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Cytochrome P450-mediated biotransformation of noscapine

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Abstract

Cytochrome P450 enzymes are promising biocatalysts for the pharmaceutical industry due to their ability to catalyse selective oxidations of drug-like substrates that are often difficult to achieve with synthetic organic chemistry. This enzyme family is, however, currently underrepresented in industrial biocatalysis due to issues with enzyme stability and cofactor requirements, as well as the poor aqueous solubility of many cytochrome P450 substrates. In this thesis, cytochrome P450-mediated biotransformation of the opium poppy alkaloid noscapine was used as a model reaction for the development and optimisation of a biotransformation process using bacterial cells. The aims of this thesis were to develop a biocatalytic method for the production of noscapine metabolites, which are of interest for the development of anticancer drugs, and to investigate strategies for the implementation of cytochrome P450s in pharmaceutical manufacturing processes.

In order to obtain a suitable P450 enzyme for the biotransformation of noscapine, a library of mutants of the enzyme P450_{BM3} from *Bacillus megaterium* was constructed using site-directed mutagenesis. This strategy identified enzyme mutants capable of producing six different noscapine metabolites, several of which may be of interest for drug development. A high selectivity for noscapine *N*-demethylation was demonstrated by several mutants. This *N*-demethylation reaction was therefore selected as a focus for further process development in a whole-cell biotransformation system.

The permeability of Gram-negative and Gram-positive bacteria to noscapine was assessed in order to minimise mass transfer limitations in a whole-cell biotransformation system. This identified *Bacillus megaterium* as a suitable expression host, as these cells enabled significantly faster reaction rates compared to the Gram-negative *Escherichia coli*. As noscapine has poor aqueous solubility, two strategies were investigated to improve substrate loading for the biotransformation. First, a biphasic biotransformation system, which uses a water-immiscible organic solvent phase to supply substrate, was developed with the use of *in silico* solvent screening based on Hansen solubility parameters. This was effective in increasing product yields but had the disadvantage of slower reaction rates due to substrate partitioning. The second approach used a cyclodextrin/polymer system to enhance aqueous noscapine solubility and proved more effective, enabling faster reaction rates. However, the stability of the mutant P450_{BM3} enzyme was found to be a major factor limiting product yield.

The use of growing cells rather than resting cells was then investigated to address the limited enzyme stability observed during biotransformation. As intracellular cofactor supply can often limit reaction rates

in growing cells, a metabolic engineering strategy based on the CRISPR/dCas9 interference (CRISPRi) system was used to redirect carbon flux towards cofactor production in the *B. megaterium* host. This provided a significant improvement in productivity compared to cells lacking the CRISPRi system, though dynamic control of gene repression was not possible due to 'leaky' expression of dCas9 from an inducible promoter. The CRISPRi-enhanced system with growing cells showed comparable productivity to the resting-cell system, with the advantage of sustained P450_{BM3} regeneration and fewer processing steps.

In summary, an enzyme and host cell system for the biocatalytic *N*-demethylation of noscapine was developed. A number of process limitations were identified and addressed to optimise the yield and productivity of the system. The limitations that were encountered during process development are common to whole-cell biotransformations using P450 enzymes, and the strategies used to address these limitations are likely to be useful for the development of other P450-catalysed biotransformation processes.

Declaration

I declare that:

- (i) this thesis comprised only my original work towards the Doctor of Philosophy degree except where indicated in the preface;
- (ii) due acknowledgement has been made in the text to all other material used; and
- (iii) the thesis is fewer than the maximum word limit in length, exclusive of tables, maps, bibliographies and appendices.

Signed:

Luke Richards

Preface

The work presented in **Chapter 3** of this thesis was published in *Biotechnology Reports* on 22 August 2019.

The work presented in **Chapter 4** was submitted for publication to *Journal of Industrial Microbiology & Biotechnology* on 18 February 2020 and is under review at the time of thesis submission.

The work presented in **Chapter 5** is unpublished material and has not been submitted for publication.

I performed all experimental work for the manuscripts presented in **Chapters 3** and **4**. The co-authors listed on these manuscripts provided:

- (i) advice on experimental design and interpretation of results;
- (ii) technical assistance with analytical and modelling work; and
- (iii) input during manuscript preparation.

No work presented in this thesis has been submitted towards another qualification and all work was carried out after enrolment in the current degree.

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1. Introduction

The use of enzymes within the pharmaceutical sector is growing rapidly, and enzymes now find frequent applications from the development of drugs at bench scale to industrial-scale manufacturing[1, 2]. The production of pharmaceutical compounds by enzymatic biotransformation can have a range of benefits compared to organic synthesis, such as waste reduction and improved process efficiency at manufacturing scale[3] to rapid drug diversification and screening during investigative stages[4]. The increasing application of enzymes within all industrial sectors is reflected in the growth of the commercial enzymes market, which was valued at US\$5.5 billion in 2018 and forecast to grow to US\$7.0 billion by 2023[5].

Enzymes can catalyse reactions with remarkable regio- and stereoselectivity, owing to their unique three-dimensional active site structures, which is of particular importance as drug compounds become more complex and multi-functionalised[6]. The significant advantage of high regioselectivity is emphasised by the high number of protection and de-protection steps required in modern industrial syntheses, providing protection against unwanted reactions at similar functional groups[7]. The use of a single regioselective biotransformation step can replace multiple synthetic steps needed to achieve a desired modification. This can reduce the use of reagents and organic solvents while improving overall process efficiency and atom economy, aligning with many of the “twelve principles of green chemistry” proposed by Anastas and Warner[8].

Many modern pharmaceuticals contain at least one chiral centre and there is an increasing market demand for enantiopure drugs[9]. During the production of chiral intermediates, the production of racemic mixtures of two enantiomers is often unavoidable, with only one of the enantiomers desired for further synthesis. Accordingly, some of the first applications of biotransformations in pharmaceutical manufacturing were for the resolution of racemates using hydrolytic enzymes such as lipases[10]. Enzymes can also avoid the need for de-racemisation altogether by the use of stereoselective biotransformations further upstream in the synthesis; for example, the use of alcohol dehydrogenases for the asymmetric reduction of ketones to chiral alcohols[11].

In addition to shortened synthetic protocols and improved yields, the favourable operating conditions of enzyme reactions are a further advantage for industrial applications. Enzymes typically catalyse reactions under mild conditions – aqueous solutions at physiological pH and ambient temperature – which can significantly reduce solvent waste and energy usage while improving the environmental impact and

human safety of manufacturing processes[12]. For example, Pfizer redeveloped the synthetic protocol for the cholesterol-lowering drug atorvastatin (Lipitor) to include a regioselective ketone reduction using an alcohol dehydrogenase enzyme. Overall, the process modification provided a 65% reduction in organic solvent waste, eliminated the use of two hazardous reagents and provided a substantial reduction in energy input, as the original process required cryogenic conditions[13].

In addition to providing significant process advantages for industrial-scale drug manufacture, biotransformations are also valuable tools for drug discovery. During the investigation of a new drug candidate, it is routine to assess the pharmacological effects of functional group substitution at various positions on the drug compound[14]. Investigating structure-activity relationships (SARs) in this way is important for understanding the biological mechanism of action of the drug, as well as for the development of derivatives of the lead compound with improved pharmacological properties[15]. The development of synthetic routes for each desired lead analogue can consume significant resources during drug research and development stages. An alternative approach is to screen the lead compound against an enzyme library to identify enzymes that produce analogues of interest[16], and libraries of engineered enzymes are becoming an increasingly popular tool for the rapid diversification of drug leads in this way[4].

Cytochrome P450 enzymes (P450s) are a particularly useful family of enzymes for drug development[17]. Some of the most relevant P450s for drug research and development are the xenobiotic-metabolising P450s found in animals[18]. In mammals, ingested drug compounds pass through the liver where they undergo oxidative reactions catalysed by microsomal P450 enzymes. A single drug compound is often converted into a range of different metabolites to reduce the concentration of the circulating drug and produce more soluble derivatives for excretion[19]. Microsomal P450s are able to recognise a very broad range of drug-like substrates and are capable of modifying them at various structural positions, often by the introduction of hydroxyl groups at inactive C-H bonds. These reactions can be of significant interest to drug development chemists, as these structures are often not be accessible from the parent drug using synthetic organic reactions and instead require a completely new synthetic route. The enzymatic addition of a hydroxyl group at a structural position of interest allows further synthetic functionalisation at this position for investigation of SARs and drug development[16] without the need for the time-consuming development of new synthetic routes.

The investigation of P450-generated drug metabolites has led to the development of several drug analogues with improved activity[20]. For example, the antihistamine agent desloratidine (Clarinox) is an

active metabolite of loratidine (Claritin) that was found to have 10-fold improved potency compared to the parent drug[21]. A lead drug candidate for cholesterol absorption inhibition was also found to produce a metabolite with improved pharmacological activity, which was subjected to further synthetic derivatisation. This led to the discovery of ezetimibe, a compound that showed 400-fold improved potency compared to the original lead compound[22]. Acetaminophen (paracetamol), one of the most widely used pain medications, was also discovered through drug metabolism studies[23].

The opium poppy alkaloid noscapine[24] is a potentially suitable target for diversification by P450 biotransformation. Noscapine has been received significant attention as a potential anticancer drug over the last two decades due to its ability to inhibit tumour cell division[25]. While this activity is fairly weak compared to similar-acting drugs in clinical use, the activity of noscapine has been significantly enhanced through the synthesis of structural analogues[26]. To date, SARs have only been investigated at three different positions of the noscapine scaffold and the selective functionalisation of other atoms by organic synthesis has not been described[26]. Studies of noscapine metabolism by microsomal P450s have identified nine different metabolites[27], several of which would be of interest for analogue development if suitable quantities could be produced.

While preparative-scale production of these metabolites for further investigation could potentially be achieved by biotransformation with mammalian cytochrome P450 enzymes, scale-up is likely to be difficult due to inherent issues with this class of P450s, including low activity, stability and solubility[28, 29]. Bacterial P450s often show more favourable properties compared to their mammalian counterparts and are more practical to use for preparative-scale applications[30]. The most well-studied bacterial P450 for drug metabolism applications is P450_{BM3} from *Bacillus megaterium*[31]. This enzyme natively functions as a fatty acid hydroxylase[32] but can be converted into a versatile drug metaboliser through protein engineering[31, 33]. The significant characterisation of the mechanistic and structural properties of this enzyme to date, including the effects of many amino acid mutations, provides an excellent source of guidance for future protein engineering efforts to metabolise new substrates using this enzyme.

Enzymes are typically employed in industrial applications as purified enzymes or contained within microbial cells, with the choice of method depending on a range of factors including enzyme stability, ease of purification and downstream processing requirements[34]. For cytochrome P450s and other monooxygenase enzymes, a key factor is the requirement for expensive NADPH cofactor, which is often needed in a large stoichiometric excess depending on the efficiency of the enzyme. The use of whole cells for an enzymatic biotransformation allows a continuous supply of NADPH via the host cell metabolism.

For this reason, as well as the potentially improved enzyme stability of many P450s within whole cells, this format is typically the method of choice[35]. Whole-cell applications can, however, have several significant limitations, including:

- poor mass transfer of drug substrates across cell membranes;
- substrate and product toxicity to the microbial host;
- oxygen limitations; and
- side-reactions with native enzymes in the host cells.

A range of reaction engineering approaches have been used to address these issues[35-38].

Whether whole cells or isolated enzymes are used, a common limitation of pharmaceutical biocatalysis is poor substrate solubility[39, 40]. Microbial cells and most enzymes require an aqueous environment to function, while most drug-like compounds show hydrophobic characteristics more compatible with organic solvents. Approaches to improve substrate loading for biotransformations include the use of biphasic systems, substrate feeding strategies, and the use of solubility enhancers such as polar co-solvents, surfactants and cyclodextrins[40].

Metabolic engineering is another way to enhance the performance of whole-cell biotransformations. This entails the use of genetic engineering to inactivate host genes, modify metabolic pathways or construct new biosynthetic pathways. Typical goals include the improvement of intracellular cofactor supply[41], inactivation of genes responsible for by-product formation[42], improvement of organic solvent tolerance[43] and enhancement of substrate uptake[44]. In recent years, more powerful synthetic biology tools have emerged for metabolic engineering, such as the use of CRISPR-based gene editing and control strategies[45]. These have significant potential for further improvement of whole-cell pharmaceutical biotransformations for industrial applications and have not yet been fully explored.

The aim of this project is to investigate the use of cytochrome P450 enzymes for the production of noscapine metabolites. Specifically, the use of engineered P450_{BM3} enzymes is explored using whole-cell biotransformations. Process development and optimisation using reaction engineering and metabolic engineering is also investigated.

2. Literature review

2.1. Scope

This review focuses on a range of important topics pertaining to the cytochrome P450-mediated biotransformation of noscapine. First, the significance of noscapine and its analogues as potential anticancer drugs is discussed. The advantages and limitations of cytochrome P450s for industrial applications are then considered, with particular emphasis on the promising enzyme P450_{BM3} from *Bacillus megaterium*. Various considerations for whole-cell biotransformations are discussed with a focus on typical process limitations and strategies for optimisation. The potential of the bacterium *B. megaterium* as a host for industrial whole-cell biotransformations is then reviewed. The use of metabolic engineering for the improvement of P450-mediated whole-cell biotransformations is also discussed.

2.2. Noscapine

2.2.1. Background

The opium poppy alkaloid noscapine (known as “narcotine” until 1958) has a range of medicinal properties that have been investigated for over a century. First characterised in 1817[46], noscapine was used as an alternative to quinine for the treatment of malaria in the 19th century[47] before being employed as a cough suppressant[48], for which it currently has widespread use. Noscapine can bind to multiple physiological receptors[49] and has shown pharmacological potential for the treatment of various conditions including stroke[50], renal ischaemia[51], polycystic ovary syndrome[52] and pulmonary fibrosis[53]. In 1998, the potential of noscapine as an anti-cancer drug was discovered[54], leading to two decades of research on the action of noscapine on a diverse range of cancer types and the characterisation of a variety of noscapine structural analogues with improved activity[26].

2.2.2. Chemistry, biosynthesis and pharmacokinetics

Noscapine (**Figure 2.1**) is part of the phthalideisoquinoline class of alkaloids[55], which includes other pharmacologically relevant drugs such as the GABA receptor antagonist bicuculline. Noscapine is a weakly basic (pKa 6.8[56]) tertiary amine with poor water solubility. The two chiral centres within noscapine are linked by a labile C-C bond, which can be readily broken by acid or base hydrolysis[57]. At neutral to alkaline pH, the lactone ring is reversibly hydrolysed to form noscapinic acid in equilibrium with the ring-closed form[56]. The solubility of noscapine is markedly improved by protonation of the amine under acidic conditions and the hydrochloride salt is commonly used for pharmaceutical applications.

Noscapine is one of the major latex alkaloids in opium poppy (*Papaver somniferum*)[58]. The biosynthesis of this compound in poppy requires twelve enzymatic steps from the branch point alkaloid (S)-reticuline and has only recently been fully elucidated[24]. The discovery of a 10-gene cluster encoding most of the required enzymes provided an initial blueprint for the biosynthetic pathway[59], and further experiments *in planta* and *in vitro*[60, 61] as well as reconstitution of the pathway in yeast[62, 63] allowed complete mapping of the sequential enzymatic steps.

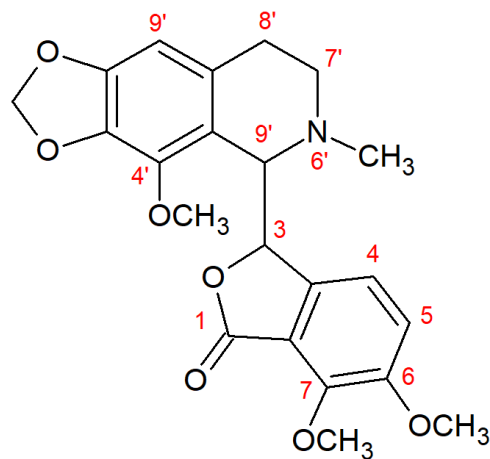


Figure 2.1. Chemical structure of noscapine with atom numbers.

Unlike the other major poppy alkaloids morphine and codeine, noscapine is non-addictive and is not a narcotic. It is of notable low toxicity at high doses[64, 65], though it is rapidly cleared by first-pass metabolism and has an absolute oral bioavailability of only around 30%[66].

2.2.3. Anticancer properties

The chemotherapeutic potential of noscapine comes from its ability to interfere with the formation of the microtubules that coordinate mammalian cell division and subsequently trigger apoptosis[54]. Although noscapine was identified as a “mitotic poison” as early as 1942[67], it was not thoroughly investigated as a chemotherapeutic candidate until five decades later. In a screen of natural compounds containing similar structural motifs to other known microtubule assembly inhibitors, such as colchicine and podophyllotoxin, Ye and colleagues noted that several cancer cell lines treated with 20 μ M noscapine displayed abnormal microtubule spindles, arrested mitosis and features consistent with apoptosis within 24 h[54]. Noscapine was demonstrated to bind stoichiometrically to tubulin, the monomer subunit of microtubules, during *in vitro* experiments. When mice implanted with several types of tumour cells were dosed with noscapine, tumour mass decreased significantly over three weeks of treatment[67]. In further studies with murine models, the group demonstrated that noscapine effectively suppressed tumour proliferation with little or no toxicity to other tissues or inhibition of immune responses[68]. The results were particularly promising because the anticancer effects of noscapine were effective with oral administration. This is an advantage compared to clinical microtubule inhibitors like paclitaxel that must be delivered intravenously due to poor intestinal absorption[69].

Studies on the mechanism of noscapine interaction with microtubules both *in vitro*[70] and *in vivo*[71] showed that noscapine causes a subtle alteration of microtubule assembly dynamics. This is in contrast with other chemotherapeutic microtubule inhibitors which either strongly promote or inhibit microtubule assembly. The effect of noscapine on microtubule assembly does not alter the overall intracellular polymer/monomer ratio of tubulin but is nonetheless sufficient to interfere with chromosomal segregation during cell division, resulting in mitotic arrest.

Further studies of noscapine identified activity against a variety of cancer types in cell lines and animal models. Noscapine was found to be capable of crossing the blood-brain barrier and has been demonstrated to have potential for the clinical treatment of glioblastoma[72-76]. Other studies have shown promising results for the treatment of liver[77], breast[78-80], ovarian[81, 82], blood[83, 84], lung[85-87], gastric[88], colorectal[89, 90], skin[71] and prostate[91-93] cancers with noscapine. Several of these studies[75, 79, 80, 82, 84, 86, 87] found that noscapine acts synergistically with other chemotherapeutics and may therefore be suitable for multi-agent chemotherapy regimens, offering broad potential for clinical applications.

2.2.4. Noscapine analogues

While many promising studies have been published, noscapine has not yet been approved for clinical use in cancer treatment. A Phase I clinical trial for relapsed or refractory multiple myeloma was terminated due to lack of clinical response[94]. Oral noscapine therapy in animal models typically requires high doses to be effective (e.g. 550 mg/kg/day[85]), which is partly due to the relatively weak binding strength of noscapine to tubulin ($k_d = 144 \mu\text{M}$ [95]) and partly due to its rapid clearance and low bioavailability[66, 96, 97]. Given the very favourable toxicity profile of noscapine, several groups have attempted to improve its anticancer potency by the synthesis of noscapine structural analogues[26].

One of the first noscapine analogues to be investigated for anticancer activity was 9'-brominated noscapine (**Figure 2.2a**), which showed improved tubulin binding (k_d 54 μM vs 144 μM for noscapine) and higher potency in arresting the division of HeLa cells (7.7 μM vs 18.4 μM to arrest 50% of cells at mitosis)[95]. The reduction of the lactone moiety of brominated noscapine to its cyclic ether was shown to further increase the antimitotic potency. These analogues were also significantly more effective than noscapine at inhibiting *in vitro* cancer cell proliferation across a range of cell lines, with 9'-bromonoscapine showing up to 40-fold higher potency in one case[98]. Like the parent compound, 9'-bromonoscapine shows no obvious toxicity towards normal tissues[99, 100]. Two other 9'-halogenated noscapine

analogues, 9'-fluoronoscapine and 9'-chloronoscapine, also showed enhanced activity against tumour cells *in vitro* and *in vivo*, including cell lines resistant to other antimitotic drugs[98, 101]. Further investigation of analogues with modifications in the 9'-position led to the discovery of 9'-nitro-noscapine, which could inhibit proliferation of multidrug-resistant lymphoma and ovarian cancer cells without any negative effects on healthy fibroblast cells[102], and 9-amino-noscapine, a rationally designed analogue with stronger antiproliferation effects than noscapine across a broad range of cell lines[103]. Another rationally designed derivative, 9'-azido-noscapine, has also been shown to have improved *in vitro* cytotoxicity compared to noscapine[104]. In another study, a range of aryl and alkyl group substitutions were made at the 9'-position, but these generally did not show any improved activity in comparison to 9'-bromonoscapine on either microtubule assembly or cancer cell mitotic arrest[105].

On the basis that tubulin binding of chemotherapeutics is enhanced by the presence of a biaryl pharmacophore, Santoshi and colleagues added a range of benzyl functional groups to the 9'-position guided by *in silico* docking studies with tubulin. In agreement with the docking studies, most of these showed enhanced tubulin binding and some showed improved antimitotic activity *in vitro*[106]. One of these analogues was tested for toxicity in mice and showed no detectable blood or tissue toxicity at large doses.

Structure-activity relationships (SARs) at the 7-position of noscapine were first investigated by Anderson and colleagues, who substituted a range of aromatic and alkyl halide

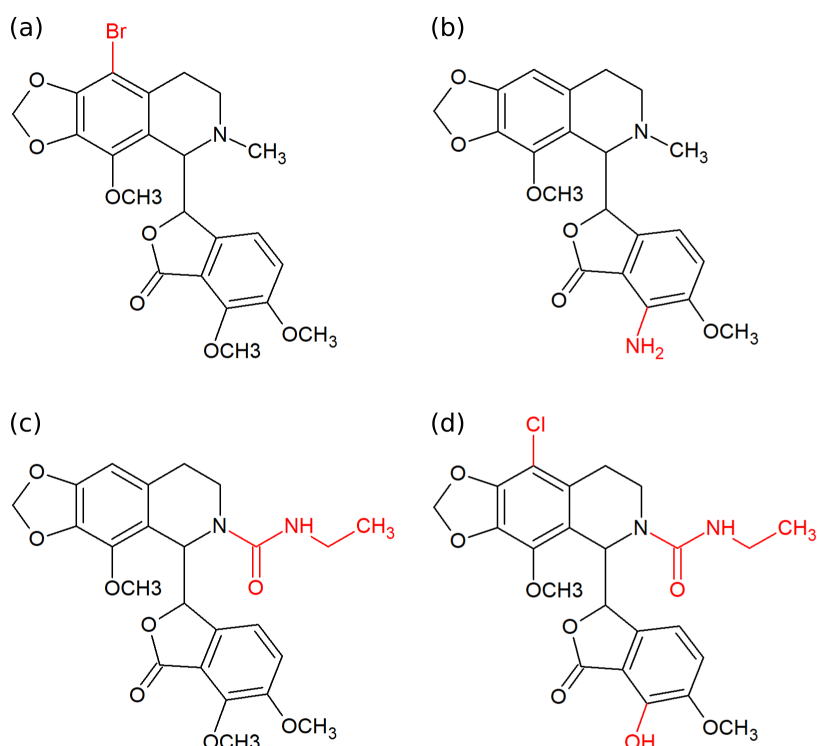


Figure 2.2. Noscapine analogues that show enhanced anticancer activity compared to noscapine. **(a)** 9'-brominated; **(b)** 7-aniline; **(c)** N-ethyl urea; **(d)** multifunctionalised.

groups at the noscapine 7-methoxy group[107]. Unexpectedly, the analogues that showed antimitotic activity were noted to arrest cells at the pre-mitotic phase (S-phase) of mitosis rather than at metaphase like the parent compound. Further investigation of substitutions at this position showed that *O*-demethylation to a phenol or the introduction of an aniline group (**Figure 2.2b**) retained metaphase arrest and significantly improved antimitotic activity, displaying one and two orders of magnitude greater potency than noscapine, respectively. These compounds also maintained a similar oral bioavailability to the parent compound[108]. Interestingly, the 7-aniline compound was later shown to interact with tubulin at a different binding site to noscapine[109]. Further work investigated the effects of acyl and carbamate ester groups at the 7-position on cytotoxicity against five cancer cell lines and identified compounds with up to 70-fold improvement in IC₅₀ values. There was a remarkable variation between cell lines, however, with one analogue showing a 250-fold difference in cytotoxicity towards two different cell types[110]. This was attributed to potential differences in tubulin isotypes between the different cell lines.

Modifications at the 7-position of noscapine were further investigated by Henary and colleagues in an effort to rationally design noscapine analogues with improved aqueous solubility[111]. The addition of alkylsulfonate groups to the 7-position of both noscapine and 9'-bromonoscapine generated structures with up to 25-fold enhanced antiproliferative activity across four cell lines compared to the parent compounds and up to two-fold enhancement in the absolute oral bioavailability of the analogues compared to noscapine.

Structure-activity relationships at the amine (6') position of noscapine have also been explored by several groups. DeBono and colleagues synthesised a range *N*-modified noscapine analogues based on the reduced lactone (cyclic ether) derivative of noscapine[112]. Of the 29 analogues studied, twelve showed significantly improved mitotic arrest of three cancer cell lines compared to noscapine, though all active compounds showed significantly less activity on colon cancer cells compared to breast and prostate cancer cell lines. The most potent compound had an ethyl aminocarbonyl group at the 6'-position (**Figure 2.2c**) and showed antimitotic activity equal to that of the clinical chemotherapeutic vincristine. Another group produced rationally designed *N*-substituted analogues based on improved *in silico* docking with tubulin, most of which displayed stronger tubulin binding *in vitro* compared to noscapine as well as enhanced inhibition of cell division across four cell lines, though there was again significant variability between cell lines.

A series of noscapine analogues functionalised at multiple positions was synthesised by DeBono and colleagues with the aim of combining several features known to improve noscapine potency; namely, an ethyl aminocarbonyl group at the 6'-position, a halogen substitution at the 9'-position and *O*-demethylation at the 7-position to produce a free phenol group (**Figure 2.2d**)[113]. The lactone group was also reduced to the cyclic ether to improve aqueous stability and several aryl group substitutions were made at the 9'-position in addition to halogen groups. Several of these compounds showed up to four-fold improvement in potency compared to an analogue with only the *N*-ethyl aminocarbonyl modification. Importantly, most analogues showed little variability across the three cell lines tested. The group also examined the susceptibility of several analogues to the multidrug resistant pumps ABCB1 and ABCG2 that are found in some breast cancer types and found no change in cytotoxicity, suggesting that the analogues are not substrates for these drug resistance pumps. This is in contrast to the chemotherapeutic agent vinblastine, which showed a 260-fold reduction in potency under the same conditions. However, the new analogues were still four- to nine-fold less potent than vinblastine toward the drug-resistant cells. Another group synthesised di-substituted noscapine analogues that had a 9'-bromine substitution combined with various modifications at the 7-position. Several of these compounds showed stronger tubulin binding *in vitro* and improved cytotoxicity against several cell lines compared to the 9'-brominated parent analogue[114].

2.2.5. Noscapine metabolites

The metabolism of noscapine has been studied in mammalian systems by several groups using both *in vivo* and *in vitro* methods. Tsunoda and Yoshimura identified several metabolites in the urine of rats, rabbits and humans and found that three different enzymatic mechanisms of metabolism were involved[115, 116]. These were:

- (i) cleavage of the central C-C bond between the phthalide and isoquinoline moieties (producing metabolites **I** and **II**; **Figure 2.3**);
- (ii) *O*-demethylation of the three methoxy groups (metabolites **III**, **IV** and **V**); and
- (iii) cleavage of the methylenedioxy bridge (metabolite **VI**).

In a later study that used high-resolution tandem mass spectrometry, Fang and colleagues tested liver microsomes purified from humans and mice and identified the same metabolites plus two hydroxylated metabolites (**VII** and **VIII**) and an *N*-demethylated metabolite (**IX**)[27]. The most abundant metabolites produced by human microsomal P450s were the cleaved methylenedioxy bridge metabolite (**VI**) and the

N-demethylated metabolite (**IX**). In the same work, experiments with pure human CYP isoforms identified CYP2C9 and CYP3A4 as the main enzymes responsible for metabolites **VI** and **IX**, respectively[27]. Metabolic profiling has also been applied to two noscapine analogues, 9'-brominated noscapine[117] and 9'-nitro-noscapine[118], which showed the same mechanisms of metabolism observed for noscapine.

It is notable that two of the demethylated metabolites, *N*-demethylated noscapine and 7-*O*-demethylated noscapine, have been synthesised as intermediates for *N*-modified and 7-modified noscapine analogues. These derivatives have also been examined for antimitotic activity and both showed improved activity against several cancer cell lines compared to noscapine[108, 119]. Syntheses for the remaining hydroxylated and demethylated metabolites have not been described and their potential anticancer activities have not been assessed.

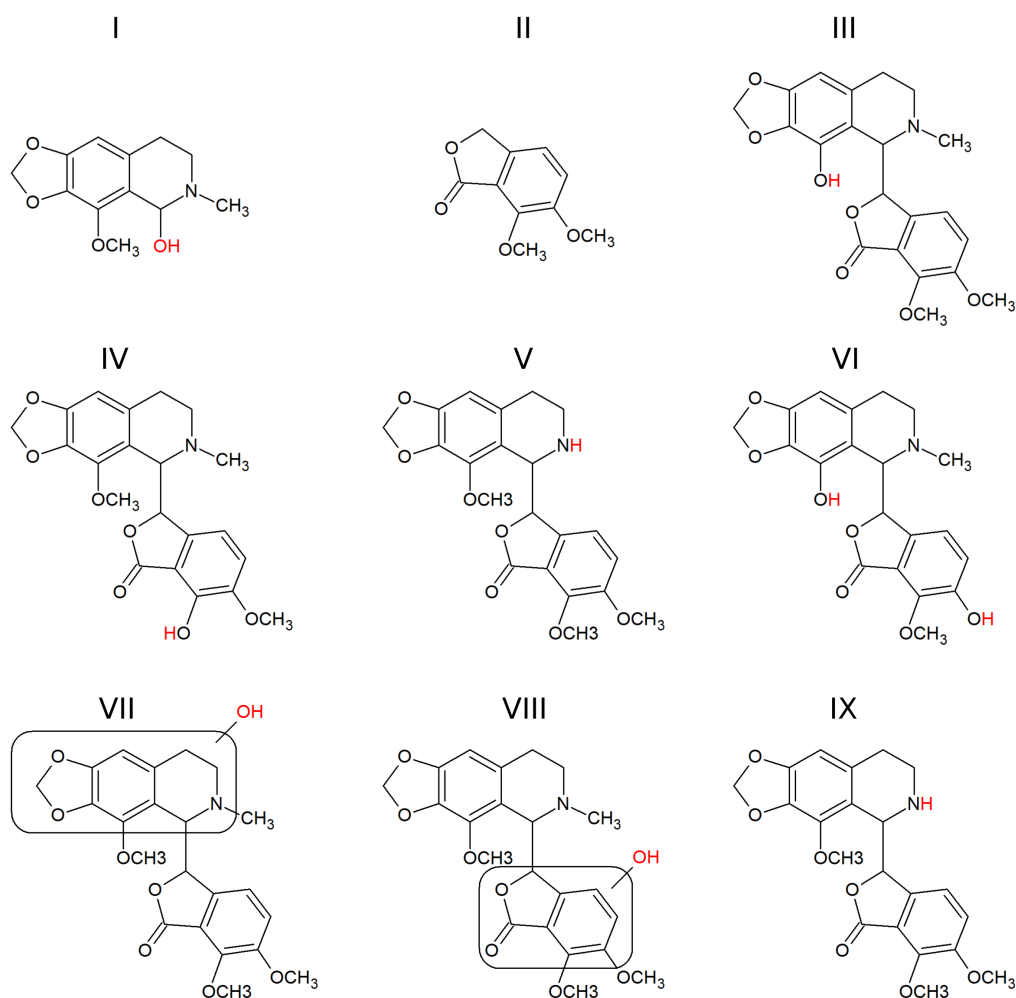


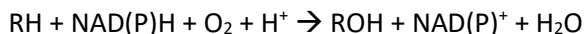
Figure 2.3. Mammalian noscapine metabolites. Functional group modifications are shown in red. The exact hydroxylation sites in metabolites VII and VIII were not determined.

2.3. Cytochrome P450 enzymes

2.3.1. Background

Cytochrome P450 enzymes (P450s) are a superfamily of haem-containing monooxygenases that are found in all kingdoms of life and have a diverse array of biological functions[120]. In mammals, P450s are well known for their role in xenobiotic metabolism[18, 121] but are also involved in steroid hormone and vitamin biosynthesis in the mitochondria[122]. Cytochrome P450s also play a dominant role in plant secondary metabolism, including the biosynthesis of structural molecules, pigments and defence compounds[123]. In bacteria and fungi, the roles of P450s include carbon utilisation, membrane maintenance and antibiotic biosynthesis[124, 125]. This remarkable functional diversity means that P450 enzymes have a substrate range that is unrivalled by any other enzyme family. In this respect, P450s have enormous potential for biotechnological applications: substrates for P450s include drugs, steroids, vitamins, insecticides, fatty acids, terpenes, alkanes, polyketides and many other compounds of relevance to the pharmaceutical and fine chemical industries[126].

The characteristic reaction of a P450 enzyme is the insertion of one oxygen atom from molecular oxygen into a C-H bond, with the second oxygen atom being reduced to water[127]. A pyridine redox cofactor, either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH), provides electrons for the reaction, with the overall reaction as follows:



Following this hydroxylation, subsequent reactions can spontaneously occur, resulting in diverse substrate modifications such as epoxidation, heteroatom dealkylation, alcohol oxidation, ring formation, and carbon-carbon bond formation and cleavage[128, 129]. These reactions often occur with high substrate specificity, regioselectivity and stereoselectivity, with the notable exception of xenobiotic-metabolising P450s, which are highly promiscuous in their substrate preference due to their role in detoxification.

2.3.2. Catalytic cycle

The active site of a P450 enzyme is located around the haem prosthetic group (**Figure 2.4**). The haem group is attached to the protein by a cysteine residue, which is the only conserved amino acid in the P450 gene family[130]. The P450 haem group is comprised of a porphyrin ring with a central six-coordinate iron ion: the four nitrogen atoms of the porphyrin ring are coordinated at the equatorial positions, the sulphur atom of the conserved cysteine residue is bound axially and one water molecule is weakly

coordinated *trans* to the cysteine[131]. In this configuration, the haem iron is able to act as a transient electron sink during P450 catalysis.

The P450 catalytic cycle[131] is shown in **Figure 2.5**. In the resting state, the haem iron is ferric (structure I) and in a low spin state. Substrate arrival at the active site displaces the axial water ligand, causing the iron(III) to become five-coordinate and high-spin, increasing the redox potential of the haem complex (II). This drives the transfer of a single electron from NAD(P)H to the iron(III), reducing it to the ferrous state (III) and resulting in the binding of molecular oxygen to form a ferric-oxo complex (IV). A second electron is transferred to the terminal oxygen, producing a ferric-peroxo complex (V), which then accepts a proton to form a ferric-hydroperoxy complex (VI). A second protonation results in cleavage of the O-O bond and the release of a water molecule, leaving a radical ferryl-oxo species (VII) which is highly reactive and thought to be the oxidising agent in most P450 reactions. In hydroxylation reactions, this species abstracts a hydrogen atom from a carbon atom on the substrate, forming a ferryl-hydroxo species and a carbon radical (VIII). The substrate radical then reacts with the iron(IV)-bound hydroxyl group, forming a hydroxylated product. The product is then released and the iron returns to its ferric resting state (IX).

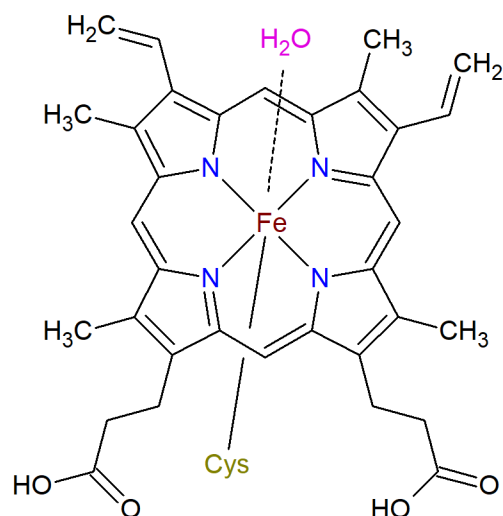


Figure 2.4. Haem prosthetic group of cytochrome P450. The haem iron is bound to the conserved cysteine residue of the enzyme and a water molecule is weakly coordinated *trans* to the cysteine.

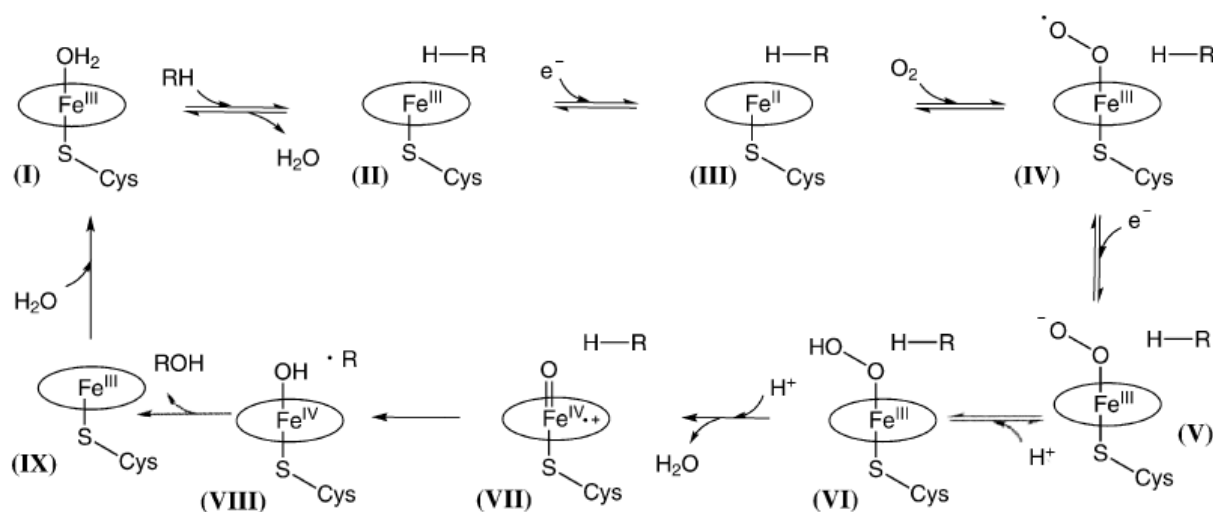


Figure 2.5. Cytochrome P450 catalytic cycle. Figure adapted from Whitehouse *et al.* (2015).

Most cytochrome P450 enzymes do not obtain reducing equivalents from NAD(P)H directly but via discrete redox partner enzymes. In bacteria, the most common P450 system is comprised of three proteins[132]:

- (i) a flavin adenine dinucleotide (FAD)-containing flavodoxin;
- (ii) a flavin mononucleotide (FMN)-containing ferredoxin; and
- (iii) the substrate-binding cytochrome P450 enzyme.

In these systems, the flavodoxin binds NAD(P)H and transfers electrons to the ferredoxin, which then shuttles the electrons to the haem group in the P450 domain. All three proteins are freely soluble in the cytoplasm. The eukaryotic mitochondrial system is analogous to the bacterial system, except the flavodoxin and P450 are membrane-bound and only the ferredoxin is soluble[133].

The microsomal P450 system, which is present in mammalian liver tissue and responsible for drug metabolism, typically consists of two protein partners: the cytochrome P450; and a cytochrome P450 reductase enzyme (CPR), which evolved as a fusion of the FAD- and FMN-containing redox proteins from bacteria[134]. Both components are bound to the membrane of the endoplasmic reticulum. Three-component microsomal systems also exist that rely on the enzyme cytochrome *b*₅ to mediate the second electron transfer of the catalytic cycle, either from CPR or its own reductase partner, cytochrome *b*₅ reductase[135].

2.3.3. Limitations of drug-metabolising cytochrome P450s

The reaction diversity and broad substrate range of cytochrome P450s make this enzyme family highly attractive for use in pharmaceutical biotransformations[136]. Regio- and stereoselective activation of C-H bonds is notoriously difficult to achieve in chemical synthesis, particularly when the target compound contains multiple functional groups[137]. Selective oxidation of complex drugs often requires tedious protection/de-protection regimes to avoid unwanted reactions at other sites on the compound[138]. Performing such modifications enzymatically, in water and at ambient temperatures, can significantly simplify syntheses, decrease manufacturing costs and provide a greener route to the product[1, 139]. Despite the appealing catalytic qualities of cytochrome P450s, however, some intrinsic properties of this enzyme family pose limitations on scale-up and integration into a viable pharmaceutical manufacturing process[28, 29, 39, 140].

Whilst the broad range of pharmaceutical substrates accepted by mammalian microsomal P450s makes them promising enzymes for drug metabolite manufacture, these enzymes typically show low rates of

catalysis with substrate turnover numbers (k_{cat}) rarely exceeding 10 min^{-1} [128, 141]. In comparison, industrially useful biocatalysts typically have k_{cat} values greater than 1 s^{-1} and sometimes several orders of magnitude higher[142, 143]. Another issue is the membrane-binding nature of these enzymes. This property can hinder high-level protein expression in bacterial hosts, as bacterial cells lack intracellular membranes to anchor the P450 membrane-binding domain[28]. While solubilisation can often be achieved by recombinantly expressing the proteins with a truncated membrane anchor, this can impact enzyme activity[144]. Additionally, microsomal P450s require one or two discrete reductase partner enzymes, which must be present at suitable concentrations for the biotransformation to occur at a useful rate. Electron transfer can be rate-limiting when P450 systems are co-expressed in heterologous hosts due to suboptimal ratios of the system components and incorrect protein-protein interactions in the host cell environment[145].

Substrate turnover by P450 enzymes requires the supply of a nicotinamide cofactor in at least equimolar amounts to the substrate. For most eukaryotic P450s, this is NADPH, which can cost several thousand dollars per gram[146]. Often, the NADPH requirement is far higher than stoichiometric amounts due to the uncoupling that can occur between NADPH oxidation and substrate turnover, resulting in premature termination of the catalytic cycle after electron transfer but before substrate oxidation[37, 147]. This effect can be significant: with microsomal P450s the uncoupling rate is typically less than 50%[148, 149], and rates of coupling efficiency as low as 1% have also been observed[150].

Substrates for drug-metabolising P450 enzymes are usually quite hydrophobic, and substrate solubility is often a major obstacle to achieving high productivity within a biotransformation system[40, 140]. Most published P450 biotransformations use sub-millimolar concentrations of substrate, which is in contrast to the molar-range concentrations achievable in chemical synthesis with organic solvents. While drug solubility can often be enhanced by the addition of water-miscible organic solvents, microsomal P450s are particularly sensitive to even small amounts (1-2%) of common co-solvents[151, 152], which makes these approaches less useful.

The use of bacterial P450 systems can potentially overcome several of the limitations discussed above to some extent. Bacterial P450s typically show higher catalytic rates than their mammalian counterparts, allowing higher volumetric productivities to be achieved[136]. Furthermore, the components of bacterial P450 systems are soluble rather than membrane-bound, which permits high protein expression, confers greater enzyme stability and facilitates protein purification[30].

A downside is that bacteria do not express an arsenal of xenobiotic-degrading enzymes like mammalian liver tissue. Bacterial P450s are often limited to primary metabolic activities, such as the utilisation of different carbon sources[153] and fatty acid biosynthesis[154]. This is, however, highly species-dependent, and some bacteria, such as *Streptomyces* sp. and other actinomycetes, are well known for producing a vast array of secondary metabolites using P450 enzymes[155-157].

Protein engineering has been applied with much greater success to improve bacterial P450 enzymes compared to mammalian P450s, as bacterial P450s are usually more amenable to high throughput screening and structural characterisation than their mammalian counterparts[17, 158]. Several groups have reported improvements in catalytic activity of bacterial P450s by several hundred-fold through rational mutagenesis and the application of directed evolution[159-161]. Protein engineering has also been used to change substrate specificity[162, 163], regioselectivity[164, 165] and cofactor preference[166], as well as to improve thermostability[167] and activity in organic solvents[168].

2.3.4. Cytochrome P450_{BM3} (CYP102A1)

In the mid-1970s, Fulco and colleagues identified a soluble, NADPH-dependent long-chain fatty acid hydroxylase from the Gram-positive bacterium *Bacillus megaterium*[169]. This was later found to be a cytochrome P450 enzyme and was named P450_{BM3}, being the third P450 to be isolated from this organism[32]. A decade after its discovery, it was confirmed by Fulco that this enzyme existed as a fusion of a P450 haem domain to a mammalian-like cytochrome P450 reductase (CPR)[170, 171]. The discovery of a P450 enzyme and its reductase partner forming a single protein was unprecedented at the time, though several others have since been discovered[172].

The self-sufficient nature of P450_{BM3} means that electron transfer to the active site is not reliant upon on a protein-protein encounter between two discrete enzyme partners. This feature is thought to contribute to the remarkably high activity of the enzyme[31]: the k_{cat} for the hydroxylation of pentanedecanoic acid is 75 s⁻¹[173], which is the highest reported turnover number for any cytochrome P450 enzyme. The P450_{BM3} enzyme is also quite unique in that the coupling efficiency between NADPH turnover and substrate oxidation is close to 100% on its natural substrates[170]. The fused nature of this P450 system, combined with its high solubility, facilitates high-level protein expression in microbial hosts, with P450 yields up to 12 μ M reported in *E. coli*[174]. In contrast, the highest reported expression yields of recombinant microsomal P450s in *E. coli* are $\sim$ 1 μ M[175], even with significant optimisation of coding sequences and culture conditions[176]. The P450_{BM3} enzyme has been highly characterised by

crystallography, NMR, and molecular dynamics simulations, and is regarded as a model cytochrome P450[31].

While P450_{BM3} is involved in lipid metabolism in *B. megaterium*, it has been shown to have modest oxidation activity on several drug substrates[177]. As the enzyme is well characterised, highly active and amenable to protein engineering, there have been significant efforts to broaden the substrate range of the enzyme to include important pharmaceutical compounds using protein engineering[31, 33].

Site-directed mutagenesis was first applied to P450_{BM3} to probe the structure-function relationships of several amino acids identified on early crystal structures as being important for substrate binding[178-180]. These results were then used by several groups to alter P450_{BM3} substrate specificity. Carmichael and Wong[181] and Li and colleagues[182] independently examined the effects of mutations in the active site and substrate access channel on the oxidation of polyaromatic hydrocarbons, such as naphthalene, and found improvements in oxidation rates of up to four orders of magnitude[182]. Further engineering was applied by Li and colleagues to alter the substrate specificity of P450_{BM3} from long-chain to medium-chain fatty acids[183]. This group also performed directed evolution experiments that evolved P450_{BM3} into an indole-metabolising enzyme[184].

Protein engineering of P450_{BM3} has also been used to emulate the drug-metabolising activity of mammalian microsomal P450s. Using key amino acid substitutions identified by others, Jan Commandeur and colleagues utilised a fluorescent screening protocol used in human P450 assays to detect mammalian-like drug metabolic activity in P450_{BM3} mutants[185]. This study identified a triple mutant capable of metabolising a range of important drugs with a metabolite profile similar to that of the human CYP3A family of enzymes[186]. This mutant was then subjected to directed evolution, resulting in a 200-fold increase in activity against dextromethorphan and methamphetamine[161]. Notably, the catalytic rates of these mutants were up to 90 times higher than those of human cytochrome CYP2D6. Four mutants from this study were also screened against diclofenac, clozapine and acetaminophen and shown to produce the same metabolite profile as human and rat microsomal P450s, with activities up to 70-fold higher[187]. This group later reduced their mutant library down to four highly promiscuous mutants that could metabolise 41 out of 43 structurally diverse drug compounds, with significant differences in regioselectivity occurring between some mutants[188]. The crystal structures of two of the most active mutants from this work have been determined, which has allowed the functional effects of the mutations to be examined using *in silico* docking and molecular dynamics simulations[189, 190]. The work by this group has identified several key P450_{BM3} residues that have been targeted by other groups in the

development of drug-metabolising mutants[191-194]. Another important study was conducted by Lewis and colleagues who used combinatorial alanine substitution[195] throughout the P450_{BM3} active site to allow the selective hydroxylation of bulky morphinan alkaloids[196]. Further work by Kille and colleagues used semi-rational site-saturation mutagenesis[197] to engineer a P450_{BM3} mutant with 97% stereoselectivity for testosterone hydroxylation from a starting mutant that produced a 50:50 racemate[198].

Other groups have used protein engineering to address other industrially relevant aspects of P450_{BM3}. Wong and colleagues applied directed evolution to improve organic co-solvent tolerance, generating a mutant with 10-fold improved activity in 25% dimethyl sulfoxide (DMSO)[168]. A crystal structure of this mutant was published, which provided insights into the mechanism of solvent deactivation of P450_{BM3}. Several rational and random engineering approaches have also been used in an effort to lower or eliminate the cost of nicotinamide cofactor in P450_{BM3} biotransformations, including changing the cofactor preference of the enzyme to the significantly cheaper NADH[166], developing peroxide-driven variants[199], and developing a hybrid P450_{BM3} enzyme driven by light instead of NADPH[200].

The combination of directed evolution and site-saturation mutagenesis studies on P450_{BM3} means that a very high number of amino acid mutations have been described for this enzyme in the literature. A comprehensive review from 2012[31] tabulated close to 1000 reported mutations throughout the haem and reductase domains of P450_{BM3} and it is likely that this number has since grown considerably. As of early 2020, there were 97 published P450_{BM3} crystal structures in the Protein Data Bank[201] with various mutations, bound substrates and hybrid domains. The enormous amount of published information regarding P450_{BM3} provides an excellent source of guidance for future engineering efforts to develop variants with industrially important qualities.

2.4. Mass transfer limitations in whole-cell biotransformations

2.4.1. Background

The mass transfer of substrate into cells is often a major limiting factor on the reaction rates of whole-cell biotransformations[38]. This is perhaps unsurprising when considering that the primary function of a cell envelope is to act as a selective permeability barrier between the intracellular and extracellular environments, permitting the influx of essential biomolecules and ions while excluding toxic compounds. The degree of permeability of a microbial host cell to a chosen biotransformation substrate depends on both:

- (i) the physicochemical properties of the substrate (size, hydrophobicity, protonation state); and
- (ii) the structure of the host cell envelope, which differs markedly between microbial species.

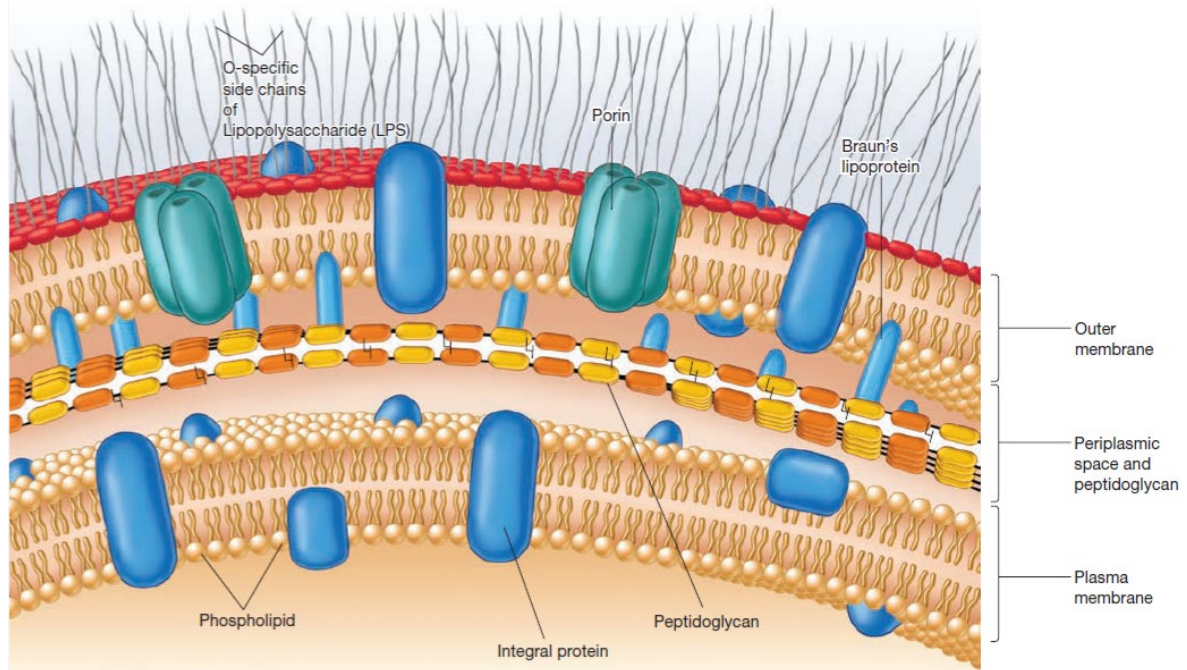
Bacteria can be broadly categorised into two groups based on their cell envelope structures: Gram-positive and Gram-negative. Gram-negative organisms, which include the popular biotransformation hosts *Escherichia coli* and *Pseudomonas* sp., have both an outer membrane as well as an inner cytoplasmic membrane (**Figure 2.6a**)[202]. The intermembrane space is referred to as periplasm and contains a layer of a mesh-like polymer called peptidoglycan, which provides structural integrity to the cell. The principal component of the outer membrane is lipopolysaccharide (LPS), which is largely responsible for the impermeability of Gram-negative organisms to many organic compounds[203]. Lipopolysaccharides are made up of:

- (i) a phospholipid component, lipid A, which forms part of the outer membrane lipid bilayer;
- (ii) a core polysaccharide; and
- (iii) a glycan polymer which forms the outermost layer of the cell envelope, known as the O-polysaccharide or O-antigen[204].

The LPS layer is chelated by divalent cations and exists in a densely packed, quasi-crystalline state[205]. As the O-polysaccharide is hydrophilic, this layer acts as a strong permeability barrier to hydrophobic molecules. Studies using hydrophobic probes have shown that small hydrophobic compounds permeate the outer membrane of Gram-negative organisms at a 50- to 150-fold slower rate compared to the cytoplasmic membrane[206].

Lipopolysaccharides are also a good barrier for hydrophilic substrates due to the hydrophobic lipid A component comprising the outer membrane layer[207]. Instead, hydrophilic small molecules can

(a)



(b)

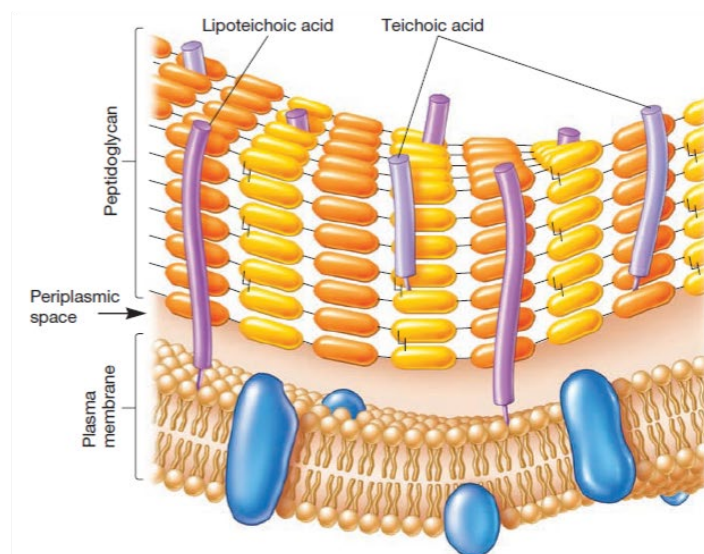


Figure 2.6. Cell wall and membrane structures of **(a)** Gram-negative and **(b)** Gram-positive bacteria. Reprinted from Prescott, Harley and Klein's *Microbiology*, 7th edition (2008) McGraw Hill.

permeate the outer membrane through protein channels called porins[203]. Many porin complexes allow the non-specific passive diffusion of hydrophilic compounds up to around 600 Da in size[208]. The inner surfaces of porin channels are typically lined with charged amino acid residues coordinated with water molecules, which makes for difficult passage of hydrophobic compounds[209, 210]; these instead mostly rely on slow diffusion through the LPS layer into the lipid membrane. Compounds that permeate the

outer membrane and enter the periplasm can then move into the intracellular space via passive diffusion across the cytoplasmic membrane or through transport channels.

Gram-positive organisms, such as *Bacillus* sp., do not have an outer membrane and are instead surrounded by a thick cell wall comprised of peptidoglycan[202] (**Figure 2.6b**). The peptidoglycan layers are covalently crosslinked, forming an open mesh-like structure, and bound to anionic polymers called teichoic acids. While this cell wall functions well in maintaining cell shape, the coarse mesh poses little barrier to hydrophobic or hydrophilic small molecules. The size exclusion limit of the cell wall has been estimated to be between 30 000 Da and 57 000 Da[211], which is much greater than the size of typical biotransformation substrates. While the cell wall has an overall negative net charge due to the presence of phosphate and carboxylate groups on the teichoic acids, these are widely interspersed and do not appear to influence the passage of charged or hydrophobic compounds[212]. The differences in cell wall structure between Gram-positive and Gram-negative organisms underlies the comparatively enhanced susceptibility of Gram-positive bacteria to many antibiotic compounds.

2.4.2. Cell permeabilisation strategies

The magnitude of mass transfer limitation posed by the cell wall and membrane structures can be assessed by comparing the activity of whole-cell biocatalysts with the activity of lysed cells. For example, hydroxylation of the hydrophobic steroid progesterone using recombinant *E. coli* showed a six-fold faster initial rate with cell lysates compared to whole cells[213]. Hydrolysis of the large hydrophilic compound nitrocefin by β -lactamase in *E. coli* was similarly reduced to 5-20% of the activity of cell lysates when conducted with whole cells[214]. Oxidation of the hydrophilic compound uridine diphosphate glucose (UDP-glucose) by UDP-glucose dehydrogenase was also reduced to 4% of the available enzyme activity using a whole cell biotransformation[215]. Baeyer-Villiger oxidation of several cyclic ketones by *E. coli* expressing cyclohexanone monooxygenase showed 1.3- to 5-fold lower initial rates compared to cell lysates[216]. In some cases, mass transfer limitations in *E. coli* have prevented any detectable enzyme activity, such as in the P450-mediated hydroxylation of the large cyclic peptide cyclosporine[217] and the hydrophobic terpenes keto-boswellic acid[218] and abietic acid[219].

A common strategy to relieve mass transfer limitations is the permeabilisation of cells through physical (e.g. freeze-thaw, sonication) or chemical (e.g. detergents, organic solvents)[38]. Permeabilisation can be very effective for this purpose but is typically destructive to the cells and the method should be optimised to maximise biotransformation productivity. The effect of permeabilization also varies significantly

depending on the host strain and substrate (**Table 2.1**). For example, the use of the detergent cetyl trimethyl ammonium bromide (CTAB) to permeabilise *E. coli* allowed the P450-mediated biotransformation of cyclosporine when no activity was detected with untreated cells[217], but product yields were decreased for the hydroxylation of the terpene abietic acid[220] and *N*-demethylation of verapamil[221] compared to untreated control cells. Other detergents such as Tween 20 and Triton X-100 have similarly been used with varying success (**Table 2.1**). A combination of freeze-thaw treatment and Triton X-100 provided a 65-fold improvement in the initial rate of biotransformation of α -pinene oxide by wild-type *Pseudomonas rhodesiae*[222], though this regime also caused cell death and significant enzyme leakage and could therefore not be applied to reactions that are dependent upon active host metabolism.

Pre-treatment of cells with organic solvents has also been shown to provide varying results. For example, acetone was used to achieve a two-fold increase in activity for the CYP153A6-mediated hydroxylation of n-octane to octanol in *E. coli*[223], but the same reaction was shown to be compromised when a similar pre-treatment process was used by a different research group[224] (**Table 2.1**), potentially due to differences in growth conditions and media prior to permeabilisation. Cell permeabilisation using toluene improved the biotransformation of the terpene abietic acid[220] but decreased or abolished P450-mediated oxidation of the 11-deoxycorticosterone[225] and n-octane[223], respectively.

Table 2.1 Comparison of cell permeabilisation strategies used to improve substrate uptake in selected whole-cell biotransformation systems.

Substrate	Host organism	Detergents				Organic solvents		Other agents		Ref.
		CTAB	Tween 20	Tween 80	Triton X-100	Acetone	Toluene	EDTA	Polymyxin B	
cyclosporine	<i>E. coli</i>	allowed detectable activity	-	-	-	-	-	-	-	[217]
abietic acid	<i>E. coli</i>	~7- to 10-fold lower yield	~2- to 5-fold lower yield	-	-	-	~2.5-fold higher yield	~3-fold higher yield	~3- to 5-fold higher yield	[220]
verapamil	<i>E. coli</i>	~2-fold lower yield	-	-	~4-fold lower yield	-	-	-	~1.5-fold higher yield	[221]
cyclic lactones	<i>E. coli</i>	-	-	-	~1.3-fold higher initial rate	-	-	-	-	[216]
pinene oxide (treatments combined with freeze-thaw)	<i>P. rhodesiae</i>	-	-	-	~65-fold higher initial rate	-	~42-fold higher initial rate	-	-	[222]
n-octane	<i>E. coli</i>	-	-	-	-	~2-fold higher initial rate	no activity detected	-	-	[223]
n-octane	<i>E. coli</i>	-	-	-	~3-fold higher initial rate but rapid activity loss	~2-fold lower initial rate	-	-	~2-fold higher initial rate but rapid activity loss	[224]
17- α -hydroxyprogesterone	<i>S. pombe</i>	-	-	~1.5-fold higher initial rate	no change in initial rate	-	-	-	-	[226]
dodecanoic acid	<i>E. coli</i>	-	-	-	-	~7-fold lower yield	-	-	-	[227]
11-deoxycorticosterone	<i>E. coli</i>	-	-	-	-	-	~75-fold lower yield	~2-fold lower yield	~25-fold lower yield	[225]
dodecanoic methyl ester	<i>E. coli</i>	-	-	-	no change in initial rate	-	-	~3-fold higher initial rate	-	[228]
cholesterol	<i>E. coli</i>	-	-	-	-	-	-	-	~4-fold higher yield	[229]

Other cell-permeabilising agents have also been explored with mixed success (**Table 2.1**). Use of the chelating agent ethylenediaminetetraacetic acid (EDTA) to disrupt outer membranes improved the biotransformation of abietic acid[220] and dodecanoic acid methyl ester[228] but caused reduced yields for the hydroxylation of 11-deoxycorticosterone[225]. Treatment of *E. coli* with low concentrations of the antibiotic polymyxin B, which forms outer membrane pores, significantly enhanced the biotransformation of abietic acid[220] and cholesterol[229] but reduced the yield for 11-deoxycorticosterone hydroxylation by ~25-fold[225]. For the hydroxylation of *n*-octane, a two- to three-fold enhancement of initial rates was achieved using either polymyxin B or Triton X-100 but in both cases the cells lost activity rapidly compared to untreated cells[224], attributed to build-up of toxic product inside the cells.

Heterologous expression of membrane transport proteins to enhance substrate uptake has also been explored as an alternative to physical or chemical permeabilisation. Co-expression of the outer membrane alkane transporter AlkL from *Pseudomonas* sp. has been used to improve oxidation rates of alkanes[230], terpenes[231], fatty acids[232] and fatty acid methyl esters[228, 233]. In the latter case, this allowed a 62-fold increase in enzymatic rate within an optimised bioreactor setup compared to cells lacking the transporter[228]. The modified cells did, however, display a more rapid decline in activity compared to control cells, attributed to the higher amounts of toxic substrate entering the cells. Protein engineering of the native *E. coli* ferredoxin hydroxamate uptake protein to produce a large passive diffusion channel also provided up to a five-fold increase in yield for the oxidation of various aromatic and terpene compounds[234] by a P450_{BM3} mutant enzyme. Another strategy involved mutations of the native *E. coli* solvent efflux pumps to increase intracellular substrate accumulation[235], allowing 16-, 7- and 2-fold productivity increases for the oxidation of the pharmaceutically important compounds compactin, calcifediol and cholestenone, respectively, by various cytochrome P450 enzymes. While allowing up to 77-fold more substrate to accumulate in the cells, this approach also meant that cell disruption was required to extract the products[235], increasing the number of processing steps.

Genetic modification of the host organism has also been used to improve substrate uptake for whole-cell biotransformations. The use of an *E. coli* strain lacking the ability to produce Braun's lipoprotein, a major outer membrane protein, provided significantly enhanced biotransformation rates for a range of compounds with differing lipophilicity and size[44, 214, 215], including up to six-fold enhancement of reaction rate for the oxidation of several small aromatic compounds by toluene dioxygenase[44] and a 14-fold improvement in the rate of UDP-glucose oxidation[215]. Cells lacking Braun's lipoprotein were found not to differ from wild-type cells in growth, carbon metabolism or membrane fatty acid

composition[236]. Modification of the cell membrane composition was also used to enhance Baeyer-Villiger oxidation by *Corynebacterium glutamicum* by treatment of the cells with ethambutol, which inhibits proteins involved in the biosynthesis of cell wall compounds and produces more permeable membrane structures[237].

Surprisingly, given the significant efforts to improve mass transfer over *E. coli* membranes and the known differences in antibiotic uptake between Gram-negative and Gram-positive organisms, the comparison of different microbial hosts to improve mass transfer is rarely investigated. For example, biotransformation of the terpenes abietic acid[219] and 11-keto- β -boswellic acid[218] using CYP106A2 in *E. coli* showed no detectable activity, but overexpression of the same enzyme in its native host, Gram-positive *Bacillus megaterium*, allowed significant productivity despite functional enzyme being produced by both hosts. This type of comparison is potentially worthy of further study for other biotransformations, where the selection of an alternative host strain may allow significant gains in biotransformation productivity without the need for cell permeabilisation.

2.5. Substrate solubility limitations in whole-cell biotransformations

2.5.1. Background

Many substrates for monooxygenase enzymes, such as pharmaceutical compounds and hydrocarbons, have poor aqueous solubility. While whole-cell biocatalytic processes can offer considerable advantages in reaction selectivity, efficiency, cost and environmental impact compared to large-scale organic synthesis, they require aqueous reaction environments to function and poor substrate solubility can significantly limit volumetric productivity. Low substrate concentrations can result in sub-optimal biotransformation activity due to a low driving force for the mass transfer of substrate across the cell membrane, as well as low enzyme activity through Michaelis-Menten kinetics. Poor solubility can necessitate large reaction volumes which drives down volumetric productivity and increases downstream processing costs, which may make a biotransformation economically unviable at industrial scale. Efforts to increase substrate loading are therefore often a standard stage in the development and scale-up of whole-cell biotransformations.

2.5.2. Biphaseic biotransformations

A popular method to enhance substrate loading is the use of a biphaseic biotransformation, where a second phase, typically a water-immiscible liquid phase such as an organic solvent, is added to the biotransformation reaction. The second phase, in which the substrate has high solubility, acts as a substrate reservoir, continuously partitioning substrate into the aqueous phase as it is consumed by the reaction. Biphaseic biotransformation systems have been investigated for over 50 years[238, 239] and have been described as one of the four most important advances in biocatalysis[240] (with the other three being recombinant DNA technology, cell disruption, and enzyme and cell immobilisation). Many biphaseic processes have been implemented at industrial scale[241, 242]. Aside from improved substrate loading, there are other advantages to biphaseic systems. Where a biotransformation substrate and/or product is toxic to the microbial host, the partitioning of these compounds between the two phases can allow sub-toxic concentrations to be maintained in the aqueous phase[243, 244]. Biphaseic systems can also provide continuous removal of product from the cells in the aqueous phase, simplifying downstream processing and enabling the re-use of biocatalyst[245].

2.5.2.1. Choice of solvent

The selection of a solvent for a biphaseic system requires a range of considerations:

- (i) The toxicity of the solvent towards the microbial host and the biotransformation enzyme must be low to allow productive and extended biocatalysis;
- (ii) The solvent should provide high solubility of the substrate to permit high mass loading, and high solubility of the product if an *in-situ* product removal process is being designed;
- (iii) Partitioning of the substrate into the aqueous phase should be sufficient to maintain good productivity. For toxic compounds, partitioning into the aqueous phase should provide sub-toxic aqueous concentrations.

For the biotransformation of some compounds, such as hydrocarbons, long-chain alcohols or esters, the substrate itself can be used as an organic second phase providing it meets the requirement of low toxicity[228, 233, 246-248]. Additionally, there are a range of other solvent characteristics that become particularly important at process scale, such as human and environmental safety, cost, and the potential of the solvent to form an undesired emulsion[249].

2.5.2.2. Solvent biocompatibility

The majority of commonly used organic solvents are toxic to microbes. While a range of mechanisms can be responsible, toxicity is typically due to the accumulation of solvent molecules in the phospholipid membrane[250]. Build-up of solvent molecules in the cell membrane increases membrane fluidity, promoting the leakage of intracellular ions, ATP and other important biomolecules[251]. Under these circumstances, the cell membrane potential, which is imperative for maintaining the energetic status of the cell, becomes disrupted or abolished completely[252]. Membrane transport proteins can also be inactivated by the solvent molecules, causing imbalances in the concentrations of critical metabolites. Continued accumulation of solvent in the bacterial membrane leads to lysis when its integrity can no longer be maintained. Solvent that passes through the membrane and into the cytoplasm can also cause enzyme inhibition and protein denaturation, and interfere with metabolic processes[250]. In addition to this 'molecular toxicity' inflicted by solvent molecules in the aqueous phase, 'phase toxicity' can also occur in biphasic systems due to the presence of an aqueous-organic interface in the biotransformation vessel[244, 253-255]. This toxicity is suggested to be caused by direct contact of the cell membranes with the interface, which can extract essential molecules from the cell envelope, remove of critical nutrients from the aqueous phase or cause nutrient limitation due to cells becoming trapped in solvent droplets[256, 257]. The degree of phase toxicity is proportional to the aqueous-organic phase ratio and can increase with a higher degree of mixing, which increases the interfacial area[255].

Extensive work has been performed to understand the relationships between solvent properties and microbial toxicity in order to establish rules for solvent biocompatibility[258-261]. A particularly useful property to predict biocompatibility is the hydrophobicity of the solvent, determined by its octanol-water partition coefficient ($\log P$)[258]. Solvents with high $\log P$ values have low aqueous solubility and therefore produce low aqueous solvent concentrations in biphasic systems. Relationships between biocatalyst activity and solvent $\log P$ typically show a sigmoidal relationship (**Figure 2.7**), where solvents with a $\log P$ above the inflection point are usually biocompatible and those below the inflection are toxic[257, 258, 261, 262]. Osborne and colleagues developed a model relating the $\log P$ at the inflection point to a critical concentration of solvent molecules partitioned into the cell membrane[263]. Unsurprisingly, given the diversity of microbial cell envelope structures and environments that microbes have evolved to tolerate, the critical $\log P$ differs between organisms but is usually around 4[257, 262]. Broadly, Gram-negative bacteria are typically more solvent-tolerant than Gram-positive bacteria, which is attributed to the presence of an outer membrane in Gram-negative organisms[251]. It is also important to consider that the $\log P$ rule does not take into account the molecular structure of the solvent, which ultimately dictates the toxic effects of solvent accumulation in the cell membrane. Using various groups of structurally similar solvents, Vermuë and colleagues demonstrated that different solvent classes have different critical $\log P$ values, with alcohols and alkylbenzenes having higher critical $\log P$ values than alkanes and ethers[257]. It is therefore important that the evaluation of solvent toxicity based on $\log P$ is accompanied by empirical testing before a solvent is selected for a biotransformation.

It is also important to note that many reported critical $\log P$ values for microorganisms are determined by evaluating cell growth or metabolic activity[257, 262, 264], whereas in a whole-cell biotransformation using recombinant enzymes, the biotransformation activity may be lost at higher or lower $\log P$ values. For reactions that are dependent on host cell metabolism, e.g. the supply of NADPH cofactor for cytochrome P450s, biotransformation activity would be expected to correlate with viability loss.

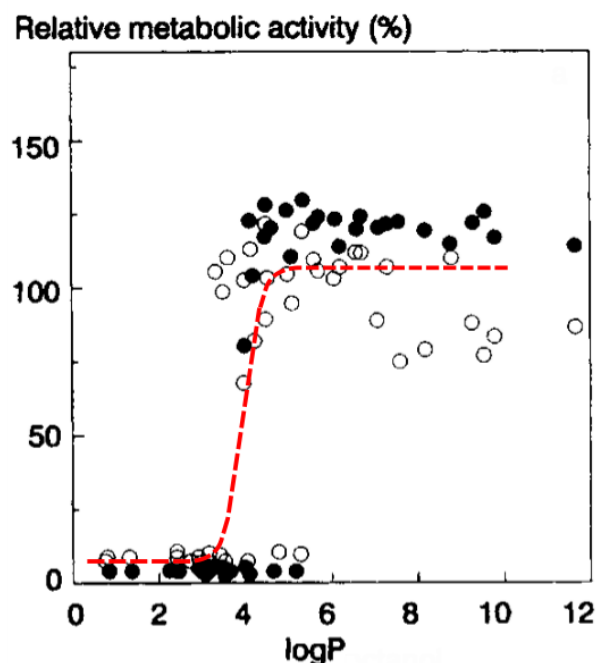


Figure 2.7. Relationship between metabolic activity and solvent $\log P$ for (●) *Arthrobacter* and (○) *Acinetobacter* species. Adapted from Vermuë *et al.* (1993) to show approximate sigmoidal curve (red).

For cofactor-independent reactions, or biotransformation systems that include a cofactor recycling partner, the reaction can continue well after cell death[217, 224, 265], providing enough membrane integrity is maintained to protect the enzyme and provide sufficient compartmentalisation of reaction components. Conversely, depending on the solvent sensitivity of the biotransformation enzyme, activity can be lost in solvents that are compatible with the growth of the host organism[263]. Another consideration regarding solvent choice is whether the solvent can act as a substrate for the enzyme, which is particularly important for biotransformations involving hydrocarbon substrates or where the enzyme shows broad substrate specificity[244], as solvent molecules entering the cytoplasm can compete with substrate for the enzyme active site and decrease the availability of cofactor for the desired reaction.

2.5.2.3. Solvent tolerance mechanisms in bacteria

While most biocompatible solvents typically have log *P* values over 4, typical biotransformation substrates, such as pharmaceuticals, often have lower hydrophobicity and therefore have suboptimal affinity with these solvents. Many studies have therefore focussed on the use of solvent-tolerant microbial strains as expression hosts for biotransformations performed in biphasic systems. Solvent-tolerant strains can either be engineered[43, 266], or wild-type strains that are naturally adapted to harsh environments can be isolated and applied[251, 267]. The most commonly employed wild-type strains are Gram-negative *Pseudomonas* sp.[254, 268-272], though some solvent-tolerant Gram-positive strains have also been used such as *Bacillus*[271, 273] and *Rhodococcus*[274] species.

Various mechanisms for solvent tolerance in bacteria exist, including changes in cell membrane composition, the active efflux of solvents, cell aggregation, and the catabolism of the solvent[275]. It is important to consider the potential impacts of solvent tolerant mechanisms on the biotransformation activity, particularly with respect to mass transfer of substrate into the cells. For example, a common mechanism of solvent tolerance in *Pseudomonas* sp. is the rigidification of the cell membrane to limit solvent partitioning into the phospholipid bilayer[276]. This adaptation may impact the permeability of cell membranes to hydrophobic substrates where passive diffusion across the membrane is the major mechanism of mass transfer. In one study, a comparison of several solvent-sensitive and solvent-resistant strains of *Pseudomonas putida* as hosts for CYP153A6-mediated biotransformation of limonene showed that all three solvent-tolerant strains had no biotransformation activity, whereas the solvent-sensitive strains showed good activity[254].

Solvent efflux pumps are a common feature of many solvent-resistant bacterial strains, particularly in *Pseudomonas* species[277], and these can also affect biotransformation activity when the substrate shares similar properties to the solvent used. For example, a study by Kuhn and colleagues using a solvent-tolerant *P. putida* strain for styrene epoxidation showed a significant loss in activity at high substrate concentrations despite the cells maintaining high viability, which was suspected to be due to active efflux of the substrate[278]. Another consideration for using strains with high efflux pump activity is that the high energy demands of active efflux may reduce the availability of NAD(P)H cofactor for the biotransformation. Some strains can significantly upregulate their metabolism to account for the increased energy demands of solvent resistance; for example, the *P. putida* strain DOT-T1E used for styrene epoxidation in an octanol biphasic system increased its NADPH-regenerating activity up to eight-fold in the presence of solvent. However, calculations showed that only 1% of the NADPH was utilised for styrene oxidation due to the high demands of cellular maintenance. The use of a more hydrophobic solvent, bis(2-ethylhexyl) phthalate (BEHP), allowed a three-fold higher rate of biotransformation due to increased cofactor availability[279].

2.5.2.4. Solvent-substrate compatibility

The solvent selected for a biphasic biotransformation system should not only be compatible with the chosen microbe but should also dissolve high amounts of the desired substrate to allow good volumetric productivity. Finding a solvent that satisfies both biocompatibility and high substrate solubility can often require extensive experimental screening and is made more difficult by the fact that many solvents that can potentially meet both requirements are not commonly used in typical laboratories. One approach to simplify solvent selection is the use of *in silico* solubility prediction using thermodynamic models, such as UNIFACS[280], NRTL[281] or Hansen parameters[282]. In this way, solvents that potentially meet biocompatibility requirements (i.e. based on log *P* or literature) can be evaluated computationally to predict solute-solvent compatibility and narrow down the number of solvents required for laboratory testing. Solubility prediction tools are widely employed for pharmaceutical applications but there are few examples of their use for biotransformation development[283, 284], despite the potential of this approach.

Partitioning of the substrate between the aqueous and organic phases is also an important factor for biotransformation productivity. As the substrate typically has a high affinity for the organic phase, the concentration of substrate partitioned into the aqueous phase is often well below that of saturation. While this is useful for applications where the substrate is toxic to the cells, a lower aqueous substrate

concentration can lead to a slower reaction rate[285, 286]. If the aim of implementing a biphasic system is to improve substrate loading, an evaluation should be made to ensure that the higher substrate loading offsets the slower reaction rate in terms of the achievable volumetric productivity. For example, Girhard and colleagues screened several water-immiscible solvents for the P450-mediated biotransformation of valencene, but the poor partitioning of substrate into the aqueous phase meant that none of the solvents improved the product yield compared to an aqueous single-phase system[287]. Alternatives to organic solvents for biphasic systems

2.5.2.5. Alternative second phases

Organic solvents are not the only option for the second phase in a biphasic biotransformation. In recent years, significant attention has been given to the use of water-immiscible ionic liquids[288, 289]. Ionic liquids are salts that exist in a liquid state at room temperature[290]. Many ionic liquids have been shown to be biocompatible with microbes[291-293] and these can offer several advantages compared to many common immiscible organic solvents. In particular, the polarity of ionic liquids means that they can dissolve more polar solutes compared to the hydrophobic organic solvents which are needed to maintain biocompatibility[294]. This is particularly useful in cases of host toxicity from polar substrates or products. Ionic liquids are, to some extent, also 'tunable' in that the solubility and partitioning of a substrate can be adjusted by, for example, varying the chain length of one of the ions[295]. Ionic liquids also offer safety advantages compared to some volatile organic solvents, and they often have superior environmental safety[296].

Aqueous two-phase systems have also been employed for whole-cell biotransformations[297]. In these systems, the two phases are aqueous solutions consisting of two incompatible polymers or polymer-salt solutions. These systems offer high biocompatibility but are typically only used for small polar solutes. A disadvantage of these systems is that the partitioning of substrates, products and cells is often difficult to predict[297, 298].

Adsorbing resins have also been used to supply substrate and sequester product in whole-cell biotransformations[213, 284, 299, 300]. For substrate supply, resins are loaded with substrate prior to the biotransformation and continuously partition free substrate into the aqueous phase as it is consumed by the reaction. These systems are often employed to reduce free aqueous substrate concentrations in cases of substrate toxicity[40]. In some cases, the cells may show an affinity to the resin[300],

necessitating the use of a specialised reactor setup where cells and resin are separated by a membrane or external column.

2.5.3. Increasing aqueous substrate solubility

An alternative to biphasic biotransformations for improving productivity with poorly water-soluble substrates is to enhance the aqueous solubility of the substrate using various aqueous modifiers. This has the advantage of driving higher reaction rates than can be achieved at saturating substrate concentrations in an unmodified aqueous solution or with a biphasic system. One approach is to modify the aqueous phase composition by adding a water-miscible polar organic solvent, such as ethanol or dimethylsulfoxide (DMSO). This method is limited by the toxicity of the solvent towards the microbial host and typically only small fractions (<10%) can be used. While some drug substrates can show reasonable solubility enhancement in this range, often only modest improvements can be achieved within the range of sub-toxic co-solvent concentrations [221, 301]. In a study by Zehentgruber and colleagues, a screen of 11 co-solvents to improve the solubility of progesterone for biotransformation by CYP21-expressing *Schizosaccharomyces pombe* showed that all of the solvents reduced biotransformation activity when used at a concentration of 10% (v/v) compared to an unmodified aqueous solution, likely due to solvent toxicity towards the host. By reducing the co-solvent concentration to 5%, only a 1.1-fold improvement in biotransformation activity could be achieved due to poor substrate solubility at this concentration[226].

A more effective approach is the use of cyclodextrins and their derivatives, which are often employed during pharmaceutical manufacture to enhance aqueous drug solubility and as excipients in drug formulations to improve biological absorption[302]. Cyclodextrins are cyclic rings of glucose subunits that are arranged so that the exterior of the ring is more hydrophilic than the interior[303]. Hydrophobic drugs can often form 'inclusion complexes' with cyclodextrins where a hydrophobic part of the drug molecule is contained inside the cyclodextrin ring, shielding it from water and decreasing the apparent hydrophobicity of the drug[304]. There are various commercial cyclodextrins available that differ in size and have functional group substitutions that can further enhance the solubility of the cyclodextrin itself or form interactions with drug compounds to improve the efficiency of complexation.

A common application of cyclodextrins is for steroid biotransformations[225, 286, 305, 306]. Many steroids have very poor water solubility, which is typically the major factor limiting product yields[307]. The use of a 20% solution of (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD) for the biotransformation of cortisone acetate by *Arthrobacter simplex* allowed a 190-fold improvement in substrate solubility, which

was more than 30-fold higher than the improvements gained by the use of co-solvents ethanol and dimethylformamide at the same concentration[308]. Importantly, at this concentration the HP- β -CD showed little toxicity towards the host cells, whereas the co-solvents tested were highly toxic. In another study, the use of HP- β -CD for the production of androstadienedione from progesterone by *Bacillus sphaericus* provided a 35-fold improvement in productivity due to the rate enhancement afforded by higher aqueous substrate concentrations[309]. Cyclodextrins have also been successfully used to enhance the biotransformation of non-steroidal substrates, such as the P450-mediated conversion of alkanes[268] and terpenes[310], suggesting this approach may have broad applicability to a range of hydrophobic substrates.

Cyclodextrins typically show good biocompatibility with microorganisms at the concentrations needed for a reasonable enhancement of substrate solubility. A significant decline in growth rate was observed for *Arthrobacter simplex* at HP- β -CD concentrations above 10%, however, and higher concentrations were shown to cause membrane damage[311]. Some specific interactions between certain cyclodextrin derivatives and microbial species can also result in higher toxicity. For example, methyl- β -cyclodextrin shows strong lytic activity against *Bacillus* species but not other Gram-positive bacteria, which is thought to be due to the extraction of *Bacillus* membrane lipids by this cyclodextrin derivative. Several cyclodextrins also showed high toxicity to the fission yeast *Scizosaccharomyces pombe*[226] but good compatibility with *Saccharomyces cerevisiae*[312]. These studies indicate that several cyclodextrin variants may need to be evaluated experimentally depending on the chosen host strain.

Hydrophilic polymers, such as polyvinylpyrrolidone (PVP) and hydroxypropylmethylcellulose (HPMC), are also common additives to pharmaceutical formulations to enhance the solubility and dissolution of hydrophobic drugs[313]. Small amounts of these polymers can also work synergistically with cyclodextrins to enhance drug solubility[314]. The use of hydrophilic polymers as solubility-enhancing agents in whole-cell biotransformations, however, is considerably unexplored. Only recently, Lee and colleagues investigated the use of several polymers to enhance the solubility of dadzein for whole-cell biotransformation by recombinant *E. coli*. A 5% solution of PVP provided a 44-fold improvement in substrate solubility with little toxicity to the host cells, allowing a significant improvement in biotransformation productivity. This study indicates the potential of using hydrophilic polymers to improve biotransformation productivity with other drug-like substrates.

2.6. Cofactor supply for whole-cell biotransformations

2.6.1. Background

A significant advantage of using whole-cell systems compared to isolated enzymes is that reaction cofactors, such as NADPH and NADH, are biosynthesised and regenerated by the host cell metabolism. Whether or not the supply is sufficient to maintain high reaction rates throughout the biotransformation depends on a range of factors such as:

- (i) the coupling efficiency and kinetic parameters of the enzyme;
- (ii) the metabolic activity of the host cells; and
- (iii) the magnitude of other process limitations such as mass transfer of substrate.

For some biotransformations, examination of the NADPH supply during process debottlenecking reveals sufficient cofactor levels are maintained throughout the process[218, 226, 286, 315], while others benefit from a cofactor regeneration strategy, which are discussed here.

2.6.2. Cofactor recycling

A simple and effective way to enhance cofactor supply is by coupling the NAD(P)H-oxidising enzyme with an NAD(P)⁺-reducing enzyme which is co-expressed within the same cell[316]. Ideally, the coupled enzyme relies on a cheap, readily available substrate and has kinetic properties that allow NAD(P)H regeneration to occur at a rate comparable to the maximal NAD(P)H turnover rate of the biotransformation enzyme. In this way, apart from the initial cofactor pools required at the start of the biotransformation, the reaction is independent of host cell metabolism and can be sustained after cell death, which is useful if toxic solvents or permeabilisation strategies are used[217, 224, 265]. The most commonly employed regeneration enzymes for this purpose are:

- (i) glucose dehydrogenases (GDH) or alcohol dehydrogenases (ADH) for NADPH; or
- (ii) glycerol dehydrogenase (GlcDH) or formate dehydrogenase (FDH) for NADH[317].

The improvements gained by these systems vary significantly depending on the degree of rate limitation caused by insufficient cofactor supply. The co-expression of GDH with P450_{SMO} from *Rhodococcus* sp. in *E. coli* provided a six-fold improvement in product yields[318]. A recombinant *Bacillus subtilis* system containing over-expressed GDH with a P450_{BM3} mutant provided a 1.4-fold increase in yields[319], and an *E. coli* system using permeabilised cells containing GDH and a cyclosporine-hydroxylating P450 demonstrated a 1.2-fold improvement[217]. As the co-expression of a second enzyme can result in

reduced expression levels of the enzyme of interest[244], Zhang and colleagues developed an *E. coli* system whereby GDH and the biotransformation enzyme were expressed in separate strains, which were permeabilised and mixed prior to biotransformation[265].

One concern with using GDH is that the majority of glucose entering *E. coli* is phosphorylated to glucose-6-phosphate by the phosphoenolpyruvate-dependent phosphotransferase system (PTS)[320], so intracellular concentrations of unphosphorylated glucose can be limiting. Schewe and colleagues addressed this issue by the co-expression of a glucose facilitator protein from *Zymomonas mobilis*, which provided a nine-fold improvement in initial reaction rates[253]. In a later study using bioreactor cultures, however, the system did not offer any improvements in yields due to lower expression levels of P450 enzyme[244].

A two-fold improvement in product yield was achieved by König and colleagues using a *Bacillus megaterium* system containing ADH from *Lactobacillus brevis* and a P450 enzyme for cortisone production[321]. In this system, the ADH produced NADPH from the reduction of exogenously supplied acetone. For another *B. megaterium* system containing ADH and the P450 enzyme CYP105A1, no improvement was gained from the cofactor recycling system[220]. In another study, Tieves and colleagues were able to improve the product enantiopurity of a P450-mediated alkane hydroxylation using *Pseudomonas putida* by exploiting the stereospecificity of ADH from *L. brevis*[268]. The initial system without cofactor recycling produced (*S*)-2-octanol with 87% enantiomeric excess (*ee*). The ADH enzyme produced NADPH from the selective reduction of the unwanted (*R*) enantiomer. While the overall yield was not improved, the enantiopurity of the product was increased to 99% *ee*[268].

For P450s that are dependent on NADH instead of NADPH, co-expression of glycerol dehydrogenase (GlcDH) is often used for cofactor recycling. For a biotransformation for camphor hydroxylation by P450cam, a 25-fold improvement in reaction rate was observed with this recycling system in *Pseudomonas putida*[285]. However, in another application using CYP153A6 in *E. coli* for octane hydroxylation, co-expression of GlcDH significantly reduced the overall performance of the system[224]. It was suggested that the excess of intracellular NADH encouraged the generation of reactive oxygen species (ROS) by CYP153A6 in the absence of substrate.

2.6.3. Metabolic engineering

Other strategies to improve NADPH supply include the use of metabolic engineering to redirect carbon flux towards NADPH production[41]. In aerobic organisms, a major source of NADPH is the pentose

phosphate (PP) pathway[322]. This pathway produces two moles of NADPH per mole of glucose-6-phosphate entering the pathway, and further NADPH can be produced by PP pathway end-products re-entering the cycle or by downstream metabolism in the tricarboxylic acid (TCA) cycle (**Figure 2.8**). The yield of NADPH produced per mole of glucose is therefore higher than what can be achieved using a cofactor recycling system with an over-expressed dehydrogenase, which produces one mole of NADPH per glucose. Not all glucose-derived flux enters the PP pathway, however, as glucose-6-phosphate (G6P) can also proceed down the Embden-Meyerhof-Parnas (EMP) pathway for glycolysis[323]. Glucose-6-phosphate isomerase, encoded by the *pgi* gene, is the first enzyme in the EMP pathway (**Figure 2.8**). Inactivation of *pgi* forces all G6P to proceed down the PP pathway and this strategy has been used by several groups to improve the cofactor supply for NADPH-dependent biotransformations[41], as summarised in **Table 2.2**.

Table 2.2. Examples of using a *pgi* gene knockout strategy to improve NADPH supply for biotransformations. ‘Product-per-glucose ratio’ refers to moles of product formed per mole of glucose consumed.

Biotransformation	Enzyme	Result	Ref.
dihydroquercetin to leucocyanidin	dihydroflavonol 4-reductase	3.8-fold improvement in product yield	[324]
propane to propanol	P450 _{BM3} mutant	2.6-fold improvement in product-per-glucose ratio	[325]
methyl acetoacetate to (<i>R</i>)-methyl 3-hydroxybutyrate	alcohol dehydrogenase	1.6-fold improvement in product-per-glucose ratio	[326]
xylose to xylitol	xylose reductase	1.2-fold improvement in product-per-glucose ratio	[327]
thymidine biosynthesis	native pathway	4-fold improvement in product yield	[328]

While the knockout of *pgi* can improve the efficiency of glucose utilisation for product formation, the mutant strains often suffer from severe growth defects. Inactivation of *pgi* was shown by one group to cause a 5.5-fold reduction in growth rate and a four-fold reduction in glucose consumption when grown on minimal medium[329], and similar results have been observed in other studies[324, 330, 331]. It is believed that this effect is largely due to a redox imbalance caused by an oversupply of NADPH, which the cells have limited capacity to oxidise back to NADP⁺. This hypothesis is supported by the observation that over-expression of the soluble transhydrogenase UdhA from *E. coli*, which catalyses the direct electron transfer from NADPH to NAD⁺, could partially restore growth defects in a *pgi* knockout strain, presumably by reducing the level of intracellular NADPH[331]. In some cases, the activity of the NADPH-dependent

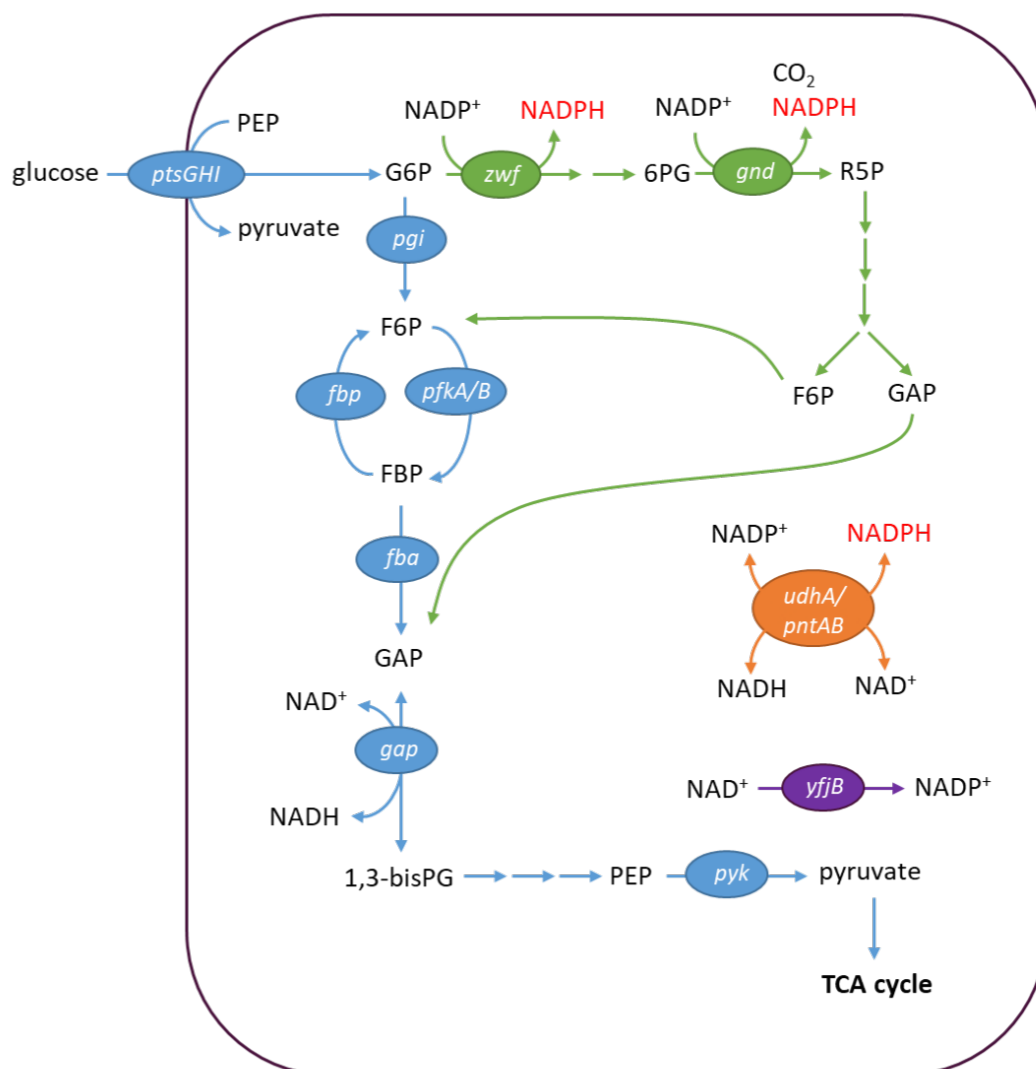


Figure 2.8. Selected metabolic reactions involved in NADPH regeneration in *E. coli*. Embden-Meyerhof-Parnas (EMP) pathway is shown in blue; pentose phosphate (PP) pathway is green; nicotinamide transhydrogenases are orange; NAD kinase is purple. Enzymes corresponding to gene names are as follows: *ptsGHI*, phosphoenolpyruvate-dependent phosphotransferase system; *pgi*, glucose-6-phosphate isomerase; *pfkA*, phosphofructokinase I; *pfkB*, phosphofructokinase II; *fbp*, fructose-1,6-bisphosphatase; *fba*, fructose-1,6-bisphosphate aldolase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pyk*, pyruvate kinase; *zwf*, glucose-6-phosphate 1-dehydrogenase; *gnd*, 6-phosphogluconate dehydrogenase; *udhA*, soluble pyridine nucleotide transhydrogenase; *pntAB*, membrane-bound nucleotide transhydrogenase system; *yjfB*, NAD kinase. Metabolite names are as follows: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; 1,3-bisPG, 1,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; 6PG, 6-phosphogluconate.

biotransformation enzyme is sufficient to partially restore growth rates by the same mechanism[327, 330]. For the biotransformation of dihydroquercetin to leucocyanidin in a *pgi* knockout strain, for example, an 11.4-fold reduction in growth rate was observed relative to a control strain but this was only 6.1-fold in the presence of the biotransformation enzyme[324].

Another target for genetic inactivation is 6-phosphofructokinase[41], which is the second enzyme in the EMP pathway (*pfkA/B*; **Figure 2.8**). Fructose-6-phosphate (F6P) is one of the end-products of the PP pathway, and the inactivation of 6-phosphofructokinase forces F6P to undergo isomerisation to G6P and re-enter the PP pathway instead of proceeding down the EMP pathway. This partial cyclisation of the PP pathway provides a theoretical maximum of six moles of NADPH produced per glucose consumed[322]. *Escherichia coli* contains a major 6-phosphofructokinase (encoded by *pfkA*), which accounts for 90-95% of total 6-phosphofructokinase activity[332], as well as a minor 6-phosphofructokinase (*pfkB*). Inactivation of *pfkA* alone provided a two-fold improvement in product-per-glucose ratio for methyl 3-hydroxybutyrate reduction, while inactivation of both *pfkA* and *pfkB* provided a 2.2-fold increase[326]. Inactivation of both enzymes, however, resulted in barely detectable growth rates on minimal medium[329]. The same research group inactivated 6-phosphofructokinase in *Corynebacterium glutamicum* for the same biotransformation and achieved a 1.5-fold improvement in yields, though the resulting strain was unable to grow on glucose on minimal medium and required complex media components for growth[333]. The *pfkA* gene was also inactivated in *E. coli* for the biotransformation of xylose to xylitol and provided a 1.5-fold increase in product-per-glucose ratio[334]. Similar to the *pgi* deletion, the observed growth defects from *pfkA* could be somewhat alleviated by the expression of xylose reductase for the biotransformation.

Another approach to the cyclisation of the PP pathway was used by Siedler and colleagues in *C. glutamicum*[333]. Glyceraldehyde 3-phosphate dehydrogenase (encoded by *gap*) catalyses the oxidative phosphorylation of glyceraldehyde 3-phosphate (GAP), which along with F6P is another end-product of the PP pathway (**Figure 2.8**). As GAP is a downstream metabolite of F6P in the EMP pathway, inactivation of *gap* prevents flux from either PP end-product from proceeding down the EMP pathway to the TCA cycle and theoretically allows complete cyclisation of the PP pathway[322, 333]. The inactivation of *gap* provided a 2.9-fold improvement of the product-per-glucose ratio but the glucose consumption rate of this strain was decreased by 7.8-fold, resulting in a 2.6-fold lower rate of product formation. One consequence of *gap* inactivation is that no phosphoenolpyruvate (PEP) is produced by the EMP pathway, which is required for the PTS-dependent uptake and phosphorylation of extracellular glucose[320]. In this strain, G6P could only be derived from PTS-independent glucose uptake and phosphorylation by less active pathways[335], resulting in poor glucose uptake and growth rates.

A different strategy targeted the *gap* gene in *E. coli* and used gene replacement to improve NADPH levels. The native *E. coli* glyceraldehyde 3-phosphate dehydrogenase enzyme reduces NAD^+ to NADH. In work

by Martinez and colleagues, the native *gap* gene was inactivated and replaced by an NADP⁺-dependent *gap* gene from *Clostridium acetobutylicum*, so that one mole of NADPH was produced per mole of glucose-6-phosphate entering the EMP pathway[336]. This approach allowed a 2.5-fold enhancement of product titre for lycopene biosynthesis and a 1.7-fold improvement of the product-per-glucose ratio for the biotransformation of cyclohexanone to ϵ -caprolactone by cyclohexanone monooxygenase.

The overexpression of the first enzyme in the PP pathway, glucose 6-phosphate 1-dehydrogenase (G6PDH; encoded by *zwf*), has also been used to enhance NADPH production. For the production of poly(3-hydroxybutyrate) (PHB) by recombinant *E. coli*, overexpression of G6PDH provided a 1.4-fold improvement in overall product titres[337]. A similar improvement in product yields was achieved using this approach for the biotransformation of cyclohexanone to ϵ -caprolactone[338]. A 1.5-fold improvement in guanosine 5'-diphosphate-L-fucose yield was also gained by this method[339]. In the same study, overexpression of the TCA cycle enzyme isocitrate dehydrogenase, the other major source of NADPH in *E. coli* alongside the PP pathway[340], provided a 1.3-fold improvement in yield[339].

Other approaches to enhance NADPH supply have targeted nicotinamide transhydrogenases[341]. The soluble transhydrogenase in *E. coli* (encoded by *udhA*) catalyses the reversible transfer of electrons from NADH to NADP⁺, forming NAD⁺ and NADPH (**Figure 2.8**). Overexpression of UdhA provided a 1.8-fold increase in PHB production in *E. coli*[342]. In another study, the overexpression of UdhA provided a two-fold improvement in product-per-glucose ratio for the product of thymidine[328]. When combined with the disruption of *pgi*, the product-per-glucose ratio was enhanced by eight-fold compared to a wild-type control. Overexpression of the other major nicotinamide transhydrogenase in *E. coli*, the membrane-bound transhydrogenase encoded by the *pntAB* operon, has also been used to improve biotransformation yields. The use of this system provided a 3.5-fold increase in overall yield for the reduction of acetophenone to (*R*)-phenylethanol[343]. Overexpression of *pntAB* in *E. coli* also provided a 1.3-fold increase in product titre for an enzyme cascade biotransformation of malonyl-CoA to 3-hydroxypropionic acid[344]. This approach was similarly used to improve fermentative production of lysine in *C. glutamicum*, which lacks a native nicotinamide transhydrogenase, and improved overall product yields by 1.1- to 3-fold depending on the carbon source used[345].

A different approach was taken by Fasan and colleagues who used metabolic engineering to increase transhydrogenase activity without overexpression of *PntAB* or *UdhA*[325]. Instead, gene deletions were used to increase intracellular NADH to drive transhydrogenase-catalysed reduction of NADP⁺. First, two NADH dehydrogenases in the *E. coli* respiratory chain were deleted. Instead of improving

biotransformation productivity, this had the unintended effect of increasing fermentative carbon flux toward ethanol and lactate. To combat this, genes encoding the NADH-dependent enzymes alcohol dehydrogenase (*adh*) and lactate dehydrogenase (*ldh*) were also disrupted. This resulted in a 3.3-fold improvement in product-per-glucose ratio for the oxidation of propane by a P450_{BM3} mutant. Another key advantage of this approach was that the glucose consumption of the mutant was not significantly affected, in contrast to the use of a *pgi* knockout in the same study, which showed a significantly lower biotransformation rate due to slower glucose utilisation[325].

While most the approaches described above aim to enhance the rate of reduction of NADP⁺ to NADPH and increase the intracellular [NADPH]/[NADP⁺] ratio, other strategies have focused on increasing the total NADPH/NADP⁺ pool. The *E. coli* NAD kinase (encoded by *yjfB*) catalyses the phosphorylation of NAD⁺ to NADP⁺[346] (**Figure 2.8**), and its overexpression in *E. coli* provided a two-fold enhancement in PHB biosynthesis and a similar increase in the product-per-glucose ratio[347]. This system also improved thymidine production by 1.3-fold and provided a total 4.8-fold improvement in yield when combined with *pgi* inactivation[328]. A similar approach used the NADH kinase from *Saccharomyces cerevisiae* which produces NADPH directly from NADH, unlike the *E. coli* NAD kinase which produces NADP⁺. This system proved a 1.5-fold improvement in GDP-L-fucose production and a two-fold yield enhancement for the biotransformation of cyclohexanone to ϵ -caprolactone in *E. coli*[348].

Many of the metabolic engineering approaches described above are effective in improving biotransformation efficiency in terms of the amount of product formed per glucose consumed. While this can reduce costs at process scale by decreasing the amount of glucose required, it does not necessarily translate to higher productivity or yield due to the microbial growth defects that are often associated with modifying central metabolic pathways. The use of more complex metabolic engineering strategies to regulate central carbon metabolism may be more effective. For example, the recently developed CRISPR interference (CRISPRi) system[349] is able to down-regulate gene expression in a dynamic and reversible manner, which may be useful for avoiding the long growth times associated with permanent inactivation of target genes in the central carbon metabolism pathways.

2.7. Microbial strains for whole-cell biotransformations

2.7.1. Considerations for choice of microbial strain

The microbial host strain selected for a whole-cell biotransformation using P450s or other monooxygenase enzymes can influence the productivity of the system in several ways. These include recombinant enzyme stability, redox homeostasis, and tolerance to toxic substrates and solvents. The most widely used expression host for biotransformations is *Escherichia coli*[36], which is unsurprising given the status of *E. coli* as a model organism, its ubiquitous use as a protein expression host across the life sciences, the wide array of commercially available specialty strains and expression vectors, and the detailed information available on its metabolism and growth characteristics.

A specific appeal of *E. coli* as a biotransformation host is the availability of vectors for high protein expression, such as the T7 phage RNA polymerase system[350]. It is important to note, however, that in many biotransformations with monooxygenase enzymes, other productivity limitations, such as mass transfer of substrate and electron transfer between redox partners, often have a greater capacity to limit the overall rate of biotransformation than the level of enzyme expression. In many instances, significantly lower intracellular enzyme concentrations do not correlate with a decrease in productivity[213, 216, 223, 224, 268, 351]. For example, a constant rate of cyclohexanone oxidation was observed for an *E. coli* whole-cell biotransformation over an 11 h period, despite an 80% loss in active enzyme concentration[315]. In some cases, high expression levels can even correlate with reduced productivity[246, 352, 353], possibly due to excessive production of reactive oxygen species.

There are no native cytochrome P450 enzymes in *E. coli*, which is advantageous in that there is a lower possibility of side-reactions of drug-like substrates with endogenous enzymes. However, this also means that the biosynthesis of haem for incorporation into heterologous P450s can be limiting and expression cultures are often supplemented with the expensive haem precursor δ -aminolevulinic acid[354]. Another consequence of the lack of native P450 enzymes is that the intracellular redox environment of *E. coli* can be less supportive of high oxygenase activity compared to organisms with multiple endogenous P450s that produce a broad range of secondary metabolites. For example, comparison of redox states between *E. coli* and *Streptomyces avermitilis* during the P450-mediated biotransformation of dadzein showed a nearly 30-fold higher [NADPH]/[NADP⁺] ratio in *S. avermitilis*, which resulted in an eight-fold higher product titre[355]. However, yield loss due to unwanted reactions with other enzymes was observed in the *S. avermitilis* system. A similar observation was also made for steroid biotransformation using a P450_{BM3} mutant in *Rhodococcus erythropolis*[356].

Overexpression of a biotransformation enzyme in its native host can offer advantages compared to heterologous expression in a non-native host. The three-component alkane monooxygenase of *Pseudomonas putida* was shown to have five- to six-fold lower activity when expressed in *E. coli* compared to *P. putida*, despite the *E. coli* system producing up to 10-fold more enzyme[357]. This was thought to result from improper interactions of one enzyme component with the *E. coli* membrane, insufficient incorporation of iron into the other two components and overall reduced stability of the enzyme system in the *E. coli* intracellular environment. When using a native host, it is important to consider the metabolic pathway in which the enzyme of interest is involved, as downstream enzymes may further metabolise the biotransformation product. For example, the styrene monooxygenase (StyAB) of *Pseudomonas* sp. forms part of the *styABCD* operon, which converts styrene to phenylacetic acid. A genetic knockout of *styC* is required for the efficient production of styrene oxide using *Pseudomonas* hosts[278].

Many drug-metabolising P450s are derived from eukaryotic hosts, in which they are natively bound to the endoplasmic reticulum. Often, these enzymes cannot be functionally expressed in bacterial hosts, which lack intracellular membranes. While enhanced solubility of eukaryotic P450s can be achieved by removing the hydrophobic membrane anchors of these enzymes, this truncation can often have negative impacts on enzyme activity[144]. Yeast hosts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are therefore often employed for biotransformations using mammalian P450s, despite these systems typically offering lower levels of recombinant protein expression than bacteria[226, 268, 279, 358, 359].

Another important consideration for the selection of a biotransformation host is the nature of the substrate and product. For toxic or hydrophobic substrates and products that may require a biphasic biotransformation strategy, solvent-tolerant strains of *Pseudomonas putida* are popular expression hosts[254, 268-272, 278, 279]. For steroids, mycobacterial species have long been the hosts of choice due to their dedicated uptake systems and metabolic pathways for these compounds[307]. An interesting application of a host strain was made by Braun and colleagues for steroid biotransformations using recombinant human P450s[360]. The lipotropic yeast *Yarrowia lipolytica* is known to grow on lipid droplets and directly import hydrophobic substrates into the endoplasmic reticulum (ER)[361]. The use of ethyl oleate, which can be utilised by *Y. lipolytica* for growth, as a solvent for a biphasic steroid biotransformation allowed higher activity and a significantly sustained period of productive biotransformation activity compared to an aqueous single-phase control[360]. This was possibly due to the import of biotransformation substrate through the ER to the ER-bound mammalian P450s.

2.7.2. *Bacillus megaterium*

2.7.2.1. Background

The Gram-positive organism *Bacillus megaterium* has long been an industrially important microbe[362, 363] but has only recently received attention as a host for whole-cell biotransformations with recombinant enzymes. This organism has many suitable traits for biotechnological applications. Its natural presence in a vast array of habitats is reflected by the ability of *B. megaterium* to utilise a broad array of different compounds as sole carbon sources. As a Gram-positive species, *B. megaterium* does not produce endotoxin, and the lack of an outer membrane allows the secretion of recombinant proteins to facilitate downstream purification[364]. The naturally low alkaline protease activity of *B. megaterium* contributes to the stability of recombinantly expressed proteins in this organism[363]. Industrially, *B. megaterium* is utilised for the production of vitamin B₁₂[365] and vitamin C[366]. It is also used as a source of industrially important enzymes including various amylases[367], glucose dehydrogenase[368], and penicillin acylase[369].

One appeal of *B. megaterium* for biotechnological applications is its remarkably high plasmid stability[370]. Most strains of *B. megaterium* naturally carry at least four plasmids[371], and some strains have been identified which carry ten[372]. Large plasmids up to 165 kb have also been identified in some strains[371]. Many native plasmids from this organism have been shown to have very similar replication proteins and recognition sequences, which in other species would typically cause plasmid incompatibility[373, 374]. A naturally plasmid-less *B. megaterium* strain, DSM-319, was identified in 1989[375] and is commonly employed in biotechnological applications to simplify cloning procedures.

The first complete *B. megaterium* genome sequence was published in 2011[376] and currently the genomes of four strains are available, including DSM-319[376-378]. The genomic information has so far allowed the identification of several novel cytochrome P450 enzymes[379-381] and the characterisation of a carotenoid biosynthesis operon[382], as well as the use of DNA microarrays for the analysis of global gene regulation[383]. The sequence information available also allows codon optimisation[384] to be applied to heterologous genes for recombinant expression in *B. megaterium*[385].

A range of modern systems biology approaches have been used to further develop the potential of *B. megaterium* for industrial-scale protein production, including transcriptomics[386], proteomics[387, 388], metabolomics[389] and flux analysis[390-392]. These methods have been applied to examine *B. megaterium* during the production of both intracellular and secreted proteins, including industrially important proteins such as antibodies[393-395] and glycosyltransferases[387]. Another study

investigated the influence of fluctuations in substrate and dissolved oxygen concentration on protein production to develop strategies for high-cell-density cultures[396]. These studies have allowed the development of nutrient feeding strategies and optimised culture conditions to maximise the growth and protein production of *B. megaterium* at industrial scale.

2.7.2.2. Recombinant protein expression systems

The most widely used system for inducible protein expression in *B. megaterium* is based on the native xylose promoter and repressor[363]. The xylose operon of *B. megaterium* was first characterised by Rygus and Hillen[397] and the promoter elements were transferred to a plasmid to drive heterologous gene expression[398]. In later work, Stammen and colleagues systematically engineered the -35 and -10 elements of the promoter, as well as the ribosome binding site, to improve the expression of green fluorescent protein (GFP) by 18-fold[364]. In addition to the optimised promoter, this group also developed signal peptide tags to allow extracellular protein secretion. This work led to the commercial availability of a range of *B. megaterium* expression vectors[399].

Several other inducible expression systems have been described for *B. megaterium* but have not found widespread use. Biedendieck *et al.* characterised the *B. megaterium* sucrose-inducible promoter, which could provide comparable protein expression levels to the native xylose promoter but also showed considerable basal expression in the absence of sucrose[400]. The T7 phage RNA polymerase system, which allows strong and tightly controlled protein expression in *E. coli*, was implemented in *B. megaterium* but the system was limited by the poor stability of the T7 RNA polymerase in this organism[401]. Further development using the K1E and SP6 phage RNA polymerases showed some improvement but did not allow higher GFP titres than the optimised xylose promoter system[402]. Recently, a synthetic cumate-inducible expression system was developed that showed high expression and tight control in *B. megaterium*[403], which is likely to be a useful alternative to xylose expression systems for future biotechnological applications with this organism.

Very recently, in 2019, Hartz and colleagues used microarray analysis to identify and characterise 15 constitutive promoters in *B. megaterium*, several of which showed comparable or higher expression levels than the optimised xylose promoter[383] and may be useful for the development of synthetic inducible expression systems. Additionally, four sugar-inducible promoters were characterised. Of these, the native arabinose promoter/repressor system showed comparable expression to the optimised xylose

promoter but with less ‘leaky’ expression in the absence of inducer[271], suggesting this is a sound alternative to xylose-inducible systems.

2.7.2.3. Genetic engineering

Genetic engineering has been used to produce several strains of *B. megaterium* with industrially relevant features. These include:

- (i) a strain deficient in extracellular proteases[404] to prevent degradation of secreted proteins;
- (ii) a sporulation-deficient strain[405];
- (iii) a UV-sensitive strain[406]; and
- (iv) a strain engineered to be deficient in xylose utilisation[407], to avoid consumption of the inducer compound during recombinant protein production with xylose-inducible systems.

The disruption, deletion and editing of genes in *B. megaterium* is somewhat more time-consuming compared to some other biotechnologically important Gram-positive organisms like *B. subtilis*[408]. This is due to the relatively poor transformation efficiency of *B. megaterium*[409]. In *B. subtilis*, for example, the high genetic competency of this organism means that a ‘scar-less’ gene deletion involves a few simple steps:

1. A linear DNA fragment flanked by regions homologous to the genomic region of interest is transformed into *B. subtilis* protoplasts and is inserted into the genome by double-crossover homologous recombination[410].
2. Colonies containing the desired insertion are then selected using an antibiotic resistance gene contained in the inserted fragment.
3. Another crossover event using a third homology region on the inserted fragment results in excision of the fragment along with the target gene for deletion. This event can be selected for by screening for loss of antibiotic resistance or, more efficiently, the loss of a ‘suicide gene’ that only permits the desired recombinants to grow[411].

Because homologous recombination events are rare, the relatively low uptake of DNA during the transformation of *B. megaterium* means that transformation with a linearised or replication-defective plasmid does not usually result in genomic integration[412]. Instead, an additional step is required[412, 413] that usually relies on the use of a temperature-sensitive origin of replication[414] contained on the insertion plasmid. A scar-less gene deletion process in *B. megaterium* (**Figure 2.9**) typically proceeds as follows:

1. Protoplasts of *Bacillus megaterium* are transformed with a freely replicating plasmid containing homology regions to the insertion site as well as the temperature-sensitive origin of replication (*ori^{ts}*; **Figure 2.9**). Transformed colonies are selected by antibiotic resistance.
2. Selected colonies are then re-streaked and incubated at 42°C, a temperature at which the plasmid is unable to replicate. The antibiotic-resistant colonies appearing after this incubation result from ‘Campbell-like’ integration[415] of the entire plasmid at one of the homology regions.
3. Selection of a second crossover event to complete the gene deletion then proceeds as described above for *B. subtilis*.

Due to the rarity of homologous recombination events, selection of a second crossover event by screening colonies for loss of antibiotic resistance can be tedious. Suicide genes like the MazF toxin from *E. coli* have effectively been used in *B. subtilis* and related species as an efficient counter-screening marker[416], providing a more efficient approach. In *B. megaterium*, the *upp* counterselection marker has also been successfully employed[42]. The *upp* gene encodes an enzyme that metabolises 5-fluorouracil to a toxic compound that prevents cell growth[417]. A crossover event that excises the inserted *upp* gene from the host genome thereby allows recombinant colonies to grow on media supplemented with 5-fluorouracil. The *upp* gene is ubiquitous in microbes, however, so the use of this selection method first requires the production of a *upp* knockout strain by some other method[417].

2.7.2.4. Whole-cell biotransformation

Whilst several unmodified, wild-type strains of *B. megaterium* have previously been used for microbial biotransformations[273, 418, 419], it is only recently that this organism has been used as a host for whole-cell biotransformations with recombinantly expressed enzymes. The work in this area has so far been performed almost exclusively by the group of Rita Bernhardt and has utilised several P450s[218, 219, 229, 286, 351, 420-424], as well as dehydrogenases[42, 321], on a diverse range of substrates.

It is important to note that the commonly used laboratory strains of *B. megaterium* contain four native P450s, and there is therefore some potential for unwanted oxidation of a chosen biotransformation substrate by these endogenous enzymes. These enzymes include the well-studied, self-sufficient P450_{BM3} (CYP102A1), which hydroxylates fatty acids[170], as well as three P450s (CYP106A1[379], CYP109E1[380], CYP109A2[381]) that can hydroxylate various steroidal compounds.

The enzyme CYP106A2, which is found in *B. megaterium* strain ATCC 13368[425] but not laboratory strains such as DSM-319, was the first P450 to be used for a recombinant whole-cell biotransformation system

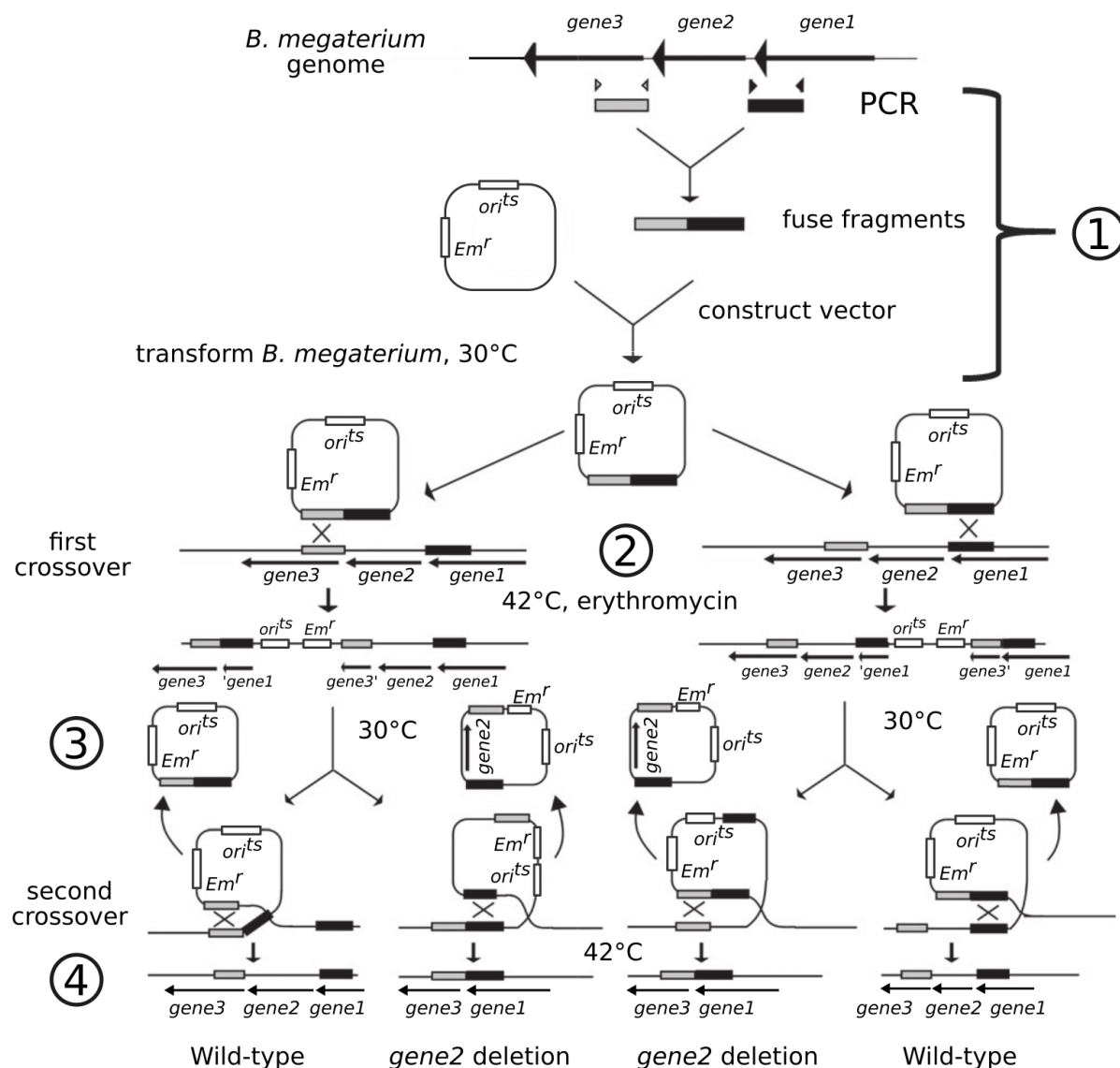


Figure 2.9. ‘Scar-less’ gene deletion in *B. megaterium*. (1) Regions flanking the target gene are cloned by PCR, fused and inserted into a plasmid containing a temperature sensitive origin of replication (*ori^{ts}*) and antibiotic resistance gene (*Em^r*). The plasmid is transformed into *B. megaterium* and colonies are selected by antibiotic resistance. (2) Colonies are re-streaked and incubated with antibiotic at 42°C, a temperature at which the plasmid cannot replicate. Antibiotic-resistant colonies result from Campbell-like integration of the plasmid at either the downstream (left-hand side) or upstream (right-hand side) homology region of the target gene. (3) Colonies are grown at 30°C without antibiotic to allow a second crossover event to occur. Depending on which of the two homology regions undergoes a crossover, this results in either a wild-type revertant or a gene deletion strain. (4) The excised plasmid is cured by growth at 42°C and double-crossover strains are selected by screening for loss of antibiotic resistance or with some other counter-selectable marker. Figure adapted from Biedendieck *et al.* (2011).

with *B. megaterium*[218]. An earlier compound library screen[219] identified the anti-inflammatory triterpene 11-keto- β -boswellic acid as a substrate for CYP106A2 but no biotransformation was detected when this enzyme was overexpressed in *E. coli*, which was suspected to be due to poor mass transfer of

the hydrophobic terpene across the *E. coli* membrane. This was resolved by overexpression of CYP106A2 in *B. megaterium*, which showed productive biotransformation activity[218]. A similar result was observed with another hydrophobic terpene acid, abietic acid[219], which also showed poor mass transfer characteristics in *E. coli*. The use of a CYP106A2-expressing *B. megaterium* catalyst was also used for the hydroxylation of the steroid hormone dehydroepiandrosterone (DHEA)[421] and corticosteroid drugs prednisone and dexamethasone[423].

The *B. megaterium* enzyme CYP109E1 has also been overexpressed in its native host and used for the whole-cell biotransformation of various statin drugs and terpenes[422]. This system was also used for the hydroxylation of cholesterol, but the *B. megaterium* system produced over three-fold less product than a recombinant *E. coli* system[229]. This was suspected to be due to accumulation of cholesterol within intracellular polyhydroxybutyrate (PHB) granules. In another study, Gerber and colleagues performed cholesterol hydroxylation in *B. megaterium* using the bovine P450 CYP11A1[426], which is natively membrane-bound. In that work, the cholesterol substrate was shown to be contained within PHB granules and the CYP11A1 enzyme was localised to the phospholipid monolayer surrounding the granules, effectively compartmentalising the enzyme with the substrate and providing high biotransformation productivity[426]. The ability to stably express natively membrane-anchored P450s on *B. megaterium* PHB granules was further established using the mitochondrial enzyme CYP27A1 for the biotransformation of cholesterol and vitamin D₃[424].

Another *B. megaterium* biotransformation system used the *Streptomyces griseolus* enzyme CYP105A1 for hydroxylation of the hypoglycaemic drugs glimepiride and glibenclamide[420]. The natural redox partners for this enzyme are unknown, so heterologous redox partners were initially co-expressed with CYP105A1. However, the system performed the same regardless of whether the redox partners were co-expressed[379], indicating that CYP105A1 activity was sufficiently supported by endogenous *B. megaterium* P450 redox partners. This feature may be useful for other 'orphaned' bacterial P450s, which have unknown redox partners, as it does not require the construction of multicistronic expression plasmids.

Extensive genetic engineering was also performed to produce a productive *B. megaterium* whole-cell biocatalyst for the production of several valuable synthetic hormones[42]. To reduce unwanted side-product formation, the native *B. megaterium* CYP106A1 gene was knocked out to prevent steroid oxidation and four putative steroid dehydrogenase enzymes identified by genome analysis were also deleted. This was followed by the overexpression of a native *B. megaterium* dehydrogenase to produce

the desired products with high yields[42]. An engineered guinea-pig steroid dehydrogenase was also expressed in *B. megaterium*, along with a cofactor recycling enzyme, to produce a highly efficient biotransformation system for cortisone production from hydrocortisone[321].

2.8. Summary and project aims

The synthesis of structural analogues of the anticancer alkaloid noscapine has provided significant enhancements of the potency of the drug, while in many cases maintaining its low toxicity towards healthy tissues. There is, however, room for improvement – many of the promising analogues tested to date show considerable variability in their activity across different cancer cell lines and their antimitotic potency is often significantly lower than clinically employed chemotherapeutics. Importantly, organic syntheses have only been developed to functionalise noscapine at three different sites on the noscapine scaffold; selective chemical modifications at other sites have not yet been described. There is therefore considerable analogue diversity that is practicably inaccessible, which limits the investigation of structure-activity relationships for further development of noscapine as a drug candidate.

It is notable that the mammalian metabolism of noscapine generates at least six derivatives that may be of interest to medicinal chemists investigating noscapine analogue development. These metabolites are formed through dealkylation and hydroxylation mechanisms by cytochrome P450 enzymes and the modifications produce potential target sites for further synthetic functionalisation. Two of these metabolites, *N*-demethylated noscapine and 7-*O*-demethylated noscapine, have been produced synthetically and show enhanced antimitotic activity compared to noscapine; they are also essential precursors for analogues modified at the *N*- and 7-positions. The preparative-scale production of noscapine metabolites by cytochrome P450 enzymes therefore has several potential outcomes, including:

- (i) the discovery of active metabolites of noscapine;
- (ii) the production of precursors for new classes of analogues;
- (iii) the development of biosynthetic routes to replace multistep organic syntheses.

The production of noscapine metabolites by mammalian microsomal P450s has so far only been performed at analytical scale and these enzymes are generally unsuitable for large-scale production. The bacterial enzyme P450_{BM3} has a range of practical advantages compared to microsomal P450s and significantly greater potential for deployment at industrial scale. Key amino acid mutations have been demonstrated to broaden the substrate range of P450_{BM3} to include a large number of drug-like substrates, but P450_{BM3} mutants have not been assessed for their activity on noscapine.

Biocatalysis using whole cells is typically the method of choice for preparative-scale biotransformations with P450 enzymes. This application has the advantage of intracellular cofactor supply from the host

organism, as well as improved enzyme stability and reduced processing steps. This method does, however, have a disadvantage in that mass transfer of substrate across cell wall structures often limits process productivity. Noscapine is a bulky and hydrophobic molecule that may have difficulty traversing the outer cell wall of common Gram-negative expression hosts like *Escherichia coli* and *Pseudomonas* species. Surprisingly, despite mass transfer being a common limitation in whole-cell biotransformations, an assessment of the substrate permeability of different potential hosts during initial process development is very rarely performed. Gram-positive organisms, which lack the outer cell membrane of Gram-negative bacteria, may be a better choice for noscapine biotransformation. The soil bacterium *Bacillus megaterium* has recently been demonstrated to have significant potential for P450-mediated pharmaceutical biotransformation processes. It is interesting to note that this organism has not previously been used for biotransformations with P450_{BM3}, despite the enzyme being native to this species. Recombinant expression of an enzyme in its native host can often offer advantages in terms of enzyme stability, higher expression levels and, for biotransformations using oxygenase enzymes, a more favourable redox environment for the reaction of interest.

Noscapine has poor aqueous solubility, which is likely to pose a limitation on achievable yields for a whole-cell biotransformation process. Biphasic biotransformation systems are a well-established method to improve substrate loading for other poorly water-soluble substrates. To achieve high productivity, it is important that the selected water-immiscible solvent shows good compatibility with both the substrate and the microbial host. Given the vast array of potential solvents, the selection process can potentially be simplified by solubility modelling and *in silico* screening prior to laboratory experiments. This approach may also identify potentially suitable solvents that are relatively uncommon and not normally considered for biotransformation applications. Solubility modelling is widely employed in pharmaceutical engineering and a range of solubility prediction methods exist; however, there are very few examples of this approach being applied for the development of biphasic biotransformations. Another effective strategy to improve substrate loading for whole-cell biotransformations is the use of cyclodextrins. This approach has been adopted from the pharmaceutical industry, where cyclodextrins are often used as solubility enhancers in drug formulations. Water-soluble polymers are also often included as solubility-enhancing additives in drug formulations but their use in whole-cell biotransformations is largely unexplored.

Metabolic engineering of microbial hosts has also been used effectively to improve biotransformation productivity. In particular, genetic engineering to improve the supply of NAD(P)H cofactor for

monooxygenase-catalysed biotransformations has proven successful in several applications, as has gene inactivation to eliminate side-reactions or improve substrate uptake. Given the complexity of cell metabolism and the vast number of genetic targets for potential manipulation, there are likely to be significant further productivity gains that can be achieved with metabolic engineering. While previously described approaches mostly rely on permanent gene inactivation, which can cause unintended growth defects in the host organism, modern metabolic engineering methods often allow dynamic control of metabolic pathways. These techniques have significant potential to optimise the performance of microbial hosts to improve biotransformation productivity.

The overall aim of this thesis is to develop a whole-cell biotransformation process for the cytochrome P450-mediated production of noscapine metabolites. To achieve this, there are three main research objectives, which form the basis of **Chapters 3-5** in this thesis:

- (i) To develop and screen P450_{BM3} mutant enzymes for their potential to produce noscapine metabolites;
- (ii) To incorporate a P450_{BM3} mutant into a whole-cell biotransformation process and perform reaction engineering to optimise productivity, including:
 - An investigation of mass transfer limitations and comparison between Gram-negative and Gram-positive hosts;
 - An improvement of substrate loading by the development of a biphasic system assisted by solubility modelling;
 - An investigation of aqueous solubility enhancers to improve reaction rates;
 - Further process debottlenecking and investigation of productivity-limiting factors.
- (iii) To investigate the use of metabolic engineering to further optimise biotransformation productivity.

The research towards these three objectives may produce several useful outcomes. The preparative-scale production of noscapine metabolites may assist in the development of novel noscapine analogues with enhanced activity. This work may also establish biosynthetic routes for potential industrial-scale analogue production. Further, the use of novel methodologies for process optimisation may be valuable for the development of other whole-cell biotransformation processes which are becoming increasingly utilised in the pharmaceutical and fine chemical industries.

3. Construction and screening of a P450_{BM3} mutant library for the production of noscapine metabolites

3.1. Paper 1

Production of metabolites of the anti-cancer drug noscapine using a P450_{BM3} mutant library

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ABSTRACT

Cytochrome P450 enzymes are a promising tool for the late-stage diversification of lead drug candidates and can provide an alternative route to structural modifications that are difficult to achieve with synthetic chemistry. In this study, a library of P450_{BM3} mutants was produced using site-directed mutagenesis and the enzymes screened for metabolism of the opium poppy alkaloid noscapine, a drug with anticancer activity. Of the 18 enzyme mutants screened, 12 showed an ability to metabolise noscapine that was not present in the wild-type enzyme. Five noscapine metabolites were detected by LC-MS/MS, with the major metabolite for all mutants being *N*-demethylated noscapine. The highest observed regioselectivity for *N*-demethylation was 88%. Two hydroxylated metabolites, a catechol and two C-C cleavage products were also detected. P450-mediated production of hydroxylated and *N*-demethylated noscapine structures may be useful for the development of noscapine analogues with improved biological activity. The variation in substrate turnover, coupling efficiency and product distribution between the active mutants was considered alongside *in silico*

docking experiments to gain insight into structural and functional effects of the introduced mutations. Selected mutants were identified as targets for further mutagenesis to improve activity, and when coupled with an optimised process may provide a route for the preparative-scale production of noscapine metabolites.

Keywords: cytochrome P450, *N*-demethylation, hydroxylation, site-directed mutagenesis, biocatalysis.

Abbreviations: P450, cytochrome P450; P450_{BM3}, CYP102A1 from *Bacillus megaterium*; IPTG, isopropyl β -D-1-thiogalactopyranoside; NADPH, nicotinamide adenine dinucleotide phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DMSO, dimethyl sulfoxide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; QTOF, quadrupole time-of-flight; WT, wild-type.

INTRODUCTION

Enzymes are emerging as a useful tool for the structural diversification of pharmaceutical drug candidates[427] and at process scale can potentially provide a safer and more environmentally friendly alternative to synthetic chemical approaches. Late-stage diversification is traditionally achieved by modifying a drug lead, such as a natural product, into a range of structural analogues with altered or additional functional groups that are then screened for improved pharmacological properties. This late-stage functionalisation is often limited to a small proportion of structural positions at which synthetic modification is practicable. For example, compounds containing multiple similar functional groups can require extensive protection/deprotection steps to achieve a chemoselective modification, and many C-H bonds are essentially inaccessible to functionalisation. A complementary strategy is to screen a library of natural or engineered enzymes for their ability to biosynthetically modify a drug compound. This approach takes advantage of the wide range of reaction chemistries and regioselectivity exhibited by enzymes and can offer rapid routes to drug analogues that would otherwise be difficult to produce with synthetic approaches, achieved in a single step under mild aqueous conditions.

The opium poppy alkaloid noscapine (**Figure 3.1**) has been a target for structural diversification to improve its native anticancer activity[428] and this may benefit from an enzymatic approach. Noscapine interferes with microtubule assembly during tumour cell division[429], although this

activity is fairly weak compared to other microtubule-binding chemotherapeutics such as paclitaxel and colchicine. Noscaphine does have the advantage, however, of very low toxicity compared to these drugs[430, 431], suggesting that structural analogues may have improved anticancer activity and retain a favourable toxicity profile. Most noscaphine analogues produced to date carry functional group

modifications at the 9'-, 7- and N-positions of the noscaphine scaffold (**Figure 3.1**), as these bonds can be selectively targeted using synthetic chemical reactions[95, 108,

112, 428, 432, 433]. While many of these analogues have been shown to have improved activity compared to the parent drug, none have progressed further than Phase I clinical trials. Investigating structure-activity relationships at alternative positions on the noscaphine backbone may therefore provide a route to new classes of analogues with enhanced activity.

Cytochrome P450 monooxygenase enzymes (P450s) offer a promising alternative to synthetic derivatisation of noscaphine and have been used to produce other drug analogues[434]. This approach mimics xenobiotic metabolism in mammals and other higher eukaryotes, where a drug compound is oxidised by microsomal P450s in the liver to produce drug metabolites. These metabolic reactions include hydroxylations, epoxidations or heteroatom dealkylations that may be difficult to achieve with chemical synthetic methods. Mammalian microsomal P450s are known to produce at least nine noscaphine metabolites through demethylation, hydroxylation and C-C cleavage mechanisms[27, 115, 435] and these compounds may be of interest for the development of noscaphine analogues. The scale-up of biosynthetic reactions with microsomal P450s, however, can be difficult due to the inherent instability, insolubility and low activity of these enzymes[29].

An alternative, more practical approach is to use the more stable bacterial fatty acid hydroxylase P450_{BM3} from *Bacillus megaterium*, an enzyme that has not yet been investigated for activity on noscaphine. This enzyme has several advantages compared to microsomal P450s. P450_{BM3} is a fusion of a P450 substrate-binding domain and a mammalian-like reductase domain[170] and is therefore 'self-sufficient' compared to microsomal P450s, which require at least two discrete enzymes to produce a functional catalyst. P450_{BM3} is also cytoplasmic, as opposed to the intracellular membrane-

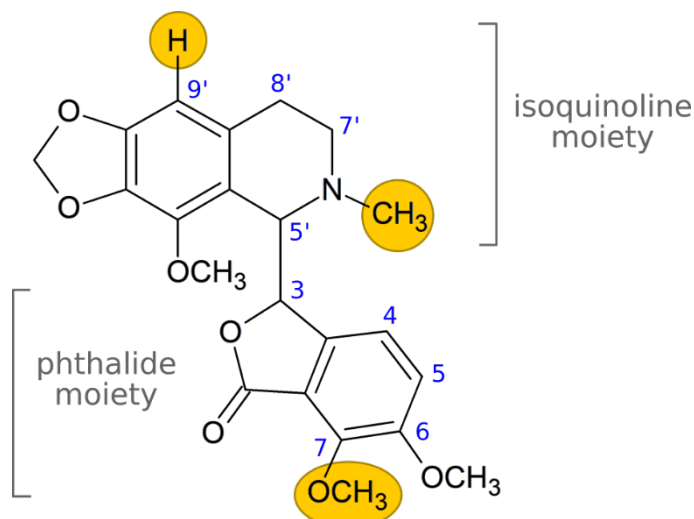


Figure 3.1. The structure of noscaphine, consisting of phthalide and isoquinoline moieties. The yellow circles highlight positions on the molecule that have been modified by others to produce analogues with enhanced biological activity.

bound location of microsomal P450s and can be expressed recombinantly at high levels in bacteria[174]. While the native substrates of P450_{BM3} are long-chain fatty acids, the wild-type enzyme is capable of metabolising some drug compounds[177]. Its substrate specificity, however, can be significantly broadened through protein mutagenesis. Both directed evolution and rational engineering approaches have been used extensively with P450_{BM3} to produce mutants capable of synthesising a wide variety of drug metabolites, as well as other industrially important fine chemicals[31, 33].

In this study, we have combined P450_{BM3} mutations known to enhance activity on bulky drug-like substrates to produce a new library of mutant enzymes. The mutant library was then screened for activity to assess the potential utility of P450_{BM3} mutants in noscapine analogue development and for enzymatic routes of analogue production.

MATERIALS AND METHODS

Chemicals and reagents

Noscapine was provided by Sun Pharmaceutical Industries (Port Fairy, Australia). The *N*-demethylated noscapine standard (*N*-nornoscapine) was a kind gift from Peter Scammells at Monash University. Molecular biology enzymes and competent cells were purchased from New England Biolabs (Ipswich, MA, USA). Primers were synthesised by GeneWorks (Thebarton, Australia). Complex growth media components were Oxoid brand (Thermo Fisher Scientific, Scoresby, Australia). Kanamycin, isopropyl β -D-1-thiogalactopyranoside (IPTG), nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (Castle Hill, Australia). Chromatographic separations were performed using deionised water (resistivity 18.2 m Ω ; MilliQ) and chromatography-grade methanol (Merck, Bayswater, Australia).

Cloning and mutagenesis

The wild-type (WT) CYP102A1 gene was cloned from *Bacillus megaterium* ATCC 14581. Cells were cultured in nutrient medium at 30°C and genomic DNA was extracted with an AxyPrep multisource genomic DNA extraction kit (Corning, Corning, NY, USA). The CYP102A1 gene was amplified by PCR using primers that introduced flanking *Bam*HI and *Xho*I restriction sites (primers *BM3_fwd_Bam*HI and *BM3_rev_Xho*I; see **Table 3.1, Supplementary Material**, for all primer sequences). The amplicon was ligated into the pET28a(+) vector (Merck, Bayswater, Australia) and the plasmid was transformed into chemically competent DH5 α *E. coli* cells for cloning. Correct sequence identity was confirmed by Sanger sequencing (AGRF, Melbourne, Australia) and alignment with the *B. megaterium* ATCC 14581 sequence in GenBank (accession CP009920.1; nucleotides 132187-135336). Plasmids containing

genes for the R47L/Y51F and GVQ (A74G/F87V/L188Q) variants were kindly provided by Stephen Bell at The University of Adelaide. The mutant genes were amplified by PCR using the primers above and ligated into the pET28a(+) vector. The order of mutant production by sequential mutation and back mutation is shown in **Figure 3.2B**. Mutants F87A, R47L/Y51F/F87A and GAQ were created by introducing the F87A mutation into WT, R47L/Y51F, and GVQ sequences, respectively, using the Q5 site-directed mutagenesis kit (New England Biolabs) and primer pair *F87A_fwd* and *F87A_rev*. The mutant sets V1-V4 and A1-A4 were created by the cumulative addition of E267V, R47L, F81I, and E64G mutations to the GVQ and GAQ templates respectively using the QuikChange multi-site-directed mutagenesis kit (Agilent, Mulgrave, Australia) with single primers for each mutation (**Table 3.1, Supplementary Material**). Variants V4A and A4A were produced by back-mutation of the A74G mutation in V4 and A4 using the same protocol. The A3I, A3D and A3S mutants were created by the addition of L86I, S72D and L437S mutations independently to the A3 template using the same method. Mutant plasmids were transformed into DH5 α F' ⁺ cells for cloning. For protein expression, all plasmids were transformed into BL21(DE3) cells to produce glycerol stocks, which were stored at -80°C.

Protein expression and lysate preparation

In trial expression experiments we noted that expression of the P450_{BM3} variants was enhanced under microaerobic (low oxygen) conditions, an observation that has been made by others for expression of P450_{BM3}[174] and mammalian P450 enzymes[175]. A microaerobic protocol was therefore employed for expression as follows. Glycerol stocks were used to inoculate 5 mL of lysogeny broth (LB) supplemented with 30 μ g/mL of kanamycin. The cultures were grown overnight at 30°C and 200 rpm on an orbital shaker and this culture then used to inoculate 100 mL of the same media in a baffled 500 mL flask to give a starting OD₆₀₀ of ~0.015. The flasks were shaken at 250 rpm at 37°C for 2-3 h until the OD₆₀₀ reached 0.6-0.8. The cells were harvested by centrifugation (3200 x *g*, 10°C, 15 min) and the pellets washed twice by resuspension/centrifugation with 10 mL volumes of MOPS minimal medium (as per Neidhardt *et al.*[436] but with FeSO₄ increased to 100 μ M) supplemented with 0.2% glucose and 0.5 mM 5- α -aminolevulinic acid. The cells were resuspended in 100 mL of the modified MOPS medium in a 125 mL round-bottomed flask with a filter cap (Corning, NY, USA) to facilitate microaerobic conditions. Prior to induction, the flasks were shaken at 20°C and 140 rpm for 5-6 h to allow growth to resume. Protein expression was induced with 0.5 mM IPTG and allowed to continue for 12-14 h. The cells were harvested by centrifugation (3200 x *g*, 4°C, 15 min), the supernatant was discarded and the pellets were snap frozen in liquid nitrogen, stored at -80°C and used within two weeks. To prepare cell lysates, the frozen pellets were resuspended in 4 mL of potassium phosphate buffer (50 mM, pH 7.4). The resuspended cells were sonicated on ice (6 mm

probe, 30% amplitude, 10 s on, 20 s off for five cycles) using a QSonica Q500 machine (QSonica LLC, Newtown, CT, USA). The lysates were then centrifuged at 18,000 x g at 4°C for 15 minutes to remove cell debris.

P450 quantification

The P450 concentration of the cell lysates was measured using carbon monoxide difference spectroscopy by the method of Omura and Sato[437] with extinction coefficients $\epsilon_{\Delta 450-490} = 91 \text{ mM cm}^{-1}$ and $\epsilon_{\Delta 420-490} = -41 \text{ mM cm}^{-1}$, with simultaneous determination of inactive P420 enzyme using extinction coefficients $\epsilon_{\Delta 450-490} = -11 \text{ mM cm}^{-1}$ and $\epsilon_{\Delta 420-490} = 110 \text{ mM cm}^{-1}$ [438].

Enzyme activity screening

Activity assays were performed at a 200 μL scale in 96-well polystyrene microtitre plates (Corning). The reaction mixture consisted of potassium phosphate buffer (50 mM, pH 7.4) containing 25 μM noscapine added in DMSO (final DMSO concentration 2%) and sufficient cell lysate to provide a P450 concentration of 500 nM. The reactions were initiated by the addition of 20 μL of an NADPH recycling system providing final concentrations of 0.2 mM NADPH, 1 mM glucose-6-phosphate and 0.5 U/mL glucose-6-phosphate dehydrogenase. For each enzyme variant, a control was included that received an additional 20 μL of buffer instead of the NADPH recycling system. A set of controls using lysate from BL21(DE3) cells containing the empty pET28a(+) vector were also included. Each assay reaction and control were performed in triplicate. The assay plates were pre-incubated at 30°C for 15 minutes prior to initiation and the reactions were allowed to proceed at 30°C for 25 minutes. To quench the reaction and extract metabolites, a multichannel pipette was used to transfer 150 μL from each well into 300 μL of ice-cold chloroform in a 500 μL deep-well polypropylene 96-well plate (Corning) and the plate was left on ice for 15 minutes. The plates were centrifuged for 10 minutes at 4°C and 3200 x g to separate the phases and 200 μL of the organic phase was transferred to a fresh 96-well plate. Each well was evaporated to dryness under a nitrogen gas stream. The extracted metabolites were reconstituted in 60 μL of 0.1% formic acid and transferred to glass vials for LC-MS/MS analysis.

LC-MS/MS analysis

Metabolites were analysed by LC-MS/MS at Metabolomics Australia (School of BioSciences, University of Melbourne) using an Agilent 6520 series QTOF mass spectrometer with a dual sprayer ESI source attached to a 1260 Infinity series HPLC system and a G1315C diode array detector. The instrument was operated in positive ion mode using the following conditions: nebuliser pressure 30 psi, gas flow rate 10 L min⁻¹, gas temperature 300 °C, capillary voltage 4000 V, fragmentor 150 V and skimmer 65 V. Metabolites were detected in targeted mode using a collision energy of 20 eV. The targeted list of

precursor ion masses and retention times is provided in **Table 3.2 (Supplementary Material)**. Proposed fragmentations are shown in **Figure 3.8 (Supplementary Material)**. Chromatography was performed using a Kinetix biphenyl column (1.7 μm , 100 $\text{\AA}$, 100 x 2.1 mm; Phenomenex, Lane Cove, Australia) maintained at 55 °C and a flow rate of 0.3 mL min⁻¹. The sample injection volume was 20 μL . A binary gradient method was developed where the mobile phase consisted of (A) 0.1% formic acid and 10 mM ammonium formate in water and (B) 0.1% formic acid and 10 mM ammonium formate in methanol. The gradient elution program was as follows: isocratic at 25% solvent B for one minute; 25 to 50% solvent B gradient over one minute; 50 to 70% solvent B gradient over nine minutes; stepped to 95% solvent B and held for one minute; then re-equilibration at 25% solvent B for seven minutes. Absorbance was monitored between 190-500 nm and a wavelength of 311 nm was used for relative metabolite quantification, assuming similar extinction coefficients of all detected metabolites at this wavelength. Ultraviolet absorption spectra for all measured metabolites are provided in **Figure 3.7 (Supplementary Material)**.

Measurement of NADPH/noscapine consumption and cofactor coupling efficiency

The coupling of NADPH consumption to product formation was measured for all BM3 variants that showed activity in the screening experiments. Assays to determine NADPH consumption were performed in triplicate in 96-well glass-bottom plates (Greiner Bio-One, Frickenhausen, Germany) at a 200 μL scale, containing 200 nM P450 enzyme from cell lysate, 100 μM noscapine added from methanol stock (final MeOH concentration 1%) and approximately 200 μM of NADPH added in a 20 μL volume to initiate the reaction. The exact NADPH concentration was determined by UV spectrophotometry ($\epsilon_{340} = 6.022 \text{ mM cm}^{-1}$) from triplicate control samples containing 180 μL potassium phosphate buffer and 20 μL of NADPH stock. For each enzyme variant, three control samples that received 20 μL buffer instead of NADPH were measured. After initiation of the reactions, the plates were placed into a FLUOstar OPTIMA plate reader (BMG Labtech, Mornington, Australia) and the NADPH concentration was monitored by reading the absorbance at 340 nm. After complete depletion of NADPH in all assay wells, a multichannel pipette was used to transfer 100 μL from each assay or control well into 100 μL of ice-cold acetonitrile in a fresh 96-well plate. The mixtures were left on ice for 15 minutes and the plates were then centrifuged at 3200 x g for 20 minutes at 4 °C to remove precipitated protein. A volume of 40 μL of supernatant from each well was then added to 60 μL of 0.5% TFA in glass HPLC vials for analysis.

The product concentrations in these assays were quantified by UHPLC analysis using a method developed on an Agilent 1290 Infinity II system using an Acquity BEH C18 column (1.7 μm , 130 $\text{\AA}$, 100 x 2.1 mm; Waters, Rydalmere, Australia) held at 60 °C. A binary gradient program was used with

mobile phase solvents comprised of (A) 0.1% trifluoroacetic acid and (B) methanol at a flow rate of 0.3 mL min⁻¹. A 20 µL injection volume was used with a gradient elution program as follows: isocratic using 20% solvent B for 0.5 minutes; a gradient of 20% to 37% solvent B gradient over 1.5 minutes; isocratic using 37% solvent B for 2.5 minutes; a gradient of 37% to 95% solvent B gradient over two minutes; isocratic using 95% solvent B for 0.5 minutes; then re-equilibration using 20% solvent B for seven minutes. The noscapine metabolite peaks were measured at a wavelength of 311 nm and quantified using a noscapine standard curve; the extinction coefficients were assumed to be similar for all measured metabolites at this wavelength (see **Figure 3.7, Supplementary Material**, for metabolite absorption spectra).

The coupling efficiency was calculated by dividing the total product concentration in the assay samples by the initial NADPH concentration.

In silico docking

Docking studies were performed using the Schrödinger software package (version 2017.4, Schrödinger, LLC, New York, NY, USA). Default settings were used unless otherwise noted. The crystal structure of the PEG-bound P450_{BM3} pentamutant R47L/F81I/F87V/L188Q/E267V (PDB ID: 4ZF6[439]) was used to model the receptor. This variant contains the same mutations as mutant V3 tested in this work with the exception of the A74G mutation, which is not in the 4ZF6 structure. To examine the effects of the F87A mutation, the Val87 residue in 4ZF6 was changed to Ala87 using the mutagenesis tool in Maestro. The Protein Preparation Wizard was used to prepare the receptor. All water molecules and the bound PEG ligand were removed. The Fe³⁺ state was selected for the haem group. The noscapine ligand was prepared using LigPrep and docked in a non-protonated state. Docking simulations were performed using the Induced Fit Docking protocol[440]. The grid box centre was selected as the centroid of the ethylene glycol molecule in the active site of the 4ZF6 structure and the box dimensions were set to 22 Å x 22 Å x 22 Å. During docking, the planarity of conjugated pi groups was enhanced. In the initial docking stage, the Leu75 and Val78 residues on the B'-helix were trimmed. The Cys400 residue, which ligates the haem iron, was excluded from side chain refinement. Docking images were generated using PyMOL (version 2.0.7, Schrödinger, LLC).

RESULTS & DISCUSSION

Mutant library development

A P450_{BM3} mutant library, shown in **Figure 3.2A**, was developed with the aim of finding mutants capable of producing useful noscapine metabolites and providing biosynthetic routes to noscapine analogue production. Our approach was to select a range of mutations shown by previous work to

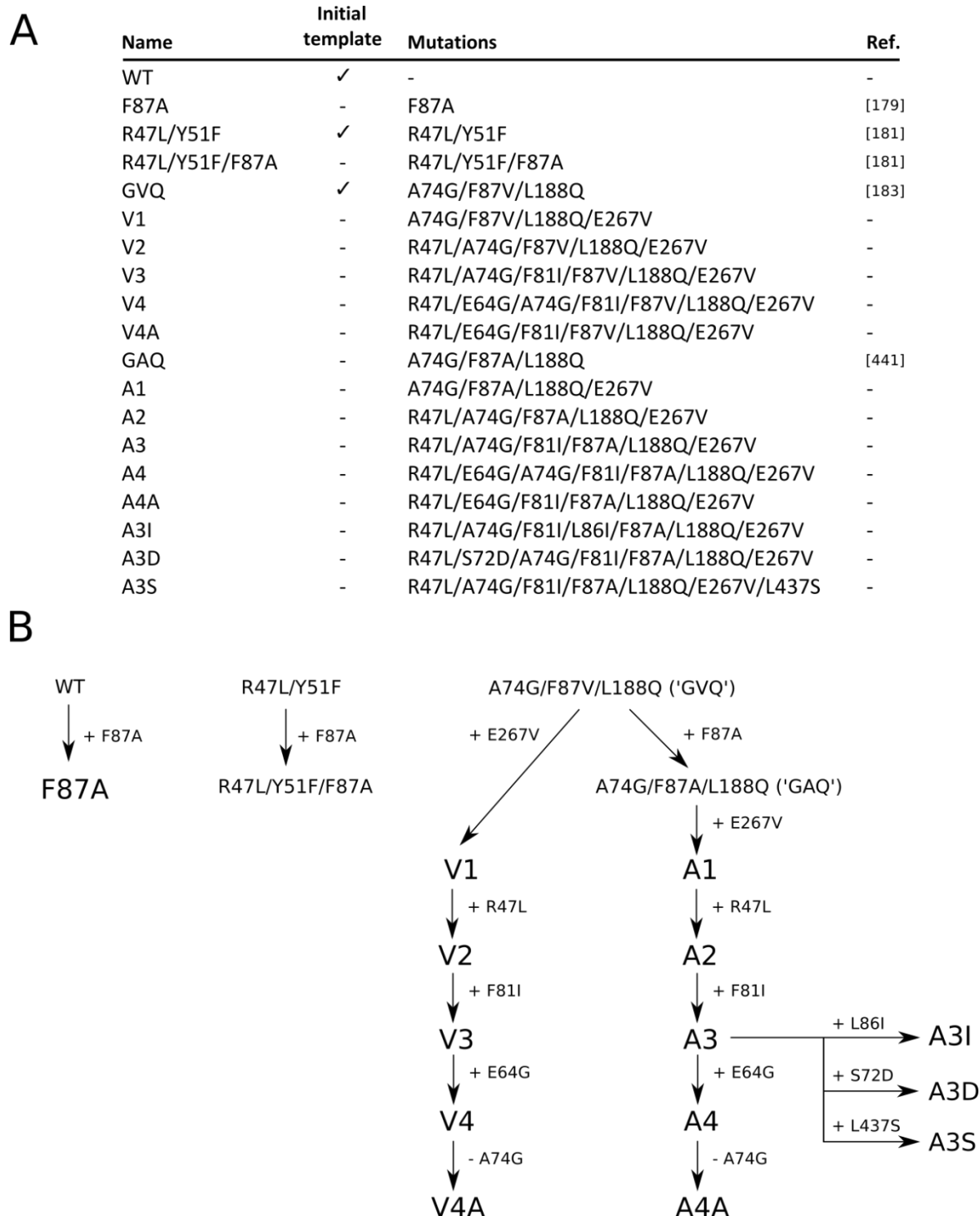


Figure 3.2. (A) Details of the P450_{BM3} mutant library used in this study, which consisted of 18 mutants and the wild-type (WT) enzyme. Variants used as starting templates for library construction are indicated in the second column. References are provided for specific combinations of mutations that have been previously described. **(B)** Mutation workflow used to create the P450_{BM3} library.

enhance P450_{BM3} oxidation of bulky, drug-like compounds and apply these in various combinations to nescapine, which has not previously been examined as a substrate for P450_{BM3} mutants. The locations of the twelve mutations at eleven sites are shown in **Figure 3.3**. A library of eighteen mutant variants was produced from combinations of the twelve mutations, together with the wild-type (WT) enzyme.

This approach makes use of the significant characterisation of P450_{BM3} mutations performed over the past two decades and demonstrates how mutations already described in the literature can be applied in new combinations to a selected target of interest. Overall, fourteen of the eighteen mutants used in the study are new to this work and four mutants, including the three starting templates and a F87A single mutant, are specific combinations described previously (**Figure 3.2A**).

The starting templates for the library were the WT enzyme and well-described R47L/Y51F[181] and GVQ[183] (A74G/F87V/L188Q) mutants. The sequence in which mutations were introduced is shown in **Figure 3.2B**. A common Phe87 substitution, F87A[179], was applied to all three templates. The resulting GAQ[441] (A74G/F87A/L188Q) mutant and original GVQ mutant were then used as templates for sequential introduction of four mutations shown to improve drug-metabolising activity, E267V, R47L, F81I and E64G[161, 181], leading to two series of four mutants, V1-V4 and A1-A4, which differ only by Phe87 substitution. The Gly74 residue was back-mutated to alanine in two mutants (V4A and A4A) to assess whether the A74G mutation in the GVQ and GAQ starting templates is beneficial for noscapine metabolism, as this mutation is usually used for hydrocarbon oxidation rather than drug metabolite production. The A3 mutant was also further mutated to give A3I, A3D and A3S

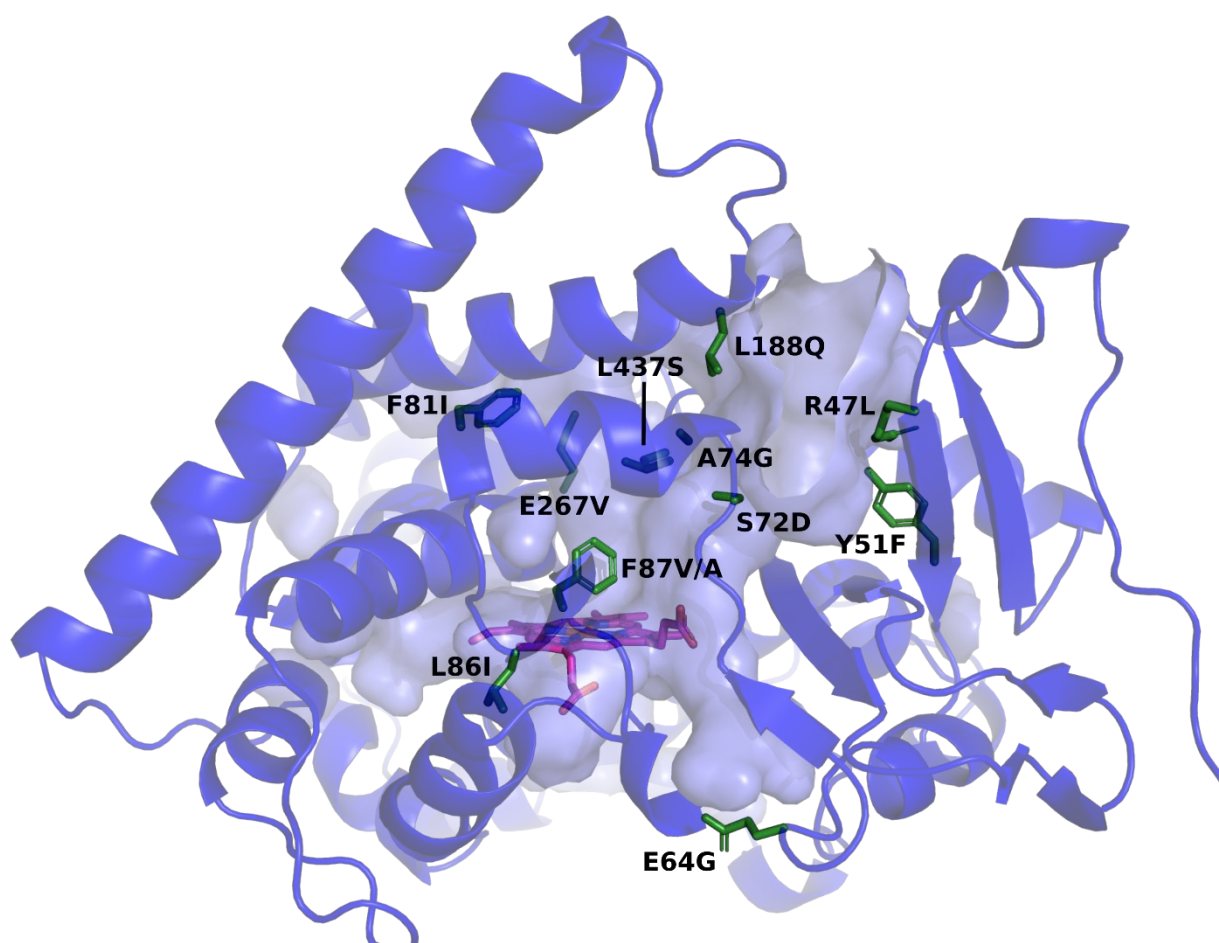


Figure 3.3. Structure of wild-type P450_{BM3} enzyme (PDB: 1BU7). Wild-type amino acids mutated in this work are coloured green and labelled with the mutations introduced. The haem group is coloured magenta.

to examine the effects of three additional mutations shown by others to enhance activity and/or modify selectivity[161, 188]. As most mutations were introduced sequentially, producing a library with a progressively increasing number of mutations, the effect of individual mutations on noscapine metabolism may be specific to the background of mutations into which they were introduced.

Mutant screening

Mutant activity

The introduced mutations successfully imparted noscapine activity to P450_{BM3}. Twelve enzymes from the panel of eighteen mutants were active on noscapine, producing the metabolites shown in **Figure 3.4**, which were detected by LC-MS/MS. The WT enzyme did not show any turnover of noscapine.

The major metabolite produced by all active mutants was *N*-demethylated noscapine (**1**), a desirable compound that can only be produced synthetically from noscapine through multiple chemical steps[112, 442]. The process of *N*-demethylation improves the *in vitro* cytotoxicity against several cancer cell lines compared to unmodified noscapine[432]. This compound is also an essential intermediate in the production of *N*-substituted noscapine analogues, several of which show further enhanced anticancer activity *in vitro*[112, 432]. The best synthetic route for *N*-demethylation of noscapine involves the oxidation of noscapine to its *N*-oxide, which is isolated as a hydrochloric acid salt, followed by treatment with an iron-containing catalyst to yield the final product. The highest reported yield for this process is 74%[112]. A one-pot, biocatalytic *N*-demethylation, as presented here, may be preferable for larger-scale syntheses, particularly as the highest-yielding synthetic protocols use chlorinated solvents. This enzymatic approach to *N*-demethylation may also be more broadly applicable to other opiate alkaloids, for which synthetic *N*-demethylation can be problematic. Five additional noscapine metabolites, including further compounds of interest, were also detected in the mutant screen. These were: two hydroxylated metabolites (**2a/2b**), a catechol product resulting from cleavage of the methylenedioxy bridge (**3**), and two metabolites produced by cleavage of the central C-C bond (cotarnine and meconine, **4** and **5**). The MS/MS fragmentation patterns of the hydroxylated metabolites **2a/2b** (**Figure 3.8, Supplementary Material**) indicated that the hydroxyl groups were attached to the isoquinoline moiety of noscapine, although the specific sites could not be resolved by this technique. Small amounts of double-oxidation products resulting from combinations of *N*-demethylation, hydroxylation and methylenedioxy cleavage were also detected (**Table 3.2** and **Figure 3.8** in **Supplementary Material**). A UHPLC chromatogram showing noscapine turnover of mutant A3 is provided in **Figure 3.10 (Supplementary Material)**.

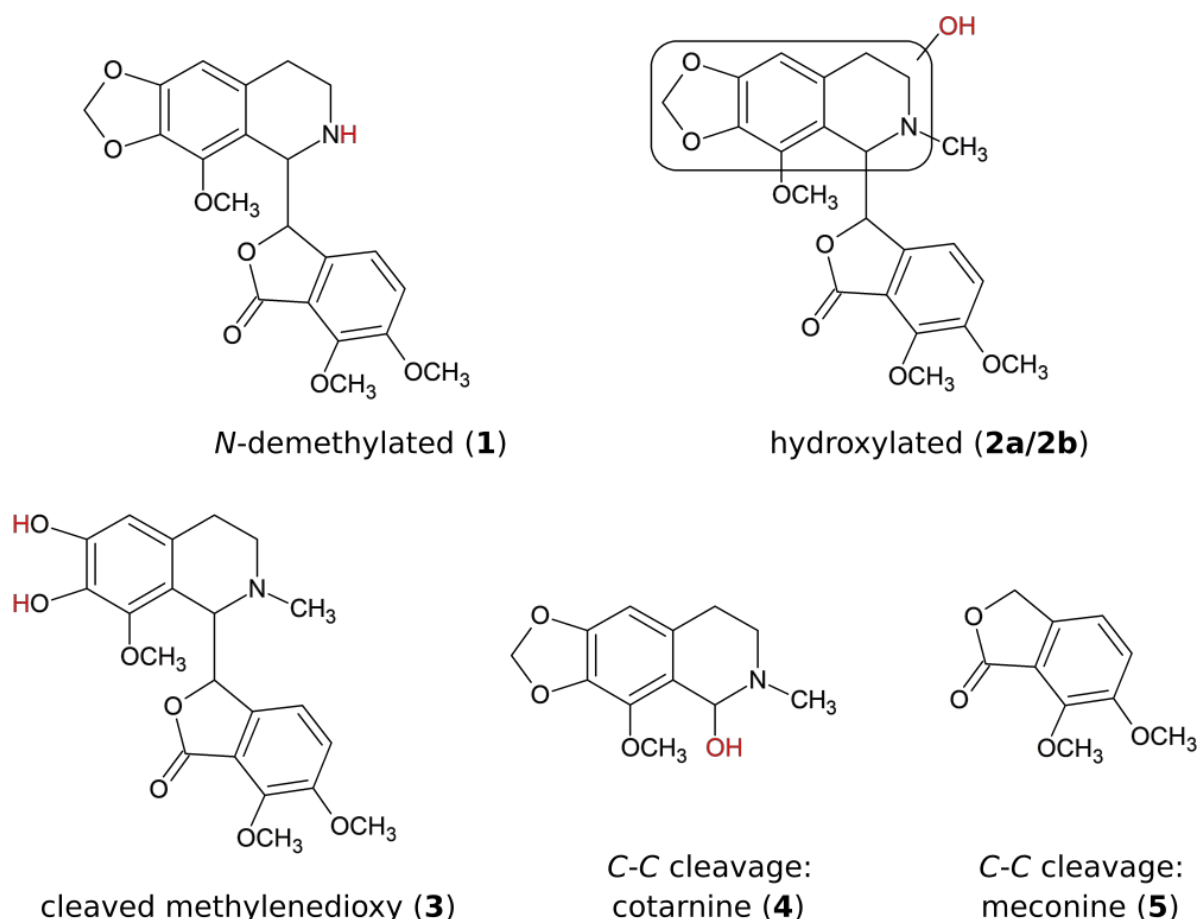


Figure 3.4. Noscapine metabolites detected by LC-MS/MS in the P450BM3 mutant screening experiments. Red text denotes modifications relative to the parent compound. The exact locations of the hydroxyl groups on metabolites **2a** and **2b** could not be resolved by the LC-MS/MS method used.

The hydroxylated metabolites (**2a/2b**) may be useful precursors for further synthetic modification in the search for noscapine analogues with enhanced anticancer activity, as structure-activity relationships (SARs) have only been studied at the 9'-, 7- and *N*-positions on the noscapine scaffold. The cleaved methylenedioxy bridge metabolite (**3**) is potentially less useful as it contains two neighbouring hydroxyl groups, which does not allow for selective functionalisation. Cleavage of the central noscapine C-C bond, producing metabolites **4** and **5**, is an undesirable reaction, as it results in loss of anticancer activity[431] and this reaction can be easily achieved by acid hydrolysis[443].

The *N*-demethylation and methylenedioxy bridge cleavage of drug compounds are common reactions in P450 drug metabolism. While the mechanism of *N*-demethylation has been debated, evidence now appears to favour the hydrogen atom transfer (HAT) reaction, whereby hydroxylation occurs on the *N*-methyl group followed by decomposition to the tertiary amine and formaldehyde[444]. Cleavage of the methylenedioxy bridge occurs by hydroxylation of the methylenedioxy carbon, followed by decomposition to the catechol metabolite and formic acid[445]. The C-C cleavage of noscapine, however, is recognised as a rare reaction in mammalian P450 metabolism[446] and is thought to

proceed via an amine cation radical[115], as proposed for the peroxidase/H₂O₂ catalysed cleavage of other isoquinoline alkaloids[447]. It is possible that C-C cleavage of noscapine is mediated by P450_{BM3} peroxidase-like activity using H₂O₂ generated by peroxide uncoupling. The reactivity introduced to P450_{BM3} by the mutations resembles that observed in mammalian microsomal P450s, with the potential advantage of greater enzyme stability. A recent study of noscapine metabolism by human and mouse microsomal P450s detected nine different metabolites[27], including compounds **1** and **3-5** that were also found here; although only one isoquinoline-hydroxylated metabolite (**2a/2b**) was reported and the specific hydroxylation site was not determined. Three *O*-demethylated structures and a phthalide-hydroxylated compound were also described that were not detected here. In this study, all detected sites of metabolism were located on the isoquinoline moiety of noscapine; qualitatively, this metabolite profile most closely resembles that of the human CYP3A4 P450 enzyme, except for the phthalide hydroxylation that was also observed with CYP3A4. Of the mammalian P450 enzymes, P450_{BM3} shares the greatest sequence homology with CYP3A4 (27%)[177] and similarities in metabolite profiles between CYP3A4 and both WT[177] and mutant P450_{BM3}[186] have previously been observed, consistent with the findings presented here.

A difference of approximately three-fold was observed in substrate turnover between the most active (A3S) and least active (V4A) variants (59% and 21%, respectively), as shown in **Figure 3.5**, which presents the noscapine turnover (**Figure 3.5A**), coupling efficiency (**Figure 3.5A**) and product distributions for the active mutants in the P450_{BM3} library (**Figure 3.5B-F**). Numerical results are also tabulated in the **Supplementary Material (Table 3.3)**.

The dominant reaction catalysed by all active mutants was *N*-demethylation, with the *N*-demethylated product **1** forming between 59-88% of the product profile (**Figure 3.5B**), with the highest yield occurring for A2. The C-C cleavage products **4** and **5** collectively varied from 6-33% of the product profile (**Figure 3.5F**), while the remaining metabolites **2a**, **2b** and **3** typically made up less than 10% of the product profile (**Figure 3.5C, D and E**, respectively). The hydroxylated metabolite **2b** was produced by only seven mutants. The highest yield of **2a** was produced by V3 and the highest yield of **2b** by A3S (considering both substrate turnover and selectivity).

The measured coupling efficiencies, defined as the molar ratio of metabolites produced to NADPH turnover, ranged between 3-22% and varied roughly in proportion to noscapine metabolism (**Figure 3.5A**). A high coupling efficiency would be desirable for preparative-scale production of noscapine metabolites and should be considered together with the substrate turnover (**Figure 3.5A**) and selectivity for the desired products (**Figure 3.5B-F**). The coupling efficiencies reported here were measured using cell lysates rather than purified enzyme, so some NADPH turnover may be attributable

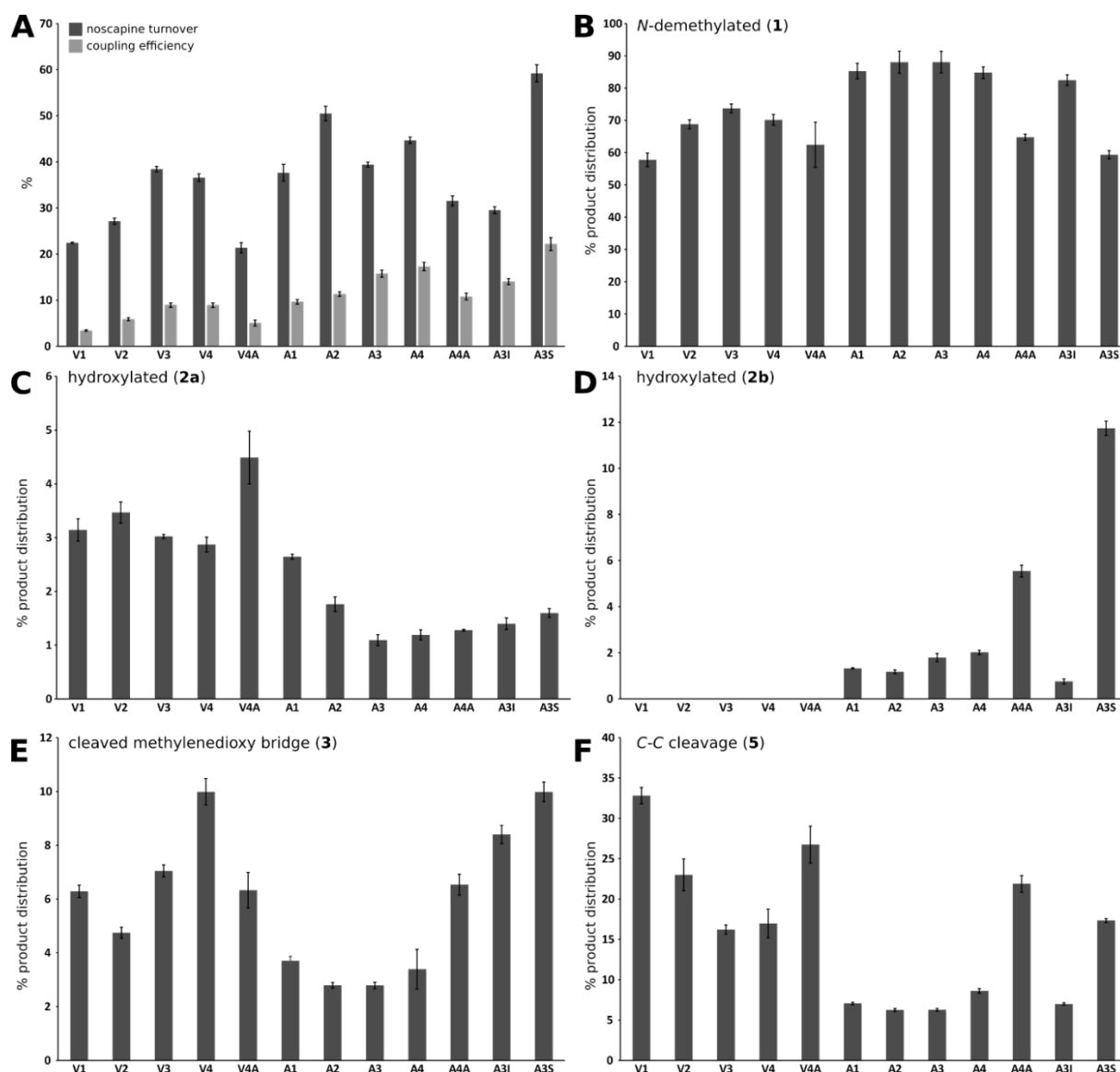


Figure 3.5. Properties of the screened mutants. **(A)** Noscapine turnover (dark bars); coupling efficiency (light bars). **(B-F)** Product distribution percentages for metabolites **1**, **2a**, **2b**, **3**, and **5**. **(C)** was generated from data for meconine (**5**) which shares a similar UV absorption spectrum with the other noscapine metabolites (**Figure 3.7, Supplementary Material**) and is produced stoichiometrically with **4**. The WT enzyme and F87A, R47L/Y51F, R47L/Y51F/F87A, GVQ, GAQ and A3D did not show detectable activity and are not included. The small amounts of double oxidation products detected (**Table 3.2, Figure 3.8 in Supplementary Material**) are not shown. Three replicates were used for each enzyme mutant; error bars are ± 1 SE. Numerical results are tabulated in the **Supplementary Material (Table 3.3)**.

to the non-specific oxidation of lysate components. Nevertheless, the observed coupling efficiencies are within the wide range reported in the literature for P450-mediated drug oxidations.

The low aqueous solubility of noscapine measured under the assay conditions applied here (approximately 100 μ M in 50 mM potassium phosphate buffer, pH 7.4) precluded the measurement of detailed kinetic and binding data for the enzymes examined. Specifically, a substrate concentration of 100 μ M was found to be near or below the k_m value of the most active mutants, preventing determination of turnover numbers (results not shown).

Effects of specific mutations

Several insights can be gained by examining the effects of the 12 mutations on nescapine metabolism across the 18 enzyme mutants. The L437S mutation produced the variant with the highest nescapine turnover in this study, A3S (**Figure 3.5A**). This mutant showed one of the lowest regioselectivities towards *N*-demethylation in the screen (59%; **Figure 3.5B**) and produced significantly more of the hydroxylated metabolite **2b** than the other mutants (12% of product distribution; **Figure 3.5D**), which may be a property of interest for further development. The A3S mutant also had the highest coupling efficiency of 22%. In a previous study using a similar background of mutations, the L437S mutation enhanced activity on most compounds in a panel of 43 drugs, significantly altering the product distribution in many cases[188]. Leu437 forms part of the β -4 loop that borders the active site cavity (**Figure 3.3**) and the polar side chain introduced by the L437S mutation may stabilise nescapine binding conformations through hydrogen bond interactions.

The E267V mutation was found to be crucial in imparting nescapine-metabolising activity to P450_{BM3}, probably due to conformational changes caused by elimination of the Glu267-Lys440 salt bridge between the I-helix and β 4 sheet. The triple-mutants GVQ and GAQ (containing A74G/F87V/L188Q and A74G/F87A/L188Q respectively) did not show any nescapine metabolism under the conditions studied but the addition of E267V to these templates (producing V1 and A1, **Figure 3.2B**) resulted in detectable nescapine metabolism of 21% and 37% respectively (**Figure 3.5A**). Select mutations are known to cause the substrate-free P450_{BM3} to adopt the structure of the substrate-bound WT enzyme[189, 448-452], a change present in two substrate-free crystal structures containing the E267V mutation (PDB: 4RSN[451] and 5E9Z[189]). It has been suggested that such mutations destabilise the substrate-free WT conformation, lowering the free energy barrier of the transition to the substrate-bound form and causing the transition to occur in the absence of substrate. This produces a “catalytically ready” structure with a significantly expanded substrate range. As two mutations shown to independently cause this rearrangement, D251G[452] and A264E[448], also disrupt interactions of the I-helix, it seems likely that the gain of function conferred by E267V occurs through the same mechanism. This conformational change was also thought to be responsible for the 18-fold increase in diclofenac metabolism when E267V was added to a R47L/Y51F/F87V/I401P template[451].

The mutations F87V and F87A were found to be important for activity, with F87A allowing greater substrate turnover, coupling efficiency, *N*-demethylation selectivity and the production of an additional hydroxylated metabolite (**2b**). All active mutants in this study possessed either an F87V or F87A mutation (derived from the GVQ or GAQ template respectively); with five pairs of active mutants that differed only in this respect (F87V/F87A respectively: V1/A1, V2/A2, V3/A3, V4/A4, V4A/A4A).

These mutations replace the bulky active-site Phe87 in the WT enzyme (**Figure 3.3**) and have been used by many groups to broaden the substrate range of P450_{BM3}[31]. **Figure 3.5A** shows that the F87A variants (A1-A4 and A4A) generally had higher noscapine turnover and coupling efficiency than their F87V-containing counterparts (V1-V4 and V4A). There were also notable changes in the product distribution: the F87A mutation was typically associated with improved *N*-demethylation selectivity, mostly due to a significant decrease in C-C bond cleavage (e.g. 26% decrease between V1 and A1), with the exception of A4A. The F87A mutation also allowed the production of the second hydroxylated metabolite (**2b**), which was absent in the product profiles of all F87V variants (V1-V4 and V4A; **Figure 3.5F**). The smaller alanine residue in the F87A-containing mutants may allow a closer approach of the noscapine *N*-methyl group to the haem centre within the enzyme active site, improving the selectivity and coupling efficiency, whilst also allowing hydroxylation of an isoquinoline carbon that is inaccessible when valine is present.

The addition of R47L to V1 and A1, producing V2 and A2 (**Figure 3.2B**), increased both the substrate turnover and coupling efficiency (**Figure 3.5A**). The R47L mutation is located at the surface of the substrate access channel (**Figure 3.3**). In the wild-type enzyme, the charged Arg47 residue interacts with the carboxyl group of fatty acid substrates[180, 453] and its replacement with the neutral leucine residue has been shown to improve activity with hydrophobic substrates, presumably by permitting a higher rate of substrate entry into the access channel[181]. The increase in noscapine turnover seen in **Figure 3.5A** is consistent with this mechanism, however, this change in substrate entry does not explain the change in product distribution seen for V2 (11% increase in *N*-demethylated noscapine compared to V1; **Figure 3.5B**) or the coupling efficiency improvements seen for both A2 and V2.

The clear benefit of the A74G mutation to noscapine metabolism was shown by the back-mutation of A74G to the wild-type Ala74 in two mutants (producing V4A and A4A; **Figure 3.2B**) and the associated 4-7% drop in coupling efficiency and 10-13% decrease in noscapine turnover (**Figure 3.5A**). The A74G mutation is at the *N*-terminus of the short B'-helix, which caps the active site (**Figure 3.3**). The mutation is not included in any available crystal structures and it is unknown if it produces significant structural changes. Removal of the Ala74 side chain may allow increased flexibility of the *N*-terminal region of the B'-helix through reduced steric interactions[183], improving noscapine accommodation within the active site.

Whilst the F81I mutation has been used to improve P450_{BM3} activity on several drug substrates[161, 188], this had varied effects depending on the F87A/V background applied here. Noscapine turnover increased in combination with F87V (i.e. between mutants V2 and V3) but decreased with F87A (between A2 and A3), yet the coupling efficiency improved in both cases (**Figure 3.5A**). Like the A74G

mutation, the F81I mutation reduces hydrophobic interactions of the B'-helix and likely permits greater flexibility of the P450_{BM3} lid region[439].

The E64G mutation also produced mixed effects, having little significant effect on noscapine turnover and coupling efficiency when combined with the F87V mutation (i.e. between V3 and V4) but increasing turnover and coupling with F87A (between A3 and A4). This mutation is located away from the active site (**Figure 3.3**) and has been suggested to affect dynamic interactions between the haem and reductase domains of P450_{BM3}[189].

The S72D mutation abolished all noscapine metabolism when introduced into the A3 mutant, producing A3D (**Figure 3.2B**; A3D is excluded from **Figure 3.5** as it showed no substrate turnover). This is in contrast to a P450_{BM3} library screen with 43 drugs, where this mutation was shown to generally improve the metabolism of uncharged substrates[188]. Ser72 is located at the junction of the access channel and active site pocket and has been suggested by computational studies to have a hydrogen bond-mediated anchoring effect on some drug substrates during catalysis[454, 455]. While noscapine has eight hydrogen bond acceptors and might therefore participate in H-bonding interactions with Ser72, it seems unlikely that eliminating these interactions would alone be responsible for the complete abolition of noscapine metabolism observed here. The introduction of a negatively charged aspartate residue at the entrance to the active site more likely obstructs the bulky and hydrophobic noscapine molecule, preventing noscapine from making a close approach to the haem.

The addition of the L86I mutation to the R47L/A74G/F81I/F87A/L188Q/E267V background of A3, producing A3I, deteriorated both the noscapine turnover and coupling efficiency (**Figure 3.5A**). This is in contrast to the enhanced *N*-demethylation of 3,4-methylenedioxymethamphetamine (MDMA)[161] observed when L86I was added to a R47L/F87V/L188Q/E267V background. This illustrates the importance of the context in which mutations are introduced.

***In silico* docking**

Molecular docking studies with selected active P450_{BM3} mutants and noscapine were performed to rationalise the enzyme activity observed in the screening experiments. An induced fit docking method was used which allows for movement of side chains in the vicinity of the docked ligand. The PDB structure 4ZF6[451] (P450_{BM3} pentamutant R47L/F87V/L188Q/E267V/F81I) was used to model the receptor, as this crystal structure contains similar mutations to the active P450_{BM3} variants examined in this work, most closely resembling V3 but without the A74G mutation. We repeated the docking after the *in silico* mutation of Val87 to alanine to test whether the docking poses supported our experimental observations, as the replacement of F87V with F87A caused significant and consistent

changes in noscapine turnover, regioselectivity and coupling efficiency (i.e. these measurements were typically higher for A1-A4 compared to V1-V4).

The docking studies produced substrate poses with the noscapine *N*-methyl group proximal to the haem iron, consistent with *N*-demethylation (**Figure 3.6A**). Replacement of Val87 with alanine allowed a closer approach of the *N*-methyl group to the haem (3.1 Å vs 3.4 Å) with the methyl group located more centrally above the iron atom (**Figure 3.6A**), consistent with the improved *N*-demethylation activity typically seen in the F87A-containing mutants (i.e. A1-A4 versus V1-V4; **Figure 3.5B**). The docking studies also predicted several orientations in which the methylenedioxy carbon was positioned close to the haem iron, an example of which is shown in **Figure 3.6B**. This position is consistent with the production of the cleaved methylenedioxy metabolite (**3**). The orientation also appeared independent of the amino acid present at position 87. The slightly lower abundance of this metabolite in the product profiles of A1-A4 compared to V1-V4 (**Figure 3.5E**) may be due to improved coupling efficiency of *N*-demethylation, shifting the product distribution towards *N*-demethylated noscapine (**1**).

The two hydroxylated metabolites (**2a/2b**) observed in the screening experiments were determined to be hydroxylated on the isoquinoline moiety of noscapine although the locations of the hydroxy groups could not be further resolved by LC-MS/MS. In the docking studies, two poses were produced with C-H groups of the isoquinoline moiety directed towards the haem: one with the carbon alpha to the nitrogen at position 7' being closest to the iron (2.9 Å; **Figure 3.6C**) and the other with the β -carbon at the 8' position closest (3.3 Å, **Figure 3.6D**). These poses are consistent with displacement of the proximal water ligand and initiation of the catalytic cycle, however the actual site of substrate oxidation depends on interactions with the ferryl oxygen of the reactive haem intermediate Compound I, which was not modelled in this work. The docking studies performed here may therefore be of limited use for the prediction of substrate oxidation sites. Other potential hydroxylated products include an *N*-oxide produced from an orientation similar to *N*-demethylation, as *N*-oxygenation has been observed alongside *N*-demethylation in the metabolism of several tertiary amine drugs by microsomal P450s[456, 457], an aromatic hydroxylation at the 9' position, or a stereoisomer pair at the 7' or 8' position.

The docking studies did not find any poses that would be consistent with C-C bond cleavage (i.e. oxidation of the 5' carbon producing metabolites **2** and **3**). This may be because the substrate orientation leading to C-C cleavage fits poorly within the active site or because these metabolites are produced through peroxide uncoupling, as discussed above.

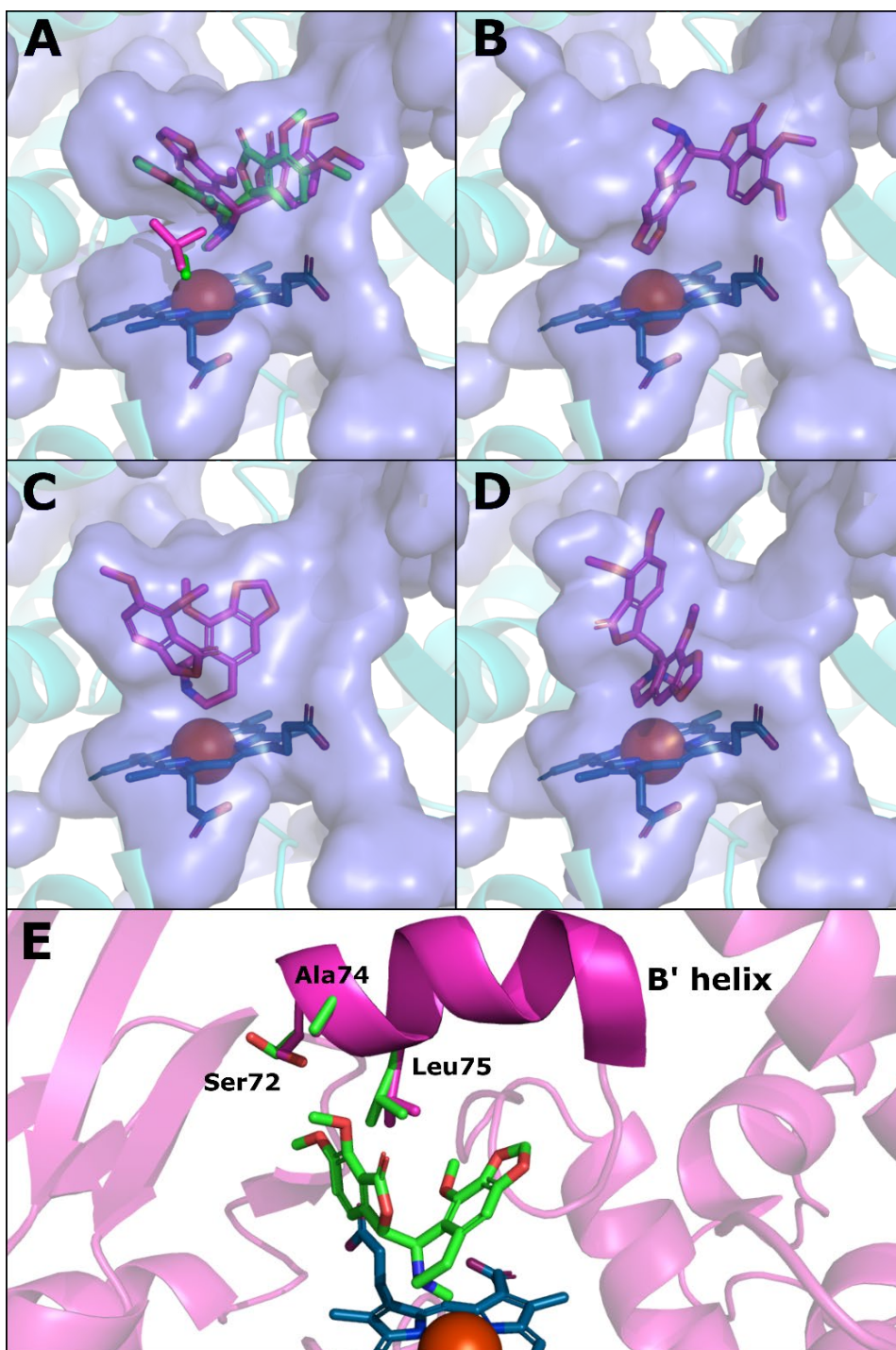


Figure 3.6. Substrate poses from induced-fit docking studies performed with P450_{BM3} mutants and noscapine. A P450_{BM3} pentamutant (R47L/F81I/F87V/L188Q/E267V; PDB: 4ZF6) was used to model the receptor. Docking runs were performed before and after *in silico* mutation of F87V to F87A; the F87A variant is shown unless otherwise noted. The blue surface represents the internal cavity of the active site. The haem group is represented as blue sticks with the iron atom shown as a brown sphere. **(A)** *N*-demethylation poses for the F87V (purple) and F87A (green) variants. The Ala87 and Val87 sidechains are shown in corresponding colours near the haem group. **(B)** One of the methylenedioxy bridge cleavage poses found. **(C)** Pose consistent with hydroxylation of the 7' carbon. **(D)** Pose consistent with hydroxylation of the 8' carbon. **(E)** *N*-demethylation pose showing greater detail of the Ser72, Ala74 and Leu75 sidechains. Sidechains shown in purple were shifted by the induced-fit protocol from the original positions shown in green. All docking poses had GlideScores between -8.5 and -6.5 kcal/mol.

The A74G mutation, which is not present in the 4ZF6 structure used for docking simulations, was shown by the enzyme screening experiments to significantly improve coupling efficiency and noscapine turnover (mutants V4A vs V4 and A4A vs A4; **Figure 3.5A**). The induced-fit docking algorithm shifted several side chains in the region of Ala74 on the B'-helix, most notably Ser72 and Leu75, to accommodate poses compatible with *N*-demethylation of noscapine (**Figure 3.6E**), suggesting that a close interaction between noscapine and this region of the active site occurs during substrate binding. Increased flexibility of this 'cap' region of the active site likely explains the improvements in turnover and coupling efficiency conferred by the A74G mutation, as well as the increase in coupling efficiency produced by the F81I mutation (mutants V3 vs V2 and A3 vs A2; **Figure 3.5A**). The close contact between noscapine and this area of the B'-helix can also explain the observation that replacing Ser72 with the larger, charged aspartate residue (S72D) noscapine activity in the A3 to A3D mutation (A3D is therefore excluded from **Figure 3.5**), as these interactions would likely be unable to occur.

The induced-fit docking studies highlight further mutations that may prove beneficial to P450_{BM3} activity. The orientation of Leu75 in the *N*-demethylation pose in **Figure 3.6E** suggests that this residue, positioned between the isoquinoline and phthalide moieties of noscapine, may be a bottleneck during substrate binding. Mutation of Leu75 to a smaller residue, such as alanine, has been shown to improve activity on several drug-like compounds[458, 459]; these mutations were not tested here and may improve noscapine activity. Recent molecular dynamics simulations suggest, however, that hydrophobic interactions between Leu75 and some drug substrates are important contributors to binding stability[460], making the interactions between this residue and noscapine difficult to predict.

Significant metabolites and mutants

In the context of noscapine analogue research, the most interesting metabolites produced in the mutant screen were *N*-demethylated noscapine (**1**) and the two hydroxylated metabolites (**2a**, **2b**). The P450_{BM3} variants with the highest regioselectivity for *N*-demethylation were A2 and A3 which both produced *N*-demethylated noscapine with a selectivity of 88% (**Figure 3.5B**). While A3 showed lower noscapine turnover than A2 (39% vs 50%), it was more efficient in terms of cofactor usage (16% vs 11%; **Figure 3.5A**) and is therefore the best candidate for producing *N*-demethylated noscapine at a preparative scale after further process optimization.

Hydroxylation at either the 7' or 8' position of noscapine, as suggested by the docking simulations, adds a new chiral centre to the noscapine scaffold which may be useful in developing more complex analogues that cannot easily be achieved by chemical synthesis. While the largest selectivity for a

hydroxylated metabolite was only 12%, observed for metabolite **2b** by mutant A3S (**Figure 3.5D**), this mutant had the highest noscapine turnover and one of the highest coupling efficiencies observed (59% and 19%, respectively; **Figure 3.5A**). Whilst the library screened here was relatively small, containing eighteen P450_{BM3} mutants, it illustrates the efficiency of applying previously described mutations to a new drug target. The introduction of further rational mutations, informed by the results presented here, may be used to further shift the enzyme selectivity towards **2a/2b** or other potential metabolites of interest.

The development and scale-up of the biotransformation presented here for the production of **1** or **2a/2b** could employ a number of strategies to further optimise yield and reduce operating costs which would be necessary for the future application of these mutants to preparative-scale *N*-demethylation. Cofactor recycling or a whole-cell biotransformation approach could be employed to reduce or eliminate the need for addition of NADPH, which is expensive above the small laboratory scale applied here. A substrate supply strategy[461], such as a two-liquid-phase system, could also be used to address the poor aqueous solubility of noscapine and improve product titres.

Conclusions

The high regioselectivity for *N*-demethylation exhibited by the P450_{BM3} mutant A3 developed here provides proof of concept for a potential alternative biocatalytic approach to the chemical synthesis of *N*-demethylated noscapine (**1**), a precursor for producing noscapine analogues with enhanced anticancer activity. Two hydroxylated metabolites were observed (**2a/2b**), with the highest production occurring for mutants V4A (**2a**) and A3S (**2b**). These metabolites may have chiral hydroxyl groups at previously uncharacterised positions on the noscapine scaffold and may be useful for the production of new classes of noscapine analogues. The study utilised a small mutant library constructed by combining mutations known to enhance P450_{BM3} activity on bulky, drug-like substrates, an approach that applies valuable information present in the literature to efficiently produce structural modifications of a desired drug target. The effects of the mutations on P450_{BM3} activity, together with docking simulations, provide functional insights into P450_{BM3}-mediated drug metabolism. A conformational transition of substrate-free P450_{BM3} to that of the wild-type, substrate-bound structure, induced by a salt-bridge disruption, appeared critical to metabolism of the non-natural noscapine substrate, as has been observed for other mutant/drug combinations. Noscapine turnover and coupling efficiency also depended on: (1) residue size at position 87, and (2) the flexibility of the active site cap region. The metabolite profile most closely resembled that of the human microsomal P450 CYP3A4 and these mutants could be used as a practical alternative to produce noscapine metabolites for drug metabolism studies. The mutant enzymes presented here

also have promise for use in a whole-cell biotransformation process, which once further optimised may allow for the efficient scale-up of metabolite production.

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Declarations of interest: None.

Author contributions: LR performed the experiments. AL assisted with metabolite analysis. DC assisted with modelling work. AL, DC, AJ, TB, GS and SG helped with experimental design and provided technical advice. LR and SG wrote the manuscript with input from all authors.

3.2. Supplementary Material

Table 3.1. List of oligonucleotide primers used in this study. For *BM3_fwd_BamHI* and *BM3_rev_XhoI*, the introduced restriction site sequences are shown in red. For all other primers, red text represents mutated bases relative to the wild-type P450_{BM3} sequence (Genbank accession CP009920.1).

Name	Sequence (5' -> 3')
<i>BM3_fwd_BamHI</i>	TAAGCA GGATCC ACAATTAAAGAAATGCCTCAGC
<i>BM3_rev_XhoI</i>	TACATA CTCGAG TTACCCAGCCCACACGTC
<i>F87A_fwd</i>	AGACGGGTTA GCG ACAAGCTGGAC
<i>F87A_rev</i>	CCTGCAAAATCACGTACAAATTTAAG
<i>E267V_fwd</i>	TGCGGGACACG TG ACAACAAGTG
<i>R47L_fwd</i>	GGCGCCTGGTCT TG GTAACGCGCT
<i>F81I_fwd</i>	TGTACGTGAT AT TGCAGGAGACG
<i>E64G_fwd</i>	AGCATGCGATG GCT CACGCTTTG
<i>G74A_fwd</i>	CTTAAGTCAAGCGCTTAAATTTG
<i>L86I_87V_fwd</i>	AGGAGACGGG ATTGCG ACAAGCTG
<i>L86I_87A_fwd</i>	AGGAGACGGG ATTG TTACAAGCTG
<i>S72D_fwd</i>	TAAAAACTTA GAT CAAG GG CTTAAATTTGTACG
<i>L437S_fwd</i>	TAAAGAACT AGC ACGTTAAACCTGAAG

Table 3.2. Identification of the noscapine metabolites detected in LC-MS/MS screening experiments of P450_{BM3} metabolism of noscapine. The chromatographic retention time is given, together with the observed mass and the name and formula of the identified compound. Metabolite numbers used throughout this work are also provided where appropriate.

Retention time (min)	Targeted precursor m/z	Formula	Observed m/z	Mass error (ppm)	Identification	Metabolite number
4.24	402.1547	[C ₂₁ H ₂₄ NO ₇] ⁺	402.1543	-1.18	Cleavage of methylenedioxy bridge	3
4.40	388.1391	[C ₂₀ H ₂₂ NO ₇] ⁺	388.1377	3.56	Cleavage of methylenedioxy bridge and demethylated	-
5.05	220.0968	[C ₁₂ H ₁₄ NO ₃] ⁺	220.0959	4.20	Cotarnine	4
5.75	195.0652	[C ₁₀ H ₁₁ O ₄] ⁺	195.0652	-0.08	Meconine	5
6.60	416.1340	[C ₂₁ H ₂₂ NO ₈] ⁺	416.1347	-1.70	Demethylated and hydroxylated	-
7.00	416.1340	[C ₂₁ H ₂₂ NO ₈] ⁺	416.1349	-2.18	Demethylated and hydroxylated	-
7.12	430.1496	[C ₂₂ H ₂₄ NO ₈] ⁺	430.1494	0.57	Hydroxylated	2a
7.32	400.1391	[C ₂₁ H ₂₂ NO ₇] ⁺	400.1389	0.45	Demethylated	1
7.70	414.1547	[C ₂₂ H ₂₄ NO ₇] ⁺	414.1558	-2.59	Noscapine	-
8.10	430.1496	[C ₂₂ H ₂₄ NO ₈] ⁺	430.1504	-1.76	Hydroxylated	2b

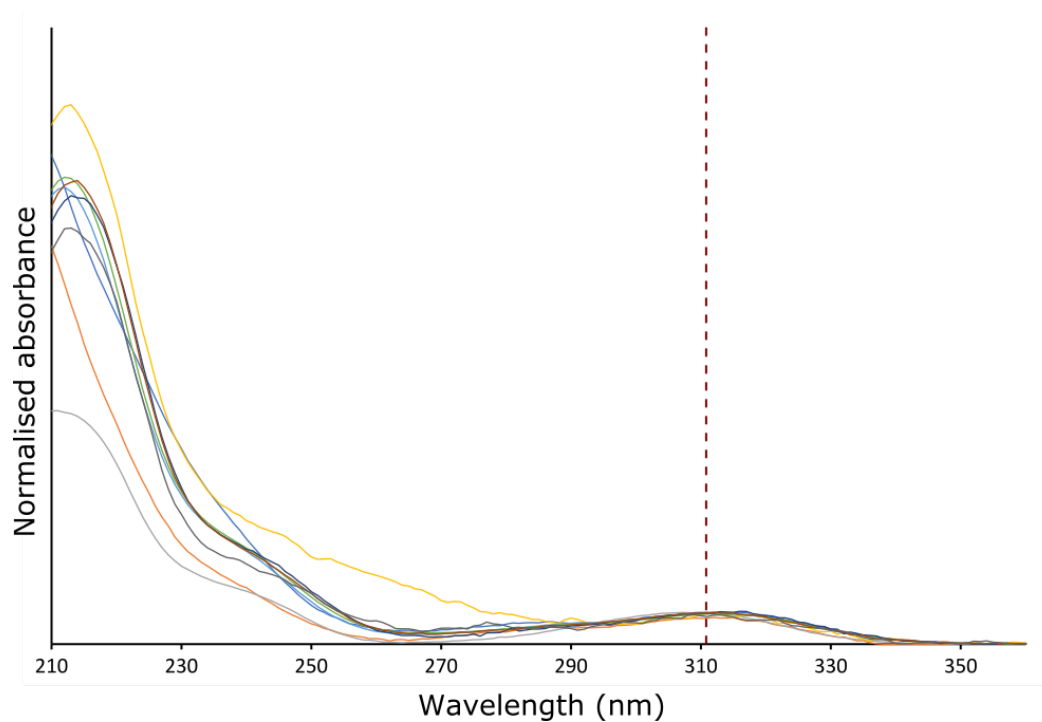


Figure 3.7. Normalised UV absorption spectra for the nine metabolites quantified by UV peak integration. Quantification was performed at 311 nm (indicated by the dashed line), as all metabolites share a similar broad peak in this region. Spectra were normalised by dividing the absorption spectrum at the peak apex by the peak height.

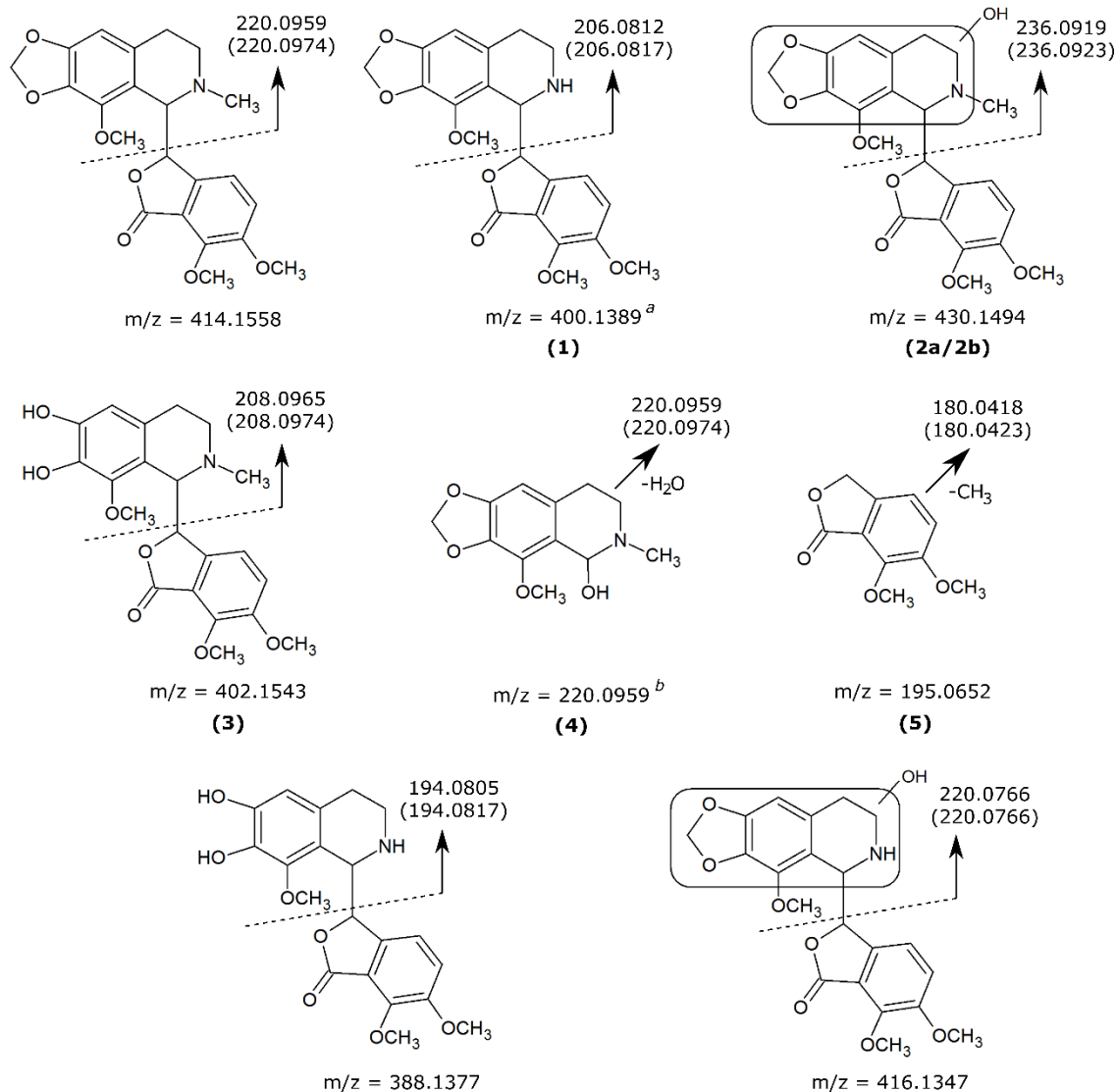


Figure 3.8. Metabolite identification based on LC-MS/MS data for the compounds listed in **Table 3.2**. The m/z of the parent compound is given below each structure. A unique MS/MS transition is shown for each metabolite, with the detected fragment indicated by a dashed line (where appropriate) and solid arrow. The observed m/z for each fragment is indicated; the calculated m/z is shown in parentheses. Metabolite numbers used throughout this work are provided in bold where appropriate. Fragments for metabolites **4** and **5** were produced by loss of a water molecule and methyl group, respectively.

^aThe demethylation site was confirmed by comparison to a *N*-demethylated noscapine standard (**Figure 3.9**).

^bThe expected m/z for cotarnine (**4**) is 238.1, however, no ion of this m/z was detected. It was shown by Tsunoda & Yashimura that cotarnine does not produce a detectable ion at m/z 238 under conditions of chemical ionisation and this metabolite instead produces a major fragment at m/z 220[115] from loss of a water molecule. This has also been observed with electrospray ionisation for noscapine[27] and noscapine analogues[117, 118].

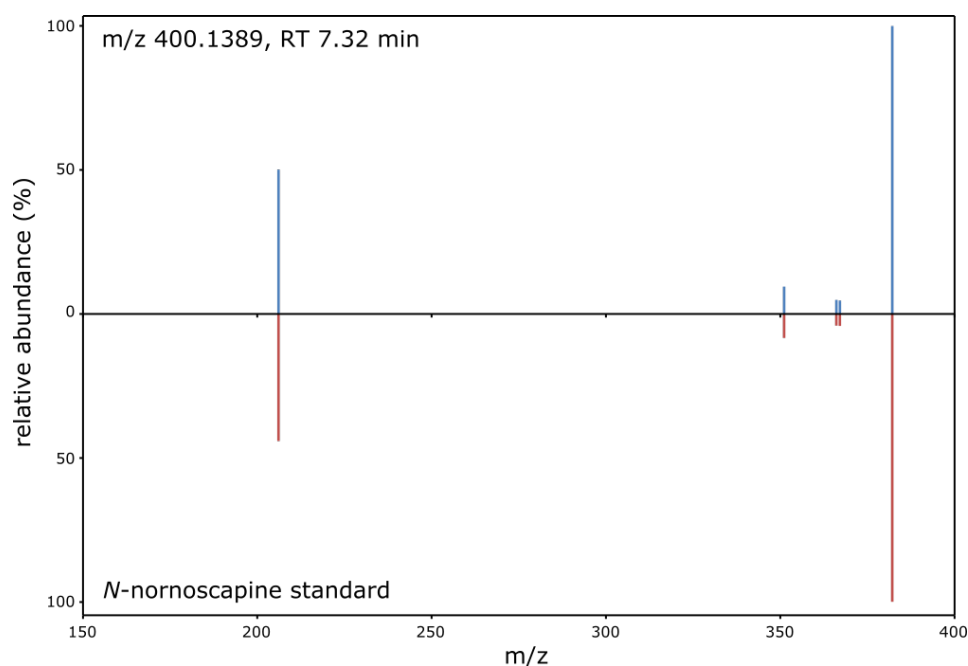


Figure 3.9. MS/MS fragment mirror comparison of the metabolite detected at RT 7.32 min (metabolite **1**) with a *N*-demethylated noscapine standard (*N*-nornoscapine). The five most abundant fragments are shown; two appear close together at m/z values of 366.1 and 367.1.

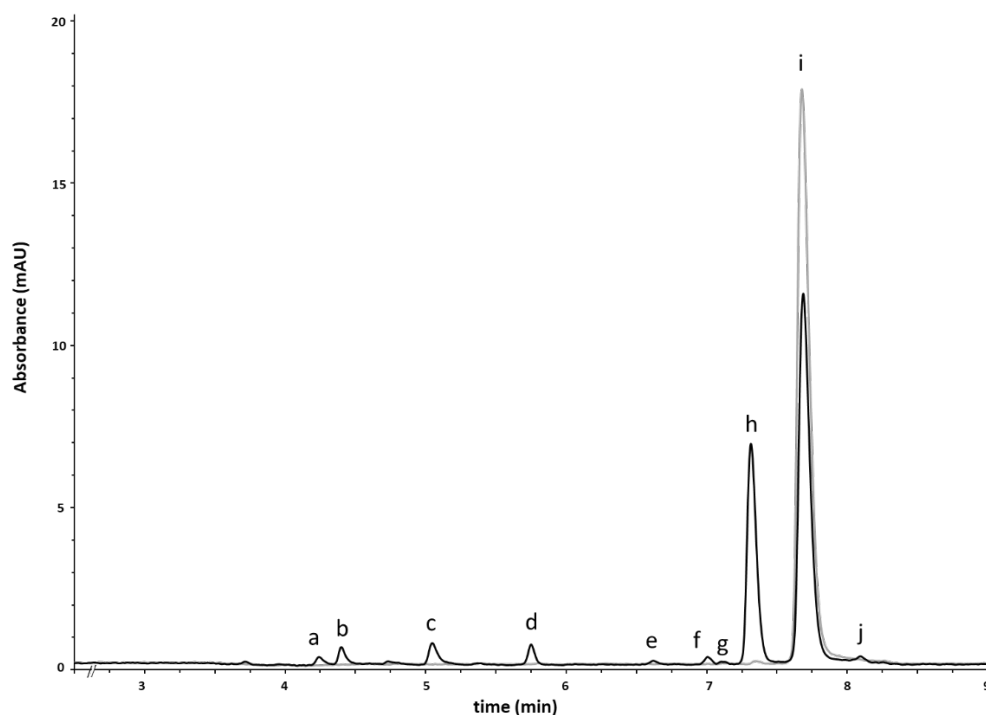


Figure 3.10. UHPLC chromatogram showing noscapine turnover by mutant A3 (black). Control without NADPH addition is shown in grey. Metabolite peaks: a, cleavage of methylenedioxy bridge (**3**); b, cleavage of methylenedioxy bridge and demethylated; c, cotarnine (**4**); d, meconine (**5**); e, demethylated and hydroxylated; f, demethylated and hydroxylated; g, hydroxylated (**2a**); h, *N*-demethylated (**1**); i, noscapine; j, hydroxylated (**2b**).

Table 3.3. Numerical data from screening experiments for the 12 active P450_{BM3} mutants. Three replicates were used for each mutant; errors are $\pm$ SE.

	% product distribution						
	Noscapine metabolised (%)	Coupling efficiency (%)	<i>N</i> -nornoscapine (1)	Hydroxylated (2a)	Hydroxylated (2b)	Cleaved methylenedioxy (3)	Meconine (5)
V1	22.3 $\pm$ 0.1	3.3 $\pm$ 0.2	57.8 $\pm$ 2.1	3.1 $\pm$ 0.2	-	6.3 $\pm$ 0.2	32.8 $\pm$ 1.0
V2	27.0 $\pm$ 0.7	5.8 $\pm$ 0.3	68.8 $\pm$ 1.4	3.5 $\pm$ 0.2	-	4.7 $\pm$ 0.2	23.0 $\pm$ 2.0
V3	38.3 $\pm$ 0.6	8.8 $\pm$ 0.5	73.7 $\pm$ 1.4	3.0 $\pm$ 0.0	-	7.1 $\pm$ 0.2	16.2 $\pm$ 0.6
V4	36.4 $\pm$ 0.8	8.8 $\pm$ 0.5	70.2 $\pm$ 1.7	2.9 $\pm$ 0.1	-	10.0 $\pm$ 0.5	17.0 $\pm$ 1.8
V4A	21.2 $\pm$ 1.1	4.9 $\pm$ 0.6	62.4 $\pm$ 7.0	4.5 $\pm$ 0.5	-	6.3 $\pm$ 0.7	26.7 $\pm$ 2.3
A1	37.5 $\pm$ 1.8	9.5 $\pm$ 0.5	85.3 $\pm$ 2.4	2.7 $\pm$ 0.0	1.3 $\pm$ 0.0	3.7 $\pm$ 0.2	7.1 $\pm$ 0.2
A2	50.4 $\pm$ 1.6	11.2 $\pm$ 0.5	88.0 $\pm$ 3.4	1.8 $\pm$ 0.1	1.2 $\pm$ 0.1	2.8 $\pm$ 0.1	6.2 $\pm$ 0.2
A3	39.3 $\pm$ 0.6	15.6 $\pm$ 0.8	88.1 $\pm$ 3.4	1.1 $\pm$ 0.1	1.8 $\pm$ 0.2	2.8 $\pm$ 0.1	6.3 $\pm$ 0.2
A4	44.6 $\pm$ 0.7	17.2 $\pm$ 0.9	84.8 $\pm$ 1.8	1.2 $\pm$ 0.1	2.0 $\pm$ 0.1	3.4 $\pm$ 0.7	8.6 $\pm$ 0.3
A4A	31.4 $\pm$ 1.1	10.7 $\pm$ 0.7	64.8 $\pm$ 1.0	1.3 $\pm$ 0.0	5.5 $\pm$ 0.3	6.5 $\pm$ 0.4	21.9 $\pm$ 1.0
A3I	29.4 $\pm$ 0.8	13.9 $\pm$ 0.6	82.5 $\pm$ 1.7	1.4 $\pm$ 0.0	0.8 $\pm$ 0.1	8.4 $\pm$ 0.3	7.0 $\pm$ 0.1
A3S	59.1 $\pm$ 1.9	22.0 $\pm$ 1.4	59.3 $\pm$ 1.3	1.6 $\pm$ 0.1	11.7 $\pm$ 0.3	10.0 $\pm$ 0.4	17.3 $\pm$ 0.3

3.3. Further experiments

Following the work described in **Paper 1**, several further mutations were made to the P450_{BM3} mutants A2 and A3 in an effort to improve the noscapine-metabolising activity of these enzymes. These new mutants were tested in both whole-cell biotransformation and *in vitro* screening experiments. While some of these mutations provided improved activity *in vitro*, they did not improve the productivity of a whole-cell biotransformation system and were not further investigated. These experiments are presented in **Appendix A** and **Appendix B**.

A further experiment was performed in which a P450_{BM3} mutant was co-expressed in *E. coli* with two reactive oxygen species (ROS) scavenger enzymes, catalase and superoxide dismutase. This was done to assess whether whole-cell biotransformation activity could be improved by negating ROS production from the P450_{BM3} mutant inside the *E. coli* cells. This strategy did not provide any improvement in biotransformation productivity and is described in **Appendix C**.

4. Development of a whole-cell biotransformation process for the *N*-demethylation of noscapine

4.1. Paper 2

Cytochrome P450-mediated *N*-demethylation of noscapine by whole-cell biotransformation: process limitations and strategies for optimisation

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ABSTRACT

Cytochrome P450 enzymes catalyse reactions of significant industrial interest but are underutilised in large-scale bioprocesses due to enzyme stability, cofactor requirements and the poor aqueous solubility and microbial toxicity of typical substrates and products. In this work, we investigate the potential for preparative-scale *N*-demethylation of the opium poppy alkaloid noscapine by a P450_{BM3} (CYP102A1) mutant enzyme in a whole-cell biotransformation system. We identify and address several common limitations of whole-cell P450 biotransformations using this model *N*-demethylation process. Mass transfer into *Escherichia coli* cells was found to be a major limitation of biotransformation rate and an alternative Gram-positive expression host *Bacillus megaterium* provided a 25-fold improvement in specific initial rate. Two methods were investigated to address poor substrate solubility. First, a biphasic biotransformation system was developed by systematic selection of potentially biocompatible solvents and *in silico* solubility modelling using Hansen solubility parameters. The best-performing biphasic system gave a 2.3-fold improvement in final product titre compared to a single-phase system but had slower initial rates of biotransformation due to low substrate concentration in the aqueous phase. The second strategy aimed to improve aqueous substrate solubility using cyclodextrin and hydrophilic polymers. This approach provided a five-fold improvement in initial biotransformation rate and allowed a 6.3-fold

increase in final product concentration. Enzyme stability and cell viability were identified as the next parameters requiring optimisation to improve productivity. The approaches used are also applicable to the development of other pharmaceutical P450-mediated biotransformations.

Keywords: P450_{BM3}, *Bacillus megaterium*, mass transfer, biphasic, cyclodextrin.

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Author contribution statement: LR performed the experiments. TB, AJ, GS and SG helped with experimental design and provided technical advice. LR and SG wrote the manuscript with input from all authors.

INTRODUCTION

Cytochrome P450 enzymes (P450s) are promising enzymes for industrial and pharmaceutical biotechnology due to their ability to catalyse regioselective oxidations that are often difficult to achieve with synthetic organic chemistry [126]. While a vast array of useful P450 reactions have been identified, this enzyme family remains underutilised in industrial biocatalysis due to a range of typical process limitations [29, 39].

The relatively poor stability of many P450 enzymes, as well as their requirement for expensive NADPH cofactor, has led to the preference of whole-cell biotransformations over *in vitro* systems for preparative applications, as cells can provide both a protective environment for the enzyme and supply NADPH through metabolic regeneration [35]. While effective in this respect, whole-cell approaches have other limitations; the mass transfer of substrate into cells can be poor and substrate and product toxicity can negatively affect the microbial host [36].

The rate of mass transfer of substrate across cell wall structures is often a significant limiting factor for biotransformations in whole-cell systems [38]. Both the rate and mechanism of mass transfer are strongly dependent on the physicochemical properties of the substrate (size, hydrophobicity, ionisation state) and the structural composition of the cell wall and membrane, which varies between organisms.

Substrates for P450s, particularly those for pharmaceutical applications, are often poorly water soluble, which is problematic for whole-cell biotransformations which take place in an aqueous environment. A common approach to tackle this issue is the use of biphasic biotransformation systems, which utilise an immiscible organic solvent phase in contact with the aqueous phase to act as a substrate reservoir [261]. Solvent selection is restricted, however, by the need for biocompatibility with the host organism, good substrate solubility in the solvent and favourable partitioning of the substrate into the aqueous phase. Other approaches to improve substrate loading focus on enhancing aqueous solubility, such as the use of miscible organic co-solvents, cyclodextrins [462] and recently the use of hydrophilic polymers [463].

In a previous work (**Paper 1**; [464]), we screened a library of mutants of the bacterial P450 enzyme P450_{BM3} (CYP102A1) and identified a variant capable of selective (88%) *N*-demethylation of the opium poppy alkaloid noscapine, a drug with anticancer properties, at analytical scale. This *N*-demethylated noscapine (*N*-nornoscapine) product is an essential precursor for *N*-modified noscapine analogues that have stronger antitumour activity [112, 119, 465] and have recently been shown to have antiparasitic activity [466]. In this work, we have investigated the potential for preparative-scale noscapine *N*-demethylation

in a whole-cell biotransformation system using this P450_{BM3} mutant. We identify key factors limiting biotransformation and test several experimental approaches to address some of these limitations relating to mass transfer and substrate solubility.

Materials and Methods

Chemicals, reagents and strains

Noscapine was provided by Sun Pharmaceutical Industries (Port Fairy, Australia). Molecular biology enzymes, nucleic acid purification kits and competent *E. coli* cells were purchased from New England Biolabs (Notting Hill, Australia). Primers for polymerase chain reactions (PCR) were synthesised by Integrated DNA Technologies (Singapore). Complex growth media components were Oxoid brand (Thermo Fisher Scientific, Scoresby, Australia). Lysozyme (chicken egg white) and achromopeptidase were purchased from Sigma-Aldrich (Castle Hill, Australia). Water used was of MilliQ grade (resistivity 18.2 mΩ). Methanol used for chromatographic separations was chromatography grade (Merck, Bayswater, Australia). Polyvinylpyrrolidone (average M_w 40,000), (hydroxypropyl)methylcellulose (average M_n 86,000), (2-hydroxypropyl)- β -cyclodextrin (average M_w 1460) and all other chemicals were purchased from Sigma-Aldrich. *Bacillus megaterium* strain ATCC 14581 was obtained from the American Type Culture Collection (Manassas, VA, USA). *B. megaterium* strain DSM-319 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The plasmid pSPYngK-hp was a gift from Dieter Jahn (Addgene plasmid #48118).

Intracellular noscapine accumulation

Accumulation of cellular noscapine was measured using a method adapted from Williams and Piddock [467]. Glycerol stocks of *B. megaterium* ATCC 14589 and *E. coli* BL121(DE3) were used to inoculate shake flasks containing 50 mL and 120 mL of lysogeny broth (LB), respectively, which were shaken at 200 rpm and 30°C for *B. megaterium* and 37°C for *E. coli* for 14 h. Cells were collected by centrifugation at 3,200 x *g* and 10°C for 20 min, washed twice with 2 x 10 mL potassium phosphate buffer (50 mM, pH 7.4) and resuspended in 1 mL of the same buffer. To measure noscapine uptake, a sample of 50 μ L of concentrated cells was added to 200 μ L of room-temperature phosphate buffer containing 100 μ M noscapine (added from methanol stock; final MeOH concentration 1%). After the appropriate incubation time, 200 μ L of this solution was rapidly cooled by mixing with 1 mL of phosphate buffer that was pre-chilled at -4°C in an ice/salt bath. The mixture was then immediately centrifuged at 20,000 x *g* for 30 s at 4°C. The supernatant was removed and the cell pellet was washed with a further 1 mL of pre-chilled buffer and centrifuged

again. The wash buffer was completely removed and the pellet was stored in an ice/salt bath at -4°C until all samples were collected. To extract the accumulated noscapine, 100 μL of ice-cold methanol was added to each cell pellet and the pellets were incubated on ice for 20 min with brief vortexing every 5 min. The samples were then centrifuged at $18,000 \times g$ for 15 min at 4°C to remove cell debris and then 30 μL of the supernatant was added to 70 μL of 0.1% trifluoroacetic acid (TFA) for analysis by ultra-high-performance liquid chromatography with a diode array detector (UHPLC-DAD) as previously described [464]. To estimate the amount of recovered noscapine that was adhered to the outside of cells rather than intracellularly located, the experiment was performed as above but the cell concentrate was added to noscapine/buffer solution that was pre-chilled at -4°C (a temperature at which no substrate is expected to cross the cell membrane [467]), incubated at -4°C for 3 min and then processed as before. Longer incubation times did not show any further increase in noscapine in the cell pellets. All noscapine accumulation experiments were performed in triplicate.

Plasmid assembly and transformation

A pET28a(+) vector (Merck, Australia) containing the gene for P450_{BM3} mutant A3 (R47L/A74G/F81I/F87A/L188Q/E267V) was constructed in previous work (**Paper 1**; [464]) and used here for recombinant expression in *E. coli* BL21(DE3). For expression of A3 in *B. megaterium*, the xylose-inducible expression vector p3STOP1632hp [364] was used as a backbone. The p3STOP1623hp backbone was obtained by PCR amplification of pSPYngK-hp [364] using a primer pair with sequences 5'-GGTACCGGCCGCATGCCG-3' and 5'-TGTACATTCACCTCCTTGATTTAAGTGAACAAGTTTATCCATCAAC-3'. The A3 coding sequence was amplified from the pET28a (+) *E. coli* expression vector by PCR using a primer pair with sequences 5'-ATCAAGGAGGTGAATGTACAATGACAATTAAAGAAATGCCTCAGC-3' and 5'-GCCGGCATGCGGCCGGTACCTTACCCAGCCCACACGTC-3'. The backbone and insert were assembled using the NEBuilder HiFi DNA assembly kit (New England Biolabs) and transformed into chemically competent DH5- α cells for cloning. An empty p3STOP1632hp control vector was made by self-ligation of the amplified backbone using KLD Enzyme Mix (New England Biolabs). Protoplasts of *B. megaterium* strain DSM-319 were prepared and transformed according to Biedendieck *et al.* [408].

Noscapine solubility

For measurement of noscapine solubility in organic solvents, excess solid noscapine was added to 1 mL of solvent in glass vials and shaken at 200 rpm at ambient temperature for 24 h. The vials were then left for a further 48 h without shaking to allow sedimentation of undissolved noscapine. The saturated solvents

were diluted into 100% methanol and noscaphine was quantified by UHPLC-DAD, as previously described [464]. For solubility measurements in cyclodextrin and polymer solutions, 40 μ L of a 100 mM noscaphine stock in 5% tartaric acid was added to 1.96 mL of cyclodextrin and/or polymer in sodium/potassium phosphate buffer (100 mM, pH 6.75) to give a final noscaphine concentration of 2 mM. Dilute tartaric acid was selected as a solvent for the noscaphine stock, due to its demonstrated ability to improve cyclodextrin solubilisation of weakly basic drugs [468]. The addition of the tartaric acid solution decreased the sample pH to 6.5. The solutions were equilibrated by shaking at 230 rpm at ambient temperature for 5 days, after which no change in noscaphine solubility was found to occur. The solutions were filtered through a 0.45 μ m nylon filter and diluted with 0.1% TFA before quantification by UHPLC-DAD.

Solubility modelling and *in silico* solvent screening

The solubility of noscaphine in organic solvents was modelled by the modified Hansen parameter method developed by Louwerse *et al.* [469] using the associated MATLAB script written by the authors (provided in Supplementary Material for [469]). Empirical noscaphine solubility measurements were made in 20 organic solvents, as described above, and used as inputs for the script program. Hansen parameters and molecular radii for solvents were taken from the HSPiP database (<https://www.hansen-solubility.com/HSPiP/>) and hydrogen bonding parameters were calculated manually, as described by Louwerse *et al.* [469]. The solubility measurements were provided as inputs to the program by grouping them into order-of-magnitude solubility intervals (i.e. 1-10 mM, 11-100 mM etc.) and providing a 'yes' or 'no' term for each tested solvent in each interval. The program was then used to estimate Hansen solubility parameters for noscaphine based on the empirical solubility measurements. These parameters were subsequently used as inputs for the program to predict noscaphine solubility in a range of water-immiscible solvents with potential utility in biphasic biotransformation systems.

Whole-cell biotransformation

Cultures of *E. coli* BL21(DE3) containing the mutant P450_{BM3} enzyme A3 were prepared as described previously [464]. For protein expression in *B. megaterium* DSM-319, 50 mL of LB medium supplemented with 10 μ g/mL tetracycline was inoculated with a glycerol stock of recombinant *B. megaterium* and shaken at 100 rpm and 37°C overnight. A 1 mL volume of the overnight culture was used to inoculate 100 mL of Terrific Broth (TB) with 10 μ g/mL tetracycline in a 500 mL shake flask and shaken at 230 rpm and 37°C until the optical density at a wavelength of 600 nm (OD₆₀₀) reached approximately 0.3. The culture was then cooled to 30°C and expression was induced by the addition of 0.5% (w/v) xylose. Expression was

continued for 20 h with 130 rpm shaking at 30°C. Following expression, cells were collected by centrifugation at 3,200 x *g* and 10°C for 20 min and the cell pellets were washed with 2 x 10 mL of nitrogen-free modified M9 medium (4.24 g/L Na₂HPO₄, 8.86 g/L KH₂PO₄, 2.5 g/L NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.4% w/v glucose; pH 6.5). The cells were then resuspended in the same medium and diluted to give an OD₆₀₀ of 5 for comparison of specific enzymatic rate with *E. coli*, or an OD₆₀₀ of 35 for all other experiments. For biotransformation in the cyclodextrin/polymer solution, cells were resuspended in the same medium supplemented with 10% (w/v) (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) and 0.01% (w/v) hydroxypropyl methylcellulose (HPMC) and with the phosphate buffer components adjusted to give an initial pH of 6.65. Biotransformations were carried out in 2 mL volumes in 28 mL glass vials (LabTek, Brendale, Australia) at 230 rpm and 25°C. Reactions were initiated by the addition of nescapine from a methanol stock (final MeOH concentration 1%) to give a nescapine concentration of 100 μM, or, for HP-β-CD/HPMC systems, by the addition of nescapine from a tartaric acid solution (final tartaric acid concentration 0.05%); the addition of tartaric acid lowered the pH to 6.5. For biphasic systems, a further addition of 0.5 mL of organic solvent saturated with nescapine was also made. At each sampling interval, 80 μL of aqueous phase and for biphasic systems, 20 μL of organic phase were removed and centrifuged at 16,000 x *g* for 5 minutes. Cleared aqueous phase was diluted into a solution of 0.1% formic acid and 25% methanol and organic phase into 100% methanol for analysis by UPLC-UV-MS as below. For colony-forming unit (CFU) counts, samples were serially diluted in 0.85% saline and plated at 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions onto LB agar and incubated at 37°C. Glucose concentrations in the culture supernatant were measured spectroscopically using a glucose hexokinase (HK) assay kit (Sigma Aldrich, Castle Hill, Australia). All biotransformation experiments were performed in triplicate.

Enzyme stability

To provide sufficient sample volumes for enzyme stability experiments, biotransformation reactions were performed essentially as above but scaled up to a volume of 13 mL and experiments conducted in 250 mL shake flasks. One set of three replicates received 1% methanol without nescapine; one set was gently agitated at 60 rpm in round-bottom flasks; reactions were otherwise carried out in baffled flasks at 25°C and shaken at 230 rpm with 100 μM nescapine added from methanol stock (final MeOH concentration 1%). At each sampling point, two 0.5 mL samples were removed for protein analysis and one 0.7 mL sample was removed for dry biomass measurement. The samples were centrifuged at 16,000 x *g* for 5 min, the supernatant removed and pellets frozen at -20°C for biomass and SDS-PAGE analysis or -80°C for cytochrome P450 measurement. For P450 quantification, the cell pellets were thawed and washed with

ice-cold potassium phosphate buffer (100 mM, pH 7.8), centrifuged and the supernatant removed. The pellets were resuspended in 50 μ L of 2 mg/mL lysozyme and 0.2 mg/mL achromopeptidase in phosphate buffer (pH 7.8) and gently agitated for 45 min at room temperature. The suspensions were then diluted with 1 mL ice-cold potassium phosphate buffer (50 mM, pH 7.4), incubated on ice for 5 min and then sonicated (1.6 mm probe, 30% amplitude, 5 s on, 10 s off for six cycles) using a QSonica Q500 machine (QSonica LLC, Newtown, CT, USA). The lysate was then separated by centrifugation at 160,000 $\times g$ for 40 min (Optima MAX ultracentrifuge; Beckman-Coulter, Mount Waverley, Australia) and the supernatant was analysed for P450 content using carbon monoxide (CO) difference spectroscopy according to the method of Omura and Sato [437]. No cytochrome P450 was detected by this method for control cells containing empty vector. For measurement of dry biomass weights, the cell pellets were resuspended in water, transferred to pre-weighed aluminium pans and dried in an oven for 24 h at 105°C. Dried biomass was cooled in a vacuum desiccator over silica beads before weighing. Cell pellets for SDS-PAGE analysis were lysed according to the method of Barg *et al.* [470] and soluble protein fractions were run on a Bolt 4-12% Bis-Tris gel (Thermo Fisher Scientific, Scoresby, Australia). Gel loading volumes were adjusted to give an equal equivalent of dry biomass weight per lane and bands were visualised with Coomassie Brilliant Blue.

Substrate and product quantification

Quantification of substrate and product concentration was performed using an ACQUITY UPLC H-Class system equipped with PDA and QDa detectors. Analytes were separated with a Kinetix biphenyl column (1.7 mm, 100 Å, 100 $\times$ 2.1 mm; Phenomenex, Lane Cove, Australia) maintained at 55°C with a flow rate of 0.3 mL/min. A binary gradient method was used, where the mobile phase consisted of (A) 0.1% formic acid and 10 mM ammonium formate in water and (B) 0.1% formic acid and 10 mM ammonium formate in methanol. The gradient elution program was as follows: isocratic at 25% solvent B for 30 s; 25 to 40% solvent B gradient over 30 s; 40 to 70% solvent B gradient over nine minutes; stepped to 95% solvent B and held for one minute; then re-equilibration at 25% solvent B for four minutes. The QDa detector was operated in positive ion mode. Absorbance was monitored between 210-400 nm and a wavelength of 311 nm was used for substrate and product quantification. The products were identified by comparison of mass and retention time data with previous qTOF LC-MS/MS experiments (**Paper 1**; [464]).

Results and discussion

Microbial host selection

Substrate permeability

Our previous screening studies using cell lysates identified a P450_{BM3} pentamutant (R47L/A74G/F81I/F87A/L188Q), designated 'A3', capable of noscapine *N*-demethylation with good (88%) selectivity. To investigate the scale-up potential of this reaction, we aimed to incorporate the A3 mutant into a whole-cell biotransformation process for the *N*-demethylation of noscapine. As the mass transfer of substrate is often a major limitation in whole-cell drug biotransformations [36, 38], we examined whether there was any difference in noscapine uptake between Gram-negative and Gram-positive organisms prior to selecting a microbial host for the biotransformation bioprocess. These experiments were performed by incubating cell suspensions of either *Escherichia coli* or *Bacillus megaterium* with noscapine and quantifying intracellular noscapine accumulation at intervals up to 90 s; this timeframe was selected as a range of antibiotics have been shown to reach equilibrium in bacterial cells within similar time periods [467, 471, 472] and our preliminary experiments showed no significant further accumulation of noscapine after 90 s of incubation.

Noscapine accumulated rapidly in *B. megaterium* cells, reaching an equilibrium within 30-45 s, as shown in **Figure 4.1a**. At the end of the assay, the accumulated noscapine was ~25-fold higher per unit biomass for *B. megaterium* cells than for *E. coli* cells (**Figure 4.1b**). As it was likely that some of the noscapine measured in the cell pellets was adhered to the surface of the cells, rather than being located within the cells, we estimated the amount of adhered

noscapine by performing extended incubations of cell suspensions with noscapine at -4°C. At this temperature, no diffusion across the cell membrane was expected to occur and all noscapine associated

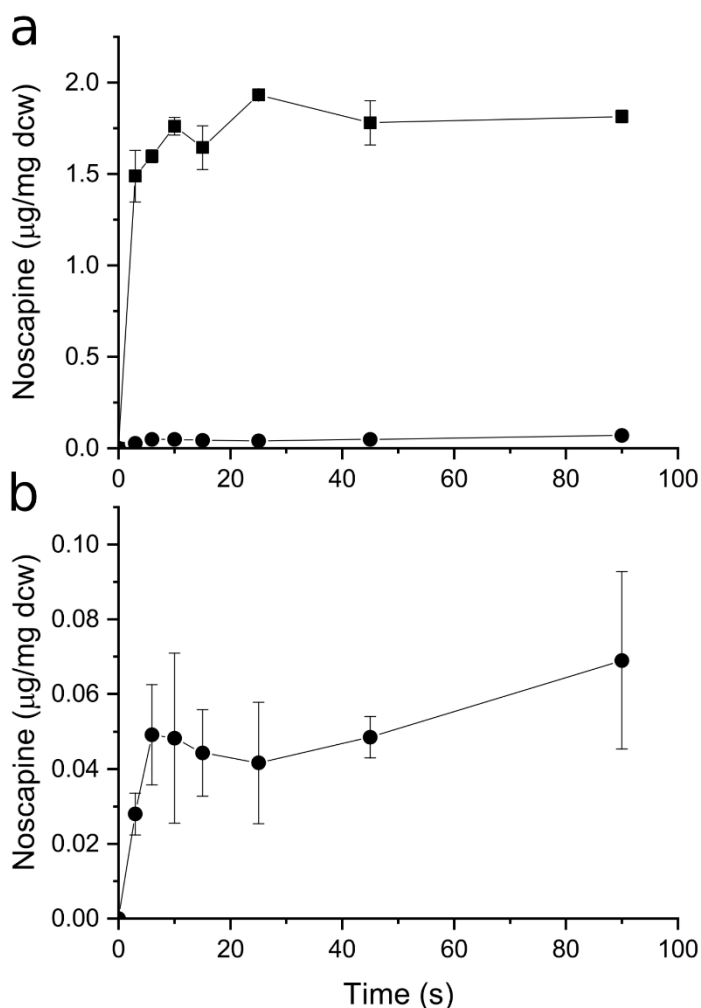


Figure 4.1. (a) Noscapine accumulation in *Bacillus megaterium* (■) vs *Escherichia coli* (●). (b) Enlarged view of noscapine accumulation in *E. coli*. Error bars are ± 1 SD and are obscured by the symbol used as a marker for *E. coli* in (a).

with the cell biomass was therefore considered to have adhered to the cell surface [467]. Under these conditions, the adhered noscapine was $0.30 \pm 0.03 \mu\text{g}/\text{mg}_{\text{dcw}}$ for *B. megaterium* and $0.05 \pm 0.01 \mu\text{g}/\text{mg}_{\text{dcw}}$ for *E. coli*. Notably, the concentration of noscapine that adhered to *E. coli* was similar to the total noscapine measured in the accumulation experiments in **Figure 4.1b**, suggesting that only a small fraction of the noscapine associated with *E. coli* passed through the outer membrane of these cells. In contrast, noscapine that had adhered to *B. megaterium* represented only ~17% of the total noscapine measured in accumulation experiments after 90 s, indicating that most of the noscapine in the cell pellets had passed through the membrane of *B. megaterium*.

These observed differences in noscapine uptake are consistent with differences in the uptake of drug-like substrates known to occur between Gram-positive and Gram-negative cells. Noscapine (pK_a 6.8) is bulky, hydrophobic and was largely uncharged under the assay conditions used here (pH 7.4). Such substrates are expected to readily diffuse through the peptidoglycan and phospholipid bilayer of Gram-positive organisms but be inhibited by the dense, hydrophilic lipopolysaccharide coating of the Gram-negative outer membrane [203]. Consequently, Gram-positive organisms may be better hosts for whole-cell biotransformation of hydrophobic drugs, where mass transfer rates often limit productivity [38].

Whole-cell biotransformation

A *B. megaterium* expression system was developed to investigate whether the significant difference in substrate uptake between this organism and *E. coli* resulted in different rates of noscapine *N*-demethylation within a whole-cell biotransformation. The commonly used DSM-319 strain was transformed with a plasmid containing the A3 mutant P450_{BM3} gene under control of an optimised xylose-inducible promoter [364]. Biotransformations were performed using these cells in a resting state and compared with biotransformations performed using resting *E. coli* BL21(DE3) cells expressing the same enzyme under the IPTG-inducible T7 expression system.

An increase of ~25-fold more *N*-nornoscapine was produced per unit enzyme in the first 30 min using the *B. megaterium* system compared to *E. coli*, resulting in a ~12-fold higher specific yield after 4 h, as shown in **Fig. 2**. All noscapine turnover for both organisms was due to the mutant P450_{BM3} enzyme, as cells containing an empty vector showed no *N*-demethylation and no production of any other noscapine metabolite (results not shown). Both systems showed the same selectivity (~88%) for noscapine *N*-

demethylation as previous cell lysate assays with this enzyme mutant (results not shown). The superior *N*-demethylation activity observed with *B. megaterium* compared to *E. coli* is presumably due to the greater permeability of *B. megaterium* cells to noscapine (**Figure 4.2**). A similar observation was made by Bleif *et al.* [219] during the development of a whole-cell system for P450-mediated hydroxylation of the hydrophobic diterpene abietic acid, where biotransformation was successful using recombinant *B. megaterium* but no

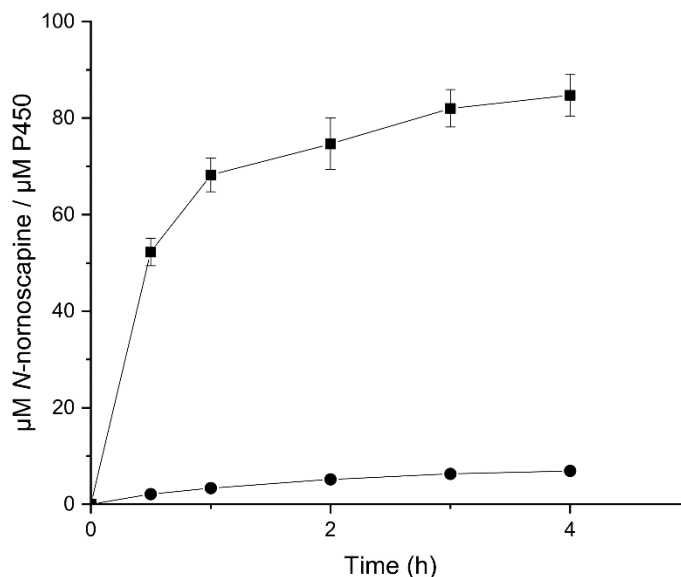


Figure 4.2. Noscapine *N*-demethylation by recombinant *Bacillus megaterium* (■) and *Escherichia coli* (●) expressing P450_{BM3} mutant A3. Error bars are ± 1 SD and are obscured by the symbol used as a marker for *E. coli*.

substrate conversion was detectable using *E. coli* despite the presence of a functional enzyme in these cells. The *E. coli* cells used here are similarly known to produce functional enzyme, as previously demonstrated with cell lysate assays (**Paper 1**; [464]).

Selection of a host organism with good native permeability to a chosen substrate has several advantages over the alternative strategy of physically permeabilising cells following protein expression, e.g. by freeze-thawing or treatment with solvents or detergents [38]. Aside from requiring extra processing steps, the cellular damage caused by permeabilisation can be detrimental in cases where functional biocatalysis depends upon the active metabolism of the host, such as for the supply of NAD(P)H in P450-catalysed reactions. The use of cell permeabilisation to improve mass transfer is also limited to resting-cell biotransformations. The choice of *B. megaterium* as a host for noscapine biotransformation therefore avoids the mass transfer limitation encountered with *E. coli* while retaining process flexibility, as growing cells can be used if desired.

This work is one of several recent examples of using *B. megaterium* as a host for whole-cell biotransformations with recombinant P450 enzymes [42, 219, 321, 420, 473]. It is also, to the best of our knowledge, the first time that this organism has been used for a biotransformation with recombinant P450_{BM3} (CYP102A1), despite the enzyme being native to this species. *Bacillus megaterium* has broad

potential as a biotransformation host due to its high capacity for heterologous protein expression and good plasmid stability [474]. We therefore chose to further investigate the potential of this host and enzyme system for preparative-scale *N*-demethylation of noscapine.

Increasing substrate supply

High concentrations of substrate are desirable in a biotransformation process to maximise volumetric productivity and reduce downstream processing requirements. Noscapine has poor aqueous solubility and low concentrations were used for the initial biotransformation experiments in **Figure 4.2** (100 μ M or 41 mg/L added from a 10 mM methanol stock), as higher concentrations resulted in rapid precipitation. We therefore investigated two strategies to increase substrate supply in the biotransformation: a biphasic system and aqueous solubility enhancers.

Biphasic biotransformation

Solvent screening

A biphasic biotransformation system consists of an immiscible organic solvent that can supply a hydrophobic substrate to the aqueous phase containing the biocatalyst. The chosen solvent must be biocompatible with the host cell system, particularly where active host metabolism is required. The solvent should also provide good substrate solubility and partitioning between the aqueous and organic phases, whilst also satisfying environmental, health and safety requirements. While the choice of potential solvents is vast, a systematic selection process can narrow down the number solvents to be tested experimentally to find an appropriate system [249].

We used a systematic screening strategy to find a suitable solvent based on published solvent properties and *in silico* solubility predictions determined using Hansen solubility parameters [282]. A similar strategy was recently shown to be effective for the development of a biphasic system for geraniol biotransformation [475]. First, a solvent database (HSPiP; <https://www.hansen-solubility.com/HSPiP/>) was used to compile a list of solvents with octanol-water partition coefficient ($\log P$) values greater than 3, as this measure provides an indication of potential biocompatibility [476]. This shortlist was then rationally narrowed to 15 solvents (**Table 4.1**) based on factors such as human and environmental safety, availability, cost, melting point and volatility. The shortlisted solvents were then assessed *in silico* for predicted noscapine solubility.

Table 4.1. Shortlist of potentially suitable solvents for biphasic biotransformation of noscapine. Log P values are from the HSPiP software database. Predicted solubilities were determined using the modified Hansen model developed by Louwerse *et al.* [469]. Solvents shaded grey were selected for experimental testing of solubility and were used for biotransformation experiments. Solubility of noscapine in water is provided for comparison.

Solvent	Log P	Predicted noscapine solubility (g/L)	Measured noscapine solubility (g/L)	Measured noscapine solubility (mM)
benzyl benzoate	4.0	159.7		
dibenzyl ether	3.3	96.8		
butyl benzoate	3.8	91.9	22.6	54.7
dibutyl maleate	4.2	16.7	19.6	47.4
octanoic acid	3.1	14.5		
decanol	4.6	13.1		
heptyl acetate	3.3	6.5	6.8	16.4
2-(ethylhexyl) acrylate	3.7	4.6		
octyl acetate	3.8	4.4		
dibutyl ether	3.2	4.1		
dibutyl sebacate	6.3	1.4		
stearic acid	8.2	0.8		
oleic acid	7.7	0.7		
isopropyl myristate	8.5	0.7	1.4	3.4
water	-2.9	-	0.1	0.2

The Hansen solubility model predicts solvent-solute compatibility based on three parameters representing dispersion (δ_D), polar (δ_P) and hydrogen-bonding (δ_H) interactions. Recently, Louwerse *et al.* [469] developed a modified Hansen model that incorporates several thermodynamic considerations for small molecule solutes and shows greater accuracy than the original model that was developed for solvent-polymer systems. The modified model and the associated MATLAB package were used to estimate Hansen parameters and predict noscapine solubility here. Noscapine solubility was initially measured in 20 readily available solvents (**Table 4.4, Supplementary Material**) and this data was used within the program for parameter estimation. A good fit ($R^2 = 0.88$) was observed between the predicted and measured noscapine solubilities for the 20-solvent training dataset using the estimated noscapine Hansen parameters ($\delta_D = 28.7$; $\delta_P = 11.3$; $\delta_H = 7.8$) (**Figure 4.8, Supplementary Material**). These Hansen parameters were then applied to predict noscapine solubility within the shortlist for potential biphasic biotransformation solvents (**Table 4.1**).

Most of the solvents shortlisted for potential use in noscapine biotransformation were esters containing aromatic or medium-to-long chain aliphatic groups. The compounds with the highest predicted noscapine solubilities (**Table 4.1**) contain either one or two aromatic functionalities; this appears to be due to the high Hansen dispersion parameter (δ_D) shared between these compounds and noscapine.

Three structurally diverse esters with good predicted noscapine solubility were selected for further investigation in a biotransformation experiment. These were: butyl benzoate, heptyl acetate and dibutyl maleate. Isopropyl myristate was also included, as this compound has a significantly higher log *P* than the other selected solvents (8.5 vs 3.3-4.2). Whilst this solvent has a comparatively low predicted noscapine solubility (0.7 g/L), its inclusion allowed an investigation of a wider range of solvent hydrophobicity. Isopropyl myristate is also known to be effective in other biphasic biotransformations using hydrophobic small molecules [475, 477-479]. To our knowledge, the other three selected solvents have not previously been investigated for use with whole-cell biphasic systems, although dibutyl maleate was recently identified in a computational screen as a potentially suitable solvent for biotransformation of flavour and fragrance compounds [480].

The measured solubility of noscapine in the four selected solvents is shown in **Table 4.1**. The measured solubilities were within 3 g/L of the predicted values, apart from butyl benzoate, for which the model gave a four-fold overestimation of noscapine solubility. These data demonstrate the potential utility of the modified Hansen model for *in silico* solvent screening for small drug molecules but also the need to validate predictions with experimental measurements.

Biphasic biotransformation

Isopropyl myristate was the most effective solvent for biphasic biotransformation, showing the highest yield of *N*-nornoscapine after 4 h compared to the use of a single aqueous phase or the three other solvents selected through *in silico* screening (**Figure 4.3**). In each case, *B. megaterium* expressing the P450_{BM3} mutant A3 was used, with either a 20% volume of the selected solvent saturated with noscapine or a single-phase system containing 100 μ M of noscapine with 1% methanol as a co-solvent. Approximately 2.3-fold more *N*-nornoscapine was produced using isopropyl myristate compared to the single-phase control, while the heptyl acetate and dibutyl maleate systems produced 6.3- and 8.8-fold less *N*-nornoscapine than the control. No substrate conversion was detected in the butyl benzoate solvent system.

The variation in product yields between the different solvent systems appeared to be primarily due to the toxicity of these solvents, as <0.1% of cells remained viable in the biphasic systems containing heptyl acetate, dibutyl maleate and butyl benzoate following 4 h of biotransformation (Table 2). Butyl benzoate, despite having a log *P* between that of heptyl acetate and dibutyl maleate (Table 4.1), appeared to be

particularly toxic as biotransformation activity was completely abolished (Figure 4.3). This is likely related to the benzyl groups of this solvent, as

alkylbenzenes have previously been shown to have higher critical log *P* for biocompatibility than other solvent classes [476]. In contrast, cell viability in the isopropyl myristate system, which produced the highest yields, appeared similar to the viability observed for the single-phase control, with 11% and 14% of viable cells remaining for these reactions respectively.

It is notable that *B. megaterium* cells containing an empty vector showed only a small viability loss under aqueous single-phase conditions (93% viable cells remaining; Table 4.2). The difference in survival between cells with the empty vector and cells containing the mutant P450_{BM3} enzyme in the single-phase control experiment (i.e. 93% vs 14% remaining viability) suggests that the expression of the enzyme lead to significant cellular toxicity. This reduction in cell viability may be due to the generation of reactive oxygen species by the P450 enzyme during non-productive catalytic cycles or toxicity associated with the *N*-nornoscapine product.

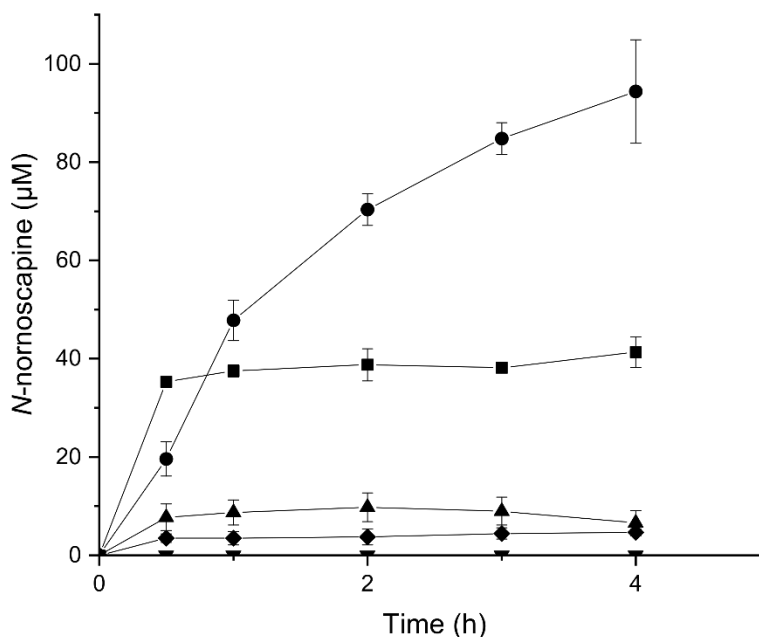


Figure 4.3. Noscapine *N*-demethylation in a biphasic biotransformation system using the solvents isopropyl myristate (●), dibutyl maleate (▲), heptyl acetate (◆) or butyl benzoate (▼). Biotransformation was also performed in a single aqueous phase as a control (■). Product concentrations are given as total product produced per volume of aqueous phase. Note that the marker for butyl benzoate (▼) appears partly obscured by the x axis, as biotransformation in this system was negligible. Error bars are ± 1 SD.

Retaining cell viability is important for the supply of NADPH cofactor to the enzyme during the biotransformation, and the retention of cell viability can also allow re-use of cells in multiple biotransformation cycles. While the general guideline is that solvents with $\log P > 4$ are non-toxic to microbial cells, there are significant deviations from this rule of thumb depending on both the microbial strain and the molecular structure of the solvent [261, 481]. Organic solvents with $\log P$ between 3-5 are considered to have less predictable toxicities than those outside this range [476]. Based on the solvents screened here, *B. megaterium* requires higher solvent hydrophobicity for biocompatibility (i.e.

isopropyl myristate); however, this comes at the cost of lower substrate loading (1.4 g/L in the organic phase), as noscapine solubility was highest in the more polar solvents that proved to be toxic (i.e. butyl benzoate and dibutyl maleate with 22.6 g/L and 19.6 g/L, respectively; **Table 4.1**).

The use of solvent-tolerant microbial strains is one approach to balance the need for good substrate solubility with biocompatibility [481]. It is important to consider, however, that some mechanisms of solvent resistance may also be detrimental to substrate uptake. For example, Gram-negative bacteria typically show higher solvent resistance than Gram-positive bacteria, attributed to the presence of an outer cell membrane. Our experiments suggest that the permeability barrier posed by the Gram-negative outer membrane is responsible for the low rate of biotransformation observed with *E. coli* (**Figure 4.2**). While some particularly solvent-tolerant *B. megaterium* strains have been isolated [273, 482, 483], the properties conferring solvent resistance may impede substrate uptake. For example, a wild-type strain used for epoxide resolution in a biphasic system was active in the presence of solvents with $\log P > 3.2$ [273] but displayed a low surface hydrophobicity and so may be less permeable to noscapine compared to the DSM-319 strain used here. Another important consideration is that many solvent resistance mechanisms in Gram-positive bacteria are adaptive responses [484]. Such responses are unlikely to occur

Table 4.2. Remaining cell viability of recombinant *B. megaterium* following 4 h of biotransformation under various conditions. Cell viability was determined by colony-forming unit (CFU) counts. Errors are given as ± 1 SD. Cross-references to figures containing the corresponding biotransformation data are also provided.

Conditions	Remaining viability (%)
Empty vector control	93 $\pm$ 13
Biphasic biotransformation (Figure 4.3)	
control (1% MeOH)	14 $\pm$ 3
isopropyl myristate	11 $\pm$ 3
dibutyl maleate	<0.1
heptyl acetate	<0.1
butyl benzoate	<0.1
HP-β-CD/HPMC (Figure 4.5)	
0.1 mM substrate	2 $\pm$ 0.5
1.0 mM substrate	3 $\pm$ 0.6
Glucose feeding (Figure 4.6)	
With glucose	2 $\pm$ 0.9
Without glucose	0.1 $\pm$ 0.07

with the nitrogen-limited, resting-cell biotransformation used here and improved tolerance may be seen with a growing-cell biotransformation process.

While the higher substrate loading in the isopropyl myristate system improved product titres compared to the single-phase system, there was an almost two-fold decrease in the initial biotransformation rate (measured after 30 min of reaction). This was likely due to a lower aqueous concentration of substrate resulting from the partitioning of noscapine between the aqueous and organic phases. The aqueous phase in the isopropyl myristate system showed a steady, low noscapine concentration of around 30 μM throughout the biotransformation (data not shown). Maintaining a low aqueous concentration is advantageous when a biphasic system is employed due to substrate toxicity, but in this case our objective was to overcome poor substrate solubility and so a decrease in reaction rate due to the limited aqueous substrate concentration is a potential disadvantage. We therefore investigated an alternative strategy of improving aqueous noscapine solubility to drive faster reaction velocities.

Improving aqueous solubility

Cyclodextrins are often employed as pharmaceutical excipients due to their ability to enhance drug solubility. This class of compounds was investigated here to assess whether the aqueous solubility of noscapine could be improved. Cyclodextrins form soluble inclusion complexes with drugs and have previously been used to improve whole-cell biotransformation rates of hydrophobic compounds such as steroids [312, 485, 486]. Cyclodextrins have also been used to enhance biotransformation by recombinant *B. megaterium* [473]. In some pharmaceutical applications, small amounts of water-soluble polymers such as polyvinylpyrrolidone (PVP) and hydroxypropyl methylcellulose (HPMC) have also been shown to improve drug-cyclodextrin complexation and allow further enhancement of drug solubility [314], though these ternary complexes have not previously been employed in whole-cell biotransformations. The cyclodextrin (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD), a widely used and highly soluble cyclodextrin derivative, as well as the polymers PVP and HPMC, were investigated here for their effects on noscapine solubility.

The combination of 10% HP- β -CD with 0.1% of polymer HPMC provided a significant increase in aqueous noscapine solubility (**Figure 4.4**), allowing a ~16-fold enhancement in noscapine concentration to 835 μM (345 mg/L) compared to the 50 μM of noscapine dissolved in the control solution containing only phosphate buffer. Solutions of 5% and 10% HP- β -CD alone gave more modest three- and four-fold enhancements in noscapine solubility, respectively, and a 0.1% solution of HPMC polymer provided a

three-fold enhancement, indicating that HP- β -CD and HPMC act synergistically to enhance noscapine solubility. This effect was still apparent when a lower polymer concentration of 0.01% HPMC was used with 10% HP- β -CD, which gave a 14.5-fold enhancement in noscapine concentration (736 μ M; 304 mg/L) with considerably less viscosity compared to solutions containing 0.1% HPMC polymer. In contrast, a solution of 0.1% PVP had negligible effect on the solubility of noscapine with or without HP- β -CD addition.

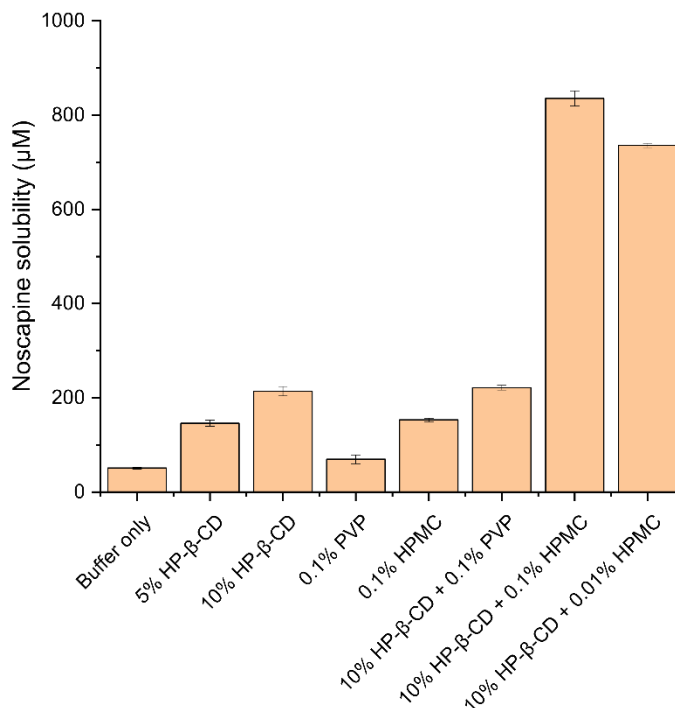


Figure 4.4. Aqueous solubility of noscapine in solutions of cyclodextrin, polymer or combinations of cyclodextrin and polymer. Abbreviations: HP- β -CD, (2-hydroxypropyl)- β -cyclodextrin; PVP, polyvinylpyrrolidone; HPMC, (hydroxypropyl)methylcellulose. Solutions were prepared in phosphate buffer (100 mM) at pH 6.5. Error bars are ± 1 SD.

The combination of cyclodextrin HP- β -CD and polymer HPMC not only provided significant enhancements in

noscapine solubility but also functioned well as a precipitation inhibitor [487] for supersaturated noscapine solutions. When measuring the equilibrium solubility of noscapine in HP- β -CD/polymer systems, we observed that the HP- β -CD/HPMC systems remained optically clear for extended periods following addition of excess noscapine, whereas precipitate was visible in all other cyclodextrin or polymer solutions within 1 h. Under the same conditions of temperature and agitation as the biotransformation experiments, a solution of 10% HP- β -CD with 0.01% HPMC could maintain a noscapine concentration of ~ 1 mM for at least 24 h (**Figure 4.9, Supplementary Material**). We therefore investigated biotransformation performance in solutions of 10% HP- β -CD with 0.01% HPMC using noscapine concentrations up to 1 mM.

The higher concentrations of noscapine enabled by HP- β -CD/HPMC addition led to increased biotransformation productivity, as shown for experiments performed using an initial substrate concentration of 100 μ M, 500 μ M or 1 mM (**Figure 4.5**). The initial rate measured at 30 min was found to increase up to eight-fold between the reactions with 100 μ M and 1 mM noscapine, allowing a final product

concentration up to 266 μM (110 mg/L). This product titre was a 6.4-fold improvement over the single-phase system in 1% methanol (**Figure 4.3**) where nescapine was used at its maximum solubility of 100 μM . This product titre was also 2.8-fold higher than the best-performing biphasic system (IPM; **Figure 4.3**).

While the aqueous solubility enhancers allowed significantly higher final yield (266 μM vs 41 μM), the initial rate of *N*-demethylation with 100 μM nescapine and HP- β -CD/HPMC (**Figure 4.5**) was slower than in the equivalent reaction with

100 μM nescapine without HP- β -CD/HPMC (**Figure 4.3**), with 1.6-fold less product at 30 min, though both systems eventually reached the same final yield. This may be because the complexation of nescapine with cyclodextrin resulted in a lower concentration of free substrate. Alternatively it may be due to decreased mass transfer from the higher viscosity of the HP- β -CD/HPMC solution [488]. A similar observation was made by Kiss *et al.* [473] for a *B. megaterium* biotransformation system using 5% HP- β -CD, which showed slower initial reaction rates with cyclodextrin compared to a control reaction with 5% DMSO in shake flask experiments. When the reaction was performed in bioreactors, however, the HP- β -CD system showed faster rates than the control, suggesting that stronger mixing may improve the initial rates in HP- β -CD/HPMC here.

While allowing higher final product titres, the HP- β -CD/HPMC solutions also showed some toxicity towards *B. megaterium* with only 2-3% of initial cell viability remaining after 4 h, whereas 14% of cells remained viable in the control biotransformation (100 μM nescapine, 1% methanol; **Table 4.2**). This difference appeared to be entirely due to the solubility enhancers as the remaining viability in the HP- β -CD/HPMC systems was the same for both 100 μM and 1 mM nescapine, indicating no additional toxicity from higher concentrations of substrate or product. While often considered to have good

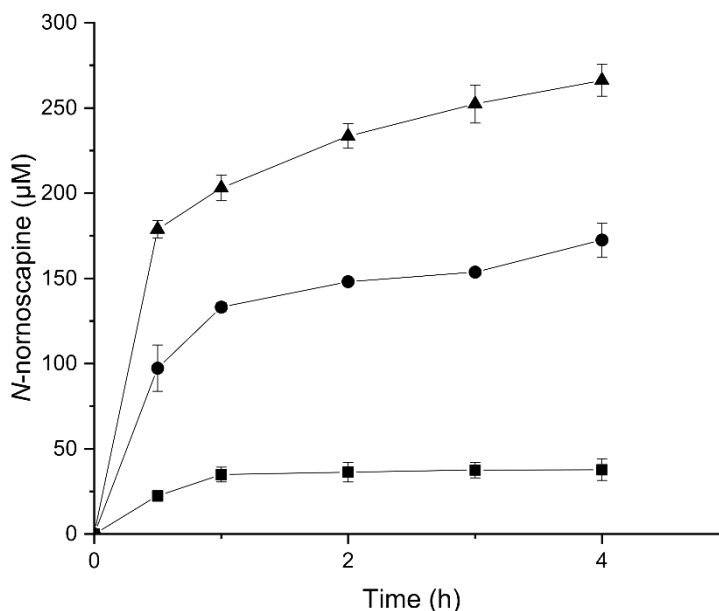


Figure 4.5. Nescapine *N*-demethylation by biotransformation in solutions of 10% (2-hydroxypropyl)- β -cyclodextrin and 0.01% (hydroxypropyl) methylcellulose with a starting substrate concentration of (■) 100 μM , (●) 500 μM or (▲) 1 mM nescapine. Error bars are ± 1 SD.

biocompatibility [489], cyclodextrins and their derivatives have been shown to be toxic to microbial cells in some cases. This appears to be highly dependent on both the microbial species and variant of cyclodextrin [226, 490, 491]. At 15% concentration, HP- β -CD has been shown to cause membrane damage and protein leakage to Gram-positive *Arthrobacter simplex* cells [491]; this is also likely responsible for the observed toxicity here. It is unlikely that the low concentration (0.01% w/v) of HPMC exerted any toxicity as this polymer is often used at higher concentrations in dietary probiotic formulations without loss of cell viability [492, 493].

Cofactor/viability limitation

While the higher substrate concentrations achieved by the addition of HP- β -CD/HPMC significantly improved initial reaction rates, the experiments indicated that another factor such as cofactor supply, cell viability or enzyme stability became limiting early in the biotransformation. After 1 h of biotransformation, product formation rates in the reactions containing high and moderate concentrations of noscapine (1 mM and 500 μ M, **Figure 4.5**) were similar, which is not in keeping with the first-order reaction kinetics expected in this substrate range. This behaviour lead us to examine cofactor supply and cell viability as potential limiting factors.

The sustained supply of NADPH to the reaction requires cells to have access to a carbon source and to be metabolically active. To investigate whether glucose was actively consumed by the host cells throughout the biotransformation and did not become exhausted, we monitored the glucose consumption and product formation in a biotransformation with 1 mM noscapine in a HP- β -CD/HPMC solution (**Figure 4.6**). Product formation was also monitored in the absence of glucose, where regeneration of NADPH is expected to be limited.

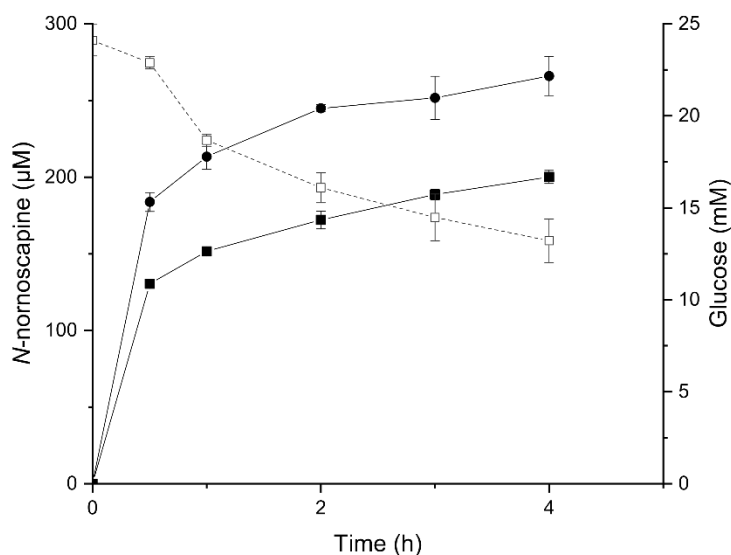


Figure 4.6. Noscapine *N*-demethylation with (●) and without (■) glucose added to the biotransformation. Glucose concentration is also shown for the relevant biotransformation (□). Error bars are ± 1 SD.

A concentration of 22 mM (0.4% w/v) glucose was found to be more than sufficient to supply the cells over a 4 h period, as shown in **Figure 4.6**. The presence of glucose resulted in 1.4-fold more product after 1 h, likely due to higher NADPH supply. At later timepoints, however, the rate of product formation was similar in the presence and absence of glucose, with an increase of $\sim 50 \mu\text{M}$ between 1-4 h despite active glucose consumption in the glucose-fed culture and an order of magnitude difference in cell viability measured after 4 h (2% vs 0.1% for cells with and without glucose, respectively; **Table 4.2**). These differences indicate that neither glucose supply nor cell viability were the key factor limiting biotransformation rate after 1 h. This suggested that the observed decline in product formation may have been due to the loss of active P450 enzyme.

Enzyme stability

The stability of the mutant P450_{BM3} enzyme was assessed during biotransformation to determine whether this factor limits the productivity of the system and could form the focus of future optimisation studies. The concentration of cytochrome P450 enzyme in cell pellets collected from a standard biotransformation was measured using carbon monoxide (CO) difference spectroscopy [437] and the concentration of soluble intracellular protein visualised using SDS-PAGE. We also investigated the potential effects of dissolved oxygen and substrate turnover on enzyme stability using reactions performed with low agitation or in the absence of substrate.

A significant loss of P450 enzyme occurred over the course of the biotransformation reactions performed under conditions of high agitation, as assessed by CO-difference spectroscopy (**Figure 4.7a**). The cytochrome P450 content of the cells dropped by four- to five-fold over 4 h, while the weight of dry biomass and the concentration of soluble P450_{BM3} protein visualised by SDS-PAGE remained relatively constant (**Figure 4.7b**). This observation likely explains the decline in product formation seen after 1 h of biotransformation in **Figures 4.5-4.6**, where high agitation was used. As the spectroscopic method quantifies P450-bound haem, it seems likely that enzyme deactivation was due to haem loss or damage rather than proteolysis or thermal denaturation and precipitation of the P450 enzyme.

A similar loss of P450 enzyme occurred both with and without substrate addition, indicating that enzyme deactivation was related to the conditions of high agitation rather than substrate turnover. In contrast, only $\sim 10\%$ of cytochrome P450 enzyme was lost in the reaction performed with gentle agitation, though subsequent biotransformation experiments using low agitation showed significantly less productivity (2.8-

fold less product after 4 h at 70 rpm compared to 230 rpm; 1 mM substrate in HP- β -CD/HPMC; **Figure 4.10, Supplementary Material**), likely due to a lower dissolved oxygen concentration under these conditions.

The cytochrome P450 haem group can be damaged by hydrogen peroxide and other reactive oxygen species (ROS) produced by the reduction of molecular oxygen during non-productive P450 catalytic cycles [494]. This can occur when product formation is poorly coupled to NADPH reduction or when no substrate is present [147]. Given that the P450_{BM3} mutant used in this work only showed only 16% coupling efficiency *in vitro* (**Paper 1**; [464]) and its stability was improved under low aeration (**Figure 4.7**), this mechanism may be responsible for the loss of activity observed during biotransformation. Production of

ROS by the recombinant P450_{BM3} enzyme may also contribute to the loss of cell viability observed during biotransformation experiments (a reduction in cell viability from 93% to 14% was observed after 4 h of reaction with P450-expressing cells compared to cells with an empty vector; **Table 4.2**). Our observations are consistent with the poor stability of other P450 enzymes which has previously been shown to be a significant factor limiting whole-cell biotransformation productivity [227, 473].

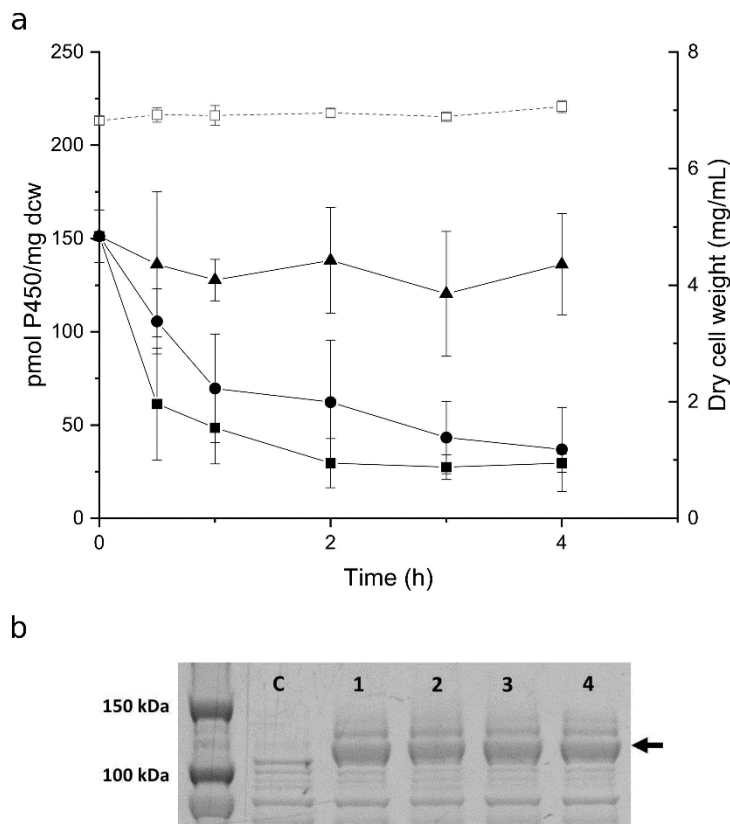


Figure 4.7. (a) Concentration of active cytochrome P450 enzyme per unit biomass in a biotransformation measured by carbon-monoxide difference spectroscopy performed with (●) high agitation and 100 μM substrate; (■) high agitation without substrate; or (▲) low agitation and 100 μM substrate. The corresponding dry biomass weight for each timepoint are also shown (□). Error bars are ± 1 SD. **(b)** SDS-PAGE gel showing the P450_{BM3} enzyme polypeptide band at 121 kDa, indicated by an arrow. Lanes are representative samples from experiments in part (A). Lane 1, before biotransformation; lanes 2-4, following 4 h of biotransformation performed at: high agitation and 100 μM substrate (lane 2); (■) high agitation without substrate (lane 3); or low agitation and 100 μM substrate (lane 4). A sample taken from cells containing an empty vector is also shown (C).

One strategy to improve both enzyme stability and cell survival would be to co-express ROS scavengers [495, 496], such as catalase or superoxide dismutase, together with the recombinant P450_{BM3} mutant. This approach would also need to consider the net benefit to biotransformation productivity, however, as co-expression strategies may result in reduced P450_{BM3} expression [244]. Additionally, these ROS scavengers are natively expressed in aerobic bacteria to mitigate oxidative stress [497], so it unclear whether the overexpression of these enzymes would have any further protective effects on the enzyme or host cell under these conditions.

Another strategy could be to change the mode of operation from a resting-cell biotransformation to a growing-cell process. All biotransformations performed in this work used a nitrogen-free medium, which does not allow for further protein expression during the biotransformation nor significant adaptive cell responses to environmental challenges. Performing the noscapine biotransformation concurrently with recombinant P450 expression and cellular growth may allow for improved product titres, as the unstable enzyme would be continuously replaced and actively growing cells may be better able to respond to ROS generation. Growing cells may also be more tolerant to organic solvents and cyclodextrins than resting cells. A common disadvantage of growing-cell processes, however, is the reduced availability of cellular cofactors, such as NADPH, due to the energetic demands of biomass formation [498]. This can be further addressed using cofactor recycling or metabolic engineering strategies [41].

These strategies, together with those already successfully employed in this study, are presented in **Table 4.3**. This table summarises the productivity and yield gains made using alternative hosts, biphasic systems and solubility enhancers. While the highest volumetric productivity achieved is one to two orders of magnitude below most reported industrial pharmaceutical biotransformations [2], the process limitations encountered in this work are typical of P450-mediated drug biotransformations. Further consideration of the factors limiting biotransformation and the strategies identified for optimisation will potentially allow improvements in enzyme activity, cofactor supply and cell viability to enhance biotransformation productivity for the *N*-demethylation of noscapine.

Conclusions

We have incorporated a drug-metabolising P450_{BM3} enzyme mutant into a whole-cell biotransformation process, showing proof of concept for preparative-scale biocatalytic *N*-demethylation of noscapine. Several factors limiting productivity were identified and strategies to increase productivity systematically assessed. The highest productivity of 27.5 mg/(L·h)) could be achieved by employing a *B. megaterium*

Table 4.3. Summary of strategies to overcome process limitations for whole-cell *N*-demethylation of noscapine. ^a Productivities are based on 4 h of biotransformation using a cell density of OD₆₀₀ = 35 (approx. 7.4 mg_{dcw}/mL).

Strategies tested in this study				
Limitation	Strategy	Improvement in biotransformation of noscapine	Highest productivity (mg/(L·h)) ^a	Considerations
Mass transfer of hydrophobic substrate across cell membrane	Alternative host (e.g. <i>B. megaterium</i>) for improved mass transfer	~25-fold higher initial rate per unit enzyme in compared to <i>E. coli</i> and ~12-fold higher final yield per unit enzyme	4.3	Potentially reduced solvent tolerance
Poor aqueous substrate solubility	Biphasic biotransformation using systematic solvent selection with <i>in silico</i> solubility screening	1.8-fold lower initial rate but 2.3-fold higher final yield using isopropyl myristate compared to aqueous single-phase system	9.8	Solvents with best noscapine solubility were toxic to <i>B. megaterium</i> ; noscapine partitioning between phases caused slower initial rates compared to aqueous single-phase system
Poor aqueous substrate solubility	Cyclodextrins and water-soluble polymers to enhance aqueous solubility	5.1-fold higher initial rate and 6.4-fold higher final yield using 1 mM noscapine compared to aqueous single-phase system at maximum noscapine solubility (100 µM); 9.1-fold higher initial rate and 2.8-fold higher final yield than best-performing biphasic system	27.5	Cyclodextrin/polymer system caused reduction in cell viability (2-3% vs 14% remaining viability after 4 h compared to aqueous buffer only)
Other strategies				
Limitation	Strategy	Improvement in biotransformation of noscapine	Highest productivity (mg/(L·h))*	Considerations
Toxicity of solvents with high noscapine solubility	Use of solvent-tolerant microbial strains [481]	-	-	Mechanisms of solvent resistance may impede substrate uptake [484]
Low stability of P450 enzyme	Co-expression of ROS scavengers (e.g. as catalase, superoxide dismutase) together with cytochrome P450 enzyme[495, 496]	-	-	Expression levels of cytochrome P450 may be reduced [244]
Low stability of P450 enzyme	Use of a growing-cell biotransformation process for renewal of inactivated P450 enzyme	-	-	Biotransformation may be limited by cofactor availability due to competition for NADPH with cellular growth processes [498]

host to overcome mass transfer limitations and cyclodextrins and polymers to enhance the aqueous solubility of noscapine. Biphasic systems developed using *in silico* solubility modelling could also be used to increase productivity (to 9.8 mg/(L·h)). Strategies that can improve the initial rates of biotransformation, such as the use of aqueous solubility enhancers, were shown to be particularly important for this system as the mutant P450_{BM3} enzyme was found to have low stability under whole-cell biotransformation conditions. Maintenance of cellular viability was also identified as a significant challenge.

This study shows that significant improvements in productivity and yield can be obtained by relatively simple changes in biotransformation parameters using a systematic debottlenecking approach. The strategies used in this work can also be applied to examine and optimise other whole-cell P450-based systems.

4.2. Supplementary Material

Cytochrome P450-mediated *N*-demethylation of noscapine by whole-cell biotransformation: process limitations and strategies for optimisation

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Table 4.4. The solubility of noscapine determined experimentally in 20 common organic solvents. This training dataset was used to estimate the Hansen solubility parameters, enabling *in silico* prediction of noscapine solubility in other solvents.

Solvent	Solubility	
	(mM)	(g/L)
chloroform	417.2	1009.6
pyridine	199.9	483.7
dimethylformamide (DMF)	114.3	276.5
benzyl alcohol	112.1	271.2
dimethylsulfoxide (DMSO)	67.0	162.1
acetylacetone	65.7	159.0
methyl ethyl ketone (MEK)	42.3	102.4
tetrahydrofuran (THF)	41.3	99.9
acetone	38.1	92.1
acetonitrile	34.8	84.2
toluene	26.4	64.0
ethyl acetate	22.4	54.3
xylene	14.5	35.1
<i>N</i> -butyl acetate	11.9	28.7
methanol	4.7	11.3
ethanol	3.8	9.1
octanol	2.9	7.1
butanol	2.4	5.7
diethyl ether	1.6	4.0
limonene	1.4	3.3

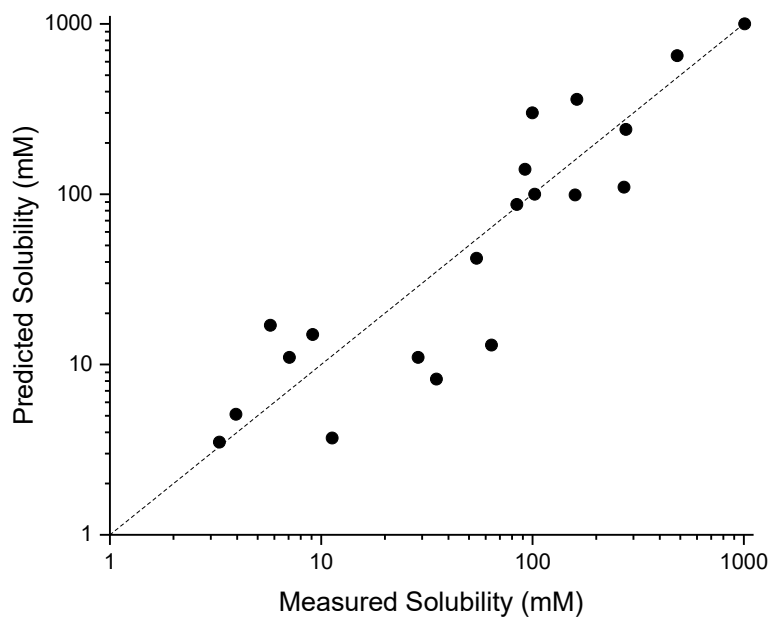


Figure 4.8. The solubility of noscapine measured experimentally and predicted using the Hansen parameters estimated for noscapine for a training dataset of 20 common organic solvents (**Table 4.4**).

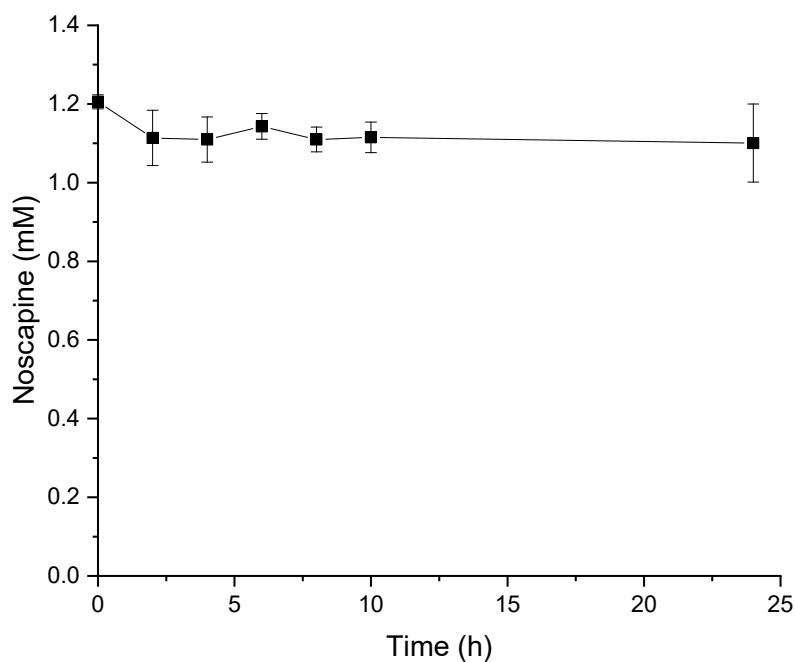


Figure 4.9. Precipitation inhibition of noscapine in a solution of 10% HP- β -CD and 0.1% HPMC. Samples were prepared and agitated using the method described in the main text. At each time interval, 1 mL of solution was removed, filtered through a 0.45 μ m nylon filter and the soluble noscapine was quantified by UHPLC-DAD. The

equilibrium solubility of noscapine in this cyclodextrin and polymer solution measured after 5 days was 0.736 mM, as described in the main text.

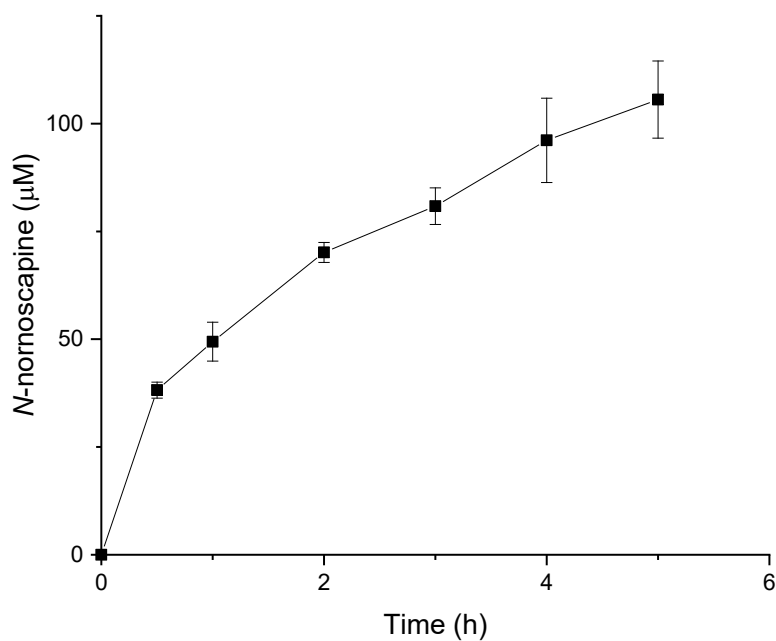


Figure 4.10. *N*-demethylation of noscapine by biotransformation performed under conditions of low agitation. The biotransformation was performed as described in the Materials and Methods section of the main text, with 1 mM noscapine added to 10% HP- β -CD and 0.1% HPMC. Agitation was reduced to 70 rpm compared to 230 rpm in other standard biotransformation experiments.

5. Metabolic engineering for enhanced cofactor supply during whole-cell biotransformation

5.1. Introduction

A key advantage of using whole cells for cytochrome P450-mediated biotransformations is that the NADPH cofactor is continuously regenerated due to the metabolism of the microbial host. As NADPH is also required for many growth-associated processes within the cell, a common approach to maximise the amount of available cofactor is to perform the biotransformation using ‘resting’ cells, where anabolic processes are minimised by placing the cells in a nitrogen-free medium following growth. A disadvantage of this approach is that there is no further biosynthesis of the enzyme of interest, which is particularly important for many cytochrome P450s that have poor stability. The alternative approach of using a growing-cell biotransformation can provide sustained turnover of recombinant enzyme, but biotransformation productivity can often be limited due to competition for NADPH with growth-associated metabolic reactions[498].

A primary source of NADPH regeneration in aerobic cells is the pentose phosphate (PP) pathway, which reduces two moles of NADP^+ to NADPH per mole of glucose-6-phosphate (G6P) entering the pathway[322]. Glucose-6-phosphate is derived directly from glucose entering the cell and is a branch-point metabolite that can enter either the Embden-Meyerhof-Parnas (EMP) pathway or the PP pathway (**Figure 5.1**). Carbon flux analysis of *B. megaterium*, for example, shows roughly equal partitioning of G6P between each pathway during exponential growth[392]. Several groups have used genetic engineering to enhance carbon flux through the PP pathway[41] to improve the ratio of NADPH generated per glucose consumed and therefore the amount of biotransformation product formed per unit of glucose (reviewed in **Chapter 2.6**).

Most efforts have been focused on *Escherichia coli* and have been successful in improving the ratio of product formation to glucose consumption for biotransformations through enhanced intracellular NADPH regeneration. Typical approaches rely on:

- overexpression of glucose-6-phosphate 1-dehydrogenase (encoded by *zwf*)[337, 338], the entry point to the PP pathway;
- genetic inactivation of *pgi* (glucose-6-phosphate isomerase)[324, 326-328] to inhibit flux through the EMP pathway; or

- the partial cyclisation of the PP pathway by inactivation of *pfkA* (6-phosphofructokinase)[326, 329, 333, 334], which forces a PP pathway end-product, fructose-6-phosphate (F6P), to re-enter the pathway through isomerisation to G6P.

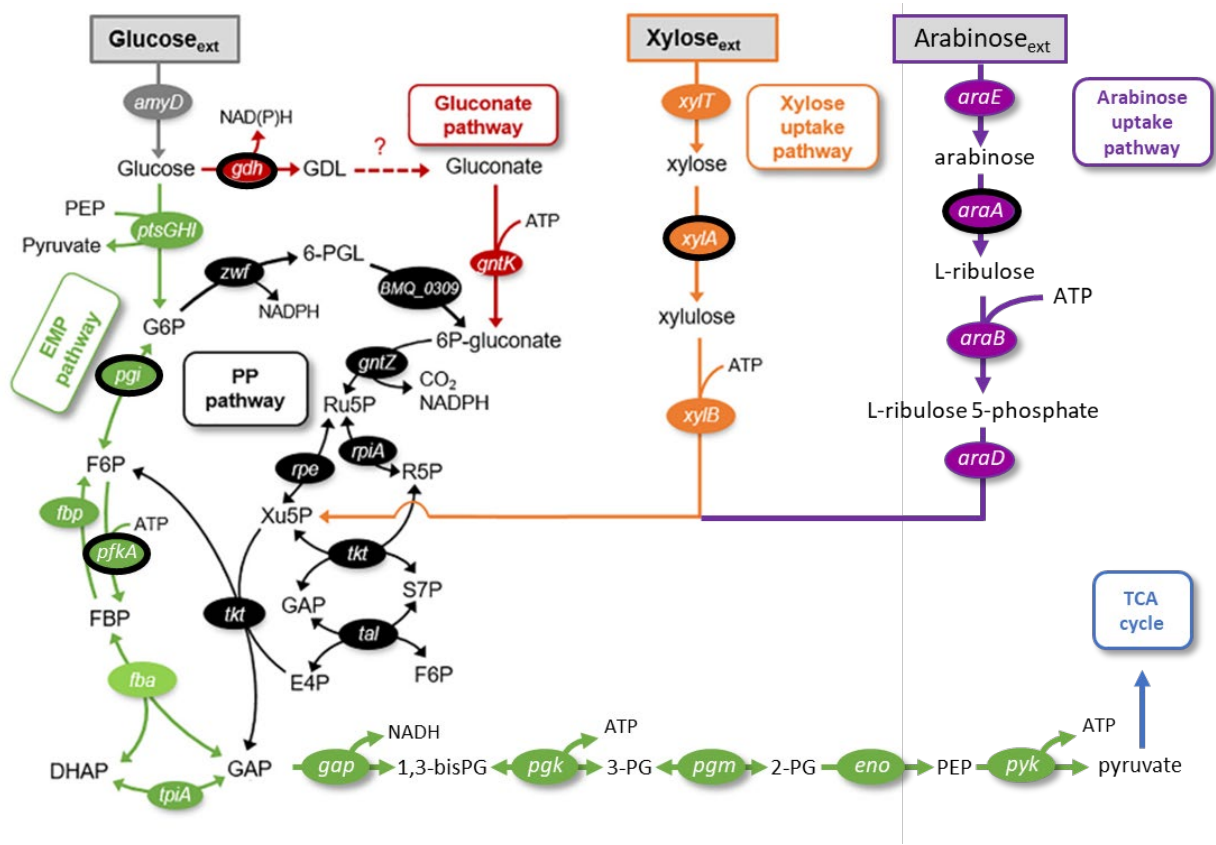


Figure 5.1. Schematic of central metabolic pathways for glucose, xylose and arabinose metabolism in *Bacillus megaterium*. Image was adapted from Wushensky *et al.* (2018) to include arabinose metabolism and remove pathways not relevant to this study. The five enzymes targeted by CRISPRi in this work (*pgi*, *pfkA*, *araA*, *xylA*) are highlighted with black circles. The metabolite abbreviations are as follows: PEP, phosphoenolpyruvate; G6P, glucose-6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone-3-phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-bisPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; GDL, glucono-1,5-lactone; 6-PGL, 6-phosphogluconolactone; 6P-gluconate, 6-phosphogluconate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; Xu5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate. Enzymes corresponding to gene names are as follows: *amyD*, carbohydrate ABC transporter permease AmyD; *ptsG*, PTS system glucose-specific transporter subunit IIBC; *ptsH*, phosphocarrier protein HPr; *ptsI*, PTS system transporter I; *pgi*, glucose-6-phosphate isomerase; *pfkA*, 6-phosphofructokinase; *fbp*, fructose-1,6-bisphosphatase; *fba*, fructose-1,6-bisphosphate aldolase; *tpiA*, triosephosphate isomerase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *pgm*, phosphoglucomutase; *eno*, enolase; *pyk*, pyruvate kinase; *gdh*, glucose 1-dehydrogenase; *gntK*, gluconate kinase; *zwf*, glucose-6-phosphate 1-dehydrogenase; *BMQ_0309*, 6-phosphogluconolactonase; *gntZ*, 6-phosphogluconate dehydrogenase; *rpiA*, ribose 5-phosphate isomerase A; *rpe*, ribulose-phosphate 3-epimerase; *tkt*, transketolase; *tal*, transaldolase; *xylT*, xylose permease; *xylA*, D-xylose isomerase; *xylB*, xylulokinase; *araE*, arabinose-proton symporter AraE; *araA*, L-arabinose isomerase; *araB*, ribulokinase; *araD*, L-ribulose-5-phosphate 4-epimerase.

One limitation of these approaches is that the inactivation of central metabolic genes causes significant growth impairments due to redox imbalance and a subsequent reduction in glucose consumption[324, 326, 327, 331]. For example, a *pgi* knockout mutant of *E. coli* showed a six-fold reduction in growth rate and four-fold reduction in glucose consumption when grown on minimal media[329]. Inactivation of *pfkA*, the major phosphofructokinase in *E. coli*, caused a three-fold reduction in both growth rate and glucose consumption. When the minor phosphofructokinase (*pfkB*) was also inactivated, growth on minimal media was barely detectable[329]. These significant growth defects not only increase the duration of the biotransformation process but also affect the biosynthesis of the enzyme of interest for the biotransformation, which is particularly important if the main goal of using a growing-cell process is to regenerate an unstable enzyme.

In recent years, more sophisticated tools for metabolic engineering have been developed that allow for the reversible regulation of target genes without requiring inheritable genetic mutations or deletions. These include antisense RNAs[499], *cis*-acting riboswitches[500] and, most recently, CRISPR interference (CRISPRi)[349]. The latter tool, developed from the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) bacterial defence system[501], is a versatile and efficient method to achieve dynamic control of multiple gene targets in both prokaryotic and eukaryotic cells. The CRISPRi system uses a catalytically 'dead' Cas9 protein (dCas9) that lacks the native Cas9 endonuclease activity. The dCas9 protein binds to a chosen target sequence on the host genome guided by a single guide RNA (sgRNA). The sgRNA consists of a 20-nucleotide sequence complementary to the target gene ('protospacer'), a Cas9 binding hairpin sequence and a transcriptional terminator. The bound dCas9-sgRNA complex then blocks transcriptional elongation by RNA polymerase at the target site, effectively silencing the target gene (**Figure 5.2**). The only requirement for the target sequence for CRISPRi is that it is directly adjacent to a protospacer adjacent motif (PAM), which for the commonly used *Streptococcus pyogenes* Cas9 protein is a nucleotide sequence of 'NGG' (where 'N' is any base).

Placing the dCas9 gene and/or sgRNA coding sequences under the control of an inducible promoter can allow temporal and reversible control of gene repression so that host cells display normal gene expression levels in the absence of inducer. Dynamic control of the degree of gene repression can often be achieved by varying the concentration of inducer added to the cells. Such a system would be potentially useful for enhancing the supply of NADPH to a whole-cell biotransformation. For example, the genes *pgi* or *pfkA* could be repressed following a normal period of cell growth and protein production, avoiding the poor

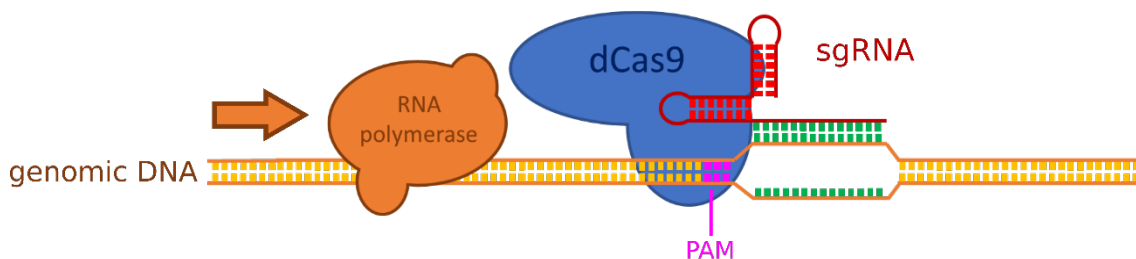


Figure 5.2. Transcriptional repression by CRISPR interference (CRISPRi). The endonuclease-deficient dCas9 protein is guided to a target sequence on the host chromosome by the single guide RNA (sgRNA). The sgRNA consists of a dCas9-binding hairpin (shown in red) and a unique protospacer sequence (green) that is complementary to the non-template strand of the target gene and directly adjacent to a protospacer-adjacent motif (PAM). The bound dCas9-sgRNA complex inhibits the elongation of mRNA transcripts by endogenous RNA polymerase.

growth rates associated with permanent inactivation of these genes. Applying only a partial degree of gene repression could also prevent severe redox imbalance and allow sufficient protein turnover to replenish inactivated P450 enzyme.

This work aimed to use CRISPRi in *B. megaterium* to improve the performance of noscapine *N*-demethylation by P450_{BM3} mutant A3 (P450_{BM3}.A3) during a growing-cell bioprocess, as this enzyme was previously shown to have poor stability in a resting-cell system (**Paper 2**). To increase NADPH production, both *pgi* and *pfkA* were selected as targets, as well as *gdh* (glucose 1-dehydrogenase), which forms an alternative pathway for glucose metabolism in this organism[392]. Unlike *E. coli*, *B. megaterium* does not possess a minor phosphofructokinase (*pfkB*), so this gene target was not considered here. The CRISPRi system has not previously been implemented in *B. megaterium*, requiring some development of the appropriate plasmids and strains for CRISPRi application, though it has been used in *Bacillus subtilis*[502] and *Bacillus methanolicus*[503]. The application of a CRISPRi system to increase the supply of NADPH to a whole-cell biotransformation process is also novel, though it has been used to repress the *pgi* gene in *Corynebacterium glutamicum* to increase NADPH regeneration during fermentative lysine[504] and arginine[505] production by native biosynthesis pathways. The CRISPRi system has also been successfully used for transcriptional repression of *pfkA* in *B. subtilis* to redirect carbon flux toward the biosynthesis of hyaluronic acid[506] and *N*-acetylglucosamine[507].

5.2. Materials & Methods

Chemicals, reagents, plasmids and strains

The plasmids and strains used in this work are described in **Table 5.1**. The primers and synthetic gene sequences used are provided in **Table 5.2**. All DNA fragment assemblies were performed using NEBuilder HiFi (NEB, Notting Hill, Australia) unless otherwise stated. Polymerase chain reaction (PCR) was performed using Q5 high-fidelity polymerase for cloning (NEB) or OneTaq (NEB) for colony PCR. Restriction enzymes and nucleic acid purification kits were NEB brand. Plasmids pAW005-02 (Addgene #85611; [502]) and pAW019-02 (Addgene #85614; [502]) were gifts from Perry Chou. Plasmid pHBintT was a gift from Dieter Jahn (Addgene #48134; [508]). Plasmid p3Shp_A3 was from previous work (**Paper 2**). Oligonucleotides and synthetic genes were manufactured by Integrated DNA Technologies (Singapore). Plasmid cloning was performed using *Escherichia coli* DH5- α competent cells (NEB). *Bacillus megaterium* strain DSM-319 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). Complex growth media components were Oxoid brand (Thermo Fisher Scientific, Scoresby, Australia). Noscapine was provided by Sun Pharmaceutical Industries (Port Fairy, Australia). All other chemicals were purchased from Sigma-Aldrich (Castle Hill, Australia).

Plasmid construction

Plasmid pLR03 (**Figure 5.3**) was constructed for the purpose of integrating a xylose-inducible dCas9 expression cassette at the *thrC* locus of *B. megaterium* DSM-319 via double-crossover homologous recombination (reviewed in **Chapter 2.7**). The pLR03 plasmid was constructed from plasmids pAW005-2, pHBintT and pAW019-2, generating the intermediate plasmids pLR01 and pLR02, as shown in Figure 3A. The pLR03 plasmid contained a temperature-sensitive origin of replication (*ori*^{ts}; **Figure 5.3**), originating from plasmid pE194ts[414] and cloned from plasmid pHBintT, to allow selection of the first crossover event during *dcas9* integration. The *mazF* toxin gene from *E. coli* was included to select for the second crossover event[416]. To facilitate cloning in *E. coli*, the *mazE* antitoxin gene under the strong constitutive *E. coli lpp* promoter (*P_{lpp}*)[416] was also included in pLR03.

To assemble pLR03, the plasmids pLR01 and pLR02 were first constructed. The backbone of the plasmid pHBintT[508], which contains the pE194ts *ori* as well as an ampicillin resistance marker (*Amp*^r) and the ColE1 *ori* for cloning in *E. coli*, was amplified using the primer pair P01/P02. The *P_{lpp}::mazE* cassette was amplified from plasmid pAW005-02[502] using primers P03/P04 and assembled with the pHBintT backbone, producing plasmid pLR01. The *B. megaterium* arabinose promoter (*P_{ara,Bm}*) was originally

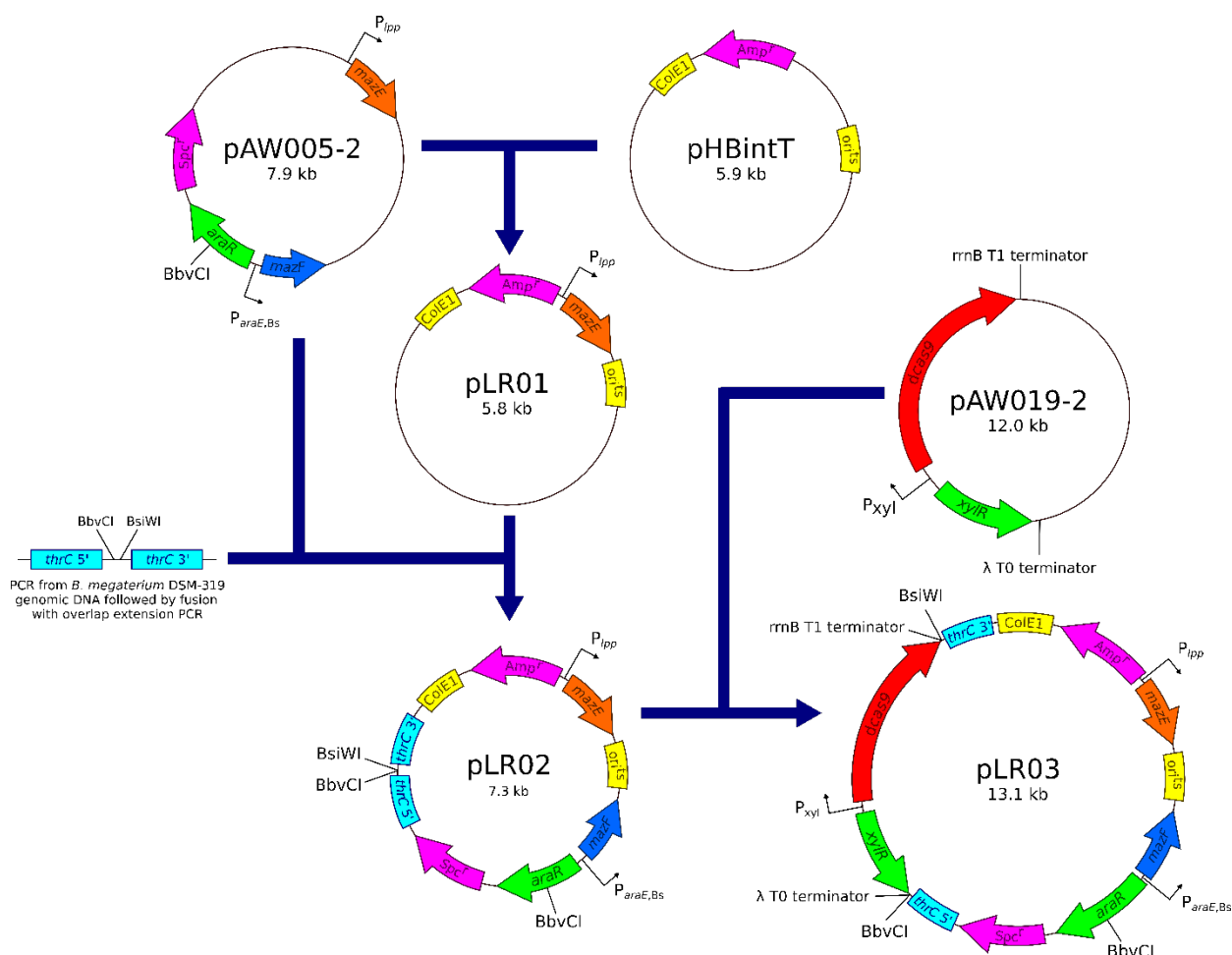
Table 5.1. Microbial strains and plasmids used in this work.

Description		Source or ref.
Strains		
<i>B. megaterium</i>		
DSM-319	Wild-type strain	DSMZ Culture Collection
LRBm01	DSM-319 <i>thrC</i> ::(<i>xylR</i> , <i>P_{xyl}</i> :: <i>dcas9</i>)	This work
Plasmids		
p3Shp_A3	For cloning of <i>P_{xyl}</i> ::P450 _{BM3} .A3 <i>xylR</i>	[464]
pHBintT	For cloning of pE194ts. <i>ori</i> Amp ^r ColE1. <i>ori</i>	[508]
pAW005-2	For cloning of <i>P_{araE, Bs}</i> :: <i>mazF</i> , <i>araR_{Bs}</i> Spc ^r <i>P_{lpp}</i> :: <i>mazE</i>	[502]
pAW019-2	For cloning of <i>P_{xyl}</i> :: <i>dcas9 xylR</i>	[502]
pLR01	Intermediate cloning vector, ColE1 Amp ^r <i>P_{lpp}</i> :: <i>mazE</i> pe194ts. <i>ori</i>	This work
pLR02	Intermediate cloning vector, pLR01 backbone, <i>P_{araE, Bs}</i> :: <i>mazF araR_{Bs}</i> Spc ^r <i>thrC</i> 5'::BbvCI.BsiWI:: <i>thrC</i> 3'	This work
pLR03	Integration vector, pLR02 backbone, <i>P_{xyl}</i> :: <i>dcas9 xylR</i>	This work
pLR04	sgRNA control vector, <i>P_{ara, Bm}</i> ::P450 _{BM3} .A3	This work
pLR05a	sgRNA expression vector, anti-(<i>araA</i> , <i>pgi</i> , <i>xylA</i>) <i>P_{ara, Bm}</i> ::P450 _{BM3} .A3	This work
pLR05a	sgRNA expression vector, anti-(<i>araA</i> , <i>pfkA</i> , <i>xylA</i>) <i>P_{ara, Bm}</i> ::P450 _{BM3} .A3	This work
pLR05b	sgRNA expression vector, anti-(<i>araA</i> , <i>gdh</i> , <i>pgi</i> , <i>xylA</i>) <i>P_{ara, Bm}</i> ::P450 _{BM3} .A3	This work
pLR05d	sgRNA expression vector, anti-(<i>araA</i> , <i>gdh</i> , <i>pfkA</i>) <i>P_{ara, Bm}</i> ::P450 _{BM3} .A3	This work

chosen to drive *mazF* expression as this was recently shown to provide tight repression in its native host[383]. To replace the *B. subtilis* arabinose promoter/repressor system that controls *mazF* on pAW005-02 (*P_{araE, Bs}*), the pAW005-02 backbone containing *mazF* was amplified using primers P05/06, the *P_{ara}/araR* system from *B. megaterium* was amplified using primers P07/08 and the two fragments were assembled. Correct clones of this assembly, however, were not able to be isolated from *E. coli*, likely due to leaky expression of MazF from *P_{ara, Bm}* following transformation, so *mazF* was left under the control of the *B. subtilis* arabinose promoter for the pLR03 plasmid. The xylose-inducible dCas9 expression cassette in plasmid pAW019-02 is flanked by transcriptional terminator hairpin sequences which precluded plasmid assembly using NEBuilder HiFi, so restriction enzyme cloning was used for the assembly of this cassette into pLR03. First, two ~500 bp fragments from the 5' and 3' regions of the *thrC* locus of *B. megaterium* were amplified from DSM-319 genomic DNA using primer pairs P09/P10 and P11/P12, respectively, and spliced together using overlap extension PCR, producing a fragment containing BbvCI and BsiWI restriction sites located between *thrC* 5' and *thrC* 3' homology arms. The cassette containing *P_{araE, Bs}*::*mazF*, *araR* and a spectinomycin resistance marker (Spc^r) was amplified from pAW005-02 with primers P13/P14; the pLR01 backbone containing *P_{lpp}*::*mazE*, Amp^r and the ColE1 and pE194ts replication origins was amplified using primers P15/P16; and these two fragments and the *thrC* homology cassette were assembled together to produce plasmid pLR02 (**Figure 5.3**). The dCas9 expression cassette

Table 5.2. List of primers and synthetic gene sequences used in this study. Lower case represents overhang sequences for NEBuilder HiFi assembly or the introduction of restriction sites. Restriction sites are shown in bold for P10, P12, P17, P18; protospacer sequences are shown in bold for P23-P32. For synthetic sequences, the *Bacillus megaterium* xylose promoter (P_{xyl}) is shown in italics and the single guide RNA (sgRNA) scaffold sequence is underlined.

Name	Sequence
Primers	
P01	ttgatgattagcTTCTTTGAGCGAACGATC
P02	gcgtccattaTTAGAGCTCTTACCAGACTTC
P03	agagctctaaTAATGGACGCAGCGCATC
P04	tcaaaagcTAATCATCAACAATCCGTTCCG
P05	agttcgtaaCTGGCGAATGGCGATTTTC
P06	cttccttactTAAGGAGGAAGCAGGTATG
P07	ttcctcctaAGTAAGGAAGCGCTTTCC
P08	cattcgccagTTAACGAACTTCATCACGC
P09	cgacatggatgaGATGTGGAAGGTCTATTAGC
P10	ttaccgttaggttagatctagttcg gctgagg GTAAGCTGTAATGTTTCCTG
P11	cgccagcaacGCCCTAAATTAGCAGAGCTAG
P12	cgaactagatctaactaacgta cgtagc ACGTCTCTTCCTCACATG
P13	actggttaagcGGATCCCTACCAATCAG
P14	acctttccacatCTCATCCATGTCGAGATC
P15	ctgctaatttagGCGTTGCTGGCGTTTTTC
P16	tggtagggatccGCTTAAACCAGTTTTCGTTGG
P17	gaatcac cctcagc AGGCTCAACTCCAAATATAGCTTG
P18	ctaag cgtagc AGCTCGGATCTCCTGCAGGATAAAAC
P19	gtcgtaacggtttatagtcTTTAAACGAACTTCATCACG
P20	gtgaacagtaCGTTTTATGTAACGCTGTTTG
P21	acataaaacgTACTGTCACTTAAATCAAGG
P22	atcacggttctacaagttagGTTAGCAAATATCACTGTTG
P23	tcttcagaacaatcgata GTTTTAGAGCTAGAAATAGC
P24	tgaaagtagctcaccattca ATCTCATCATATACAAAATAAATG
P25	tgaatggtgagctactttca GTTTTAGAGCTAGAAATAGC
P26	aactagtactaataaaatta ATCTCATCATATACAAAATAAATG
P27	ggtatgaatcctgcggttcg ATCTCATCATATACAAAATAAATG
P28	cgaaccgcaggattcatacc GTTTTAGAGCTAGAAATAGC
P29	ttaaagtgacgtaacagtt ATCTCATCATATACAAAATAAATG
P30	aactgttacgtcacctttaa GTTTTAGAGCTAGAAATAGC
P31	tgaaagtagctcaccattca ATCTCATCATATACAAAATAAATG
P32	ggtatgaatcctgcggttcg ATCTCATCATATACAAAATAAATG
P33	taattttattagtactagtt GTTTTAGAGCTAGAAATAGC
P34	tatccgattgttctgcaaga ATCTCATCATATACAAAATAAATG
P35	ATCCTTTACCTTGTCTACAAAC
P36	AAGATTTGCCAAACGAAAC
P37	GGAATTTGTGTTCCAGGC
P38	TGTGAAAGGAAGCGAATTAG
P39	AACTTGAATCGGAGTTTGTG
P40	CATTCTCATGACACTCTACTG
Synthetic genes	
<i>P_{xyl}_scaffold</i>	CTCACTTGTAGAACGGTGATCAGCCTGTGCTCTAGAGCCTGATAGTTGAGCGATACACACATATTATAACTA AATTTTCTAAAAAACATTGAAATAAACATTTATTTGTATATGATGAGATGTTTAGAGCTAGAAATAGCA AGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCAGATCGGTGCTTTTTGTGCTAACGT TTATAGTCT
<i>scaffold_P_{xyl}</i>	GTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCAGATCG GTGCTTTTTTATATTATAACTAAATTTCTAAAAAACATTGAAATAAACATTTATTTGTATATGATGAGAT



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Plasmid pLR04 and the four plasmids pLR05a, pLR05b, pLR05c and pLR05d (pLR05a-d; **Figure 5.4**) were constructed for the plasmid-based expression of CRISPRi sgRNAs and the P450_{BM3} mutant A3 (P450_{BM3}-A3). These plasmids were derived from the previously described plasmid p3Shp_A3 (**Paper 2**), which was used for xylose-inducible expression of P450_{BM3}-A3 in *B. megaterium*. As the chromosomally integrated *dcas9* gene was also inducible by xylose, the P450_{BM3}-A3 gene was placed under control of the *B. megaterium* arabinose induction system in pLR04 and pLR05a-d. The $P_{ara,Bm}$ and *araR* regions were amplified from DSM-319 genomic DNA using primers P19/P20, and the p3Shp_A3 backbone including the P450_{BM3}-A3 coding sequence was amplified with primers P21/22. These two fragments were assembled with a gBlock (*Pxyl_scaffold*) consisting of the *B. megaterium* xylose promoter (P_{xyl}) directly upstream of the sgRNA scaffold (consisting of the Cas9-binding hairpin sequence and a transcriptional terminator[509]) to produce pLR04 (**Figure 5.4**). The resulting plasmid was used as a control for CRISPRi in *B. megaterium*, as it lacks a protospacer sequence in the sgRNA cassette, as well as a template for construction of plasmids pLR05a-d. Protospacer sequences for CRISPRi-mediated repression of the five genes *pgi* (glucose-6-phosphate isomerase), *pfkA* (6-phosphofructokinase), *gdh* (glucose 1-dehydrogenase), *xylA* (xylose isomerase) and *araA* (L-arabinose isomerase) consisted of 20 bp sequences complementary to the non-template strand of the target genes within 200 bp of the start codon and directly adjacent to a PAM site (NGG) (**Figure 5.6**). These sequences were selected with the assistance of the MegaBac v9 database (<http://megabac.tu-bs.de/>). For the construction of sgRNA arrays for pLR05a-d, the STAgR (String Assembly gRNA cloning) method developed by Breunig *et al.*[510] was used. A PCR template consisting of the sgRNA scaffold directly upstream of the P_{xyl} sequence (*scaffold_Pxyl*) was produced by gene synthesis (Integrated DNA Technologies, Singapore). The *scaffold_Pxyl* template was then amplified using various primer pairs (P23/P24, P25/P26, P23/P27, P28/P26, P23/P29, P30/P31, P30/P32) where each primer had a 20 bp overhang consisting of either the sense or antisense sequence of the desired sgRNA protospacers. The primers were paired so that the forward primer for each amplicon contained the sense sequence of a protospacer and the reverse primer contained the antisense sequence for the adjacent protospacer in the sgRNA array (**Figure 5.5**). In this way, fragments were generated with 20 bp overlaps of protospacer sequences to facilitate assembly using the NEBuilder HiFi system. The entire pLR04 plasmid was then amplified by inverse PCR from the junction of P_{xyl} and the sgRNA scaffold using primers P33/P34, which had overhangs containing the first and final sgRNA protospacers in the array, respectively. This backbone was then assembled with the appropriate *scaffold_Pxyl* amplicons to produce plasmids pLR05a-d, which each contained arrays of three to four sgRNA cassettes (**Figure 5.4**). Transformed

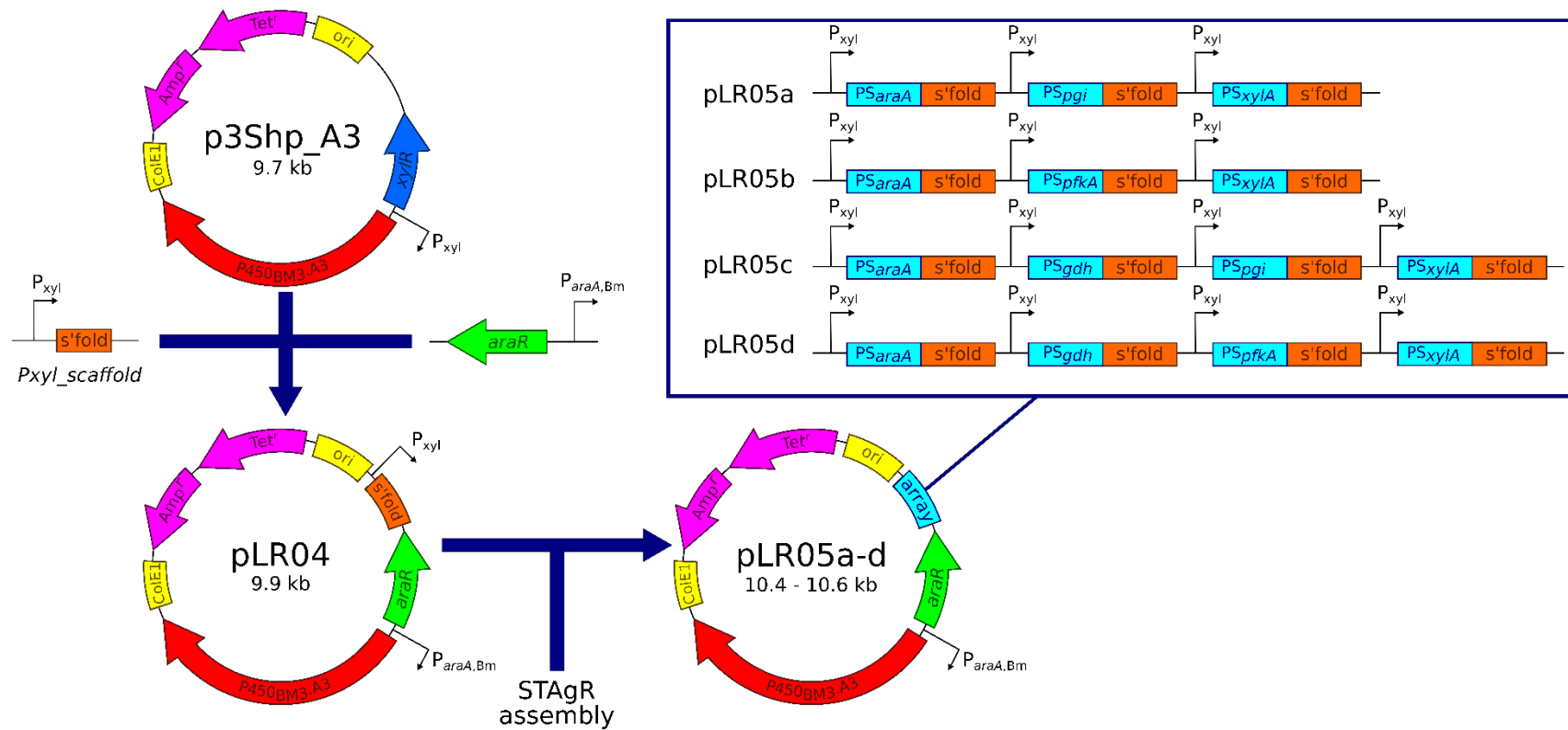


Figure 5.4. Construction of the four dual expression plasmids pLR05a-d for the expression of arabinose-inducible P450_{BM3} mutant A3 (P450_{BM3}.A3) and constitutive expression of sgRNA arrays. The plasmid pLR04 contains no protospacer for CRISPRi and was used as a control plasmid; plasmids pLR05a-d contain three or four protospacer sequences, as shown in the inset. For the sgRNA arrays (inset), ‘P_{xyl}’ refers to the *B. megaterium* xylose promoter; ‘PS’ refers to the unique 20-bp protospacer for the gene name which follows in subscript; and ‘s’fold’ refers to the sgRNA scaffold sequence consisting of the dCas9-binding hairpin and a transcriptional terminator.

pgi 5' TACATACTTACGTGATG**CCG**TGAAAGTAGCTCACCATTCAATTCATGAGAAAACAGGTGC 3'
 (153) (212)

pfkA 5' CAAGCGGCGAGATTCT**CCA**GGTATGAATCCTGCGGTTCTGTGCTGTTGTGCGTAAAGCAA 3'
 (23) (82)

gdh 5' CGGCGGAGAGGCAATTG**CCG**TTAAAGGTGACGTAACAGTTGAGTCTGACGTGATCAATTT 3'
 (165) (224)

araA 5' CGGCGGAGAGGCAATTG**CCG**TTAAAGGTGACGTAACAGTTGAGTCTGACGTGATCAATTT 3'
 (-12) (51)

xylA 5' GGGAAATGACAAATGGT**CCA**AAGTAGTACTAATAAAATTAAATCATTTTGAAAGCGCAAAC 3'
 (116) (175)

Figure 5.5. Protospacer sequences used for CRISPRi-mediated gene repression of the genes *pgi*, *pfkA*, *gdh*, *araA* and *xylA*. The protospacer sequences are complementary to the target sequences highlighted in grey. Nucleotides complementary to the protospacer-adjacent motifs (PAMs) are shown in bold. Base numbers in brackets are relative to the first translated nucleotide for each sequence.

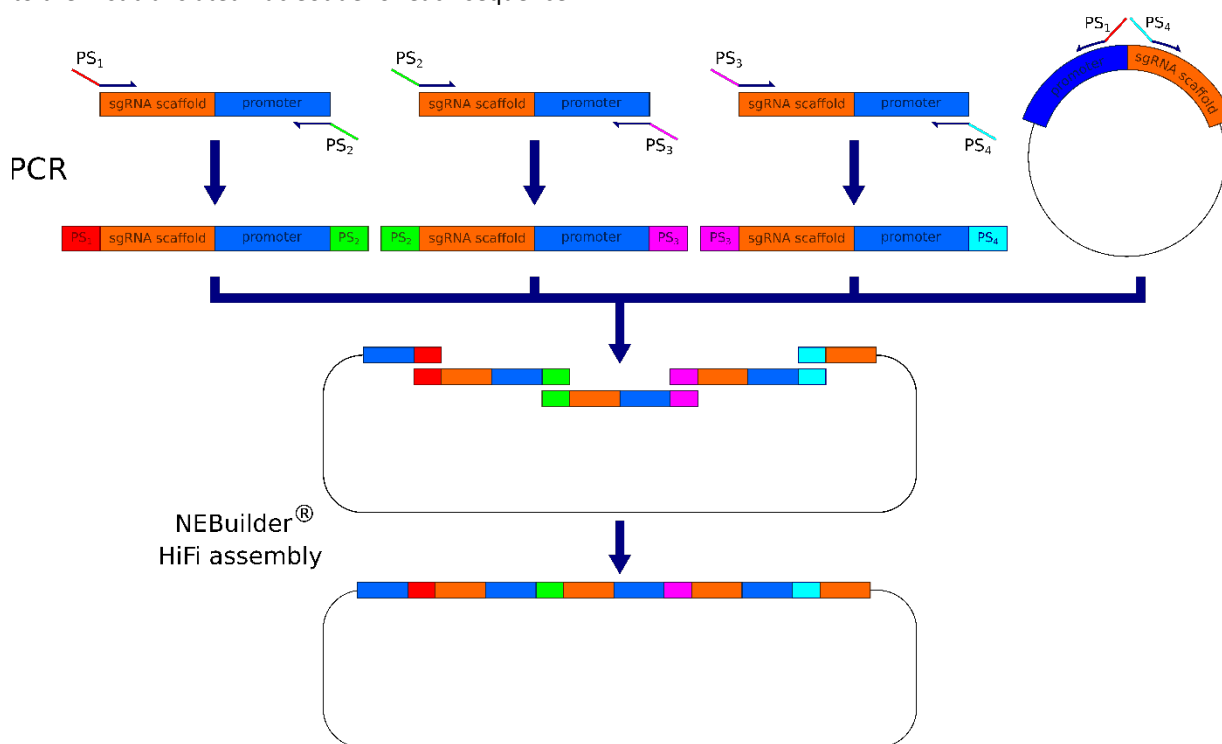


Figure 5.6. Schematic of the String Assembly gRNA Cloning (STAgR) method developed by Breunig *et al.*[510] for simultaneous assembly of multiplexed sgRNA arrays. A synthetic gene sequence consisting of the sgRNA scaffold (orange) followed by a promoter region (dark blue) is used as a template for PCR. The PCR primers contain overhangs (PS₁, PS₂, etc.) where the forward primer of each pair contains the sense sequence of one protospacer and the reverse primer contains the antisense sequence of the next protospacer in the array. The plasmid backbone contains the promoter sequence followed by the sgRNA scaffold sequence and is amplified by inverse PCR using primers with overhangs corresponding to the first and last protospacers in the array. The backbone and amplified PCR fragments are then assembled with NEBuilder HiFi using the protospacer sequences as complementary overlaps.

colonies were screened by colony PCR using primer pair P35/P36. Colonies that produced DNA electrophoresis bands showing the expected sgRNA cassette lengths were selected and the correct cassette sequences were confirmed by Sanger sequencing (Australian Genome Research Facility, Parkville, Australia).

Protoplast transformation

Preparation and transformation of *B. megaterium* DSM-319 protoplasts was performed using the minimal medium protocol developed by Moore *et al.*[511], with the following modifications: cultures for protoplast production were grown in the MOPS minimal medium described by Neidhardt[436] supplemented with 1% glucose and 0.05% yeast extract; Tris buffer in the hypertonic protoplast medium was also replaced by 40 mM MOPS buffer. Protoplast regeneration was performed at 30°C. Agar plates for regeneration were supplemented with either spectinomycin (for pLR03; 85 µg/mL) or tetracycline (pLR04, pLR05a-d; 10 µg/mL).

Construction of strain LRBm01

Following transformation of the *B. megaterium* DSM-319 protoplasts with the *dcas9* integration plasmid pLR03, transformant colonies were plated onto MOPS minimal medium agar supplemented with spectinomycin, 1% glucose, 0.02% casamino acids and 40 µg/mL threonine and incubated overnight at 42°C. At this temperature, replication from the pE194ts *ori* of plasmid pLR03 does not occur and the resulting spectinomycin-resistant colonies resulted from single-crossover homologous recombination of the pLR03 plasmid into the *B. megaterium* DSM-319 genome.

Spectinomycin-resistant colonies were screened by colony PCR using primers P37/P38 and P39/P40, which were specific to a single crossover at either the *thrC* 5' or 3' homology regions of pLR03, respectively. One colony demonstrating a single crossover at the *thrC* 5' site was then used to produce a strain resulting from a second crossover event at the *thrC* 3' homology arm, as described below. This second event resulted in the eviction of the pLR03 backbone and left the xylose-inducible dCas9 expression cassette at the *thrC* genomic locus.

A screening strategy based on the loss of spectinomycin resistance was used to select for the second crossover event, as the alternative approach of using counterselection based on the arabinose-inducible MazF toxin in plasmid pLR03 was not possible due to the spontaneous generation of MazF-resistant colonies. The single-crossover colony selected after growth at 42°C was used to inoculate 5 mL of nonselective MOPS minimal medium supplemented with 0.5% glucose and 40 µg/mL threonine, which

was cultured for 24 h at 30°C with shaking at 200 rpm. The culture was then diluted 1/100 into 5 mL of fresh medium and grown for a further 12 h. The dilution process was repeated and the culture was grown for a further 12 h. The culture was then serially diluted, plated onto LB medium and incubated at 42°C overnight to prevent the replication of any excised pLR03 backbone vector. The resulting colonies were then replica-plated onto LB agar with and without spectinomycin. Colonies demonstrating spectinomycin sensitivity were then screened by colony PCR using primers P39/P40 for a crossover event at *thrC* 3' homology region. One colony that showed a *thrC* 3' crossover was selected and the correct *dcas9* sequence was confirmed to be present at the *thrC* locus by Sanger sequencing. This strain was named LRBm01; the strain was then transformed with the sgRNA expression plasmids pLR04 and pLR05a-d using the protoplast transformation protocol described above.

Whole-cell biotransformation

Strains of LRBm01 containing the plasmids pLR04 (sgRNA control) and pLR05c (*pgi*, *gdh*, *araA* and *xylA* protospacers) were inoculated in 50 mL of minimal medium (containing per L: 4.86 g KH₂PO₄; 4.87 g Na₂HPO₄; 2 g NH₄Cl; 1 g casamino acids; 500 mg NaCl; 896 mg Tricine; 214 mg MgSO₄; 11 mg CaCl₂; 38 mg FeSO₄·7H₂O; 100 µg biotin; 1 mL 1000X trace metals[436]) and grown at 30°C and 100 rpm shaking overnight. Each culture was diluted 1/100 into 150 mL of the same medium in a 500 mL round-bottom flask. The flasks were then shaken at 30°C and 200 rpm until the OD₆₀₀ reached 0.3-0.4. Each 150-mL culture was split into 3 x 50 mL cultures in 250 mL flasks and expression of P450_{BM3}.A3 was induced by the addition of arabinose at a concentration of 0.5% (w/v). Six hours after induction, dCas9 expression in two of the three flasks of each set was induced using xylose at 0.5% and 1.5% (w/v), while the third flask received no xylose addition. Four hours later, biphasic biotransformation of noscapine was commenced by the addition of 100 µM noscapine from a methanol stock (1% final MeOH concentration) and 12.5 mL (20% total volume) of isopropyl myristate saturated with noscapine. Following 12 h of biotransformation, samples of the aqueous and organic layers from each flask were removed and analysed for product formation by UHPLC-DAD, as described in **Paper 2**.

5.3. Results and Discussion

The first step in establishing the CRISPRi system in *B. megaterium* DSM-319 was to integrate an inducible copy of the *dcas9* gene into the *B. megaterium* genome. Plasmids for sgRNA and P450_{BM3} expression were then constructed and transformed into the *dcas9* integration strain to allow CRISPRi-mediated gene repression to occur during the biotransformation of noscapine (**Figure 5.7**).

Construction of a *dcas9* integration strain

The sequence of steps required to integrate the *dcas9* gene into the *B. megaterium* DSM-319 by homologous recombination is shown in **Figure 5.7**. Previous studies with other bacteria have shown that the expression of dCas9 from a single copy within the genome is preferable to plasmid-based expression, as ‘leaky’ expression from multicopy plasmids can result in significant target gene repression in the absence of an inducer[512]. A single genomic copy can also provide better dynamic control of dCas9 expression (and therefore target gene repression) by varying the concentration of inducer.

The details of the double-crossover homologous recombination process (reviewed in **Chapter 2.7**) used for genomic integration of the *dcas9* gene are shown in **Figure 5.8**. This use of a double-crossover integration approach allows marker-less integration of the desired gene so that selectable markers (e.g. antibiotic resistance) do not remain in the host genome following a genetic engineering event, allowing these to be re-used for future gene editing. Another advantage of using a double-crossover strategy is that it avoids the genetic instability associated with the integration of a plasmid using only a single crossover (i.e. Campbell-like integration[415]), as a single crossover generates two homologous repeats that can recombine and result in spontaneous excision of the integrated plasmid.

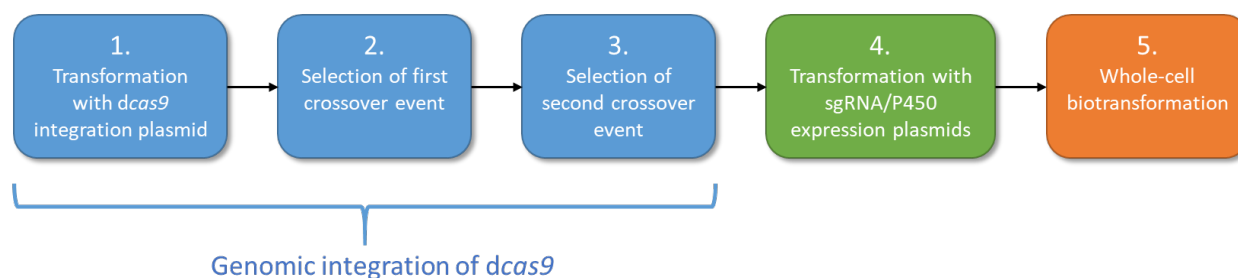


Figure 5.7. Workflow used for CRISPRi-mediated gene repression during whole-cell biotransformation of noscapine.

The three-step approach used for double-crossover homologous recombination in *B. megaterium*[404, 412, 413] consisted of:

- (i) transformation of *B. megaterium* with a freely-replicating plasmid;
- (ii) selection of single-crossover plasmid integration; and
- (iii) selection of a second crossover event resulting in excision of the plasmid backbone.

This approach was necessary as the transformation efficiency of *B. megaterium* is relatively low compared to other commonly used bacteria such as *B. subtilis* and *E. coli*[409]. Consequently, homologous recombination events rarely accompany transformation[412]. Gene editing strategies that accomplish transformation and integration in a single step, such as transformation with a linearised or replication-defective plasmid, are therefore not suitable for the genetic engineering of this organism.

The plasmid pLR03 used for double-crossover homologous recombination is shown in **Figure 5.3**. This plasmid was constructed for the integration of *dcas9* at the threonine synthase locus (*thrC*), as this locus has been shown to provide good recombination frequency in *Bacillus* species[513]. The plasmid contained a spectinomycin resistance gene (*Spc^R*) to allow for the selection of transformed colonies in the first step of the gene integration strategy. The second step made use of a temperature-sensitive origin of replication (*ori^{ts}*) which allowed the free replication of pLR03 at temperatures under ~37°C but not above 40°C[414]. Colonies transformed with pLR03 were sub-cultured at 42°C and the resulting spectinomycin-resistant contained a genomic copy of pLR03 at the *thrC* locus, as confirmed by colony PCR. A single-crossover strain produced in this way was then used to generate a double-crossover strain in the third step of the process.

The MazF toxin from *E. coli* was chosen as a positive selection marker for a second recombination event[416]. MazF is an endoribonuclease that cleaves free mRNA, inhibiting protein production[514]. The *mazF* gene was placed under arabinose-inducible control outside the *thrC* homology arms on pLR03 so that it would be excised by a second recombination event (**Figure 5.8**). Arabinose-resistant colonies derived from a single-crossover strain would therefore be expected to have undergone a second crossover, resulting in either a *dcas9* integration strain (shown in **Figure 5.8**) or reversion to the wild-type strain (not shown). As the leaky expression of MazF from inducible promoters has been shown to render plasmids non-transformable in *E. coli*[416, 502, 515, 516], it was necessary to include the *mazE* antitoxin gene on the pLR03 backbone to allow plasmid construction and cloning in this species. The antitoxin gene was placed under control of the strong constitutive *E. coli* promoter *P_{lpp}*[416].

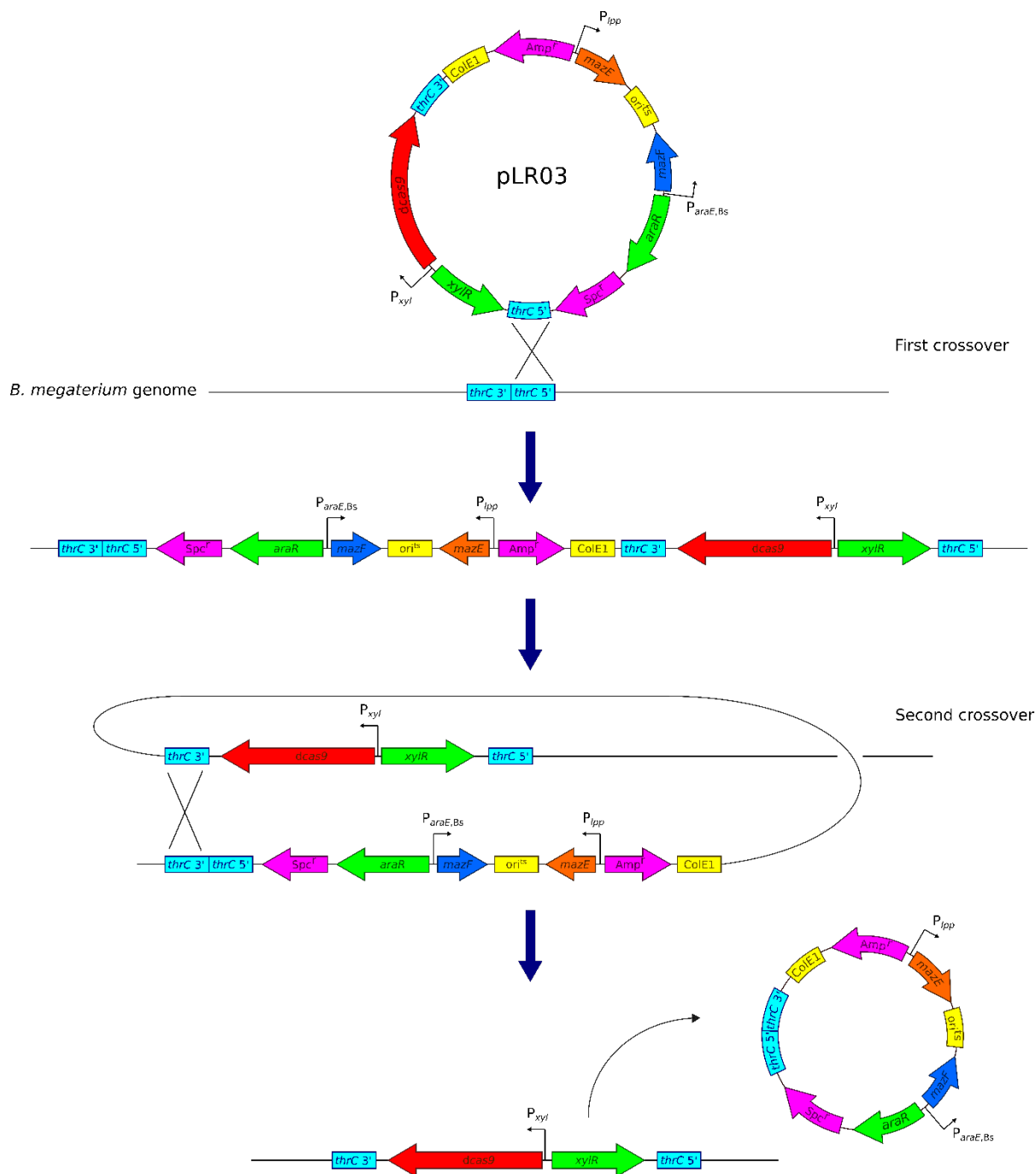


Figure 5.8. Chromosomal integration of the xylose-inducible dCas9 expression cassette by double-crossover homologous recombination at the threonine synthase (*thrC*) locus of *Bacillus megaterium* DSM-319. The plasmid pLR03 was integrated into the *B. megaterium* genome by homologous recombination at the *thrC 5'* locus. The resulting single-crossover was isolated by growth on spectinomycin at 42°C, a temperature at which the free plasmid is unable to replicate. A subsequent crossover event occurred between the homologous *thrC 3'* regions, resulting in the eviction of the plasmid backbone. The double-crossover strain containing the expression cassette at the *thrC* locus (strain LRBm01) was isolated by screening for spectinomycin-sensitive colonies and confirmed using PCR.

The MazF toxin gene made the construction of the pLR03 plasmid difficult in *E. coli*. Specifically, transformation efficiency with the intermediate construct pLR02 (**Figure 5.3**) was found to be poor, and sequencing of the *mazF* region of the plasmid from resulting colonies typically showed transposon insertions or deleterious mutations within the coding sequence or promoter. This appeared to be due to MazF toxicity caused by basal expression from the *B. subtilis* arabinose promoter ($P_{araE,Bs}$), despite constitutive expression of the *mazE* antitoxin from this plasmid. Transformation efficiency was improved by incubating colonies at room temperature (instead of at 37°C) following transformation in an effort to reduce leaky MazF expression. Arabinose-sensitive colonies containing plasmids with functional *mazF* expression cassettes were successfully selected by replica-plating the transformed colonies onto antibiotic media with and without arabinose. The same strategy was then used to isolate *E. coli* colonies containing the plasmid pLR03 following the insertion of the xylose-inducible *dcas9* cassette between the *thrC* homology arms of pLR02 (**Figure 5.3**). The transformation efficiency of pLR03 into *B. megaterium* DSM-319 was also poor, again likely due to leaky MazF expression, though spectinomycin-resistant colonies could be successfully isolated.

Difficulties were also encountered using the MazF toxin to screen for a second crossover event in *B. megaterium* due to the generation of spontaneous MazF resistance. A single-crossover colony was cultured overnight and then plated on arabinose to allow for the positive selection of colonies that had lost the *mazF* cassette. Unexpectedly, all arabinose-resistant colonies also showed spectinomycin resistance, indicating that the pLR03 backbone was still present and that the expression of the MazF toxin had failed to cause cell death. This was despite arabinose sensitivity being evident following initial transformation with the pLR03 plasmid. Sequencing of the *mazF* cassette in selected arabinose-resistant colonies showed no mutations in the *mazF* coding sequence or promoter, indicating that MazF resistance was not due to direct genetic inactivation. The generation of spontaneous MazF resistance has been reported for the use of this counterselection system in *B. subtilis* by others, although at a lower frequency[416, 516, 517]. These integration strategies, however, utilised a combined transformation and single-crossover step and therefore required less sub-culturing steps than the approach used here.

The mechanism for MazF resistance is not clear. While it is possible that resistance was conferred by expression of the MazE antitoxin on the pLR03 backbone, this seems unlikely because:

- (i) arabinose sensitivity was evident following transformation;

- (ii) the P_{lpp} promoter driving MazE expression, while strong in *E. coli*, was shown to be weak in *Bacillus* sp.[518]; and
- (iii) spontaneous MazF resistance arises in other MazF counterselection systems that do not utilise the mazE antitoxin[516, 517].

MazF resistance is usually attributed to low-level expression of the toxin from leaky promoters prior to overexpression in the counterselection stage, a problem that has been overcome by the use of more tightly regulated promoters[517]. The *B. subtilis* arabinose promoter used here for MazF expression may therefore have not been sufficiently tightly regulated in *B. megaterium*, despite supplementation of growth media with glucose for further repression via the catabolite-responsive element (*cre*) in the promoter sequence[519]. The requirement here for promoters that show tight regulation in both *E. coli* and *B. megaterium* makes promoter selection difficult given the lack of characterised promoters for *B. megaterium*. The *B. megaterium* arabinose promoter was recently studied and shown to provide tight control of expression in its native host[383] but initial attempts to use this promoter for MazF expression here failed due to poor regulation in *E. coli*. Similarly, the *B. megaterium* xylose promoter[397], the most widely used promoter for recombinant expression in this organism, is poorly controlled in *E. coli*[401, 520] and attempts by others to use it to drive MazF expression failed to produce transformable plasmid constructs during cloning[502, 516]. Further characterisation of promoters for recombinant expression *B. megaterium* may allow the successful use of the MazF counterselection in this organism in the future.

An alternative approach was used to detect a second crossover event where colonies were screened for the loss of spectinomycin resistance via replica plating. To do this, a single-crossover colony showing a crossover at the *thrC* 5' locus was grown in nonselective liquid medium at 30°C and sub-cultured three times to allow sufficient opportunity for a second crossover event to occur. Samples were then plated on nonselective agar and grown at 42°C to prevent the replication of excised plasmid backbone. Colonies were then re-plated onto agar in parallel both with and without spectinomycin. Of the 152 colonies screened, six (4%) were unable to grow on spectinomycin. Four of these were found to have undergone a crossover at the *thrC* 3' homology region when assessed by colony PCR, leaving the xylose-inducible *dcas9* cassette in the *B. megaterium* genome (**Figure 5.8**). One colony was selected for sequencing and shown to contain a $P_{xyI}::dcas9$ cassette with the correct sequence; this strain was named LRBm01 and was used for all subsequent work.

Construction of sgRNA/P450 expression vectors

Four plasmids (pLR05a to pLR05d; **Figure 5.4**) were constructed to allow CRISPRi-mediated target gene repression to occur alongside noscapine *N*-demethylation by P450_{BM3} mutant A3 (P450_{BM3}.A3).

Several genes were selected as targets for CRISPRi-mediated repression to enhance carbon flux through the pentose phosphate pathway. These were: glucose-6-phosphate isomerase (*pgi*), 6-phosphofructokinase (*pfkA*) and glucose 1-dehydrogenase (*gdh*) (**Figure 5.1**). The sgRNA arrays on plasmids pLR05a-d were designed to enable the repression of *pgi* and *pfkA* to be examined individually, with and without additional repression of *gdh* (inset, **Figure 5.4**). Each cassette also contained sgRNA sequences for the repression of both xylose isomerase (*xylA*) and L-arabinose isomerase (*araA*) to minimise consumption of the sugars used to induce dCas9 and P450_{BM3}.A3 expression, respectively. While the active consumption of glucose by *B. megaterium* typically limits the utilisation of these sugars through carbon catabolite repression (CCR)[521], these sgRNAs were included because repression of either *pgi* or *pfkA* may decrease the intracellular concentration of the main regulator of CCR, fructose 1,6-bisphosphate (FBP; **Figure 5.1**), to levels low enough to relieve CCR, potentially allowing consumption of these inducing sugars. This would allow carbon flux derived from xylose or arabinose to enter the central carbon metabolism after the oxidative stage of the pentose phosphate pathway in which NADPH is generated (as xylulose 5-phosphate, **Figure 5.1**). A recent study using *Bacillus subtilis* found that CRISPRi-mediated repression of *pfkA* permitted diauxic growth on glucose and xylose, likely through this mechanism[507]. Additionally, repression of *xylA* and *araA* would limit consumption of xylose and arabinose inducers if glucose became depleted during biotransformation.

The plasmids pLR05a-d were designed to allow constitutive expression of sgRNAs using the *B. megaterium* xylose promoter (P_{xyl}) without its associated repressor binding sequence. Each of the individual sgRNA cassettes within the sgRNA arrays of the plasmids consisted of the P_{xyl} sequence upstream of a 20-bp protospacer sequence complementary to the target gene, followed by the sgRNA scaffold which consisted of the Cas9-binding hairpin structure and a transcriptional terminator (inset, **Figure 5.4**). The protospacer sequences were selected to be complementary to the non-template strand of the target gene, within 200 bp of the start codon and directly adjacent to a PAM site ('NGG'), as shown in **Figure 5.5**. The four sgRNA arrays were assembled into the expression plasmids using the STaGR method developed by Breunig *et al.*[510], which allows for simultaneous incorporation of multiplex sgRNA arrays into a plasmid backbone (**Figure 5.6**).

The P450_{BM3}.A3 enzyme for noscapine *N*-demethylation was also included in the sgRNA expression plasmids pLR05a-d. In previous work (**Paper 2**), expression of P450_{BM3}.A3 in *B. megaterium* was under

xylose-inducible control; this was changed to arabinose induction here to allow independent induction of dCas9 and P450_{BM3}.A3 expression. The arabinose promoter of *B. megaterium* was recently characterised by Hartz and colleagues for the expression of recombinant proteins and showed strong induction with low basal expression[383]. In the work here, examination of the promoter region used by Hartz *et al.* revealed a putative catabolite-responsive element (*cre*)[522] that may cause repression of target gene expression in the presence of glucose (**Figure 5.9**). This fragment of the arabinose promoter of *B. megaterium* was therefore excluded when the promoter was incorporated into expression plasmids in this work to maximise P450_{BM3}.A3 expression during growth on glucose.

Whole-cell biotransformation

To allow the whole-cell biotransformation of noscapine, the *dcas9* integration strain LRBm01 was transformed with plasmids pLR05a-d. In parallel, the strain was also transformed with the sgRNA control plasmid pLR04, which lacks a protospacer sequence in its sgRNA cassette. Transformed colonies were successfully obtained for pLR05a, pLR05c and pLR04. Plasmids pLR05b and pLR05d, which both contain sgRNA sequences targeting *pfkA*, did not produce viable transformants. The lack of transformants for these plasmids may have been due to basal expression of dCas9 in LRBm01 causing repression of *pfkA* in the absence of induction, which may have been detrimental for protoplast regeneration and colony formation. In *B. subtilis*, inactivation of *pfkA* has been shown to cause reduced growth rate and glucose uptake in minimal medium[523]. The protoplast transformation and regeneration protocol followed here used glucose-supplemented minimal medium, which may have contributed to this outcome. The use of complex medium may therefore provide better results for the transformation of LRBm01 with these plasmids.

Preliminary biotransformation experiments performed with the successful transformants demonstrated that the CRISPRi system could improve the productivity of noscapine *N*-demethylation by P450_{BM3}.A3. Experiments were performed with strain LRBm01 containing plasmid pLR05c, which had sgRNA protospacer sequences targeting *pgi*, *gdh*, *xylA* and *araA* (inset, **Figure 5.4**).



Figure 5.9. Identification of a putative catabolite-responsive element (*cre*) in the region of the *Bacillus megaterium* arabinose promoter described by Hartz *et al.* (2019) which could potentially cause repression of gene expression during active glucose metabolism. This region was excluded when the promoter was incorporated into plasmids pLR04 and pLR05a-d. The suspected *cre* is aligned with the *Bacillus subtilis* *cre* consensus sequence derived by Miwa *et al.* (2000). The region is located 278 bases upstream of the start codon of *BMD_3532* in the *B. megaterium* DSM-319 genome. The letter N represents any base; W represents A or T; R represents G or C. Base mismatches are represented by dots.

Biotransformation performance was compared to cells containing the control plasmid pLR04, which had no protospacer sequences. The biotransformation experiments utilised a biphasic system as used in previous work (**Paper 2**).

The CRISPRi system improved product titres by three- to four-fold after 12 h of biotransformation in the pLR05c cultures compared to the pLR04 control strain, as shown in **Figure 5.10**. The improvement in biotransformation yield was similar for the three levels of xylose used to induce the dCas9 gene (0, 0.5 and 1.5% w/v; **Figure 5.10**), indicating that the sgRNA array on pLR05c conferred higher biotransformation productivity with or without dCas9 induction. It is likely that this result was due to leaky expression of dCas9 in the absence of inducer. This low-level expression may have caused sufficient target gene repression to alleviate the NADPH limitation of the P450 reaction and suggests a high efficiency of the CRISPRi system in *B megaterium*.

In the preliminary experiments performed here, the CRISPRi system allowed a growing-cell biotransformation process to produce higher specific yields than previously developed resting-cell systems. The previously described biphasic resting-cell process using isopropyl myristate as a solvent (**Paper 2**) is the best system for comparison as

this biphasic system was also used for the growing-cell experiments here. The resting-cell process consisted of 20 h of P450_{BM3}.A3 expression followed by cell harvesting and concentration prior to substrate addition. The reaction was performed in a nitrogen-free medium that did not allow for further cell growth or protein expression. In the growing-cell process, substrate was added after 10 h of P450_{BM3}.A3 expression, and growth and expression continued throughout the 12 h biotransformation period. Given that the cell culture for the resting-cell process was concentrated five-fold prior to biotransformation, it is reasonable to compare the two processes based on product yield per

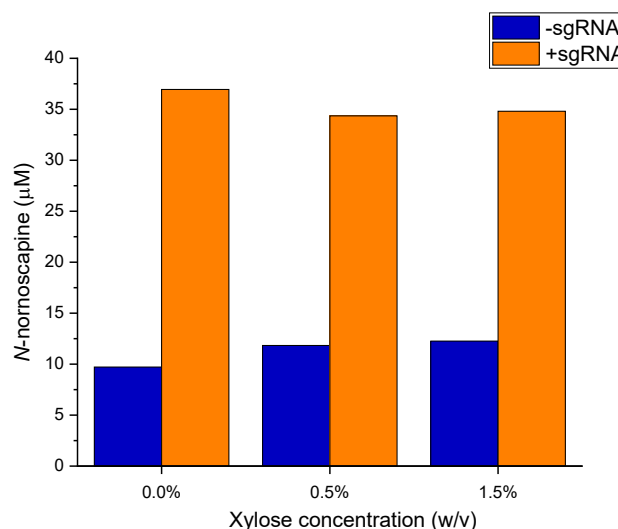


Figure 5.10. Noscapine biotransformation experiments using the *dcas9* integration strain LrBm01 containing either the control plasmid pLR04 (-sgRNA) or plasmid pLR05c (anti- *pgi*, *gdh*, *araA*, *xylA*; +sgRNA). Expression of P450_{BM3}.A3 was induced by arabinose for 6 h followed by the addition of 0.0%, 0.5% or 1.5% (w/v) xylose to induce expression of dCas9. After 4 h, noscapine was added in saturated isopropyl myristate (20% final volume) to commence biphasic biotransformation. The biotransformation reactions were stopped after 12 h. Experiments were preliminary and not replicated.

original culture volume. On that basis, the resting-cell process produced a final *N*-nornoscapine concentration of $\sim 94 \mu\text{M}$ which corresponds to a specific product yield of $\sim 7.9 \text{ mg per L}$ of original culture volume. For the growing-cell processes, cells containing the CRISPRi system produced a final product yield of $\sim 14.5 \text{ mg/L}$ ($35 \mu\text{M}$) while the control culture without CRISPRi produced an average of $\sim 4.5 \text{ mg/L}$ ($11 \mu\text{M}$) (**Table 5.3**). This represents an almost two-fold improvement in specific product yield in the CRISPRi-enhanced growing-cell system compared to the resting-cell process.

The use of the CRISPRi system also allowed a reduced process time and the elimination of processing steps in addition to improving product yields. Productivity was higher in the resting-cell biotransformation, which ran for 4 h, corresponding to a specific productivity of $\sim 1.9 \text{ mg/(L}\cdot\text{h)}$ when adjusted for original culture volume, while the growing-cell CRISPRi process had a lower productivity of $\sim 1.2 \text{ mg/(L}\cdot\text{h)}$ (**Table 5.3**). However, when considering the total process time from inoculation of the expression culture to termination of the biotransformation reaction, the resting-cell process took longer at 28 h while the growing-cell process ran for 25 h and did not require cell harvesting, washing or concentration steps.

Leaky expression of dCas9, as observed here, can often cause significant repression of target genes[505, 524-526], in some cases reducing target gene activity by 90-99% compared to control cultures[526-528] due to the high efficiency of CRISPRi in bacteria. In the work performed here, the degree of repression of target genes with and without xylose addition was not characterised. The experiments suggest that the biotransformation reaction was NADPH-limited in the control cultures and basal expression of dCas9 was sufficient to alleviate this limitation (**Figure 5.10**). It is possible that higher levels of dCas9 expression further enhanced the intracellular NADPH concentration but the productivity was limited by some other factor such as enzyme kinetics or P450_{BM3} concentration. Further work is required to assess whether xylose induction of dCas9 in the CRISPRi system developed here can provide any dynamic control over

Table 5.4. Comparison of key process metrics for biphasic (isopropyl myristate) growing-cell processes with and without the CRISPRi system compared to the biphasic (isopropyl myristate) resting-cell biotransformation process described in Chapter 2. The data for the growing-cell processes are the average yields from experiments with 0.0%, 0.5% and 1.5% xylose addition, depicted in Figure 7. Errors are $\pm 1 \text{ SD}$.

	Resting cells	Growing cells (control)	Growing cells (CRISPRi)
Final product conc. (μM)	94 ± 11	11 ± 1	35 ± 1
Final product conc. (mg/L)	39.5 ± 5	4.5 ± 0.4	14.5 ± 0.4
Cell concentration factor	5X	1X	1X
Yield per L original culture volume (mg/L)	7.9 ± 1	4.5 ± 0.4	14.5 ± 0.4
Biotransformation productivity (mg/(L·h))	1.9 ± 0.3	0.4 ± 0.03	1.2 ± 0.03
Total process time (h)	28	25	25

gene repression. This could be examined by quantifying target gene mRNA transcripts or measuring the enzyme activity of target gene products such as glucose-6-phosphate isomerase. Nevertheless, this study provides a proof of concept that CRISPRi-mediated repression can be applied to enhance biotransformation productivity in *B. megaterium*.

Chromosomal integration of sgRNA arrays in the system developed here may help to reduce gene repression in absence of inducer. The expression of dCas9 was controlled by the *B. megaterium* xylose promoter in this work. This promoter has been used by others to drive dCas9 expression in *B. subtilis* with minimal target gene repression in the absence of inducer[502, 507]. In these cases, however, both the *dcas9* expression cassette and the sgRNA arrays were integrated into the host chromosome. Here, the sgRNAs were transcribed from multicopy plasmids and target gene repression by basally expressed dCas9 may have been enhanced by an abundance of sgRNA transcripts. The most straightforward solution would be to place *dcas9* under the control of a more tightly regulated promoter, as done by others[525, 528], but this approach is made difficult by the lack of characterised promoters for recombinant *B. megaterium* systems. Other strategies to tackle leaky CRISPRi systems include engineering of the promoter and ribosomal binding site sequences to reduce basal dCas9 expression[528].

Whilst leaky dCas9 expression may make this system unsuitable for applications where CRISPRi gene targets cause significant growth or viability defects, in this case the biotransformation productivity was significantly enhanced with no obvious disadvantages from constitutive induction of the CRISPRi system. A slightly reduced growth rate was observed prior to induction of P450_{BM3}-A3 expression, with the pLR05c culture taking approximately 1 h longer than the control culture to reach a suitable cell density for induction, although both cultures had a similar final cell density following 12 h of biotransformation. The lack of inducer requirements in this system also has the advantage of reducing costs for large-scale bioprocesses.

The CRISPRi system developed and implemented in *B. megaterium* here shows promise for enhancing the performance of noscapine *N*-demethylation by growing cells and further work may allow greater improvements in productivity. Initially, examination of gene repression with and without induction of dCas9 could be investigated by mRNA quantification. Intracellular NADPH concentrations could be compared between CRISPRi and control strains to quantify the improvement in cofactor supply using the CRISPRi system. Growth rates and glucose consumption could also be investigated to further understand the metabolic changes produced by CRISPRi implementation. The biotransformation yields here were only measured at one timepoint and more data could be collected to investigate the kinetics of the

biotransformation and optimise the productivity of the system. If transformation with plasmids pLR04b and pLR04d can be achieved, these strains could also be investigated to see whether the repression of *pfkA* provides improvements in biotransformation productivity compared to *pgi* repression.

5.4. Conclusions

A CRISPR-interference system was established for the first time in *B. megaterium* to enhance the metabolic supply of NADPH cofactor during cytochrome P450-mediated whole-cell biotransformation. The system was comprised of chromosomally integrated *dcas9* and plasmid-based sgRNA arrays. Preliminary results showed a three- to four-fold enhancement in noscapine *N*-demethylation productivity using multiplexed gene repression of *pgi*, *gdh*, *xylA* and *araA*. Improvement in product titres occurred both with and without induction of dCas9 expression, indicating that basal expression of dCas9 from the *B. megaterium* xylose promoter was sufficient to alleviate NADPH limitation of the P450 reaction in growing cells. The use of the CRISPRi system allowed growing cells to produce approximately two-fold higher specific product titres than a previously developed resting-cell biotransformation system while reducing overall process time and eliminating processing steps. Constitutive CRISPRi activation was also not found to significantly impair cell growth. Further refinement of leaky dCas9 expression will require the investigation of new promoters for recombinant protein expression in *B. megaterium*, as few have been characterised in this organism. This chapter shows that the use of CRISPRi to enhance the performance of a P450-mediated biotransformation is a promising strategy. Further investigation will allow a more complete characterisation of the system presented here and provide further potential to enhance biotransformation productivity.

6. Conclusions and future work

A combination of protein engineering (**Chapter 3**), reaction engineering (**Chapter 4**) and strain engineering (**Chapter 5**) was used to develop a cytochrome P450-mediated whole-cell biotransformation process for the production of noscapine metabolites. The workflow used was as follows:

1. Construction and screening of a library P450_{BM3} mutants to find enzymes capable of metabolising noscapine (**Chapter 3**);
2. Systematic assessment of process limitations for a whole-cell biotransformation using a P450_{BM3} mutant, including debottlenecking of mass transfer and substrate solubility limitations (**Chapter 4**);
3. Metabolic engineering of the microbial host strain to address cofactor supply limitation (**Chapter 5**).

Engineering at each stage of the project provided gains in biotransformation productivity, for example:

- (i) Protein engineering produced active mutants from the inactive wild-type enzyme. Sequential introduction of further mutations provided up to 2.7-fold enhanced activity, 6.7-fold improved coupling efficiency and 1.5-fold improvement in *N*-demethylation selectivity;
- (ii) The use of Gram-positive *Bacillus megaterium* as a biotransformation host provided a 25-fold improvement in specific initial rate due to superior mass transfer compared to Gram-negative *E. coli*;
- (iii) The use of a biphasic biotransformation for enhanced substrate supply provided a two-fold improvement in product yields;
- (iv) Improved substrate solubility using a cyclodextrin/polymer system provided a five-fold increase in initial reaction rate;
- (v) Redirection of carbon flux towards NADPH production provided a three-fold improvement in productivity for a growing-cell biotransformation.

The process limitations identified and addressed throughout the project - mass transfer, substrate supply, enzyme stability and cofactor supply - are common issues for whole-cell biotransformations with cytochrome P450s and the approaches developed here may be useful for the improvement of P450-mediated biotransformations with other substrates. It is important to note, however, that the highest product yield achieved in this project (~110 mg/L, **Chapter 4**) is likely to be insufficient for the practical

production and purification of *N*-nornoscapine for use in drug development and is far below the target of 20 g/L recommended for an industrially viable P450-based biomanufacturing process[529]. In this respect, several areas are identified for future optimisation.

The most important target for further improvement is the stability of the mutant P450_{BM3} enzyme used for the whole-cell biotransformations. The enzyme was found to destabilise rapidly under the biotransformation conditions applied in **Chapter 4**, with the functional P450 concentration decreasing by four- to five-fold over 4 hours. This was suspected to be due to the generation of reactive oxygen species (ROS) by the mutant. Unfortunately, this characteristic is likely to be a consequence of the broadened substrate specificity introduced to P450_{BM3} through mutagenesis. It was shown in **Chapter 3** that a crucial mutation for noscapine-metabolising activity in P450_{BM3}, the E267V mutation, caused a structural transition of the substrate-free enzyme to resemble that of the substrate-bound, wild-type enzyme. This transition means that the significant structural rearrangement normally induced by the binding of natural fatty acid substrates is no longer required to facilitate electron transfer to the haem iron and initiate the catalytic cycle. Another key mutation, F78A, significantly opened the active site cavity to allow bulky non-natural substrates to approach the haem group. As a result, P450_{BM3} variants containing these mutations can metabolise a wide range of drug-like substrates but those that fit poorly within the active site have a high propensity to cause premature termination of the catalytic cycle[530], resulting in ROS generation. Many P450_{BM3} mutants also show a high NADPH turnover in the absence of substrate due to less stringent requirements for electron transfer to the substrate-binding domain[531]. While it appears that some improvements in noscapine binding were achieved in **Chapter 3** by the addition of further mutations, as suggested by an increase in coupling efficiency, the poor stability of the enzyme in whole-cell applications as well as the apparent toxicity of the expressed enzyme towards the *B. megaterium* host (**Chapter 4**) suggests that substantial ROS generation occurs.

A broad substrate range is a key feature of mammalian microsomal P450s that enables them to detoxify a wide variety of xenobiotic compounds, and the resulting ROS generation is negated by high levels of ROS scavenger enzymes in liver tissue[532]. It is notable that many of the mutations used in this study have previously been used by others to produce P450_{BM3} mutants that emulate the metabolite-producing activity of microsomal P450s but with significantly higher rates than their mammalian counterparts, so it is perhaps not surprising that high ROS generation often accompanies this strategy. In this work, noscapine is the only desired substrate, so the best approach to improve P450_{BM3} mutant stability is to focus on increasing the binding affinity of the enzyme for noscapine. In this way, the coupling efficiency

will be improved and noscapine will be more successful in competing with other intracellular compounds for the enzyme active site, potentially reducing ROS generation induced by other molecules within the host cell cytoplasm.

It is unlikely that making further mutations to the current library of mutants will offer major improvements in biotransformation productivity. As demonstrated in **Appendix A** and **Appendix B**, some additional mutations were tested and shown to provide improved activity *in vitro* but decreased performance in a whole-cell system. This result may have been due to further destabilisation of the enzyme by the cumulative effects of many mutations. An alternative approach would be to start with one of the original templates (e.g. F87A/V) and use a directed evolution[533] or semi-rational site-saturation mutagenesis[198] together with a screening protocol that considers the binding affinity of the enzyme for noscapine, which could be assessed by spectroscopic measurement of the dissociation constant (K_d)[534]. However, these approaches require significantly more resources than the rational mutant library constructed in this work. A further suggested improvement for mutant screening is the use of whole cells rather than the cell lysates used for screening here. While *in vitro* screening can identify improved kinetic parameters, the performance of an enzyme in a whole-cell system is due to a combination of factors, such as expression efficiency, host toxicity and enzyme stability under process conditions. While a high-throughput screen of whole-cell biotransformation activity may not provide information on these factors individually, it would be a better overall indicator of process suitability and would be more informative than the turnover number *in vitro*, which was assessed here (**Chapter 3**).

An alternative strategy to obtain a high-affinity noscapine-metabolising enzyme is to seek one in nature. This ‘bioprospecting’ approach was recently used to identify an *N*-demethylating enzyme with high affinity for the poppy morphinan alkaloids, such as thebaine and codeine, as well as some minor activity on noscapine[535]. The enzyme was found in a bacterial species that was isolated from a waste stream at a poppy processing facility and is able to grow on these alkaloids as sole carbon. A similar approach has also previously been used to identify a microbial morphine dehydrogenase enzyme[536] and several caffeine *N*-demethylases[537]. Enzymes forming part of a catabolic pathway like these often have high binding affinity (low K_m) for their substrates, ensuring the efficient sequential breakdown of the compound and allowing the organism to survive under conditions with limited carbon sources. It is conceivable that a similar strategy could be used to identify a noscapine-catabolising enzyme in an organism isolated from a noscapine extraction facility or from microbiota associated with the poppy plant.

A further approach could be to modify an enzyme involved in noscapine biosynthesis in opium poppy. In the noscapine biosynthesis pathway, there are two P450s and a reductase that specifically react with noscapine intermediates that have structural features similar to noscapine[24]. While these enzymes have high specificity for the intermediate compounds, it may be possible to use a mutagenesis approach to permit the metabolism of noscapine. As the active sites of the enzymes already accommodate very similar compounds, this may have the advantage of requiring less dramatic structural changes than those needed for P450_{BM3} which resulted in low specificity and high uncoupling of cofactor turnover. A disadvantage, however, is the lack of prior structural studies and mutagenesis work on these enzymes compared to the large amount of published work on P450_{BM3}.

In regard to reaction engineering of the whole-cell biotransformation, the use of a different host with improved substrate permeability (**Chapter 4**) was a simple way to gain over an order of magnitude in improvement of specific reaction rate (i.e. from 2 to 52 μM product/(μM P450·h)) without any further engineering of the enzyme. The fact that *E. coli* is typically the default organism of choice for whole-cell biotransformations suggests that other processes with drug-like substrates could gain similar rate improvements in this way. A disadvantage of using a Gram-positive organism, however, is that their lack of an outer membrane typically confers a higher sensitivity to organic solvents compared to Gram-negative bacteria. In **Chapter 4**, three of the four water-immiscible solvents that were tested for use in biphasic biotransformations have log *P* values between 3.3 and 4.2, and all showed significant toxicity towards *B. megaterium*. The fourth solvent, isopropyl myristate, has a log *P* of 8.5 and was shown to be biocompatible. Noscapine solubility in this solvent was only 1.4 g/L, however, whereas concentrations of noscapine up to 22.6 g/L were observed in more polar solvents. To improve substrate loading, the *in silico* solvent screening strategy could be repeated using an expanded list of solvents with log *P* values between 4 and 6. This may enable the identification of solvents that meet both high solubility and biocompatibility requirements. This activity could be carried out alongside a more detailed examination of the solvent tolerance characteristics of *B. megaterium* to identify any particular structural classes of solvents that are better tolerated than others.

A solvent engineering strategy may also be useful in improving substrate loading. In this approach, smaller amounts of more polar solvents are blended with a biocompatible hydrophobic solvent to improve substrate solubility while maintaining cellular biocompatibility. This has previously been shown to be effective, provided that the polar solvent is sufficiently sequestered by the organic phase to prevent toxicity toward host organism[259]. The Hansen solubility model is suitable for assessing solvent blends,

so this approach could be used with the same *in silico* screening strategy developed in **Chapter 4**. Alternatively, the use of water-immiscible ionic liquids[289] or substrate-loaded resins[40] could also be explored to optimise substrate loading and *B. megaterium* viability.

While the enhancement of aqueous noscapine solubility using a cyclodextrin/polymer system was highly effective in improving the reaction rate of the biotransformation (**Chapter 4**), the usefulness of this approach for the large-scale production of noscapine metabolites is more limited. The maximum noscapine concentration achieved (0.41 g/L), while 10-fold greater than the solubility of noscapine in water alone, was still far below the grams-per-litre substrate concentrations that are typically employed industrially[529]. The cyclodextrin/polymer system developed here may, however, be suitable for preparative-scale production of *N*-nornoscapine for research use. In this case, the system should be further optimised, as the use of (2-hydroxypropyl)- β -cyclodextrin at a concentration of 10% showed toxicity towards *B. megaterium*. This cyclodextrin derivative is expensive to use at this concentration. Further study with other cyclodextrin derivatives at different concentrations may be able to reduce the cost of the system and allow higher cell viability.

To address the limited stability of the P450_{BM3} mutant under resting-cell biotransformation conditions, a growing-cell process was investigated which allowed for recombinant enzyme expression to continue throughout the biotransformation (**Chapter 5**). As cofactor availability can often limit the productivity of growing-cell processes[41], a CRISPR-interference (CRISPRi) system was implemented in *B. megaterium* to enhance carbon flux through the pentose phosphate pathway. Preliminary testing of this strategy demonstrated significant potential, with an approximately three-fold improvement in product yields compared to a control lacking the CRISPRi system. This improvement provided comparable biotransformation productivity to resting cells without the need for cell harvest and washing steps. This system requires significant further characterisation, however, to fully optimise the productivity of noscapine *N*-demethylation and to extend the use of CRISPRi in *B. megaterium* to other applications (see **Chapter 5**). A particular focus for the future development of *B. megaterium* as a microbial cell factory could be the development of more tightly regulated promoter systems, as the commonly used xylose-inducible promoter limited the dynamic control of gene repression achievable by CRISPR interference here. The use of metabolic engineering as an approach to enhance whole-cell biotransformations with *B. megaterium* may also benefit from the recent development of a genome-scale metabolic network model for the DSM-319 strain used in this work[538]. This may allow for simulation-based identification of other targets for genetic engineering to enhance biotransformation productivity.

Finally, it is useful to consider the degrees of improvement required for the process developed here to be viable at industrial scale. Recent guidelines proposed by Lundemo and Woodley[529] for P450-based whole cell biotransformations suggest targets of 20 g/L product yield and 2 g/(L·h) productivity for a growing-cell process. Regarding the target yield, it is essential that the substrate loading can be enhanced to provide at least 20 g/L of noscapine. On this basis, a biphasic system seems to be the most promising approach. Considering that typical volume fractions of the organic phase in biphasic biotransformations are often 20-40% of the total reaction volume, this corresponds to a target noscapine solubility in the organic phase of 50-100 g/L. While the isopropyl myristate solvent used to improve productivity in this work (**Chapter 4**) had a noscapine solubility of only 1.4 g/L, this solvent was highly hydrophobic. It is conceivable that with further screening of more polar solvents, potentially combined with a solvent engineering approach as described above, a substrate loading in the required range could be achieved.

A volumetric productivity of ~1 mg/(L·h) was measured for the CRISPRi-enhanced, growing-cell biphasic system described in **Chapter 5**. With respect to the productivity target of 2 g/(L·h) for a growing cell process, this suggests an improvement of three orders of magnitude is required for industrial viability. Considering the reactions were conducted in shake flasks and the biotransformation conditions were not optimised, it is plausible that the use of a high-cell-density culture in a fed-batch bioreactor system combined with the systematic optimisation of reaction conditions could provide a ~10-fold improvement in productivity. This then leaves an improvement of around two orders of magnitude required for the activity of the host cell enzyme, assuming mass transfer limitations are minimal with the *B. megaterium* host. As discussed above, high substrate-binding affinity should be a key target for enzyme improvement. This kinetic parameter becomes even more important when considering that the use of a biphasic system reduces the aqueous noscapine concentration to below its already poor solubility limit due to phase partitioning. It was demonstrated in **Chapter 4** that the high aqueous concentration of noscapine concentration allowed by the use solubility enhancers drove an initial reaction rate around nine-fold faster than that of the best-performing biphasic system. A high-affinity enzyme that can maintain maximal reaction rates at low substrate concentrations is therefore crucial for achieving industrially relevant biotransformation productivity.

In conclusion, a cytochrome P450-mediated biotransformation process has been developed for the production of metabolites of noscapine, a natural product with anticancer properties. A range of novel approaches were used to investigate and optimise the process and these are applicable to the

development of other pharmaceutical biotransformation systems. Several areas have been identified for future improvement and strategies have been proposed to further optimise yields and productivity.

7. Bibliography

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8. Appendices

Appendix A. Whole-cell biotransformations with other P450_{BM3} mutants

Following the *in vitro* P450_{BM3} mutant screening experiments in **Chapter 3**, various other P450_{BM3} mutations were introduced to the A2 and A3 mutant templates to screen for enhanced activity or modified regioselectivity of noscapine metabolism. With the exception of the mutants discussed in **Appendix B**, all testing of these mutants was performed using whole-cell biotransformations only. Unlike the *in vitro* screen, differences in productivity between mutants in whole-cell biotransformations is caused not only by differences in enzyme activity but also in enzyme stability, protein expression rate and toxicity to the host organism. These individual factors were not specifically investigated in the experiments presented here; rather, the experiments served as a fast test to identify improved enzyme mutants that would be suitable for use in a whole-cell system i.e. for application in **Chapter 4**.

The preliminary results shown in this Appendix were not replicated and were often performed under different process conditions; comparisons of mutants are therefore only valid between biotransformation runs presented within the same Figure. No mutations tested produced a significantly altered product profile or demonstrated the production of metabolites other than those found in **Chapter 3**, so the results presented here show *N*-demethylation productivity only. All amino acid mutations were performed by PCR mutagenesis, as described in **Chapter 3**.

Mutations at Ala82

In an effort to improve the binding of non-natural substrates to P450_{BM3}, Huang and colleagues made several amino acid substitutions at the Ala82 position[539]. Their aim was to replace Ala82 with larger residues to fill a hydrophobic pocket above the active site that was suspected to bind substrates in a non-productive manner. Both A82F and A82W substitutions significantly improved the hydroxylation of indole, and work by others demonstrated that the A82W mutation improves the activity and selectivity of P450_{BM3} on several steroid substrates[540].

Here, both the A82F and A82W mutations were introduced individually to the A2 mutant and were shown to cause a significant reduction in productivity (**Figure 1**), showing ~33% and ~14% of the activity of the parent mutant, respectively.

Mutations in the electron transport chain of P450_{BM3}

It was recently shown by Darimont and colleagues[541] that significant improvements in P450_{BM3}-mediated hydroxylation of aniline could be achieved by mutations to amino acid residues proposed to form part of the electron transport pathway, between the reductase and haem domains of the enzyme. By combining the mutations of F393A and M490A, located in the haem and reductase domains, respectively, a 32-fold improvement in coupling efficiency and a 13-fold improvement in specific activity was observed in this previous study. These two mutations were introduced into the A2 template here, both individually and in combination. The resulting biotransformation data are shown in **Figure 2**. The F393A mutation did not alter the biotransformation performance, producing a similar concentration of *N*-nornoscapine to A2, but both of the enzymes containing the M490A mutation (M490A and F393A/M490A; **Figure 2**) showed ~15% of the productivity of the parent enzyme. It is likely that the M490A mutation caused a significant reduction in enzyme stability, as the dark red cell pellets that are normally observed after P450_{BM3} overexpression in *E. coli* were not evident following expression of these mutants.

Back-mutation of L188Q and E267V

Both the L188Q and E267V mutations were introduced early during the mutant library construction detailed in **Chapter 3** and were present in all active mutants. As work by Geronimo and colleagues suggested that both of these mutations have structural destabilising effects when introduced into P450_{BM3}[439], these were back-mutated to their wild-type residues individually in the A2 mutant. **Figure 3** shows that the E267V mutation significantly contributes to the activity of mutant A2, as back-mutation reduced the productivity to ~12% of the parent mutant (E267V BM, **Figure 3**). This is likely due to the altered salt bridge interactions caused by this mutation, as discussed in **Chapter 3**. The removal of the L188Q mutation, however, improved the rate of product formation (L188Q BM, **Figure 3**). This improved performance was confirmed in a further biotransformation experiment, shown in **Figure 4**, where this mutation provided a 1.3-fold improvement in final product titre. The A3 mutant from **Chapter 3** was also included in this experiment and was shown to perform better than the other two mutants tested, despite this mutant showing lower activity than A2 *in vitro* (**Chapter 3**). Both the L188Q back-mutation and the A3 mutant were selected for further *in vitro* characterisation, as detailed in **Appendix B**.

Mutations at Leu75

The *in silico* docking studies performed in **Chapter 3** suggested that the replacement of the Leu75 side chain of P450_{BM3} with a smaller residue, such as alanine, may improve the binding of noscapine in the active site. To test this hypothesis, Leu75 was replaced by alanine, valine and isoleucine (L75A, L75V, L75I)

in mutant A2L (A2 with L188Q back-mutation). The results, shown in **Figure 5**, suggested a potential improvement in productivity from the L75A mutation, a decrease in activity from the L75I mutation and no significant change from the L75V mutation. While these unreplicated results show an increasing trend of productivity with smaller residues at this position, the differences in productivity were minor and this may have been due to variation. To investigate further, the L75A mutation was selected for further characterisation *in vitro*, as discussed in **Appendix B**.

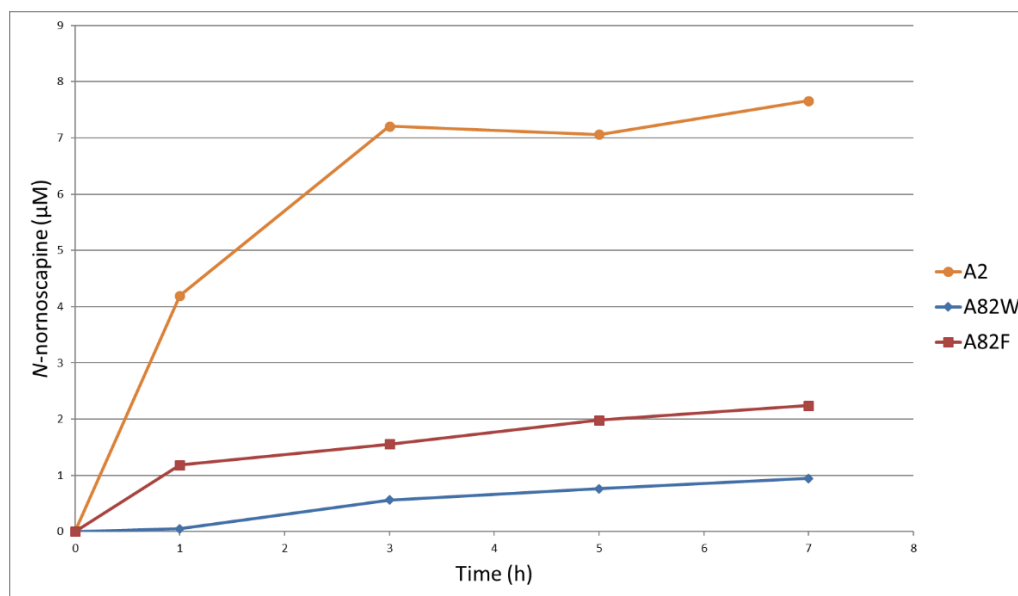


Figure 1. Mutations at Ala82. Biotransformations were performed in *E. coli* and compared to the parent A2 strain.

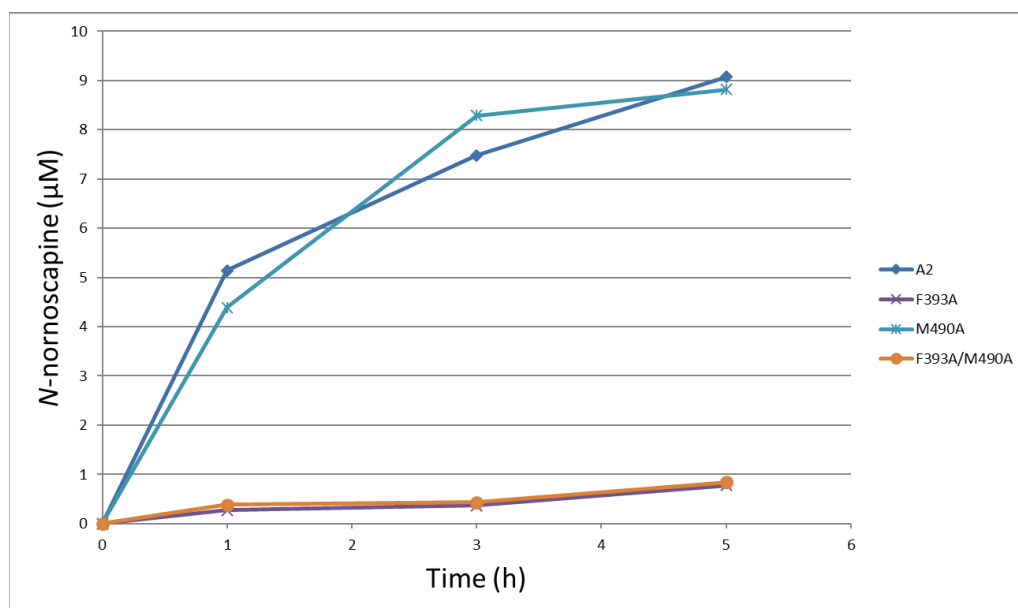


Figure 2. Mutations in the P450_{BM3} electron transport chain. Biotransformations were performed in *E. coli* and compared to the parent A2 strain.

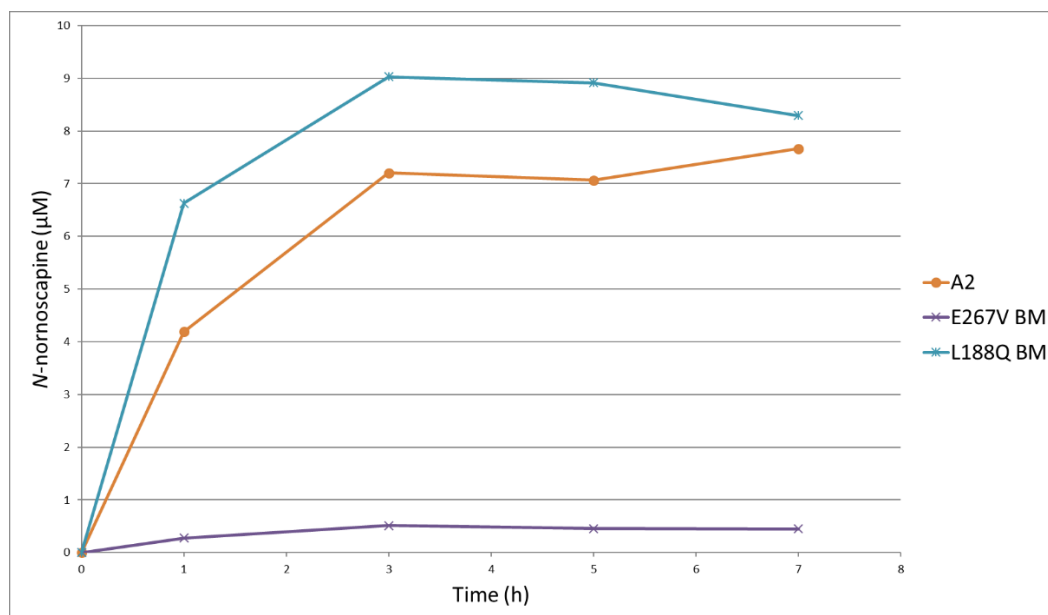


Figure 3. Back-mutation (BM) of E267V and L188Q to wild-type residues. Biotransformations were performed in *E. coli* and compared to the parent A2 strain.

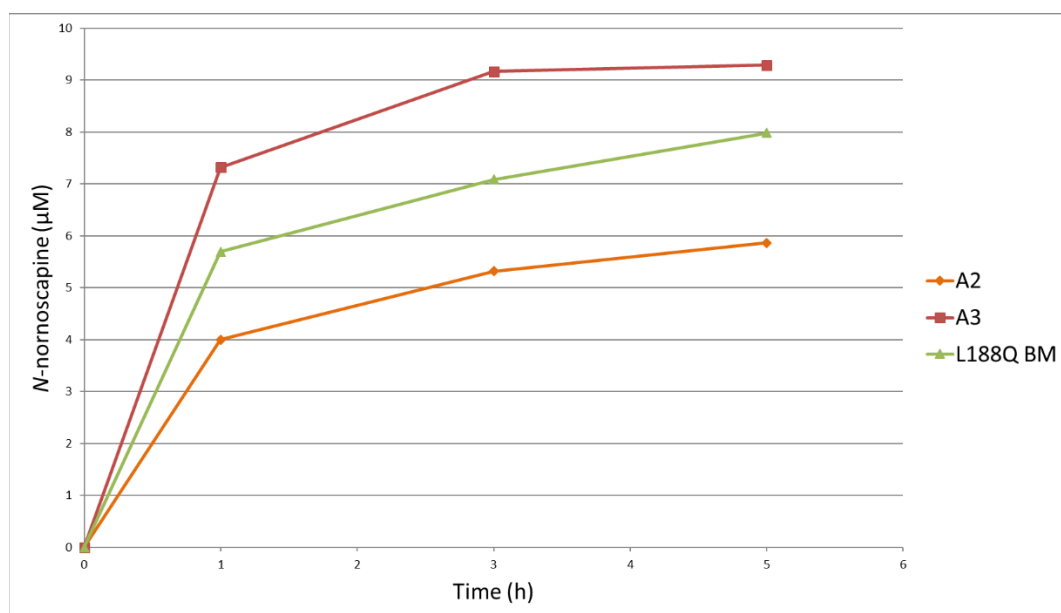


Figure 4. Confirmation of improved performance of L188Q back-mutation. Mutant A3 was shown to perform better than A2 despite lower activity *in vitro*. Biotransformations were performed in *E. coli* and compared to the parent A2 strain.

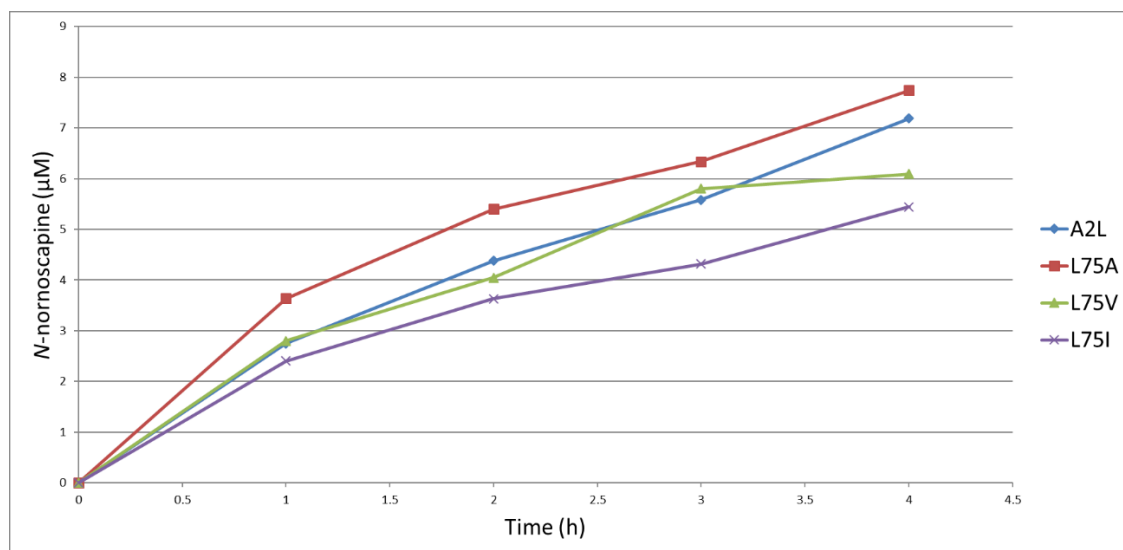


Figure 5. Mutations at the Leu75 residue. Mutations were added to variant A2L (A2 with L188Q back-mutation). Biotransformations were performed in *E. coli*.

Appendix B. Further screening of promising mutations

Screening of mutations *in vitro*

Following the P450_{BM3} mutant screening experiments detailed in **Chapter 3** and preliminary whole-cell biotransformation experiments in **Appendix A**, the effects of two further mutations were characterised by *in vitro* assessment of noscapine turnover, *N*-demethylation selectivity and coupling efficiency. The mutants selected for *in vitro* screening were: the back-mutation of L188Q to the wild-type leucine residue, and the mutation of Leu75 to alanine (L75A). The rationale for performing these mutations is outlined in **Appendix A**. These mutations were added sequentially to the A2 and A3 mutants (producing mutants A2L and A2LA, and A3L and A3LA, respectively) as these both showed high activity, selectivity and coupling efficiency in the initial mutant library screen (**Chapter 3**).

The screening experiments were performed essentially as described in **Chapter 3**, except the total reaction time for turnover experiments was shortened from 25 min to 15 min, and the co-solvent for noscapine addition was changed from DMSO (5% final volume) to methanol (1% final volume). The latter change was made as methanol was found to cause less NADPH turnover by the mutants in the absence of substrate (results not shown).

Some slight differences in activity were noted between A2 and A3 in these experiments (**Figure 1**) compared to the screening experiment in **Chapter 3**. In the original screen, A2 showed a 1.3-fold higher relative noscapine turnover compared to A3. In the work here, A3 performed slightly better than A2. This may be due to different co-solvent effects, as DMSO was used in the previously screening whereas methanol was used here. There was also some change in the observed coupling efficiency for A2 between the original screen in **Chapter 3** showing 8.3% in the original screen and 11.6% here, which may have been due to similar co-solvent effects.

The results of the preliminary screening study are shown in **Figure 1**. The *N*-demethylation selectivity did not significantly change between the mutants, remaining high at an average of 92%. Back-mutation of L188Q to leucine in mutant A2, producing mutant A2L, provided a 1.2-fold increase in noscapine turnover, while coupling efficiency remained the same. The increase in activity was similar to that observed in whole-cell biotransformation experiments for the same mutation (**Appendix A**). When this mutation was made to A3, producing A3L, no significant change in turnover or coupling efficiency was observed.

The L75A mutation provided significant and consistent enhancements in both turnover and coupling efficiency when added to the A2L and A3L mutants (producing A2LA and A3LA, respectively). The

noscapine turnover of both enzymes increased 1.3-fold and coupling efficiency increased 1.4-fold. The *in silico* docking studies performed in **Chapter 3** showed the Leu75 residue to be in close contact with noscapine in the active site. It is possible that this residue hindered substrate binding and that this was relieved by the replacement of Leu75 with the smaller alanine sidechain.

Whole-cell biotransformation performance

Following the promising *in vitro* results shown in **Figure 1**, the mutants A3, A3L and A3LA were tested in a *B. megaterium* whole-cell biotransformation system with enhanced substrate supply using cyclodextrin. Despite showing superior activity *in vitro*, the A3LA mutant performed the worst of the three enzymes tested, showing around ~60% of the productivity of the A3 parent mutant (**Figure 2**). This may have been due to reduced enzyme stability. While the replacement of leucine with alanine is a relatively minor substitution, particularly as this side-chain is directed into the solvent in the active site rather than involved in hydrophobic interactions in the protein interior, the compounded effects of multiple mutations to the wild-type structure may be significant enough to cause substantial structural instability under whole-cell biotransformation conditions. Interestingly, the A3L mutant also performed worse than the A3 mutant in whole-cell biotransformations, showing ~80% of the productivity of A3, despite this mutation being a reversion to the wild-type residue and producing no activity changes *in vitro* here (**Figure 1**). The reason for this difference is unclear but highlights the fact that enzyme performance can differ significantly between *in vitro* experiments and the complex environment within a cell.

As no mutants investigated in **Appendix A** or **Appendix B** were shown to provide enhanced whole-cell biotransformation activity compared to the A3 mutant developed in **Chapter 3**, the A3 mutant was selected for all further biotransformation work in this thesis.

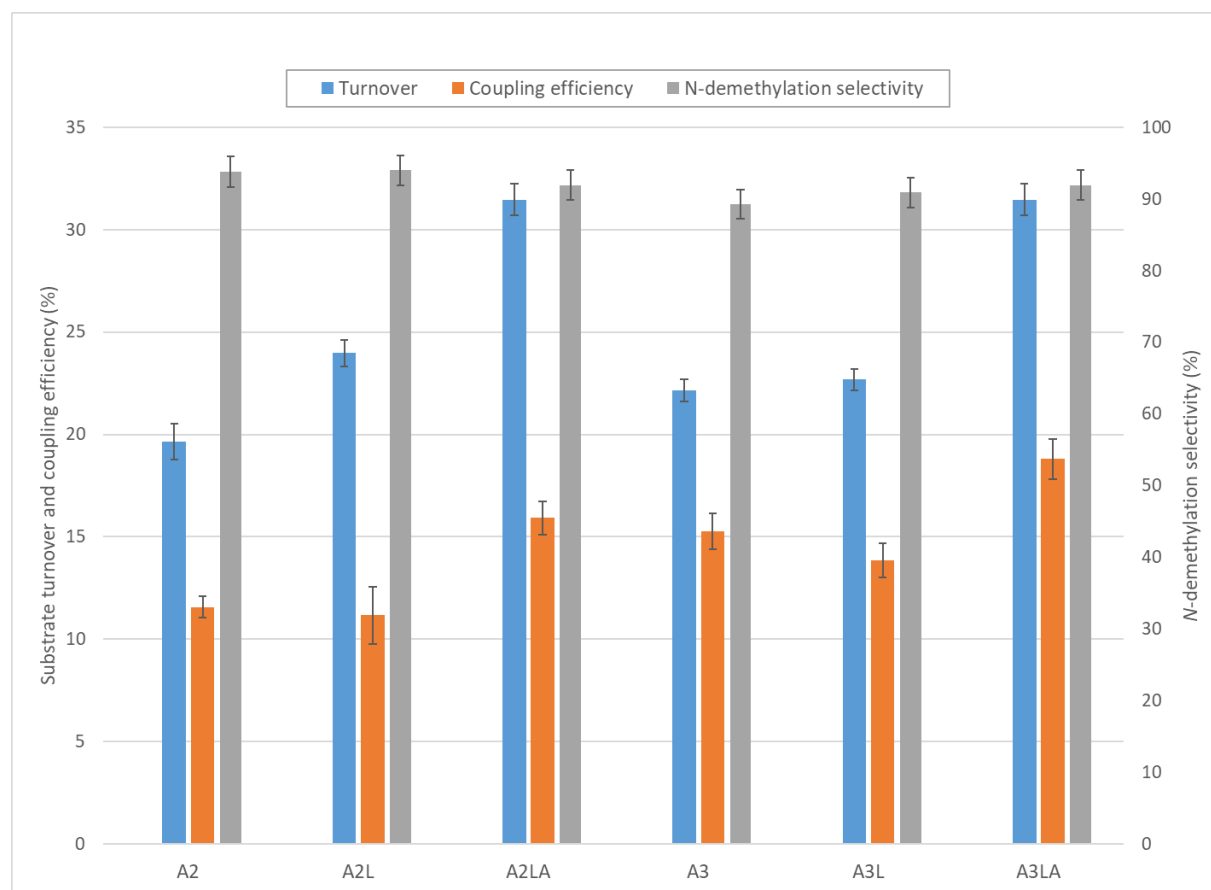


Figure 1. *In vitro* screening experiments assessing the performance of further P450_{BM3} mutations. Experiments were performed in triplicate. Error bars are ± 1 SD.

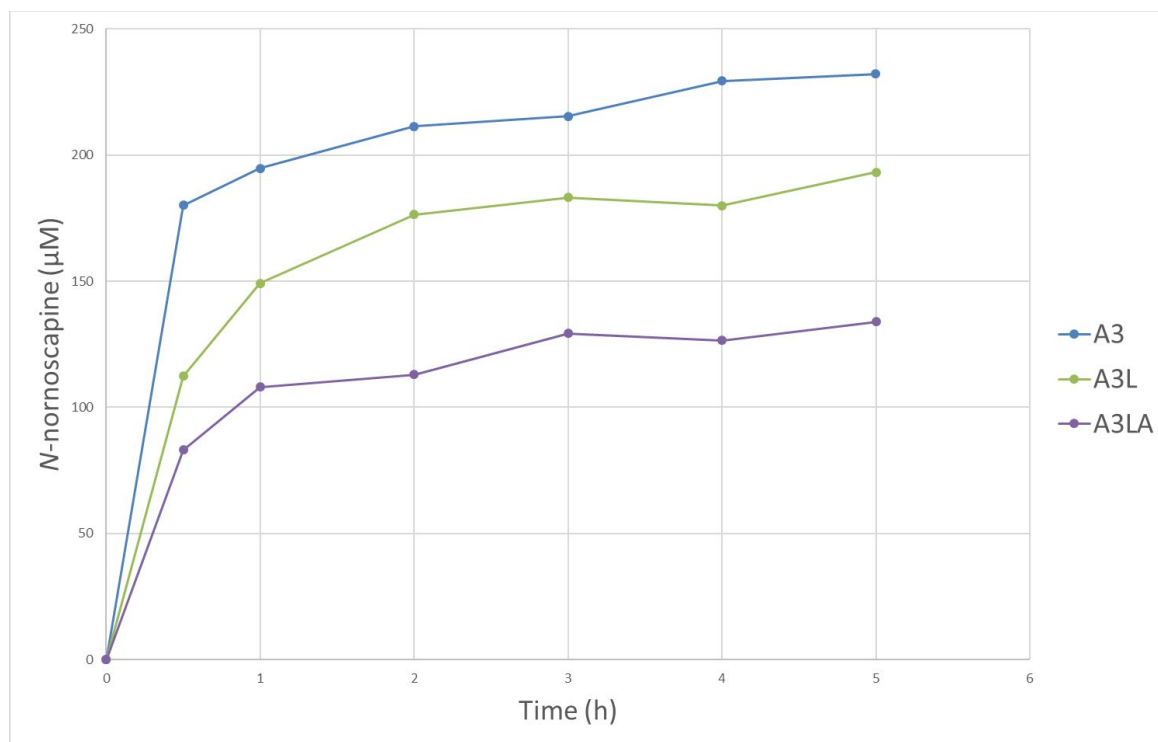


Figure 2. Whole-cell biotransformation experiment comparing mutants A3, A3L (A3 with L188Q back-mutation) and A3LA (A3L with L75A mutation). Biotransformations were performed in *B. megaterium* with noscapine solubility enhancement using cyclodextrin.

Appendix C. Co-expression of ROS scavengers during whole-cell biotransformation

Background

Cytochrome P450 enzymes can produce significant amounts of reactive oxygen species (ROS) when NADPH turnover is poorly coupled to product formation. Depending on the mechanism of uncoupling, either hydrogen peroxide (H_2O_2) or superoxide (O_2^-) can be produced, both of which can damage the haem and cause enzyme inactivation. Additionally, in whole-cell applications, ROS can cause oxidative damage to other cellular components and affect the viability of the host cell.

To assess whether the generation of ROS was a limiting factor in the whole-cell biotransformation of noscapine using P450_{BM3} mutants, experiments were performed in which ROS scavenger proteins were co-expressed with P450_{BM3} mutant A2 in *E. coli* BL21(DE3). The ROS scavengers used for this strategy were KatE, the native *E. coli* catalase HPII[542], or SodA, the *E. coli* manganese superoxide dismutase[543]. KatE catalyses the decomposition of hydrogen peroxide into water and molecular oxygen, while SodA produces molecular oxygen and hydrogen peroxide from superoxide.

Materials & Methods

Plasmids were designed for the bicistronic or tricistronic co-expression of P450_{BM3} mutant A2 with either or both of KatE and SodA (**Figure 1**). The pET28a(+) vector was used as a plasmid backbone. The *katE* and *sodA* genes were cloned from *E. coli* DH5- α genomic DNA and included their native ribosomal binding sites. All primer sequences are provided in **Table 1**. Plasmids were assembled by NEBuilder HiFi assembly. For all plasmids, the pET28a(+) vector backbone was amplified by PCR using primers *pET28a_rev* and *pET28a_fwd*. For plasmid pET28a_{BM3}_katE, the P450_{BM3} mutant A2 gene was cloned from the expression plasmid described in **Chapter 1** using primers *BM3_fwd* and *BM3_rev1*. The *katE* gene was amplified using primers *katE_fwd* and *katE_rev1*. For plasmid pET28a_{BM3}_sodA, the A2 gene was amplified using primers *BM3_fwd* and *BM3_rev2* and the *sodA* gene was amplified using primers *sodA_fwd1* and *sodA_rev*. For the tricistronic expression vector pET28a_{BM3}_katE_sodA, the A2 gene was amplified using *BM3_fwd* and *BM3_rev1*, the *katE* gene was amplified using *katE_fwd* and *katE_rev2*, and the *sodA* gene was amplified using *sodA_fwd2* and *sodA_rev*. Plasmids were cloned in *E. coli* DH5- α and then transformed into *E. coli* BL21(DE3) for whole-cell biotransformation experiments. Protein expression and whole-cell biotransformations were performed as described in **Chapters 1 and 2**.

Results & Discussion

Co-expression of P450_{BM3} mutant A2 with ROS scavenger proteins katE and sodA, both individually and together, from the three different expression plasmids was confirmed by SDS-PAGE (**Figure 2**).

The performance of the three co-expression strains in a whole-cell biotransformation experiment is shown in **Figure 3**. All co-expression strains showed a similar decrease in productivity of ~50% compared to the control strain expressing the P450_{BM3} mutant A2 only. This is likely due to lower expression levels of mutant A2 in these cultures, resulting from the overexpression of multiple proteins. Cultures containing plasmids pET28a_BM3_katE, pET28a_BM3_sodA and pET28a_BM3_katE_sodA showed 34%, 60% and 49%, respectively, of the cytochrome P450 concentration of the control strain containing pET28a_BM3, as assessed by carbon monoxide difference spectroscopy (results not shown). Cytochrome P450 concentration may be particularly affected by the co-expression of KatE because both enzymes require a haem prosthetic group. Regardless of the decreased A2 expression levels, the co-expression cultures do not show any prolonged *N*-demethylation activity compared to the control culture at later time points. This suggests that either (i) enzyme inactivation due to ROS formation is not a major limiting factor for the biotransformation in *E. coli*, or (ii) over-expression of ROS scavengers does not offer any further protection above the native expression levels of these endogenous enzymes. As this strategy did not result in an improvement in biotransformation performance, it was not pursued further.

Table 1. Primers used in this study. Overlapping regions for assembly by NEBuilder HiFi are shown in lower case.

Primer	Sequence
<i>pET28a_rev</i>	GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTC
<i>pET28a_fwd</i>	GATCCGGCTGCTAACAAGC
<i>BM3_fwd</i>	ctttaagaaggagatataccATGACAATTAAAGAAATGCCTCAGC
<i>BM3_rev1</i>	tgttttgggtcTTACCCAGCCCACACGTC
<i>katE_fwd</i>	ggctgggtaaGACCAAAACAGCGGCCCTTTC
<i>katE_rev1</i>	gctttgtagcagccgatcTCAGGCAGGAATTTGTCAATCTTAG
<i>BM3_fwd2</i>	ctttaagaaggagatataccATGACAATTAAAGAAATGCCTCAGC
<i>sodA_fwd1</i>	ggctgggtaaCTTACGCGGCATTAACAATC
<i>sodA_rev</i>	gctttgtagcagccgatcTTATTTTTTCGCCGCAAAAC
<i>katE_rev2</i>	aatgccgcgtaagTCAGGCAGGAATTTGTCAATCTTAG
<i>sodA_fwd2</i>	aattcctgcctgaCTTACGCGGCATTAACAATC

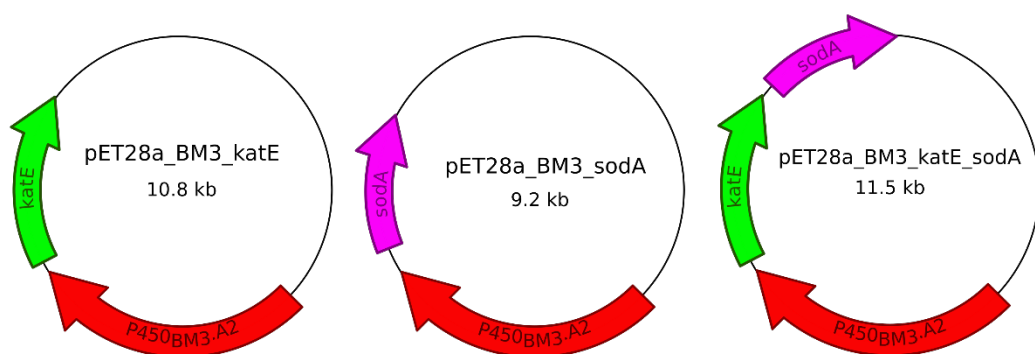


Figure 1. Plasmids used for bicistronic and tricistronic co-expression of P450_{BM3} mutant A2, KatE and SodA.

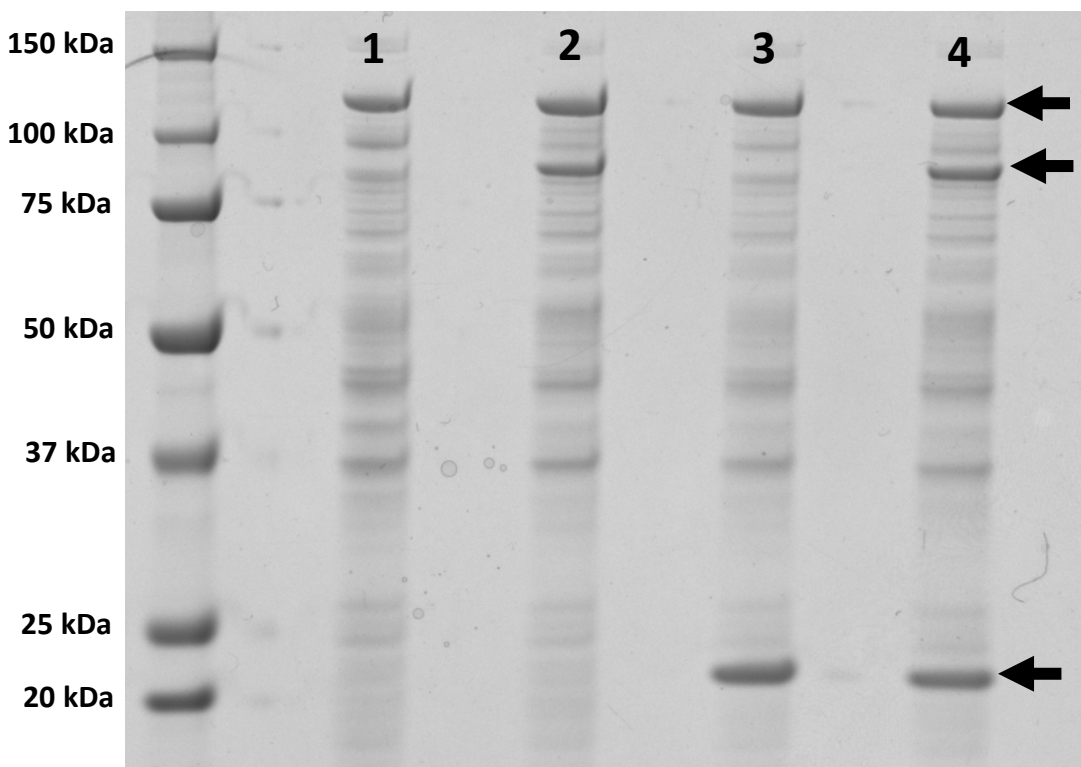


Figure 2. SDS-PAGE analysis of soluble cell lysates from *E. coli* strains co-expressing the three enzymes of interest in three different constructs. Lane 1, control vector pET28a_BM3. Lane 2, pET28a_BM3_katE expressing katE. Lane 3, pET28a_BM3_sodA expressing sodA. Lane 4, pET28a_BM3_katE_sodA expressing katE and sod A. The molecular weight of P450_{BM3} is 121 kDa; KatE is 84 kDa; sodA is 23 kDa.

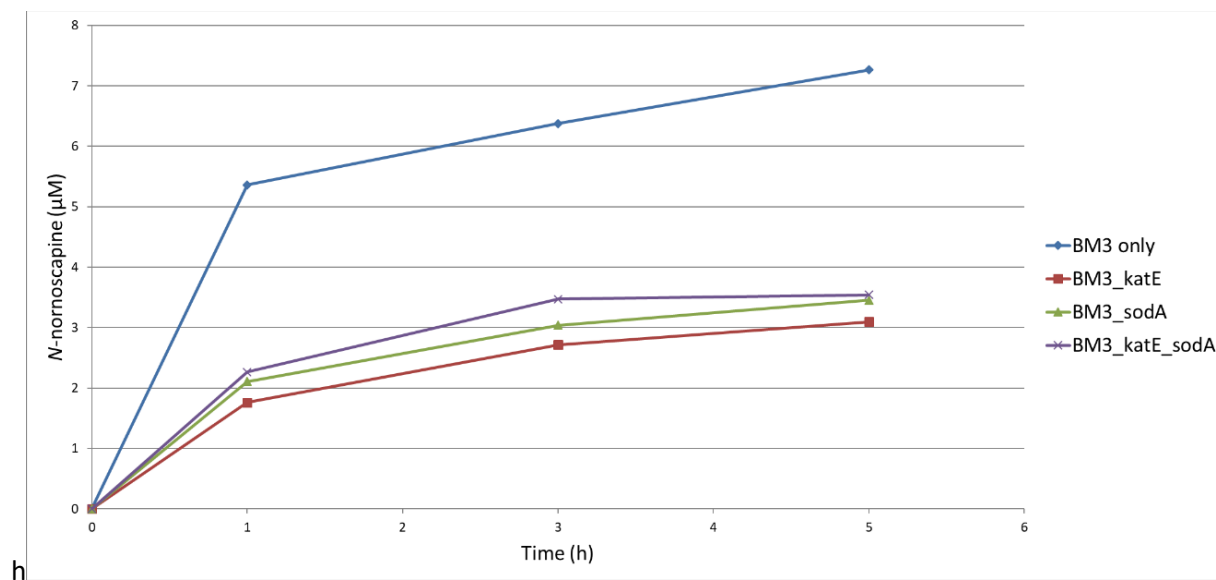


Figure 3. Biotransformation of noscapine with co-expression of ROS scavenger proteins katE, AodA or both KatE and SodA compared to the control strain expressing P450_{BM3} mutant A2 only.