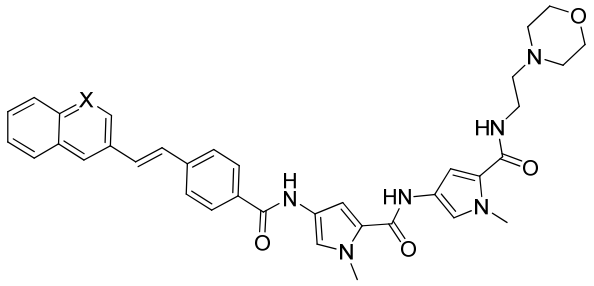
62

The first compounds shown are analogues of compound **33** as described in section 2.15. Compound **61** is of particular interest due to the fact that it is biologically active and also that it is neutral at physiological pH. **62** is also biologically active due to the guanidine tail group which will be almost exclusively protonated at physiological pH. Both of these compounds are of interest therefore it was important to determine if the biological activity shown would translate to binding using thermal denaturation experiments. It has been previously thought that a basic tail group that would be protonated at physiological pH was essential to binding, however the results show that compound **61** causes a T_m increase of 16°C therefore

implying strong binding to DNA. Compound **62** however has a tail group that is almost permanently protonated , this compound shows a T_m increase of 29°C which incidentally is the highest T_m increase recorded from our library of compounds thus far. Interestingly though, the biological activities of the two compounds are very similar and in fact **61** is seen to be slightly more active than **62**. One possible explanation for this is bioavailability. Since compound **61** is essentially neutral this would be of great benefit to membrane permeability, this may be why a compound that shows modest binding strength has such high biological activity. Conversely the fact that compound **62** is protonated permanently may be detrimental to bioavailability as it would make passive diffusion through the cell membrane more difficult. This may explain why it would appear to have stronger DNA binding than **61** yet lower biological activity. It is also important to note that both compounds are analogues of the lead compound **33** therefore would to a certain degree be expected to have biological activity.

The importance of the presence of nitrogen in the head group of lead compound 33

Compounds **33** and **39**



39 (X=H), **33** (X=N)

Another interesting comparison between biological activity and measured binding strength can be made between **33** and **39**. These compounds are identical structurally apart from the quinoline head group present in **33** which is replaced with a naphthalene group in **39**. This is essentially a difference of one nitrogen atom, removing it and replacing it with a carbon atom should give us some information about the requirements of binding in this area. Biological results shown earlier in this report show that **33** has some of the best biological activity of all the MGB compounds tested thus far and has been used as a lead compound for several

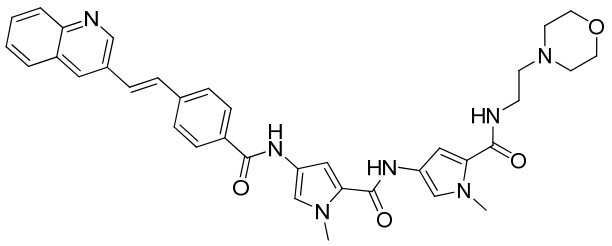
other MGBs. Conversely **39** has shown no biological activity whatsoever; this is a very significant result as it demonstrates the importance of the nitrogen atom to activity in this compound. Obviously it would be very interesting to test the binding strength of these two compounds to see if the biological data and the binding data correlate. Compound **33** has caused an increase to the T_m of the oligomer of almost 20^oC, one of the highest increases observed from this class of compound. **39** however showed no increase at all suggesting that no binding is taking place.[73] This data would also strongly suggest that the presence of the nitrogen atom in the head group is vital to binding. It also is further evidence of the link between strong binding to DNA and biological activity.

3.5.7 DNA Footprinting Results

Compound **33** along with its diazo (**63**) and amide (**64**) analogues were sent to Prof. Fox for analysis by DNA footprinting. This data has yet to be published. DNA footprinting would tell us the preferred binding site of our MGBs.

DNA footprinting is a process by which a ligand, in this case an MGB, is added to a portion of DNA. A cleaving agent is then added, in this case DNase 1, this enzyme binds to the DNA minor groove and cleaves the phosphodiester backbone. Having an MGB bound in the minor groove will disrupt the action of the enzyme. The sample is then run using polyacrylamide gel electrophoresis. The section of the DNA that does not have an MGB bound to it will be cut into fragments by the enzyme producing a ladder-like distribution when it is run on the gel. The section of the DNA with bound MGB will result in a break in the ladder distribution where the DNA has been protected from cleavage this producing a footprint indicating where binding has taken place.

Along with these compounds, **42** (page 135) and **57** (page 140) were also tested. At the time of writing these are the only compounds relating to this thesis that have been tested however several other compounds have been sent and are awaiting analysis.



33

The results confirm that compound **33** has strong binding to DNA with footprints shown in several sites. The binding sites are typically runs of between four and six A and T repeating unit. It is worth noting that in several of the binding sites there are single G and C bases in between the AT runs however there are no footprints shown in continuous GC sequences. The amide analogue of this compound (**64**)

shows no apparent binding to DNA; this is slightly surprising since while the CE data showed no evidence of binding, the UV data from this compound suggested binding was taking place. The diazo analogue of these compounds (**63**) showed good evidence of binding in both the UV and CE experiments, this was confirmed by footprinting results which showed binding to several sites. The specific binding sites for this compound are TTTAAA, TATATAT and ATATGTA which is most unusual as these are generally the weakest AT-sites for standard AT-selective groove binders (see fig. 63).

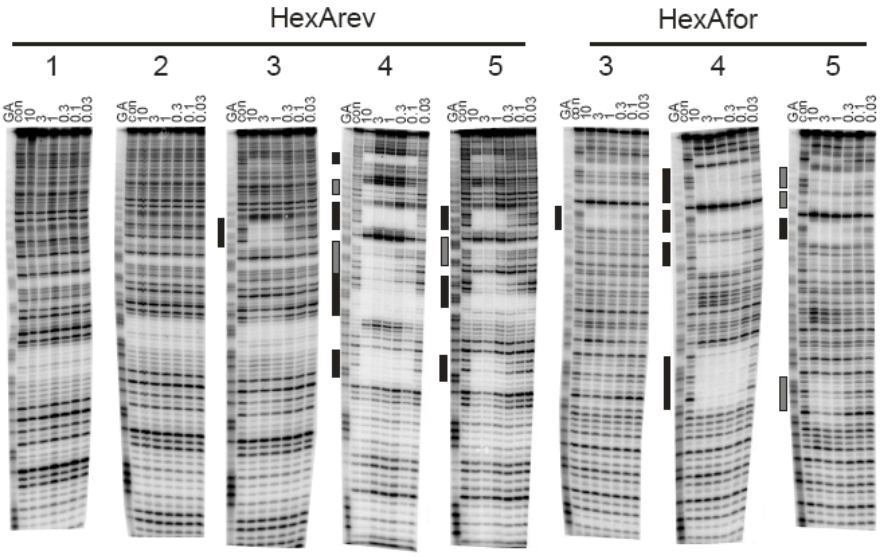


Figure 63

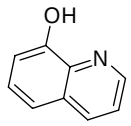
Footprinting results (Key: 1 = **57**; 2 = **64**; 3 = **63**; 4 = **33**; 5 = **42**) Ligand concentrations (μM) are shown at the top of each gel lane. GA corresponds to marker lanes specific for purines, while 'con' is cleavage in the absence of added ligand. The filled boxes show the location of the best binding sites

It is important to note that binding in this compound is considerably weaker than in compound **33** with far fewer binding sites; however each of the binding sites for **63** is also a binding site of **33**. This would suggest that changing from an alkene to a diazo or amide certainly has an effect on affinity for binding, however the selectivity of the compounds appears to be unchanged. This observation, that alkene linkages in this position give stronger binding than amide or diazo, is consistent with previous data (antibacterial UV and CE data). Compound **42** also has several binding sites with its strength of binding less than that of compound **33** but higher than that of compound **63**. The selectivity for this compound also seems to be

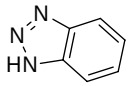
similar to compound **33**. The final compound that was tested in this batch was compound **57**, this showed no binding to DNA which is consistent with the available biological and binding data. In order to provide a good comparison it would also be useful to have the footprinting data for compound **56** which is the mono-alkene analogue to this compound and we are currently in the process of obtaining this data.

3. 6 Additional Compounds

In addition to the lead compound analogues synthesised in during this project it was also decided to attempt the synthesis of MGBs with the stilbene head group motif that incorporated the 8- Hydroxyquinoline and benzatriazole groups (fig. 64).



8-hyroxyquinoline

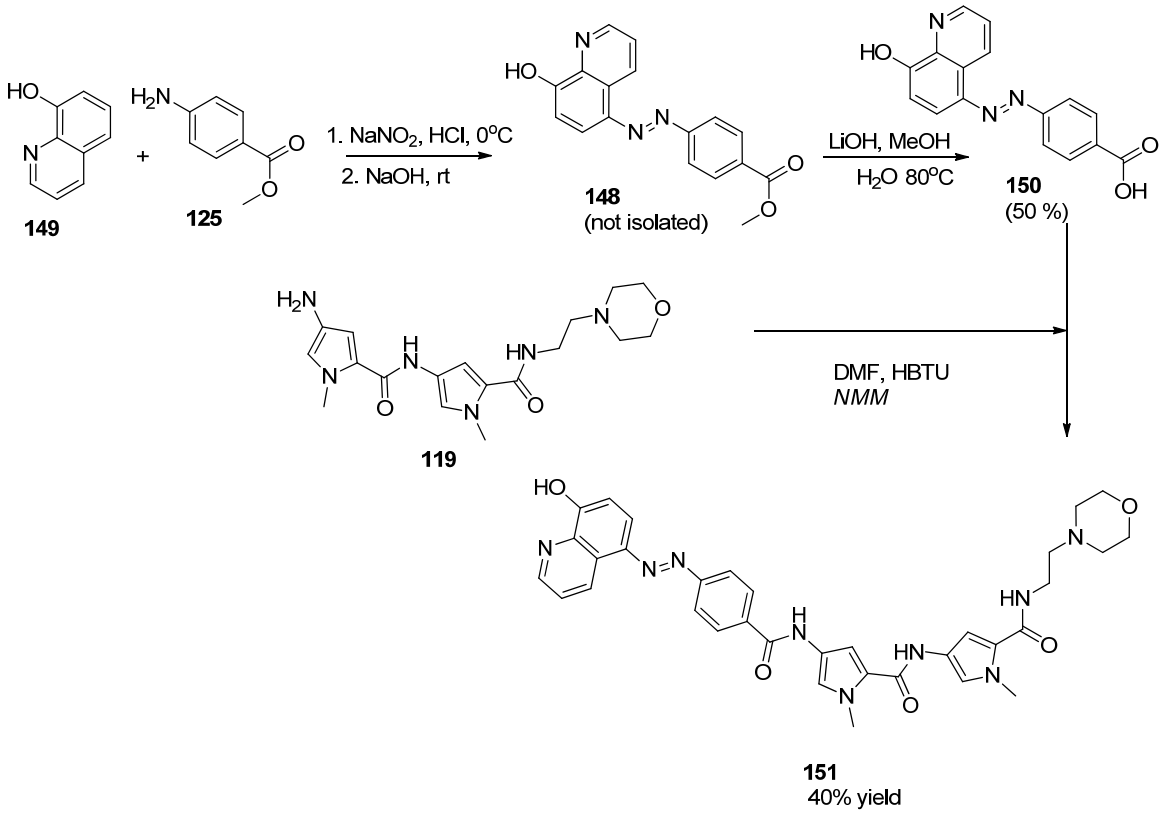


Benzatriazole

Figure 64

Hydroxyquinoline and benzatriaole head groups

The first synthesis attempted was that of the 8-hydroxyquinoline, it was decided that a diazo link could be formed at the position para to the hydroxyl group via a diazotisation reaction to give the following head group **148** and resulting MGB **152** (scheme 34). The structure of this molecule is a slight departure from the usual design of MGBS particularly those containing quinoline head groups. Typically MGBs of this type would be connected to the quinoline group via the 2 or 3 positions, this is largely due to the fact that this would maintain the curvature of the molecule that is widely regarded as important for binding to DNA. By connecting the head group at the 5 position as in this molecule it leaves part of the head group 'sticking out' which would possibly be a hindrance to binding. However it was decided to synthesise this structure anyway as it was considered the only way to incorporate an 8-hydroxyquinoline group into a MGB of this type. Consequently it was necessary to synthesise the following head group **148**. The synthesis of this dimer would require a diazotisation reaction on the amine group of methyl-4-aminobenzoate followed by electrophilic aromatic substitution of this diazo salt on quinolin-8-ol **149**. Both of these starting materials are commercially available which meant the synthesis would be relatively straightforward. This is shown schematically in scheme 33.



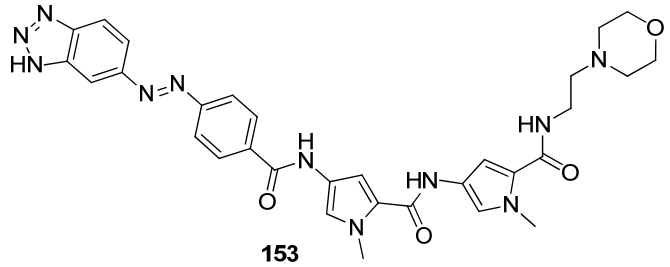
Scheme 33:

Synthesis of compound **151**

The product of this reaction was found to be a mixture of the product **148** and the corresponding carboxylic acid **150**, it was therefore decided to take this material forward to the next step to convert all of the product to compound **151**. With the carboxylic acid **150** in hand it was now possible to attempt an amide coupling reaction between this dimer and the morpholino tail group **90** described earlier.

As in previous reactions the nitro group was reduced to the amine which was used immediately in the coupling step. HPLC was then carried out directly on the reaction mixture to give the desired product **151** in 43% yield (scheme 33).

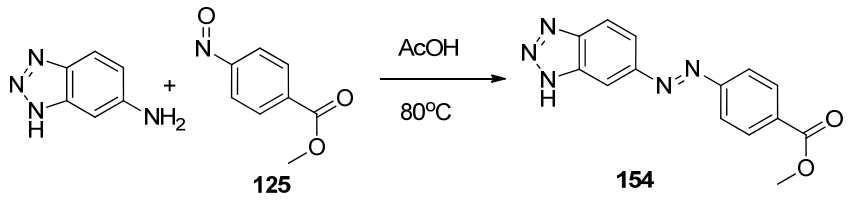
Compound **153** shows the full structure of the benzotriazole containing MGB.



153

As before, this would involve the synthesis of the following dimer **154**. Similar to the diazo analogue for AIK 19/56-2 this molecule could not be synthesised by the diazonium salt reaction described earlier in the report. This is again due to the lack of a strongly electron donating group on either of the benzene rings.

This meant that a condensation reaction would have to be carried out between commercially available benzotriazole-6-amine and the nitroso compound **125** described previously. The reaction was therefore carried out in the presence of AcOH, although preliminary mass spec data suggests the product **154** had been formed this has not been confirmed at the time of writing. (scheme 34).



125 **154**

Scheme 34

Condensation of amine and nitroso

.

4. Conclusions

There are now several MGBs synthesised by the Suckling group that test the hypothesis that hydrophobic binding plays a major role in DNA binding by having non-polar groups replacing the amide linkage. In particular in this thesis describes an effective route to incorporate diazo linkages directly into the structure of an MGB creating several novel compounds.

This approach to MGB synthesis has led to the discovery of several lead compounds (**33** and **35**). Techniques developed for synthesising diazo containing MGBs as well as existing techniques to synthesise alkene and amide containing MGBs have been used in order to design a series of compounds that would compare the effectiveness of different lining groups. By creating a series of analogues that differ only the the linking group of the head portion (i.e. amide, diazo and alkene) the importance of H-bonding in this area of the molecule can be effectively tested. Several other areas of the molecule have also been explored including the positioning of substituents on the head group (analogues of compound **33**; **136 - 138)**, the use of different aliphatic groups in heterocycle groups (analogues of compounds **42** and **33**; **46 - 53**), positioning of hetero atoms on the head group (compounds **33** and **39**) and differing tail groups (compounds **61** and **62**). Cherry-picking existing compounds from the Suckling library and performing analysis on them has allowed a large amount of insight to be gained as to the nature of DNA binding.

In order to successfully compare different compounds not only in terms of biological activity but also in terms of DNA binding necessitated the use of a variety of different techniques. Capillary electrophoresis, Thermal denaturation and ITC were used effectively to gain information about the nature and strength of binding while collaboration with research partners allowed a large amount of information to be gleaned from NMR and DNA footprinting.

In conclusion; synthetic techniques along with information from biological testing and a variety of analytical techniques has allowed valuable information to be gained

as to the exact nature of the binding of these compounds to DNA. This will, along with future work along similar lines, undoubtedly provide effective guidelines for future synthesis of active minor groove binders.

Future work

Several important conclusions have been drawn from the experiments in this thesis regarding the binding of MGBs to DNA. We now have a greater insight into various aspects of DNA binding, for example: hydrogen bonding in several key areas of the MGB; tolerance to larger alkyl groups; the use of alternative linking groups and the type and placement of head group substituents. One of the obvious areas in which this work could be expanded is in the use of these insights to synthesise MGBs with specific features designed to test and confirm them. In other words, the knowledge gained here could be used to design more effective MGBs.

Many of the MGBs produced by the Suckling group have been tested for biological activity and DNA binding and have provided valuable information. There are however many others that could provide the same information if they were analysed with the techniques used in this thesis. In addition to this the MGBs tested have not all been tested fully. This is due to practical reasons such as time constraints and dependence on outside help. For example it has previously been stated that DNA footprinting is a very important method of analysis for these compounds. Footprinting of all the MGBs available would certainly give valuable insight into the binding of these compounds. However for this particular technique we are dependant on the help of outside partners such as Prof. Fox therefore it has not been practical to test all the compounds. Likewise techniques such as capillary electrophoresis and thermal denaturation are limited by the fact that oligomers chosen are not specific to each MGB. Use of a wider range of oligomers could provide more insight into binding however due to time constraints this work has not yet been carried out. Again time constraints and instrument availability have limited the range of the experiments. Future work in this area may include more detailed analysis of the MGBs, thus expending on the information already gathered.

There are also several areas of the project that have yet to be expanded on. For example there is a possibility that several of these compounds may be useful as SERRS tools, further work in this area may give interesting results. There are also several other areas where the coloured/ fluorescent nature of these compounds

could be exploited. This is an aspect of the project with a lot of potential which has yet to be fully explored.

6. Experimental

6.1 Experimental Specifications

Instrumental:-

m.p: Reichert hot stage melting point apparatus

NMR: Bruker spectrospin 400 MHz operating frequency for ^{1}H NMR and ^{13}C NMR. Chemical shifts are quoted in ppm and measured relative to the residual proton/carbon from the solvent. Coupling constants, *J*, are given in Hertz (Hz)

IR: Mattson 1000 FTIR spectrometer as KBr discs. Frequencies are quoted in cm^{-1}.

MS: Recorded on a Jeol JMS AX505 mass spectrometer in either FAB or EI mode

Microanalysis: Perkin Elmer 2400, analyser series 2

Chromatography:-

TLC: Merck 0.25mm silica gel 60 F254. Visualisation used UV radiation at 254 nm or 2% aqueous potassium permanganate.

Column: Silica gel mesh size 230-400, (40-60 μm). Flash column chromatography was carried using standard procedures. [88]

HPLC: HPLC chromatography carried on a Waters system, with a Waters 1525 Binary HPLC pump, and a Waters 2487 dual λ absorbance detector at 254 nm using a C18 Luna column with the following gradient.

Time (mins)	A	B	Flow rate (ml/min)
0	90	10	4
28	30	70	4
33	10	90	4
38	90	10	4
40	90	10	0

Table 16

Program used for HPLC.

Mobile phase

A = Water + 0.1% TFA

B = Acetonitrile + 0.1% TFA

6.2 Experimental procedures for binding studies

Thermal denaturation experiments

DNA oligomers and their complements were melted at a rate of 0.5°C/min in 10mM PBS buffer soln. (pH 7.4) with 50mM NaCl on a Cary 300 BIO UV-visible spectrophotometer fitted with a peltier temperature controller. Programs were set and data was processed using Cary WinUV software.
Each oligomer made to a concentration of $6x10^{-6}$ M was mixed with sufficient MGB to give the appropriate ratio. Samples were heated from 10°C to 80°C and cooled from 80°C to 10°C with the spectra being recorded at 260 nm during both of these cycles. The melting temperatures (T_m) of the hybrids were determined from the derivative maxima. This process was repeated a total of 4 times to ensure repeatability of the experiment. [89]

Capillary electrophoresis experiments

All measurements were taken on a Beckman Coulter P/ACE™ MDQ, Diode-Array Detector Module, Sample Temperature Control. Running buffer was 0.22 M total borate concentration (TBC) buffer, pH 7.5. Capillaries used were 50μm internal diameter and 375μm external diameter bare fused silica capillary with an effective length of 32 cm and a total length of 40 cm.

Samples were made to a volume of 50 uL containing 2×10^{-5} M of the appropriate oligomer plus complement with 20 mM NaCl. Ligand was added in appropriate concentration to give the desired ratio of Ligand:oligomer.

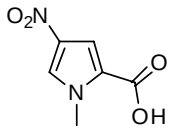
A plug of the sample solution was introduced into the anodic end of the capillary by a pressure injection (15 psi for 7 sec). Electrophoresis was driven by a high-voltage power supply. The separation voltage was set at 25 kV and the capillary temperature was set at 20 ± 0.1°C. Electropherograms were recorded at 260 nm. The data obtained were analysed using 32 Karat software. In order to obtain good reproducibility, the capillary was purged between runs by flushing for 5 mins with

doubly distilled water, then 3 mins with 0.1 M NaOH, 2 mins with double-distilled water and 10 mins with the running buffer.

6.3 Experimental Procedures for synthesis

Monomers and Tail groups

1-Methyl-4-nitro-1*H*-pyrrole-2-carboxylic acid (81)



Acetic anhydride (40 mL) was cooled to 0 ^{o}C using an ice bath with sodium chloride salt, fuming nitric acid (4 mL; 1.2 equiv) was then added to this mixture dropwise via a dropping funnel with stirring. Meanwhile a solution of methyl-1H-pyrrole-2-carboxylic acid, **80** (10 g; 80 mmol), in acetic anhydride (40 mL) was stirred and cooled to – 45 ^{o}C using an acetone/dry ice bath, temperature was regulated by regular addition of small pieces of dry ice. The nitric acid/acetic anhydride was then slowly added to this solution using a side arm dropping funnel. The dropwise addition took approximately 45 min to complete. When addition was complete, the temperature was allowed to rise to room temperature; this temperature rise was carried out very slowly and regulated with the addition of dry ice when necessary. The solution was then cooled back down to –20^{o}C at which point a yellow precipitate was formed. Collection of the precipitate by filtration gave the nitrated product, **81**, as a yellow solid.

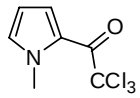
Yield: 4 g; 29%.

δ_H: (DMSO-d_6) 13.1 (1H, s, OH); 8.2 (1H, d, J = 1.9, CH aromatic); 7.30 (1H, d, J = 1.9, CH aromatic); 4.01 (3H, s, CH_3).

LCMS: Found 171 calculated for $C_6H_7O_4N_2^+$ 171.

m.p: 198-200^{o}C (lit - 199-200^{o}C) [90].

2,2,2-Trichloro-1-(1-methyl-1*H*-pyrrol-2-yl)ethanone (83)



N-Methylpyrrole **82** (1.78 g; 22 mmol) was dissolved in dry dichloromethane (10 mL), this mixture was stirred at room temperature. Trichloroacetyl chloride (4 g; 22 mmol) was also dissolved in dichloromethane (20 mL; dry), this solution was placed in a side arm dropping funnel and added dropwise to the *N*-methylpyrrole solution. The resulting solution was stirred at room temperature for 2 h. The dichloromethane was then removed under reduced pressure to give a red/brown residue. This residue was then redissolved in the minimum volume of dichloromethane and then filtered through a short silica column (dichloromethane as eluent). The yellow liquid was then collected and concentrated under reduced pressure to give the product, **83**, as a yellow solid.

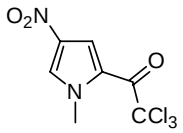
Yield: 3 g; 61%.

δ_H: ($CDCl_3$) 7.51 (1H, dd, J = 2.8, J = 4.4, CH aromatic); 6.97 (1H, d, J = 1.6, CH aromatic); 6.23 (1H, dd, J = 2.4, J = 4.4, CH aromatic); 3.99 (3H, s, CH_3).

ν_{max} (KBr): 3422, 3120, 2953, 1657, 1642, 1457,1401 cm^{-1}.

m.p: 65-68^oC (lit - 64-65^oC) [91].

2,2,2-Trichloro-1-(1-methyl-4-nitro-1*H*-pyrrol-2-yl)ethanone (84)



Nitration was carried out on compound **83** (9 g; 40 mmol). The procedure used was the same as that previously described for nitration in the preparation of compound **81**. The required product, **84**, was obtained as a yellow solid.

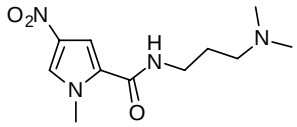
Yield: 5.5 g; 51%.

δ_H: ($CDCl_3$) 7.95 (1H, d, *J* = 1.7, CH aromatic); 7.76 (1H, d, *J* = 1.7, CH aromatic); 4.06 (3H, s, CH_3).

ν_{max} (KBr): 3144, 1694, 1495, 1515, 1423 cm^{-1}.

m.p: 132-135°C (lit - 135-140°C) [91]

_N_-[3-(Dimethylamino)propyl]-1-methyl-4-nitro-1_H_-pyrrole-2-carboxamide (85)



2,2,2-Trichloro-1-(1-methyl-4-nitro-1*H*-pyrrol-2-yl)ethanone, **84** (2 g; 7.4 mmol), was dissolved with stirring in dry dichloromethane (10 mL) at room temperature. *N,N*-dimethylaminopropane-1,3-diamine (1.5 g; 14 mmol) was also dissolved in dichloromethane (20 mL; dry) and the solution was then added dropwise to the original solution and stirred at room temperature for 2 h. The dichloromethane was then removed under reduced pressure to give an orange solid. This residue was the purified by flash column chromatography (eluant: 1:1 ethyl acetate/methanol with 0.5% TEA). Removal of the solvents gave the pyrole monomer, **85**, as an orange solid.

Yield: 1.1 g; 59%

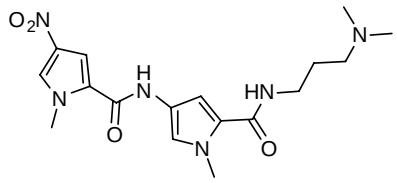
δ_H: ($CDCl_3$) 8.63 (1H, s, NH); 7.52 (1H, d, *J* = 1.6, CH aromatic); 7.01 (1H, d, *J* = 1.6, CH aromatic); 4.05 (3H, s, NCH_3); 3.50 (2H, q, *J* = 5.4, CH_2); 2.58 (2H, t, *J* = 5.8, CH_2); 2.37 (6H, s, $N(CH_3)_2$); 1.78 (2H, q, *J* = 5.9, CH_2).

LCMS: Found 255 calculated for $C_{11}H_{19}O_3N_4^+$ 255.

ν_{max} (KBr): 3115, 2560, 1650, 1490 cm^{-1}.

m.p: 124-125°C (lit - 126-127°C) [90].

***N*-[3-(Dimethylamino)propyl]-1-methyl-4-{[(1-methyl-4-nitro-1*H*-pyrrol-2-yl)carbonyl]amino}-1*H*-pyrrole-2-carboxamide (88)**



3-Nitro-*N*-methylpyrrole-2-carboxylic acid **81** (0.2 g; 0.8 mmol) was dissolved in thionyl chloride and the solution was heated to 80^{o}C with stirring under nitrogen for 3 h. The excess thionyl chloride was then removed under reduced pressure to give the corresponding acid chloride as a grey solid.

Compound **85** (0.136 g; 0.8 mmol) was dissolved in methanol and palladium catalyst (10% by weight on activated carbon; 0.1 g) added. The reaction vessel was then evacuated of air and refilled with hydrogen gas. The reaction mixture was then stirred for 3 h. The reaction mixture was then filtered through Kieselguhr, the filtrate was collected and the solvents removed under reduced pressure to give the aminopyrrole, **87** as a glassy solid.

Both the amine and acid chloride were used immediately.

The acid chloride was dissolved in dichloromethane (10 mL), the amine (**87**) was dissolved in dichloromethane (10 mL) and added dropwise. *N*-Methylmorpholine (0.1 mL; 0.8 mmol) was then added to the reaction mixture which was sealed and stirred at room temperature for 24. The dichloromethane was then removed under reduced pressure and the resulting residue was partitioned between ethyl acetate (with 5% methanol, 50 mL) and aq. sodium hydroxide (10% w/v, 50 mL). The organic layer was removed, dried ($MgSO_4$), and concentrated under reduced pressure to give a dark yellow solid. This solid was then purified by flash column chromatography (eluant: 1:1 methanol/ ethyl acetate with 1% TEA). Removal of the solvents gave the product, **88**, as a yellow solid.

Yield: 0.2 g; 66%.

δ_H: (DMSO-d_6) 10.25 (1H, s, NH); 8.16 (1H, d, J = 1.7, CH aromatic); 8.10 (1H, t, J = 5.6, NH); 7.57 (1H, d, J = 1.8, CH aromatic); 7.20 (1H, d, J = 1.7, CH aromatic); 6.81 (1H, d, J = 1.7, CH aromatic); 4.12 (3H, s, NCH_3); 3.85 (3H, s,

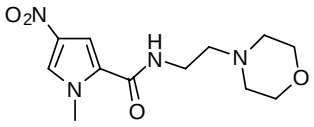
NCH_3); 3.19 (2H, q, J = 5.6, CH_2); 2.23 (2H, t, J = 7.1, CH_2); 2.18 (6H, s, $N(CH_3)_2$); 1.62 (2H, quintet, J = 7, CH_2).

LCMS: Found 377 calculated for $C_{17}H_{25}O_4N_6^+$ 377.

ν_{max} (KBr): 3286, 2941, 1665, 1623, 1573 cm^{-1}.

m.p: 190-192°C (lit 190-191°C) [90]

1-Methyl-*N*-[2-(4-morpholinyl)ethyl]-4-nitro-1*H*-pyrrole-2-carboxamide (89)



3-Nitro-*N*-methylpyrrole-2-carboxylic acid, **81,** (1 g; 5.9 mmol) was dissolved in thionyl chloride (5 mL), and the solution heated under reflux at 80°C for 2h. Excess thionyl chloride was then removed under reduced pressure to give a grey solid. This was dissolved in dry dichloromethane (10 mL) and added dropwise to a solution of 4-(2-aminoethyl)morpholine (0.77 g; 5.9 mmol) in dry dichloromethane (10 mL). The resulting solution was stirred at room temperature for 24 h during which time a yellow solid precipitate was formed. The precipitate was collected by filtration and washed with dichloromethane to give the required product, **89**, as a pale yellow solid.

Yield: 1.43 g; 86%.

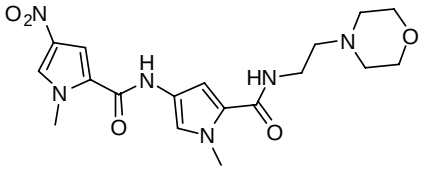
δ_H: ($CDCl_3$) 8.85 (1H, s, NH); 8.14 (1H, d, J = 1.8 Hz, CH aromatic); 7.36 (1H, d, J = 1.8 Hz, CH aromatic); 3.88 (3H, s, CH_3); 3.56 - 3.59 (4H, m, CH_2); 3.31 – 3.33 (2H, m, CH_2); 2.41-2.44 (6H, m, CH_2).

LCMS: Found 283 calculated for $C_{12}H_{19}O_4N_4^+$ 283.

ν_{max} (KBr): 3287, 3144, 3091, 2932. 2568, 2467,_1659, 1548, 1526 cm^{-1}.

m.p: 142-143°C (lit– 143-145°C) [92].

1-Methyl-4-{[(1-methyl-4-nitro-1*H*-pyrrol-2-yl)carbonyl]amino}-*N*-[2-(4-morpholinyl)ethyl]-1*H*-pyrrole-2-carboxamide (90)



3-Nitro-*N*-methylpyrrole-2-carboxylic acid **81** (0.6 g; 3.5 mmol) was dissolved in thionyl chloride (4 mL), this solution was heated to 80°C and stirred under nitrogen for 3 h. The thionyl chloride was then removed under reduced pressure to give the corresponding acid chloride as a grey solid.

1-Methyl-*N*-[2-(4-morpholinyl)ethyl]-4-nitro-1*H*-pyrrole-2-carboxamide, **89** (1 g; 3.5 mmol), was dissolved in methanol (30 mL), palladium catalyst (10% by weight on activated carbon; 0.5 g) was then added. Hydrogenation was then carried out as previously described (compound **88**) to give the corresponding amine as a glassy solid. Both the amine and acid chloride were used immediately. The acid chloride was dissolved in dichloromethane (10 mL), the amine was dissolved in dichloromethane (10 mL) and added dropwise to this. *N*-Methylmorpholine (0.4 mL; 1.41 mmol) was then added to the reaction mixture which was sealed and stirred at room temperature for 24 h. Dichloromethane was then removed under reduced pressure, the resulting residue was partitioned between ethyl acetate (with 1% methanol, 50 mL)) and aq. sodium hydroxide (10% w/v, 50 mL). The resulting organic layer was removed, dried ($MgSO_4$), and concentrated under reduced pressure to give a pale yellow solid. This solid was then purified by flash column chromatography (eluant: 1:1 methanol/ethyl acetate with 1% TEA). Removal of the solvents then gave the product, **90,** as a yellow solid.

Yield: 0.5 g; 35%

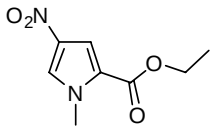
δ_H: (DMSO-d_6) 10.22 (1H, s, NH); 8.18 (1H, d, J = 2.0, CH aromatic); 7.95(1H, t, J = 6.2 Hz, NH); 7.58 (1H, d, J = 2.0, CH aromatic); 7.21 (1H, d, J = 1.9, CH aromatic); 6.83 (1H, d, J = 1.9, CH aromatic); 3.96 (3H, s, CH_3); 3.85 (3H, s, CH_3); 3.57 – 3.58 (4H, m, CH_2); 3.32 – 3.35 (2H, m, CH_2), 2.40 - 2.44 (6H, m, CH_2).

LCMS: Found 403 (-ve ion) calculated for $C_{18}H_{24}N_6O_5$ 404.

ν_{max} (KBr): 3286, 3135, 2955, 2808. 2760, 1666, 1635, 1536cm^{-1}

m.p: > 230^{o}C (lit > 230^{o}C) [92]

Ethyl 1-methyl-4-nitro-1*H*-pyrrole-2-carboxylate (104)



2,2,2-Trichloro-1-(1-methyl-4-nitro-1*H*-pyrrol-2-yl)ethanone**, 84** (1 g; 3.7 mmol), was dissolved in ethanol (dried; 15 mL), sodium ethoxide (0.25 g; 1 equiv) was the added to this mixture which was heated to 110^{o}C under reflux in a nitrogen atmosphere. After 1 h, TLC showed 100% conversion to the ethyl ester. The ethanol was removed under reduced pressure, the resulting residue was partitioned between dichloromethane (50 mL) and water (50 mL); the organic layer was removed and dried ($MgSO_4$). Removal of the dichloromethane gave the pyrrole **104** as a white solid.

Yield: 0.71 g; 97%.

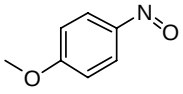
δ_H: ($CDCl_3$) 7.59 (1H, d, *J* = 2, CH aromatic); 7.42 (1H, d, *J* = 1.9, CH aromatic); 4.37 (2H, q, *J* = 7.1, C$\underline{H_2}$CH$_3$); 4.04 (3H, s, CH$_3$); 1.39 (3H, t, *J* = 7.1, CH$_2$C$\underline{H_3}$).

LCMS: Found 199 calculated for $C_8H_{11}N_2O_4^+$ 199.

ν_{max} (KBr): 3138, 2997, 1696, 1505, 1313 cm^{-1}.

m.p. 113-114^{o}C (lit -113-115^{o}C) [90].

1-Methoxy-4-nitrosobenzene



p-Anisidine (0.25 g; 2 mmol) was dissolved in methanol (2 mL) and stirred at room temperature. H_2O_2 (2 mL; 4 equivalents) in water (2 mL) was then added causing

the amine to precipitate as fine needles. MoO_3 (0.06 g; 0.2 equivalents), potassium hydroxide (0.025 g in 1 mL water; 0.1 equivalents) and hexane (5 mL) were the added to the solution which was sealed and stirred vigorously at 0^o C for 24 hours. The layer formed by the hexane was then decanted and the remaining aqueous layer was extracted with hexane (2x 20 mL). The organics were combined and dried ($MgSO_4$) then concentrated in vacuo to give a brown oil. TLC analysis of the solid showed a mixture of the nitroso product and the amine starting material. The oil was therefore purified by flash column chromatography (30% ethyl acetate in hexane as eluant) Removal of the solvents gave the nitroso as a green solid.

Yield: 0.1 g; 37% yield.

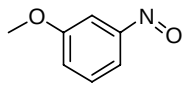
δ_H: ($CDCl_3$) 7.93 (2H, d, CH aromatic, J = 7.9); 7.02 (2H, d, CH aromatic, J = 7.9); 3.95 (3H, s, CH_3).

LCMS: Found 138 calculated for $C_7H_8NO_2^+$ 138.

ν_{max} (KBr): 3067, 2836, 1604, 1529, 1483 cm^{-1}

m.p: 28-30^{o}C (lit - 30^{o}C) [76].

1-Methoxy-3-nitrosobenzene (106)



m-Anisidine (0.5 g; 4 mmol) was dissolved in methanol (2 mL) and stirred at room temperature. H_2O_2 (10 % soln; 3 mL; 4 equivalents) in water (2 mL) were the added causing the amine to precipitate as fine needles. Molybdenum trioxide (0.06 g; 0.1 equivalents), potassium hydroxide (0.05 g in 1 mL water; 0.1 equivalents) and hexane (5 mL) were the added to the solution which was sealed and stirred vigorously at 0 ^{o}C for 24 h. The layer formed by the hexane was then decanted and the remaining aqueous layer was extracted with hexane (2 x 20 mL). The hexane layers were combined and dried ($MgSO_4$), then concentrated under reduced pressure to give a brown oil. TLC analysis of the solid showed a mixture of the nitroso product and the amine starting material. The oil was therefore purified by

flash column chromatography (30% ethyl acetate in hexane as eluant). Removal of the solvents gave the nitroso, **106**, as a green solid.

Yield: 0.24 g; 44%.

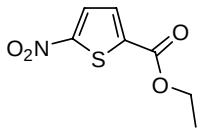
δ_H: ($CDCl_3$) 8.01 (1H, d, *J* = 7.7, CH aromatic); 7.62 (1H, t, *J* = 8, NH); 7.27 - 7.30 (1H, m, CH aromatic); 6.92 (1H, t, *J* = 2.1, CH aromatic); 3.91 (3H, s, CH_3).

LCMS: Found 138 calculated for $C_7H_8NO_2^+$ 138.

ν_{max} (KBr): 3124, 1520, 1487cm^{-1}.

m.p: 45-46^oC (lit - 48^oC) [76].

Ethyl 5-nitro-2-thiophenecarboxylate (112)



5-Nitrothiophene-2-carboxylic acid (**110**, 1g ; 5.8 mmol) was dissolved in thionyl chloride (5 mL) and the solution was heated to 80 ^oC under reflux and stirred for 3 h. After this time TLC showed the reaction was completed. The excess thionyl chloride was removed under reduced pressure to give the acid chloride **111** as a solid. The solid was then dissolved in ethanol and the reaction mixture was stirred overnight. During this time a solid was formed in the solution, this was then cooled to 0 ^oC causing more solid to precipitate. This was collected by filtration to give the thiophene monomer, **112**, as a white solid.

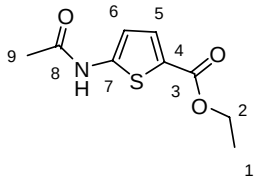
Yield: (0.3g; 26% yield).

δ_H: ($CDCl_3$) 7.87 (1H, d, *J* = 4.3, CH aromatic); 7.7 (1H, d , *J* = 4.3, CH aromatic); 4.26 (2H, q, *J* = 7.1, $C\underline{H}_2CH_3$); 1.43 (3H, t, *J* = 7.1, $CH_2C\underline{H}_3$).

ν_{max} (KBr): 3115, 2981, 1716, 1540, 1508 cm^{-1}.

m.p: 60-61^oC (lit - 63-64^oC)[74].

Ethyl 5-(acetylamino)-2-thiophenecarboxylate (113)[74]



Ethyl 5-nitro-2-thiophenecarboxylate, **112**, (0.1 g; 0.5 mmol) was dissolved in a 1:1 mixture of acetic acid and acetic anhydride (20 mL). Iron powder (0.14 g; 0.5 mmol) was then added to this and the mixture was heated to 100 ^{o}C for 24 h. After this time, the entire solution was poured into ice water. The solution was then neutralised with sat. sodium bicarbonate solution. Ethyl acetate (100 mL) was added to this mixture and the product extracted into the organic layer which was separated, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL) and dried ($MgSO_4$). Removal of the solvents gave the product **113** as white solid.

Yield: 0.1 g; 94 %.

δ_H: ($CDCl_3$) 8.45 (1H, s, NH); 7.87 (1H, d, *J* = 4.1, CH aromatic); 7.7 (1H, d, *J* = 4.1, CH aromatic); 4.26 (2H, q, *J* = 7.1, $C\underline{H}_2CH_3$); 2.19 (3H, s, CH_3); 1.43 (3H, t, *J* = 7.1, $CH_2C\underline{H}_3$).

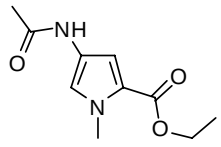
δ_C: (DMSO-d_6) 14.6 (C1); 22.9 (C9); 60.6 (C2); 111.5 (C6); 122.3 (C4); 132.1 (C5); 146.8 (C7); 162.6 (C3); 167.6 (C8).

HREIMS: found 213.0459 calculated for $C_9H_{11}O_3NS^+$ 213.0460

ν_{max} (KBr): 3209, 3089, 3002, 2967, 1712, 1470cm^{-1}.

m.p: 162-164^{o}C.

Ethyl 4-(acetylamino)-1-methyl-1*H*-pyrrole-2-carboxylate (115)



The amine ethyl 4-amino-1-methyl-1H-pyrrole-2-carboxylate (**104**, 0.14 g; 0.7 mmol) was dissolved in a 1:1 mixture of acetic acid and acetic anhydride (20 mL).

Iron powder (0.2 g; 5 equiv) was then added to this and the mixture was heated to 100^{o}C for 24 hours. After this time, the entire solution was poured into ice water. The solution was then neutralised with sat. sodium bicarbonate solution. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL) and dried ($MgSO_4$). Removal of the solvents gave the required product **115** as white solid.

Yield: 0.1 g; 68%.

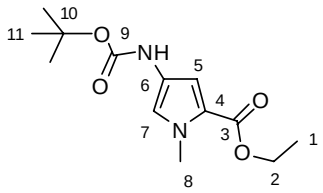
δ_H: (DMSO-d_6) 9.8 (1H, s, NH); 7.27 (1H, d, J = 1.9, CH aromatic); 6.7 (1H, d, J = 1.9, CH aromatic); 4.2 (2H, q, J = 7.1, $C\underline{H}_2CH_3$); 3.8 (3H, s, CH_3); 2.06 (3H, s, CH_3); 1.24 (3H, t, J = 7.1, $C\underline{H}_3CH_2$).

LCMS: Found 211 calculated for $C_{10}H_{15}N_2O_3^+$ 211.

ν_{max} (KBr): 3208, 3058, 1710, 1656, 1567, 1521 cm^{-1}.

m.p: 148-150^{o}C (lit 149-151^{o}C) [74].

Ethyl 4-[(*tert*-butoxycarbonyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylate (117) [86]



A solution of ethyl 4-amino-1-methyl-1*H*-pyrrole-2-carboxylate (0.2 g; 1 mmol) in methanol (25 mL) was cooled in an ice/salt bath to 0 ^{o}C with stirring under a nitrogen atmosphere. To this was added palladium (10 % wt in activated carbon; 0.2 g) as catalyst slowly in small portions. Boc anhydride (0.2 g; 1 equiv) was then added to this mixture. The reaction vessel was evacuated of air under vacuum then filled with hydrogen from a cylinder. The reaction mixture was then sealed and left to stir vigorously for 24h.The reaction mixture was then filtered under suction through Kieselguhr to remove any solid material from the liquid. The liquid was

then collected and the volatile Methanol was removed under reduced pressure to give a brown oil. This oil was purified using flash column chromatography (30% ethyl acetate in hexane). Removal of the solvents gave the required product, **117**, as a white solid.

Yield: 0.195 g; 73%.

δ_H: ($CDCl_3$) 7.0 (1H, s, NH); 6.65 (1H, d, J = 1.9, CH aromatic); 6.21 (1H, s, CH aromatic); 4.28 (2H, q, J = 7.1, CH_2); 3.88 (3H, s,CH_3); 1.53 (9H, s, $(CH_3)_3$); 1.37 (3H, t, J = 7.1, CH_3).

δ_C: ($CDCl_3$) 14.8 (C1); 28.8 (C11); 37.1 (C8); 60.2 (C2); 108.2 (C10); 117.3 (C5); 120.1 (C4); 121.4 (C7); 123.5 (C6); 144.2 (C9); 163.2 (C3).

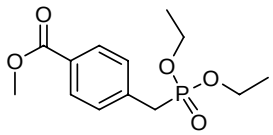
HREIMS: found 268.1424 calculated for $C_{13}H_{20}O_4N_2^+$ 268.1423.

m.p: 78-80 ^oC.

ν_{max} (KBr): 3344, 2966, 1713, 1681 cm^{-1}.

Microanalysis Calcd. For $C_{13}H_{20}O_4N_2$: C, 58.19; H, 7.51; N, 10.44. Found: C, 57.56, H, 7.16; N, 10.13.

Methyl 4-[(diethoxyphosphoryl)methyl]benzoate (121)



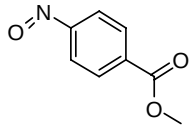
Methyl 4-(bromomethyl)benzoate (0.5 g; 2.2 mmol) was dissolved in triethyl phosphite (3 mL) and the solution was heated to 160 ^oC and stirred for 5 h. The volatile triethyl phosphite was then removed under reduced pressure to give the product, **121**, as a clear colourless oil.

Yield: 0.6 g, 95 %.

δ_H (DMSO-d_6): 1.26 (6H, t, J = 7.1), 3.17 (1H, s), 3.22 (1H, s), 3.9 (3H, s), 4.00 – 4.02 (4H, m), 7.38 (2H, d, J = 8.2), 7.99 (2H, d, J = 8.2).

LRMS: found 287 calculated for $C_{13}H_{20}O_5P^+$ 287.

Methyl 4-nitrosobenzoate (125)



Methyl 4-aminobenzoate **124** (0.5 g; 3.3 mmol) was dissolved in methanol (2 mL) and stirred at room temperature. Hydrogen peroxide (10 % soln; 2.5 mL; 13.2 mmol; 4 equivalents) in water (2 mL) were the added causing the amine to precipitate as fine needles. Molybdenum trioxide (0.02 g; 0.33 mmol; 0.1 equivalents) and potassium hydroxide (0.05 g in 1 mL water; 0.3 mmol; 0.1 equivalents) were the added to the solution which was sealed and stirred at room temperature for 72 h. During this time a yellow solid was observed to be forming in the reaction mixture. This solid was collected by filtration and washed with water (3 x 20 mL) then dried in a heating pistol. TLC analysis of the solid showed a mixture of the nitroso product, **125**, and the amine starting material. The solid was therefore purified by flash column chromatography (30% ethyl acetate in hexane as eluant). Evaporation of the solvents gave the required nitroso **125** as a yellow solid.

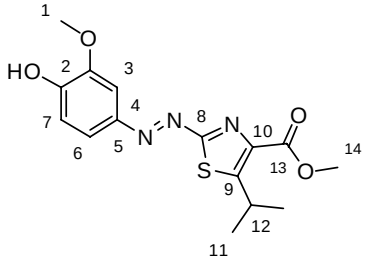
Yield: 0.2 g; 37%

δ_H: ($CDCl_3$) 8.31 (2H,d, J = 8.4, CH aromatic); 7.96 (2H, d, J = 8.4, CH aromatic); 3.9(3H, s, CH_3).

m.p: 130-132^{o}C (lit -129-131^{o}C) [76].

Head groups

Methyl 2-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-5-isopropyl-1,3-thiazole-4-carboxylate (77)



Methyl 2-amino-5-isopropylthiazole-4-carboxylate **76** (0.5 g; 2.5 mmol) was dissolved in hydrochloric acid solution (20% of 12M hydrochloric acid in water; 20 mL). This mixture was cooled to 0^{o}C using an ice/salt bath with vigorous stirring. Sodium nitrite (0.17 g; 2.5 mmol) was then dissolved in water (1 mL) and then added dropwise to the above solution. This solution was then stirred at 0 ^{o}C for 30 min to allow formation of the diazonium salt to occur. During this time, guaiacol (0.3 mL; 2.5 mmol) dissolved in sodium hydroxide (10% soln. in water; 20 mL) was cooled to 0 ^{o}C. This solution was then added dropwise to the diazonium salt solution. A brown precipitate was observed in the mixture upon addition of the phenol solution. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$) then concentrated under reduced pressure to give a brown solid. The brown solid was purified by flash column chromatography (50% ethyl acetate in hexane as eluant; product identified by strong orange colour on the column). The required diazo dimer, **77**, was obtained as an orange solid on evaporation of the solvent.

Yield: 0.62 g; 73%.

δ_H: ($CDCl_3$) 7.6 (1H, d, J = 8.3, CH aromatic, H^6); 7.59 (1H, d, J = 2.1 , CH aromatic, H^4); 7.07 (1H, d, J = 8.3, CH aromatic, H^7); 4.25 (1H, sept, J = 6.8, $CH(CH_3)_2$); 4.1 (3H, s, CH_3); 4.00 (3H, s, CH_3); 1.39 (6H, d, J = 6.8, $CH(CH_3)_2$).

δ_c: (DMSO-d_6) 24.9 (C11); 28.2 (C12); 55.9 (C14); 57.2 (C1); 103.1 (C4); 116.1 (C7); δ124 (C5); 139.7 (C6); 144.8 (C10); 149.2 (C2); 153.8 (C8); 159.9 (C3) 162.4 (C9); 171.7 (C13).

HRFABMS: found: 336.1025, calculated for $C_{15}H_{18}O_4N_3S$ $(M+H)^+$ 336.1018.

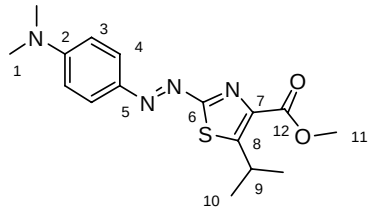
ν_{max} (KBr): 3402, 3357, 2959, 1716, 1590, 1484 cm^{-1},

m.p: 82-84 ^{o}C.

Microanalysis. Calcd. for $C_{15}H_{17}O_4N_3S$: C, 53.72; H, 5.11; N, 12.53; S, 9.56.

Found: C, 53.82; H, 5.05, N, 12.18; S, 9.14 %.

Methyl 2-{(*E*)-[4-(dimethylamino)phenyl]diazenyl}-5-isopropyl-1,3-thiazole-4-carboxylate (78)



Methyl 2-amino-5-isopropylthiazole-4-carboxylate **76** (0.5 g; 2.5 mmol) was dissolved in hydrochloric acid solution (20% of 12M hydrochloric acid in water; 20 mL). This mixture was cooled to 0^{o}C using an ice/salt bath with vigorous stirring. Sodium nitrite (0.17 g; 2.5 mmol) was then dissolved in water (1 mL) and then added dropwise to the above solution. This solution was then stirred at 0 ^{o}C for 30 min to allow formation of the diazonium salt to occur. During this time, *N,N*-dimethylaniline (0.3 g; 2.5 mmol) dissolved in methanol (5 mL) and sodium hydroxide (10% soln. in water; 20 mL) was cooled to 0 ^{o}C. This solution was then added dropwise to the diazonium salt solution. A brown precipitate was observed in the mixture upon addition of the solution.

Ethyl acetate was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$) then concentrated under reduced pressure to give a brown solid. The brown solid was purified by flash column chromatography (50% ethyl acetate in hexane as eluant; product identified by strong red colour on the column). The required diazo dimer, **78,** was obtained as an orange solid on evaporation of the eluates.

Yield: 0.62 g; 75%.

δ_H: ($CDCl_3$) 7.95 (2H, d, *J* = 8.4, CH aromatic); 7.27 (2H, d, *J* = 8.4, CH aromatic); 4.2 (1H, sept, *J* = 6.7, $CH(CH_3)_2$); 4.1 (3H, s, CH_3); 3.1 (6H, s, $(CH_3)_2$); 1.34 (6H, d, *J* = 6.7, $CH(CH_3)_2$).

δ_c : ($CDCl_3$) 25.11 (C10); 28.1 (C9); 40.2 (C1); 52.4 (C11); 112 (C3); 126.9 (C5); 138.8 (C4); 143.5 (C7); 155.5 (C2); 160.4 (C6); 163.5 (C8); 173 (C12).

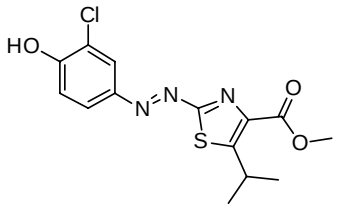
HREIMS: found 332.1309, calculated for $C_{16}H_{20}O_2N_4S^+$ 332.1307.

ν_{max} (KBr): 2958, 2858, 1704, 1602 cm^{-1}.

m.p: 178-180 ^{o}C.

Purity by HPLC: 95 % (See appendix 2 for sample HPLC trace)

Methyl 2-[(*E*)-(3-chloro-4-hydroxyphenyl)diazenyl]-5-isopropyl-1,3-thiazole-4-carboxylate (79)



A solution of sulphuric acid (0.16 mL; 2 equiv) in water (4 mL) was cooled to –5 ^{o}C using an ice bath with salt. A slurry of methyl 2-amino-5-isopropylthiazole-4-carboxylate **76** (0.2g; 1 equiv) in water (1 mL) was then added to this solution. To this was added a solution of a solution of sodium nitrite (0.172 g; 2.5 equiv) in water (1 mL). Finally, 2-chlorophenol (0.1 mL; 1 equiv) in ethanol (1 mL) and water (1 mL) was added to this mixture. The reaction mixture was then removed from the ice bath and stirred under a N_2 atmosphere as it gradually warmed to room temperature. A 1% solution of sodium hydroxide in water was then used to bring the pH of the solution to ~9 producing a strong red colour. Ethyl acetate (100mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$), then concentrated under reduced pressure to give a brown solid. The sat. sodium bicarbonate wash was also collected and left to stand at room temperature for several hours, during this time a white precipitate formed. The mixture was filtered and the solid collected, washed with water and dried to give the required product, **79,** as a yellow powder.

Yield: 20mg; 4%.

δ_H: ($CDCl_3$): 8.1(1H, s, CH aromatic, H^2); 7.9 (1H, d, J = 8.2, CH aromatic, H^6); 7.2 (1H, d, J = 8.2, CH aromatic, H^5); 6 (1H, s, O<u>H</u>); 4.25 (1H, sept, J = 6.8, C<u>H</u>$(CH_3)_2$); 4.0 (3H, s, OC<u>H</u>$_3$); 1.4 (6H, d, J = 6.8, CH(C<u>H</u>$_3)_2$).

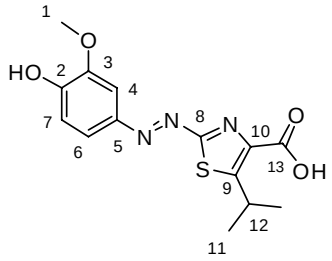
LRMS: Found: 340 calculated for $C_{14}H_{15}ClN_3O_3S^+$ 340.

ν_{max} (KBr): 1723, 3345 cm^{-1}.

m.p: No discernible melting point obtained.

Insufficient material for complete analysis

2-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-5-isopropyl-1,3-thiazole-4-carboxylic acid (96)



The foregoing ester **77** (0.07 g; 0.2 mmol) was dissolved in methanol (2 mL), to this was added a solution of lithium hydroxide (0.01 g; 0.4 mmol) in water (10 mL) causing the ester to precipitate out of solution. The solution was then heated to 70 ^oC and stirred for 24 h during which time a strong red colour was produced. TLC analysis of the mixture showed 100% conversion to the lithium salt of the acid. The solution was then acidified to pH 2 using hydrochloric acid (10% solution in water) causing a red precipitate to form. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL) and dried ($MgSO_4$). Evaporation of the solvents than gave carboxylic acid **96** as a red solid.

Yield: 0.06 g; 93%.

$\delta_{H:}$ ($CDCl_3$) 7.6 (1H, d, *J* = 8.4, CH aromatic, H^6); 7.46 (1H, d, *J* = 2.1, CH aromatic, H^4); 7.03 (1H, d, *J* = 8.4, CH aromatic, H^7); 4.23 (1H, sept, J = 6.8, $CH(CH_3)_2$); 4.14 (3H, s, CH_3); 1.41 (6H, d, *J* = 6.8, $CH(CH_3)_2$).

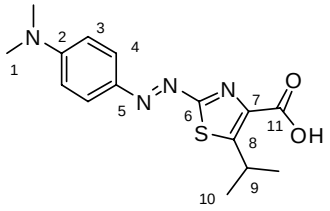
HRFABMS: Found 322.0789 calculated for $C_{14}H_{16}N_3O_4S$ $(M+H)^+$ 322.0794.

ν_{max} (KBr): 3231, 2963, 1695, 1592, 1503 cm^{-1}.

m.p: No discernible melting point obtained.

Purity by HPLC: 94 %

2-{(*E*)-[4-(dimethylamino)phenyl]diazenyl}-5-isopropyl-1,3-thiazole-4-carboxylic acid (98)



The foregoing ester **78** (0.4 g; 1.2 mmol) was dissolved in methanol (2 mL). To this was added a solution of lithium hydroxide (0.057 g; 2.4 mmol) in water (10 mL) causing the ester to precipitate out of solution. The solution was then heated to 70 ^{o}C and stirred for 24 h during which time a strong red colour was produced. TLC analysis of the mixture showed 100% conversion to the lithium salt of the acid. The solution was then acidified to pH 2 using hydrochloric acid (10% solution in water) causing a red precipitate to form. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL) and dried ($MgSO_4$. Removal of the solvents gave the required carboxylic acid **98** as a red solid.

Yield: 0.36 g; 94%

δ_H: ($CDCl_3$) 7.93 (2H, d, *J* = 9.2, CH aromatic, H^4); 7.27 (2H, d, *J* = 9.2, CH aromatic, H^3); 4.3 (1H, sept, *J* = 6.8, $CH(CH_3)_2$); 3.1 (6H, s, $(CH_3)_2$); 1.39 (6H, d, *J* = 6.8, $CH(CH_3)_2$).

^{13}C NMR: (DMSO-d_6) 25.5 (10); 28.5(9); 40.65 (1); δ112.9 (3); 127.9 (5); 141.04 (4); 142.4 (7); 154.9 (2); 158.2 (6); 164.1 (8); 173.02 (11).

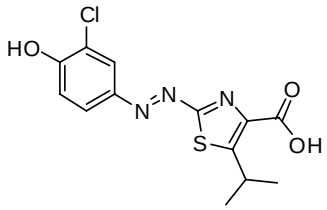
HRFABMS: found 319.1230 calculated for $C_{15}H_{19}O_2N_4S$ $(M+H)^+$ 319.1229.

ν_{max} (KBr): 2971, 2706, 2602 ,1680 , 1605 cm^{-1}.

m.p: No discernible melting point obtained

Purity by HPLC: 95 %.

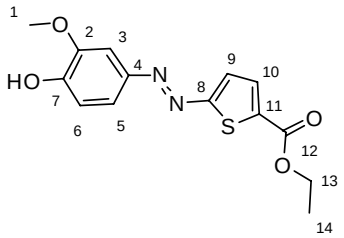
2-[(*E*)-(3-Chloro-4-hydroxyphenyl)diazenyl]-5-isopropyl-1,3-thiazole-4-carboxylic acid (93)



The foregoing ester **79** (17 mg) was dissolved in methanol (20 mL). Lithium hydroxide (1.44 mg; 1.2 equiv) was added to the mixture which was then stirred at room temperature for 24 hours. The reaction mixture was then dried using a freeze/dryer to give the required carboxylic acid **93** as a yellow solid.

LCMS: found 326 calculated for $C_{13}H_{12}ClN_3O_3S$ 325.
This material was used without any further purification.

Ethyl 5-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-2-thiophenecarboxylate (114)



Ethyl 5-(acetylamino)-2-thiophenecarboxylate, **113**, (0.1 g; 0.47 mmol) was dissolved in 12M hydrochloric acid (5 mL), this solution was then heated to 80 ^{o}C and stirred for 1h. This mixture was cooled to 0 ^{o}C using an ice/salt bath with vigorous stirring. Sodium nitrite (0.032 g; 0. 47 mmol) was dissolved in water (1mL) and added dropwise to the above solution. This solution was then stirred at 0^{o} C for 30 min to allow formation of the diazonium salt to occur. During this time, guaiacol (0.058 g; 0.47 mmol) dissolved in sodium hydroxide (10% soln. in water; 20 mL) was cooled to 0^{o}C. This solution was then added dropwise to the diazonium salt solution. A brown precipitate was observed in the mixture upon addition of the phenol solution. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$) then concentrated under reduced pressure to give a brown solid. The brown solid was purified by flash column

chromatography (50% ethyl acetate in hexane as eluant; product identified by strong orange colour on the column). Removal of the solvents gave the required product **114** as an orange solid.

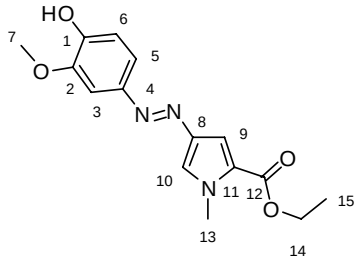
Yield: 0.02 g; 14%.

δ_H: ($CDCl_3$) 7.9 (1H, d, J = 4.0, CH aromatic); 7.81 (1H, d, J = 4.0, CH aromatic); 7.77 (1H, d, J = 8.4, CH aromatic, H^6); 7.57 (1H, s, CH aromatic); 7.06 (1H, d, J = 8.4, CH aromatic, H^5), 6.01 (1H, s, OH), 4.4 (2H, q, J = 7.1, CH_2CH_3); 3.99 (3H, s, CH_3); 1.25 (3H, t, J = 7.1, CH_2CH_3).

HREIMS: found 306.0673, calculated for $C_{14}H_{14}O_4N_2S^+$ 306.0674.

Microanalysis: Insufficient material for analysis

Ethyl 4-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-1-methyl-1*H*-pyrrole-2-carboxylate (116)



Compound **117** (0.26g; 1 mmol) was dissolved in 12M hydrochloric acid (5 mL), this solution was then heated to 80 ^oC and stirred for 1hour. This mixture was cooled to 0 ^oC using an ice/salt bath with vigorous stirring. Sodium nitrite (0.07 g; 1 mmol) was then dissolved in water (1 mL) and then added dropwise to the above solution. This solution was then stirred at 0 ^oC for 30 min to allow formation of the diazonium salt to occur. During this time, guaiacol (0.13 g; 1 mmol) dissolved in sodium hydroxide (10% soln. in water; 20 mL) was cooled to 0 ^oC. This solution was then added dropwise to the diazonium salt solution. A brown precipitate was observed in the mixture upon addition of the phenol solution.

Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$) then concentrated under reduced pressure to give a brown solid. The brown solid was purified by flash column chromatography (50% ethyl acetate in hexane as eluant; product identified by strong orange colour on the

column). The required product **116** was obtained as an orange solid on evaporation of the solvents

Yield: 0.1 mg; 33%

δ_H: ($CDCl_3$) 7.48 – 7.54 (3H, m, CH aromatic); 7.45 (1H, d, *J* = 1.8, CH aromatic, H^{10}); 7.03 (1H, d, *J* = 8.3, CH aromatic); 4.35 (2H, q, *J* = 7.1, CH_2CH_2); 4.02 (3H, s, CH_3); 3.96 (3H, s, CH_3); 1.37 (3H, t, *J* = 7.1, CH_3CH_2)

δ_C: ($CDCl_3$) 14.59 (C15); 37.6(C13); 56.25 (C7); 60.48 (C14); 102.12 (C8); 114.4 (C3); 119.8 (C6); 123.9 (C11); 128.5 (C4); 141.9 (C5); 147.11 (C1); 147.28 (C10); 148.1 (C2); 161.6 (C12).

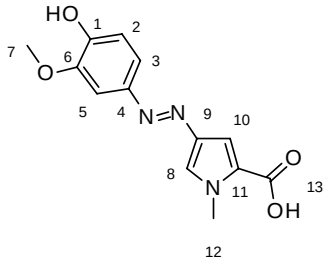
HRCIMS: found 304.1294, calculated for $C_{15}H_{18}O_4N_3$ $(M+H)^+$ 304.1297.

ν_{max} (KBr): 3496, 2987, 1701, 1597, 1540, 1499 cm^{-1}.

m.p: 118-120 ^oC.

Microanalysis: Insufficient material for analysis

4-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-1-methyl-1*H*-pyrrole-2-carboxylic acid (118)



The ester compound from the previous preparation, **116,** (0.08 g; 0.26 mmol) was dissolved in methanol (2 mL). To this was added a solution of Sodium hydroxide (0.026 g; 2equiv) in water (10 mL) causing the ester to precipitate out of solution. The solution was then heated to 70^{o}C and stirred for 24 hours during which time a strong red colour was produced. TLC analysis of the mixture showed 100% conversion to the lithium salt of the acid. The solution was then acidified to pH 2 using hydrochloric acid (10% solution in water) causing a red precipitate to form. Ethyl acetate was added to this mixture (100 mL), the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x

50 mL) and dried ($MgSO_4$). Removal of the solvents gave the required diazo dimer, **118**, as a red solid.

Yield: 0.065g; 91%.

δ_H: ($CDCl_3$) 9.64 (1H, s, CH aromatic, H^5); 7.83 (1H, d, J = 1.9, CH aromatic, H^8); 7.31 (1H, d, J = 8.2, CH aromatic, H^3); 7.28 (1H, d, J = 1.9, CH aromatic, H^{10}); 6.91 (1H, d, J = 8.2, CH aromatic, H^2); 4.02 (3H, s, CH_3); 3.87 (3H, s, CH_3).

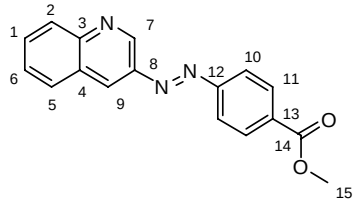
δ_C: (DMSO-d_6) 37.4 (C12); 55.9 (C7); 103.2 (C9); 106.9 (C10); 115.6 (C5); 119.03 (C2); 124.2 (C4); 129.7 (C3); 141.3 (C11); 146 (C8); 148.7 (C1); 149.5 (C6); 162.5 (C13).

HRCIMS: found 276.0986 calculated for $C_{13}H_{14}O_4N_3$ $(M+H)^+$ 276.0984.

m.p: No discernible melting point obtained

Microanalysis: Insufficient material for analysis

Methyl 4-[(*E*)-3-quinolinyldiazenyl]benzoate (126)



3-Aminoquinoline (0.05 g; 0.34 mmol) was dissolved in glacial acetic acid and stirred at room temperature. Methyl 4-nitrosobenzoate, **125** (0.06 g; 0.34 mmol), was then dissolved in glacial acetic acid (2 mL) and added to the 3-aminoquinoline solution. The mixture was then stirred at 70 ^oC under a nitrogen atmosphere for 24 hours. During this time a brown precipitate formed and the solution became almost solid. The solid was collected by filtration. The filtrate was neutralised with sat. sodium bicarbonate soln (50 mL). Ethyl acetate (100 mL) was then added to this and the resulting organic layer was removed, washed with brine, and dried ($MgSO_4$) then concentrated under reduced pressure to give a brown solid which was combined with the solid obtained by filtration. The combined solid products were then purified by flash column chromatography (20% ethyl acetate in hexane as eluant) to give the required diazo compound, **126**, as an orange solid.

Yield: 0.06 g; 60%.

δ_H: ($CDCl_3$) 9.53 (1H, s, CH aromatic); 8.68 (1H, s, CH aromatic); 8.25 (2H, d, *J* = 8.4, CH aromatic); 8.22 (1H, d, *J* = 8.4, CH aromatic), 8.02 - 8.06 (3H, m, CH aromatic); 7.85 (1H, t, *J* = 6.9, CH aromatic); 7.65 (1H, t, *J* = 7.4, CH aromatic); 3.98 (3H, s, CH_3).

δ_C: ($CDCl_3$) 52.3 (C15); 123.12 (C6); 124.02 (C9); 127.9 (C10); 129.6 (C13); 129.67 (C4); 130.9 (C11); 131.5 (C5); 132.4 (C2); 142.3 (C1); 144.6 (C12); 145.1 (C8); 155.2 (C7); 166.7 (C3); 178.3 (C14).

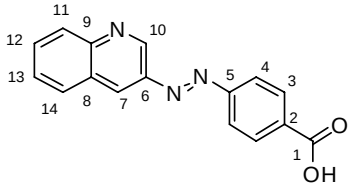
HRFABMS: found 292.1082 calculated for $C_{17}H_{14}O_2N_3$ $(M+H)^+$ 292.1086.

m.p: 150-152^{o}C.

ν_{max} (KBr): 3402, 1727, 1603, 1573, 1497, 1462 cm^{-1}.

Purity by HPLC: 95 %

4-[(*E*)-3-quinolinyldiazenyl]benzoic acid (127)



The foregoing methyl ester **126** (0.08 g; 0.275mmol) was dissolved in methanol (2 mL) and to this was added a solution of lithium hydroxide (0.014g; 2 equiv) in water (10 mL) causing the ester to precipitate out of solution. The solution was then heated to 70 ^{o}C and stirred for 24 h during which time a strong red colour was produced. TLC analysis of the mixture showed 100% conversion to the lithium salt of the acid. The solution was then acidified to pH 6 using hydrochloric acid (10% solution in water). Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (50 mL) and sat. sodium bicarbonate soln (2 x 50 mL and dried ($MgSO_4$). Removal of the solvents then gave the acid, **127**, as an orange solid.

Yield: 0.075 g; 98%.

δ_H: ($CDCl_3$) 9.45 (1H, s, CH aromatic); 8.93 (1H, s, CH aromatic); 8.36 (1H, d, J = 8.3), 8.27 (1H, d, J = 8.1, CH aromatic), 8.05 – 8.19 (4H, m, CH aromatic); 7.95 (1H, t, CH aromatic, *J* = 7.6); 7.69 (1H, t, CH aromatic, *J* = 7.5).

δ_C: (DMSO-d_6) 167.4 (C1); 166.8 (C9); 154.3 (C6); 149.2 (C10); 144.8 (C5); 131.2 (C7); 130.7 (C3); 130.5 (C2); 130.1 (C14); 129.7 (C12); 128.3 (C11); 125.1 (C4); 123.1 (C8); 122.9 (C13).

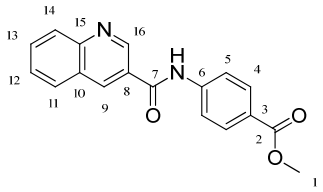
HRFABMS: found 278.0925 calculated for $C_{16}H_{12}O_2N_3$ $(M+H)^+$ 278.0924.

ν_{max} (KBr): 3421, 1634, 1462, 1425 cm^{-1}.

m.p: > 230 ^oC

Purity by HPLC: 96 %

Methyl 4-[(3-quinolinylcarbonyl)amino]benzoate (128)



3-Quinoline carboxylic acid (0.2 g, 1.15 mmol) was dissolved in methanol (5 mL) and DMF (1 mL); methyl 4-aminobenzoate (0.17 g, 1.15 mmol) was then added followed by DMT-MM (0.3 g, 1.15 mmol). The mixture was then stirred at room temperature for 24h. During this time the product, **128**, formed as a white precipitate which was collected by filtration and dried under reduced pressure.

Yield: 0.25 g, 71%.

δ_H (DMSO-d_6): 10.9 (1H, s, NH), 9.36 (1H, d, *J* = 2.3, H^{16}), 8.9 (1H, d, *J* = 1.9, H^9), 7.99 – 7.02 (4H, m, CH aromatic), 7.89 – 7.91 (1H, m, H^{12}), 7.74 (1H, t, *J* = 7.5, H^{13}), 3.85 (3H, s, CH_3).

δ_C: (DMSO-d_6) 51.9 (C1); 119.5 (C5);124.5 (C3); 126.3 (C8); 127.3 (C12); 127.6 (C14); 128.8 (C11); 129.2 (C10);130.2 (C4); 131.5 (C13); 136.3 (C9); 143.4 (C6); 148.6 (C15); 149.1 (C16); δ164.6 (C7); 165.8 (C2).

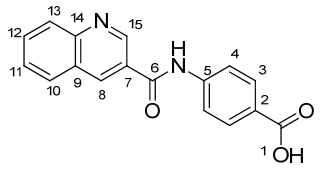
HREIMS: found 306.1004 calculated for $C_{18}H_{14}N_2O_3$ $(M+H)^+$ 306.1004.

ν_{max} (KBr**):** 3360, 2994, 1715, 1676, 1600, 1478 cm^{-1}.

m.p. >230^oC (lit value: >230^oC)[72]

Purity by HPLC: 96 %

4-[(3-Quinolinylcarbonyl)amino]benzoic acid (129)



Methyl 4-[(3-quinolinylcarbonyl)amino]benzoate, **128**, (0.15 g, 0.51 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4 mL) and added to this. This suspension was heated to 80 ^{o}C and stirred for 5h. The solution was then acidified using dilute hydrochloric acid causing the precipitation of a solid. This was collected by filtration and dried under reduced pressure to give the carboxylic acid, **129**, as a brown solid.

Yield: 0.14g, 94%.

δ_H (DMSO-d_6): 10.9 (1H, s, NH), 9.38 (1H, d, J = 1.8, H^{15}), 9.03 (1H, d, J = 1.8 H^{8}), 8.13 -8.19 (2H, m, CH aromatic), 7.91 – 7.99 (5H, m, CH aromatic), 7.75 (1H, t, J = 7.1, H^{12}).

δ_C: (DMSO-d_6) 119.6(C4);125.8 (C2); 126.5 (C7);127.2 (C11);127.4 (C13);128.1 (C10);129.5 (C9);130.2 (C3);132.4 (C12);138.3 (C8);142.9 (C5);146.5 (C14);148.2 (C15);163.9 (C6);166.8 (C1).

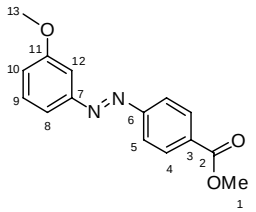
HRFABMS: found 293.0924 calculated for $C_{17}H_{13}O_3N_2$ $(M+H)^+$ 293.0921.

ν_{max} (KBr): 3320, 3052, 2928, 2473, 1930, 1695, 1542 cm^{-1}.

m.p. >230^{o}C (lit value: >230^{o}C)[72]

Purity by HPLC: 94 %

Methyl 4-[(*E*)-(3-methoxyphenyl)diazenyl]benzoate (132)



m-Anisidine (0.23 g; 1.8 mmol) was dissolved in acetic acid (glacial, 5 mL); methyl 4-nitrosobenzoate **125** (0.3 g; 1.8 mmol) was then dissolved in acetic acid (glacial, 5mL) and added to this solution. The mixture was then heated to reflux and stirred under nitrogen for 24 h. Acetic acid was the removed under reduced pressure and the resulting residue was partitioned between ethyl acetate (100 mL) and aqueous sodium hydroxide solution (10% w/v, 100 mL). The resulting organic layer was removed, dried ($MgSO_4$), and concentrated under reduced pressure to give a purple residue. This residue was purified using flash column chromatography (30% ethyl acetate/ hexane R_f = 0.8). Removal of the solvents gave the product, **132**, as an orange solid.

Yield: 0.19g, 39%.

δ_H ($CDCl_3$): 8.18 - 8.21 (2H, m, CH aromatic), 7.98 (2H, d, *J* = 8.4, CH aromatic), 7.61 (1H, d, *J* = 7.6, CH aromatic), 7.43 - 7.49 (2H, m, CH aromatic), 7.1 (1H, dd, *J* = 8.2, *J* = 1.8, CH aromatic), 3.99 (3H, s, OCH_3), 3.91(3H, s, OCH_3).

δ_C: ($CDCl_3$) 52.5 (C1); 55.7 (C13); 106.0 (C12); 117.8 (C10); 118.7 (C8); 122.8 (C5); 130.1 (C4); 130.8 (C9); 132.1 (C3); 154.1(C7); 155.3 (C6); 160.6 (C11), 166.6 (C2).

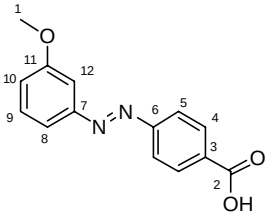
HREIMS: found 270.1002 calculated for $C_{15}H_{14}N_2O_3^+$ 270.1004.

ν_{max} (KBr): 2958, 1727, 1600, 1435 cm^{-1}.

m.p. 93-95^oC.

Microanalysis Calcd. For $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.4; 5.24; N, 10.01.

[(*E*)-(3-Methoxyphenyl)diazenyl]benzoic acid (133)



Methyl 4-[(*E*)-(3-methoxyphenyl)diazenyl]benzoate (**132**, 0.12 g, 0.44 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4 mL) and added to this. This suspension was heated to 80 ^{o}C with stirring for 5h. The solution was then acidified using dilute hydrochloric acid causing the precipitation of an orange solid. This solid which was collected by filtration and dried under reduced pressure to give the product, **133**, as an orange solid.

Yield: 0.096g, 85%.

δ_H (DMSO-d_6): 8.13 – 8.16 (2H, m, CH aromatic), 7.97 (2H, dd, J = 6.8, J = 1.9, CH aromatic), 7.54 – 7.66 (2H, m, CH aromatic), 7.45 – 7.46 (1H, m, CH aromatic), 7.18 - 7.21 (1H, m, CH aromatic), 3.99 (3H, s, OCH_3).

δ_C: (DMSO-d_6) 56.3 (C1); 107.1 (C12); 117.5 (C10); 119.4 (C8); 123.4 (C5); 131.2 (C4); 131.5 (C9); 133.8 (C3); 154.0 (C7); 155.1 (C6); 161.0 (C11), 167.5 (C2)

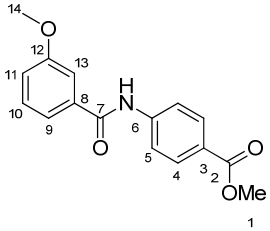
HRFABMS: found 257.0928 calculated for $C_{14}H_{13}N_2O_3$ $(M+H)^+$ 257.0926.

ν_{max} (KBr): 3414, 2934, 2834, 1680, 1600, 1429 cm^{-1}.

m.p. > 230^{o}C

Microanalysis Calcd. For $C_{14}H_{12}N_2O_3$: C, 65.62, H, 4.72, N, 10.93. Found: C, 65.06, H, 3.96, N, 10.00

Methyl 4-[(3-methoxybenzoyl)amino]benzoate (134)



3-Methoxybenzoyl chloride (0.2 g, 1.17 mmol) was dissolved in dichloromethane (5 mL, dry) to which a solution of methyl 4-aminobenzoate (0.18g, 1.17 mmol) in dichloromethane (5mL, dry) was added followed by *N*-methylmorpholine (0.25 mL, 2.34 mmol). The solution was then stirred at room temperature for 3 h. Sodium hydroxide solution (aq. 10% w/v, 20 mL) was then added to the solution, the resulting dichloromethane layer was removed, dried ($MgSO_4$), and the solvent removed under reduced pressure to give a pale brown solid. This solid was purified using silica gel column chromatography (R_F = 0.30 30% ethyl acetate/n-hexane). Removal of the solvent gave the product, **134**, as a brown solid.

Yield: 0.2 g, 60%.

δ_H ($CDCl_3$): 8.07 (2H, d, *J* = 8.7, CH aromatic), 7.75 (2H, dd, *J* = 7.0, *J* = 1.9, CH aromatic), 7.45 (1H, s, CH aromatic), 7.40 – 7.41 (2H, m, CH aromatic), 7.10 - 7.12 (1H, m, CH aromatic), 3.92 (3H, s, OCH_3), 3.85 (3H, s, OCH_3).

δ_C: (DMSO-d_6) 51.9 (C1); 55.3 (C14); 113.6 (C13); 113 (C11); 117.6 (C9); 119.6 (C5); 124.3 (C3); 139.6 (C10); 130.1 (C4); 135.9 (C8); 143.6 (C6); 153.5 (C12); 159.2 (C7); 166.3 (C2).

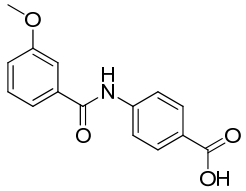
HRFABMS: found 286.1079 calculated for $C_{16}H_{16}NO_4$ $(M+H)^+$ 286.1081.

ν_{max} (KBr): 3356, 2942, 1709, 1666, 1522 $cm.^{-1}$.

m.p. 88-90^oC (lit value 79 -81^oC)[73]

Purity by HPLC: 96 %

4-[(3-Methoxybenzoyl)amino]benzoic acid (136)



The foregoing methyl ester, **134,** (0.13 g, 0.46 mmol) was dissolved in methanol (5mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4 mL) and added to this solution. This suspension was heated to 80 ^oC with stirring for 5 h. The solution was then acidified using dilute hydrochloric acid causing the

precipitation of a brown solid. This was collected by filtration and dried under reduced pressure to give the carboxylic acid, **136**, as a brown solid.

Yield: 0.1g, 80%.

δ_H (DMSO-d_6): 7.90 – 7.95 (4H, m, CH aromatic), 7.40 - 7.62 (3H, m, CH aromatic), 7.16 – 7.18 (1H, m, CH aromatic), 3.85 (3H, s, OCH_3).

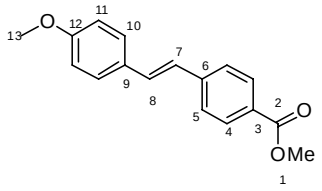
HRFABMS: found 272.0922 calculated for $C_{15}H_{14}O_4N$ $(M+H)^+$ 272.0923

ν_{max} (KBr): 3401, 2924, 2854, 1681, 1632, 1598, 1513, 1462, 1424 $cm.^{-1}$

m.p. >230°C (lit value: >230°C)[73]

Microanalysis Calc. For $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.16. Found: C, 65.47; H, 4.95; N, 5.13.

Methyl 4-[(*E*)-2-(4-methoxyphenyl)ethenyl]benzoate (137-A)



Diethyl (4-(methoxycarbonyl)phenyl)methylphosphonate **121** (0.2 g; 0.7 mmol) was dissolved in THF (dry, 5 mL), and the solution was the cooled to 0 °C. Sodium hydride (0.13 g; 8 equiv) was then added slowly in small portions to this solution which was then stirred for 20 min. *p*-Anisaldehyde (0.1 g; 0.7 mmol) was then dissolved in THF (dry, 3 mL) and added dropwise to this solution. The resulting mixture was stirred at room temperature for 4 h. The reaction was then quenched with water (10 mL) causing a white solid to precipitate solution; this was collected by filtration and dried under reduced pressure to give the product, **137-A**.

Yield: 0.11g, 59%.

δ_H (DMSO-d_6): 3.79 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.99 (d, 2H, J = 8.7, CH aromatic), 7.21 (1H, d, J = 16.4, CH=CH), 7.4 (d, 1H, J= 16.4, CH=CH), 7.6 (2H, d, J = 8.7, CH aromatic), 7.71 (2H, d, J = 8.4, CH aromatic), 7.95 (2H, d, J = 8.4, CH aromatic).

δ_C: (DMSO-d_6) 52.01 (C1); 55.17 (C13); 114.232 (C11); 124.85 (C5); 126.21 (C7); 127.71 (C8); 128.25 (C9); 129.16 (C10); 129.56 (C3); 130.99 (C4); 142.25 (C6); 159.43 (C12); 165.99 (C2).

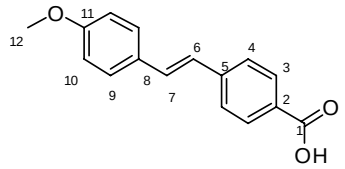
HRCIMS: found 269.1181 calculated for $C_{17}H_{17}O_3$ $(M+H)^+$ 269.1178.

ν_{max} (KBr); 2955, 2925, 1719, 1600, 1575 cm^{-1}.

m.p. 196-198^oC. (lit value: 201 – 202^oC)[93]

Purity by HPLC: 94 %

4-[(*E*)-2-(3-Methoxyphenyl)ethenyl]benzoic acid (137-B)



Methyl 4-[(*E*)-2-(4-methoxyphenyl)ethenyl]benzoate **137-A** (0.10 g, 0.37 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4mL) and added to this. This suspension was heated to 80 ^oC and stirred for 5h. The solution was then acidified using dilute hydrochloric acid causing the product, **137-B** to precipitate. This precipitate was collected by filtration and dried under reduced pressure to give the product as an orange solid.

Yield: 0.085 g, 90%.

δ_H (DMSO-d_6): 3.79 (3H, s, OCH_3), 6.99 (d, 2H, *J* = 8.3, CH aromatic), 7.15 (1H, d, *J* = 16.4, CH=CH), 7.38 (d, 1H, *J*= 16.4, CH=CH), 7.6 (2H, d, *J* = 8.7, CH aromatic), 7.68 (2H, d, *J* = 8.3, CH aromatic), 7.93 (2H, d, *J* = 8.7, CH aromatic).

δ_C: (DMSO-d_6) 55.16 (C12); 114.22(C10); 124.03 (C4); 126.04 (C6); 128.18 (C7); 129.23 (C8); 129.71 (C9); 129.72 (C2); 130.57 (C3); 142.03 (C5); 159.35 (C11); 165.83 (C1)

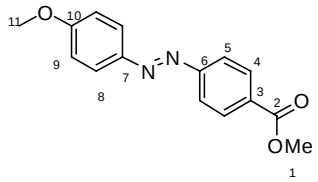
HRCIMS: found 255.1017 calculated for $C_{16}H_{15}O_3$ $(M+H)^+$ 255.1021.

m.p. > 230^oC (lit value: 250 – 251^oC)[94]

ν_{max} (KBr): 3433, 2923, 1601, 1552, 1431 cm^{-1}.

Purity by HPLC: 95 %

Methyl 4-[(*E*)-(4-methoxyphenyl)diazenyl]benzoate) (138-A)



p-Anisidine (0.23 g; 1.8 mmol) was dissolved in acetic acid (glacial, 5 mL), methyl 4-nitrosobenzoate (0.3g; 1.8mmol) was then dissolved in acetic acid (glacial, 5 mL) and added to this solution. The mixture was then heated to reflux and stirred under nitrogen for 24. Acetic acid was the removed under reduced pressure and the resulting residue was partitioned between ethyl acetate and aqueous sodium hydroxide solution (10% w/v). The resulting organic layer was removed and dried ($MgSO_4$), and concentrated under reduced pressure to give a purple residue. This residue was purified using flash column chromatography (30% ethyl acetate/ hexane R_f = 0.8). Removal of the solvents gave the diazo dimer, **138-A**, as an orange solid.

Yield: 0.14g, 29%

δ_H (DMSO-d_6): 8.16 (d, 2H, *J* = 8.6, CH aromatic), 7.93 - 7.96 (m, 4H, CH aromatic), 7.18 (2H, d, *J* = 8.9, CH aromatic), 3.90 (3H, s, OCH_3), 3.89 (3H, s, OCH_3)

δ_C: (DMSO-d_6) 52.35 (C1); 55.73 (C11); 114.76 (C9); 122.36 (C5) ; 125.05 (C8); 130.48 (C4); 130.86 (C3); 146.21 (C7); 154.61 (C6); 162.68 (C10); 165.66 (C2).

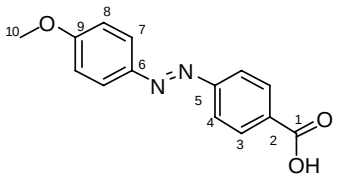
LCMS: found 271.1 calculated for $C_{15}H_{15}N_2O_3^+$ 270.1

ν_{max} (KBr): 2951, 1717, 1603, 1500 $cm.^{-1}$

m.p. 163-165 ^oC (lit value: 170 – 171^oC)[95]

Microanalysis Calcd. For $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.39; H, 5.21; 10.03

4-[(*E*)-(4-Methoxyphenyl)diazenyl]benzoic acid (138-B)



Methyl 4-[(*E*)-(4-methoxyphenyl)diazenyl]benzoate (**138-A** 0.12 g, 0.44 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4 mL) and added to this solution. This suspension was heated to 80 ^{o}C with stirring for 5h. The solution was then acidified using dilute hydrochloric acid causing the precipitation of an orange solid. This was collected by filtration and dried under reduced pressure to give the product, **138-B**, as an orange solid.

Yield: 0.1g; 89%.

δ_H (DMSO-d_6): 8.13 (d, 2H, J = 8.6, CH aromatic), 7.91 – 7.95 (m, 4H, CH aromatic), 7.18 (2H, d, J = 8.7, CH aromatic), 3.92 (3H, s, OCH_3).

δ_C: (DMSO-d_6) 55.69 (C10); 114.71 (C8); 122.19 (C4) ; 125.97 (C7); 130.55 (C3); 132.16 (C2); 146.19 (C6); 154.411 (C5); 162.57 (C9); 166.73 (C1);

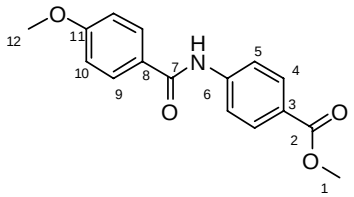
LCMS: found 257.1 calculated for $C_{14}H_{13}N_2O_3^+$ 257.09

ν_{max} (KBr): 3446, 2844, 1683, 1598, 1506, 1427 cm^{-1}.

m.p. > 230^{o}C (lit value: 256 – 258^{o}C)[96]

Microanalysis Calcd. For $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 64.89; H, 4.36; N, 9.99.

Methyl 4-[(4-methoxybenzoyl)amino]benzoate (139-A)



4-Methoxybenzoyl chloride (0.2 g, 1.17 mmol) was dissolved in dichloromethane (5 mL, dry) to which a solution of methyl 4-aminobenzoate (0.18 g, 1.17 mmol) in dichloromethane (5 mL, dry) was added followed by *N*-methylmorpholine (0.25 mL, 2.34 mmol). The solution was then stirred at room temperature for 3 hours. Sodium hydroxide solution (10% w/v in H_2O, 20 mL) was then added to the solution, the resulting dichloromethane layer was removed, dried ($MgSO_4$), and the solvent removed under reduced pressure to give a pale brown solid. This solid was purified using silica gel column chromatography (R_F = 0.30 30% ethyl acetate/n-hexane). Removal of the solvents gave the dimer, **139-A**, as a brown solid.

Yield: 0.2g, 60%.

δ_H (DMSO-d_6): 7.97 – 7.98 (2H, m, CH aromatic), 7.93 – 7.95 (4H, m, CH aromatic), 7.06 – 7.08(2H, m, CH aromatic); 3.82 (3H, s, OCH_3), 3.84 (3H, s, OCH_3).

δ_C: (DMSO-d_6) 51.8 (C1); 55.4 (C12); 113.6 (C10); 119.4 (C5); 123.9 (C3); 126.4 (C8); 129.7 (C9); 130.1 (C4); 143.8 (C6); 162.1 (C11); 165.3 (C7), 165.8 (C2).

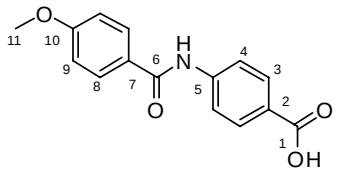
HRCIMS: found 286.1079 calculated for $C_{16}H_{16}NO_4$ $(M+H)^+$ 286.1080.

ν_{max} (KBr): 3250, 1716, 1599, 1532 cm^{-1}.

m.p. 170-172^{o}C (lit value: 205 – 206^{o}C)[97]

Purity by HPLC: 97 %

4-[(3-methoxybenzoyl)amino]benzoic acid (139-B)



Methyl 4-[(4-methoxybenzoyl)amino]benzoate **139-A** (0.10 g, 0.35 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.02 g, 0.92 mmol) was dissolved in water (4 mL) and added to this. This suspension was heated to 80^{o}C and stirred for 5h. The solution was then acidified using dilute hydrochloric acid causing the precipitation of an orange solid. This was collected by filtration and dried under reduced pressure to give the required product, **139-B**.

Yield: 0.08; 84%.

δ_H (DMSO-d_6): 10.32 (1H, s, NH), 7.98 (2H, d, J = 8.6, CH aromatic), 7.85 – 7.89 (4H, m, CH aromatic), 7.07 (2H, d, J = 8.6); 3.84 (3H, s, OCH_3).

δ_C: (DMSO-d_6) 55.4 (C11); 113.6 (C9); 119.3 (C4); 126.6 (C2); 126.9 (C7); 129.7 (C8); 130.1 (C3); 143.9 (C5); 162.1 (C10); 165.3 (C6), 165.8 (C1).

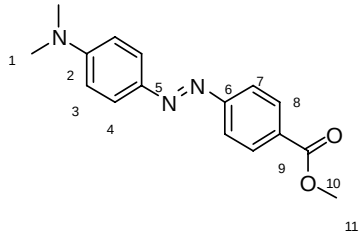
HRFABMS: found 272.0918 calculated for $C_{15}H_{14}O_4N$ $(M+H)^+$ 272.0917.

m.p. >230^{o}C (lit value: 305^{o}C)[98]

Microanalysis. Calc. For $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.16. Found: C, 65.47; H, 4.95; N, 5.13.

Purity by HPLC: 94 %

Methyl 4-{(*E*)-[4-(dimethylamino)phenyl]diazenyl}benzoate (141)[99]



Methyl 4-aminobenzoate (0.38 g; 2.5 mmol) was dissolved in hydrochloric acid solution (20% of 12M hydrochloric acid in water; 20 mL). This mixture was cooled to 0 ^{o}C using an ice/salt bath with vigorous stirring. Sodium nitrite (0.17 g; 2.5 mmol) was then dissolved in water (1 mL) and added dropwise to the above solution. This solution was then stirred at 0 ^{o}C for 30min to allow formation of the diazonium salt to occur. During this time, *N,N*-dimethylaniline (0.3g; 2.5 mmol) dissolved in methanol (5 mL) and sodium hydroxide (10% soln. in water; 20mL) was cooled to 0^{o}C. This solution was then added dropwise to the diazonium salt solution. The solution was then taken to pH 6 with the addition of 10% sodium hydroxide solution. Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL), dried ($MgSO_4$), then concentrated under reduced pressure to give a brown solid. The brown solid was purified by flash column chromatography (10% EtOAc in hexane as eluant; product identified by strong red colour on the column). Removal of the solvents gave the product **141** as a red solid.

Yield: 0.42 g; 60%.

δ_H : ($CDCl_3$) 8.16 (2H, dd, J = 6.8, J = 1.7, CH aromatic, H^4); 7.86 - 7.92 (4H, m, CH aromatic); 6.78 (2H, d, J = 9.2, CH aromatic, H^7); 4.14 (3H, s, CH_3); 3.11 (6H, s, $(CH_3)_2$).

δ_C: (DMSO-d_6) 35.9 (C1); 47.2 (C11); 107.2 (C3); 117.7 (C7); 121.2 (C4); 125.7 (C8); 126.2 (C9); 139.4 (C5); 148 (C2); 151.6 (C6); 162.5 (C10).

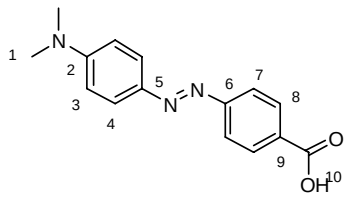
HREIMS: found 283.1320 calculated for $C_{16}H_{17}O_2N_3^+$ 283.1321.

ν_{max} (KBr): 3358; 2905; 1710; 1606 cm^{-1}.

m.p: 183-184 ^oC.

Microanalysis Calcd. For. $C_{16}H_{17}O_2N_3$: C, 67.83; H, 6.05; N, 14.83. Found: 66.92; H, 5.7, N, 13.47

4-{(*E*)-[4-(Dimethylamino)phenyl]diazenyl}benzoic acid (142)



The foregoing ester **141** (0.2 g; 0.7 mmol) was dissolved in methanol (2 mL), to this was added a solution of lithium hydroxide (0.025 g; 1.2 equiv) in water (10 mL) causing the ester to precipitate out of solution. The solution was then heated to 70^{o}C and stirred for 24 hours during which time a strong red colour was produced. TLC analysis of the mixture showed 100% conversion to the lithium salt of the acid. The solution was then acidified to pH 6 using hydrochloric acid (10% solution in water). Ethyl acetate (100 mL) was added to this mixture, the resulting organic layer was removed, washed with brine (1 x 50 mL) and sat. sodium bicarbonate solution (2 x 50 mL) and dried ($MgSO_4$). Removal of the solvents gave the required diazo compound, **142**, as a red solid.

Yield: 0.1g; 53 %.

δ_H: (DMSO-d_6) 8.08 (2H, d, J = 8.8, CH aromatic, H^3); 7.81 - 7.84 (4H, m, CH aromatic); 6.86 (2H, d, J = 9.2, CH aromatic, H^7); 3.08 (6H, s, $(CH_3)_2$).

δ_C: (DMSO-d_6) 40.4 (C1), 112.4 (C3); 122.5 (C7); 126.1 (C4); 131.4 (C8); 131.7 (C9); 143.5 (C5); 153.88 (C2); 155.9 (C6); 168.2 (C10).

HREIMS: found 269.1162 calculated for $C_{15}H_{15}O_2N_3^+$ 269.1164.

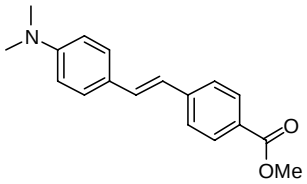
ν_{max} (KBr): 3374, 3076, 2653, 1681, 1597, 1521 cm^{-1}.

m.p: >230^oC (lit value: 271 – 273^oC)[100]

Microanalysis Calcd. For $C_{15}H_{15}O_2N_3$: C, 66.9; H, 5.61; N, 15.6. Found: C, 64.49; H, 4.72; 13.47

Purity by HPLC: 95 %

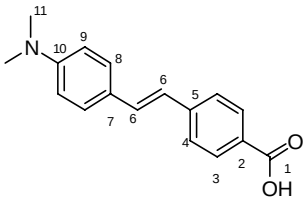
Methyl 4-{(*E*)-2-[4-(dimethylamino)phenyl]ethenyl}benzoate (143)[93]



Diethyl (4-(methoxycarbonyl)phenyl)methylphosphonate **121** (0.1 g; 0.35 mmol) was dissolved in THF (dry, 5 mL) and the solution was cooled to 0 ^oC. Sodium hydride (0.13 g; 8 equiv) was then added slowly in small portions to this solution which was then stirred for 20 min. 4-(Dimethylamino)benzaldehyde (0.052 g; 0.35 mmol) was dissolved in THF(dry, 3 mL) and added dropwise to this solution. The resulting mixture was stirred at room temperature for 4 h. The reaction was then quenched with water (10 mL) causing a white solid to precipitate. The precipitate was collected by filtration and dried under reduced pressure.

LRMS: 282 and 268 (mixture of product **143** and carboxylic acid **144**)

4-{(*E*)-2-[4-(dimethylamino)phenyl]ethenyl}benzoic acid (144)



Methyl 4-{(*E*)-2-[4-(dimethylamino)phenyl]ethenyl}benzoate **143** (0.045 g, 0.15 mmol) was dissolved in methanol (5mL) and a solution of lithium hydroxide (0.01

g, 0.92 mmol) water (4 mL) and added. The resulting suspension was heated to 80 ^{o}C and stirred for 5h. The solution was then acidified using dilute hydrochloric acid causing the product, **144**, to precipitate as a brown solid, which was collected by filtration and dried under reduced pressure

Yield:0.034 g, 85%.

δ_H (DMSO-d_6): 7.9 (2H, d, J = 7.8, H^8), 7.64 (2H, d, J = 7.4, H^3), 7.49 (2H, d, J = 7.4, H^4), 7.32 (1H, d, J =16.8, CH=CH), 7.09 (1H, d, J = 16.8, CH=CH), 6.83 (2H, s (broad), H^9), 2.96 (6H, s, $(CH_3)_2$).

δ_C: (DMSO-d_6) 38.8(C11); 125.7 (C7); 125.8 (C9); 127.8 (C8); 127.9 (C4); 128.6 (C2); 129.6 (C3); 129.7 (C6); 130.9 (C5); 142.03 (C10); 167.1 (C1).

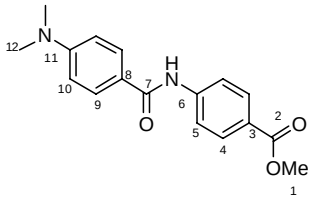
HRFABMS: found 268.1341 calculated for $C_{17}H_{18}NO_2$ $(M+H)^+$ 268.1338

ν_{max} (KBr): 3429, 2925, 1681 1595cm^{-1}.

m.p. >230 ^{o}C. (lit value: 330^{o}C)[101]

Purity by HPLC: 96 %

Methyl 4-{[4-(dimethylamino)benzoyl]amino}benzoate (145)[102]



4-Dimethylaminobenzoyl chloride (0.2 g, 1.1 mmol) was dissolved in dichloromethane (5 mL, dry) to which a solution of methyl 4-aminobenzoate (0.16 g, 1.11 mmol) in dichloromethane (5 mL, dry) was added followed by *N*-Methylmorpholine (0.25 mL, 2.34 mmol). The solution was then stirred at room temperature for 3 h. A white solid precipitate was formed during this time. This solid was filtered off and purified using silica gel column chromatography (R_F = 0.6 30% ethyl acetate/*n*-hexane) to give the product, **145**, as a white solid.

Yield: 0.1 g, 30%.

δ_H (DMSO-d_6): 10.26 (1H, s, NH), 7.90 - 7.97 (6H, m, CH aromatic), 6.83 – 6.86 (2H, m, CH aromatic), 3.8 (3H, s, CH_3), 3.05 (6H, s, $(CH_3)_2$).

δ_C: (DMSO-d_6) 38.8 (C12);51.8 (C1);110.7 (C10);119.2 (C5);120.4 (C8);123.5 (C3);129.3 (C9); 129.9 (C4); 144.3 (C6);152.6 (C11);165.5 (C7);165.8 (C2).

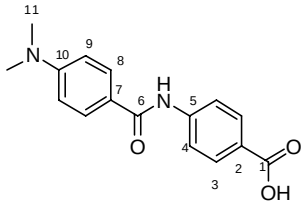
HRCIMS: found 299.1390 calculated for $C_{17}H_{19}N_2O_3$ $(M+H)^+$ 299.1396.

ν_{max} (KBr): 3336, 1711, 1655, 1609, 1517 cm^{-1}.

m.p. 208-210 ^oC.

Purity by HPLC: 94 %

4-{[4-(Dimethylamino)benzoyl]amino}benzoic acid (147)



Methyl 4-{[4-(dimethylamino)benzoyl]amino}benzoate **145** (0.045 g, 0.15 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.01 g, 0.92 mmol) was dissolved in water (4 mL) and added to this solution. The resulting suspension was heated to 80 ^oC and stirred for 5h. The solution was then acidified using dilute hydrochloric acid causing the product, **147**, to precipitate as a brown solid which was collected by filtration and dried under reduced pressure.

Yield: 0.036 g, 85 %.

δ_H (DMSO-d_6): 10.12 (1H, s, NH), 7.86 – 7.89 (6H, m, CH aromatic), 6.86 (2H, d, *J* = 8.8, CH aromatic), 3.00 (6H, s, $N(CH_3)_2$).

δ_C: (DMSO-d_6) 38.8 (C11); 111.3 (C9); 119.2 (C6); 119.3 (C7); 124.7 (C2); 129.4 (C8); 130.1 (C3); 143.8 (C5); 152.1 (C10); 165.4 (C6); 166.9 (C1).

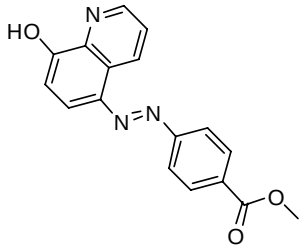
HRFABMS: found 285.1242 calculated for $C_{16}H_{17}N_2O_3$ $(M+H)^+$ 285.1239.

ν_{max} (KBr): 3341, 2561, 1675, 1606, 1502 cm^{-1}.

m.p. >230^oC.

Purity by HPLC: 94 %

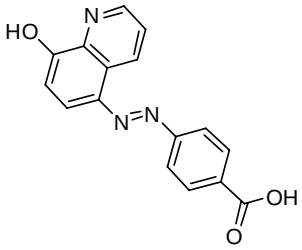
Methyl 4-[(*E*)-(8-hydroxy-5-quinolinyl)diazenyl]benzoate (148)



8-Hydroxyquinoline **149** (0.23 g; 1.8 mmol) was dissolved in acetic acid (glacial, 5 mL); methyl 4-nitrosobenzoate (0.3 g; 1.8 mmol) was then dissolved in acetic acid (glacial, 5 mL) and added to this solution. The mixture was then heated to reflux and stirred under nitrogen for 24 h. Acetic acid was the removed under reduced pressure and the resulting residue was partitioned between ethyl acetate and aqueous sodium hydroxide solution (10% w/v). The resulting organic layer was separated, dried ($MgSO_4$), and concentrated under reduced pressure to give the required diazo compound **148** as a purple residue

LRMS: 308 and 294 corresponding to product **148** and carboxylic acid **150** respectively.
This mixture was taken forward to the next stage without further purification.

4-[(*E*)-(8-Hydroxy-5-quinolinyl)diazenyl]benzoic acid (150)[103]



Methyl 4-[(*E*)-(8-hydroxy-5-quinolinyl)diazenyl]benzoate **148** (0.045 g, 0.15 mmol) was dissolved in methanol (5 mL); lithium hydroxide (0.01 g, 0.92 mmol) was dissolved in water (4 mL) and added to this solution. The resulting suspension was heated to 80 ^{o}C and stirred for 5 h. The solution was then acidified using dilute

hydrochloric acid causing the product, **150**, to precipitate as a brown solid which was collected by filtration and dried under reduced pressure.

Yield: 0.022 g; 50 %

δ_H (DMSO-d_6): 9.45 (1H, s, CH aromatic), 9.05 (1H, s, CH aromatic), 8.11 – 8.15 (6H, m, CH aromatic), 7.9 (1H, s, CH aromatic), 7.34(1H, s, broad, OH)

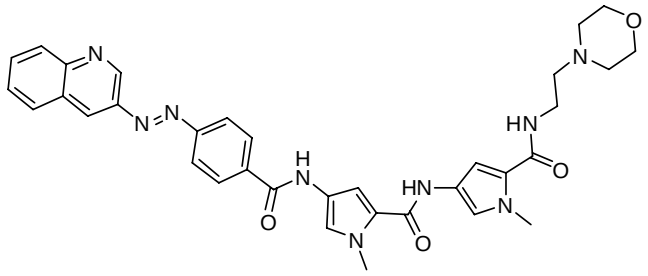
LRMS: found 294, expected 293

HRFABMS: found 294.0873 calculated for $C_{16}H_{12}O_3N_3$ $(M+H)^+$ 294.0873

(Insufficient material for complete analysis)

Minor Groove Binders

1-Methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-4-({4-[(*E*)-3-quinolinyldiazenyl]benzoyl}amino)-1*H*-pyrrole-2-carboxamide (63)



A solution of the dimer **90** (0.06 g; 0.15 mmol) in methanol (25 mL) was cooled in an ice/ salt bath to 0 ^oC with stirring under a nitrogen atmosphere. To this was added palladium (10 % wt in activated carbon; 25 mg) as catalyst slowly in small portions. The reaction vessel was evacuated of air under vacuum then filled with hydrogen from a cylinder. The reaction mixture was then sealed and left to stir vigorously for 3 hours. After 3 hours of stirring the reaction mixture was removed from stirring and filtered under suction through keiselguhr to remove any solid material from the liquid. The liquid was then collected and the volatile methanol was removed under reduced pressure (care was taken to ensure the resulting solid was completely free of solvent) to give compound **119**. This material was use in the next stage without purification or analysis. This aminopyrrole dimer was then

dissolved in dry DMF (1 mL) to which was added HBTU (0.11 g; 0.3 mmol; 2 equiv), *N*-methylmorpholine (0.025mL; 0.45mmol; 3equiv) and the dimer **127** (0.04 g; 0.15 mmol). The reaction was stirred until all the solid material had dissolved. The reaction was stoppered and left overnight without stirring. Purification was then carried out directly on the reaction mixture using HPLC. Collection of appropriate fractions and evaporation of the solvent under reduced pressure gave the required product, **63,** as an orange solid.

Purity by HPLC: > 95%.

Yield: 0.033 g; 35%.

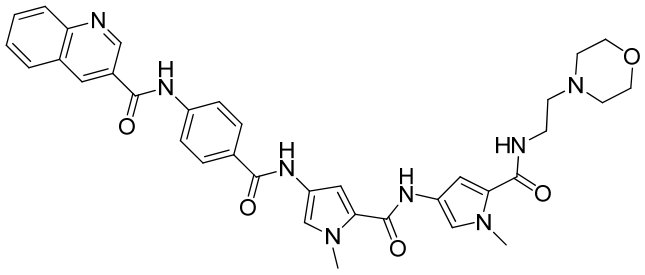
δ_H (DMSO-d_6) 10.57 (1H, s, NH); 10.0 (1H, s, NH); 9.46 (1H, d, *J* = 2.4, CH aromatic); 8.94 (1H, d, *J* = 2.4, CH aromatic); 8.28 – 8.29 (1H, m, NH); 8.22 - 8.27 (2H, m, CH aromatic); 8.10 – 8.16 (4H, m, CH aromatic); 7.9 – 7.94 (1H, m, CH aromatic); 7.74 – 7.78 (1H, m, CH aromatic); 7.37 (1H, d, *J* = 1.7, CH aromatic); 7.23 (1H, d, *J* = 1.8, CH aromatic); 7.16 (1H, d, *J* = 1.8, CH aromatic); 7.01 (1H, d, *J* = 1.7 CH aromatic); 3.99 – 4.02 (2H, m, CH_2); 3.92 (3H, s, CH_3); 3.89 (3H, s, CH_3); 3.55 – 3.70 (6H, m, 3 x CH_2); 3.26 -3.29 (2H, m, CH_2); 3.12 -3.16 (2H, m, CH_2).

HRFABMS: found 634.2889 calculated for $C_{34}H_{36}O_4N_9$ $(M+H)^+$ 634.2890.

ν_{max} (KBr): 3394, 1678, 1554, 1464 cm^{-1}.

m.p: No discernible melting point obtained.

_N_-[4-({[1-methyl-5-({[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1_H_-pyrrol-3-yl]amino}carbonyl)-1_H_-pyrrol-3-yl]amino}carbonyl)phenyl]-3-quinolinecarboxamide (64)[72]



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **129** (0.03 g; 0.1 mmol) was carried out according to the procedure used for compound **63.** The reaction mixture was purified by HPLC to give the product, **64**, as a white solid.

Purity by HPLC: > 95%.

Yield: 0.01g, 15%.

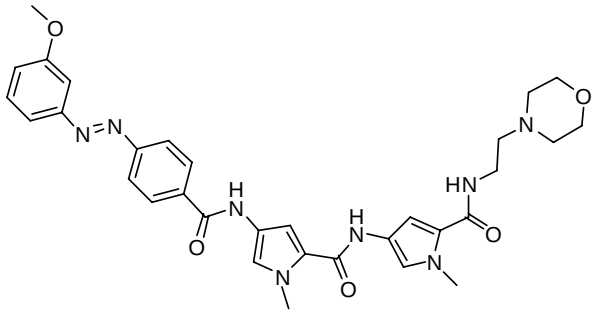
δ_H (DMSO-d_6): 10.87 (1H, s, NH); 10.28 (1H, s, NH), 9.98 (1H, s, NH), 9.39 (1H, d, *J* = 2.4, CH aromatic), 9.01 (1H, d, *J* = 1.6, CH aromatic), 8.20 – 8.22 (1H, m, NH), 8.12 – 8.19 (2H, m, CH aromatic), 7.80 – 7.90 (5H, m, CH aromatic), 7.75 – 7.76 (1H, m, CH aromatic), 7.33 (1H, d, *J* = 1.8, CH pyrrole), 7.22 (1H, d, *J* = 1.8, CH pyrrole), 7.12 (1H, d, *J* = 1.6, CH pyrrole), 7.0 (1H, d, *J* = 1.6, CH pyrrole), 3.99 – 4.0 (2H, m, CH_2), 3.88 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.69 – 3.73 (2H, m, CH_2), 3.54 – 3.55 (4H, m, 2 x CH_2), 3.26 – 3.29 (2H, m, CH_2); 3.11 – 3.15 (2H, m, CH_2).

HRFABMS: found 649.2887 calculated for $C_{35}H_{37}N_8O_5$ $(M+H)^+$ 649.2902.

ν_{max} (KBr): 3421, 1676, 1530, 1436, 1406 cm^{-1}.

m.p. no discernible melting point

-({4-[(*E*)-(3-Methoxyphenyl)diazenyl]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (65)



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **133** (0.026g; 0.1 mmol) was carried out according

to the procedure used for compound **63.** The reaction was then purified by HPLC to give the product, **65**, as an orange solid.

Purity by HPLC: > 95%.

Yield: 0.021g (34%).

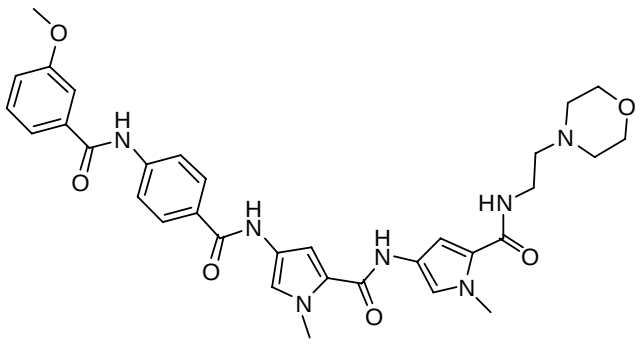
δ_H (DMSO-d_6): 10.56 (1H, s, NH), 10.03 (1H, s, NH) 8.20 – 8.26 (1H, m, NH) 8.2 (2H, d, *J* = 8.4, CH aromatic), 8.03 (2H, d, *J* = 8.4, CH aromatic), 7.53 - 7.57 (2H, m, CH aromatic), 7.46 (1H, s, CH pyrrole), 7.37 (1H, s, CH pyrrole), 7.21 – 7.24 (2H, m, CH aromatic), 7.15 (1H, s, CH pyrrole), 7.01 (1H, s, CH pyrrole), 4.00 - 4.01 (2H, m, CH_2), 3.89 (3H, s, CH_3), 3.88 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.67 – 3.70 (2H, m, CH_2), 3.53 -3.57 (4H, m, 2 x CH_2), 3.26 -3.28 (2H, m, CH2); 3.13 – 3.17 (2H, m, CH_2)

HRFABMS: found 613.2899 calculated for $C_{32}H_{37}N_8O_5$ $(M+H)^+$ 613.2887.

ν_{max} (KBr): 3414, 3190, 1678, 1600, 1526, 1436 cm^{-1}.

m.p. no discernible melting point.

4-({4-[(3-Methoxybenzoyl)amino]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (66)[72]



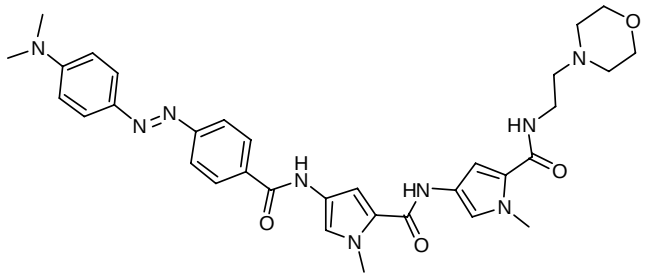
Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **136** (0.026g; 0.1mmol) was carried out according to the procedure used for compound **63.** The reaction mixture was then purified by HPLC to give the product, **66**, as a white solid.

Purity by HPLC: > 95%.

Yield: 12 mg, 19%.

δ_H (DMSO-d_6): 10.45 (1H, s, NH), 10.24 (1H, s, NH), 9.97 (1H, s, NH), 8.25 (1H, s, NH), 7.91 – 7.98 (4H, m, CH aromatic), 7.47 – 7.56 (3H, m, CH aromatic), 7.32 (1H, d, J = 1.6, CH pyrrole), 7.20 - 7.22 (2H, m, CH aromatic), 7.11 (1H, d, J = 1.6, CH pyrrole), 7.00 (1H, d, J = 1.6, CH pyrrole), 4.00 – 4.03 (2H, m, CH_2), 3.88 (3H, s, CH_3), 3.86 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.64 – 3.72 (2H, m, CH_2), 3.54 – 3.56 (4H, m, 2 x CH_2), 3.25 – 3.29 (2H, m, CH_2), 3.12 – 3.16 (2H, m ,CH_2)
HRFABMS: found 628.2879 calculated for $C_{33}H_{38}N_7O_6$ $(M+H)^+$ 628.2884.
ν_{max} (KBr): 3421, 1673, 1592, 1525 $cm.^{-1}$.
m.p. no discernible melting point.

4-[(4-{(*E*)-[4-(Dimethylamino)phenyl]diazenyl}benzoyl)amino]-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (67)

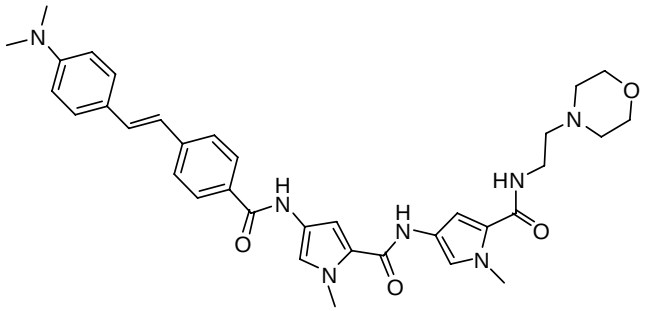


Hydrogenation of compound **90** (0.06 g; 0.14 mmol) to compound **119** followed by HBTU coupling with compound **142** (0.04 g; 0.14 mmol) was carried out according to the procedure used for compound **63**. Purification was then carried out by HPLC directly on the reaction mixture to give the product, **67**, as a red solid.

Purity by HPLC: >95 %.
Yield: 0.035 g; 40 %.
δ_H: (DMSO-d_6) 10.43 (1H, s, NH); 9.98 (1H, s, NH); 8.23 (1H, t, J = 6.1, NH); 8.1 (2H, d, J = 8.6, CH aromatic); 7. 22 - 7.88 (4H, m, CH aromatic); 7.34 (1H, s, CH pyrrole); 7.21 (1H, s, CH pyrrole); 7.13 (1H, s, CH pyrrole); 7.0 (1H, s, CH pyrrole); 6.8 (2H, d, J = 9.3, CH aromatic), 3.99 – 4.02 (2H, m, CH_2), 3.88 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.54 – 3.71 (6H, m, 3 x CH_2), 3.27 – 3.29 (2H, m, CH_2), 3.12 – 3.15 (2H, m, CH_2), 3.09 (6H, s, $(CH_3)_2$).
HRFABMS: found 626.3204 calculated for $C_{33}H_{40}O_4N_9$ $(M+H)^+$ 626.3203.
ν_{max} (KBr): 3422, 2927, 1681, 1647, 1596 cm^{-1}.

m.p: No discernible melting point obtained.

4-[(4-{(*E*)-2-[4-(Dimethylamino)phenyl]ethenyl}benzoyl)amino]-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (68)



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **144** (26.8mg; 0.1 mmol)was carried out according to the procedure used for compound **63**. The reaction mixture was purified by HPLC to give the product, **68**, as a brown solid.

Purity by HPLC: >95 %.

Yield: 29 mg, 46 %.

δ_H (DMSO-d_6): 10.35 (1H, s, NH), 10.03 (1H, s, NH), 10.00 (1H, s, broad, TFA), 8.24 – 8.27 (1H, m, NH), 7.94 (2H, d, *J* = 8.2, CH aromatic), 7.92 (2H, d, *J* = 8.2, CH aromatic), 7.47 – 7.49 (2H, m, CH aromatic), 7.00 – 7.42 (5H, m), 6.77 (2H, d, J = 8.8, CH aromatic), 6.57 (1H, s, CH aromatic), 4.02- 4.04 (2H, m, CH_2), 3.9 (3H, s, CH_3), 3.88 (3H, s, CH_3), 3.67 – 3.73 (2H, m, CH_2), 3.53 – 3.57 (4H, m, 2 x CH_2), 3.26 -3.30 (2H, m, CH_2), 3.11 – 3.15 (2H, m, CH_2), 2.96 (6H, s, $(CH_3)_2$).

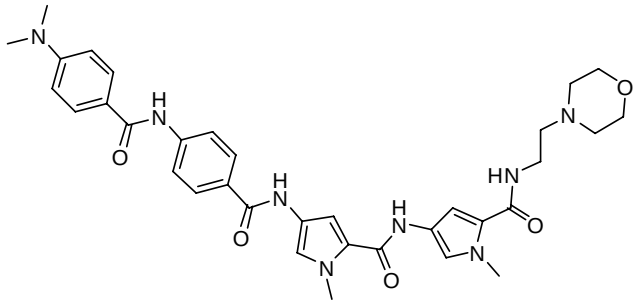
LCMS: found 624 calculated for $C_{35}H_{41}N_7O_4$ 623.

HRFABMS: found 624.0345 calculated for $C_{35}H_{42}N_7O_4$ $(M+H)^+$ 624.0320

ν_{max} (KBr): 3421, 3330, 1678, 1594, 1524, 1434, 1400 cm^{-1}.

m.p. no discernible melting point.

4-[(4-{[4-(Dimethylamino)benzoyl]amino}benzoyl)amino]-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (69)



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **147** (0.027 g; 0.1mmol) was carried out according to the procedure used for compound **63**. The reaction mixture was purified by HPLC to give the product, **69**, as a brown solid.

Purity by HPLC: >95 %. Yield: 0.012 g, 19%.

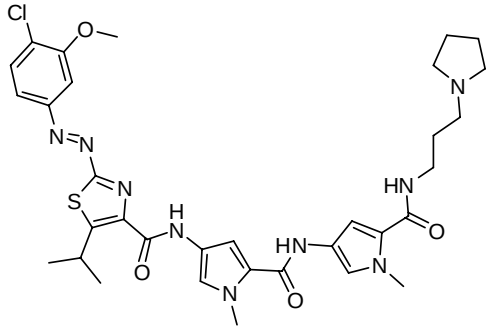
δ_H (DMSO-d_6): 10.19 (1H, s, NH), 10.06 (1H, s, NH), 9.96 (1H, s, NH), 8.23 (1H, s, NH), 7.88- 7.91 (6H, m, CH aromatic), 7.31 (1H, s, CH pyrrole), 7.21 (1H, s, CH pyrrole), 7.10 (1H, s, CH pyrrole), 7.00 (1H, s, CH pyrrole), 6.7 (2H, d, J = 8.8, CH aromatic), 3.99 - 4.0 (2H, m, CH_2), 3.87 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.64 -3.72 (2H, m, CH_2), 3.54 - 3.58 (4H, m, 2 x CH_2), 3.26 – 3.29 (2H, m, CH_2), 3.11 -3.15 (2H, m, CH_2), 3.01 (6H, s, $(CH_3)_2$).

HRFABMS: Found 641.3197 calculated for $C_{34}H_{41}N_8O_5$ $(M+H)^+$ 641.3200

ν_{max} (KBr): 3414, 2963, 1650, 1604, 1536, 1437, cm^{-1}.

m.p. no discernible melting point

2-[(*E*)-(4-chloro-3-methoxyphenyl)diazenyl]-*N*-[5-({[5-({[3-(pyrrolidin-1-y)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-5-isopropyl-1,3-thiazole-4-carboxamide (94)



A solution of the dimer **91** (20 mg; 0.05 mmol) in methanol (25 mL) was cooled in an ice/salt bath to 0^{o}C with stirring under a nitrogen atmosphere. To this was added palladium (10 % wt in activated carbon; 10 mg) as catalyst slowly in small portions. The reaction vessel was evacuated of air under vacuum, then filled with hydrogen from a cylinder. The reaction mixture was then sealed and left to stir vigorously for 3 h. The reaction mixture was then filtered under suction through keiselguhr to remove any solid material from the liquid. The liquid was then collected and the volatile methanol was removed under reduced pressure (care was taken to ensure the resulting solid was completely free of solvent) to give the corresponding amine **92**. This material was use in the next stage without purification or analysis. The solid material from the previous step was dissolved in dry DMF (1.5 mL), to this was added HBTU (0.038 g; 0.1 mmol; 2equiv), *N*-methylmorpholine (0.015 mL; 0.15 mmol; 3equiv) and the thiazole dimer **93** (0.017 g; 1 equiv). The reaction was stirred until all the solid material had dissolved at which point a homogeneous mixture was formed. The reaction was stoppered and left overnight without stirring. Purification was carried out by HPLC directly on the reaction mixture without any work up using an acetonitrile/water (0.1% TFA) eluant. The appropriate fractions were collected and dried using a freeze drier to give compound **94** as a white solid.

Purity by HPLC: > 95%

Yield: 0.0035 g; 10 %

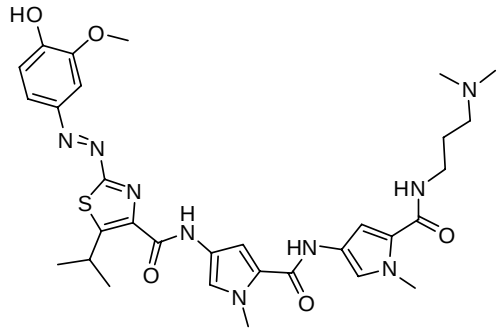
δ_H: (DMSO-d_6: Insufficient material for analysis.

ν_{max} (KBr): 1720, 1747, 1893, 3041, 3250 cm^{-1}.

m.p: No discernible melting point obtained

LCMS: found 694 calculated for $C_{33}H_{40}ClN_9O_4S$ 693.

N-[5-({[5-({[3-(dimethylamino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-2-[(E)-(4-hydroxy-3-methoxyphenyl)diazenyl]-5-isopropyl-1,3-thiazole-4-carboxamide (97)



A solution of the dimer **88** (70 mg; 0.18 mmol) in methanol (25 mL) was cooled in an ice/salt bath to 0^{o}C with stirring under a nitrogen atmosphere. To this was added palladium (10 % wt in activated carbon; 40 mg) as catalyst slowly in small portions. The reaction vessel was evacuated of air under vacuum then filled with hydrogen from a cylinder. The reaction mixture was then sealed and left to stir vigorously for 3 hours. After 3 hours of stirring the reaction mixture was removed from stirring and filtered under suction through keiselguhr to remove any solid material from the liquid. The liquid was then collected and the volatile methanol was removed under reduced pressure (care was taken to ensure the resulting solid was completely free of solvent) to give compound **95**. This material was use in the next stage without purification or analysis The solid material (compound **95**) from the previous step was dissolved in dry DMF (1.5 mL), to this was added HBTU (0.145 g; 0.4 mmol; 2 equiv), *N*-methylmorpholine (0.062 mL; 0.6 mmol; 3 equiv) and the acid **96** (0.06 g; 1 equiv). The reaction was stirred until all the solid material had dissolved. The reaction was stoppered and left overnight without stirring. Purification was carried out by HPLC directly on the reaction mixture without any work up. The appropriate fractions were collected and dried using a freeze drier to give compound **97** as an orange solid.

Purity by HPLC: >95%.

Yield: 0.025g; 21%.

δ_H: (DMSO-d_6) 10.5 (1H, s, NH); 9.9 (1H, s, NH); 8.1 (1H, s, NH); 7.70 – 7.75 (3H, m, CH aromatic); 7.33 (1H, s, CH aromatic); 7.29 (1H, s, CH aromatic); 7.16

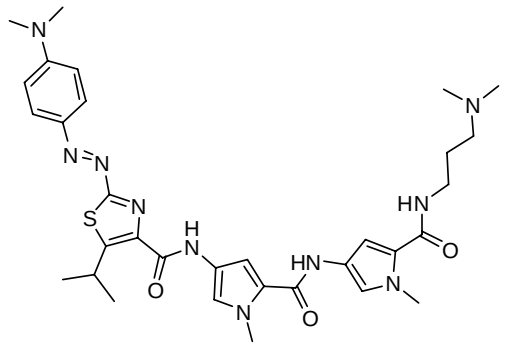
(1H, s, CH aromatic); 6.99 (1H, s, CH aromatic); 4.30 (1H, sept, J = 6.6, C<u>H</u>$(CH_3)_2$); 4.0 (3H, s, OCH_3); 3.87 (3H, s, NCH_3); 3.82 (3H, s, NCH_3); 3.20 (2H, q, J = 6.1, CH_2); 3.16 (2H, t, J = 7.0, CH_2); 2.78 (6H, s, $N(CH3)_2$); 1.81 (2H, quintet, J = 7.0, CH_2); 1.38 (6H, d, J = 6.6, $CH(C\underline{H}_3)_2$).

HRFABMS: found 650.2861 calculated for $C_{31}H_{40}O_5N_9S$ $(M+H)^+$ 650.2900.

ν_{max} (KBr): 3320, 1681, 1586 cm^{-1}.

m.p: No discernible melting point obtained.

2-{(*E*)-[4-(dimethylamino)phenyl]diazenyl}-*N*-[5-({[5-({[3-(dimethylamino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-5-isopropyl-1,3-thiazole-4-carboxamide (99)



The nitropyrrole dimer **88** (70 mg; 0.18 mmol) was hydrogenated to amine **95** using palladium (10 % wt in activated carbon; 40 mg) as described previously in the preparation of compound **97**. This material was use in the next stage without purification or analysis. The solid material (compound **95**) from the previous step was dissolved in dry DMF (1.5 mL), to this was added HBTU (0.15 g; 2 equiv), *N*-methylmorpholine (0.062 mL; 2 equiv) and the acid **98** (0.06 g; 1 equiv). The reaction was stirred until all the solid material had dissolved. The reaction was stoppered and left overnight without stirring. Purification was carried out by HPLC directly on the reaction mixture. The appropriate fractions were collected and dried using a freeze drier to give compound **99** as a red solid.

Purity by HPLC: >95%

Yield: 0.038g; 33%

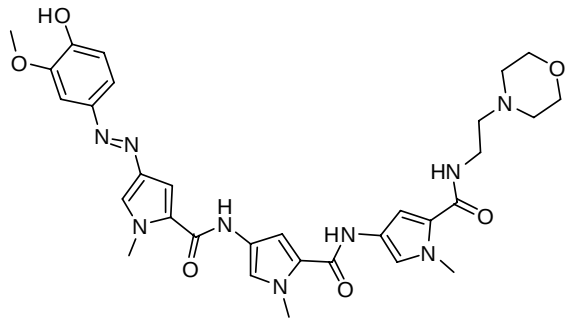
δ_H: (DMSO-d_6) 10.36 (1H, s, NH); 9.91 (1H, s, NH); 8.147 (1H, t, *J* = 6.1, NH); 7.81 (2H, d, *J* = 9.1, CH aromatic); 7.33 (1H,s, CH aromatic); 7.25 (1H,s, CH aromatic); 7.17 (1H, s, CH aromatic); 6.96 (1H, s, CH aromatic); 6.91 (2H, d, *J* = 9.2, CH aromatic); 4.3 (1H, sept, *J* = 6.7 Hz, C<u>H</u>$(CH_3)_2$); 3.92 (3H, s, NCH_3); 3.86 (3H, s, NCH_3); 3.25 (2H, q, *J* = 6.2, CH_2); 3.14 (6H,s, $N(CH_3)_2$); 3.06 (2H, t, *J* = 7.0, CH_3); 2.79 (6H, s, $N(CH_3)_2$); 1.9 (2H, quintet, *J* = 7.1, CH_2); 1.34 (6H, d, *J* = 6.7, $CH(C\underline{H}_3)_2$).

HRFABMS: found 647.3213 calculated for $C_{32}H_{43}O_3N_{10}S$ $(M+H)^+$ 647.3285.

ν_{max} (KBr): 3421, 1678, 1602, 1531cm^{-1}.

m.p: No discernible melting point obtained

4-[(*E*)-(4-hydroxy-3-methoxyphenyl)diazenyl]-1-methyl-*N*-[1-methyl-5-({[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (120)



A solution of the dimer **90** (45 mg; 0.11 mmol) in methanol (25 mL) was cooled in an ice/salt bath to 0^oC with stirring under a nitrogen atmosphere. To this was added palladium (10 % wt in activated carbon; 25 mg) as catalyst slowly in small portions. The reaction vessel was evacuated of air under vacuum then filled with hydrogen from a cylinder. The reaction mixture was then sealed and left to stir vigorously for 3 hours. After 3 hours of stirring the reaction mixture was removed from stirring and filtered under suction through keiselguhr to remove any solid material from the liquid. The liquid was then collected and the volatile methanol was removed under reduced pressure (care was taken to ensure the resulting solid was completely free of solvent) to give compound **119**. This material was use in the next stage without purification or analysis. This aminopyrrole dimer was then dissolved in dry DMF (1

mL) to which was added HBTU (0.083 g; 0.22 mmol; 2 equiv), *N*-methylmorpholine (0.025mL; 0.66mmol; 3equiv) and the dimer **118** (0.03 g; 1 equiv). The reaction was stirred until all the solid material had dissolved. The reaction was stoppered and left overnight without stirring. Purification was then carried out directly on the reaction mixture using HPLC. Collection of appropriate fractions and evaporation of the solvent under reduced pressure gave the required product, **120**, as an orange solid.

Purity by HPLC: >95%.

Yield: 0.025 g; 36%.

δ_H (DMSO-d_6): 10.23 (1H, s, NH), 9.96 (1H, s, NH), 8.23 – 8.25 (1H, m, NH), 8.00 (1H, d, *J* = 1.7, CH aromatic), 7.58 (1H, d, *J* = 8.4, CH aromatic), 7.50 – 7.52 (2H, m, CH aromatic), 7.35 (1H, d, *J* = 1.8, CH aromatic), 7.25 (1H, d, *J* = 1.7, CH aromatic), 7.01 (1H, d, *J* = 1.7, CH aromatic), 6.98 (1H, d, *J* = 1.8, CH aromatic), 6.94 (1H, d, *J* = 1.7, CH aromatic), 4.01 (3H, s, CH_3), 3.99 (3H, s, CH_3), 3.86 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.81 – 3.83 (2H, m, CH_2), 3.68 – 3.70 (2H, m, CH_2) 3.54 – 3.55 (4H, m, 2 x CH_2), 3.26 – 3.29 (2H, m, CH_2), 3.11 – 3.14 (2H, m, CH_2)

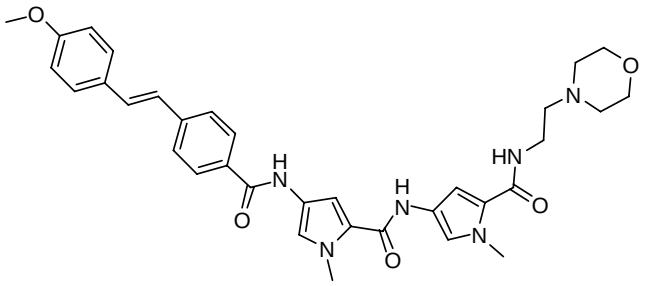
HRFABMS: found 632.2946 calculated for $C_{31}H_{38}O_6N_9$ $(M+H)^+$ 632.2945.

ν_{max} (KBR): 1715, 3240 cm^{-1}.

m.p.: No discernible melting point obtained.

(Insufficient material for complete analysis)

4-({4-[(*E*)-2-(3-Methoxyphenyl)ethenyl]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (137) – Attempted synthesis.



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **137-B** (0.0255g; 0.1 mmol) was carried out

according to the procedure used for compound **63**. The reaction mixture was purified by HPLC to give the product, **137**, as a yellow solid.

Purity by HPLC: > 95 %

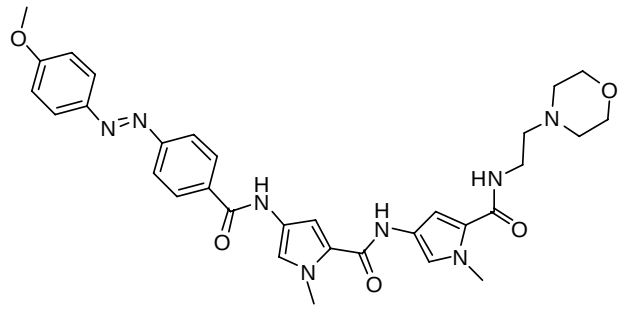
Yield: 18 mg; 30%

δ_H (DMSO-d_6): NMR data inconclusive.

HRFABMS: Found 611. 2988 calculated for $C_{34}H_{39}N_6O_5$ $(M+H)^+$ 611. 2982

m.p. no discernible melting point

4-({4-[(*E*)-(3-Methoxyphenyl)diazenyl]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (138)



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **138-B** (0.026g; 0.1 mmol) was carried out according to the procedure used for compound **63.** The reaction was purified by HPLC to give the product, **138**, as an orange solid.

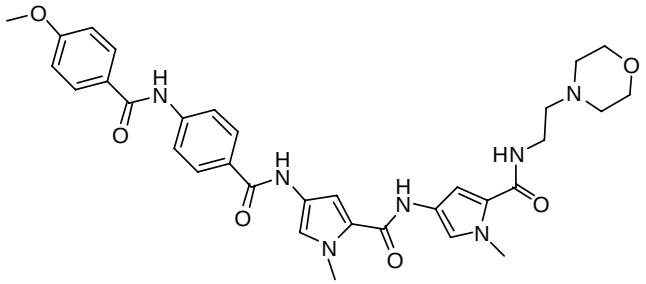
Yield: 0.024g; 39%.

δ_H (DMSO-d_6): 10.5 (1H, s, NH), 9.9 (1H, s, NH), 8.23 – 8.24 (1H, m, NH), 8.12 - 8.14 (2H, m), 7.89 - 7.96 (4H, m), 7.14 – 7.30 (3H, m, CH aromatic), 6.87 – 7.00 (3H, m, CH aromatic), 3.99 – 4.02 (2H, m, CH_2), 3.88 (3H, s, CH_3), 3.85 (3H, s, CH_3), 3.82 (3H, s, CH_3), 3.62 – 3.68 (2H, m, CH_2), 3.53 – 3.58 (4H, m, 2 x CH_2), 3.25-3.28 (2H, m, CH_2), 3.10 -3.14 (2H, m, CH_2).

HRFABMS: found 613.2886 calculated for $C_{32}H_{37}N_8O_5$ $(M+H)^+$ 613.2887.

m.p. no discernible melting point.

4-({4-[(3-Methoxybenzoyl)amino]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (139)



Hydrogenation of compound **90** (0.04 g; 0.1 mmol) to compound **119** followed by HBTU coupling with compound **139-B** (0.03 g; 0.1 mmol) was carried out according to the procedure used for compound **63.** The reaction mixture was purified by HPLC to give the product, **139**, as a pale yellow solid.

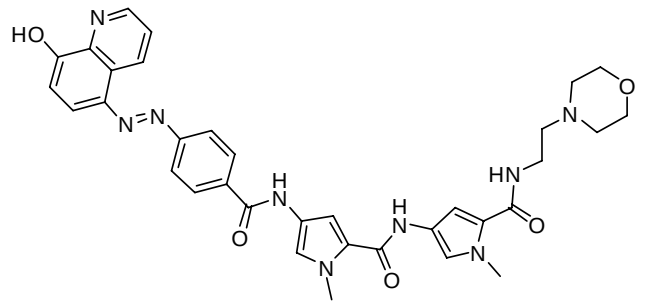
Purity by HPLC: > 95%.

Yield: 0.015 g, 24%.

δ_H (DMSO-d_6): 10.3 (1H, s, NH), 10.2 (1H, s, NH), 9.9 (1H, s, NH), 8.21 (1H, s, NH), 7.99 - 7.97 (2H, m, CH aromatic), 7.93- 7.96 (2H, m, CH aromatic), 7.89 – 7.92 (2H, m, CH aromatic), 7.31 (1H, d, J = 1.7, CH pyrrole), 7.21 (1H, d, J = 1.7, CH pyrrole), 7.05 - 7.10 (3H, m, CH aromatic), 6.99 (1H, s, CH aromatic), 3.98 – 4.0 (2H, m, CH_2), 3.86 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.82 (3H, s, CH_3), 3.65 - 3.71 (2H, m, CH_2), 3.54 – 3.58 (4H, m, 2 x CH_2), 3.26 – 3.30 (2H, m, CH_2), 3.10 – 3.15 (2H, m, CH_2)

HRFABMS: found 628.2875 calculated for $C_{33}H_{38}N_7O_6$ $(M+H)^+$ 628.2878.

4-({4-[(*E*)-(8-Hydroxy-5-quinolinyl)diazenyl]benzoyl}amino)-1-methyl-*N*-[1-methyl-5-({[2-(4-morpholinyl)ethyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide (151)



An HBTU coupling reaction was carried out as described previously between compound **119** (0.04g; 0.1mmol) and compound **150** (30 mg; 0.1mmol). The reaction mixture was purified by HPLC to give the product as a red solid.

Yield: 0.026g, 40%

Purity by HPLC: > 95 %

δ_H (DMSO-d_6): 10.51 (1H, s, NH) 10.00 (1H, s, NH), 9.37 – 9.39 (1H, m, CH aromatic), 9.0 – 9.1 (1H, m, CH aromatic), 8.22 – 8.23 (1H, m, NH), 8.05-8.17 (6H, m, CH aromatic), 7.80 - 7.84 (1H, m, CH aromatic), 7.36 (1H, d, *J* = 1.8, CH pyrrole), 7.21 (1H, d, *J* = 1.8, CH pyrrole), 7.15 (1H, d, *J* = 1.8, CH pyrrole), 7.01 (1H, d, *J* = 1.8, CH pyrrole), 4.00 – 4.03 (2H, m, CH_2), 3.90 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.56 -3.69 (6H, m, 3 x CH_2), 3.26 - 3.30 (2H, m, CH_2), 3.12- 3.16 (2H, m, CH_2).

LCMS: found 650 calculated for 650 $C_{34}H_{36}N_9O_5^+$

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