
Downloaded from: http://sure.sunderland.ac.uk/id/eprint/6561/

Usage guidelines

Please refer to the usage guidelines at http://sure.sunderland.ac.uk/policies.html or alternatively contact sure@sunderland.ac.uk.
SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL CHROMOGENIC SUBSTRATES FOR THE ENHANCED DETECTION OF PATHOGENIC BACTERIA

Alexandre Bedernjak

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, Dr 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 Dr 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).
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™ 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.
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
Et₂O diethyl ether
EtOAc ethyl acetate
EtOH ethanol
Et₃N 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
TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION

1.1 Bacterial detection 1
1.1.1 Classical detection method 2
  1.1.1.1 The Gram stain 2
1.1.2 New methods of identification 5
  1.1.2.1 Polymerase chain reaction (PCR) 5
  1.1.2.2 Bacteriophages 6
  1.1.2.3 Enzyme-linked immunosorbent assay (ELISA) 8
  1.1.2.4 Chromogenic media 9
    1.1.2.4.1 Agar-based media 9
    1.1.2.4.2 Micro gallery and automated systems 10
1.1.3 Chromogenic and fluorogenic dyes 11
  1.1.3.1 DNA fluorescent dyes 11
  1.1.3.2 pH indicators 12
  1.1.3.3 Redox indicators 13
  1.1.3.4 Enzyme substrates 14
    1.1.3.4.1 Simultaneous capture chromogenic substrates 15
    1.1.3.4.2 Post-incubation coupling chromogenic substrates 19
    1.1.3.4.3 Intramolecular rearrangement/electron conjugation chromogenic substrates 21
    1.1.3.4.4 Self coloured chromogenic/fluorogenic substrates 23
  1.1.3.4.1 Fluorogen 24
  1.1.3.4.2 Chromogens 25
1.1.4 Enzyme targets 27
  1.1.4.1 Esterases and lipases 27
  1.1.4.2 Glycosidases 28
    1.1.4.2.1 β-D-Glucuronidase 28
    1.1.4.2.2 β-D-Galactosidase 29
    1.1.4.2.3 α-D-Galactosidase 30
    1.1.4.2.4 β-D-Glucosidase 30
1.1.4.2.5 $\alpha$-D-Glucosidase
1.1.4.2.6 $\beta$-Hexoaminidase
1.1.4.3 Phosphatase
1.1.4.3.1 Phosphatidylinositol phospholipase C (PI-PLC)
1.1.4.4 Peptidase
1.1.4.4.1 Pyroglutamyl aminopeptidase
1.1.4.4.2 L-Alanine aminopeptidase
1.1.4.4.3 $\beta$-Alanine aminopeptidase
1.1.4.5 Nitroreductase enzymes

1.2 Occurrence of the phenoxazinone core
1.2.1 Natural products
1.2.1.1 Ommochromes
1.2.1.2 Mould metabolites
1.2.1.3 Actinomycins
1.2.1.4 Biosynthesis of the phenoxazinone core
1.2.2 Phenoxazinone dyes
1.2.2.1 Litmus and orceins

1.3 Chemical synthesis of the phenoxazinone core
1.3.1 Oxidative condensation of $\alpha$-aminophenol
1.3.2 Oxidative mixed condensation of $\alpha$-aminophenol
1.3.3 Condensation of $\alpha$-aminophenols with hydroxyquinones
1.3.4 Condensation of $\alpha$-aminophenol with quinolin-5,8-dione
1.3.5 Condensation of $\alpha$-aminophenols with halogenated quinones
1.3.6 Condensation of $p$-nitrosoaniline and $\alpha$-nitrosophenols with phenols
1.3.7 Condensation of benzoquinonechlorodiimide with resorcinol
1.3.8 Reductive cyclisation of 5-hydroxy-2'-nitrodiphenyl ethers
1.3.9 Oxidative cyclisation of 2,5-dinitro-2',5'-dihydroxydiphenylether
1.3.10 Oxidation of phenoxazines

1.4 Aims
CHAPTER TWO: SYNTHESIS OF PHENOXAZINONE SUBSTRATES

2.1 Synthesis of halogenophenoxazin-3-one substrates 60

2.1.1 Preparation of nitro-1,2,4-trihalogenophenoxazin-3-ones 130α-γa-c 60

2.1.1.1 Nitro-1,2,4-trifluorophenoxazin-3-ones 130α-γa 63

2.1.2 Synthesis of 8-amino-1,2,4-trihalogenophenoxazin-3-ones 165a-c 67

2.2 Synthesis of phenoxazinones via oxidative cyclisation 68

2.2.1 Previous work 68

2.2.2 Substitution strategy 69

2.2.3 Retrosynthetic analysis 70

2.2.4 Synthesis of 2,5-dimethoxybenzaldehyde 74

2.2.4.1 From hydroquinones 173a-c 74

2.2.4.2 From 2-hydroxy-5-methoxybenzaldehyde 182 74

2.2.4.2.1 Sonogashira cross-coupling 76

2.2.4.2.2 Suzuki cross-coupling 79

2.2.4.3 From 1,4-dimethoxy-2-bromobenzene 174 81

2.2.5 Synthesis of 2,5-dimethoxyphenols 150a-f 83

2.2.6 Bromination of 3,5-dimethoxyphenol 177 85

2.2.7 Oxidation of olivetol 181 85

2.2.8 Synthesis of diphenylethers 169a-g, 175b-c and 152a-b 87

2.2.8.1 Reaction with 2,4-dinitro-1-fluorobenzene 168 87

2.2.8.2 Reaction with 2,5-dinitro-1-fluorobenzene 151 90

2.2.9 Attempted substitution of diphenylether 92

2.2.9.1 From 2,4-dinitro-3'-bromo-2',5'-dimethoxy-diphenylether 92

2.2.9.2 From 2,4-dinitro-2',5'-dimethoxy-diphenylether 169g 94

2.2.10 Deprotection of diphenylethers 169b,g and 227b with BBr₃ 95

2.2.11 Oxidative demethylation of diphenylethers 169a,c,d,f and 152c-d using of cerium (IV) ammonium nitrate 97

2.2.12 Synthesis of 7- and 8-aminophenoxazin-3-ones 101

2.2.12.1 From 2,4-dinitro-2',5'-dihydroxydiphenylethers 170a-b 101

2.2.12.2 From 2,4 and 2,5-dinitrophenoxyn-2',5'-benzoquinones 103
CHAPTER THREE: MICROBIOLOGICAL RESULTS

3.1 Nitroreductase activity 126
3.1.1 Results and discussion 127
3.2 Testing of β-alanyl halogenated phenoxazinone derivatives 135
3.2.1 Toxicity screening 136
3.1.3 Results and discussion 137
3.3 Testing of β-alamylalkylphenoxazin-3-one derivatives 141
3.3.1 Results and discussion 142
3.3.2.1 Lipophilicity 148
3.4 Conclusion 149

CHAPTER FOUR: CONCLUSION

4.1 Conclusion and future work 151
4.1.1 Halogenophenoxazinone substrates 151
CHAPTER FIVE: EXPERIMENTAL

5.1 General experimental

5.2 Synthesis

5.2.1 General procedure for the preparation of

\textit{nitro-1,2,4-trihalogeno-3H-phenoxazin-3-one} 130α-γ a-c

5.2.1.1 7-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one \textit{130αa} 161

5.2.1.2 7-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one \textit{130αb} 162

5.2.1.3 7-Nitro-1,2,4-tribromo-3H-phenoxazin-3-one \textit{130αc} 163

5.2.1.4 8-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one \textit{130βa} 163

5.2.1.5 8-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one \textit{130βb} 165

5.2.1.6 8-Nitro-1,2,4-tribromo-3H-phenoxazin-3-one \textit{130βc} 165

5.2.1.7 9-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one \textit{130γa} 166

5.2.1.8 9-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one \textit{130γb} 166

5.2.2 General procedure for the preparation of

\textit{8-amino-1,2,4-trihalogeno-3H-phenoxazin-3-one} 165a-c

5.2.2.1 8-Amino-1,2,4-trifluoro-3H-phenoxazin-3-one \textit{165a} 167

5.2.3 General procedure for the preparation of

\textit{8-N-(N'-butoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-3H-phenoxazin-3-ones} 236g-i

5.2.3.1 \textit{8-N-(N'-Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3H-phenoxazin-3-one} \textit{236g} 168

5.2.3.2 \textit{8-N-(N'-Butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-3H-phenoxazin-3-one} \textit{236h} 170

5.2.3.3 \textit{8-N-(N'-Butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3H-phenoxazin-3-one} \textit{236i} 171
5.2.4 General procedure for the deprotection of 8-N-(N'-tbutoxycarbonyl-β-alanyl)amino-1,2,4-halogeno-3H-phenoxazin-3-one 237g-i 172

5.2.4.1 8-N-(β-Alanyl)amino-1,2,4-trifluoro-3H-phenoxazin-3-one TFA salt 237g 172

5.2.4.2 8-N-(β-Alanyl)amino-1,2,4-trichloro-3H-phenoxazin-3-one TFA salt 237h 173

5.2.4.3 8-N-(β-Alanyl)amino-1,2,4-tribromo-3H-phenoxazin-3-one TFA salt 237i 173

5.2.5 General procedure for the preparation of 8-N-(N'-tbutoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-10H-phenoxazin-3-yl 3''-(N'-tbutoxycarbonyl-amino)propanoate 239a-b 174

5.2.5.1 8-N-(N'-tButoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-10H-phenoxazin-3-yl 3''-(N'-tbutoxycarbonylamino)propanoate 239a 175

5.2.5.2 8-N-(N'-tButoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-10H-phenoxazin-3-yl-3''-(N'-tbutoxycarbonylamino)propanoate 239b 175

5.2.6 General procedure for the deprotection of 8-N-(N'-tbutoxycarbonyl-β-alanyl)amino-1,2,4-trihalogeno-10H-phenoxazin-3-yl-3''-(N'-tbutoxycarbonyl-amino)propanoate 240a-b 176

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 177

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 177

5.2.7 General procedure for the preparation of dimethoxybenzenes 172a-c 178

5.2.7.1 1,4-Dimethoxy-2,3-dimethylbenzene 172a 179

5.2.7.2 1,4-Dimethoxy-2,3,5-trimethylbenzene 172b 179

5.2.7.3 1,4-Dimethoxy-2-tbutylbenzene 172c 180

5.2.8 Procedure for the lithiation of 2-bromo-1,4-dimethoxybenzene 178 180

5.2.8.1 1,4-Dimethoxy-2-pentylbenzene 196 180

5.2.9 General procedure for the formylation of substituted dimethoxybenzene 172a-c, 196 and 189 via the Duff reaction 181

5.2.9.1 2,5-Dimethoxy-3,4-dimethylbenzaldehyde 149a 182
5.2.9.2 2,5-Dimethoxy-3,4,6-trimethylbenzaldehyde 149b  182
5.2.9.3 2,5-Dimethoxy-4-t-butylbenzaldehyde 149c  183
5.2.9.4 2,5-Dimethoxy-4-pentylbenzaldehyde 197  183
5.2.9.5 2,4-Dinitro-2',5'-dimethoxy-4'-carbaldehydediphenylether 211  184
5.2.10 Halogenation of 2-hydroxy-5-methoxybenzaldehyde 182  185
5.2.10.1 3-Bromo-2-hydroxy-5-methoxybenzaldehyde 183  185
5.2.10.2 3-Iodo-2-hydroxy-5-methoxybenzaldehyde 186a and 2,2'-dihydroxy-5,5'-dimethoxybiphenyl-3,3'-dicarboxaldehyde 186b  186
5.2.11 Protection of 3-halogeno-2-hydroxy-5-methoxybenzaldehyde 187  187
5.2.11.1 3-Bromo-2,5-dimethoxybenzaldehyde 178 and 2,5-dimethoxybenzaldehyde 149  187
5.2.11.2 3-Iodo-2,5-dimethoxybenzaldehyde 185  188
5.2.12 Sonogoshira cross-couping  189
5.2.12.1 Preparation of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde 184  189
5.2.13 Suzuki cross-coupling  191
5.2.13.1 Preparation of 2,5-dimethoxybiphenyl-3-carbaldehyde 194  191
5.2.13.2 Attempted preparation of 3-(2',4'-dinitrophenoxy)-2,5-dimethoxybiphenyl 169f  191
5.2.14 General procedure for the Baeyer-Villiger oxidation of benzaldehydes 149a-d, 178, 194 and 197  192
5.2.14.1 2,5-Dimethoxyphenol 150g  193
5.2.14.2 2,5-Dimethoxy-3,4-dimethylphenol 150a  194
5.2.14.3 2,5-Dimethoxy-3,4,6-trimethylphenol 150b  194
5.2.14.4 2,5-dimethoxy-4-t-butylphenol 150c  195
5.2.14.5 2,5-Dimethoxy-4-pentylphenol 150d  197
5.2.14.6 3-Bromo-2,5-dimethoxyphenol 150e  197
5.2.14.7 2,5-Dimethoxy-biphenyl-3-ol 150f  198
5.2.15 Bromination  198
5.2.15.1 2,6-Dibromo-3,5-dimethoxyphenol 176  198
5.2.15.1 2,4,6-Tribromoolivetol 205  199
5.2.16 Oxidation of tribromoolivetol 205: 2,6-dibromo-3-hydroxy-5-pentyl-1,4-benzoquinone 206  200
5.2.17 4-Nitro-2-fluoroaniline 210a and 6-nitro-2-fluoroaniline 210b  201
5.2.18 General procedure for the oxidation of fluoroanilines  
202
5.2.18.1 2,5-Dinitro-1-fluorobenzene 151  
203
5.2.19 General procedure for the preparation of biarylethers  
169a-g and 152c-d  
203
5.2.19.1 2,4-Dinitro-2',5'-dimethoxydiphenylether 169g  
204
5.2.19.2 2,4-Dinitro-2',5'-dimethoxy-3',4'-dimethylidiphenylether 169a  
205
5.2.19.3 2,4-Dinitro-2',5'-dimethoxy-3',4',6'-trimethylidiphenylether 169b  
205
5.2.19.4 2,4-Dinitro-2',5'-dimethoxy-4'-butyldiphenylether 169c  
206
5.2.19.5 2,4-Dinitro-2',5'-dimethoxy-3'-pentyldiphenylether 169d  
207
5.2.19.6 2,4-Dinitro-6'-bromo-2',5'-dimethoxydiphenylether 169e  
207
5.2.19.7 3-(2',4'-Dinitrophenoxy)-2,5-dimethoxybiphenyl 169f  
208
5.2.19.8 2,4-Dinitro-3',5'-dimethoxydiphenylether 175c  
209
5.2.19.9 2,4-Dinitro-2',5'-dihydroxydiphenylether 170a  
210
5.2.19.10 2,4-Dinitro-2',5'-dihydroxy-3',4',6'-trimethyl-1',4'-benzoquinone 179b  
213
5.2.19.11 2,4-Dinitro-2',5'-dihydroxy-3',5'-dihydroxydiphenylether 214
5.2.20 General procedure for the deprotection of substituted 2,4-dinitro-2',5'-dimethoxydiphenyl ethers 169b and 169g  
212
5.2.20.1 2,4-Dinitro-2',5'-dihydroxydiphenylether 170a  
213
5.2.20.2 2,4-Dinitro-2',5'-dihydroxy-3',4',6'-trimethylidiphenylether 170b  
and 2,4-dinitrophenoxo-3',4',6'-trimethyl-1',4'-benzoquinone 179b  
213
5.2.20.3 2,4-dinitro-2',6'-dibromo-3'-hydroxy-5'-methoxydiphenylether 212
and 2,4-dinitro-2',6'-dibromo-3',5'-dihydroxydiphenylether 214
5.2.21 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 215
5.2.21.2 2,4-Dinitrophenoxo-3',4'-dimethyl-2',5'-benzoquinone 179a  
216
5.2.21.3 2,4-Dinitrophenoxo-4',5'-butyl-2',5'-benzoquinone 179c  
216
5.2.21.4 2,4-Dinitrophenoxo-3'-phenyl-2',5'-benzoquinone 179d  
217
5.2.21.5 2,4-Dinitrophenoxo-4'-pentyl-2',5'-benzoquinone 179e  
218
5.2.21.6 2,5-Dinitrophenoxo-4'-butyl-2',5'-benzoquinone 219a  
218
5.2.21.7 2,5-Dinitrophenoxo-4'-pentyl-2',5'-benzoquinone 219b  
219
5.2.22 General procedure for the trapping of 2,4-diamino-2',5'-dihydroxydiphenylether 220a and diaminophenoxy-2',5'-benzoquinone 171a,c,d and 154a

5.2.22.1 2,4-Diacetamido-2',5'-dihydroxydiphenylether 222

5.2.22.2 2,4-Diacetamido-3',4'-dimethyl-2',5'-benzoquinone 223a

5.2.22.3 2,4-Diacetamido-4'-butyl-2',5'-benzoquinone 223b

5.2.22.4 2,4-Diacetamido-4'-pentyl-2',5'-benzoquinone 223c

5.2.22.5 2,5-Diacetamido-2',5'-dihydroxy-4'-butyldiphenylether 258

5.2.22.6 2-Acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone

5.2.23 General procedure for the preparation of 8-amino-alkyl-3H-phenoxazin-3-ones 167a-e and 7-amino-2-alkyl-3H-phenoxazin-3-ones 59d-e

5.2.23.1 8-Amino-3H-phenoxazin-3-one 167a

5.2.23.2 8-Amino-1,2-dimethyl-3H-phenoxazin-3-one 167b

5.2.23.3 8-Amino-1,2,4-trimethyl-3H-phenoxazin-3-one 167c

5.2.23.4 8-Amino-2',4'-butyl-3H-phenoxazin-3-one 167d

5.2.23.5 8-Amino-2-pentyl-3H-phenoxazin-3-one 167e

5.2.23.6 8-Amino-1-phenyl-3H-phenoxazin-3-one 167f

5.2.23.7 7-Amino-2',4'-butyl-3H-phenoxazin-3-one 59d

5.2.23.8 7-Amino-2-pentyl-3H-phenoxazin-3-one 59e

5.2.24 General procedure for the preparation of 7-N and 8-N-(N'-t-butoxycarbonyl-β-alanyl)amino-3H-phenoxazin-3-ones 234a-b and 236a-f

5.2.24.1 8-N-(N'-t-Butoxycarbonyl-β-alanyl)amino-3H-phenoxazin-3-one 236a

5.2.24.2 8-N-(N'-t-Butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3H-phenoxazin-3-one 236b

5.2.24.3 8-N-(N'-t-Butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3H-phenoxazin-3-one 236c

5.2.24.4 8-N-(N'-t-Butoxycarbonyl-β-alanyl)amino-2',4'-butyl-3H-phenoxazin-3-one 236d

5.2.24.5 8-N-(N'-t-Butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one 236e
5.2.24.6 $8-N'$(Butoxycarbonyl-$\beta$-alanyl)amino-$1$-phenyl-$3H$-phenoxazin-3-one 236f

5.2.24.7 $7-N'$(Butoxycarbonyl-$\beta$-alanyl)amino-$2$-$t$-butyl-$3H$-phenoxazin-3-one 234a

5.2.24.8 $7-N'$(Butoxycarbonyl-$\beta$-alanyl)amino-$2$-pentyl-$3H$-phenoxazin-3-one 234b

5.2.25 General procedure for the deprotection of $8-N'$(Butoxycarbonyl-$\beta$-alanyl)amino-alkyl-$3H$-phenoxazin-3-one 236a-f and $7-N'$(Butoxycarbonyl-$\beta$-alanyl)amino-alkyl-$3H$-phenoxazin-3-one 268a-b

5.2.25.1 $8-N'$(Alanyl)amino-$3H$-phenoxazin-3-one TFA salt 237a

5.2.25.2 $8-N'$(Alanyl)amino-$1,2$-dimethyl-$3H$-phenoxazin-3-one TFA salt 237b

5.2.25.3 $8-N'$(Alanyl)amino-$1,2,4$-trimethyl-$3H$-phenoxazin-3-one TFA salt 237c

5.2.25.4 $8-N'$(Alanyl)amino-$2$-$t$-butyl-$3H$-phenoxazin-3-one TFA salt 237d

5.2.25.5 $8-N'$(Alanyl)amino-$2$-pentyl-$3H$-phenoxazin-3-one TFA salt 237e

5.2.25.6 $8-N'$(Alanyl)amino-$1$-phenyl-$3H$-phenoxazin-3-one TFA salt 237f

5.2.25.7 $7-N'$(Alanyl)amino-$2$-$t$-butyl-$3H$-phenoxazin-3-one TFA 235a

5.2.25.8 $7-N'$(Alanyl)amino-$2$-pentyl-$3H$-phenoxazin-3-one TFA salt 235b

5.3 Microbiological testing

5.3.1 Preparation of the medium

5.3.1.1 Nitrophenoxazinones substrates

5.3.1.2 Trihalogenophenoxazin-3-one substrates 237g-i and 240a-b

5.3.1.3 Alkylphenoxazin-3-one substrates 237a-f and 235a-b

5.3.2 Inoculation

5.3.2.1 Individual inoculation

5.3.2.2 Multipoint inoculation
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.

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 \(N\)-acetylg glucosamine (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.

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).
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.

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 specificity 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 (FMNH₂) (Scheme 1.1, 1991MR123). Monitoring of the resulting light emission is therefore indicative of the presence of the bacterium.


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](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
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). 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).

![Diagram of DNA fluorescent dyes]

\[
\lambda_{\text{ex/em}} = 500 \text{ nm} / 526 \text{ nm (DNA)} \\
\lambda_{\text{ex/em}} = 510 \text{ nm} / 595 \text{ nm} \\
\lambda_{\text{ex/em}} = 530 \text{ nm} / 625 \text{ nm} \\
\lambda_{\text{ex/em}} = 509 \text{ nm} / 527 \text{ nm}
\]

*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 \( \lambda_{\text{em}} \) whether it is bound to single stranded RNA (\( \lambda_{\text{em}} = 650 \text{nm} \)) or double stranded DNA (\( \lambda_{\text{em}} = 526 \text{nm} \)). 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).

\[
\begin{align*}
10, \quad \text{pH} & < 6.8 : \text{yellow} \\
\text{pH} & > 8.4 : \text{fuschia}
\end{align*}
\]

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).
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.](image)

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.](image)
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.](image-url)
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 \(\beta\)-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).

Scheme 1.7: Enzymatic hydrolysis of CHE-\(\beta\)-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.
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.

\[
\text{Scheme 1.8: Formation of the blue indophenol } 24 \text{ upon enzymatic catalysis.}
\]

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 \(\alpha\)-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 \(\alpha\)-naphthol have rendered possible the detection of osidase and amidase microbial activities via this reaction (2002US6340573B1). The most
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.

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).

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.](image)

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.](image)

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.](image)

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).

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-2009M101). 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-10H-indolo-[1,2a]-indol-10-one 51 (Scheme 1.14).

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-2009M101, with D'. 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 $\pi$-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
1.1.3.4.1 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).

![Structure of common fluorogens.](image)

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
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). 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. 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).

\[
\begin{align*}
R_1 &= n\text{-pentyl}, \quad R_2 = R_3 = H, \quad \text{red/pink} \\
R_1 &= R_2 = CH_3, \quad R_3 = H, \quad \text{red/pink} \\
R_1 &= R_2 = R_3 = CH_3, \quad \text{red/pink} \\
R_1 &= R_2 = -(CH)_4 -, \quad R_3 = H, \quad \text{red/pink}
\end{align*}
\]

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).

\[
\begin{align*}
60, \quad \text{Pink} \\
61, \quad \text{Red-Pink} \\
62, \quad \text{Red-Pink} \\
63a, \quad R_1 = R_2 = OCH_3, \quad \text{burgundy} \\
63b, \quad R_1 = OCH_3, \quad R_2 = H, \quad \text{red}
\end{align*}
\]

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 substrates 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).
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 3-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 activity of E. coli.

1.1.4.2.2 β-D-Galactosidase

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 3-nitrophenol, 6-bromo-2-naphtol (1967MI395), 5-bromo-4-chloro-3-indoxyl (1990AEM301), alizarin (2000LAM336), CHE (1996AEM3868), 3-naphtholbenzoin (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). Streptococcus 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 enterococci (1997JAM532, 2006JAM410), Listeria spp. (2004MI1), Vibrio spp. (2005MI1454), Candida spp. (2009JMM139) and members of the Enterobacteriaceae family (1997JAM532, 2006JAM410).

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 occurrence 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 conditions, 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)-quinolinium bromide (VLPA-GlcNAc, 68, Scheme 1.18, 2002AEM3622), which produced red colonies of *C. albicans*. 
Acidic phosphatase, which has optimum activity at pH 4-5 and was found to be 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).

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: *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, **68**, pale orange **63b**, red).
Merck, 2000JF205), 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 (2000JF205) or Rapid L’MONO, Sanofi (2004MI1)).

Shashidhar et al. reported the use of 2-naphthol-myoinositol-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-myoinositol-1-phosphate 71 (2000US6051391, Scheme 1.19).

![Scheme 1.19: PI-PLC hydrolysis of 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate 71.](image)

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.

<table>
<thead>
<tr>
<th>Alanyl</th>
<th>Glycine</th>
<th>Lysyl-alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Alanyl</td>
<td>Glycyl-arginine</td>
<td>Methionine</td>
</tr>
<tr>
<td>Alanyl-alanine</td>
<td>Glycyl-glycine</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Alanyl-phenylalanyl-proline</td>
<td>Glycyl-proline</td>
<td>Proline</td>
</tr>
<tr>
<td>Arginine</td>
<td>Glycyl tryptophan</td>
<td>Pyrrolidionyl</td>
</tr>
<tr>
<td>Arginy-arginine</td>
<td>Histidine</td>
<td>Serine</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Hydroxyproline</td>
<td>Seryl-tyrosine</td>
</tr>
<tr>
<td>Citrulline</td>
<td>Isoleucine</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>α-Glutamic acid</td>
<td>Leucine</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Glutamyl-glutamic</td>
<td>Leucyl-glycine</td>
<td></td>
</tr>
<tr>
<td>Glutamyl-glycyl-arginine</td>
<td>Lysine</td>
<td></td>
</tr>
</tbody>
</table>

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-pyroglutamynitroanilide (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).

![Aminopeptidase](image)

**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).

**1.1.4.3 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).

![β-ALA](image)

**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, involving nitroso and hydroxylamine intermediates (Scheme 1.23). Experiments involving the monitoring of molecular hydrogen absorbance by bacteria during reduction confirmed this hypothesis (1976AEM949).

\[
\begin{align*}
\text{Scheme 1.23: Suggested enzymatic reduction pathway of nitro compounds (1954BCJ42).}
\end{align*}
\]

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.

\[
\begin{align*}
\text{Scheme 1.24: Nitroreductase activity evidenced by 7-nitrocoumarin-3-carboxylate (2001LAM403).}
\end{align*}
\]
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). 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...
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.](image)

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 Pycnoporus cinnabarinus: pycnoporin 85d. These phenoxazinones were isolated as red crystalline solids, responsible for the bright red-orange pigmentation of the fungi. 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 Actinomycyes (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 C1 (D) (86a, 1964N384), actinomycin C2 (86b) and actinomycin C3 (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).
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 E₁ (86d) and E₂ (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, cinnabarinic acid and its analogues, as well as actinomycin, all share a common core structure, namely 2-amino-3H-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 \(\alpha\)-aminophenol moieties \(94\) to 2-aminophenoxazin-3-one \(95\) is known to occur with different enzymes, phenoxyazinone 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 ceryluloplasmin (1998BBA268) have all been reported to catalyse the reaction (Scheme 1.27).

\[ \text{Scheme 1.27: Formation of 2-aminophenoxazin-3-one } 90 \text{ via an } \alpha\text{-aminophenol oxidative cascade (1989B6323).} \]
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 phenoxyazinone chemistry, particularly for their preparation.

1.1.2.2 Phenoxyazinone 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 phenoxyazinone and phenoxyazimine 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 (K₂CO₃) and gypsum (CaSO₄) 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) NH₃, aerial oxidation; ii) NH₃, aerial oxidation, Ca(OH)₂, K₂CO₃, CaSO₄ (2003MI289)](image)

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 phenoxyazinone 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).
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 to 2-aminophenoxazin-3-one in acidic solution using ferric chloride or potassium dichromate (Scheme 1.30, 1890MI2792).

![Scheme 1.30: Oxidative condensation of o-aminophenol](image)

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 C₃ (1960N230) and actinomycin C₁ (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).
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).

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 ferroXone (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).

Figure 1.19: Example of metal complexes used for the catalytic oxidation of o-aminophenols.
1.3.2 Oxidative mixed condensation of \( \text{o-aminophenol} \)

The first mixed condensation of \( \text{o-aminophenol} \) 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](image)

Ruan et al. reported another type of mixed condensation, between \( \text{o-aminophenols} \) \( 112a-d \) 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[3,2a]phenoxazin-5-one](image)

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

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 aminodinaphthoxazine (120, 2006US0121551). As an example, the synthesis of aminodinaphthoxazine 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).

\[
\text{Scheme 1.37: Preparation of aminodinaphthoxazone 119}
\]
\[\text{i) AcOH, reflux; ii) aq. NH}_3,\text{ reflux; yield not given (2006US0121551).}\]

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 5H-pyrido[3,4a]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).

\[
\text{Scheme 1.38: Preparation of 5H-pyrido[3,4a]phenoxazin-5-one 122}
\]
\[\text{i) Zn(CH}_3\text{COO)}_2,\text{ AcOH, reflux, 2h; yield not given.}\]

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 5H-pyrido[3,2a]phenoxazin-5-one (130, Scheme 1.39).
1.3.5 Condensation of α-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, θ = 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).](image)

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](image)

Scheme 1.42: Preparation of nitro-1,2,4-trihalogeno-3H-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](image)

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).
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. H₂SO₄ 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).

**Scheme 1.45:** Condensation of fluorinated 4-nitrosoresorcinol 137a-c and resorcinol 132b

i) conc. H₂SO₄, EtOH, Δ = 80 °C; 138a, 16.7%; 138b, 41.3%; 138c, 48.5% (2008USP7432372).

### 1.3.7 Condensation of benzoquinonechlorodiimide with resorcinol

Nietzki and Maeccker reported the preparation of 7-amino-1-methylphenoxazin-3-one 133a via the condensation of benzoquinonechlorodiimide 139 and orcinol 132a in conc. H₂SO₄, 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.
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

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 NH₄Cl and zinc dust, resulted in an intramolecular cyclisation of the nitrosodiphenyl ether 147 to a phenoxazin-3-one (81, Scheme 1.48).
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-3H-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 BBr₃. 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).
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 1.49: Preparation of 7-aminophenoxazin-3-one derivatives \(59b\)-c

i) MMPP, MeOH; ii) 2M NaOH, CH\(_3\)OH; \(150a\), 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).

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)pentafluoriodobenzene (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](image)

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).
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 \textit{via} two different synthetic pathways and is divided into four parts. The first part is related to the preparation and characterisation of nitrohalogenophenoxazinones \textit{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 \textit{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 \(\beta\)-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 \(\beta\)-alanine derivatives.

\section*{2.1 Synthesis of halogenophenoxazin-3-ones}

\subsection*{2.1.1 Preparation of nitro-1,2,4-trihalogenophenoxazin-3-ones 130a-c}

The condensation of nitroaminophenols 124\(\beta\) 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 \textit{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 124a-c 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 130αβ-c, 130ββ-c and 130γβ in moderate to excellent yields (Scheme 2.1). The case of fluoranil was slightly different and will be discussed later.

![Chemical structure](attachment:structure.png)

**Scheme 2.1**: Preparation of nitro-1,2,4-trihalogeno-3H-phenoxazin-3-ones

i) EtOH, NaOAc, R.T.

The $^1$H NMR spectra of the resulting nitro-1,2,4-trihalogenophenoxazin-3-ones 130αβ-c, 130ββ-c and 130γβ) displayed only three characteristic deshielded aromatic protons, two doublets and a doublet of doublets in the case of 130αβ-c and 130ββ-c and two doublets of doublets and a triplet in the case of 130γβ (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 130αβ-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 130\(\beta\)-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 130\(\gamma\), 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 130\(\alpha\)-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).

<table>
<thead>
<tr>
<th>(\delta_h) NMR (ppm)</th>
<th>130(\alpha)(\beta)-c</th>
<th>130(\beta)-c</th>
<th>130(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-H 8.44-8.55 (1H, d, (J = 2.4) Hz)</td>
<td>6-H 7.82-7.95 (1H, d, (J = 9.0) Hz)</td>
<td>6-H 8.09 (1H, dd, (J = 7.5, 2.1) Hz)</td>
<td></td>
</tr>
<tr>
<td>8-H 8.29-8.32 (1H, dd, (J = 8.7, 2.4) Hz)</td>
<td>7-H 8.47-8.57 (1H, dd, (J = 9.0-9.3, 2.7) Hz)</td>
<td>7-H 7.97 (1H, t, (J = 8.4) Hz)</td>
<td></td>
</tr>
<tr>
<td>9-H 8.21-8.24 (1H, d, (J = 8.7-9.0) Hz)</td>
<td>9-H 8.60-8.73 (1H, d, (J = 2.7) Hz)</td>
<td>8-H 8.02 (1H, dd, (J = 8.4, 2.1) Hz)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(\delta_c) NMR (ppm)</th>
<th>130(\alpha)(\beta)-c</th>
<th>130(\beta)-c</th>
<th>130(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-C 170.0-171.5</td>
<td>3-C 171.1-171.6</td>
<td>3-C 171.0</td>
<td></td>
</tr>
<tr>
<td>4-C 103.4-111.8</td>
<td>4-C 103.2-111.6</td>
<td>4-C 111.2</td>
<td></td>
</tr>
<tr>
<td>5a-C 136.6-137.1</td>
<td>5a-C 147.7-148.2</td>
<td>5a-C 143.7</td>
<td></td>
</tr>
<tr>
<td>9a-C 143.5-143.6</td>
<td>9a-C 132.6-133.0</td>
<td>9a-C 125.3</td>
<td></td>
</tr>
<tr>
<td>C-(\text{NO}_2) 149.7-149.8</td>
<td>C-(\text{NO}_2) 145.3</td>
<td>C-(\text{NO}_2) 147.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(\nu_{\text{max}}) (cm(^{-1}))</th>
<th>130(\alpha)(\beta)-c</th>
<th>130(\beta)-c</th>
<th>130(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=O 1641-1646</td>
<td>C=O 1638-1649</td>
<td>C=O 1647</td>
<td></td>
</tr>
<tr>
<td>(\text{NO}_2) 1529-1519 and 1347-1346</td>
<td>(\text{NO}_2) 1510-1508 and 1331-1328</td>
<td>(\text{NO}_2) 1532 and 1330</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Key NMR and I.R. spectral features of nitro-1,2,4-trihalogenophenoxazin-3-ones 130\(\alpha\)\(\beta\)-c, \(\gamma\)
I.R. spectroscopy confirmed the presence of a nitro group with the two characteristic stretches around within the 1532-1519 and 1347-1330 cm\(^{-1}\) region (Table 2.1). The C=O stretch observed was of a relatively low absorbance (1646-1638 cm\(^{-1}\)) 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.](image)

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

The condensation of nitro-2-aminophenol 124α-γ with fluoranil 129α 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-γα (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 phenoxyazinone 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 triphenodioxazinone even at R.T.; furthermore, nitro-1,2,4-trifluorophenoxazinones 130α-γα would also show an increased reactivity towards a second condensation.
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-γα followed by cyclisation to 162α-γα, 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-γα.
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- (130αα) and 8-nitro-1,2,4-trifluorophenoxazin-3-one 130βa spectra was made following the same reasoning, the value of δC 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 $\delta_F = -266$ppm corresponding to 1-F, a dd at $\delta_F = -271$ppm corresponding to 2-F and a triplet at $\delta_F = -282$ppm corresponding to 4-F.

<table>
<thead>
<tr>
<th>$\delta_F$ NMR (ppm)</th>
<th>1-F</th>
<th>-266.07- -265.41 (1F, dd, $J = 9.3, 2.0-2.5$Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d$_6$-THF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-F</td>
<td>-271.89 -271.43 (1F, dd, $J = 9.6-9.0, 1.7-2.3$Hz)</td>
<td></td>
</tr>
<tr>
<td>4-F</td>
<td>-282.53 -282.17 (1F, t, $J = 2.3-2.8$Hz)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$\delta_C$ NMR (ppm)</th>
<th>1-C</th>
<th>142.1-142.2 (dd, $J = 273.4-274.7, 12.0-12.2$Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d$_6$-THF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-C</td>
<td>141.3-141.4 (dt, $J = 272.7-273.4, 6.8-6.9$Hz)</td>
<td></td>
</tr>
<tr>
<td>3-C</td>
<td>166.7-166.8 (dt, $J = 21.0-21.4, 5.4-5.6$Hz)</td>
<td></td>
</tr>
<tr>
<td>4-C</td>
<td>135.8-136.0 (dd, $J = 261.9-262.7, 6.1-6.5$Hz)</td>
<td></td>
</tr>
<tr>
<td>4a-C</td>
<td>140.7-141.7 (ddd, $J = 18.1-18.3, 4.8-5.5, 1.1-1.2$Hz)</td>
<td></td>
</tr>
<tr>
<td>10a-C</td>
<td>129.85-130.1 (ddd, $J = 8.5-10.0, 5.0-5.2, 1.2-1.4$Hz)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$\nu_{max}$ (cm$^{-1}$)</th>
<th>C=O</th>
<th>1637-1634</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO$_2$</td>
<td>1531-1518 and 1312-1308</td>
</tr>
<tr>
<td></td>
<td>C-F</td>
<td>1004-1003</td>
</tr>
</tbody>
</table>

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

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-3H-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.

![Scheme 2.5: Preparation of 8-amino-1,2,4-trihalogeno-3H-phenoxazin-3-one 165a-c](image)

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%, Et3N and a H2 pressure of 3.4 bar, the more sensitive brominated analogue led to the isolation of 8-amino-3H-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.

![Scheme 2.6: Hydro-dehalogenation of 8-nitro-1,2,4-tribromo-3H-phenoxazin-3-one 130βc](image)

67
The Pd/C 5% catalyst was replaced by Pt₂O 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-3H-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 1630-1640 cm⁻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).

<table>
<thead>
<tr>
<th></th>
<th>vₘₐₓ (cm⁻¹) NH₂</th>
<th>vₘₐₓ (cm⁻¹) C=O</th>
<th>m/Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>165a X =F</td>
<td>3503, 3358 and 3230</td>
<td>1638</td>
<td>264.9 (M⁻)</td>
</tr>
<tr>
<td>165b X = Cl</td>
<td>3387,3321 and 3226</td>
<td>1634</td>
<td>312.8 (94%), 314.8 (100%), 316.8 (42%), 318.8 (28%) (M⁻)</td>
</tr>
<tr>
<td>165c X = Br</td>
<td>3376, 3319 and 3216</td>
<td>1628</td>
<td>447 (37%), 449 (100%), 451 (91%), 453 (29%) (MH)⁺</td>
</tr>
</tbody>
</table>

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).

The commercially available 2,5-dimethoxybenzaldehyde 149d was oxidised with magnesium monoperxyphthalate (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 Et3N. Demethylation of the diphenylether 169 using BBr3 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-3H-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.

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).
Route A

Route B

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-ethyl-1,4-hydroquinone (173c) (Scheme 2.10).

\[
\begin{array}{c}
\text{OH} \\
\text{4} \\
\text{3} \\
\text{2} \\
\text{1} \\
\text{OCH}_3
\end{array}
\quad \text{i, ii)} \quad
\begin{array}{c}
\text{OCH}_3 \\
\text{5} \\
\text{4} \\
\text{3} \\
\text{2} \\
\text{1} \\
\text{R}
\end{array}
\quad \text{iii)} \quad
\begin{array}{c}
\text{OCH}_3 \\
\text{6} \\
\text{5} \\
\text{4} \\
\text{3} \\
\text{2} \\
\text{1} \\
\text{R}
\end{array}
\]

**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](image)

\[ \text{i) } \text{Br}_2, \text{AcOH, } \text{AcONa}, \text{75.1%}; \text{ ii) } \text{DMF, } \text{K}_2\text{CO}_3, (\text{MeO})_2\text{SO}_2, \text{99.7%}. \]

The structure was confirmed by NMR spectroscopy: the $^1$H NMR spectrum revealed 2 aromatic protons, a doublet at 7.31 ppm of $J = 3.0$ Hz (6-H) and another doublet at 7.51 ppm of $J = 3.0$ Hz (4-H), a singlet at 10.08 ppm corresponded to the formyl group, and a singlet at 10.65 ppm corresponded to the hydroxyl group, as well as an aliphatic signal at 3.78 ppm that corresponded to the methoxy group. The coupling constant of 3.0 Hz 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 hydrodehalogenation producing 2,5-dimethoxybenzaldehyde 149 as a by-product (Scheme 2.12).

![Scheme 2.12: Protection of 2-hydroxy-5-methoxybenzaldehyde 183](image)

\[ \text{i) } \text{NaH, DMF, ii) } \text{MeI, } 45^\circ\text{C}; \text{ 178, 50.7%; 149d, 14.3% or }\]

\[ \text{i) Dimethylsulphate, } \text{K}_2\text{CO}_3, \text{DMF; 178, 99.7%}. \]
This method was replaced by protection using dimethyl sulphate in a suspension of K₂CO₃ 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 Et₃N, Cul, [Ph₃P]₂PdCl₂ and 1-pentyne (Scheme 2.13); however even after 48h reflux in an inert atmosphere of N₂ 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).

![Scheme 2.13: Attempted Sonogashira coupling on 2,5-dimethoxy-3-bromobenzaldehyde 178](image)

i) 1-pentyne, Cul, Pd[PPPh₃]Cl₂, Et₃N, N₂ atm., reflux, 48h, 10.7% or i) 1-pentyne, Cul, Pd[PhCN]₂Cl₂, (Bu)₃P, (Pr)₂NH, dioxane, R.T., 48h, 13.2%.

The use of tris-⁴-butylphosphine ligand along with [PhCN]₂PdCl₂ as a catalyst, in dioxane and di-isopropylamine 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-⁴-butylphosphine ligand is thought to accelerate the rate of oxidative addition of the aryl bromide to [PhCN]₂PdCl₂. 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](image)

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 diacetoxiodate 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$_2$CO$_3$/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](image)

**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 pentyn 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 $J = 3.0$Hz, 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 preparing 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 Na₂CO₃, Pd(PPh₃)₄, DME, EtOH, Δ = 100°C, 85%.

The product was evidenced by NMR analysis: ¹H 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: ¹H 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 metallation 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).

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-dimethoxyphenyltrimethyl lithium 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 \( \text{NH}_4\text{Cl} \) 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).

\[ \text{H}_2\text{C} - \text{O} \quad \text{H} \quad \text{C} - \text{O} \]

196

\[ \text{H}_2\text{C} - \text{O} \quad \text{H} \quad \text{C} - \text{O} \]

197

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

\[ \text{H}_2\text{C} - \text{O} \quad \text{H} \quad \text{C} - \text{O} \]

197

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-pentylphenoxyazinones, and allow a comparison of their performances to the gold standard 7-amino-1-pentylphenoxyazin-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 monoperoxypthalate (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-butybenzaldehyde 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).

![Diagram](image_url)

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 3416-3263 cm\(^{-1}\) region of the I.R. spectra, characteristic of an O-H stretch (Table 6). The hydroxyl proton could also be observed on the \(^1\)H NMR spectra as singlet within the 5.77-6.10 ppm 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 \textit{meta} to each other.

<table>
<thead>
<tr>
<th>(\delta_\text{H, NMR (ppm)})</th>
<th>213a-g</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCH(_3) (7-H)</td>
<td>3.40-3.90</td>
</tr>
<tr>
<td>OCH(_3) (8-H)</td>
<td>3.65-3.90</td>
</tr>
<tr>
<td>-OH</td>
<td>5.55-5.90</td>
</tr>
</tbody>
</table>

| \(\nu_{\text{max}} \text{ (cm}^{-1}\) | 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-\(^1\)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 triisopropylborate. The resulting boronic acid could then be oxidised to the desired phenol using a 35% w/v aqueous solution of \(\text{H}_2\text{O}_2\) (Scheme 2.20).

**Scheme 2.20**: Preparation of 2,5-dimethoxy-4-\(^1\)butylphenol 150c
i) 2.5M \(n\)-BuLi in hexane, dry THF, -78 °C; ii) [isoPrO]_3B, -78 °C to R.T.; iii) sat. aq. \(\text{NH}_3\)Cl; iv) 35% w/w \(\text{H}_2\text{O}_2\), 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).

\[ \text{Scheme 2.21: Bromination of 3,5-dimethoxyphenol 177} \]

i) \( \text{Br}_2, \text{DCM}, 72.6\% \).

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-OH 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 \textit{para} substituents, required for the preparation of 2-hydroxy-6-pentyl-1,4-benzoquinone 180 from olivetol 181 (Route D), was potassium nitrosodisulphonate (Fremy’s salt, 201), with many reports of this type of oxidation in the literature (1971CRV229).

A first attempt to oxidise olivetol, adapted from the procedure described by Musso (1958CB349, Scheme 2.22), in CH\(_3\)CN using a K\(_2\)HPO\(_4\) buffer was unsuccessful and only olivetol was recovered.

\[ \text{Scheme 2.22: Oxidation of orcinol (1958CB349)} \]

i) Fremy’s salt, H\(_2\)O, K\(_2\)HPO\(_4\).

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.

\[ R_1 - R_2 \quad \xrightarrow{i)} \quad R_1 - R_2 - R_3 \]

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Structure</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>203a</td>
<td>( R_1 = R_2 = H, R_3 = Cl )</td>
<td>86%</td>
</tr>
<tr>
<td>203b</td>
<td>( R_1 = R_3 = Cl )</td>
<td>83%</td>
</tr>
<tr>
<td>203c</td>
<td>( R_1 = R_2 = R_3 = Br )</td>
<td>96%</td>
</tr>
</tbody>
</table>

**Scheme 2.23:** Oxidation of orcinol derivatives 203a-c

i) Fremy’s salt, K$_2$HPO$_4$, H$_2$O, 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 tribromoolivetol. 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 $^1$H NMR spectroscopy, with only one singlet in the aromatic region, at 6.10ppm, which disappeared after a D$_2$O 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.

\[ 181 \quad \xrightarrow{i)} \quad 205 \quad \xrightarrow{ii)} \quad 206 \]

**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$_2$HPO$_4$, H$_2$O, CH$_3$CN, R.T., 81.6%.
The oxidation of 2,4,6-tribromoolivetol 205 was attempted again, with a slight modification, as CH₃CN was used to ease the dissolution of 2,4,6-tribromoolivetol in the K₂HPO₄ 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 ¹³C 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 Et₃N 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 Et₃N. 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.

![Scheme 2.25: Preparation of 2,4-dinitro-2',5'-dimethoxydiphenylether 169a-g](image)

i) DMSO, Et₃N, R.T., 5h-18h (b: DMSO, DBU, R.T., 18h).

87
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](image)

i) DMSO, Et$_3$N, 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 3.32-3.755 ppm for 7'-H and within 3.63-3.82 ppm for 8'-H. I.R. spectroscopy showed the characteristic absorption bands for nitro groups within the 1337-1345 cm$^{-1}$ and 1529-1540 cm$^{-1}$ (Table 2.5).

<table>
<thead>
<tr>
<th>$\delta_\text{H}$ NMR (ppm)</th>
<th>$\nu_{\text{max}}$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d$_6$-DMSO</td>
<td>NO$_2$</td>
</tr>
<tr>
<td>3-H</td>
<td>8.73-8.94 (1H, d, $J = 2.7$-3.0 Hz)</td>
</tr>
<tr>
<td>5-H</td>
<td>8.15-8.39 (1H, dd, $J = 9.0$-9.3, 2.7-3.0 Hz)</td>
</tr>
<tr>
<td>6-H</td>
<td>6.69-7.05 (1H, d, $J = 9.0$-9.3 Hz)</td>
</tr>
<tr>
<td>7'-CH$_3$</td>
<td>3.32-3.755 (3H, s)</td>
</tr>
<tr>
<td>8'-CH$_3$</td>
<td>3.63-3.82 (3H, s)</td>
</tr>
</tbody>
</table>

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.

\[
\begin{array}{c}
\text{Scheme 2.27: Attempted preparation of 2,4-dinitrophenoxy-4',6'-dibromo-3'-pentyl-2',5'-benzoquinone 208} \\
i) \text{DMSO, Et}_3\text{N, R.T. or NaH, DMF, 50°C, 72h.}
\end{array}
\]

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.
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 HNO₃ and conc. H₂SO₄, followed by hydrolysis of the amide bond using aq. NaOH (Scheme 2.29). Nitration mainly occurred in the para position to the acetonilide group, but the ortho isomer 210b was also isolated in low yield (1976ACB141, 1989JFC245, Scheme 2.29).

![Scheme 2.29: Nitrination of 2-fluoroacetanilide 209](image)

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 NaBO₃·4H₂O, 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 trifluoroacetic 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 H₂O₂. Such a high concentration of H₂O₂ is not available anymore due to its known hazard and use of a 35% w/v aqueous H₂O₂ 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 H₂O₂ solution with a urea-hydrogen peroxide adduct (UHPA) to generate the TFPAA (1992TL4835).

2-Fluoro-4-nitroaniline was then oxidised in CH₃CN using TFPAA, previously generated by addition of trifluoroacetic anhydride to an CH₃CN suspension of urea-hydrogen peroxide adduct, to the expected 2,5-dinitrofluorobenzene 151 in good yield (Scheme 2.30).

![Scheme 2.30: Oxidation of 2-fluoro-4-nitroaniline 210a](image)

i) UHPA, TFAA, CH₃CN, -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 NH₂ (Table 2.6).

<table>
<thead>
<tr>
<th></th>
<th>210a</th>
<th>151</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ₀ NMR (ppm)</td>
<td>3-H 7.87-7.93 (2H, m)</td>
<td>8.42 (1H, dd, J = 9.0, 7.5 Hz)</td>
</tr>
<tr>
<td></td>
<td>5-H 7.87-7.93 (2H, m)</td>
<td>8.26 (1H, ddd, J = 9.0, 2.4, 1.5 Hz)</td>
</tr>
<tr>
<td></td>
<td>6-H 6.85 (1H, d, J = 8.7 Hz)</td>
<td>8.53 (1H, dd, J = 10.5, 2.4 Hz)</td>
</tr>
<tr>
<td></td>
<td>NH₂ 6.77 (2H, br. s)</td>
<td>-</td>
</tr>
<tr>
<td>ν max (cm⁻¹)</td>
<td>NH₂ 3493 and 3395</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NO₂ 1522 and 1314</td>
<td>1549 and 1343</td>
</tr>
</tbody>
</table>

*Table 2.6: Key ¹H 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 3395 cm\(^{-1}\) 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\(_3\)N 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.

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.

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
dry THF, 2.5M n-BuLi, N₂, -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).

i) Phenylboronic acid, Pd(PPh₃)₄, 2M Na₂CO₃, EtOH, DME, ∆ = 100°C, 24h, N₂ 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.

![Scheme 2.34: Formylation of 2,4-dinitro-2',5'-dimethoxydiphenylether 169g](image)

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 BBr₃

The diphenylethers prepared were deprotected using BBr₃. 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 ¹³C 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).

![Chemical structure](Image)

**Scheme 2.35:** Deprotection of 2,4-dinitro-2',5'-dimethoxydiphenylether 169b and 169g  
i) 1M BBr₃ in DCM, dry DCM, N₂ atm., -75°C to R.T., ii) H₂O.

The isolation of quinone 179b was confirmed by the presence on the ¹³C 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',5'-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 BBr₃ 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).
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 tribromodiphenyl 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.

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$_3$OH 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).](image)

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).
The quinone C=O stretches were also visible as two distinct absorption bands within the 1647-1651 cm\(^{-1}\) and 1665-1676 cm\(^{-1}\) 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 1341-1347 cm\(^{-1}\) and 1526-1544 cm\(^{-1}\) region of the I.R. spectra confirmed the integrity of the dinitrophen oxybenzoquinone ring (Table 2.7).

<table>
<thead>
<tr>
<th></th>
<th>179a,c,d,e</th>
<th>219a-b</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\delta_h) NMR (ppm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-H</td>
<td>8.88-8.94 (1H, d, (J = 2.7Hz))</td>
<td>3-H</td>
</tr>
<tr>
<td>5-H</td>
<td>8.38-8.60 (1H, dd, (J = 9.0-9.3, 2.7Hz))</td>
<td>4-H</td>
</tr>
<tr>
<td>6-H</td>
<td>7.31-7.90 (1H, d, (J = 9.0-9.3Hz))</td>
<td>6-H</td>
</tr>
<tr>
<td>(\delta_c) NMR (ppm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-C</td>
<td>179.9-181.4</td>
<td>2'-C</td>
</tr>
<tr>
<td>5'-C</td>
<td>186.6-187.6</td>
<td>5'-C</td>
</tr>
<tr>
<td>(\nu_{max}) (cm(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C=O</td>
<td>1647-1649 and 1665-1676</td>
<td>C=O</td>
</tr>
<tr>
<td>NO(_2)</td>
<td>1341-1347 and 1526-1543</td>
<td>NO(_2)</td>
</tr>
</tbody>
</table>

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).

![HMQC spectrum of 2,4-dinitrophenoxy-4'-pentyl-2',5'-benzoquinone 179d.](image)

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.
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-dinitrophenoxyno-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-dinitrophenoxyno-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 BBr₃ 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 BBr₃, 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](image)

i) H₂, 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, Et₃N 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, Ag₂O or MnO₂) was added to promote the reaction (Scheme 2.40).

![Scheme 2.40: Oxidative cyclisation of 2,4-diamino-2’,5’-dihydroxydiphenylether derivatives](image)

i) Et₃N, ii) [O] with AgO, Ag₂O or MnO₂.
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$^{-1}$.

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 occurring 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.](image)

### 2.2.12.2 From 2,4 and 2,5-dinitrophenoxo-2',5'-benzoquinones

Dinitrophenox-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 Ag₂O or MnO₂ (Scheme 2.42).

![Scheme 2.42: Catalytic hydrogenation of dinitrophenoxo-2',5'-benzoquinone 179a-d,f and 219a-b](image)

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 Et₃N 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 $^1$H 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).

<table>
<thead>
<tr>
<th>δ$_H$ NMR (ppm)</th>
<th>167b-f</th>
<th>59d-e</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-H</td>
<td>7.05-7.20 (1H, d, $J = 8.7$-9.3Hz)</td>
<td>6.475-6.51 (1H, d, $J = 2.4$Hz)</td>
</tr>
<tr>
<td>7-H</td>
<td>6.80-6.90 (1H, dd, $J = 8.7$-9.0, 2.7Hz)</td>
<td>6.69-6.71 (1H, dd, $J = 8.7$-9.0, 2.4Hz)</td>
</tr>
<tr>
<td>8-H</td>
<td>6.77-6.96 (1H, d, $J = 2.7$Hz)</td>
<td>9-H</td>
</tr>
<tr>
<td>9-H</td>
<td>6.77-6.96 (1H, d, $J = 2.7$Hz)</td>
<td>7.43-7.48 (1H, d, $J = 8.7$Hz)</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>5.31-5.41 (2H, br.s)</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>3-C</td>
<td>184.1-184.5</td>
<td>3-C</td>
</tr>
<tr>
<td>4-C</td>
<td>104.0-111.75</td>
<td>4-C</td>
</tr>
<tr>
<td>184.3-184.6</td>
<td>105.1-106.5</td>
<td></td>
</tr>
<tr>
<td>C=O</td>
<td>1634-1649</td>
<td>C=O</td>
</tr>
<tr>
<td>1643-1661</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-H</td>
<td>3355-3414, 3308-3337 and 3198-3225</td>
<td>3408-3420, 3317-3329 and 3205-3226</td>
</tr>
</tbody>
</table>

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-dinitrophenoxycarbonitrile and 2,5-dinitrophenoxycarbonitrile.

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](image)

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

![Scheme 2.44: Trapping of the 2,4-aminophenoxy-2',5'-benzoquinones intermediates via acetylation](image)

Scheme 2.44: Trapping of the 2,4-aminophenoxy-2',5'-benzoquinones intermediates via acetylation

1 i) MeOH, Pd/C 5%, H₂, P = 2.4 bar; ii) Acetic anhydride.

Acetylation was evidenced on the \(^1\)H NMR spectrum, as described above, by two singlets in the 2.0-2.1 ppm region, each integrating for three protons, and two deuterium exchangeable protons in the 9.0 ppm and 10.0 ppm region. The carbonyls were observed as two deshielded signals in the 165-170 ppm region of \(^13\)C NMR spectrum, and as a sharp peak within the 1697-1726 cm\(^{-1}\) 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 180 ppm and 186 ppm, and on the I.R. spectrum with two absorption bands around 1660 cm\(^{-1}\) and 1650 cm\(^{-1}\) (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 186 ppm, respectively, following the reasoning discussed in section 2.2.11.
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).

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

<table>
<thead>
<tr>
<th>Range for 223a-c</th>
<th>2a-CH₃ and 2b-CH₃</th>
<th>2.03-2.06 (3H, s) and 2.055-2.07 (3H, s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ₂ NMR (ppm)</td>
<td>2-NH</td>
<td>8.63-9.33 (1H, s)</td>
</tr>
<tr>
<td></td>
<td>4-NH</td>
<td>9.31-10.07 (1H, s)</td>
</tr>
<tr>
<td>δc NMR (ppm)</td>
<td>1a-C and 1b-C</td>
<td>165.8-168.8 and 166.3-169.2</td>
</tr>
<tr>
<td></td>
<td>2'-C</td>
<td>181.0-182.1</td>
</tr>
<tr>
<td></td>
<td>5'-C</td>
<td>186.6-187.8</td>
</tr>
<tr>
<td>νmax (cm⁻¹)</td>
<td>C=O (quinone)</td>
<td>1639-1648 and 1655-1665</td>
</tr>
<tr>
<td></td>
<td>C=O (amide)</td>
<td>1697-1726</td>
</tr>
<tr>
<td></td>
<td>N-H</td>
<td>3270-3403</td>
</tr>
</tbody>
</table>

Scheme 2.45: Formation of 2-acetoxy-5-acetamido-1-phenylamino-3',4'-dimethyl-2',5'-benzoquinone 224
i) MeOH, Pd/C 5%, H₂, 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-NH).](image)

The correlation between one of the NH 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'-butyl-2',5'-benzoquinone 219a and trapping of the resulting intermediate was carried out following the same conditions described for 2,4-dinitrophen oxybenzoquinones 179a, c and e (Scheme 2.47). The resulting product was 2,5-diacetamido-4'-butyl-2',5'-dihydroxydiphenylether 227, providing evidence for the formation of 2,5-diamino-2',5'-dihydroxy-4'-butyldiphenylether 154a as an intermediate (Scheme 2.47).

Scheme 2.47: Catalytic hydrogenation of 2,5-dinitrophenoxy-4'-butyl-2',5'-benzoquinone 219a
i) MeOH, Pd/C 5%, H₂, P = 2.4 bar; ii) Acetic anhydride; 97.5%.

Unlike its 2,4-regioisomer 179c, 2,5-dinitrophenoxy-4'-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 8.4 ppm, respectively (Figure 2.11).

![Figure 2.11: Comparison of sections of 1H NMR spectrum and 13C NMR spectrum for 222b and 226 in d6-DMSO.](image)

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-dinitrophenoxazinone 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](image)

| i) | MeOH, Pd/C 5%, H₂, P = 2.4bar, ii) Ag₂O, 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 MnO₂, 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).
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 βoc protected β-alanine via a standard peptide coupling procedure. The addition of TFA, required to remove the βoc 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-3H phenoxazin-3-one, showed satisfactory results when an active mixed anhydride was used for the coupling procedure. In the case of 7-amino-3H-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-3H-phenoxazin-3-ones 59d-e were reduced to 7-amino-3-hydroxy-3H-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).
Scheme 2.50: Reduction of 7-aminophenoxazin-3-ones to 7-amino-3-hydroxy-3H-phenoxazin-3-ones.

The active mixed anhydride 232 was prepared in situ by reacting isobutylchloroformate 231 with N-\(^1\)Boc-\(\beta\)-alanine 230 under dry conditions in the presence of a base (Scheme 2.51). Although N-methylmorpholine or Et\(_3\)N 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-3H-phenoxazin-3-one chromogens prepared.

\[
\begin{align*}
230 & \quad + \quad 231 & \quad \xrightarrow{i)} \quad 232 \\
NCH(\text{Boc})-\text{COOH} & \quad + \quad \text{Cl}-\text{CH}_{\text{R}} & \quad \xrightarrow{i)} \quad \text{NCH(\text{Boc})-CO-}\text{CO-CH}_{\text{R}}
\end{align*}
\]

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-\(^1\)Boc-\(\beta\)-alanyl)aminophenoxazin-3-ones 234a-b (Scheme 2.52).

Coupling of the protected \(\beta\)-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-NH attested the formation of the peptide bond. The broad singlet at 6.80ppm was assigned to NH of the \(^1\)Boc protected amino acid chain. The \(\beta\)-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 \(^1\)Boc protecting group (Figure 2.12).
Scheme 2.52: Preparation of the 7-N-(β-alanyl)amino-2-alkylenoxazinone-3-one 269a-b
i) H₂, 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, 10 min, R.T., 235a, 94.4%, 235b, 88.5%.

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

Figure 2.12: ¹H NMR of 7-N-(Boc-β-alanyl)-amino-2-pentylphenoxazinone-3-one 234b.
Deprotection of the substrate was effected smoothly using neat TFA (Scheme 2.52). Removal of the ‘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 NH-$^t$Boc signal at 6.80ppm and the C(CH$_3$)$_3$ signal at 1.30ppm, along with the appearance of a very broad singlet integrating for three protons at 7.965ppm, characteristic of NH$_3^+$ (Figure 2.13).

![Figure 2.13: $^1$H NMR spectrum of 7-$N$-(β-alanyl)-amino-2-pentylphenoxazin-3-one TFA salt 235b.](image)

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).
The characteristic spectral features, attesting to the coupling to N\textsuperscript{Boc}-\beta-alanine, were the singlet integrating for nine hydrogen atoms within the 1.30-1.42ppm region, corresponding to C(CH\textsubscript{3})\textsubscript{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 \beta-alanine protected amino group (Table 2.10). The formation of a peptide bond resulted in a dramatic deshielding of the 8-NH proton, observed within the 9.90-10.33ppm region (Table 2.10). Both carbonyls resulting from the linking to N\textsuperscript{Boc}-\beta-alanine could be seen, on the 13C 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\textsuperscript{-1} region (Table 2.10).

The deprotection of substrates 236a-i with TFA resulted in the disappearance of the C(CH\textsubscript{3})\textsubscript{3}, NH and C=O (4'-C) signals belonging to the \textsuperscript{6}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 1H NMR spectra, and as a broad absorption band within the 3032-3077cm\textsuperscript{-1} region of the I.R. spectra (Table 2.10).
### Table 2.10: Key NMR and I.R. spectroscopic features of 8-\(N'-\text{Boc-\(\beta\)-alanyl})\text{aminophenoxazin-3-ones 236a-i} and their corresponding TFA salt 237a-i resulting from deprotection.

<table>
<thead>
<tr>
<th>(\delta) _NMR (ppm)</th>
<th>(\delta) _C (ppm)</th>
<th>(\nu_{\text{max}}) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d_6)-DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(CH(_3))(_3)</td>
<td>1.30-1.42 (9H, s)</td>
<td></td>
</tr>
<tr>
<td>2'-H</td>
<td>2.34-2.57 (2s, (J = 6.6-7.8)Hz)</td>
<td>2.67-2.77 (2s, (J = 6.3-6.6)Hz)</td>
</tr>
<tr>
<td>3'-H</td>
<td>3.175-3.32 (2s, (J = 6.6-7.2)Hz)</td>
<td>3.05-3.30 (2s, (J = 6.3-6.6)Hz)</td>
</tr>
<tr>
<td>8-NH</td>
<td>9.90-10.33 (1H, s)</td>
<td>10.33-10.56 (1H, s)</td>
</tr>
<tr>
<td>(NH\text{boc})</td>
<td>6.49-6.90 (1H, br. s)</td>
<td></td>
</tr>
<tr>
<td>(NH_3^+)</td>
<td>-</td>
<td>7.75-7.98 (3H, br. s)</td>
</tr>
<tr>
<td>1'-C</td>
<td>170.05-170.4</td>
<td>168.9-169.3</td>
</tr>
<tr>
<td>4'-C</td>
<td>156.0-156.1</td>
<td></td>
</tr>
<tr>
<td>C=O</td>
<td>1679-1713</td>
<td>1670-1678</td>
</tr>
<tr>
<td>(N^+\text{-H})</td>
<td>-</td>
<td>3032-3077</td>
</tr>
<tr>
<td>N-H</td>
<td>3263-3422</td>
<td>3255-3408</td>
</tr>
</tbody>
</table>

### 2.3.2.2 \(N\)- and \(O\)- di-\(\beta\)-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\(_2\)O, in an attempt to re-oxidise the expected 8-\(N\'(\text{N'-Boc-\(\beta\)-alanyl})\text{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.
NMR analysis of the isolated product 240b clearly showed the presence of two $N$-$\text{tBoc}$-$\beta$-alanine groups (Figure 2.14), along with two extra aromatic protons to those expected, one attributed to the second $N$-$\text{tBoc}$-$\beta$-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.
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 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$_2$O. 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 4.19 ppm along with the 3 aromatic protons at 6.69, 6.95 and 7.28 ppm 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.](image)

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 50\(\mu\)M in MeOH in order to quantify the difference of colouration between 7- and 8-aminophenoxazin-3-ones. With a \(\lambda_{\text{max}}\) 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 \((\lambda_{\text{max}} = 536-540\text{nm})\). The extinction coefficient \(\varepsilon\) of 7-aminophenoxazinones was considerably higher than that of 8-aminophenoxazinones (Table 2.11, Figure 2.16).

<table>
<thead>
<tr>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>A</th>
<th>(\varepsilon) (dm(^3).mol(^{-1}).cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>167d</td>
<td>536</td>
<td>0.295</td>
</tr>
<tr>
<td>167e</td>
<td>540</td>
<td>0.322</td>
</tr>
<tr>
<td>59d</td>
<td>533</td>
<td>1.804</td>
</tr>
<tr>
<td>59e</td>
<td>528</td>
<td>1.554</td>
</tr>
</tbody>
</table>

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).

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).

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).

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( \Delta \lambda ) (nm)</th>
<th>( \lambda )</th>
<th>( \varepsilon ) (dm(^3).mol(^{-1}).cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>167d</td>
<td>536</td>
<td>64</td>
<td>0.295</td>
<td>5900</td>
</tr>
<tr>
<td>237c</td>
<td>472</td>
<td></td>
<td>0.348</td>
<td>6960</td>
</tr>
<tr>
<td>167e</td>
<td>540</td>
<td>65</td>
<td>0.322</td>
<td>6400</td>
</tr>
<tr>
<td>237d</td>
<td>475</td>
<td></td>
<td>0.388</td>
<td>7760</td>
</tr>
<tr>
<td>59d</td>
<td>533</td>
<td>75</td>
<td>1.804</td>
<td>36080</td>
</tr>
<tr>
<td>235a</td>
<td>458</td>
<td></td>
<td>0.922</td>
<td>18440</td>
</tr>
<tr>
<td>59e</td>
<td>528</td>
<td>71</td>
<td>1.554</td>
<td>31080</td>
</tr>
<tr>
<td>235b</td>
<td>457</td>
<td></td>
<td>0.861</td>
<td>17220</td>
</tr>
</tbody>
</table>

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-d 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 \( \lambda_{\text{max}} \) 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 nitrophoxazinones 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.

Scheme 3.1: Expected nitroreductase enzyme activity and subsequent reaction of substrates 130α-γ a-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).
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).

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 $^{130}\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\a
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>130αa</th>
<th>130αb</th>
<th>130αc</th>
<th>130γa</th>
<th>130γb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>++ -</td>
<td>++ Red +/-</td>
<td>++ Orange (halo) +</td>
<td>++ -</td>
<td>++ -</td>
<td>++ -</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td>++ -</td>
<td>++ -</td>
<td>++ Orange (halo) +</td>
<td>++ -</td>
<td>++ -</td>
<td>++ -</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>++ -</td>
<td>++ Red +/-</td>
<td>++ -</td>
<td>++ -</td>
<td>++ -</td>
<td>++ -</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ -</td>
<td>++ -</td>
<td>++ -</td>
</tr>
<tr>
<td><em>M. morganii</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/</td>
</tr>
<tr>
<td><em>P. rettgeri</em></td>
<td>++ -</td>
<td>++ Red +</td>
<td>++ Orange (halo) +</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/-</td>
<td>++ Brown halo +/</td>
</tr>
<tr>
<td>Background colour</td>
<td>None</td>
<td>Sepia</td>
<td>Orange/brown</td>
<td>Yellow/brown</td>
<td>Sepia</td>
<td>Lilac</td>
</tr>
</tbody>
</table>

**Table 3.2:** Multi-point screening results for substrates 130αa-c and 130γα-β 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:**

![Substrate Image](image-url)
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>130αa</th>
<th>130αb</th>
<th>130αc</th>
<th>130γa</th>
<th>130γb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. faecium</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>St. epidermidis</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>Tr.</td>
<td>-</td>
</tr>
<tr>
<td>St. aureus</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>MRSA</td>
<td>+</td>
<td>-</td>
<td>Tr.</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Red   +/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C. albicans</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Background colour</td>
<td>None</td>
<td>Sepia</td>
<td>Orange/brown</td>
<td>Yellow/brown</td>
<td>Sepia</td>
<td>Lilac</td>
</tr>
</tbody>
</table>

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**: 

![Substrate Diagram](image)
## 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:**

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>130βa</th>
<th>130βb</th>
<th>130βc</th>
<th>242</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative</strong></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>C. freundii</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M. morganii</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>P. rettgeri</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Background colour</td>
<td>None</td>
<td>Sepia</td>
<td>Orange/brown</td>
<td>Yellow/brown</td>
<td>Orange</td>
</tr>
</tbody>
</table>
### 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

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>130βa</th>
<th>130βb</th>
<th>130βc</th>
<th>242</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive</strong></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>+</td>
<td>-</td>
<td>Tr.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E. faecium</td>
<td>+</td>
<td>-</td>
<td>Tr.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>St. epidermidis</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>St. aureus</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MRSA</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C. albicans</td>
<td>+</td>
<td>-</td>
<td>Tr.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>+</td>
<td>-</td>
<td>NG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Background colour</strong></td>
<td>None</td>
<td>Sepia</td>
<td>Orange/brown</td>
<td>Yellow/brown</td>
<td>Orange</td>
</tr>
</tbody>
</table>

**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 \textbf{130α-γ a-c} and \textbf{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 \textbf{130αa}, \textbf{130βa} and \textbf{130γa}.

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 \textbf{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 \textbf{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).

Nitroreductase activity was similar, but of a much weaker intensity, for the rest of the 7-nitrophenoxazin-3-one series, substrates \textbf{130αb-c}. Several factors could contribute to the observed reduced enzymatic activity for substrates \textbf{130αb-c}: the reduced availability of the substrate in the medium and the low solubility compared to \textbf{130αa} may have limited the quantity of substrate permeated into the bacteria, resulting in the production of weak colour.
The series of 9-nitrophenoaxin-3-one substrates 130γα-β showed similar results; both substrates 130γα and 130γβ were reduced by most of the Gram negative organisms (Table 3.2 and Table 3.4). The colour generated (brown, substrate 130γα and purple, substrate 130γβ) was sufficient to justify enzymatic activity; however visualisation was impaired by a weak contrast with the background (Figures 3.4 and Figure 3.5) and extensive diffusion in the medium (130γβ, Figure 3.5). The diffusion was particularly noticeable for colonies 8, 9 and 10, for which nitroreductase activity seems to be the more pronounced (130γβ, Figure 3.5), with the formation of a large halo around the colonies.

Interestingly, none of the substrates belonging to the 8-nitro-1,2,4-trihalogenophenoxazine series 130βα-γ 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 3.4: Screening of 9-nitro-1,2,4-trifluoro-3H-phenoxazin-3-one for nitroreductase activity.](image1)

![Figure 3.5: Screening of 9-nitro-1,2,4-trichloro-3H-phenoxazin-3-one for nitroreductase activity.](image2)

![Figure 3.6: substituted para nitrobenzenes tested for nitroreductase activity.](image3)

R = NH₂, OCH₃, COOH, OH, Cl, Br, SO₂H, CHO, COCH₃, SO₂NH₂, CO-Ar, NO₂, COOCH₃, 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-γ-c and 7-nitrocoumarin-3-carboxylate.

The electron density present on the 8-position in the case of substrate 130β-c was considerably higher compared to the 7- and 9- position in the case of substrate 130α-c, 130γ-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 130β-c, while substrates 130α-c, 130γ-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 130αα-c, 130ββ-a-b and 130γγ-a-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 130αα-c, 130ββ-a-c and 130γγ-a-b, the colour generated was still weak (Figure 3.7), suggesting such substrates have little application for microbiological use.

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.
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-3H-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 130αα and 130γα 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.
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>237g</th>
<th>237h</th>
<th>237i</th>
<th>240a</th>
<th>240b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>M. morganii</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>P. rettgeri</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Background colour</td>
<td>None</td>
<td>Brown-orange</td>
<td>V. faint orange</td>
<td>V. faint orange</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

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
### 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

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Control</th>
<th>237g</th>
<th>237h</th>
<th>237i</th>
<th>240a</th>
<th>240b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
</tr>
<tr>
<td><strong>Gram positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>St. epidermidis</em></td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>St. aureus</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>MRSA</em></td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Background colour</strong></td>
<td>None</td>
<td>Brown-orange</td>
<td>V. faint orange</td>
<td>V. faint orange</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**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:**

![237g-i Substrate](image1)

![240a-b Substrate](image2)
An important concern with substrates 240a and 240b was the release of the actual chromogen, 8-amino-1,2,4-trihalogeno-3H-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.

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-alanylaminophenoxazinone 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).
As for substrates 237c, the substrates 237a-f 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**
<table>
<thead>
<tr>
<th></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. marcescens</em></th>
<th></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. marcescens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>237a</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><strong>237b</strong></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td><strong>237f</strong></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><strong>237c</strong></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

*Table 3.8:* Testing of substrates *237a,b,c,f* after 24h of incubation at 37°C. Concentration 50mg/L.
<table>
<thead>
<tr>
<th></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. marcescens</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. marcescens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>237d</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>237e</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Control</th>
<th>237a</th>
<th>237b</th>
<th>237c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25mg/L</td>
<td>50mg/L</td>
<td>100mg/L</td>
</tr>
<tr>
<td><strong>Growth</strong></td>
<td><strong>Colour</strong></td>
<td><strong>Growth</strong></td>
<td><strong>Colour</strong></td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Background colour</strong></td>
<td>None</td>
<td>Faint yellow</td>
<td>Orange</td>
</tr>
<tr>
<td><strong>Growth</strong></td>
<td><strong>Colour</strong></td>
<td><strong>Growth</strong></td>
<td><strong>Colour</strong></td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>+/− violet (diff.)</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Background colour</strong></td>
<td>None</td>
<td>Yellow</td>
<td>Orange</td>
</tr>
</tbody>
</table>

**Table 3.10** Results for substrates 237a,b,c,f after 24h of incubation at 37°C.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>237d</th>
<th>237e</th>
<th>235a</th>
<th>235b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+/- violet (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+/- violet (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Background colour

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>237d</th>
<th>237e</th>
<th>235a</th>
<th>235b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+ pink (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+ pink (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Background colour

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>237d</th>
<th>237e</th>
<th>235a</th>
<th>235b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
<td>Colour</td>
<td>Growth</td>
</tr>
<tr>
<td>E. coli</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+ pink (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+ pink (diff.)</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

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.

The dimethyl 237b, trimethyl 237c, 4-′butyl 237d 8-amino phenoxazinone substrates and 7-amino-4-′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-′butylphenoxazinone 235a (Table 3.8 and Table 3.9). This activity was more pronounced for the dimethyl and 4-′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-\textsuperscript{t}butylphenoxazinone \textbf{235a} exhibited a better contrast with the background compared to 8-amino-4-\textsuperscript{t}butylphenoxazinone \textbf{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 (\textbf{237e} and \textbf{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 \textbf{235b} compared to the isomeric 8-amino-2-pentylphenoxazinone substrate \textbf{237e}. Better results were obtained after 48h of incubation (Figure 3.11).

![Figure 3.11: Performance of substrate 74, 235b and 237e with \textit{Ps. aeruginosa} at 50mg/L after 48h of incubation A: 74, B: 235b, C: 237e](image)

The colouration was weaker compared to the lead compound 7-amino-1-pentylphenoxazin-3-one \textbf{74} in both instances, but the toxicity was also considerably lower: individual colonies were easily observed with \textbf{235b} and \textbf{237e}, but almost absent in the case of \textbf{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 \textbf{235a-b} and \textbf{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 \textbf{237a} corresponded to the absence of any obvious hydrolysis observed, whereas the highest calculated value found for \textbf{235b} and \textbf{237e} corresponded to a good level of hydrolysis. The high value obtained for \textbf{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.

| 237a, 8-position, R₁ = R₂ = R₃ = H | 0.658 |
| 237b, 8-position, R₁ = R₂ = CH₅, R₃ = H | 1.606 |
| 237c, 8-position, R₁ = R₂ = R₃ = CH₃ | 2.105 |
| 235a, 7-position, R₁ = R₂ = 'bu, R₃ = H | 2.484 |
| 237d, 8-position, R₁ = R₂ = 'bu, R₃ = H | 2.547 |
| 237f, 8-position, R₁ = R₃ = Ph, R₂ = H | 3.273 |
| 235b, 7-position, R₁ = R₂ = penty, R₃ = H | |
| 237e, 8-position, R₁ = R₂ = penty, R₃ = H | |

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 \textit{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 \textit{79} and \textit{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).

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{NH}_2 \quad \text{OH} \quad \text{X} \quad \text{X} \quad \text{X} \quad \text{X} \\
\xrightarrow{\text{i)} \quad \text{NaOAc, EtOH, R.T.}} & \quad \text{O}_2\text{N} \quad \text{N} \quad \text{O} \quad \text{X} \quad \text{X} \quad \text{X} \\
\text{130α-γa-c} & \quad \text{H}_2 \quad \text{Pd/C 5% or Pt}_2\text{O, MeOH/EtOAc, R.T.}} \quad \text{iv)} \quad \text{Ag}_2\text{O, MeOH, 48-72h, R.T.}\quad \text{v)} \quad \text{CF}_3\text{COO^− H}_3\text{N^+}} \quad \text{237g-h}
\end{align*}
\]

Scheme 4.1: Recapitulative synthesis of substrates 130α-γa-b and substrates 237g-h
i) NaOAc, EtOH, R.T.; ii) H₂, Pd/C 5% or Pt₂O, MeOH/EtOAc; iii) Ag₂O, MeOH; iv) 232, dry THF, N-methylpiperidine, 48-72h, R.T.; v) neat TFA.
*Only substrates 130αa-c were subjected to reduction

The nitrophenoxyazinone 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).

<table>
<thead>
<tr>
<th>Hydrolysis</th>
<th>Colour</th>
<th>Toxicity</th>
<th>Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram negative</td>
<td>Gram positive</td>
<td>Gram negative</td>
</tr>
<tr>
<td>7-NO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130αa X = F</td>
<td>YES</td>
<td>NO</td>
<td>Red</td>
</tr>
<tr>
<td>130αb X = Cl</td>
<td>YES</td>
<td>NO</td>
<td>Orange</td>
</tr>
<tr>
<td>130αc X = Br</td>
<td>YES</td>
<td>NO</td>
<td>Brown</td>
</tr>
<tr>
<td>8-NO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130βa X = F</td>
<td>NO</td>
<td>NO</td>
<td>-</td>
</tr>
<tr>
<td>130βb X = Cl</td>
<td>NO</td>
<td>NO</td>
<td>-</td>
</tr>
<tr>
<td>130βc X = Br</td>
<td>NO</td>
<td>NO</td>
<td>-</td>
</tr>
<tr>
<td>9-NO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>130γa X = F</td>
<td>YES</td>
<td>NO</td>
<td>Brown</td>
</tr>
<tr>
<td>130γb X = Cl</td>
<td>YES</td>
<td>NO</td>
<td>Violet</td>
</tr>
</tbody>
</table>

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-3H-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.
**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](image)

i) EtOH, base or AcOH, reflux

The presence of an alkyl substituent in the 2-position (R₂) 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 phenoaxinone.

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-3H-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-3H-phenoxazine 229c would also result in a reduced colouration.
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-\text{butoxycarbonyl-β-alanyl})amino-1-pentyl-3H-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-dinitrophenoxoxybenzoquinones 179a,c,d,f and 219a-b. Finally, catalytic hydrogenation of 2,4- or 2,5-
dinitrophenoxynbenzoquinones 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$_3$N, DMSO or NaH 60% w/w, DMF, iv) CAN, H$_2$O : CH$_3$CN, v) H$_2$, Pd/C 5%, EtOAc : MeOH, vi) Ag$_2$O, 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-dinitrophenoxynbenzoquinone 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 dinitrophenoxynbenzoquinones 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-dinitrophenoxynbenzoquinones 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-diaminophenoxazinone \(171\) 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](image)

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

With 2,5-dinitrophenoxynbenzoquinones, 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-diaminophenoxynbenzoquinone intermediate, and reason for the reduction of the quinone ring in this case (Scheme 4.6) could not be fully rationalised.

![Scheme 4.6](image)

**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).

<table>
<thead>
<tr>
<th></th>
<th>Hydrolysis/Toxicity</th>
<th>Colour</th>
<th>Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ps. aeruginosa</em></td>
<td><em>B.cepacia</em></td>
<td><em>S.marcrescens</em></td>
</tr>
<tr>
<td>237a</td>
<td>R₁ = R₂ = R₃ = H</td>
<td>NO / NO</td>
<td>- / -</td>
</tr>
<tr>
<td>237f</td>
<td>R₁ = Ph, R₂ = R₃ = H</td>
<td>NO / NO</td>
<td>- / -</td>
</tr>
<tr>
<td>237b</td>
<td>R₁ = R₂ = CH₃, R₃ = H</td>
<td>YES / NO</td>
<td>- / -</td>
</tr>
<tr>
<td>237c</td>
<td>R₁ = R₂ = R₃ = CH₃</td>
<td>YES / NO</td>
<td>- / -</td>
</tr>
<tr>
<td>237d</td>
<td>R₁ = R₃ = H, R₂ = tbu</td>
<td>YES / NO</td>
<td>- / -</td>
</tr>
<tr>
<td>237e</td>
<td>R₁ = R₃ = H, R₂ = pentyl</td>
<td>YES / NO</td>
<td>NO / NO</td>
</tr>
</tbody>
</table>

Table 4.2: Summary of microbiological testing results for substrates 237a-e (at 50mg/L)
- : Data not available
### Table 4.3: Summary of microbiological testing results for substrates 59d-e (at 50mg/L)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis</th>
<th>Colour</th>
<th>Diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>59d</td>
<td>YES/NO, NO/NO, YES/NO, NO</td>
<td>Pink</td>
<td>YES</td>
</tr>
<tr>
<td>59e</td>
<td>YES/NO, -/-, YES/NO, NO</td>
<td>Pink</td>
<td>NO</td>
</tr>
</tbody>
</table>

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-Nβ-alanylamino-1-pentylphenoxazin-3-one 79, and with 8-Nβ-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).
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) BuLi, Δ, iii) n-BuLi, THF, -78 °C, iv) DMF, -78 °C to R.T., v) m-CPBA, DCM, vi) MeOH, NaOH, vii) Et3N, 2,4-dinitrofluorobenzene, viii) CAN oxidation, ix) H2, 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 phenoaxin-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 derivativalisation 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-Haen 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-trihalogeno-3H-phenoxazin-3-one 130\(\alpha\)-\(\gamma\)-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-3H-phenoxazin-3-one 130αα

The title compound was prepared from p-fluranil 129α (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 (MgSO₄). 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-3H-phenoxazin-3-one 130αα 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%. C₁₂H₅F₃N₂O₄ 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 (NO₂), 1003 (C-F); δ_H (300 MHz, δ₋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, δ₋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, δ₋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-3H-phenoxazin-3-one 130αβ (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-3H-phenoxazin-
3-one 130\textit{ab} was isolated as a purple solid (4.06g, 11.75mmol, 90.4%). Purple lustrous plates of 130\textit{ab} were obtained from glacial acetic acid; \textit{m.p.}: 239-241°C [lit.: 238-240°C (from AcOH, 1958ZOB2977)]; \textit{m/z} 343.8 (85%), 345.8 (100%), 347.7 (26%), 348.9 (15%) (M\textsuperscript{+}); (found: C, 41.7; H, 0.9; N, 7.9%. C\textsubscript{12}H\textsubscript{3}Cl\textsubscript{3}N\textsubscript{2}O\textsubscript{4} requires: C, 41.7; H, 0.9; N 8.1%); \textit{ν\textsubscript{max}}/cm\textsuperscript{-1} 3094 (C=O), 1646 (C=O), 1584 (C=C), 1519 and 1347 (NO\textsubscript{2}); \textit{δ\textsubscript{H}} (300 MHz, d\textsubscript{6}-DMSO) 8.24 (1H, d, \textit{J} = 8.7Hz, 9-H), 8.32 (1H, dd, \textit{J} = 8.7, 2.4Hz, 8-H), 8.51 (1H, d, \textit{J} = 2.4Hz, 6-H); \textit{δ\textsubscript{C}} (75 MHz, d\textsubscript{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., 2x 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-3H-phenoxazin-3-one 130\textit{ac}

\begin{center}
\includegraphics[width=0.2\textwidth]{image1}
\end{center}

The title compound was prepared from p-bromanil 129\textit{c} (1.03g, 2.42mmol) and 2-aminophenol 124\textit{a} (0.34g, 2.20mmol); 7-nitro-1,2,4-tribromo-3H-phenoxazin-3-one 130\textit{ac} was isolated as a bright red solid (1.10g, 2.30mmol, 95.0%). A microcrystalline dark red solid of 130\textit{ac} was obtained from acetic acid, \textit{m.p.}: 263-264°C; (found: C, 30.1; H, 0.7; N, 5.6%. C\textsubscript{12}H\textsubscript{3}Br\textsubscript{3}N\textsubscript{2}O\textsubscript{4} requires: C, 30.1; H, 0.6; N 5.85%); \textit{m/z} 475.6 (33%), 477.6 (100%), 479.7 (84%), 481.7 (28%) (M\textsuperscript{+}); \textit{ν\textsubscript{max}}/cm\textsuperscript{-1} 3101 (C=H), 1641 (C=O), 1529 and 1346 (NO\textsubscript{2}); \textit{δ\textsubscript{H}} (300 MHz, d\textsubscript{6}-DMSO) 8.21 (1H, d, \textit{J} = 9.0Hz, 9-H), 8.29 (1H, dd, \textit{J} = 8.7, 2.4Hz, 8-H), 8.44 (1H, d, \textit{J} = 2.4Hz, 6-H); \textit{δ\textsubscript{C}} (75 MHz, d\textsubscript{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 130\textit{ba}

\begin{center}
\includegraphics[width=0.2\textwidth]{image2}
\end{center}
**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 MgSO₄. 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) : Et₂O (50:50 to 0:100). 8-Nitro-1,2,3-trifluoro-3H-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 MgSO₄. The solvent was evaporated in vacuo and the residue was purified by column chromatography using a gradient of petroleum ether (60-80°C) : EtOAc (75:25 to 60:40). 8-Nitro-1,2,3-trifluoro-3H-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%. C₁₂H₃F₃N₂O₄ requires C, 48.7; H, 1.0; N, 9.5%); m/z 295.9 (M⁺); νmax/cm⁻¹ 3093 (C-H), 1648 (C=O), 1608 (C=C), 1518 and 1308 (NO₂), 1004 (C-F); δH (300 MHz, d₈-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); δC (75 MHz, d₈-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); δF (282 MHz, d₈-THF) -282.05 (1F, t, J = 2.8Hz, 4-F), -271.73 (1F, dd, J = 9.3, 2.5Hz, 1-F).
5.2.1.5 8-Nitro-1,2,4-trichloro-3H-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-3H-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%. C₁₂H₃Cl₃N₂O₄ 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⁻); νmax/cm⁻¹ 3088 (C-H), 1649 (C=O), 1597 (C=C), 1510 and 1331 (NO₂); δH (300 MHz, d₆-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); δC (75 MHz, d₆-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-3H-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-3H-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%. C₁₂H₃Br₃N₂O₄ 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⁻); νmax/cm⁻¹ 3078 (C-H), 1638 (C=O), 1581 (C=C), 1508 and 1328 (NO₂); δH (300 MHz, d₆-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); δC (75 MHz, d₆-DMSO) 103.4 (quat., 4-C), 118.1 (CH, 6-C), 125.6 (CH, 9-C),

165
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-3H-phenoxazin-3-one 130\(\gamma\)a

![Chemical Structure](image)

The title compound was prepared from \(p\)-fluoranil 129\(c\) (0.90g, 5.00mmol) and 2-amino-3-nitrophenol 124\(\gamma\) (0.74g, 4.82mmol); 9-nitro-1,2,4-trifluoro-3H-phenoxazin-3-one 130\(\gamma\)a was isolated as a dark red solid (0.22g, 0.73mmol, 15.2%). Ruby red prisms of 130\(\gamma\)a 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%. \(C_{12}H_{3}F_{3}N_{2}O_{4}\) requires C, 48.7; H, 1.0; N, 9.5%); \(m/z\) 295.9 (\(M^+\)); \(v_{\text{max}}/\text{cm}^{-1}\) 3084 (C-H), 1649 (C=O), 1603 (C=C), 1530 and 1313 (NO\(_2\)), 1004 (C-F); \(\delta_{H}\) (300 MHz, \(d_8\)-THF) 6.04-6.13 (m, 3H, 6-H, 7-H and 8-H); \(\delta_{C}\) (75 MHz, \(d_8\)-THF) 117.6 (CH, 8-C), 117.9 (CH, 6-C), 123.0 (quat., 9a-C), 130.0 (quat., ddd, \(J = 10.0, 5.0, 1.2\) Hz, 10a-C), 131.5 (CH, 7-C), 135.8 (quat., dd, \(J = 261.9, 6.3\) Hz, 4-C), 141.4 (quat., dt, \(J = 273.2, 6.8\) Hz, 2-C), 142.1 (quat., dd, \(J = 274.7, 12.2\) Hz, 1-C), 140.7 (quat., ddd, \(J = 18.2, 4.8, 1.2\) Hz, 4a-C), 141.5 (quat., d, \(J = 1.7\) Hz, 5a-C), 146.4 (quat., 9-C), 166.7 (quat., td, \(J = 21.1, 5.5\) Hz, 3-C); \(\delta_{F}\) (282 MHz, \(d_8\)-THF) -282.53 (1F, t, \(J = 2.3\) Hz, 4-F), -271.43 (1F, dd, \(J = 9.0, 2.3\) Hz, 1-F), -265.36 (1F, dd, \(J = 9.3, 2.3\) Hz, 2-F).

5.2.1.8 9-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one 130\(\gamma\)b

![Chemical Structure](image)

The title compound was prepared from \(p\)-chloranil 129\(c\) (1.17g, 7.61mmol) and 2-amino-3-nitrophenol 124\(\gamma\) (1.07g, 6.92mmol); 9-nitro-1,2,4-trichloro-3H-phenoxazin-3-one 130\(\gamma\)b was isolated as a dark purple solid (1.08g, 3.13mmol, 45.3%). Lustrous dark purple needles of 130\(\gamma\)b were obtained from acetic acid, m.p.: 254-258°C; (found: C, 41.6; H, 0.9; N, 7.8%. \(C_{12}H_{5}Cl_{3}N_{2}O_{4}\) 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); $v_{\text{max}}/\text{cm}^{-1}$ 3100 (C-H), 1647 (C=O), 1590 (C=C), 1532 and 1331 (NO$_2$); $\delta_\text{H}$ (300 MHz, d$_6$-DMSO) 7.97 (1H, t, $J = 8.4$Hz, 7-H), 8.02 (1H, dd, $J = 8.4$, 2.1Hz, 8-H), 8.09 (1H, dd, $J = 7.5$, 2.1Hz, 6-H); $\delta_\text{C}$ (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-3H-phenoxazin-3-one 165a-c

Nitro-1,2,3-trihalogeno-3H-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-3H-phenoxazin-3-one 165a

The title compound was prepared from 8-nitro-1,2,3-trifluoro-3H-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-3H-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$_5$F$_3$N$_2$O$_2$
requires C, 54.15; H, 1.9; N, 10.5%); \( m/2 \) 264.9 (\( M^- \); \( \nu_{\text{max}} \)/cm\(^{-1} \) 3503, 3358 and 3230 (NH\(_2\)), 1638 (C=O), 1610, 1575 and 1513 (C=C), 1002 (C-F); \( \delta_H \) (500 MHz, d\(_1\)-TFA) 8.17 (1H, d, \( J = 9.0 \text{Hz} \), 6-H), 8.35 (1H, dd, \( J = 9.0, 2.0 \text{Hz} \), 7-H), 8.74 (1H, d, \( J = 1.5 \text{Hz} \), 9-H); \( \delta_C \) (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, \( J = 16.5, 3.9 \text{Hz} \), 10a-C), 132.8 (quat., 8-C), 138.2 (quat., dd, \( J = 263.5, 5.4 \text{Hz} \), 4-C), 141.6 (quat., dd, \( J = 18.0, 7.8 \text{Hz} \), 4a-C), 143.9 (quat., dt, \( J = 275.0, 5.4 \text{Hz} \), 2-C), 144.2 (quat., 5a-C), 144.4 (quat., dd, \( J = 279.2, 13.2 \text{Hz} \), 1-C), 172.3 (quat., td, \( J = 19.0, 6.6 \text{Hz} \), 3-C).

5.2.3 General procedure for the preparation of 8-\( N^t\)-butoxycarbonyl-\( \beta \)-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 130\( \beta \)-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 \textit{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-\( \beta \)-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 isobutylchloroformate (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 \textit{via} syringe to the mixed
anhydride. The resulting mixture was stirred under nitrogen for 48-72h. A 10% aq. solution of Na₂CO₃ 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 Na₂CO₃ (100mL), once with water (100mL), once with brine (100mL), and dried (MgSO₄). 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-(N¹-Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3H-phenoxazin-3-one 236g

The title compound was prepared as described from 8-amino-1,2,4-trifluoro-3H-phenoxazin-3-one 165a (0.30g, 1.13mmol). Elution used a gradient mixture of petroleum ether (60-80°C) : EtOAc (25:75 to 0:100). 8-N-(N¹-Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3H-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%. C₂₀H₁₈F₃N₃O₅ requires C, 54.9; H, 4.15; N, 9.6%); m/z 436.0 (M-H⁻); νmax/cm⁻¹ 3362 and 3304 (N-H), 1680 (C=O), 1654 (C=O), 1632 (C=O), 1158 (C-O), 1001 (C-F); δH (300 MHz, d₅-DMSO) 1.40 (9H, s, C(CH₃)₃), 2.53 (2H, t, J = 7.2Hz, 2¹H), 3.27 (2H, q, J = 6.9Hz, 3¹H), 6.88 (1H, br. s, NH¹Boc), 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-NH); δC (75 MHz, d₅-DMSO) 28.7 (3 × CH₃, 6'/7'/8'-C), 36.9 (CH₂, 3¹-C), 37.3 (CH₂, 2¹-C), 78.2 (quat., 5¹-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., 4¹-C), 168.7 (quat., dt, J = 20.4, 6.6Hz, 3-C), 170.4 (quat., 1¹-C); δF (282 MHz, d₅-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'-Butoxycarbonyl-β-alanylamino-1,2,4-trichloro-3H-phenoxazin-3-one 236h

8-Amino-1,2,4-trichloro-3H-phenoxazin-3-one 165b was prepared as described earlier from 8-nitro-1,2,4-trichloro-3H-phenoxazin-3-one 130βb (0.16g, 0.46mmol). 8-Amino-1,2,4-trichloro-3H-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+) ; νmax/cm⁻¹ 3387, 3321 and 3226 (NH₂), 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-3H-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'-butoxycarbonyl-β-alanylamino-1,2,4-trichloro-3H-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%. C₂₀H₁₈Cl₅N₃O₅ 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); νmax/cm⁻¹ 3359 and 3318 (N-H), 1681 (C=O), 1652 (C=O), 1628 (C=O), 1165 (C-O); δH (300 MHz, d₅-DMSO) 1.40 (9H, s, C(CH₃)₃), 2.54 (2H, t, J = 6.9Hz, 2'-H), 3.27 (2H, q, J = 6.6Hz, 3'-H), 6.89 (1H, br. s, NH'-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-NH); δC (75 MHz, d₅-DMSO) 28.7 (3 x CH₃, 6'/7'/8'-C), 36.9 (CH₂, 3'-C), 37.4 (CH₂, 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′-Butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3H-phenoxazin-3-one 236i

8-Amino-1,2,4-tribromo-3H-phenoxazin-3-one 165c was prepared as described from 8-nitro-1,2,4-tribromo-3H-phenoxazin-3-one 130βc (0.43g, 0.89mmol). 8-Amino-1,2,4-tribromo-3H-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⁺); νmax/cm⁻¹ 3376, 3319 and 3216 (NH₂), 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-3H-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′-butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3H-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%. C₂₀H₁₉Br₉N₃O₅ 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-H⁺); νmax/cm⁻¹ 3357 (N-H), 1685 (C=O), 1678 (C=O), 1613 (C=O), 1170 (C-O); δH (300 MHz, d₆-DMSO) 1.40 (9H, s, C(CH₃)₃), 2.54 (2H, t, J = 6.9Hz, 2'-H), 3.27 (2H, q, J = 6.6Hz, 3'-H), 6.89 (1H, br. s, NH-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-NH); δC (75 MHz, d₆-DMSO) 28.7 (3 × CH₃, 6'/7'/8'-C), 36.9 (CH₂, 3'-C), 37.4 (CH₂, 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′-butoxycarbonyl-β-alanyl)amino-1,2,4-halogeno-3H-phenoxazin-3-one 237g-i (adapted from 20080BC682)

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 Et₂O, which produced a lasting turbidity. The cloudy suspension was left overnight and the microcrystals formed were collected by filtration and washed with Et₂O. The filtrate was concentrated in vacuo to recover more product, which was triturated and washed with Et₂O.

5.2.4.1 8-N-(β-Alanyl)amino-1,2,4-trifluoro-3H-phenoxazin-3-one TFA salt 237g

The title compound was prepared as described earlier from 8-N-(N′-butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-3H-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°C (dec.); (Found (M)+, 338.0749. Calc. for C₁₅H₁₁F₃N₃O₃: (M)+, 338.0747); m/z 338.1 (MH)+, 350.1 (MNa)+; νmax/cm⁻¹ 3255 (N-H), 3032 (N-H), 1663 (C=O), 1642 (C=O), 1131 (C-O), 1007 (C-F); δH (500 MHz, d₆-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, NH₃+), 8.32 (1H, d, J = 2.5Hz, 9-H), 10.68 (1H, s, NH-Ar); δC (125 MHz, d₆-DMSO) 33.8 (CH₂, 2'-C), 35.3 (CH₂, 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); δF (282 MHz, d₆-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-3H-phenoxazin-3-one TFA salt 237h

![Reaction Diagram]

The title compound was prepared as described earlier from 8-N-(N′-butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-3H-phenoxazin-3-one 236h (0.07g, 0.14mmol). 8-N-(β-alanyl)amino-1,2,4-trichloro-3H-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 C₁₅H₁₁₃Cl₃N₃O₃: (M⁺), 385.98605); m/z 386.0 (100%), 388.0 (97%), 390.0 (42%), 392.0 (10%) (MH⁺); νmax/cm⁻¹ 3348 (N-H), 3073 (N-H). δH (300 MHz, d₆-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, NH₂), 7.77 (1H, dd, J = 9.0, 2.1Hz, 7-H), 8.28 (1H, d, J = 2.1Hz, 9-H), 10.51 (1H, s, NH-Ar); δC (75 MHz, d₆-DMSO) 33.9 (CH₂, 2'-C), 35.35 (CH₂, 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-3H-phenoxazin-3-one TFA salt 237i

![Reaction Diagram]

The title compound was prepared as described earlier from 8-N-(N′-butoxycarbonyl-β-alanyl)amino-1,2,4-tribromo-3H-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 C₁₅H₁₁₇Br₃N₃O₃: (M⁺), 517.8345); m/z 517.8 (35%), 519.8 (100%), 521.8 (89%), 523.8 (35%) (MH⁺); νmax/cm⁻¹ 3344 (N-H), 3060 (N⁺-H), 1663 (C=O), 1609 (C=O), 1140 (C-O); δH (300 MHz, d₆-DMSO) 2.77 (2H, t, J = 6.3Hz, 2'-H), 3.13 (2H,
t, $J = 6.0\text{Hz, 3'}-\text{H}$), 7.65 (1H, d, $J = 9.0\text{Hz, 6-H}$), 7.84 (1H, dd, $J = 9.0, 3.0\text{Hz, 7-H}$), 7.96 (3H, br. s, NH$_3^+$), 8.36 (1H, d, $J = 3.0\text{Hz, 9-H}$), 10.69 (1H, s, NH-Ar); $\delta_C (75\text{MHz, d$_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-$\beta$-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-$3H$-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-$\beta$-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°C. The DMF solution of 8-amino-3-hydroxy-1,2,4-trihalogeno-$10H$-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°C 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 x 70 mL). The combined organic layers were successively washed with a 5% aqueous solution of LiCl (100mL), a 10% aqueous Na$_2$CO$_3$ 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\textsuperscript{1}-Butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-10H-phenoxazin-3-yl-3"-(N\textsuperscript{1}-butoxycarbonylamino)propanoate 239a

The title compound was prepared as described earlier from 8-amino-1,2,4-trifluoro-3H-phenoxazin-3-one 165a (0.285g, 1.07mmol), N\textsuperscript{1}-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\textsubscript{28}H\textsubscript{33}F\textsubscript{3}N\textsubscript{4}O\textsubscript{8} requires C, 55.1; H, 5.45; N, 9.2%); m/z 609.2 (M-H\textsuperscript{+}; m/z 611.2 (M\textsuperscript{+}H\textsuperscript{+}), 633.3 (MN\textsubscript{a})\textsuperscript{+}; ν\textsubscript{max}/cm\textsuperscript{-1} 3399, 3321 and 3367 (N-H), 2978 (C-H), 1777 (C=O), 1679 (C=O), 1565 (C=O), 1170 (C-O), 1119 (C-O), 994 (C-F); δ\textsubscript{H} (300 MHz, (CD\textsubscript{3})\textsubscript{2}CO) 1.27 (9H, s, (CH\textsubscript{3})\textsubscript{3}), 1.29 (9H, s, (CH\textsubscript{3})\textsubscript{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-NH or 3'a-NH\textsubscript{2}), 5.99 (1H, br. s, 3'a-NH or 3'a-NH\textsubscript{2}), 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-NH\textsubscript{2}), 8.95 (1H, s, NH-Ar); δ\textsubscript{C} (75 MHz, (CD\textsubscript{3})\textsubscript{2}CO) 27.7 (3 × CH\textsubscript{3}, 6''/7''/8''-C or 6''/7''/8''-C), 27.8 (3 × CH\textsubscript{3}, 6''/7''/8''-C or 6''/7''/8''-C), 33.4 (CH\textsubscript{2}, 2''-C), 36.2 (CH\textsubscript{2}, 3''-C), 37.0 (CH\textsubscript{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\textsubscript{F} (282 MHz, (CD\textsubscript{3})\textsubscript{2}CO) -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-\(\text{N}^\prime\)-Butoxycarbonyl-\(\beta\)-alanyl)amino-1,2,4-trichloro-10\(H\)-phenoxazin-3-yl 3''-(\(N^\prime\)-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), \(N^\prime\)-Boc-\(\beta\)-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\(_{29}\)H\(_{33}\)Cl\(_3\)N\(_2\)O\(_8\) requires C, 51.0; H, 5.0; N, 8.5%); m/z 657.0 (82%), 659.0 (100%), 661.1 (35%), 663 (12%); \(\nu\)max/cm\(^{-1}\) 3374 (N-H), 3319 (N-H), 1758 (C=O), 1686 (C=O), 1672 (C=O), 1166 (C-O), 1129 (C-O); \(\delta\)\(_H\) (300 MHz, d\(_6\)-DMSO) 1.32 (18H, s, 2 \times (CH\(_3\)_2)), 2.37 (2H, t, \(J = 6.9\)Hz, 2''-H), 2.76 (2H, t, \(J = 6.9\)Hz, 2'-H), 3.14 (2H, q, \(J = 6.9\)Hz, 3''-H), 3.22 (2H, q, \(J = 6.9\)Hz, 3'-H), 6.59 (1H, d, \(J = 8.7\)Hz, 6-H), 6.73 (2H, br. dd, \(J = 8.7\), 2.1Hz, 7-H and 3a'-NH or 3a''-NH), 6.87 (1H, br s, 3a'-NH or 3a''-NH), 7.32 (1H, d, \(J = 2.1\)Hz, 9-H), 8.43 (1H, s, 10-NH), 9.70 (1H, s, NH-Ar); \(\delta\)\(_C\) (75 MHz, d\(_6\)-DMSO) 28.7 (6 \times 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-\(\text{N}^\prime\)-butoxycarbonyl-\(\beta\)-alanyl)amino-1,2,4-trihalogeno-10\(H\)-phenoxazin-3-yl-3''-(\(N^\prime\)-butoxycarbonylamino)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 Et₂O.

5.2.6.1 3-O-(8-N-(β-Alanyl)amino-1,2,4-trifluoro-10H-phenoxazin-3-yloxy)-1''-oxopropane-3''-aminium difluoracetate salt 240a

The title compound was prepared as described earlier from 8-N-(N''-butoxycarbonyl-β-alanyl)amino-1,2,4-trifluoro-10H-phenoxazin-3-yl-3''-(N''-butoxycarbonylamino)propanoate 239a (0.10g, 0.155mmol). The product 240a was isolated as an off-white solid (0.075g, 0.12mmol, 70.0%); m.p.: 120-125°C; (Found (M-C₃H₆NO)⁺, 340.0906. Calc. for C₁₅H₁₃F₃N₃O₃: (M-C₃H₆NO)⁺, 340.0935); m/z 338.1 (M-C₃H₆NO)⁺, 340.0 (M-C₃H₆NO)⁺; v_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, (CD₃)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, (CD₃)₂CO) -165.93 (1F, dd, J = 21.4, 7.3Hz, 1-F), -159.70 (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 difluoracetate salt 240b

The title compound was prepared as described earlier from 8-N-(N''-butoxycarbonyl-β-alanyl)amino-1,2,4-trichloro-10H-phenoxazin-3-yl-3''-(N''-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 difluoracetate 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%), \((M-H)^+\); \(v_{max}/cm^{-1}\) 3378 (N-H), 3041 (N-H), 1759 (C=O), 1670 (C=O), 1198 and 1180 (C-O); \(\delta_{H}(300 MHz, d_6-DMSO)\) 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\textsubscript{3}+), 8.10 (3H, br. s, NH\textsubscript{3}+), 8.59 (1H, s, 10-NH), 10.08 (1H, s, NH-Ar); \(\delta_{C}(75 MHz, d_6-DMSO)\) 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\textsubscript{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 (5.57 g, 40.31 mmol); elution used petroleum ether (60-80 °C) : Et₂O (95:5). 1,4-Dimethoxy-2,3-dimethylbenzene 172a was isolated as a white solid (6.11 g, 36.78 mmol, 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%. C₁₀H₁₄O₂ requires C, 72.3; H, 8.5%); m/z 166.0 (M⁺, 179.1 (MNa⁺); νmax/cm⁻¹ 2956 (C-H), 1462 (C=C), 1257 (C-O), 1094 (C-O); δH (300 MHz, CDCl₃) 2.25 (6H, s, 2 x CH₃, 8-H and 9-H), 3.85 (6H, s, 2 x OC₃H₃, 7-H and 10-H), 6.74 (2H, s, 5-H and 6-H); δC (75 MHz, CDCl₃) 12.1 (CH₃, 8-C and 9-C), 56.1 (CH₃, 7-C and 10-C), 107.9 (CH₃, 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 (9.06 g, 59.53 mmol); elution used petroleum ether (60-80 °C) : Et₂O (95:5). 1,4-Dimethoxy-2,3,5-trimethylbenzene 172b was isolated as a white solid (9.05 g, 50.20 mmol, 84.4%); m.p. (petroleum ether 60-80 °C): 34-35.5 °C [lit.: 35-36 °C (from MeOH, 1962JOC841)]; νmax/cm⁻¹ 2990 (C-H), 1485 (C=C), 1229 (C-O), 1092 (C-O); δH (300 MHz, CDCl₃) 2.19 (3H, s, 8-H), 2.24 (3H, s, 9-H), 2.35 (3H, s, 11-H), 3.72 (3H, s, OCH₃, 7-H or 10-H), 3.85 (3H, s, OCH₃, 7-H or 10-H), 6.60 (1H, s, 5-H); δC (75 MHz, CDCl₃) 12.5 (CH₃, 8-C), 13.1 (CH₃, 9-C), 16.75 (CH₃, 11-C), 56.3 (CH₃, 7-C), 60.55 (CH₃, 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-\textsuperscript{t}butylbenzene 172c (2000MI924)

![Chemical structure of 1,4-Dimethoxy-2-\textsuperscript{t}butylbenzene]

The title compound was prepared as described from \textsuperscript{t}butylhydroquinone 173c (9.99g, 60.10mmol); elution used petroleum ether (60-80°C) : Et\textsubscript{2}O (98:2). 1,4-Dimethoxy-2-\textsuperscript{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\textsubscript{max}/cm\textsuperscript{-1} 2951 (C-H), 1583 (C=C), 1217 (C-O); δ\textsuperscript{H} (300 MHz, CDCl\textsubscript{3}) 1.28 (9H, s, C(CH\textsubscript{3})\textsubscript{3}), 3.675 (3H, s, OCH\textsubscript{3}, 7-H or 12-H), 3.70 (3H, s, OCH\textsubscript{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\textsuperscript{C} (75 MHz, CDCl\textsubscript{3}) 29.7 (3 × CH\textsubscript{3}, 10/11/12-C), 35.0 (quat., 9-C), 55.7 (2 × CH\textsubscript{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)

![Chemical structure of 1,4-Dimethoxy-2-pentylbenzene]

A 250mL flame dried round bottomed flask, flushed with N\textsubscript{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 N₂ 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 Et₂O (2 x 200mL). The combined organic layers were successively washed with water (200mL) and brine (200mL), and dried (MgSO₄). The solvent was removed in vacuo and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et₂O (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 (M⁺); (found: C, 74.65; H, 9.5%. C₁₃H₂₀O₂ requires C, 75.0; H, 9.7%); νmax/cm⁻¹ 2832-2928 (C-H), 1498 (C=C), 1219 (C-O), 1048 (C-O); δH (300 MHz, CDCl₃) 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, OCH₃, 7-H or 8-H), 3.68 (3H, s, OCH₃, 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); δC (75 MHz, CDCl₃) 14.1 (CH₃, 5'-C), 22.6 (CH₂, 3'-C or 4'-C), 29.6 (CH₂, 2'-C), 30.3 (CH₂, 1'-C), 31.8 (CH₂, 3'-C or 4'-C), 55.7 (CH₃, 8'-C), 56.0 (CH₃, 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 Na₂CO₃. When no more evolution of CO₂ was observed following addition of Na₂CO₃, the resulting mixture was diluted with water and then extracted with Et₂O (3 x 80mL to 3 x 200mL), washed with brine (1 x 100mL to 1 x 200mL) and dried (MgSO₄). 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) : Et₂O (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%. C₁₁H₁₄O₃ requires C, 68.0; H, 7.3%); m/z 195.0 (M⁺), 217.0 (MNa⁺); νₓ cm⁻¹ 2957 and 2855 (C-H), 1681 (C=O), 1593 (C=C), 1128 and 1091 (C-O); δ_H (300 MHz, CDCl₃) 2.22 (3H, s, CH₃, 9-H); 2.26 (3H, s, CH₃, 10-H); 3.83 (3H, s, OCH₃, 5-H); 3.85 (3H, s, OCH₃, 8-H); 7.15 (1H, s, 6-H); 10.35 (1H, s, 7-H); δ_C (75 MHz, CDCl₃) 12.1 (CH₃, 10-C), 12.9 (CH₃, 9-C), 55.7 (CH₃, 8-C), 63.8 (CH₃, 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⁺); νₓ 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, CDCl₃) 2.13 (3H, s, CH₃, 11-H), 2.19 (3H, s, CH₃, 10-H); 2.42 (3H, s, CH₃, 7-H); 3.57 (3H, s, OCH₃, 9-H); 3.70 (3H, s, OCH₃, 12-H); 10.41 (1H, s, CHO, 8-H); δ_C (75 MHz, CDCl₃) 12.1 (CH₃, 11-C), 12.8 (CH₃, 7-C), 13.7 (CH₃, 10-C), 60.2 (CH₃, 9-C), 63.2 (CH₃, 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): Et₂O (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%. C₁₃H₁₈O₃ requires C, 70.25; H, 8.2%); m/z 223.1 (MH)⁺, 245.05 (MNa)⁺; νmax/cm⁻¹ 2965 and 2832 (C-H), 1673 (C=O), 1611 (C=C), 1200 and 1043 (C-O); δH (300 MHz, CDCl₃) 1.42 (9H, s, C(CH₃)₃), 3.87 (3H, s, OC₃H₃, 8-H), 3.93 (3H, s, OCH₃, 13-H), 6.98 (1H, s, 3-H), 7.32 (1H, s, 6-H), 10.43 (1H, s, CHO, 7-H); δC (75 MHz, CDCl₃) 29.4 (3 × CH₃, 10/11/12-C), 35.9 (quat., 9-C), 55.5 (CH₃, 8-C), 56.2 (CH₃, 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) : Et₂O (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. C₁₄H₂₀O₃ requires C,
71.2; H, 8.5%); \( m/z \) 237.0 (\( MH^+ \)), 259.0 (\( MNa^+ \); \( \nu_{\text{max}}/\text{cm}^{-1} \) 2929 and 2859 (C-H), 1675 (C=O), 1610 (C=C), 1210 and 1040 (C-O); \( \delta_H \) (300 MHz, CDCl₃) 0.94 (3H, t, \( J = 6.9\) Hz, 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, \( J = 7.5\) Hz, 1'-H), 3.85 (3H, s, OCH₃, 8-H), 3.92 (3H, s, OCH₃, 9-H), 6.82 (1H, s, 3-H), 7.29 (1H, s, 6-H), 10.43 (1H, s, CHO, 7-H); \( \delta_C \) (75 MHz, CDCl₃) 14.0 (CH₃, 5'-C), 22.5 (CH₂, 3'-C or 4'-C), 29.2 (CH₂, 2'-C), 31.0 (CH₂, 1'-C), 31.8 (CH₂, 3'-C or 4'-C), 55.8 (CH₃, 8-C), 56.2 (CH₃, 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

![Structure of 2,4-Dinitro-2',5'-dimethoxy-4'-carbaldehydediphenylether](image)

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%. C₁₅H₁₂N₂O₈ requires C, 51.7; H, 3.5; N, 8.0%); \( m/z \) 349.0 (\( MH^+ \); \( \nu_{\text{max}}/\text{cm}^{-1} \) 2997 and 2882 (C-H), 1682 (C=O), 1603, 1518 and 1500 (C=C), 1539 and 1345 (NO₂), 1215 and 1035 (C-O); \( \delta_H \) (300 MHz, d₁-TFA) 3.30 (3H, s, OCH₃, 8'-H), 3.40 (3H, s, OCH₃, 9'-H), 6.455 (1H, s, 6'-H), 6.57 (1H, d, \( J = 9.3\) Hz, 6-H), 7.14 (1H, s, 3'-H), 7.89 (1H, dd, \( J = 9.3, 2.7\) Hz, 5-H), 8.41 (1H, d, \( J = 2.7\) Hz, 3-H), 9.75 (1H, s, CHO, 7'-H); \( \delta_C \) (75 MHz, d₁-TFA) 55.7 (CH₃, 9'-C), 56.0 (CH₃, 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 Br₂ (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 (Na₂S₂O₃), 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 (MgSO₄). 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)]; \( \nu_{\text{max/cm}^{-1}} \) 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%). C₉H₇BrO₃ requires C, 41.6; H, 3.05%); \( \delta_H \) (300 MHz, d₆-DMSO) 3.78 (3H, s, OCH₃), 7.31 (1H, d, J = 3.0Hz, 6-H), 7.51 (1H, d, J = 3.0Hz, 4-H), 10.08 (1H, s, CHO), 10.65 (1H, s, O-H); \( \delta_C \) (75 MHz, d₆-DMSO) 56.5 (CH₃, 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)

![Chemical structures](image)

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 (MgSO₄). 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; \textbf{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%); \(\nu_{\text{max}}/\text{cm}^{-1}\) 3062 (O-H), 3003, 2939, 2887 and 2836 (C-H), 1641 (C=O), 1311, 1235, 1124 and 1034 (C-O); \(\delta_H\) (300 MHz, CDCl₃) 3.75 (3H, s, OC\(_3\)H₃, 8-C), 6.99 (1H, d, \(J = 3.0\)Hz, 6-H), 7.54 (1H, d, \(J = 3.0\)Hz, 4-H), 9.66 (1H, s, CHO, 7-H), 11.26 (1H, s, OH); \(\delta_C\) (75 MHz, CDCl₃) 56.2 (CH₃, 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, \textbf{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%); \(\nu_{\text{max}}/\text{cm}^{-1}\) 3047 (O-H), 2871 and 2835 (C-H), 1643 (C=O), 1599 (C=C), 1257, 1147 and 1050 (C-O); \(\delta_H\) (300 MHz, d₆-DMSO) 3.81 (6H, s, OCH₃, 8-H), 7.21 (2H, d, \(J = 3.3\)Hz, 4-H), 7.32 (2H, d, \(J = 3.0\)Hz, 6-H), 10.12 (2H, s, CHO, 7-H), 10.48 (2H, s, OH); \(\delta_C\) (75 MHz, d₆-DMSO) 56.2 (CH₃, 8-C), 114.9
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 CaCl₂ 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 H₂ 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 Et₂O (3 × 300mL). The combined organic layers were washed with water (2 × 250mL), 5% aqueous solution of LiCl (2 × 200mL) and brine (200mL), and dried (MgSO₄). The solvent was evaporated in vacuo and the residue purified by column chromatography, eluting with petroleum ether (60-80°C): Et₂O (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)]; δH (300 MHz, CDCl₃) 3.73 (3H, s, OCH₃, 8-H), 3.82 (3H, s, OCH₃, 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); δC (75 MHz, CDCl₃) 55.8 (CH₃, 8-C), 56.2 (CH₃, 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$_2$CO$_3$ (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$_2$O (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$_9$H$_9$BrO$_3$ requires C, 41.1; H, 3.7%); $\nu_max$/cm$^{-1}$ 2935, 2854 and 2832 (C-H), 1685 (C=O), 1600 (C=C), 1225, 1205 and 1042 (C-O); $\delta_H$ (300 MHz, CDCl$_3$) 3.85 (3H, s, OCH$_3$, 8-H), 3.97 (3H, s, OCH$_3$, 9-H), 7.30 (1H, d, $J = 3.0$Hz, 6-H), 7.41 (1H, d, $J = 3.0$Hz, 4-H), 10.34 (1H, s, CHO); $\delta_C$ (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$_9$H$_9$IO$_3$ requires C, 37.0; H, 3.1%); $m/z$ 315.1 (MNa$^+$); $\nu_max$/cm$^{-1}$ 2934, 2858 and 2745 (C-H), 1680 (C=O), 1592 (C=C), 1208 and 1043 (C-O); $\delta_H$ (300 MHz, CDCl$_3$) 3.85 (3H, s, OCH$_3$, 8-H), 3.93 (3H, s, OCH$_3$, 9-H), 7.34 (1H, d, $J = 3.0$Hz, 6-H), 7.63 (1H, d, $J = 3.3$Hz, 4-H), 10.305 (1H, s, CHO, 7-H); $\delta_C$ (75 MHz, CDCl$_3$) 56.02 (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).
5.2.12 Sonogoshira cross-coupling

5.2.12.1 Preparation of 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde

Method A (adapted from 1975TL4467): A flame dried sealed tube flushed with N₂ was charged 3-bromo-2,5-dimethoxybenzaldehyde 178 (0.21g, 0.84mmol), [Ph₃P]₂PdCl₂ (0.014g, 0.02mmol), pentyne (0.15mL, 1.50mmol) and dried Et₃N (3mL). The resulting suspension was stirred and heated at 40°C for 15 min, before addition of Cul (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 Et₂O (2 x 10mL). The combined organic extracts were washed with brine (15mL) and dried (MgSO₄). The solvent was removed in vacuo and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et₂O (90:10). The more mobile fraction gave 2,5-dimethoxy-1-pentyn-1'-ylbenzaldehyde 184 as a yellow coloured oil, which crystallised 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 N₂ was charged with 3-bromo-2,5-dimethoxybenzaldehyde 178 (0.21g, 0.86mmol), [PhCN]₂PdCl₂ (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- butylphosphine (0.22mL, 0.06mmol) and (iPr)₂NH (0.18mL). The resulting dark solution was stirred for 48h at R.T. under a N₂ atmosphere. The mixture was then diluted with EtOAc (10mL), washed with 10% aq. HCl (10mL) and brine (10mL). The organic layer was dried (MgSO₄) 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) : Et₂O (90:10). The more mobile fraction gave 2,5-dimethoxy-1-pentyn-1′-ylbenzaldehyde 184 as a slightly brown coloured oil, which crystallised 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 N₂ atmosphere. It was then charged with 3-iodo-2,5-dimethoxybenzaldehyde 185 (1.58g, 5.42mmol), [Ph₃P]₂PdCl₂ (0.08g, 0.11mmol), 1-pentyne (1.10mL, 0.74g, 10.83mmol), dry Et₃N (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 N₂ 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 Et₂O. The filtrate was diluted with 100mL of Et₂O, washed with a saturated solution of NH₄Cl (2 × 50mL), a 5% aq. solution of NaHCO₃ (2 × 50mL) and water (100mL), and dried (MgSO₄). The solvent was removed in vacuo and the residue purified by column chromatography eluting with petroleum ether (60-80°C) : Et₂O (90:10). 2,5-Dimethoxy-1-pentyn-1′-ylbenzaldehyde 184 was isolated as a yellow coloured oil, which crystallised 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%. C₁₄H₁₆O₃ requires C, 72.4; H, 6.9%); **m/z** 233.0 (MH)⁺, 255.0 (MNa)⁺; **νmax/cm⁻¹**: 2961 and 2867 (C-H), 2236 (C≡C), 1680 (C=O), 1591 (C=C), 1244 and 1047 (C-O); **δH (300 MHz, CDCl₃)** 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, OCH₃, 8-H), 3.93 (3H, s, OCH₃, 9-H), 7.09 (1H, d, J = 3.3Hz, 4-H), 7.15 (1H, d, J = 3.0Hz, 6-H), 10.26 (1H, s, CHO, 7-H); **δC (75 MHz, CDCl₃)** 13.5 (CH₃, 5'-C), 21.6 (CH₂, 3'-C), 22.0 (CH₂, 4'-C), 55.8 (CH₃, 8-C), 63.0 (CH₃, 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$_2$CO$_3$ (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$_{oil\,bath} = 100^\circ C$), 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$_2$O (80:20). 2,5-Dimethoxybiphenyl-3-carbaldehyde 194 was isolated as clear cubic white plates (2.29g, 9.43mmol, 85.0%); m.p. (Et$_2$O : 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 ($MNa^+$); $\nu_{\text{max}}$/cm$^{-1}$ 2877 and 2754 (C-H), 1680 (C=O), 1596 (C=C), 1229 and 1041 (C-O); $\delta_H$ (300 MHz, CDCl$_3$) 3.51 (3H, s, OC$_3$H$_3$, 9-H), 3.89 (3H, s, OC$_3$H$_3$, 8-H), 7.22 (1H, d, $J = 3.3$Hz, 6-H), 7.36 (1H, d, $J = 3.3$Hz, 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, CHO, 7-H); $\delta_C$ (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, 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) : Et₂O (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 (1.7 mol 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 NaHCO₃ (3 x 100mL). The DCM layer was then dried (MgSO₄) 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 NaHCO₃ (100mL), water (100mL) and brine (100mL), and dried (MgSO₄). 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)

![Chemical structure of 2,5-Dimethoxyphenol](image)

**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; νmax/cm⁻¹ 3416 (O-H), 1596 and 1504 (C=C), 1232, 1147 and 1039 (C-O); δH (300 MHz, CDCl₃) 3.67 (3H, s, OCH₃, 8-H), 3.76 (3H, s, OCH₃, 7-H), 5.58 (1H, s, OH), 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); δC(75 MHz, CDCl₃)
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$_2$O (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$^+$; $\nu_{\text{max}}$/cm$^{-1}$ 3263 (O-H), 1598 and 1508 (C=C), 1330, 1262, 1214 and 1038 (C-O); $\delta$$_H$ (300 MHz, CDCl$_3$) 2.12 (3H, s, CH$_3$, 8-H), 2.26 (3H, s, CH$_3$, 9-H), 3.77 (3H, s, OCH$_3$, 7-H), 3.81 (3H, s, OCH$_3$, 10-H), 5.77 (1H, br. s, OH), 6.48 (1H, s, 6-H); $\delta$$_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 °C) 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 °C): Et₂O (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 °C); m.p.: 105-106 °C [lit.:105-106 °C (2008OBC682)]; (found C, 67.4; H, 8.2%. C₁₁H₁₆O₃ requires C, 67.3; H, 8.2%); m/z 219.0 (MNa)⁺; νν νν νν νν νν /cm⁻¹ 3401 (O-H), 1612 (C=C), 1309, 1264 and 1070 (C-O); δH (300 MHz, d₆-DMSO) 2.18 (3H, s, CH₃, 9-H), 2.23 (3H, s, CH₃, 7-H), 2.23 (3H, s, CH₃, 10-H), 3.71 (3H, s, OC₃H₃, 11-H), 3.77 (3H, s, OC₃H₃, 8-H), 5.71 (1H, s, OH); δC (75 MHz, d₆-DMSO) 9.1 (CH₃, 10-C), 12.0 (CH₃, 7-C), 12.5 (CH₃, 9-C), 60.2 (CH₃, 11-C), 60.9 (CH₃, 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 °C): 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 °C): Et₂O (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 (MgSO₄) and the solvent removed under reduced pressure. The residue was dissolved in THF (20mL) and H₂O₂ 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 (MgSO₄). The solvent was removed in vacuo and the residue was subjected to column chromatography on silica eluting with petroleum ether (60-80°C) : Et₂O (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 crystalline solid; m.p.: 52-54°C [lit.:54-55°C (from petroleum ether, 1968JC1438)]; (found C, 68.6; H, 8.7%. C₁₂H₁₈O₃ requires C, 68.55; H, 8.6%); νmax/cm⁻¹ 3381, 3280 (O-H), 1597 and 1511 (C=C), 1300, 1192 and 1043 (C-O); δH (300 MHz, CDCl₃) 1.41 (9H, s, C(CH₃)₃), 3.83 (3H, s, OCH₃, 7-H), 3.90 (3H, s, OCH₃, 12-H), 5.60 (1H, s, OH), 6.63 (1H, s, 6-H), 6.90 (1H, s, 3-H); δC (75 MHz, CDCl₃) 30.0 (CH₃, 9-C, 10-C and 11-C), 34.5 (quat., 8-C), 55.7 (CH₃, 7-C), 57.0 (CH₃, 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) : Et₂O (70:30). 2,5-Dimethoxy-4-pentylphenol 150d was isolated as a white crystalline solid (2.66g, 11.87mmol, 81.4%). Recrystallisation from Et₂O : petroleum ether (60-80°C) produced white needles, m.p.: 33-35°C; (found: C, 69.55; H, 9.0%). C₁₃H₂₀O₃ requires C, 69.6; H, 9.0%); m/z 225.0 (MH⁺); νmax/cm⁻¹ 3354 (O-H), 2953 and 2930 (C-H), 1598 and 1515 (C=C), 1192 and 1038 (C-O); δH (300 MHz, CDCl₃) 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, OCH₃, 8-H), 3.89 (3H, s, OCH₃, 7-H), 5.61 (1H, s, OH), 6.60 (1H, s, 6-H), 6.73 (1H, s, 3-H); δC (75 MHz, CDCl₃) 14.1 (CH₃, 5'-C), 22.6 (CH₂, 3'-C or 4'-C), 29.8 (CH₂, 1'-C), 30.1 (CH₂, 2'-C), 31.8 (CH₂, 3'-C or 4'-C), 56.1 (CH₃, 8-C), 56.8 (CH₃, 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) : Et₂O (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%. C₁₂H₁₈O₃ requires C, 41.2; H, 3.8%); νmax/cm⁻¹ 3377 (O-H), 1609 and 1573
(C=C), 1323, 1165 and 1041 (C-O); \(\delta_H\) (300 MHz, CDCl\(_3\)) 3.78 (3H, s, 7-H, OCH\(_3\)), 3.89 (3H, s, 8-H, OCH\(_3\)), 5.90 (1H, s, OH), 6.55 (1H, d, \(J = 3.0\) Hz, 6-H), 6.67 (1H, d, \(J = 2.7\) Hz, 4-H); \(\delta_C\) (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

![structure](image)

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\(_2\)O (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\(^+\)); \(\nu_{\text{max}}/\text{cm}^{-1}\) 3396 (O-H), 1619, 1591 and 1573 (C=C), 1206 and 1049 (C-O); \(\delta_H\) (300 MHz, CDCl\(_3\)) 3.44 (3H, s, OCH\(_3\), 7-H), 3.85 (3H, s, OCH\(_3\), 8-H), 6.10 (1H, s, OH), 6.51 (1H, d, \(J = 3.0\) Hz, 6-H), 6.64 (1H, d, \(J = 3.0\) Hz, 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); \(\delta_C\) (75 MHz, CDCl\(_3\)) 55.6 (CH\(_3\), 8-C), 60.9 (CH\(_3\), 7-C), 106.6 (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)

![structure](image)

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 x 100mL) and dried (MgSO₄). The solvent was removed under reduced pressure and the residue was recrystallised from a solvent mixture of petroleum ether (60-80°C) : CHCl₃. 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): CHCl₃, 1989CJC335)]; (found C, 30.75; H, 2.55%. C₈H₈Br₂O₃ requires C, 30.8; H, 2.6%); νν νν νmax/cm⁻¹ 3488 (O-H), 1583 and 1567 (C=C), 1343 and 1065 (C-O); δH (300 MHz, CDCl₃) 3.93 (6H, s, 2 x OCH₃, 7-H and 8-H), 6.06 (1H, br s, OH), 6.20 (1H, s, 4-H); δC (75 MHz, CDCl₃) 56.7 (CH₃, 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 Br₂ (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 Na₂S₂O₃ (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 Et₂O and the resulting solution was dried (MgSO₄), before removing the solvent in vacuo. The residue was purified by column chromatography, eluting with petroleum ether (60-80°C) : Et₂O (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%. C₁₁H₁₃Br₃O₂ requires C, 31.7; H, 3.2%); νmax/cm⁻¹ 3585-3405 (O-H), 2952-2854 (C-H), 1622, 1573 and 1558 (C=C), 1210 and 1195 (C-O), 676 (C-Br); δH (300 MHz, CDCl₃) 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 x OH); δC (75 MHz, CDCl₃) 14.0 (CH₃, 5'-C), 22.4 (CH₂, 4'-C), 27.7 (CH₂, 2'-C), 31.8 (CH₂, 3'-C), 37.6 (CH₂, 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 tribromooolivetol 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-tribromooolivetol 205 (1.11g, 2.67mmol) was dissolved in a mixture of a 0.1M K₂HPO₄ phosphate buffer (50mL) and CH₃CN (70mL). Potassium nitrosodisulfonate 201 (2.15g, 8.00mmol, prepared according to 1970CR229) was dissolved in 0.1M K₂HPO₄ phosphate buffer (50mL) and the resulting deep purple solution was added to the 2,4,6-tribromooolivetol 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 x 70mL) and the combined organic extracts were washed with brine (100mL) and dried (MgSO₄). 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%. C₁₁H₁₂Br₂O₅ requires C, 37.5; H, 3.4%); m/z 348.7 (41%), 350.7 (100%), 352.7 (43%) (M-H⁻); νmax/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); δH (300 MHz, CDCl₃) 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, OH); δC (75 MHz, CDCl₃) 13.9 (CH₃, 5'-C), 22.3 (CH₂, 3'-C or
4'-C), 27.1 (CH₂, 2'-C), 30.5 (CH₂, 1'-C), 31.8 (CH₂, 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)

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{F} \quad \text{NH}_2 \\
\text{F} & \quad \text{NH}_2 \\
\text{F} & \quad \text{NH}_2
\end{align*}
\]

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 HNO₃ (55mL) and conc. H₂SO₄ (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 (MgSO₄) 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%). C₆H₅FNO₂ requires C, 46.2; H, 3.2; N, 17.9%); ν_max/cm⁻¹ 3487 and 3366 (NH₂), 1583 (C=C), 1525 and 1329 (NO₂), 1068 (C-F); δH (300 MHz, d₆-DMSO) 6.62 (1H, td, J = 8.7, 5.4Hz, 4-H), 7.22 (2H, br. s, NH₂), 7.42 (1H, dd, J = 10.2, 7.8Hz, 3-H), 7.82 (1H, d, J = 8.7Hz, 5-H); δC (75 MHz, d₆-DMSO) 113.95 (CH, d, J = 7.9Hz, 4-C), 120.2 (CH, d, J =
18.3 Hz, 3-C), 121.5 (CH, d, J = 3.3 Hz, 5-C), 132.4 (quat., d, J = 5.1 Hz, 6-C), 136.45 (quat., d, J = 16.6 Hz, 1-C), 152.2 (quat., d, J = 242.7 Hz, 2-C). Further elution gave 4-nitro-2-fluoroaniline 210a (11.61 g, 74.37 mmol, 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)]; νννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννν

5.2.18 General procedure for the oxidation of fluoroanilines (adapted from
1992TL4835)
In a 250 mL 3 necked flask equipped with a pressure equalizing dropping funnel
and a thermometer, urea hydrogen peroxide adduct (12 mol equivalent) was
suspended in acetonitrile (100 mL) 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 (10 mol
equivalent) in acetonitrile (20 mL), 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 (1 mol equivalent) was prepared in acetonitrile (50 mL) 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 Na₂CO₃
solution, until no more evolution of CO₂ was observed. The resulting mixture
was extracted with Et₂O (3 x 80 mL) and the combined organic layers washed
once with water (100 mL) and brine (100 mL), and dried (MgSO₄). 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$_8$H$_3$FN$_2$O$_4$ requires C, 38.7; H, 1.6; N,15.05%); $\nu_{\text{max}}$/cm$^{-1}$ 3118 (C-H), 1549 and 1343 (NO$_2$); $\delta_{\text{H}}$ (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); $\delta_{\text{C}}$(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$_3$N (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 x 100mL) and the combined DCM extracts were successively washed with a 10% aqueous solution of sodium hydroxide (3 x 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°C 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 (MgSO₄). The solvent was removed in vacuo and the residue subjected to column chromatography, eluting with a solvent mixture of petroleum ether (60:80°C) : 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°C; m/z 343.1 (MNa)+ (found C, 52.4; H, 3.7; N, 8.7%. C₁₄H₁₂N₂O₆ requires C, 52.5; H, 3.8; N, 8.75%); νmax /cm⁻¹ 1604 (C=C), 1506 and 1340 (NO₂), 1274, 1035 and 1014 (C-O); δH (300 MHz, d₆-DMSO) 3.69 (3H, s, OCH₃, 7'-H), 3.74 (3H, s, OCH₃, 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); δC(75 MHz, d₆-DMSO) 56.2 (CH₃, 7'-C), 56.8 (CH₃, 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

![Structure of 2,4-Dinitro-2',5'-dimethoxy-3',4'-dimethyldiphenylether 169a](image)

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°C) : 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°C; (found C, 55.4; H, 4.6; N, 8.0%). C$_{16}$H$_{16}$N$_2$O$_7$ requires C, 55.2; H, 4.6; N, 8.0%; $m/z$ 371.0 (MNa)$^+$; $v_{\text{max}}$/cm$^{-1}$ 1606 (C=C), 1529 and 1345 (NO$_2$), 1263, 1233 and 1026 (C=O); $\delta$$_{\text{H}}$ (300 MHz, d$_6$-DMSO) 2.13 (3H, s, CH$_3$, 9'-H), 2.19 (3H, s, CH$_3$, 8'-H), 3.60 (3H, s, OCH$_3$, 7'-H), 3.77 (3H, s, OCH$_3$, 10'-H), 6.89 (1H, s, 6'-H), 7.01 (1H, d, $J$ = 9.0Hz, 6-H), 8.38 (1H, dd, $J$ = 9.3, 3.0Hz, 5-H), 8.88 (1H, d, $J$ = 3.0Hz, 3-H); $\delta$$_{\text{C}}$ (75 MHz, 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

![Structure of 2,4-Dinitro-2',5'-dimethoxy-3',4',6'-trimethyldiphenylether 169b](image)

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$_3$N. Elution with petroleum ether (60-80°C) : 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°C; (found C, 56.4; H, 5.05; N, 7.7%. C$_{17}$H$_{19}$N$_2$O$_7$ requires C, 56.35; H, 5.0; N, 7.7%); $m/z$ 385.1 (MNa)$^+$; $v_{\text{max}}$/cm$^{-1}$
1609 (C=C), 1540 and 1345 (NO₂), 1260, 1250 and 1072 (C-O); δₜ (300 MHz, CDCl₃) 2.04 (3H, s, CH₃, 7'-H), 2.12 (3H, s, CH₃, 10'-H), 2.16 (3H, s, CH₃, 9'-H), 3.59 (3H, s, OCH₃, 8'-H), 3.63 (3H, s, OCH₃, 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); δₜ (75 MHz, CDCl₃) 9.6 (CH₃, 7'-C), 12.4 (CH₃, 9'-C or 10'-C), 12.8 (CH₃, 9'-C or 10'-C), 60.5 (CH₃, 8'-C), 61.2 (CH₃, 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'-butylidiphenylether 169c

The title compound was prepared as described (method A) from 2,5-dimethoxy-4'-butylphenol 150c (3.55g, 16.88mmol). Elution with petroleum ether (60-80°C) : Et₂O (70:30) yielded 2,4-dinitro-2',5'-dimethoxy-4'-butylidiphenylether 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%). C₁₈H₂₀N₂O₇ requires C, 57.4; H, 5.4; N, 7.4%; m/z 399.1 (MNa)+; νmax/cm⁻¹ 1608 (C=C), 1535 and 1342 (NO₂), 1209 and 1031 (C-O); δₜ (300 MHz, CDCl₃) 1.32 (9H, s, C(CH₃)₃), 3.64 (3H, s, OCH₃, 7'-H), 3.72 (3H, s, OCH₃, 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); δₜ (75 MHz, CDCl₃) 29.7 (3 × CH₃, 9'/10'/11'-C), 35.2 (quat., 8'-C), 55.8 (CH₃, 7'-C), 57.1 (CH₃, 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

![Chemical structure](image)

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_{2}O_{7} requires C, 58.5; H, 5.7; N, 7.2%); m/z 390.1 (MH)^+; 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, OCH_{3}, 7''-H), 3.82 (3H, s, OCH_{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

![Chemical structure](image)

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_{14}BrN_{2}O_{7} requires C, 42.1; H, 2.8; N, 7.0%); m/z 421.0 (95%), 423.0 (100%) (MNa)^+; ν\text{max/cm}^{-1} 1601, 1567 and 1529 (C=C), 1528 and 1337 (NO\_2 (300 MHz, CDCl\_3) 3.79 (3H, s, OCH\_3, 7'-H), 3.84 (3H, s, OCH\_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

![Chemical Structure](image)

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_{2}O_{7} requires C, 60.6; H, 4.1; N, 7.1%); m/z 397.1 (MH)^+; 419.1 (MNa)^+; ν\text{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, OCH\_3, 7-H), 3.75 (3H, s, OCH\_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%. C₁₄H₁₂N₂O₇ 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 (NO₂), 1261 and 1055 (C-O); δ_H (300 MHz, CDCl₃) 3.71 (6H, s, 2 × OCH₃, 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, CDCl₃) 55.7 (CH₃, 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%. C₁₄H₁₀Br₂N₂O₇ 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 (NO₂), 1215 and 1093 (C-O); δ_H (300 MHz, d₆-DMSO) 4.00 (6H, s, 2 × OCH₃, 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, d₆-DMSO) 57.7 (CH₃, 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'-butyl-2',5'-dimethoxydiphenylether 152c (2008OBC682)

The title compound was prepared as described (method B) from 2,5-dimethoxy-4'-butylphenol 150c (1.88g, 8.92mmol). 2,5-Dinitro-4'-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_{2}O_{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-pentyphenol 150d (2.07g, 9.25mmol). The product was purified by column chromatography, eluting with petroleum ether (60-80 °C) : Et_{2}O (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=O), 1531 and 1347 (NO\(_2\)).

5.1.19.12 Attempted preparation of 2,4-dinitro-3'-penty-2',5'-dimethoxydiphenylether 169p

![Chemical structure of the compound](image)

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 consumed, the resulting solution was refluxed for 10 min. In the mean time, a flame dried 100mL two-necked round-bottomed flask flushed with N₂ 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-pyrroldione (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 N₂, the substituted biarylether (1mol equivalent) was dissolved in dry DCM (70mL). The resulting solution was cooled to -78°C in a dry ice/acetone bath. Once the temperature reached -78°C, a 1M DCM solution of BBr₃ (3.8mol equivalents) was slowly added to the biarylether solution. The temperature was kept below -55°C 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°C and more 1M DCM solution of BBr₃ (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 BBr₃, 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 (MgSO₄). The solvent was removed in vacuo and the residue subjected to column chromatography.
5.1.20.1 2,4-Dinitro-2',5'-dihydroxydiphenylether 170a

![Chemical Structure of 170a](image)

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°C) : 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°C; (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-H_2NO_2), 290.9 (M-H); v_{max}/cm^{-1} 3401 and 3347 (OH), 1600 (C=O), 1506 and 1340 (NO_2); 5\textsubscript{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'-OH), 9.84 (1H, s, 2'-OH); 5\textsubscript{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'-trimethylidiphenylether 170b and 2,4-dinitrophenoxy-3',4',6'-trimethyl-2',5'-benzoquinone 179b

![Chemical Structures of 170b and 179b](image)

The title compounds were prepared as described from 2,4-dinitro-2',5'-dimethoxy-3',4',6'-trimethylidiphenylether (3.38g, 9.32mmol); elution used petroleum ether (60-80°C) : EtO (70:30). The collected fractions were combined and left at room temperature until crystals of 2,4-dinitro-2',5'-dihydroxy-3',4',6'-trimethylidiphenylether 170b formed. The crystals were removed by filtration yielding 2,4-dinitro-2',5'-dihydroxy-3',4',6'-trimethylidiphenylether 170b (1.67g, 4.99mmol, 53.5%) as cream coloured prisms; m.p. : 131-139°C; (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-H_2NO_2), 332.9 (M-H); v_{max}/cm^{-1}
3431 (O-H), 1602 and 1517 (C=C), 1537 and 1348 (NO₂), 1244 (C-O); δH (300 MHz, d₆-DMSO) 1.99 (3H, s, CH₃, 7'-H), 2.09 (6H, s, 2 × CH₃, 8'-H and 9'-H), 6.83 (1H, d, J = 9.6 Hz, 6'-H), 7.965 (1H, s, 5'-OH), 8.40 (1H, dd, J = 9.6, 3.0 Hz, 5-H), 8.79 (1H, s, 2'-OH), 8.84 (1H, d, J = 3.0 Hz, 3-H); δC (75 MHz, d₆-DMSO) 10.2 (CH₃, 7'-C), 12.7 (CH₃, 8'-C or 9'-C), 12.8 (CH₃, 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; νmax/cm⁻¹ 1666 and 1648 (C=O), 1602 (C=C), 1526 and 1343 (NO₂), 1249 (C-O); (found: C, 54.2; H, 3.6; N, 8.4%. C₁₂H₇NO₆ requires C, 54.2; H, 3.6; N, 8.4%); m/z 330.9 (M-H); δH (300 MHz, d₆-DMSO) 1.95 (3H, d, J = 1.2 Hz, CH₃, 7'-H), 1.97 (3H, s, CH₃, 9'-H), 2.04 (3H, d, J = 0.9 Hz, CH₃, 8'-H), 7.54 (1H, d, J = 9.3 Hz, 6-H), 8.38 (1H, dd, J = 9.3, 2.7 Hz, 5-H), 8.89 (1H, d, J = 2.7 Hz, 3-H); δC (75 MHz, d₆-DMSO) 9.85 (CH₃, 9'-C), 12.2 (CH₃, 7'-C), 12.9 (CH₃, 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) : Et₂O (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%. C₁₃H₈Br₂N₂O₇ requires C, 33.65; H, 1.7; N, 6.0%); m/z 460.9 (52%), 462.8 (100%), 464.8 (48%) (M-H); νmax/cm⁻¹ 3487
(O-H), 1593 and 1518 (C=C), 1533 and 1349 (NO₂), 1091 (C-O), 682 (C-Br); δH (300 MHz, d₆-DMSO) 3.88 (3H, s, OCH₃), 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'-OH); δC (75 MHz, d₆-DMSO) 57.1 (CH₃, 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%. C₁₂H₅Br₃N₂O₇ 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-H); νmax/cm⁻¹ 3364 (O-H), 1606 (C=C), 1518 and 1344 (NO₂), 1183 and 1068 (C-O), 682 (C-Br); δH (300 MHz, d₆-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 × OH); δC (75 MHz, d₆-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 CH₃CN (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. CH₃CN 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 (MgSO₄) 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 (MgSO₄). 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%. C₁₄H₁₀N₂O₇ requires C, 52.8; H, 3.2; N, 8.8%); m/z 316.9 (M-H); νmax/cm⁻¹ 1666 and 1648 (C=O, quinone), 1600 (C=C), 1537 and 1347 (NO₂), 1190 (C-O); δH (300 MHz, d₆-DMSO) 2.01 (6H, s, 2 x CH₃, 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); δC (75 MHz, d₆-DMSO) 12.3 (CH₃, 7'-C or 8'-C), 12.6 (CH₃, 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'-butyl-2',5'-benzoquinone 179c

The title compound was prepared as described from 2,4-dinitro-4'-butyl-2',5'-dimethoxydiphenylether 169c (2.87g, 7.69mmol). The crude product was recrystallised from MeOH yielding 2,4-dinitrophenoxy-4'-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_{2}O_{7} requires C, 55.5; H, 4.1; N, 8.1%); \nu_{\text{max}}/\text{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); \delta_H (300 MHz, d_6-DMSO) 1.27 (9H, s, C(CH_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); \delta_C (75 MHz, d_6-DMSO) 29.5 (CH, 3-C), 114.5 (C-O);

5.1.21.4 2,4-Dinitrophenoxyl-4'-penty1-2',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-Dinitrophenoxyl-2',5'-dimethoxy-4'-penty1benzoquinone 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_{2}O_{7} requires C, 56.7; H, 4.5; N, 7.8%); m/\nu 358.95 (M-H); \nu_{\text{max}}/\text{cm}^{-1} 2927 (C-H), 1673 and 1649 (C=O), 1610 (C=C), 1530 and 1342 (NO_2), 1224 (C-O); \delta_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, 12Hz, 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); \delta_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

![Diagram of the molecule]

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%). C₁₈H₁₀N₂O₇ requires C, 59.0; H, 2.75; N, 7.65%; m/₂ 364.9 (M-H); νmax/cm⁻¹ 1676 and 1649 (C=O, quinone), 1590 and 1523 (C=C), 1543 and 1347 (NO₂), 1226 and 1082 (C-O); δH (300 MHz, d₆-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); δC (75 MHz, d₆-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'-butyl-2',5'-benzoquinone 219a

![Diagram of the molecule]

The title compound was prepared as described from 2,5-dinitro-4'-butyl-2',5'-dimethoxydiphenylether 152c (1.57g, 4.18mmol). 2,5-Dinitrophenoxy-4'-butyl-2',5'-benzoquinone 219a was isolated as a pale yellow solid (1.35g, 3.89mmol, 93.2%). Recrystallisation from EtOH produced a pale yellow microcrystalline solid; m.p.: 165-168°C; (found: C, 55.1; H, 4.0; N, 8.0%. C₁₆H₁₄N₂O₇ requires C, 55.5; H, 4.1; N, 8.1%); νmax/cm⁻¹ 2952 (C-H), 1672 and 1650 (C=O, quinone), 1594 (C=C), 1540 and 1479 (C=O, quinone).
1347 (NO$_2$), 1237 (C-O); $\delta_H$ (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, $J = 9.0$Hz, 3-H), 8.51 (1H, d, $J = 2.4$Hz, 6-H); $\delta_C$ (75 MHz, d$_6$-DMSO) 29.5 (3 × CH$_3$, 8'/9'/10'-C), 55.8 (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., 2'-C), 187.8 (quat., 5'-C).

5.1.21.6 2,5-Dinitrophenoxo-4'-pentyl-2',5'-benzoquinone 219b

The title compound was prepared as described from 2,4-dinitro-2',5'-dimethoxy-3'-pentyl diphenylether 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-Dinitrophenoxo-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$_2$O$_7$ requires C, 56.7; H, 4.5; N, 7.8%); $\nu_{max}$/cm$^{-1}$ 2870-2957 (C-H), 1676 and 1651 (C=O, quinone), 1603 (C=C), 1544 and 1343 (NO$_2$), 1230 (C-O); $\delta_H$ (300 MHz, d$_6$-DMSO) 0.79 (3H, t, $J = 6.9$Hz, 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, $J = 6.9$Hz, 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, $J = 9.0$Hz, 3-H), 8.41 (1H, d, $J = 2.4$Hz, 6-H); $\delta_C$ (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 dianinophenoxy-2',5'-benzoquinone 171a,c,d and 152a

Either 2,4-dinitro-2',5'-dihydroxydiphenylether 170a (1 mol equivalent) or the correctly substituted 2,4-dinitrophenoxy-2',5'-benzoquinone 179 (1 mol 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 2.4 bar. The usual hydrogenation time was about 3 h or when no more evolution of the H$_2$ pressure could be noted. Acetic anhydride (10 mol 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$_3$ (70 mL) and brine (100 mL), 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 (0.57 g, 1.955 mmol). The crude product was purified by column chromatography, eluting with EtOAc (100%). 2,4-Diacetamido-2',5'-dihydroxydiphenylether 222 (0.49 g, 1.555 mmol; 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$_2$O$_5$ requires C, 60.75; H, 5.1; N, 8.9%); $m/z$ 314.9 (M-H)$^-$; $\nu_{\text{max}}$/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); $\delta_H$(300 MHz, d$_6$-DMSO) 2.025 (3H,
s, COCH₃, 1b-H), 2.11 (3H, s, COCH₃, 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'-OH), 8.88 (1H, s, 5'-OH), 9.44 (1H, s, 2-NH), 9.89 (1H, s, 4-NH); δC (75 MHz, d₆-DMSO) 24.2 (CH₃, 2a-C or 2b-C), 24.3 (CH₃, 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%. C₁₈H₁₈N₂O₅ requires C, 63.15; H, 5.3; N, 8.2%); m/z 298.9 (M-CH₃CO)⁺, 340.9 (M-H)⁺; νmax/cm⁻¹ 3403 and 3277 (N-H), 1711 (C=O), 1655 and 1648 (C=O, quinone), 1606 (C=C), 1207 (C-O); δH (300 MHz, d₈-THF) 2.00 (3H, d, J = 1.2Hz, 7'-H), 2.05-2.06 (6H, m, 2 x CH₃, 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-NH), 9.31 (1H, s, 4-NH); δC (75 MHz, d₈-THF) 9.1 (CH₃, 7'-C or 8'-C), 9.3 (CH₃, 7'-C or 8'-C), 21.1 (CH₃, 2a-C or 2b-C), 21.25 (CH₃, 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'-butyl-2',5'-benzoquinone 223b

The title compound was prepared as described from 2,5-dinitrophenoxy-4'-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°C) : EtOAc (20 : 80). 2,4-Diacetamido-4'-butyl-2',5'-benzoquinone 223b was isolated as a yellow-brown solid (0.19g, 0.51mmol, 99.0%). Recrystallisation 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_{2}O_{5} requires C, 64.85; H, 6.0; N, 7.6%); m/z 371.2 (MH)^+; v_{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(CH_{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-NH), 10.07 (1H, s, 4-NH); 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'-penty1-2',5'-benzoquinone 223c

The title compound was prepared as described from 2,5-dinitrophenoxy-4'-penty1-2',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′-penty1-2′,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%. C21H24N2O5 requires C, 65.6; H, 6.3; N, 7.3%); m/z 341.0 (M-CH3CO), 383.0 (M-H+); νmax/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); δH (300 MHz, d6-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-NH), 9.31 (1H, s, 4-NH); δC (75 MHz, d6-THF) 13.3 (CH3, 5″-C), 22.3 (CH2, 3″-C or 4″-C), 23.0 (CH3, 2a-C or 2b-C), 23.1 (CH3, 2a-C or 2b-C), 27.85 (CH2, 2″-C), 28.6 (CH2, 1″-C), 31.5 (CH2, 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′-butyldiphenylether 227

The title compound was prepared as described from 2,5-dinitrophenoxo-4′-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′-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%. C20H24N2O5 requires C, 64.5; H, 6.5; N, 7.5%); m/z 371.0 (M-H+); νmax/cm⁻¹ 3345 (N-H), 3259 (N-H), (br., O-H), 2957 (C-H), 1701 (C=O), 1667 (C=O), 1605 (C=C); δH (300 MHz, d6-DMSO) 1.25 (9H, s, C(CH3)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, \( J = 1.8 \)Hz, 6-H), 7.25 (1H, dd, \( J = 8.4, 1.5 \)Hz, 4-H), 7.60 (1H, d, \( J = 8.7 \)Hz, 3-H), 8.44 (1H, s, 2'-OH), 8.76 (1H, s, 5'-OH), 9.33 (1H, s, 2'-NH), 9.77 (1H, s, 5-NH); \( \delta_C \) (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%). Recrystallisation from EtOAc : petroleum ether (68-80°C) yielded a red microcrystalline solid.

Method B. The title compound was prepared 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%). Recrystallisation from EtOAc : petroleum ether (68-80°C) produced a microcrystalline red solid; \( \nu_{\text{max}} / \text{cm}^{-1} \) 3334 (N-H), 1754 (C=O), 1697 (C=O), 1662 and 1644 (C=O), 1186 (C-O); \( \delta_H \) (300 MHz, d\(_6\)-DMSO) 1.95 (3H, d, \( J = 0.9 \)Hz, CH\(_3\), 7'-H or 8'-H), 1.98 (3H, d, \( J = 1.2 \)Hz, CH\(_3\), 7'-H or 8'-H), 2.065 (3H, s,
CH₃, 2b-H), 2.22 (3H, s, CH₃, 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-NH), 10.09 (1H, s, 5-NH); δC (75 MHz, d₆-DMSO) 12.3 (CH₃, 7'-C or 8'-C), 12.8 (CH₃, 7'-C or 8'-C), 21.1 (CH₃, 2a-C), 24.45 (CH₃, 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-3H-phenoxazin-3-ones 167a-e and 7-amino-2-alkyl-3H-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 H₂ pressure was set to 2.4bar and reset to this pressure when necessary. Once the H₂ pressure was steady, the reacting mixture was filtered through celite to remove the catalyst and the celite was washed several times with small portions of MeOH. Either AgO, Ag₂O or MnO₂ (3mol equivalent) was then added quickly to the filtrate, followed by the addition of Et₃N (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 H₂ pressure was set to 2.4bar and reset to this pressure when necessary. Once the H₂ 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 Ag₂O (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-3H-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-3H-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 Et₃N (1mL) were then added to the resulting suspension. The resulting reaction mixture was hydrogenated for 48h in a Berghof apparatus; the initial H₂ 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. Ag₂O 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-3H-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⁺); νmax/cm⁻¹ 3387, 3320, 3200 (NH₂), 1640 (C=O), 1591 (C=C); δH (300 MHz, d₆-DMSO) 5.45 (2H, br. s, NH₂), 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); δC (75 MHz, d₆-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-3H-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-3H-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\textsubscript{max}\,/\text{cm}^{-1} 3414, 3332 and 3225 (NH\textsubscript{2}), 2851-2920 (C-H), 1638 (C=O), 1570 (C=C); δ\textsubscript{H} (300 MHz, d\textsubscript{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\textsubscript{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\textsubscript{C} (75 MHz, d\textsubscript{6}-DMSO) 13.1 (CH\textsubscript{3}, 11-C or 12-C), 13.2 (CH\textsubscript{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-3H-phenoxazin-3-one 167c

Method A. The title compound was prepared as described earlier from 2,4-dinitrofenoxyn-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-3H-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'- trimethyl diphenylether 170b (0.33g, 0.98mmol); elution used petroleum ether (60-80 °C) : EtOAc (30:70), yielding 8-amino-1,2,4-trimethyl-3H-
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⁺); νmax/cm⁻¹ 3355, 3332 and 3219 (NH₂), 2850-2920 (C-H), 1649 (C=O), 1595, 1577 and 1505 (C=C); δH (300 MHz, d6-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, NH₂), 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); δC (75 MHz, d6-DMSO) 8.08 (CH, 1-C), 13.0 (CH, 11-C), 13.4 (CH₃, 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-2-5'-butyl-3H-phenoxazin-3-one 167d

![Image of molecule structure]

The title compound was prepared as described (method B) from 2,4-dinitrophenoxy-4'-5'-butyl-2',5'-benzoquinone 179b (0.62g, 1.78mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60) yielding 8-amino-2-5'-butyl-3H-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%. C₁₆H₁₈N₂O₂ requires C, 71.6; H, 6.0; N, 10.4%); m/z 269.0 (MH⁺); νmax/cm⁻¹ 3420 and 3337 (NH₂), 2952 (C-H), 1649 (C=O), 1601 and 1575 (C=C); δH (300 MHz, CDCl₃) 1.31 (9H, s, C(CH₃)₃), 3.74 (2H, br. s, NH₂), 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); δC (75 MHz, CDCl₃) 29.3 (CH₃, 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-3H-phenoxazin-3-one 167e

![Image of molecule structure]
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-3H-phenoaxin-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_{2}O_{2} requires C, 72.2; H, 6.4; N, 9.9%); \( m/z \) 283.1 (MH)^+; \( \nu_{\max}/\text{cm}^{-1} \) 3400, 3337 and 3222 (N-H), 2853 and 2928 (C-H), 1639 (C=O), 1585, 1566 and 1508 (C=C); \( \delta_{H} (300 \text{ MHz, } \text{d}_{6}-\text{DMSO}) \) 0.87 (3H, t, \( J = 6.9 \text{Hz} \), 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, \( J = 6.9 \text{Hz} \), 1'-H), 5.41 (2H, br. s, N-H), 6.17 (1H, s, 3'-H), 6.90-6.93 (2H, m, 6-H and 7-H), 7.21 (1H, d, \( J = 9.3 \text{Hz} \), 6-H), 7.25 (1H, s, 1-H); \( \delta_{C} (75 \text{ MHz, } \text{d}_{6}-\text{DMSO}) \) 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-3H-phenoaxin-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 occurred in the presence of Ag_{2}O (0.16g, 0.71mmol) and Et_{3}N (1mL); elution used petroleum ether (60-80 °C) : EtOAc (30:70) yielded 8-amino-1-phenyl-3H-phenoaxin-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)^+; \( \nu_{\max}/\text{cm}^{-1} \) 3421, 3308 and 3198 (N-H), 1634 (C=O), 1586, 1560 and 1502 (C=C); \( \delta_{H} (300 \text{ MHz, } \text{d}_{6}-\text{DMSO}) \) 5.31 (2H, br. s, NH_{2}), 6.18 (1H, d, \( J = 2.1 \text{Hz} \), 4-H), 6.68 (1H, d, \( J = 2.1 \text{Hz} \), 2-H), 6.77 (1H, d, \( J = 2.7 \text{Hz} \), 9-H), 6.87 (1H, dd, \( J = 8.7 \text{Hz} \), 7-H), 7.17 (1H, d, \( J = 8.7 \text{Hz} \), 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); \( \delta_{C} (75 \text{ MHz, } \text{d}_{6}-\text{DMSO}) \) 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-buty-3H-phenoxazin-3-one 59d

![Chemical structure](image)

The title compound was prepared as described from 2,5-dinitrophenoxy-4-buty-2',5'-benzoquinone 219a (1.01g, 2.91mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60), yielding 7-amino-2-buty-3H-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_{2}O_{2} requires C, 71.6; H, 6.0; N, 10.4%); m/z 269.1 (MH)^+; ν\text{max/cm}^{-1} 3408, 3317 and 3205 (NH₂), 2944 (C-H), 1643 (C=O), 1589, 1568 and 1547 (C=C); δ_H (300 MHZ, d_6-DMSO) 1.31 (9H, s, C(CH₃)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, NH₂), 7.14 (1H, s, 1-H), 7.43 (1H, d, J = 8.7Hz, 9-H); δ_c (75 MHZ, d_6-DMSO) 29.4 (CH₃, 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-3H-phenoxazin-3-one 59e

![Chemical structure](image)

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-3H-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\text{max/cm}^{-1} 3420, 3329 and 3226 (NH₂), 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, NH$_2$), 7.24 (1H, s, 1-H), 7.48 (1H, d, J = 8.7Hz, 9-H); $\delta$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-3H-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 isobutylchloroformate (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-3H-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-3H-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'-Butoxycarbonyl-β-alanyl)amino-3H-phenoxazin-3-one 236a

The title compound was prepared as described (method B) from 8-amino-3H-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'-butoxycarbonyl-β-alanyl)amino-3H-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_{26}H_{21}N_{3}O_{5} requires C, 62.65; H, 5.5; N, 11.0%); m/2 384.1 (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(CH_{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, NH\textsubscript{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, NH\textsubscript{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 x 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'-Butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3H-phenoxazin-3-one 236b

The title compound was prepared as described (method B) from 8-amino-1,2-dimethyl-3H-phenoxazin-3-one 167b (0.27g, 1.14mmol). Elution with petroleum ether (60-80 °C) : EtOAc : (30:70) yielded 8-N-(N'-butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3H-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_{3}O_{5} requires C, 64.2; H, 6.1; N, 10.2%); m/2 412.1 (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); δH (300 MHz, d6-DMSO) 1.31 (9H, s, C(CH3)3), 1.95 (3H, s, CH3, 12-H), 2.24 (3H, s, CH3, 11-H), 2.425 (2H, t, J = 7.2 Hz, CH2, 2'-H), 3.175 (2H, q, J = 7.2 Hz, 3'-H), 6.06 (1H, s, 4-H), 6.78 (1H, br. s, NH Boc), 7.28 (1H, d, J = 8.7 Hz, 6-H), 7.53 (1H, dd, J = 9.0, 2.4 Hz, 7-H), 8.08 (1H, d, J = 2.4 Hz, 9-H), 10.06 (1H, s, NH Ar); δC (75 MHz, d6-DMSO) 13.0 (CH3, 11-C or 12-C), 13.15 (CH3, 11-C or 12-C), 28.7 (CH3, 6'/7'/8'-C), 37.0 (CH2, 3'-C), 37.3 (CH2, 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'-Butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3H-phenoxazin-3-one 236c

The title compound was prepared as described (method B) from 8-amino-1,2,4-trimethyl-3H-phenoxazin-3-one 167c (0.67g, 2.65mmol). Elution with petroleum ether (60-80 °C) : EtOAc (30:70) yielded 8-N-(N'-butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3H-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)+; νmax/cm−1 3333 (N-H), 1713 (C=O), 1690 (C=O), 1650 (C=O), 1167 (C-O); δH (300 MHz, d6-DMSO) 1.42 (9H, s, C(CH3)3), 1.98 (3H, s, 13-H), 2.07 (3H, s, 12-H), 2.33 (3H, s, 11-H), 2.545 (2H, t, J = 6.9 Hz, 2'-H), 3.32 (2H, q, J = 6.6 Hz, 3'-H), 6.49 (1H, br. s, NH Boc), 7.32 (1H, d, J = 8.7 Hz, 6-H), 7.64 (1H, dd, J = 8.7, 2.1 Hz, 7-H), 8.09 (1H, d, J = 1.8 Hz, 9-H), 9.90 (1H, s, Ar-NHCO); δC (75 MHz, d6-DMSO) 7.9 (CH3, 13-C), 12.8 (CH3, 11-C), 13.2 (CH3, 12-C), 28.8 (CH3, 6'/7'/8'-C), 37.3 (CH2, 3'-C), 37.5 (CH2, 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'-Butoxycarbonyl-β-alanyl)amino-2'-butyl-3H-phenoxazin-3-one 236d

The title compound was prepared as described (method B) from 8-amino-2'-butyl-3H-phenoxazin-3-one 167d (0.53g, 1.96mmol); elution used petroleum ether (60-80°C) : EtOAc (30:70) yielded 8-N-(N'-butoxycarbonyl-β-alanyl)amino-4'-butyl-3H-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<sub>24</sub>H<sub>25</sub>N<sub>3</sub>O<sub>5</sub> requires C, 65.6; H, 6.65; N, 9.6%); m/z 438.1 (M-H)<sup>+</sup>; \( \nu_{\text{max}} \) cm<sup>-1</sup> 3340 (N-H), 3304 (N-H), 2962 (OH), 1682 (C=O), 1648 (C=O), 1167 (C-O); \( \delta \) <sub>d</sub> (300 MHz, <sup>d</sup> - DMSO) 1.375 (9H, s, 12/13/14-H, C(CH<sub>3</sub>)), 1.44 (9H, s, 6'/7'/8'-H, C(CH<sub>3</sub>)), 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, NH-fBuoc), 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, NH-Ar); \( \delta \) <sub>c</sub> (75 MHz, <sup>d</sup> - DMSO) 28.7 (CH<sub>3</sub>, 6'/7'/8'-C), 29.4 (CH<sub>3</sub>, 12/13/14-C), 35.7 (quat., 11-C), 37.0 (CH<sub>2</sub>, 3'-C), 37.3 (CH<sub>2</sub>, 2'-C), 78.1 (quat., 5'-C), 107.4 (CH<sub>3</sub>, 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'-Butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one 236e

The title compound was prepared as described (method B) from 8-amino-2-pentyl-3H-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'-butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-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<sub>25</sub>H<sub>31</sub>N<sub>3</sub>O<sub>5</sub> requires C, 66.2; H, 6.9; N, 9.3%); m/z 454.3 (MH)<sup>+</sup>, 476.3 (MNa)<sup>+</sup>; \( \nu_{\text{max}} \) cm<sup>-1</sup> 3322
5.1.24.6 8-β-Butoxycarbonyl-β-alanyl)amino-1-phenyl-3H-phenoxazin-3-one 236f

The title compound was prepared as described (method B) from 8-amino-1-phenyl-3H-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'-β-butoxycarbonyl-β-alanyl)amino-1-phenyl-3H-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_{28}N_{2}O_{5} requires C, 68.0; H, 5.5; N, 9.1%); m/z 460.2 (MH)^+; \nu_{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(CH_{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, NHfBoc), 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, NHfAr); 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), 130.5 (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''-Butoxycarbonyl-β-alanyl)amino-2'-butyl-3H-phenoxazin-3-one 234a

The title compound was prepared as described (method A) from 7-amino-2'-butyl-3H-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''-butoxycarbonyl-β-alanyl)amino-2'-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_{3}O_{5} requires C, 65.6; H, 6.65; N, 9.6%); m/z 438.1 (M-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(CH_{3})_{3}), 1.39 (9H, s, 6'/7'/8'-H, C(CH_{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, NHfBoc), 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, NH-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''-Butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one 234b
The title compound was prepared as described (method A) from 7-amino-2-pentyl-3H-phenoxazin-3-one 59e (0.48g, 1.69mmol); elution used petroleum ether (60-80°C) : EtOAc (40:60) yielding 7-N-({\textit{N'}-t-butoxycarbonyl-β-alanyl})amino-2-pentyl-3H-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_{3}O_{5} requires C, 66.2; H, 6.9; N, 9.3%); m/z 453.2 (M-H)^{+}; 454.3 (MH)^{+}; v_{\text{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, NH\text{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, NH\text{-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-({\textit{N'}-t-butoxycarbonyl-β-alanyl})amino-alkyl-3H-phenoxazin-3-one 236a-f and 7-N-({\textit{N'}-t-butoxycarbonyl-β-alanyl})amino-alkyl-3H-phenoxazin-3-one 234a-b

To the 7-N- or 8-N-({\textit{N'}-t-butoxycarbonyl-β-alanyl})amino-alkyl-3H-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_{2}O. The cloudy suspension was left standing overnight to allow for the formation of a microcrystalline solid. The latter was filtered and washed with Et_{2}O. The filtrate was evaporated under reduced pressure for further recovery of the product.
5.1.25.1 8-N-(β-Alanyl)amino-3H-phenoxazin-3-one TFA salt 237a

The title compound was prepared as described from 8-N-(N'-butoxycarbonyl-β-alanyl)amino-3H-phenoxazin-3-one 236a (0.16g, 0.43mmol). 8-N-(β-Alanyl)amino-3H-phenoxazin-3-one TFA salt 237a was isolated as a dark red solid (0.14g, 0.35mmol, 80.7%); m.p.: 167-170°C; (Found (M)+, 284.1031. Calc. for C15H14N3O3: (M)+, 284.1030); m/z 284.0 (M)+; νmax/cm⁻¹ 3408-2907 (N⁺-H), 3053 (N-H), 1671 (C=O), 1643 (C=O); δH (300 MHz, d₆-DMSO) 2.77 (2H, t, J = 6.6 Hz, 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, NH₃⁺), 8.15 (1H, d, J = 2.4Hz, 9-H), 10.56 (1H, s, NH-Ar); δC (75 MHz, d₆-DMSO) 33.8 (CH₂, 2'-C), 35.4 (CH₂, 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-3H-phenoxazin-3-one TFA salt 237b

The title compound was prepared as described from 8-N-(N'-butoxycarbonyl-β-alanyl)amino-1,2-dimethyl-3H-phenoxazin-3-one 236b (0.20g, 0.49mmol). 8-N-(β-Alanyl)amino-1,2-dimethyl-3H-phenoxazin-3-one TFA salt 237b was isolated as an orange-red solid (0.20g, 0.47mmol, 96.1%); m.p.: 202-207°C; (Found (M)+, 312.1344. Calc. for C₁₇H₁₉N₃O₃: (M)+, 312.1343); m/z 312.2 (M)+; νmax/cm⁻¹ 3282 (N-H), 3082 (N⁺-H), 1671 (C=O), 1645 (C=O); δH (300 MHz, d₆-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, NH₃⁺), 8.11 (1H, d, J = 1.8 Hz, 9-H), 10.46 (1H, s, NH-Ar); δC (75
MHZ, d₆-DMSO) 13.0 (CH₃, 11-C or 12-C), 13.05 (CH₃, 11-C or 12-C), 33.75 (CH₂, 2'-C), 35.4 (CH₂, 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-3H-phenoxazin-3-one TFA salt 237c

Prepared from 8-N-(N⁻⁴-butoxycarbonyl-β-alanyl)amino-1,2,4-trimethyl-3H-phenoxazin-3-one 236c (0.06g, 0.16mmol). 8-N-(β-Alanyl)amino-1,2,4-trimethyl-3H-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 C₁₈H₂₀N₃O₅: (M⁺), 326.1499); m/z 326.2 (M⁺); νmax/cm⁻¹ 3322 (N-H), 3054 (N⁻⁺-H), 1670 (C=O), 1649 (C=O); δH (300 MHz, d₆-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, NH₃⁺), 8.15 (1H, d, J = 2.1Hz, 9-H), 10.40 (1H, s, NH-Ar); δC (75 MHz, d₆-DMSO) 7.9 (CH₃, 13-C), 12.8 (CH₃, 12-C), 13.2 (CH₃, 11-C), 33.9 (CH₂, 2'-C), 35.8 (CH₂, 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'-butyl-3H-phenoxazin-3-one TFA salt 237d

The title compound was prepared as described from 8-N-(N⁻⁴-butoxycarbonyl-β-alanyl)amino-4'-butyl-3H-phenoxazin-3-one 236d (0.16g, 0.37mmol). 8-N-(β-Alanyl)amino-4'-butyl-3H-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$_3$O$_5$: (M$^+$, 340.1656); m/$\zeta$ 340.2 (M$^+$); $\nu_{\text{max}}$/cm$^{-1}$ 3462 (N-H), 3308-2965 (N$^+$-H) and (C-H), 1678 (C=O), 1649 (C=O); $\delta_H$ (300 MHz, d$_6$-DMSO) 1.32 (9H, s, C(CH$_3$)$_3$, 2.78 (2H, t, $\nu = 6.6$Hz, 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, $\nu = 9.0$Hz, 6-H), 7.74 (1H, dd, $\nu = 9.0$, 1.8Hz, 7-H), 7.97 (3H, br. s, NH$_3^+$), 8.13 (1H, d, $\nu = 1.8$Hz, 9-H); $\delta_C$ (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$_3$, 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-3H-phenoxazin-3-one TFA salt 237e**

![Chemical Structure](image)

The title compound was prepared as described from 8-N-(N$^-$-butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one **236e** (0.08g, 0.18mmol). 8-N-(β-Alanyl)amino-2-pentyl-3H-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$_3$O$_5$: (M$^+$, 354.1812); m/$\zeta$ 354.2 (M$^+$); $\nu_{\text{max}}$/cm$^{-1}$ 3312 (N-H), 3077 (N$^+$-H), 2932-2861 (C-H), 1672 (C=O), 1649 (C=O); $\delta_H$ (300 MHz, d$_6$-DMSO) 0.80 (3H, t, $\nu = 6.6$Hz, 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, $\nu = 8.1$Hz, 1'-H), 2.67 (2H, t, $\nu = 6.6$Hz, 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, $\nu = 9.0$Hz, 6-H), 7.65 (1H, dd, $\nu = 9.0$, 2.4Hz, 7-H), 7.75 (3H, br. s, NH$_3^+$), 8.08 (1H, d, $\nu = 2.4$Hz, 9-H), 10.39 (1H, s, NH-Ar); $\delta_C$ (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$_3$, 1'-C), 31.5 (CH$_2$, 3'-C or 4'-C), 33.9 (CH$_2$, 2''-C), 35.4 (CH$_3$, 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-3H-phenoxazin-3-one TFA salt 237f

The title compound was prepared as described from 8-N-(N′-butoxycarbonyl-β-alanyl)amino-1-phenyl-3H-phenoxazin-3-one 236f (0.05g, 0.12mmol). 8-N-(β-Alanyl)amino-1-phenyl-3H-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 C₂₃H₁₈N₃O₅: (M⁺), 360.1343); m/z 360.2 (M⁺); vₓmax/cm⁻¹ 3262 (N-H), 3068 (N⁺-H), 2903 (C-H), 1670 (C=O), 1640 (C=O); δₓH (300 MHz, d₆-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), 7.62 (1H, dd, J = 8.7, 1.8Hz, 7-H), 7.80 (3H, s, NH₃⁺), 8.03 (1H, d, J = 1.2Hz, 9-H), 10.33 (1H, s, NH-Ar); δₓC (75 MHz, d₆-DMSO) 33.8 (CH₂, 2"-C), 35.5 (CH₂, 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'-butyl-3H-phenoxazin-3-one TFA salt 235a

The title compound was prepared as described from 7-N-(N′-butoxycarbonyl-β-alanyl)amino-2'-butyl-3H-phenoxazin-3-one 234a (0.11g, 0.25mmol). 7-N-(β-Alanyl)amino-2'-butyl-3H-phenoxazin-3-one TFA salt 235a (Found (M⁺), 340.1657. Calc. for C₁₉H₂₂N₃O₅: (M⁺), 340.1656); m/z 340.2 (M⁺); vₓmax/cm⁻¹ 3180 (N-H), 3028 (N⁺-H), 2961 (C-H), 1672 (C=O); δₓH (300 MHz, d₆-DMSO) 1.235 (9H, s, C(CH₃)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, NH₃⁺), 10.69 (1H, s, NH-Ar); δₓC (75 MHz, d₆-DMSO)
29.4 (CH₃, 12/13/14-C), 34.1 (CH₂, 2'-C), 35.3 (CH₂, 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-3H-phenoxazin-3-one TFA salt 235b

The title compound was prepared as described from 7-N(N-butoxycarbonyl-β-alanyl)amino-2-pentyl-3H-phenoxazin-3-one 234b (0.14g, 0.30mmol). 7-N-(β-Alanyl)amino-2-pentyl-3H-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°C; (Found (M⁺, 354.1815. Calc. for C₂₀H₂₉N₃O₃: (M⁺, 354.1812); m/z 354.2 (M⁺)

ν<sub>max</sub>/cm⁻¹ 3263 (N-H), 3188-3060 (N⁻H), 2928 (C-H), 1674 (C=O), 1651 (C=O); δ<sub>H</sub> (300 MHz, d₆-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, NH₃⁺), 10.83 (1H, s, NH-Ar); δ<sub>C</sub> (75 MHz, d₆-DMSO) 14.4 (CH₃, 5'-C), 22.4 (CH₂, 3'-C or 4'-C), 28.1 (CH₂, 2'-C), 29.4 (CH₂, 1'-C), 31.5 (CH₂, 3''-H or 4''-H), 34.1 (CH₂, 2''-C), 35.2 (CH₂, 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 preferred, 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 130α-a-c, 130β-a-c, 130γ-a-b and 242 were prepared following the general method described above and according to Table 5.1.
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-3H-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.

### Table 5.1: Preparation of the medium for substrates 130αα-αα, 130ββ-ββ, 130γγ-γγ and 242

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mass (mg)</th>
<th>V (NMP) (µL)</th>
<th>V (Tween 20) (µL)</th>
<th>V (Columbia agar) (mL)</th>
<th>Final conc. (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130αα</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.017</td>
</tr>
<tr>
<td>130αβ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.0145</td>
</tr>
<tr>
<td>130αγ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.010</td>
</tr>
<tr>
<td>130βα</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.017</td>
</tr>
<tr>
<td>130ββ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.0145</td>
</tr>
<tr>
<td>130βγ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.010</td>
</tr>
<tr>
<td>130γα</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.017</td>
</tr>
<tr>
<td>130γβ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.0145</td>
</tr>
<tr>
<td>130γγ</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.015</td>
</tr>
<tr>
<td>242</td>
<td>5</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.2: Preparation of the medium for substrates 237g-i and 240a-b

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mass (mg)</th>
<th>V (NMP) (µL)</th>
<th>V (Tween 20) (µL)</th>
<th>V (Columbia agar) (mL)</th>
<th>Final conc. (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>237g</td>
<td>5</td>
<td>200</td>
<td>-</td>
<td>100</td>
<td>0.11</td>
</tr>
<tr>
<td>237h</td>
<td>5</td>
<td>400</td>
<td>-</td>
<td>100</td>
<td>0.10</td>
</tr>
<tr>
<td>237i</td>
<td>5</td>
<td>200</td>
<td>300</td>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>240a</td>
<td>5</td>
<td>200</td>
<td>-</td>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>240b</td>
<td>5</td>
<td>200</td>
<td>-</td>
<td>100</td>
<td>0.07</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V (stock sol.) µL</th>
<th>V (agar) mL</th>
<th>Conc. (mmol/L)</th>
<th>V (stock sol.) µL</th>
<th>V (agar) mL</th>
<th>Conc. (mmol/L)</th>
<th>V (stock sol.) µL</th>
<th>V (agar) mL</th>
<th>Conc. (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>237a</td>
<td>200</td>
<td>80</td>
<td>0.063</td>
<td>400</td>
<td>80</td>
<td>0.126</td>
<td>800</td>
<td>80</td>
<td>0.252</td>
</tr>
<tr>
<td>237b</td>
<td>200</td>
<td>80</td>
<td>0.053</td>
<td>400</td>
<td>80</td>
<td>0.106</td>
<td>800</td>
<td>80</td>
<td>0.211</td>
</tr>
<tr>
<td>237c</td>
<td>200</td>
<td>80</td>
<td>0.059</td>
<td>400</td>
<td>80</td>
<td>0.118</td>
<td>800</td>
<td>80</td>
<td>0.235</td>
</tr>
<tr>
<td>237d</td>
<td>200</td>
<td>80</td>
<td>0.057</td>
<td>400</td>
<td>80</td>
<td>0.114</td>
<td>800</td>
<td>80</td>
<td>0.228</td>
</tr>
<tr>
<td>237e</td>
<td>200</td>
<td>80</td>
<td>0.055</td>
<td>400</td>
<td>80</td>
<td>0.110</td>
<td>800</td>
<td>80</td>
<td>0.221</td>
</tr>
<tr>
<td>237f</td>
<td>200</td>
<td>80</td>
<td>0.053</td>
<td>400</td>
<td>80</td>
<td>0.107</td>
<td>800</td>
<td>80</td>
<td>0.214</td>
</tr>
<tr>
<td>235a</td>
<td>200</td>
<td>80</td>
<td>0.055</td>
<td>400</td>
<td>80</td>
<td>0.110</td>
<td>800</td>
<td>80</td>
<td>0.221</td>
</tr>
<tr>
<td>235b</td>
<td>200</td>
<td>80</td>
<td>0.053</td>
<td>400</td>
<td>80</td>
<td>0.107</td>
<td>800</td>
<td>80</td>
<td>0.214</td>
</tr>
</tbody>
</table>

**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).

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

**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°C for 24h, before recording the results.

Figure 5.1: Inoculation pattern of a Petri dish.
CHAPTER SIX:

REFERENCES
6.1 References

1884MI185 C. Gram., *Fortschr. Med.*, 1884, 2, 185.


<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
</table>


...c’est l’atterrissage.