Heterocyclic NO-donors as anti-cancer agents

A thesis submitted by

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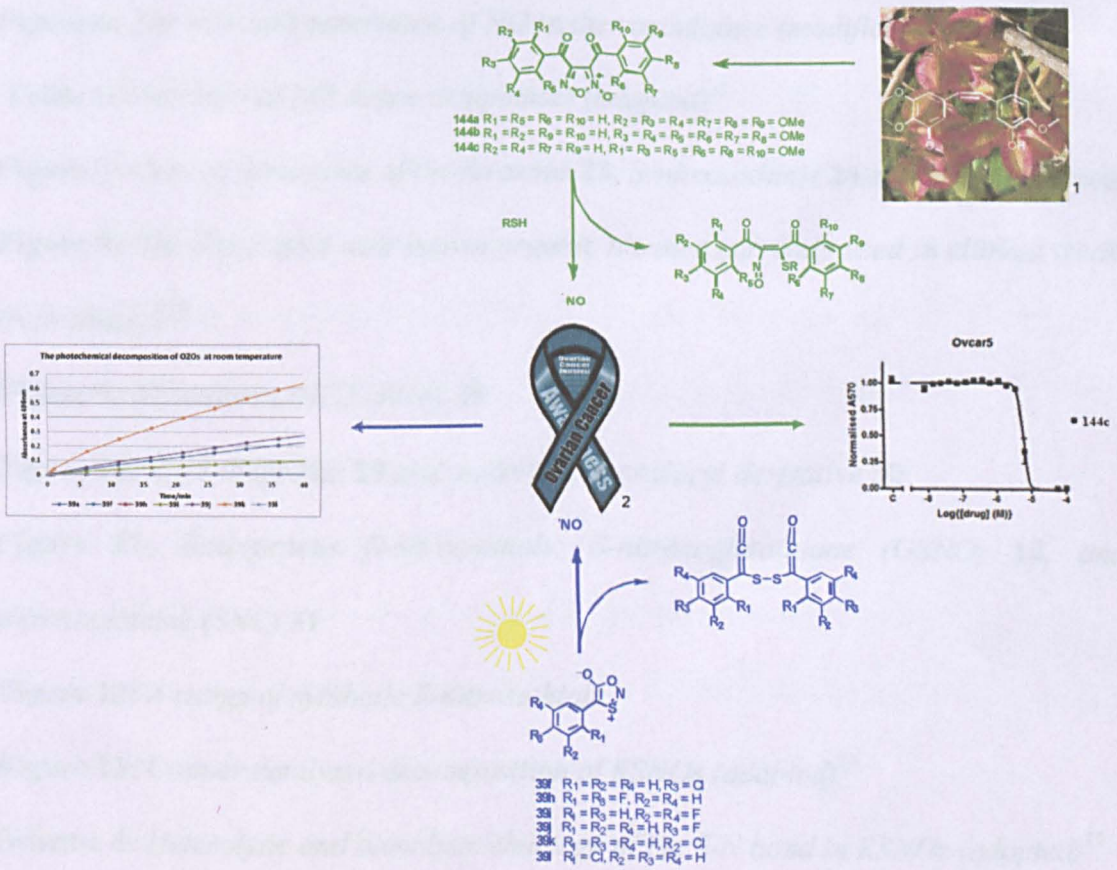
"I have not failed. I've just found 10,000 ways that won't work."
— Thomas A. Edison

Abstract

Nitric oxide (NO) is involved in numerous biological processes including cancer, where this small diatomic radical can exert both pro- and anti-cancer effects. Two series of novel NO-donors were synthesised in this work, the first representing an extension to the S-nitrosothiols (RSNO) class, with the second utilising the popular 1,2,5-oxadiazole-2-oxide (furoxan) functionality. The oxathiazolylium-5-olates **39a**, **39f, 39h-l**, were successfully made *via* improved seven-step synthesis whilst a series of combretastatin-like furoxans **144a-c** (NO-hybrids) were generated in a one-pot reaction. All compounds, including byproducts from failed alternative synthetic routes, had their cytotoxic activity evaluated. From this study NO-hybrid **144c** showed the most promising biological profile when tested against eight different ovarian cancer cell lines.



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Abbreviations

Ac	Acetyl
Ar	Aryl
ASAP	As soon as possible
Bu	Butyl
CA-4	Combretastatin A-4
cAMP	3'-5'-cyclic adenosine monophosphate
cGMP	Cyclic guanosine-3,5-monophosphate
Cat.	Catalytic
Conc.	Concentrated
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
d	Doublet
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDRF	Endothelium derived relaxing factor

EI	Electron impact
eNOS	Endothelial nitric oxide synthase or NOSIII nitric oxide synthase 3
ERK	Extracellular signal-regulated kinases
Et	Ethyl
Eq.	Equivalents
Furoxans	1,2,5-oxadiazole-2-oxides
FVT	Flash vacuum thermolysis
GC- MS	Gas Chromatography- Mass Spectrometry
GC	Guanylyl cyclase
GSNO	*S*-Nitrosoglutathione
GTN	Nitroglycerin or trinitroglycerin or glyceryl trinitrate
GTP	Guanosine triphosphate
Hrs	Hours
HCl	Hydrochloric acid
HIF-1α	Hypoxia-inducible factor alpha
i-Bu	*iso*-butyl
IC_{50}	Half maximal inhibitory concentration
iNOS	Inducible nitric oxide synthase or NOSII nitric oxide synthase 2
i-Pr	*iso*-Propyl

IR	Infra red Spectroscopy
LD_{50}	Lethal Dose, 50% or median lethal dose. The amount of the substance required (usually per body weight) to kill 50% of the test population
m	Multiplet
MDR	Multi-Drug-Resistant
Me	Methyl
min	Minutes
ml	Millilitre
mmol	Millimoles
mp	Melting point
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NEDD	*N*-(1-Napthyl)ethylenediamine
NMR	Nuclear Magnetic Resonance spectroscopy
nNOS	Neuronal nitric oxide synthase or NOSI nitric oxide synthase 1
NO	Nitric oxide
NSCLC	Non-small-cell lung carcinoma
NSAIDs	Nonsteroidal anti-inflammatory drugs
Nu	Nucleophile

OZO	Oxathiazolylium-5-olates
Ph	Phenyl
ppm	Parts per million
RSNOs	*S*-nitrosothiols
s	Singlet
SAR	Structure-Activity Relationship
SNAG	*S*-Nitroso-1-thio-2,3,4,6–tetra-*O*-acetyl-β-D-glucopyranose
SNC	*S*-Nitrosocysteine
SNAP	*S*-Nitroso-*N*-acetyl-penicillamine
SNAC	*S*-Nitroso-*N*-acetyl-cysteine
SULF	Sulfanamide
t	Triplet
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilane
UV	Ultraviolet spectrophotometry
VEGF	Vascular endothelial growth factor
VDAs	Vascular Disrupting Agents

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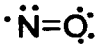
1 Introduction

1.1 Nitric oxide

Nitric oxide **1** (NO) is a small diatomic molecule that exists as a gas. It readily oxidises to form NO_2, nitrogen dioxide, a molecule more commonly associated with smog and cigarette smoke, and other *N*-oxides, and as a result, prior to 1987 NO was usually mentioned in this negative context.

Figure 1:



Nitric oxide molecule **1**

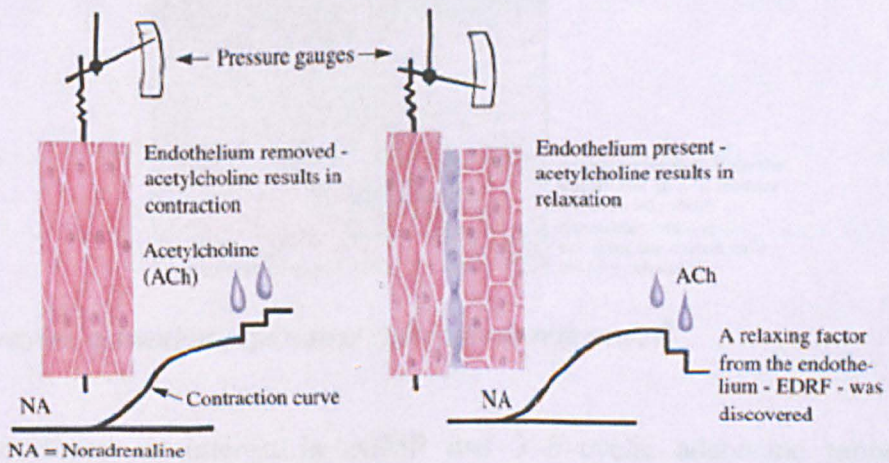
The discovery of NO as an important biological molecule generated huge scientific interest into this relatively stable free radical and this fascination continues to grow. Literature searches using "nitric oxide" as the key word returns an amazing 600,000 hits on the Web of Science[1] and 225,000 hits on Sciencedirect.[2] These published articles contain research on NO from within a wide range of areas such as chemistry, biology, medicine and physics. Given that there are so many areas where NO plays an important role, it is beyond the scope of this thesis to try to review all the literature nor is it possible to detail all the processes and functions of NO in human biology. As a result this work will concentrate on NO in relation to cancer and the chemistry of two key NO-donors; the *S*-nitrosothiols and the furoxans.

1.1.1 The discovery of NO as an important signalling agent

The discovery of the biological importance of NO is a story that has been told many times and therefore only a brief overview of the early work will be covered. The 1998 Nobel Prize in Medicine/Physiology was awarded to Robert F Furchgott, Louis J Ignarro and Ferid Murad for their work towards identifying "nitric oxide as a signalling molecule in the cardiovascular system".[3–6]

Furchgott had been working on vasoactive drugs for many years[4] when he discovered the effect of an intact endothelium on vasodilation. He used two different pieces of rabbit aorta in his experiment, one that had an intact endothelium, and one where the endothelium had been removed. Upon introducing acetylcholine to both strips of aorta (Figure 2), he found that only the strip with the endothelium intact reversed the effects of noradrenaline (NA). Furchgott suggested that a vasodilating substance, which he called endothelium derived relaxing factor (EDRF), diffused from the endothelial cells through to the smooth muscle cells when stimulated by acetylcholine.[4]

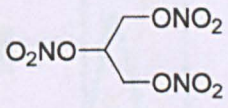
Figure 2:



Furchgott's sandwich experiment (taken from reference)[7]

Murad's work found that nitroglycerin **2** (Figure 3) relaxed smooth muscle cells by activating guanylyl cyclase (GC) which increased cyclic guanosine-3,5-monophosphate (cGMP). Murad wanted to investigate whether or not nitroglycerin acted via the release of NO. He conducted an experiment where he passed NO gas through GC containing tissue, and found an increase in cGMP (Figure 4). This confirmed that nitroglycerin **2** (Figure 3) acted via the release of NO.[5]

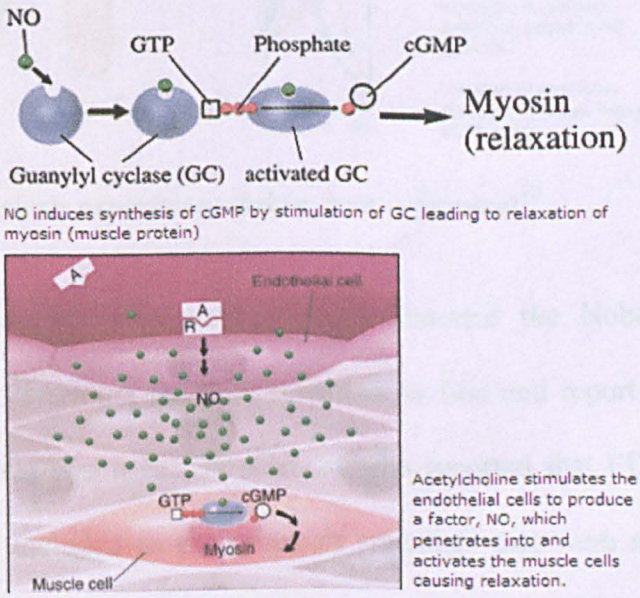
Figure 3:



2

Nitroglycerin or trinitroglycerin or glyceryl trinitrate (GTN) **2**

Figure 4:

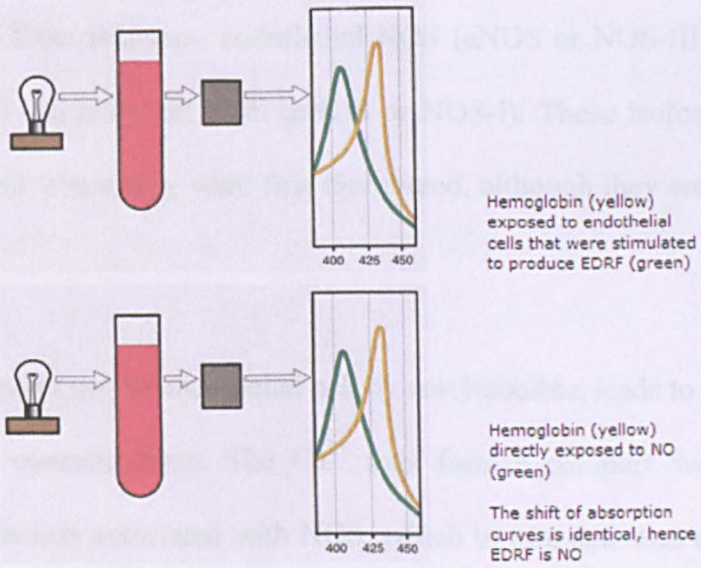


Murad's enzyme activation experiment (taken from reference)[8]

Ignarro developed an interest in cGMP and 3'-5'-cyclic adenosine monophosphate (cAMP), and following the work of Murad and others, he decided to test if the relaxing

effect of nitroglycerin was due to the release of NO. He also wanted to determine whether or not NO had vasodilating effects on its own and he tested this by using UV-Vis spectrophotometry. Ignarro conducted an experiment that proved EDRF to be NO by exposing haemoglobin to both EDRF and NO and comparing the spectral data (Figure 5). He found that the absorption shifts were identical; thus EDRF was NO.[9]

Figure 5:



Ignarro's spectral analysis experiment (taken from reference)[10]

Although Ignarro, Murad and Furchgott were awarded the Nobel Prize for their discovery, these three were not the only scientists to find and report the importance of NO. Salvador Moncada was a British scientist who reported that EDRF was NO[11] but surprisingly was not included in the group of scientists that were awarded the Nobel Prize. Concurrently with the research carried out by these scientists on the vasodilating effects of NO, Hibbs and co-workers were investigating a substance which was important in anti-pathogen and anti-tumour response. It was discovered that this substance was NO

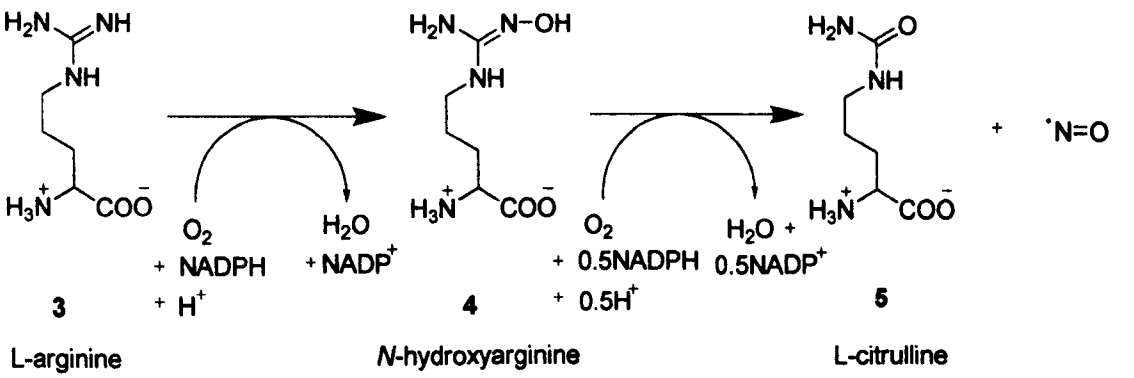
and shortly after these initial studies it was also found that leukaemia cells were killed by NO released from macrophages.[12]

1.1.2 Endogenous synthesis of NO

The endogenous production of NO results from a biological cascade and involves a large number of enzymes, co-enzymes and other factors. Activation of nitric oxide synthase (NOS), present in most cells, initiates the formation of NO. Nitric oxide synthase enzymes exists in three isoforms, endothelial NOS (eNOS or NOS-III), inducible NOS (iNOS or NOS-II) and neuronal NOS (nNOS or NOS-I). These isoforms were initially named after the cell where they were first discovered, although they are present in many other cell types.[13–15]

Activation of receptors on the endothelial cell by acetylcholine, leads to an increase in the intracellular Ca^{2+} concentrations. The Ca^{2+} ions form a complex with calmodulin, a calcium binding protein associated with NOS, which in turn activates eNOS. Scheme 1 illustrates the conversion of L-arginine **3** and oxygen to L-citrulline **5** and NO by NOS.[13–15]

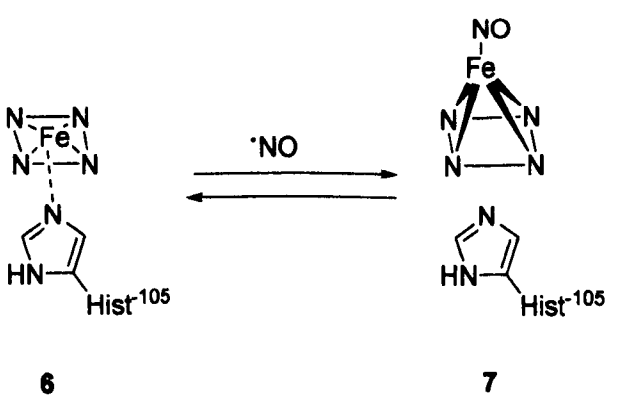
Scheme 1:



A balanced equation for conversion of L-arginine into L-citrulline and NO (reproduced)[14]

The conversion from L-arginine **3** via *N*-hydroxyarginine **4** to L-citrulline **5** consists of two oxygenations where the electrons required are derived from the cofactor nicotinamide adenine dinucleotide phosphate (NADPH).[14] Once NO has been synthesised in the endothelium it can diffuse through to the smooth muscle where NO binds to the Fe(II) component of guanylyl cyclase (GC) as outline in scheme 2.

Scheme 2:

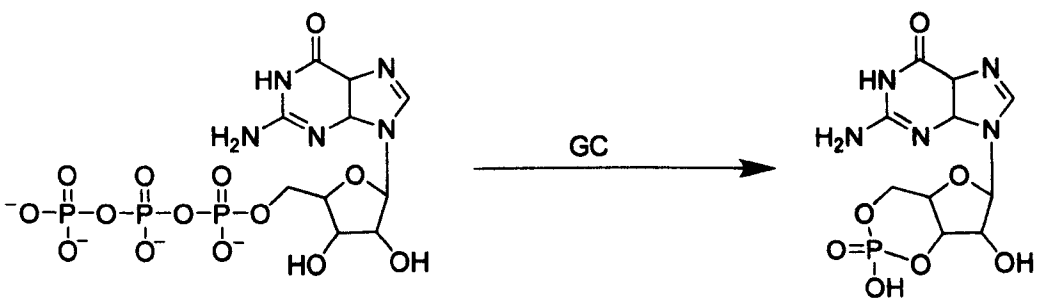


6 **7**

Activation of guanylyl cyclase by NO (reproduced)[14]

Activation of guanylyl cyclase leads to the production of cyclic guanosine-3,5-monophosphate (cGMP) **9**, from guanosine triphosphate **8** (GTP) (Scheme 3). This results in a range of reactions such as protein phosphorylation, and an increase in cGMP which reduces the level of Ca^{2+} in the smooth muscle cell, which in turn leads to relaxation and an increase in blood flow.

Scheme 3:

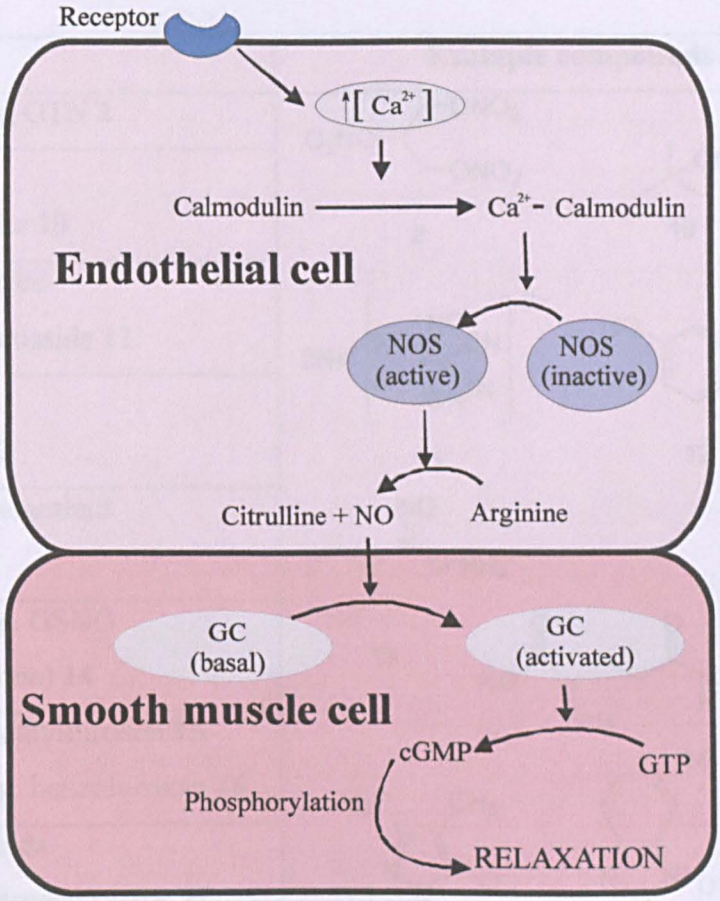


8 **9**

The conversion of GTP **8** *to cGMP* **9** *(reproduced)*[14]

The biosynthesis of NO and sequence of events leading to relaxation of the smooth muscle is summarised in Figure 6.

Figure 6:

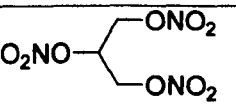
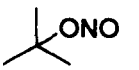
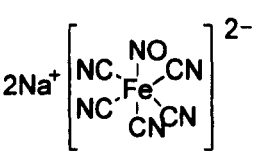
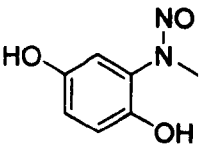
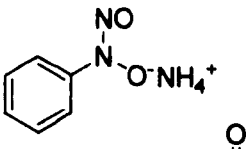
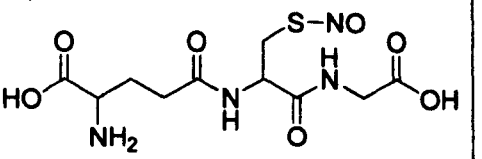
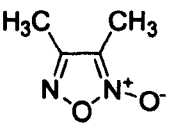
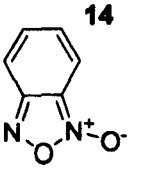
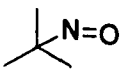
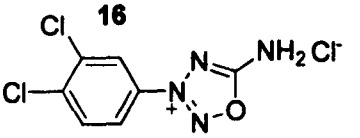
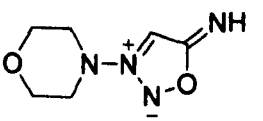
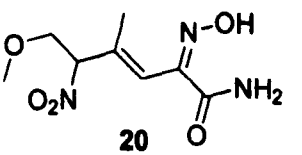
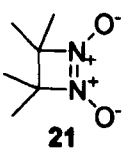


The role and generation of NO in the vasculature (modified)[14]

1.2 Key NO donors

As the interest into NO increased with rapid intensity due to the discovery of its beneficial effects, such as those on vasodilatation and cancer, studies into compounds which could release NO grew accordingly. Such NO donating compounds can be characterised by many different classes. For a compound to be deemed NO donating, it must have the ability to release NO, either spontaneously or after interaction with an initiator such as light, heat, enzymes or compounds such as thiols or metals. Table 1 lists a selection of the currently known NO-donor compounds including some specific examples.

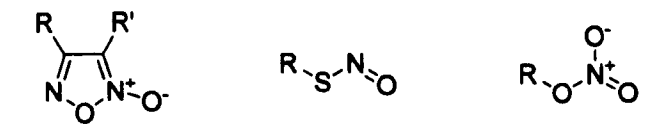
Table 1:

Name	Example compounds
Organic nitrates e.g GTN **2**	 **2**
Organic nitrites e.g. *tert*-butyl nitrite **10**	 **10**
Metal NO complexes e.g. sodium nitroprusside **11**	 **11**
N-nitrosoamines e.g. Dephostatin **12**	 **12**
N-Hydroxyl nitrosoamines e.g. Cupferron **13**	 **13**
S-Nitrosothiols e.g. GSNO (*S*-nitrosoglutathione) **14**	 **14**
Furoxans e.g. dimethylfuroxan **15** Benzofuroxans e.g. benzofuroxan **16**	 **15**  **16**
C-nitroso compounds e.g. 2-methyl-2-nitrosopropane **17**	 **17**
Oxatriazole-5-imines e.g. GEA 3162 (1,2,3,4-oxatriazolium-5-amino-3 (3,4-dichlorophenyl)chloride **18**	 **18**
Sydonimines e.g. SIN-1 (3-morpholinosydinomine) **19**	 **19**
Oximes e.g. NOR-1 **20**	 **20**
Diazetin dioxides e.g. 3,3,4,4-tetramethyl-1,2-diazetine 1,2 dioxide **21**	 **21**
NO-donor hybrid drugs (Generic structure) **22**	 **22**

Overview of NO donor compounds (adapted)[16]

Over the last two decades three NO-donor classes have received a great deal of attention: the furoxans, **23**, the *S*-nitrosothiols, **24**, and the organic nitrates, **25**. The organic nitrates **25** have been researched greatly as these compounds are the oldest class of NO-donors. GTN **2** has been used clinically in the treatment of angina since it was suggested by William Murrell in 1879, which is clearly long before the realisation of how NO acts as a vasodilator.[14,17]

Figure 7:



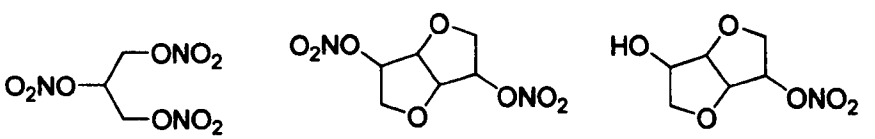
23 **24** **25**

R = R' or R ≠ R'

Generic structures of the furoxans **23**, *S-nitrosothiols* **24** *and organic nitrates* **25**

GTN **2** is currently employed as a first-line treatment for patients suffering from angina[18] and other clinically used nitrates include isosorbide dinitrate (ISDN) **26** and isosorbide mononitrate (ISMN), **27**. The use of organic nitrates can result in tolerance after prolonged use, but the mechanism of this is still unclear.[18–20]

Figure 8:



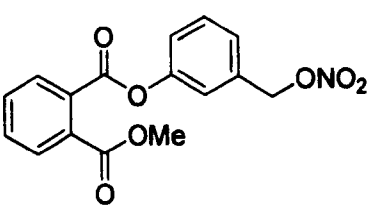
2 **26** **27**

GTN ISDN ISMN

The three most well-known organic nitrates currently used in clinical treatment (reproduced)[19]

The *S*-nitrosothiols (RSNOs) **24** have received a lot of attention particularly since RSNOs have been found *in vivo*[21–25] and because of their usually brightly coloured solutions which are favoured by biologists as a lack of colour is a useful indicator the decomposition of RSNO **24** to the inactive disulphides (section 1.2.1). The third class of NO-donors, the furoxans **23**, are generally more stable than the RSNOs and the synthetic route to these compounds is relatively straight forward. Despite the drawback of tolerance, organic nitrates **25** are currently one of the most used NO-donors in NO hybrid drugs as the nitrates have the advantage of well established profiles when used *in vivo*.[16] Over the last decade or so the NO-donor hybrid drugs have gained an increasing popularity with promising results from NO-NSAIDs (NO containing non-steroidal anti inflammatory drugs), which have shown a reduction in gastric side effects compared to traditional NSAIDs.[16,26,27] In addition, NO-aspirin (NCX-4016) **28**, is one example, which has recently been investigated in a phase I trial as a chemopreventive agent in colorectal cancer.[28]

Figure 9:



28

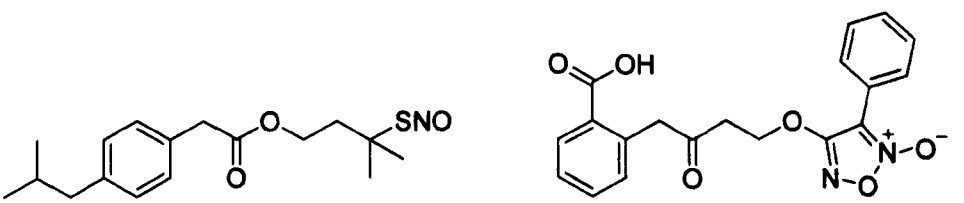
NCX-4016

NO-aspirin (NCX-4016) **28**

The success with the nitrate hybrids have resulted in a drive to synthesis hybrid drugs containing other NO-donors which do not produce tolerance. NO-donor hybrid drugs

have also been synthesised using *S*-nitrosothiols as the NO-donor, with promising results.[16] Recently a series of furoxanylacyl derivatives of aspirin was synthesised and preliminary biological testing showed a decrease in gastrotoxicity in a rat lesion model.[29] Figure 10 show SNO-ibuprofen **29** and furoxanylacyl derivative of aspirin **30**.

Figure 10:



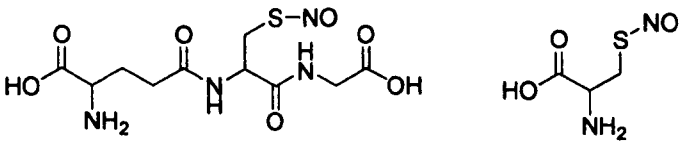
29 **30**

SNO-ibuprofen **29** *and aspirin furoxanylacyl derivative* **30**

1.2.1 *S*-Nitrosothiols (RSNOs)

S-Nitrosothiols are a class of NO donors which are of particular interest as these exist both as synthetic compounds and as naturally occurring compounds. *S*-Nitrosoalbumin, *S*-nitrosohaemoglobin, *S*-nitrosoglutathione, **14,** and *S*-nitrosocysteine, **31** (Figure 11), are all *S*-nitrosothiols (RSNOs) which have been found *in vivo*.[21–25] The exact amount of *S*-nitrosothiols which exist *in vivo* is debatable, as currently no techniques are available to accurately measure the presence of these compounds in biological fluids and tissue.[21–24] It is their endogenous nature which has prompted much research into this class of NO-donor since it is widely acknowledged that they are unlikely to produce any significant toxicity within the body. In addition, these compounds do not require metabolic degradation in order to release NO, and therefore they are not expected to produce tolerance.[30]

Figure 11:



14 **31**

Endogenous S-nitrosothiols: S-nitrosoglutathione (GSNO) **14**, *and S-nitrosocysteine (SNC)* **31**

1.2.1.1 Synthesis of *S*-nitrosothiols

The reaction between NO and free thiol does not give the *S*-nitrosothiol (RSNO), but instead yields the disulphide.[31] In the presence of oxygen on the other hand, NO can be oxidised to form nitrogen oxides such as NO_2, N_2O_4 and N_2O_3 which can then nitrosate the thiol. One of the most common methods for synthesising RSNOs is the use of sodium nitrite in an acidic environment (Equations 1 and 2).[16,15,22,25]

$$HNO_2 + H^+ \longrightarrow NO^+ + H_2O \qquad \text{Equation 1}$$

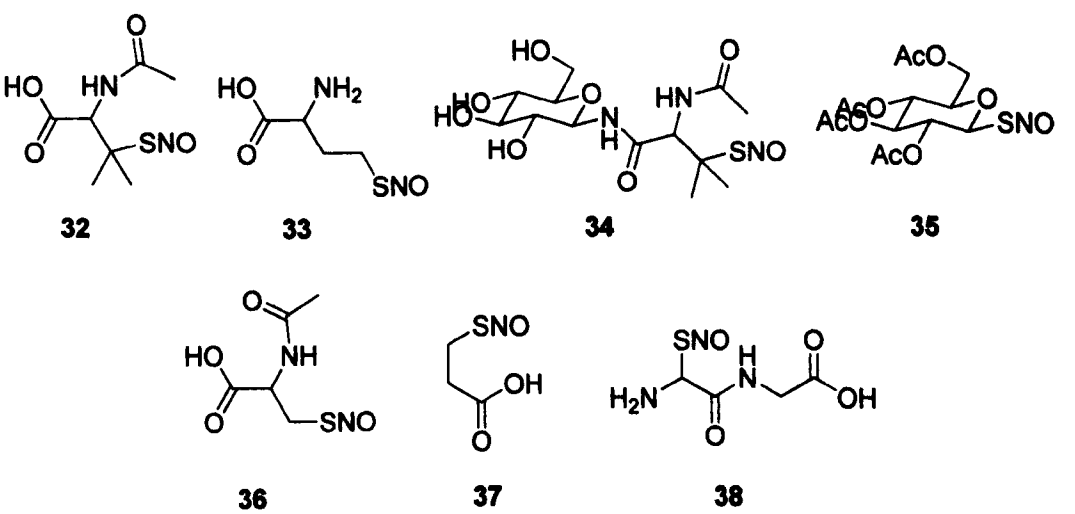
$$RSH + NO^+ \longrightarrow RSNO + H^+ \qquad \text{Equation 2}$$

With thiols that are not water soluble or have functional groups susceptible to modification by nitrite, such as amines or alcohols in proteins, alkyl nitrites such as *tert*-butyl nitrite in a suitable organic solvent can be used.[16,22] Formation of the RSNO **24** results in a colour change, which usually produces a red colour, for primary or secondary RSNOs, or a green colour, for tertiary RSNOs.[16] The colour of the RSNO is often used to monitor their decomposition and the rate of release of NO using UV-Vis spectrophotometry.[16]

1.2.1.2 Stability and decomposition of *S*-nitrosothiols (RSNOs)

The isolation of a pure RSNO **24** is a challenging task as the presence of trace metals, such as copper, or exposure to heat or light can result in their decomposition and release of NO to give the corresponding disulphide. It is possible to isolate RSNOs by precipitation, provided they are stable in the solid state. *S*-Nitroso-*N*-acetyl-penicillamine (SNAP) **32**, will precipitate from acidified aqueous solution to give a green solid. Similarly, *S*-nitrosoglutathione (GSNO) **14** precipitates out with the addition of acetone to give a pink solid.[22] Much confusion surrounds the stability of RSNOs although it is clear that their stability is governed by the nature of the R-group. While GSNO **14** and SNAP **32** are relatively stable in the solid form, other small RSNOs such as *S*-nitrosocysteine **31** and *S*-nitrosohomocysteine **33** are not stable enough to be isolated, although if kept at low temperature kinetic experiments can be carried out.[25] Larger molecules such as the protein RSNOs and the synthesised sugar derivatives, sugar SNAP **34**, and *S*-nitroso-1-thio-2,3,4,6–tetra-*O*-acetyl-β-D-glucopyranose (SNAG)[32] **35** are considered more stable than the smaller RSNOs.[33]

Figure 12:



32 **33** **34** **35**

36 **37** **38**

A range of synthetic S-nitrosothiols

Ab initio studies on the thermal stability of SNAP **32** and *S*-nitroso-*N*-acetyl-cysteine, SNAC **36**, suggests that the steric factors are important in the increased stability of SNAP **32** over SNAC **36**.[34] The dimerisation required for the formation of the disulphide is hindered by the adjacent methyl groups in the SNAP molecule resulting in a decreased rate of decomposition and thus increased stability.[34] Tullett and co-workers found that at pH 7.4 *S*-nitroso-3-mercaptopropionic acid **37** and *S*-nitroso-*N*-acetyl-L-cysteine **36** were more stable than *S*-nitroso-L-cysteinylglycine **38** and *S*-nitrosocysteine **31** and suggested that this decomposition was affected by the rate-limiting formation of Cu^{+} from Cu^{2+}. Addition of the copper chelating agent DTPA (diethylenetriaminpenta-acetic acid) had a more pronounced effect on *S*-nitroso-L-cysteinylglycine **38** and *S*-nitrosocysteine **31** with an 24-fold increase in half life from 0.54 hours to 13.10 hours for *S*-nitrosocysteine **31** and an 18-fold increase in the half life from 0.31 hours to 5.67 hours for *S*-nitroso-L-cysteinylglycine **38**.[35]

The role of copper and other metals in the decomposition of RSNOs has been extensively studied.[36–39] In 1993 it was shown that copper catalyses the decomposition of RSNOs in solution and prior to this it was difficult to reproduce decomposition studies.[37] The initial work on copper catalysed decomposition of RSNOs was carried out by Butler and Williams by monitoring the decrease in absorbance of SNAP **32** relative to the concentration of Cu^{2+} in the presence and absence of copper chelating agent EDTA.[37] Extensive research into the role of copper as a mediator of RSNO decomposition has revealed that it is the reduced metal ion (Cu^{+}) rather than the oxidised metal ions (Cu^{2+}) that initiates the release of NO.[38,40] Butler and Williams employed the use of a specific Cu^{+}-chelating agent to determine which form of the copper was involved in the decomposition of SNAP **32**. Addition the Cu^{+}-chelating agent, neocuprine, to a solution

of SNAP at pH 7.4 with added Cu^{2+} reduced the reaction rate of the decomposition until, at equimolar amounts of SNAP **32** and neocuprin, the reaction was completely suppressed.

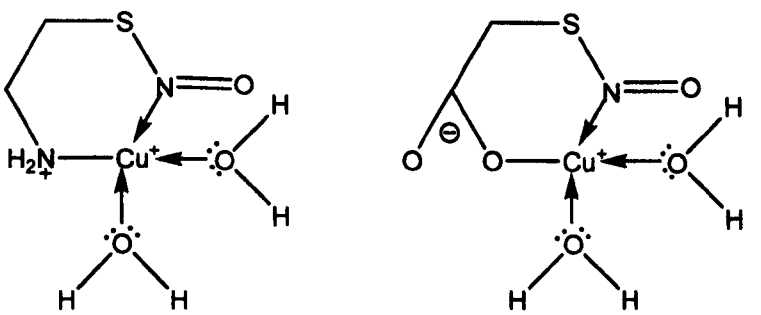
$$RSNO + H_2O \rightleftharpoons RS^- + NO_2^- + 2H^+ \quad \text{Equation 3}$$

$$Cu^{2+} + RS^- \longrightarrow Cu^+ + RS^{\cdot} \quad \text{Equation 4}$$

$$Cu^+ + RSNO \rightleftharpoons [X] \longrightarrow Cu^{2+} + RS^- + {}^{\cdot}NO \quad \text{Equation 5}$$

$$2RS^{\cdot} \longrightarrow RSSR \quad \text{Equation 6}$$

Figure 13:



Copper catalysed decomposition of RSNOs (adapted)[16]

The mechanism proposed by Butler and Williams presumes that the initial reduction of Cu^{2+} to Cu^+ is facilitated by either trace amounts of thiolate impurity or by hydrolysis of the RSNO to give the thiolate. The two complex intermediates **A** and **B** in figure 13 (represented as X in Equation 5) are formed by the binding of Cu^+ to the nitrogen of the NO group and another electron rich atom present within the structure of the RSNO. This leads to the production of NO and RS^- and oxidation of Cu^+ to give Cu^{2+}, followed by oxidation of the thiolate to give $RS^{\cdot}$ (Equation 4) which dimerises to give the corresponding disulphide (Equation 6).[38] Computational work supports this and suggests formation of a Cu^+-RSNO intermediate (X, see Equation 5 and Figure 13) that results in

strengthening of the NO bond and weakening of the SN bond, thus releasing NO.[41] It has also been reported that iron (Fe^{2+}) catalyses RSNO decomposition,[42] whereas other metal ions such as Ca^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, Zn^{2+}, Mg^{2+} or Ni^{2+} do not.[25]

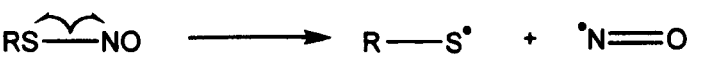
In addition to copper, and other metals, a number of initiators such as heat, light, thiols and enzymes can trigger the decomposition of RSNOs to give the corresponding disulphide and release NO (Equation 7).[25,43,15]

$$2RSNO \xrightarrow[\text{RSH, Cu+, or enzyme}]{\text{hv,heat}} RSSR + 2\,^{\bullet}NO \qquad \text{Equation 7}$$

The release of NO involves either homolytic or heterolytic bond cleavage of the S-NO bond as shown in Scheme 4.

Scheme 4:

Homolytic decomposition



Heterolytic decomposition

Heterolytic and homolytic cleavage of the S-N bond in RSNOs (adapted)[16]

As previously mentioned RSNOs can decompose when exposed to heat. The mechanism through which this occurs is believed to be by homolytic cleavage of the S-NO bond

which produces a thiyl radical and the NO radical (Scheme 4). The second step of thermal decomposition is dimerisation of two thiyl radical to give the disulphide (Equation 6).[16] In the solid form the decomposition of SNAP **32** was only seen after heating to 150°C whereas in methanol SNAP **32** decomposed readily at 90°C to give the disulphide after 2 hours.[34,44]

A similar reaction occurs when RSNOs are exposed to light (Equation 8). Irradiation of GSNO **14** at 340 nm or 540-545 nm results in homolytic cleavage of the S-NO bond (Equation 8). The reaction between the thiyl radical and GSNO results in the formation of the disulphide and the release of NO (Equation 9). In the presence of oxygen the peroxy radical (GSOO·) is formed (Equation 10) which can react with GSNO in a similar fashion to the thiyl radical, giving the disulphide along with the release of NO and oxygen (Equation 11).[13,25]

A fourth important route of decomposition for RSNOs is the release of NO through transnitrosation.[25] In this reaction between a RSNO and a free thiol, NO is transferred from one thiol to the next (Equation 12). If the second thiol is less stable, the presence of reducing agents may result in the release of NO. This reaction is believed to be a possible route for transportation of NO *in vivo*.[15,45,46] Protein modification *via* transnitrosation is also considered as a potential way to activate enzymes *in vivo*.[47]

$$RSNO + R'SH \rightleftharpoons RSH + R'SNO \qquad \text{Equation 12}$$

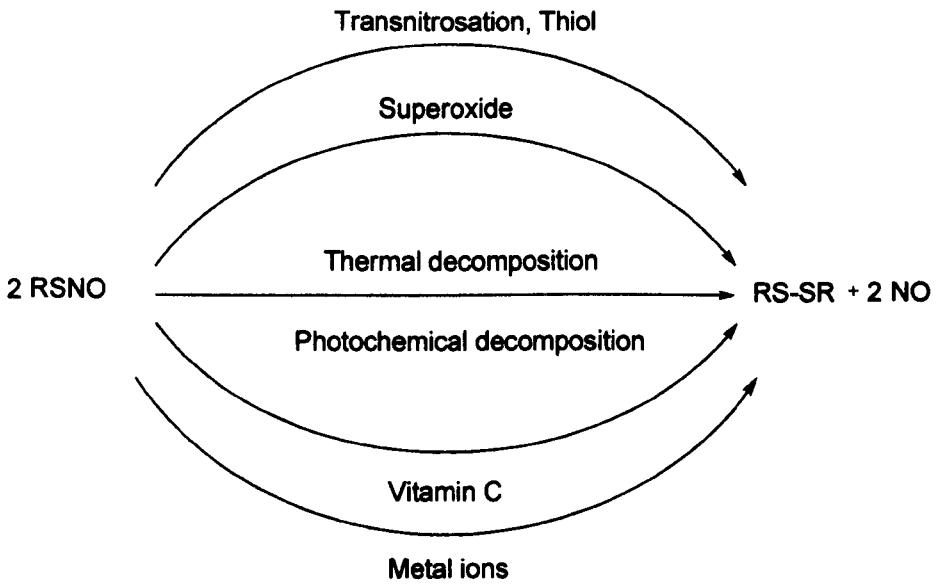
It has also been shown that ascorbate (vitamin C) can react with RSNOs in two different ways.[16] The reduction of Cu^{2+} to Cu^{+} by ascorbate at low concentrations, can initiate the copper catalysed reaction of RSNOs as described previously. In addition, at higher concentrations, ascorbate can act as a nucleophile producing the thiolate and NO from an electrophilic nitrosation reaction with RSNO (Equation 13).[13]

$$2RSNO + \text{Ascorbate} \longrightarrow 2RS^{-} + 2NO + \text{dehydroascorbic acid} \qquad \text{Equation 13}$$

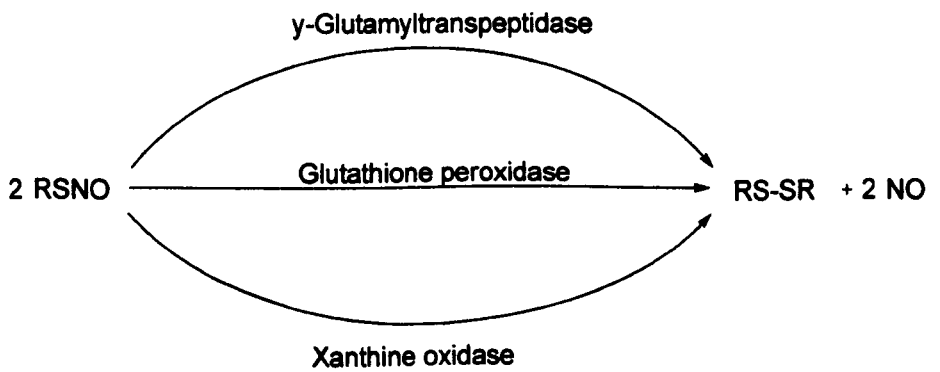
Other *in vivo* routes leading to the release of NO include enzymatic degradation by superoxide dismutase,[48] γ-glutamyltranspeptidase,[23] glutathione peroxidase[49] and xanthine oxidase.[23] An overview of the possible ways RSNOs can degrade, thus releasing NO, is outlined in Scheme 5.

Scheme 5:

Nonenzymatic



Enzymatic

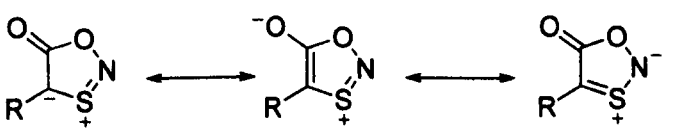


Possible routes for the decomposition of S-nitrosothiols (RSNOs) in vivo (adapted)[23]

1.2.2 Aryl-1,3,2-oxathiazolylium-5-olates (OZOs)

A recent attempt[50] to improve the stability of *S*-nitrosothiols involved investigations into the oxathiazolylium-5-olates, **39**. These mesoionic heterocycles have had very few mentions in the literature since their synthesis was first reported by Bacchetti and Alemagna in 1961.[51] In the early 1970's Gotthardt described a different synthetic route to these compounds.[52,53] One application for these five-membered heterocycles as NO-donor compounds was suggested in 1992 when Glaxo Inc patented the OZOs for use as cardiovascular agents.[54,55] In 2007 Wang published a new synthetic route to these compounds.[50] Prior to Glaxo's patent and Wang's publication the main focus for the synthesis of the OZOs, oxathiazolylium-5-olates, was to employ the mesoionic ring structure to synthesise other heterocycles. The OZOs are a particularly interesting group of NO donors as they lock the SNO moiety into a ring system and have been shown to have anticancer activity.[56]

Figure 14:



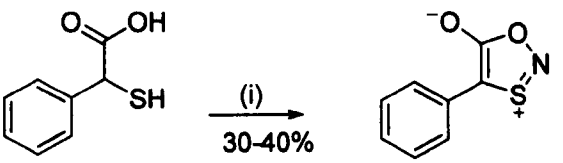
39

Mesoionic oxathiazolylium-5-olates **39** *and their resonance structures*

1.2.2.1 Synthesis of Aryl-1,3,2-oxathiazolylium-5-olates (OZOs)

The first OZO synthesis was reported by Bacchetti and Alemagna and involved the treatment of α-aryl-α-mercaptoacetic acid **40a** (Scheme 6) with sodium nitrite, acetic anhydride and sulphuric acid to give the desired product as a yellow crystalline material **39a** in a 30-40% yield.[51]

Scheme 6:



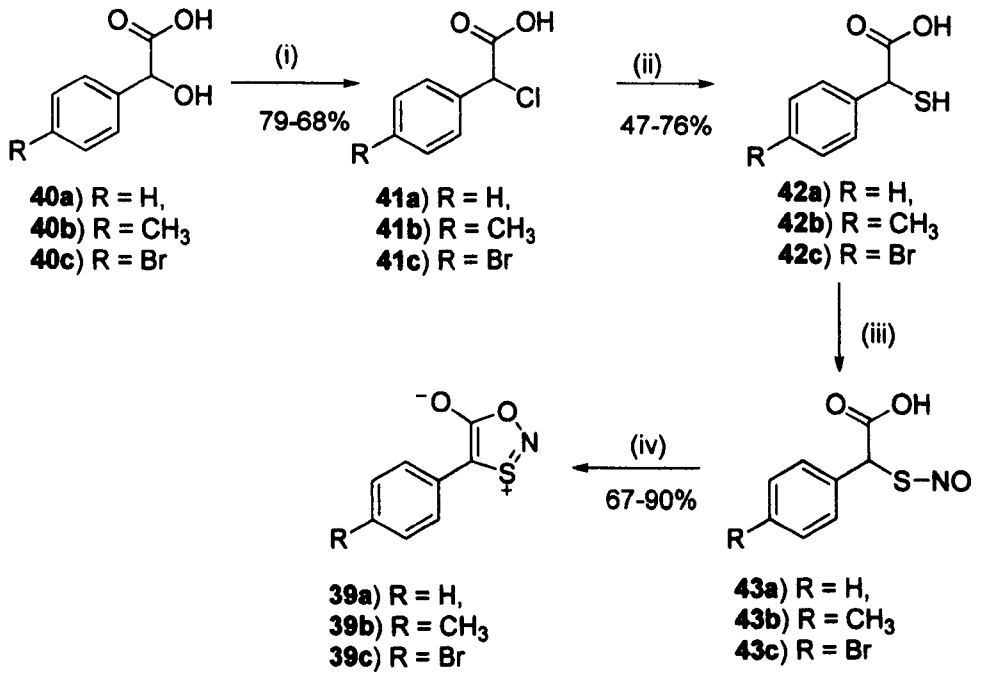
40a **39a**

Reagents and conditions: (i) $NaNO_2$, Ac_2O, H_2SO_4, -5-0°C

Bacchetti and Alemagna's synthesis of OZOs[51]

A decade later, in the early 1970s, Gotthardt reported the synthesis of three OZO compounds (scheme 7) from the α-aryl-α-mercaptoacetic acids **40a-c** using sulphuric acid and ethylnitrite to give the red nitroso compounds **43a-c**, followed by a ring closure reaction involving *N'N*-dicyclohexylcarboiimide (DCC) to produce the yellow needles of the OZOs **39a-c** in yields of 67-90% after recrystallisation.

Scheme 7:

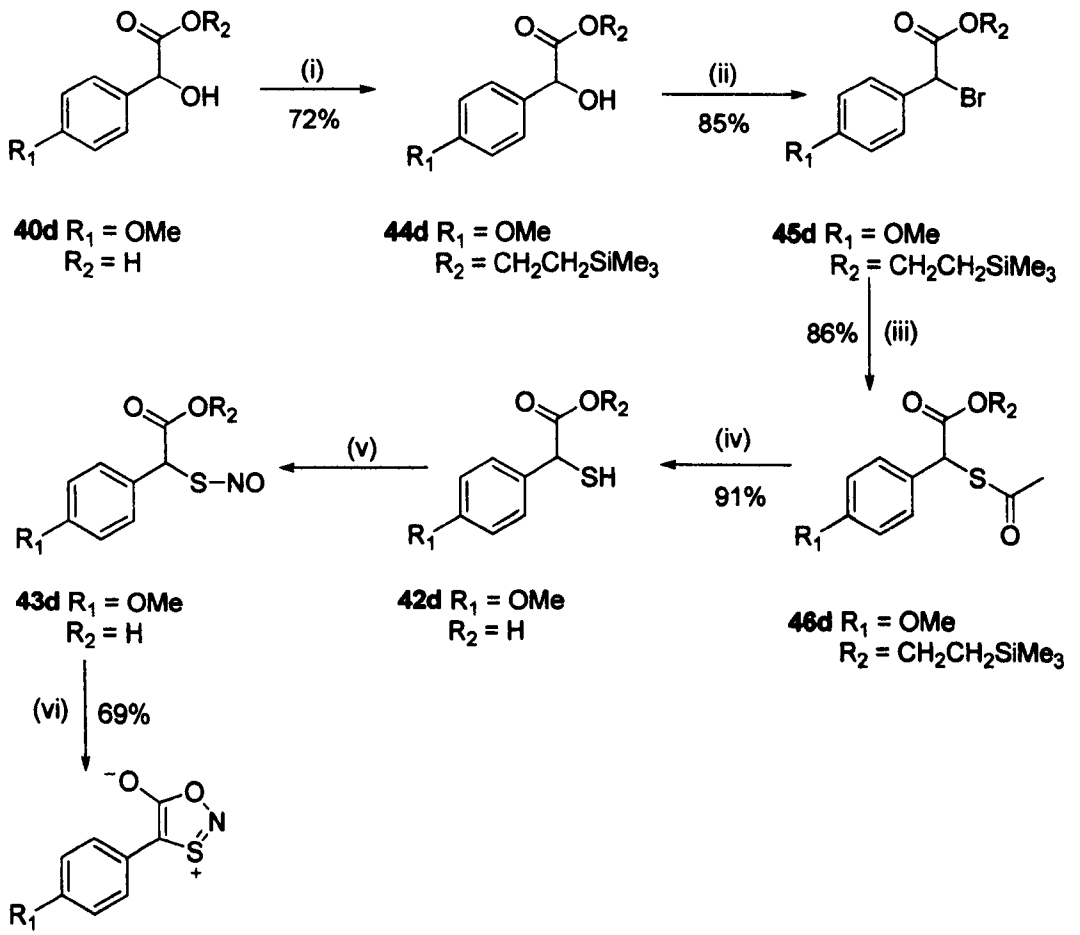


Reagents and conditions: (i) 1.3-2.5 eq. $SOCl_2$, pyridine (ii) K_2S, EtOH (iii) 1.3eq. EtONO, H_2SO_4, 0°C (iv) 2.2eq. DCC, benzene, 0°C

Gotthardt's synthesis of OZOs[53]

Some twenty years later Shaffer and Thompson proposed a different route to the OZO compound **39d**, including the synthesis of the mercaptoacetic acid **42d** from the mandelic acid **40d** (Scheme 8).[54,55] From the substituted acetic ester **44d**, the halide acetic ester **45d** was generated by using PBr_3 in refluxing dichloromethane, followed by *S*-acetylation using KSAc in ethanol to give the *S*-acetyl-acetic ester **46d**. Following treatment with NaOH in refluxing ethanol the mercaptoacetic acid **42d** was generated and nitrosated with *tert*-butyl nitrite. Dehydration using EDC gave the desired OZO compound **39d** as an orange solid.[55]

Scheme 8:



40d R_1 = OMe, R_2 = H

44d R_1 = OMe, R_2 = $CH_2CH_2SiMe_3$

45d R_1 = OMe, R_2 = $CH_2CH_2SiMe_3$

46d R_1 = OMe, R_2 = $CH_2CH_2SiMe_3$

42d R_1 = OMe, R_2 = H

43d R_1 = OMe, R_2 = H

39d R_1 = OMe

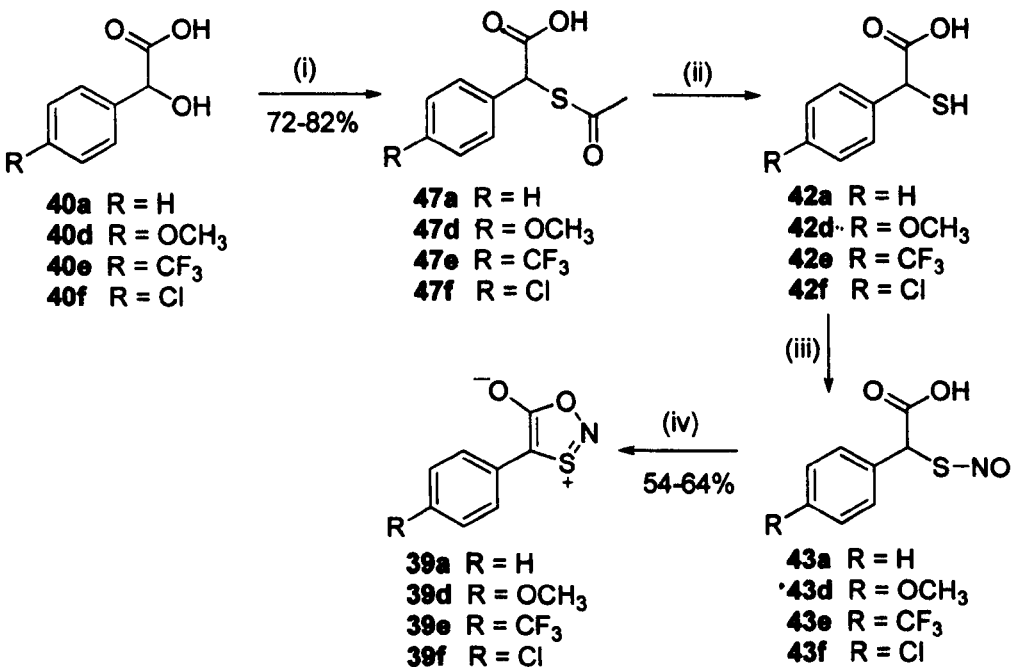
Reagents and conditions: (i) 2-Trimethylsilylethyl-*N*,*N*-diisopropyl-pseudourea, dioxane, 90°C, 8 hrs (ii) PBr_3, $CHCl_3$, r.t., 45 min, reflux, 45 min (iii) KSAc, EtOH, r.t, 4.5 hrs (iv) EtOH, NaOH, r.t., 1.5 hrs, 55°C, 5.5 hrs (v) *t*-BuONO, DCM, 20 min, 0°C (vi) EDC, DCM, 0°C 1 hr, r.t., 2 hrs.

Shaffer and Thompson's synthesis of OZOs

The most recently reported synthesis of these mesoionic compounds was published in 2007 by Wang *et. al.*[50] who employed a Mitsunobu-type reaction to synthesise the *S*-acetylated phenylacetic acid **47** from the mandelic acid derivatives **40** using triphenyl phosphine, diisopropyl azodicarboxylate (DIAD) and thiolacetic acid (Scheme 9). Cleavage of the acetate group was achieved using sodium methoxide and the resulting mercaptoacetic acid **42** was then nitrosated and cyclised using isobutyl nitrite and *N*'*N*-

dicyclohexylcarboiimide. Wang reported that this synthesis improved the yield of the mercaptoacetic acid derivatives **42** compared to previous procedures by Gotthardt, and made purification of the compounds easier.[50]

Scheme 9:



Reagents and conditions: (i) 2 eq. HSAc, 2 eq. PPh_3, 2 eq. DIAD, THF, 0°C, 4 hrs (ii) NaOMe, MeOH, r.t., 5 hrs (iii) *i*BuONO, DCM, 0°C, 2 hrs (iv) DCC, DCM 0°C, 2 hrs.

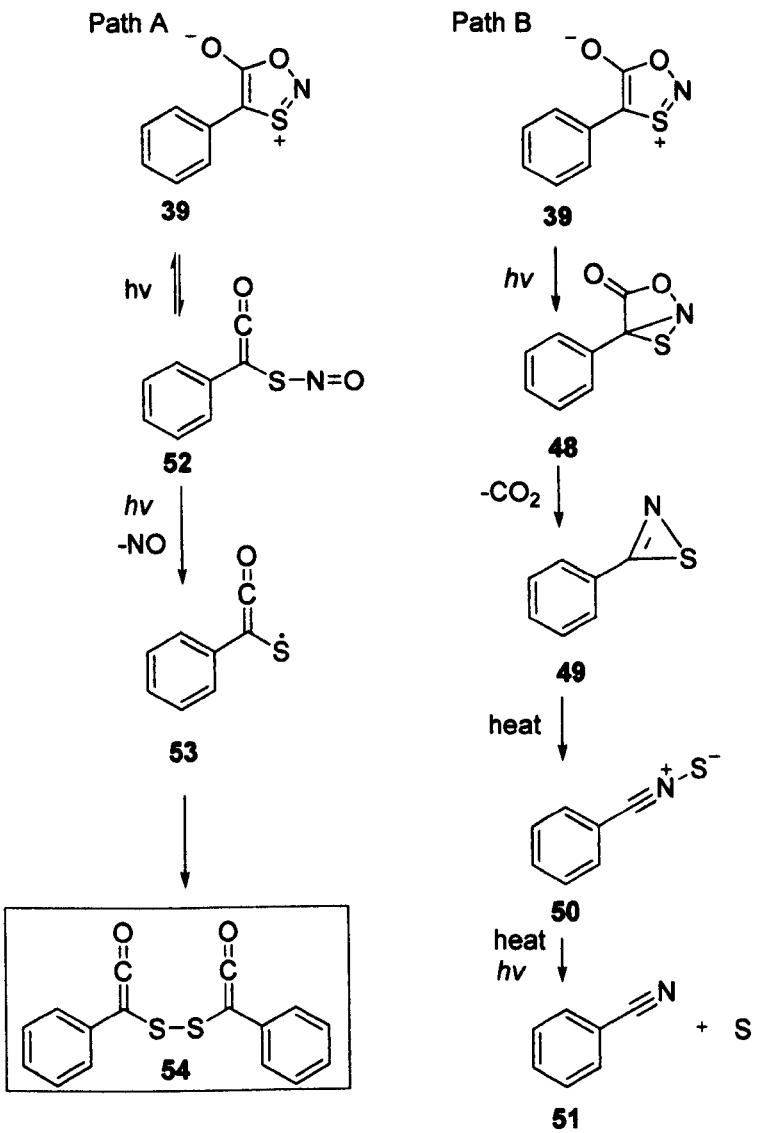
Wang's synthesis of the OZOs.[50]

Wang's synthesis of the OZOs provides the best overall yields with a range of 39-50% for the four derivatives **39a** and **39d-f** compared to Shaffer and Thompson with 44% yield for compound **39d**. Gotthardt's route, despite having fewer steps compared to Shaffer and Thompson has an overall yield of 24-25%. These four published routes to the OZO compounds employ different intermediates and chemistry despite all using the mandelic acid starting material with the most promising being Wang's route with fewer steps and good overall yields.

1.2.2.2 Stability and decomposition of 4-aryl-1,3,2-oxathiazolylium-5-olates (OZOs)

The stability and decomposition of 4-aryl-1,3,2-oxathiazolium-5-olates (OZOs) has attracted much interest, particularly in relation to their photochemical degradation. The initial work in this area was carried out by Gotthardt (Scheme 10).[52,53] Irradiation of the OZOs with UV light, 404.5-407.8 nm, resulted in the release of CO_2, and the production of a nitrile sulphide intermediate **50** which decomposes to give elemental sulphur and benzonitrile **51** (Scheme 10, path B)

Scheme 10:



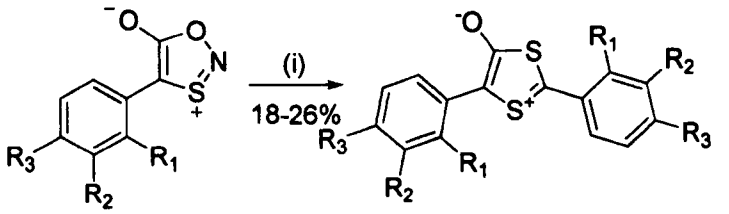
Photolysis of OZOs (adapted)[53,57,58]

The production of the benzonitrile sulphide **50** has been confirmed by trapping experiments[52,53] and UV spectroscopy,[59,60] thus supporting the photolytic decomposition of OZOs through path B. Although there is no evidence supporting the formation of structure **48** and **49**, these hypothetical intermediates are usually accepted in the photochemistry of mesoionic compounds.[57]

Path A occurs through a ring opening of the OZO to give a nitrosothioketene **52.** Holm and co-workers found that the fate of the nitrosothioketene **52** depends on the experimental parameters.[60] When the photolysis was carried out under an atmosphere of ^{15}NO, the equilibrium between the OZO and the nitrosothioketene **52** shifted towards the OZO so the product of the reaction was the benzonitrile **51** resulting from the competing elimination reaction from path B. Photolysis under an argon environment produced only half the amount of the benzonitrile **51**, suggesting that the remaining starting material decomposed through a different route. The formation of a disulphide species **54** from the thiyl radical produced by homolytic cleavage of the S-NO bond is likely to support path A and is similar to analogous research on *S*-nitrosothiols.[16,24,33,50]

Tono and co-workers[61] refluxed three OZO derivatives **39a, 39c** and **39g** under an atmosphere of argon to give a different mesoionic compound believed to be 2,5-diphenyl-1,3-dithiolylium-4-olate **55** (Scheme 11).

Scheme 11:



39a $R_1 = R_2 = R_3 = H$
39c $R_1 = R_2 = H$, $R_3 = Br$
39g $R_1 = R_2 = R_3 = OMe$

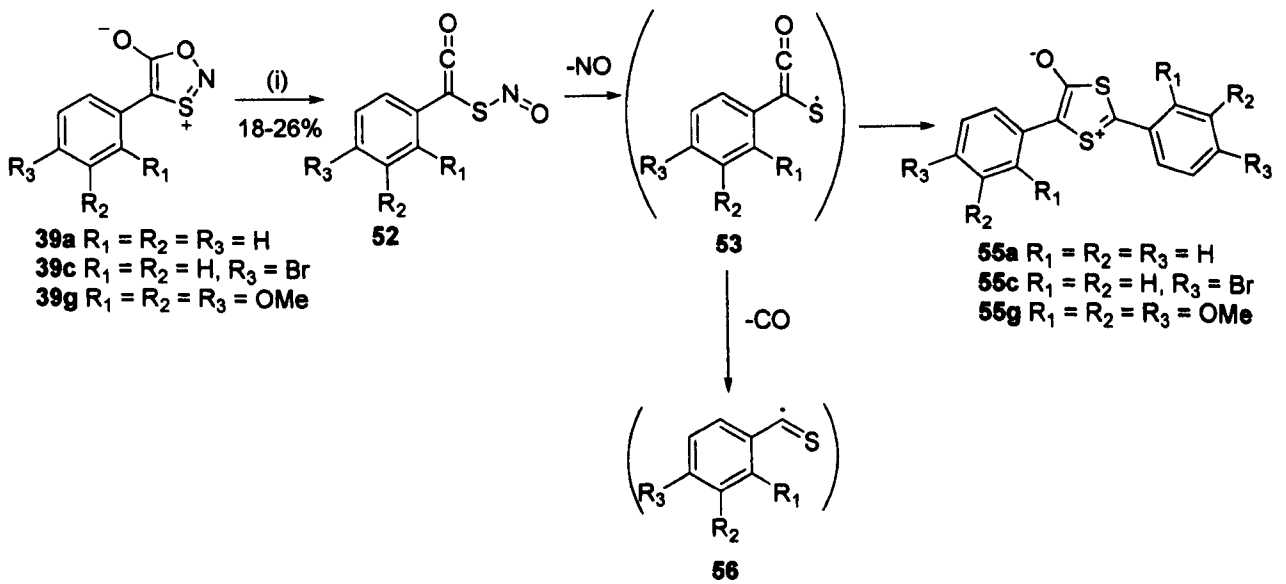
55a $R_1 = R_2 = R_3 = H$
55c $R_1 = R_2 = H$, $R_3 = Br$
55g $R_1 = R_2 = R_3 = OMe$

Reagents and conditions: (i) Xylene, under Ar, reflux

The thermolysis of OZOs[61]

Compared to path B (Scheme 10) it was found that only a small amount of benzonitrile **51** was formed when the OZO compounds were refluxed under argon, suggesting a different mechanism of decomposition. Experimental evidence demonstrates that this reaction occurs through a radical mechanism since in the presence of a radical scavenger, compound **55** was not formed (Scheme 12).[61]

Scheme 12:



39a $R_1 = R_2 = R_3 = H$
39c $R_1 = R_2 = H$, $R_3 = Br$
39g $R_1 = R_2 = R_3 = OMe$

52

53

55a $R_1 = R_2 = R_3 = H$
55c $R_1 = R_2 = H$, $R_3 = Br$
55g $R_1 = R_2 = R_3 = OMe$

56

Reagents and conditions: (i) Xylene, under Ar, reflux

A radical mechanism for the thermolysis of OZOs (adapted)[61]

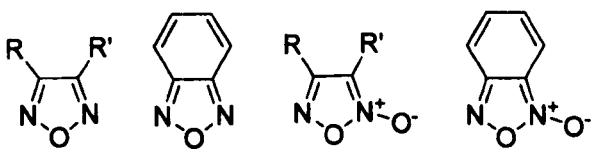
Wang and co-workers studied the decomposition of OZO compound **39a** and found that in aqueous solution minimal decomposition occurred, only 7%, when exposing the solution to stray light. Similarly, in acetonitrile only minimal decomposition of **39a** was reported when heated at 37°C over a period of 2 hours. It was identified that the decomposition of **39a** was pH dependent and the rate of decomposition increased with decreasing pH. In addition, decomposition studies on all four OZOs, **39a**, **39d-f**, at pH 5 revealed a substituent effect where the electron-donating group (OMe) in compound **39d** was more stable with a half-life of 130 minutes compared to the derivative with an electron-withdrawing group (CF_3) **39e** which had a half life of 1 minute.

From the available data the OZO compounds appear more stable than the *S*-nitrosothiols as high temperatures or irradiation with UV light is required for the OZO compounds to decompose. In addition, all reported OZO compounds appear as solid, crystalline materials unlike the RSNOs which can be difficult to obtain in solid form, as described previously (Section 1.2.1).

1.2.3 1,2,5-Oxadiazole-2-oxides (Furoxans)

Interest in furoxans has greatly increased since the discovery, in the early 1990s, that their biological activity was related to their ability to release NO.[62] The furoxans **23** are formally called the 1,2,5-oxadiazole-2-oxides, which also includes their benzo derivatives, the benzofuroxans **16**. The furoxans are the *N*-oxides of the 1,2,5-oxadiazoles, which are commonly called the furazans. The furazans, **57**, and their benzo derivatives, benzofurazan **58** have been found to have biological activity although this is unrelated to the release of NO.[63]

Figure 15:

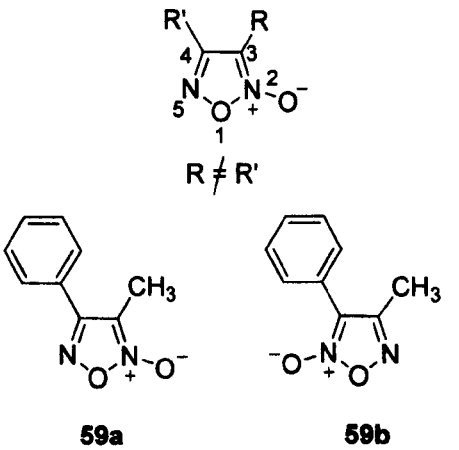


57 **58** **23** **16**

Generic structures of the furoxans and furazans

Synthesis of the monocyclic furoxans **23** can generate two isomers provided that the substituents (R and R') are different, due to the ability of the furoxans to isomerise when heated or exposed to light (section 1.2.3.2). The naming of these isomers is based on the position of the substituent R-groups relative to the position of the exocyclic oxygen. The unsymmetrical furoxan **59a** in figure 16 is 3-methyl-4-phenyl furoxan, and the other isomer, furoxan **59b**, is 4-methyl-3-phenyl furoxan.

Figure 16:



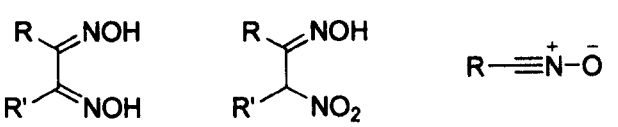
59a **59b**

The two isomers of methylphenylfuroxan, 3-methyl-4-phenyl furoxan **59a**, *and 3-phenyl-4-methyl furoxan* **59b**

1.2.3.1 The synthesis of furoxans

Construction of the 1,2,5-oxadiazole-2-oxide ring can be achieved through a number of synthetic routes. The three main routes are oxidative cyclisation of α-dioximes **60**, dehydration of α-nitroketoximes **61** and dimerisation of nitrile oxides **62** (figure 17).

Figure 17:



60 **61** **62**

Generic structures of α-dioximes **60**, *α-nitroketoximes* **61**, *and nitrile oxides* **62**

Oxidation of α-dioximes **60** can be carried out using a wide range of reagents, some of which are outlined in Table 2 with representative yields where reported.

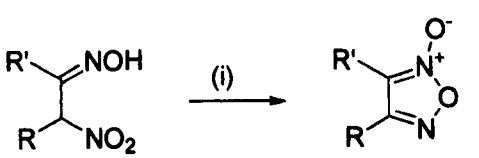
Table 2:

Reagent	Yield (%)	Reference
copper	83-96	64
potassium ferracyanide	50	65
halogens or alkali hypohalites	67(Br), 90-95 (Cl), 80 (sodium hypochlorite), 27-50 (sodium hypochlorite)	66, 67, 68 68
nitric acid	48.5, 82	69, 70
nitrogen oxides	40-42, 25-81	71, 70
ceric ion	34	67
potassium hexacyanoferrate(III)	96	72
lead tetraacetate	23-25, 62	73
MnO_2	N/A	74
Electrochemical oxidation.	52-89	75

Overview of oxidising agents employed in the synthesis of furoxans from their parent α-dioximes **60**

Dehydration of α-nitroketoximes **61** can be achieved by reaction with acidic alumina and several furoxans have been synthesised with yields ranging from 75-93% using this procedure (Scheme 13).[76]

Scheme 13:



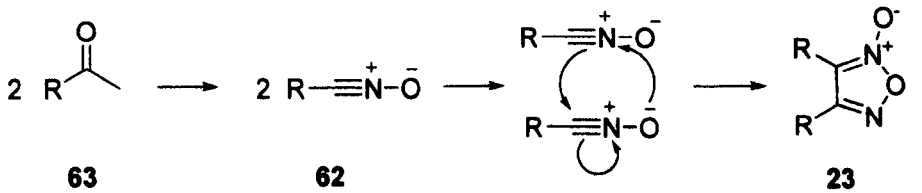
61 **23**

Reagents and conditions: (i) Acidic Al_2O_3, CH_3CN, 1-5 hrs, 60°C

Synthesis of furoxans **23** *from α-nitroketoximes* **61** *using acidic alumina*

The third most commonly employed synthetic route to the furoxans is through dimerisation of nitrile oxides **62** which can be produced from the corresponding methyl ketones **63**. The 1,2,5-oxadiazole-2-oxide ring system is constructed *via* a 1,3 dipolar cycloaddition reaction from the nitrile oxides which can easily dimerise (Scheme 14).

Scheme 14:

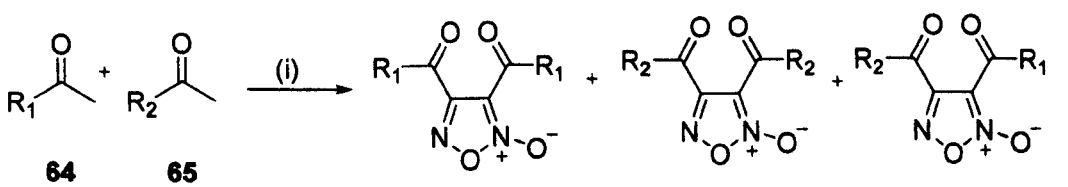


63 **62** **23**

Synthesis of furoxan **23** *from the methyl ketone* **63** *via the nitrile oxide* **62**

Although this latter approach can be used to synthesise unsymmetrical furoxans, the yields are generally low due to a mixture of products. In general, great care must be taken when selecting the synthetic approach for the production of unsymmetrical furoxans to avoid a mixture of products. For example, the synthesis of an unsymmetrical furoxan from two different methyl ketones could in theory give three different products as shown in scheme 15.

Scheme 15:



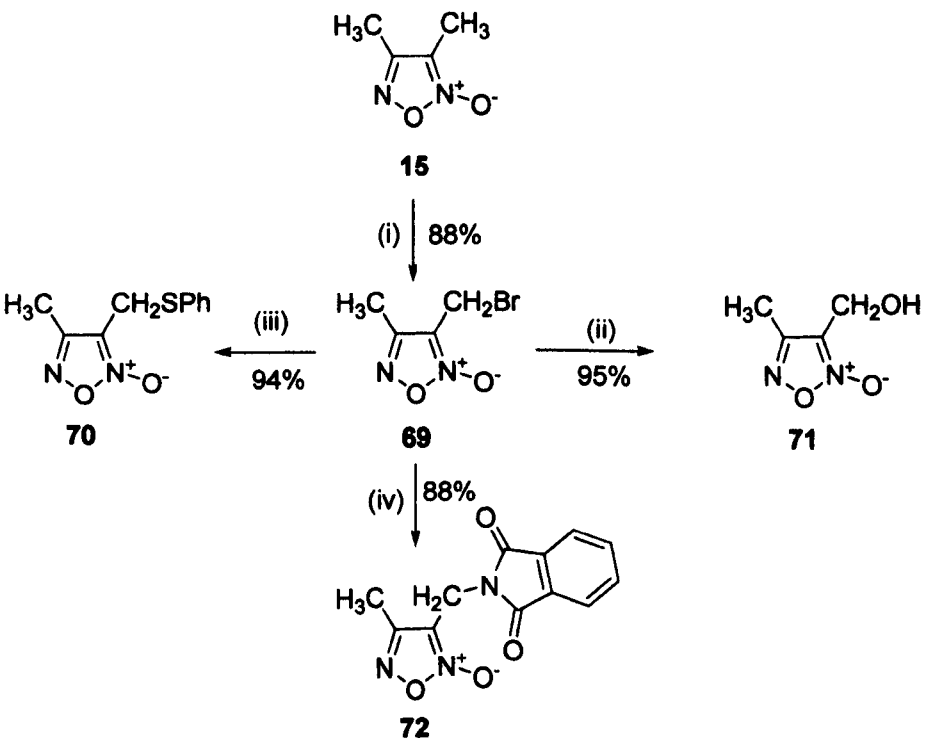
64 **65** **66** **67** **68**

Reagents and conditions: (i) N_2O_3

Synthesis of furoxan **66-68** *from the methyl ketone* **64** *and* **65**

Such problems can be avoided as reported by Gasco[77] who synthesised the symmetrical dimethyl furoxan derivative **15** (Scheme 16), which was then successfully brominated in the 3-position using radical chemistry to give 3-bromomethylfuroxan **69**. Further reactions with compound **69** gave a range of different unsymmetrical furoxans **70-72**. Similarly, Gasco has also employed benzensulphone-substituted furoxans as a means of overcoming the problem of generating a mixture of isomers.[78]

Scheme 16:



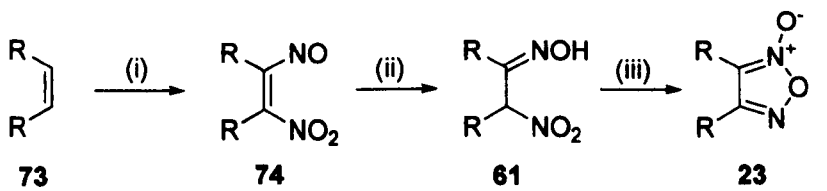
Reagents and conditions: (i) 1.1eq. NBS, CCl_4, cat. benzoylperoxide, 24hrs, 60°C, (ii) Calcium carbonate, dioxane/H_2O, heat (iii) PhSH, EtOH, -10°C, (iv) Potassium phthalamide, benzyltriethylammonium chloride, $CHCl_3$

Gasco's synthesis of unsymmetrical furoxans **69-72**[77]

Another synthetic approach that generates the furoxans uses the Wieland synthesis where alkenes are reacted with dinitrogen trioxide (Scheme 17). Such reactions generate α-

nitroketoximes **61** *in situ* which undergo a dehydration and cyclisation reaction to give the furoxans.[79]

Scheme 17:



73 **74** **61** **23**

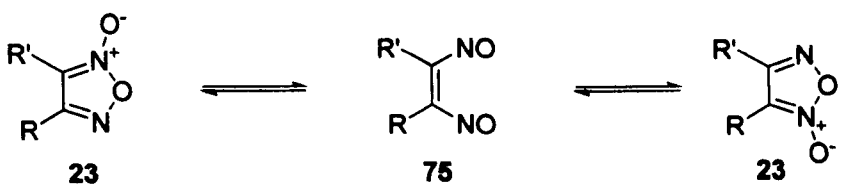
Reagents and conditions: (i) N_2O_3 (ii) Heat (iii) $-H_2O$

Wieland synthesis of furoxans **23** *from the alkenes* **73**

1.2.3.2 The stability and decomposition of furoxans

One of the widely reported features of furoxans is their ability to undergo photochemical and thermal isomerisation and it is generally believed that the isomerisation of the furoxans occurs through a ring opening-closing mechanism with a 1,2-dinitrosoalkene **75** as an intermediate (Scheme 18).[80,63]

Scheme 18:



23 **75** **23**

$R' \neq R$

Isomerisation of furoxans **23** *via the 1,2-dinitrosoalkene* **75**

Ab inito MO calculations support the mechanism in scheme 18 and recently Himmel and co-workers found evidence for the formation of the 1,2-dinitrosoalkene **75** as a photochemical decomposition product of dimethylfuroxan **15** (Scheme 19).[81,82]

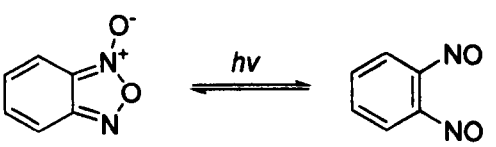
Scheme 19:

15 **76**

Photochemical decomposition of dimethylfuroxan **15**

Similarly, 1,2-dinitrosobenzene **16** (Scheme 20) was characterised by UV and IR spectroscopy for the closely related benzofuroxans[83,84] from matrix isolation experiments and theoretical calculations.[82,85,86]

Scheme 20:

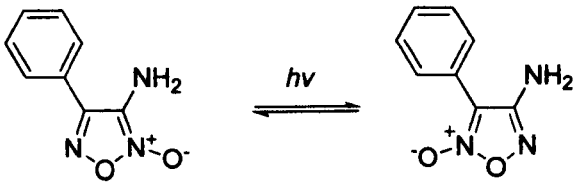


16 **77**

Photolysis of benzofuroxan **16** *at 14 K*

Several examples of photochemical isomerisation have been published. One example is aminophenylfuroxan, **78**, which isomerises completely to the 4-amino isomer **78b** when irradiated at 300 nm and above, whereas irradiation at 254 nm gave a 1:1 ratio of the isomers (Scheme 21).[87]

Scheme 21:

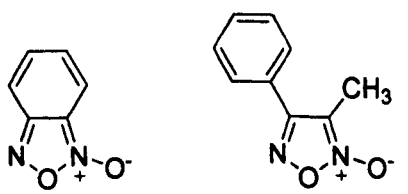


78a **78b**

The photochemical isomerisation of aminophenylfuroxan **78**

The rate at which the isomerisation occurs has been investigated for a number of furoxans and several reviews have covered the topic.[63,87,88,80] Early reports found the rate to be substituent dependent[89,90] and monocyclic furoxans were very slow to isomerise whereas electron donating substituents[67,90] and fusion to aromatic rings[91] (such as in **16**) resulted in an increased rate. The isomerisation in benzofuroxans **16** occurs at room temperature, whereas 3-methyl-4-phenylfuroxan **59** requires temperatures of approximately 100°C (figure 18).[63,80] The position of the equilibrium tends to favour the 4-isomer in the monocyclic series when the substituents are electron donating however, the reverse is not seen for electron withdrawing substituents.[67,90]

Figure 18:

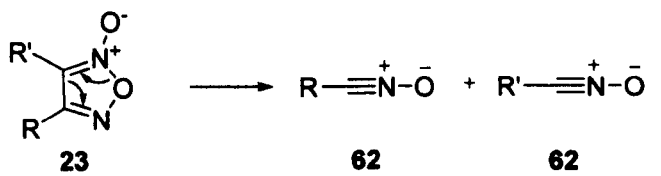


16 **59**

Benzofuroxan **16** *and 3-methyl-4-phenylfuroxan* **59**

In a similar fashion to the OZOs, the furoxans undergo thermal and photochemical ring cleavage. The thermal ring cleavage is the reverse of one of the nitrile oxide dimerisation and can be seen as a retro 1,3-dipolar cycloaddition reaction (Scheme 22).

Scheme 22:

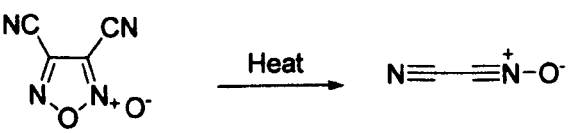


23 **62** **62**

Thermal decomposition of furoxan **23** *via a retro 1,3-dipolar cycloaddition reaction to give two nitrile oxide fragments* **62**

Generally, thermal ring cleavage requires high temperatures to form two nitrile oxide **62** fragments although with bulky substituents or compounds with ring strain the reaction occurs more readily at lower temperatures.[92] The formation of the nitrile oxide **62** fragment is supported by flash vacuum pyrolysis (FVT) which is a particularly important technique due to the formation of by-products in liquid mediums.[93,94] The FVT of dicyanofuroxan **79** resulted in the formation of cyanogen *N*-oxide **80** (Sceheme 23).[95]

Scheme 23:

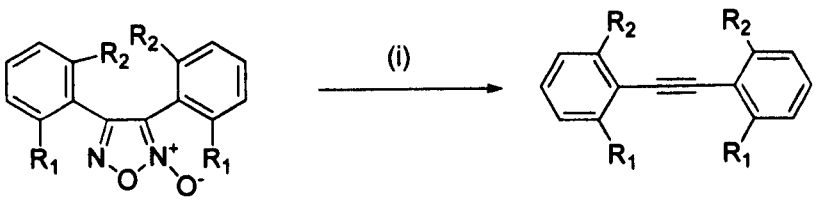


79 **80**

Flash vacuum thermolysis of dicyanofuroxan **79**

The photochemical decomposition of furoxans has also been investigated. Diphenylfuroxans **81a-c** decompose to give the diphenylacetylenes **82a-c** when irradiated with 254 nm light (Scheme 24).[96]

Scheme 24:



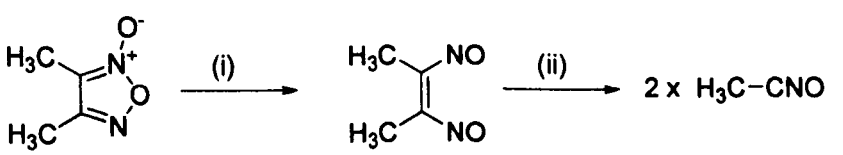
81a, $R_1 = R_2 = H$ **82a**, $R_1 = R_2 = H$
81b, $R_1 = H$, $R_2 = Cl$ **82b**, $R_1 = H$, $R_2 = Cl$
81c, $R_1 = R_2 = Cl$ **82c**, $R_1 = R_2 = Cl$

Reagents and conditions: (i) Hexane, 254nm, 1hr

Photochemical decomposition of diphenylfuroxans **81**

Prolonged exposure at 254 nm or 700 nm causes dimethylfuroxan **15** to form photostable acetonitrile oxide **83** (Scheme 25), which was isolated by argon matrix at 12 K.[81]

Scheme 25:



15 **76** **83**

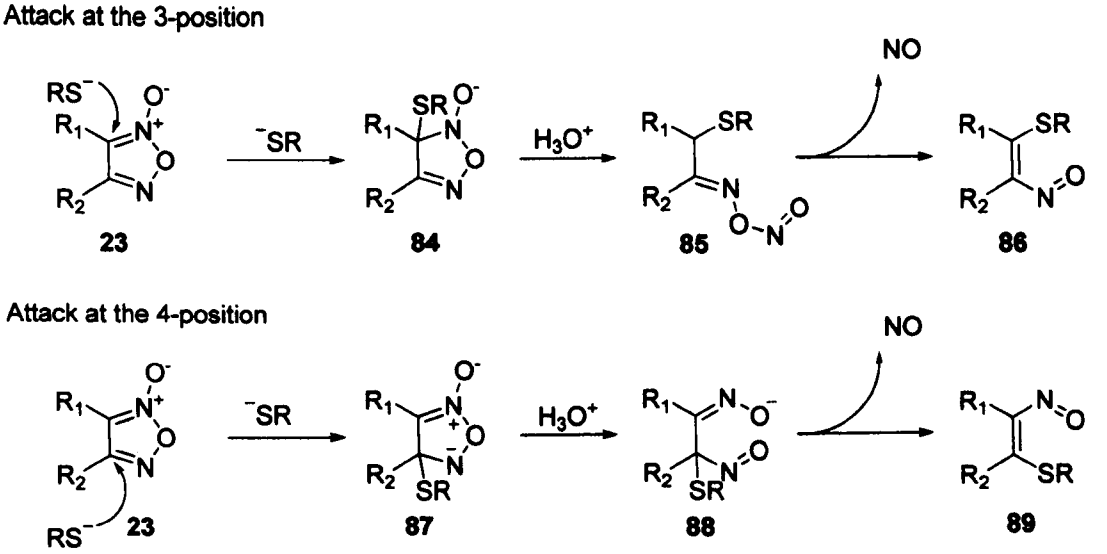
Reagents and conditions: (i) Argon matrix, 254 nm (ii) Ar matrix, 700 nm, 20 min – 2 hrs

Photochemical decomposition of dimethylfuroxan **15**

Compared to the RSNOs, furoxans are considered thermally stable compounds as high temperatures are usually required for their decomposition. In addition, furoxans require a thiol cofactor to initiate the release of NO. Scheme 26 shows the mechanism of

decomposition as proposed by Gasco and co-workers where attack by thiolate in the 3 or 4 position of the furoxan ring results in ring opening of the furoxan ring and the release of NO.[97]

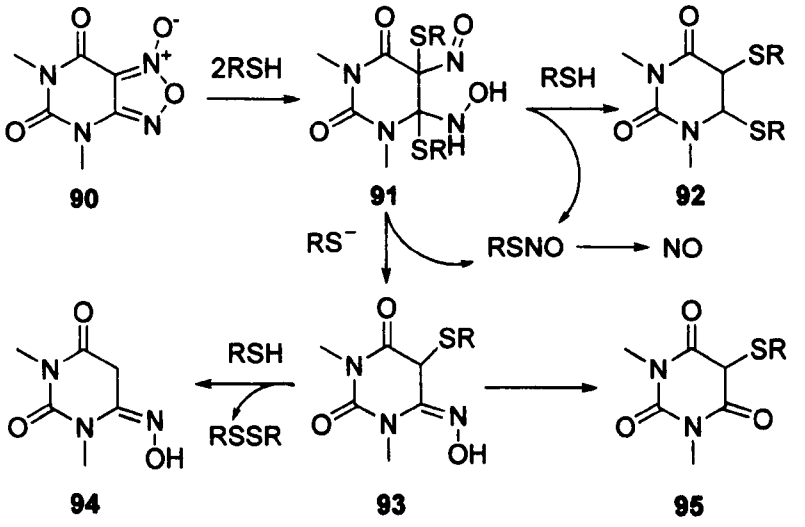
Scheme 26:



Decomposition of furoxans in the presence of thiols at physiological pH (Adapted)[97]

In an oxygen rich environment formation of N_2O_3 and NO_2 from NO is plausible and further hydrolysis forms the nitrosating anions NO_2^- and NO_3^-. The ring opened nitroso compounds **85** and **88** may form *S*-nitrosothiols by reaction with the thiol cofactor in addition to nitrosation by the anions NO_2^- and NO_3^-. Sako *et. al.*[98,69] further investigated thiol mediated NO release from furoxans using 4H-[1,2,5]oxadiazolo[3,4-d]pyrimidine-5,7-dione 1-oxides **90**. The group's findings supported the mechanism proposed by Gasco, and by using ^{15}N labelled compounds the group was also able to determine that the nitrogen of the *N*-oxide was incorporated into the RSNO product (Scheme 27).

Scheme 27:



90 **91** **92** **93** **94** **95**

The thiol-mediated release of NO from furoxan (adapted)[69]

The furoxans can release NO when treated with thiols under physiological conditions and compared to the RSNOs (and OZOs), the furoxans can be viewed as thermally and photochemically stable NO-donors. Although there are examples of furoxans which decompose when exposed to heat and light, this usually requires high temperatures and there are only a few examples where furoxans photochemically decompose.

1.3 NO and cancer

The role of NO in inflammatory responses within the body is well established and therefore it is probably not so surprising that NO is also involved in the body's fight against cancer. NO in cancer biology has been researched greatly over the last few years.[99–105] Since there are many different types of cancer the role of NO in cancer

biology is complex. In an effort to review the area in a unified manner, this section will focus on both the pro- and anti-cancer effects of NO.

1.3.1 Endogenous NO and cancer

iNOS (NOSII) has been associated with a number of cancer types including breast,[106] malanoma,[107] pancreatic carcinomas,[108] prostate,[109] lung,[110] brain[111] and colorectal[112] tumours. In breast cancer tumours a correlation between the degree of malignancy and expression of iNOS was found.[106] In cases of malignant neoplasmas in the central nervous system the expression of iNOS in the tumour cells was linked to a higher tumour grade[111] and similarly, in patients with a melanoma it was found that a high expression of iNOS resulted in higher fatality.[107] On the other hand, low expression of iNOS was also found to result in metastasis for patients with a melanoma[113] whilst Blumberg and co-workers have reported that with a increased expression of iNOS the effectiveness of radiation treatment of colorectal cancer is increased.[114] These are only a few examples, of many, which emphasise the dichotomous role of iNOS and NO in cancer biology.

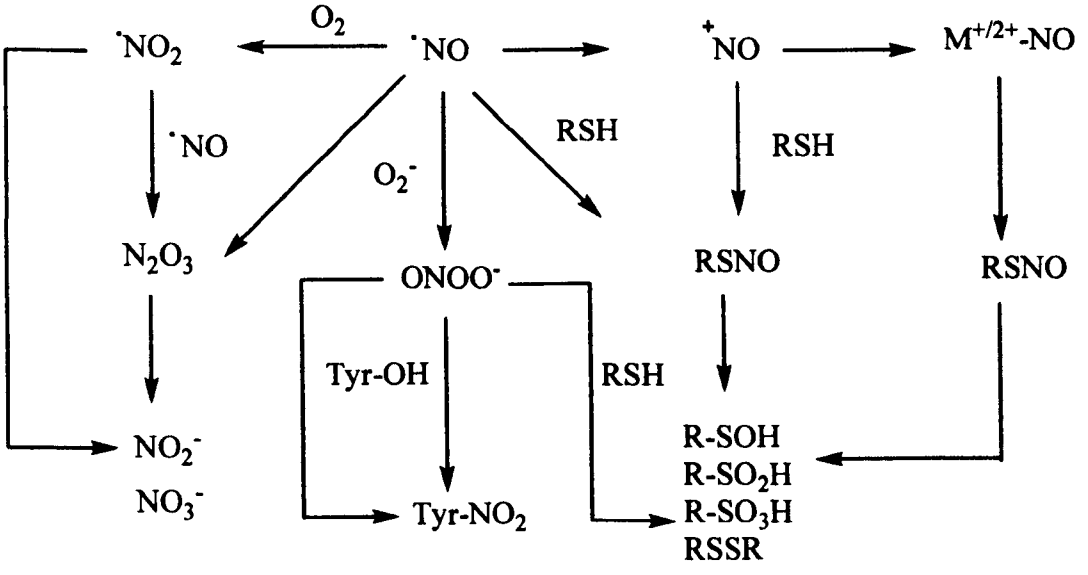
There is evidence of eNOS (NOSIII) being involved in a number of cancer related events such as angiogenesis, apoptosis, invasion and metastasis.[115] Studies on eNOS in breast cancer found this NOS isoform to be expressed in 56% of the *in situ* lesions and in 61% of the invasive lesions.[116] There is also a correlation between eNOS expression and vascular invasion in both throphoblastic disease[117] and gastric cancer.[118] In terms of cancer biology, there have not been many studies on the third isoform nNOS (NOSI).[119]

The concentration of NO produced in response to cancer cells, differs for each NOS isoform. Unlike nNOS and eNOS, iNOS can produce a toxic amount of NO which can

last for a prolonged period of time. eNOS and nNOS on the other hand generate low concentrations of NO which have a short period of action and act directly on the target.[120,121]

In order to understand how NO can be both pro cancer and anti-cancer, it is important to understand the possible biochemical reactions of NO *in vivo* (Scheme 28).

Scheme 28:



Reactions of NO in vivo (adapted)[122]

Whether the NO is endogenously produced or delivered via a NO-donor, free NO can undergo a number of reactions *in vivo*. In the presence of O_2, NO is oxidised to give $^{\cdot}NO_2$ which can form N_2O_3 and then give NO_2^{-} and NO_3^{-}. NO can also react with superoxide to produce peroxynitrite $ONOO^{-}$, which is capable of nitrating tyrosine residues.[121] NO can also nitrosate thiols to give *S*-nitrosothiols which are believed to be responsible of the transportation of NO in the body.[122] Nitrolysation of cysteine sulphenic acids, RSOH, sulphenic acids, RSO_2H, and the formation of disulphide bonds in addition to reactions

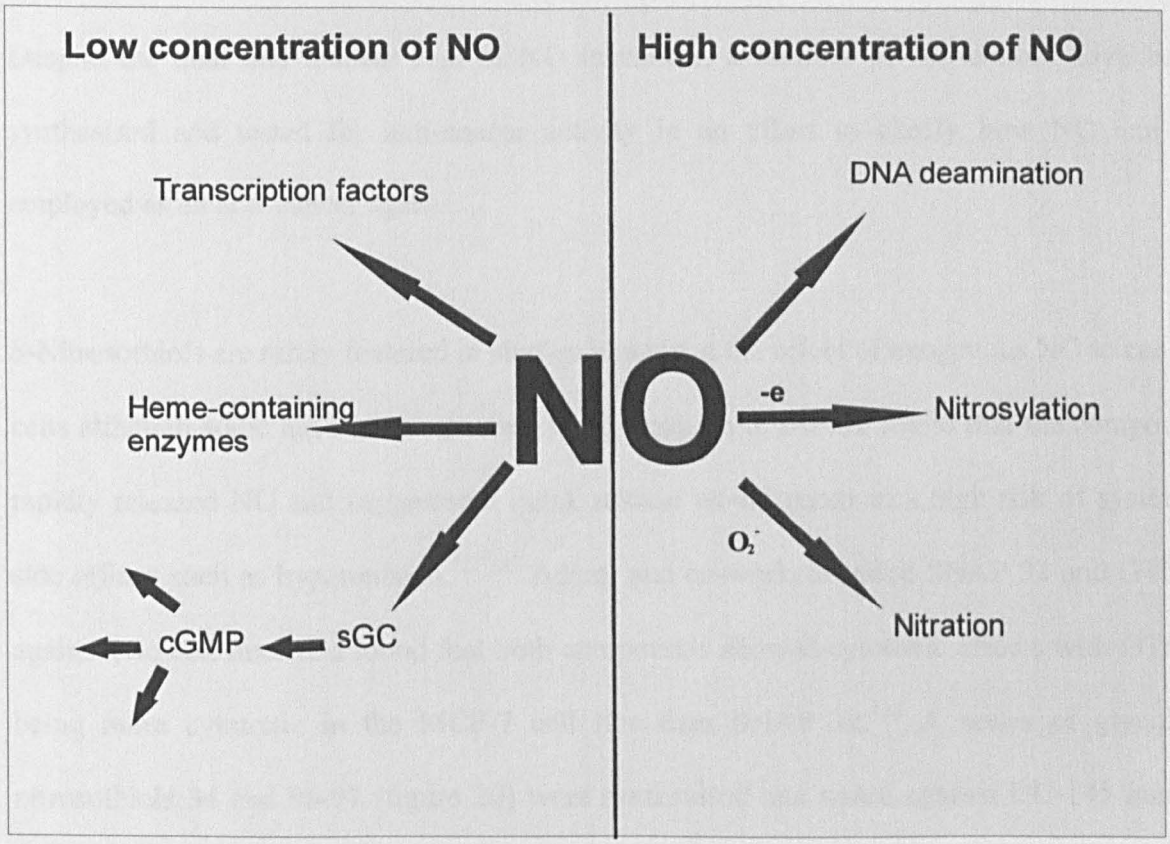
with metals, can result in altered protein structure and disruption to signalling cascades.[122]

It has been shown that peroxynitrite can increase cancer cell growth by nitration of key proteins[123] wilst reactive oxygen species such as peroxynitrite have been linked to a number of cancers including ovarian,[124] leukaemia,[125] melanoma[126] and cervical forms of the disease.[127]

NO has been found to increase activation of caspase-3 by nitrosylating the heme group on cytochrome c, resulting in apoptosis (programmed cell death).[128] The addition of GTN **2** to colon cancer cells resulted in activation of caspases 1 and 10 and apoptosis,[129] whereas nitrolysation of caspases 9 and 3 resulted in inhibiton.[130]

The concentration of NO produced or supplied determines the activation or deactivation of a number of enzymes and other cellular signalling agents.[121] Wink and co-workers found that supplying low concentrations of NO (10-50 nM) to breast cancer cells resulted in phosphorylation of signalling protein ERK (extracellular signal-regulated kinases) and intermediate NO levels (150-300 nM) caused the accumulation of HIF-1α (hypoxia-inducible factor alpha). High concentrations of NO (300-700 nM) resulted in increased expression of transcription factor p53.[131] Activation by phosphorylation of ERK results in promotion of cancer growth. HIF-1α is a transcription factor usually associated with a pro-tumour effect whereas increased expression of transcription factor p53 P-Ser-15, on the other hand, correlates to the induction of apoptosis.[121] The duration also had an impact with the phosphorylation of ERK being transient whereas a sustained supply of NO resulted in accumulation of HIF-1α and p53 P-Ser-15.[131]

Figure 19:



Concentration dependence of NO on cellular activation (adapted)[121]

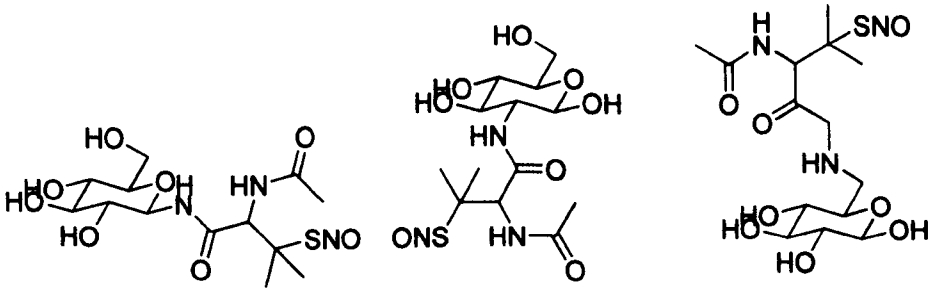
There are a number of possible ways for NO to act as an anti- or pro-cancer agent (Figure 19). The location of NO is another factor that determines its effects, if present in cancer cells at high concentrations it is plausible that DNA damage to the cancer cells is likely to induce cell death. On the other hand, DNA damage to healthy non-cancerous cells might result in cancer formation.

1.3.2 Exogenous NO and cancer

Despite the dual and unclear role of NO in cancer, a number of NO-donors have been synthesised and tested for anti-cancer activity in an effort to clarify how NO can be employed as an anti-cancer agent.

S-Nitrosothiols are rarely featured in studies looking at the effect of exogenous NO to cancer cells although some have been reported. Early work on SNAP **32** found that the compound rapidly released NO and suggested a quick release would result in a high risk of systemic side effects such as hypertension.[132,133] Adami and co-workers tested SNAP **32** and GTN **2** against two cell lines and found that both compounds showed cytotoxic effects with GTN **2** being more cytotoxic in the MCF-7 cell line than SNAP **32**.[132] A series of glycol-*S*-nitrosothiols **34** and **96-97** (figure 20) were synthesised and tested against DU-145 human prostate cancer cells and found to be more cytotoxic than SNAP **32** on its own.[133,134] The increased cytotoxicity of the sugar derivatives is attributed to the ability to permeate cell membranes.[134,135]

Figure 20:

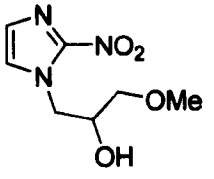


Glycol-S-nitrosothiols **34** *and* **96-97**

Wang and co-workers synthesised a series of four OZO compounds **39a** and **39d-f** (Scheme 9, section 1.2.2.1) which were tested against K562 leukaemia and Panc-1 breast cancer cell lines. Three of these four OZO derivatives showed cytotoxicity in the μM range.[56]

As early as 1968 the antileukemic properties of furoxans was established when a number of benzofurazans and benzofuroxans showed inhibitory activity against lymphocytes.[136–138] The furoxans are a very interesting group as the five-membered heterocyclic ring is structurally related to the nitroimidazoles (Figure 21), which are a group of hypoxia-selective cytotoxins.[139–141]

Figure 21:

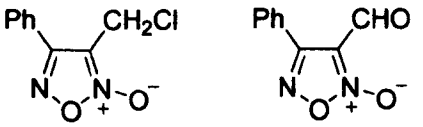


98

2-Nitroimidazole misonidazole **98** *- a hypoxia-selective cytotoxin*

On this basis a series of furoxans were synthesised and tested as selective hypoxia cytotoxins (Figure 22).[141] Boiani and co-workers found the furoxans to be non-selective although one compound was cytotoxic at μM concentrations against Chinese hamster lung fibroblasts. The corresponding furazans were also synthesised and biological testing of these resulted in decreased activity suggesting that the release of NO is key to their activity.[140,141]

Figure 22:

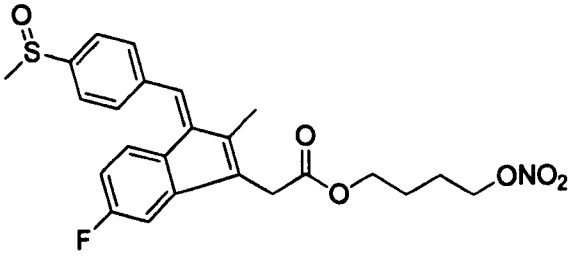


99 **100**

Furoxans **99** *and* **100** *tested as selective hypoxia cytotoxins*

In recent years the NO-donor hybrid drugs have gained popularity and a number of hybrids have been synthesised and tested for anti-cancer activity including NO-aspirin (NCX-4016) **28**, which has recently been investigated in a phase I trial as a chemopreventive agent in colorectal cancer.[28] Earlier work on this compound, in a rat model, showed that **28** reduced the incidence of colon cancer by 85% with normal aspirin only reducing it by 64%.[142] Other NO-NSAIDs such as NO-sulinadac **101** has also been tested against cancer cells and was found to be cytotoxic against HT29-D4 colon cancer cells (Figure 23).[143]

Figure 23:



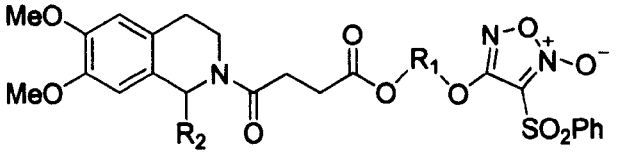
101

NO-sulinadac **101**

Recently Zou and co-workers synthesised a series of tetrahydroisoquinoline furoxan derivatives in an effort to combine the multidrug resistance reversal ability of the tetrahydroisoquinolines with the NO releasing ability of the furoxans[144] (Figure 24). Compounds **102-104** were found to be less cytotoxic than the reference chemotherapy agent

but showed potent multidrug resistance reversal activity in human erythroleukemia cell lines.[144]

Figure 24:

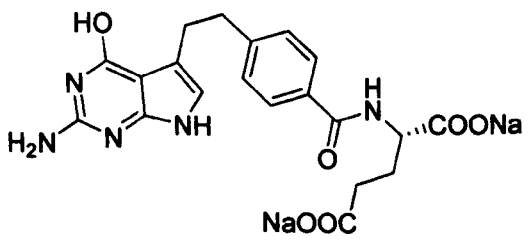


102 $R_1 = CH_2C{=}C{=}CH_2$, R_2 =a-Naphthylmethyl
103 $R_1 = CH_2CH_2$, R_2 = 3,4-Dimethoxybenzyl
104 $R_1 = CH_2(CH_2)_2CH_2$, R_2 = a-Naphthylmethyl

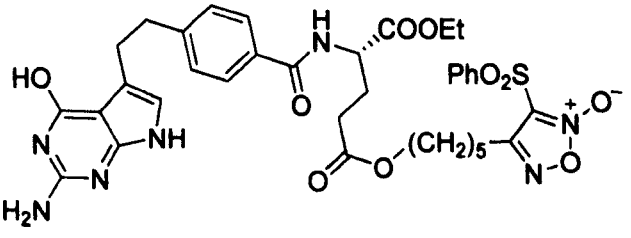
Tetrahydroisoquinoline furoxan derivatives **102-104**

In a similar way Min and co-workers synthesised and tested the biological activity of a series of premetrexed furoxan derivatives (Figure 25). These compounds were based on a multi-targeted folate analogue **105**, which has shown promising results as an anti-cancer agent in clinical trials due to its ability to suppress tumour growth by impeding both DNA synthesis and folate metabolism. Cytotoxicity assays showed that furoxan hybrid **106** had more potent activity to that of **105** in one cell line with the parent compound being more active in the other three cell lines tested although all the compounds showed activity in the μM range.[145]

Figure 25:



105

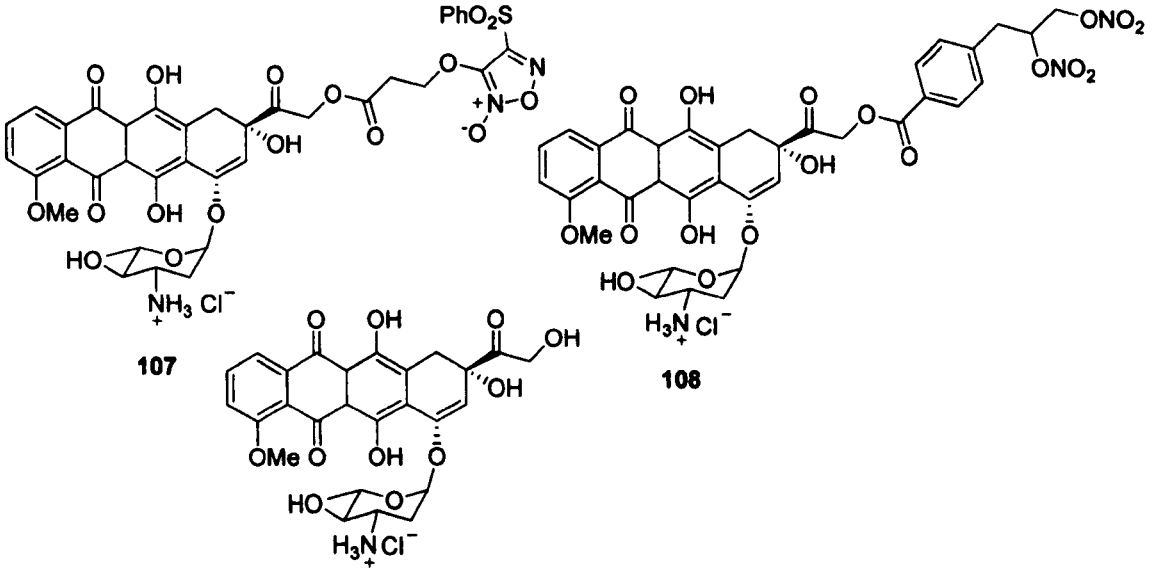


106

Pemetrexed disodium **105** *and premetrexed furoxan derivative* **106**

Gasco and co-workers synthesised two NO-donor hybrids **107-108** of the chemotherapy drug doxorubicin **109** and tested these compounds against doxorubicin-resistant human colon cancer cells (Figure 26). Preliminary biological testing showed that the NO-donor hybrids accumulated in the cells leading to high cytotoxicity possibly due to the nitration of tyrosine residues.[146]

Figure 26:



NO-donor doxorubicin hybrids **107-108** *and doxorubicin* **109**

The use of NO-donors as therapeutic agents has shown promising results. However, more research must be done in order to determine under which conditions NO exerts its anti-cancerous effects for the full potential of this powerful diatomic radical to be completly understood. Overall, a number of factors must be considered when explaining the multiple roles of NO in cancer and, as such, it appears that each set of data must be viewed on a case by case basis.

1.3.3 Ovarian cancer

Ovarian cancer is the fourth most common cause of cancer-related deaths in women in the UK according to cancer research UK.[147] Mortality statistics from 2008 show that approximately 4400 women died from ovarian cancer, the majority of which were women over the age 50, and approximately 6500 women were diagnosed the same year. [147] Although there has been a marked increase in survival statistics over the last few decades, the relative survival rates are still low. The one-year survival rate is 70% with the five-year survival rate reported as only 41%.[148,147]

The cause of ovarian cancer is still largely unknown despite many studies holding the view that this cancer develops "*de novo*".[149] The lack of knowledge into the origin of this disease results in most cases being detected at a later stage and relapses are very common, particularly in patients in the later stages of the disease.[150] Common symptoms are pain in the abdomen and pelvic regions, abdominal bloating and decreased appetite.[151] Other symptoms include urinary and bowel changes, fatigue, back pain, postmenopausal bleeding and rectal bleeding.[152] These symptoms can often be confused with other diseases which may lead to the late detection of ovarian cancer, and as a result, as many as 70% of sufferers are diagnosed with late stage cancer.[152,153] Table 3 outlines the different stages used in classifying ovarian cancer according to the International Federation of Obstetricians and Gynaecologists (FIGO) which is used to stage any cancer found after a diagnostic laparotomy of the patient.[147]

Table 3:

Stage	Description
Stage I	Tumour confined to the ovaries
IA	Tumour limited to one ovary; no tumour on external surface; capsule intact. No malignant cells in ascites or peritoneal washings
IB	As above, but tumour limited to both ovaries
IC	Tumour limited to one or both ovaries with any of the following: tumour on external surface; ruptured capsule; malignant cells in ascites or peritoneal washings
Stage II	Tumour involving one or both ovaries with pelvic extension
IIA	Extension and/or implants in uterus and/or fallopian tubes. No malignant cells in ascites or peritoneal washing
IIB	Extension to other pelvic organs. No malignant cells in ascites or peritoneal washings
IIC	Tumour staged either IIA or IIB with malignant cells in ascites or peritoneal washings

Stages in ovarian cancer (adapted)[147]

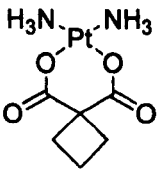
Stage I and stage II are classed as being early stage disease whereas stage III and stage IV are the later stages of the disease. Stage I and II tumours are usually slow growing and tend to be restricted to the ovaries, stage III and IV, on the other hand, are rapidly proliferating tumours often found in other parts of the pelvis.[149]

The first line of treatment in ovarian cancer is a combination of surgery and chemotherapy, depending on the disease stage at the point of discovery and depending on the menopausal status of the patient. For early stage disease, surgery to remove all of the tumour is often enough for a cure although many also undergo chemotherapy treatment in addition to surgery to remove any hidden tumour.[154] In the later stages of the disease

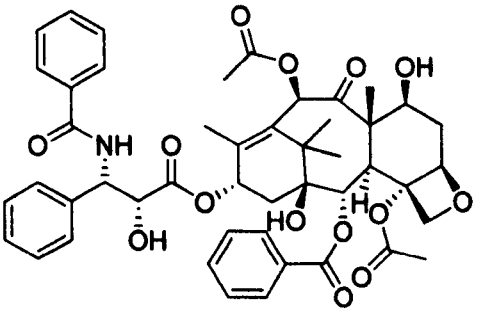
surgery to remove as much of the tumour(s) as possible, known as "debulking" or cytoreductive surgery is performed, which is then usually followed by chemotherapy.[147,155]

A variety of different drugs are available as chemotherapy agents, although carboplatin **110** is most commonly is used on its own, or in combination with paclitaxel **111**, as a first line chemotherapy treatment (Figure 27).[156]

Figure 27:



110

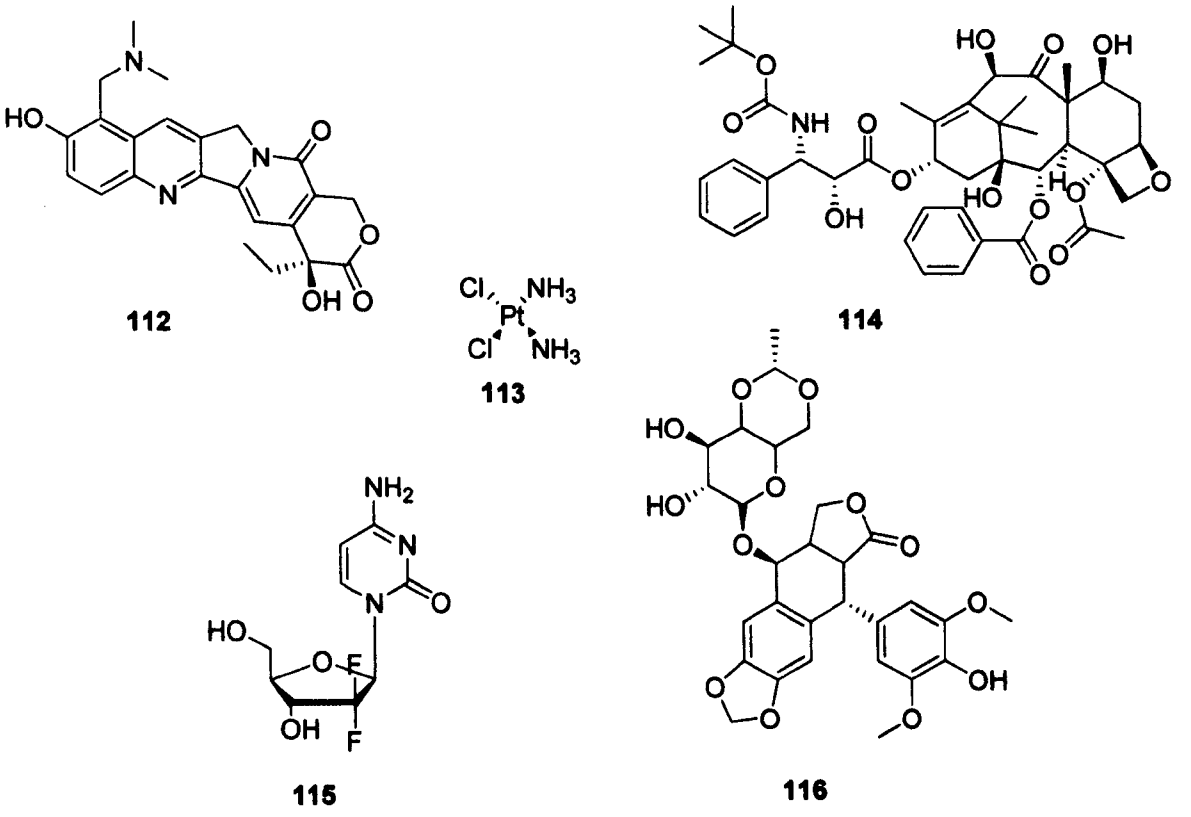


111

Carboplatin **110** *and paclitaxel* **111**

Other drugs which may be used either in first line treatment or in cases of relapse are topotecan (Hycamtin®) **112**, liposomal doxorubicin (Caelyx®, Myocet®) **109** , cisplatin **113**, docetaxel (Taxotere®) **114** , gemcitabine (Gemzar®) **115** and etoposide (VP-16®, Etopophos® Vepesid®) **116** (Figure 28).[156]

Figure 28:



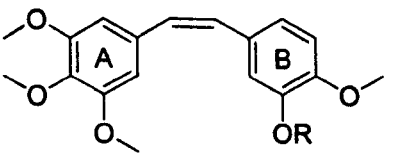
Chemotherapy agents used in the treatment of ovarian cancer

One of the challenges in the treatment of ovarian cancer is the development of drug resistance. Over 90% of ovarian cancer deaths are attributed to drug resistance.[150] In cases of recurrent ovarian cancer where there has been development of resistance to platinum based chemotherapy, combretastatin A-4 phosphate, **117** (See figure 29) is particularly interesting. Two recent phase trials, phase Ib and phase II, have shown promising results, with a higher response rate to the combination of combretastatin with carboplatin **110** and paclitaxel **111**, than with carboplatin **110** and paclitaxel **111** on their own.[157,158]

1.3.4 Combretastatin

Combretastatin A-4 **118** (CA-4) is one of several compounds which was extracted and isolated in 1989 from the bark of the African willow tree, *C. Caffrum*, by Pettit and co-workers at Arizona State University (Figure 29).[159]

Figure 29:



117 R = PO_3Na_2
118 R = H

Combretastatin A-4 **118** *Combretastatin A-4 phosphate* **117** *(sharing the A- and B-ring)*

The combretastatins have been found to be antimitotic agents which rapidly bind to tubulin in competition with colchicines and inhibit tubulin polymerisation.[160] The combretastatin analogues are part of a group of compounds known as small molecule vascular disrupting agents (VDAs). The VDAs work by targeting the vasculature of the tumour and disruption of the tumour blood flow ultimately causes necrosis.[161,162]

The ability of cells to divide and multiply is the result of a number of complex stages in the cell cycle. Microtubules are cylindrical organelles composed of αβ-tubulin heterodimers and these are essential to the cellular division of eukaryotic cells. Polymerisation and depolymerisation of the tubulins into microtubules is a vital part of mitosis (cell divison) and any changes in this process lead to the cell being unable to progress past the mitosis point which in turn results in apoptosis (programmed cell death).[163] The αβ-tubulin heterodimer has two small molecule binding sites, one for vinca

alkaloids and one for colchicines, with a third binding site on the polymerised microtubule, known as the taxoid site.[161,163]

It has been shown that combretastatin A-4, **118**, competes with colchicine for binding to tubulin, and unlike colchicine, the reversible binding is both rapid and not temperature dependent.[160] CA-4 **118** was found to be cytotoxic against a number of cancer cell lines but further *in vitro* studies were difficult due to its lack of solubility in water.[163,162] As a result a phosphate derivative CA-4-P **117** (Figure 29) of CA-4 **118** was synthesised to overcome these solubility issues.[161,162,164]

1.3.4.1 Studies on the structure activity relationship (SAR) for Combretastatin

Combretastatin A-4 **118** (Figure 29) has two aryl rings separated by a double bond, where ring A has three methoxy groups in positions 3, 4 and 5 and ring B has a methoxy group in the 4-position and a hydroxyl group in the 3-position. The biological data for combretastatin A-4-P **118** as an anticancer agent and the promising results emerging from clinical trials has inspired the synthesis of a large number of analogues. Structure activity studies have been performed, revealing several structural features which are important to combretastatins activity. The presence of the trimethoxy A-ring is believed to be key to the cytotoxic activity of the drug,[159] and similarly, it was found that a methoxy or methyl group in the 4-position of the B-ring is important for strong cytotoxic activity.[165] The hydroxyl group in the 3-position on the B-ring on the other hand, is not required[166] and an additional hydroxyl group on the 2-position decreases the activity.[167] Melero and co-workers[168] showed that the B-ring could be altered without necessarily impacting the cytotoxicity. Gussio and co-workers employed docking studies and molecular dynamic

simulations to construct a pharmacophore for the colchicine binding site. A small set of structurally different antitubulin inhibitors were used and a seven point pharmacophore was found. [169] The key features of this are three hydrogen acceptors, one hydrogen bond donor, two hydrophobic centres and a planar group.[169]

Table 4 shows a number of examples where the B ring has been altered or modified.

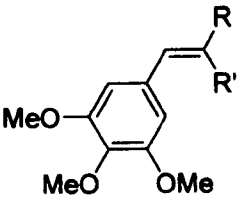
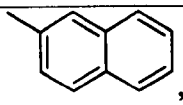
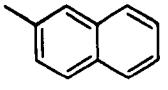
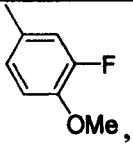
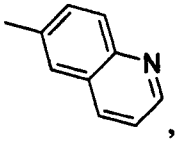
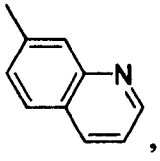
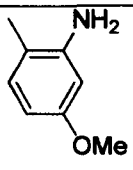
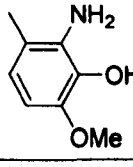
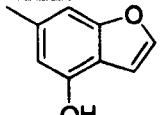
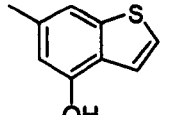


Table 4:

Compound No.	R / R'	IC_{50} values
119[170] **120**[170]	R' =  , R = H R' = H, R = 	IC_{50} = 15.8nM (P388, A-549, HT29, MEL-28 cancer lines) IC_{50} = 15.8nM (P388, A-549, HT29, MEL-28 cancer lines)
121[171]	R'=  , R = H	IC_{50} = 4.0 nM (K562 cell-line)
122[168] **123**[168]	R' =  , R = H R' =  , R = H	IC_{50} = 31.6 nM (P388, A-549, HT29, MEL-28, H116 cell lines) IC_{50} = 158.5 nM (P388, A-549, HT29, MEL-28, H116 cell lines)

Compound No.	R / R'	IC_{50}
124[172]	 R' = , R = H	IC_{50} = 0.0080 μM
125[172]	 R' = , R = H	IC_{50} = 0.017μM
126[173]	 R' = , R = H	IC_{50} = 28 nM (MCF-7 dx)
127[173]	 R' = , R = H	IC_{50} = 54 nM (MCF-7 dx)

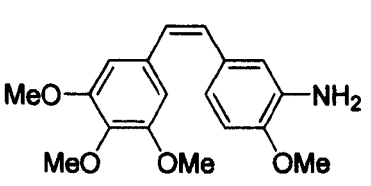
Summary of combretastatin analogues with modifications on the B-ring

The cytotoxic potency of **119** and **120** is similar to CA-4-P **117** whereas the compounds are less active as tubulin binding agents with **119** 40 times more potent than **120** but 5 times less potent than CA-4-P **117** when tested against microtubules in leukaemia cells.[170] The fluorinated analogue **121** showed cytotoxic activity similar to CA-4P, further confirmation that the hydroxyl group in the 3-position on the B-ring is not essential for activity.[171] The addition of fused five-membered heterocyclic ring such as in **126** and **127** revealed that cytotoxic potency of these compounds is not necessarily related to their anti-tubulin activity.[173]

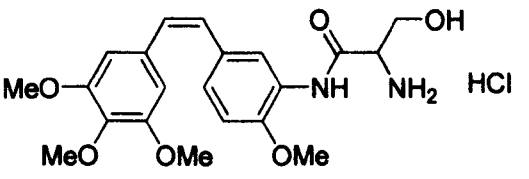
With the many different alterations investigated for the B-ring, two are particularly interesting. Oshumi and co-workers synthesised a derivative with an amine in the meta position on the B-ring **128** (Figure 30) which proved to be very active.[174] Currently, patients are being recruited for a phase III trial using compound **129**, which is the prodrug of **128**, in the treatment of soft tissue sarcoma (ClinicalTrials.gov Identifier:

NCT00699517).[175] Similarly, patients are being recruited for a phase I trial using the diphosphate ester of combretastatin A-1 **130** (ClinicalTrials.gov Identifier: NCT01085656)[176], thus making derivatives with substituents in both meta position of the B-ring seem very promising.

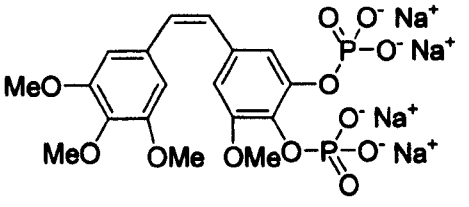
Figure 30:



128



129



130

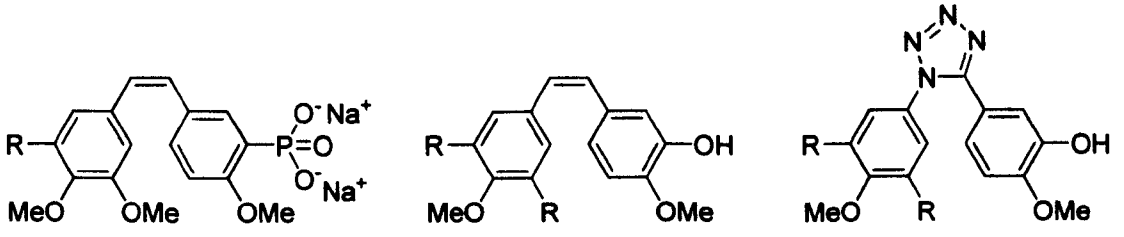
Combretastatin derivatives **128-130** *with modifications on the B-ring*

Early work by Hadfield and co-workers showed that cytotoxicity decreased when replacing the methoxy groups on the A-ring with methyl groups and ethoxy groups.[165,177] The trimethyl derivative retained the anti-tubulin potency whilst a trifluoro derivative was not cytotoxic but showed a small degree of anti-tubulin activity.[177] As this early work emphasised the importance for the three methoxy groups on the A-ring, very few examples include modifications to this ring. Of the few cases that exist, Pettit's work, is worth a mention, where one of the meta methoxy groups on the A-ring were replaced with halogens.[178] These chloro-, fluoro-, and bromo-combretastatin **131-133**, were

equally potent when compared to CA-4 **118** in terms of cytotoxicity and in their ability to inhibit tubulin binding.[178]

More recent work by Ley and co-workers showed equal or greater activity, compared to CA-4 **118** when replacing both meta methoxys with either a bromine or iodine **134-135** (Figure 31).[179] Further work by this group involved replacing the *cis* bridge with a tetrazole ring which reduced the potency compared to CA-4 **118**. Dihalogenation using bromine or iodine in the meta positions **136-137** restored the potency to that of CA-4 **118**.[180]

Figure 31:



131 R = Cl
132 R = F
133 R = Br

134 R = Br
135 R = I

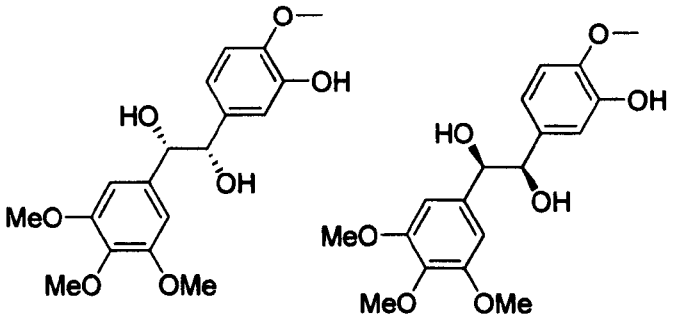
136 R = Br
137 R = I

Combretastatin derivatives **131-135** *with modifications on the A-ring and combretastatin derivatives* **136-137** *with modifications on the A-ring and the rigid bridge*

One of the most common modifications in the combretastatin series involves altering the rigid ethane bridge structure. Some of the earliest work details the effect of the *cis* versus the *trans* isomer in terms of cytotoxicity and antitubulin polymerisation.[166,174] The cytotoxicity and anti-tubulin results consistently show that the *cis* isomer is more active than the *trans*.[166,174,181] Pettit and co-workers prepared two *trans* diol isomers (Figure 32), **138** and **139**. Anti-tubulin assays revealed that **138** was inactive while **139** showed

inhibition with a reported IC_{50} of 22μM but neither compound had any significant activity against cancer cell lines when compared to CA-4 **118**.[182]

Figure 32:

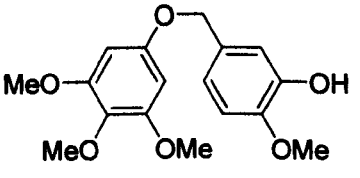


138 **139**

Combretastatin derivatives **138-139** *with modifications on the rigid bridge*

Lawrence and co-workers replaced the double bond with an ether giving compound **140**, which exhibited potent activity against K652 human leukaemia cell line (Figure 33).[183]

Figure 33:



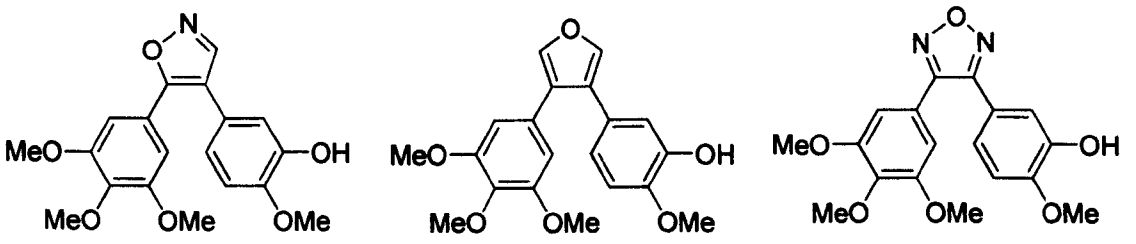
140

Combretastatin derivative **140** *with a modification to the rigid bridge*

Having reviewed the literature, the most common change to the bridge involves the use of 5-membered or 6-membered heterocyclic ring to retain the rigidity and maintain the *cis* conformation. Kaffy and co-workers[184] synthesised a number of derivatives with nitrogen containing 5-membered rings as the bridge and reported that the position of the heteroatoms within the 5-membered ring had an impact on the biological activity. One of

the isoxazole compounds, **141** (Figure 34) had a greater anti-tubulin activity than CA-4 **118**, with IC_{50} = 0.75μM compared to IC_{50} = 1.2μM for CA-4 **118**.

Figure 34:



141 **142** **143**

Combretastatin derivative **141-143** *with modifications to the rigid bridge*

Other analogues where the alkene bridge has been replaced by a 5-membered heterocycle are **142** and **143**. The furan analogue, **142**, was synthesised by Pirali and co-workers and exhibited an cytotoxic potency similar to CA-4 **118**.[185] The diarylfurazan, **143**, was tested against SH-SY5Y neuroblastoma cells with IC_{50} values in the low nanomolar range, suggesting slightly better potentcy than CA-4 **118**.[186] These are only a few of the combretastatin derivatives synthesised over the last two decades and the results from these compounds are promising.

In summary, it appears there are a few common structural features that are important to the biological activity of the combretastatins and their synthetic analogues. The 3,4,5 trimethoxy substitution on the A ring is important although replacement of the meta positions with halogens are promising alternatives. The methoxy group in the para position of the B ring appears to be vital for activity with amine groups in the meta position also providing benefit. The alteration of the alkene bridge provides an array of

possibilities so long as the *cis* conformation is retained, as achieved with the 5-membered ring systems which provide a particularly attractive modification.

1.3.4.2 Combretastatin clinical trials

Combretastatin A-4-P **117** was the first vascular disrupting agent to undergo a phase I clinical trial.[187,188] The trial was conducted with 25 patients with advanced solid tumours. CA-4-P **117** was given by i.v. infusion with a starting drug dosage of 18mg/m^2 over a 10 min and 60 min period. With the absence of any severe side effects, the dosage was escalated.[188] With the lower dosages only low grade side effects such as hot flush, nausea and abdominal cramps were reported. With the higher dosages cardiotoxic events were reported and included one case of acute shortness of breath, at 90mg/m^2, and one case of acute myocardial infarction, also at 90mg/m^2. Two other severe cardio events were reported, both at 60mg/m^2 over 10 minutes, one case with heart rates changes, prolonged QTc interval, and a second case with myocardial ischemia.[188] One patient went into complete remission and remained disease free for 30 months after treatment with CA-4-P **117**, one patient showed no progression of the disease for 6 months, two other patients did not have any progression of the disease while undergoing treatment with CA-4-P **117**, a fifth patient was progression free for 12 months and a sixth patient had a decrease in disease by 34% but developed progressive disease at increased doses.[188]

Rustin and co-workers[189] investigated the toxicity profile of CA-4-P **117** in a phase I trial with 26 patients. Similar results were reported with cardiotoxicity being the most severe side effect and 18 patients (53%) experiencing tachycardia, eight patients experiencing bradycardia and 12 patients (35%) presenting with hypertension.[189] Of these events only

four, all of which occurred at high doses, were deemed as severe with the remaining incidents being treatable. None of the patients showed any response, in terms of improvement of their condition, to the treatment with CA-4-P **117**, although two patients had stable disease initially, their disease later progressed. Based on these results the investigators recommended a dosage range for a phase II clinical trial as 52 to 68 mg/m^2.[189]

A third phase I clinical trial, which also employed CA-4-P **117** as a single agent, was carried out by Stevenson and co-workers in 2003.[190] The study enrolled 37 patients with solid tumours which were treated with CA-4-P **117** intravenously over 10 minutes for 5 consecutive days, every three weeks, with dosages ranging from $5mg/m^2$ to $75mg/m^2$. Unlike the previous two phase I trials cardiotoxicity was not reported as the most severe side effect although there was one reported instance of hypertension. In this study the most severe side effect was pain at the site of the tumour which was seen at $75mg/m^2$ and found to be dose dependent.[190] A partial response to the treatment was seen in one patient while 14 others remained stable after two cycles with CA-4-P **117**.[190]

CA-4-P **117** was investigated as a single agent in a phase II trial by Conney and co-workers.[188] A dose of $45mg/m^2$ of CA-4-P **117** was administered intravenously over 10 minutes on day 1, 8 and 15 of a 28 day cycle. Of the 18 patients in the study, 6 were disease stable for over 3 months while 12 patients showed progression in their disease. No severe cardiotoxicity was observed with the most severe side effect being tumour pain which was resolved after 24 hours. The investigators recommended a study into the combination of CA-4-P **117** with radiation or chemotherapy.[191]

Three phase I trials with CA-4-P **117** in combination with other treatments have been performed and published. In 2006, Bilenker and co-workers[192] investigated CA-4-P **117** in combination with carboplatin. Sixteen patients were enrolled into a dose escalation study with a drug regimen of CA-4-P **117** and carboplatin **110** administered intravenously on day 1 in a 21 day cycle. Three different doses were tested 27/5, 36/5 and 36/4 (CA-4-P mg/m^2/ carboplatin mg x min/mL) with six of the patients showing a stable disease while the other 10 exhibited progression. The study was halted early due to haematological toxicities, in particular severe thrombocytophenia (low red blood cell count) and neutrophenia (low white blood cell count) which in some patients led to transfusions and hospitalisation. It is possible that these side effects were due to drug-drug interactions and the investigators recommended a different regimen.[192]

Ng and co-workers[193] reported in the same year the preliminary result of a phase I trial with CA-4-P **117** and radiation therapy. The recently published complete trial result reports a response in 7 of 18 patients with non-small-cell lung cancer (NSCLC), and 3 of 18 patients with prostate cancer, which have had a prostate specific antigen relapse within 3 years. Two patients with prostate cancer experienced reversible ataxia and oculomotor nerve palsy as a dose limiting response at 63mg/m^2, while at 50mg/m^2, combined with cetuximab, a patient with NSCLC, experienced cardiac ischaemia.[193]

A phase I study looking at CA-4-P **117** in combination with VEGF (vascular endothelial growth factor) inhibitor bevacizumab resulted in a stable disease profile over 3 months in 9 of 14 patients with another 3 patients stabilised for over 6 months. Two dose limiting toxicities were reported, one patient withdrew after developing transient asymptomatic atrial fibrillation while another experienced a severe haemorrhage. The recommended

phase II dosage arising from this study was 63mg/m^2 CA4P + 10mg/kg bevacizumab and it appears the initial beneficial effect of CA-4-P **117** is maintained by treatment with bevacizumab.[194]

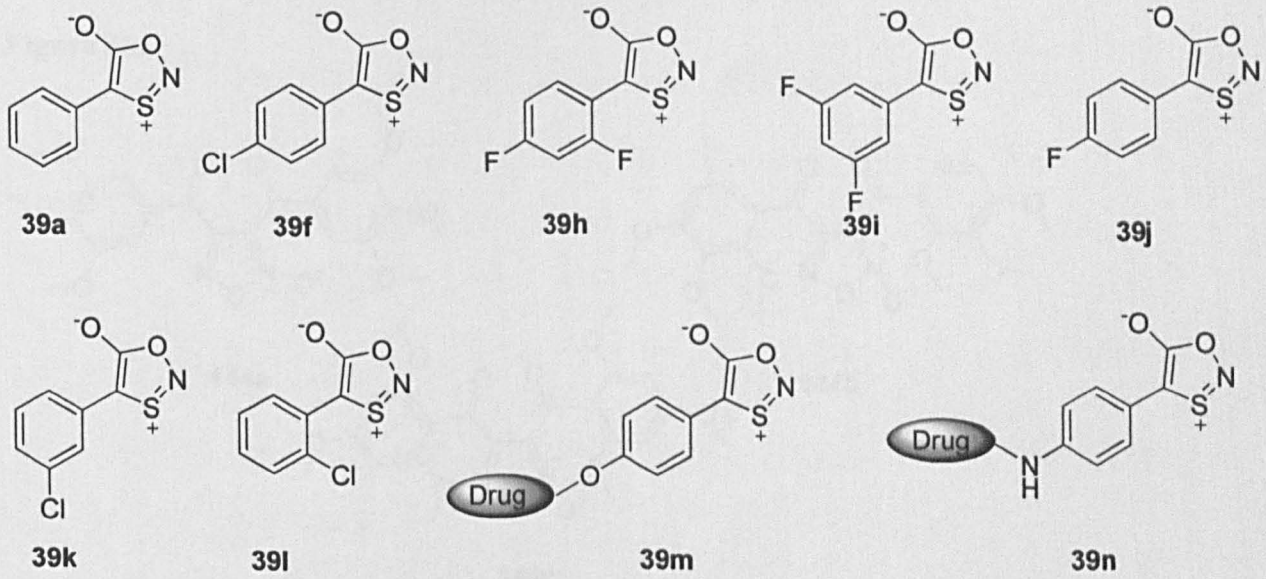
Following the results from Bilenker and co-workers[192] a phase Ib study looking at the combination of CA-4-P **117** with carboplatin **110** and paclitaxel **111** was carried out with patients with advanced cancer.[157] This dose escalation study was performed on three groups, one where the patients were given all three drugs, a second where CA-4-P **117** was combined with carboplatin **110** and a third where CA-4-P **117** was combined with paclitaxel **111**. Each group was divided into several cohorts that were given different doses. The dose limiting toxicities for CA-4-P **117** were seen at 72mg/m^2, with ataxia and arterial hypertension. Haematological results similar to that of Bilenker[192] were not seen when carboplatin **110** was administered at least 20 hours after CA-4-P **117**, although it was observed in higher dosages of CA-4-P **117** (up to 72mg/m^2).[157] For 7 of the 18 patients in the study with ovarian cancer there was a response to the drug regime, and 3 of 30 patients with other cancers showed a partial remission.[157]

Preliminary results from an ongoing phase II trial with CA-4-P **117**, carboplatin **110**, paclitaxel **111** and bevacizumab report an increased overall survival rate when including CA-4-P **117**, compared to results from the control groups. Adverse effects included hypertension, which were also present in the control group with three cases of cardiac ischemia which did not require hospitalisation.[195] Currently, a phase II trial (ClinicalTrials.gov identifier: NCT01305213) is recruiting patients to study CA-4-P **118** in combination with bevacizumab for the treatment of recurrent or persistent epithelial ovarian, fallopian tube or primary peritoneal carcinoma.[196]

1.4 Project overview

The work published by Wang,[50] which re-visited the synthetic routes to the OZOs **39a-39d-f**, opened an intriguing avenue in terms of the stability issues encountered when synthesising *S*-nitrosothiols. The SNO fragment locked in a 5-membered heterocyclic ring suggests a way of overcoming the stability issues of the traditional *S*-nitrosothiols. Wang's work also showed the potential for altering the rate of release of NO by varying the substituents on the aromatic ring. The ability to control how much NO is released is an important tool as the beneficial or toxic effects of NO in cancer appear to depend on the concentration of free NO. Based on this a number of OZO derivatives, **39a**, **39f** and **39h-l**, were synthesised in order to investigate how altering the substituents and their position on the ring alters the rate of NO-release (Figure 35). In addition, two potential NO-donor hybrid drugs, **39m-n**, was targeted, alongside the series of OZO derivatives, to allow for their cytotoxic activity against ovarian cancer cell lines to be compared.

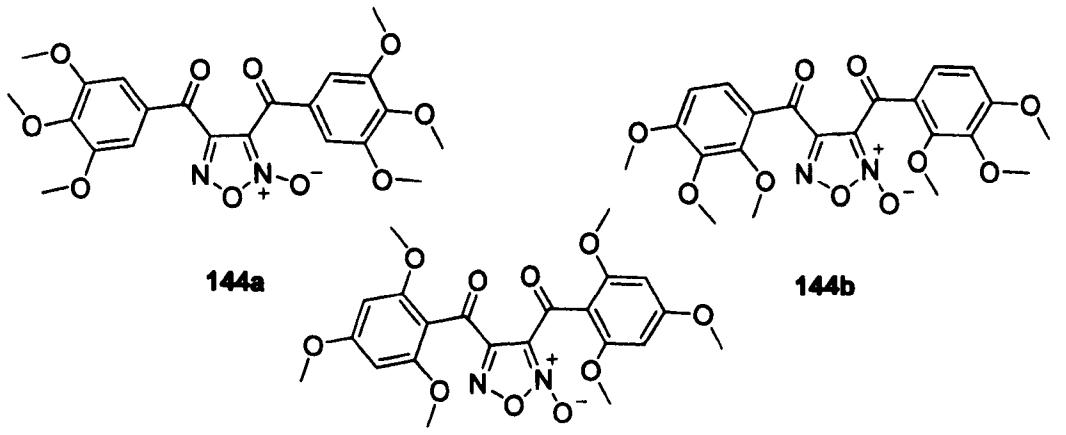
Figure 35:



The series of target OZO compounds **39a**, **39f** *and* **39h-n**

As described previously, combretastatin A-4-P **117** is an exciting anticancer agent which is currently undergoing clinical trials and has great promise as an anticancer agent. The cardiotoxicity associated with combretastatin is controlled by medication such as GTN or careful patient selection.[197] Combining combretastatin A-4 **118** with a NO-donor such as a furoxan is a very appealing idea and such a compound should be able to combat any cardiotoxicity associated with the VDAs with the added potential for increased cytotoxicity due to the release of NO. The heterocyclic furoxan ring will retain the rigidity of the bridge between the A- and B-rings without compromising the free rotation of the rings, in addition to potentially improving the water solubility of the parent compound. A number of derivatives can be synthesised from methyl ketones (as shown in scheme 15) in a one-pot synthesis compared to an 8-step synthesis for compounds **136-137**. A wide range of aryl methyl ketones are commercially available as starting materials thus ensuring that the A- and B-rings can be altered. Based on this a number of symmetrical target compounds (Figure 36) have been designed, synthesised and tested against several ovarian cancer cell lines.

Figure 36:



144a **144b** **144c**

Series of symmetrical combretastatin like furoxan target compounds **144a-c**

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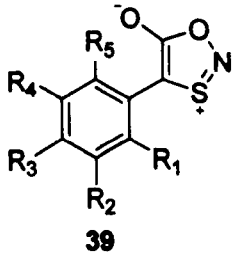
2 Results and discussion – chemistry

2.1 4-Aryl-1,3,2-oxathiazolium-5-olates (OZOs)

The first group of NO donors investigated herein are the 4-aryl-1,3,2-oxathiazolium-5-olates, **39** (Figure 1). These compounds were first synthesised in 1961 by Bacchetti and Alemagna[1] and further investigated in the early 1970s and 1980s by Gotthardt and co-workers, primarily to take advantage of their mesoionic ring structure as a route to other heterocycles.[2–8] It was the realisation that these compounds could be employed as NO-donors that sparked a renewed interest in the OZO compounds, initially by Schaffer and Thompson[9,10] in the early 1990s, and in 2007 Wang and co-workers[11] published a novel synthesis and reported that these NO-donors also had anticancer properties. Aside from their favourable biological properties this group of NO-donors are particularly interesting as they appear to overcome the stability issues associated with classic *S*-nitrosothiols by enclosing the NO fragment in a 5-membered ring.

Figure 1 :

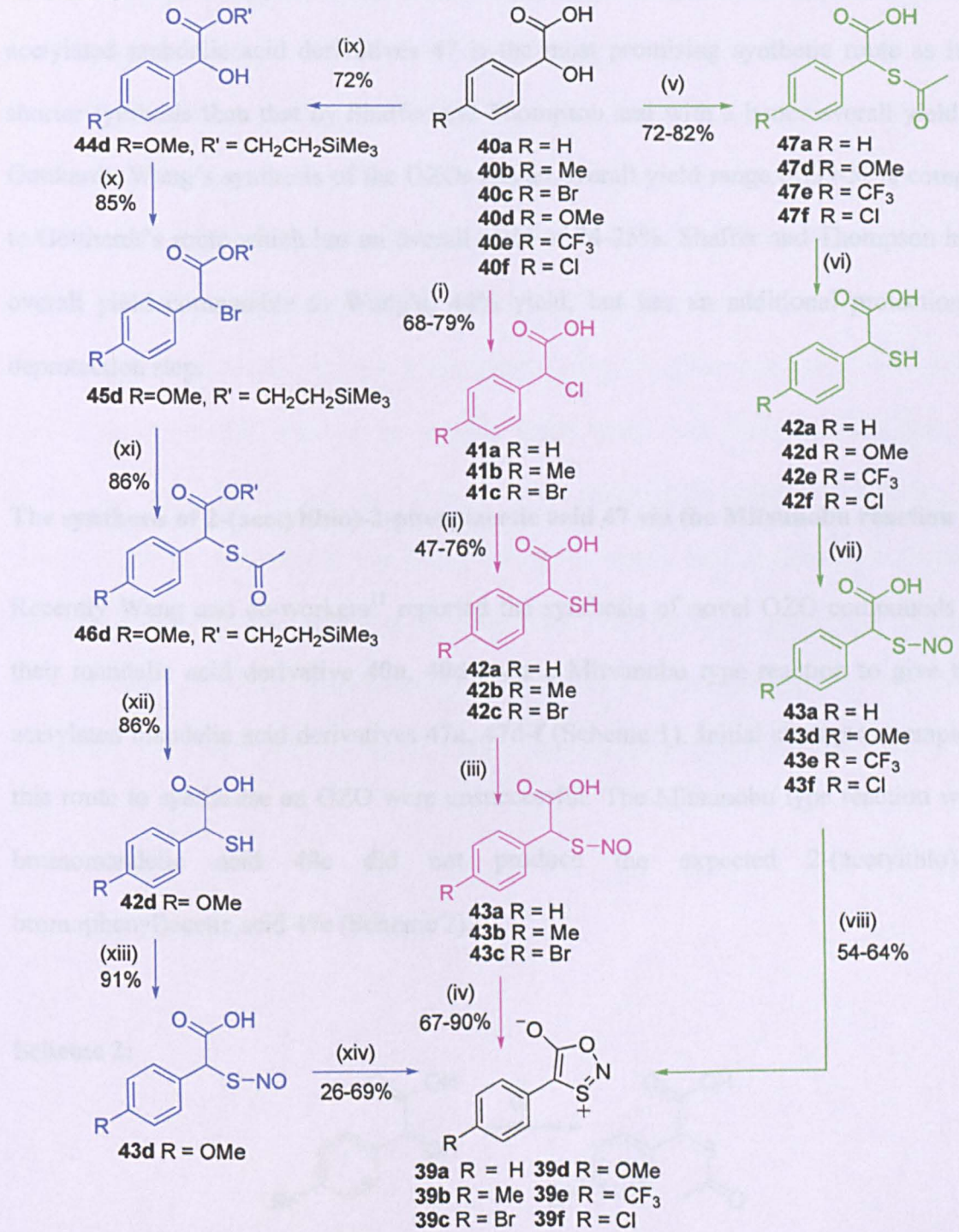


39

The general OZO structure **39**

Scheme 1 shows the three published procedures employed to synthesise the OZOs from their mandelic acid derivatives. Highlighted in green is the procedure by Wang, the procedure by Gotthardt is in purple and Schaffer and Thompsons' route is shown in blue.

Scheme 1:



Reagents and conditions: (i) 1.3-2.5eq. $SOCl_2$, pyridine (ii) K_2S, EtOH (iii) 1.3eq. EtONO, H_2SO_4, 0°C (iv) 2.2eq. DCC, 0°C (v) 2.0eq. HSAc, 2.0eq. PPh_3, 2.0eq. DIAD, THF, 0°C (vi) NaOMe/MeOH, r.t., 5hrs (vii) 2.0eq. *i*BuONO, DCM, 0°C, 2hrs (viii) 3.0eq. DCC, DCM, 0°C, 2hrs (ix) 2-trimethylsilylethyl-*N*,*N*-diisopropyl-pseudourea, dioxane, 90°C, 8hrs (x) PBr_3, $CHCl_3$, r.t., 45min, reflux 45min (xi) KSAc, EtOH, r.t,, 4.5hrs (xii) NaOH, EtOH, r.t., 1.5hrs, 55°C, 5.5hrs (xiii) *t*-BuONO, DCM, 20min, 0°C (xiv) EDC, DCM, 0°C 1hr, r.t 2hrs

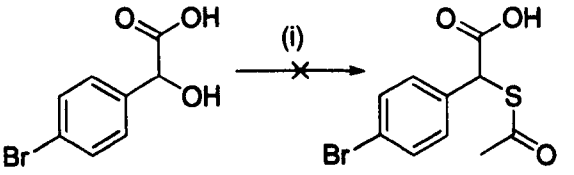
The three published synthetic approaches to the synthesis of the OZOs **39**

Of the three known synthetic routes to the OZOs, Wang's novel approach to the *S*-acetylated mandelic acid derivatives **47** is the most promising synthetic route as it is a shorter synthesis than that by Shaffer and Thompson and with a better overall yield than Gotthardt. Wang's synthesis of the OZOs has an overall yield range of 39-50% compared to Gotthardt's route which has an overall yield of 24-25%. Shaffer and Thompson has an overall yield comparable to Wang's, 44% yield, but has an additional protection and deprotection step.

2.1.1 The synthesis of 2-(acetylthio)-2-phenylacetic acid 47 via the Mitsunobu reaction

Recently Wang and co-workers[11] reported the synthesis of novel OZO compounds from their mandelic acid derivative **40a**, **40d-f** via a Mitsunobu type reaction to give the *S*-acetylated mandelic acid derivatives **47a**, **47d-f** (Scheme 1). Initial attempts at employing this route to synthesise an OZO were unsuccessful. The Mitsunobu type reaction with 4-bromomandelic acid **40c** did not produce the expected 2-(acetylthio)-2-(4-bromophenyl)acetic acid **47c** (Scheme 2).

Scheme 2:



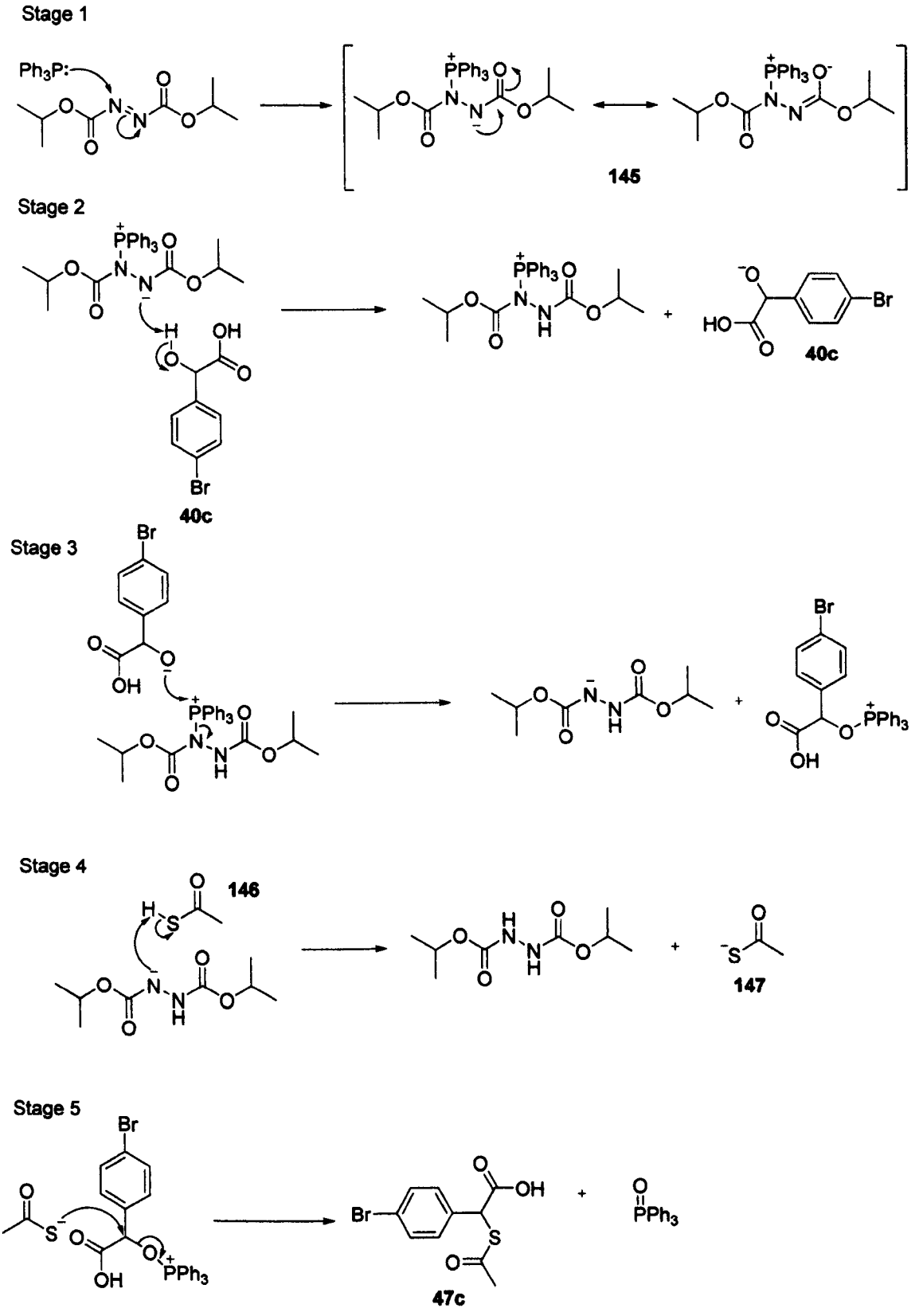
40c **47c**

Reagents and conditions: (i) 2.0eq. HSAc, 2.0eq. PPh_3, 2.0eq. DIAD, THF, 0°C

Wang's synthesis of 2-(acetylthio)-2-(2-bromophenyl)acetic acid **47c**

The mechanism of the Mitsunobu reaction[12] (Scheme 3) relies on the ability of DIAD to deprotonate the hydroxyl group. In order for the deprotonation of the hydroxyl group to occur the triphenyl phosphine and DIAD must first form complex **145** (See Scheme 3). The reaction between the complex and the mandelic acid does not require the presence of the thiolacetic acid and therefore the procedure was altered to include the addition of the thiolacetic acid at a later stage. This modified Mitsunobu reaction did not produce the desired product when the reaction was repeated using literature compound **40a**. The last step of the mechanism requires the sulphur nucleophile, from thiolacetic acid **146**, to be easily deprotonated to the thiolate **147** to allow the nucleophilic substitution to occur. With this in mind, the nucleophile was change from thiolacetic acid to potassium thioacetate (KSAc), which as the salt should easily form the thiolate anion and attack the phosphonium ion. In two parallel reactions KSAc was added with the other reagents and alternatively at a later stage. However, neither reaction gave the *S*-acetylated mandelic acid **47a**.

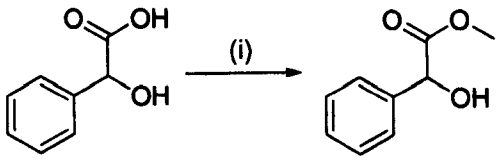
Scheme 3:



The Mitsunobu reaction mechanism[12]

The mandelic acid can clearly be deprotonated at two sites by DIAD and this specific reaction assumes a preferential deprotonation at the secondary alcohol with no removal of the more acidic carboxylic acid proton. Based on the different pK_a values the decision was made to protect the acid group to prevent any competing deprotonation reactions. The mandelic acid **40a** was treated with sulphuric acid in methanol to give the methyl ester **148a** in a 90% yield and without the need for further purification (Scheme 4).

Scheme 4:



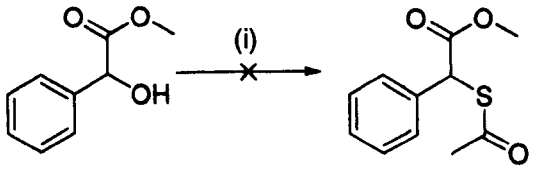
40a **148a**

Reagents and conditions: (i) H_2SO_4, MeOH, 60°C, 4hrs

The synthesis of methyl mandelate **148a**

Unfortunately, the revised Mitsunobu reaction which was carried out using the methyl mandelate **148a** proved to be unsuccessful despite protecting the more acidic proton (Scheme 5). As with the previous attempts the recovered material was identified as starting material **148a.**

Scheme 5:



148a **149a**

Reagents and conditions: (i) 2eq. HSAc, 2 eq. PPh_3, 2eq. DIAD, THF, 0°C

The revised Mitsunobu reaction using methyl mandelate **148a**

Table 1 summarises a number of unsuccessful attempts to synthesis the *S*-acetylated mercaptoacetic acid / ester from 4-bromomandelic acid, **40c**, mandelic acid, **40a**, and methyl mandelate, **148a**.

Table 1:

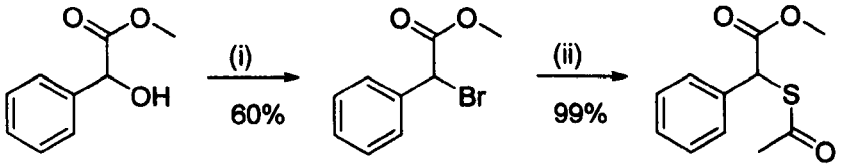
Starting material	Nucleophile	Order of addition	Number of equivalents of each reagent
40c	HSAc	No change	2 eq.
40c	HSAc	HSAc added at later stage	2 eq.
40a	HSAc	No change	2 eq.
40a	HSAc	HSAc added at later stage	2 eq.
40a	KSAc	KSAc added at later stage	2 eq.
40a	HSAc	HSAc added at later stage	3 eq.
148a	HSAc	No change	2 eq.
148a	KSAc	KSAc added at later stage	3 eq.
148a	HSAc	HSAc added at later stage	1 eq.
148a	KSAc	KSAc added at later stage	3 eq.

Summary of experiments for the synthesis of 2-(acetylthio)-2-phenylacetic acid/ester employing the Mitsunobu reaction

2.1.2 The revised synthesis of 2-(acetylthio)-2-phenylacetic acid 47

Given the many unsuccessful attempts at synthesising the 2-(acetylthio)-2-phenylacetic acid/ester **47a/47c/149a** *via* the Mitsunobu reaction, the decision was made to try a different synthetic route. With the acid group protected, as the methyl ester **148a**, the benzylic alcohol was brominated to create a better leaving group which could then be *S*-acetylated with potassium thioacetate (Scheme 6).

Scheme 6:



148a **150a** **149a**

Reagents and conditions: (i) HBr, reflux, 90°C (ii) 1.1eq. KSAc, MeOH, r.t., 24hrs

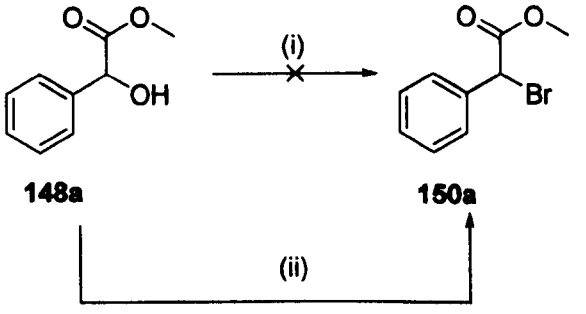
A different synthetic route to the 2-(acetylthio)-2-phenylacetic ester **149a**

The first step was carried out successfully to give **150a** in a 60% yield and after co-evaporation with toluene to remove the remaining HBr, no further purification was required. The bromo-derivative **150a** was then reacted with KSAc to give the 2-(acetylthio)-2-phenyalacetic ester **149a** in an excellent yield of 99%, again without any further purification as the KBr byproduct was simply removed by filtration.

The bromination reaction was repeated and scaled up using 10 grams of **148a** in order to bring forward more material, but rather than giving **150a** as previously, the analysis revealed that the white product was mandelic acid **40a**. This was anticipated given the choice of acid protecting group, since strong acids such as HBr are often used to deprotect methyl esters. A different reagent to selectively brominate the benzylic hydroxyl group

was required with phosphorus tribromide being the obvious choice (Scheme 7). When using phosphorus tribromide in carbon tetrachloride, TLC analysis showed that the reaction had gone to completion after stirring at room temperature for 48 hours. After removing any excess phosphorous tribromide with an aqueous wash, the solution was filtered through a pad of silica to give the bromophenylacetic ester **150a** as a clear oil in an 80% yield.

Scheme 7:



Reagents and conditions: (i) HBr, reflux, 90°C (ii) 1.1 eq. PBr_3, CCl_4, r.t., 48hrs

Bromination of methyl mandelate **148a**

The rationale for our new strategy of protecting the acid and brominating the benzylic alcohol prior to the *S*-acetylation step was supported by the synthetic route designed by Schaffer and Thompson (Scheme 1).[9,10] Here the authors protected the acid group, brominated, *S*-acetylated and then deprotected to give the free acid and the free thiol in one step. The compound **42d** was then nitrosated and dehydrated to give the OZO derivative **39d** in an overall yield of 44%. Due to a combination of reasons such as, health and safety, and availability, a solvent other than carbon tetrachloride had to be chosen for the synthesis to be feasible to use for a complete series of target compounds. Shaffer and Thompson synthesised the bromophenylacetic derivative **45d** by using phosphorus tribromide in refluxing chloroform. Attempts were made to repeat these conditions for methyl mandelate

with the reaction being monitored by TLC to identify the reaction time required for a complete transformation. The reaction time (6 days) was significantly longer than what was reported (1.5 hours) with a yield of 60%, compared to 85% in the literature.[10] Four different solvents were tried, as shown in Table 2, and those with a shorter reaction time were also tested at different temperatures. Due to the reactivity of phosphorus tribromide with water to form HBr, only dry or anhydrous solvents were used.

Table 2:

Solvent	Conditions	Yield
CCl_4	21°C	80%
THF	21°C	52%
CH_2Cl_2	21°C	65%
CH_2Cl_2	0°C	72%
CH_2Cl_2	40°C	60%
$CHCl_3$	60°C	60%
$CHCl_3$	21°C	75%

The solvents and temperatures used for the test reactions using PBr_3 for the bromination of methyl mandelate **148a**

From the solvents and temperatures tested, chloroform at room temperature was the best compromise based on yield and reaction time. With the solvent selected, more material was brought forward for the *S*-acetylation reaction. The bromophenylacetic ester **150a** was dissolved in methanol and then reacted with 1.1 equivalents of potassium thioacetate. The reaction was left to stir at room temperature and was complete after 4 hours as shown by

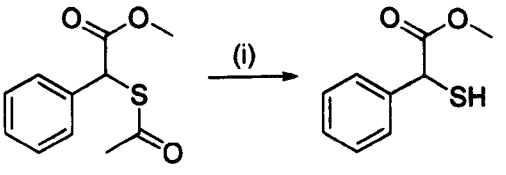
TLC analysis and precipitation of the KBr salt. Vacuum filtration of the oil gave the pure product **149a** in an excellent yield of 95%.

Compared to the synthetic routes of Wang[11] and Shaffer and Thompson,[9,10] the strength of this revised route to the 2-(acetylthio)-2- phenylacetic ester **149a** is that no further purification such as column chromatography is required. Each step in this synthesis gives pure products in good yields, with an overall yield of 48%. Wang's one step synthesis reports excellent yields, 72-82%, for the *S*-acetylated derivatives after column chromatography but we were unable to reproduce these results. Shaffer and Thompson's route gives an overall yield of 54%, however purification by column chromatography is required after two of the three steps.

2.1.3 De-*S*-acetylation and deprotection of the methyl ester to give mercaptoacetic acid 42a

With the synthetic procedure for 2-(acetylthio)-2-phenylacetic ester **149a** perfected the next step was to deprotect the sulphur to give the free thiol. Employing Wang's route the de-*S*-acetylation of **149a** was successfully done using sodium methoxide in methanol, to give the methyl 2-mercapto-2-phenylacetate **151a** in excellent yield, 90% (Scheme 8).

Scheme 8:



149a **151a**

Reagents and conditions: (i) NaOMe/MeOH, r.t, 5hrs

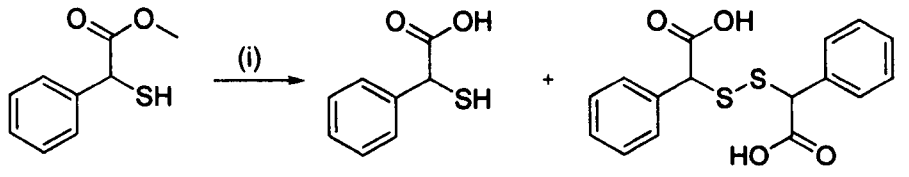
De-S-acetylation of 2-(acetylthio)-2-phenylacetic ester **149a** *to give methyl 2-mercapto-2-phenylacetate* **151a**

^{1}H NMR spectroscopy of **151a** showed the diagnostic doublet expected for the thiol (J = 7.70 Hz) at 2.51 ppm and the benzylic proton appeared as a doublet at 4.61 ppm (J = 7.70 Hz). This is an upfield shift for the benzylic proton compared to the singlet at 5.30 ppm, in compound **149a**, for the same proton. The reaction did not produce any of the corresponding disulphide and no purification was required.

By far the most difficult part of the synthesis was the deprotection of the methyl group to give the acid. Although refluxing in HBr had proven successful in removing the methyl group previously (Scheme 7, Section 2.1.2), this procedure yielded mostly disulphide and required purification. When performing column chromatography using a mixture of 10:1 petroleum ether : ethyl acetate, only the disulphide was isolated. Due to the small amount of material, recrystallisation proved ineffective. Therefore alternative deprotection methods were explored.

Shaffer and Thompson reported the use of sodium hydroxide in ethanol to remove both protecting groups (Scheme 1) to give the mercaptoacetic acid **42d** in a high yield of 91%.[10] In compound **46d** the acid is protected with a trimethylsilylether group, although this group is different from the methyl ester, sodium hydroxide should still be able to remove the methyl ester. When the methyl 2-mercapto-2-phenylacetate **151a** was reacted under these conditions (Scheme 9), it gave the desired acid **42a**, but also disulphide **152a** which was difficult to remove using a combination of column chromatography and recrystallisation techniques. This procedure was also used on 2-(acetylthio)-2-phenylacetic ester **149a** in an effort to remove both protecting groups in one step, although this also resulted in the formation of disulphide, **152a**.

Scheme 9:



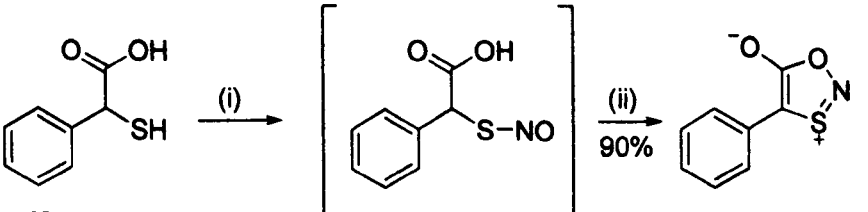
151a **42a** **152a**

Reagents and conditions: (i) 1M NaOH, EtOH, r.t., 1.5hrs, 55°C, 5.5hrs

Deprotection of the methyl ester **151a** *to give mercaptoacetic acid* **42a** *using sodium hydroxide*

Since ^{1}H NMR spectroscopy suggested the mercaptoacetic acid **42a** was present as the major product, the mixture was reacted on using Wang's method[11] to give 4-phenyl-1,3,2-oxathiazol-3-ium-5-olate (OZO) **39a** (Scheme 10). Using the impure mercaptoacetic acid **42a** contaminated with disulphide lead to the need for column chromatography at this stage, however, the disulphide co-eluted with the target OZO compound **39a** and attempts to purifying this further by recrystallisation were unsuccessful. ^{1}H NMR spectroscopy revealed the disulphide to be a minor impurity, representing approximately 10% of the total sample when peak integrals were analysed.

Scheme 10:



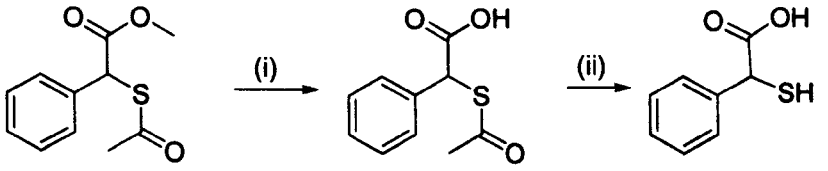
42a **43a** Not isolated **39a**

Reagents and conditions: (i) 3eq. *i*-BuONO, DCM, 0°C, 2hrs (ii) 2eq. DCC, DCM, 0°C, 2hrs

Nitrosation and ring-closure to give 4-phenyl-1,3,2-oxathiazolium-5-olate (OZO) **39a**

With the purity of the mercaptoacetic acid, compound **42**, key to the overall synthesis, a change in the order of synthetic steps was explored (Scheme 11). Removal of the methyl ester using lithium hydroxide in THF/H_2O gave 2-(acetylthio)-2-phenylacetic acid **47a** and the de-*S*-acetylation of this compound was done using sodium methoxide in methanol to give the mercaptoacetic acid **42a**. The change in order of reactions did not improve the purity and disulphide was still formed in the reaction.

Scheme 11:



149a **47a** **42a**

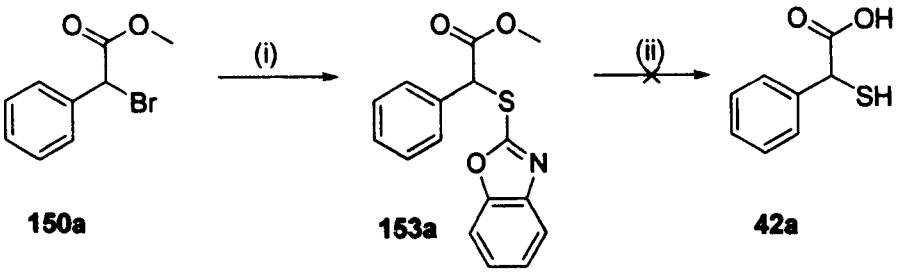
Reagents and conditions: (i) 1.1eq. LiOH, THF/H_2O, r.t., 12hrs (ii) NaOMe/MeOH, r.t., 5hrs

Altering the deprotection strategy for the synthesis of mercaptoacetic acid **42a**

With little success in isolating pure mercaptoacetic acid **42a** a different group was used to protect the sulphur. Rather than reacting the bromophenylacetic ester **47a** with potassium thioacetate, 2-mercaptobenzoxazole was used to give a protecting group which could be removed simultaneously with the methyl ester (Scheme 12). In a small scale reaction bromophenylacetic ester **150a** was dissolved in DMF with 1.1 equivalents of 2-mercaptobenzoxazole and 1.3 equivalents of potassium carbonate. The reaction was then stirred at room temperature and the progress of the reaction was monitored by TLC. After 4 hours TLC analysis showed the reaction had gone to completion. The DMF was evaporated off to give an oily residue which was dissolved in ethyl acetate and after an aqueous work-up gave **153a** as a pale white oil. ^{1}H NMR spectroscopy revealed the expected addition of four aromatic protons attributable to the mercaptobenzoxazole ring.

With the new protecting group in place the deprotection was carried out using 2 equivalents sodium hydroxide in an ethanol : water solvent system. After refluxing for 5.5 hours the product was extracted to give an insoluble white material.

Scheme 12:



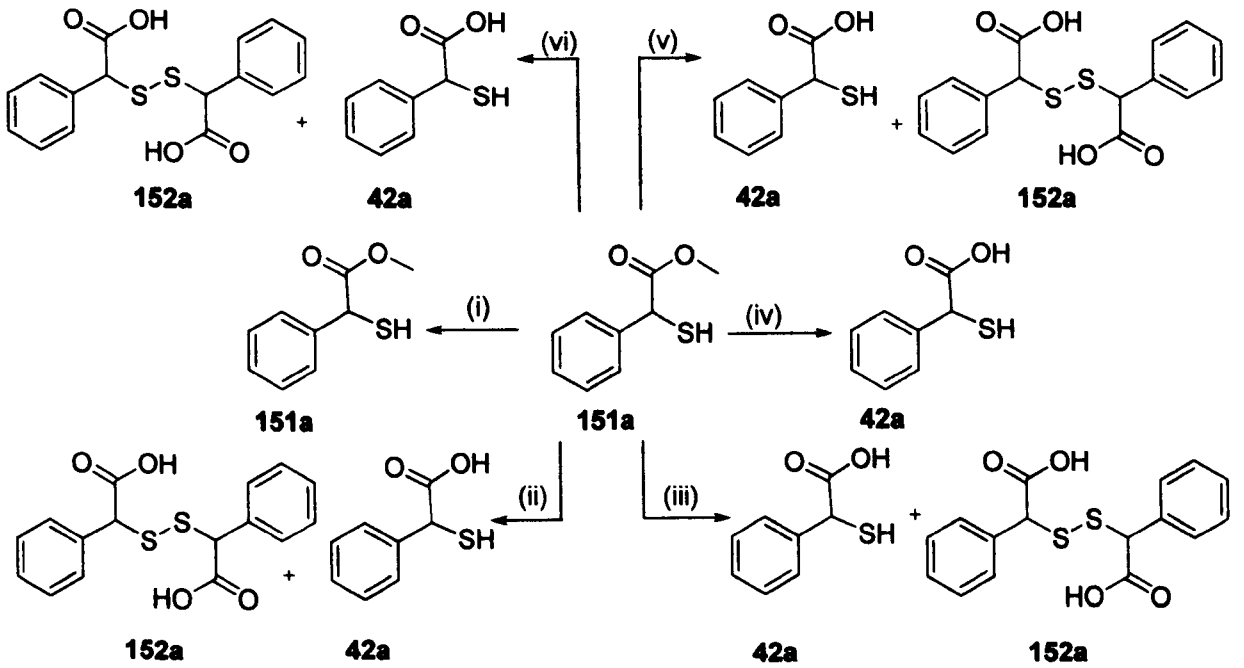
150a **153a** **42a**

Reagents and conditions: (i) 1.1eq. 2-mercaptobenzoxazole, DMF, K_2CO_3, r.t., 4hrs (ii) 2eq. NaOH, EtOH/H_2O, 75°C

Synthesis and deprotection of methyl 2-(benzoxazol-2-ylthio)-2-phenylacetate **153a**

Since altering the sulphur protecting group did not give the desired result, the focus was turned towards optimising the deprotection of the methyl ester without forming any disulphide. Scheme 13 shows the parallel reactions employed to test several other reagents that were likely to deprotect the acid without formation of disulphide. The products obtained from the reactions using borane tribromide and lithium hydroxide both had a high presence of the disulphide, whereas the reaction using hydrochloric acid only gave starting material. The best reagent seemed to be the stronger acid, sulphuric acid, at room temperature which only had traces of disulphide.

Scheme 13:



Reagents and conditions: (i) HCl/H_2O, r.t., 24hrs (ii) LiOH, THF/H_2O, r.t., 12hrs (iii) BBr_3, DCM, r.t, 12hrs (iv) H_2SO_4/H_2O, 0°C, 24hrs (v) H_2SO_4/H_2O, r.t., 24hrs (vi) 1M NaOH, EtOH, r.t., 1.5hrs, 55°C, 5.5hrs

Different deprotection strategies

The conditions giving the most disulphide all included either heating the reaction or an aqueous work-up in which heat was generated. The best conditions, resulting in no disulphide product included the addition of sulphuric acid/H_2O at 0°C.

2.1.4 Nitrosation and ring-closure to give 4-phenyl-1,3,2-oxathiazolium-5-olate 39a

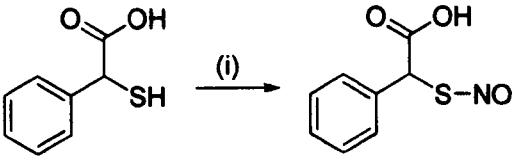
With the problematic synthesis of mercaptoacetic acid **42a** solved and a high degree of purity obtained, 4-phenyl-1,3,2-oxathiazolium-5-olate **39a** was finally synthesised following Wang's nitrosation and ring-closure method. The crude yellow/orange material was first filtered through celite to remove any excess isobutyl nitrite and then columned using 10:1 petroleum ether : ethyl acetate to remove the excess DCC. When performing

column chromatography the bright yellow OZO compound, lost its colour and ^{1}H NMR spectroscopy of the isolated compound showed that the target compound had decomposed to the disulphide when exposed to the light in the lab. In order to avoid this, all future columns were carried out in a darkened environment or the columns were covered with aluminium foil to limit light exposure. When scaling up the synthesis and purification of the target compound, a different problem was encountered. The DCC repeatedly co-eluted with the desired product. To remove the dehydrating agent and also to limit the time of exposure of the compound to light, the DCC was replaced by the water soluble reagent EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide. After an aqueous wash the majority of the EDC was removed without the need for column chromatography but the yield was considerably lower, with the most likely explanation being the OZO compounds partial solubility in the aqueous layer. With the DCC providing a better yield but clearly being more difficult to remove from the crude mixture, the polymer-bound DCC reagent was trialed. This proved very successful and the DCC polymer beads were easily removed by filtration to give the OZO compound in an efficient purification step and in an excellent yield of 90%.

All three procedures described to synthesis the OZO compounds (Scheme 1) used alkyl nitrites such as *tert*-butyl nitrite, isobutyl nitrite and ethylnitrite to nitrosate the mercaptoacetic acid derivatives prior to dehydration using EDC or DCC. In an effort to ensure all synthesised compounds were fully characterised and isolated the mercaptoacetic acid **42a** was dissolved in deuterated chloroform and nitrosated using isobutyl nitrite. The resulting pink solution was filtered through celite to remove excess nitrite prior to obtaining a ^{1}H NMR spectrum. The suspected instability of the *S*-nitrosothiol **43a** was

confirmed with the loss of colour with ^{1}H NMR spectroscopy only showing the presence of disulphide. A second attempt to isolate the *S*-nitrosothiol was carried out using Hart's method to nitrosate the mercaptoacetic acid (Scheme 14).[13] This reaction was successful in nitrosating the thiol **42a** to give a pale pink solution but with no precipitation formed it was not possible to isolate the *S*-nitrosothiol or complete Hart's methodology, which relies heavily on solid formation.[13]

Scheme 14:



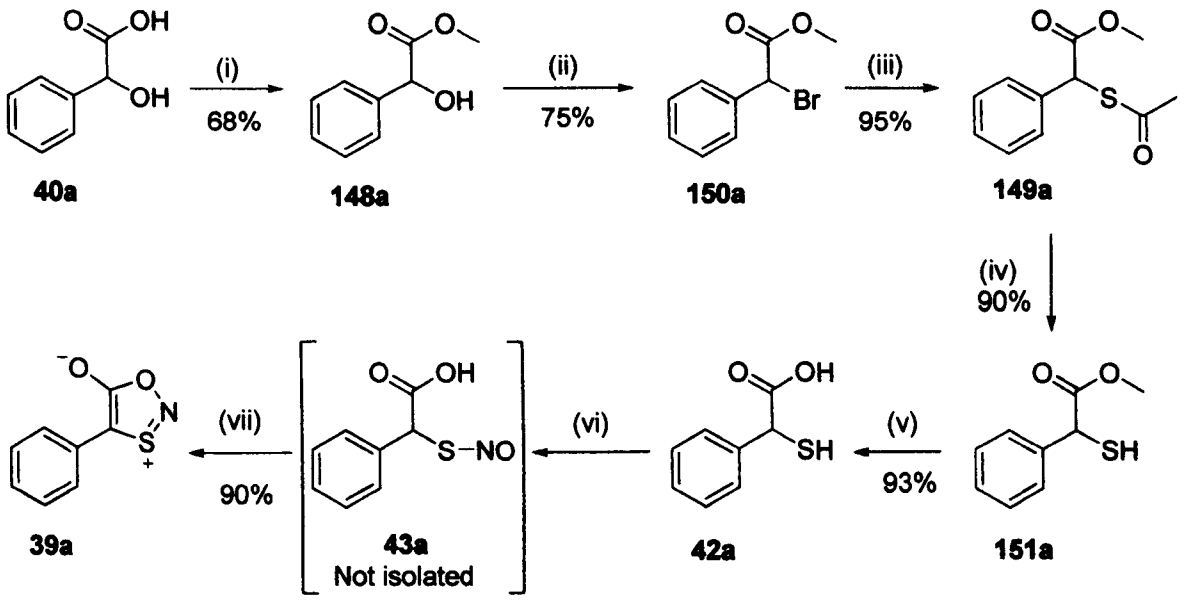
22a **23a**

Reagents and conditions: (i) $NaNO_2$, HCl, 0°C, 2hrs

Nitrosation using Hart's method

A third possible route to *S*-nitrosothiols from thiols involves the fuming method where the reaction with sodium nitrite and concentrated hydrochloric acid produces N_2O_3 as a dark blue gas, which is bubbled through a solution of the thiol in ethanol. However due to the ability of ethanol to become reversibly nitrosated this method can be problematic. Scheme 15 shows our revised seven-step synthesis for the OZO compound **39a** with an overall yield of 13%, showing the isolated yields of pure material and the conditions required for each step.

Scheme 15:



Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 60°C, 4hrs (ii) 1.1eq. PBr_3, $CHCl_3$, r.t., 96hrs (iii) 1.1eq. KSAc, MeOH, r.t., 4hrs (iv) NaOMe/MeOH, r.t., 5hrs (v) H_2SO_4/H_2O, 0°C, 24hrs (vi) 2eq. *i*-BuONO, DCM, 0°C, 2hrs (vii) 1eq. polymer bound-DCC, DCM, 0°C, 2hrs, dark

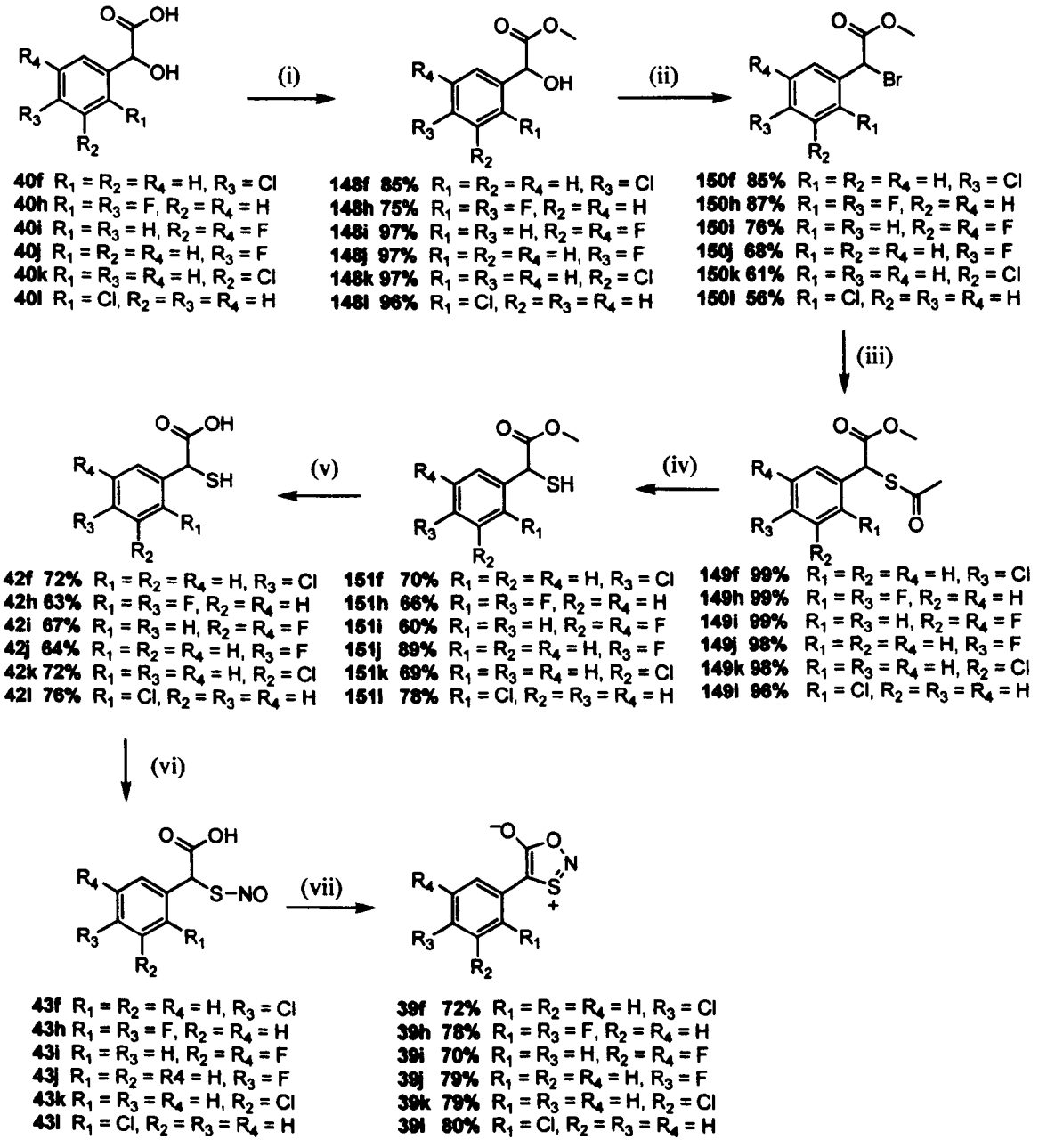
The revised seven-step synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate **39a**

2.1.5 Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate halogen derivatives 39f, 39h-l

The synthesis of the halogen derivatives **39f, 39h-l** was accomplished by employing the synthetic route derived from the work with the mandelic acid derivative (Scheme 15) as outlined in scheme 16. Each compound was synthesised from the corresponding starting material in acceptable yields (Scheme 16), and as with the mandelic derivative **39a**, no further purification was required until the final step. For compounds **39h**, **39k** and **39l** the mercaptoacetic acids, **42h**, **42i** and **42l**, showed a small amount of starting material, (methyl esters **148h**, **148i** and **148l**) was still evident by ^{1}H NMR spectroscopy. Altering the conditions and quantities of sulphuric acid and water used resulted in disulphide formation, which was previously proven to be difficult to remove in the synthesis of

compound **39a**. As a result the mercaptoacetic acids were used as intermediates and the small amount of starting material, estimated to be 2-5% by ^{1}H NMR spectroscopy, was removed by column chromatography after synthesising the OZO compounds **39f, 39h-l**.

Scheme 16:



Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 60°C, 4hrs (ii) 1.1eq. PBr_3, $CHCl_3$, r.t., 96hrs (iii) 1.1eq. KSAc, MeOH, r.t., 4hrs (iv) NaOMe/MeOH, r.t. 5hrs (v) H_2SO_4/H_2O, 0°C, 24hrs (vi) 2eq. *i*BuONO/DCM, 0°C, 2hrs (vii) 1eq. polymer -DCC/DCM, 0°C, 2hrs
The synthetic route for the halogenated OZO series **39f, 39h-l**

Table 3 shows a summary of all the derivatives synthesised in this series, with overall yields and sharp melting point data. All seven OZO compounds **39a, 39f, 39h-l** were bright yellow and crystalline with the exception of **39a** which was a darker yellow/orange colour with λ_{max} = 408nm compared to the other six derivatives which all had λ_{max} = 402nm.

Table 3:

Compound	m.p./°C	Overall yield
39a	128.8 – 131.2	13%
39f	124.5 – 126.8	12%
39h	96.6 - 99.8	10%
39i	119.4 – 121.8	28%
39j	109.3 - 111.4	20%
39k	104.6 – 106.9	5%
39l	94.2 – 96.7	4%

The overall yield and m.p. for the series of 4-phenyl-1,3,2-oxathiazolium-5-olate derivatives **39a, 39f, 39h-l**

As detailed in this chapter, number of alterations were made to the original synthetic route planned for these compounds, which was based on Wang's work in 2007.[11] After numerous attempts at employing the Mitsunobu reaction described by Wang to synthesise the *S*-acetylated mandelic acid derivatives, the approach was abandoned. As described previously, several test reactions were carried out to determine which solvent was the most suitable for the bromination of the benzylic alcohol using phosphorous tribromide.

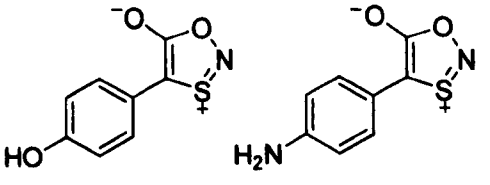
Similarly, test reactions were carried out to determine which reagent and conditions gave pure mercaptoacetic acid without any disulphide formation.

Compared to the Wang's synthetic route[11] this revised synthesis includes additional steps and although the overall yield is lower, 4-28% compared to 39-50%, the mercaptoacetic acids which Wang used as intermediates, have been isolated and fully characterised in our work. Shaffer and Thompson's route[9,10] has a similar strategy and one less step with an overall yield of 44%. Compared to both routes the strength of this revised route is that only one step requires column chromatography. In addition, the use of polymer bound DCC makes the purification step easier and improves the yield of the ring-closure step.

2.1.6 Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate linker derivatives 39m and 39n

With the success in synthesising the halogenated derivatives, the decision was made to try to synthesis an OZO compound with the capability of linking to a known anticancer agent to give a NO-hybrid drug via an oxygen or nitrogen linkage. Figure 2 shows the two target OZOs for use as linker compounds, these being 4-(4-hydroxyphenyl)-1,3,2-oxathiazolium-5-olate **39m** and 4-(4-aminophenyl)-1,3,2-oxathiazolium-5-olate **39n**.

Figure 2:



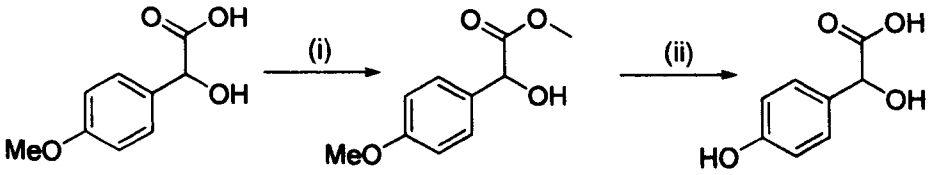
39m **39n**

4-phenyl-1,3,2-oxathiazolium-5-olate linker derivatives **39m** *and* **39n**

2.1.6.1 Synthesis of 4-(4-hydroxyphenyl)-1,3,2-oxathiazolium-5-olate 39m

Given that 4-methoxymandelic acid **40d** was commercially available with a methyl protecting group on the phenolic oxygen, a test reaction was carried out to ensure that both the methyl ester and the aryl methyl ether could be hydrolysed. First, methyl 2-hydroxy-2-(4-methoxyphenyl)acetate **148d** was synthesised in good yield (90%) from the 2-hydroxy-2-(4-methoxyphenyl)acetic acid **40d** (Scheme 17) using esterifying conditions.

Scheme 17:



40d **148d** **40m**

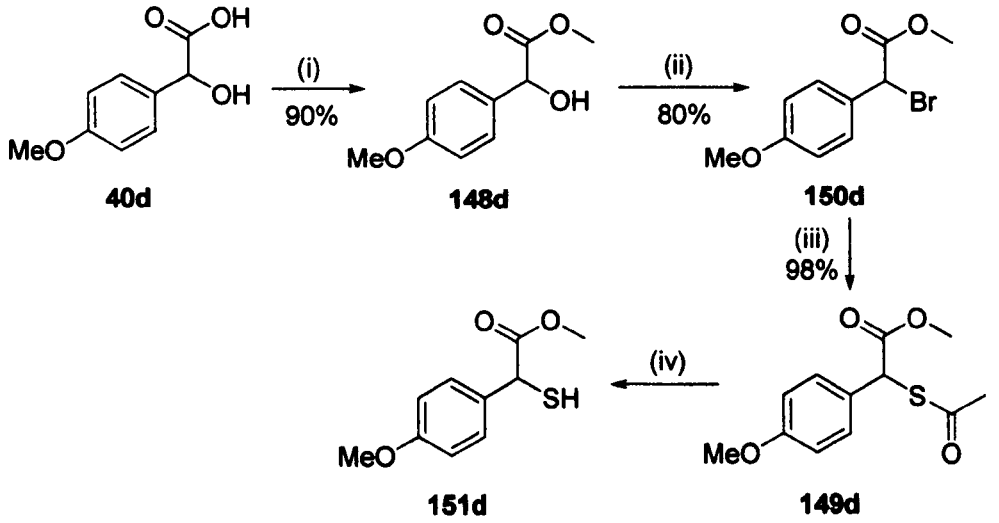
Reagents and conditions: (i) H_2SO_4, MeOH, 60°C, 4hrs (ii) H_2SO_4/H_2O , 0°C, 24hrs

Protection and deprotection of 2-hydroxy-2-(4-methoxyphenyl)acetic acid **40d**

With both the phenolic and carboxylic positions protected, a test reaction was carried out using our established procedure for removing the methyl ester, with the expectation that this reaction would remove both protecting groups (Scheme 17). The reaction successfully gave the 2-hydroxy-2-(4-hydroxyphenyl)acetic acid **40m** as a pale pink oil in moderate yield (50%). ^{1}H NMR spectroscopy of the oil **40m** was consistent with the commercially available 2-hydroxy-2-(4-hydroxyphenyl)acetic acid **40m**.

With a procedure in place for the deprotection in one-pot at both sites, the synthesis of the linker derivative was performed. Following the steps established for the mandelic and halogen derivatives the linker derivative was carried through to the methyl 2-(acetylthio)-2-(4-methoxyphenyl)acetate **149d** (Scheme 18).

Scheme 18:



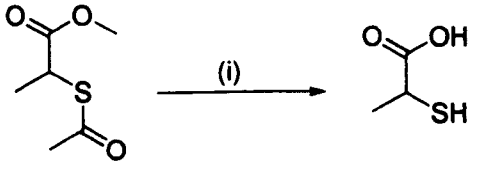
40d **148d** **150d** **151d** **149d**

Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 60°C, 4hrs (ii) 1.1eq. PBr_3, $CHCl_3$, r.t., 96hrs (iii) 1.1eq. KSAc, MeOH, r.t., 4hrs (iv) NaOMe/MeOH, r.t. 5hrs

Synthesis of methyl 2-mercapto-2-(4-methoxyphenyl)acetate **151d**

Unlike the synthesis for the previous target compounds, **151a, 151f, 151h-l** (see Scheme 16), the de-*S*-acetylation step provided a mixture of the methyl 2-mercapto-2-(4-methoxyphenyl)acetate **151d** and the corresponding disulphide. This prompted a look at other potential reagents. Suzukamo and co-workers[14] reported the synthesis of 2-mercaptopropanoic acid **154** from methyl 2-(acetylthio)propanoate **155** by refluxing in HCl (Scheme 19).

Scheme 19:



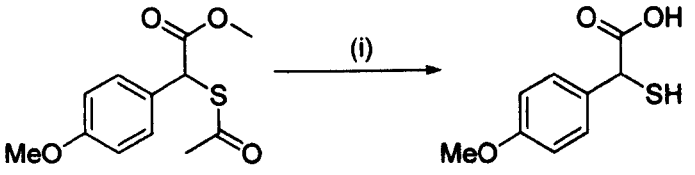
155 **154**

Reagents and conditions: (i) conc. HCl, 80°C, 2hrs

The synthesis of 2-mercaptopropanoic acid **154** *under acid reflux*

Despite the previous issues with **42a** in respect to heating the thiol, these reaction conditions proved successful with none of the disulphide formed in the process (Scheme 20). ^{1}H NMR analysis confirmed that the material formed was the 2-hydroxy-2-(4-methoxyphenyl)acetic acid **42d**, with the two diagnostic doublets attributed to the thiol and the benzylic proton, when compared to the data reported by Shaffer and Thompson.[9,10]

Scheme 20:



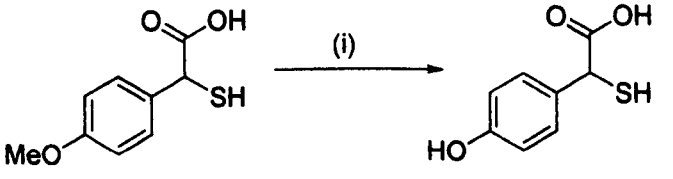
149d **42d**

Reagents and conditions: (i) conc. HCl, 80°C, 2hrs

The synthesis of 2-hydroxy-2-(4-methoxyphenyl)acetic acid **42d** *under acid reflux*

The mercaptoacetic acid **42d** was carried through to the next step however, once the remaining protecting group had been removed (Scheme 21), the polar material showed a strong preference for the aqueous layer in the washing phase and despite numerous attempts at extracting the product, this was not possible.

Scheme 21:



42d **42m**

Reagents and conditions: (i) H_2SO_4/H_2O, 0°C, 24hrs

Synthesis of 2-hydroxy-2-(4-hydroxyphenyl)acetic acid **42m**

Some of the protected starting material was extracted but the desired product remained in the aqueous layer. After co-evaporating with toluene proved unsuccessful, the aqueous layer was freeze-dried but this did not remove enough of the water for any analysis or further reactions to be carried out. At this point it was decided to abandon this linker derivative until the solubility issue could be solved.

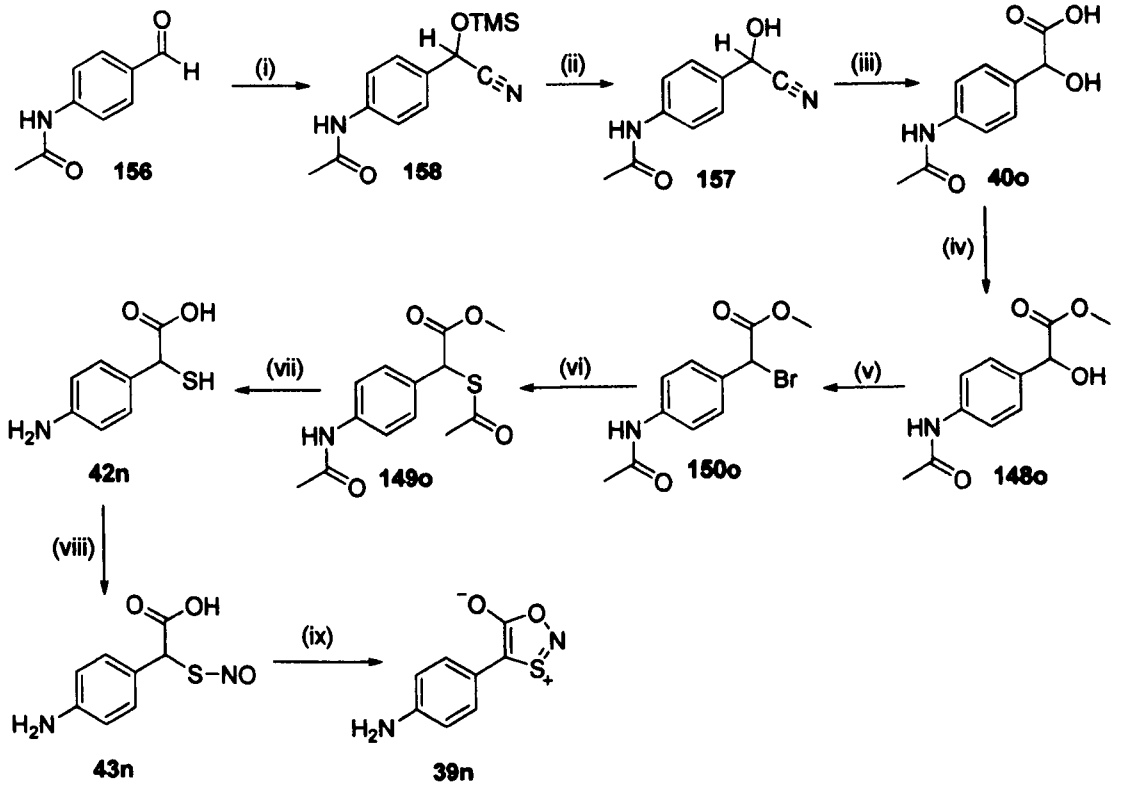
2.1.6.2 Synthesis of 4-(4-aminophenyl)-1,3,2-oxathiazolium-5-olate 39n

A second attempt to synthesis a linker derivative was performed using commercially available 4-acetaminobenzaldehyde **156** as starting material. As illustrated in Scheme 22, the proposed synthesis involved the formation of the cyanohydrin **157** which could be hydrolysed to give 4-acetamino mandelic acid **40o**. The mandelic acid derivative **40o** could then be reacted on following the procedure established for the previously synthesised OZO derivatives.

Synthesis of the cyanohydrin **157** was performed using the procedure by Robertson.[15] The aldehyde **156** was added to a solution of potassium metabisulfite in water and stirred at 0° prior to the dropwise addition of an aqueous solution of potassium cyanide. After stirring at room temperature for 2 hours, diethyl ether was used to extract the product from the aqueous phase. After washing the organic layer with 5M hydrochloric acid and brine, the ether was evaporated off to give the product as a pale orange oil in very poor yield, 3%. ^{1}H NMR showed the product to contain a mixture of starting material and product in a 1:1 ratio. The reaction was repeated but no improvement was seen in the yield or the purity of the compound. It is possible that cyanohydrin remained in the aqueous layer, however, due to the possibility of forming HCN gas, the reaction was not repeated a third time. Instead a

different synthetic approach was taken using trimethylsilyl cyanide following the procedure by Gassman and Talley.[16] The synthesis of trimethylsilyl cyanohydrin **158** was successfully achieved by reacting benzaldehyde **159** with trimethylsilyl cyanide and a catalytic amount of zinc iodide in dichloromethane at room temperature. TLC monitoring of the reaction showed it had gone to completion after 48 hours, and after an aqueous wash the reaction gave trimethylsilyl cyanohydrin **158** as an oil. ^{1}H NMR spectroscopy showed the expected peaks with the TMS giving a peak at 0 ppm alongside the loss of the aldehyde peak. ^{13}C NMR spectroscopy provided further confirmation with the loss of the carbonyl peak.

Scheme 22:

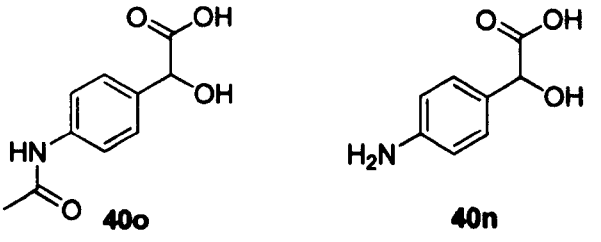


Reagents and conditions: (i) 1.2eq. TMSCN, DCM, ZnI, r.t., 48hrs (ii) 2M HCl, r.t. 8hrs (iii) HCl, r.t., 24hrs (iv) H_2SO_4, MeOH, reflux, 60°C, 4hrs (v) PBr_3, $CHCl_3$, r.t., 96hrs (vi) KSAc, MeOH, r.t., 4hrs (vii) HCl, reflux, 2hrs (viii) *i*-BuONO, DCM, 0°C, 2hrs (ix) polymer-bound DCC, DCM, 0°C, 2hrs

Proposed synthesis for the OZO linker derivative 4-(4-aminophenyl)-1,3,2-oxathiazolium-5-olate **39n**

The trimethylsilyl cyanohydrin **158** was dissolved in 2M hydrochloric acid and stirred at room temperature for 8 hours to give the deprotected cyanohydrin **157** in 90% yield. After co-evaporation of the acid with toluene the cyanohydrin **157** appeared as a yellow solid. Hydrolysis to give the 4-acetamino-mandelic acid **40o** was carried out by refluxing in concentrated hydrochloric acid (Scheme 22). This step of the synthesis proved to be challenging as ^{1}H NMR analysis of the crude material revealed that the acid hydrolysis resulted in partial deacetylation to give a 1:1 mixture of 2-(4-aminophenyl)-2-hydroxyacetic acid **40n** and 2-(4-acetamidophenyl)-2-hydroxyacetic acid, **40o** (Figure 3).

Figure 3:

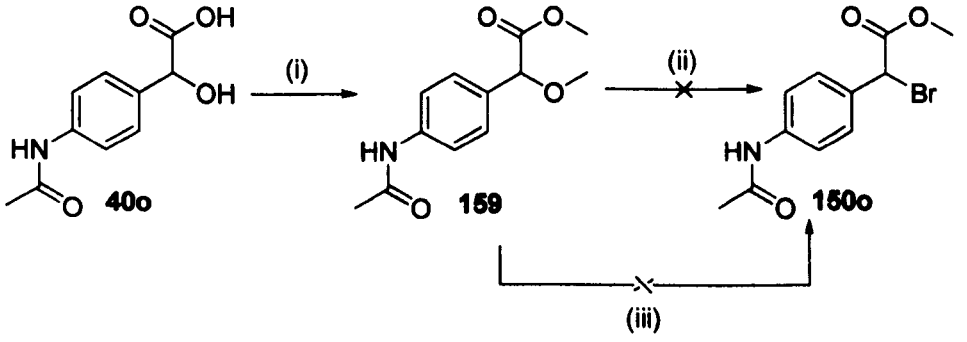


2-(4-acetamidophenyl)-2-hydroxyacetic acid **40o** *and 2-(4-aminophenyl)-2-hydroxyacetic acid* **40n**

The two products were columned using a 10: 1 methanol : dichloromethane solvent system but despite having a good separation on the TLC plate, the products repeatedly co-eluted. Unable to separate the two products, the hydrolysis was repeated but rather than heating, the reaction was stirred at room temperature overnight. This gave the *N*-acetylated compound **40o** as the only product. The next step in the synthesis was performed by dissolving the acid in methanol and refluxing with a few drops of concentrated sulphuric acid (Scheme 23). The white solid obtained after a base wash was analysed by ^{1}H NMR spectroscopy, which revealed three methyl groups rather than the two that were expected.

This suggested that both the benzylic hydroxyl group and the carboxylic hydroxyl group had been methylated giving compound **159** rather than **148o**, which was confirmed by high resolution mass spectrometry. Yoo and Pagel[17] reported the successful bromination of a methoxy group to give the bromine derivative using phosphorus tribromide in carbon tetrachloride at room temperature.[17] Similarly, hydrobromic acid is a known reagent for this bromination and therefore two test reactions were carried out using the benzylic methyl ether as shown in scheme 23. NMR spectroscopy and GC-MS analysis of the reactions showed only starting material **159**, with no product being formed.

Scheme 23:



Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 60°C, 4hrs (ii) PBr_3, $CHCl_3$, r.t., 96hrs (iii) HBr, reflux, 80°C, 6hrs

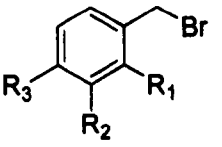
Synthesis of methyl 2-(4-acetamidophenyl)-2-bromoacetate **150o**

At this point it was decided to focus on other *S*-nitrosothiol and furoxan work until a different route to the 4-(4-aminophenyl)-1,3,2-oxathiazolium-5-olate **39n** could be found.

2.2 Synthesis of nitrosated phenylmethanethiol derivatives

In order to effectively compare the stability of the OZO series to that of the structurally similar ring opened *S*-nitrosothiols, a series of commercially available benzylic bromides were selected. These benzylic bromides had halogen substituents on the ring in the same positions as those in the OZO series (Figure 4).

Figure 4:

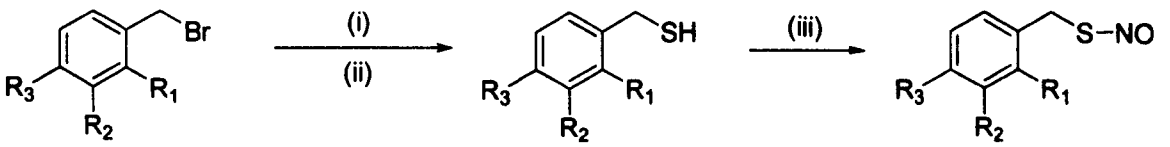


160a $R_1 = R_2 = H$, $R_3 = F$
160b $R_1 = R_3 = F$, $R_2 = H$
160c $R_1 = R_2 = H$, $R_3 = Cl$
160d $R_1 = R_3 = H$, $R_2 = Cl$

The selected benzylic bromide starting materials **160a-d**

Traditional methods for synthesising *S*-nitrosothiols from halides usually include *S*-acetylation using thioacetate salts such as potassium thioacetate followed by de-*S*-acetylation to give the free thiol as already discussed. The de-*S*-acetylation step often includes harsh reagents or conditions such as NaOMe in refluxing methanol,[18] and in particular, strong bases such as NaOH,[19] KOH,[20] and NaOMe[19] are used. Recently, Han and Balakumer[21] reported a novel one-pot method to convert benzylic bromides to the benzylic thiols using mild conditions with high yields (Scheme 24). Thiolacetic acid is used to give the *S*-acetylated compound which is then de-*S*-acetylated by the addition of a second portion of methanol, to increase the solubility of the mild base, K_2CO_3, resulting in the formation of free thiol (Scheme 24).

Scheme 24:



160a $R_1 = R_2 = H$, $R_3 = F$
160b $R_1 = R_3 = F$, $R_2 = H$
160c $R_1 = R_2 = H$, $R_3 = Cl$
160d $R_1 = R_3 = H$, $R_2 = Cl$

161a $R_1 = R_2 = H$, $R_3 = F$
161b $R_1 = R_3 = F$, $R_2 = H$
161c $R_1 = R_2 = H$, $R_3 = Cl$
161d $R_1 = R_3 = H$, $R_2 = Cl$

162a $R_1 = R_2 = H$, $R_3 = F$
162b $R_1 = R_3 = F$, $R_2 = H$
162c $R_1 = R_2 = H$, $R_3 = Cl$
162d $R_1 = R_3 = H$, $R_2 = Cl$

Reagents and conditions: (i) 1.2 eq. HSAc , 2.2eq. K_2CO_3, THF, 0.5hrs, N_2 (ii) MeOH, 0.5hrs, N_2 (iii) 2eq. *i*-BuONO, DCM, 0°C, 0.5hrs

The proposed synthesis of benzylic S-nitrosothiols **162a-d**

The mild conditions in the procedure successfully gave the benzylic thiols **161a-d** in good to excellent yields ranging from 60-90%. For compound **161a** ^{1}H NMR analysis showed the expected doublet due to the benzylic methylene at 3.64 ppm (J = 7.62 Hz), and the diagnostic triplet due to the thiol was seen at 1.69 ppm (J = 7.62 Hz). Similar results were found for the other three derivatives and this was found to be consistent with data reported for other benzylic thiols.[22]

Unfortunately, the thiols appeared to be particularly unstable, removal of trace amounts of solvent on the high vacuum trap resulted in the formation of the corresponding disulphide. In an attempt to avoid this, the crude thiol was nitrosated *in situ* with isobutyl nitrite to give a very pale pink solution but none of the nitrosated thiol could be fully analysed as it readily decomposed to the corresponding disulphide. This observation alone, highlights a significant difference in the stability of these compounds in comparison to their ring-closed OZO derivatives. It also reinforces why the free RSNOs could not be easily isolated in the synthetic pathway described in Scheme 15 and 16.

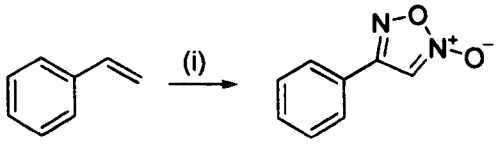
2.3 The 1,2,5-oxadiazole-2-oxides (Furoxans)

Unlike the OZOs, a great deal of research has been conducted on the furoxans, with a vast number successfully synthesised over the last 30 years.[23–26] These 5-membered heterocycles, have some structural resemblance to the OZOs and are clearly more stable than the ring-open *S*-nitrosothiols, requiring a thiol cofactor for NO to be released. The furoxan family are known to be thermally stable, with high temperatures needed to decompose them and only a few examples demonstrating their photochemical decomposition.[27] The furoxans are also known to undergo photochemical and thermal isomerisation. As described in chapter 1, the naming of these isomers is based on the position of the substituent R-groups relative to the position of the exocyclic oxygen (Figure 16, Chapter 1).

2.3.1 The synthesis of a series of unsymmetrical furoxans

The increased stability of the *S*-nitrosothiols when incorporated into a 5-membered ionic ring prompted a look at other NO-donors with 5-membered ring systems. The comparison of the OZOs to these systems would in theory provide greater insight into the substituent effects of the halogens decorating the benzene ring. The group chosen for comparison were the 1,2,5-oxadiazole-2-oxides, which are also known as the furoxans. The main reason for choosing this group of NO-donor was the apparently straightforward synthesis from the terminal alkenes using acetic acid and sodium nitrite (Scheme 25).

Scheme 25 :



163a **164**

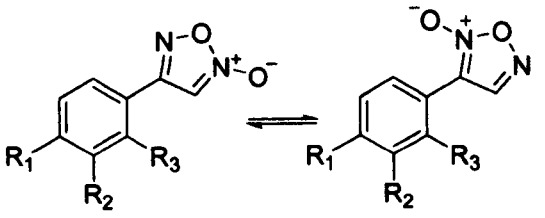
Reagents and conditions: (i) 5eq. $NaNO_2$, $AcOH/H_2O$

The synthesis of furoxan **164** *from alkene* **163a**

This synthetic approach to the furoxans is known as the Wieland synthesis,[28,27] where the alkenes **75** are reacted with dinitrogen trioxide formed from the reaction between sodium nitrite and acetic acid. This generates α-nitroketoximes **76** *in situ* which then undergo a dehydration and cyclisation reaction to give the furoxans **59** (See Scheme 17, chapter1).

A series of novel furoxans with halogen substituents were synthesised based on the commercial availability of the terminal alkenes, whilst ensuring this would allow a direct comparison to the OZO series in terms of stability and the rate of release of NO (Figure 5). As the furoxan structure is unsymmetrical there is the possibility of two isomers being formed, the 4-phenyl isomer **164-169a** and the 3-phenyl isomer **164-169b**, both of which are represented in Figure 5.

Figure 5:



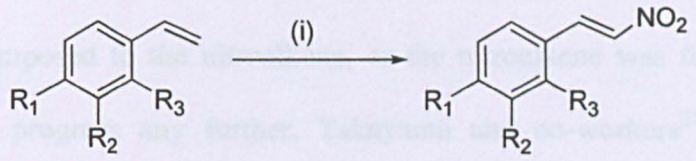
164-169a **164-169b**

$R_1 = R_2 = H$, $R_3 = Cl$ **164**
$R_1 = R_3 = H$, $R_2 = Cl$ **165**
$R_1 = Cl$, $R_3 = R_2 = H$ **166**
$R_1 = R_2 = H$, $R_3 = F$ **167**
$R_1 = R_3 = H$, $R_2 = F$ **168**
$R_1 = F$, $R_3 = R_2 = H$ **169**

A series of unsymmetrical furoxan target compounds **164-169**

The twelve furoxan derivatives **164-169** were synthesised following the procedure by Gasco.[29] The alkenes were dissolved in acetic acid and a 5M excess of sodium nitrite in water was added while the reaction mixtures were kept at a low temperature. The reaction mixtures were columned and recrystallised from petroleum ether to give what was presumed to be the unsymmetrical furoxans and the initial low resolution mass spectrometry data supported this. However, high resolution mass spectrometry data suggested that the corresponding nitroalkenes **170b-g** (Scheme 26), also known as the β-nitrostyrenes, rather than the furoxans were formed, which was supported by X-ray crystallography of **170d** and **170g** (Figures 6 and 7).

Scheme 26:



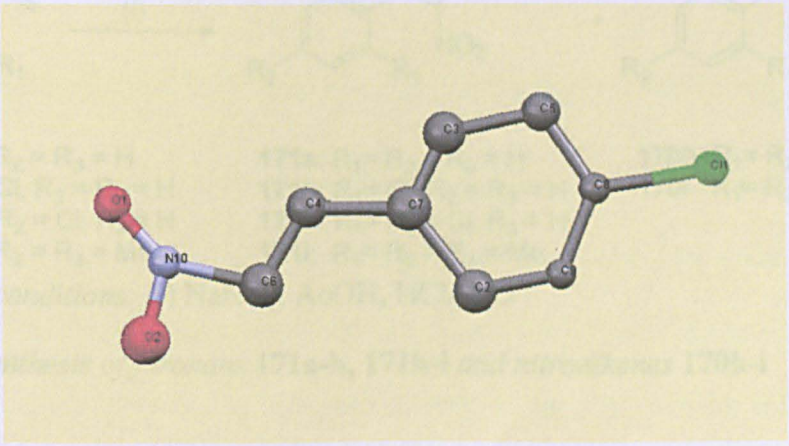
163b-g **170b-g**

$R_1 = R_2 = H, R_3 = Cl$ **163b**	$R_1 = R_2 = H, R_3 = Cl$ **170b**
$R_1 = R_3 = H, R_2 = Cl$ **163c**	$R_1 = R_3 = H, R_2 = Cl$ **170c**
$R_1 = Cl, R_3 = R_2 = H$ **163d**	$R_1 = Cl, R_3 = R_2 = H$ **170d**
$R_1 = R_2 = H, R_3 = F$ **163e**	$R_1 = R_2 = H, R_3 = F$ **170e**
$R_1 = R_3 = H, R_2 = F$ **163f**	$R_1 = R_3 = H, R_2 = F$ **170f**
$R_1 = F, R_3 = R_2 = H$ **163g**	$R_1 = F, R_3 = R_2 = H$ **170g**

Reagents and conditions: (i) 5eq. $NaNO_2$, $AcOH/H_2O$

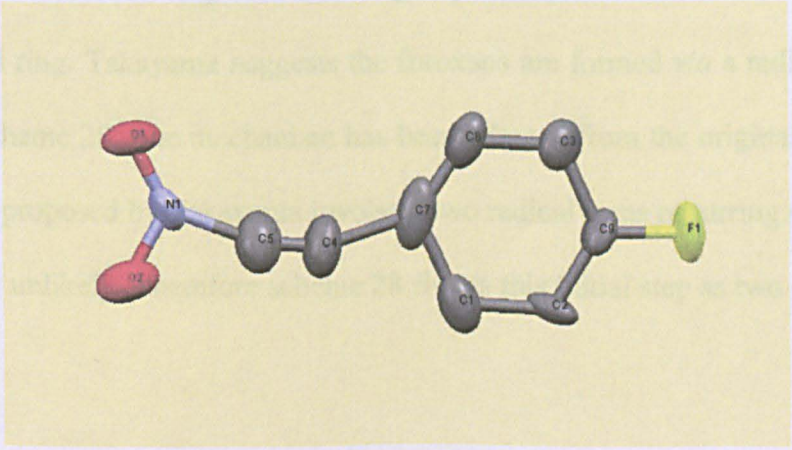
The synthesis of β-nitrostyrenes **170b-g**

Figure 6:



X-Ray crystal structure of **170d**

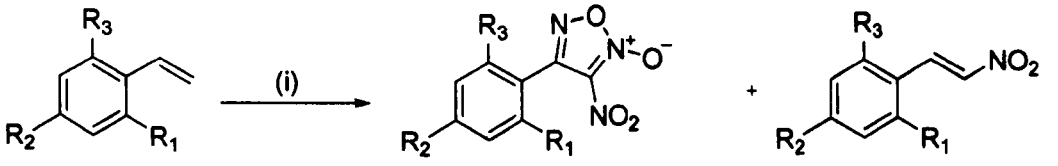
Figure 7:



X-Ray crystal structure of **170g**

There are two plausible explanations for this alternative product, either the furoxan was formed and decomposed to the nitroalkene, or the nitroalkene was formed first and the reaction did not progress any further. Takayama and co-workers[28] found that when employing the Wieland synthesis to terminal alkenes some of the derivatives formed the corresponding nitroalkene, in addition to the desired 3-phenyl-4-nitrofuroxan (Scheme 27), in particular, this was seen when compounds had substituents in the ortho and para positions of the aromatic ring.

Scheme 27:



163a: $R_1 = R_2 = R_3 = H$
163b: $R_1 = Cl$, $R_2 = R_3 = H$
163h: $R_1 = R_2 = Cl$, $R_3 = H$
163i: $R_1 = R_2 = R_3 = Me$

171a: $R_1 = R_2 = R_3 = H$
171b: $R_1 = Cl$, $R_2 = R_3 = H$
171h: $R_1 = R_2 = Cl$, $R_3 = H$
171i: $R_1 = R_2 = R_3 = Me$

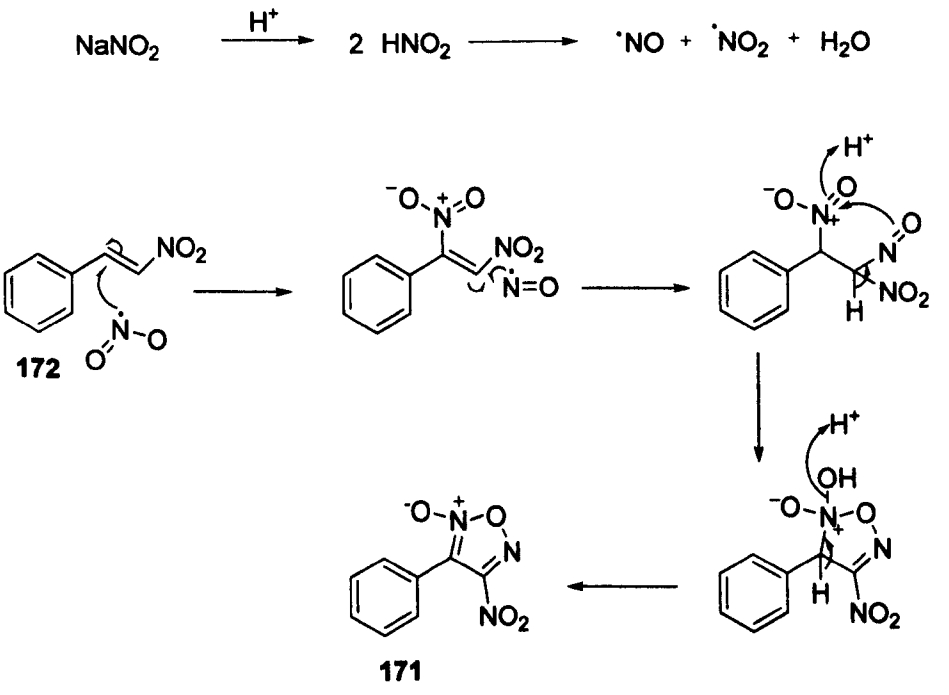
170h $R_1 = R_2 = Cl$, $R_3 = H$
170i $R_1 = R_2 = R_3 = Me$

Reagents and conditions: (i) $NaNO_2$, AcOH, HCl/H_2O

Takayama's synthesis of furoxans **171a-b, 171h-i** *and nitroalkenes* **170h-i**

For the six alkenes **163b-g** reacted using the Wieland route, none of the products showed any evidence of a furoxan ring with a nitro group attached to the 3- or 4- position of the five membered ring. Takayama suggests the furoxans are formed *via* a radical mechanism as shown in scheme 28. The mechanism has been adapted from the original mechanism as the initial step proposed by Takayama involved two radical steps occurring simultaneously, which is rather unlikely. Therefore scheme 28 shows this initial step as two different steps.

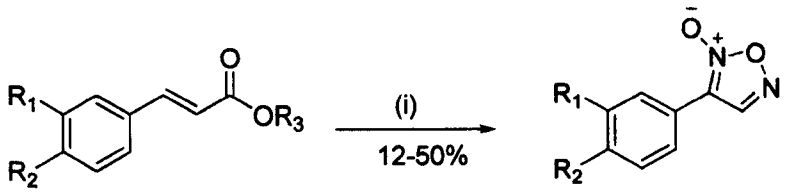
Scheme 28:



Takayama's mechanism for the formation of furoxans **171a-b, 171h-i** *(adapted)*[28]

The literature only describes a few examples of monosubstituted furoxans similar to those shown in figure 5, and to our knowledge only a few have been synthesised by nitrosation of an alkene. Cotelle and Vizin reported monosubstituted **173a** (Scheme 29) as the main product from the reaction of caffeic methyl ester **174a** with sodium nitrite at low pH. However, only ^{1}H NMR data of the compound was obtained as the furoxan **173a** rapidly decomposed at room temperature.[30] Similar reactions carried out by Kikugawa also resulted in the formation of a monosubstituted furoxan **173b** from **174b**,[31] and other work by Torres and Rosazza showed formation of **173c** from **174c**.[32]

Scheme 29:



174a R_1 = OH, R_2 = OH, R_3 = CH_3
174b R_1 = H, R_2 = OH, R_3 = H
174c R_1 = OCH_3, R_2 = OH, R_3 = H

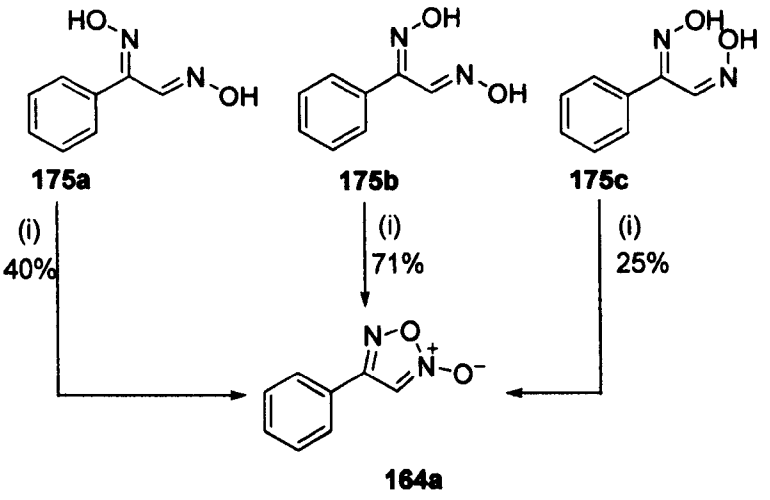
173a R_1 = OH, R_2 = OH
173b R_1 = H, R_2 = OH
173c R_1 = OCH_3, R_2 = OH

Reagents and conditions: (i) $NaNO_2$, pH 1-3 buffers

Synthesis of monosubstituted furoxans **173a-c** *from alkenes* **174a-c**

Compund **173a** synthesised by Cotelle and Vizin and reported to be unstable was also synthesised by Napolitano and d'Ischia through the same reaction of **174a** with sodium nitrite at low pH.[33] The resulting furoxan was fully characterised but its stability was not mentioned, nor was it for compounds **173b** or **173c**. This data presents a confusing picture of the stability of monosubstituted furoxans as no extensive stability studies for these compounds have been published. The earlier work on these compounds, in the early 1900s, was carried out before the exact structure of the furoxan ring was determined and prior to reliable structure determination instrumentation, which has resulted in some earlier reports of monosubstituted furoxans being discredited.[34] More recently Burakevich and co-workers reported the synthesis of 4-phenylfuroxan-2-oxide **164a** from the three isomers of phenylglycoxime **175a-c** (Scheme 30).[35]

Scheme 30:

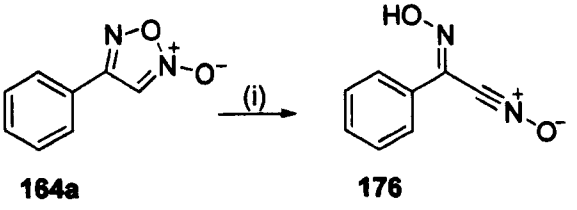


Reagents and conditions: (i) N_2O_4, ether, 15 min

Synthesis of monosubstituted 4-phenylfuroxan-2-oxide **164a** *from phenylglycoximes* **175a-c**

Further work on compound **164a** revealed it to be unstable and to readily decompose to the nitrile oxide **176** when dissolved in certain solvents such as acetone or alcohol-water. Similar instability was observed when **164a** was exposed to base (Scheme 31).[36]

Scheme 31:



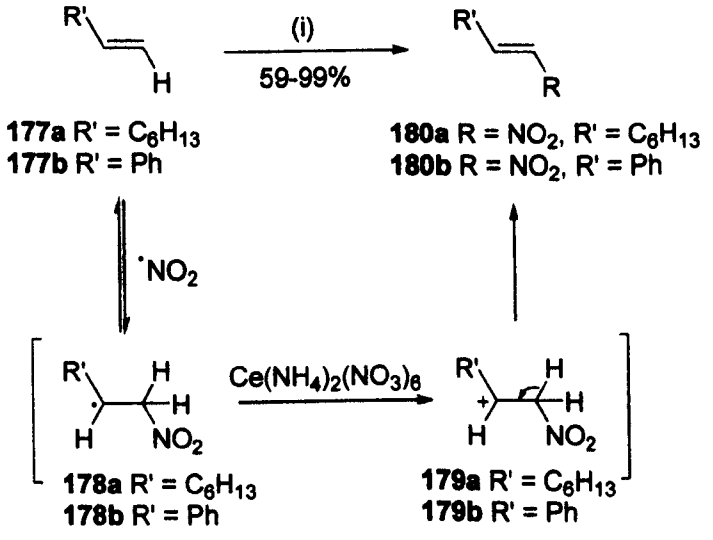
Reagents and conditions: (i) base

Decomposition of 4-phenylfuroxan-2-oxide **164a** *to the corresponding nitrile oxide* **176**

There are no reports for the decomposition of a furoxan to give a nitroalkene, except for a proposal by Takayama, although it is not clear if the nitroalkenes were produced by the decomposition of the furoxans or formed as a by-product unrelated to the furoxans.[28]

Hwu and co-workers synthesised nitroalkenes **180a-b** from their corresponding alkenes **177a-b** using $NaNO_2$ and acetic acid in chloroform in combination with the oxidising agent cerium (IV) ammonium nitrate (CAN). After sonication for four hours at 25-73°C, the nitroalkenes **180a-b** were obtained from the corresponding alkene in 54-81% yield (Scheme 32). Hwu and co-workers proposed a radical route to the β-nitrostyrene where radical attack at the β-carbon, followed by oxidation gives the nitroalkene. The authors also report the successful synthesis of the nitroalkenes in the absence of the oxidising agent CAN, albeit in a lower yield of 45% compared to 81%.

Scheme 32:



Reagents and conditions: (i) $NaNO_2$, AcOH, $CHCl_3$, CAN, 4hrs, 25-73°C

The synthesis of nitroalkenes **180a-b** *from the corresponding styrenes* **177a-b** *(Adapted)*[37]

As mentioned previously another possibility for the formation of the nitroalkenes is when the reaction does not progress beyond nitrosation of the terminal β-carbon of the styrene. Unlike the procedure by Takayama (Scheme 27), our procedure did not utilise hydrochloric acid and it is possible that the chloride is required on the α-carbon to act as a leaving group for a nitrosation step, followed by a dehydration step to finally give the furoxan.

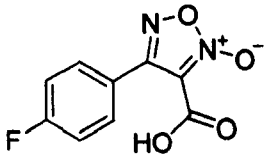
The apparent inherent instability of the monosubstituted furoxans suggests that the substituents attached to either side of the furoxan ring may determine the overall stability. This is consistent with the substituent effects observed for the thermal and photochemical isomerisation of the furoxans (Chapter 1, Section 1.2.3.2).

The synthesis of nitroalkenes, also referred to as the β-nitrostyrenes, such as those reported herein is generally difficult when starting from the parent styrene. This is due to the favoured nitration of the aromatic ring[38] and the formation of a mixture of compounds.[39] In addition, the reported procedures often require either expensive[40] or dangerous reagents.[41] Our treatment of styrenes with sodium nitrite in acetic acid is a novel and mild method to the synthesis of β-nitrostyrenes from the corresponding styrenes. This procedure gives the nitroalkenes in moderate to high yields (30-70%) with a reaction time of only 1-3 hours.

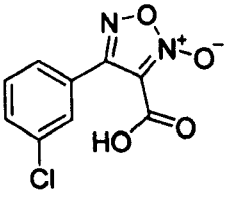
2.3.2 The synthesis of cinnamic furoxans

In the search for a series of furoxans which could be easily compared to the OZOs, two new target compounds were proposed (Figure 8). These novel target compounds **181** and **182** contain an acid group, which allows for the possibility of easily linking the furoxan moiety to an anti-cancer drug via acid chloride chemistry.

Figure 8:



181



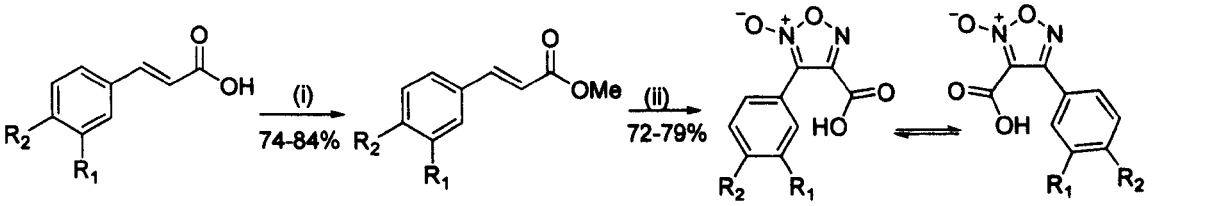
182

Two cinnamic furoxans **181** *and* **182**

The synthesis of the unsymmetrical furoxans **181** and **182** (Scheme 33) proved difficult as the cinnamic acids **183** and **184** were insoluble in the acetic acid, and the initial attempt at the synthesis produced only starting material. In order to overcome this solubility issue, the methyl esters **185** and **186** was synthesised in high yield using sulphuric acid and methanol (Scheme 33). However, once the methyl esters **185** and **186** were dissolved in the acetic acid, a white precipitate formed, which ^{1}H NMR spectroscopy showed to be the cinnamic acids **183** and **184**. A second attempt at synthesising the furoxans was carried out dissolving the methyl ester in a mixture of acetic acid and sulphuric acid prior to addition of the aqueous solution of sodium nitrite. Both reactions were left to stir at room temperature overnight after the addition of 5 equivalents of sodium nitrite. The aqueous solutions were extracted with dichloromethane since the crystals obtained by precipitation

in ice-cold water were too unstable to filter. Both red solids obtained from these reactions were recrystallised from dichloromethane to give a yellow solid for **181** and a white solid for **182**.

Scheme 33:



183 R_1 = H, R_2 = F
184 R_1 = Cl, R_2 = H

185 R_1 = H, R_2 = F
186 R_1 = Cl, R_2 = H

181a R_1 = H, R_2 = F
182a R_1 = Cl, R_2 = H

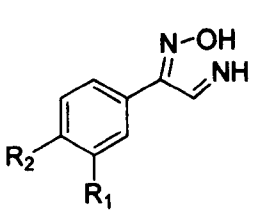
181b R_1 = H, R_2 = F
182b R_1 = Cl, R_2 = H

Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 60°C, 4hrs (ii) $NaNO_2$, H_2O, $AcOH/H_2SO_4$

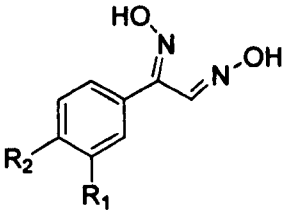
Synthesis of cinnamic furoxans **181** *and* **182**

The loss of the alkene protons in the ^1H NMR spectrum supported the formation of the furoxan ring and the ^{13}C NMR spectrum showed that the carbonyl group was still in place. However, mass spectrometry of the two furoxans was not in accordance with the NMR analysis suggesting that the two furoxans had decomposed to give ring opened products as shown in figure 9. These decomposition products **187** and **188** can exist as isomer similar to the furoxans, however it is not possible to determine which isomers are present from the available data.

Figure 9:



188 R_1 = Cl, R_2 = H

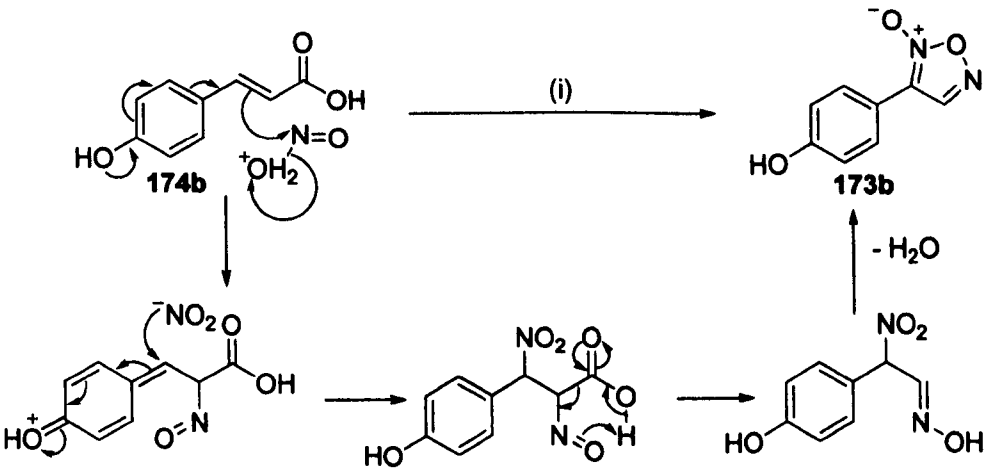


187 R_1 = H, R_2 = F

Decomposition products **187** *and* **188**

As shown in section 2.3.1 (scheme 29) a similar synthesis on cinnamic acids **174a-c** gave the monosubstituted furoxans **173a-c**. Torres and Rosazza suggests that the nitrosation of the double bond leads to decarboxylation of **174c** to give compound **173c** (Scheme 34).[32] Similar proposals are also made by Kikugawa and co-workers[31] and Cotelle and Vezin[30] for the formation of compounds **173a** and **173b** from compounds **174a-b**.

Scheme 34:



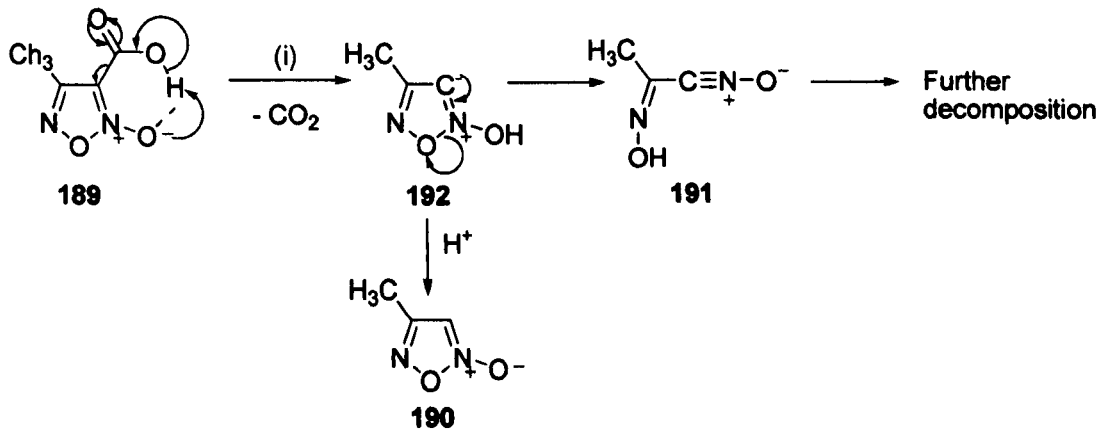
Reagents and conditions: (i) $NaNO_2$, pH 1-3 buffers

Synthesis of monosubstituted furoxan **173b** *from alkene* **174b** *(Adapted)*[30,32]

Neither the suggested mechanism nor the reaction product in scheme 34 is in accordance with that found in our synthetic work as ^1H and ^{13}C NMR analysis showed no indication of

decarboxylation. A more probable route to the decomposition of **181** and **182** is that suggested by Gasco,[42] as shown in scheme 35.

Scheme 35:



Reagents and conditions: (i) Xylene, 120°C, 2 hrs

Decomposition of 4-methyl-3-furoxancarboxylic acid **189** *to give monosubstituted furoxan* **190** *and the nitrile oxide* **191** *(Adapted)*[42]

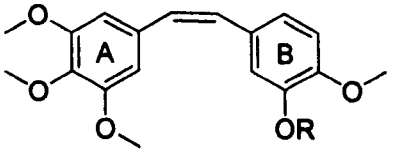
Regardless of the atctual mechanism, the attempted synthesis of cinnamic furoxans **181** and **182** has proved to be unsuccessful, possible due to the products being too unstable for any chemical or biological analysis. Decarboxylation is the most likely explanation, resulting in the monosubstituted furoxans, which have been shown to be unstable in previous studies (Section 2.3.1).[36]

2.3.3 The synthesis of symmetrical furoxans embedded within a combretastatin core structure – NO-hybrids

Within the last decade there has been a substantial increase in the number of NO-donors being tested and showing activity against a wide variety of cancer cell lines.[43–47] Whether the NO molecule itself is anti-cancerous or not, is still not clear (See chapter 1, section 1.3). Regardless of this, many NO-donor compounds are showing anti-cancer activity and therefore it is still an area of intense research.[43,48–50]

Combretastatin A-4 **118** is a potent anti cancer drug, with a LD_{50} value of 0.007μM against murin L1210 leukaemia cell lines. This compound is also active against colon and lung cancers.[51,52] The water-soluble phosphate derivative **117** which acts as a prodrug, is currently undergoing Phase II trials in the USA and the UK (Figure 10) as described in chapter 1.[53]

Figure 10:



117 R = PO_3Na_2
118 R = H

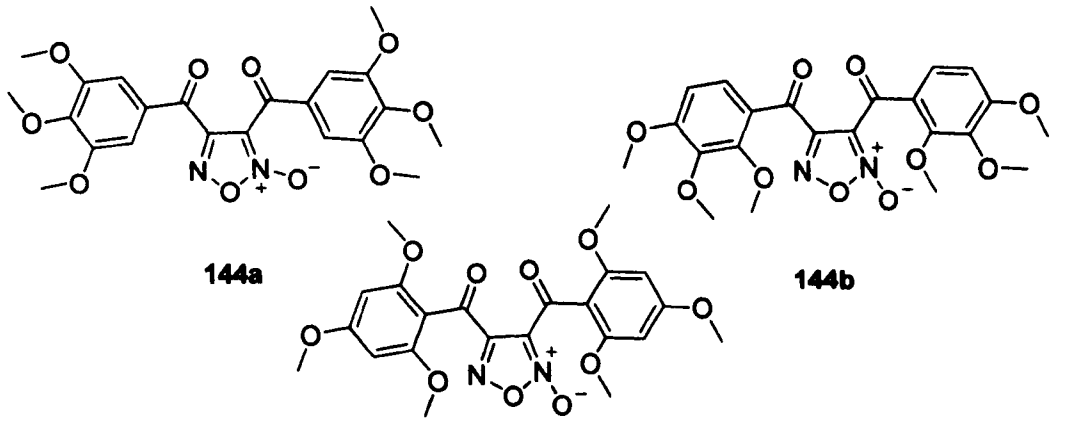
Combretastatin A-4 **118** *and its phosphate prodrug* **117**

Combretastatin A-4 **118** was extracted and isolated in 1989 from the bark of African willow tree *C. Caffrum* by Pettit and co-workers at Arizona State University.[53] A number of structure–activity studies have been performed on the combretastatins, revealing several key structural features which are important to their biological activity (Chapter 1, section

1.3.4.1). Combretastatin A-4 **118** (Figure 10) has two aryl rings separated by a rigid spacer, in this case a double bond, with ring A having three methoxy groups in positions 3, 4 and 5 and ring B has a methoxy group in the 4-position and a hydroxyl group in the 3-position. It is widely acknowledged that these structural features are needed for optimum activity.[54]

Based on the structure-activity information and known synthetic approaches a series of novel combretastatin-like furoxans were designed (Figure 11).

Figure 11:



144a **144b**

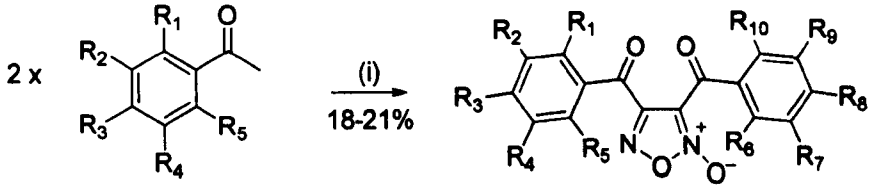
144c

Symmetrical combretastatin furoxan (combretafuroxans) target compounds **144a-c**

Following similar chemistry by Snyder and Boyer,[55] the symmetrical trimethoxy aryl furoxans **144a-c** were synthesised from the aryl methyl ketones **193a-b** using acetic acid, nitric acid and a catalytic amount of sodium nitrite (Scheme 36). In compound **144a** the A-ring is kept as in combretastatin A-4 **118** with methoxy groups in the 3-, 4- and 5-positions, but the rigidity of the alkene is replaced with a furoxan ring flanked by carbonyl groups with the B-ring being symmetrical with the A-ring.

3,4,5-Trimethoxyacetophenone **193a** was dissolved in acetic acid and concentrated nitric acid was added dropwise while heating the reaction to 60°C. After the reaction mixture had reached 60°C a catalytic amount of $NaNO_2$ was added, resulting in a colour change from pale orange to bright red. The reaction mixture was heated for 2 hours before it was quenched by pouring it onto ice-water.

Scheme 36:



193a $R_1 = R_5 = H$, $R_2 = R_3 = R_4 = OMe$ **144a** $R_1 = R_5 = R_6 = R_{10} = H$, $R_2 = R_3 = R_4 = R_7 = R_8 = R_9 = OMe$
193b $R_1 = R_2 = H$, $R_3 = R_4 = R_5 = OMe$ **144b** $R_1 = R_2 = R_9 = R_{10} = H$, $R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = OMe$
193c $R_2 = R_4 = H$, $R_1 = R_3 = R_5 = OMe$ **144c** $R_2 = R_4 = R_7 = R_9 = H$, $R_1 = R_3 = R_5 = R_6 = R_8 = R_{10} = OMe$

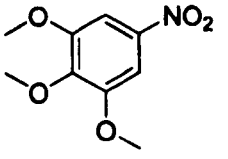
Reagents and conditions: (i) HNO_3/AcOH, $NaNO_2$, 60°C, 2hrs

The synthesis of novel symmetrical combretastatin furoxan (combretafuroxans) **144a-c**

The reaction work-up produced two products, a yellow solid obtained from pouring the reaction mixture onto ice-water, and a yellow oil obtained by extracting the aqueous filtrate with dichloromethane. ^1H NMR analysis of the two crude products showed similar signals, suggesting the oil and the solid contained the same material and TLC analysis suggested the presence of three main products in each sample. The crude materials were combined and purified by column chromatograpy using 1:1, ethyl acetate : petroleum ether. The first fraction to elute was analysed by ^1H NMR and found to be starting material, whilst the second fraction to elute off the column showed product with traces of starting material. These fractions were recrystallised from petroleum ether to give small yellow crystals in a yield of 10%. ^1H NMR spectroscopy of the yellow crystals of compound **144a** showed

three new peaks in the aromatic region, which suggested the presence of four aromatic protons by integration. In addition the spectrum showed singlets at 3.78-3.89 attributable to the methoxy groups and ^{13}C NMR spectroscopy revealed a second carbonyl group and a peak at 107.9ppm attributed to the carbon at the 3-position of the furoxan ring. This combined with the loss of the peak at 2.58ppm attributed to the CH_3 of the methyl ketone suggested the formation of the furoxan ring which was confirmed by high resolution mass spectrometry. The third compound to elute off the column was analysed and found to be a nitrobenzene compound **194** (Figure 12), which was confirmed by low resolution mass spectrometry, NMR spectroscopy and a melting point which was consistent with literature data.[56] The formation of such nitrobenzene compounds from the reaction with nitric acid is not uncommon and was reported as early as 1927 by Wheeler and Harris.[57,58]

Figure 12:



194

Nitrobenzene byproduct **194**

The formation of the nitrobenzene compound as a by-product and the presence of unreacted starting material prompted another attempt at the synthesis of this compound. Snyder and Boye[55] reported the formation of the nitrobenzene compounds when performing this type of chemistry with concentrated nitric acid, therefore, it was decided to test if using diluted nitric acid would improve the yield of the product. A scaled up reaction using dilute nitric acid (11.2 ml of HNO_3 in 34 ml H_2O) produced the same three main

products without any reduction in the amount of the nitrobenzene **194**. After column chromatography and recrystallisation from petroleum ether, the furoxan **144a** was isolated in a 7% yield. In an effort to improve the yield the reaction was heated at 60°C for 1 hour while the nitric acid was added dropwise and then left to stir for 2 hours at this temperature before cooling to room temperature and stirring for two days. There was no significant increase in the overall yield of the furoxan nor in the amount of nitrobenzene produced although, the quantity of starting material recovered from the column decreased. The procedure was repeated using the two other acetophenones, **144b** and **144c**, which gave the novel furoxans in comparable yields to that of compound **144a**, with yields ranging from 9-11% after purification by column chromatography and several rounds of recrystallisation.

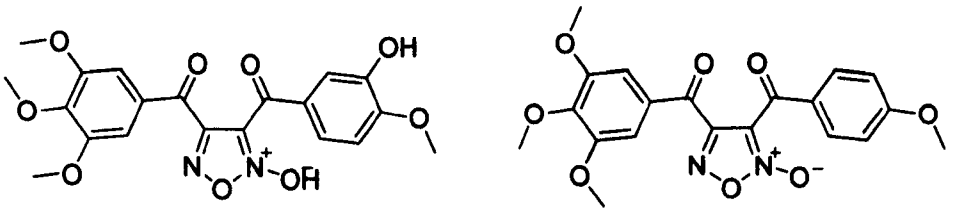
Compared to other heterocyclic combretastatin analogues, such as those obtained from a eight-step synthesis, compounds **136** and **137**, Chapter 1, section 1.3.4.1, Figure 3) by Ley and co-workers,[59] the synthetic route to our combretastatin-like furoxans is straightforward and achievable in one step. In addition, our compounds have the added benefit of potentially releasing NO whilst maintaining many of the key combretastatin features.

2.4 Future work

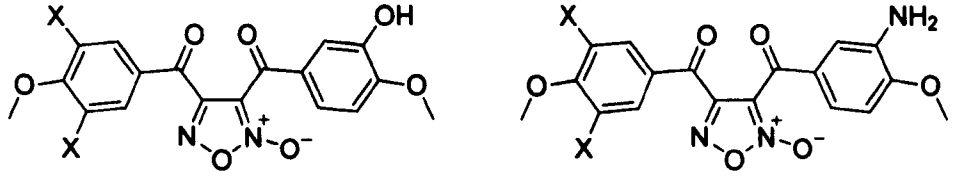
The novel combretastatin-like furoxans **144a-c** were successfully synthesised from the corresponding methyl ketones **193a-c** as described in section 2.3.3. The ease of synthesis for these compounds combined with the wide range of commercially available methyl ketones introduces the potential for a number of similar symmetrical and asymmetrical compounds to be synthesised and tested for anti-cancer activity. The mixing of methyl

ketone starting materials will clearly provide a route to asymmetrical derivatives. Figure 13 shows a number of future targets based on the structure-activity relationship studies for combretastatin A-4 **119** as described in Chapter 1, section 1.3.4.1. Particularly interesting is the proposed dihalogenation in the 3- and 5-position of the A-ring and replacing the hydroxyl group in the 3-position on the B-ring with an amine.

Figure 13:



148e **148f**



148g X = Br
148h X = F
148i X = I
148j X = Cl

148k X = Br
148l X = F
148m X = I
148n X = Cl

Future target compounds based on recent literature findings

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3 Results and discussion – Chemical stability and biological data

As early as 1988 the anti-cancerous effects of NO were reported by Hibbs and co-workers,[1] shortly after the role of NO in the vasculature was revealed.[2–4] More than two decades later the role of NO in cancer is filled with conflicting information and there are more questions than answers.[5–7] However, several NO-donors have been tested and found to be active anti-cancer agents and therefore this area of research continues to grow.[8–14]

Ovarian cancer is the fourth most common cause of cancer-related deaths among women in the UK, with over 4000 deaths in 2008. Although there has been a marked increase in survival statistics over the last few decades, the relative survival rates are still low. The one-year survival rate is 70% with the five-year survival rate reported as only 41%.[15,16] The biggest challenge concerned with the treatment of ovarian cancer is the high occurrence of relapse and the development of resistance to platinum based chemotherapy agents such as carboplatin **110** (Chapter 1, section 1.3.3, figure 27). Two recent phase trials, phase Ib and phase II, have shown promising results for the treatment of ovarian cancer, with a higher response rate to the combination of combretastatin A-4 phosphate, **117** (Chapter 1, section 1.3.3, figure 29) with carboplatin **110** and paclitaxel **111**, than with carboplatin **110** and paclitaxel **111** on their own.[17,18]

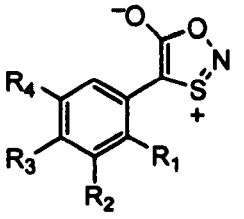
As detailed in chapter 2, a number of NO-donors have been synthesised based on compounds which have been shown to have an anti-cancer effect. The OZOs, β-nitrostyrenes and combretastatin-like furoxans were submitted for anticancer testing.

3.1 Stability studies

With the focus on the planned *in vitro* work for the target compounds synthesised, as detailed in chapter 2, a number of UV-Vis spectroscopy experiments were performed to determine what precautions and handling instructions were required to prevent decomposition of the NO-donors prior to testing.

3.1.1 Stability studies - Aryl-1,3,2-oxathiazolylium-5-olates

Figure 1:



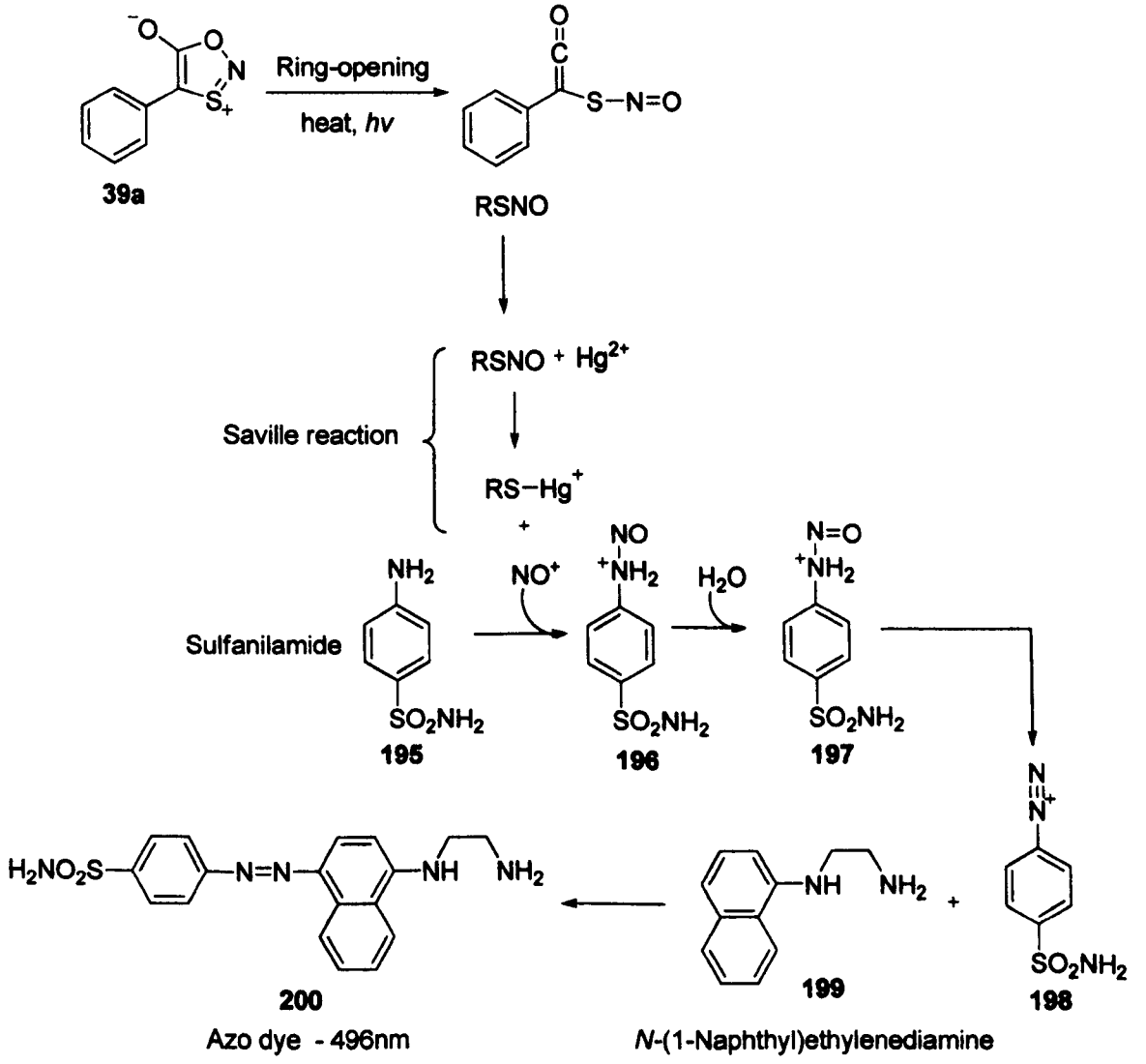
39a $R_1 = R_2 = R_3 = R_4 = H$
39f $R_1 = R_2 = R_4 = H$, $R_3 = Cl$
39h $R_1 = R_3 = F$, $R_2 = R_4 = H$
39i $R_1 = R_3 = H$, $R_2 = R_4 = F$
39j $R_1 = R_2 = R_4 = H$, $R_3 = F$
39k $R_1 = R_3 = R_4 = H$, $R_2 = Cl$
39l $R_1 = Cl$, $R_2 = R_3 = R_4 = H$

The aryl-1,3,2-oxathiazolylium-5-olates, OZOs, synthesised for anti-cancer testing

A number of factors can affect and promote the decomposition of *S*-nitrosothiols, such as heat,[19–21] light,[19,21] metals,[19,22–28] thiols[19,21,29–31] and enzymes.[19–21,32–34] In order to quantify the amount of NO released, the Griess test was employed. Scheme 1 illustrates the chemical method used to quantify NO levels using the formation of an azo dye which is directly related to the amount of NO present in the sample. The initial step involves the ring opening of the OZO **39a** initiated by heat or light to give the ring RSNO, followed by the Saville reaction where mercuric chloride in water gives Hg^{2+}. The metal ion binds to

the sulphur of the RSNO molecule causing the release of NO in the form of NO^+. Sulfanamide **195** (SULF) reacts with the generated NO^+ to give the diazonium ion **198** which then reacts with *N*-(1-Napthyl)ethylenediamine **199** (NEDD), to give the azo dye **200** (Scheme 1).[35]

Scheme 1:



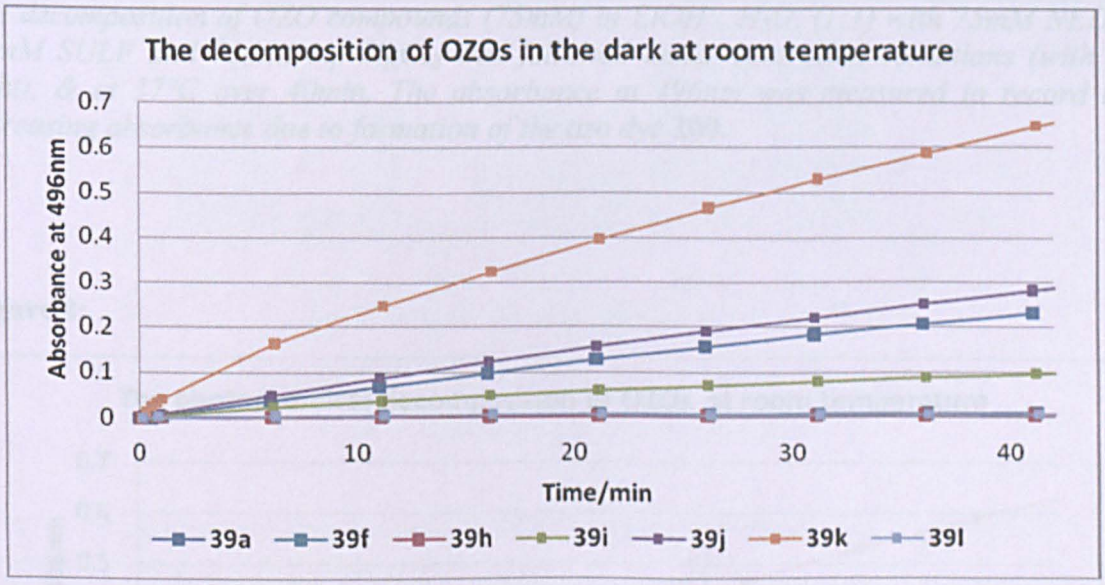
Saville and Griess reaction used to quantify the amount of NO released (Adapted)[36]

The UV-Vis work was carried out in ethanol : H_2O, 1:1, and two key conditions were investigated for their effect on the decomposition of the OZOs; exposure to heat and light.

Two different temperatures were investigated, lab temperature (20°C) and physiological temperature (37°C).

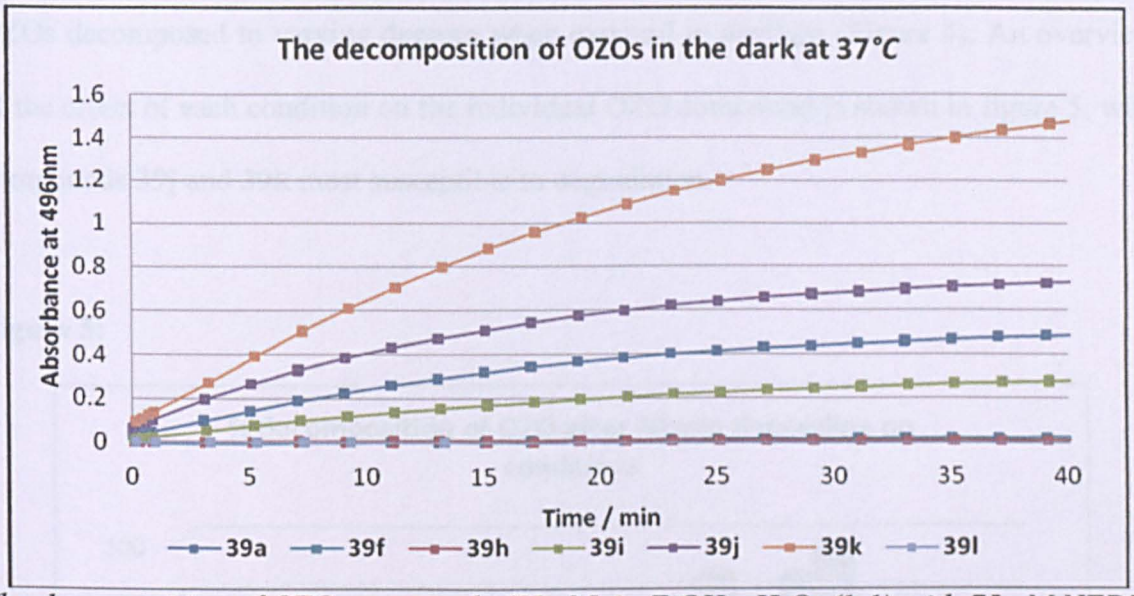
As illustrated in figure 2 even at 20°C there was a significant effect on some of the OZO compounds, specifically **39a**, **39i**, **39j** and **39k**. The same trend can be seen in figure 3, with the same four OZO compounds rapidly decomposing and the remaining three (**39f**, **39h** and **39l**) showing no significant decomposition.

Figure 2:



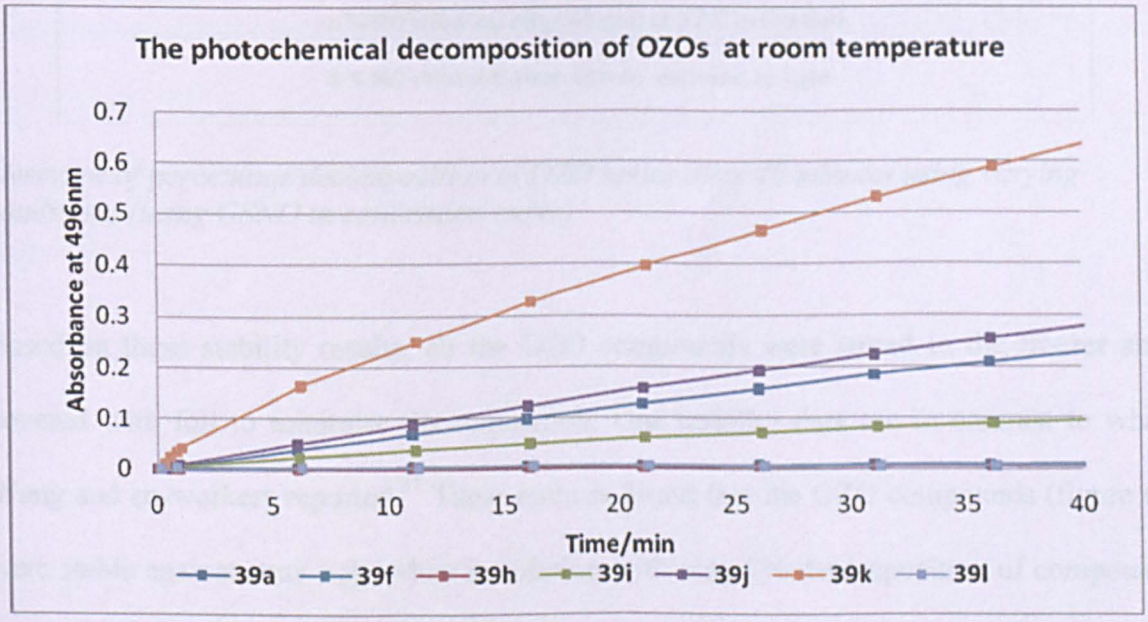
The decomposition of OZO compounds (75mM) in EtOH : H_2O, (1:1) with 75mM NEDD, 75mM SULF and 75mM aq. $HgCl_2$ was followed under controlled conditions (with no light), & at room temperature over 40min. The absorbance at 496nm was measured to record the increasing absorbance due to formation of the azo dye **200**.

Figure 3:



The decomposition of OZO compounds (75mM) in EtOH : H_2O, (1:1) with 75mM NEDD, 75mM SULF and 75mM aq. $HgCl_2$ was followed under controlled conditions (with no light), & at 37°C over 40min. The absorbance at 496nm was measured to record the increasing absorbance due to formation of the azo dye **200**.

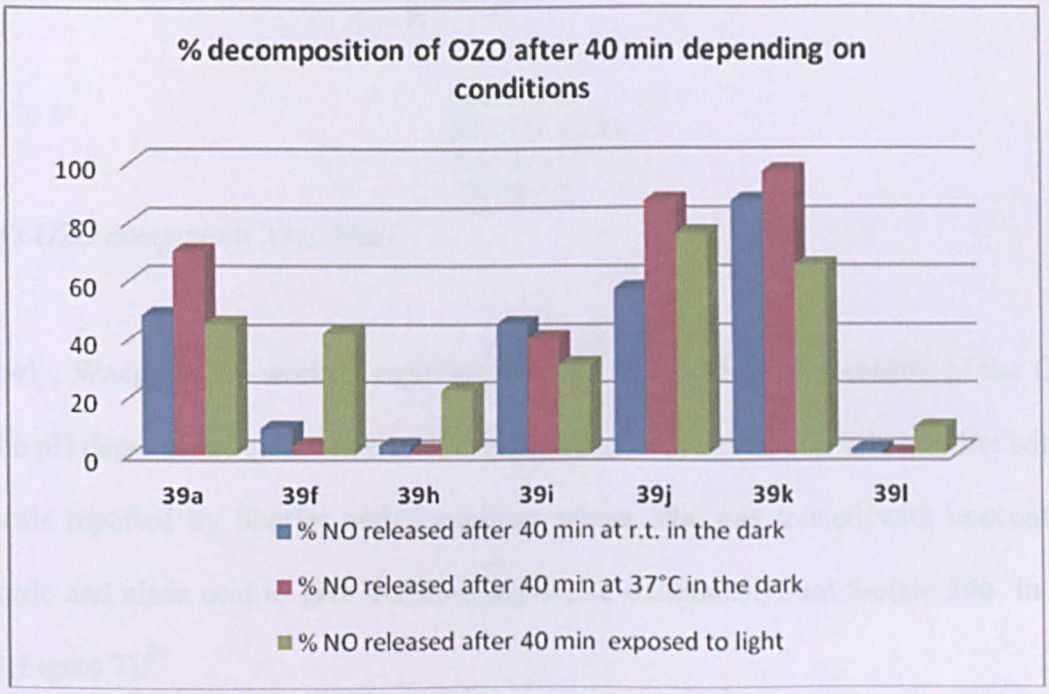
Figure 4:



The decomposition of OZO compounds (75mM) in EtOH : H_2O, (1:1) with 75mM NEDD, 75mM SULF and 75mM aq. $HgCl_2$ was followed when exposed to light at room temperature over 40min. The absorbance at 496nm was measured to record the increasing absorbance due to formation of the azo dye **200**.

Photochemical decomposition was shown to be the degradation route for where all the OZOs decomposed to varying degrees when exposed to daylight (Figure 4). An overview of the effect of each condition on the individual OZO compound is shown in figure 5, with compounds **39j** and **39k** most susceptible to degradation.

Figure 5:

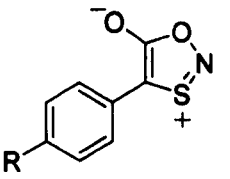


Overview of percentage decomposition of OZO series after 40 minutes using varying conditions (using GSNO in calibration curve)

Based on these stability results, all the OZO compounds were stored in the freezer and covered with foil to minimise decomposition. Our stability data are in contrast to what Wang and co-workers reported.[37] These authors found that the OZO compounds (figure 6) were stable against stray light when in solution with only 7% decomposition of compound **39a** over a period of 3 hours, whilst in the solid form, at room temperature, no decomposition was seen over several months.[37] Gotthardt on the other hand, reports the

decomposition of OZO compound **39a** to give benzonitrile as the final compound after exposing a solution of **39a** to visible light.[38]

Figure 6:

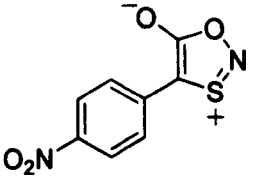


39a R = H
39d R = OCH_3
39e R = CF_3
39f R = Cl

Wang's OZO compounds **39a, 39d-f**

Similarly, Wang and co-workers reported the acid catalysed decomposition of the OZOs and the pH dependence on the rate of decomposition.[37] However, this is in conflict with the synthesis reported by Shaffer and Thompson where **39a** was treated with concentrated sulphuric and nitric acid to give 4-nitro-4-aryl-1,3,2-oxathiazolylium-5-olate **39o** in good yield (Figure 7).[39]

Figure 7:



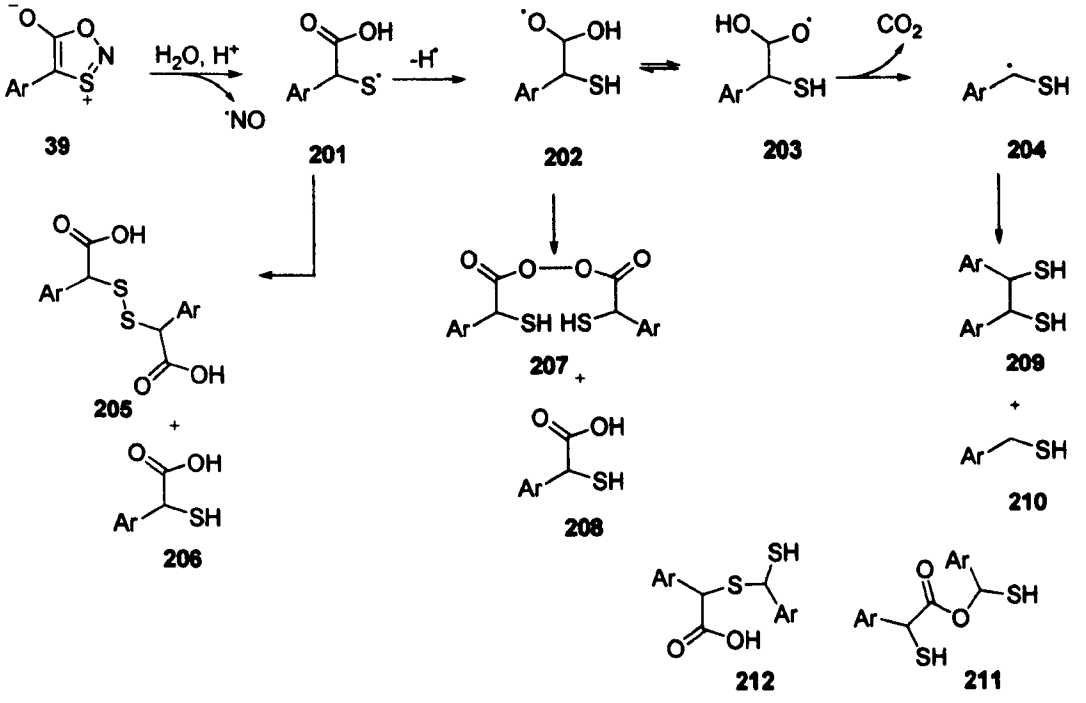
39p

4-Nitro-4-aryl-1,3,2-oxathiazolylium-5-olate **39p**

We investigated the significance of pH on the rate of decomposition of our compounds but found that dissolving the OZOs in buffer solutions gave complex UV-Vis spectra which

were difficult to analyse. Based on the electrospray ionisation mass spectrometric analysis of **39a**, both before and after acidification, Wang and co-workers assigned the fragmentation pattern to a number of decomposition products (Scheme 2). The structures of compounds **201-212** were assigned based on their respective molecular ions found in the fragmentation pattern of acidified **39a**. Compounds **211** and **212** are produced from the reaction of **201** or **202/203** with **204**.

Scheme 2:



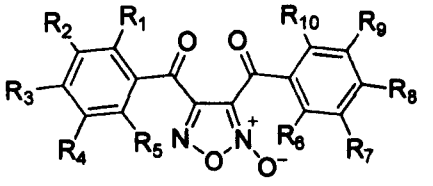
Acid catalysed decomposition of OZOs and decomposition products (Adapted)[37]

The complexity of our UV-Vis spectra was explained by the numerous potential degradation species which can be formed when the OZO compounds decompose. As a result this investigation was not continued as the optimisation of the synthetic routes for our other target compounds was deemed more important.

3.1.2 Stability studies - symmetrical furoxans as Combretastatin A-4 anticancer derivatives

Unlike the *S*-nitrosothiols, the furoxans are thermally and often photochemically stable, and require the presence of a thiol cofactor for the ring opening and release of NO to occur.

Figure 8:



144a $R_1 = R_5 = R_6 = R_{10} = H$, $R_2 = R_3 = R_4 = R_7 = R_8 = R_9 = OMe$
144b $R_1 = R_2 = R_9 = R_{10} = H$, $R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = OMe$
144c $R_2 = R_4 = R_7 = R_9 = H$, $R_1 = R_3 = R_5 = R_6 = R_8 = R_{10} = OMe$

Combretastatin analogues based on a furoxan core **144a-c**

The furoxan containing combretastatin derivatives **144a-c** are a series of diarylfuroxans similar to those synthesised by Auricchio, which were shown to decompose to the diphenylacetylenes **82a-c** when irradiated at 254nm (Chapter 1, section 1.2.3.2, Scheme 24).[40] In order to determine the photochemical stability of compounds **144a-c**, they were dissolved in deuterated chloroform, irradiated at 254nm and monitored by ^1H NMR spectroscopy at regular intervals to identify any decomposition to the corresponding diphenylacetylenes. None of the three compounds showed any decomposition over a two week period suggesting that under these conditions the furoxans were relatively stable.

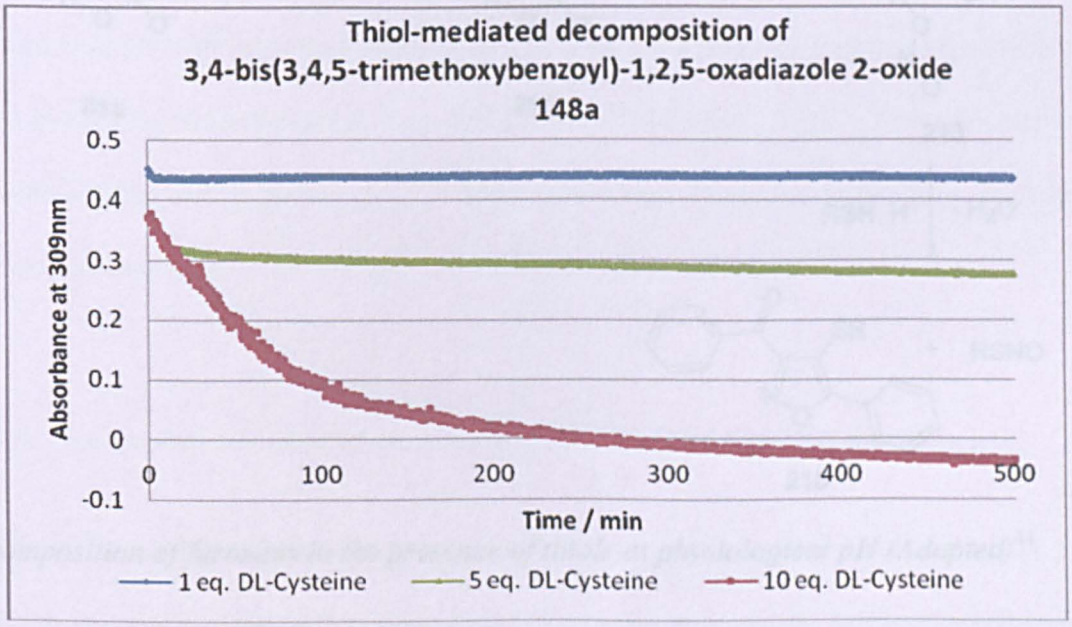
The thermal stability of the furoxans was also investigated by monitoring the λ_{max} for **144a-c** by UV-Vis spectroscopy at both room temperature and at 37°C. None of the compounds showed any decomposition over a period of 24 hours.

Attempts were also made to analyse the amount of NO release in the presence of thiols using the method by Nirode and co-workers.[41] The three furoxans were dissolved in pH 7.4 buffer with EDTA added to prevent any decomposition of the resulting nitrosated thiol due to traces of copper in the buffer solution. This was followed by the addition of the thiol (5 equivalents of DL-cysteine) and the Greiss test reagents (Scheme 1). By UV-Vis spectrophotomety no absorbance in the 540nm region attributable to the azo dye **200** could be seen. Although 496nm was the wavelength used for detection in the azo dye **200** for the OZO compounds, 540nm is used here due to the presence of pH buffers as the OZOs were analysed under pH neutral conditions.[36] The investigation into the thiol-mediated release of NO from these compounds was complicated by a number of factors. The biggest problem in this study was the solubility of these compounds as only a concentration of 0.01mM was possible in buffer solution and ethanol : H_2O, 1:1. The analysis was further complicated by the disturbance to the UV-Vis reading by both the EDTA and the DL-cysteine in the 200-300nm region where the furoxans absorbed, despite both reagents being added to the blank buffer sample. Nirode and co-workers found that the diarylfuroxans **81a-c** release between 3.4% and 18% NO from the furoxans in the thiol-mediated reaction.[41] Given the low concentration of the combretastatin furoxans **144a-c** it is possible that the amount of *S*-nitrosothiol formed, and thus any resulting dye was below the detection limits of the UV-Vis spectrophotometer.

The reaction was repeated using *N*-acetylcysteamine, in the hope that this would produce less interference in the UV-Vis region allowing the decomposition of the furoxans to be monitored by the decrease of absorbance by the individual furoxan. Changing the thiol did not alter the observations, with no azo dye **200** detected, and no improvement in the appearance in the UV-Vis spectra in the 200-300nm region.

However, for compound **144a** a clear decrease in its absorbance at 309nm was observed with the addition of the thiols, as seen in figure 9. Three different concentrations of thiol were investigated and the greatest drop in absorbance correlates to increasing levels of thiol. These results are in accordance with those found by Gasco showing that the rate of release of NO from the furoxans is dependent upon the concentration of the thiol co-factor.[42]

Figure 9:

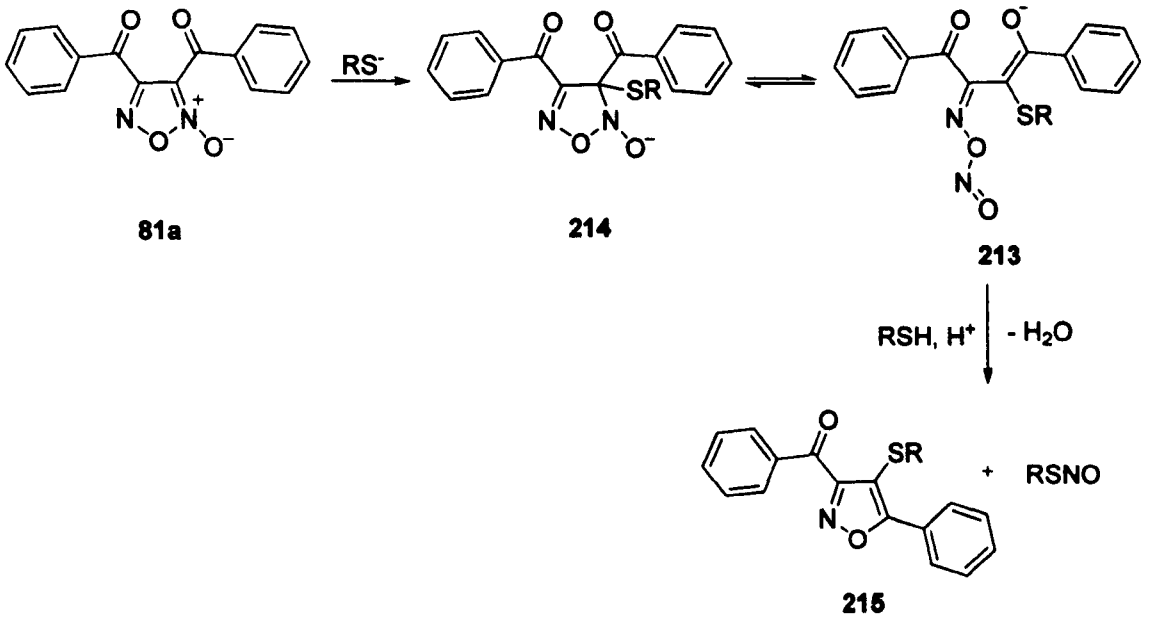


Thiol-mediated decomposition of 3,4-bis(3,4,5-trimethoxybenzoyl)-1,2,5-oxadiazole 2-oxide **144a**

The addition of 10 equivalents of thiol showed the largest drop in absorbance. As shown in scheme 3 the reaction between thiol and furoxan **81a** leads to the production of RSNO following the release of NO from **213**. For this process to occur, two moles of RS^- are required where one will bond with the furoxan **81a** and one will form the RSNO. However, there is also the possibility for the RS^- to attack the RSNO to give the corresponding

disulphide, RSSR which will reduce the amount of RS^- available to attack the furoxan **81a**. The release of NO prior to disulphide formation will again lead to the formation of a second RSNO which will further reduce the available RS^- required attack the furoxan **81a**. With 10 equivalents of thiol present the amount of RS^- is significant enough for all these competing reactions to occur without limiting the attack on the furoxan by the RS^-.

Scheme 3:



Decomposition of furoxans in the presence of thiols at physiological pH (Adapted)[41]

The combretastatin-like furoxans **144a-c** were found to be thermally and photochemically stable with the expected decomposition when treated with thiol, although we were unable to confirm the exact quantity of NO release from this decomposition, due to solubility issues.

3.2 *In vitro* cytotoxicity

Pure samples of **39a**, **39f**, **39h-l**, **170b-g** and **144a-c** from the OZO, β-nitrostyrene and combretastatin work, respectively were submitted for anticancer testing in DMSO. The samples were treated in accordance with the data obtained from the stability studies and were therefore kept in the freezer, and covered with foil, to avoid decomposition.

The initial tests carried out on all the compounds **39a**, **39f**, **39h-l**, **170b-g** and **144a-c** determined their IC_{50} values using a cell proliferation assay and the procedures previously published.[43] Cells from ovarian cancer cell lines such as Skov-3 were added in a growth medium to a 96-well plate and after 24 hours in an incubator our target compounds were added in varying concentrations from 100 μM to 0.0085 μM (8.5 nM). The cells were then incubated again for a period of 72 hours and were then analysed. Any compounds requiring a concentration of 100 μM, or more, were deemed inactive.

3.2.1 In vitro cytotoxicity - Aryl-1,3,2-oxathiazolylium-5-olates 39a, 39f, 39h-l

In 2008 Wang and co-workers reported the anticancer activity of a series of 4-aryl-1,3,2-oxathiazolylium-5-olates, OZOs, **39a, 39d-f** (Figure 6) in the K562 leukaemia cell line and the Panc-1 breast cancer cell line.[44] Compound **39a** was the most potent of these compounds with an IC_{50} of 62μM in the Panc-1 cell line and 42 μM in the K562 cell line. Two other derivatives, **39d** and **39e** had values in the 120-150μM region with **39f** having a value above 200μM. Their activity was shown to be related to inhibiton of the enzyme GST, Glutathione-*S*-transferases, in the cancer cells.[44]

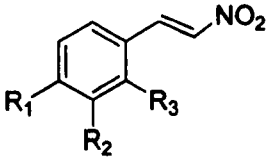
Compounds **39a**, **39f**, **39h-l** (Figure 1) were tested against eight ovarian cancer cell lines, Igrov, Skov, Ovcar3, Ovcar4, Ovcar5, Ovcar8, A2780 and cisA2780. None of the

compounds displayed any cytotoxic activity below 100μM, and as such all were deemed inactive.

3.2.2 *In vitro* cytotoxicity – nitroalkenes (β-nitrostyrene) 170b-g

The nitroalkenes **170b-g** were all tested in the cell proliferation assays using the same eight cell lines as those used to test the OZO compounds (See section 3.2.1).

Figure 10:



170b-g

$R_1 = R_2 = H$, $R_3 = Cl$ **170b**
$R_1 = R_3 = H$, $R_2 = Cl$ **170c**
$R_1 = Cl$, $R_3 = R_2 = H$ **170d**
$R_1 = R_2 = H$, $R_3 = F$ **170e**
$R_1 = R_3 = H$, $R_2 = F$ **170f**
$R_1 = F$, $R_3 = R_2 = H$ **170g**

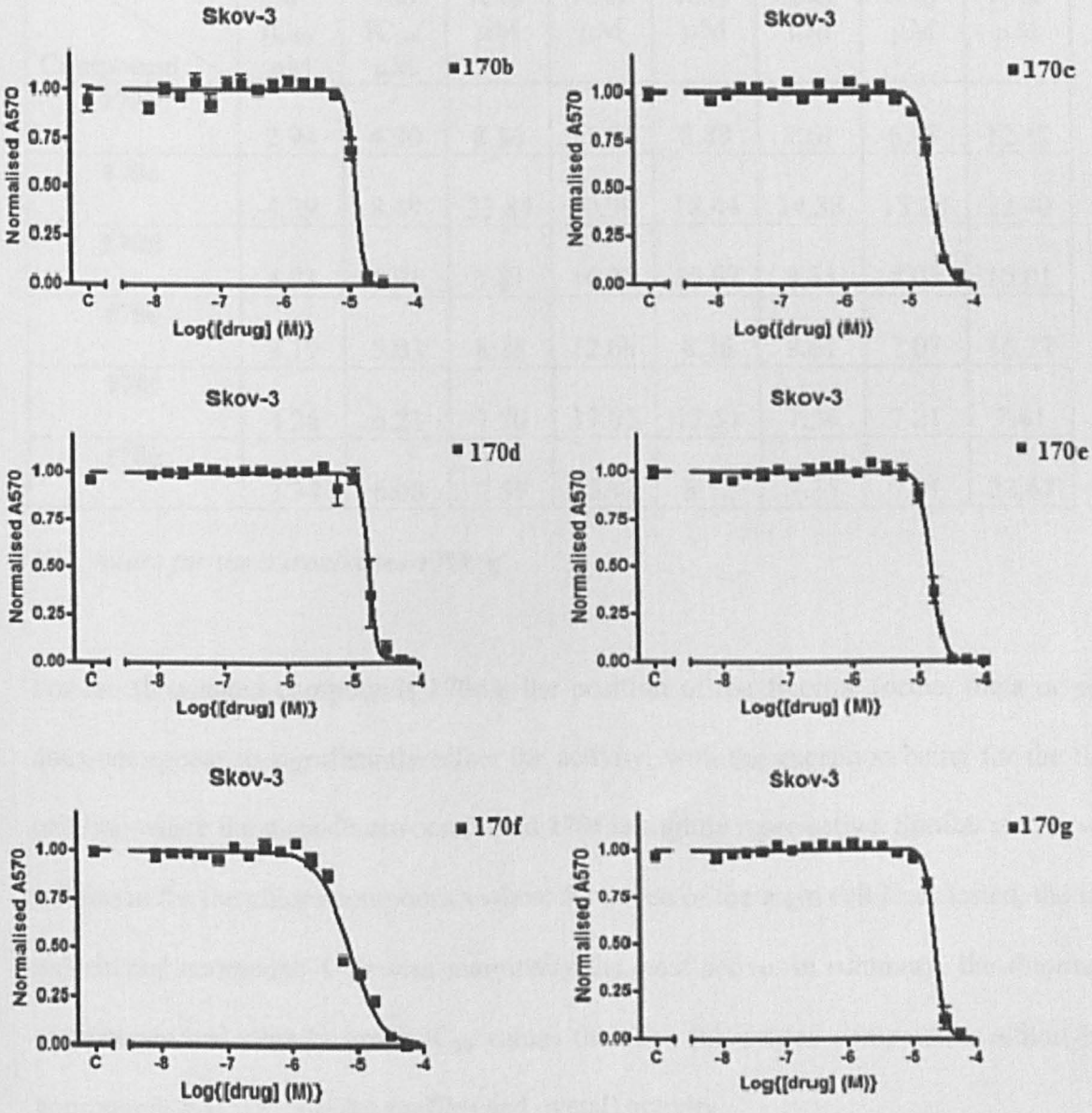
The β-nitrostyrenes **170b-g**

A representative set of IC_{50} plots are shown in figure 11, illustrating the change in % cell viability relative to the concentration of drug supplied for each of the six nitroalkenes **170b-g**. The IC_{50} profiles show that all six compounds inhibit the growth of the ovarian cancer cells and cause death with no viable cells.

The representative profiles for the six nitroalkenes **174b-g** in figure 11 were tested in cell line Skov-3. This cell line, and the others used for the cytotoxicity testing (See appendix 1 and table 1 for all available data), is one of the 59 cell lines from the NCI60 cell lines set and part of the cancer genome project which is run by the Wellcome Trust.[45] The Skov-3

cells are resistant to a number of cytotoxic drugs such as cis-platin **113** (Chapter 1, section 1.3.3, Figure 28).[46]

Figure 11:



Representative IC_{50} graphs for nitroalkenes **170b-g** *in cell line Skov-3*

The IC_{50} values for the nitroalkenes are all in the μM range with a slight variation across the various cell lines tested (Table 1).

Table 1:

Cell line / Compound	A2780 IC_{50}/ μM	cisA2780 IC_{50}/ μM	ov3 IC_{50}/ μM	ov4 IC_{50}/ μM	ov5 IC_{50}/ μM	ov8 IC_{50}/ μM	Igrov IC_{50}/ μM	Skov IC_{50}/ μM
170b	2.94	4.50	8.56	11.31	8.88	7.61	6.99	12.32
170c	4.29	8.49	33.84	30.99	18.44	14.38	15.28	22.40
170d	4.83	6.91	7.81	16.25	10.62	8.65	8.03	15.01
170e	3.19	5.03	8.18	12.66	8.26	8.61	7.07	16.77
170f	4.26	6.21	7.70	17.03	12.51	7.34	7.21	7.61
170g	3.34	6.08	7.59	13.97	8.12	7.35	7.91	23.67

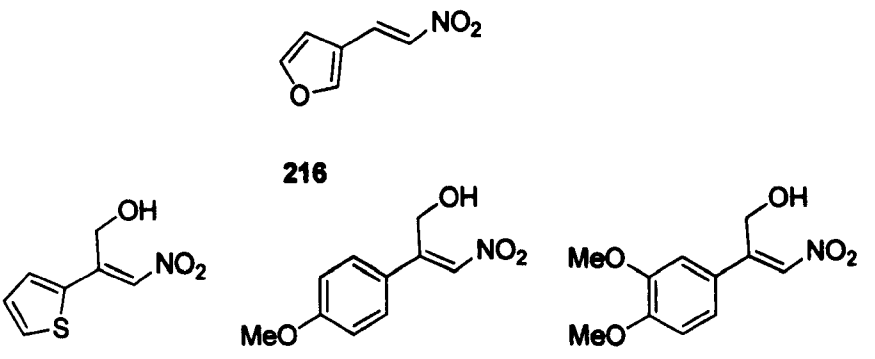
IC_{50} values for the nitroalkenes **170b-g**

For the fluorinated compounds **170e-g** the position of the fluorine (ortho, meta or para) does not appear to significantly affect the activity, with the exception being for the Skov cell line where the meta-fluoro compound **170f** is slightly more active. Similar results were not found for the chloro compounds where for seven of the eight cell lines tested, the meta chlorinated compound **170c** was marginally the least active. In summary, the fluorinated compounds had slightly lower IC_{50} values than the chlorinated compounds, although all compounds had very similar profiles and overall activity.

This data is comparable with the values found for other nitroalkenes tested by Mohan and co-workers on human cervical cancer cell line He-La, which gave IC_{50} values ranging from 2-38μM.[47] A number of nitroalkenes and their α-hydroxylated adducts were tested both for

their anti-proliferating effects against He-La cells and the most potent compounds **219-222** (Figure 12) were also tested for their effect on tubulin polymerisation. These nitroalkenes were found to inhibit tubulin polymerisation by binding to tubulin at a site other than the known colchicine and vinblastine sites.[47]

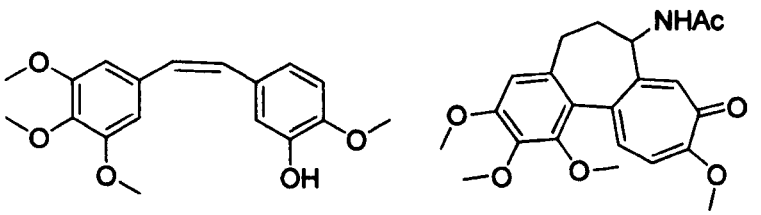
Figure 12:



The most potent nitroalkene **216** *and α-hydroxylated nitroalkenes* **217-219** against HeLA cells

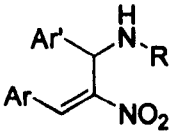
Despite these compounds being much smaller than other tubulin binding agents such as combretastatin A-4 **118** and colchicine **220** (Figure 13) they are potent antitubulin agents. Mohan and co-workers also synthesised a series of α-aminoalkylated nitroalkenes (Figure 13) which displayed a similar biological activity to the α-hydroxylated nitroalkenes due to their ability to bind to tubulin.[48]

Figure 13:



combretastatin A-4
118

Colchicine
220



aminoalkylated nitroalkenes

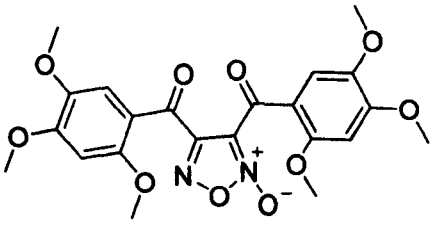
Combretastatin A-4 **118** *and colchicine* **220** *and the generic structure for α-aminoalkylated nitroalkenes*

Based on these results it is plausible that our nitroalkenes **170b-g** may owe their cytotoxic activity to their ability to bind to tubulin, although further experiments are clearly required to confirm this.

3.2.3 *In vitro* cytotoxicity – combretastatin-like symmetrical furoxans

Combretastatin like furoxans **144a-c** were tested in seven ovarian cancer cell lines for cytotoxicity using combretastatin A-4 **118** as a positive control. Of our three novel compounds only **144c** and compound **144d** (Figure 14) (which was synthesised by another member of the group using the optimised route devised in this PhD thesis) showed cytotoxicity below 100μM. The IC_{50} values obtained for the furoxans are summarised in table 2.

Figure 14:



144d

Combretastatin analogue based on a furoxan core **144d**

Table 2:

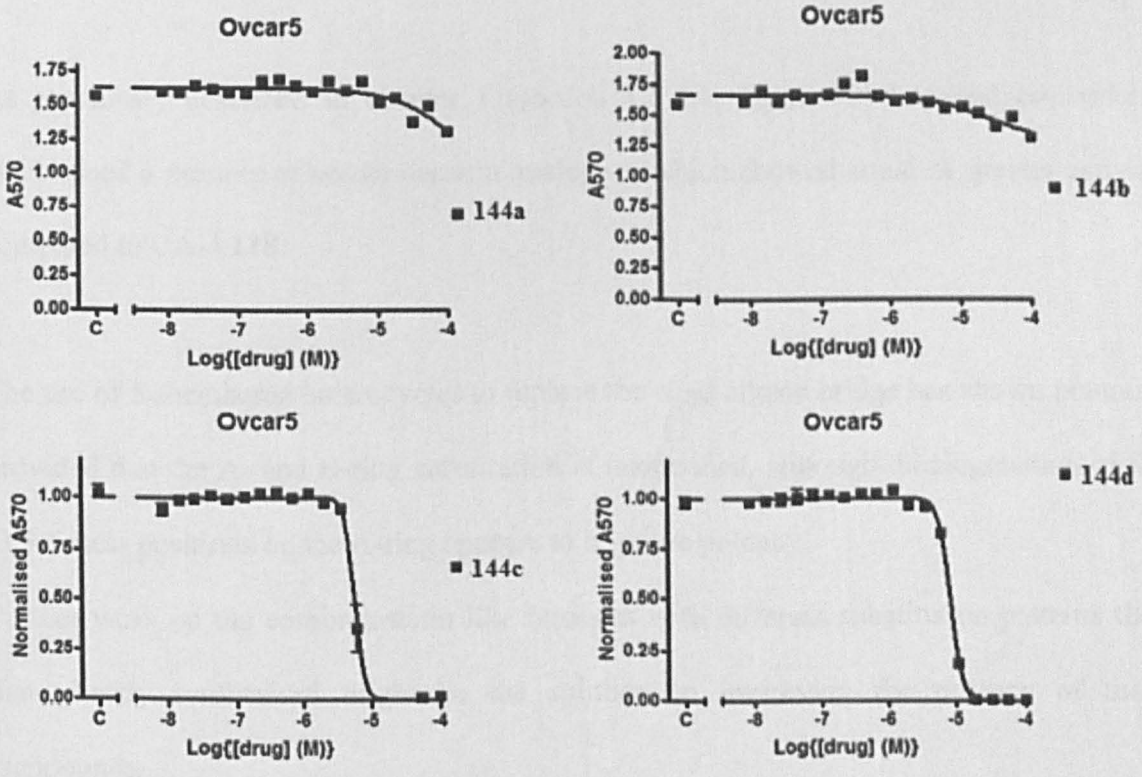
Cell line / Compound	A2780 IC_{50}/ μM	cisA2780 IC_{50}/ μM	ov3 IC_{50}/ μM	ov4 IC_{50}/ μM	ov5 IC_{50}/ μM	ov8 IC_{50}/ μM	Igrov IC_{50}/ μM	Skov IC_{50}/ μM
144a	>100μM	>100μM	>100μM	>100μM	>100μM	>100μM	>100μM	-
144b	>100μM	>100μM	>100μM	>100μM	>100μM	>100μM	>100μM	-
144c	3.64	4.64	11.22	9.89	7.75	4.81	4.25	18.47
144d	8.04	10.19	-	17.84	8.36	7.23	8.72	-
118	0.003	0.004	-	0.007	0.004	0.005	0.005	0.033

IC_{50} values for the combretastatin like furoxans **144a-d** *and* **118**. *The results quoted are the mean of 3 independent experiments*

Compound **144c** had the highest potency in this series with IC_{50} values in the low μM range for all eight cell lines. Given that the structure activity studies all indicate the 3,4,5-substitution pattern of the A-ring to be important for the cytotoxic activity of combretastatin analogues, the lack of activity by **144a** was surprising. However, given that these compounds are symmetrical it suggests that the 3,4,5-arrangment of methoxy groups on the B-ring may be detrimental to biological activity. This is supported by the lack of

activity for **144b**, which also has a methoxy group in the 3-position on the B-ring, and by the activity of compound **144c** without methoxy groups in the 3,5-positions on either ring. On the other hand, compound **144d** has a 2,4,5 substitution pattern (Figure 14) of methoxy groups and this compound is only slightly less reactive than **144c**. This observation creates a confusing picture of the structure activity relationship of the combretastatin-like furoxans which emphasises the need for further work as shown in chapter 2 (section 2.4, figure 14). Figure 15 shows representative IC_{50} plots for both the active and the inactive combretastatin-like analogues (all IC_{50} plots for **144c** and **144d** for the cell lines mentioned in table 2 can be found in appendix 2).

Figure 15:



R

epresentative IC_{50} graphs for combretastatin like furoxans **144a-d** *in cell line Ovcar5*

Replacing the rigid alkene bridge with a furoxan ring did not improve the potency relative to combretastatin A-4 **118**, which as a positive control in our assays consistently showed IC_{50} values in the nM range compared to our derivatives with IC_{50} values in the low μM range. These observations are consistent with those reported in the literature[49] where the alkene bridge has been replaced with a 5-membereed ring and in addition, the substitution pattern on the A and B ring is not the same as in combretastatin A-4 **118** which may exert an additional negative effect on cytotoxicity. LeBlanc and co-workers replaced the rigid bridge with pyrazole and epoxide rings and simultaneously altered the substituents on the B-ring. This gave a number of derivatives, most of which showed cytotoxicity in the μM range, which is similar to that found for our compounds.[49]

As previously described in chapter 1 (section 1.3.4.1, figure 32) Ley and co-workers synthesised a number of combretastatin analogues which showed equal or greater activity compared to CA-4 **118**.

The use of 5-membered heterocycles to replace the rigid alkene bridge has shown potential provided that the A- and B-ring substitution is maintained, although dihalogenation of the (3,5-) meta positions on the A-ring appears to improve potency.

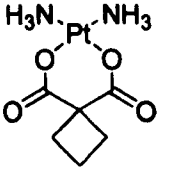
Further work on the combretastatin like furoxans with different substitution patterns than that already synthesised might be the solution to improving the potency of these compounds.

3.3 Caspase assay

With both the nitroalkenes **170b-h** and the combretastatin-like furoxans **144c-d** showing cytotoxic activity clarification was needed into the method by which they exert their effect. Apoptosis is the programmed death of cells and is required within developmental processes as the growth of new cells requires the death of "old cells" to maintain the steady state. Apoptosis is initiated by several signals such as DNA damage, lack of growth factors and the presence of death signal proteins. Any of these signals results in the activation of cytoplasmic protease enzymes called caspases. Increased activity of these enzymes can be measured and an increased presence indicates that cytotoxic activity is due to caspase dependent apoptosis.

Carboplatin **110** acts by binding to DNA, which causes DNA damage and ultimately results in apoptosis *via* the caspase pathway (Figure 16). It is used as a positive control in caspase assays and the effect of the compounds being investigated is measured relative to carboplatin **110**.

Figure 16:

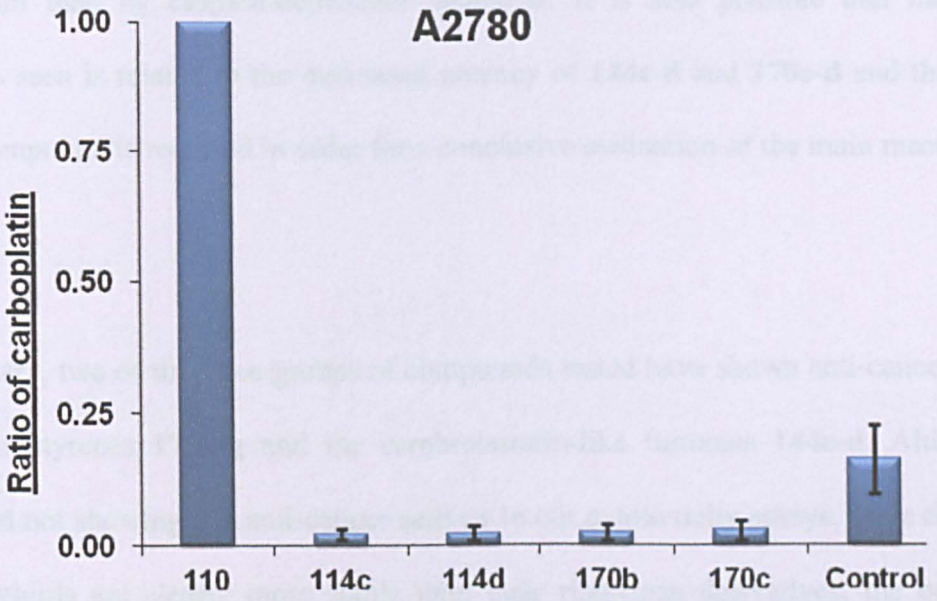


110

Carboplatin **110**

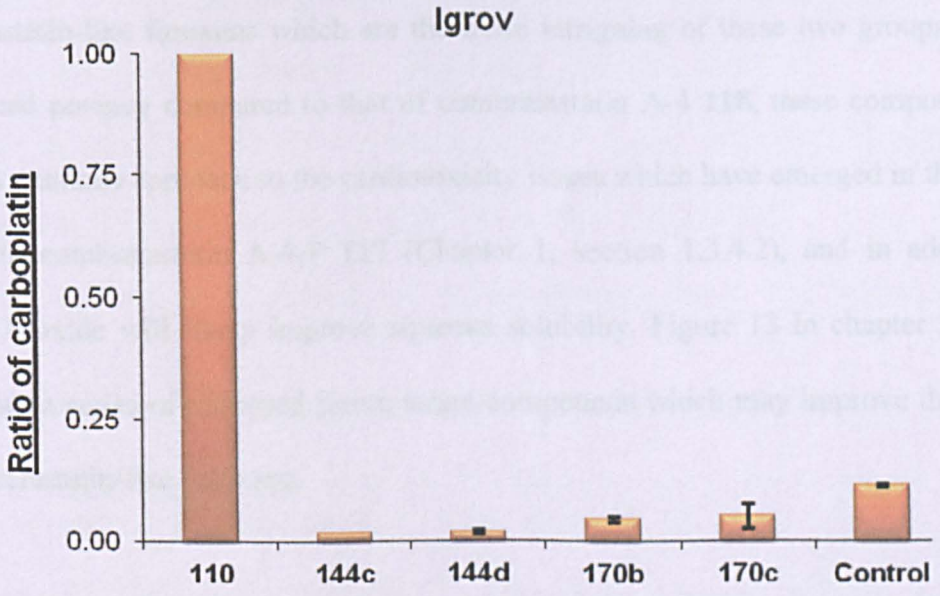
Figures 17 and 18 show the results of the caspase assays in A2780 and Igrov. These results are the average of three different assays, using the procedure previously described in literature,[43] for each cell line with limited caspases being found when treated with compounds **144c, 144d** and **170c-d.**

Figure 17:



Caspase assay showing the amount of caspases 3 and 7 present after inducing cell death by treating A2780 cells with compounds **144c, 144d** and **170c-d** *(relative to the amount of caspases 3 and 7 present after inducing cell death by treating A2780 cells with carboplatin* **110***)*

Figure 18:



Caspase assay showing the amount of caspases 3 and 7 present after inducing cell death by treating Igrov cells with compounds **144c, 144d** and **170c-d** *(relative to the amount of caspases 3 and 7 present after inducing cell death by treating Igrov cells with carboplatin* **110***)*

The lack of caspases found suggests that compounds **144c-d** and **170c-d** act via a different mechanism than by caspase-dependent apoptosis. It is also possible that the lack of apoptosis seen is related to the decreased potency of **144c-d** and **170c-d** and that a more potent compound is required in order for a conclusive evaluation of the main mechanism of action.

In summary, two of the three groups of compounds tested have shown anti-cancer activity, the β-nitrostyrenes **170b-g** and the combretastatin-like furoxans **144c-d**. Although the OZOs did not showing any anti-cancer activity in our cytotoxicity assays, these ring-closed *S*-nitrosothiols are clearly more stable than their ring-open derivatives, the benzylic *S*-nitrosothiols **162a-d**, and therefore we are keen to explore the biological activity of these compounds in other assays.

The β-nitrostyrenes **170b-g** were synthesised via a novel procedure to give a series of small molecules with a somewhat surprising biological activity. However, it is the combretastatin-like furoxans which are the more intriguing of these two groups. Despite the reduced potency compared to that of combretastatin A-4 **118**, these compounds may represent a unique approach to the cardiotoxicity issues which have emerged in the clinical trials with combretastatin A-4-P **117** (Chapter 1, section 1.3.4.2), and in addition the charged *N*-oxide will likely improve aqueous solubility. Figure 13 in chapter 2 (section 2.4) shows a series of proposed future target compounds which may improve the potency of combretastatin-like furoxans.

3.4 References

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4 Conclusions

From the target compounds listed in figure 35 of in chapter 1 (section 1.4), only **39m** and **39n** were not successfully synthesised (chapter 2, section 2.1.6). These linker compounds would have been interesting to test given the success seen with NO-donor hybrids in anticancer research (section 1.3.2). However, compounds **39a** and **39f**, **39h-l** were successfully synthesised *via* a revised synthetic approach as detailed in chapter 2 (section 2.1.5) and it was found that these ring-closed *S*-nitrosothiols are more stable than their ring-open benzylic derivatives. In addition, altering the substituents on the aryl ring had a pronounced effect on the rate of release of NO from the OZO compounds **39a** and **39f-k**. Figures 2, 3 and 4 in chapter 3 (Section 3.1.1) show how the rate of decomposition changes when the substituent or the position of the substituent is altered. In addition, it was found that contrary to reports in literature, exposure to light and heat does result in the decomposition of the OZOs and the release of NO.

From the target compounds listed in figure 36 of chapter 1 (section 1.4), all three combretastatin-like furoxans **144a-c** were successfully synthesised from the corresponding acetophenones (Chapter 2, section 2.3.3). Stability studies revealed that these compounds were thermally and photochemically stable under the conditions employed. For compound **144a** the addition of thiol at physiological pH resulted in a decrease in absorbance when monitored by UV-Vis spectrophotometry, which illustrated the thiol-mediated release of NO.

In an effort to synthesis monosubstituted furoxans **164-169** for comparison against the OZOs, in terms of stability, a series of β-nitrostyrenes **170b-g** were obtained in good yields through a novel procedure employing mild reaction conditions.

All compounds synthesised in this study in a pure form, were evaluated for their anti-cancerous activity by employing cytotoxicity assays using a range of ovarian cancer cells lines. Despite their previously reported cytotoxicity, the OZOs proved to be inactive in our cell lines. The β-nitrostyrenes **170b-g**, on the other hand, somewhat surprisingly showed activity in all the cell lines they were tested in. The mechanism by which these compounds exert their anti-cancer activity is unclear, although caspase assays revealed the mechanism is not caspase-dependent apoptosis. Of the three combretastatin-like furoxans, compound **144c** showed the most promising anti-cancer activity, albeit with a reduced potency when compared to combretastatin A-4 **118**. With only a few compounds tested, a sound structure-activity relationship for the combretastatin furoxan analogues is not possible. However, due to the many available starting materials and the straightforward synthesis for these compounds, future work (Chapter 2, section 2.4, Figure 13) such as that involving the 3,5-dihalogenated analogues, may produce more potent compounds.

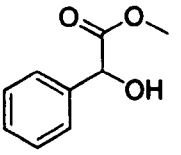
5 Experimental

5.1. Materials

- Commercial solvents were used and dried where appropriate.
- All reagents used were 96% in purity or above.
- TLC plates used were silica gel 60 F_{254} (Merck) and detection was using UV light or phosphomolybdic acid hydrate in methanol.
- Melting point was determined using a Bibby Stuart Scientific melting point apparatus (uncorrected).
- IR spectra were recorded using Thermo Nicolet FT-IR Nexus with an Avatar Smart Omni Sampler.
- UV-Vis spectra were recorded on a Varian – Cary 50 Bio UV-Visible spectrophotometer fitted with a waterbath.
- ^{1}H NMR and ^{13}C NMR spectra were obtained using Spectrospin and Bruker (300MHz, 52MM), and referenced using the following internal standards δ_H ($CDCl_3$ 7.3 ppm, DMSO 2.2 ppm), δ_C(CDC_{l3} 77.36 ppm, DMSO 40.45 ppm)
- Accurate mass spectrometry data was obtained from NMSSC (EPSRC National Mass Spectrometry Service Centre). The instrument used to obtain accurate mass data is noted after the mass data. LTQ Orbitrap refers to Thermofisher LTQ Orbitrap XL (High resolution instrument giving accurate mass measurement over the full mass range in electropray), MAT 95 refers to Finnigan MAT 95 XP (EI, CI, LSIMS, ESI and APCI capability), ASAP refers to Atmospheric-pressure Solids Analysis Probe.

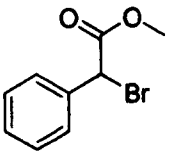
5.2. 4 -Aryl-1, 3, 2-oxathiazolium-5-olate 39a

5.2.1 Preparation of methyl 2-hydroxy-2-phenylacetate **148a**[1]



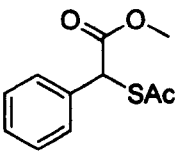
A few drops of conc. H_2SO_4 were added to a stirred solution of mandelic acid (4.56 g, 29 mmol) in methanol (50 ml). The reaction was refluxed for 3 hours, after which the solvent was removed and the residue dissolved in ethyl acetate (25 ml). The product was washed with a 10% aqueous K_2CO_3 solution (2 x 50 ml) and then with brine (2 x 50 ml). The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a white crystalline solid, 3.23 g (19 mmol, 68%). Mp. 54-55°C (Lit. 54-55°C)[2]. R_f = 0.59 (ethyl acetate : petroleum ether, 10:1, solvent front: 49 mm). δ_H(300MHz, DMSO) 3.63 (3H, s, CH_3), 5.18-5.520 (1H, d, *J* = 5.0 Hz, C*H*OH), 6.14-6.16 (1H, d, *J* = 5.0 Hz, O*H*), 7.33-7.47 (5H, m, Ar*H*). δ_C(75MHz, DMSO) 51.77 (CH_3), 72.35 (CH), 126.63, 127.87, 128.25, 139.58 (Ar*C*), 173.05 (C=O).

5.2.2 Preparation of methyl 2-bromo-2-phenylacetate **150a**



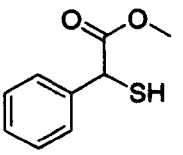
Methyl 2-hydroxy-2-phenylacetate **148a** (5.00 g, 30 mmol) was dissolved in $CHCl_3$ (50 ml) and 2 equivalents of PBr_3 (8.09 ml, 60 mmol). This was then stirred at r.t. for 4 days. Upon completion the reaction was washed with water (50 ml), dried ($MgSO_4$) and then passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 5.00 g (26 mmol, 75%). R_f = 0.21 (hexane : ethyl acetate, 10:1, solvent front: 32 mm). υ_{max}/cm^{-1} 1745 (br, s) (C=O), 1496 (w) (C-H), 1215 (s) (C-O), 694 (s) (C-Br). δ_H(300MHz, $CDCl_3$) 3.58 (3H, s, OC*H*$_3$), 5.19 (1H, s, C*H*Br), 7.15-7.19 (3H, m, Ar*H*), 7.34-7.37 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 46.59 (O-*C*H$_3$), 53.43 (*C*-Br), 128.72, 128.82, 129.29, 135.77 (Ar*C*), 168.82 (*C*=O). $C_9H_9BrO_2$ requires 227.9786, found 227.9779 (M^+, 50%, EI).

5.2.3 Preparation of methyl 2-(acetylthio)-2-phenylacetate **149a**



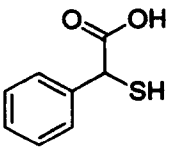
Potassium thioacetate (2.0 g, 17 mmol) was added to methyl 2-bromo-2-phenylacetate **150a** (2.0 g, 8.9 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4 hours and the KBr salt was then filtered off, before the filtrate was reduced to give the title compound as a light orange oil, 1.90 g (8.4 mmol, 95%). R_f = 0.29 (hexane : ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 1741 (w) (C=O), 1693 (w) (C=C), 1526 (s) (C=C), 1134 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.35 (3H, s, SCOC*H*$_3$), 3.64 (3H, s, OC*H*$_3$), 5.30 (1H, s, C*H*SAc), 7.35- 7.36 (5H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.98 (S-*C*H$_3$), 51.05 (O-*C*H$_3$), 53.15 (*C*H), 128.39, 128.53, 128.98, 134.79 (Ar*C*), 170.48 (*C*=O), 193.98 (S-*C*=O). $C_{11}H_{12}O_3S$ requires 224.0507, found 224.0503 (M^+, 50%, EI).

5.2.4 Preparation of methyl 2-mercapto-2-phenylacetate **151a**[3]



Methyl 2-(acetylthio)-2-phenylacetate **149a** (2.0 g, 8.9 mmol) was dissolved in dry methanol (120 ml) and flushed for 5 min with argon. Sodium methoxide was added to give a pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was then removed after filtering off the resin, giving the title compound as a dark brown oil, 1.5 g (8.0 mmol, 90%). R_f = 0.80 (hexane : ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 1736 (w) (C=O), 1152 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.62-2.65 (1H, d, *J* = 7.7 Hz, S*H*), 3.75 (3H, s, OCH_3), 4.72-4.74 (1H, d, *J* = 7.7 Hz, C*H*SH), 7.32-7.49 (5 H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 45.67 (*C*-SH), 53.10 (O-CH_3), 127.76, 128.26, 128.87, 138.25 (Ar*C*), 171.95 (*C*=O). $C_9H_{10}O_2S$ requires 182.0402, found 182.0394 (M^+, 50%, EI).

5.2.5 Preparation of methyl 2-hydroxy-2-phenylacetate **42a**



Method A:

Methyl 2-mercapto-2-phenylacetate **151a** (1.0 g, 5.4 mmol) was refluxed at 90°C for 24 hrs with 48% aqueous HBr (10 ml). The reaction mixture was dissolved in ethyl acetate (50 ml), dried ($MgSO_4$) and reduced to give a dark brown oil, 0.4 g (2.3 mmol, 43%). δ_H(300MHz, $CDCl_3$) 2.50-5.3 (1H, d, *J* = 7.7 Hz, S*H*), 4.59-4.61 (1H, d, *J* = 7.7 Hz, C*H*SH), 7.15-7.37 (5H, m, Ar*H*), 9.56 (1H, br.s, O*H*). δ_C(75MHz, $CDCl_3$) 44.96 (*C*-SH), 127.70, 128.19, 128.39, 128.59, 128.63, 135.68 (Ar*C*), 172.43.21 (*C*=O).

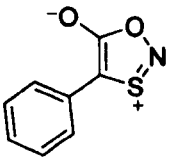
Method B:

Methyl 2-mercapto-2-phenylacetate **151a** (0.5 g, 2.7 mmol) was dissolved in THF (18 ml) and water (8 ml). Lithium hydroxide (0.17 g, 7.1 mmol) was added and the reaction stirred for 20hrs at r.t. The solvent was evaporated off and water (30 ml) was added before acidifying the solution with 2M HCl and washing with ethyl acetate (25 ml). The organic layer was dried ($MgSO_4$) and reduced to give a dark brown oil, 0.5 g (2.9 mmol, 93%). δ_H(300MHz, $CDCl_3$) 2.553-2.56 (1H, d, *J* = 7.6 Hz, S*H*), 4.65-7.73 (1H, d, *J* = 7.6 Hz, C*H*SH), 7.22-7.40 (5H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.96 (*C*-S), 127.08, 128.25, 128.39, 128.59, 128.63, 135.68 (Ar*C*), 172.44 (*C*=O).

Method C:

Methyl 2-mercapto-2-phenylacetate **151a** (0.5 g, 2.7 mmol) was partly dissolved in H_2O (18 ml) and conc. H_2SO_4 was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.5 g (2.9 mmol, 93%). R_f = 0.33 (hexane : ethyl acetate, 10:1, solvent front: 37 mm). υ_{max}/cm^{-1} 2952 (w) (C-H), 1735 (w) (C=O). δ_H(300MHz, $CDCl_3$) 2.53-2.55 (1H, d, *J* = 7.6 Hz, S*H*), 4.62-4.64 (1H, d, *J* = 7.6 Hz, C*H*SH), 7.18-7.40 (5H, m, Ar*H*). δ_C(75MHz, $CDCl_3$), 45.51 (*C*-S), 127.79, 128.31, 128.59, 128.79, 128.95, 137.14 (Ar*C*), 177.93 (*C*=O). $C_8H_8O_2S$ requires 168.0245, found 168.0241 (M^+, 50%, EI).

5.2.6 Preparation of 4-phenyl-1,3,2-oxathiazolium-5-olate **39a**[3]

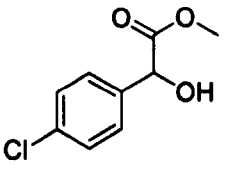


2-Mercapto-2-phenylacetic acid **42a** (0.40 g, 2.30 mmol) was dissolved in dry DCM (50ml) and cooled in an ice-bath before the addition of isobutylnitrite (1.9ml, 4.6mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), 3 equivalents of polymer bound DCC (1.4 g, 6.9 mmol) was added and the reaction stirred for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml) the DCC was filtered off and the reaction mixture was passed through a Celite pad. The filtrate was reduced to give the title compound as yellow crystals. The filtrate was reduced to give a yellow solid which was purified by column chromatography using hexane : ethyl acetate (10:1) to give the title compound as dark orange/yellow crystals, 0.37 g (2.07 mmol, 90%). Mp. (128.8–131.2)°C. R_f = 0.25 (hexane : ethyl acetate, 10:1, solvent front: 44 mm). υ_{max}/cm^{-1} 2988 (w) (CH), 1699 (m) (C=O), 1054 (m) (C-O). δ_H(300MHz, $CDCl_3$) 7.50-7.53 (3H, m, Ar*H*), 7.88-7.93 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$), 121.56, 125.95, 126.35, 129.57(2), 130.69 (Ar*C*), 175.08 (*C*=O). $C_8H_6NO_2S$ requires 180.0114, found 180.0113 ($[M+H]^+$, 100%). LTQ Orbitrap.

5.3. Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate halogen derivatives 39f, 39h-l

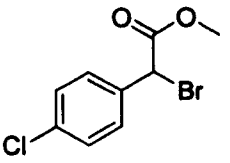
5.3.1 Preparation of 4-(4-chlorophenyl)-1,3,2-oxathiazolium-5-olate **39f**[3]

5.3.1.1 Preparation of methyl 2-(4-chlorophenyl)-2-hydroxyacetate **148f**[1]



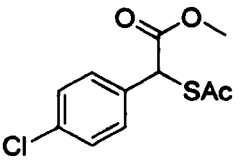
A few drops of conc. H_2SO_4 were added to a stirred solution of 4-chloromandelic acid (4.50 g, 24.11 mmol) in methanol (50 ml). The reaction was refluxed for 3 hours, after which the solvent was evaporated off and the residue dissolved in diethyl ether (50 ml). The product was washed with a 10% aqueous K_2SO_3 solution (3 x 50ml) followed by brine (3 x 50 ml). The organic layer was dried ($MgSO_4$) and the solvent removed to give the title compound as a pale yellow oil, 4.73 g (23.58 mmol, 97%). R_f = 0.12 (ethyl acetate : petroleum ether, 5:1, solvent front: 49 mm). υ_{max}/cm^{-1} 3334.43 (br.) (OH), 1734.97 (vs) (C=O), 1088.46 (s) (CO). δ_H(300MHz, DMSO) 3.61 (3H, s, C*H*$_3$), 5.22 (1H, s,C*H*OH), 7.39-7.48 (4H, m, Ar*H*). δ_C(75MHz, DMSO) 51.82 (O-*C*H$_3$), 71.66 (*C*-OH), 128.22, 128.44, 128.65, 128.73, 132.54 (Ar*C*), 138.53 (*C*-Cl), 172.69 (*C*=O). $C_9H_9ClO_3Na$ requires: 223.0138, found 223.0132 ($[M+Na]^+$, 100%) NESP, Orbitrap.

5.3.1.2 Preparation of methyl 2-bromo-2-(4-chlorophenyl)acetate **150f**



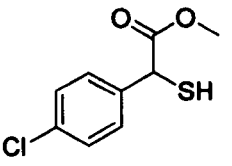
Methyl 2-(4-chlorophenyl)-2-hydroxyacetate **148f** (4.40 g, 21.54 mmol) was dissolved in $CHCl_3$ (50 ml) with 2 equivalents of PBr_3 (4.05 ml, 43.08 mmol). The solution was left to stir at r t. for 4 days before being washed with water (50 ml), dried ($MgSO_4$) and passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 3.85 g (14.60 mmol, 68%). R_f = 0.20 (ethyl acetate : petroleum ether, 10:1, solvent front: 52 mm). υ_{max}/cm^{-1} 1743.39 (s) (C=O), 1143.73 (s) (CO). δ_H(300MHz, $CDCl_3$) 3.78 (3H, s, OC*H*$_3$), 5.35 (1H, s, C*H*Br), 7.31-7.38 (2H, m, Ar*H*), 7.49-7.51 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 45.38 (*C*-Br), 53.53 (O-*C*H$_3$), 128.98, 129.06, 130.14, 130.29, 134.29 (Ar*C*), 135.30 (*C*-Cl), 168.51 (*C*=O). $C_9H_9BrClO_2$ requires: 262.9469, found 262.9470 ($[M+H]^+$, 100%) ASAP, Orbitrap.

5.3.1.3 Preparation of methyl 2-(acetylthio)-2-(4-chlorophenyl)acetate **151f**



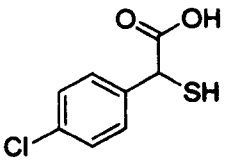
Potassium thioacetate (1.58 g, 13.84 mmol) was added to methyl 2-bromo-2-(4-chlorophenyl)acetate **150f** (3.65 g, 13.84 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4hrs before the KBr salt was filtered off and the filtrate was reduced to give the title compound as a light orange oil, 3.50 g (13.53 mmol, 98%). R_f = 0.35 (ethyl acetate : petroleum ether, 10:1, solvent front: 52 mm). υ_{max}/cm^{-1} 1741.05 (vs) (C=O), 1133.56 (s) (CO). δ_H(300MHz, $CDCl_3$) 2.34 (3H, s, SCOCH_3), 3.73 (3H, s, OCH_3), 5.29 (1H, s, C*H*SAc), 7.28-7.34 (4H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.98 (*C*-S), 50.26 (-(C=O)CH_3), 53.23 (O-CH_3), 128.88, 129.02, 129.09, 129.80, 133.59 (Ar*C*), 134.47 (*C*-Cl), 170.08 (*C*=O), 193.55 (S-*C*=O). $C_{11}H_{12}O_3SCl$ requires: 259.0196, found 259.0195 ($[M+H]^+$, 100%) ASAP, Orbitrap.

5.3.1.4 Preparation of methyl 2-(4-chlorophenyl)-2-mercaptoacetate **151f**[3]



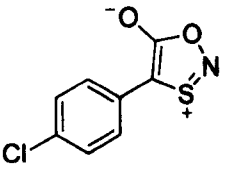
Methyl 2-(acetylthio)-2-(4-chlorophenyl)acetate **149f** (1.60 g, 6.18 mmol) was dissolved in dry methanol (120 ml) and flushed for 5 min with argon. Sodium methoxide was added to give pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was the removed after filtering off the resin, giving the title compound as a dark brown oil, 1.20 g (5.54 mmol, 89%). R_f = 0.56 (hexane: ethyl acetate, 10:1, solvent front: 38 mm). υ_{max}/cm^{-1} 1736.46 (s) (C=O), 1154.66(vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.52-2.55 (1H, d, *J* = 7.7 Hz, S*H*), 3.67 (3H, s, CH_3), 4.58-4.60 (1H, d, *J* = 7.7 Hz, C*H*SH), 7.23-7.33 (4H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.95 (*C*-SH), 53.24 (O-*C*H_3), 127.96, 128.90, 129.07, 129.19, 133.07 (Ar*C*), 136.63 (*C*-Cl), 171.66 (*C*=O). $C_9H_{10}O_2SCl$ requires 216.0012, found 216.0005 ($[M+H]^+$, 100%) ASAP, Orbitrap.

5.3.1.5 Preparation of 2-(4-chlorophenyl)-2-mercaptoacetic acid **42f**



Methyl 2-(4-chlorophenyl)-2-mercaptoacetate **151f** (0.20 g, 0.92 mmol) was partly dissolved in H_2O (10 ml) and conc. H_2SO_4 (10ml) was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.12 g (0.59 mmol, 64%). R_f = 0.38 (hexane : ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 2968.80 (br) (OH), 2359.57 (w) (CH), 1716.17 (m) (C=O), 1099.96 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.54-2.57 (1H, d, *J* = 7.6 Hz, S*H*), 4.58-4.61 (1H, d, *J* = 7.6Hz, C*H*SH), 7.24-7.35 (4H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.95 (*C*-SH), 128.92, 129.00, 129.19, 129.32, 134.54 (Ar*C*), 136.62 (*C*-Cl), 176.75 (*C*=O). $C_8H_6ClO_2S$ requires: 200.9793, found 200.9786 ($[M-H]^-$, 100%) ASAP, Orbitrap.

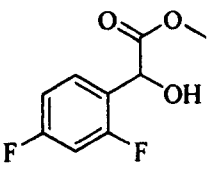
5.3.1.6 Preparation of 4-(4-chlorophenyl)-1,3,2-oxathiazolium-5-olate **39f**[3]



2-(4-Chlorophenyl)-2-mercaptoacetic acid **42f** (0.12 g, 0.59 mmol) was dissolved in dry DCM (50 ml) and cooled in an ice bath before the addition of isobutylnitrite (1.9 ml, 4.6 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), 1 equivalent of polymer bound DCC (0.26 g, 0.59 mmol) was added and the reaction stirred for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml), the DCC was filtered off and the reaction mixture was passed through a celite pad. The filtrate was reduced to give a yellow solid which was purified by column chromatography (hexane : ethyl acetate, 10:1) to give the title compound as yellow crystals, 0.10 g (0.47 mmol, 79%). Mp. 124.5 –126.8°C. R_f = 0.19 (hexane: ethyl acetate, 10:1, solvent front: 34 mm). υ_{max}/cm^{-1} 3085, 2952 (w) (CH), 1090 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 7.07-7.12 (2H, m, Ar*H*), 7.77-7.82 (2H, m, Ar*H*) δ_C(75MHz, $CDCl_3$) 119.24 (*C*-S), 123.81, 126.02, 129.13, 129.16, 130.34 (Ar*C*), 135.59 (*C*-Cl), 169.05 (*C*-O^-). $C_8H_5O_2SNCl$ requires: 213.9730, found 213.9718 ($[M+H]^+$, 100%) ASAP, Orbitrap.

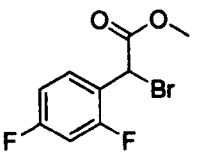
5.3.2 Preparation of 4-(2,4-difluorophenyl)-1,3,2-oxathiazolium-5-olate **39h**[3]

5.3.2.1 Preparation of methyl 2-(2,4-difluorophenyl)-2-hydroxyacetate **148h**[1]



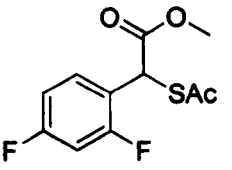
A few drops of conc. H_2SO_4 were added to a stirred solution of 2,4-difluromandelic acid (4.5 g 23.91 mmol) in methanol (50 ml). The reaction was refluxed for 3 hours before the solvent was removed and the residue dissolved in ethyl acetate (50 ml). The product was washed with a 10% aqueous K_2CO_3 solution (2 x 50 ml) and then brine (2 x 50 ml). The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a white solid 3.67 g (18.16 mmol, 75%). Mp. 48.6–50.8°C. R_f = 0.13 (hexane: ethyl acetate, 10:1, solvent front: 45 mm). υ_{max}/cm^{-1} 3435.20 (w, br.) (OH), 1741.32 (s) (C=O), 1607.82 (w) (C=C), 1504.86 (m) (C-H), 1217.96 (s) (C-O). δ_H(300MHz, DMSO) 3.60 (3H, s, C*H*$_3$), 5.32-5.344 (1H, d, *J* = 7.0 Hz, O*H*), 6.29-6.31 (1H, d, *J* = 7.0 Hz, C*H*OH), 7.00–7.06 (1H, m, Ar*H*), 7.10-7.17 (1H, m, Ar*H*), 7.46-7.54 (1H, m, Ar*H*). δ_C(75MHz, DMSO) 51.81 (O-*C*H$_3$), 66.13 (*C*-OH), 103.67, 111.56, 123.52, 130.11(Ar*C*), 157.87, 160.25 (d, *C*-F), 161.17, 163.68 (d, *C*-F), 172.12 (*C*=O). $C_9H_8O_3F_2$ requires: 202.0442, found 202.0436 (M^+, 50%, EI).

5.3.2.2 Preparation of methyl 2-bromo-2-(4-fluorophenyl)acetate **150h**



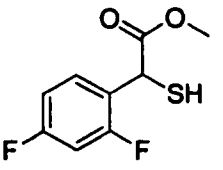
Methyl 2-(2,4-difluorophenyl)-2-hydroxyacetate **148h** (2.88 g, 14.24 mmol) was dissolved in $CHCl_3$ (50 ml) and 2 equivalents of PBr_3 (2.67 ml, 28.49 mmol) were added. The solution was left to stir at r.t. for 4 days before being was washed with water (100 ml), dried ($MgSO_4$) and passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 3.30 g (12.43 mmol, 87%). R_f = 0.32 (hexane: ethyl acetate, 10:1, solvent front: 45 mm). υ_{max}/cm^{-1} 1748.90 (s) (C=O), 1617.81 (w) (C=C), 1504.36 (m) (C-H), 1142.60 (s) (C-O). δ_H(300MHz, $CDCl_3$) 3.73 (3H, s, OC*H*$_3$), 5.59 (1H, s, C*H*Br), 6.71-6.77 (1H, m, Ar*H*), 6.83-6.88 (1H, m, Ar*H*) 7.60-7.68 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 37.59 (*C*-Br), 53.61 (O-*C*H$_3$), 104.02, 112.17, 119.75, 132.08 (Ar*C*), 158.11, 159.12 (d, *C*-F), 161.45, 165.63 (d, *C*-F), 168.05 (*C*=O). $C_9H_7BrF_2O_2$ requires: 263.9597, found 263.9594 (M^+, 50%, EI).

5.3.2.3 Preparation of methyl 2-(acetylthio)-2-(2,4-difluorophenyl)acetate **148h**



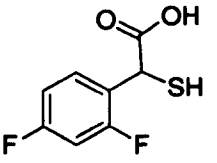
Potassium thioacetate (0.95 g, 8.33 mmol) was added to methyl 2-bromo-2-(4-fluorophenyl)acetate **150h** (2.206 g, 8.33 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4 hrs before the KBr salt was filtered off and the solvent removed to give the title compound as a light orange oil, 2.40 g (9.21 mmol, 99%). υ_{max}/cm^{-1} 1726.61 (s) (C=O), 1527.06 (m) (C-H), 1139.57 (s) (C-O). δ_H(300MHz, $CDCl_3$) 2.29 (3H, s, SCOC*H*$_3$), 3.67 (3H, s, OC*H*$_3$), 5.51 (1H, s, *H*), 6.72-6.82 (2H, m, Ar*H*), 7.27-7.35 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.89 (*C*H$_3$), 43.92 (*C*-S), 53.37 (O-*C*H$_3$), 104.31, 111.86, 119.03, 131.11 (Ar*C*), 158.73, 161.30 (d, *C*-F), 162.07, 164.62 (d, *C*-F), 169.53 (*C*=O), 193.20 (S-*C*=O). $C_{11}H_{10}F_2O_3S$ requires: 260.0319, found 260.0315, (M^+, 50%, EI).

5.3.2.4 Preparation of methyl 2-(2,4-difluorophenyl)-2-mercaptoacetate **151h**[3]



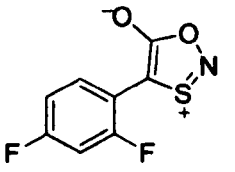
Methyl 2-(acetylthio)-2-(2,4-difluorophenyl)acetate **148h** (1.8 g, 6.9 mmol) was dissolved in dry methanol (120 ml) and the solution flushed for 5 min with argon. Sodium methoxide was added to give pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). The acid resin was then added to give pH = 2-3 and removed by filtration to give the title compound, after solvent evaporation, as a dark brown oil, 0.99 g (4.5 mmol, 66%). R_f = 0.16 (hexane: ethyl acetate, 10:1, solvent front: 45mm). υ_{max}/cm^{-1} 1739.50 (w) (C=O), 1503.12 (s) (C=C), 1141.36 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.63-2.66 (1H, d, *J* = 8.0 Hz, S*H*), 3.76 (3H, s, OCH_3), 4.96-4.99 (1H, d, *J* = 8.0 Hz, C*H*SH), 6.82-6.92 (2H, m, Ar*H*), 7.52-7.62 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 37.69 (*C*-SH), 53.08 (O-CH_3), 103.69, 112.01, 121.80, 130.27 (Ar*C*), 158.17, 164.21 (d, *C*-F), 169.74, 169.90 (d, *C*-F), 171.19 (*C*=O). $C_9H_8F_2O_2S$ requires: 218.0213, found 218.0207, (M^+, 50%, EI).

5.3.2.5 Preparation of 2-(2,4-difluorophenyl)-2-mercaptoacetic acid **42h**



Methyl 2-(2,4-difluorophenyl)-2-mercaptoacetate **151h** (0.18 g, 0.8 mmol) was partly dissolved in H_2O (18 ml) and conc. H_2SO_4 (18 ml) was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale yellow oil, 0.11 g (0.5 mmol, 63%). R_f = 0.1 (hexane : ethyl acetate, 10:1, solvent front: 45 mm). υ_{max}/cm^{-1} 3081.06 (OH), 2359.30 (w) (SH), 1713.05 (C=O), 1508.21 (vs) (C-H). δ_H(300MHz, $CDCl_3$) 2.57-2.59 (1H, d, *J* = 8.0 Hz, S*H*), 4.88-4.91 (1H, d, *J* = 8.0 Hz, C*H*SH), 6.72-6.87 (2H, m, Ar*H*), 7.44-7.52 (1H, m, Ar*H*), 10.38 (1H, br s, O*H*). δ_C(75MHz, $CDCl_3$) 37.82 (*C*-S), 104.07, 112.04, 120.82, 130.49 (Ar*C*), 158.30, 161.24 (d, *C*-F), 163.20, 164.40 (d, *C*-F), 176.84 (*C*=O). $C_8H_5F_2O_2S$ requires: 202.9978, found 202.9986, ($[M-H]^-$, 100%) LTQ Orbitrap.

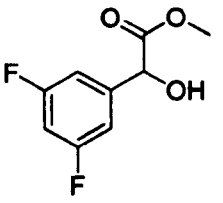
5.3.2.6 Preparation of 4-(2,4-difluorophenyl)-1,3,2-oxathiazolium-5-olate **39h**[3]



2-(2,4-Difluorophenyl)-2-mercaptoacetic acid **42h** (0.4 g, 2.3 mmol) was dissolved in dry DCM (50 ml) and cooled in an ice-bath before the addition of isobutylnitrite (1.9 ml, 4.6 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), polymer bound DCC (1.4 g, 6.9 mmol) was added and the reaction was left to stir for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml), the DCC was removed by filtration and the reaction mixture was passed through a celite pad. The filtrate was reduced to give yellow crystals which were purified by column chromatography using hexane : ethyl acetate (10:1) to give the title compound as yellow crystals 0.38 g (1.79 mmol, 78%). Mp. 96.6–99.8°C. R_f = 0.33 (hexane: ethyl acetate, 10:1, solvent front: 44 mm). υ_{max}/cm^{-1} 3061 (w) (CH), 1701 (s) (C=O), 1050 (s) (C-O). δ_H(300MHz, $CDCl_3$) 6.92-7.04 (2H, m, Ar*H*), 8.52-8.60 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 104.30 (*C*-S), 112.70 (2), 126.53, 126.57 (Ar*C*), 157.62, 161.13 (d, *C*-F), 161.74, 165.14 (d, *C*-F), 175.31 (*C*-O^-). $C_8H_3F_2NO_2S$ requires: 214.9853, found, ([M–H]$^-$, 100%) LTQ Orbitrap.

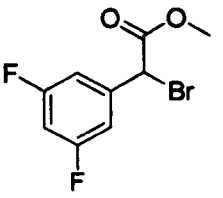
5.3.3 Preparation of 4-(3,5-difluorophenyl)-1,3,2-oxathiazolium-5-olate **39i**[3]

5.3.3.1 Preparation of methyl 2-(3,5-difluorophenyl)-2-hydroxyacetate **148i**[1]



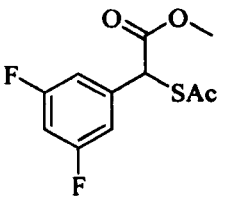
A few drops of conc. H_2SO_4 was added to a stirred solution of 3,5-difluoromandelic acid (4.5 g, 23.91 mmol) in methanol (30 ml). The reaction was refluxed for 3 hours, after which the solvent was evaporated off and the residue dissolved in ethyl acetate (25 ml). The product was washed with a 10% aqueous K_2CO_3 solution (50 ml) followed by brine (25 ml). The organic layer was dried ($MgSO_4$) and the solvent removed to give the title compound as a white crystalline solid, 4.7 g (23.24 mmol, 97%). Mp. 56.5-58.9°C. R_f = 0.59 (ethyl acetate : petroleum ether, 5:1, solvent front: 49 mm). υ_{max}/cm^{-1} 3554.98 (OH), 1736.37 (C=O), 1621.80 (s) (C-H), 1124.30 (CO). δ_H(300MHz, DMSO) 3.64 (3H, s, OC*H*$_3$), 5.23-5.25 (1H, d, *J* = 5.0 Hz, C*H*OH), 6.41-6.43 (1H, d, *J* = 5.0 Hz, O*H*), 7.10-7.21 (3H, m, Ar*H*). δ_C(75MHz, DMSO) 52.09 (O-*C*H$_3$), 71.26 (*C*-OH), 103.29, 109.63, 109.86, 144.00 (Ar*C*), 160.61, 163.87(d, *C*-F), 172.03 (*C*=O). $C_9H_9F_2O_3$ requires: 203.0520, found 203.0515 ($[M+H]^+$, 100%) LTQ Orbitrap.

5.3.3.2 Preparation of methyl 2-bromo-2-(3,5-difluorophenyl)acetate **150i**



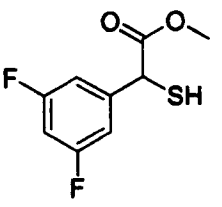
Methyl 2-(3,5-difluorophenyl)-2-hydroxyacetate **148i** (4.42 g, 21.89 mmol) was dissolved in $CHCl_3$ (50 ml) with 1.1 equivalents of PBr_3 (4.4 ml, 23.76 mmol). The solution was left to stir at r.t. for 4 days before being washed with water (50 ml), dried ($MgSO_4$) and passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 4.4 g (16.60 mmol, 76%). R_f = 0.23 (ethyl acetate : petroleum ether, 5:1, solvent front: 49 mm). υ_{max}/cm^{-1} 1747.42 (m) (C=O), 1623.69 (m) (C-H), 1147.75 (s) (CO). δ_H(300MHz, $CDCl_3$) 3.73 (3H, s, OC*H*$_3$), 5.20 (1H, s, C*H*Br), 6.69-6.76 (1H, m, Ar*H*), 7.00-7.05(2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.41 (*C*-Br), 53.67 (O-*C*H$_3$), 104.87, 111.80, 112.15, 139.06 (Ar*C*), 161.23, 164.55 (d, *C*-F), 168.00 (*C*=O). $C_9H_8BrF_2O_2$ requires: 264.9676, found 264.9671 ($[M+H]^+$, 100%) CI Orbitrap.

5.3.3.3 Preparation of methyl 2-(acetylthio)-2-(3,5-difluorophenyl)acetate **149i**



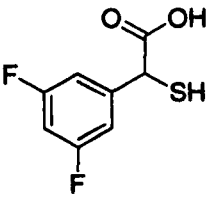
Potassium thioacetate (1.02 g, 8.89 mmol) was added to methyl 2-bromo-2-(3,5-difluorophenyl)acetate **150i** (2.36 g, 8.89 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4hrs before the KBr salt was filtered off and the filtrate was reduced to give the title compound as a light yellow oil, 2.28 g (8.77 mmol, 99%). R_f = 0.33 (ethyl acetate : petroleum ether, 5:1, solvent front: 48 mm). υ_{max}/cm^{-1} 1743.07 (m) (C=O), 1623.14 (m) (C-H), 1122.34 (s) (CO). δ_H(300MHz, $CDCl_3$) 2.29 (3H, s, SCOC*H*$_3$), 3.68 (3H, s, OC*H*$_3$), 5.21 (1H, s, C*H*SAc), 7.19-7.26 (2H, m, Ar*H*), 7.31-7.33 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$29.97 (*C*-S), 50.10 ((C=O)-*C*H$_3$), 53.39 (O-*C*H$_3$), 104.07, 111.58, 111.81, 138.76 (Ar*C*), 161.37, 164.68 (d, *C*-F), 169.46 (*C*=O), 193.06 (S-*C*=O). $C_{11}H_{10}F_2O_3S$ requires: 260.0319, found 260.0315, (M^+, 50%, EI).

5.3.3.4 Preparation of methyl 2-(3,5-difluorophenyl)-2-mercaptoacetate **151i**[3]



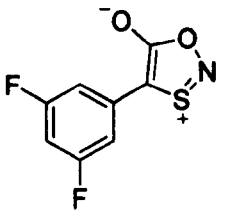
Methyl 2-(acetylthio)-2-(3,5-difluorophenyl)acetate **149i** (2.00 g, 7.68 mmol) was dissolved in dry methanol (120 ml) and flushed for 5 min with argon. Sodium methoxide was added to give pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was then removed after filtering off the resin, giving the title compound as a dark brown oil, 1.00 g (4.58 mmol, 60%). R_f = 0.25 (ethyl acetate : petroleum ether, 5:1, solvent front: 32 mm). υ_{max}/cm^{-1} 1734 (m) (C=O), 1596 (s) (C=C), 1118 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.61-2.64 (1H, d, *J* = 7.6 Hz, S*H*), 3.75 (3H, s, OCH_3), 4.67-4.69 (1H, d, *J* = 7.6 Hz, C*H*SH), 7.31-7.41 (3H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.90 (*C*-SH), 53.45 (O-CH_3), 104.14, 111.16, 111.39, 140.64 (Ar*C*), 161.40, 164.54 (d, *C*-F), 176.52 (*C*=O). $C_9H_9F_2O_2S$ requires: 219.0286, found 219.0283, ($[M+H]^+$, 100%, CI) LTQ Orbitrap.

5.3.3.5 Preparation of 2-(3,5-difluorophenyl)-2-mercaptoacetic acid **42i**



Methyl 2-(3,5-difluorophenyl)-2-mercaptoacetate **151i** (0.30 g, 1.37 mmol) was partly dissolved in H_2O (18 ml) and conc. H_2SO_4 (18 ml) was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.19 g (0.9 mmol, 67%). R_f = 0.28 (ethyl acetate : petroleum ether, 10:1, solvent front: 42 mm). υ_{max}/cm^{-1} 3088 (OH), 2590 (w) (SH), 1712 (C=O), 1463 (vs) (C-H). δ_H(300MHz, $CDCl_3$) 2.57-2.59 (1H, d, *J* = 8.0 Hz, S*H*), 4.56-4.59 (1H, d, *J* = 8.0 Hz, C*H*SH), 6.68-6.77 (1H, m, Ar*H*), 6.94-6.97 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 43.69 (*C*-S), 103.09, 109.99, 110.34, 139.64 (Ar*C*), 160.37, 163.37 (d, *C*-F), 175.13 (*C*=O). $C_8H_5F_2O_2S$ requires: 202.9984, found 202.9983, ([M-H]$^-$, 100%, CI) LTQ Orbitrap.

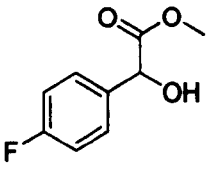
5.3.3.6 Preparation of 4-(3,5-difluorophenyl)-1,3,2-oxathiazolium-5-olate **39i**[3]



2-(3,5-difluorophenyl)-2-mercaptoacetic acid **42i**, (0.15 g, 0.73 mmol) was dissolved in dry DCM (50 ml)) and cooled in an ice-bath before the addition of isobutylnitrite (1.9 ml, 4.6 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), polymer bound DCC (0.6g, 0.73mmol) was added and the reaction was left to stir for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml), the DCC was removed by filtration and the reaction mixture was passed through a celite pad. The filtrate was reduced to give yellow crystals which were purified by column chromatography using hexane : ethyl acetate (10:1) to give the title compound as yellow crystals, 0.11 g (0.51 mmol, 70%). Mp. 119.4-121.8°C. R_f = 0.16 (hexane : ethyl acetate, 10:1, solvent front: 44 mm). υ_{max}/cm^{-1} 2957 (w) (CH), 1710 (s) (C=O), 1126 (s) (C-O). δ_H(300MHz, $CDCl_3$) 6.80-6.90 (1H, m, Ar*H*), 7.35-7.38 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 104.48 (*C*-S), 105.70, 108.67, 112.09, 128.87 (Ar*C*), 161.63, 164.92 (d, *C*-F), 174.40 (*C*=O). $C_8H_3F_2O_2SNH$ requires: 215.9925, found 215.9924 ($[M + H]^+$, 29%) ASP Orbitrap.

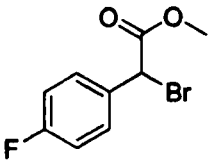
5.3.4 Preparation of 4-(4-fluorophenyl)-1,3,2-oxathiazolium-5-olate **39j**[3]

5.3.4.1 Preparation of methyl 2-(4-fluorophenyl)-2-hydroxyacetate **148j**[1]



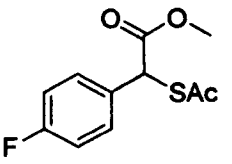
A few drops of conc. H_2SO_4 were added to a stirred solution of 4-fluromandelic acid (4.5 g, 26.44 mmol) in methanol (50 ml). The reaction was refluxed for 3 hours, after which the solvent was removed and the residue dissolved in ethyl acetate (50 ml). The product was washed with a 10% aqueous K_2CO_3 solution (2 x 50 ml) and then with brine (2 x 50 ml). The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a white crystalline solid, 4.18 g (22.71 mmol, 85%). Mp. 39.1-41.5°C. R_f = 0.13 (hexane : ethyl acetate, 10:1, solvent front: 30 mm). υ_{max}/cm^{-1} 3464.72 (w, br.) (OH), 1741.41 (vs) (C=O), 1601.80 (w) (C=C), 1506.57 (m) (C-H), 1083.91 (s) (C-O). δ_H(300MHz, DMSO) 3.5 (3H, s, C*H*$_3$), 5.17 (1H, d, *J* = 5.17 Hz, C*H*OH), 6.15 (1H, d, *J* = 5.17 Hz, O*H*), 7.14-7.22 (2H, m, Ar*H*), 7.39-7.48(2H, m, Ar*H*). δ_C(75MHz, DMSO) 51.68 (O-*C*H$_3$), 71.68 (*C*-OH), 114.83, 115.12, 128.58, 128.69 (Ar*C*), 135.78(Ar*C*), 160.17, 163.40 (d, *C*-F), 172.93 (*C*=O). $C_9H_9FO_3$ requires: 184.0536, found: 184.0529 (M^+, 50%,) EI.

5.3.4.2 Preparation of methyl 2-bromo-2-(4-fluorophenyl)acetate **150j**



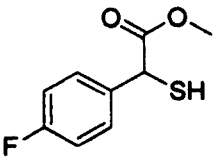
Methyl 2-(4-fluorophenyl)-2-hydroxyacetate **148j** (3.38 g, 18.35 mmol) was dissolved in $CHCl_3$ (50 ml) and 2 equivalents of PBr_3 (3.45 ml, 36.67 mmol). This was then stirred at r.t. for 4 days. Upon completion the reaction was washed with water (50 ml), dried ($MgSO_4$) and then passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 3.86 g (15.62 mmol, 85%). $R_f = 0.51$ (hexane: ethyl acetate, 10:1, solvent front: 30mm). υ_{max}/cm^{-1} 1745.10 (br, m) (C=O), 1604.26 (m) (C=C), 1510.07 (m) (C-H), 1224.10 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 3.78 (3H, s, OCH_3), 5.37 (1H, s, C*H*Br), 7.02-7.08 (2H, m, Ar*H*), 7.53-7.58 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.96 (*C*-Br), 53.42 (O-CH_3), 115.71, 116.75, 130.64, 131.30, 131.73 (Ar*C*), 161.39, 164.69 (d, *C*-F) 168.69 (*C*=O). $C_9H_8BrFO_2$ requires: 245.9692, found: 245.9684 (M^+, 50%) EI.

5.3.4.3 Preparation of methyl 2-(acetylthio)-2-(4-fluorophenyl)acetate **149j**



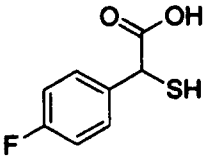
Potassium thioacetate (1.55 g, 13.61 mmol) was added to methyl 2-bromo-2-(4-fluorophenyl)acetate **150j**, (3.36 g, 13.61 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4 hrs and the KBr salt was then filtered off, before the filtrate was reduced to give the title compound as a light orange oil, 3.26 g (13.47 mmol, 99%). R_f = 0.46 (hexane: ethyl acetate, 10:1, solvent front: 30 mm). υ_{max}/cm^{-1} 1740.63 (br, m) (C=O), 1603.80 (m) (C=C), 1508.36 (vs) (C-H), 1224.81 (s) (C-O). δ_H(300MHz, $CDCl_3$) 2.37 (3H, s, SCOC*H*$_3$), 3.75 (3H, s, OC*H*$_3$), 5.31 (1H, s, C*H*SAc), 7.01- 7.08 (2H, m, Ar*H*), 7.35- 7.42 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.98 (*C*H$_3$), 50.18 (*C*-S), 53.19 (O-*C*H$_3$), 115.76, 116.05, 130.15, 130.26, 130.85 (Ar*C*), 161.01, 164.30 (d, *C*-F), 169.76 (*C*=O), 193.71 (S-*C*=O). $C_{11}H_{11}FO_3S$ requires 242.0413, found 242.0403 (M^+, 50%) EI.

5.3.4.4 Preparation of methyl 2-(acetylthio)-2-(4-fluorophenyl)acetate **151j**[3]



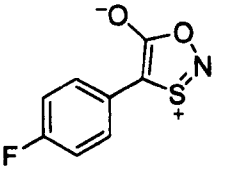
Methyl 2-(acetylthio)-2-(4-fluorophenyl)acetate **149j** (1.00 g, 4.13 mmol) was dissolved in dry methanol (120 ml) and flushed for 5 min with argon. Sodium methoxide was added to give a pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was then removed after filtering off the resin, giving the title compound as a dark brown oil, 0.65 g (2.89 mmol, 70%). R_f = 0.43 (hexane: ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 1736.13 (s) (C=O), 1507.99 (vs) (C-H), 1224.22 (s) (C-O). δ_H(300MHz, $CDCl_3$) 2.61-2.64 (1H, d, *J* = 7.7 Hz, S*H*), 3.76 (3H, s, OCH_3), 4.69-4.71 (1H, d, *J* = 7.7 Hz, *H*), 7.02-7.08 (2H, m, Ar*H*), 7.42-7.48 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.62 (*C*-SH), 53.13 (O-CH_3), 115.59, 115.96, 129.51, 129.62, 133.91 (Ar*C*), 160.84, 164.12 (d, *C*-F), 171.80 (*C*=O). $C_9H_9FO_2S$ requires: 200.0307, found 200.0302 (M^+, 50%) EI.

5.3.4.5 Preparation of 2-(4-fluorophenyl)-2-mercaptoacetic acid **42j**



Methyl 2-(acetylthio)-2-(4-fluorophenyl)acetate, **151j** (0.25 g, 1.25 mmol) was partly dissolved in H_2O (18 ml) and conc. H_2SO_4 was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.17 g (0.9 mmol, 72%). R_f = 0.11 (hexane: ethyl acetate, 10:1, solvent front: 31mm). υ_{max}/cm^{-1} 2988.58 (br.) (COOH), 2558.83 (w) (SH), 1725.23 (C=O), 1510.46 (vs) (C-H). δ_H(300MHz, $CDCl_3$) 2.54-2.58 (1H, d, *J* = 7.0 Hz, S*H*), 4.60-4.64 (1H, d, *J* = 7.0 Hz, C*H*SH), 6.93-7.00 (2H, m, Ar*H*), 7.33-7.40 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$), 44.68 (*C*-S), 115.72, 116.01, 129.72, 129.83, 132.8 (Ar*C*), 161.01, 164.30 (d, *C*-F), 177.67 (*C*=O). $C_8H_6FO_2S$ requires: 185.0078, found 185.0080 $[M-H]^-$. LTQ Orbitrap

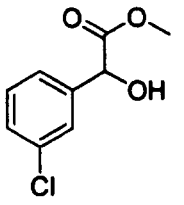
5.3.4.6 Preparation of 4-(4-fluorophenyl)-1,3,2-oxathiazolium-5-olate **39j**[3]



2-(4-Fluorophenyl)-2-mercaptoacetic acid **42j** (0.131 g, 0.70 mmol) was dissolved in dry DCM (50 ml) and cooled in an ice-bath before the addition of isobutylnitrite (0.24 ml, 2.1 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), 1 equivalent of polymer bound DCC (0.3 g, 0.7 mmol) was added and the reaction stirred for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml) the DCC was filtered off and the reaction mixture was passed through a celite pad. The filtrate was reduced to give a yellow solid which was purified by column chromatography using hexane : ethyl acetate (10:1) to give the title compound as yellow crystals, 0.10 g (0.50 mmol, 72%). Mp. 109.3–111.4°C. R_f = 0.15 (hexane: ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 2962 (w) (CH), 1683 (m) (C=O), 1015 (s) (C-O). δ_H(300MHz, $CDCl_3$) 7.07-7.12 (2H, m, Ar*H*), 7.76-7.83 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$), 115.73 (*C*-S), 116.03, 119.55, 121.57, 121.63, 127.00 (Ar*C*), 161.02, 164.39 (d, *C*-F), 174.06 (*C*-O^-). $C_8H_5FNO_2S$ requires: 198.0020, found 198.0017 ($[M+H]^+$, 100%). LTQ Orbitrap.

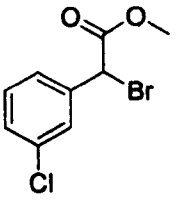
5.3.5 Preparation of 4-(3-chlorophenyl)-1,3,2-oxathiazolium-5-olate **39k**[3]

5.3.5.1 Preparation of methyl 2-(3-chlorophenyl)-2-hydroxyacetate **148k**[1]



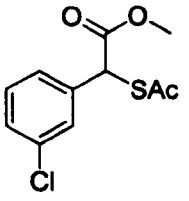
A few drops of conc. H_2SO_4 were added to a stirred solution of 3-chloromandelic acid (5.90 g, 31.61 mmol) in methanol (50 ml). The reaction was refluxed for 3 hours, after which the solvent was removed and the residue dissolved in ethyl acetate (25 ml). The product was washed with a 10 % aqueous K_2CO_3 solution (2 x 50 ml) and then brine (2 x 50 ml). The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a yellow crystalline solid, 6.17 g (30.75 mmol, 97%). Mp. 72.9-75.1°C. R_f = 0.12 (ethyl acetate : petroleum ether, 5:1, solvent front: 49 mm). υ_{max}/cm^{-1} 3447.90 (br) (OH), 1737.87 (vs) (C=O), 1189.37 (vs) (C-O). δ_H(300MHz, DMSO) 3.62 (3H, s, OC*H*$_3$), 5.20 (1H, d, *J* = 5.46 Hz, C*H*OH), 6.27 (1H, d, *J* = 5.46 Hz, CHO*H*), 7.3-7.4 (3H, m, Ar*H*), 7.35-7.48 (1H, m, Ar*H*). δ_C(75MHz, DMSO) 51.94 (O-*C*H$_3$), 71.61 (*C*-OH), 125.29, 126.40, 129.82, 130.17, 132.94 (Ar*C*), 141.99 (*C*-Cl), 172.51(*C*=O). $C_9H_9ClO_3Na$ requires: 223.0130, found 223.0130 (M^+, 100%) LTQ Orbitrap.

5.3.5.2 Preparation of methyl 2-bromo-2-(3-chlorophenyl)acetate **150k**



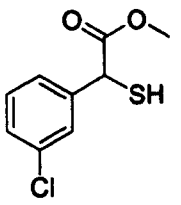
Methyl 2-(3-chlorophenyl)-2-hydroxyacetate **148k** (5.82 g, 29.01 mmol) was dissolved in $CHCl_3$ (50 ml) with 2 equivalents of PBr_3 (5.45ml, 58.02mmol). The solution was left to stir at r.t. for 4 days before being washed with water (50 ml), dried ($MgSO_4$) and passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 4.66 g (17.67 mmol, 61%). R_f = 0.19 (ethyl acetate : petroleum ether, 10:1, solvent front: 32 mm). υ_{max}/cm^{-1} 1746.39 (s) (C=O), 1145.24 (m) (C-O). δ_H(300MHz, $CDCl_3$) 3.79 (3H, s, OC*H*$_3$), 5.31 (1H, s, C*H*Br), 7.26-7.31 (2H, m, Ar*H*), 7.40-7.43 (1H, m, Ar*H*), 7.55-7.56 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 45.26 (*C*-Br), 53.60 (O-*C*H$_3$), 126.90, 128.88, 129.46, 130.10, 134.62 (Ar*C*), 137.56 (*C*-Cl), 168.38 (*C*=O). $C_9H_9BrClO_2$ requires: 262.9474, found 262.9469 ($[M + H]^+$, 80%) CI, Orbitrap ASAP.

5.3.5.3 Preparation of methyl 2-(acetylthio)-2-(3-chlorophenyl)acetate **149k**



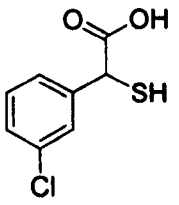
Potassium thioacetate (1.80 g, 15.78 mmol) was added to methyl 2-bromo-2-(3-chlorophenyl)acetate **150k** (4.16 g, 15.78 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4hrs before the KBr salt was filtered off and the filtrate was reduced to give the title compound as a dark orange oil, 4.00 g (15.46mmol, 98%). R_f = 0.26 (ethyl acetate : petroleum ether, 10:1, solvent front: 30 mm). υ_{max}/cm^{-1} 1740.85 (vs) (C=O), 1133.03 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.37 (3H, s, SCOC*H*$_3$), 3.76 (3H, s, OC*H*$_3$), 5.30 (1H, s, C*H*SAc), 7.27-7.32 (3H, m, Ar*H*), 7.41 (1H, s, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.98 (*C*-SH), 50.39 (-(C=O)*C*H$_3$), 53.30 (O-*C*H$_3$), 126.54, 128.64, 128.72, 130.15, 134.67 (Ar*C*), 136.93 (*C*-Cl), 169.94 (*C*=O), 193.50 (S-*C*=O). $C_{11}H_{11}ClO_3S$ requires: 259.0190, found 259.0194 ($[M+H]^+$, 100%, CI) HNESP, Orbitrap.

5.3.5.4 Preparation of methyl 2-(3-chlorophenyl)-2-mercaptoacetate **151k**[3]



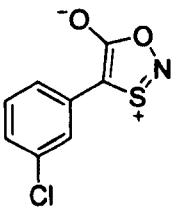
Methyl 2-(acetylthio)-2-(3-chlorophenyl)acetate **149k** (1.5g, 5.7mmol) was dissolved in dry methanol (120ml) and flushed for 5 min with argon. Sodium methoxide was added to give pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was then removed after filtering off the resin, giving the title compound as a dark brown oil, 0.86 g (3.9 mmol, 69%). R_f = 0.35 (hexane: ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 3499.86 (br) (SH), 2360.30 (m), CH), 1735.84 (s) (C=O), 1156.74 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.62-2.65 (1H, d, *J* = 7.8 Hz, S*H*), 3.75 (3H, s, OCH_3), 5.20-5.23 (1H, d, *J* = 7.8 Hz, C*H*SH), 7.21-7.39 (3H, m, Ar*H*), 7.57-7.60 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 43.13 (*C*-SH), 53.29 (O-CH_3), 128.01, 128.47, 128.68, 134.68 (Ar*C*), 140.09 (*C*-Cl), 169.92 (*C*=O). $C_9H_8ClO_2S$ requires: 214.9934, found 214.9940 ([M-H]$^-$, 100%) HNES, Orbitrap.

5.3.5.5 Preparation of 2-(3-chlorophenyl)-2-mercaptoacetic acid **42k**



Methyl 2-(3-chlorophenyl)-2-mercaptoacetate **151k** (0.097 g, 0.45 mmol) was partly dissolved in H_2O (10 ml) and conc. H_2SO_4 (10ml) was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.066 g (0.33 mmol, 72%). R_f = 0.17 (hexane : ethyl acetate, 10:1, solvent front: 31 mm). υ_{max}/cm^{-1} 2924.92 (br) (OH), 1712.01 (vs) (C=O), 1188.12 (m) (C-O). δ_H(300MHz, $CDCl_3$) 2.55-2.58 (1H, d, *J* = 7.8 Hz, S*H*), 4.58-4.61 (1H, d, *J* = 7.8 Hz, C*H*SH), 7.17-7.44 (4H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 43.87 (*C*-SH), 125.98, 127.92, 127.98, 129.05, 133.64 (Ar*C*), 137.96 (*C*-Cl), 175.85 (*C*=O). $C_8H_6ClO_2S$ requires: 200.9777, found 200.9785 ($[M-H]^-$, 100%) LTQ Orbitrap.

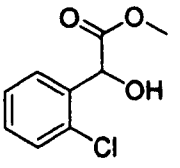
5.3.5.6 Preparation of 4-(3-chlorophenyl)-1,3,2-oxathiazolium-5-olate **39k**[3]



2-(3-Chlorophenyl)-2-mercaptoacetic acid **42k** (0.066 g, 0.33 mmol,) was dissolved in dry DCM (50 ml) and cooled in an ice bath before the addition of isobutylnitrite (1.9 ml, 4.6 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), 1 equivalent of polymer bound DCC (0.14 g, 0.33 mmol) was added and the reaction stirred for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml), the DCC was filtered off and the reaction mixture was passed through a celite pad. The filtrated was reduced to give a yellow solid which was purified by column chromatography (hexane : ethyl acetate, 10:1) to give the title compound as yellow crystals, 0.056 g (0.26 mmol, 79%). Mp. 104.6–106.9°C. R_f = 0.17 (hexane: ethyl acetate, 10:1, solvent front: 37 mm). υ_{max}/cm^{-1} 2922 (w) (CH), 1694 (m) (C=O), 1081 (s) (C-O). δ_H(300MHz, $CDCl_3$) 7.33 - 7.37 (2 H, m, Ar*H*), 7.64-7.68 (1 H, m, Ar*H*), 7.80 - 7.83 (1 H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 119.24 (*C*-S), 124.01, 125.64, 127.97, 130.59, 130.78 (Ar*C*), 135.70 (*C*-Cl), 169.45 (*C*=O). $C_8H_5O_2SNCl$ requires: 213.9730, found 213.9717 ($[M+H]^+$, 100%) ASAP, Orbitrap.

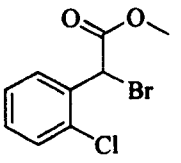
5.3.6 Preparation of 4-(2-chlorophenyl)-1,3,2-oxathiazolium-5-olate **391**[3]

5.3.6.1 Preparation of methyl 2-(2-chlorophenyl)-2-hydroxyacetate **1481**[1]



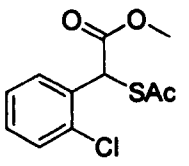
A few drops of conc. H_2SO_4 were added to a stirred solution of 2-chloromandelic acid (4.50 g, 24.12 mmol) in methanol (30 ml). The reaction was refluxed for 3 hours, after which the solvent was removed and the residue dissolved in ethyl acetate (25 ml). The product was washed with a 10 % aqueous K_2CO_3 solution (2 x 50 ml) and then brine (2 x 50 ml). The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a clear oil, 4.73 g (23.58 mmol, 97%). R_f = 0.12 (ethyl acetate : petroleum ether, 10:1, solvent front: 49 mm). υ_{max}/cm^{-1} 3451.70 (br.) (OH), 1738.74 (m) (C=O), 1221.21 (m) (CO). δ_H(300MHz, DMSO) 3.63 (3H, s, OC*H*$_3$), 5.52 (1H, s, C*H*OH), 7.30-7.39 (2H, m, Ar*H*), 7.43-7.46 (1H, m, Ar*H*), 7.57-7.60 (1H, m, Ar*H*). δ_C(75MHz, DMSO) 51.88 (O-*C*H$_3$), 69.56 (*C*-OH), 127.25, 128.76, 129.18, 129.55, 132.10 (Ar*C*), 137.32 (*C*-Cl), 172.11 (*C*=O). $C_9H_9ClO_3Na$ requires: 223.0138, found 223.0130 ([M+Na]$^+$, 100%) LTQ Orbitrap.

5.3.6.2 Preparation of methyl 2-bromo-2-(2-chlorophenyl)acetate **1501**



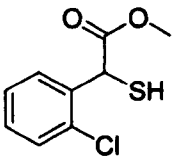
Methyl 2-(2-chlorophenyl)-2-hydroxyacetate **1481** (4.30 g, 21.43 mmol) was dissolved in $CHCl_3$ (50 ml) with 2 equivalents of PBr_3 (4.05 ml, 43.08 mmol). The solution was left to stir at r.t. for 4 days before being washed with water (50 ml), dried ($MgSO_4$) and passed through a silica pad. The $CHCl_3$ was removed to give the title compound as a clear oil, 3.14 g (11.91 mmol, 56%). R_f = 0.09 (ethyl acetate : petroleum ether, 10:1, solvent front: 29 mm). υ_{max}/cm^{-1} 1748.57 (m) (C=O), 11147.06 (s) (CO). δ_H(300MHz, $CDCl_3$) 3.72 (3H, s, OC*H*$_3$), 5.83 (1H, s, C*H*Br), 7.18-7.32 (3H, m, Ar*H*), 7.65-7.69 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 42.92 (*C*-Br), 53.64 (O-*C*H$_3$), 127.61, 129.77, 130.43, 130.84, 133.23 (Ar*C*), 133.74 (*C*-Cl), 168.30 (*C*=O). $C_9H_9BrClO_2$ requires: 262.9469, found 262.9469 ($[M+H]^+$, 80%) ASAP, Orbitrap.

5.3.6.3 Preparation of methyl 2-(acetylthio)-2-(2-chlorophenyl)acetate **1491**



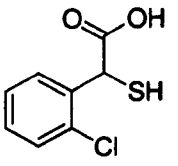
Potassium thioacetate (1.27 g, 11.15 mmol) was added to methyl 2-bromo-2-(2-chlorophenyl)acetate **1501** (2.94 g, 11.15 mmol) dissolved in methanol (50 ml). The solution was stirred at r.t. for 4hrs before the KBr salt was filtered off and the filtrate was reduced to give the title compound as a light orange oil, 2.77 g (10.71 mmol, 96%). R_f = 0.26 (ethyl acetate : petroleum ether, 10:1, solvent front: 27 mm). υ_{max}/cm^{-1} 1741.18 (s) (C=O), 1132.87 (m) (C-O). δ_H(300MHz, $CDCl_3$) 2.38 (3H, s, SCOC*H*$_3$), 3.76 (3H, s, OC*H*$_3$), 5.79 (1H, s, C*H*SAc), 7.24-7.28 (2H, m, Ar*H*), 7.39-7.44 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 29.89 (S-*C*H$_3$), 48.51 (*C*-S), 53.38 (O-*C*H$_3$), 127.22, 129.70, 129.87, 130.37, 133.43 (Ar*C*), 133.87 (*C*-Cl), 169.84 (*C*=O), 193.52 (S-*C*=O). $C_{11}H_{12}ClO_3S$ requires: 259.0190, found 259.0194 9 ($[M+H]^+$, 100%) HNESP, Orbitrap.

5.3.6.4 Preparation of methyl 2-(2-chlorophenyl)-2-mercaptoacetate **1511**[3]



Methyl 2-(2-chlorophenyl)-2-hydroxyacetate **1491** (0.20 g, 0.77 mmol) was dissolved in dry methanol (120 ml) and flushed for 5 min with argon. Sodium methoxide was added to give pH = 9-10 before the reaction was stirred at room temperature for 5 hours under N_2(g). Acid resin was then added to ensure a pH = 2-3 and the solvent was then removed after filtering off the resin, giving the title compound as a dark brown oil, 0.13 g (0.59 mmol, 78%). R_f = 0.20 (ethyl acetate : petroleum ether, 10:1, solvent front: 27 mm). υ_{max}/cm^{-1} 3415.48 (br) (SH), 1737.68 (s) (C=O), 1155.87 (vs) (C-O). δ_H(300MHz, $CDCl_3$) 2.61-2.64 (1H, d, *J* = 7.9 Hz, S*H*), 3.79 (3H, s, OCH_3), 5.21-5.24 (1H, d, *J* = 7.9 Hz, C*H*SH), 7.24-7.32 (2H, m, Ar*H*), 7.40-7.42 (1H, m, Ar*H*), 7.57-7.60 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 42.34 (*C*-SH), 53.16 (O-*C*H_3), 129.52, 129.11, 129.76, 130.33, 133.98 (Ar*C*), 136.22 (*C*-Cl), 171.60 (*C*=O). $C_9H_{10}ClO_2S$ requires: 217.0085, found 217.0083 ($[M+H]^+$, 100%) ASAP, Orbitrap.

5.3.6.5 Preparation of 2-(2-chlorophenyl)-2-mercaptoacetic acid **421**



Methyl 2-(2-chlorophenyl)-2-mercaptoacetate **1511** (0.58 g, 2.6 mmol) was partly dissolved in H_2O (18 ml) and conc. H_2SO_4 (18 ml) was added dropwise until the oil had gone into solution. The reaction was then stirred for 12 hrs at 0°C. After this time the solution was washed with ethyl acetate (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a pale brown oil, 0.40 g (1.9 mmol, 76%). R_f = 0.15 (ethyl acetate : petroleum ether, 10:1, solvent front: 25 mm). υ_{max}/cm^{-1} 2952.33 (br) (OH), 1711.35 (s) (C=O), 1156.88 (m) (C-O). δ_H(300MHz, $CDCl_3$) 2.55-2.58 (1H, d, *J* = 7.9 Hz, S*H*), 5.14-5.17 (1H, d, *J* = 7.9 Hz, C*H*SH), 7.16-7.21 (2H, m, Ar*H*), 7.31-7.33 (1H, m, Ar*H*), 7.50-7.55 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 41.07 (*C*-SH), 12.47, 128.22, 128.58, 128.73, 132.27 (Ar*C*), 134.26 (*C*-Cl), 174.79 (*C*=O). $C_8H_6ClO_2S$ requires: 200.9783, found: 200.9785 ($[M-H]^-$, 100%) LTQ Orbitrap.

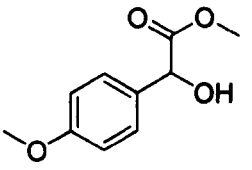
5.3.6.6 Preparation of 4-(2-chlorophenyl)-1,3,2-oxathiazolium-5-olate **391**[3]

2-(2-Chlorophenyl)-2-mercaptoacetic acid **421** (0.40 g, 2.3 mmol) was dissolved in dry DCM (50 ml) and cooled in an ice-bath before the addition of isobutylnitrite (1.9 ml, 4.6 mmol). The reaction was protected from light while stirring for 2 hrs. After diluting the reaction with dry DCM (200 ml), 3 equivalents of polymer bound DCC (1.4 g, 6.9 mmol) were added and the reaction stirred for a further 2 hrs. After quenching the reaction with H_2O (0.3 ml), the DCC was filtered off and the reaction mixture was passed through a celite pad. The filtrate was reduced to give a yellow solid which was purified by column chromatography (hexane : ethyl acetate, 10:1) to give the title compound as yellow crystals 0.39 g (1.84 mmol, 80%). Mp. 94.2–96.7°C. R_f = 0.22 (hexane : ethyl acetate, 10:1, solvent front: 34 mm). υ_{max}/cm^{-1} 2961 (w) (CH), 1738 (m) (C=O), 1009 (s) (C-O). δ_H(300MHz, $CDCl_3$) 7.28-7.41 (2H, m, Ar*H*), 7.45-7.49 (1H, m, Ar*H*), 8.60-8.64 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 115.25 (*C*-S), 126.59, 126.85, 127.68, 130.08, 130.63 (Ar*C*), 132.02 (*C*-Cl), 175.60 (*C*-O^-). $C_8H_5ClNO_2S$ requires: 213.9724, found 213.9718 ($[M+H]^+$, 100%) ASAP Orbitrap.

5.4. Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate linker derivatives **39m** and **39n**

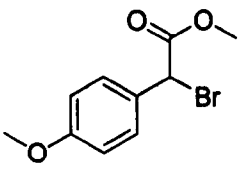
5.4.1. Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate linker derivative **39m**

5.4.1.1 Preparation of methyl 2-hydroxy-2-(4-methoxyphenyl)acetate **148d**[1]



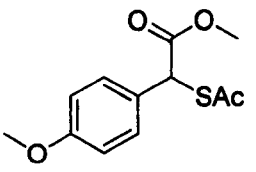
2-Hydroxy-2-(4-methoxyphenyl)acetic acid (2.5 g, 13.72 mmol) was dissolved in MeOH (50ml) followed by a few drops of conc. H_2SO_4. The reaction was refluxed for 12 hrs before the solvent reduced to give a residue that was diluted in ethyl acetate (50 ml), and washed with a 10% aqueous K_2CO_3 solution. Drying ($MgSO_4$) and removal of the solvent gave the title compound as a colourless oil, 2.42 g (12.33 mmol, 90%). R_f = 0.5 (petroleum ether : ethyl acetate, 1:1 ,solvent front: 35 mm). υ_{max}/cm^{-1} 3459 (br, w) (OH), 2954, 2838 (w) (C-H), 1732 (m) (C=O). δ_H(300MHz, $CDCl_3$) 3.75 (3H, s, OC*H*$_3$), 3.81 (3H, s, COOC*H*$_3$), 5.14 (1H, s, C*H*OH), 6.88-6.91 (2H, m, Ar*H*), 7.32-7.35 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 52.95 (O-*C*H$_3$), 55.60 (ArO-*C*H$_3$), 72.51 (*C*H), 114.08 (2), 127.94 (2), 130.11 (Ar*C*), 159.74 (Ar*C*-O), 174.34 (*C*=O). $C_{10}H_{16}O_4N$ requires: 214.1074, found 214.1076 $[M+NH_4]^+$. LTQ Orbitrap.

5.4.1.2 Preparation of methyl 2-bromo-2-(4-methoxyphenyl)acetate **150d**



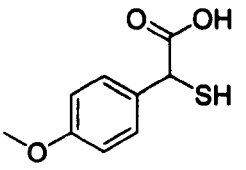
Methyl 2-hydroxy-2-(4-methoxyphenyl)acetate **148d** (1.94 g, 9.88 mmol) was dissolved in $CHCl_3$ (50 ml) with 1.1 equivalents of PBr_3 (1.02 ml, 10.86 mmol). The reaction was left to stir at r.t. for 4 days before being washed with water (100 ml), dried ($MgSO_4$) and reduced to give the title compound as a orange oil, 2.07 g (7.99 mmol, 80%). R_f = 0.3 (petroleum ether : ethyl acetate, 1:1, solvent front: 25 mm). υ_{max}/cm^{-1} 2839 (br. w) (C-H), 1712 (s) (C=O) 522 (s) (C-Br). δ_H(300MHz, $CDCl_3$) 3.80-3.82 (6H, 2 x s, 2 x OC*H*$_3$), 5.38 (1H, s, C*H*Br), 6.88-6.91 (2H, m, Ar*H*), 7.49-7.52 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 46.51 (O-*C*H$_3$), 53.34 (*C*-Br), 55.37 (ArO-*C*H$_3$), 114.26 (2), 127.68 (2), 130.12 (Ar*C*), 160.32 (Ar*C*-O), 168.95 (*C*=O). $C_{10}H_{11}BrO_3$ requires: 257.9879, found 257.9870 $[M]^+$. EI MAT.

5.4.1.3 Preparation of methyl 2-(acetylthio)-2-(4-methoxyphenyl)acetate **149d**



Methyl 2-bromo-2-(4-methoxyphenyl)acetate **150d** (1.87 g, 7.21 mmol) was dissolved in methanol (50 ml) with 1.1 equivalents of KSAc (0.9 g, 7.88 mmol) and left to stir at r.t. for 12 hrs. The KBr salt was filtered off and the filtrate was reduced to give the title compound as an orange oil, 1.8 g (7.08 mmol, 98%). R_f = 0.6 (petroleum ether : ethyl acetate, 1:1, solvent front: 32 mm). υ_{max}/cm^{-1} 3010, 2958 (w) (C-H), 1737 (m) (C=O). δ_H(300MHz, $CDCl_3$) 2.34 (3H, s, $SCOCH_3$), 3.73 (3H, s, OCH_3), 3.79 (3H, s, $COOCH_3$), 5.27 (1H, s, C*H*), 6.85-6.88 (2H, m, Ar*H*), 7.30-7.33 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 52.31 (O-CH_3), 55.31 (*C*-S), 57.14 (ArO-CH_3), 114.14 (2), 128.21, 129.89 (Ar*C*), 159.99 (Ar*C*-O), 171.40 (*C*=O). $C_{12}H_{15}O_4S$ requires: 255.0691, found 255.0695 ($[M+H]^+$, 100%) LTQ Orbitrap.

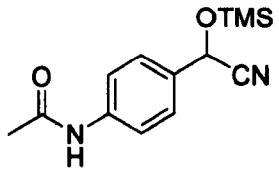
5.4.1.4 Preparation of 2-mercapto-2-(4-methoxyphenyl)acetic acid **42d**[4]



Methyl 2-(acetylthio)-2-(4-methoxyphenyl)acetate **149d** 0.25 g (1.17 mmol) was dissolved in conc. HCl, 2 ml, and refluxed for 2hrs at 80°C. The product was extracted with DCM, dried ($MgSO_4$) and the solvent evaporated off to give the title compound as an oil, 0.15 g (0.75 mmol, 65%). R_f = 0.19 (petroleum ether : ethyl acetate solvent front: 25 mm). δ_H(300MHz, $CDCl_3$) 2.51-2.54 (1H, d, *J* = 7.39 Hz, S*H*), 3.73 (3H, s, Ar-OCH_3), 4.59-4.61 (1H, d, *J* = 7.35 Hz, C*H*SH), 6.76-6.84 (2H, m, Ar*H*), 7.17-7.34 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 44.82 (*C*-S), 55.36 (ArO-CH_3), 114.25, 114.36, 129.13 (2) (Ar*C*), 162.34 (Ar*C*-O), 177.37 (*C*=O). υ_{max}/cm^{-1} 2961 (w) (C-H), 1709 (m) (C=C). $C_9H_{11}O_3S$ requires: 199.0429, found 199.0432 $[M+H]^+$. LTQ Orbitrap.

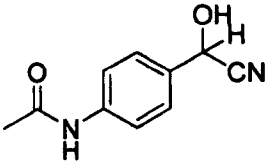
5.4.2. Synthesis of 4-phenyl-1,3,2-oxathiazolium-5-olate linker derivative **39n**

5.4.2.1 Preparation of *N*-(4-(cyano(trimethylsilyloxy)methyl)phenyl)acetamide **158**[5]



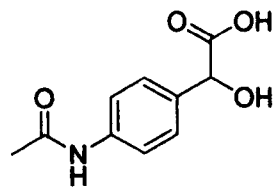
4-Acetaminoaldehyde **156** (5.5 g, 33.7 mmol) and a catalytic amount of ZnI was dissolved in dry DCM (150 ml). 1.2 equivalents of TMSCN (5 ml, 40.4 mmol) was added before the reaction was left to stir at r.t. for 48 hrs. The organic layer was washed with H_2O (150 ml), dried ($MgSO_4$) and reduced to give the title compound as a dark yellow oil, 6.25 g (23.82 mmol, 71%). Mp: 99-102°C. R_f = 0.47 (petroleum ether : ethyl acetate, 1:1, solvent front: 37mm). υ_{max}/cm^{-1} 3309 (w) (NH), 2959 (w) (CH_3), 1668 (m) (C=O), 1077 (br. w) (OTMS). δ_H(300MHz, $CDCl_3$) 0.00 (9H, s, TMS), 2.00 (3H, s, $COCH_3$), 5.24 (1H, s, C*H*CN), 7.17-7.20 (2H, m, Ar*H*), 7.33-7.36 (2H, m, Ar*H*), 7.42 (1H, br s, N*H*). δ_C(75MHz, $CDCl_3$) 0.00 (3 x Si-CH_3), 24.89 (CH_3), 63.48 (*C*-O), 119.44 (*C*N), 120.38, 127.45, 131.41, 132.29 (Ar*C*) 138.93 (Ar*C*-NH), 169.27 (*C*=O). $C_{13}H_{19}N_2O_2Si$ requires: 263.1210, found 263.1214 $[M+H]^+$. LTQ Orbitrap.

5.4.2.2 Preparation of *N*-(4-(cyano(hydroxy)methyl)phenyl)acetamide **157**[5]



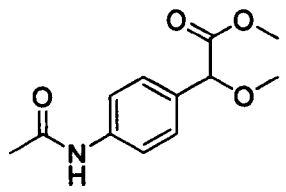
N-(4-(Cyano(trimethylsilyloxy)methyl)phenyl)acetamide **158** (6.25 g, 23.82 mmol) was dissolved in 2M HCl (20 ml) and stirred at r.t. for 24 hrs. The reaction was extracted with diethyl ether, dried ($MgSO_4$) and reduced to give the alcohol as a yellow solid, 4.1 g (21.56 mmol, 90%). Mp: 123-125°C. R_f = 0.39 (petroleum ether : ethyl acetate, 5:1, solvent front: 38 mm). υ_{max}/cm^{-1} 3261 (br. s) (OH), 2159 (w) (CH), 1672 (m) (C=O). δ_H(300MHz, MeOD) 2.06 (3H, s, COC*H*$_3$), 5.51 (1H, s, C*H*CN), 7.38-7.41 (2H, m, Ar*H*), 7.55-7.58 (2H, m, Ar*H*). δ_C(75MHz, MeOD) 23.97 (*C*H$_3$), 63.50 (*C*-OH), 121.01, 121.30, 128.33, 133.67 (Ar*C*), 140.79 (Ar*C*-NH), 172.10 (*C*=O). $C_{10}H_9N_2O_2$ requires: 189.0670, found 189.0671 $[M-H]^+$. LTQ Orbitrap.

5.4.2.3 Preparation of 2-(4-acetamidophenyl)-2-hydroxyacetic acid **40o**[6]



N-(4-(Cyano(hydroxy)methyl)phenyl)acetamide **157** (2.0 g, 10.57 mmol) was dissolved in conc. HCl (5 ml) and stirred at r.t. for 24 hrs. The acid was removed by co-evaporating with toluene to give the title compound as an orange solid, 1.5 g (7.21 mmol, 68%). Mp: 105-106°C. R_f = 0.37 (MeOH : DCM, 1:9, solvent front: 27 mm). υ_{max}/cm^{-1} 3300 (w, s) (O-H), 1714 (m) (C=O), 1636 (m) (C=O). δ_H(300MHz, MeOD) 2.15 (3H, s, COC*H*$_3$), 5.13 (1H, s, C*H*OH), 7.40-7.47 (2H, m, Ar*H*), 7.56-7.68 (2H, m, Ar*H*). δ_C(75MHz, MeOD) 23.97 (*C*H$_3$), 71.68 (*C*-OH), 118.61 (2), 122.81, 126.94, 127.84 (Ar*C*), 139.92 (Ar*C*-NH), 163.75 (N-*C*=O), 173.73 (*C*=O). $C_{10}H_{10}NO_4$ requires: 208.0615, found 208.0617 $[M-H]^+$. LTQ Orbitrap.

5.4.2.4 Preparation of methyl 2-(4-acetamidophenyl)-2-methoxyacetate **159**[1]

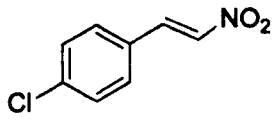


2-(4-Acetamidophenyl)-2-hydroxyacetic acid **40o** (0.5 g, 2.39 mmol) was dissolved in MeOH (50 ml) with a few drops of conc. H_2SO_4 before being refluxed for 12 hrs. After this time the solvent was removed and the residue was dissolved in ethyl acetate (50 ml), washed with a 10% aqueous K_2CO_3 solution and dried ($MgSO_4$) to give the title compound as a white solid, 0.4 g (1.69 mmol, 70%). Mp: 119-121°C. R_f = 0.66 (petroleum ether : ethyl acetate, 1:2, solvent front: 45mm). υ_{max}/cm^{-1} 2961 (w) (C-H), 1743 (m) (C=O), 1732 (m) (C=O). δ_H(300MHz, MeOD) 2.13 (3H, s, NCOC*H*$_3$), 3.32 (3H, s, OC*H*$_3$), 3.64 (3H, s, CO*CH*$_3$), 4.68 (1H, s, C*H*OMe), 7.30-7.33 (2H, m, Ar*H*), 7.45-7.48 (2H, m, Ar*H*), 7.92 (1H, s, N*H*). δ_C(75MHz, MeOD) 29.71 (*C*H$_3$), 52.39 (O*C*H$_3$), 57.29 ((C=O)-O*C*H$_3$), 119.92 (2), 128.02, 131.90 (Ar*C*), 138.31 (Ar*C*-NH), 168.33 (N-*C*=O), 170.02 (*C*=O). $C_{12}H_{15}NO_4NH_4$ requires: 255.1339, found 255.1342 $[M+NH_4]^+$. LTQ Orbitrap.

5.5. Synthesis of 1,2,5-oxadiazole-2-oxides

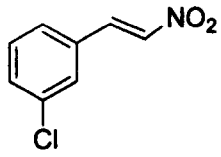
5.5.1. Synthesis of unsymmetrical 1,2,5-oxadiazole-2-oxides

5.5.1.1 Preparation of (*E*)-1-chloro-4-(2-nitrovinyl)benzene **170d**[7]



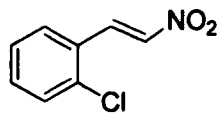
4-Chlorostyrene (2.0 g, 14.43 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (4.97 g, 72.15 mmol). After diluting the reaction mixture with water (200 ml) the reaction mixture was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as a yellow crystalline material 1.09 g (5.94 mmol, 41%). Mp: 101-103°C. R_f = 0.28 (petroleum ether : DCM, 7:3, solvent front: 21mm). υ_{max}/cm^{-1} 3039 (w) (C-H), 1635 (m) (C-NO_2), 1335 (m) (C-NO_2), 968 (s) (C=C), 816 (s) (C-Cl). δ_H(300MHz, $CDCl_3$) 7.35-7.47 (4H, m, Ar*H*), 7.47-7.51 (1H, d, *J* = 13.7 Hz, C*H*), 7.87-7.92 (1H, d, *J* = 13.7 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 128.53 (2), 128.82, 129.79, 130.28 (Ar*C*), 137.43 (*C*-Cl), 137.71 (*C*H-NO_2), 138.38 (*C*H). $C_8H_6ClNO_2$ requires: 184.0160, found: 184.0163 ($[M+H]^+$, 100%, ASAP)

5.5.1.2 Preparation of (*E*)-1-chloro-3-(2-nitrovinyl)benzene **170c**[7]



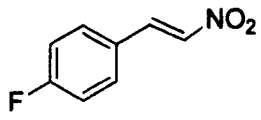
3-Chlorostyrene (2.0 g, 14.43 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (4.97 g, 72.15 mmol). After diluting the reaction mixture with water (200 ml) the reaction mixture was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as yellow crystals, 1.88 g (10.24 mmol, 70%). Mp: 56-58°C. R_f = 0.21 (petroleum ether : DCM, 7:3, solvent front: 21 mm). υ_{max}/cm^{-1} 3109 (w) (C-H), 1636 (m) (C-NO_2), 1337 (m) (C-NO_2), 962 (s) (C=C), 820 (s) (C-Cl). δ_H(300MHz, $CDCl_3$) 7.27-7.42 (4H, m, Ar*H*), 7.44-7.49 (1H, d, *J* = 13.7 Hz, C*H*), 7.80-7.85 (1H, d, *J* = 13.7 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 128.41, 128.77, 129.01, 129.61, 130.74 (Ar*C*), 135.72 (*C*-Cl), 136.48 (*C*H-NO_2), 136.98 (*C*H). $C_8H_6ClNO_2$ requires: 184.0160, found: 184.0161 ($[M+H]^+$, 100%, ASAP)

5.5.1.3 Preparation of (*E*)-1-chloro-2-(2-nitrovinyl)benzene **170b**[7]



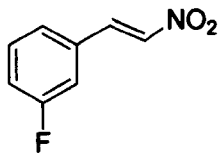
2-Chlorostyrene (2.0 g, 14.43 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (4.97 g, 72.15 mmol). After diluting the reaction mixture with water (200 ml) the reaction mixture was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as a yellow oil, 0.9 g (4.9 mmol, 33%). R_f = 0.26 (petroleum ether: DCM, 7:3, solvent front: 21 mm). υ_{max}/cm^{-1} 2917 (w) (C-H), 1631 (m) (C-NO_2), 1337 (m) (C-NO_2), 962 (s) (C=C), 749 (s) (C-Cl). δ_H(300MHz, $CDCl_3$) 7.19-7.55 (5H, m, C*H* & Ar*H*), 8.32-7.37 (1H, d, J = 13.7 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 127.14 (2), 127.79, 128.08, 130.73 (Ar*C*), 136.01 (*C*-Cl), 137.91 (*C*H-NO_2), 138.81 (*C*H). $C_8H_6ClNO_2$ requires: 184.0160, found: 184.0161 ($[M+H]^+$) ASAP.

5.5.1.4 Preparation of (*E*)-1-fluoro-4-(2-nitrovinyl)benzene **170g**[7]



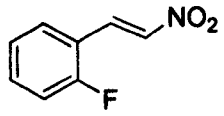
4-Fluorostyrene (2.0 g, 16.33 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (5.65 g, 81.88 mmol). After diluting the reaction mixture with water (200 ml) it was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as yellow crystals, 1.0 g (5.98 mmol, 37%). Mp: 104-105°C. R_f = 0.44 (petroleum ether : DCM, 7:3, solvent front: 27 mm). υ_{max}/cm^{-1} 3112 (w) (C-H), 1635 (m) (C-NO_2), 1336 (m) (C-NO_2), 964 (s) (C=C), 724 (m) (C-F). δ_H(300MHz, $CDCl_3$) 7.05-7.11 (2H, m, Ar*H*), 7.44-7.52 (3H, m, C*H* & Ar*H*), 7.89-7.93 (1H, d, *J* = 13.7 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 116.65, 116.95, 126.34, 131.39, 131.48 (Ar*C*), 136.84 (*C*H-NO_2), 137.89 (*C*H), 163.25, 166.63 (d, *C*-F). $C_8H_6FNO_2$ requires: 168.0455, found: 168.0454 ($[M + H]^+$) ASAP.

5.5.1.5 Preparation of (*E*)-1-fluoro-3-(2-nitrovinyl)benzene **170f**[7]



3-Fluorostyrene (2.0 g, 16.33 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (5.65 g, 81.88 mmol). After diluting the reaction mixture with water (200 ml) it was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as yellow crystals, 1.08 g (6.46 mmol, 40%). Mp: 85-86°C. R_f = 0.11 (petroleum ether : DCM, 7:3, solvent front: 34 mm). υ_{max}/cm^{-1} 3112 (w) (C-H), 1636 (m) (C-NO_2), 1343 (m) (C-NO_2), 962 (s) (C=C), 782 (m) (C-F). δ_H(300MHz, $CDCl_3$) 7.09-7.19 (2H, m, Ar*H*), 7.25-7.28 (1H, m, Ar*H*), 7.33-7.40 (1H, m, Ar*H*), 7.46-7.41 (1H, d, *J* = 13.7 Hz, C*H*), 7.86-7.90 (1H, d, *J* = 13.7 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 115.25, 115.55, 118.95, 125.19, 130.58 (Ar*C*), 137.71 (*C*H-NO_2), 137.74 (*C*H), 161.32, 164.61 (d, *C*-F). $C_8H_6FNO_2$ requires: 168.0454, found: 168.0455 ($[M+H]^+$) ASAP.

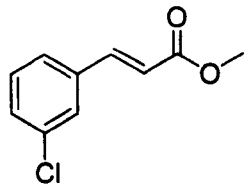
5.5.1.6 Preparation of (*E*)-1-fluoro-2-(2-nitrovinyl)benzene **170e**[7]



2-Fluorostyrene (2.0 g, 16.33 mmol) was dissolved in acetic acid (25 ml) and stirred at 14°C whilst adding 5 eq. of a saturated solution of sodium nitrite (5.65 g, 81.88 mmol). After diluting the reaction mixture with water (200 ml) it was stirred at r.t. until a yellow solid precipitate formed. The solid was filtered off, dissolved in DCM (50 ml), dried ($MgSO_4$) and reduced to give a yellow oil. The filtrate was washed repeatedly with DCM (200 ml), before the organic layer was dried ($MgSO_4$) and reduced to give a yellow oil. Column chromatography (petroleum ether : DCM, 7:3) of the combined oils, followed by recrystallisation from petroleum ether gave the title compound as yellow crystals, 1.08 g (6.46 mmol, 40%). Mp: 86-88°C . R_f = 0.20 (petroleum ether : DCM, 7:3, solvent front: 34 mm). υ_{max}/cm^{-1} 1648 (m) (C-NO_2), 1342 (m) (C-NO_2), 835 (s) (C=C), 757 (m) (C-F). δ_H(300MHz, $CDCl_3$) 7.06-7.19 (2H, m, Ar*H*), 7.37-7.47 (2H, m, Ar*H*), 7.61-7.66 (1H, d, *J* = 13.8 Hz, C*H*), 7.93-7.98 (1H, d, *J* = 13.8 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 115.25, 115.55, 118.95, 125.19, 130.58 (Ar*C*), 137.71 (*C*H-NO_2), 137.74 (*C*H), 161.32, 164.61 (d, *C*-F). $C_8H_6FNO_2$ requires: 168.0455, found: 168.0454 ($[M+H]^+$) ASAP.

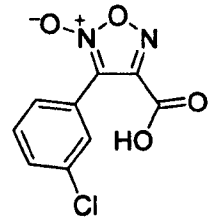
5.5.2. Synthesis of cinnamic furoxans

5.5.2.1 Preparation of (*E*)-methyl 3-(3-chlorophenyl)acrylate **186**

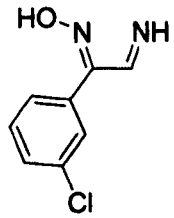


3-Chlorocinnamic acid (0.5 g, 2.74 mmol) was dissolved in MeOH (20 ml) with 2-3 drops of conc. H_2SO_4 and allowed to relux for 12 hrs. The solvent was reduced and the residue dissolved in ethyl acetate (50 ml) before being washed with a 10% aqueous K_2CO_3 solution. The organic layer was dried ($MgSO_4$) and reduced to give the title compound as a white solid, 0.4g (2.03mmol, 74%), Mp: 27-29°C. R_f = 0.86, (petroleum ether : ethyl acetate 1:1, solvent front: 22 mm). υ_{max}/cm^{-1} 3075,2959 (w) (C-H), 1709 (s) (C=C), 1644 (C=O), 791 (s) (C-Cl). δ_H(300MHz, $CDCl_3$) 3.82 (3H, s, OCH_3), 6.42-6.48 (1H, d, J = 16.1 Hz, C*H*), 7.27-7.45 (3H, m, Ar*H*), 7.51 (1H, s, Ar*H*), 7.61-7.66 (1H, d, J = 16.1 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 51.88 (OCH_3), 119.25 (*C*H), 126.26, 127.80, 130.15 (2) (Ar*C*), 136.18 (*C*-Cl), 134.91 (Ar*C*), 143.27 (*C*H), 167.04 (*C*=O). $C_{10}H_{10}ClO_2$ requires: 197.0364, found 197.0360 $[M+H]^+$. ASAP.

5.5.2.2 Preparation of 3-carboxy-4-(3-chlorophenyl)-1,2,5-oxadiazole 2-oxide **182**

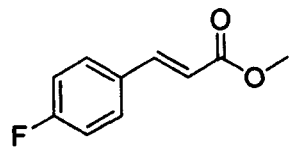


3-Chlorocinnamic methyl ester **186** (0.34 g, 1.73 mmol) was dissolved in sulphuric acid (25 ml) before a saturated solution of $NaNO_2$ (100 ml) was added and the reaction mixture left to stir at r.t. for 24 hrs. The mixture was extracted with DCM (50 ml), dried ($MgSO_4$) and reduced to give a red solid. The crude product was recrystallised from DCM to give the title compound as a pale yellow solid, 0.3 g (1.24 mmol, 72%), Mp: 53-55°C. R_f = 0.67 (petroleum ether : ethyl acetate 1:1, solvent front: 38 mm). υ_{max}/cm^{-1} 3100-2823 (br. m) (OH), 1682 (s) (C=O), 1573 (s) (C=N), 791 (s) (C-Cl). δ_H(300MHz, $CDCl_3$) 7.33-7.40 (1H, m, Ar*H*), 7.51-7.55 (1H, m, Ar*H*), 7.91-7.96 (1H, m, Ar*H*), 8.01-8.05 (1H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 128.34 (2), 129.88 (2), 130.28 (Ar*C*), 130.93 (*C*-Cl), 133.94 (*C*=N^+), 134.72 (*C*=N), 170.89 (*C*=O). $C_9H_5ClN_2O_4$ requires: 239.9938, found 182.9857 $[M-H]^-$. LTQ Orbitrap. (Decomposition to **188**.)



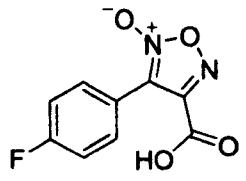
188

5.5.2.3 Preparation of (*E*)-methyl 3-(4-fluorophenyl)acrylate **185**

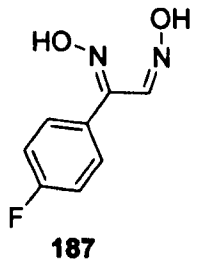


4-Fluorocinnamic acid (4.54 g, 27.32 mmol) was dissolved in MeOH (200 ml) with 5-6 drops of H_2SO_4 and refluxed for 12 hrs. After this time the solvent was removed and the residue was dissolved in ethyl acetate (150 ml), and washed with a 10% aqueous K_2CO_3 solution before being dried ($MgSO_4$) and reduced to give the title compound as a white solid, 4.14 g (22.86 mmol, 84%), Mp: 52-54°C. R_f = 0.87, (petroleum ether : ethyl acetate 1:1, solvent front: 39 mm). υ_{max}/cm^{-1} 3034, 2955 (w) (C-H), 1704 (m) (C=C), 1632 (C=O). δ_H(300MHz, $CDCl_3$) 3.81 (3H, s, OC*H*$_3$), 6.34-6.40 (1H, d, *J* = 16.0 Hz, C*H*), 7.05-7.12 (2H, m, Ar*H*), 7.49-7.55 (2H, m, Ar*H*), 7.63-7.69 (2H, d, *J* = 16.0 Hz, C*H*). δ_C(75MHz, $CDCl_3$) 51.75 (O*C*H$_3$), 115.90, 116.19 (Ar*C*), 117.50 (*C*H), 129.89, 130.58, 130.63 (Ar*C*), 143.56 (*C*H), 162.23, 165.56 (d, C-F) 167.32 (*C*=O). $C_{11}H_9FO_2$ requires: 181.0659, found 181.0655 $[M+H]^+$. ASAP.

5.5.2.4 Preparation of 3-carboxy-4-(4-fluorophenyl)-1,2,5-oxadiazole 2-oxide **181**



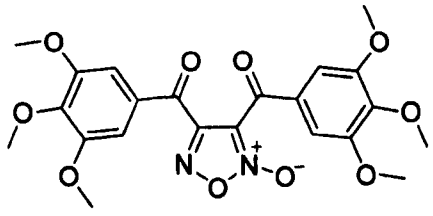
4-Fluorocinnamic methyl ester **185** (2.0 g, 10.17 mmol) was dissolved in sulphuric acid (25 ml). A saturated solution of $NaNO_2$ was added to the solution and left to stir at r.t. for 24 hrs. The mixture was extracted with DCM (50 ml), dried ($MgSO_4$) and reduced to give a red solid. The crude product was recrystallised from DCM to give the title compound as a white solid, 1.8 g (8.04 mmol, 79%), Mp: 79-81°C. R_f = 0.58 (petroleum ether : ethyl acetate, 1:1, solvent front: 32 mm). υ_{max}/cm^{-1} 3100-2553 (br. m) (OH), 1672 (s) (C=O), 1603 (s) (C=N). δ_H(300MHz, $CDCl_3$) 7.04-7.13 (2H, m, Ar*H*), 8.04-8.10 (2H, m, Ar*H*). δ_C(75MHz, $CDCl_3$) 115.62, 115.91, 125.46 (2), 125.50 (Ar*C*), 132.81 (*C*=N^+), 132.97 (*C*=N), 164.66, 168.05 (d, *C*-F), 171.02 (*C*=O). $C_9H_4N_2O_4$ requires: 223.0155, found 182.9854 $[M-H]^-$. LTQ Orbitrap. (Decomposition to **187**.)



187

5.5.3. Synthesis of symmetrical furoxans as Combretastatin A-4 anticancer derivatives

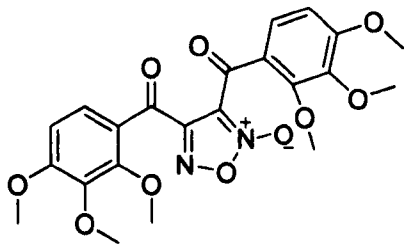
5.5.3.1 Preparation of 3,4-bis(3,4,5-trimethoxybenzoyl)-1,2,5-oxadiazole 2-oxide **144a**[8]



Diluted HNO_3 (2.8 ml in 8.5 ml H_2O) was added dropwise to 3,4,5-trimethoxyacetophenone (2.5 g, 11.9 mmol) acetic acid (12.5 ml) whilst heating the mixture to 60°C. Having added a catalytic amount of $NaNO_2$ the reaction was heated (60°C) and stirred for 2 hrs, going from a pale yellow to a bright red in the process. The reaction was halted by pouring the mixture onto ice-water and the solid precipitate was then filtered off whilst the aqueous filtrate was extracted with DCM (3 x 50 ml) dried ($MgSO_4$) and reduced to give a red oil. Both precipitate and oil were combined and columned using 1:1, petroleum ether : ethyl acetate, to give a dark orange solid of the title compound, which was recrystallised from DCM to give yellow needle-like crystals, 0.5g (1.05 mmol, 21%). Mp: 116-118°C. R_f = 0.2 (petroleum ether : ethyl acetate, 1:1, solvent front: 21 mm). υ_{max}/cm^{-1} 2947 (w) (C-H), 1660 (w) (C=O), 1617 (w) (C=N), 1459 (m) (O-N-O), 1332 (m) (N-O), 1185, 989, 853 (m) (furoxan ring). δ_H(300MHz, $CDCl_3$) 3.78-3.91 (18H, m, 6 x OC*H*$_3$), 7.02 (1H, s, Ar*H*), 7.40 (1H, s, Ar*H*), 7.485 (2H, s, Ar*H*). δ_C(75MHz, $CDCl_3$) 56.19 (2 x O*C*H$_3$), 56.35 (2 x O*C*H$_3$), 61.09 (2 x O*C*H$_3$), 101.24 (2) Ar*C*) , 107.93 (*C*=N$^+$), 128.58, 128.72 (Ar*C*), 143.37, 143.66 (Ar*C*), 144.77 (*C*=N), 152.83 (2 x Ar*C*-OCH$_3$), 153.23 (2 x Ar*C*-OCH$_3$), 153.43 (Ar*C*-OCH$_3$), 154.64 (Ar*C*-OCH$_3$), 179.03

(*C*=O), 180.31 (*C*=O). $C_{22}H_{22}N_2O_{10}H$ requires: 475.1340, found 475.1347 $[M+H]^+$. ASAP.

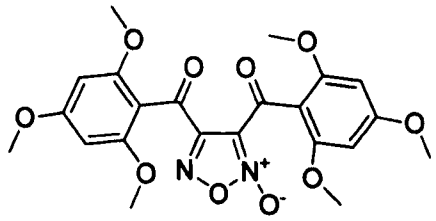
5.5.3.2 Preparation of 3,4-bis(2,3,4-trimethoxybenzoyl)-1,2,5-oxadiazole 2-oxide **144b**[8]



Diluted HNO_3 (2.8 ml in 8.5 ml H_2O) was added dropwise to 2,3,4-trimethoxyacetophenone (2.5 g, 11.9 mmol) in acetic acid (12.5 ml) whilst heating the mixture to 60°C. Having added a catalytic amount of $NaNO_2$ the reaction was heated (60°C) and stirred for 2 hrs, going from a pale yellow to a bright orange in the process. The reaction was halted by pouring the mixture onto ice-water and the solid precipitate was then filtered off whilst the aqueous filtrate was extracted with DCM (3 x 50 ml) dried ($MgSO_4$) and reduced to give a red oil. Both precipitate and oil were combined and columned using 1:1, petroleum ether : ethyl acetate, to give a dark orange solid of the title compound, which was recrystallised from DCM to give yellow needle-like crystals, 0.6g (1.26mmol, 21%). Mp: 94-96°C. R_f = 0.19 (petroleum ether : ethyl acetate, 1:1, solvent front: 23 mm). υ_{max}/cm^{-1} 2947 (w) (C-H), 1660 (w) (C=O), 1617 (w) (C=N), 1459 (m) (O-N-O), 1332 (m) (N-O), 1185, 989, 853 (m) (furoxan ring). δ_H(300MHz, $CDCl_3$) 3.82-4.07(18H, m, 6 x OC*H*$_3$), 6.73-6.78 (2H, d, *J* = 8.9 Hz, Ar*H*), 7.82-7.85 (2H, d, *J* = 8.9 Hz, Ar*H*). δ_C(75MHz, $CDCl_3$) 56.26 (2 x OCH_3), 61.25 (2 x OCH_3), 62.59 (2 x OCH_3), 103.59 (2) (Ar*C*), 108.05 (*C*=N^+), 114.26 (2), 128.45 (2) (Ar*C*), 141.21 (*C*=N), 152.93 (3 x

Ar*C*-OCH_3), 158.30 (3 x Ar*C*-OCH_3), 165.52 (*C*=O), 198.82 (*C*=O). $C_{22}H_{22}N_2O_{10}H$ requires: 475.1342, found 475.1347 $[M+H]^+$. ASAP.

5.5.3.3 Preparation of 3,4-bis(2,4,6-trimethoxybenzoyl)-1,2,5-oxadiazole 2-oxide **144c**[8]



Diluted HNO_3 (2.8 ml in 8.5 ml H_2O) was added dropwise to 2,4,6-trimethoxyacetophenone (2.5 g, 11.9 mmol) in acetic acid (12.5 ml) whilst heating the mixture to 60°C. Having added a catalytic amount of $NaNO_2$ the reaction was heated (60°C) and stirred for 2 hrs, going from a pale yellow to a dark red colour in the process. The reaction was halted by pouring the mixture onto ice-water and the solid precipitate was then filtered off whilst the aqueous filtrate was extracted with DCM (3 x 50 ml) dried ($MgSO_4$) and reduced to give a red oil. Both precipitate and oil were combined and columned using 1:1, petroleum ether : ethyl acetate, to give a dark red solid of the title compound, which was recrystallised from DCM to give red needle-like crystals, 0.5g (1.05mmol, 18%). Mp: 173-175°C. R_f = 0.26, (petroleum ether : ethyl acetate, 1:1, solvent front: 21mm). υ_{max}/cm^{-1} 2946 (w) (C-H), 2837 (w) (OMe), 1678 (m) (C=O), 1618 (w) (C=N), 1457 (m) (O-N-O), 1329 (m) (N-O), 1183, 989, 854 (m) (furoxan ring). δ_H(300MHz, $CDCl_3$) 3.84 (18H, s, 6 x O*CH*$_3$), 5.87 (4H, s, Ar*H*). δ_C(75MHz, $CDCl_3$) 56.52 (6 x OCH_3), 107.44 (*C*=N^+), 113.81 (4) (Ar*C*), 157.31 (*C*=N), 164.02 (2) (Ar*C*),

176.72 (6 x Ar*C*-OCH_3), 186.89 (*C*=O), 193.41 (*C*=O). $C_{23}H_{22}N_2O_{10}$ requires: 476.1370, found 476.1379 $[M+H]^+$. ASAP.

5.6 References

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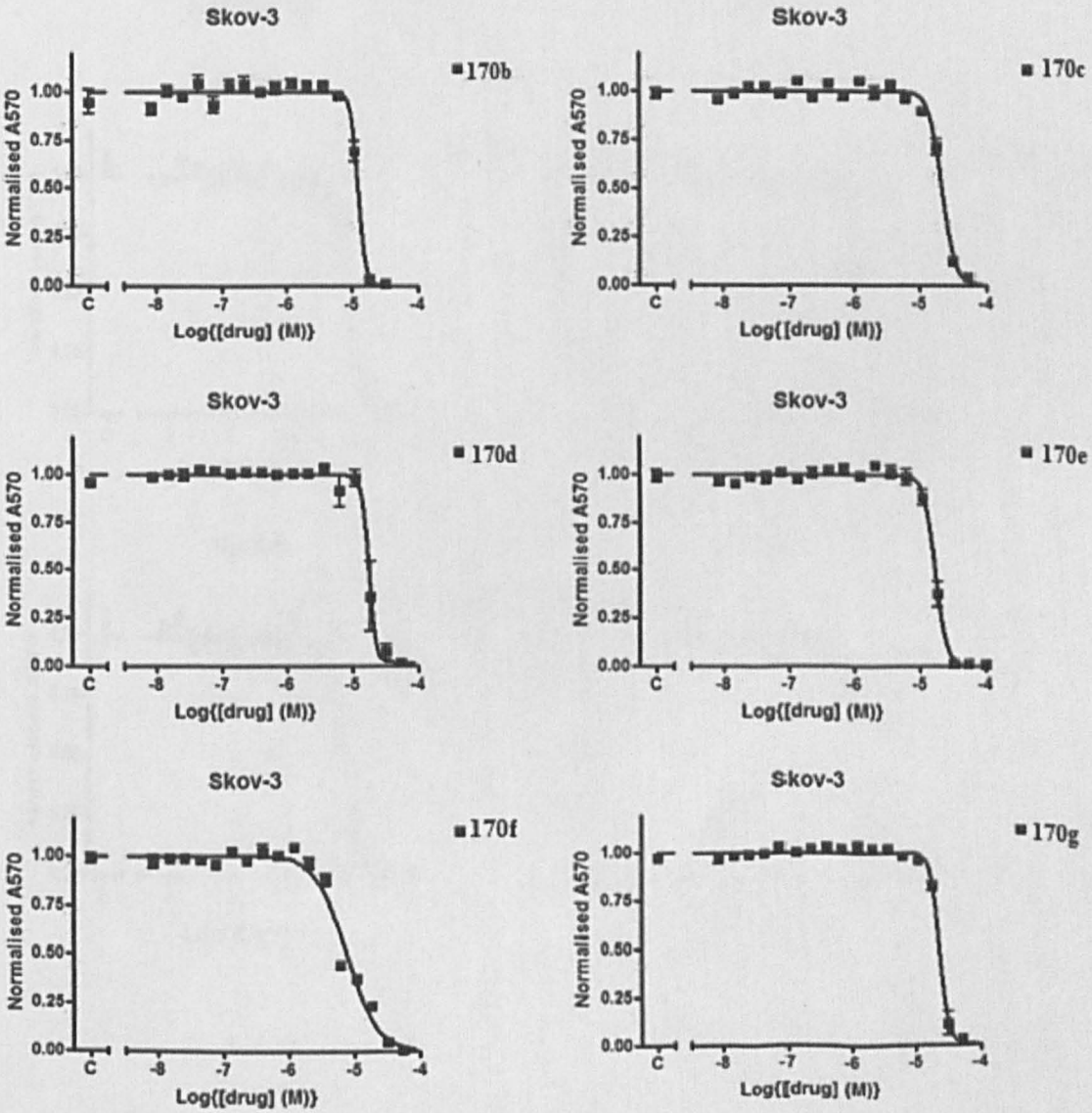
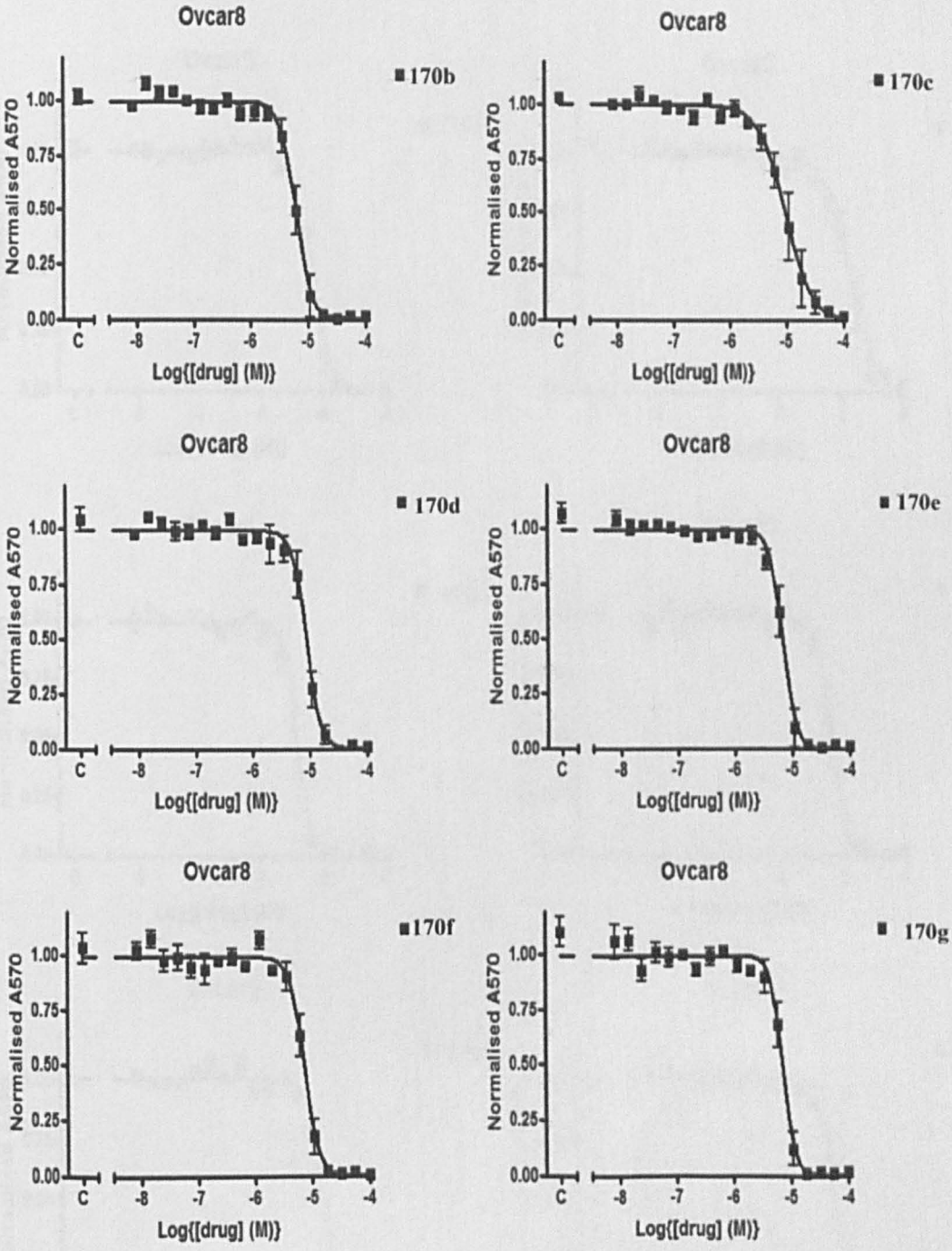
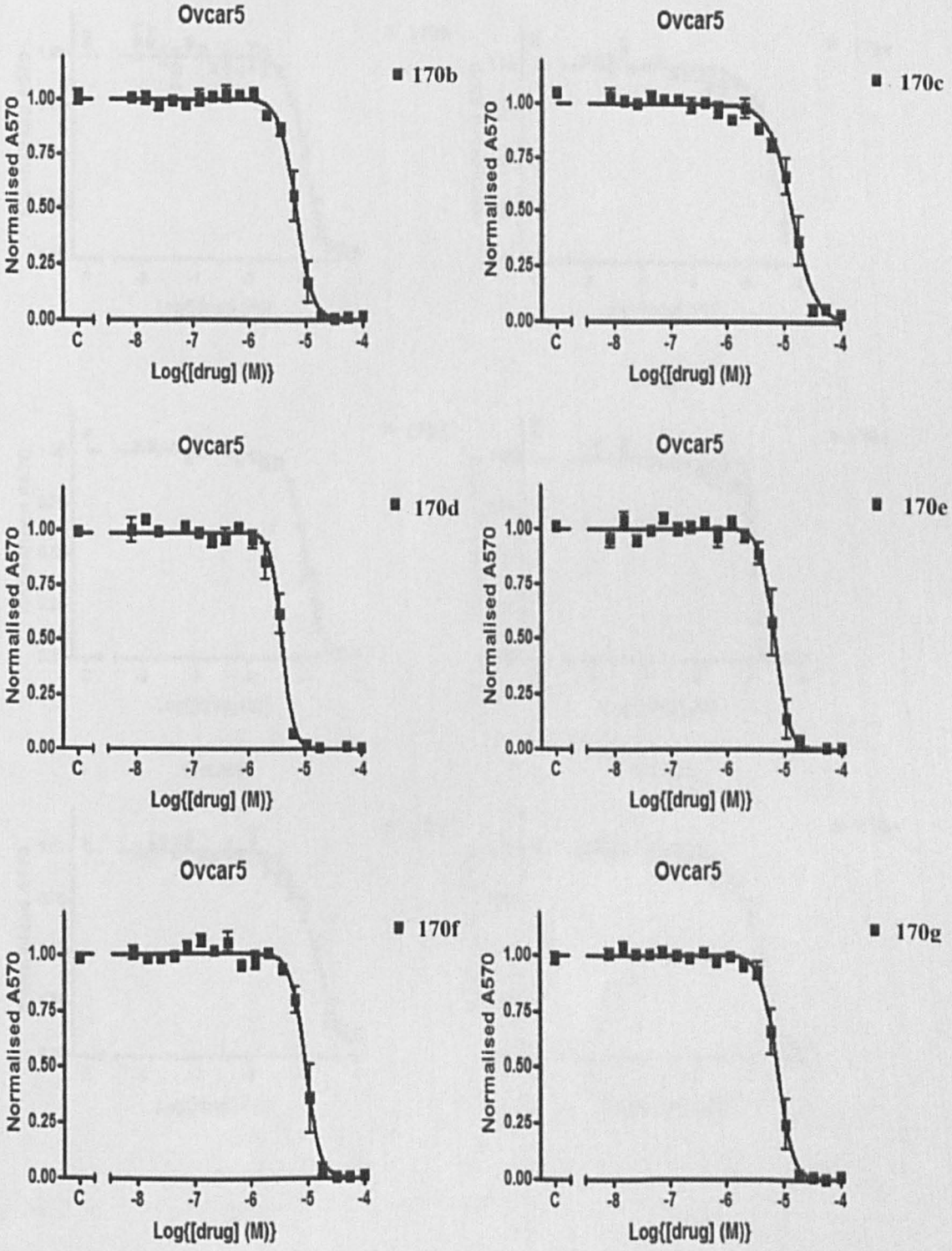
Appendix 1:

IC_{50} profiles for nitroalkenes **170b-g** in cell lines Skov-3, Ovcar8, Ovcar5, Ovcar4, Igrov, cisA2780 and A2780.




Ovcar8
170b
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar8
170c
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar8
170d
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar8
170e
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar8
170f
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar8
170g
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))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Normalised A570
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0.75
0.50
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C
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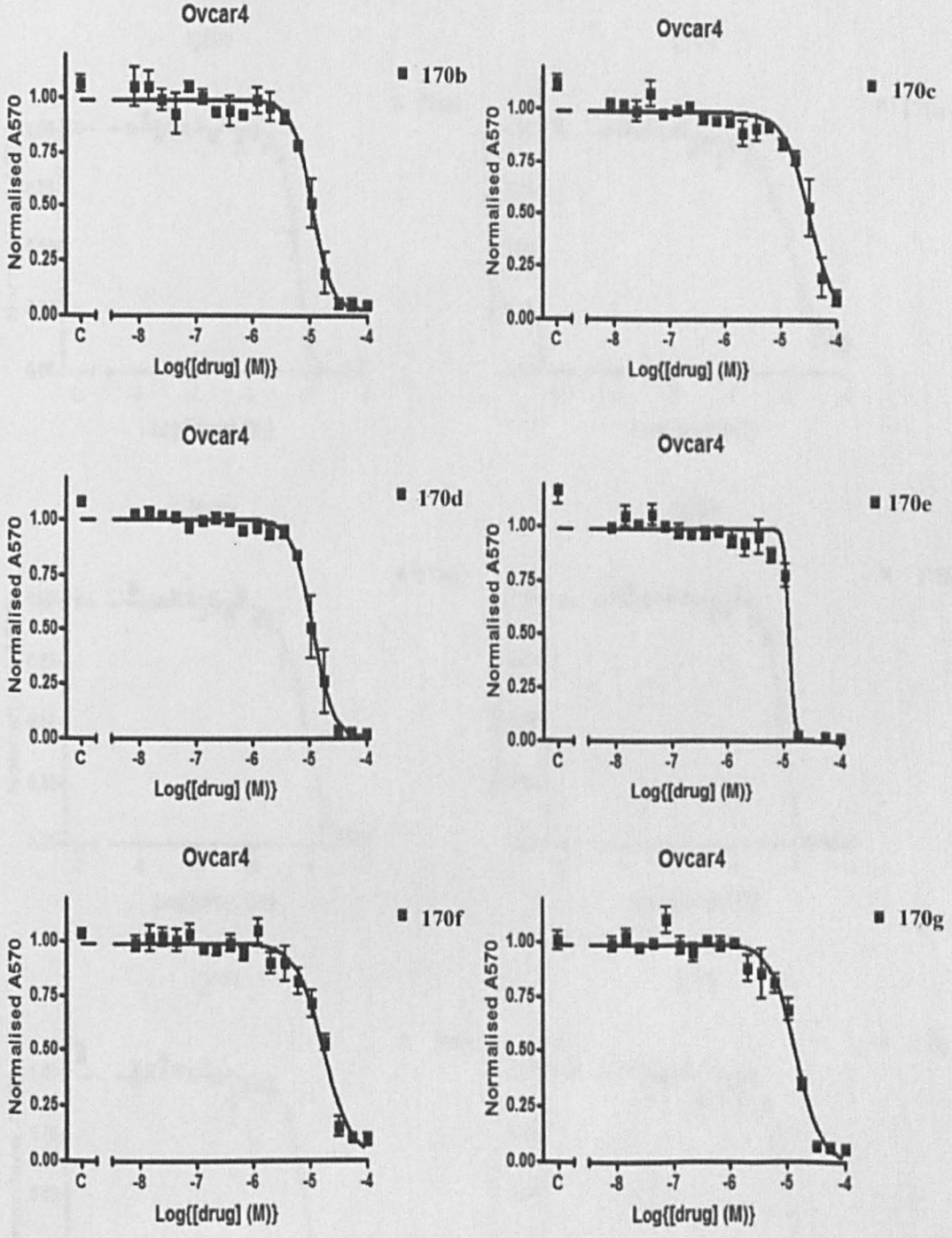
-7
-6
-5
-4
Log{[drug] (M)}
170b
Ovcar5
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
170c
Ovcar5
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
170d
Ovcar5
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
170e
Ovcar5
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
170f
Ovcar5
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
170g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0.25
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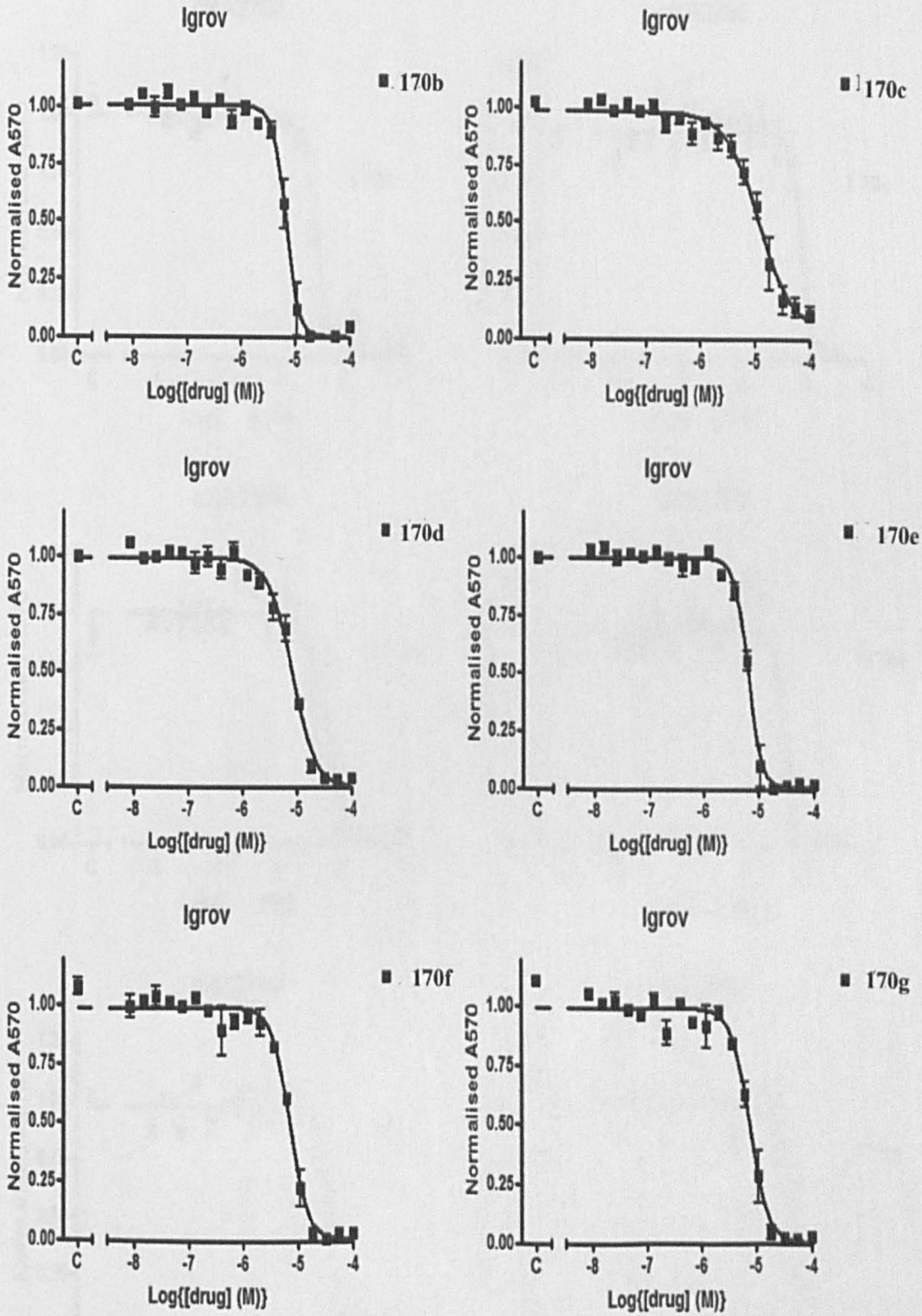
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar4
170c
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar4
170d
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar4
170e
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar4
170f
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))
Ovcar4
170g
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log([drug] (M))


Igrov
170b
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
Igrov
170c
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
Igrov
170d
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
Igrov
170e
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
Igrov
170f
Normalised A570
1.00
0.75
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}
Igrov
170g
Normalised A570
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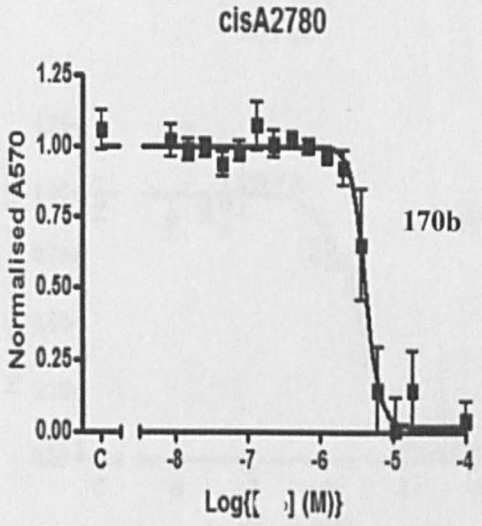
0.50
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[drug] (M)}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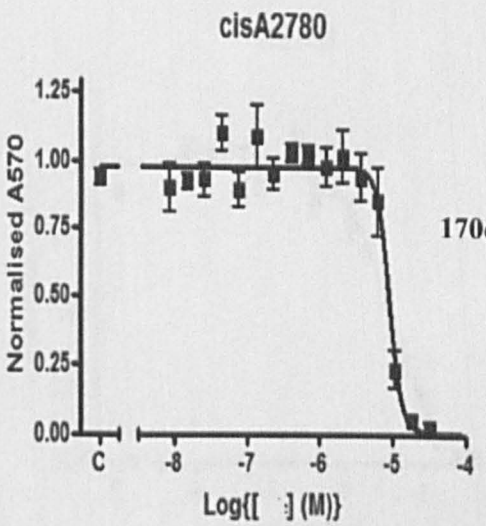
0.00
C
-8
-7
-6
-5
-4
Log{[ ] (M)}
170b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Normalised A570
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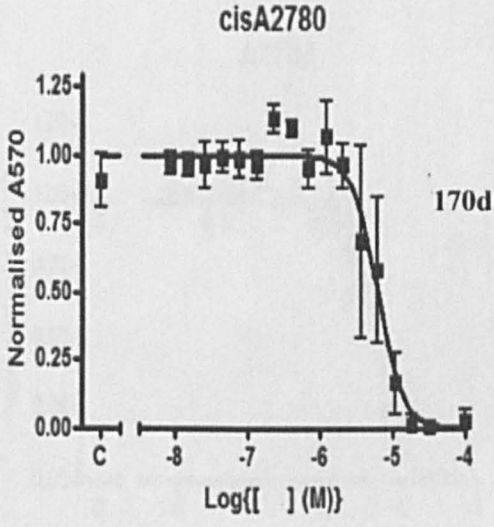
0.25
0.00
C
-8
-7
-6
-5
-4
Log{[ ] (M)}
170c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0.25
0.00
C
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-7
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-5
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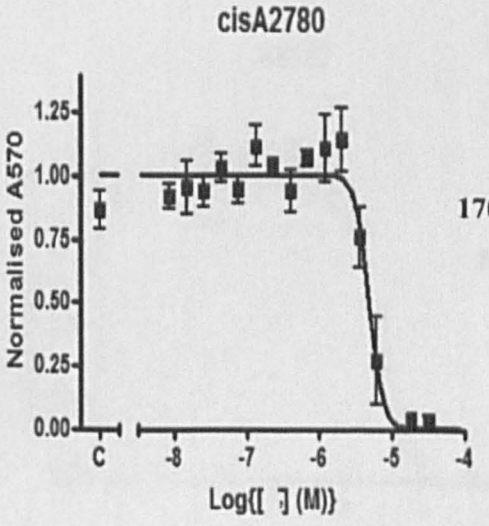
Log{[ ] (M)}
170d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0.00
C
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-7
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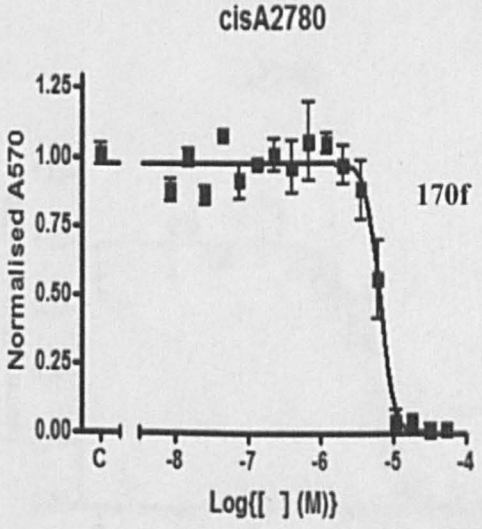
-5
-4
Log{[ ] (M)}
170e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C
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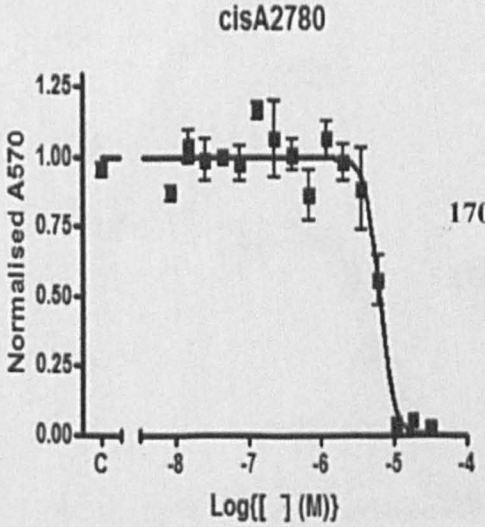
-6
-5
-4
Log{[ ] (M)}
170f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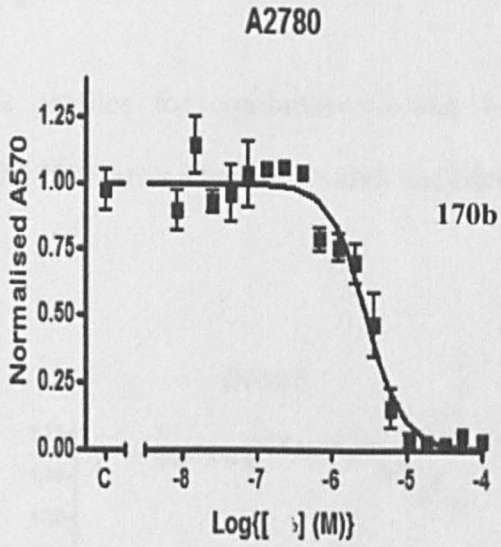
-4
Log{[ ] (M)}
170g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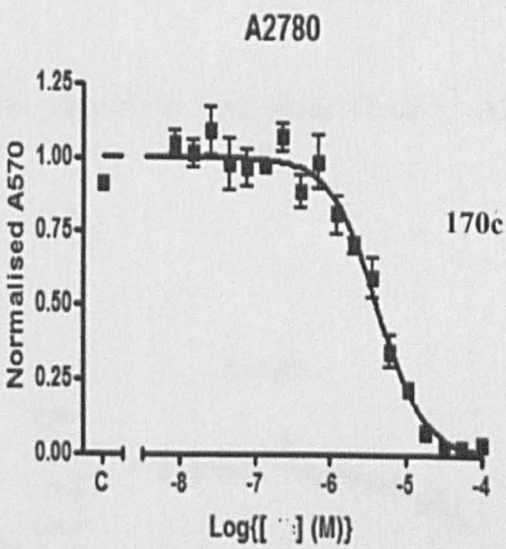
-4
Log([ ] (M))
170b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Normalised A570
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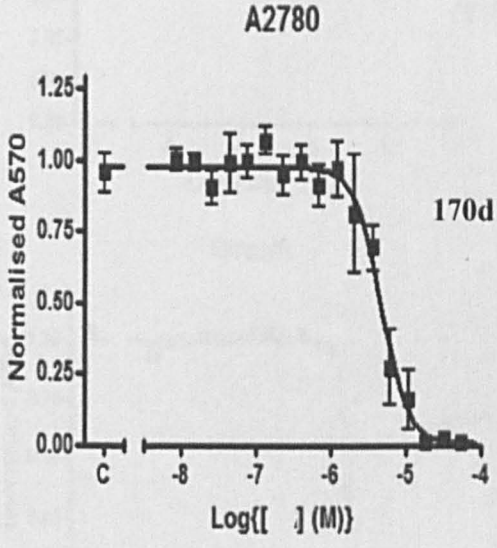
0.25
0.00
C
-8
-7
-6
-5
-4
Log([ ] (M))
170c


A2780
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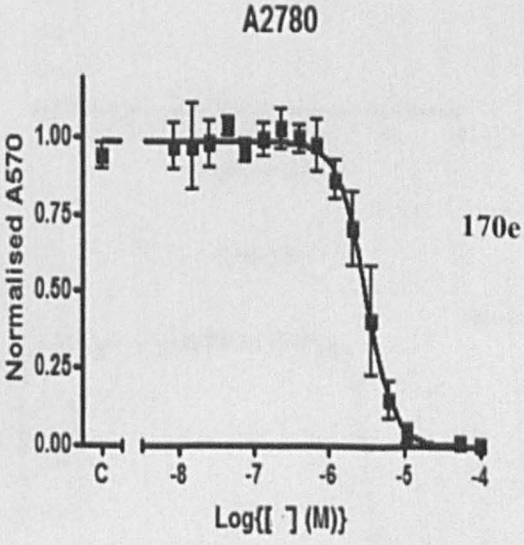
0.00
C
-8
-7
-6
-5
-4
Log([ ] (M))
170d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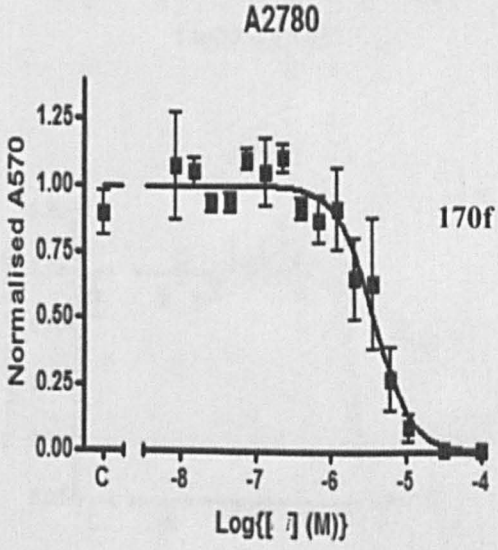
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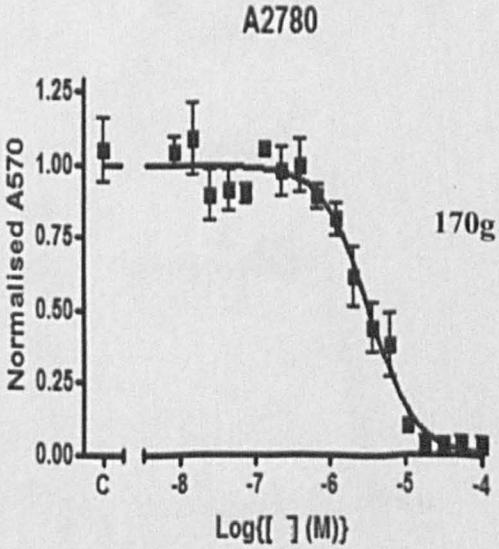
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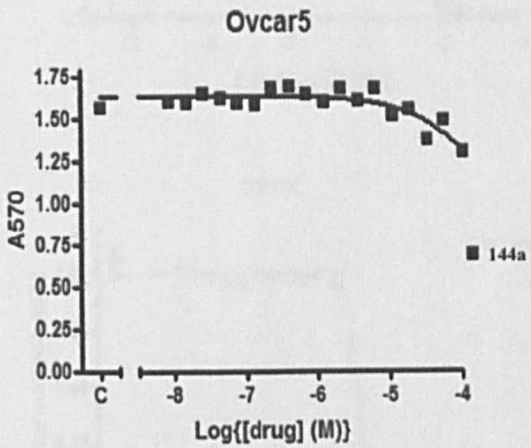
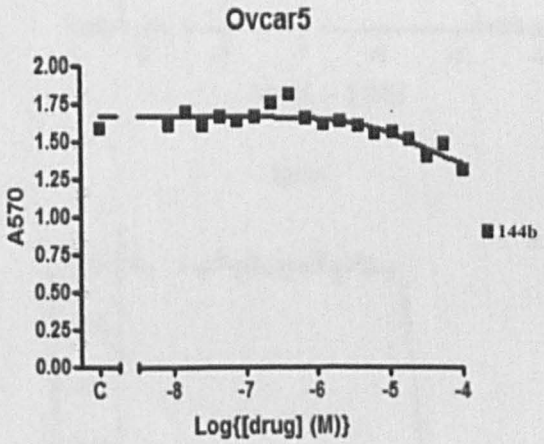
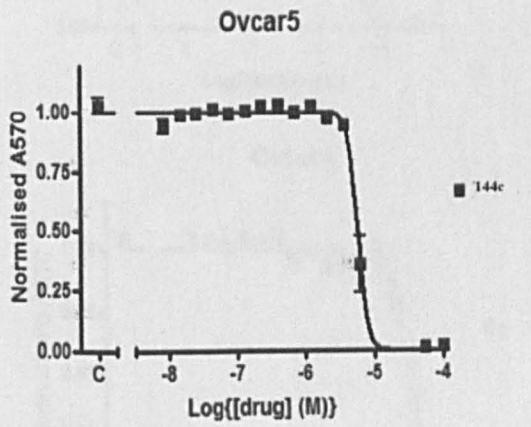
0.00
C
-8
-7
-6
-5
-4
Log([ ] (M))
170g

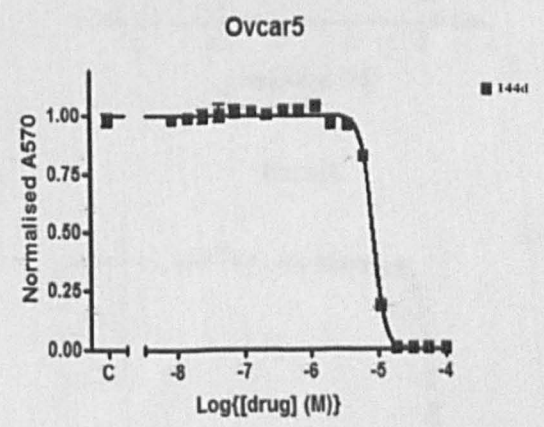
Appendix 2:

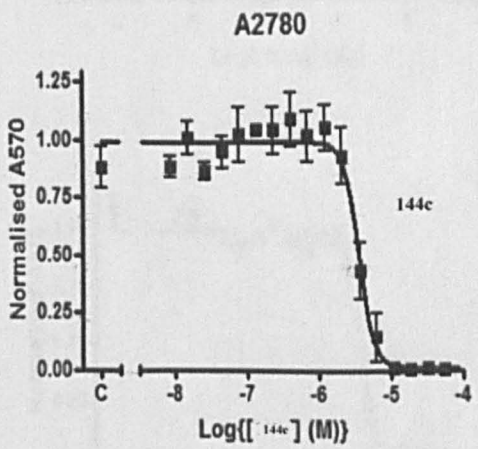
IC_{50} profiles for combretastatin-like furoxans **144c-d** in cell lines Ovcar5, A2780, cisA2780, Igrov, Ovcar4, Ovcar8 and Skov.

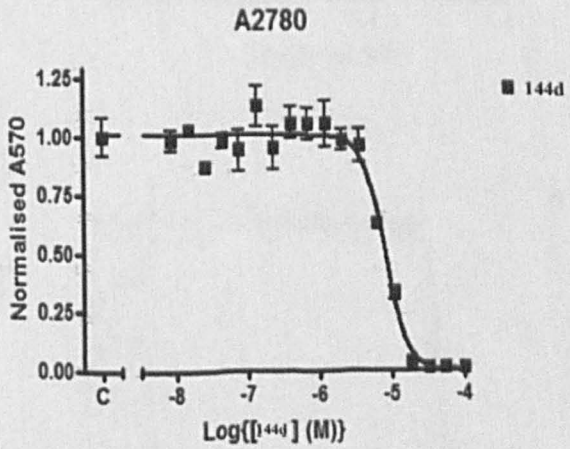


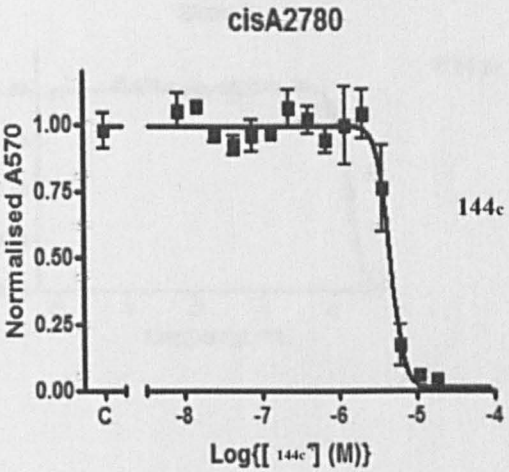








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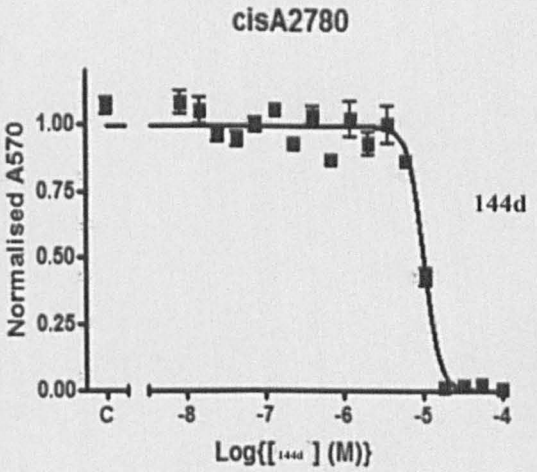
144c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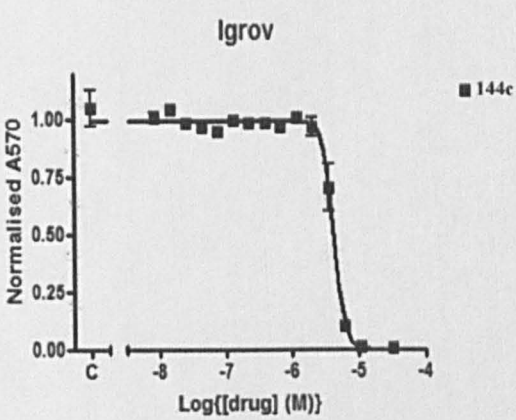
Log{[144d] (M)}
144d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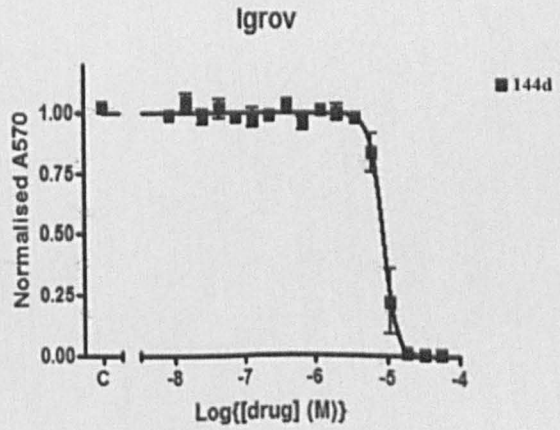
144c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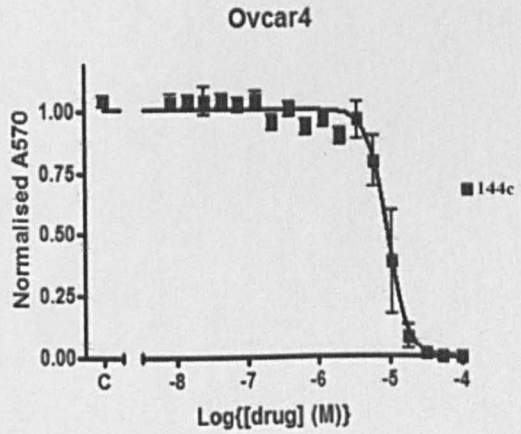
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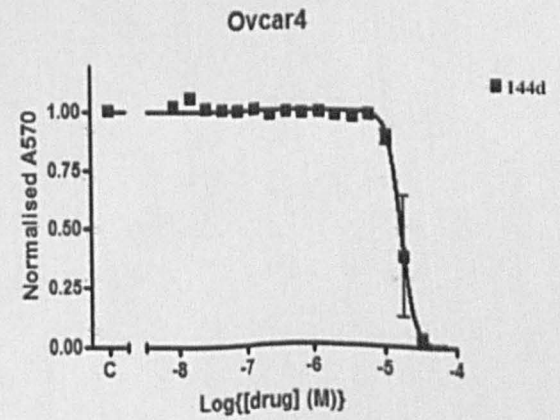
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C
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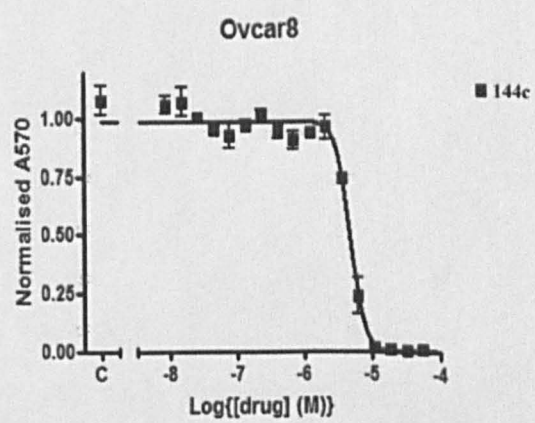
Log{[drug] (M)}
144d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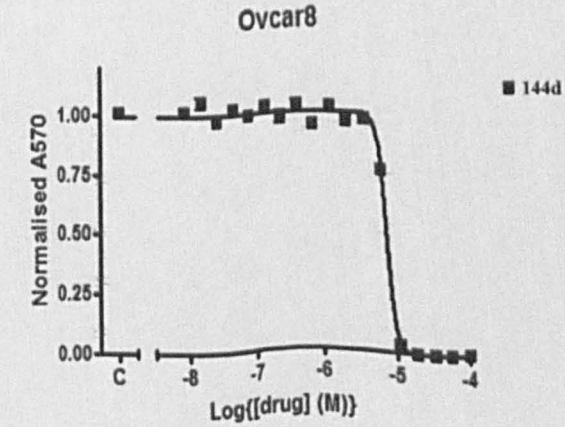
Log{[drug] (M)}
144c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C
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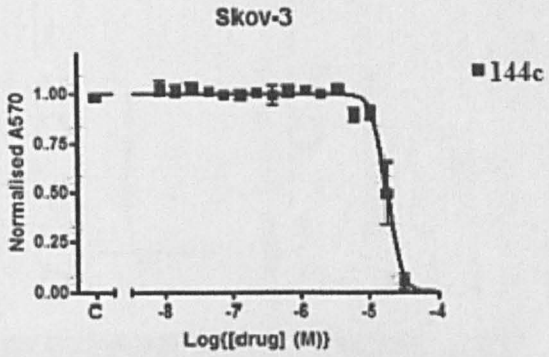
Log{[drug] (M)}
144d


Skov-3
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C
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-7
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-5
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Log([drug] (M))

A NOVEL SERIES OF NITRIC OXIDE RELEASING COMPOUNDS

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m.eilertsen@epsam.keele.ac.uk

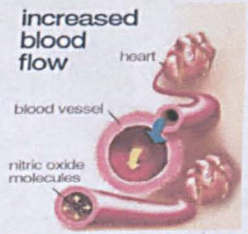
Appendix 3

What is Nitric Oxide?

Nitric Oxide is a colourless diatomic gas. In the late 1980's it was discovered that NO is an important chemical signalling agent *in vivo*.

Why is NO important?

Nitric Oxide has many functions in the human body. It relaxes smooth muscles, inhibits platelet aggregation, acts as a signalling agent in the brain and is a cytotoxic agent involved in inflammatory responses. It is synthesised in the body when needed and has very localised effects.



Source: www.iecwealth.com

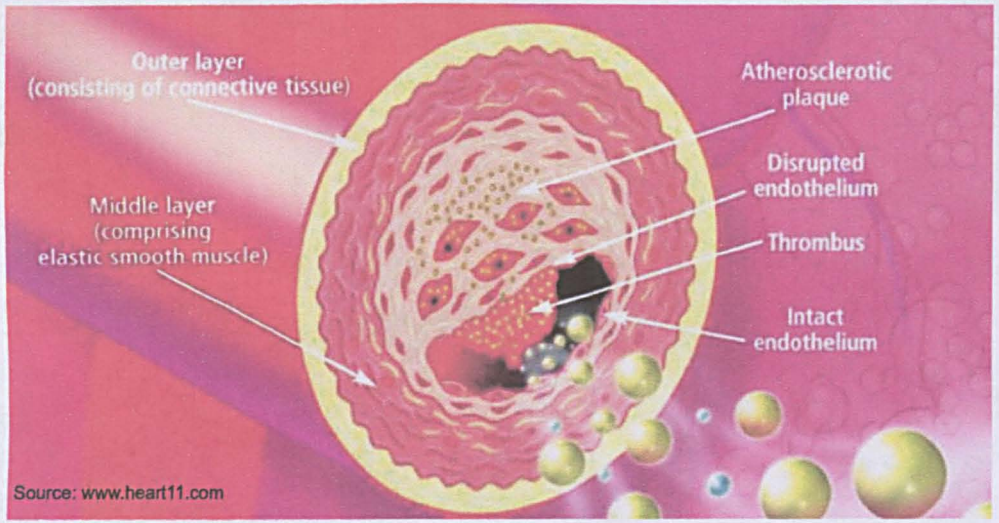
Nitric oxide donor drugs supply exogenous NO to the body, which results in a biological cascade, ultimately leading to relaxation of the smooth muscle and thus increased blood flow.

Why use *S*-nitrosothiols?

This characteristic colour of *S*-nitrosothiols (RSNO's) makes it possible to monitor the rate of release of NO using UV-Vis spectroscopy. RSNO's are found in a range of tissues and body fluids so, due to their endogenous nature, they are unlikely to produce any toxic effects in the body. In addition, RSNO's do not require metabolic degradation and are therefore expected to be tolerated *in vivo*.

However, RSNO's are not very stable, and exposure to light, heat, enzymes or metals such as copper, results in decomposition to the corresponding disulphide (RSSR) and the release of NO.

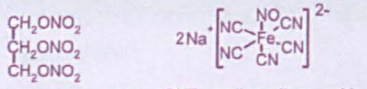
$$2RSNO \rightarrow RSSR + 2NO^{\bullet}$$



Source: www.heart11.com

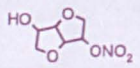
Why try to synthesis new NO donor drugs?

In order to understand the need for new NO donor drugs, it is necessary to review the NO donor drugs currently avaliable for clinical use in the UK.



GTN - glyceryl trinitrate

SNP - sodium nitroprusside



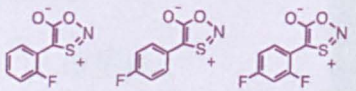
ISMN - isosorbide mononitrate

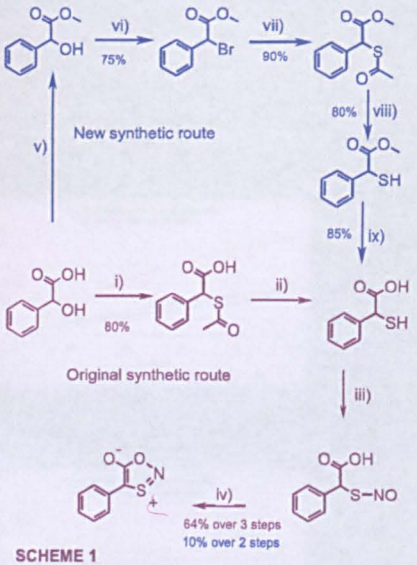
GTN and ISMN are used in treatment of acute and chronic angina, respectively. SNP is used to lower blood pressure in hospitalised patients. However, there is a need for new drugs as use of GTN and ISMN result in increased tolerance, while SNP can give cyanosis due to the reported release of cyanide.

Next generation *S*-nitrosothiols

Recently, P.G. Wang *et al.* published work on a new generation *S*-nitrosothiols, 4-Aryl, 1,3,2-Oxathiazolylium-5-olates (AZOs) which showed promising chemical and biological results. The aim of this project is to synthesis novel NO donors based on these compounds and carry out tests to evaluate the suitability of these compounds as future NO donor drugs.

Scheme 1 illustrates the synthetic route used to synthesise the azo compounds. Future target molecules include a series of fluorinated compounds, some of which are shown below.





SCHEME 1

i) Ph_3P, DIAD, HSAc
ii) NaOMe/MeOH
iii) $^tBuONO/CH_2Cl_2$
iv) DCC/CH_2Cl_2
v) H_2SO_4, MeOH, reflux, 24hr
vi) PBr_3, $CHCl_3$, rt., 4d
vii) KSAc, MeOH, rt., 4hr
viii) NaOMe/MeOH
ix) LiOH, THF, H_2O, rt., 20hr

Acknowledgments

We would like to thank EPSAM for funding this project.

The author would like to thank Dr Russell Pearson and Prof Steve Allin for their support and supervision. We would also like to thank John Clews, Liam Duffy and the other members of the Medicinal and Pharmaceutical Sciences research cluster for practical advice and input.

KEELE UNIVERSITY

Heterocyclic Nitric Oxide Donors - Making Drugs

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[1]School of Physical and Geographical Sciences, Keele University
[2]School of Pharmacy, Keele University

Appendix 4

What is Nitric Oxide?

$^{\cdot}N{=}O$ $^{\cdot}NO$

Since the discovery of nitric oxide (NO) as an important biological messenger, this small gaseous molecule has generated interest from all branches of the scientific community. NO has many functions in the human body. It relaxes smooth muscles, prevents clotting of the blood, acts as a signalling agent in the brain and is involved in inflammatory responses. It is synthesised in the body when needed and has very localised effects. Due to its biological activity, research on both the biological/medicinal and chemical nature of NO has steadily increased in recent years.

Why synthesise heterocyclic NO donors?

The focus of this research is to design a compound which can deliver exogenous NO and deliver this to a biological system at a controlled rate. The reason for making heterocyclic NO donors is that these compounds are more stable and can act as prodrugs. The different classes of heterocyclic NO donors (Figure 1) release NO under different conditions, the OZOs (1) release NO when exposed to heat or light. The furoxans (2) release NO when exposed to light of a specific wavelength or when in contact endogenous compounds such as thiolates.

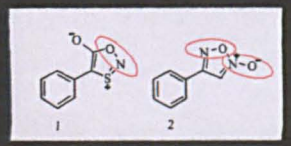
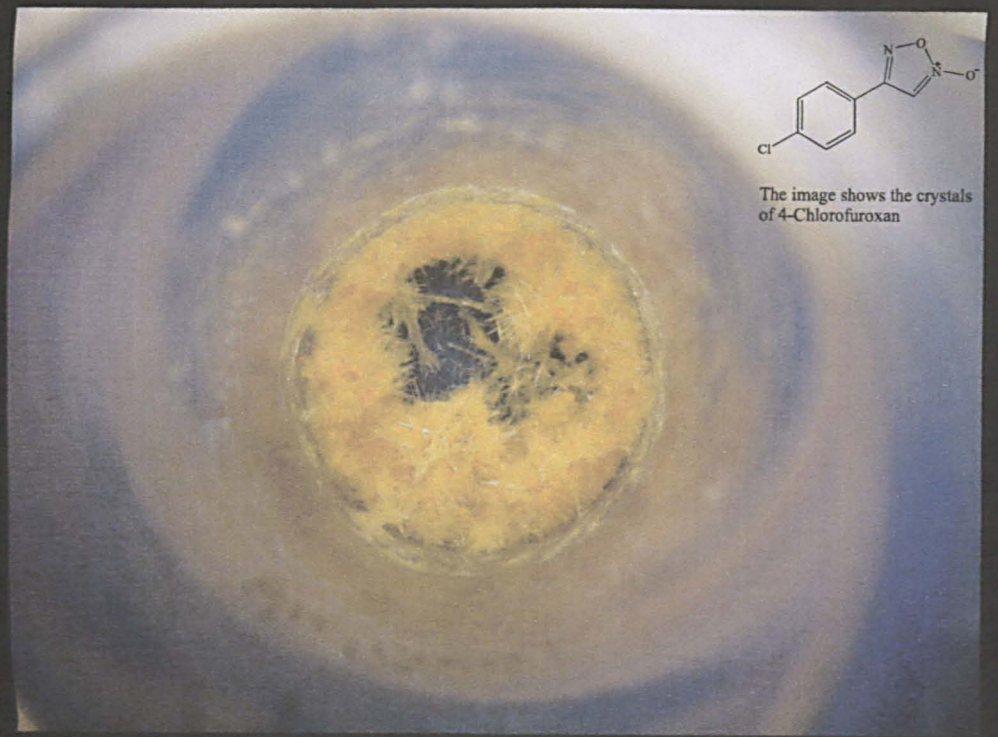
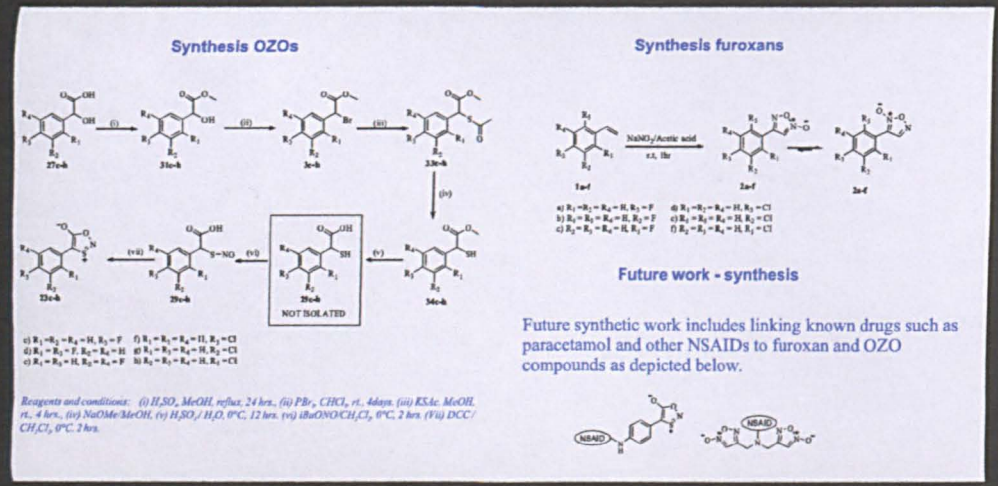


Figure 1: Heterocyclic NO donors: Structure 1 is an 4-Aryl 1,3,2-Oxathiazolylium-5-olate (OZO) and structure 2 is a 3-phenyl-1,2,5-oxadiazole 2-oxide (furoxan)

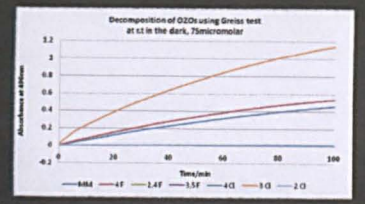


The image shows the crystals of 4-Chlorofuroxan

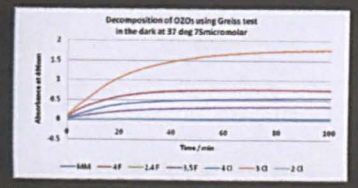


Preliminary Results

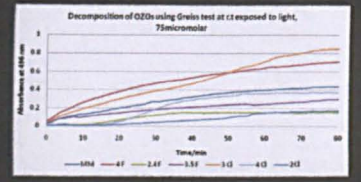
UV-Vis analysis of the OZOs show how the rate of release of NO can be varied by altering the substituents on the six-membered ring. This ability is important as this facilitates the release of NO depending on the demand.



Graph 1: Decomposition of OZOs at room temperature in the dark. 3 Cl OZO, 4 F OZO and mandelic OZO have the fastest rates of release while the other OZOs show little or no decomposition over the selected time frame.



Graph 2: Decomposition of OZOs at 37°C (biological temperature) in the dark. 3 Cl OZO, 4 F OZO, 3,5 F OZO and mandelic OZO have the fastest rates of release respectively, while the other OZOs show little or no release over the selected time frame.



Graph 3: Decomposition of OZOs at room temperature while exposed to light. 3 Cl OZO and 4 F OZO have the fastest rates of release. And unlike previous graphs the other OZOs show increased release of NO when exposed to light over the selected time frame.

Acknowledgments

We would like to thank EPSAM for funding this project. The author would like to thank Dr Russell Pearson and Prof Steve Allin for their support and supervision. We would also like to thank John Clews, Liam Duffy and the other members of the Medicinal and Pharmaceutical Sciences research cluster for practical advice and input.