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Thiol-reactive small molecules to accentuate oxidative stress in mitochondria

Patrick A. Cardwell, MChem

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy in Chemistry (PhD)



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July 2025

Abstract

Mitochondria are the hubs of cellular metabolism and redox signalling. Reactive oxygen species (ROS), produced as by-products of normal metabolism, regulate many signalling pathways essential to cell function. However, when present at supraphysiological levels, referred to as oxidative stress, ROS damage vital biomolecules, which threatens cell survival and contributes to the onset of various diseases. Compounds that accentuate mitochondrial oxidative stress can help to provide an understanding of redox signalling and can serve as leads for drugs that aim to exert a toxic effect towards malfunctioning mitochondria in disease states. One such compound, MitoCDNB, is the mitochondria-targeted analogue of 1-chloro-2,4-dinitrobenzene (CDNB), a common substrate for glutathione *S*-transferase (GST) enzymes. Owing to its triphenylphosphonium (TPP) cation, MitoCDNB accumulates into the mitochondrial matrix, where it selectively depletes mitochondrial glutathione (mGSH) via catalysis by mitochondrial GSTs (mGSTs). This disrupts mitochondrial thiol redox status and increases ROS production. The primary aim of this work was to build upon the MitoCDNB platform by leveraging the GSH/GST mechanism to selectively deliver probes and drugs to mitochondria. The initial focus was to deliver biguanides to induce ROS generation through inhibition of complex I. Chapter 2 explores the expression of three mGST isoforms and the synthesis of chloronitrobenzene derivatives for kinetics studies to investigate the enzymes' substrate specificity. hGSTA1-1 was found to be the most active enzyme for the derivatives, while MitoCDNB was the most reactive mitochondria-targeted substrate. Chapter 3 describes the synthesis and kinetics studies of sulfonamide derivatives, largely based on the structure of MitoCDNB, which aimed to release amine and phenol payloads. Although the release of a biguanide or an arylamine was unsuccessful, alkanesulfonamides enabled GSH/GST-mediated release of alkylamines. Subsequently, sulfonamides, which were attached to a phenolic fluorophore via an ethylenediamine linker, were synthesised to investigate the release of phenols. Kinetic studies showed that these ethylenediamine sulfonamides reacted rapidly with GSH in the absence of GST to release the fluorophore. Following this, ethylenediamine sulfonamide prodrugs of two phenolic mitochondrially bioactive compounds were synthesised. Chapter 4 explores the synthesis of two photoaffinity probes of a biguanide for use in a chemoproteomics study to identify the protein targets of biguanides. These probes are expected to advance the understanding of biguanides' complex mechanisms of action and may uncover new therapeutic targets.

Declaration

I certify that the thesis presented here for examination for the PhD degree at the University of Glasgow is solely my own work, except where explicitly stated otherwise.

I declare that Chapter 2.0 of this thesis is an expanded version of the publication, Cardwell, P. A.; Del Moro, C.; Murphy, M. P.; Lapthorn, A. J.; Hartley R. C. Human mitochondrial glutathione transferases: Kinetic parameters and accommodation of a mitochondria-targeting group in substrates, *Bioorg. Med. Chem.* **2024**, *104*, 117712, <https://doi.org/10.1016/j.bmc.2024.117712>, for which I am the first author. I confirm that Chapter 2.0 adheres to the University's copyright policy and the publication's Creative Commons (CC) BY 4.0 license. I was assigned first author and corresponding author of the publication for conducting all the laboratory research and data processing, writing the original draft of the whole paper and editing subsequent versions. The full list of author contributions can be found in the publication, but in brief: Del Moro trained me in protein isolation, Lapthorn and Hartley are my academic supervisors and Murphy is an academic collaborator, who is an expert in mitochondria.

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Abbreviations

a.u	Arbitrary unit
Ac	Acetyl
Acetyl CoA	Acetyl coenzymeA
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Ar	Aromatic
ATP	Adenosine triphosphate
Bn	Benzyl
Boc	<i>Tert</i> -Butyloxycarbonyl
Br	Broad
Bu	Butyl
CDNB	1-Chloro-2,4-dinitrobenzene
CYP	Cytochrome P ₄₅₀
Cys	Cysteine
Cyt c	Cytochrome c
d	Doublet
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCDMH	1,4-Dichloro-5,5-dimethylhydantoin
DCM	Dichloromethane
DEPT	Distortionless enhancement polarization transfer
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
eq	Equivalents
ER	Endoplasmic reticulum
ESI	Electrospray
ETC	Electron transport chain
F	Faraday constant

FAD(H ₂)	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FMN	Flavin mononucleotide
g	Gram
GPx	Glutathione peroxidase
Grx	Glutaredoxin
GSH	Glutathione
GSR	Glutathione reductase
GSSG	Glutathione disulfide
GST	Glutathione S-Transferase
h	Hour
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
HIF-1	Hypoxia-inducible factor
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
Hz	Herz
IC ₅₀	Half maximal inhibitory concentration
IMM	Inner mitochondrial membrane
IR	Ischemia reperfusion
IMS	Intermembrane space
K	Kelvin
k _{cat}	Catalytic rate constant
K _M	Michaelis Menten constant
LCMS	Liquid chromatography-mass spectrometry
m	Multiplet
M	Molar
min	Minute
mGSH	Mitochondrial glutathione
mGST	Mitochondrial glutathione S-transferase
mL	Milliliters
mmol	Millimoles
mM	Millimolar
mV	Millivolt
MeCN	Acetonitrile

Mp	Melting point
MRC	Medical Research Council
Ms	Mesyl
MS	Mass spectrometry
NAD(H)	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NHS	N-Hydroxysuccinimide
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
p	Pentet
P	Product
PDH	Pyruvate dehydrogenase
PEG	Polyethylene glycol
Ph	Phenyl
Prx	Peroxiredoxin
PUFA	Polyunsaturated fatty acid
q	Quartet
Q	Ubiquinone
QH ₂	Ubiquinol
R	Universal gas constant
R _F	Retention factor
RET	Reverse electron transport
RI	Reperfusion injury
ROS	Reactive oxygen species
RT	Room temperature
s	Singlet
SDH	Succinate dehydrogenase
sept	Septet

SM	Starting material
S _N 1	Nucleophilic unimolecular substitution
S _N 2	Nucleophilic bimolecular substitution
S _N Ar	Nucleophilic aromatic substitution
SOD	Superoxide dismutase
t	Triplet
Temp.	Temperature
TCA	Tricarboxylic acid cycle
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TPP	Triphenylphosphonium
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UV	Ultraviolet
Vis	Visible
z	Charge
Å	Angstrom
$\Delta\psi_m$	Membrane potential
Δp	Proton motive force
ΔpH	pH gradient
μL	Microlitre
μM	Micromolar
°C	Degrees centigrade

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Chapter 1 Introduction

Before beginning a detailed introduction to the work of this thesis, a brief outline of the key aim of the work is provided to offer context for the discussion that follows. Mammalian mitochondria are equipped with multiple antioxidant systems to protect cells from oxidative damage and chemical toxicity. Antioxidants neutralise oxidants, such as reactive oxygen species (ROS), which in excess, cause damage to essential biomolecules. Glutathione (GSH), a key non-enzymatic antioxidant, scavenges various oxidants through several mechanisms. One major mechanism involves Glutathione-S-Transferase (GST) enzymes, which catalyse the conjugation of GSH to toxic electrophilic metabolites and xenobiotics, facilitating their breakdown and excretion. Compared to healthy cells, cancer cells exhibit elevated ROS levels, counterbalanced by upregulated antioxidants, including GSTs and GSH. Overexpression of GSTs contributes to drug detoxification via GSH conjugation, while overproduction of GSH promotes tumour growth. Hence, a promising strategy for cancer therapy involves selectively depleting mitochondrial GSH (mGSH) in cancer cells, which should induce oxidative damage and cell death. This can be achieved by delivering a compound to the mitochondria that reacts with mGSH via mitochondrial GST (mGST) catalysis. Additionally, a mGST-activated prodrug that simultaneously depletes mGSH and releases an anti-cancer drug could circumvent mGST-mediated drug detoxification. There is a particular interest to selectively deliver a prodrug of metformin to the mitochondria. Metformin, an antihyperglycemic biguanide with poor bioavailability, exerts apoptotic effects in cancer cells, primarily by mitochondrial complex I inhibition. Thus, the initial objective of the research described in this thesis was to develop mitochondrial-targeted prodrugs that kill cancer cells by mGST-catalysed mGSH depletion and metformin release.

1.1 Mitochondrial bioenergetics and metabolism

1.1.1 General structure and functions of mitochondria

Mitochondria are major ancient endomembrane systems in eukaryotic cells. According to the widely accepted endosymbiotic theory, mitochondria once existed as aerobic prokaryotes around two billion years ago, until they were engulfed by a predecessor of

the modern eukaryotic cell.¹ The symbiotic relationship, in which the bacteria could respire in host cells that had depended on glycolysis and fermentation for energy production, meant mitochondria evolved considerably. They have retained their double membrane character and key role of adenosine triphosphate (ATP) production, but have otherwise acquired a very different structure and a plethora of new functions.² As a result, the genomic material of the α -proteobacteria was lost or transferred to the nuclear genome.³ Mitochondria have been termed ‘powerhouses of the cell’ due to their importance in cellular bioenergetics, but they also play a crucial role in cell signalling, contributing to apoptotic cell death and many other complex cellular processes.^{4, 5} The number, shape and size of mitochondria can vary widely by cell type, and they are constantly remodelled by processes collectively referred to as mitochondrial membrane dynamics.⁵ However, all mitochondria are composed of the same five parts (Figure 1).

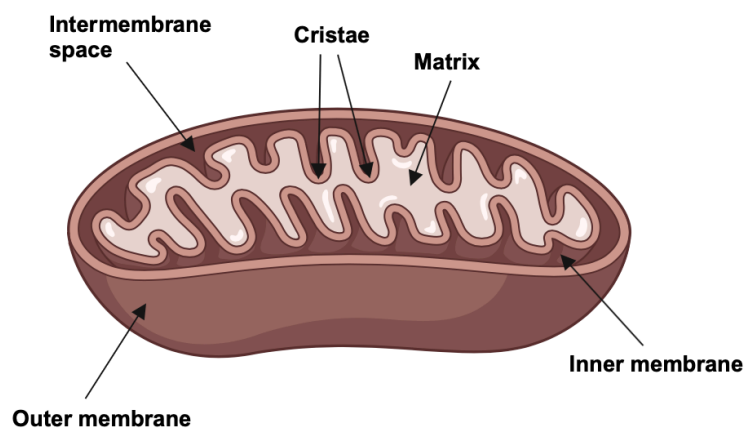


Figure 1. General structure of a mitochondrion.

They consist of an outer mitochondria membrane (OMM) and an inner mitochondrial membrane (IMM) which surround the intermembrane space (IMS). The IMM forms folds, named cristae, which expand the surface area of the IMM for ATP synthesis and contain the electron transport chain (ETC) for oxidative phosphorylation (OXPHOS).⁶ Within the IMM lies the mitochondrial matrix where the tricarboxylic acid (TCA) cycle, mitochondrial DNA replication and protein biosynthesis occur. The OMM is similarly lipophilic to the cell membrane and contains proteins called porins, which allow ions, molecules and small proteins to pass through.^{5, 7} The OMM controls release of signalling molecules from the mitochondria to the rest of the cell, interacting via membrane contact sites with other

subcellular organelles, such as the endoplasmic reticulum (ER) membrane in calcium signalling.⁸ The ratio of ATP to the diphosphate (ADP) or monophosphate (AMP) forms is the energy exchange factor that connects anabolism and catabolism, but also fuels processes such as muscle contraction, phosphorylations and active transport. Cellular metabolism primarily relies on glucose, which is catabolized to ATP in four successive processes: glycolysis, pyruvate oxidation, the TCA cycle and OXPHOS (Figure 2).⁹

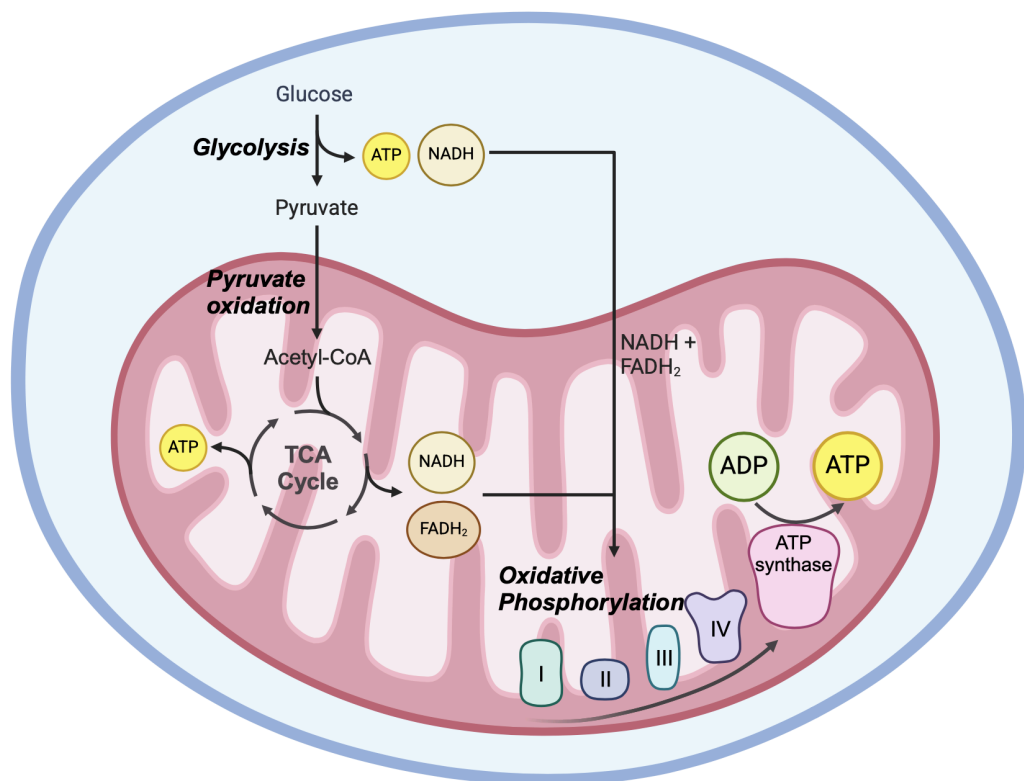


Figure 2. Cellular aerobic respiration consisting of glycolysis, pyruvate oxidation, the TCA cycle and oxidative phosphorylation. In addition to generating a small amount of ATP, glycolysis, pyruvate oxidation and the TCA cycle produce the reducing agent molecules, NADH and FADH₂, which are used in OXPHOS to produce a large amount of ATP.

Glycolysis, which occurs in the cytosol, breaks down one glucose molecule into two pyruvate molecules, while also generating two NADH and two ATP molecules.⁹ Pyruvate is then transported to the matrix where it is coupled to coenzyme A by pyruvate dehydrogenase to afford acetyl coenzyme A (acetyl-CoA), while simultaneously converting NAD⁺ to NADH and releasing CO₂. The TCA cycle completely oxidises acetyl-

CoA via a series of biochemical reactions into two CO_2 molecules, alongside generating three NADH, one FADH_2 and one ATP per turn of the cycle.⁹ The NADH and FADH_2 produced from glycolysis and the TCA cycle serve as electron donors for OXPHOS, the main source of ATP production. In total, approximately 30-32 ATP molecules are produced per cycle of cellular respiration.

1.1.2 Oxidative Phosphorylation (OXPHOS)

OXPHOS is the phosphorylation of ADP to ATP, driven by the membrane potential and proton gradient across the IMM established by the electron transport chain (ETC) (Figure 3).

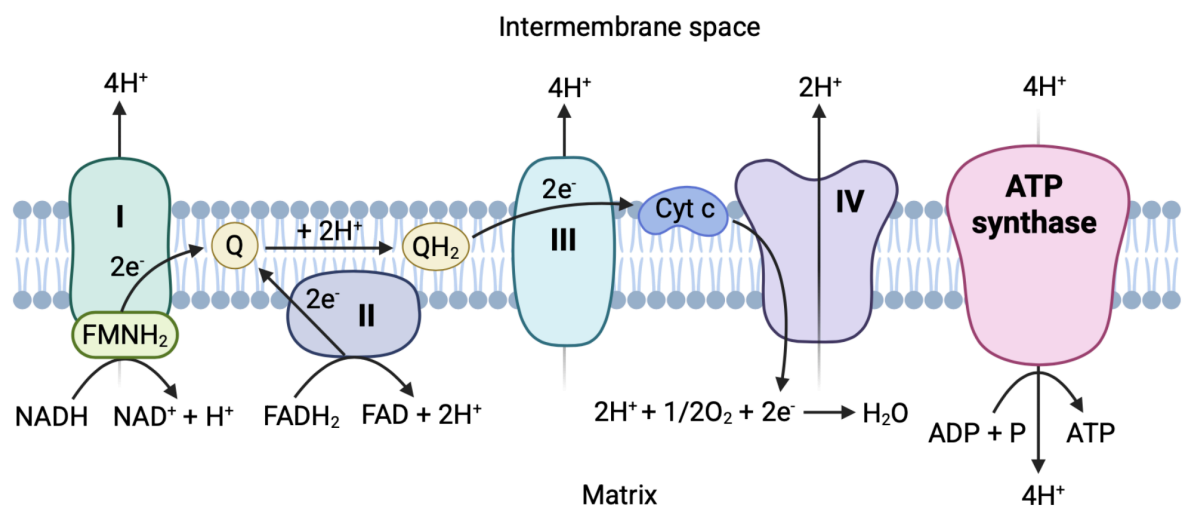
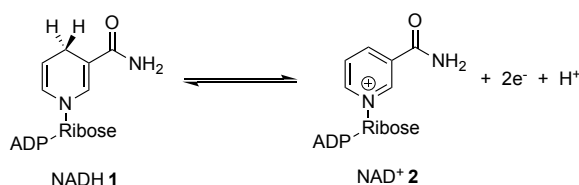


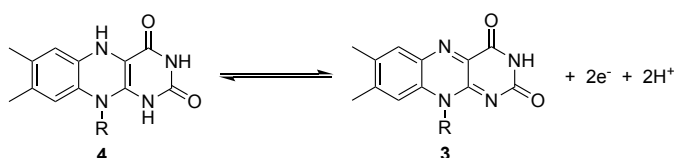
Figure 3. The electron transport chain and ATP synthase in OXPHOS.

As electrons move through the ETC, protons are pumped from the matrix to the IMS, creating an electrochemical gradient that powers ATP synthesis via ATP synthase. The ETC, embedded within the cristae of the IMM, consists of four protein complexes and a range of cofactors that shuttle the electrons through the complexes via a series of redox reactions to complex IV where they reduce O_2 to H_2O . The protein complexes contain non-protein prosthetic groups essential for electron transfer. The process begins at Complex I where NADH 1 is oxidised to NAD^+ 2 (Scheme 1), coupled with the reduction of the flavin moiety 3 of the flavin mononucleotide (FMN) prosthetic group to the dihydroflavin 4 form

(FMNH₂) (Scheme 2). The two electrons are then passed down a chain of iron-sulfur (Fe-S) clusters, regenerating FMN.

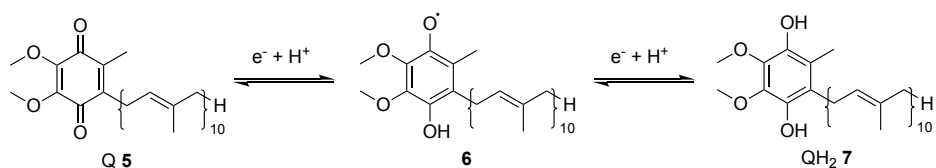


Scheme 1. Reversible oxidation of NADH 2 to NAD⁺ 1.



Scheme 2. Reversible oxidation of the dihydroflavin redox centre 4 of FMNH₂ and FADH₂ to the flavin form 3 of FMN and FAD, respectively.

Complex II is a parallel electron transport pathway to complex I. It initially plays a part in the TCA cycle, catalytically oxidising succinate to fumarate, generating FADH₂ as a side product.^{10, 11} FADH₂, which has the same dihydroflavin redox centre 4 as FMNH₂ (Scheme 2), is then oxidised to the flavin form 3, FAD, and the two electrons are transferred to a Fe-S cluster. The electrons from the Fe-S clusters of complexes I and II are passed onto a mobile electron carrier called coenzyme Q (or ubiquinone) 5, which is reduced first to semiubiquinone 6, and then to ubiquinol (QH₂) 7, as part of the Q-cycle (Scheme 3).¹² Overall, the transfer of electrons from complex I to Q 5 results in the net active transport of four protons from the matrix to the IMS, but complex II does not directly contribute to proton pumping.^{9, 13-15} Once in the Q-cycle, the electrons arrive at complex III, where QH₂ 7 is oxidised back to Q 5.¹⁵ The process transfers two electrons, via a Fe-S cluster, to two molecules of the electron carrier, cytochrome c. As a result, four more protons are pumped into the IMS.^{9, 16} The reduced cytochrome c ferries electrons to complex IV, where O₂ is reduced via a redox-active heme to two molecules of water.¹⁶ The reduction of O₂ to H₂O results in the pumping of four protons to the IMS for every H₂O molecule generated, but two are consumed in the process.^{9, 16}



Scheme 3. The Q-cycle. Redox reactions between Q 5 and QH₂ 7 occur via ubisemiquinone 6.

Overall, the transfer of electrons via redox reactions across the four complexes is coupled with the pumping of ten protons from the matrix to the IMS. The accumulation of protons in the IMS generates a proton motive force (Δp) comprised of the mitochondrial membrane potential ($\Delta\psi_m$), which equals -120 to -180 mV, and a small pH gradient (ΔpH).¹⁷ Ultimately, Δp drives ATP synthesis as protons that were originally pumped into the IMS by the ETC, re-enter the matrix through ATP synthase. This proton movement facilitates a rotary motion of the ATP synthase that results in the addition of a phosphate molecule to ADP to generate ATP.¹⁵

1.1.3 The production of reactive oxygen species (ROS)

Mitochondria are a major source of reactive oxygen species (ROS) generated by the incomplete reduction of O₂. ROS can exist as radicals, such as superoxide (O₂^{•-}), or non-radicals, including hydrogen peroxide (H₂O₂) and hydroperoxide (HOO[•]) (Figure 4). The different types of ROS vary greatly in their reactivity, lifespan, membrane permeability and ability to generate further downstream reactive species.¹⁸ At physiological levels, ROS play a role in redox signalling, but at pathological levels they can cause oxidative damage to important biomolecules, such as DNA, proteins and lipids.¹⁹ Oxidative damage occurs when the levels of ROS exceed a cell's antioxidant capacity.

Radicals	Non-radicals
$O_2^{\bullet-}$ superoxide	$^1O_2$ singlet oxygen
$HO^{\bullet}$ hydroxyl	HO^- hydroxide
$HOO^{\bullet}$ hydroxyperoxyl	HOO^- hydroperoxide
$RO^{\bullet}$ alkoxyl	H_2O_2 hydrogen peroxide

Figure 4. Common reactive oxygen species.

Superoxide ($O_2^{\bullet-}$) is the primary ROS formed by the monoelectronic reduction of O_2 . It is largely produced in the mitochondria as a by-product of cellular respiration due to electron leaking from the ETC (Figure 5).^{15, 20} Under physiological conditions, approximately 0.2 - 2% of the electrons in the ETC leak to produce $O_2^{\bullet-}$.¹⁶ The rate of electron leaking and $O_2^{\bullet-}$ production is influenced by factors, such as local O_2 tension, Δp , electron flux and the activity of ATPases, which hydrolyse ATP to ADP.¹⁶ Experiments by Treberg *et al.*²¹ on the mitochondria of wild-type rat skeletal muscle demonstrated that roughly 63% of ROS are produced in the mitochondrial matrix. In complex I, $O_2^{\bullet-}$ can be generated in the matrix at both the FMN site (I_F) and Q 5 binding site (I_Q) during forward electron transport (FET) from NADH 1 to Q 5. Rotenone and piericidin are I_Q inhibitors that interrupt electron transfer to Q 5 and increase ROS production at I_F . Complex I can also generate $O_2^{\bullet-}$ at the I_F and I_Q sites by reverse electron transport (RET), which occurs when the Q pool is highly reduced and Δp is high.^{16, 20} Under these conditions, electron transport is driven against the redox potential gradient of the ETC and electrons are forced back from QH_2 7 into complex I. Compared to complex I, the amount of $O_2^{\bullet-}$ produced directly by complex II at the succinate dehydrogenase site (II_F) is negligible under normal conditions.¹⁵ However, under pathological conditions, such as a period of ischemia or hypoxia, complex II triggers RET responsible for $O_2^{\bullet-}$ production at complex I (Figure 5).²²⁻²⁴ Ischemia causes a dramatic increase in succinate and stalls ATP synthesis.

Upon reperfusion, oxygen is reintroduced and rapidly oxidises the accumulated succinate to fumarate at II_F , generating a highly reduced Q pool and a high Δp , both of which drive electrons back to the I_F site in complex I.

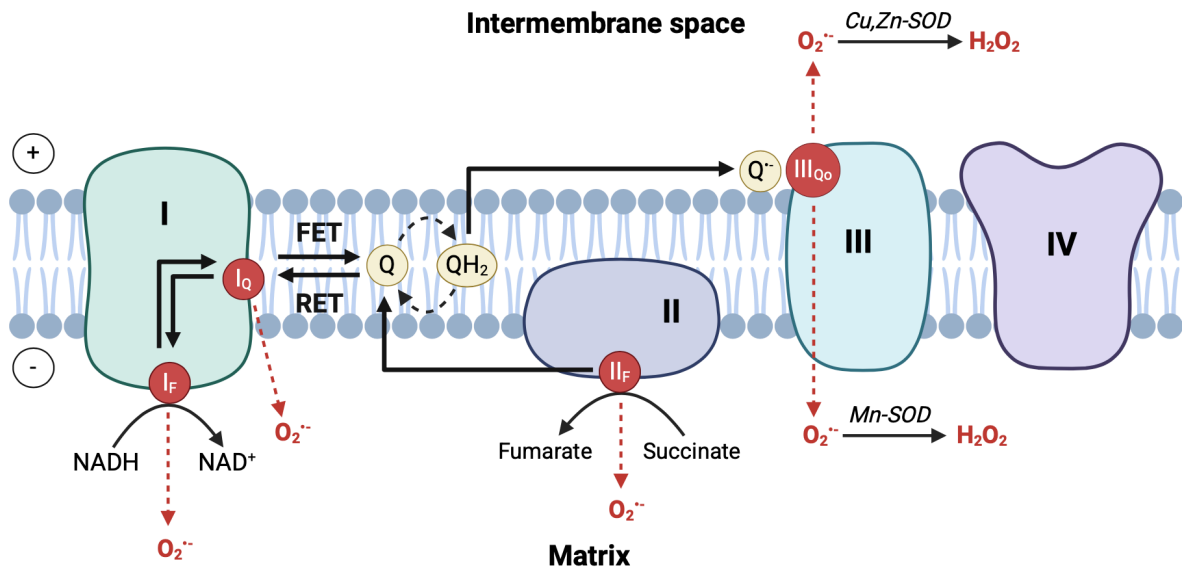
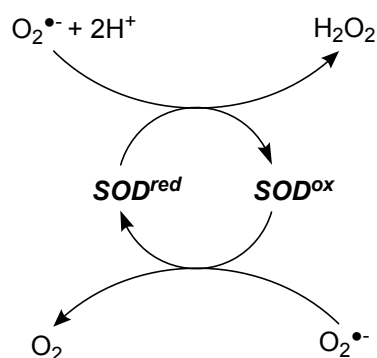


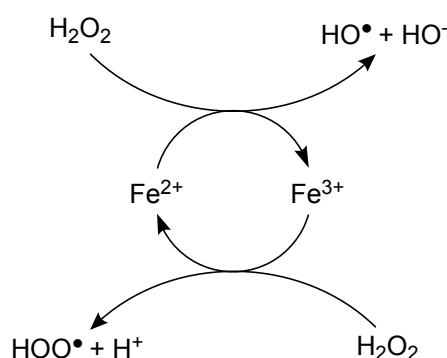
Figure 5. $\text{O}_2^{\bullet-}$ production in the ETC by forward electron transport (FET) and reverse electron transport (RET).

Complex III also generates ROS via the Q-cycle (Figure 5). QH_2 7 can lose an electron at the III_{Q_0} site to form semiquinone, $\text{Q}^\bullet$ 6, which reacts with O_2 to produce $\text{O}_2^{\bullet-}$ in both the matrix and IMS.²⁵⁻²⁷ Complex IV efficiently reduces oxygen to water and is not considered prone to ROS production.¹⁵ Most of the $\text{O}_2^{\bullet-}$ generated in the ETC is dismutated to the more stable and membrane-permeable hydrogen peroxide (H_2O_2) and O_2 by the superoxide dismutase (SOD) enzymes: Cu,Zn-SOD in the IMS and MnSOD in the matrix (Scheme 4).^{28, 29} Alternatively, mitochondria-generated $\text{O}_2^{\bullet-}$ can be released into the cytosol through the voltage-dependent anion channel that spans the OMM, where it can participate in cytoplasmic signalling processes.³⁰



Scheme 4. Superoxide dismutase catalyses the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2 .

H_2O_2 generated in the mitochondria has many possible fates. H_2O_2 in the IMS can diffuse across the OMM into the cytosol where it acts as a signalling molecule. H_2O_2 in the matrix is largely metabolised by antioxidant systems (see Section 1.1.5), but it can also undergo Fenton chemistry in the presence of ferrous or other transition metal ions to form the highly reactive hydroxyl radical, $HO^{\bullet}$, which reacts with nearby biomolecules indiscriminately.¹⁸ Fenton chemistry describes the disproportionation of H_2O_2 by Fe^{2+}/Fe^{3+} to produce $HO^{\bullet}$, $HOO^{\bullet}$ and water (Scheme 5).³¹



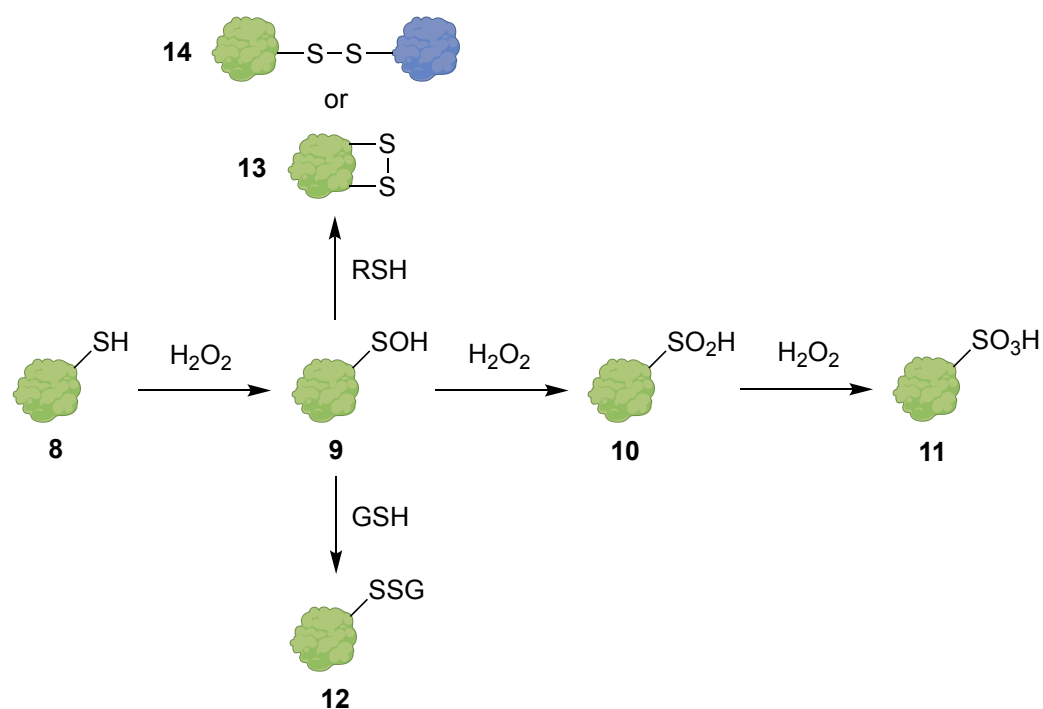
Scheme 5. The cycle of the Fenton reaction. Fe^{2+} is oxidised to Fe^{3+} by reaction with H_2O_2 , which is reduced to $HO^{\bullet}$ and HO^- . Fe^{3+} is reduced back to Fe^{2+} when another molecule of H_2O_2 is oxidised to $HOO^{\bullet}$ and a proton.

Overall, complex I is largely responsible for the production of mitochondrial ROS. $O_2^{\bullet-}$ production is significantly increased under conditions of high NADH/NAD⁺, which induces

FMN reduction, or a high QH₂ 7/Q 5 ratio and high Δp , which trigger RET.^{32, 33} A small decrease in Δp is ideal to reduce ROS production while sustaining optimum ATP production. Miwa *et al.*³⁴ reported that a 10 mV decrease in $\Delta\psi_m$, during RET, could abolish roughly 70% of ROS produced by complex I. However, any decrease in Δp , which arises from the leaking of protons to the matrix without passing through ATP synthase, makes ATP production less efficient.^{35, 36}

1.1.4 ROS in redox-signalling

ROS is often referred to as a ‘double-edged sword’. At physiological levels, mitochondria-generated ROS function as crucial redox signalling molecules, regulating intracellular pathways in a range of important biological areas, including cellular differentiation,³⁷⁻³⁹ oxygen sensing,⁴⁰⁻⁴² the immune system,^{43, 44} hormone signalling,^{45, 46} mitochondrial homeostasis,⁴⁷ and microorganism lifespan.⁴⁸⁻⁵¹ However, excessive production of ROS contributes to several degenerative processes. Whether ROS have protective or harmful effects depends on a delicate balance between their production and disposal, maintained by antioxidant defences (see Section 1.1.5). ROS mediate cellular function by the oxidative modification of receptors, phosphatases, ion channels and transcription factors.^{15, 52} The most important ROS in redox signalling is H₂O₂, which influences networks that control the cell cycle, stress responses, energy metabolism, and redox homeostasis.^{53, 54} While other ROS, such as O₂[•], can participate in redox signalling independently of H₂O₂, their role is limited due to a shorter lifetime and poorer diffusion across membranes compared to H₂O₂.⁵⁵ ROS signalling primarily occurs through the oxidation of metal cofactors or thiol groups in cytosolic proteins, reversibly or irreversibly altering their function.³⁰ In response to H₂O₂, protein thiols **8** initially form the sulfenic acid **9** (Scheme 6), which can undergo further post-translational modifications. The sulfenic acid **9** can undergo subsequent reactions with H₂O₂ to produce sulfinic acid **10** and sulfonic acid **11**, which are generally formed irreversibly and are considered oxidative damage. Alternatively, the sulfenic acid **9** can react with other endogenous thiols, an adjacent thiol residue or a different protein thiol to form disulfides **12**, **13** and **14**, respectively.⁵⁵ These post-translational modifications serve as ‘redox switches’.⁵⁵



Scheme 6. ROS-mediated redox signalling through protein thiol modifications.

Among the many biological mechanisms regulated by mitochondrial ROS, their role in O₂ sensing is particularly intriguing. During hypoxia, mitochondria release H₂O₂ into the cytosol, stabilising hypoxia-inducible factor 1 α (HIF-1 α),⁴⁰ which transcribes genes encoding proteins that enable cell survival. At the organism level, hypoxia also triggers the contraction of pulmonary artery smooth muscle cells through a ROS-mediated increase in cytosolic Ca²⁺.^{41, 42} In the nervous system, ROS act as secondary messengers in synaptic plasticity processes, particularly long term-potential, a form of synaptic enhancement involved in mammalian learning and memory.^{56, 57} In the hippocampus, H₂O₂ enhances hippocampal plasticity by releasing Ca²⁺ from intracellular neuronal stores.⁵⁸⁻⁶⁰ Additionally, low levels of ROS regulate transcriptional factors and several protein kinase cascades involved in the cross-talk of autophagy and apoptosis in cells.⁶¹

1.1.5 ROS in pathology

At supraphysiological levels, ROS induce oxidative damage to important biomolecules, threatening cell survival. There is good evidence that they contribute to physiological and

premature aging, and age-related diseases.⁵³ Normal levels of ROS may become disrupted under conditions of hypoxia, ischemia-reperfusion (IR), inflammation, or impaired antioxidant defences.^{15, 19, 53, 62} Ischemia-reperfusion (IR) injury refers to tissue damage caused by ROS, which is overproduced when blood flow is restored to a tissue (reperfusion) after a period of ischemia (described in Section 1.1.3). The oxidative burst, combined with Ca^{2+} accumulation during ischaemia, triggers the induction of the mitochondrial permeability transition pore (MPTP), leading to cell death (Figure 6).⁶³⁻⁶⁶ The release of mitochondrial contents through the MPTP activates an inflammatory response, which can cause tissue scarring and remodelling.^{63, 67} Unfortunately, IR injury is an inevitable consequence of organ transplantation, necessitating strategies to minimise its impact and prevent organ failure.⁶⁷⁻⁶⁹ IR injury is responsible for heart failure and brain damage following myocardial infarction,⁷⁰ and stroke,⁷¹ respectively.

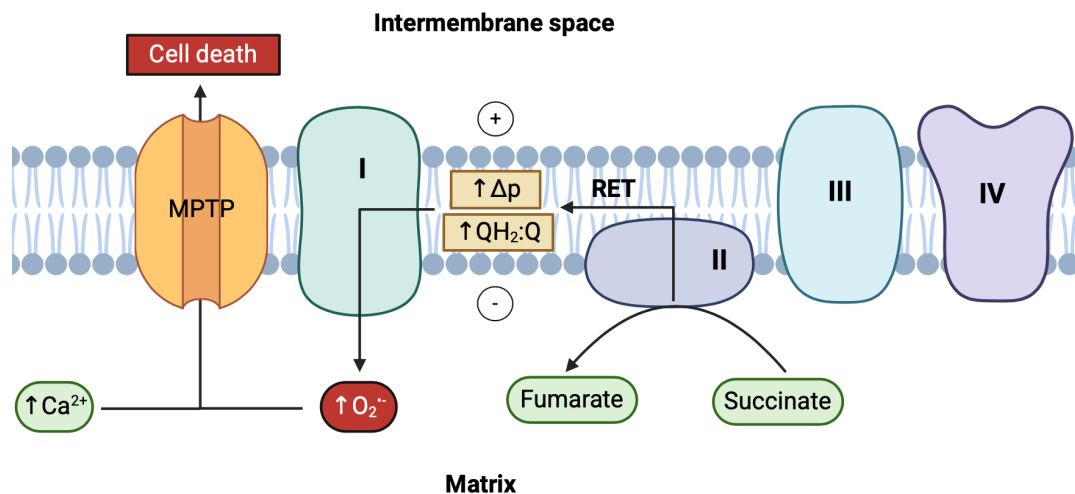
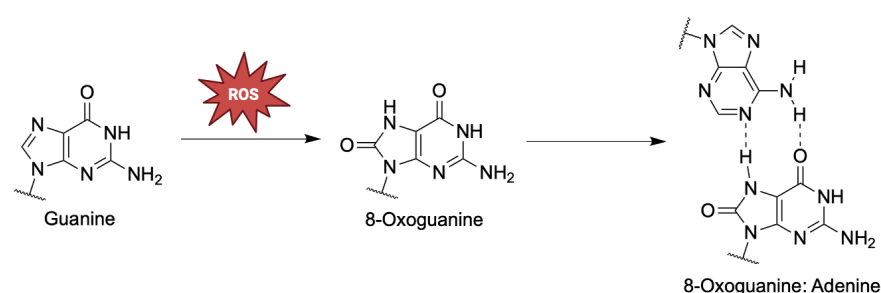


Figure 6. The process of IR injury. Ischaemia results in an accumulation of succinate and Ca^{2+} . Upon reperfusion, succinate is rapidly oxidised to fumarate. This leads to a highly reduced Q pool and a high Δp , driving RET to complex I, which increases $\text{O}_2^{\bullet-}$ production. In conjunction with Ca^{2+} accumulation, the oxidative damage caused by $\text{O}_2^{\bullet-}$ leads to the induction of the MPTP, resulting in cell death.

ROS-induced oxidative damage affects nucleic acids, lipids and proteins. Highly reactive species, such as $\text{HO}^{\bullet}$, react with nuclear and mitochondrial DNA bases, generating over 20 different base lesions, which cause genome instability and mutation, and consequently, cell apoptosis.^{72, 73} Due to pathological conditions and ageing, the balance between

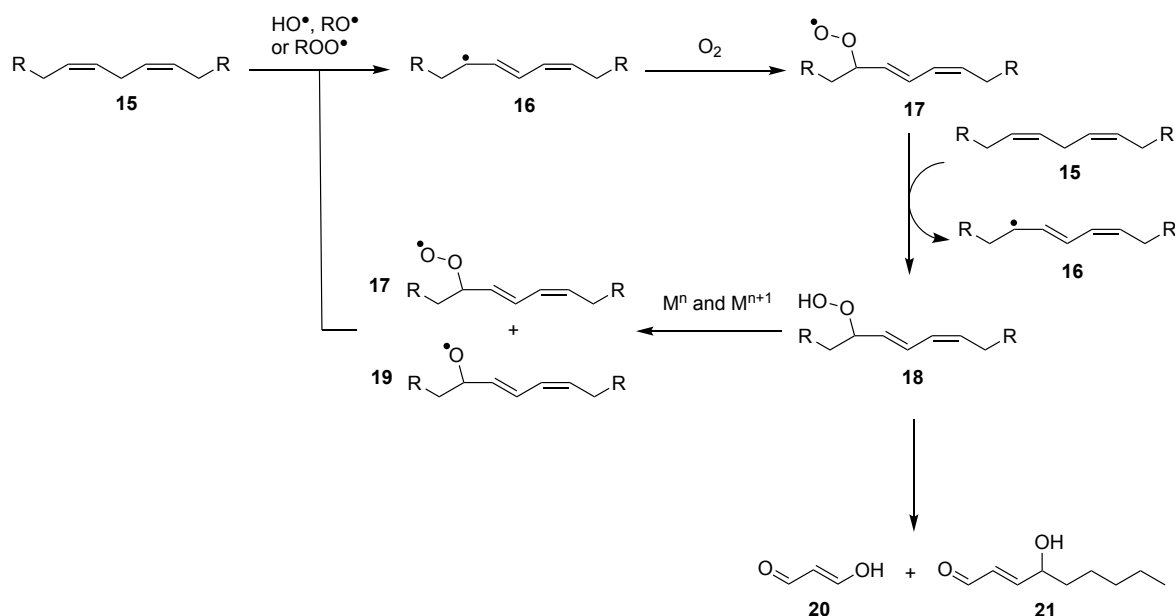
oxidative DNA damage and DNA repair processes is disrupted, promoting the development of age-related diseases.⁷² The most widely studied base susceptible to oxidative damage is guanine, which forms 8-oxoguanine (8-oxoG) (Scheme 7).^{72, 73} 8-oxoG is highly mutagenic, pairing to adenine and causing a guanine-to-thymine mutation during DNA replication,⁷⁴ which can disrupt genome function by several mechanisms.⁷³ Elevated levels of DNA lesions, including 8-oxoG, are particularly linked to cancers, such as lung,⁷⁵⁻⁷⁷ breast,^{78, 79} stomach,^{80, 81} and leukaemia,⁸² as well as noncancerous disorders, including Parkinson's,⁸³ diabetes,⁸⁴ and cardiovascular diseases.^{85, 86}



Scheme 7. Oxidation of guanine to 8-oxoguanine, which pairs to adenine rather than cytosine.

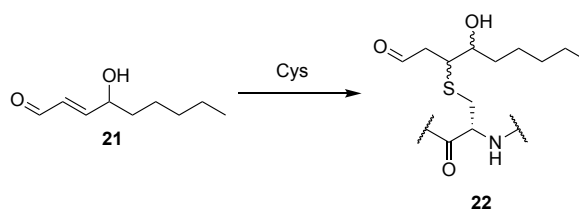
Lipid peroxidation, a hallmark of oxidative stress, also contributes to cell dysfunction and cell death by apoptosis.⁸⁷ Phospholipids form the lipid bilayer of cell membranes and are comprised of esters of polyunsaturated fatty acids (PUFAs), which are prone to oxidation by $\text{HO}^\bullet$ and other free radicals. The overall process of lipid peroxidation consists of three steps (Scheme 8): initiation, propagation and termination. In the initiation step, free radicals, such as $\text{HO}^\bullet$, abstract an allylic hydrogen from a PUFA **15** forming a lipid radical **16**. During propagation, the lipid radical **16** reacts with O_2 to generate a peroxy radical **17**, which can abstract another allylic hydrogen from a different PUFA **15** to form a lipid hydroperoxide **18**.⁸⁸ Lipid hydroperoxides **18** can also be re-converted into lipid peroxy radical **17** and lipid alkoxyl **19** radicals by redox cycling of transition metals.⁸⁸ These radicals propagate chain reactions that attack other lipids.⁸⁸ Termination occurs when antioxidants, such as vitamin E, donate a hydrogen atom to the lipid peroxy radical **17**, generating a vitamin E radical that reacts with a water-soluble vitamin C molecule, thereby forming non-radical products (see Scheme 10, Section 1.1.6). Further oxidative decomposition of lipid hydroperoxides **18** produces a variety of toxic carbonyl species,

such as malondialdehyde (MDA) **20** and 4-hydroxynonenal (4-HNE) **21**, which can easily diffuse across membranes, inactivating membrane-bound proteins and threatening membrane integrity.⁸⁸



Scheme 8. Lipid peroxidation forms lipid hydroperoxides and toxic secondary metabolites.

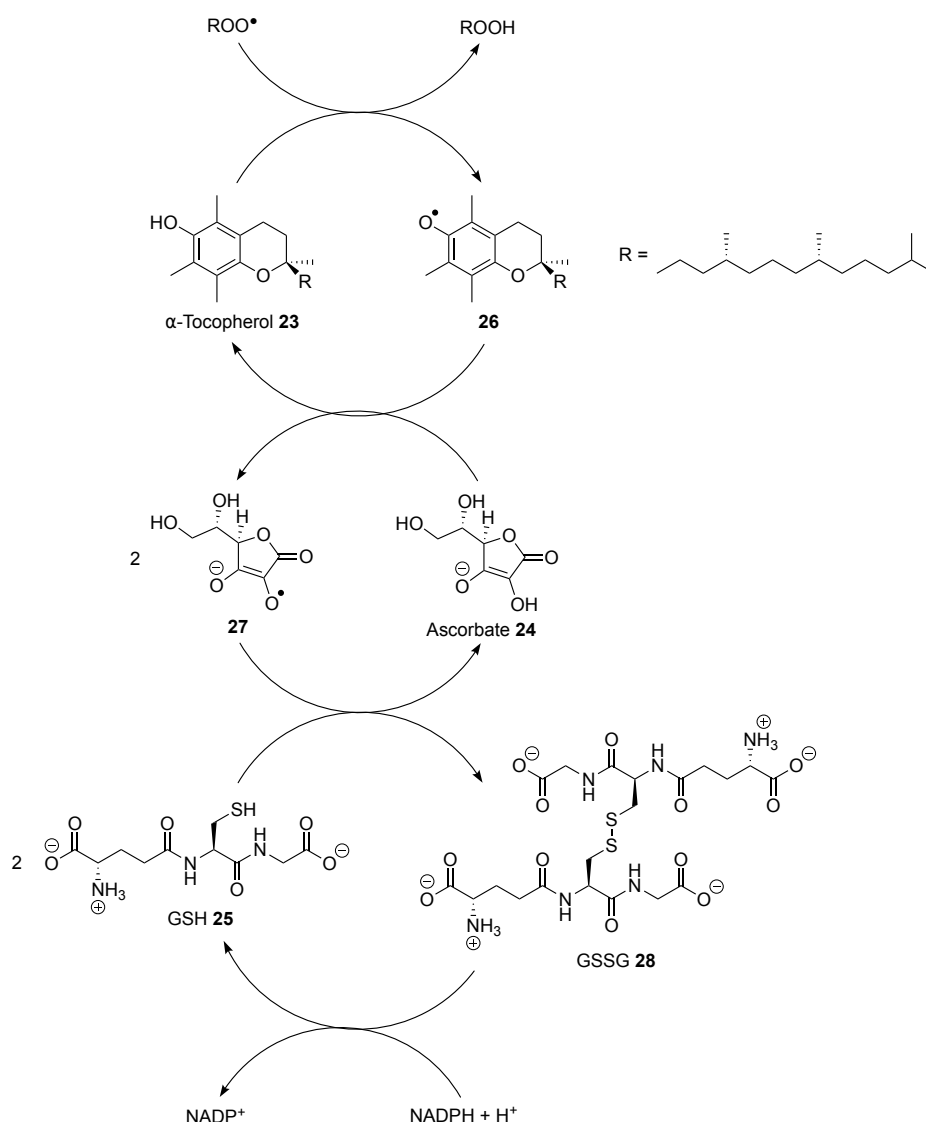
While protein oxidation, typically of cysteine residues, is a natural consequence of H_2O_2 -mediated redox-signalling, $\text{HO}^\bullet$ and $\text{RO}^\bullet$ radicals indiscriminately attack amino acids, impairing a wide variety of enzymatic processes and growth factors critical to cell function.⁸⁹ Additionally, secondary reactions between protein residues, such as cysteine, and lipid peroxidation products, such as 4-HNE **21**, generate protein carbonyl adducts **22** (Scheme 9), which are biomarkers of protein oxidative damage.⁸⁸⁻⁹⁰ Increased levels of lipid peroxidation products and protein carbonyl adducts are strongly associated with cancer,⁹¹⁻⁹⁴ diabetes,⁹⁵⁻⁹⁷ cardiovascular diseases,⁹⁸ and neurodegenerative diseases.⁹⁹⁻¹⁰¹



Scheme 9. Reaction between HNE **21** and a cysteine residue to generate a carbonyl adduct **22**.

1.1.6 Antioxidant defence systems

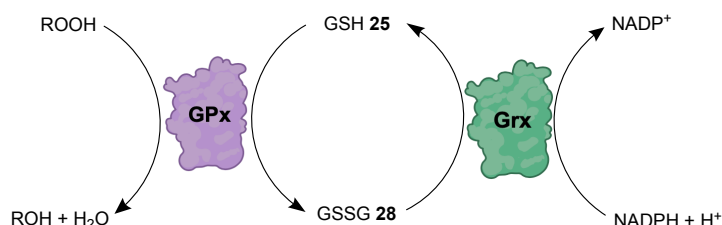
ROS levels in the cell can be seen as a balance between ROS generation and ROS detoxification by antioxidants. Biological systems contain enzymatic and non-enzymatic antioxidants in various subcellular compartments that can prevent oxidative damage by reducing ROS to more stable forms.¹⁰² Nonenzymatic antioxidants can be endogenous or supplied by dietary nutrients. They include α -tocopherol **23** (the main component of Vitamin E), ascorbate (Vitamin C) **24** and glutathione (GSH) **25**. These three antioxidants regulate one another to scavenge peroxy radicals ($\text{ROO}^\bullet$) (Scheme 10), especially those generated by lipid peroxidation.^{19, 103} α -Tocopherol **23**, which resides in the lipid phase of the cell membrane, can donate a hydrogen atom to $\text{ROO}^\bullet$, generating the tocopheryl radical **26**. α -Tocopherol **23** can be regenerated from reaction of the tocopheryl radical **26** with ascorbate **24** or GSH **25**. Ascorbate **24** scavenges ROS in intracellular and extracellular environments and can reduce tocopheryl radical **26** to α -tocopherol **23**, simultaneously generating the ascorbate radical **27**. Ascorbate **24** can be regenerated by a termination reaction, such as a reaction between one ascorbate radical **27** and another or a ROS radical.¹⁰⁴ Alternatively, GSH **25** can act as a reducing agent to convert tocopheryl radical **26** or ascorbic radical **27** back to α -tocopherol **23** and ascorbate **24**, respectively, forming the disulfide GSSG **28** in the process.¹⁰⁵ Additionally, GSH **25** is a cofactor used in many enzymatic detoxifications, explored in Section 1.1.7.



Scheme 10. Peroxyl radicals scavenged by α -tocopherol 23. The resulting α -tocopherol radical 26 is reduced back to α -tocopherol 23 by either ascorbate 24 or GSH 25. If ascorbate radical 27 is generated, GSH 25 will reduce it to ascorbate 24, forming disulfide GSSG 28 in the process. Electrons donated from NADPH regenerate GSH 25 from GSSG 28.

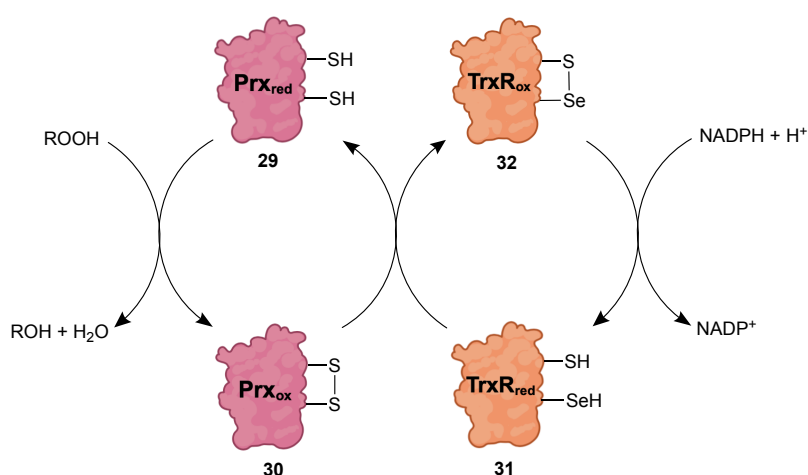
There are also a variety of antioxidant enzymes that regulate ROS levels. Introduced in Section 1.1.3, SOD enzymes dismutate mitochondria-generated $\text{O}_2^\bullet$ to the less reactive H_2O_2 and O_2 . Levels of intracellular H_2O_2 and organic peroxides are regulated by two major enzymatic antioxidant systems that exist in the mitochondria and cytoplasm: the glutathione peroxidase (GPx)/glutathione reductase (Grx) system (Scheme 11) and the peroxiredoxin (Prx)/thioredoxin reductase (TrxR) system (Scheme 12).^{16, 102} GPx enzymes

catalyse the oxidation of GSH **25** to GSSG **28** which reduces H_2O_2 to H_2O or a lipid hydroperoxide to a lipid hydroxyl and H_2O_2 .^{106, 107} Grx enzymes catalyse the reduction of GSSG **28** back to GSH **25**, using electrons donated by NADPH.¹⁰⁸



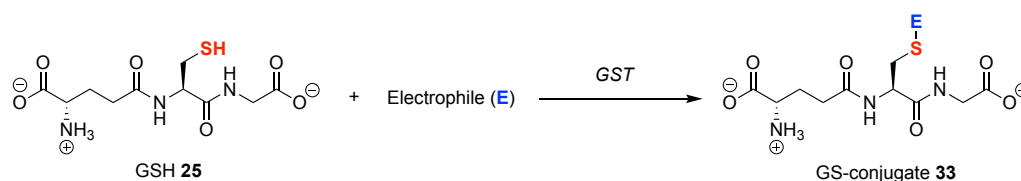
Scheme 11. The reduction of hydroperoxides (ROOH) by glutathione peroxidase (GPx), using glutathione (GSH) **25** as a reductant.

Prx enzymes **29** contain a cysteine residue in their catalytic site, which is oxidised by hydroperoxides, forming a disulfide bridge **30** with a neighbouring cysteine.¹⁰⁹ The Prx disulfide **30** is reduced back to the thiol **29** through a disulfide exchange reaction with a TrxR enzyme **31**, which is oxidised to the selenosulfide **32** in the process.¹⁰⁹⁻¹¹¹ Like Grxs, TrxRs harness electrons from NADPH, reducing the selenosulfide **32** back to the thiol-selenol **31**.^{110, 111}



Scheme 12. The reduction of hydroperoxides (ROOH) by peroxiredoxin (Prx) and thioredoxin reductase (TrxR).

Another major family of antioxidant enzymes are glutathione *S*-transferases (GSTs), which scavenge a range of products of oxidative damage. Their main function is the detoxification of electrophilic xenobiotics and secondary products of lipid peroxidation. GSTs catalyse the conjugation of GSH **25** to toxic electrophiles (Scheme 13), generating a less-reactive GS-conjugate for excretion from the cell. The functions, structure and cellular distribution of GSTs will be explored in greater detail in Section 1.2.



Scheme 13. GST-catalysed conjugation of GSH **25** to a toxic electrophile, generating a glutathione-conjugate **33** which is excreted from the cell.

1.1.7 Targeting mitochondria

As discussed, mitochondrial dysfunction and its irregular production of ROS contribute to many common pathologies, including cancer, cardiovascular diseases and neurological disorders. Therefore, mitochondria are an important target for probes to understand the biological mechanisms responsible for dysfunction and for drugs to manipulate these mechanisms and prevent disease. Since the mitochondrial matrix is the primary home of oxygen metabolism and ROS production, it is the desired target for small molecule therapeutics. Delivering a small molecule to the matrix requires crossing of the cell plasma membrane and both mitochondrial membranes. In contrast to the OMM, the IMM is highly impermeable, which presents a challenge of developing bioactive compounds with the necessary properties to penetrate the IMM. During the last two decades, the covalent modification of compounds into mitochondrial-targeted conjugates has gained great traction due to straightforward chemical synthesis and high targeting efficiency.¹¹² Currently, the most effective way to deliver a cargo to the matrix is by covalent linking to a lipophilic cation (Figure 7), which exploits the large negative membrane potential ($\Delta\psi_m$) across the IMM.¹¹³ Such cations include membrane penetrating peptides **34**, rhodamine esters **35** and triphenylphosphonium (TPP) **36** (Figure 8).¹¹⁴

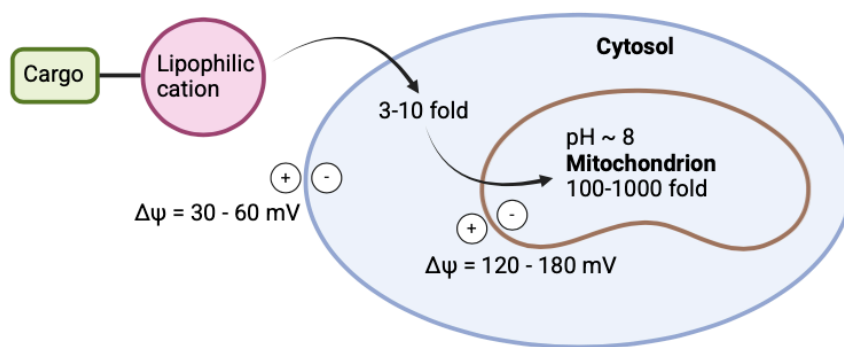


Figure 7. Accumulation of lipophilic cationic conjugates into the mitochondrial matrix in response to the membrane potentials.

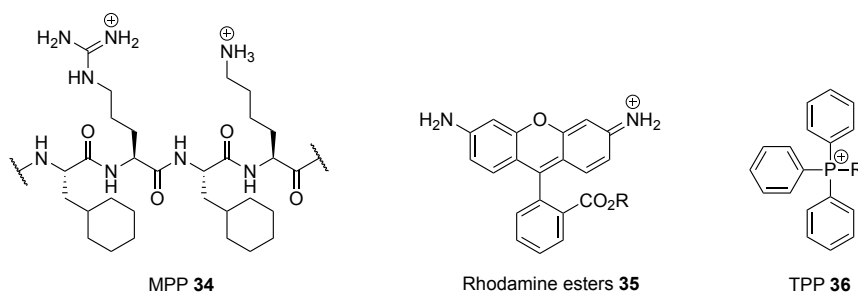


Figure 8. Lipophilic cations to deliver cargos to the mitochondrial matrix.

The accumulation of cations into the mitochondrial matrix can be estimated using the Nernst equation (Equation 1), where $\Delta\psi_m$ is the membrane potential, R is the gas constant, T is the temperature, z is the charge of the cation, and F is Faraday's constant. For a monocation and assuming a temperature of 300 K, Equation 1 can be rearranged for the ratio of cation inside the mitochondrial matrix relative to outside and simplified to Equation 2. According to Equation 2, a 60 mV increase in membrane potential affords a 10-fold increase in matrix accumulation. Therefore, given the $\Delta\psi_m$ is typically around 120-180 mV, Equation 2 predicts a 100-1000-fold accumulation of a monocation in the matrix.

$$(1) \quad \Delta\psi \text{ (in mV)} = \frac{2.303RT}{zF} \log_{10} \left(\frac{[\text{cation}_{\text{in}}]}{[\text{cation}_{\text{out}}]} \right)$$

$$(2) \quad \frac{[\text{cation}_{\text{in}}]}{[\text{cation}_{\text{out}}]} = 10^{\left(\frac{zF\Delta\psi}{2.303RT}\right)} = 10^{\left(\frac{\Delta\psi}{60}\right)}$$

TPP cations **36** are typically used because their large hydrophobic surface area enables rapid crossing of biological membranes by lowering the activation energy for movement through the membrane.^{112, 115} Increasing the lipophilicity of the cations helps with passing through the hydrophobic core of the IMM through the hydrophobic effect. Therefore, adding hydrocarbon chains to TPP cations **36** or decorating the phenyl groups with methyl substituents further enhances their delivery. Alkylated TPP cations **36** were first used as probes to study the mechanism of mitochondrial coupling, and were subsequently used to determine the $\Delta\psi_m$.¹¹² The use of TPP-conjugates of biomolecules in mitochondrial biology was reinvented and refined by Murphy and co-workers to target probes, antioxidants, and pharmacophores to mitochondria.¹¹⁶

1.2 Glutathione-S-Transferases (GSTs)

1.2.1 Function, classification and structure

The Glutathione-S-Transferase (GST) enzyme superfamily, which are expressed and distributed across several cellular compartments, protects cells against oxidative stress and chemical toxicity.¹¹⁷ As described in Section 1.1.6, GSTs play a major role in the phase II detoxification of electrophilic endogenous toxins and xenobiotics (Figure 9).^{118, 119} Lipophilic toxic molecules can freely diffuse across the plasma membrane of cells where they are processed by enzymes, such as cytochrome P450s, as part of Phase I metabolism. Alternatively, they can proceed directly to Phase II metabolism. Often the metabolites produced in Phase I are more harmful than the parent molecules. In Phase II, GST enzymes catalyse the conjugation of GSH **25** to the metabolite to form a less harmful hydrophilic glutathione (GS)-conjugate. The GS-conjugate is then actively transported out of the cell by transmembrane efflux pumps in Phase III metabolism. GS-conjugates are typically eliminated by the mercapturic pathway, being broken down by a γ -glutamyltransferase and a dipeptidase, followed by *N*-acetylation via a *N*-acetyltransferase, and finally, excreted in the urine.^{118, 119}

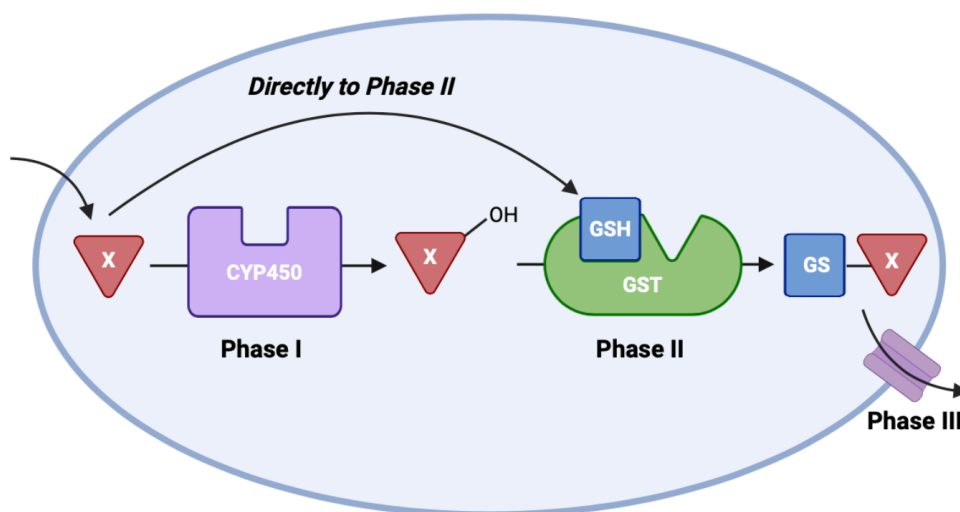
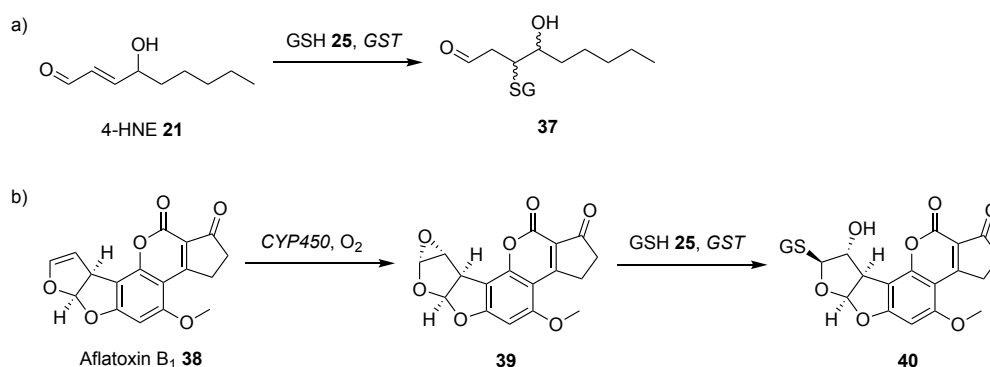


Figure 9. The Phase I-III metabolism of xenobiotics and endogenous metabolites.

GST enzymes can catalyse a range of reactions, including epoxide ring openings, Michael additions, nucleophilic aromatic substitutions (S_NAr), isomerisations and peroxidations. The toxic end-product of lipid peroxidation, 4-HNE **21**, damages DNA and proteins by formation of covalent adducts. Its major detoxification pathway involves the Michael addition of GSH **25** to form GS-conjugate **37**, catalysed by GST enzymes (Scheme 14a).¹²⁰



Scheme 14. Detoxification of a) endogenous 4-HNE **21** and b) exogenous Aflatoxin B₁ **38** by GST-catalysed GSH **25** conjugation.

Aflatoxin B₁ **38** is an exogenous hepatocarcinogen found in cereals and groundnuts, which can freely diffuse across the plasma membrane of cells. It is metabolised by cytochrome

P450 enzymes to form the epoxide metabolite **39** (Scheme 14b), which can intercalate DNA and induce mutations.^{118, 119} Epoxide **39** is detoxified in an epoxide ring opening reaction with GSH **25** to form GS-conjugate **40**, catalysed by GST enzymes.¹²¹ The GST-catalysed GSH-conjugation mechanism can be exploited to deliver anti-cancer drugs to cells from prodrugs, encouraging the development of GST-activated prodrugs.¹²² On the other hand, GST enzymes can detoxify anti-cancer drugs by GSH **25** conjugation and the up-regulation of GST expression in tumours is often associated with cancer drug resistance, which has prompted research into GST inhibitors.¹²³ GSTs also demonstrate other types of enzymatic activity, such as that akin to glutathione peroxidases (GPx), catalysing the reduction of organic hydroperoxides, including phospholipids and DNA hydroperoxides, to their corresponding alcohols.¹¹⁸ Some GSTs exhibit thiol transferase activity, catalysing isomerisation reactions, including carbon-carbon double bond shifts, intramolecular redox reactions and cis-trans isomerisations, for amino acid degradation and steroid hormone biosynthesis.^{117, 118, 124} Beyond their enzymatic functions, GSTs also regulate signalling pathways in cell survival and apoptosis through protein-protein interactions.¹¹⁸ In particular, GSTP1-1 protects tumour cells from apoptosis signals by inhibiting the JNK kinase, which belongs to the mitogen-activated protein kinase (MAPK) family.^{125, 126}

In eukaryotes, GSTs are divided according to their cellular localization into three major families: cytosolic, mitochondrial and membrane-bound microsomal. The largest and most-studied family, cytosolic GSTs, are in turn divided into several classes, such as alpha (A, α), mu (M, μ), pi (π) and omega (ω) among others, based on chemical and structural properties.¹¹⁸ Most GSTs exist as homodimers, but in some cases, heterodimers made of two different chains from the same class are found. Typically, cytosolic GST isoenzymes within a class share >40% structural identity, and those between classes share <25% identity.¹¹⁷ In humans, the highest cytosolic GST activity level is present in the liver, whereas the kidney (78% of liver), lung (34% of liver), and intestine (37% of liver) have lower activity levels.¹²⁷ Additionally, different GST enzymes are expressed in varying amounts depending on the human tissue. For example, GSTA1-1 is highly expressed in the liver, kidney and testicles, whereas GSTP1-1 is highly expressed in the brain and lung.¹²⁸ Each GST subunit is composed of an N-terminal and a C-terminal domain (Figure 10). The N-terminal domain, which is quite conserved among the different GST classes, adopts a thioredoxin-like fold and comprises most of the GSH-binding site (G-site).^{118, 122} In contrast, the C-terminal domain adopts an α -helix structure, and, along with a small portion of the N-terminus, forms the binding site for hydrophobic electrophiles (H-site).

The C-terminal domain is more divergent across the GSTs, and even slight variations in hydrophobic amino acid residues of the H-site, have a great influence on substrate selectivity.^{118, 122} In most GSTs, including the alpha, mu and pi classes, the key residue involved in GSH **25** binding is tyrosine, while other classes, such as kappa, use a serine.¹¹⁷ The hydroxyl group of the tyrosine or serine residue forms a hydrogen bond with the sulfhydryl group of the GSH **25** to promote formation and stabilisation of the thiolate anion.

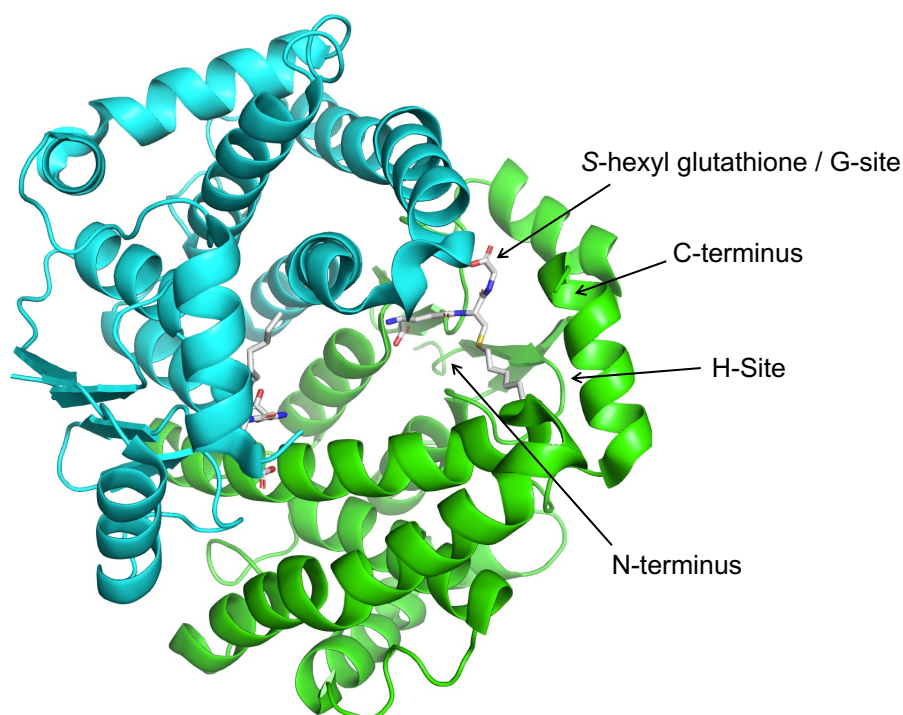
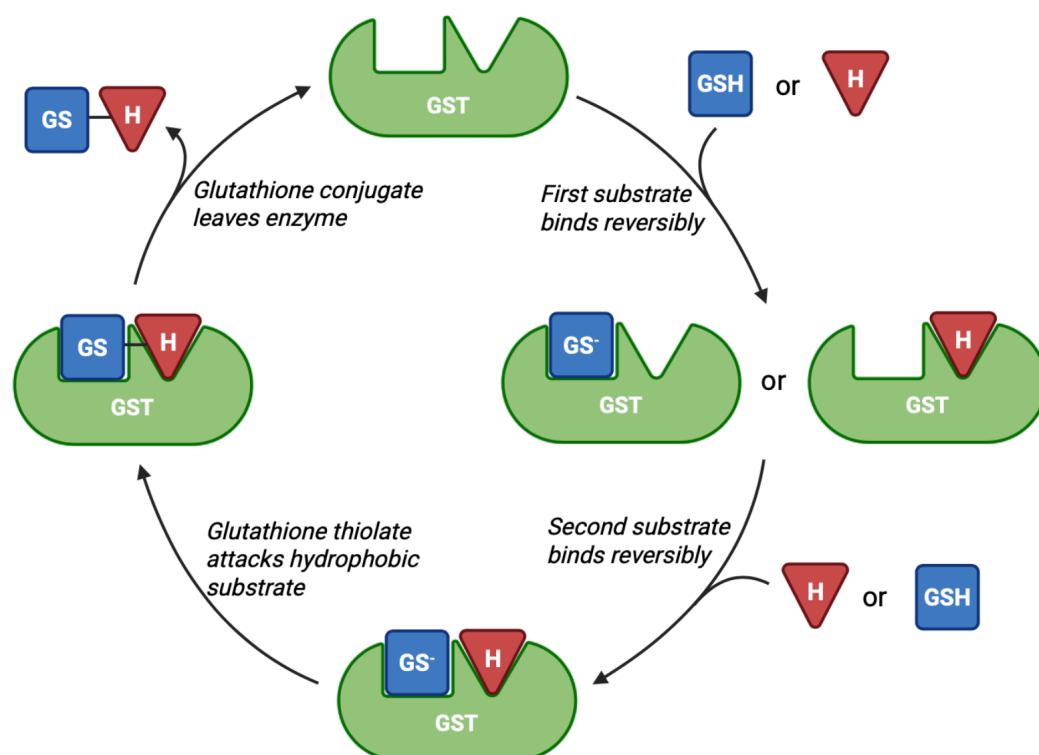


Figure 10. Crystal structure of the hGSTA1-1 (PDB: 1K3L) homodimer with the ligand S-hexyl. The individual chains of the protein are shown in green and blue.

Several kinetics studies have shown that the catalytic mechanism of GSTs follows a random sequential Bi Bi model.¹²⁹⁻¹³² In this model, GSH **25** or the hydrophobic substrate bind to their respective substrate binding sites in either order (Scheme 15). Upon binding of GSH **25**, the thiol is deprotonated to form the thiolate anion. Once the ternary complex is formed, the nucleophilic thiolate attacks the electrophilic centre of the hydrophobic substrate to form the GS-conjugate. Finally, the GS-conjugate is released from the active site via the C-terminus.^{133, 134}



Scheme 15. Catalytic cycle of GST enzymes.

1.2.2 Mitochondrial GST pool

Mammalian mitochondria are reported to contain a diverse pool of GST enzymes with varying substrate specificities. The main mitochondrial GST is the kappa enzyme (GSTK1-1), which is the only enzyme of its class, and is also located in the peroxisome. Molecular cloning and crystallography have revealed that its three-dimensional fold is structurally distinct from cytosolic GSTs and more similar to bacterial 2-hydroxychromene-2-carboxylate isomerase, a GSH-dependent oxidoreductase.^{131, 135, 136} In contrast to cytosolic GSTs, the GSTK1-1 is widely and uniformly expressed in human tissues. GSTK1-1 has a putative cleavable N-terminal signal for mitochondrial translocation and a C-terminal signal sequence for peroxisomal targeting.¹³⁷ There is also evidence of alpha, mu and pi GSTs in the mitochondria, which translocate from the cytosol (Figure 11).¹³⁸⁻¹⁴¹ The precise mechanisms for their translocation are yet to be elucidated. In general, mitochondrial-imported proteins have N-terminal cleavable residues rich in positively charged and hydroxylated amino acids which form an amphipathic helix essential for interaction with negatively charged residues on the translocase of the OMM.¹⁴² Other

proteins, such as cytochrome P450s, are targeted to the mitochondria by hyperphosphorylation of key serine residues, which increases the affinity for binding to chaperones, such as the heat shock proteins Hsp70 and Hsp90, resulting in increased mitochondrial translocation.^{143, 144} One study found that mouse GSTA4-4 is targeted to the mitochondria by hyperphosphorylation and Hsp70 binding, especially in response to oxidative stress.¹⁴⁵ This indicates that mitochondrial GSTA4-4 has a physiological role in protecting mitochondria under oxidative stress. Alpha GSTs A1-1 and A2-2, mu GSTs M1-1 and M2-2, and GSTPi have also been identified in mouse and human liver mitochondria.^{140, 141, 146, 147}

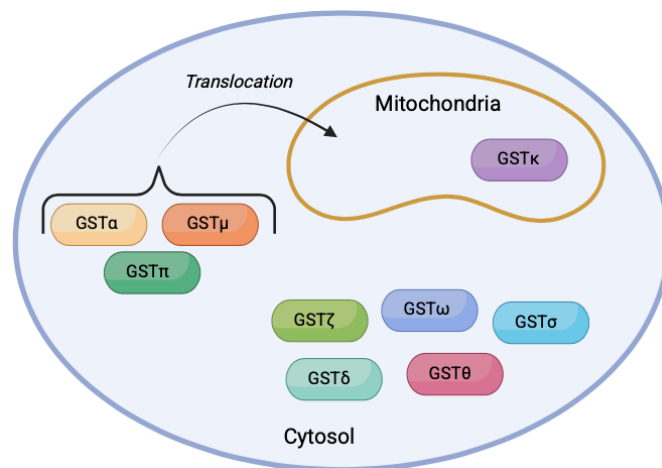


Figure 11. Cellular localisation of GST enzymes. Several cytosolic GSTs belonging to the alpha, mu and pi classes also translocate to the mitochondria, often in response to oxidative stress.

1.2.3 GST overexpression in cancer

Chemotherapy is one of the most effective treatments for cancer, but tumours can develop multidrug resistance (MDR) through various biological mechanisms. One form of MDR is linked to GST overexpression, which can result in direct detoxification of chemotherapeutics.¹¹⁹ GSTs cooperate with efflux transporters and MDR proteins to protect cancer cells from cytotoxic drugs.¹¹⁹ Furthermore, GST enzymes, especially GSTP1-1, form protein-protein interactions with enzymes in the MAPK pathway. This inhibits the MAPK pathway at multiple points, suppressing cell death pathways to ensure cell survival. There have been many cancer studies to investigate the role of GSTs in MDR

and to develop new therapeutic strategies. One study found that multiple GST enzymes were overexpressed in 60 human tumour lines, with GSTP1-1 being the most prevalent.^{128, 148} Different GST enzymes are overexpressed in different tumours, which offers a unique opportunity to design highly selective therapeutics. For example, GSTP1-1 is overexpressed in ovarian, breast, colon, pancreas, lung and lymphoma tumours.^{128, 149-151} In contrast, GSTA1-1 is overexpressed in colorectal, breast and gastric tumours.^{128, 152-155} Overexpressed GSTs cause cancer cell drug resistance to several chemotherapeutics, including cisplatin,¹⁵⁶ doxorubicin,¹⁵⁷ busulfan,¹⁵⁸ and dichloroacetate.¹⁵⁹ As a result, one anti-cancer strategy to reduce MDR involves targeting GST enzymes with inhibitors. This strategy requires inhibitors that selectively target the specific GST enzymes overexpressed in a tumour and avoid disruption to GST-mediated cellular maintenance of normal cells. Although ethacrynic acid **41** (Figure 12) is a potent inhibitor of GSTP, GSTA and GSTM enzymes, its clinical application is limited by poor specificity and diuretic side effects.^{119, 128} Selective inhibition of GSTP1-1 is better achieved with Ezatiostat **42** (Figure 12), which underwent Phase II clinical trials for treatment of blood cancers.^{119, 128, 160, 161} It showed activity against myelodysplastic syndrome, but appears to have lacked an industrial sponsor to progress it.¹⁶¹ Benzophenone analogue **43** (Figure 12) is an interesting competitive inhibitor of GSTA1-1 with an IC_{50} of 1.8 μ M and a LC_{50} of 31 μ M in human colon adenocarcinoma (Caco-2) cells.¹⁶² However, selectivity for this isoform alone was not tested and the potency was not sufficient for drug development of this compound.¹⁶² Alternatively, the overexpression of GST enzymes in cancers has been utilised to metabolise prodrugs that selectively deliver an anti-cancer drug to a specific tumour, explored further in Section 1.2.5.

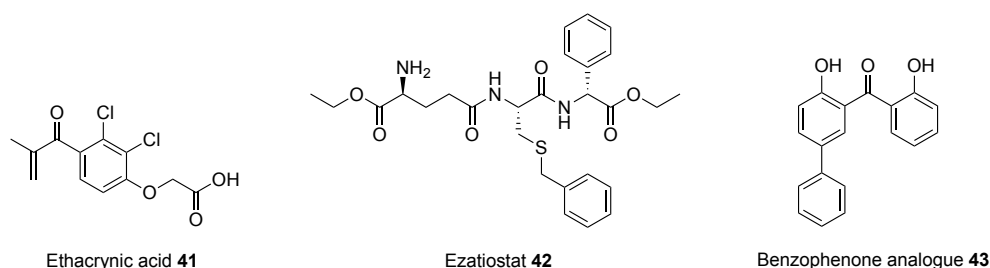


Figure 12. Structures of current GST inhibitors.

1.2.4 Intracellular GSH in pathology

Intracellular GSH **25** status is closely associated with ROS regulation, as discussed in Section 1.1.6. Changes in the cytosolic GSH (cGSH) and mitochondrial GSH (mGSH) pools are associated with numerous oxidative stress related disorders including cancer, diabetes, neurodegenerative diseases and liver disease.^{138, 163-166} GSH **25** is only produced in the cytosol, which is home to around 85-90% of the cellular GSH **25**.¹³⁸ The remaining 10-15% is imported in the mitochondria via two major anion carriers, dicarboxylate and oxoglutarate, which mediate the exchange of GSH **25** for phosphate and dicarboxylates, respectively, across the IMM.^{138, 163} The concentration of GSH **25** in the mitochondrial matrix is approximately 10-12 mM.¹³⁸ Hence, mGSH levels are regulated by a combination of mGSH transport activity and GSH **25** metabolism by enzymes, including GPx, Grxs and GSTs (see Section 1.1.6). cGSH has a rapid turnover of 2-3 h while mGSH has a relatively longer half-life of 30 h.¹³⁸ Under experimental or pathological conditions, the mGSH and cGSH pools can be selectively depleted, and the mGSH pool has been found to take a significantly longer time to recover.^{137, 164} A depleted mGSH pool promotes the oxidation of critical thiols of the IMM and leads to mitochondrial permeabilization and cytochrome c release associated with apoptosis.¹⁶⁷ An increasing number of pathologies are being linked with marked depletion and/or oxidation of the mGSH pool, such as alcohol liver disease and type II diabetes.¹⁶³ In particular, the former has been attributed to decreased expression and activity of GSH **25** carriers.¹⁶⁸ Gradual loss of mGSH is also a hallmark of the aging process.¹⁶⁹ Therefore, a sustained mGSH pool is advantageous to minimize ROS-induced oxidative damage during physiological and pathological metabolism of oxygen in the mitochondria. In cancer, ROS production is significantly increased which, on the one hand, can promote tumour growth by activating signalling pathways, but on the other, can result in an accumulation of large amounts of oxidised biomolecules, which threatens cancer cell survival. As an adaptive response, cancer cells up-regulate levels of ROS-scavenging molecules, including GSH **25**. Elevated levels of GSH **25** in cancers, including breast, lung, colon, and ovarian, play multiple roles in promoting tumour initiation and metastasis.^{165, 166} As in normal cells, GSH **25** maintains redox homeostasis of cancers cells, scavenging endogenous ROS and harmful metabolites, as well as detoxifying xenobiotics by GST catalysis, which confers resistance to chemotherapeutic drugs. As a result, attention has focused on developing drugs that selectively reduce GSH **25** levels of cancer cells to improve the efficacy of targeted therapy. For example, disulfiram **44** (Figure 13) induces melanoma cell apoptosis by shifting the ratio of GSH **25**/GSSG **28** towards its

oxidised state and has been studied in a range of phase I/II clinical trials, including for treatment of metastatic melanoma.¹⁷⁰ However, there has been no convincing efficacy against cancer in clinical trials, perhaps due to poor pharmacokinetics.

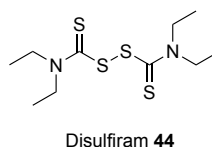
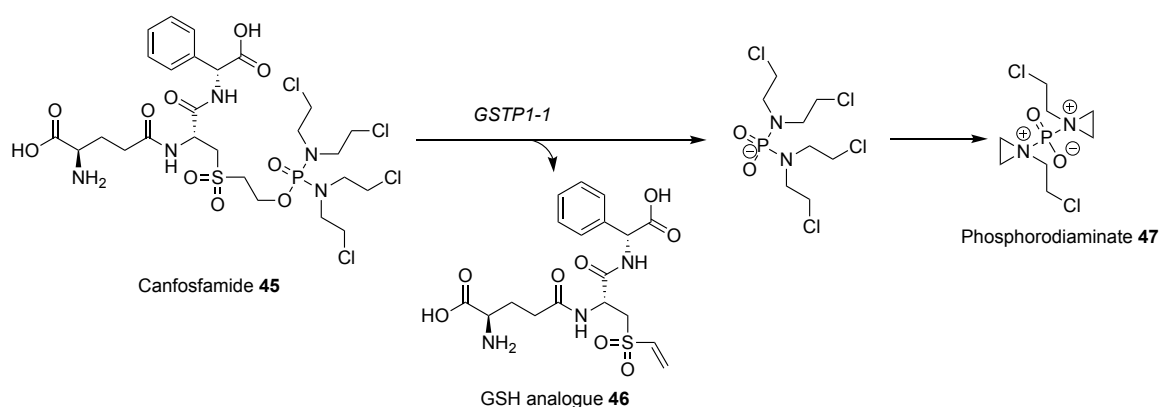


Figure 13. Structure of disulfiram 44, which depletes intracellular GSH 25.

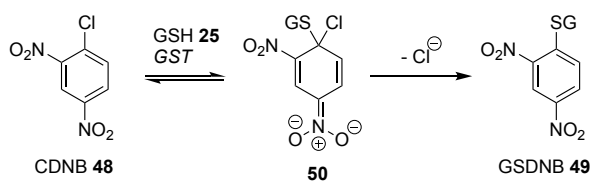
1.2.5 GST-activated probes and prodrugs

Prodrugs are pharmacologically inactive molecules that are converted into their active parent drugs *in vivo* after chemical modifications, often catalysed by enzymes. They are designed to improve the bioavailability of drugs, while increasing their selectivity and reducing off-target effects. The overexpression of GST enzymes in cancer cells has been exploited to metabolise GST-activated prodrugs to deliver high concentrations of active drugs to tumours. There are two different classes of GST-activated prodrug designs commonly found in the literature: i) molecules containing a GSH 25 or a GSH-like moiety and ii) molecules containing an electrophilic moiety whose enzymatic activation occurs via formation of a GS-conjugate. The most notable GST-activated prodrug to date is Canfosfamide 45, which belongs to the first class. Similar in structure to GSH 25, Canfosfamide 45 is activated by GSTP1-1 (Scheme 16), adopting a conformation that undergoes E1cB to release a GSH analogue 46 and phosphorodiaminate 47, an alkylating metabolite that forms covalent linkages with DNA and induces cancer cell death.^{171, 172} The GSH analogue 46 may also have effects as a competitive inhibitor of GSH-dependent enzymes and as an alkylating agent. Canfosfamide 45 was investigated in Phase I-III trials to evaluate its activity against ovarian and non-small cell lung cancers, including as a combination therapy with standard chemotherapeutics, but it was ultimately determined to be insufficiently effective.¹⁷³⁻¹⁷⁵



Scheme 16. Activation of canfosfamide **45** by GSTP1-1 to form GSH analogue **46** and phosphorodiaminate **47**.

There are many GST-activated probes and prodrugs based on an electron-poor electrophilic moiety, especially 1-chloro-2,4-dinitrobenzene (CDNB) **48** (Scheme 17). Many GSTs catalyse the conjugation of GSH **25** to CDNB **48** to form GSDNB **49** by nucleophilic aromatic substitution (S_NAr).¹¹⁷ Consequently, CDNB **48** is routinely used as a substrate to measure the activity of GSTs.¹⁷⁶ The reaction follows a classical two-step addition-elimination mechanism, in which formation of the Meisenheimer intermediate **50** is the rate-determining step.¹⁷⁷



Scheme 17. Mechanism of GST-catalysed S_NAr reaction of CDNB **48** and GSH **25**.

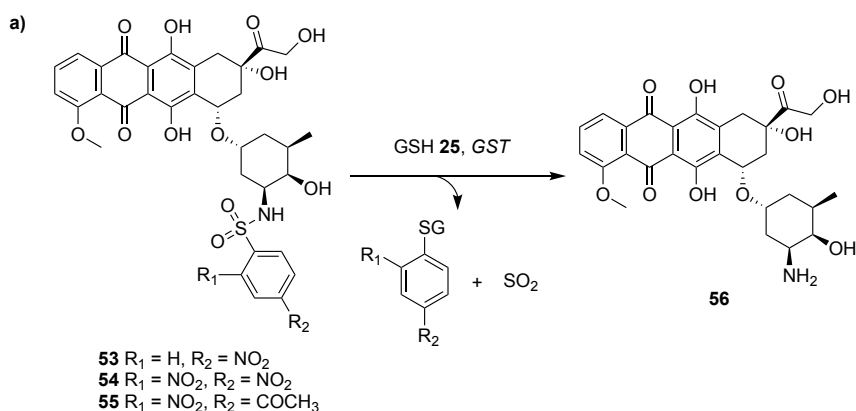
Booty *et al.*¹⁷⁸ developed MitoCDNB **51** (Scheme 18), a GST-activated molecule that selectively disrupts mitochondrial thiol redox status. MitoCDNB **51** is the mitochondria-targeted analogue of CDNB **48** where the CDNB moiety is linked to a TPP cation, which targets the molecule to the mitochondrial matrix. mGSTs catalyse the conjugation of mGSH to MitoCDNB **51** to produce MitoGSDNB **52**, which is rapidly exported from the cell. As a result, MitoCDNB **51** selectively depletes the mGSH, as well as inhibiting TrxR2 and

Prx3, which are involved in GSH **25** regulation (see Section 1.1.6).¹⁷⁸ mGSH-depleting molecules such as MitoCDNB **51** have potential in cancer therapy to selectively enhance oxidative damage and cell death of cancer cells which already have high levels of ROS.



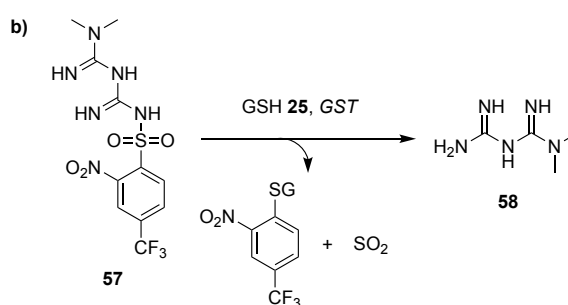
Scheme 18. mGST-catalysed S_NAr reaction of MitoCDNB **51** and mGSH.

Many prodrugs in the literature attach the drug to an electron-poor aromatic ring, similar to CDNB **48**, via a sulfonamide bond, which is readily cleaved by GST-catalysed reaction with GSH **25**.¹⁷⁹ Gisbergen *et al.*¹⁸⁰ developed three doxorubicin prodrugs **53-55** (Scheme 19) based on three different electron-poor rings, which were activated by GST enzymes to release doxorubicin **56** and the corresponding GS-conjugates. Interestingly, prodrugs **53-55** displayed preferences for different GST enzymes.¹⁸⁰ Doxorubicin **56** is a common chemotherapeutic that inhibits topoisomerase II, but it is susceptible to detoxification by several GST enzymes in cancer cells. Prodrugs **53-55** overcame resistance to doxorubicin **56** and successfully killed GSTA1-1, GSTP1-1 and MGST1-1 overexpressing tumour cells.¹⁸⁰ Additionally, prodrugs **53-55** depleted GSH **25** levels and inhibited TrxR1.¹⁸⁰



Scheme 19. GST-catalysed S_NAr reaction between doxorubicin analogues **53-55** and GSH **25** to release doxorubicin **56**.

Rautio *et al.*¹⁸¹ developed a metformin analogue **57** (Scheme 20) based on an *ortho*-nitro *para*-trifluoromethyl benzene ring and demonstrated its GST-catalysed quantitative conversion to metformin **58** in rat and human liver fractions (which contain a variety of phase I and II metabolising enzymes), as well as with recombinant human GST enzymes. Metformin **58** is a widely-prescribed anti-diabetic drug, but it also exhibits anti-cancer effects toward a variety of tumours, particularly in patients also diagnosed with diabetes. Although metformin **58** is an effective drug with minimal side-effects, its oral absorption is poor, which has necessitated metformin prodrugs with improved lipid membrane permeability. The pKaH of metformin **58** is 12.4, meaning it almost completely exists as the protonated form at physiological pH 7.0. In addition to increasing lipophilicity, sulfonamide prodrugs of metformin, such as analogue **57**, lower the pKa of the metformin moiety reducing the amount of the molecule in its charged form.^{181, 182}



Scheme 20. GST-catalysed S_NAr reaction between metformin analogue **57** and GSH **25** to release metformin **58**.

1.3 Michaelis-Menten Kinetics

1.3.1 The model

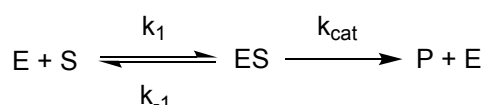
Optimising prodrugs to be activated by an enzyme, such as a GST in the case of this thesis, requires a consideration of enzyme kinetics. Enzyme kinetics is the study of the rates of enzyme-catalysed chemical reactions and the conditions that affect them. When characterising an enzyme, it is important to obtain parameters that describe how conditions, such as enzyme concentration, substrate concentration, pH and temperature define its activity for a reaction. For many chemical reactions, the rate is proportional to

the concentration of a reactant A.¹⁸³ The order of a reaction with regard to a reactant is related to the exponent of that reactant. Hence, in Equation 3, the reaction is first order with respect to [A], meaning that the reaction rate is directly proportional to [A]. In Equation 4, the reaction is zero order with respect to [A], so the reaction rate is independent of [A].

$$(3) \quad v = k[A]^1 = k[A]$$

$$(4) \quad v = k[A]^0 = k$$

In the simplest type of an enzyme-catalysed reaction (Scheme 21), an enzyme (E) reacts reversibly with a single substrate (S) to form an enzyme-substrate complex (ES), which can then then form the product (P) and regenerate enzyme (E).



Scheme 21. A single-enzyme single-substrate reaction.

Many enzymatic reactions, including the majority of GST-catalysed GSH 25 conjugation reactions, can be accurately described by Michaelis-Menten kinetics. This model describes enzymatic reactions using the steady-state approximation, which refers to a process in which the formation and consumption rates of a reactive intermediate are identical.¹⁸⁴ As a result, the concentration of the reactive intermediate is assumed to remain constant. In an enzyme-catalysed reaction, E, S and ES are assumed to be in rapid equilibrium.¹⁸³ Considering only the first step, the equilibrium is expressed by the dissociation constant, K_d , for the ES complex (Equation 5).

$$(5) \quad K_d = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1}$$

Considering both steps and assuming the second-step is slow and rate-determining, the steady-state approximation can be applied. ES is considered to be the reactive intermediate and its concentration [ES] is assumed to be constant.¹⁸⁴ Additionally, for a typical enzyme-catalysed reaction, in which [S] is much larger than [E], the change in [S] is also negligible.¹⁸⁴ Under these assumptions, the contemporary Michaelis-Menten equation (Equation 6) relates the rate (v) of an enzyme-catalysed reaction to two key kinetic parameters, $V_{\max}$ and K_M :

$$(6) \quad v = \frac{V_{\max}[S]}{K_M + [S]}$$

The Michaelis-Menten equation (Equation 6) explains the hyperbolic behaviour typically observed for an enzymatic reaction when the initial rate of the formation of the product is plotted as a function of substrate concentration (Figure 14). At low substrate concentrations ($[S] \ll K_M$), the rate increases linearly with increasing substrate concentration, obeying first-order kinetics. At high substrate concentration which saturates the enzyme ($[S] \gg K_M$), there is minimal increase in the initial rate when the substrate concentration is increased further, and zero-order kinetics are observed.

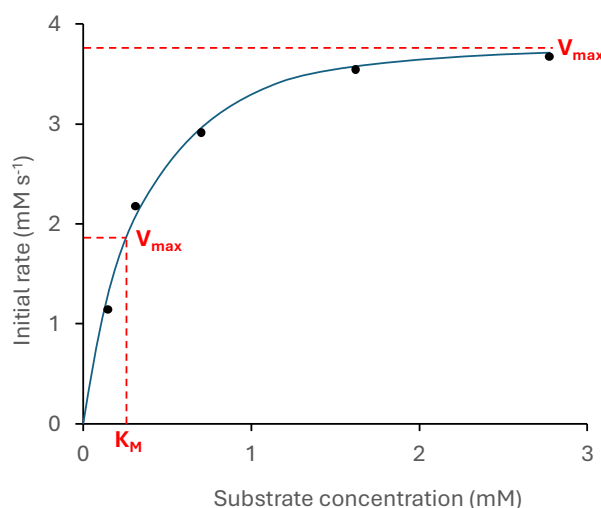


Figure 14. General graph for the initial rate of an enzyme-catalysed reaction as a function of substrate concentration. $V_{\max}$ is the maximal velocity. K_M is the substrate concentration at half the $V_{\max}$.

$V_{\max}$ is the maximal velocity and is the product of the catalytic rate constant k_{cat} multiplied by the enzyme concentration (Equation 7). $V_{\max}$ refers to the hypothetical case in which all the enzyme molecules are bound to substrate. Also known as the turnover number, k_{cat} , is normally expressed in s^{-1} and refers to the maximal number of substrate molecules converted to product per enzyme molecule per unit time. Hence, the higher the value of k_{cat} , the faster the enzyme catalyses the productive reaction.

$$(7) \quad V_{\max} = k_{\text{cat}}[E]$$

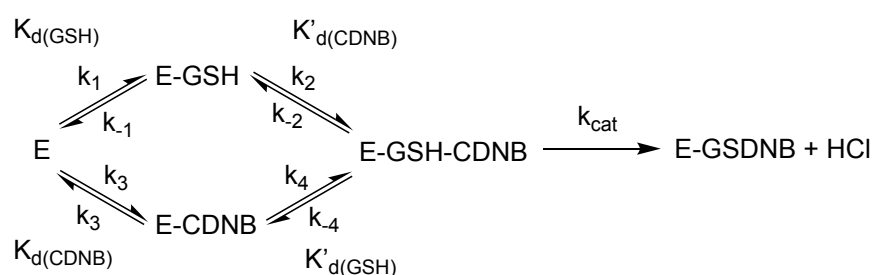
The Michaelis constant, K_M , normally expressed in mM, is the substrate concentration at half the maximal velocity ($1/2 V_{\max}$). In other words, it is the concentration of substrate when half the enzyme is free and half is occupied with substrate. It is derived from the ratio of rate constants for the decomposition of ES to E and S or E and P, and formation of ES (Equation 8). Therefore, it describes the binding affinity of a substrate for an enzyme: the lower the value of K_M , the better the substrate binds to the enzyme. K_M is unique to the enzyme and is affected by conditions such as temperature and pH, but not the concentration or purity of the enzyme. It is important to note that K_M is distinct from K_d , because it depends on k_{cat} . However, in cases where the dissociation rate is much faster than the productive rate ($k_{-1} \gg k_{\text{cat}}$), $K_M \approx K_d$.

$$(8) \quad K_M = \frac{k_{-1} + k_{\text{cat}}}{k_1}$$

The specificity of an enzyme for different substrates is usually compared by calculating the specificity constant (k_{cat}/K_M). Typically expressed in $\text{mM}^{-1} \text{s}^{-1}$, the specificity constant measures how efficiently an enzyme converts substrate into product. Hence, the higher the value of k_{cat}/K_M , the better the substrate for the enzyme.

1.3.2 Bisubstrate reactions

The kinetics of multisubstrate enzyme-catalysed reactions are more complex but they often still obey the Michaelis-Menten model. Consider the second-order bisubstrate reaction between CDNB **48** and GSH **25** which is catalysed by a GST enzyme to form GSDNB **49** as the main product and HCl as a side-product (Scheme 22).



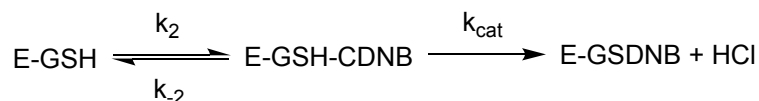
Scheme 22. GST-catalysed bisubstrate reaction between CDNB **48** and GSH **25**.

In this case, the catalytic constant, k_{cat} , refers to the rate constant of the productive reaction when the enzyme is saturated with both CDNB **48** and GSH **25**. GSH **25** can bind to the enzyme first ($K_d(\text{GSH})$), followed by CDNB **48** ($K'_d(\text{CDNB})$). Alternatively, CDNB **48** can bind to the enzyme first ($K_d(\text{CDNB})$), followed by GSH **25** ($K'_d(\text{GSH})$). For each route towards the formation of E-GSH-CDNB, the product of their rate constants is equal (Equation 9). However, the binding of one substrate can affect the binding of the second substrate, so $K_d(\text{GSH}) \neq K'_d(\text{GSH})$ and $K_d(\text{CDNB}) \neq K'_d(\text{CDNB})$.

$$(9) \quad K_d(\text{GSH}) \times K'_d(\text{CDNB}) = K_d(\text{CDNB}) \times K'_d(\text{GSH})$$

If the enzyme is saturated with respect to one of the substrates, such as GSH **25** (provided the substrate is soluble in aqueous solution at a very high concentration), the reaction can be treated as first-order with respect to the other substrate, CDNB **48** (Scheme 23). Hence, using the Michaelis-Menten equation (Equation 6), the V_{max} and K_M values for the substrate of interest can be measured while the other substrate is at saturating

concentration, and vice-versa. Provided that saturating conditions were achieved in both cases, the $V_{\max}$ calculated for each of the substrates should be equal.



Scheme 23. Simplified reaction between GSH-saturated enzyme and CDNB 48.

1.3.3 Graphical methods to determine kinetic parameters

For an enzyme-catalysed reaction, determining values for $V_{\max}$ and K_M is crucial to characterise an enzyme. There are many graphical methods that can estimate $V_{\max}$ and K_M . Generally, non-linear regression analysis is the preferred approach, provided the rate data exhibit non-linear behaviour across the measured substrate concentrations, because it fits non-transformed initial rate data to the Michaelis-Menten equation (Equation 6).¹⁸⁴ Non-linear regression algorithms generate a best-fit curve for the observed data, typically using the least-squares method, which minimises the sum of squared residuals (a residual being the difference between an observed value and fitted value provided by the model). These algorithms combine gradient-based optimisation with grid search techniques, which exhaustively evaluate all possible combinations of $V_{\max}$ and K_M to identify the optimal fit. Several commercially available software packages, including Microsoft Excel®, GraphPad Prism® and SigmaPlot®, can be used for non-linear regression analysis. Before the advent of modern statistical computing, linear transformations of the Michaelis-Menten equation were used to estimate kinetic parameters. One such method, the Lineweaver-Burk plot (Figure 15), remains widely used. In this double reciprocal plot, $1/v$ is plotted against $1/[S]$ to obtain a linear line where the slope and y-intercept describe $K_M/V_{\max}$ and $V_{\max}$, respectively. The advantage of the Lineweaver-Burk plot over non-linear methods is its applicability to both linear and non-linear rate data. Additionally, generating a linear line of best-fit is simpler than fitting a curve, making it easier to obtain estimated parameters. However, it is generally less accurate for parameter estimation because rates measured at low substrate concentration lie to the far right of the graph, and consequently, have a larger effect on the gradient of the line. Additionally, the Lineweaver-Burk plot poorly illustrates experimental error. Errors in the rate data at low substrate concentrations

translate into large errors in the reciprocal values and vice-versa. A simulation study by Cho *et al.*,¹⁸⁵ compared various non-linear and linear regression methods for estimating $V_{\max}$ and K_M , concluding that non-linear approaches provide the most accurate parameters and errors. Ultimately, if the data permits, it is good practice to use both non-linear and linear methods to compare the parameters they generate.

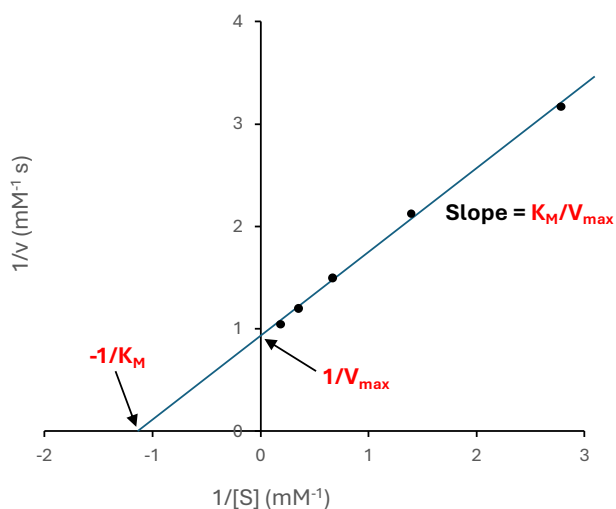


Figure 15. Example of a Lineweaver-Burk plot. The slope of the line is equal to $K_M/V_{\max}$. The y-intercept is equal to $1/V_{\max}$. The x-intercept is equal to $-1/K_M$.

1.4 Biguanides

1.4.1 Sites of action

The original aim of the work in this thesis was to use GST-activated prodrugs to deliver metformin **58** to the mitochondrial matrix in the hope of achieving anti-cancer activity. Metformin **58** is the first-line oral medication for type II diabetes and belongs to a class anti-diabetic biguanide compounds with broad therapeutic potential. The more potent biguanides, phenformin **59** and buformin **60** (Figure 16), were the preferred anti-diabetic drugs until the 1970s when they were withdrawn from most markets for causing high risk of lactic acidosis and replaced by the safer metformin **58**.¹⁸⁶ Initially, the liver was identified as the major site of action for biguanides, where they suppress glucose production.¹⁸⁷ However, recent research has highlighted additional sites of action,

including the gastrointestinal tract, gut microbiota and tissue-resident immune cells.¹⁸⁷ Despite extensive study, the molecular mechanisms underlying the therapeutic effects of biguanides remain complex and only partially characterised. These mechanisms can vary depending on biguanide dosage and treatment duration.^{187, 188}

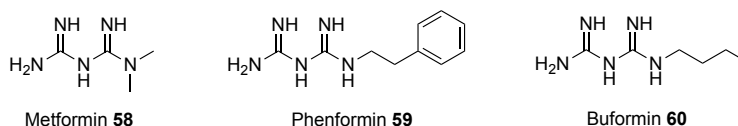


Figure 16. Structures of Metformin 58, Phenformin 59 and Buformin 60.

Metformin **58**, a basic hydrophilic compound with a pKaH of 12.4, exists primarily as a cation at physiological pH and is absorbed in the small intestine. Its absorption and cellular uptake require organic cation transporters (OCTs).¹⁸⁶ Its positive charge facilitates accumulation within the mitochondrial matrix, where it inhibits mitochondrial complex I as its main target (Figure 17).^{186, 189-191} Overall, metformin **58** exhibits poor absolute bioavailability of 50-60%. A cryo-electron microscopy and enzyme kinetics study by Bridges *et al.*¹⁹² identified three possible biguanide binding sites within complex I, with the primary site located in the Q-binding channel. Weak, reversible inhibition of mitochondrial complex I by biguanides disrupts OXPHOS and decreases ATP production, causing an increase in the AMP to ATP ratio. This rise inhibits AMP-regulated enzymes, such as adenylate cyclase, which consequently, reduces ATP-dependent hepatic gluconeogenesis.¹⁸⁷ Simultaneously, AMP binds to and activates the cellular energy sensor, AMP-activated protein kinase (AMPK), which restores energy homeostasis to the cell by transforming it from an ATP-consuming anabolic state into an ATP-producing catabolic state.¹⁸⁶⁻¹⁸⁸ However, the physiological relevance of AMP-dependent AMPK activation has been debated as many studies have used suprapharmacological concentrations of metformin **58**. At lower, clinically relevant doses, metformin **58** has been found to activate AMPK via an AMP-independent mechanism in the lysosome of mouse hepatocytes (Figure 17).^{193, 194} Metformin **58** binds to presenilin enhancer-2 (PEN-2),¹⁹⁵ a subunit of the γ -secretase intramembrane protease complex, inhibiting vacuolar H⁺-ATPase (v-ATPase) and forming a supercomplex that activates AMPK.^{193, 194} Metformin-induced activation of AMPK in lysosomes has been implicated in reducing hepatic lipid accumulation, via acetyl-

CoA carboxylase (ACC) inhibition,¹⁹⁶ and in lowering glucose levels in the gut, via increasing glucagon-like peptide1 (GLP1) secretion.¹⁹⁵

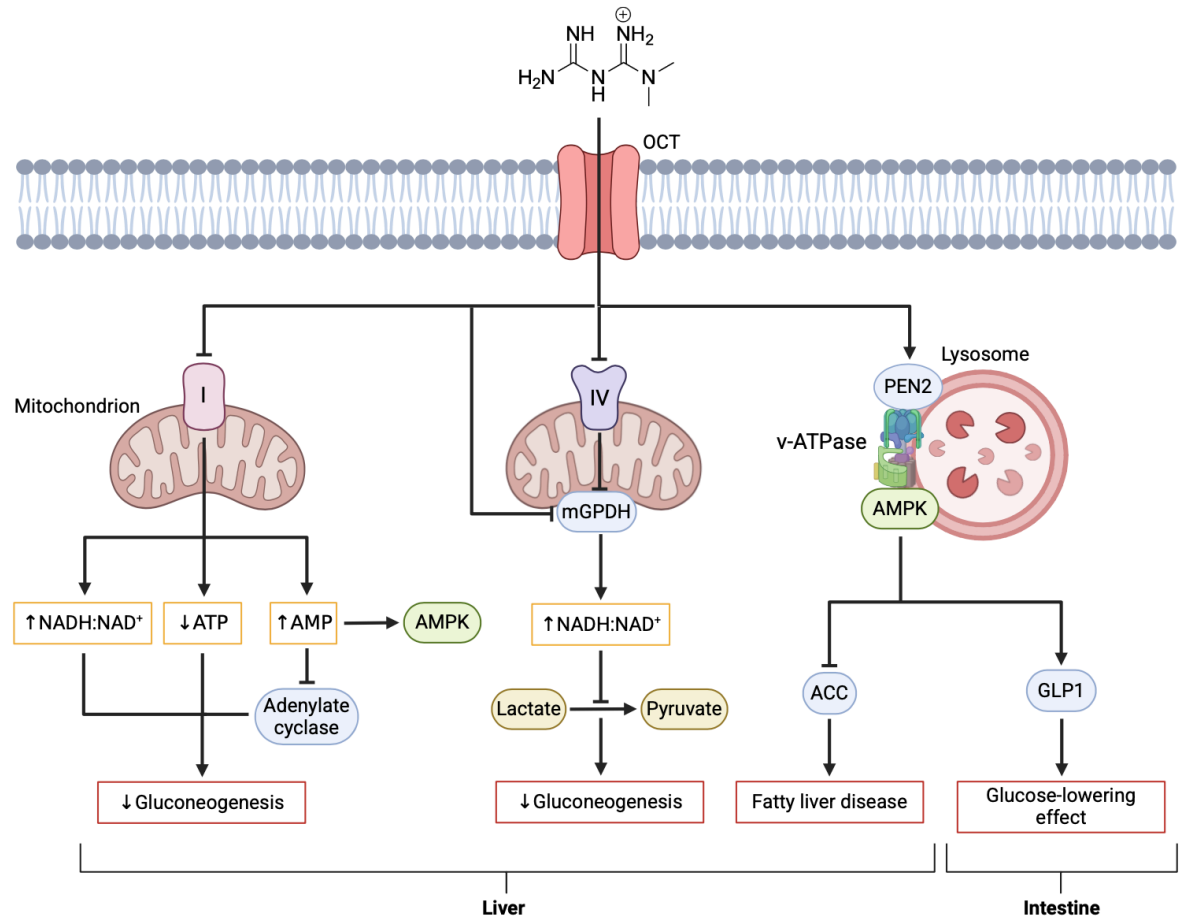


Figure 17. Proposed anti-diabetic mechanisms of action of metformin, adapted from Foretz *et al.*¹⁸⁷ Left: Metformin 58 inhibits mitochondrial complex I, leading to a decrease in ATP synthesis and an increase in AMP and NADH levels, all of which reduce gluconeogenesis. Increased AMP levels lead to inhibition of adenylate cyclase involved in gluconeogenesis. The increase in the AMP/ATP ratio also activates AMPK, but this has no direct effect on glucose production. Middle: mGPDH is inhibited directly by metformin 58, but also indirectly, via metformin-induced complex IV inhibition. This causes an increase in NADH and consequently reduces gluconeogenesis from lactate. Right: Low-dose metformin 58 binds to PEN2, which is recruited to v-ATPase to form a supercomplex containing other proteins and AMPK. Subsequent activation of AMPK reduces lipid accumulation in the liver via ACC inhibition and increases GLP1 secretion in the gut to reduce blood glucose levels.

Biguanides have also been demonstrated to bind to other proteins to decrease gluconeogenesis via mechanisms independent of changes to AMP levels and AMPK activation. Low doses of metformin **58** are reported to directly and indirectly inhibit mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) (Figure 17),^{197, 198} an enzyme essential for the glycerol-3-phosphate shuttle system, which transfers electrons from cytosolic NADH to the mitochondrial ETC for ATP synthesis. One study suggested that metformin **58** and phenformin **59** directly inhibit complex IV of the ETC,¹⁹⁸ leading to indirect mGPDH inhibition. Inhibition of mGPDH increases the cytosolic NADH to NAD⁺ ratio, thereby reducing gluconeogenesis from lactate and glycerol.

While inhibition of complex I remains the predominant molecular mechanism for anti-diabetic effects of biguanides, ongoing research continues to uncover novel targets and pathways, adding complexity to the field's understanding of their therapeutic effects. Given its multiple sites and mechanisms of action, along with its high safety profile, metformin **58** has been actively explored for repurposing to treat areas beyond type II diabetes, including cancer,^{199, 200} cardiovascular diseases^{201, 202} and age-related disorders.²⁰³⁻²⁰⁶

1.4.2 Anti-cancer potential

Metformin **58** first gained attention for its potential anti-cancer properties following a epidemiological report in 2005 that identified a reduction in cancer risk among diabetic patients using the drug.¹⁹⁹ Subsequent epidemiological and retrospective studies have demonstrated that metformin **58** reduces tumour incidence and/or mortality in diabetic patients across various cancer types, including liver, colorectal, pancreas and gastric cancers.²⁰⁷ Cellular and pre-clinical studies have provided mechanistic insights into the anti-proliferative and apoptotic effects of biguanides on tumour cells. One key mechanism involves the regulation of systemic glucose metabolism. As discussed in Section 1.4.1, biguanides lower blood glucose levels through both AMPK-dependent and independent pathways, leading to a reduction in circulating insulin. Given that insulin functions as a mitogen for numerous cancer cell types, this reduction consequently inactivates the pro-tumorigenic phosphoinositide 3-kinase (PIK3) signalling pathway in insulin-sensitive tumour cells.^{186, 189, 208, 209} Additionally, biguanides exert anti-cancer activity by directly disrupting tumour cell energy metabolism, with inhibition of complex I emerging as a critical determinant in tumour suppression (Figure 18). Notably,

metformin-induced inhibition of complex I has been shown to trigger apoptosis in glucose-deprived cancer cells.²¹⁰ Inhibition of complex I also activates AMPK, which attenuates cancer cell proliferation via multiple downstream mechanisms. The most significant consequence of AMPK activation is the suppression of mammalian target of rapamycin (mTOR), an important kinase protein which regulates many cellular processes including growth, proliferation and protein synthesis.^{186, 187, 208} Biguanides have also been reported to promote anti-tumour immunity by modulating the tumour immune microenvironment.¹⁸⁷ The signal transducer and activator of transcription 3 (Stat3), which is associated with mTOR and plays a crucial role in immune regulation, is inactivated upon AMPK activation by metformin **58** in human oesophageal carcinoma cells.²¹¹ Its inactivation leads to an increase in tumour-infiltrating cytotoxic CD8⁺ T cells and a decrease in tumour-promoting macrophages.²¹¹ Metformin **58** has also been shown to suppress mTOR and Stat3 via AMPK-independent mechanisms.^{186, 187}

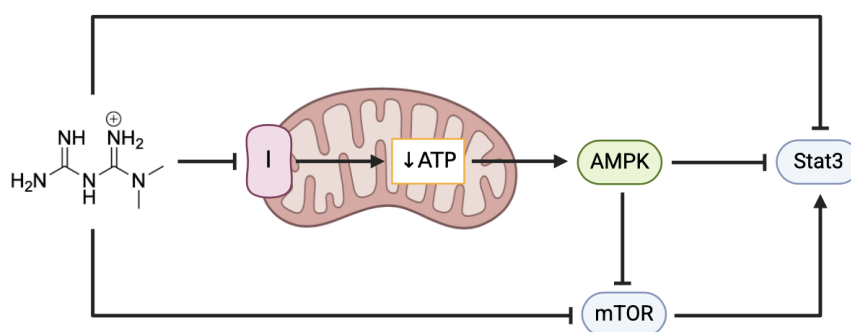


Figure 18. Metformin **58** inhibits mTOR and Stat3 signalling pathways by AMPK-dependent and independent mechanisms.

Notably, phenformin **59** exhibits stronger anti-cancer potency than metformin **58**, as its superior lipophilicity facilitates greater mitochondrial uptake in tumour cells. In various experimental models, including cell lines, mouse models and breast cancer xenografts, phenformin **59** has demonstrated anti-neoplastic effects at lower concentrations than metformin **58**.^{189, 210, 212, 213} Hence, despite its greater risk of lactic acidosis, phenformin **59** is still worth exploring for cancer therapy. Overall, the biguanides exhibit anti-cancer activity primarily through OXPHOS inhibition and associated AMPK-dependent and independent mechanisms. However, many of these mechanisms are subjects of ongoing debate and a critical challenge remains in translating these findings to clinical relevance.

Many *in vitro* studies used high concentrations of biguanide, raising concerns about whether these mechanisms can fully account for the observed clinical benefits. Therefore, further research is necessary to determine whether biguanides are suitable for use in cancer therapy as single agents or as adjuvants.

1.4.3 Metformin derivatives

Several derivatives of metformin have been designed to understand its mechanisms of action, but also to improve its pharmacokinetic and pharmacological properties. The chemical structure of metformin **58** makes it challenging to derivatize, particularly for prodrug design, as generating amide and carbamate bonds results in cyclisation to form 5- and 6-membered rings.²¹⁴ Many non-prodrug metformin derivatives have shown greater anti-tumour activity in different cancer cells compared to metformin **58**. For example, non-prodrug metformin derivative IM176 **61** (Figure 19) activates AMPK and inhibits mTOR to kill prostate cancer cells more effectively than metformin **58**.²¹⁵ Kalyanaraman *et al.*, designed a series of anti-cancer mitochondria-targeted derivatives of metformin in which metformin **58** was conjugated to a TPP cation via alkyl chains of different length.²¹⁶ The lead compound, Mito-Met₁₀ **62** (Figure 19), inhibited complex I and pancreatic cancer cell respiration 1000-fold compared to metformin **58** and reduced the growth of pancreatic tumour in mice.²¹⁶ Additionally, when used in combination with ionizing radiation, Mito-Met₁₀ **62** behaved as an effective radiosensitizer of pancreatic cancer cells.²¹⁶

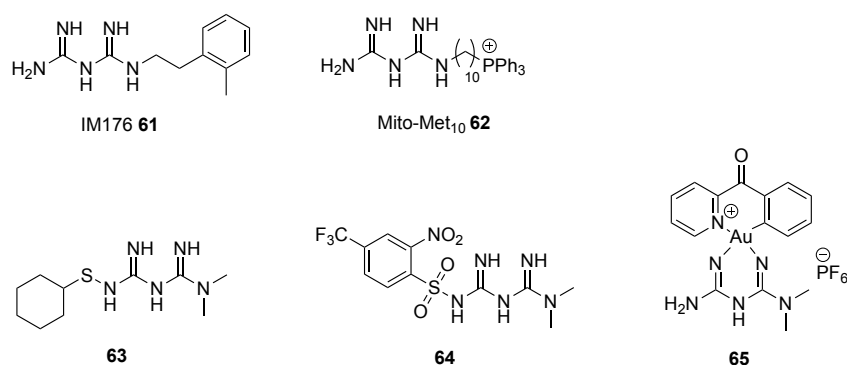


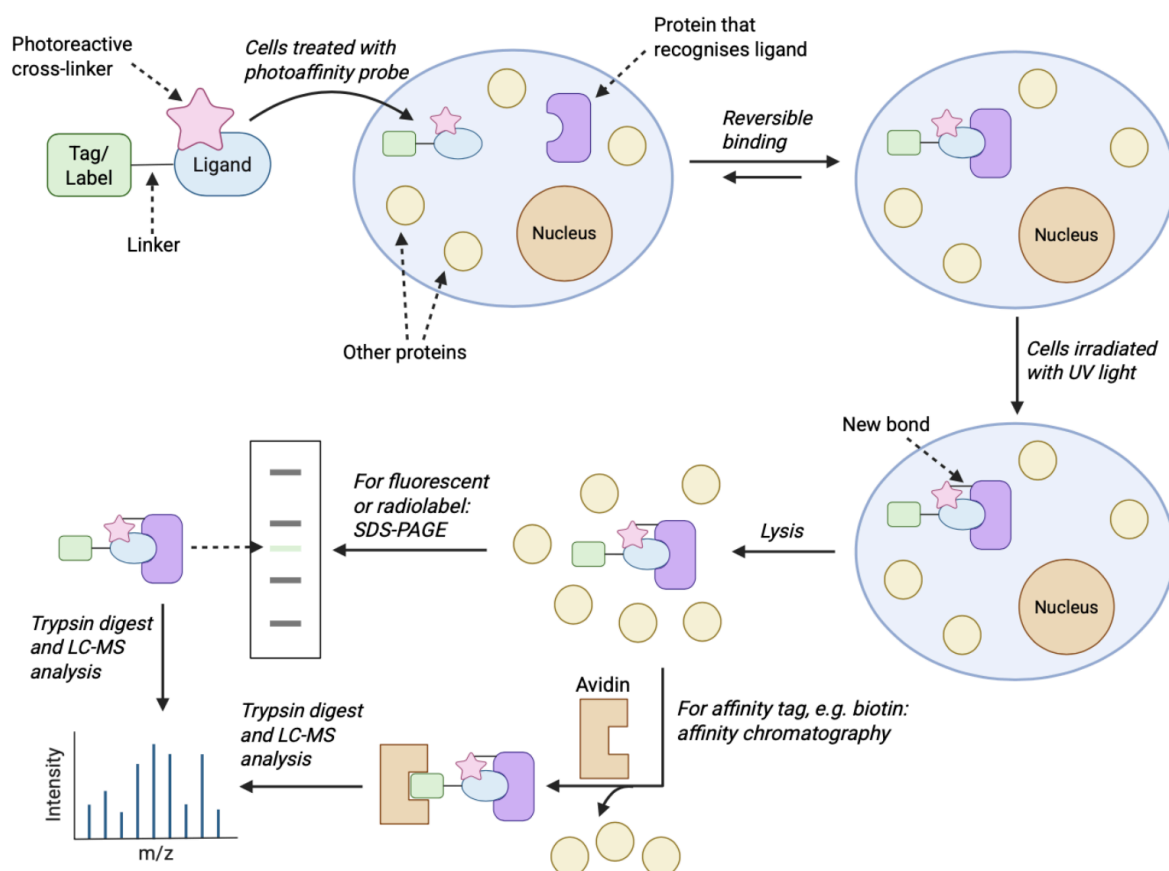
Figure 19. Examples of metformin derivatives and prodrugs.

The first reported metformin prodrugs were sulfenamide derivatives, including sulfenamide **63** (Figure 19), which is bioconverted to metformin **58** via a sulfide exchange reaction with endogenous thiols such as GSH **25** and cysteine residues.^{217, 218} However, no preclinical or clinical studies have reported improved pharmacological properties compared to metformin **58** to date. The sulfonamide functional group has long featured in drug design for its anti-bacterial, anti-diabetic and anti-cancer activities. Many non-prodrug sulfonamide derivatives of metformin have demonstrated improved cellular uptake and variable pharmacological effects compared to metformin **58**.²¹⁴ Additionally, the sulfonamide bond has been employed in the design of metformin prodrugs such as prodrug **64** (Figure 19), which is activated by GST-catalysed reaction with GSH **25** (see Section 1.2.5 for GST-activated prodrugs). Babak *et al.*²¹⁹ reported Au^{III}-cyclometalated prodrugs of metformin and phenformin (Figure 19), which release the biguanides in breast cancer cells. In particular, metformin complex **65** demonstrated over 6000-fold greater *in vitro* activity and significantly reduced the growth of breast tumours in mice compared to metformin **58**.²¹⁹ It would be desirable to design a mitochondria-targeted prodrug of metformin to increase its bioavailability and pharmacological activity for use in cancer therapy. Cancer cells often exhibit a higher mitochondrial membrane potential compared to normal cells, which should drive accumulation of a prodrug targeted by a lipophilic cation into the mitochondrial matrix.

1.5 Photoaffinity labelling

Since the biological targets of biguanide drugs are not fully understood, it would be helpful to detect all the proteins they bind to. Small molecules serve not only as effective therapeutics, but also as valuable tools for probing biological processes and identifying new drug targets. In biological systems, molecular interactions, particularly those between proteins or between proteins and small molecules, are dynamic and dependent on their native environments, making their characterisation challenging.²²⁰ Photoaffinity labelling (PAL) is a powerful technique that utilises photo-activated small molecule probes to covalently capture these interactions while preserving the native cellular context.²²⁰ The resulting covalent probe-protein conjugates can then be analysed by mass spectrometry-based proteomics and imaging methods. A typical photoaffinity probe consists of three key components (Scheme 24): a small molecule ligand, a photoreactive cross-linking group and an identification tag for isolation of the probe-protein adduct.²²¹

The length and composition of the spacer between these components are also critical for efficient cross-linking. The general strategy of PAL begins with cellular uptake of the probe, followed by reversible binding of the ligand to its target protein. Irradiation of the photo-cross-linking group generates a highly reactive intermediate, which forms a covalent bond with a residue of the protein. Following cell lysis, the probe-protein conjugate is enriched by affinity chromatography, digested into peptides and analysed by mass spectrometry for protein identification.



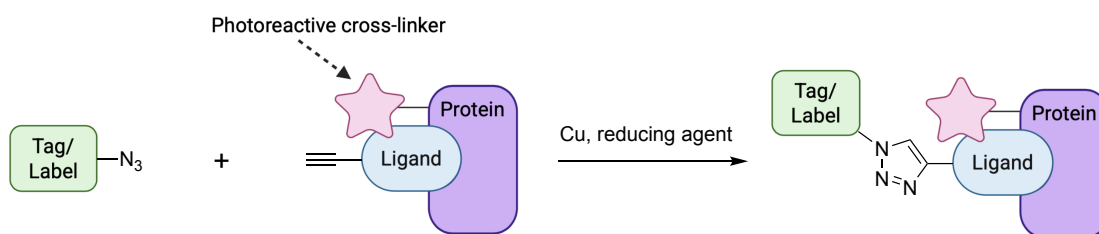
Scheme 24. General design and mechanism of action of a photoaffinity probe.

The ideal photoaffinity probe must have structural and functional similarity to the parent ligand, stability in the dark at a range of pHs, and a photo-cross-linking group that activates at a wavelength that minimises damage to biomolecules while also forming stable covalent conjugates with the protein.²²¹ However, designing an optimal probe that

meets these criteria often requires extensive structure-activity relationship (SAR) studies.²²¹

1.5.1 Identification and purification

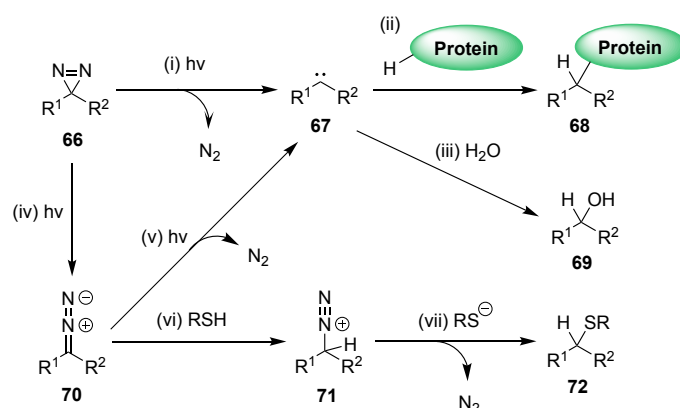
There are many moieties that can be incorporated either directly or indirectly into the probe to assist identification and purification of protein targets. The affinity tag, biotin, is commonly used to isolate probe-protein conjugates due to its high affinity for avidin. Alternatively, a variety of fluorescent labels can be used to visualise the probe-protein conjugates by fluorescence microscopy and gel-based imaging. However, moieties used for identification and purification purposes are often large, which means their direct incorporation in the original probe can change the pharmacological activity of the ligand moiety and sterically hinder binding to proteins, leading to inaccurate identification of protein targets. A polyethylene glycol linker is often incorporated to provide space between the affinity tag or fluorescent label and the rest of the molecule, but it makes the probe even larger and less cell-permeable.²²¹ Instead, the affinity tag or fluorescent label can be introduced at a later stage using complementary tags and bioorthogonal chemistry. One way of doing this is to take advantage of the copper-catalysed reaction between an azido tag and an alkyne tag. In addition to the ligand and photo-cross-linking group, the probe can include an alkyne tag (Scheme 25). After photo-cross-linking and cell lysis, a copper catalyst, reducing agent and an azide-tagged fluorophore or affinity tag is added to the lysate to link the probe-protein conjugate to the fluorophore or affinity tag through triazole formation.²²¹



Scheme 25. Cu-catalysed cycloaddition between an azide-tagged fluorescent label or affinity tag and alkyne-tagged probe-protein conjugate to form a triazole.

1.5.2 Photo-cross-linking groups

Aryl diazirines **66** are widely used as photo-cross-linkers due to their stability against nucleophiles, acids and bases. They are irradiated at around 350 nm to generate a highly reactive, short-lived carbene intermediate **67** (Scheme 26), which can insert into C-H or heteroatom-H bonds to form a covalent protein conjugate **68**. A competing reaction with water produces alcohol **69**, which reduces photoaffinity yields, but minimises nonspecific binding. While the majority of the diazirine **66** is directly converted to the carbene **67**, approximately 30% generates a diazo intermediate **70**, which has a longer lifetime but can still form the carbene **67**.^{221, 222} Alternatively, the diazo intermediate **70** can react with endogenous thiols, generating a diazonium cation **71**, which undergoes nucleophilic attack by the thiolate to afford a sulfide **72**. To mitigate this, many probes incorporate an electron-withdrawing trifluoromethyl group adjacent to the diazirine, reducing the anionic character of the α -carbon and thereby decreasing its likelihood to be protonated.



Scheme 26. Photoirradiation pathways of the diazirine group.

Prior to this thesis, the only photoaffinity probe for investigating the targets of biguanide drugs was Met-P1 **73** (Figure 20), a metformin probe developed by Ma *et al.*,¹⁹⁵ which binds to PEN2 and activates the lysosomal AMPK pathway.¹⁹⁵ The diazirine moiety was photoactivated to form a covalent bond with PEN2 and the probe-protein conjugate was isolated following a click reaction between the alkyne group and azide-tagged biotin.¹⁹⁵

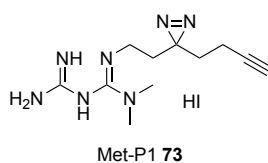
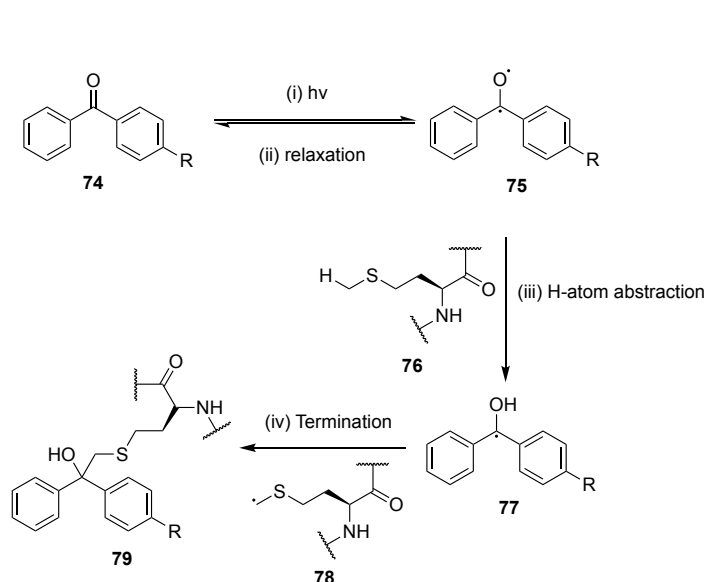


Figure 20. Structure of Met-P1 **73**, a photoaffinity probe of metformin by Ma *et al.*¹⁹⁵

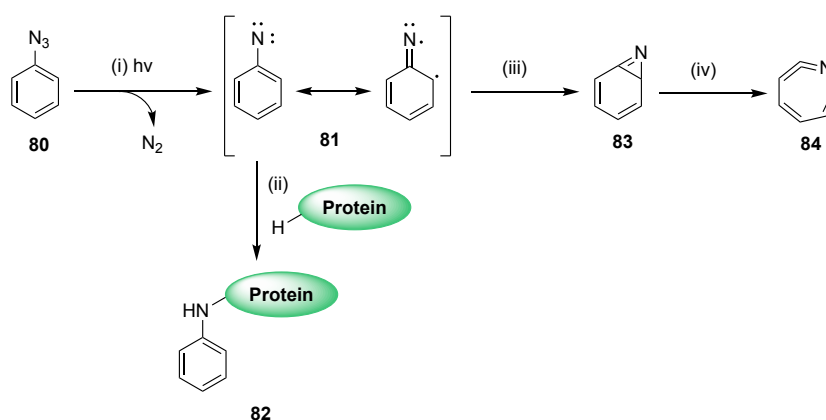
Benzophenones **74** are also activated around 350 nm to reversibly generate a reactive diradical **75** that inserts into C-H bonds (Scheme 27).²²² The reaction follows a two-step process in which the diradical **75** abstracts a hydrogen atom from a protein residue, such as methionine **76**, to form a single radical **77**, which then combines with the residue radical **78** to form the protein conjugate **79**. The diradical **75** exhibits a preference for methionine residues because the intermediate radical is stabilised by the lone pairs of the sulfur atom.^{220, 223} Unlike aryl diazirines **66**, benzophenones **74** undergo many excitation-relaxation cycles, requiring a longer irradiation period which presents the diradical with more opportunities to cross-link to proteins, but also increases the risk of nonspecific labelling.²²⁰⁻²²² Additionally, their larger size can interfere with ligand binding.



Scheme 27. Photoirradiation of the benzophenone group.

Although aryl azides **80** are frequently used as photo-cross-linkers due to their straightforward synthesis and chemical stability, their photoaffinity yields are often less

than 30%.²²² Unlike aryl diazirines **66** and benzophenones **74**, aryl azides **80** require irradiation at shorter wavelengths typically lower than 280 nm, which can damage biological molecules.^{220, 222} Additionally, their activation can form highly reactive intermediates that label proteins non-specifically.^{220, 222} Irradiation initially generates a nitrene **81** (Scheme 28), which can insert into C-H or N-H protein bonds to form an amine **82**. However, the nitrene **81** can also rearrange to a benzazirine intermediate **83**, and subsequently, a dehydroazepin **84**, which is prone to nucleophilic attack by endogenous thiolates.²²²



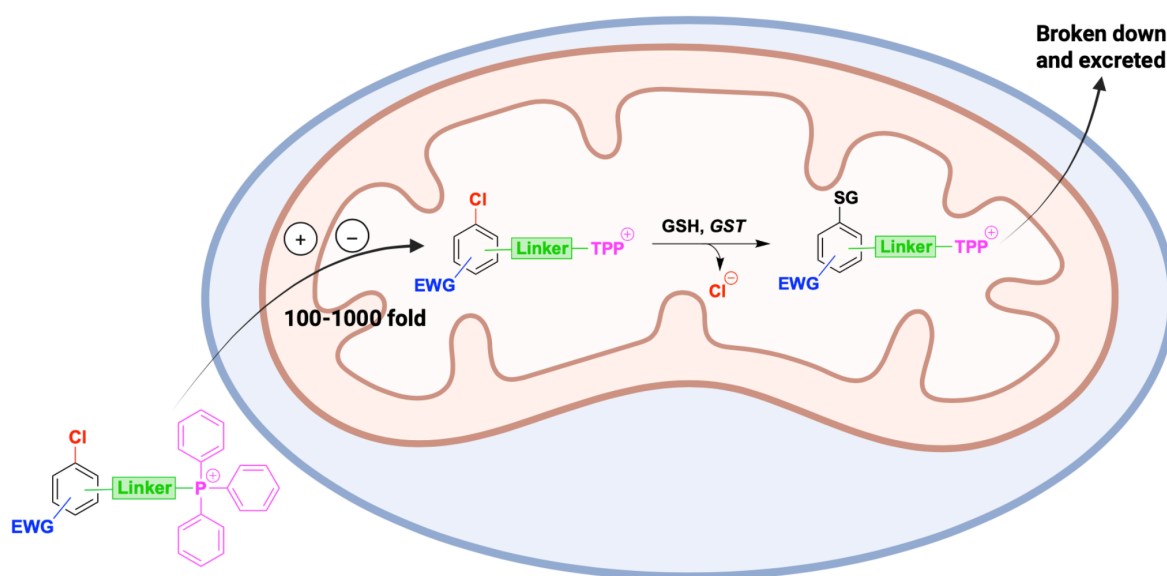
Scheme 28. Photoirradiation pathways of the aryl azide group.

1.6 Research objectives

1.6.1 Characterisation of mitochondrial GSTs

As discussed in Section 1.2, mGSH and mGST enzymes play crucial roles in antioxidant defence, supporting cell survival and function in both normal and diseased tissues. Many cancers exhibit elevated levels of GSH **25** and overexpression of GST enzymes. The mammalian mitochondrial matrix contains several antioxidant GST isoforms, including GSTK1-1, GSTA1-1 and GSTA4-4, each with distinct substrate specificities. Notably, the mitochondrial localisation of GSTA1-1 and GSTA4-4 increases under oxidative stress, a hallmark of cancer cells. Using GST isoforms overexpressed in the mitochondria of cancer cells could potentially be exploited to selectively deplete mGSH in cancer cells by mGST-catalysed reaction with mitochondria-targeted electrophiles. Depleting mGSH could

enhance oxidative damage and promote cancer cell death. Cancer cells often have higher levels of ROS and poor repair mechanisms, so they are more susceptible to additional oxidative stress. Moreover, mitochondria are the main source of ROS, which makes them an attractive target for cancer therapy. Chapter 2 focuses on the characterisation of these GST isoforms by determining their kinetic parameters for the common substrates CDNB **48** and GSH **25**.

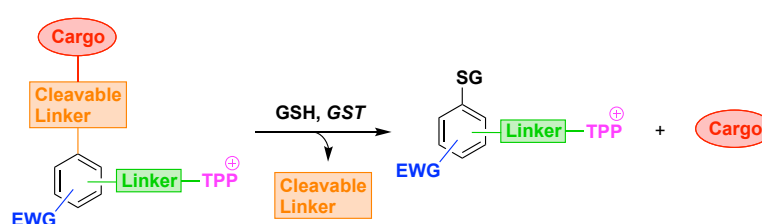


Scheme 29. General design of chloronitrobenzene derivatives and proposed reaction with mGSH catalysed by mGSTs to form the corresponding GS-conjugate.

Additionally, a series of electrophilic chloronitrobenzene derivatives would be designed based on the structures of CDNB **48** and MitoCDNB **51**, differing by their electronic properties and the position of a mitochondria-targeting TPP group. The GST-catalysed GSH-conjugation kinetics of these compounds would be assessed for each GST isoform to identify effective substrates. Among these, the mitochondria-targeted derivatives would be expected to accumulate within the mitochondrial matrix and deplete mGSH by mGST catalysis (Scheme 29).

1.6.2 Mitochondria-targeted GST-activated prodrug strategy

Dr Rebecca Taig, a former PhD student in the Hartley group, focused on developing mitochondria-targeted GST-activated prodrugs based on MitoCDNB **51** to selectively deplete mGSH and deliver different cargos.²²⁴ The combined effects of mGSH depletion and targeted delivery of a mitochondria-damaging anti-cancer agent to cancer cells should induce irreversible oxidative damage and cell death. Instead of a chloride substituent, the prodrug targets incorporated a cleavable linker to attach the cargo to the electron-poor aromatic ring. Like the chloronitrobenzene derivatives (Scheme 29), the prodrugs would be expected to accumulate within the mitochondrial matrix and undergo mGST-catalysed conjugation with mGSH (Scheme 30). The linker was anticipated to cleave, releasing the cargo while forming the corresponding GS-conjugate. However, several of Dr Taig's prodrug targets could not be synthesised due to synthetic limitations.²²⁴ Building on her research, Chapter 3 explores the development of mitochondria-targeted GST-activated prodrugs based not only on MitoCDNB **51** but also on other electron-poor aromatic scaffolds identified in Chapter 2. Various cleavable linkers were investigated to release different cargos. Metformin **58** was chosen as the primary cargo of interest for its widely-reported anti-cancer properties, including anti-proliferative and apoptotic effects through inhibition of mitochondrial complex I and disruption of energy metabolism (see Section 1.5).

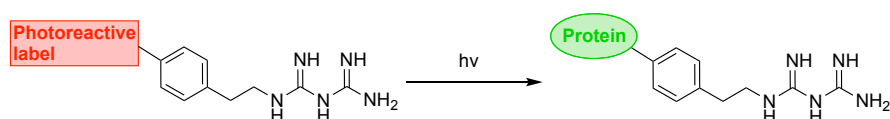


Scheme 30. General prodrug design and proposed reaction with mGSH catalysed by mGSTs to form the cargo and corresponding GS-conjugate.

1.6.3 Development of photoaffinity probes of phenformin

As discussed in Section 1.5, the anti-diabetic biguanides metformin **58** and phenformin **59** show therapeutic potential for cancer treatment. In addition to inhibiting

mitochondrial complex I at multiple sites, they are reported to target several other proteins. However, the mechanisms of action underlying their anti-cancer effects remain widely debated and incompletely characterised. As explained in Section 1.4, photoaffinity labelling (PAL) is a powerful tool for probing biological processes and identifying protein targets of small molecules. The use of PAL in studying metformin **58** has been demonstrated with Met-P1 **73**, the only reported photoaffinity probe for metformin to date.¹⁹⁵ However, metformin **58** has a small chemical structure which means incorporation of a photoreactive label and click handle as in Met-P1 **73** poses a risk of altering its pharmacological properties. Compared to metformin **58**, phenformin **59** is a more potent complex I inhibitor and exhibits greater anti-cancer activity in various experimental models. Although it carries a higher risk of lactic acidosis, it is still a promising candidate for cancer therapy. Phenformin's **59** larger, more lipophilic structure makes it more amenable to chemical modification with a lower likelihood of altering its pharmacological activity. However, no photoaffinity probes of phenformin have been reported to date. Chapter 3 focuses on the design of phenformin-based photoaffinity probes which explore different photo-cross-linkers. At this stage, no tag or label would be used for identification or purification to minimise modification of the structure (Scheme 31). Theoretically, the probe-protein conjugates could be distinguishable from the rest of the proteome using proteomics. Alternatively, radiolabelled derivatives might be used since tritium adds no bulk.



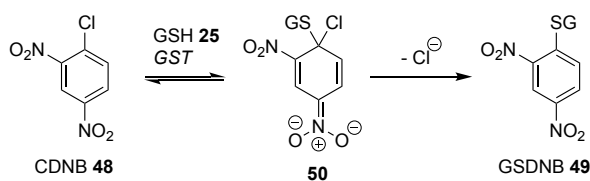
Scheme 31. General design of photoaffinity probe of phenformin which upon photoactivation should cross-link to a nearby protein.

Chapter 2 Preparation of chloronitrobenzene derivatives to study GST substrate specificities

2.1 Approach

As discussed in Section 1.2.2, mammalian mitochondria contain a diverse pool of GST enzymes, including GSTs K1-1, A1-1 and A4-4. In particular, mitochondrial translocation of GSTs A1-1 and A4-4 from the cytosol is reported to increase during oxidative stress, which is a hallmark of cancer cells.^{139, 141} Hence, the different substrate specificity of these mGSTs presents an opportunity to selectively deplete mitochondrial glutathione (mGSH) in cancer cells. Disruption of the mGSH pool in cancers cells should induce irreversible oxidative damage, and consequently, cell death.

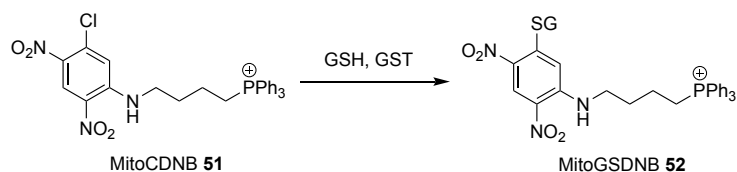
The first objective of the research focused on the expression of the human GST isoforms A1-1, A4-4 and K1-1 in *Escherichia coli* (*E.coli*). The activity of the enzymes for the GSH-conjugation reaction with the common substrate CDNB **48** (Scheme 32) would be determined by the calculation of key kinetic parameters. As discussed in Section 1.2.5, the reaction between CDNB **48** and GSH **25** proceeds via formation of the Meisenheimer intermediate **50** as part of the classical S_NAr mechanism. The Meisenheimer intermediate **50** then collapses to form a GS-dinitrophenyl conjugate, GSDNB **49**.



Scheme 32. Mechanism for GST-catalysed nucleophilic aromatic substitution (S_NAr) of CDNB **48** and GSH **25**.

The next objective would be to investigate the substrate specificity of the GST enzymes for untargeted and mitochondria-targeted CDNB analogues. Previously developed by the Hartley group, MitoCDNB **51** is the mitochondria-targeted analogue of CDNB **48** (Scheme

33), which selectively depletes mGSH through formation of MitoGSDNB **52** catalysed by mGSTs (see Section 1.2.5).¹⁷⁸



Scheme 33. GST-catalysed nucleophilic aromatic substitution (S_NAr) of MitoCDNB **51** and GSH **25** to form MitoGSDNB **52**.

In order to support the design of products activated by GSTs (see Chapter 3), there was an interest to determine how changing the position of the TPP group and varying the electronic properties of the aromatic ring would influence the rate of GST-catalysed GSH **25** conjugation for each of the enzymes. Therefore, a series of chloronitrobenzene derivatives **85a-c** (Figure 21) and **86a-c** (Figure 22) based on the structures of CDNB **48** and MitoCDNB **51** were devised. In the absence of enzyme, all the compounds were expected to react with GSH **25** via the classical S_NAr mechanism (Scheme 32). Electrophiles, containing electron-withdrawing groups, such as nitro groups, at the *ortho* and *para* positions on the aromatic ring, favour the S_NAr reaction. One nitro group in CDNB **48** would be replaced with an electron-withdrawing methyl ester group (as in **85a-c**, Figure 21). Similarly, one nitro group in MitoCDNB **51** would be replaced with an electron-withdrawing amide group through which the TPP cation was attached (as in **86a-c**, Figure 22). The position of substituents on the aromatic ring would be varied to study the effects on the reactivity in the uncatalysed and GST-catalysed S_NAr reactions and the substitution patterns are denoted as **a**, **b** and **c**. In substitution pattern **a**, the nitro group is *ortho* and the ester or amide group is *para* relative to the chloride. In substitution pattern **b**, the nitro group is *para* and the ester or amide group is *ortho* relative to the chloride. In substitution pattern **c**, the nitro group is *para* and the ester or amide group is *meta* relative to the chloride. Ultimately, the ester analogues **85a-c** would assess intrinsic reactivity, while the amide analogues **86a-c** would be used to determine whether the enzyme active site could accommodate the linker to the TPP targeting group.

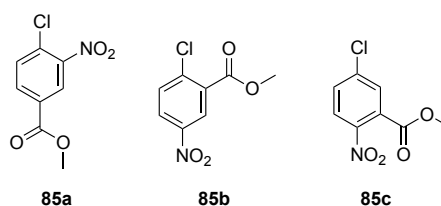


Figure 21. Structure of chloronitrobenzene ester analogues **85a-c**.

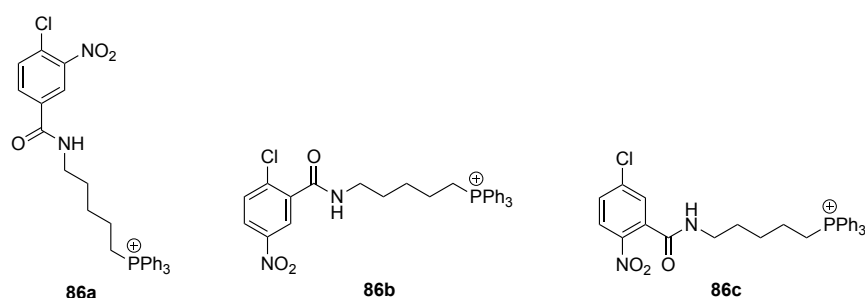


Figure 22. Structure of mitochondria-targeted chloronitrobenzene amide analogues **86a-c**.

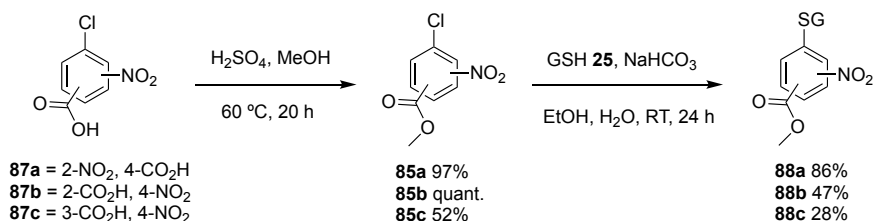
If any of the mitochondria-targeted compounds **86a-c** proved to be effective substrates, the substitution pattern could potentially be used in anti-cancer prodrugs. Challenges in synthesising sulfonamide prodrugs based on MitoCDNB **51** were previously encountered by Dr Taig.²²⁴ These issues likely arose from the highly electron-poor ring system of MitoCDNB **51**. Therefore, novel derivatives with modified reactivity may be necessary for prodrug development.

2.2 Synthesis

2.2.1 Synthesis of chloronitrobenzene esters and their GS conjugates

The ester analogues **85a-c** were synthesised (Scheme 34) in yields ranging from moderate to quantitative through acidic esterification of their respective commercially available benzoic acids **87a-c**. To perform kinetic measurements of the GST-catalysed GSH **25** conjugation of ester analogues **85a-c** by UV-Vis absorbance, their corresponding GS-conjugates **88a-c** also needed to be synthesised as standards. The ester analogues **85a-c**

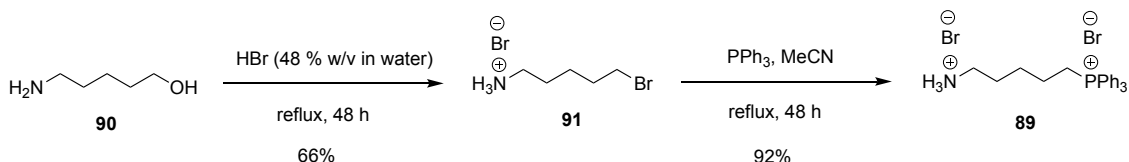
were reacted with GSH **25** in a S_NAr reaction to obtain the corresponding GS-conjugates **88a-c** in yields ranging from low to excellent.



Scheme 34. Synthesis of chloronitrobenzene esters **85a-c** and their corresponding GS-conjugates **88a-c**.

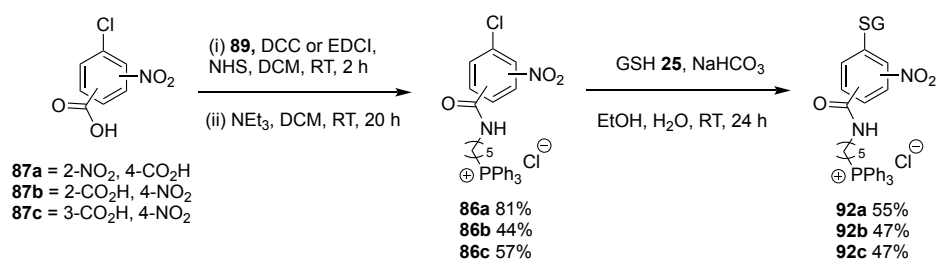
2.2.2 Synthesis of chloronitrobenzene amides and their GS conjugates

The synthesis of the amide analogues **86a-c** began with the preparation of the TPP linker **89** (Scheme 35). Commercially available 5-aminopentan-1-ol **90** was reacted with HBr in a nucleophilic substitution to give the bromide **91** in a moderate yield. Subsequent nucleophilic substitution of bromide **91** with triphenylphosphine afforded the TPP linker **89** in excellent yield.



Scheme 35. Synthesis of TPP-linker **89**.

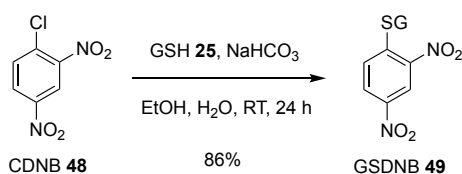
The benzoic acids **87a-c** were then treated with the TPP linker **89** in an amide coupling reaction with either DCC or EDCI as the coupling agent to afford the amide analogues **86a-c** in moderate to good yields (Scheme 36). Finally, the amide analogues **86a-c** were reacted with GSH **25** in a S_NAr reaction to obtain the corresponding GS-conjugates **92a-c** standards in moderate yields.



Scheme 36. Synthesis of mitochondria-targeted chloronitrobenzene amides **86a-c** and their corresponding GS-conjugates **92a-c**.

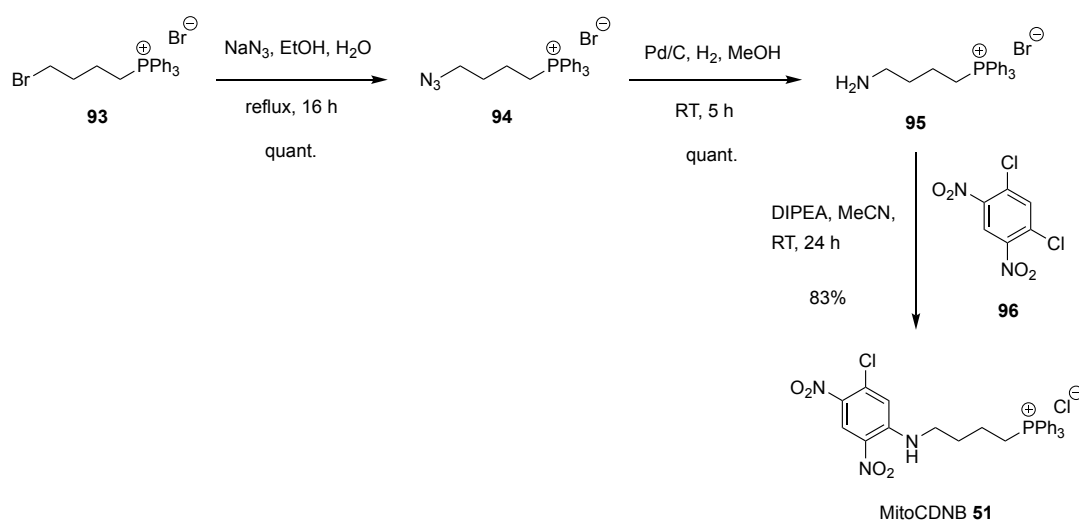
2.2.3 Synthesis of GSDNB and MitoCDNB

To measure the kinetics of the GST-catalysed GSH **25** conjugation of CDNB **48** by UV-Vis absorbance, GSDNB **49** was synthesised for use as standard in excellent yield by a S_NAr reaction between CDNB **48** and GSH **25** (Scheme 37).



Scheme 37. Synthesis of GSDNB **49** from CDNB **48** and GSH **25**.

MitoCDNB **51** was synthesised following the procedure by Booty *et al* (Scheme 38).¹⁷⁸ Commercially available bromide **93** was reacted with sodium azide to produce azide **94** in quantitative yield. Hydrogenation of azide **94** afforded amine **95**, also in quantitative yield. Finally, a S_NAr reaction between amine **95** and 1,3-dichloro-2,4-dinitrobenzene **96** afforded MitoCDNB **51** in good yield. A batch of the corresponding GS-conjugate, MitoGSDNB **52**, was previously prepared by Dr Stuart Caldwell in the Hartley group and was used for the kinetic studies.



Scheme 38. Synthesis of MitoCDNB 51 following the procedure of Booty *et al.*¹⁷⁸

2.3 Expression and purification of GST enzymes

Commercially available DNA sequences encoding hGSTK1-1, hGSTA1-1 and hGSTA4-4 were purchased and cloned into an expression vector pNIC-28-Bsal. This vector introduces a 6 × histidine-tag and a tobacco etch virus (TEV) protease recognition sequence at the N-terminus of each protein. The polyhistidine tag would enable the expressed proteins to be purified by nickel affinity chromatography. Following purification, the TEV sequence could be cleaved by TEV protease to remove the polyhistidine tag. The genes were expressed in *Escherichia coli* by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were lysed by sonication and the proteins were purified by nickel affinity chromatography. Overall, the GST enzymes were obtained in high yields of 28 mg for GSTK1-1, 59 mg for GSTA1-1, and 42 mg for GSTA4-4, from 0.6 L cultures. The purity of the proteins was verified by SDS-PAGE analysis (Figure 23). The polyhistidine tag was not cleaved from the proteins after determining that it did not interfere with enzyme activity (see section 2.4).

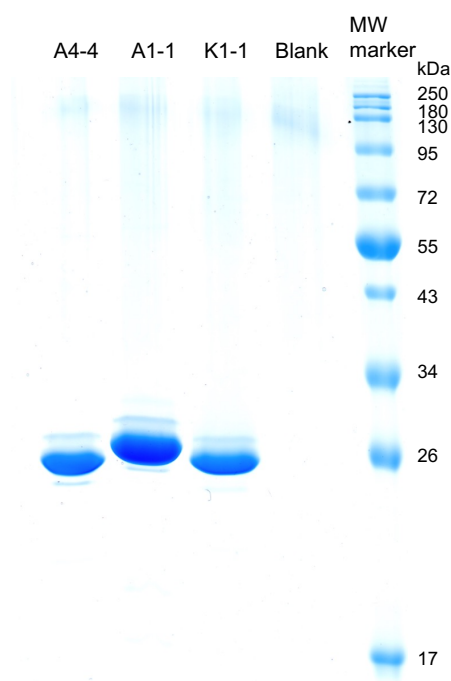


Figure 23. SDS-PAGE of purified hGSTs K1-1, A1-1 and A4-4. MW marker = Molecular weight marker. The molecular weights for hGSTA1-1, hGSTA4-4 and hGSTK1-1 are 28184.80, 28256.83 and 28049.58 Da, respectively. See Section 5.2.16 for experimental details.

2.4 Kinetics

2.4.1 Characterisation of GST enzymes for CDNB and GSH

The Michaelis-Menten kinetic parameters for the GST-catalysed reaction between CDNB **48** and GSH **25** were determined by UV-Vis absorbance assays, which is the standard protocol in the literature. These assays are typically carried out at pH 6.5 because the background rate with GSH **25** is negligible, despite most GST enzymes exhibiting optimum activity above pH 7.0. The kinetics of hGSTA1-1 and hGSTA4-4 were measured at pH 6.5 to compare the calculated kinetic parameters with those reported in the literature. By contrast, kinetic parameters for hGSTK1-1 had only been previously reported at pH 8.0. Furthermore, the kinetics of all three enzymes were also measured at pH 8.0, the approximate pH of the mitochondrial matrix. In the assays, the concentration of the substrate of interest was varied, while the concentration of the other substrate was fixed at a near-saturating concentration if possible. Initial rates of reaction were measured by monitoring the formation of GSDNB **49** at 340 nm where the GS-conjugate absorbs strongly

and CDNB **48** absorbs weakly (Figure 24). These rates, recorded as changes in absorbance per unit time, were converted to concentration per unit time using the Beer-Lambert Law and the difference in molar extinction coefficients between CDNB **48** and GSDNB **49**, $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$, which was determined by comparison of their UV-Vis absorbance spectra.

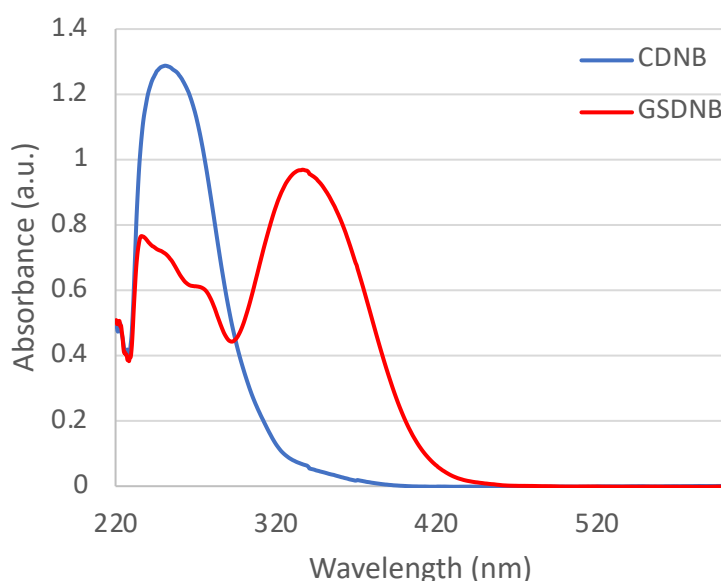


Figure 24. The UV-Vis absorbance spectra of CDNB **48** (100 μM) and GSDNB **49** (100 μM) at pH 8.0. CDNB **48**: $\epsilon_{340\text{nm}} = 0.578 \text{ mM}^{-1}\text{cm}^{-1}$. GSDNB **49**: $\epsilon_{340\text{nm}} = 9.60 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$.

The background rates were subtracted from the observed rates to obtain the enzyme-catalysed rates, and the data were fitted to the Michaelis-Menten equation to generate non-linear regression plots and Lineweaver-Burk plots. The non-linear regression plot and Lineweaver-Burk plot of hGSTA1-1 for GSH **25** at pH 6.5 are shown as examples in Figure 25 and Figure 26, respectively. All UV-Vis absorbance spectra and kinetics plots can be found in the Appendix.

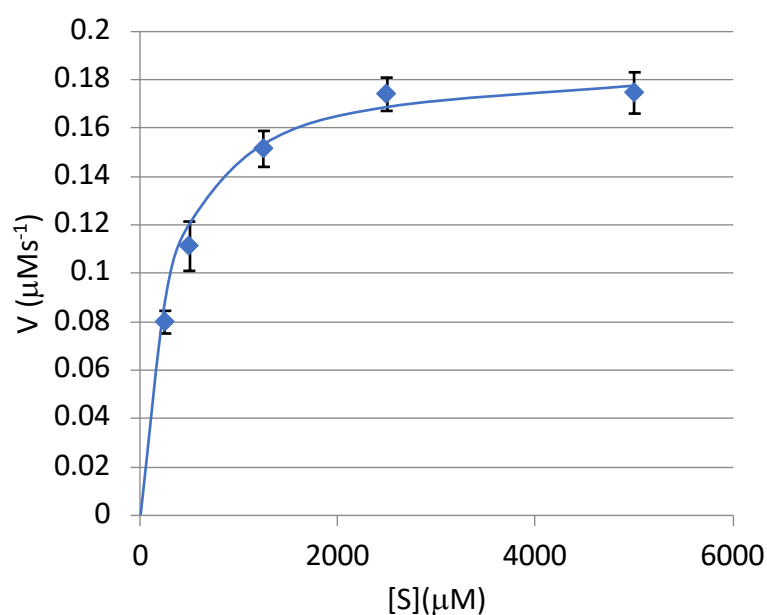


Figure 25. Non-linear plot of hGSTA1-1 for GSH 25 at pH 6.5, 30 °C. Data are expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Concentration of CDNB 48 = 1 mM. Concentration of enzyme = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

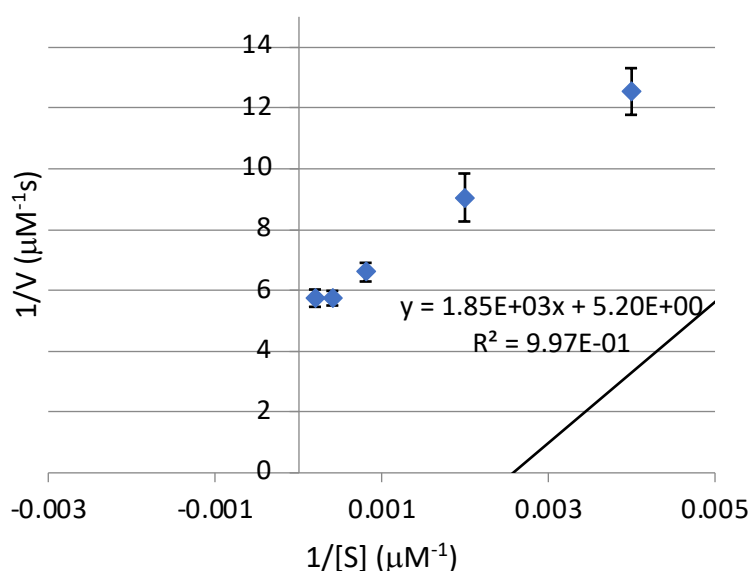


Figure 26. Lineweaver-Burk plot of hGSTA1-1 for GSH 25 at pH 6.5, 30 °C. Data are expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Concentration of CDNB 48 = 1 mM. Concentration of enzyme = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

From the plots, the catalytic constant (k_{cat}), the Michaelis-Menten constant (K_M), and the specificity constant (k_{cat}/K_M) were determined for each substrate when its concentration was varied and the other was fixed. The kinetic parameters of the GST enzymes for CDNB **48** and GSH **25**, presented in Table 1, were derived from the non-linear regression plots. As discussed in Section 1.3.3, non-linear regression methods rely on data that illustrates non-linear behaviour but are generally more interpretable and accurate than the Lineweaver-Burk plot for the determination of Michaelis-Menten parameters.

The GST enzymes were saturated with 10 mM GSH **25** in order to obtain accurate kinetic parameters for CDNB **48**. However, for the determination of kinetic parameters for GSH **25**, the enzymes were unable to be saturated with respect to CDNB **48** due to its poor water solubility. These assays used 1 mM of CDNB **48**, which was the maximum concentration that did not result in visible precipitation. Hence, the kinetic parameters calculated for GSH **25** are given as 'apparent' values. In the literature, comparable concentrations of CDNB **48** have been used for the calculation of kinetic parameters for GSH **25**.^{136, 225}

A higher value of k_{cat} indicates a greater rate of turnover of substrate to product. A lower value of K_M indicates better substrate binding with the enzyme. Consequently, the higher the ratio of k_{cat}/K_M , the more efficiently the enzyme converts substrate to product and the greater the preference of the enzyme for the substrate.

Substrate	pH	hGSTK1-1			hGSTA1-1			hGSTA4-4		
		k_{cat} (s ⁻¹)	K_{M} (mM)	$k_{\text{cat}} / K_{\text{M}}$ (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_{M} (mM)	$k_{\text{cat}} / K_{\text{M}}$ (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_{M} (mM)	$k_{\text{cat}} / K_{\text{M}}$ (mM ⁻¹ s ⁻¹)
CDNB 48	6.5	NM	NM	NM	35.4 ± 0.9	0.492 ± 0.036	71.9 ± 3.7	14.9 ± 2.4	5.20 ± 1.1	2.93 ± 0.15
	8.0	14.6 ± 1.5	1.90 ± 0.32	7.67 ± 0.53	45.3 ± 1.7	0.128 ± 0.018	353 ± 38	4.94 ± 0.36	0.253 ± 0.052	19.5 ± 2.7
GSH* 25	6.5	NM	NM	NM	36.1 ± 1.0	0.276 ± 0.034	130 ± 13	1.80 ± 0.05	1.14 ± 0.99	1.59 ± 0.11
	8.0	4.83 ± 0.07	0.652 ± 0.048	7.41 ± 0.48	49.1 ± 0.9	0.233 ± 0.024	211 ± 19	6.10 ± 0.23	0.580 ± 0.85	10.5 ± 1.2

Table 1. Kinetic constants of human GST enzymes for CDBN 48 and GSH 25 at pH 6.5 and 8.0, at 30°C. When measuring kinetics for CDBN 48, for GSTs A1-1, A4-4, and K1-1, the saturating substrate, GSH 25 was fixed at 10 mM. When measuring kinetics for GSH 25, the saturating substrate, CDBN 48 was fixed at 1 mM. Background reaction initial rates were subtracted from the enzyme-catalysed reaction initial rates prior to calculation of the kinetic constants. Kinetic constants were calculated based on molecular weights of 28184.80, 28256.83 and 28049.58 Da and stock concentrations of 2.91, 2.11 and 1.42 mg mL⁻¹ for GSTs A1-1, A4-4 and K1-1, respectively. *Apparent kinetic constants were calculated for GSH 25 because the enzyme could not be saturated with CDBN 48 due to its low aqueous solubility: $k_{\text{cat}}^{\text{app}}$, $K_{\text{M}}^{\text{app}}$ and $(k_{\text{cat}}/K_{\text{M}})^{\text{app}}$. NM = Not measured.

All three GST enzymes exhibited higher activity for CDNB **48** and GSH **25** at pH 8.0 compared to pH 6.5, but they showed strikingly different activities for the two substrates. For both CDNB **48** and GSH **25**, the enzyme activity followed the order: hGSTA1-1 > hGSTA4-4 > hGSTK1-1. The kinetic parameters of hGSTA1-1 at pH 6.5 are comparable to those reported by Zhao *et al.*²²⁶ The $k_{\text{cat}}/K_{\text{M}}$ of hGSTA1-1 for CDNB **48** at pH 8.0 is 5-fold greater than at pH 6.5. The kinetic parameters of hGSTA4-4 for CDNB **48** are broadly consistent with those of Hubatsch *et al.*,²²⁷ although they do not specify the pH of their measurements. The $k_{\text{cat}}/K_{\text{M}}$ of hGSTA4-4 for CDNB **48** at pH 8.0 is 6.6-fold greater than at pH 6.5. hGSTA1-1 exhibited a significantly greater $k_{\text{cat}}/K_{\text{M}}$ for CDNB **48** in comparison to hGSTA4-4, which is unsurprising given their distinct substrate specificities described in the literature. Although the two enzymes share 76% sequence similarity and 54% sequence identity, hGSTA1-1 exhibits significant catalytic activity towards electrophilic substrates with diverse structures, while hGSTA4-4 is distinguished by its specificity for alkenals, such as 4-HNE **21**, a toxic end-product of lipid peroxidation.²²⁸⁻²³² The substrate promiscuity of hGSTA1-1 is evident in the kinetic study by Zhao *et al.*,²²⁶ who determined that the $k_{\text{cat}}/K_{\text{M}}$ of hGSTA1-1 was 2.5-fold greater for CDNB **48** compared to 4-HNE **21**. In contrast, Hubatsch *et al.*²²⁷ determined that the $k_{\text{cat}}/K_{\text{M}}$ of hGSTA4-4 was 392-fold greater for 4-HNE **21** compared to CDNB **48**, highlighting the enzyme's substrate preferences. This difference in substrate specificity has been further explored in several site directed mutagenesis and structural studies. The H-site of hGSTA1-1 and hGSTA4-4 is formed from three regions of the protein (Figure 27): the B1- α 1 loop (residues 9-16), the C-terminal part of the α 4 helix (residues 104-111), and the α 9 helix at the C terminus (residues 210-220).²³³ The α 9 helix, in particular, has been found to play a crucial role in determining substrate specificity.²²⁸⁻²³¹ The H-site of GSTA1-1 has a highly flexible α 9 helix, enabling accommodation of a wide variety of substrates.²³³ In addition, molecular dynamics simulations and probes of the two enzymes have highlighted that GSTA1-1 exhibits greater solvent accessibility throughout its sequence and greater conformation heterogeneity among the key residues at its active site compared to GSTA4-4.²²⁸ To explore changes in substrate specificity, Blikstad *et al.*²³⁰ designed a mutant of hGSTA1-1, GIMFhelix, incorporating hGSTA4-4 residues 12, 107, 108, and 111, along with its C-terminus (residues 208-222), into the structure of hGSTA1-1. Despite retaining 94% sequence identity with hGSTA1-1, the mutant displayed significant substrate specificity for alkenals, akin to hGSTA4-4, including a >300-fold increase in activity for nonenal and a >10 fold decreased activity for CDNB **48**.²³⁰

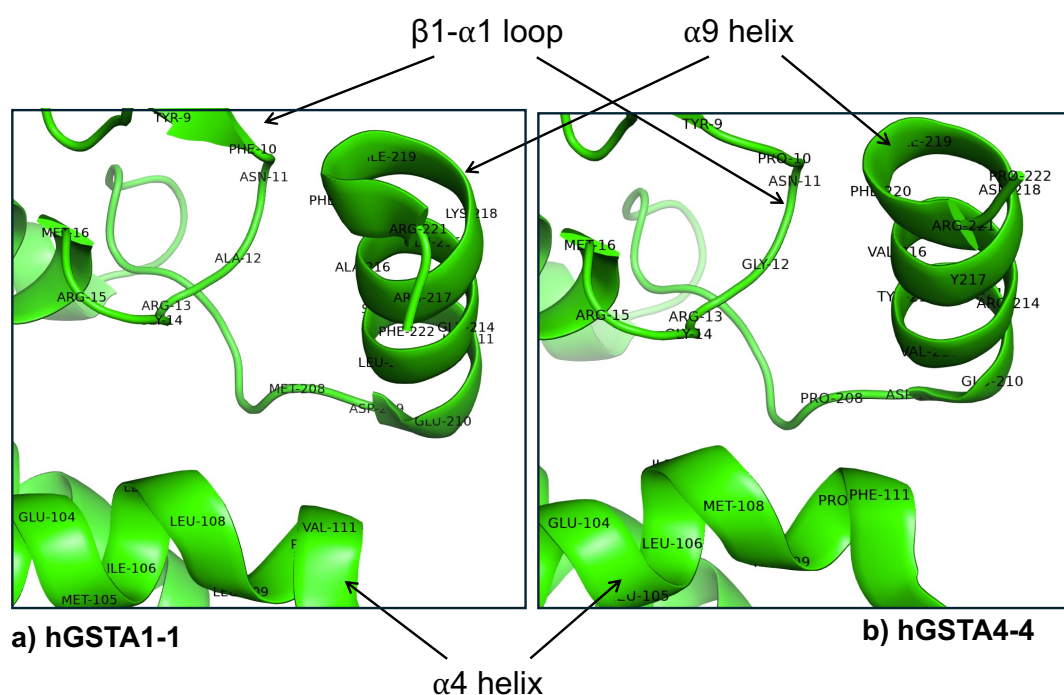


Figure 27. Differences in the H-site of a) hGSTA1-1 and b) hGSTA4-4, formed from the B1- α 1 loop (residues 9-16), the α 4 helix (residues 104-111) and the α 9 helix (residues 210-220).

Hyperbolic Michaelis-Menten character was observed for hGSTK1-1 for both CDNB **48** and GSH **25**, consistent with Wang *et al.*,¹³¹ but in contrast to Robinson *et al.*,¹³⁶ who reported hyperbolic Michaelis-Menten character for GSH **25** and sigmoidal character for CDNB **48**. The kinetic parameters of hGSTK1-1 determined at pH 8.0 are similar to those reported by Wang *et al.*,¹³¹ although their kinetic measurements were performed at pH 7.4. Nevertheless, these results indicate that hGSTK1-1 has moderate affinity for GSH **25**, but low affinity for CDNB **48**, which suggests that hGSTK1-1 may exhibit better GSH-conjugating activity for other substrates. By comparison, Robinson *et al.*¹³⁶ reported low affinities for both CDNB **48** and GSH **25**, providing kinetic parameters for the reaction at pH 8.0. They also carried out a pH dependence study, demonstrating that hGSTK1-1 exhibited optimum activity at approximately pH 9.0.¹³⁶

Interestingly, murine and rat GSTK1-1 show a significantly higher activity for CDNB **48** than the human isoform as well as many other human GSTs.²³⁴ This difference in activity could potentially be explained by comparison of their amino acid sequences. Murine and rat GSTK1-1 share 86% sequence identity,²³⁴ but only a 71% and 69% sequence identity, respectively, with hGSTK1-1.¹³⁷ Structural studies have revealed that the G-site of

hGSTK1-1 utilises many of the same key amino acids for binding to GSH **25** as rat and murine GSTK1-1, as well as human cytosolic GSTs.^{131, 136} By contrast, the amino acids involved in the H-site of hGSTK1-1 are less conserved compared to the rodent isoforms and are strikingly different compared to the cytosolic GSTs.^{131, 136} Additionally, a crystal structure study by Wang *et al.*¹³¹ demonstrated that hGSTK1-1 exhibits unique conformational changes upon substrate binding. Unlike cytosolic GSTs, the G- and H-sites of hGSTK1-1 are connected via a shared structural element, the $\alpha 2$ - $\alpha 3$ loop.¹³¹ Therefore, a conformational change at one site upon substrate binding can induce a dramatic conformational change at the other site, which significantly governs the binding of the second substrate. In the apo form of hGSTK1-1, both the G- and H-sites are accessible to the solvent, enabling random binding of substrates and GSH **25** (Figure 28a).¹³¹ Upon binding of GSH analogues, the $\alpha 2$ - $\alpha 3$ and $\alpha 3$ - $\alpha 4$ loops become ordered, closing the H-site (Figure 28b).¹³¹ The $\alpha 3$ - $\alpha 4$ loop that forms part of the H-site still exhibits a degree of flexibility, which may enable binding of hydrophobic substrates.¹³¹ However, in the case of CDNB **48** and GSH **25**, the structural and kinetic data indicate that the G- and H-sites of hGSTK1-1 do not cooperate. Furthermore, hGSTK1-1 has been suggested to constitute a distinct family of the thioredoxin (Trx) fold protein superfamily. The structure of its active sites and its overall secondary structure are more comparable to the bacterial enzymes, 2-hydroxychromene-2-carboxylic acid (HCCA) isomerase and disulfide-bond-forming (DsbA) oxidoreductase, than with cytosolic GSTs.^{131, 136, 235} Whereas all cytosolic GSTs possess a helical domain at the C-terminus, which is external to the thioredoxin fold, the helical domain in hGSTK1-1, HCCA isomerase and DsbA is inserted within the thioredoxin fold.^{136, 235}

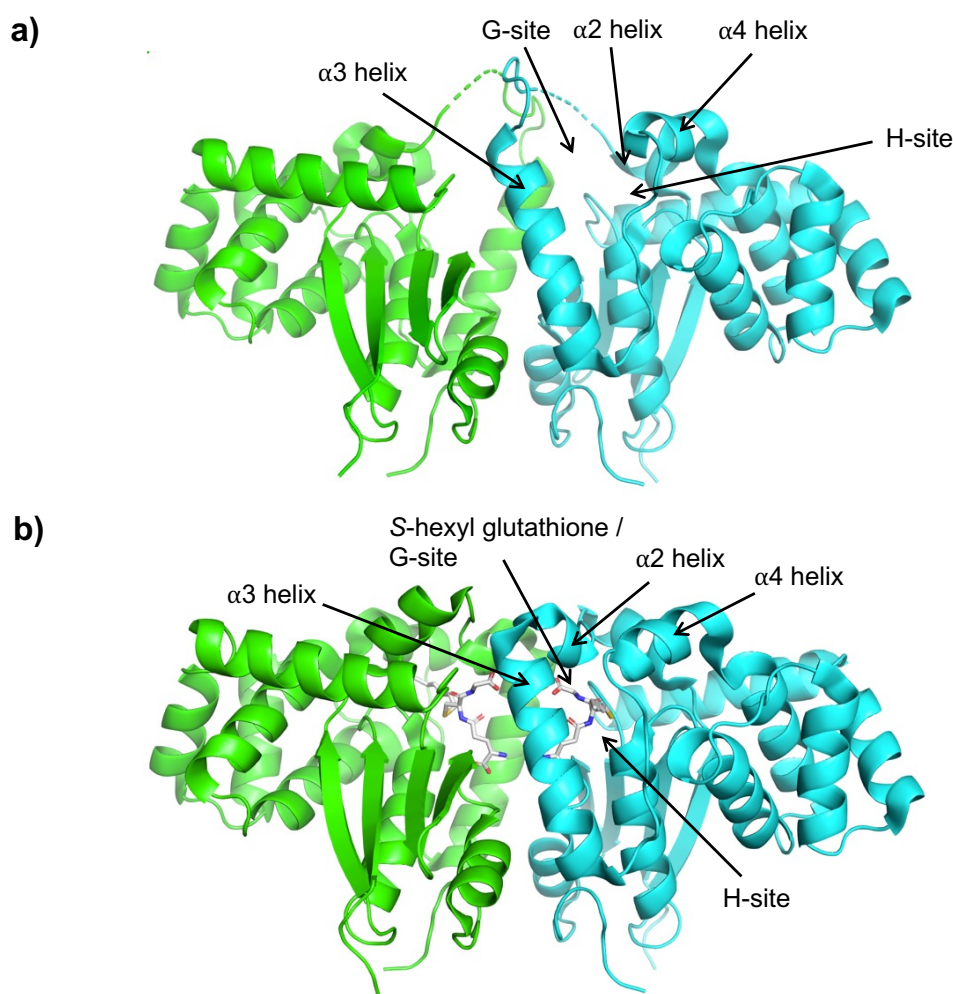


Figure 28. a) Homodimeric hGSTK1-1 in the apo form (PDB: 3RPP). The disordered $\alpha 2$ - $\alpha 3$ loop is shown by the dashed line. b) Homodimeric hGSTK1-1 in the closed form in complex with S-hexyl glutathione (PDB: 3RPN).

Overall, the kinetic studies provide a comparative assessment of the activity of three hGST enzymes likely involved in mitochondrial antioxidant defence. Extending the kinetic characterisation of these enzymes to pH 8.0, the approximate pH of the mitochondrial matrix, gives a more accurate indication of their role in detoxifying different electrophiles. The kinetic parameters of hGSTA1-1 for CDNB **48** align with literature values, confirming its high activity for electrophilic compounds that undergo S_NAr reactions. In contrast, hGSTA4-4 demonstrated significantly lower activity for CDNB **48**, consistent with its widely-reported high specificity for Michael-addition-type electrophiles, such as 4-HNE **21**. The kinetic data for hGSTK1-1 is largely in agreement

with prior studies and further highlights that it is a structurally distinct GST with a unique substrate specificity compared to cytosolic GSTs. These kinetics serve as a platform to investigate a range of CDNB-related compounds to explore the scope for specific GSH **25** depletion within the mitochondrial matrix.

2.4.2 Kinetics of chloronitrobenzene derivatives with GSH

Next, the effect of the different substituents in the chloronitrobenzene derivatives on the rate of the uncatalysed reaction with GSH **25** was investigated. Initial rates of reaction were measured for substrates **48**, **51**, **85a-c** and **86a-c** at varying concentrations in the presence of excess GSH **25** at pH 8.0 and 30 °C. As in Section 2.4.1, the concentration of GSH **25** was fixed at 10 mM, so that the pseudo-first-order reaction could be observed with respect to the substrates. For each chloronitrobenzene derivative, the reaction was monitored by measuring the increase of absorbance at or near the wavelength of maximum absorbance of the corresponding GS conjugate. At this wavelength, there was minimal overlap between the absorbance spectra of the derivative and GS conjugate. Second-order rate constants were calculated on the assumption that the reaction is second-order for all substrates (Table 2). This assumption was based on the well-established S_NAr mechanism of CDNB **48** and the observed pseudo-first-order kinetics for the reactions of the chloronitrobenzene derivatives at different concentrations. Background reactions were only detected for CDNB **48**, MitoCDNB **51**, and ester **85a**. Therefore, among compounds with a nitro group *ortho* to the chloro substituent, reactivity followed the order: *para*-nitro **48** > *para*-carboxylic ester **85a** > *para*-amide **86a**. The results contrast with those of Miller *et al.*,²³⁶ who found that amides, similar in structure to amide **86a**, were more favourable for S_NAr reaction than ester **85a** when reacted with sodium methoxide in methanol solvent. This is potentially a result of contrasting nucleophilicity; glutathione is a soft nucleophile, whereas methoxide is a hard nucleophile.

Compound	Substituents				Background rate constant k' ($\text{mM}^{-1}\text{s}^{-1}$)
	2-	3-	4-	5-	
CDNB 48	NO_2	H	NO_2	H	$(5.05 \pm 0.40) \times 10^{-5}$
MitoCDNB 51	NO_2	H	NO_2	$\text{NH}(\text{CH}_2)_4\text{TPP}$	$(1.50 \pm 0.18) \times 10^{-5}$
85a	NO_2	H	CO_2CH_3	H	$(2.92 \pm 0.10) \times 10^{-6}$
86a	NO_2	H	$\text{CONH}(\text{CH}_2)_5\text{TPP}$	H	ND
85b	CO_2CH_3	H	NO_2	H	ND
86b	$\text{CONH}(\text{CH}_2)_5\text{TPP}$	H	NO_2	H	ND
85c	H	CO_2CH_3	NO_2	H	ND
86c	H	$\text{CONH}(\text{CH}_2)_5\text{TPP}$	NO_2	H	ND

Table 2. Second-order background rate constants k' of the compounds for the reaction with GSH 25 at 10 mM at pH 8 at 30 °C. Data presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements of four rate constants determined at four different concentrations. ND = No reaction detected.

However, the reactivity trend of the chloronitrobenzene derivatives is consistent with the Hammett parameters of electron-withdrawing *para* substituents which primarily stabilise negative charge by conjugation: $\sigma_p = +0.78$ for a nitro group, $\sigma_p = +0.45$ for CO_2Me and $\sigma_p = +0.35$ for CONH_2 and CONMe_2 , respectively (Table 3).²³⁷ The trend is expected for a $\text{S}_{\text{N}}\text{Ar}$ reaction because both *ortho* and *para* electron-withdrawing groups stabilise the negative charge in the transition state of the rate-determining step and in the resulting Meisenheimer intermediate by direct conjugation. The background rate constant MitoCDNB **51** is >3-fold smaller than that of CDNB **48**, due to the electron-donating amino group at the *meta* position, which reduces electron-withdrawal to the nitro groups. However, despite the amino group, the two nitro groups in MitoCDNB **51** make it >5-fold more reactive than ester **85a**, which only has one nitro group. When the nitro and ester groups are switched, the resulting ester **85b** is unreactive. This is attributed to the nitro group's greater effect at the *ortho* position than at the *para* position because it combines resonance stabilisation with stronger electron-withdrawal by induction. The inductive effect of a nitro group is greater than that of the ester and amide groups as evident from the Hammett parameters for *meta* substituents where resonance is not possible: $\sigma_m = +0.71$ for a nitro group, $\sigma_m = +0.37$ for CO_2Me and $\sigma_m = +0.35$ for CONHMe .²³⁷ The lack of reactivity of compounds **85c** and **86c** can be explained by the presence of the *meta* carbonyl groups, which are weakly electron-withdrawing by induction and cannot exert a mesomeric effect. Although, GSH **25** exists primarily in its thiol form at pH 8, the reaction proceeds via the thiolate because it is by far the more nucleophilic species and approximately one tenth of the GSH **25** is in this deprotonated form (the thiol $\text{pK}_a = 8.94$ or 9.27 depending on whether the amino group is protonated or not).²³⁸ Any solvation effects on the rate of reaction of the chloronitrobenzene derivatives with GSH **25** are likely to be negligible. Solvation of the thiolate by coordination to the NH of the amide or activation of the thiol by coordination to the oxygen atom of the carbonyl or nitro group have no significant effect because water solvates both positive and negative charges well.²³⁹

Substituent	σ_m	σ_p
NO ₂	+0.71	+0.78
CO ₂ Me	+0.37	+0.45
CONH ₂	+0.28	+0.36
CONHMe	+0.35	+0.36

Table 3. Hammett Substitution Constants.²³⁷

2.4.3 Kinetics of chloronitrobenzene derivatives with GST enzymes

The reactivity of the chloronitrobenzene derivatives in the GST-catalysed reaction with each of the enzymes was investigated using the same conditions described in Section 2.4.2. To ensure pseudo-first-order kinetics with respect to the substrates, the enzymes were near-saturated with 10 mM of GSH **25**, which is >15-fold greater than the apparent K_M of each enzyme for GSH **25**. The formation of the GS-conjugates **49**, **52**, **88a-c** and **92a-c** was monitored in the same way as in Section 2.4.2. The background rates were subtracted from the observed reaction rates to obtain the enzyme-catalysed rates. For compounds where clear non-linear behaviour was observed for the range of concentrations measured, the Michaelis-Menten constants were derived from the nonlinear regression plot. For compounds where linear (or near-linear) behaviour was observed, the constants were derived from the Lineweaver-Burk plot. The kinetic constants, k_{cat}/K_M , k_{cat} and K_M , for the chloronitrobenzene derivatives are presented in Tables 4, 5 and 6, respectively, and the chosen plot is specified for each case.

Compound	Substituents				$k_{\text{cat}}/K_{\text{M}}$ ($\text{mM}^{-1}\text{s}^{-1}$)		
	2-	3-	4-	5-	hGSTA1-1	hGSTA4-4	hGSTK1-1
CDNB 48	NO ₂	H	NO ₂	H	353 ± 38 ^(a)	19.5 ± 2.7 ^(a)	7.67 ± 0.53 ^(a)
MitoCDNB 51	NO ₂	H	NO ₂	NH(CH ₂) ₄ TPP	101 ± 10 ^(a)	32.3 ± 1.5 ^(a)	0.287 ± 0.038 ^(a)
85a	NO ₂	H	CO ₂ CH ₃	H	4.32 ± 0.28 ^(b)	0.146 ± 0.003 ^(b)	0.351 ± 0.010 ^(b)
86a	NO ₂	H	CONH(CH ₂) ₅ TPP	H	21.6 ± 0.5 ^(b)	2.57 ± 0.02 ^(b)	ND
85b	CO ₂ CH ₃	H	NO ₂	H	1.38 ± 0.04 ^(b)	(5.91 ± 0.17) × 10 ^{-2(b)}	(6.13 ± 0.12) × 10 ^{-2(b)}
86b	CONH(CH ₂) ₅ TPP	H	NO ₂	H	(6.49 ± 0.16) × 10 ^{-2(b)}	ND	ND
85c	H	CO ₂ CH ₃	NO ₂	H	ND	ND	ND
86c	H	CONH(CH ₂) ₅ TPP	NO ₂	H	ND	ND	ND

Table 4. Catalytic efficiency $k_{\text{cat}}/K_{\text{M}}$ calculated for the compounds for the GST-catalysed reaction with GSH 25 at pH 8.0 and 30 °C. GSH 25 concentration was fixed at 10 mM. Data presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements. Where necessary, enzyme concentrations were varied to observe a reaction with a measurable initial rate and accounted for in any calculations. Kinetic parameters were calculated either by (a) non-linear curve fit to the Michaelis-Menten plot or (b) best line fit to the double reciprocal Lineweaver-Burk plot. All plots provided in the appendix. ND = No reaction detected.

Compound	Substituents				k_{cat} (s^{-1})		
	2-	3-	4-	5-	hGSTA1-1	hGSTA4-4	hGSTK1-1
CDNB 48	NO ₂	H	NO ₂	H	45.3 ± 1.7 ^(a)	4.94 ± 0.36 ^(a)	4.94 ± 0.36 ^(a)
MitoCDNB 51	NO ₂	H	NO ₂	NH(CH ₂) ₄ TPP	14.7 ± 0.6 ^(a)	4.59 ± 0.08 ^(a)	4.59 ± 0.08 ^(a)
85a	NO ₂	H	CO ₂ CH ₃	H	8.85 ± 2.79 ^(b)	0.521 ± 0.203 ^(b)	0.521 ± 0.203 ^(b)
86a	NO ₂	H	CONH(CH ₂) ₅ TPP	H	9.50 ± 0.45 ^(b)	2.13 ± 0.05 ^(b)	2.13 ± 0.05 ^(b)
85b	CO ₂ CH ₃	H	NO ₂	H	2.31 ± 0.55 ^(b)	(5.61 ± 0.37) × 10 ^{-2(b)}	(5.61 ± 0.37) × 10 ^{-2(b)}
86b	CONH(CH ₂) ₅ TPP	H	NO ₂	H	(4.67 ± 0.19) × 10 ^{-2(b)}	ND	ND
85c	H	CO ₂ CH ₃	NO ₂	H	ND	ND	ND
86c	H	CONH(CH ₂) ₅ TPP	NO ₂	H	ND	ND	ND

Table 5. Catalytic rate constant k_{cat} calculated for the compounds for the GST-catalysed reaction with GSH 25 at pH 8 and 30 °C. GSH 25 concentration was fixed at 10 mM. Data presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements. Where necessary, enzyme concentrations were varied to observe a reaction with a measurable initial rate and accounted for in any calculations. Kinetic parameters were calculated either by (a) non-linear curve fit to the Michaelis-Menten plot or (b) best line fit to the double reciprocal Lineweaver-Burk plot. All plots provided in the appendix. ND = No reaction detected.

Compound	Substituents				K _M (mM)		
	2-	3-	4-	5-	hGSTA1-1	hGSTA4-4	hGSTK1-1
CDNB 48	NO ₂	H	NO ₂	H	0.128 ± 0.018 ^(a)	0.253 ± 0.052 ^(a)	hGSTK1-1
MitoCDNB 51	NO ₂	H	NO ₂	NH(CH ₂) ₄ TPP	0.146 ± 0.020 ^(a)	0.142 ± 0.009 ^(a)	1.90 ± 0.32 ^(a)
85a	NO ₂	H	CO ₂ CH ₃	H	2.05 ± 0.77 ^(b)	3.58 ± 0.09 ^(b)	0.360 ± 0.067 ^(a)
86a	NO ₂	H	CONH(CH ₂) ₅ TPP	H	0.441 ± 0.010 ^(b)	0.826 ± 0.005 ^(b)	0.301 ± 0.008 ^(b)
85b	CO ₂ CH ₃	H	NO ₂	H	1.67 ± 0.05 ^(b)	0.949 ± 0.027 ^(b)	ND
86b	CONH(CH ₂) ₅ TPP	H	NO ₂	H	0.720 ± 0.017 ^(b)	ND	1.54 ± 0.03 ^(b)
85c	H	CO ₂ CH ₃	NO ₂	H	ND	ND	ND
86c	H	CONH(CH ₂) ₅ TPP	NO ₂	H	ND	ND	ND

Table 6. Michaelis Menten constant K_M calculated for the compounds for the GST-catalysed reaction with GSH 25 at pH 8 and 30 °C. GSH 25 concentration was fixed at 10 mM. Data presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements. Where necessary, enzyme concentrations were varied to observe a reaction with a measurable initial rate and accounted for in any calculations. Kinetic parameters were calculated either by (a) non-linear curve fit to the Michaelis-Menten plot or (b) best line fit to the double reciprocal Lineweaver-Burk plot. All plots provided in the appendix. ND = No reaction detected.

Several conclusions can be drawn from the kinetic data for compounds **48**, **51**, **85a-c** and **86a-c** with the different GST enzymes, particularly the $k_{\text{cat}}/K_{\text{M}}$ values, which give a good indication of enzyme specificity and substrate reactivity. Comparison of the $k_{\text{cat}}/K_{\text{M}}$ values for the three enzymes reveals that hGSTA1-1 is the most active for all the substrates, highlighting its promiscuity for electrophilic compounds that undergo $\text{S}_{\text{N}}\text{Ar}$ reactions. In contrast, the activities of hGSTA4-4 and hGSTK1-1 are significantly lower, and in most cases, comparable to one another, indicating their preference for other types of substrates. Overall, these results complement the findings from the kinetics of CDNB **48** (see Section 2.4.1).

In line with the uncatalysed reaction (see Section 2.4.2), compounds **48**, **51**, **85a** and **86a**, where the nitro group is *ortho* to the chloro substituent, are more reactive than derivatives **85b**, **85c**, **86b**, and **86c**, where the nitro group is *para*. This reflects the combination of strong inductive and mesomeric electron withdrawal by the *ortho* nitro group, which stabilises the negative charge in the Meisenheimer intermediate. Hydrogen bonding between the *ortho* nitro group and the thiolate is unlikely to contribute to the rate of reaction because it would have to compete with the specific interactions between the thiol of the GSH **25** and the GST enzyme, which lowers its pKa to 6 or 7.^{239,177}

MitoCDNB **51** is an excellent substrate for hGSTA1-1 and hGSTA4-4, as evident in the $k_{\text{cat}}/K_{\text{M}}$ values (Table 4). However, in the case of hGSTA1-1, CDNB **48** is 3.5-fold more reactive than MitoCDNB **51**, which appears to be the result of CDNB **48**'s greater electrophilicity. This is supported by the 3-fold higher k_{cat} for CDNB **48** (Table 5), which mirrors the 3.4-fold higher background rate (Table 2). Additionally, the minimal difference between the K_{M} values of the two substrates (Table 6) suggests that they exhibit similar binding affinity for the active site. In contrast, for hGSTA4-4, MitoCDNB **51** is a slightly better substrate than CDNB **48**, with a $k_{\text{cat}}/K_{\text{M}}$ value 1.7-fold higher (Table 3). Despite CDNB **48**'s greater electrophilicity, their k_{cat} values are surprisingly similar (Table 5). However, comparison of their K_{M} values (Table 6) indicates that MitoCDNB **51** binds more efficiently to the enzyme than CDNB **48**. The alkyl chain in MitoCDNB **51** may restrict unproductive binding conformations that are otherwise accessible to the smaller structure of CDNB **48**, thereby offsetting the effect of the latter's greater electrophilicity. Compared to ester **85a**, MitoCDNB **51** is 23-fold and 221-fold more reactive in the hGSTA1-1 and hGSTA4-4 catalysed reactions, respectively (Table 4). These large differences in reactivity are partly due to MitoCDNB **51**'s greater electrophilicity. The k_{cat} values for MitoCDNB **51** are 3-fold and 9-fold greater than those for ester **85a** for hGSTA1-1 and hGSTA4-4, respectively (Table 5), which roughly corresponds to the 5-fold greater

reactivity of MitoCDNB **51** in the background reaction (Table 2). However, the main contribution to MitoCDNB **51**'s superior reactivity for hGSTA1-1 and hGSTA4-4 is its greater binding affinity with the two enzymes. MitoCDNB **51** exhibits a 14-fold lower K_M for hGSTA1-1 and a 25-fold lower K_M for hGSTA4-4 compared to ester **85a** (Table 6). Clearly the alkyl chain is well accommodated by these enzymes when at the 5-carbon position, perhaps due to the hydrophobic effect. In contrast, the k_{cat}/K_M for the hGSTK1-1-catalysed reaction of MitoCDNB **51** is 28-fold slower than that of CDNB **48** and comparable to that of ester **85a** (Table 4), indicating that the alkyl chain is not well accommodated by hGSTK1-1. The significantly greater reactivity of MitoCDNB **51** with hGSTA1-1 and hGSTA4-4 compared to hGSTK1-1 suggests that it may be more effective at selectively depleting mGSH in cells under oxidative stress, where mitochondrial import of hGSTA1-1 and hGSTA4-4 is elevated, than in normal cells.

Interestingly, attachment of the TPP-alkyl chain via an amide at the *para* position results in moderate substrate reactivity in the hGSTA1-1 and hGSTA4-4 catalysed reactions. Amide **86a** exhibits k_{cat}/K_M values that are 5-fold and 18-fold higher than those of its analogue, ester **85a**, for hGSTA1-1 and hGSTA4-4, respectively (Table 4). Additionally, the k_{cat} values of amide **86a** for these enzymes are higher than those of ester **85a** (Table 5), contrary to expectations based on the uncatalysed reaction rates (Table 2) and the Hammett parameters, which indicate that ester **85a** is slightly more electrophilic than amide **86a**. Clearly the TPP chain at the *para* position is efficiently accommodated by the enzymes, as reflected by amide **86a**'s 4.6-fold and 4.3-fold higher K_M values, for hGSTA1-1 and hGSTA4-4, respectively (Table 6). However, the accommodation of the TPP chain at the *para* position does not compensate for the weaker electron withdrawal of a *para* amide compared to the *para* nitro group in CDNB **1**, which is significantly more reactive than amide **86a** for all three enzymes. Attachment of the TPP chain at the *ortho* position appears to confer no advantages, demonstrated by the fact that amide **86b** is less reactive ester **85b**. These results align with the Hammett parameters and the rates of S_NAr reactions of ester **85b** and amides similar to amide **86b** with sodium methoxide in methanol reported by Miller et al.²³⁶ Finally, ester **85c** and amide **86c** showed no detectable reaction with the GST enzymes, which indicates that the compounds were insufficiently electrophilic and that *meta* attachment of the TPP chain provided no benefit.

The specific interactions which make MitoCDNB **51** a particularly good substrate for hGSTA1-1 and hGSTA4-4 remain unclear. However, crystallographic studies on mu and pi class GSTs report that H-bonding interactions with key tyrosine residues stabilise the

Meisenheimer complex of CDNB **48**.²⁴⁰⁻²⁴² A comprehensive structural study of hGSTA1-1 by Gildenhuis *et al.*²⁴³ indicates that Arg-15, which is located at the interface of the G- and H-sites and conserved in many alpha class GSTs, plays a crucial role in reducing the pK_a of the thiol of GSH **25** and stabilising the Meisenheimer complex. In addition, tyrosine residues, particularly Tyr9, stabilise the Meisenheimer complex of CDNB analogues by hydrogen bonding to the *ortho* nitro group.²⁴³ This interaction could account for the high reactivity of MitoCDNB **4**, as well as the superior reactivity of ester **85a** and amide **86a** relative to their regioisomers. Unfortunately, there are no available crystal structures of hGSTA4-4 or hGSTK1-1 in complex with CDNB analogues. However, a docking study of hGSTK1-1 bound to GSDNB **49**, suggest that hydrophobic interactions between the CDNB moiety and several hydrophobic residues in the H-site are important in substrate binding.¹³¹

2.5 Conclusions

In summary, the kinetic measurements demonstrate that mitochondrial GST enzymes can bind to and catalyse the GSH **25** conjugation of several different electrophilic substrates. The hGSTA1-1 isoform was the most active enzyme towards the chloronitrobenzene derivatives, which supports findings in the literature that hGSTA1-1 is highly active for S_NAr-type electrophiles and can accommodate a variety of substrate structures. In contrast, the hGSTA4-4 enzyme exhibited lower activity for CDNB **48** and the chloronitrobenzene derivatives **85a-c** and **86a-c**, which compliments literature reports that it prefers Michael-addition-type substrates.²²⁹ The hGSTK1-1 enzyme showed the poorest activity for CDNB **48** and the chloronitrobenzene derivatives, which supports structural studies describing an active site that is strikingly different to cytosolic GST enzymes and more comparable to prokaryotic enzymes, such as HCCA isomerase.^{131, 136}

The kinetic measurements indicated that dinitro compounds CDNB **48** and MitoCDNB **51** were the most reactive substrates for the GST enzymes, primarily attributed to the high electrophilicity of their electron-poor dinitro aromatic systems. However, novel substrates **85a** and **86a**, in which the nitro was placed *ortho* and the ester or TPP-linked amide group was placed *para*, respectively, also exhibited moderate reactivity. When the TPP alkyl chain is attached in the *meta* position, as in MitoCDNB **51** (Figure 29), or in the *para* position, as in amide **86a**, it increases the reaction rate for hGSTA4-4. This suggests that the active site of hGSTA4-4 is flexible enough to accommodate an alkyl chain at

different points. MitoCDNB **51** and amide **86a** are good substrates for both hGSTA1-1 and hGSTA4-4, but GSH **25** conjugation by hGSTK1-1 is far less efficient.

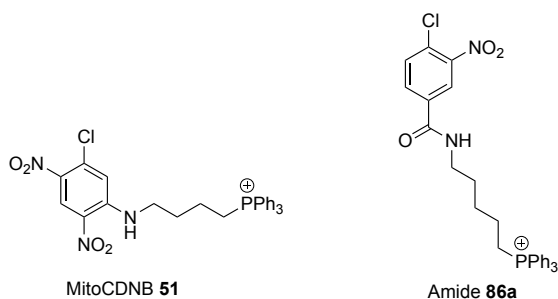


Figure 29. Lead compounds MitoCDNB **51** and Amide **86a** for selective mGSH depletion in cancer cells.

MitoCDNB **51** has been shown to selectively deplete mGSH with high efficiency via mGST catalysis.¹⁷⁸ The kinetic measurements indicate that MitoCDNB **51** may be particularly effective for mGSH depletion in cells under oxidative stress, during which hGSTA1-1 and hGSTA4-4 translocate to the mitochondria. Amide **86a**, which demonstrated moderate reactivity when catalysed by the alpha enzymes, but no reactivity with hGSTK1-1, may also serve as a selective mGSH depleter under these conditions. Hence, MitoCDNB **51** and amide **86a** are promising lead compounds for the selective depletion of mGSH in cancer cells, where the expression and distribution of GST isoforms often differs from healthy cells. There is also potential to develop these compounds into prodrugs that combine mGSH depletion with the release of an anti-cancer drug. The research proceeded in this direction, but before moving on to discuss these studies in Chapter 3, it is worth proposing some future work based on the discoveries described in this chapter.

2.6 Future work

The inactivation of mitochondrial thiol redox homeostasis by MitoCDNB **51** in cells and *in vivo* has already been validated,¹⁷⁸ but it would be valuable to carry out the same experiments on amide **86a** for comparison. Additionally, neither compound has been tested on *in vivo* cancer models to study their selectivity for the depletion of mGSH in

tumours. It would be interesting to observe the efficacy of the compounds against various tumour types overexpressing different GST enzymes. To complement our kinetic measurements, molecular docking calculations on the chloronitrobenzene derivatives with each GST enzyme would be beneficial. Furthermore, to achieve a more comprehensive understanding of substituent effects on substrate binding and reactivity with GST enzymes, both docking and kinetic studies should be performed on MitoCDNB analogues **97** and amide analogues **98** (Figure 30), in which one or both nitro groups are replaced with alternative electron-withdrawing substituents, such as cyano or trifluoromethyl groups. The Hammett parameters for these groups at the *para* position are as follows: $\sigma_p = +0.78$ for nitro, $\sigma_p = +0.66$ for cyano, $\sigma_p = +0.54$ for CF_3 , $\sigma_p = +0.45$ for CO_2Me and $\sigma_p = +0.36$ for CONHMe .²³⁷ Such computational and kinetic analyses could reveal key interactions that promote high substrate reactivity and provide valuable insights for the rational design of future mGSH-depleting compounds.

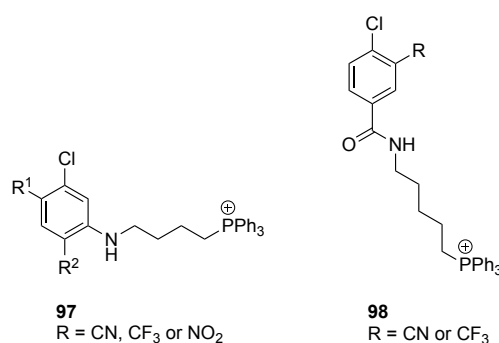


Figure 30. Proposed analogues **97** and **98** of MitoCDNB **51** and amide **86a**, respectively, in which a nitro group is replaced with a cyano or trifluoromethyl substituent.

Developing GST-active compounds that are not based on $\text{S}_{\text{N}}\text{Ar}$ -type substrates would also be valuable. It is well known that GSTA4-4 shows high activity towards Michael acceptor alkenals, such as HNE **21** and ethacrynic acid **41**.^{229, 234} Preparation of mitochondria-targeted analogues of these molecules represents one potential strategy to develop novel substrates for mGSH depletion which are specific to hGSTA4-4. The relative proportions of different GSTs in mammalian mitochondria are yet to be determined. Therefore, since hGSTK1-1 is mitochondria-specific, it remains the most compelling target for the discovery of novel substrates for GSH-conjugation. Future efforts should prioritise high throughput screening of electrophilic small molecules for hGSTK1-1.

Chapter 3 Preparation of GSH/GST-activated sulfonamides

3.1 Approaches

Mitochondria-targeted GSH/GST-activated prodrugs were designed based on the structures of CDNB **48**, MitoCDNB **51** and the TPP-conjugated chloronitrobenzene derivatives **86a-c** explored in Chapter 2. The objective of the prodrugs was to selectively deplete mGSH and release an active amine-bearing anti-cancer drug in cancer cells. Three different prodrug approaches, A, B and C, were designed (Figure 31). In each approach, the prodrug scaffold incorporated a TPP moiety to drive their accumulation into the mitochondrial matrix. Each prodrug should be activated by GSH **25**, with or without GST enzyme, to form the corresponding GS-conjugate, which is expected to be degraded and exported from the mitochondria in the same way as MitoGSDNB **5**.

Building on the research by Dr Taig,²²⁴ approaches A and B aimed to release an active cargo via a cleavable sulfonamide bond attached to an electron-poor aromatic ring. In approach A, the cargo is directly attached to the sulfonamide bond, which upon activation by GSH **25**, should generate sulfur dioxide and release the active cargo.

In approach B, the cargo is attached to the sulfonamide bond via an ethylenediamine self-immolative linker. Activation by GSH **25** is expected to generate sulfur dioxide and the amine intermediate which should undergo an intramolecular cyclisation to form a cyclic urea by-product, thereby releasing the active cargo. This approach has the advantage of separating the cargo from the site of S_NAr reaction so that it does not influence reactivity. Dr Taig had found that aryl arenesulfonate analogues of MitoCDNB were too reactive to be accessed synthetically,²²⁴ but this approach may overcome the problem and allow delivery of phenols. Approach C incorporates a mercaptopropyl sulfonyl self-immolative linker between the electron-poor aromatic ring and the cargo. Following activation by GSH, the thiolate intermediate should undergo an intramolecular cyclisation to form a cyclic thiosulfonate by-product and release the active cargo.

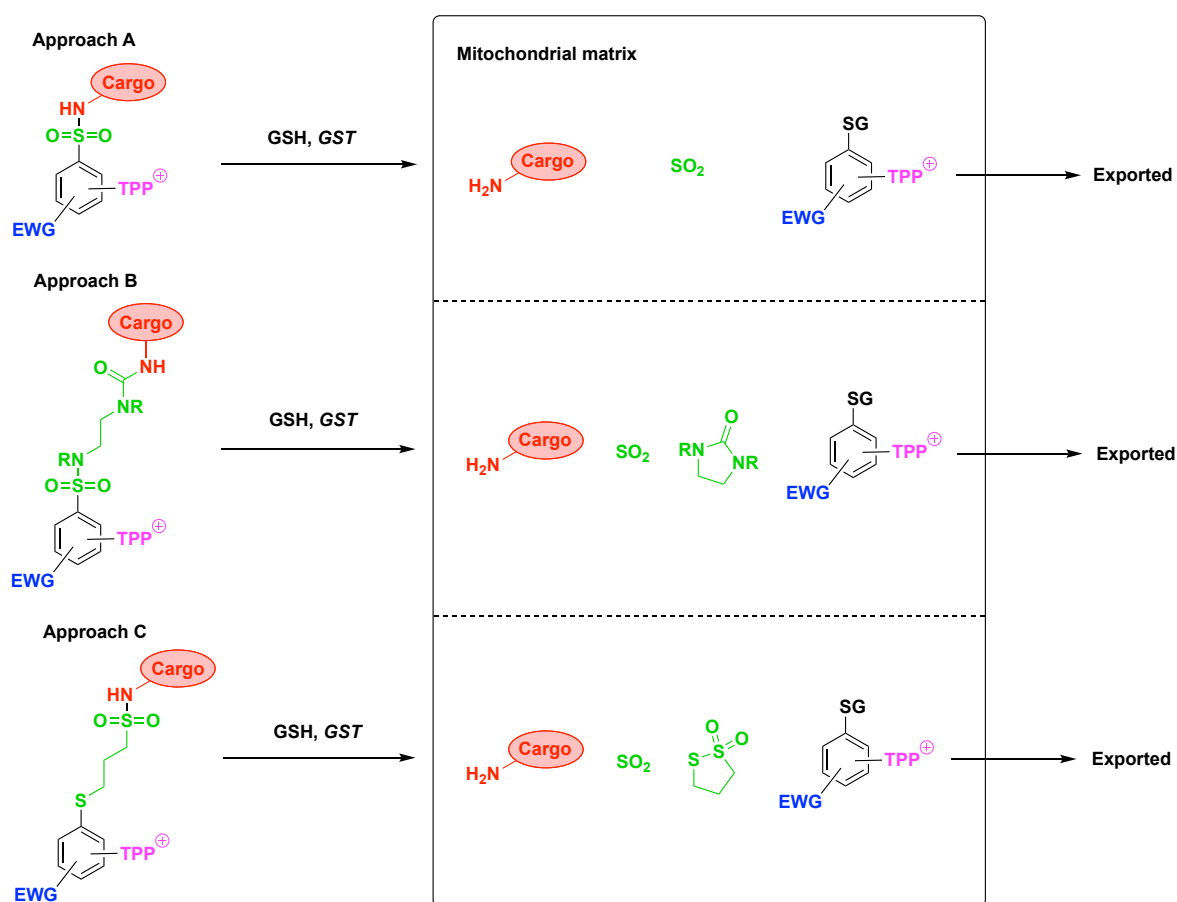


Figure 31. Three approaches for a TPP-conjugated GSH/GST-activated prodrug to deliver an active cargo to the mitochondrial matrix and deplete the mGSH pool by formation of a GS-conjugate. In Approach A, the cargo is directly attached to the electron-poor ring via a cleavable sulfonamide bond. In Approach B, the cargo is linked via a carbamate to an ethylenediamine self-immolative linker, which in turn is attached to the electron-poor ring via a cleavable sulfonamide bond. In Approach C, the cargo is linked via a sulfonamide bond to a propyl sulfide self-immolative linker, which in turn is attached to the electron-poor ring.

To investigate the potential of the different approaches, we explored attaching a variety of cargos, including fluorophores and mitochondria-damaging anti-cancer drugs, to the prodrug systems. We were especially interested to develop a prodrug of metformin because of its anti-cancer properties revealed in several pre-clinical studies (see Section 1.4.2).^{199, 210, 213, 219, 244-246} Metformin **58** is a mitochondrial complex I inhibitor, although it is suspected to have several other targets responsible for its therapeutic effects (see Section 1.4.1).^{188, 192, 247} Crucially, it has never been released in the mitochondria from a prodrug. These prodrug systems present a promising strategy for treating cancer by

delivering a polypharmacological effect. Cancer cells often contain dysfunctional mitochondria with increased mitochondrial membrane potentials,²⁴⁸ which in theory, would provide greater accumulation of TPP-conjugated prodrugs. Compared to normal cells, cancer cells are typically associated with enhanced production of ROS, necessitating an increased antioxidant defence system, including elevated GSH **25** levels, to maintain redox balance.^{163, 166, 249} Moreover, GSH **25** can detoxify cancer drugs which contributes to anticancer drug resistance.²⁴⁹ The GSH/GST-activated prodrug systems aim to deplete the mGSH pool while simultaneously releasing an active anti-cancer drug, thereby circumventing undesirable detoxification. Overall, the delivery of an anti-cancer drug that damages mitochondrial homeostasis, combined with mGSH depletion, should compromise a cancer cell's antioxidant defence, leading to irreversible oxidative damage and consequently, cell death.

3.2 Approach A

For a general aryl sulfonamide **99** (Figure 32), there are two potential electrophilic sites that are susceptible to nucleophilic attack by GSH **25**: C-S (a) and S (b). It is possible to manipulate where nucleophilic attack occurs by altering the electronic properties of the aromatic ring and the R group. Approaches A-C require attack at C-S (a) by a S_NAr reaction, which should be favoured by strongly electron-withdrawing substituents on the aromatic ring. Attack at S (b) should be favoured when the aromatic ring is electron-poor and when the amine moiety is a good leaving group due to the presence of electron-withdrawing groups. Furthermore, in an aryl metformin sulfonamide **100** (Figure 32), the biguanide moiety is strongly electron-withdrawing by induction, which increases the electrophilicity of S (b) for nucleophilic attack. Attack at C = NH (c) by GSH **25** to form a S-thiocarbamate is unfavourable because the pKa of GSH **25** is lower than that of an amine. For an aryl arenesulfonamide **101** (Figure 32), in addition to the C-S (a) and S (b) electrophilic sites, nucleophilic attack is also possible at C-N (c). As before, attack at C-S (a) should be favoured by strong electron-withdrawing substituents on ring A, while attack at S (b) should be favoured by an electron-poor ring A and by the leaving group ability of the aniline derived from ring B, also enhanced by electron-withdrawing substituents. Attack at C-N (c) by a S_NAr reaction should be favoured by electron-withdrawing substituents on ring B. It is important to note that in GST-catalysed reactions, the substrate specificity of the GST enzymes will also affect the site of attack.

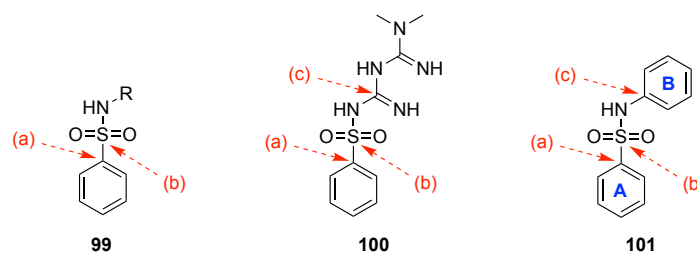


Figure 32. Possible sites of nucleophilic attack by GSH **25** on an aryl sulfonamide **99**, aryl metformin sulfonamide **100** and aryl arene sulfonamide **101**.

In Chapter 2, the electron-poor aromatic systems of amide **86a** and MitoCDNB **51** were shown to undergo S_NAr reactions with GSH **25**, with reaction rates greatly enhanced by GST catalysis. Building on the structures of amide **86a** and MitoCDNB **51**, two sulfonamide scaffolds, **102** and **103**, respectively, were trialled (Figure 33). In these scaffolds, attack at C-S was expected to be favoured due to the strong electron withdrawing substituents on the aromatic ring. The sulfonamide compounds were anticipated to exhibit greater S_NAr reactivity with GSH **25** than their chloride analogues due to the superior electron withdrawal of a sulfonamide substituent. Ideally, the sulfonamide prodrugs would be stable to the uncatalysed reaction with GSH **25**, allowing selective depletion of GSH **25** by GST catalysis without reacting with other cellular thiols. Achieving this may require reducing the electrophilicity of the highly electron-poor dinitro system in MitoCDNB **51**. Therefore, of particular interest was scaffold **102**, which features only one nitro group *ortho* to the sulfonamide, and an amide group in the *para* position, resulting in a less electron-poor aromatic system than in scaffold **103**. To provide proof of concept for the release of amine-bearing payloads from a sulfonamide, the fluorophore 7-aminocoumarin **22**, was selected as a reporter. Metformin **58** was the drug of interest due to its widely-reported anti-cancer properties through inhibition of complex I (see Section 1.4.2).

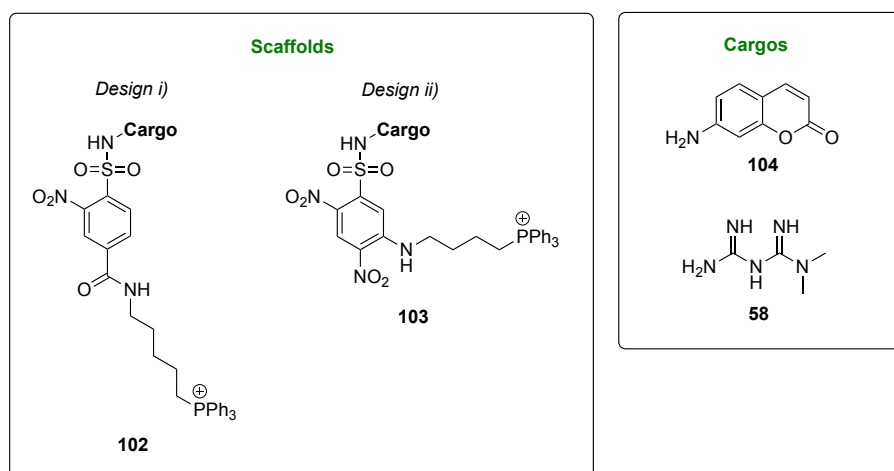
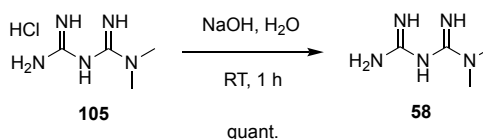


Figure 33. Prodrug scaffold designs and cargos of interest. In design i), scaffold 102 is based on the substitution pattern of amide 86a. In design ii), scaffold 103 is based on the substitution pattern of MitoCDNB 51. For the cargos, aminocoumarin 104 and metformin 58 were to be used as a fluorescent reporter and an anticancer drug, respectively.

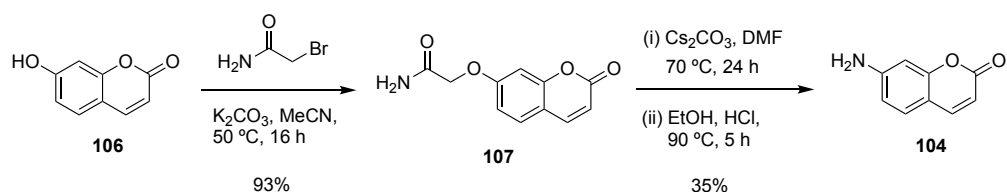
3.2.1 Synthesis of cargos

First the cargos to be attached in prodrug designs i) and ii) were prepared. Metformin hydrochloride **105** was commercially available and was treated with sodium hydroxide to afford basic metformin **58** in quantitative yield (Scheme 39).



Scheme 39. Synthesis of basic metformin **58** from metformin hydrochloride **105**.

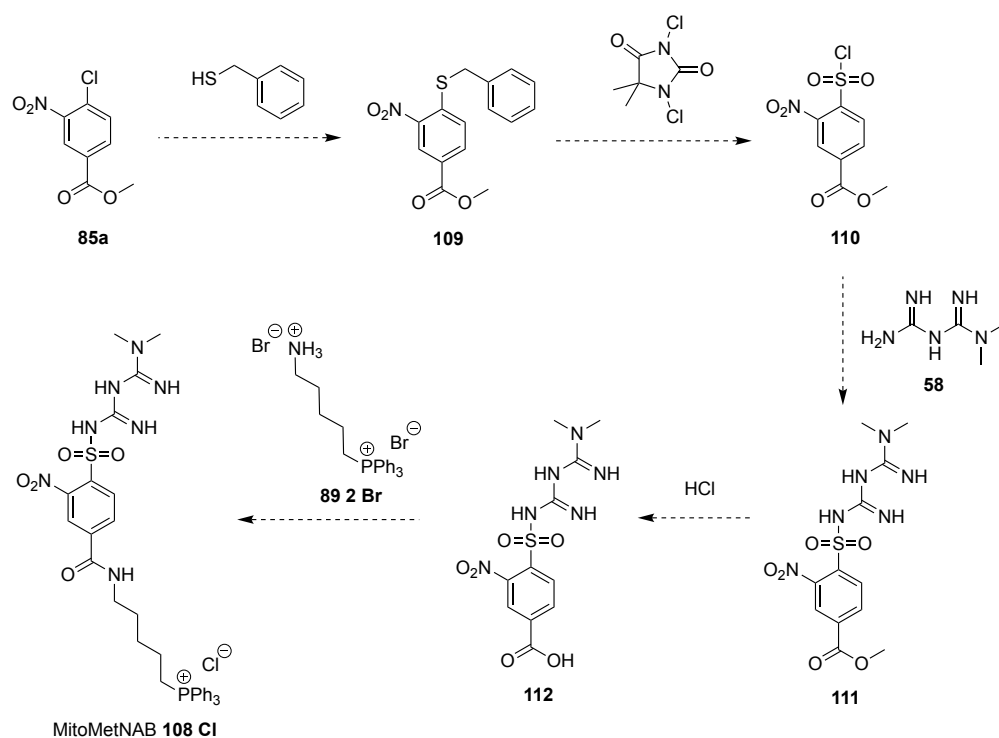
7-Aminocoumarin **104** was not commercially available and was prepared in two steps by adapting the procedure of Lippe *et al* (Scheme 40).²⁵⁰ 7-Hydroxycoumarin **106** was reacted with bromoacetamide in a nucleophilic substitution reaction to afford the ether **107** in excellent yield, followed by a Smiles rearrangement to obtain 7-aminocoumarin **104**, albeit in poor yield.



Scheme 40. Synthesis of 7-aminocoumarin **104** adapting the procedure of Lippe *et al.*²⁵⁰

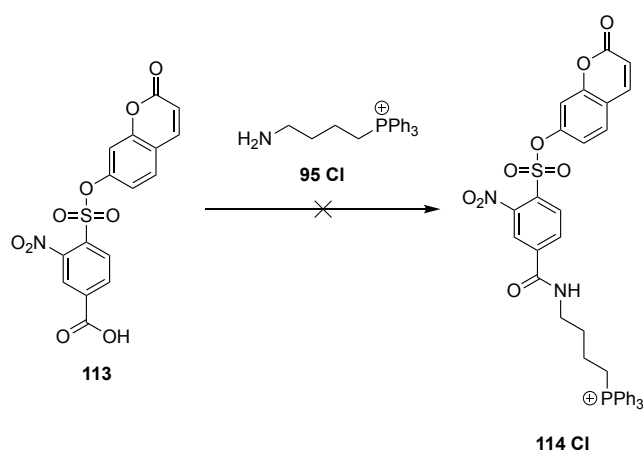
3.2.2 Synthesis of prodrug design i)

Preparation of a metformin prodrug based on design i), which was named MitoMetNAB **108** (Scheme 41), was attempted by two different synthetic routes. These routes were adapted from routes originally devised and trialled by Dr Taig,²²⁴ whose target compounds incorporated the 4-aminobutyl TPP linker **95**, as opposed to the 4-aminopentyl TPP linker **89** which features in the structures of amide **86a** and MitoMetNAB **108**. The first route introduces the cargo before the TPP targeting group because this allows the reactivity to be investigated early in the sequence (Scheme 41). The synthesis would begin with ester **85a**, which was previously synthesised in Section 2.2.1. Ester **85a** would undergo a S_NAr reaction with benzyl mercaptan to afford sulfide **109**, followed by oxidative chlorination with 1,3-dichloro-5,5-dimethylhydantoin (DCDMH) to generate sulfonyl chloride **110**, adapting the method of Xin *et al.*²⁵¹ Metformin **58** would then be coupled to sulfonyl chloride **110** to produce metformin sulfonamide **111**. Subsequent acidic hydrolysis would generate the carboxylic acid **112**. Finally, amide coupling of carboxylic acid **112** with the ammonium salt **89**, previously synthesised in Section 2.2.2, would give MitoMetNAB **108**.



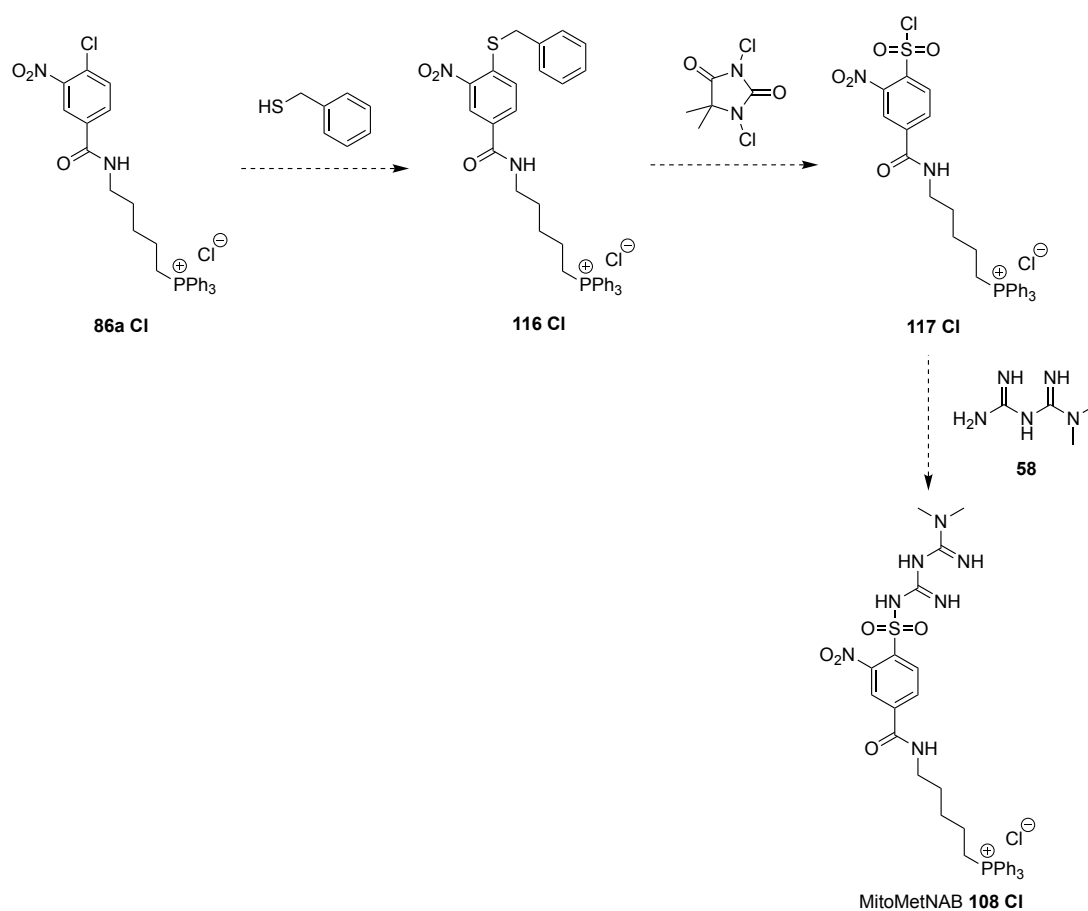
Scheme 41. First proposed synthesis route towards MitoMetNAB 108 based on prodrug design i), which introduces the cargo earlier in the synthesis.

Following the route in Scheme 41, Dr Taig managed to obtain the carboxylic acid **112** of the metformin sulfonamide and the carboxylic acid **113** of a 7-hydroxycoumarin sulfonamide (Scheme 42) in 56% yield and 53% yield, respectively, over a total of four steps.²²⁴ Dr Taig's attempted coupling of carboxylic acid **113** to 4-aminobutyl TPP **95** resulted in the cleavage of 7-hydroxycoumarin **106** and returned a mixture of decomposed products, which could not be isolated.²²⁴ Dr Taig did not attempt coupling of **112** to 4-aminobutyl TPP **95** out of anticipation that the same result would occur. However, it would be valuable to determine whether MitoMetNAB **108** is synthetically accessible via this route.



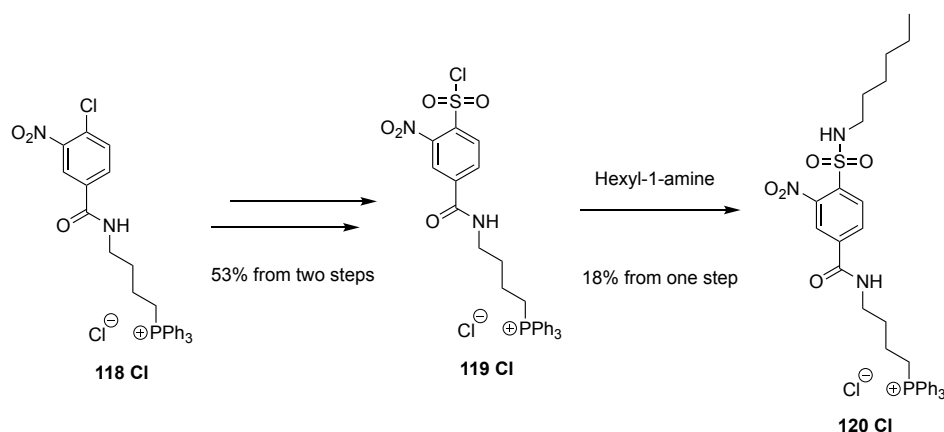
Scheme 42. Previous work by Dr Taig: 7-hydroxycoumarin sulfonamide **113** was found to decompose upon attempted coupling with amine **95**.²²⁴

Applying the proposed synthesis in Scheme 41, ester **85a** was reacted with benzyl mercaptan to afford sulfide **109** in excellent yield (Scheme 43). Oxidative chlorination of sulfide **109** with DCDMH afforded sulfonyl chloride **110** in excellent yield. To confirm formation of sulfonyl chloride **110**, it was coupled to commercially available hexyl-1-amine to obtain the hexyl sulfonamide **115** in poor yield. The sulfonyl chloride **110** was then coupled with metformin **58** to obtain the metformin sulfonamide **111**, also in poor yield. Acid hydrolysis of the ester produced a mixture of compounds, despite the same conditions resulting in pure carboxylic acid **112** in the hands of Dr Taig.²²⁴ However, Dr Taig did not provide NMR data for carboxylic acid **112** due to hydrogen-deuterium exchange.²²⁴ Therefore, a comparison could not be made with the NMR spectra of the crude material, which indicated a mixture of two metformin compounds. The two compounds could not be separated by TLC, and the route was paused while the second route towards MitoMetNAB **108** was investigated.



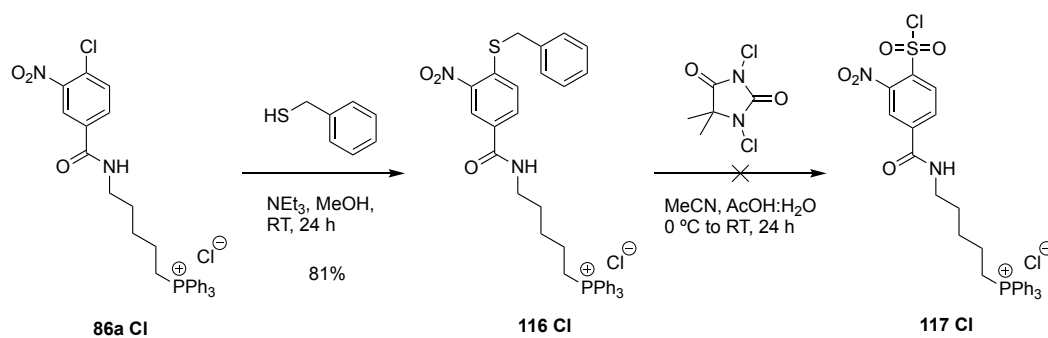
Scheme 44. Proposed second synthesis route towards MitoMetNAB 108 based on design i), which introduces the cargo in the final step.

As highlighted above, Dr Taig's target compounds incorporated a 4-aminobutyl TPP linker **95** instead of a 5-aminopentyl TPP linker **89**.²²⁴ Hence, starting from the chloride **118** and following a route parallel to the one in Scheme 44, Dr Taig managed to obtain the sulfonyl chloride **119** as the chloride salt in 53% yield from two steps, which was then coupled to 1-hexylamine to afford the hexylsulfonamide **120** in 18% yield (Scheme 45).²²⁴ However, Dr Taig did not attempt coupling of sulfonyl chloride **119** to metformin **58**.



Scheme 45. Previous work by Dr Taig: Parallel to the synthesis route in Scheme 6, sulfonyl chloride **119** was prepared from chloride **118**.²²⁴

Applying the proposed synthetic route from Scheme 44, amide **86a** was reacted with benzyl mercaptan to afford sulfide **116** in good yield (Scheme 46). However, reaction of sulfide **116** with DCDMH did not afford the desired sulfonyl chloride **117**.



Scheme 46. Synthesis towards sulfonyl chloride **117**.

After purification of the reaction mixture, the major product, which was only soluble in polar protic solvents, was identified by mass spectrometry to be sulfonic acid **121** (Figure 34) likely due to over-oxidation.

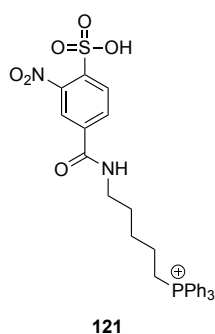
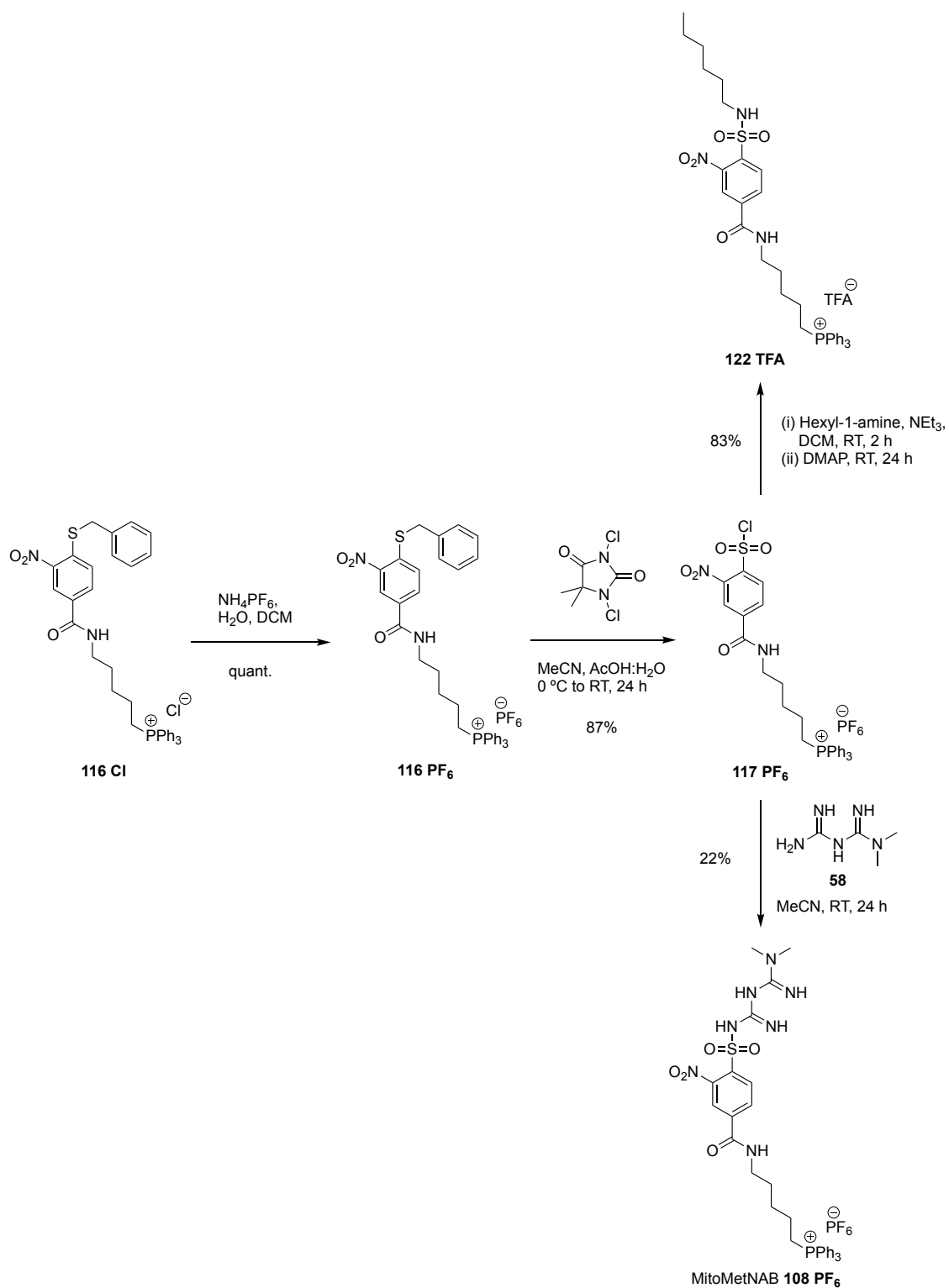


Figure 34. Structure of sulfonic acid **121**, the undesired product from the oxidation chlorination of sulfide **116**.

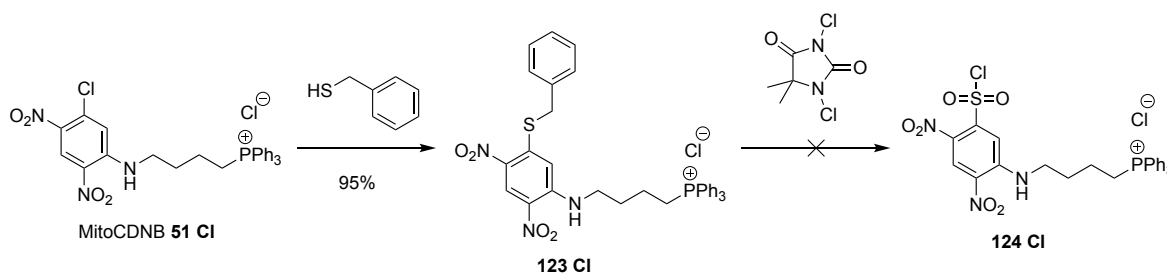
To improve sulfide **116**'s solubility in acetonitrile and its reactivity with DCDMH, the chloride counter anion was exchanged for the lipophilic and weakly coordinating hexafluorophosphate anion, PF_6^- , counter ion. Sulfide **116** as the chloride salt was dissolved in dichloromethane and washed with excess ammonium hexafluorophosphate in water to afford sulfide **116** as the hexafluorophosphate salt (Scheme 47). Reaction of sulfide **116** with DCDMH afforded sulfonyl chloride **117** as the hexafluorophosphate salt in excellent yield. To confirm formation of sulfonyl chloride **117**, it was coupled to hexyl-1-amine to obtain the hexylsulfonamide **122** in good yield. Finally, sulfonyl chloride **117** was reacted with metformin **58**, which afforded the desired MitoMetNAB **108** as the hexafluorophosphate salt, albeit in poor yield. The reactivity of MitoMetNAB **108** and hexylsulfonamide **122** for GSH **25** conjugation by GST-catalysis was then investigated (see Sections 3.2.6 and 3.2.7).



Scheme 47. Second synthesis route towards MitoMetNAB 108.

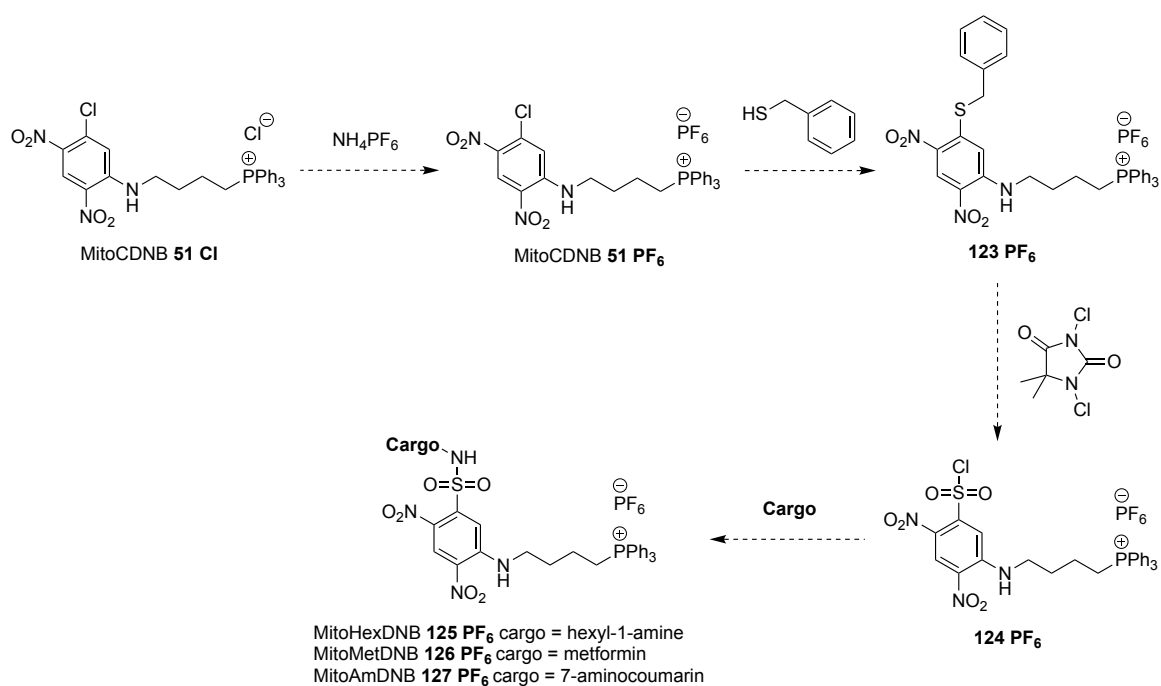
3.2.3 Synthesis of prodrug design ii)

The proposed synthetic route for design ii) was based on the route attempted by Dr Taig towards the same design (Scheme 48) and the optimised route for MitoMetNAB **108** (Scheme 47).²²⁴ In Dr Taig's original route,²²⁴ sulfide **123** as the chloride salt was prepared from MitoCDNB **51** as the same salt. However, oxidative chlorination with DCDMH to form sulfonyl chloride **124** instead led to decomposition of sulfide **123**.



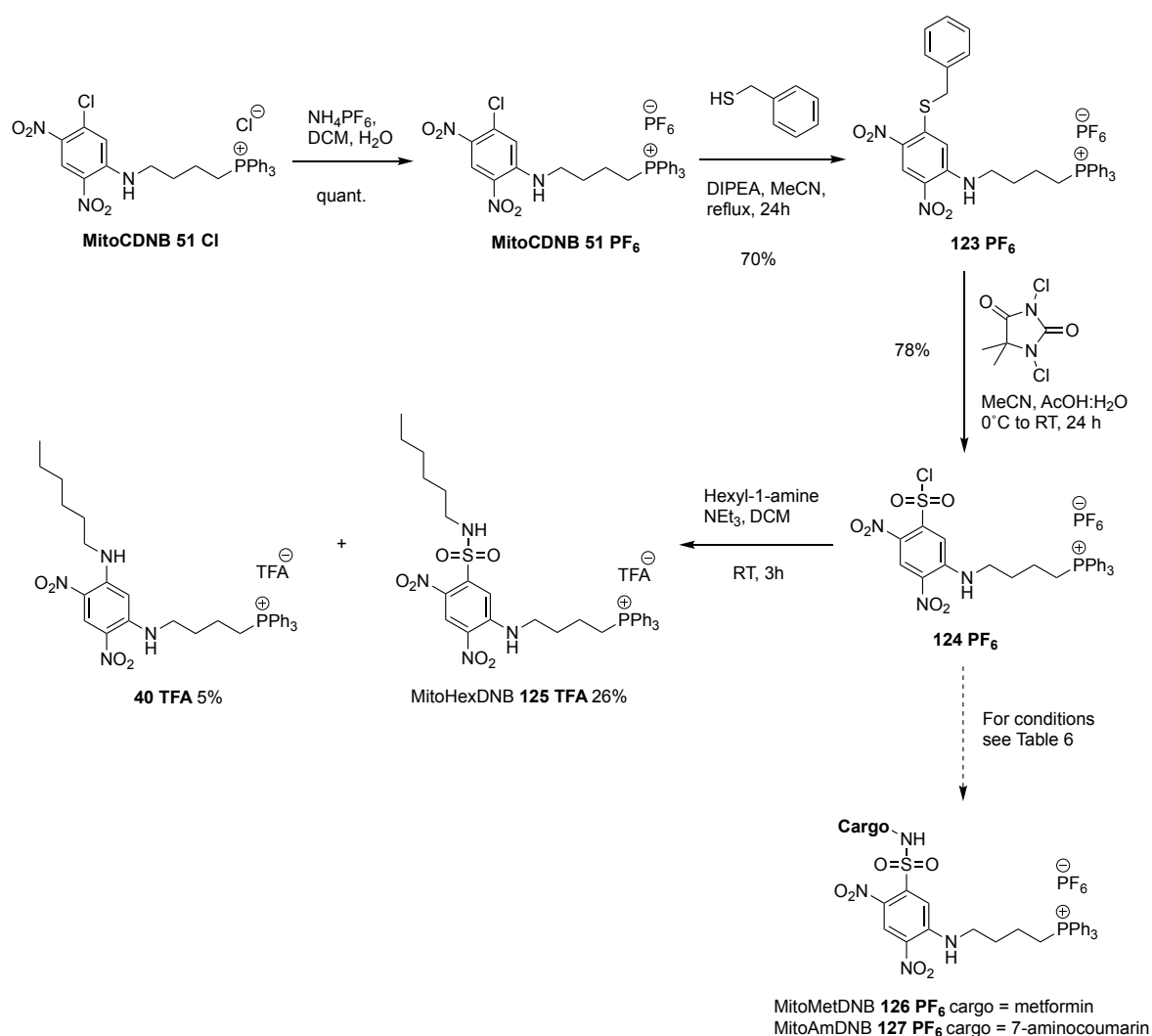
Scheme 48. Previous work by Dr Taig: Oxidative chlorination of sulfide **123** as the chloride salt resulted in decomposition.²²⁴

Building on the successful use of the hexafluorophosphate counterion in the synthesis of sulfonyl chloride **118** (Scheme 47), Dr. Taig's route was adapted to develop the revised route shown in Scheme 49. Synthesis would begin from MitoCDNB **51**, previously prepared in Section 2.2.3 by following the procedure of Booty *et al.*¹⁷⁸ First, MitoCDNB **51** as the chloride salt would be ion-exchanged with ammonium hexafluorophosphate to afford MitoCDNB **51** as the hexafluorophosphate salt. This would be reacted with benzyl mercaptan to obtain sulfide **123** as the hexafluorophosphate salt. Oxidative chlorination of sulfide **123** with DCDMH would generate the sulfonyl chloride **124**, followed by coupling with the relevant cargos to afford the desired sulfonamides, MitoHexDNB **125**, MitoMetDNB **126** and MitoAmDNB **127**.



Scheme 49. Proposed synthesis route towards prodrug design ii).

Applying the proposed synthesis in Scheme 59, MitoCDNB **51** was first ion-exchanged with ammonium hexafluorophosphate to obtain MitoCDNB **51** as the hexafluorophosphate salt in quantitative yield (Scheme 50). MitoCDNB **51** was then reacted with benzyl mercaptan, which afforded sulfide **123** in good yield, followed by treatment with DCDMH to generate the sulfonyl chloride **124**, also in good yield. Formation of the sulfonyl chloride **124** was validated by its successful coupling to hexyl-1-amine to afford MitoHexDNB **125**, albeit in poor yield. Aniline **40** was formed as a side-product due to the displacement of the sulfonyl chloride moiety by hexyl-1-amine in a S_NAr reaction. Semi-preparative HPLC was required to separate MitoHexDNB **125** and aniline **40**.



Scheme 50. Synthesis route towards prodrug design ii).

Unfortunately, coupling of sulfonyl chloride **124** to metformin **58** or 7-aminocoumarin **104** did not afford the desired products MitoMetDNB **126** or MitoAmDNB **127**, respectively. The conditions and results of these reactions are presented in Table 7. For the attempted reactions with metformin **58** (Entries 1 and 2), LCMS analysis of the crude material indicated a mixture primarily consisting of sulfonic acid **128** (Figure 35) and metformin **58** starting material, but also a small amount of MitoCDNB **51**. For the attempted reaction with 7-aminocoumarin **104** (Entry 3), LCMS analysis of the crude material indicated a mixture of sulfinic acid **129** (Figure 35) and 7-aminocoumarin **104** starting material. The individual compounds were not isolated from the crude material. The formation of sulfonic acid **128** and sulfinic acid **129** indicate that the sulfonyl chloride **124** was prone

to hydrolysis and reduction, respectively. However, there was no obvious reducing agent present in the reaction with 7-aminocoumarin **104**. The formation of sulfonic acid **128** may result directly from hydrolysis of sulfonyl chloride **124**, or it may be that sulfonamide MitoMetDNB **126** was initially generated *in situ* but was unstable to moisture in the reaction mixture or upon work-up. An explanation for the formation of sulfinic acid **129** in the reaction with 7-aminocoumarin **104** is less obvious. Although MitoCDNB **51** was a minor product of the reaction of sulfonyl chloride **124** with metformin **58**, it suggests that sulfonyl compounds attached to the electron-poor dinitro ring are susceptible to S_NAr . SO_2 produced by decomposition of such compounds may be the reductant (Scheme 51).

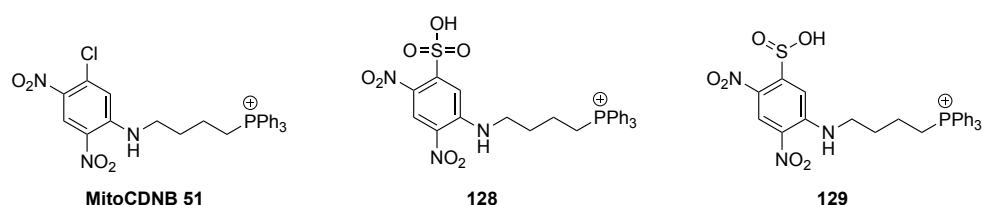
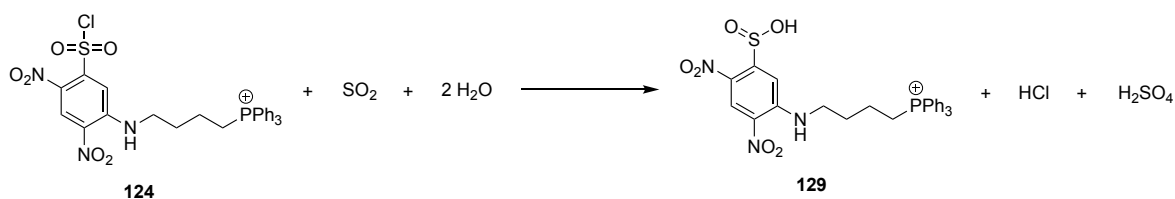


Figure 35. Structures of the side-products from the coupling of sulfonyl chloride **124** with metformin **58** or 7-aminocoumarin **104**.

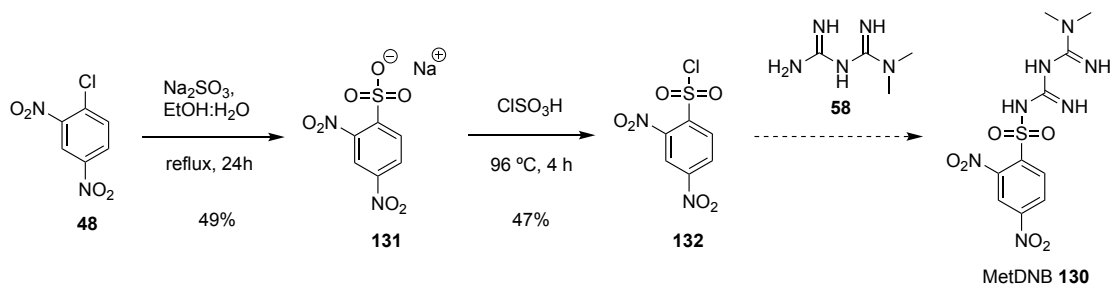
Entry	Starting material	Reagents	Solvent	Temp.	Time	Result
1	Sulfonyl chloride 124	Metformin 58 (2 eq.)	MeCN	RT	8 h	Metformin 58 , sulfonic acid 128 , trace MitoCDNB 51
2	Sulfonyl chloride 124	Metformin 58 (1 eq.), NEt ₃ (2 eq.)	MeCN	RT	24 h	Metformin 58 , sulfonic acid 128 , trace MitoCDNB 51
3	Sulfonyl chloride 124	7-Aminocoumarin 104 (1 eq.), NEt ₃ (2 eq.)	MeCN	RT	24 h	7-Aminocoumarin 104 , sulfinic acid 129

Table 7. Reaction conditions and results for the coupling of sulfonyl chloride **124** with metformin **58** or 7-aminocoumarin **104**.



Scheme 51. Proposed formation of sulfinic acid 129 where SO₂ acts as a reducing agent.

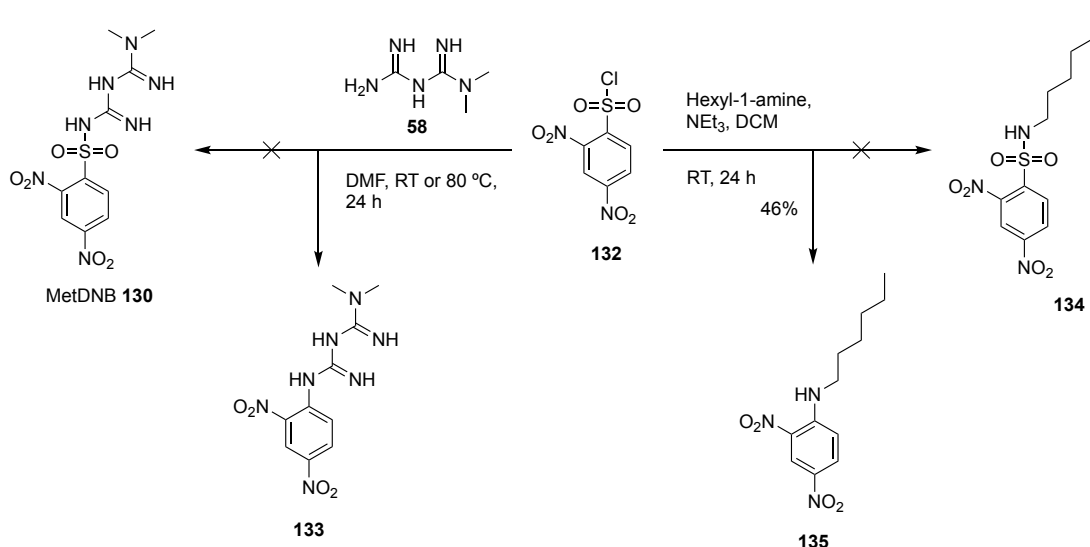
An attempt was then made to investigate whether the electron-poor nature of the dinitro aromatic ring or steric interference from the TPP-linker was responsible for the failed synthesis of MitoMetDNB **126**. The synthesis of a simpler sulfonamide of metformin, MetDNB **130**, based on the structure of CDNB **48** was undertaken (Scheme 52). Synthesis began with an S_NAr reaction between CDNB **48** and sodium sulfite, yielding sulfonate **131** in moderate yield. Subsequent chlorination with chlorosulfonic acid produced sulfonyl chloride **132**, also in moderate yield. However, due to the absence of NMR data for sulfonyl chloride **132** in the literature for comparison and the inability of ESI^{+/·} mass spectrometry to detect a corresponding mass ion, the synthesis of this compound was uncertain.



Scheme 52. Attempted synthesis of MetDNB 130.

The coupling of sulfonyl chloride **132** to metformin **58** was attempted in DMF at RT and at 80 °C because it appeared to be the best solvent to solubilise both reactants. However, in both cases, ¹H NMR and LCMS analysis of the crude material revealed no trace of MetDNB **130**. Aniline **133** was identified as the major product, likely due to an S_NAr reaction in which metformin **58** displaced the sulfonyl chloride group. Sulfonyl chloride **132** was also reacted with hexyl-1-amine in an attempt to obtain sulfonamide **134**

(Scheme 53). However, column chromatography of the crude material only returned a single product, which ^1H NMR analysis indicated to be aniline **135**, likely due to an $\text{S}_{\text{N}}\text{Ar}$ reaction in which hexyl-1-amine displaced the sulfonyl chloride group. These results demonstrated that the dinitro ring system was too reactive to synthesise stable sulfonamide prodrugs.



Scheme 53. Reaction of sulfonyl chloride **131** and metformin **58** produced aniline **133** instead of the desired MetDNB **130**. Reaction of sulfonyl chloride **131** and hexyl-1-amine produced aniline **135** instead of the desired sulfonamide **134**.

Overall, despite being unable to synthesise a sulfonamide prodrug of metformin or 7-aminocoumarin based on design ii), a prodrug-like compound, MitoHexDNB **125**, was prepared. This was used to study the kinetics of this related prodrug design for GST-catalysed GSH **25** conjugation (see Section 3.2.5 and 3.2.6).

3.2.4 Tuning the reactivity of prodrug design ii) to access stable sulfonamides

Given the synthetic challenges of obtaining a stable sulfonamide prodrug of metformin based on design (ii), likely due to the highly electron-poor dinitro system, an attempt was made to reduce the scaffold's reactivity by replacing one nitro group with a less electron-withdrawing substituent. This built on the work of Rautio *et al.*,¹⁸¹ who successfully

synthesised a moderately reactive GSH/GST-activated sulfonamide metformin prodrug **136** featuring *ortho* nitro and *para* trifluoromethyl substituents (Figure 36, also see Section 1.2.5). Hence, the aim was to develop a mitochondria-targeted analogue of sulfonamide **136**, which was named MitoMetNTB **137** (Figure 36), by incorporating the TPP-linker at the *meta* position in the same way as in prodrug design ii).

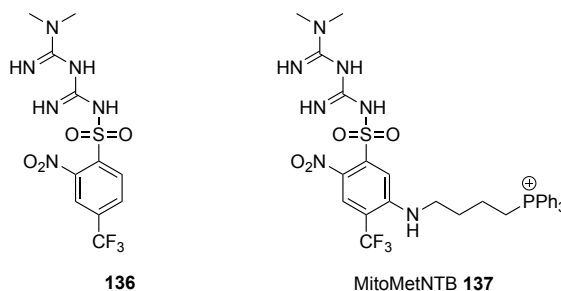
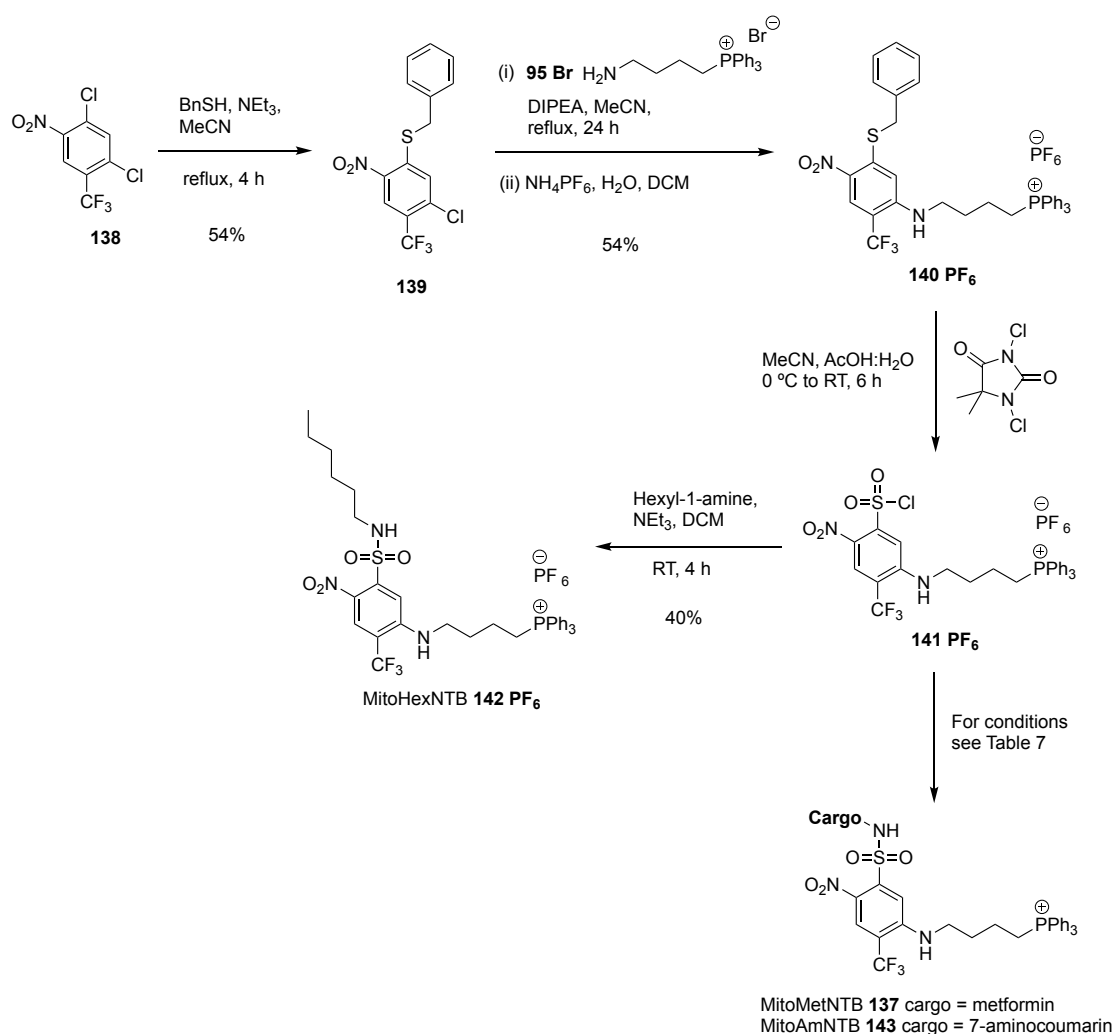


Figure 36. Structures of metformin prodrug **136** prepared by Rautio *et al.*¹⁸¹ and the proposed TPP-conjugated analogue MitoMetNTB **137**.

The devised synthesis route was based on the routes towards designs i) and ii). 1,3-Dichloro-2-nitro-4-trifluoromethylbenzene **138** was treated with benzyl mercaptan in a $\text{S}_{\text{N}}\text{Ar}$ reaction, which afforded sulfide **139** in moderate yield. Another $\text{S}_{\text{N}}\text{Ar}$ reaction with (4-aminobutyl)TPP bromide **95** (previously synthesised in Section 2.2.3), followed by an ion-exchange with ammonium hexafluorophosphate afforded the TPP-conjugated sulfide **140** as the hexafluorophosphate salt, also in moderate yield (Scheme 54). Oxidative chlorination with DCDMH generated the sulfonyl chloride **141**, but it was only partially purified by column chromatography. Formation of the sulfonyl chloride **141** was validated by its successful coupling to hexyl-1-amine to afford the sulfonamide MitoHexNTB **142** in moderate yield.



Scheme 54. Synthesis route towards CF₃ analogues of prodrug design ii).

Unfortunately, as was the case for the dinitro analogue, MitoMetDNB **126**, coupling of sulfonyl chloride **141** to metformin **58** or 7-aminocoumarin **104** did not afford the desired sulfonamides, MitoMetNTB **137** or MitoAmNTB **143**, respectively. For each of the reactions of sulfonyl chloride **141** with metformin **58** and 7-aminocoumarin **104**, LCMS analysis of the crude material indicated a complex mixture including the cargo starting material, sulfonic acid **144**, the sulfinic acid **145**, the chloride **146** (Figure 37, Table 8) and two other sulfonyl-related side-products whose structures could not be identified. None of these compounds were isolated. Sulfonic acid **144** and sulfinic **145** are the hydrolysis and reduction products of sulfonyl chloride **141**. The formation of chloride **146** indicates that sulfonyl compounds attached to the *ortho*-nitro *para*-trifluoromethyl ring, like the dinitro system, are susceptible to S_NAr, which again suggests that SO₂ may be the reductant.

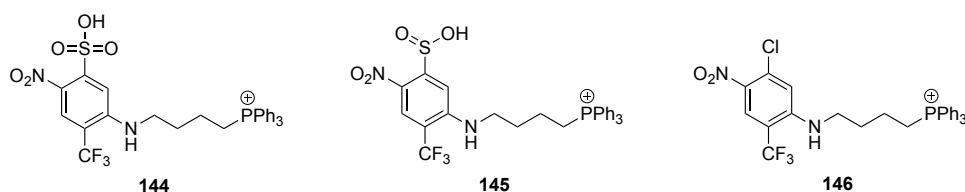
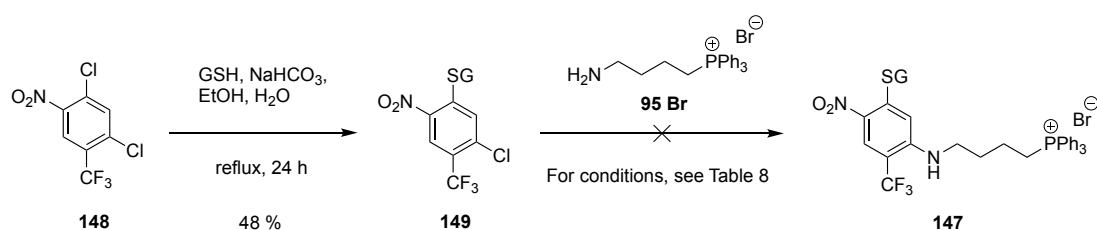


Figure 37. The major side-products sulfonic acid **144**, sulfinic acid **145** and chloride **146** from the reactions of sulfonyl chloride **141** with metformin **58** or 7-aminocoumarin **104**.

Entry	Starting material	Reagents	Solvent	Temp.	Time	Result
2	Sulfonyl chloride 141	Metformin 58 (2 eq.)	MeCN	RT	24 h	Metformin 58 , sulfonic acid 144 , sulfinic acid 145 , chloride 146
3	Sulfonyl chloride 141	7-aminocoumarin 104 (1 eq), NEt ₃ (2 eq)	MeCN	RT	3 h	7-aminocoumarin 104 , sulfonic acid 144 , sulfinic acid 145 , chloride 146

Table 8. Reaction conditions and results for the coupling of sulfonyl chloride **141** with metformin **58** or 7-aminocoumarin **104**.

Given that Rautio *et al.*¹⁸¹ successfully prepared metformin sulfonamide **136**, it is surprising that its TPP-conjugated analogue **137** could not be synthesised. The presence of the electron-donating amino substituent would be expected to make the aromatic ring less electron-poor, reducing its susceptibility to S_NAr. MitoHexNTB **142** was still of interest for kinetic studies of its GST-catalysed GSH **25** conjugation reaction, but this required the synthesis of the corresponding GS-conjugate **147** (Scheme 55). Hence, 1,3-dichloro-2-nitro-4-trifluoromethylbenzene **148** was reacted with GSH **25** in a S_NAr reaction, affording the GS-conjugate **149** in moderate yield. However, subsequent S_NAr reaction of GS-conjugate **149** with (4-aminobutyl)TPP bromide **95** (previously synthesised in Section 2.2.3) under various conditions did not produce any of the desired product **147**, returning only starting materials (Table 9).



Scheme 55. Attempted synthesis of GS-conjugate 147.

Entry	Starting material	Reagents	Solvent	Temp.	Time	Result
1	GS-conjugate 149	Amine 95 (1 eq.), DIPEA (1 eq.)	MeCN	reflux	24 h	Starting materials
2	GS-conjugate 149	Amine 95 (1 eq.), DIPEA (1 eq.)	DMF	80 °C	24 h	Starting materials
3	GS-conjugate 149	Amine 95 (1.3 eq), NaHCO ₃ (3 eq)	EtOH:H ₂ O 1:1	reflux	24 h	Starting materials

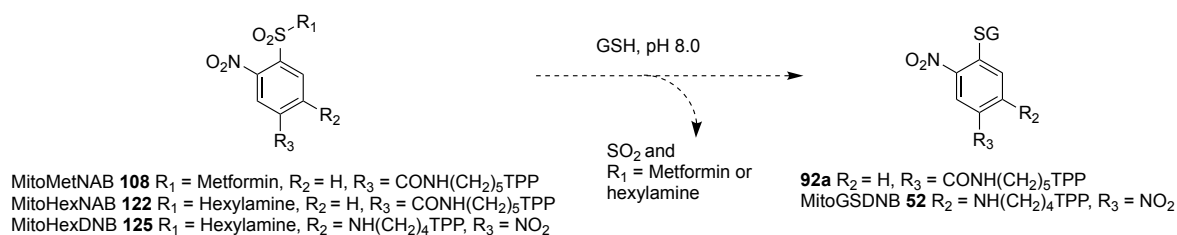
Table 9. Reaction conditions and results for the attempted synthesis of GS-conjugate 147.

This outcome is somewhat surprising since the chloride *ortho* to the trifluoromethyl substituent in benzyl sulfide **139** had been successfully displaced with amine **95** (Scheme 54), which has comparable ring electronics to GS-conjugate **149**. The reaction certainly suffers from solubility issues given the water-solubility of GS-conjugate **61** and hydrophobicity of amine **12**. To address this, reaction was attempted in both aprotic and protic polar solvents with heating, but without success. A more suitable solvent system may be required to effectively solubilise both reactants. Ultimately, this result meant that the kinetics of MitoHexNTB **142** could not be studied without GS-conjugate **147**. Given the time constraints of the project, further synthesis was paused and the kinetics of the GST-catalysed GSH **25** conjugation of the existing sulfonamides was investigated (see Section 3.2.5 and 3.2.6).

3.2.5 Kinetics of sulfonamides with GSH

The next step was to investigate the uncatalysed and GST-catalysed reactions between sulfonamides MitoMetNAB **108**, MitoHexNAB **122** and MitoHexDNB **125**. First, the second-order rate constant (k) for the background reaction of each of the sulfonamides was measured with GSH **25** in the absence of GST enzyme (Scheme 56, Table 10). The concentration of sulfonamide and GSH **25** was fixed at 0.1 mM and 2 mM, respectively, so that the pseudo-first-order reaction could be observed with respect to the sulfonamides. The background rate constants for CDNB **48**, MitoCDNB **51** and amide **86a**, previously determined in Section 2.4.2, are provided again for comparison (Table 10).

Overall, the sulfonamide analogues exhibited greater reactivity with GSH **25** than the chloride analogues. MitoHexDNB **125** exhibited very high reactivity, with a k approximately 60-fold greater than that of its chloride analogue MitoCDNB **51**. The high reactivity of MitoHexDNB **125** aligns with a study by Zhao *et al.*,²⁵² which found that sulfonamides conjugated to ortho, para-dinitrophenyls were rapidly cleaved by GSH **25** in the absence of GST enzyme. Similarly, MitoHexNAB **125** demonstrated high reactivity with GSH **25**, whereas its chloride analogue, amide **86a**, showed no detectable reactivity. These results indicate that the sulfonamide analogues are more electrophilic than their chloride counterparts.



Scheme 56. Expected reaction of MitoMetNAB **108**, MitoHexNAB **122** and MitoHexDNB **125** with GSH **25** at pH 8.0 to form corresponding GS-conjugates **92a** or MitoGSDNB **52**.

Compound	Substituents				Second-order rate constant k ($\times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$)
	1-	2-	4-	5-	
CDNB 48	Cl	NO ₂	NO ₂	H	5.05 $\pm$ 0.40 ^(a)
MitoCDNB 51	Cl	NO ₂	NO ₂	NH(CH ₂) ₄ TPP	1.50 $\pm$ 0.18 ^(a)
MitoHexDNB 125	SO ₂ NH(CH ₂) ₅ CH ₃	NO ₂	NO ₂	NH(CH ₂) ₄ TPP	89.7 $\pm$ 2.4 ^(b)
86a	Cl	NO ₂	CONH(CH ₂) ₅ TPP	H	ND
MitoHexNAB 122	SO ₂ NH(CH ₂) ₅ CH ₃	NO ₂	CONH(CH ₂) ₅ TPP	H	6.52 $\pm$ 0.51 ^(b)
MitoMetNAB 108	SO ₂ Metformin	NO ₂	CONH(CH ₂) ₅ TPP	H	NS

Table 10. Second-order background rate constants (k) for the reactions of compounds **48**, **51**, **125**, **86a**, **122** and **108** (ordered by substitution pattern) with GSH **25** at pH 8 and 30 °C. (a) Calculated using 10 mM GSH **25**, presented as mean values (and errors as $\pm$ 1 standard deviation) of triplicate measurements of four rate constants determined at four different concentrations. (b) Calculated using 2 mM GSH **25**, presented as means values (and errors as $\pm$ 1 standard deviation) of triplicate measurements of one rate constant determined at 0.1 mM of compound. ND = No reaction detected. NS = Not stable.

The high electrophilicity of the sulfonamide moiety is further highlighted by comparison of sulfonamide and chloride analogues of different substitution patterns. MitoHexDNB **125** is almost 18-fold more reactive than CDNB **48**, despite the presence of the *meta* electron-donating amino group. Additionally, the *k* of MitoHexNAB **122** is slightly greater than that of CDNB **48** and approximately 4-fold greater than that of MitoCDNB **51**, even though its *para* amide group is less electron-withdrawing than a *para* nitro group. The greater rate of the sulfonamide compounds arises because the sulfonamide moiety exerts stronger electron-withdrawal by induction compared to a chloride group. Electron-withdrawing groups at the *ipso* position increase its electrophilicity for nucleophilic attack during the rate-determining step of S_NAr reactions. Although Hammett parameters do not describe the electronic effects of substituents at the *ipso* position, comparing the σ_m of substituents gives a good insight into their relative electron-withdrawal by induction since resonance is not possible. Chloride (Cl: $\sigma_m = +0.37$) is electronegative and small in size, which minimises steric hindrance for nucleophilic attack.²³⁷ Sulfonamides (SO_2NH_2 : $\sigma_m = +0.53$), though bulkier than a chloride, are far more electron-withdrawing by inductive effects due to the presence of electronegative oxygen and nitrogen atoms,^{237, 253} making them more reactive for S_NAr reactions.

The relative reactivity of the sulfonamide analogues broadly reflects that of the chloride analogues. MitoHexDNB **125** is >13-fold more reactive than MitoHexNAB **122**. Similarly, MitoCDNB **51** is moderately reactive, while amide **86a** shows no detectable reactivity. These results are expected as MitoHexDNB **125** is more electrophilic than MitoHexNAB **122**. Despite the presence of the electron-donating *meta* amino substituent, MitoHexDNB **125** has a *para* nitro group which is more electron-withdrawing by mesomeric effects than the *para* amide group in MitoHexNAB **122**.

It was initially surprising that MitoMetNAB **108** showed no detectable reaction with GSH **25**, especially given the high reactivity of MitoHexNAB **122**. On the one hand, MitoMetNAB **108** would be expected to react faster than MitoHexNAB **122** since the biguanide moiety is more inductively electron-withdrawing than hexylamine. On the other hand, the biguanide moiety may sterically impede nucleophilic attack by GSH **25**. However, subsequent observation by HPLC of MitoMetNAB **108** in HEPES (0.1 M) pH 8.0 buffer in the absence of GSH **25** or GST enzyme revealed slow decomposition of the compound. After 24 h, LCMS analysis (Figure 38) revealed almost complete conversion of MitoMetNAB **108** into a mixture of guanide compounds **150** and **151** (Scheme 57).

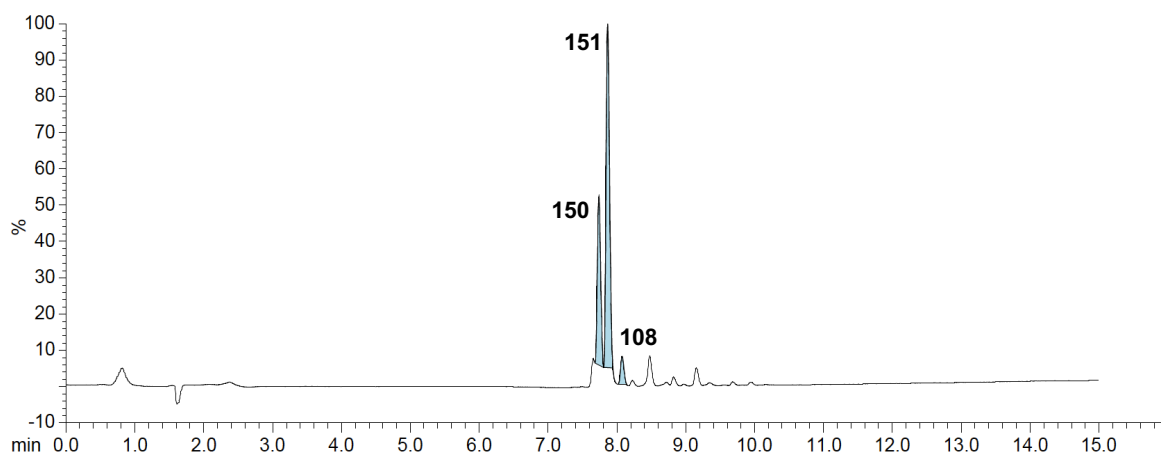
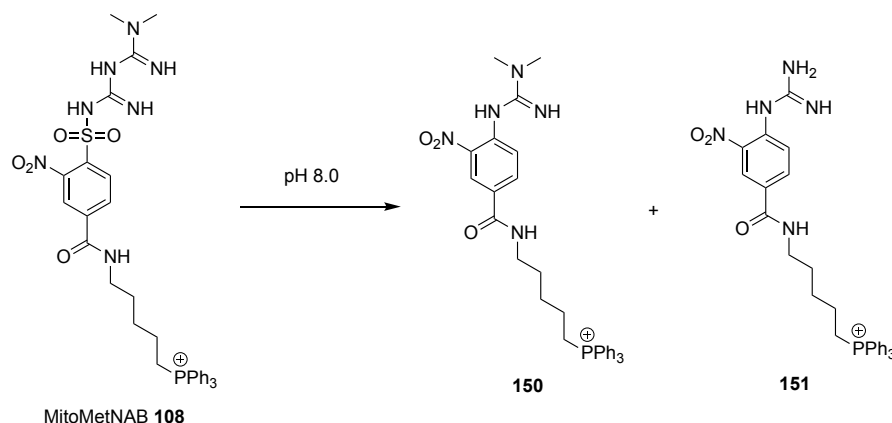


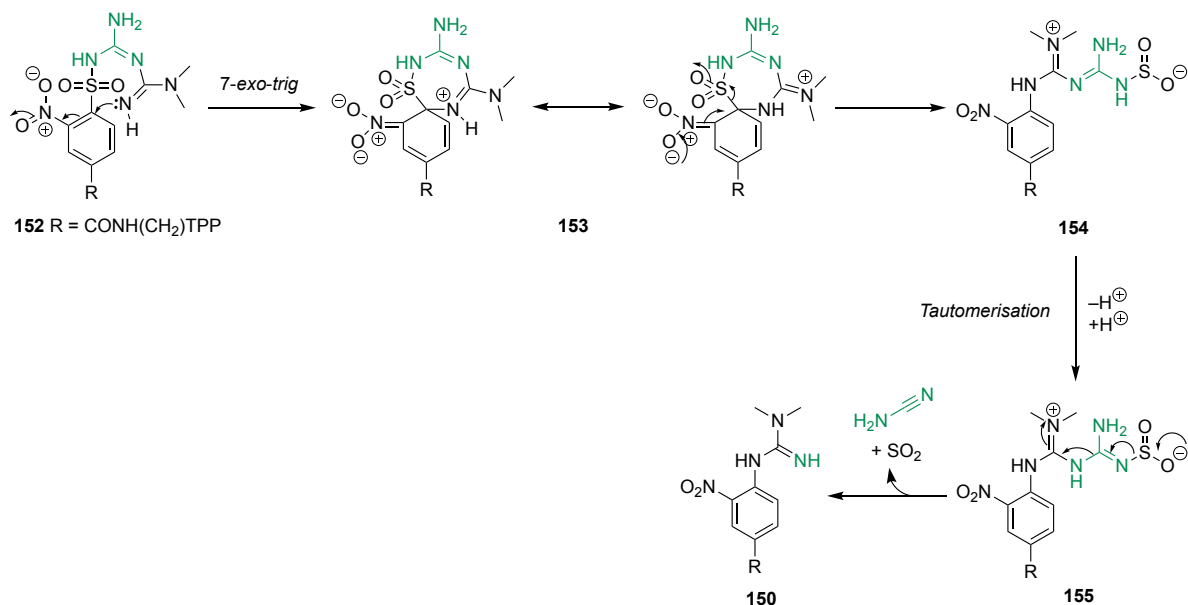
Figure 38. LCMS chromatogram at 254 nm after 24 h of incubation of MitoMetNAB 108 in pH 8.0 HEPES (0.1 M) buffer. Method: H₂O (0.1% formic acid): MeCN 10-90% 15 min.



Scheme 57. Decomposition of MitoMetNAB 26 in pH 8.0 HEPES (0.1 M) buffer to guanide compounds 62 and 63.

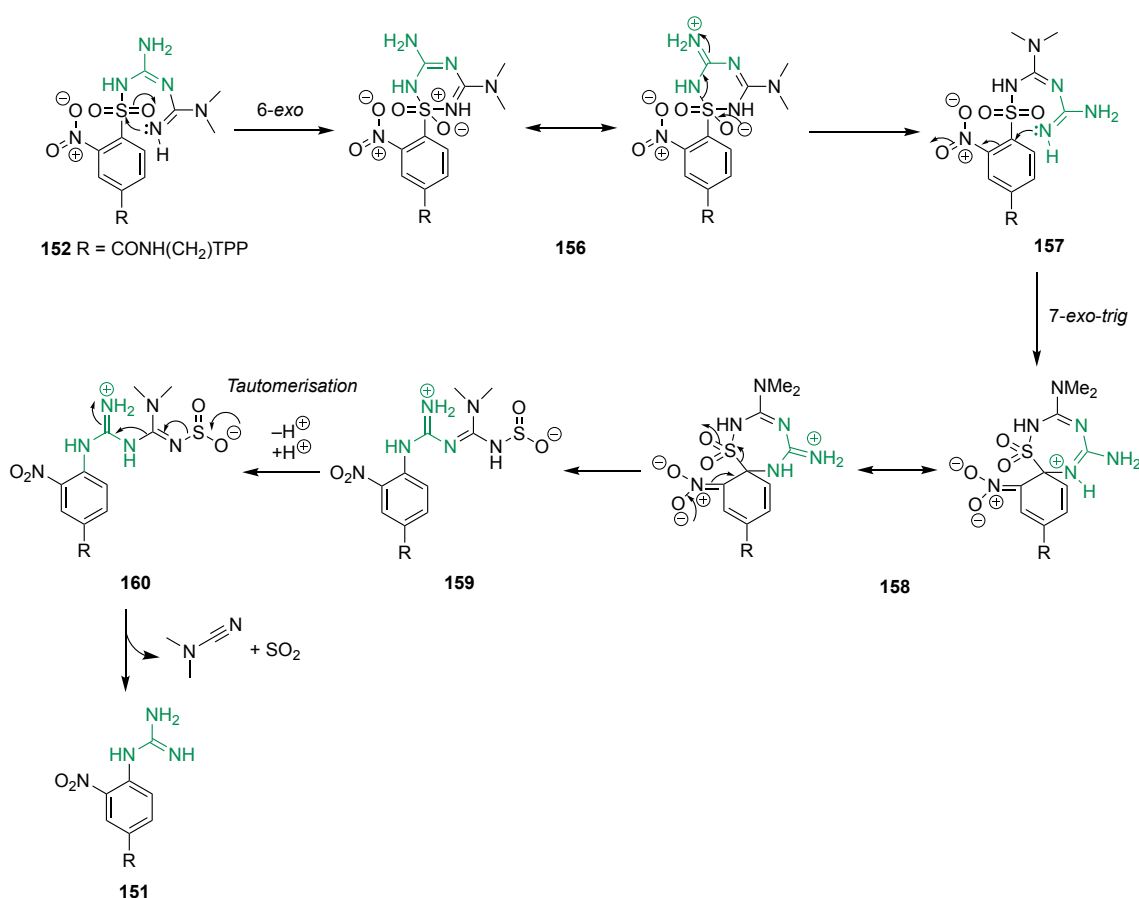
The mechanisms for the formation of guanide **150** (Scheme 58) and guanide **151** (Scheme 59) likely involve a S_NAr-type Smiles rearrangement, in which a nucleophilic imine nitrogen attacks the electrophilic *ipso* position of the electron-poor aromatic ring. The proposed mechanism for guanide **150** (Scheme 58) begins with a Smiles rearrangement of the tautomer of MitoMetNAB **152**. The most Lewis basic site of the biguanide moiety is the nitrogen of the imine furthest away from the sulfonyl group and its lone pair can attack the *ipso* position in a 7-*exo-trig* ring closure to form the 7-membered ring

Meisenheimer intermediate **153**, which can be represented by different resonance structures. In the second step, the sulfonyl group of the Meisenheimer intermediate **153** leaves the aromatic ring, producing biguanide **154**. Biguanide **154** can undergo tautomerisation to generate tautomer **155**, which subsequently fragments to SO₂, cyanamide and guanide **150**.



Scheme 58. Proposed mechanism for the decomposition of MitoMetNAB tautomer **152** to guanide **150**. The guanide moiety shown in green makes tracking of the process easier.

The proposed mechanism for the formation of guanide **151** (Scheme 59) is slightly more complex. Its first step competes with the Smiles rearrangement that leads to guanide **150**. Instead of attacking the *ipso* position, the nitrogen lone pair can attack the electrophilic sulfur atom in a 6-*exo* ring closure. This forms the 6-membered ring **156**, which can be represented by different resonance structures, and in which the sulfur atom has a trigonal bipyramidal geometry. Regeneration of the sulfonamide opens the ring to produce biguanide **157**. As before, the most Lewis basic imine nitrogen is situated furthest from the sulfonyl group and can attack the *ipso* position via a 7-*exo*-trig Smiles rearrangement to generate the Meisenheimer intermediate **158**, and subsequently, biguanide **159**. Biguanide **159** can undergo tautomerisation to generate tautomer **160**, which then fragments to SO₂, dimethylcyanamide and guanide **151**.



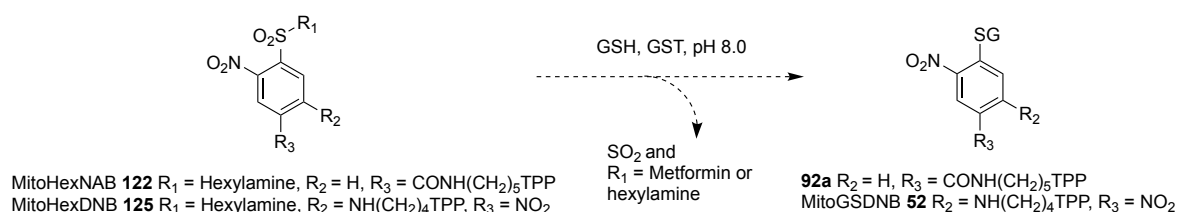
Scheme 59. Proposed mechanism for the decomposition of tautomer MitoMetNAB 152 to guanide 151. The guanide moiety shown in green makes tracking of the process easier.

Ultimately, the discovery that MitoMetNAB 108 is unstable in alkaline aqueous conditions indicated that it is unfeasible to develop a stable GST-activated prodrug in which metformin was linked to an electron-poor ring via a sulfonamide bond.

3.2.6 Kinetics of sulfonamides with GST enzymes

The next step was to measure the GST-catalysed GSH 25 conjugation reaction for sulfonamides MitoHexNAB 122 and MitoHexDNB 125 (Scheme 60) with the GST enzymes, hGSTA1-1, hGSTA4-4 and hGSTK1-1, previously expressed (see Section 2.3). Although the Michaelis-Menten parameters (k_{cat} , K_{M} and $k_{\text{cat}}/K_{\text{M}}$) provide valuable insights into an enzyme's activity for a substrate, their calculation requires numerous measurements at varying substrate concentrations, which consumes significant amounts of substrate

material. Given the limited quantities of compounds **122** and **125** available, their specific activity with the GST enzymes was calculated instead, as this requires rate measurements at only a single substrate concentration. Specific activity is a widely used parameter in enzyme kinetics that measures an enzyme's activity for a substrate at a specific concentration, defined as the amount of substrate in moles converted per minute per milligram of enzyme. Since specific activity depends on substrate concentration, all kinetic measurements were performed on 100 μM of substrate to allow for direct comparison of their reactivity. Prior to determining the GSTs' Michaelis-Menten parameters for the chloronitrobenzene derivatives CDNB **48**, MitoCDNB **51**, **85a-c**, and **86a-c** (see Section 2.4.3), their specific activities had been calculated, along with those of the sulfonamides **122** and **125**, using a GSH **25** concentration of 2 mM to provide a quick assessment of reactivity. Only after these measurements and the determination of the GSTs' K_M values for GSH **25** (see Section 2.4.1), were the Michaelis-Menten parameters calculated for the chloronitrobenzene derivatives using 10 mM GSH **25** to ensure that the GSTs were as close to saturation with respect to GSH **25** as possible. A GSH **25** concentration of 2 mM is still at least 3-fold higher than the apparent K_M values of each GST enzyme for GSH **25**, so it can be assumed that, under these conditions, the majority of GST enzyme active sites are occupied by GSH **25**. Several other studies have calculated Michaelis-Menten constants and specific activities of GST enzymes using sub-saturating concentrations of GSH **25**.^{131, 225, 254, 255} Overall, the specific activity measurements for sulfonamides **122** and **125** were straightforward to calculate, and give a reasonable indication of substrate reactivity. The background rates were subtracted from the observed rates to obtain the enzyme-catalysed rates, from which the specific activity values were calculated (Table 11). The specific activities for CDNB **48**, MitoCDNB **51** and amide **86a** were also calculated using 100 μM substrate and 2 mM GSH **25** (Table 11).



Scheme 60. Expected reaction of sulfonamides MitoHexNAB **122** and MitoHexDNB **125** with GSH **25** catalysed by GST enzyme at pH 8.0 to form corresponding GS-conjugates **92a** or MitoGSDNB **52**.

Compound	Substituents				Specific activity ($\mu\text{mol}\cdot\text{min}^{-1}\text{mg}^{-1}$)		
	1-	2-	4-	5-	hGSTA1-1	hGSTA4-4	hGSTK1-1
CDNB 48	Cl	NO ₂	NO ₂	H	105 $\pm$ 0.7	9.64 $\pm$ 0.58	1.51 $\pm$ 0.13
MitoCDNB 51	Cl	NO ₂	NO ₂	NH(CH ₂) ₄ TPP	73.8 $\pm$ 2.9	16.1 $\pm$ 1.0	(4.59 $\pm$ 0.06) $\times 10^{-2}$
MitoHexDNB 125	SO ₂ NH(CH ₂) ₅ CH ₃	NO ₂	NO ₂	NH(CH ₂) ₄ TPP	45.1 $\pm$ 8.1	33.0 $\pm$ 2.9	(5.76 $\pm$ 0.56) $\times 10^{-2}$
86a	Cl	NO ₂	CONH(CH ₂) ₅ TPP	H	5.25 $\pm$ 0.63	0.545 $\pm$ 0.023	ND
MitoHexNAB 122	SO ₂ NH(CH ₂) ₅ CH ₃	NO ₂	CONH(CH ₂) ₅ TPP	H	0.762 $\pm$ 0.131	4.07 $\pm$ 0.07	0.440 $\pm$ 0.041

Table 11. Specific activities of the GST enzymes for the reaction of compounds 48, 51, 125, 86a, 122 and 108 (ordered by substitution pattern) with 2 mM GSH 25 at pH 8.0 HEPES (0.1 M) buffer and 30 °C. Specific activities are presented as mean values (and errors as $\pm$ 1 standard deviation) of triplicate measurements. ND = No reaction detected. NS = Not stable in buffer.

The specific activity data indicate that CDNB **48** is a better substrate than MitoCDNB **51** for hGSTA1-1 and hGSTK1-1, but not for hGSTA4-4. Both CDNB **48** and MitoCDNB **51** outperform amide **86a** as substrates for all enzymes. These trends align with those observed from the compounds' $k_{\text{cat}}/K_{\text{M}}$ values (see Table 4, Section 2.4), validating the use of specific activity as a measure of substrate reactivity.

For each enzyme, the specific activities of MitoCDNB **51** and MitoHexDNB **125** are of the same order of magnitude, indicating comparable reactivity. The reactivity trend of the two compounds for the enzymes follows hGSTA1-1 > hGSTA4-4 > hGSTK1-1. Like MitoCDNB **51**, MitoHexDNB **124** exhibits particularly high reactivity with hGSTA1-1 and hGSTA4-4, which indicates that the *meta* TPP-alkylamino chain is well-accommodated by these enzymes. However, of these two enzymes, only hGSTA4-4 shows greater activity for MitoHexDNB **125** than MitoCDNB **51**: the specific activity of the former is twice that of the latter. In contrast, the specific activity of hGSTA1-1 for MitoHexDNB **125** is 1.6-fold lower than for MitoCDNB **51**. This is contrary to expectations given that MitoHexDNB **125** is intrinsically far more reactive than MitoCDNB **51**, as evidenced by its 60-fold greater background rate with GSH **25** (see Table 10, Section 3.2.5). These results suggest that the hexylsulfonamide moiety introduces steric hindrance that impairs favourable binding to both hGSTA1-1 and hGSTA4-4. However, in the case of hGSTA4-4, the steric effects are compensated by the sulfonamide's greater intrinsic reactivity and any additional hydrophobic interactions of the hexyl chain. As expected, replacing the *para* nitro group in MitoCDNB **51** and MitoHexDNB **125** for a less electron-withdrawing *para* amide group in amide **86a** and MitoHexNAB **122** reduces substrate reactivity. Interestingly, hGSTA1-1 exhibits approximately 7-fold lower specific activity for MitoHexNAB **122** than for amide **86a**, despite MitoHexNAB **122**'s greater intrinsic reactivity. This suggests that hGSTA1-1 does not accommodate large substituents placed *para* to one another effectively. In contrast, hGSTA4-4 shows >7-fold higher specific activity for MitoHexNAB **122** than for amide **86a**, consistent with its greater intrinsic reactivity and indicates that the substitution pattern is well-accommodated by the enzyme. Although MitoHexNAB **122** is a poorer substrate for hGSTK1-1 than for the alpha enzymes, it is >7-fold more reactive than MitoHexDNB **125**, despite the latter's greater intrinsic reactivity (see Table 10, Section 3.2.5). This indicates that hGSTK1-1 better accommodates large hydrophobic substituents when placed *para* to one another than when they are *meta* to one another. Overall, the results from the syntheses and kinetic measurements of the sulfonamide compounds present clear conclusions. First, in most cases, the dinitro ring system is critical for high substrate reactivity with GSH/GST. Second, this high reactivity renders

the dinitro ring too unstable to develop a metformin or arylamino prodrug by directly linking to this ring via a sulfonamide moiety. Third, a prodrug of metformin (or perhaps any biguanide drug) linked to an electron-poor ring via a sulfonamide moiety is prone to decomposition in alkaline aqueous solution. Fourth, sulfonamides with a straight aliphatic chain conjugated to the dinitro ring system, as in MitoHexDNB **125**, are synthetically accessible, stable in alkaline aqueous solution, and highly reactive for GSH **25** conjugation with or without GST catalysis.²⁵² Consequently, attention shifted to approaches B and C, focusing on prodrug designs in which the cargo is separated from the dinitro ring by a straight-chain self-immolative linker.

3.3 Approach B

Triggered cyclative cleavage of an ethylenediamine derived carbamate is a widely used strategy to release hydroxyl-bearing payloads.²⁵⁶⁻²⁶⁰ There was an interest to investigate this strategy to release phenoxy- and amine-bearing cargos by GSH/GST activation from a sulfonamide based on the general structure in Figure 39. There are four possible sites of nucleophilic attack by the glutathione thiolate. As explored in Section 3.2, attack at (a) is expected to be favoured over attack at (b) for an electron-poor ring A. Attack at (c) to form a *S*-thiocarbamate is unlikely because the pK_a of glutathione is lower than that of a typical phenol or amine, although it depends on the specific cargo. Attack at (d) by S_NAr relies on an electron poor ring B.

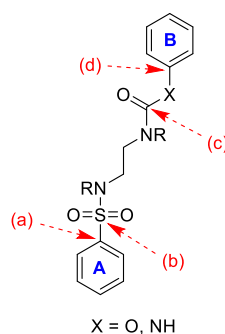
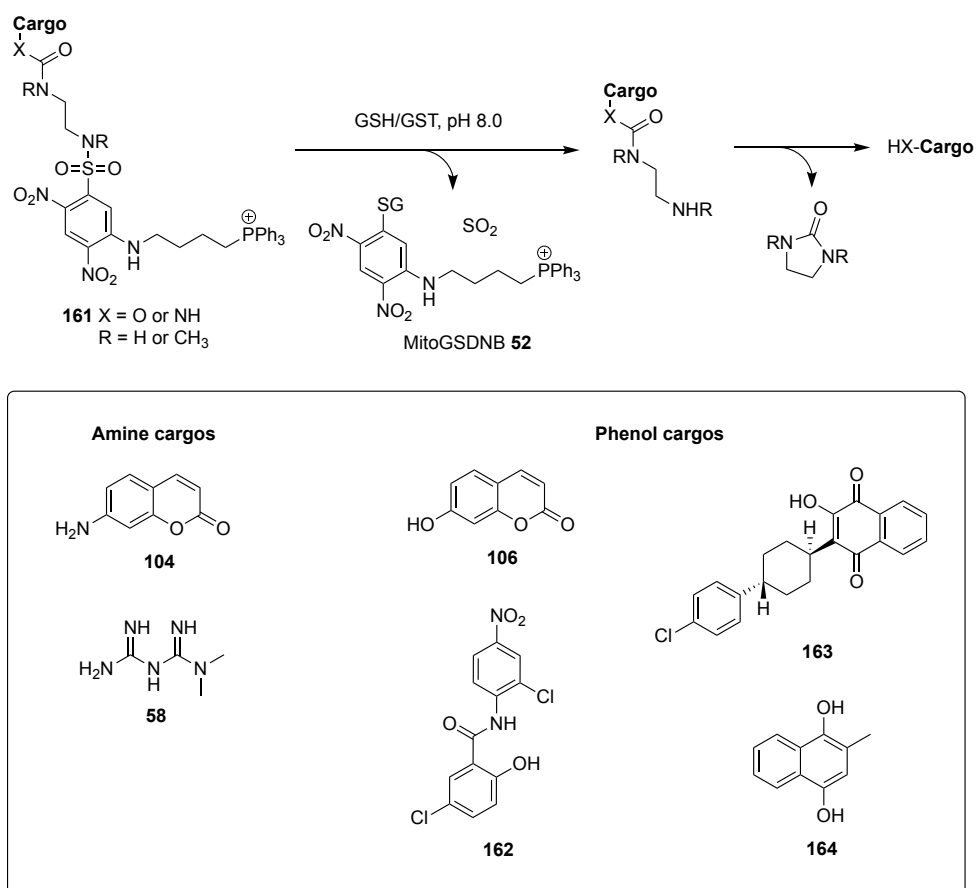


Figure 39. Possible sites of nucleophilic attack by a thiol on an aryl arenesulfonate ester or aryl arenesulfonamide.

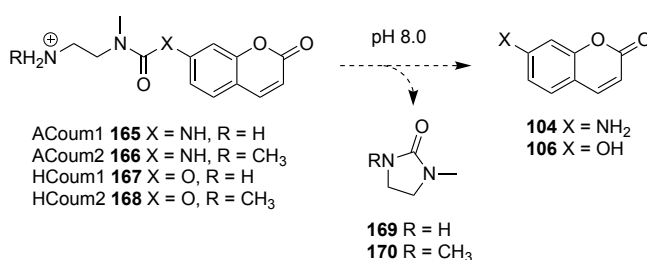
The general structure in Figure 39 was applied to design prodrug scaffold **161** (Scheme 61), which is based on the structures of the highly reactive substrates MitoCDNB **51** and MitoHexDNB **125**. Glutathione attack at (a) is favoured by mesomeric withdrawal to the *ortho* and *para* nitro groups and inductive withdrawal by the sulfonamide. The reaction of prodrug design **161** with GSH **25** is expected to proceed in two main steps (Scheme 61). In the first step, conjugation of GSH **25** by S_NAr is expected to produce MitoGSDNB **52**. Release of sulfur dioxide to generate the amine intermediate is assumed to be fast. In the second step, the ethylenediamine fragment cyclises under basic aqueous conditions to form a 5-membered cyclic urea, thereby releasing the active cargo.



Scheme 61. Prodrug design **161** for approach B. Activation of prodrug **161** by GSH **25** with or without GST catalysis should release MitoGSDNB **52**, the ethylenediamine intermediate and SO₂. Under alkaline aqueous conditions, the intermediate should form the 5-membered cyclic urea, releasing the amine or phenol cargo.

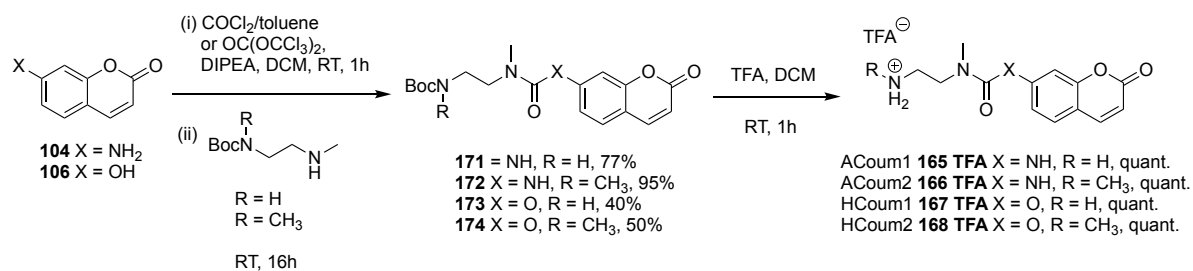
3.3.1 Synthesis of untargeted ethylenediamine coumarin derivatives

First, the release of phenol and amine cargos would be investigated by using simple precursors **165-168** of the fluorophores 7-aminocoumarin **104** and 7-hydroxycoumarin **106**. ACoum1 **165** and ACoum2 **166** were designed to release 7-aminocoumarin **104**, while HCoum1 **167** and HCoum2 **168** were designed to release 7-hydroxycoumarin **106** (Scheme 62). Cyclisation of compounds **165** and **167**, and **166** and **168**, was expected to form cyclic urea **169** or **170**, respectively, as the by-product.



Scheme 62. Proposed reaction of ethylenediamine coumarins derivatives **165-168** at pH 8.0.

Saari *et al.*²⁵⁶ had reported that a methyl group attached to either one or both of the nitrogen atoms of the ethylenediamine spacer increased the rate of cyclisation. Hence, analogues were prepared, containing either a *N*-methyl carbamate or a *N*-methyl urea at the fluorophore end and either a primary amine (as in ACoum1 **165** and HCoum1 **167**) or *N*-methyl secondary amine (as in ACoum2 **166** and HCoum2 **168**) at the terminal nitrogen. The Boc derivatives **171-174** were prepared from the appropriate coumarins **104** and **106** by reaction with phosgene or triphosgene, followed by coupling with either *N*-boc-*N*-methylethylenediamine or tert-butyl methyl(2-(methylamino)ethyl)carbamate, which were commercially available (Scheme 63). Ureas **171** and **172** were obtained in a good and excellent yield, respectively, while carbamates **173** and **174**, were both obtained in a moderate yield. Boc deprotection with trifluoroacetic acid afforded the ethylenediamine coumarin analogues **165-168** as trifluoroacetate salts in quantitative yield.



Scheme 63. Synthesis of ethylenediamine coumarin derivatives 165-168.

3.3.2 Kinetics of untargeted ethylenediamine coumarin derivatives

The release of the coumarin cargo when the ethylenediamine coumarin derivatives **165-168** were treated with pH 8.0 buffer was studied. The reaction was followed by monitoring the increase in emission from the released fluorescent coumarins **104** and **106**. A calibration curve was created for coumarins **104** and **106** by measuring the emission intensity of the coumarin at different concentrations (see Appendix). When 7-aminocoumarin **104** was excited at the wavelength of maximum absorbance, $\lambda_{\text{ex}} = 348 \text{ nm}$, maximum emission was observed at $\lambda_{\text{em}} = 454 \text{ nm}$ (Figure 40). When 7-hydroxycoumarin **106** was excited at the wavelength of maximum absorbance, $\lambda_{\text{ex}} = 342 \text{ nm}$, maximum emission was observed at $\lambda_{\text{em}} = 454 \text{ nm}$ (Figure 40).

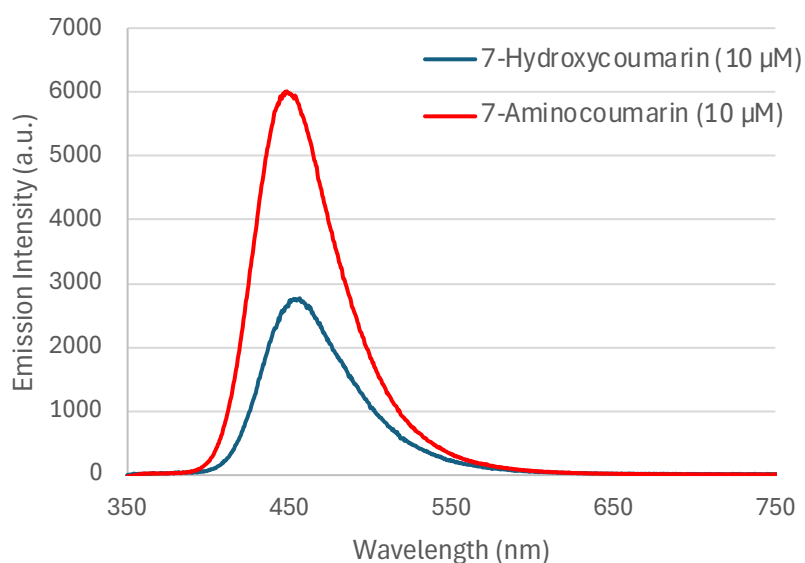


Figure 40. Emission spectra of 7-aminocoumarin 104 (10 μM) and 7-hydroxycoumarin 106 (10 μM) in pH 8.0 HEPES (0.1 M) buffer. 7-Aminocoumarin: λ_{ex} = 348 nm, λ_{em} = 454 nm. 7-hydroxycoumarin 106: λ_{ex} = 342 nm, λ_{em} = 454 nm.

For the reactions of coumarin analogues **165-168** (Figure 41) in pH 8.0 buffer, the initial rate was measured for 120 s with arbitrary units of emission per second. This was converted to concentration per second using the appropriate calibration curve for the coumarin being released. Finally, the rate was divided by the concentration of the prodrug used in the assay to obtain the first-order rate constant, k (Table 12).

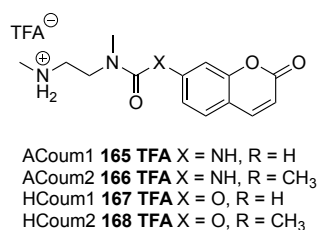


Figure 41. Structures of ethylenediamine coumarin derivatives **165-168**.

Compound	X group	R group	First-order rate constant k ($\times 10^{-3} \text{ s}^{-1}$)
ACoum1 165	NH	H	ND
ACoum2 166	NH	CH ₃	ND
HCoum1 167	O	H	0.770 ± 0.085
HCoum2 168	O	CH ₃	3.76 ± 0.09

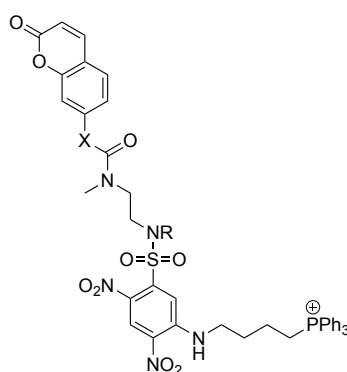
Table 12. First-order rate constant (k) for the reaction of coumarin prodrugs **165-168** in pH 8.0 buffer, presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements. ND = No reaction detected.

ACoum1 **165** and ACoum2 **166** did not release 7-aminocoumarin **104** in pH 8.0 buffer, but HCoum1 **167** and HCoum2 **168** successfully released 7-hydroxycoumarin **106**. This discovery supports the literature that the ethylenediamine strategy can only be used to release hydroxy-bearing payloads. The rate constant k for the cyclisation of di-*N*-methyl derivative **168** is approximately 5-fold greater than that of mono-*N*-methyl derivative **167**. This is in broad agreement with the study by Saari *et al.*²⁵⁶ on the release of 4-hydroxyanisole from various carbamates derivatives of the ethylenediamine spacer. The study found that release of 4-hydroxyanisole was approximately 8-times faster at pH 7.4 when both nitrogen atoms were methylated as opposed to only a methyl group on the carbamate nitrogen.²⁵⁶ It is possible that when both nitrogen atoms are *N*-methylated, the methyl groups pre-organise the ethylenediamine fragment into a favourable conformation for cyclisation in water.

3.3.3 Synthesis of TPP-conjugated ethylenediamine coumarin derivatives

After determining that the ethylenediamine prodrug strategy could be used to release phenols but not amines (see Section 3.3.2), efforts focused on preparing mitochondria-targeted GSH-activated derivatives of 7-hydroxycoumarin **106** based on prodrug scaffold **161**. Given the high un-catalysed reactivity of MitoHexDNB **125** with GSH **25** (see Section 3.2.4), it seemed that GST enzymes may not be necessary for fast activation of these compounds. As discussed, the release of the cargo from scaffold **161** is expected to

proceed in two major steps: first, reaction with GSH **25**, and second, cyclisation of the intermediate under alkaline aqueous conditions. In the case of 7-hydroxycoumarin precursors based on scaffold **161**, the effective rate constants for the second step were calculated in Section 3.3.2. However, there was also an interest to obtain the rate constants for the initial reaction with GSH **25** to establish which of the two steps is rate-determining. Therefore, two TPP-conjugated derivatives of 7-aminocoumarin, MitoACoum1 **175** and MitoACoum2 **176** (Figure 42), would be prepared to allow the kinetics of the first step with GSH **25** to be studied. These compounds were expected to react with GSH **25** initially to produce the deprotonated form of coumarins ACoum1 **165** and ACoum2 **166**, respectively, which do not cyclise as shown above (see Section 3.3.2). Additionally, TPP-conjugated derivatives of 7-hydroxycoumarin, MitoHCoum1 **177** and MitoHCoum2 **178** (Figure 42), would be synthesised, which should react with GSH **25** to initially produce the deprotonated form of HCoum1 **167** and HCoum2 **168**, respectively, and consequently cyclise to release 7-hydroxycoumarin **106**.

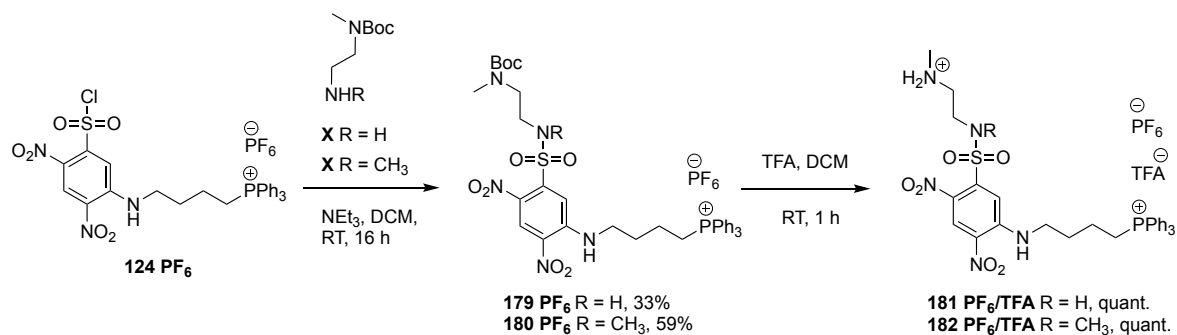


MitoACoum1 **175** X = NH, R = H
MitoACoum2 **176** X = NH, R = CH₃
MitoHCoum1 **177** X = O, R = H
MitoHCoum2 **178** X = O, R = CH₃

Figure 42. Structure of mitochondria-targeted ethylenediamine coumarin derivatives 175-178.

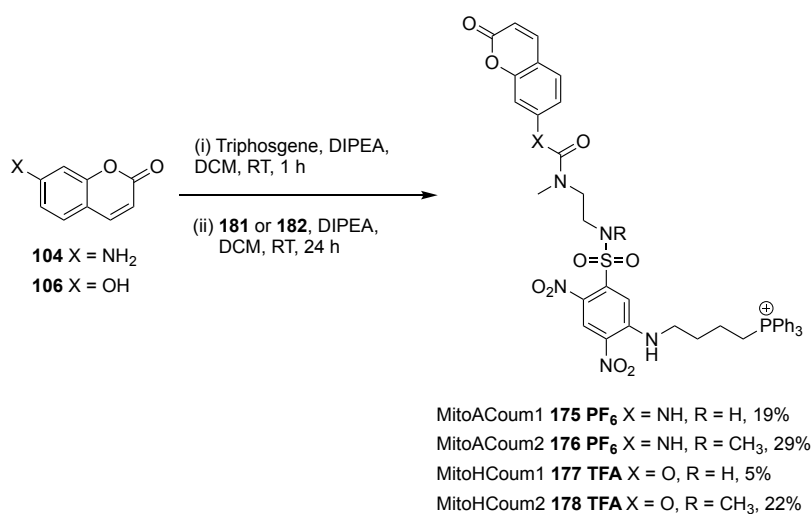
Starting from sulfonyl chloride **124**, previously synthesised in five steps (see Section 3.2.1), coupling with N-Boc-N-methylethylenediamine or tert-butyl methyl(2-(methylamino)ethyl)carbamate afforded carbamates **179** and **180**, respectively, in low to moderate yields (Scheme 64). Boc-deprotection with trifluoroacetic acid afforded the

ammonium compounds **181** and **182** as a mixture of the hexafluorophosphate and trifluoroacetate salts in quantitative yield.



Scheme 64. Synthesis of TPP-conjugated ammonium salts **181** and **182**.

Reaction of 7-aminocoumarin **104** with triphosgene, followed by addition of ammonium salt **181** or **182**, produced 7-aminocoumarin precursors MitoACoum1 **175** and MitoACoum2 **176**, respectively, as hexafluorophosphate salts in poor yield (Scheme 65). Reaction of 7-hydroxycoumarin **106** with triphosgene, followed by addition of ammonium salt **181** or **182**, produced 7-hydroxycoumarin precursors MitoHCoum1 **177** and MitoHCoum2 **178**, respectively, as trifluoroacetate salts in poor yield.



Scheme 65. Synthesis of TPP-conjugated coumarin derivatives **175-178**.

3.3.4 Kinetics of TPP-conjugated ethylenediamine coumarin derivatives

The next step was to test the reactivity of the TPP-conjugated coumarin derivatives **175-178** with GSH **25**. The compounds **175-178** were incubated at a concentration of 10 μ M in pH 8.0 buffer at 30 °C and treated with excess GSH **25** so that the reaction was pseudo-first-order. As before, the reaction was measured in triplicate by monitoring the release of the coumarin by its fluorescence. Initial rates were then converted into concentration per unit time using the calibration curve for the appropriate coumarin (see Appendix). MitoCoum1 **175** and MitoCoum2 **176** were expected to release ACoum1 **165** and ACoum2 **166**, respectively, which were previously synthesised (see Section 3.3.1) and found to be stable in pH 8.0 buffer (see Section 3.3.2). ACoum1 **165** and ACoum2 **166** were fluorescent in pH 8.0 buffer, meaning their release could be monitored by fluorescence. Calibration curves were created for ACoum1 **165** and ACoum2 **166** by measuring the emission intensity of the coumarin at different concentrations (see Appendix). The fluorescence of compounds **175-178** in pH 8.0 buffer was quenched. The emission spectra of compounds **175-178** and their corresponding coumarin cargos are presented in Figures 43-45. The kinetics for the conversion of MitoACoum1 **175** to ACoum1 **165** were measured by exciting at the wavelength of maximum absorbance of the ACoum1 **165**, $\lambda_{\text{ex}} = 330$ nm, and monitoring emission at the wavelength of maximum emission, $\lambda_{\text{em}} = 414$ nm (Figure 43, for calibration curve see Appendix).

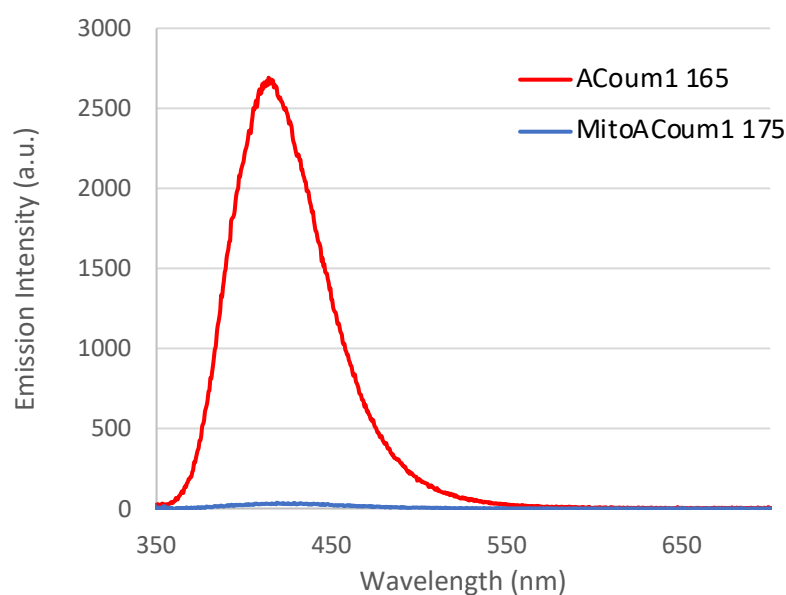


Figure 43. Emission spectra of MitoACoum1 175 (10 μM) and ACoum1 165 (10 μM) in pH 8 HEPES (0.1 M) buffer. Excitation wavelength, λ_{ex} = 330 nm. For ACoum1 165, the wavelength of maximum emission was 414 nm.

The kinetics for the conversion of MitoACoum2 176 to ACoum2 166 were measured by exciting at the wavelength of maximum absorbance of the ACoum2 166, λ_{ex} = 328 nm, and monitoring emission at the wavelength of maximum emission, λ_{em} = 454 nm (Figure 44, for calibration curve see Appendix).

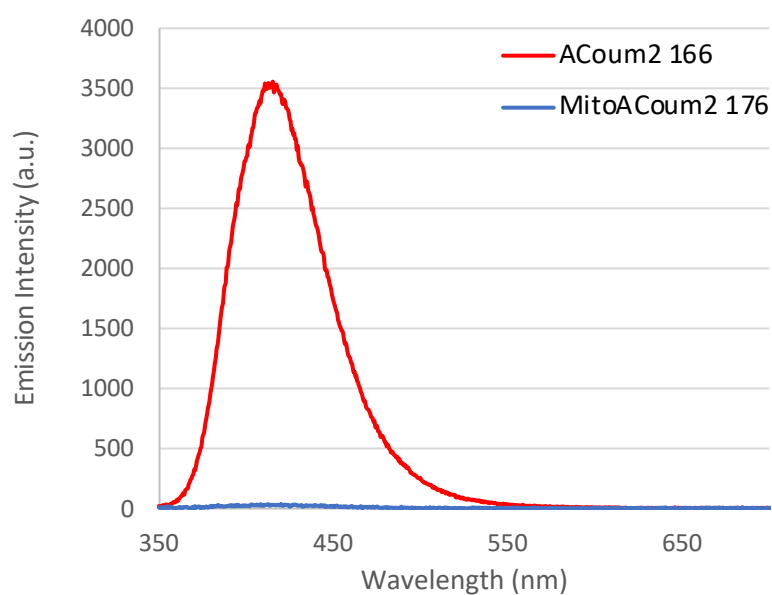


Figure 44. Emission spectra of MitoACoum2 176 (10 μ M) and ACoum2 166 (10 μ M) in pH 8 HEPES (0.1 M) buffer. Excitation wavelength, λ_{ex} = 328 nm. For ACoum2 166, the wavelength of maximum emission was 415 nm.

The kinetics for the conversion of compounds MitoHCoum1 177 and MitoHCoum2 178 to 7-hydroxycoumarin 106 were measured by exciting at the wavelength of maximum absorbance of 7-hydroxycoumarin 106, λ_{ex} = 342 nm, and monitoring emission at the wavelength of maximum emission, λ_{em} = 454 nm (Figure 45, for calibration curve see Appendix).

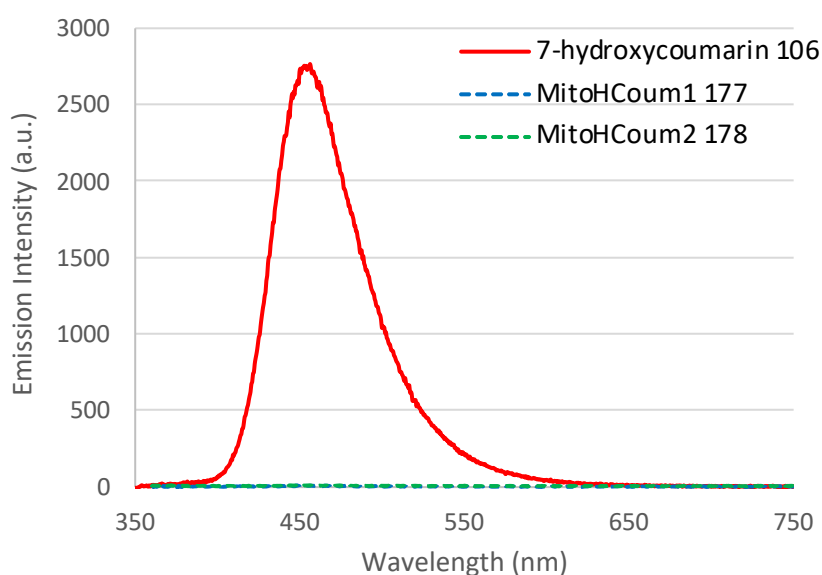


Figure 45. Emission spectra of MitoHCoum1 177 (10 μM), MitoHCoum2 178 (10 μM) and 7-hydroxycoumarin 106 (10 μM) in pH 8 HEPES (0.1 M) buffer. Excitation wavelength, λ_{ex} = 342 nm. For 7-hydroxycoumarin 106, the wavelength of maximum emission was 454nm.

From the initial rate data, pseudo-first-order and second-order rate constants were calculated for the TPP-conjugated coumarin derivatives 175-178 (Figure 46, Table 13).

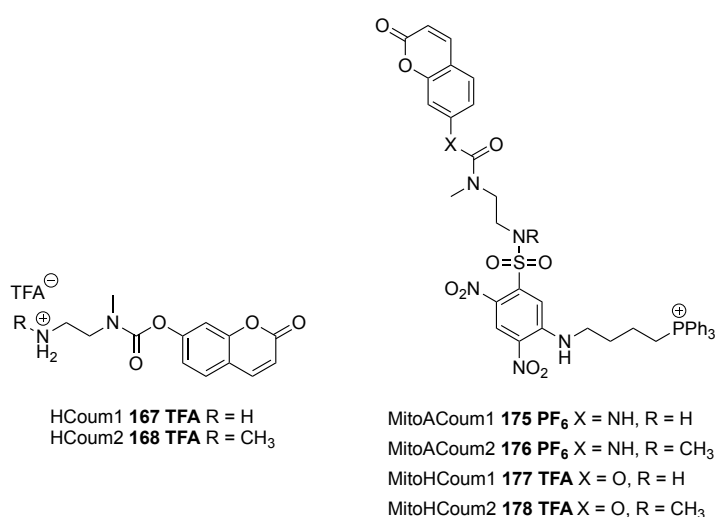
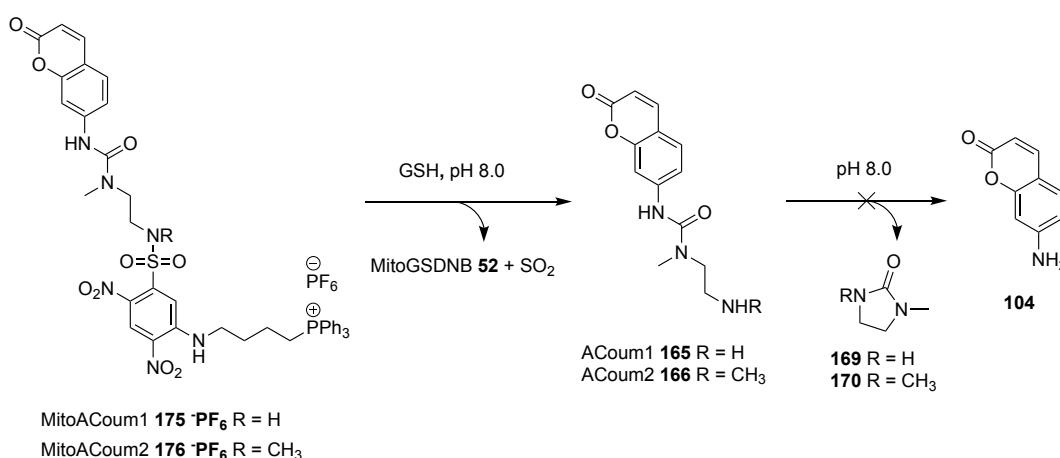


Figure 46. Structures of untargeted coumarin derivatives, HCoum1 167 and HCoum2 168, and TPP-conjugated coumarin derivatives 175-178.

Compound	X group	R group	First-/pseudo-first-order rate constant k ($\times 10^{-3} \text{ s}^{-1}$)	Second-order rate constant k' ($\text{M}^{-1}\text{s}^{-1}$)	First-/pseudo-first-order rate constant k ($\times 10^{-3} \text{ s}^{-1}$) adjusted for 10 mM GSH
HCoum1 167	O	H	$0.770 \pm 0.085^{(a)}$	N/A	$0.770 \pm 0.085^{(a)}$
HCoum2 168	O	CH ₃	$3.76 \pm 0.09^{(a)}$	N/A	$3.76 \pm 0.09^{(a)}$
MitoACoum1 175	NH	H	$1.28 \pm 0.3^{(b)}$	$0.640 \pm 0.153^{(b)}$	$6.40 \pm 1.53^{(b)}$
MitoACoum2 176	NH	CH ₃	$3.23 \pm 0.19^{(b)}$	$1.61 \pm 0.09^{(b)}$	$16.1 \pm 0.9^{(b)}$
MitoHCoum1 177	12	H	$0.532 \pm 0.019^{(c)}$	$0.0532 \pm 0.0019^{(c)}$	$0.532 \pm 0.019^{(c)}$
MitoHCoum2 178	O	CH ₃	$3.39 \pm 0.01^{(c)}$	$0.339 \pm 0.001^{(c)}$	$3.39 \pm 0.01^{(c)}$

Table 13. Rate constants for the reactions of coumarin derivatives 167-168 and 175-78 at pH 8.0 with or without excess GSH 25. Rate constants are presented as mean values (and errors as ± 1 standard deviation) of triplicate measurements. Conditions: (a) 10 μM compound, pH 8.0 HEPES (0.1 M) buffer, 30 °C. (b) 10 μM compound, 2 mM GSH 25, pH 8.0 HEPES (0.1 M) buffer, 30 °C. (c) 10 μM compound, 10 mM GSH 25, pH 8.0 HEPES (0.1 M) buffer, 30 °C.

When TPP-conjugated aminocoumarin derivatives MitoACoum1 **175** and MitoACoum2 **176** were treated with 10 mM GSH **25** to mimic the GSH **25** concentration in the mitochondrial matrix, the reaction was too fast to measure an accurate initial rate, so 2 mM GSH **25** was used instead. The kinetic results demonstrated that MitoACoum1 **175** and MitoACoum2 **176** react rapidly with GSH **25** to release the stable ethylenediamine coumarins, ACoum1 **165** and ACoum2 **166**, respectively (Scheme 66, Table 13). The initial rate graphs for MitoACoum1 **175** and MitoACoum2 **176** can be found in the Appendix.



Scheme 66. Reaction of MitoACoum1 **175** and MitoACoum2 **176** with GSH **25** to produce ACoum1 **165** and ACoum2 **166**, respectively, which were stable to release of 7-aminocoumarin **104** at pH 8.0.

Interestingly, MitoACoum2 **176** reacts with GSH **25** approximately 2.5-fold faster than MitoACoum1 **175**. This difference in reactivity may be attributed to the hydrogen-bonding potential of the secondary sulfonamide in MitoACoum1 **175** (Figure 47). The hydrogen atom of the secondary sulfonamide could form a H-bond with either of the oxygen atoms of the *ortho* nitro group, which may make the sulfonamide group less electron-withdrawing by induction, leading to a slower $\text{S}_{\text{N}}\text{Ar}$ reaction. In contrast, MitoACoum2 **77** is a tertiary sulfonamide, meaning this interaction is not possible.

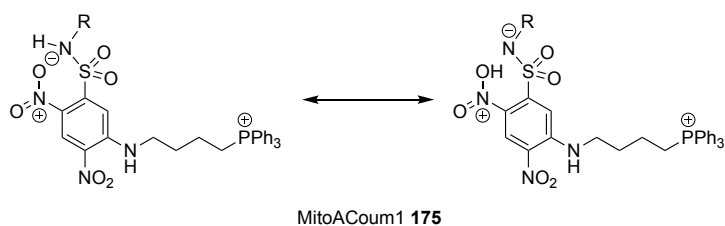
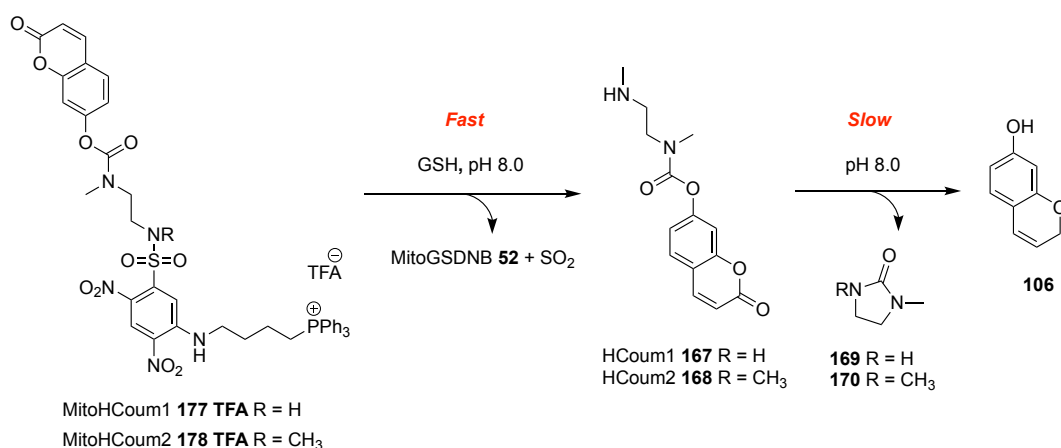


Figure 47. Possible H-bonding between NH and NO₂ may make the sulfonamide group in MitoACoum1 **76** less electron-withdrawing by induction.

The pseudo-first-order rate constant k of MitoACoum1 **175** and MitoACoum2 **176** was adjusted for 10 mM GSH **25** to compare the rate of GSH **25** activation with the rate of the cyclisation of HCoum1 **167** and HCoum2 **168** at physiological conditions in the mitochondrial matrix. At 10 mM GSH **25**, the k of MitoACoum1 **175** is approximately 8.3-fold greater than that of HCoum1 **167**. At 10 mM GSH **25**, the k of MitoACoum2 **176** is approximately 4.3-fold greater than that of HCoum2 **168**. Therefore, in both cases, GSH **25** activation is faster than cyclisation. As expected, the TPP-conjugated 7-hydroxycoumarin derivatives MitoHCoum1 **177** and MitoHCoum2 **178** reacted with 10 mM GSH **25** in pH 8.0 buffer to release 7-hydroxycoumarin **106** via a two-step reaction (Scheme 67). The initial rate of MitoHCoum1 **177** was measured between 120 and 300 s where the reaction was linear after an initial period of lag due to the GSH **25** activation step (see Appendix). No lag was observed in the reaction of MitoHCoum2 **178**, allowing the initial rate to be measured between 0 and 180 s (see Appendix). Analysis of the kinetic constants indicates that, for the reactions of MitoHCoum1 **177** and MitoHCoum2 **178** with 10 mM GSH **25**, the cyclisation of the intermediates HCoum1 **167** and HCoum2 **168** to release 7-hydroxycoumarin **106** is the rate-determining step. The k of MitoHCoum1 **177** is smaller but similar to that of its analogue HCoum1 **167**. This indicates that the rate of reaction of MitoHCoum1 **177** is largely dependent on the cyclisation of the intermediate HCoum1 **167**, but that there is minor contribution from the first step, the reaction with GSH **25**. Similarly, the k of MitoHCoum2 **178** is only slightly smaller than that of its analogue HCoum2 **168**, which indicates that there is very little contribution to the rate from the GSH **25** activation step. In theory, if the concentration of GSH **25** is high enough, conversion of the MitoHCoum1 **177** and MitoHCoum2 **178** to HCoum1 **167** and HCoum2 **168**, respectively, should be instantaneous. Therefore, the rate of reaction of MitoHCoum1 **177** and MitoHCoum2 **178** should be equal to that of HCoum1 **167** and HCoum2 **168**, respectively, assuming that the release of SO₂ is also instantaneous.

Comparison with the corresponding 7-aminocoumarin analogues MitoACoum1 **175** and MitoACoum2 **176** further supports that the cyclisation step is rate-determining. The second-order rate constant k' (or k adjusted to 10 mM GSH **25**) of MitoACoum1 **175** is approximately 12-fold greater than that of MitoHCoum1 **177**, which reflects the comparison between MitoACoum1 **175** and HCoum1 **167** (see above). The k' (or k adjusted to 10 mM GSH **25**) of MitoACoum2 **176** is approximately 4.7-fold greater than that of MitoHCoum2 **178**, which mirrors the comparison between MitoACoum2 **176** and HCoum2 **168** (see above).



Scheme 67. Reaction of MitoHCoum1 **177** and MitoHCoum2 **178** with 10 mM GSH **25** in pH 8.0 buffer. Reaction with GSH **25** is rapid to produce HCoum1 **167** or HCoum2 **168**. Cyclisation to release 7-hydroxycoumarin **106** is slower, and thus, the rate-determining step.

The reactions of MitoHCoum1 **177** and MitoHCoum2 **178** with 10 mM GSH **25** were also monitored to completion (Figure 48 and Figure 49, respectively). MitoHCoum2 **178** reacted rapidly and was measured in triplicate, whereas MitoHCoum1 **177** was much slower and measured only once. Both reactions appeared to demonstrate pseudo-first-order kinetics with respect to the cyclisation step, indicating that it is rate-determining. To validate this, the reactions of MitoHCoum1 **177** and MitoHCoum2 **178** were treated as first-order and their predicated graphs were calculated (Figure 48 and Figure 49, respectively) using the integrated rate law for a first-order reaction and their respective k experimentally determined at 10 mM GSH **25**. The observed reactions of MitoHCoum1 **177** and MitoHCoum2 **178** closely resemble their predicted reactions when treated as first-order, highlighting that the cyclisation step is rate-determining at 10 mM GSH **25**.

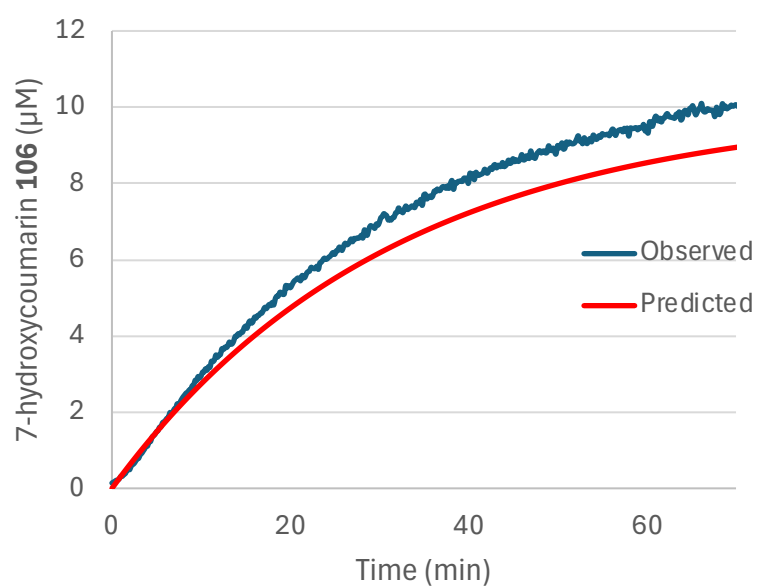


Figure 48. Blue line shows the observed single-run of MitoHCoum1 177 (10 μ M) in GSH 25 (10 mM), HEPES (100 mM) pH 8.0 buffer at 30 $^{\circ}$ C. Red line shows the predicted reaction calculated using the integrated rate law for a first-order reaction and $k = (0.532 \times 0.019) \times 10^{-3} \text{ s}^{-1}$ determined from the reaction of MitoHCoum1 177 with 10 mM GSH 25.

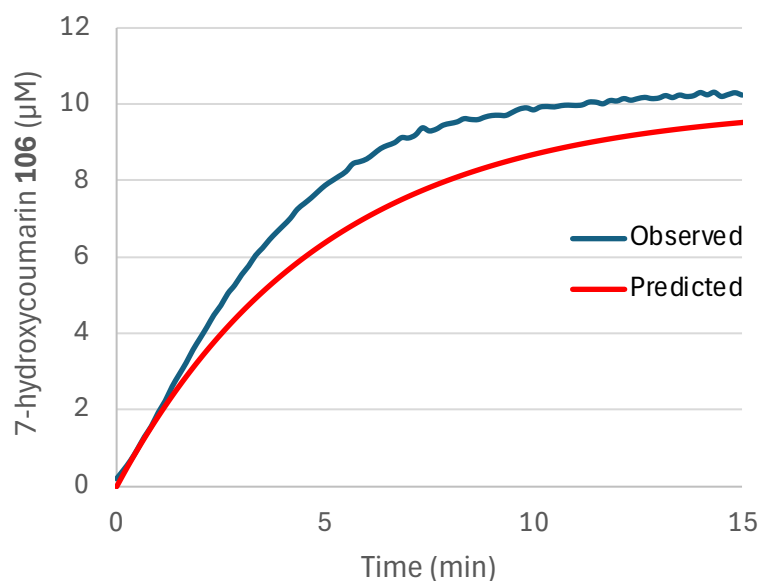


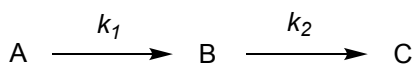
Figure 49. Blue line shows the observed triplicate-run of MitoHCoum2 178 (10 μ M) in GSH 25 (10 mM), HEPES (100 mM) pH 8.0 buffer at 30 $^{\circ}$ C. Red line shows the predicted reaction calculated using the integrated rate law for a first-order reaction and $k = (3.39 \times 0.01) \times 10^{-3} \text{ s}^{-1}$ determined from the reaction of MitoHCoum2 178 with 10 mM GSH 25.

In conclusion, the ethylenediamine spacer is an effective self-immolative linker to release phenol cargos but not amines from a sulfonamide attached to an electron-poor aromatic system. At physiological conditions in the mitochondrial matrix, the release of the phenol cargo by cyclisation is rate-determining, meaning that GST catalysis is not important. The next objective for the research was to use the ethylenediamine linker in prodrugs designed to deliver mitochondrially active drugs to the mitochondrial matrix. However, before describing this work (in Section 3.3.6) it is worth considering whether more information about the release of the cargo can be acquired from the model compounds MitoACoum1 175, MitoACoum2 176, HCoum1 167 and HCoum2 168 using a more complex analysis.

3.3.5 Consecutive reaction model

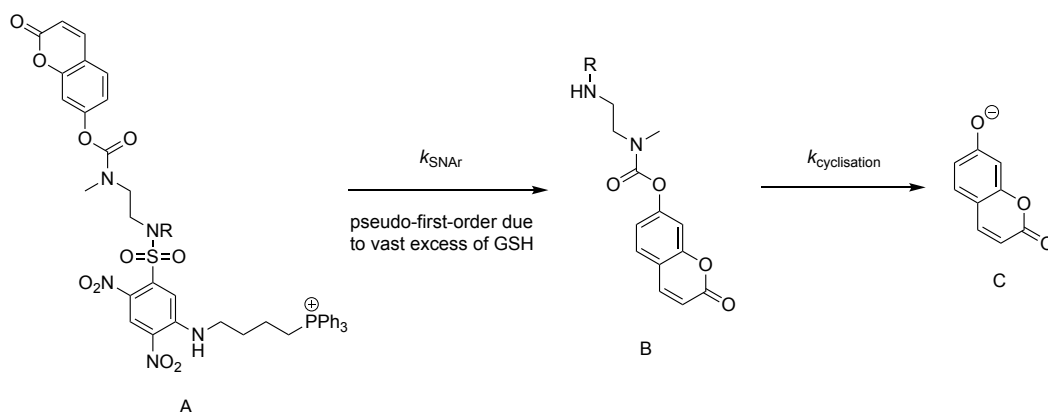
Many chemical reactions consist of multiple steps, meaning the rate equation is more complex than for a single elementary reaction. Consecutive reactions are a series of

single-step reactions. In the simplest case, reactant A converts to intermediate B in a first-order reaction, which in turn, converts to product C, in another first-order reaction (Scheme 68).



Scheme 68. A simple consecutive reaction.

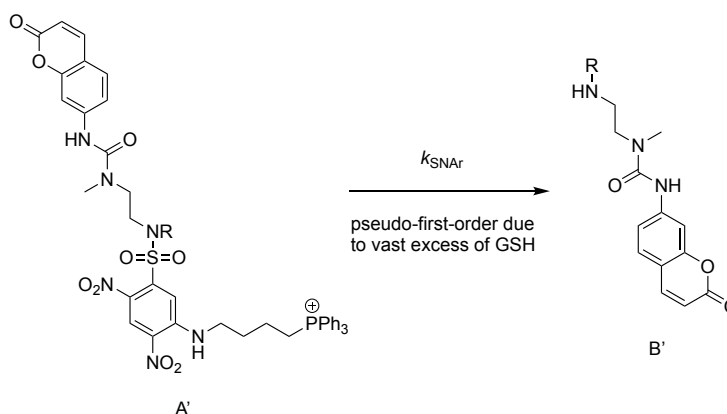
Treating a complex reaction as a consecutive reaction enables the relative rates of the individual steps and the reaction mechanism to be determined. The reaction of MitoHCoum1 **177** and MitoHCoum2 **178** can be treated as an A to B to C consecutive reaction (Scheme 69).



Scheme 69. Consecutive reaction model of MitoHCoum1 **177** and MitoHCoum2 **178** collectively defined as A, which undergo S_NAr to HCoum1 **167** and HCoum2 **168**, respectively, collectively defined as intermediate B, which in turn cyclises to 7-hydroxycoumarin **106**, defined as product C.

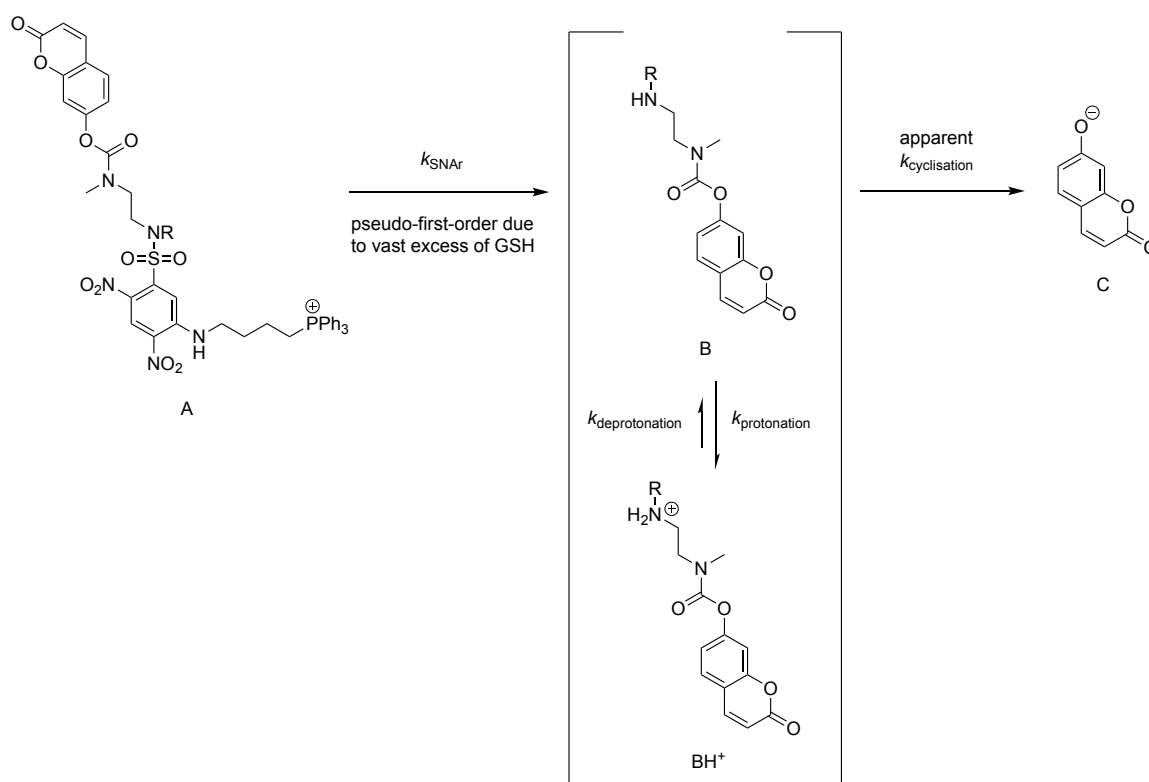
At a high concentration of GSH **25**, the S_NAr reaction of A to B becomes pseudo-first-order. The reaction kinetics of A to B can be considered identical to the reaction kinetics of model compounds MitoACoum1 **175** and MitoACoum2 **176** (Scheme 70), collectively

defined as A', which undergo S_NAr reaction to ACoum1 165 and ACoum2 166, respectively, collectively defined as B' (see Section 3.3.4 for kinetics).



Scheme 70. Reaction of MitoACoum1 175 and MitoACoum2 176, collectively defined as A', which undergo S_NAr to ACoum1 165 and ACoum2 166, respectively, collectively defined as B'.

At pH 8.0, the equilibrium of intermediate B likely favours the protonated form BH⁺ (Scheme 71). A typical alkyl amine has a pK_{aH} of 9.5 - 11.0, although the pK_{aH} of B is likely to be closer to 9.0 due to the carbamate which is electron-withdrawing by inductive effects. Furthermore, if the $k_{\text{protonation}}$ of B is much greater than the $k_{\text{cyclisation}}$, the equilibrium between B and BH⁺ is rapidly established. Therefore, the rate constant for cyclisation of B to C is in fact an apparent rate constant that includes a term for the ratio of B/BH⁺ at a given pH.



Scheme 71. Expanded consecutive reaction of A to B to C, where the equilibrium of B favours the protonated form at pH 8.0.

For an A to B to C consecutive reaction, equation 10 can be used to plot the time-dependent variation of the concentration of product C, where k_1 is the first-order rate constant of step one and k_2 is the first-order rate constant of step two.

$$(10) \quad C_c = C_0 \left[1 - \frac{(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})}{(k_2 - k_1)} \right]$$

To determine whether the observed reactions of MitoHCoum1 **177** and MitoHCoum2 **178** with 10 mM GSH **25** in pH 8.0 buffer can be accurately described by the consecutive reaction model, equation 1 was used to calculate the predicted reaction for each compound. For the predicted reaction of MitoHCoum1 **177** (Figure 50), the pseudo-first-order rate constant of MitoACoum1 **175** adjusted for 10 mM GSH **25** and the first-order rate constant of HCoum1 **167** were used as k_{SNAr} and apparent $k_{\text{cyclisation}}$, respectively

(previously determined in Section 3.3.4). For the predicted reaction of MitoHCoum2 **178** (Figure 51), the pseudo-first-order rate constant of MitoACoum2 **176** adjusted for 10 mM GSH **25** and the first-order rate constant of HCoum2 **168** were used as the k_{SNAr} and apparent $k_{\text{cyclisation}}$, respectively (previously determined in Section 3.3.4). It is important to note that in the assays of HCoum1 **167** and HCoum2 **168**, the compounds were protonated to begin with, so their first-order rate constants include the equilibrium between B and BH⁺.

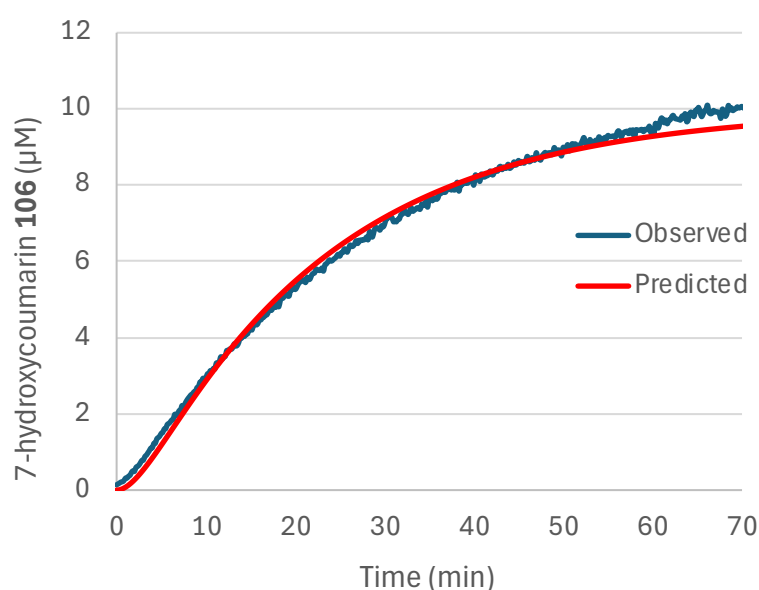


Figure 50. Blue line shows the single-run of MitoHCoum1 **177** (10 µM) in GSH **25** (10 mM), HEPES (100 mM) pH 8.0 buffer at 30 °C. Red line shows the predicted reaction calculated using the consecutive reaction equation 1, $k = 6.40 \times 10^{-3} \text{ s}^{-1}$ of MitoACoum1 **175** at 10 mM GSH **25** (determined by $k' = 0.640 \times 10^{-3} \text{ mM}^{-1}\text{s}^{-1}$ multiplied by 10 mM) and $k = 0.770 \times 10^{-3} \text{ s}^{-1}$ of HCoum1 **167**.

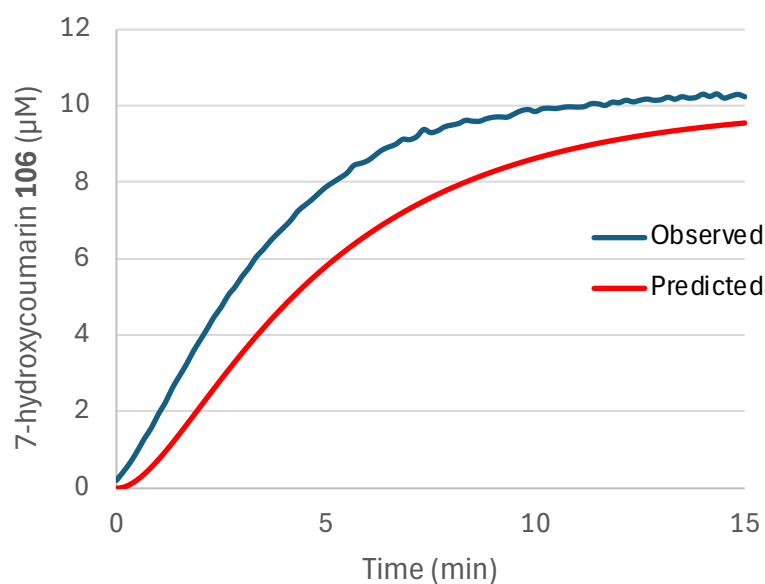


Figure 51. Blue line shows the triplicate-run of MitoHCoum2 178 (10 μM) in GSH 25 (10 mM), HEPES (100 mM) pH 8.0 buffer at 30 $^{\circ}\text{C}$. Red line shows the predicted reaction calculated using the consecutive reaction equation 1, $k = 16.1 \times 10^{-3} \text{ s}^{-1}$ of MitoACoum2 176 at 10 mM GSH 25 (determined by $k' = 1.61 \times 10^{-3} \text{ mM}^{-1}\text{s}^{-1}$ multiplied by 10 mM) and $k = 3.76 \times 10^{-3} \text{ s}^{-1}$ of HCoum2 168.

The observed and predicted reactions of MitoHCoum1 177 with 10 mM GSH 25 are nearly identical (Figure 50). Moreover, the reaction of MitoHCoum1 177 near perfectly fits the consecutive reaction model and its rate constants k_{SNAr} and apparent $k_{\text{cyclisation}}$ match those of MitoACoum1 175 and HCoum1 167, respectively. This implies that upon formation of B in the first step, its protonation is much faster than cyclisation and the equilibrium between B and BH^+ is rapidly established.

The reaction of MitoHCoum2 178 with 10 mM GSH 25 also fits well to the consecutive reaction model; its observed reaction is similar to but slightly faster than the predicted reaction (Figure 51). Given that the apparent $k_{\text{cyclisation}}$ of HCoum2 168 is approximately 5-fold greater than that of HCoum1 167 (as determined in Section 3.3.4), it is possible that in the reaction of MitoHCoum2 178, where non-protonated HCoum2 168 is generated in the first step, the cyclisation competes to some extent with protonation. This competition would account for the faster observed reaction, indicating that the apparent $k_{\text{cyclisation}}$ of MitoHCoum2 178 is greater than the apparent $k_{\text{cyclisation}}$ of HCoum2 168.

The model appears to break down when the reaction is performed at a much lower concentration of GSH **25**, despite it still being in large excess of compound A. Single-run reactions of MitoHCoum1 **177** and MitoHCoum2 **178** were measured with 0.2 mM GSH **25** (Figure 52 and Figure 53, respectively). The predicted reactions were calculated as before, except this time, the $k_{\text{S}_{\text{N}}\text{Ar}}$ of MitoACoum1 **175** and MitoACoum2 **176** was adjusted for 0.2 mM. In both cases, the observed reaction is significantly faster than the predicted reaction, which suggests that there is another reaction independent of GSH **25** concentration that is competing with the GSH-dependent $\text{S}_{\text{N}}\text{Ar}$ bimolecular reaction. It is possible that at very high GSH **25** concentration, the bimolecular reaction dominates. However, at significantly lower GSH **25** concentration, the bimolecular reaction rate decreases to a level where a unimolecular reaction competes. The unimolecular reaction could potentially involve a GS^- counter-ion of the TPP^+ salt, which is directly delivered to carry out the $\text{S}_{\text{N}}\text{Ar}$ reaction (Figure 54).

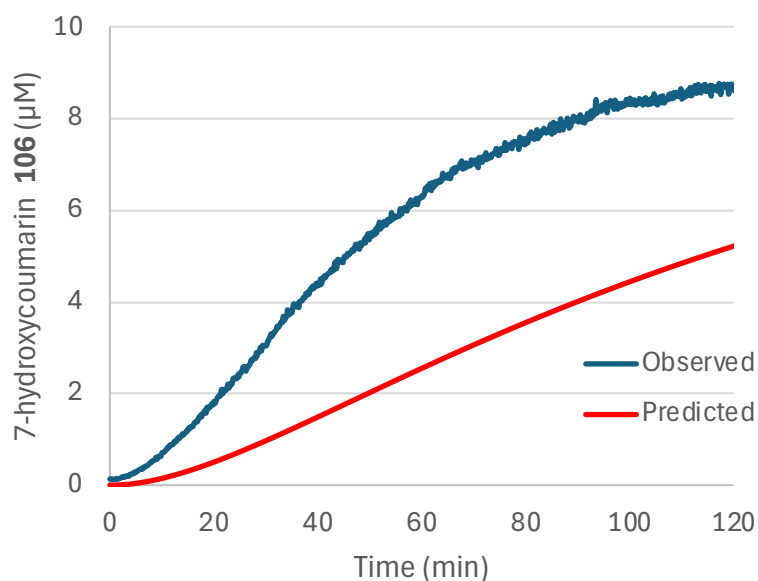


Figure 52. Blue line shows the single-run of MitoHCoum1 **177** (10 μM) in GSH **25** (0.2 mM), HEPES (100 mM) pH 8.0 buffer at 30 $^{\circ}\text{C}$. Red line shows the predicted reaction calculated using the consecutive reaction equation 1, $k = 0.128 \times 10^{-3} \text{ s}^{-1}$ of MitoACoum1 **175** at 0.2 mM GSH **25** (determined by $k' = 0.640 \times 10^{-3} \text{ mM}^{-1}\text{s}^{-1}$ multiplied by 0.2 mM) and $k = 0.770 \times 10^{-3} \text{ s}^{-1}$ of HCoum1 **167**.

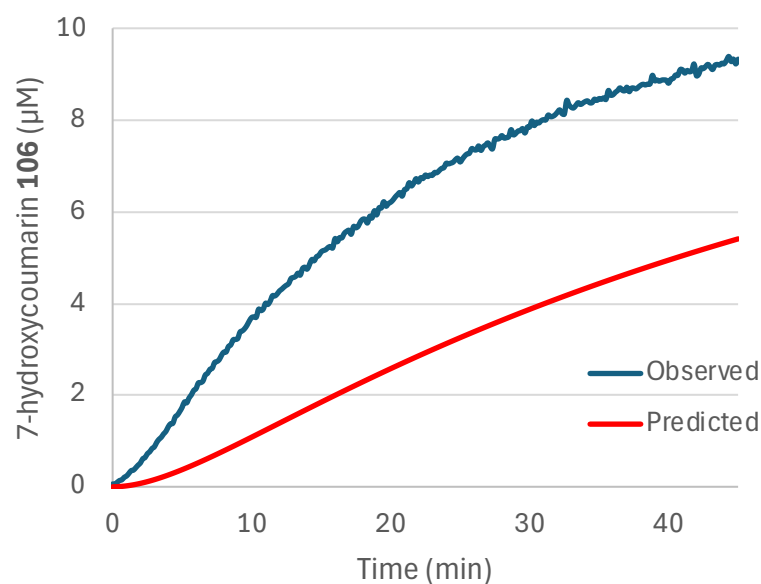


Figure 53. Blue line shows the triplicate full run of MitoHCoum2 178 (10 μM) in GSH 25 (0.2 mM), HEPES (100 mM) pH 8.0 buffer at 30 $^{\circ}\text{C}$. Red line shows the predicted reaction calculated using the consecutive reaction equation 1, $k = 0.322 \times 10^{-3} \text{ s}^{-1}$ of MitoACoum2 176 at 0.2 mM GSH 25 (determined by $k' = 1.61 \times 10^{-3} \text{ mM}^{-1}\text{s}^{-1}$ multiplied by 0.2 mM) and $k = 3.76 \times 10^{-3} \text{ s}^{-1}$ of HCoUm2 168.

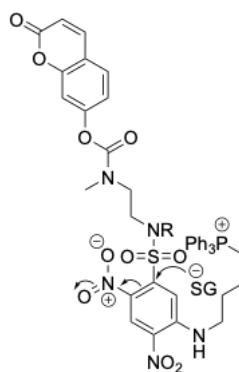


Figure 54. Proposed unimolecular delivery of GS^- counterion of TPP^+ .

Overall, the consecutive reaction model accounts for the reactions of MitoHCoum1 177 and MitoHCoum2 178 with 10 mM GSH 25 and pH 8.0 which mimic the physiological conditions in the mitochondrial matrix. These scaffolds thus offer a viable strategy for delivery of phenol cargos to the mitochondrial matrix and could be adapted to deliver

phenol drugs from prodrugs. Given the pH of the mitochondrial matrix, the non-TPP amine intermediates produced by these scaffolds are expected to exist predominantly in their protonated forms, slowing diffusion across membranes and ensuring their retention in the matrix. Additionally, the two scaffolds present an opportunity to release phenol drugs at different rates depending on the therapeutic needs. The secondary sulfonamide scaffold of MitoHCoum1 **177** provides a slower, more sustainable release: the half-life ($t_{1/2}$) of its intermediate HCoUm1 **167** is 900 s (15 min). In contrast, the tertiary sulfonamide scaffold of MitoHCoum2 **178** enables much faster release: its intermediate HCoUm2 **168** has $t_{1/2}$ = 184 s (~ 3 min).

3.3.6 Synthesis of prodrugs of phenols

After designing reactive GSH-activated prodrug-like compounds of 7-hydroxycoumarin, attention turned to the preparation of prodrugs of anti-cancer phenolic drugs based on the same scaffolds. The drugs of interest were niclosamide **162**, atovaquone **163** and menadiol **164** (Figure 55) for their damaging effects on the mitochondria and anti-cancer properties. Niclosamide **162** is an anti-parasitic drug which induces mitochondrial uncoupling and is suspected to inhibit tumour proliferation of several cancer types via mitochondrial-dependent apoptosis.²⁶¹⁻²⁶⁶ Atovaquone **163** is an anti-malarial drug which inhibits mitochondrial complex III and has shown antiproliferative effects in several cancer cells.^{267, 268} Menadiol **164** is the reduced form of menadione **183** (Figure 55), which generates ROS by redox cycling and induces apoptosis at high concentrations.^{269, 270}

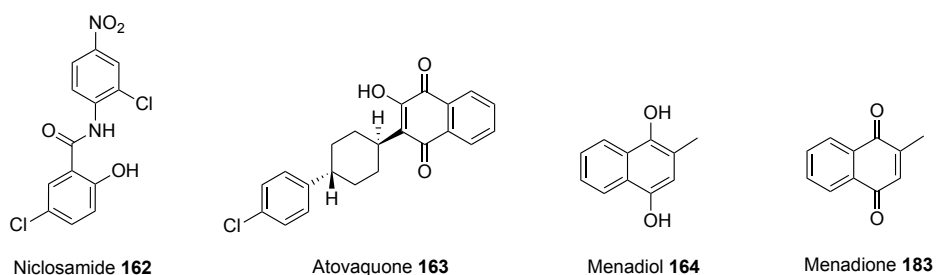
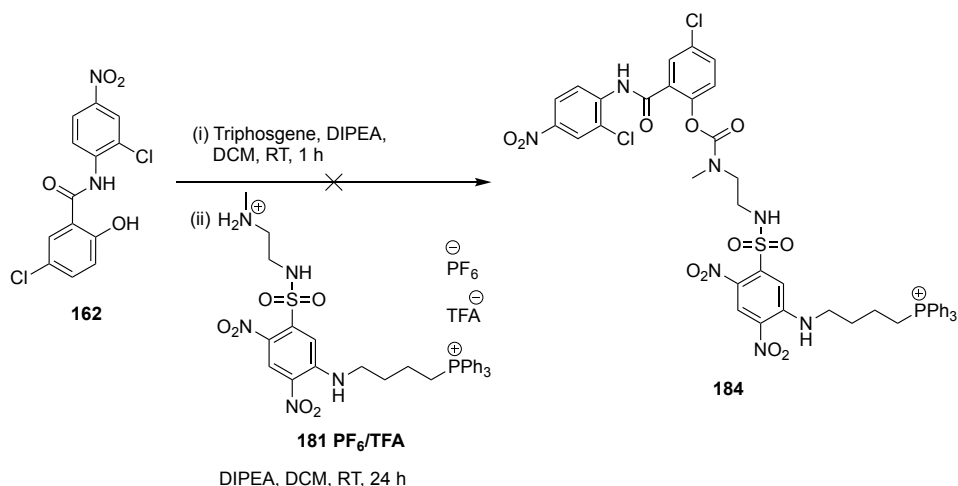


Figure 55. Anti-cancer phenolic drugs: niclosamide **162**, atovaquone **163** and menadiol **164** (and its oxidised form, menadione **183**).

Niclosamide **162** is commercially available. Coupling of niclosamide **162** to ammonium salt **181** (previously synthesised in Section 3.3.3) failed to yield the corresponding carbamate **184** (Scheme 72).



Scheme 72. Attempted synthesis of niclosamide prodrug **184**.

LCMS analysis of the crude material revealed a complex mixture of niclosamide **162**, ammonium starting material **181**, aniline side-product **185** and trace amounts of ether **186** (Figure 56). The individual compounds were not isolated. The aniline **185** likely formed via a Smiles rearrangement under the reaction's basic conditions. The ether **186** likely formed via a S_NAr reaction in which niclosamide **162** displaced the sulfonyl moiety. It is possible that niclosamide **162** did not react with triphosgene to generate the intermediate in the first step. The poor nucleophilicity of the niclosamide phenoxide arises from two factors: electron donation into the aromatic ring to stabilise the electron-poor system caused by the electron-withdrawing chloride and amide substituents, and hydrogen-bonding between the phenoxide and the amide NH. Given this poor nucleophilicity, niclosamide prodrugs were rejected as targets.

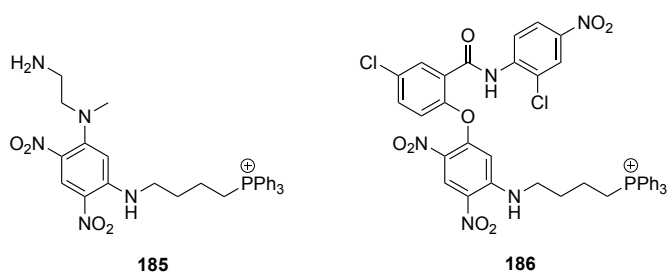
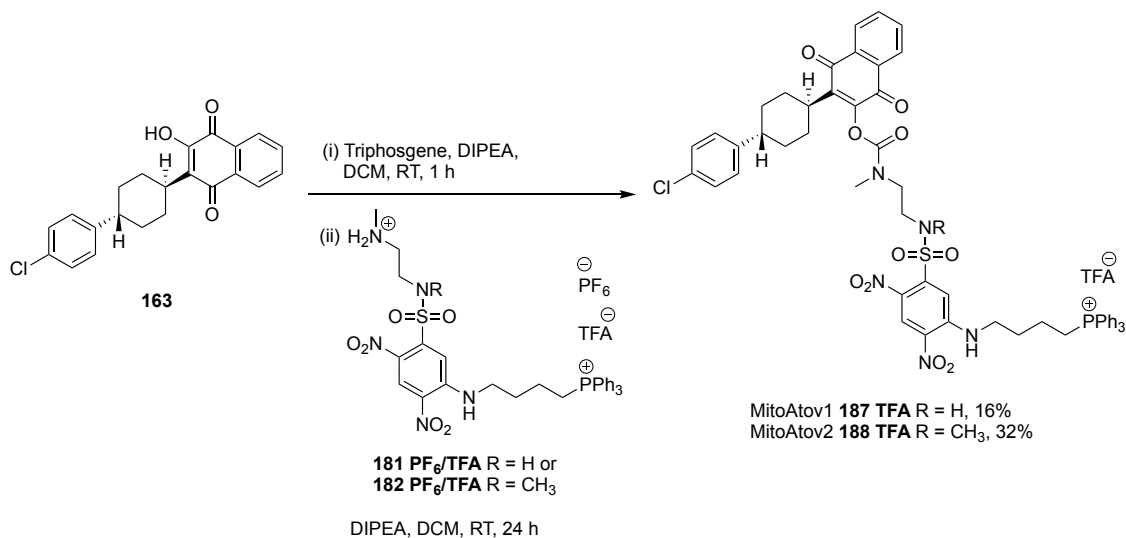


Figure 56. Aniline **185** produced by a Smiles rearrangement and ether **186** produced by S_NAr reaction during the attempted reaction in Scheme 37.

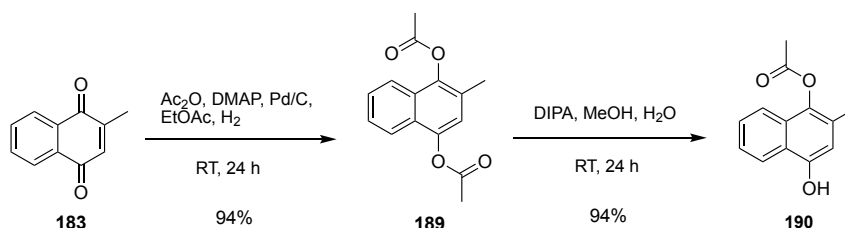
Atovaquone **163** is also commercially available. Coupling of atovaquone **163** to ammonium scaffolds **181** or **182** afforded carbamates, MitoAtov1 **187** or MitoAtov2 **188**, respectively, albeit in poor yield (Scheme 73).



Scheme 73. Synthesis of atovaquone prodrugs MitoAtov1 **187** and MitoAtov2 **188**.

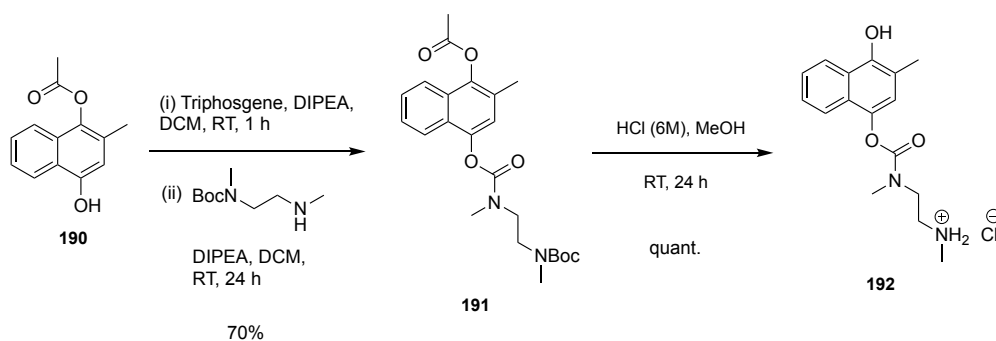
Menadione **183** is also commercially available. However, menadiol **164** is unstable and spontaneously oxidises to menadione **183**, so it was coupled to the prodrug scaffold as the monoacetylated analogue and then finally deacetylated. Following a patent procedure,²⁷¹ menadione **183** was reduced and acetylated *in situ* to form the diacetate

189 in excellent yield (Scheme 74). Diacetate **189** was hydrolysed under alkaline conditions to obtain the monoacetate **190** in excellent yield.



Scheme 74. Synthesis of monoacetate 185.

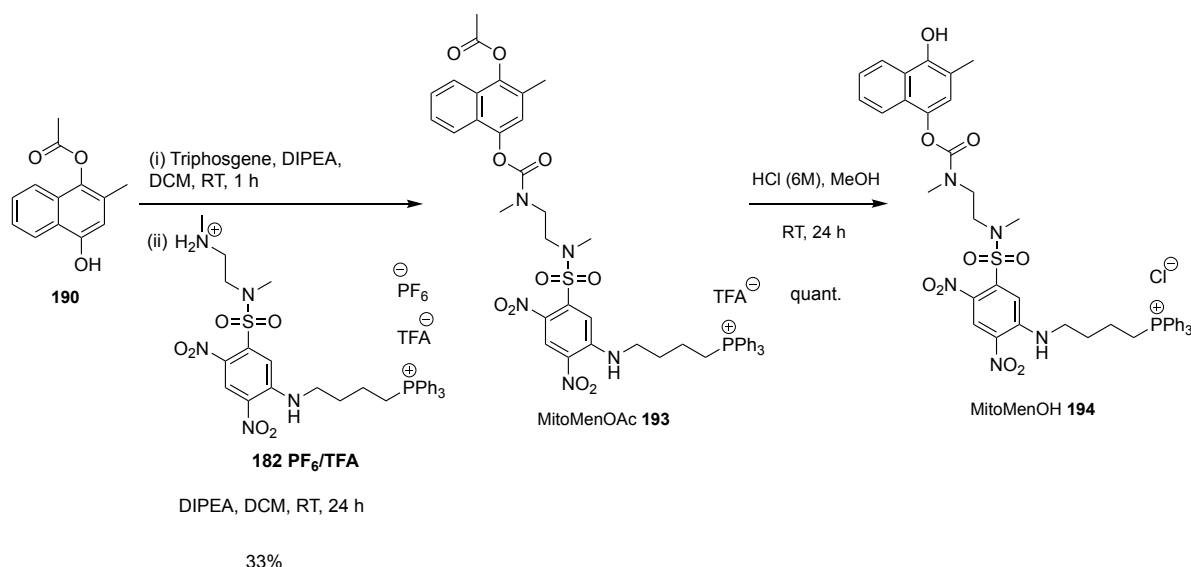
The monoacetate **190** was coupled to *tert*-butyl methyl(2-(methylamino)ethyl)carbamate to give carbamate **191** in good yield (Scheme 75). Acidic hydrolysis of the acetate and Boc group afforded a quantitative yield of ammonium salt **192**. The ammonium salt **192** would be useful in kinetic studies but could not be used to attach the TPP-conjugate because it would fragment in base.



Scheme 75. Synthesis of a simple ethylenediamine prodrug of menadiol 192.

Instead, the monoacetate **190** was coupled to the faster self-immolative linker, ammonium scaffold **182**, to afford the double prodrug MitoMenOAc **193**, albeit in poor yield (Scheme 76). Some of MitoMenOAc **193** was hydrolysed under acidic conditions to obtain a quantitative yield of the single prodrug, MitoMenOH **194**. In addition to activation

by GSH **25**, MitoMenOAc **193** would require hydrolysis of the acetate protecting group by a mitochondrial esterase-type enzyme to release menadiol **164**.



Scheme 76. Synthesis of menadiol prodrugs MitoMenOAc **193** and MitoMenOH **194**.

3.3.7 Kinetics of atovaquone prodrugs

Due to the time constraints of the project, only the kinetics of the atovaquone prodrugs, MitoAtov1 **187** and MitoAtov2 **188**, were measured. Initially, the aim was to measure the kinetics of the reaction between the atovaquone prodrugs and GSH **25** by monitoring the release of free atovaquone **163**, as had been done for the coumarin prodrugs, MitoHCoum1 **177** and MitoHCoum2 **178** (see Section 3.3.4 and 3.3.5). Since atovaquone **163** is not fluorescent, its formation would be monitored by absorbance. However, due to the poor solubility of atovaquone at 100 μ M in pH 8.0 buffer, the assays were conducted on 10 μ M of MitoAtov1 **187** and MitoAtov2 **188**. This concentration would allow for direct comparison of the kinetics of the atovaquone prodrugs with the coumarin prodrugs. However, at such a low concentration, the absorbance of atovaquone **163** was weak. Furthermore, the absorption spectrum of atovaquone **163** overlapped with that of MitoGSDNB **52**. Consequently, the kinetic measurements were obtained using LCMS. Both MitoAtov1 **187** and MitoAtov2 **188** were stable in pH 8 buffer during the 24 h period measured. However, when MitoAtov1 **187** and MitoAtov2 **188** were treated with 2 mM

GSH **25**, a variety of compounds were detected by LCMS analysis (Figures 57 and 58, respectively). Alongside the desired products, MitoGSDNB **52** and atovaquone **163**, a glutathione-atovaquone conjugate **195** was also identified. This suggests that the C-O bond of the atovaquone moiety is susceptible to S_NAr by GSH **25** due to the electron-withdrawing properties of the naphthoquinone (Scheme 77). The formation of the GS-atovaquone conjugate **195** likely results in the fragmentation of the carbamate bond to produce CO_2 and the ammonium cation **181** or **182**. However, the ammonium cation **181** or **182** were not detected by LCMS, suggesting that they are short-lived and undergo rapid S_NAr with GSH **25** to form MitoGSDNB **52**. The LCMS chromatogram of the reaction of the mono-*N*-methylated MitoAtov1 **187** after 1 h (Figure 57) gave a considerably higher ratio of GS-atovaquone **195** to atovaquone **183**, than MitoAtov2 **188** (Figure 58). This suggests that the tertiary sulfonamide, MitoAtov2 **188**, undergoes S_NAr with GSH **25** more rapidly than the secondary sulfonamide, MitoAtov1 **187**. As discussed in Section 3.3.4, the tertiary sulfonamide, MitoACoum2 **176**, is twice as reactive as the secondary sulfonamide, MitoACoum1 **175**. Furthermore, cyclisation of intermediate. Furthermore, cyclisation of intermediate **182** would be expected to be faster than intermediate **181**, so reaction with GSH **25** at this point to generate the GS-atovaquone conjugate **195** is also less likely.

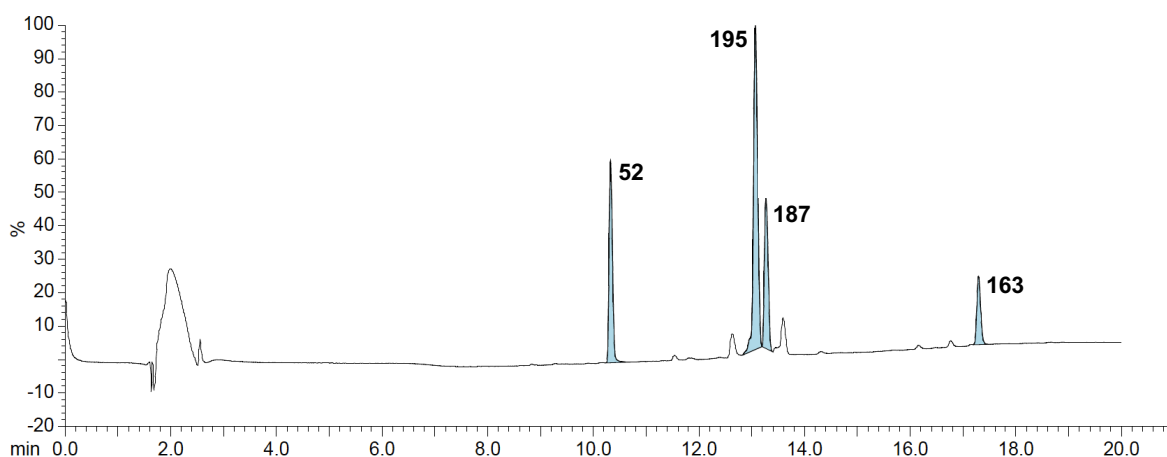


Figure 57. LCMS chromatogram at 254 nm after 1 h of incubation of MitoAtov1 **187** with 2 mM GSH **25** in pH 8.0 HEPES (0.1 M) buffer. Method: H_2O (0.1% formic acid): MeCN 5-95% 20 min.

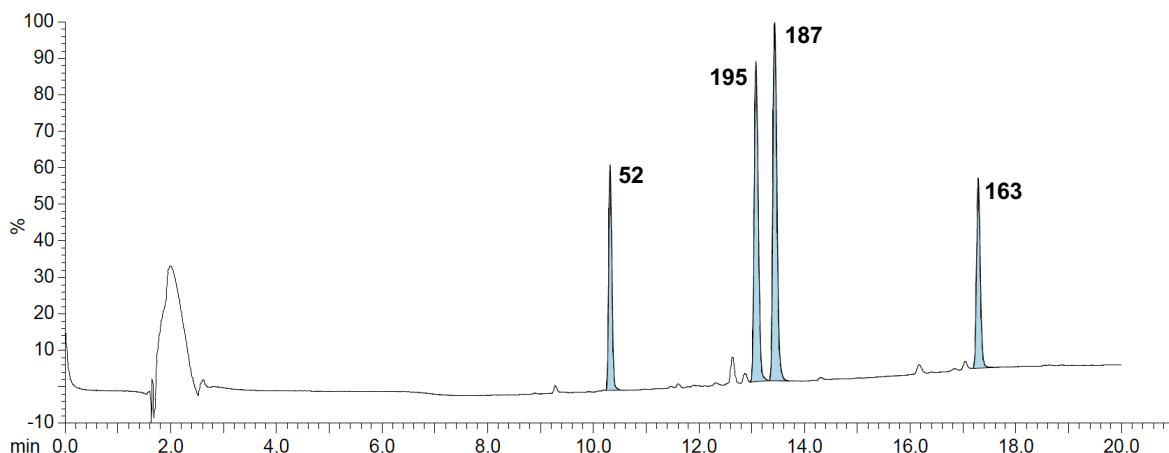
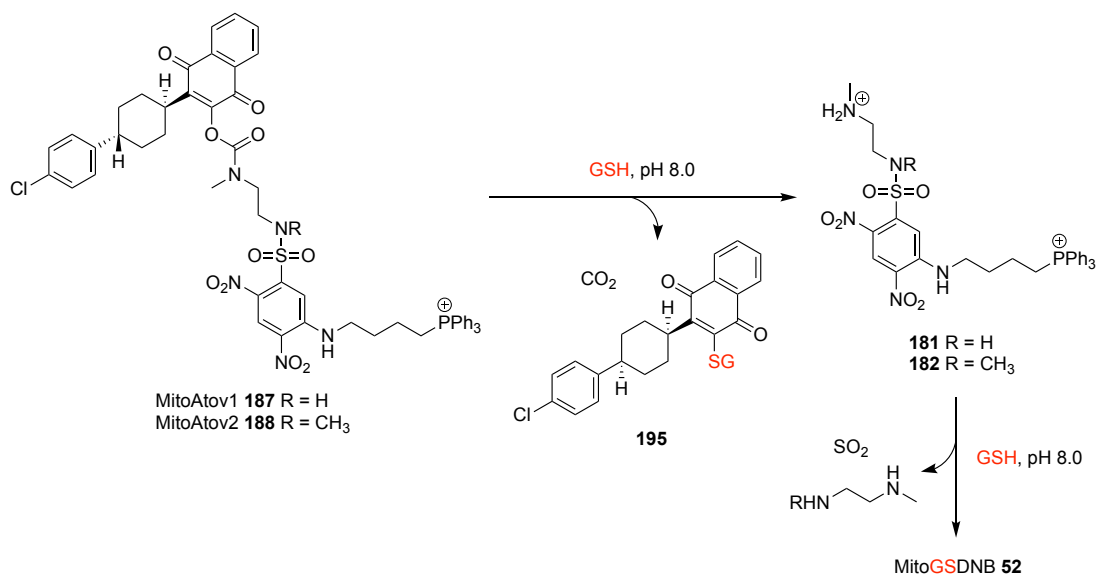


Figure 58. LCMS chromatogram at 254 nm after 1 h of incubation of MitoAtov2 **187** with 2 mM GSH **25** in pH 8.0 HEPES (0.1 M) buffer. Method: H₂O (0.1% formic acid): MeCN 5-95% 20 min.



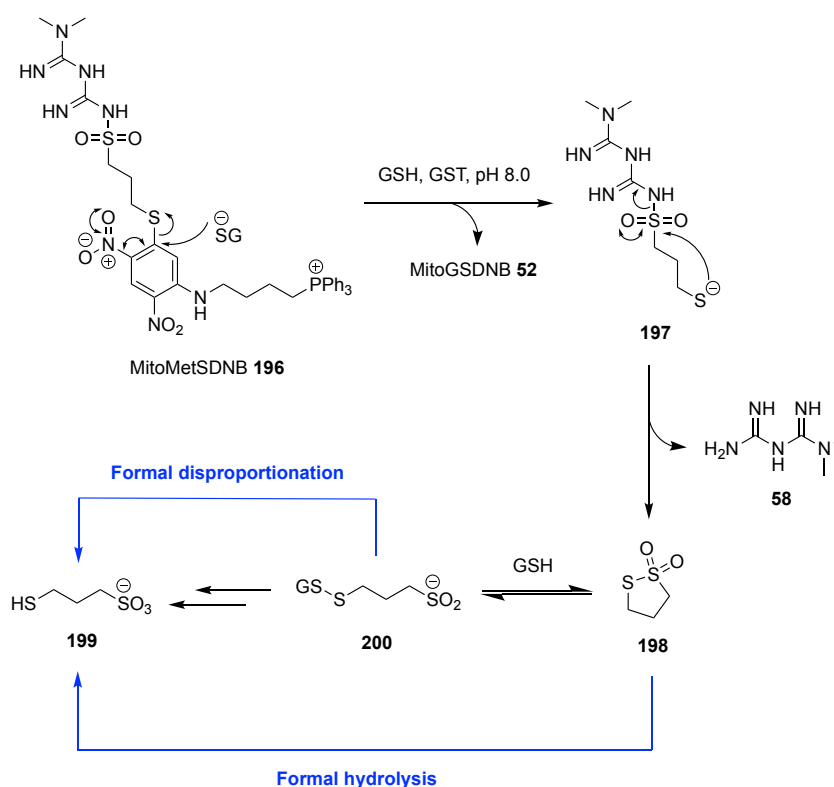
Scheme 77. Proposed reactions that generate GS-atovaquone side-product **195** and MitoGSDNB **52** from the reaction of MitoAtov1 **187** or MitoAtov2 **188** with GSH **25**.

However, surprisingly, the reactions between prodrugs **187** and **188** and GSH **25** did not reach complete conversion even after several hours of monitoring, despite the large excess of glutathione. After the first hour, approximately 19% of MitoAtov1 **187** and 25% of MitoAtov2 **188** remained unreacted and did not undergo further conversion when

incubation was continued for a further 2 h. While the exact reason for this is unclear, it is possible that the prodrug molecules aggregate to form micelles due to the high lipophilicity of the atovaquone moiety and the positive charge of the triphenylphosphonium moiety. Unfortunately, there was not sufficient time to fully investigate this hypothesis. Menadiol **164** is electron-rich, and MitoMenOAc **193** and MitoMenOH **194** are less lipophilic than MitoAtov1 **187** and MitoAtov2 **188**. Furthermore, MitoMenOAc **193** and MitoMenOH **194** are expected to completely convert to MitoGSDNB **5** and menadiol **164** without formation of undesirable side-products.

3.4 Approach C

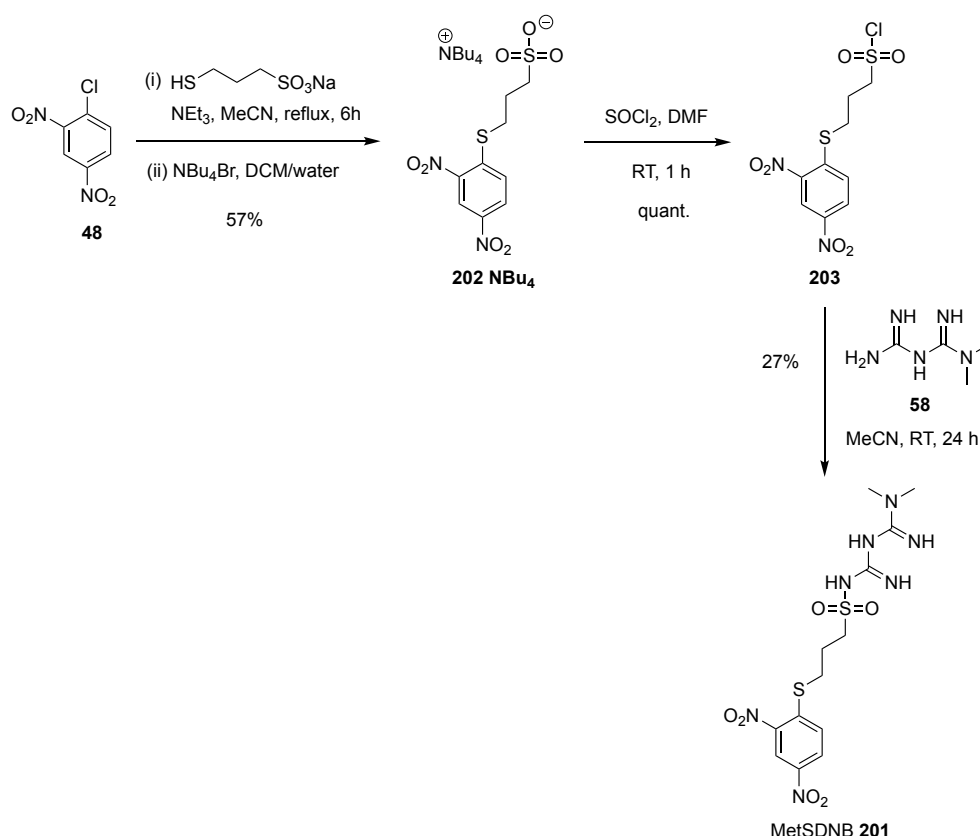
Approach C was developed with the specific aim of delivering metformin **58** from a novel prodrug design MitoMetSDNB **196** (Scheme 78). The strategy employed a sulfonyl propylsulfide self-immolative linker, similar to the widely used disulfide linker system,^{272, 273} where the disulfide is reduced by thiols such as GSH **25**. The prodrug included the electron-poor dinitro ring of MitoCDNB **51**. Although sulfur is much less electronegative than chlorine, it was hoped that the prodrug would be react with GSH **25** under catalysis. Upon S_NAr by GSH **25** to produce MitoGSDNB **52**, the resulting thiolate intermediate **197** was expected to undergo intramolecular cyclisation to form a cyclic thiosulfonate **198**, thereby releasing the active cargo. The cyclic thiosulfonate **198** could potentially undergo formal hydrolysis to yield a sulfonate anion **199**, which would act as an antioxidant and remain trapped in the mitochondria. The hydrolysis would likely proceed via a disulfide sulfinic acid intermediate **200**,²⁷⁴ which would also be retained in the mitochondria (given that the pKa of sulfinic acids is approximately 2).²⁷⁵ Sulfinate anions are more effective antioxidants than thiols at neutral pH, as they react more rapidly with hydrogen peroxide due to their almost complete deprotonation.²⁷⁶ This process may ultimately generate the disulfide of the sulfonate thiol **199**.



Scheme 78. Structure of novel prodrug design MitoMetSDNB 196 and proposed mechanism of reaction with GSH 25 to release metformin.

3.4.1 Synthesis of an untargeted control prodrug

A synthetic route was developed to create an untargeted control prodrug of metformin, MetSDNB 201, modelled on the structure of CDNB 48, to evaluate the effectiveness of the linker in releasing metformin 58 upon GSH/GST activation (Scheme 79). This route could later be adapted for synthesis of the mitochondria-targeted analogue, MitoMetSDNB 196. Synthesis began with a S_NAr reaction between CDNB 48 and sodium 3-mercaptopropanesulfonate, followed by a work-up with tetrabutylammonium bromide, to obtain sulfonate 202 as the tetrabutylammonium salt in moderate yield. The sulfonate 202 was reacted with thionyl chloride to generate sulfonyl chloride 203 in quantitative yield, which was then coupled to metformin 58 to afford the MetSDNB 201, albeit in poor yield.



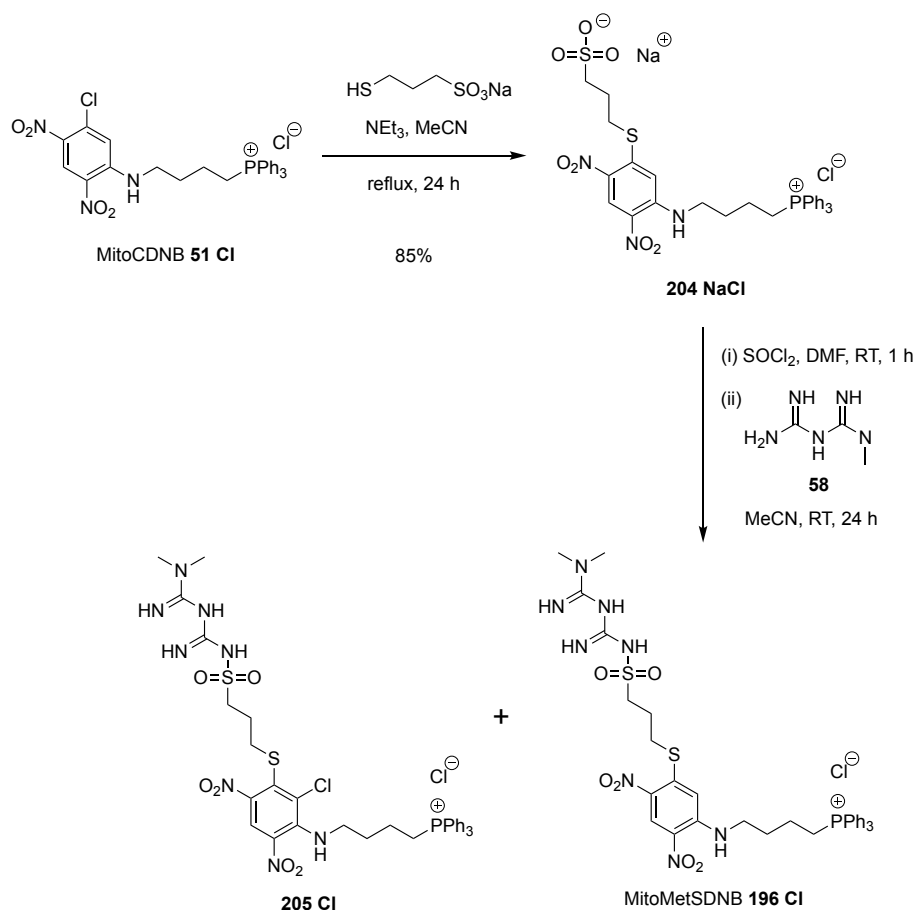
Scheme 79. Synthesis of untargeted control prodrug, MetSDNB 201.

The next step would have been to measure the kinetics of the catalysed reaction between MetSDNB **201** and GSH **25** in the presence of GST enzymes. However, at a concentration of 100 μM , MetSDNB **201** exhibited poor solubility in pH 8.0 buffer. Even at 10 μM , it was not certain that MetSDNB **201** was fully dissolved, and this concentration was likely too low for reliable signal detection by UV-Vis absorbance. Given the straightforward synthesis of MetSDNB **201**, the focus was shifted to the preparation of MitoMetSDNB **196**, which was anticipated to have greater water solubility.

3.4.2 Synthesis of MitoMetSDNB via route A

Applying the synthetic route outlined in Section 3.4.1 (Scheme 79), the preparation of the TPP-conjugated prodrug, MitoMetSDNB **196** (Scheme 80), began with a $\text{S}_{\text{N}}\text{Ar}$ reaction between MitoCDNB **51** (previously prepared in Section 2.2.3) and sodium 3-mercaptopropanesulfonate, affording sulfonate **204** in excellent yield. Sulfonate **204** was treated

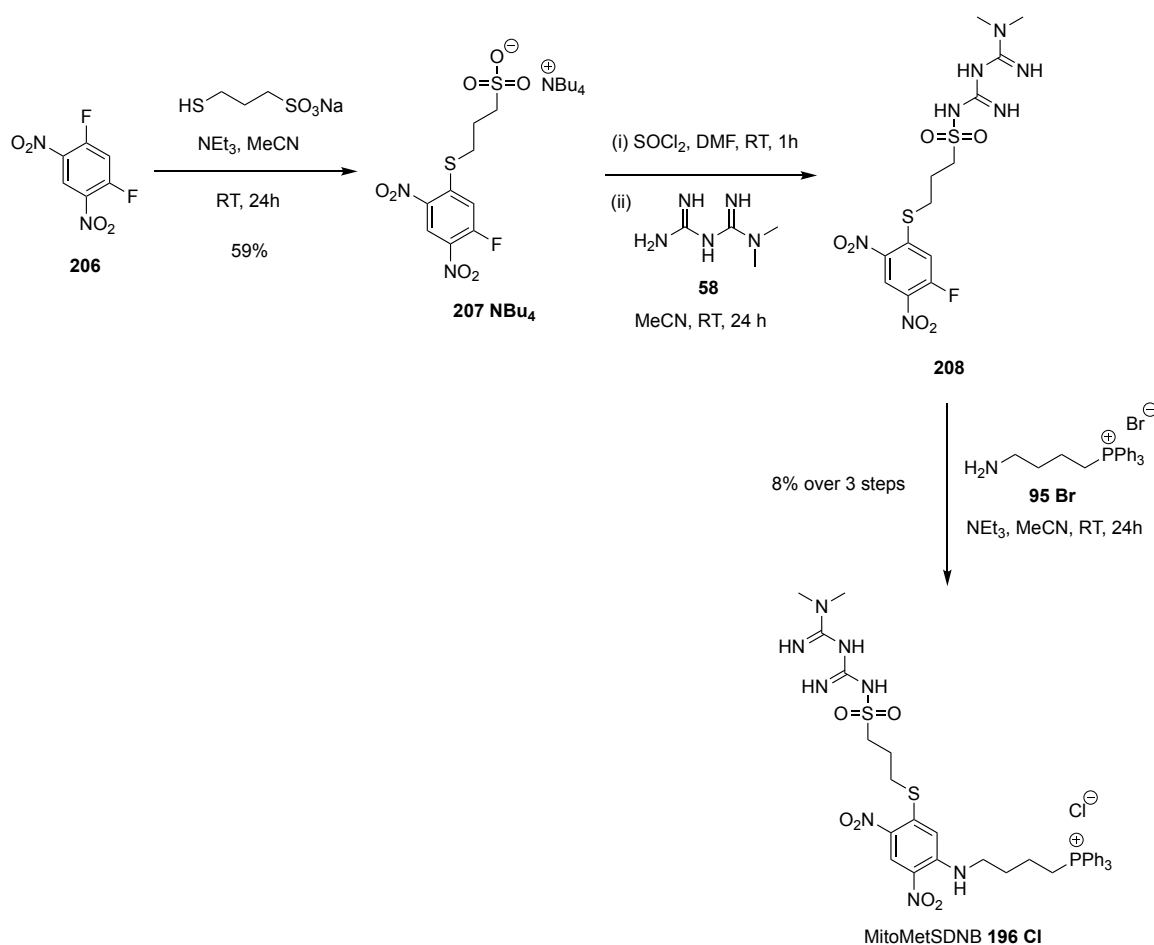
with thionyl chloride to generate the sulfonyl chloride, which was taken onto the next step without characterisation. Subsequently coupling with metformin **58** yielded a mixture of the desired product, MitoMetSDNB **196**, and a chlorinated analogue **205** as a side-product. The chloride substituent in side-product **205** is likely a result of an electrophilic aromatic substitution during the reaction between sulfonate **204** and excess thionyl chloride. Chlorination appeared to have occurred at the 6-position rather than the 3-position, as evidenced by the disappearance of the more up-field proton signal in the ^1H NMR spectrum. The reaction was attempted again using one equivalent of thionyl chloride, but this failed to prevent formation of side-product **205**. Due to the similar retention times of MitoMetSDNB **96** and side-product **205**, they could not be separated by flash chromatography or semi-preparative RP-HPLC. As a result, the synthetic route to MitoMetSDNB **96** was redesigned.



Scheme 80. Synthesis route A towards MitoMetSDNB **196**, which could not be separated from side-product **205**.

3.4.3 Synthesis of MitoMetSDNB via route B

The new synthetic route towards MitoMetSDNB **196** (Scheme 81) aimed to attach metformin **58** to the scaffold earlier in the synthesis, before addition of the TPP group. This way, if reaction with thionyl chloride generated any undesirable chlorinated side-products they could more easily be separated from the product. Synthesis began with a S_NAr reaction between 1,5-difluoro-2,4-dinitrobenzene **206** and sodium 3-mercapto-1-propanesulfonate, followed by a work-up with tetrabutylammonium bromide, to obtain sulfonate **207** as the tetrabutylammonium salt in moderate yield. Sulfonate **207** was reacted with thionyl chloride to generate the sulfonyl chloride which was taken onto the next step without characterisation. Coupling with metformin **58** afforded a mixture of metformin sulfonamide **208** as the major product with some minor impurities after flash chromatography. The impure mixture was taken on to the final step, a S_NAr reaction with amine **95** (previously synthesised in Section 2.2.3). This obtained the pure product, MitoMetSDNB **196**, in an 8% yield over three steps.

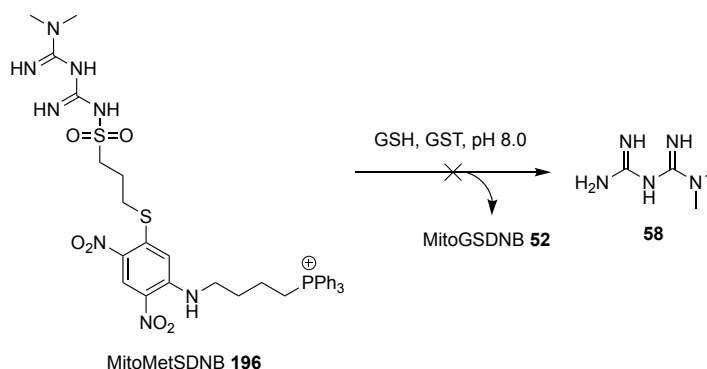


Scheme 81. Synthesis route B towards MitoMetSDNB **196**. Sulfonamide **208** could not be completely purified after flash chromatography and was taken onto the final step.

3.4.4 Kinetics of MitoMetSDNB

Next, the kinetics of the reaction between MitoMetSDNB **196** and GSH **25** with and without GST enzyme were investigated. The UV-Vis absorbance spectra of MitoMetSDNB **196** and MitoGSDNB **52** were too similar to monitor the formation of MitoGSDNB **52** by UV-Vis absorbance kinetics, so reaction was monitored for both MitoGSDNB **52** and metformin **58** by HPLC at regular time points. MitoMetSDNB **196** at a concentration of 100 μM appeared soluble in pH 8.0 buffer. However, MitoMetSDNB **196** showed no detectable reaction with GSH **25** in pH 8.0 buffer at 30 $^{\circ}\text{C}$ before or after treatment with GSTA1-1, GSTA4-4 or GSTK1-1 enzyme (Scheme 82). Since the Pauling electronegativities of carbon and sulfur are similar (2.55 and 2.58, respectively),²⁷⁷ there is very little inductive effect by the sulfur atom in MitoMetSDNB **196**. In the absence of an electron-withdrawing group at the

ipso position, the electron-poor dinitro ring is insufficient to promote reaction of MitoMetSDNB **196** with the glutathione thiolate. It is also worth noting that the sulfonamide of metformin is also stable to nucleophilic substitution by the glutathione thiolate. Unfortunately, given the limited time for further laboratory work, there was no opportunity to explore other self-immolative prodrug strategies to release metformin **58**.



Scheme 82. MitoMetSDNB **196** did not react with GSH **25** with or without GST enzyme to release metformin **58**.

3.5 Conclusions

Three approaches, A, B and C, were investigated to develop TPP-conjugated sulfonamide prodrugs that would target the mitochondria as part of a general strategy to treat cancer. The prodrugs would be activated by mGSH, potentially catalysed by mGSTs, to release hydroxy- or amine-bearing payloads. The combination of the release of a mitochondria-damaging anti-cancer drug and the depletion of mGSH by formation of a glutathione conjugate should generate irreparable oxidative damage, and consequently, cancer cell death. From approaches A-C, a range of prodrug-like sulfonamide compounds have been successfully synthesised (Figure 59).

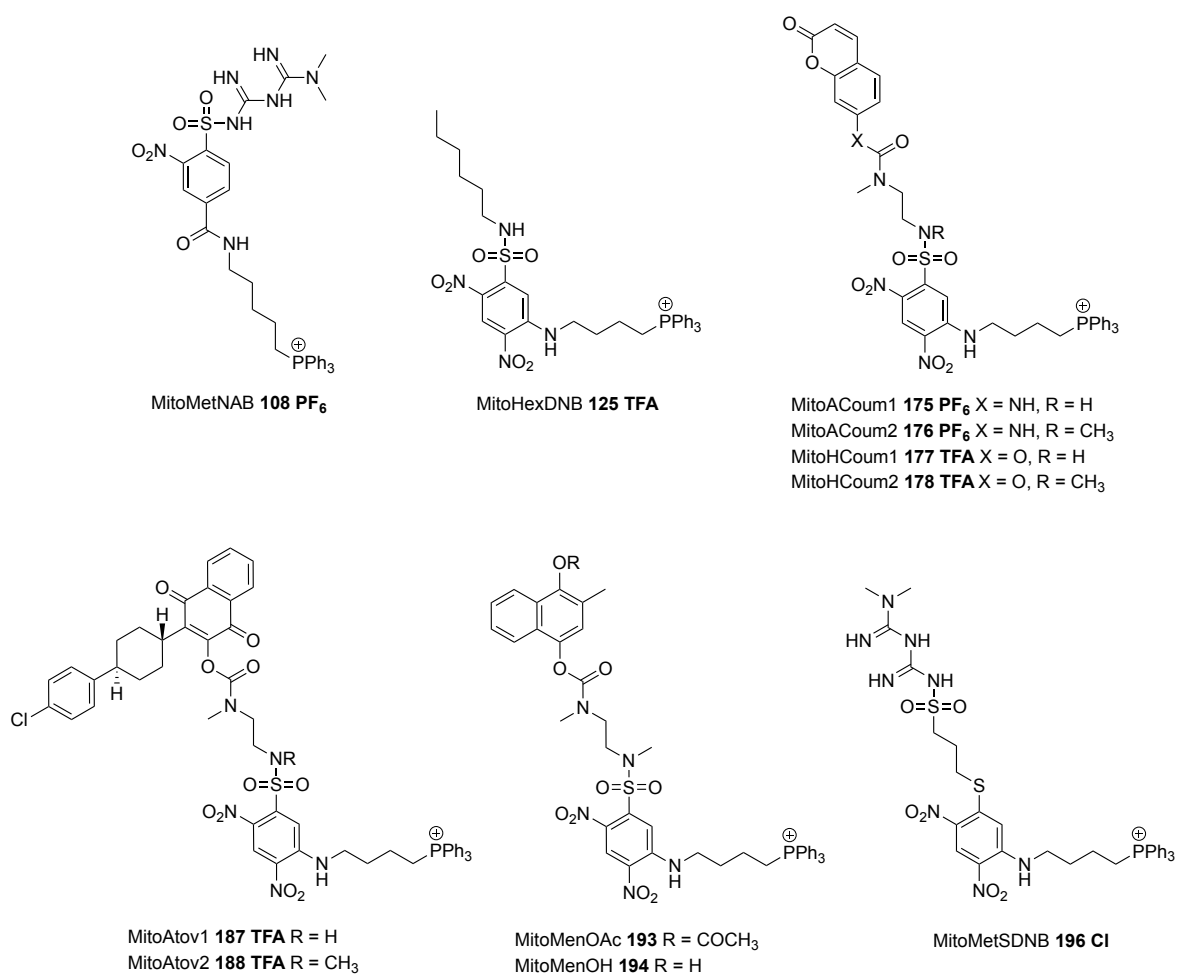


Figure 59. Successfully synthesised prodrug-like sulfonamide compounds.

Approach A aimed to attach an FDA approved drug, via a sulfonamide bond, to an electron-poor ring based on the structures of CDNB **48** and MitoCDNB **51**. Metformin **58** was the primary cargo of interest due to its complex I inhibitory effects and anti-cancer activity. When the prodrug is in the presence of GSH **25** and GST enzymes, a glutathione-conjugate was expected to form via a S_NAr reaction at C-S to release SO₂ and the cargo. Two prodrug designs i) and ii) were explored. Design i) utilised an *ortho*-nitro, *para*-amide aromatic system, previously recognised as a GST enzyme substrate (see Section 2.6). Here, the chloride substituent was replaced with a sulfonamide cargo. The target compound, MitoMetNAB **108**, was successfully synthesised. However, it was found to be unstable in pH 8.0 buffer due to a Smiles rearrangement of the metformin moiety onto the ring's *ipso* position. Design ii) used the dinitro aromatic system of MitoCDNB **51**, with the chloride substituent similarly replaced by a sulfonamide cargo. Unfortunately, this

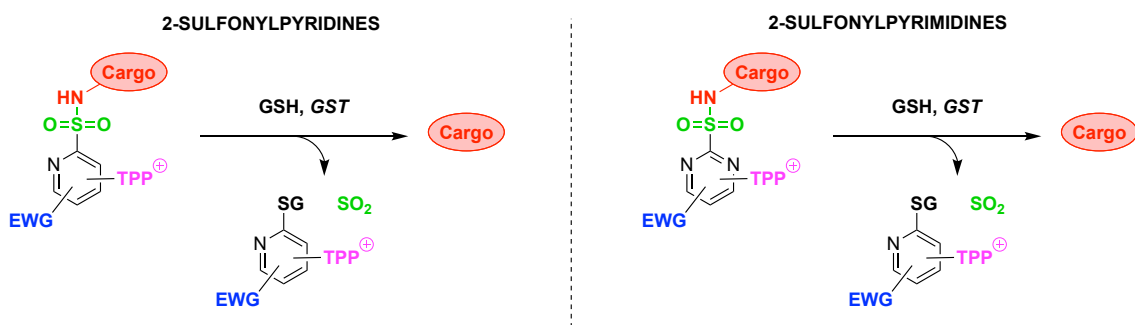
scaffold was identified to be too reactive to obtain stable sulfonamide prodrugs of the cargos of interest. However, a prodrug-like compound, MitoHexDNB **125**, featuring a straight aliphatic lipophilic chain, was successfully synthesised and found to be highly reactive with GSH **25** and GST enzymes. This demonstrates that alkyl amine cargos could be delivered via this prodrug strategy. Attention then shifted to approach B, which incorporated an ethylenediamine spacer between the electron-poor ring (via a sulfonamide bond) and a phenol or arylamine payload (via a carbamate or urea linkage, respectively). Upon activation by mGSH/mGSTs, the intermediate was expected to cyclise in alkaline aqueous conditions, releasing the payload. To explore this strategy for phenol or arylamine payloads, 7-aminocoumarin prodrugs (MitoACoum1 **175** and MitoACoum2 **176**) and 7-hydroxycoumarin prodrugs (MitoHCoum1 **177** and MitoHCoum2 **178**) were prepared. Fluorescence was quenched for each compound in pH 8.0 buffer. Kinetic analysis revealed rapid reaction with GSH **25**, independent of GST catalysis. However, only MitoHCoum1 **177** and MitoHCoum2 **178** released the coumarin payload, demonstrating that this self-immolative strategy was only effective to release phenol payloads. Subsequently, prodrugs MitoAtov1 **187** and MitoAtov2 **188** were prepared to release atovaquone **163**, a mitochondrial complex III inhibitor with anticancer potential. While these prodrugs reacted with GSH **25** to produce MitoGSDNB **51** and release atovaquone **163**, a glutathione-atovaquone conjugate **195** was also generated by a competing S_NAr reaction on the electron-poor atovaquone moiety. Additionally, the prodrugs were not completely converted, perhaps due to micelles. The double prodrug, MitoMenOAc **193**, and the single prodrug, MitoMenOH **194**, were also prepared to release menadiol **164**, which is the electron-rich and reduced form of menadione **183**, a ROS-generator by redox cycling. Both prodrugs require activation by GSH **25**, but MitoMenOAc **193** must also be hydrolysed by a mitochondrial enzyme with esterase activity to cleave the acetate protecting group. These prodrugs will potentially induce the combination of GSH **25** depletion with ROS generation, which is expected to be lethal towards the mitochondria of cancer cells. Furthermore, prodrugs MitoMenOAc **193** and MitoMenOH **194** are expected to be sent down to the MRC Mitochondrial Biology Unit in Cambridge for biological studies. Finally, approach C investigated metformin **58** release from a different self-immolative system. A sulfonamide of metformin was attached to a dinitro ring via a sulfonamide bond and a propyl sulfide linker in a prodrug MitoMetSDNB **196**. The design envisaged that cleavage of the C-S bond by S_NAr with GSH **25** would yield a thiolate intermediate, which would cyclise to a thiosulfonate and release metformin **58**.

alongside SO₂. Although the target compound, MitoMetSDNB **196**, was successfully synthesised, it was unreactive with GSH **25**, even in the presence of GST enzymes.

3.6 Future Work

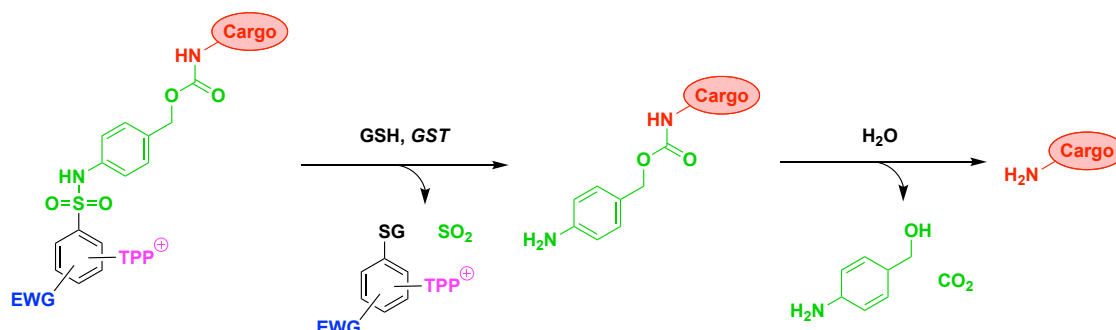
Future work can be divided into work using the compounds reported in this thesis and the exploration of new avenues. In the first of these, the uptake of compounds MitoHCoum1 **177**, MitoHCoum2 **178**, MitoMenOAc **193**, and MitoMenOH **194** into mitochondria and their transformation to release their payloads will be investigated in isolated mitochondria, cell lines, and *in vivo* models. MitoHCoum1 **177** and MitoHCoum2 **178** will serve as proof-of-concept compounds, demonstrating both mitochondrial accumulation and GSH-mediated activation to release 7-hydroxycoumarin **106**, detectable via fluorescence microscopy. In parallel, MitoMenOAc **193**, and MitoMenOH **194** will be studied for their effect on mitochondrial thiol redox status and oxidative stress.

Following a new avenue of research, there is potential to explore other S_NAr-type sulfonamide systems based on heteroaromatic rings as opposed to nitrobenzenes (Scheme 83). Several literature studies have developed GST or GSH-activated sulfonyl compounds based on electron-poor 2-sulfonylpyridines and 2-sulfonylpyrimidines.^{179, 252, 278, 279} In particular, the 2-sulfonyl-5-cyanopyridinyl moiety has been employed to release a number of drugs because it is sufficiently stable in the reaction with GSH **25**, but efficiently activated by GST enzymes.²⁵²



Scheme 83. Novel designs for GSH/GST-activated sulfonyl heteroaromatic prodrugs.

The GSH-induced release of alkylamines by S_NAr on electron-poor arylsulfonamides was demonstrated with MitoHexDNB **125**, MitoACoum1 **175** and MitoACoum2 **176**. Ultimately, MitoMetNAB **108** demonstrated that electron-poor rings attached to metformin sulfonamides are prone to Smiles rearrangement in alkaline aqueous conditions, highlighting the need for a spacer. The ethylenediamine spacer facilitated the release of phenol payloads but was ineffective for arylamine payloads. This prompts exploration of alternative self-immolative strategies for release of metformin **58**. One such approach employs the widely used 4-aminobenzyl alcohol system.^{280, 281} Emulating approaches A and B, this design would link the amine group to the electron-poor ring via a sulfonamide and attach the hydroxy group to the drug through a carbamate (Scheme 84). Activation by GSH **25** would generate SO_2 and an aniline intermediate, which would undergo a 1,6-elimination to produce 4-aminobenzyl alcohol, CO_2 and the free amine drug. Like the ethylenediamine linker, this linker separates the amine cargo from the site of S_NAr reaction. It may also allow formation of prodrugs of electron-poor arylamines, such as of 7-aminocoumarin **104**, which could not be developed into the prodrugs MitoAmDNB **127** and MitoAmNTB **143** in approach A (see Sections 3.2.3 and 3.2.4).



Scheme 84. Novel prodrug design featuring a self-immolative 4-aminobenzyl alcohol.

Kinetic measurements of the S_NAr -activated prodrugs revealed high reactivity with GSTA1-1 and GSTA4-4 enzymes but significantly lower reactivity with GSTK1-1. This is likely attributed to its distinct substrate-binding site. As the primary GST enzyme in mitochondria, GSTK1-1 remains an appealing target for substrate binding and GSH **25** conjugation. The literature indicates that among the substrates tested for GSH **25** conjugation with GSTK1-1, CDNB **48** exhibits the highest reactivity.^{136, 234} However, CDNB **48** remains a considerably less efficient substrate for GSTK1-1 than GSTA1-1 and GSTA4-

4. Therefore, there is a need to identify novel, more efficient substrates for GSTK1-1, potentially through high-throughput screening approaches.

Chapter 4 Preparation of photoaffinity probes of phenformin

4.1 Approach

In Chapter 3, attempts to prepare suitable prodrugs for the selective delivery of metformin **58** to the mitochondrial matrix were described. However, as alluded to in Section 1.4, metformin **58** may have many targets inside and outside the mitochondria. To investigate this, a close analogue of metformin **58** was considered, Phenformin **59** (Figure 60). Phenformin **59** belongs to a class of biguanides with strong therapeutic potential to treat several different diseases. It was formerly a worldwide frontline antidiabetic drug before it was withdrawn from most markets due to a high risk of lactic acidosis. It has since been replaced by the safer analogue, metformin **58**.²⁸² Phenformin **59** and metformin **58** have been the subject of extensive research for their repurposing to treat cancer and numerous pre-clinical studies have identified their antiproliferative properties toward several tumour types (see Section 1.4).^{199, 210, 212, 213, 244-246} However, the mechanisms underlying their therapeutic action are complex and still not fully understood.^{187, 188} There is also evidence to indicate that the mechanisms are dose-dependent.^{187, 283} It is generally agreed that both phenformin **59** and metformin **58** inhibit mitochondrial respiratory complex I, a 1-MDa multiprotein assembly.^{187, 188, 190-192} However, biguanides have been reported to target many cellular proteins.^{195, 197, 198, 284, 285} The aim of this project was to investigate the target proteins of key biguanides in normal cells and cancer cells by photoaffinity labelling. Metformin **58** is smaller and more water-soluble than phenformin **59** which means modification of its structure using organic synthesis is difficult and poses a higher risk of altering its bioactivity. Additionally, studies show that phenformin **59** is a stronger inhibitor of complex I and a more potent anticancer drug in various tumour types, compared to metformin **58**,^{192, 198, 212, 213} which makes it an attractive biguanide to investigate. Two photoreactive cross-linking derivatives of phenformin **59** would be developed, incorporating at the 4-position of the phenyl ring either a trifluoromethyl diazirine or a benzophenone in Phenformin-DZ **209** or Phenformin-BZ **210**, respectively (Figure 60). Trifluoromethyl diazirines and benzophenones are widely used as photo-cross-linking groups and provide different advantages (see Chapter 1.5.2).

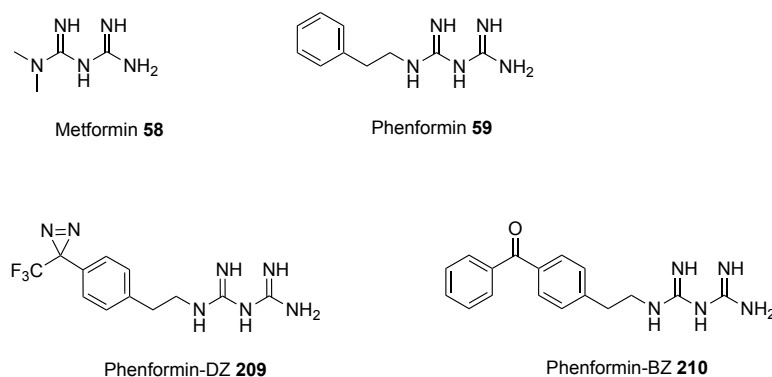
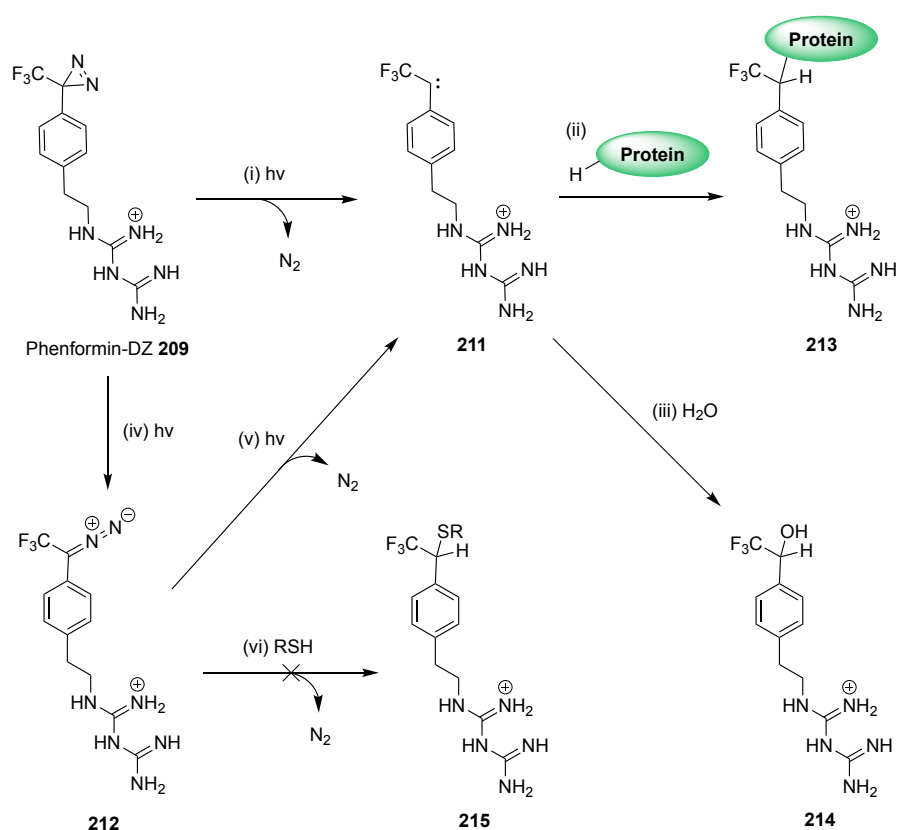


Figure 60. Structures of metformin 58, phenformin 59, trifluoromethyl-diazirine phenformin derivative, Phenformin-DZ 209 and benzoyl phenformin derivative. Phenformin-BZ 210.

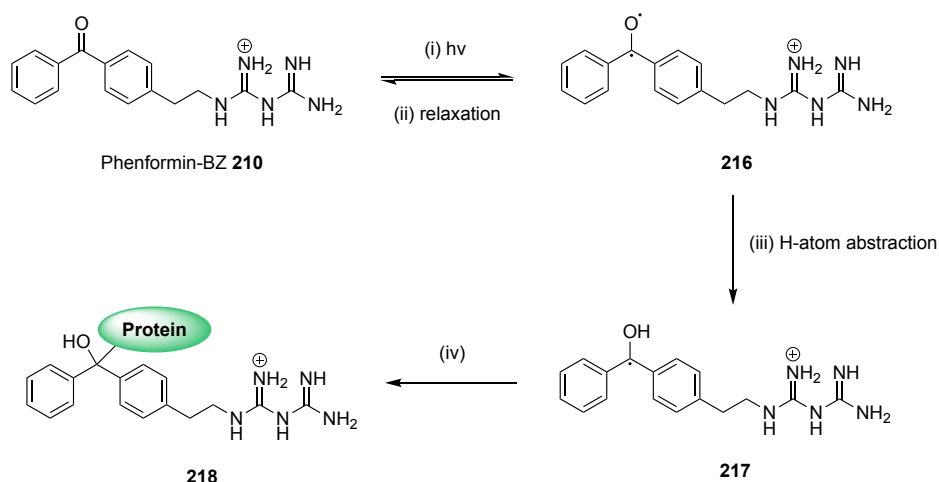
Initially, a tag for biorthogonal chemistry was not included in the design of the molecules to minimize structural modification to the phenformin moiety, which might alter its interactions with protein targets. In theory, comparing a cell's proteome before and after treatment with Phenformin-DZ 209 or Phenformin-BZ 210 using mass spectrometry should reveal the cross-linked proteins. Therefore, the objective was to collaborate with a company specializing in advanced proteomics.

Irradiation of Phenformin-DZ 209 or Phenformin-BZ 210 at approximately 350 nm is expected to generate a highly reactive species capable of inserting into C-H or X-H bonds of the protein.^{220, 223} In the case of Phenformin-DZ 209 (Scheme 85), irradiation should primarily convert the diazirine into the highly reactive carbene intermediate 211, while approximately 30% will form the diazo compound 212, which has a longer lifetime but can still generate carbene 211.^{221, 222} The carbene intermediate 211 can insert into the C-H or X-H bonds of the protein to produce the cross-linked protein 213 or react with water to form alcohol 214. The presence of the electron-withdrawing trifluoromethyl moiety should limit the reactivity of diazo compound 212 with intracellular thiols to form sulfide 215, thereby reducing off-target cross-linking.



Scheme 85. Proposed photoreactivity of Phenformin-DZ **209** in cells.

Upon photoirradiation of Phenformin-BZ **210**, the carbonyl group is expected to generate the reactive diradical **216** (Scheme 86). In its singlet state, diradical **216** can relax to Phenformin-BZ **210**, whereas in its triplet state, it can abstract a hydrogen atom from the C-H bond of the protein. The resulting ketyl radical **217** can then combine with the protein carbon centred radical, leading to the formation of the cross-linked protein **218**.

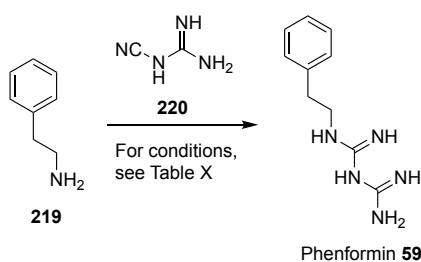


Scheme 86. Proposed photoreactivity of Phenformin-BZ **210** in cells.

4.2 Synthesis

4.2.1 Synthesis of phenformin

The synthesis of phenformin **59** involved reacting commercially available phenylethylamine **219** with 2-cyanoguanidine **220** (Scheme 87) by adapting two literature procedures (Table 14). Using the method of Cao *et al.* (Entry 1),²⁸⁶ the reaction did not reach completion, and the triflate salt of the desired phenformin product **59** was only partially separated from the triflate salt of phenylethylamine **219** via column chromatography, resulting in a low yield of 13%. In contrast, when the procedure of Fortun *et al.* (Entry 2) was used,²⁸⁷ the reaction proceeded to completion, and quenching with excess HCl precipitated the double chloride salt of phenformin product **59**, which was isolated without the need for column chromatography.



Scheme 87. Synthesis of phenformin **59** from phenylethylamine **219** and 2-cyanoguanidine **220**.

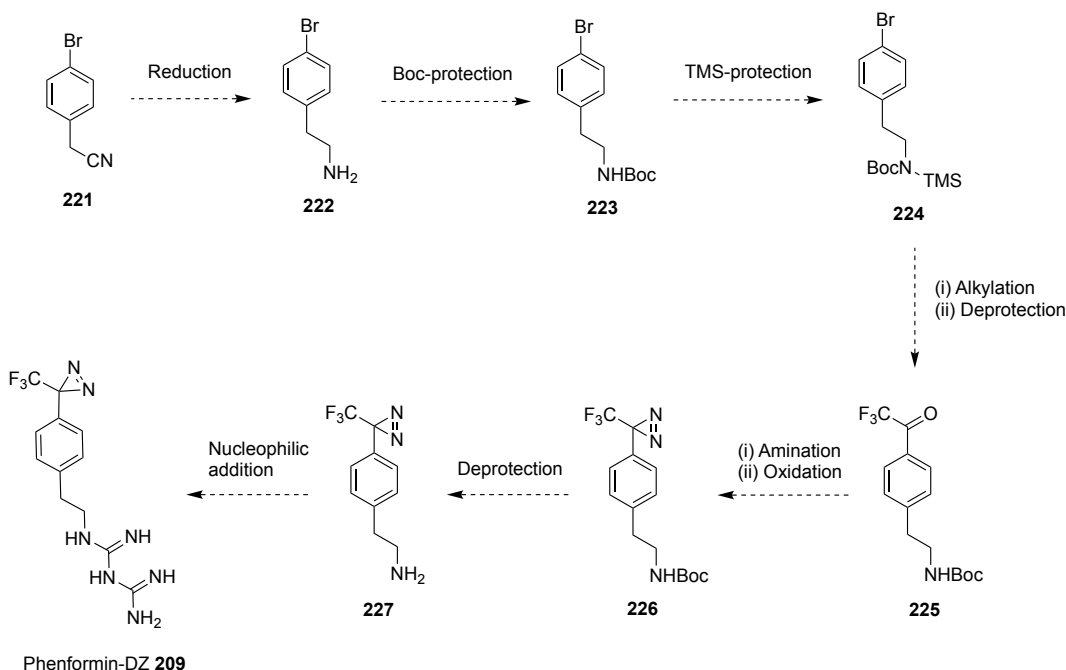
Entry	Starting material	Reagents	Solvent	Temp.	Time	Result
1	Phenylethylamine 219	(i) TMSOTf (ii) 2-cyanoguanidine 220	Dichloroethane	(i) RT (ii) Reflux	(i) 0.5 h (ii) 6 h	Mostly SM, 13% product 59
2	Phenylethylamine 219	(i) 2-cyanoguanidine 220 , FeCl ₃ (ii) HCl (3 eq.)	Dioxane	(i) Reflux (ii) RT	(i) 1.5 h	91% product 59

Table 14. Reaction conditions used to synthesise phenformin **59** from phenylethylamine **219** and 2-cyanoguanidine **220**. Entry 1 adapts the procedure by Cao *et al.*²⁸⁶ Entry 2 adapts the procedure by Forun *et al.*²⁸⁷

4.2.2 Synthesis of phenformin photo-cross-linkers

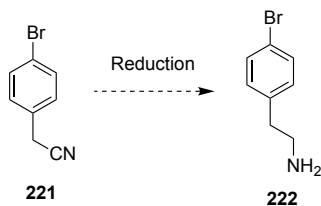
Next, a synthetic route for Phenformin-DZ **209** was developed (Scheme 88), starting from commercially available 4-bromophenylacetonitrile **221**. The nitrile would be reduced to the corresponding amine **222**, which would then undergo two protections: first, Boc-protection to form carbamate **223**, and second, TMS-protection to yield silyl carbamate **224**. Alkylation of silyl carbamate **224** would introduce the trifluoromethyl ketone moiety, followed by an acidic work-up to remove the TMS group, affording ketone **225**. A one-pot amination and oxidation reaction should generate diazirine **226**. Finally, Boc deprotection would yield amine **227**, which, under the same nucleophilic addition

conditions used in the synthesis of Phenformin **59**, should provide the target compound, Phenformin-DZ **209**.



Scheme 88. Proposed synthesis route towards Phenformin-DZ **109**.

The reduction of 4-bromophenylacetonitrile **221** to amine **222** (Scheme 89) was attempted using a variety of standard reducing conditions from the literature (Table 15). Neither borane (Entry 1) nor Raney Nickel/ H_2 (Entry 2) produced any detectable amine product, and analysis of the crude material by ^1H NMR indicated only the presence of the nitrile starting material **221**. Similarly, reduction by LiAlH_4 (Entry 3) yielded primarily unreacted nitrile **221**, with only a small amount of impure amine **222** isolated.



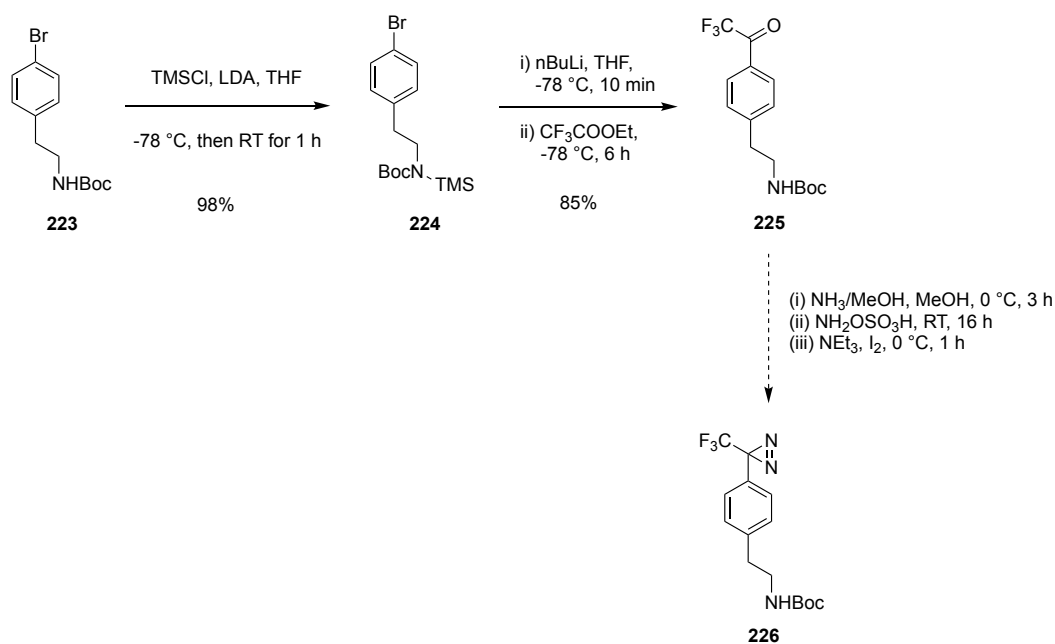
Scheme 89. Attempted reduction of nitrile **221** to amine **222**.

Entry	Starting material	Reagents	Solvent	Temp.	Time	Result
1	4-bromophenyl-acetonitrile 221	BH ₃ .THF	THF	0 °C, then reflux	24 h	Starting materials
2	4-bromophenyl-acetonitrile 221	Raney Nickel, H ₂	MeOH	RT	24 h	Starting materials
3	4-bromophenyl-acetonitrile 221	LiAlH ₄ .THF	THF	0 °C, then reflux	24 h	Mostly nitrile 221 , impure product 222

Table 15. Reaction conditions used to prepare amine **222** from nitrile **221**.

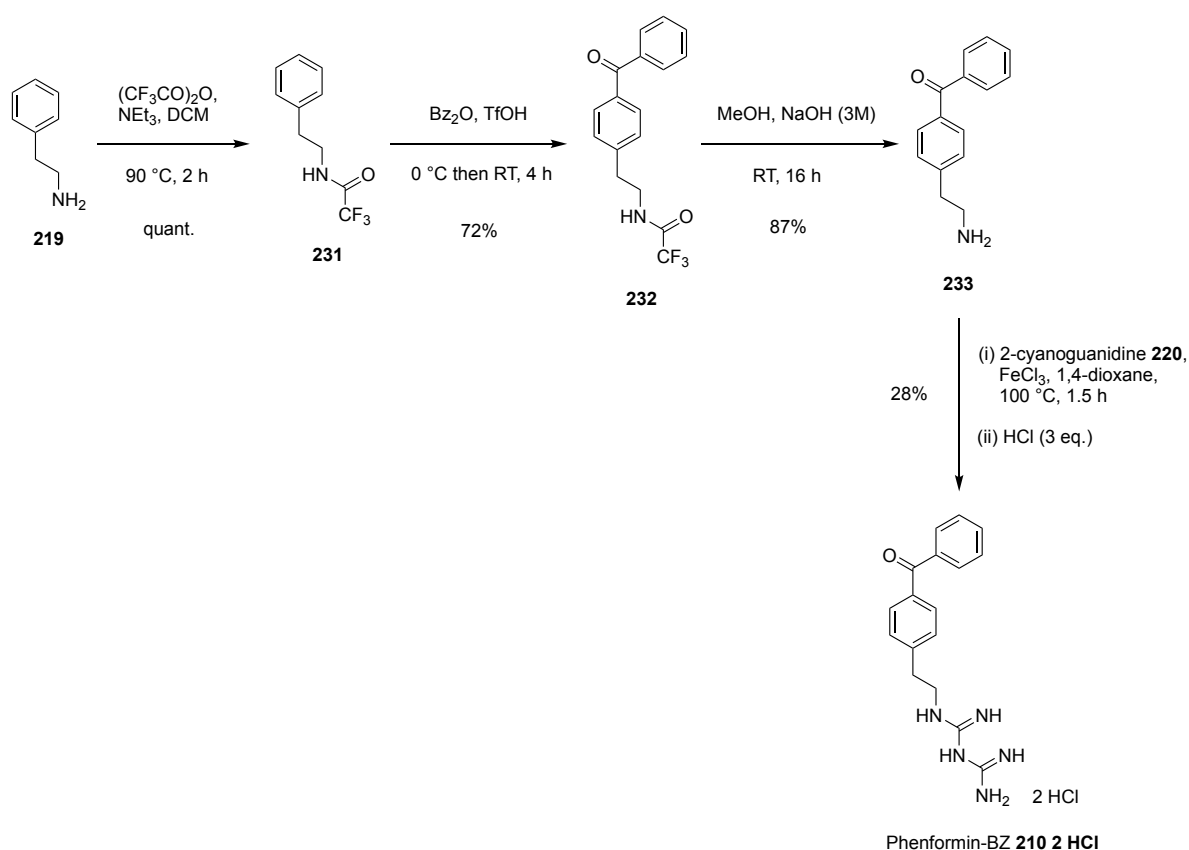
Given the challenges of reducing 4-bromophenylacetonitrile **221** to amine **222**, and the time limitations for the project, attention was turned to a study by Murai *et al.*,²⁸⁸ who had developed diazirine photo-cross-linkers based on the structure of 2-phenylethylamine **219**, starting from the Boc-protected amine **223**. Although more expensive than 4-bromophenyl-acetonitrile **221**, the Boc-protected amine **223** was also commercially available.

The synthetic route was resumed by beginning two steps later with the TMS-protection of the Boc-protected amine **223** (Scheme 90), which afforded silyl carbamate **224** in excellent yield. Lithiation of the aryl ring of silyl carbamate **224** with *n*-butyllithium, followed by reaction with ethyl trifluoroacetate produced ketone **225** in excellent yield. In a one-pot reaction, ketone **225** was first treated with ammonia/methanol and hydroxylamine-*O*-sulfonic acid, followed by oxidation with iodine in an attempt to obtain the diazirine **226**. ¹H NMR analysis of the crude material indicated a mixture consisting primarily of ketone **225** and a small amount of diazirine **226**, which were inseparable by column chromatography.



Scheme 90. Synthesis route one towards diazine 125.

After the limited success of the one-pot reaction, diazine **226** was instead prepared in four steps (Scheme 91) by following the exact procedure of Murai *et al.*²⁸⁸ Ketone **225** was reacted with hydroxylamine hydrochloride to obtain the oxime **228** in excellent yield, followed by tosylation with tosyl chloride to afford the *O*-sulfonate **228** in quantitative yield. Liquid ammonia was generated from reaction between NaOH and NH_4Cl , and added to the *O*-sulfonate **229**, which produced the diaziridine **230** in moderate yield. Oxidation of diaziridine **230** with MnO_2 afforded the diazine **226** in excellent yield. The Boc group of the diazine **226** was deprotected with trifluoroacetic acid, followed by treatment with NaOH to obtain the free amine **227** in excellent yield. Finally, the amine was reacted under the same conditions used in the preparation of Phenformin **59** above, and adapting the method of Fortun *et al.*²⁸⁷ gave Phenformin-DZ **209** as the double HCl salt, albeit in poor yield.



Scheme 92. Synthesis of Phenformin-BZ **210**.

4.3 Conclusions

Overall, two photoaffinity probes of Phenformin were synthesised (Figure 61): Phenformin-DZ **209**, incorporating a trifluoro-diazirine photo-cross-linker, and Phenformin-BZ **210**, incorporating a benzophenone photo-cross-linker. A proteomics study on these compounds should help identify key protein targets, providing further insight into the mechanisms of action of the biguanide therapeutic class. To preserve the structural (and therefore pharmacological) integrity of Phenformin **209**, neither cross-linker included a tag for biorthogonal chemistry. However, as a result, identifying the proteins that these molecules bond to will require advanced mass spectrometry and significant computational analysis.

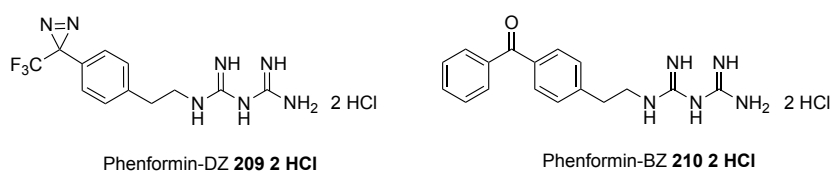
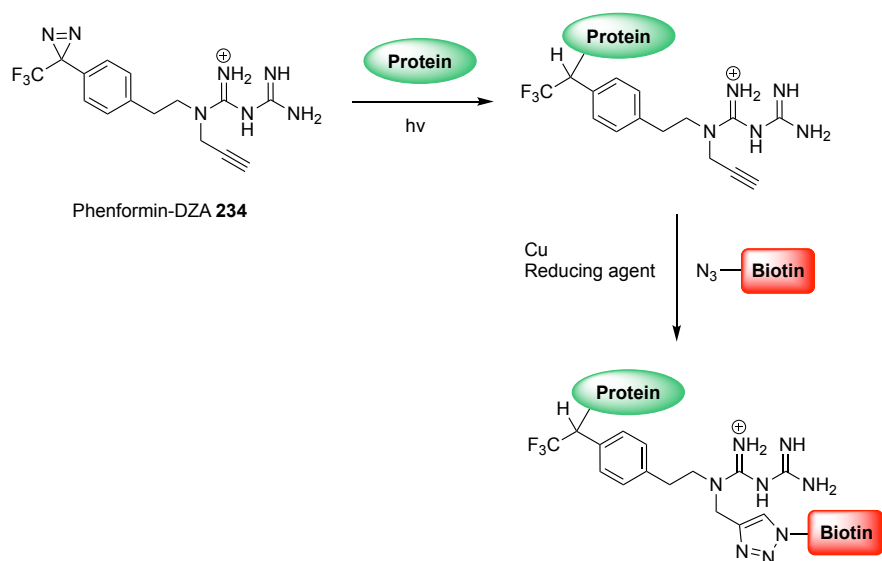


Figure 61. Structures of the prepared photoaffinity probes Phenformin-DZ 209 and Phenformin-BZ 210 as the double HCl salts.

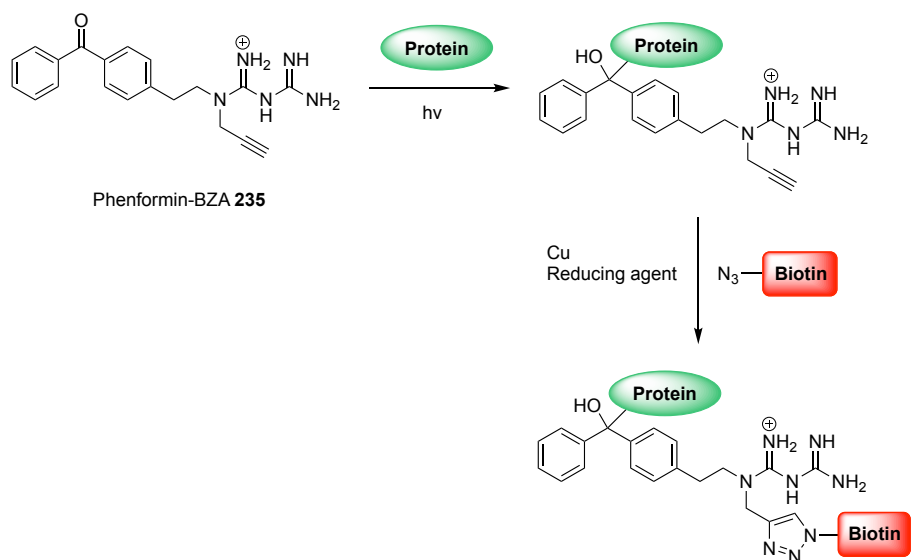
To first assess the *in vitro* pharmacological activity of Phenformin-DZ 209 and Phenformin-BZ 210 in comparison to Phenformin 58, all three compounds have been sent to Prof. Angus Lamond's research group at the University of Dundee. Following this, there are plans to collaborate with a specialized proteomics company to identify the target proteins.

4.4 Future Work

Future synthesis efforts should focus on developing photoaffinity probes of phenformin that incorporate a tag for biorthogonal chemistry, such as an alkyne group, to make target protein identification easier. Upon photoirradiation, the probe would covalently capture the target protein, after which cells would be lysed and an azide-containing affinity tag would be introduced. Biotin is commonly used due to its high affinity for avidin, enabling efficient isolation of cross-linked proteins.²²¹ The alkyne group must be positioned to minimize interference with both the photo-cross-linker and the guanidine ligand moiety of the molecule. Considering these factors, as well as the synthetic challenge of incorporating an alkyne group onto the phenformin scaffold, Phenformin-DZA 234 (Scheme 93) and Phenformin-BZA 235 (Scheme 94) would appear to be sensible targets.



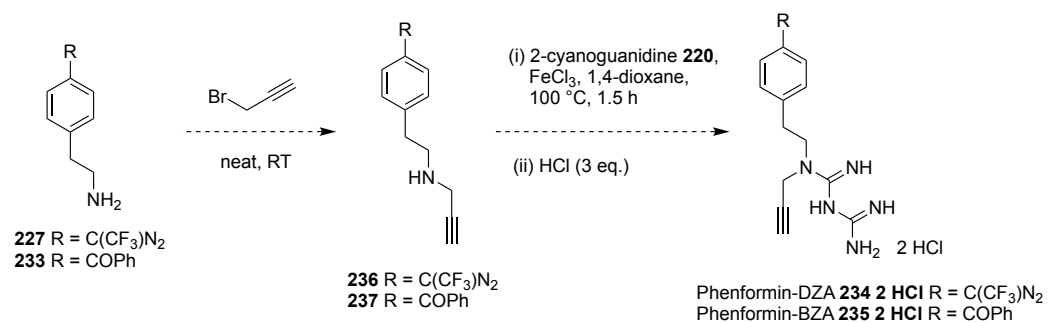
Scheme 93. Proposed mechanism of action for novel photoaffinity probe Phenformin-DZA **234**.



Scheme 94. Proposed mechanism of action for novel photoaffinity probe Phenformin-BZA **235**.

Photoaffinity probes **234** and **235** could be synthesised starting from the previously prepared amines **227** and **233**, respectively (Scheme 95). Nucleophilic substitution of propargyl bromide with amine **126** or **132**, adapting literature procedure,²⁸⁹ should yield alkyne **135** or **136**, respectively. Finally, reaction of alkynes **135** or **136** with 2-cyanoguanidine, adapting the method of Fortun *et al.*,²⁸⁷ should afford the desired

photoaffinity probes Phenformin-DZA **234** or Phenformin-BZA **235**, respectively, as the double HCl salts.



Scheme 95. Proposed synthesis of photoaffinity probes Phenformin-DZA **234** and Phenformin-BZA **235**.

Chapter 5 Experimental

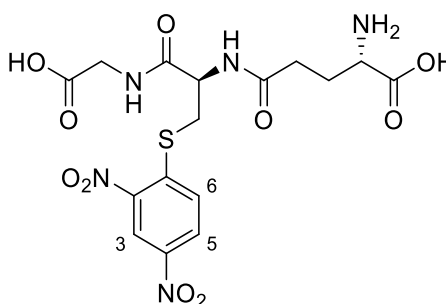
5.1 Chemical Synthesis

General

All reagents and starting materials were obtained from commercial suppliers. All reactions were performed in flame or oven-dried flasks. Anhydrous solvents tetrahydrofuran, dichloromethane, diethyl ether, toluene and acetonitrile were obtained from a PurSolv 500 MD solvent purification system. All other anhydrous solvents were obtained from commercial vendors in septum sealed bottles under an inert atmosphere. Merck aluminium-backed plates pre-coated with silica gel 60 (UV254) were used for thin layer chromatography and were visualised under ultraviolet light or by staining with permanganate, ninhydrin, vanillin, ceric sulfate or dragendorff reagent. Column chromatography was performed either manually using fluorochem 60 Å (particle size 40–63 µm) silica gel or, using a Biotage® Isolera™ One Flash Chromatography system with Biotage® SNAP Ultra, Biotage® KIP or Agela silica gel cartridges (4, 10, 20, 35, 40 or 70 g). Purification and reaction kinetics by HPLC was performed using a Shimadzu LC-20AD Liquid Chromatograph fitted with an SPD-20A UV-VIS detector with absorbance measured at 254 and 280 nm. The column used for purification was a Luna Omega PS C18 (250 x 10 mm), with a solvent flow of 5 mL/min. The column used for analysis was a Luna Omega PS (250 x 4.6 mm), with a solvent flow of 1 mL/min. The mobile phase consisted of a mixture of HPLC-grade H₂O (0.1 % TFA) and HPLC-grade acetonitrile. Melting points were determined using a Gallenkamp MFB.595.010M equipped with an internal thermometer. UV-Visible spectra were measured on a Jasco V550 UV/Vis spectrometer or a Horiba Duetta. UV-Visible absorbance kinetics were measured on a Jasco V550 UV/Vis spectrometer. Fluorescence spectra and fluorescence kinetics were measured on a Horiba Duetta. Infrared spectra were recorded using a Shimadzu FTIR-8400S spectrometer. NMR spectra were recorded on either 400 MHz (Bruker AVIII, Bruker AVIII HD or Bruker DPX) or 500 MHz (Bruker AVIII HD) spectrometers. Chemical shift values are reported in ppm relative to tetramethylsilane (δ_{H} 0.00 and δ_{C} 0.00) and referenced to the relevant deuterated solvent residual peaks. NMR data are reported in the format: chemical shift (integral, multiplicity [s = singlet, d = doublet, dd= doublet of doublets, t = triplet, td=

triplet of doublets, q = quartet, quin = qn, m = multiplet, sept = septet, br = broad], coupling constant in Hz, assignment). Coupling constants are as observed and are not corrected to match other signals. ^{13}C NMR data are reported to 2 d.p. to distinguish between observed signals that differ by less than 0.1 ppm. It is recognised that the absolute value for the second decimal place may not be accurate. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of DEPT, COSY, HSQC, HMBC and NOESY experiments. Mass spectra were recorded using a Bruker MicroTOFq High Resolution Mass Spectrometer coupled to a time-of-flight (TOF) analyser for positive or negative mode electrospray ($\text{ESI}^{+/-}$) or atmospheric pressure chemical ionisation ($\text{APCI}^{+/-}$).

***S*-(2,4-Dinitrophenyl)glutathione (GSDNB) 49**

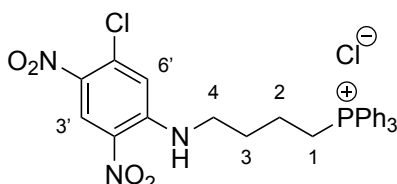


Glutathione (228 mg, 0.741 mmol, 1.50 eq.) was added to a solution of 1-chloro-2,4-dinitrobenzene (100 mg, 0.494 mmol, 1.00 eq.) and NaHCO_3 (124 mg, 1.48 mmol, 3.00 eq.) in $\text{EtOH}:\text{H}_2\text{O}$ (1:1, 6 mL). The reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography [SiO_2 , water:acetonitrile 0-100%] to afford **49** as an orange solid (195 mg, 86%).

Mp: Decomposition at 210 °C. ^1H NMR (400 MHz, D_2O): δ_{H} 9.12 - 9.06 (1H, m, H-3), 8.54 - 8.46 (1H, m, H-5), 7.95 - 7.87 (1H, m, H-6), 4.87 - 4.75 (1H, m, Cys CH), 3.87 - 3.62 (4H, m, Gly CH_2 , Gln CH, and $\text{CH}_\text{A}\text{CH}_\text{B}\text{S}$), 3.47 (1H, dd, $J = 14.2, 9.3$ Hz, $\text{CH}_\text{A}\text{CH}_\text{B}\text{S}$), 2.47 (2H, t, $J = 7.7$ Hz, CH_2CONH), 2.08 (2H, q, $J = 7.4$ Hz, $\text{CH}_2\text{CH}_2\text{CONH}$). ^{13}C NMR (101 MHz, D_2O): δ_{C} 176.14 (C), 174.99 (C), 174.56 (C), 170.96 (C), 145.37 (C), 144.44 (C), 144.28 (C), 128.14 (CH), 127.71 (CH), 121.89 (CH), 54.18 (CH), 51.55 (CH), 43.36 (CH_2), 33.51 (CH_2), 31.45 (CH_2), 26.53 (CH_2). HRMS (ESI^+): $\text{C}_{16}\text{H}_{20}\text{N}_5\text{O}_{10}\text{S}$ requires 474.0925 found 474.0931 ($\text{M} + \text{H}$) $^+$. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT

experiments. The ^1H and ^{13}C NMR data is in broad agreement with that previously reported in DMSO.²⁹⁰

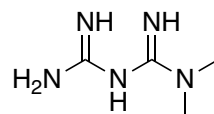
**{4-[(5'-Chloro-2',4'-dinitrophenyl)amino]butyl}triphenylphosphonium chloride
(MitoCDNB) 51**



Following the procedure of Booty *et al.*,¹⁷⁸ 1,5-dichloro-2,4-dinitrobenzene (631 mg, 2.66 mmol, 1.10 eq.) and anhydrous *N,N*-diisopropylethylamine (842 μL , 4.84 mmol, 2.00 eq.) were added to a solution of **95** (1.00 g, 2.42 mmol, 1.00 eq.) in anhydrous acetonitrile (5 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the solid residue was redissolved in dichloromethane (40 mL). The organic layer was washed with HCl (30 mL, 1 M) and the aqueous layer was extracted with dichloromethane (3 $\times$ 25 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **51** as an orange hygroscopic foam (1.26 g, 83%).

^1H NMR (400 MHz, CDCl_3): δ_{H} 8.93 (1H, s, H-3'), 8.42 (1H, t, J = 5.4 Hz, NH), 7.91 - 7.83 (6H, m, Ph), 7.82 - 7.74 (3H, m, Ph), 7.73 - 7.65 (6H, m, Ph), 7.16 (1H, s, H-6'), 4.01 (2H, m, CH_2 -1), 3.72 (2H, q, J = 6.5 Hz, CH_2 -4), 2.26 - 2.04 (2H, qn, J = 7.0 Hz, CH_2 -2), 1.94 - 1.65 (2H, m, CH_2 -3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 146.90 (C), 136.11 (C), 135.27 (d, J = 3.1 Hz, 3 $\times$ CH), 134.56 (C), 133.83 (d, J = 10.0 Hz, 6 $\times$ CH), 130.67 (d, J = 12.6 Hz, 6 $\times$ CH), 128.97 (C), 126.81 (C), 118.57, 117.35 (d, J = 73.9 Hz, 3 $\times$ C), 42.73 (CH_2), 28.85 (d, J = 17.0 Hz, CH_2), 22.58 (d, J = 50.9 Hz, CH_2), 19.86 (d, J = 4.0 Hz, CH_2). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 24.68 (1P, s, PPh_3). HRMS (ESI $^+$): $\text{C}_{28}\text{H}_{26}^{35}\text{ClN}_3\text{O}_4\text{P}$ requires 534.1344 found 534.1345 (M^+). The ^1H and ^{13}C NMR data is in agreement with that previously reported.¹⁷⁸

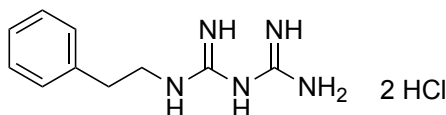
1,1-Dimethylbiguanide (Metformin) **58**



NaOH (120 mg, 3.02 mmol, 1.00 eq.) in water (2.5 mL) was added dropwise to metformin hydrochloride (500 mg, 3.02 mmol, 1.00 eq.) in water (2.5 mL) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure. Methanol (10 mL) was added to the solid residue and the mixture was filtered under vacuum. The filtrate was collected and the solvent was removed under reduced pressure to afford **58** as a white solid (513 mg, quant.).

Mp 108 - 110 °C. ¹H NMR (400 MHz, DMSO): δ_{H} 6.47 (5H, br s, 5 x NH), 2.83 (6H, s, 2 x CH₃). ¹³C NMR (101 MHz, DMSO): δ_{C} 160.64 (C), 160.27 (C), 36.94 (2 x CH₃). HRMS (ESI⁺): C₄H₁₂N₅ requires 130.1087 found 130.1087 (M + H)⁺. The ¹H NMR data is in agreement with that previously reported.²⁹¹ The ¹³C NMR data is in broad agreement with that previously reported in D₂O.²⁹²

N-Phenylethylbiguanide bishydrochloride (Phenformin) **59**

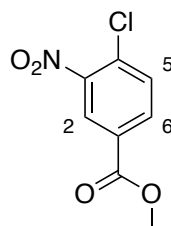


Adapting the procedure of Fortun *et al.*,²⁸⁷ dicyandiamide (138 mg, 1.59 mmol, 1.00 eq.) and iron (III) chloride (258 mg, 1.59 mmol, 1.00 eq.) were added to a solution of phenylethylamine (200 μ L, 1.59 mmol, 1.00 eq.) in anhydrous 1,4-dioxane (1.59 mL). The reaction was stirred at reflux for 1.5 h and then allowed to cool to room temperature. To the reaction was added concentrated hydrochloric acid (398 μ L, 12 M), followed by ethyl acetate (5 mL). The suspension was filtered under vacuum and washed with ethyl acetate (20 mL) to afford **59** as a white hygroscopic solid (350 mg, 91%).

ν_{max} (ATR): 2593 - 3440 (N-H), 1684 (C=N), 1680 (C=N) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_{H} 7.40 - 7.24 (5H, m, Ph), 3.60 (2H, t, *J* = 7.5 Hz, NCH₂), 2.98 (2H, t, *J* = 7.6 Hz, PhCH₂).

^{13}C NMR (101 MHz, CD_3OD): δ_{C} 156.74 (C), 154.28 (C), 138.89 (C), 129.86 (2 x CH), 129.77 (2 x CH), 127.91 (CH), 45.24 (CH_2), 34.93 (CH_2). HRMS (ESI^+): $\text{C}_{10}\text{H}_{16}\text{N}_5$ requires 206.1400 found 206.1402 ($\text{M} + \text{H}$) $^+$. The ^1H and ^{13}C NMR data is in broad agreement with that previously reported in DMSO.²¹⁹

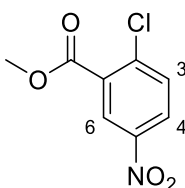
Methyl 4-chloro-3-nitrobenzoate **85a**



Following the procedure of Haydon *et al.*,²⁹³ sulfuric acid (0.40 mL) was added to a solution of 4-chloro-3-nitrobenzoic acid (2.50 g, 12.4 mmol) in methanol (15 mL) and the reaction was stirred at 60 °C for 20 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was redissolved in ethyl acetate (30 mL). The organic layer was washed with water (50 mL) and the aqueous was extracted with ethyl acetate (3 x 30 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure to afford **85a** as a white solid (2.60 g, 97%).

Mp: 77 - 78 °C. ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.52 (1H, dd, J = 2.0 Hz, H-2), 8.17 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.65 (1H, dd, J = 8.4 Hz, H-5), 3.97 (3H, s, CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 164.37 (C), 148.06 (C), 133.73 (CH), 132.35 (CH), 131.86 (C), 130.20 (C), 126.77 (CH), 53.09 (CH_3). Mass spectrometry by ESI^+ failed to identify the product. The ^1H and ^{13}C NMR data is in agreement with that previously reported.²⁹³

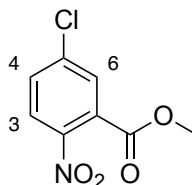
Methyl 2-chloro-5-nitrobenzoate **85b**



Adapting the procedure of Haydon *et al.*,²⁹³ sulfuric acid (0.20 mL) was added to a solution of 2-chloro-5-nitrobenzoic acid (500 mg, 2.48 mmol) in methanol (7.5 mL) and the reaction was stirred at 60 °C for 20 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was redissolved in dichloromethane (20 mL). The organic layer was washed with HCl (20 mL, 1 M) and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford **85b** as a white solid (541 mg, quant.).

Mp 66 - 67 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 8.68 (1H, d, *J* = 2.7 Hz, H-6), 8.25 (1H, dd, *J* = 8.8, 2.7 Hz, H-4), 7.64 (1H, d, *J* = 8.8 Hz, H-3), 3.98 (3H, s, CH₃). ¹³C NMR (101 MHz, CDCl₃): δ_C 164.02 (C), 146.21 (C), 140.83 (C), 132.45 (CH), 131.08 (C), 126.92 (CH), 126.71 (CH), 53.17 (CH₃). Mass spectrometry by ESI⁺ failed to identify the product. The ¹H and ¹³C NMR data is in agreement with that previously reported.²⁹⁴

Methyl 5-Chloro-2-nitrobenzoate **85c**

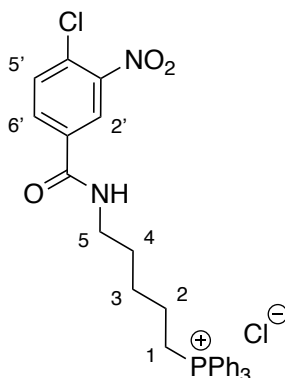


Adapting the procedure of Haydon *et al.*,²⁹³ sulfuric acid (0.40 mL) was added to a solution of 5-chloro-2-nitrobenzoic acid (1.00 g, 6.96 mmol) in methanol (15 mL) and the reaction was stirred at 60 °C for 20 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was redissolved in dichloromethane (20 mL). The organic layer was washed with HCl (20 mL, 1 M) and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined and dried with MgSO₄, and the solvent removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane:dichloromethane 20-80%] to afford **85c** as a white solid (774 mg, 52%).

Mp 48 - 49 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 7.90 (1H, d, *J* = 8.7 Hz, H-3), 7.68 (1H, d, *J* = 2.3 Hz, H-6), 7.58 (1H, dd, *J* = 8.7, 2.3 Hz, H-4), 3.93 (3H, s, CH₃). ¹³C NMR (101 MHz,

CDCl₃): δ_c 164.85 (C), 146.25 (C), 139.80 (C), 131.71 (CH), 129.93 (CH), 129.49 (C), 125.59 (CH), 53.69 (CH₃). Mass spectrometry by ESI⁺ failed to identify the product. The ¹H and ¹³C NMR data is in agreement with that previously reported.²⁹⁵

{5-[(4'-Chloro-3'-nitrophenyl)formamido]pentyl}triphenylphosphonium chloride **86a**

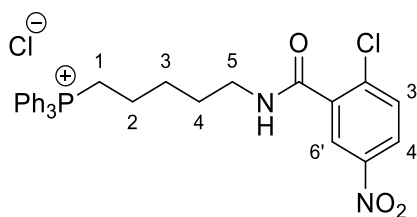


Adapting the procedure of Freeman *et al.*,²⁹⁶ *N*, *N*-dicyclohexylcarbodiimide (506 mg, 2.45 mmol, 1.25 eq.) was added to a stirring solution of **85a** (396 mg, 1.96 mmol, 1 eq.) and *N*-hydroxysuccinimide (249 mg, 2.16 mmol, 1.10 eq.) in anhydrous dichloromethane (8 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 2 h. A solution of **89** (1.00 g, 1.96 mmol, 1.00 eq.) and triethylamine (547 μ L, 3.92 mmol, 2.00 eq.) in anhydrous dichloromethane (2 mL) was added and the reaction was stirred at room temperature for an additional 19 h under argon. The mixture was filtered, and the filtrate was washed with an aqueous solution of HCl (20 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane: methanol 0-20%] to afford **86a** as a pale-yellow hygroscopic foam (906 mg, 81%).

$U_{\max}$ (ATR): 3217 - 2874 (N-H), 1651 (C=O), 1531 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 9.34 (1H, t, *J* = 5.6 Hz, NH), 8.78 (1H, dd, *J* = 8.4, 2.1 Hz, H-6'), 8.62 (1H, d, *J* = 2.1 Hz, H-2'), 7.86 - 7.65 (15H, m, Ph), 7.59 (1H, d, *J* = 8.4 Hz, H-5'), 3.67 - 3.55 (2H, m, CH₂-1), 3.49 (2H, q, *J* = 5.8 Hz, CH₂-5), 1.88 - 1.70 (6H, m, CH₂-2, CH₂-3 and CH₂-4). ¹³C NMR (101 MHz, CDCl₃): δ_c 164.32 (C), 147.72 (C), 135.18 (d, *J* = 3.1 Hz, 3 x CH), 134.47 (C), 133.57 (d, *J* = 9.9 Hz, 3 x CH), 132.67 (CH), 131.71 (CH), 130.55 (d, *J* = 12.5 Hz, 6 x

CH), 128.82 (C), 125.75 (CH), 118.15 (d, $J = 86.0$ Hz, 3 x C), 38.88 (CH₂), 27.12 (d, $J = 16.8$ Hz, CH₂), 27.10 (CH₂), 22.72 (d, $J = 50.4$ Hz, CH₂), 21.65 (d, $J = 4.4$ Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 23.98 (1P, s, PPh₃). HRMS (ESI⁺): C₂₉H₂₇³⁵ClN₂O₃P requires 531.1599 found 531.1606 (M⁺).

{5-[(2'-Chloro-5'-nitrophenyl)formamido]pentyl}triphenylphosphonium chloride 86b

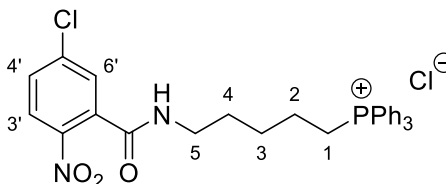


1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (169 mg, 0.844 mmol, 1.50 eq.) and *N*-hydroxysuccinimide (89.4 mg, 0.844 mmol, 1.50 eq.) were added to a solution of **85b** (154 mg, 0.766 mmol, 1.30 eq.) in anhydrous dichloromethane (4 mL) at 0 °C. The reaction was allowed to warm to room temperature and stirred for 2 h under argon. A solution of **89** (300 mg, 0.589 mmol, 1.00 eq.) and triethylamine (246 μ L, 1.77 mmol, 3.00 eq.) in dichloromethane (4 mL) was added and the reaction was stirred for an additional 19 h at room temperature. The reaction was filtered, and the filtrate was washed with an aqueous solution of HCl (20 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane: methanol 0-20%] to afford **86b** as yellow hygroscopic foam (149 mg, 44%).

$\nu_{\max}$ (ATR): 3156 (N-H), 1648 (C=O), 1609 (Aryl C=C), 1555 (NO₂), 1521 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 8.69 (1H, t, $J = 5.5$ Hz, NH), 8.36 (1H, d, $J = 2.7$ Hz, H-6'), 8.11 (1H, dd, $J = 8.8, 2.7$ Hz, H-4'), 7.84 - 7.75 (9H, m, Ph), 7.72 - 7.65 (6H, m, Ph), 7.50 (1H, d, $J = 8.8$ Hz, H-3'), 3.72 - 3.61 (2H, m, CH₂-1), 3.47 (2H, q, $J = 5.8$ Hz, CH₂-5), 1.87 - 1.78 (4H, m, CH₂-4 and CH₂-3), 1.76 - 1.64 (2H, m, CH₂-2). ¹³C NMR (101 MHz, CDCl₃): δ_C 165.53 (C), 146.10 (C), 138.37 (C), 137.80 (C), 135.13 (d, $J = 3.1$ Hz, 3 x CH), 133.62 (d, $J = 10.0$ Hz, 6 x CH), 130.90 (CH), 130.55 (d, $J = 12.6$ Hz, 6 x CH), 124.90 (CH), 124.73 (CH), 118.25 (d, $J = 85.9$ Hz, 3 x C), 38.87 (CH₂), 27.37 (CH₂), 27.08 (d, $J = 16.8$ Hz, CH₂), 22.59 (d, J

= 50.4 Hz, CH₂), 21.73 (d, *J* = 4.2 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 24.29 (1P, s, PPh₃). HRMS (ESI⁺): C₂₉H₂₇³⁵ClN₂O₃P requires 531.1599 found 531.1621 (M⁺).

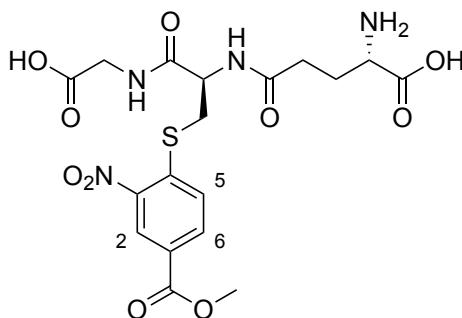
{5-[(5'-Chloro-2'-nitrophenyl)formamido]pent-1-yl}triphenylphosphonium chloride
86c



Adapting the procedure of Freeman *et al.*,²⁹⁶ *N*, *N*-dicyclohexylcarbodiimide (294 mg, 1.43 mmol, 1.25 eq.) and *N*-hydroxysuccinimide (127 mg, 1.25 mmol, 1.10 eq.) were added to a stirring solution of **85c** (230 mg, 1.14 mmol, 1 eq.) in anhydrous dichloromethane (8 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 2 h. A solution of **89** (549 mg, 1.07 mmol, 1.00 eq.) and triethylamine (318 μL, 2.28 mmol, 2.00 eq.) in anhydrous dichloromethane (2 mL) was added and the reaction was stirred at room temperature for an additional 19 h under argon. The mixture was filtered, and the filtrate was washed with an aqueous solution of HCl (20 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL) and the organic layers were combined, dried with MgSO₄, and the solvent removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane: methanol 0-20%] to afford **86c** as an off-white hygroscopic foam (371 mg, 57%).

U_{max} (ATR): 3491 (N-H), 1738 (C=O), 1660 (Aryl C=C), 1524 (NO₂). ¹H NMR (400 MHz, CDCl₃): δ_H 9.19 (1H, t, *J* = 5.5 Hz, NH), 7.91 (1H, d, *J* = 8.7 Hz, H-3'), 7.84 - 7.73 (9H, m, Ph), 7.73 - 7.63 (7H, m, H-6' and Ph), 7.41 (1H, dd, *J* = 8.7, 2.3 Hz, H-4'), 3.70 - 3.53 (2H, m, CH₂-1), 3.47 (2H, q, *J* = 5.8 Hz, CH₂-5), 1.93 - 1.65 (m, 6H, CH₂-2, CH₂-3, CH₂-4). ¹³C NMR (101 MHz, CDCl₃): δ_C 165.78 (C), 145.05 (C), 140.11 (C), 135.25 (C), 135.15 (d, *J* = 3.0 Hz, 3 x CH), 133.70 (d, *J* = 9.9 Hz, 6 x CH), 130.61 (d, *J* = 12.5 Hz, 6 x CH), 129.87 (CH), 129.77 (CH), 125.69 (CH), 118.44 (d, *J* = 85.9 Hz, 3 x C), 38.99 (CH₂), 27.27 (CH₂), 27.11 (CH₂), 22.51 (d, *J* = 50.4 Hz, CH₂), 21.93 (d, *J* = 4.3 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 24.22 (1P, s, PPh₃). HRMS (ESI⁺): C₃₀H₂₉³⁵ClN₂O₃P requires 531.1599 found 531.1614 (M⁺).

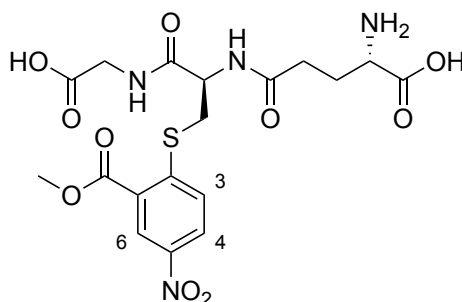
Methyl 4-(*S*-glutathionyl)-3-nitrobenzoate **88a**



Glutathione (214 mg, 0.696 mmol, 1.50 eq.) and NaHCO₃ (117 mg, 1.39 mmol, 3.00 eq.) were added to a solution of **85a** (100 mg, 0.464 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 6 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-100%) to afford **88a** as a yellow solid (195 mg, 86%).

Mp: Decomposition at 192 °C. $\nu_{\max}$ (ATR): 1723 (C=O), 1603 (Aryl C=C), 1516 (NO₂) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_{H} 8.69 (1H, d, J = 1.6 Hz, H-2), 8.17 (1H, dd, J = 8.5, 1.6 Hz, H-6), 7.74 (1H, d, J = 8.6 Hz, H-5), 4.75 (1H, dd, J = 9.3, 4.8 Hz, Cys CH), 3.99 - 3.95 (3H, s, CH₃), 3.82 - 3.66 (4H, m, Gly CH₂, Gln CH, and CH_ACH_BS), 3.40 (1H, dd, J = 14.2, 9.3 Hz, CH_ACH_BS), 2.47 (2H, t, J = 6.6 Hz, CH₂CONH), 2.08 (2H, q, J = 7.2 Hz, CH₂CH₂CONH). ¹³C NMR (101 MHz, D₂O): δ_{C} 176.13 (C), 174.90 (C), 174.36 (C), 171.03 (C), 166.77 (C), 145.63 (C), 141.43 (C), 133.81 (CH), 127.55 (CH), 127.13 (CH), 126.86 (C), 54.16 (CH), 53.01 (CH₃), 51.71 (CH), 43.35 (CH₂), 33.34 (CH₂), 31.42 (CH₂), 26.42 (CH₂). HRMS (ESI⁺): C₁₈H₂₃N₄O₁₀S requires 487.1129 found 487.1137 (M + H)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

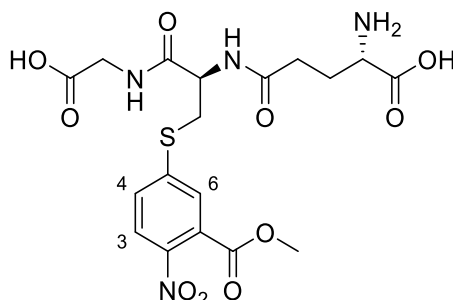
Methyl 2-(*S*-glutathionyl)-5-nitrobenzoate **88b**



Glutathione (214 mg, 0.696 mmol, 1.50 eq.) and NaHCO₃ (117 mg, 1.39 mmol, 3.00 eq.) were added to a solution of **85b** (100 mg, 0.464 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 5.0 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-100%) to afford **88b** as a pale-orange solid (105 mg, 47%).

Mp: Decomposition at 190 °C. U_{max} (ATR): 1718 (C=O), 1645 (C=O), 1598 (Aryl C=C), 1507 (NO₂) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_H 8.71 (1H, d, *J* = 2.6 Hz, H-6), 8.33 (1H, dd, *J* = 9.1, 2.7, H-4), 7.69 (1H, d, *J* = 9.0 Hz, H-3), 4.76 (1H, dd, *J* = 9.2, 4.7 Hz, Cys CH), 3.96 (3H, s, *J* = 1.0 Hz, CH₃), 3.83 - 3.63 (4H, m, Gly CH₂, Gln CH and SCH_ACH_B), 3.38 (1H, dd, *J* = 13.9, 9.1 Hz, SCH_ACH_B), 2.47 (2H, t, *J* = 7.8 Hz, CH₂CONH), 2.14 - 2.02 (2H, m, CH₂CH₂CONH). ¹³C NMR (101 MHz, D₂O): δ_C 176.13 (C), 174.90 (C), 174.39 (C), 171.12 (C), 166.61 (C), 148.78 (C), 144.08 (C), 127.53 (C), 126.88 (CH), 126.61 (CH), 126.45 (CH), 54.18 (CH), 53.08 (CH₃), 51.83 (CH), 43.37 (CH₂), 33.04 (CH₂), 31.47 (CH₂), 26.45 (CH₂). HRMS (ESI⁺): C₁₈H₂₃N₄O₁₀S requires 487.1129 found 487.1139 (M + H)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

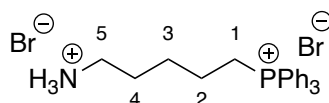
Methyl 5-(*S*-glutathionyl)-2-nitrobenzoate **88c**



Glutathione (214 mg, 0.696 mmol, 1.50 eq.) and NaHCO₃ (117 mg, 1.39 mmol, 3.00 eq.) were added to a solution of **85c** (100 mg, 0.464 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 6 mL) and the reaction was stirred at 40 °C for 24 h under argon. The reaction was allowed to cool to room temperature. The solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-100%) to afford **88c** as an off-white solid (195 mg, 86%).

Mp: Decomposition at 187 °C. ν_{max} (ATR): 1731 (C=O), 1589 (Aryl C=C), 1522 (NO₂) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_{H} 8.12 - 8.05 (1H, m, H-3), 7.74 - 7.67 (2H, m, H-4 and H-6), 4.71 (1H, dd, J = 8.0, 5.2 Hz, Cys CH), 3.98 (3H, s, CH₃), 3.76 - 3.60 (4H, m, Gly CH₂, Gln CH, and CH_ACH_BS), 3.46 (1H, dd, J = 14.6, 8.2 Hz, CH_ACH_BS), 2.49 - 2.36 (2H, m, CH₂CONH), 2.06 (2H, q, J = 7.3 Hz, CH₂CH₂CONH). ¹³C NMR (101 MHz, D₂O): δ_{C} 176.05 (C), 174.86 (C), 174.59 (C), 170.99 (C), 168.22 (C), 144.99 (C), 143.69 (C), 130.27 (CH), 128.11 (C), 127.44 (CH), 125.17 (CH), 54.21 (CH), 54.03 (CH₃), 52.58 (CH), 43.29 (CH₂), 33.33 (CH₂), 31.45 (CH₂), 26.48 (CH₂). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. HRMS (ESI⁺): C₁₈H₂₃N₄O₁₀S requires 487.1129 found 487.1136 (M + H)⁺.

(5-Aminopentyl)triphenylphosphonium bromide hydrobromide **89**

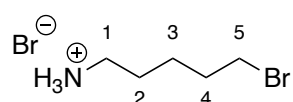


Triphenylphosphine (1.70 g, 6.48 mmol, 2.00 eq.) was added to a solution of **91** (800 mg, 3.24 mmol, 1.00 eq) in anhydrous acetonitrile (20.0 mL) and the reaction was stirred

under reflux for 48 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was dissolved in chloroform and triturated three times with diethyl ether to afford **89** as a brown hygroscopic foam (2.89 g, 92%).

^1H NMR (400 MHz, CDCl_3): δ_{H} 8.22 (3H, br s, NH_3) 7.90 - 7.63 (15H, m, Ph), 3.80 - 3.64 (2H, m, CH_2 -1), 3.18 - 2.94 (2H, m, CH_2 -5), 2.08 - 1.92 (2H, m, CH_2 -2), 1.92 - 1.76 (2H, m, CH_2 -4), 1.76 - 1.59 (2H, m, CH_2 -3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 135.16 (d, $J = 2.9$ Hz, 3 x CH), 133.78 (d, $J = 10.0$ Hz, 6 x CH), 130.63 (d, $J = 12.5$ Hz, 6 x CH), 118.15 (d, $J = 86.1$ Hz, C x 3), 39.38 (CH_2), 26.82 (d, $J = 16.5$ Hz, CH_2), 25.66 (CH_2), 22.32 (d, $J = 50.9$ Hz (CH_2), 21.63 (CH_2). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 24.25 (1P, s, PPh_3). HRMS (ESI $^+$): $\text{C}_{23}\text{H}_{28}\text{NP}$ requires 174.5974 found 174.5981 (M^{2+}). The ^1H and ^{13}C NMR data is in broad agreement with that previously reported in CDCl_3 .²⁹⁷

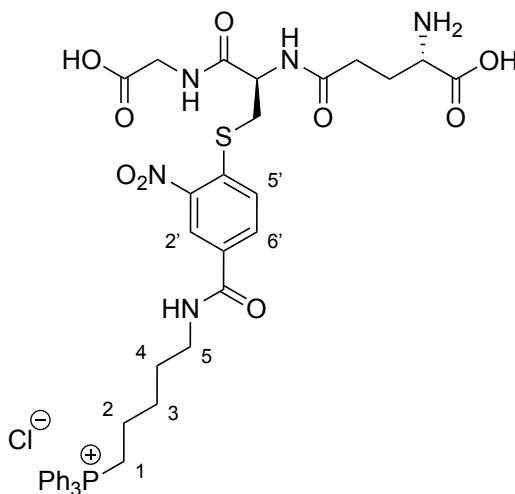
5-Bromopentylammonium bromide **91**



Following the procedure of Kai *et al.*,²⁹⁸ a solution of 5-amino-1-pentanol (0.530 g, 5.10 mmol, 1.00 eq) in aqueous hydrogen bromide (48% w/v in water, 5.00 mL) was stirred at reflux for 48 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. Recrystallisation of the crude material from ethanol and ethyl acetate afforded **91** as a brown hygroscopic foam (828 mg, 66%).

^1H NMR (400 MHz, CD_3OD): δ_{H} 3.47 (2H, t, $J = 6.6$ Hz, CH_2 -5), 2.98 - 2.89 (2H, m, CH_2 -1), 1.96 - 1.83 (2H, m, CH_2 -4), 1.75 - 1.61 (2H, m, CH_2 -2), 1.61 - 1.48 (2H, m, CH_2 -3). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 39.19 (CH_2), 32.38 (CH_2), 31.86 (CH_2), 26.33 (CH_2), 24.62 (CH_2). HRMS (ESI $^+$): $\text{C}_5\text{H}_{13}^{79}\text{BrN}$ requires 166.0226 found 166.0227 (M^+). The ^1H and ^{13}C NMR data is in agreement with that previously reported.²⁹⁸

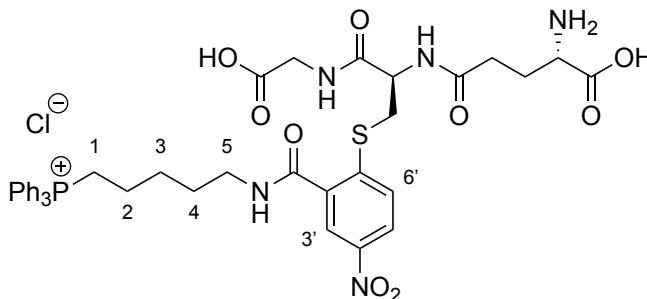
{5-[(4'-{*S*-glutathionyl}-3'-nitrophenyl)formamido]pent-1-yl}triphenylphosphonium chloride **92a**



Glutathione (62.8 mg, 0.204 mmol, 2.00 eq.) and NaHCO₃ (28.3 mg, 0.337 mmol, 3.30 eq) were added to a solution of **86a** (58 mg, 0.102 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 3 mL). The reaction was stirred at room temperature for 3 h under argon. The solvent was removed under reduced pressure and the crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-40%) to afford **92a** as a yellow hygroscopic foam (46.8 mg, 55%).

U_{max} (ATR): 1739 (C=O), 1637 (Aryl C=C), 1604 (Aryl C=C), 1516 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_H 8.54 (1H, d, *J* = 2.0 Hz, H-2'), 8.09 (1H, dd, *J* = 8.5, 2.0 Hz, H-6'), 7.91 - 7.70 (16H, m, Ph and H-5'), 4.70 (1H, dd, *J* = 8.4, 5.2 Hz, Cys CH), 3.76 (2H, s, Gly CH₂), 3.66 (1H, dd, *J* = 14.0, 5.2 Hz, CH_ACH_BS), 3.60 (1H, t, *J* = 6.1 Hz, Gln CH), 3.50 - 3.25 (5H, m, CH_ACH_BS, CH₂-1 and CH₂-5), 2.59 - 2.40 (2H, m, CH₂CONH), 2.10 (2H, q, *J* = 7.0 Hz, CH₂CH₂CONH), 1.81 - 1.60 (6H, m, CH₂-2, CH₂-3 and CH₂-4). ¹³C NMR (101 MHz, CD₃OD): δ_C 176.10 (C), 175.35 (C), 174.53 (C), 171.22 (C), 166.81 (C), 147.01 (C), 141.65 (C), 136.26 (d, *J* = 3.0 Hz, 3 x CH), 134.83 (d, *J* = 10.0 Hz, 6 x CH), 132.82 (CH), 132.27 (C), 131.52 (d, *J* = 12.6 Hz, 6 x CH), 128.40 (CH), 126.06 (CH), 119.99 (d, *J* = 86.3 Hz, 3 x C), 55.63 (CH), 53.57 (CH), 44.72 (CH₂), 40.50 (CH₂), 34.58 (CH₂), 33.07 (CH₂), 29.54 (CH₂), 28.96 (d, *J* = 16.8 Hz, CH₂), 28.15 (CH₂), 23.31 (d, *J* = 3.9 Hz, CH₂), 22.72 (d, *J* = 51.1 Hz, CH₂). ³¹P NMR (162 MHz, CD₃OD): δ_P 23.71 (1P, s, PPh₃). HRMS (ESI⁺): C₄₀H₄₅N₅O₉PS requires 802.2670 found 802.2676 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

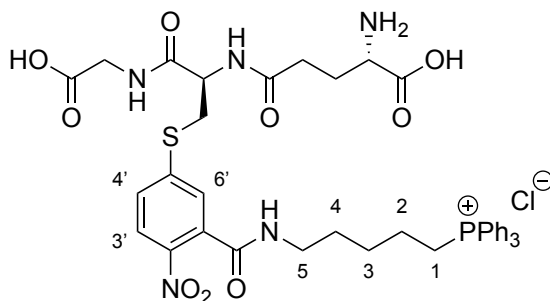
{5-[(2'-{*S*-glutathionyl}-5'-nitrophenyl)formamido]pent-1-yl}triphenylphosphonium chloride **92b**



Glutathione (43.3 mg, 0.141 mmol, 2.00 eq.) and NaHCO₃ (19.5 mg, 0.233 mmol, 3.30 eq.) were added to a solution of **86b** (40.0 mg, 0.0705 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 3 mL) and the reaction was stirred at room temperature for 3 h under argon. The solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water: acetonitrile 0-100%) to afford **92b** as an orange hygroscopic foam (27.8 mg, 47%).

U_{max} (ATR): 3379 (N-H), 1646 (C=O), 1595 (Aryl C=C), 1507 (NO₂) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_H 8.23 (1H, dd, *J* = 8.8, 2.5 Hz, H-4'), 8.06 (1H, d, *J* = 2.5 Hz, H-6'), 7.86 - 7.59 (16H, m, Ph and H-3'), 4.59 (1H, dd, *J* = 8.5, 5.2 Hz, Cys CH), 3.73 - 3.53 (3H, m, Gly CH₂ and SCH_ACH_B), 3.42 - 3.18 (6H, m, Gln CH, SCH_BCH_A, CH₂-1 and CH₂-5), 2.38 - 2.20 (2H, m, CH₂CONH), 1.92 - 1.79 (2H, m, CH₂CH₂CONH), 1.78 - 1.67 (2H, m, CH₂-4), 1.66 - 1.50 (4H, m, CH₂-2 and CH₂-3). ¹³C NMR (101 MHz, D₂O): δ_C 175.88 (C), 175.39 (C), 170.78 (C), 168.40 (C), 144.56 (C), 143.98 (C), 135.43 (C), 134.96 (d, *J* = 2.6 Hz, 3 x CH), 133.44 (d, *J* = 10.0 Hz, 6 x CH), 130.03 (d, *J* = 12.5 Hz, 6 x CH), 127.77 (CH), 125.30 (CH), 122.48 (CH), 118.07 (d, *J* = 86.6 Hz, 3 x C), 54.91 (CH), 52.29 (CH), 43.32 (CH₂), 39.11 (CH₂), 33.12 (CH₂), 31.95 (CH₂), 28.82 (CH₂), 27.40 (CH₂), 27.22 (d, *J* = 16.0 Hz, CH₂), 21.72 (d, *J* = 51.9 Hz, CH₂), 21.37 (d, *J* = 4.1 Hz, CH₂). ³¹P NMR (162 MHz, D₂O): δ_P 23.80 (1P, s, PPh₃). HRMS (ESI⁺): C₄₀H₄₅N₅O₉PS requires 802.2670 found 802.2684 (*M*⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. One signal corresponding to a C was not identified in the ¹³C NMR spectrum, perhaps due to coincidence with another signal.

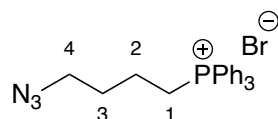
{5-[(5'-[*S*-glutathionyl]-2'-nitrophenyl)formamido]pent-1-yl}triphenylphosphonium chloride **92c**



Glutathione (75.8 mg, 0.247 mmol, 2.00 eq.) and NaHCO₃ (28.3 mg, 0.337 mmol, 3.30 eq.) were added to a solution of **86c** (70.0 mg, 0.123 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 3 mL). The mixture was stirred at room temperature for 3 h under argon and the solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-100%) to afford **92c** as a yellow hygroscopic foam (40.6 mg, 47%).

U_{max} (ATR): 1739 (C=O), 1659 (Aryl C=C), 1521 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_H 8.02 (1H, d, *J* = 8.6 Hz, H-3'), 7.93 - 7.71 (15H, m, Ph), 7.60 (1H, dd, *J* = 8.8, 2.1 Hz, H-4'), 7.47 (1H, d, *J* = 2.1 Hz, H-6'), 4.64 (1H, dd, *J* = 8.6, 5.0 Hz, Cys CH), 3.74 - 3.57 (4H, m, Gly CH₂, Gln CH and CH_ACH_BS), 3.51 - 3.39 (2H, m, CH₂-1), 3.39 - 3.32 (3H, m, CH₂-5 and CH_ACH_BS), 2.57 - 2.40 (2H, m, CH₂CONH), 2.14 - 2.03 (2H, m, CH₂CH₂CONH), 1.82 - 1.60 (6H, m, CH₂-2, CH₂-3 and CH₂-4). ¹³C NMR (101 MHz, CD₃OD): δ_C 175.80 (C), 175.36 (C), 171.27 (C), 168.94 (C), 146.81 (C), 144.95 (C), 136.27 (d, *J* = 3.1 Hz, 3 x CH), 134.85 (d, *J* = 10.0 Hz, 6 x CH), 134.76 (C), 131.55 (d, *J* = 12.6 Hz, 6 x CH), 129.54 (CH), 127.70 (CH), 126.21 (CH), 120.00 (d, *J* = 86.4 Hz, 3 x C), 55.53 (CH), 53.86 (CH), 44.56 (CH₂), 40.08 (CH₂), 34.53 (CH₂), 33.18 (CH₂), 29.12 (CH₂), 28.58 (d, *J* = 17.0 Hz, CH₂), 27.89 (CH₂), 23.11 (d, *J* = 4.0 Hz, CH₂), 22.74 (d, *J* = 51.6 Hz, CH₂). ³¹P NMR (162 MHz, CD₃OD): δ_P 23.99 (1P, s, PPh₃). HRMS (ESI⁺): C₄₀H₄₅N₅O₉PS requires 802.2670 found 802.2676 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. One signal corresponding to a C was not identified in the ¹³C NMR spectrum, perhaps due to coincidence with another signal.

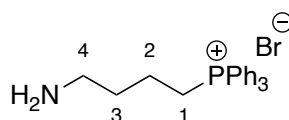
(4-Azidobutyl) triphenylphosphonium bromide **94**



Following the procedure of Dr Taig,²²⁴ sodium azide (1.22 g, 18.8 mmol, 3.00 eq.) was added to a solution of (4-bromobutyl)triphenylphosphonium bromide (3.00 g, 6.27 mmol, 1.00 eq.) in EtOH:H₂O (2:1, 70 mL). The reaction was stirred at reflux for 16 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was redissolved in dichloromethane (50 mL) and washed with water (30 mL). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (3 x 25 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford **94** as a white hygroscopic foam (2.94 g, quant.).

¹H NMR (400 MHz, CDCl₃): δ_H 7.89 - 7.81 (6H, m, Ph), 7.81 - 7.74 (3H, m, Ph), 7.72 - 7.65 (6H, m, Ph), 4.01 - 3.89 (2H, m, CH₂-1), 3.43 (2H, t, *J* = 6.2 Hz, CH₂-4), 2.07 - 1.97 (2H, m, CH₂-2), 1.79 - 1.64 (2H, m, CH₂-3). ¹³C NMR (101 MHz, CDCl₃): δ_C 135.05 (d, *J* = 3.1 Hz, 3 x CH), 133.71 (d, *J* = 10.1 Hz, 6 x CH), 130.52 (d, *J* = 12.5 Hz, 6 x CH), 118.26 (d, *J* = 85.9 Hz, 3 x CH), 50.63 (CH₂), 29.17 (d, *J* = 16.8 Hz, CH₂), 22.07 (d, *J* = 50.5 Hz, CH₂), 19.87 (d, *J* = 3.9 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 24.41 (1P, s, PPh₃). HRMS (ESI⁺): C₂₂H₂₃N₃P requires 360.1624 found 360.1626 (M⁺). The ¹H and ¹³C NMR data is in agreement with that previously reported.²²⁴

(4-Aminobutyl)triphenylphosphonium bromide **95**

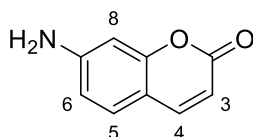


Following the procedure of Dr Taig,²²⁴ **94** (1.50 g, 3.41 mmol) was dissolved in anhydrous methanol (50 mL) and 5% Pd/C (100 mg) was added. The reaction was purged with a balloon of H₂ and the mixture was stirred at room temperature for 5 h under H₂. The

catalyst was removed by vacuum filtration over Celite, and the solvent was removed under reduced pressure to afford **95** as an off-white hygroscopic foam (1.24 g, quant.).

^1H NMR (400 MHz, CD_3OD): 7.95 - 7.72 (15H, m, Ph), 3.62 - 3.48 (2H, m, CH_2 -1), 3.02 - 2.94 (2H, m, CH_2 -4), 2.01 - 1.89 (2H, m, CH_2 -3), 1.87 - 1.72 (2H, m, CH_2 -2). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 136.38 (d, $J = 3.1$ Hz, 3 x CH), 134.91 (d, $J = 10.0$ Hz, 6 x CH), 131.61 (d, $J = 12.6$ Hz, 6 x CH), 119.67 (d, $J = 86.6$ Hz, 3 x CH), 39.94 (CH_2), 29.48 (d, $J = 17.9$ Hz, CH_2), 22.63 (d, $J = 52.4$ Hz, CH_2), 20.88 (d, $J = 3.7$ Hz, CH_2). ^{31}P NMR (162 MHz, CD_3OD): δ_{P} 23.65 (1P, s, PPh_3). HRMS (ESI^+): $\text{C}_{22}\text{H}_{25}\text{NP}$ requires 334.1719 found 334.1720 (M^+). The ^1H and ^{13}C NMR data is in agreement with that previously reported.²²⁴

7-Aminocoumarin **104**

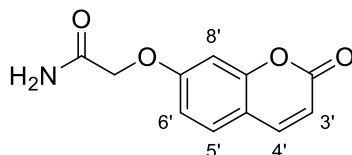


Adapting the procedure of Lippe *et al.*,²⁵⁰ Cs_2CO_3 (2.25 g, 6.92 mmol, 1.20 eq.) was added to a solution of **105** (1.26 g, 5.77 mmol, 1.00 eq.) in anhydrous dimethylformamide (58 mL) and the reaction was stirred at 70 °C for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was redissolved in ethanol (58 mL) and HCl (18 mL, 6 M). The reaction was stirred at 90 °C for 5 h under argon. The solvent was removed under reduced pressure and the solid residue was redissolved in dichloromethane (50 mL) and washed with a saturated solution of NaHCO_3 (50 mL). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 x 40 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane: methanol 0-10%] to afford **104** as an off-white solid (325 mg, 35%).

Mp: 202 - 204 °C. ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{H} 7.72 (1H, d, $J = 9.4$ Hz, H-4), 7.31 (1H, d, $J = 8.4$ Hz, H-5), 6.64 (1H, dd, $J = 8.4, 2.1$ Hz, H-6), 6.52 (1H, d, $J = 2.1$ Hz, H-8), 5.96 (1H, d, $J = 9.4$ Hz, H-3), 5.62 (2H, s, NH_2). ^{13}C NMR (101 MHz, $(\text{CD}_3)_2\text{CO}$): δ_{C} 161.58 (C), 157.63 (C), 153.81 (C), 153.75 (C), 144.93 (CH), 130.14 (CH), 112.31 (CH), 110.09 (CH),

100.23 (CH). HRMS (APCI⁺): C₉H₈NO₂ requires 162.0550 found 162.0554 (M + H)⁺. The ¹H and ¹³C NMR data is in broad agreement with that reported in the literature in DMSO.²⁹⁹

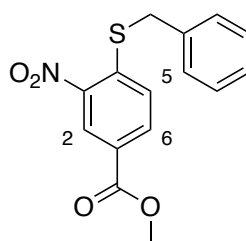
2-[(2'-Oxo-2H-chromen-7'-yl)oxy]acetamide **105**



Adapting the procedure of Lippe *et al.*,²⁵⁰ bromoacetamide (1.02 g, 7.40 mmol, 1.20 eq.) and K₂CO₃ (1.02 g, 7.40 mmol, 1.00 eq.) were added to a solution of 7-hydroxycoumarin (1.00 g, 0.62 mmol, 1.00 eq.) in anhydrous acetonitrile (40 mL) and the reaction was stirred at 50 °C for 16 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was washed with water (2 x 40 mL) and filtered under vacuum to afford **105** as a white solid (1.26 g, 93%).

Mp: 219 - 220 °C. U_{max} (ATR): 3437 (N-H), 1716 (C=O), 1674 (C=O) cm⁻¹. ¹H NMR (400 MHz, DMSO): δ_H 7.99 (1H, d, *J* = 9.5 Hz, H-4'), 7.65 (1H, d, *J* = 8.5 Hz, H-5'), 7.61 (1H, s, NH), 7.43 (1H, s), 6.99 (1H, dd, *J* = 8.5, 2.5 Hz, H-6'), 6.96 (1H, d, *J* = 2.4 Hz, H-8'), 6.31 (1H, d, *J* = 9.5 Hz, H-3'), 4.56 (2H, s, CH₂). ¹³C NMR (101 MHz, DMSO): δ_C 169.16 (C), 160.83 (C), 160.18 (C), 155.13 (C), 144.24 (CH), 129.46 (CH), 112.80 (CH), 112.76 (C), 112.73 (CH), 101.65 (CH), 66.92 (CH₂). HRMS (APCI⁺): C₁₂H₁₀NO₄ requires 220.0604 found 220.0605 (M + H)⁺.

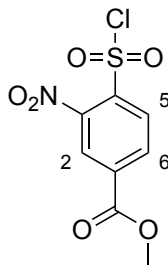
Methyl 4-(benzylsulfanyl)-3-nitrobenzoate **109**



Following the procedure of Xin *et al.*,³⁰⁰ benzyl mercaptan (900 μ L, 7.65 mmol, 1.10 eq.) and K_2CO_3 (2.88 g, 20.9 mmol, 3.00 eq.) were added to a solution of **85a** (1.5 g, 6.96 mmol, 1.00 eq.) in methanol (20 mL) and the reaction was stirred at room temperature for 24 h under argon. The reaction was filtered and washed with methanol (20 mL). The solid was dissolved in dichloromethane (20 mL) and the solvent was removed under reduced pressure to afford **109** as a yellow solid (1.88 g, 89%).

Mp: 136 - 137 °C. ^1H NMR (400 MHz, $CDCl_3$): δ_H 8.86 (1H, d, J = 1.9 Hz, H-2), 8.14 (1H, dd, J = 8.5, 1.9 Hz, H-6), 7.52 (1H, d, J = 8.5 Hz, H-5), 7.46 - 7.28 (5H, m, Ph), 4.25 (2H, s, CH_2), 3.95 (3H, s, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ_C 165.09 (C), 145.27 (C), 144.00 (C), 134.37 (C), 133.77 (CH), 129.22 (2 x CH), 129.13 (2 x CH), 128.19 (CH), 127.45 (CH), 126.88 (C), 126.67 (CH), 52.78 (CH_3), 37.73 (CH_2). Mass spectrometry by ESI $^{+/-}$ failed to identify the product. The ^1H and ^{13}C NMR data is in agreement with that previously reported.³⁰⁰

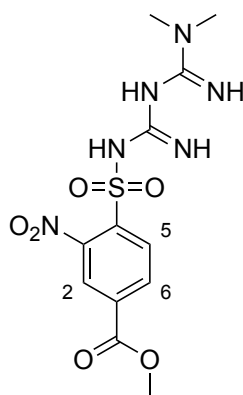
Methyl 4-(chlorosulfonyl)-3-nitrobenzoate **110**



Following the procedure of Xin *et al.*,³⁰⁰ 1,3-dichloro-5,5-dimethylhydantoin (650 mg, 3.3 mmol, 2.00 eq.) was added to a solution of **109** (500 mg, 1.65 mmol, 1.00 eq.) in a solvent mixture of acetonitrile:acetic acid:water (8 mL: 0.4 mL: 0.4 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 20 h under argon. The solvent was removed under reduced pressure and the crude material was redissolved in dichloromethane (50 mL). The organic layer was washed with an aqueous solution of HCl (50 mL, 1 M) and the aqueous layer was extracted with dichloromethane (3 x 30 mL). The organic layers were combined, dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , hexane:dichloromethane 20-80%] to afford **110** as a white solid (448 mg, 97%).

Mp: 90 - 92 °C. ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.48 (1H, d, J = 1.7 Hz, H-2), 8.45 (1H, dd, J = 8.3, 1.7 Hz, H-6), 8.34 (1H, d, J = 8.3 Hz, H-5), 4.04 (3H, s, CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 163.12 (C), 147.43 (C), 138.82 (C), 137.76 (C), 133.57 (CH), 130.93 (CH), 126.32 (CH), 53.76 (CH_3). Mass spectrometry by ESI $^{+/-}$ failed to identify the product. The ^1H and ^{13}C NMR data is in agreement with that previously reported.³⁰⁰

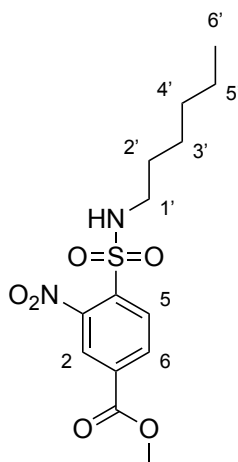
Methyl 4-[[*N,N'*-dimethylcarbamimidoyl]carbamimidamido]sulfonyl}-3-nitrobenzoate 111



1,1-Dimethylbiguanide **58** (92.4 mg, 0.715 mmol, 2.00 eq.) was added to a solution of **110** (100 mg, 0.358 mmol, 1.00 eq.) in anhydrous acetonitrile (4 mL) and the reaction was stirred at room temperature for 20 h under argon. The solvent was removed under reduced pressure and the crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **111** as a yellow solid (36.9 mg, 28%).

Mp 189 - 191 °C. U_{max} (ATR): 3418 - 3376 (N-H), 1724 (C=O), 1578 (NO_2) cm^{-1} . ^1H NMR (400 MHz, DMSO): δ_{H} 8.32 (1H, d, J = 1.5 Hz, H-2), 8.26 (1H, dd, J = 8.2, 1.7 Hz, H-6), 8.21 (1H, d, J = 8.3 Hz, H-5), 3.91 (3H, s, CH_3), 2.92 (6H, s, $\text{N}(\text{CH}_3)_2$). ^{13}C NMR (101 MHz, DMSO): δ_{C} 163.81 (C), 158.89 (C), 158.38 (C), 147.33 (C), 139.50 (C), 133.37 (C), 132.17 (CH), 129.75 (CH), 124.40 (CH), 52.99 (CH_3), 36.67 (2 x CH_3). HRMS (ESI $^+$): $\text{C}_{12}\text{H}_{17}\text{N}_6\text{O}_6\text{S}$ requires 373.0930 found 373.0917 ($\text{M} + \text{H}$) $^+$.

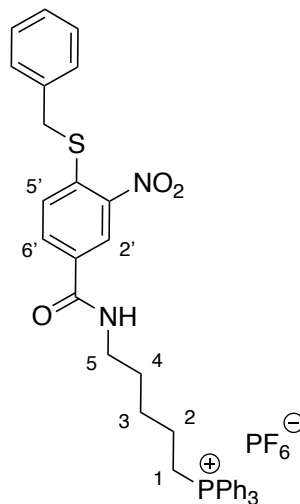
Methyl 4-(hexylaminosulfonyl)-3-nitrobenzoate **115**



Triethylamine (50.0 μ L, 0.380 mmol, 2.00 eq.) and hexyl-1-amine (29.0 μ L, 0.210 mmol, 1.10 eq.) were added to a solution of **110** (50.0 mg, 0.180 mmol, 1.00 eq.) in anhydrous dichloromethane (2 mL) and the reaction was stirred at room temperature for 20 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , hexane:dichloromethane 20-100%] to afford **115** as a pale-yellow solid (25.0 mg, 40%).

Mp 93 - 94 $^{\circ}\text{C}$. U_{max} (ATR): 3335 (N-H), 1735 (C=O), 1547 (NO_2) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.46 (1H, d, J = 1.6 Hz, H-2), 8.35 (1H, dd, J = 8.1, 1.6 Hz, H-6), 8.21 (1H, d, J = 8.1 Hz, H-5), 5.32 - 5.23 (1H, m, NH), 4.01 (3H, s, OCH_3), 3.11 (2H, td, J = 7.1, 6.0 Hz, CH_2 -1'), 1.57 - 1.41 (2H, m, CH_2 -2'), 1.38 - 1.14 (6H, m, CH_2 -3', CH_2 -4' and CH_2 -5'), 0.89 - 0.80 (3H, t, J = 6.8, CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 163.80 (C), 148.21 (C), 137.50 (C), 135.23 (C), 133.48 (CH), 131.51 (CH), 126.38 (CH), 53.41 (CH_3), 44.09 (CH_2), 31.29 (CH_2), 29.70 (CH_2), 26.24 (CH_2), 22.55 (CH_2), 14.05 (CH_3). HRMS (ESI^-): $\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_6\text{S}$ requires 343.0964 found 343.0977 ($M - \text{H}$) $^-$. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

(5-[[4'-(Benzylsulfanyl)-3'-nitrophenyl]formamido}pentyl)triphenylphosphonium hexafluorophosphate **116**

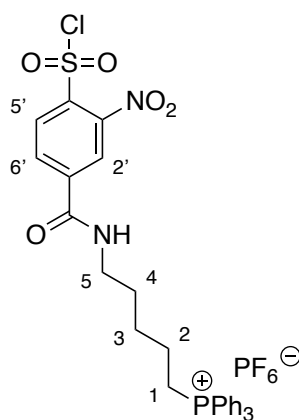


Adapting the procedure of Xin *et al.*,³⁰⁰ benzyl mercaptan (134 μ L, 1.15 mmol, 1.3 eq.) and triethylamine (368 μ L, 2.64 mmol, 3.00 eq.) were added to a solution of **86a** (500 mg, 0.880 mmol, 1.00 eq.) in anhydrous methanol (10 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the solid residue was redissolved in dichloromethane (15 mL). A solution of ammonium hexafluorophosphate (1.00 g, 6.13 mmol, 7.00 eq.) in water (15 mL) was added and the biphasic mixture was stirred at room temperature for 15 mins. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO_4 and solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **116** as a yellow hygroscopic foam (545 mg, 81%).

U_{max} (ATR): 1651 (C=O), 1516 (NO_2) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.67 (1H, d, J = 2.0 Hz, H-2'), 8.04 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 7.83 - 7.75 (3H, m, Ph), 7.74 - 7.62 (12H, m, Ph), 7.55 (1H, d, J = 8.6 Hz, H-5'), 7.46 - 7.39 (2H, m, Ph), 7.39 - 7.25 (3H, m, Ph), 7.08 (1H, t, J = 5.7 Hz, NH), 4.21 (2H, s, SCH_2), 3.41 (2H, q, J = 5.4 Hz, CH_2 -5), 3.27 - 3.12 (2H, m, CH_2 -1), 1.77 - 1.66 (6H, m, CH_2 -2, CH_2 -3 and CH_2 -4). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 164.97 (C), 145.19 (C), 141.19 (C), 135.30 (d, J = 3.0 Hz, 3 x CH), 134.61 (C), 133.30 (d, J = 9.9 Hz, 6 x CH), 131.40 (CH), 131.06 (C), 130.62 (d, J = 12.5 Hz, 6 x CH), 129.32 (2 x CH), 128.86 (2 x CH), 127.85 (CH), 126.85 (CH), 125.57 (CH), 117.83 (d, J =

86.1 Hz, 3 x C), 39.53 (CH₂), 37.55 (CH₂), 27.72 (CH₂), 27.30 (d, *J* = 16.7 Hz, CH₂), 22.08 (d, *J* = 51.9 Hz, CH₂), 21.91 (d, *J* = 4.3 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 23.62 (1P, s, PPh₃), -127.25 - -158.26 (1P, sept, *J* = 713.1 Hz, PF₆). HRMS (ESI⁺): C₃₇H₃₆N₂O₃PS requires 619.2179 found 619.2165 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

(5-[[4'-(Chlorosulfonyl)-3'-nitrophenyl]formamido}pentyl)triphenylphosphonium hexafluorophosphate 117

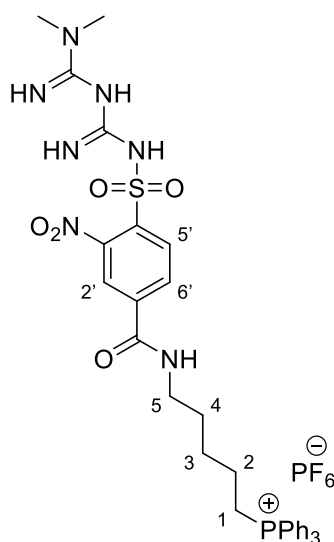


Adapting the procedure of Xin *et al.*,³⁰⁰ 1,3-dichloro-5,5-dimethylhydantoin (258 mg, 1.31 mmol, 2.00 eq.) was added to a solution of **116** (500 mg, 0.655 mmol, 1.00 eq.) in a solvent mixture of acetonitrile: acetic acid: water (4 mL: 0.2 mL: 0.2 mL), at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 24 h. The solvent was removed under reduced pressure and the residue was redissolved in dichloromethane (30 mL). The organic layer was washed with an aqueous solution of HCl (30 mL, 1 M) and the aqueous layer was extracted with dichloromethane (3 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. Purification of the crude material by column chromatography [SiO₂, dichloromethane: methanol 0-25%] afforded **117** as a white hygroscopic foam (421 mg) with minor impurities. It was characterised as the impure mixture and taken onto the next step of the synthesis.

U_{max} (ATR): 1659 (C=O), 1543 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 8.31 (2H, m, H-5' and H-6'), 8.20 (1H, d, *J* = 7.3 Hz, H-2'), 7.78 (3H, m, Ph), 7.73 - 7.56 (12H, m, Ph), 3.54 - 3.28 (2H, m, CH₂-5), 3.25 - 2.97 (2H, m, CH₂-1), 1.80 - 1.53 (6H, m, CH₂-2, CH₂-3 and

CH₂-4). ¹³C NMR (101 MHz, CDCl₃): δ_c 163.17 (C), 147.03 (C), 142.35 (C), 136.66 (C), 135.40 (d, *J* = 3.0 Hz, 3 x CH), 133.32 (d, *J* = 9.8 Hz, 6 x CH), 131.35 (CH), 130.68 (d, *J* = 12.6 Hz, 6 x CH), 128.42 (d, *J* = 72.4 Hz, CH), 124.81 (CH), 117.77 (d, *J* = 86.2 Hz, 3 x C), 44.70 (CH₂), 39.88 (CH₂), 27.56 (CH₂), 27.34 (d, *J* = 16.6 Hz, CH₂), 21.93 (d, *J* = 3.6 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_p 23.50 (1P, s, PPh₃), -144.21 (1P, sept, *J* = 713.7 Hz, PF₆). HRMS (ESI⁺): C₃₀H₂₉³⁵ClN₂O₅PS requires 595.1218 found 595.1208 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

{5-[(4-{[*N,N,N*-dimethylcarbamimidoyl]carbamimidamido}sulfonyl}-3-nitrophenyl)formamido]pentyl}triphenylphosphonium hexafluorophosphate (MitoMetNAB) 108

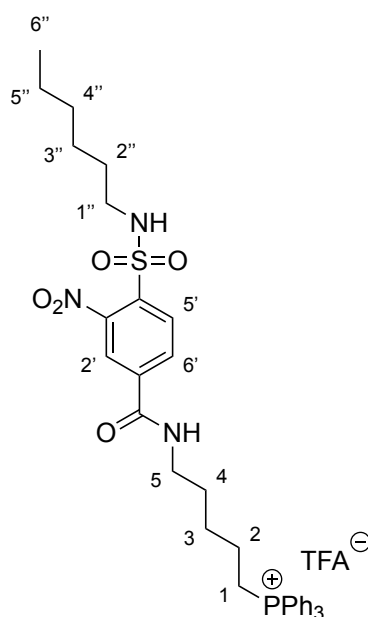


1,1-Dimethylbiguanide **58** (31.9 mg, 0.245, 2.00 eq.) was added to a solution of **117** (91.4 mg, 0.123 mmol, 1.00 eq.) in anhydrous dichloromethane (5 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-30%] to afford **108** as a pale-yellow hygroscopic foam (22.3 mg, 22%).

U_{max} (ATR): 1651 (C=O), 1543 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_H 8.19 (1H, d, *J* = 8.8 Hz, H-2'), 8.10 - 8.06 (2H, m, H-5' and H-6'), 7.88 (3H, m, Ph), 7.84 - 7.71 (12H, m, Ph), 3.47 - 3.36 (4H, m, CH₂-1 and CH₂-5), 3.01 (6H, s, 2 x CH₃), 1.82 - 1.56 (6H, m, CH₂-

2, CH₂-3 and CH₂-4). ¹³C NMR (101 MHz, CD₃OD): δ_c 165.07 (C), 159.68 (C), 159.07 (C), 147.84 (C), 138.36 (d, *J* = 3.6 Hz, CH), 134.86 (d, *J* = 3.0 Hz, 3 x CH), 133.38 (d, *J* = 9.9 Hz, 6 x CH), 130.12 (d, *J* = 12.5 Hz, 6 x CH), 129.58 (d, *J* = 23.9 Hz, CH), 122.62 (CH), 118.51 (d, *J* = 86.3 Hz, 3 x C), 39.23 (CH₂), 35.92 (CH₂), 27.98 (CH₂), 27.46 (d, *J* = 16.7 Hz, CH₂), 21.80 (d, *J* = 4.4 Hz, CH₂), 21.27 (d, *J* = 51.3 Hz, CH₂). ³¹P NMR (162 MHz, CD₃OD): δ_p 23.65 (1P, s, PPh₃), -144.57 (1P, sept, *J* = 708.0 Hz, PF₆). HRMS (ESI⁺): C₃₄H₃₉N₇O₅PS requires 688.2466 found 688.2481 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

(5-{[4'-(Hexylsulfamoyl)-3'-nitrophenyl]formamido}pentyl)triphenylphosphonium hexafluorophosphate (MitoHexNAB) 122

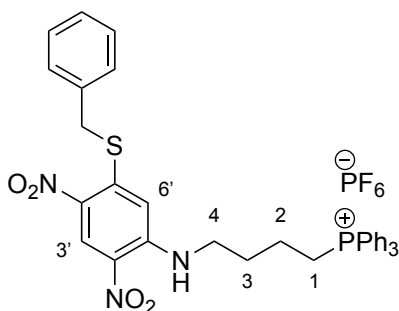


Anhydrous triethylamine (16.5 μL, 81.0 μmol, 2.00 eq.) and hexylamine (9.4 μL, 71.3 μmol, 1.10 eq.) were added to a solution of **117** (40.0 mg, 64.8 μmol, 1.00 eq.) in anhydrous dichloromethane (2 mL) and the reaction was stirred at room temperature for 2 h under argon. 4-(Dimethylamino)pyridine (0.900 mg, 5.00 μmol, 0.125 eq.) was added and the reaction was stirred at room temperature for 48 h under argon. The reaction was diluted with dichloromethane (10 mL) and washed with an aqueous solution of HCl (10 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL) and the organic layers were combined, dried with MgSO₄, and solvent removed under reduced pressure. The crude material was purified by column chromatography [SiO₂,

dichloromethane: methanol 0-15%], followed by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 10-90%] to afford **122** as an orange hygroscopic foam (47.5 mg, 83%).

$U_{\max}$ (ATR): 1614 (C=O) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 9.43 (1H, t, J = 5.5 Hz, CONH), 8.56 - 8.45 (2H, m, H-2' and H-6'), 8.08 (1H, d, J = 8.0 Hz, H-5'), 7.84 - 7.74 (3H, m, Ph), 7.71 - 7.59 (12H, m, Ph), 5.36 (1H, t, J = 6.0 Hz, SO_2NH), 3.46 (2H, q, J = 5.7 Hz, CH_2 -5), 3.41 - 3.27 (2H, m, CH_2 -1), 3.04 (2H, q, J = 6.8 Hz, CH_2 -1''), 1.81 - 1.62 (6H, m, CH_2 -2, CH_2 -3 and CH_2 -4), 1.50 (2H, qn, J = 7.2 Hz, CH_2 -2''), 1.25 (6H, m, CH_2 -3'', CH_2 -4'' and CH_2 -5''), 0.84 (3H, t, J = 6.7 Hz, CH_3 -6''). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 164.16 (C), 148.04 (C), 140.18 (C), 135.40 (d, J = 3.0 Hz, 3 x CH), 134.69 (C), 133.49 (d, J = 9.9 Hz, 6 x CH), 131.71 (CH), 131.24 (CH), 130.68 (d, J = 12.5 Hz, 6 x CH), 125.34 (CH), 118.13 (d, J = 86.1 Hz, 3 x C), 44.04 (CH_2), 39.78 (CH_2), 31.31 (CH_2), 29.65 (CH_2), 27.66 (d, J = 16.7 Hz, CH_2), 27.27 (CH_2), 26.26 (CH_2), 22.57 (CH_2), 22.02 (CH_2), 21.98 (d, J = 56.2 Hz, CH_2), 14.06 (CH_3). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 23.95 (s, 1P, PPh_3). ^{19}F NMR (377 MHz, CDCl_3): δ_{F} -75.16 (3F, s, TFA). HRMS (ESI^+): $\text{C}_{36}\text{H}_{43}\text{N}_3\text{O}_5\text{PS}$ requires 660.2656 found 660.2667 (M^+). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA^- anion were not observed in the ^{13}C NMR spectrum.

(4-[[5'-(Benzylsulfanyl)-2',4'-dinitrophenyl]amino]butyl)triphenylphosphonium hexafluorophosphate **123**

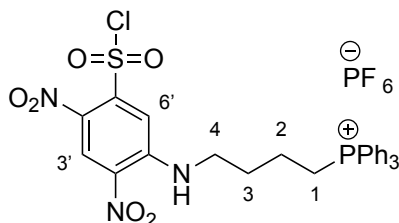


A solution of ammonium hexafluorophosphate (964 mg, 5.92 mmol, 5.00 eq.) in water (15 mL) was added to a solution of MitoCDNB **51** (805 mg, 1.18 mmol, 1.00 eq.) in dichloromethane (15 mL) and the biphasic mixture was stirred at room temperature for 15 mins. The organic layer was separated, and the aqueous layer was extracted with

dichloromethane (2 x 30 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford MitoCDNB **51** as the hexafluorophosphate salt as an orange hygroscopic foam (1.01 g, 88%). Benzyl mercaptan (349 μ L, 2.98 mmol, 2.00 eq.) and anhydrous *N,N*-diisopropylethylamine (519 μ L, 2.98 mmol, 2.00 eq.) were added to a solution of MitoCDNB **51** as the hexafluorophosphate salt in anhydrous acetonitrile (30 mL) and the reaction was stirred at reflux for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was re-dissolved in dichloromethane (30 mL), washed with a saturated solution of NH₄Cl (25 mL) and the aqueous layer was extracted with dichloromethane (2 x 30 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford **123** as an orange hygroscopic foam (806 mg, 70%).

$U_{\max}$ (ATR): 1605 (Aryl C=C), 1576 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃CN): δ_{H} 9.03 (1H, s, H-3'), 8.30 (1H, m, NH), 7.85 (3H, m, Ph), 7.71 - 7.62 (12H, m, Ph), 7.50 - 7.43 (2H, m, Ph), 7.38 - 7.29 (2H, m, Ph), 7.29 - 7.23 (1H, m, Ph), 6.58 (1H, s, H-6'), 4.30 (2H, s, SCH₂), 3.33 (2H, q, J = 6.4 Hz, CH₂-4), 3.22 - 3.10 (2H, m, CH₂-1), 1.78 (2H, qn, J = 6.8 Hz, CH₂-2), 1.66 (2H, qn, J = 7.9 Hz, CH₂-3). ¹³C NMR (101 MHz, CD₃CN): δ_{C} 147.62 (C), 147.34 (C), 136.20 (d, J = 3.0 Hz, 3 x CH), 135.95 (C), 134.61 (d, J = 10.0 Hz, 6 x CH), 134.26 (C), 131.29 (d, J = 12.6 Hz, 6 x CH), 130.01 (CH), 129.96 (d, J = 9.8 Hz, CH), 128.87 (CH), 127.54 (CH), 119.05 (d, J = 86.5 Hz), 110.10 (CH), 42.69 (CH₂), 37.90 (CH₂), 29.65 (d, J = 16.6 Hz, CH₂), 22.34 (d, J = 52.2 Hz, CH₂), 20.46 (d, J = 3.9 Hz, CH₂). ³¹P NMR (162 MHz, CD₃CN): δ_{P} 23.69 (1P, s, PPh₃), -144.62 (1P, sept, J = 705.8, PF₆). HRMS (ESI⁺): C₃₅H₃₃N₃O₄PS requires 622.1940 found 622.1937 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

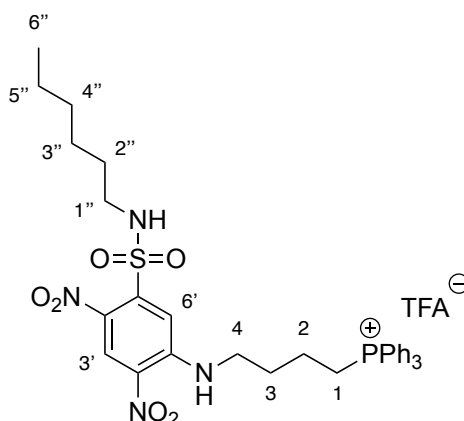
(4-[[5'-(Chlorosulfonyl)-2',4'-dinitrophenyl]amino]butyl)triphenylphosphonium hexafluorophosphate 124



Adapting the procedure of Xin *et al.*,³⁰⁰ 1,3-dichloro-5,5-dimethylhydantoin (142 mg, 0.719 mmol, 2.00 eq.) was added to a solution of **123** (276 mg, 0.359 mmol, 1.00 eq.) in a solvent mixture of acetonitrile: acetic acid: water (2 mL: 0.1 mL: 0.1 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the residue was redissolved in dichloromethane (30 mL). The organic layer was washed with water (30 mL) and the aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford **124** an orange hygroscopic foam (209 mg, 78%).

$\nu_{\max}$ (ATR): 1615 (Aryl C=C), 1541 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃CN): δ_{H} 8.89 (1H, s, H-3'), 8.61 (1H, m, NH), 7.87 (3H, m, Ph), 7.77 - 7.62 (13H, m, H-6' and Ph), 3.57 (2H, q, J = 6.6 Hz, CH₂-4), 3.27 - 3.15 (2H, m, CH₂-1), 1.92 - 1.85 (2H, m, CH₂-2), 1.80 - 1.68 (2H, m, CH₂-3). ¹³C NMR (101 MHz, CD₃CN): δ_{C} 147.90 (C), 142.38 (C), 136.22 (d, J = 3.0 Hz, 3 x CH), 134.66 (d, J = 10.0 Hz, 6 x CH), 131.31 (d, J = 12.6 Hz, 6 x CH), 128.43 (CH), 119.68 (C), 119.09 (d, J = 86.5 Hz, C), 43.26 (CH₂), 29.86 (d, J = 17.0 Hz, CH₂), 22.41 (d, J = 52.1 Hz, CH₂), 20.45 (d, J = 4.0 Hz, CH₂). ³¹P NMR (162 MHz, CD₃CN): δ_{P} 23.61 (1P, s, PPh₃), -144.63 (1P, sept, J = 704.2 Hz, PF₆). HRMS (ESI⁺): C₂₈H₂₆ClN₃O₆PS requires 598.0963 found 598.0850 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. Two signals corresponding to C were not identified in the ¹³C spectrum, perhaps due to coincidence with another signal.

(4-{[5'-(hexylsulfamoyl)-2',4'-dinitrophenyl]amino}butyl)triphenylphosphonium trifluoroacetate (MitoHexDNB) 125

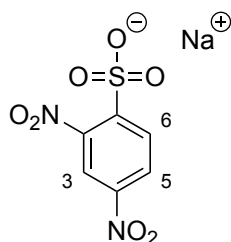


Hexyl-1-amine (8.9 μ L, 0.067 mmol, 1.00 eq.) and triethylamine (18.9 μ L, 0.134, 2.00 eq.) were added to a solution of **124** (50.0 mg, 0.067 mmol, 1.00 eq.) in anhydrous dichloromethane (1 mL) and the reaction was stirred at room temperature for 3 h under argon. The reaction was diluted with dichloromethane (20 mL) and washed with HCl (20 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO_4 and the solvent removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:ethyl acetate 0-40%], followed by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 47% isocratic] to afford **125** as a yellow hygroscopic foam (14.3 mg, 26%).

ν_{max} (ATR): 3355 (N-H), 1614 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.87 (1H, s, H-3'), 8.71 - 8.64 (1H, m, NH), 7.81 (3H, m, Ph), 7.76 - 7.64 (12H, m, Ph), 7.63 (1H, s, H-6'), 6.16 (1H, t, J = 5.9 Hz, SO_2NH), 3.67 (2H, m, CH_2 -4), 3.53 (2H, m, CH_2 -1), 3.10 (2H, q, J = 6.7 Hz, CH_2 -1''), 2.10 - 1.98 (2H, m, CH_2 -3), 1.88 - 1.71 (2H, m, CH_2 -2), 1.52 (2H, qn, J = 7.0 Hz, CH_2 -2''), 1.34 - 1.14 (6H, m, CH_2 -3'', CH_2 -4'' and CH_2 -5''), 0.84 (3H, t, J = 6.6 Hz, CH_3 -6''). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 146.94 (C), 141.34 (C), 135.46 (d, J = 3.0 Hz, 3 x CH), 134.21 (C), 133.55 (d, J = 10.0 Hz, 6 x CH), 130.75 (d, J = 12.6 Hz, 6 x CH), 130.53 (C), 127.12 (CH), 117.92 (d, J = 86.1 Hz, 3 x C), 117.66 (CH), 44.15 (CH_2), 42.20 (CH_2), 31.37 (CH_2), 29.85 (CH_2), 29.12 (d, J = 16.8 Hz, CH_2), 26.26 (CH_2), 22.59 (CH_2), 21.90 (d, J = 52.4 Hz, CH_2), 19.66 (d, J = 3.7 Hz, CH_2), 14.08 (CH_3). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 24.02 (1P, s, PPh_3). ^{19}F NMR (377 MHz, CDCl_3): δ_{F} -75.60 (3F, s, TFA). HRMS (ESI $^+$): $\text{C}_{34}\text{H}_{40}\text{N}_4\text{O}_6\text{PS}$ requires 663.2401 found 633.2406 (M^+). Assignment of ^1H and ^{13}C NMR

signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

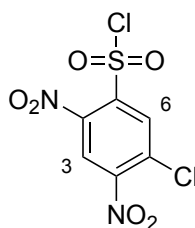
Sodium 2,4-dinitrobenzene-1-sulfonate **131**



Adapting the procedure of Dr Taig,²²⁴ sodium sulfite (315 mg, 2.50 mmol, 1.00 eq.) was added to a solution of 1-chloro-2,4-dinitrobenzene (315 mg, 2.50 mmol, 1.00 eq.) in ethanol:water (5:1, 13 mL) and the reaction was stirred at 80 °C for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane: methanol 0-30%] to afford **131** as a yellow solid (309 mg, 49%).

Mp: Decomposition at 220 °C. ν_{max} (ATR): 1601 (Aryl C=C), 1550 (NO₂), 1524 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_{H} 8.53 (1H, d, J = 2.2 Hz, H-3), 8.48 (1H, dd, J = 8.6, 2.3 Hz, H-5), 8.28 (1H, d, J = 8.6 Hz, H-6). ¹³C NMR (101 MHz, CD₃OD): δ_{C} 149.97 (C), 149.60 (C), 144.08 (C), 132.26 (CH), 126.86 (CH), 120.04 (CH). Mass spectrometry by ESI⁻ failed to identify the product.

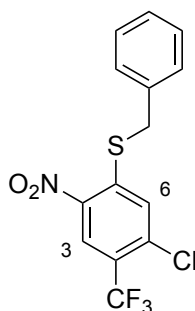
2,4-Dinitrobenzene-1-sulfonyl chloride **132**



Adapting the procedure of Dr Taig,²²⁴ sodium 2,4-dinitrobenzene-1-sulfonate **131** (200 mg, 0.992 mmol, 1.00 eq.) was dissolved in chlorosulfonic acid (5 mL) and the reaction was stirred at 96 °C for 4 h under argon. The reaction was allowed to cool to room temperature and added dropwise to ice-water (100 mL). The precipitate was filtered under vacuum and re-dissolved in dichloromethane (20 mL). The organic layer was washed with water (20 mL) and the aqueous layer extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford **132** as a white solid (126 mg, 47%).

Mp: 94 - 96 °C. ν_{max} (ATR): 1538 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_{H} 8.83 (1H, d, J = 2.6 Hz, H-3), 8.48 (1H, dd, J = 8.8, 2.6 Hz, H-5), 7.96 (1H, d, J = 8.9 Hz, H-6). ¹³C NMR (101 MHz, CDCl₃): δ_{C} 151.42 (C), 140.13 (C), 134.54 (C), 132.44 (CH), 127.45 (CH), 120.98 (CH). Mass spectrometry by ESI^{+/−} failed to identify the product.

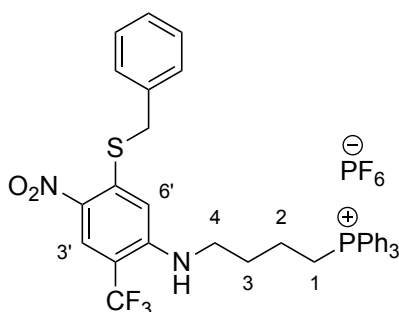
1-(Benzylsulfanyl)-5-chloro-2-nitro-4-(trifluoromethyl)benzene **139**



Benzyl mercaptan (0.225 mL, 1.92 mmol, 1.00 eq.) and triethylamine (0.536 mL, 3.84 mmol, 2.00 eq.) were added to a solution of 2,4-dichloro-5-nitrobenzotrifluoride (500 mg, 1.92 mmol, 1.00 eq.) in anhydrous acetonitrile (9.6 mL). The reaction was stirred at reflux under argon for 4 h. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was re-dissolved in dichloromethane (30 mL) and washed with HCl (30 mL, 1 M). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude was purified by column chromatography [SiO₂, hexane: dichloromethane 5-40%] to afford **139** as yellow solid (364 mg, 54%).

Mp 105 - 107 °C. $U_{\max}$ (ATR): 1594 (Aryl C=C), 1549 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_{H} 8.57 (1H, s, H-3), 7.59 (1H, s, H-6), 7.47 - 7.29 (5H, m, Ph), 4.24 (2H, s, CH₂). ¹³C NMR (101 MHz, CDCl₃): δ_{C} 144.76 (C), 142.86 (C), 138.02 (C), 133.59 (C), 129.28 (2 x CH), 129.27 (2 x CH), 129.02 (CH), 128.48 (CH), 125.77 (q, J = 5.5 Hz, CH), 124.66 (q, J = 33.6 Hz, C), 121.91 (q, J = 273.1 Hz, C), 37.91 (CH₂). ¹⁹F NMR (377 MHz, CDCl₃): δ_{F} -62.66 (3F, s, CF₃). Mass spectrometry by APCI^{+/•} failed to identify the product.

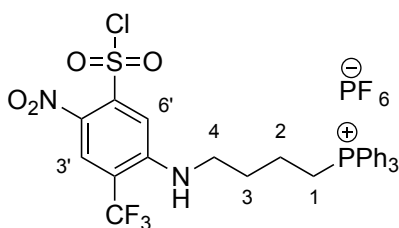
(4-{[5'-(Benzylsulfanyl)-4'-nitro-2'-(trifluoromethyl)phenyl]amino}butyl) triphenylphosphonium hexafluorophosphate 140



N,N-diisopropylethylamine (0.300 mL, 1.73 mmol, 2.00 eq.) and **139** (300 mg, 0.863 mmol, 1.00 eq.) were added to a solution of **95** (357 mg, 0.863 mmol, 1.00 eq.) in anhydrous acetonitrile (8.6 mL) and the reaction was stirred at reflux for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The solid residue was re-dissolved in dichloromethane (30 mL) and washed with HCl (30 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude was purified by column chromatography [SiO₂, dichloromethane: methanol 0-20%] to afford **140** as the chloride salt (329 mg, 56%). A solution of ammonium hexafluorophosphate (393 mg, 2.42 mmol, 5.00 eq.) in water (15 mL) was added to a solution of **140** as the chloride salt (329 mg, 0.483 mmol, 1.00 eq.) in dichloromethane (15 mL) and the biphasic mixture was stirred at room temperature for 15 mins. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford **140** as the hexafluorophosphate salt as a yellow hygroscopic foam (368 mg, 54%).

$\nu_{\max}$ (ATR): 1611 (Aryl C=C), 1560 (Aryl C=C), 1521 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_{H} 8.35 (1H, s, H-3'), 7.83 - 7.74 (3H, m, Ph), 7.70 - 7.58 (12H, m, Ph), 7.50 - 7.43 (2H, m, Ph), 7.31 - 7.27 (2H, m, Ph), 7.22 - 7.12 (1H, m, Ph), 6.41 (1H, s, H-6'), 4.86 (1H, t, J = 4.7 Hz, NH), 4.19 (2H, s, SCH₂), 3.24 - 3.10 (4H, m, CH₂-1 and CH₂-4), 1.83 (2H, qn, J = 6.7 Hz, CH₂-2), 1.65 (2H, h, J = 7.8 Hz, CH₂-3). ¹³C NMR (101 MHz, CDCl₃): δ_{C} 148.19 (C), 145.62 (C), 135.03 (C), 135.00 (d, J = 3.0 Hz, 3 x CH), 133.70 (d, J = 10.1 Hz, 6 x CH), 133.32 (C), 130.45 (d, J = 12.6 Hz, 6 x CH), 128.89 (2 x CH), 128.88 (2 x CH), 127.65 (CH), 126.69 (q, J = 5.6 Hz, CH), 123.59 (q, J = 272.3 Hz, C), 118.20 (d, J = 85.9 Hz, 3 x C), 109.56 (q, J = 32.2 Hz, C), 107.21 (CH), 41.52 (CH₂), 37.37 (CH₂), 28.05 (d, J = 17.2 Hz, CH₂), 22.25 (d, J = 50.4 Hz, CH₂), 19.30 (d, J = 4.1 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_{P} 23.73 (1P, s, PPh₃), -144.22 (sept, J = 713.8 Hz, PF₆). ¹⁹F NMR (377 MHz, CDCl₃): δ_{F} -62.56 (3F, s, CF₃), -72.18 (6F, d, J = 714.1 Hz, PF₆). HRMS (ESI⁺): C₃₆H₃₃F₃N₂O₂PS requires 645.1947 found 645.1956 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

(4-[[5'-(Chlorosulfonyl)-4'-nitro-2'-(trifluoromethyl)phenyl]amino}butyl) triphenylphosphonium hexafluorophosphate 141

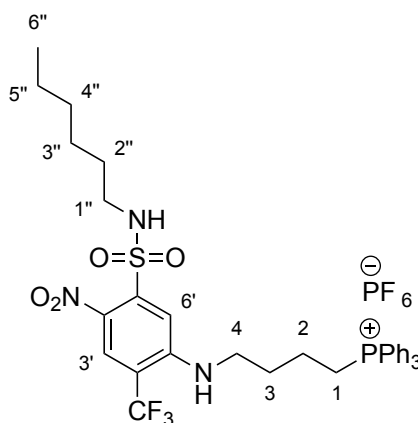


Adapting the procedure of Xin *et al.*,³⁰⁰ 1,3-dichloro-5,5-dimethylhydantoin (51.3 mg, 0.253 mmol, 2.00 eq.) was added to a solution of **140** (100 mg, 0.126 mmol, 1.00 eq.) in a solvent mixture of acetonitrile:acetic acid:water (1.2 mL: 0.050 mL: 0.050 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 6 h under argon. The solvent was removed under reduced pressure and the residue was re-dissolved in dichloromethane (20 mL). The organic layer was washed with an aqueous solution of HCl (20 mL, 1 M) and the aqueous layer was extracted with dichloromethane (3 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. Purification of the crude material by column chromatography [SiO₂, dichloromethane: methanol 0-10%] afforded **141** as a yellow

hygroscopic foam (29.4 mg) with minor impurities. It was characterised as the impure mixture and taken onto the next step of the synthesis.

^1H NMR (400 MHz, CDCl_3): δ_{H} 8.21 (1H, s, H-3'), 7.86 - 7.77 (3H, m, Ph), 7.75 - 7.61 (12H, m, Ph), 7.38 (1H, s, H-6'), 5.88 - 5.69 (1H, br s, NH), 3.46 (2H, q, J = 6.5 Hz, CH_2 -4), 3.28 - 3.18 (2H, m, CH_2 -1), 2.02 - 1.95 (2H, m, CH_2 -2), 1.81 - 1.69 (2H, m, CH_2 -3). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 23.56 (1P, s, PPh_3), -144.34 (sept, J = 712.9 Hz, PF_6). ^{19}F NMR (377 MHz, CDCl_3): δ_{F} -64.23 (3F, s, CF_3), -72.44 (d, J = 713.9 Hz, PF_6).

(4-{[5'-(Hexylsulfamoyl)-4'-nitro-2'-(trifluoromethyl)phenyl]amino}butyl) triphenylphosphonium hexafluorophosphate (MitoHexNTB) 142

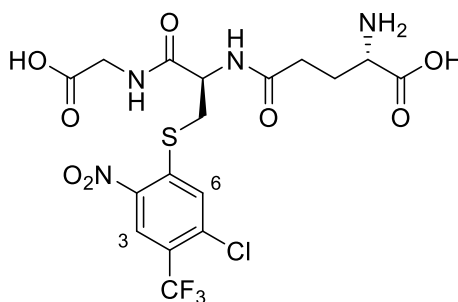


Anhydrous triethylamine (10.6 μL , 75.6 μmol , 2.00 eq.) and hexyl-1-amine (5.0 μL , 37.8 μmol , 1.00 eq.) were added to a solution of **141** (29.0 mg, 37.8 μmol , 1.00 eq.) in anhydrous dichloromethane (0.4 mL) and the reaction was stirred at room temperature for 4 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane: methanol 0-10%], to afford **142** as a yellow hygroscopic foam (12.5 mg, 40%).

U_{max} (ATR): 1612 (Aryl C=C), 1572 (NO_2) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.19 (1H, s, H-3'), 7.86 - 7.76 (3H, m, Ph), 7.74 - 7.61 (12H, m, Ph), 7.33 (1H, s, H-6'), 5.69 (1H, t, J = 6.0 Hz, SO_2NH), 5.60 (1H, t, J = 4.0 Hz, NH), 3.46 (2H, q, J = 6.5 Hz, CH_2 -4), 3.24 (2H, m,

CH₂-1), 3.08 (2H, q, $J = 6.6$ Hz, CH₂-1''), 1.99 (2H, qn, $J = 7.1$ Hz, CH₂-2), 1.76 (2H, qn, $J = 8.0$ Hz, CH₂-3), 1.51 (2H, qn, $J = 6.9$ Hz, CH₂-2''), 1.36 - 1.17 (6H, m, CH₂-3'', CH₂-4'' and CH₂-5''), 0.83 (3H, t, $J = 6.6$ Hz, CH₃-6''). ¹³C NMR (101 MHz, CDCl₃): δ_c 149.03 (C), 140.01 (C), 135.51 (d, $J = 3.0$ Hz, CH), 134.46 (C), 133.48 (d, $J = 9.9$ Hz, CH), 130.82 (d, $J = 12.6$ Hz, CH), 127.44 (q, $J = 5.4$ Hz, CH), 117.72 (d, $J = 86.4$ Hz, C), 114.23 (q, $J = 32.0$ Hz, C), 113.90 (CH), 44.15 (CH₂), 42.46 (CH₂), 31.36 (CH₂), 29.73 (CH₂), 28.96 (d, $J = 16.9$ Hz, CH₂), 26.26 (CH₂), 22.58 (CH₂), 22.19 (d, $J = 52.3$ Hz, CH₂), 19.78 (d, $J = 4.0$ Hz, CH₂), 14.07 (CH₃). ³¹P NMR (162 MHz, CDCl₃): δ_P 23.68 (1P, s, PPh₃), -144.32 (sept, $J = 712.7$ Hz, PF₆). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -63.75 (3F, s, CF₃), -72.46 (d, $J = 713.9$ Hz, PF₆). HRMS (ESI⁺): C₃₅H₄₀F₃N₃O₄PS requires 668.2318 found 668.2310 (M^+). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signal corresponding to CF₃ was not identified in the ¹³C NMR spectrum, perhaps because the spectrum was too weak.

1-(*S*-Glutathionyl)-5-chloro-2-nitro-4-(trifluoromethyl)benzene **149**

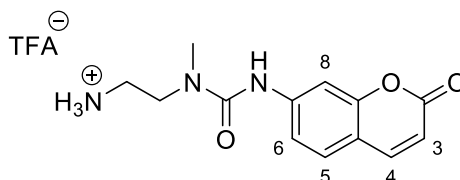


Glutathione (591 mg, 1.92 mmol, 1.00 eq.) and NaHCO₃ (323 mg, 3.85 mmol, 2.00 eq) were added to a solution of 1,4-dichloro-2-nitro-4-(trifluoromethyl)benzene (500 mg, 1.92 mmol, 1.00 eq.) in EtOH:H₂O (1:1, 19.2 mL) and the reaction was stirred at reflux for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was purified by reversed phase chromatography (SiO₂, water:acetonitrile 0-100%) to afford **149** as a yellow hygroscopic solid (491 mg, 48%).

U_{max} (ATR): 1602 (Aryl C=C), 1549 (Aryl C=C), 1509 (NO₂) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_H 8.59 (1H, s, H-3), 7.86 (1H, s, H-6), 4.77 (1H, dd, $J = 8.9, 3.5$ Hz, Cys CH), 3.84 - 3.65 (4H, m, Gly CH₂, SCH_ACH_B, Gln CH), 3.43 (1H, dd, $J = 14.1, 8.6$ Hz, SCH_BCH_A), 2.49 (2H, m,

CH_2CONH), 2.10 (2H, m, $\text{CH}_2\text{CH}_2\text{CONH}$). ^{13}C NMR (101 MHz, D_2O): δ_{C} 176.08 (C), 174.94 (C), 174.55 (C), 170.80 (C), 143.45 (C), 142.24 (C), 137.71 (C), 129.51 (CH), 125.93 (q, $J = 5.4$ Hz, CH), 124.41 (q, $J = 33.4$ Hz, C), 121.80 (q, $J = 272.3$ Hz, C), 54.20 (CH), 51.64 (CH), 43.35 (CH_2), 33.37 (CH_2), 31.46 (CH_2), 26.52 (CH_2). ^{19}F NMR (377 MHz, D_2O): δ_{F} -62.72 (3F, s, CF_3). HRMS (ESI $^+$): $\text{C}_{17}\text{H}_{19}^{35}\text{ClF}_3\text{N}_4\text{O}_8\text{S}$ requires 531.0559 found 531.0579 ($\text{M} + \text{H}$) $^+$. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

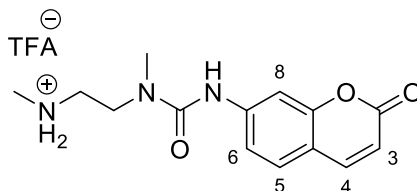
3-(2-Azaniumylethyl)-3-methyl-1-(2-oxo-2H-chromen-7-yl)urea trifluoroacetate (ACoum1) 165



Trifluoroacetic acid (0.6 mL) was added to a solution of **171** (20.0 mg, 0.0553 mmol, 1.00 eq.) in anhydrous dichloromethane (0.6 mL) and the reaction was stirred at room temperature for 0.5 h under argon. The solvent was removed under reduced pressure to afford **185** as a white hygroscopic foam (14.5 mg, quant.).

ν_{max} (ATR): 2757 - 3257 ($\text{N}^+\text{-H}$), 1748 (C=O), 1649 (Aryl C=C), 1598 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, D_2O): δ_{H} 7.87 (1H, d, $J = 9.5$ Hz, H-4), 7.47 (1H, d, $J = 8.5$ Hz, H-5), 7.31 (1H, d, $J = 2.0$ Hz, H-8), 7.20 (1H, dd, $J = 8.5, 2.1$ Hz, H-6), 6.30 (1H, d, $J = 9.4$ Hz, H-3), 3.69 (2H, t, $J = 5.9$ Hz, NH_3CH_2), 3.24 (2H, t, $J = 6.0$ Hz, CH_3NHCH_2), 3.09 (3H, s, CH_3). ^{13}C NMR (101 MHz, D_2O): δ_{C} 164.39 (C), 162.75 (q, $J = 35.7$ Hz, C), 157.33 (C), 153.67 (C), 145.57 (CH), 142.48 (C), 128.61 (CH), 117.24 (CH), 116.22 (q, $J = 291.3$ Hz, C), 114.35 (C), 112.55 (CH), 107.06 (CH), 46.08 (CH_2), 37.82 (CH_2), 34.65 (CH_3). ^{19}F NMR (377 MHz, D_2O): δ_{F} -75.63 (3F, s, TFA). HRMS (ESI $^+$): $\text{C}_{13}\text{H}_{16}\text{N}_3\text{O}_3$ requires 262.1186 found 262.1191 ($\text{M} + \text{H}$) $^+$. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

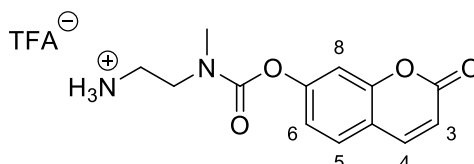
3-Methyl-3-[2-(methyldiazaniumyl)ethyl]-1-(2-oxo-2H-chromen-7-yl)urea trifluoroacetate (ACoum2) 166



Trifluoroacetic acid (2 mL) was added to a solution of **172** (75.0 mg, 0.200 mmol, 1.00 eq.) in anhydrous dichloromethane (2 mL) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure to afford **166** as a white hygroscopic foam (74.0 mg, quant.).

$U_{\max}$ (ATR): 3138 (N⁺-H), 3067 (N⁺-H), 1682 (C=O), 1608 (Aryl C=C) cm⁻¹. ¹H NMR (400 MHz, D₂O): δ_{H} 7.85 (1H, d, J = 9.5 Hz, H-4), 7.45 (1H, d, J = 8.5 Hz, H-5), 7.30 (1H, d, J = 2.0 Hz, H-8), 7.18 (1H, dd, J = 8.5, 2.1 Hz, H-6), 6.29 (1H, d, J = 9.5 Hz, H-3), 3.70 (2H, t, J = 5.7 Hz, CH₃NH₂CH₂), 3.28 [2H, t, J = 5.7 Hz, CON(CH₃)CH₂], 3.09 (3H, s, NH₂CH₃), 2.76 (3H, s, CONCH₃). ¹³C NMR (101 MHz, D₂O): δ_{C} 164.50 (C), 162.81 (q, J = 35.7 Hz, C), 157.55 (C), 153.78 (C), 145.64 (CH), 142.44 (C), 128.69 (CH), 117.57 (CH), 116.24 (q, J = 291.5 Hz, C), 114.58 (CH), 112.76 (CH), 107.47 (CH), 47.45 (CH₂), 45.13 (CH₂), 34.58 (CH₃), 33.07 (CH₃). ¹⁹F NMR (377 MHz, D₂O): δ_{F} -75.63 (3F, s, TFA). HRMS (ESI⁺): C₁₄H₁₈N₃O₃ requires 276.1343 found 276.1345 (M + H)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

2-Oxo-2H-chromen-7-yl N-(2-azaniumylethyl)-N-methylcarbamate trifluoroacetate (HCoum1) 167

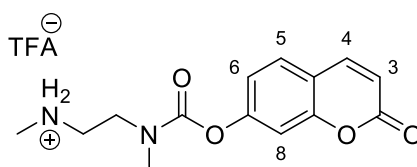


Trifluoroacetic acid (0.4 mL) was added to a solution of **173** (15.0 mg, 0.0414 mmol, 1.00 eq.) in anhydrous dichloromethane (0.4 mL) and the reaction was stirred at room

temperature for 1 h under argon. The solvent was removed under reduced pressure to afford **167** as a white hygroscopic foam (16.1 mg, quant.).

NMR spectroscopy indicated a 1.8:1 mixture of diastereomers (rotamers) A:B. $U_{\max}$ (ATR): 1691 (C=O), 1619 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ_{H} 7.96 (1H, d, J = 9.6 Hz, H-4^{A and B}), 7.67 (1H, d, J = 8.5 Hz, H-5^{A and B}), 7.29 - 7.23 (1H, m, H-8^{A and B}), 7.19 (1H, dd, J = 8.5, 2.2 Hz, H-6^{A and B}), 6.41 (1H, d, J = 9.6 Hz, H-3^{A and B}), 3.80 (2H, t, J = 6.1 Hz, $\text{CH}_3\text{NCH}_2^{\text{B}}$), 3.68 (2H, t, J = 5.8 Hz, $\text{CH}_3\text{NCH}_2^{\text{A}}$), 3.26 (2H, t, J = 6.2 Hz, $\text{NH}_3\text{CH}_2^{\text{B}}$), 3.21 (2H, t, J = 5.8 Hz, $\text{NH}_3\text{CH}_2^{\text{A}}$), 3.18 (3H, s, CH_3^{A}), 3.07 (3H, s, CH_3^{B}). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 162.40 (C^{A and B}), 156.47 (C^{A and B}), 155.84 (C^{A and B}), 155.33 (C^{A and B}), 145.10 (CH^{A and B}), 130.11 (CH^{A and B}), 119.86 (CH^B), 119.73 (CH^A), 118.01 (C^{A and B}), 116.46 (CH^B), 116.39 (CH^A), 111.35 (CH^B), 111.26 (CH^A), 39.01 (CH₂^{A and B}), 38.87 (CH₂^{A and B}), 35.51 (CH₃^{A and B}). ^{19}F NMR (377 MHz, CD_3OD): δ_{F} -76.87 (3F, s, TFA). HRMS (ESI⁺): $\text{C}_{13}\text{H}_{15}\text{N}_2\text{O}_4$ requires 263.1026 found 263.1027 (M^+). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA⁻ anion were not observed in the ^{13}C NMR spectrum.

2-Oxo-2H-chromen-7-yl N-methyl-N-[2-(methylazaniumyl)ethyl]carbamate trifluoroacetate (HCoum2) 168

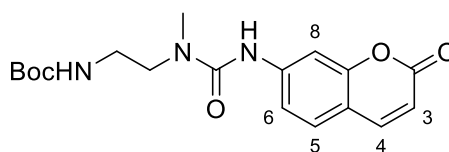


Trifluoroacetic acid (0.74 mL) was added to a solution of **174** (23.8 mg, 0.0736 mmol, 1.00 eq.) in anhydrous dichloromethane (0.7 mL) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure to afford **168** as a white hygroscopic solid (23.7 mg, quant.).

NMR spectroscopy indicated a 3:1 mixture of diastereomers (rotamers) A:B. $U_{\max}$ (ATR): 1725 (C=O), 1700 (C=O), 1618 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 9.38 (2H, br s, NH_2^{B}), 9.20 (2H, br s, NH_2^{A}), 7.68 (1H, d, J = 9.6 Hz, H-4^{A and B}), 7.46 (1H, d, J = 8.5 Hz, H-5^{A and B}), 7.18 (1H, d, J = 2.2 Hz, H-8^{A and B}), 7.11 (1H, dd, J = 8.5, 2.2 Hz, H-6^{A and B}), 6.37 (1H, d, J = 9.5 Hz, H-4^{A and B}), 3.79 [2H, t, J = 5.4 Hz, $\text{O}_2\text{CN}(\text{CH}_3)\text{CH}_2^{\text{B}}$], 3.68 [2H, t, J = 5.4

Hz, O₂CN(CH₃)CH₂^A], 3.28 - 3.16 (2H, m, CH₃NH₂CH₂^{A and B}), 3.11 (3H, s, O₂CNCH₃^A), 3.00 (3H, s, O₂CNCH₃^B), 2.70 (3H, s, NH₂CH₃^B), 2.67 (3H, s, NH₂CH₃^A). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -75.66 (3F, s, TFA). ¹³C NMR (101 MHz, CDCl₃): δ_C 160.61 (C^{A and B}), 155.15 (C^{A and B}), 154.44 (C^{A and B}), 153.69 (C^{A and B}), 143.29 (CH^B), 143.10 (CH^A), 128.50 (CH^{A and B}), 118.68 (CH^A), 118.46 (CH^B), 116.44 (C^{A and B}), 115.84 (CH^A), 115.60 (CH^B), 110.56 (CH^A), 110.31 (CH^B), 47.27 (CH₂^A), 47.12 (CH₂^B), 46.03 (CH₂^A), 45.60 (CH₂^B), 35.38 (CH₃^B), 35.13 (CH₃^A), 33.47 (CH₃^B), 33.28 (CH₃^A). HRMS (ESI⁺): C₁₄H₁₇N₂O₄ requires 277.1183 found 277.1175 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

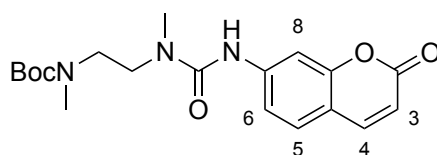
***Tert*-butyl N-(2-{methyl[(2-oxo-2H-chromen-7-yl)carbamoyl]amino}ethyl)carbamate**
171



Anhydrous phosgene solution (15 wt. % in toluene) (244 µL, 0.341 mmol, 1.10 eq.) and triethylamine (87.0 µL, 0.620 mmol, 2.00 eq.) were added to a solution of **104** (50.0 mg, 0.310 mmol, 1.00 eq.) in anhydrous dichloromethane (3.1 mL) at 0 °C. The reaction was stirred at 0 °C for 1 h under argon. The solvent was removed under reduced pressure. The solid residue was re-dissolved in anhydrous acetonitrile (3.1 mL) and triethylamine (87.0 µL, 0.620 mmol, 2.00 eq.) and *N*-boc-*N*-methylethylenediamine (54.0 mg, 0.341 mmol, 1.10 eq.) were added. The reaction was stirred at room temperature for 18 h under argon. The solvent was removed under reduced pressure and the solid residue re-dissolved in dichloromethane (20 mL). The organic layer was washed with an aqueous solution of HCl (20 mL, 1 M) and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford **171** as a white hygroscopic foam (86.1 mg, 77%).

$U_{\max}$ (ATR): 1667 (C=O) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.28 (1H, s, ArNH), 7.63 - 7.48 (3H, m, H-4, H-6 and H-8), 7.29 (1H, d, J = 8.5 Hz, H-5), 6.18 (1H, d, J = 9.4 Hz, H-3), 5.03 (1H, s, BocNH), 3.40 (2H, t, J = 7.2 Hz, CH_3NCH_2), 3.27 - 3.19 (2H, m, BocNHCH₂), 2.98 (3H, s, NCH₃), 1.40 (9H, s, 3 x CH₃). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 161.80 (C), 157.32 (C), 155.28 (C), 155.14 (C), 144.32 (C), 143.62 (CH), 128.19 (CH), 115.84 (CH), 113.66 (C), 113.58 (CH), 106.39 (CH), 80.71 (C), 48.13 (CH₂), 39.67 (CH₂), 35.35 (CH₃), 28.53 (3 x CH₃). HRMS (ESI⁺): $\text{C}_{18}\text{H}_{23}\text{N}_3\text{O}_5\text{Na}$ requires 384.1530 found 384.1532 ($\text{M} + \text{Na}$)⁺. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

Tert*-butyl *N*-methyl-*N*-(2-{methyl[(2-oxo-2H-chromen-7-yl)carbamoyl]amino}ethyl) carbamate **172*

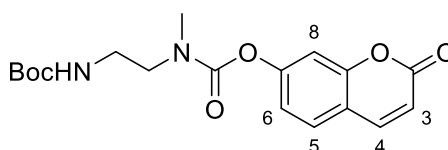


Triphosgene (30.7 mg, 0.103 mmol, 0.330 eq.) and anhydrous *N,N*-diisopropylethylamine (81.0 μL , 0.465 mmol, 1.50 eq.) were added to a solution of **104** (50.0 mg, 0.310 mmol, 1.00 eq.) in anhydrous dichloromethane (3.1 mL). The reaction was stirred at room temperature for 1 h under argon. A solution of *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (64.0 mg, 0.341 mmol, 1.10 eq.) and anhydrous *N,N*-diisopropylethylamine (81.0 μL , 0.465 mmol, 1.50 eq.) in anhydrous dichloromethane (3.1 mL) was added to the reaction, which was stirred at room temperature for an additional 3 h under argon. The reaction was diluted with dichloromethane (20 mL). The organic layer was washed with a saturated solution of NH_4Cl (20 mL) and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , hexane:ethyl acetate 10-100%] to afford **172** as a white hygroscopic foam (112 mg, 95%).

$U_{\max}$ (ATR): 1697 (C=O), 1663 (Aryl C=C), 1620 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 7.68 (1H, br s, H-8), 7.65 - 7.56 (2H, m, H-4 and H-6), 7.33 (1H, d, J = 8.5 Hz, H-5), 6.22 (1H, d, J = 9.4 Hz, H-3), 3.49 - 3.41 (2H, m, BocNCH₂ or CH₃NCH₂), 3.40 - 3.31 (2H, m, BocNCH₂ or CH₃NCH₂), 3.04 (3H, s, OCNCH₃ or BocNCH₃), 2.93 (3H, s, OCNCH₃ or

BocNCH₃), 1.48 (9H, s, 3 x CH₃). ¹³C NMR (101 MHz, CDCl₃): δ_c 161.76 (C), 156.89 (C), 155.15 (2 x C), 144.59 (C), 143.57 (CH), 128.11 (CH), 115.74 (CH), 113.50 (C), 113.45 (CH), 106.29 (CH), 80.96 (C), 48.25 (CH₂), 46.60 (CH₂), 36.22 (CH₃), 35.51 (CH₃), 28.54 (3 x CH₃). HRMS (ESI⁺): C₁₉H₂₆N₃O₅ requires 376.1867 found 376.1863 (M + H)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

2-Oxo-2H-chromen-7-yl N-(2-[[(*tert*-butoxy)carbonyl]amino]ethyl)-N-methylcarbamate **173**

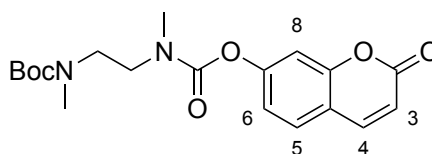


Triphosgene (30.2 mg, 0.102 mmol, 0.330 eq.) and anhydrous *N,N*-diisopropylethylamine (53.7 μL, 0.308 mmol, 1.00 eq.) were added to a solution of 7-hydroxycoumarin (50.0 mg, 0.308 mmol, 1.00 eq.) in a anhydrous solvent mixture of tetrahydrofuran: dichloromethane (1:1, 6 mL). The reaction was stirred at room temperature for 1 h under argon. A solution of *N*-boc-*N*-methylethylenediamine (64.5 mg, 0.370 mmol, 1.20 eq.) and anhydrous *N,N*-diisopropylethylamine (64.4 μL, 0.370 mmol, 1.20 eq.) were added to the reaction, which was stirred at room temperature for an additional 1 h under argon. The solvent was removed under reduced pressure and the solid residue was re-dissolved in dichloromethane (20 mL). The organic layer was washed with a saturated solution of NH₄Cl (20 mL) and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane:ethyl acetate 10-80%] to afford **173** as a white hygroscopic foam (45.2 mg, 40%).

NMR spectroscopy indicated a 1.2:1 mixture of diastereomers (rotamers) A:B. U_{max} (ATR): 3393 (N-H), 1726 (C=O), 1696 (C=O), 1617 (Aryl C=C) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 7.68 (1H, d, *J* = 9.5 Hz, H-4^A and ^B), 7.46 (1H, d, *J* = 8.5 Hz, H-5^A and ^B), 7.21 - 7.11 (1H, m, H-8^A and ^B), 7.10 (1H, m, H-6^A), 7.08 (1H, m, H-6^B) 6.37 (1H, d, *J* = 9.5 Hz, H-3^A and ^B), 3.56 (2H, t, *J* = 6.0 Hz, CH₃NCH₂^B), 3.48 (2H, t, *J* = 6.1 Hz, CH₃NCH₂^A), 3.42 - 3.33 (2H, m, BocNHCH₂^A and ^B), 3.14 (3H, s, CH₃^A), 3.05 (3H, s, CH₃^B), 1.43 (9H, s, 3 x CH₃^A and ^B). ¹³C NMR (101 MHz, CDCl₃): δ_c 160.55 (C^A), 160.62 (C^B), 156.15 (C^A or ^B), 155.92 (C^A or ^B), 154.81 (C^A

and B), 154.40 (C^B), 154.16 (C^A), 154.15 (C^A), 153.90 (C^B), 143.07 (CH^A and B), 128.46 (CH^A and B), 118.71 (CH^B), 118.61 (CH^A), 116.39 (C^B), 116.31 (C^A), 115.78 (CH^B), 115.85 (CH^A), 110.62 (CH^B), 110.44 (CH^A), 79.96 (C^B), 79.68 (C^A), 49.27 (CH₂^A), 49.13 (CH₂^B), 38.80 (CH₂^B), 38.64 (CH₂^A), 35.57 (CH₃^A), 35.50 (CH₃^B), 28.52 (3 x CH₃^A), 28.50 (3 x CH₃^B). HRMS (ESI⁺): C₁₈H₂₂N₂O₆Na requires 385.1370 found 385.1367 (M + Na)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

2-Oxo-2H-chromen-7-yl N-methyl-N-(2-[(*tert*-butoxy)carbonyl]amino)ethyl)-N-methylcarbamate **174**

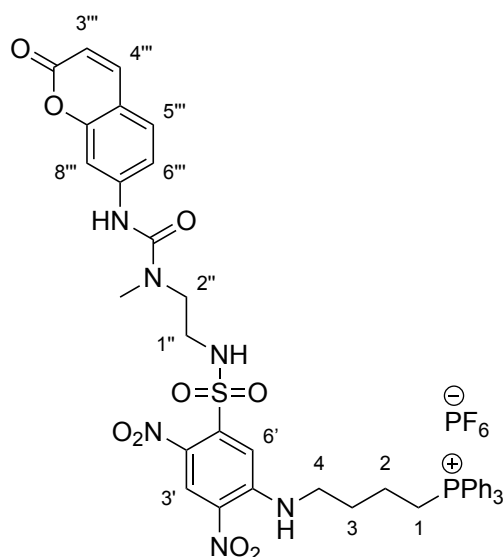


Triphosgene (61.0 mg, 0.206 mmol, 0.330 eq.) and anhydrous *N,N*-diisopropylethylamine (107.4 μ L, 0.616 mmol, 1.00 eq.) were added to a solution of 7-hydroxycoumarin (100 mg, 0.616 mmol, 1.00 eq.) in anhydrous dichloromethane (6 mL). The reaction was stirred at room temperature for 1 h under argon. A solution of *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (139 mg, 0.740 mmol, 1.20 eq.) and anhydrous *N,N*-diisopropylethylamine (129 μ L, 0.740 mmol, 1.20 eq.) in anhydrous dichloromethane (6 mL) was added to the reaction, which was stirred at room temperature for an additional 1 h under argon. The solvent was removed under reduced pressure and the solid residue was re-dissolved in dichloromethane (20 mL). The organic layer was washed with a saturated solution of NH₄Cl (20 mL), and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane:ethyl acetate 10-80%] to afford **174** as a white hygroscopic foam (115 mg, 50%).

NMR spectroscopy indicated a mixture of four diastereomers A:B:C:D, but the ¹H and ¹³C NMR spectra were too complex to differentiate between them and determine their ratio with confidence. $\nu_{\max}$ (ATR): 1724 (C=O), 1685 (C=O) cm⁻¹. ¹H NMR (400 MHz, CO(CD₃)₂): δ_{H} 7.97 (1H, d, *J* = 9.6 Hz, H-4^{A, B, C and D}), 7.68 (1H, d, *J* = 8.4 Hz, H-5^{A, B, C and D}), 7.21 - 7.11 (2H, m, H-6^{A, B, C and D} and H-8^{A, B, C and D}), 6.37 (1H, d, *J* = 9.6 Hz, H-3^{A, B, C and D}), 3.72 - 3.39

[4H, m, BocNCH₂^{A, B, C and D} and CO₂N(CH₃)CH₂^{A, B, C and D}], 3.20 – 2.99 (3H, m, BocNCH₃^{A, B, C and D} or O₂CNCH₃^{A, B, C and D}), 2.98 – 2.85 (3H, m, BocNCH₃^{A, B, C and D} or O₂CNCH₃^{A, B, C and D}), 1.50 – 1.40 (9H, m, 3 x CH₃^{A, B, C and D}). HRMS (ESI⁺): C₁₉H₂₄N₂O₆Na requires 399.1527 found 399.1530 (M + Na)⁺. Assignment of ¹H NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The ¹³C NMR spectra was too complex to interpret with confidence.

[4-({5'-[(2''-{Methyl[(2'''-oxo-2H-chromen-7'''-yl)carbamoyl]amino}ethyl)sulfamoyl]-2,4-dinitrophenyl}amino)butyl]triphenylphosphonium hexafluorophosphate (MitoACoum1) 175

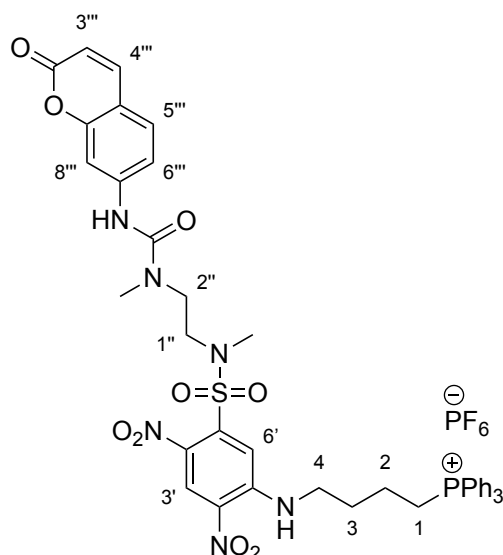


Anhydrous phosgene solution (15% wt.% in toluene) (89 µL, 0.124, 1.10 eq.) and triethylamine (31.7 µL, 0.226 mmol, 2.00 eq.) were added to a solution of **104** (18.2 mg, 0.113 mmol, 1.00 eq.) in anhydrous dichloromethane (1.1 mL) and the reaction was stirred at 0 °C for 1 h under argon. The solvent was removed under reduced pressure. To the solid residue was added a solution of **181** (88.3 mg, 0.113, 1.00 eq.) in anhydrous acetonitrile (1.1 mL), followed by triethylamine (31.7 µL, 0.226 mmol, 2.00 eq.) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the solid residue was re-dissolved in dichloromethane (15 mL). The organic layer was washed with an aqueous solution of HCl (15 mL, 1 M) and the aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The

crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-30%] to afford the **175** as a yellow hygroscopic foam (20.7 mg, 19%).

$\nu_{\max}$ (ATR): 1615 (C=O) cm⁻¹. ¹H NMR (400 MHz, CD₃CN): δ_{H} 8.61 (1H, s, H-3'), 8.42 (1H, t, J = 5.8 Hz, CH₂NH), 7.89 - 7.78 (3H, m, Ph), 7.74 (1H, d, J = 9.5 Hz, H-4'''), 7.72 - 7.59 (12H, m, Ph), 7.51 (1H, s, H-6'), 7.48 (1H, d, J = 2.1 Hz, H-8'''), 7.36 (1H, d, J = 8.5 Hz, H-5'''), 7.19 (1H, s, OCNH), 7.12 (1H, dd, J = 8.5, 2.1 Hz, H-6'''), 6.47 (1H, t, J = 4.8 Hz, O₂SNH), 6.21 (1H, d, J = 9.4 Hz, H-3'''), 3.53 (2H, q, J = 6.6 Hz, CH₂-4), 3.47 - 3.40 (4H, m, CH₂-1'' and CH₂-2''), 3.32 - 3.18 (2H, m, CH₂-1), 2.90 (3H, s, CH₃), 1.90 (2H, m, CH₂-2), 1.82 - 1.69 (2H, m, CH₂-3). ¹³C NMR (101 MHz, CD₃CN): δ_{C} 161.79 (C), 155.96 (C), 155.75 (C), 148.03 (C), 144.61 (CH), 144.55 (C), 141.66 (C), 136.16 (d, J = 3.1 Hz, 3 x CH), 134.64 (d, J = 10.0 Hz, 6 x CH), 134.15 (C), 131.27 (d, J = 12.6 Hz, 6 x CH), 131.24 (C), 129.27 (CH), 127.90 (CH), 119.05 (d, J = 86.6 Hz, 3 x C), 118.25 (CH), 115.98 (CH), 114.59 (C), 114.38 (CH), 106.28 (CH), 49.26 (CH₂), 43.08 (CH₂), 42.72 (CH₂), 35.90 (CH₃), 29.82 (d, J = 16.9 Hz, CH₂), 22.38 (d, J = 52.2 Hz, CH₂), 20.47 (d, J = 3.8 Hz, CH₂). ³¹P NMR (162 MHz, CD₃CN): δ_{P} 23.64 (1P, s, PPh₃), -144.62 (1P, sept, J = 706.3 Hz, PF₆). ¹⁹F NMR (377 MHz, CD₃CN): δ_{F} -72.91 (6F, d, J = 707.3 Hz, PF₆). HRMS (ESI⁺): C₄₁H₄₀N₆O₉PS requires 823.2310 found 823.2314 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

[4-({5'-[Methyl(2''-{methyl[(2'''-oxo-2H-chromen-7'''-yl)carbamoyl]amino}ethyl)sulfamoyl]-2,4-dinitrophenyl}amino)butyl]triphenylphosphonium hexafluorophosphate (MitoACoum2) 176

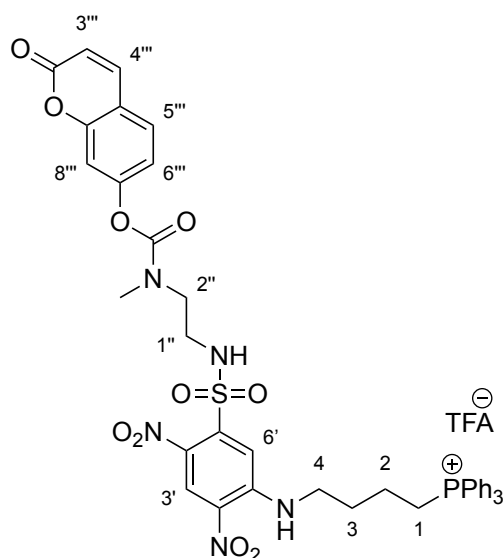


Triphosgene (6.1 mg, 0.0207 mmol, 0.333 eq.) and anhydrous *N,N*-diisopropylethylamine (10.8 μ L, 0.0621 mmol, 1.00 eq.) were added to a solution of **104** (10.0 mg, 0.0621 mmol, 1.00 eq.) in anhydrous dichloromethane (0.6 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of **182** (55.5 mg, 0.0621 mmol, 1.00 eq.) in anhydrous dichloromethane (0.6 mL) was added, followed by anhydrous *N,N*-diisopropylethylamine (21.6 μ L, 0.124 mmol, 2.00 eq.), and the reaction was stirred at room temperature for an additional 24 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-30%] to afford **176** as a yellow hygroscopic foam (16.8 mg, 29 %).

U_{max} (ATR): 1727 (C=O), 1614 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 8.63 (1H, s, H-3'), 8.22 (1H, t, $J = 5.8$ Hz, CH_2NH), 7.87 - 7.80 (3H, m, Ph), 7.76 (1H, d, $J = 9.5$, H-3'''), 7.72 - 7.64 (12H, m, Ph), 7.60 (1H, d, $J = 2.1$ Hz, H-8'''), 7.39 (1H, d, $J = 8.5$ Hz, H-5'''), 7.36 (1H, s, H-6'), 7.19 (1H, s, CONH), 7.15 (1H, dd, $J = 8.5, 2.1$ Hz, H-6'''), 6.21 (1H, d, $J = 9.5$ Hz, H-4'), 3.61 - 3.51 (4H, m, CH_2 -1'' and CH_2 -2''), 3.48 (2H, q, $J = 6.5$ Hz, CH_2 -4), 3.28 - 3.18 (2H, m, CH_2 -1), 3.07 (3H, s, O_2SNCH_3 or OCNCH_3), 2.96 (3H, s,

O₂SNCH₃ or OCNCH₃), 1.87 (2H, qn, *J* = 7.0 Hz, CH₂-2), 1.80 - 1.68 (2H, m, CH₂-3). ¹³C NMR (101 MHz, CD₃CN): δ_c 161.88 (C), 155.81 (C), 155.78 (C), 147.34 (C), 144.73 (C), 144.71 (CH), 140.67 (C), 136.16 (d, *J* = 3.1 Hz, 3 x CH), 135.02 (C), 134.64 (d, *J* = 10.1 Hz, 6 x CH), 131.39 (C), 131.27 (d, *J* = 12.6 Hz, 6 x CH), 129.28 (CH), 126.94 (CH), 119.48 (d, *J* = 85.7 Hz, 3 x C), 118.86 (CH), 116.18 (CH), 114.57 (C), 114.30 (CH), 106.44 (CH), 48.97 (CH₂), 47.53 (CH₂), 43.02 (CH₂), 36.56 (CH₃), 35.59 (CH₃), 29.90 (d, *J* = 17.0 Hz, CH₂), 22.39 (d, *J* = 52.1 Hz, CH₂), 20.49 (d, *J* = 3.8 Hz, CH₂). ³¹P NMR (162 MHz, CD₃CN): δ_p 23.62 (1P, s, PPh₃), -144.63 (1P, sept, *J* = 706.0 Hz, PF₆). ¹⁹F NMR (377 MHz, CD₃CN): δ_F -72.93 (6F, d, *J* = 707.4 Hz, PF₆). HRMS (ESI⁺): C₄₂H₄₂N₆O₉PS requires 837.2466 found 837.2477 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

(4-{[5'-({2''-[Methyl({[(2'''-oxo-2H-chromen-7'''-yl)oxy]carbonyl})amino]ethyl} sulfamoyl)-2,4-dinitrophenyl]amino}butyl)triphenylphosphonium trifluoroacetate (MitoHCoum1) 177

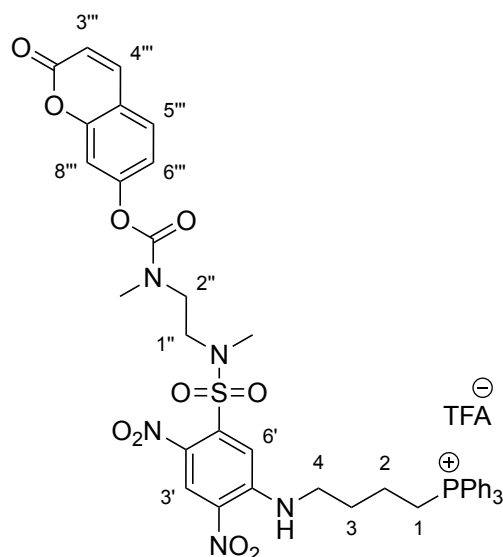


Triphosgene (15.3 mg, 0.0524 mmol, 0.333 eq.) and anhydrous *N,N*-diisopropylethylamine (26.9 µL, 0.154 mmol, 1.00 eq.) were added to a solution of 7-hydroxycoumarin (25.0 mg, 0.154 mmol, 1.00 eq.) in dichloromethane (3.0 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of **181** (113 mg, 0.154 mmol, 1.00 eq.) in anhydrous dichloromethane (3 mL) was added, followed by anhydrous *N,N*-diisopropylethylamine (53.7 µL, 0.308 mmol, 2.00 eq.), and the reaction was stirred at

room temperature for an additional 24 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by several rounds of column chromatography: first by normal phase column chromatography [SiO_2 , dichloromethane:methanol 0-20%], then by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 35-50%], and finally another round of normal phase column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **177** as a yellow hygroscopic foam (9.0 mg, 5%).

NMR spectroscopy indicated a 1.2:1 mixture of diastereomers (rotamers) A:B. U_{max} (ATR): 1719 ($\text{C}=\text{O}$), 1613 (Aryl $\text{C}=\text{C}$) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.94 (1H, t, $J = 6.0$ Hz, $\text{O}_2\text{SNH}^{\text{A}}$), 8.69 (1H, s, $\text{H}-3'^{\text{B}}$), 8.67 (1H, t, $J = 6.0$ Hz, $\text{O}_2\text{SNH}^{\text{B}}$), 8.59 (1H, s, $\text{H}-3'^{\text{A}}$), 8.48 (1H, t, $J = 5.6$ Hz, $\text{CH}_2\text{NH}^{\text{B}}$), 8.42 (1H, t, $J = 5.8$ Hz, $\text{CH}_2\text{NH}^{\text{A}}$), 8.08 (1H, s, $\text{H}-6'^{\text{A}}$), 8.02 (1H, s, $\text{H}-6'^{\text{B}}$), 7.88 - 7.61 (16H, m, $\text{Ph}^{\text{A and B}}$, and $\text{H}-4''^{\text{A and B}}$), 7.41 (1H, s, $\text{H}-8''^{\text{B}}$), 7.39 (1H, s, $\text{H}-8''^{\text{A}}$), 7.19 - 7.07 (4H, m, $\text{H}-5''^{\text{A and B}}$, and $\text{H}-6''^{\text{A and B}}$), 6.31 (1H, d, $J = 9.4$ Hz, $\text{H}-3''^{\text{B}}$), 6.27 (1H, d, $J = 9.4$ Hz, $\text{H}-3''^{\text{A}}$), 3.99 - 3.80 (4H, m, $\text{CH}_2-1^{\text{A and B}}$, and $\text{CH}_2-4^{\text{A and B}}$), 3.70 (2H, t, $J = 6.1$ Hz, $\text{CH}_2-2''^{\text{A}}$), 3.58 (2H, t, $J = 5.6$ Hz, $\text{CH}_2-2''^{\text{B}}$), 3.52 - 3.37 (4H, m, $\text{CH}_2-1''^{\text{A and B}}$), 3.22 (3H, s, CH_3^{B}), 3.08 (3H, s, CH_3^{A}), 2.05 (2H, m, $\text{CH}_2-2^{\text{A and B}}$), 1.81 (2H, m, $\text{CH}_2-3^{\text{A and B}}$). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 160.82 (C^{A}), 160.79 (C^{B}), 154.63 (C^{B}), 154.57 (C^{A}), 154.19 (C^{B}), 153.82 (C^{A}), 147.05 (C^{B}), 146.95 (C^{A}), 143.38 ($\text{CH}^{\text{A or B}}$), 143.33 ($\text{CH}^{\text{A or B}}$), 142.33 (C^{A}), 142.11 (C^{B}), 135.20 (d, $J = 3.0$ Hz, 3 x $\text{CH}^{\text{A and B}}$), 133.83 (d, $J = 10.1$ Hz, 6 x $\text{CH}^{\text{A and B}}$), 130.62 (d, $J = 12.6$ Hz, 6 x $\text{CH}^{\text{A and B}}$), 130.07 ($\text{C}^{\text{A and B}}$), 128.43 ($\text{CH}^{\text{A or B}}$), 128.40 ($\text{CH}^{\text{A or B}}$), 125.75 (CH^{B}), 125.23 (CH^{A}), 119.07 (CH^{A}), 119.04 (CH^{B}), 118.18 (d, $J = 86.1$ Hz, 3 x $\text{C}^{\text{A and B}}$), 117.71 (CH^{A}), 117.49 (CH^{B}), 116.08 (C^{A}), 116.04 (C^{B}), 115.45 ($\text{CH}^{\text{A or B}}$), 115.35 ($\text{CH}^{\text{A or B}}$), 110.85 (CH^{A}), 110.77 (CH^{B}), 50.34 (CH_2^{B}), 49.79 (CH_2^{A}), 42.65 ($\text{CH}_2^{\text{A and B}}$), 41.84 ($\text{CH}_2^{\text{A and B}}$), 36.72 (CH_3^{B}), 36.01 (CH_3^{A}), 29.41 (d, $J = 18.0$ Hz, $\text{CH}_2^{\text{A and B}}$), 22.05 (d, $J = 53.6$ Hz, $\text{CH}_2^{\text{A and B}}$), 19.55 (d, $J = 4.0$ Hz, $\text{CH}_2^{\text{A and B}}$). ^{31}P NMR (162 MHz, CD_3CN): δ_{P} 24.46 (1P, s, $\text{PPh}_3^{\text{A or B}}$), 24.45 (1P, s, $\text{PPh}_3^{\text{A or B}}$). ^{19}F NMR (377 MHz, CD_3CN): δ_{F} -75.23 (3F, s, TFA). HRMS (ESI⁺): $\text{C}_{41}\text{H}_{39}\text{N}_5\text{O}_{10}\text{PS}$ requires 824.2150 found 824.2161 (M^+). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. Two signals corresponding to two C carbons were not identified in the ^{13}C NMR spectrum, perhaps due to coincidence with other signals. The signals corresponding to the TFA⁻ anion were not observed in the ^{13}C NMR spectrum.

[4-({5-[Methyl({2-[methyl({[(2-oxo-2H-chromen-7-yl)oxy]carbonyl})amino]ethyl})sulfamoyl]-2,4-dinitrophenyl}amino)butyl] triphenylphosphonium trifluoroacetate (MitoHCoum2) **178**

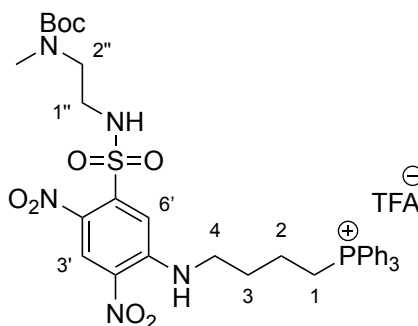


Triphosgene (15.3 mg, 0.0524 mmol, 0.333 eq.) and anhydrous *N,N*-diisopropylethylamine (26.9 μ L, 0.154 mmol, 1.00 eq.) were added to a solution of 7-hydroxycoumarin (25.0 mg, 0.154 mmol, 1.00 eq.) in anhydrous dichloromethane (1.5 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of **182** (138 mg, 0.154 mmol, 1.00 eq.) in anhydrous dichloromethane (1.5 mL) was added, followed by anhydrous *N,N*-diisopropylethylamine (53.7 μ L, 0.308 mmol, 2.00 eq.), and the reaction was stirred at room temperature for an additional 24 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%], followed by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 35-45%] to afford **178** as a yellow hygroscopic foam (27.9 mg, 22 %).

NMR spectroscopy indicated a 1.1:1 mixture of diastereomers (rotamers) A:B. U_{max} (ATR): 1719 (C=O), 1615 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 8.69 (1H, s, H-3'^A), 8.68 (1H, s, H-3'^B), 8.44 - 8.35 (1H, m, NH^{A and B}), 7.90 - 7.79 (5H, m, Ph^{A and B}), 7.74 - 7.62 (11H, m, Ph^{A and B} and H-4''^{A and B}), 7.58 (1H, d, J = 8.4 Hz, H-5''^B), 7.57 (1H, d, J = 8.4 Hz, H-5''^A), 7.37 (1H, s, H-6'^A), 7.34 (1H, s, H-6'^B), 7.15 - 7.09 (2H, m, H-8''^{A and B} and H-6''^A

or B), 7.08 (1H, dd, $J = 8.4, 2.2$ Hz, H-6''''^{A or B}), 6.33 (1H, d, $J = 9.6$ Hz, H-3''''^B), 6.32 (1H, d, $J = 9.5$ Hz, H-3''''^A), 3.69 - 3.45 (6H, m, H-4^{A and B}, H-1''^{A and B} and H-2''^{A and B}), 3.26 - 3.14 (2H, m, H-1^{A and B}), 3.10 (3H, s, O₂SNCH₃^{A or B} or O₂CNCH₃^{A or B}), 2.98 (6H, m, O₂SNCH₃^{A and B} and O₂CNCH₃^{A or B}, or, O₂SNCH₃^{A or B} and O₂CNCH₃^{A and B}), 1.92 - 1.81 (2H, m, H-2^{A and B}), 1.80 - 1.66 (2H, m, H-3^{A and B}). ¹³C NMR (101 MHz, CD₃CN): 161.23 (C^{A and B}), 155.54 (C^{A and B}), 155.21 (C^{A or B}), 155.14 (C^{A or B}), 155.08 (C^{A or B}), 154.63 (C^{A or B}), 147.45 (C^{A and B}), 144.41 (CH^{A and B}), 140.41 (C^{A or B}), 140.35 (C^{A or B}), 136.17 (d, $J = 3.1$ Hz, 6 x CH^{A and B}), 135.42 (C^{A or B}), 135.35 (C^{A or B}), 134.62 (d, $J = 10.0$ Hz, 6 x CH^{A and B}), 131.73 (C^{A and B}), 131.28 (d, $J = 12.6$ Hz, 6 x CH^{A and B}), 129.85 (CH^{A and B}), 126.98 (CH^{A and B}), 119.63 (CH^{A or B}), 119.32 (CH^{A or B}), 119.06 (d, $J = 86.4$ Hz, 6 x C^{A and B}), 118.48* (CH^{A or B}), 118.17* (CH^{A or B}), 117.46 (C^B), 117.36 (C^A), 116.43 (CH^B), 116.36 (CH^A), 111.02 (CH^{A or B}), 110.92 (CH^{A or B}), 49.42 (CH₂^B), 48.72 (CH₂^A), 47.55 (CH₂^{A or B}), 47.50 (CH₂^{A or B}), 42.94 (CH₂^{A and B}), 35.82 (CH₃^A), 35.77 (CH₃^B), 35.64 (CH₃^B), 35.47 (CH₃^A), 29.89 (d, $J = 16.8$ Hz, CH₂^{A and B}), 22.35 (d, $J = 52.2$ Hz, CH₂^B), 22.34 (d, $J = 52.2$ Hz, CH₂^A), 20.44 (CH₂^{A and B}). ³¹P NMR (162 MHz, CD₃CN): δ_P 23.62 (1P, s, PPh₃). ¹⁹F NMR (377 MHz, CD₃CN): δ_F -76.59 (3F, s, TFA). HRMS (ESI⁺): C₄₂H₄₁N₅O₁₀PS requires 838.2306 found 838.2208 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. *Signals were hidden behind the solvent signal in the ¹³C NMR spectrum, but were observed in the DEPT spectrum. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

[4-({5'-[(2''-{[*N*-Boc](methyl)amino}ethyl)sulfamoyl]-2,4-dinitrophenyl}amino)butyl]triphenylphosphonium trifluoroacetate 179

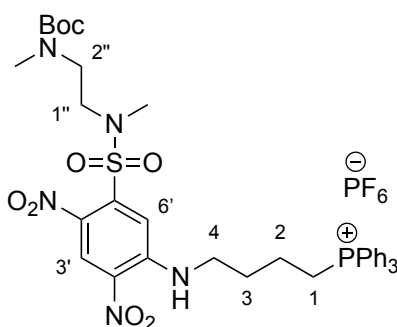


A solution of *N*-*boc*-*N*-methylethylenediamine (11.7 mg, 0.0672 mmol, 1.00 eq.) and triethylamine (18.8 μL, 0.134 mmol, 2.00 eq.) in anhydrous dichloromethane (0.4 mL) was added to a solution of **124** (50.0 mg, 0.0672 mmol, 1.00 eq.) in anhydrous dichloromethane (0.4 mL) and the reaction was stirred at room temperature for 3 h under

argon. The reaction was diluted with dichloromethane (10 mL) and washed with an aqueous solution of HCl (10 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%], followed by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 30-70%] to obtain **179** as the trifluoroacetate salt as a yellow hygroscopic foam (18.9 mg, 33%).

U_{max} (ATR): 1683 (C=O), 1614 (Aryl C=C) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 8.87 (1H, s, H-3'), 8.58 (1H, t, *J* = 5.5 Hz, NH), 7.82 (3H, m, Ph), 7.75 - 7.59 (12H, m, Ph), 7.57 (1H, s, H-6'), 3.65 - 3.55 (2H, m, CH₂-4), 3.45 - 3.33 (4H, m, CH₂-1 and CH₂-2''), 3.27 (2H, q, *J* = 5.5 Hz, CH₂-1''), 2.83 (3H, s, NHCH₃), 2.09 - 2.01 (2H, m, CH₂-2), 1.88 - 1.76 (2H, m, CH₂-3), 1.42 (9H, s, 3 x CH₃). ¹³C NMR (101 MHz, CDCl₃): δ_C 160.19 (C), 146.94 (C), 140.97 (C), 135.57 (d, *J* = 3.0 Hz, 3 x CH), 134.13 (C), 133.47 (d, *J* = 10.0 Hz, 6 x CH), 130.78 (d, *J* = 12.6 Hz, 6 x CH), 130.57 (C), 127.21 (CH), 117.74 (d, *J* = 86.3 Hz, 3 x C), 117.59 (CH), 48.21 (CH₂), 42.36 (2 x CH₂), 35.19 (CH₃), 29.20 (d, *J* = 17.0 Hz, CH₂), 28.43 (3 x CH₃), 22.12 (d, *J* = 52.4 Hz, CH₂), 19.81 (d, *J* = 3.8 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 23.72 (1P, s, PPh₃). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -75.76 (3F, s, TFA). HRMS (ESI⁺): C₃₆H₄₃N₅O₈PS requires 736.2564 found 736.2589 (*M*⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signal corresponding to C(*t*-Bu) was not identified in the ¹³C NMR spectrum, perhaps due to coincidence with the solvent signal. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

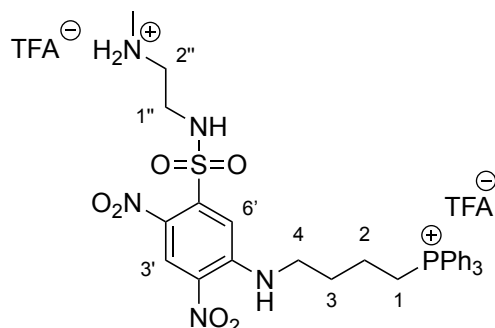
[4-({5'-[(2''-[(*tert*-butoxy)carbonyl](methyl)amino}ethyl)(methyl)sulfamoyl]-2',4'-dinitrophenyl}amino)butyl]triphenylphosphonium hexafluorophosphate **180**



Tert-butyl methyl(2-(methylamino)ethyl)carbamate (97.7 mg, 0.519 mmol, 0.900 eq.) and triethylamine (162 μ L, 1.15 mmol, 2.00 eq.) were added to a solution of **124** (429 mg, 0.576 mmol, 1.00 eq) in anhydrous dichloromethane (5.8 mL) and the reaction was stirred at room temperature for 3 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (3 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **180** as an orange hygroscopic foam (303 mg, 59%).

NMR spectroscopy indicated a 1:1 mixture of diastereomers (rotamers) A:B. U_{max} (ATR): 1685 (Aryl C=C), 1616 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.61 (1H, s, H-3'^A and B), 8.40 (1H, t, J = 5.3 Hz, $\text{NH}^{\text{A and B}}$), 7.84 - 7.59 (15H, m, $\text{Ph}^{\text{A and B}}$), 7.37 (1H, s, H-6'^A or B), 7.35 (1H, s, H-6'^A or B), 3.52 (2H, q, J = 6.2 Hz, $\text{CH}_2\text{-4}^{\text{A and B}}$), 3.49 - 3.37 (4H, m, $\text{CH}_2\text{-1}''^{\text{A and B}}$ and $\text{CH}_2\text{-2}''^{\text{A and B}}$), 3.31 - 3.20 (2H, m, $\text{CH}_2\text{-1}^{\text{A and B}}$), 2.98 (3H, s, $\text{O}_2\text{SNCH}_3^{\text{A and B}}$ or $\text{BocNCH}_3^{\text{A and B}}$), 2.84 (3H, s, $\text{O}_2\text{SNCH}_3^{\text{A and B}}$ or $\text{BocNCH}_3^{\text{A and B}}$), 2.05 (2H, qn, J = 7.7 Hz, $\text{CH}_2\text{-2}^{\text{A and B}}$), 1.90 - 1.74 (2H, m, $\text{CH}_2\text{-3}^{\text{A and B}}$), 1.43 (9H, s, 3 x $\text{CH}_3^{\text{A or B}}$), 1.41 (9H, s, 3 x $\text{CH}_3^{\text{A or B}}$). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 155.99 ($\text{C}^{\text{A or B}}$), 155.61 ($\text{C}^{\text{A or B}}$), 146.49 ($\text{C}^{\text{A and B}}$), 140.50 ($\text{C}^{\text{A or B}}$), 140.40 ($\text{C}^{\text{A or B}}$), 135.48 (d, J = 3.0 Hz, 3 x $\text{CH}^{\text{A and B}}$), 134.72 ($\text{C}^{\text{A and B}}$), 133.49 (d, J = 9.9 Hz, 6 x $\text{CH}^{\text{A and B}}$), 130.79 (d, J = 12.6 Hz, 6 x $\text{CH}^{\text{A and B}}$), 130.33 ($\text{C}^{\text{A and B}}$), 126.04 ($\text{CH}^{\text{A and B}}$), 117.76 ($\text{CH}^{\text{A or B}}$), 117.71 (d, J = 86.0 Hz, 3 x $\text{C}^{\text{A and B}}$), 117.62 ($\text{CH}^{\text{A or B}}$), 79.98 ($\text{C}^{\text{A or B}}$), 79.65 ($\text{C}^{\text{A or B}}$), 48.62 ($\text{CH}_2^{\text{A or B}}$), 48.10 ($\text{CH}_2^{\text{A or B}}$), 47.08 ($\text{CH}_2^{\text{A or B}}$), 46.30 ($\text{CH}_2^{\text{A or B}}$), 42.46 ($\text{CH}_2^{\text{A and B}}$), 35.39 ($\text{CH}_3^{\text{A or B}}$), 35.24 ($\text{CH}_3^{\text{A or B}}$), 34.89 ($\text{CH}_3^{\text{A or B}}$), 34.49 ($\text{CH}_3^{\text{A or B}}$), 29.25 (d, J = 16.8 Hz, $\text{CH}_2^{\text{A and B}}$), 28.49 (3 x $\text{CH}_3^{\text{A and B}}$), 22.07 (d, J = 52.6 Hz, $\text{CH}_2^{\text{A and B}}$), 19.92 (d, J = 3.5 Hz, $\text{CH}_2^{\text{A and B}}$). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 23.63 (1P, s, PPh_3), -144.38 (sept, J = 713.2 Hz, PF_6). HRMS (ESI^+): $\text{C}_{37}\text{H}_{45}\text{N}_5\text{O}_8\text{PS}$ requires 750.2721 found 750.2734 (M^+). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

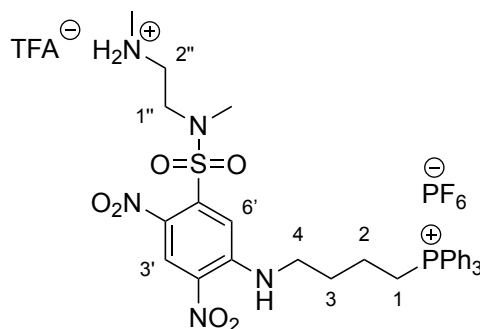
{4-[(5'-{[2''-(Methylamino)ethyl]sulfamoyl}-2',4'-dinitrophenyl)amino]butyl}triphenylphosphonium bistrifluoroacetate **181**



Trifluoroacetic acid (0.9 mL) was added to a solution of **179** (100 mg, 0.120 mmol, 1.00 eq.) in anhydrous dichloromethane (0.9 mL) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure and the crude material purified by column chromatography [SiO_2 , dichloromethane:methanol 0-30%] to afford **181** as a yellow hygroscopic foam (79.8 mg, 91%).

U_{max} (ATR): 1671 (Aryl C=C), 1616 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 9.52 (2H, br s, CH_3NH_2), 8.75 (1H, s, H-3'), 8.49 (2H, t, $J = 5.9$ Hz, PhNH), 8.27 (1H, br s, O_2SNH), 7.89 - 7.79 (3H, m, Ph), 7.75 - 7.64 (12H, m, Ph), 7.58 (1H, s, H-6'), 3.60 (2H, q, $J = 6.5$ Hz, CH_2 -4), 3.46 (2H, t, $J = 5.6$ Hz, CH_2 -1''), 3.34 - 3.22 (2H, m, CH_2 -1), 3.14 (2H, t, $J = 5.6$ Hz, CH_2 -2''), 2.61 (3H, s, CH_3), 1.90 (2H, m, CH_2 -2), 1.82 - 1.67 (2H, m, CH_2 -3). ^{13}C NMR (101 MHz, CD_3CN): δ_{C} 148.02 (C), 141.23 (C), 136.12 (d, $J = 3.0$ Hz, 3 x CH), 135.08 (C), 134.64 (d, $J = 10.1$ Hz, 6 x CH), 131.57 (C), 131.25 (d, $J = 12.6$ Hz, 6 x CH), 127.48 (CH), 119.16 (d, $J = 86.5$ Hz, 3 x C), 50.10 (CH_2), 42.89 (CH_2), 40.80 (CH_2), 33.73 (CH_3), 29.92 (d, $J = 16.9$ Hz, CH_2), 22.25 (d, $J = 52.0$ Hz, CH_2), 20.33 (d, $J = 3.8$ Hz, CH_2). ^{31}P NMR (162 MHz, CD_3CN): δ_{P} 23.77 (1P, s, PPh_3). ^{19}F NMR (377 MHz, CD_3CN): δ_{F} -75.85 (6F, s, 2 x TFA). HRMS (ESI $^+$): $\text{C}_{31}\text{H}_{35}\text{N}_5\text{O}_6\text{PS}$ requires 636.2040 found 636.2066 (M^+). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA^- anions were not observed in the ^{13}C NMR spectrum.

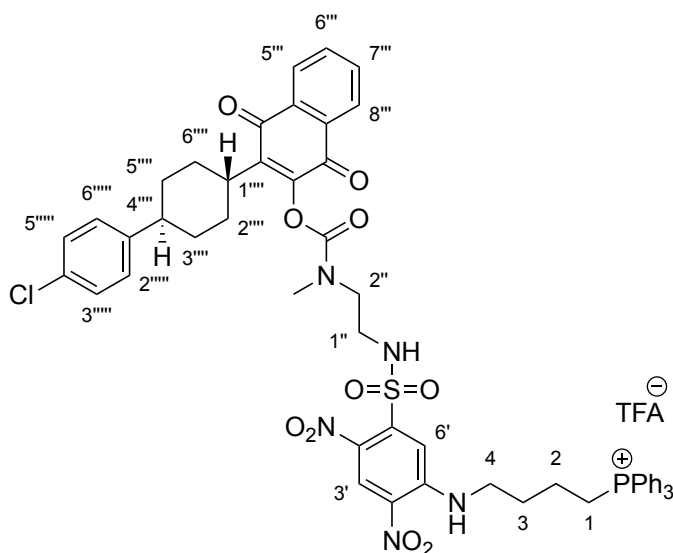
{4-[(5'-{Methyl[2''-(methyldiazaniumyl)ethyl]sulfamoyl}-2',4'-dinitrophenyl)amino]butyl}triphenylphosphonium trifluoroacetate hexafluorophosphate **182**



Trifluoroacetic acid (1.7 mL) was added to a solution of **180** (303 mg, 0.338 mmol, 1.00 eq.) in anhydrous dichloromethane (1.7 mL) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure to afford **182** as a yellow hygroscopic foam (286 mg, quant.).

$U_{\max}$ (ATR): 1616 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 8.70 (1H, s, H-3'), 8.43 (1H, t, $J = 5.9$ Hz, PhNH), 7.92 - 7.82 (3H, m, Ph), 7.73 - 7.63 (12H, m, Ph), 7.55 (2H, s, CH_3NH_2), 7.29 (1H, s, H-6'), 3.61 (2H, t, $J = 5.7$ Hz, CH_2 -1''), 3.55 (2H, q, $J = 6.3$ Hz, CH_2 -2''), 3.27 - 3.15 (4H, m, CH_2 -1 and CH_2 -4), 3.03 (2H, s, O_2SNCH_3), 2.70 (3H, t, $J = 5.4$ Hz, NH_2CH_3), 1.92 - 1.85 (2H, m, CH_2 -2), 1.75 (2H, qn, $J = 8.1$ Hz, CH_2 -3). ^{13}C NMR (101 MHz, CD_3CN): δ_{C} 160.07 (q, $J = 37.6$ Hz, C), 147.51 (C), 139.50 (C), 136.19 (d, $J = 3.0$ Hz, 3 x CH), 135.44 (C), 134.64 (d, $J = 10.0$ Hz, 6 x CH), 131.94 (C), 131.28 (d, $J = 12.6$ Hz, 6 x CH), 127.07 (CH), 119.09 (d, $J = 86.5$ Hz, 3 x C), 118.20 (CH), 47.48 (CH_2), 47.43 (CH_2), 42.90 (CH_2), 36.31 (CH_3), 34.33 (CH_3), 29.84 (d, $J = 17.0$ Hz, CH_2), 22.35 (d, $J = 52.2$ Hz, CH_2), 20.41 (d, $J = 3.8$ Hz, CH_2). ^{31}P NMR (162 MHz, CDCl_3): δ_{P} 23.65 (1P, s, PPh_3), -144.62 (1P, sept, $J = 707.6$ Hz, PF_6). ^{19}F NMR (377 MHz, CD_3CN): δ_{F} -72.88 (6F, d, $J = 707.6$ Hz, PF_6), -76.67 (3F, s, TFA). HRMS (ESI $^{+/-}$): $\text{C}_{32}\text{H}_{38}\text{N}_5\text{O}_6\text{PS}$ requires 651.2269 found 586.2587 corresponding to the aniline $\text{C}_{32}\text{H}_{37}\text{N}_5\text{O}_4\text{P}$ likely due to fragmentation during ionisation. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signal corresponding to CF_3COO^- of the TFA $^-$ anion was not observed in the ^{13}C NMR spectrum.

[4-({5'-[(2''-[(1''',4'''-Dioxo-3'''-[(1r,4r)-4''''-(4'''''-chlorophenyl)cyclohexyl]-1''',4'''-dihydronaphthalen-2''-yl]oxy)carbonyl](methyl)amino}ethyl)sulfamoyl]-2',4'-dinitrophenyl]amino)butyl]triphenylphosphonium trifluoroacetate (**MitoAtov1**)
187

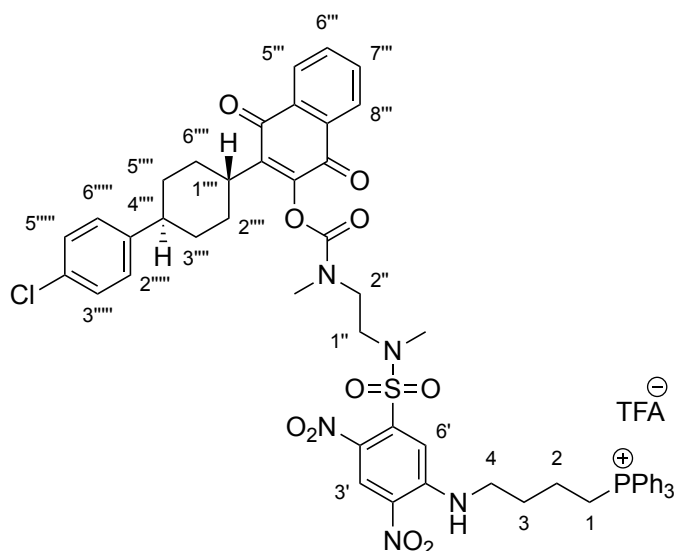


Triphosgene (13.5 mg, 0.0450 mmol, 0.333 eq.) and anhydrous *N,N*-diisopropylethylamine (23.7 μ L, 0.136 mmol, 1.00 eq.) were added to a solution of atovaquone (50.0 mg, 0.136 mmol, 1.00 eq.) in anhydrous dichloromethane (1.5 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of **181** (100 mg, 0.136 mmol, 1.00 eq.) in anhydrous dichloromethane (1.5 mL) was added, followed by anhydrous *N,N*-diisopropylethylamine (47.5 μ L, 0.273 mmol, 2.00 eq.) and the reaction was stirred at room temperature for an additional 24 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-20%] to afford **187** as a yellow hygroscopic foam (24.3 mg, 16%).

NMR spectroscopy indicated a 1:1 mixture of diastereomers (rotamers) A:B. U_{max} (ATR): 1725 (C=O), 1671 (C=O), 1614 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 9.32 (1H, t, $J = 5.4$ Hz, $\text{O}_2\text{SNH}^{\text{A or B}}$), 9.12 (1H, t, $J = 5.7$ Hz, $\text{O}_2\text{SNH}^{\text{A or B}}$), 8.67 (1H, s, H-3' $^{\text{A or B}}$), 8.55 (1H, s, H-3' $^{\text{A or B}}$), 8.50 (1H, t, $J = 6.0$ Hz, $\text{PhNH}^{\text{A or B}}$), 8.43 (1H, t, $J = 5.9$ Hz, $\text{PhNH}^{\text{A or B}}$), 8.06 (1H, m, H-5'' $^{\text{A and B}}$ or H-8'' $^{\text{A and B}}$), 8.00 (1H, s, H-6' $^{\text{A or B}}$), 7.98 (1H, s, H-6' $^{\text{A or B}}$), 7.95

- 7.89 (1H, m, H-5''''A and B or H-8''''A and B), 7.86 - 7.56 (17H, m, H-6''''A and B, H-7''''A and B, Ph^A and B), 7.31 - 7.12 (4H, m, H-2''''A and B, H-3''''A and B, H-5''''A and B H-6''''A and B), 3.88 - 3.62 (4H, m, CH₂-4^A and B and CH₂-2''A and B), 3.62 - 3.47 (2H, m, CH₂-1^A and B), 3.39 (2H, q, *J* = 6.5 Hz, CH₂-1''A or B), 3.27 - 3.17 (5H, m, CH₃^A or B and CH₂-1''A or B) 3.19 - 3.03 (1H, m, H-1''''A and B), 2.99 (3H, s, CH₃^A or B), 2.66 (1H, tt, *J* = 12.3, 3.2 Hz H-4''''A or B), 2.56 (1H, tt, *J* = 12.3, 3.2 Hz H-4''''A or B), 1.92 - 1.65 (8H, m, CH₂-2^A and B, CH₂-3^A and B, H-2''''A and B, ax and eq, H-6''''A and B, ax and eq, H-3''''A and B, ax or eq and H-5''''A and B, ax or eq), 1.61 - 1.43 (2H, m, H-3''''A and B, ax or eq and H-5''''A and B, ax or eq). ¹³C NMR (101 MHz, CD₃CN): δ_C 185.63 (C^A or B), 185.60 (C^A or B), 180.47 (C^A or B), 180.34 (C^A or B), 154.12 (C^A or B), 153.79 (C^A or B), 152.38 (C^A or B), 152.27 (C^A or B), 148.17 (C^A or B), 148.15 (C^A or B), 147.58 (C^A or B), 147.48 (C^A or B), 142.52 (C^A or B), 142.44 (C^A or B), 142.02 (C^A or B), 141.90 (C^A or B), 135.96 (d, *J* = 3.2 Hz, 6 x CH^A and B), 135.63 (C^A and B), 135.42 (CH^A or B), 135.35 (CH^A or B), 134.86 (CH^A and B), 134.82 (d, *J* = 10.4 Hz, 6 x CH^A or B), 134.78 (d, *J* = 10.0 Hz, 6 x CH^A or B), 133.42 (C^A or B), 133.38 (C^A or B), 131.85 (C^A and B), 131.57 (C^A or B), 131.45 (C^A or B), 131.31 (C^A or B), 131.23 (d, *J* = 12.6 Hz, 6 x CH^A or B), 131.22 (d, *J* = 12.6, 6 x CH^A or B), 130.99 (C^A or B), 129.62 (CH^A or B), 129.47 (CH^A or B), 129.15 (CH^A or B), 129.12 (CH^A or B), 127.53 (CH^A and B), 127.02 (CH^A or B), 126.90 (CH^A or B), 126.78 (CH^A or B), 126.76 (CH^A or B), 119.32 (d, *J* = 86.3 Hz, 6 x C^A and B), 117.94 (CH^A or B), 117.71 (CH^A or B), 50.46 (CH₂^A or B), 50.02 (CH₂^A or B), 43.78 (CH^A or B), 43.70 (CH^A or B), 43.58 (CH₂^A or B), 43.55 (CH₂^A or B), 42.54 (CH₂^A or B), 42.09 (CH₂^A or B), 36.56 (CH^A or B), 36.34 (CH^A or B), 36.21 (CH₃^A or B), 36.20 (CH₃^A or B), 34.89 (CH₂^A or B), 34.79 (CH₂^A or B), 30.69 (CH₂^A and B), 30.02 (d, *J* = 16.9 Hz, CH₂^A and B), 22.04 (d, *J* = 51.6 Hz, CH₂^A or B), 21.93 (d, *J* = 51.2 Hz, CH₂^A or B), 20.33 (d, *J* = 3.2 Hz, CH₂^A and B). ³¹P NMR (162 MHz, CD₃CN): δ_P 23.45 (1P, s, PPh₃^A or B), 23.40 (1P, s, PPh₃^A or B). ¹⁹F NMR (377 MHz, CD₃CN): δ_F -74.86 (3F, s, TFA). HRMS (ESI⁺): C₅₄H₅₂ClN₅O₁₀PS requires 1028.2856 found 1028.2863 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

[4-({5'-[(2''-[(1''',4'''-Dioxo-3'''-[(1*r*,4*r*)-4''''-(4'''''-chlorophenyl)cyclohexyl]-1''',4'''-dihydronaphthalen-2''-yl]oxy)carbonyl](methyl)amino}ethyl)(methyl)sulfamoyl]-2',4'-dinitrophenyl]amino)butyl]triphenylphosphonium trifluoroacetate (MitoAtov2) **188**

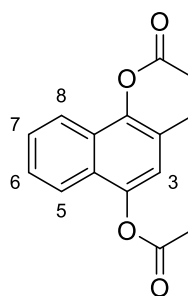


Triphosgene (6.2 mg, 0.0207 mmol, 0.333 eq.) and anhydrous *N,N*-diisopropylethylamine (10.8 μ L, 0.622 mmol, 1.00 eq.) were added to a solution of atovaquone (22.8 mg, 0.0622 mmol, 1.00 eq.) in anhydrous dichloromethane (0.6 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of **182** (52.6 mg, 0.0622 mmol, 1.00 eq.) in anhydrous dichloromethane (0.6 mL) was added, followed by anhydrous *N,N*-diisopropylethylamine (21.7 μ L, 0.124 mmol, 2.00 eq.), and the reaction was stirred at room temperature for an additional 24 h under argon. The reaction was diluted with dichloromethane (15 mL) and washed with an aqueous solution of HCl (15 mL, 1 M). The aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane: methanol 0-20%] to afford **188** as a yellow hygroscopic foam (22.9 mg, 32 %).

NMR spectroscopy indicated a 1.3:1 mixture of diastereomers (rotamers) A:B. ν_{max} (ATR): 1726 (C=O), 1672 (C=O), 1613 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 8.68 (1H, s, H-3'^A), 8.65 (1H, s, H-3'^B), 8.53 (1H, m, NH^{A and B}), 8.12 - 8.05 (1H, m, H-8''''^{A and B}), 7.99 (1H, m, H-5''''^{A and B}), 7.87 - 7.59 (18H, m, H-6'^{A and B}, H-6''''^{A and B}, H-7''''^{A and B} and Ph^{A and B}), 7.33 - 7.10 (4H, m, H-2''''^{A and B}, H-3''''^{A and B}, H-5''''^{A and B} and H-6''''^{A and B}), 3.91 - 3.31

(8H, m, CH₂-1^A and B, CH₂-4^A and B, CH₂-2''^A and B and CH₂-1''^A and B), 3.22 (3H, s, O₂SNCH₃^A or O₂CNCH₃^A), 3.18 - 3.09 (2H, m, H-1''''^A and B), 3.06 (3H, s, O₂SNCH₃^B or O₂CNCH₃^B), 3.04 (3H, s, O₂SNCH₃^B or O₂CNCH₃^B), 3.00 (3H, s, O₂SNCH₃^A or O₂CNCH₃^A), 2.73 - 2.55 (1H, m, H-4''''^A and B), 2.11 - 1.97 (2H, m, H-2''''^A and B, ax or eq, H-6''''^A and B, ax or eq), 1.92 - 1.71 (6H, m, CH₂-2^A and B, H-2''''^A and B, ax or eq, H-3''''^A and B, ax or eq, H-5''''^A and B, ax or eq, H-6''''^A and B, ax or eq), 1.66 (2H, m, CH₂-3^A and B), 1.54 (2H, m, H-3''''^A and B, ax or eq and H-5''''^A and B, ax or eq). ¹³C NMR (101 MHz, CD₃CN): δ_C 185.28 (C^A and B), 180.11 (C^B), 179.99 (C^A), 153.84 (C^A), 153.49 (C^B), 151.99 (C^A), 151.83 (C^B), 147.27 (C^A or B), 147.04 (C^A or B), 147.02 (C^A or B), 146.97 (C^A or B), 142.21 (C^B), 142.12 (C^A), 140.04 (C^B), 139.98 (C^A), 135.66 (d, *J* = 3.0 Hz, 3 x CH^A and B), 135.09 (CH^A), 134.95 (CH^B), 134.90 (C^A and B), 134.50 (CH^B), 134.47 (CH^A), 134.28 (d, *J* = 9.9 Hz, 6 x CH^B), 134.26 (d, *J* = 9.9 Hz, 6 x CH^A), 133.10 (C^A or B), 133.04 (C^A or B), 131.46 (C^A or B), 131.39 (C^A or B), 131.36 (C^A or B), 131.26 (C^A or B), 131.22 (C^A or B), 131.01 (C^A or B), 130.81 (d, *J* = 12.6 Hz, 6 x CH^A and B), 129.15 (CH^A), 128.99 (CH^B), 128.74 (CH^A and B), 127.13 (CH^B), 127.11 (CH^A), 126.70 (CH^B), 126.48 (CH^A), 126.39 (CH^A and B), 118.80 (d, *J* = 86.4 Hz, 3 x C^B), 118.76 (d, *J* = 86.4 Hz, 3 x C^A), 117.43 (CH^A), 117.09 (CH^B), 49.21 (CH₂^B), 48.41 (CH₂^A), 48.05 (CH₂^B), 46.99 (CH₂^A), 43.30 (CH^A), 43.24 (CH^B), 42.41 (CH₂^B), 42.32 (CH₂^A), 36.10 (CH^A and B), 35.77 (CH₃^A or B), 35.59 (CH₃^A or B), 35.42 (CH₃^A or B), 35.17 (CH₃^A or B), 34.44 (CH₂^A and B), 30.24 (CH₂^A and B), 29.19 (d, *J* = 17.0 Hz, CH₂^A and B), 21.89 (d, *J* = 51.5 Hz, CH₂^B), 21.85 (d, *J* = 51.5 Hz, CH₂^A), 19.91 (d, *J* = 3.9 Hz, CH₂^A or B), 19.87 (d, *J* = 3.9 Hz, CH₂^A or B). ³¹P NMR (162 MHz, CD₃CN): δ_P 23.81 (1P, s, PPh₃^B), 23.76 (1P, s, PPh₃^A). ¹⁹F NMR (377 MHz, CD₃CN): δ_F -75.23 (3F, s, TFA). HRMS (ESI⁺): C₅₅H₅₄N₅O₁₀PS requires 1042.3012 found 1042.3033 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

2-Methyl-1,4-naphthohydroquinone diacetate 189

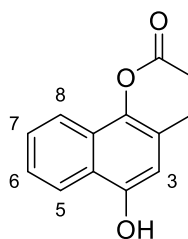


Following the patent procedure by Sunny Pharmtech,²⁷¹ Ac₂O (2.75 mL, 29.1 mmol, 2.50 eq.), DMAP (142 mg, 1.16 mmol, 0.100 mmol) and Pd/C (62.0 mg, 0.580 mmol, 0.0500

eq.) were added to a solution of menadione (2.00 g, 11.6 mmol, 1.00 eq.) in ethyl acetate. The reaction was stirred at room temperature for 24 h under H₂. The reaction was filtered through celite under vacuum and washed with ethyl acetate (50 mL). The filtrate was washed with brine (2 x 50 mL). The organic layer was separated, dried with MgSO₄ and the solvent removed under reduced pressure to afford **189** as a white solid (2.81 g, 94%).

Mp: 108 - 110 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 7.84 - 7.70 (2H, m, H-6 and H-7), 7.56 - 7.41 (2H, m, H-5 and H-8), 7.15 (1H, s, H-3), 2.48 (3H, s, O₂CCH₃), 2.46 (3H, s, O₂CCH₃), 2.33 (3H, s, PhCH₃). ¹³C NMR (101 MHz, CDCl₃): δ_C 169.57 (C), 169.04 (C), 144.26 (C), 142.28 (C), 128.05 (C), 127.24 (CH), 126.65 (C), 126.31 (C), 126.18 (CH), 121.61 (CH), 121.31 (CH), 120.87 (CH), 21.15 (CH₃), 20.74 (CH₃), 16.66 (CH₃). HRMS (ESI⁺): C₁₅H₁₈NO₄ requires 276.1230 found 276.1210 (M⁺). The ¹H NMR data is in agreement with that previously reported.²⁷¹

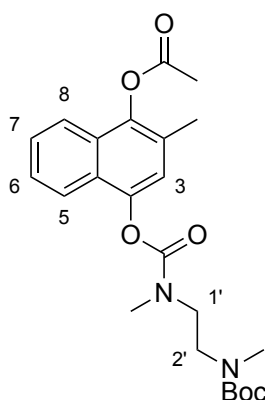
2-Methyl-1,4-naphthohydroquinone monoacetate **190**



Following the patent procedure of Sunny Pharmtech,²⁷¹ anhydrous diisopropylamine (0.273 mL, 1.94 mmol, 1.00 eq.) was added to a suspension of **189** (500 mg, 1.94 mmol, 1.00 eq.) in methanol (2.5 mL) and water (0.3 mL). The reaction was stirred at room temperature for 3 h under argon. The reaction was diluted with ethyl acetate (30 mL) and washed with an aqueous solution of HCl (30 mL, 1 M). The aqueous layer was extracted with ethyl acetate (20 mL). The organic layers were combined and washed with brine (50 mL). The organic layer was separated again, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane: ethyl acetate 5-40%] to afford **190** as a brown solid (392 mg, 94%).

Mp: 118 - 120 °C. ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.02 (1H, d, J = 8.0 Hz, H-8), 7.66 (1H, d, J = 8.3 Hz, H-5), 7.54 - 7.37 (2H, m, H-7 and H-6), 6.49 (1H, s, H-3), 5.44 (1H, bs, OH), 2.48 (3H, s, O_2CCH_3), 2.22 (3H, s, PhCH_3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 170.17 (C), 149.39 (C), 137.86 (C), 127.82 (C), 127.19 (CH), 126.57 (C), 124.88 (CH), 123.90 (C), 122.19 (CH), 120.63 (CH), 111.11 (CH), 20.80 (CH_3), 16.52 (CH_3). Mass spectrometry by $\text{ESI}^{+/-}$ failed to identify the product. The ^1H NMR data is in agreement with that previously reported.²⁷¹

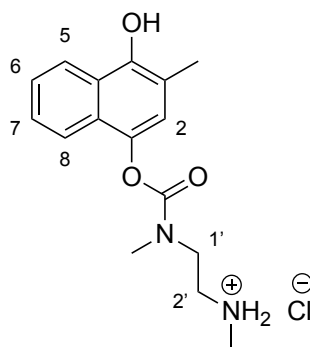
4-[[2'-{[(*Tert*-butoxy)carbonyl](methyl)amino}ethyl](methyl)carbamoyl]oxy}-2-methylnaphthalen-1-yl acetate **191**



Triphosgene (22.9 mg, 0.0770 mmol, 0.330 eq.) and anhydrous *N,N*-diisopropylethylamine (40.2 μL , 0.231 mmol, 1.00 eq.) were added to a solution of **190** (50.0 mg, 0.231 mmol, 1.00 eq.) in anhydrous dichloromethane (1.2 mL) and the reaction was stirred at room temperature for 1 h under argon. A solution of *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (43.5 mg, 0.231 mmol, 1.00 eq.) and *N,N*-diisopropylethylamine (40.2 μL , 0.231 mmol, 1.00 eq.) in anhydrous dichloromethane (1.2 mL) was added to the reaction, which was stirred at room temperature for an additional 1 h under argon. The reaction was diluted with dichloromethane (20 mL). The organic layer was washed with a saturated solution of NH_4Cl (20 mL), and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , hexane:ethyl acetate 10-50%] to afford **191** as a colourless oil (70.1 mg, 70%).

NMR spectroscopy indicated a 2:1:1 mixture of diastereomers (rotamers) A:B:C. $U_{\max}$ (ATR): 1719 (C=O), 1685 (C=O) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 7.93 – 7.79 (1H, m, H-5^A, B and C), 7.77 – 7.69 (1H, m, H-8^A, B and C), 7.55 – 7.42 (2H, m, H-6^A, B and C and H-7^A, B and C), 7.19 (1H, s, H-3^B and C), 7.17 (1H, s, H-3^A), 3.77 – 3.41 (4H, m, CH_2 -1'^A, B and C and CH_2 -2'^A, B and C), 3.28 (3H, s, $\text{O}_2\text{CNCH}_3^{\text{B and C}}$ or $\text{BocNCH}_3^{\text{B and C}}$), 3.10 (3H, s, $\text{O}_2\text{CNCH}_3^{\text{A}}$ or $\text{BocNCH}_3^{\text{A}}$), 2.96 (3H, s, $\text{O}_2\text{CNCH}_3^{\text{A}}$ or $\text{BocNCH}_3^{\text{A}}$), 2.95 – 2.88 (3H, m, $\text{O}_2\text{CNCH}_3^{\text{B and C}}$ or $\text{BocNCH}_3^{\text{B and C}}$), 2.47 (3H, s, $\text{O}_2\text{CCH}_3^{\text{A, B and C}}$), 2.32 (3H, s, $\text{PhCH}_3^{\text{A, B and C}}$), 1.56 – 1.43 (9H, m, 3 x $\text{CH}_3^{\text{A, B and C}}$). HRMS (ESI⁺): $\text{C}_{23}\text{H}_{34}\text{N}_3\text{O}_6$ requires 448.2442 found 448.2451 ($\text{M} + \text{NH}_4$)⁺. Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The ^{13}C NMR spectra was too complex to interpret.

4-Hydroxy-3-methylnaphthalen-1-yl N-methyl-N-[2-(methyldiazaniumyl)ethyl] carbamate chloride **192**

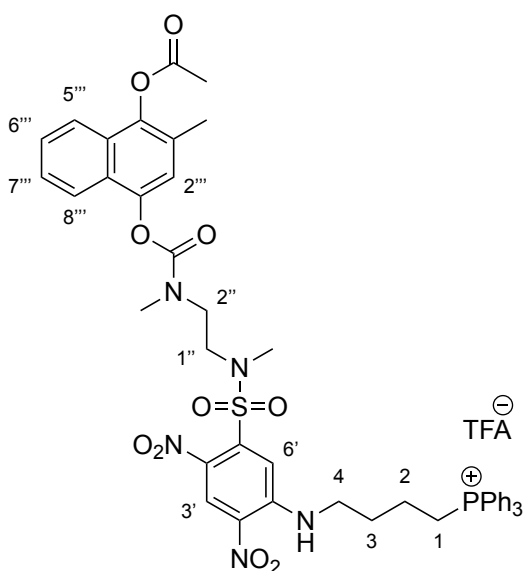


An aqueous solution of HCl (0.3 mL, 6 M) was added to a solution of **191** (25.0 mg, 0.0581 mmol) in methanol (0.3 mL) and the reaction was stirred at reflux for 24 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure to afford **192** as a white hygroscopic foam (18.5 mg, quant.).

NMR spectroscopy indicated a 2.3:1 mixture of diastereomers (rotamers) A:B. $U_{\max}$ (ATR): 1696 (C=O) cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ_{H} 8.26 – 8.16 (1H, m, H-8^{A and B}), 7.84 – 7.71 (1H, m, H-5^{A and B}), 7.50 – 7.42 (2H, m, H-6^{A and B} and H-7^{A and B}), 7.11 (1H, s, H-2^{A and B}), 3.98 (2H, t, J = 6.0 Hz, CH_2 -1'^B), 3.73 (2H, t, J = 5.8 Hz, CH_2 -1'^A), 3.41 (1H, t, J = 5.5 Hz, CH_2 -2'^B), 3.32 – 3.28 (5H, m, CH_2 -1'^A and $\text{O}_2\text{CNCH}_3^{\text{A}}$), 3.10 (3H, s, $\text{O}_2\text{CNCH}_3^{\text{B}}$), 2.82 (3H, s, $\text{NH}_2\text{CH}_3^{\text{B}}$), 2.74 (3H, s, $\text{NH}_2\text{CH}_3^{\text{A}}$), 2.40 (3H, s, $\text{PhCH}_3^{\text{A and B}}$). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 158.11 ($\text{C}^{\text{A and B}}$), 148.97 ($\text{C}^{\text{A and B}}$), 140.80 ($\text{C}^{\text{A and B}}$), 127.81 ($\text{C}^{\text{A and B}}$), 127.55 ($\text{C}^{\text{A and B}}$), 126.60

(CH^A and B), 126.36 (CH^A and B), 123.27 (CH^B), 123.18 (CH^A), 122.78 (CH^B), 122.61 (CH^A), 121.91 (CH^A), 121.83 (CH^B), 118.98 (C^A and B), 48.64* (CH₂^A), 48.45* (CH₂^B), 47.24 (CH₂^A), 47.00 (CH₂^B), 35.59 (CH₃^A and B), 34.12 (CH₃^B), 34.01 (CH₃^A), 16.43 (CH₃^A and B). HRMS (ESI⁺): C₁₆H₂₁N₂O₃ requires 289.1547 found 289.1556 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. *Signals were hidden behind the solvent signal in the ¹³C NMR spectrum but were observed in the DEPT spectrum.

(4-{[5'-({2''-([4'''-(Acetyloxy)-3'''-methylnaphthalen-1'''-yl]oxy)carbonyl(methyl)amino]ethyl}(methyl)sulfamoyl)-2',4'-dinitrophenyl]amino}butyl) triphenylphosphonium hexafluorophosphate (MitoMenOAc) **193**



Triphosgene (50.2 mg, 0.169 mmol, 0.330 eq.) and anhydrous *N,N*-diisopropylethylamine (88.7 μ L, 0.508 mmol, 1.00 eq.) were added to a solution of **190** (110 mg, 0.508 mmol, 1.00 eq.) in anhydrous dichloromethane (2.5 mL). The reaction was stirred at room temperature for 1 h under argon. A solution of **182** (454 mg, 0.508 mmol, 1.00 eq.) and anhydrous *N,N*-diisopropylethylamine (177 μ L, 1.02 mmol, 2.00 eq.) in dichloromethane (2.5 mL) was added to the reaction, which was stirred at room temperature for an additional 3 h under argon. The reaction was diluted with dichloromethane (20 mL). The organic layer was washed with a saturated solution of NH₄Cl (20 mL), and the aqueous layer was extracted with dichloromethane (2 x 15 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude

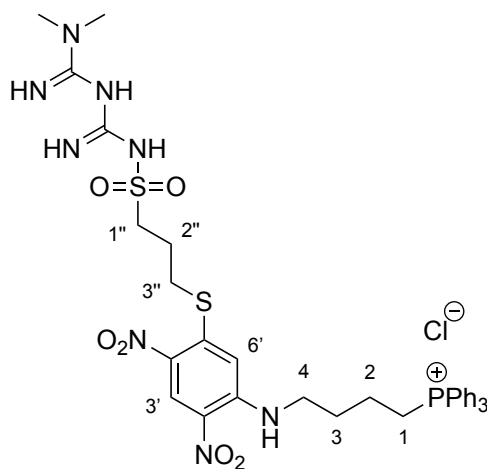
material was purified by semi-preparative RP-HPLC [0.1% TFA/water: acetonitrile, 10-90%] to afford **193** as an orange hygroscopic foam (179 mg, 36%).

NMR spectroscopy indicated a 1.3:1 mixture of diastereomers (rotamers) A:B. $\nu_{\max}$ (ATR): 1719 (C=O), 1685 (C=O) cm^{-1} . ^1H NMR (400 MHz, CD_3CN): δ_{H} 8.69 (1H, s, H-3'^A), 8.65 (1H, s, H-3'^B), 8.39 (1H, t, J = 5.9 Hz, NH^A), 8.25 (1H, t, J = 5.8 Hz, NH^B), 8.00 - 7.77 (5H, m, H-5''''^{A and B}, H-8''''^{A and B} and Ph^{A and B}), 7.74 - 7.60 (12H, m, Ph^{A and B}), 7.57 - 7.42 (2H, m, H-6''''^{A and B} and H-7''''^{A and B}), 7.32 (1H, s, H-6'^B), 7.32 (1H, s, H-6'^A), 7.19 (1H, s, H-2''^A), 7.18 (1H, s, H-2''^B), 3.75 (2H, t, J = 6.0 Hz, CH₂-2''^{A or B}), 3.67 (2H, t, J = 5.8 Hz, CH₂-2''^{A or B}), 3.63 - 3.51 (2H, m, CH₂-1''^{A and B}), 3.41 (2H, q, J = 6.4 Hz, CH₂-4^{A or B}), 3.30 (2H, q, J = 6.3 Hz, CH₂-4^{A or B}), 3.26 (3H, s, O₂SNCH₃^A or O₂CNCH₃^A), 3.23 - 3.08 (2H, m, CH₂-1^{A and B}), 3.06 (3H, s, O₂SNCH₃^B or O₂CNCH₃^B), 3.02 (3H, s, S O₂SNCH₃^B or O₂CNCH₃^B), 3.00 (3H, s, O₂SNCH₃^A or O₂CNCH₃^A), 2.46 (3H, s, O₂CCH₃^{A and B}), 2.27 (3H, s, PhCH₃^{A and B}), 1.75 (2H, p, J = 6.6 Hz, CH₂-2^{A and B}), 1.64 (2H, p, J = 7.8 Hz, CH₂-3^{A and B}). ^{13}C NMR (101 MHz, CD_3CN): δ_{C} 170.51 (C^B), 170.41 (C^A), 155.92 (C^A), 155.36 (C^B), 147.41 (C^A), 147.25 (C^B), 145.99 (C^A), 145.91 (C^B), 142.86 (C^{A or B}), 142.78 (C^{A or B}), 140.48 (C^B), 140.39 (C^A), 136.14 (d, J = 2.9 Hz, 3 x CH^{A and B}), 135.40 (C^A), 135.30 (C^B), 134.61 (d, J = 10.0 Hz, 6 x CH^{A and B}), 131.68 (C^A), 131.64 (C^B), 131.26 (d, J = 12.6 Hz, 6 x CH^{A and B}), 128.67 (C^{A and B}), 128.21 (CH^{A and B}), 127.93 (C^B), 127.80 (C^A), 127.71 (C^{A or B}), 127.67 (C^{A or B}), 127.11 (CH^{A and B}), 127.05 (CH^B), 126.99 (CH^A), 122.99 (CH^A), 122.67 (CH^B), 122.18 (CH^B), 122.06 (CH^A), 122.02 (CH^B), 121.99 (CH^A), 119.08 (d, J = 86.8 Hz, 3 x C^{A and B}), 118.41* (CH^B), 118.29* (CH^A), 49.22 (CH₂^B), 48.79 (CH₂^A), 47.66 (CH₂^B), 47.52 (CH₂^A), 42.75 (CH₂^A), 42.67 (CH₂^B), 35.96 (CH₃^B), 35.89 (CH₃^A), 35.87 (CH₃^B), 35.64 (CH₃^A), 29.65 (d, J = 16.7 Hz, CH₂^{A and B}), 22.46 (d, J = 52.1 Hz, CH₂^{A or B}), 22.38 (d, J = 51.8 Hz, CH₂^{A or B}), 20.87 (CH₃^B), 20.86 (CH₃^A), 20.31 (CH₂^{A and B}), 16.50 (CH₃^{A and B}). ^{31}P NMR (162 MHz, CD_3CN): δ_{P} 23.69 (1P, s, PPh₃^A), 23.67 (1P, s, PPh₃^B). ^{19}F NMR (377 MHz, CD_3CN): δ_{F} -75.18 (3F, s, TFA). HRMS (ESI⁺): C₄₆H₄₇N₅O₁₀PS requires 892.2776 found 892.2791 (M⁺). Assignment of ^1H and ^{13}C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. *Signals were hidden behind the solvent signal in the ^{13}C NMR spectrum but were observed in the DEPT spectrum. The signals corresponding to the TFA⁻ anion were not observed in the ^{13}C NMR spectrum.

{4-[(5'-{[2''-(4'''-Hydroxy-3'''-methylnaphthalen-1'''-yl)oxy]carbonyl}(methyl)amino)ethyl](methyl)sulfamoyl}-2',4'-dinitrophenyl)amino]butyl}triphenylphosphonium chloride (MitoMenOH) 194

(C^A or B), 119.25 (d, $J = 86.3$ Hz, $3 \times$ C^A or B), 119.21 (d, $J = 86.4$ Hz, $3 \times$ C^A or B), 118.71 (CH^A or B), 118.68 (CH^A or B), 48.81 (CH₂^A or B), 48.63 (CH₂^A or B), 47.70 (CH₂^A or B), 47.65 (CH₂^A or B), 42.64 (CH₂^A or B), 42.57 (CH₂^A or B), 36.11 (CH₃^A or B), 36.07 (CH₃^A or B), 36.05 (CH₃^A or B), 35.90 (CH₃^A or B), 29.46 (d, $J = 16.5$ Hz, CH₂^A or B), 29.36 (d, $J = 16.8$ Hz, CH₂^A or B), 22.19 (d, $J = 51.4$ Hz, CH₂^A or B), 22.01 (d, $J = 51.7$ Hz, CH₂^A or B), 20.20 (CH₂^A and B), 17.23 (CH₃^A and B). ³¹P NMR (162 MHz, CD₃CN): δ_P 23.86 (1P, s, PPh₃^A and B). HRMS (ESI⁺): C₄₄H₄₅N₅O₉PS requires 850.2670 found 850.2680 (M⁺). Assignment of ¹H and ¹³C signals was supported by analysis of COSY, HSQC and DEPT experiments. *Signals were hidden behind the solvent signal in the ¹³C NMR spectrum but were observed in the DEPT spectrum from which their shift was estimated. The signals corresponding to the TFA⁻ anion were not observed in the ¹³C NMR spectrum.

[4-({2',4'-Dinitro-5'-[(3''-{*N,N*-dimethylbiguanide}propyl)sulfanyl]phenyl}amino)butyl]triphenylphosphonium chloride (MitoMetSDNB) 196

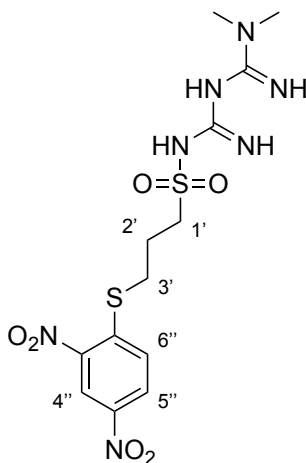


Anhydrous dimethylformamide (10 μ L) was added to a solution of **207** (50.0 mg, 0.0859 mmol, 1.00 eq.) in thionyl chloride (86 μ L) and the reaction was stirred at room temperature for 1 h under argon. The solvent was removed under reduced pressure. The residue was redissolved in anhydrous acetonitrile (0.7 mL) and **58** (18.0 mg, 0.139 mmol, 2.00 eq.) was added. The reaction was stirred at room temperature for 16 h. The solvent was removed under reduced pressure. The crude material was partially purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford the desired biguanide intermediate with some minor impurities, which was taken onto the next step. **95** (6.5 mg, 0.0156 mmol, 1.10 eq) and triethylamine (4.0 μ L, 0.0284 mmol, 2.00 eq.) were added

to a solution of the intermediate (6.4 mg, approx. 0.0142 mmol, 1.00 eq.) in anhydrous acetonitrile (0.5 mL) and the reaction was stirred at room temperature for 3 h. The solvent was removed under pressure. The residue was re-dissolved in dichloromethane (15 mL) and washed with HCl (3 x 15 mL, 1 M). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford **196** as a yellow hygroscopic foam (4.6 mg, 8% over three steps).

$\nu_{\max}$ (ATR): 1604 (Aryl C=C), 1566 (Aryl C=C) cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ_{H} 9.07 (1H, d, J = 1.0 Hz, H-3'), 8.51 (1H, t, J = 5.6 Hz, NH), 7.94 - 7.68 (15H, m, Ph), 6.70 (1H, s, H-6'), 3.66 - 3.44 (4H, m, CH₂-1 and CH₂-1''), 3.28 - 3.17 (4H, m, CH₂-4 and CH₂-3''), 2.98 (6H, s, 2 x CH₃), 2.20 (2H, qn, J = 6.8 Hz, CH₂-2''), 2.06 - 1.95 (2H, m, CH₂-2), 1.92 - 1.80 (2H, m, CH₂-3). ¹³C NMR (101 MHz, CD₃OD): δ_{C} 160.57 (C), 158.89 (C), 146.19 (C), 134.90 (d, J = 3.0 Hz, 3 x CH), 133.46 (d, J = 10.1 Hz, 6 x CH), 130.15 (d, J = 12.6 Hz, 6 x CH), 127.79 (C), 126.22 (CH), 118.37 (d, J = 86.4 Hz, 3 x C), 108.07 (CH), 51.62 (CH₂), 41.71 (CH₂), 35.85 (2 x CH₃), 29.84 (CH₂), 29.11 (d, J = 16.6 Hz, CH₂), 22.79 (CH₂), 21.00 (d, J = 51.8 Hz, CH₂), 19.64 (d, J = 4.0 Hz, CH₂). ³¹P NMR (162 MHz, CD₃OD): δ_{P} 23.96 (1P, s, PPh₃). HRMS (ESI⁺): C₃₅H₄₂N₈O₆PS₂ requires 765.2401 found 765.2426 (M⁺). Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments. The signals corresponding to the biguanide carbons were not observed in the ¹³C NMR spectrum, perhaps because it was too weak.

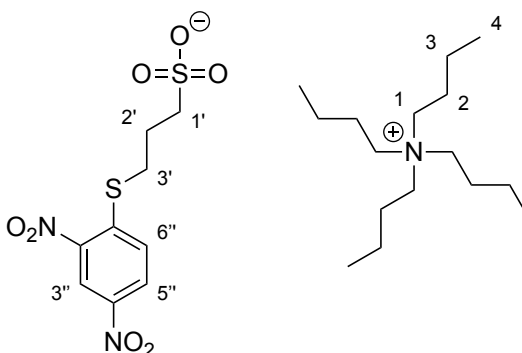
1-(*N*'-{3'-[(2'',4''-Dinitrophenyl)sulfanyl]propanesulfonyl}carbamimidamido)-*N,N*'-dimethylmethanimidamide 201



58 (73.1 mg, 0.566 mmol, 2.00 eq.) was added to a solution of **203** (96.4 mg, 0.283 mmol, 1.00 eq.) in anhydrous acetonitrile (3 mL) and the reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure and the crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-30%] to afford **201** as a yellow solid (33 mg, 27%).

Mp: 124-126 °C. ν_{max} (ATR): 3451 (N-H), 3342 (N-H), 1606 (Aryl C=C), 1590 (Aryl C=C) cm⁻¹. ¹H NMR (400 MHz, DMSO): δ_{H} 8.88 (1H, d, J = 2.5 Hz, H-3''), 8.43 (1H, dd, J = 9.0, 2.6 Hz, H-5''), 7.95 (2H, br s, 2 x NH), 7.87 (1H, d, J = 9.1 Hz, H-6''), 6.77 (2H, br s, 2 x NH) 3.37 - 3.28 (2H, m, CH₂-1'), 3.17 - 3.06 (2H, m, CH₂-3'), 2.91 (6H, s, 2 x CH₃), 2.10 - 1.96 (2H, m, CH₂-2'). ¹³C NMR (101 MHz, DMSO): δ_{C} 160.04 (C), 158.25 (C), 144.94 (C), 144.75 (C), 143.63 (C), 127.95 (CH), 127.41 (CH), 121.45 (CH), 51.46 (CH₂), 36.51 (2 x CH₃), 29.74 (CH₂), 22.63 (CH₂). HRMS (ESI⁺): C₁₃H₁₉N₇O₆S₂Na requires 456.0730 found 456.0740 (M + Na)⁺.

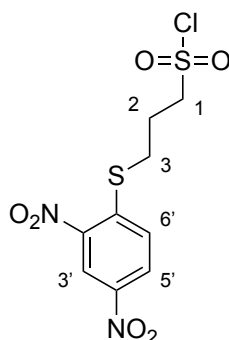
Tetrabutylammonium ([[(2'',4''-dinitrophenyl)sulfanyl]butane-1'-sulfonate **202**



Sodium 3-mercapto-1-propanesulfonate (1.00 g, 4.94 mmol, 1.50 eq.) and triethylamine (1.32 g, 7.41 mmol, 3.00 eq.) were added to a solution of 1-chloro-2,4-dinitrobenzene (1.00 g, 4.94 mmol, 1.00 eq.) in acetonitrile (5 mL) and the reaction was stirred at reflux for 6 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The residue was re-dissolved in a biphasic mixture of dichloromethane (20 mL) and tetrabutylammonium bromide (1.00 g) in water (20 mL), which was stirred at room temperature for 10 mins. The organic layer was separated and washed with water (3 x 20 mL). The organic layer was dried with MgSO₄ and the solvent was removed under reduced pressure to afford **202** as an orange gel (963 mg, 57%).

ν_{max} (ATR): 1587 (Aryl C=C), 1514 (NO_2) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 9.01 (1H, d, J = 2.5 Hz, H-3''), 8.32 (1H, dd, J = 9.0, 2.5 Hz, H-5''), 7.91 (1H, d, J = 9.1 Hz, H-6''), 3.40 - 3.34 (2H, m, CH_2 -1'), 3.32 - 3.23 (8H, m, 4 x CH_2 -1), 2.95 (2H, t, J = 6.6 Hz, CH_2 -3'), 2.30 - 2.17 (2H, m, CH_2 -2'), 1.65 (8H, m, 4 x CH_2 -2), 1.44 (8H, sept, J = 7.4 Hz, 4 x CH_2 -3), 0.98 (12H, t, J = 7.3 Hz, 4 x CH_3 -4). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 147.72 (CH), 144.82 (CH), 143.62 (CH), 128.07 (C), 127.24 (C), 121.67 (C), 59.09 (4 x CH_2), 50.20 (CH_2), 31.53 (CH_2), 24.43 (CH_2), 24.23 (4 x CH_2), 19.89 (4 x CH_2), 13.81 (4 x CH_3). HRMS (ESI $^-$): $\text{C}_9\text{H}_9\text{N}_2\text{O}_7\text{S}_2$ requires 320.9857 found 320.9857 (M^-).

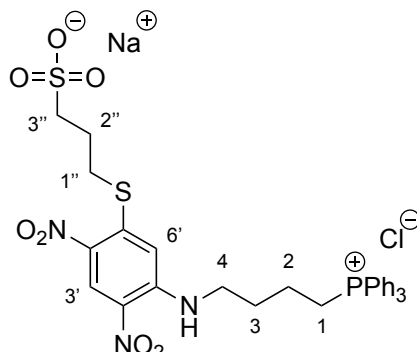
3-[(2',4'-Dinitrophenyl)sulfanyl]propane-1-sulfonyl chloride **203**



Anhydrous dimethylformamide (10 μL) was added to a solution of **202** (50.0 mg, 0.0887 mmol, 1.00 eq.) in thionyl chloride (0.9 mL) and the reaction was stirred at reflux for 2 h under argon. The reaction was allowed to cool to room temperature and diluted with ice-water (30 mL) and dichloromethane (30 mL). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (20 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure to afford **203** as a yellow solid (30.2 mg, quant.).

^1H NMR (400 MHz, CDCl_3): δ_{H} 9.09 (1H, d, J = 2.5 Hz, H-3'), 8.42 (1H, dd, J = 9.0, 2.5 Hz, H-5'), 7.59 (1H, d, J = 9.0 Hz, H-6'), 3.92 (2H, t, J = 7.0 Hz, CH_2 -1), 3.33 (2H, t, J = 7.2 Hz, CH_2 -3), 2.53 (1H, p, J = 7.1 Hz, CH_2 -2). The compound was taken onto the next step without further characterisation.

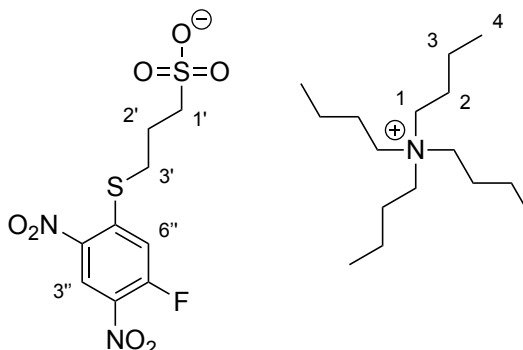
[4-({2',4'-dinitro-5'-[(3''-sulfonatopropyl)sulfanyl]phenyl}amino)butyl]triphenylphosphonium 204



Sodium 3-mercapto-1-propanesulfonate (124 mg, 0.698 mmol, 1.10 eq.) and anhydrous triethylamine (0.177 mL, 1.27 mmol, 2.00 eq.) were added to a solution of MitoCDNB **51** (0.400 g, 0.635 mmol, 1.00 eq) in anhydrous acetonitrile (7 mL) and the reaction was stirred at reflux for 20 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, dichloromethane:methanol 0-20%] to afford **204** as an orange hygroscopic foam (385 mg, 85%).

U_{max} (ATR): 1604 (Aryl C=C), 1570 (Aryl C=C), 1533 (NO₂) cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ_H 9.16 (1H, s, CH-3'), 8.24 (1H, t, *J* = 4.7 Hz, NH), 7.88 - 7.65 (15H, m, Ph), 7.09 (1H, s, H-6'), 3.94 - 3.85 (2H, m, CH₂-4), 3.75 - 3.64 (2H, m, CH₂-1), 3.51 - 3.42 (2H, t, *J* = 8.1 Hz, CH₂-3''), 3.04 - 2.97 (2H, t, *J* = 5.1 Hz, CH₂-1''), 2.27 - 2.17 (2H, m, CH₂-2''), 2.05 - 1.86 (4H, m, CH₂-2 and CH₂-3). ¹³C NMR (101 MHz, CDCl₃): δ_C 147.93 (C), 146.88 (C), 135.27 (d, *J* = 3.0 Hz, 3 x CH), 134.41 (C), 133.81 (d, *J* = 10.0 Hz, 6 x CH), 130.71 (d, *J* = 12.5 Hz, 6 x CH), 127.65 (C), 127.05 (CH), 118.21 (d, *J* = 86.1 Hz, 3 x C), 108.91 (CH), 50.51 (CH₂), 42.37 (CH₂), 30.98 (CH₂), 29.90 (d, *J* = 16.6 Hz, CH₂), 25.33 (CH₂), 21.97 (d, *J* = 51.4 Hz, CH₂), 19.90 (d, *J* = 4.0 Hz, CH₂). ³¹P NMR (162 MHz, CDCl₃): δ_P 24.66 (1P, s, PPh₃). HRMS (ESI⁺): C₃₁H₃₃N₃O₇PS₂ requires 654.1492 found 654.1497 (*M* + H)⁺. Assignment of ¹H and ¹³C NMR signals was supported by analysis of COSY, HSQC and DEPT experiments.

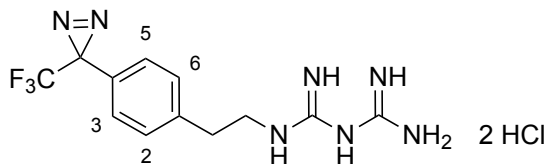
Tetrabutylammonium 3'-(5''-fluoro-2'',4''-dinitro-phenylsulfanyl)-propane-1-sulfonate **207**



Sodium 3-mercapto-1-propanesulfonate (87.3 mg, 0.490 mmol, 1.00 eq.) and triethylamine (137 μ L, 0.980, 2.00 mmol) were added to a solution of 1,5-difluoro-2,4-dinitrobenzene (100 mg, 0.490 mmol, 1.00 eq.) in anhydrous acetonitrile (4.9 mL). The reaction was stirred at room temperature for 24 h under argon. The solvent was removed under reduced pressure. The residue was redissolved in a biphasic mixture of dichloromethane (15 mL) and an excess of tetrabutylammonium chloride in water (15 mL), which was stirred at room temperature for 15 mins. The organic layer was washed with water (3 x 15 mL), dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , dichloromethane:methanol 0-10%] to afford **207** as a yellow oil (128 mg, 59%).

U_{max} (ATR): 1601 (Aryl C=C), 1587 (Aryl C=C), 1531 (NO_2) cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ_{H} 9.02 (1H, d, $J = 7.6$ Hz, H-3''), 7.78 (1H, d, $J = 12.1$ Hz, H-6''), 3.41 - 3.32 (2H, m, CH_2 -1'), 3.35 - 3.26 (8H, m, 4 x CH_2 -1), 2.97 (2H, t, $J = 6.4$ Hz, CH_2 -3'), 2.30 - 2.19 (2H, m, CH_2 -2'), 1.73 - 1.60 (8H, m, 4 x CH_2 -2), 1.45 (8H, h, $J = 7.4$ Hz, 4 x CH_2 -3), 1.00 (12H, t, $J = 7.3$ Hz, 4 x CH_3 -4''). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 158.80 (C), 156.08 (C), 150.30 (d, $J = 10.2$ Hz, C), 140.37 (C), 125.14 (CH), 116.57 (d, $J = 26.6$ Hz, CH), 58.98 (4 x CH_2), 49.92 (CH_2), 31.74 (CH_2), 24.23 (CH_2), 24.15 (4 x CH_2), 19.85 (4 x CH_2), 13.77 (4 x CH_3). ^{19}F (^1H coupled) NMR (377 MHz, CDCl_3): δ_{F} -107.91 (dd, $J = 12.2, 7.3$ Hz). HRMS (ESI^-): $\text{C}_9\text{H}_8\text{FN}_2\text{O}_7\text{S}_2$ requires 338.9762 found 338.9773 (M^-).

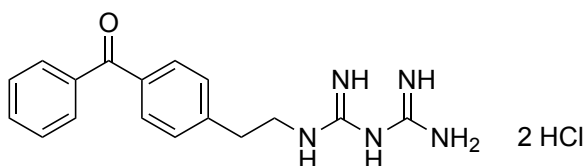
2-{4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl}ethylbiguanide bishydrochloride (Phenformin-DZ) 209



Adapting the procedure of Fortun *et al.*,²⁸⁷ dicyandiamide (73.4 mg, 0.873 mmol, 1.00 eq.) and iron (III) chloride (142 mg, 0.873 mmol, 1.00 eq.) were added to a solution of **227** (200 mg, 0.873 mmol, 1.00 eq.) in anhydrous 1,4-dioxane (0.9 mL). The reaction was stirred at reflux for 1.5 h under argon and then allowed to cool to room temperature. To the reaction was added concentrated hydrochloric acid (218 μ L, 12 M) and the solvent was removed under reduced pressure. The crude material was purified by reversed phase column chromatography [SiO_2 , water:acetonitrile 0-100%] to afford (**109**) as a yellow hygroscopic solid (44.6 mg, 13%).

U_{max} (ATR): 3167 - 3338 (N-H), 1638 (C=N), 1541 (Aryl C=C) cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ_{H} 7.37 (2H, d, J = 8.5 Hz, H-3 and H-5), 7.20 (2H, d, J = 7.7 Hz, H-2 and H-6), 3.47 (2H, t, J = 7.1 Hz, NCH_2), 2.89 (2H, t, J = 7.1 Hz, PhCH_2). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 142.80 (C), 130.78 (2 x CH), 128.07 (C), 127.77 (q, J = 1.3 Hz, 2 x CH), 123.66 (q, J = 273.7 Hz, C), 43.48 (CH_2), 36.13 (CH_2), 29.42 (q, J = 40.3 Hz, C). HRMS (ESI^+): $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}$ requires 310.1662 found 310.1663 ($\text{M} + \text{H}^+$). The signals corresponding to the biguanide carbons were not observed in the ^{13}C NMR spectrum, perhaps because the spectrum was too weak.

***N*-(4-Benzoylphenethyl)biguanide bishydrochloride (Phenformin-BZ) 210**

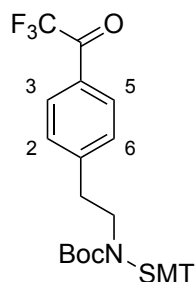


Adapting the procedure of Fortun *et al.*,²⁸⁷ dicyandiamide (74.6 mg, 0.888 mmol, 1.00 eq.) and iron (III) chloride (144 mg, 0.888 mmol, 1.00 eq.) were added to a solution of **233** (200 mg, 0.888 mmol, 1.00 eq.) in anhydrous 1,4-dioxane (0.9 mL). The reaction was

stirred at reflux for 1.5 h and then allowed to cool to room temperature. To the reaction was added concentrated hydrochloric acid (222 μ L, 12 M) and the solvent was removed under reduced pressure. The crude material was purified by reversed phase column chromatography [SiO_2 , water:acetonitrile 0-100%] to afford **210** as a white hygroscopic solid (84.8 mg, 28%).

U_{max} (ATR): 3199 - 3336 (N-H), 1684 (C=N), 1636 (C=N), 1544 (Aryl C=C). ^1H NMR (400 MHz, CD_3OD): δ_{H} 7.80 - 7.39 (9H, m, Ph), 3.54 (2H, t, J = 7.2 Hz, NCH_2), 2.97 (2H, t, J = 7.2 Hz, PhCH_2). ^{13}C NMR (101 MHz, CD_3OD): δ_{C} 198.36 (C), 163.24 (C), 162.90 (C), 145.87 (C), 138.95 (C), 137.05 (C), 133.76 (CH), 131.43 (2 x CH), 130.92 (2 x CH), 130.08 (2 x CH), 129.51 (2 x CH), 43.46 (CH_2), 36.55 (CH_2). HRMS (ESI^+): $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}$ requires 310.1662 found 310.1663 ($\text{M} + \text{H}$) $^+$.

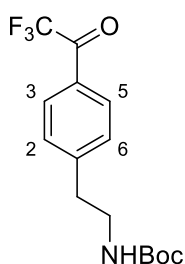
Tert*-butyl 4-(2',2',2'-Trifluoroacetyl)phenethyltrimethylsilylcarbamate **224*



Anhydrous diisopropylamine (1.69 mL, 12.0 mmol, 1.20 eq.) was added to a solution of anhydrous *n*-butyllithium (2.5M in tetrahydrofuran, 4.80 mL, 12.0 mmol, 1.20 eq.) in anhydrous tetrahydrofuran (25 mL) to generate lithium diisopropylamine. The solution of lithium diisopropylamine was added dropwise to a solution of *N*-boc-2-(4-bromophenyl)ethylamine (3.00 g, 9.99 mmol, 1.00 eq.) and trimethylsilyl chloride (1.52 mL, 12.0 mmol, 1.20 eq.) in anhydrous tetrahydrofuran (25 mL) at -78 $^{\circ}\text{C}$ under argon. The reaction was allowed to warm to room temperature and stirred for 1 h. The solvent was removed under reduced pressure. The solid residue was washed with diethyl ether (50 mL) and filtered under vacuum. The filtrate was collected and the solvent was removed under reduced pressure to afford **224** as a white solid (3.65 g, 98%).

^1H NMR (400 MHz, CDCl_3): δ_{H} 7.39 (2H, d, $J = 7.8$ Hz, H-3 and H-5), 7.06 (2H, d, $J = 7.8$ Hz, H-2 and H-6), 3.26 (3H, t, $J = 7.7$ Hz, NCH_2), 2.72 (3H, t, $J = 7.8$ Hz, PhCH_2), 1.48 [9H, s, $\text{O}_2\text{C}(\text{CH}_3)_3$], 0.17 [9H, s, $\text{Si}(\text{CH}_3)_3$]. The compound was taken onto the next step without further characterisation.

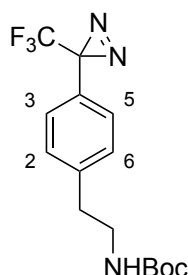
Tert*-butyl 4-(2',2',2'-Trifluoroacetyl)phenethylcarbamate **225*



n-Butyllithium (2.5 M in tetrahydrofuran; 3.92 mL, 9.81 mmol, 1.00 eq.) was added to a solution of **224** (3.65 g, 9.81 mmol, 1.00 eq.) in anhydrous tetrahydrofuran (49 mL) at -78 °C under argon. The reaction was stirred at -78 °C for 10 min, after which ethyl trifluoroacetate (2.32 mL, 19.6 mmol, 2.00 eq.) was added. The reaction was stirred at -78 °C for an additional 6 h under argon. The reaction was quenched with a saturated solution of NH_4Cl (50 mL). The organic layer was separated, and the aqueous layer was extracted with diethyl ether (2 x 40 mL). The organic layers were combined, dried with MgSO_4 and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO_2 , hexane:ethyl acetate 5-40%], to afford **225** as a white solid (2.66 g, 85%).

Mp: $56 - 58$ °C. ^1H NMR (400 MHz, CDCl_3): δ_{H} 8.02 (2H, d, $J = 7.9$ Hz, H-3 and H-5), 7.38 (2H, d, $J = 8.0$ Hz, H-2 and H-6), 4.56 (1H, s, NH), 3.42 (2H, q, $J = 7.0$ Hz, NCH_2), 2.91 (2H, t, $J = 7.0$ Hz, PhCH_2), 1.43 (9H, s, 3 x CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 180.08 (q, $J = 35.0$ Hz, C), 155.90 (C), 148.01 (C), 130.61 (q, $J = 1.5$ Hz, 2 x CH), 129.77 (2 x CH), 128.42 (C), 116.85 (q, $J = 291.4$ Hz, C), 79.72 (C), 41.41 (CH_2), 36.71 (CH_2), 28.50 (3 x CH_3). ^{19}F NMR (377 MHz, CDCl_3): δ_{F} -71.37 (CF_3). HRMS (ESI^+): $\text{C}_{11}\text{H}_{11}\text{F}_3\text{NO}_3$ (lost *t*-Bu group) requires 262.0686 found 266.0689 ($\text{M} + \text{H}$) $^+$. The ^1H and ^{13}C NMR data are in agreement with that previously reported.³⁰¹

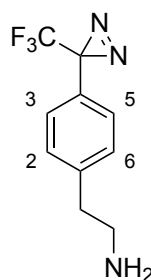
Tert*-butyl 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenethylcarbamate **226*



Following the procedure of Murai *et al.*,³⁰¹ excess activated MnO₂ was added to a solution of **229** (290 mg, 0.875 mmol, 1.00 eq.) in anhydrous diethyl ether (8.8 mL) and the reaction was stirred at room temperature for 1 h under argon. The reaction was filtered over celite under vacuum. The filtrate was collected, and the solvent was removed under reduced pressure to afford **226** as a white solid (245 mg, 85 %).

Mp: 54 - 56 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 7.22 (2H, d, *J* = 8.3 Hz, H-3 and H-5), 7.13 (2H, d, *J* = 8.1 Hz, H-2 and H-6), 4.54 (1H, s, NH), 3.35 (2H, q, *J* = 6.9 Hz, NCH₂), 2.80 (2H, t, *J* = 7.0 Hz, PhCH₂), 1.42 (9H, s, 3 x CH₃). ¹³C NMR (101 MHz, CDCl₃): δ_C 155.93 (C), 141.10 (C), 129.46 (2 x CH), 127.38 (C), 126.84 (2 x CH), 122.30 (q, *J* = 274.5 Hz, C), 79.54 (C), 41.66 (CH₂), 36.11 (CH₂), 28.50 (3 x CH₃), 28.47 (q, *J* = 40.3 Hz, C). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -65.31 (3F, s, CF₃). HRMS (ESI⁺): C₁₁H₁₁F₃N₃O₂ (lost *t*-Bu group) requires 274.0798 found 274.0793 (*M* + H)⁺. The ¹H and ¹³C NMR data is in agreement with that previously reported.³⁰¹

2-{4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenyl}ethanamine **227**

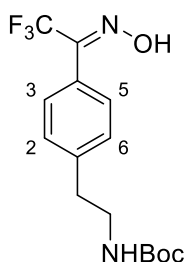


Trifluoroacetic acid (3 mL) was added to a solution of **230** (201 mg, 0.609 mmol, 1.00 eq.) in dichloromethane (3 mL) and the reaction was stirred at 0 °C for 2 h under argon.

The solvent was removed under reduced pressure. The solid residue was re-dissolved in methanol (3 mL) and NaOH (3 mL, 3 M), and the reaction was stirred at room temperature for 1 h under argon. The reaction was diluted with ethyl acetate (20 mL) and washed with brine (20 mL). The organic layer was dried with MgSO₄ and the solvent was removed under reduced pressure to afford **227** as a pale-yellow oil (133 mg, 96%).

¹H NMR (400 MHz, CDCl₃): δ_H 7.23 (2H, d, *J* = 8.4 Hz, H-3 and H-5), 7.13 (2H, d, *J* = 8.0 Hz, H-2 and H-6), 2.96 (2H, t, *J* = 6.9 Hz, NCH₂), 2.76 (2H, t, *J* = 6.9 Hz, PhCH₂), 1.84 (2H, br s, NH₂). ¹³C NMR (101 MHz, CDCl₃): δ_C 141.68 (C), 129.43 (2 x CH), 127.22 (C), 126.78 (q, *J* = 1.3 Hz, 2 x CH), 122.30 (q, *J* = 274.6 Hz, C), 43.21 (CH₂), 39.40 (CH₂), 28.47 (q, *J* = 40.4 Hz, C). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -65.32 (3F, s, CF₃). HRMS (ESI⁺): C₁₀H₁₀F₃N₃ requires 274.0900 found 230.0901 (*M* + H)⁺. The ¹H and ¹³C NMR data is in agreement with that previously reported.³⁰¹

Tert*-butyl 4-(2,2,2-trifluoroacetyl)phenethylcarbamate **228*

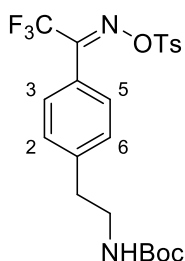


Following the procedure of Murai *et al.*,³⁰¹ hydroxylamine hydrochloride (657 mg, 9.45 mmol, 3.00 eq.) was added to a solution of **225** (1.00 g, 3.15 mmol, 1.00 eq.) in pyridine (15.8 mmol) and the reaction was stirred at reflux for 3 h under argon. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude material was re-dissolved in diethyl ether (30 mL) and washed with HCl (30 mL, 1 M), and then with brine (30 mL). The organic layer was dried with MgSO₄ and the solvent was removed under reduced pressure to afford **228** as a white solid (988 mg, 94%).

NMR spectroscopy indicated a 1:1 mixture of diastereomers (rotamers) A:B. ¹H NMR (400 MHz, (CD₃)₂CO): δ_H 11.97 (1H, s, OH^A or B), 11.70 (1H, s, OH^A or B), 7.55 - 7.23 (4H, m, Ph^A and B), 6.05 (1H, s, NH^A and B), 3.41 - 3.26 (2H, m, PhCH₂^A and B), 2.92 - 2.80 (2H, m, NCH₂^A and B).

B), 1.39 (9H, s, 3 x CH₃^{A and B}). ¹³C NMR (101 MHz, (CD₃)₂CO): δ_C 156.65 (C^{A and B}), 146.84 (q, *J* = 31.5 Hz, C^{A or B}), 146.71 (q, *J* = 29.0 Hz, C^{A or B}), 143.03 (C^{A or B}), 142.81 (C^{A or B}), 129.82 (CH^{A or B}), 129.80 (CH^{A or B}), 129.58 (CH^{A and B}), 129.16 (CH^{A and B}), 125.70 (C^{A and B}), 122.23 (q, *J* = 273.5 Hz, C^{A or B}), 119.76 (q, *J* = 282.3 Hz, C^{A or B}), 78.61 (C^{A and B}), 42.40 (CH₂^{A or B}), 42.35 (CH₂^{A or B}), 36.70 (CH₂^{A or B}), 36.58 (CH₂^{A or B}), 28.62 (3 x CH₃^{A and B}). ¹⁹F NMR (377 MHz, (CD₃)₂CO): δ_F -62.96 (CF₃^{A or B}), -66.61 (CF₃^{A or B}). The ¹H and ¹³C NMR data is in agreement with that previously reported.³⁰¹ The compound was taken onto the next step without further characterisation.

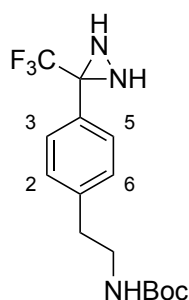
Tert*-butyl 4-(2,2,2-trifluoroacetyl)phenethylcarbamate **229*



Following the procedure of Murai *et al.*,³⁰¹ tosyl chloride (1.13 g, 5.95 mmol, 2.00 eq.) and triethylamine (1.24 mL, 8.92 mmol, 3.00 eq.) were added to a solution of **228** in acetone (14.9 mL) at 0 °C. The reaction was stirred at this temperature for 1 h under argon. The solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane:ethyl acetate 5-40%], to afford **229** as a white solid (1.47 g, quant).

¹H NMR spectroscopy indicated a 1.3:1 mixture of diastereomers (rotamers) A:B. ¹H NMR (400 MHz, CDCl₃): δ_H 7.95 - 7.85 (2H, m, 2 x H-3^{A and B}), 7.43 - 7.09 (6H, m, 2 x H-2^{A and B} and Ts^{A and B}), 4.58 (1H, s, NH^B), 4.53 (1H, s, NH^A), 3.46 - 3.28 (2H, m, NCH₂^{A and B}), 2.84 (2H, q, *J* = 6.6 Hz, PhCH₂^{A and B}), 2.48 (3H, s, CH₃^B), 2.46 (3H, s, CH₃^A), 1.43 (9H, s, 3 x CH₃^B), 1.42 (9H, s, 3 x CH₃^A). The ¹H NMR data is in agreement with that previously reported.³⁰¹ The compound was taken onto the next step without further characterisation.

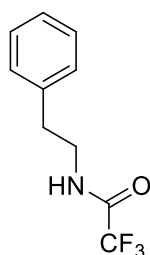
Tert*-butyl 4-[3-(Trifluoromethyl)diaziridin-3-yl]phenethylcarbamate **230*



Following the procedure of Murai *et al.*,³⁰¹ a solution of **229** (1.90 g, 3.88 mmol, 1.00 eq.) in anhydrous diethyl ether (39 mL) was added to a flask containing liquid ammonia (approx. 5 mL) at -78 °C. The reaction was stirred at -78 °C for 1 h, before it was allowed to warm to room temperature and stirred for an additional 4 h. The excess ammonia gas was removed in a fumehood before the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane:dichloromethane (70-100%), then hexane:ethyl acetate (50:50)] to afford **230** as a white solid (652 mg, 51%).

Mp: 54 - 56 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 7.47 (2H, d, *J* = 7.9 Hz, H-3 and H-5), 7.17 (2H, d, *J* = 7.9 Hz, H-2 and H-6), 3.30 (2H, q, *J* = 6.8 Hz, NCH₂), 2.73 (3H, m, PhCH₂ and F₃CCNH), 2.14 (1H, d, *J* = 8.8 Hz, F₃CCNH), 1.35 (9H, s, 3 x CH₃). ¹³C NMR (101 MHz, CDCl₃): δ_C 155.92 (C), 141.50 (C), 129.94 (C), 129.32 (2 x CH), 128.43 (2 x CH), 123.65 (q, *J* = 278.3 Hz, C), 79.47 (C), 57.96 (q, *J* = 35.9 Hz, C), 41.68 (CH₂), 36.15 (CH₂), 28.48 (3 x CH₃). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -75.57 (3F, s, CF₃). HRMS (ESI⁺): C₁₁H₁₃F₃N₃O₂ (lost *t*-Bu group) requires 276.0954 found 276.0957 (*M* + *H*)⁺. The ¹H and ¹³C NMR data is in agreement with that previously reported.³⁰¹

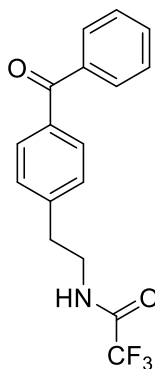
***N*-Trifluoroacetyl-*B*-phenethylamine 231**



Trifluoroacetic anhydride (1.32 mL, 9.53 mmol, 1.20 eq.) and triethylamine (2.22 mL, 15.9 mmol, 2.00 eq.) were added to a solution of phenylethylamine in anhydrous dichloromethane (12 mL) and the reaction was stirred at room temperature for 16 h. The reaction was diluted with dichloromethane (30 mL) and washed with a saturated solution of NaHCO₃ (30 mL). The aqueous layer was extracted with dichloromethane (2 x 20 mL). The organic layers were combined, dried with MgSO₄ and the solvent was removed under reduced pressure to afford **231** as a yellow solid (1.77g, quant.).

Mp: 43 - 44 °C. ¹H NMR (400 MHz, CDCl₃): δ_H 7.38 - 7.15 (5H, m, Ph), 6.29 (1H, br s, NH), 3.63 (2H, q, *J* = 6.6 Hz, NCH₂), 2.89 (2H, t, *J* = 7.0 Hz, PhCH₂). ¹³C NMR (101 MHz, CDCl₃): δ_C 157.23 (q, *J* = 36.9 Hz, C), 137.58 (C), 128.92 (2 x CH), 128.69 (2 x CH), 127.02 (CH), 115.81 (q, *J* = 287.8 Hz, C), 41.05 (CH₂), 34.96 (CH₂). ¹⁹F NMR (377 MHz, CDCl₃): δ_F -76.03 (3F, s, CF₃). HRMS (ESI⁺): C₁₀H₉F₃NO requires 216.0642 found 216.0643 (*M* - H)⁺. The ¹H and ¹³C NMR data is in agreement with that previously reported.³⁰¹

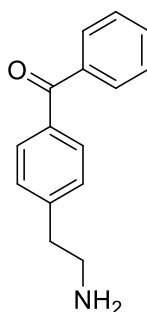
N-(4-Benzoylphenethyl)-2,2,2-trifluoroacetamide **232**



Following the procedure of Murai *et al.*,³⁰¹ benzoic anhydride (1.25 g, 5.53 mmol, 2.00 eq.) was added to a solution of **231** (600 mg, 2.77 mmol, 1.00 eq.) in triflic acid (2 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and stirred for 4 h under argon. The reaction was quenched with ice-water (30 mL) and ethyl acetate (30 mL) was added. The organic layer was separated and washed with brine (30 mL). The organic layer was separated again, dried with MgSO₄ and the solvent was removed under reduced pressure. The crude material was purified by column chromatography [SiO₂, hexane: ethyl acetate 5-30%] to afford **232** as a pale-yellow solid (638 mg, 72%).

Mp: 45 - 46 °C. ^1H NMR (400 MHz, CDCl_3): δ_{H} 7.82 - 7.28 (9H, m, Ph), 6.54 (1H, s, NH), 3.67 (2H, q, J = 6.8 Hz, NCH_2), 2.99 (2H, t, J = 7.1 Hz, PhCH_2). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 196.47 (C), 157.44 (q, J = 37.2 Hz, C), 142.66 (C), 137.63 (C), 136.50 (C), 132.65 (CH), 130.83 (2 x CH), 130.10 (2 x CH), 128.79 (2 x CH), 128.47 (2 x CH), 115.88 (q, J = 287.8 Hz, C), 40.88 (CH_2), 35.13 (CH_2). ^{19}F NMR (377 MHz, CDCl_3): δ_{F} -75.59 (3F, s, CF_3). HRMS (ESI^+): $\text{C}_{17}\text{H}_{15}\text{F}_3\text{NO}_2$ requires 322.1049 found 322.1056 ($\text{M} + \text{H}$) $^+$. The ^1H and ^{13}C NMR data is in agreement with that previously reported.³⁰¹

[4-(2-Aminoethyl)phenyl](phenyl)methanone **233**



Following the procedure of Murai *et al.*,³⁰¹ **232** (1.12 g, 3.47 mmol, 1.00 eq.) was dissolved in methanol (9 mL) and NaOH (3M, 9 mL), and the reaction was stirred at room temperature for 16 h. The reaction was diluted with ethyl acetate (50 mL) and washed with brine (2 x 50 mL). The organic layer was separated, dried with MgSO_4 and the solvent was removed under reduced pressure to afford **233** as a yellow oil (686 mg, 87%).

^1H NMR (400 MHz, CDCl_3): δ_{H} 7.84 - 7.29 (9H, m, Ph), 3.03 (2H, t, J = 6.9 Hz, NCH_2), 2.84 (2H, t, J = 6.9 Hz, PhCH_2). ^{13}C NMR (101 MHz, CDCl_3): δ_{C} 196.58 (C), 145.19 (C), 137.95 (C), 135.81 (C), 132.41 (CH), 130.59 (2 x CH), 130.12 (2 x CH), 128.92 (2 x CH), 128.39 (2 x CH), 43.44 (CH_2), 40.28 (CH_2). HRMS (ESI^+): $\text{C}_{15}\text{H}_{16}\text{NO}$ requires 226.1226 found 226.1231 ($\text{M} + \text{H}$) $^+$. The ^1H and ^{13}C NMR data is in agreement with that previously reported in the literature.³⁰¹

5.2 Protein Chemistry

5.2.1 DNA Sequences

The human GST A1-1, A4-4 and K1-1 DNA sequences were purchased as GeneArt Strings double stranded DNA fragments from ThermoFisher Scientific.

5.2.2 Luria Bertani (LB) media for agar plates

Bacterial cultures were grown on agar plates made using an agar solution of 30 μ M kanamycin in Luria Bertani (Lennox) broth, which contained the following reagents: 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar per 1 L of deionised water. The agar solution was pipetted onto sterile culture plates to a depth of approximately 0.5 cm in a laminar flow hood. Plates were allowed to cool until the agar became solid and then stored at 4 °C.

5.2.3 Terrific Broth (TB) media for bacterial growth

The Terrific Broth (TB) media used for all bacterial growths was made by the following reagents: 6 g Na₂HPO₄, 3 g KH₂PO₄, 10 g tryptone, 5 g yeast extract and 5g NaCl per 1 L deionised water, autoclaved at 126 °C for 45 min.

5.2.4 Acrylamide gel for SDS-PAGE

The following running buffer recipe made enough for two 15% acrylamide gels: 4.58 mL water, 5.0 mL Tris pH 8.8, 10.0 mL acrylamide (30%), 0.2 mL SDS (10%), 0.2 mL APS (10%) and 0.025 mL TEMED added in the order as listed. The stacking buffer was made by the following recipe: 5.98 mL water, 2.5 mL Tris pH 8.8, 1.30 mL acrylamide (30%), 0.1 mL SDS (10%), 0.1 mL APS (10%) and 0.025 mL TEMED added in the order as listed. The acrylamide gel was prepared by first pouring the running buffer in between two glass plates fitted to a PAGE apparatus. The top was covered with a few drops of isopropanol to eliminate bubbles from the meniscus. After the separation gel had polymerised, the

isopropanol was discarded and the stacking gel poured on top of the solid separation gel and a sample comb inserted in between the glass plates. After the stacking gel had polymerised, the combs were removed to create wells for samples.

5.2.5 Enzyme restriction of pNIC-28-*Bsa*I vector

2 µg pNIC-28-*Bsa*I plasmid, 2 µL 10X buffer, 1 µL *Bsa*I fast-enzyme and 1 µL ECO105I fast-enzyme were combined in an Eppendorf tube and made up with sterile water to 20 µL, making sure to add the enzymes last. The reaction was incubated at 37 °C for 10 minutes and then inactivated at 65 °C for 10 minutes.

5.2.6 T4 DNA polymerase treatment of vector

4 µL 5X New England Biolabs buffer, 600 ng *Bsa*I-digested vector, 0.5 µL dGTP (100 mM), 1 µL DTT (100 mM) and 0.4 µL T4 DNA polymerase (3U/µL) were combined in an Eppendorf tube and made up with sterile water to a volume of 20 µL, making sure to add the polymerase last. The reaction was incubated at 18 °C for 35 minutes and then inactivated at 75 °C for 20 minutes.

5.2.7 T4 DNA polymerase treatment of the DNA insert

4 µL 5X New England Biolabs buffer, 0.2 pmol DNA insert, 0.5 µL dCTP (100 mM), 1 µL DTT (100 mM) and 0.4 µL T4 DNA polymerase (3U/µL) were combined in an Eppendorf tube and made up with sterile water to a volume of 20 µL, making sure to add the polymerase last. The reaction was incubated at 18 °C for 35 minutes and then inactivated at 75 °C for 20 minutes.

5.2.8 Annealing of the insert and the vector

1 µL T4 polymerase-treated vector and 2 µL T4 polymerase-treated DNA insert were combined in an Eppendorf tube and the annealing mixture was incubated at 18 °C for 10

minutes. 1 μ L EDTA (25 mM) was added, and the mixture incubated at 18 °C for 10 minutes.

5.2.9 Transformation

1 μ L of the annealing mixture was added to a new Eppendorf tube and incubated on ice for 5 minutes. 50 μ L of *E. coli* BL21 (DE3) competent cells was added and the mixture incubated on ice for 30 minutes. The mixture was then heat-shocked in a water bath at 42 °C for 45 seconds. The mixture was again incubated on ice for 2 minutes. 200 μ L of SOC medium was added to the Eppendorf tube, and the mixture incubated at 37 °C in an orbital shaker for 1 hour, at a speed of 250 rpm. The solution was then pipetted onto the centre of a warmed LB agar plate containing 30 μ g/mL kanamycin and spread evenly across the surface using a sterile glass rod. The plates were incubated overnight at 37 °C. A negative control plate was also prepared, following the same procedure, but with the omission of plasmid DNA.

5.2.10 Overnight growth of a bacterial colony

A single colony was selected from the LB agar plates (or from the glycerol strains, see below), looped using a sterile plastic loop and added to 10 mL of LB media in a universal vessel. This was then incubated in an orbital shaking incubator overnight at 37 °C.

5.2.11 Storage of bacterial strains

Bacterial colonies grown on selective resistant agar plates were sealed using Parafilm and stored at 4 °C for up to 4 weeks. For long term storage an overnight culture was grown from a single colony, mixed with 100 % glycerol to a final concentration of 80:20 glycerol/culture (v/v) and stored at -80 °C.

5.2.12 Overexpression of protein

1.8 mL of a 1 M stock solution of kanamycin was added to 1.8 L of TB media to give a 30 μ M concentration of antibiotic, followed by addition of 18 mL of 60 % glycerol solution and 9 mL of 10% glucose solution. The TB was divided into 300 mL portions in 6 x 2 L conical flasks and placed in an orbital shaking incubator at 220 rpm and 37 °C. When the TB media had reached the required temperature, 5 mL of the overnight growth solutions at 37 °C were pipetted into separate conical flasks. The top of each flask was covered using aluminium foil and all flasks were returned to the incubator until cell density reached an OD₆₀₀ of approximately 0.8. At this point, 0.1 mM isopropyl- β -D-thiogalactoside (IPTG) was added to induce expression of the plasmid, and all flasks were shaken at 220 rpm and 15 °C overnight. Each solution was then decanted into two centrifuge pots and centrifuged at 4000 rpm (3400 g) for 20 min with a Sigma 4 K15 centrifuge at 20 °C. The supernatant was discarded while the pellets containing the same proteins were combined and resuspended in 35 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl buffer pH 8.0).

5.2.13 Cell lysing

A spatula tip of lysozyme crystals and 2 μ L benzonase nuclease (25 kunits) were added to the solutions and inverted over 10 minutes. The solutions were then sonicated (20 kHz, 125 W at half power, 15 s on with 30 s off for 30 cycles) on ice to prevent overheating. After sonification the solution was centrifuged at 24,000 rpm (50,870 g) with a Sigma 3 K30 centrifuge at 4 °C for 30 min. The soluble fraction was then decanted into a clean storage vessel and the insoluble cell material discarded.

5.2.14 Affinity column purification

The soluble fraction was loaded onto a gravity flow Ni-NTA agarose column and the proteins purified by immobilized nickel ion affinity chromatography. The column was first equilibrated with 20 mL of Buffer A (50 mM Tris-HCl, 250 mM NaCl, pH 8.0). The protein solution was then passed through the column and the flow-through collected. The column was washed with a further 5 mL of Buffer A and collected with the flow through. The

column was then washed with 10 mL of Buffers B (50 mM Tris-HCl, 250 mM NaCl, 50 mM Imidazole, pH 8.0), C (50 mM Tris-HCl, 250 mM NaCl, 75 mM Imidazole, pH 8.0) and D (50 mM Tris-HCl, 250 mM NaCl, 250 mM Imidazole, pH 8.0) and these fractions collected in separate containers. The concentration and activity of fractions from Buffer B, C and D were analysed using a nanodrop ND-1000 spectrophotometer.

5.2.15 Dialysis and storage of proteins

The 250 mM imidazole fractions, which contained the most protein, were buffer-exchanged with a low salt buffer (50 mM Tris-HCl, 150 mM NaCl at pH 8) by dialysis. Protein concentration was measured by UV absorption at 280nm (using $\epsilon_{280\text{nm}} = 1.291$, 0.772 and 0.665 for GSTs K1-1, A1-1 and A4-4, respectively), before storage at -14 °C in 50% glycerol with 1 μL of dithiothreitol (1M) added as a reducing agent. The final concentration of the GST enzyme solutions was 2.93 mg/mL of GSTA1-1, 2.11 mg/mL of GSTA4-4 and 1.43 mg/mL of GSTK1-1. Each GST enzyme was obtained in high yield with 28 mg of GSTK1-1, 59 mg of GSTA1-1, and 42 mg of GSTA4-4, recovered from 0.6 L cultures. The vector derived 6xHistidine-tag and TEV (Tobacco Etch Virus Protease) recognition sequence were not cleaved from the proteins.

5.2.16 SDS-PAGE

The agarose gel was prepared from the running and stacking buffers (recipe described in 5.2.4). The SDS-PAGE running buffer (3.02 g Tris, 14.4 g glycine and 0.8 g SDS per 1 L of water) was then added to the tank until the gel was submerged to an appropriate depth. The outermost wells of the gel were loaded with 4 μL molecular weight marker while the inside wells were loaded with 15-20 μL protein sample. To make the protein samples, 15 μL protein stock solution (0.26 mg/mL) was mixed with 15 μL of a DTT stock (100 mM) in 2x sample buffer in an Eppendorff tube. The protein solutions were placed on a heat block at 80 °C for 5 minutes and then centrifuged at 5,000 rpm for 30 seconds. The gel was run at 300 V and 20 mA for 1h 30min. The gel was removed from the glass plates, rinsed with water and stained in Coomassie Brilliant Blue on an orbital shaker overnight, which confirmed each protein had been isolated in good purity.

5.2.17 Plasmid purification for DNA sequencing

Plasmid DNA was isolated using the New England BioLabs Monarch Miniprep kit. The compositions of the solutions included are proprietary and so cannot be included. 5 mL of overnight bacteria culture solution was centrifuged at 5,000 rpm for 30 seconds. The supernatant was discarded and the pellet resuspended in 200 μ L Plasmid Resuspension Buffer. The solution was then transferred to an Eppendorf tube, 200 μ L Plasmid Lysis Buffer added and mixed by inverting five times. The dark pink solution was incubated for no longer than 5 minutes, until the solution became transparent and viscous. 400 μ L Plasmid Neutralisation Buffer was added, inverted to mix and incubated for 2 minutes, by which a precipitate formed. The solution was then centrifuged at 13,000 rpm for 5 minutes at room temperature. A spin column was inserted into a 2 mL collection tube and the cleared lysate from the centrifuged solution was decanted into the spin column. This was then centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the spin column re-inserted into the collection tube. The spin column was then washed with 200 μ L of Plasmid Wash Buffer 1 and centrifuged at 13,000 rpm for 1 minute, followed by washing with 400 μ L of Plasmid Wash Buffer 2 and centrifugation again. The spin column was transferred to a new Eppendorf tube. The plasmid DNA was eluted with 30 μ L of nuclease free water, and after incubation for 1 minute, the DNA was centrifuged at 13,000 rpm for 1 minute. The spin column was discarded and the plasmid concentration determined using a nanodrop ND-1000 spectrophotometer. The DNA solution was diluted to approximately 100 ng/ μ L and sent for Sanger sequencing to confirm successful cloning.

5.3 Kinetics

5.3.1 UV/Vis absorbance kinetics and calculation of enzyme kinetic constants

UV-Vis absorbance spectra and kinetics were performed using a Jasco V550 UV/Vis spectrometer at 30 °C with quartz cuvettes (0.3-1 mL in volume, 0.1-1 cm in pathlength). Assays were conducted in either a 0.1 M sodium phosphate buffer (pH 6.5) (diluted from a 1 M stock of 1 M NaH_2PO_4 and 1 M Na_2HPO_4 in a 68.5:31.5 ratio), or a 0.1 M HEPES-HCl buffer (pH 8.0). The buffers were adjusted to the desired pH with HCl or NaOH. The reaction rate was monitored by measuring the initial rate of formation of the glutathione (GS) conjugate.

To fully characterise the GST enzymes with respect to CDNB **48** and GSH **25**, the concentration of one substrate was kept constant at near-saturating concentrations (if possible), while the concentration of the other substrate was varied. In the assays for CDNB **48** and the other chloronitrobenzene derivatives (MitoCDNB **51**, esters **85a-c** and amides **86a-c**), GSH **25** concentration was fixed at 10 mM to ensure saturation. In the assays for GSH **25**, CDNB **48** concentration was fixed at 1 mM due to its poor aqueous solubility above this concentration. This was likely insufficient to saturate the enzymes, so kinetic constants derived under non-saturating conditions were reported as ‘apparent’. In the assays for the sulfonamides, MitoHexNAB **122** and MitoHexDNB **125** were fixed at a concentration of 0.1 mM and GSH **25** was fixed at 2 mM. To calculate the background rates and enzyme-catalysed rates for the compounds, a suitable absorbance wavelength and molar extinction coefficient needed to be determined. The UV-Vis absorbance spectra were measured between 220 nm and 600 nm for the compounds and their corresponding GS-conjugates, both at 0.1 mM. From the spectra a suitable wavelength for measurement was identified at which there was minimal overlap between the compound and GS-conjugate.

The molar extinction coefficients for the chloronitrobenzene derivatives were:

- CDNB **48**: 0.578 mM⁻¹cm⁻¹ at 340 nm
- MitoCDNB **51**: 7.88 mM⁻¹cm⁻¹ at 328 nm
- **85a**: 2.05 mM⁻¹cm⁻¹ at 286 nm
- **85b**: 0.704 mM⁻¹cm⁻¹ at 343 nm
- **85c**: 0.780 mM⁻¹cm⁻¹ at 348 nm
- **86a**: 1.16 mM⁻¹cm⁻¹ at 284 nm
- **86b**: 0.687 mM⁻¹cm⁻¹ at 342 nm
- **86c**: 0.979 mM⁻¹cm⁻¹ at 345 nm.

The molar extinction coefficients for the sulfonamides were:

- MitoHexNAB **122**: 3.23 mM⁻¹cm⁻¹ at 284 nm
- MitoHexDNB **125**: 3.11 mM⁻¹cm⁻¹ at 328 nm.

The molar extinction coefficients for the GS-conjugates were:

- GSDNB **50**: 9.60 mM⁻¹cm⁻¹ at 340 nm
- MitoGSDNB **52**: 17.3 mM⁻¹cm⁻¹ at 328 nm
- **88a**: 11.4 mM⁻¹cm⁻¹ at 286 nm

- **88b**: 14.1 mM⁻¹cm⁻¹ at 343 nm
- **88c**: 7.48 mM⁻¹cm⁻¹ at 348 nm
- **92a**: 7.88 mM⁻¹cm⁻¹ at 284 nm
- **92b**: 11.2 mM⁻¹cm⁻¹ at 342 nm
- **92c**: 5.91 mM⁻¹cm⁻¹ at 345 nm.

All assay mixtures were prepared by adding the GSH **25**, followed by the GST enzyme, to the compound dissolved in buffer and mixed by shaking *in situ*. The rate of the reaction was measured for at least 60 s. The initial rate of reaction was determined using the Jasco V550 software, selecting initial data to maximise the correlation with a straight line. Background rates were measured once at pH 6.5, while at pH 8.0, the background rate was appreciable and were measured in triplicate. For the chloronitrobenzene derivatives, the enzyme-catalysed reaction rates were measured at a variety of compound concentrations in triplicate. For the sulfonamides, MitoHexNAB **122** and MitoHexDNB **125**, the background chemical and enzyme-catalysed rates were measured for 0.1 mM of compound in triplicate. For a given concentration of the compound of interest, the averaged background rate was subtracted from the averaged enzyme-catalysed reaction rate to obtain the real enzyme-catalysed reaction rate in A s⁻¹. Using the Beer-Lambert Law and the difference in molar extinction coefficients between the compound and the corresponding GS-conjugate, the real enzyme-catalysed rate was converted to mM s⁻¹. To calculate the catalytic rate constant, k_{cat} , and the Michaelis-Menten constant, K_{M} , the initial rate data (in mM s⁻¹) was fitted in Microsoft Excel[®] to the Michaelis-Menten equation using non-linear regression for compounds where non-linear behaviour was observed, or, to the Lineweaver-Burk model where linear (or near-linear) behaviour was observed. Standard errors were calculated using the method of Kitaoka for non-linear regression,³⁰² and the LINEST function (in Microsoft Excel[®]) for linear regression. To calculate specific activity (in μmol.min⁻¹mg⁻¹) at a single concentration of a given compound, the initial rate data (in μM min⁻¹) was divided by the total volume of the assay solution (in μL) and the amount of enzyme (in mg). Errors in the mean specific activity were calculated as the ±1 standard deviation of three specific activities determined from the triplicate measurements.

5.3.2 Fluorescence kinetics and calculation of rate constants

UV-Vis absorbance spectra, fluorescence spectra and fluorescence kinetics were measured for the coumarin-containing compounds: 7-aminocoumarin **104**, 7-hydroxycoumarin **105**, ACoum1 **165**, ACoum2 **166**, HCoum1 **167**, HCoum2 **168**, MitoACoum1 **175**, MitoACoum2 **176**, MitoHCoum1 **178** and MitoHCoum2 **179**. Measurements were performed at 30 °C using a Horiba Duetta spectrometer and a quartz cuvette with a pathlength of 1 cm. UV-Vis absorbance spectra were recorded for 100 μM solutions of each compound in 0.1 M HEPES-HCl buffer (pH 8.0) between 220 nm and 800 nm. Fluorescence emission spectra were then obtained for 10 μM solutions, using the wavelength of maximum absorbance as the excitation wavelength, and measuring between 350 nm and 750 nm. Calibration curves were plotted for 7-aminocoumarin **104**, 7-hydroxycoumarin **106**, ACoum1 **165**, ACoum2 **166** in pH 8.0 buffer by measuring fluorescence at six different concentrations (ranging between 0.625 and 25 μM). Fluorescence intensity was plotted against concentration to produce a linear line. For ACoum1 **165**, ACoum2 **166**, HCoum1 **167**, HCoum2 **168**, fluorescence assays were performed in triplicate using 10 μM of compound in buffer at 30 °C. For MitoACoum1 **175**, MitoACoum2 **176**, MitoHCoum1 **178** and MitoHCoum2 **179**, assays were performed in triplicate under the same conditions but in the presence of excess GSH **25** to ensure that the reactions observed pseudo-first order behaviour. For the reactions of MitoHCoum1 **177** or MitoHCoum2 **178**, the assays were performed using 10 mM GSH **25** to emulate the concentration within the mitochondrial matrix. For the reactions of MitoACoum1 **175** and MitoACoum2 **176** with 10 mM GSH **25**, the reaction rate was too fast to obtain an accurate initial rate, so 2 mM GSH **25** was used instead. The reaction was monitored by measuring the initial rate of formation of the appropriate coumarin by fluorescence: 7-aminocoumarin **104** (for ACoum1 **165** and ACoum2 **166**), 7-hydroxycoumarin **106** (for HCoum1 **167**, HCoum2 **168**, MitoHCoum1 **177** and MitoHCoum2 **178**), ACoum1 **165** (for MitoACoum1 **175**) and ACoum2 **166** (for MitoACoum2 **176**). The excitation wavelength was the wavelength of maximum absorbance and the emission wavelength was the wavelength of maximum emission for the appropriate coumarin. Fluorescence intensity was recorded every 10 s for at least 60 s, and the initial rate data (in arbitrary units per second, a.u. s^{-1}) was converted to $\mu\text{M s}^{-1}$ using the appropriate calibration curve. For each of the triplicate measurements, the initial rate data was plotted against time to produce a linear line. The first (or pseudo-first) order rate constant was determined by dividing the gradient of the line by the concentration of the compound. The second-order rate

constant was calculated by dividing the pseudo-first order rate constant by the concentration of GSH **25**. Errors in the rate constants were calculated as the ± 1 standard deviation of three rate constants determined from triplicate measurements.

5.3.3 HPLC and LCMS kinetics

HPLC-based reaction kinetics were performed using a Shimadzu LC-20AD Liquid Chromatograph fitted with an SPD-20A UV-Vis detector. LCMS-based reaction kinetics were performed using a nominal mass Agilent 6125B mass spectrometer attached to an Agilent 1290 Infinity UHPLC, fitted with a Diode Array Detector (DAD) and electrospray (ESI) source. Absorbance was recorded at 254 and 280 nm. To measure the kinetics of MitoMetNAB **108** by HPLC and LCMS, 100 μM of compound was treated with pH 8.0 buffer at 30 °C and a 10 μL aliquot was injected into the instrument every hour for 24 h. To measure the kinetics of MitoMetSDNB **96** by HPLC, 100 μM of compound was treated with 10 mM GSH **25** (with or without 1 μL of the GST enzyme solutions described in Section 5.2.15) in pH 8.0 buffer at 30 °C. A 10 μL aliquot was injected into the instrument every hour for 24 h. To measure the kinetics of MitoAtov1 **187** and MitoAtov2 **188** by LCMS, 10 μM of compound was treated with 2 mM GSH **25** in pH 8.0 buffer at 30 °C and a 100 μL aliquot was injected into the instrument every 20 min for 180 min.

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Appendix

7.1 UV-Vis spectra

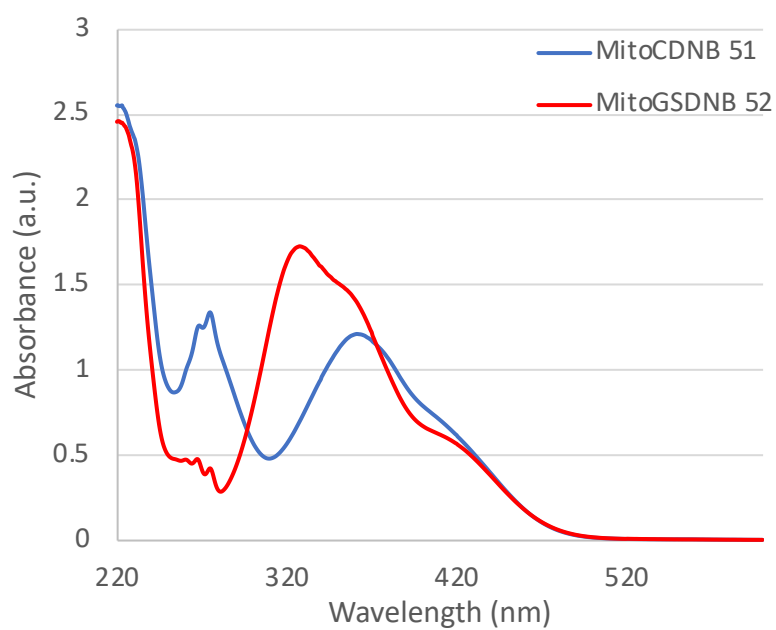


Figure 62. The UV-Vis absorbance spectra of MitoCDNB 51 (100 μM) and MitoGSDNB 52 (100 μM) in HEPES (0.1 M) pH 8.0 buffer. MitoCDNB 51: $\epsilon_{328\text{nm}} = 7.88 \text{ mM}^{-1}\text{cm}^{-1}$. MitoGSDNB 52: $\epsilon_{328\text{nm}} = 17.3 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{338\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$.

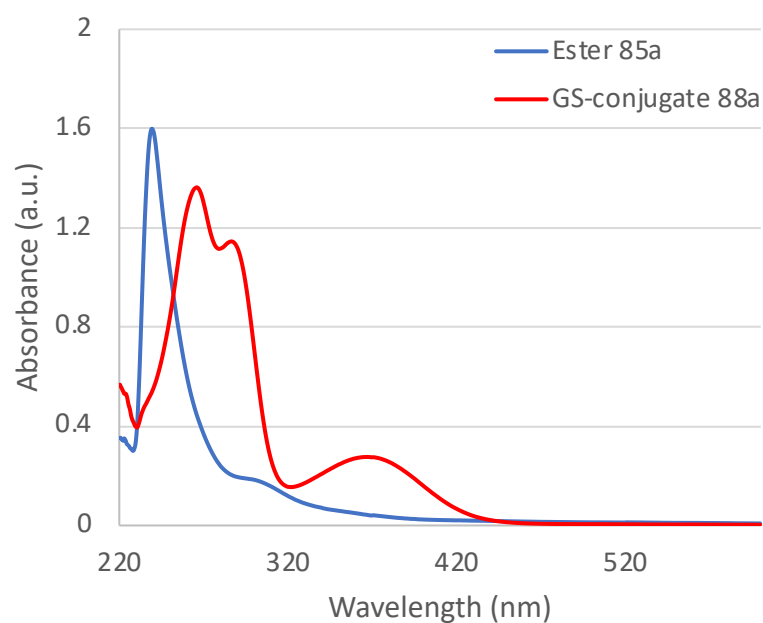


Figure 63. The UV-Vis absorbance spectra of compound 85a (100 μM) and corresponding GS-conjugate 88a (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 85a: $\epsilon_{286\text{nm}} = 2.05 \text{ mM}^{-1}\text{cm}^{-1}$. 88a: $\epsilon_{286\text{nm}} = 11.4 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{286\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$.

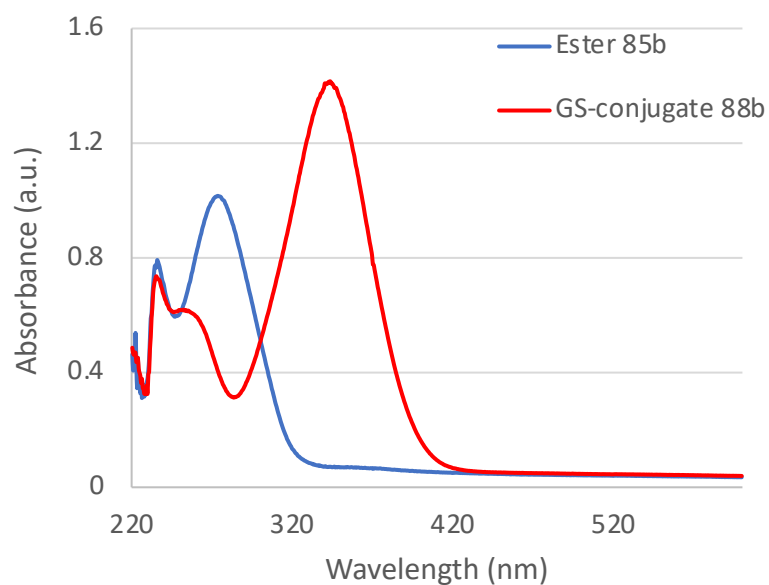


Figure 64. The UV-Vis absorbance spectra of compound 85b (100 μM) and corresponding GS-conjugate 88b (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 85b: $\epsilon_{343\text{nm}} = 0.704 \text{ mM}^{-1}\text{cm}^{-1}$. 88b: $\epsilon_{343\text{nm}} = 14.1 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{343\text{nm}} = 13.5 \text{ mM}^{-1}\text{cm}^{-1}$.

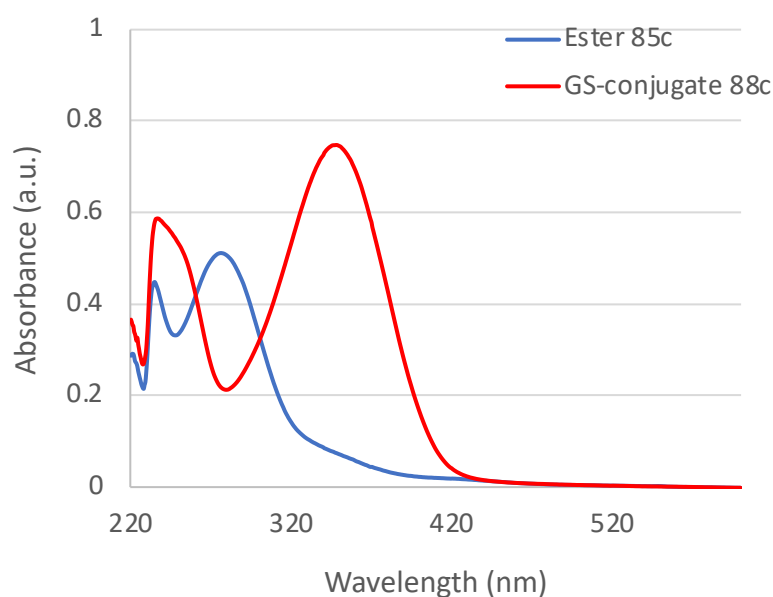


Figure 65. The UV-Vis absorbance spectra of compound 85c (100 μM) and corresponding GS-conjugate 88c (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 85c: $\epsilon_{348\text{nm}} = 0.780 \text{ mM}^{-1}\text{cm}^{-1}$. 88c: $\epsilon_{348\text{nm}} = 7.48 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{348\text{nm}} = 6.70 \text{ mM}^{-1}\text{cm}^{-1}$.

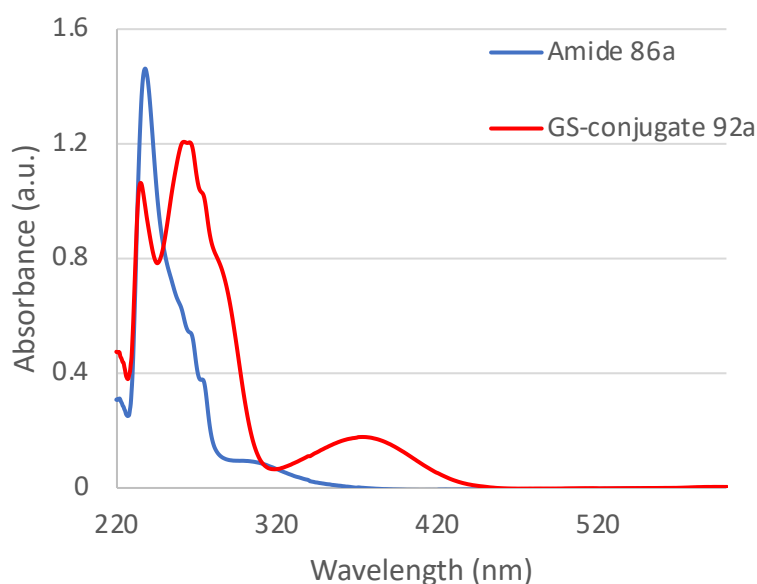


Figure 66. The UV-Vis absorbance spectra of compound 86a (100 μM) and corresponding GS-conjugate 92a (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 86a: $\epsilon_{284\text{nm}} = 1.16 \text{ mM}^{-1}\text{cm}^{-1}$. 92a: $\epsilon_{284\text{nm}} = 7.88 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{284\text{nm}} = 6.72 \text{ mM}^{-1}\text{cm}^{-1}$.

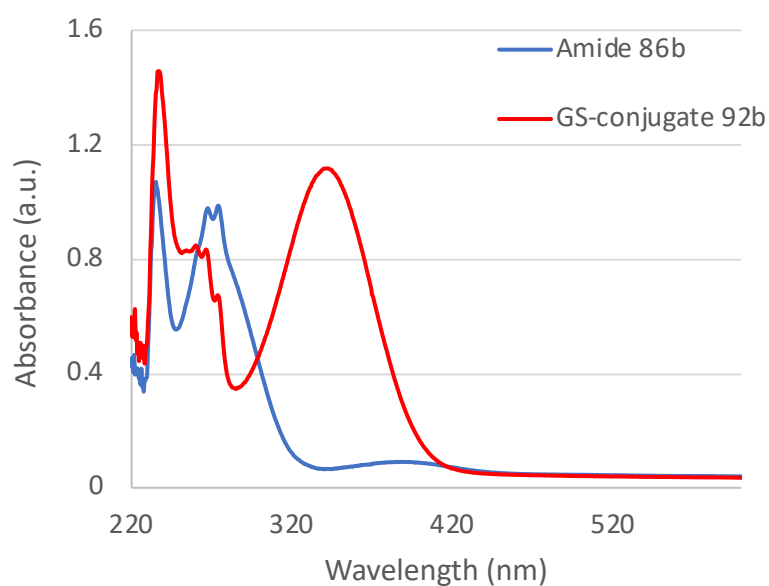


Figure 67. The UV-Vis absorbance spectra of compound 86b (100 μM) and corresponding GS-conjugate 92b (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 86b: $\epsilon_{342\text{nm}} = 0.687 \text{ mM}^{-1}\text{cm}^{-1}$. 92b: $\epsilon_{342\text{nm}} = 11.2 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{342\text{nm}} = \text{mM}^{-1}\text{cm}^{-1}$.

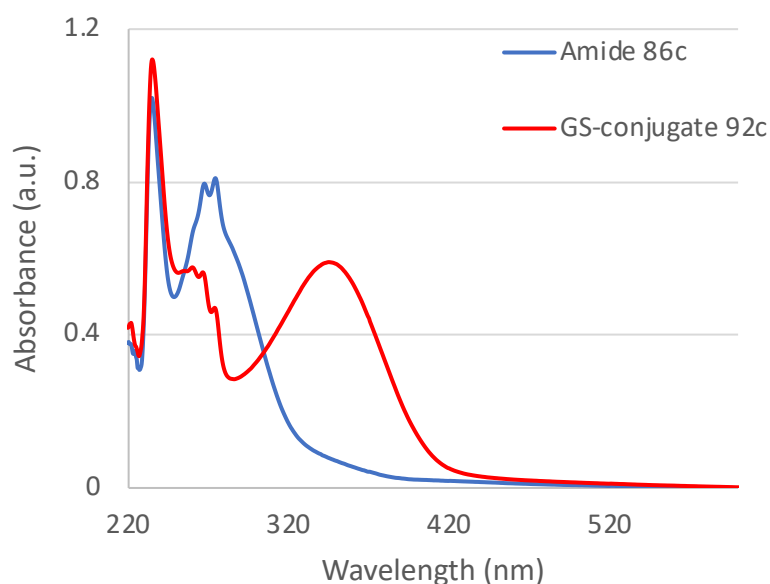


Figure 68. The UV-Vis absorbance spectra of compound 86c (100 μM) and corresponding GS-conjugate 92c (100 μM) in HEPES (0.1 M) pH 8.0 buffer. 86c: $\epsilon_{345\text{nm}} = 0.979 \text{ mM}^{-1}\text{cm}^{-1}$. 92c: $\epsilon_{345\text{nm}} = 5.91 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{345\text{nm}} = 4.93 \text{ mM}^{-1}\text{cm}^{-1}$.

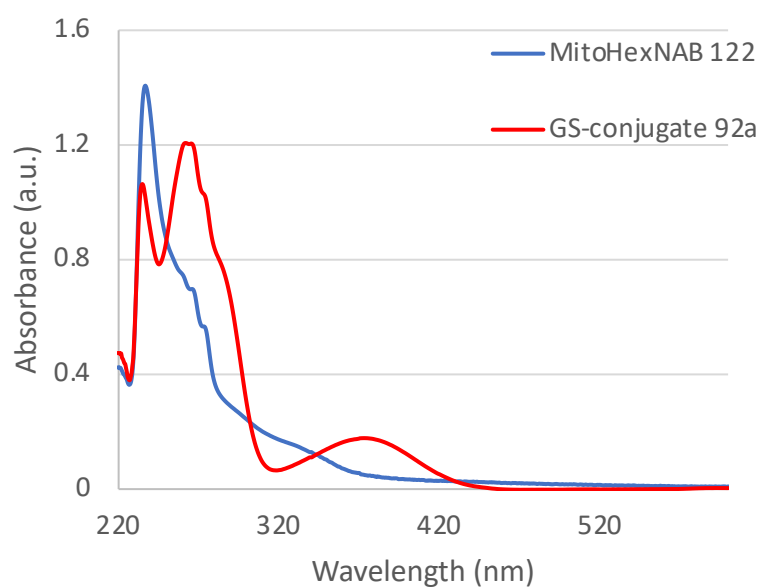


Figure 69. The UV-Vis absorbance spectra of MitoHexNAB 122 (100 μM) and GS-conjugate 92a (100 μM) at pH 8.0. MitoHexNAB 122: $\epsilon_{284\text{nm}} = 3.23 \text{ mM}^{-1}\text{cm}^{-1}$. GS-conjugate 92a: $\epsilon_{284\text{nm}} = 7.88 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{284\text{nm}} = 4.65 \text{ mM}^{-1}\text{cm}^{-1}$.

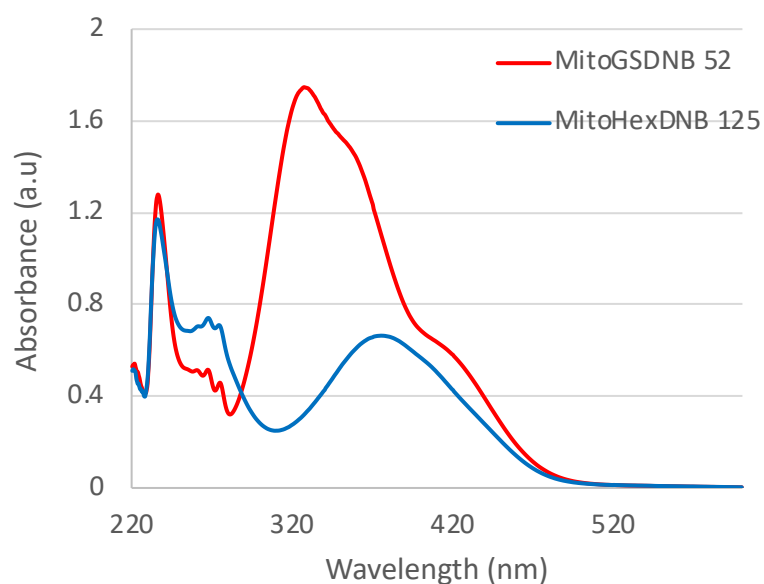


Figure 70. The UV-Vis absorbance spectra of MitoHexDNB 125 (100 μM) and MitoGSDNB 52 (100 μM) at pH 8.0. MitoCDNB 51: $\epsilon_{326\text{nm}} = 3.1 \text{ mM}^{-1}\text{cm}^{-1}$. MitoGSDNB 52: $\epsilon_{326\text{nm}} = 17.4 \text{ mM}^{-1}\text{cm}^{-1}$. $\Delta\epsilon_{326\text{nm}} = 14.3 \text{ mM}^{-1}\text{cm}^{-1}$.

7.2 Non-linear regression and Lineweaver-Burk plots

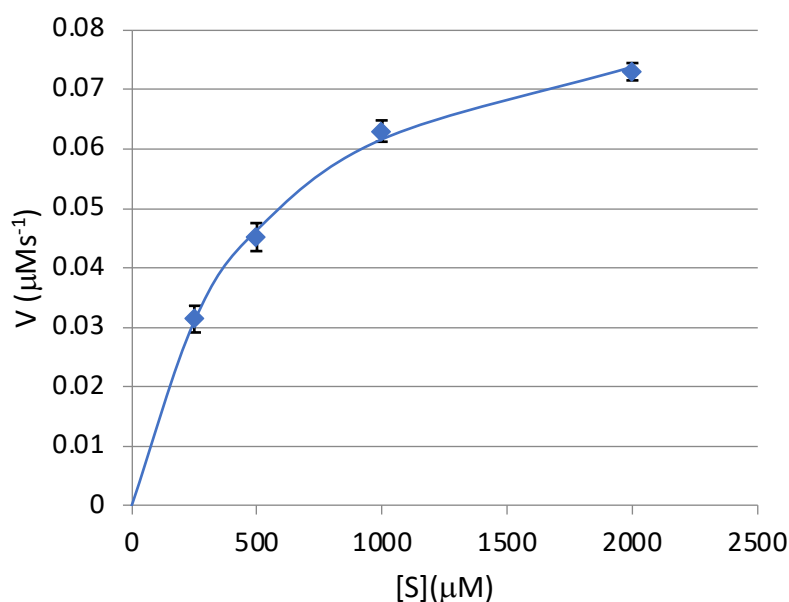


Figure 71. Non-linear plot of hGSTA1-1 for CDNB 48 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $2.60 \times 10^{-3} \text{ } \mu\text{M}$.

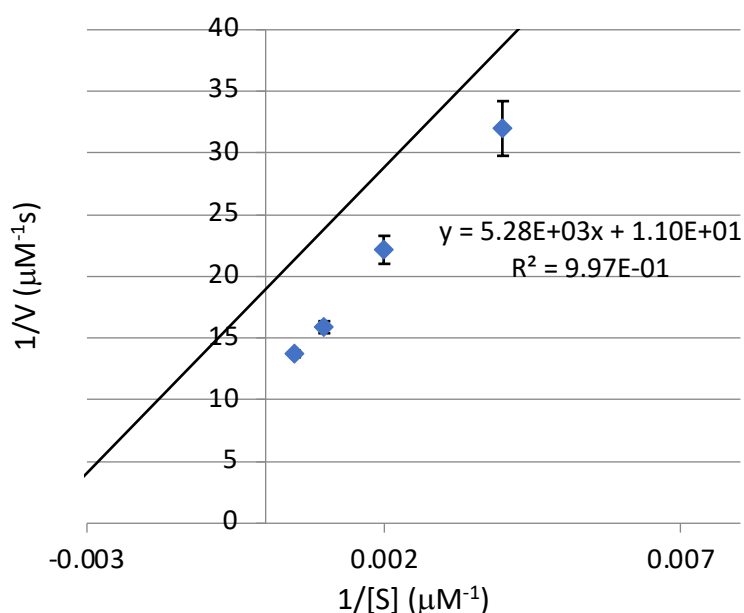


Figure 72. Lineweaver-Burk plot of hGSTA1-1 for CDNB 48 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $2.60 \times 10^{-3} \text{ } \mu\text{M}$.

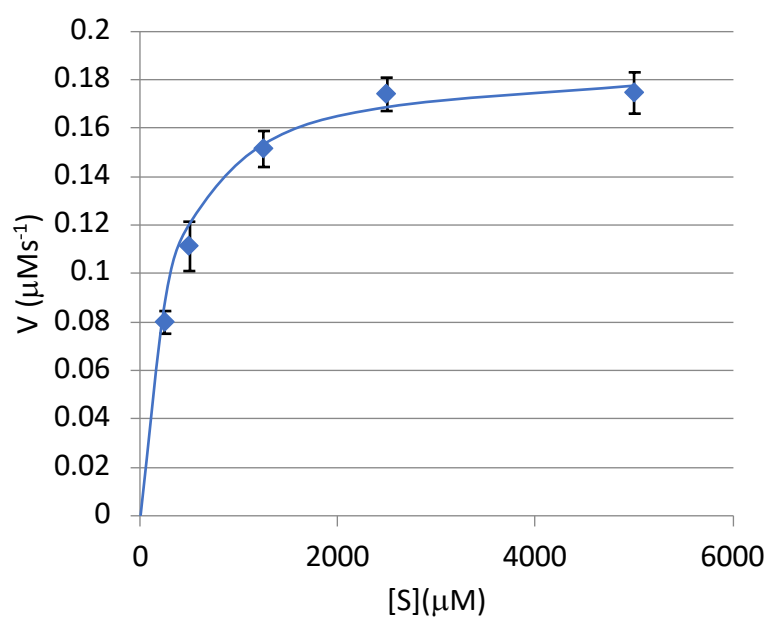


Figure 73. Non-linear plot of hGSTA1-1 for GSH 25 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

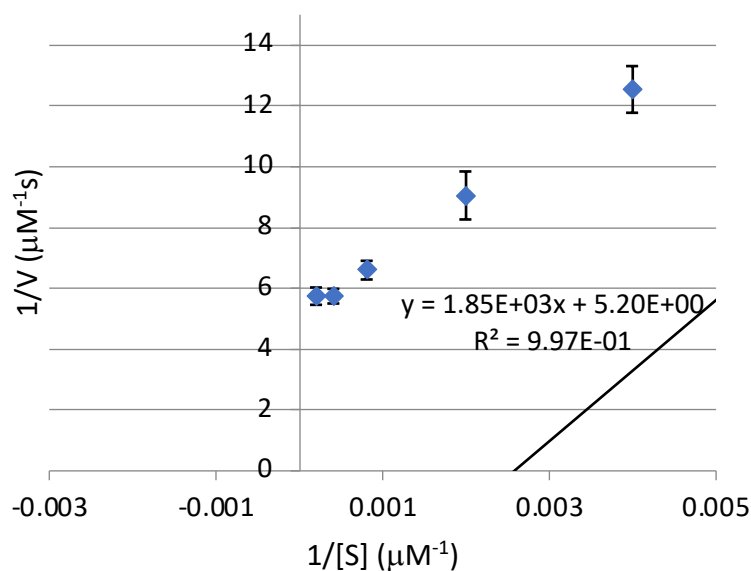


Figure 74. Lineweaver-Burk plot of hGSTA1-1 for GSH 25 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

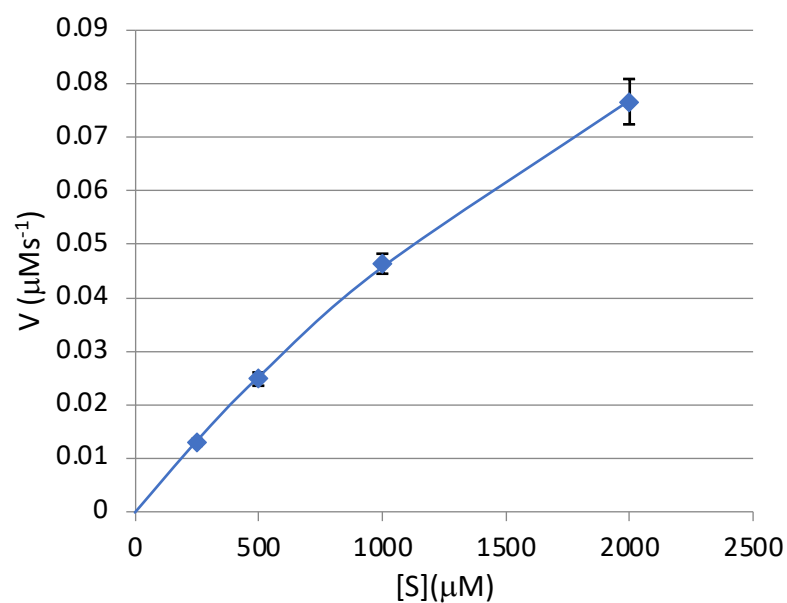


Figure 75. Non-linear plot of hGSTA4-4 for CDNB 48 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $1.87 \times 10^{-3} \text{ } \mu\text{M}$.

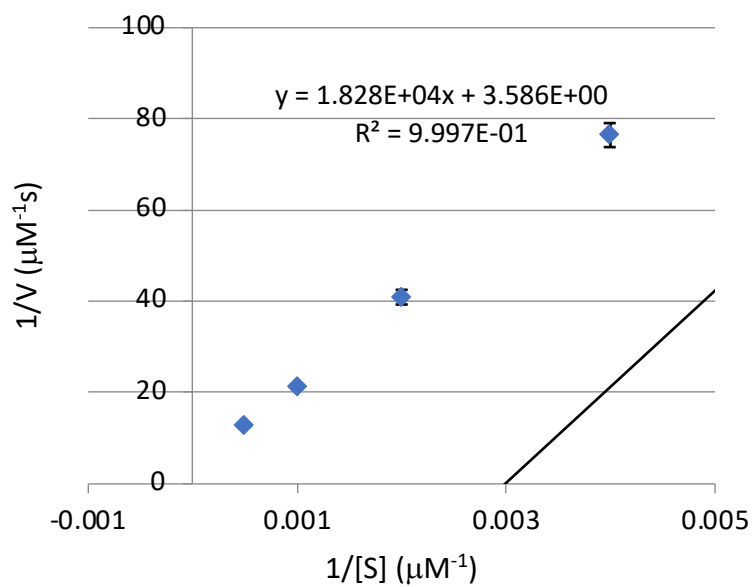


Figure 76. Lineweaver-Burk plot of hGSTA4-4 for CDNB 48 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $1.87 \times 10^{-3} \text{ } \mu\text{M}$.

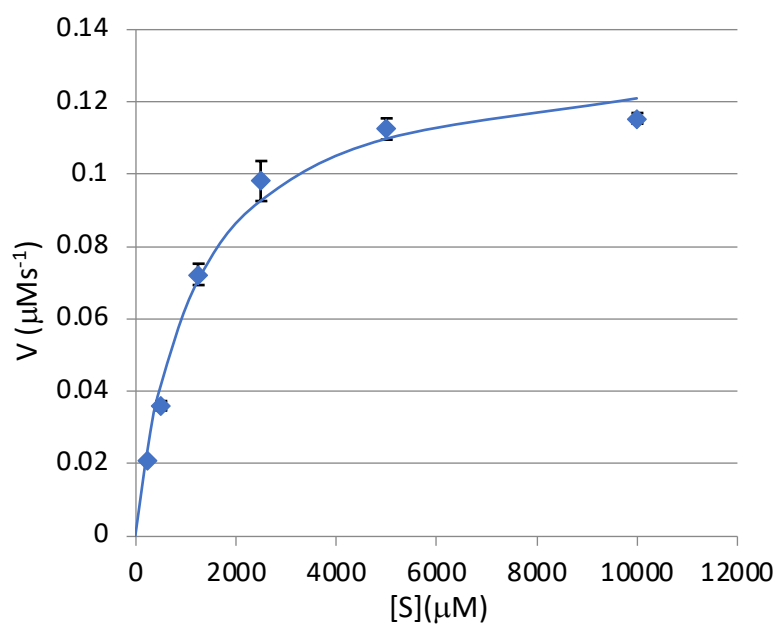


Figure 77. Non-linear plot of hGSTA4-4 for GSH 25 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $7.47 \times 10^{-3} \text{ } \mu\text{M}$.

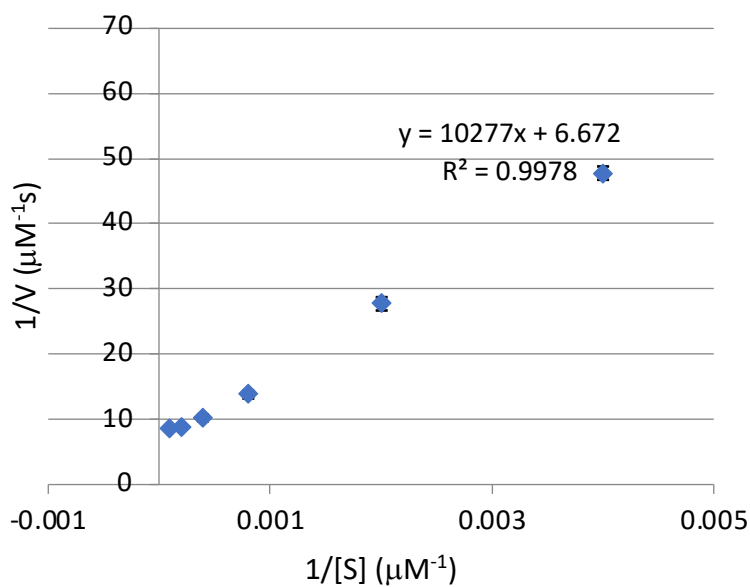


Figure 78. Lineweaver-Burk plot of hGSTA4-4 for GSH 25 at pH 6.5, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $7.47 \times 10^{-3} \text{ } \mu\text{M}$.

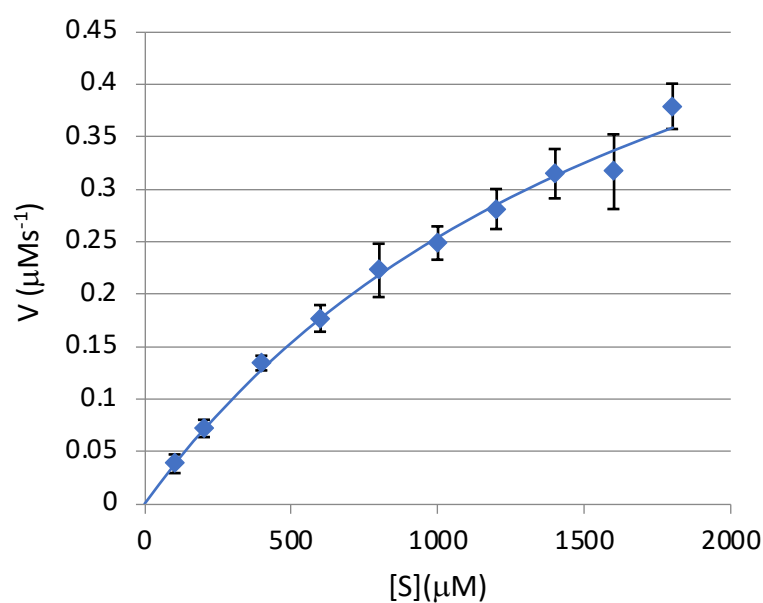


Figure 79. Non-linear plot of hGSTK1-1 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.06 \times 10^{-2} \mu\text{M}$.

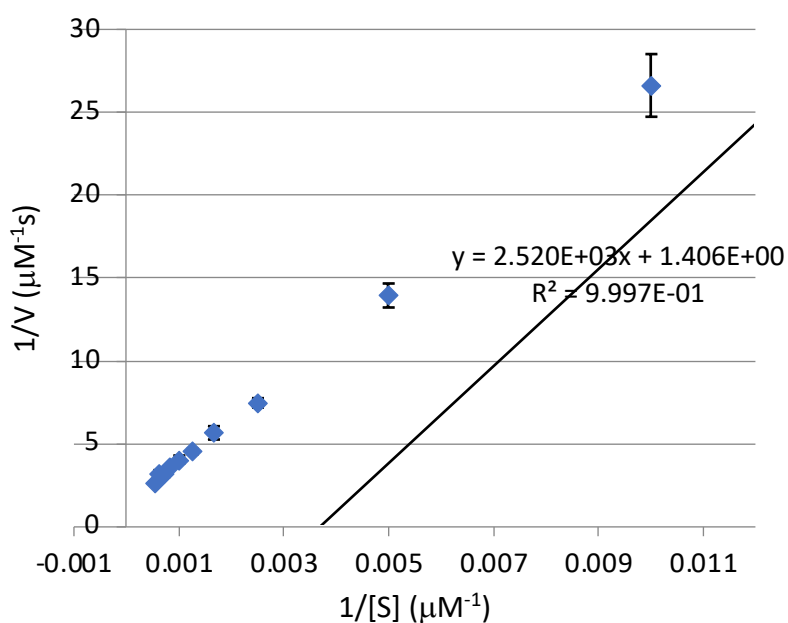


Figure 80. Lineweaver-Burk plot of hGSTK1-1 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.06 \times 10^{-2} \mu\text{M}$.

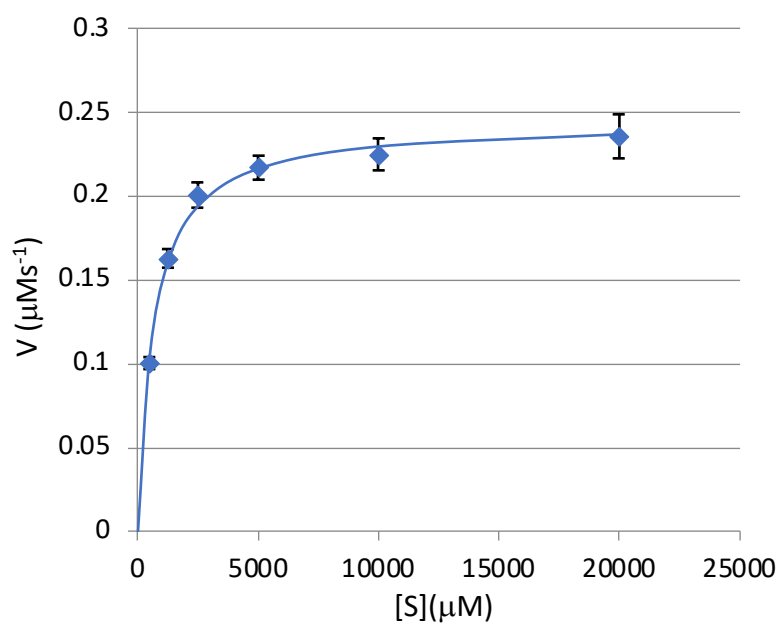


Figure 81. Non-linear plot of hGSTK1-1 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.06 \times 10^{-2} \text{ } \mu\text{M}$.

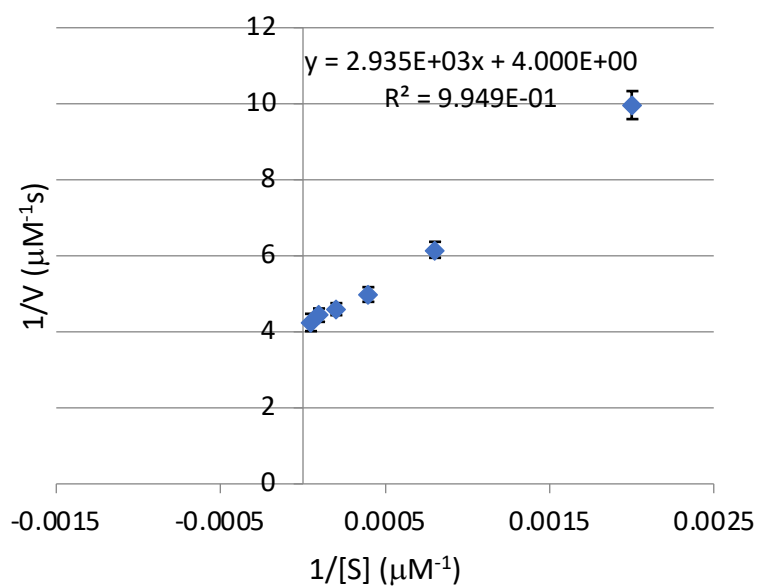


Figure 82. Lineweaver-Burk plot of hGSTK1-1 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.06 \times 10^{-2} \text{ } \mu\text{M}$.

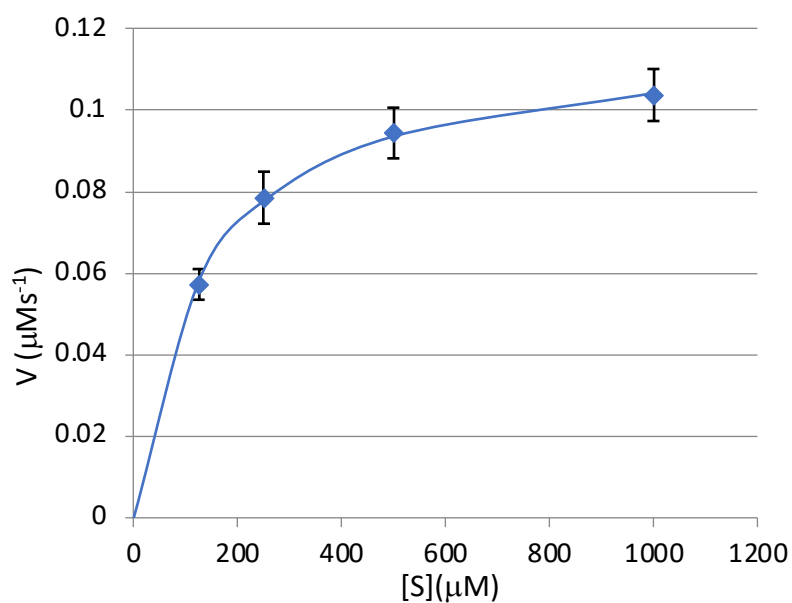


Figure 83. Non-linear plot of hGSTA1-1 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $2.60 \times 10^{-3} \text{ } \mu\text{M}$.

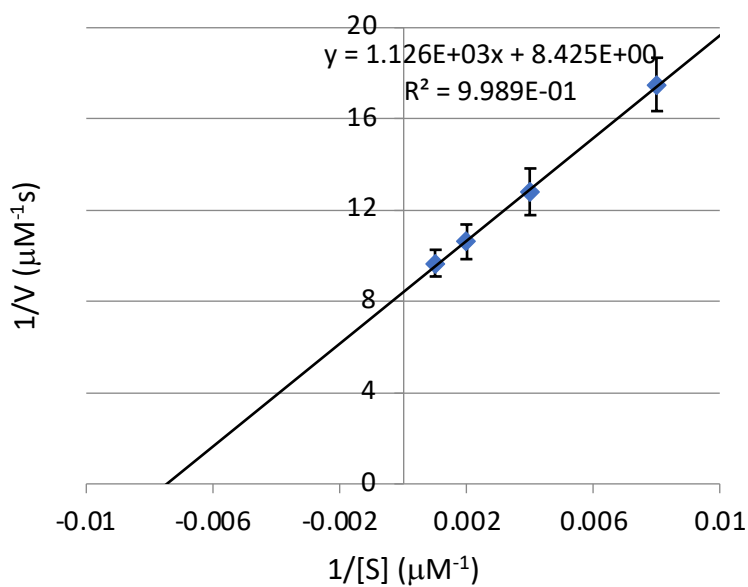


Figure 84. Lineweaver-Burk plot of hGSTA1-1 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $2.60 \times 10^{-3} \text{ } \mu\text{M}$.

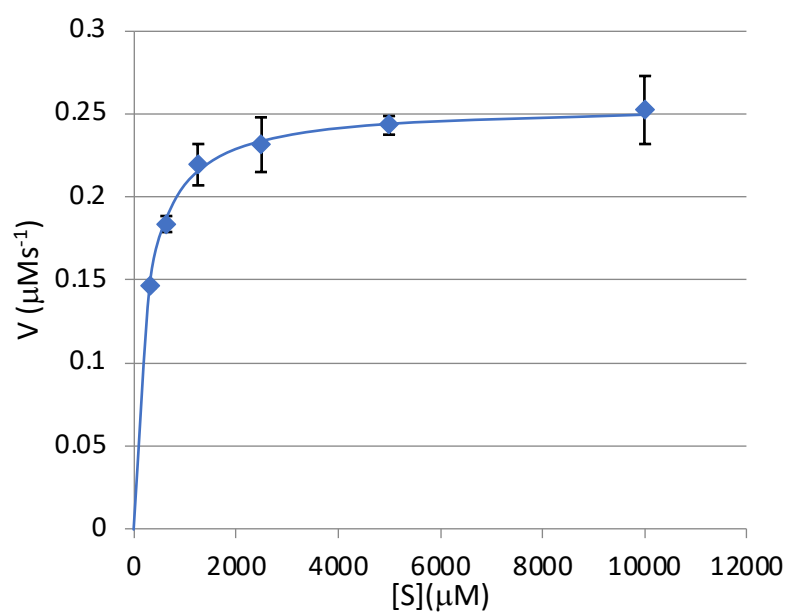


Figure 85. Non-linear plot of hGSTA1-1 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

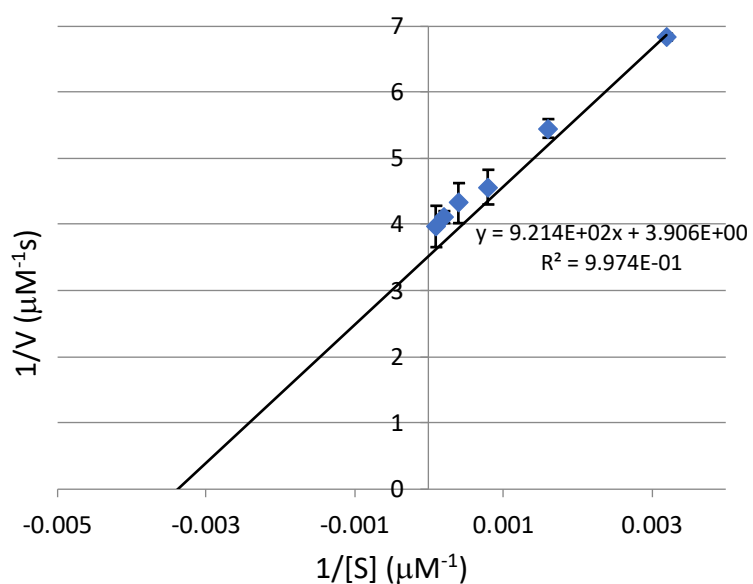


Figure 86. Lineweaver-Burk plot of hGSTA1-1 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $5.20 \times 10^{-3} \text{ } \mu\text{M}$.

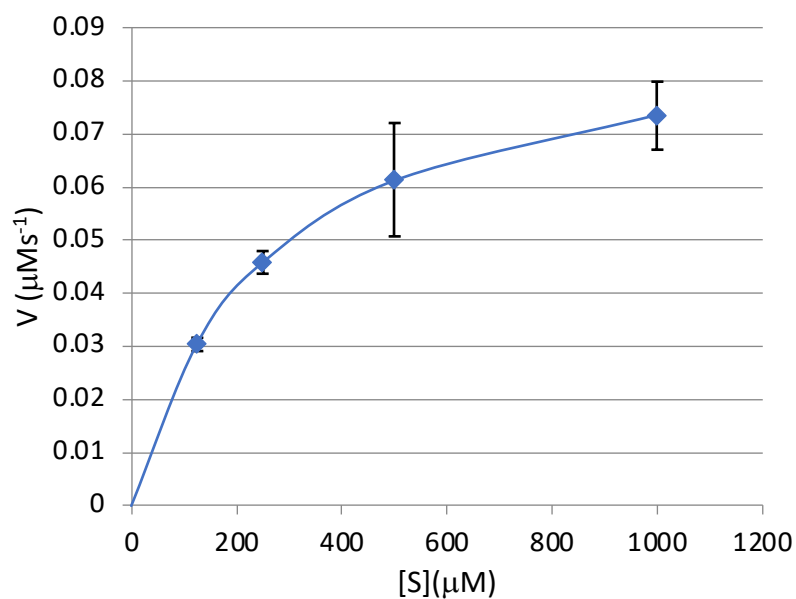


Figure 87. Non-linear plot of hGSTA4-4 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $1.87 \times 10^{-3} \text{ } \mu\text{M}$.

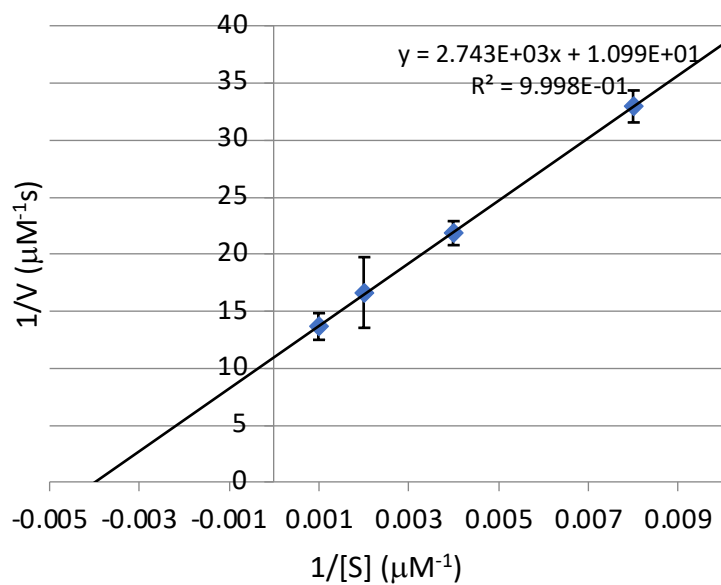


Figure 88. Lineweaver-Burk plot of hGSTA4-4 for CDNB 48 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $1.87 \times 10^{-3} \text{ } \mu\text{M}$.

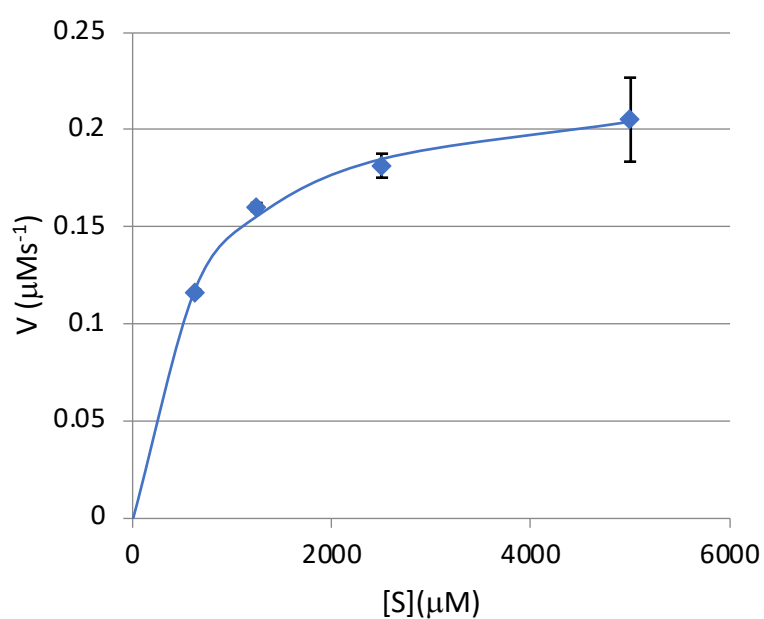


Figure 89. Non-linear plot of hGSTA4-4 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $3.73 \times 10^{-3} \text{ } \mu\text{M}$.

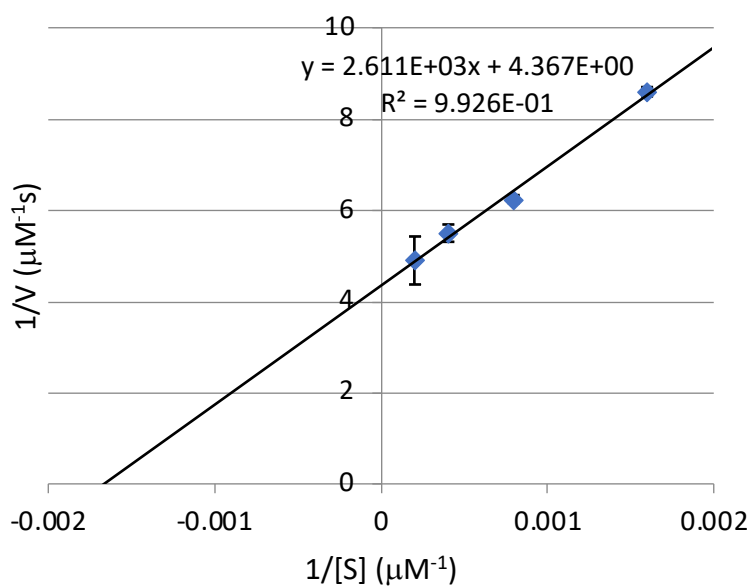


Figure 90. Lineweaver-Burk plot of hGSTA4-4 for GSH 25 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{340\text{nm}} = 9.02 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = $3.73 \times 10^{-3} \text{ } \mu\text{M}$.

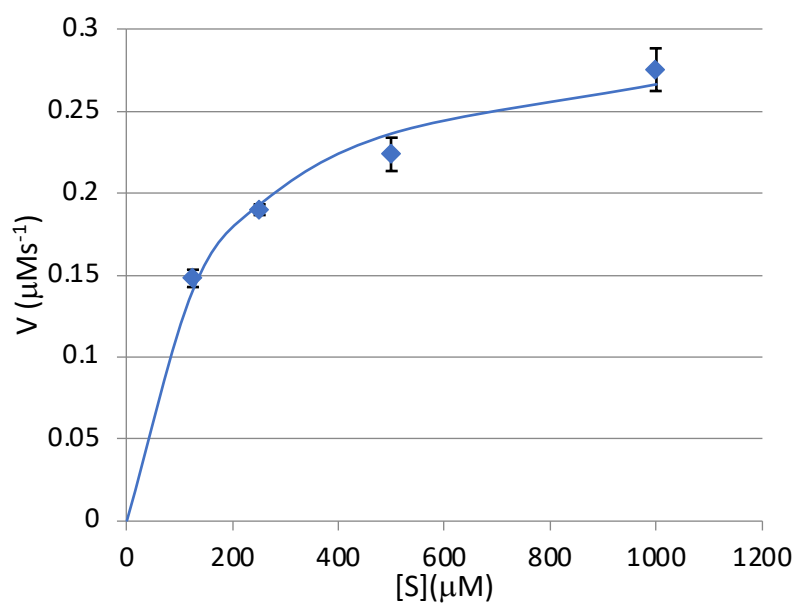


Figure 91. Non-linear plot of hGSTA1-1 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = $2.08 \times 10^{-2} \mu\text{M}$.

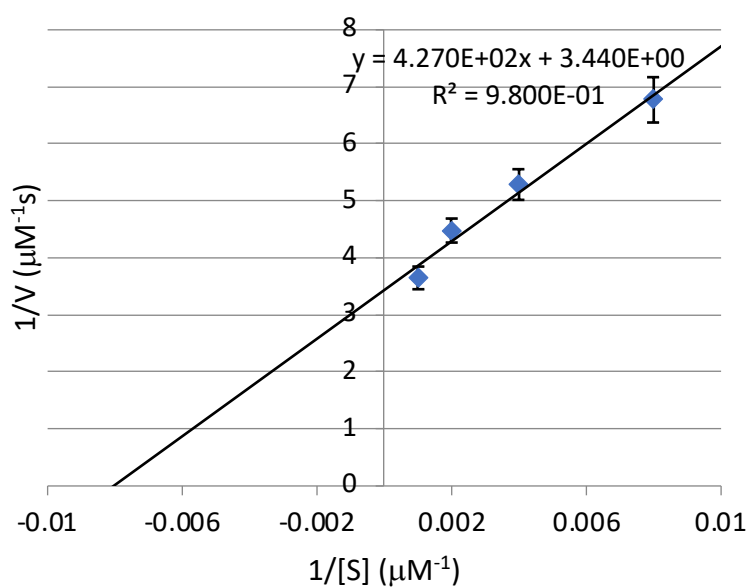


Figure 92. Lineweaver-Burk plot of hGSTA1-1 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = $2.08 \times 10^{-2} \mu\text{M}$.

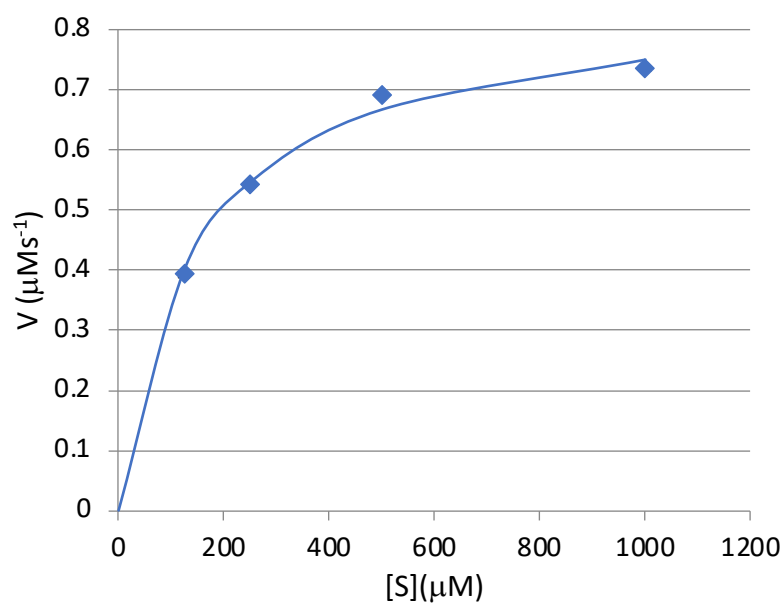


Figure 93. Non-linear plot of hGSTA4-4 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = 0.187 μM .

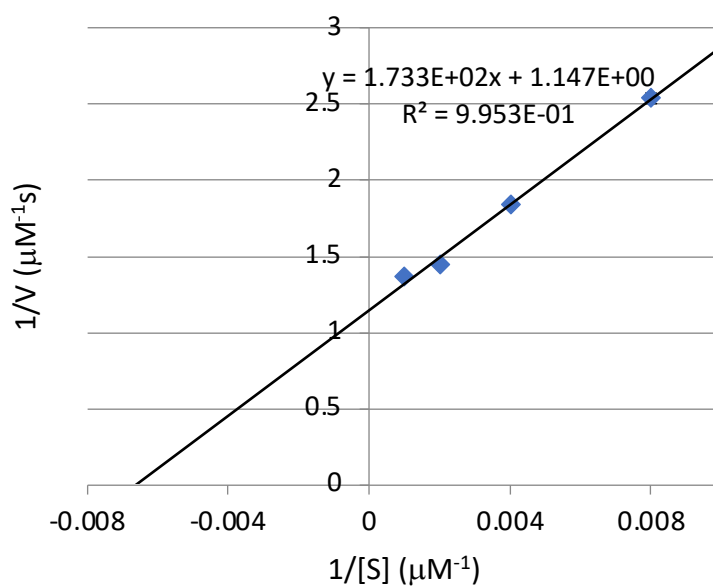


Figure 94. Lineweaver-Burk plot of hGSTA4-4 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = 0.187 μM .

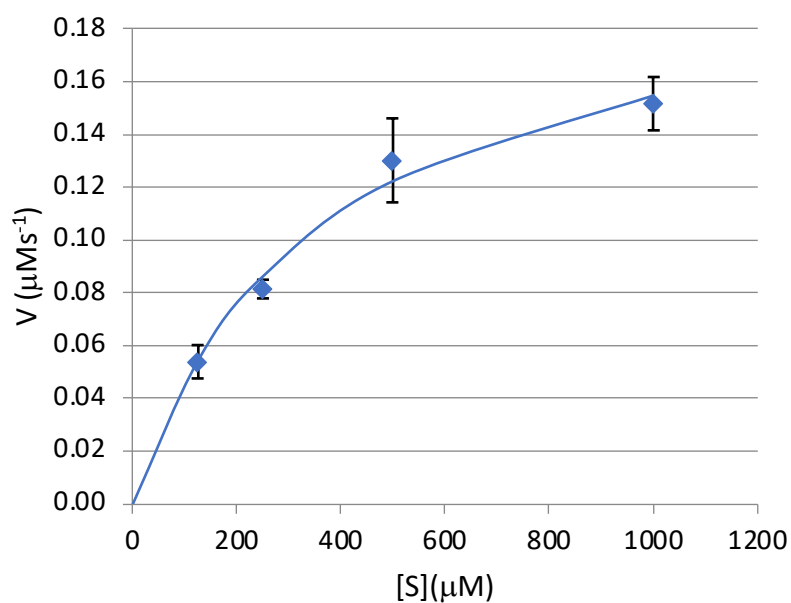


Figure 95. Non-linear plot of hGSTK1-1 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = 2.04 μM .

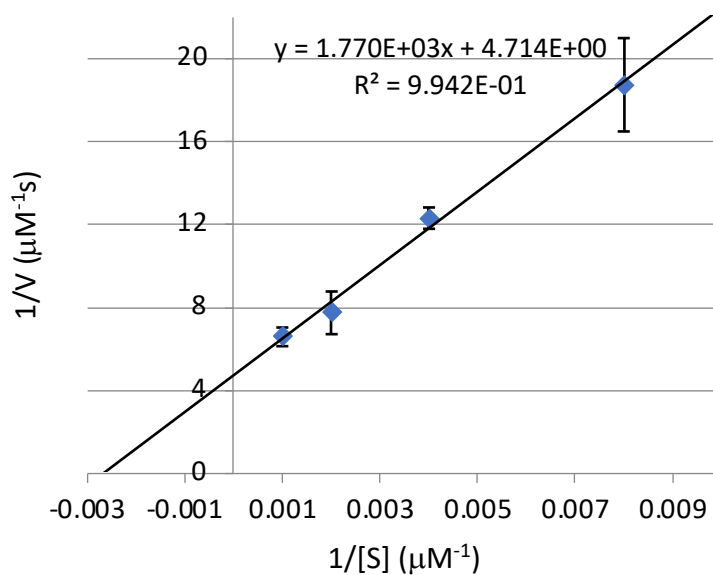


Figure 96. Lineweaver-Burk plot of hGSTK1-1 for MitoCDNB 51 at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{328\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.1 cm. Enzyme conc. = 2.04 μM .

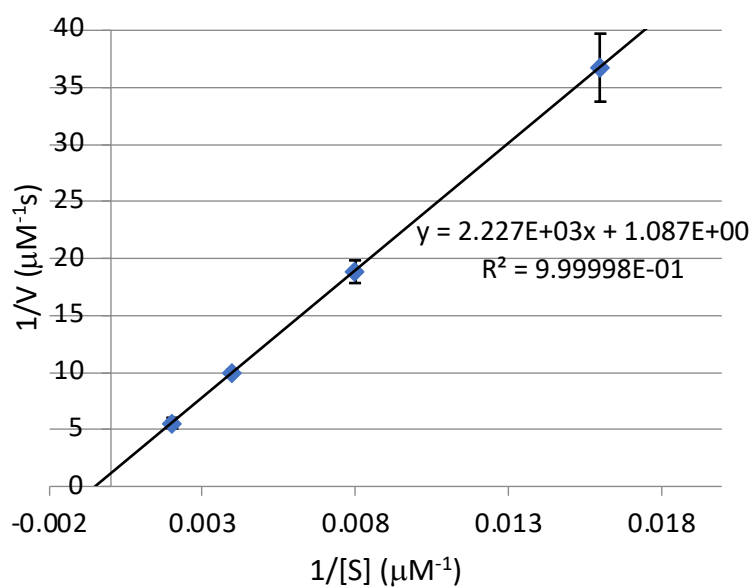


Figure 97. Lineweaver-Burk plot of hGSTA1-1 for compound 85a at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{286\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1 cm. Enzyme conc. = 0.104 μM .

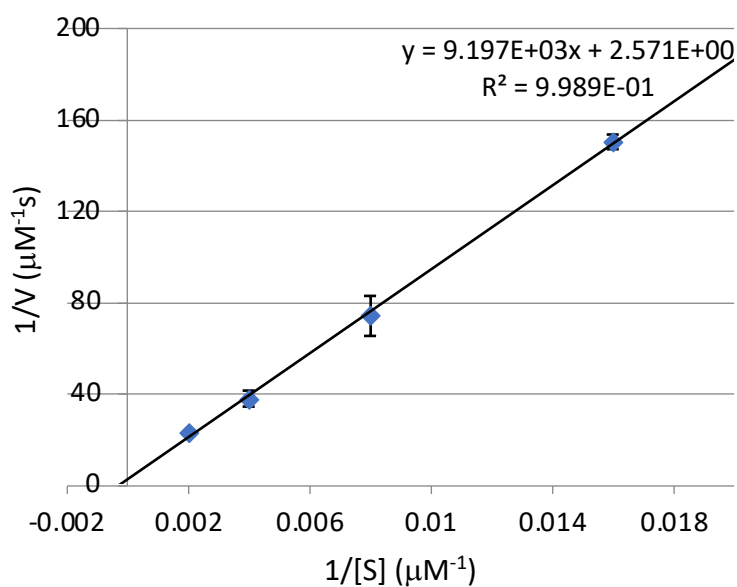


Figure 98. Lineweaver-Burk plot of hGSTA4-4 for compound 85a at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{286\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1 cm. Enzyme conc. = 0.747 μM .

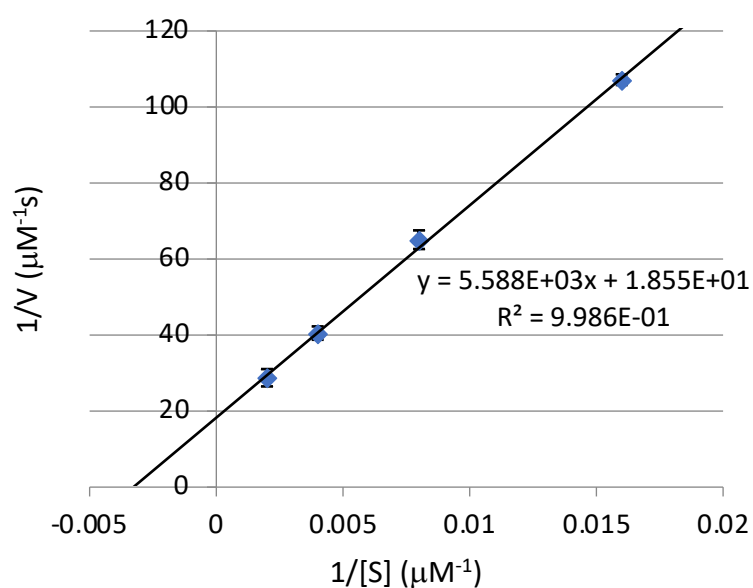


Figure 99. Lineweaver-Burk plot of hGSTK1-1 for compound 85a at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{286\text{nm}} = 9.38 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1 cm. Enzyme conc. = 0.510 μM .

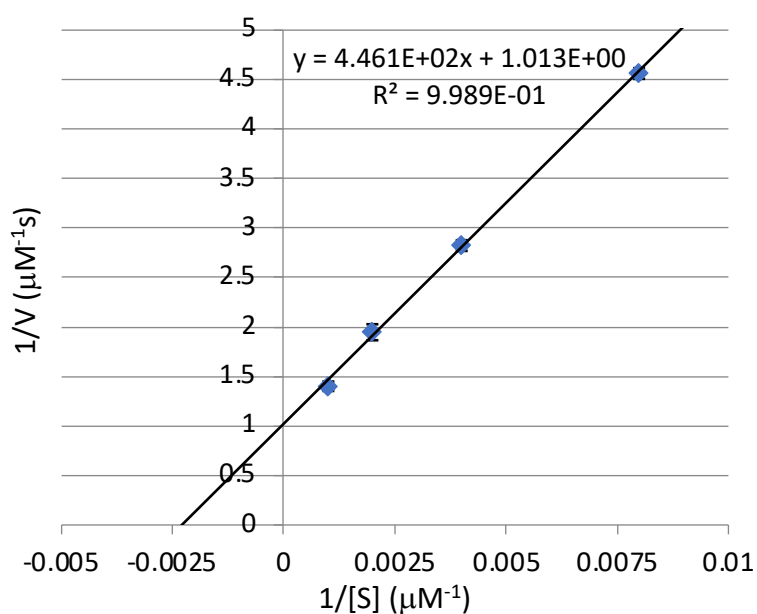


Figure 100. Lineweaver-Burk plot of hGSTA1-1 for compound 86a at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{284\text{nm}} = 6.72 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.5 cm. Enzyme conc. = 0.104 μM .

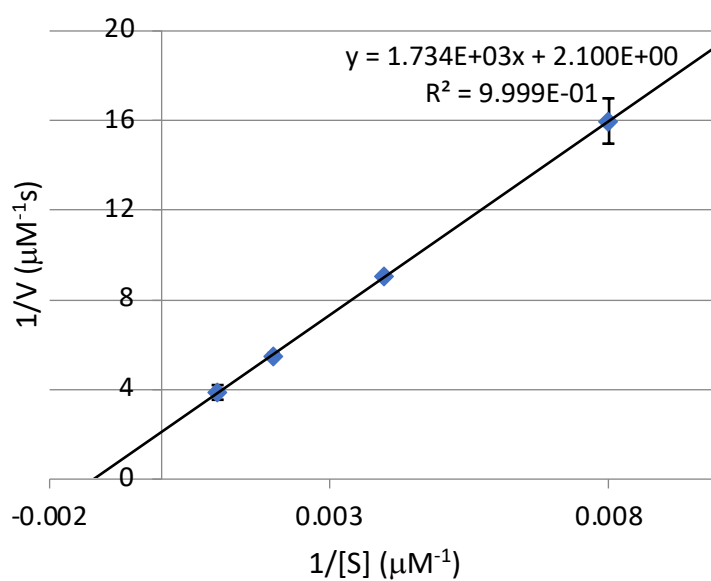


Figure 101. Lineweaver-Burk plot of hGSTA4-4 for compound 86a at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{284\text{nm}} = 6.72 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 0.5 cm. Enzyme conc. = 0.224 μM .

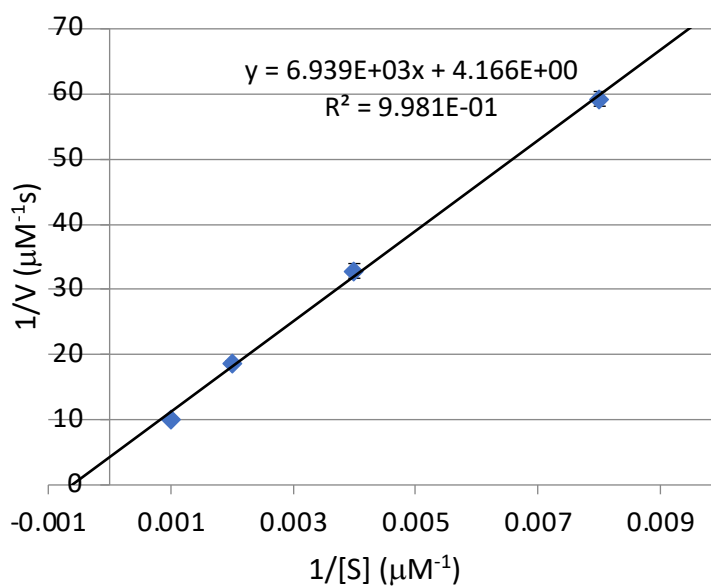


Figure 102. Lineweaver-Burk plot of hGSTA1-1 for compound 85b at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{343\text{nm}} = 13.5 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = 0.104 μM .

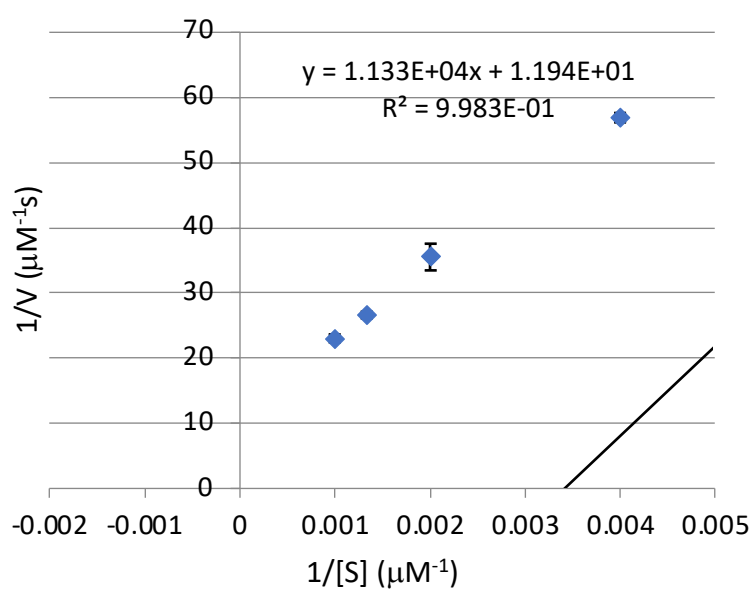


Figure 103. Lineweaver-Burk plot of hGSTA4-4 for compound 85b at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{343\text{nm}} = 13.5 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = 1.49 μM .

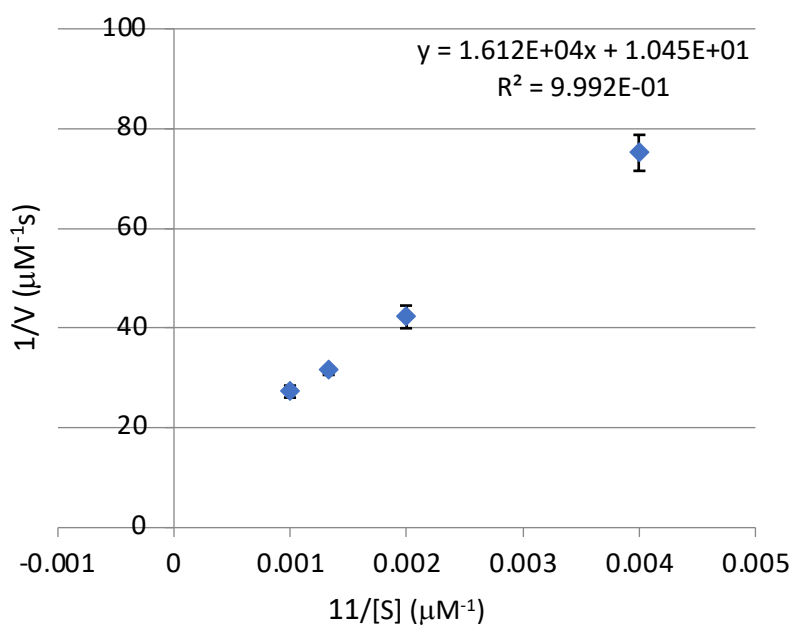


Figure 104. Lineweaver-Burk plot of hGSTK1-1 for compound 85b at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. (n = 3). $\Delta\epsilon_{343\text{nm}} = 13.5 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = 1.01 μM .

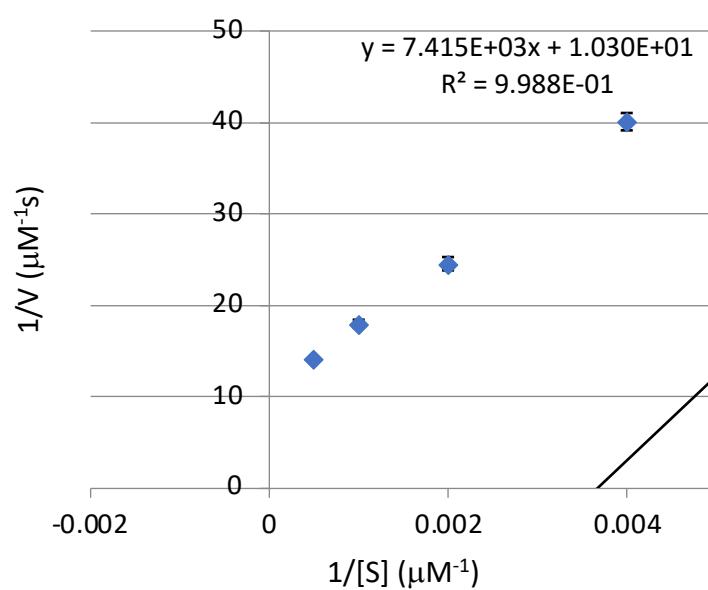


Figure 105. Lineweaver-Burk plot of hGSTA1-1 for compound 86b at pH 8.0, 30 °C. Data expressed as means $\pm$ S.D. ($n = 3$). $\Delta\epsilon_{342\text{nm}} = 10.5 \text{ mM}^{-1}\text{cm}^{-1}$. Pathlength = 1.0 cm. Enzyme conc. = 2.08 μM .

7.3 Calibration curves

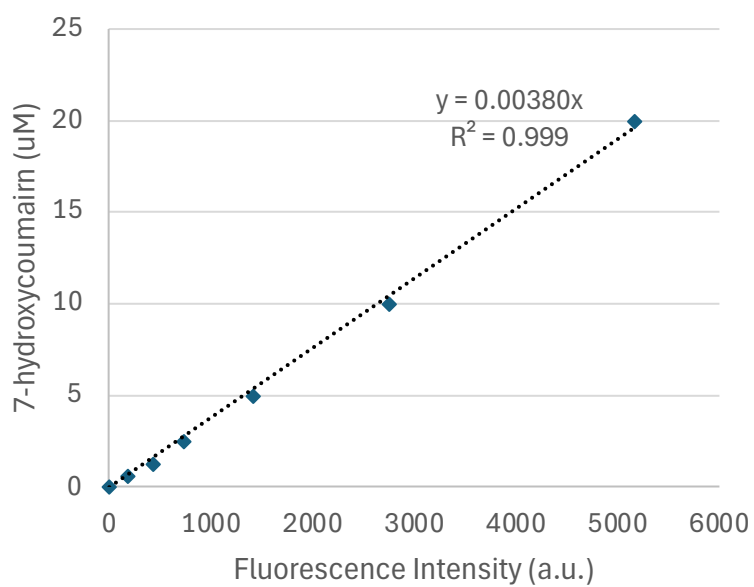


Figure 106. Calibration curve of 7-hydroxycoumarin 106 at pH 8.0, 30 °C.

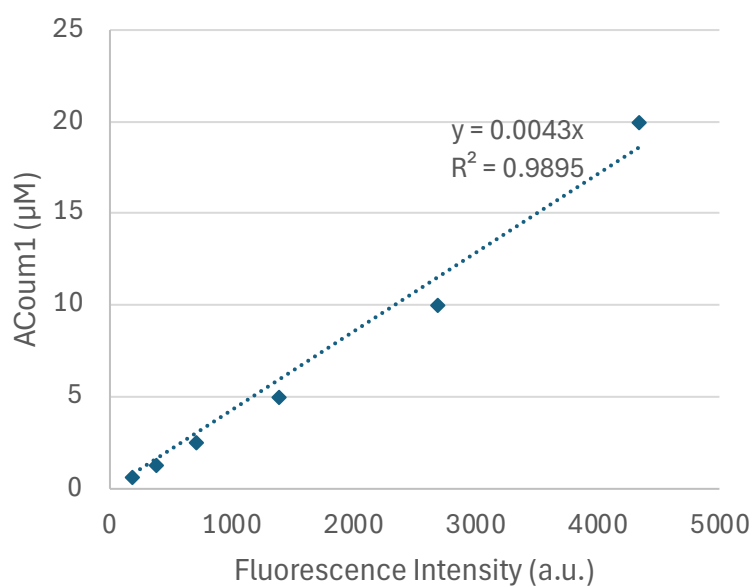


Figure 107. Calibration curve of ACoum1 165 at pH 8.0, 30 °C.

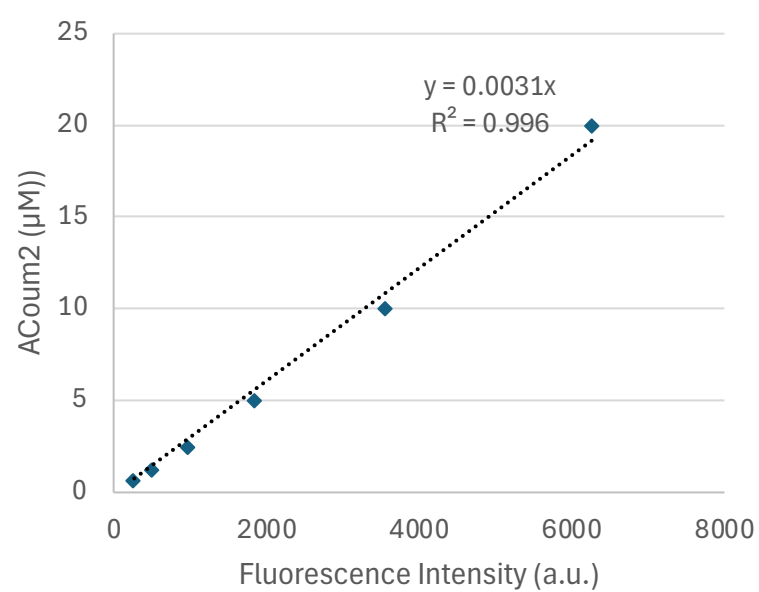


Figure 108. Calibration curve of ACoum2 166 at pH 8.0, 30 °C.

7.4 Initial rates of coumarin derivatives

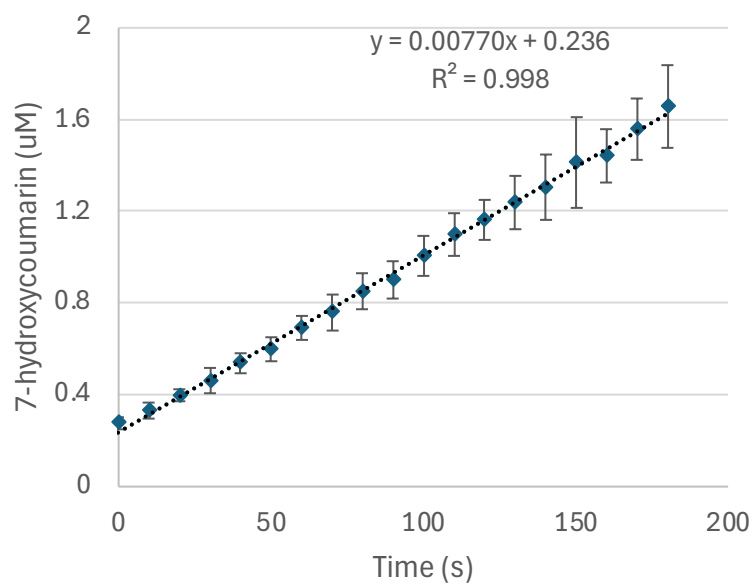


Figure 109. Triplicate initial rate of HCOuM1 167 (10 μ M) at pH 8.0, 30 $^{\circ}$ C.

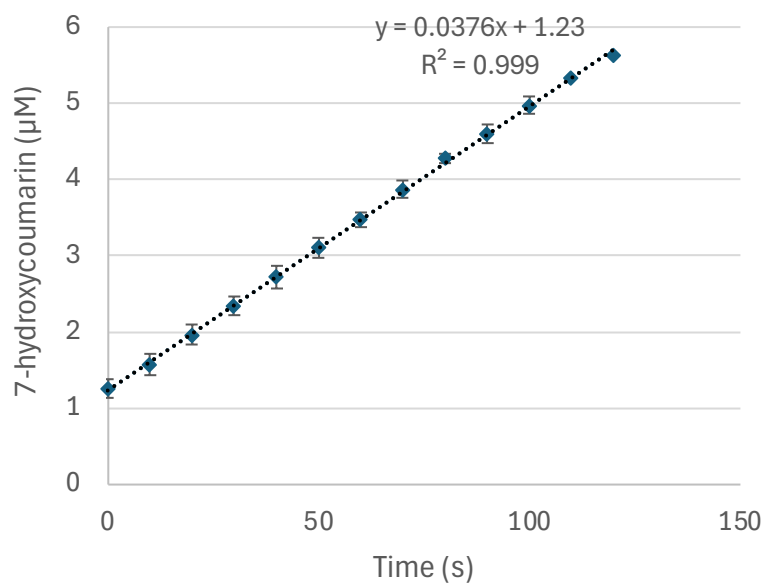


Figure 110. Triplicate initial rate of HCOuM2 168 (10 μ M) at pH 8.0, 30 $^{\circ}$ C.

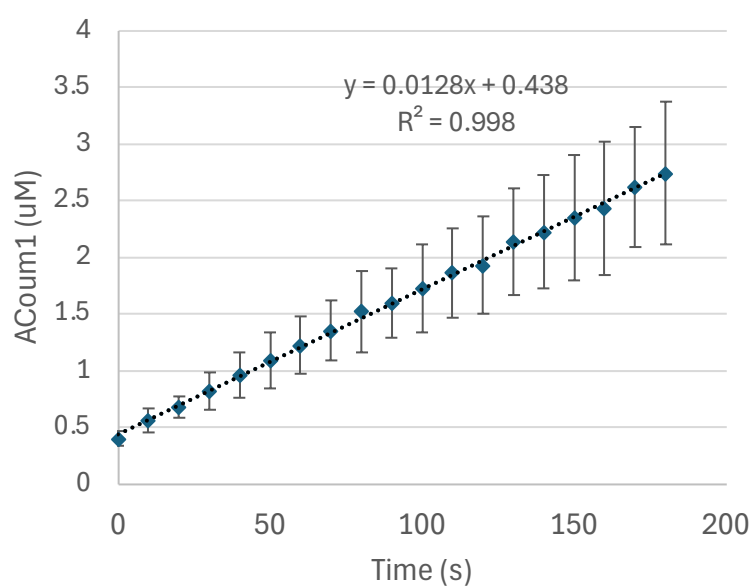


Figure 111. Triplicate initial rate of MitoACoum1 175 (10 μ M) in GSH 25 (2 mM) at pH 8.0, 30 $^{\circ}$ C.

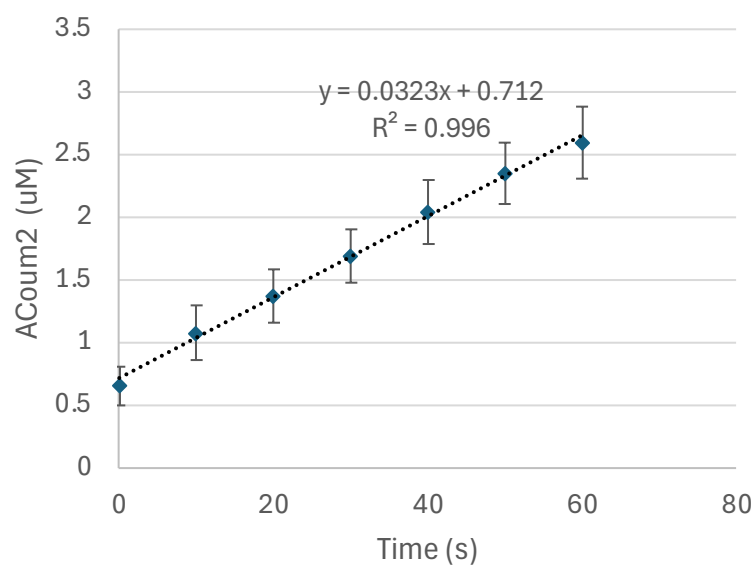


Figure 112. Triplicate initial rate of MitoACoum1 176 (10 μ M) in GSH 25 (2 mM) at pH 8.0, 30 $^{\circ}$ C.

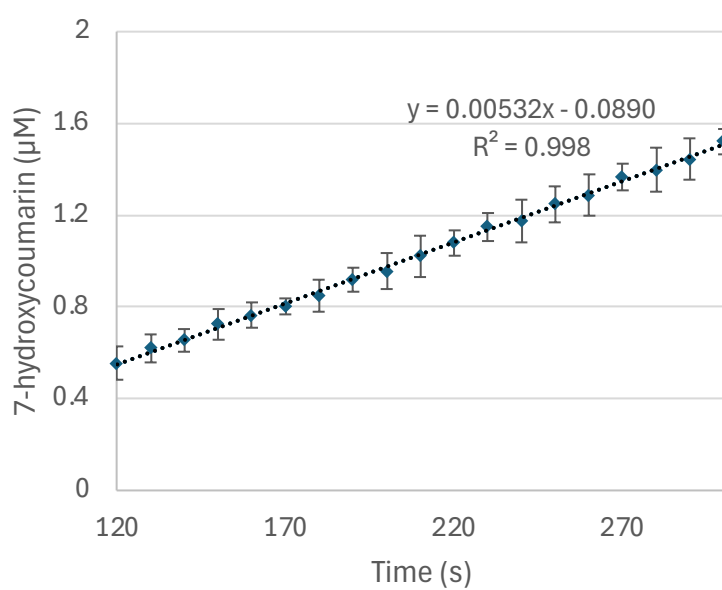


Figure 113. Triplicate initial rate of MitoHCoum1 177 (10 μM) in GSH 25 (10 mM) at pH 8.0, 30 $^{\circ}\text{C}$.

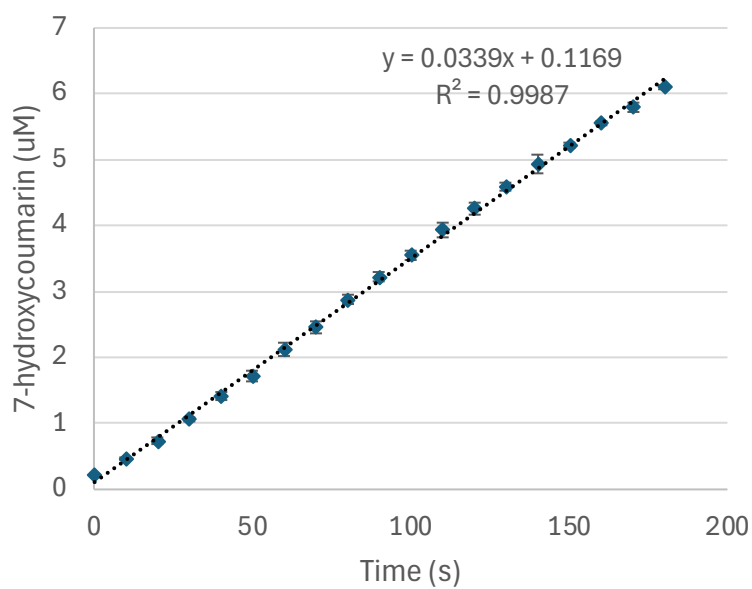


Figure 114. Triplicate initial rate of MitoHCoum2 178 (10 μM) in GSH 25 (10 mM) at pH 8.0, 30 $^{\circ}\text{C}$.