Towards targeted nitric oxide delivery using β-lactamase antibody conjugates

Andrew Wu

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Towards targeted nitric oxide delivery using β-lactamase antibody conjugates

Andrew Wu

Supervisors:
Professor Michael Kelso
Doctor Charlotte Williams

This thesis is presented in fulfilment of the requirements for the award of the degree
Master of Philosophy (Chemistry)

University of Wollongong
School of Chemistry and Molecular Bioscience

August 2020
Declaration

I, Andrew Wu, declare that this thesis, submitted in fulfilment of the requirements for the award of the degree Master of Philosophy (Chemistry) in the School of Chemistry and Molecular Bioscience at the University of Wollongong, is my own work unless otherwise referenced or acknowledged.
Acknowledgement

I would like to express my appreciation to my supervisors, Professor Michael Kelso at the University of Wollongong and Dr. Charlotte Williams at CSIRO, for their support, knowledge and guidance throughout the project. I would like to thank them for their patience, giving me a chance to learn new techniques and helping me to become an independent scientist.

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Finally, I would like to thank my mother, Diane Tao, for her loving support, interest and advice during my studies.
Abstract

Nitric oxide (NO) is a lipophilic, freely-diffusing, reactive (free radical) gas that acts as a signalling molecule in the cardiovascular, nervous and immune systems. Since the discovery that NO acts as a powerful vasodilator, several clinically useful NO-donor drugs have been developed in cardiovascular medicine; for example, nitroglycerin and isosorbide dinitrate for the treatment of acute angina. However, all the drugs developed to date have been spontaneous NO donors that non-specifically release NO in physiological milieu, leading to systematic exposure of host tissues to NO. Thus, there is an unmet opportunity to create innovative NO technologies that selectively target NO to specific tissue locations in the body. Antibody-directed enzyme prodrug therapy (ADEPT) is one promising strategy that could enable such tissue-specific NO delivery. In ADEPT, an antibody or antibody fragment that recognises a tissue-specific antigen is linked to an enzyme capable of activating some prodrug. Administration of the antibody-enzyme bioconjugate localises the enzyme activity to the tissue site bound by the attached antibody and administration of the prodrug then leads to selective revelation of active drug at the tissue site. A major advantage of ADEPT is its ability to reduce unwanted side-effects. In this Masters research project, an new ADEPT strategy was explored that uses an antibody-β-lactamase bioconjugate in combination with a cephalosporin-3’-diazeniumdiolate (C3D) NO-donor prodrug, a class of highly drug-like compounds that rapidly release NO upon reaction with β-lactamases, as a strategy for targeted NO therapy.

A possible application for a targeted NO therapy of this type is as an emergency intervention during heart attacks caused by atherosclerotic plaques, where the plaque occludes a coronary vessel and causes myocardial ischemia. Patients suffering from this type of heart attack could be given ADEPT NO therapy by paramedics that triggers vasodilation directly at the site of the occlusion. The increased cardiac perfusion and reduced myocardial ischemia could preserve the cardiac tissue and buy time for patients to undergo emergency surgery to
remove the blockage. ADEPT under this scenario could use an atheroma-targeting antibody (e.g. anti\textsubscript{LIBS} scFv, Prof. Karlheinz Peter) β-lactamase conjugate in combination with a C3D.

My Masters project aimed to provide the first molecular proof-of-concept towards this goal by constructing a model antibody-β-lactamase conjugate and demonstrating that such a conjugate can cause NO release from a C3D. The specific aims were: 1) design, clone and express a suitable TEM-1 β-lactamase, 2) conjugate the TEM-1 β-lactamase to the model antibody fragment Fab’ 528, 3) synthesise and purify a C3D (DEA-C3D 1) and 4) confirm NO release from DEA-C3D 1 is triggered by the Fab’ 528-β-lactamase conjugate.

The TEM-1 β-lactamase was cloned and expressed in good yield in \textit{E. coli} cells and the protein was purified using an immobilised metal affinity chromatography/size exclusion chromatography sequence. The TEM-1 β-lactamase was successfully coupled to the thiol-bearing antibody fragment Fab’ 528 \textit{via} a sulfo-SMCC linker to give a TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate. A fresh sample of DEA-C3D 1 was then synthesised, purified and characterised using the previously reported methods. Importantly, NO measurements showed that the unconjugated TEM-1 β-lactamase and the TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate both triggered NO release from DEA-C3D 1, although the conjugate showed lower activity. This study demonstrated for the first time that it is possible to produce a β-lactamase-Fab’ 528 conjugate that triggers NO release from C3Ds and opens the way for studies aimed at achieving tissue selective NO therapy via this ADEPT approach.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$^{13}$C NMR</td>
<td>Carbon nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>ADEPT</td>
<td>Antibody directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>C3D</td>
<td>Cephalosporin-3’-diazeniumdiolates</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CPG2</td>
<td>Carboxypeptidase G2</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Commonwealth scientific and industrial research organisation</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DEA</td>
<td>Diethylamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSF</td>
<td>Differential scanning fluorimetry</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelial derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum β-lactamase</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GFC</td>
<td>Gel filtration chromatography</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced form)</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GTG</td>
<td>GoTaq® Green</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IAESTE</td>
<td>International association for the exchange of students for technical experience</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>MALDI- TOF</td>
<td>Matrix-assisted laser desorption/ionization-time of flight mass spectrometry</td>
</tr>
<tr>
<td>$m/z$</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>NHS</td>
<td>$N$-hydroxysuccinimide</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
</tbody>
</table>
NONOates             Diazeniunmioiates
NOS                 Nitric oxide synthase
PBPs                Penicillin-binding proteins
PBS                 Phosphate buffered saline
PCR                 Polymerase chain reaction
PKG                 Protein kinase G
PMB                 Para-methoxybenzyl
RNA                 Ribonucleic acid
ROS                 Reactive oxygen species
SDS-PAGE            Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sGC                 Soluble guanylyl cyclase
SMCC                Succinimidyl-(N-maleimidomethyl) cyclohexane carboxylate
SHV                 Sulfhydryl variable type 1
TAE                 Tris-acetate-EDTA
TB                  Terrific broth
TCEP                Tris(2-carboxyethyl) phosphine
TEV                 Tobacco etch virus
TFA                 Trifluoroacetic acid
TLC                 Thin layer chromatography
U                   Enzyme units
UV                  Ultraviolet
YT                  Yeast extract tryptone
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Chapter 1: Introduction
1.1 Discovery of nitric oxide

Nitric oxide (NO) was first discovered in 1772 by Joseph Priestley as an atmospheric pollutant and possible cause of acid rain. In the 1980s, NO started to be investigated for medical applications and cell signalling. The Nobel Prize in Medicine or Physiology was awarded to Robert F. Furchgott, Ferid Murad and Louis J. Ignarro in 1998 for their discoveries related to “nitric oxide as a signalling molecule in the cardiovascular system”. In 1980, Furchgott was studying the effects of acetylcholine on vasodilation and noticed that blood vessels relaxed if endothelial cells were present. He proposed that a small factor produced by the endothelial cells relaxes the blood vessels and named it Endothelial Derived Relaxing Factor (EDRF).¹ At around the same time, Murad was investigating the biological activities of nitroglycerin when he noted that it decomposes to NO and causes relaxation of smooth muscle.² Ignarro independently discovered that EDRF was actually the gaseous molecule NO that causes vasodilation.³ These three discoveries motivated a great deal of research into the role of NO signalling in the cardiovascular system and led to several drugs for treating cardiovascular diseases, including glyceryl trinitrate for heart failure and molsidomine to treat angina pectoris.⁴

1.2 Physiological roles of nitric oxide

Nitric oxide is a colourless gas at room temperature and has a melting point of -163.6 °C and a boiling point of -151.8 °C.⁵ NO is a heteronuclear diatomic compound that exists as a free radical due to an unpaired electron.⁵ The gas is highly lipophilic and intrinsically reactive, with a half-life of only a few seconds in solution before it decomposes into oxygen and nitrogen gas. Nitric oxide readily diffuses through biological membranes without assistance from channels or receptors. It can be synthesised in the laboratory by reduction of nitric acid or
oxidation of ammonia. Relevant to this research project, NO is also a product of the spontaneous decomposition of diazeniumdiolates in aqueous physiological solutions.6-9

Nitric oxide acts as a potent signalling molecule involved in regulating the cardiovascular, nervous and immune systems.10 NO can also react with the thiol group of cysteine residues of some proteins; a non-enzymatic protein modification, that leads to S-nitrosylation.11,12 NO is involved in maintaining physiological homeostasis, controlling aspects of vascular tone, neuronal communication, cell differentiation and apoptosis (Figure 1).

**Physiological Roles of NO**

- **NO** → Control vascular tone
- **Smooth muscle cell replication**
- **Cell differentiation and apoptosis**
- **Blood vessel wall**
  - Platelets → Inhibit platelet adhesion and aggregation
  - Neuronal communication
  - Defence against pathogens

**Figure 1.** Activities of NO in the cardiovascular, nervous and immune systems.

### 1.3 Nitric oxide signalling

Endogenous NO is produced by nitric oxide synthases (NOS) from L-arginine via oxidation of the guanidine side chain.13 The oxidation process forms NO and L-citrulline via two successive mono-oxygenation reactions, with N-hydroxy L-arginine serving as an
Three NOS isoforms are involved in regulating endogenous NO in the body: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NOS and eNOS are constitutive NO generators, producing basal and short-lasting NO levels, whereas iNOS produces greater and longer-lasting NO concentrations. The resulting NO modulates many cell signalling pathways, such as the NO/sGC/cGMP, serine threonine protein kinase B(AKT), Ca^{2+}/eNOS and nuclear factor kappa B(NF-κB)/iNOS. Constitutive NO is produced in the blood vessels by eNOS and is regulated by calcium and calmodulin levels. Changes in regulation can occur via phosphorylation, negative feedback and interactions with regulatory enzymes. In contrast, iNOS regulation occurs via inflammatory cytokines, oxidative stress and hypoxia.

The two pathways that regulate constitutive NOS both involve release of calcium from subsarcolemmal storage sites. The first pathway begins when shearing forces applied to the vascular endothelium by blood flow cause release of calcium and subsequent NOS activation. The second involves stimulation of endothelial receptors by a variety of ligands, causing calcium release and NO production. Induction of iNOS occurs during inflammation and can cause NO levels to increase 1000-fold above constitutive levels. iNOS becomes active in several inflammatory states, including atherosclerosis, however, its role in cardiovascular inflammatory processes remains unclear. NO produced constitutively and by iNOS in endothelial cells diffuses into adjacent smooth muscle, where it binds to its principal receptor soluble guanylate cyclase (sGC). NO binds to the β1 subunit of the α1β1 sGC heterodimer, activating the enzyme to convert GTP into cGMP (Figure 2). Another NO pathway converts the thiol group of protein cysteine residues to nitrosothiols, which serve as natural ligands for soluble guanylyl cyclase(sGC). Both pathways impact downstream signalling targets, such as cGMP-dependent protein kinase G (PKG), ultimately leading to vasodilation and decreased platelet aggregation.
Sheer forces on endothelial cells cause an increase in intracellular Ca\(^{2+}\) release that activates NOS. NOS triggers the conversion of L-arg to L-cit and production of NO. The NO diffuses to and activates sGC, which converts GTP to cGMP. Ensuing PKG activation triggers a cascade that ultimately leads to vasodilation.

**Figure 2.** Sheer forces on endothelial cells cause an increase in intracellular Ca\(^{2+}\) release that activates NOS. NOS triggers the conversion of L-arg to L-cit and production of NO. The NO diffuses to and activates sGC, which converts GTP to cGMP. Ensuing PKG activation triggers a cascade that ultimately leads to vasodilation.

1.4 Nitric oxide and cardiovascular disease

In the cardiovascular system, NO regulates vascular tone and myocardial contractility and acts to limit platelet aggregation.\(^{25,26}\) Vasodilation through the sGC–cGMP pathway is a prominent feature of NO signalling in the cardiovascular system. NO also affects circulating platelets and white blood cells and controls various cellular events, including platelet activation, mitochondrial function, ion transport, inflammation, angiogenesis and cell proliferation, all of which are important in cardiovascular homeostasis. NO regulates cardiovascular function through two distinct pathways: an indirect pathway that involves activation of sGC and downstream stimulation of PKG, and a direct pathway acting via S-nitrosylation of proteins.\(^{27}\) NO bioavailability is highly regulated by NOS and disruptions
can be caused by decreasing availability of L-arginine and NOS cofactors, increased levels of NO inhibitors or increased oxidative stress, causing NO degradation. Dysregulation of NO metabolism is a contributing factor in several vascular diseases, including atherosclerosis, hypertension and septic shock.\textsuperscript{28}

**1.5 Current treatments using nitric oxide**

There are three main ways to influence NO signalling in the body: (1) increase NO formation, (2) reduce NO breakdown and (3) directly stimulate NO downstream signalling targets. Increased NO-mediated effects can be achieved by direct exposure to NO gas, use of inorganic nitrates, organic nitrates or other types of NO-donor compounds that spontaneously release NO in solution.

**1.5.1 Inhaled NO**

The use of inhaled NO was first proposed in 1991 as a method for achieving selective pulmonary vasodilation. Experiments were initially performed using animal (lamb) models of pulmonary hypertension and subsequently in humans.\textsuperscript{29} Selective pulmonary vasodilation is achievable partly because NO is scavenged by haemoglobin after diffusion into the blood. Inhaled NO reaching well-ventilated areas of the lungs improves ventilation–perfusion matching and increases blood oxygenation. Approximately 70\% of inhaled NO is removed within 2 days of inhalation as nitrate in the urine.\textsuperscript{30} Inhaled NO is used today for the treatment of pulmonary hypertension in newborns, as well as in patients with congenital heart disease and after cardiac transplantation in adults.\textsuperscript{31,32} The gas can be delivered \textit{via} a face mask, nasal cannula or endotracheal tube.\textsuperscript{33}
1.5.2 Inorganic nitrites

Nitrite has been known to cause vasodilation since Furchgott showed in 1953 that acidified sodium nitrite solutions are able to relax pre-constricted rabbit aortic strips.\(^34\) It is now known that nitrite contributes to the regulation of blood pressure and flow via its conversion to NO. Acidification of nitrite produces nitrous acid, which spontaneously decomposes to NO and other nitrogen oxides.\(^35\) The process is aided by iron or molybdenum metalloprotein oxidoreductases through electron transfer.\(^36\) There are at least three known sources of nitrite in mammals. Firstly, nitrite is an oxidation product of NOS-derived NO. Secondly, nitrite is present in some food products, including processed (cured) meats and green leafy vegetables.\(^37\) Thirdly, nitrite is generated by bacteria in the digestive system by nitrate reduction.\(^38\) Interestingly, NO generation from nitrite is known to be increased by vitamin C.\(^39\)

1.5.3 Organic nitrates

Organic nitrates are nitric acid esters of mono- or polyhydric alcohols and represent the oldest clinically approved NO-based treatments.\(^40\) Nitrates essentially act as NO donor prodrugs and are commonly used to treat angina and coronary artery disease. They exert their effects through enzyme bioactivation, producing NO that serves to relax vascular smooth muscle in both the arterial and venous circulation via the NO/sGC/cGMP pathway. Organic nitrates can enter the circulation by absorption across mucous membranes, the gastrointestinal tract or the skin. Commonly used organic nitrates are nitroglycerin, isosorbide dinitrate, and isosorbide mononitrate. One of the issues with use of organic nitrates is the development of tolerance with prolonged therapy.\(^41\) Two hypothesis have been proposed for how tolerance develops: 1) superoxide anions produced during nitrate therapy cause inactivation of NO; 2) depletion of tissue protein thiols required for liberation of NO and vasodilation.\(^42\)
1.6 Clinically used NO donor drugs

Nitroglycerin (Figure 3) is a dense, colourless liquid that is typically produced by reacting glycerol with fuming nitric acid. The compound was discovered in 1847 and was originally used as the active ingredient in gunpowder and explosives. For the last 130 years, nitroglycerin has been used medically as a potent vasodilator in patients with angina, hypertension and heart failure.\(^{41}\) Due to its half-life of 4 min in plasma and its biologically active metabolites half-life of 40 minutes,\(^{43}\) the drug can be delivered orally as a tablet, sublingually as a spray or transdermally as an ointment or patch. Release of NO from nitroglycerin is mediated by mitochondrial alcohol dehydrogenases. Although it contains three nitrate groups, nitroglycerin releases only one mole equivalent of NO from one of its terminal nitrates after bioactivation.\(^{44}\)

Isosorbide dinitrate (Figure 3), first synthesised in 1939, is used as a vasodilator in the treatment of heart failure, oesophageal spasms and chest pain. It is usually taken orally, and its mechanism of action involves conversion to NO. Studies have shown that isosorbide dinitrate in combination with hydralazine is effective in treating African American patients with advanced heart failure.\(^{45-47}\)

Sodium nitroprusside (Figure 3) is an arterial and venous vasodilator that has been used clinically for over 40 years. The compound acts as a prodrug, where it reacts with sulfhydryl groups to release nitric oxide, causing rapid vasodilation and acutely lowering blood pressure for up to 10 minutes. The drug is used in hypertensive crises, heart failure and paediatric/vascular/cardiac surgery.\(^{48}\) Experiments using frog hearts concluded that sodium nitroprusside activity can be enhanced by exposure to laser light.\(^{49,50}\) The metabolism of sodium nitroprusside to produce cyanide and promote oxidative stress has limited its clinical utility.\(^{48}\)
Figure 3. Structures of nitroglycerin (top left), isosorbide dinitrate (top right) and sodium nitroprusside (bottom).

Another important NO-donor drug is molsidomine; a sydnonimine that has been used to treat angina pectoris for over 50 years. Molsidomine is a long-lasting, oral prodrug that releases the metabolite SIN-1 following reaction with liver esterases. SIN-1 then acts as a potent vasodilator through spontaneous release of NO (Scheme 1). The drug is also used to treat early stage atherosclerosis, where it reduces adhesion of plaques onto the vascular endothelium.51

Scheme 1. Liver esterases promotes formation of NO from molsidomine via the intermediate SIN-1.

1.7 Targeted delivery of NO

The clinical NO-donor drugs described above are non-selective agents that can affect all blood vessels exposed to the NO they produce. This can sometimes lead to a dangerous
lowering of blood pressure and potentially DNA damage.\textsuperscript{44} Accordingly, there has been significant research into the development of targeted therapies that can provide tissue-specific exposure to NO, with a focus in the areas of cancer therapy and inflammation.

\textbf{1.7.1 Targeted NO therapy in cancer}

The effects of NO on tumours is somewhat paradoxical. In some circumstances, high NO concentrations have been shown to be cytotoxic, while low concentrations have been shown in other studies to promote tumour growth.\textsuperscript{52} At low NO concentrations, NO promotes angiogenesis and anti-apoptotic effects. At high concentrations, NO can lead to cell-cycle arrest and apoptosis through DNA strand breaking, nitrosylation of enzyme cysteine residues and inhibition of mitochondrial activity.\textsuperscript{53} NO is known to react with superoxide to form peroxynitrite, which can react with tumour DNA leading to single strand breaks that trigger apoptosis.\textsuperscript{54} NO can also activate certain tumour-suppressing genes that reduce metastasis. At low concentrations, NO acts as a modulator of the tumour microenvironment, causing increased tumour size. At high concentrations, NO increases production of superoxide, hydrogen peroxide and peroxynitrite, which can damage ATP synthases in tumours leading to cell death. Peroxynitrite has been shown to nitrate tyrosine residues, generating 3-nitrotyrosines in proteins such as actin and superoxide dismutase. Nitrotyrosine is currently used as a biomarker for endogenous peroxynitrite activity.\textsuperscript{55}

In recent years there has been increasing interest in trying to incorporate NO into nanomaterials as a way of providing targeted NO therapy, where NO or NO-based prodrugs are locally released at tumour sites.\textsuperscript{56,57} Drug-loaded nanomaterials carry the advantages of being able to target tumours directly, resulting in reduced renal excretion, increased drug half-life and generating high local drug concentrations at the tumour site. In one example, S-nitrosoglutathione was loaded into calcium carbonate-mineralised nanoparticles, which allow
the NO donor to achieve much longer blood circulation times and provide a more sustained NO release, compared to direct administration of NO donors. The acid environment of endosomes in cancer cells serves to activate S-nitrosogluthathione and generate high concentrations of NO at the tumour site. The NO can diffuse to the surrounding blood vessels where it promotes vasodilation, providing better access for the nanoparticles and anti-cancer drug to the tumour leading to apoptosis (Figure 4).

A selection of hybrid drugs that contain NO have been developed for targeted vasodilation, such as COX-inhibitor-NO and statin-inhibitor-NO. The purpose of these hybrid drugs is to maintain the potency of the native drug action and achieve the right concentration of NO at the site of action. The materials containing NO prodrugs are designed to enhance endothelial cell growth, decrease smooth muscle cell proliferation and cellular adhesion. NBS-1120 is a NO/H₂S releasing hybrid that inhibits proliferation by affecting the cell cycle and is a promising anticancer agent.
1.7.2 Targeted NO therapy in cardiovascular disease

In section 1.6, clinically used NO-donor drugs were described that provide indirect NO treatment of cardiovascular diseases. In the last decade, new targeted NO therapies have been designed as more direct treatments. Nicorandil is a dual-acting nicotinamide nitrate ester that acts as a NO donor and targets ATP-sensitive potassium channels and is used to treat angina. The appended nitrate ester moiety acts as a source of NO that stimulates soluble guanylyl cyclase activity. The compound also acts as an ATP-sensitive potassium channel opener causing surrounding veins and coronary arteries to dilate. Nicorandil has been shown to provide cardioprotective properties for up to 1.6 years by improving fibrinolysis and reducing the risk of thrombus formation.\textsuperscript{61}

![Nicorandil](image)

**Figure 5.** Structure of nicorandil, a dual-action NO donor drug used to treat angina.

1.8 Diazeniumdiolates (NONOates): a versatile class of NO donors

The first diazeniumdiolate (NONOate) NO donor compound was synthesised in 1960 as an adduct formed in the reaction between diethylamine and NO gas under pressure.\textsuperscript{62} Diazeniumdiolates consist of a diolate group [N(O-) N=O] bound to an amine. Potential medical applications were discovered in the 1990s when it was found that the diazeniumdiolates spontaneously decompose in aqueous solution at physiological pH and temperature to generate up to 2 mole equivalents of NO (Scheme 2).\textsuperscript{63} Keefer and co-workers have described a range of diazeniumdiolates with half-lives varying from seconds to hours,\textsuperscript{6-9,64} whose rates of NO release follow first-order kinetics. The released NO can influence a
range of biological effects, including vasodilation, inhibition of platelet aggregation and blood coagulation. Diethylamino diazeniumdiolate (DEA-NONOate) was shown to prevent and reverse vasospasm in a primate model of subarachnoid haemorrhage without affecting systemic blood pressure. A clinical focus for NONOates has been in the prevention of thrombosis and neointimal formation following vascular injury. A recent study described a ‘bump and hole’ strategy for delivering targeted NO using an enzyme-triggered prodrug; galactosidase-galactosyl-NONOate, allowing for more efficient tissue repair and recovery of function with reduced side-effects.

**Scheme 2.** Mechanism of NO release from diazeniumdiolates following protonation in aqueous solution.

Recently, a metal NONOate Ni(SalPipNONO) was shown to have potential antitumor activity. When Ni(SalPipNONO) enters tumour cells, the NO released causes a burst of intracellular reactive oxygen species (ROS), leading to apoptosis and arrested migration. More than 90% of cells were killed by Ni(SalPipNONO) compared to untreated controls and reduced blood vessel development was also observed.

JS-K is a targeted NONOate carrying an O-arylated diazeniumdiolate. JS-K contains the 2,4-dinitrophenyl group, which is cleaved off in the presence of glutathione (GSH) in a reaction catalysed by glutathione-S-transferase (GST). GST is overexpressed in tumour cells; thus JS-K is designed to be selectively activated in tumours. Release of 2 moles of NO causes an increase in ROS that changes the redox environment of the tumour, leading to apoptosis (Scheme 3).
1.9 \textbf{β-lactamases}

β-lactamases are enzymes that catalyse the irreversible inactivation of β-lactam antibiotics by hydrolytic opening of the azetidin-2-one (β-lactam) ring. Antibiotics containing a β-lactam ring (such as cephalosporins, see 1.10) interfere with the biosynthesis of peptidoglycan in bacterial cells by binding and acylating the active site serine nucleophiles of penicillin-binding proteins (PBPs), thus preventing cell wall cross-linking.\textsuperscript{69} The weakened bacterial cells lyse due to an inability to counter intracellular osmotic pressure. Bacteria have evolved the ability to destroy these antibiotics through the production of β-lactamases, which inactivate the antibiotics (through β-lactam hydrolysis) before they can react with PBPs.\textsuperscript{70}

β-lactamases employ a simple mechanism whereby nucleophilic addition at the carbonyl carbon of the β-lactam ring occurs through reaction with an active-site serine in the β-lactamase, leading to cleavage of the β-lactam C-N bond.\textsuperscript{71} The serine is subsequently deacylated \textit{via} water-mediated hydrolysis to reactivate the β-lactamase for further reaction with another β-lactam molecule (Scheme 4).\textsuperscript{72}
**Scheme 4.** Mechanism for β-lactam ring opening by active-site serine nucleophiles in β-lactamases.

β-lactamases are divided into four classes (A, B, C, D) based on their amino acid sequences. β-lactamases can be divided mechanistically into the serine enzymes and zinc enzymes. Serine β-lactamase contain a serine residue in the active site and form an acyl-enzyme intermediate during catalysis. Zinc β-lactamases form a non-covalent bond with the β-lactam through interactions between the carbonyl group and a zinc metal ion. Serine β-lactamases are sub-divided into three classes; A, C and D, while zinc β-lactamase are all from class B.

**1.10 Cephalosporins**

Cephalosporins are an important class of clinically useful antibiotics that contain a β-lactam fused to a dihydrothiazine ring. Cephalosporins exert antibacterial activity by binding to bacterial penicillin-binding proteins (PBP). Cephalosporins undergo β-lactam ring-opening when they acylate transpeptidases and a cis-conformation at the C6 and C7 positions in the β-lactam ring is required for activity.

Cephalosporins are classified into five generations based on their era of development and their ability to treat Gram-positive and Gram-negative infections. The older, first generation cephalosporins are typically more effective against Gram-positive bacteria, while later generation compounds are active against Gram-negatives. First generation cephalosporins, such as cephalexin and cephalothin (Figure 6), are used to treat Gram-positive cocci. They can be used orally to treat tissue infections, have a half-life of more than 1 hour and are typically administered every 6-8 hours. Many bacteria have developed β-lactamase-mediated resistance to cephalosporins, making early generation cephalosporin treatments less
effective these days. Side-chain modification of cephalosporins can alter antimicrobial activity and resistance to β-lactamases, as was done to create the later generation compounds.\textsuperscript{77}

![Cephalosporins Structures](image)

**Figure 6.** Structures of two first-generation cephalosporins.

Cephalosporins can be used as prodrug scaffolds, where effector drugs are attached at the C3'-position. When the cephalosporin is hydrolysed by PBPs, β-lactamases or other nucleophiles, the cephalosporin undergoes β-lactam ring opening, causing the effector drug attached at the C3'-position to be released via a conjugate elimination reaction.

### 1.11 Cephalosporin-3’-diazeniumdiolate NO donor prodrugs

Cephalosporin-based NO-donor prodrugs have been a major research area in the Kelso Research Group at The University of Wollongong for the past decade. The group has created prodrugs that release a diazeniumdiolate NO-donor from cephalosporins upon reaction with β-lactamases. These cephalosporin-3’-diazeniumdiolates (C3Ds) are being explored as a new way of treating chronic infections due to the ability of NO to trigger dispersion of bacterial biofilms.\textsuperscript{78,79} The diazeniumdiolate NO-donor moiety is covalently attached to the cephalosporin 3’-position via its terminal oxygen, creating a chemically stable prodrug. The diazeniumdiolate is released from the prodrug after cleavage of the O-C bond, which occurs via rapid conjugation elimination following β-lactam hydrolysis (Scheme 5). The diazeniumdiolate then spontaneously fragments to produce 2 moles of NO and the constituent...
secondary amine (e.g. pyrrolidine in PYRRO-NO). Some diazeniumdiolates have a very short NO-release half-lives at pH 7.4 (e.g. PYRRO-NO $t_{1/2} = 2$ sec).\(^{80}\)

Scheme 5. Mechanism of $\beta$-lactamase-triggered NO release from cephalosporin-3'-diazeniumdiolate NO donor prodrug PYRRO-C3D.\(^{78}\)

1.12 Antibody dependent enzyme prodrug therapy (ADEPT)

Antibody-directed enzyme–prodrug therapy is a drug targeting approach where an enzyme is targeted to a tissue in the body by conjugation to a tissue-specific antibody. Once localised at the tissue, the enzyme serves to site-selectively activate a prodrug.\(^1\) The ADEPT concept was first hinted at in 1906 by Paul Ehrlich when he proposed the ‘magic bullet’ theory of targeting therapeutic agents to specific tissues to increase their potency and selective activity and to reduce toxicity. ADEPT wasn’t really considered further until Bagshawe demonstrated the concept in the late 1980s.\(^{81-83}\) Here, Bagshawe conjugated bacterial carboxypeptidase G2 (CPG2), an enzyme that catalyses the conversion of folates to pteroates and L-glutamate, to a $\text{F(ab')}_2$ antibody fragment of a monoclonal antibody that had been raised to human chorionic gonadotrophin. The antibody portion served to target the conjugate to colon and rectal tumours when injected into mice. After the antibody CPG2 conjugate had localised at the tumour site and excess conjugate was cleared from the blood, para-$N$-bis(2-chloroethyl) amino benzoylglutamic acid (a nitrogen mustard cytotoxin prodrug) was introduced, which was cleaved to the active mustard cytotoxin by the CPG2 localised at the tumour via the antibody. A few years later, Bagshawe and Senter independently reported ADEPT strategies using
alkaline phosphatase conjugated to two tumour-targeting monoclonal antibodies, L6 and 1F5. The conjugates were able to dephosphorylate the prodrugs mitomycin phosphate and etoposide phosphate, respectively, into the active cytotoxins mitomycin and etoposide. The prodrugs were shown to be less toxic to cells than the parent cytotoxins and stronger antitumor responses were seen with the conjugates.\textsuperscript{84}

ADEPT techniques have explored conjugates of monoclonal antibodies with a wide range of modalities, ranging from enzymes, drugs and radionuclides to genetically engineered fusion proteins. All ADEPT techniques are designed to produce higher concentrations of active drug at the target site than other tissues in the body in order to lower exposure of non-target tissues to the drug.\textsuperscript{85} When ADEPT is used in cancer, selectivity for the tumour is achieved by the antibody binding to an antigen that is uniquely expressed on the surface of the tumour cells.\textsuperscript{86} The approach carries several advantages in cancer treatment, including : 1) a bystander effect, where an active small molecule drug diffuses throughout the tumour, thereby killing surrounding tumour cells that may not express the tumour antigen\textsuperscript{87} 2) an amplification step, where one enzyme molecule locally converts many prodrug molecules into their active form and 3) systemic toxicity is reduced because the active drug is generated selectively at the tumour site.\textsuperscript{85} The main disadvantage of ADEPT is that antibody-enzyme conjugates can be immunogenic. Other challenges for ADEPT include drug resistance, toxicity from the immune system and antigen downregulation.\textsuperscript{88}

An example of ADEPT using a cephalothin-primaquine prodrug with an antibody-β-lactamase conjugate is provided in Figure 7.\textsuperscript{89} The first step in this approach involves injecting a monoclonal antibody-β-lactamase conjugate with high affinity for a targeted antigen into the host. One administered, the cephalothin-primaquine prodrug is converted to the active drug primaquine by the β-lactamase localised at the target site by the attached antibody.
Figure 7. Example of ADEPT using a cephalothin-primaquine prodrug with an antibody-β-lactamase conjugate.\textsuperscript{89}

ADEPT efforts have focussed on achieving greater tissue selectivity through modification of the monoclonal antibody (or fragments) to increase binding to the target of interest. Antibodies ‘humanized’ by replacing the murine regions with complementarity determining regions from human antibodies have also been investigated,\textsuperscript{90} along with antibodies simplified into single chains, F(\text{ab}')\textsubscript{2} fragments or variable region fragments (Fv).\textsuperscript{91}

Recently, ADEPT conjugates have been produced where enzymes are fused to a single-chain antibody (scFv).\textsuperscript{92,93} This type of fusion protein has the same selectivity as the whole monoclonal antibody but slows circulatory clearance and minimises immunogenicity. Recombinant fusion proteins can be reproducibly expressed in useful quantities and can be tailored to control pharmacokinetics and overcome other hurdles, such as tumour distribution.\textsuperscript{94} A novel glyceridase with epitopes removed to bypass the immune system recently opened up new possibilities for ADEPT.\textsuperscript{95} In one study, a conjugate was made by fusing a single chain
fragment from a second-generation murine antibody to a β-lactamase, where the enzyme acts as a reporter for gene expression.\textsuperscript{96}.

1.13 Long term goals

Our long-term goal is to create an antibody-enzyme conjugate for use in ADEPT that provides targeted delivery of vasodilating NO for the emergency treatment of acute-atheroma-induced coronary artery occlusions that cause heart attacks. We propose that a single chain antibody fragment that targets atherosclerotic plaques (designed by Karlheinz Peter)\textsuperscript{97-101} could be conjugated to a β-lactamase. Upon binding of the conjugate to a coronary artery-occluding plaque, the β-lactamase portion could serve to activate a co-administered C3D, causing locally raised NO concentrations around the plaque site. This concentrated burst of NO could produce a powerful vasodilation event around the plaque, potentially restoring blood flow through the blocked artery and providing temporary relief from myocardial hypoxia. If used as an emergency intervention during an atheroma-induced heart attack, the approach could keep patients alive long enough to undergo emergency surgery to physically resolve the blockage (Figure 8).
Figure 8. ADEPT strategy for emergency intervention during of heart attacks caused by atheroma-induced coronary artery occlusion. An atheroma-targeting scFv-β-lactamase conjugate and is used with a C3D to target vasodilating NO to occluded sites.

1.14 Thesis Aims

Since the discovery of NO as a potent vasodilator, many clinically useful drugs have been developed, including nitroglycerin and sodium nitroprusside, to treat cardiovascular diseases. There is, however, an unmet need for targeted NO technologies that can selectively release NO at specific tissue locations. ADEPT is a technology that could enable such targeted delivery. In this project, a new ADEPT strategy was explored that uses a C3D NO-donor with a β-lactamase conjugated to an antibody. The specific aims of this project were to: 1) design, clone and express useable quantities of a suitable β-lactamase, 2) conjugate the β-lactamase to a model Fab'-528 antibody fragment, 3) synthesise and purify a cephalosporin-3′-diazeniumdiolate (DEA-C3D) and 4) demonstrate NO release from DEA-C3D in the presence of the Fab'-528-β-lactamase conjugate. The overarching goal was to show for the first time that a β-lactamase enzyme conjugated to a (model) antibody fragment can trigger NO release from a C3D. This preliminary proof-of-concept study, if successful, would motivate
downstream translational ADEPT studies aimed at producing a clinical intervention for atheroma-induced heart attacks.
Chapter 2: Cloning, expression and purification of a recombinant TEM-1 \(\beta\)-lactamase
2.1 Introduction

As described in section 1.9, there are several classes of β-lactamase that catalyse the ring opening of β-lactam antibiotics, including cephalosporins. TEM-1 β-lactamase was selected for use in this study due to its high expression levels and cephalosporinase activity, being a class A enzyme. A soluble, highly purified, functionally active and stable recombinant TEM-1 β-lactamase was required for the protein bioconjugation and NO release studies. For this work, electrocompetent bacterial cells, *E. coli*, were used for gene cloning and protein expression. *E. coli* cells were chosen due to their well-established culturing methods, simple nutrient requirement, easy of scalability and affordability. A flowchart depicting the cloning, expression and purification of a recombinant TEM-1 β-lactamase is provided in Figure 9.

![Flowchart showing the steps of transformation, expression and purification used to obtain a recombinant TEM-1 β-lactamase.](image)

**Figure 9.** Flowchart showing the steps of transformation, expression and purification used to obtain a recombinant TEM-1 β-lactamase.
2.2 Gene and Vector design

I acknowledge Stewart Nuttall (CSIRO) for design of the vectors used for TEM-1 β-lactamase production. A suitable β-lactamase gene for cloning was obtained from published research that used this gene to express TEM-1 β-lactamase protein that was then used for X-ray crystallographic studies, an X-ray crystal structure was reported.\textsuperscript{102,103} Protein sequences were searched \textit{via} the protein database and Uniprot. TEM-1 β-lactamase with a 100% confirmed primary sequencing was reported by Adachi.\textsuperscript{104} The protein was modified to replace the N-terminal NusA solubility tag with an N-terminal hexa-histidine (Hisx6) tag followed by a Tobacco Etch Virus (TEV) protease cleavage site upstream of the main gene. The signal peptide, identified as amino acids 1-23 in the β-lactamase sequence, was removed to allow the enzyme to be expressed intracellularly and preventing the β-lactamase from exiting the cell. As the β-lactamase is produced intercellularly, the project can be safely conducted in-house. The wild-type TEM-1 β-lactamase vector was chemically synthesised by GenScript and provided as a lyophilised powder. The sequence of the protein was:

\begin{verbatim}
MHHHHHHSSGVDLGTENLYQ ▼ SNGSHPETLVKVKDAEDQLGARVGYIELDLNSG
KILESFRPEERPMMSTFKVLLCGAVLSRDAGQEQLGRRIHYSQNDLVEYSPVTEK
HLDGTVRELCASAAITMSDNTAANLTTTIGGPKELTAFLHNMGDHVTRLDRWEP
ELNEAIIPNDERDTTMAPAMATTLRKLLTGEILTASRQQLIDWMEADKVAGPLLRS
ALPGWFIAADKSGAGERGSRGIIAALGPDGKPSRVIVVIYTTGSQATMDERNRQIAEIG
ASLIKHWAS**
\end{verbatim}

The construct contained the N-terminal Hisx6-tag (red) to facilitate purification of the recombinant protein using immobilised metal affinity chromatography (IMAC). The TEV protease cleavage site (blue) allowed for removal of the Hisx6-tag from the purified protein by
the highly specific Tobacco Etch Virus (TEV) cysteine protease. The construct also contained a \textit{BamH}I restriction enzyme site (purple) before the main protein sequence (green) and ended with a \textit{NheI} restriction enzyme site (orange) followed by stop codons. Theoretical data for the TEM-1 \(\beta\)-lactamase are displayed in Table 1 (Note: prior to TEV cleavage). A start codon (M) was also included before the His tag, along with a few amino acids between the His tag and TEV cleavage site to allow the Hisx6-tag to protrude from the main protein structure and bind to nickel during IMAC purification. Three TEM-1 \(\beta\)-lactamase constructs were originally considered for use in the study (Figure 10).

\textbf{Construct 1:}

\textbf{Construct 2:}

\textbf{Construct 3:}

\textbf{Figure 10.} Constructs 1, 2 and 3 proposed by Stewart Nuttall (CSIRO) for cloning and expression of a recombinant TEM-1 \(\beta\)-lactamase. Theoretical data (i.e. molecular weight, pI,
number of cysteines and extinction coefficients) for the proteins coded by the constructs are provided in Table 1.

Table 1. Theoretical properties of the three constructs of TEM-1 β-lactamase (values obtained from ProtParam).

<table>
<thead>
<tr>
<th>Constructs</th>
<th>His-TEV-TEM-β-lactamase (Construct 1)</th>
<th>His-TEV-SUMO-GGCC-β-lactamase (Construct 2)</th>
<th>His-TEV-SUMO-GGGG-β-lactamase (Construct 3)</th>
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<tr>
<td>No. of amino acids</td>
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<td>Molecular weight</td>
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<td>42721.38</td>
<td>42675.29</td>
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<td>5.73</td>
<td>5.50</td>
<td>5.50</td>
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<tr>
<td>No. of cysteines</td>
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<td>2 (bonded) 1 (free)</td>
<td>2 (bonded)</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>29575</td>
<td>30940</td>
<td>31065</td>
</tr>
<tr>
<td>Stability index</td>
<td>39.86 (stable)</td>
<td>43.57 (unstable)</td>
<td>44.21 (unstable)</td>
</tr>
</tbody>
</table>

The modified pET43.1a vector was kindly provided by CSIRO. This vector is similar to the pET 43.1 a⁺ created by Novagen (Appendix 1), except GGGG and GGCG tags were added to the N-terminus just before the main protein sequence. The vector also contains a SUMO gene between the TEV gene and the GGGG or GGCG tags to aid protein solubility. The vector contains the origin of replication (F1), a lac promotor site that is induced using IPTG and an ampicillin resistance gene, which allows the vector to express in E. coli. The pET43.1a vector is a simple way to create the three proposed constructs of TEM-1 β-lactamase. Construct 1 was designed as a β-lactamase control. Construct 2 was designed to allow intermolecular disulfide bonding through the thiol group of the cysteines in β-lactamase with another protein containing a free thiol group. Construct 3 was designed to enable Sortase-mediated bioconjugation (5.2.1.2). Different selection markers were considered, such as ampicillin, capreomycin and kanamycin, however, ampicillin was ultimately chosen as the selective marker as unpublished studies from CSIRO shows that ampicillin provides the highest cell viability count compared to capreomycin and kanamycin (CSIRO, unpublished results).
2.3 Digestion of recombinant TEM-1 β-lactamase insert and vector

I acknowledge Regina Surjadi for assistance in digesting the TEM-1 β-lactamase insert and vector and Luisa Pontes-Braz and Anna Raicevic for help in troubleshooting. Recombinant TEM-1 β-lactamase was expressed through a cloning cell line created by digesting the gene from the cloning vector provided by Genscript. The cloning vector containing the TEM-1 β-lactamase insert was mixed with restriction enzymes, BamH1 and Nde1 to excise the vector at the restriction enzyme sites (2.2). The BamH1 and Nde1 restriction enzymes were used at 20 U/µL. The optimal concentrations of restriction enzymes were 10 units of enzyme per µg DNA for a 1-hour digest. For a vector concentration of 160 ng/µL, 10 U of both restriction enzymes were used for 1.5 hours to ensure that the insert was cleanly cut from the vector. The digestion was performed at 37 °C as this is the best temperature for restriction enzyme activity. The digested mixture was loaded onto a standard 1% agarose DNA gel with 1:10,000 SYBR safe dye. SYBR safe dye binds to DNA like ethidium bromide but is less toxic. A DNA ladder was loaded into another lane (100 bps to 1517 bps). For complete digestion, the insert band was expected at 870 bps. The DNA gel was run at 110 V for 30 minutes and imaged at 500 to 600 nm (Figure 11). This TEM-1 β-lactamase insert was recovered from the DNA gel and used for ligation. Similarly, the modified pET43.1a vectors containing the GGCG and GGGG tags (constructs 2 and 3, respectively) were digested and the open vectors recovered using the same procedure and conditions as construct 1. The insert from the pET43.1a vectors were not required for this project.
Figure 11. DNA agarose gel showing the digestion of the TEM-1 β-lactamase cloning vector using *BamH1* and *Nde1*. Lane 1 shows the DNA ladder with a bps marker ladder on the side. Lane 2 is the digested sample showing a clean insert band with the expected size (~870 bps).

2.4 Agarose gel electrophoresis and DNA fragment purification

I acknowledge Regina Surjadi for assistance in the DNA fragment purification of the TEM-1 β-lactamase vector. Agarose gel electrophoresis is the preferred method for determining the size of DNA and for extracting DNA fragments. DNA fragment samples and PCR mixtures were mixed with 4x DNA loading dye and loaded in separate wells on an agarose gel. All experiments were performed in 1x TAE buffer with a GeneRuler 1kB Plus DNA ladder included and were run at 150 V for 40 minutes. The gels were visualised using an Odyssey Fc imaging system, LI-COR, at 600 nm. Positive clones were identified as higher running bands on the gel than the parental clones (negative controls). Sizes of the plasmids were confirmed
by comparison to the DNA ladder. DNA fragments were excised under a UV lamp using a gel
knife to isolate individual bands. The DNA fragment was purified using NucleoSpin Gel and
PCR Clean-Up according to the manufacturer's instructions. The kit contains a high-salt buffer
that dissolves the gel containing the DNA. The DNA is then bound to the silica column and
ethanol solution is used to wash away any impurities. The pure DNA is then eluted under low
ionic strength conditions with slightly alkaline buffer (5 mM Tris/HCl, pH 8.5).

### 2.5 Quantification of DNA insert and vector

DNA insert and vector concentrations can be quantified using the Nanodrop 2000
spectrometer (Thermo Fisher). DNA concentrations are calculated from the absorbance at 260
nm using the Beer-Lambert Law, (A= εcl) and DNA purity is determined by the absorbance
ratio at 280 nm and 260 nm. The size of the DNA is determined by the position where the DNA
stops migrating in an agarose gel and comparison to DNA molecular weight (MW) markers.
As DNA has a negatively charged phosphate backbone and moves towards the anode, smaller
DNA strands move faster through the gel and larger DNA fragments run more slowly. A DNA
MW ladder run in a separate lane is used to determine the size of each DNA band. High quality
DNA should have a ratio between 1.8-2.0 for the 280 nm/260 nm ratio which inform the correct
AT to CG base pairs. A sample of 1-2 µL was loaded onto the Nanodrop to measure the DNA
concentration. TEM-1 β-lactamase insert, and open vector concentration are displayed in Table
2.

<table>
<thead>
<tr>
<th>DNA sample</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactamase insert</td>
<td>116</td>
</tr>
<tr>
<td>pET43.1a GGCG vector</td>
<td>103</td>
</tr>
</tbody>
</table>
2.6 Cloning recombinant TEM-1 β-lactamase

I acknowledge Regina Surjadi for assistance in cloning the TEM-1 β-lactamase. The purified insert DNA and open vector DNA could be ligated together due to their compatible sticky ends since the same restriction enzymes were used for digestion. The TEM-1 β-lactamase insert was used to ligate to both opening vectors. A T4 DNA ligase is used to join the phosphate backbones when the complementary bases are aligned. Molar ratios of 1:1, 3:1 and 5:1 of insert: vector were examined but only the 5:1 ratio gave successful ligation, as determined by DNA electrophoresis gel (see 2.4). The enzyme activity of T4 ligase used in all trials was 10 kU.

Transformation is the process wherein a newly designed vector is introduced into host bacterial cells in order to express the recombinant protein of interest. Construct 1, 2 and 3 plasmids were transformed into DH5-α E. coli cells. The competent DH5-α cells have a porous membrane when heat-shocked, which allows the supercoiled plasmids to enter the cells with high transformation efficiency. A standard heat shock method was applied that involved placing the cells on ice for 10 minutes, applying a heat shock at 42 °C for 45 seconds and returning to the ice for another 2 minutes. The transformed cells were supplemented with nutrient rich 2x YT with ampicillin. The cells were streaked onto YT-Amp agar plates with the ampicillin selective marker to develop single colonies overnight. The plates were incubated upside down in the 37 °C incubator to prevent condensation of water and contamination. The doubling time for E. coli is 20 minutes and a visible colony can develop on the agar plates within 16 hours. A negative control plate was also incubated using undigested pET 43.1 a. Colonies were formed as spherical white nodules on the agar plates and random colonies were selected for colony screening.
2.7 Colony screening for positive TEM-1 β-lactamase clones

I acknowledge Regina Surjadi for assistance in colony screening for the TEM-1 β-lactamase vector. Potential positive clones were identified through colony PCR on the transformants. Random colonies from each transformation plate, along with the control plates, were selected from 2.5 and screened for positive TEM-1 β-lactamase clones. A Master Mix was created containing 5 µL of 2xGoTaq® Green (GTG), 0.5 µL of T7 Forward primers (10 µM), 0.5 µL of Coli Down, a reverse primer, (10 µM) and 4 µL of distilled water for each colony to undergo PCR. The reverse primer binds to the stop codon of the β-lactamase gene and read the codon backwards for PCR. Each colony was dipped into separate 10 µL aliquots of the Master Mix in PCR tubes and placed into the PCR machine. Annealing temperatures for the PCR reactions were obtained using an online calculator that estimates the melting temperature based on the percentage of cytosine and guanine bases in the primers. Each colony in the reaction mixture was amplified using 25 cycles using the conditions outlined in Table 3.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time per sample (sec)</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>240</td>
<td>1</td>
</tr>
</tbody>
</table>

Colonies were streaked onto YT-Amp agar patch plates and incubated at 37 °C overnight. The reaction mixtures were analysed on a 1% (w/v) agarose gel with 1:10,000 SYBR Safe dye made up in Tris-acetate-EDTA (TAE) buffer to determine the positive clones. A bacterial colony with positive transformation was streak purified and stored long-term in LB medium containing 50% glycerol at -80 °C.
2.8 DNA Sequencing

I acknowledge Regina Surjadi for assistance in DNA sequencing. DNA sequencing was used to confirm whether the entire sequence had inserted correctly into the expressing vector. Samples of the positive clone identified from the agarose gels in 2.7 were grown up in sterile YT-Amp media overnight at 37 °C from the overnight patch plate. The DNA was then extracted by centrifugation at 4,000 rpm for 10 min at 4 °C and a Miniprep was performed on the pellet. Polymerase Chain Reaction (PCR) (Perkin Elmer GeneAmp 9600 PCR System Thermal Cycler) was performed on the purified DNA to amplify the DNA concentration. Purified DNA of positive clones was sent to Micromon (Monash University, Victoria, Australia) for Sanger DNA sequencing. The DNA sequencing was performed using the T4 reverse primer as the β-lactamase was at the downstream end of the plasmid. 10 µL samples of DNA were sent fresh from the Miniprep extraction as the DNA degrades over time. The rest of the samples were stored in the -20 °C freezer until completion of the sequencing. DNA sequencing chromatograms analysed using Chromas. Chromas provided three different sequences, one for each reading frame. The correct reading frame was aligned to the theoretical DNA sequence using BLAST. DNA that aligned correctly indicated that the corresponding cell colony had transformed successfully and could be used for protein expression. DNA sequencing results for the β-lactamase constructs 1-3 are provided in Appendix 2.

2.9 Analytical protein expression studies

I acknowledge Regina Surjadi for assistance in small-scale analytical protein expression. A small-scale analytical protein expression with the positive clones was performed to determine whether the three β-lactamase constructs express the correct, enzymatically active protein. The DNA plasmids confirmed by Sanger sequencing from 2.8 were transformed into
*E. coli* BL21-DE3 cells using the heat-shock method and incubated on YT-Amp plates overnight at 37 °C. One overnight colony from each construct was selected and grown in fresh 5 mL of 2 x YT media at 37 °C until an OD of 0.8 was reached before the sample was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The overnight colony plates were sealed and kept in the refrigerator for further expression experiments. IPTG causes overexpression of the β-lactamase construct at 16 °C overnight and allows correct folding of the enzyme. Cells were centrifuged and the pellets lysed with Bugbuster to extract the overexpressed β-lactamase protein. The lysate was centrifuged briefly and the supernatant containing the β-lactamase protein was extracted. Sodium dodecyl sulfate (SDS)-gel and Western blot analyses were performed to determine whether the expressed enzyme had the correct sizes. The SDS gel and Western blot showing the expression of the three β-lactamase constructs are shown in Figure 12.
Figure 12. (A) SDS gel and (B) Western blot of the analytical expression of the three constructs respectively. Samples were loaded in duplicate. NuPAGE 4-12% precast gel was used. Lane 1 = SeeBlue 2 Ladder. Lane 2 and 3 = duplicate samples of the expressed His-TEV-β-lactamase construct. Lane 4 and 5 = His-TEV-SUMO-GGGG-β-lactamase construct. Lane 6 and 7 = His-TEV-SUMO-GGCG-β-lactamase construct.

SDS gel and Western blot were performed on the crude lysates from the *E. coli* cells. In lanes 2 and 3 in the SDS gel, a dark band above the 28-kDa marker corresponded to the His-TEV-β-lactamase. The corresponding lanes in the anti-His tag Western blot showed the same size band, confirming that the His-TEV-β-lactamase construct was expressing well. A band below the 49 kDa marker in lanes 2 and 3 in both the SDS gel and Western blot suggested that the sample may contain endogenous proteins with a high number of histidine residues or that the expressed β-lactamase is aggregating to some extent. Lanes 4 and 5 showed a major band just below the 49 kDa marker, which corresponded to the His-TEV-SUMO-GGGG-β-lactamase (construct 3). Similarly, lanes 6 and 7 showed the same band as in lane 4, consistent with the very small difference in the constructs 2 and 3 (i.e. substitution of one glycine residue to a cysteine residue). However, the two constructs also showed a band around 98 kDa in both
the SDS gel and Western blot, suggesting that the proteins were forming dimers. This was more prominent in lanes 6 and 7, which may indicate that the newly introduced cysteine residue was forming an intermolecular disulfide bond. It is possible that a small amount of protein leaked from the adjacent well and that the band at 49 kDa is not aggregation. We suspect that increasing the size of the protein by adding the SUMO gene may have contributed to increased dimerisation. Due to this dimerization issue, only the His-TEV-β-lactamase construct 1 was advanced to large-scale protein expression and bioconjugation studies.

2.10 SDS-Page Gels and Western Blotting

I acknowledge Dr. Bill McKinstry for his expert tips on SDS-PAGE gels and Western blots. SDS-gel and Western blot analysis was used to detect whether the BL21 DE3 cells were expressing the correct TEM-1 β-lactamase. SDS denatures proteins and coats them with a negative charge, allowing migration through gels based on molecular size when a voltage is applied. The size of the protein determines how fast and how far down the gel the protein migrates, with smaller proteins moving further than larger proteins. All SDS-gels and Western blots were performed using commercially available NuPAGE 6-12% Bis-Tris gels. A commercial standard See Plus 2 MW marker ladder was added to the first lane of each gel. This ladder contains a mixture of proteins of defined molecular weights and was used to estimate the sizes of proteins, including the expressed TEM-1 β-lactamase. Coomassie stain was used to visualise the proteins on the SDS gel. Washing with 10 % ethanol/7.5 % glacial acetic acid (v/v) to remove excess stain allowed clear visualisation of the protein bands and ladder.

Western blot was performed to confirm the protein of interest within the gel using a specific antigen conjugated to a reporter enzyme. Chemiluminescent Western blot was used to detect the TEM-1 β-lactamase as it is a sensitive assay. The SDS gel was transferred to a
nitrocellulose membrane using a Western blot transfer tank running at 25 V for 90 minutes. The nitrocellulose membrane was blocked with milk powder solution as a cheap, arbitrary protein source. Detection used a, anti-His- horseradish peroxidase (HRP) antibody conjugate that recognises and binds to poly-histidine epitopes (Hisx6 and Hisx10). The only protein on the membrane with a poly-His tag and expected to be bound by the antibody is the TEM-1 β-lactamase. HRP chemiluminescence substrate (Super-signal West Pico PLUS) was then added to the membrane. The chemiluminescence substrate contains luminol, which is the limiting substrate for HRP and produces a light-emitting product. The membrane was imaged immediately at 425 nm. The band that lit up from the imaging system corresponded to the TEM-1 β-lactamase. The purity of the sample was determined by comparing the Western blot and the Coomassie blue-stained SDS gels.

2.11 Large scale TEM-1 β-lactamase expression

I acknowledge Dr. Bill McKinstry for assistance in the large scale β-lactamase expression. The SDS-gel of the small-scale expression of the three constructs (section 2.9) showed that only the wild type His-TEV-β-lactamase had one major protein band, compared to the other constructs. Therefore, only the His-TEV-β-lactamase (hereafter referred to as TEM-1 β-lactamase) was carried forward to large scale expression and purification. The YT-Amp colony plate of TEM-1 β-lactamase used for the small-scale expression was thawed and another colony picked and incubated with 12.5 mL of 2x YT-Amp medium overnight at 37 °C. The overnight culture was used to inoculate one litre of TB media containing 10% potassium chloride salt. The potassium phosphate was included in the media to act as a buffer to delay alkalization and entry of cells into the death phase. E. coli cells were incubated in TB as it contains glycerol as a carbon source, releasing acid when metabolised and delaying alkalization. The OD for the TB starting culture was 0.1, corresponding to the bacteria’s growth.
phase. The one litre culture was split into two 500 mL Turnair flasks and shaken in the incubator at 180 rpm at 37 °C to allow better aeration. Shaking and aeration are required to prevent the cells from settling to the bottom of the flask, which can increase cell death due to nutrient shortage and low oxygen. The cultures were grown to OD 0.7-0.8 was achieved, where the bacteria are in mid-logarithmic growth phase. At this point, the culture has an abundance of healthy cells and ample nutrients remaining in the media for continued growth. The cultures were then induced with 0.5 mM IPTG and moved to a 16 °C overnight shaker. IPTG triggers a change in the conformation of the lac repressor on the lac operon in the BL21 DE3 genome, causing it to dissociate and allow binding of the T7 RNA polymerase. This commences expression of the TEM-1 β-lactamase. Expression was tested at both 16 °C and 30 °C to establish the best temperature for correct protein folding. Expression at 16 °C was ultimately found to provide more homogeneous and higher quantity TEM-1 β-lactamase than at 30 °C. The overnight cultures were centrifuged down, the bacteria pellets isolated and lysed to release the TEM-1 β-lactamase into solution.

2.12 Extraction and purification of TEM-1 β-lactamase

I acknowledge Dr. Bill McKinstry for assistance in extracting the β-lactamase and Lesley Pearce for guidance in protein purification. TEM-1 β-lactamase was extracted from the bacterial cell pellets and then purified from the other bacterial proteins. Cell lysis can be achieved using mechanical techniques, such as sonication and cell crushing using a French press, or non-mechanical techniques like alkaline chemical lysis or enzymatic lysis (2.12.1). Combinations of mechanical and non-mechanical techniques were used here to extract the TEM-1 β-lactamase from the pellets (2.12.2). After the bacterial cells were lysed, the expressed TEM-1 β-lactamase was purified using immobilized metal affinity chromatography (IMAC). Adding the cell lysate to the column leads to the TEM-1 β-lactamase becoming immobilised
through binding of the His tag to the nickel column matrix; this step removes most non-His tagged proteins. Further purification by gel filtration chromatography was performed to separate the homogenous TEM-1 β-lactamase from protein aggregate mixtures (2.12.4).

2.12.1 Chemical and enzymatic extraction

Pellets obtained in 2.11 were lysed on ice using chemical and enzymatic lysis buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 2 mM MgCl₂, 10 mM imidazole, 0.5 mg/mL lysozyme, 5 ul benzonase, 1 mM PMSF and 3 tablets of protease inhibitor pellets) in a ratio of 10 mL of lysis buffer to 1 gram of cells. Sodium azide (0.02%) was included to prevent microbial contamination and sodium chloride was used to raise the ionic strength of the buffer. This helps to break down protein complexes and causes cells to ‘implode’ due to the increased external osmotic pressure. Lysozyme was used to break up the cell wall and benzonase and MgCl₂ were used to separate the DNA and RNA from the cells and degrade them. PMSF and a cocktail of protease inhibitors were used to inhibit the functions of proteases and protect the TEM-1 β-lactamase. A low concentration of imidazole was added to prevent the His tag from forming a complex with adventitious metal ions. The pellet in the lysis buffer was stirred at 4 °C until the sample became a homogeneous mixture, after which the samples were treated using the cell crusher or sonicator.

2.12.2 Cell crushing and sonication

Occasionally, cells are not broken down by chemical or enzymatic treatment and require further mechanical action using a cell crusher or sonicator. Cell crushing involves passing cells through a French press at high pressure (15000 to 16000 psi) multiple times, where the shear stresses cause the cells to rupture. The French press can handle large amounts of cellular matter; however, it is time-consuming and is plagued by blockages in valve that are
difficult to clean. The French press, EmulsiFlex C5 high pressure homogeniser is covered with ice to keep it cool. Alternatively, a sonicator S4000 fitted with a 20 kHz converter and ½ inch probe with a replaceable tip, can be used to disrupt the membranes of cells and break them open. The cell matter was placed in the sonicator vessel and sonicated using a titanium probe. The vessel needed to be kept on ice as sonication generates heat. Rest phases were used to allow the vessel to cool down between the pulses. The sonicator can only handle small volumes of cellular matter in one cycle of pulse and rest and constant use can cause overheating and damage to the proteins. Both methods were trialled for obtaining cell lysates for the protein purification steps.

2.12.3 Immobilized metal affinity chromatography

The lysed samples from 2.12.2 were centrifuged and separated into supernatant and pellet. A 5 mL His-Trap column was attached to a protein purification instrument (Bio-Rad Profinia™ Affinity Chromatography Protein Purification System) and the supernatant was loaded onto the column. The initial binding phase adheres the TEM-1 β-lactamase and some non-specific proteins to the nickel matrix in the His-Trap column, while non-His-tagged proteins are collected in the flowthrough. Two washes were performed using PBS with 0.02% NaN₃, 150 mM NaCl, 10 mM imidazole followed by PBS with 0.02% NaN₃, 150 mM NaCl, 20 mM imidazole to remove any non-specific binding proteins from the column. The TEM-1 β-lactamase was then eluted with PBS containing 0.02% NaN₃, 150 mM NaCl and 250 mM imidazole. A chromatogram for the IMAC purification is shown in Figure 13.
Figure 13. Chromatogram from the IMAC purification of TEM-1 β-lactamase from crude cell lysate supernatant. Green lines show the UV intensity at 280 nm and pink lines show conductivity. 1A, 1B, 1C and 1D indicate the collected sample tubes.

During the first 10 minutes, there was a small jump in the conductivity as the system is switched to binding buffer. There is a small jump in the UV signal as any previous proteins and impurities are washed out. The UV intensity at A280 nm then rises sharply (tube 1A) and becomes saturated at 1.2. This is caused by the large amount of unbound protein flowing off the column. At this stage, the His-tagged and other proteins with affinity for the nickel column matrix were bound and all other proteins had eluted into tube 1A. In tube 1B, the UV decreases back to baseline as all the proteins that were not immobilised on the nickel matrix had exited the column. Tube 1C shows a small increase in the UV signal, possibly due to proteins with low affinity for the nickel matrix are eluted. A large peak (tube 1D) was collected that appeared to correspond to the TEM-1 β-lactamase. The crude lysate and each collected tube were analysed by SDS-PAGE gel electrophoresis to determine whether the IMAC purification had been successful (Figure 14).
Figure 14. SDS-gel of samples collected from the IMAC purification of TEM-1 β-lactamase. Lane 1 = SeeBlue 2 standard protein ladder. Lane 2 = crude cell lysate s. Lanes 3 and 4 = lysate supernatant and pellet, respectively. Lane 5 = flowthrough samples from IMAC (tube 1A). Lane 6 and 7 = tubes 1B and 1C, respectively. Lane 8 = tube 1D containing the TEM-1 β-lactamase.

The cell lysate (Lane 2) and supernatant fraction (Lane 3) showed similar proteins, including a concentrated band above the 28 kDa marker. The pellet fraction (Lane 4) showed almost no protein compared to the lysate and supernatant, suggesting that the lysis buffer and the cell crusher had successfully extracted all of the cellular proteins, including the expressed TEM-1 β-lactamase, and that these proteins were soluble. The flowthrough (Fraction 1A) showed a similar pattern of proteins as the lysate and supernatant fractions, except the concentrated band above 28 kDa was not visible, suggesting the HisTrap column had trapped the His-tagged TEM-1 β-lactamase. The wash 1 fraction (Fraction 1B) and wash 2 fraction (Fraction 1C) showed faint bands above the 28 kDa marker, suggesting some weaker binding N-His tagged protein had dissociated from the column. This elution fraction 1D showed only a single concentrated band above the 28 kDa marker, consistent with the His-tagged TEM-1 β-lactamase.
The elution fraction 1D was collected and its protein concentration measured using a Nanodrop spectrophotometer (2.4), with elution buffer serving as the blank, (extinction coefficient: 28,085, MW: 29,410 Da). The concentration of 1D was determined to be 1.2 mg/mL. Due to the volume of the elution fraction being greater than the maximum loading capacity on the gel filtration column, elution fraction 1D was concentrated using an Amicon® Ultra-15 10,000 MW cut-off spin concentrator. Sequential concentration of 5 mL aliquots reduced the volume from 110 mL to 30 mL. Nanodrop concentration measurements at 280 nm using PBS as a blank were performed to determine if any TEM-1 β-lactamase had been lost during the protein concentration step. The initial amount of pooled TEM-1 β-lactamase eluted from the multiple runs of IMAC columns were 196 mg at a concentration of 3.76 mg/mL. The pooled TEM-1 β-lactamase obtained after final concentration was 187 mg at 6.23 mg/mL. This indicated that most of the TEM-1 β-lactamase had been retained in solution and not precipitated during concentration. The concentrated TEM-1 β-lactamase samples were advanced to final purification by gel filtration chromatography.

2.12.4 Gel filtration chromatography

Gel filtration chromatography (GFC) is routinely used to separate protein mixtures based on molecular size. Small proteins can strongly interact with the matrix as they are small enough to enter the pores of the gel, slowing their migration compared to larger proteins. Proteins therefore elute in order of decreasing molecular weight, that is, larger proteins elute earlier from the column and smaller proteins elute later. GFC can also separate protein aggregates from their monomeric forms, as protein aggregates have a higher molecular weight.

GFC was used as the final step in purifying the TEM-1 β-lactamase and to ensure that the protein isolated is a homogenous monomer. The concentrated samples were loaded in 4 cycles of 7.5 mL aliquots onto a Superdex 200, 26/60 column attached to an AKTA Pure
chromatography instrument. The elution buffer was PBS that had been 0.22 µm sterile filtered and 1 mL fractions were collected in 96 deep-well plates. Elution of proteins was monitored at A280 nm. An example chromatogram obtained from the GFC purification is shown in Figure 15.

**Figure 15.** Gel filtration chromatogram of TEM-1 β-lactamase purification. The chromatogram is zoomed between 80 - 280 mL where all the proteins eluted. Conductivity (Cond) is shown in orange. Collected fractions 1E and 1F corresponded to protein aggregates. 1G was the intermediate between the protein aggregates and the monomeric TEM-1 β-lactamase (1H).

The eluted proteins were collected in tubes labelled 1E, 1F, 1G and 1H. A drop in conductivity was shown at retention time of 110 mL indicating the salts from the injected sample were being eluted. The main peak was located between retention volumes 210-255 mL, which was collected in tube 1H. It was likely that the peaks designated as 1E and 1F were aggregated and dimerised protein, respectively. Tube 1G corresponded to the eluent between the main protein peak and the dimerised protein peak. An SDS-PAGE gel (Figure 16) run on every 5th fraction collected across the gel filtration chromatogram allowed determination of
protein purity and confirmation of size. All sample concentration were measured using the Nanodrop spectrophotometer (2.4).

Figure 16. SDS-PAGE gel of samples collected from the first GFC run. Lane 1 = SeeBlue 2 MW Ladder. Lanes 2-4 = every 5th sample in Tube 1E. Lanes 5 and 6 = samples from Tube 1F. Lanes 7-12 = samples from Tube 1H.

Lane 1 contained the commercial See Blue 2 protein MW ladder. Lanes 2-4 corresponded to every 5th sample in tube 1E. Lanes 5 and 6 contained samples from 1F. Lanes 7-12 came from tube 1H. 1G was not run in the gel due to its low protein content. Each lane showed a protein band at around the 28 kDa MW marker, with the bands becoming more concentrated with each progressing fraction. Lanes 2-5 indicate that the samples loaded contained protein around the 62 kDa MW marker and above, suggesting that larger proteins or aggregates were eluting with the TEM-1 β-lactamase. Moving from left to right across the SDS gel, these other proteins or aggregates diminished in intensity until only the pure protein band at 28 kDa remained.
In comparing Figures 15 and 16, tube 1H contained most of the protein, as evidenced by the high UV absorbance at 280 nm. Protein absorption at this wavelength is caused by the aromatic amino acids, tyrosine, tryptophan and phenylalanine. The gel lanes corresponding to tube 1H confirmed it contained almost pure protein with the correct mass of 28 kDa. Fractions from 1H were next subjected to mass spectrometry to confirm the correct mass of the TEM-1 β-lactamase.

2.12.5 Mass spectrometry

MALDI-TOF MS analysis was performed on the protein samples from tube 1H by Dr. Tom Nebl at CSIRO, Parkville. A representative mass spectrum obtained is displayed in Figure 17.

![Mass Spectrum](image)

**Figure 17.** MALDI-TOF mass spectrum of protein (1 mg/mL) contained from tube 1H. Figure provided by Dr Tom Nebl.

From Table 1, the expected molecular weight of the His-tagged TEM-1 β-lactamase was 31,876.11 Da. The deconvoluted MALDI-TOF mass spectrum (Figure 17) showed a major peak corresponding to a protein of 31,876.0 Da. This molecular weight was identical to the theoretical weight, confirming that tube 1H contained the desired, His-tagged TEM-1 β-
lactamase. A small peak at around 32,053.1 Da indicated the possible presence of a small amount of gluconoylated protein and addition of 80.4 Da suggests potentially further phosphorylation.

2.13 TEM-1 β-lactamase: stability and activity studies

After successfully cloning, expressing and purifying the TEM-1 β-lactamase, the next step was to determine if the protein had suitable enzymatic activity and stability. Differential Scanning Fluorimetry (DSF) studies, performed by Dr. Janet Newman from the Collaborative Crystallisation Centre at CSIRO, were performed to establish the stability of the TEM-1 β-lactamase under a range of pH and buffer conditions. DSF measures unfolding of proteins from the changes in fluorescence that occur as the temperature is increased. The melting temperature (Tm) is used to indicate when half of the protein has unfolded, with higher Tm corresponding to increased conformation stability. The results of these studies are shown in Figure 18.
Figure 18. Melting temperature (Tm) of purified TEM-1 β-lactamase under a range of pH and buffer conditions. Study performed and figure provided by Dr. Janet Newman, CSIRO.

These experiments helped to understand the stability of the protein over the pH range 5.0-9.0 under low NaCl (50 mM) and high NaCl (200 mM) conditions in various buffers. The Tm of the β-lactamase in PBS was measured at 37.21 °C. Figure 18 shows that the Tm under all conditions was higher than in PBS, and that the enzyme is more stable at lower pH and that salt concentration has minimal effects on stability.

The catalytic activity of β-lactamases can be measured by reacting the enzyme with the chromogenic cephalosporin substrate nitrocefin, which gives rise to a coloured product when the β-lactam ring opens (Scheme 6). A nitrocefin assay was performed on the TEM-1 β-lactamase according to the guidelines provided by the manufacturer (Biovision) against a standard curve (Appendix 3). The β-lactamase was found to show an activity of 3600 U. One
unit (U) of TEM-one β-lactamase activity is the amount of enzyme that generates 1 µmol of nitrocefin per minute at pH 7.0 at 25°C.

![Scheme 6](image).

**Scheme 6.** Ring opening of nitrocefin by β-lactamases causes a colour change from yellow to red that is used to quantify enzyme activity.

### 2.14 Cleavage of the His-TEV tag

I acknowledge Dr. Bill McKinstry for assistance in cleaving the TEM-1 β-lactamase His tag. The final step in preparing the β-lactamase prior to use in bioconjugation studies was removal of the His-TEV purification tag. This was achieved by incubating the His-TEV-β-lactamase with His-TEV protease in the presence of the reducing agent dithiothreitol (DTT) overnight in a refrigerator. DTT was required to activate the His-TEV protease. The cleaved β-lactamase was purified from the His tag and the protease using an IMAC column. The mixture was added to the IMAC column and the cleaved β-lactamase was collected in the flowthrough. PBS with increasing concentrations of imidazole (10 mM, 20 mM, 250 mM) was then used to subsequently wash the other components from the column. SDS-PAGE gel and Western blot analysis were used to confirm removal of the His-TEV tag. The cleavage was run in two batches and the results are shown in Figure 19.
Figure 19. (A) SDS-PAGE gel and (B) Western blot from the His-TEV cleavage reaction. Lane 1 = SeeBlue 2 MW ladder. Lane 2 = uncleaved His-TEV-β-lactamase. Lane 3 = TEV protease. Lane 4 = flowthrough for batch 1. Lane 5 first wash for batch 1. Lane 6 = second wash from batch 1. Lane 7 = elution for batch 1. Lane 8 = flowthrough for batch 2. Lane 9 = first wash for batch 2. Lane 10 = second wash for batch 2. Lane 11 = elution for batch 2.
The SDS-PAGE gel for the cleavage reaction showed that the protein band in the flowthrough and washes (lanes 3-6) were different from the His-tagged β-lactamase control (lane 2). This was expected as loss of the tag (2 kDa) causes a decrease in the protein’s molecular weight, making it run faster through the gel. The corresponding Western blot depicted a concentrated band for the His-tagged enzyme (lane 2), and a concentrated band in the elution sample, suggesting a large amount of uncleaved His-tagged β-lactamase was still present. The N-His tag on its own is only around 2 kDa and does not show on the Western Blot. The Western blot also showed a negative result for wash 1, which had an equivalent strong band in the SDS-PAGE-gel. This result suggested that wash 1 contained the cleaved β-lactamase, while the uncleaved β-lactamase had come through in the flowthrough. In the second batch, a band with the correct size occurred in lane 8 in the SDS-PAGE gel with no equivalent signal present in the Western blot, indicating that the cleaved β-lactamase was present in the flowthrough this time. Lane 9 showed a band at 29 kDa that had an equivalent band in the Western blot, corresponding to the His-TEV protease. The reasons for the anomalous elution profiles are unknown but were likely due to human error. Nevertheless, samples from Lanes 4 and 8 containing the pure, His-tag cleaved TEM-1 β-lactamase were buffer-exchanged into PBS using Amicon®-15 10,000 MW cut off spin filters and mass spectra were acquired to confirm the correct molecular weight (Figure 20).
The theoretical molecular weight of the TEM-1 β-lactamase after removal of the tag was 29410.47 Da (calculated using ProtParam). The mass spectra from the two matches, showed closely matching peaks at 29408.7 and 29410.0, respectively, confirming that the target TEM-1 β-lactamase had been successfully isolated. The pure protein was stored in PBS buffer at 2.39 mg/mL in the fridge (4 °C). Enzyme activity was not performed on the cleaved TEM-1 β-lactamase due to time restriction. It is known that the tag was on the N-terminus and away from the active site therefore the enzyme activity should remain the same as the tagged enzyme (3600 U).

### 2.15 Summary

A TEM-1 β-lactamase was designed and cloned in *E. coli*. Expression of the enzyme occurred at 16 °C overnight with induction by IPTG. Purification of the enzyme was achieved by lysing the cells using a combination of chemical and mechanical techniques, followed by sequential IMAC and GFC columns. Mass spectrometry confirmed that the expressed protein had the correct molecular weight. The DSF assay showed that the enzyme is more stable at lower pH, which was important for planning bioconjugation strategies (Chapter 3) that would keep the β-lactamase active.
Chapter 3: Bioconjugation of TEM-1 β-lactamase to the model antibody fragment Fab' 528
3.1 Introduction

Bioconjugation is the chemical coupling of two molecules together via a covalent linker, with at least one of the molecules being a biomolecule, such as a protein. For this study, the TEM-1 β-lactamase was to be conjugated to the antibody fragment Fab' 528. This particular antibody fragment was chosen for this work as a model protein and representative of a range of possible antibody fragments and, more generally, cysteine-containing proteins that are amenable to thio-based conjugation chemistry. The antibody fragment Fab' 528 was derived from the monoclonal IgG antibody, referred to as 528. Fab' 528 has been shown to reduce expression of Epidermal Growth Factor Receptor (EGFR) in malignant human cell lines.\textsuperscript{106} Fab' 528 is routinely prepared by reduction of 528-F(ab')\textsubscript{2} using the mild reducing agent, tris(2-carboxyethyl) phosphine (TCEP). Bioconjugation reactions can utilise the nucleophilic thiol groups on Fab' 528 fragment that are solvent exposed after the reduction (Figure 21).

\textbf{Figure 21.} Disulfide reduction of 528-F(ab')\textsubscript{2} with TCEP to give Fab' 528. Two solvent accessible thiols are revealed after reduction at the hinge region of the antibody fragment and can be used in bioconjugation reactions.

The strategy for conjugating the Fab' 528 fragment to the TEM-1 β-lactamase involved use of the small water soluble crosslinker sulfo-succinimidyl-(N-maleimidomethyl) cyclohexane carboxylate (sulfo-SMCC), as summarised in Scheme 7.
Scheme 7. Conjugation chemistry plan. TEM-1 β-lactamase is first attached to the sulfo-SMCC-linker via amide coupling and the Fab’ 528 fragment is subsequently appended using thiol-maleimide chemistry, forcing the β-lactamase-Fab’ 528 conjugate.

Sulfo-SMCC is a commercially available heterofunctional cross-linker that is commonly used in bioconjugation chemistry. In general, bioconjugation using this crosslinker does not give rise to site-specific protein modification. This is because the N-hydroxysuccinimide (NHS) ester group of sulfo-SMCC can form amide bonds with any solvent-exposed amines on a protein, such as those on surface lysines and, in some circumstances, the N-terminal primary amino group. Similarly, the maleimide group can react with the thiol groups on any of a biomolecule’s solvent-exposed cysteine residues. The sulfo-SMCC group is a relatively short linker at 8.3 Å in length. The hydrophilic sulfonate group enhances aqueous solubility of sulfo-SMCC, allowing the compound to be soluble at concentrations up to 10 mM in water or buffer.

The TEM-1 β-lactamase contains no solvent-exposed cysteines. and can therefore only react with the NHS group of sulfo-SMCC via its amino groups. TEM-1 β-lactamase contains two cysteines that forms a disulfide bond located in the centre of the enzyme. The Fab’ 528 antibody fragment, when first reduced, contains two solvent accessible cysteines. Thus, the Fab’ 528 can be conjugated via these hinge region exposed thiols to the maleimide group of the sulfo-SMCC crosslinker. The sulfo-SMCC was thus reacted first with the TEM-1 β-lactamase
via the NHS group and the functionalised β-lactamase subsequently conjugated to Fab’ 528 via the maleimide group. This conjugation method can lead to heterogenous products as the NHS active ester can react with any of the solvent assemble lysine amine residues on the TEM-1 β-lactamase surface and the maleimide group can react with either of the two exposed thiols on the Fab’ 528. Site-specific bioconjugation (5.2.1) can be performed to prevent this potential issue.

Solvent and pH were important considerations in the bioconjugation reactions. NHS esters react with protein amines at pH 7-9 to form stable amide bonds. The maleimide group reacts with protein thiol groups at pH 6.5-7.5, to form stable thioether bonds. In section 2.13, TEM-1 β-lactamase was shown to have good stability across the pH range 5.0-7.0 and in PBS buffer. Previous studies from the Williams research group at CSIRO described conjugation of the Fab’ 528 fragment to PEGylated doxorubicin constructs in PBS at pH 7.2.106

3.2 Molar ratios in the reaction of TEM-1 β-lactamase with sulfo-SMCC

Experiments were performed to determine the optimal number of molar equivalents of sulfo-SMCC required for one-to-one attachment of the sulfo-SMCC crosslinker to the TEM-1 β-lactamase. Ratios of 1:1, 1:5 and 1:10 TEM-1 β-lactamase (2.39 ng/mL) to sulfo-SMCC (dissolved in water to give 4.36 mg/mL) were reacted at room temperature for 1-2 hours before passing each through a gel filtration column (S200 10/300 increase) using PBS as eluent (2.12.4). Initial ratio experiments were performed with 4 nanomoles of TEM-1 β-lactamases. The chromatograms obtained are displayed in Figure 22.
Figure 22. Gel filtration chromatograms of crude reaction mixtures obtained during molar ratio experiments with TEM-1 β-lactamase and sulfo-SMCC.

The overlaid chromatograms of the crude reaction mixtures showed that in all experiments the protein peak appeared to remain unchanged relative to the unreacted control TEM-β-lactamase sample (purple), which eluted at 17 mL. Excess and unreacted sulfo-SMCC and the NHS leaving group eluted later from the column, giving a peak at 20 mL. A small shoulder in the protein peak (~16 mL) suggested that some of the β-lactamase may have become aggregated. Fractions collected across the 17 mL peak were pooled, concentrated and analysed by mass spectrometry to determine if the sulfo-SMCC group had been conjugated to the protein.

The molecular weight of the His tag-cleaved TEM-1 β-lactamase is 29,410.47 Da. When one sulfo-SMCC molecule reacts via its NHS ester with a protein amino group, the molecular weight increases by 218 Da. The mass spectra indicated that the molar ratios of 1:5 and 1:10 (protein: crosslinker) both led to addition of one sulfo-SMCC linker to the TEM-1 β-lactamase (Appendix 4-6). All subsequent reactions were therefore performed using a protein: crosslinker molar ratio of 1:10. After performing the reactions, the linker-installed protein was purified on a 1 mL HiTrap desalting column to remove the excess SMCC/NHS by-products.
The TEM-1 β-lactamase-SMCC was used immediately with the Fab' 528 to avoid hydrolysis of the maleimide.

### 3.3 Reduction of 528-F(ab')2 to Fab' 528

I thank Dr. Judith Scoble at CSIRO for providing me with the 528-F(ab')2 antibody. The Fab' 528 antibody fragment was obtained by routine reduction of the complete 528-F(ab')2 antibody using TCEP (Figure 21). The concentration of TCEP used to selectively reduce the solvent exposed, hinge region disulfides, needed to be controlled so that the intramolecular disulfide bonds between the light and heavy chains of the Fab' 528 fragment were not also reduced. The molar ratio of 528-F(ab')2 to TCEP used in the reaction was trialled from 1 molar equivalent of the reducing reagent to 100-fold excess. It was found that a 100-fold excess of TCEP provided the largest amount of Fab' 528 and did not lead to over-reduction of the intramolecular disulfide bonds. Using this ratio, 528-F(ab')2 was reacted with TCEP for 1 hour at 4 °C before isolating the Fab' 528 fragment using GFC (Figure 23).
Figure 23. Representative gel filtration chromatogram for purification of the Fab’ 528 antibody fragment following reduction of 528-F(ab’)2 with TCEP.

The GFC showed that three main A280 nm peaks eluted from the GFC column after loading the crude reaction mixture. The peak at 15 mL corresponded to unreacted (or re-oxidised) 528-F(ab’)2. The major peak at 17 mL corresponded to the reduced Fab’ 528 fragment and the peak at 21 mL was due to salts and small molecules, such as TCEP. Fractions were collected across the peak at 17 mL and pooled. Protein MW standards eluting at 17 mL are known to be around 50 kDa, corresponding to the MW of Fab’ 528 (MW = 49527.51 g/mol). The pooled Fab’ 528 fractions were quickly submitted to reactions with the TEM-1 β-lactamase-SMCC conjugate obtained in 3.2 because over time the Fab’ 528 slowly air-oxidises back to 528-F(ab’)2.

3.4 Conjugation of the TEM-1 β-lactamase-sulfo-SMCC linker conjugate to Fab’ 528

The concentrated TEM-1 β-lactamase-sulfo-SMCC linker conjugate and pooled Fab’ 528 solutions were mixed together to react the maleimide group of sulfo-SMCC with the reduced hinge thiols of the Fab’ 528 antibody fragment. The maleimide group on the sulfo-SMCC linker can also hydrolyse in water, making it unreactive towards crosslinking. Michael addition of protein thiols to the maleimide leads to stable conjugates. Conditions for the bioconjugation reaction were explored using incubation periods of 1-24 hours at two temperatures; 25 and 4 °C. Reactions stirred for 2 hours at room temperature followed by overnight at 4 °C were found to provide reasonable yields of conjugation products. The final target TEM-1 β-lactamase-Fab’ 528 conjugate was isolated from reaction mixtures and purified by GFC using PBS as eluent. (Figure 24).
Figure 24. Gel filtration chromatogram from the isolation of TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 from the maleimide conjugation reaction.

The GFC showed two major peaks at 14.6 mL and 17 mL and a very broad peak from 9-14 mL. The samples were pooled within the indicated fraction lines and collected into tubes labelled 2A-2E. Fractions 2A-2D were concentrated and analysed using SDS-PAGE gel electrophoresis to identify the location of the conjugate. (Figure 25).
Figure 25. SDS-PAGE gel of the concentrated fractions from the GFC purification of the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate. Lane 1 = SeeBlue 2 MW Protein Ladder, Lane 2 = Tube 2A, Lane 3 = 2B, Lane 4 = 2C, Lane 5 = 2D.

The gel showed that no protein was present in lanes 2 and 3 (Coomassie blue detection). The high A280 nm absorption observed for tubes 2A and 2B suggested that protein aggregates may have been forming during the purification. Lane 4 showed a protein band between the 62 kDa and 98 kDa MW markers (red rectangle), consistent with the theoretical weight of the conjugate (79,174.23 Da). Lane 5 showed a protein band around the 28 kDa marker, consistent with the unconjugated TEM-1 β-lactamase-SMCC linker (MW = 29,626.5 Da). A MALDI-TOF mass spectrum was acquired on the tube 2C fractions and a sample was analysed by SDS-PAGE gel electrophoresis to confirm that they contained the desired conjugate.

During SDS-PAGE-gel electrophoresis run under reducing conditions, all intra- and inter-molecular disulfide bonds are reduced. As the free thiol used for the conjugation was located on the heavy chain of the Fab’ 528 fragment (Figure 25), reduction of the disulfide bond between the light (V\textsubscript{l}) and heavy (V\textsubscript{h}) chains would yield the antibody fragment V\textsubscript{l} chain and the V\textsubscript{h} chain conjugated to the TEM-1 β-lactamase-SMCC. Both Fab’ 528 fragments (V\textsubscript{l} and V\textsubscript{h}) have approximate molecular weights of 20-25 kDa. Thus, unreacted light chain should run at around 20-25 kDa relative to the SDS-PAGE gel MW markers. When conjugated to the 29 kDa β-lactamase, the heavy chain should run at around 54 kDa.

3.5 Mass spectral analysis of the TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 conjugate

A MALDI-TOF mass spectrum of the conjugate was acquired by Dr. Tom Nebl in the CSIRO mass spectrometry lab. The spectrum (Figure 26) showed a peak at 79,139.6 Da (after deconvolution), which matched the theoretical molecular weight of the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate (49,513.1 Da Fab’ 528 plus 29,626.5 Da β-lactamase-sulfo-SMCC).
Figure 26. Deconvoluted MALDI-TOF mass spectrum of the TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate. The spectrum was acquired, and the figure provided by Dr. Tom Nebl, CSIRO.

3.6 Reducing SDS-PAGE gel electrophoresis of the TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 conjugate

SDS-PAGE gel analysis was performed under reducing and non-reducing conditions on the TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate sample obtained from the pooled, concentrated tube 2C fractions (Figure 24). Reducing conditions were achieved by addition of DTT (1 mM) to the appropriate samples and heating prior to loading (Figure 27).
Figure 27. SDS-PAGE gel of the TEM-1 β-lactamase-SMCC-Fab' 528 conjugate run under non-reducing and reducing conditions. Lane 1 = SeeBlue 2 protein ladder. Lanes 2-4 = non-reduced TEM-1 β-lactamase, 528-F(ab')2 and TEM-1 β-lactamase-SMCC-Fab' 528 conjugate, respectively. Lane 6, 7, 8 = the same respective samples under TCEP reducing conditions.

Lane 2 in the gel showed the expected band at approximately 28 kDa for the His-tag cleaved TEM-1 β-lactamase control (MW = 29410.47 g/mol). The band did not change under reducing conditions (Lane 6). Lane 3 showed one major band for the 528-F(ab')2 antibody control (MW = 99055.02 g/mol) at around 98 kDa and Lane 4 showed one major band for the conjugate (MW = 79174.23 g/mol) at around 75 kDa. Reduction of the 528-F(ab')2 antibody with TCEP produced two bands at around 50 kDa and 25 kDa (Lane 7), consistent with cleavage to the Vh and Vil fragments. TCEP reduction of the conjugate produced a light chain Vil band at ~25 kDa and a ~50 kDa band for the TEM-1 β-lactamase-SMCC-Vh fragment (Lane 8). The combination of mass spectral and SDS-PAGE gel data confirmed that the desired TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 conjugate had been obtained.

When tested using the nitrocefin assay (2.13), the TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 conjugate showed enzymatic activity of 2.5 kU. This represented only 69.4% of the activity of the (His-tagged) TEM-1 β-lactamase predecessor (3.6 kU). It is likely that the non-site-specific nature of the conjugation reaction between sulfo-SMCC and the TEM-1 β-lactamase (i.e. non-selective amide coupling to solvent exposed lysines/N-terminus) were responsible for the loss of activity, possibly through steric blocking of substrate access to the active site. At this stage, the expressed TEM-1 β-lactamase and TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 bioconjugate were ready to be tested for their ability to cause NO release from C3Ds (4.4).
Chapter 4: Synthesis of DEA-C3D and nitric oxide release studies
The next part of the work aimed to test the expressed TEM-1 β-lactamase and synthesised TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 conjugate for their ability to trigger NO release from C3Ds. The Kelso Group at UOW have synthesised several C3Ds over the past decade and demonstrated that they release NO when treated with β-lactamases. These studies have focussed on the antibacterial and antibiofilm properties of the compounds since NO acts as a biofilm disperser. None of these previous studies have investigated NO release from C3Ds in the presence of a β-lactamase that had been conjugated to another protein. This chapter describes the re-synthesis of DEA-C3D 1, a cephalosporin-3′-diazeniumdiolate that incorporates the diethylaminodiazeniumdiolate (DEA-NONOate). This particular C3D was chosen for its rapid NO release properties in the presence of β-lactamases (half-life = 2 minutes).

The synthetic approach to 1 is depicted in Scheme 8. The first step involves freshly preparing the sodium diazeniumdiolate salt DEA-NONOate 2. This salt is then reacted with the commercially available cephalosporin PMB-ester 3 via a sodium iodide-promoted (Finkelstein) halogen substitution reaction to give the penultimate product 4. PMB-ester deprotection of 4 gives the target compound DEA-C3D 1. The mechanism of how the TEM-1 β-lactamase and β-lactamase-sulfo-SMCC-Fab' 528 conjugate would be expected to generate NO from 1 was outlined in Scheme 5 (for the closely-related analogue PYRRO-C3D).
4.1 Synthesis of DEA-NONOate 2

I acknowledge Dr Ardeshir Rineh for assisting in the synthesis of DEA-NONOate. The sodium salt DEA-NONOate 2 was synthesised according to the method reported by the Kelso group. The diazeniumdiolate needed to be freshly prepared prior to reaction with cephalosporin 3 as it is unstable and quickly degrades at room temperature or in the freezer. The reaction involved adding anhydrous diethylamine (DEA), NaOMe, MeOH and Et₂O to a sealed Parr-Knorr vessel and shaking under a constant pressure of NO gas (50 psi) for 3 days. The precipitate was collected by filtration (under a stream of dry argon), washed with ether and dried under vacuum. The white powder was stored under argon in a sealed dry flask wrapped in foil in the freezer and used within 2-3 days. This reaction was performed once on a 7 g scale and produced DEA-NONOate 2 in 25% yield. The mechanism of this reaction involves nucleophile attack of the DEA onto the electron deficient NO radical) to form the N-N bond. Radical-radical coupling followed by hydrogen atom transfer and final hydrogen atom abstraction by NaOMe produces DEA-NONOate 2 (Scheme 9).
Secondary amine diazeniumdiolates are powerful and versatile NO donors under physiological conditions. A wide range of secondary amines have been used for preparing diazeniumdiolates that show NO release half-lives varying from seconds to days. The NO-donor DEA-NONOate 2 becomes stable when coupled to cephalosporins and does not release NO. The diazene double bond can theoretically adopt both the syn (Z) and anti (E) configurations, however, the reaction produces only the more thermodynamically stable Z isomer. Photochemical studies suggest that O-linked diazeniumdiolates (e.g. DEA-C3D) can photo decompose, thus all reactions must be performed in flasks covered in foil. In the presence of light the compound could decompose to form NO and the secondary amine. Two pathways have been suggested for O-linked diazeniumdiolate decomposition (shown in Scheme 10 for O-benzyl-DEA-NONOate).
In the presence of light, the minor pathway (5%) involves cleavage at the N-O bond, forming nitrous oxide (N\(_2\)O), as well as aminyl and alkoxyl radicals that ultimately form amines, aldehydes and alcohols. The major reaction pathway (95%) involves photochemical cleavage of the N=N bond to form the nitrosamine and an oxygen-substituted nitrene. The oxynitrene structure was inferred from the production of abundant oxime, occurring via rearrangement of the oxynitrene to a C-nitroso compound and subsequent tautomerization to the stable oxime.\(^\text{111}\)

### 4.2 Synthesis of PMB-protected DEA-C\(_3\)D 4

I acknowledge Dr. Ardeshir Rineh for assisting in the synthesis of compound 4. The reaction involved \(O\)-alkylation of DEA-NONOate 2 with the commercially available cephalosporin precursor 3. It is well known that alkylation occurs on the terminal oxygen of diazeniumdiolates.\(^7\) The Kelso group identified that direct alkylation of sodium diazeniumdiolates with 3 does not produce the \(O\)-alkylated product and that an \textit{in situ} Finkelstein reaction is required to first convert the chloride to the more reactive iodide.\(^{78,80}\) The mechanism for the Finkelstein-promoted alkylation reaction is shown in Scheme 11.

\[ \text{Scheme 10. Decomposition pathways for } O\text{-benzyl-DEA-NONOate in the presence of light.} \]
Scheme 11. Mechanism for the formation of PMB-protected DEA-C3D 4.

As with all cephalosporins, C3D esters like 3 and the product 4 are prone to double bond migration during 3’-substitution reactions involving basic nucleophiles, leading to variable formation of the Δ2 isomer. This isomer is unable to undergo the conjugate elimination reaction and release the diazeniumdiolate (and hence NO) after β-lactam ring opening. Due to the difficulties in separating this isomer from the desired Δ3 isomer, isomerisation needed to be avoided during the reaction. To lower the likelihood of Δ2 isomer formation, the Finkelstein/diazeniumdiolate substitution reaction was performed under scrupulously anhydrous conditions under argon in dry acetone and the product 4 was stored in the freezer at -20 °C.

The alkylation reaction was performed four times on 0.5-1.0 g scale using one molar equivalent of DEA-NONOate 2 at room temperature in each case. Yields of 20-24% of 4 were obtained after silica gel column chromatography. Cephalosporin PMB-ester 3 was first reacted with sodium iodide in acetone to substitute the chloride for iodide under Finkelstein conditions. The choice of acetone allowed dissolution of the soluble NaI while causing the by-product NaCl to precipitate from the reaction, driving the chloride to iodide substitution to completion. Solid 2) was then added to the reaction in one shot. The low solubility of DEA-NONOate 2 in acetone served to reduce the reagent’s basicity, limiting deprotonation of the acidic sulphide α-protons and resulting double bond migration to form the Δ2 isomer. Only one of the four reactions showed any formation of the isomer (Reaction 4, 5-10% by 1H NMR analysis of the crude reaction product). Results from the reactions performed are summarised in Table 4 and the mechanism for the potential isomerisation is shown in Scheme 12.
Scheme 12. Mechanism of isomerisation to the Δ2 isomer via double bond migration triggered by abstraction of the acidic sulfide α-proton by basic nucleophiles.

Table 4. Summary of reactions performed to produce 4.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Starting mass of 3 (g)</th>
<th>Yield 4 (mg)</th>
<th>Yield (%)</th>
<th>Δ2 isomer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>130</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>126</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>278</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>284</td>
<td>24</td>
<td>5-10</td>
</tr>
</tbody>
</table>

The purified (silica gel column chromatography) samples of 4 were analysed by $^1$H and $^{13}$C NMR spectroscopy and their spectra compared to those published previously by the Kelso Group (Figures 28 and 29).
Figure 28. Top: $^1$H NMR spectrum of 4 published by the Kelso group.$^{60}$ Bottom: $^1$H NMR (500 MHz, CDCl$_3$) spectrum of 4 synthesised in this study.

The $^1$H NMR signals observed for the material prepared in this study perfectly matched the chemical shifts and multiplicity patterns in the published data.
Figure 29. Top: $^{13}$C NMR spectrum of 4 published by the Kelso group.\textsuperscript{50} Bottom: $^{13}$C NMR (125 MHz, DMSO-$d_6$) spectrum of 4 synthesised in this study.

The $^{13}$C NMR spectrum showed the correct number of signals and similar chemical shifts to the published data. However, the spectrum was recorded in DMSO-$d_6$ as opposed to CDCl$_3$, causing the chemical shifts to move slightly. Thus, the $^1$H NMR and $^{13}$C NMR spectra confirmed that compound 4 synthesised in this study was spectroscopically identical to that previously produced by the Kelso group.
4.3 Deprotection of 4 to give DEA-C3D 1

I acknowledge Dr Ardeshir Rineh for assistance in the deprotection reaction. The final step in the synthesis involved removal of the p-methoxybenzyl (PMB) ester from 4 to give the free carboxylic acid. Deprotection was achieved by stirring 4 for 1-1.5 hours with neat trifluoroacetic acid (TFA) in molten phenol at 45 °C under argon. Purification of 1 was achieved using preparative reverse phase HPLC or reverse phase silica gel column chromatography (H2O, CH3CN mixtures), with the final product obtained after freeze-drying. A mechanism for the ester deprotection reaction is proposed in Scheme 13.

Scheme 13. Mechanism of PMB-ester deprotection using TFA in phenol to produce the target C3D 1. The molten phenol serves to scavenge the quinone methide cation formed in the reaction.

This mild TFA/phenol PMB-deprotection method was originally reported by Torii et al.113 and was adapted by the Kelso group for use with PMB-protected C3Ds.109 The molten phenol functions as the solvent for the reaction and also serves to rapidly scavenge the highly reactive quinone methide cation formed during loss of the PMB group. The deprotection reaction was monitored using reverse phase TLC, where loss of the starting material spot and appearance of a new product spot were clearly observable. Once the reaction was completed, ether was added, and the reaction flask was placed on ice. Unexpectedly, no precipitate of the crude 1 formed at this stage. Initially, it was thought that the volume of ether added was too
high, preventing precipitation. Further concentration under reduced pressure at room temperature did not trigger precipitation. Changing the solvent to petroleum spirit successfully precipitated 1 and it was isolated using a centrifugation. The crude was loaded with a small amount of CH₂Cl₂ onto a reverse-phase silica gel column and eluted using increasing concentrations of acetonitrile in water. Fractions containing the pure 1 by reverse phase TLC analysis were pooled and freeze-dried overnight to give a fluffy white powder. A summary of the deprotection reactions performed is shown in Table 5.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Starting amount of 4 (mg)</th>
<th>Mass of 1 (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>23</td>
<td>48</td>
</tr>
</tbody>
</table>

The first attempt at the reaction gave only 10 mg of 1 from 100 mg of starting ester 4 (13%) after reverse-phase column chromatography. The second attempt yielded 23 mg of 1 from 60 mg of 4 (48%). The increased yield was attributed to improved techniques after having performed the reaction once already. The Kelso group reported recovery of 160 mg of 1 (81%) from 250 mg of 4 using their original procedure (neat TFA at room temperature). The lower yield obtained in this study was appeared to be caused by degradation of 4 reverse-phase column chromatography. Nevertheless, sufficient quantities of pure, freeze-dried 1 were produced for this study.

The purified (silica gel column chromatography) samples of 1 were analysed by ¹H and ¹³C NMR spectroscopy and their spectra compared to those published previously by the Kelso Group (Figures 30 and 31).
Figure 30. Top: $^1$H NMR spectrum of 1 published by the Kelso group.\textsuperscript{80} Bottom: $^1$H NMR (500 MHz, CD$_3$OD) spectrum of 1 synthesised in this study.
The $^1$H NMR signals observed for the material prepared in this study closely matched
the chemical shifts and multiplicity patterns in the published data with minor impurity except
the solvent resident peak (3.31, CD$_3$OD) was more intense for this study than the published
data.

![Image of 1H NMR spectrum]

Figure 31. Top: $^{13}$C NMR spectrum of 1 published by the Kelso group. Bottom: $^{13}$C NMR
with Proton Decoupling (125 MHz, DMSO-$d_6$) spectrum of 1 synthesised in this study.

The $^{13}$C NMR with proton decoupling spectrum contained the signals and similar
chemical shifts to the published data. However, the spectrum was recorded in DMSO-$d_6$ as
opposed to CD$_3$OD, causing the chemical shifts to move slightly. There were also addition
signal peaks in the $^{13}$C NMR with proton decoupling spectrum which may be caused by
degradation of 1 or impurities in the DMSO-$d_6$. Attempts to isolate and characteristic these
peaks from the $^{13}$C NMR with proton decoupling spectrum were unsuccessful as the $^1$H NMR
spectrum shows a complete match to the published data, concluding that the sample was pure when submitted to the NMR analysis.

4.4 Nitric oxide release studies

I acknowledge Dr Ardeshir Rineh for training and assistance with the Apollo Free Radical Analyser used for NO release studies. At this stage of the project, the pure C3D 1 (4.3), TEM-1 β-lactamase (2.15) and TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 conjugate (3.4) were in-hand and ready for proof-of-concept NO release studies. The objective of these studies was to compare NO production from 1 following exposure to the two β-lactamase and establish whether addition of the SMCC-linker and Fab’ 528 protein affects the activity. The experimental setup involved connecting a NO-selective probe to the free radical analyser. The sensitive probe was placed in the middle of a two-neck round-bottom flask containing MilliQ water and the flask was sealed. The range of the sensor was set to 10 nA and the probe selector set to NO. The setup was stabilized by stirring overnight or until a stable baseline signal was obtained.

After equilibrating the probe, the instrument was calibrated to enable accurate determination of NO concentrations in the test solutions. This was achieved using standard solutions containing known concentrations of nitric oxide in water. Saturated nitric oxide solution (1.91 mM), generated by continuously bubbling the gas into MilliQ water, was diluted 10-fold to 191 µM. Test solutions of different concentration were prepared by adding increasing amounts of 191 µM stock solution and added to the flask containing the probe and the change in current was recorded. The NO calibration curve was generated by transforming the current signal to [NO] (Figure 32).
Figure 32. Top: Measurement of NO levels in standard aqueous solutions to generate a standard curve. Aliquots of 100 µL, 200 µL, 400 µL, 800 µL, 1600 µL of a standard NO solution (191 µM) were added to 20 mL of water and the NO signal (current pA) was recorded. The corresponding NO concentration is shown on the right axis. (B) Standard curve for NO concentrations in water.

After calibrating the probe, the water in the flask was replaced with 20 mL of fresh phosphate-buffered saline (PBS). The calibrated NO probe was left in the stirring PBS solution until the signal stabilised. While the probe was stirring DEA-C3D 1, TEM-1 β-lactamase and the TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 conjugate were taken out of the freezer and
allowed to warm to room temperature. TEM-1 β-lactamase and the conjugate were diluted from 3600 U and 2500 U, respectively, to 30 U. Once the signal had stabilised, 2 mg of DEA-C3D 1 was added to the stirring PBS and the signal was recorded for 10 minutes. 100 µL of 30 U TEM-1 β-lactamase was then injected into the solution and NO was rapidly detected. When the signal reached a plateau, a 200 µL aliquot was added. Additional aliquots with doubling volumes were subsequently added when the signal from the previous aliquot had reached a plateau. The process was repeated until the NO signal saturated, usually within 1-1.5 hours after addition of 3 aliquots. The same method was repeated on the TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 conjugate. The NO signal reaches the maximum presumably when all the DEA-C3D substrate has been ring-opened by the β-lactamase and all of the DEA-NONOate released. Plots of [NO] versus time the two β-lactamases are shown in Figure 33.
Figure 33. NO concentrations in PBS solutions containing DEA-C3D 1 (100 mM) following addition of Top: TEM-1 β-lactamase. Arrows indicate addition of the following to reaction vials: (a) 100 µL of 30 U/mL TEM-1 β-lactamase, (b) 200 µL, (c) 400 µL. Bottom: TEM-1 β-lactamase-sulfo-SMCC-Fab’528 conjugate. Arrows indicate addition of the following to reaction vials: (d) 100 µL of 30 U/mL TEM-1 β-lactamase-sulfo-SMCC-Fab’528, (e) 200 µL, (f) 400 µL.

The TEM-1 β-lactamase and the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate both clearly catalysed the hydrolysis of 1, leading to release of NO into the solution. The NO concentration steadily increased over time until reaching a plateau. The first NO plateau for the TEM-1 β-lactamase occurred after 40 minutes and for the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate it occurred after 80 minutes. Since the same amount of enzyme activity units were added to each reaction (30 U/mL according to nitrocefin assay, 2.13) the lower rate of NO production by the conjugate suggests its β-lactamase component is less active towards the C3D substrate 1 than the unconjugated TEM-1 parent enzyme. Second injections of the enzymes caused another increase in the NO concentration before again reaching a plateau. Third
injection produced very small responses, suggesting that all the DEA-C3D 1 had been consumed.

It is believed that the further releases of NO into the solution from the initial reaction is caused by NO reaching a steady state. This means that the rate of NO produced is equal to the rate of NO decomposition. Additional β-lactamase injection increases the rate of NO produced until the system reaches steady state again. Another theory is that the acylated β-lactamase is being hydrolysed only slowly, thus the rate of NO production is slowed. A third possibility is that the β-lactamase is being covalently modified by the NO being produced, leading to inactivation. Further studies are required to confirm these theories.

The initial rates of reaction were obtained from the gradients of the curves after addition of the respective enzymes. The amount of DEA-C3D 1 consumed during the experiments was determined for each enzyme by dividing the area under the curve by two, since each mole of 1 produces two moles of NO (Figures 34 and 35).

<table>
<thead>
<tr>
<th>Time interval (min)</th>
<th>Rate of reaction (nmol/min)</th>
<th>Estimated NO released (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.42</td>
<td>2.4</td>
<td>6.0</td>
</tr>
<tr>
<td>42.63</td>
<td>1.4</td>
<td>8.4</td>
</tr>
<tr>
<td>63.73</td>
<td>0.8</td>
<td>4.6</td>
</tr>
</tbody>
</table>
Figure 34. NO release from 1 by TEM-1 β-lactamase. Gradients and areas under the curve after each injection of the enzyme are shown in the table.

![Graph showing NO release from TEM-1 β-lactamase](image)

<table>
<thead>
<tr>
<th>Time interval (min)</th>
<th>Rate of reaction (nmol/min)</th>
<th>Estimated NO release (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-80</td>
<td>0.9</td>
<td>1.91</td>
</tr>
<tr>
<td>80-130</td>
<td>0.8</td>
<td>3.23</td>
</tr>
<tr>
<td>130-161</td>
<td>0.1</td>
<td>2.34</td>
</tr>
</tbody>
</table>

Figure 35. NO release from 1 by TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate. Gradients and areas under the curve after each injection of the enzyme are shown in the table.

From the plots and tables, it was clear that the TEM-1 β-lactamase shows a higher rate of reaction with DEA-C3D 1 than the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate. The rates of reactions were obtained by creating tangent lines at the Km of each addition. The Km was determined by halving the NO concentration at the plateau, where the sample almost reached Vmax. The estimated Km chosen for the wild-type β-lactamase plot was extracted at 20 minutes for the first aliquot, 45 minutes for the second aliquot and 65 minutes in the third. Similarly, the estimated Km for the β-lactamase-sulfo-SMCC-Fab’ 528 conjugate was taken at 40 minutes for the first aliquot, 100 minutes for the second and 145 minutes for the third. The
gradient of the tangent at the estimated Km was used as the rate of reaction, as shown in the tables in Figures 34 and 35. The areas under the three aliquot curves were also calculated for the enzymes. This represented the total NO released during that reaction period. It is noted, however, that NO is lost from the solutions with time due to its reactivity, meaning that the extracted values underestimate the actual amount of NO released. It was seen that the TEM-1 β-lactamase produced almost three times the amount of NO after the first and second aliquots, compared to the β-lactamase-sulfo-SMCC-Fab′ 528 conjugate. The β-lactamase-sulfo-SMCC-Fab′528 conjugate also took twice as long to reach saturation. It is postulated that the reduced activity arises because of the non-specific bioconjugation method that was used to create the conjugate, which may sterically impede access of the DEA-C3D substrate to the β-lactamase active site.
Chapter 5: Conclusions and future directions
5.1 Conclusions

The aims of this project were: (1) to clone, express and purify a TEM-1 β-lactamase, (2) conjugate it to a model antibody fragment using a bifunctional linker, (3) synthesise the cephalosporin-3′-diazeniumdiolate DEA-C3D 1 and (4) use 1 as substrate and testing for NO release by the TEM-1 β-lactamase and β-lactamase-sulfo-SMCC-Fab’ 528 conjugate. The TEM-1 β-lactamase was expressed in good yield and conjugated to the thiol-bearing antibody fragment Fab’ 528 using a sulfo-SMCC crosslinker. DEA-C3D 1 was synthesised, isolated and characterised using the previously reported methods. NO release experiments showed that the TEM-1 β-lactamase produced more NO from DEA-C3D 1 and at a faster rate than the TEM-1 β-lactamase-SMCC-Fab’ 528 conjugate. Overall, the study has demonstrated for the first time that it is possible to produce a β-lactamase-Fab’ 528 conjugate that triggers NO release from C3Ds opening the way for further studies aimed at achieving tissue selective NO delivery via an ADEPT strategy. A summary of the project outcomes is provided in Scheme 14.
Scheme 14. Summary of aims completed in the project. (1) Cloning, expression and purification of a TEM-1 β-lactamase (2) conjugation of the TEM-1 β-lactamase to the antibody fragment Fab' 528 to produce a TEM-1 β-lactamase-SMCC-Fab' 528 conjugate (3) synthesis of DEA-C3D 1 (4) measurement and comparison of the catalytic activity of the two β-lactamases in generating NO from 1.

5.2 Future directions

While the first molecular proof-of-concept has been achieved in the project, a wide variety of improvements could now be pursued. One possibility would be to explore more site-specific protein bioconjugation methods using different mutants of the TEM-1 β-lactamase,
aiming to improve enzyme activity. Once optimal conjugation chemistry has been identified, functional proteins of interest could then be attached, including a monoclonal antibody but also fluorescent proteins, peptides, polymers or single chain antibody fragments. Alternative C3Ds with different β-lactamase susceptibilities could also be explored, especially if the approached moved closer towards clinical application where in vivo drug properties of the C3D would be an important consideration. Ultimately, our long-term goal is to explore an ADEPT strategy employing the plaque-targeting antiLIBS scFv antibody with a C3D to create a targeted NO treatment for emergency interventions in heart attacks caused by atherosclerotic plaques (1.12).

5.2.1 Site-specific bioconjugation

In this project, the crosslinker chosen for protein bioconjugation was sulfo-SMCC, which gave rise to non-site-specific TEM-1 β-lactamase functionalisation through lysine side chain amines and (and/or N-terminus). Two site-specific bioconjugation methods routinely used in the Williams Laboratory at CSIRO making use of click chemistry and Sortase-mediated reactions could be investigated.

5.2.1.1 Click chemistry approach

Bioconjugation using Click chemistry has been widely published. A mild (i.e. protein-friendly), bio-orthogonal copper-free click chemistry approach could potentially be used to conjugate proteins of interest (e.g. antiLIBS scFv antibody) to TEM-1 β-lactamase (Scheme 15). In this approach, the β-lactamase could be reacted with an NHS or tetrafluorophenol (TFP) ester PEGₙ azide. These are commercially available heterobifunctional crosslinking reagents that come in a range of PEG chain lengths. The antiLIBS scFv antibody engineered with a C-terminal cysteine residue from Karlheinz Peter laboratory, could then be reacted with a maleimide-PEGₙ dibenzocyclooctyne (DBCO)
linker. Conjugation of the azide-modified β-lactamase and the DBCO-modified antiLIBS scFv antibody would proceed via the well documented, strain-promoted 1, 2-dipolar cycloaddition of the DBCO group and the organic azide. Different length PEG linkers could be investigated, the choice of which would be guided by the measured activity of both the β-lactamase and antiLIBS scFv antibody portions. The opposite combination of cross-linkers could also be explored, where the DBCO group is installed on the TEM-1 β-lactamase and the azide on the antiLIBS scFv antibody. Although the reaction to lysine amines on the β-lactamase in the initial step, by reaction with the TFP ester, can be heterogeneous (multiple Lysine amine residues on the BLA protein), the azide group that has been installed is a stable reagent and is selective to reaction with alkyne groups such as the DBCO group. This method is more likely to lead to a homogenous conjugate and purification is expected to be easier.

Scheme 15. Copper-free Click chemistry bioconjugation approach to a TEM-1 β-lactamase-antiLIBS scFv antibody conjugate
5.2.1.2 Sortase-mediated approach

Sortase-mediated bioconjugation uses the enzyme Sortase A to covalently link two molecules, usually proteins. The Sortase A enzyme recognises and selectively binds to a penta-peptide recognition sequence, LPETG, which can be added to almost any protein. Sortase A has a conserved cysteine residue in its active site that reacts with the carbonyl carbon of the threonine residue (T) in the pentapeptide motif to form a thioester intermediate. The thioester is then attacked by a nucleophile, which in nature is triglycine with the N-terminal primary amine forming an amide bond at the threonine carbonyl. The C-terminal glycine residue of the LPETG motif is cleaved off in the process and the Sortase A active site regenerated for further reactions. A β-lactamase engineered to contain a GGGG sequence (Construct 3, 2.2) could potentially be used in a Sortase A-mediated bioconjugation strategy. A scFv has been engineered to contain the LPETG Sortase-recognition sequence in our collaborator’s laboratory (Prof Karlheinz Peter). This LPETG-modified antibody fragment could be submitted to Sortase-mediated bioconjugation with a GGGG-tagged TEM-1 β-lactamase to produce a scFv-TEM-1 β-lactamase conjugate. (Figure 36). This method would likely be cleaner and more site-specific than the SMCC, thiol-maleimide and Click chemistry methods. Extra amino acids could be added to either the scFv or the TEM-1 β-lactamase in front of the LPETG or GGGG sequences to lengthen the linker, if needed.

Figure 36. Sortase A-mediated bioconjugation approach using a LPETG-modified scFv and GGGG-tagged β lactamase.
5.2.2 β-lactamase mutants

In this project, a wild-type TEM-1 β-lactamase was chosen as the first model β-lactamase for exploring antibody conjugation and NO release with C3Ds. However, there are several mutant variants of TEM-1 β-lactamase that are known, which could potentially be used to refine enzymatic efficiency and/or alter protein stability. One useful mutant contains a single point mutation (E166N) that lowers β-lactamase activity by 1,000,000-fold. This ‘inactive’ mutant could serve as an appropriate negative control in NO release experiments moving forward, both in vitro and in vivo. Another mutant, E166Y, has been shown to have 100-times higher cephalosporinase activity than the wild-type enzyme. This ‘super-lactamase’ mutant could be used to prepare higher activity conjugates. Studies involving conjugation of proteins of interest to β-lactamases from other families, such as the extended spectrum β-lactamases (ESBL) or the SHV strain, could also be explored.

5.2.3 Modifying the C3D structure

In C3Ds, the β-lactam and diazeniumdiolate portions are crucial functional components. However, the group attached at the C7 position and the diazeniumdiolate structure can be modified to increase susceptibility to β-lactamases and modulate drug properties. In this work, a phenacyl group was attached at C7 in DEA-C3D 1 and the diazeniumdiolate was derived from diethylamine. The Kelso group have synthesised and studied several C3D analogues carrying different C7 groups diazeniumdiolates. Examples like 5-8 (Figure 37) could be explored for targeted NO delivery using ADEPT.
Figure 37. Examples of C3Ds from the Kelso group carrying variations in the C7 side chain and diazeniumdiolate.

5.2.4 Long term goal: An emergency intervention during heart attacks caused by atherosclerotic plaques

As discussed earlier, a possible medical application of a NO-targeting ADEPT strategy could be in the treatment of heart attacks caused by atherosclerotic plaques, where the plaque occludes a coronary vessel leading to myocardial ischemia. An emergency ADEPT intervention could perhaps be given by paramedics to patients suffering this type of heart attack that aims to achieve localised NO therapy and vasodilation directly at the site of the artery occlusion. The vasodilation could increase blood flow around the occlusion and reduce myocardial ischemia, keep the patient alive long enough to undergo emergency surgery to remove the blockage. The ADEPT therapy could comprise a β-lactamase conjugated to the plaque-targeting antiLIBS scFv antibody (Prof Karlheinz Peter) and a C3D. When administered to the patient, the antiLIBS scFv-β-lactamase conjugate would localise β-lactamase activity to the immediate vicinity of the artery blockage. After some minutes, administration of the C3D would lead to NO delivery exactly at the required site, triggering vasodilation and increased blood flow (Figure 38). In addition to this potential ADEPT application, one can imagine
targeting NO to just about any tissue through tissue-specific antigen-antibody interactions, where a relevant antibody is linked to a $\beta$-lactamase and used with a C3D.

**Figure 38.** ADEPT approach for targeting vasodilating NO to occluded artery sites during atheroma-induced heart attacks.
Chapter 6: Experimental
6.1 General

The TEM-1 β-lactamase DNA and plasmids were designed by Dr. Stewart Nuttall and expressed by Genscript. DH5-α cells were provided by CSIRO. TB Broth was made by dissolving yeast extract (2.4% w/v), tryptone (2.0% w/v) and glycerol (0.4% w/v) in sterile water and sterile phosphate buffer (10% v/v). PBS was formulated using Na₂HPO₄ (10 mM), NaCl (137 mM), KH₂PO₄ (1.8 mM) and KCl (2.7 mM) in MilliQ water. 2 x YT medium was made from 1.6% (w/v) tryptone, 1.0% (w/v) yeast extract and 0.5% NaCl in sterile water. All buffers and media used for cell culturing were filtered through a 0.22 µm sterile filter before use. Miniprep kits were purchased from Qiagen and the NucleoSpin Gel and PCR Clean-Up kit was obtained from Macherey Nagel. All restriction enzymes, primers and DNA ladders were from New England BioLabs. SYBR Safe DNA Gel Stain was supplied by Thermo Fisher. DNA gels were run on BioRad electrophoresis instruments and analysed using a LICOY Odyssey imaging system.

Protein gels were run using 4-12% Bis Tris Pre-Cast gel 10 or 15 wells from NuPAGE, Life Technologies on an XCell SureLock Mini cell. Protein concentrations were measured using a Nanodrop spectrophotometer at 280 nm. MALDI-TOF mass spectra of proteins were acquired by Dr. Tom Nebl at Parkville, CSIRO. Nitrocefin was purchased from Biovision. SMCC was purchased from Thermo Fisher. Protein samples were concentrated using Amicon® Ultra-4 spin or Amicon® Ultra-15 spin filters with a 30 kDa molecular weight cut-off. Protein purification chromatography was monitored at 280 nm, 254 nm and 214 nm.

All chemical reactions were performed under an argon atmosphere in oven-dried glassware with magnetic stirring. 7-Phenylacetamido-3-chloromethyl-3-cephem-4-carboxylic acid-p-methoxybenzyl ester (3 was purchased from AK Scientific. Phenol, sodium iodide, molecular sieves (3 Å and 4 Å) were purchased from Sigma-Aldrich. Molecular sieves were activated by drying overnight in a muffle furnace at 300 °C. Anhydrous ether was distilled from
sodium benzophenone ketyl under argon. Anhydrous methanol was heated at reflux over 4 Å molecular sieves for 4 hours and distilled onto 3 Å molecular sieves under argon. Diethylamine was heated at reflux over CaH$_2$ for 2 hours before distillation onto 4 Å molecular sieves under argon. Anhydrous acetone was obtained by heating analytical grade acetone over CaSO$_4$ (Drierite) granules for 1-2 hours under argon before distilling onto 4 Å molecular sieves. All other solvents were analytical reagent (AR) grade and used without further purification. NO$_{(\text{g})}$ was purchased from Sigma Aldrich. Concentration under reduced pressure was performed by rotatory evaporation at 40 °C unless otherwise stated. The term petroleum spirit refers to hydrocarbon mixtures with a boiling range of 40-60 °C.

Silica gel column chromatography was performed using SiliaFlash P60 230-400 mesh silica gel (Silicycle) with the indicated eluents. Reverse-phase silica gel column chromatography was performed using C18 (Carbon 17%) 60 Å 40-63 μm silica gel. TLC analysis for reaction monitoring was performed using SiliaPlate TLC aluminium-backed 200 μm plates (Silicycle). Compounds were visualised on TLC plates under UV light at 254 nm. Samples from reverse-phase silica gel column chromatography purifications were freeze-dried on a CHRIST Alpha 1-2 LD plus instrument at -53 °C and 0.15 mbar. $^1$H NMR and $^{13}$C NMR were recorded on either Varian Unity-300 MHz or Varian-Inova-500 MHz spectrometers in the indicated deuterated solvents (DMSO-$d_6$ or CD$_3$OD). The spectra were referenced to the chemical shifts of the residual undeuterated solvent signals. Melting points were determined using a Buchi Melting Point M-560 apparatus and are uncorrected. Optical rotations were measured using the Jasco P-2000 Polarimeter.
6.2 Molecular Biology

6.2.1 Digestion of TEM-1 β-lactamase wild type inserts

Lyophilised TEM-1 β-lactamase DNA was resuspended in distilled water (100 µL) and transformed into DH5-α cells. The DH5-α cells were cultured in TB Broth (5 mL) with ampicillin (100 µg/mL) overnight. An aliquot of the cells was mixed with 50% glycerol and stored as a backup in -80 °C. The rest of cells were spun down at 4,000 rpm for 10 min at 4 °C and the supernatant discarded. Mini preps were performed according to the manufacturer's instructions on the pellet to extract the high-quality DNA.

The insert was obtained by double digesting the high-quality DNA (160 ng/uL) vector with the restriction enzymes BamH1-HF (20 U/ul) and NheI-HF (20 U/ul) in (10x) CutSmart Buffer for 1.5 hours at 37 °C. A 1% (w/v) agarose gel with 1:10,000 SYBR Safe dye was prepared in Tris-acetate-EDTA (TAE) buffer (Tris base (2 M), glacial acetic acid (1 M), 0.5 M EDTA pH 8.0). The digested vector was loaded onto the 1% agarose gel and run for 30 minutes at 150 V with 1 kB and 500 bp DNA ladder present. The insert band was excised under UV with a gel knife and the DNA extracted from the gel using the NucleoSpin Gel and PCR Clean-Up kit according to the manufacturer’s instructions. The insert DNA (14.3 ng/µL) was stored in the -20 °C freezer.

6.2.2 Digestion of the pET43.1α vector

The pET43.1 vector (556 ng/µL) was double digested with the restriction enzymes BamH1-HF (20 U/ul) and NheI-HF (20 U/ul) in (10x) CutSmart Buffer for 1.5 hours at 37 °C. A 1% (w/v) agarose gel with 1:10,000 SYBR Safe dye was prepared in Tris-acetate-EDTA (TAE) buffer (Tris base (2M), glacial acetic acid (1M), 0.5M EDTA pH 8.0). The digested plasmid was loaded on to the 1% agarose gel and run for 30 minutes at 150 V with 1 kB and 500 bp DNA ladders. The vector band was excised under UV light and extracted from the gel.
using the NucleoSpin Gel and PCR Clean-Up following the manufacturer’s protocol. The pET43.1α vector (6 ng/µL) was stored in the -20 °C freezer.

6.2.3 Ligating TEM-1 β-lactamase inserts into open pET43.1a vectors

Digested insert DNA, digested vector DNA, T4 ligase buffer (10x), T4 DNA ligase and MilliQ water were added into a microeppendorf at room temperature for 15 minutes, as indicated in Table 6. A negative control containing no insert was included.

Table 6. Reagent volumes for ligation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample volume (µL)</th>
<th>Control volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA (14.3 ng/µL)</td>
<td>4.2</td>
<td>-</td>
</tr>
<tr>
<td>Vector DNA (6 ng/µL)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>T4 DNA Ligase (20 KU)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T4 Ligase buffer (10x)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>1.8</td>
<td>6</td>
</tr>
</tbody>
</table>

6.2.4 Transformation into competent DH5-α E. coli cells

Ligated vector (10 µL) was gently mixed with 50 µL of competent DH5-α E. coli cells on ice for 10 minutes. The cells were subjected to heat shock at 42 °C for 45 seconds and returned to ice for 2 minutes. The transformed cells were supplemented with 100 µL 2x YT medium with ampicillin (100 µg/mL). The transformed sample and the control were spread evenly on YT-Amp plates. The plates were incubated overnight at 37 °C with the agar side up.
6.2.5 Agarose gel DNA electrophoresis

A 1% (w/v) agarose gel was prepared in TAE buffer (Tris base (2 M), glacial acetic acid (1 M), 0.5M EDTA pH 8.0) with 1:10,000 SYBR Safe dye. DNA samples were mixed with 6x DNA loading dye (Thermo Fisher) and loaded onto the agarose gel well comb. DNA ladders (10 µL) 1 Kbp and 500 bp DNA were added to other lanes in the gel. The gel was run in 1x TAE buffer at 110 V for 30 minutes and visualised under UV light at 600 nm. The gel was placed on a UV box and the DNA of interest was excised using a gel knife. The DNA was recovered using the NucleoSpin Gel and PCR Clean-Up kit.

6.2.6 Colony screening PCR

Using a sterile inoculation loop, 6 random colonies from the positive transformation plate and one control colony were selected, dipped into separate empty PCR tubes and streaked onto new patch plates (YT-Amp), which were grown overnight at 37 °C. A Master Mix was created containing 5 µL of 2xGoTaq® Green (GTG, Promega), 0.5 µL of T7 Forward primers (10 µM), 0.5 µL of Coli Down (10 µM) and 4 µL of distilled water per colony on ice. 10 µL of Master Mix was added to each empty PCR tube on ice. PCR reactions were performed on the colonies’ sample using the following PCR parameters (Table 3). 1% (w/v) agarose gel with 1:10,000 SYBR Safe dye in Tris-acetate-EDTA (TAE) buffer was used to analyse the positive clones as described in section 6.2.5. Gels were analysed on the LICOY Odyssey imaging system.

6.2.7 Sequencing PCR

A colony sample of the positive clones from the patch plates was inoculated in fresh 5 mL of sterile YT-Amp media and incubated overnight at 37 °C. An aliquot was taken from the overnight colonies and mixed with 50% glycerol (v/v) to store at -80 °C as a backup sample.
The overnight samples were spun down at 4,000 rpm for 10 min at 4 °C and the supernatant discarded. Minipreps were performed on the pellets, according to the manufacturer's instructions, to extract the DNA from the cells. The DNA samples were sent to Micromon (Monash University, Victoria, Australia) for Sanger DNA sequencing. DNA sequencing chromatograms were analysed using Chromas.

6.3 Expression and purification of recombinant TEM-1 β-lactamase

6.3.1 Analytical protein expression studies

Positive clone DNA (0.5 µL, 82 ng/µL) was added to 50 µL BL21-DE3 E. coli cells. The cells were incubated for 10 minutes on ice, treated for 45 seconds with heat shock at 42 °C and then placed on ice for another 2 minutes. 2 x YT media (100 µL) supplemented with Amp (100 µg/mL) was then mixed with the sample on ice. The samples were grown on separate YT-Amp plates overnight at 37 °C. A single positive colony from the overnight plates was picked and inoculated into 5 mL 2xYT media containing 100 µg/mL Amp overnight at 37 °C with shaking at 150 rpm. The overnight cultures were used to inoculate another 5 mL fresh 2xYT-Amp media with a starting OD$_{600nm}$ of 0.1. The OD$_{600nm}$ was measured on a spectrophotometer (Eppendorf) and cultures were grown in a shake incubator (Raytek) at 37 °C with shaking 180 rpm until the culture OD$_{600nm}$ reached 0.7-0.8. TEM-1 β-lactamase expression was induced by addition of 0.5 mM IPTG into the culture. The induced culture was incubated at 16 °C overnight with shaking at 180 rpm in a HT Ecotron shaking incubator with integral cooling (Infors). The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4 °C using a JA 25.50 centrifuge (Beckman Coulter). The bacterial cell pellets were lysed using 500 µL Bugbuster Protein Extraction Reagent (Merck). Both the soluble fraction and the insoluble fraction (dissolved in 1% SDS solution) were analysed by SDS-PAGE and Western blotting.
6.3.2 Protein SDS-Page Gels and Western Blotting

Proteins were analysed by SDS–PAGE under non-reducing conditions on a 4–12% Bis–Tris NuPAGE gel using MES electrophoresis buffer (50 mM MES, 50 mM TRIS, 0.1 % SDS, 1 mM EDTA pH 7.3). For reducing conditions, DTT (5 mM) was added to the 4x LDS sample buffer. The samples were incubated with 4x LDS sample buffer and heated to 70 °C for 5 minutes prior to loading into separate wells of the gels. 5 µl of See Blue 2 pre-stained molecular weight ladder was added in lane 1 to estimate the molecular weight of proteins. Gels were placed in a Surelock MiniCell filled with fresh MES running buffer and run at 150 V for 40 minutes. Gels were then stained with Coomassie Brilliant Blue to detect the denatured proteins (0.1 % w/v Coomassie Brilliant Blue R250 dissolved in 40 % v/v ethanol/10 % v/v glacial acetic acid) for 30 mins at room temperature on a rocking platform followed by multiple rounds of de-staining with 10 % v/v ethanol/7.5 % v/v glacial acetic acid at room temperature (on a rocking platform).

Western blots were performed by transferring the replicate gel onto a nitrocellulose membrane (Protran 0.45 µm nitrocellulose membrane, Perkin Elmer) using transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA pH 7.2 and 10 % v/v methanol) at 25 V for 90 mins. Transferred membranes were blocked in PBS containing 5% (w/v) skim milk powder and 0.1% Tween-20 (v/v) (Blotto) for 30 minutes, and incubated with an anti-His tagged monoclonal antibody horseradish peroxidase conjugate (Sigma) diluted 1:4000 with Blotto to detect His tagged proteins. The membranes were washed several times with PBS containing 0.1% Tween-20 (v/v) before being developed using a chemiluminescent substrate (Super-signal West Pico PLUS chemiluminescent substrate, (ThermoScientific) and imaged using an Amersham Imager 680 (GE Healthcare).
6.3.3 Large scale TEM-1 β-lactamase expression

The positive colony picked from the overnight small-scale expression plate was inoculated into fresh 2 x YT media (12.5 mL) supplemented with Amp (100 µg/mL). The culture was grown overnight at 37 °C with shaking at 150 rpm. The overnight culture was used to inoculate 1 L TB media containing 10% potassium chloride. The media was decanted into two 500 mL sample volumes in 2 x 2 L Tunair shake flasks and incubated at 37 °C with shaking at 180 rpm. Cells were grown until the OD_{600nm} reached 0.7-0.8. Control samples were taken for SDS-gel and Western Blotting. IPTG (0.5 mM) was used to induce the cultures. The induced cultures were moved into a 16 °C incubator and shaken at 180 rpm overnight. The cells were spun down at 6000 g at 4 °C for 10 minutes on a F500 rotor, the supernatant discarded, and the pellets frozen at -80 °C.

6.3.4 Extraction and purification of TEM-1 β-lactamase

6.3.4.1 Cell lysis

Cell pellets from section 6.3.3 were resuspended in lysis buffer (PBS with 0.02 % NaN₃, 150 mM NaCl, 2 mM MgCl₂, 10 mM imidazole, 0.5 mg/mL lysozyme, 5 ul benzonase, 1 mM PMSF and 3 tablets of protease inhibitor pellets) in a proportion of 10 mL buffer per gram of cells. The lysis buffer was stirred for 10-20 minutes at 4 °C or until the mixture was homogeneous. Cells were then placed in the chilled EmulsiFlex C5 high pressure homogeniser (Avestin) three times at 15,000 psi before the flowthrough was poured into 50 mL centrifuge tubes and spun down in a JA25.50 rotor at 18,000 g for 20 minutes at 4 °C. The supernatant was collected and concentrated using Amicon®-15 10,000 MW cut off spin filters. The pellet, supernatant and uninduced control samples were analysed by SDS-PAGE gel electrophoresis under non-reducing conditions (6.3.2). For sonication as an alternative to the cell crusher, the
solution was sonicated on ice using a program with the amplitude set to 60 Hz and the sample subjected to a 30 second pulse followed by a 60 second rest for 10 cycles.

6.3.4.2 Immobilized metal affinity chromatography

Concentrated supernatant was mixed with imidazole (10 mM) before loading onto a 5 mL HiTrap column (GE Healthcare Life Sciences) connected to a Profinia™ Affinity Chromatography Protein Purification System (BioRad). TEM-1 β-lactamase were eluted using 20 column volume (cV) binding buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 10 mM imidazole), followed by 20 cV wash buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 20 mM imidazole) and then 5 cV elution buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 250 mM imidazole). The eluted fractions were concentrated, and SDS-PAGE gel analysis was performed on each step of the column purification under non-reducing conditions.

6.3.4.3 Protein purification

Elution fractions containing protein samples from IMAC were injected onto a HiLoad® 26/600 Superdex® 200 column connected to an AKTA Pure system and eluted using PBS. The column was run at 2 mg/min for 1.5 cV. A standard molecular weight ladder run on the same column was used to estimate the molecular mass of eluting peaks (Table 7). Peak fractions were pooled and concentrated. Samples were run on SDS-PAGE gels and Western Blots under non-reducing and reducing conditions (6.3.2) and mass spectrometry was performed.

Table 7. Standard molecular weight ladder for protein purification.

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular Weight (Da)</th>
<th>Sigma-Aldrich</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin (bovine)</td>
<td>670,000</td>
<td>T9145-1VL 35 mg</td>
</tr>
<tr>
<td>γ-globulin (bovine)</td>
<td>158,000</td>
<td>G5009-1g</td>
</tr>
<tr>
<td>Ovalbumin (chicken)</td>
<td>44,000</td>
<td>A5503-1g</td>
</tr>
<tr>
<td>Myoglobin (horse)</td>
<td>17,000</td>
<td>M1882-250 mg</td>
</tr>
</tbody>
</table>
6.3.5 TEM-1 β-lactamase stability and function studies

Protein samples at 1 mg/mL and PBS were examined for buffer stability and pH stability by the CSIRO Collaborative Crystallisation Centre labs (Parkville, Melbourne).

6.3.6 Enzyme activity

Enzyme activity of the TEM-1 β-lactamase was measured by nitrocefin assay using the protocol supplied by BioVision. A standard curve was obtained to quantitate the activity (Appendix 3).

6.3.7 His-TEV tag removal

TEM-1 His-TEV-β-lactamase (3.46 mg/mL) were incubated with in-house His-TEV protease at a molar ratio of 1:15 with DTT (1 mM) for 12 hours in the refrigerator. Imidazole (20 mM) was added and the cleaved TEM-1 β-lactamase was purified using IMAC on a 1 mL His tag column using the Profinia system. The column was washed using 5 cV of binding buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 20 mM imidazole), followed by 5 cV wash buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 20 mM imidazole) and ended with 5 cV elution buffer (PBS with 0.02% NaN₃, 150 mM NaCl, 250 mM imidazole). The collected fractions were analysed using SDS-PAGE gel electrophoresis at 150 V for 40 minutes. A western blot using anti-His mouse antibody was performed to confirm removal of the His-tag.

6.4 Bioconjugation chemistry

6.4.1 Synthesis of TEM-1 β-lactamase-sulfo-SMCC-Fab' 528 conjugate

TEM-1 β-lactamase (50 µL, 0.016 µmol, 2 mg/mL) was reacted with sulfo-SMCC (3.2 µl, 0.16 µmol, 22 mg/mL) in a final volume of 100 µL PBS for 1 hour at room temperature.
The reaction was desalted using a 5 mL HiTrap desalting column (GE) attached to an AKTA pure system using PBS as eluent at a flow rate of 5 mL/min. The peak containing the TEM-1 β-lactamase-SMCC adduct was pooled and concentrated using Amicon® Ultra-4 spin filters. The protein concentration was measured using a Nanodrop spectrophotometer at 280 nm.

Dr. Judy Scoble from CSIRO generously provided the antibody fragment Fab′ 528. Fab′ 528 (180 µL, 0.001 µmol, 0.56 mg/mL) was reduced using TCEP (20 µL, 0.1 µmol, 5 mM) for 1 hour at 4 °C. The reaction was desalted using a 5 mL HiTrap desalting column attached to an AKTA pure system using PBS as eluent at a flow rate of 5 mL/min. Peaks containing the Fab′ 528 were pooled and concentrated using Amicon® Ultra-4 spin filters and the protein concentration was measured using a Nanodrop spectrophotometer at 280 nm.

TEM-1 β-lactamase-SMCC (0.5 mg/mL) and Fab′ 528 (0.5 mg/mL) were mixed at room temperature overnight. The sample was purified on a Superdex 200 increase 10/300 GL column (GE) attached to AKTA Pure using PBS as eluent at a flow rate of 1 mL/min. Samples containing the protein were pooled, concentrated and SDS-PAGE gels were performed under non-reducing and reducing conditions. Mass spectrometry analysis was also performed.

6.5 Chemical synthesis

6.5.1 Synthesis of DEA-NONOate 2

\[
\text{HN} \quad \text{NO gas, MeONa} \quad \text{NaO}^+ \text{O}^- \quad \text{O}^\cdot \quad \text{O}^- \quad \text{O}^- \quad \text{N}^- \quad \text{N}^- \quad \text{HN}
\]

Sodium methoxide solution was freshly prepared by dissolving sodium metal (2.5 g) into dry methanol (25 mL) and sonicateing for 1 hour until clear. Anhydrous diethylamine (7
g, 0.1 mol) was then added to the sodium methoxide solution followed by anhydrous diethyl ether, bringing the solution to a total volume of 300 mL. The solution was decanted into a Parr-Knorr shaker attached to a NO cylinder and reacted for 3 days with shaking at 50-55 psi. After completion of the reaction, the solution was carefully depressurised and unsealed and vacuum filtered under a stream of dry nitrogen to obtain 2 as a white solid. The solid 2 was washed with copious dry diethyl ether, dried under high vacuum and stored under argon in a sealed dry flask wrapped in foil in the freezer (3.8 g, 25%).

6.5.2 Synthesis of PMB protected-DEA-C3D 4

7-Phenylacetamido-3-chloromethyl-3-cephem-4-carboxylicacid-p-methoxybenzyl ester 3 (0.5 g, 1 mmol) was stirred with sodium iodide (0.154 g, 1 mmol) in dry acetone (8 mL) under argon in the dark at room temperature for 1 hour. Freshly prepared sodium DEA-NONOate 2 (0.160 g, 1 mmol) was added in one shot to the reaction mixture and the reaction stirred for another 1.5 hours under nitrogen while monitoring by TLC analysis (petroleum spirit: ethyl acetate, 2:1). Upon completion of the reaction, the solvent was removed under reduced pressure and the residue purified by gradient silica gel column chromatography (0-50% ethyl acetate in petroleum spirit) to give 4 (126 mg, 20%) as an off-white solid.

$^1$H NMR: (500 MHz, CDCl$_3$): $\delta$ 7.36-7.24 (m, 7H), 6.88 (d, 2H, $J = 9$ Hz), 6.08 (d, 1H, $J = 10$ Hz), 5.81 (dd, 1H, $J = 10, 4.5$ Hz), 5.33 and 4.98 (AB$_q$, 2H, $J = 14$ Hz), 5.17 (s, 2H), 4.88 (d,
1H, J = 5 Hz), 3.79 (s, 3H), 3.67 and 3.62 (ABq, 2H, J = 9 Hz), 3.44 and 3.42 (ABq, 2H, J = 18 Hz), 3.10 (q, 4H, J = 7 Hz), 1.05 (m, 6H, J = 7 Hz).

$^1\text{H}^\text{NMR}$: (125 MHz, CDCl$_3$): $\delta$ 171.1, 164.6, 161.2, 159.9, 133.6, 130.7, 129.4, 129.2, 127.8, 126.7, 126.4, 125.5, 114.0, 72.0, 68.1, 59.2, 57.5, 55.2, 48.4, 43.3, 26.0, 11.5.

FTIR: (cm$^{-1}$, Neat) 3287, 3031, 2967, 2920, 2854, 1894, 1730, 1717, 1531, 1374, 1350, 1258, 1157, 1076, 1057, 915.

$[^\text{D}]\alpha$: (c = 1.0, CHCl$_3$) = -12.69

MP: 123-123.4°C

ESI-MS (m/z): Calculated: 584.22[M + H]$^+$ C$_{28}$H$_{34}$N$_5$O$_7$S, Found 584.51.

6.5.3 Synthesis of DEA-C-D 1

PMB protected-DEA-C3D 4 (60 mg, 0.1 mmol) was stirred with TFA (0.8 mL) in molten phenol (3 g) at 45 °C in a dry flask for 1-1.5 hours under argon. Reaction progress was monitored by reverse-phase TLC analysis (30% acetonitrile in water). Upon completion, the crude product was precipitated out of solution by adding petroleum spirit (30-40 mL) and placed on ice for 2 hours. The mixture was centrifuged at 3500 g for 5 minutes causing the precipitate to form a tight cake. The solvent was decanted, and the cake washed twice with petroleum spirit using vortex/centrifugation cycles. The precipitate was redissolved in 1-2 mL DMSO and purified using C18-reverse phase silica gel column chromatography (0-30% acetonitrile in water). Fractions containing the pure product (by reverse-phase TLC analysis)
were pooled and freeze-dried overnight to give DEA-C3D 1 (23 mg, 48%) as a fluffy white powder. The compound was stored in the freezer in a dry flask wrapped in foil under argon.

**H NMR:** (500 MHz, CD$_3$OD): δ 7.35-7.26 (m, 5H), 5.75 (d, 1H, J = 4.8, Hz), 5.36 and 5.07 (AB$_q$, 2H, J = 12.9 Hz), 5.05 (d, 1H, J = 4.8 Hz), 5.03 (m, 1H), 3.68-3.45 (m, 4H), 3.19 (q, 4H, J = 7.0 Hz), 1.07 (t, 6H, J = 7.0 Hz).

**13C NMR:** (125 MHz, DMSO-d$_6$): δ 171.4, 165.5, 161.8, 136.2, 130.7, 131.0, 129.5, 128.8, 123.0, 74.6, 61.5, 59.8, 50.1, 44.0, 27.7, 12.6

**FTIR:** (cm$^{-1}$, Neat) 3277, 3030, 2977, 2938, 2114, 1716, 1660, 1532, 1495, 1380, 1339, 1229, 1176, 1073, 1055, 838

**[α]$_D$:** (c= 1.0, CHCl$_3$) = -32.5

**ESI-MS** (m/z) Calculated: 464.16 (C$_{20}$H$_{26}$N$_5$O$_6$S) [M + H]$^+$, Found 464.21

**MP:** 81.8-83.7°C

### 6.5.4 Amperometric measurements of nitric oxide release

Nitric oxide was detected amperometrically using a TBR 4100 free radical analyser (World Precision Instruments) with an ISO-NOP microsensor probe. The probe was first polarised, and the baseline stabilised by placing in a stirring solution of distilled water (200 mL) in a room temperature water bath for 12 hours. The probe was calibrated using a saturated solution of NO generated by bubbling the pure gas through water according to the manufacturer’s protocol. The saturated aqueous NO solution (1.91 mM) was diluted 1:10 to give a 191 µM stock solution. Aliquots of 100 µL, 200 µL, 400 µL, 800 µL and 1600 µL of the 191 µM stock solution were injected sequentially after stabilisation of the NO signal. A standard curve was generated and used to measure NO concentrations produced from cleavage of DEA-C3D 1 by the β-lactamases.
The probe was placed in PBS (20 mL, 10 mM Na₂HPO₄, 137 mM NaCl, 1.8 mM KH₂PO₄, 2.7 mM KCl) and stirred at room temperature to stabilise the baseline. The NO signal was recorded for 15 minutes or until a stable baseline was evident and DEA-C3D 1 (2 mg) was added to the stirring solution. Aliquots of TEM-1 β-lactamase (30 U) were added and the NO signal changes recorded over 1-1.5 hour. The analogous experiment was performed using the TEM-1 β-lactamase-sulfo-SMCC-Fab’ 528 conjugate. Experimental data were processed and normalised in Microsoft Excel.
Chapter 7: References


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 Appendices
Appendix 1: Vector of pET43.1 a+ obtained from Novagen.

**pET43-N-His-TEV-TEM1**

![Diagram of pET43-N-His-TEV-TEM1 vector](image)

**pET43-N-His-SUMO G4-2 TEV-TEM1**

![Diagram of pET43-N-His-SUMO G4-2 vector](image)

**pET43-N-His-SUMO G4-2 Cys TEV-TEM1**

![Diagram of pET43-N-His-SUMO G4-2 Cys vector](image)
Appendix 2: DNA sequences for the three designed constructs of TEM-1 β-lactamase.

Appendix 3: Standard curve for nitrocefin assay.

Appendix 4: Mass spectrum of 1:1 mole ratio reaction of TEM-1 β-lactamase: sulfo-SMCC.
Appendix 5: Mass spectrum of 1:5 mole ratio reaction of TEM-1 β-lactamase: sulfo-SMCC.

Appendix 6: Mass spectrum of 1:10 mole ratio reaction of TEM-1 β-lactamase: sulfo-SMCC.