SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL CHROMOGENIC SUBSTRATES FOR THE ENHANCED DETECTION OF PATHOGENIC BACTERIA

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A thesis submitted in partial fulfillment of the requirements of the University of Sunderland for the degree of Doctor of Philosophy

This research programme was carried out in collaboration with the Freeman Hospital (Newcastle-upon-Tyne) and bioMérieux (La Balme-les-Grottes, France)

June 2010

L’important c’est pas la chute…

ACKNOWLEDGEMENTS

I would like to express the deepest and most sincere thanks to my supervisors Prof. Roz Anderson and Prof. Paul Groundwater for their constant help, support, encouragement, sharing of knowledge and infinite patience.

I also would like to thank my external supervisor Prof. John Perry for letting me use his facilities at the Freeman Hospital and for his precious help with microbiological testing.

Thanks to bioMérieux for financing this project, and thanks to the bioMérieux team, D^{r} Sylvain Orenga, Céline Roger-Dalbert, and Marie Cellier for carrying the microbiological testing.

Thanks also go to Prof. Arthur James for his advice and to his wife Gill for welcoming us into their home in the Lake District for the most enjoyable team meetings.

Special thanks go to D^{r} Andrey Zaytsev for his guidance, precious help and advice during my first year.

Thanks to the chemiSpec team, Nicolas and Andrea, for their most precious help with NMR spectroscopy, mass spectrometry and elemental analysis. Thanks to the University of Sunderland technical staff, Arun, Jeff, Joan, Mick and Barry for their help and letting me borrow equipment, glassware and chemicals. Thanks also to Norman Turner for his most precious help in ordering chemicals.

Special thanks go to Rebecca for her precious help and support.

Thanks also to all my colleagues of lab 2.06 and lab 2.10, Adam, Alice, Gabi, Linda, Lisa, Liz, Nagendra, Neil, Ning, Sam, Satya, Surresh, Pratap, Yu and Yang for making these four years very enjoyable.

Merci aussi à Benjam et à Jérôme de s'être assuré que le retour au pays se fasse toujours bien dans les règles de l'art.

Et finalement, un grand merci à mon père et à ma mère pour l'éducation qu'ils m'ont donné, je me doute que ça ne devait pas être facile tous les jours, mais bon les fruits d'un dur labeur sont là (ou presque).

ABSTRACT

The present work investigated the preparation of phenoxazinone derivatives and evaluated their performances for the detection of pathogenic bacteria.

The first method investigated the condensation of nitroaminophenol with tetrahalogenobenzoquinones; the corresponding nitrohalogenophenoxazinones were all characterised and evaluated for the detection of nitroreductase activity in a range of clinically important microorganisms. The detection of nitroreductase activity has been previously suggested for the monitoring of bacterial growth; however, nitrohalogenophenoxazinones were proven to be less suitable for this purpose than other, previously reported, nitroreductase substrates.

The second route investigated the synthesis of phenoxazinone derivatives *via* the oxidative cyclisation of diamino-dihydroxydiphenylethers and of diaminobenzoquinones. The reactive intermediates were trapped and characterised in order to rationalize the mechanism of formation of aminophenoxazinones *via* this route. 7- and 8-Aminophenoxazinones derivatives were prepared and further derivatised with β-alanine. Similarly, some nitrohalogenophenoxazinones were reduced to their corresponding aminophenoxazinones and derivatised with β-alanine.

Thirteen new chromogenic substrates were prepared, characterised and evaluated for their sensitivity to detect β-alanine aminopeptidase on agar medium; this enzyme is expressed by only three types of bacteria, the most important being *Pseudomonas aeruginosa*, a pathogen commonly known to affect cystic fibrosis sufferers. Their performance for the detection of *Pseudomonas aeruginosa* were compared to the lead compound (7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one), the substrate contained in a commercially available medium, chromID^{TM} ID *Ps. aeruginosa*. The substrates, if hydrolysed, resulted in a low colouration of the colonies when compared to the lead compound; however, 2-pentyl substituted aminophenoxazinones were found to be less toxic and had an excellent affinity for the bacterial colonies.

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SYMBOLS AND ABBREVIATIONS

calc.	calculated
cat.	catalytic
conc.	concentrated
DBU	1,8-diazabicyclo[5-4-0]undec-7-ene
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
dt	doublet of triplets
DCM	dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DNA	deoxyribonucleic acid
DME	dimethoxyethane
DMF	dimethylformamide
DMS	dimethylsulfate
DMSO	dimethylsulfoxide
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
Et_3N	triethylamine
h	hour
[H]	reduction
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
Hz	hertz
IBCF	*iso*-butylchloroformate
J	coupling constant
M	molarity
M^+	positive molecular ion
M^-	negative molecular ion
MeOH	methanol
min	minute

mL	millilitre
mol	mole
mmol	mmole
mg	milligram
m.p.	melting point
MMPP	magnesium monoperoxyphthalate
MRSA	meticillin-resistant *Staphylococcus aureus*
MW	molecular weight
$m/_Z$	mass over charge of the ion
n-BuLi	*n*-butyl lithium
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
NMP	*N*-methyl-2-pyrrolidone
[O]	oxidation
PCR	polymerase chain reaction
ppm	parts per million
q	quartet
quat.	quaternary
RNA	ribonucleic acid
s	singlet
sex.	sextuplet
T	temperature
t	triplet
td	triplet of doublets
^{t}Boc	*tert*-butoxycarbonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
U.V.	ultra-violet
v/v	volumic percentage
w/w	weight percentage
δ	NMR chemical shift
Δ	heat
ε	molar extinction coefficient

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CHAPTER ONE:

INTRODUCTION

Microorganisms are ubiquitous in our environment: they colonise and adapt to the most extreme environment (extremophiles) or live in symbiosis with other organisms, such as the human commensal bacteria *Staphylococci* (skin, 2001JID170), *Streptococci* (saliva and teeth, 1996AOB1133) or *Bacteroid*, *Eubacterium* and *Bifidobacterium* (colon, 2005CPD1047) to name a few. If the presence of such bacteria is well tolerated by, and even necessary to, humans (nutrients; immune system stimulation), the proliferation of exogenous pathogenic bacteria can lead to life-threatening conditions.

The presence of pathogenic bacteria can have disastrous consequences in hospitals, food and water, to name three highly significant areas of our health and well-being. Their identification in such domains is necessary and has become a corner stone of such areas.

The present work focuses on the development of specific chromogenic enzyme substrates allowing for the rapid detection of nosocomial bacteria. Identification using chromogenic enzyme substrates has acquired a significant impact for the detection of human pathogens. Their simplicity and reliability, shorter identification process and fewer complementary tests required, respond to the need for quick results in a hospital context. Indeed, the successful treatment and eradication of a pathogen rely on its quick and correct identification.

Many methods have been developed, and are still under development, for the accurate and rapid detection of microorganisms (1996MI455, 2000MI549). Full details of each method are out of the scope of the present work and only a brief overview of the existing and most relevant techniques will be given here. The description of techniques using fluorogenic and, more particularly, chromogenic enzyme substrates as a means of detection will be more carefully detailed with an insight into their properties, structures and mode of action. The second part of this introduction will be dedicated to the occurrence and preparation of the phenoxazin-3-one core, which is the core of the new chromogenic substrates prepared herein.

1.1 Bacterial detection

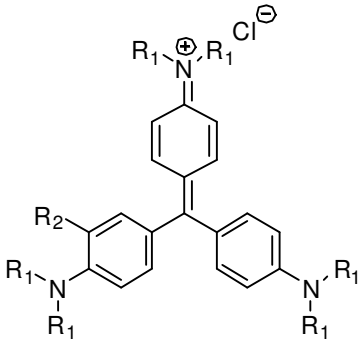
Identification of a bacterium requires quantifying a relationship based on its morphology, physiology and chemical structure (B-1993MI01). Bacteria are characterized, defined and named after the appearance of their colonies, the

morphology of a single cell and respective arrangement of several cells, their growth characteristics, their biochemistry and their reaction to inhibitory tests, the need for specific nutrients or conditions for growth, their reaction to antiserum and their genetics. The detection of a bacterium involves a series of tests allowing the observation of a determinant character allowing its relation to a previously described genus and species. It is generally admitted that no single test provides a definitive identification of an unknown microorganism. The traditional identification methods involve culture of the bacteria for enrichment and isolation of the subsequent bacterial colonies, followed by screening using a series of biochemical tests and serological confirmation (1990MI497).

1.1.1 Classical detection methods

1.1.1.1 The Gram stain

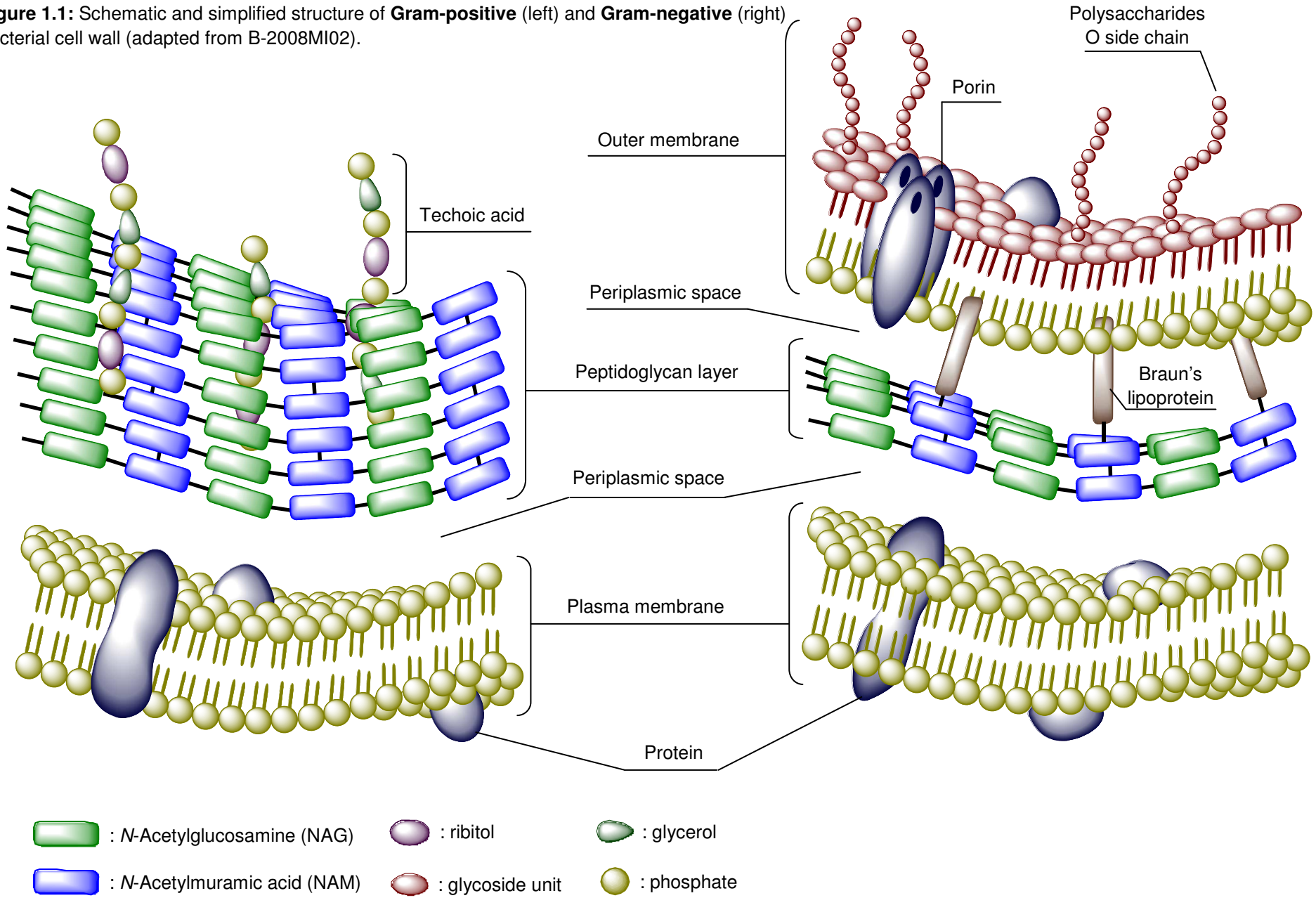
The Gram stain, developed in 1884 by Christian Gram, was the first taxonomic test of its kind allowing such an important differentiation within the bacterial kingdom. The Gram stain procedure (1884MI185) involves the use of an aqueous solution of gentian violet (crystal violet, **1**), along with a saturated solution of iodine and potassium iodide as mordant. Decolorisation of the bacterial cell is then attempted by washing with ethanol or acetone, leaving Gram-positive bacteria stained in violet. A counter stain of carbol fuschin **2** is finally applied, dying any Gram-negative cells in red. Several factors can influence the outcome of the Gram stain, such as the age of the culture and the composition of the cultivation medium (1990JAB822). Errors in determining the Gram reaction have been observed, with bacteria, known as Gram variable, showing a variable behaviour toward the Gram stain.



1, $R_1 = CH_3$, $R_2 = H$
2, $R_1 = H$, $R_2 = CH_3$

The ability of Gram-positive organisms to retain the dye more effectively than Gram-negative organisms when exposed to a solvent is related to major differences within the structure of the bacterial cell wall. Gram-positive bacterial cell walls (**Figure 1.1**) consist of a thick homogeneous layer of peptidoglycan, covalently bound to linear anionic polymeric teichoic acids (ribitol and glycerol units linked by phosphodiesters, 2002MI46S), situated outside the plasma membrane.

Figure 1.1: Schematic and simplified structure of **Gram-positive** (left) and **Gram-negative** (right) bacterial cell wall (adapted from B-2008MI02).



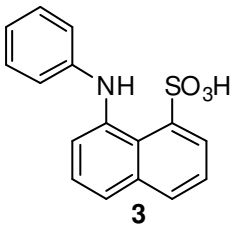
Peptidoglycan is a mesh-like polymer composed of covalently bound *N*-acetylglucosamine (NAG) and *N*-acetyl muramic acid (NAM) subunits.

The peptidoglycan layer is highly crosslinked in Gram positive organisms; crosslinking occurs *via* the carboxylic acid residues of NAM. With Gram-positive bacteria, crystal violet is thought to be retained behind the peptidoglycan layer, which pores are being shrunk upon the decolorisation process.

The Gram-negative bacterial cell wall is a relatively more complex structure (**Figure 1.1**). It consists of a thin, poorly crosslinked peptidoglycan layer recovered by an outer membrane; the coherence of this structure is ensured by Braun's lipoproteins (**Figure 1.1**). The outer membrane, which is the main self-protective structure of Gram-positive bacteria against toxic compounds, is composed of three main elements: the phospholipid layer, the lipopolysaccharide layer and the polysaccharide *O*-side chain. This highly lipophilic external structure exercises a control over the permeation of hydrophilic compounds, whereas the porin channels it contains (**Figure 1.1**) allow the permeation of small hydrophilic molecules such as nutrients (2001MI215).

With Gram-negative bacteria, the thin peptidoglycan layer and the outer membrane, which permeability is affected by ethanol, fail to retain crystal violet upon the decolorisation process, leaving the cells colourless.

Other, yet less popular, dye-based Gram differentiation methods have since been developed. The growth of bacteria on medium containing 8-anilino-1-naphthalene sulphonic acid (ANS, **3**), a hydrophobic fluorescent dye, was found to differentiate Gram-negative bacteria from Gram-positive by selective fluorescence. The hydrophobic interactions occurring upon adsorption of the dye onto the bacterial proteins, present in the lipopolysaccharide layer of the Gram-negative outer membrane, result in a dramatic increase of ANS quantum yield (1980AEM372). The lack of interaction with the outer peptidoglycan layer of Gram-negative bacteria left the latter non-fluorescent, the intrinsic quantum yield of ANS being very low in aqueous environment.



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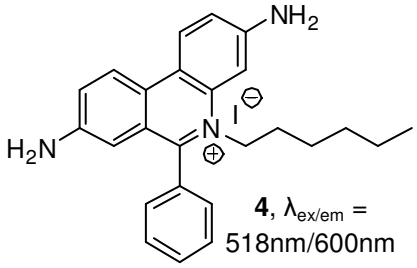
More recently, Mason *et al.* reported a live Gram stain method using two fluorescent nucleic acid binding dyes, hexidium iodide (HI, **4**) and SYTO 13 (1998AEM2681).



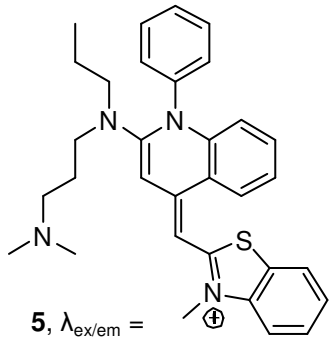
4, $\lambda_{ex/em}$ = 518nm/600nm

While Gram-positive bacteria did permeate both HI and SYTO 13, Gram-negative only permeated SYTO 13. This resulted in the observation of an orange fluorescence for Gram-positive bacteria, as the simultaneous presence of both dyes resulted in a quenching of SYTO 13 fluorescence by HI (1998AEM2681), and a green fluorescence for Gram-negative bacteria. The outer lipopolysaccharide layer is thought to be responsible for the exclusion of HI (1998AEM2681).

1.1.2 New methods of identification

1.1.2.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is defined as "a primer-mediated enzymatic amplification of specifically cloned or genomic DNA sequence" (B-2002MI03). The PCR process involves denaturation of a DNA sequence, hybridization of the primers (short oligonucleotides sequences) and polymerization of the desired sequence by a thermostable DNA polymerase, *Taq* (from the thermophile *Thermus aquaticus*). These three successive steps, which constitute a cycle, are realized in a thermal cycler, allowing each step to take place at a different, optimum temperature: 95°C (denaturation), 60°C (primer hybridization) and 72°C (polymerization). This cycle is typically repeated 20 to 40 times, at which point billions of copies of the gene sequence are available (number of cloned sequence = $2^{n\ cycles}$). Interpretation of the results is done by staining the PCR product with a phenantridium dye (analogous to **4**), followed by separation using gel electrophoresis. The recent emergence of real-time PCR can by-pass this interpretation step by monitoring the increase of fluorescence during the amplification process using double-stranded DNA specific dyes such as SYBR Green I **5**.



5, $\lambda_{ex/em}$ = 494nm/521nm

The ability to replicate a unique gene sequence has proven to be highly useful in the identification of pathogenic bacteria and PCR is becoming increasingly important as a rapid means of detection (1998JCM2810). Virtually any species-specific nucleotide sequence can be selected for amplification, but replication of gene sequence determinants of the pathogenic character of a bacterium is sometimes key to identification: the *vtx1* and *vtx2* genes encoding for the production of verotoxin, responsible for the hemorrhagic colitis linked to *E. coli* O157 : H7 infection (2010MI7), can easily discriminate *E. coli* O157 : H7 from non

virulent *E. coli* strains. The *mecA* gene, encoding for the production of penicillin-binding protein 2a (1997CMR781), is another example of useful gene marker which can discriminate meticillin-resistant *Staphylococcus aureus* (MRSA) from non-resistant *Staph. aureus* species.

The concomitant use of several pairs of primers (multiplex PCR) has been successfully applied to the simultaneous detection of some nosocomial pathogens, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Klebsiella pneumonia* (2009JMM329), and common food contaminants, *Salmonella* spp. (2009MI348, 2009MI43), *Listeria monocytogenes* (2006MI763) and *Campylobacter jejuni* (2003AEM1383).

1.1.2.2 Bacteriophages

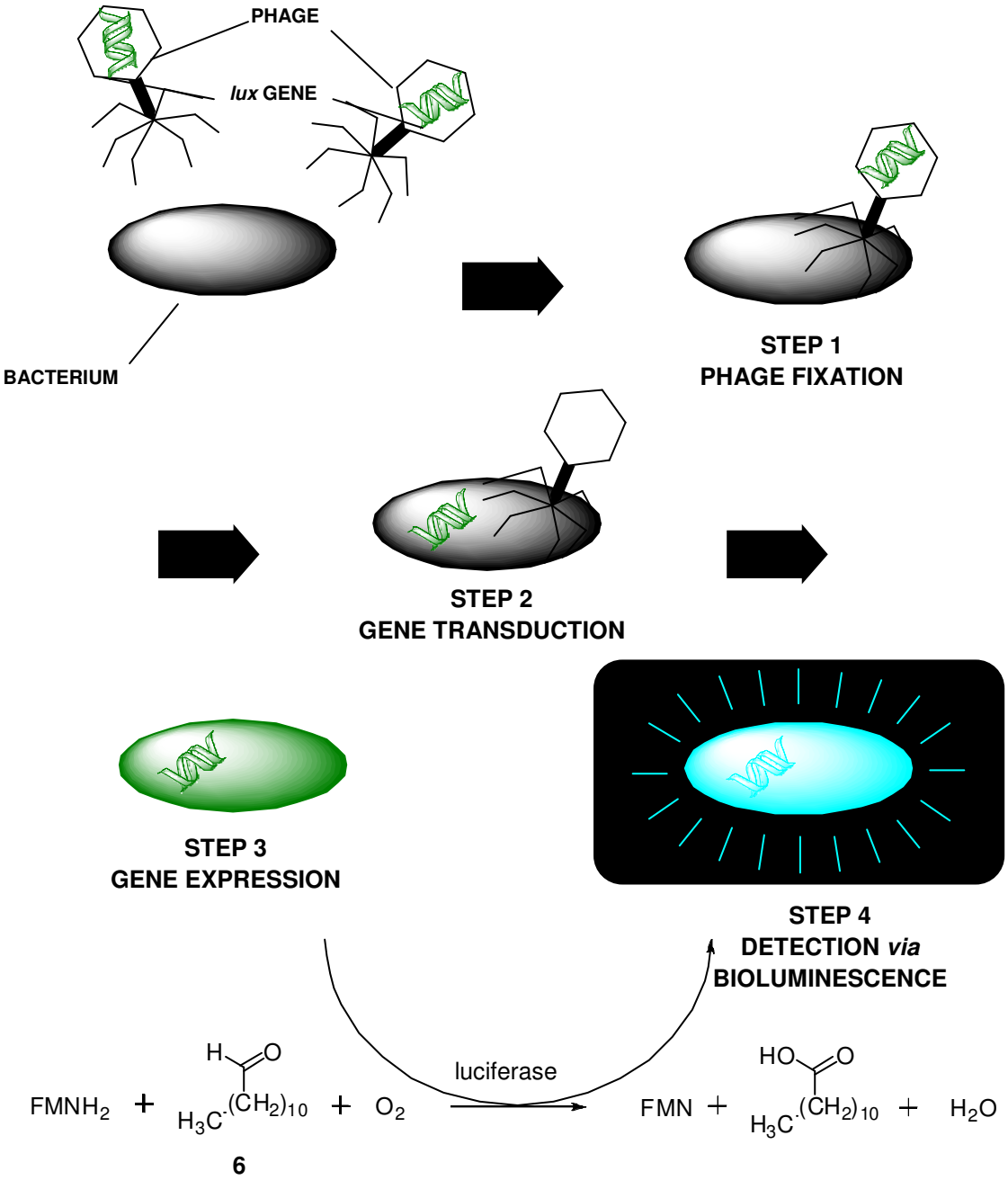
Bacteriophages or phages are viruses that infect bacteria cells only. They can recognize and bind to specific receptors on the outer membrane of bacterial cells, *i.e.* an amino acid sequence of a surface protein. The high specifity of bacteriophages for their respective host has led to the development of an original approach to bacterial detection, which was first demonstrated by Ulitzur and Kuhn (B-1987MI04).

For the purpose of pathogen detection, phages are genetically engineered to include a reporter gene, the majority being bioluminescence genes (*lux*) from naturally bioluminescent species, such as *Vibrio fischeri*. Upon infection of the bacterium by the genetically modified phage, the *lux* reporter gene is introduced along with the phage DNA into the bacterial host and then expressed (**Scheme 1.1**). As the *lux* gene encodes for the production of luciferase (2002FRI863), the infected bacterium becomes bioluminescent. The production of bacterial bioluminescence results from the aerobic luciferase-catalysed oxidation of an aliphatic aldehyde (e.g. dodecanal **6**) in the presence of reduced flavin mononucleotide ($FMNH_2$) (**Scheme 1.1**, 1991MR123). Monitoring of the resulting light emission is therefore indicative of the presence of the bacterium.

Assays using luciferase reporter phages have been reported for the detection of the following pathogens: *E. Coli* O157 : H7 (1996MI152), *Listeria monocytogenes* (1997AEM2961), *Mycobacterium tuberculosis* (2001JCM3883), *Salmonella spp.* (1996JFP908), *Staphylococcus aureus* or *Yersinia pestis* (2009JCM3889).

Bacterial detection using bacteriophages is a reliable and specific method; however, it requires the genetic engineering of specific phages, which are as yet only available

for a few common pathogens. Long assay times are usually required for the production of detectable luminous signal, especially when few cells of the pathogen are available.

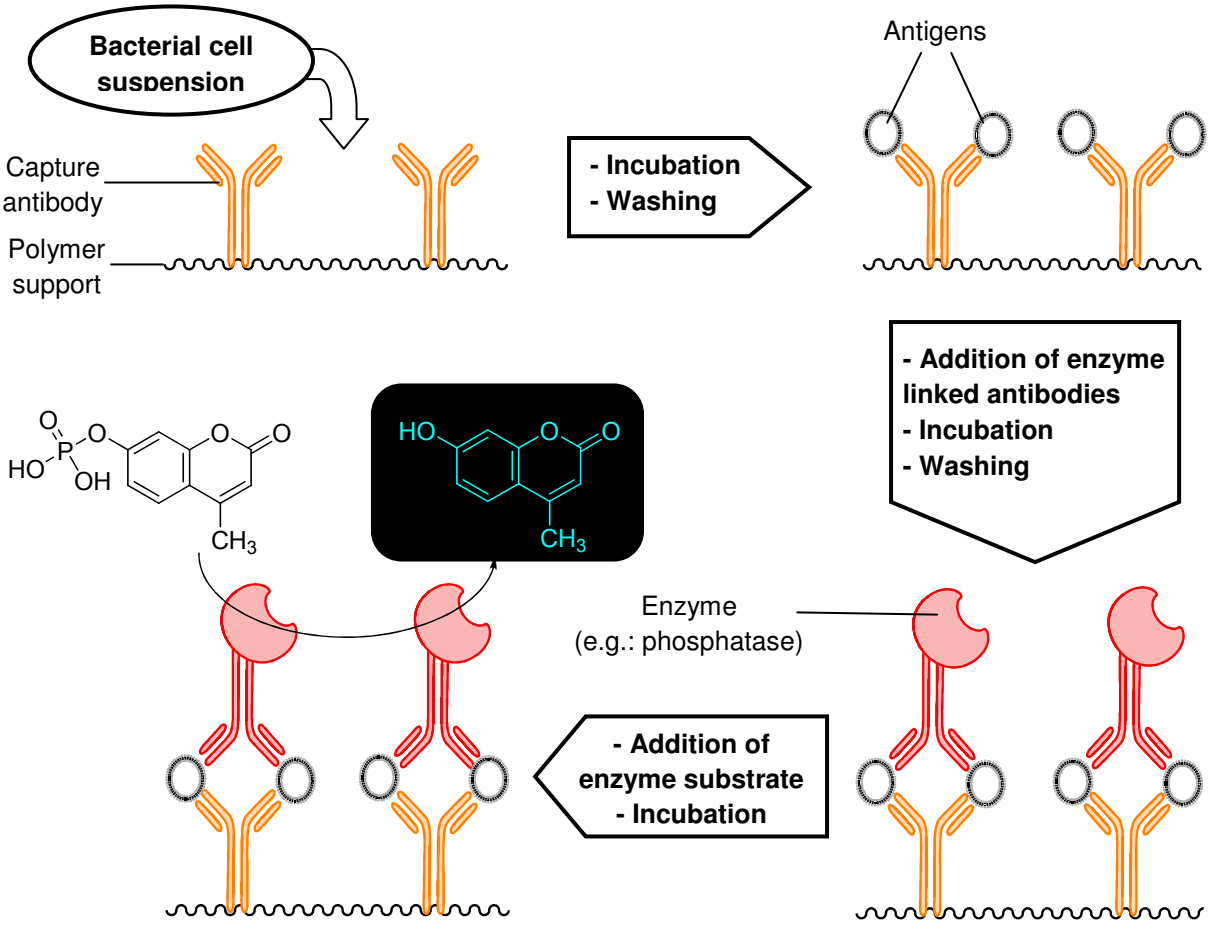


Scheme 1.1: Principle of detection using genetically engineered bacteriophage and enzymatic reaction resulting in bioluminescence (adapted from 2002FRI863).

1.1.2.3 Enzyme-linked immunosorbent assay (ELISA)

Immunoassay utilises the specific relationship between antibodies and antigen (1990MI497). The specificity and sensitivity of immunoassay has been enhanced by the use of monoclonal antibodies (specific to one particular type of bacterial antigen) and the combination of enzyme labels and substrates (1990MI497).

The most popular and widely used type of immunoassay is probably enzyme-linked immunosorbent assay (ELISA). The principle of ELISA, illustrated in **Scheme 1.2**, uses microplate wells coated with capture antibodies.



Scheme 1.2: Bacterial detection using sandwich ELISA
(Last accessed 23/03/2010 and adapted from:
http://64.202.120.86/upload/image/articles/2006/biopen/biopen-elisa-schematic.jpg).

After incubation of the bacteria and capture of the antigens, enzyme-linked antibodies are added to the wells and bind to the fixed antigen (**Scheme 1.2**). A fluorogenic enzyme substrate is finally added and, if the correct antigens are present

in the resulting ELISA sandwich complex, further incubation results in the detection of fluorescence.

The most sensitive enzymes used with ELISA are horseradish peroxidase, phosphatase and β-galactosidase (2005ABI227). This technique has been applied to the detection of *L. monocytogenes* (1992LAM26), *Ps. fluorescens* (1993JAB394), *E. coli* O157:H7 (2009BBE1641) or *Salmonella spp*. (2009BBE1641).

1.1.2.4 Chromogenic media

Chromogenic media utilise enzymes as taxonomic markers.

1.1.2.4.1 Agar-based media

Chromogenic agar-based media are an evolution of the traditional, general purpose, growth media used in microbiology, which only allow a presumptive identification of the microorganisms based on their colonial appearance (e.g. pigmentation, morphology) and require the use of further biochemical test for definitive identification (2007JAM2046).

The addition of chromogenic enzyme substrates in those media has allowed a more direct identification of the suspected pathogen, *via* the evidence of a specific enzymatic activity. As chromogenic media are destined to be used directly on clinical samples, a combination of enzyme substrates is often used to differentiate the pathogen from other commensal bacteria, or even to allow the identification of several pathogens simultaneously. **Figure 1.2** depicts the example of a chromogenic medium (chromID CPS3, bioMérieux) allowing the simultaneous detection of the most common urinary tract pathogens: *Escherichia coli* (purple), detected *via* β-glucuronidase activity, *Proteus mirabilis* (brown), detected *via* deaminase activity and enterococci (blue), detected *via* β-glucosidase activity. More detail will be given later on the exact nature of such enzyme substrates. These media also tend to promote the development of

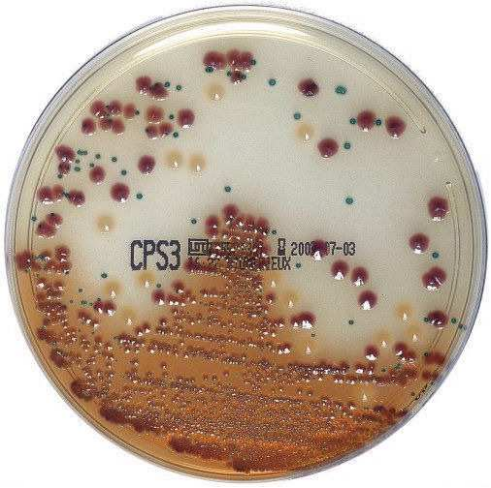


Figure 1.2: Example of a chromogenic medium (chromID CPS3) allowing multiple detection: here *E. coli* (purple), *P. mirabilis* (brown) and enterococci (blue). (Picture taken from http://www.biomerieux.fr/servlet/srt/bio/france/dynPage?open=FRN_CLN_PRD&doc=FRN_CLN_PRD_G_PRD_CLN_20&pubparams.sform=2&lang=fr, last accessed 04/04/2010).

the microorganism under investigation and the expression of the enzyme necessary to hydrolyse the substrate, while inhibiting the growth of competing microorganisms, to facilitate identification and avoid false-positive results (2007BSM96). Thus chromogenic media can by-pass the time consuming procedure of isolating a pure culture prior to carrying out further identification tests.

Many media have been developed for the identification of specific pathogens (2007JAM2046, 2009JMM139), including the detection of antibiotic-resistant pathogens such as vancomycin-resistant enterococci (VRE, 2009JCM4113, 2009JMM124) and MRSA (2004JCM4519, 2010JCM215).

Chromogenic media confer the advantage to permit procedures and readings free from any specific and costly equipment.

1.1.2.4.2 Micro gallery and automated system

Micro galleries, such as API gallery (1988AEM2838, 1993ACB81), combine a wide range of enzyme substrates. A gallery can correctly identify a microorganism within a given family or genera for which the gallery has been designed. This process has been automated recently, with systems such as VITEK®2 (bioMérieux), which only require the manual preparation of the inoculum. Inoculation of the wells, incubation and interpretation of the results are carried out by the system itself. **Figure 1.3** depicts the type of card used in VITEK®2 system after incubation, with a card designed for Gram positive bacteria, showing positive results corresponding to presence of *Staphylococcus epidermis*. Tests are available for the identification of *Enterobacteriaceae* (2003JCM2096), yeasts (2007JCM1087) or *Bacillus* species (2010LAM120).

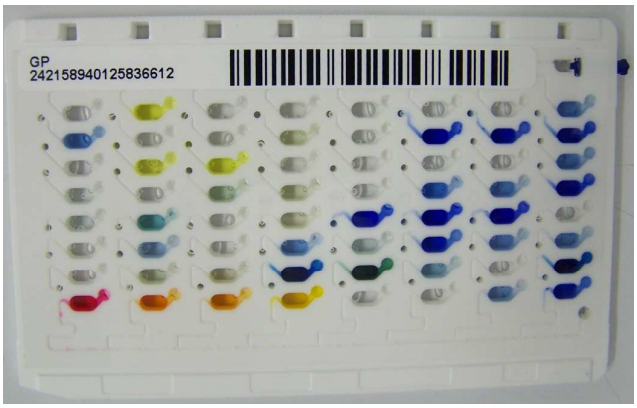


Figure 1.3: Example of a VITEK®2 card displaying results for *St. epidermis*.

The use of galleries allows for the identification of a much wider range of microorganisms, compared to the agar-based media mentioned previously; however these techniques of identification usually require the isolation of a pure culture, which implies longer identification procedures. Automated systems are costly and can only accommodate a limited number of samples at a time.

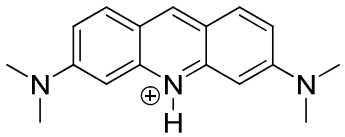
1.1.3 Chromogenic and fluorogenic dyes

Chromogenic and fluorogenic dyes have been implemented in many detection techniques, to enhance the visualisation of results by providing a strong and easily identifiable signal.

They have been divided into 4 classes (B-1980MI05, 2007BSM96) based on their mode of interaction with microorganisms: DNA fluorescent dyes, pH indicators, redox indicators and enzyme substrates.

1.1.3.1 DNA fluorescent dyes

DNA fluorescent dyes typically have a low intrinsic fluorescence upon excitation; however, the electronic alteration resulting from interaction with DNA base pairs results in a dramatic increase of the quantum yield for the resulting DNA-fluorogen complex (B-2006MI06). The nature of the interaction is for most dyes, non-covalent, intercalative binding. DNA fluorescent dyes are usually cationic aromatic heterocycles, with a planar structure facilitating insertion between the stacked base pairs of the DNA duplex (B-2006MI06).



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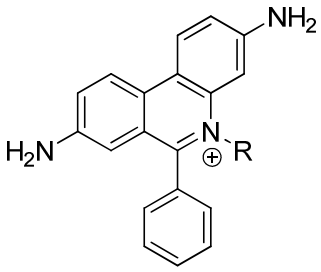
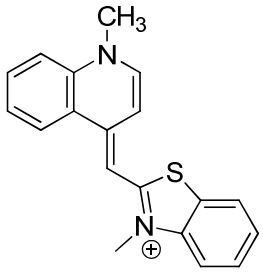
$\lambda_{ex/em}$ = 500 nm / 526 nm (DNA)



8a,R = CH_2CH_3
$\lambda_{ex/em}$ = 510 nm / 595 nm
8b, R = $(CH_2)_3$-N^+-$(CH_3)(CH_2CH_3)_2$
$\lambda_{ex/em}$ = 530 nm / 625 nm



9, $\lambda_{ex/em}$ = 509 nm / 527 nm

Figure 1.4: Examples of some common DNA fluorescent dyes.

Acridine orange **7** (**Figure 1.4**) is perhaps one of the oldest examples, known to have two distinct λ_{em} whether it is bound to single stranded RNA (λ_{em}= 650nm) or double stranded DNA (λ_{em}= 526nm). Phenanthridium dyes **8a-b**, mentioned above in section **1.1.1.1** and section **1.1.2.1**, are widely used DNA stains; their membrane permeability can be tuned by modification of the nitrogen substituent (**8a-b**, **Figure 1.4**).

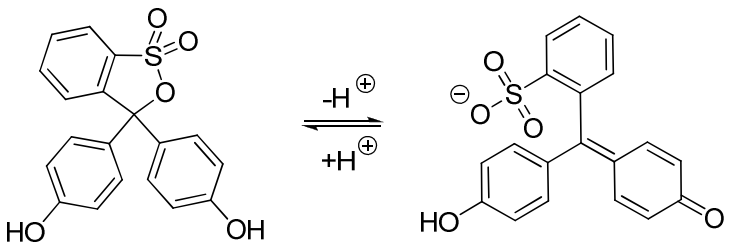
Thiazole orange **9**, an old photographic dye belonging to the class of cyanine dyes, has shown excellent nucleic acid binding properties associated with an important increase of its quantum yield upon excitation. This discovery led the preparation of many analogues such as the SYBR Green I **5** or SYTO dyes (1995US5436134), mentioned in section **1.1.1.1** and section **1.1.2.1**.

These dyes have gained much popularity due to their implementation into modern detection techniques, which are not limited to microorganism identification, such as PCR, DNA probes or epifluorescence microscopy to enhance the visualisation of genetic material or the microorganism itself.

1.1.3.2 pH indicators

Bacterial growth often results in biochemical changes within the growth medium, some resulting in a noticeable variation of the pH. The addition of a pH indicator to the growth medium is often used to monitor the production of acid or the release of a base.

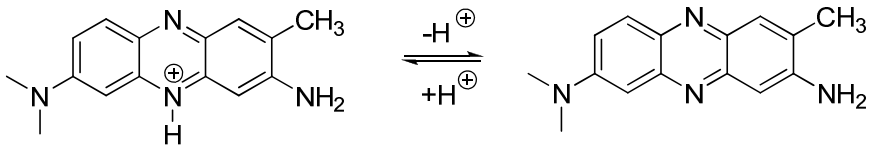
A significant increase of the pH in the growth medium is characteristic of urease activity or of L-amino-oxidase (deaminase) activity, such as phenylalanine deaminase, which are both characterised by the release of ammonia. A common biochemical test used for the detection of these enzymes is the addition of phenol red (**10**, **Scheme 1.3**) into the medium, which results in a change of the medium colour from yellow to red-fuschia (**Scheme 1.3**).



10, pH < 6.8 : yellow pH > 8.4 : fuschia

Scheme 1.3: Acidic and basic forms of phenol red.

A decrease in pH is usually characteristic of sugar fermentation, which results in the production of lactic acid. MacConkey agar uses neutral red **11** to specifically identify bacteria that ferment lactose to lactic acid (**Scheme 1.4**, pH transition: 6.8-8.0).

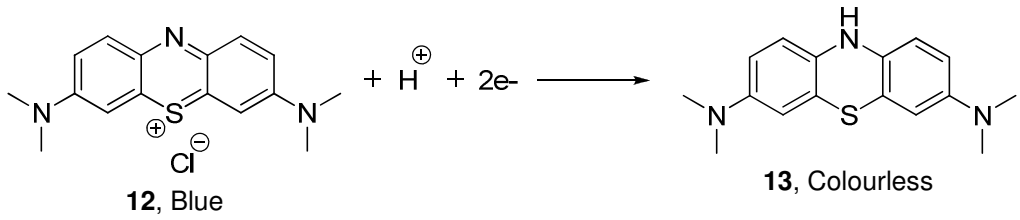


Scheme 1.4: Acidic and basic forms of neutral red.

1.1.3.3 Redox indicators

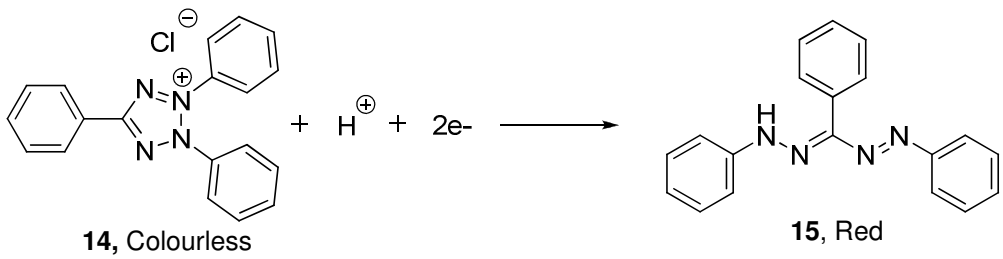
Redox indicators detect oxidative enzyme systems, present in all living organisms, by acting as artificial electron acceptors (2002MI63). They present little specificity and are used as general bacterial growth indicators.

Methylene blue **12** has been widely used for this purpose, with micro organism growth monitored by the disappearance of the blue colour resulting from the formation of leucomethylene blue **13** (**Scheme 1.5**).



Scheme 1.5: Reduction of methylene blue to the colourless leucomethylene blue.

Alternatively, 2,3,5-triphenyltetrazolium chloride (TTC, **14**, **Scheme 1.6**) is also commonly used as a growth indicator (1987JAB551, 2002MI63) and for the enumeration of bacterial colonies. TTC is a colourless water soluble salt which forms, upon reduction, 1,3,5-triphenylformazan **15**, a red insoluble solid, which allows clear visualisation of bacterial development.



Scheme 1.6: Formation of insoluble 1,3,5-triphenylformazan upon reduction of 2,3,5-triphenyltetrazolium chloride.

The ability of 2,3,5-triphenyltetrazolium chlorides to form 1,3,5-triphenylformazan upon reduction has been widely exploited as a marker of biological activity; the production of a range of colours can be achieved by substitution or replacement of the phenyl ring with various heterocyclic moieties.

1.1.3.4 Enzyme substrates

An enzyme substrate is composed of two moieties: a chromophore/fluorophore, or a precursor susceptible to form one of the former upon a further reaction taking place after enzymatic hydrolysis, and a biological molecule, usually a peptide or a sugar. Enzyme substrate dyes comprise an auxochrome, a substituent which enables linkage to the biological molecule and the formation of an intense colour *via* electron conjugation, chelation with a metal, or reaction with a secondary reagent (B-2008MI07). The two main types of auxochromic groups encountered are the hydroxyl group, which allows for the detection of osidase, esterase, phosphatase and sulfatase activities, or the amino group which permits the detection of aminopeptidase activities.

The efficiency of enzyme substrates has been quantified by the following features:

- The absorption maximum of the free chromophore/fluorophore must be significantly different from that of the substrate. Ideally the enzymatic substrate will be non-coloured and/or non U.V. active to allow clear identification of enzymatic activity;

- The chromophore/fluorophore must be readily cleaved and must dissociate from the enzyme to avoid any interference with the enzymatic activity.

- The chromophore/fluorophore must have a high molar absorptivity to allow detection of weak enzyme activity;

- The chromophore/fluorophore and its corresponding substrate should be of a low toxicity to the microorganism(s) under investigation;

- The chromophore/fluorophore and its corresponding substrate should be stable to the conditions used for bacterial growth: the colour/fluorescence must develop only upon enzymatic action. Moreover, the chromophore/fluorophore itself should not undergo any further modification induced by side enzymatic reaction that would attenuate its colour/fluorescence;

- The substrate should have a good water solubility to ease its incorporation into the medium and favour its enzymatic hydrolysis;

-The chromophore/fluorophore should accurately localise to the bacterial colony with little or no diffusion of the colour when used in a solid medium.

The overall performances of an enzyme substrate in bacterial identification will be discussed in terms of sensitivity and specificity; these two criteria are determined by screening the substrate against a large number of bacterial strains. The sensitivity of a chromogenic medium is defined as the percentage of positive results for the overall strains of bacteria expressing the enzyme activity. The specificity is defined as the percentage of organisms producing a colour being the actual targeted bacterial strains.

Enzyme substrates have been subdivided into four groups by virtue of their mode of action (B-1980MI02): simultaneous capture chromogenic substrates, post-incubation coupling chromogenic substrates, intra-molecular rearrangement/electron conjugation chromogenic substrates and self-coloured chromogenic/fluorogenic substrates. Examples of traditional and recently developed enzyme substrates follow to illustrate this classification.

1.1.3.4.1 Simultaneous capture chromogenic substrates

The product resulting from enzymatic hydrolysis of the substrate, called the primary reaction product, is further reacted (capture reaction) with a second reagent present in the medium to form a highly coloured product, called the final reaction product.

Capture reaction has been widely applied to metal chelators such as esculetin (1987MI188, **16**), cyclohexenoesculetin (1996AEM3868, 1997JAM532, 1999US6008008, CHE, **17**), 8-hydroxyquinoline (1987MI410, **18**), alizarin (2000LAM336, 2006US7052863, **19**), 3-hydroxyflavone (2007MI410, **20**) and 3',4'-dihydroxyflavone (2007MI410, **21**, **Figure 1.5**).

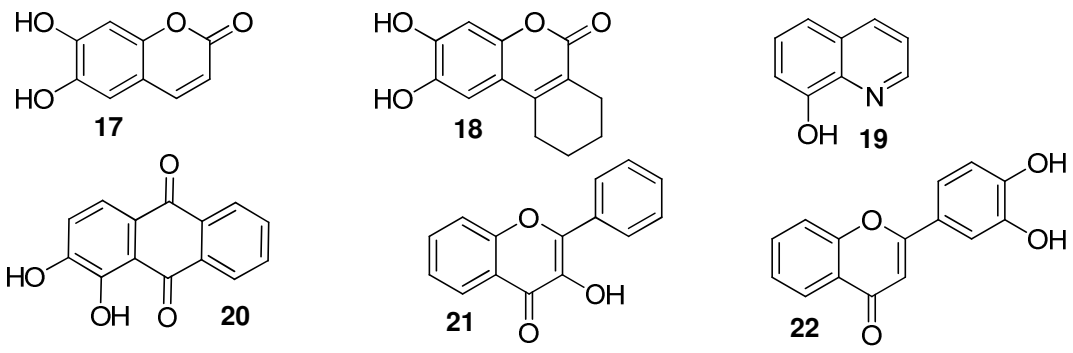


Figure 1.5: Structures of metal chelators used as simultaneous capture chromogenic substrates.

Ferric ion, usually incorporated as ferric citrate in the medium, is the metal ion of choice due to its low toxicity toward microorganisms. The resulting iron complexes are black for all ligands depicted in **Figure 1.5**, excepted alizarin, for which the resulting iron complex is bright violet (2000LAM336).

The capture reaction using ferric ion is depicted in **Scheme 1.7** with the example of β-D-glycosidic derivatives of cyclohexenoesculetin **23** (1996AEM3868, 1997JAM532, 1999US6008008). Upon enzymatic hydrolysis, chelation of the primary reaction product, cyclohexenoesculetin (CHE, **18**), with ferric ions, results in the formation of the black iron complex **24** (2007BMC1172, **Scheme 1.7**). The resulting iron complex **24** precipitates out of the medium and locates, with great accuracy, the site of hydrolysis (**Figure 1.6**).

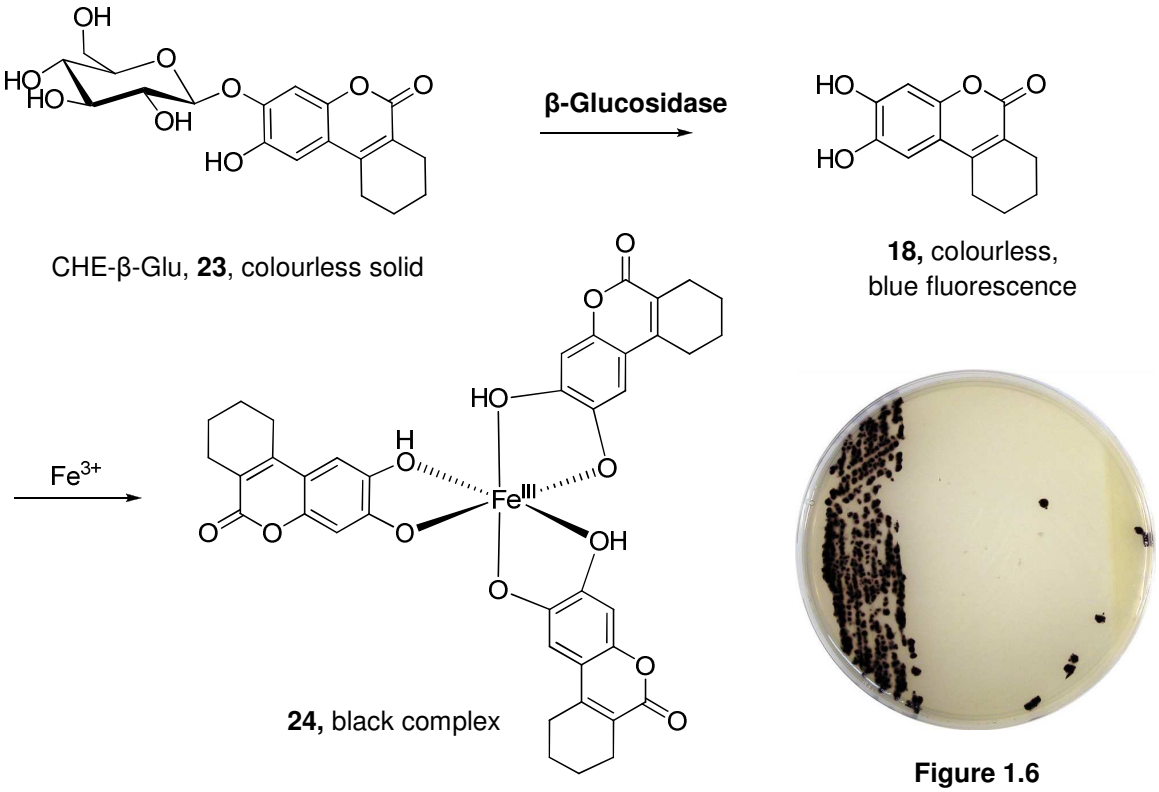


Figure 1.6

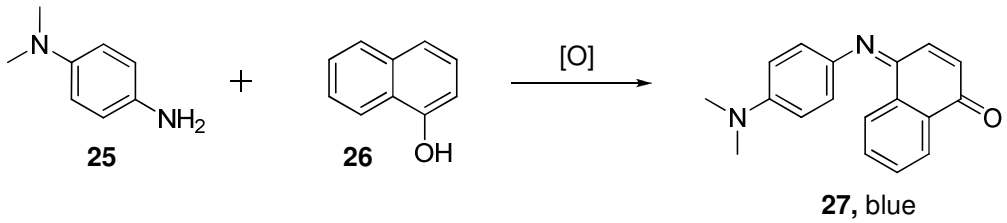
Scheme 1.7: Enzymatic hydrolysis of CHE-β-Glu **23**, followed by chelation of CHE with ferric ion with **Figure 1.6** illustrating the formation of **24** for the detection of *Clostridium NC1MB 10697*.

The accurate location of the site of hydrolysis, and hence the bacterial colonies, greatly depends upon the diffusion factor of the primary reaction product and the rate constant of the capture reaction to form the final reaction product.

Figure 1.7: Detection of *Listeria monocytogenes* on agar medium using esculetin-β-D-glucoside (esculin, left) and CHE-β-D-glucoside (right) (L-2008MI01).

This issue is well illustrated while comparing esculin **17** and CHE **18** (**Figure 1.7**), the presence of an extra cyclic carbon chain confers CHE with an increased lipophilicity resulting in low diffusion within the medium (**Figure 1.7**).

An alternative to metal chelators in simultaneous capture chromogenic substrates is the coupling of the primary reaction product to a secondary organic reagent. The NADI reaction (**Scheme 1.8**), reported by Ehrlich in 1885 (B-1885MI08), was appropriately adapted to simultaneous capture reactions.



Scheme 1.8: Formation of the blue indophenol **24** upon enzymatic catalysis

This reaction was initially used to detect the presence of cytochrome c oxidase (1954JBC733) *via* the oxidative addition of *N,N*-dimethyl-*p*-phenylenediamine **25** and α-naphthol **26**, resulting in the formation of the blue coloured indophenol **27** (**Scheme 1.8, Figure 1.6**). Appropriate derivatisation of *N,N*-dimethyl-*p*-phenylenediamine, or an analogous compound, and α-naphthol have rendered possible the detection of osidase and amidase microbial activities *via* this reaction (2002US6340573B1). The most

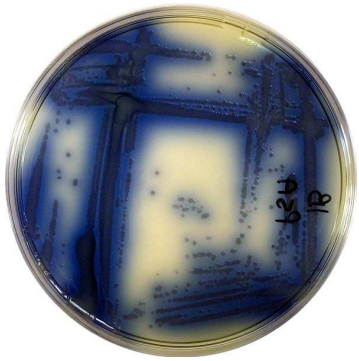
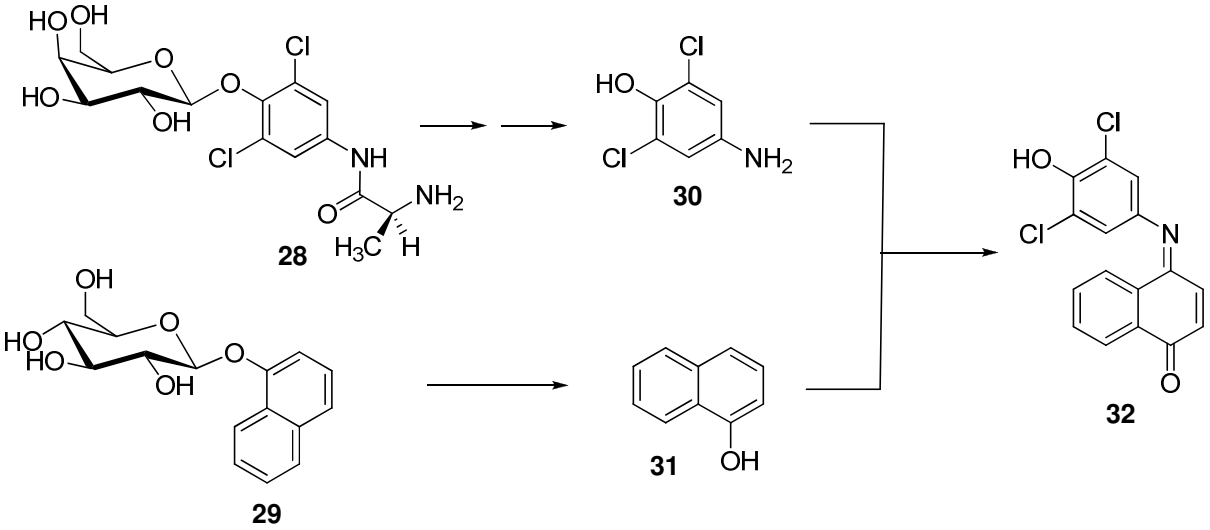


Figure 1.8: Formation of blue indophenol on agar medium with *Pseudomonas aeruginosa.*

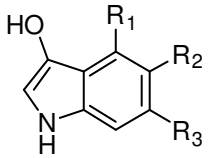
advanced example reported allows the detection of up to three different enzyme activities with the two enzyme substrates L-alanyl-4-amino-2,6-dichlorophenyl-β-D-galactopyranoside **28** and α-naphthyl-β-D-glucopyranoside **29** (2002US6340573B1, **Scheme 1.9**). Formation of the indodichlorophenol dye **32** only occurred upon full enzymatic hydrolysis and formation of both primary reaction products, 2,6-dichloro-4-aminophenol **30** and α-naphthol **31** (**Scheme 1.9**) simultaneously, allowing high selectivity amongst bacteria.



Scheme 1.9: Detection of three different enzyme activities: L-alanine-aminopeptidase and β-galactosidase activities to hydrolyse **28** and β-glucosidase activity to hydrolyse **29**.

This reaction is advantageous in terms of specificity compared to metal chelators; however, it shows less affinity with the site of hydrolysis on solid media where metal complexes are more accurate, and is best suited for liquid media.

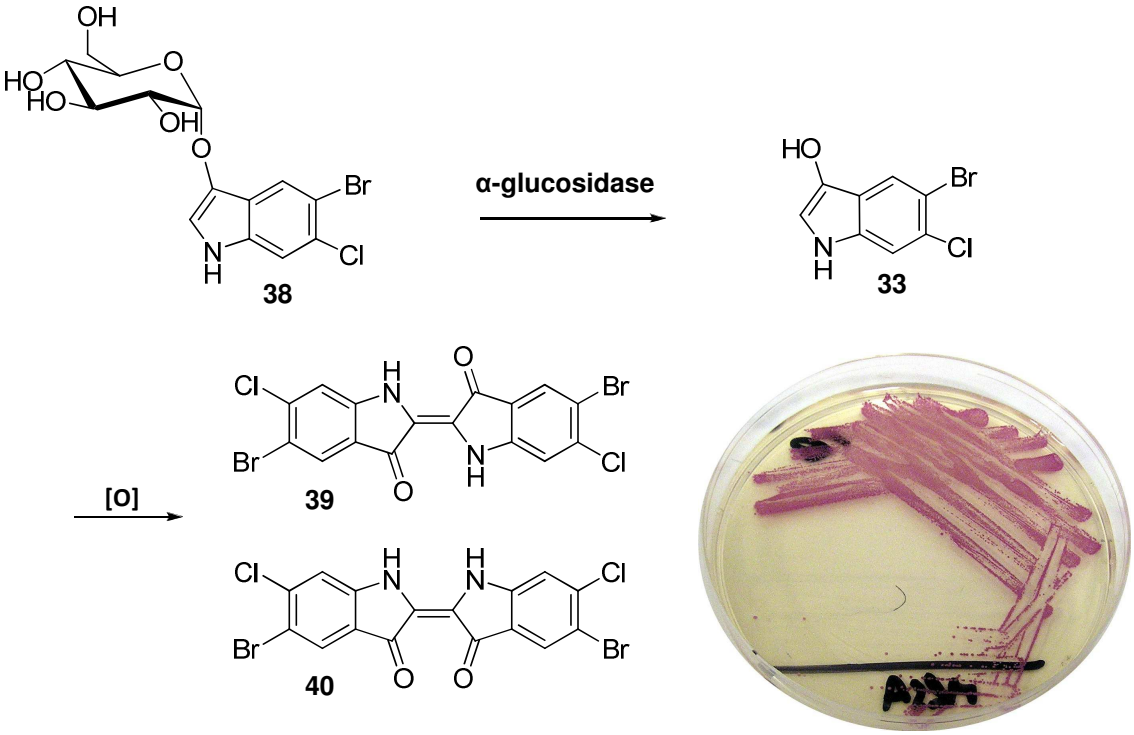
The final and most popular example of this type of substrate are indoxyl derivatives **33-37** (1961T236, 1961JMC574, 1988CJM690); these substrates are without contest the most widely used chromogens for the detection of enzymatic activity and are currently implemented in most commercially available chromogenic media (2009JMM139).



33, R_1 = H, R_2 = Br, R_3 = Cl, 'Magenta'
34, R_1 = Cl, R_2 = Br, R_3 = H, turquoise ('X')
35, R_1 = R_2 = H, R_3 = Cl, 'Rose/Salmon'
36, R_1 = R_3 = H, R_2 = Br, blue
37, R_1 = R_2 = R_3 = H, indigo blue

Scheme 1.10 illustrates the example of 5-bromo-6-chloroindoxy-α-glucoside **38**, which, upon hydrolysis of the α-glucoside moiety, releases the free indoxyl **33**,

spontaneously oxidised to a brightly coloured mixture of indigo **39** and other indigogenic by-products in minor quantities, such as indirubin **40** (**Scheme 1.10**). In this instance, this could be termed a simultaneous self-capture reaction. The resulting chromogenic mixture formed is highly insoluble and precipitates out of the medium, allowing a very clear identification of α-glucosidase expressing bacteria (**Figure 1.9**).



Scheme 1.10 and Figure 1.9: Enzymatic hydrolysis of 5-bromo-6-chloroindoxyl (Magenta) α-glucoside and formation of the brightly coloured indigo and indirubin derivatives.

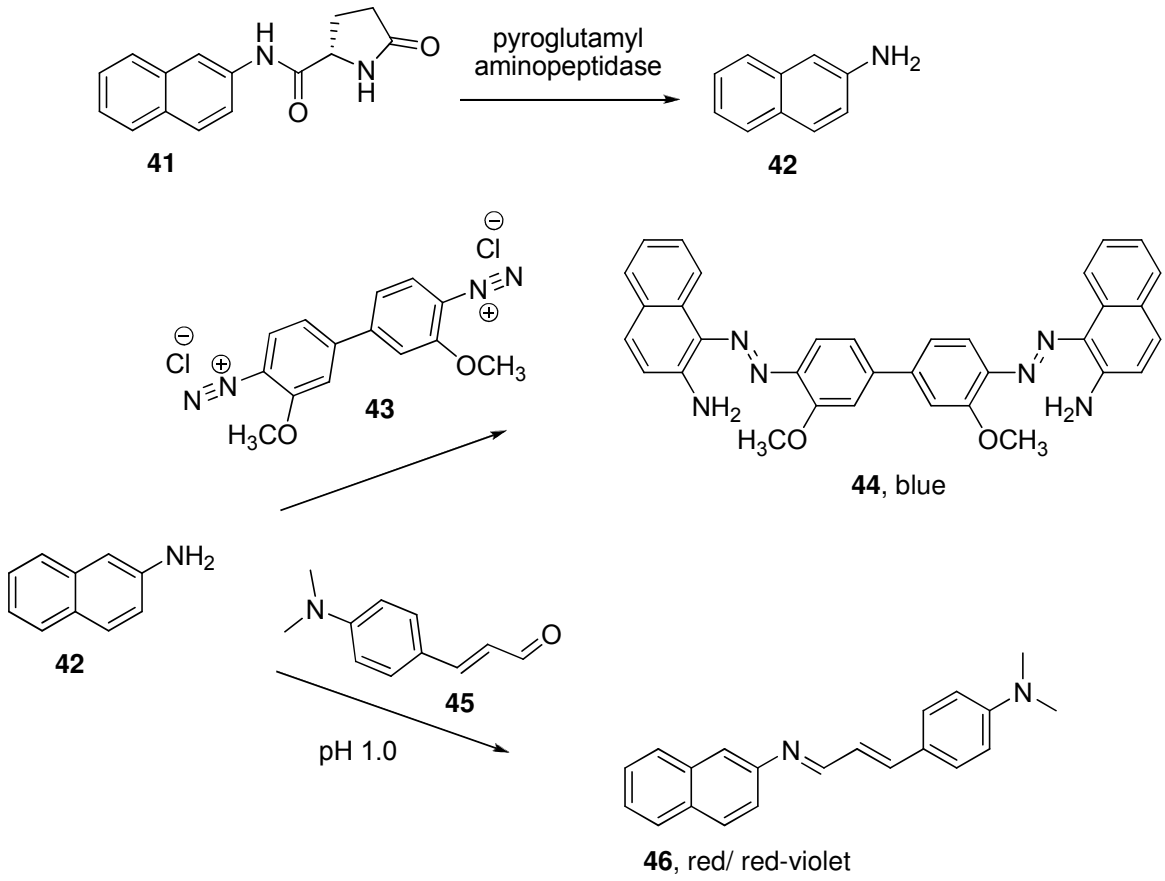
Indoxyls **33-37** are very sensitive substrates with a low toxicity (2007JAM2046); they are suitable for the detection of osidases, esterases and phosphatases. Moreover the position and nature of the halogen substituent on the indoxyl moiety offers a large range of colours, which explains their popularity in microbial detection. Their synthesis is, however, difficult to achieve and their use is limited to aerobic microorganisms, as the presence of oxygen is necessary to the formation of the final reaction product.

1.1.3.4.2 Post-incubation coupling chromogenic substrates

The conditions necessary for the coupling reaction, when using post-incubation coupling chromogenic substrates, require addition of the secondary reagent after enzyme hydrolysis, as the growth conditions are strongly affected by the reaction.

This method has been mostly applied to azo-coupling reactions, where the secondary reagent, a diazonium salt, is toxic to microorganisms and inhibitory toward enzymatic activities (B-1980MI02).

The detection of pyroglutamyl amidase with the substrate pyroglutamyl-β-naphthylamide **41** is a good illustration of this method: the primary reaction product, naphthylamine **42**, can be coupled with tetrazotized *o*-anisidine (**43**, Fast blue B), resulting in the formation of the blue azo dye **44** (**Scheme 1.11**, B-1980MI02).

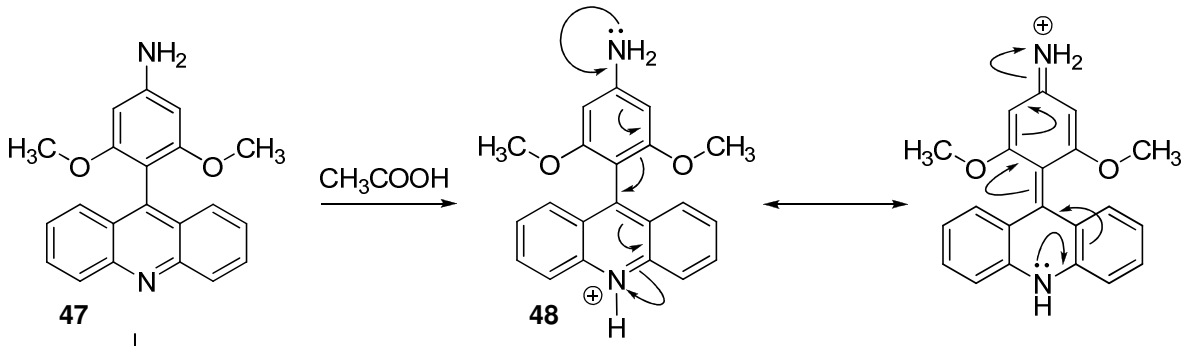


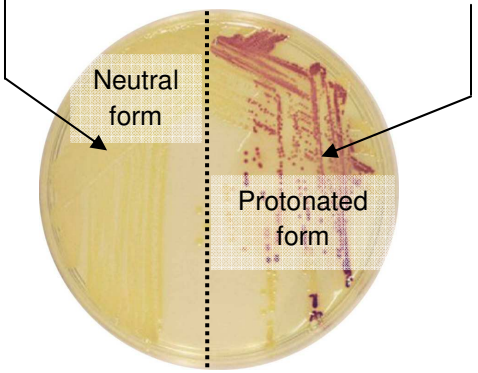
Scheme 1.11: Enzymatic hydrolysis of pyroglutamyl-β-naphthylamide **41** and capture of the resulting product, β-naphthylamine **42**.

An alternative capture reaction, using an acidic solution of *p*-dimethylaminocinnamaldehyde **45** to form the highly coloured red ene-imine product **46** is also possible (**Scheme 1.11,** 1987JCM1805, 1996JCM1811).

In both cases the toxicity of the final reaction product forbids any further assay on the microorganism under investigation, and this resulted in research on other, less toxic, substrates (1967MI500, 2002US6340573B1).

More recently, the example of 9-(4'-aminophenyl)acridine substrates was introduced as a means of detection for peptidase activities (2007BMCL1418). Although not a true coupling reaction, the primary reaction product **47** is weakly coloured and requires the addition of acetic acid to yield the colourful 9-(4'-aminophenyl)acridinium ion (**48**, **Scheme 1.12**, **Figure 1.10,** 2007BMCL1418).





Scheme 1.12: Ionisation of 9-(4'-amino-2',6'-dimethoxyphenyl)acridine with acetic acid and consequent formation of a purple colour (2007BMLC1418).
Figure 1.10: Importance of ionisation of **33** for the visualisation of the bacterial colonies.

Acidification of the medium is only possible after incubation; as such a low pH is inhibitory to the growth of most bacteria.

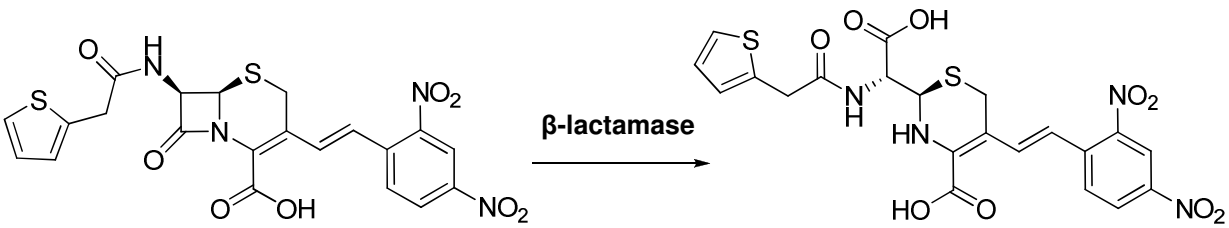
Post-incubation coupling chromogenic substrates are now seldom used; the important range of other enzyme substrates available now can usually provide a better alternative to post-coupling reactions.

1.1.3.4.3 Intramolecular rearrangement/electron conjugation chromogenic substrates

In this instance, the enzyme induces a structural modification, such as an intramolecular rearrangement, forcing a change of the electron conjugation within the molecule and a shift in the wavelength of light absorption.

An example of this type of substrate is nitrocefin **47**, which is utilised in the detection of β-lactamase enzyme producing bacteria (2005JOC367). Upon hydrolysis of the

β-lactam ring in nitrocefin **47** by β-lactamase activity, the altered electron density in cephalothin **48** induces a dramatic change of the wavelength absorbance, permitting visible colony detection (**Scheme 1.13**).

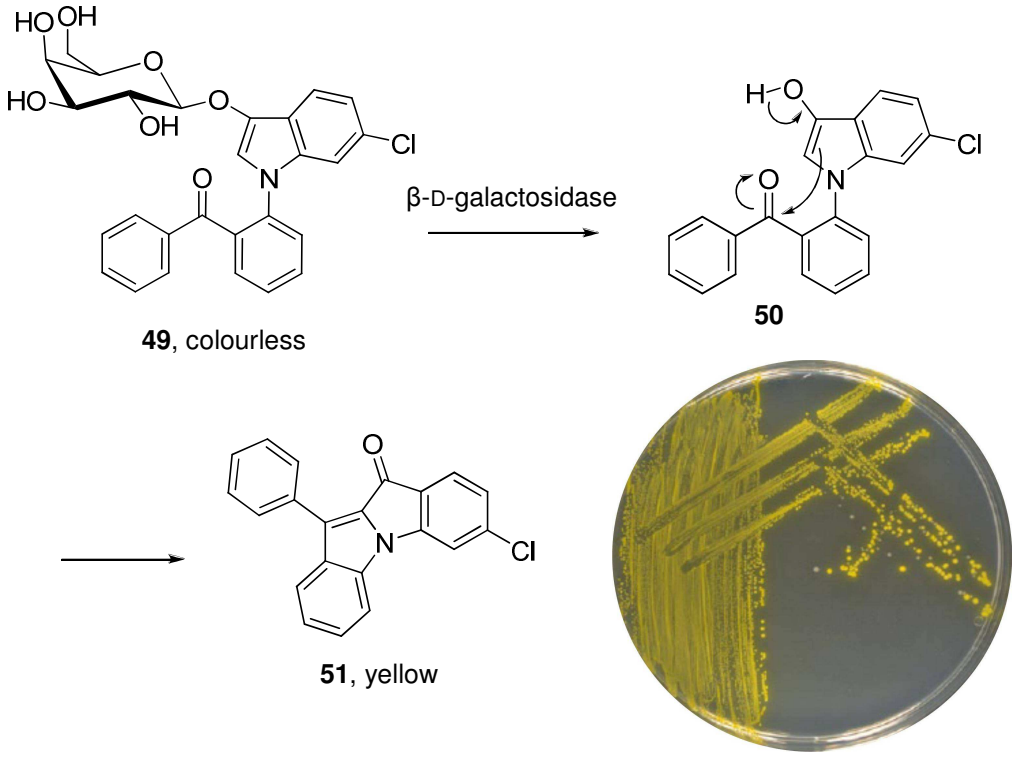


47, yellow, λ_{max}: 390nm

48, red, λ_{max}: 486nm

Scheme 1.13: Opening of the nitrocefin β-lactam ring by β-lactamase enzymatic activity.

Recently, researchers from Biosynth® have exploited this technique by developing a range of indoxyl enzyme substrates, such as the ALDOL™-455-β-D-galactosidase **49** (P-2009MI01). Upon enzymatic hydrolysis of **49**, the colourless primary reaction product **50** undergoes an intramolecular Aldol-type condensation to produce a yellow insoluble dye, 7-chloro-10-phenyl-10*H*-indolo-[1,2a]-indol-10-one **51** (**Scheme 1.14**).



49, colourless

50

51, yellow

Scheme 1.14 and Figure 1.11: Intramolecular rearrangement of ALDOL™-455-β-D-galactosidase **49** upon enzymatic hydrolysis and resulting yellow colonies of *Enterobacter cloacae* (P-2009MI01, with D^{r}. L.M. Wick's authorisation).

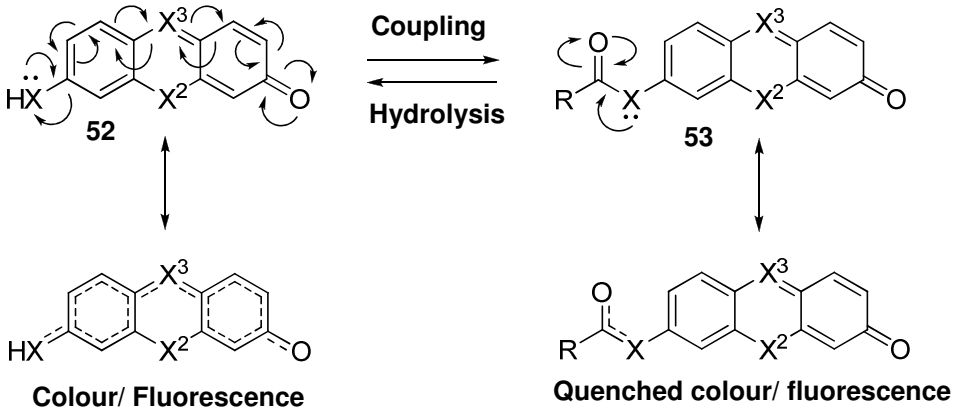
The rearrangement appears to be instantaneous, and the precipitation of the resulting product **51** locates the bacterial colonies with precision (**Figure 1.11**). The low toxicity and absence of background of these substrates is also notable (**Figure 1.11**).

Very few examples of intramolecular rearrangement substrates are available yet, presumably due to the difficulty of finding an adequate rearrangement reaction, suitable to the bacterial growth condition.

1.1.3.4.4 Self coloured chromogenic/fluorogenic substrates

The primary reaction product itself is coloured/fluorescent suppressing the need of any other reagents.

The colouration/fluorescence of this type of enzyme substrates results from the conjugation of an available lone pair of electrons, belonging to the auxochrome, into the π-system of the chromophore/fluorophore. The linkage of a biological molecule to the auxochromic group translates into a dramatic quenching of the colour/fluorescence for the resulting enzyme substrate, as a result of important changes within the electron conjugation. **Scheme 1.15** depicts the electron movement within the core structure **52** common to some popular chromogenic and fluorogenic dyes, and the restricted conjugation occurring within the bond formed between the dye and the biological molecule, in the corresponding enzyme substrate **53**. Cleavage of the biological marker by enzymatic hydrolysis restores its initial colour/fluorescence.



Scheme 1.15: Electron movements and quenching of this effect in some common structure of self-coloured enzyme substrates:
X = O or N, X^2 = O or $C(CH_3)_2$, X^3 = N, C-Ph or C-PhCOOH, R = Alkyl chain or amino acid

1.1.3.4.4.1 Fluorogen

The three most common fluorogenic cores (**Figure 1.12**) used as enzyme substrates are fluorescein **54**, 7-hydroxyphenoxazin-3-one (resorufin, **55**) and, probably the most important class, the coumarin core, including 7-hydroxy-4-methylcoumarin (4-methylumbelliferone, 4-MU, **56a**) and 7-amino-4-methylcoumarin (7-AMC, **56b**) (2009JMM139). Recently, benzoxazole **57a** and benzothiazole **57b** derivatives have been used successfully as enzyme substrates (2008WO152306).

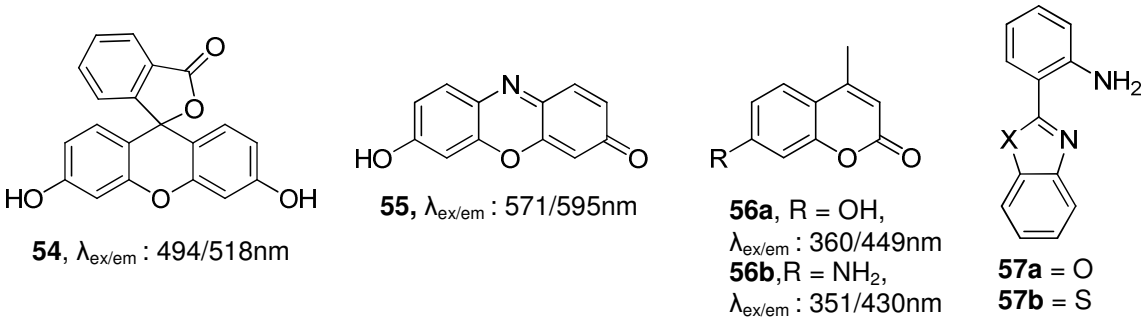


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Figure 1.12: Structure of common fluorogens.

The most widely used fluorogen is probably 4-MU, partly due to its low toxicity to microorganisms, ease of preparation and bright blue fluorescence under U.V. excitation. It is commonly utilised for the detection of microbial sugar hydrolase activity. However, the use of 4-MU (pKa = 7.80) has several disadvantages. The pH dependence is an important issue, as full or partial dissociation of the phenolic proton is essential to enhance electron conjugation and reach maximum fluorescence intensity. This issue has been addressed by the preparation of several 7-hydroxycoumarin derivatives possessing a lower pKa and allowing full dissociation of the phenolic proton at relatively low pH: fluorinated coumarins and particularly 6,8-difluoro-4-methylumbelliferone (1997BMC1985, 1998US5830912) as well as chlorinated coumarin analogues (2006JAM977), showed higher efficiency and sensitivity than the traditional 4-MU enzyme substrate. If the full dissociation of the phenolic proton improves the

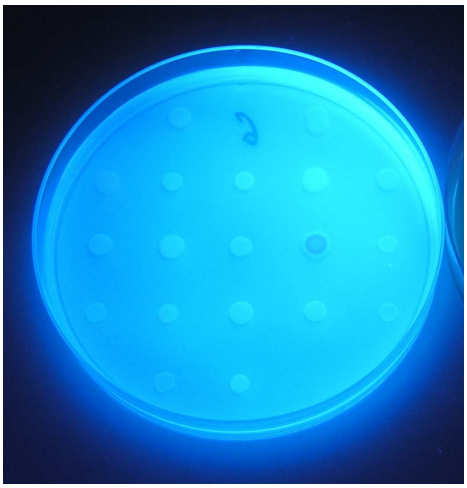


Figure 1.13: Diffusion of 4-MU on a multipoint agar plate.

emission intensity of the fluorogen, it consequently augments its solubility in aqueous media, which dramatically increases the diffusion of the fluorogen on agar media, considerably limiting its usefulness in locating bacterial colonies on solid media. As an example, the screening of organisms *via* multipoint inoculation is rendered extremely difficult due to the diffusion of 4-MU in the medium (**Figure 1.13**).

The relatively low pKa of fluorescein (pKa = 6.4) and of resorufin (pKa = 6.0) results in partial deprotonation at physiological pH, and causes the same diffusion phenomenon when these substrates are used on agar media. Resorufin-β-D-glucuronide is commonly used for the detection of *E. coli*; however, the product of enzymatic hydrolysis shows poor localisation of the bacterial colonies (**Figure 1.14**). Its derivative, 2-dodecylresorufin is reported to show high affinity for lipid regions of cells (B-2005MI04); however, its use as an enzyme substrate has not been reported yet. The preparation of fluorinated resorufin analogues has also been reported to improve the fluorogenic properties of these dyes (2008US7432372).

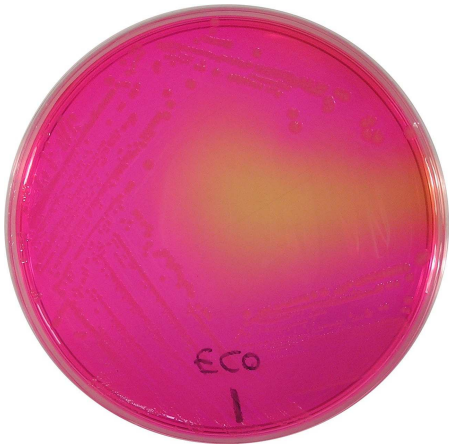
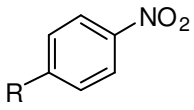


Figure 1.14: Detection of *E. coli* using resorufin-β-D-glucuronide.

Despite the disadvantages mentioned above, fluorogenic substrates are notoriously very sensitive with diagnostic results achievable within hours. Early readings are not achievable with the naked eye and require instrumentation to measure weak fluorescence. Moreover, their use in solid media has been restricted in favour of chromogenic substrates (2007BSM96).

1.3.4.4.2 Chromogens

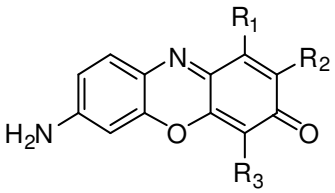
Early development of self-coloured enzyme substrates involved the use of *p*-nitroaniline **58a** (1961ABB271, 1967MI415) and *p*-nitrophenol **58b** (1961BBA460, 1967SCI1451). Enzyme activity was witnessed by the appearance of a yellow colour at the site of hydrolysis. Although, the colour is relatively weak compared to other chromogens, these enzyme substrates are still used in various test kits, as they are cheap and easy to produce.



58a, R = NH_2, yellow
58b, R = OH, yellow

More recently, several 7-aminophenoxazin-3-one derivatives **59a-d** have been reported for the detection of aminopeptidase activity on agar medium (2008OBC682).

The presence of a fused benzene ring (2006US0121551), or alkyl substituent (2006WO030119, 2008OBC682), and the absence of a significant pH dependency, successfully limited the colour diffusion from the site of hydrolysis in solid media. 7-Amino-1-pentylphenoxazin-3-one **59a** is a particularly efficient substrate, with high affinity for the bacterial colonies (**Figure 1.15**). The main drawback reported for 7-aminophenoxazin-3-one based enzyme substrates is the orange background colouration generated by the substrate itself (**Figure 1.15**).



59a, R_1 = *n*-pentyl, R_2 = R_3 = H, red/pink
59b, R_1 = R_2 = CH_3, R_3 = H, red/pink
59c, R_1 = R_2 = R_3 = CH_3, red/pink
59d, R_1 = R_2 = -$(CH)_4$-, R_3 = H, red/pink

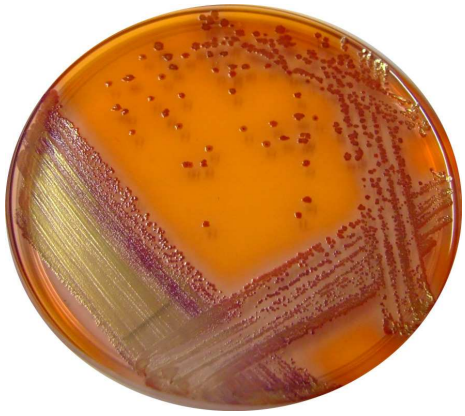
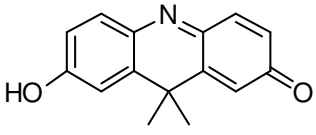
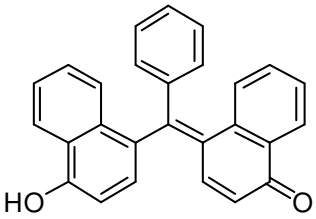


Figure 1.15: Detection of *Ps .aeruginosa* using 7-*N*-β-alanylamino-1-pentylphenoxazin-3-one.

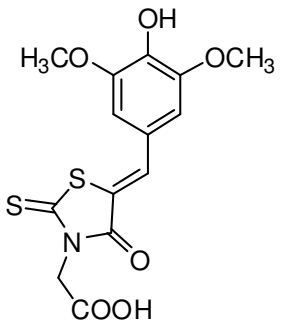
Other, less popular, chromogens were reported for detection of enzymatic activity: acridinone **60**, (1989US4810636, 1991AGE1646), naphtholbenzein **61** (2000AEM5521), 5-(4-hydroxy-3,5-dimethoxyphenylmethylene)-2-thioxothia-zolidin-4-one-3-ethanoic acid (SRA, **62**, 2009FML10) and the 3-methoxy and 3,5-dimethoxy derivatives of 4-[2-(4-hydroxy-3,5-dimethoxyphenyl)-vinyl]-quinolinium-1-(propan-3-yl carboxylic acid) (**63a** [SLPA] and **63-b** [VLPA], 1990CARc5, 1999AEM807, 2002AEM3622) proven to be efficient for bacteria identification; colourfull colonies were formed upon hydrolysis of their corresponding substrates (**Figure 1.16**).



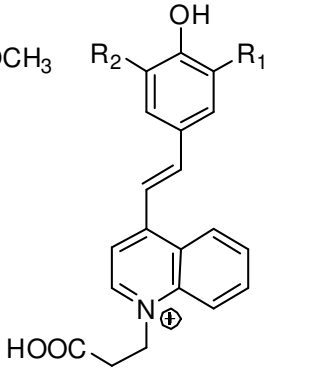
60, Pink



61, Red-Pink



62, Red-Pink



63a, R_1 = R_2 = OCH_3, burgundy
63b, R_1 = OCH_3, R_2 = H, red

Figure 1.16: Structure of chromogens recently developed for the detection of enzymatic activity.

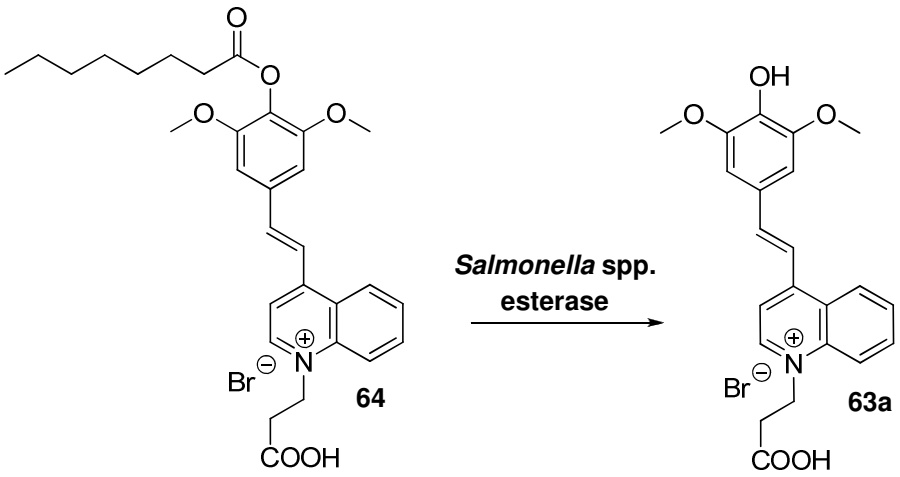
1.1.4 Enzyme targets

Knowledge of the most common enzymes expressed by pathogenic and non-pathogenic microorganisms is essential to the successful development of efficient enzyme substrates. Identification and characterisation of all the different enzymes produced by a bacterium is a long and tedious process (2007BSM96) and such information is not always available. However, a good knowledge of the most important bacterial enzymes has been acquired. A description of enzyme classes and their use in microbiology for the differentiation of microorganisms has been given by Bascomb (1987MM105) and more recently by Orenga *et al.* (2009JMM139). The following review will attempt to highlight briefly the main enzyme activities exploited in chromogenic media for the detection of some important pathogens.

1.1.4.1 Esterases and lipases

Esterase enzymes are ubiquitous in all living organisms. Esterases can hydrolyse substrates with a short carbon chain, whereas lipase enzymes hydrolyse long carbon chain esters and trialkylglycerol fatty acid esters to glycerol and the constituent fatty acids. Hydrolysis of short esterase substrates, such as various fluorescein diacetate derivatives, has been widely used for the monitoring of microorganism viability and activity by flow cytometry (1995FML1, 2003JMM379). Variation of the ester hydrocarbon chain has been exploited to achieve higher specificity, for example, use of the fluorogenic substrate 4-methylumbelliferyl butyrate has been suggested as a complementary test for the differentiation of various microorganisms, such as *Branhamella catarrhalis* and *Neisseria* spp. (1988JCM1227) or *Mycobacterium fortuitum* and *Mycobacterium chelonei* (1977MI147).

The most important application of chromogenic subtrates to esterase activity is probably for the detection in stool samples of *Salmonella* spp., the pathogen responsible for the majority of food poisoning episodes in the United Kingdom (1999JCM766). *Salmonella* spp. are known to be some of the few *Enterobacteriaceae* able to hydrolyse fatty acid esters with carbon chain lengths of C-7 to C-10 (2007JAM2046). Cooke *et al.* studied the sensitivity of various esters of 4-[2-(4-hydroxy-3,5-dimethoxyphenyl)-vinyl]-quinolinium-1-(propan-3-yl carboxylic acid) bromide (SLPA, **63a**), differing in chain length (C-4 to C-10), and found the octanoate ester to be most sensitive for the detection of *Salmonella* spp. (**64**, **Scheme 1.16**, 1999AEM807).



Scheme 1.16: Hydrolysis of SLPA octanoate **63** by *Salmonella* spp. esterase (1999AEM807).

Consequently, many recent commercial chromogenic substrates rely on the octanoate esterase activity to detect *Salmonella* spp.: as an example, indoxyl magenta caprylate is present in Oxoid-*Salmonella*-Chromogen-Agar (OSCM, Oxoid), Rapid'*Salmonella* (BioRad), Compass *Salmonella* (Biokar diagnostic), *Salmonella*-Agar-Plate (ASAP, AES), *Salmonella*-Medium-Identification-Detection (SM ID2, bioMérieux) or HiCrome *Salmonella* agar (HIMEDIA) (2007BCM96). A comparative study carried by Perez *et al.* (2003JCM1130) confirmed the high specificity and selectivity of such detection media for the elucidation of *Salmonella* spp. within clinical specimens.

The lipase enzyme activity has been seldom exploited in the field of enzyme substrates, presumably due to the difficulty of incorporating highly lipophilic enzyme substrates into an aqueous medium. The hydrolysis of dialkylglycerol fatty acid esters of resorufin by a free lipase from *Pseudomonas cepacia* (2006JMCE76) has, however, been reported, suggesting potential applications.

1.1.4.2 Glycosidases

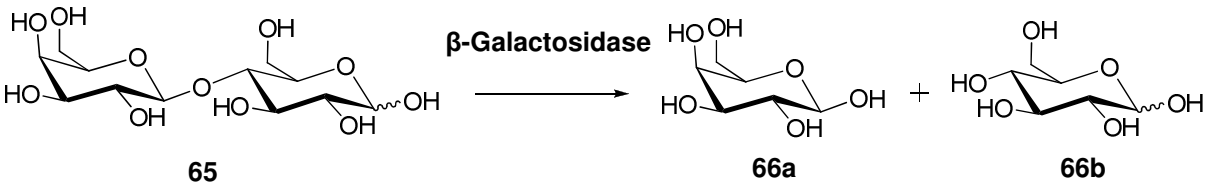
1.1.4.2.1 β-D-Glucuronidase

Bacterial β-D-glucuronidase plays a role in the decomposition process of the host connective tissue during the infectious process (1973AM863).

β-D-Glucuronidase activity is relatively limited amongst bacteria; it has been detected mainly in *Escherichia coli* (94 to 96% of clinical isolates), but also in some *Shigella* species (44 to 58% of clinical isolates) and *Salmonella* species (20 to 29% of clinical isolates) (1991MR335). The prevalence of this enzyme in *E. coli* (1990AEM1203) has

generated a strong interest in the preparation of β-D-glucuronic acid derivatives: with chromogens, such as *p*-nitrophenol (1984JCM1177, 1990AEM2021), 5-bromo-4-chloroindoxyl (1988CJM690, 1988AEM1874), or fluorogens, such as 4-methylumbelliferone (1984JFS1186, 1986JCM368, 1988JCM2682) and its 6-chloro derivative (2006JAM977). These substrates can differentiate with high specificity *E. coli* (the most common urinary tract pathogen) amongst other *Enterobacteriaceae* present in urine samples (1995JCM199, 2009JMM139). Current commercial chromogenic agars such as chromID coli (bioMérieux) or Oxoid *Brilliance*™ E.coli/coliform Selective Agar (Oxoid) exploit the β-D-glucuronidase acitivity of *E. coli*.

. **1.1.4.2.2 β-D-Galactosidase**



65 **66a** **66b**

Scheme 1.17: Lactose hydrolysed into β-D-galactose and α/β-D-glucose.

β-D-Galactosidase, also called lactase, catalyses the breakdown of lactose **65** into its monosaccharide constituents β-D-galactose **66a** and α/β-D-glucose **66b** (**Scheme 1.17**), a step involved in the fermentation of sugar. This enzyme is mainly distributed within the coliform group (*Enterobacteriaceae*), which are common water pollutants; assay of β-D-galactosidase activity is therefore part of the national guidelines for the microbiological examination of water (2001JAM1118).

The detection of β-D-galactosidase activity with chromogenic substrates was first introduced by Aizawa using *o*-nitrophenyl-β-D-galactoside (1939MI321). Numerous β-D-galactopyranosyl substrates have been prepared since then, using *p*-nitrophenol, 6-bromo-2-naphtol (1967MI395), 5-bromo-4-chloro-3-indoxyl (1990AEM301), alizarin (2000LAM336), CHE (1996AEM3868), *p*-naphtholbenzein (2000AEM5521) as chromogens, and 4-methylumbelliferone (4-MU) or 7-hydroxycoumarin-3-carboxylate (2001JAM1118) as fluorogens.

Specific chromogenic media for some virulent member of the coliform group such as *E. coli* 0157:H7 or vancomycin resistant enterrococci (VRE) are based on a β-D-galactopyranosyl derivative of indoxyl or alizarin (2009JMM139).

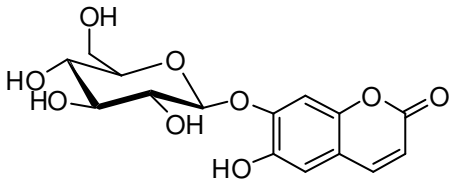
1.1.4.2.3 α-D-Galactosidase

Little information is available on the prevalence of α-D-glycosidase, however, α-D-galactopyranoside activity seems to occur in some species of the genus *Streptococcus* and *Enterococcus* (1989JCM1719). *Streptoccocus bovis*, which is linked with gastrointestinal neoplasia, was differentiated from other streptococci *via* α-D-galactopyranoside activity using 4-MU (1983AEM622) or *p*-nitrophenol (1989JCM1719).

Perry *et al.* reported this enzyme to be predominant in *Salmonella* spp., and consequently developed an agar medium (ABC medium, 1999JCM766) including an α-D-galactoside chromogen, to differentiate *Salmonella* spp. from the various *Enterobacteriaceae* present in stool samples.

1.1.4.2.4 β-D-Glucosidase

The primary role of β-D-glucosidase is the hydrolysis of gluco-oligosaccharides into single glucose units. This enzyme is widely distributed amongst bacteria and has found application for the detection of some important human pathogens (2009JMM139). β-D-Glucosidase is prevalent in enteroccoci (1997JAM532, 2006JAM410), *Listeria* spp. (2004MI1), *Vibrio* spp. (2005MI1454), *Candida* spp. (2009JMM139) and members of the *Enterobacteriaceae* family (1997JAM532, 2006JAM410).



67

The use of the natural substrate esculin **67** for the detection of β-D-glucosidase activity has been known for more than a century (1909MI547), but the issue of diffusion discussed in section **1.3.4.1** (**Figure 1.7**) led to the use of more efficient substrates. Perry *et al.* tested most known chromogen for the detection of β-D-glucosidase activity: indoxyls, 8-hydroxyquinoline, esculetin, CHE, 3-hydroxyflavone, 3',4'-dihydroxyflavone and alizarin, and highlighted the variation of sensitivity of a same substrate amongst bacterial species and the necessity to select carefully a chromogen for targeting a particular pathogen (1997JAM532).

1.1.4.2.5 α-D-Glucosidase

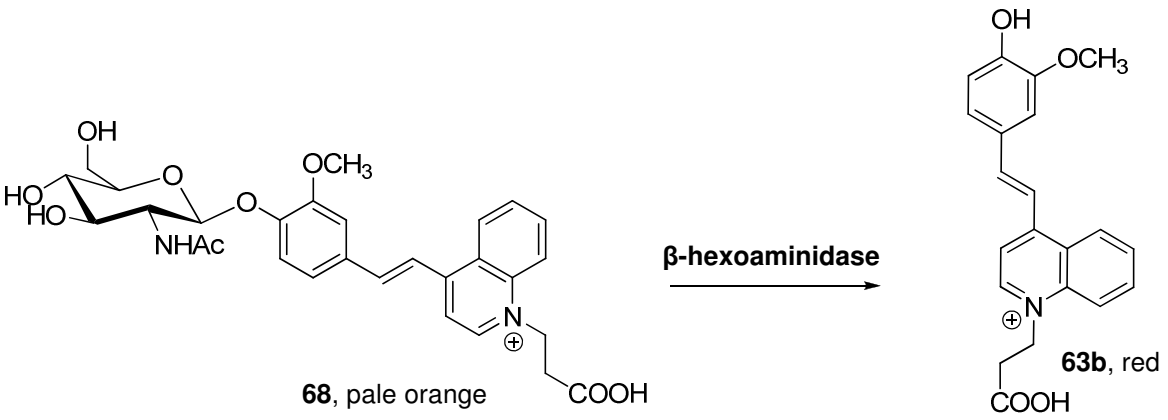
Sadler *et al.* demonstrated the occurence of α-D-glucosidase activity in the genus *Bacilli* using *p*-nitrophenyl-α-D-glucoside (1984JCM594) and differentiated the pathogenic *Bacilli anthracis*, responsible for the disease commonly called anthrax, from other non pathogenic *Bacilli* (1984JCM594).

More recent applications have exploited α-D-glucosidase activity to differentiate *Enterococcus faecalis* from *Enterococcus faecium* (VRE-BMX, bioMérieux, 2007JCM1556), to identify specifically *Staphylococcus aureus* (S. aureus ID, bioMérieux, 2003JCM5695), or *Chronobacter sakazakii* (2007AEM48), an occasional contaminant of powdered infant formula milk. All media used indoxyl-α-D-glucoside derivatives as the chromogen.

1.1.4.2.6 β-Hexoaminidase

β-Hexoaminidase enzyme is prevalent mainly in *Candida albicans*, a commensal yeast of the digestive mucosa, which can proliferate under certain condition, causing severe mycoses. This enzyme is expressed weakly, if at all, by other *Candida* species (2002AEM3622, 2006JCM3340), and provides the ideal tool for the differentiation of *C. albicans*.

Commercially available chromogenic media, such as CHROMagar *Candida* (CHROMagar, 1996JCM454), *Candida ID* 2 (CAID2, bioMérieux, 2006JCM3340), *Candida* diagnostic agar (CDA, PPR diagnostic limited, 2002AEM3622) and CandiSelect4 (CS4, Bio-Rad, 2008JMM89) exploit β-hexoaminidase activity for the identification of *C. albicans.* Cooke *et al.* reported the use of a new substrate, ammonium 4-{2-[4-(2-acetamido-2-deoxy-β-D-glucopyranosyloxy)-3-methoxyphenyl]-vinyl}-1-(propan-3-yl-oate)-quinolium bromide (VLPA-GlcNAc, **68**, **Scheme 1.18**, 2002AEM3622), which produced red colonies of *C. albicans*.



Scheme 1.18: Enzymatic hydrolysis of VLPA-GlcNAc by *Candida albicans* (2002AEM3622).

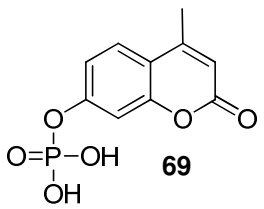
Several comparative studies (1996JCM454, 1998JMM623, 2001MI9, 2002AEM3622) have highlighted the high sensitivity, high specificity and time-saving of such chromogenic media, suggesting them as an advantageous replacement of conventional *C. albicans* tests in microbiological routine tests.

1.1.4.3 Phosphatase

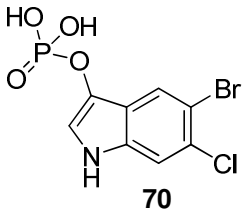
Phosphatase enzymes are involved in several key processes and, hence, are widely distributed amongst organisms. Their use in the domain of bacterial identification is limited to the hydrolases of phosphate ester, the latter being divided into two categories, according to their pH optimum:

- Acidic phosphatase, which has optimum activity at pH 4-5 and was found to be specific to hexose phosphates (1987MM105);
- Alkaline phosphatase, which has optimum activity at pH 8.5-9.5 and hydrolyses all phosphomonoesters (1987MM105).

The most relevant examples of microorganism detection *via* phosphatase activity are:



69



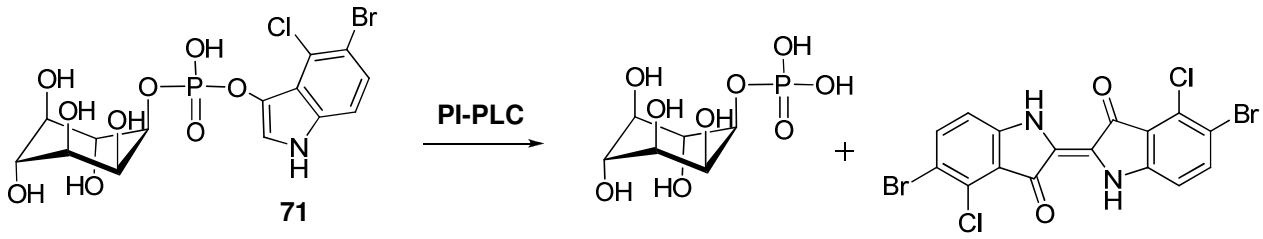
70

Clostridium perfringens, which expresses acidic phosphatase and is detected using 4-methylumbelliferyl-phosphate **69** in combination with a selectively toxic antimicrobial agent, D-cycloserine (Fluorocult TSC-agar,

Merck, 2000IJF205), and *Staphylococcus aureus*, which is detected using phosphatase substrates such as 5-bromo-6-chloro-3-indoxyl phosphate **70** (CHROMagar *Staph. aureus*, CHROMagar, 2000US6548268, 2000JCM1587).
Phosphatase substrates have also been included for non-direct detection of *Candida albicans*, *i.e.* to allow a better differentiation of *C. albicans* by tagging other *Candida* species in different colors (CHROMagar *Candida,* 1996JCM454).

1.1.4.3.1 Phosphatidylinositol phospholipase C (PI-PLC)

Phosphatidylinositol phospholipase C (PI-PLC) is a periplasmic enzyme secreted by several bacteria including pathogenic strains of *Listeria* spp. (*L. monocytogenes* and *L. ivanovii*), *Staphylococcus* spp., *Bacillus* spp. and *Clostridium* spp. (2000US6051391). Its main application in microbial detection is the differentiation of *L. monocytogenes*, the food-borne pathogen causative of meningitis, septicaemia and miscarriage in pregnant women (1996MI195), from other less pathogenic *Listeria* species (BCM-LMDS, Biosynth (2000IJF205) or Rapid L'MONO, Sanofi (2004MI1)).
Shashidhar *et al.* reported the use of 2-naphthol-*myo*-inositol-1-phosphate for the detection of PI-PLC activity (1991ABI10); however, it has been replaced by the more effective 5-bromo-4-chloro-3-indoxyl-*myo*-inositol-1-phosphate **71** (2000US6051391, **Scheme 1.19**).

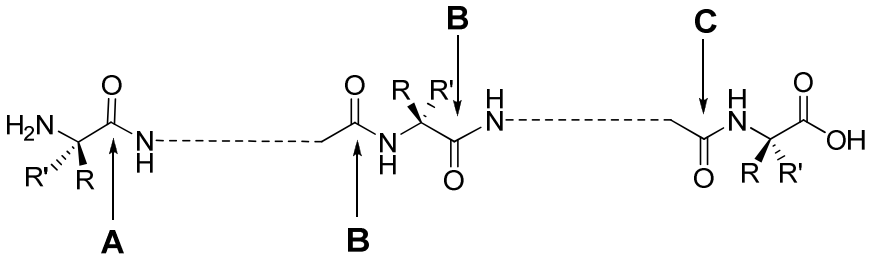


Scheme 1.19: PI-PLC hydrolysis of 5-bromo-4-chloro-3-indoxyl-*myo*-inositol-1-phosphate **71**.

1.1.4.4 Peptidase

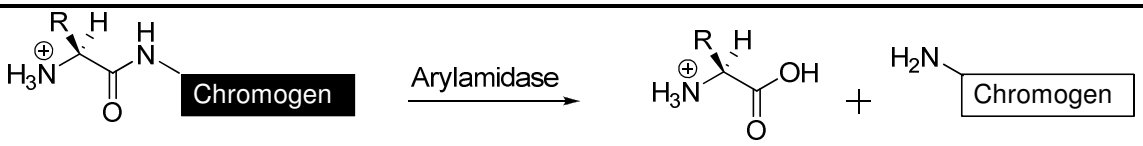
Peptidases are ubiquitous in bacteria, as they participate in the degradation of peptides into free amino acids and, hence, provide the essential supply of amino acids for the synthesis of new proteins (1996FMR319).
Peptidases can be classified into two groups: endopeptidases and exopeptidases. Endopeptidases hydrolyse peptide bonds anywhere within a peptide chain. Exopeptidases hydrolyse peptide bonds only at the extremities of a peptide chain;

after hydrolysis, carboxypeptidases leave a free carboxylic acid group on the *C*-terminal end of the peptide chain, while aminopeptidases leave a free amino residue at the *N*-terminal end of the polypeptide (**Scheme 1.20**, 1996FMR319).



Scheme 1.20: Peptidase classification: **A**, aminopeptidase (exopeptidase); **B**, endopeptidase; **C**, carboxypeptidase (exopeptidase).

Some peptidases have promiscuous activity, others are highly specific for peptide bonds to a few particular amino acids (1996FMR319). **Table 1.1** summarise the wide range of arylamidase enzymes actually used in manual and automated enzyme test kits, mentioned in section **1.1.2.5.2**.



Alanyl	**Glycine**	**Lysyl-alanine**
β-Alanyl	**Glycyl-arginine**	**Methionine**
Alanyl-alanine	**Glycyl-glycine**	**Phenylalanine**
Alanyl-phenylalanyl-proline	**Glycyl-proline**	**Proline**
Arginine	**Glycyl tryptophan**	**Pyrrolidonyl**
Arginyl-arginine	**Histidine**	**Serine**
Aspartic acid	**Hydroxyproline**	**Seryl-tyrosine**
Citrulline	**Isoleucine**	**Tryptophan**
α-Glutamic acid	**Leucine**	**Tyrosine**
Glutamyl-glutamic	**Leucyl-glycine**	
Glutamyl-glycyl-arginine	**Lysine**	

Table 1.1: Known arylamidase activities which are exploited for bacterial detection (2009JMM139).

1.1.4.4.1 Pyroglutamyl aminopeptidase

L-Pyroglutamyl aminopeptidase or L-pyrrolidonyl aminopeptidase, more commonly named PYRase, was first described in *Bacillus subtilis* (1969EJB63). This enzyme is prevalent in group A streptococci, *Streptococcus pyogenes*, and group D enterococci, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus avium* and *Enterococcus durans* (1983JCM1275, 1987JCM1805, 1996JCM1811). As a result, PYRase substrates have been suggested as a complementary test for the identification of these species (1987MI283). The PYRase activity test using L-pyroglutamyl-β-naphthylamide paper strip is extremely quick; incubation of the bacterial colonies on a paper strip containing the enzyme substrate usually gives a positive reaction within 10-20 minutes (1987JCM1805, 1996JCM1811). Although L-pyroglutamyl-β-naphthylamide has been the most commonly reported substrate (1991MR335), L-pyroglutamyl-7-amido-4-methylcoumarin (1978JB1145) and its 4-trifluoromethyl derivative (1990APF326) or L-pyroglutamylnitroanilide (1978JB1145) have also been used successfully.

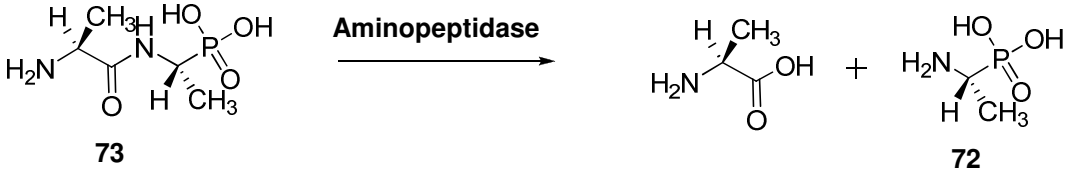
1.1.4.4.2 L-Alanine aminopeptidase

L-Alanyl aminopeptidase (L-ALA) is localised in the cell wall of Gram-negative bacteria (1990JAB822). Cerny reported the prevalence of L-ALA in Gram-negative bacteria and the absence of the latter in Gram-positive bacteria using L-alanyl-*p*-nitroanilide (1976EJM223). Manafi *et al.* screened for L-ALA against a wide range of Gram-negative and Gram-positive positive organisms using four different L-ALA aminopeptidase substrates, and suggested this test was a reliable alternative to the Gram stain (1990JAB822).

The following chromogens were reported for the detection of Gram-negative organisms: 4-methoxy-β-naphthylamine (1990JAB822), 2-aminoacridone (1990JAB822), 7-amino-4-methylcoumarin (1990JAB822) and derivatives of 9-(4'-aminophenyl)-10-methylacridinium salts (2008BML832). The Gram-Sure test (Remel, USA) is an example of a commercially available test for the differentiation of Gram-positive and Gram-negative bacteria, based on L-alanyl aminopeptidase fluorogenic substrates.

An alternative use of L-ALA activity, although not directly related to chromogenic substrates, is the selective release of the antibiotic, fosfalin **72**. Alafosfalin **73**, which has a general low toxicity, is known to be stereospecifically recognised by bacteria

(1979AAC677). The antibiotic L-α-aminoethylphosphonic acid (fosfalin, **73**), is released upon selective aminopeptidase hydrolysis (**Scheme 1.21**), and interferes with alanine racemase, which plays an essential role in the crosslinking of the peptidoglycan layer, resulting in growth inhibition (2002JCC3913).



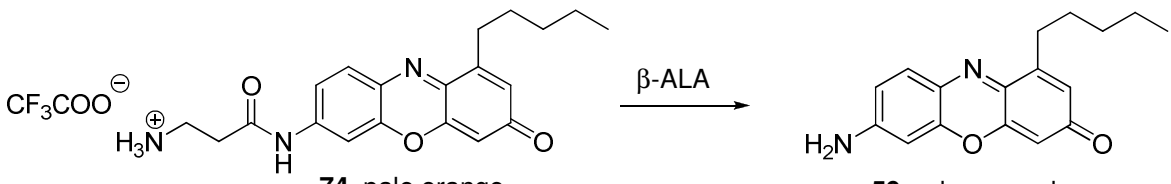
73 **72**

Scheme 1.21: Release of L-α-aminoethylphosphonic acid *via* aminopeptidase activity.

Perry *et al.* have demonstrated that *Salmonella* spp. are resistant to alafosfalin, possibly due to non-permeation of alafosfalin through the cell membrane, and have exploited those results to develop a selective medium for the detection of *Salmonella* in stool samples (2002JCC3913, 2002WO24725).

1.1.4.4.3 β-Alanine aminopeptidase

β-Alanine aminopeptidase (β-ALA) was recently isolated from *Pseudomonas* sp. (2005MI3075) and appeared to be very specific to β-alanine dipeptides. The preparation of β-alanine derivatives of 9-(4'-aminophenyl)acridines (2007BML1418), 9-(4'-aminophenyl)-10-methylacridinium salts (2008BML832), and 7-aminophenoxazinones (2008OBC682) have confirmed the occurrence of β-ALA in *Ps. aeruginosa*, but also detected β-ALA activity in *Serratia marcescens* and in *Burkholderia cepacia*. Consequently, a chromogenic medium for the specific detection of *Ps. aeruginosa* has been developed (PS-ID, bioMérieux, 2009MI143), using the β-alanine derivative **74** of the potent chromogen 7-amino-1-pentylphenoxazin-3-one (**59a**, **Scheme 1.22**).

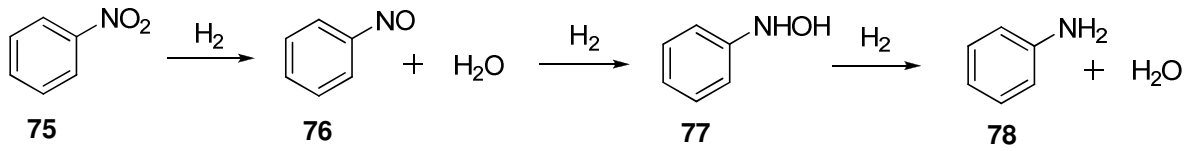


74, pale orange **59a**, deep purple with metallic sheen

Scheme 1.22: Detection of β-alanine aminopeptidase using 7-amino-1-pentylphenoxazin-3-one (2008OBC682).

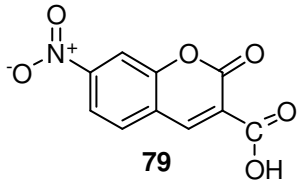
1.1.4.5 Nitroreductase enzymes

It appears that the reduction of 2,4,6-trinitrotoluene by bacteria was the first nitroreductase activity reported in the literature by Channon (1944BJ70). A three-step pathway has been suggested by Yamashina (1954BCJ42) for the enzymatic reduction of nitroaromatic compounds **75**, involving nitroso **76** and hydroxylamine **77** intermediates (**Scheme 1.23**). Experiments involving the monitoring of molecular hydrogen absorbance by bacteria during reduction confirmed this hypothesis (1976AEM949).

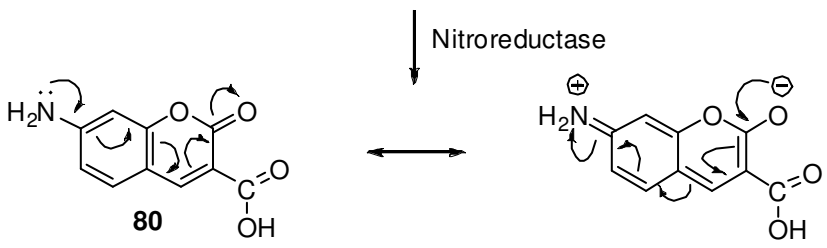


Scheme 1.23: Suggested enzymatic reduction pathway of nitro compounds (1954BCJ42).

The nitroreductase enzymes have been subdivided (1970JBA1126, 1979JBC4009, 1980CJM81) into two different classes, namely “reductase I” and “reductase II” types, based on their sensitivity to oxygen. The “reductase I” type corresponds to an oxygen-insensitive enzyme, following the three step reduction pathway described above (**Scheme 1.23**), while the “reductase II” type is an oxygen-sensitive enzyme (1979JBC4009), with a reduction pathway initiated with a nitro radical anion, extremely reactive toward molecular oxygen.



No emission under long range U.V. excitation



Bright blue fluorescence under long range U.V. excitation

Scheme 1.24: Nitroreductase activity evidenced by 7-nitrocoumarin-3-carboxylate (2001LAM403).

James *et al.* evidenced nitroreductase activity using several 7-nitrocoumarin derivatives (2001LAM403). Amongst the 7-nitrocoumarins tested, 7-nitrocoumarin-3-carboxylic acid (**79**, **Scheme 1.24**) is reduced, upon enzymatic activity, to the highly fluorescent 7-aminocoumarin-3-carboxylic acid (**80**, **Scheme 1.24**).

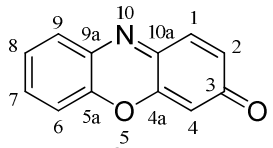
Nitroreductase activity was hence evidenced in a wide range of clinically important bacteria and yeasts. 7-Nitrocoumarins have potential application as growth indicators, due their general activity amongst microbial genera and their stability as fluorescent markers.

1.2 Occurrence of the phenoxazinone core

1.2.1 Natural products

1.2.1.1 Ommochromes

The phenoxazinone core **81** was first discovered occurring as a natural pigment. These pigments were referred to as ommochromes, the name originating from its first localisation: Becker isolated this new type of pigment from the eyes of the arthropod *Ephestia kühniella* (1939MI597, 1941N237).



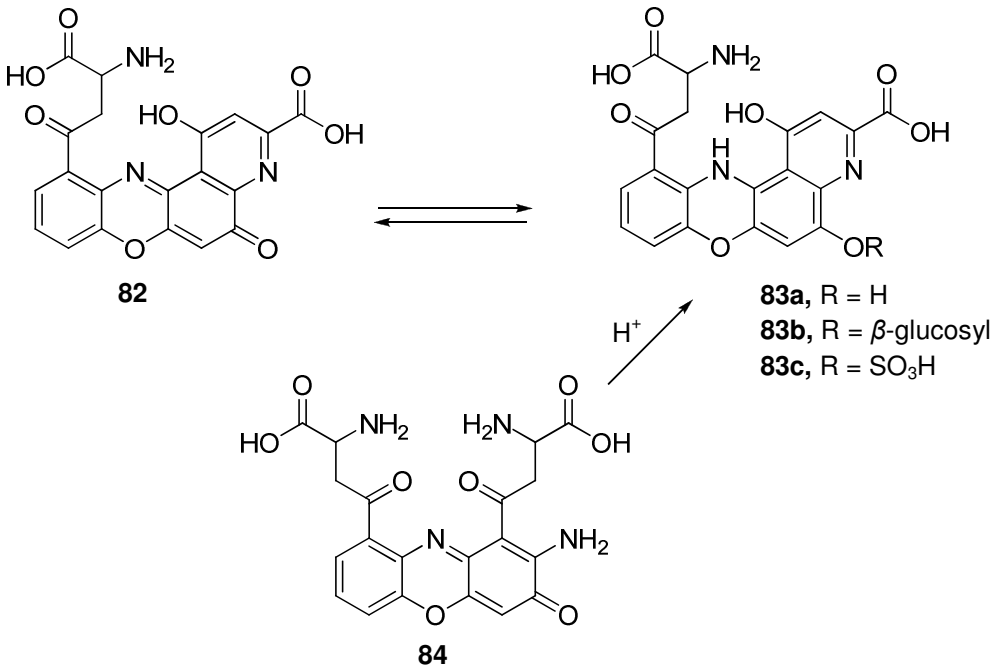
81

They have been isolated since in a wide range of insects from the order of *Lepidoptera*, *Diptera*, *Odonata*, *Orthoptera*, *Phasmida* and *Hymenoptera* (B-1974MI10).

Much work was undertaken by Butenandt who later successfully elucidated (1954LA217, 1954LA229) and synthesised (1954LA106) xanthommatin **82** (**Scheme 1.25**). Xanthommatin is a yellow pigment which is readily and reversibly reduced to a red pigment, 5,12-dihydroxyxanthomatin **83a** (**Scheme 1.25**). This transformation is thought to occur *via* an enzymatic process, possibly *via* a specific xanthommatin reductase enzyme, as reported by Santoro *et al.* (1986MI169). Both ommochromes (**82** and **83a**), amongst other type of pigments, are present in arthropods' eyes, more precisely located between the

Figure 1.16: Eye pigmentation of *Drosophila melanogaster* (1998G1551): Wild type (left) and mutant lacking ommochrome pigmentation (right) (with the authorisation of Prof. Kent Golic)

individual ommatidium (1972MI616) and are responsible for the eye colouration (**Figure 1.16**). Xanthommatin and 5,12-dihydroxyxanthomatin act mainly as screening pigments: screening pigments affect light perception by lowering the sensitivity of photoreceptor cells, resulting in a decreased "background illumination" of the photoreceptors, hence increasing acuity (B-1974MI11). This role was confirmed by experiments showing that *Drosophila* mutants devoid of ommochromes had reduced contrast perception.



Scheme 1.25: Xanthommatin **82**, its reduced **83a** and trapped reduced forms **83b**, **83c**.

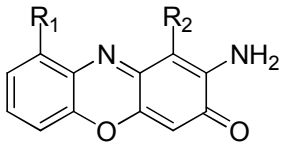
The 5,12-dihydroxyxanthomatin form **83a** is responsible for the formation of two other ommochromes, namely rhodommatin **83b**, the β-*O*-glucoside derivative of dihydroxyxanthomatin, and ommatin D **83c**, the sulphate ester derivative of dihydroxyxanthomatin (**Scheme 1.25**). These pattern pigments have been isolated from the wings of various butterflies (1997MI215).

A fifth type of pigment, **84**, was isolated by Bolognese from the eyes and skin of cephalopods (1988JHC1243). This pigment was recognised as a precursor of 5,12-dihydroxyxanthomatin, which was produced when **84** was stirred under acidic conditions (1988JHC1243, **Scheme 1.25**).

There is no reported biological activity for ommochromes and study of those compounds was mostly related to the understanding of their biosynthetic pathways, and their distribution in living entities.

1.2.1.2 Mould metabolites

Cinnabaric acid **85a**, cinnabarin **85b** and tramesanguin **85c** were isolated by Cavill *et al.* (1959T275, 1961T139) and Gripenberg *et al.* (1957ACB1485, 1958ACB603, 1963ACB703) from wood-rotting fungi, namely *Coriolus sanguineus*, various species of *Polystictus sanguineus* and *Trametes cinnabarina*. Later, Sullivan *et al.* isolated the same pigments from three other fungi belonging to the *Pycnoporus* species (1971JPS1097). Very recently, Dias *et al.* (2009NPC489) isolated a novel phenoxazinone pigment from *Picnoporus cinnabarinus* : pycnoporin **85d**. These phenoxazinones were isolated as red crystalline solids, responsible for the bright red-orange pigmentation of the fungi.

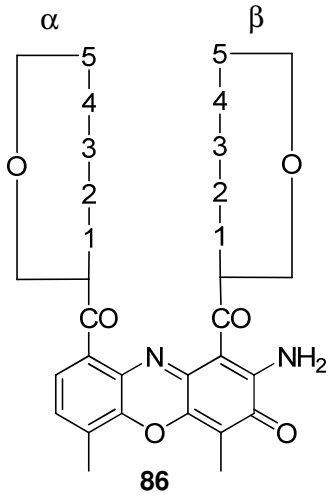


85a $R_1 = R_2 = COOH$
85b $R_1 = CH_2OH$, $R_2 = COOH$
85c $R_1 = COOH$, $R_2 = CHO$
85d $R_1 = CH(OCH_3)OH$, $R_2 = COOH$

Cinnabarin was reported to possess anti-viral and anti-microbial activity (1998MI317, 1999MI89, 2003MI1069), as well as some significant antitumor activity (2009NPC489).

1.2.1.3 Actinomycins

Actinomycins (**Figure 1.17**, **86**) are, without contest, the most well known and studied structure related to the phenoxazinone core. They were first isolated by Waksman and Woodruff from cultures of *Actinomyces* (*Streptomyces*) *antibioticus* (1940MI609), who observed high bacteriostatic and bactericidal activities over a range of both Gram-positive and Gram-negative bacteria. The structure of actinomycins was classified by Brockmann as a chromopeptide (1950N494): it is composed of a chromophore moiety, 2-amino-1,9-dicarboxyl-4,6-dimethylphenoxazin-3-one, and two cyclopeptide chains (**86**). Three particularly well known actinomycins are actinomycin C_1 (D) (**86a**, 1964N384), actinomycin C_2 (**86b**) and actinomycin C_3 (**86c**, 1960N230); all show high anti-tumour activity in human cancer (1974MI49). Actinomycin D is currently used to treat a variety of paediatric cancers (B-2006MI12).



86

Figure 1.17: General structure of actinomycins

86a- Actinomycin C_1 (or D)
α=β: 1 = L-Thr, 2 = D-Val, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-L-Val
86b- Actinomycin C_2
α: 1 = L-Thr, 2 = D-Val, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-L-Val
β: 1 = L-Thr, 2 =L-Ile, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-L-Val
86c- Actinomycin C_3
α=β: 1 = L-Thr, 2 =L-Ile, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-L-Val
86d- Actinomycin E_1
α: 1 = L-Thr, 2 = D-Val, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-L-Val
β: 1 = L-Thr, 2 =L-Ile, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-Ile
86e- Actinomycin E_2
α=β: 1 = L-Thr, 2 =L-Ile, 3 = L-Pro, 4 = Sar, 5 = *N*-methyl-Ile

However, actinomycins use is limited due to their high toxicity toward normal cells and many derivatives have been made by either modifying, *via* chemical synthesis, the chromophore moiety (1966JOC3694, 1975JMC1175, 1990LA1269), or the sequence of amino acids (1964TL3523, 1966TL2331, 1972JACS4759), in an attempt to lower its toxicity.

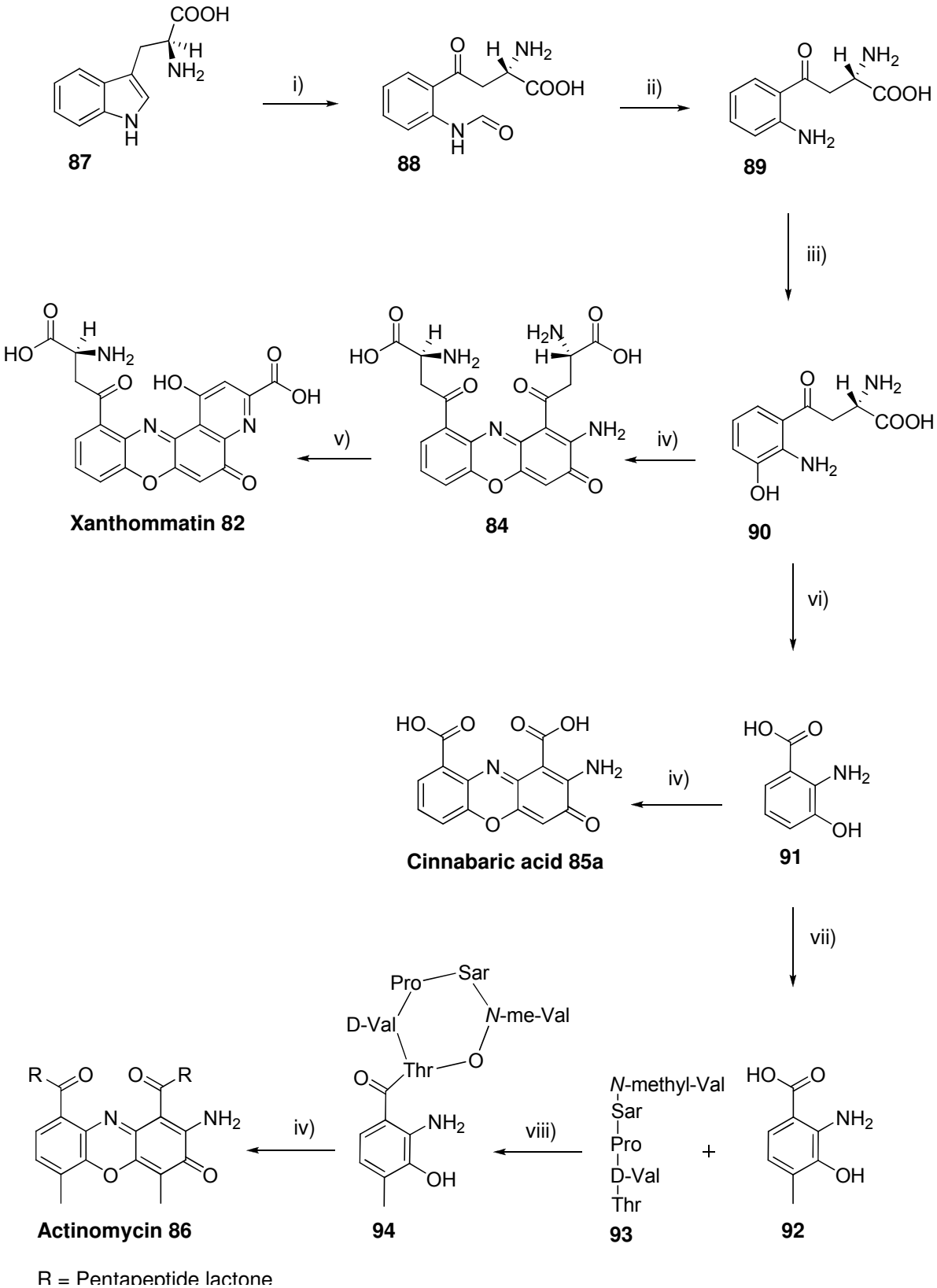
The amino acid sequence was also successfully modified *via* controlled biosynthesis, a technique involving the introduction of similar amino acids to the ones present in the actinomycin peptide chain into the microorganism growing medium (1974CRV625). As an example, actinomycin E_1 (**86d**) and E_2 (**86e**) (**Figure 1.17**) were produced by *Streptomyces antibioticus* in the presence of DL-*iso*-leucine.

The efficiency of actinomycin is tightly bound to its complex structure and the way it specifically intercalates with DNA (1974CRV625); this was impaired with the introduction into the 4,6-positions of a bulkier alkyl chain or a methoxy group, the absence of the methyl group, modification of the amino group in the 2-position, or modification of the cyclopentapeptide chain and resulted in a considerable decrease of activity for the resulting analogues (1974CRV625).

Despite intensive research in the preparation of new actinomycin derivatives, no analogues with superior activity to that of actinomycin D were discovered and research in the field is now less important.

1.2.1.5 Biosynthesis of the phenoxazinone core

It is worthy to note that ommochromes, cinnabaric acid and its analogues, as well as actinomycin, all share a common core structure, namely 2-amino-3*H*-phenoxazin-3-one.



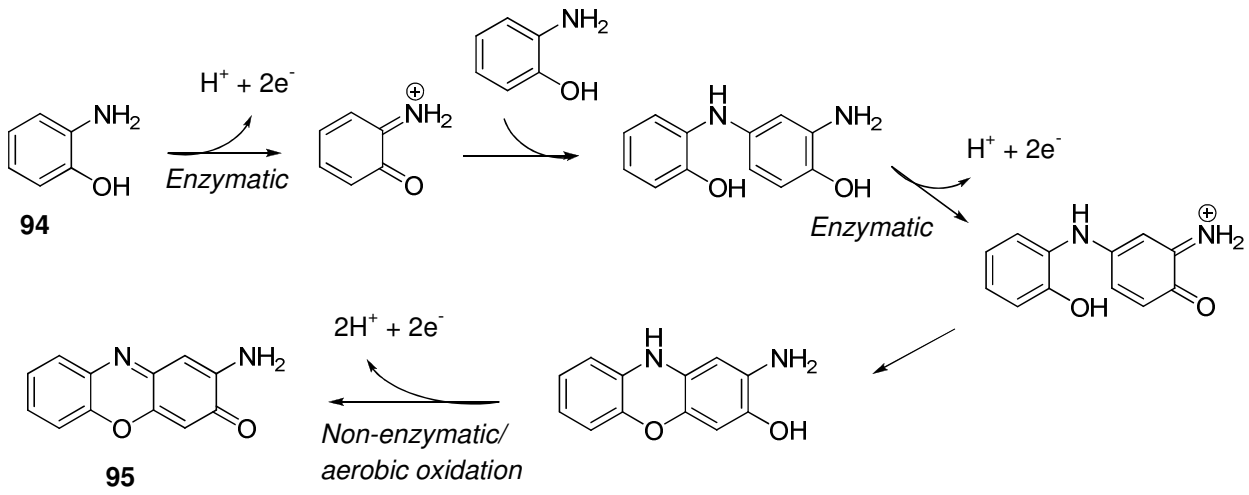
Scheme 1.26: Analogy in the biosynthesis of xanthommatin, cinnabaric acid and actinomycins (1965JBC4377, 1974MI47, 1974CRV625):
i) Tryptophan pyrrolase, ii) Kynurenine formamidase, iii) Kynurenine-3-hydroxylase, iv) Phenoxazinone synthetase, v) [Oxidative-cyclisation], vi) Kynureninase, vii) [Methylation], viii) [Peptide formation].

This similarity arises from their common precursor, tryptophan **87**, as well as a very similar biosynthetic pathway (**Scheme 1.26**), although they are all produced by different living entities.

Tryptophan is enzymatically opened to *N*-formylkynurenine **88**, followed by *N*-deformylation to produce kynurenine **89**. Subsequent hydroxylation leads to 3-hydroxykynurenine **90**, which is the known precursor for both enzymatic (1974MI47) and chemical (1954LA75) preparation of xanthommatin **82**, *via* the phenoxazin-3-one intermediate **84** (**Scheme 1.26**). 3-Hydroxykynurenine **90** is further transformed to 3-hydroxyanthranilic acid **91**, the precursor of cinnabaric acid **85a** (**Scheme 1.26**).

In the biosynthesis of actinomycin, 3-hydroxyanthranilic acid **91** is further methylated by methionine, to give 4-methyl-3-hydroxyanthranilic acid (4-MHAA, **94**), which is linked to a pentapeptide chain **93** followed by lactonisation of the latter once attached to 4-MHAA, leading to 4-MHAA pentapeptide lactone **94** (1974CRV625, 1993JBC10612). The oxidative coupling of two identical 4-MHAA pentapeptide lactone moieties leads to actinomycin **86** (**Scheme 1.26**).

The enzymatic oxidative coupling of two *o*-aminophenol moieties **94** to 2-aminophenoxazin-3-one **95** is known to occur with different enzymes, phenoxazinone synthase (phsA) being responsible for the formation of actinomycin in *Streptomyces antibioticus* (1961JBCPC16, 1962JBC882, 1989B6323), but laccase (1999MI141), tyrosinase (1998BBA268), peroxidase (2008MI579), cytochrome c and cytochrome oxidase (1992B8090) and cerruloplasmin (1998BBA268) have all been reported to catalyse the reaction (**Scheme 1.27**).



Scheme 1.27: Formation of 2-aminophenoxazin-3-one **90** via an *o*-aminophenol **112** oxidative cascade (1989B6323).

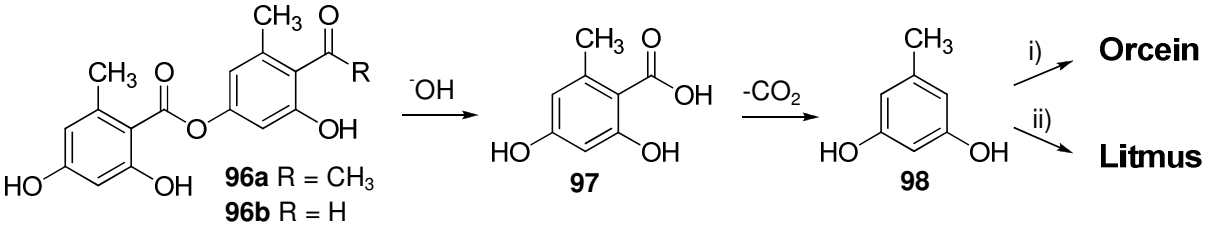
Barry *et al.* studied the enzymatic oxidation of *o*-aminophenol and its derivatives and proposed a cascade of three consecutive 2-electron oxidations for the biosynthetic formation of 2-aminophenoxazin-3-ones (1989B6323, **Scheme 1.27**).

The understanding of the enzymatic pathway, and the chemistry developed to identify and prepare the intermediates, allowed for an important advance in the field of phenoxazinone chemistry, particularly for their preparation.

1.1.2.2 Phenoxazinone dyes

1.1.2.2.1 Litmus and orceins

Litmus and orcein refer to a variable and relatively poorly defined mixture of dyes, mainly composed of phenoxazinone and phenoxazimine derivatives (B-2002MI05), and was traditionally manufactured from *Roccella*, *Lecanora* and *Varialaria* lichens. These lichens contain orsellinic acid depsides **96a-b** which, upon basic hydrolysis release *o*-orsellinic acid **97**. Decarboxylation leads to the production of orcinol **98**, which, upon treatment with ammonia and air, give the dye named orcein and, in the case of litmus, lime ($Ca(OH)_2$), potash (K_2CO_3) and gypsum ($CaSO_4$) were also added (**Scheme 1.28**, 2003MI289).



Scheme 1.28: Chemical reactions occurring during the extraction and manufacturing of orcein and litmus from lichens: i) NH_3, aerial oxidation; ii) NH_3, aerial oxidation, $Ca(OH)_2$, K_2CO_3, $CaSO_4$ (2003MI289)

Musso and co-workers studied extensively the oxidation mechanism of orcinol in ammonia (1965CB3952, 1965CB3964, 1963CB1588) and succeeded in the separation and characterisation of the low molecular weight oxidation products (1963CB1579, 1963CB1593), which are part of the litmus and orcein dye mixture. Amongst the products characterised, four general structures related to phenoxazinone were isolated: α-orcein **99a-b**, β- and γ-orcein **100a-b**, β- and γ-hydroxyorcein-monoquinone **101**, β- and γ-hydroxyorcein-diquinone **102** (**Figure 1.18**).

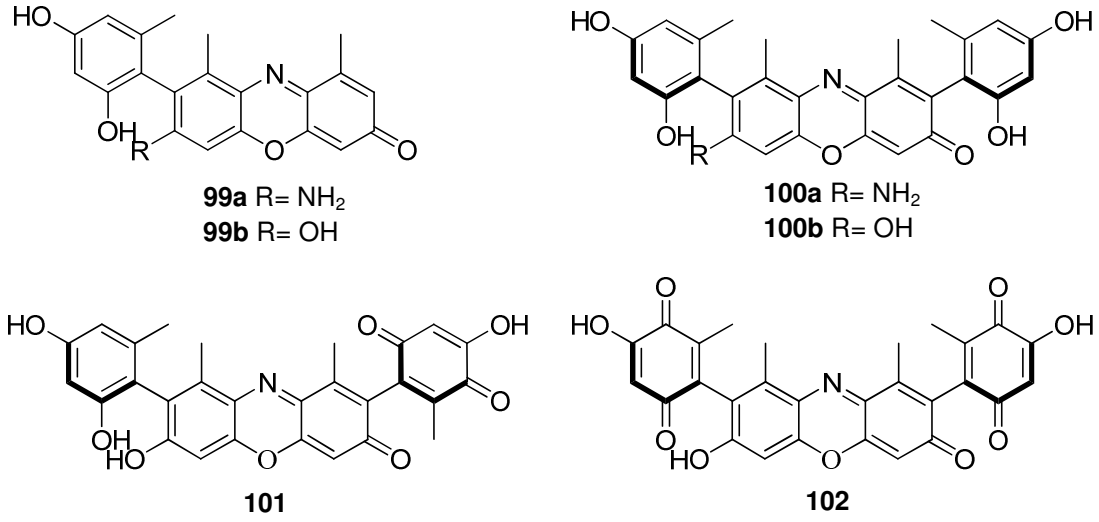
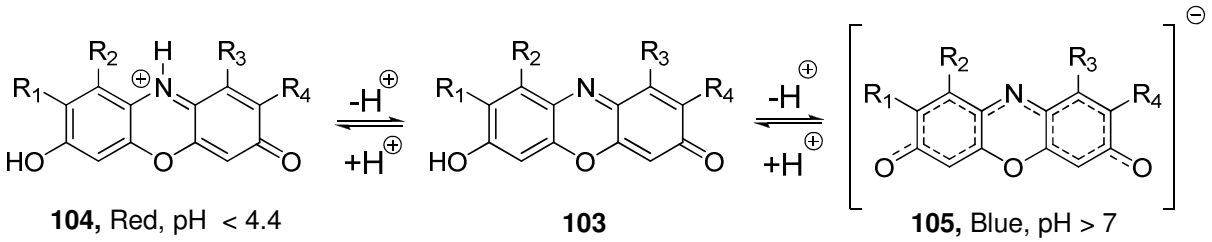


Figure 1.18: Orceins resulting from the oxidation of orcinol in ammonia

The core structure was either 7-amino or 7-hydroxy-1,9-dimethylphenoxazinone. The two isomers designated by β- and γ- refer to the relative position of the two orcinol substituents: β- designates the *trans* isomer, represented on structure **101**, and γ- the *cis* isomer, represented on structures **100** and **102**.

Orcein and litmus were originally used to dye silk and wool, and are nowadays used as a biological stain or as pH indicator; they have also been described in hair dyeing preparations (2006FR2907005).

The colour change observed upon pH variation was demonstrated to be caused by hydroxyphenoxazinones **103**, which is protonated at acidic pH (pH 4.4) to generate the red cation **104** and above pH 7 is deprotonated to afford the blue-violet cation **105** (**Scheme 26**, 2003MI289).



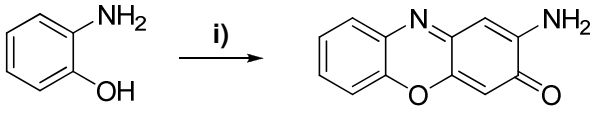
Scheme 1.29: pH indicator species present in litmus and their corresponding acid and basic ions (2003MI289).

1.3 Chemical synthesis of the phenoxazinone core

The preparation of phenoxazinones has been reviewed previously by Pearson (B-1960MI12), Landquist (B-1979MI13), and Schäfer (1964POC135). The present review will attempt to classify the synthesis of phenoxazinones based upon their mechanism of formation, as well as give an update of the most recent synthesis. However, interest in the field of phenoxazinones has decreased over the last two decades, possibly due to the lack of new discoveries since the actinomycins, and very few novel methods have been developed regarding their synthesis.

1.3.1 Oxidative condensation of *o*-aminophenol

The most important and widely applied synthesis of phenoxazinones is probably the oxidative condensation of *o*-aminophenol and *o*-aminophenol derivatives. This reaction mimics the biological formation of phenoxazin-3-one *via* the use of various oxidising agents. The first reaction was reported by Fischer *et al.*, with the autooxidation of *o*-aminophenol **94** to 2-aminophenoxazin-3-one **94** in acidic solution using ferric chloride or potassium dichromate (**Scheme 1.30**, 1890MI2792).

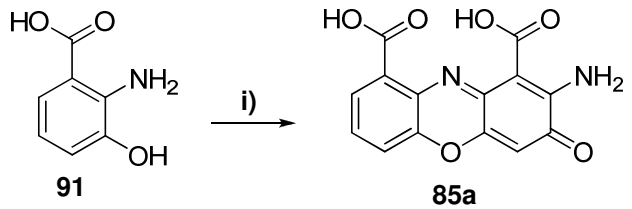


94 **95**

Scheme 1.30: Oxidative condensation of *o*-aminophenol
i) $FeCl_3$ or $K_2Cr_2O_7$, CH_3COOH; yield not given (1890MI2792).

The same results were achieved in neutral solution using mercuric oxide (1894CB2784) or lead dioxide (1902MI2816). The mechanism of formation is analogous to that of the biological synthesis described previously in section **1.2.1.5** (**Scheme 1.28**). This analogy has been highly exploited for the preparation of naturally occurring phenoxazin-3-ones: actinomycin C_3 (1960N230) and actinomycin C_1 (D) (1964N384), as well as various actinomycin-related analogues (1966JOC3694, 1971JHC989, 1975JMC1175, 1990LA1269), were prepared *via* oxidative condensation of a single substituted *o*-aminophenol unit.

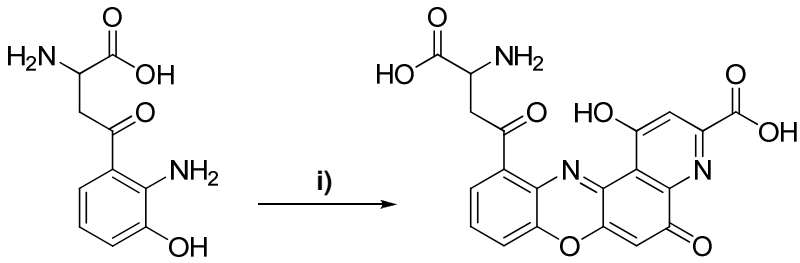
The preparation of the mould metabolite cinnabarinic acid **85a** was achieved *via* the oxidative condensation of 3-hydroxyanthranilic acid **91** with manganese dioxide (1978CA22792d, **Scheme 1.31**).



91 **85a**

Scheme 1.31: Oxidative condensation of 3-hydroxyanthranilic acid **91**
i) MnO_2, 92% (1978CA22792d).

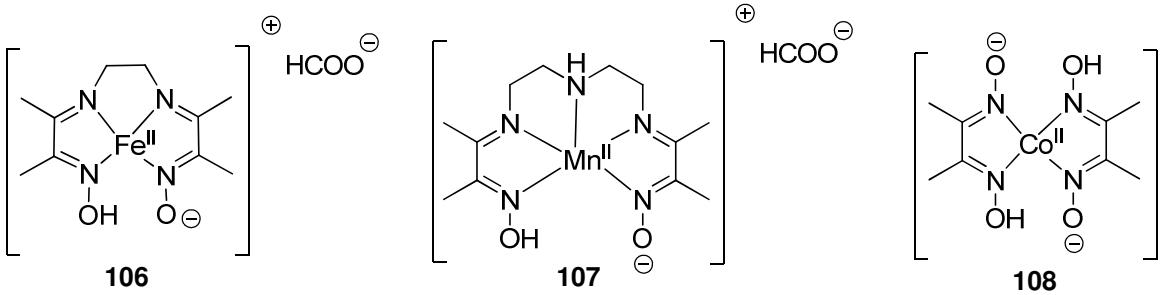
Another remarkable example of the application of the oxidative condensation of *o*-aminophenol for the preparation of natural products is the synthesis of xanthommatin (**Scheme 1.32**, 1954LA75).



90 **82**

Scheme 1.32: Synthesis of xanthommatin **82**
i) $K_3Fe(CN)_6$, K_3PO_4, H_2O (pH = 7.1) (1954LA75).

More recent studies on the oxidative condensation of *o*-aminophenol have focussed on mimicking the enzyme phenoxazinone synthase *via* the use of various metal complexes, such as ferroxime (II) (2004JCD1056, 2006ICA2329, **Figure 1.19**, **106**), dioximatomanganese (II) (2006JMO(A)270, (**Figure 1.19**, **107**), or cobaloxime (II) (1996JCD473, 1998JCD3275, **Figure 1.19**, **108**).

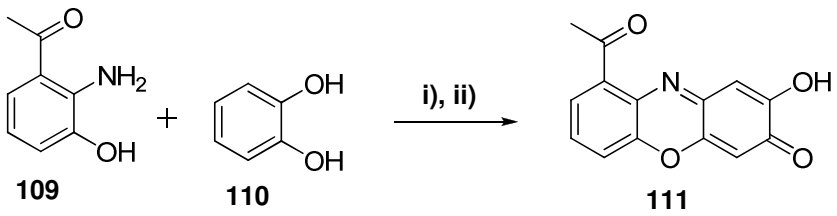


106 **107** **108**

Figure 1.19: Example of metal complexes used for the catalytic oxidation of *o*-aminophenols.

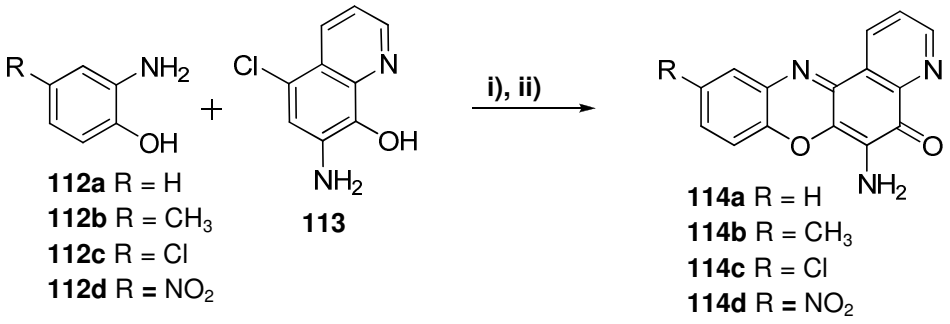
1.3.2 Oxidative mixed condensation of *o*-aminophenol

The first mixed condensation of *o*-aminophenol was observed by Butenandt *et al.* (1957LA72) who reported the formation of 2-hydroxy-1-acetylphenoxazin-3-one **111** in moderate yield when 2-amino-3-hydroxyacetophenone **109** was reacted with catechol **110** in acetic acid, using potassium ferricyanide (**Scheme 1.33**).



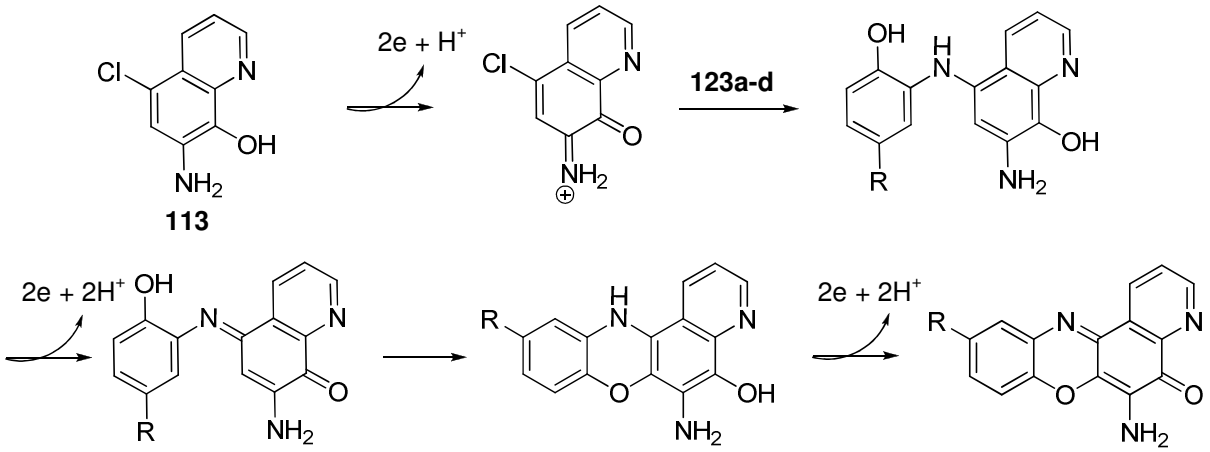
Scheme 1.33: Synthesis of 2-hydroxy-1-acetylphenoxazin-3-one
i) $K_3(FeCN_6)$, AcOH; ii) H_2O; 43% (1957LA72).

Ruan *et al.* reported another type of mixed condensation, between *o*-aminophenols **112a-d** and 7-amino-5-chloroquinolin-8-ol **113**, in a recent study on the antiproliferative activities of some phenoxazin-3-one derivatives **114a-d** (**Scheme 1.34**, 2006CCL1141).



Scheme 1.34: Synthesis of 6-amino-5*H*-pyrido[3,2*a*]phenoxazin-5-one (2006CCL1141)
i) $NaIO_3$, acetone, H_2O, 2h; ii) $NaIO_3$, H_2O, 20h, R.T.; 40-58%, specific yields not given.

The 6-amino-5-*H*-pyrido[3,2*a*]phenoxazin-5-ones **114a-d** were produced in moderate yield by condensing the *o*-aminophenols **112a-d** in aqueous acetone using sodium periodate (**Scheme 1.34**).

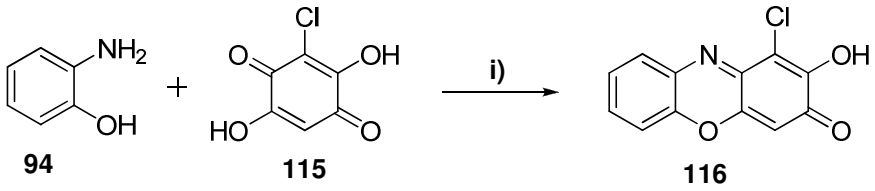


Scheme 1.35: Suggested mechanism of the condensation between *o*-aminophenols **112a-d** and 7-amino-5-chloroquinolin-8-ol **113**.

No study of the mechanism was performed; however, the author suggested the condensation occurred *via* three 2-electron oxidation steps and two conjugate addition processes (**Scheme 1.35**), similar to the mechanism of the self-condensation of *o*-aminophenols.

1.3.3 Condensation of *o*-aminophenols with hydroxyquinones

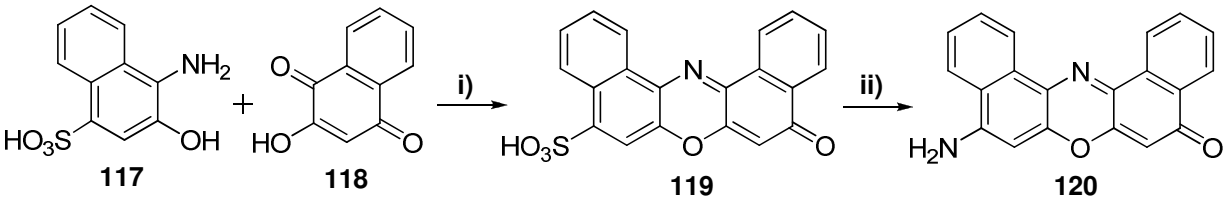
The condensation of *o*-aminophenol **94** with 2,5-dihydroxy-3-chlorobenzoquinone **115** to 1-chloro-2-hydroxyphenoxazin-3-one **116** was initially reported by Kehrmann and Messinger (**Scheme 1.36**, 1893CB2375).



Scheme 1.36: Condensation of *o*-aminophenol with 2,5-dihydroxy-3-chlorobenzoquinone i) aq. HCl, yield not given (1893CB2375).

Kehrmann (1895CB353, 1924HCA973) and later Butenandt *et al.* (1960MI143) extensively studied the condensation between *o*-aminophenol and 2-hydroxynaphthoquinone. This reaction was later adapted to the synthesis of xanthommatin (1954LA75), fluorescent probes (2001AC2920), or the synthesis of aminodinaphthoxazone (**120**, 2006US0121551). As an example, the synthesis of aminodinaphthoxazone **120** was achieved *via* the condensation of 1-amino-2-

naphthol-4-sulphonic acid **117** and 2-hydroxynaphthoquinone **118** in AcOH. The resulting dinaphthoxazonesulphonic acid **119** was then refluxed in the presence of ammonium to yield aminodinaphthoxazone **120** (2006US0121551, **Scheme 1.37**).

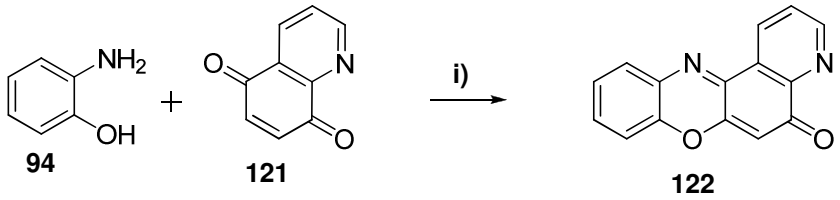


Scheme 1.37: Preparation of aminodinaphthoxazone **119**
i) AcOH, reflux; ii) aq. NH_3, reflux; yield not given (2006US0121551).

Similarly the condensation of *o*-aminophenol and 2-hydroxybenzoquinone to phenoxazin-3-one was also reported by Schäfer *et al.* (1971CB3937, 1971T4721, 1972T3811). However, as discussed by Schäfer *et al.*, the outcomes of this condensation depend on the substitution pattern of the *o*-aminophenol, the hydroxyquinone and the conditions used.

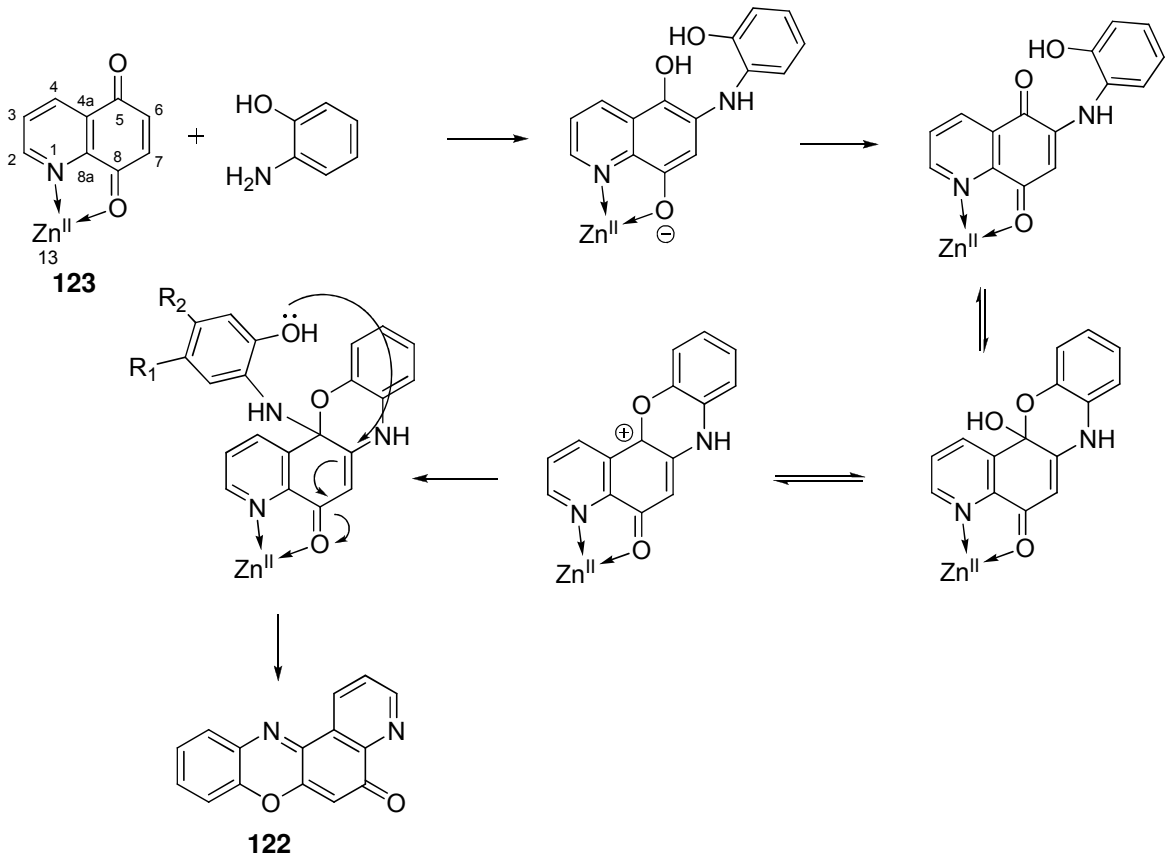
1.3.4 Condensation of *o*-aminophenol with quinolin-5,8-dione

Bolognese *et al.* described the formation of 5*H*-pyrido[3,4*a*]phenoxazin-5-one **122** by condensing *o*-aminophenol with quinolin-5,8-dione **121** in the presence of zinc acetate in refluxing acetic acid (**Scheme 1.38**, 2002JME5205, 2002JME5217).



Scheme 1.38: Preparation of 5*H*-pyrido[3,4a]phenoxazin-5-one **122**
i) $Zn(CH_3COO)_2$, AcOH, reflux, 2h; yield not given.

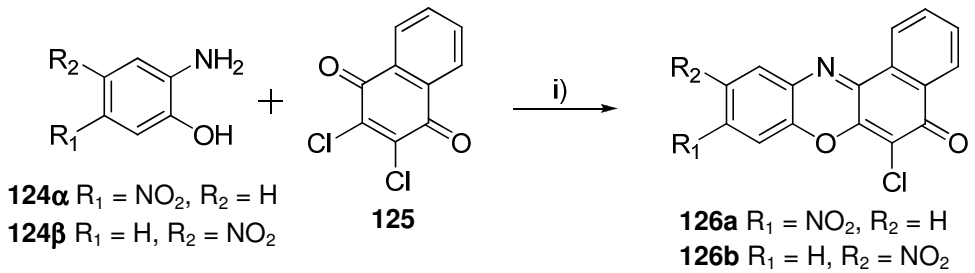
Once in solution, the zinc cation forms a complex **123** with quinolin-5,8-dione **121** which, according to the authors, has an increased electron density at the 7- position, hence favouring attack of the *o*-aminophenol in the 6-position. The mechanism of condensation involved sequential attack of two *o*-aminophenol molecules followed by elimination of one *o*-aminophenol molecule to give the 5*H*-pyrido[3,2*a*]phenoxazin-5-one (**130**, **Scheme 1.39**).



Scheme 1.39: Suggested mechanism for the formation of 5*H*-pyrido[3,4*a*]phenoxazin-5-one (2002JME5205)

1.3.5 Condensation of *o*-aminophenols with halogenated quinones

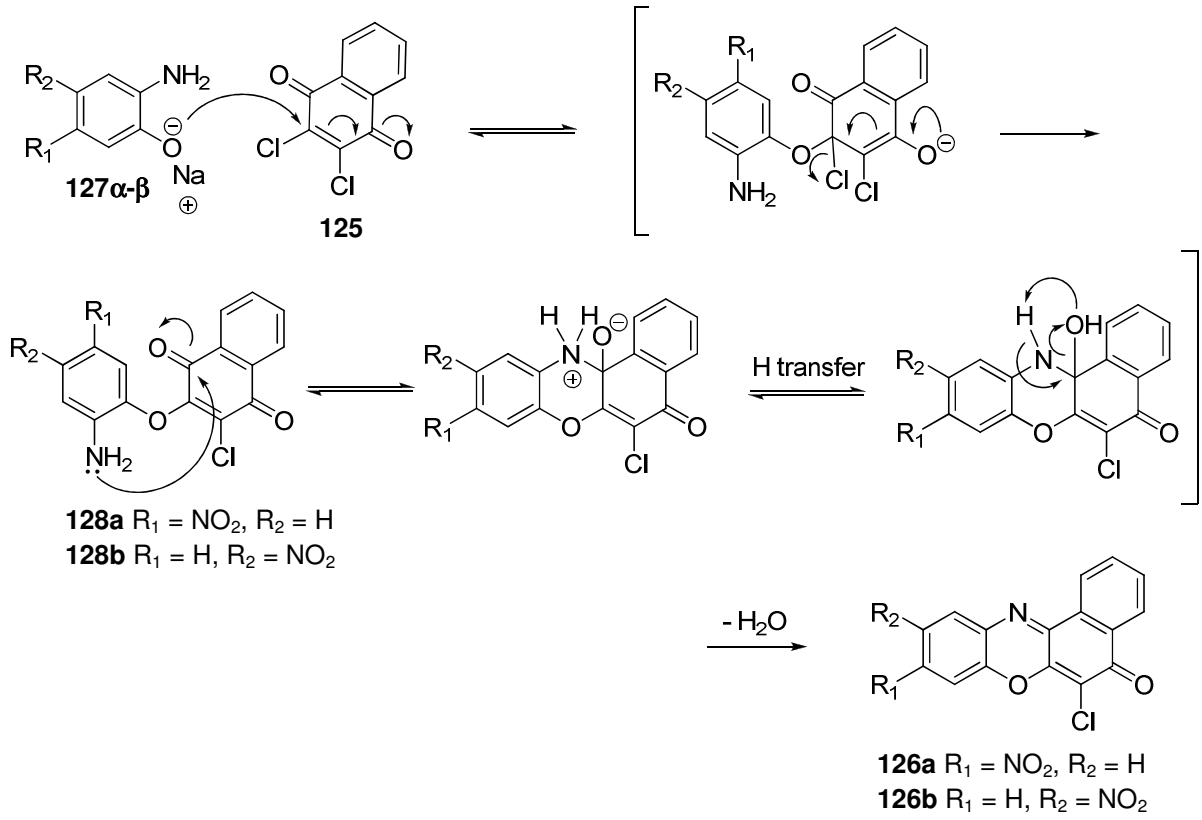
The condensation between 2-hydroxy-nitroaniline **124α-β** and 2,3-dichloro-1,4-naphthoquinone **125** in EtOH with NaOAc, yielded the corresponding nitro-6-chlorobenzo[*a*]phenoxazin-5-one **126a-b** in good yield (**Scheme 1.40**).



Scheme 1.40: Preparation of nitro-6-chlorobenzo[*a*]phenoxazin-5-one **126a-b**
i) EtOH, KOAc , Δ = 90-100°C; **126a**, 76% ; **126b**, 78% (1980JOC2155).

The products had potential application as dyes (1933US2020651) and have also been used as intermediates in the preparation of chromogenic substrates (2006US0121551).

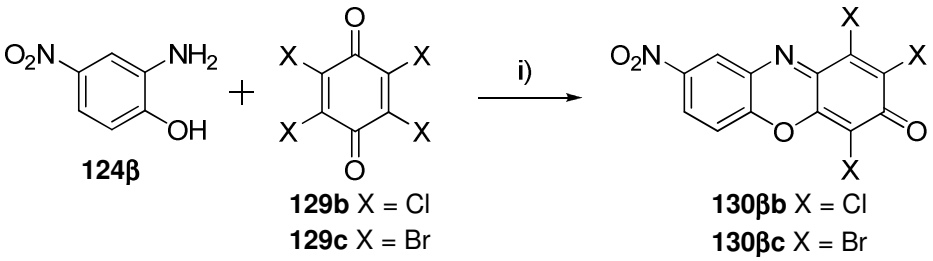
The mechanism of this condensation was studied by Agarwal and Schäfer (1980JOC2155) who suggested the formation of a nitro-*o*-aminophenoxide anion **127α-β** when sodium or potassium acetate was used. This resulted in a nucleophilic attack on 2,3-dichloronaphthoquinone by sodium nitroaminophenolate **127α-β**, forming the 2-phenoxy-3-chloronaphthoquinone intermediate **128a-b**, which underwent ring closure to form nitro-6-chlorobenzo[*a*]phenoxazin-5-one **126a-b** (**Scheme 1.41**).



Scheme 1.41: Suggested mechanism for the formation of nitro-6-chlorobenzo[*a*]phenoxazin-5-one (1980JOC2155).

An alternative, similar condensation was initially reported in 1933 (1933US2020651) and later by Mital and Jain (1971JC1875), with a slight modification of the conditions. This reaction involves a condensation between aminophenol **124β** and tetrahalogenated-1,4-benzoquinones (bromanil **129c**, chloranil **129b**), in a

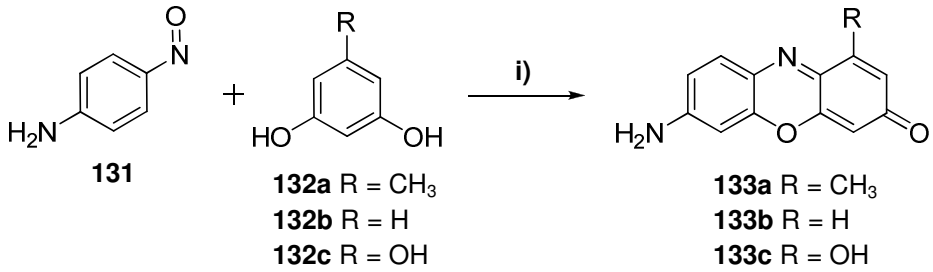
EtOH/NaOAc system, and yielded 8-nitrophenoxazinones **130βb-c** (**Scheme 1.42**). Little information is known about the mechanism of this reaction, but it is very likely to occur through a similar pathway to the 2,3-dichloro-1,4-naphthoquinone condensation.



Scheme 1.42: Preparation of nitro-1,2,4-trihalogeno-3*H*-phenoxazin-3-one
i) EtOH, NaOAc, 85°C; **130βb**, 58.0%; **130βc**, 62.0%; (1971JC1875).

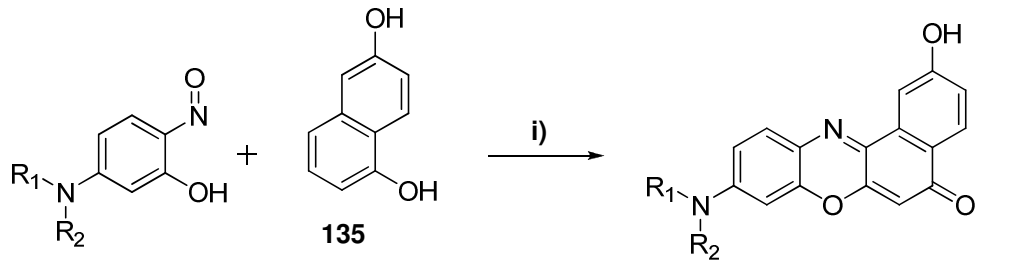
1.3.6 Condensation of *p*-nitrosoaniline and *o*-nitrosophenol derivatives with phenols

The first example of this type of condensation was reported by Fischer and Schäfer (1895LA145), who noted the condensation of *p*-nitrosoaniline **131** and orcinol **132a** to 7-amino-1-methylphenoxazin-3-one **133a** (**Scheme 1.43**). This method was later applied to other resorcinol derivatives **132b-c** by Stužka *et al.* (1969M1670, **Scheme 1.43**).



Scheme 1.43: Condensation of *p*-nitrosoaniline and resorcinol derivatives
i) conc. HCl, EtOH, reflux; **133a**, 1.5%; **133b**, 14.8%; **133c**, yield not given (1969M1670).

Jose *et al.* described a synthesis of Nile red derivatives **136a-b** by condensing *N,N*-disubstituted-4-amino-nitrosophenols **134a-c** with 1,6-dihydroxynaphthalene **135** in DMF or in acidic methanol (**Scheme 1.44**, 2006JOC7835, 2006T11021).

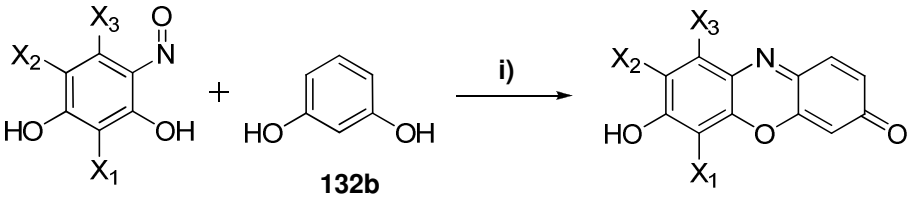


134a $R_1=R_2= (CH_2)_3OH$
134b $R_1=R_2= (CH_2)_2COOCH_3$
134c $R_1= (CH_2)_3SO_3H$
$R_2= (CH_2)_2COOH$

136a $R_1=R_2= (CH_2)_3OH$
136b $R_1=R_2= (CH_2)_2COOCH_3$
136c $R_1= (CH_2)_3SO_3H$
$R_2= (CH_2)_2COOH$

Scheme 1.44: Condensation of *p*-nitrosoaniline derivatives **134a-c** and 1,6-dihydroxynaphthalene **135**
i) DMF, reflux; **136a**, 54%; **136c**, 53%; or MeOH, H^+, reflux; **136b**, 53% (2006JOC7835, 2006T11021)

The method has also been extended to the preparation of fluorinated resorufin derivatives **138a-c**: the condensation of nitroso-fluororesorcinol **137a-c** with resorcinol **132b** in conc. H_2SO_4 led to fluorinated 7-hydroxyphenoxazin-3-ones **138a-c** (**Scheme 1.45**). The resulting resorufins were further derivatised to prepare substrates sensitive to peroxidase activities (2008USP7432372).



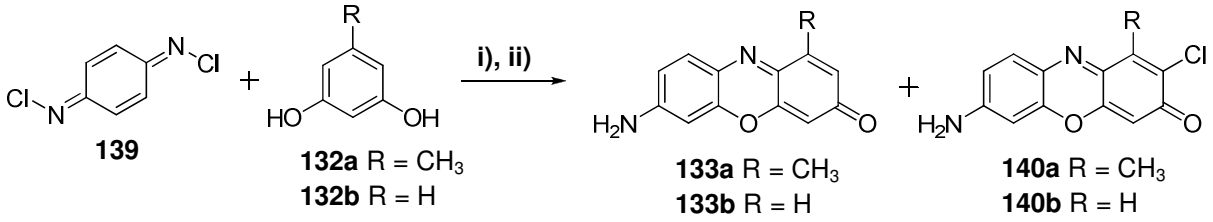
137a $X_1 = X_3 = H, X_2 = F$
137b $X_1 = X_2 = F, X_3 = H$
137c $X_1 = X_2 = X_3 = F$

138a $X_1 = X_3 = H, X_2= F$
138b $X_1 = X_2 = F, X_3 = H$
138c $X_1 = X_2 = X_3 = F$

Scheme 1.45: Condensation of fluorinated 4-nitrosoresorcinol **137a-c** and resorcinol **132b**
i) conc. H_2SO_4, EtOH, Δ = 80°C; **138a**, 16.7%; **138b**, 41.3%; **138c**, 48.5% (2008USP7432372).

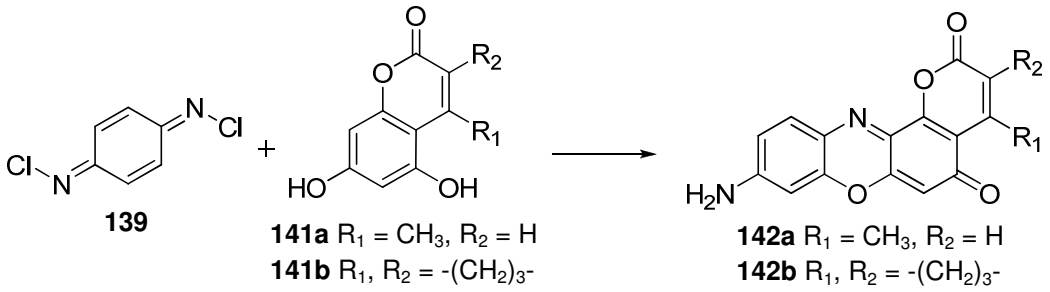
1.3.7 Condensation of benzoquinonechlorodiimide with resorcinol

Nietzki and Maeckler reported the preparation of 7-amino-1-methylphenoxazin-3-one **133a** *via* the condensation of benzoquinonechlorodiimide **139** and orcinol **132a** in conc. H_2SO_4, followed by neutralisation with an aqueous ammoniacal solution (1890CB718, **Scheme 1.46**). When repeating the experiment, Musso and Wager also isolated 7-amino-2-chloro-1-methylphenoxazin-3-one **140a** as a by-product in relatively important yield (1961CB2551, **Scheme 1.46**). Formation of the chlorinated by-product **140b** was also notable in the preparation of 7-aminophenoxazin-3-one **133b**.



Scheme 1.46: Condensation of benzoquinonechlorodiimide **139** and resorcinol derivatives **132a-b** i) conc. H_2SO_4, Δ ; ii) aq. NH_3; **133a**, 6.1%; **140a**, 1.6%; **133b**, 4.7%; **140b**, 13.5% (1961CB2551).

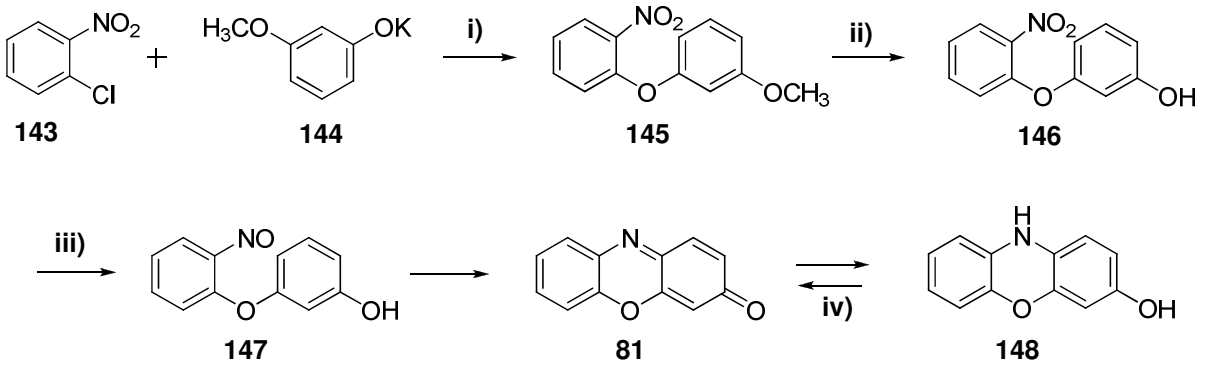
The preparation of several 7-aminophenoxazin-3-one derivatives was successfully achieved, following a similar method, by condensing benzoquinonechlorodiimide **139** with 5,7-dihydroxy-3,4-substituted coumarins **141a-b** in refluxing EtOH or MeOH, yielding 7-amino-1,2-(3',4'-disubstituted-2'-pyranyl)phenoxazin-3-one (**142a-b**, **Scheme 1.47**, 2006US0121551). The substitution in the 1,2-position of the phenoxazin-3-one avoided the formation of the chlorinated by-product.



Scheme 1.47: Condensation of benzoquinonechlorodiimide **139** and 5,7-dihydroxysubstituted coumarins **141a-b**
i) EtOH or MeOH, reflux; **142a**; **142b**; yields not given (2006US0121551).

1.3.8 Reductive cyclisation of 5-hydroxy-2'-nitrodiphenyl ethers

Studies undertaken by Bird and Latif (1979T529, 1979T1813, 1986JNSM95) concerned the cyclisation of 2-nitro-3'-hydroxydiphenyl ether **146**. The phenoxazinone core **81** was accessed *via* the initial formation of an ether bridge between 2-chloronitrobenzene **143** and potassium *m*-methoxyphenoxide **144**. Deprotection of the resulting diphenyl ether **145**, followed by reduction of the nitro group with NH_4Cl and zinc dust, resulted in an intramolecular cyclisation of the nitrosodiphenyl ether **147** to a phenoxazin-3-one (**81**, **Scheme 1.48**).

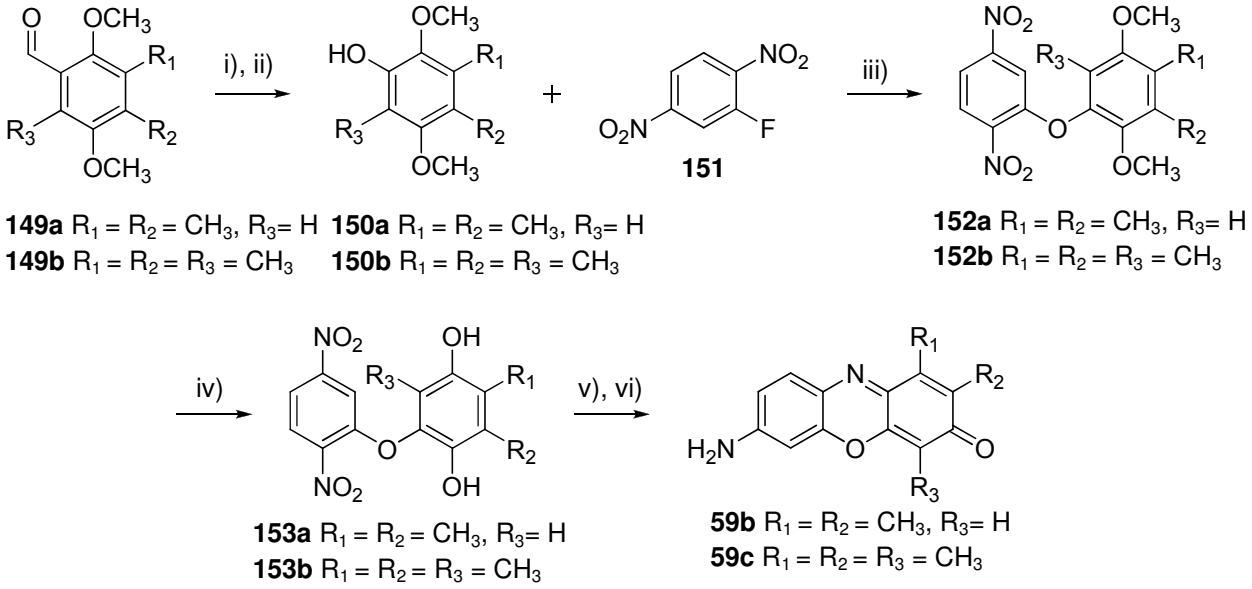


Scheme 1.48: Preparation of phenoxazin-3-one **81** *via* reductive cyclisation of 2-nitro-3'-hydroxydiphenyl ether **146**.
i) 140-150°C, 75%; ii) BCl_3, CH_2Cl_2, -80°C to R.T. to reflux, 66%; iii) NH_4Cl, Zn dust, aq. DME, 40°C; iv) Aerial oxidation, 67%.

The nitrosodiphenylether **147**, generated during the reduction step, was proposed as the reacting species initiating the intramolecular cyclisation to phenoxazin-3-one **81**. However, the resulting phenoxazin-3-one is easily reduced to 3-hydroxy-3*H*-phenoxazine **148** under such conditions and phenoxazin-3-one could be isolated only after the removal of zinc followed by aerial oxidation.

1.3.9 Oxidative cyclisation of 2,5-dinitro-2',5'-dihydroxydiphenylether

Groundwater *et al.* reported the synthesis of 7-aminophenoxazin-3-one analogues **59a-b** *via* the cyclisation of 2,5-dinitro-2',5'-dihydroxydiphenylethers (2008OBC682). The synthesis was achieved by the initial preparation of appropriately substituted 2,5-dimethoxyphenols **150a-b** *via* Baeyer-Villiger oxidation of the corresponding 2,5-dimethoxybenzaldehydes **149a-b**. Coupling of the 2,5-dimethoxyphenols **150a-b** to 2,5-dinitro-1-fluorobenzene **151** afforded 2,5-dinitro-2,5-dimethoxybenzene **152a-b**, which were further dealkylated using BBr_3. The resulting 2,5-dinitro-2',5'-dihydroxydiphenylethers **153a-b** were reduced *via* catalytic hydrogenation; subsequent aerial oxidation of the solution resulted in formation of the desired 7-aminophenoxazin-3-ones **59a-b** (**Scheme 1.49**, 2008OBC682).

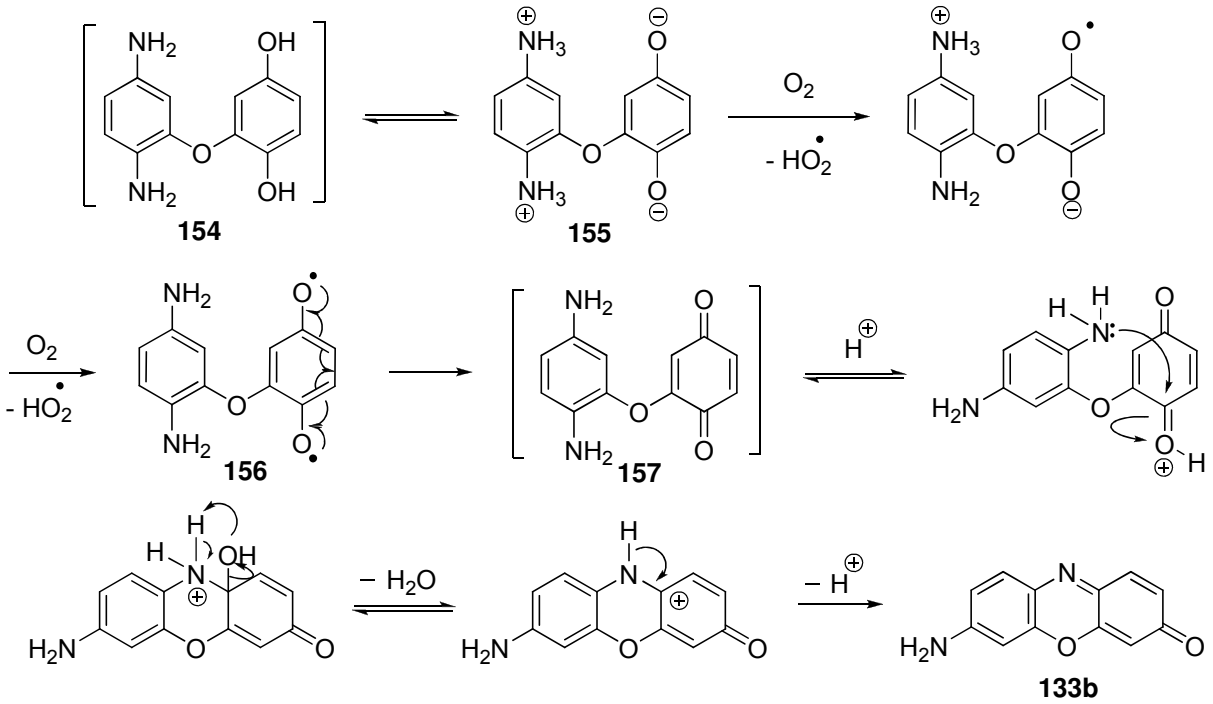


149a $R_1 = R_2 = CH_3$, R_3= H **150a** $R_1 = R_2 = CH_3$, R_3= H **152a** $R_1 = R_2 = CH_3$, R_3= H
149b $R_1 = R_2 = R_3 = CH_3$ **150b** $R_1 = R_2 = R_3 = CH_3$ **152b** $R_1 = R_2 = R_3 = CH_3$

153a $R_1 = R_2 = CH_3$, R_3= H **59b** $R_1 = R_2 = CH_3$, R_3= H
153b $R_1 = R_2 = R_3 = CH_3$ **59c** $R_1 = R_2 = R_3 = CH_3$

Scheme 1.49: Preparation of 7-aminophenoxazin-3-one derivatives **59b-c**
i) MMPP, MeOH; ii) 2M NaOH, CH_3OH; **150a**, 23.0%; **150b**, 75.0%; iii) NaH, DMF; **152a**, 76%; **152b**, 89%; iv) 1M BBr_3 in DCM, dry DCM, -75°C to R.T.; v) H_2, Pd/C 5%, MeOH; vi) Air, MeOH, R.T.; **59b**, 68%; **59c**, 75%; (2008OBC682).

The proposed mechanism suggested the formation of 2,5-diamino-2',5'-dihydroxydiphenylether **154** and its existence as a zwitterion **115**. Subsequent attack of **155** by 2 mol of oxygen results in the formation of the hypothetical di-radical **156**, which self-terminates into the 2,5-diaminophenoxybenzoquinone **157** (**Scheme 1.50**).

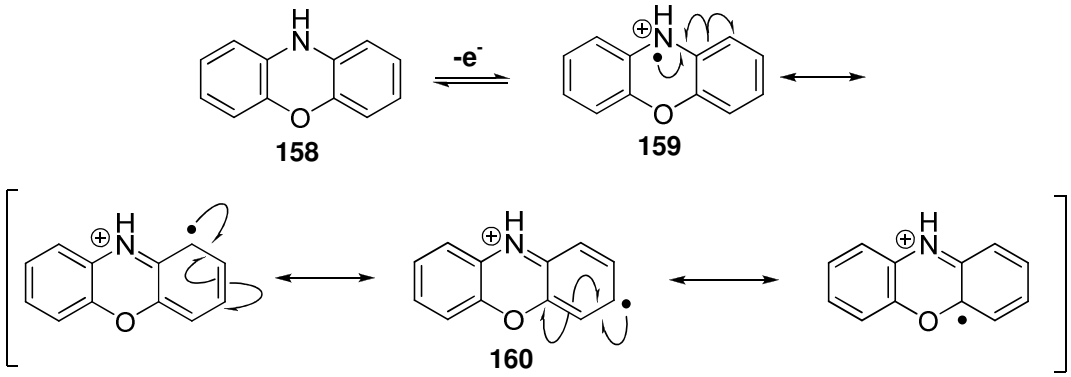


Scheme 1.50: Suggested mechanism for the cyclisation of 2,5-diamino-2',5'-dihydroxydiphenylether to 7-aminophenoxazin-3-one **133b** (2008OBC682).

Finally, intra-molecular cyclisation of 2,5-diaminophenoxybenzoquinone **157** yields the 7-aminophenoxazin-3-one (**Scheme 1.50**).

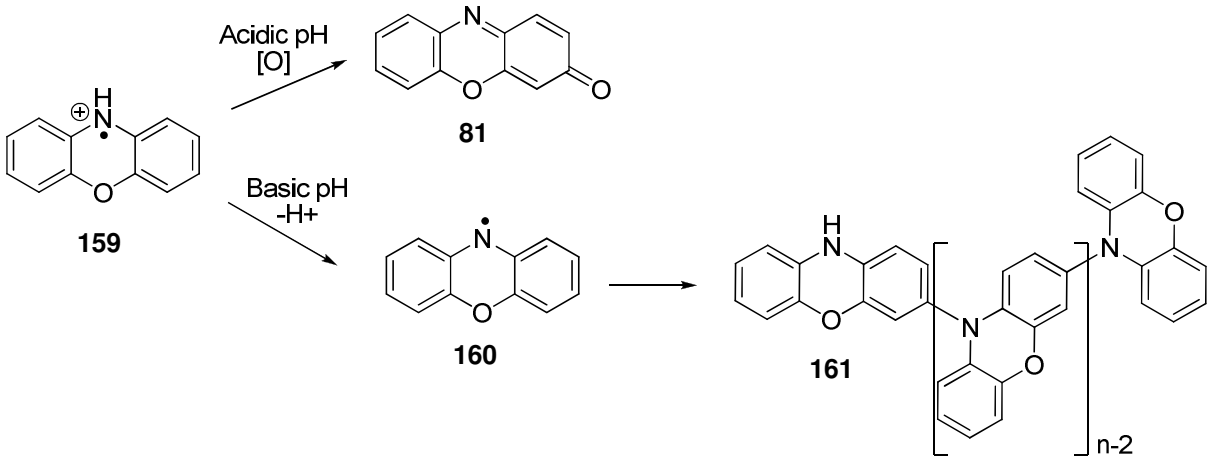
1.3.10 Oxidation of phenoxazines

Preparation of the phenoxazin-3-one **81** was also achieved *via* oxidation of phenoxazine **158** (**Scheme 1.51**). The first oxidations were carried out using potassium nitrosodisulfonate, or ferric chloride in acetic acid (1902CB341). Other oxidants, such as iodoxylbenzene with vanadium oxide acetyl acetonate (1990SC1543), bis-(trifluoroacetoxy)pentafluoroiodobenzene (1991MC323) and ozone (1984BCJ2526) successfully oxidised phenoxazine in good yield. Study of the mechanism suggested that phenoxazine was oxidised *via* a one-electron step, giving the radical cation **159**. Conjugation through the benzene ring gives the phenoxazonium radical cation **160** (**Scheme 1.51**), the species responsible for the oxidation to phenoxazin-3-one **81**.



Scheme 1.51: Formation of the phenoxazine radical cation **160**

The oxidation is pH dependent and formation of phenoxazinone occurs only at acidic pH. Higher pH results in loss of a proton from the phenoxazonium ion **159**, generating the radical **160**, and resulted in dimerisation or polymerisation of the phenoxazine unit (**161**, **Scheme 1.52**).



Scheme 1.52: pH dependance of the phenoxazine oxidation.

1.4 Aims

The aims of this work were to synthesise and test novel chromogenic enzyme substrates suitable for the specific detection of the β-alanine aminopeptidase. The enzyme substrates of interest will be based on the 8-aminophenoxazin-3-one core and their synthesis will be achieved mainly by exploration of the newly developed pathway reported above in section **1.3.9**. The preparation of 8-amino-1-pentylphenoxazin-3-one is of interest as a possible replacement for 7-amino-1-pentylphenoxazin-3-one in current use for the detection of β-alanine aminopeptidase. Preparation of other 8-aminophenoxazinone derivatives, would be desirable, in search of better performing enzyme substrates. Optimisation and shortening of the pathway reported in section **1.3.9**, would also be of interest.

All phenoxazinone substrates prepared will be derivatised with β-alanine and tested against *Ps. aeruginosa* and *B. cepacia* to evaluate their efficiency in the detection of those pathogens.

CHAPTER TWO:

SYNTHESIS OF PHENOXAZINONE SUBSTRATES

The present chapter will discuss the preparation of new phenoxazinones chromogens *via* two different synthetic pathways and is divided into four parts.

The first part is related to the preparation and characterisation of nitrohalogenophenoxazinones *via* a condensation type reaction. The synthesis of some of the corresponding aminohalogenophenoxazinones derivatives will also be discussed.

The second part will discuss the preparation of novel 7- and 8-aminophenoxazinone derivatives *via* an oxidative cyclisation reaction. Several strategies will be explored for the introduction of a substituent onto the phenoxazin-3-one core. Due to the similarities of the synthetic routes explored, the preparation of each key precursor will be presented stepwise, in a grouped manner, rather than discussing each synthetic route separately.

The third part will discuss the preparation of β-alanine enzyme substrates using the 7- and 8-aminophenoxazinones derivatives previously prepared in the first and second part.

Finally, the fourth part will evaluate and compare the U.V.-Visible characteristics of some of the 7- and 8-aminophenoxazinones derivatives prepared along with their corresponding β-alanine derivatives.

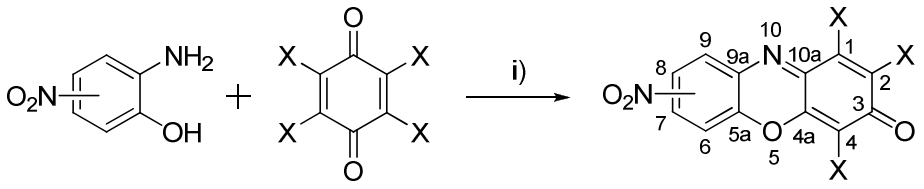
2.1 Synthesis of halogenophenoxazin-3-ones

2.1.1 Preparation of nitro-1,2,4-trihalogenophenoxazin-3-ones 130α-γa-c

The condensation of nitroaminophenols **124β** with tetrahalogenobenzoquinones **129b-c** (**Scheme 1.40**), described earlier in section **1.3.5**, seemed particularly attractive to prepare quickly several phenoxazin-3-one derivatives. The amino group necessary for the preparation of enzyme substrate could be introduced readily *via* reduction of the nitro group.

The necessary starting materials, including the three halogenobenzoquinones fluoranil **124a**, chloranil **124b** and bromanil **124c**, were readily available and allowed the direct preparation of 7-nitro, 8-nitro and 9-nitro-1,2,4-trihalogenophenoxazin-3-ones. The reaction was attempted using the original procedure described (1935US2020651), without modification. The condensation occurred smoothly in an ethanolic NaOAc suspension of the correctly substituted nitro-2-aminophenol **124α-γ** and tetrahalogenobenzoquinone **129a-c** at room temperature, with rapid formation of

the product as a bright red precipitate. The precipitate was conveniently collected by filtration and purified by recrystallisation from glacial AcOH, yielding the expected nitro-1,2,4-trihalogenophenoxazinones **130αb-c**, **130βb-c** and **130γb** in moderate to excellent yields (**Scheme 2.1**). The case of fluoranil was slightly different and will be discussed later.



124α 5-NO_2 **129a** X = F, Fluroanil **130αa-c** 7-NO_2 12.4-38.5%
124β 4-NO_2 **129b** X = Cl, Chloranil **130βa-c** 8-NO_2 45.3-98.6%
124γ 3-NO_2 **129c** X = Br, Bromanil **130γa-b** 9-NO_2 72.3-95.0%

Scheme 2.1: Preparation of nitro-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones
i) EtOH, NaOAc, R.T.

The ^{1}H NMR spectra of the resulting nitro-1,2,4-trihalogenophenoxazin-3-ones **130αb-c**, **130βb-c** and **130γb**) displayed only three characteristic deshielded aromatic protons, two doublets and a doublet of doublets in the case of **130αb-c** and **130βb-c** and two doublets of doublets and a triplet in the case of **130γb** (**Table 2.1**).

The formation of the phenoxazin-3-one core was supported by the ^{13}C NMR spectra, with the presence of 12 carbon signals; two particular carbons were peculiar to the formation of phenoxazinone. Firstly, the deshielded signal within the 171.0-171.6ppm region was characteristic of a carbonyl and was assigned to 3-C (**Table 2.1**). Secondly, the relatively shielded aromatic carbon within the 103.3-111.6ppm region, was attributed to 4-C (**Table 2.1**). This shielded value was characteristic of the 4-position, *ortho* to the oxygen bridge and α to the carbonyl 3-C. The four remaining carbons present on the quinoid ring could not be assigned with certainty.

The three quaternary carbons present on the benzenoid part of the ring were assigned regarding their electronic environment; hence assignment depended upon the nitro group position.

With 7-nitrophenoxazin-3-ones **130αb-c** the cumulated effects of electron withdrawal at 7-C from both the *ipso* nitro group and the imino-quinone justified the assignment of the signal at 149.7-149.8ppm to 7-C (**Table 2.1**). The *para* position of the nitro group to 9a-C had a significant deshielding effect and the value of 143.5-143.6ppm

was assigned to 9a-C. The remaining value of 136.6 was assigned to 5a-C (**Table 2.1**).

With 8-nitrophenoxazin-3-ones **130βb-c** the most deshielded value of 147.7-148.5ppm was attributed to 5a-C, as a result of the cumulated electron withdrawing effect from the *ispo* oxygen atom and the *para* nitro substituants (**Table 2.1**). The value of 145.3ppm was assigned to 8-C, and the remaining value of 132.6-133.0ppm to 9a-C, as in this case the *meta* nitro substituent could not affect the chemical shift of 9a-C (**Table 3**).

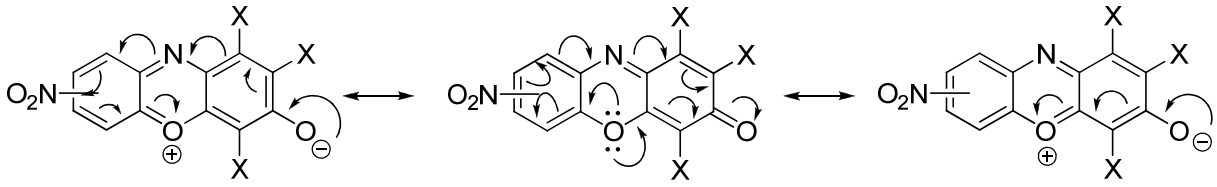
With 9-nitrophenoxazinone **130γb**, the *ipso* position of the nitro group to 9-C resulted in electronic conditions similar to that described above for 7-C with 8-nitrophenoxazinones **130αb-c**, such that the value of 147.8ppm was assigned to 9-C. The two remaining signals at 143.7 and 125.3ppm were assigned to 5a-C and 9a-C respectively (**Table 2.1**).

		130αb-c		**130βb-c**		**130γb**
δ_H NMR (ppm) d_6-DMSO	6-H	8.44-8.55 (1H, d, *J* = 2.4Hz)	6-H	7.82-7.95 (1H, d, *J* = 9.0Hz)	6-H	8.09 (1H, dd, *J* = 7.5, 2.1Hz)
	8-H	8.29-8.32 (1H, dd, *J* = 8.7, 2.4Hz)	7-H	8.47-8.57 (1H, dd, *J* = 9.0-9.3, 2.7Hz	7-H	7.97 (1H, t, *J* = 8.4Hz)
	9-H	8.21-8.24 (1H, d, *J* = 8.7-9.0Hz)	9-H	8.60-8.73 (1H, d, *J* = 2.7Hz)	8-H	8.02 (1H, dd, *J* = 8.4, 2.1Hz)
δ_C NMR (ppm) d_6-DMSO	3-C	170.0-171.5	3-C	171.1-171.6	3-C	171.0
	4-C	103.4-111.8	4-C	103.2-111.6	4-C	111.2
	5a-C	136.6-137.1	5a-C	147.7-148.2	5a-C	143.7
	9a-C	143.5-143.6	9a-C	132.6-133.0	9a-C	125.3
	C-NO_2	149.7-149.8	C-NO_2	145.3	C-NO_2	147.8
ν_{max} (cm^{-1})	C=O	1641-1646	C=O	1638-1649	C=O	1647
	NO_2	1529-1519 and 1347-1346	NO_2	1510-1508 and 1331-1328	NO_2	1532 and 1330

Table 2.1: Key NMR and I.R. spectral features of nitro-1,2,4-trihalogenophenoxazin-3-ones **130α-βb-c**, **γb**

I.R. spectroscopy confirmed the presence of a nitro group with the two characteristic stretches around within the 1532-1519 and 1347-1330cm^{-1} region (**Table 2.1**).

The C=O stretch observed was of a relatively low absorbance (1646-1638cm^{-1}) for a carbonyl (**Table 2.1**). The conjugations within the phenoxazin-3-one ring (**Scheme 2.1**), were thought to be responsible for the weakening of the double bond strength and its resulting low absorption.



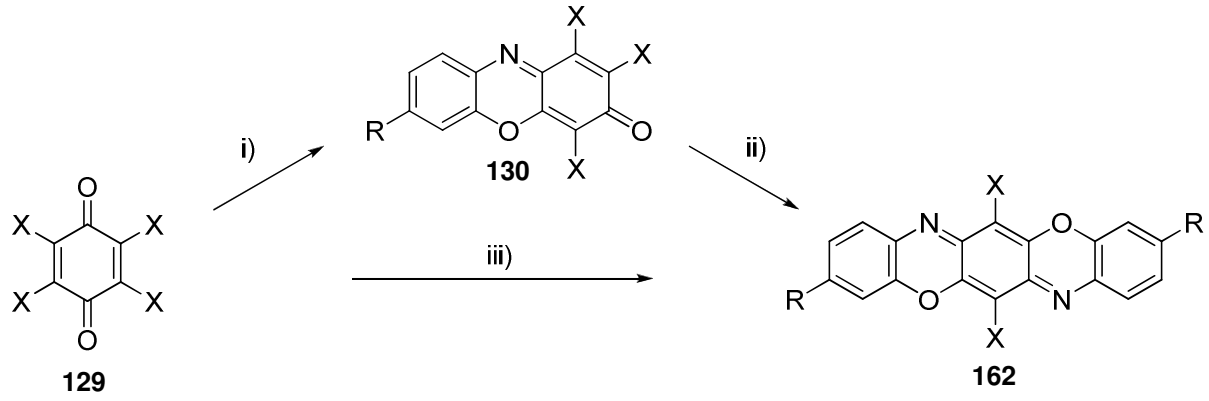
Scheme 2.2: Electron conjugations within the phenoxazin-3-one core responsible for the weakening of the C=O bond.

2.1.1.1 Nitro-1,2,4-trifluorophenoxazin-3-ones 130α-γa

The condensation of nitro-2-aminophenol **124α-γ** with fluoranil **129a** gave the corresponding nitro-1,2,4-trifluorophenoxazinones in poor yields (12-38%, **Scheme 2.1**). In this particular case, the formation of an insoluble material, suspected to be a triphenodioxazinone, seemed to dominate the formation of nitro-1,2,4-trifluorophenoxazinone **130α-γa** (**Scheme 2.1**). This was not observed with brominated or chlorinated derivatives under the present conditions at room temperature.

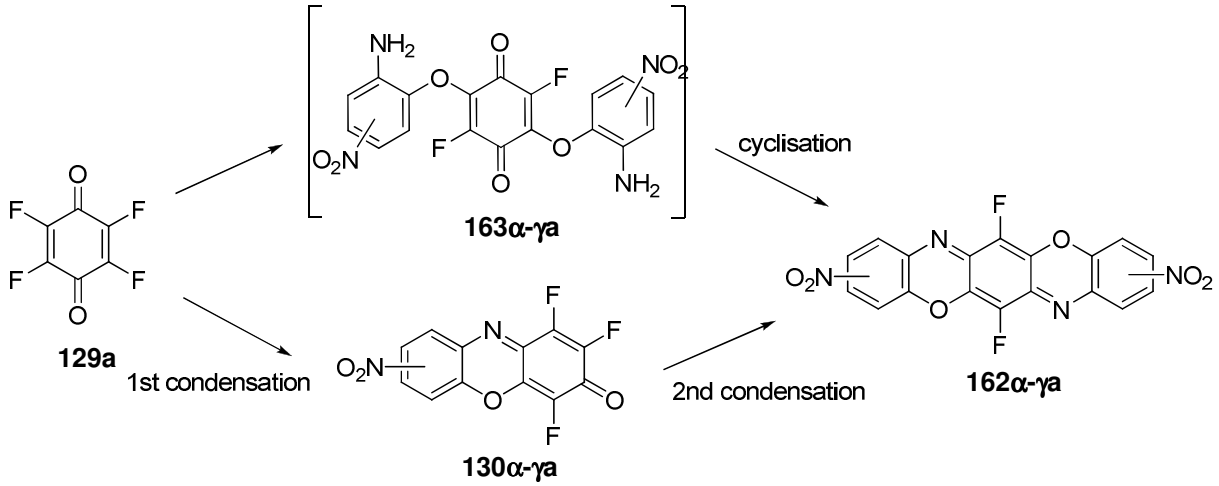
Mital and Jain (1971JC1875) reported the formation of such triphenodioxazinones **162** from chloranil or bromanil and various aminophenols derivatives under the following conditions: either by (i) refluxing two equivalents of aminophenols with one equivalent of the halogenated quinone and NaOAc, or (ii) by reacting a trihalogenated phenoxazinone **130** with another equivalent of aminophenol and NaOAc under reflux conditions (**Scheme 2.3**).

The strong electronegative character of fluorine atoms would suggest that fluoranil would be sensitive toward nucleophilic attack, allowing the formation of a triphendioxazinone even at R.T.; furthermore, nitro-1,2,4-trifluorophenoxazinones **130α-γa** would also show an increased reactivity towards a second condensation.



Scheme 2.3: Formation of triphenodioxazinone **162**
i) 1eq. *o*-aminophenol, EtOH, NaOAc; ii) 1eq. *o*-aminophenol, EtOH, NaOAc, reflux;
iii) 2eq. *o*-aminophenol, EtOH, NaOAc, reflux;
X = Br or Cl, R = H, CH_3, F, Cl, Br, OMe, OEt (1971JC1875).

Hence two possibilities could be envisaged for the low yield of nitro-1,2,4-trifluorophenoxazin-3-ones: a concomitant attack of fluoranil **130a** by two molecule of nitroaminophenol, resulting in the formation of the quinone intermediate **163α-γa** followed by cyclisation to **162α-γa**, or formation of nitro-1,2,4-trifluorophenoxazinone, followed by a condensation with a second molecule of nitroaminophenol (**Scheme 2.4**). The possibility of both pathways occurring simultaneously was envisaged.
However, attempts to avoid the formation of by-products, by lowering the reaction temperature to -15°C using a salt/ice bath, along with a dropwise addition of the nitro-2-aminophenol solution, failed to improve significantly the yield of **130α-γa**.



Scheme 2.4: Hypothetic formation of dinitro-6,13-difluorotriphenodioxazinones **162α-γa.**

The formation of nitro-1,2,4-trifluorophenoxazinones **130α-γa,** was evidenced by NMR analysis using ^{13}C and ^{19}F spectra, the presence of fluorine atoms being

strongly confirmed by ^{19}F-^{13}C coupling in the ^{13}C NMR spectrum. In the example of 9-nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one **130γa** (**Figure 2.1**), 1-C appeared as a doublet of doublets with a $^1J_{CF}$ of 274.7Hz, which corresponded to direct ^{19}F-^{13}C coupling, and a $^2J_{CF}$ of 12.2Hz, generated by the neighbouring fluorine on 2-C. A doublet of triplets was observed from 2-C, exhibiting a $^1J_{CF}$ of 273.2Hz and a $^2J_{CF}$ of 6.8Hz, both fluorine atoms at 1-C and 4-C also coupling with 2-C. The carbonyl 3-C was shown as a triplet of doublets with a $^2J_{CF}$ of 21.1Hz, due to the two neighbouring fluorine atoms at 2-C and 4-C, and a $^3J_{CF}$ of 5.5Hz generated by the fluorine at 1-C. The carbon 4-C exhibited a doublet of doublets with a $^1J_{CF}$ of 261.9Hz and a $^2J_{CF}$ of 6.3Hz. The ^{19}F-^{13}C coupling was extremely useful in fully assigning the carbon atoms: a $^1J_{CF}$ of 260-270Hz indicating the carbons bearing a fluorine atom and the value of $^2J_{CF}$ indicating the relative position to other fluorine atoms.

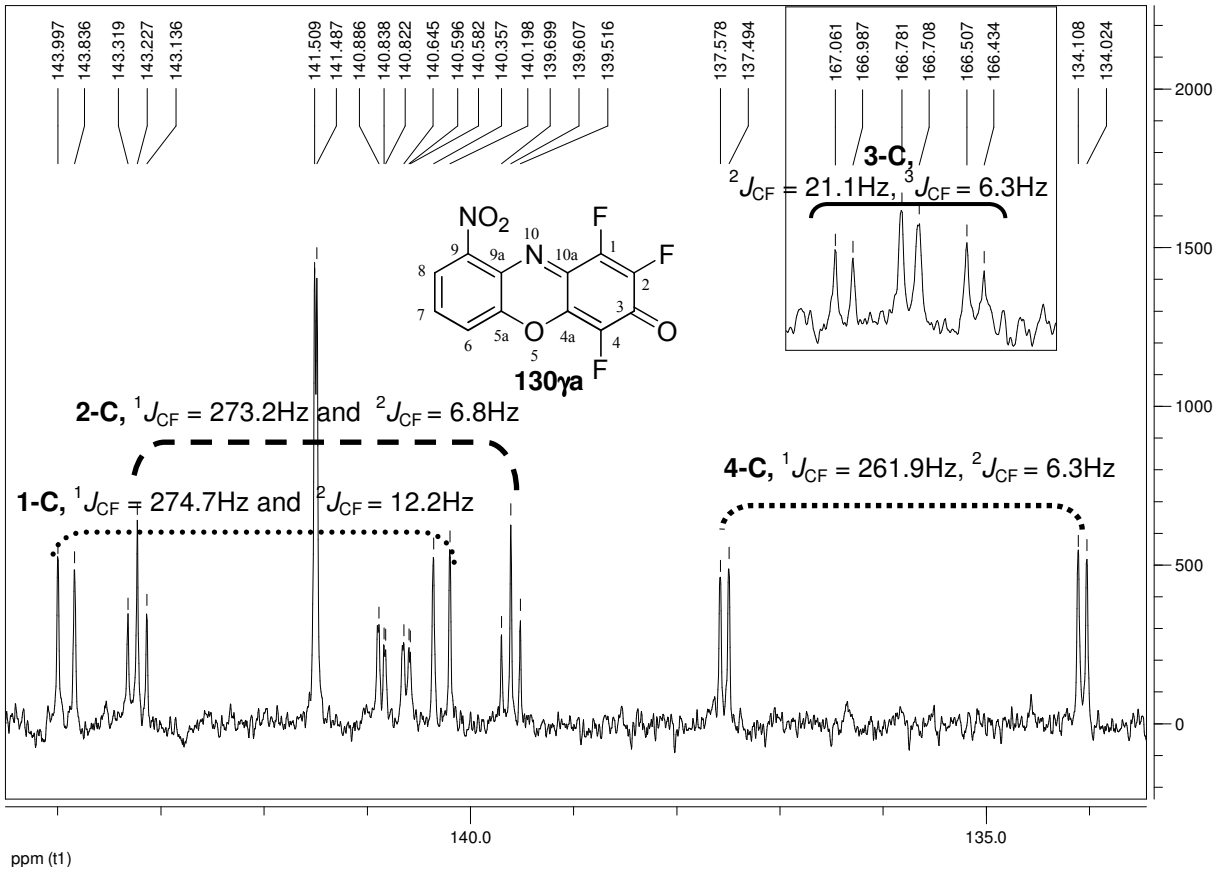


Figure 2.1: ^{13}C NMR spectrum of 9-nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one 2.3γa within the δ133.5-144.5ppm region and an expansion of 3-C (δ166.10-167.30ppm).

The assignment of the 7- (**130αa**) and 8-nitro-1,2,4-trifluorophenoxazin-3-one **130βa** spectra was made following the same reasoning, the value of δ_C on the quinoid ring

remaining unaffected by the position of the nitro group on the benzenoid ring (**Table 2.2**). The ^{19}F revealed three signals, a dd at δ_F = -266ppm corresponding to 1-F, a dd at δ_F = -271ppm corresponding to 2-F and a triplet at δ_F = -282ppm corresponding to 4-F.

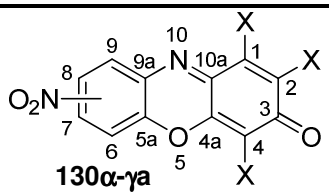
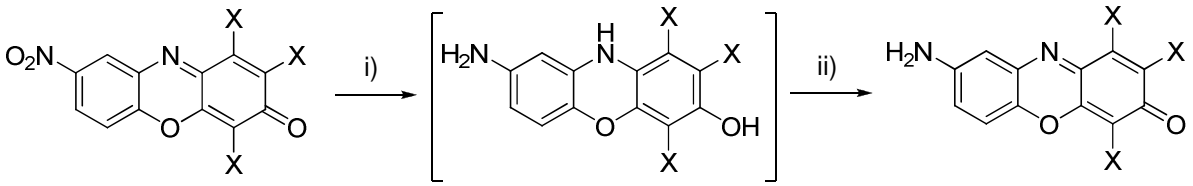
		 **130α-γa**
δ_F NMR (ppm) d_8-THF	1-F	-266.07- -265.41 (1F, dd, *J* = 9.3, 2.0-2.5Hz)
	2-F	-271.89- -271.43 (1F, dd, *J* = 9.6-9.0, 1.7-2.3Hz)
	4-F	-282.53- -282.17 (1F, t, *J* = 2.3-2.8Hz)
δ_C NMR (ppm) d_8-THF	1-C	142.1-142.2 (dd, *J* = 273.4-274.7, 12.0-12.2Hz)
	2-C	141.3-141.4 (dt, *J* = 272.7-273.4, 6.8-6.9Hz)
	3-C	166.7-166.8 (dt, *J* = 21.0-21.4, 5.4-5.6Hz)
	4-C	135.8-136.0 (dd, *J* = 261.9-262.7, 6.1-6.5Hz)
	4a-C	140.7-141.7 (ddd, *J* = 18.1-18.3, 4.8-5.5, 1.1-1.2Hz)
	10a-C	129.85-130.1 (ddd, *J* = 8.5-10.0, 5.0-5.2, 1.2-1.4Hz)
ν_{max} (cm^{-1})	C=O	1637-1634
	NO_2	1531-1518 and 1312-1308
	C-F	1004-1003

Table 2.2: Key NMR and I.R. spectral features of nitro-1,2,4-trifluorophenoxazin-3-one **130α-γa**.

The main features observed by I.R. spectroscopy were the low C=O stretch within the 1637-1634cm^{-1} region, the NO_2 stretches within the 1531-1518 and 1312-1308cm^{-1} region and the strong absorption band within 1004-1003cm^{-1} corresponding to the C-F stretch (**Table 2.2**).

2.1.2 Synthesis of 8-amino-1,2,4-trihalogenophenoxazin-3-ones 165a-c

The next step towards the preparation of amino-1,2,4-trihalogenophenoxazin-3-one substrates required reduction of the nitro group. Catalytic hydrogenation was chosen, mainly because of the simplicity of the procedure and work up. Subsequently, catalytic hydrogenation of the isolated 8-nitro-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones **130βa-c** was conducted smoothly in an EtOAc:MeOH solvent mixture (**Scheme 2.5**), but care was required when reducing the chlorinated **130βb** and brominated **130βc** derivatives, as discussed below.



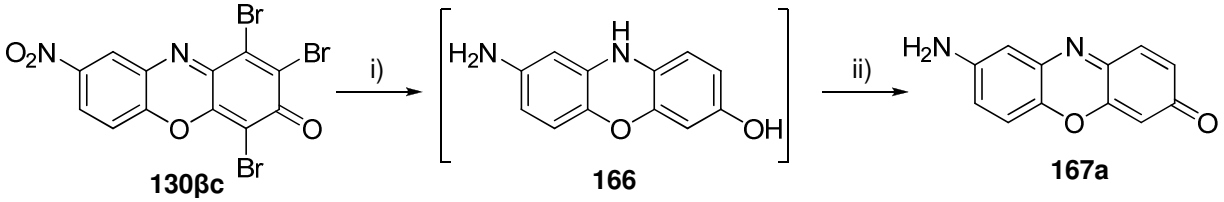
130βa X = F
130βb X = Cl
130βc X = Br

164a X = F
164b X = Cl
164c X = Br

165a X = F
165b X = Cl
165c X = Br

Scheme 2.5: Preparation of 8-amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-one **165a-c**
i) H_2, Pd/5%C or PtO_2, MeOH/EtOAc; ii) AgO or Ag_2O or MnO_2, MeOH; **165a**: 98.0%; **165b**: 97.6%, **165c**: 94.8%.

When the hydrogen pressure was set above 2.0 bar during the catalytic reduction, a mixture of colourful products resulted after the re-oxidation step (observed by TLC) in the case of **130βb** and **130βc**, which was possibly the result of hydro-dehalogenation. This hypothesis seems likely as, under forcing conditions, using Pd/C 10%, Et_3N and a H_2 pressure of 3.4 bar, the more sensitive brominated analogue led to the isolation of 8-amino-3*H*-phenoxazin-3-one **167a** in moderate yield, after re-oxidation and purification (**Scheme 2.6**). No attempt was made to improve the yield; however, milder conditions could possibly reduce the amount of tarring observed.



130βc **166** **167a**

Scheme 2.6: Hydro-dehalogenation of 8-nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one **130βc**
i) H_2, Pd/C 10%, Et_3N, MeOH/EtOAc; ii) AgO, MeOH, 54.1%.

The Pd/C 5% catalyst was replaced by Pt_2O for the reduction of **130βb-c**, allowing a better control of the hydrogenation outcome, and avoiding hydro-dehalogenation. The conditions used to reduce the nitro group in **130βa-c** irremediably reduced the phenoxazin-3-one imino-quinone system. The 8-amino-3-hydroxy-1,2,4-trihalogeno-3*H*-phenoxazine intermediates **165a-c** were not isolated but the colourless solution obtained after hydrogenation, which slowly turned to deeply coloured solution upon removal of the catalyst and aeration, strongly suggest this species was formed. The aerial oxidation was rather slow and, for synthetic convenience, the oxidation process was accelerated by the use of an oxidising agent, such as silver (I) oxide, silver (II) oxide or manganese (IV) dioxide (**Scheme 2.5**).

The resulting 8-amino-1,2,4-trihalogenophenoxazin-3-ones **165a-c** showed a general low solubility in organic solvents, which did not permit their characterisation by NMR spectroscopy, nor a full assessment of their purity, except for the trifluorinated analogue **165a**. However, I.R. spectroscopy could confirm the reduction of the nitro to an amino group and the presence of the imino-quinone, with the characteristic I.R. absorption bands within the 3200-3500 cm^{-1} and 1630-1640cm^{-1} regions respectively (**Table 2.3**). Mass spectrometry also gave peaks with masses corresponding to the expected products, with the expected isotopic ratio for **165b** and **165c** (**Table 2.3**).

	ν_{max} (cm^{-1}) NH_2	**ν_{max} (cm^{-1}) C=O**	**m/Z**
165a X =F	3503, 3358 and 3230	1638	264.9 (M^-)
165b X = Cl	3387,3321 and 3226	1634	312.8 (94%), 314.8 (100%), 316.8 (42%), 318.8 (28%) (M^-)
165c X = Br	3376, 3319 and 3216	1628	447 (37%), 449 (100%), 451 (91%), 453 (29%) $(MH)^+$

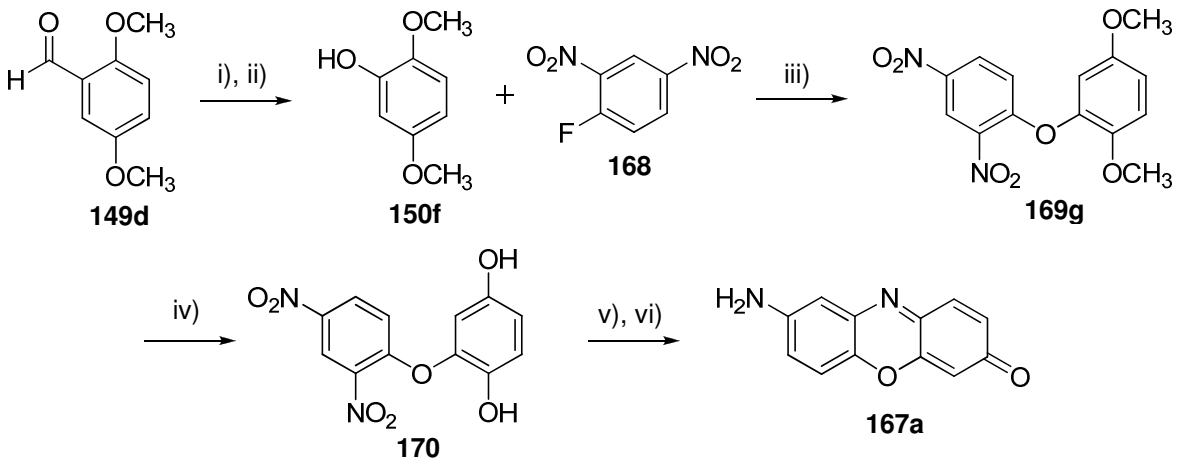
Table 2.3: Key I.R. spectral features of 8-amino-1,2,4-trihalogenophenoxazin-3-ones **165a-c**.

2.2 Synthesis of phenoxazinones *via* oxidative cyclisation

2.2.1 Previous work

Previous work undertaken at the University of Sunderland to synthesize 7-aminophenoxazin-3-one chromogenic substrates led to the development of a new synthetic pathway (2008OBC682, 2006WO030119), described earlier in section

1.3.9. Before the synthesis of 7-aminophenoxazin-3-ones, Zaytsev (T-2006MI00) developed a model study leading to the synthesis of 8-aminophenoxazin-3-one **167a** (**Scheme 2.7**).



Scheme 2.7: Preparation of 8-aminophenoxazin-3-one **167a**
i) MMPP, MeOH; ii) 2M NaOH, CH_3OH, ; iii) Et_3N, DMSO; iv) 1M BBr_3 in DCM, dry DCM, -75℃ to R.T.; v) H_2, Pd/C 5%, MeOH; vi) Air, MeOH, R.T. (T-2006MI00).

The commercially available 2,5-dimethoxybenzaldehyde **149d** was oxidised with magnesium monoperoxyphthalate (MMPP) in MeOH and the resulting formate ester hydrolysed to 2,5-dimethoxyphenol **150f** in basic methanol. 2,4-dinitro-2',5'-dimethoxy diphenylether **169** was formed by reacting 2,5-dimethoxyphenol **150f** with 2,4-dinitro-1-fluorobenzene **168** in DMSO using Et_3N. Demethylation of the diphenylether **169** using BBr_3 and reduction of the resulting 2,4-dinitro-2',5'-dihydroxydiphenylether **170**, followed by aerial oxidation, led to the formation of 8-aminophenoxazin-3-one **167a**. This pathway was then modified in order to prepare the reported 7-aminophenoxazin-3-one derivatives. 8-Aminophenoxazin-3-one **167a** itself was never derivatised with β-alanine, nor were any of its potential analogues prepared. The current work considers the preparation of 8-aminophenoxazin-3-one derivatives and the synthesis of the corresponding β-alanyl chromogenic substrates.

2.2.2 Substitution strategy

The main difficulty of the previously mentioned pathway was the introduction of a specific substituent on the quinoid part of the phenoxazinone ring, to prepare mono, di- or trisubstituted phenoxazinones at the 1-,2- or 4- position (**Figure 2.2**).

Substituents were sought to increase the lipophilicity of the molecule; short to long alkyl chains, trifluoromethyl or perfluoroalkyl chains, or alkoxy chains were considered to be good candidates. Based on the efficiency of 7-amino-1-pentyl-3*H*-phenoxazin-3-one, efforts were focused onto the introduction of a pentyl chain in the 1-position. Substituents on the benzenoid part of the phenoxazinone, other than the auxochrome required for electron conjugation and derivatisation with an amino acid, were not desirable as it would be neighbouring the amino group and might have impaired binding to the enzyme active site resulting in poor hydrolysis of the substrate. Furthermore, substituting the benzenoid moiety within the pathway investigated would require modification of the dinitrofluorobenzene starting material which would considerably lengthen the synthesis. This possibility was not investigated.

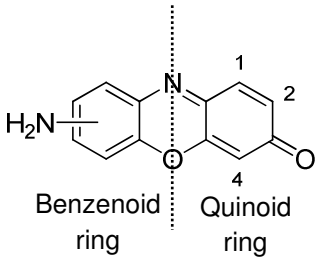
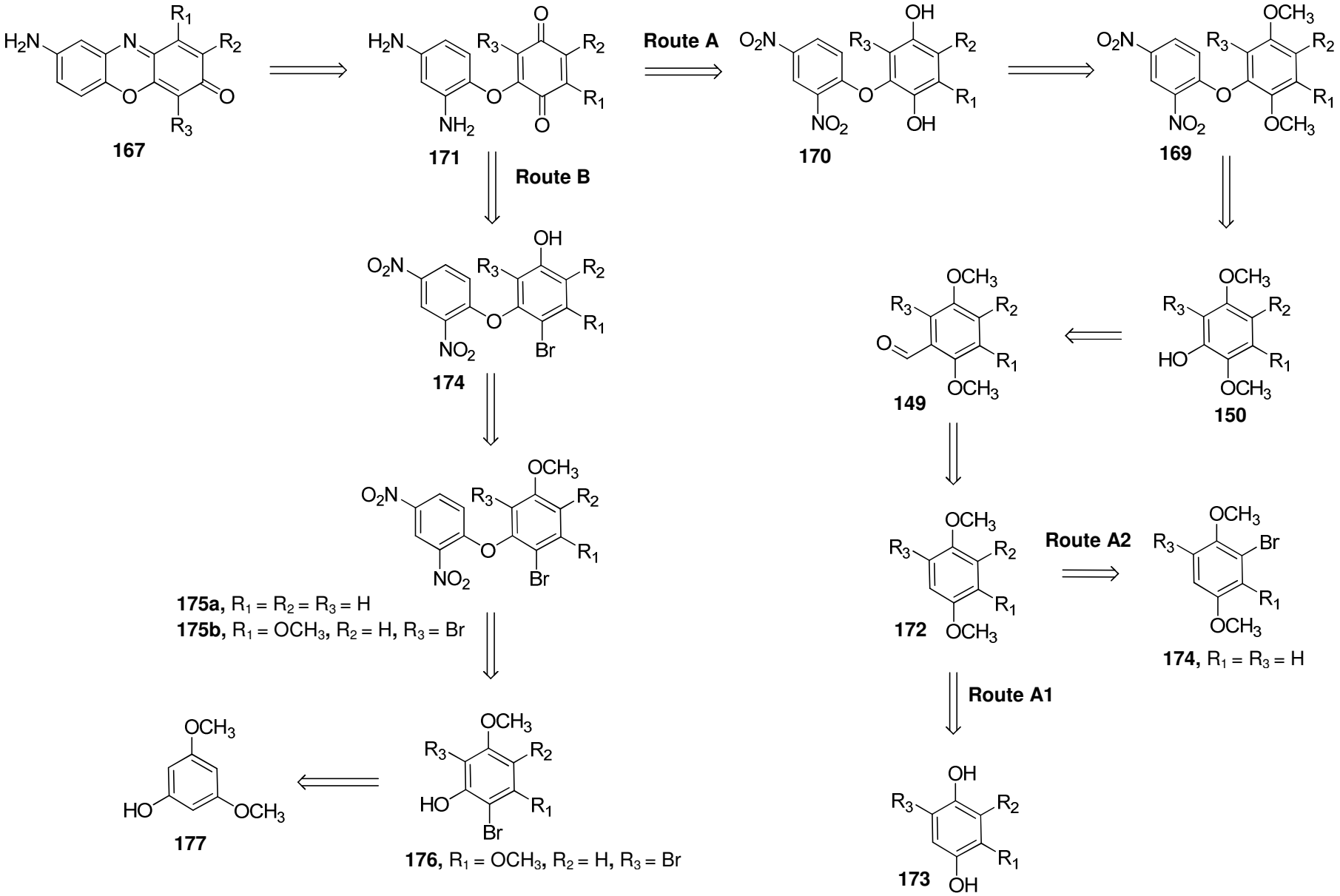


Figure 2.2

2.2.3 Retrosynthetic analysis

All disconnection approaches discussed here are based on the formation of a diphenylether or a similar species and involve a 2,4-diaminophenoxy-2',5'-benzoquinone derivative **171** as a direct precursor of 8-aminophenoxazinones.

The first synthetic route to be explored, **route A**, considers the preparation of the 2,4-diaminophenoxy-2',5'-benzoquinone **171** *via* a 2,4-dinitro-2',5'-dihydroxydiphenylether **170**, itself available from a 2,4-dinitro-2',5'-dimethoxydiphenylether **169** (**Scheme 2.8**). The ether bridge would arise from a 2,5-dimethoxyphenol **150**, prepared from the corresponding 2,5-dimethoxybenzaldehyde **149** (**Scheme 2.8**). The appropriately substituted 2,5-dimethoxybenzaldehydes **149** would be prepared from the corresponding 1,4-dimethoxybenzenes **172** (**Scheme 2.8**). Two alternatives would be possible here: either the preparation of substituted 1,4-dimethoxybenzenes from the corresponding hydroquinones **173** (**route A1**) which is limiting in terms of analogues available, or the preparation of 2-substituted 1,4-dimethoxybenzenes from the readily available 1,4-dimethoxy-2-bromobenzene **174** (**route A2**) which restricts to the preparation of 2-substituted 8-aminophenoxazinones (**Scheme 2.8**).



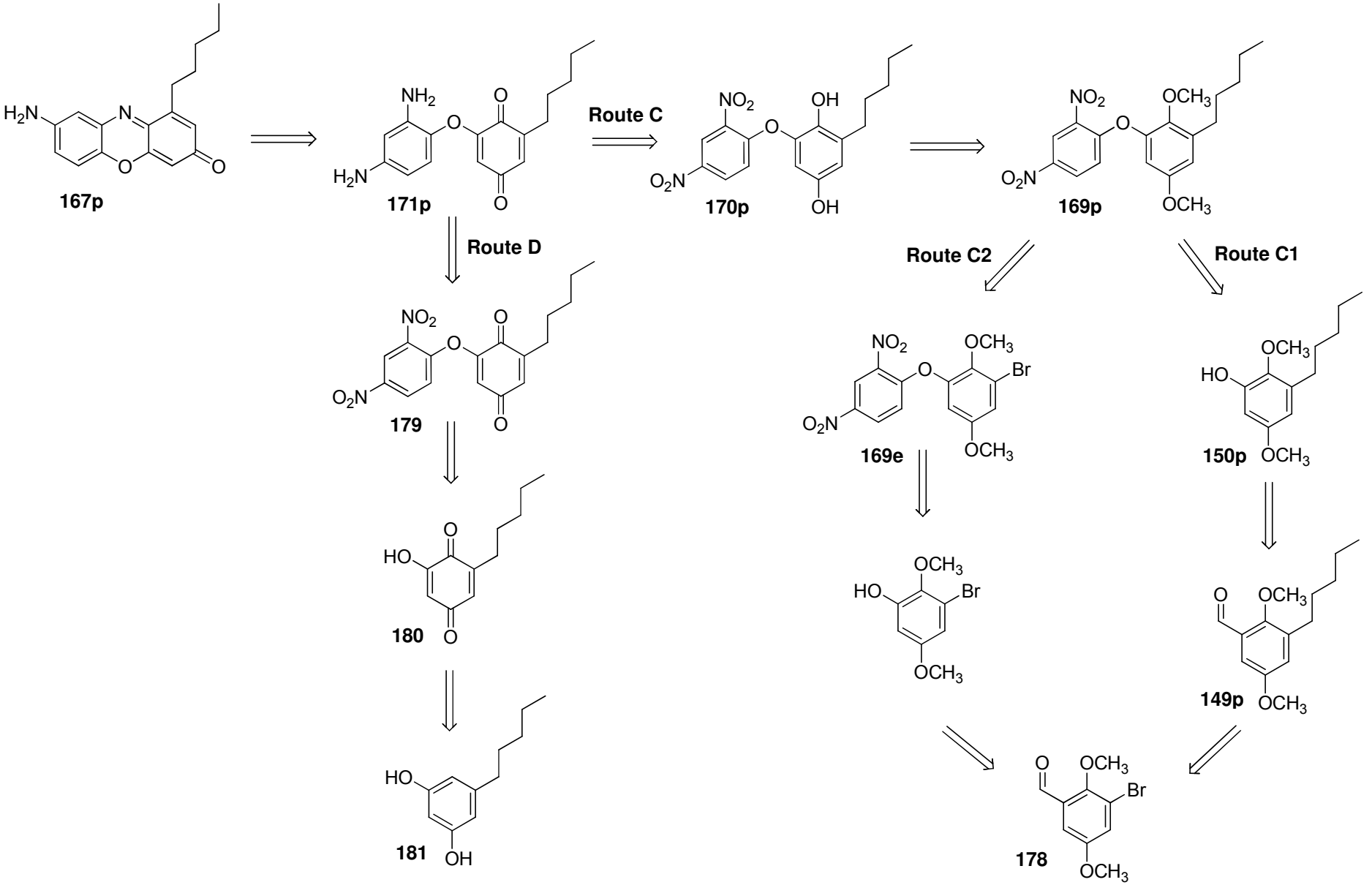
Scheme 2.8: Retrosynthetic analysis on potential 8-amino-phenoxazin-3-ones derivatives.

An alternative to this route, **route B**, considers the possible formation of 2,4-diaminophenoxy-2',5'-benzoquinone **171** from a 2,4-dinitro-2'-bromo-5'-hydroxydiphenylether **174** (**Scheme 2.8**). The latter could be prepared from 2,4-dinitro-2'-bromo-5'-methoxydiphenylether **175a**. For a more convenient synthesis, the latter could be replaced by 2,4-dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether **175b**, achievable in two step from the readily available 3,5-dimethoxyphenol **177** (**Scheme 2.8**).

The next analysis focuses on the synthesis of 8-amino-1-pentylphenoxazinone **167p** *via* a diphenylether approach. Two main routes (**Route C** and **D**) were considered to reach the 2,4-diaminophenoxy-3'-pentyl-2',5'-benzoquinone **171a** required for cyclisation (**Scheme 2.9**).

Route C involved the preparation of 2,4-dinitro-3'-pentyl-2',5'-dimethoxydiphenylether **169p** which was not achievable *via* the two previous route described (**Route A** and **B**, **Scheme 2.8**). Two alternatives were possible to prepare the latter, either the synthesis of 2,5-dimethoxy-6-pentylphenol **150p (Route C1**) or the synthesis of 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether **169e** (**Route C2**, **Scheme 2.9**). Both routes relied on the introduction of a pentyl substituent by substitution of the bromine atom, and both shared 2,5-dimethoxy-6-bromobenzaldehyde **178** as a common precursor (**Scheme 2.9**). The preparation of **169e** seemed advantageous, as it could allow a quicker preparation of diphenylether derivatives *via* substitution of the bromine, hence allowing a quicker preparation of other 8-amino-1-alkylphenoxazin-3-one derivatives.

Route D considered a shorter approach to 2,4-diaminophenoxy-3'-pentyl-2',5'-benzoquinone **171p** *via* the direct preparation of 2,4-dinitrophenoxy-3'-pentyl-2',5'-benzoquinone **179** from 2-hydroxy-6-pentyl-1,4-benzoquinone **180** (**Scheme 2.9**). The latter could be prepared from the readily available olivetol **181** (**Scheme 2.9**), hence avoiding the tedious introduction of a pentyl chain.

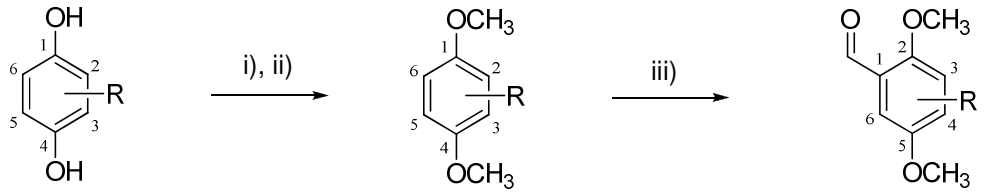


Scheme 2.9: Retrosynthetic analysis on 8-amino-1-pentylphenoxazin-3-one **167p**.

2.2.4 Synthesis of 2,5-dimethoxybenzaldehydes

2.2.4.1 From hydroquinones 173a-c

The preparation of substituted 2,5-dimethoxybenzaldehydes **149a-c**, following **Route A1**, started with the three following commercially available substituted hydroquinones: 2,3-dimethyl-1,4-hydroquinone (**173a**), 2,3,5-trimethyl-1,4-hydroquinone (**173b**) and 2-^{t}butyl-1,4-hydroquinone (**173c**) (**Scheme 2.10**).



173a R = 2,3-diMe
173b R = 2,3,5-triMe
173c R = 2-^{t}butyl

172a R = 2,3-diMe, 91.2%
172b R = 2,3,5-triMe, 84.4%
172c R = 2-^{t}Butyl, 78.5%

149a R = 3,4-diMe, 62.6%
149b R = 3,4,6-triMe, 85.3%
149c R = 4-^{t}Butyl, 56.7%

Scheme 2.10: Preparation of substituted 2,5-dimethoxybenzaldehyde **149a-c** from commercially available hydroquinones **173a-c**
i) NaH, DMF; ii) MeI, 45°C, 3h; iii) TFA, hexamethylenetetraamine, 90°C.

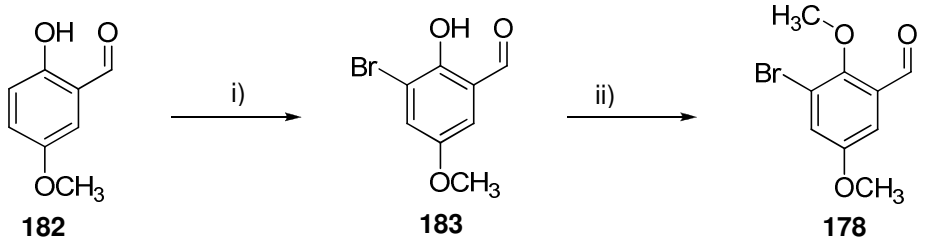
Protection of the hydroquinone (**173a-c**) was effected in DMF using NaH, followed by addition of MeI, as previously described (2008OBC682), giving the dimethoxybenzene (**172a-c**) in excellent yield (**Scheme 2.10**). The methylation of hydroquinone **173a-c** was confirmed by the presence of two singlets integrating each for 3 protons (or one singlet integrating for 6 protons) within the 3.65-3.85ppm region of the ^{1}H NMR spectra, and the absence of a characteristic O-H stretch on the I.R. spectra. Introduction of a formyl substituent was conveniently achieved under Duff reaction conditions (1972JOC3972, 1968T5001), giving the substituted 2,5-dimethoxybenzaldehyde (**149a-c**) in good to excellent yield (**Scheme 2.10**). Introduction of the formyl substituent was confirmed by the presence of a deshielded singlet within the 10.35-10.45ppm region of the ^{1}H NMR spectra and the deshielded carbon signal within the 189-193ppm region of the ^{13}C NMR spectra.

2.2.4.2 From 2-hydroxy-5-methoxybenzaldehyde 182

The preparation of 3-bromo-2,5-dimethoxybenzaldehyde **178** (**Route C**) was reported in the literature and proceeded *via* bromination of 2-hydroxy-5-methoxybenzaldehyde **182**, followed by methylation of the resulting 2-hydroxy-3-bromo-5-methoxybenzaldehyde **183** (**Scheme 2.11**, 1925JCS1998, 525OL2004).

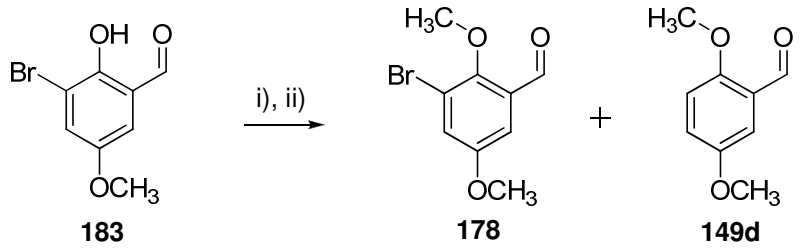
Direct bromination of the readily available 2,5-dimethoxybenzaldehyde **149d** would lead to 2,5-dimethoxy-4-bromobenzaldehyde, as previously reported (2003TL3281), most probably due to cumulated steric hindrance at the 3-position, overcoming the *meta* directing effect of the formyl group.

Bromination of 2-hydroxy-5-methoxybenzaldehyde **195** was effected smoothly using bromine in a glacial acetic acid/sodium acetate mixture, giving the desired 2-hydroxy-3-bromo-5-methoxybenzaldehyde **183** in excellent yield (**Scheme 2.11**).



Scheme 2.11: Preparation of 2,5-dimethoxy-3-bromobenzaldehyde **178**
i) Br_2, AcOH, AcONa, 75.1%; ii) DMF, K_2CO_3, $(MeO)_2SO_2$, 99.7%.

The structure was confirmed by NMR spectroscopy: the ^{1}H NMR spectrum revealed 2 aromatic protons, a doublet at 7.31ppm of J = 3.0Hz (6-H) and another doublet at 7.51ppm of J = 3.0Hz (4-H), a singlet at 10.08ppm corresponded to the formyl group, and a singlet at 10.65ppm corresponded to the hydroxyl group, as well as an aliphatic signal at 3.78ppm that corresponded to the methoxy group. The coupling constant of 3.0Hz for both doublets was characteristic of a *meta* aromatic coupling, hence confirming the 3-position of bromine in **183**. Protection of the free hydroxyl group was first attempted using sodium hydride in DMF, followed by addition of methyl iodide; the desired product was isolated in moderate yield due to some hydro-dehalogenation producing 2,5-dimethoxybenzaldehyde **149** as a by-product (**Scheme 2.12**).



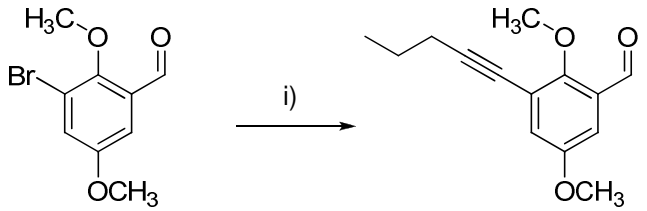
Scheme 2.12: Protection of 2-hydroxy-5-methoxybenzaldehyde **183**
i) NaH, DMF, ii) MeI, 45°C; **178**, 50.7%; **149d**, 14.3% or
i) Dimethylsulphate, K_2CO_3, DMF; **178**, 99.7%.

This method was replaced by protection using dimethyl sulphate in a suspension of K_2CO_3 in DMF, giving 2,5-dimethoxy-3-bromobenzaldehyde **178** in near quantitative yield (2004OL525, **Scheme 2.12**).

2.2.4.2.1 Sonogashira cross-coupling

One direct method to introduce an alkyl substituent onto 2,5-dimethoxy-3-bromobenzaldehyde **178,** in order to prepare the desired 2,5-dimethoxy-6-pentylbenzaldehyde **149p** (**Route C1**), was a cross-coupling reaction, and the well-known Sonogashira conditions were considered first. If successful, this would result in the preparation of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** rather than 2,5-dimethoxy-6-pentylbenzaldehyde **149p**; however the 1-pentyne substituent could be reduced to a pentane chain further along the synthetic pathway.

Replacement of the bromine by 1-pentyne was first attempted using the original Sonogashira conditions (1975TL4467), using Et_3N, CuI, $[Ph_3P]_2PdCl_2$ and 1-pentyne (**Scheme 2.13**); however even after 48h reflux in an inert atmosphere of N_2 in a sealed tube, only a small amount of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** could be recovered, along with unreacted starting material (**Scheme 2.13**).

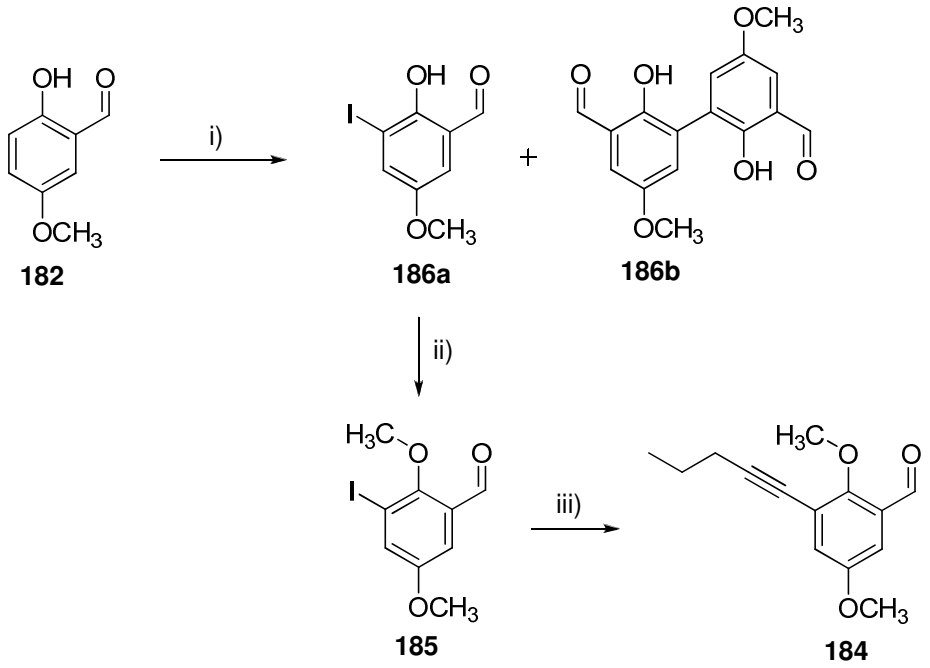


178 **184**

Scheme 2.13: Attempted Sonogoshira coupling on 2,5-dimethoxy-3-bromobenzaldehyde **178**
i) 1-pentyne, CuI, $Pd[PPh_3]Cl_2$, Et_3N, N_2 atm., reflux, 48h, 10.7% or
i) 1-pentyne, CuI, $Pd[PhCN]_2Cl_2$, $(^tBu)_3P$, $(^iPr)_2NH$, dioxane, R.T., 48h, 13.2%.

The use of tris-*t*butylphosphine ligand along with $[PhCN]_2PdCl_2$ as a catalyst, in dioxane and di-*iso*propylamine has been reported to be an extremely efficient system for Sonogashira cross-couplings on less reactive aryl bromides at room temperature (2000OL1729). The electron rich tris-*t*butylphosphine ligand is thought to accelerate the rate of oxidative addition of the aryl bromide to $[PhCN]_2PdCl_2$. However, no improvement was noticeable on the yield of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** under these conditions (**Scheme 2.13**), and 2,5-dimethoxy-3-bromobenzaldehyde **178** was the main product recovered.

The replacement of the slow reacting bromine by the notoriously more reactive iodine was then considered, and the preparation of 2,5-dimethoxy-3-iodobenzaldehyde **185** was attempted, again using 2-hydroxy-5-methoxybenzaldehyde **182** as starting material. The only procedure reported in the literature describes the consequent formation of the dimer **186b** (**Scheme 2.14**) along with the desired 2-hydroxy-3-iodo-5-methoxybenzaldehyde **186a**, when iodination of 2-hydroxy-5-methoxybenzaldehyde **182** was attempted (1996T3841).



Scheme 2.14: Preparation of 2,5-dimethoxy-3-iodobenzaldehyde **185**
i) bis-(2,4,6-trimethylpyridine) iodine (I), DCM, -15°C, **186a**, 47.4%; **186b**, 22.8%; ii) DMF, K_2CO_3, $(MeO)_2SO_2$, 93.5%; iii) $[Ph_3P]_2PdCl_2$, CuI, 1-pentyne, dry Et_3N, dry DMF, N_2 atm., 95°C, 24h, 84.6%.

According to Hart *et al.* (1996T3841), the procedure requires an efficient and non-oxidative iodinating agent, as oxidative condition will favour the formation of **186b**. An attempt to improve the procedure was made by utilising bis-(2,4,6-trimethylpyridine) iodine (I), a very efficient iodinating agent for phenol (1995TL8217). Hence, iodination of 2-hydroxy-5-methoxybenzaldehyde **182** was effected in DCM at -15°C, producing the expected 2-hydroxy-3-iodo-5-methoxybenzaldehyde **186a** in moderate yield; however, the formation of some dimer **186b** could not be avoided (**Scheme 2.14**). These results were slightly poorer to those reported by Hart *et al.* using tetraethylammonium diacetoxyiodate in DCM at -15°C (**186a**, 54%; **186b**, 14%, 1996T3841); however no further attempt were made to improve the yield, and **186a**

was methylated in excellent yield using dimethyl sulphate in a K_2CO_3/DMF suspension to give 2,5-dimethoxy-3-iodobenzaldehyde **185** (**Scheme 63**).

The Sonogashira conditions were again attempted on 2,5-dimethoxy-3-iodobenzaldehyde **185**, using dry DMF as a co-solvent, with this time a total conversion of the starting material and isolation of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** in excellent yield (**Scheme 2.14**).

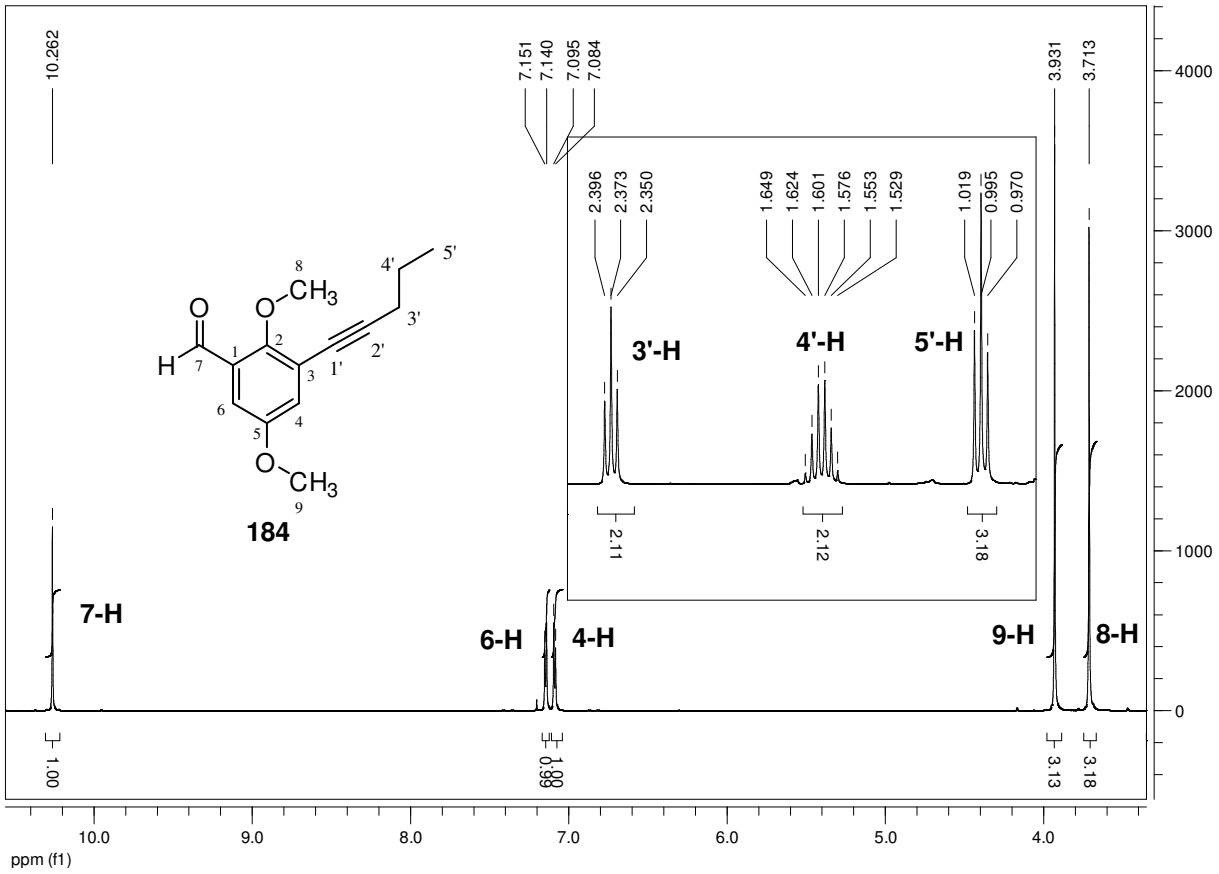
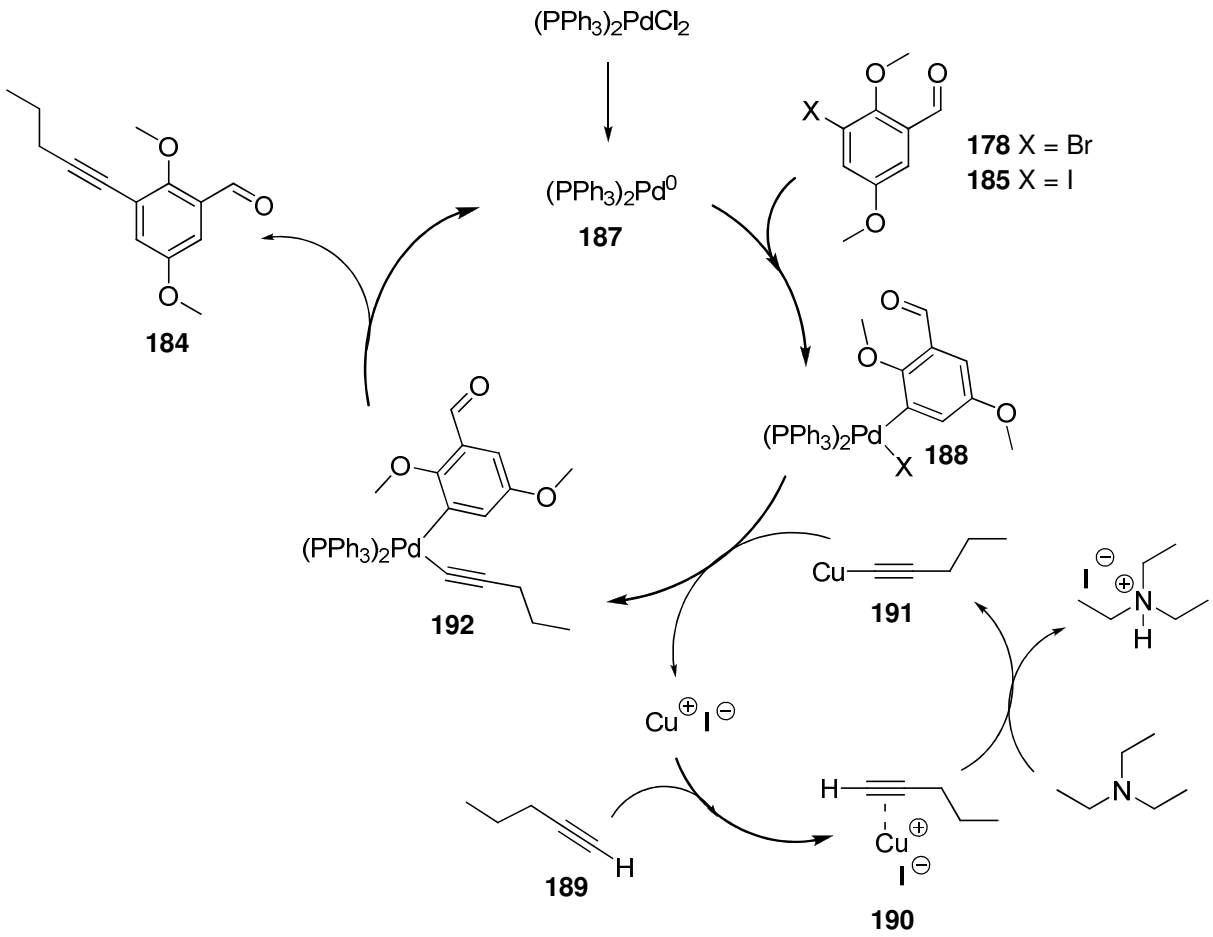


Figure 2.3: ^{1}H NMR spectrum of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** within the 3.40-10.60ppm region, with an inset expansion of the 0.50-2.50ppm region.

The ^{1}H NMR clearly evidenced the presence of the pentyne chain, with a triplet at 0.995ppm corresponding to 5'-H, a sextuplet at 1.59ppm corresponding to 4'-H and a triplet at 2.37ppm corresponding to 3'-H (**Figure 2.3**). The two singlets at 3.71ppm and 3.93ppm belonged to the two methoxy groups and the aromatic region showed two doublets of J = 3.0Hz, at 7.09ppm and 7.15ppm corresponding to 4-H and 6-H respectively and a singlet at 10.26ppm corresponding to the formyl group (**Figure 2.3**).

The replacement of the bromine by iodine had a positive impact on the reaction outcome, confirming the difficulties encountered in prepararing **184** from **178** were largely inherent to the leaving group. 3-Iodo-2,5-dimethoxybenzaldehyde **185** is likely to undergo the oxidative addition to bis(diphenylphosphine)palladium (0) **187** more easily than its brominated analogue **178** (**Scheme 2.15**). The transmetalation step of pentynyl copper **191** to **188** is also likely to be eased by the presence of iodine (**Scheme 2.15**).



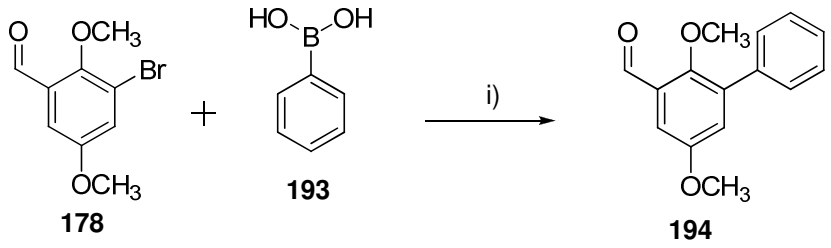
Scheme 2.15: Hypothetic catalytic cycle of the Sonogashira cross-coupling reaction.

2.2.4.2.2 Suzuki cross-coupling

The Suzuki reaction is another interesting cross-coupling reaction, involving the reaction of an aryl boronic acid and an aryl halide. It was considered as an alternative to synthesise 1-substituted 8-aminophenoxazinones *via* **Route C1**.

Kawada *et al.* reported a Suzuki type coupling between protected *p*-hydroxyboronic acids and 2,5-dimethoxy-3-bromobenzaldehyde (1998JOC5831, 1998AGE973).

Using the reported conditions, Suzuki coupling was attempted on 2,5-dimethoxy-3-bromobenzaldehyde **178** with benzene boronic acid **193**, successfully yielding 2,5-dimethoxybiphenyl-3-carbaldehyde **194** in excellent yield (**Scheme 2.16**).



Scheme 2.16: Preparation of 2,5-dimethoxybiphenyl-3-carbaldehyde **194**
i) 2M Na_2CO_3, $Pd(PPh_3)_4$, DME, EtOH, Δ = 100°C, 85%.

The product was evidenced by NMR analysis: ^{1}H showing two multiplets at 7.41-7.63ppm and 7.60-7.64ppm corresponding to protons 3'-H, 4'-H and 5'-H and protons 2'-H and 6'-H of the additional benzene ring (**Figure 2.4**).

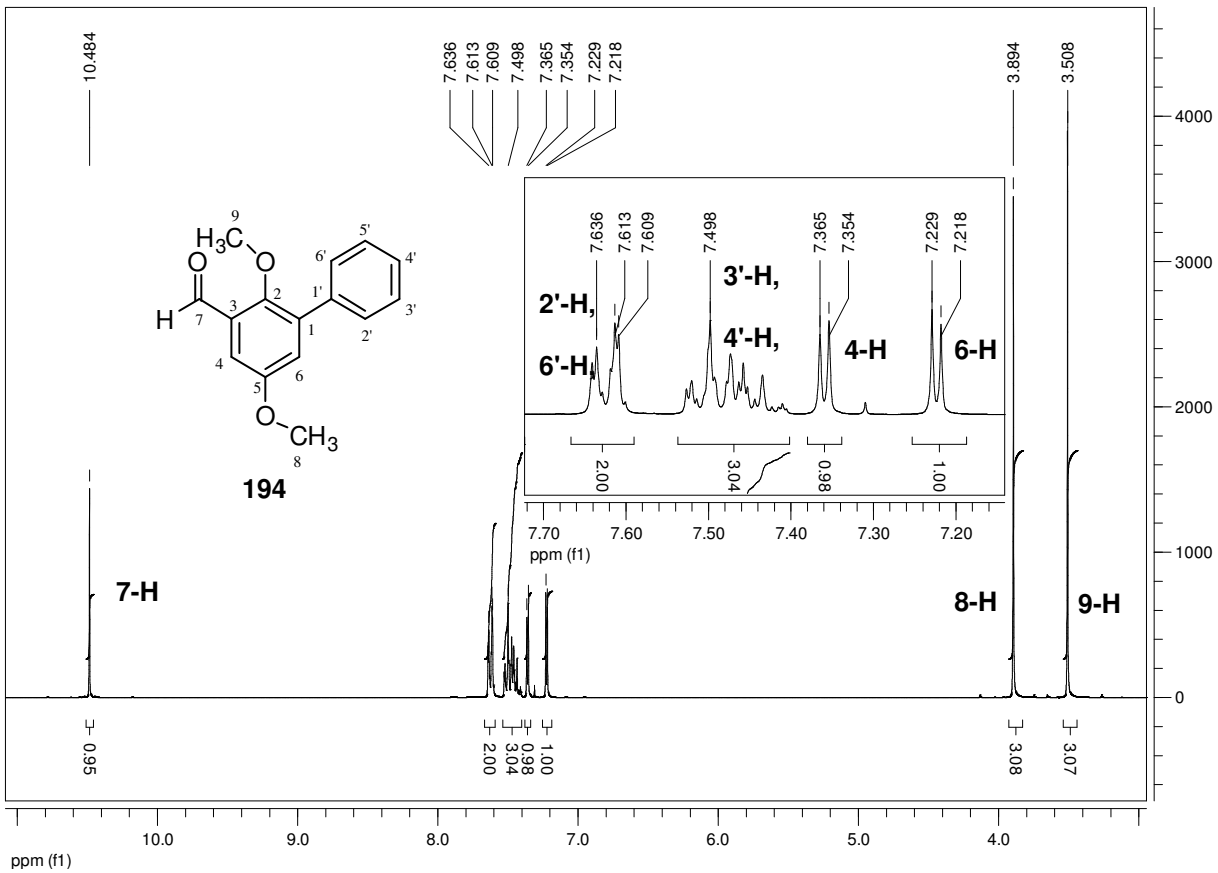


Figure 2.4: ^{1}H NMR spectrum of 2,5-dimethoxybiphenyl-3-carbaldehyde **194** within the 3.00-11.00ppm region, with an inset expansion of the 7.24-7.72ppm region.

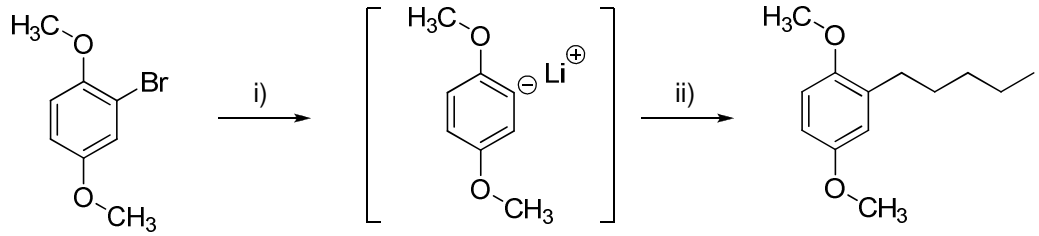
This reaction opened a route to a wide range of 2,5-dimethoxybiphenyl-3-carbaldehydes analogues *via* the use of substituted boronic acids; however, this alternative was not explored any further due to the poor results obtained from testing the corresponding phenoxazin-3-one chromogenic substrate.

2.2.4.3 From 1,4-dimethoxy-2-bromobenzene 174

The *ortho*-directed metalation of some 1,4-dimethoxy-2-bromobenzene analogues using *n*-butyl lithium (*n*-BuLi) has been reported by Carmen *et al.* (1997TA913). The resulting lithiated intermediates were reacted with variety of electrophiles, including methyl iodide.

This method seemed ideal for the substitution of 1,4-dimethoxy-2-bromobenzene **174** with an alkyl substituent, and particularly a pentyl substituant. The resulting product would then lead to 2-substituted 8-aminophenoxazinones via **Route A2**.

Hence, alkylation of 1,4-dimethoxy-2-bromobenzene **174** was attempted, using *n*-BuLi and tetramethylenediamine (TMEDA) in dry THF, followed by addition of 1-bromopentane (**Scheme 2.17**).



178 **195** **196**

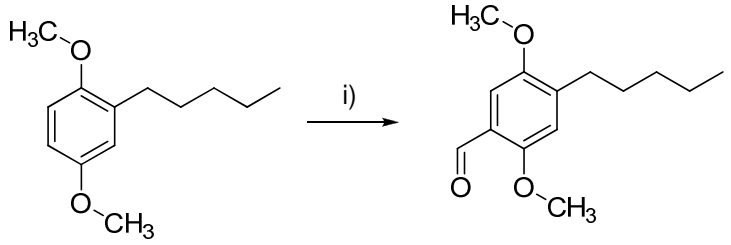
Scheme 2.17: Alkylation of 1,4-dimethoxy-2-bromobenzene **178**
i) 2.5M n-BuLi, dry THF, TMEDA, dry THF, N_2 atm., -78°C, 1h;
ii) Bromopentane, -78°C to R.T. to reflux, 15h, 85.7%.

In a review on direct *ortho* metallation, Snieckus (1990CRV879) reported the use of TMEDA to increase the nucleophilicity of *n*-BuLi, mainly due to a modification of its solubilised form in the presence of TMEDA.

Initially, after formation of 2,5-dimethoxyphenyllithium **195** and addition of neat bromopentane, the reaction was allowed to warm up to room temperature. However, even after a prolonged reaction time, the starting material could not be fully converted to 1,4-dimethoxy-2-pentylbenzene **196** and quenching of the reaction with NH_4Cl resulted in the major formation of 1,4-dimethoxybenzene. It appeared that 1,4-

dimethoxy-2-bromobenzene **178** was fully lithiated, but that nucleophilic attack of bromopentane was too slow to be completed at room temperature. The weak electrophilic character of bromopentane could be responsible for the slow kinetics of the reaction; overnight reflux was necessary for the full conversion of the starting material to 1,4-dimethoxy-2-pentylbenzene **196**.

Formylation of the resulting 1,4-dimethoxy-2-pentylbenzene **196** was effected smoothly using the Duff conditions described previously, producing the expected 2,5-dimethoxy-4-pentylbenzaldehyde **211** in excellent yield (**Scheme 2.18**).



Scheme 2.18: Formylation of 1,4-dimethoxy-2-bromobenzene **196**
i) TFA, urotropine, reflux, 82.4%.

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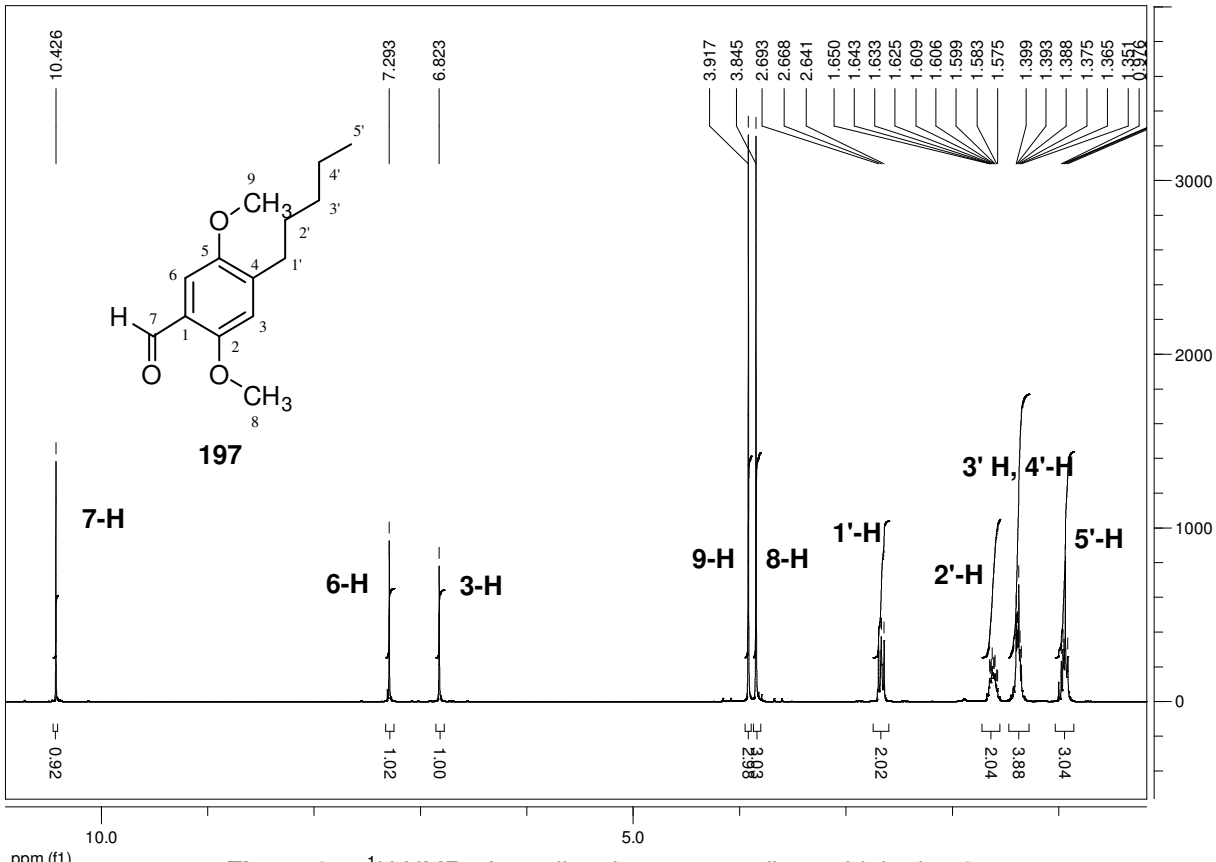


Figure 2.5: ^{1}H NMR of 2,5-dimethoxy-4-pentylbenzaldehyde **197**.

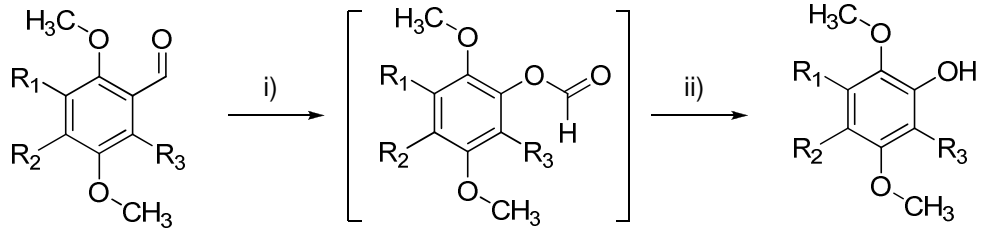
The ^{1}H NMR (**Figure 2.5**) revealed two aromatic singlets at 6.82ppm and 7.29ppm corresponding to 3-H and 6-H, respectively. The absence of coupling between the two protons, unlike the situation with benzaldehydes **178** and **194**, each with two *meta* aromatic protons, confirmed that in the case of **197**, formylation had occurred *para* to the pentyl chain and the two aromatic protons were in 3- and 6-position (**Figure 2.5**).

The successful synthesis of 2,5-dimethoxy-4-pentylbenzaldehyde was expected to lead to 2-pentylphenoxazinones, and allow a comparison of their performances to the gold standard 7-amino-1-pentylphenoxazin-3-one.

2.2.5 Synthesis of 2,5-dimethoxyphenols 150a-f

The Baeyer-Villiger oxidation was employed to convert the substituted 2,5-dimethoxybenzaldehydes to the corresponding substituted 2,5-dimethoxyphenols *via* the formate ester.

The initial conditions, using a suspension of magnesium monoperoxyphthalate (MMPP) in MeOH, proved to be efficient in the case of 2,5-dimethoxybenzaldehyde **149d** and 2,5-dimethoxy-3,4,6-trimethylbenzaldehyde **149b**, giving the corresponding phenols in good yield (60.1% and 62%, respectively). However, the oxidation of 2,5-dimethoxy-3,4-dimethylbenzaldehyde **149a** and 2,5-dimethoxy-4-^{t}butylbenzaldehyde **149c**, using the same conditions, gave unsatisfactory yields for the conversion to the corresponding phenols (17.3% and 20.1%). The use of MMPP in MeOH was replaced by *m*-CPBA in DCM with a great improvement in the conversion to the 2,5-dimethoxyphenols **150a-g** (**Scheme 2.19**).



149a $R_1 = R_2 = Me, R_3 = H$
149b $R_1 = R_2 = R_3 = Me$
149c $R_2 = R_3 = H, R_2 = {}^t$Butyl
178 $R_2 = R_3 = H, R_2 = Br$
197 $R_1 = R_3 = H, R_2 = n$-pentyl
194 $R_2 = R_3 = H, R_2 =$ phenyl
149d $R_1 = R_2 = R_3 = H$

198a $R_1 = R_2 = Me, R_3 = H$
198b $R_1 = R_2 = R_3 = Me$
198c $R_2 = R_3 = H, R_2 = {}^t$Butyl
198d $R_2 = R_3 = H, R_2 = Br$
198e $R_1 = R_3 = H, R_2 = n$-pentyl
198f $R_2 = R_3 = H, R_2 =$ phenyl
198g $R_1 = R_2 = R_3 = H$

150a $R_1 = R_2 = Me, R_3 = H$, 79.8%
150b $R_1 = R_2 = R_3 = Me$, 83.0%
150c $R_2 = R_3 = H, R_2 = {}^t$butyl, 54.15%
150d $R_1 = R_3 = H, R_2 = n$-pentyl, 81.4%
150e $R_2 = R_3 = H, R_2 = Br$, 70.4%
150f $R_2 = R_3 = H, R_2 =$ phenyl, 74.2%
150g $R_1 = R_2 = R_3 = H$, 60.9%

Scheme 2.19: Preparation of 2,5-dimethoxyphenols **150a-g**
i) 75% *m*-CPBA, DCM, 18-24h, R.T.; ii) 10% aq. NaOH, MeOH.

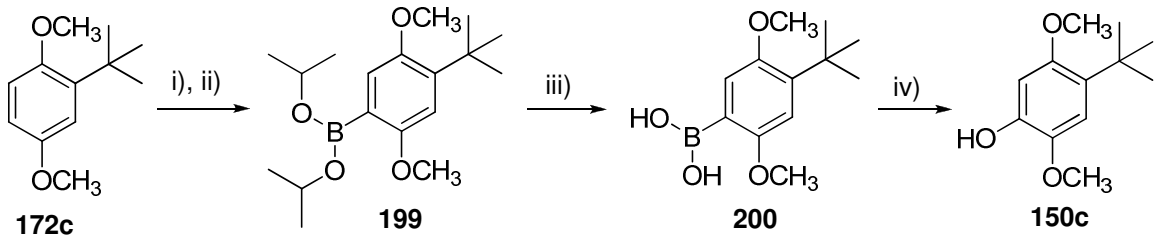
The 2,5-dimethoxybenzylformate intermediates **198a-g** were not isolated, but immediately hydrolysed to the corresponding 2,5-dimethoxyphenols **150a-g** in MeOH using 10% aq. NaOH in MeOH (**Scheme 2.19**). All phenols were isolated in good to excellent yields (**Scheme 2.19**).

The formation of a phenol was confirmed in all cases by I.R. spectroscopy with the presence of an absorption band within the 3416-3263cm^{-1} region of the I.R. spectra, characteristic of an O-H stretch (**Table 6**). The hydroxylic proton could also be observed on the ^{1}H NMR spectra as singlet within the 5.77-6.10ppm region; correlation of this singlet with 1-C, 2-C, 5-C and 6-C on the HMBC spectrum occurred only in the case of **213e** and **213f** were the two aromatic proton were positioned *meta* to each other.

		213a-g
δ_H NMR (ppm)	OC*H*$_3$ (7-H)	3.40-3.90
	OC*H*$_3$ (8-H)	3.65-3.90
	-O*H*	5.55-5.90
ν_{max} (cm^{-1})	O-H	3416-3263

Table 2.4: Key ^{1}H NMR and I.R. features of substituted 2,5-dimethoxyphenols **150a-g**.

In the case of 1,4-dimethoxy-2-*t*butylbenzene, the phenol was also prepared in good yield *via* the introduction of a boronic acid group using a solution of 2.5M *n*-BuLi in hexane followed by the addition of tri*iso*propylborate. The resulting boronic acid could then be oxidised to the desired phenol using a 35% w/v aqueous solution of H_2O_2 (**Scheme 2.20**).

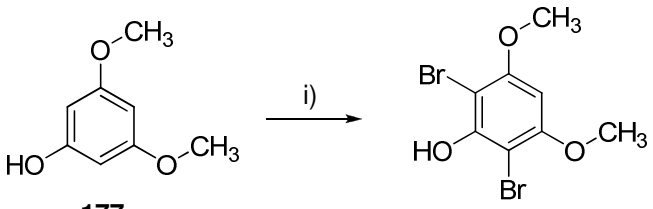


172c **199** **200** **150c**

Scheme 2.20: Preparation of 2,5-dimethoxy-4-*t*butylphenol **150c**
i) 2.5M *n*-BuLi in hexane, dry THF, -78°C; ii) [*iso*PrO]$_3$B, -78°C to R.T.;
iii) sat. aq. NH_4Cl; iv) 35% w/w H_2O_2, THF, R.T., 53.4%

2.2.6 Bromination of 3,5-dimethoxyphenol 177

The preparation of 2,6-dibromo-3,5-dimethoxyphenol **176** from 3,5-dimethoxyphenol **177** was achieved in good yield by simple addition of two equivalents of bromine to a DCM solution of 3,5-dimethoxyphenol **177**, according to the literature (**Scheme 2.21**, 1989CJC335).



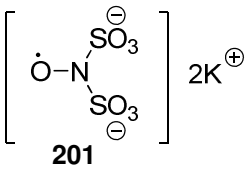
177 **176**

Scheme 2.21: Bromination of 3,5-dimethoxyphenol **177**
i) Br_2, DCM, 72.6%.

The main evidence for the 2,6-disubstitution was the presence of only two aromatic signals at 6.06 and 6.20ppm, each integrating for one hydrogen, corresponding to 1-O*H* and 4-H respectively.

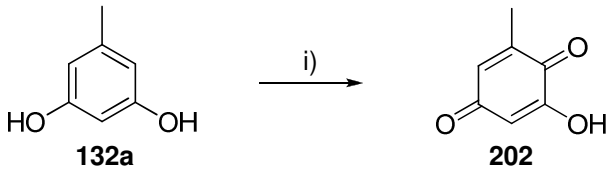
2.2.7 Oxidation of olivetol 181

The oxidant of choice for the synthesis of 1,4-benzoquinones from phenols with no *para* substituents, required for the preparation of 2-hydroxy-6-pentyl-1,4-benzoquinone **180** from olivetol **181** (**Route D**), was potassium nitrosodisulfonate (Fremy's salt, **201**), with many reports of this type of oxidation in the literature (1971CRV229).



201

A first attempt to oxidise olivetol, adapted from the procedure described by Musso (1958CB349, **Scheme 2.22**), in CH_3CN using a K_2HPO_4 buffer was unsuccessful and only olivetol was recovered.

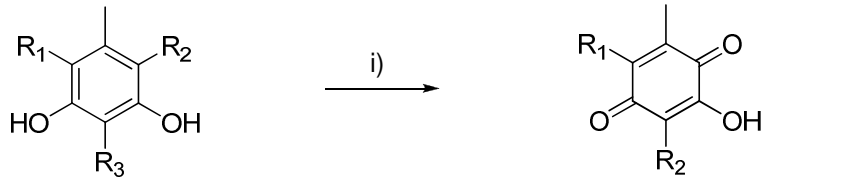


132a **202**

Scheme 2.22: Oxidation of orcinol (1958CB349)
i) Fremy's salt, H_2O, K_2HPO_4.

Oxidation of various halogenated orcinol derivatives (**203a-c**) to the corresponding 2-hydroxy-1,4-benzoquinone (**204a-c**) has previously been reported (1983J(P1)2595,

Scheme 2.23) using potassium nitrosodisulfonate; all conversions were realised in excellent yield.

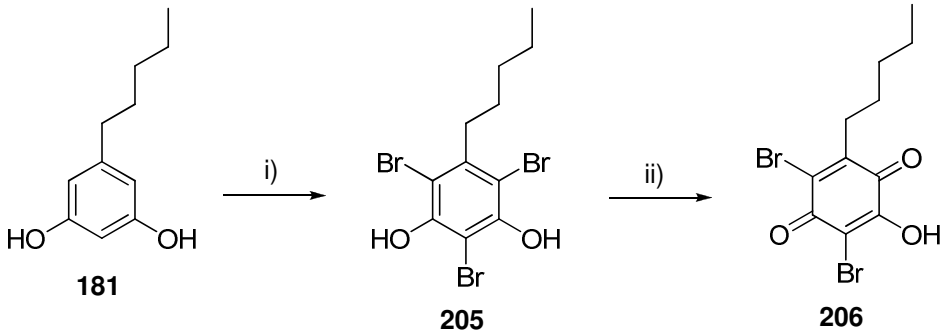


203a $R_1 = R_2 = H$, $R_3 = Cl$
203b $R_1 = R_2 = R_3 = Cl$
203c $R_1 = R_2 = R_3 = Br$

204a $R_1 = H$, $R_2 = Cl$, 86%
204b $R_1 = R_2 = Cl$, 83%
204c $R_1 = R_2 = Br$, 96%

Scheme 2.23: Oxidation of orcinol derivatives **203a-c**
i) Fremy's salt, K_2HPO_4, H_2O, R.T. (1983J(P1)2595).

Preparation of a bromo olivetol derivative was then attempted in order to drive the oxidation to 2-hydroxy-6-pentyl-1,4-benzoquinone. The 4-bromo-5-pentylresorcinol required to prepare the latter *via* oxidation, was not achievable using olivetol. Addition of one equivalent of Br_2 to **181** occurred primarily at the 2-position, but the high reactivity of olivetol led to an inseparable mixture of olivetol, mono-, di and tri-bromoolivetol. The preparation of tribromoolivetol **205**, previously reported in the literature (1936CB1643), using an excess of Br_2 within a suspension of NaOAc in glacial AcOH (**Scheme 2.24**), was chosen to avoid the formation of partially brominated olivetol analogues. Tribromoolivetol **205** was isolated in excellent yield (**Scheme 2.24**); the full substitution of olivetol was confirmed by ^1H NMR spectroscopy, with only one singlet in the aromatic region, at 6.10ppm, which disappeared after a D_2O shake, as is characteristic of phenolic protons. The presence of unwanted bromine atoms was thought not be a problem, as they could be removed readily during the later catalytic hydrogenation step.



181 **205** **206**

Scheme 2.24: Preparation of 2,6-dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone **206**
i) Br_2, AcOH, NaOAc, 0°C to R.T., 5h, 92.2%; ii) Fremy's salt, K_2HPO_4, H_2O, CH_3CN, R.T., 81.6%.

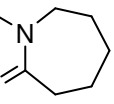
The oxidation of 2,4,6-tribromoolivetol **205** was attempted again, with a slight modification, as CH_3CN was used to ease the dissolution of 2,4,6-tribromoolivetol in the K_2HPO_4 buffer. The expected 2,6-dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone **206** was isolated in excellent yield, although some starting material remained unreacted. The conversion could not be pushed to completion, even after further addition of Fremy's salt.

The oxidation to a quinone was evidenced by the presence of two deshielded signals on the ^{13}C NMR spectrum at 172.9 and 178.1 ppm and by the two quinone C=O stretch present at 1638 and 1669 cm^{-1} the I.R. spectrum

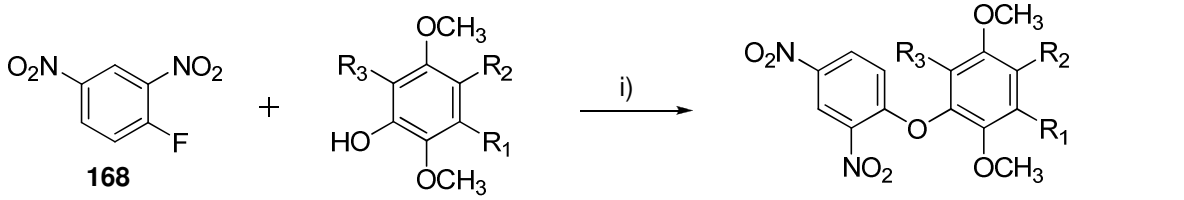
2.2.8 Synthesis of diphenylethers 169a-g, 175b-c and 152c-d

2.2.8.1 Reaction with 2,4-dinitro-1-fluorobenzene 168

The preparation of 2,4-dinitro-2',5'-dimethoxydiphenylethers **169a-g** was achieved as previously described (2008OBC682), using 2,4-dinitro-1-fluorobenzene **168**, commonly called Sanger's reagent. The 2,5-dimethoxyphenols **150a-e** previously prepared were dissolved in DMSO, followed by the addition of Et_3N and 2,4-dinitro-1-fluorobenzene, giving the diphenylethers **169a-g** as white to yellow crystalline solids in excellent yield (**Scheme 2.25**). The exception was 3,4,6-trimethylphenol, which failed to react in the presence of Et_3N. This issue was addressed by the use of a much stronger organic base, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, **207**), allowing the formation of the desired diphenylether **169b** in excellent yield.



207

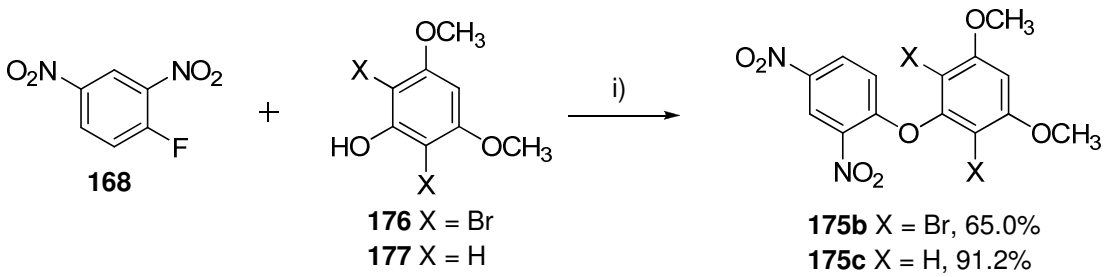


150a $R_1 = R_2 = Me$, $R_3 = H$
150b $R_1 = R_2 = R_3 = Me$
150c $R_2 = R_3 = H$, $R_2 = {}^t$butyl
150d $R_1 = R_3 = H$, $R_2 = n$-pentyl
150e $R_2 = R_3 = H$, $R_1 = Br$
150f $R_2 = R_3 = H$, $R_1 =$ phenyl
150g $R_1 = R_2 = R_3 = H$

169a $R_1 = R_2 = Me$, $R_3 = H$, 61.6%
169b $R_1 = R_2 = R_3 = Me$, 85.7% b
169c $R_2 = R_3 = H$, $R_2 = {}^t$butyl, 95.8%
169d $R_1 = R_3 = H$, $R_2 = n$-pentyl, 92.4%
169e $R_2 = R_3 = H$, $R_1 = Br$, 76.6%
169f $R_2 = R_3 = H$, $R_1 =$ phenyl, 91.8%
169g $R_1 = R_2 = R_3 = H$, 88.2%

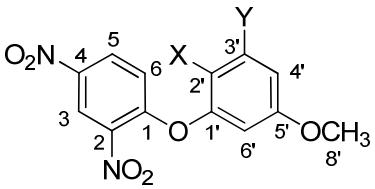
Scheme 2.25: Preparation of 2,4-dinitro-2',5'-dimethoxydiphenylether **169a-g**
i) DMSO, Et_3N, R.T., 5h-18h (b: DMSO, DBU, R.T., 18h).

Likewise, 3,5-dimethoxyphenol and its dibromoanalogue were coupled to 2,4-dinitro-1-fluorobenzene yielding 2,4-dinitro-3',5'-dimethoxydiphenylethers (**175b-c**, **Scheme 2.26**).



Scheme 2.26: Preparation of 2,4-dinitro-3,5-dimethoxydiphenylether **175b-c**
i) DMSO, Et_3N, R.T., 5h-18h.

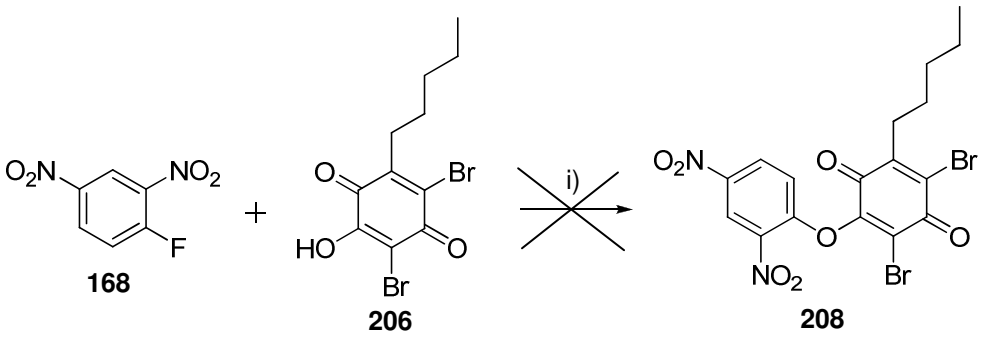
The formation of a diphenylether product was confirmed by the presence of twelve aromatic carbon signals on the ^{13}C NMR spectrum. The ^{1}H NMR spectrum provided evidence of the 2,4-dinitrobenzene ring with the three deshielded aromatic protons (3-H, 5-H and 6-H) and evidence of dimethoxybenzene ring with the two methoxy signals within 3.32-3.755ppm for 7'-H and within 3.63-3.82ppm for 8'-H. I.R. spectroscopy showed the characteristic absorption bands for nitro groups within the 1337-1345cm^{-1} and 1529-1540cm^{-1} (**Table 2.5**).



		169a-g (X = OCH_3, Y = H) and **175b-c** (X = H, Y = OCH_3)
δ_H NMR (ppm) d_6-DMSO	3-H	8.73-8.94 (1H, d, *J* = 2.7-3.0 Hz)
	5-H	8.15-8.39 (1H, dd, *J* = 9.0-9.3, 2.7-3.0 Hz)
	6-H	6.69-7.05 (1H, d, *J* = 9.0-9.3 Hz)
	7'-C*H*$_3$	3.32-3.755 (3H, s)
	8'-C*H*$_3$	3.63-3.82 (3H, s)
ν_{max} (cm^{-1})	NO_2	1337-1345 and 1529-1540

Table 2.5: Key ^{1}H NMR and I.R. spectral features of 2,4-dinitro-2,5-dimethoxydiphenylethers **169a-g** and 2,4-dinitro-3,5-dimethoxydiphenylethers **175b-c**.

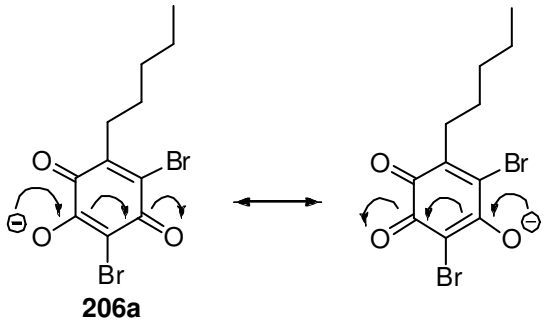
However, when a similar coupling was attempted on 2,6-dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone **206**, none of the expected 2,4-dinitrophenoxy-4',6'-dibromo-3'-pentyl-2',5'-benzoquinone **208** could be isolated, only starting material was recovered (**Scheme 2.27**). Even under more forcing conditions, using NaH in DMF with heat over 72h, none of the expected product was formed.



Scheme 2.27: Attempted preparation of 2,4-dinitrophenoxy-4',6'-dibromo-3'-pentyl-2',5'-benzoquinone **208** i) DMSO, Et_3N, R.T. or NaH, DMF, 50°C, 72h.

2,6-Dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone was easily deprotonated, as witnessed upon the addition of a base by the formation of a deep purple colour generated by the corresponding anion **206a** (**Scheme 2.28**).

2,4-Dinitro-1-fluorobenzene itself particularly favours aromatic nucleophilic substitution, as the nitro groups *ortho* and *para* to the fluorine atom strongly activate the carbon in position 1- to nucleophilic attack. The failure of this reaction was possibly linked to the conjugation and subsequent stabilisation of the anion **206a**, as well as some steric hindrance generated by the two bromine atoms, ultimately hindering nucleophilic attack on 2,4-dinitro-1-fluorobenzene **168**. The use of simple 2-hydroxy-1,4-benzoquinones for this particular reaction could confirm their low reactivity; however, this option was not further investigated and no more attempt to prepare 8-aminophenoxazin-3-ones *via* **Route D** were made.

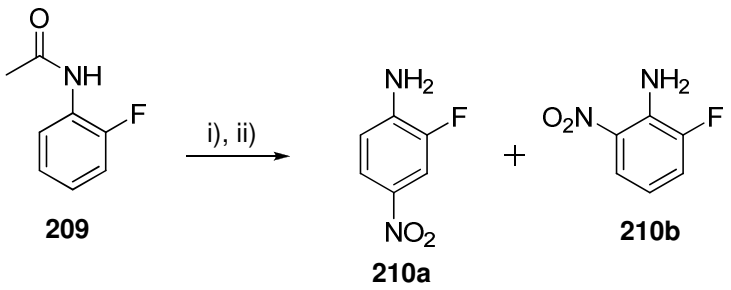


Scheme 2.28: Stabilisation of 3,5-dibromo-3-hydroxylate-6-pentylbenzoquinone anion **206a**.

2.2.8.2 Reaction with 2,5-dinitro-1-fluorobenzene 151

Although the synthesis of 7-aminophenoxazin-3-one was not the primary target, some of the 2,5-dimethoxyphenols prepared previously could lead to new, potentially interesting, substituted 7-aminophenoxazin-3-ones.

Under the route currently being investigated, 2,5-dinitro-2',5'-dimethoxydiphenylethers **152** were the required precursors to the corresponding 7-aminophenoxazin-3-ones. The 2,5-dinitro-1-fluorobenzene **151** required for their synthesis, was not readily available, and had to be prepared from 2-fluoroacetanilide **209**, as previously described in the literature (2002TL3221). Hence, 2-fluoroacetanilide **209** was nitrated using a mixture of fuming HNO_3 and conc. H_2SO_4, followed by hydrolysis of the amide bond using aq. NaOH (**Scheme 2.29**). Nitration mainly occurred in the *para* position to the acetanilide group, but the *ortho* isomer **210b** was also isolated in low yield (1976ACB141, 1989JFC245, **Scheme 2.29**).

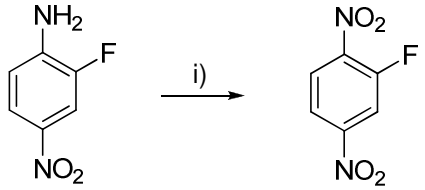


Scheme 2.29: Nitration of 2-fluoroacetanilide **209**
i) fuming HNO_3, conc. H_2SO_4, glacial AcOH, 0℃, ii) 10% aq. NaOH, MeOH; **210a**, 67.5%; **210b**,15.3%.

The following step, involving the oxidation of aniline to a nitro group, was previously reported in the literature for 4-nitro-2-fluoroaniline **210a**, using dimethyldioxirane (2002TL3221); however, the yield was not given. The oxidation method previously used by the University of Sunderland research team (T-2006MI00) was effected in glacial AcOH using $NaBO_3.4H_2O$, affording the desired 2,5-dinitro-1-fluorobenzene **151** in 49.0% yield. A new method was desirable to improve the yield and the use of trifluoroperacetic acid (TFPAA) was investigated. Emmons reported the oxidation of several aniline derivatives to their corresponding nitrobenzene derivatives, in excellent yield, using TFPAA (1954JACS3470). The method described the generation of TFPAA by the addition of trifluoroacetic anhydride to a DCM solution of 90% w/v aqueous H_2O_2. Such a high concentration of H_2O_2 is not available anymore due to its known hazard and use of a 35% w/v aqueous H_2O_2 solution was reported

to lower considerably the yield of aniline oxidation, the formation of by-products being important in this case (1954JACS3470). This issue was addressed by replacing the aqueous H_2O_2 solution with a urea-hydrogen peroxide adduct (UHPA) to generate the TFPAA (1992TL4835).

2-Fluoro-4-nitroaniline was then oxidised in CH_3CN using TFPAA, previously generated by addition of trifluoroacetic anhydride to an CH_3CN suspension of urea-hydrogen peroxide adduct, to the expected 2,5-dinitrofluorobenzene **151** in good yield (**Scheme 2.30**).



210a **151**

Scheme 2.30: Oxidation of 2-fluoro-4-nitroaniline **210a**
i) UHPA, TFAA, CH_3CN, -15°C to R.T., 70.2%.

The formation of 2,5-dinitrofluorobenzene **151** was confirmed firstly by a general deshielding of all aromatic protons, particularly 3-H (numbered as 6-H in the starting material **210a**) positioned *ortho* to the transformed substituent, and secondly by the absence of the broad singlet integrating for the two protons of N*H*$_2$ (**Table 2.6**).

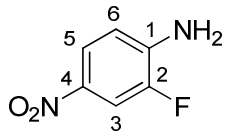
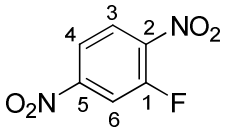
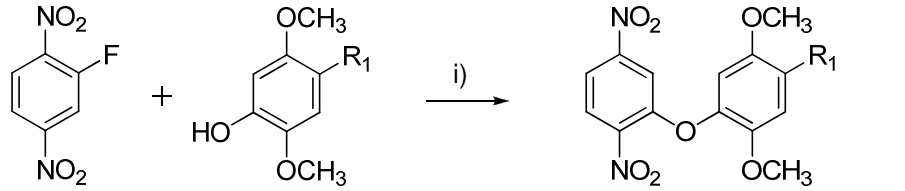
		 **210a**	 **151**
δ_H NMR (ppm) d_6-DMSO	3-H	7.87-7.93 (2H, m)	8.42 (1H, dd, *J* = 9.0, 7.5 Hz)
	5-H	7.87-7.93 (2H, m)	8.26 (1H, ddd, *J* = 9.0, 2.4, 1.5 Hz)
	6-H	6.85 (1H, d, *J* = 8.7 Hz)	8.53 (1H, dd, *J* = 10.5, 2.4 Hz)
	N*H*$_2$	6.77 (2H, br. s)	-
ν_{max} (cm^{-1})	NH_2	3493 and 3395	-
	NO_2	1522 and 1314	1549 and 1343

Table 2.6: Key ^{1}H NMR and I.R. spectral features of 2-fluoro-4-nitroaniline **210a** and 2,5-dinitro-1-fluorobenzene **151**.

The oxidation of the amine substituent was also notable on the I.R. spectrum of 2,5-dinitrofluorobenzene **151** since the two N-H stretches at 3493 and 3395cm^{-1} could not be observed anymore (**Table 2.6**).

2,5-Dinitro-1-fluorobenzene **151** does not benefit from the same reactivity toward nucleophilic aromatic substitution as 2,4-dinitro-1-fluorobenzene **168**, as only the nitro group *ortho* to the fluorine activates the carbon in the 1-position. Attempts to couple 2,5-dimethoxyphenol using Et_3N in DMSO gave poor results, as mostly starting materials were recovered, even after 72h. The use of NaH in DMF improved the yields of the 2,5-dinitro-2',5'-dimethoxydiphenylether **152c-d** (**Scheme 2.31**), but they remained comparatively much lower than the corresponding 2,4-dinitro-2',5'-dimethoxydiphenylethers **169c-d**.



151

150c R_2 = *t*butyl
150d R_2 = *n*-pentyl

152c R_1 = *t*butyl, 47.4%
152d R_1 = *n*-pentyl, 46.8%

Scheme 2.31: Preparation of 2,5-dinitro-2,5-dimethoxydiphenylether **152c-d**.

2.2.9 Attempted substitution of diphenylether

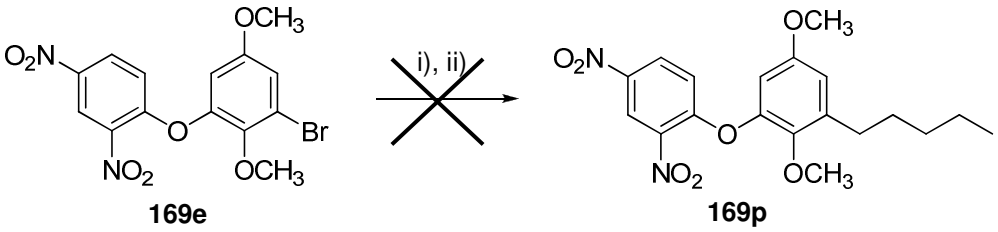
2.2.9.1 From 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether

A series of reactions were attempted to substitute the bromine atom on 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether **169e**, with the objective of shortening the synthesis of 8-aminophenoxazin-3-ones derivatives (**Route C2**).

A Grignard-type reaction was first attempted, as described in the literature, using ferric acetylacetonate ($Fe(acac)_3$) (2005OS33). The conditions described were applied to 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether with bromopentane, but failed to yield any product (**Scheme 2.32**).

Bromine-lithium exchange was attempted in the same fashion as for 1,4-dimethoxy-2-bromobenzene, using 2.5M *n*-BuLi in dry THF followed by the addition of bromopentane (**Scheme 2.32**). *n*-BuLi was revealed as too strong a base for the

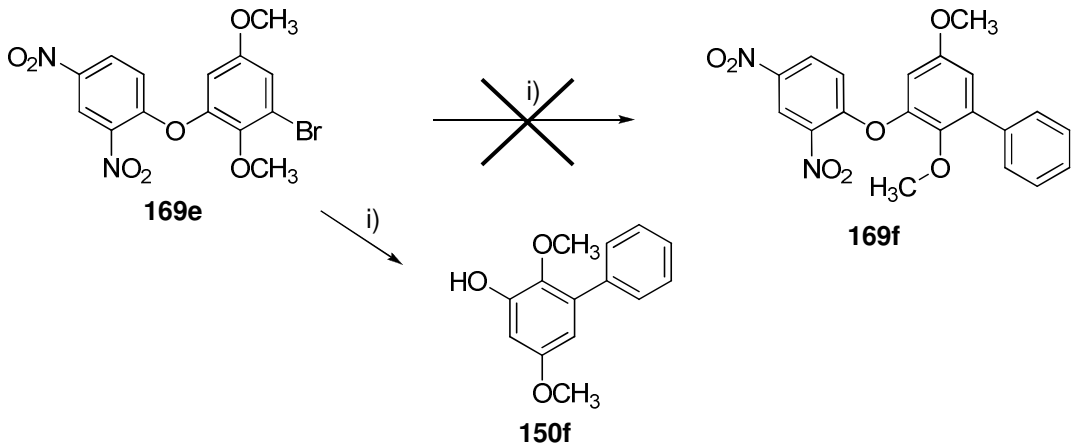
diphenylether moiety, as a considerable amount of tarring occurred, probably initiated by cleavage of the diphenylether bond, followed by further side reactions.



169e **169p**

Scheme 2.32: Attempted preparation of 2,4-dinitro-2',5'-dimethoxy-3'-pentyldiphenylether **169p**
i) Bromopentane, Mg, dry THF, 1,2-dibromoethane; ii) $Fe(acac)_3$, dry NMP, or
i) dry THF, 2.5M *n*-BuLi, N_2, -75°C; ii) Bromopentane -75°C to R.T.

Suzuki coupling was also attempted; by analogy with the results obtained from the trials effected previously on 2,5-dimethoxy-3-bromobenzaldehyde **178**, it seemed that boronic acid reacted more readily with the 2,5-dimethoxy-3-bromobenzene moiety. Using the conditions described above, 2,4-dinitro-2',5'-dimethoxydiphenylether **169e** was thus subjected to a coupling reaction with benzeneboronic acid **193** (**Scheme 2.33**).



169e **169f** **150f**

Scheme 2.33: Suzuki coupling of 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether **169e**
i) Phenylboronic acid, $Pd(PPh_3)_4$, 2M Na_2CO_3, EtOH, DME, Δ = 100°C, 24h, N_2 atm., 93.3%.

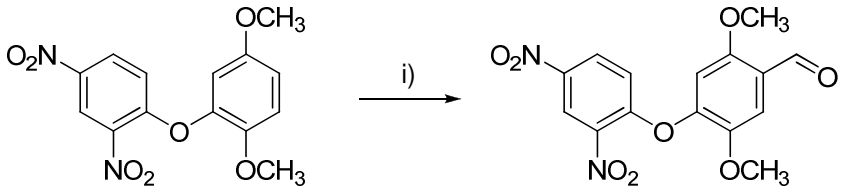
After 24h reflux, no more starting material could be observed by TLC in the reaction mixture. NMR analysis of the isolated product revealed the presence of the expected extra aryl group, but the absence of signals corresponding to the 2,4-dinitrobenzene moiety and the presence of a sharp singlet at 6.10ppm. This strongly indicated that

coupling of the boronic acid did occur along with a cleavage of diphenylether. Comparison with the spectra of 2,5-dimethoxy-biphenyl-3-ol **150f** previously prepared from 2,5-dimethoxybiphenyl-3-carbaldehyde **194** confirmed the formation of 2,5-dimethoxy-biphenyl-3-ol during this reaction in 93.3% yield.

This unsuccessful series of reactions undertaken on 2,4-dinitro-2',5'-dimethoxy-3'-bromodiphenylether **169e** proved this diphenylether to be unsuitable for the desired type of substitutions, i.e. introduction of alkyl substituents and this pathway was not further investigated.

2.2.9.2 From 2,4-dinitro-2',5'-dimethoxydiphenylether 169g

In a last attempt to substitute on the diphenylether ring, a formyl group was introduced on the 2,4-dinitro-2',5'-dimethoxydiphenylether **169g**, using the Duff conditions described previously and leading to the isolation of 2,4-dinitro-4'-formyl-2',5'-dimethoxydiphenylether **211** in good yield (**Scheme 2.34**). The ^{1}H NMR spectrum exhibited a deshielded singlet at 9.75ppm, characteristic of a formyl group, further confirmed by a very deshielded peak in the ^{13}C NMR spectrum at 193.5ppm. Five aromatic protons, a doublet at 8.41ppm, a doublet of doublets at 7.89ppm, a singlet at 7.14ppm, a doublet at 6.57ppm and a singlet at 6.455ppm confirmed the presence of the two aromatic rings. The two singlets were part of the dimethoxybenzene ring and confirmed their relative *para* position and the introduction of the formyl group into the 4-position.



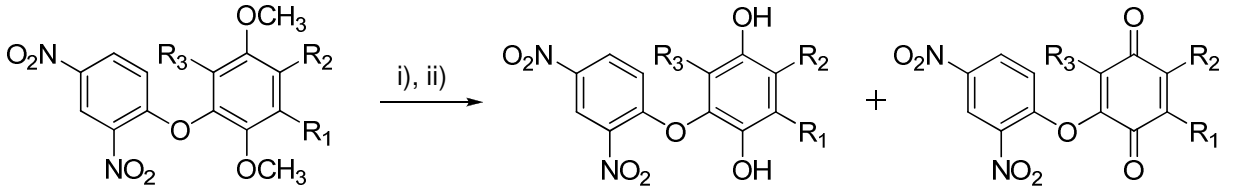
169g **211**

Scheme 2.34: Formylation of 2,4-dinitro-2',5'-dimethoxydiphenylether **169g**
i) Hexamethylenetetramine, TFA, 95°C, 48h, 73.2%.

The successful preparation of 2,4-dinitro-4'-formyl-2',5'-dimethoxydiphenylether **211** opened a route to new derivatives, as the formyl group could be transformed into a wide range of products, within the limitations previously experienced with diphenylethers, *i.e.* use of an inorganic base or strong organic base would have to be avoided.

2.2.10 Demethylation of diphenylether 169b,g and 227b using BBr_3

The diphenylethers prepared were deprotected using BBr_3. This reagent has been used previously on similar structures (2008OBC682) and has also been reported to be very effective for the demethylation of various aryl methyl ethers (1968T2289). This method was successfully applied to the demethylation of 2,4-dinitro-2',5'-dimethoxydiphenylether **169g** and 2,4-dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether **169b**. Deprotection was confirmed by the disappearance of the two methoxy signals in the aliphatic region of the ^1H NMR and ^{13}C NMR spectra and the formation of two deshielded singlets within the 9.50-9.80ppm region for **170a** and within the 8.80-8.90ppm region for **170b**. The I.R. spectra of both **170a** and **170b** also showed absorption band within the 3300-3400cm^{-1} region, characteristic of phenols. In the case of 2,4-dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether, a notable amount of the corresponding quinone **179b** was also isolated (**Scheme 2.35**).



169b $R_1 = R_2 = R_3 = CH_3$
169g $R_1 = R_2 = R_3 = H$

170a $R_1 = R_2 = R_3 = H$, 66.5%
170b $R_1 = R_2 = R_3 = CH_3$, 53.5%

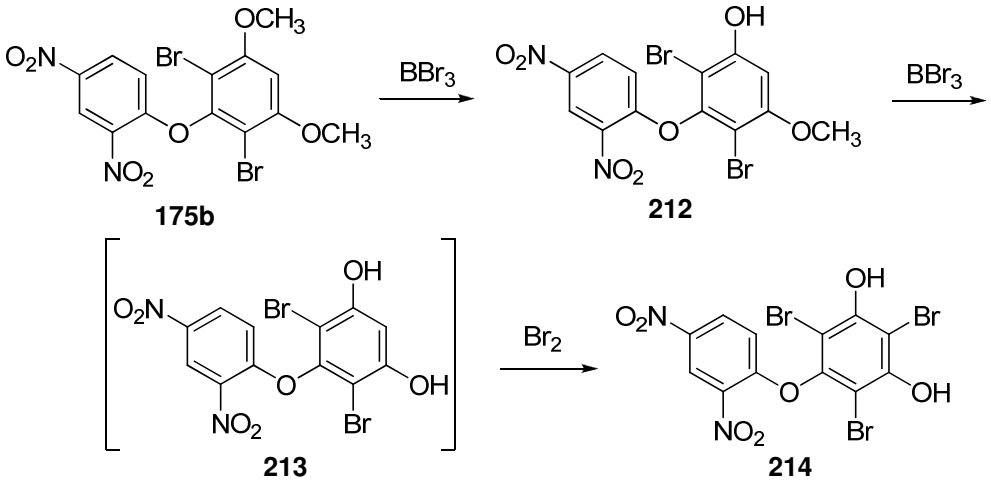
179b $R_1 = R_2 = R_3 = CH_3$, 13.0%

Scheme 2.35: Deprotection of 2,4-dinitro-2',5'-dimethoxydiphenylether **169b** and **169g**
i) 1M BBr_3 in DCM, dry DCM, N_2 atm., -75°C to R.T., ii) H_2O.

The isolation of quinone **179b** was confirmed by the presence on the ^{13}C NMR spectrum of two strongly deshielded carbon signals at 180.5ppm and 186.9ppm and the two quinone carbonyl stretches observed at 1648 and 1666cm^{-1} on the I.R. spectrum.

This method of deprotection showed limitations when deprotection of 2,4-dinitro-2',5'-dimethoxy-4'-^{t}butyldiphenylether **169c** was attempted, as only a small amount of the expected product could be isolated, along with a complex mixture of by-products.

Further experiments with the BBr_3 demethylation procedure and 2,4-dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether **175b** also proved to be problematic. Two products were isolated: the mono-deprotected diphenylether **212** and the 2,4-dinitro-2',5'-dihydroxy-3',4',6'-tribromodiphenylether **214** (**Scheme 2.36**).



Scheme 2.36: Deprotection of 2,4-dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether **175b**.

The isolation of **212** and **214** was rationalised as follows: as soon as the second demethylation occurred, generating **213**, the electron rich carbon at the 4'-position reacted immediately with bromine to form the triibromodiphenyl ether **214**, avoiding any possible isolation of the desired 2,4-dinitro-2',5'-dihydroxy-3',6'-dibromodiphenylether **213.** The isolation of **214** suggested that sequential demethylation of the diphenylether occurred, one methyl group being removed at a time.

The formation of **214** was suspected as the aromatic region only showed 3 aromatic protons on the ^{1}H NMR spectrum, a doublet at 7.10ppm, a doublet of doublets at 8.33ppm and a doublet at 8.95ppm, corresponding to 6-H, 5-H and 3-H respectively. The addition of a third bromine atom was confirmed by mass spectrometry (**Figure 2.6**): the four peaks corresponding to the different isotopic mass of **214**, with a 1:4:4:1 ratio, correlated with the mass of **214** and the presence of three bromine atoms.

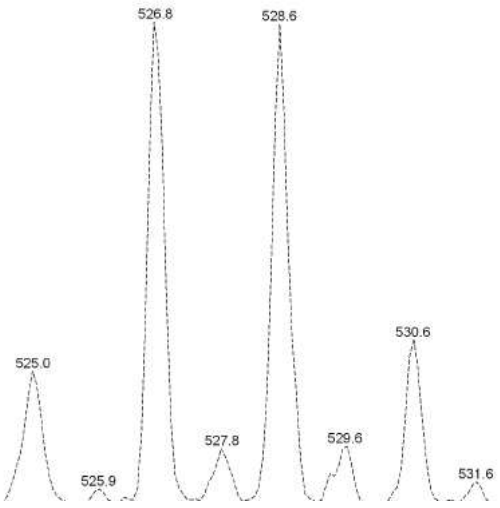
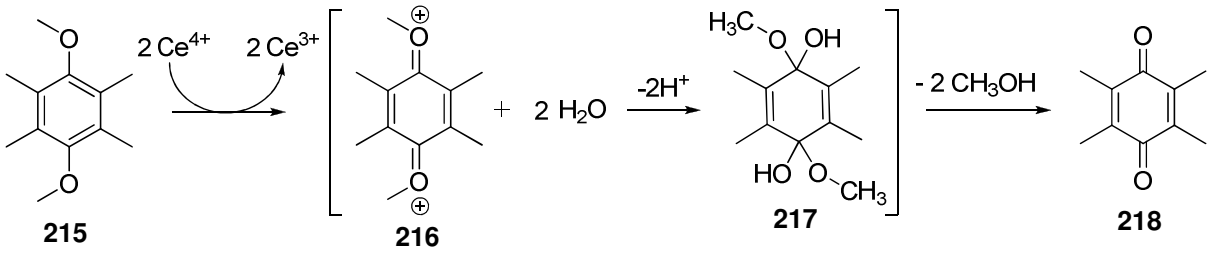


Figure 2.6: Mass spectrum representing the $(M\text{-}H)^-$ peaks of the different bromine isotopes of **214**.

The preparation of 8-aminophenoxazin-3-one derivatives from 2,4-dinitro-2',5'-dihydroxy-3',6'-dibromodiphenylether **213** (**Route B**), was abandoned here due to the difficulties encountered to prepare this intermediate.

2.2.11 Oxidative demethylation of diphenylethers 169a,c,d,f and 152c-d using cerium (IV) ammonium nitrate

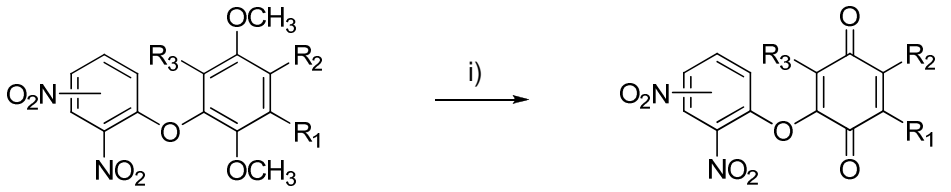
Consequently, an alternative method for the deprotection of diphenylethers was sought. The formation of a range of substituted *p*-quinones in high yield from substituted *p*-dimethoxybenzenes *via* oxidative demethylation using cerium (IV) ammonium nitrate (CAN), reported by Jacob *et al.* (1976JOC3627), seemed advantageous. 1,4-Dimethoxybenzene-2,3,5,6-tetramethylbenzene **215** is thought to be first oxidised by cerium (IV) to the di-cation **216**, followed by addition of water to form the tetramethylcyclohexadiene intermediate **217** (**Scheme 2.37**). Subsequent loss of CH_3OH leads to the formation of duroquinone **218** (1976JOC3627, **Scheme 2.37**).



Scheme 2.37: Suggested intermediates for the formation of duroquinone *via* CAN oxidation of 1,4-dimethoxybenzene-2,3,5,6-tetramethylbenzene **215** (1976JOC3627).

Oxidative demethylation was hence attempted, by addition of an aqueous solution of CAN to an acetonitrile solution of the correctly substituted diphenylethers (**Scheme 2.38**). The expected 2,4- (**179a,c,d,e**) and 2,5-dinitrophenoxy-2',5'-benzoquinones (**219a-b**) were all isolated in good to excellent yield (**Scheme 2.38**).

The oxidative-demethylation was evidenced by the absence of signals corresponding to the two methoxy groups within the 3.30-3.82ppm region of the ^{1}H NMR spectrum. The key characteristics were the quinone carbonyls, observed as two deshielded signals, on the ^{13}C NMR spectra, within 179.9-181.4ppm and 186.6-187ppm for quinones **179a,c,d,e** and within 181.0-181.4ppm and 187.65-187.8ppm for quinones **219a-b** (**Table 7**).



169a 4-NO_2, R_1 = R_2 = Me, R_3 = H
169c 4-NO_2, R_2 = R_3 = H, R_2 = ^{t}butyl
169d 4-NO_2, R_1 = R_3 = H, R_2 = *n*-pentyl
169f 4-NO_2, R_2 = R_3 = H, R_1 = phenyl
152c 5-NO_2, R_2 = R_3 = H, R_2 = ^{t}butyl
152d 5-NO_2, R_1 = R_3 = H, R_2 = *n*-pentyl

179a 4-NO_2, R_1 = R_2 = Me, R_3 = H, 90.9%
179c 4-NO_2, R_2 = R_3 = H, R_2 = ^{t}butyl, 63.0%
179d 4-NO_2, R_1 = R_3 = H, R_2 = *n*-pentyl, 96.7%
179e 4-NO_2, R_2 = R_3 = H, R_1 = phenyl, 84.6%
219a 5-NO_2, R_2 = R_3 = H, R_2 = ^{t}butyl, 93.2%
219b 5-NO_2, R_1 = R_3 = H, R_2 = *n*-pentyl, 91.4%

Scheme 2.38: Oxidative demethylation of substituted 2,4- and 2,5-dinitro-2',5'dimethoxydiphenylethers i) CAN, CH_3CN, H_2O, R.T.

The quinone C=O stretches were also visible as two distinct absorption bands within the 1647-1651cm^{-1} and 1665-1676cm^{-1} region of the I.R. spectra. The three deshielded aromatic protons (3-H, 5-H and 6-H for **179a,c,d,e** or 3-H, 4-H and 6-H for **219a-b**) and the absorbance characteristic of nitro groups within the 1341-1347cm^{-1} and 1526-1544cm^{-1} region of the I.R. spectra confirmed the integrity of the dinitrophenoxybenzoquinone ring (**Table 2.7**).

		179a,c,d,e		**219a-b**
δ_H NMR (ppm) d_6-DMSO	3-H	8.88-8.94 (1H, d, *J* = 2.7Hz)	3-H	8.32-8.44 (1H, d, *J* = 9.0Hz)
	5-H	8.38-8.60 (1H, dd, *J* = 9.0-9.3, 2.7Hz)	4-H	8.23-8.33 (1H, dd, *J* = 9.0, 2.4Hz)
	6-H	7.31-7.90 (1H, d, *J* = 9.0-9.3Hz)	6-H	8.41-8.51 (1H, d, *J* = 2.4Hz)
δ_C NMR (ppm)	2'-C	179.9-181.4	2'-C	181.0-181.4
	5'-C	186.6-187.6	5'-C	187.65-187.8
ν_{max} (cm^{-1})	C=O	1647-1649 and 1665-1676	C=O	1650-1651 and 1672-1676
	NO_2	1341-1347 and 1526-1543	NO_2	1343-1347 and 1540-1544

Table 2.7: Key spectral features of substituted 2,4- and 2,5-dinitrophenoxy-2'-5'-benzoquinones **179a,c,d,e** and **219a-b**.

The two carbon signals at 180ppm and 187ppm were assigned to 2'-C and 5'-C, respectively (**Figure 2.7**), the assignments being justified by the HMQC spectrum of 2,4-dinitrophenoxy-4'-pentyl-2',5'-benzoquinone **179d** (**Figure 2.7**).

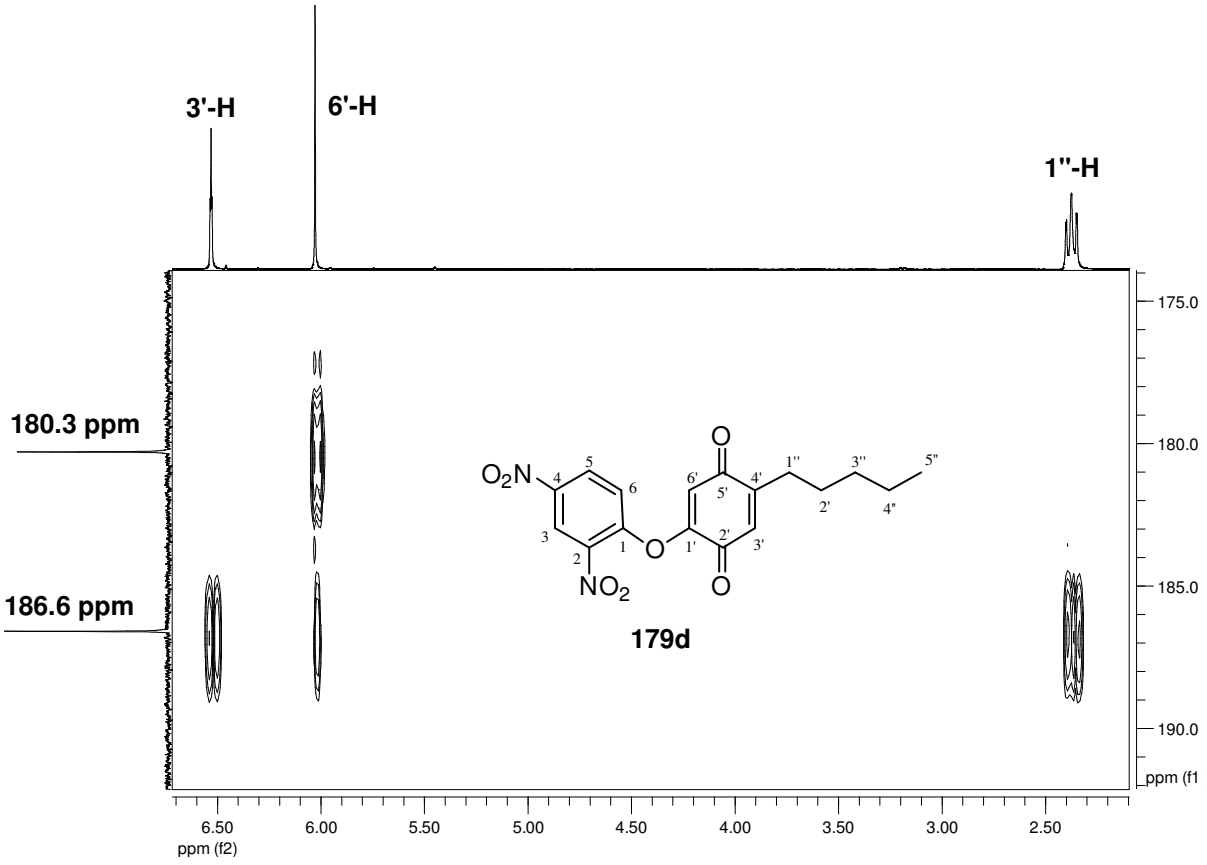


Figure 2.7: HMQC spectrum of 2,4-dinitrophenoxy-4'-pentyl-2',5'-benzoquinone **179d**.

Firstly, a strong correlation between the carbon signal at 180.3ppm and 6'-H could be seen, as well as a similar correlation between the carbon signal at 186.6ppm and 3'-H. This suggested a close proximity between 6'-H and the carbon at 180.3ppm and between 3'-H and the carbon at 186.6ppm. However, a strong correlation was also seen between 1''-H, belonging to the pentyl chain substituent, and the carbon at 186.6ppm, but no correlation between the same 1''-H and the carbon signal at 180.3ppm. A three bond correlation is much more likely to be seen on an HMQC spectrum than a four bond correlation, suggesting that the signal at 186.6 ppm could be assigned to 5'-C and the signal at 180.3ppm could be assigned to 2'-C. The HMQC spectrum of 2,4-dinitrophenoxy-3',4',6'-trimethyl-2',5'-benzoquinone **179b** (**Figure 2.8**) led to the same conclusion regarding the assignment of 2'-C and 5'-C.

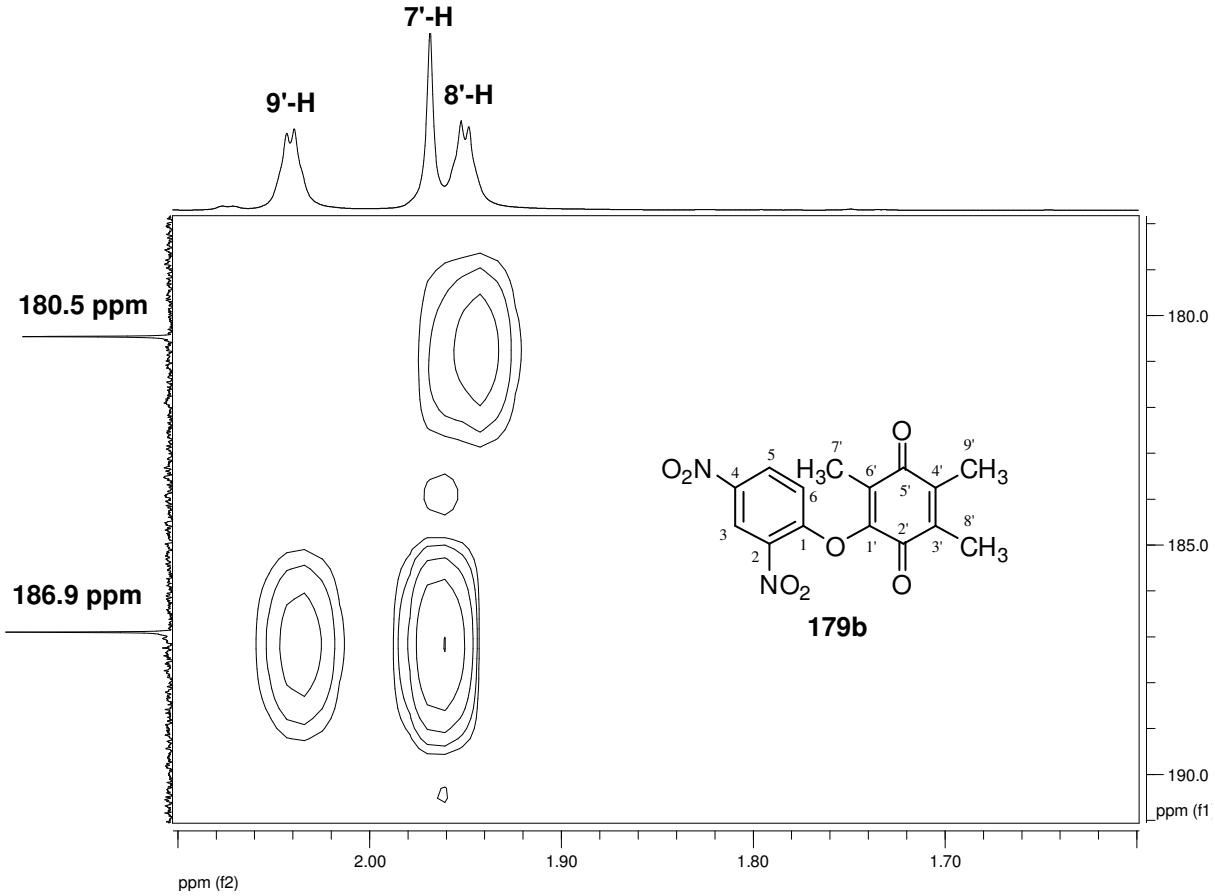


Figure 2.8: HMQC of 2,4-dinitrophenoxy-3',4',5'-trimethyl-2',5'-benzoquinone **179b**.

A correlation between the methyl group 8'-H and the carbon signal at 180.5ppm and correlation between the two methyl groups 7'-H and 9'-H and the carbon signal at 186.9ppm also strongly suggested that the 2'-C signal was at 180.5ppm and the 5'-C signal was at 186.9ppm.

This strategy could not be applied to the assignment of 2'-C and 5'-C of the other 2,4- and 2,5-dinitrophenoxy-2',5'-benzoquinones, mainly due to the absence of determinant correlations between the substituents of the quinoid ring and the 2'-C and 5'-C signals. It was considered reasonable to assign the signal around 180ppm to 2'-C and the signal around 187ppm to 5'-C for all the 2,4-dinitro and 2,5-dinitrophenoxy-2',5'-benzoquinones prepared, by extrapolation of the signals assigned to **179b** and **179d**. Considering the nature of their substituents, it is very unlikely that the chemical shift of 2'-C and 5'-C would be inverted.

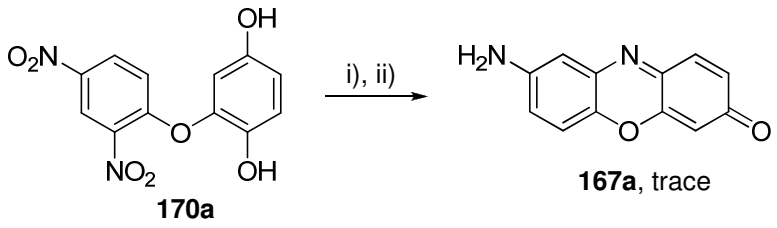
Overall, the CAN oxidative-demethylation proved to be superior to the BBr_3 demethylation for 2,4- and 2,5-dinitro-2',5'-dimethoxydiphenylethers. Cerium (IV) ammonium nitrate presented the advantage of being easily handled, compared to the

moisture sensitive BBr_3, resulting in a simple reaction procedure, providing crystalline solids in high yield.

2.2.12 Synthesis of 7- and 8-aminophenoxazin-3-ones

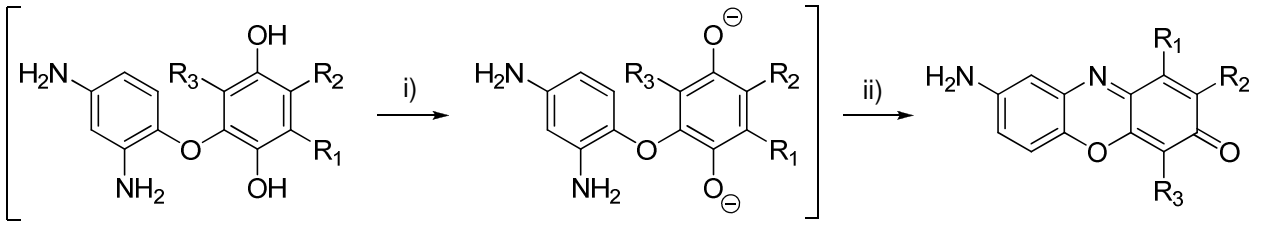
2.2.12.1 From 2,4-dinitro-2',5'-dihydroxydiphenylethers 170a-b

The catalytic hydrogenation of 2,4-dinitro-2',5'-dihydroxydiphenylether **170a**, followed by removal of the catalyst and aeration of the resulting solution, was reported to give 8-aminophenoxazin-3-one **167a** in excellent yield (T-2006MI00, **Scheme 2.39**). This procedure was attempted several times to prepare 8-aminophenoxazin-3-one **167a**, with little success, as 8-aminophenoxazin-3-one **167a** was only isolated in poor yield, if at all (**Scheme 2.39**).



Scheme 2.39: Oxidative cyclisation of 2,4-dinitro-2',5'-dihydroxydiphenylether **170a**
i) H_2, Pd/C 5%, MeOH, ii) Aerial oxidation.

After examination of the suggested mechanism based upon the oxidation of duroquinone (1938JACS98), it seemed necessary that the hydroquinone was fully deprotonated for the oxidation to occur. Hence, Et_3N was added after the reduction of 2,4-dinitro-2',5'-dihydroxydiphenylether **170a** to promote the formation of **221a**, but this was not sufficient to drive the oxidative-cyclisation and a metal oxide (AgO, Ag_2O or MnO_2) was added to promote the reaction (**Scheme 2.40**).



220a $R_1 = R_2 = R_3 = H$
220b $R_1 = R_2 = R_3 = CH_3$

221a $R_1 = R_2 = R_3 = H$
221b $R_1 = R_2 = R_3 = CH_3$

167a $R_1 = R_2 = R_3 = H$, 91.3%
167c $R_1 = R_2 = R_3 = CH_3$, 86.7%

Scheme 2.40: Oxidative cyclisation of 2,4-diamino-2',5'-dihydroxydiphenylether derivatives
i) Et_3N, ii) [O] with AgO, Ag_2O or MnO_2.

Using these conditions, 8-aminophenoxazin-3-one **167a** and 8-amino-1,2,4-trimethylphenoxazin-3-one **167c** were isolated in good yield (**Scheme 2.40**). The formation of the phenoxazinone ring could be witnessed by the appearance of a deep purple colour. The 8-aminophenoxazin-3-one structure was confirmed mainly by two key pieces of information, namely, the presence of a deshielded carbon signal around 185ppm on the ^{13}C NMR spectrum and the presence of one amino group, as shown on the ^{1}H NMR spectrum by a broad singlet around 5.3ppm, integrating for two protons (**Figure 2.9**) and on the I.R. spectrum by three bands characteristic of an aromatic amine within 3200-3370cm^{-1}.

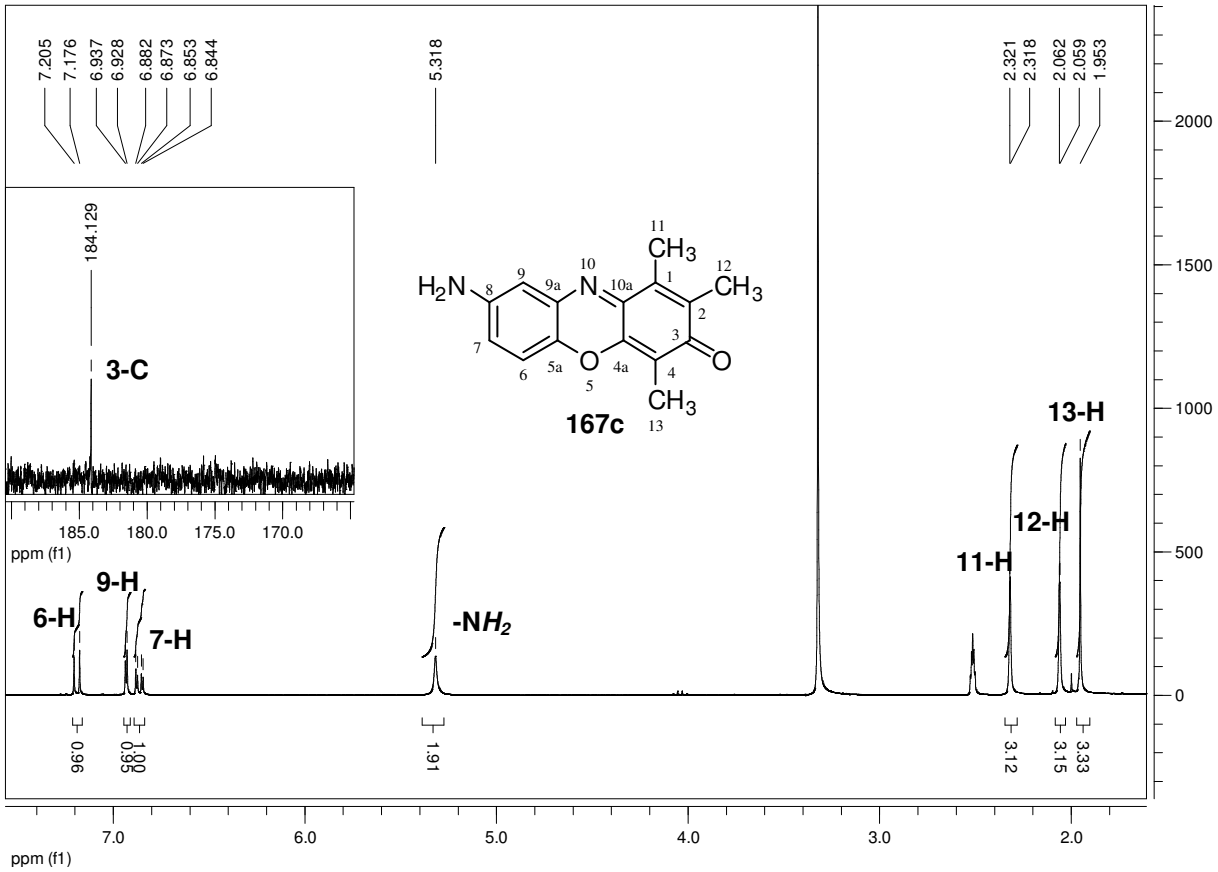
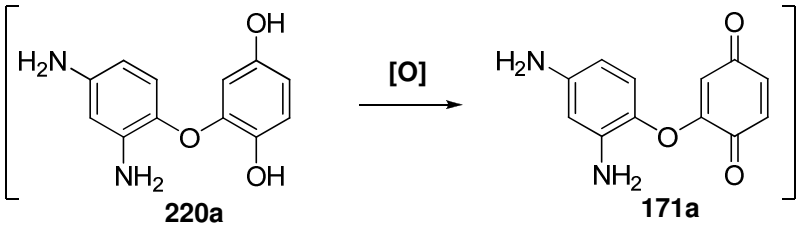


Figure 2.9: ^{1}H NMR spectrum of 8-amino-1,2,4-trimethylphenoxazin-3-one **167c** and expansion of the ^{13}C NMR spectrum within the 165-190ppm region (inset).

Experiments showed that neutral conditions were insufficient to drive the cyclisation of **220a** to **167a**. The formation of a zwitterion, as suggested in section **1.3.9** (**Scheme 1.50**), seemed not to be occuring with **220a-b**; furthermore, aerial oxidation was not sufficient to drive the cyclisation of **220a** to **167a** (**Scheme 2.40**), even after the potential formation of **221a** and stronger oxidising conditions were required to

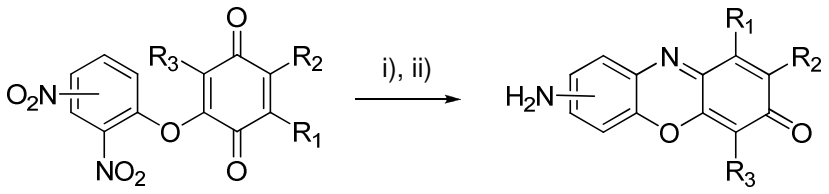
isolate **167a**. These observations are in favour with the formation of **171a** as a key intermediate, and highlight the need to favour the oxidation of **220a** to **171a** (**Scheme 2.41**) for optimum formation of 8-aminophenoxazin-3-ones.



Scheme 2.41: Oxidation of 2,4-diamino-2',5'-dihydroxydiphenylether **220a**.

2.2.12.2 From 2,4 and 2,5-dinitrophenoxy-2',5'-benzoquinones

Dinitrophenoxy-2',5'-benzoquinones **179a-e** and **219a-b** were reduced by catalytic hydrogenation using Pd/C 5% as a catalyst. A solvent mixture of EtOAc : MeOH (1:1) was used to improve the solubility of the starting material. The reduction typically required 2h for completion and, after removal of the catalyst, cyclisation to the phenoxazin-3-one moiety occurred rapidly upon addition of a metal oxide such as Ag_2O or MnO_2 (**Scheme 2.42**).



179a 4-NO_2, $R_1 = R_2 = CH_3$, $R_3 = H$
179b 4-NO_2, $R_1 = R_2 = R_3 = CH_3$
179c 4-NO_2, $R_1 = R_3 = {}^t$bu, $R_2 = H$
179d 4-NO_2, $R_1 = R_3$ = pentyl, $R_2 = H$
179e 4-NO_2, R_1 = Ph, $R_2 = R_3 = H$
219a 5-NO_2, $R_1 = R_3 = {}^t$bu, $R_2 = H$
219b 5-NO_2, $R_1 = R_3$ = pentyl, $R_2 = H$

167b 8-NH_2, $R_1 = R_2 = CH_3$, $R_3 = H$, 94.0%
167c 8-NH_2, $R_1 = R_2 = R_3 = CH_3$, 92.0%
167d 8-NH_2, $R_1 = R_3 = {}^t$bu, $R_2 = H$, 86.8%
167e 8-NH_2, $R_1 = R_3$ = pentyl, $R_2 = H$, 94.7%
167f 8-NH_2, R_1 = Ph, $R_2 = R_3 = H$, 73.4%
59d 7-NH_2, $R_1 = R_3 = {}^t$bu, $R_2 = H$, 97.5%
59e 7-NH_2, $R_1 = R_3$ = pentyl, $R_2 = H$, 97.6%

Scheme 2.42: Catalytic hydrogenation of dinitrophenoxy-2',5'-benzoquinone **179a-d,f** and **219a-b**
i) MeOH/EtOAc (1:1), Pd/C 5%, H_2, P = 2.4bar; ii) MeOH, Ag_2O or MnO_2

Cyclisation was witnessed by the production a deep violet coloured solution for the 8-aminophenoxazin-3-ones **167b-f** and deep pink-red colour for the 7-aminophenoxazin-3-one **59d-e**. The resulting 7- and 8-aminophenoxazin-3-ones

167b-f and **59d-e** were all formed in excellent yield (**Scheme 2.42**); removal of the catalyst and evaporation of the solvent gave a product free of impurities. Exception was made for 2,4-dinitrophenoxy-2',5'-dimethoxy-3'-phenylbenzoquinone **167f,** for which reduction required modified conditions (Pd/C 10% in THF), longer hydrogenation time (48h), and the subsequent cyclisation required the use of Et_3N to yield **167f**.

The key characteristics for all the phenoxazinones prepared were first an important shielding of the aromatic protons situated on the benzene ring (6-H, 7-H and 9-H or 6-H, 8-H and 9-H, **Table 2.8**), as a result of both nitro groups reduction. The observation of only one amino group, seen as a broad singlet within the 5.31-5.41ppm region of the ^1H NMR spectra for 8-aminophenoxazinones and within 6.81-6.83ppm for 7-aminophenoxazinones (**Table 2.8**), as well as a carbonyl within the 184.1-184.6ppm region of the ^{13}C NMR spectra, attested for the formation of a cyclised product. The presence of three absorption bands within 3355-3420, 3308-3329 and 3198-3226cm^{-1} (range for both 7- and 8-aminophenoxazinones) and of an absorption band within 1634-1649cm^{-1} (8-aminophenoxazinone) or 1643-1661cm^{-1} (7-aminophenoxazinone), provided further evidence for the formation of an amino and a carbonyl group (**Table 2.8**).

		167b-f		**59d-e**
δ_H NMR (ppm) **d_6-DMSO**	6-H	7.05-7.20 (1H, d, *J* = 8.7-9.3Hz)	6-H	6.475-6.51 (1H, d, *J* = 2.4Hz)
	7-H	6.78-6.90 (1H, dd, *J* = 8.7-9.0, 2.7Hz)	8-H	6.69-6.71 (1H, dd, *J* = 8.7-9.0, 2.4Hz)
	9-H	6.77-6.96 (1H, d, *J* = 2.7Hz)	9-H	7.43-7.48 (1H, d, *J* = 8.7Hz)
	NH_2	5.31-5.41 (2H, br.s)	NH_2	6.81-6.83 (2H, br.s)
δ_C NMR (ppm)	3-C	184.1-184.5	3-C	184.3-184.6
	4-C	104.0-111.75	4-C	105.1-106.5
ν_{max} (cm^{-1})	C=O	1634-1649	C=O	1643-1661
	N-H	3355-3414, 3308-3337 and 3198-3225	N-H	3408-3420, 3317-3329 and 3205-3226

Table 2.8: Key NMR and I.R. spectral data for 7- and 8-aminophenoxazin-3-ones **167b-f**, **59d-e**.

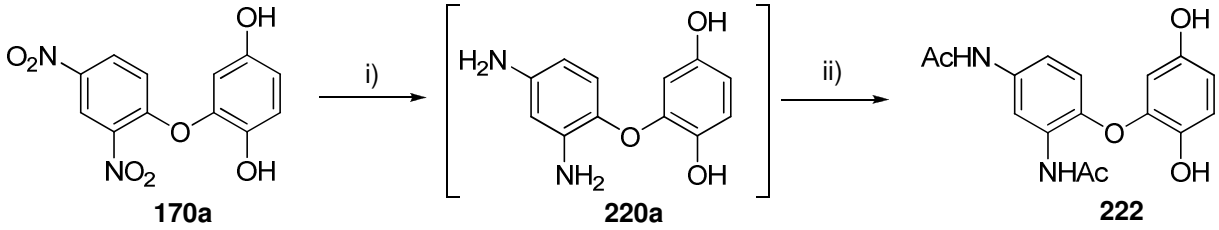
Finally, the shielded aromatic carbon (4-C), present within 104.0-111.75ppm region of the ^{13}C NMR spectrum, resulting from its position *ortho* to the oxygen bridging atom and the carbonyl 3-C, provided further evidence for the quinoid nature of the product (**Table 2.8**).

2.2.13 Trapping of reactive species

A series of experiments was undertaken to confirm the nature of the intermediates involved in the cyclisation of phenoxazin-3-ones. Three different starting materials were investigated, 2,4-dinitro-2,5-dihydroxydiphenylether, 2,4-dinitrophenoxybenzoquinones and 2,5-dinitrophenoxybenzoquinone.

2.2.13.1 2,4-Diamino-2',5'-dihydroxydiphenylether 220a

The first species, 2,4-diamino-2,5-dihydroxydiphenylether **250**, which was expected from catalytic hydrogenation of 2,4-dinitro-2,5-dihydroxydiphenylether, could not be fully characterised due to its poor stability. The addition of acetic anhydride immediately after catalytic hydrogenation of **170a** provided the expected 2,4-diacetamido-2',5'-dihydroxydiphenylether **222** in good yield (**Scheme 2.43**), providing evidence for 2,4-diamino-2,5-dihydroxydiphenylether **220a** as an intermediate.



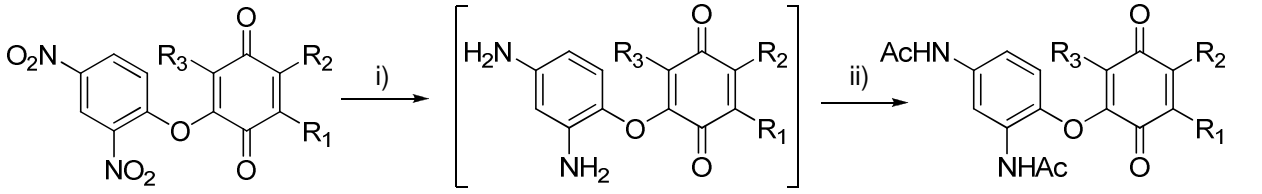
Scheme 2.43: Catalytic hydrogenation of 2,4-dinitro-2',5'-dihydroxydiphenylether **170a**
i) MeOH, Pd/C 5%, H_2, P = 2.4bar; ii) Acetic anhydride; 79.5%.

Acetylation was evidenced by the presence of two singlets each integrating for 3 protons at 2.025 and 2.11ppm on the ^{1}H NMR spectrum, along with the two carbonyls at 168.5 and 169.1ppm on the ^{13}C NMR spectrum. The four deshielded singlets at 8.67, 8.88, 9.44 and 9.89ppm, each integrating for one proton, were readily exchangeable with deuterium and were assigned to the two hydroxylic protons and the two amide protons, respectively. Acetylation was further confirmed by I.R. spectroscopy, the sharp absorption bands at 1698 and 1665cm^{-1} were thought

to arise forml the C=O stretch, the broad absorption band from 2900 to 3500cm^{-1} was thought to arise from the O-H stretch, and the overlapping sharp peaks at 3416 and 3317cm^{-1} were thought to arise from the N-H stretch.

2.2.13.2 2,4-Diaminophenoxybenzoquinones 171a,c,d

The 2,4-dinitrophenoxy-2',5'-benzoquinones **179a**, **c** and **d** were dissolved in a solvent mixture of EtOAc and MeOH and subjected to catalytic reduction using Pd/C 5%, directly followed by addition of acetic anhydride. In each case, the corresponding 2,4-diacetamido-2',5'-benzoquinones **223a-c** were isolated in excellent yield (**Scheme 2.44**), providing evidence for the formation of 2,4-diaminophenoxybenzoquinones **171a,c,d** as intermediates.



179a $R_1 = R_2 = CH_3$, $R_3 = H$
179c $R_1 = R_3 = H$, $R_2 = {}^t$butyl
179d $R_1 = R_3 = H$, R_2 = pentyl

171a $R_1 = R_2 = CH_3$, $R_3 = H$
171c $R_1 = R_3 = H$, $R_2 = {}^t$butyl
171d $R_1 = R_3 = H$, R_2 = pentyl

223a $R_1 = R_2 = CH_3$, $R_3 = H$, 95.8%
223b $R_1 = R_3 = H$, $R_2 = {}^t$butyl, 99.0%
223c $R_1 = R_3 = H$, R_2 = pentyl, 98.3%

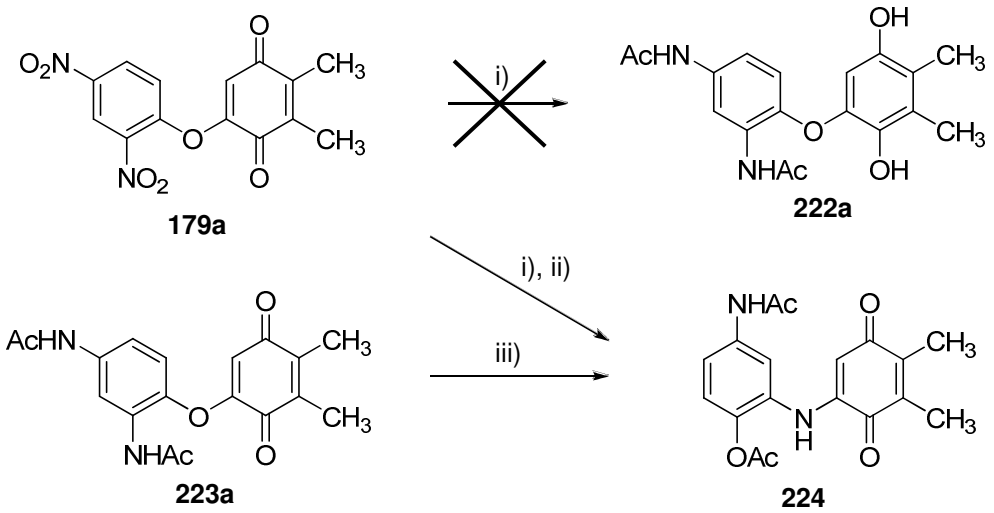
Scheme 2.44: Trapping of the 2,4-aminophenoxy-2',5'-benzoquinones intermediates *via* acetylation
i) MeOH, Pd/C 5%, H_2, P = 2.4bar; ii) Acetic anhydride.

Acetylation was evidenced on the ^{1}H NMR spectrum, as described above, by two singlets in the 2.0-2.1ppm region, each integrating for three protons, and two deuterium exchangeable protons in the 9.0ppm and 10.0ppm region. The carbonyls were observed as two deshielded signals in the 165-170ppm region of ^{13}C NMR spectrum, and as a sharp peak within the 1697-1726cm^{-1} region of the I.R. spectrum (**Table 2.9**). The conservation of the quinone ring was evident on the ^{13}C NMR spectrum, with the two deshielded signals around 180ppm and 186ppm, and on the I.R. spectrum with two absorption bands around 1660cm^{-1} and 1650cm^{-1} (**Table 2.9**). Those spectral features were similar to that of the corresponding starting material **179a**, **c** and **d**, and the quinone carbonyls were assigned as 2'-C and 5'-C for the signals at 180 and 186ppm, respectively, following the reasoning discussed in section **2.2.11**.

		Range for **223a-c**
δ_H NMR (ppm)	2a-CH_3 and 2b-CH_3	2.03-2.06 (3H, s) and 2.055-2.07 (3H, s)
	2-N*H*	8.63-9.33 (1H, s)
	4-N*H*	9.31-10.07 (1H, s)
δ_C NMR (ppm)	1a-C and 1b-C	165.8-168.8 and 166.3-169.2
	2'-C	181.0-182.1
	5'-C	186.6-187.8
ν_{max} (cm^{-1})	C=O (quinone)	1639-1648 and 1655-1665
	C=O (amide)	1697-1726
	N-H	3270-3403

Table 2.9: Key NMR and I.R. spectroscopic features of 2,4-diacetamido-2',5'-benzoquinones **223a-c**.

Attempts to reduce the quinone ring on **179a** with a much longer hydrogenation time (48h) failed to produce the desired product **222a** or even the previously isolated quinone **223a**, but led to an unexpected result (**Scheme 2.45**). The same analytical data were obtained when 2,4-diacetamidophenoxybenzoquinone **223a** was refluxed in MeOH (**Scheme 2.45**).



Scheme 2.45: Formation of 2-acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone **224** i) MeOH, Pd/C 5%, H_2, P = 2.4bar, 48h; ii) Acetic anhydride Δ or iii) MeOH, Δ.

The ^{1}H NMR spectrum appeared very much similar to the data obtained for **223a**, with the presence of two acetyl groups, two methyl group, four aromatic protons and two deshielded, exchangeable protons. The slight difference in chemical shift could not be taken into account, as both NMR spectra could not be run in the same solvent. The ^{13}C NMR spectrum revealed the possible quinoid nature of the product, with the presence of two deshielded signals at 183.6 and 185.8ppm. The major difference arose from the examination of the HMBC spectrum, which revealed key correlations between the singlet at 8.26ppm and carbon atoms belonging to both the benzene and the quinone ring (**Figure 2.10**).

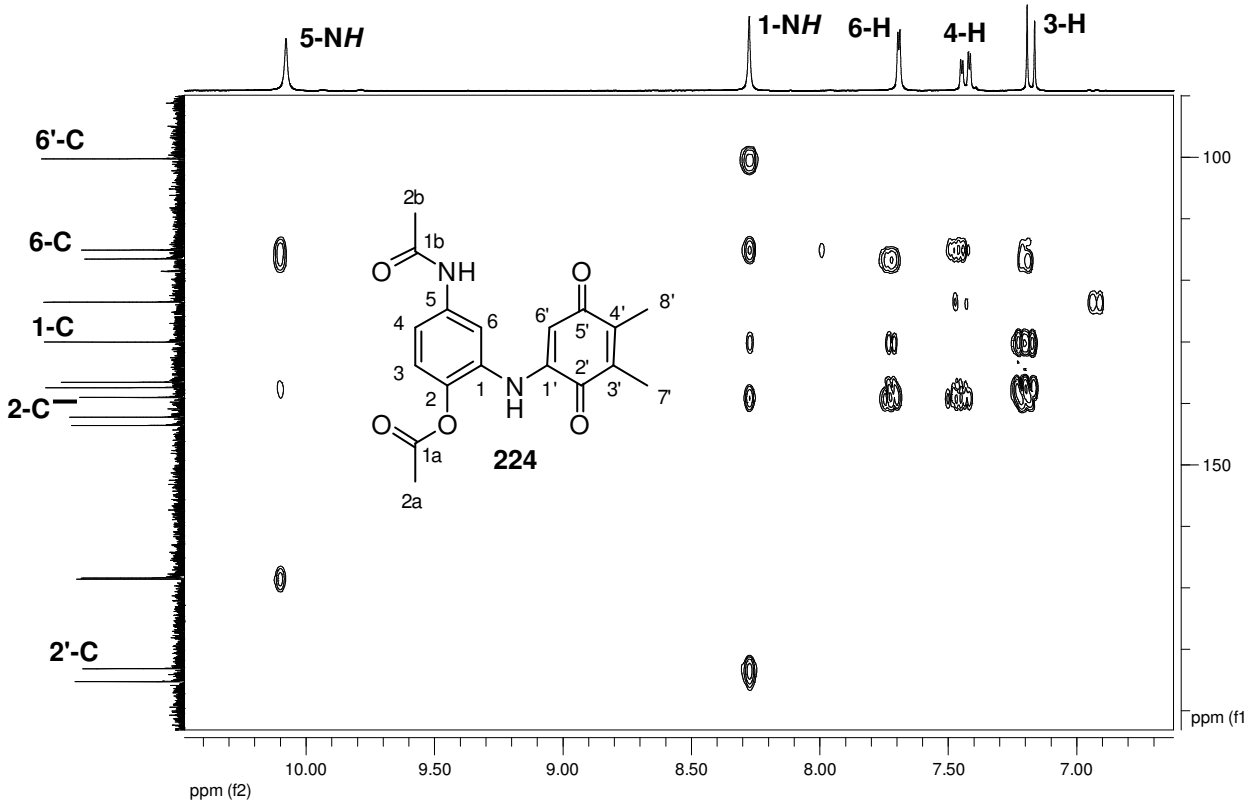
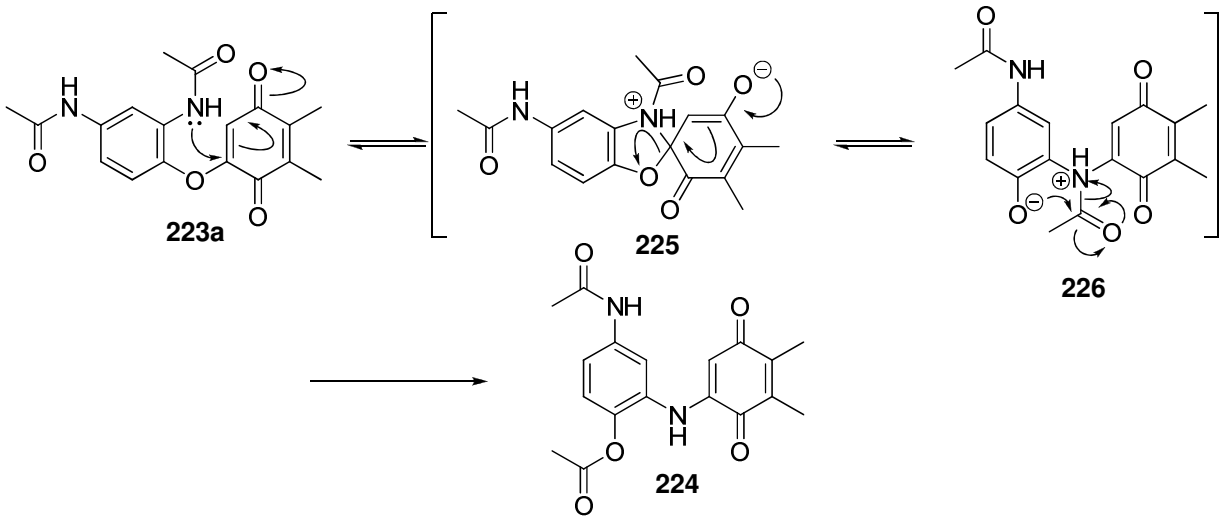


Figure 2.10: HMBC spectrum of the probable **224** (assigned carbons correlate with 1-N*H*).

The correlation between one of the N*H* and 6-C, 1-C, 2-C and 2'-C could only be possible in a structure where the nitrogen would be the bridging atom between both rings (**Figure 2.10**). The proposed structure **224** matched appropriately the NMR data and correlation observed; however, X-ray crystallography would be required for a definitive confirmation of the proposed structure **224**.

The formation of the quinone **224** is thought to be possible *via* a Smiles rearrangement type reaction: nucleophilic attack of the nitrogen atom in the 2-position

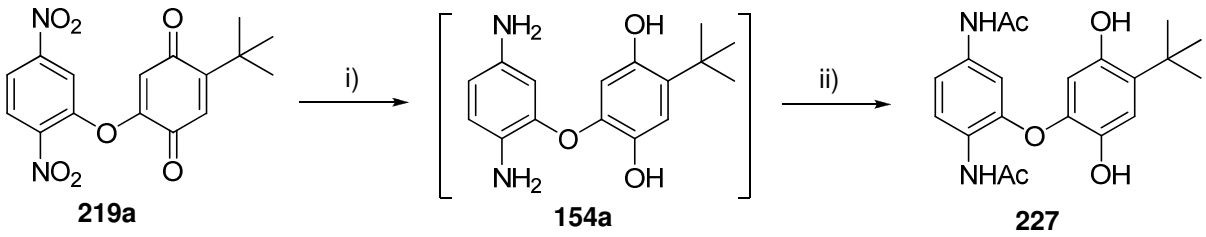
ipso to the oxygen bridging atom could lead to the intermediate **225** with subsequent rearrangement to **225**, followed by transfer of the acetyl group to form 2-acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone **224** (**Scheme 2.46**).



Scheme 2.46: Suggested mechanism for the formation of 2-acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone **224**.

2.2.13.3 2,5-Diamino-2',5'-dihydroxydiphenylether 154a

The reduction of 2,5-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **219a** and trapping of the resulting intermediate was carried out following the same conditions described for 2,4-dinitrophenoxybenzoquinones **179a**, **c** and **e** (**Scheme 2.47**). The resulting product was 2,5-diacetamido-4'-^{t}butyl-2',5'-dihydroxydiphenylether **227**, providing evidence for the formation of 2,5-diamino-2',5'-dihydroxy-4'-^{t}butyldiphenylether **154a** as an intermediate (**Scheme 2.47**).



Scheme 2.47: Catalytic hydrogenation of 2,5-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **219a** i) MeOH, Pd/C 5%, H_2, P = 2.4bar; ii) Acetic anhydride; 97.5%.

Unlike its 2,4-regioisomer **179c**, 2,5-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **219a** was fully reduced (nitro groups and quinone ring) under catalytic reductive conditions:

comparison of both acetylated product **223b** and **227** evidence the reduction of the quinone ring for **226**, with the absence of the two quinone carbonyls 5'-C and 2'-C around 187 and 182 ppm and the appearance of the two hydroxylic protons 2'-OH and 5'-OH at 8.8 and 8.4ppm, respectively (**Figure 2.11**).

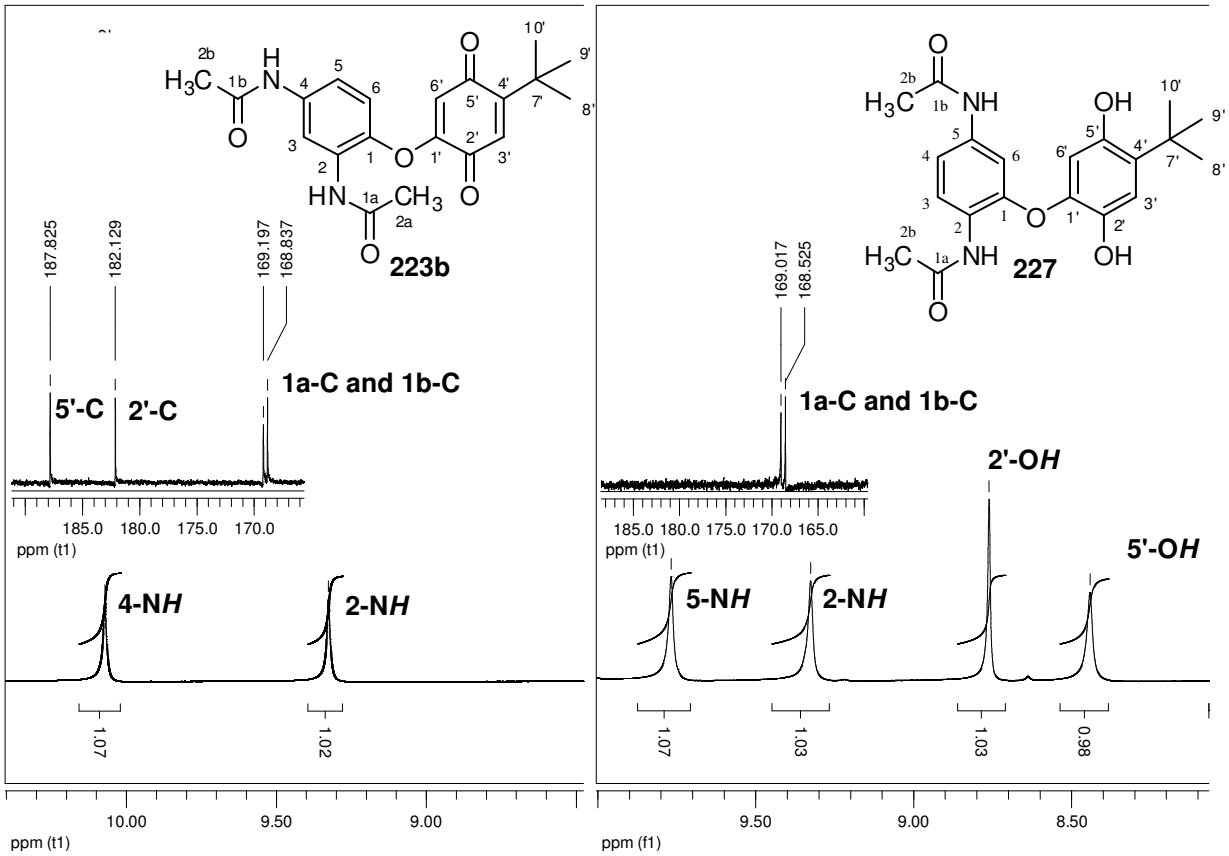


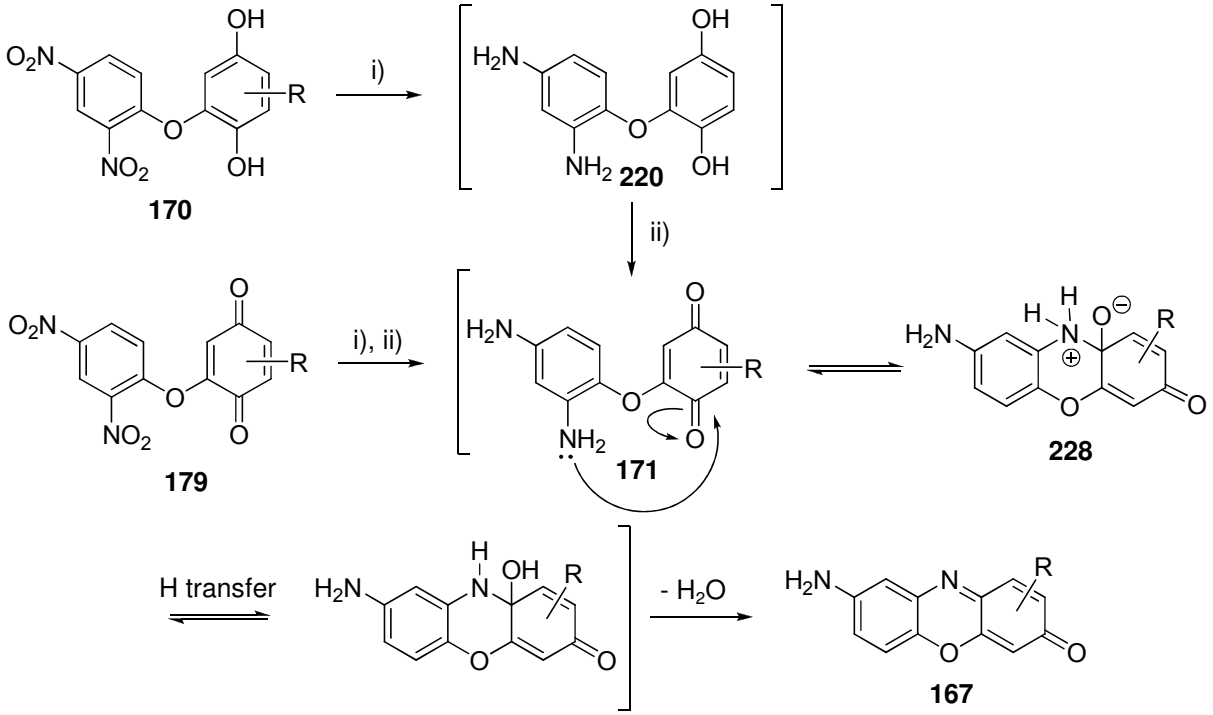
Figure 2.11: Comparison of sections of ^{1}H NMR spectrum and ^{13}C NMR spectrum for **222b** and **226** in d_6-DMSO.

The reasons for the reduction of the quinone ring in the case of **227** could not be rationalised considering the hydrogenation conditions used. However, it appeared evident that the relative position of the two nitro groups was determinant of the hydrogenation outcomes.

2.2.14 Mechanism of cyclisation

In the case of 8-aminophenoxazin-3-ones **167**, 2,4-diaminophenoxybenzoquinones **171** have been confirmed to be key intermediates. They are direct intermediates in the reduction of 2,4-dinitrophenoxybenzoquinones **179**, but are also likely to arise from the oxidation of 2,4-diamino-2',5'-dihydroxydiphenylether **220**, intermediates

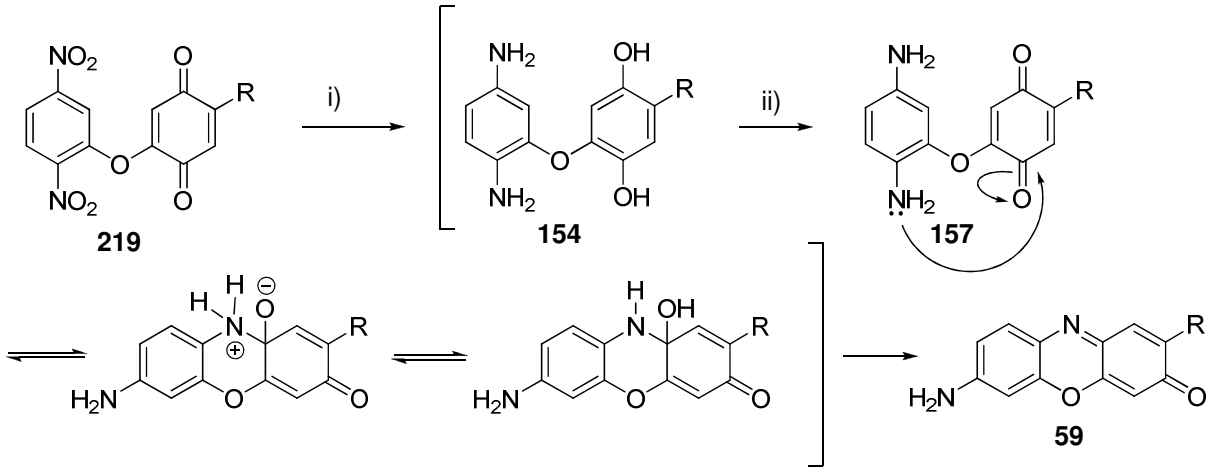
resulting from the reduction of 2,4-dinitro-2',5'-dihydroxydiphenylether **170** (**Scheme 2.48**). This last hypothesis, oxidation of 2,4-diamino-2',5'-dihydroxydiphenylether **220** to 2,4-diaminophenoxybenzoquinone **171** (**Scheme 2.48**), is supported by the necessity to have oxidising conditions to favour the formation of 8-aminophenoxazin-3-one from this route (**Scheme 2.48**).



Scheme 2.48: Possible mechanism of formation of 8-aminophenoxazin-3-ones **167**
i) MeOH, Pd/C 5%, H_2, P = 2.4bar, ii) Ag_2O, MeOH.

The cyclisation is likely to occur from 2,4-diaminophenoxybenzoquinone **171** *via* nucleophilic attack of the amino group onto the quinone carbonyl, giving to the intermediate **228** (**Scheme 2.48**). Proton transfer and subsequent loss of water would then yield to 8-aminophenoxazin-3-ones **167** (**Scheme 2.48**).

In the case of 2,5-dinitrophenoxybenzoquinones, only the formation of 2,5-diamino-2',5'-dihydroxydiphenylethers **154** could be proven. However, after addition of MnO_2, **154** are likely to be oxidised to 2,5-diaminophenoxy-2',5'-benzoquinones **157**, before undergoing the same cyclisation as described previously (**Scheme 2.49**).



Scheme 2.49: Possible mechanism of formation of 7-aminophenoxazin-3-ones **59**
i) MeOH, Pd/C 5%, H_2, P = 2.4bar, ii) Ag_2O, MeOH.

It appears that the oxidation of 2,5-diamino-2',5'-dihydroxydiphenylether **154** is easier under mild conditions than the oxidation of 2,4-diamino-2',5'-dihydroxydiphenylether **220**.

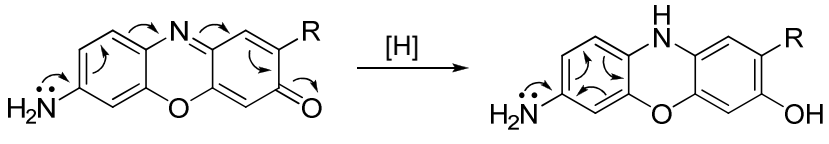
2.3 Synthesis of β-alanine substrates 235a-b and 237a-i

After preparation, the 7- and 8-aminophenoxazin-3-ones needed to be derivatised with ^{t}boc protected β-alanine *via* a standard peptide coupling procedure. The addition of TFA, required to remove the ^{t}Boc protecting group, had the advantage of producing a salt, facilitating the incorporation of the resulting β-alanine aminopeptidase substrate into aqueous medium.

2.3.1 From 7-aminophenoxazin-3-ones 59d-e

Previous work (2008OBC613) effected on the lead compound, 7-amino-1-pentyl-3*H*-phenoxazin-3-one, showed satisfactory results when an active mixed anhydride was used for the coupling procedure. In the case of 7-amino-3*H*-phenoxazin-3-ones, the position of the amino group considerably reduces its nucleophilic character, due to the extended conjugation of the nitrogen lone pair of electrons (**59d-e**, **Scheme 2.50**).

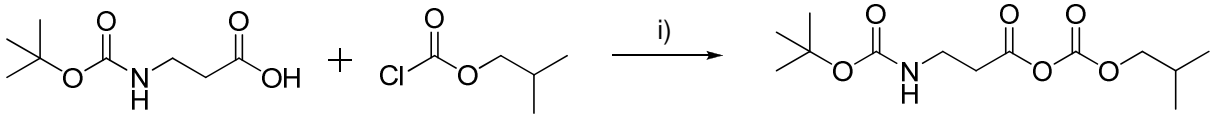
For that reason, 7-amino-3*H*-phenoxazin-3-ones **59d-e** were reduced to 7-amino-3-hydroxy-3*H*-phenoxazines **229a-b**, using catalytic reductive conditions, prior to peptide coupling, in order to limit conjugation and increase the amino group nucleophilicity (**Scheme 2.50**).



59d-e **229a** R = ^{t}butyl
229b R = pentyl

Scheme 2.50: Reduction of 7-aminophenoxazin-3-ones to 7-amino-3-hydroxy-3*H*-phenoxazin-3-ones.

The active mixed anhydride **232** was prepared *in situ* by reacting *iso*-butylchloroformate **231** with *N*-^{t}Boc-β-alanine **230** under dry conditions in the presence of a base (**Scheme 2.51**). Although *N*-methylmorpholine or Et_3N is usually the base of choice, it has been reported that *N*-methylpiperidine limits the formation of urethane by-products, resulting from a reaction on the unwanted side of the mixed anhydride (1983JOC2939). *N*-Methylpiperidine was therefore adopted as the base of choice for the coupling of all the 7- and 8-amino-3*H*-phenoxazin-3-one chromogens prepared.

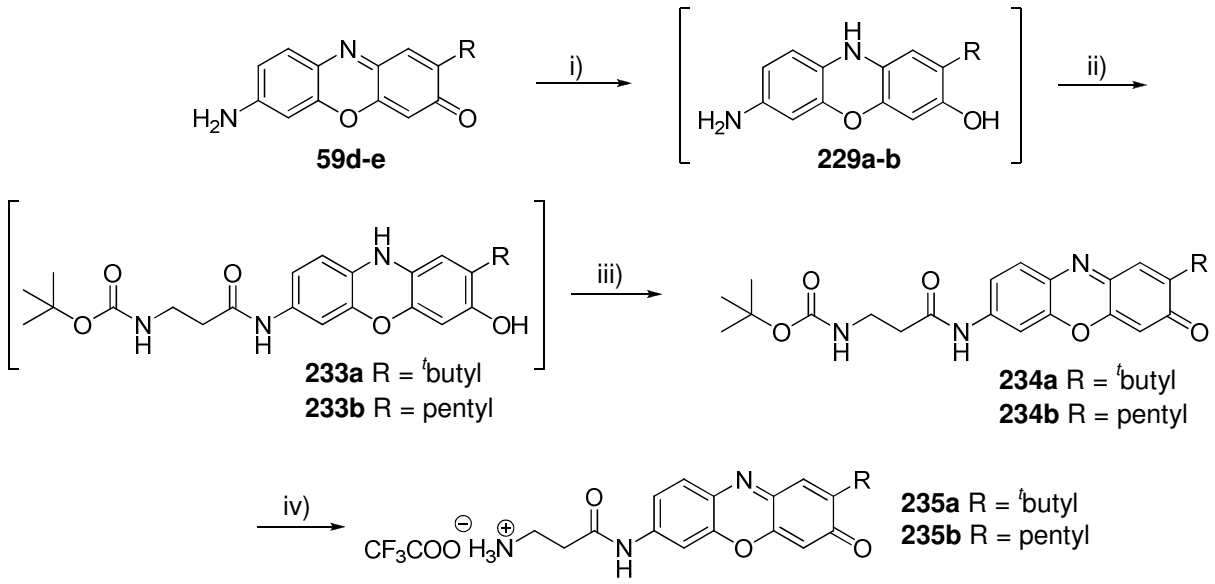


230 **231** **232**

Scheme 2.51: Preparation of the reactive mixed anhydride **232**
i) Dry THF, *N*-methylpiperidine, 0°C.

Once the mixed anhydride had formed, the 7-amino-3-hydroxyphenoxazines **229a-b** were added under a N_2 atmosphere to avoid re-oxidation and the resulting mixture was stirred under N_2 until no more of the phenoxazine starting material **229a-b** remained (**Scheme 2.52**). Aeration of the solution quickly re-oxidised the intermediates **233a-b** to the desired 7-*N*-(*N'*-^{t}Boc-β-alanyl)aminophenoxazin-3-ones **234a-b** (**Scheme 2.52**).

Coupling of the protected β-alanine was confirmed by ^{1}H NMR spectroscopy. In the case of **234b,** the presence of a deshielded proton at 10.41ppm corresponding to 7-N*H* attested the formation of the peptide bond. The broad singlet at 6.80ppm was assigned to N*H* of the ^{t}Boc protected amino acid chain. The β-alanyl signals appeared at 3.16ppm as a quartet and at 2.47ppm as a triplet, corresponding to 3''-H and 2''-H, respectively. Finally, the signal corresponding to 9H at 1.30ppm was characteristic of the ^{t}Boc protecting group (**Figure 2.12**).



Scheme 2.52: Preparation of the 7-*N*-(β-alanyl)amino-2-alkylphenoxazin-3-one **269a-b**
i) H_2, Pd/C 5%, DMF, 1h30; ii) **232**, dry THF, *N*-methylpiperidine, 48h; iii) Aerial oxidation, 1h, R.T.; **234a**, 45.3%, **234b**, 46.0%; iv) neat TFA, 10min, R.T., **235a**, 94.4%, **235b**, 88.5%.

Re-oxidation of the phenoxazin-3-one ring was confirmed by the characteristic carbonyl signal at 185.1ppm on the ^{13}C NMR spectrum.

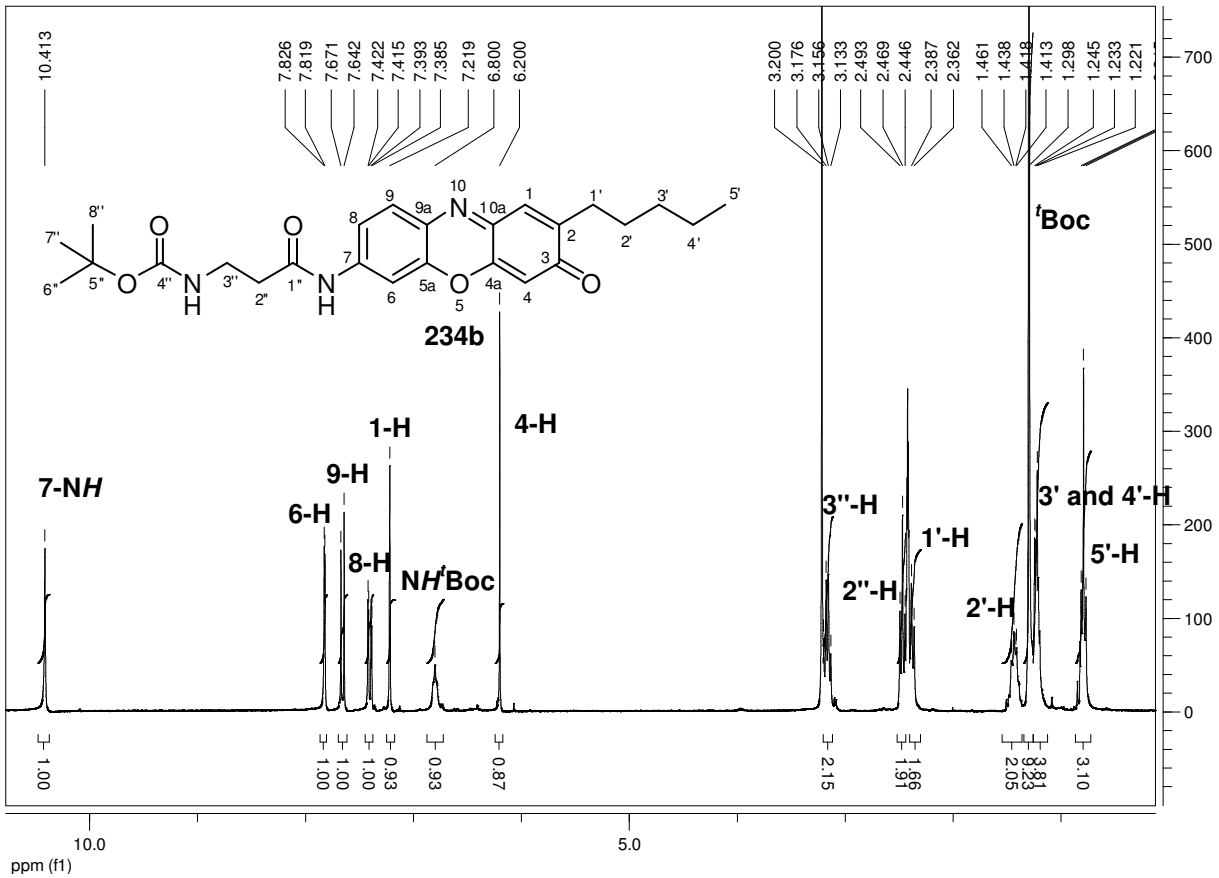


Figure 2.12: ^1H NMR of 7-*N*-(*t*Boc-β-alanyl)-amino-2-pentylphenoxazin-3-one **234b**.

Deprotection of the substrate was effected smoothly using neat TFA (**Scheme 2.52**). Removal of the *t*Boc protecting group was readily observed by ^{1}H NMR spectroscopy. For example, in the case of **235b**, deprotection was confirmed by the disappearance of the N*H*-*t*Boc signal at 6.80ppm and the C(C*H*$_3$)$_3$ signal at 1.30ppm, along with the appearance of a very broad singlet integrating for three protons at 7.965ppm, characteristic of N*H*$_3^+$ (**Figure 2.13**).

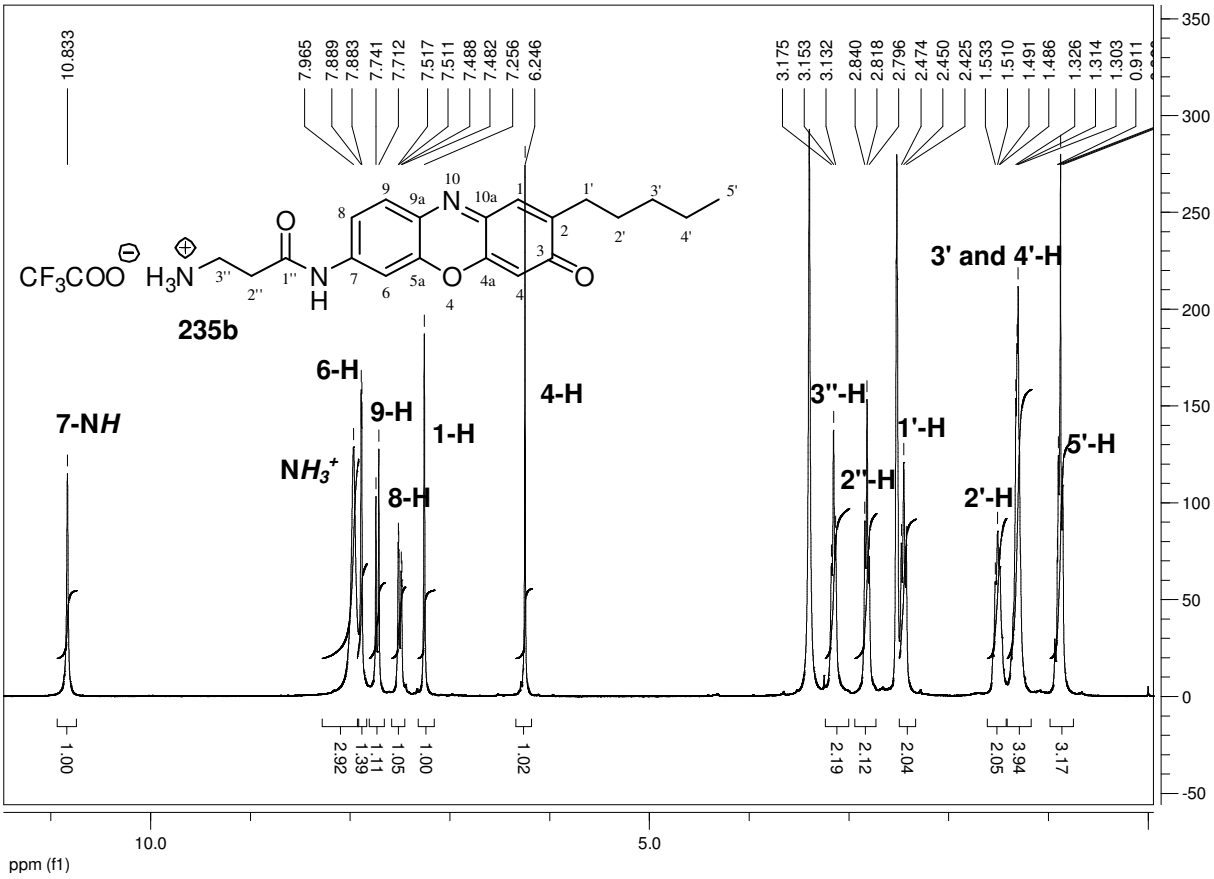
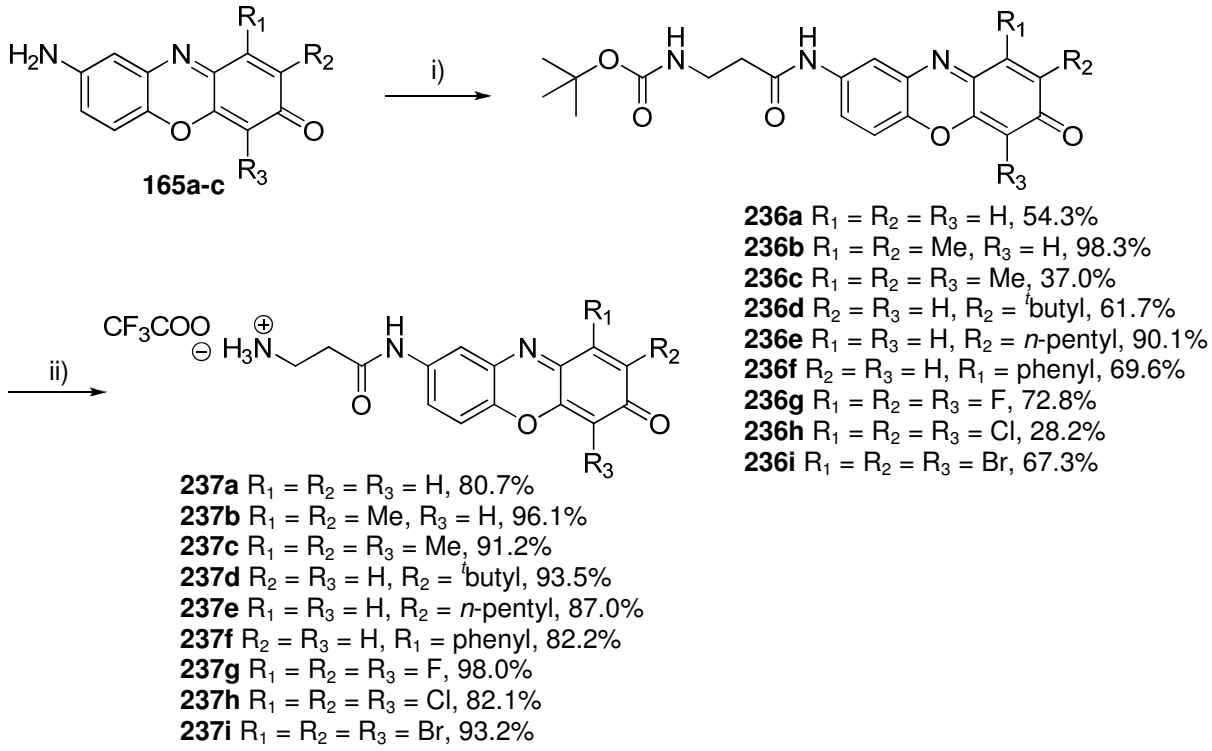


Figure 2.13: ^{1}H NMR spectrum of 7-*N*-(β-alanyl)-amino-2-pentylphenoxazin-3-one TFA salt **235b**.

2.3.2 From 8-aminophenoxazin-3-ones 165a-c and 167a-f

2.3.2.1 *N*-β-Alanine derivatives

The 8-aminophenoxazin-3-ones **165a-c** and **167a-f** were coupled to *N*-*t*Boc-β-alanine following the procedure described for 7-aminophenoxazin-3-ones **59c-d**, except that the reduction step was not necessary (**Scheme 2.53**), as the nucleophilicity of the amino group is much higher in this case. Consequently, isolated yields of the resulting *N*-*t*Boc-β-alanine substrates were generally higher for 8-aminophenoxazin-3-ones (**Scheme 2.53**).



Scheme 2.53: Preparation of 8-*N*-(β-alanyl)aminophenoxazin-3-ones **237a-i**
i) **232**, dry THF, *N*-methylpiperidine, 48h, R.T.; ii) neat TFA, 10min, R.T.

The characteristic spectral features, attesting to the coupling to *N*^{t}Boc-β-alanine, were the singlet integrating for nine hydrogen atoms within the 1.30-1.42ppm region, corresponding to C(C*H*$_3$)$_3$, the triplet integrating for two hydrogens within 2.34-2.57ppm, assigned to 2'-H, the quartet integrating for two hydrogens within 3.175-3.32ppm, assigned to 3'-H and the broad singlet within the 6.49-6.90ppm, assigned to the β-alanine protected amino group (**Table 2.10**). The formation of a peptide bond resulted in a dramatic deshielding of the 8-N*H* proton, observed within the 9.90-10.33ppm region (**Table 2.10**). Both carbonyls resulting from the linking to *N*^{t}Boc-β-alanine could be seen, on the ^{13}C NMR spectra, within 156.0-156.1ppm for 4'-C and within 170.05-170.4ppm for 1'-C, and on the I.R. spectra within the 1679-1713cm^{-1} region (**Table 2.10**).

The deprotection of substrates **236a-i** with TFA resulted in the disappearance of the C(C*H*$_3$)$_3$, N*H* and C=O (4'-C) signals belonging to the ^{t}Boc protecting group (**Table 2.10**). The resulting free, protonated amine, could be seen as broad singlet integrating for three protons within the 7.75-7.98ppm region of the ^{1}H NMR spectra, and as a broad absorption band within the 3032-3077cm^{-1} region of the I.R. spectra (**Table 2.10**).

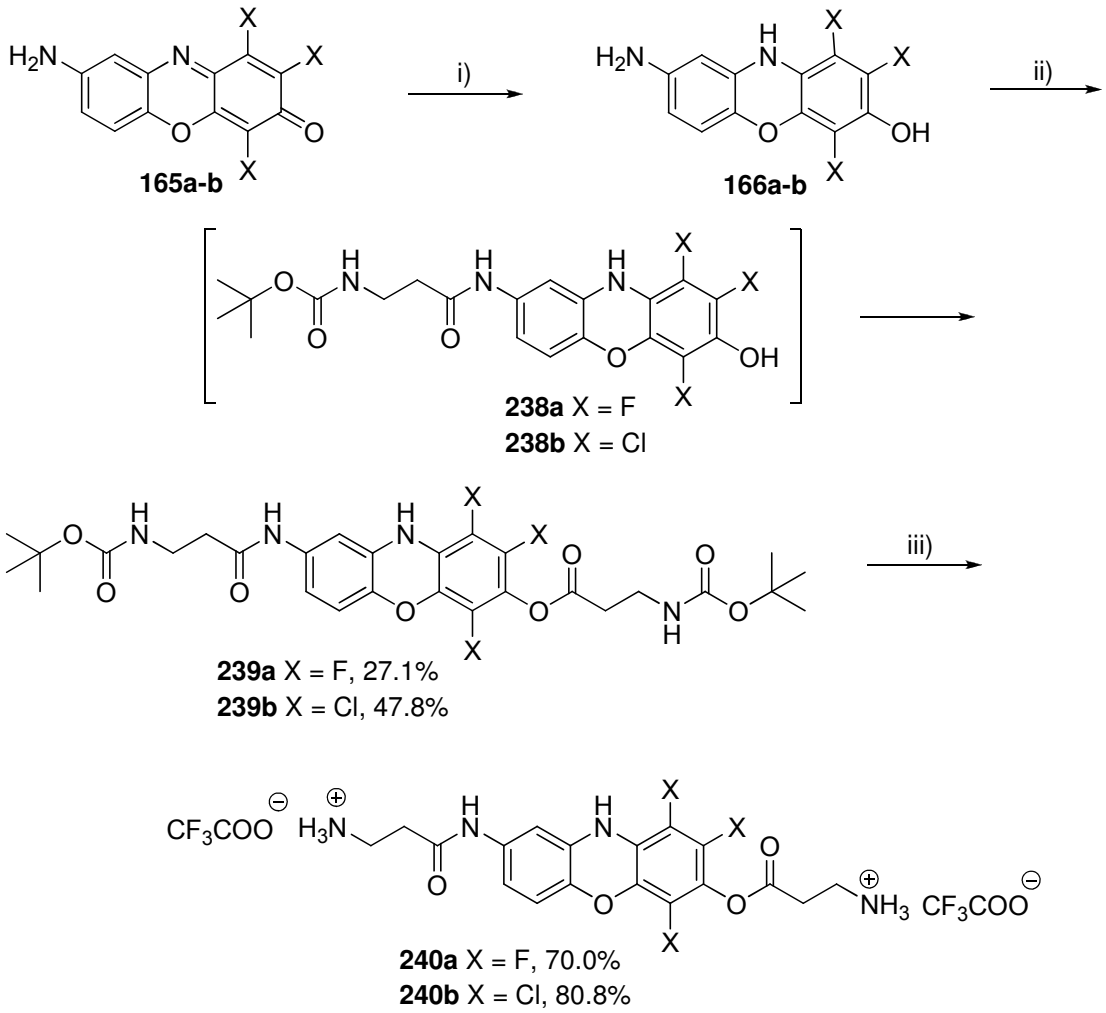
		236a-i	237a-i
δ_H NMR (ppm) **d_6-DMSO**	C(CH_3)$_3$	1.30-1.42 (9H, s)	-
	2'-H	2.34-2.57 (2s, t, *J* = 6.6-7.8Hz)	2.67-2.77 (2s, t, *J* = 6.3-6.6Hz)
	3'-H	3.175-3.32 (2s, q, *J* = 6.6-7.2Hz)	3.05-3.30 (2s, t, *J* = 6.3-6.6Hz)
	8-N*H*	9.90-10.33 (1H, s)	10.33-10.56 (1H, s)
	N*H*^{t}boc	6.49-6.90 (1H, br. s)	-
	NH_3^+	-	7.75-7.98 (3H, br. s)
δ_C NMR (ppm)	1'-C	170.05-170.4	168.9-169.3
	4'-C	156.0-156.1	-
ν_{max} (cm^{-1})	C=O	1679-1713	1670-1678
	N^+-H	-	3032-3077
	N-H	3263-3422	3255-3408

Table 2.10: Key NMR and I.R. spectroscopic features of 8-*N*-(*N*'-^{t}Boc-β-alanyl)aminophenoxazin-3-ones **236a-i** and their corresponding TFA salt **237a-i** resulting from deprotection.

2.3.2.2 *N*- and *O*- di-β-alanine derivatives

It is notable that amino-3-hydroxy-3*H*-phenoxazines exhibit a better general solubility than their corresponding oxidised forms, the amino-3*H*-phenoxazin-3-ones.

In an attempt to increase the yield of the peptide coupling step for the 8-aminophenoxazin-3-ones **165a-c**, 8-amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **165b** was reduced to 8-amino-3-hydroxy-1,2,4-trichloro-3*H*-phenoxazines **166b** and reacted with an excess of the mixed anhydride **232** under a nitrogen atmosphere (**Scheme 2.54**), according to the procedure described for the coupling of 7-amino-3*H*-phenoxazin-3-ones. After reacting **166b** and **232** for 48h, an aliquot of the reaction mixture was shaken with some Ag_2O, in an attempt to re-oxidise the expected 8-*N*-(*N*-^{t}Boc-β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazine **238b** to the corresponding phenoxazin-3-one **236i**, but this failed to yield the expected orange coloured product.



Scheme 2.54: Trapping of 8-amino-3-hydroxy-1,2,4-trihalogeno-3*H*-phenoxazines **166a-b**
i) H_2, Pd/C 5%, DMF; ii) **232**, dry THF, *N*-methylpiperidine, 0°C to R.T., N_2 atm, 48h; iii) DCM, TFA.

NMR analysis of the isolated product **240b** clearly showed the presence of two *N*-^{t}Boc-β-alanine groups (**Figure 2.14**), along with two extra aromatic protons to those expected, one attributed to the second *N*-^{t}Boc-β-alanine group, the other to the bridging N-*H* of the phenoxazine ring, confirming the formation of **240b** in fair yield (**Scheme 2.54**). The reaction was also successfully carried out on the trifluorinated analogue **240a**.

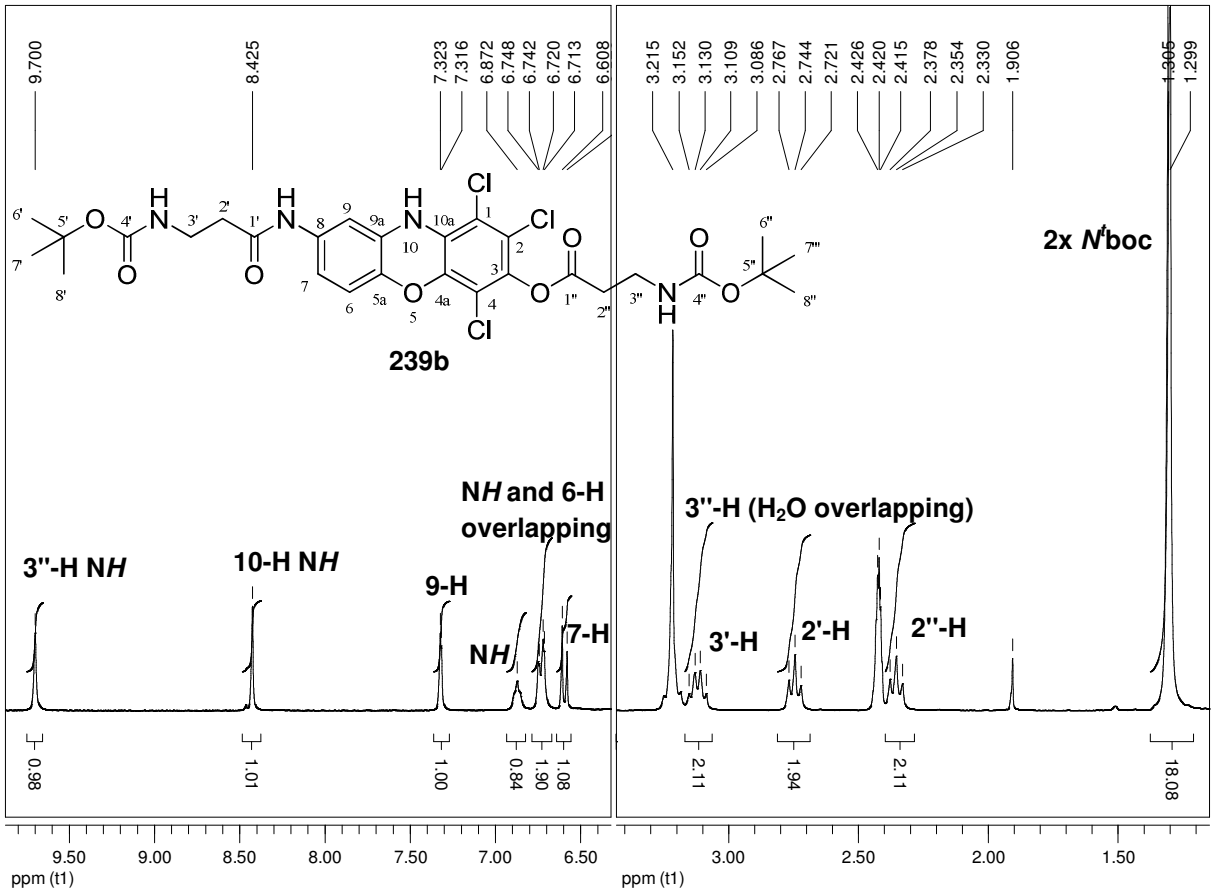


Figure 2.14: ^{1}H NMR spectrum of **239b** in d_6-DMSO.

The subsequent deprotection step needed to be carried out carefully, as use of neat TFA led to an almost complete hydrolysis of the ester bond. A suspension of **239a-b** in DCM followed by addition of a slight excess of TFA, allowed removal of both ^{t}Boc protecting groups in excellent yield, without any hydrolysis of the sensitive ester bond. The resulting products **240a** and **240b** showed a relatively low stability, particularly **240a** which could not be fully characterised by NMR due to its decomposition in d_6-DMSO, d_6-acetone and D_2O. Only the ^{1}H NMR spectrum was obtained, which confirmed the presence of two β-alanine groups with the presence of four triplets at 2.98, 3.47, 4.10 and 4.19ppm along with the 3 aromatic protons at 6.69, 6.95 and 7.28ppm corresponding to a doublet (6-H), a doublet of doublets (7-H) and a doublet (9-H).

The mass corresponding to **240a** could not be observed by mass spectrometry; however, the two main peaks observed at $m/_Z$ 340.1 and 338.1 are likely to

correspond to **241**, resulting from the ester hydrolysis of **240a**, and to **237h**, resulting from the oxidation of **241** (**Figure 2.15**), respectively.

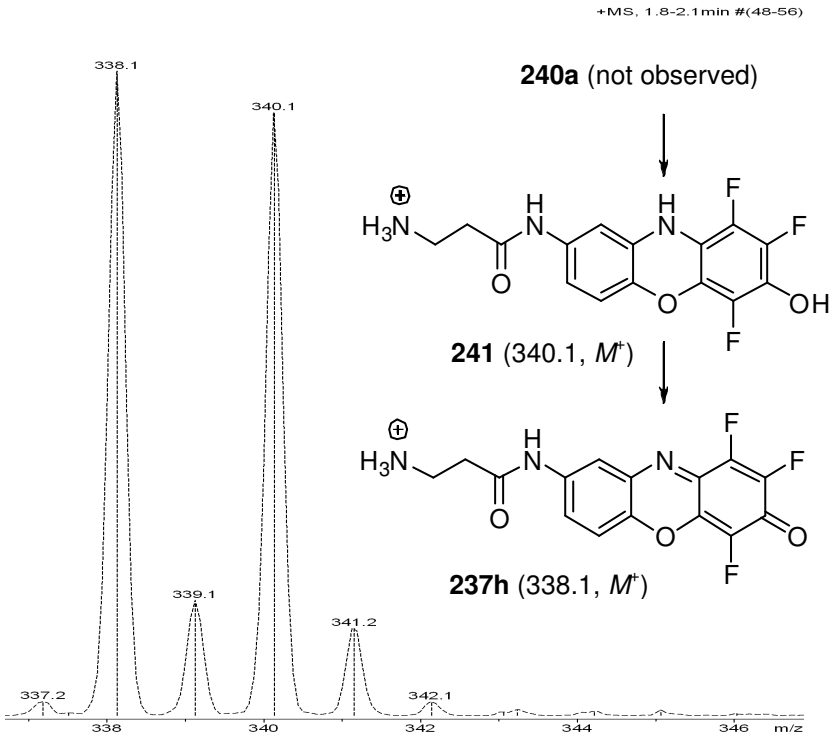


Figure 2.15: Mass spectrum of **240a**, positive ionisation mode.

The preparation of the substrates **240a-b** offered several advantages for the detection of enzymatic activity: firstly, the reduction of the imino-quinone system resulted in a total suppression of the substrate colour, hence avoiding any background colouration. Secondly, the presence of a double TFA salt dramatically increased the general solubility of substrates **240a-b**. However, the apparent lack of stability of substrates **240a-b** presented a certain disadvantage.

2.4 U.V.-Visible absorption

2.4.1 Comparison of 7- and 8-aminophenoxazin-3-ones 59d-e and 167d-e

The U.V. – visible absorbance spectra of 7- and 8-amino-4-^{t}butylphenoxazin-3-one **5d** and **167d**, as well as 7- and 8-amino-2-pentylphenoxazin-3-one **59e** and **167e**, were recorded at a concentration of 50µM in MeOH in order to quantify the difference of colouration between 7- and 8-aminophenoxazin-3-ones. With a λ_{max} ranging between 528-533nm (**Table 2.11**), 7-aminophenoxazin-3-ones **59d-e** produced a

bright red-pink coloured methanolic solution, whereas the 8-aminophenoxazin-3-ones **167d-e** produced in a violet coloured methanolic solution (λ_{max} = 536-540nm). The extinction coefficient ε of 7-aminophenoxazinones was considerably higher than that of 8-aminophenoxazinones (**Table 2.11**, **Figure 2.16**).

	λ_{max} (nm)	A	ε ($dm^3.mol^{-1}.cm^{-1}$)
167d	536	0.295	5900
167e	540	0.322	6400
59d	533	1.804	36080
59e	528	1.554	31080

Table 2.11: Visible absorption characteristics of 7- and 8-aminophenoxazin-3-ones **59d-e** and **167d-e**.

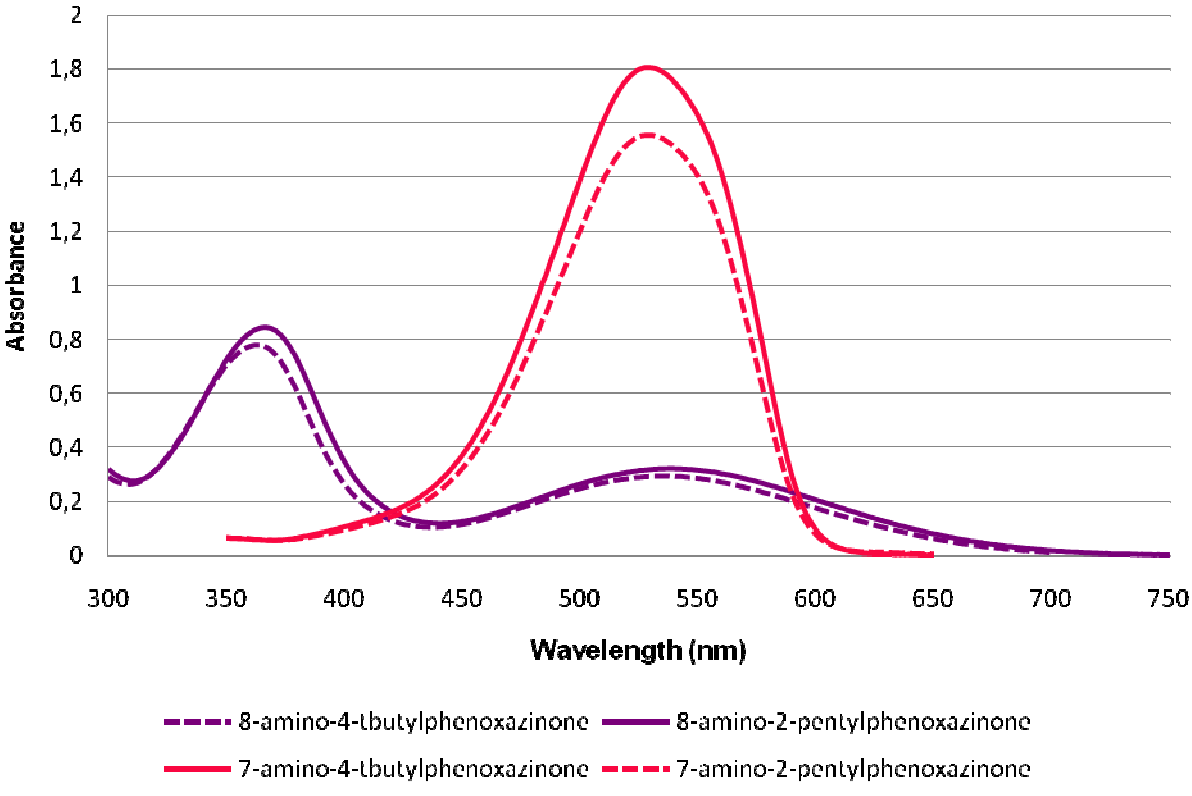
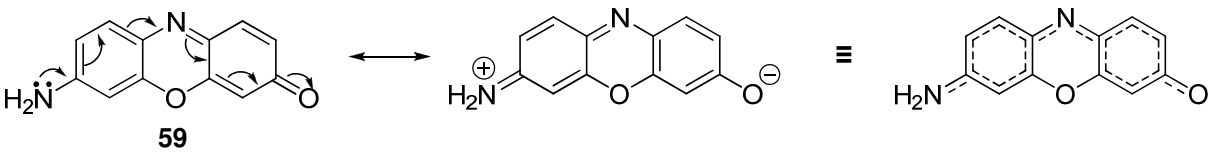


Figure 2.16: U.V.-Visible absorption of 7- and 8-aminophenoxazin-3-ones **59d-e** and **167d-e** at 50µM in MeOH.

The high ε observed for 7-aminophenoxazin-3-one was rationalised by the existence of the extended electronic conjugation generated by the position of the auxochrome (**Scheme 2.55**), as evidenced by the single strong absorption band in the visible region (**Figure 2.16**).



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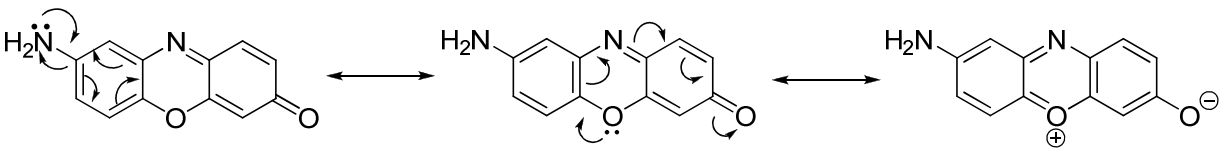
Scheme 2.55: Electron conjugation within 7-amino-3*H*-phenoxazin-3-one.

The presence of two absorption bands on the U.V. – visible spectra of 8-aminophenoxazin-3-ones **167d-e** (**Figure 2.16**) attest of their lesser conjugated character (**Scheme 2.56**): absorption in the U.V. region is thought to be related to electron movement within the benzene ring and absorption in the visible region could be related to electron movement within the quinoid ring of the phenoxazin-3-one (**Scheme 2.56**).



167

Scheme 2.56: Electron conjugation within 8-amino-3*H*-phenoxazin-3-one.

This comparison highlights the importance of the auxochrome position on the chromophore in order to generate a potent chromogen. In the case of the two aminophenoxazin-3-one isomers studied, the ideal position for the amino group appears to be the 7-position.

This also suggests that 8-aminophenoxazin-3-ones will be potentially less sensitive compared to 7-aminophenoxazin-3-ones for the detection of enzymatic activity.

2.4.2 Quenching effect of β-alanine for substrates 235a-b and 237c-d

The U.V.-visible absorbances of four substrates prepared (**235a-b** and **237c-d**) were directly compared to their corresponding 7- and 8-aminophenoxazinones (**59d-e**, **167d-e**), in order to quantify the hypsochromic shift resulting from derivatisation with β-alanine (**Table 2.12**).

	λ_{max} (nm)	$\Delta\lambda$ (nm)	A	ε ($dm^3.mol^{-1}.cm^{-1}$)
167d	536	64	0.295	5900
237c	472		0.348	6960
167e	540	65	0.322	6400
237d	475		0.388	7760
59d	533	75	1.804	36080
235a	458		0.922	18440
59e	528	71	1.554	31080
235b	457		0.861	17220

Table 2.12: Comparison of visible absorption characteristic of aminophenoxazin-3-ones **59c-d** and **167d-e** with their corresponding β-alanine substrates **235a-b** and **237c-db** at 50μM in MeOH.

The hypsochromic shifts resulting from β-alanine derivatisation were 64 and 65nm for **237c** and **237d**, respectively (**Table 2.12**). This resulted in an important enough change of colour. The molar absorptivity corresponding to the visible λ_{max} was slightly higher for both β-alanine derivatives (**Table 2.12**, **Figure 2.17**).

Comparatively, the hypsochromic shifts resulting from β-alanine derivatisation were slightly more important for **235a** and **235b** with value of 75 and 71nm, respectively (**Table 2.13**). The most remarkable effect was the decrease of molar absorptivity by nearly a factor two for the β-alanine derivatives **235a-b** (**Table 2.13**, **Figure 2.18**). This attests for the important quenching of electron movement due to the formation of a peptide bond and was the effect sought for chromogenic substrates.

The effect of β-alanine derivatisation is more promising for the 7-aminophenoxazin-3-one substrates **235a** and **235b**, compared to 8-aminophenoxazin-3-one substrates **237c** and **237d**, and is the result of a more dramatic change in electron movement for **235a-b**. This suggests **235a** and **235b** will show a more important contrast once hydrolysed and could be more sensitive to weak enzymatic activity.

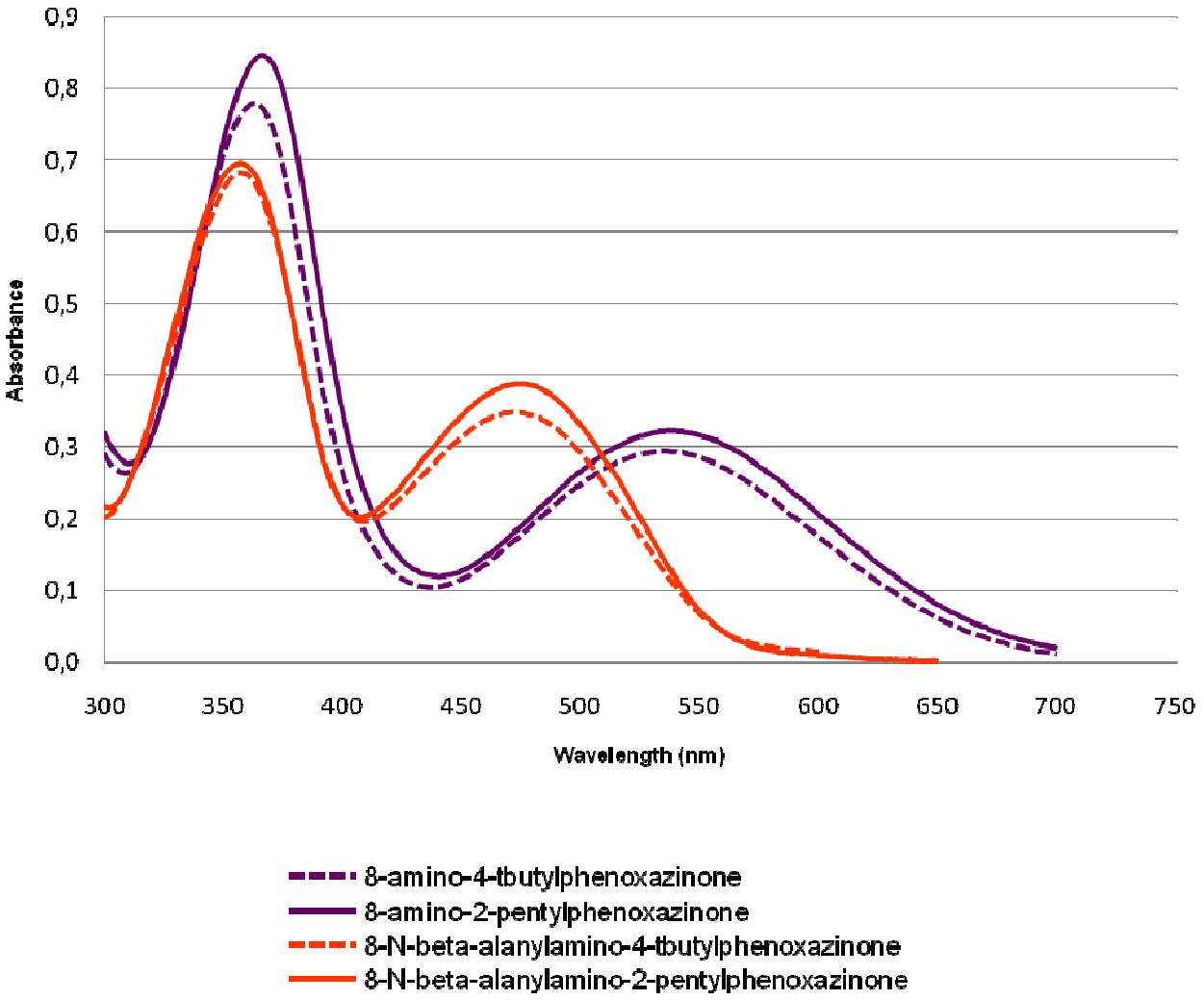


Figure 2.17: U.V.-Vis spectra of 8-aminophenoxazin-3-ones **167d-e** and their corresponding β-alanyl substrate **237c-d** at 50μM in MeOH.

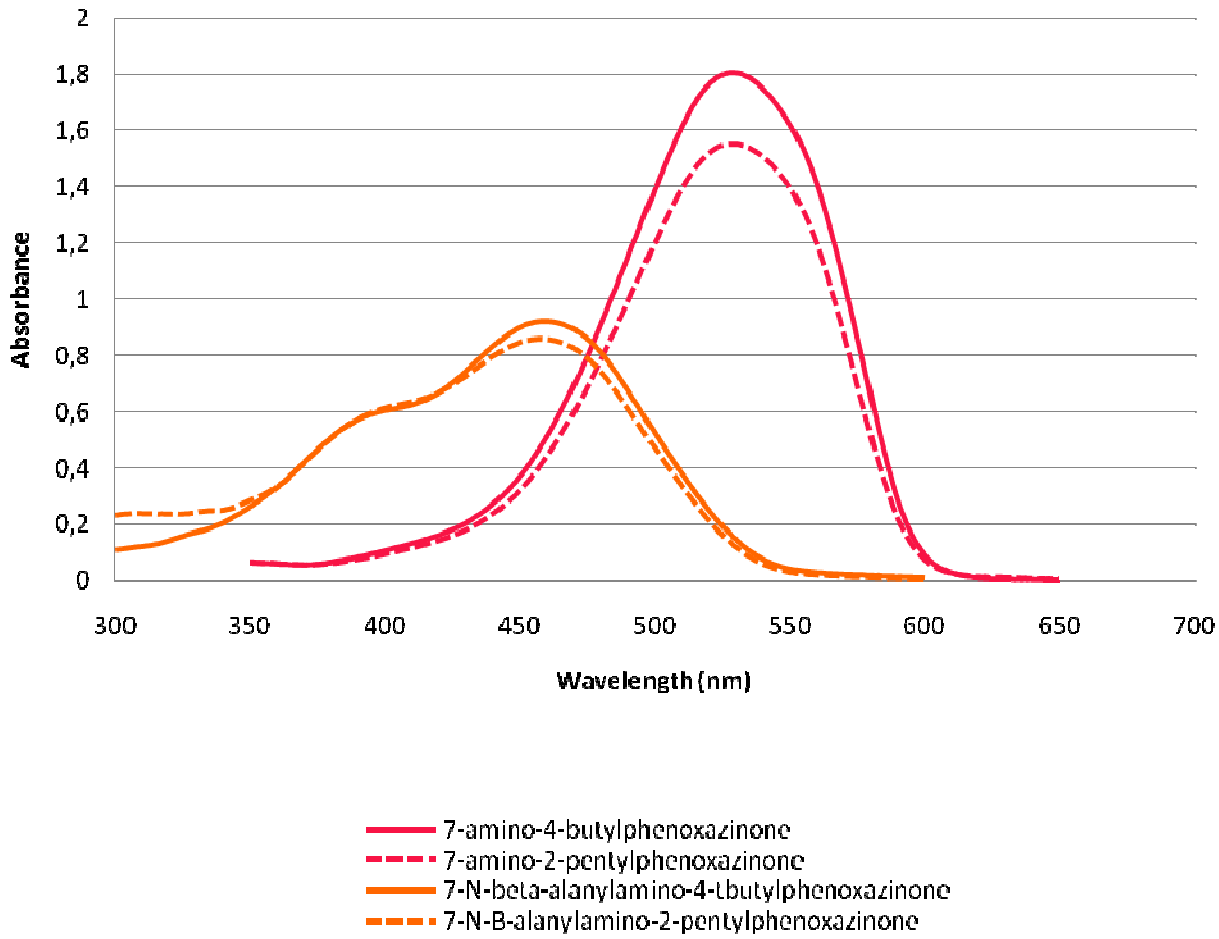


Figure 2.18: U.V.-Vis spectra of 7-aminophenoxazin-3-ones **59d-e** and their corresponding β-alanyl substrates **235a-b** at 50μM in MeOH.

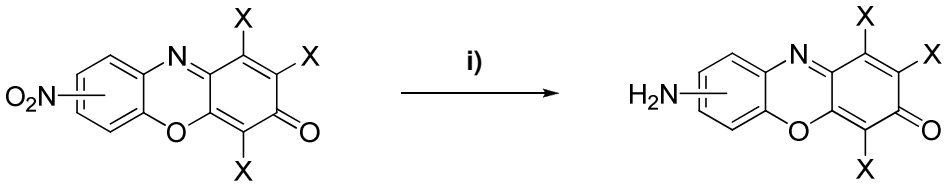
CHAPTER THREE:

MICROBIOLOGICAL TESTING

The chromogenic substrates prepared were divided into 3 different sets. Nitro-1,2,4-trihalogenophenoxazin-3-ones were tested against general nitroreductase activity; testing was undertaken at the Freeman Hospital (Newcastle). The 8-*N*-β-alanylaminotrihalogenophenoxazin-3-ones and their corresponding trapped reduced forms were tested for β-alanine aminopeptidase activity; testing was again undertaken at the Freeman Hospital (Newcastle). The third set, which included the alkyl derivatives of 7-*N*- and 8-*N*-β-alanylaminophenoxazin-3-ones was also tested for β-alanine aminopeptidase activity; the testing of this set was undertaken by bioMérieux (La Balme-Les-Grottes, France).

3.1 Nitroreductase activity

The reaction sought was the reduction of nitrophenoxazinones **130α-γ a-c** to their corresponding aminophenoxazinones **165α-γ a-c** (**Scheme 3.1**) by an unspecified nitroreductase enzyme, resulting in a dramatic change of colour at the site of hydrolysis.



130αa-c, orange
130βa-c, orange
130γa-b, orange

165αa-c, pink-red
165βa-c, blue-violet
165γa-b, brown-purple

Scheme 3.1: Expected nitroreductase enzyme activity and subsequent reaction of substrates **130αa-c**, **130βa-c** and **130γa-b**
i) Nitroreductase

The nine substrates were screened against a set of twenty clinically important microorganisms (ten Gram negative bacteria, eight Gram positive bacteria, two yeasts, **Table 3.1**).

Gram negative	Ref.	Gram positive	Ref.
1 *E. coli*	NCTC 10418	11 *B. subtilis*	NCTC 9372
2 *S. marcescens*	NCTC 10211	12 *E. faecalis*	NCTC 775
3 *Ps. aeruginosa*	NCTC 10662	13 *E. faecium*	NCTC 7171
4 *B. cepacia*	LMG 1222	14 *St. epidermidis*	NCTC 11047
5 *Y. enterocolitica*	NCTC 11176	15 *St. aureus*	NCTC 6571
6 *S. typhimurium*	NCTC 74	16 *MRSA*	NCTC 11939
7 *C. freundii*	46262 (wild)	17 *S. pyogenes*	NCTC 8306
8 *M. morganii*	462403 (wild)	18 *L. monocytogenes*	NCTC 11994
9 *E. cloacae*	NCTC 11936	19 *C. albicans*	ATCC 90028
10 *P. rettgeri*	NCTC 7475	20 *C. glabrata*	NCPF 3943

Table 3.1: Strains of microorganism tested by multipoint inoculation.

The presence of a nitroreductase enzyme in microorganisms was demonstrated, using fluorogens such as 7-nitrocoumarin-3-carboxylic acid or 7-nitro-4-methylcoumarin derivatives (**Figure 3.1**), by James *et al.*, who reported nitroreductase activity in every strain tested over a sample of thirty microorganisms (2001LAM403). This suggested a wide distribution of nitroreductase activity amongst bacteria and the use of nitroreductase enzyme substrates as general microbial growth indicators (2001LAM403).

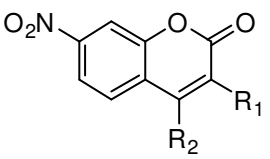


Figure 3.1: Nitroreductase substrates (2001LAM403)
R_1 = COOH, R_2 = H
R_1 = $COOCH_3$, R_2 = H
R_1 = CH_3, R_2 = H
R_1 = CH_3, R_2 = CH_2CH_3

Most bacteria used for the screening of nitrophenoxazin-3-ones (**Table 3.1**) are already known to express nitroreductase activity, and were expected to reduce the substrates **130αa-c**, **130βa-c** and **130γa-b**, and hence allowing a comparison of the performance with previously reported nitroreductase substrates (**Figure 3.1**).

3.1.1 Results and discussion

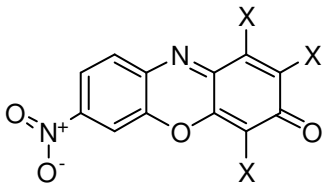
Bacterial Strain	**Control**		**130αa**		**130αb**		**130αc**		**130γa**		**130γb**	
Gram negative	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown halo +	++	Violet +
S. marcescens	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown halo +	++	Violet halo +
Ps. aeruginosa	++	-	++	Red +/-	++	Orange (halo) +	++	-	++	Brown +/-	++	-
B. cepacia	++	-	++	-	++	-	++	-	++	-	++	-
Y. enterocolitica	++	-	++	Red +/-	++	-	++	-	++	-	++	Violet +/-
S. typhimurium	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown +/-	++	Violet halo +
C. freundii	++	-	++	Red +	++	Orange (halo) +	++	-	++	Brown halo +	++	Violet +
M. morganii	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown halo +	++	Violet halo +
E. cloacae	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown halo +	++	Violet +
P. rettgeri	++	-	++	Red +	++	Orange (halo) +	++	Brown halo +/-	++	Brown halo +	++	Violet halo +/-
Background colour	None		Sepia		Orange/brown		Yellow/brown		Sepia		Lilac	

Table 3.2: Multi-point screening results for substrates **130αa-c** and **130γa-b** against Gram negative bacteria after 24h of incubation at 37°C

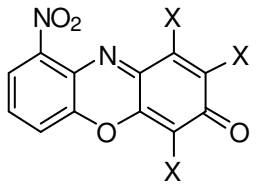
Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



130αa X = F
130αb X = Cl
130αc X = Br



130γa X = F
130γb X = Cl

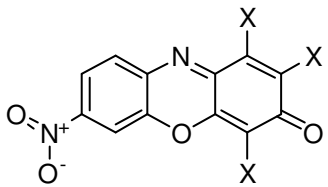
Bacterial Strain	**Control**		**130αa**		**130αb**		**130αc**		**130γa**		**130γb**	
Gram positive	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
B. subtilis	+	-	NG	-	+	-	+	-	NG	-	+	-
E. faecalis	+	-	+	-	+	-	+	-	+	-	+	Violet +/-
E. faecium	+	-	+	-	+	-	+	-	+	-	+	-
St. epidermidis	+	-	NG	-	Tr.	-	Tr.	-	NG	-	NG	-
St.aureus	+	-	NG	-	NG	-	Tr.	-	NG	-	NG	-
MRSA	+	-	Tr.	-	+	-	+	-	Tr.	-	+	Violet +
S. pyogenes	+	-	NG	-	NG	-	NG	-	NG	-	NG	-
L. monocytogenes	+	-	+	Red +/-	+	-	+	-	+	-	+	-
C. albicans	+	-	+	-	+	-	+	-	Tr.	-	+	-
C. glabrata	+	-	+	-	+	-	+	-	+	-	+	-
Background colour	None		Sepia		Orange/brown		Yellow/brown		Sepia		Lilac	

Table 3.3: Multi-point screening results for substrates **130αa-c** and **130γa-b** against Gram positive bacteria and yeasts after 24h of incubation at 37°C

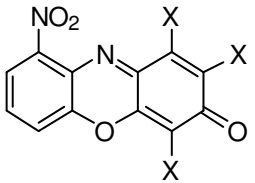
Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



130αa X = F
130αb X = Cl
130αc X = Br



130γa X = F
130γb X = Cl

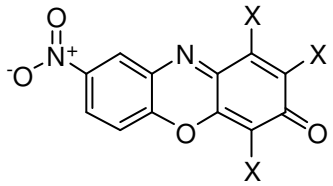
Bacterial Strain	**Control**		**130βa**		**130βb**		**130βc**		**242**	
Gram negative	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	++	-	++	Salmon +
S. marcescens	++	-	++	-	++	-	++	-	++	Salmon +
Ps. aeruginosa	++	-	++	-	++	-	++	-	++	-
B. cepacia	++	-	++	-	++	-	++	-	++	-
Y. enterocolitica	++	-	++	-	++	-	++	-	++	-
S. typhimurium	++	-	++	-	++	-	++	-	++	Salmon +/-
C. freundii	++	-	++	-	++	-	++	-	++	Salmon +/-
M. morganii	++	-	++	-	++	-	++	-	++	Salmon +/-
E. cloacae	++	-	++	-	++	-	++	-	++	Salmon +
P. rettgeri	++	-	++	-	++	-	++	-	++	Salmon +
Background colour	None		Sepia		Orange/brown		Yellow/brown		Orange	

Table 3.4: Multi-point screening results for substrates **130βa-c** and **276** against Gram negative bacteria after 24h of incubation at 37°C

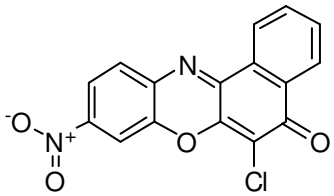
Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



130βa X = F
130βb X = Cl
130βc X = Br



242

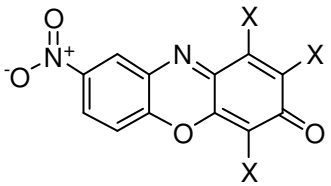
Bacterial Strain	**Control**		**130βa**		**130βb**		**130βc**		**242**	
Gram positive	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
B. subtilis	+	-	NG	-	+	-	+	-	+	-
E. faecalis	+	-	Tr.	-	+	-	+	-	+	Salmon +/-
E. faecium	+	-	Tr.	-	+	-	+	-	+	Salmon +/-
St. epidermidis	+	-	NG	-	+	-	+	-	Tr.	-
St.aureus	+	-	NG	-	+	-	+	-	+	Salmon +
MRSA	+	-	NG	-	+	-	+	-	+	Salmon +
S. pyogenes	+	-	NG	-	NG	-	+	-	+	-
L. monocytogenes	+	-	NG	-	+	-	+	-	+	Salmon +
C. albicans	+	-	Tr.	-	+	-	+	-	+	-
C. glabrata	+	-	NG	-	+	-	+	-	+	-
Background colour	None		Sepia		Orange/brown		Yellow/brown		Orange	

Table 3.5: Multi-point screening results for substrates **237h-c** and **240a-b** against Gram positive bacteria and yeasts after 24h of incubation at 37°C

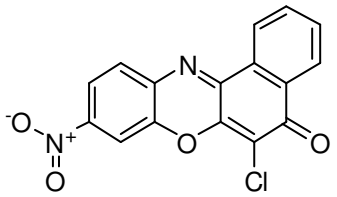
Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



130βa X = F
130βb X = Cl
130βc X = Br



242

The growth control did not indicate any particular inhibition linked to the use of NMP and Tween 20. The growth of Gram negative bacteria was not affected by any of the substrates **130α-γ a-c** and **242** (**Table 3.2** and **Table 3.4**). However, these substrates showed a higher toxicity for Gram positive microorganisms (**Table 3.3** and **Table 3.5**), particularly the nitro-trifluorophenoxazin-3-ones **130αa**, **130βa** and **130γa**.

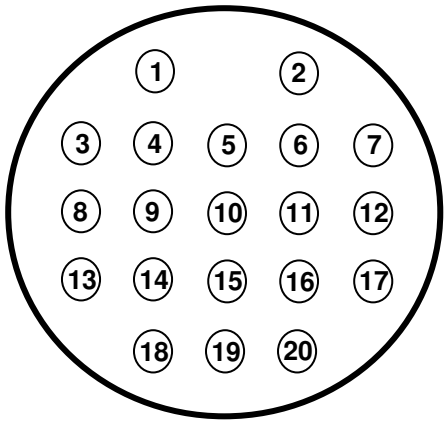


Figure 3.2: Inoculation pattern.

Nitroreductase enzymatic activity was evidenced by the appearance of a colour contrasting from the background within and around the bacterial colonies. The highest activity was recorded for 7-nitro-1,2,4-trifluorophenoxazin-3-one **130αa** with the appearance of red colonies on dark yellow background (**Figure 3.3**, microorganism arrangement as depicted on **Figure 3.2**). Diffusion of the colour was significant around those colonies where the activity was more pronounced. Substrate **130αa** seemed to have a low affinity for the bacterial cell walls, despite the presence of fluorine atoms that usually increase lipophilicity. The substrate seemed to be reduced mainly by Gram negative bacteria, although strains **3**, **4**, and **5** showed little to no activity at all (**Figure 3.3**, **Table 3.2**). No obvious reduction of the substrate was noted for Gram positive microorganisms, possibly due to the important growth inhibition attributed to the substrate itself (**Table 3.3**).

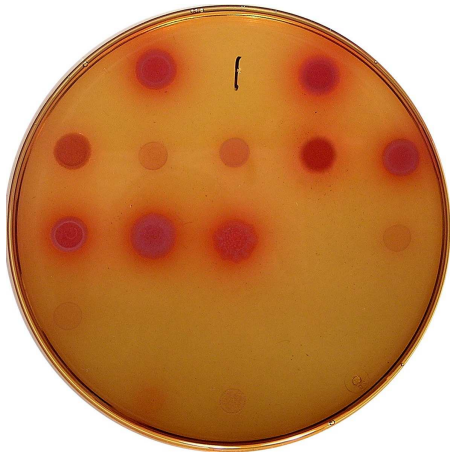


Figure 3.3: Screening of 7-nitro-1,2,4-trifluorophenoxazinones for nitroreductase activity.

Nitroreductase activity was similar, but of a much weaker intensity, for the rest of the 7-nitrophenoxazin-3-one series, substrates **130αb-c**. Several factors could contribute to the observed reduced enzymatic activity for substrates **130αb-c**: the reduced availability of the substrate in the medium and the low solubility compared to **130αa** may have limited the quantity of substrate permeated into the bacteria, resulting in the production of weak colour.

The series of 9-nitrophenoxazin-3-one substrates **130γa-b** showed similar results; both substrates **130γa** and **130γb** were reduced by most of the Gram negative organisms (**Table 3.2** and **Table 3.4**). The colour generated (brown, substrate **130γa** and purple, substrate **130γb**) was sufficient to justify enzymatic activity; however visualisation was impaired by a weak contrast with the background (**Figure 3.4** and **Figure 3.5**) and extensive diffusion in the medium (**130γb, Figure 3.5**). The diffusion was particularly noticeable for colonies **8**, **9** and **10**, for which nitroreductase activity seems to be the more pronounced (**130γb**, **Figure 3.5**), with the formation of a large halo around the colonies.

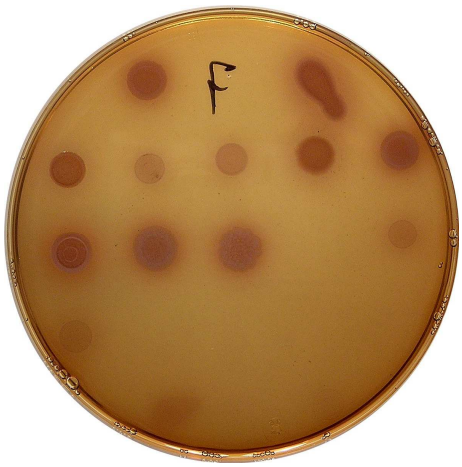


Figure 3.4: Screening of 9-nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one for nitroreductase activity.

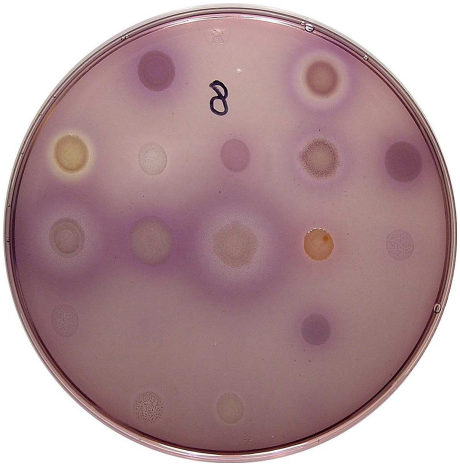


Figure 3.5: Screening of 9-nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one for nitroreductase activity.

Interestingly, none of the substrates belonging to the 8-nitro-1,2,4-trihalogenophenoxazinone series **130βa-c** were reduced at all, despite a good growth for all Gram negative and Gram positive microorganisms tested.

The position of the nitro group seemed to be the determinant factor responsible for the absence of activity. Kitamura (1983JPD18) tested a wide range of *para*-substituted nitrocompounds against nitroreductase activity and demonstrated the important difference of activity between nitrocompounds bearing an electron

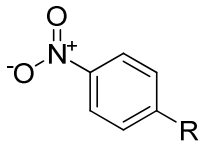
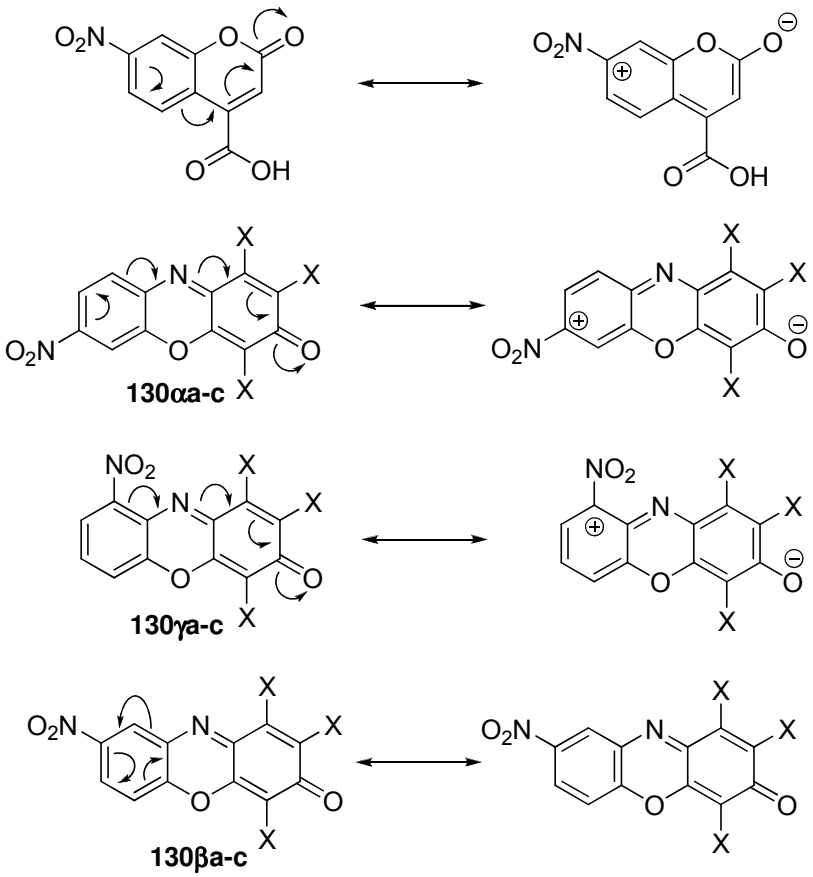


Figure 3.6: substituted *para* nitrobenzenes tested for nitroreductase activity. R = NH_2, OCH_3, COOH, OH, Cl, Br, SO_3H, CHO, $COCH_3$, SO_2NH_2, CO-Ar, NO_2, $COOCH_3$, ranked by increasing nitroreductase activity (1983JPD18).

withdrawing group and an electron donating group: nitroreductase activity was much higher for *para*-nitrobenzenes bearing electron withdrawing groups (**Figure 3.6**).



Scheme 3.2: Resonance forms in 7-, 8- and 9-nitrophenoxazin-3-ones **130α-γa-c** and 7-nitrocoumarin-3-carboxylate.

The electron density present on the 8-position in the case of substrate **130βa-c** was considerably higher compared to the 7- and 9- position in the case of substrate **130αa-c**, **130γa-b** and **242** respectively (**Scheme 3.2**). The 7-nitrocoumarin-3-carboxylate previously reported to be efficiently reduced by microorganisms (2001LAM403) presented a similar reduced electron density at the 7- position, as illustrated by the resonance form (**Scheme 3.2**). These observations correlated well with Kitamura's experiments and could explain the absence of visible reduction for substrates **130βa-c**, while substrates **130αa-c**, **130γa-b** and **242** were reduced to some extent.

9-Nitro-6-chlorobenzo[*a*]phenoxazin-5-one, **242** (kindly provided by Prof. A.L. James), is a commercially available substrate for nitroreductase activity (B-2005MI09). The company claim the substrate to be efficient for the detection of some

bacterial nitroreductase activity with no further specification. Smaill *et al.* (2008WO030120) tested this compound for nitroreductase activity in mammalian cells and found decreased performance under aerobic conditions; however, no report on its microbiological application in agar media seems to have been undertaken. This substrate showed higher activity compared to substrate **130αa-c**, **130βa-b** and **130γa-c**, with visible activity for both Gram negative and Gram positive bacteria (**Table 3.4**, **Table 3.5**, **Figure 3.7**). The presence of a fused benzene ring seemed to contribute to the higher activity of this substrate, as well as a better retention of the colour by the bacteria (**Figure 3.7**). However, although the general performance was higher than for substrates **130αa-c**, **130βa-c** and **130γa-b**, the colour generated was still weak (**Figure 3.7**), suggesting such substrates have little application for microbiological use.

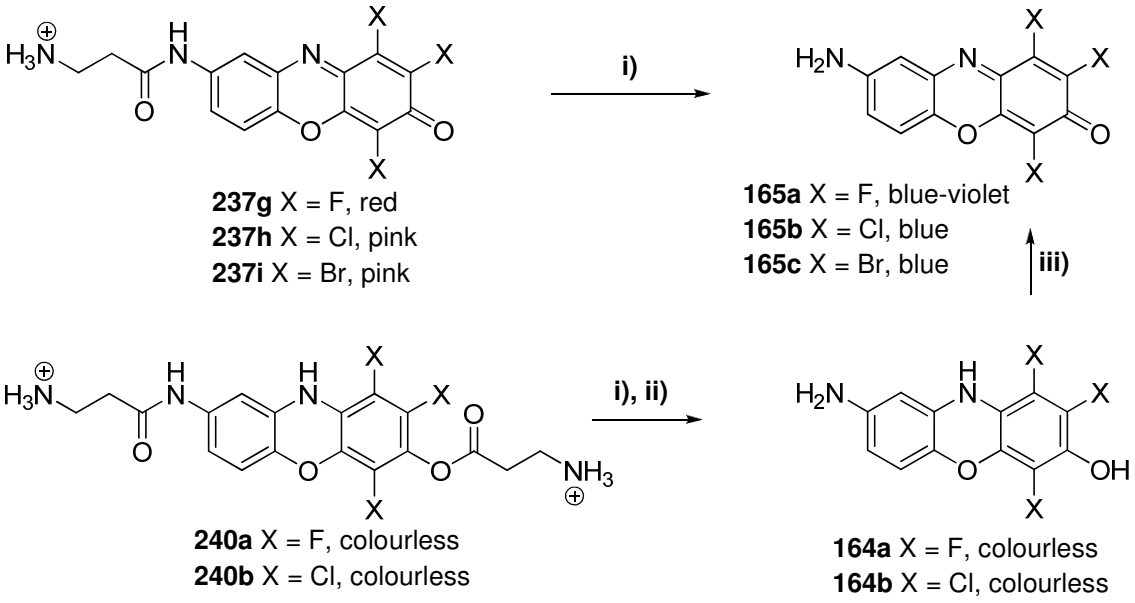
Figure 3.7: Screening of 9-nitro-6-chlorobenzo[a]phenoxazin-5-one for nitroreductase activity.

3.2 Testing of substrates 237g-i and 240a-b for β-alanyl aminopeptidase

For substrates **237g-i**, aminopeptidase activity was required to release the chromogen **165a-c**. Substrates **240a-b** required aminopeptidase, along with carboxypeptidase or esterase, activity, followed by oxidation of the 3-hydroxy-8-aminophenoxazine to produce the colourful 8-amino-1,2,4-trihalogenophenoxazin-3-one (**Scheme 3.3**).

As described previously in section **1.4.4.3**, β-alanylaminopeptidase activity is very specific, and hydrolysis of the substrates **237g-i** and **240a-b** was expected only for the three bacteria known to express this activity: *S. marcescens*, *B. cepacia* and *Ps. aeruginosa*.

Considering the novelty of substrates **237g-i** and **240a-b**, they were also screened against the set of microorganisms described in **Table 3.1**, to check for any unexpected enzymatic reactions.



Scheme 3.3: Expected enzyme activities and subsequent reaction of substrates **237g-i** **i)** β-Alanyl aminopeptidase, **ii)** Carboxypeptidase or esterase, **iii)** Oxidation.

3.2.1 Toxicity screening

Toxicity screenings were carried out to determine the concentration required for optimum observation of enzymatic activity: growth inhibition, colouration of the background and contrast of the bacterial colonies upon enzymatic hydrolysis were the main factors considered.

The toxicity screening was carried out with the substrates **237g-i**, at respective concentrations of 25mg/L, 50mg/L and 100mg/L. The results, depicted in **Figure 3.8** for substrate **237g**, clearly showed that a concentration of 100mg/L (**Figure 3.8**, **C**) was inappropriate for any testing, as it fully inhibited the growth of *Ps. aeruginosa* and produced a significant background colour.

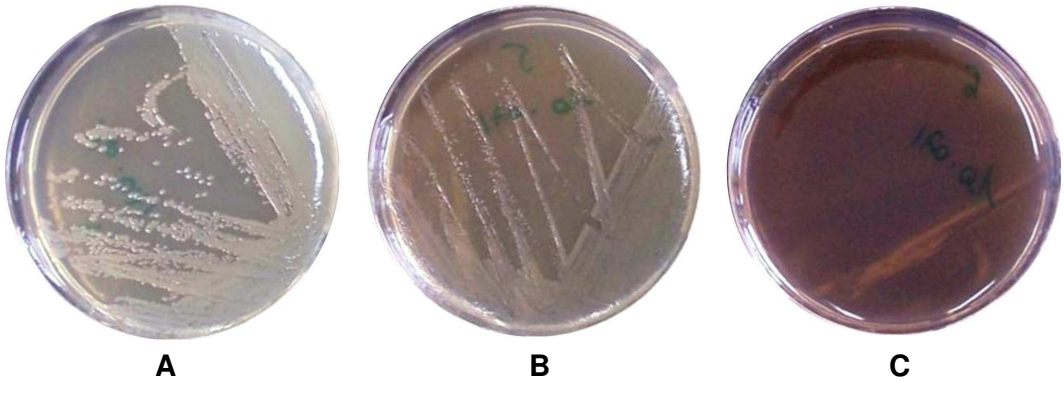


Figure 3.8: Toxicity screening of 8-*N*-(β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one TFA salt **237g** against *Ps. aeruginosa* at concentrations of A : 25mg/L, B : 50mg/L and C : 100mg/L.

The concentrations of 25mg/L (**Figure 3.8**, **A**) and 50mg/L (**Figure 3.8**, **B**) allowed a good growth of *Ps. aeruginosa* with little background colour and were considered for further testing. The absence of any enzyme activity for *Ps. aeruginosa* was also notable, but this issue was to be further investigated with screening against a wider range of microorganisms.

3.1.3 Results and discussion

A good general growth was observed for most microorganisms with substrates **237g-i** and **240a-b** (**Table 3.6 and Table 3.7**); only some mild inhibition was noticed with substrates **237j** and **240b** for certain Gram positive strains (**Table 3.7**), particularly *Streptococcus pyogenes*. However, none of the initially expected enzymatic activity with *S. marcescens*, *Ps. Aeruginosa* and *B. cepacia* could be detected with substrates **237g-i** or **240a-b**. Furthermore, the multi-point screening test did not reveal any unexpected enzyme activity by other microorganisms.

Previous results obtained with 7- and 9-nitro-1,2,4-trihalogenophenoxazinones **130αa-c** and **130γa-b** showed that those substrates were reduced to some extent by *S. marcescens* and *Ps. aeruginosa* (**Table 3.2**), indicating the substrates permeated the bacteria. Hence, it is reasonable to postulate that the absence of hydrolysis for substrates **237g-i** is not related to a permeation problem, as the core of the molecule is also 1,2,4-trihalogenophenoxazin-3-one, but rather link to the enzyme itself. The possibility of enzyme inhibition, as a result of a nucleophilic substitution from an amino residue on the 1-position for substrates **237g-i**, was considered; however this hypothesis could not be verified.

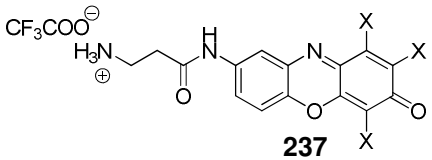
Bacterial Strain	Control		237g		237h		237i		240a		240b	
Gram negative	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	++	-	++	-	++	-
S. marcescens	++	-	++	-	++	-	++	-	++	-	++	-
Ps. aeruginosa	++	-	++	-	++	-	++	-	++	-	++	-
B. cepacia	++	-	++	-	++	-	++	-	++	-	++	-
Y. enterocolitica	++	-	++	-	++	-	++	-	++	-	++	-
S. typhimurium	++	-	++	-	++	-	++	-	++	-	++	-
C. freundii	++	-	++	-	++	-	++	-	++	-	++	-
M. morganii	++	-	++	-	++	-	++	-	++	-	++	-
E. cloacae	++	-	++	-	++	-	++	-	++	-	++	-
P. rettgeri	++	-	++	-	++	-	++	-	++	-	++	-
Background colour	None		Brown-orange		V. faint orange		V. faint orange		None		None	

Table 3.6: Multi-point screening results at 50mg/L for substrates **237g-i** and **240a-b** against Gram negative bacteria after 24h of incubation at 37°C

Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

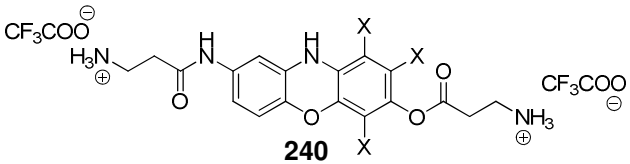
Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



237

237g X = F
237h X = Cl
237i X = Br



240

240a X = F
240b X = Cl

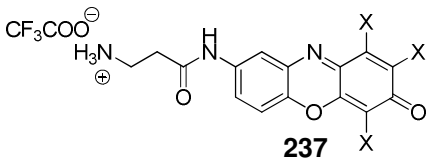
Bacterial Strain	Control		237g		237h		237i		240a		240b	
Gram positive	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
B. subtilis	++	-	++	-	++	-	++	-	++	-	+	-
E. faecalis	+	-	+	-	+	-	+	-	+	-	+	-
E. faecium	+	-	+	-	+	-	+	-	+	-	+	-
St. epidermidis	++	-	+	-	+	-	+	-	++	-	Tr.	-
St.aureus	++	-	++	-	++	-	++	-	++	-	++	-
MRSA	++	-	++	-	++	-	++	-	++	-	Tr.	-
S. pyogenes	+	-	+	-	+	-	NG	-	+	-	NG	-
L. monocytogenes	++	-	+	-	+	-	++	-	++	-	+	-
C. albicans	++	-	+	-	+	-	++	-	++	-	Tr.	-
C. glabrata	+	-	+	-	+	-	Tr.	-	+	-	Tr.	-
Background colour	None		Brown-orange		V. faint orange		V. faint orange		None		None	

Table 3.7: Multi-point screening results for substrates **237g-i** and **240a-b** against Gram positive bacteria and yeasts after 24h of incubation at 37°C

Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

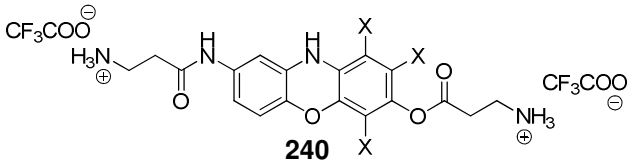
Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

Substrate:



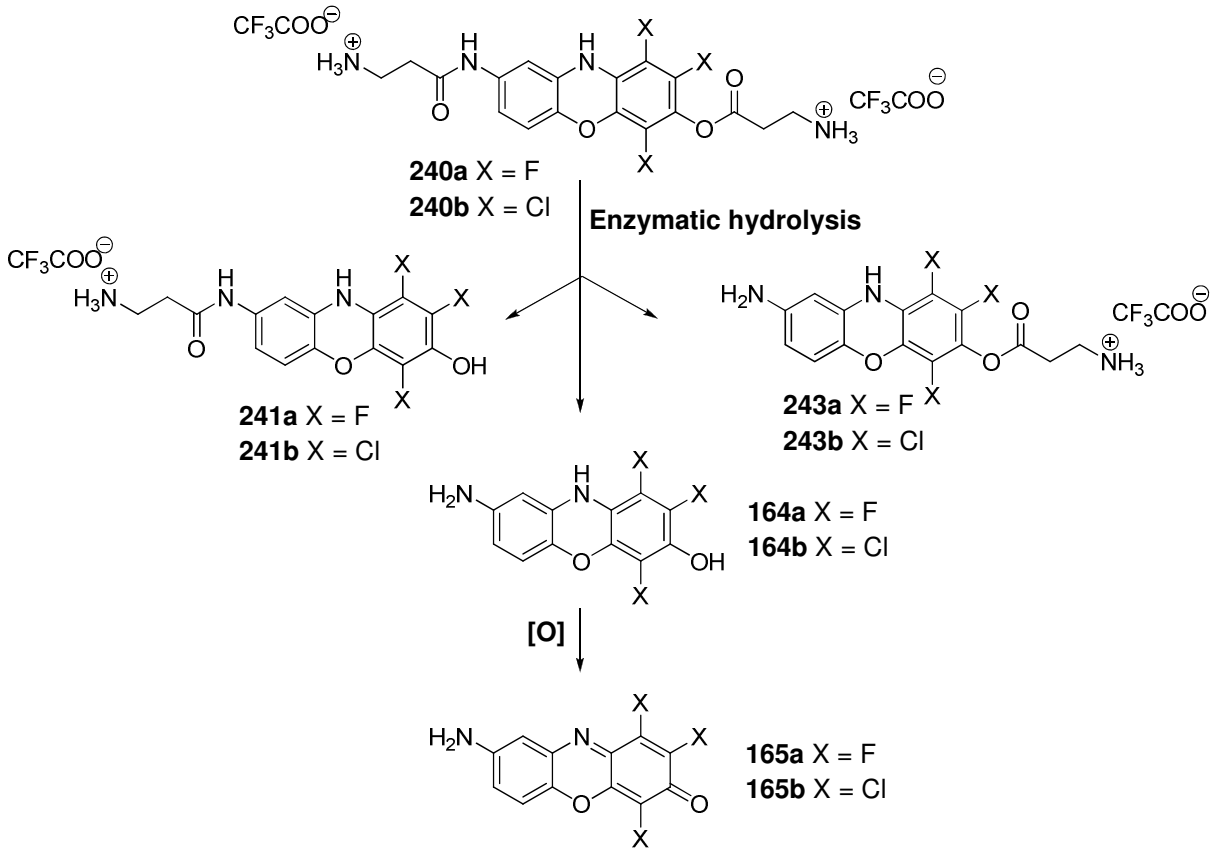
237

237g X = F
237h X = Cl
237i X = Br



240

240a X = F
240b X = Cl



Scheme 3.4: Possible outcomes from the enzymatic hydrolysis of **237a-b**.

An important concern with substrates **240a** and **240b** was the release of the actual chromogen, 8-amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones **165a-b**. Three possible outcomes were considered with the hypothetical enzymatic hydrolysis of substrates **240a** and **240b** (**Scheme 3.4**). First, hydrolysis of either the ester or peptide bond alone would produce **241** or **243**, respectively (**Scheme 3.4**). These two possibilities were unlikely, considering that the substrate had permeated into the bacteria; aminopeptidase activity would be expected to occur along with carboxypeptidase activity. The remaining possibility was the actual expression of both desired enzyme activities, resulting in the formation of **164** (**Scheme 3.4**) as expected, but a failure to re-oxidise **164** to the colourful **165**. As discussed earlier, it was noted that 8-amino-3-hydroxy-1,2,4-trihalogenophenoxazines **164a-b** slowly re-oxidised to 8-amino-1,2,4-trihalogenophenoxazinones **165a-b** under aerobic conditions. The hypothesis of **164** being present in the medium could be verified by adding a few drops of a 1% solution of sodium periodate in distilled water over the colonies of *Pseudomonas aeruginosa*. If **164** was present, a coloration of the *Pseudomonas aeruginosa* colonies would be expected. However, after a period of 15

minutes, no evolution of colour could be noticed (**Figure 48**). These observations, including the absence of colouration for substrate **237h-j**, led to the conclusion that none of the substrates were hydrolysed.

One positive observation resulting from this series of tests was the effective reduction of the background colour for the substrate **240a** in comparison to **237g**, in which the imino-quinone was not reduced (**Figure 3.10**). This also suggests that, despite the relatively low stability of **240a** noticed during its preparation, it was not converted to **237h** during preparation and incubation of the agar plates. This was unfortunately not of any use for substrates **240a-b**, as no hydrolysis occurred, but the technique is potentially adaptable to other, more efficient aminophenoxazinone substrates.

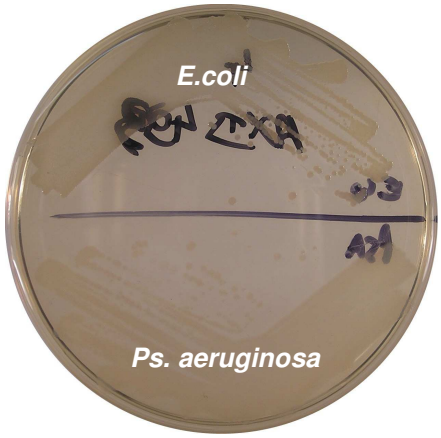


Figure 3.9: Substrate **240a** against *E. coli* and *Ps. aeruginosa* after 24h of incubation and addition of 1% aq. $NaIO_4$.

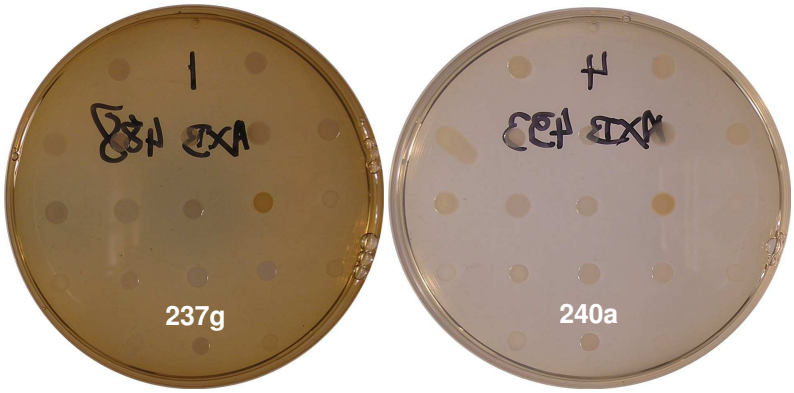
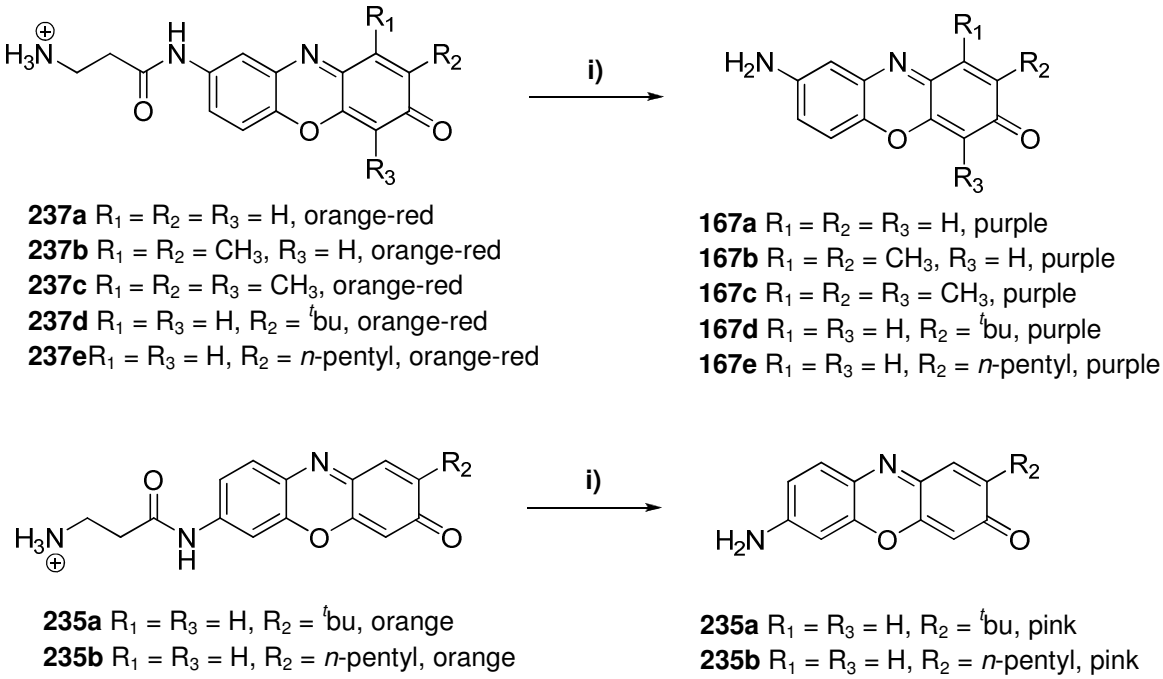


Figure 3.10: Background comparison between substrate **237g** and its reduced analogue **240a**.

3.3 Testing of substrates 237a-f and 235a-b for β-alanine aminopeptidase

The enzymatic activity required was the hydrolysis of 7-*N*-β-alanyl-aminophenoxazinone **235a-b** and 8-*N*-β-alanyl-aminophenoxazinone **237a-f** by β-alanine aminopeptidase to release the 7- and 8-aminophenoxazin-3-one chromogens, respectively (**Scheme 3.5**).



237a $R_1 = R_2 = R_3 = H$, orange-red
237b $R_1 = R_2 = CH_3$, $R_3 = H$, orange-red
237c $R_1 = R_2 = R_3 = CH_3$, orange-red
237d $R_1 = R_3 = H$, $R_2 = {}^t$bu, orange-red
237e $R_1 = R_3 = H$, $R_2 = n$-pentyl, orange-red

167a $R_1 = R_2 = R_3 = H$, purple
167b $R_1 = R_2 = CH_3$, $R_3 = H$, purple
167c $R_1 = R_2 = R_3 = CH_3$, purple
167d $R_1 = R_3 = H$, $R_2 = {}^t$bu, purple
167e $R_1 = R_3 = H$, $R_2 = n$-pentyl, purple

235a $R_1 = R_3 = H$, $R_2 = {}^t$bu, orange
235b $R_1 = R_3 = H$, $R_2 = n$-pentyl, orange

235a $R_1 = R_3 = H$, $R_2 = {}^t$bu, pink
235b $R_1 = R_3 = H$, $R_2 = n$-pentyl, pink

Scheme 3.5: Expected reaction upon enzymatic hydrolysis of substrates **237a-f** and **235a-b**
i) β-alanine aminopeptidase

As for substrates **237h-c**, the substrates **237a-c** and **235a-b** were tested against *S. marcescens*, *Ps. Aeruginosa* and *B. cepacia*, for which enzyme activity was expected, and *E. coli*, which was used as the control.

3.3.1 Results and discussion

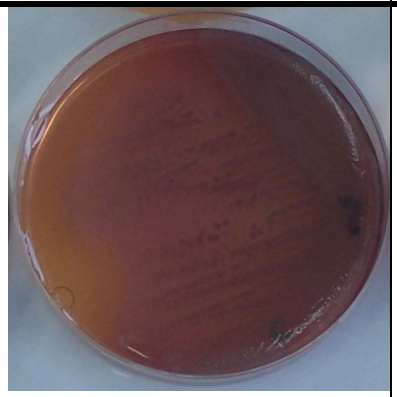
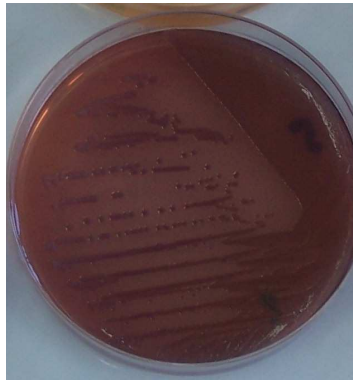
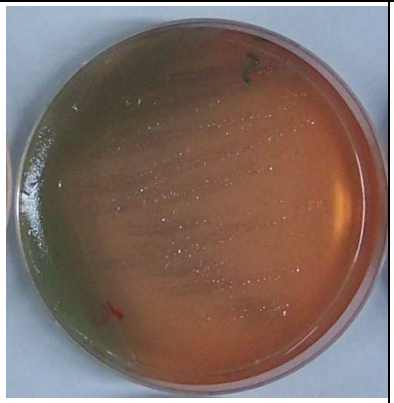
	P. aeruginosa	*S. marcescens*		*P. aeruginosa*	*S. marcescens*
237a			**237b**		
237f			**237c**		

Table 3.8: Testing of substrates **237a,b,c,f** after 24h of incubation at 37℃. Concentration 50mg/L.

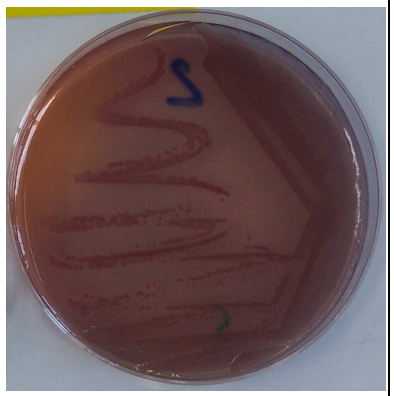
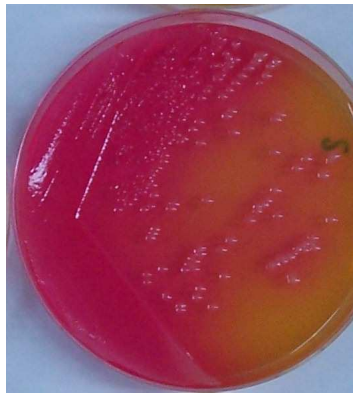
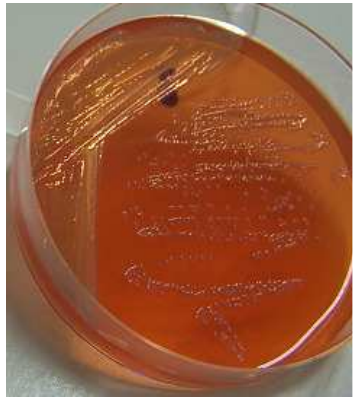
	P. aeruginosa	*S. marcescens*		*P. aeruginosa*	*S. marcescens*
237d			**235a**		
237e			**235b**		

Table 3.9: Testing of substrates **237d,e** and **235a-b** after 24h of incubation at 37℃. Concentration: 50mg/L.

	Control		237a						237f					
			25mg/L		50mg/L		100mg/L		25mg/L		50mg/L		100mg/L	
	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	++	+/- grey	++	-	++	-	++	+/- grey
S. marcescens	++	-	++	-	++	-	++	+/- grey	++	-	++	-	++	+/- grey
Ps. aeruginosa	++	-	++	-	++	-	++	+/- grey	++	-	++	-	++	+/- grey
B. cepacia	++	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Background colour	None		Faint yellow		Orange		Red-orange		Faint orange		Orange		Red-orange	

	Control		237b						237c					
			25mg/L		50mg/L		100mg/L		25mg/L		50mg/L		100mg/L	
	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	+	-	++	-	++	-	++	-
S. marcescens	++	-	++	+/- violet (diff.)	++	+ violet (diff.)	+	++ violet (diff.)	++	+/- violet	++	+/- violet	Tr.	+/- violet
Ps. aeruginosa	++	-	++	+/- violet (diff.)	++	+ violet (diff.)	+	++ violet (diff.)	++	+/- violet	++	+/- violet	Tr.	+/- violet
B. cepacia	++	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Background colour	None		Yellow		Orange		Orange		Faint yellow		Faint orange		Faint orange	

Table 3.10: Results for substrates **237a,b,c,f** after 24h of incubation at 37°C

Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour

	Control		237d						237e					
			25mg/L		50mg/L		100mg/L		25mg/L		50mg/L		100mg/L	
	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	++	-	++	-	++	-	++	-
S. marcescens	++	-	++	+/- violet (diff.)	++	++ violet (diff.)	Tr.	-	++	+/- purple	++	+ purple	++	++ purple
Ps. aeruginosa	++	-	++	+/- violet (diff.)	++	++ violet (diff.)	Tr.	-	++	+/- purple	++	+ purple	++	++ purple
B. cepacia	++	-	NA	NA	NA	NA	NA	NA	++	-	++	-	++	-
Background colour	None		Faint orange		Orange		Red-orange		Faint yellow		Orange		Orange	
	Control		**235a**						**235b**					
			25mg/L		**50mg/L**		**100mg/L**		**25mg/L**		**50mg/L**		**100mg/L**	
	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour	Growth	Colour
E. coli	++	-	++	-	++	-	++	-	++	-	++	-	++	-
S. marcescens	++	-	++	+ pink (diff.)	++	++ pink (diff.)	++	++ pink (diff.)	++	+ pink	++	+ pink	++	+ pink
Ps. aeruginosa	++	-	++	+ pink (diff.)	++	++ pink (diff.)	++	++ pink (diff.)	++	+ pink	++	+ pink	++	+ pink
B. cepacia	++	-	++	-	++	-	++	+/- pink	NA	NA	NA	NA	NA	NA
Background colour	None		Yellow		Orange		Orange		Orange		Orange		Red	

Table 3.11: Results for substrates **237d-e** and **235a-b** after 24h of incubation at 37°C

Growth: NG: No growth, **Tr.**: Traces of growth, only a few colonies visible, **+**: Good growth, colonies are multiple and clearly visible, **++**: Excellent growth, thick agglomeration of colonies

Colour: -: No colour; **+/-**: Weak colour, uncertain; **+**: Obvious colour; **++**: Bright colour, (diff.): diffusion

The optimum concentration for hydrolysis of substrates **237a-f** and **235a-b** was found to be 50mg/L. A greater concentration of 100mg/L produced a more intense colouration for **237b**, **237e** and **235a** upon hydrolysis; however, the background colour generated was rather intense thus impeding visual result interpretation and growth inhibition was notable, particularly for substrates **237b**, **237c** and **237d** (**Table 3.10** and **Table 3.11**).

Both substrates **237a** and **237f** failed to show any obvious enzymatic hydrolysis (**Table 3.8** and **Table 3.10**). Some darkening of the bacterial colonies was noticeable (**Table 3.8**), along with a significant background coloration; however, the control plates (*E. coli*) showed a similar phenomenon. This was more obvious at a concentration of 100mg/L, particularly after 48h of incubation, as shown for substrate **237f** (**Table 3.12**). These results suggested that substrates **237a** and **237f** were not suitable for the detection of β-alanine aminopeptidase activity, as the weak coloration observed could not be attributed to specific enzyme activity with certainty.

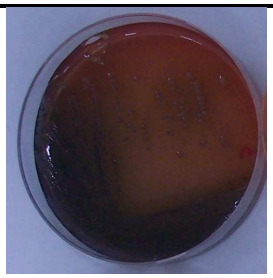
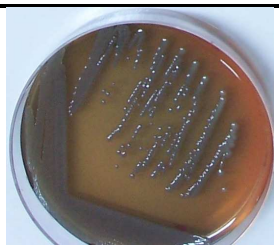
	E. coli	***S. marcescens***
24h		
48h		

Table 3.12: Results for substrate **237f** at 100mg/L after 48h of incubation at 37°C.

The dimethyl **237b**, trimethyl **237c**, 4-^{t}butyl **237d** 8-amino phenoxazinone substrates and 7-amino-4-^{t}butylphenoxazinone **235a** substrates showed obvious enzymatic hydrolysis, with the production of a purple colour for 8-aminophenoxazin-3-ones **237b-d** and of a pink colour for 7-amino-4-^{t}butylphenoxazinone **235a** (**Table 3.8** and **Table 3.9**). This activity was more pronounced for the dimethyl and ^{t}butyl substituted substrates with noticeable colour diffusion around the site of hydrolysis. The trimethyl substituted phenoxazinone **237c** was less sensitive to enzymatic activity, with the production of faint purple colonies, but showed a better retention of the colour in the bacteria compared to **237b** and **237d**.

7-Amino-4-^{t}butylphenoxazinone **235a** exhibited a better contrast with the background compared to 8-amino-4-^{t}butylphenoxazinone **237d** (**Table 3.9**), as this has been suggested in the U.V.-visible absorption study as discussed in section **2.4**.

Both the 7- and 8-amino-2-pentylphenoxazinone substrates (**237e** and **235b)**, showed enzymatic hydrolysis with good retention of the colour by the cells. The contrast with the background was weak in the case of 7-amino-2-pentylphenoxazinone substrate **235b** compared to the isomeric 8-amino-2-pentylphenoxazinone substrate **237e**. Better results were obtained after 48h of incubation (**Figure 3.11**).

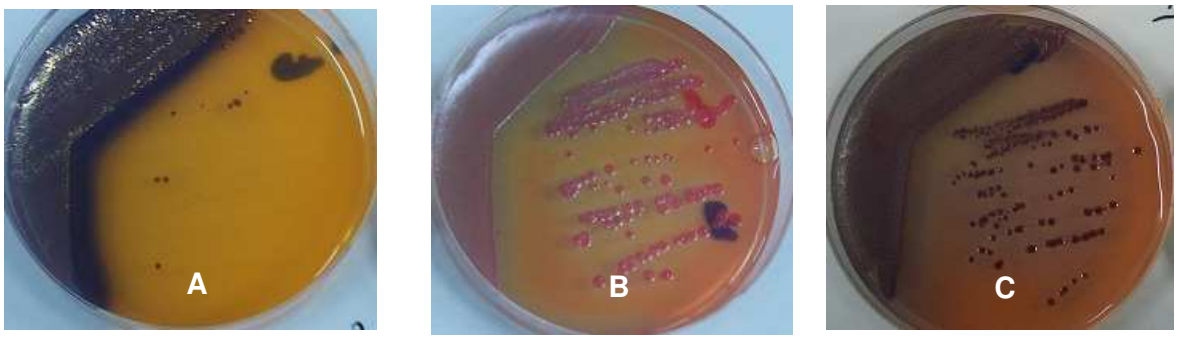


Figure 3.11: Performance of substrate **74**, **235b** and **237e** with *Ps. aeruginosa* at 50mg/L after 48h of incubation
A: **74**, B: **235b**, C: **237e**

The colouration was weaker compared to the lead compound 7-amino-1-pentylphenoxazin-3-one **74** in both instances, but the toxicity was also considerably lower: individual colonies were easily observed with **235b** and **237e**, but almost absent in the case of **74** (**Figure 3.11**).

3.3.2 Lipophilicity

An approximation of the lipophilicity of each substrate tested was made by generating a calculated LogP value using ChemBioDraw (**Table 3.12**). The following values were calculated to give an order of comparison for substrates **235a-b** and **237a-e**.

The resulting values showed some correlation with the level of hydrolysis and of colour retention: the comparatively low value for the non-substituted 8-aminophenoxazin-3-one **237a** corresponded to the absence of any obvious hydrolysis observed, whereas the highest calculated value found for **235b** and **237e** corresponded to a good level of hydrolysis. The high value obtained for **235b** and

237e also corresponded to the absence of colour diffusion within the medium (**Table 3.9** and **Table 3.12**). The high value found for **237f**, suggested that the absence of any obvious hydrolysis in this case could be caused by steric hindrance from the phenyl substituent.

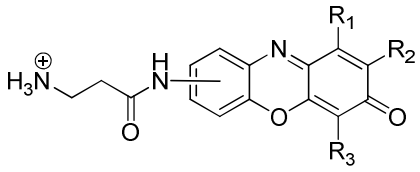
	**CLogP**
237a, 8-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = $\mathbf{R_3}$ = H	0.658
237b, 8-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = CH_3, $\mathbf{R_3}$ = H	1.606
237c, 8-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = $\mathbf{R_3}$ = CH_3	2.105
235a, 7-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = ^{t}bu, $\mathbf{R_3}$ = H **237d,** 8-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = ^{t}bu, $\mathbf{R_3}$ = H	2.484
237f, 8-position, $\mathbf{R_1}$ = $\mathbf{R_3}$ = Ph, $\mathbf{R_3}$ = H	2.547
235b, 7-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = pentyl, $\mathbf{R_3}$ = H **237e,** 8-position, $\mathbf{R_1}$ = $\mathbf{R_2}$ = pentyl, $\mathbf{R_3}$ = H	3.273

Table 3.12: Calculated LogP for substrates **235a-b** and **237a-f**. Values generated using ChemBioDraw Ultra v. 11.0.

3.4 Conclusion

The preparation of 7- and 9-nitrophenoxazinone substrates showed positive results for the detection of nitroreductase activity; however, their performances were considered poor compared to existing substrates. 8-Nitrophenoxazinones failed to show any hydrolysis of the substrate; the position of the nitro group and resulting electron density was thought to be the cause for absence of reduction.

All halogenated phenoxazinone substrates synthesised to detect β-alanine aminopeptidase failed to produce any colouration of the colonies. These included the trapped reduced form, which presented a higher solubility in the agar medium, indicating that the solubility of the substrate was not the cause for the absence of hydrolysis. Unlike the nitrophenoxazinone substrates, the position of the auxochrome could not be the causative factor here, as testing of substrates **237b-e** showed positive results for β-alanine aminopeptidase. The possibility of enzyme inhibition was suggested.

All 7- and 8-aminophenoxazinone substrates substituted with an aliphatic alkyl chain produced coloured colonies. However, none of the substrates prepared performed as well as the lead compound, 7-amino-1-pentylphenoxazin-3-one **79**. The lipophilicity of the substrates was linked to colour retention, and to a lesser extent the level of substrate hydrolysis. The position of the alkyl substituent also seemed to have important consequences on the level of hydrolysis as well as chromogen toxicity; this was evident with substrates **79** and **235a**, and the relative position of the pentyl substituent in the 1- and 2-position, respectively.

CHAPTER FOUR:

CONCLUSION

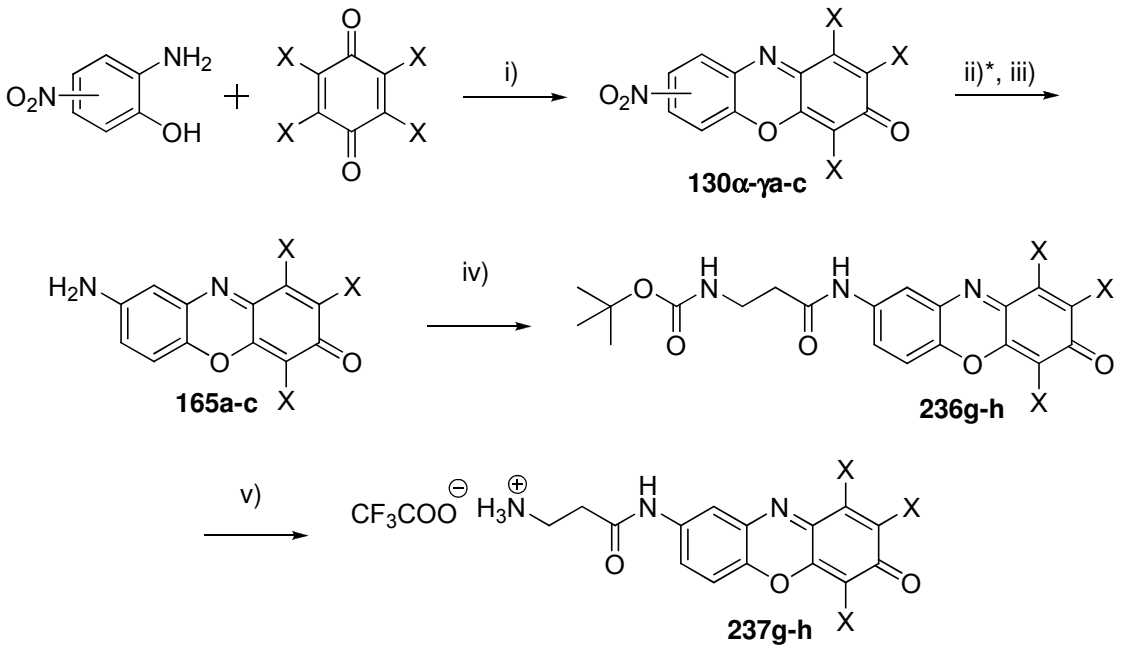
4.1 Conclusion and future work

Two synthetic routes were successfully explored within this work for the preparation of 8-aminophenoxazinone derivatives: the condensation of nitroaminophenols with halogenobenzoquinones and the reductive cyclisation of dinitrophenoxybenzoquinones.

4.1.1 Halogenophenoxazinone substrates

4.1.1.1 Summary

The condensation of nitroaminophenol with tetrahalogenobenzoquinones presented the advantage of being a short synthetic pathway (**Scheme 4.1**), using readily available reagents. It was, however, limiting in terms of the range of analogues accessible from this synthetic route (**Scheme 4.1**).



Scheme 4.1: Recapitulative synthesis of susbtrates **130α-γa-b** and substrates **237g-h**
i) NaOAc, EtOH, R.T.; ii) H_2, Pd/C 5% or Pt_2O, MeOH/EtOAc; iii) Ag_2O, MeOH; iv) **232**, dry THF, *N*-methylpiperidine, 48-72h, R.T.; v) neat TFA.
*Only substrates **130αa-c** were subjected to reduction

The nitrophenoxazinone intermediates **130α-γa-c** tested for nitroreductase activity clearly showed some enzymatic reduction for the 7-nitro and 9-nitro series (**Table 4.1**). The absence of hydrolysis for the 8-nitro series was thought to be linked to the electronic configuration of the phenoxazin-3-one. The chromogenic properties of these compounds were relatively poor compared to previously reported substrates

such as 7-nitrocoumarin or nitrobenzoxazole derivatives. The background colouration, weak enzymatic activity and significant colour diffusion suggested nitrohalogenophenoxazinones were not suitable for the detection of nitroreductase enzymatic activity (**Table 4.1**).

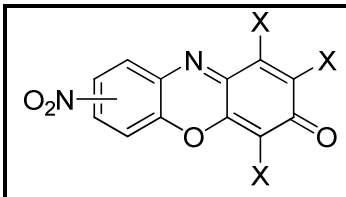
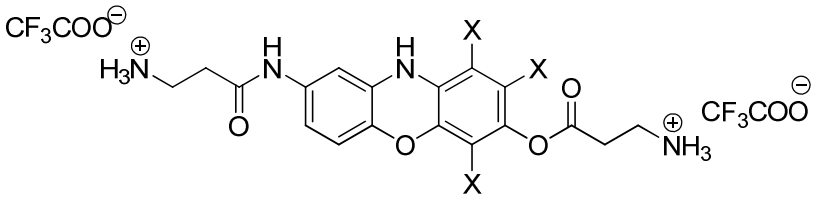
		Hydrolysis		Colour	Toxicity		Diffusion
		Gram negative	Gram positive		Gram negative	Gram positive	
7-NO_2	**130αa** X = F	YES	NO	Red	NO	YES	YES
	130αb X = Cl	YES	NO	Orange	NO	YES	YES
	130αc X = Br	YES	NO	Brown	NO	YES	YES
8-NO_2	**130βa** X = F	NO	NO	-	NO	YES	-
	130βb X = Cl	NO	NO	-	NO	YES	-
	130βc X = Br	NO	NO	-	NO	YES	-
9-NO_2	**130γa** X = F	YES	NO	Brown	NO	YES	YES
	130γb X = Cl	YES	NO	Violet	NO	YES	YES

Table 4.1: Summary of microbiological testing results for substrates **130α-γ a-c**
- : Data not available

The series of 8-amino-1,2,4-trihalogenophenoxazin-3-ones **237g-h** prepared from this route failed to evidence any enzymatic activity; the nature of the substituent was thought to possibly result in some enzyme inhibition.

The corresponding trapped reduced forms of 8-amino-3-hydroxy-3*H*-phenoxazines, substrates **240a-b**, also failed to evidence enzymatic activity; however the reduction of the imino-quinone resulted in a total suppression of the background colour, and an improved solubility in agar medium.

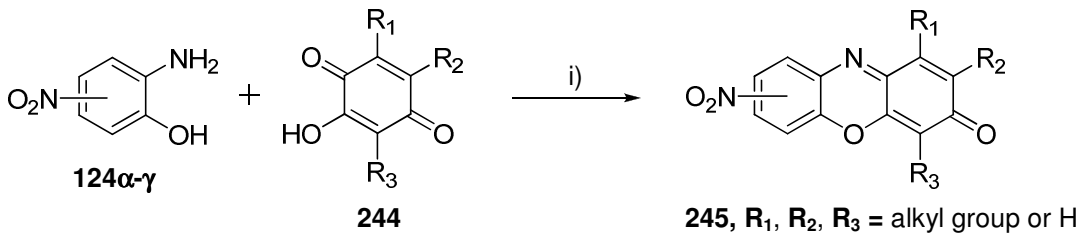


240a X = F
240b X = Cl

4.1.1.2 Future work

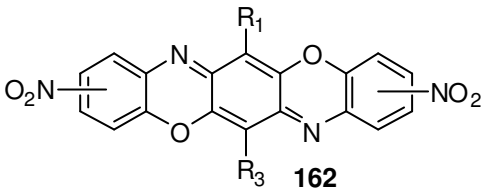
The preparation of alkyl substituted nitrophenoxazinones would be interesting to complete this study. The relation made between lipophilicity and colour retention suggests nitroalkylphenoxazinones could produce better results for the chromogenic detection of bacterial nitroreductase activity, compared to their halogenated analogues.

The preparation of nitrophenoxazinones bearing alkyl substituents **245** could be undertaken *via* condensation of nitroaminophenols **130α-γ** and substituted hydroxylbenzoquinones **244**. As described in section **1.3.3**, numerous examples of condensations between aminophenols and hydroxybenzoquinones have been reported. However, some research might be required to find satisfactory conditions for the reaction described in **Scheme 4.2**.



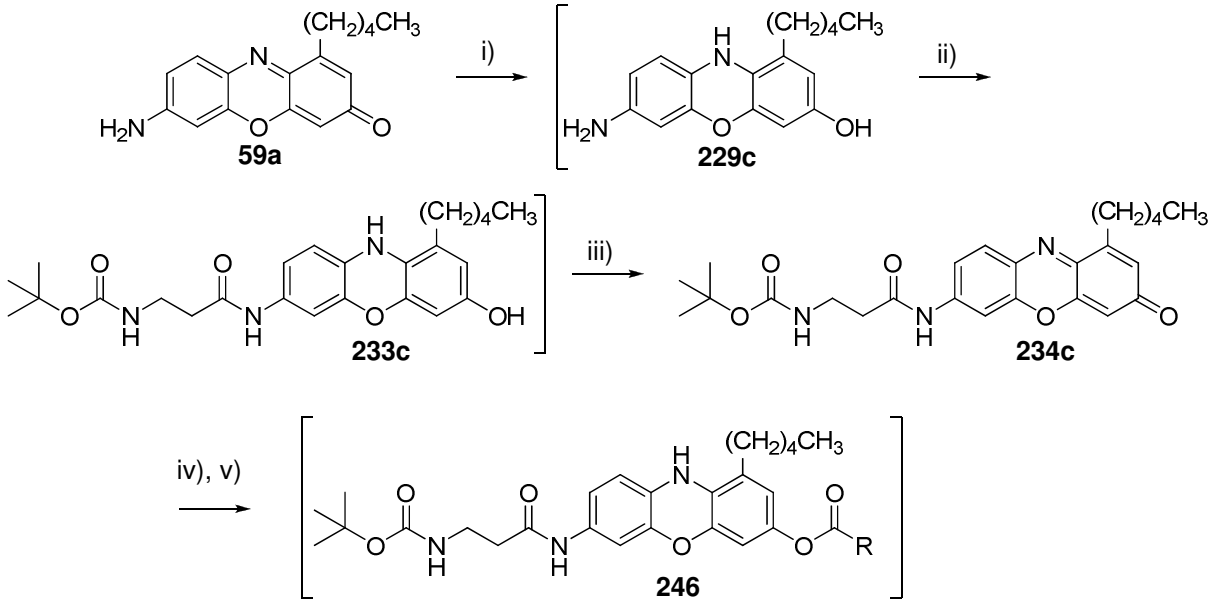
Scheme 4.2: Possible preparation of nitroalkylphenoxazin-3-ones **245**
i) EtOH, base or AcOH, reflux

The presence of an alkyl substituent in the 2-position (R_2) may be desirable to avoid the formation of triphendioxazinone by-products **162**, discussed in section **2.1.1.1**, which result from subsequent nucleophilic attack of the aminophenol derivative **124α-γ** onto the formed phenoxazinone.



If successful, the prepared nitroalkylphenoxazinones **245** could also be reduced readily to aminoalkylphenoxazin-3-ones, leading to further potential substrates for microbial aminopeptidase detection.

The lead compound, 7-*N*-β-alanylamino-1-pentyl-3*H*-phenoxazin-3-one **74**, produces a strong orange background colour. The absence of background colouration described for substrate **240a-b**, suggests a similar trapping of 7-amino-3-hydroxy-1-pentyl-3*H*-phenoxazine **229c** would also result in a reduced colouration.



Scheme 4.3: Possible preparation of a reduced 7-*N*-β-alanylamidophenoxazin-3-one i) [H], ii) Mixed anhydride, iii) aerial [O], iv) [H], v) acyl chloride.

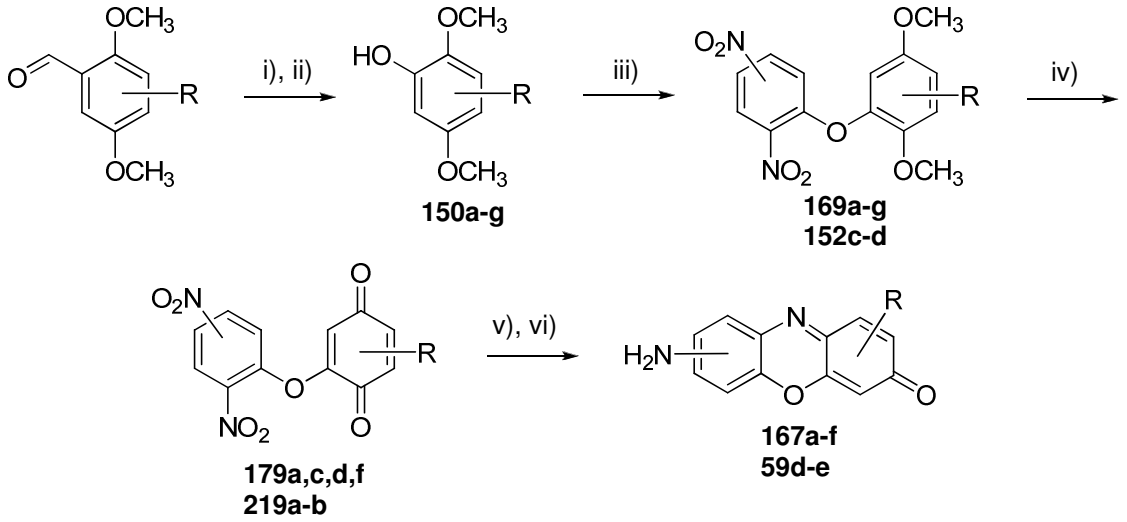
The method would require modification of the reaction conditions, as preparation of **74** involved reduction to the 7-amino-3-hydroxyphenoxazine species **229c**, where only the amino group was found to react with the amino acid mixed anhydride, unlike 8-amino-3-hydroxy-1,2,4-trihalogenophenoxazines **165a-c**. Isolation of 7-*N*-(*N*-^{t}butoxycarbonyl-β-alanyl)amino-1-pentyl-3*H*-phenoxazin-3-one **234c** followed by reduction and reaction with an acyl chloride could lead to the weakly coloured molecule **246** (**Scheme 4.3**).

4.1.2 Alkylphenoxazinone substrates

4.1.2.1 Summary

The preparation of 7- and 8-aminophenoxazin-3-one derivatives **59c-d** and **167a-f** was achieved. The general synthetic route adopted can be summarised as follows: preparation of 2,5-dimethoxybenzaldehydes, Baeyer-Villiger oxidation of the latter to 2,5-dimethoxyphenols **150a-g**, coupling of the latter with 2,4 or 2,5-dinitrofluorobenzene **168** or **151** to yield 2,4- or 2,5-dinitrodiphenylethers **169a-g** and **152c-d**, oxidation of the latter using CAN to 2,4- or 2,5-dinitrophenoxybenzoquinones **179a,c,d,f** and **219a-b**. Finally, catalytic hydrogenation of 2,4- or 2,5-

dinitrophenoxybenzoquinones **169a,c,d,f** and **219a-b**, followed by oxidation led to the corresponding 7- or 8-aminophenoxazin-3-one (**Scheme 4.4**).



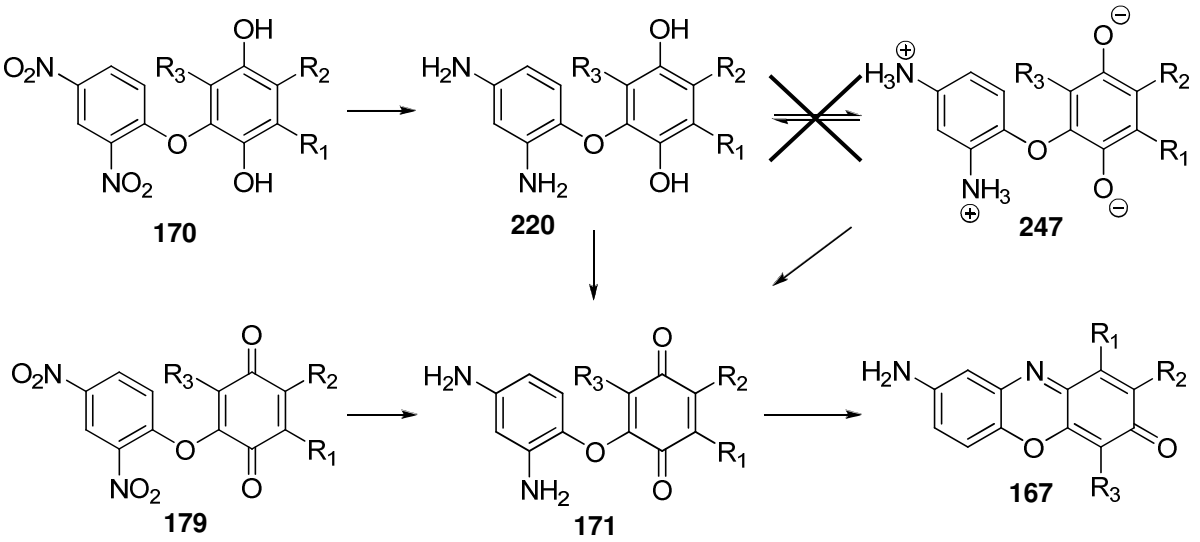
Scheme 4.4: Recapitulative synthesis of phenoxazinones **167a-f and 59d-e**
i) DCM, *m*-CPBA, ii) aq. NaOH, MeOH, iii) Et_3N, DMSO or NaH 60% w/w, DMF, iv) CAN, H_2O : CH_3CN, v) H_2, Pd/C 5%, EtOAc : MeOH, vi) Ag_2O, MeOH.

Various strategies were explored to introduce substituents onto the phenoxazinone core; the most successful proved to be the derivatisation of 1,4-dimethoxy-2-bromobenzene or 2,5-dimethoxy-3-bromobenzaldehyde. However, the resulting synthetic route required numerous steps to introduce specific substituents at a particular position. Attempts to shorten the pathway, *via* the direct preparation of a 2,4-dinitrophenoxybenzoquinone **208**, from the coupling of 2,4-dinitro-1-fluorobenzene and a 2-hydroxybenzoquinone derivative failed, as a result of the poor nucleophilic character of the latter. Attempts to substitute 2,4-dinitro-2',5'-dimethoxy-3'-bromodiphenylether failed, firstly due to its poor reactivity, secondly due to its susceptibility to some of the reagents, notably strong bases.

The preparation of dinitrophenoxybenzoquinones **179** and **219**, proved to be more convenient than the preparation of dinitro-2,5-dihydroxydiphenylethers, initially used within this synthetic route.

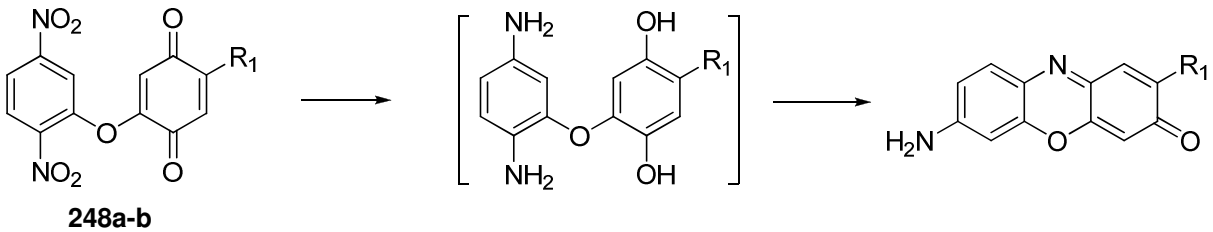
With 2,4-dinitrophenoxybenzoquinones **179**, evidence was given for the formation of 2,4-diaminophenoxybenzoquinone **171**, as a result of catalytic hydrogenation, confirming the species undergoes cyclisation to the 8-aminophenoxazin-3-one core **167** (**Scheme 4.5**).

With 2,4-dinitro-2,5-dihydroxydiphenylethers **150**, evidence was given for the formation of 2,4-diamino-2',5'-dihydroxydiphenylether **220**, as a result of catalytic hydrogenation (**Scheme 4.5**). The oxidative conditions used to produce 8-aminophenoxazinone from 2,4-diamino-2',5'-dihydroxydiphenylether favoured the formation of 2,4-diaminophenoxybenzoquinone **171** from 2,4-diamino-2',5'-dihydroxydiphenylether **220** (**Scheme 4.5**). The suggested participation of a zwitterion **247** in the oxidation of diamino-2',5'-dihydroxydiphenylether (**Scheme 1.50**, section **1.3.9**) seemed unlikely in the case of **220**, as neutral conditions did not favour cyclisation to 8-aminophenoxazin-3-one **167** (**Scheme 4.5**).



Scheme 4.5: Relation between the evidenced intermediates and their role in the cyclisation of 8-aminophenoxazin-3-one **167**.

With 2,5-dinitrophenoxybenzoquinones, evidence was given for the formation of 2,5-diamino-2',5'-dihydroxydiphenylether, as the product resulting from catalytic hydrogenation (**Scheme 4.6**). No evidence could be given here for the formation of a 2,5-diaminophenoxybenzoquinone intermediate, and reason for the reduction of the quinone ring in this case (**Scheme 4.6**) could not be fully rationalised.



Scheme 4.6: Evidenced intermediate in the cyclisation of 7-aminophenoxazin-3-ones **59**.

The microbiological testing of the corresponding β-alanine substrates prepared *via* this route did allow the successful detection of β-alanine aminopeptidase activity in most cases. The contrast resulting from the release of 8-aminophenoxazinones into the agar medium was significant enough for accurate observation of enzyme activity, despite the results suggested from the U.V.-visible study. However, the release of 7-aminophenoxazinones resulted in a brighter colour, improving the quality of the readings, and may be considered more suitable to the detection of enzyme activity.
Several factors influenced the hydrolysis of the substrates: it appeared evident the nature of the chromogen substituent and its position on the phenoxazin-3-one ring were two determining factors (**Table 4.2** and **Table 4.3**). The reduced lipophilic character of 8-*N*-β-alanyl-aminophenoxazinone **271a** was thought to prevent hydrolysis, whereas the enhanced lipophilic character of substrates **271-f** clearly improved enzymatic hydrolysis (**Table 4.2** and **Table 4.3**).

	Hydrolysis/Toxicity				Colour	Diffusion
	Ps. aeruginosa	*B. cepacia*	*S. marcescens*	*E. coli*		
237a $R_1 = R_2 = R_3 = H$	NO / NO	- / -	NO / NO	NO	-	-
237f $R_1 = Ph, R_2 = R_3 = H$	NO / NO	- / -	NO / NO	NO	-	-
237b $R_1 = R_2 = CH_3, R_3 = H$	YES / NO	- / -	YES / NO	NO	Violet	YES
237c $R_1 = R_2 = R_3 = CH_3$	YES / NO	- / -	YES / NO	NO	Violet	YES
237d $R_1 = R_3 = H, R_2 = {}^t bu$	YES / NO	- / -	YES / NO	NO	Violet	YES
237e $R_1 = R_3 = H, R_2 =$ pentyl	YES / NO	NO / NO	YES / NO	NO	Violet	NO

Table 4.2: Summary of microbiological testing results for substrates **237a-e** (at 50mg/L)
- : Data not available

	Hydrolysis				Colour	Diffusion
	Ps. aeruginosa	*B. cepacia*	*S. marcescens*	*E. coli*		
59d $R_2 = {}^t$bu	YES / NO	NO / NO	YES / NO	NO	Pink	YES
59e $R_1 = R_3 = H$, R_2 = pentyl	YES / NO	- / -	YES / NO	NO	Pink	NO

Table 4.3: Summary of microbiological testing results for substrates **59d-e** (at 50mg/L)
- : Data not available

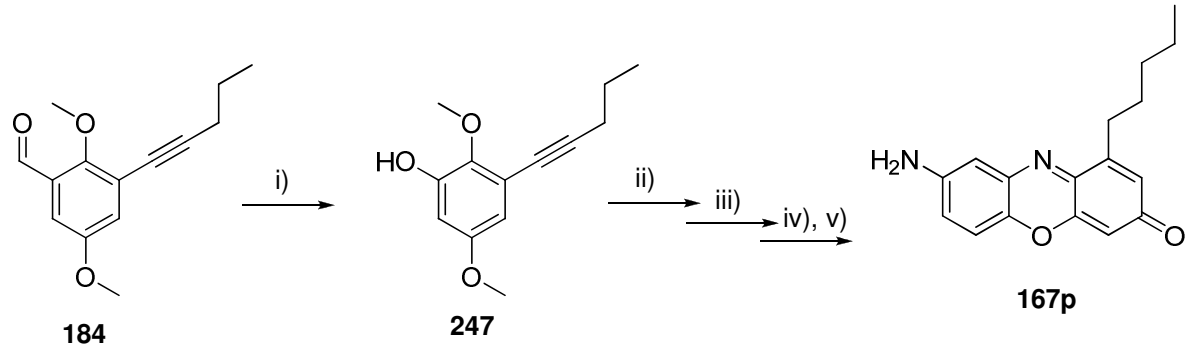
Hence the lipophilicity of substrates **237a-f** and **269a-b** was thought to be an important property for good permeation through the Gram positive bacterial outer-membrane and hydrolysis of the substrate. However, as demonstrated with the two isomers 7-*N*-β-alanylamino-2-pentylphenoxazin-3-one **269b** and 7-*N*-β-alanylamino-1-pentylphenoxazin-3-one **79,** and with 8-*N*-β-alanylamino-1-phenylphenoxazin-3-one **271b**, the lipophilic character of a substrate does not always permit good hydrolysis: in the instance of **269b**, the 2-position considerably reduced the level of hydrolysis, and in the case of **271b**, the presence of a phenyl group resulted in absence of hydrolysis, possibly due to some steric hindrance.

The overall performance of all β-alanine substrates prepared did not match the efficiency of 7-*N*-β-alanylamino-1-pentylphenoxazin-3-one **74**.

4.1.2.2 Future work

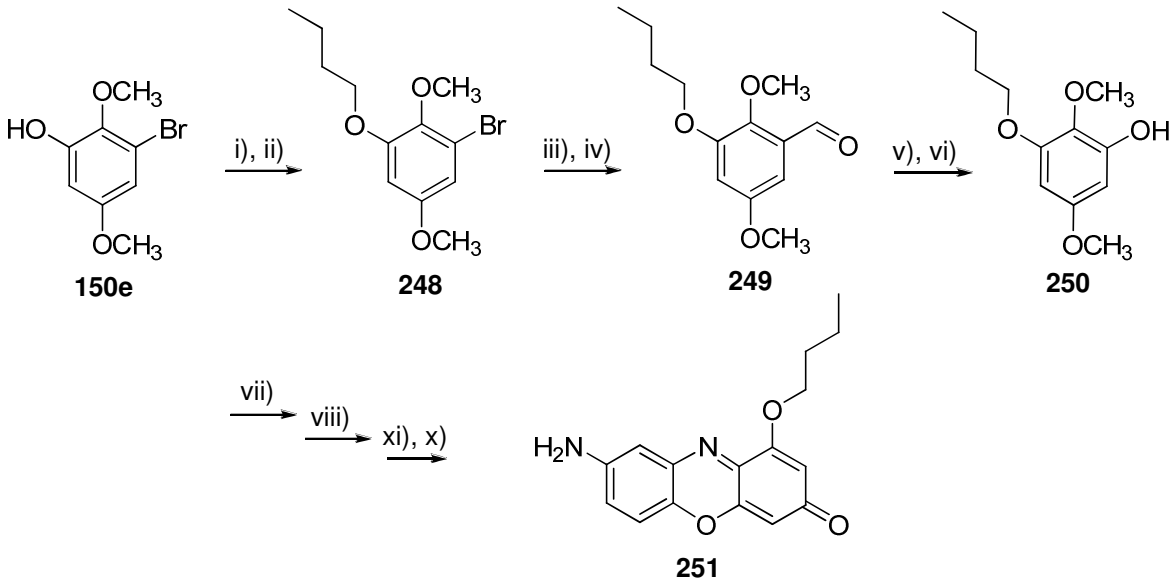
In view of the results obtained within this work, preparation of future substrates should focus on long alkyl substituents, as shorter alkyl chain substrates cannot precisely localise the site of hydrolysis, and preferably on the 1-position of the phenoxazin-3-one ring, as this seemed to favour hydrolysis.

The preparation of 8-amino-1-pentylphenoxazin-3-one **167p**, which could not be achieved despite numerous attempts, is now thought to be possible *via* the 2,5-dimethoxy-6-pentynylbenzaldehyde **184** prepared, following the synthetic pathway summarised below (**Scheme 4.7**).



Scheme 4.7: Possible route to 8-amino-1-pentylphenoxazin-3-one **167p**
i) Baeyer-Villiger oxidation, ii) Base, 2,4-dinitrofluorobenzene, iii) CAN oxidation, iv) [H], v) [O]

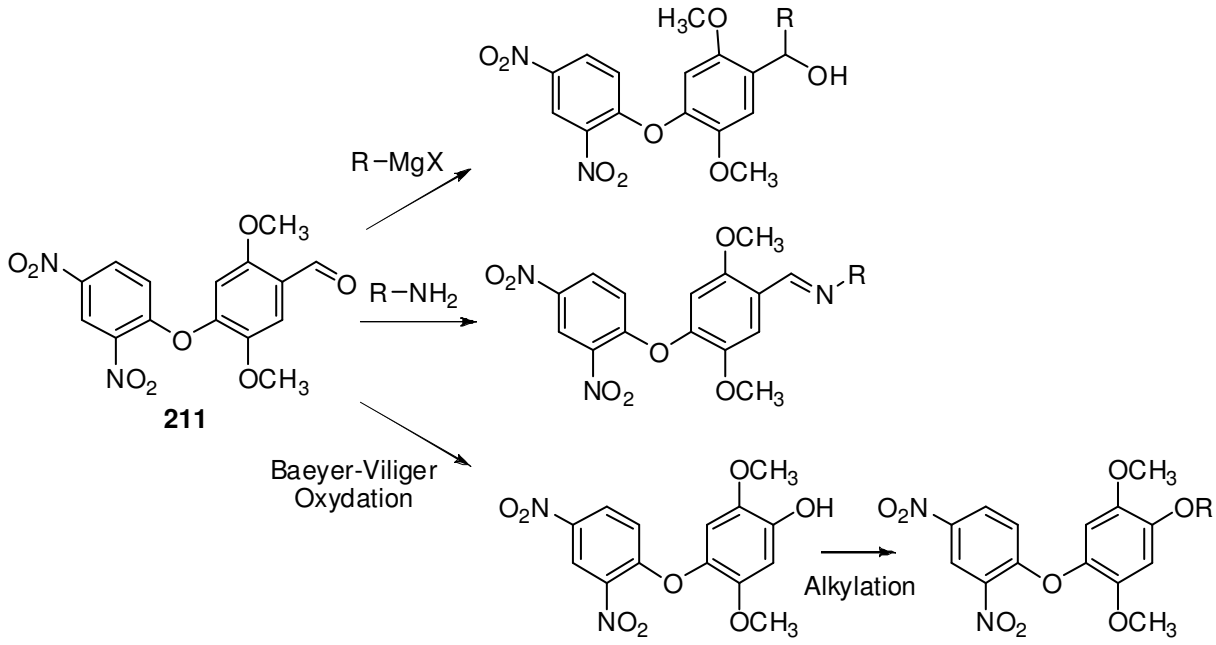
An analogous compound, 8-amino-1-butoxyphenoxazinone **251** has also been envisaged, using 3-bromo-2,5-dimethoxyphenol **150e**. Alkylation of the latter using NaH and butyl iodide, followed by introduction of a formyl group on the resulting 3-bromo-1-butoxy-2,5-dimethoxybenzene **248** using *n*-BuLi and DMF would lead to 3-butoxy-2,5-dimethoxybenzaldehyde **249** (**Scheme 4.8**). Preparation of 3-butoxy-2,5-dimethoxyphenol **287** from **286** could lead to 8-amino-1-butoxyphenoxazinone **288** following the pathway summarised below (**Scheme 4.8**).



Scheme 4.8: Possible route to 8-amino-1-butoxyphenoxazin-3-one **251**
i) NaH, DMF, ii) BuI, Δ, iii) *n*-BuLi, THF, -78°C, iv) DMF, -78°C to R.T., v) m-CPBA, DCM, vi) MeOH, NaOH, vii) Et_3N, 2,4-dinitrofluorobenzene, viii) CAN oxidation, xi) H_2, Pd/C 5%, MeOH, x) [O].

The preparation of 2,4-dinitro-2',5'-dimethoxy-3'-carbaldehydediphenylether **211** offers a solution to the issue concerning the lack of versatile precursor allowing a quick preparation of several phenoxazin-3-one derivatives.

Several interesting derivatives are thought to be accessible *via* reaction of the formyl group (**Scheme 4.9**). The resulting intermediates would lead to preparation of novel 2-substituted 8-aminophenoxazinone derivatives suitable for testing as bacterial enzyme substrates.



Scheme 4.8: Example of possibly interesting derivativatisation of 2,4-dinitro-2',5'-dimethoxy-3'-carbaldehydediphenylether **238**.

CHAPTER FIVE:

EXPERIMENTAL

5.1 General experimental

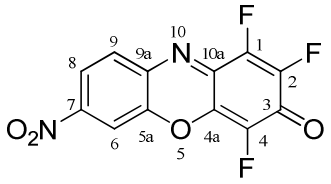
All commercially available reagents and solvents were obtained from Sigma-Aldrich, Fluka or Riedel-de-Haan and were used without any further purification. In the case of dry conditions, solvents, amines and other reagents were dried according to the procedures described in the literature (B-2003MI07). All yields were recorded after purification by column chromatography, unless this technique is not mentioned. Melting points were recorded on a Reichart-Kofler hot-stage microscope apparatus and are uncorrected. Infrared spectra were recorded in the range 4000 – 600 cm^{-1} using a Perkin Elmer Spectrum BX FT-IR instrument with internal calibration and a Pike sampling system. NMR spectra were obtained using a Bruker Ultrashield 300 spectrometer at 300 MHz for ^{1}H spectra or at 75 MHz for ^{13}C spectra or a Bruker Ultrashield 500 spectrometer at 500 MHz for ^{1}H spectra or at 125 MHz for ^{13}C spectra. Signals are described as being broad (br), singlet (s), doublet (d), double doublet (dd), triplet of doublets (dt), doublet of double doublets (ddd), triplet (t), doublet of triplets (dt), quartet (q) or multiplet (m), as appropriate. Low-resolution mass spectra were recorded on a Bruker Esquire 3000plus analyser using an electrospray source in either positive or negative ion mode. Elemental analyses were performed using an Exeter Analytical CE-440 Elemental Analyzer.

5.2 Synthesis

5.2.1 General procedure for the preparation of nitro-1,2,4-trihalegeno-3H-phenoxazin-3-one 130α-γ a-c (1933USP2020651)

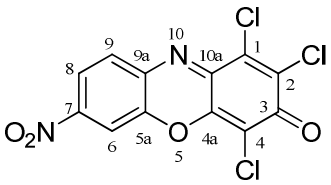
2-Aminonitrophenol (1mol equivalent), *p*-tetrahalogenobenzoquinone (1.1mol equivalent) and sodium acetate (1.2mol equivalent) were suspended in ethanol in a round bottomed flask. The solution was stirred for 24 hours or until no more 2-aminonitrophenol was detected by TLC. A red precipitate (for brominated and chlorinated derivatives) progressively appeared as the reaction neared completion. Water was then added to precipitate completely the red solid out of solution. The solid was recovered by filtration and washed with water and a little ethanol, then either recrystallised from acetic acid or purified by column chromatography.

5.2.1.1 7-Nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one 130α**a**



The title compound was prepared from *p*-fluoranil **129a** (1.90g, 10.57mmol) and 2-amino-5-nitrophenol **124α** (1.42g, 9.61mmol). Water (100mL) was added to the reaction mixture and the resulting mixture was extracted with EtOAc (3 × 60mL). The emulsion was filtered through a sintered funnel and the insoluble residue was washed several times with EtOAc. The aqueous layer was discarded and the combined EtOAc filtrates and layer were washed with water (150mL) and brine (150mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue subjected to column chromatography on silica, eluting with EtOAc : petroleum ether 60-80° (20 : 80). 7-Nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one **130α a** was isolated as a dark purple solid (0.35g, 1.19mmol, 12.4%). Dark red-purple needle-like crystals were obtained from acetic acid, **m.p.**: 218-220°C; (found: C, 48.5; H, 1.05; N, 9.15%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); *m/Z* 295.9 $(M)^-$; ν_{max}/cm^{-1} 3104 (C-H), 1660 (C=O), 1596 (C=C), 1531 and 1312 (NO_2), 1003 (C-F); **δ_H (300 MHz, d_8-THF)** 6.41 (1H, d, *J* = 8.7Hz, 9-H), 6.58 (1H, dd, *J* = 8.7, 2.4Hz, 8-H), 6.74 (1H, d, *J* = 2.4Hz, 6-H); **δ_C (75 MHz, d_8-THF)** 110.3 (CH, 6-C), 118.6 (CH, 8-C), 129.4 (CH, 9-C), 130.1 (quat., ddd, *J* = 8.5, 5.2, 1.4Hz, 10a-C), 134.1 (quat., 9a-C), 136.0 (quat., dd, *J* = 262.7, 6.1Hz, 4-C), 141.3 (quat., dt, *J* = 273.4, 6.9Hz, 2-C), 142.2 (quat., dd, *J* = 273.4, 12.2Hz, 1-C), 141.4 (quat., d, *J* = 1.5Hz, 5a-C), 141.7 (quat., ddd, *J* = 18.1, 4.8, 1.2Hz, 4a-C), 147.9 (quat., 7-C), 166.7 (quat., dt, *J* = 21.4, 5.4Hz, 3-C); **δ_F (282 MHz, d_8-THF)** -282.17 (1F, t, *J* = 2.3Hz, 4-F), -271.89 (1F, dd, *J* = 9.3, 2.0Hz, 2-F), -265.41 (1F, dd, *J* = 9.3, 2.0Hz, 1-F).

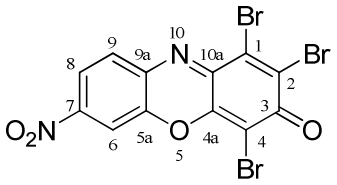
5.2.1.2 7-Nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one 130α**b**(1958ZOB2977)



The title compound was prepared from *p*-chloranil **129b** (3.52g, 14.32mmol) and 2-amino-5-nitrophenol **124α** (1.97g, 12.78mmol); 7-nitro-1,2,4-trichloro-3*H*-phenoxazin-

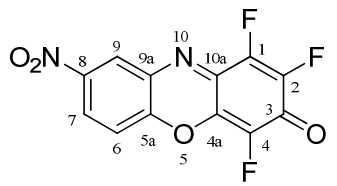
3-one **130αb** was isolated as a purple solid (4.06g, 11.75mmol, 90.4%). Purple lustrous plates of **130αb** were obtained from glacial acetic acid; **m.p.**: 239-241℃ [lit.: 238-240℃ (from AcOH, 1958ZOB2977]; *m/Z* 343.8 (85%), 345.8 (100%), 347.7 (26%), 348.9 (15%) $(M)^-$; (found: C, 41.7; H, 0.9; N, 7.9%. $C_{12}H_3Cl_3N_2O_4$ requires: C, 41.7; H, 0.9; N 8.1%); ν_{max}/cm^{-1} 3094 (C-H), 1646 (C=O), 1584 (C=C), 1519 and 1347 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 8.24 (1H, d, *J* = 8.7Hz, 9-H), 8.32 (1H, dd, *J* = 8.7, 2.4Hz, 8-H), 8.51 (1H, d, *J* = 2.4Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 111.6 (quat., 4-C), 112.7 (CH, 6-C), 121.5 (CH, 8-C), 132.1 (CH, 9-C), 136.6 (quat., 7-C), 137.2 (quat.,1-C or 2-C), 138.5 (quat., 1-C or 2-C), 143.5 (quat., 9a-C), 146.4 (quat., 2 × C, 4a-C or 10a-C), 149.8 (quat., 7-C), 171.0 (quat., 3-C).

5.2.1.3 7-Nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one 130αc



The title compound was prepared from *p*-bromanil **129c** (1.03g, 2.42mmol) and 2-amino-5-nitrophenol **124α** (0.34g, 2.20mmol); 7-nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one **130αc** was isolated as a bright red solid (1.10g, 2.30mmol, 95.0%). A microcrystalline dark red solid of **130αc** was obtained from acetic acid, **m.p.**: 263-264℃; (found: C, 30.1; H, 0.7; N, 5.6%. $C_{12}H_3Br_3N_2O_4$ requires: C, 30.1; H, 0.6; N 5.85%); *m/Z* 475.6 (33%), 477.6 (100%), 479.7 (84%), 481.7 (28%) $(M)^-$; ν_{max}/cm^{-1} 3101 (C-H), 1641 (C=O), 1529 and 1346 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 8.21 (1H, d, *J* = 9.0Hz, 9-H), 8.29 (1H, dd, *J* = 8.7, 2.4Hz, 8-H), 8.44 (1H, d, *J* = 2.4Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 103.2 (quat., 4-C), 112.45 (CH, 6-C), 121.4 (CH, 8-C), 131.9 (CH, 9-C), 134.9 (quat., 1-C or 2-C), 137.0 (quat., 1-C or 2-C), 137.1 (quat., 5a-C), 143.6 (quat., 9a-C), 147.2 (quat., 4a-C or 10a-C), 148.3 (quat., 4a-C or 10a-C), 149.7 (7-C), 171.5 (quat., 3-C).

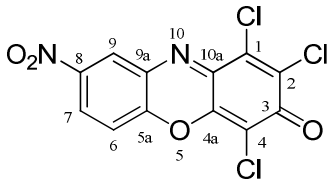
5.2.1.4 8-Nitro-1,2,4-trifluoro-*3H*-phenoxazin-3-one 130βa



Method A. The title compound was prepared from *p*-fluoranil **129a** (1.51g, 8.39mmol) and 2-amino-4-nitrophenol **124β** (1.27g, 8.22mmol) according to the general procedure. The precipitate was dissolved in EtOAc (100mL) and the organic layer was washed with 10% aq. NaOH solution (4 x 100mL) and water (1 x 100mL), and dried with $MgSO_4$. The solvent was removed under reduced pressure and the residue subjected to column chromatography, eluting with a gradient mixture of petroleum ether (60-80°C) : Et_2O (50:50 to 0:100). 8-Nitro-1,2,3-trifluoro-3*H*-phenoxazin-3-one **130βa** was isolated as a red solid (0.94g, 3.17mmol, 38.5%). Analytical data were identical to that obtained for the product of ***method B*** below.

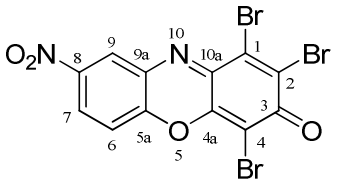
Method B. In a 100mL 2 neck round bottomed flask, equipped with a pressure equalizing dropping funnel, was dissolved *p*-fluoranil (1.48g, 8.22mmol) in dry THF (50mL). A solution of 2-amino-4-nitrophenolate was prepared, by adding DBU (0.82mL, 5.48mmol) to a solution of 2-amino-4-nitrophenol **124β** (0.84g, 5.45mmol) in dry THF (15mL), and transferred into the pressure equalizing dropping funnel. The *p*-fluoranil solution was cooled with an ice-bath and to it was added dropwise the phenolate solution. The resulting solution was stirred for 48 hours. Water (150mL) was then added and the resulting mixture was extracted with EtOAc (3 x 80mL). The insoluble solid was discarded and the organic layer was washed once with water (100mL) and dried with $MgSO_4$. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography using a gradient of petrolem ether (60-80°C) : EtOAc (75:25 to 60:40). 8-Nitro-1,2,3-trifluoro-3*H*-phenoxazin-3-one **130βa** was isolated as a red solid (0.44g, 1.49mmol, 27.3%). An orange crystalline solid **130βa** of was obtained from acetic acid, **m.p.**: 212-214°C; (found: C, 48.6; H, 1.05; N, 9.1%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); $m/_Z$ 295.9 $(M)^-$; ν_{max}/cm^{-1} 3093 (C-H), 1648 (C=O), 1608 (C=C), 1518 and 1308 (NO_2), 1004 (C-F); **δ_H (300 MHz, d_8-THF)** 6.03 (1H, d, *J* = 9.3Hz, 6-H), 6.80 (1H, dd, *J* = 9.0, 2.7Hz, 7-H), 7.06 (1H, d, *J* = 2.7Hz, 9-H); **δ_C (75 MHz, d_8-THF)** 115.4 (CH, 6-C), 123.8 (CH, 9-C), 126.2 (CH, 7-C), 129.85 (quat., ddd, *J* = 9.6, 5.1, 1.3Hz, 10a-C), 130.2 (quat., 9a-C), 136.0 (quat., dd, *J* = 262.4, 6.5Hz, 4-C), 141.3 (quat., dt, *J* = 272.7, 6.9Hz, 2-C), 142.2 (quat., dd, *J* = 273.9, 12.0Hz, 1-C), 140.8 (quat., ddd, *J* = 18.3, 5.5Hz, 1.1Hz, 4a-C), 143.4 (quat., 8-C), 145.3 (quat., d, *J* = 1.6Hz, 5a-C), 166.8 (quat., td, *J* = 21.0, 5.6Hz, 3-C); **δ_F (282 MHz, d_8-THF)** -282.05 (1F, t, *J* = 2.8Hz, 4-F), -271.73 (1F, dd, *J* = 9.6, 1.7Hz, 2-F), -266.07 (1F, dd, *J* = 9.3, 2.5Hz, 1-F).

5.2.1.5 8-Nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one 130βb (1971JC1875)



The title compound was prepared from *p*-chloranil **129b** (1.74g, 7.07mmol) and 2-amino-4-nitrophenol **124β** (0.991g, 6.43mmol); 8-nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one **130βb** was isolated as a red solid (2.19g, 6.34mmol, 98.6%). Red plates of **130βb** were obtained from glacial acetic acid; **m.p.**: 261-263°C [lit.: 248-250°C (from acetone, 1971JC1875)]; (found: C, 41.7; H, 0.9; N, 7.9%. $C_{12}H_3Cl_3N_2O_4$ requires: C, 41.7; H, 0.9; N 8.1%); *m/z* 343.9 (92%), 345.8 (100%), 347.7 (32%), 348.9 (13.2%) $(M)^-$; ν_{max}/cm^{-1} 3088 (C-H), 1649 (C=O), 1597 (C=C), 1510 and 1331 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 7.95 (d, 1H, *J* = 9.0Hz, 6-H), 8.57 (dd, 1H, *J* = 9.0, 2.7Hz, 7-H), 8.73 (d, 1H, *J* = 2.7Hz, 9-H); **δ_C (75 MHz, d_6-DMSO)** 111.8 (quat., 4-C), 118.3 (CH, 6-C), 125.85 (CH, 9-C), 129.1 (CH, 7-C), 132.6 (quat., 9a-C), 137.2 (quat., 1-C or 2-C), 138.2 (quat., 1-C or 2-C), 145.3 (quat., 8-C), 145.6 (quat., 4a-C or 10a-C), 146.2 (quat., 4a-C or 10a-C), 147.7 (quat., 5a-C), 171.1 quat., 3-C).

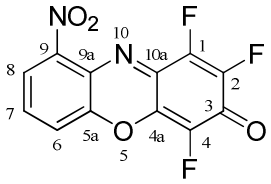
5.2.1.6 8-Nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one 130βc (1971JC1875)



The title compound was prepared from *p*-bromanil **129c** (4.05g, 9.57mmol) and 2-amino-4-nitrophenol **124β** (1.34g, 8.70mmol). Elution with EtOAc : petroleum ether 60-80 ° (40:60) gave 8-nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one **130βc** as a red solid (3.01g, 6.29mmol, 72.3%). Lustrous cardinal red crystals of **130βc** were obtained from acetic acid, **m.p.**: 285-287°C [lit.: 287°C (from acetone, 1971JC1875)]; found: C, 30.1; H, 0.6; N, 5.6%. $C_{12}H_3Br_3N_2O_4$ requires: C, 30.1; H, 0.6; N 5.85%); *m/z* 475.8 (29%), 477.7 (100%), 479.7 (95%), 481.7 (27%) $(M)^-$; ν_{max}/cm^{-1} 3078 (C-H), 1638 (C=O), 1581 (C=C), 1508 and 1328 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 7.82 (1H, d, *J* = 9.0 Hz, 6-H), 8.47 (1H, dd, *J* = 9.3, 2.7 Hz, 7-H), 8.60 (1H, d, *J* = 2.7 Hz, 9-H); **δ_C (75 MHz, d_6-DMSO)** 103.4 (quat., 4-C), 118.1 (CH, 6-C), 125.6 (CH, 9-C),

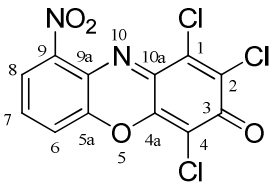
129.0 (CH, 7-C), 133.0 (quat., 9a-C), 134.8 (quat., 1-C or 2-C), 136.5 (quat., 1-C or 2-C), 145.3 (quat., 8-C), 146.45 (quat., 4a-C or 10a-C), 147.9 (quat., 4a-C or 10a-C), 148.2 (quat., 5a-C), 171.6 (quat., 3-C).

5.2.1.7 9-Nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one 130γa



The title compound was prepared from *p*-fluoranil **129c** (0.90g, 5.00mmol) and 2-amino-3-nitrophenol **124γ** (0.74g, 4.82mmol); 9-nitro-1,2,4-trifluoro-3*H*-phenoxazin-3-one **130γa** was isolated as a dark red solid (0.22g, 0.73mmol, 15.2%). Ruby red prisms of **130γa** with a metallic lustre were obtained from glacial acetic acid, **m.p.**: 216-218°C; (found: C, 48.5; H, 1.0; N, 9.2%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); *m/Z* 295.9 (M^-); ν_{max}/cm^{-1} 3084 (C-H), 1649 (C=O), 1603 (C=C), 1530 and 1313 (NO_2), 1004 (C-F); **δ_H (300 MHz, d_8-THF)** 6.04-6.13 (m, 3H, 6-H, 7-H and 8-H); **δ_C (75 MHz, d_8-THF)** 117.6 (CH, 8-C), 117.9 (CH, 6-C), 123.0 (quat., 9a-C), 130.0 (quat., ddd, *J* = 10.0, 5.0, 1.2Hz, 10a-C), 131.5 (CH, 7-C), 135.8 (quat., dd, *J* = 261.9, 6.3Hz, 4-C), 141.4 (quat., dt, *J* = 273.2, 6.8Hz, 2-C), 142.1 (quat., dd, *J* = 274.7, 12.2Hz, 1-C), 140.7 (quat., ddd, *J* = 18.2, 4.8, 1.2Hz, 4a-C), 141.5 (quat., d, *J* =1.7Hz, 5a-C), 146.4 (quat., 9-C), 166.7 (quat., td, *J* = 21.1, 5.5Hz, 3-C); **δ_F (282 MHz, d_8-THF)** -282.53 (1F, t, *J* = 2.3Hz, 4-F), -271.43 (1F, dd, *J* = 9.0, 2.3Hz, 1-F), -265.36 (1F, dd, *J* = 9.3, 2.3Hz, 2-F).

5.2.1.8 9-Nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one 130γb



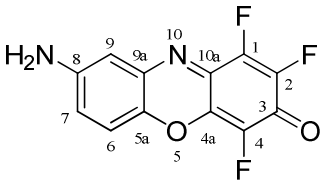
The title compound was prepared from *p*-chloranil **129c** (1.17g, 7.61mmol) and 2-amino-3-nitrophenol **124γ** (1.07g, 6.92mmol); 9-nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one **130γb** was isolated as a dark purple solid (1.08g, 3.13mmol, 45.3%). Lustrous dark purple needles of **130γb** were obtained from acetic acid, **m.p.**: 254-258°C; (found: C, 41.6; H, 0.9; N, 7.8%. $C_{12}H_3Cl_3N_2O_4$ requires C, 41.7; H, 0.9; N, 8.1%); *m/Z*

343.9 (100%), 345.8 (91%), 347.7 (56%), 348.9 (21%) $(M)^-$; ν_{max}/cm^{-1} 3100 (C-H), 1647 (C=O), 1590 (C=C), 1532 and 1331 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 7.97 (1H, t, *J* = 8.4Hz, 7-H), 8.02 (1H, dd, *J* = 8.4, 2.1Hz, 8-H), 8.09 (1H, dd, *J* = 7.5, 2.1Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 111.2 (quat., 4-C), 120.6 (CH, 8-C), 120.8 (CH, 6-C), 125.3 (quat., 9a-C), 134.6 (CH, 7-C), 136.9 (quat., 1-C or 2-C), 138.6 (quat., 1-C or 2-C), 143.7 (quat., 5a-C), 145.5 (quat., 4a-C or 10a-C), 146.3 (quat., 4a-C or 10a-C), 147.8 (quat., 9-C), 171.0 (quat., 3-C).

5.2.2 General procedure for the preparation of 8-amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-one 165a-c

Nitro-1,2,3-trihalogeno-3*H*-phenoxazin-3-ones **130α-γ a-c** were dissolved in a solvent mixture of EtOAc : MeOH (1:1) and either Pd/C 10% or PtO_2 (10% of the reactant mass) was added to the solution. The reaction mixture was then hydrogenated for 2 to 20 hours, the initial hydrogen pressure being set around 2.4bars. Hydrogenation was continued until the hydrogen pressure was steady, after which the catalyst was removed from the reaction mixture by filtration through Celite 451. The Celite cake was washed several times with methanol. MnO_2 (3mol equivalent) was added to the clear solution and the resulting mixture stirred until no more amino-3-hydroxyphenoxazine was observed by TLC. The reaction mixture changed to a deep colour, ranging from blue to blue-violet, and was again filtered through Celite 451 to remove inorganic insoluble materials and the cake was washed several times with THF, until the filtrate was clear of colour. The solvent mixture was evaporated *in vacuo* and the residue was subjected to column chromatography on silica.

5.2.2.1 8-Amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one 165a



The title compound was prepared from 8-nitro-1,2,3-trifluoro-3*H*-phenoxazin-3-one **130βa** (0.38g, 1.29mmol) using Pd/C 10%. Elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (30:70 to 10:90). 8-Amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **165a** was isolated as a dark violet solid (0.34g, 1.27mmol, 98.0 %); **m.p.** : 277°C (dec.); (found: C, 54.2; H, 2.1; N, 10.1%. $C_{12}H_5F_3N_2O_2$

requires C, 54.15; H, 1.9; N, 10.5%); *m/z* 264.9 $(M)^-$; ν_{max}/cm^{-1} 3503, 3358 and 3230 (NH_2), 1638 (C=O), 1610, 1575 and 1513 (C=C), 1002 (C-F); **δ_H (500 MHz, d_1-TFA)** 8.17 (1H, d, *J* = 9.0Hz, 6-H), 8.35 (1H, dd, *J* = 9.0, 2.0Hz, 7-H), 8.74 (1H, d, *J* = 1.5Hz, 9-H); **δ_C (125 MHz, d_1-TFA)** 119.1 (CH, 6-C), 125.7 (CH, 9-C), 127.1 (quat., 9a-C), 129.4 (CH, 7-C), 132.5 (quat., dd, *J* = 16.5, 3.9Hz, 10a-C), 132.8 (quat., 8-C), 138.2 (quat., dd, *J* = 263.5, 5.4Hz, 4-C), 141.6 (quat., dd, *J* = 18.0, 7.8Hz, 4a-C), 143.9 (quat., dt, *J* = 275.0, 5.4Hz, 2-C), 144.2 (quat., 5a-C), 144.4 (quat., dd, *J* = 279.2, 13.2Hz, 1-C), 172.3 (quat., td, *J* = 19.0, 6.6Hz, 3-C).

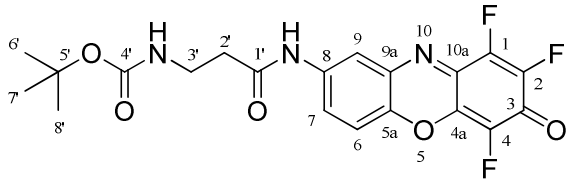
5.2.3 General procedure for the preparation of 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones 236g-i (adapted from 2008OBC682)

8-Nitro-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones **130βb-c** were dissolved in a solvent mixture of EtOAc / MeOH (1:1) and PtO_2 (10% of the reactant mass) was added to the solution. The reaction mixture was then hydrogenated for 2h, the initial hydrogen pressure being set around 2.4bars. Hydrogenation was continued until the hydrogen pressure was steady, after which the catalyst was removed from the reaction mixture by filtration through Celite 451. The Celite cake was washed several times with methanol. MnO_2 (3mol equivalent) was added to the clear solution and the resulting mixture stirred until no more 8-amino-1,2,4-trihalogeno-3-hydroxyphenoxazine **165a-c** was observed by TLC. The reaction mixture changed to a deep blue colour and was again filtered through Celite 451 to remove inorganic insoluble materials. The celite cake was washed several times with THF, until the filtrate was clear of colour. The solvent mixture was evaporated *in vacuo* and the crude product was used without further purification for peptide coupling.

In a 100mL two necked flame dried flask flushed with nitrogen, *N*-^{t}Boc-β-alanine (3.5mol equivalent) was dissolved in dry THF (15mL). The solution was cooled in an ice bath to 2-3°C (internal temperature) and *N*-methylpiperidine (3.7mol equivalent) was added slowly. The resulting solution was stirred for 10-15min before *iso*butylchloroformate (3.5mol equivalent) was added dropwise; the temperature rose to 5°C and a white solid precipitated. The resulting mixture was stirred at 3°C for 1h 30min to allow the mixed anhydride to form. A solution of the appropriate 8-amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-one **165a-c** was prepared in dry THF in a round bottomed flask flushed with nitrogen and then added *via* syringe to the mixed

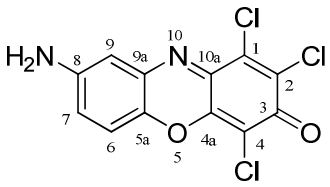
anhydride. The resulting mixture was stirred under nitrogen for 48-72h. A 10% aq. solution of Na_2CO_3 was added to quench the reaction and the resulting mixture was extracted with EtOAc (3 x 70mL). The combined organic layers were washed once with a 10% aq. solution of Na_2CO_3 (100mL), once with water (100mL), once with brine (100mL), and dried ($MgSO_4$). The solvent mixture was removed *in vacuo* and the residue was subjected to column chromatography using a gradient mixture of petroleum ether (60-80°C) : EtOAc.

5.2.3.1 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one 236g

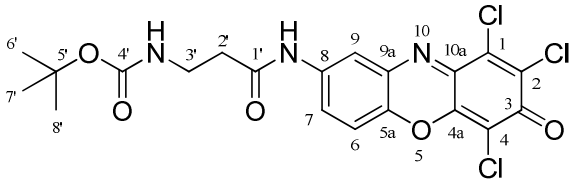


The title compound was prepared as described from 8-amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **165a** (0.30g, 1.13mmol). Elution used a gradient mixture of petroleum ether (60-80°) : EtOAc (25:75 to 0:100). 8-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **236g** was isolated as a cherry red solid (0.36g, 0.82mmol, 72.8%); **m.p.:** 195-196°C (dec.); (found: C, 54.75; H, 4.2; N, 9.4%. $C_{20}H_{18}F_3N_3O_5$ requires C, 54.9; H, 4.15; N, 9.6%); *m/Z* 436.0 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3362 and 3304 (N-H), 1680 (C=O), 1654 (C=O), 1632 (C=O), 1158 (C-O), 1001 (C-F); **δ_H (300 MHz, d_6-DMSO)** 1.40 (9H, s, C(C*H*$_3$)$_3$), 2.53 (2H, t, *J* = 7.2Hz, 2'-H), 3.27 (2H, q, *J* = 6.9Hz, 3'-H), 6.88 (1H, br. s, N*H*-^{t}Boc), 7.64 (1H, d, *J* = 9.3Hz, 6-H), 7.83 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 8.26 (1H, d, *J* = 2.1Hz, 9-H), 10.33 (1H, s, Ar-N*H*); **δ_C (75 MHz, d_6-DMSO)** 28.7 (3 × CH_3, 6'/7'/8'-C), 36.9 (CH_2, 3'-C), 37.3 (CH_2, 2'-C), 78.2 (quat., 5'-C), 117.1 (CH, 6-C), 119.1 (CH, 9-C), 125.95 (CH, 7-C), 132.4 (quat., 9a-C), 133.1 (quat., dd, *J* = 9.2, 5.1Hz, 10a-C), 137.65 (quat., dd, *J* = 253.7, 6.6Hz, 4-C), 137.95 (quat., 5a-C), 138.8 (quat., 8-C), 140.1 (quat., dd, *J* = 16.7, 3.3Hz, 4a-C), 143.1 (quat., dt, *J* = 267.1, 7.5Hz, 2-C), 144.0 (quat., dd, *J* = 270.0, 11.9Hz, 1-C), 156.1 (quat., 4'-C), 168.7 (quat., dt, *J* = 20.4, 6.6Hz, 3-C), 170.4 (quat., 1'-C); **δ_F (282 MHz, d_6-DMSO)** -163.18 (1F, s, 4-F), -149.40 (1F, d, *J* = 11.9Hz, 1-F), -145.69 (1F, dd, *J* = 11.9, 2.5Hz, 2-F).

5.2.3.2 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one 236h

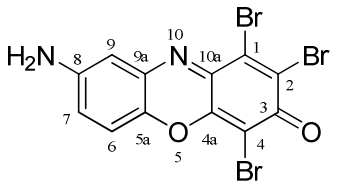


8-Amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **165b** was prepared as described earlier from 8-nitro-1,2,4-trichloro-3*H*-phenoxazin-3-one **130βb** (0.16g, 0.46mmol). 8-Amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **165b** (0.14g, 0.45mmol, 97.6%) was isolated as a dark blue solid, **m.p.:** >290°C; *m/Z* 312.8 (94%), 314.8 (100%), 316.8 (42%), 318.8 (28%) (*M*)$^-$; ν_{max}/cm^{-1} 3387, 3321 and 3226 (NH_2), 1634 (C=O), 1616, 1570 and 1502 (C=C). Due to the poor solubility of the product, no NMR data could be obtained.

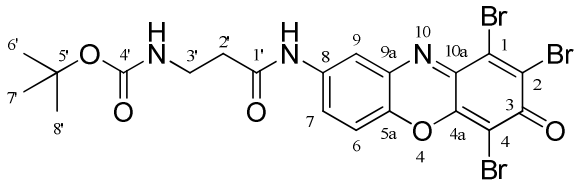


The title compound was prepared as described earlier from 8-amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **165b** (0.173g, 0.55mmol). Elution using a gradient mixture of petroleum ether (60-80°C) : EtOAc (30:70 to 0:100) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **236h** as a dark pinkish solid (0.075g, 0.155mmol, 28.2%); **m.p.:** 220°C (dec.); (found: C, 49.3; H, 3.7; N, 8.4%. $C_{20}H_{18}Cl_3N_3O_5$ requires C, 49.35; H, 3.7; N, 8.6%); *m/Z* 485.9 (73%), 487.9 (100%), 489.8 (24%), 491.9 (5%) (*M-H*)$^-$; ν_{max}/cm^{-1} 3359 and 3318 (N-H), 1681 (C=O), 1652 (C=O), 1628 (C=O), 1165 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.40 (9H, s, C(C*H*$_3$)$_3$), 2.54 (2H, t, *J* = 6.9Hz, 2'-H), 3.27 (2H, q, *J* = 6.6Hz, 3'-H), 6.89 (1H, br. s, N*H*-^{t}Boc), 7.64 (1H, d, *J* = 9.0Hz, 6-H), 7.83 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 8.32 (1H, d, *J* = 2.4Hz, 9-H), 10.32 (1H, s, Ar-N*H*); **δ_C (75 MHz, d_6-DMSO)** 28.7 (3 x CH_3, 6'/7'/8'-C), 36.9 (CH_2, 3'-C), 37.4 (CH_2, 2'-C), 78.2 (quat., 5'-C), 110.2 (quat., 4-C), 116.9 (CH, 6-C), 119.1 (CH, 9-C), 126.3 (CH, 7-C), 132.9 (quat., 9a-C), 136.6 (quat., 1-C or 2-C), 137.4 (quat., 1-C or 2-C), 138.1 (quat., 8-C), 139.3 (quat., 5a-C), 143.3 (quat., 4a-C or 10a-C), 146.4 (quat., 4a-C or 10a-C), 156.1 (quat., 4'-C), 170.4 (quat., 1'-C), 170.6 (quat., 3-C).

5.2.3.3 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3*H*-phenoxazin-3-one 236i



8-Amino-1,2,4-tribromo-3*H*-phenoxazin-3-one **165c** was prepared as described from 8-nitro-1,2,4-tribromo-3*H*-phenoxazin-3-one **130βc** (0.43g, 0.89mmol). 8-Amino-1,2,4-tribromo-3*H*-phenoxazin-3-one **165c** (0.38g, 0.85mmol, 94.8%) was isolated as a dark blue solid, **m.p.**: > 290°C; *m/z* 447 (37%), 449 (100%), 451 (91%), 453 (29%) $(MH)^+$; ν_{max}/cm^{-1} 3376, 3319 and 3216 (NH_2), 1628 (C=O). Due to the poor solubility of the product, no NMR data could be obtained.

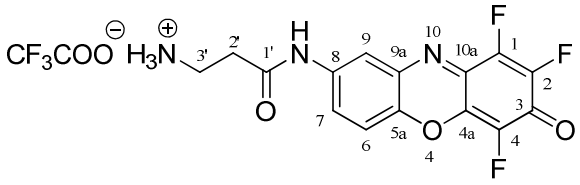


The title compound was prepared as described from 8-amino-1,2,4-tribromo-3*H*-phenoxazin-3-one **165c** (0.28g, 0.62mmol). Elution using a gradient mixture of petroleum ether (60-80°C) : EtOAc (50:50 to 0:100), then EtOAc : MeOH (95:5), yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3*H*-phenoxazin-3-one **236i** as a dark pinkish solid (0.26g, 0.42mmol, 67.3%), **m.p.:** 245°C (dec.); (found: C, 38.8; H, 3.0; N, 6.5%. $C_{20}H_{18}Br_3N_3O_5$ requires C, 38.7; H, 2.9; N, 6.8%); *m/z* 615.8 (25%), 617.8 (100%), 619.8 (92%), 621.8 (33%) $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3357 (N-H), 1685 (C=O), 1678 (C=O), 1613 (C=O), 1170 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.40 (9H, s, C(C*H*$_3$)$_3$), 2.54 (2H, t, *J* = 6.9Hz, 2'-H), 3.27 (2H, q, *J* = 6.6Hz, 3'-H), 6.89 (1H, br. s, N*H*-^{t}Boc), 7.61 (1H, d, *J* = 8.7Hz, 6-H), 7.82 (1H, dd, *J* = 8.7, 2.1Hz, 7-H), 8.33 (1H, d, *J* = 2.1Hz, 9-H), 10.30 (1H, s, Ar-N*H*); **δ_C (75 MHz, d_6-DMSO)** 28.7 (3 × CH_3, 6'/7'/8'-C), 36.9 (CH_2, 3'-C), 37.4 (CH_2, 2'-C), 78.2 (quat., 5'-C), 101.5 (quat., 4-C), 116.8 (CH, 6-C), 119.0 (CH, 9-C), 126.2 (CH, 7-C), 133.4 (quat., 9a-C), 134.2 (quat., 1-C or 2-C), 135.7 (quat., 1-C or 2-C), 138.0 (quat., 8-C), 139.5 (quat., 5a-C), 144.2 (quat., 4a-C or 10a-C), 148.3 (quat., 4a-C or 10a-C), 156.1 (quat., 4'-C), 170.4 (quat., 1'-C), 171.0 (quat., 3-C).

5.2.4 General procedure for the deprotection of 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-halogeno-3*H*-phenoxazin-3-one 237g-i (adapted from 2008OBC682)

To the protected substrate in a round bottomed flask, an excess of neat TFA (3-4mL) was added and the resulting dark solution was swirled for a minute and left standing for another 5 min. The TFA was then removed *in vacuo*. The residue was taken into methanol and the solvent from the resulting mixture was removed *in vacuo*. This procedure was repeated until most of the remaining TFA was removed. The residue was then redissolved in a minute amount of MeOH and the resulting solution was diluted with Et_2O, which produced a lasting turbidity. The cloudy suspension was left overnight and the microcrystals formed were collected by filtration and washed with Et_2O. The filtrate was concentrated *in vacuo* to recover more product, which was triturated and washed with Et_2O.

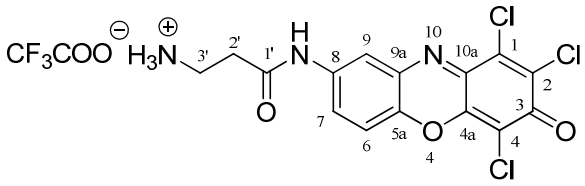
5.2.4.1 8-*N*-(β-Alanyl)amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one TFA salt 237g



The title compound was prepared as described earlier from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **236g** (0.152g, 0.348mmol). 8-*N*-(β-Alanyl)amino-1,2,4-trifluoro-3H-phenoxazin-3-one TFA salt **237g** was isolated as a red solid (0.154g, 0.341mmol, 98.0%); **m.p.**: 220℃ (dec.); (Found $(M)^+$, 338.0749. Calc. for $C_{15}H_{11}F_3N_3O_3$: $(M)^+$, 338.0747); *m/z* 338.1 $(MH)^+$, 350.1 $(MNa)^+$; ν_{max}/cm^{-1} 3255 (N-H), 3032 (N^+-H), 1663 (C=O), 1642 (C=O), 1131 (C-O), 1007 (C-F); **δ_H (500 MHz, d_6-DMSO)** 2.79 (2H, t, *J* = 7.0Hz, 2'-H), 3.15 (2H, br. s, 3'-H), 7.72 (1H, d, *J* = 9.0Hz, 6-H), 7.87 (1H, dd, *J* = 8.4, 2.0Hz, 7-H), 7.91 (3H, br. s, NH_3^+), 8.32 (1H, d, *J* = 2.5Hz, 9-H), 10.68 (1H, s, N*H*-Ar); **δ_C (125 MHz, d_6-DMSO)** 33.8 (CH_2, 2'-C), 35.3 (CH_2, 3'-C), 117.3 (CH, 6-C), 119.4 (CH, 7-C), 126.05 (CH, 9-C), 132.5 (quat., 9a-C), 133.25 (quat., dd, *J* = 9.4, 5.6Hz, 4a-C), 137.65 (quat., dd, *J* = 253.6, 6.3Hz, 4-C), 137.6 (quat., 8-C), 139.1 (quat., 5a-C), 140.35 (quat., d, *J* = 16.9Hz, 10a-C), 143.1 (quat., dt, *J* = 267.3, 11.6Hz, 2-C), 144.1 (quat., dd, *J* = 267.3, 11.6Hz, 1-C), 168.8 (quat., td, *J* = 20.0, 5.7Hz, 3-C), 169.3 (quat., 1'-C); **δ_F (282 MHz, d_6-DMSO)**

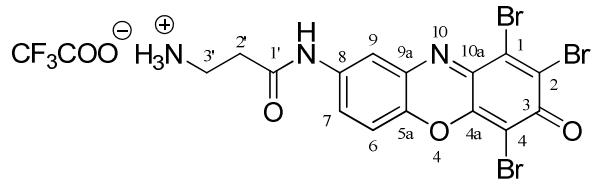
-163.31 (1F, d, *J* = 2.8 Hz, 4-F), -149.43 (1F, dd, *J* = 12.1 Hz, 2.0 Hz , 1-F), -145.65 (1F, dd, *J* = 12.1 Hz, 3.4 Hz, 2-F).

5.2.4.2 8-*N*-(β-Alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one TFA salt 237h



The title compound was prepared as described earlier from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **236h** (0.07g, 0.14mmol). 8-*N*-(β-alanyl)amino-1,2,4-trichloro-3*H*-phenoxazin-3-one TFA salt **237h** was obtained as a purple solid (0.06g, 0.115mmol, 82.1%); **m.p.**: > 290°C; (Found (*M*)$^{+}$, 385.98635. Calc. for $C_{15}H_{11}{}^{35}Cl_3N_3O_3$: (*M*)$^{+}$, 385.98605); *m/z* 386.0 (100%), 388.0 (97%), 390.0 (42%), 392.0 (10%) (*MH*)$^{+}$; ν_{max}/cm^{-1} 3348 (N-H), 3073 (N-H^{+}), 1671 (C=O), 1619 (C=O), 1124 (C-O); **δ_H (300 MHz, d_6-DMSO)** 2.69 (2H, t, *J* = 6.9Hz, 2'-H), 3.06 (2H, br. s, 3'-H), 7.64 (1H, d, *J* = 9.0Hz, 6-H), 7.72 (3H, br. s, N*H*$_3^{+}$), 7.77 (1H, dd, *J* = 9.0, 2.1Hz, 7-H), 8.28 (1H, d, *J* = 2.1Hz, 9-H), 10.51 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 33.9 (CH_2, 2'-C), 35.35 (CH_2, 3'-C), 110.2 (quat., 4-C), 117.0 (CH, 6-C), 119.3 (CH, 9-C), 126.35 (CH, 7-C), 132.9 (quat., 9a-C), 136.6 (quat., 1-C or 2-C), 137.4 (quat., 1-C or 2-C), 137.8 (quat., 8-C), 139.5 (quat., 5a-C), 143.4 (quat., 4a-C), 146.4 (quat., 10a-C), 169.3 (quat., 1'-C), 170.6 (quat., 3-C).

5.2.4.3 8-*N*-(β-Alanyl)amino-1,2,4-tribromo-3*H*-phenoxazin-3-one TFA salt 237i



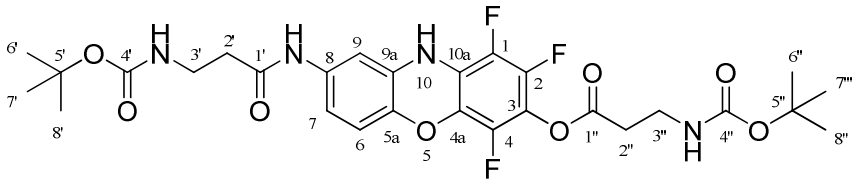
The title compound was prepared as described earlier from 8-*N*-(*N*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3*H*-phenoxazin-3-one **236i** (0.18g, 0.29mmol). 8-*N*-(β-Alanyl)amino-1,2,4-tribromo-3H-phenoxazin-3-one TFA salt **237i** was obtained as a purple solid (0.17g, 0.27mmol, 93.2%); **m.p.**: >290°C (dec.); (Found (*M*)$^{+}$, 517.8351. Calc. for $C_{15}H_{11}{}^{79}Br_3N_3O_3$: (*M*)$^{+}$, 517.8345); *m/z* 517.8 (35%), 519.8 (100%), 521.8 (89%), 523.8 (35%) (*MH*)$^{+}$; ν_{max}/cm^{-1} 3344 (N-H), 3060 (N^{+}-H), 1663 (C=O), 1609 (C=O), 1140 (C-O); **δ_H (300 MHz, d_6-DMSO)** 2.77 (2H, t, *J* = 6.3Hz, 2'-H), 3.13 (2H,

t, *J* = 6.0Hz, 3'-H), 7.65 (1H, d, *J* = 9.0Hz, 6-H), 7.84 (1H, dd, *J* = 9.0, 3.0Hz, 7-H), 7.96 (3H, br. s, NH_3^+), 8.36 (1H, d, *J* = 3.0Hz, 9-H), 10.69 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 33.8 (CH_2, 2'-C), 35.4 (CH_2, 3'-C), 101.5 (quat., 4-C), 117.0 (CH, 6-C), 119.3 (CH, 9-C), 126.3 (CH, 7-C), 133.4 (quat., 1-C or 2-C), 134.3 (quat., 9a-C), 135.7 (quat., 1-C or 2-C), 137.7 (quat., 5a-C), 139.7 (quat., 8-C), 144.4 (quat., 4a-C), 148.4 (quat., 10a-C), 169.3 (quat., 1'-C), 171.1 (quat., 3-C).

5.2.5 General procedure for the preparation of 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-*10H*-phenoxazin-3-yl 3''-(*N*-^{t}butoxycarbonyl-amino)propanoate 239a-b (adapted from 2008OBC682)

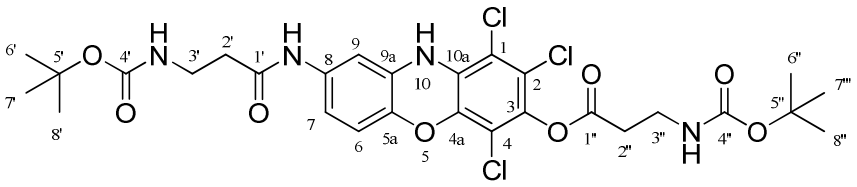
8-Amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-ones **165a-b** (1mol eq.) were dissolved in DMF (10mL) in a 20mL round bottomed flask. Pd/C 5% was added to the solution and the resultant mixture was hydrogenated for 2h. In the meantime, in a flame-dried two necked flask flushed with nitrogen, *N*-^{t}Boc-β-alanine (4mol eq.) was dissolved in dry THF (10mL) and the resulting solution was cooled in an ice-bath. *N*-Methylpiperidine (4mol eq.) was then added dropwise and the resulting solution was stirred for 5 min, before IBCF (4mol eq.) was added dropwise. A white precipitate appeared upon addition of IBCF and the resulting mixture was stirred for a further 1h at 0℃. The DMF solution of 8-amino-3-hydroxy-1,2,4-trihalogeno-10*H*-phenoxazines **164a-b** was protected by a N_2 atmosphere to avoid reoxidation and was transferred *via canula* to the THF mixture, now containing a mixed anhydride. The resulting mixture was left stirring at 0℃ for another hour and under a continuous N_2 flow at room temperature for 72h. The reacting mixture was diluted with MeOH and filtered through celite to remove the catalyst. The cake was washed several times with MeOH. The filtrate was evaporated *in vacuo* and the residue was diluted with a 5% aqueous solution of LiCl (100mL). The resulting mixture was extracted with EtOAc (3 × 70 mL). The combined organic layers were successively washed with a 5% aqueous solution of LiCl (100mL), a 10% aqueous Na_2CO_3 solution (100mL), a 10% aqueous citric acid solution (100mL), water (100mL), brine (100mL) and then dried ($MgSO_4$). The solvent was evaporated *in vacuo* and the residue subjected to column chromatography on silica gel.

5.2.5.1 8-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-*10H*-phenoxazin-3-yl-3''-(*N*-^{t}butoxycarbonylamino)propanoate 239a



The title compound was prepared as described earlier from 8-amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **165a** (0.285g, 1.07mmol), *N*-^{t}Boc-β-alanine (1.22g, 6.43mmol), IBCF (0.83mL, 6.43mmol) and 1-methylpiperidine (0.85mL, 6.99mmol). Elution with petroleum ether (60-80°) : EtOAc (40:60) gave **239a** as an off-white solid (0.17g, 0.28mmol, 27.1%), **m.p.**: 168-170°C; (found: C, 55.1; H, 5.4 ; N, 8.9%. $C_{28}H_{33}F_3N_4O_8$ requires C, 55.1; H, 5.45; N, 9.2%); *m/z* 609.2 $(M\text{-}H)^-$; *m/z* 611.2 $(MH)^+$, 633.3 $(MNa)^+$; ν_{max}/cm^{-1} 3399, 3321 and 3367 (N-H), 2978 (C-H), 1777 (C=O), 1679 (C=O), 1657 (C=O), 1170 (C-O), 1119 (C-O), 994 (C-F); **δ_H (300 MHz, $(CD_3)_2CO$)** 1.27 (9H, s, $(CH_3)_3$), 1.29 (9H, s, $(CH_3)_3$), 2.40 (2H, t, *J* = 6.6Hz, 2'-H), 2.79 (2H, t, *J* = 6.6Hz, 2''-H), 3.24 (2H, q, *J* = 6.6Hz, 3'-H), 3.34 (2H, q, *J* = 6.6Hz, 3''-H), 5.86 (1H, br. s, 3''a-N*H* or 3'a-N*H*), 5.99 (1H, br. s, 3''a-N*H* or 3'a-N*H*), 6.53 (1H, d, *J* = 8.7Hz, 6-H), 6.71 (1H, dd, *J* = 8.7, 2.4Hz, 7-H), 7.21 (1H, d, *J* = 2.1Hz, 9-H), 7.71 (1H, s, 10-N*H*), 8.95 (1H, s, N*H*-Ar); **δ_C (75 MHz, $(CD_3)_2CO$)** 27.7 (3 × CH_3, 6'/7'/8'-C or 6''/7''/8''-C), 27.8 (3 × CH_3, 6'/7'/8'-C or 6''/7''/8''-C), 33.4 (CH_2, 2''-C), 36.2 (CH_2, 3''-C), 36.7 (CH_2, 3'-C), 37.0 (CH_2, 2'-C), 77.9 (quat., 5'-C or 5''-C), 78.1 (quat., 5'-C or 5''-C), 106. 4 (CH, 9-C), 112.6 (CH, 7-C), 115.6 (CH, 6-C), 120.4 (quat., td, *J* = 14.6, 3.1Hz, 3-C), 121.3 (quat., dt, *J* = 14.3, 3.1Hz, 10a-C), 128.7 (quat., dt, *J* = 13.1, 3.8Hz, 4a-C), 129.4 (quat., 9a-C), 134.8 (quat., ddd, *J* = 239.0, 14.9, 3.8Hz, 1-C), 136.4 (quat., 8-C), 137.4 (quat., 5a-C), 139.9 (quat., dt, *J* = 244.1, 3.9Hz, 2-C), 140.1 (quat., ddd, *J* = 243.5, 13.1, 4.8Hz, 4-C), 155.7 (quat., 4''-C), 155.8 (quat., 4'-C), 168.5 (quat., 1''-C), 169.5 (quat., 1'-C); **δ_F (282 MHz, $(CD_3)_2CO$)** -166.27 (1F, dd, *J* = 21.5, 7.3Hz, 1-F), -160.16 (1F, dd, *J* = 21.5, 4.0Hz, 2-F), -156.56 (1F, br s, 4-F).

5.2.5.2 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-*10H*-phenoxazin-3-yl 3''-(*N''*-^{t}butoxycarbonylamino)propanoate 239b



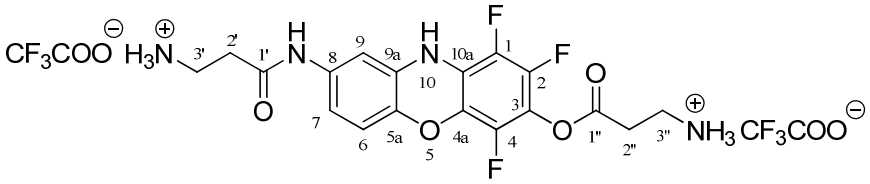
The title compound was prepared as described earlier from 8-amino-1,2,4-trichloro-3*H*-phenoxazin-3-one **165b** (0.40g, 1.28mmol), *N*-^{t}Boc-β-alanine (0.97g, 5.12mmol), IBCF (0.66mL, 5.12mmol) and 1-methylpiperidine (0.62mL, 5.12mmol). Elution with petroleum ether (60-80°C) : EtOAc (40:60) gave **239b** as a white solid (0.40g, 0.61mmol, 47.8%); **m.p.** (from EtOAc : hexane): 150-151°C; (found: C, 50.9; H, 5.1; N, 8.7%. $C_{28}H_{33}Cl_3N_4O_8$ requires C, 51.0; H, 5.0; N, 8.5%); *m/z* 657.0 (82%), 659.0 (100%), 661.1 (35%), 663 (12%); ν_{max}/cm^{-1} 3374 (N-H), 3319 (N-H), 1758 (C=O), 1686 (C=O), 1672 (C=O), 1166 (C-O), 1129 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.32 (18H, s, 2 × (C*H*$_3$)$_3$), 2.37 (2H, t, *J* = 6.9Hz, 2''-H), 2.76 (2H, t, *J* = 6.9Hz, 2'-H), 3.14 (2H, q, *J* = 6.9Hz, 3''-H), 3.22 (2H, q, *J* = 6.9Hz, 3'-H), 6.59 (1H, d, *J* = 8.7Hz, 6-H), 6.73 (2H, br. dd, *J* = 8.7, 2.1Hz, 7-H and 3a'-N*H* or 3a''-N*H*), 6.87 (1H, br s, 3a'-N*H* or 3a''-N*H*), 7.32 (1H, d, *J* = 2.1Hz, 9-H), 8.43 (1H, s, 10-N*H*), 9.70 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 28.7 (6 × CH_3, 6'/7'/8'-C and 6''/7''/8''-C), 33.8 (CH_2, 3''-C), 36.45 (CH_2, 2''-C), 37.05 (CH_2, 2'-C or 3'-C), 37.2 (CH_2, 2'-C or 3'-C), 78.1 (quat., 5'-C or 5''-C), 78.3 (quat., 5'-C or 5''-C), 107.1 (CH, 9-C), 113.0 (CH, 7-C), 113.85 (quat., 1-C or 2-C), 113.95 (quat., 1-C or 2-C), 115.6 (CH, 6-C), 120.7 (quat., 4-C), 130.0 (quat., 9a-C), 130.5 (quat., 10a-C), 136.6 (quat., 8-C), 136.9 (quat., 3-C), 137.4 (quat., 5a-C), 139.6 (quat., 4a-C), 155.9 (quat., 4'-C or 4''-C), 156.0 (quat., 4'-C or 4''-C), 168.8 (quat., 1'-C), 169.6 (quat., 1''-C).

5.2.6 General procedure for the deprotection of 8-*N*-(*N*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-*10H*-phenoxazin-3-yl-3''-(*N*-^{t}butoxycarbonyl-amino)propanoate 239a-b

The protected substrates **239a-b** were suspended in DCM (15mL) and TFA (10 mol eq.) was added dropwise to the mixture. Upon addition of TFA, the solid fully dissolved and this was accompanied by a slight change of colour, from colourless to faint brown. The resulting solution was left with stirring for 24-48h while cloudiness started to appear. Once completion of the reaction was indicated by TLC, the DCM

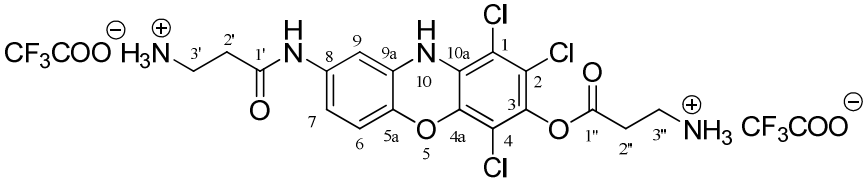
was removed under reduced pressure and the resulting solid triturated several times with dry Et_2O.

5.2.6.1 3-*O*-(8-*N*-(β-Alanyl)amino-1,2,4-trifluoro-*10H*-phenoxazin-3-yloxy)-1''-oxopropane-3''-aminium ditrifluoroacetate salt 240a



The title compound was prepared as described earlier from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-*10H*-phenoxazin-3-yl-3''-(*N''*-^{t}butoxycarbonylamino)propanoate **239a** (0.10g, 0.17mmol). The product **240a** was isolated as an off-white solid (0.075g, 0.12mmol, 70.0%); **m.p.:** 120-125°C; (Found $(M\text{-}C_3H_6NO)^+$, 340.0906. Calc. for $C_{15}H_{13}F_3N_3O_3$: $(M\text{-}C_3H_6NO)^+$, 340.09035); *m/z* 338.1 $(M\text{-}C_3H_8NO)^+$, 340.0 $(M\text{-}C_3H_6NO)^+$; ν_{max}/cm^{-1} 3290 (N-H), 3226 (N-H), 3026 (N^+-H), 1769 (C=O), 1664 (C=O), 1201 and 1180 (C-O), 1126 (C-O), 1001 (C-F); **δ_H (500 MHz, $(CD_3)_2CO$)** 2.98 (2H, t, *J* = 6.5Hz, 3' or 3''-H), 3.47 (2H, t, *J* = 6.5Hz, 3' or 3''-H), 4.10 (2H, t, *J* = 6.5Hz, 2' or 2''-H), 4.19 (2H, t, *J* = 6.5Hz, 2' or 2''-H), 6.69 (1H, d, *J* = 8.5Hz, 6-H), 6.95 (1H, dd, *J* = 8.5, 2.5Hz, 7-H), 7.28 (1H, d, *J* = 2.5Hz, 9-H); **δ_F (282 MHz, $(CD_3)_2CO$)** -165.93 (1F, dd, *J* = 21.4, 7.3Hz, 1-F), -159.70 (1F, dd, *J* = 21.5, 4.2Hz, 2-F), -156.31 (1F, dd, *J* = 4.8, 2.8Hz, 4-F).

5.2.6.2 3-*O*-(8-*N*-(β-Alanyl)amino-1,2,4-trichloro-*10H*-phenoxazin-3-yloxy)-1''-oxopropane-3''-aminium ditrifluoroacetate salt 240b



The title compound was prepared as described earlier from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-*10H*-phenoxazin-3-yl-3''-(*N''*-^{t}butoxycarbonylamino)-propanoate **239b** (0.10g, 0.155mmol). 3-*O*-(8-*N*-(β-alanyl)amino-1,2,4-trichloro-*10H*-phenoxazin-3-yloxy)-1''-oxopropane-3''-aminium ditrifluoroacetate salt **240b** was isolated as an off-white solid (0.09g, 0.125mmol, 80.8%); **m.p.:** 150-153°C; (Found

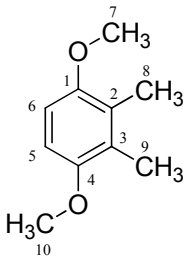
(M-H)$^+$, 459.0395. Calc. for $C_{18}H_{18}{}^{35}Cl_3N_4O_4$: ($M$-$H$)$^+$, 459.0388); $m/_Z$ 230.1 (90%), 231.1 (100%), 232.1 (38%), (M)$^{2+}$; 388.1 (100%), 390.1 (94%), 392.1 (28%), 394.1 (3%), (M-$COC_2H_4NH_2$)$^+$; 459.1 (93%), 461.1 (100%), 463.1 (29%), 465.1 (4%), (M-H)$^+$; ν_{max}/cm^{-1} 3378 (N-H), 3041 (N$^+$-H), 1759 (C=O), 1670 (C=O), 1198 and 1180 (C-O), 1128 (C-O); **δ_H (300 MHz, d_6-DMSO)** 2.69 (2H, t, J = 6.6Hz, 2'-H), 3.07-3.12 (4H, m, 3'-H and 2''-H), 3.18 (2H, q, J = 6.3Hz, 3''-H), 6.71 (1H, d, J = 8.7Hz, 6-H), 6.89 (1H, dd, J = 8.7, 2.1Hz, 7-H), 7.36 (1H, d, J = 2.1Hz, 9-H), 7.92 (3H, br. s, NH_3^+), 8.10 (3H, br. s, NH_3^+), 8.59 (1H, s, 10-NH), 10.08 (1H, s, NH-Ar); **δ_C (75 MHz, d_6-DMSO)** 31.3 (CH_2, 3'-C), 33.7 (CH_2, 2'-C), 34.8 (CH_2, 3''-C), 35.5 (CH_2, 2''-C), 107.2 (CH, 9-C), 113.2 (CH, 7-C), 113.7 (quat., 1-C or 2-C), 114.0 (quat., 1-C or 2-C), 115.7 (CH, 6-C), 120.7 (quat., 4-C), 130.0 (quat., 9a-C), 130.7 (quat., 10a-C), 136.4 (quat., 8-C), 136.6 (quat., 3-C), 137.55 (quat., 5a-C), 139.6 (quat., 4a-C), 168.1 (quat., 1''-C), 168.5 (quat., 1'-C).

5.2.7 General procedure for the preparation of dimethoxybenzenes 172a-c

(2008OBC682)

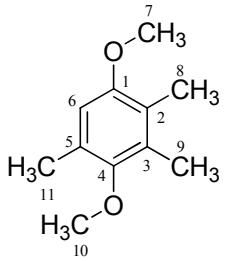
In a 3 necked round-bottomed flask equipped with a reflux condenser, a calcium chloride drying tube, a pressure equalizing dropping funnel (all the glassware was dried prior to use) and a thermometer, the substituted hydroquinone **173a-c** (1mol equivalent) was dissolved in DMF (50mL to 180mL depending on the batch size) and NaH in an oil dispersion 60% w/w (2.2mol equivalent) was added portion-wise to the hydroquinone solution. Once the evolution of hydrogen had stopped, methyl iodide was introduced in the pressure equalizing dropping funnel and added dropwise to the phenolate solution. The rate of addition was adjusted to maintain the temperature below 40°C. Once the addition was finished, the solution was heated at 50°C for 3 hours. After cooling, brine (200 to 400mL) was added to the reaction mixture and then extracted with diethyl ether (3 x 70mL to 3 x 200mL). The combined organic layers were washed once with water (100mL to 300mL), once with a 5% aqueous solution of LiCl (100mL to 300mL) and once with brine (100mL to 300mL). It was then dried with $MgSO_4$, the solvent was removed *in vacuo* and the residue was subjected to column chromatography on silica.

5.2.7.1 1,4-Dimethoxy-2,3-dimethylbenzene 172a (2004T9131)



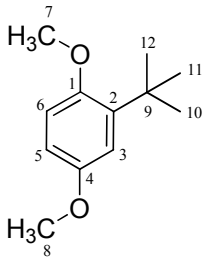
The title compound was prepared as described from 2,3-dimethylhydroquinone **173a** (5.57g, 40.31mmol); elution used petroleum ether (60-80°C) : Et_2O (95:5). 1,4-Dimethoxy-2,3-dimethylbenzene **172a** was isolated as a white solid (6.11g, 36.78mmol, 91.2%). White plates were obtained from petroleum ether (60-80°C); **m.p.**: 78-79°C [lit.: 78°C (from MeOH, 2004T9131)]; (found: C, 72.3; H, 8.6%. $C_{10}H_{14}O_2$ requires C, 72.3; H, 8.5%); *m/Z* 166.0 $(M)^+$, 179.1 $(MNa)^+$; ν_{max}/cm^{-1} 2956 (C-H), 1462 (C=C), 1257 (C-O), 1094 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.25 (6H, s, 2 x C*H*$_3$, 8-H and 9-H), 3.85 (6H, s, 2 x OC*H*$_3$, 7-H and 10-H), 6.74 (2H, s, 5-H and 6-H); **δ_C (75 MHz, $CDCl_3$)** 12.1 (CH_3, 8-C and 9-C), 56.1 (CH_3, 7-C and 10-C), 107.9 (CH, 5-C and 6-C), 126.8 (quat., 2-C and 3-C), 152.0 (quat., 1-C and 4-C).

5.2.7.2 1,4-Dimethoxy-2,3,5-trimethylbenzene 172b (1962JOC841)



The title compound was prepared as described from 2,3,4-trimethylhydroquinone **173b** (9.06g, 59.53mmol); elution used petroleum ether (60-80°C) : Et_2O (95:5). 1,4-Dimethoxy-2,3,5-trimethylbenzene **172b** was isolated as a white solid (9.05g, 50.20mmol, 84.4%); **m.p.** (petroleum ether 60-80°): 34-35.5°C [lit.: 35-36°C (from MeOH, 1962JOC841)]; ν_{max}/cm^{-1} 2990 (C-H), 1485 (C=C), 1229 (C-O), 1092 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.19 (3H, s, 8-H), 2.24 (3H, s, 9-H), 2.35 (3H, s, 11-H), 3.72 (3H, s, OC*H*$_3$, 7-H or 10-H), 3.85 (3H, s, OC*H*$_3$, 7-H or 10-H), 6.60 (1H, s, 6-H); **δ_C (75 MHz, $CDCl_3$)** 12.5 (CH_3, 8-C), 13.1 (CH_3, 9-C), 16.75 (CH_3, 11-C), 56.3 (CH_3, 7-C), 60.55 (CH_3, 10-C), 110.9 (CH, 6-C), 124.4 (quat., 5-C), 128.2 (quat., 2-C or 3-C), 130.1 (quat., 2-C or 3-C), 151.2 (quat., 1-C or 4-C), 154.1 (quat., 1-C or 4-C).

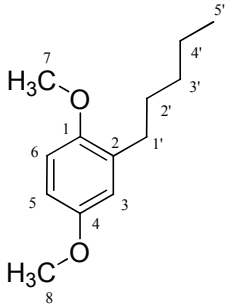
5.2.7.3 1,4-Dimethoxy-2-^{t}butylbenzene 172c (2000MI924)



The title compound was prepared as described from ^{t}butylhydroquinone **173c** (9.99g, 60.10mmol); elution used petroleum ether (60-80°C) : Et_2O (98:2). 1,4-Dimethoxy-2-^{t}butylbenzene **172c** was isolated as a pale yellow oil (9.17g, 47.19mol, 78.5%); **b.p.** (0.4mbar): 58-62°C [lit.: 240°C (at 66.7mbar, 2000MI924)]; ν_{max}/cm^{-1} 2951 (C-H), 1583 (C=C), 1217 (C-O); **δ_H (300 MHz, $CDCl_3$)** 1.28 (9H, s, C(C*H*$_3$)$_3$), 3.675 (3H, s, O*CH*$_3$, 7-H or 12-H), 3.70 (3H, s, O*CH*$_3$, 7-H or 12-H), 6.60 (1H, dd, *J* = 8.7, 3.0Hz, 5-H), 6.71 (1H, d, *J* = 8.7Hz, 6-H), 6.80 (1H, d, *J* = 3.0Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 29.7 (3 × CH_3, 10/11/12-C), 35.0 (quat., 9-C), 55.7 (2 × CH_3, 7-C and 8-C), 109.9 (CH, 5-C), 112.4 (CH, 6-C), 114.3 (CH, 3-C), 139.95 (quat., 2-C), 153.0 (quat.,1-C or 4-C), 153.35 (quat., 1-C or 4-C).

5.2.8 Procedure for the lithiation of 2-bromo-1,4-dimethoxybenzene 178 (adapted from 1997TA913)

5.2.8.1 1,4-Dimethoxy-2-pentylbenzene 196 (1972CPB1968)



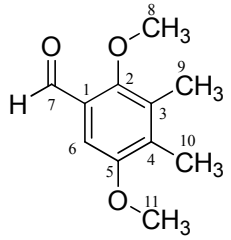
A 250mL flame dried round bottomed flask, flushed with N_2, was charged with 1-bromo-2,5-dimethoxybenzene **178** (4.0mL, 5.78g, 26.63mmol), TMEDA (8.03mL, 6.18g, 53.20mmol) and dry THF (150mL). The resulting solution was cooled to -75°C using a dry ice-acetone bath, followed by a dropwise addition of a 2.5M hexane solution of *n*-BuLi (24mL, 60.00mmol). The lithium-halogen exchange was allowed to take place during 1h at -75°C and after this period of time, neat 1-bromopentane (18.65mL, 150.0mmol) was added dropwise. The resulting solution was left with

stirring at -75°C under N_2 flow during 1h, allowed to warm-up to room temperature, and then refluxed for a period of 15h. The formation of a white precipitate was noted after the reflux. The resulting mixture was allowed to cool to room temperature, quenched with water (200mL) and extracted with Et_2O (2 × 200mL). The combined organic layers were successively washed with water (200mL) and brine (200mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et_2O (95:5). 1,4-Dimethoxy-2-pentylbenzene **196** was isolated as a pale yellow oil (4.75g, 22.80mmol, 85.7%), **b.p.**: 81-82°C at 0.2mbar [lit.: 114°C (at 6.7mbar, 1972CPB1968)]; $m/_Z$ 209.0 $(MH)^+$; (found: C, 74.65; H, 9.5%. $C_{13}H_{20}O_2$ requires C, 75.0; H, 9.7%); ν_{max}/cm^{-1} 2832-2928 (C-H), 1498 (C=C), 1219 (C-O), 1048 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.81 (3H, t, *J* = 6.9Hz, 5'-H), 1.24-1.28 (4H, m, 3'-H and 4'-H), 1.45-1.52 (2H, m, 2'-H), 2.49 (2H, t, *J* = 7.5Hz, 1'-H), 3.67 (3H, s, OCH_3, 7-H or 8-H), 3.68 (3H, s, OCH_3, 7-H or 8-H), 6.59 (1H, dd, *J* = 8.7, 3.0Hz, 5-H), 6.65 (1H, d, *J* = 3.0Hz, 3-H), 6.67 (1H, d, *J* = 8.7Hz, 6-H); **δ_C (75 MHz, $CDCl_3$)** 14.1 (CH_3, 5'-C), 22.6 (CH_2, 3'-C or 4'-C), 29.6 (CH_2, 2'-C), 30.3 (CH_2, 1'-C), 31.8 (CH_2, 3'-C or 4'-C), 55.7 (CH_3, 8'-C), 56.0 (CH_3, 7'-C), 110.6 (CH, 5-C), 111.3 (CH, 6-C), 116.3 (CH, 3-C), 132.8 (quat., 2-C), 151.9 (quat., 1-C), 153.55 (quat., 4-C).

5.2.9 General procedure for the formylation of substituted dimethoxybenzene **172a-c**, **196 and 169g** *via* **the Duff reaction** (1972JOC3972)

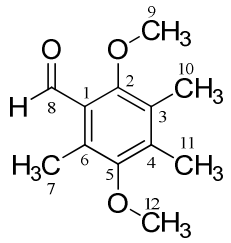
In a 250mL round bottomed flask equipped with a reflux condenser, the appropriately substituted dimethoxybenzene (1mol equivalent) was dissolved in TFA (50 to 120mL) and hexamethylene tetramine (2mol equivalent) was added to the resulting solution which was gently refluxed (temperature oil bath: 95°C) overnight (15 to 20 hours). The solution was allowed to cool to room temperature; most of the TFA was removed *in vacuo* and the residue was neutralised by the portion-wise addition of solid Na_2CO_3. When no more evolution of CO_2 was observed following addition of Na_2CO_3, the resulting mixture was diluted with water and then extracted with Et_2O (3 x 80mL to 3 x 200mL), washed with brine (1 x 100mL to 1 x 200mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue subjected to column chromatography.

5.2.9.1 2,5-Dimethoxy-3,4-dimethylbenzaldehyde **149a** (1983T781)



The title compound was prepared as described from 1,4-dimethoxy-2,3-dimethylbenzene **172a** (4.08g, 24.55mmol); elution used petroleum ether (60-80°C) : Et_2O (80:20). 2,5-Dimethoxy-3,4-dimethylbenzaldehyde **149a** was isolated as a white solid (2.99g, 15.39mmol, 62.6%). Crystals were obtained from petroleum ether (60-80°C); **m.p.**: 68-69°C [lit.: 72°C (from hexane, 1983T781)]; (found: C, 67.9; H, 7.2%. $C_{11}H_{14}O_3$ requires C, 68.0; H, 7.3%); *m/Z* 195.0 $(M)^+$, 217.0 $(MNa)^+$; ν_{max}/cm^{-1} 2957 and 2855 (C-H), 1681 (C=O), 1593 (C=C), 1128 and 1091 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.22 (3H, s, C*H*$_3$, 9-H), 2.26 (3H, s, C*H*$_3$, 10-H), 3.83 (3H, s, OC*H*$_3$, 11-H), 3.85 (3H, s, OC*H*$_3$, 8-H), 7.15 (1H, s, 6-H), 10.35 (1H, s, 7-H); **δ_C (75 MHz, $CDCl_3$)** 12.1 (CH_3, 10-C), 12.9 (CH_3, 9-C), 55.7 (CH_3, 8-C), 63.8 (CH_3, 11-C), 105.0 (CH, 6-C), 126.6 (quat., 1-C), 131.9 (quat., 3-C), 135.3 (quat., 4-C), 154.3 (quat., 2-C), 156.5 (quat., 5-C), 189.9 (CH, 7-C).

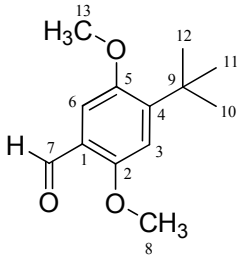
5.2.9.2 2,5-Dimethoxy-3,4,6-trimethylbenzaldehyde **149b** (1998S1153)



The title compound was prepared as described from 1,4-dimethoxy-2,3,5-trimethylbenzene **172b** (7.94g, 44.05mmol). Recrystallisation from petroleum ether (60-80°C) gave 2,5-dimethoxy-3,4,6-trimethylbenzaldehyde **149b** as cream coloured plates (7.83g, 37.60mmol, 85.3%); **m.p.**: 79-80°C [lit.: 80°C (from aq. EtOH, 1998S1153)] *m/Z* 209.0 $(MH)^+$; ν_{max}/cm^{-1} 2925 and 2867 (C-H), 1681 (C=O), 1586 and 1560 (C=C), 1452, 1254 and 1075 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.13 (3H, s, C*H*$_3$, 11-H), 2.19 (3H, s, C*H*$_3$, 10-H), 2.42 (3H, s, C*H*$_3$, 7-H), 3.57 (3H, s, OC*H*$_3$, 9-H), 3.70 (3H, s, OC*H*$_3$, 12-H), 10.41 (1H, s, C*H*O, 8-H), **δ_C (75 MHz, $CDCl_3$)** 12.1 (CH_3, 11-C), 12.8 (CH_3, 7-C), 13.7 (CH_3, 10-C), 60.2 (CH_3, 9-C), 63.2 (CH_3, 11-C), 126.2 (quat., 1-

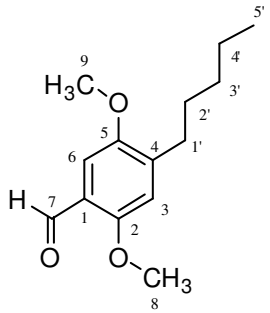
C), 129.0 (quat., 4-C), 131.0 (quat., 6-C), 138.4 (quat., 3-C), 153.6 (quat., 2-C), 159.0 (quat., 5-C), 192.8 (CH, 8-C).

5.2.9.3 2,5-Dimethoxy-4-*t*butylbenzaldehyde 149c (1974JME1100)



The title compound was prepared as described from 1,4-dimethoxy-2-*t*butylbenzene (10.14g, 52.20mmol); elution used a gradient mixture of petroleum ether (60-80°C): Et_2O (95:5 to 70:30). 2,5-Dimethoxy-4-*t*butylbenzaldehyde was isolated as a white solid (6.58g, 29.58mmol, 56.7%). Long translucent plates were obtained from petroleum ether (60-80°C); **m.p.**: 125-126°C [lit. 125°C (1974JME1100)]; (found: C, 70.3; H, 8.2%. $C_{13}H_{18}O_3$ requires C, 70.25; H, 8.2%); *m/Z* 223.1 $(MH)^+$, 245.05 $(MNa)^+$; ν_{max}/cm^{-1} 2965 and 2832 (C-H), 1673 (C=O), 1611 (C=C), 1200 and 1043 (C-O); **δ_H (300 MHz, $CDCl_3$)** 1.42 (9H, s, C(CH_3)$_3$), 3.87 (3H, s, OCH_3, 8-H), 3.93 (3H, s, OCH_3, 13-H), 6.98 (1H, s, 3-H), 7.32 (1H, s, 6-H), 10.43 (1H, s, C*H*O, 7-H); **δ_C (75 MHz, $CDCl_3$)** 29.4 (3 × CH_3, 10/11/12-C), 35.9 (quat., 9-C), 55.5 (CH_3, 8-C), 56.2 (CH_3, 13-C), 109.3 (CH, 6-C), 111.4 (CH, 3-C), 122.9 (quat., 1-C), 147.9 (quat., 4-C), 152.9 (quat., 2-C), 156.5 (quat., 5-C), 189.1 (CH, 7-C).

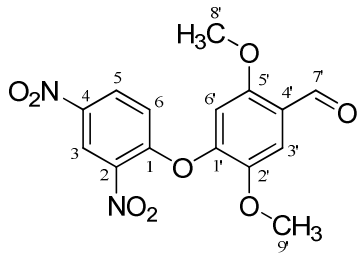
5.2.9.4 2,5-Dimethoxy-4-pentylbenzaldehyde 197



The title compound was prepared as described from 2,5-dimethoxy-1-pentylbenzene **196** (4.49g, 21.56mmol). The product was purified by column chromatography, eluting with a mixture of petroleum ether (60-80°C) : Et_2O (80:20). 2,5-Dimethoxy-4-pentylbenzaldehyde **197** was isolated as a pale yellow oil (4.19g, 17.73mmol, 82.4%); **b.p.**: 119-120°C at 0.2mbar; (found C, 71.0; H, 8.5. $C_{14}H_{20}O_3$ requires C,

71.2; H, 8.5%); *m/Z* 237.0 $(MH)^+$, 259.0 $(MNa)^+$; ν_{max}/cm^{-1} 2929 and 2859 (C-H), 1675 (C=O), 1610 (C=C), 1210 and 1040 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.94 (3H, t, *J* = 6.9Hz, 5'-H), 1.35-1.42 (4H, m, 3'-H and 4'-H), 1.60-1.65 (2H, m, 2'-H), 2.67 (2H, t, *J* = 7.5Hz, 1'-H), 3.85 (3H, s, OC*H*$_3$, 8-H), 3.92 (3H, s, OC*H*$_3$, 9-H), 6.82 (1H, s, 3-H), 7.29 (1H, s, 6-H), 10.43 (1H, s, C*H*O, 7-H); **δ_C (75 MHz, $CDCl_3$)** 14.0 (CH_3, 5'-C), 22.5 (CH_2, 3'-C or 4'-C), 29.2 (CH_2, 2'-C), 31.0 (CH2, 1'-C), 31.8 (CH_2, 3'-C or 4'-C), 55.8 (CH_3, 8-C), 56.2 (CH_3, 9-C), 108.1 (CH, 6-C), 113.8 (CH, 3-C), 122.9 (quat., 1-C), 141.2 (quat., 4-C), 151.8 (quat., 2-C), 156.7 (quat., 5-C), 189.15 (quat., 7-C).

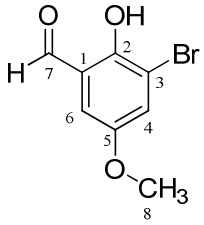
5.2.9.5 2,4-Dinitro-2',5'-dimethoxy-4'-carbaldehydediphenylether 211



The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxydiphenylether **169g** (1.99g, 6.20mmol). The crude product was recrystallised from EtOAc, giving 2,4-dinitro-2',5'-dimethoxy-4'-carbaldehydediphenylether **211** as amber prisms (1.58g, 4.54mmol, 73.2%), **m.p.**: 202-205°C; (found: C, 51.6; H, 3.55; N, 8.0%. $C_{15}H_{12}N_2O_8$ requires C, 51.7; H, 3.5; N, 8.0%); *m/Z* 349.0 $(MH)^+$; ν_{max}/cm^{-1} 2997 and 2882 (C-H), 1682 (C=O), 1603, 1518 and 1500 (C=C), 1539 and 1345 (NO_2), 1215 and 1035 (C-O); **δ_H (300 MHz, d_1-TFA)** 3.30 (3H, s, OC*H*$_3$, 8'-H), 3.40 (3H, s, OC*H*$_3$, 9'-H), 6.455 (1H, s, 6'-H), 6.57 (1H, d, *J* = 9.3Hz, 6-H), 7.14 (1H, s, 3'-H), 7.89 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.41 (1H, d, *J* = 2.7Hz, 3-H), 9.75 (1H, s, C*H*O, 7'-H); **δ_C (75 MHz, d_1-TFA)** 55.7 (CH_3, 9'-C), 56.0 (CH_3, 8'-C), 106.15 (CH, 6'-C), 113.6 (CH, 3'-C), 118.9 (CH, 6-C), 121.4 (quat., 4'-C), 122.3 (CH, 3-C), 129.3 (CH, 5-C), 142.1 (quat., 2-C), 142.1 (quat., 4-C), 144.8 (quat., 5'-C), 150.5 (quat., 1'-C), 155.3 (quat., 1-C), 159.9 (quat., 2'-C), 193.5 (CH, 7'-C).

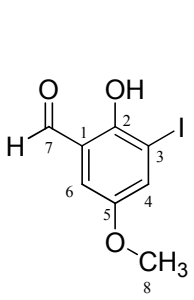
5.2.10 Halogenation of 2-hydroxy-5-methoxybenzaldehyde 182

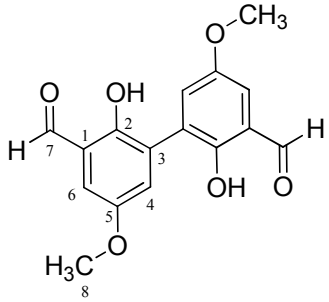
5.2.10.1 3-Bromo-2-hydroxy-5-methoxybenzaldehyde 183 (2004OL525)



In a 500mL 3 necked round bottomed flask equipped with a thermometer and a pressure equalizing dropping funnel, 2-hydroxy-5-methoxybenzaldehyde **182** (11.51g, 75.65mmol) was dissolved in glacial AcOH (300mL). To this solution was added AcONa (10.34g, 126.05mmol) and the resulting solution was cooled with a water/ice bath. A solution of Br_2 (4.91mL, 95.33mmol) in AcOH (50mL) was then introduced into the pressure equalizing dropping funnel and added dropwise to the 2-hydroxy-4-methoxybenzaldehyde solution. Once the addition was finished (1h), the ice/water bath was removed and the resulting orange solution was left with stirring overnight. The reaction was quenched with a 10% aqueous solution of sodium thiosulfate ($Na_2S_2O_3$), resulting in the instantaneous formation of a yellow precipitate. It was recovered by filtration, washed several times with water and dried. More product was recovered from the filtrate by extraction with DCM (4 × 200mL). The combined organic layers were washed with water (2 × 150mL) and brine (150mL), and dried ($MgSO_4$). The solvent was evaporated *in vacuo* and the residual yellow-brown crystals were recrystallised from aq. EtOH. 3-Bromo-2-hydroxy-5-methoxybenzaldehyde **183** was isolated as a yellow crystalline solid (12.86g, 55.66mmol, 75.1%). Small crescent-like yellow plates were obtained from aq. EtOH, **m.p.**: 108-109°C [lit.: 108°C (from EtOH, 2004OL525)]; ν_{max}/cm^{-1} 3071 (O-H), 3006, 2944, 2901 and 2840 (C-H), 1644 (C=O), 1609 and 1582 (C=C), 1315, 1237, 1129 and 1036 (C-O); (found C, 41.6; H, 2.95%. $C_8H_7BrO_3$ requires C, 41.6; H, 3.05%); **δ_H (300 MHz, d_6-DMSO)** 3.78 (3H, s, O*CH*$_3$), 7.31 (1H, d, *J* = 3.0Hz, 6-H), 7.51 (1H, d, *J* = 3.0Hz, 4-H), 10.08 (1H, s, C*H*O), 10.65 (1H, s, O*H*) ; **δ_C (75 MHz, d_6-DMSO)** 56.5 (CH_3, 8-C), 112.4 (quat., 3-C), 114.7 (CH, 6-C), 123.3 (quat., 1-C), 126.6 (CH, 4-C), 151.4 (quat., 2-C), 153.1 (quat., 5-C), 194.5 (CH, 7-C).

5.2.10.2 3-Iodo-2-hydroxy-5-methoxybenzaldehyde 186a and 2,2'-dihydroxy-5,5'-dimethoxybiphenyl-3,3'-dicarboxaldehyde 186b (1996T3841)



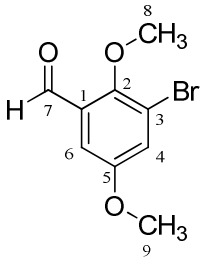


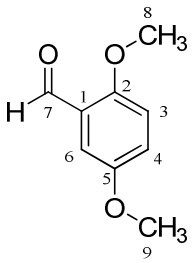
Procedure adapted from 1995TL8217. 2-Hydroxy-5-methoxybenzaldehyde **182** (2.48g, 16.30mmol) was dissolved in DCM (120mL) in a 250mL round-bottomed flask equipped with a thermometer and the resulting solution was cooled to -15°C (internal temperature) using an ice and salt bath. Bis(*sym*-collidine)iodine (I) hexafluorophosphate (8.38g, 16.30mmol; prepared according to the literature [2004OSC122]) was added portion-wise to the resulting solution, keeping the temperature below 10°C. The reaction was left with stirring at -15°C for further 3h after the addition. The resulting brown solution was washed with 10% aq. HCl (2 × 150mL) and brine (150mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : EtOAc (70:30). 3-Iodo-2-hydroxy-5-methoxybenzaldehyde **186a** was isolated as a yellow crystalline solid (2.26g, 7.72mmol, 47.4%). Recrystallisation from EtOH produced yellow needles; **m.p.**: 102-103.5°C [lit.: 102-104°C (1996T3841)]; (found: C, 34.6; H, 2.6%. $C_8H_7IO_3$ requires C, 34.6; H, 2.5%); $\boldsymbol{\nu_{max}}$/cm^{-1} 3062 (O-H), 3003, 2939, 2887 and 2836 (C-H), 1641 (C=O), 1311, 1235, 1124 and 1034 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.75 (3H, s, OC*H*$_3$, 8-C), 6.99 (1H, d, *J* = 3.0Hz, 6-H), 7.54 (1H, d, *J* = 3.0Hz, 4-H), 9.66 (1H, s, C*H*O, 7-H), 11.26 (1H, s, O*H*); **δ_C (75 MHz, $CDCl_3$)** 56.2 (CH_3, 8-C), 85.65 (quat., 3-C), 116.9 (CH, 6-C), 119.5 (quat., 1-C), 133.4 (CH, 4-C), 153.3 (quat., 5-C), 154.9 (quat., 1-C), 195.5 (CH, 7-C). Further elution gave 2-hydroxy-5-methoxybenzaldehyde dimer **186b** as an orange-brown solid (0.56g, 1.86mmol, 22.8%). Recrystallisation from EtOAc/EtOH produced small orange/brown plates, **m.p.:** 212-214°C [lit.: 217-218°C (1996T3841)]; (found: C, 63.5; H, 4.7%. $C_{16}H_{14}O_6$ requires C, 63.6; H, 4.7%); $\boldsymbol{\nu_{max}}$/cm^{-1} 3047 (O-H), 2871 and 2835 (C-H), 1643 (C=O), 1599 (C=C), 1257, 1147 and 1050 (C-O); **δ_H (300 MHz, d_6-DMSO)** 3.81 (6H, s, OC*H*$_3$, 8-H), 7.21 (2H, d, *J* = 3.3Hz, 4-H), 7.32 (2H, d, *J* = 3.0Hz, 6-H), 10.12 (2H, s, C*H*O, 7-H), 10.48 (2H, s, O*H*); **δ_C (75 MHz, d_6-DMSO)** 56.2 (CH_3, 8-C), 114.9

(CH, 6-C), 122.1 (quat., 1-C), 126.4 (CH, 4-C), 127.2 (quat., 3-C), 152.3 (quat., 5-C), 153.0 (quat., 2-C), 196.1 (CH, 7-C).

5.2.11 Protection of 3-halogeno-2-hydroxy-5-methoxybenzaldehyde

5.2.11.1 3-Bromo-2,5-dimethoxybenzaldehyde 178 (2004OL525) and **2,5-dimethoxybenzaldehyde 149** (2006T3550)

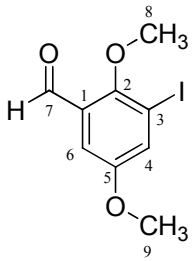




Method A (adapted from 2008OBC682). In an oven dried 250mL two necked round bottomed flask, equipped with a condenser and $CaCl_2$ drying tube, 3-bromo-2-hydroxy-5-methoxybenzaldehyde **183** (10.52g, 45.55mmol) was dissolved in dry DMF (300mL) and NaH in an oil dispersion 60%w/w was added portion-wise to the resulting solution. A strong evolution of H_2 was observed, and as soon as it stopped, methyl iodide was added slowly to the reaction mixture. The reaction was heated at 50°C (oil bath) for 5h and then allowed to cool to room temperature. The reaction was quenched with brine (300mL) and extracted with Et_2O (3 × 300mL). The combined organic layers were washed with water (2 × 250mL), 5% aqueous solution of LiCl (2 × 200mL) and brine (200mL), and dried ($MgSO_4$). The solvent was evaporated *in vacuo* and the residue purified by column chromatography, eluting with petroleum ether (60-80°C) : Et_2O (70 : 30). The more mobile fraction gave 3-bromo-2,5-dimethoxybenzaldehyde **178** as a white crystalline solid (5.65g, 23.05mmol, 50.7%). Analytical data were identical to that obtained for the product of ***method B*** below. Continued elution gave 2,5-dimethoxybenzaldehyde **149** as pale yellow oil which crystallised upon standing (1.08g, 6.50mmol, 14.3%); **m.p.**:47-48°C [lit.: 48-50°C (2006T3550)]; **δ_H (300 MHz, $CDCl_3$)** 3.73 (3H, s, OCH_3, 8-H), 3.82 (3H, s, OCH_3, 9-H), 6.87 (1H, d, *J* = 9.0 Hz, 3-H), 7.06 (1H, dd, *J* = 9.0, 3.3 Hz, 4-H), 7.26 (1H, d, *J* = 3.3 Hz, 6-H), 10.375 (1H, s, 8-H); **δ_C (75 MHz, $CDCl_3$)** 55.8 (CH_3, 8-C), 56.2 (CH_3, 9-C), 110.5 (CH, 6-C), 113.4 (CH, 3-C), 123.4 (CH, 4-C), 125.0 (quat., 1-C), 153.7 (quat., 5-C), 156.7 (quat., 2-C), 189.5 (CH, 8-C).

Method B (2004OL525). An oven dried 500mL round bottomed flask was charged with 3-bromo-2-hydroxy-5-methoxybenzaldehyde **183** (12.86g, 55.70mmol), K_2CO_3 (10.78g, 77.98mmol) and dry DMF (300mL). The resulting mixture was stirred for 5 min and DMS (7.38mL, 77.98mmol) was then added dropwise. The reaction was left overnight, with stirring, at ambient temperature. The resulting olive green coloured mixture was quenched with water resulting in the formation of a white precipitate. This mixture was extracted with Et_2O (4 × 300mL). The combined organic layers were washed with brine (3 × 250mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and 3-bromo-2,5-dimethoxybenzaldehyde **178** was isolated as a white crystalline solid (13.61g, 55.54mmol, 99.7%). White crystals were obtained from petroleum ether (60-80°C); **m.p.**: 62.5-63.5°C [lit.: 68°C (2004OL525)]; (found: C, 41.1; H, 3.6%. $C_9H_9BrO_3$ requires C, 41.1; H, 3.7%); $\boldsymbol{\nu_{max}}$/cm^{-1} 2935, 2854 and 2832 (C-H), 1685 (C=O), 1600 (C=C), 1225, 1205 and 1042 (C-O); $\boldsymbol{\delta_H}$ **(300 MHz, $CDCl_3$)** 3.85 (3H, s, O*CH*$_3$, 8-H), 3.97 (3H, s, O*CH*$_3$, 9-H), 7.30 (1H, d, *J* = 3.0Hz, 6-H), 7.41 (1H, d, *J* = 3.0Hz, 4-H), 10.34 (1H, s, C*H*O); $\boldsymbol{\delta_C}$ **(75 MHz, $CDCl_3$)** 56.0 (CH_3, 8-C), 63.8 (CH_3, 9-C), 110.3 (CH, 6-C), 118.6 (quat., 3-C), 126.5 (CH, 4-C), 130.6 (quat., 1-C), 154.4 (quat., 5-C), 156.5 (quat., 2-C), 189.0 (CH, 7-C).

5.2.11.2 3-Iodo-2,5-dimethoxybenzaldehyde 185

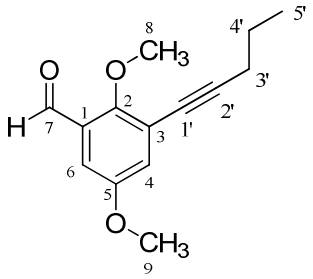


The title compound was prepared from 3-iodo-2-hydroxy-5-methoxybenzaldehyde **186a** (1.98g, 7.12mmol) following ***method B***. 3-Iodo-2,5-dimethoxybenzaldehyde **185** was isolated as a white crystalline solid (1.95g, 6.66mmol, 93.5%). White plates were obtained from petroleum ether (60-80°C); **m.p.**: 61.5-63.5°C; (found C, 37.0; H, 3.0. $C_9H_9IO_3$ requires C, 37.0; H, 3.1%); *m/Z* 315.1 $(MNa)^+$; $\boldsymbol{\nu_{max}}$/cm^{-1} 2934, 2858 and 2745 (C-H), 1680 (C=O), 1592 (C=C), 1208 and 1043 (C-O); $\boldsymbol{\delta_H}$ **(300 MHz, $CDCl_3$)** 3.85 (3H, s, OC*H*$_3$, 8-H), 3.93 (3H, s, OC*H*$_3$, 9-H), 7.34 (1H, d, *J* = 3.0Hz, 6-H), 7.63 (1H, d, *J* = 3.3Hz, 4-H), 10.305 (1H, s, C*H*O, 7-H); $\boldsymbol{\delta_C}$ **(75 MHz, $CDCl_3$)** 56.02 (CH_3,

8-C), 64.3 (CH_3, 9-C), 93.1 (quat., 3-C), 111.5 (CH, 6-C), 129.7 (quat., 1-C), 132.4 (CH, 4-C), 156.8 (quat., 2-C), 157.1 (quat., 5-C), 189.3 (CH, 7-C).

5.2.12 Sonogoshira cross-couping

5.2.12.1 Preparation of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde 184



Method A (adapted from 1975TL4467): A flame dried sealed tube flushed with N_2 was charged 3-bromo-2,5-dimethoxybenzaldehyde **178** (0.21g, 0.84mmol), $[Ph_3P]_2PdCl_2$ (0.014g, 0.02mmol), pentyne (0.15mL, 1.50mmol) and dried Et_3N (3mL). The resulting suspension was stirred and heated at 40°C for 15 min, before addtion of CuI (0.008g, 0.04mmol). The resulting mixture turned brown and was heated at 80°C for 48h. The reaction mixture was allowed to cool to room temperature, quenched with 10% aq. HCl (10mL) and extracted with Et_2O (2 × 10mL). The combined organic extracts were washed with brine (15mL) and dried ($MgSO_4$). The solvent was removed in vacuo and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et_2O (90:10). The more mobile fraction gave 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** as a yellow coloured oil, which crystalised upon standing (0.021g, 0.09mmol, 10.7%). Analytical data were identical to that obtained for the product of ***method C*** below. Continued elution gave the starting material 3-bromo-2,5-dimethoxybenzaldehyde **178** as a white solid (0.16g, 0.67mmol, 79.8%).

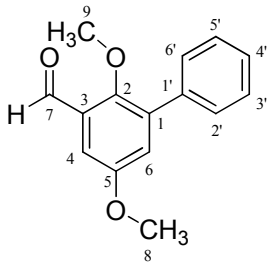
Method B (adapted from 2000OL1729): A 25mL, flame dried round bottomed flask, flushed with N_2 was charged with 3-bromo-2,5-dimethoxybenzaldehyde **178** (0.21g, 0.86mmol), $[PhCN]_2PdCl_2$ (0.01g, 0.03mmol), pentyne (0.13mL, 1.29mmol) and dry dioxane (1mL), followed by successive addition of a 0.25M dioxane solution of tris-^{t}butylphosphine (0.22mL, 0.06mmol) and $(i\text{Pr})_2NH$ (0.18mL). The resulting dark solution was stirred for 48h at R.T. under a N_2 atmosphere. The mixture was then diluted with EtOAc (10mL), washed with 10% aq. HCl (10mL) and brine (10mL). The organic layer was dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The solvent

was removed in vacuo and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et_2O (90:10). The more mobile fraction gave 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** as a slightly brown coloured oil, which crystalised upon standing (0.03g, 0.11mmol, 13.2%). Analytical data were identical to that obtained for the product of ***method C*** below. Continued elution gave the starting material 3-bromo-2,5-dimethoxybenzaldehyde as a white solid (0.17g, 0.71mmol, 82.5%).

Method C (adapted from 1975TL4467): A 100mL two-necked flask equipped with a reflux condenser was flame dried and kept under an N_2 atmosphere. It was then charged with 3-iodo-2,5-dimethoxybenzaldehyde **185** (1.58g, 5.42mmol), $[Ph_3P]_2PdCl_2$ (0.08g, 0.11mmol), 1-pentyne (1.10mL, 0.74g, 10.83mmol), dry Et_3N (10mL) and dry DMF (15mL). The resulting mixture was stirred and heated at 40°C for 5min, before the addition of copper (I) iodide. The resulting mixture turned slightly brown, then was heated at 95°C under N_2 for 24h. The reaction mixture was allowed to cool to room temperature and was then filtered through celite and the celite washed several times with Et_2O. The filtrate was diluted with 100mL of Et_2O, washed with a saturated solution of NH_4Cl (2 × 50mL), a 5% aq. solution of $NaHCO_3$ (2 × 50mL) and water (100mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et_2O (90:10). 2,5-Dimethoxy-1-pentyn-1'-ylbenzaldehyde **184** was isolated a a yellow coloured oil, which crystalised upon standing (1.06g, 4.58mmol, 84.6%). Recrystallisation from petroleum ether 60-80°C produced white prisms; **m.p.**: 31-33°C; (found: C, 72.2; H, 6.8%. $C_{14}H_{16}O_3$ requires C, 72.4; H, 6.9%); *m/z* 233.0 $(MH)^+$, 255.0 $(MNa)^+$; ν_{max}/cm^{-1} 2961 and 2867 (C-H), 2236 (C≡C), 1680 (C=O), 1591 (C=C), 1244 and 1047 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.995 (3H, t, *J* = 7.5Hz, 5'-H), 1.59 (2H, sex., *J* = 7.5Hz, 4'-H), 2.37 (2H, t, *J* = 6.9Hz, 3'-H), 3.71 (3H, s, OC*H*$_3$, 8-H), 3.93 (3H, s, OC*H*$_3$, 9-H), 7.09 (1H, d, *J* = 3.3Hz, 4-H), 7.15 (1H, d, *J* = 3.0Hz, 6-H), 10.26 (1H, s, C*H*O, 7-H); **δ_C (75 MHz, $CDCl_3$)** 13.5 (CH_3, 5'-C), 21.6 (CH_2, 3'-C), 22.0 (CH_2, 4'-C), 55.8 (CH_3, 8-C), 63.0 (CH_3, 9-C), 75.5 (quat., 1'-C), 96.55 (quat., 2'-C), 110.1 (CH, 6-C), 120.1 (quat., 3-C), 126.5 (CH, 4-C), 129.5 (quat., 1-C), 155.4 (quat., 2-C), 158.5 (quat., 5-C), 189.5 (CH, 7-C).

5.2.13 Suzuki cross-coupling

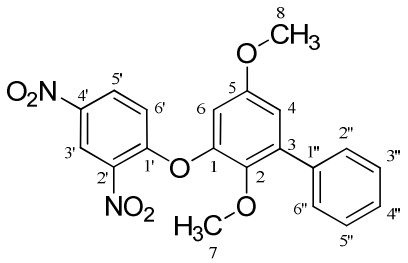
5.2.13.1 Preparation of 2,5-dimethoxybiphenyl-3-carbaldehyde 194



A 250mL two necked round bottomed flask equipped with a reflux condenser was charged with 3-bromo-2,5-dimethoxybenzaldehyde **178** (2.72g, 11.10mmol), phenylboronic acid (1.62g, 13.29mmol) and DME (75mL). The resulting solution was degassed with N_2, under stirring, over 15min. A separate round bottomed flask was charged with a 2M aqueous solution of Na_2CO_3 (20mL) and EtOH (20mL); the resulting mixture was also degassed with N_2 over 15min. It was then transferred, along with $Pd(Ph_3)_4$ (0.64g, 0.56mmol), into the two necked round bottomed flask containing the 3-bromo-2,5-dimethoxybenzaldehyde **178** solution. The resulting mixture was brought to reflux ($T_{oil\ bath}$ = 100°C), while keeping a constant N_2 flow over the reaction. The reflux was stopped after 23h and the reaction cooled to room temperature. Once at room temperature, the reaction was cooled with an ice bath and acidified with a 10% aqueous solution of HCl (pH=1-2). The resulting mixture was diluted with EtOAc and filtered through celite. The celite was washed several times with small portions of EtOAc. The filtrate was decanted into a separating funnel and the aqueous layer was further extracted with EtOAc (3 × 50mL). The combined organic layers were washed with water (100mL) and brine (100mL), and dried ($MgSO_4$). The solvent was evaporated *in vacuo* and the residue subjected to column chromatography, eluting with petroleum ether (60-80°C) : Et_2O (80:20). 2,5-Dimethoxybiphenyl-3-carbaldehyde **194** was isolated as clear cubic white plates (2.29g, 9.43mmol, 85.0%); **m.p.** (Et_2O : petroleum ether 60-80°C): 64.5-65.5°C; (found: C, 74.3; H, 5.9%. $C_{15}H_{14}O_3$ requires C, 74.4; H, 5.8%); *m/z* 243.0 $(MH)^+$, 265.0 $(MNa)^+$; ν_{max}/cm^{-1} 2877 and 2754 (C-H), 1680 (C=O), 1596 (C=C), 1229 and 1041 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.51 (3H, s, OCH_3, 9-H), 3.89 (3H, s, OCH_3, 8-H), 7.22 (1H, d, *J* = 3.3Hz, 6-H), 7.36 (1H, d, *J* = 3.3Hz, 4-H), 7.41-7.53 (3H, m, 3'-H, 4'-H and 5'-H), 7.60-7.64 (2H, m, 2'-H and 6'-H), 10.48 (1H, C*H*O, 7-H); **δ_C (75 MHz, $CDCl_3$)** 55.8 (CH_3, 8-C), 62.9 (CH_3, 9-C), 109.1 (CH, 4-C), 124.75 (CH, 6-C), 127.9

(CH, 4'-C), 128.6 (CH, 3'-C and 5'-C), 128.9 (CH, 2'-C and 6'-C), 130.1 (quat., 3-C), 136.9 (quat., 1-C), 137.3 (quat., 1'-C), 155.4 (quat., 2-C), 156.0 (quat., 5-C), 190.05 (CH, 7-C).

5.2.13.2 Attempted preparation of 3-(2',4'-dinitrophenoxy)-2,5-dimethoxybiphenyl 169f



Preparation of the title compound was attempted following the method described above from 2,4-dinitro-2',5'-dimethoxy-3'-bromodiphenylether **169e** (0.65g, 1.64mmol) and phenyl boric acid **193** (0.27g, 2.13mmol); elution used petroleum ether (60-80°C) : Et_2O (80:30) yielding 2,5-dimethoxy-biphenyl-3-ol **150f** as a pale yellow oil which crystallised upon standing (0.35g, 1.53mmol, 93.3%). Analytical data where identical to that obtained for the product resulting from Baeyer-Villiger oxidation of 2,5-dimethoxybiphenyl-3-carbaldehyde **194**.

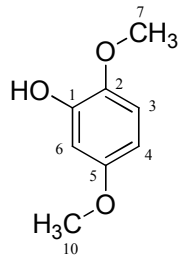
5.2.14 General procedure for the Baeyer-Villiger oxidation of benzaldehydes 149a-d, 178, 194 and 197

Method A (adapted from 2000J(P1)2681). In a two necked round bottomed flask equipped with a dropping funnel, a suspension of magnesium monoperoxyphthalate (0.55mol equivalent) in methanol (50mL) was prepared. A few drops of concentrated sulphuric acid were added to the suspension until all the MMPP dissolved in the resulting acidic methanolic solution. It was then cooled with an ice-bath and a solution of the substituted benzaldehyde (1mol equivalent) in DCM (50mL) was added dropwise to the MMPP solution. Once the addition was finished, the resulting solution was left with stirring overnight. The solvent mixture was then removed under reduced pressure and the residue was redissolved in a 10% aqueous potassium carbonate solution (70mL). The resulting mixture was extracted with ethyl acetate (2x100mL) and the combined organic layers washed once with brine (100mL). The solvent was removed under reduced pressure and the residue subjected to column

chromatography on silica. Recrystallisation was achieved from petroleum ether (60-80°C).

Method B (adapted from 1987T2653). In a two necked round bottomed flask equipped with a reflux condenser, the correctly substituted benzaldehyde (1 mol equivalent) was dissolved in DCM (70 mL). The resulting solution was cooled with an ice bath and *meta*-chloroperbenzoic acid (1.7mol equivalent) was added portion-wise. The ice bath was removed 1h after the end of the addition and the resulting solution left with stirring overnight at room temperature. The reaction progress was monitored by TLC and the reaction was refluxed for 5-6h to drive it to completion, when necessary. The reaction was then allowed to cool to room temperature and washed several times with a 5% aqueous solution of $NaHCO_3$ (3 x 100mL). The DCM layer was then dried ($MgSO_4$) and the solvent was removed *in vacuo*. The residual oil was dissolved in methanol (20mL) and a 10% aqueous solution of NaOH (excess) was added to hydrolyse the formyl phenylate ester formed; the resulting dark solution was stirred for 2-3 hours and was then acidified (pH 1-2) with a 10% aqueous solution of HCl. The resulting mixture was extracted with DCM (3 x 80mL) and the combined organic extracts were successively washed with a 5% aqueous solution of $NaHCO_3$ (100mL), water (100mL) and brine (100mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue subjected to column chromatography on silica gel. The product was recrystallised from petroleum ether (60-80°C).

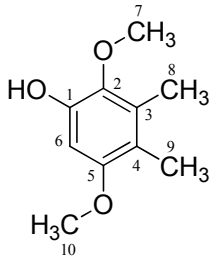
5.2.14.1 2,5-Dimethoxyphenol 150g (1987T2653)



Method A. The title compound was prepared as described from 2,5-dimethoxybenzaldehyde **149d** (4.14g, 24.91mmol). 2,5–Dimethoxyphenol **150g** (2.33g, 15.12mmol, 60.7%) was isolated as a pale yellow oil; ν_{max}/cm^{-1} 3416 (O-H), 1596 and 1504 (C=C), 1232, 1147 and 1039 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.67 (3H, s, O*CH*$_3$, 8-H), 3.76 (3H, s, O*CH*$_3$, 7-H), 5.58 (1H, s, O*H*), 6.30 (1H, dd, *J* = 8.9, 3.0Hz, 4-H), 6.49 (1H, d, *J* = 3.0Hz, 6-H), 6.69 (1H, d, *J* = 8.7Hz, 3-H); **δ_C(75 MHz, $CDCl_3$)**

56.1 (CH_3, 8-C), 57.0 (CH_3, 7-C), 102.2 (CH, 6-C), 104.7 (CH, 4-C), 112.0 (CH, 3-C), 141.4 (quat., 2-C), 146.9 (quat., 1-C), 155.0 (quat., 5-C).

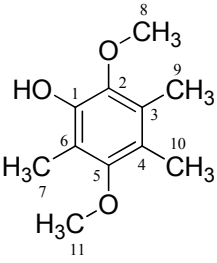
5.2.14.2 2,5-Dimethoxy-3,4-dimethylphenol 150a (2008OBC682)



Method A. The title compound was prepared as described from 2,5-dimethoxy-3,4-dimethylbenzaldehyde **149a** (0.19g, 1.02mmol). Elution with petroleum ether (60-80°C) : Et_2O (70:30) yielded 2,5-dimethoxy-3,4-dimethylphenol as a white solid (0.03g, 0.18mmol, 17.3%). Analytical data were identical to that obtained for the product of ***method B***.

Method B. The title compound was prepared as described from 2,5-dimethoxy-3,4-dimethylbenzaldehyde **149a** (1.04g, 5.35mmol). Elution with petroleum ether (60-80°C) : EtOAc (70:30) yielded 2,5-dimethoxy-3,4-dimethylphenol **150a** as a white solid (0.78g, 4.27mmol, 79.8%). White needles were obtained from petroleum ether (60-80°C); **m.p.**: 69-71°C [lit.: 69-71°C (2008OBC682)]; (found C, 65.9; H, 7.7%. $C_{10}H_{14}O_3$ requires C, 65.9; H, 7.7%); *m/z* 183.2 $(MH)^+$; ν_{max}/cm^{-1} 3263 (O-H), 1598 and 1508 (C=C), 1330, 1262, 1214 and 1038 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.12 (3H, s, C*H*$_3$, 8-H), 2.26 (3H, s, C*H*$_3$, 9-H), 3.77 (3H, s, OC*H*$_3$, 7-H), 3.81 (3H, s, OC*H*$_3$, 10-H), 5.77 (1H, br. s, O*H*), 6.48 (1H, s, 6-H); **δ_C (75 MHz, $CDCl_3$)** 11.4 (CH_3, 8-C), 12.7 (CH_3, 7-C), 55.8 (CH_3, 9-C), 61.1 (CH_3, 10-C), 96.7 (CH, 6-C), 117.0 (quat., 4-C), 130.2 (quat., 3-C), 139.1 (quat., 2-C), 146.7 (quat., 1-C), 154.3 (quat., 5-C).

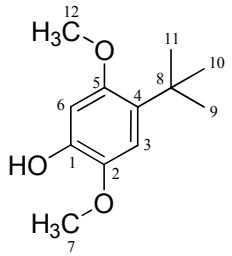
5.2.14.3 2,5-Dimethoxy-3,4,6-trimethylphenol 150b (2008OBC682)



Method A. The title compound was prepared as described from 2,5-dimethoxy-3,4,6-trimethylbenzaldehyde **149b** (1.11g, 5.32mmol). Elution with petroleum ether (60-80℃) EtOAc : (80:20) yielded 2,5-dimethoxy-3,4,6-trimethylphenol **150b** as a white solid (0.65g, 3.29mmol, 61.8%). Analytical data were identical to that obtained for the product of ***method B***.

Method B. The title compound was prepared as described from 2,5-dimethoxy-3,4,6-trimethylbenzaldehyde **149b** (2.61g, 12.53mmol). Elution with petroleum ether (60-80℃): Et_2O (70:30) yielded 2,5-dimethoxy-3,4,6-trimethylphenol **150b** as a white solid (2.09g, 10.65mmol, 85.0%). White needles were obtained from petroleum ether (60-80℃); **m.p.**: 105-106℃ [lit.:105-106℃ (2008OBC682)]; (found C, 67.4; H, 8.2%. $C_{11}H_{16}O_3$ requires C, 67.3; H, 8.2%); *m/z* 219.0 $(MNa)^+$; $\boldsymbol{\nu_{max}}$/cm^{-1} 3401 (O-H), 1612 (C=C), 1309, 1264 and 1070 (C-O); $\boldsymbol{\delta_H}$ **(300 MHz, $\boldsymbol{d_6}$-DMSO)** 2.18 (3H, s, C*H*$_3$, 9-H), 2.23 (3H, s, C*H*$_3$, 7-H), 2.23 (3H, s, C*H*$_3$, 10-H), 3.71 (3H, s, OC*H*$_3$, 11-H), 3.77 (3H, s, OC*H*$_3$, 8-H), 5.71 (1H, s, O*H*); $\boldsymbol{\delta_C}$ **(75 MHz, $\boldsymbol{d_6}$-DMSO)** 9.1 (CH_3, 10-C), 12.0 (CH_3, 7-C), 12.5 (CH_3, 9-C), 60.2 (CH_3, 11-C), 60.9 (CH_3, 8-C), 115.0 (quat., 6-C), 121.0 (quat., 4-C), 126.8 (quat., 3-C), 141.7 (quat., 2-C), 145.3 (quat., 1-C), 153.4 (quat., 5-C).

5.2.14.4 2,5-dimethoxy-4-^{t}butylphenol 150c (1968JC1438)

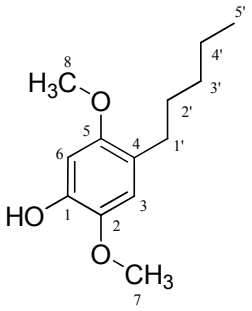


Method A. The title compound was prepared as described from 2,5-dimethoxy-4-^{t}butylbenzaldehyde **149c** (2.03g, 9.10mmol); elution used petroleum ether (60-80℃): EtOAc (70:30). 2,5-Dimethoxy-4-^{t}butylphenol **150c** was obtained as a white solid (0.39g, 1.87mmol, 20.5%). Analytical data were identical to that obtained for the product of ***method C***.

Method B. The title compound was prepared as described from 2,5-dimethoxy-4-^{t}butylbenzaldehyde **149c** (1.59g, 7.13mmol). Elution with petroleum ether (60-80℃): Et_2O (60:40). 2,5-Dimethoxy-4-^{t}butylphenol **150c** was obtained as a white solid (0.82g, 3.86mmol, 54.15%). Analytical data were identical to that obtained for the product of ***method C***.

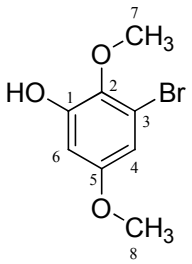
Method C (2008OBC). In a flame dried 250mL 2 neck round bottomed flask, flushed with argon, 1,4-dimethoxy-2-^{t}butylbenzene **172c** (6.84g, 35.21mmol) was dissolved in dry THF (75mL). The solution was cooled in a dry ice-acetone bath to -75°C and a solution of 2.5 M *n*-BuLi (24mL, 60.0mmol) was added slowly to it. The resulting orange solution was allowed to warm up to room temperature and stirred for a further 45min. The orange-brown coloured solution was cooled again to -75°C and triisopropyl borate was added to it. The solution was left overnight under Ar with the dry-ice/acetone bath, so that the temperature slowly warmed up to room temperature overnight. The clear yellow solution was then quenched with a 10% aqueous ammonium chloride solution and extracted with diethyl ether (3 x 150mL). The combined organic layers were dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was dissolved in THF (20mL) and H_2O_2 35% (20mL) was added to the resulting solution and the emulsion was stirred for 1 hour (exothermic reaction). Water was added to the reaction mixture, which was then extracted with diethyl ether (3 x 150mL) and the combined organic extracts were washed once with brine (150mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was subjected to column chromatography on silica eluting with petroleum ether (60-80°C) : Et_2O (70:30). Residual starting material was recovered as a yellow oil (1.13g, 5.84 mmol, 16.6%) and 2,5-dimethoxy-4-^{t}butylphenol **150c** was isolated as a white solid (3.9527g, 18.79mmol, 53.4%). Recrystallisation from petroleum ether (60-80°C) yielded a white crystaline solid; **m.p.**: 52-54°C [lit.:54-55°C (from petroleum ether, 1968JC1438)]; (found C, 68.6; H, 8.7%. $C_{12}H_{18}O_3$ requires C, 68.55; H, 8.6%); ν_{max}/cm^{-1} 3381, 3280 (O-H), 1597 and 1511 (C=C), 1300, 1192 and 1043 (C-O); δ_H **(300 MHz, $CDCl_3$)** 1.41 (9H, s, C(C*H*$_3$)$_3$), 3.83 (3H, s, OC*H*$_3$, 7-H), 3.90 (3H, s, OC*H*$_3$, 12-H), 5.60 (1H, s, O*H*), 6.63 (1H, s, 6-H), 6.90 (1H, s, 3-H); δ_C **(75 MHz, $CDCl_3$)** 30.0 (CH_3, 9-C, 10-C and 11-C), 34.5 (quat., 8-C), 55.7 (CH_3, 7-C), 57.0 (CH_3, 12-C), 100.6 (CH, 6-C), 110.9 (CH, 3-C), 129.7 (quat., 4-C), 139.6 (quat., 5-C), 144.2 (quat., 1-C), 153.2 (quat., 2-C).

5.2.14.5 2,5-Dimethoxy-4-pentylphenol 150d



Method B. The title compound was prepared as described from 2,5-dimethoxy-4-pentylbenzaldehyde **197** (3.44g, 14.58mmol); elution used petroleum ether (60-80°C) : Et_2O (70:30). 2,5-Dimethoxy-4-pentylphenol **150d** was isolated as a white crystalline solid (2.66g, 11.87mmol, 81.4%). Recrystallisation from Et_2O : petroleum ether (60-80°C) produced white needles, **m.p.**: 33-35°C; (found: C, 69.55; H, 9.0%. $C_{13}H_{20}O_3$ requires C, 69.6; H, 9.0%); *m/z* 225.0 $(MH)^+$; ν_{max}/cm^{-1} 3354 (O-H), 2953 and 2930 (C-H), 1598 and 1515 (C=C), 1192 and 1038 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.96 (3H, t, *J* = 6.9Hz, 5'-H), 1.38-1.415 (4H, m, 3'-H and 4'-H), 1.58-1.63 (2H, m, 2'-H), 2.59 (2H, t, *J* = 7.5Hz, 1'-H), 3.81 (3H, s, OC*H*$_3$, 8-H), 3.89 (3H, s, OC*H*$_3$, 7-H), 5.61 (1H, s, O*H*), 6.60 (1H, s, 6-H), 6.73 (1H, s, 3-H); **δ_C (75 MHz, $CDCl_3$)** 14.1 (CH_3, 5'-C), 22.6 (CH_2, 3'-C or 4'-C), 29.8 (CH_2, 1'-C), 30.1 (CH_2, 2'-C), 31.8 (CH_2, 3'-C or 4'-C), 56.1 (CH_3, 8-C), 56.8 (CH_3, 7-C), 99.5 (CH, 6-C), 113.3 (CH, 3-C), 122.3 (quat., 4-C), 140.1 (quat., 2-C), 144.1 (quat., 1-C), 152.0 (quat., 5-C).

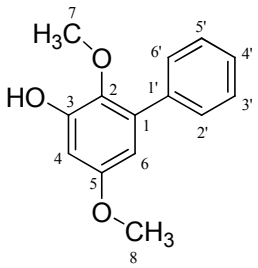
5.2.14.6 3-Bromo-2,5-dimethoxyphenol 150e(1998JOC5831)



The title compound was prepared as described from 3-bromo-2,5-dimethoxybenzaldehyde **178** (5.24g, 21.38mmol); elution used petroleum ether (60-80°C) : Et_2O (70:30) yielded 3-bromo-2,5-dimethoxyphenol **150e** as a white solid (3.51g, 15.06mmol, 70.4%). White needle-like plates were obtained from petroleum ether (60-80°C); **m.p.**: 70.5-72.0°C [lit.: 73-74°C (1998JOC5831)]; (found C, 41.2; H, 3.8%. $C_{12}H_{18}O_3$ requires C, 41.2; H, 3.8%); ν_{max}/cm^{-1} 3377 (O-H), 1609 and 1573

(C=C), 1323, 1165 and 1041 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.78 (3H, s, 7-H, OC*H*$_3$), 3.89 (3H, s, 8-H, OC*H*$_3$), 5.90 (1H, s, O*H*), 6.55 (1H, d, *J* = 3.0Hz, 6-H), 6.67 (1H, d, *J* = 2.7Hz, 4-H); **δ_C (75 MHz, $CDCl_3$)** 55.8 (CH_3, 7-C), 61.35 (CH_3, 8-C), 101.5 (CH, 6-C), 109.9 (CH, 4-C), 115.8 (quat., 3-C), 138.7 (quat., 5-C), 150.3 (quat., 1-C), 157.0 (quat., 2-C).

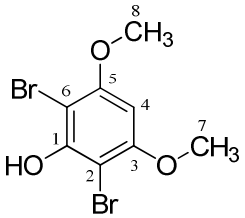
5.2.14.7 2,5-Dimethoxy-biphenyl-3-ol 150f



The title compound was prepared as described from 2,5-dimethoxybiphenyl-3-carbaldehyde **194** (2.02g, 8.34mmol); elution used petroleum ether (60-80°C) : Et_2O (70:30) yielded 2,5-dimethoxy-biphenyl-3-ol **150f** as a white crystalline solid (1.43g, 6.19mmol, 74.2%). White needles were obtained from petroleum ether (60-80°C); **m.p.**: 67-69°C; (found: C, 73.1; H, 6.2%. $C_{14}H_{14}O_3$ requires C, 73.0; H, 6.1%); *m/z* 231.0 $(MH)^+$; ν_{max}/cm^{-1} 3396 (O-H), 1619, 1591 and 1573 (C=C), 1206 and 1049 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.44 (3H, s, OC*H*$_3$, 7-H), 3.85 (3H, s, OC*H*$_3$, 8-H), 6.10 (1H, s, O*H*), 6.51 (1H, d, *J* = 3.0Hz, 6-H), 6.64 (1H, d, *J* = 3.0Hz, 4-H), 7.39-7.52 (3H, m, 3'-H, 5'-H and 4'-H), 7.64-7.67 (2H, m, 2'-H and 6'-H); **δ_C (75 MHz, $CDCl_3$)** 55.6 (CH_3, 8-C), 60.9 (CH_3, 7-C), 100.6 (CH, 4-C), 107.2 (CH, 6-C), 127.6 (CH, 4'-C), 128.5 (CH, 3'-C and 5'-C), 128.8 (CH, 2'-C and 6'-C), 134.7 (quat., 1-C), 138.0 (quat., 1'-C), 138.5 (quat., 2-C), 149.9 (quat., 3-C), 156.45 (quat., 5-C).

5.2.15 Bromination

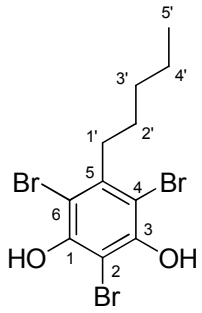
5.2.15.1 2,6-Dibromo-3,5-dimethoxyphenol 176 (1989CJC335)



3,5-Dimethoxyphenol **177** (2.01g, 13.04mmol) was dissolved in DCM (40mL) and a solution of bromine (1.34mL, 26.02mmol) in DCM (20mL) was added dropwise to the

3,5-dimethoxyphenol solution. The resulting solution was stirred during 6 hours, then washed with water (2 × 100mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue was recrystallised from a solvent mixture of petroleum ether (60-80°C) : $CHCl_3$. 2,6-Dibromo-3,5-dimethoxyphenol **176** was isolated as white crystals (2.97g, 9.52mmol, 72.6%); **m.p.** : 163-164°C [lit.: 164-165°C (from petroleum ether (65-110°C): $CHCl_3$, 1989CJC335)]; (found C, 30.75; H, 2.55%. $C_8H_8Br_2O_3$ requires C, 30.8; H, 2.6%); ν_{max}/cm^{-1} 3488 (O-H), 1583 and 1567 (C=C), 1343 and 1065 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.93 (6H, s, 2 x OC*H*$_3$, 7-H and 8-H), 6.06 (1H, br s, O*H*), 6.20 (1H, s, 4-H); **δ_C (75 MHz, $CDCl_3$)** 56.7 (CH_3, 7-C and 8-C), 89.6 (CH, 4-C), 91.1 (quat., 2-C and 6-C), 150.8 (quat., 1-C), 156.4 (quat., 3-C and 5-C).

5.2.15.2 2,4,6-Tribromoolivetol 205 (1936CB1643)

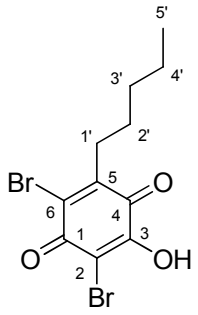


In a 250mL two-necked round bottomed flask equipped with a pressure equalizing dropping funnel, olivetol **181** (4.92g, 27.30mmol) was dissolved in glacial AcOH (150mL). To this solution was added NaOAc (8.28g, 100.94mmol) and the resulting suspension was cooled with an ice-bath. The pressure equalizing funnel was charged with a solution of Br_2 (4.62mL, 89.70mmol) in glacial AcOH (30mL), which was then added dropwise to the olivetol suspension. After the end of the addition, the resulting solution was allowed to warm up to room temperature and stirred for a further 5h. The reaction was quenched with a 10% aq. solution of $Na_2S_2O_3$ (300mL), resulting in the formation of a white precipitate. The precipitate was filtered off, washed several times with water, and dried under vacuum. It was then redissolved in Et_2O and the resulting solution was dried ($MgSO_4$), before removing the solvent *in vacuo*. The residue was purified by column chromatography, eluting with petroleum ether (60-80°C) : Et_2O (75 : 25), giving 2,4,6-tribromoolivetol **205** as a white solid (10.49g, 25.17mmol, 92.2%). 2,4,6-Tribromoolivetol **205** was recrystallised from petroleum ether (60-80°C), producing a white fluffy solid; **m.p.**: 80-82°C [lit.: 87°C

(from AcOH, 1936CB1643)]; (found C, 31.4; H, 3.2%. $C_{11}H_{13}Br_3O_2$ requires C, 31.7; H, 3.2%); ν_{max}/cm^{-1} 3585-3405 (O-H), 2952-2854 (C-H), 1622, 1573 and 1558 (C=C), 1210 and 1195 (C-O), 676 (C-Br); **δ_H (300 MHz, $CDCl_3$)** 0.975 (3H, t, *J* = 7.2Hz, 5'-H), 1.43-1.49 (4H, m, 3'-H and 4'-H), 1.57-1.68 (2H, m, 2'-H), 3.00 (2H, t, *J* = 7.8Hz, 1'-H), 6.10 (2H, s, 2 × O*H*); **δ_C (75 MHz, $CDCl_3$)** 14.0 (CH_3, 5'-C), 22.4 (CH_2, 4'-C), 27.7 (CH_2, 2'-C), 31.8 (CH_2, 3'-C), 37.6 (CH_2, 1'-C), 95.0 (quat., 2-C), 103.1 (quat., 4-C and 6-C), 141.2 (quat., 5-C), 149.4 (quat., 1-C and 3-C).

5.2.16 Oxidation of tribromoolivetol 205 (adapted from 1983J(P1)2595):

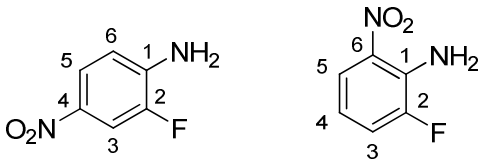
2,6-Dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone 206



Into a 250mL round-bottomed flask, 2,4,6-tribromoolivetol **205** (1.11g, 2.67mmol) was dissolved in a mixture of a 0.1M K_2HPO_4 phosphate buffer (50mL) and CH_3CN (70mL). Potassium nitrosodisulfonate **201** (2.15g, 8.00mmol, prepared according to 1970CR229) was dissolved in 0.1M K_2HPO_4 phosphate buffer (50mL) and the resulting deep purple solution was added to the 2,4,6-tribromoolivetol solution. The resulting solution was left with stirring at room temperature for 5h. The reaction was then quenched with 10% aq. HCl, resulting in the formation of an orange oil. The mixture was extracted with DCM (2 × 70mL) and the combined organic extracts were washed with brine (100mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the oily residue was recrystallised from *n*-hexane, yielding 2,6-dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone **206** as bright orange plates (0.77g, 2.18mmol, 81.6%); **m.p.:** 81-83°C; (found: C, 37.6; H, 3.4%. $C_{11}H_{12}Br_2O_5$ requires C, 37.5; H, 3.4%); *m/z* 348.7 (41%), 350.7 (100%), 352.7 (43%) (*M-H*)$^-$; ν_{max}/cm^{-1} 3186 (O-H), 2853-2931 (C-H), 1660 and 1638 (C=O, quinone), 1591 (C=C), 1348, 1270 and 1245 (C-O), 727 (C-Br), 686 (C-Br); **δ_H (300 MHz, $CDCl_3$)** 0.95 (3H, t, *J* = 6.9Hz, 5'-H), 1.39-1.44 (4H, m, 3'-H and 4'-H), 1.53-1.58 (2H, m, 2'-H), 2.75 (2H, t, *J* = 7.5Hz, 1'-H), 7.54 (1H, br. s, O*H*); **δ_C (75 MHz, $CDCl_3$)** 13.9 (CH_3, 5'-C), 22.3 (CH_2, 3'-C or

4'-C), 27.1 (CH_2, 2'-C), 30.5 (CH_2, 1'-C), 31.8 (CH_2, 3'-C or 4'-C), 106.0 (quat., 3-C), 137.8 (quat., 5-C), 145.7 (quat., 6-C), 153.2 (quat., 2-C), 172.9 (quat., 4-C), 178.1 (quat., 1-C).

5.2.17 4-Nitro-2-fluoroaniline 210a and 6-nitro-2-fluoroaniline 210b (1976ACB141)



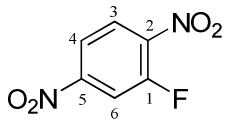
A 500mL 3 necked round bottomed flask equipped with a dropping funnel and a thermometer was charged with 2-fluoroacetanilide **209** (16.8651g, 0.1101mol) and glacial acetic acid (90mL). The resulting solution was cooled to 10°C (internal T). The dropping funnel was charged with a solution of fuming HNO_3 (55mL) and conc. H_2SO_4 (55mL), which was added dropwise to the 2-fluoroacetanilide solution. The temperature was maintained at 10°C during the addition (1h). The ice-bath was then removed and the resulting solution was stirred for a further 1h 30min. The reaction mixture was then poured into ice, resulting in the formation of a cream coloured precipitate. The precipitate was recovered by filtration, washed several times with water until the filtrate was of a clear colour, and dried under vacuum. The resulting solid was partially redissolved in EtOH (250mL) and a 10% aq. solution of NaOH (150mL) was added to the mixture. The resulting orange solution was stirred for 5h or until the acetyl product was fully hydrolysed. The reaction was then quenched with 10% aq. HCl (200mL) resulting in the formation of a yellow precipitate. The precipitate was recovered by filtration, washed several times with water and dried. It was then redissolved into EtOAc, dried ($MgSO_4$) and purified by column chromatography, eluting with petroleum ether (60-80°C) : EtOAc (70:30). The more mobile band gave 6-nitro-2-fluoroaniline **210b** (2.64g, 16.91mmol, 15.3%) as a yellow solid. Recrystallisation from EtOH produced yellow plates; **m.p.**: 71.5-72.5°C [lit. 75.5-76.5°C (1976ACB141)]; (found: C, 46.0; H, 3.2; N, 17.9%. $C_6H_5FN_2O_2$ requires C, 46.2; H, 3.2; N, 17.9%); ν_{max}/cm^{-1} 3487 and 3366 (NH_2), 1583 (C=C), 1525 and 1329 (NO_2), 1068 (C-F); **δ_H (300 MHz, d_6-DMSO)** 6.62 (1H, td, *J* = 8.7, 5.4Hz, 4-H), 7.22 (2H, br. s, N*H*$_2$), 7.42 (1H, dd, *J* = 10.2, 7.8Hz, 3-H), 7.82 (1H, d, *J* = 8.7Hz, 5-H); **δ_C (75 MHz, d_6-DMSO)** 113.95 (CH, d, *J* = 7.9Hz, 4-C), 120.2 (CH, d, *J* =

18.3Hz, 3-C), 121.5 (CH, d, *J* = 3.3Hz, 5-C), 132.4 (quat., d, *J* = 5.1Hz, 6-C), 136.45 (quat., d, *J* = 16.6Hz, 1-C), 152.2 (quat., d, *J* = 242.7Hz, 2-C). Further elution gave 4-nitro-2-fluoroaniline **210a** (11.61g, 74.37mmol, 67.5%) as a bright yellow crystalline solid. Recrystallisation from ethanol gave small yellow needles; **m.p.**: 130-131°C [lit. 135-136°C (1976ACB141)]; ν_{max}/cm^{-1} 3493 and 3395 (NH_2), 1626 (C=C), 1523 and 1314 (NO_2), 1079 (C-F); **δ_H (300 MHz, d_6-DMSO)** 6.77 (2H, br. s, N*H*$_2$), 6.85 (1H, t, *J* = 8.7Hz, 6-H), 7.87-7.93 (2H, m, 3-H and 5-H); **δ_C (75 MHz, d_6-DMSO)** 111.8 (CH, d, *J* = 22.3Hz, 3-C), 114.3 (CH, d, *J* = 5.4Hz, 6-C), 122.7 (CH, d, *J* = 1.8Hz, 5-C), 135.3 (quat., d, *J* = 7.8Hz, 4-C), 144.8 (quat., d, *J* = 13.1Hz, 1-C), 148.4 (quat., d, *J* = 241.2Hz, 2-C).

5.2.18 General procedure for the oxidation of fluoroanilines (adapted from 1992TL4835)

In a 250mL 3 necked flask equipped with a pressure equalizing dropping funnel and a thermometer, urea hydrogen peroxide adduct (12mol equivalent) was suspended in acetonitrile (100mL) and the resulting suspension was cooled in a salt-ice bath to -10°C (internal temperature). The pressure equalizing dropping funnel was charged with a solution of trifluoroacetic anhydride (10mol equivalent) in acetonitrile (20mL), which was added dropwise to the urea hydrogen peroxide adduct suspension. The rate of the addition was adjusted in such a way that the temperature did not rise above 5°C. The resulting clear solution was then stirred at -5°C during 45 min. A solution of the correctly substituted fluoroaniline (1mol equivalent) was prepared in acetonitrile (50mL) and then added dropwise *via* the pressure equalizing dropping funnel to the trifluoroperacetic acid solution. The temperature was kept around -5°C during the addition by adjusting the addition rate. Once the addition was finished, the cooling bath was removed and the resulting solution left with stirring at ambient temperature overnight. The solution colour ranged from emerald green to blue during addition of the fluoroaniline and then slowly turned to yellow toward completion of the reaction. Most of the acetonitrile was then removed under reduced pressure and the acidic residue was neutralised by addition of a 10% aqueous Na_2CO_3 solution, until no more evolution of CO_2 was observed. The resulting mixture was extracted with Et_2O (3 x 80mL) and the combined organic layers washed once with water (100mL) and brine (100mL), and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue subjected to column chromatography.

5.2.18.1 2,5-Dinitro-1-fluorobenzene 151 (2002TL3221)



The title compound was prepared from 2-fluoro-4-nitroaniline **210a** (9.90g, 63.40mmol). The crude product was recrystallised from petroleum ether (60-80°C) using decolourising charcoal. 2,5-Dinitro-1-fluorobenzene **151** (6.97g, 37.45mmol, 59.1%) was isolated as large pale yellow plates; **m.p.**: 72-74°C [lit.: 74-75°C (from hexane, 2002TL3221)]; (found: C, 38.5; H, 1.6; N, 14.7%. $C_6H_3FN_2O_4$ requires C, 38.7; H, 1.6; N,15.05%); ν_{max} /cm^{-1} 3118 (C-H), 1549 and 1343 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 8.26 (1H, ddd, *J* = 9.0, 2.4, 1.5 Hz, 4-H), 8.42 (1H, dd, *J* = 9.0, 7.5 Hz, 3-H), 8.53 (1H, dd, *J* = 10.5, 2.4 Hz, 6-H); **δ_C(75 MHz, d_6- DMSO)** 115.05 (CH, d, *J* = 26.0 Hz, 6-C), 120.75 (CH, d, *J* = 4.45 Hz, 4-C), 128.2 (CH, d, *J* = 2.5 Hz, 3-C), 141.2 (quat., d, *J* = 7.9 Hz, 5-C), 151.2 (quat., d, *J* = 8.5 Hz, 2-C), 154.8 (quat., d, *J* = 264.9 Hz, 1-C).

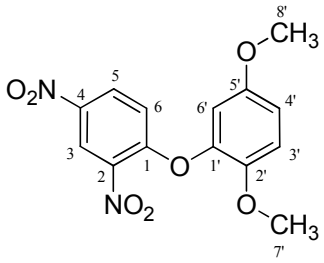
5.2.19 General procedure for the preparation of biarylethers 169a-g and 152c-d

***Method A: 2,4-dinitrofluorobenzene* 168** (adapted from 1957JOC1743). The correctly substituted phenol (1mol equivalent) was dissolved in DMSO (50mL) in a round bottomed flask and a base, Et_3N (1mol equivalent) was added to the resulting solution. The resulting solution was stirred for 5min, before 2,4-dinitro-1-fluorobenzene (1mol equivalent) was added; the solution colour turned to orange-red. The reaction was left with stirring for 5 hours, then water (200mL) was added, resulting in the formation of a precipitate. The mixture was then extracted with DCM (3 × 100mL) and the combined DCM extracts were successively washed with a 10% aqueous solution of sodium hydroxide (3 × 100mL), water (100mL) and brine (150mL), and then dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was either subjected to column chromatography or directly recrystallised from ethanol.

***Method B: 2,5-dinitrofluorobenzene* 151** (adapted from 2008OBC682). The correctly substituted phenol (1mol equivalent) was dissolved in dry DMF (30mL) in an oven dried two necked flask equipped with reflux condenser and $CaCl_2$ drying tube. NaH in an oil dispersion 60% w/w (2.2mol equivalent) was then added portion-wise and, once evolution of H_2 had stopped, a solution of 2,5-dinitrofluorobenzene (1mol

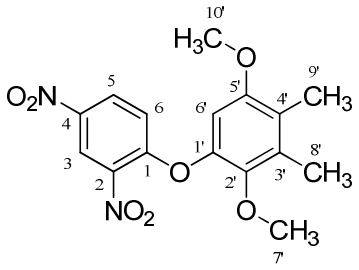
equivalent) was added in DMF (10mL). The resulting deep red solution was left with stirring for 5h and then heated at 50℃ for another 5h. The reaction was then allowed to cool down to room temperature and quenched with brine (100mL). The resulting mixture was extracted with DCM (3 × 80 mL) and the combined DCM layers washed with water (100mL), brine (2 × 100mL) and then dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue subjected to column chromatography, eluting with a solvent mixture of petroleum ether (60:80℃) : EtOAc. The product was recrystallised from EtOH.

5.2.19.1 2,4-Dinitro-2',5'-dimethoxydiphenylether 169g



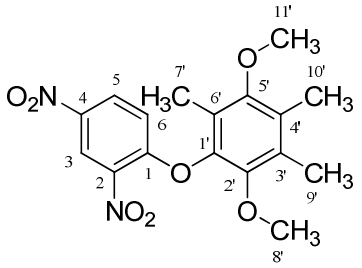
The title compound was prepared as described (**method A**) from 2,5-dimethoxyphenol **150g** (6.38g, 41.37mmol) and 2,4-dinitrofluorobenzene **168** (7.70g, 41.37mmol). 2,4–Dinitro–2,5–dimethoxydiphenylether **169g** (11.68g, 36.47mmol, 88.2%) was isolated as crescent shaped yellow crystals from EtOH; **m.p.** 95-96℃; *m/z* 343.1 $(MNa)^+$; (found C, 52.4; H, 3.7; N, 8.7%. $C_{14}H_{12}N_2O_6$ requires C, 52.5; H, 3.8; N, 8.75%); ν_{max} /cm^{-1} 1604 (C=C), 1506 and 1340 (NO_2), 1274, 1035 and 1014 (C-O); **δ_H (300 MHz, d_6-DMSO)** 3.69 (3H, s, O*CH*$_3$, 7'-H), 3.74 (3H, s, O*CH*$_3$, 8'-H), 6.92 (1H, dd, *J* = 9.0, 3.0Hz, 4'-H), 6.98 (1H, d, *J* = 2.7Hz, 6'-H), 6.99 (1H, d, *J* = 9.3Hz, 6-H), 7.20 (1H, d, *J* = 9.0Hz, 3'-H), 8.39 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.86 (1H,d, *J* = 3.0Hz, 3-H); **δ_C(75 MHz, d_6-DMSO)** 56.2 (CH_3, 7'-C), 56.8 (CH_3, 8'-C), 109.1 (CH, 6'-C), 113.1 (CH, 4'-C), 115.3 (CH, 3'-C), 117.9 (CH, 6-C), 122.2 (CH, 3-C), 129.9 (CH, 5-C), 138.6 (quat., 2-C), 141.4 (quat., 4-C), 141.7 (quat., 1'-C), 145.1 (quat., 2'-C), 154.3 (quat., 5'-C), 155.6 (quat., 1-C).

5.1.19.2 2,4-Dinitro-2',5'-dimethoxy-3',4'-dimethyldiphenylether 169a



The title compound was prepared as described (**method A**) from 2,5-dimethoxy-3,4-dimethylphenol **150a** (0.65g, 3.56mmol). Elution with petroleum ether (60-80℃) : EtOAc (70:30) yielded 2,4-dinitro-2',5'-dimethoxy-3',4'-dimethyldiphenylether **169a** as an orange solid (0.76g, 2.19mmol, 61.6%). Orange crystals were obtained from aqueous ethanol, **m.p.**: 100℃; (found C, 55.4; H, 4.6; N, 8.0%. $C_{16}H_{16}N_2O_7$ requires C, 55.2; H, 4.6; N, 8.0%); *m/z* 371.0 $(MNa)^+$; $\boldsymbol{\nu_{max}}$/cm^{-1} 1606 (C=C), 1529 and 1345 (NO_2), 1263, 1233 and 1026 (C-O); $\boldsymbol{\delta_H}$ **(300 MHz, $\boldsymbol{d_6}$-DMSO)** 2.13 (3H, s, CH_3, 9'-H), 2.19 (3H, s, C*H*$_3$, 8'-H), 3.60 (3H, s, OC*H*$_3$, 7'-H), 3.77 (3H, s, OC*H*$_3$, 10'-H), 6.89 (1H, s, 6'-H), 7.01 (1H, d, *J* = 9.0Hz, 6-H), 8.38 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.88 (1H, d, *J* = 3.0Hz, 3-H); $\boldsymbol{\delta_C}$ **(75 MHz, $\boldsymbol{d_6}$-DMSO)** 12.3 (CH_3, 9'-C), 12.8 (CH_3, 8'-C), 56.5 (CH_3, 10'-C), 61.4 (CH_3, 7'-C), 103.4 (CH, 6'-C), 118.0 (CH, 6-C), 122.1 (CH, 3-C), 124.4 (quat., 4'-C), 129.9 (CH, 5-C), 132.9 (quat., 3'-C), 138.7 (quat., 2-C), 141.3 (quat., 4-C), 143.2 (quat., 1'-C), 143.7 (quat., 2'-C), 154.4 (quat., 5'-C), 155.7 (quat., 1-C).

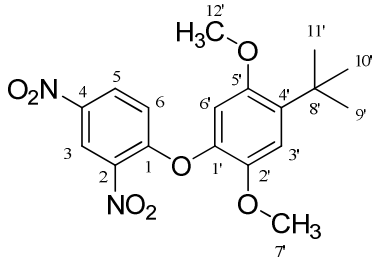
5.1.19.3 2,4-Dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether 169b



The title compound was prepared as described (**method A**) from 2,5-dimethoxy-3,4,6-trimethylphenol **150b** (0.37g, 1.89mmol), using 1,8-diazabicyclo[5-4-0]undec-7-ene (DBU) (0.30mL, 2.00mmol) instead of Et_3N. Elution with petroleum ether (60-80℃) : EtOAc (30:70) yielded 2,4-dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether **169b** as an off-white solid (0.59g, 1.63mmol, 85.7%). Long off-white needles were obtained from ethanol; **m.p.**: 142℃; (found C, 56.4; H, 5.05; N, 7.7%. $C_{17}H_{18}N_2O_7$ requires C, 56.35; H, 5.0; N, 7.7%); *m/z* 385.1 $(MNa)^+$; $\boldsymbol{\nu_{max}}$/cm^{-1}

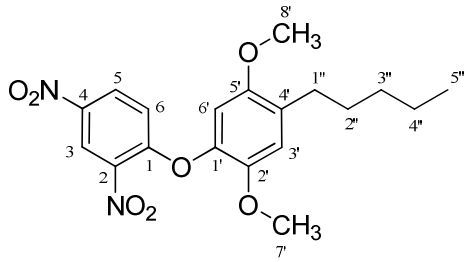
1609 (C=C), 1540 and 1345 (NO_2), 1260, 1250 and 1072 (C-O); **δ_H (300 MHz, $CDCl_3$)** 2.04 (3H, s, C*H*$_3$, 7'-H), 2.12 (3H, s, C*H*$_3$, 10'-H), 2.16 (3H, s, C*H*$_3$, 9'-H), 3.59 (3H, s, OC*H*$_3$, 8'-H), 3.63 (3H, s, OC*H*$_3$, 11'-H), 6.69 (1H, d, *J* = 9.3Hz, 6-H), 8.15 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.76 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 9.6 (CH_3, 7'-C), 12.4 (CH_3, 9'-C or 10'-C), 12.8 (CH_3, 9'-C or 10'-C), 60.5 (CH_3, 8'-C), 61.2 (CH_3, 11'-C), 116.6 (CH, 6-C), 122.0 (CH, 3-C), 122.1 (quat., 6'-C), 128.9 (CH, 5-C), 129.6 (quat., 3'-C or 4'-C), 130.0 (quat., 3'-C or 4'-C), 138.15 (quat., 2-C), 141.0 (quat., 4-C), 142.2 (quat., 1'-C), 146.1 (quat., 2'-C), 153.5 (quat., 5'-C), 155.8 (quat., 1-C).

5.1.19.4 2,4-Dinitro-2',5'-dimethoxy-4'-*t*butyldiphenylether 169c



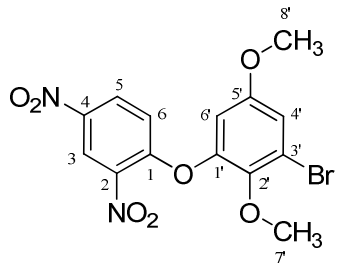
The title compound was prepared as described (***method A***) from 2,5-dimethoxy-4-*t*butylphenol **150c** (3.55g, 16.88mmol). Elution with petroleum ether (60-80°C) : Et_2O (70:30) yielded 2,4-dinitro-2',5'-dimethoxy-4'-*t*butyldiphenylether **169c** as a yellow crystalline solid (6.06g, 16.09mmol, 95.8%). Recrystallisation from EtOH produced small bright yellow needles; **m.p.**: 131-132°C; (found C, 57.45; H, 5.4; N, 7.4%. $C_{18}H_{20}N_2O_7$ requires C, 57.4; H, 5.4; N, 7.4%); *m/z* 399.1 (*MNa*)$^+$; **ν_{max}**/cm^{-1} 1608 (C=C), 1535 and 1342 (NO_2), 1209 and 1031 (C-O); **δ_H (300 MHz, $CDCl_3$)** 1.32 (9H, s, C(C*H*$_3$)$_3$), 3.64 (3H, s, OC*H*$_3$, 7'-H), 3.72 (3H, s, OC*H*$_3$, 12'-H), 6.66 (1H, s, 6'-H), 6.84 (1H, d, *J* = 9.3Hz, 6-H), 6.94 (1H, s, 3'-H), 8.17 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.74 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 29.7 (3 × CH_3, 9'/10'/11'-C), 35.2 (quat., 8'-C), 55.8 (CH_3, 7'-C), 57.1 (CH_3, 12'-C), 106.5 (CH, 6'-C), 113.8 (CH, 3'-C), 117.5 (CH, 6-C), 122.0 (CH, 3-C), 128.6 (CH, 5-C), 138.1 (quat., 4'-C), 138.3 (quat., 2-C), 139.5 (quat., 1'-C), 141.0 (quat., 4-C), 143.8 (quat., 2'-C), 153.4 (quat., 5'-C), 156.5 (quat., 1-C).

5.1.19.5 2,4-Dinitro-2',5'-dimethoxy-3'-pentyldiphenylether 169d



The title compound was prepared as described (**method A**) from 2,5-dimethoxy-4-pentylphenol **150d** (2.71g, 12.09mmol). 2,4-Dinitro-2',5'-dimethoxy-3'-pentyldiphenylether **169d** was isolated as a yellow crystalline solid (4.36g, 11.17mmol, 92.4%). Recrystallisation from EtOH yielded a yellow crystalline solid; **m.p.** (EtOH): 85-87°C; (found: C, 58.3; H, 5.7; N, 7.2%. $C_{19}H_{22}N_2O_7$ requires C, 58.5; H, 5.7; N, 7.2%); *m/z* 390.1 $(MH)^+$, 413.1 $(MNa)^+$; ν_{max}/cm^{-1} 2855-2957 (C-H), 1608, 1508 (C=C), 1536 and 1342 (NO_2), 1212 and 1037 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.96 (3H, t, *J* = 6.9Hz, 5''-H), 1.39-1.43 (4H, m, 3''-H or 4''-H), 1.62-1.67 (2H, m, 2''-H), 2.66 (2H, t, *J* = 7.5Hz, 1''-H), 3.755 (3H, s, OC*H*$_3$, 7'-H), 3.82 (3H, s, OC*H*$_3$, 8'-H), 6.76 (1H, s, 6'-H), 6.91 (1H, s, 3'-H), 6.96 (1H, d, *J* = 9.3Hz, 6-H), 8.31 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.87 (1H, d, *J* = 3.0Hz, 3-H); **δ_C(75 MHz, $CDCl_3$)** 14.1 (CH_3, 5''-C), 22.6 (CH_2, 3''-C or 4''-C), 29.6 (CH_2, 2''-C), 30.1 (CH_2, 1''-C), 31.75 (CH_2, 3''-C or 4''-C), 56.1 (CH_3, 8'-C), 56.9 (CH_3, 7'-C), 105.6 (CH, 6'-C), 115.85 (CH, 3'-C), 117.4 (CH, 6-C), 122.0 (CH, 3-C), 128.65 (CH, 5-C), 131.0 (quat., 4'-C), 138.3 (quat., 2-C), 139.3 (quat., 1'-C), 141.0 (quat., 4-C), 144.3 (quat., 2'-C), 152.2 (quat., 5'-C), 156.6 (quat., 1-C)

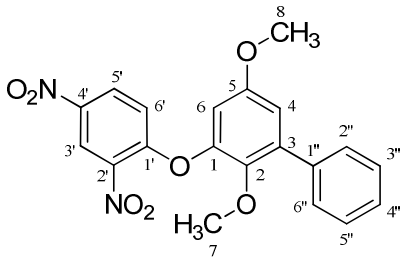
5.1.19.6 2,4-Dinitro-6'-bromo-2',5'-dimethoxydiphenylether 169e



The title compound was prepared as described (**method A**) from 3-bromo-2,5-dimethoxyphenol **150e** (0.70g, 2.99mmol); elution used petroleum ether (60-80°C) : EtOAc (70:30). 2,4-Dinitro-6'-bromo-2',5'-dimethoxydiphenylether **169e** was isolated as a white crystalline solid (0.87g, 2.17mmol, 72.6%). Recrystallisation from EtOH

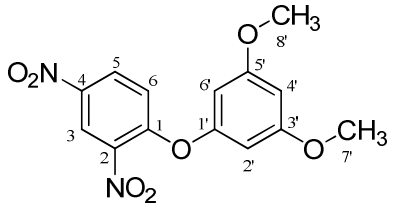
produced white plates; **m.p.** (EtOH): 109-110°C; (found: C, 42.1; H, 2.75; N, 6.8%. $C_{14}H_{11}BrN_2O_7$ requires C, 42.1; H, 2.8; N, 7.0%); *m/z* 421.0 (95%), 423.0 (100%) $(MNa)^+$; ν_{max}/cm^{-1} 1601, 1567 and 1529 (C=C), 1528 and 1337 (NO_2), 1270, 1228 and 1039 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.79 (3H, s, O*CH*$_3$, 7'-H), 3.84 (3H, s, O*CH*$_3$, 8'-H), 6.76 (1H, d, *J* = 3.0Hz, 6'-H), 6.96 (1H, d, *J* = 9.3Hz, 6-H), 7.13 (1H, d, *J* = 2.7Hz, 4'-H), 8.33 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.88 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 56.1 (CH_3, 7'-C), 61.7 (CH_3, 8'-C), 108.2 (CH, 6'-C), 116.7 (CH, 4'-C), 117.7 (CH, 6-C), 119.2 (quat., 3'-C), 122.0 (CH, 3-C), 128.9 (CH, 5-C), 138.7 (quat., 2-C), 141.7 (quat., 4-C), 143.0 (quat., 2'-C), 146.9 (quat., 1-C), 155.3 (quat., 1'-C), 156.9 (quat., 5'-C).

5.1.19.7 3-(2',4'-Dinitrophenoxy)-2,5-dimethoxybiphenyl 169f



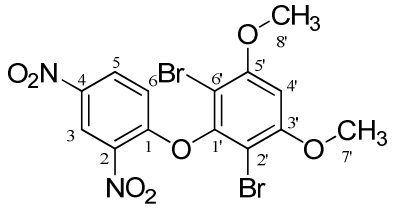
The title compound was prepared as described (**method A**) from 2,5-dimethoxybiphenyl-3-ol **150f** (0.98g, 4.25mmol). The crude product was recrystallised from EtOH. 3-(2',4'-Dinitrophenoxy)-2,5-dimethoxybiphenyl **169f** was isolated as cream plates (1.55g, 3.96mmol, 91.8%); **m.p.**: 146-148°C; (found: C, 60.6; H, 4.1; N, 7.0%. $C_{20}H_{16}N_2O_7$ requires C, 60.6; H, 4.1; N, 7.1%); *m/z* 397.1 $(MH)^+$, 419.1 $(MNa)^+$; ν_{max}/cm^{-1} 1610 (C=C), 1529 and 1337 (NO_2), 1271 and 1048 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.32 (3H, s, OC*H*$_3$, 7-H), 3.75 (3H, s, OC*H*$_3$, 8-H), 6.68 (1H, d, *J* = 3.0Hz, 6-H), 6.81 (1H, d, *J* = 3.0Hz, 4-H), 7.01 (1H, d, *J* = 9.3Hz, 6'-H), 7.26-7.37 (3H, m, 3''-H, 5''-H and 4''-H), 7.42 (2H, m, 2''-H and 6''-H), 8.25 (1H, dd, *J* = 9.3, 2.7Hz, 5'-H), 8.76 (1H, d, *J* = 2.7Hz, 3'-H); **δ_C (75 MHz, $CDCl_3$)** 55.95 (CH_3, 8-C), 61.4 (CH_3, 7-C), 107.2 (CH, 6-C), 114.3 (CH, 4-C), 117.7 (CH, 6'-C), 121.95 (CH, 3'-C), 128.0 (CH, 4''-C), 128.5 (CH, 3''-C and 5''-C), 128.9 (CH, 5'-C), 129.0 (CH, 2''-C and 6''-C), 136.9 (quat., 3-C), 138.1 (quat., 1''-C), 138.6 (quat., 4'-C), 141.4 (quat., 2'-C), 142.7 (quat., 2-C), 146.7 (quat., 1-C), 156.1 (quat., 1'-C), 156.4 (quat., 5-C).

5.1.19.8 2,4-Dinitro-3',5'-dimethoxydiphenylether 175c



The title compound was prepared as described (**method A**) from 3,5-dimethoxyphenol **177** (3.14g, 20.37mmol). Recrystallisation from EtOH yielded 2,4-dinitro-3',5'-dimethoxydiphenylether **175c** (5.96g, 18.61mmol, 91.2%) as pale yellow needles; **m.p.**: 105-107°C; (found: C, 52.3; H, 3.8; N, 8.75%. $C_{14}H_{12}N_2O_7$ requires C, 52.5; H, 3.8; N, 8.75%); *m/Z* 321.1 $(MH)^+$; ν_{max}/cm^{-1} 1598 and 1582 (C=C), 1535 and 1343 (NO_2), 1261 and 1055 (C-O); **δ_H (300 MHz, $CDCl_3$)** 3.71 (6H, s, 2 × OCH_3, 7'-H and 8'-H), 6.20 (2H, d, *J* = 2.4Hz, 2'-H and 6'-H), 6.32 (1H, t, *J* = 2.1Hz, 4'-H), 7.05 (1H, d, *J* = 9.3Hz, 6-H), 8.24 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.73 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 55.7 (CH_3, 7'-C and 8'-C), 98.5 (CH, 4'-C), 99.0 (CH, 2'-C and 6'-C), 118.8 (CH, 6-C), 122.0 (CH, 3-C), 128.8 (CH, 5-C), 139.5 (quat., 4-C), 141.5 (quat., 2-C), 155.2 (quat., 1'-C), 156.0 (quat., 1-C), 162.3 (quat., 3'-C and 5'-C).

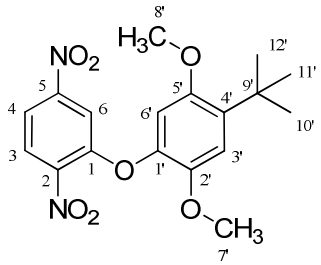
5.1.19.9 2,4-Dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether 175b



The title compound was prepared as described (**method A**) from 2,6-dibromo-3,5-dimethoxyphenol **176** (2.00g, 6.41mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (70:30 to 50:50) yielded 2,4-dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether **175b** as an off white solid (1.99g, 4.17mmol, 65.0%). White crystals were obtained from petroleum ether (60-80°C) : EtOAc; **m.p.** : 236-239°C; (found: C, 35.1; H, 2.15; N, 5.8%. $C_{14}H_{10}Br_2N_2O_7$ requires C, 35.2; H, 2.1; N, 5.9%); *m/Z* 477.0 (43%), 479.0 (100%), 481.1 (45%) $(MH)^+$; ν_{max}/cm^{-1} 1614 and 1579 (C=C), 1535 and 1345 (NO_2), 1215 and 1093 (C-O); **δ_H (300 MHz, d_6-DMSO)** 4.00 (6H, s, 2 x OCH_3, 7'-H and 8'-H), 6.96 (1H, s, 4'-H), 6.96 (1H, d, *J* = 9.0Hz, 6-H), 8.39 (1H, dd, *J* = 9.0, 3.0Hz, 5-H), 8.94 (1H, d, *J* = 3.0Hz, 3-H); **δ_C (75 MHz, d_6-DMSO)** 57.7 (CH_3, 7'-C and 8'-C), 97.2 (quat., 2'-C and 6'-C), 97.3 (CH, 4'-C), 117.0 (CH,

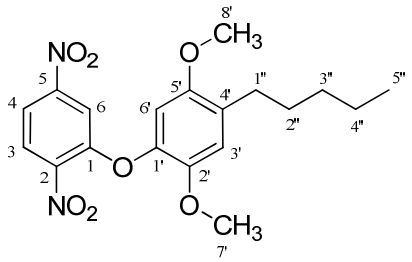
6-C), 122.6 (CH, 3-C), 130.1 (CH, 5-C), 138.7 (quat., 2-C), 142.3 (quat., 4-C), 147.9 (quat., 1'-C), 152.9 (quat., 1-C), 157.7 (quat., 3'-C and 5'-C).

5.1.19.10 2,5-Dinitro-4'-^{t}butyl-2',5'-dimethoxydiphenylether 152c (2008OBC682)



The title compound was prepared as described (**method B**) from 2,5-dimethoxy-4-^{t}butylphenol **150c** (1.88g, 8.92mmol). 2,5-Dinitro-4'-^{t}butyl-2',5'-dimethoxydiphenylether **152c** was isolated as an orange solid (1.59g, 4.23mmol, 47.4%). Recrystallisation from EtOH produced bright orange plates; **m.p.** (EtOH): 138°C; (found: C, 57.4; H, 5.35; N, 7.25%. $C_{18}H_{20}N_2O_7$ requires C, 57.4; H, 5.4; N, 7.4%); *m/Z* 399.1 $(MNa)^+$; ν_{max}/cm^{-1} 1586 (C=C), 1537 and 1348 (NO_2), 1206 and 1038 (C-O); **δ_H (300 MHz, $CDCl_3$)** 1.33 (9H, s, C(CH_3)$_3$), 3.64 (3H, s, OCH_3, 7'-H), 3.72 (3H, s, OCH_3, 8'-H), 6.66 (1H, s, 6'-H), 6.94 (1H, s, 3'-H), 7.56 (1H, d, *J* = 3.0Hz, 6-H), 7.82 (1H, dd, *J* = 8.7, 2.1Hz, 4-H), 7.92 (1H, d, *J* = 8.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 29.7 (CH_3, 10'/11'/12'-C), 35.2 (quat., 4'-C), 55.8 (CH_3, 8'-C), 57.0 (CH_3, 7'-C), 106.5 (CH, 6'-C), 112.8 (CH, 6-C), 113.8 (CH, 3'-C), 116.3 (CH, 4-C), 126.1 (CH, 3-C), 137.9 (quat., 4'-C), 139.7 (quat., 1'-C), 142.8 (quat., 5-C), 143.9 (quat., 2'-C), 150.4 (quat., 2-C), 152.3 (quat., 1-C), 153.4 (quat., 5'-C).

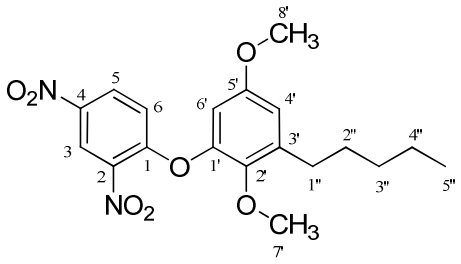
5.1.19.11 2,5-Dinitro-2',5'-dimethoxy-3'-pentyldiphenylether 152d



The title compound was prepared as described (**method B**) from 2,5-dimethoxy-4-pentylphenol **150d** (2.07g, 9.25mmol). The product was purified by column chromatography, eluting with petroleum ether (60-80°C) : Et_2O (75:25). 2,5-Dinitro-2',5'-dimethoxy-3'-pentyldiphenylether **152d** was isolated as a bright orange solid

(1.69g, 4.33mmol, 46.8%). Recrystallisation from EtOH produced small yellow needles; **m.p.**: 77-79°C; (found: C, 58.3; H, 5.65; N, 7.2%. $C_{19}H_{22}N_2O_7$ requires C, 58.5; H, 5.7; N, 7.2%); *m/Z* 391.2 $(MH)^+$, 413.2 $(MNa)^+$; ν_{max}/cm^{-1} 2853-2952 (C-H), 1629 (C=C), 1531 and 1347 (NO_2), 1209 and 1038 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.84 (3H, t, *J* = 6.9Hz, 5''-H), 1.25-1.30 (4H, m, 3''-H and 4''-H), 1.48-1.55 (2H, m, 2''-H), 2.54 (2H, t, *J* = 8.1Hz, 1''-H), 3.63 (3H, s, OC*H*$_3$, 7'-H), 3.70 (3H, s, OC*H*$_3$, 8'-H), 6.63 (1H, s, 6'-H), 6.785 (1H, s, 3'-H), 7.53 (1H, d, *J* = 2.4Hz, 6-H), 7.83 (1H, dd, *J* = 9.0, 2.4Hz, 4-H), 7.93 (1H, d, *J* = 8.7Hz, 3-H); **δ_C (75 MHz, $CDCl_3$)** 14.0 (CH_3, 5''-C), 22.6 (CH_2, 3''-C or 4''-C), 29.6 (CH_2, 2''-C), 30.1 (CH_2, 1''-C), 31.8 (CH_2, 3''-C or 4''-C), 56.1 (CH_3, 8'-C), 56.9 (CH_3, 7'-C), 105.6 (CH, 6'-C), 112.6 (CH, 6-C), 115.9 (CH, 3'-C), 116.25 (CH, 4-C), 126.1 (CH, 3-C), 130.8 (quat., 4'-C), 139.5 (quat., 1'-C), 142.8 (quat., 5-C), 144.4 (quat., 2'-C), 150.4 (quat., 2-C), 152.2 (quat., 5'-C), 152.4 (quat., 1-C).

5.1.19.12 Attempted preparation of 2,4-dinitro-3'-pentyl-2',5'-dimethoxydiphenylether 169p



Method A (adapted from 1997TA913): Preparation of the title compound was attempted from 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether **169e** (0.69g, 1.72mmol) following the method described earlier for 1,4-dimethoxy-2-pentylbenzene **196**. Overnight reaction resulted in the formation of a black, inextractable tar. No products could be isolated.

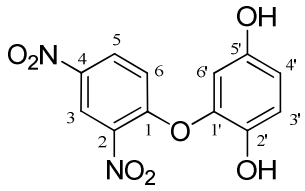
Method B (adapted from 2005OS33): A 100mL two-necked round-bottomed flask equipped with a pressure equilizing dropping funnel and a reflux condenser was charged with Mg turnings (0.08g, 3.07mmol) and flame dried under a flow of N_2. Dry THF (10mL) and 1,2-dibromoethane (0.5mL) were then successively added to the Mg turnings and the resulting suspension stirred. The pressure equilizing dropping funnel was charged with a solution of THF (10mL) and 1-bromopentane (0.31mL, 2.50mmol), which was added dropwise to the Mg turnings suspension. The resulting mixture was gently heated with an air gun until the Mg turning started to react; once

all Mg turnings were consummed, the resulting solution was refluxed for 10min. In the mean time, a flame dried 100mL two-necked round-bottomed flask flushed with N_2 was charged with 2,4-dinitro-3'-bromo-2',5'-dimethoxydiphenylether **169e** (0.77g, 1.92mmol), $Fe(acac)_3$ (0.035g, 0.10mmol), dry THF (30mL) and dry *N*-methyl-2-pyrrolidone (NMP, 5mL). The resulting orange solution was cooled with an ice bath and the previously prepared solution of pentylmagnesium bromide was added to it *via* canula. This resulted in the formation of a white precipitate and the resulting mixture was allowed to warm to R.T. and stirred for 24h. TLC monitoring of the reaction showed no evolution of the reaction, and quenching of the reaction with water (100mL) followed by extraction with EtOAc (2 × 50mL) resulted in full recovery of the starting materials.

5.1.20 General procedure for the deprotection of substituted 2,4-dinitro-2',5'-dimethoxydiphenyl ethers 169b and 169g

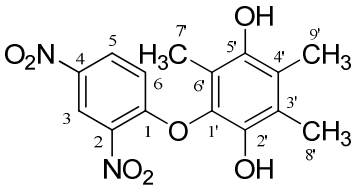
In a 250 mL flame dried 2 necked round bottomed flask equipped with a thermometer and flushed with N_2, the substituted biarylether (1mol equivalent) was dissolved in dry DCM (70mL). The resulting solution was cooled to -78℃ in a dry ice/acetone bath. Once the temperature reached -78℃, a 1M DCM solution of BBr_3 (3.8mol equivalents) was slowly added to the biarylether solution. The temperature was kept below -55℃ during the addition. The resulting solution was stirred with the dry ice/acetone bath over 40 min, then was allowed to warm up to room temperature and left with stirring overnight under a nitrogen atmosphere. After 18-20 hours the solution was cooled again to -78℃ and more 1M DCM solution of BBr_3 (1.6 mol equivalents) was added. The resulting solution was allowed to warm to room temperature and stirred for a further 5 hours. It was then quenched with ice to neutralise the excess BBr_3, and the DCM was removed under reduced pressure. The resulting mixture was extracted with diethyl ether (3x100mL) and the combined organic extracts were washed once with brine (200mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue subjected to column chromatography.

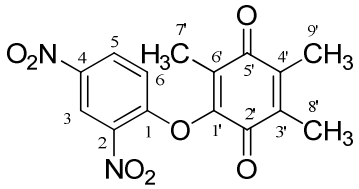
5.1.20.1 2,4-Dinitro-2',5'-dihydroxydiphenylether 170a



The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxydiphenylether **169g** (4.01g, 12.50mmol). The product was purified by column chromatography, eluting with petroleum ether (60-80℃) : EtOAc (30:70). 2,4–Dinitro–2',5'–dihydroxydiphenylether **170a** was isolated as an amorphous hygroscopic yellow solid (3.53g, 12.10mmol, 96.7%) and was further purified by precipitation from DCM; **m.p.**: 113-115℃; (found: C, 49.0; H, 2.8; N, 9.55%. $C_{12}H_8N_2O_6$ requires C, 49.3; H, 2.8; N, 9.6%); *m/Z* 243.9 $(M\text{-}H_2NO_2)^-$, 290.9 $(M\text{-}H)^-$; ν_{max}/ cm^{-1} 3401 and 3347 (OH), 1600 (C=C), 1506 and 1340 (NO_2); **δ_H (300 MHz, d_6-DMSO)** 6.29 (1H, dd, *J* = 8.7, 2.7Hz, 4'-H), 6.49 (1H, d, *J* = 2.7Hz, 6'-H), 6.97 (1H, d, *J* = 3.3Hz, 6-H), 7.00 (1H, d, *J* = 2.7Hz, 3'-H), 8.39 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.82 (1H, d, *J* = 3.0Hz, 3-H), 9.49 (1H, s, 5'-O*H*), 9.84 (1H, s, 2'-O*H*); **δ_C (75 MHz, d_6-DMSO)** 104.8 (CH, 6'-C), 107.2 (CH, 4'-C), 117.6 (CH, 6-C), 122.1 (CH, 3-C), 123.2 (CH, 3'-C), 129.8 (CH, 5-C), 133.0 (quat., 2'-C), 138.5 (quat., 2-C), 140.85 (quat., 4-C), 149.8 (quat., 1'-C), 156.7 (quat., 5'-C), 157.1 (quat., 1-C).

5.1.20.2 2,4-Dinitro-2',5'-dihydroxy-3',4',6'-trimethyldiphenylether 170b and 2,4-dinitrophenoxy-3',4',6'-trimethyl-2',5'-benzoquinone 179b



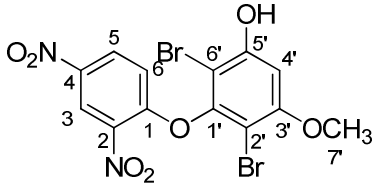


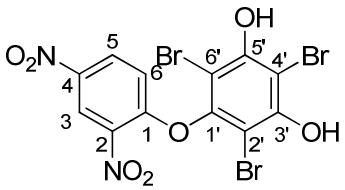
The title compounds were prepared as described from 2,4-dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether (3.38g, 9.32mmol); elution used petroleum ether (60-80℃) : Et_2O (70:30). The collected fractions were combined and left at room temperature until crystals of 2,4-dinitro-2',5'-dihydroxy-3',4',6'-trimethyldiphenylether **170b** formed. The crystals were removed by filtration yielding 2,4-dinitro-2',5'-dihydroxy-3',4',6'-trimethyldiphenylether **170b** (1.67g, 4.99mmol, 53.5%) as cream coloured prisms; **m.p.** : 131-139℃; (found: C, 53.8; H, 4.2; N, 8.3%. $C_{15}H_{14}N_2O_7$ requires C, 53.9; H, 4.2; N, 8.4%); *m/Z* 285.9 $(M\text{-}H_2NO_2)^-$, 332.9 $(M\text{-}H)^-$; ν_{max}/cm^{-1}

3431 (O-H), 1602 and 1517 (C=C), 1537 and 1348 (NO_2), 1244 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.99 (3H, s, C*H*$_3$, 7'-H), 2.09 (6H, s, 2 × C*H*$_3$, 8'-H and 9'-H), 6.83 (1H, d, *J* = 9.6Hz, 6-H), 7.965 (1H, s, 5'-O*H*), 8.40 (1H, dd, *J* = 9.6, 3.0Hz, 5-H), 8.79 (1H, s, 2'-O*H*), 8.84 (1H, d, *J* = 3.0Hz, 3-H); **δ_C (75 MHz, d_6-DMSO)** 10.2 (CH_3, 7'-C), 12.7 (CH_3, 8'-C or 9'-C), 12.8 (CH_3, 8'-C or 9'-C), 111.9 (quat., 3'-C), 115.8 (quat., 4'-C), 116.85 (CH, 6-C), 122.2 (CH, 3-C), 126.1 (quat., 6'-C), 130.0 (CH, 5-C), 132.7 (quat., 1'-C), 138.6 (quat., 4-C), 140.8 (quat., 2-C), 145.1 (quat., 2'-C), 152.0 (quat., 5'-C), 156.6 (quat., 1-C).

The filtrate was evaporated under reduced pressure and the residue was recrystallised from MeOH and 2,4-dinitrophenoxy-3',4',6'-trimethyl-2',5'-benzoquinone **179b** was isolated as orange fluffy crystals (0.40g, 1.22 mmol, 13.0%); **m.p.**: 142-145°C; **ν_{max}**/cm^{-1} 1666 and 1648 (C=O), 1602 (C=C), 1526 and 1343 (NO_2), 1249 (C-O); (found: C, 54.2; H, 3.6; N, 8.4%. $C_{12}H_8N_2O_6$ requires C, 54.2; H, 3.6; N, 8.4%); *m/Z* 330.9 (*M-H*)$^-$; **δ_H (300 MHz, d_6-DMSO)** 1.95 (3H, d, *J* = 1.2Hz, CH_3, 7'-H), 1.97 (3H, s, CH_3, 9'-H), 2.04 (3H, d, *J* = 0.9Hz, CH_3, 8'-H), 7.54 (1H, d, *J* = 9.3Hz, 6-H), 8.38 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.89 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, d_6-DMSO)** 9.85 (CH_3, 9'-C), 12.2 (CH_3, 7'-C), 12.9 (CH_3, 8'-C), 119.2 (CH, 6-C), 122.3 (CH, 5-C), 129.7 (CH, 3-C), 134.3 (quat., 6'-C), 138.6 (quat., 2-C), 139.1 (quat., 4'-C), 141.7 (quat., 3'-C), 142.3 (quat., 4-C), 149.0 (quat., 1'-C), 154.1 (quat., 1-C), 180.5 (quat., 2'-C), 186.9 (quat., 5'-C).

5.1.20.3 2,4-dinitro-2',6'-dibromo-3'-hydroxy-5'-methoxydiphenylether 212 and 2,4-dinitro-2',6'-dibromo-3',5'-dihydroxydiphenylether 214





The two products were prepared as described from 2,4-dinitro-2',6'-dibromo-3',5'-dimethoxydiphenylether **175b** (1.90g, 3.98mmol); elution used petroleum ether (60-80°C) : Et_2O (50 : 50). The more mobile fraction yielded 2,4-dinitro-2',6'-dibromo-3'-hydroxy-5'-methoxydiphenylether **212** (0.7516g, 1.620mmol, 40.7%) as a white solid. Recrystallisation from aq. EtOH produced small cream coloured needles; **m.p.** : 150.5-152.5°C; (found: C, 33.4; H, 1.7; N, 5.9%. $C_{13}H_8Br_2N_2O_7$ requires C, 33.65; H, 1.7; N, 6.0%); *m/Z* 460.9 (52%), 462.8 (100%), 464.8 (48%) (*M-H*)$^-$; **ν_{max}**/cm^{-1} 3487

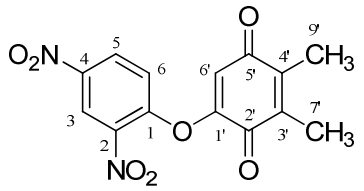
(O-H), 1593 and 1518 (C=C), 1533 and 1349 (NO_2), 1091 (C-O), 682 (C-Br); **δ_H (300 MHz, d_6-DMSO)** 3.88 (3H, s, OC*H*$_3$), 6.78 (1H, s, 4'-H), 6.98 (1H, d, *J* = 9.3Hz, 6-H), 8.43 (1H, dd, *J* = 9.3, 2.7Hz, 5-H), 8.945 (1H, d, *J* = 2.7Hz, 3-H), 11.14 (1H, s, 5'-O*H*); **δ_C (75 MHz, d_6-DMSO)** 57.1 (CH_3, 7'-C), 95.6 (quat., 6'-C), 96.0 (quat., 2'-C), 99.6 (CH, 4'-C), 117.0 (CH, 6-C), 122.5 (CH, 3-C), 130.1 (CH, 5-C), 138.6 (quat., 2-C), 142.2 (quat., 4-C), 148.0 (quat., 1'-C), 153.0 (quat., 1-C), 156.6 (quat., 5'-C), 157.05 (quat., 3'-C). Continued elution gave 2,4-dinitro-2',4',6'-tribromo-3',5'-hydroxydiphenylether **214** (0.30g, 0.49mmol, 12.3%) as a cream solid. Recrystallisation from EtOAc : petroleum ether (60-80°C) gave cream coloured needles; **m.p.**: 174.5-176.5°C; (found: C, 27.6; H, 1.1; N, 5.0%. $C_{12}H_5Br_3N_2O_7$ requires C, 27.25; H, 0.95; N, 5.3%); *m/Z* 525.0 (27%), 526.8 (100%), 528.6 (100%), 530.6 (34%) $(M\text{-}H)^-$; **ν_{max}**/cm^{-1} 3364 (O-H), 1606 (C=C), 1518 and 1344 (NO_2),1183 and 1068 (C-O), 682 (C-Br); **δ_H (300 MHz, d_6-DMSO)** 7.10 (1H, d, *J* = 9.3Hz, 6-H), 8.33 (1H, dd, *J* = 9.3, 3.0Hz, 5-H), 8.95 (1H, d, *J* = 9.0Hz, 3-H), 10.53 (2H, br. s, 2 × O*H*); **δ_C (75 MHz, d_6-DMSO)** 97.7 (quat., 2'-C and 6'-C), 101.85 (quat., 4'-C), 117.2 (CH, 6-C), 122.5 (CH, 3-C), 130.1 (CH, 5-C), 138.6 (quat., 2-C), 142.3 (quat., 4-C), 146.7 (quat., 1'-C), 152.8 (quat., 1-C), 153.0 (quat., 3'-C and 5'-C).

5.1.21 General procedure for the oxidation of the substituted 2,4-dinitro-2',5'-dimethoxydiphenylether 169a,c,d,f and 2,4-dinitro-2',5'-dimethoxydiphenylether 219a-b (adapted from 1976JOC3627)

The correctly substituted dinitro-2',5'-dimethoxydiphenylether (1mol equivalent) was dissolved in CH_3CN (50 to 500 mL) in a 2 necked round bottomed flask equipped with a thermometer and a dropping funnel. Cerium (IV) ammonium nitrate (3mol equivalent) was dissolved in the minimum amount of water and the resulting solution charged into the dropping funnel. This solution was then added slowly to the biaryl ether solution. The yellow solution usually darkened upon addition of the cerium (IV) ammonium nitrate solution, followed by the formation of a yellow precipitate soon after the addition was finished. The resulting mixture was stirred for a further 1h to ensure completion of the reaction. CH_3CN was removed under reduced pressure and, whenever possible, the precipitate formed was collected by filtration and washed with water. It was then redissolved in DCM, the resulting solution dried ($MgSO_4$) and the solvent was then removed *in* vacuo. The resulting solid was recrystallised from ethanol. When no precipitate, but a yellow oil/syrup, was observed, the reaction

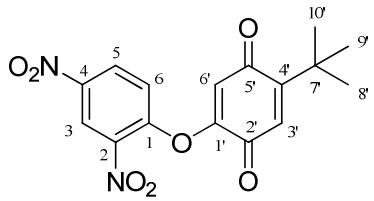
mixture was extracted twice with DCM and the combined organic layers washed once with brine and dried ($MgSO_4$). The solvent was then removed under reduced pressure and the residue either subjected to column chromatography or recrystallised from EtOH.

5.1.21.1 2,4-Dinitrophenoxy-3',4'-dimethyl-2',5'-benzoquinone 179a



The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxy-3',4'-dimethyldiphenylether **169a** (0.57g, 1.63mmol). Elution with petroleum ether (60:80°C) : EtOAc (30:70) yielded 2,4-dinitrophenoxy-3',4'-dimethyl-2',5'-benzoquinone **179a** as a yellow solid (0.47g, 1.48mmol, 90.9%). Recrystallisation from EtOH produced yellow prisms; **m.p.**: 110-111°C; (found: C, 52.8; H, 3.2; N, 8.7%. $C_{14}H_{10}N_2O_7$ requires C, 52.8; H, 3.2; N, 8.8%); *m/z* 316.9 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 1666 and 1648 (C=O, quinone), 1600 (C=C), 1537 and 1347 (NO_2), 1190 (C-O); **δ_H (300 MHz, d_6-DMSO)** 2.01 (6H, s, 2 x CH_3, 7'-H and 8'-H), 6.46 (1H, s, 6'-H), 7.77 (1H, d, *J* = 9.0 Hz, 6-H), 8.55 (1H, dd, *J* = 9.0, 2.7 Hz, 5-H), 8.91 (1H, d, *J* = 2.7 Hz, 3-H); **δ_C (75 MHz, d_6-DMSO)** 12.3 (CH_3, 7'-C or 8'-C), 12.6 (CH_3, 7'-C or 8'-C), 117.9 (CH, 6'-C), 122.5 (CH, 3-C), 123.5 (CH, 6-C), 130.3 (CH, 5-C), 139.5 (quat., 3'-C), 140.5 (quat., 2-C), 141.4 (quat., 4'-C), 144.1 (quat., 4-C), 152.1 (quat., 1-C), 155.2 (quat., 1'-C), 180.8 (quat., 2'-C), 187.1 (quat., 5'-C).

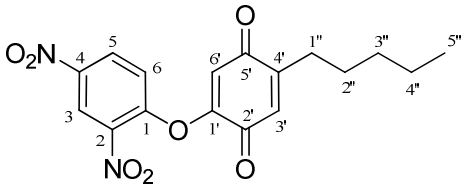
5.1.21.2 2,4-Dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone 179c



The title compound was prepared as described from 2,4-dinitro-4'-^{t}butyl-2',5'-dimethoxydiphenylether **169c** (2.87g, 7.69mmol). The crude product was recrystallised from MeOH yielding 2,4-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **179c** as a yellow fluffy solid (1.68g, 4.85mmol, 63.0%); **m.p.**: 179.5-182.5°C; (found:

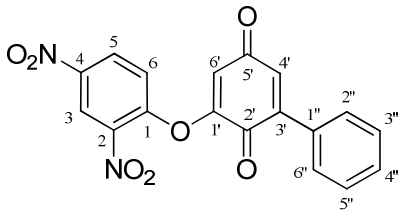
C, 55.5; H, 4.1; N, 8.1%. $C_{16}H_{14}N_2O_7$ requires C, 55.5; H, 4.1; N, 8.1%); ν_{max}/cm^{-1} 2870-2957 (C-H), 1665 and 1647 (C=O, quinone), 1611 (C=C), 1591 (C=C), 1533 and 1341 (NO_2), 1145 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.27 (9H, s, C(C*H*$_3$)$_3$), 6.40 (1H, s, 6'-H), 6.67 (1H, s, 3'-H), 7.82 (1H, d, *J* = 9.3Hz, 6-H), 8.56 (1H, dd, *J* = 9.0, 2.7Hz, 5-H), 8.92 (1H, d, *J* = 2.7Hz, 3-H); **δ_C (75 MHz, d_6-DMSO)** 29.5 (CH_3, 8'/9'/10'-C), 35.5 (quat., 7'-C), 119.6 (CH, 6'-C), 122.5 (CH, 3-C), 123.9 (CH, 6-C), 130.3 (CH, 3'-C or 5-C), 130.5 (CH, 3'-C or 5-C), 140.6 (quat., 2-C), 144.3 (quat., 4-C), 151.8 (quat., 1-C), 154.5 (quat., 1'-C), 156.3 (quat., 4'-C), 181.4 (quat., 2'-C), 187.6 (quat., 5'-C).

5.1.21.4 2,4-Dinitrophenoxy-4'-pentyl-2',5'-benzoquinone 179d



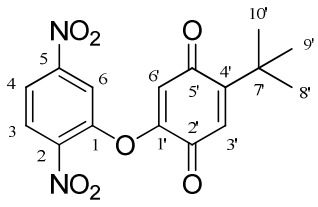
The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxy-3'-pentyldiphenylether **169d** (2.65g, 6.79mmol). The crude product was purified by column chromatography, eluting with petroleum ether (60-80°C) : EtOAc (70:30). 2,4-Dinitrophenoxy-2',5'-dimethoxy-4'-pentylbenzoquinone **179d** was isolated as a yellow solid (2.36g, 6.56mmol, 96.7%). Recrystallisation from EtOH produced shiny yellow plates; **m.p.**: 110-114°C; (found: C, 56.5; H, 4.4; N, 7.7%. $C_{17}H_{16}N_2O_7$ requires C, 56.7; H, 4.5; N, 7.8%); *m/Z* 358.95 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 2927 (C-H), 1673 and 1649 (C=O), 1610 (C=C), 1530 and 1342 (NO_2), 1224 (C-O); **δ_H (300 MHz, $CDCl_3$)** 0.83 (3H, t, *J* = 6.9Hz, 5"-H), 1.26-1.29 (4H, m, 3"-H and 4"-H), 1.43-1.48 (2H, m, 2"-H), 2.38 (2H, td, *J* = 7.8, 1.2Hz, 1"-H), 6.03 (1H, s, 6'-H), 6.53 (1H, t, *J* = 1.5Hz, 3'-H), 7.31 (1H, d, *J* = 9.0Hz, 6-H), 8.43 (1H, dd, *J* = 9.0, 2.7Hz, 5-H), 8.88 (1H, d, *J* = 2.7Hz, 3-H); **δ_C(75 MHz, $CDCl_3$)** 13.9 (CH_3, 5"-C), 22.4 (CH_2, 3"-C or 4"-C), 27.6 (CH_2, 1"-C), 28.9 (CH_2, 2"-C), 31.45 (CH_2, 3"-C or 4"-C), 116.3 (CH, 6'-C), 122.5 (CH, 3-C), 123.6 (CH, 6-C), 129.4 (CH, 5-C), 130.6 (CH, 3'-C), 140.7 (quat., 2-C), 144.5 (quat., 4-C), 151.2 (quat., 4'-C), 151.8 (quat., 1-C), 155.3 (quat., 1'-C), 180.3 (quat., 2'-C), 186.6 (quat., 5'-C).

5.1.21.3 2,4-Dinitrophenoxy-3'-phenyl-2',5'-benzoquinone 179e



The title compound was prepared as described from 3-(2',4'-dinitrophenoxy)-2,5-dimethoxybiphenyl **169f** (1.48g, 3.97mmol). Elution with petroleum ether (60-80°C) : EtOAc (20:80) yielded 2,4-dinitrophenoxy-2',5'-dimethoxy-3'-phenylbenzoquinone **179e** as a yellow crystalline solid (1.23g, 3.36mmol, 84.6%). Recrystallisation from EtOH/EtOAc produced golden orange plates; **m.p.**: 158.5-160°C; (found: C, 58.8; H, 2.8; N, 7.6%. $C_{18}H_{10}N_2O_7$ requires C, 59.0; H, 2.75; N, 7.65%); *m/Z* 364.9 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 1676 and 1649 (C=O, quinone), 1590 and 1523 (C=C), 1543 and 1347 (NO_2), 1226 and 1082 (C-O); **δ_H (300 MHz, d_6-DMSO)** 6.59 (1H, d, *J* = 2.4Hz, 6-H), 7.00 (1H, d, *J* = 2.4Hz, 4-H), 7.48-7.51 (3H, m, 3''-H, 5''-H and 4''-H), 7.54-7.58 (2H, m, 2''-H and 6''-H), 7.90 (1H, d, *J* = 9.3Hz, 6'-H), 8.60 (1H, dd, *J* = 9.3, 2.7Hz, 5'-H), 8.94 (1H, d, *J* = 2.7Hz, 3'-H); **δ_C (75 MHz, d_6-DMSO)** 117.95 (CH, 6'-C), 122.5 (CH, 3-C), 123.6 (CH, 6-C), 128.8 (CH, 3''-C and 5''-C), 129.8 (CH, 2''-C and 6''-C), 130.4 (CH, 5-C and 4''-C), 132.7 (quat., 1''-C), 133.15 (CH, 4'-C), 140.6 (quat., 2-C), 144.1 (quat., 4-C), 144.7 (quat., 3'-C), 152.2 (quat., 1-C), 155.9 (quat., 1'-C), 179.9 (quat., 2'-C), 187.3 (quat., 5'-C).

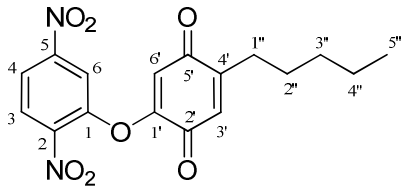
5.1.21.5 2,5-Dinitrophenoxy-4'-*t*butyl-2',5'-benzoquinone 219a



The title compound was prepared as described from 2,5-dinitro-4'-*t*butyl-2',5'-dimethoxydiphenylether **152c** (1.57g, 4.18mmol). 2,5-Dinitrophenoxy-4'-*t*butyl-2',5'-benzoquinone **219a** was isolated as a pale yellow solid (1.35g, 3.89mmol, 93.2%). Recrystalisation from EtOH produced a pale yellow microcrystalline solid; **m.p.**: 165-168°C; (found: C, 55.1; H, 4.0; N, 8.0%. $C_{16}H_{14}N_2O_7$ requires C, 55.5; H, 4.1; N, 8.1%); ν_{max}/cm^{-1} 2952 (C-H), 1672 and 1650 (C=O, quinone), 1594 (C=C), 1540 and

1347 (NO_2), 1237 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.26 (9H, s, C(CH_3)$_3$), 6.19 (1H, s, 6'-H), 6.66 (1H, s, 3'-H), 8.33 (1H, dd, *J* = 9.0, 2.4Hz, 4-H), 8.41 (1H, d, *J* = 9.0Hz, 3-H), 8.51 (1H, d, *J* = 2.4Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 29.5 (3 × CH_3, 8'/9'/10'-C), 35.4 (quat., 7'-C), 116.5 (CH, 6'-C), 119.9 (CH, 6-C), 122.3 (CH, 4-C), 127.9 (CH, 3-C), 130.4 (CH, 3'-C), 145.3 (quat., 2-C), 146.65 (quat., 5-C), 151.1 (quat., 1-C), 155.8 (quat., 1'-C), 156.1 (quat., 4'-C), 181.4 (quat., 2'-C), 187.8 (quat., 5'-C).

5.1.21.6 2,5-Dinitrophenoxy-4'-pentyl-2',5'-benzoquinone 219b

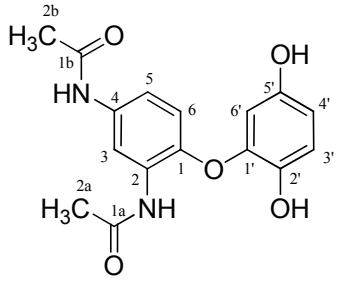


The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxy-3'-pentyldiphenylether **152d** (1.41g, 3.62mmol). The crude product was purified by column chromatography eluting with petroleum ether (60-80°C) : EtOAc (75:25). 2,5-Dinitrophenoxy-2',5'-dimethoxy-4'-pentylbenzoquinone **219b** was isolated as a yellow solid (1.19g, 3.31mmol, 91.4%). Recrystallisation from EtOH produced golden prisms; **m.p.** (EtOH): 91-94°C; (found: C, 56.4; H, 4.5; N, 7.8%. $C_{17}H_{16}N_2O_7$ requires C, 56.7; H, 4.5; N, 7.8%); **ν_{max}**/cm^{-1} 2870-2957 (C-H), 1676 and 1651 (C=O, quinone), 1603 (C=C), 1544 and 1343 (NO_2), 1230 (C-O); **δ_H (300 MHz, d_6-DMSO)** 0.79 (3H, t, *J* = 6.9Hz, 5"-H), 1.24-1.19 (4H, m, 3"-H and 4"-H), 1.42-1.35 (2H, m, 2"-H), 2.29 (2H, t, *J* = 6.9Hz, 1"-H), 6.18 (1H, s, 6'-H), 6.675 (1H, s, 3'-H), 8.23 (1H, dd, *J* = 9.0, 2.4Hz, 4-H), 8.32 (1H, d, *J* = 9.0Hz, 3-H), 8.41 (1H, d, *J* = 2.4Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 14.3 (CH_3, 5"-H), 22.3 (CH_2, 3"-C or 4"-C), 27.75 (CH_2, 2"-C), 28.6 (CH_2, 1"-C), 31.4 (CH_2, 3"-C or 4"-C), 115.1 (CH, 6'-C), 119.8 (CH, 6-C), 122.2 (CH, 4-C), 127.9 (CH, 3-C), 131.2 (CH, 3'-C), 145.25 (quat., 2-C), 146.7 (quat., 5-C), 150.2 (quat., 4'-C), 151.1 (quat., 1-C), 156.6 (quat., 1'-C), 181.0 (quat., 2'-C), 187.65 (quat., 5'-C).

5.1.22 General procedure for the trapping of 2,4-diamino-2',5'-dihydroxydiphenylether 220a and diaminophenoxy-2',5'-benzoquinone 171a,c,d and 152a

Either 2,4-dinitro-2',5'-dihydroxydiphenylether **170a** (1mol equivalent) or the correctly substituted 2,4-dinitrophenoxy-2',5'-benzoquinone **179** (1mol equivalent) was dissolved in MeOH or an EtOAc:MeOH (1:1) solvent mixture and Pd/C 5% (10% w/w of starting material) was added to the resulting solution. The resulting mixture was hydrogenated in a Berghof apparatus, the H_2 pressure being set to 2.4bar. The usual hydrogenation time was about 3h or when no more evolution of the H_2 pressure could be noted. Acetic anhydride (10mol equivalent) was added as soon as the hydrogenation was stopped and the resulting mixture was stirred at ambient atmosphere overnight. The reaction mixture was filtered through a pad of celite, the celite washed several times with MeOH and the filtrate was evaporated *in vacuo*. A 5% aq. solution of $NaHCO_3$ was added to the residue and the resulting mixture extracted with EtOAc (2 × 60 mL). The combined organic layers were washed with a 5% aq. solution of $NaHCO_3$ (70mL) and brine (100mL), and dried ($MgSO_4$). The solvent was removed *in vacuo* and the crude product was purified by column chromatography.

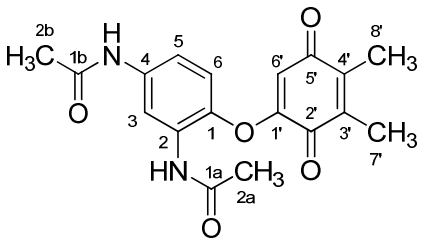
5.1.22.1 2,4-Diacetamido-2',5'-dihydroxydiphenylether 222



The title compound was prepared as described from 2,4-dinitro-2',5'-dihydroxydiphenylether **170a** (0.57g, 1.955mmol). The crude product was purified by column chromatography, eluting with EtOAc (100%). 2,4-Diacetamido-2',5'-dihydroxydiphenylether **222** (0.49g, 1.555mmol, 79.5%) was isolated as a white solid. Recrystallisation was achieved from petroleum ether (60-80°C) : EtOAc; **m.p.**: 194.5-196°C; (found: C, 60.5; H, 5.1; N, 8.65%. $C_{16}H_{16}N_2O_5$ requires C, 60.75; H, 5.1; N, 8.9%); *m/z* 314.9 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3416 (N-H), 3317 (N-H), (br., O-H), 1698 (C=O), 1665 (C=O), 1616 (C=C), 1240 and 1205 (C-O); **δ_H (300 MHz, d_6-DMSO)** 2.025 (3H,

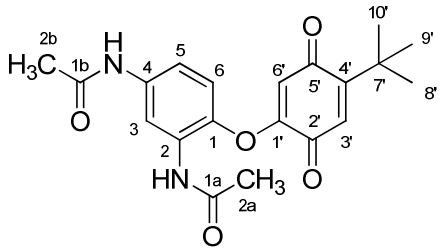
s, COC*H*$_3$, 1b-H), 2.11 (3H, s, COC*H*$_3$, 1a-H), 6.28 (1H, d, *J* = 2.7Hz, 6'-H), 6.45 (1H, dd, *J* = 8.7, 2.7Hz, 4'-H), 6.70 (1H, d, *J* = 8.7Hz, 6-H), 6.78 (1H, d, *J* = 8.7Hz, 3'-H), 7.35 (1H, dd, *J* = 9.0, 2.4Hz, 5-H), 8.12 (1H, d, *J* = 2.1Hz, 3-H), 8.67 (1H, s, 2'-O*H*), 8.88 (1H, s, 5'-O*H*), 9.44 (1H, s, 2-N*H*), 9.89 (1H, s, 4-N*H*); **δ_C (75 MHz, d_6-DMSO)** 24.2 (CH_3, 2a-C or 2b-C), 24.3 (CH_3, 2a-C or 2b-C), 108.3 (CH, 6'-C), 111.85 (CH, 4'-C), 114.9 (CH, 3-C), 116.1 (CH, 5-C), 117.0 (CH, 6-C), 118.0 (CH, 3'-C), 128.85 (quat., 4-C), 134.6 (quat., 2-C), 141.3 (quat., 2'-C), 144.0 (quat., 1-C and 1'-C), 150.8 (quat., 5'-C), 168.5 (quat., 1b-C), 169.1 (quat., 1a-C).

5.1.22.2 2,4-Diacetamido-3',4'-dimethyl-2',5'-benzoquinone 223a



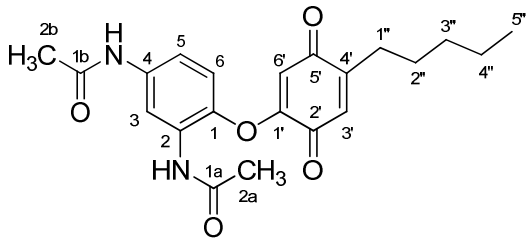
The title compound was prepared as described from 2,4-dinitrophenoxy-3',4'-dimethyl-2',5'-benzoquinone **179a** (0.26g, 0.88mmol) and acetic anhydride (2.5mL). The crude product was purified by column chromatography on silica eluting with a gradient mixture of petroleum ether (60-80°C) : EtOAc (10:90 to 0:100). 2,4-Diacetamido-3',4'-dimethyl-2',5'-benzoquinone **223a** was isolated as a bright orange crystalline solid (0.26g, 0.77mmol, 95.8%). Recrystallisation from EtOAc : hexane produced small orange prisms, **m.p.** : 161°C (dec.); (found: C, 63.4; H, 5.4; N, 8.1%. $C_{18}H_{18}N_2O_5$ requires C, 63.15; H, 5.3; N, 8.2%); *m/Z* 298.9 (*M*-CH_3CO)$^-$, 340.9 (*M*-*H*)$^-$; ν_{max}/cm^{-1} 3403 and 3277 (N-H), 1711 (C=O), 1655 and 1648 (C=O, quinone), 1606 (C=C), 1207 (C-O); **δ_H (300 MHz, d_8-THF)** 2.00 (3H, d, *J* = 1.2Hz, 7'-H), 2.05-2.06 (6H, m, 2 × C*H*$_3$, 8'-H and 2a-H or 2b-H), 2.08 (3H, s, 2a-H or 2b-H), 5.655 (1H, s, 6'-H), 6.98 (1H, d, *J* = 9.0Hz, 6-H), 7.915 (1H, dd, *J* = 8.7, 2.1Hz, 5-H), 8.30 (1H, d, *J* = 2.4Hz, 3-H), 8.63 (1H, s, 2-N*H*), 9.31 (1H, s, 4-N*H*); **δ_C (75 MHz, d_8-THF)** 9.1 (C*H*$_3$, 7'-C or 8'-C), 9.3 (C*H*$_3$, 7'-C or 8'-C), 21.1 (C*H*$_3$, 2a-C or 2b-C), 21.25 (C*H*$_3$, 2a-C or 2b-C), 108.5 (CH, 6'-C), 110.45 (CH, 3-C), 113.0 (CH, 5-C), 118.8 (CH, 6-C), 129.5 (quat., 2-C), 135.35 (quat., 4-C), 136.2 (quat., 1-C), 136.6 (quat., 3'-C), 138.8 (quat., 4'-C), 156.1 (quat., 1'-C), 165.8 (quat., 1a-C or 1b-C), 166.3 (quat., 1a-C or 1b-C), 179.0 (quat., 2'-C), 184.3 (quat., 5'-C).

5.1.22.3 2,4-Diacetamido-4'-^{t}butyl-2',5'-benzoquinone 223b



The title compound was prepared as described from 2,5-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **179c** (0.18g, 0.51mmol). The crude product was purified by column chromatography on silica gel eluting with petroleum ether (60-80°) : EtOAc (20 : 80). 2,4-Diacetamido-4'-^{t}butyl-2',5'-benzoquinone **223b** was isolated as a yellow-brown solid (0.19g, 0.51mmol, 99.0%). Recrystalisation from petroleum ether (60-80°C) : EtOAc produced light brown micro-crystalline solid; **m.p.**: 174-178°C; (found: C, 64.9; H, 6.1; N, 7.5%. $C_{20}H_{22}N_2O_5$ requires C, 64.85; H, 6.0; N, 7.6%); *m/z* 371.2 $(MH)^+$; ν_{max}/cm^{-1} 3330 (N-H), 2997-2868 (C-H), 1697 (C=O), 1665 and 1639 (C=O, quinone), 1594 and 1534 (C=C), 1168 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.25 (9H, s, C(C*H*$_3$)$_3$), 2.03 (3H, s, 2a-H or 2b-H), 2.055 (3H, s, 2a-H or 2b-H), 5.42 (1H, s, 6'-H), 6.63 (1H, s, 3'-H), 7.13 (1H, d, *J* = 9.0Hz, 6-H), 7.55 (1H, dd, *J* = 9.0, 2.4Hz, 5-H), 8.18 (1H, d, *J* = 2.4Hz, 3-H), 9.33 (1H, s, 2-N*H*), 10.07 (1H, s, 4-N*H*); **δ_C (75 MHz, d_6-DMSO)** 24.1 (CH_3, 2a-C or 2b-C), 24.4 (CH_3, 2a-C or 2b-C), 29.5 (CH_3, 8'/9'/10'-C), 35.3 (quat., 7'-C), 112.2 (CH, 6'-C), 115.2 (CH, 3-C), 116.2 (CH, 5-C), 121.9 (CH, 6-C), 130.4 (CH, 3'-C), 130.9 (quat., 2-C), 137.9 (quat., 1-C), 138.5 (quat., 4-C), 155.7 (quat., 4'-C), 157.6 (quat., 1'-C), 168.8 (quat., 1a-C or 1b-C), 169.2 (quat., 1a-C or 1b-C), 182.1 (quat., 2'-C), 187.8 (quat., 5'-C).

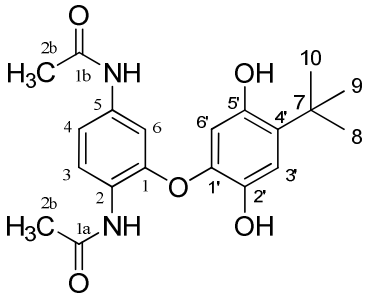
5.1.22.4 2,4-Diacetamido-4'-pentyl-2',5'-benzoquinone 223c



The title compound was prepared as described from 2,5-dinitrophenoxy-4'-pentyl-2',5'-benzoquinone **179d** (0.46g, 1.26mmol). The crude product was purified by column chromatography on silica eluting with petroleum ether (60-80°C) : EtOAc

(90:10). 2,4-Diacetamido-4'-pentyl-2',5'-benzoquinone **223c** was isolated as a dark orange crystalline solid (0.48g, 1.24mmol, 98.3%). Recrystallisation from EtOAc : hexane produced red prisms, **m.p.**: 90-93°C; (found: C, 65.6; H, 6.4; N, 7.2%. $C_{21}H_{24}N_2O_5$ requires C, 65.6; H, 6.3; N, 7.3%); *m/z* 341.0 $(M\text{-}CH_3CO)^-$, 383.0 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3357 (N-H), 3269 (N-H), 2955 (C-H), 2929 (C-H), 1726 (C=O), 1663 and 1647 (C=O, quinone), 1599 (C=C), 1206 (C-O); **δ_H (300 MHz, d_8-THF)** 0.931 (3H, t, *J* = 6.9Hz, 5''-H), 1.34-1.39 (4H, m, 3''-H and 4''-H), 1.48-1.55 (2H, m, 2''-H), 2.04 (3H, s, 2a-H or 2b-H), 2.07 (3H, s, 2a-H or 2b-H), 2.40 (2H, dt, *J* = 7.5, 1.2Hz, 1''-H), 5.67 (1H, s, 6'-H), 6.59 (1H, br. s, 3'-H), 6.98 (1H, d, *J* = 8.7Hz, 6-H), 7.91 (1H, dd, *J* = 9.0, 2.1Hz, 5-H), 8.28 (1H, d, *J* = 2.1Hz, 3-H), 8.63 (1H, s, 2-N*H*), 9.31 (1H, s, 4-N*H*); **δ_C (75 MHz, d_8-THF)** 13.3 (CH_3, 5''-C), 22.3 (CH_2, 3''-C or 4''-C), 23.0 (CH_3, 2a-C or 2b-C), 23.1 (CH_3, 2a-C or 2b-C), 27.85 (CH_2, 2''-C), 28.6 (CH_2, 1''-C), 31.5 (CH_2, 3''-C or 4''-C), 110.8 (CH, 6'-C), 112.5 (CH, 3-C), 114.9 (CH, 5-C), 120.7 (CH, 6-C), 130.45 (CH, 3'-C), 131.3 (quat., 2-C), 137.15 (quat., 1-C), 138.2 (quat., 4-C), 149.8 (quat., 4'-C), 158.2 (quat., 1'-C), 167.7 (quat., 1a-C or 1b-C), 168.1 (quat., 1a-C or 1b-C), 181.0 (quat., 2'-C), 186.6 (quat., 5'-C).

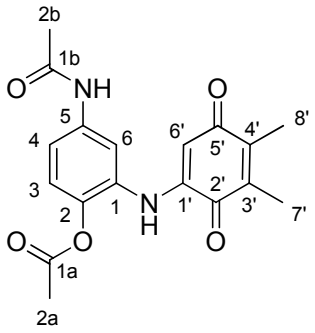
5.1.22.5 2,5-Diacetamido-2',5'-dihydroxy-4'-*t*butyldiphenylether 227



The title compound was prepared as described from 2,5-dinitrophenoxy-4'-*t*butyl-2',5'-benzoquinone **219a** (0.11g, 0.32mmol). The crude product was purified by column chromatography on silica eluting with petroleum ether (60-80°C) : EtOAc (20:80). 2,5-Diacetamido-2',5'-dihydroxy-4'-*t*butyldiphenylether **227** (0.12g, 0.31mmol, 97.5%) was isolated as a white solid. Recrystallisation from petroleum ether (60-80°C) : EtOAc produced a white crystalline solid; **m.p.**: 148-152°C; (found: C, 64.7; H, 6.55; N, 7.6%. $C_{20}H_{24}N_2O_5$ requires C, 64.5; H, 6.5; N, 7.5%); *m/z* 371.0 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3345 (N-H), 3259 (N-H), (br., O-H), 2957 (C-H), 1701 (C=O), 1667 (C=O), 1605 (C=C); **δ_H (300 MHz, d_6-DMSO)** 1.25 (9H, s, $C(CH_3)_3$), 1.87 (3H, s, 2a-H or 2b-H),

1.99 (3H, s, 2a-H or 2b-H), 6.36 (1H, s, 3'-H), 6.69 (1H, s, 6'-H), 6.85 (1H, d, *J* = 1.8Hz, 6-H), 7.25 (1H, dd, *J* = 8.4, 1.5Hz, 4-H), 7.60 (1H, d, *J* = 8.7Hz, 3-H), 8.44 (1H, s, 2'-O*H*), 8.76 (1H, s, 5'-O*H*), 9.33 (1H, s, 2-N*H*), 9.77 (1H, s, 5-N*H*); **δ_C (75 MHz, d_6-DMSO)** 24.0 (CH_3, 2a-C or 2b-C), 24.3 (CH_3, 2a-C or 2b-C), 29.9 (CH_3, 8'/9'/10'-C), 34.5 (quat., 7'-C), 107.1 (CH, 6-C), 109.7 (CH, 3'-C), 113.1 (CH, 4-C), 115.9 (CH, 6'-C), 123.55 (quat., 2-C), 124.2 (CH, 3-C), 132.8 (quat., 4'-C), 136.9 (quat., 5-C), 140.4 (quat., 1'-C), 140.7 (quat., 2'-C), 148.8 (quat., 5'-C), 149.7 (quat., 1-C), 168.5 (quat., 1a-C or 1b-C), 169.0 (quat., 1a-C or 1b-C).

5.1.22.6 2-Acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone 224



Method A. The title compound was prepared from 2,4-dinitrophenoxy-3',4'-dimethyl-2',5'-benzoquinone **179a** (0.13g, 0.43mmol) following the general trapping method described above and using an hydrogenation time of 48h. The residue was purified by column chromatography, eluting with a gradient mixture of petroleum ether (60-80°C) : EtOAc (10 : 90) to (0 : 100). The title compound **224** was isolated as a red solid (0.13g, 0.38mmol, 88.1%). Recrystalisation from EtOAc : petroleum ether (68-80°C) yielded a red microcrystalline solid.

Method B. The title compound was prapred by refluxing 2,4-diacetamido-3',4'-dimethyl-2',5'-benzoquinone **257a** (0.15g, 0.44mmol) in MeOH (10mL) for 12h. The solvent was then evaporated in vacuo and the residue purified by column chromatography, eluting with a gradient mixture of petroleum ether (60-80°C) : EtOAc (10 : 90) to (0 : 100). The title compound **224** was isolated as a red solid (0.11g, 0.32mmol, 73.2%). Recrystalisation from EtOAc : petroleum ether (68-80°C) produced a microcrystalline red solid; ν_{max}/cm^{-1} 3334 (N-H), 1754 (C=O), 1697 (C=O), 1662 and 1644 (C=O), 1186 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.95 (3H, d, *J* = 0.9Hz, C*H*$_3$, 7'-H or 8'-H), 1.98 (3H, d, *J* = 1.2Hz, C*H*$_3$, 7'-H or 8'-H), 2.065 (3H, s,

C*H*$_3$, 2b-H), 2.22 (3H, s, C*H*$_3$, 2a-H), 5.61 (1H, s, 6'-H), 7.18 (1H, d, *J* = 8.7Hz, 6-H), 7.45 (1H, dd, *J* = 8.7, 2.4Hz, 4-H), 7.71 (1H, d, *J* = 2.4Hz, 3-H), 8.26 (1H, s, 1-N*H*), 10.09 (1H, s, 5-N*H*); **δ_C (75 MHz, d_6-DMSO)** 12.3 (CH_3, 7'-C or 8'-C), 12.8 (CH_3, 7'-C or 8'-C), 21.1 (CH_3, 2a-C), 24.45 (CH_3, 2b-C), 100.8 (CH, 6'-C), 115.4 (CH, 6-C), 117.0 (CH, 4-C), 124.0 (CH, 3-C), 130.5 (quat., 1-C), 137.1 (quat., 4'-C), 138.0 (quat., 5'-C), 139.5 (quat., 2-C), 142.8 (quat., 3'-C), 144.0 (quat., 1'-C), 169.0 (quat., 1a-C or 1b-C), 169.1 (quat., 1a-C or 1b-C), 183.6 (quat., 2'-C), 185.8 (quat., 5'-C).

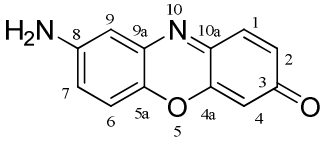
5.1.23 General procedure for the preparation of 8-amino-alkyl-3*H*-phenoxazin-3-ones 167a-e and 7-amino-2-alkyl-3*H*-phenoxazin-3-ones 59d-e

Method A. The correctly substituted 2,4-dinitro-2',5'-dihydroxydiphenyl ether **170** was dissolved in MeOH. Pd/C 5% (10% w/w of starting material) was added to the solution and the resulting mixture was hydrogenated in a Berghof apparatus. The H_2 pressure was set to 2.4bar and reset to this pressure when necessary. Once the H_2 pressure was steady, the reacting mixture was filtered through celite to remove the catalyst and the celite washed several time with small portions of MeOH. Either AgO, Ag_2O or MnO_2 (3mol equivalent) was then added quickly to the filtrate, followed by the addition of Et_3N (1mL), which rapidly produced a dark purple-violet suspension after stirring. Once the oxidative-cyclisation was complete (TLC monitoring), the inorganic materials were removed by filtration through celite and the celite was washed several times with THF and MeOH, until the filtrate was of a clear colour. The combined filtrates were evaporated *in vacuo* and the residue subjected to column chromatography.

Method B. The correctly substituted 2,4-dinitrophenoxy-2',5'-benzoquinone **179a-e** or 2,5-dinitrophenoxy-2',5'-benzoquinone **219a-b** was dissolved in an EtOAc : MeOH (1:1) solvent mixture. Pd/C 5% (10% w/w of the starting material) was added to the solution and the resulting mixture was hydrogenated in a Berghof apparatus. The H_2 pressure was set to 2.4bar and reset to this pressure when necessary. Once the H_2 pressure was steady, the reacting mixture was filtered through celite to remove the catalyst and the celite was washed several times with MeOH. Either AgO or Ag_2O (3mol equivalent) were then added quickly to the filtrate, which rapidly produced a dark purple-violet solution for 8-aminophenoxazinone derivatives and a dark red-purple fluorescent solution for 7-aminophenoxazinone derivatives during stirring. Once the oxidative-cyclisation was complete (TLC monitoring), the inorganic

materials were removed by filtration through celite and the celite was washed several times with THF and MeOH, until the filtrate was of a clear colour. The combined filtrates were evaporated *in vacuo* and the residue subjected to column chromatography.

5.1.23.1 8-Amino-3*H*-phenoxazin-3-one 167a

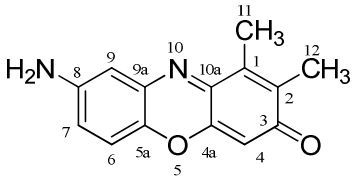


Method A. The title compound was prepared as described earlier from 2,4-dinitro-2'-5'-dihydroxydiphenylether **170a** (0.56g, 1.91mmol). The crude product was purified by column chromatography on silica, eluting with a gradient mixture of petroleum ether (60-80°C) : EtOAc (70:30 to 100:0), yielding 8-amino-3*H*-phenoxazin-3-one **167a** as a dark purple solid (0.37g, 1.75mmol, 91.3%). Analytical data were identical to that obtained for the product of ***method C*** below.

Method C. 8-Nitro-1,2,4-tribromophenoxazin-3-one (0.30g, 0.62mmol) was dissolved in 20mL of MeOH : EtOAc (1:1) solvent mixture. Pd/C 10% (60mg) and Et_3N (1mL) were then added to the resuting suspension. The resulting reaction mixture was hydrogenated for 48h in a Berghof apparatus; the initial H_2 pressure was set to 3.4bar. After 48h, the catalyst was removed by filtering the reaction mixture over celite, and the celite was washed several times with small portions of MeOH. Ag_2O was added to the filtrate, which rapidly produced a dark violet solution. The resulting suspension was again filtered over celite, and the celite was washed several times with small portions of THF. The filtrate was evaporated *in vacuo*, and the residue was purified by column chromatography on silica, eluting with a gradient mixture of petroleum ether (60-80°C) : EtOAc (70:30 to 100:0), yielding 8-amino-3*H*-phenoxazin-3-one **167a** as a dark purple solid (0.07g, 0.34mmol, 54.1%); **m.p.** >290°C; *m/z* 213.0 $(MH)^+$ and 234.9 $(MNa)^+$; $\boldsymbol{\nu_{max}}$/cm^{-1} 3387, 3320, 3200 (NH_2), 1640 (C=O), 1591 (C=C); **δ_H (300 MHz, d_6-DMSO)** 5.45 (2H, br. s, NH_2), 6.21 (1H, d, *J* = 2.1Hz, 4-H), 6.82 (1H, dd, *J* = 9.6, 2.1Hz, 2-H), 6.97 (2H, m, 7-H and 9-H), 7.24 (1H, d, *J* = 9.6Hz, 6-H), 7.49 (1H, d, *J* = 9.9Hz, 1-H); **δ_C (75 MHz, d_6-DMSO)** 105.3 (CH, 4-C), 111.6 (CH, 9-C), 116.7 (CH, 6-C), 121.0 (CH, 7-C), 134.3 (quat., 8-C), 135.0

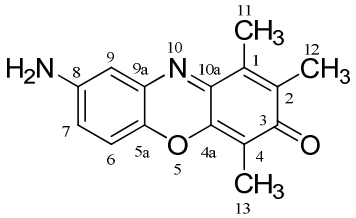
(CH, 1-C and 2-C), 135.5 (quat., 9a-C), 147.4 (quat., 5a-C), 148.2 (quat., 10a-C), 150.4 (quat., 4a-C), 185.6 (quat., 3-C).

5.1.23.2 8-Amino-1,2-dimethyl-3*H*-phenoxazin-3-one 167b



The title compound was prepared as described (***method B***) from 2,4-dinitrophenoxy-3',4'-dimethyl-2',5'-benzoquinone **179a** (0.41g, 1.28mmol); elution used petroleum ether (60-80°C) : EtOAc yielded 8-amino-1,2-dimethyl-3*H*-phenoxazin-3-one **167b** (0.28g, 1.18mmol, 92.0%) as a dark violet solid; **m.p.**: 260-265°C; *m/z* 241.0 $(MH)^+$; ν_{max}/cm^{-1} 3414, 3332 and 3225 (NH_2), 2851-2920 (C-H), 1638 (C=O), 1570 (C=C); **δ_H (300 MHz, d_6-DMSO)** 2.05 (3H, d, *J* = 0.9 Hz, 12-H), 2.35 (3H, d, *J* = 0.9Hz, 11-H), 5.38 (2H, br. s, NH_2), 6.12 (1H, s, 4-H), 6.90 (1H, dd, *J* = 9.0, 2.7Hz, 7-H), 6.96 (1H, d, *J* = 2.7Hz, 9-H), 7.20 (1H, d, *J* = 8.7Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 13.1 (CH_3, 11-C or 12-C), 13.2 (CH_3, 11-C or 12-C), 104.0 (CH, 4-C), 112.0 (CH, 9-C), 116.3 (CH, 6-C), 120.1 (CH, 7-C), 133.7 (quat., 8-C), 135.3 (quat., 9a-C), 137.5 (quat., 1-C), 139.1 (quat., 2-C), 147.1 (quat., 5a-C), 148.2 (quat., 10a-C), 149.8 (quat., 4a-C), 184.5 (quat., 3-C).

5.1.23.3 8-Amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one 167c

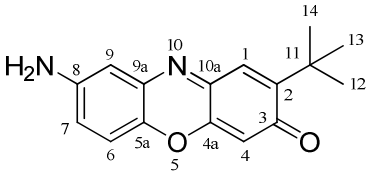


Method A. The title compound was prepared as described earlier from 2,4-dinitrophenoxy-3',4',6'-trimethylbenzoquinone **179b** (0.27g, 0.81mmol); elution used petroleum ether (60-80°C) : EtOAc (30:70), yielding 8-amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one **167c** (0.19g, 0.77mmol, 94.0%) as a dark violet solid. Analytical data were identical to that obtained for the product of ***method B*** below.

Method B. The title compound was prepared as described earlier from 2,4-dinitro-2',5'-dihydroxy-3',4',6'-trimethyldiphenylether **170b** (0.33g, 0.98mmol); elution used petroleum ether (60-80°C) : EtOAc (30:70), yielding 8-amino-1,2,4-trimethyl-3*H*-

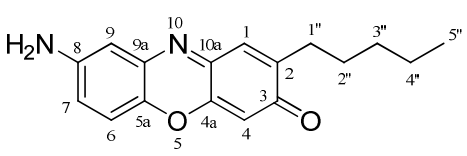
phenoxazin-3-one **167c** (0.22g, 0.85mmol, 86.7%) as a dark violet solid; **m.p.**: 235-246°C; *m/Z* 255.0 $(MH)^+$; ν_{max}/cm^{-1} 3355, 3332 and 3219 (NH_2), 2850-2920 (C-H), 1649 (C=O), 1595, 1577 and 1505 (C=C); **δ_H (300 MHz, d_6-DMSO)** 1.95 (3H, s, 13-H), 2.06 (3H, d, *J* = 0.9Hz, 12-H), 2.32 (3H, d, *J* = 0.9Hz, 11-H), 5.32 (2H, br. s, N*H*$_2$), 6.86 (1H, dd, *J* = 8.7, 2.7Hz, 7-H), 6.93 (1H, d, *J* = 2.7Hz, 9-H), 7.19 (1H, d, *J* = 8.7Hz, 6-H); **δ_C (75 MHz, d_6-DMSO)** 8.08 (CH_3, 13-C), 13.0 (CH_3, 11-C), 13.4 (CH_3, 12-C), 111.75 (quat., 4-C), 112.1 (CH, 9-C), 116.3 (CH, 6-C), 119.6 (CH, 7-C), 133.3 (quat., 8-C), 135.8 (quat., 9a-C), 136.6 (quat., 1-C or 2-C), 138.4 (quat., 1-C or 2-C), 145.9 (quat., 4a-C), 146.8 (quat., 5a-C), 148.1 (quat., 10a-C), 184.1 (quat.,3-C).

5.1.23.4 8-Amino-2-^{t}butyl-3*H*-phenoxazin-3-one 167d



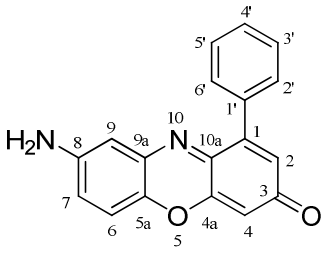
The title compound was prepared as described (***method B***) from 2,4-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **179b** (0.62g, 1.78mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60) yielding 8-amino-2-^{t}butyl-3*H*-phenoxazin-3-one **167d** (0.41g, 1.55mmol, 86.8%) as a dark violet solid; **m.p.**: 217-226°C; (found: C, 71.6; H, 6.3; N, 10.1%. $C_{16}H_{16}N_2O_2$ requires C, 71.6; H, 6.0; N, 10.4%); *m/Z* 269.0 $(MH)^+$; ν_{max}/cm^{-1} 3420 and 3337 (NH_2), 2952 (C-H), 1649 (C=O), 1601 and 1575 (C=C); **δ_H (300 MHz, $CDCl_3$)** 1.31 (9H, s, C(C*H*$_3$)$_3$), 3.74 (2H, br. s, N*H*$_2$), 6.12 (1H, s, 4-H), 6.78 (1H, dd, *J* = 8.7, 2.7Hz, 7-H), 6.94 (1H, d, *J* = 2.7Hz, 9-H), 7.05 (1H, d, *J* = 8.7Hz, 6-H), 7.20 (1H, s, 1-H); **δ_C (75 MHz, $CDCl_3$)** 29.3 (CH_3, 12/13/14-C), 35.8 (quat., 11-C), 107.6 (CH, 4-C), 113.6 (CH, 9-C), 116.5 (CH, 6-C), 119.9 (CH, 7-C), 129.4 (CH, 1-C), 134.25 (quat., 8-C), 137.0 (quat., 9a-C), 143.9 (quat., 5a-C), 148.7 (quat., 4a-C or 10a-C), 149.8 (quat., 4a-C or 10a-C), 154.9 (quat., 2-C), 185.9 (quat., 3-C).

5.1.23.5 8-Amino-2-pentyl-3*H*-phenoxazin-3-one 167e



The title compound was prepared as described (***method B***) from 2,4-dinitrophenoxy-2',5'-dimethoxy-4'-pentyl-2',5'-benzoquinone **179d** (0.38g, 1.07mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (50 : 50 to 35 : 65), yielding 8-amino-2-pentyl-3*H*-phenoxazin-3-one **167e** as a dark violet solid (0.29g, 1.01mmol, 94.7%), **m.p.**: 141-144°C; (found: C, 71.9; H, 6.6; N, 9.65%. $C_{17}H_{18}N_2O_2$ requires C, 72.2; H, 6.4; N, 9.9%); *m/z* 283.1 $(MH)^+$; ν_{max}/cm^{-1} 3400, 3337 and 3222 (N-H), 2853 and 2928 (C-H), 1639 (C=O), 1585, 1566 and 1508 (C=C); **δ_H (300 MHz, d_6-DMSO)** 0.87 (3H, t, *J* = 6.9Hz, 5'-H), 1.28-1.32 (4H, m, 3'-H and 4'-H), 1.46-1.56 (2H, m, 2'-H), 2.45 (2H, t, *J* = 6.9Hz, 1'-H), 5.41 (2H, br. s, NH_2), 6.17 (1H, s, 4-H), 6.90-6.93 (2H, m, 6-H and 7-H), 7.21 (1H, d, *J* = 9.3Hz, 6-H), 7.25 (1H, s, 1-H); **δ_C (75 MHz, d_6-DMSO)** 14.3 (CH_3, 5'-C), 22.4 (CH_2, 3'-C or 4'-C), 28.1 (CH_2, 2'-C), 29.5 (CH_2, 1'-C), 31.5 (CH_2, 3'-C or 4'-C), 105.0 (CH, 4-C), 111.55 (CH, 9-C), 116.55 (CH, 6-C), 120.3 (CH, 7-C), 130.6 (CH, 1-C), 134.3 (quat., 8-C), 135.3 (quat., 9a-C), 147.25 (quat., 2-C or 5a-C), 147.3 (quat., 2-C or 5a-C), 148.4 (quat., 4a-C), 149.7 (quat., 10a-C), 184.8 (quat., 3-C).

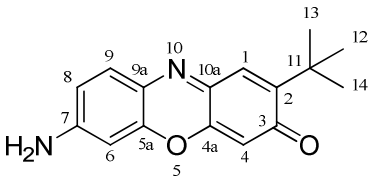
5.1.23.6 8-Amino-1-phenyl-3*H*-phenoxazin-3-one 167f



The title compound was prepared by reduction of 2,4-dinitrophenoxy-2',5'-dimethoxy-3'-phenylbenzoquinone **179e** (0.065g, 0.18mmol), using THF as solvent (***method A***). Hydrogenation time of 48h was required; cyclisation occured in the presence of Ag_2O (0.16g, 0.71mmol) and Et_3N (1mL); elution used petroleum ether (60-80°C) : EtOAc (30:70) yielded 8-amino-1-phenyl-3*H*-phenoxazin-3-one **167f** as a dark violet solid (0.04g, 0.13mmol, 73.4%); **m.p.**: 205°C (dec.); *m/z* 289.0 $(MH)^+$, 301.1 $(MNa)^+$; ν_{max}/cm^{-1} 3421, 3308 and 3198 (N-H), 1634 (C=O), 1586, 1560 and 1502 (C=C); **δ_H (300 MHz, d_6-DMSO)** 5.31 (2H, br. s, NH_2), 6.18 (1H, d, *J* = 2.1Hz, 4-H), 6.68 (1H, d, *J* = 2.1Hz, 2-H), 6.77 (1H, d, *J* = 2.7Hz, 9-H), 6.87 (1H, dd, *J* = 8.7, 2.7Hz, 7-H), 7.17 (1H, d, *J* = 8.7Hz, 6-H), 7.38-7.40 (3H, m, 3'-H, 4'-H and 5'-H), 7.48-7.53 (2H, m, 2'-H and 6'-H); **δ_C (75 MHz, d_6-DMSO)** 105.15 (CH, 4-C), 112.0 (CH, 9-C), 116.5 (CH, 6-C), 121.15 (CH, 7-C), 128.3 (CH, 3'-C and 5'-C), 129.1 (CH, 4'-C), 130.6 (CH, 2'-C

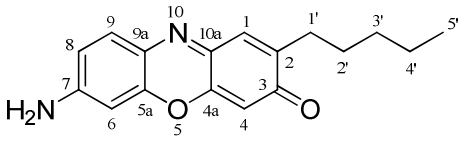
and 6'-C), 133.0 (CH, 2-C), 133.9 (quat., 8-C), 135.3 (quat., 9a-C), 135.5 (quat., 1-C), 144.9 (quat., 1'-C), 147.2 (quat., 5a-C or 10a-C), 147.3 (quat., 5a-C or 10a-C), 150.6 (quat., 4a-C), 184.5 (quat., 3-C).

5.1.23.7 7-Amino-2-^{t}butyl-3*H*-phenoxazin-3-one 59d



The title compound was prepared as described from 2,5-dinitrophenoxy-4'-^{t}butyl-2',5'-benzoquinone **219a** (1.01g, 2.91mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60), yielding 7-amino-2-^{t}butyl-3*H*-phenoxazin-3-one **59d** as a dark solid with a metallic shine (0.76g, 2.84mmol, 97.5%); **m.p.**: 255-260°C; (found: C, 71.5; H, 6.1; N, 10.4%. $C_{16}H_{16}N_2O_2$ requires C, 71.6; H, 6.0; N, 10.4%); *m/Z* 269.1 $(MH)^+$; ν_{max}/cm^{-1} 3408, 3317 and 3205 (NH_2), 2944 (C-H), 1643 (C=O), 1589, 1568 and 1547 (C=C); **δ_H (300 MHz, d_6-DMSO)** 1.31 (9H, s, C(CH_3)$_3$), 6.04 (1H, s, 4-H), 6.475 (1H, d, *J* = 2.4Hz, 6-H), 6.69 (1H, dd, *J* = 9.0, 2.4Hz, 8-H), 6.81 (2H, br. s, NH_2), 7.14 (1H, s, 1-H), 7.43 (1H, d, *J* = 8.7Hz, 9-H); **δ_C (75 MHz, d_6-DMSO)** 29.4 (CH_3, 12/13/14-C), 35.4 (quat., 11-C), 97.9 (CH, 6-C), 106.5 (CH, 4-C), 113.6 (CH, 8-C), 126.4 (quat., 7-C), 129.45 (CH, 1-C), 132.0 (CH, 9-C), 140.7 (quat., 10a-C), 146.6 (quat., 9a-C), 149.0 (quat., 4a-C), 150.4 (quat., 2-C), 155.1 (quat., 5a-C), 184.6 (quat., 3-C).

5.1.23.8 7-Amino-2-pentyl-3*H*-phenoxazin-3-one 59e



The title compound was prepared as described from 2,5-dinitrophenoxy-4'-pentyl-2',5'-benzoquinone **219b** (0.80g, 2.23mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (40:60 to 0:100), yielding 7-amino-2-pentyl-3*H*-phenoxazin-3-one **59e** as a dark violet solid (0.62g, 2.18mmol, 97.6%); **m.p.**: 250-254°C; *m/Z* 283.2 $(MH)^+$; ν_{max}/cm^{-1} 3420, 3329 and 3226 (NH_2), 2923 (C-H), 1654 (C=O), 1599 and 1565 (C=C); **δ_H (300 MHz, d_6-DMSO)** 0.88 (3H, t, *J* = 6.9Hz, 5'-H),

1.29-1.33 (4H, m, 3'-H and 4'-H), 1.47-1.54 (2'-H), 2.45 (2H, t, *J* = 7.5Hz, 1'-H), 6.17 (1H, s, 4-H), 6.51 (1H, d, *J* = 2.4Hz, 6-H), 6.71 (1H, dd, *J* = 8.7, 2.4Hz, 8-H), 6.83 (2H, br. s, N*H*$_2$), 7.24 (1H, s, 1-H), 7.48 (1H, d, *J* = 8.7Hz, 9-H); **δ_C (75 MHz, d_6-DMSO)** 14.4 (CH_3, 5'-C), 22.4 (CH_2, 3'-C or 4'-C), 28.3 (CH_2, 2'-C), 29.5 (CH_2, 1'-C), 31.55 (CH_2, 3'-C or 4'-C), 97.8 (CH, 6-C), 105.1 (CH, 4-C), 113.8 (CH, 8-C), 126.4 (quat., 7-C), 130.8 (CH, 1-C), 132.1 (CH, 9-C), 140.6 (quat., 9a-C), 144.0 (quat., 4a-C), 146.7 (quat., 10a-C), 149.8 (quat., 2-C), 155.1 (quat., 5a-C), 184.3 (quat., 3-C).

5.1.24 General procedure for the preparation of 7-*N* and 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-3*H*-phenoxazin-3-ones 234a-b and 236a-f

(2008OBC682)

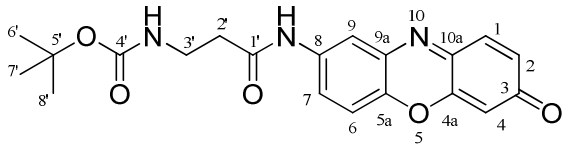
In a 250mL flame dried flask flushed with nitrogen, *N*-^{t}boc-β-alanine (3 mol equivalent) was dissolved in dry THF (10mL) and the solution was cooled with an ice bath to 2-3°C (internal temperature). *N*-Methylpiperidine (3.5mol equivalent) was then added dropwise, the resulting solution stirred for 5min and *iso*butylchloroformate (3mol equivalent) was added dropwise (temperature rose to 5°C). The resulting cloudy solution was stirred at 3°C for 1h to allow the mixed anhydride to form.

Method A. In the mean time, the correctly substituted 7-amino-3*H*-phenoxazin-3-one (1mol equivalent) was dissolved in dry DMF (5mL) in a 10mL round-bottomed flask, and Pd/C 5% (10% w/w of the starting material) was added to the resulting solution. The resulting mixture was hydrogenated in a Berghof apparatus for 1h with the H_2 pressure initially set at 2 bar. The flask was immediately sealed after hydrogenation and protected with an N_2 atmosphere. The resulting 7-amino-3-hydroxy-phenoxazine was added *via* canula to anhydride mixture.

Method B. The correctly substituted 8-amino-3*H*-phenoxazin-3-one (1mol equivalent) was directly added as a solid to the anhydride mixture.

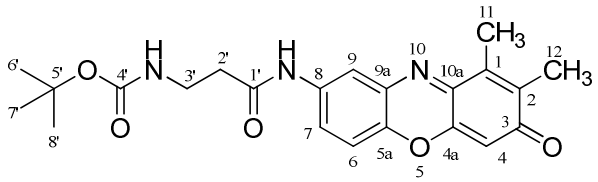
The resulting mixture (***method A*** or ***method B***) was stirred under nitrogen for 48-72h. The reaction mixture was quenched with a 5% aq. solution of $NaHCO_3$ and extracted with EtOAc (3 x 70 mL). The combined organic extracts were washed once with a 5% aq. solution of $NaHCO_3$ (100mL), once with water (100mL) and once with brine (100mL), then dried ($MgSO_4$). The solvent was evaporated *in vacuo* and the residue was subjected to column chromatography on silica.

5.1.24.1 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-3*H*-phenoxazin-3-one 236a



The title compound was prepared as described (***method B***) from 8-amino-3*H*-phenoxazin-3-one **167a** (0.37g, 1.75mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (20:80 to 0:100) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-3*H*-phenoxazin-3-one **236a** as a dark orange solid (0.36g, 0.95mmol, 54.3%); **m.p.**: 213-215°C; (found: C, 62.3; H, 5.6; N, 10.7%. $C_{20}H_{21}N_3O_5$ requires C, 62.65; H, 5.5; N, 11.0%); *m/z* 384.1 $(MH)^+$, 406.1 $(MNa)^+$; ν_{max}/cm^{-1} 3385 (N-H), 3304 (N-H), 1683 (C=O), 1644 (C=O), 1172 and 1136 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.38 (9H, s, C(C*H*$_3$)$_3$), 2.51 (2H, t, *J* = 7.8Hz, 2'-H), 3.25 (2H, q, *J* = 6.9Hz, 3'-H), 6.24 (1H, d, *J* = 2.1Hz, 4-H), 6.81 (1H, dd, *J* = 9.9, 2.1Hz, 2-H), 6.86 (1H, br. s, N*H*^{t}Boc), 7.42 (1H, d, *J* = 8.7Hz, 6-H), 7.52 (1H, d, *J* = 9.9Hz, 1-H), 7.73 (1H, dd, *J* = 9.0, 2.7Hz, 7-H), 8.14 (1H, d, *J* = 2.7Hz, 9-H), 10.20 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 28.75 (CH_3, 6'/7'/8'-C), 37.0 (CH_2, 3'-C), 37.35 (CH_2, 2'-C), 78.2 (quat., 5'-C), 106.1 (CH, 4-C), 116.6 (CH, 6-C), 119.3 (CH, 9-C), 124.7 (CH, 7-C), 133.3 (quat., 9a-C), 135.3 (2 × CH, 1-C and 2-C), 137.1 (quat., 8-C), 139.65 (quat., 5a-C), 149.2 (quat., 10a-C), 150.1 (quat., 4a-C), 156.1 (quat., 4'-C), 170.2 (quat., 1'-C), 185.9 (quat., 3-C).

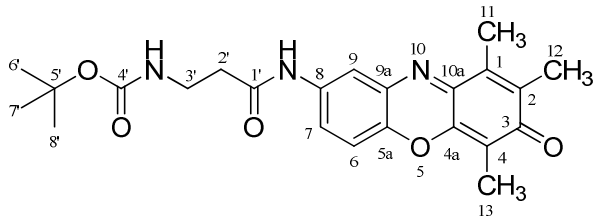
5.1.24.2 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3*H*-phenoxazin-3-one 236b



The title compound was prepared as described (***method B***) from 8-amino-1,2-dimethyl-3*H*-phenoxazin-3-one **167b** (0.27g, 1.14mmol). Elution with petroleum ether (60-80°C) : EtOAc : (30:70) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3*H*-phenoxazin-3-one **236b** as a bright orange solid (0.46g, 1.12mmol, 98.3%). Recrystallisation from EtOAc produced a microcrystalline orange solid; **m.p.**: 216-217°C; (found: C, 64.2; H, 6.2; N, 10.2%. $C_{22}H_{25}N_3O_5$ requires C, 64.2; H, 6.1; N, 10.2%); *m/z* 412.1 $(MH)^+$, 434.1 $(MNa)^+$; ν_{max}/cm^{-1} 3337 (N-H), 2973 and 2927

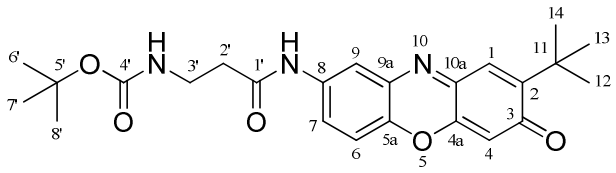
(C-H), 1700 (C=O), 1679 (C=O), 1647 (C=O), 1166 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.31 (9H, s, C(C*H*$_3$)$_3$), 1.95 (3H, s, CH_3, 12-H), 2.24 (3H, s, CH_3, 11-H), 2.425 (2H, t, *J* = 7.2 Hz, CH_2, 2'-H), 3.175 (2H, q, *J* = 7.2 Hz, 3'-H), 6.06 (1H, s, 4-H), 6.78 (1H, br. s, N*H*^{t}Boc), 7.28 (1H, d, *J* = 8.7Hz, 6-H), 7.53 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 8.08 (1H, d, *J* = 2.4Hz, 9-H), 10.06 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 13.0 (CH_3, 11-C or 12-C), 13.15 (CH_3, 11-C or 12-C), 28.7 (CH_3, 6'/7'/8'-C), 37.0 (CH_2, 3'-C), 37.3 (CH_2, 2'-C), 78.15 (quat., 5'-C), 104.9 (CH, 4-C), 116.2 (CH, 6-C), 119.3 (CH, 9-C), 123.8 (CH, 7-C), 132.7 (quat., 9a-C), 136.8 (quat., 8-C), 137.8 (quat., 1-C), 139.4 (quat., 2-C or 5a-C), 139.5 (quat., 2-C or 5a-C), 149.0 (quat., 10a-C), 149.4 (quat., 4a-C), 156.05 (quat., 4'-C), 170.1 (quat., 1'-C), 184.8 (quat., 3-C).

5.1.24.3 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one 236c



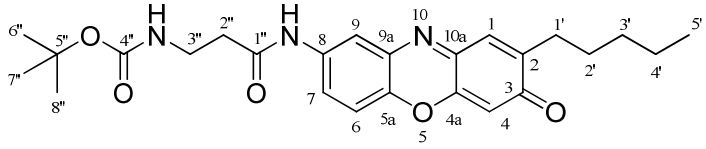
The title compound was prepared as described (***method B***) from 8-amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one **167c** (0.67g, 2.65mmol). Elution with petroleum ether (60-80°C) : EtOAc (30:70) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one **236c** (0.38g, 0.98mmol, 37.0%) as a dark orange solid ; **m.p.**: 195-198°C; *m/Z* 426.2 (*MH*)$^+$, 448.2 (*MNa*)$^+$; **ν_{max}**/cm^{-1} 3333 (N-H), 1713 (C=O), 1690 (C=O), 1650 (C=O), 1167 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.42 (9H, s, C(C*H*$_3$)$_3$), 1.98 (3H, s, 13-H), 2.07 (3H, s, 12-H), 2.33 (3H, s, 11-H), 2.545 (2H, t, *J* = 6.9Hz, 2'-H), 3.32 (2H, q, *J* = 6.6Hz, 3'-H), 6.49 (1H, br. s, N*H*^{t}Boc), 7.32 (1H, d, *J* = 8.7Hz, 6-H), 7.64 (1H, dd, *J* = 8.7, 2.1Hz, 7-H), 8.09 (1H, d, *J* = 1.8Hz, 9-H), 9.90 (1H, s, Ar-N*H*CO); **δ_C (75 MHz, d_6-DMSO)** 7.9 (CH_3, 13-C), 12.8 (CH_3, 11-C), 13.2 (CH_3, 12-C), 28.8 (CH_3, 6'/7'/8'-C), 37.3 (CH_2, 3'-C), 37.5 (CH_2, 2'-C), 78.3 (quat., 5'-C), 113.0 (quat., 4-C), 116.0 (CH, 6-C), 119.8 (CH, 9-C), 123.7 (CH, 7-C), 132.55 (quat., 9a-C), 136.55 (quat., 1-C or 2-C), 136.9 (quat., 8-C), 138.9 (quat., 1-C or 2-C), 140.1 (quat., 5a-C), 145.6 (quat., 4a-C), 149.1 (quat., 10a-C), 156.0 (quat., 4'-C), 170.1 (quat., 1'-C), 184.4 (quat., 3-C).

5.1.24.4 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one 236d



The title compound was prepared as described (***method B***) from 8-amino-2-^{t}butyl-3*H*-phenoxazin-3-one **167d** (0.53g, 1.96mmol); elution used petroleum ether (60-80°C) : EtOAc (30:70) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-4-^{t}butyl-3*H*-phenoxazin-3-one **236d** (0.53g, 1.21mmol, 61.7%) as a dark orange solid; **m.p.**: 191.5-193.5°C; (found: C, 65.5; H, 6.8; N, 9.5%. $C_{24}H_{29}N_3O_5$ requires C, 65.6; H, 6.65; N, 9.6%); *m/z* 438.1 $(M\text{-}H)^-$; ν_{max}/cm^{-1} 3340 (N-H), 3304 (N-H), 2962 (C-H), 1682 (C=O), 1648 (C=O), 1167 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.375 (9H, s, 12/13/14-H, C(C*H*$_3$)$_3$), 1.44 (9H, s, 6'/7'/8'-H, C(C*H*$_3$)$_3$), 2.57 (2H, t, *J* = 7.2Hz, 2'-H), 3.31 (2H, q, *J* = 7.2Hz, 3'-H), 6.21 (1H, s, 4-H), 6.90 (1H, br. s, N*H*^{t}Boc), 7.29 (1H, s, 1-H), 7.78 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 8.17 (1H, d, *J* = 2.4Hz, 9-H), 10.24 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 28.7 (CH_3, 6'/7'/8'-C), 29.4 (CH_3, 12/13/14-C), 35.7 (quat., 11-C), 37.0 (CH_2, 3'-C), 37.3 (CH_2, 2'-C), 78.1 (quat., 5'-C), 107.4 (CH, 4-C), 116.5 (CH, 6-C), 119.1 (CH, 9-C), 124.1 (CH, 7-C), 129.8 (CH, 1-C), 133.5 (quat., 9a-C), 137.0 (quat., 8-C), 139.4 (quat., 5a-C), 148.0 (quat., 4a-C or 10a-C), 149.6 (quat., 4a-C or 10a-C), 154.05 (quat., 2-C), 156.05 (quat., 4'-C), 170.1 (quat., 1'-C), 185.5 (quat., 3-C).

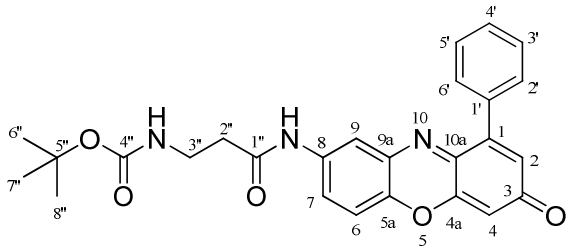
5.1.24.5 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one 236e



The title compound was prepared as described (***method B***) from 8-amino-2-pentyl-3*H*-phenoxazin-3-one **167e** (0.50g, 1.78mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (35:65 to 20:80) yielded 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **236e** as an orange solid (0.73g, 1.60mmol, 90.1%); **m.p.**:176-179°C; (found: C, 66.2; H, 6.8; N, 9.1%. $C_{25}H_{31}N_3O_5$ requires C, 66.2; H, 6.9; N, 9.3%); *m/z* 454.3 $(MH)^+$, 476.3 $(MNa)^+$; ν_{max}/cm^{-1} 3322

(N-H), 2954 and 2930 (C-H), 1684 (C=O), 1658 (C=O), 1648 (C=O), 1171 (C-O); **δ_H (300 MHz, d_6-DMSO)** 0.78 (3H, t, *J* = 6.6Hz, 5'-H), 1.21-1.23 (4H, m, 3'-H and 4'-H), 1.30 (9H, s, C(C*H*$_3$)$_3$), 1.38-1.40 (2H, m, 2'-H), 2.34 (2H, t, *J* = 7.2Hz, 1'-H), 2.42 (2H, t, *J* = 6.6Hz, 2''-H), 3.19 (2H, q, *J* = 6.6Hz, 3''-H), 6.095 (1H, s, 4-H), 6.77 (1H, br. s, N*H*^{t}Boc), 7.15 (1H, s, 1-H), 7.28 (1H, d, *J* = 8.7Hz, 6-H), 7.60 (1H, dd, *J* = 8.7, 2.4Hz, 7-H), 8.00 (1H, d, *J* = 2.1Hz, 9-H), 10.08 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 14.3 (CH_3, 5'-C), 22.4 (CH_2, 3'-C or 4'-C), 28.0 (CH_2, 2'-C), 28.7 (CH_3, 6''/7''/8''-C), 29.4 (CH_2, 1'-C), 31.5 (CH_2, 3'-C or 4'-C), 36.95 (CH_2, 3''-C), 37.3 (CH_2, 2''-C), 78.1 (quat., 5''-C), 105.8 (CH, 4-C), 116.5 (CH, 6-C), 119.0 (CH, 9-C), 124.0 (CH, 7-C), 130.7 (CH, 1-C), 133.3 (quat., 9a-C), 137.0 (quat., 8-C), 139.3 (quat., 5a-C), 147.6 (quat., 2-C), 149.2 (quat., 4a-C or 10a-C), 149.3 (quat., 4a-C or 10a-C), 156.0 (quat., 4''-C), 170.05 (quat., 1''-C), 185.1 (quat., 3-C).

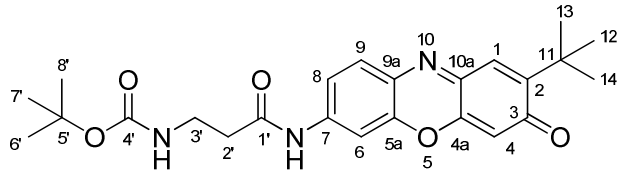
5.1.24.6 8-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-1-phenyl-3*H*-phenoxazin-3-one 236f



The title compound was prepared as described (***method B***) from 8-amino-1-phenyl-3*H*-phenoxazin-3-one **167f** (0.12g, 0.39mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (50:50 to 40:60) yielding 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1-phenyl-3*H*-phenoxazin-3-one **236f** as an orange-red solid (0.12g, 0.27mmol, 69.6%); **m.p.**: 241-243°C; (found: C, 67.7; H, 5.6; N, 8.9%. $C_{26}H_{25}N_3O_5$ requires C, 68.0; H, 5.5; N, 9.1%); *m/Z* 460.2 $(MH)^+$; **ν_{max}**/cm^{-1} 3422 (N-H), 3263 (N-H), 1679 (C=O), 1639 (C=O), 1167 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.385 (9H, s, C(C*H*$_3$)$_3$), 2.50 (2H, t, *J* = 7.2Hz, 2''-H), 3.24 (2H, q, *J* = 6.9Hz, 3''-H), 6.32 (1H, d, *J* = 2.1Hz, 4-H), 6.80 (1H, d, *J* =2.1Hz, 2-H), 6.86 (1H, br. s, N*H*^{t}Boc), 7.45 (1H, d, *J* = 9.0Hz, 6-H), 7.49-7.51 (3H, m, 3'-H, 4'-H and 5'-H), 7.59-7.63 (2H, m, 2'-H and 6'-H), 7.68 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 8.12 (1H, d, *J* = 2.4Hz, 9-H), 10.17 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 28.7 (CH_3, 6''/7''/8''-C), 37.0 (CH_2, 3''-C), 37.3 (CH_2, 2''-C), 78.1 (quat., 5''-C), 106.0 (CH, 4-C), 116.5 (CH, 6-C), 119.6 (CH, 9-C), 124.8 (CH, 7-C), 128.4 (CH, 3'-C and 5'-C), 129.3 (CH, 4'-C), 130.55 (CH, 2'-C and 6'-C), 133.0

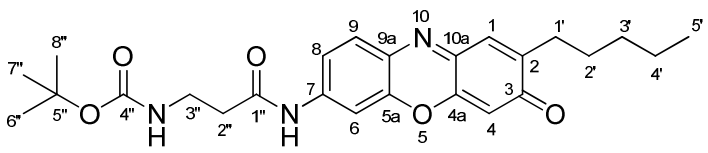
(quat., 9a-C), 133.2 (CH, 2-C), 135.2 (quat., 1-C), 136.95 (quat., 8-C), 139.5 (quat., 5a-C), 145.2 (quat., 1'-C), 148.25 (quat., 10a-C), 150.4 (quat., 4a-C), 156.0 (quat., 4''-C), 170.1 (quat., 1''-C), 184.9 (quat., 3-C).

5.1.24.7 7-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one 234a



The title compound was prepared as described (***method A***) from 7-amino-2-^{t}butyl-3*H*-phenoxazin-3-one **59d** (0.52g, 1.92mmol); elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (50:50 to 30:70) yielded 7-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-^{t}butyl-3H-phenoxazin-3-one **234a** as an orange solid (0.38g, 0.87mmol, 45.3%); **m.p.**: 194.5-197.5°C; (found: C, 65.3; H, 6.7; N, 9.4%. $C_{24}H_{29}N_3O_5$ requires C, 65.6; H, 6.65; N, 9.6%); *m/z* 438.1 $(M\text{-}H)^-$; 440.2 $(MH)^+$; ν_{max}/cm^{-1} 3373 (N-H), 3311 (N-H), 3001-2883 (C-H), 1712 (C=O), 1685 (C=O), 1644 (C=O), 1165 (C-O); **δ_H (300 MHz, d_6-DMSO)** 1.32 (9H, s, 12/13/14-H, C(C*H*$_3$)$_3$), 1.39 (9H, s, 6'/7'/8'-H, C(C*H*$_3$)$_3$,), 2.56 (2H, t, *J* = 6.9Hz, 2'-H), 3.26 (2H, q, *J* = 6.9Hz, 3'-H), 6.16 (1H, s, 4-H), 6.88 (1H, br. s, N*H*^{t}Boc), 7.20 (1H, s, 1-H), 7.47 (1H, dd, *J* = 8.7, 2.1Hz, 8-H), 7.69 (1H, d, *J* = 8.7Hz, 9-H), 7.87 (1H, d, *J* = 2.1Hz, 6-H), 10.485 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 28.7 (CH_3, 12/13/14-C), 29.4 (CH_3, 6'/7'/8'-C), 35.6 (quat., 11-C), 36.8 (CH_2, 3'-C), 37.5 (CH_2, 2'-C), 78.2 (quat., 5'-C), 105.35 (CH, 6-C), 107.5 (CH, 4-C), 116.7 (CH, 8-C), 129.7 (quat., 7-C), 129.8 (CH, 1-C), 130.7 (CH, 9-C), 143.5 (quat., 9a-C), 144.4 (quat., 5a-C), 146.9 (quat., 4a-C), 148.75 (quat.,10a-C), 153.1 (quat., 2-C), 156.05 (quat., 1'-C), 170.8 (quat., 4'-C), 185.3 (quat., 3-C).

5.1.24.8 7-*N*-(*N'*-^{t}Butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one 234b

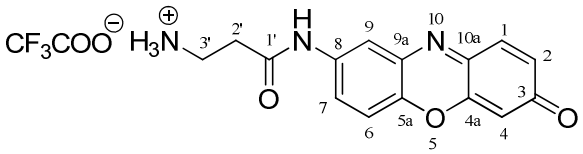


The title compound was prepared as described (***method A***) from 7-amino-2-pentyl-3*H*-phenoxazin-3-one **59e** (0.48g, 1.69mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60) yielding 7-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **234b** as an orange solid (0.36g, 0.78mmol, 46.0%). Recrystallisation from EtOAc produced microcrystalline orange solid with a green metallic shine, **m.p.**: 205-209°C; (found: C, 66.35; H, 6.9; N, 9.25%. $C_{25}H_{21}N_3O_5$ requires C, 66.2; H, 6.9; N, 9.3%); *m/z* 453.2 $(M\text{-}H)^-$; 454.3 $(MH)^+$; ν_{max}/cm^{-1} 3263 and 3190 (N-H), 2951 and 2922 (C-H), 1710 (C=O), 1651 (C=O); **δ_H (300 MHz, d_6-DMSO)** 0.79 (3H, t, *J* = 6.9Hz, 5'-H), 1.20-1.245 (4H, m, 3'-H and 4'-H), 1.30 (9H, s, C(CH_3)$_3$), 1.39-1.46 (2H, m, 2'-H), 2.39 (2H, t, *J* = 7.5Hz, 1'-H), 2.47 (2H, t, *J* = 7.2Hz, 2''-H), 3.17 (2H, q, *J* = 7.2Hz, 3''-H), 6.20 (1H, s, 4-H), 6.80 (1H, br. s, N*H*^{t}Boc), 7.22 (1H, s, 1-H), 7.40 (1H, dd, *J* = 8.7, 2.1Hz, 7-H), 7.66 (1H, d, *J* = 8.7Hz, 9-H), 7.82 (1H, d, *J* = 2.1Hz, 6-H), 10.41 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 14.3 (CH_3, 5'-C) , 22.4 (CH_2, 3'-C or 4'-C), 28.1 (CH_2, 2'-C), 28.7 (CH_2, 1'-C), 29.4 (CH_3, 6''/7''/8''-C), 31.5 (CH_2, 3'-C or 4'-C), 36.8 (CH_2, 3''-C), 37.5 (CH_2, 2''-C), 78.2 (quat., 5''-C), 105.3 (CH, 6-C), 106.05 (CH, 4-C), 116.8 (CH, 8-C), 129.6 (quat.,7-C), 130.7 (CH, 9-C), 130.9 (CH, 1-C), 143.4 (quat., 9a-C), 144.5 (quat., 5a-C), 146.7 (quat., 10a-C and 2-C), 149.5 (quat., 4a-C), 156.0 (quat., 4''-C), 170.8 (quat., 1''-C), 185.1 (quat., 3-C).

5.1.25 General procedure for the deprotection of 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-alkyl-3*H*-phenoxazin-3-one 236a-f and 7-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-alkyl-3*H*-phenoxazin-3-one 234a-b

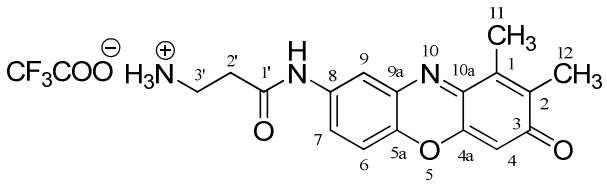
To the 7-*N*- or 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-alkyl-3*H*-phenoxazin-3-one in a round bottomed flask, an excess of neat TFA was added and the resulting solution was swirled for one minute. TFA was evaporated *in vacuo*, and MeOH was added to the residue. The resulting solution was again evaporated *in vacuo*, and the procedure was repeated several times until no more TFA remained. The residue was then redissolved in a minute amount of MeOH and the resulting solution diluted with Et_2O. The cloudy suspension was left standing overnight to allow for the formation of a microcrystalline solid. The latter was filtered and washed with Et_2O. The filtrate was evaporated under reduced pressure for further recovery of the product.

5.1.25.1 8-*N*-(β-Alanyl)amino-3*H*-phenoxazin-3-one TFA salt 237a



The title compound was prepared as described from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-3*H*-phenoxazin-3-one **236a** (0.16g, 0.43mmol). 8-*N*-(β-Alanyl)amino-3*H*-phenoxazin-3-one TFA salt **237a** was isolated as a dark red solid (0.14g, 0.35mmol, 80.7%); **m.p.:** 167-170℃; (Found $(M)^+$, 284.1031. Calc. for $C_{15}H_{14}N_3O_3$: $(M)^+$, 284.1030); $m/_Z$ 284.0 $(M)^+$; ν_{max}/cm^{-1} 3408-2907 (N^+-H), 3053 (N-H), 1671 (C=O), 1643 (C=O); **δ_H (300 MHz, d_6-DMSO)** 2.77 (2H, t, *J* = 6.6Hz, 2'-H), 3.14 (2H, br. s, 3'-H), 6.245 (1H, d, *J* = 1.8Hz, 4-H), 6.835 (1H, dd, *J* = 9.9, 1.5Hz, 2-H), 7.44 (1H, d, *J* = 9.0Hz, 6-H), 7.52 (1H, d, *J* = 9.9Hz, 1-H), 7.77 (1H, dd, *J* = 9.0, 2.1Hz, 7-H), 7.98 (3H, br. s, NH_3^+), 8.15 (1H, d, *J* = 2.4Hz, 9-H), 10.56 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 33.8 (CH_2, 2'-C), 35.4 (CH_2, 3'-C), 106.1 (CH, 4-C), 116.7 (CH, 6-C), 119.4 (CH, 9-C), 124.7 (CH, 7-C), 133.3 (quat., 9a-C), 135.3 (CH, 1-C and 2-C), 136.8 (quat., 8-C), 139.8 (quat., 5a-C), 149.2 (quat., 10a-C), 150.1 (quat., 4a-C), 169.05 (quat., 1'-C), 185.95 (quat., 3-C).

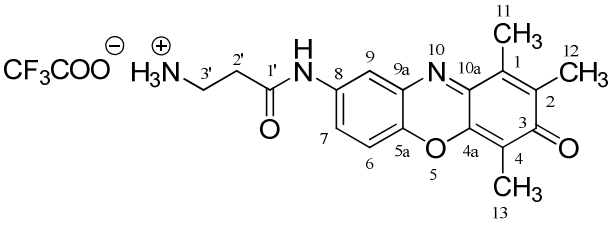
5.1.25.2 8-*N*-(β-Alanyl)amino-1,2-dimethyl-3*H*-phenoxazin-3-one TFA salt 237b



The title compound was prepared as described from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3*H*-phenoxazin-3-one **236b** (0.20g, 0.49mmol). 8-*N*-(β-Alanyl)amino-1,2-dimethyl-3*H*-phenoxazin-3-one TFA salt **237b** was isolated as an orange-red solid (0.20g, 0.47mmol, 96.1%); **m.p.**: 202-207℃; (Found $(M)^+$, 312.1344. Calc. for $C_{17}H_{18}N_3O_3$: $(M)^+$, 312.1343); $m/_Z$ 312.2 (M^+); ν_{max}/cm^{-1} 3282 (N-H), 3082 (N^+-H), 1671 (C=O), 1645 (C=O); **δ_H (300 MHz, d_6-DMSO)** 2.00 (3H, s, 12-H), 2.26 (3H, s, 11-H), 2.77 (2H, t, *J* = 6.6 Hz, 2'-H), 3.16 (2H, t, *J* = 6.6 Hz, 3'-H), 6.08 (1H, s, 4-H), 7.32 (1H, d, *J* = 8.7 Hz, 6-H), 7.60 (1H, dd, *J* = 8.7, 1.8 Hz, 7-H), 7.99 (3H, br. s, NH_3^+), 8.11 (1H, d, *J* = 1.8 Hz, 9-H), 10.46 (1H, s, N*H*-Ar); **δ_C (75**

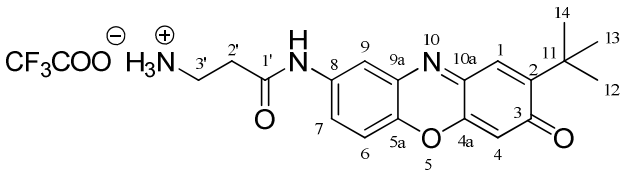
MHz, d_6-DMSO) 13.0 (CH_3, 11-C or 12-C), 13.05 (CH_3, 11-C or 12-C), 33.75 (CH_2, 2'-C), 35.4 (CH_2, 3'-C), 104.9 (CH, 4-C), 116.25 (CH, 6-C), 119.3 (CH, 9-C), 123.7 (CH, 7-C), 132.6 (quat., 9a-C), 136.5 (quat., 8-C), 137.7 (quat., 1-C or 2-C), 139.4 (quat., 5a-C or 1-C or 2-C), 139.45 (quat., 5a-C or 1-C or 2-C), 148.9 (quat., 4a-C or 10a-C), 149.2 (quat., 4a-C or 10a-C), 168.9 (quat., 1'-C), 184.7 (quat., 3-C).

5.1.25.3 8-*N*-(β-Alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one TFA salt 237c



Prepared from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one **236c** (0.06g, 0.16mmol). 8-*N*-(β-Alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one TFA salt **237c** was isolated as a red solid (0.06g, 0.14mmol, 91.2%); **m.p.**: 231-235°C; (Found $(M)^+$, 326.1501. Calc. for $C_{18}H_{20}N_3O_3$: $(M)^+$, 326.1499); *m/Z* 326.2 (M^+); $\boldsymbol{\nu_{max}}$/cm^{-1} 3322 (N-H), 3054 (N^+-H), 1670 (C=O), 1649 (C=O); $\boldsymbol{\delta_H}$ **(300 MHz, d_6-DMSO)** 1.97 (3H, s, 13-H), 2.07 (3H, s, 12-H), 2.33 (3H, s, 11-H), 2.76 (2H, t, *J* = 6.6Hz, 2'-H), 3.15 (2H, t, *J* = 6.3Hz, 3'-H), 7.41 (1H, d, *J* = 8.7Hz, 6-H), 7.64 (1H, dd, *J* = 8.7, 2.1Hz, 7-H), 7.855 (3H, br. s, NH_3^+), 8.15 (1H, d, *J* = 2.1Hz, 9-H), 10.40 (1H, s, N*H*-Ar); $\boldsymbol{\delta_C}$ **(75 MHz, d_6-DMSO)** 7.9 (CH_3, 13-C), 12.8 (CH_3, 12-C), 13.2 (CH_3, 11-C), 33.9 (CH_2, 2'-C), 35.8 (CH_2, 3'-C), 113.2 (quat., 4-C), 116.1 (CH, 6-C), 120.0 (CH, 9-C), 123.8 (CH, 7-C), 132.6 (quat., 9a-C), 136.2 (quat., 8-C), 136.9 (quat., 1-C or 2-C), 139.0 (quat., 1-C or 2-C), 140.2 (quat., 5a-C), 145.5 (quat., 4a-C), 149.15 (quat., 10a-C), 169.0 (quat., 1'-C), 184.4 (quat., 3-C).

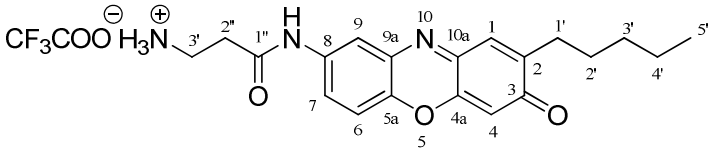
5.1.25.4 8-*N*-(β-Alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one TFA salt 237d



The title compound was prepared as described from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-4-^{t}butyl-3*H*-phenoxazin-3-one **236d** (0.16g, 0.37mmol). 8-*N*-(β-Alanyl)amino-4-^{t}butyl-3*H*-phenoxazin-3-one TFA salt **237d** was obtained as an

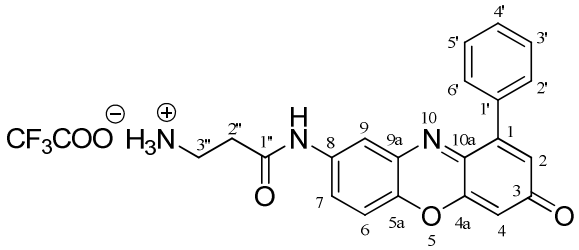
orange-brown solid (0.16g, 0.35mmol, 93.5%); **m.p.**: 119-126°C; (Found $(M)^+$, 340.1657. Calc. for $C_{19}H_{22}N_3O_3$: $(M)^+$, 340.1656); *m/z* 340.2 (M^+); ν_{max}/cm^{-1} 3462 (N-H), 3308-2965 (N^+-H) and (C-H), 1678 (C=O), 1649 (C=O); **δ_H (300 MHz, d_6-DMSO)** 1.32 (9H, s, C(C*H*$_3$)$_3$), 2.78 (2H, t, *J* = 6.6Hz, 2'-H), 3.30 (2H, br. s, 3'-H), 6.15 (1H, s, 4-H), 7.225 (1H, s, 1-H), 7.42 (1H, d, *J* = 9.0Hz, 6-H), 7.74 (1H, dd, *J* = 9.0, 1.8Hz, 7-H), 7.97 (3H, br. s, N*H*$_3^+$), 8.13 (1H, d, *J* = 1.8Hz, 9-H); **δ_C (75 MHz, d_6-DMSO)** 29.4 (CH_3, 12/13/14-C), 33.8 (CH_2, 2'-C), 35.4 (quat., 11-C), 35.7 (CH_2, 3'-C), 107.4 (CH, 4-C), 116.6 (CH, 6-C), 119.3 (CH, 9-C), 124.2 (CH, 1-C), 129.8 (CH, 7-C), 133.5 (quat., 9a-C), 136.6 (quat., 8-C), 139.5 (quat., 5a-C), 148.65 (quat., 4a-C or 10a-C), 149.6 (quat., 4a-C or 10a-C), 154.1 (quat., 2-C), 169.0 (quat., 1'-C), 183.2 (quat., 3-C).

5.1.25.5 8-*N*-(β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt 237e



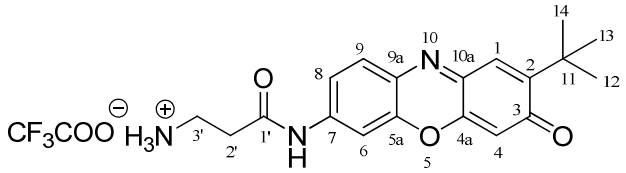
The title compound was prepared as described from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **236e** (0.08g, 0.18mmol). 8-*N*-(β-Alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt **237e** was isolated as a dark red solid (0.07g, 0.15mmol, 87.0%); **m.p.**: 132-139°C; (Found $(M)^+$, 354.1813. Calc. for $C_{20}H_{24}N_3O_3$: $(M)^+$, 354.1812); *m/z* 354.2 (M^+); ν_{max}/cm^{-1} 3312 (N-H), 3077 (N^+-H), 2932-2861 (C-H), 1672 (C=O), 1649 (C=O); **δ_H (300 MHz, d_6-DMSO)** 0.80 (3H, t, *J* = 6.6Hz, 5'-H), 1.22-1.25 (4H, m, 3'-H and 4'-H), 1.425-1.47 (2H, m, 2'-H), 2.41 (2H, t, *J* = 8.1Hz, 1'-H), 2.67 (2H, t, *J* = 6.6Hz, 2''-H), 3.05 (2H, br. s, 3''-H), 6.21 (1H, s, 4-H), 7.27 (1H, s, 1-H), 7.40 (1H, d, *J* = 9.0Hz, 6-H), 7.65 (1H, dd, *J* = 9.0, 2.4Hz, 7-H), 7.75 (3H, br. s, N*H*$_3^+$), 8.08 (1H, d, *J* = 2.4Hz, 9-H), 10.39 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 14.3 (CH_3, 5'-C), 22.4 (CH_2, 3'-C or 4'-C), 28.1 (CH_2, 2'-C), 29.4 (CH_2, 1'-C), 31.5 (CH_2, 3'-C or 4'-C), 33.9 (CH_2, 2''-C), 35.4 (CH_2, 3''-C), 105.9 (CH, 4-C), 116.7 (CH, 6-C), 119.2 (CH, 9-C), 124.1 (CH, 7-C), 130.9 (CH, 1-C), 133.4 (quat., 9a-C), 136.7 (quat., 8-C), 139.6 (quat., 5a-C), 147.75 (quat., 2-C), 149.4 (quat., 4a-C or 10a-C), 149.5 (quat., 4a-C or 10a-C), 169.0 (quat., 1''-C), 185.2 (quat., 3-C).

5.1.25.6 8-*N*-(β-Alanyl)amino-1-phenyl-3*H*-phenoxazin-3-one TFA salt 237f



The title compound was prepared as described from 8-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-1-phenyl-3*H*-phenoxazin-3-one **236f** (0.05g, 0.12mmol). 8-*N*-(β-Alanyl)amino-1-phenyl-3*H*-phenoxazin-3-one TFA salt **237f** was isolated as a dark red solid (0.05g, 0.10mmol, 82.2%); **m.p.**: 236-243°C; (Found $(M)^+$, 360.1345. Calc. for $C_{21}H_{18}N_3O_3$: $(M)^+$, 360.1343); *m/z* 360.2 $(M)^+$; ν_{max}/cm^{-1} 3262 (N-H), 3068 (N^+-H), 2903 (C-H), 1670 (C=O), 1640 (C=O); **δ_H (300 MHz, d_6-DMSO)** 2.67 (2H, t, *J* = 6.3Hz, 2''-H), 3.05 (2H, t, *J* = 6.3Hz, 3''-H), 6.23 (1H, d, *J* = 1.5Hz, 4-H), 6.72 (1H, d, *J* = 1.5Hz, 2-H), 7.37-7.42 (4H, m, 3'-H, 4'-H, 5'-H and 6-H), 7.52-7.53 (2H, m, 2'-H and 6'-H), 7.62 (1H, dd, *J* = 8.7, 1.8Hz, 7-H), 7.80 (3H, s, NH_3^+), 8.03 (1H, d, *J* = 1.2Hz, 9-H), 10.33 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)** 33.8 (CH_2, 2''-C), 35.5 (CH_2, 3''-C), 106.1 (CH, 4-C), 116.5 (CH, 6-C), 120.0 (CH, 9-C), 125.0 (CH, 7-C), 128.35 (CH, 3'-C and 5'-C), 129.2 (CH, 4'-C), 130.5 (CH, 2'-C and 6'-C), 133.0 (quat., 9a-C), 135.2 (quat., 2-C), 136.6 (quat., 1-C), 139.7 (quat., 8-C), 145.3 (quat., 5a-C), 148.4 (quat., 10a-C), 150.35 (quat., 4a-C), 169.1 (quat., 1''-C), 184.9 (quat., 3-C).

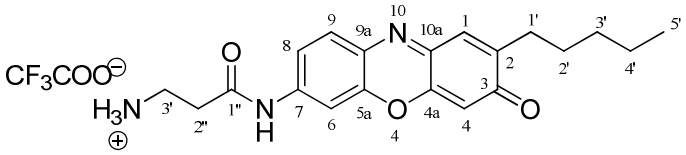
5.1.25.7 7-*N*-(β-Alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one TFA salt 235a



The title compound was prepared as described from 7-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one **234a** (0.11g, 0.25mmol). 7-*N*-(β-Alanyl)amino-2-^{t}butyl-3*H*-phenoxazin-3-one TFA salt **235a** (Found $(M)^+$, 340.1657. Calc. for $C_{19}H_{22}N_3O_3$: $(M)^+$, 340.1656); *m/z* 340.2 (M^+); ν_{max}/cm^{-1} 3180 (N-H), 3028 (N^+-H), 2961 (C-H), 1672 (C=O); **δ_H (300 MHz, d_6-DMSO)** 1.235 (9H, s, C(CH_3)$_3$), 2.73 (2H, t, *J* = 6.6Hz, 2'-H), 3.07 (2H, t, *J* = 6.6Hz, 3'-H), 6.07 (1H, s, 4-H), 7.12 (1H, s , 1-H), 7.42 (1H, dd, *J* = 8.7, 1.8Hz, 8-H), 7.62 (1H, d, *J* = 8.7Hz, 9-H), 7.77 (1H, d, *J* = 2.1Hz, 6-H), 7.84 (3H, br. s, NH_3^+), 10.69 (1H, s, N*H*-Ar); **δ_C (75 MHz, d_6-DMSO)**

29.4 (CH_3, 12/13/14-C), 34.1 (CH_2, 2'-C), 35.3 (CH_2, 3'-C), 35.6 (quat., 11-C), 105.7 (CH, 6-C), 107.6 (CH, 4-C), 116.9 (CH, 8-C), 129.8 (CH, 1-C), 129.89 (quat., 7-C), 130.7 (CH, 9-C), 143.1 (quat., 7-C), 144.4 (quat., 5a-C), 147.2 (quat., 10a-C), 148.7 (quat., 4a-C), 153.3 (quat., 2-C), 169.7 (quat., 1'-C), 185.35 (quat., 3-C).

5.1.25.8 7-*N*-(β-Alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt 235b



The title compound was prepared as described from 7-*N*-(*N'*-^{t}butoxycarbonyl-β-alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one **234b** (0.14g, 0.30mmol). 7-*N*-(β-Alanyl)amino-2-pentyl-3*H*-phenoxazin-3-one TFA salt **235b** was isolated as a brown-red solid with a green metallic shine (0.12g, 0.27mmol, 88.5%); **m.p.**: 213-223℃; (Found $(M)^+$, 354.1815. Calc. for $C_{20}H_{24}N_3O_3$: $(M)^+$, 354.1812); $m/_Z$ 354.2 (M^+) $\boldsymbol{\nu_{max}}$/cm^{-1} 3263 (N-H), 3188-3060 (N^+-H), 2928 (C-H), 1674 (C=O), 1651 (C=O); $\boldsymbol{\delta_H}$ **(300 MHz, d_6-DMSO)** 0.88 (3H, t, *J* = 6.9Hz, 5'-H), 1.30-1.33 (4H, m, 3'-H and 4'-H), 1.485-1.53 (2H, m, 2'-H), 2.45 (2H, t, *J* = 7.5Hz, 1'-H), 2.82 (2H, t, *J* = 6.3Hz, 2''-H), 3.15 (2H, t, *J* = 6.3Hz, 3''-H), 6.25 (1H, s, 4-H), 7.26 (1H, s, 1-H), 7.50 (1H, dd, *J* = 8.7, 1.8Hz, 8-H), 7.73 (1H, d, *J* = 8.7Hz, 9-H), 7.89 (1H, d, *J* = 1.8Hz, 6-H), 7.97 (3H, br. s, NH_3^+), 10.83 (1H, s, N*H*-Ar); $\boldsymbol{\delta_C}$ **(75 MHz, d_6-DMSO)** 14.4 (CH_3, 5'-C), 22.4 (CH_2, 3'-C or 4'-C), 28.1 (CH_2, 2'-C), 29.4 (CH_2, 1'-C), 31.5 (CH_2, 3'-H or 4'-H), 34.1 (CH_2, 2''-C), 35.2 (CH_2, 3''-C), 105.5 (CH, 6-C), 106.1 (CH, 4-C), 116.8 (CH, 8-C), 129.8 (quat., 7-C), 130.8 (quat., 9-C or 1-C), 130.9 (quat., 9-C or 1-C), 143.1 (quat., 9a-C), 144.4 (quat. 5a-C), 146.7 (quat., 2-C or 10a-C), 146.8 (quat., 2-C or 10a-C), 149.4 (quat., 4a-C), 169.7 (quat., 1''-C), 185.1 (quat., 3-C).

5.3 Microbiological testing

5.3.1 Preparation of the medium

Standard Columbia agar medium was used and the solutions were prepared according to the manufacturer's recommendations. The resulting agar solution was sterilised by either autoclaving or microwave irradiation and maintained at around 40°C in a water bath before incorporation of the substrate.

The substrate solutions were prepared in *N*-methylpyrrolidone (NMP), with the solvent volume kept to an absolute minimum (below 0.6% v/v of agar solution) to avoid any significant growth inhibition. When the substrate's poor solubility resulted in precipitation during incorporation to the agar solution, new solutions were prepared by either increasing the volume of NMP or using a combination of NMP and a surfactant (Tween 20). The use of Tween 20 along with NMP was prefered, as this combination always resulted in the production of a clear homogeneous medium. This quality was essential to maximise substrate uptake by bacteria and to ensure clear reading of the results.

When relatively large volume of NMP and/or Tween were used, a control plate containing the highest volume of NMP and of Tween 20 used was prepared to monitor any resulting growth inhibition.

Screening for enzyme activity using novel substrates is generally performed at a standard concentration of 100mg/L of agar solution (communication, Prof. John Perry); however, due to the strong background coloration generated by phenoxazinones substrates at 100mg/L, a concentration of 50mg/L was usually preferred. Tests at 25mg/L and 100mg/L were also undertaken for comparison of the performances.

The final media solutions were poured into separate Petri dishes and left to settle into a gel. All plates were then dried at 110°C for 5 minutes to remove extra moisture before inoculation.

5.3.1.1 Nitrophenoxazinones substrates

The chromogenic media containing the nitrophenoxazinones **130αa-c**, **130βa-c**, **130γa-b** and **242** were prepared following the general method described above and according to **Table 5.1**.

Substrate	Mass (mg)	V (NMP) (µL)	V (Tween 20) (µL)	V (Columbia agar) (mL)	Final conc. (mmol/L)
130αa	5	100	100	100	0.017
130αb	5	100	100	100	0.0145
130αc	5	100	100	100	0.010
130βa	5	100	100	100	0.017
130βb	5	100	100	100	0.0145
130βc	5	100	100	100	0.010
130γa	5	100	100	100	0.017
130γb	5	100	100	100	0.0145
242	5	200	100	100	0.015

Table 5.1: Preparation of the medium for substrates **130αa-c**, **130βa-c**, **130γa-b** and **242**

The final substrate concentration in the medium for all nitroreductase substrates was within the range 0.010 mmol/L to 0.017 mmol/L (**Table 5.1**).

5.3.1.2 Trihalogenophenoxazin-3-one substrates 237g-i and 240a-b

The chromogenic media containing the 8-*N*-(β-alanyl)amino-1,2,4-trihalogeno-3*H*-phenoxazin-3-one **237g-i** and 3-*O*-(8-*N*-(β-alanyl)amino-1,2,4-trifluoro-*10H*-phenoxazin-3-yloxy)-1''-oxopropane-3''-aminium **240a-b** were prepared following the general method described above and according to**Table 5.2**.

Substrate	Mass (mg)	V (NMP) (µL)	V (Tween 20) (µL)	V (Columbia agar) (mL)	Final conc. (mmol/L)
237g	5	200	-	100	0.11
237h	5	400	-	100	0.10
237i	5	200	300	100	0.08
240a	5	200	-	100	0.08
240b	5	200	-	100	0.07

Table 5.2: Preparation of the medium for substrates **237g-i** and **240a-b**

The final concentration in the medium for substrates **237g-i** and **240a-b** was within the range 0.07mmol/L to 0.11mmol/L (**Table 5.2**). This factor was considered regarding the availability of each substrate in the medium.

5.3.1.3 Alkylphenoxazin-3-one substrates 237a-g and 235a-b

The chromogenic media containing the 7-*N*- and 8-*N*-(β-alanyl)amino-alkyl-3*H*-phenoxazin-3-one **235a-b** and **237a-f** were prepared following the general method described above, starting from a stock solution matching a concentration of 10g/L, and according to **Table 5.3** to match a final concentration of 25mg/L, 50mg/L and 100mg/L.

Substrate	V (stock sol.) µL	V (agar) mL	Conc. (mmol/ L)	V (stock sol.) µL	V (agar) (mL)	Conc. (mmol/L)	V (stock sol.) µL	V (agar) µL	Conc. (mmol/L)
237a	200	80	0.063	400	80	0.126	800	80	0.252
237b	200	80	0.053	400	80	0.106	800	80	0.211
237c	200	80	0.059	400	80	0.118	800	80	0.235
237d	200	80	0.057	400	80	0.114	800	80	0.228
237e	200	80	0.055	400	80	0.110	800	80	0.221
237f	200	80	0.053	400	80	0.107	800	80	0.214
235a	200	80	0.055	400	80	0.110	800	80	0.221
235b	200	80	0.053	400	80	0.107	800	80	0.214

Table 5.3: Preparation of medium for substrates **235a-b** and **237a-f**

The final concentration in the medium for substrates **235a-b** and **237a-f** ranged from 0.053 to 0.063 mmol/L (25mg/L), 0.106 to 0.126 mmol/L (50mg/L) and 0.211 to 0.252 mmol/L (100mg/L).

5.3.2 Inoculation

5.3.2.1 Individual inoculation

Separate suspensions of *Ps. aeruginosa*, *B. cepacia*, *S. marcescens* and *E. coli*, corresponding to a turbidity of 0.5 McFarland (1.5×10^8 organisms/mL), were made up in distilled water in McFarland tubes.

Each petri dish containing the solidified chromogenic medium was then inoculated separately, using plastic disposable loops, with strains of *Ps. aeruginosa*, *B. cepacia*, *S. marcescens* and *E. coli*.

The preparation of Petri dishes containing directly the control *E. coli* was also possible by inoculating one side of the Petri dish with colonies of *E. coli* and the strain of interest on the other.

The Petri dishes were then incubated for 24h, before recording the results. When required, the Petri dishes were incubated for a further 24h before a second recording.

5.3.2.2 Multipoint inoculation

Multipoint inoculation was used to screen the activity of novel substrates over a relatively large range of microorganisms, and, to allow for the detection of any unexpected enzymatic activity.

The substrates were screened against twenty different microorganisms, a set of ten Gram negative bacteria and a set of ten Gram positive microorganisms (eight bacteria strains and two yeast strains, **Table 5.4**).

Gram negative	Ref.	Gram positive	Ref.
1 *E. coli*	NCTC 10418	11 *B. subtilis*	NCTC 9372
2 *S. marcescens*	NCTC 10211	12 *E. faecails*	NCTC 775
3 *Ps. aeruginosa*	NCTC 10662	13 *E. faecium*	NCTC 7171
4 *B. cepacia*	LMG 1222	14 *St. epidermidis*	NCTC 11047
5 *Y. enterocolitica*	NCTC 11176	15 *St. aureus*	NCTC 6571
6 *S. typhimurium*	NCTC 74	16 *MRSA*	NCTC 11939
7 *C. freundii*	46262 (wild)	17 *S. pyogenes*	NCTC 8306
8 *M. morganii*	462403 (wild)	18 *L. monocytogenes*	NCTC 11994
9 *E. cloacae*	NCTC 11936	19 *C. albicans*	ATCC 90028
10 *P. rettgeri*	NCTC 7475	20 *C. glabrata*	NCPF 3943

Table 5.4: Strains of microorganism tested by multipoint inoculation.

A suspension of each microorganism, corresponding to a turbidity of 0.5 McFarland (1.5×10^8 organisms/mL), was made up in distilled water in McFarland tubes. Each Petri dish containing the medium was then inoculated using a multipoint inoculator, receiving the twenty strains concurrently, organised in the pattern depicted on **Figure**

5.1. The Petri dishes were then incubated at 37℃ for 24h, before recording the results.

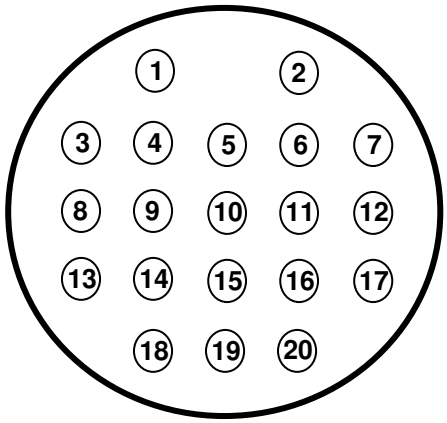


Figure 5.1: Inoculation pattern of a Petri dish.

CHAPTER SIX:

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…c’est l’atterrissage.