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Biocatalysis

Enzymatic Late-Stage Oxidation of Lead Compounds with Solubilizing Biomimetic Docking/Protecting groups

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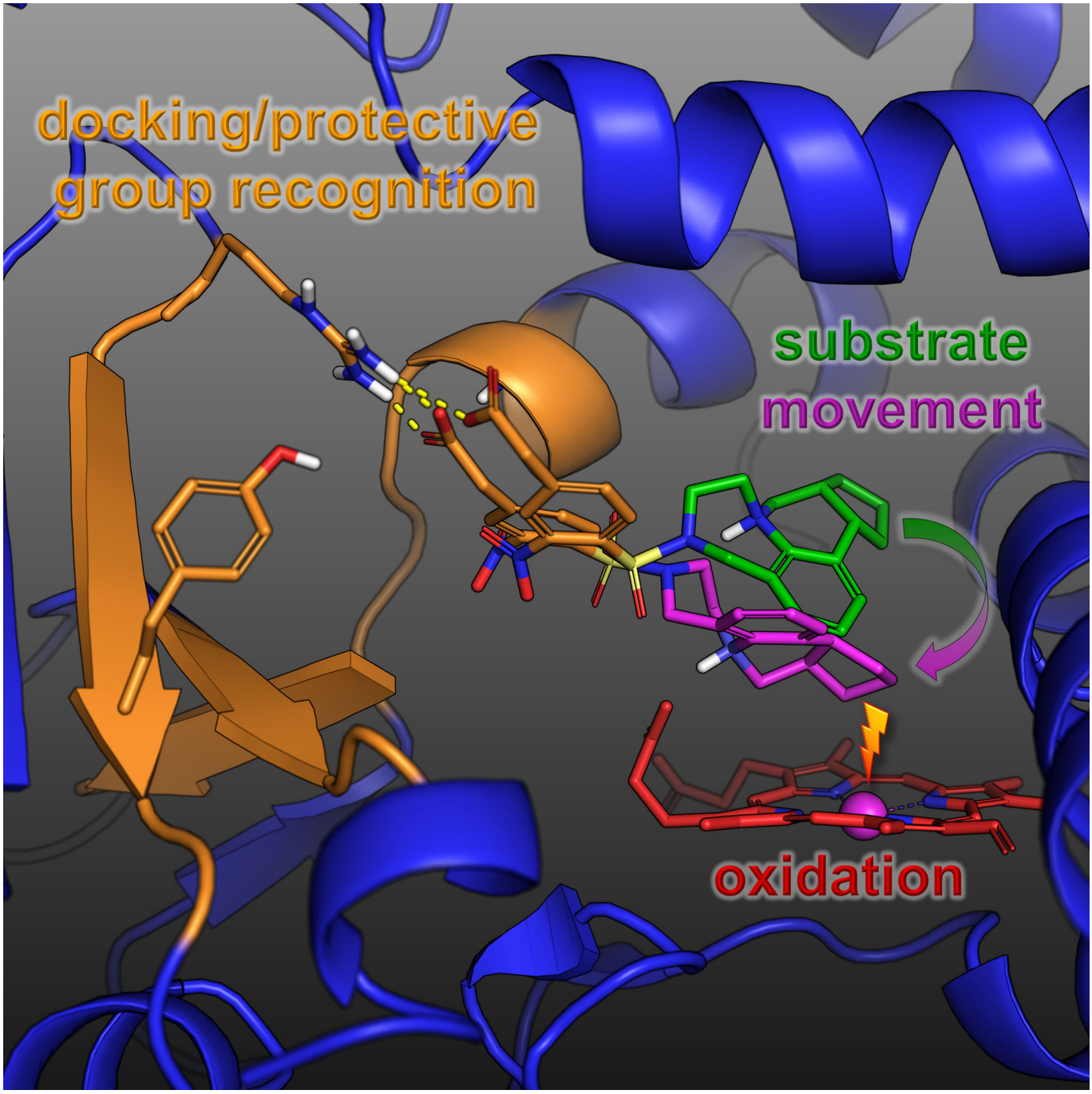

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Abstract: Late-stage functionalization of lead compounds is of high interest in drug discovery since it offers an easy access to metabolites and derivatives of a lead compound without the need to redesign an often long multistep synthesis. Owing to their high degree of chemoselectivity, biocatalytic transformations, enzymatic oxidations in particular, are potentially very powerful because they could allow the synthesis of less lipophilic derivatives of a lead compound. In the majority of cases, enzymatic oxidations have been used in an empirical way as their regioselectivity is difficult to predict. In this publication, the concept of using docking/protecting groups in a biomimetic fashion was investigated, which could help steer the regioselectivity of a $P450_{BM3}$-mediated oxidation. A novel set of docking/protecting groups was designed that can be cleaved under very mild conditions and address the often problematic aqueous solubility of the substrates. Vabicaserin was used as tool compound containing typical groups such as basic, aliphatic, and aromatic moieties. The results were rationalized with the help of in silico docking and molecular dynamic studies.

Introduction

Late-stage oxidation (LSO) of complex molecular structures is increasingly used in lead optimization of drug candidates, where the aim is to improve the physicochemical properties of a promising lead molecule, to introduce a synthetic handle for further structural diversification, or to produce a specific metabolite for toxicity or biological studies.[1] Whilst there are several chemical methods which can be employed for LSO,[2] biotransformations using either whole cells or isolated enzymes can offer a complementary method for LSO, often yielding alternative oxidation products to those produced by chemical means.[3] Additionally the ability to perform bio-LSO (LSO using whole cells or isolated enzymes) under mild pHs at room temperature, allows chemo-selective C–H bond oxidation on highly functionalized scaffolds.[4]

Chemists utilizing biotransformations for LSO of drug candidates may come across various obstacles such as limited substrate scope, poor substrate aqueous solubility, or unwanted metabolism of reactive functionality (e.g., amines, thiols, or C–H bonds alpha to heteroatoms).[5] As many biologically active molecules contain amine functionalities, the reactivity of amines can be particularly problematic when considering these structures for bio-LSO modification as not only unstable metabolites are formed, but amines and their metabolites can inhibit P450 enzymes (see Figure 1).[6]

Whilst protein engineering can be used to address the issues of substrate scope,[8] solubility[9] and regioselectivity,[8d,10] this strategy is not often conveniently available in most organic chemistry laboratories. Commercially available kits of engineered P450 mutants could address these issues for certain substrates, however these represent a black box concerning substrate scope as vendors do not provide information as to the precise modifications made to the P450s, additionally a lack of diversity within such panels can limit their generality and wider application.[11] In this respect, more accessible substrate engineering strategies have been reported which facilitate the reaction of unnatural substrates with various P450s, and generally involve covalently attaching a chemical auxiliary which can act as a docking group.[11b,12] If the binding interactions between the enzyme and natural substrate are known the incorporation of a specific recognition motif within the docking group can mimic these interactions and thus lead to improved enzymatic recognition and improved reactivity.[13] Additionally, the docking group can function as a protecting group for reactive functionality, the dual role giving rise to the term "docking/protecting groups" or "d/p groups",[14] which

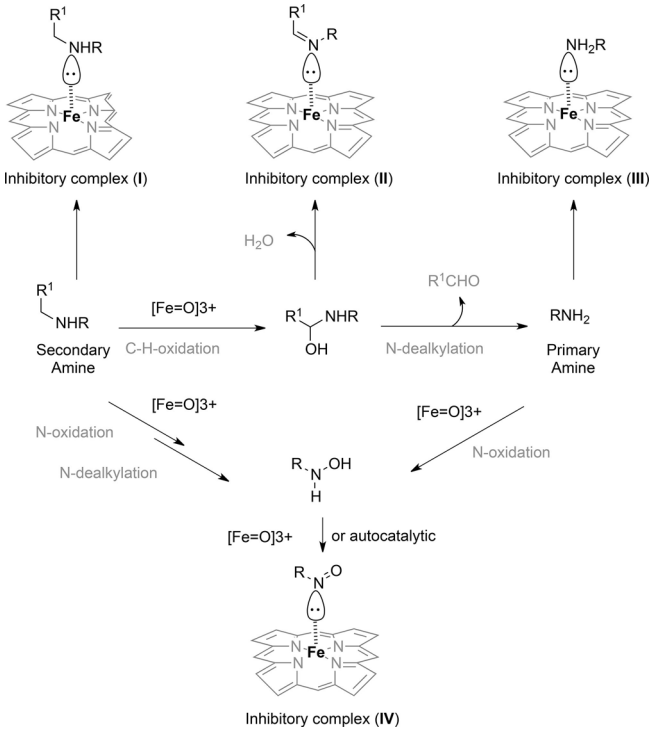

Figure 1. Possible oxidation of 1° and 2° amines by P450s and inhibitory pathways of nucleophilic amines and their metabolites. Other inhibitory complexes may also be possible with the metabolic intermediates preceding the nitroso-complex (**IV**) but are not shown here.[7]

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serve to shift the chemo-/regioselectivity of the oxidation away from reactive functionality by modifying one or more of the regioselectivity determining factors such as C–H bond reactivity, accessibility, and proximity to the heme group[15] (Figure 2).

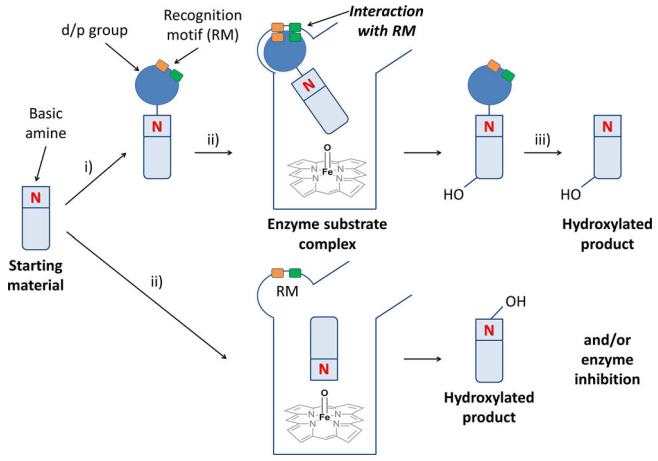

Figure 2. Schematic of the docking/protecting group (d/p group) strategy showing the three steps: (i) protection of the basic amine, (ii) biooxidation, and (iii) deprotection yielding the final hydroxylated product.

Additionally, in the case of nucleophilic amines, an electron withdrawing protecting group would eliminate the propensity of this functionality towards inhibition of P450 enzymes via the pathways shown in Figure 1.[18,19]

Classical amine protecting groups such as Bn (benzyl), Bz (benzoyl), BOC (tert-butoxycarbonyl), Cbz (carboxybenzyl) or trifluoroacetyl groups[12b,20] have been reported to have also increased reactivity of the protected substrates. This observation could result from either increased hydrophobic interactions between the hydrophobic d/p group and the active site's hydrophobic residues, or via polar interactions of the amide/carbamate moiety.[21] However, these protecting groups are not ideal d/p groups for bio-LSO as not only do they decrease the aqueous solubility of the substrates but the conditions required for deprotection may not be compatible with the oxidized products, for example, dehydration of hydroxylated products or hydrogenation of alkene products. Therefore, a d/p group which can be modified to incorporate a functionality which enhances aqueous solubility and also offers the opportunity to control the regioselectivity of oxidation via specific and predictable interactions with polar active site residues could be superior in comparison to previously reported d/p groups. Considering the latter aspect, the choice of biocatalyst used for the oxidation will determine which functional groups should be incorporated for regioselectivity control.

Of the known P450s which are suitable for use as biocatalysts, that is, are easily produced and stable enough to be used synthetically, the well-studied C12–C16 fatty acid metabolizing P450 from *Bacillus megaterium*, CYP102A1, $P450_{BM3}$,[22] offers an attractive starting point for development of this strategy as the natural substrate recognition motif is known. A tyrosine Y51,[23] and arginine, R47,[16] have been reported to facilitate binding of carboxylate containing substrates in $P450_{BM3}$ (see Figure 3).[11a,17,24] Thus, incorporation of a carboxylate moiety into the d/p group which mimics that of the natural substrate could therefore facilitate the docking of the non-natural substrate, potentially changing the site of oxidation with

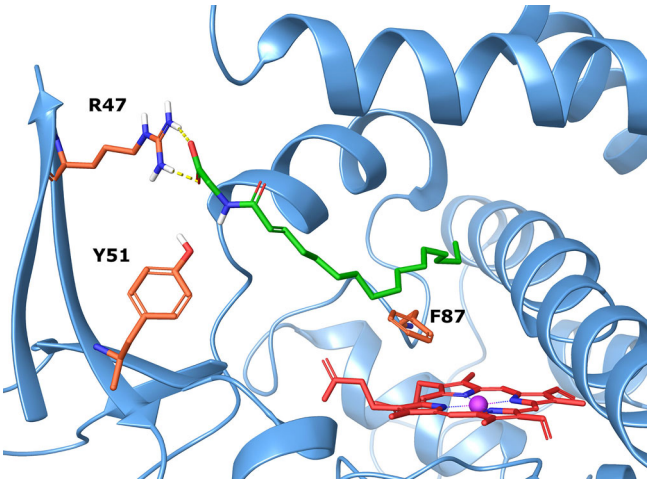

Figure 3. Crystal structure $P450_{BM3}$ complex with *N*-palmitoylglycine (4KPA), showing interactions between the ligand's carboxylate and the active site's Y51 and R47 (the carboxylate recognition motif).[16] The original substrate, palmitic acid, was modified via amide formation with glycine which increased both the conversion and aqueous solubility.[12c,17]

respect to that observed with the original non-protected substrate. Indeed, it has been reported that the modification of palmitic acid via amide formation with glycine not only enhanced the aqueous solubility but also increased conversion with $P450_{BM3}$ by acting as a docking groups (Figure 3).[25,26] The reverse of this d/p concept, using the $P450_{PikC}$ from *Streptomyces venezuelae*, which has an amine rather than a carboxylate recognition motif as in $P450_{BM3}$, was used in conjunction with various amine containing d/p groups for the biooxidation of menthol and was successful in producing different metabolites depending on the nature of the of the d/p group.[13] The advantage of developing a d/p group which can be used with P450 systems containing a carboxylate recognition motif is that it can be used in conjunction with the commercially available P450 kits based on $P450_{BM3}$ and will provide a convenient methodology which can be exploited in synthetic chemistry laboratories.

As mentioned above the conditions required for deprotection must be mild enough to avoid dehydration of the hydroxylated product, therefore strongly acidic conditions should be avoided. Considering the two issues of ease of deprotection and versatility with respect to carboxylate incorporation, nitrophenylsulfonamides (Nosyl) groups are of interest as they can be removed under mildly basic conditions with a thiolate via a nucleophilic aromatic substitution (see Figure 4),[27] and also offers four different sites for introduction of a carboxylate moiety.

Herein, we report the development of a series of novel carboxylated 2-Ns d/p groups (see Figure 5), based on the d/p concept described above, which have the potential to enable

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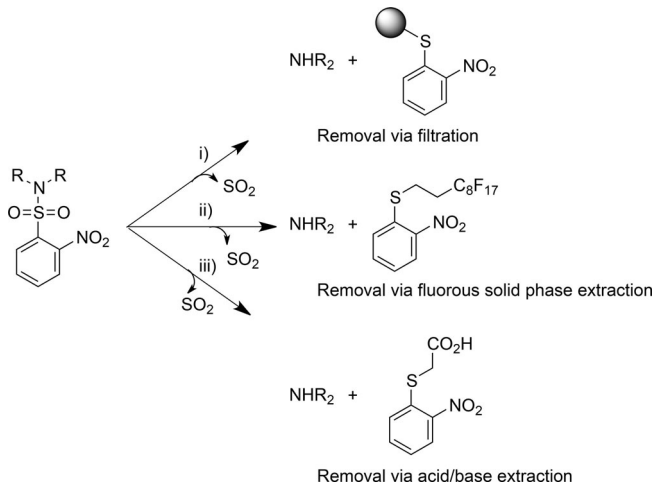

Figure 4. Nosyl deprotection conditions allowing facile isolation of the deprotected amine from the mercaptan by-product: (i) solid supported thiolate; (ii) fluorous thiolate; (iii) mercaptoacetic acid and base.

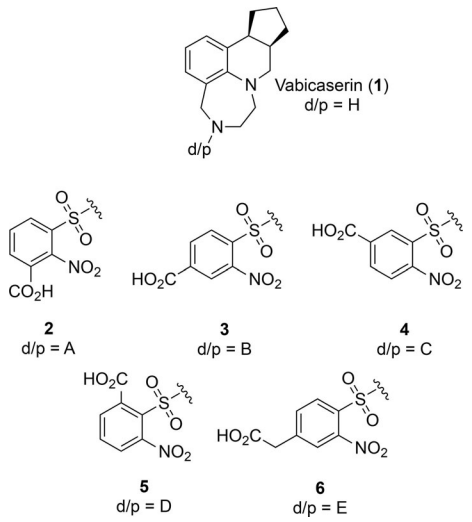

Figure 5. Docking/protecting (d/p) groups used in this study in combination with Vabicaserin (**1**) as the model substrate.

the synthesis of different hydroxylated compounds from a substrate using the same or a set of P450 enzymes possessing a carboxylate recognition motif. For a feasibility study Vabicaserin (**1**) was chosen as model substrate as the structure offers a variety of C–H bonds of differing reactivity for potential oxidation (e.g., C–H bonds alpha to amines in addition to aromatic, benzylic, and non-activated aliphatic C–H bonds).[28]

A broad range of computational methods have successfully been used to rationalize and predict substrate binding and metabolism for various CYP (cytochrome P450) proteins in the last decade.[29,30] Today, state-of-the-art computer-aided approaches are capable of predicting sites of metabolism (SOMs) among the three highest-ranked atom positions in 70–90% of all cases. However, computational structure-based approaches like docking are facing several hurdles when applied to P450 proteins. The $P450_{BM3}$ protein is designed to accommodate a wide variety of substrates and has in contrast to many other substrate-recognizing proteins, a fairly large lipophilic and highly flexible binding site which exhibits ligand-induced conformational changes during substrate binding.[25] Binding modes retrieved from docking experiments therefore heavily vary and rely on the underlying X-ray protein conformation. In recent years molecular dynamic simulations (MDS) have been shown to provide a much deeper insight in the CYP structure-function compared to standard docking approaches by taking into account the conformational flexibility of CYPs as well as the role of water. For example, while the metabolic regio- and stereoselectivity of the $P450_{BM3}$ substrate in Figure 3 cannot be explained by the binding mode of the X-Ray structure alone, the application of MDS on the crystal structure successfully identified the relevant binding mode and predicted the correct regioselective SOMs.[31] In addition, approaches combining docking with MDS showed improved prediction capabilities compared to simple static docking experiments.[29,32]

The experimentally observed SOMs using $P450_{BM3\text{-}F87V}$ mutants, biomimetic d/p groups (A–E) and Vabicaserin (**1**) as model substrate were structurally rationalized with the help of a combined docking/MDS approach. The feasibility to guide the design of d/p ligands applying this concept was also evaluated.

Results and Discussion

Modified Nosyl groups: protection/deprotection conditions

An initial feasibility study regarding the ability of the modified 2-Ns protecting groups (d/p = A—E in Figure 5) to act as protecting groups, that is, facile conditions for protection and deprotection, was carried out (see Figure 6 and 7). In most cases

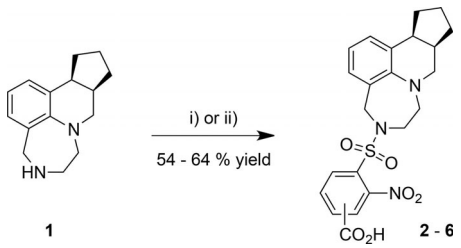

Figure 6. Nosylamide preparation. Reaction conditions: d/p = A–C and E (i) Amine (1 equiv), CO_2H-Ns-Cl (2 equiv), DIPEA (5 equiv), in 2% TPGS-750 M, 10 min at room temperature;[33] d/p = D (ii) Amine (1 equiv), CO_2Me-Ns-Cl (2 equiv), DIPEA (5 equiv), in CH_2Cl_2, followed by ester hydrolysis with NaOH (1 M) in MeOH/MeCN.

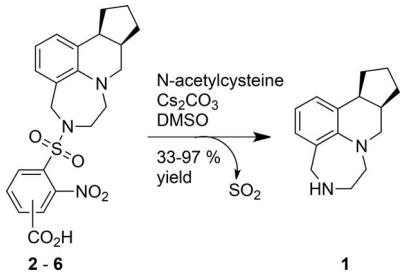

Figure 7. Nosyl group cleavage with *N*-acetylcysteine.

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protection of the amine with the carboxylated Ns-Cl reagent was directly achieved in one step. Only d/p group D required the use of the corresponding ester and a two-step protocol.

The use of polymer bound thiols provides an attractive deprotection method for small scale reactions owing to the simple work-up consisting of filtration followed by evaporation of solvent.

Deprotection of protected Vabicaserin analogues (**2**–**6**) with *N*-acetylcysteine under mildly basic conditions (Cs_2CO_3) in a polar aprotic solvent (DMSO) at room temperature gave quantitative conversion to the free amine, the product being isolated in good to moderate yields (see Figure 7).[34,35] However, deprotection of compound (**2**) required elevated temperatures and resulted in a complex mixture of products.

Modified Nosyl (2-Ns) groups: effect on solubility

Aqueous substrate solubility is important in biocatalysis as poor solubility can result in irreproducible screening results and the need to develop special formulation techniques for preparative reactions.[36]

The aqueous solubility of protected Vabicaserin analogues (**2**–**6** shown in Figure 5) under the conditions required for the bio-oxidation reaction was compared with Vabicaserin protected with common electron withdrawing protecting groups (Boc, Cbz and 2-Ns) and unprotected Vabicaserin (**1**) (see Figure 8).

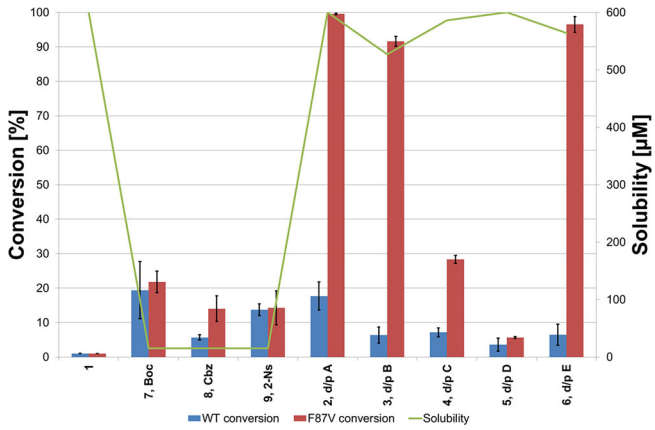

Figure 8. The effect of the protecting groups on conversion of Vabicaserin (**1**) with $P450_{BM3\text{-}WT}$ and $P450_{BM3\text{-}F87V}$. Conversion is based on substrate depletion as measured in by LCMS using an internal standard. Measurements were performed in triplicate.[37]

The free amine was highly soluble (>600 μM), however the solubility of the Boc (**7**), Cbz (**8**) and 2-Ns (**9**) protected Vabicaserin derivatives was very low (<5 μM). The use of the carboxylated 2-Ns groups (d/p = A–E) led to a much increased aqueous solubility with levels approaching that of the free amine (>500 μM) corresponding to over a 100-fold increase in solubility for all five derivatives.

Modified Nosyl (2-Ns) groups: effect on bio-oxidation conversion, number of metabolites formed and region of oxidation

The series of carboxylated 2-Ns groups (d/p = A–E, in Figure 5) were investigated for their effect on the biocatalytic oxidation of Vabicaserin with $P450_{BM3}$ and the $P450_{BM3\text{-}F87V}$ mutant, a mutation which has been shown to broaden the substrate scope to include bulkier drug-like substrates.[8f,11a,38]

The conversion of Vabicaserin as the free amine was very low (≈1%) with both the wild type and mutant enzyme, however once the amine was protected conversion increased with both enzymes (see Figure 8). As expected the F87V mutant gave higher conversion as a result of increased access to the heme which was confirmed by the molecular dynamics simulations that indicated in the $P450_{BM3}$ wild type F87 had to be rotated to open the heme site for the substrate.[39] The observed general increase in conversion of the all protected analogues tested compared to that of the free amine results from the amine being rendered non-nucleophilic by the electron withdrawing nature of the d/p groups and thus being unable to participate in the inhibitory pathways outlined in Figure 1. Vabicaserin (**1**) caused a type-II shift in the UV spectrophotometric assay for the heme absorbance band indicating the formation of an inhibitory complex with the $P450_{BM3\text{-}F87V}$ mutant.[40] Additionally, in general all the d/p groups (A–E) increase the potential for formation enzyme-substrate complex stabilizing interactions resulting in increased binding affinity which could contribute to the increase in conversion. Aqueous solubility appears to be a prerequisite for conversion, with the poorly soluble compounds (**7**, **8** and **9**) being poorly converted. Interestingly, whilst all five of the modified 2-Ns groups (d/p = A–E) increased solubility of the protected compounds (**2**–**6**) compared to Boc (**7**), Cbz (**8**) and 2-Ns (**9**), a significant increase in conversion (>90-fold increase in conversion with the F87V mutant compared with the conversion of the free amine) was only observed in three out of the five protected Vabicaserin analogues (**2**, **3** and **6**), with the remaining two compounds (**4** and **5**) having conversions comparable with that of the analogues protected with the common protecting groups [Boc (**7**), Cbz (**8**) and 2-Ns (**9**)]. This observation suggests that the modified 2-Ns groups (A–E) can only function as a d/p group when the carboxylate recognition motif is positioned in the enzyme's active site in such a way that a C–H bond of the protected substrate is held close enough to the heme (c.f. MDS section below).

The positioning of the d/p group carboxylate moiety also had an impact on the oxidation pattern, with the different protecting groups each having a different regioselectivity and thus led to a different mixture of aliphatic, aromatic and other oxidation products (illustrated in Figure 9).

The site/region of oxidation was determined by LC-MS/MS analysis of the crude reaction mixtures. Monohydroxylation (M + 16) was favored over other possible oxidation products. Interestingly, in the reactions of analogues **2** or **3** and **6**, the favored region of oxidation was inverted, with oxidation at an

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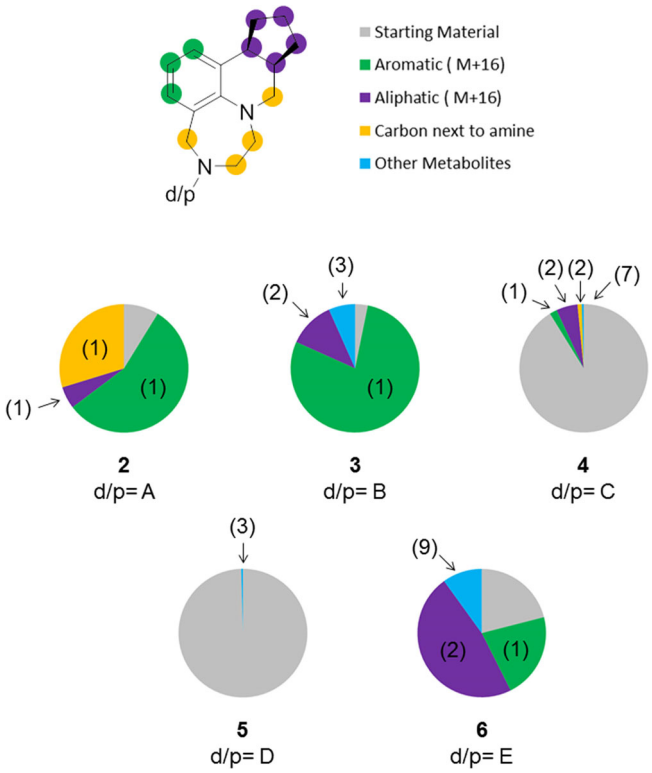

Figure 9. Composition of $P450_{BM3\text{-}F87V}$ oxidation reactions with protected Vabicaserin analogues (**2**–**6**). The region of oxidation (%AUC as determined by LC-MS/MS) is dependent on the positioning of the protecting group carboxylate moiety. The numbers in brackets refer to the number of metabolites found within each regional classification (stereoisomers could not be separated).

aromatic C–H bond being favored for **2** and **3**, and aliphatic C-H oxidation dominating the reaction of **6**.

In order to investigate the factors affecting the observed regioselectivity further, and determine the finite site of oxidation by NMR, the reactions of protected compounds **3** and **6** with $P450_{BM3\text{-}F87V}$ mutant were scaled up (see Figure 10 and 11). The reaction of compound **3** was very selective for aromatic oxidation at the most activated position in the aromatic ring of Vabicaserin (**10** in Figure 10), whereas compound **6** gave a mixture of four M+16 oxidation products of which the four major

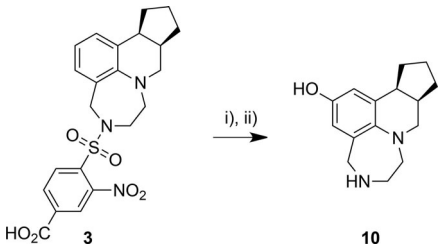

Figure 10. Preparative reaction with the F87V mutant and protected Vabicaserin analogue **3**. The product was purified after the protecting group was removed. Reaction conditions: (i) Substrate (0.25 mM), $P450_{BM3\text{-}F87V}$ mutant (10 mg mL^{-1}), Potassium phosphate buffer (pH 8, 100 mM), MeCN (1.25%), $NADP^+$ (1 mM), Glucose (25 mM), Glucose Dehydrogenase (0.5 mg mL^{-1}), at r.t. (ii) *N*-acetyl cysteine, Cs_2CO_3, DMSO, r.t., 20.8% yield over two steps.

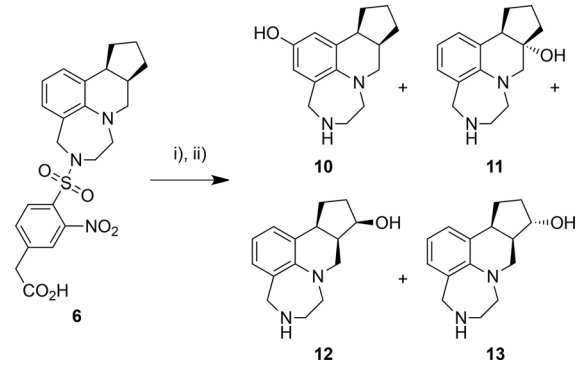

Figure 11. Preparative reaction with the F87V mutant and protected Vabicaserin analogue **6**. Four major M+16 products (**10**–**13**) were isolated from the mixture after removal of the protecting group. Reaction conditions: (i) Substrate (0.25 mM), $P450_{BM3\text{-}F87V}$ mutant (10 mg mL^{-1}), Potassium phosphate buffer (pH 8, 100 mM), MeCN (1.25%), $NADP^+$ (1 mM), Glucose (25 mM), Glucose Dehydrogenase (0.5 mg mL^{-1}), at r.t. (ii) *N*-acetyl cysteine, Cs_2CO_3, DMSO, r.t., yielding compounds **10**, **11**, **12** and **13** in 12%, 7%, 10% and 8% yield, respectively over two steps.

products could be isolated and characterized to show that in addition to the phenol product (**10**), the aliphatically hydroxylated compounds **11**, **12** and **13** were produced (Figure 11).

Computer-aided rationalization of d/p group-related regioselectivity

To rationalize the observed oxidation patterns of two of the substrates, docking and molecular dynamic simulation studies were initiated. In a first step, protected Vabicaserin analogues **3** and **6** were docked including two different protonation states into two corresponding F87V mutants of the crystal structure representations of $P450_{BM3}$ (1FAG: X-ray of cytochrome $P450_{BM3}$ with fatty acid substrate palmitoleic acid, 4KPA: X-ray of cytochrome $P450_{BM3}$ in complex with *N*-palmitoylglycine). In all cases the Vabicaserin moiety was orientated towards the heme group in a reactive distance between 4.4 and 5.8 Å from the iron atom while the carboxylated protection group was located next to the entrance of the substrate binding site mimicking the carboxylate group of the endogenous substrates. Compared to unprotected Vabicaserin (**1**) additional hydrogen bonds and van der Waals interactions between the d/p group and the entrance of the binding pocket increase the substrate affinity of **3** and **6** to the enzyme which is also in line with the significantly higher conversion of **3** and **6**.

All top poses of analogue **3** in the F87V mutant of 1FAG and 4KPA, respectively, revealed the same binding mode, orientating the aromatic ring of Vabicaserin in an almost orthogonal position to the heme group positioning the terminal (*para*) C–H bond to the iron ion, respectively.[41] In the $4KPA_{F87V}$ mutant the substrate was bound deeper into the active site leading to a slightly different orientation of the protection group and to a shorter distance between the terminal aromatic C–H bond and the iron of 4.2 Å (Figure 12, green pose) compared to the C-Fe distance of 5.7 Å in $1FAG_{F87V}$ mutant.[39] These results demon-

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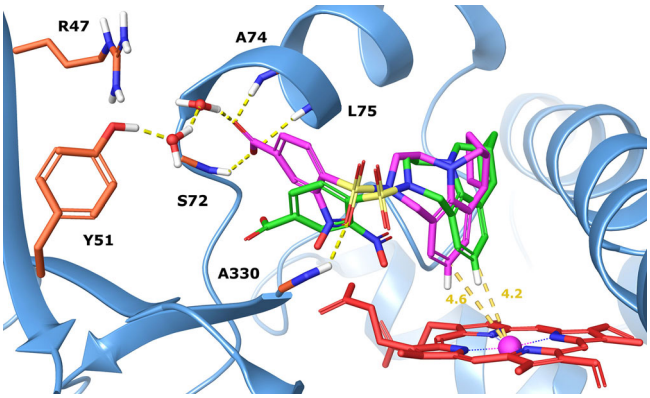

Figure 12. Comparison of best docking pose of compound **3** (green) and the refined MDS result (purple) on 4KPA $P450_{BM3\text{-}F87V}$.

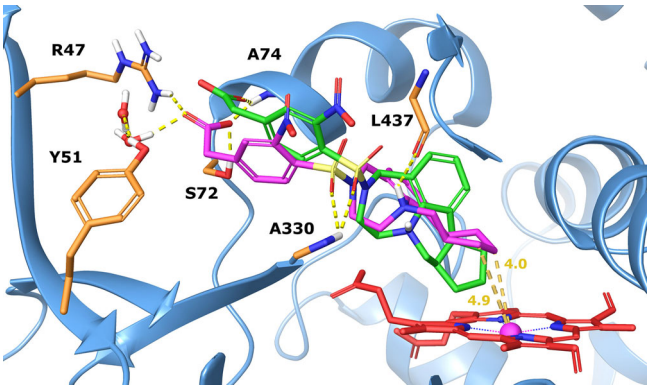

Figure 14. Comparison of best docking pose of compound **6** (green) and its corresponding MDS retrieved binding mode (purple) on 4KPA $P450_{BM3\text{-}F87V}$.

strate that automated docking was not only sufficient to predict the experimentally favored region of oxidation (Figure 9 and 10), but also identified the regioselective site of metabolization for this substrate.

During the subsequent 10 ns Molecluar Dynamics Simulation (MDS) a significant improvement of the initial docking pose was observed (Figure 12, magenta pose). While the Vabicaserin part was only translated slightly (RMSD 1.26 Å), without changing the orientation of the correctly identified aromatic C–H bond towards the heme iron, the formerly non-interacting protection group was re-orientated completely establishing a set of new hydrogen bond interactions with the backbone amines of S72, A74, L75, Y51 (water mediated) and A330. The distance of the aromatic C-H atom only increased very slightly from 4.2 Å to 4.6 Å without weakening the regioselective prediction power.

Automated docking of Vabicaserin analogue **6** on the 1FAG (Figure 13) and 4KPA F87V structures (Figure 14) revealed a significantly different docking behavior compared to the docking results of analogue **3.** While all top poses of analogue **3** showed a conserved binding mode, docking experiments with analogue **6** resulted in many different binding modes covering poses similar to analogue **3** (aromatic ring oriented towards the heme) but also binding modes where the aliphatic part of

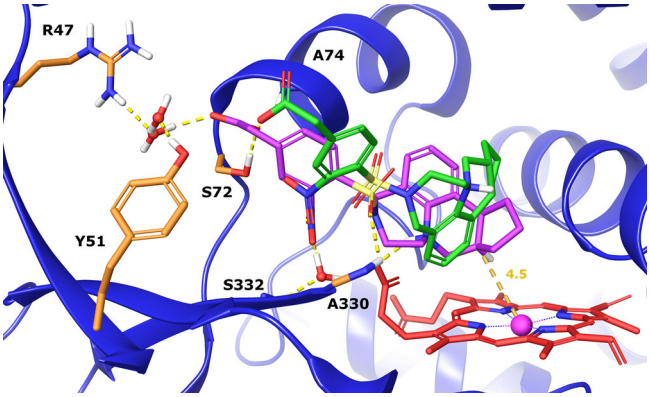

Figure 13. Best automatic docking solution of compound **6** on 1FAG $P450_{BM3\text{-}F87V}$ (green) compared to the refined binding mode (purple) after a 10 ns MDS.

Vabicaserin was positioned towards the prosthetic group or the Vabicaserin ring system was oriented in parallel to the heme.[42] Interestingly, these "fuzzy" in silico observations were in alignment with the experimental results which showed a much larger number (Figure 11) and less biased ratio (Figures 9 and 10) of the resulting oxidation products. However, the correct identification of the experimentally found SOMs (Figure 11) could not be obtained from these binding modes. Subsequent MDS was performed to analyze, if the right SOMs and products could be identified after the simulation.[26]

In contrast to compound **3**, most of the docking poses of analogue **6** showed significantly rearrangements during the MDS. The substrate often moved deeper into the binding pocket leading to decreased distances between the experimentally found SOM and the heme. This is exemplarily described below for the top pose[26] of analogue **6** on $1FAG_{F87V}$ and $4KPA_{F87V}$. The best docking pose of compound **6** on $1FAG_{F87V}$ (Figure 13, green pose) exhibited a very similar binding mode like analogue **3**. In addition the docking pose revealed an interaction with Y51 because of the slightly longer carboxylate chain showing that the d/p protection group is mimicking the carboxylate of the endogenous substrate.[43] The aromatic ring was positioned towards the heme (5.2 Å between aromatic (*para*) C–H bond and iron) which was in agreement with the experimental finding that one out of four observed oxidations took place on the aromatic ring system (Figure 11, compound **10**). Despite the similar docking pose compound **3** only showed small rearrangements during the simulation (Figure 12)), MDS of analogue **6** on $1FAG_{F87X}$ revealed a significant rearrangement (Figure 13, magenta pose). The Vabicaserin core rotated by 180 degrees positioning the cyclopentane ring towards the prosthetic group and moving the aromatic ring away from the heme. The final binding pose (RMSD 4.39 Å) revealed numerous direct and water mediated hydrogen bonds between the protection group and the protein (Y51, R47, S72, A74, A330, S332). Even more interestingly it placed the correct ring-bridging C–H bond, which was the major SOM in the $P450_{BM3\text{-}F87V}$-mediated oxidation yielding compound **11**, to the heme iron with the shortest distance of 4.5 Å. Surprisingly the MDS on the second-best pose, which almost shared the same binding mode but only differed by

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the protonation pattern of the Vabicaserin nitrogen (different sides),[43] resulted in a completely different simulation trajectory. As observed for compound **3**, the orientation of the aromatic moiety of the Vabicaserin did not change during the simulation and therefore supported the oxidation of the terminal aromatic (*para*) C-H and explaining the presence of compound **10**.[44] This indicates that even small modifications can have a large impact on the binding mode and SOM.

The best docking pose of compound **6** on $4KPA_{F87V}$ showed a completely different binding pose compared to those found for compound **3** and compound **6** on $1FAG_{F87V}$.[45] The aliphatic cyclopentane ring was positioned towards the prosthetic group revealing a short distance of 3.8 Å between the terminal C–H bond and the iron (Figure 14, green pose). Also this observation was supported by the experimental findings showing that the aliphatic ring system was the primary oxidation region of compound **6** (Figures 9 and 11). The succeeding MDS led to an improved binding mode showing many additional interactions (R47, Y51, A74, S72, A330 and L437). More importantly the Vabicaserin core rotated by 90 degrees (Figure 14, magenta pose). While the core was aligned orthogonal to the heme in the starting pose, the cyclopentane ring was now in parallel to the prosthetic group facing the aliphatic C–H bonds towards the iron heme. Despite the correct rotation of the ring, the methylene group that was oxidized leading to compound **13** was not the closest to the iron (distance to Fe 4.9 Å). Instead the terminal methylene group of the cyclopentane ring revealed the shortest distance to the heme (4.0 Å).

In summary, the combined docking/MDS approach proved to be more reliable to rationalize the biomimetic d/*p*-group related regioselectivity behavior compared to static docking solutions alone. Especially for compound **6** substantial reorientations of the Vabicaserin moiety could be found allowing better explanations of the experimental oxidation pattern. Nevertheless, it also exhibits a few limitations regarding its prospective application in d/*p*-group and $P450_{BM3}$ design.[46] The large size of d/*p*-protected Vabicaserin analogues limited the number of possible docking poses and also restricted the rearrangement of the Vabicaserin core during the MDS. In addition, it could be observed that very small structural differences between crystal structures of P450 (1FAG and 4KPA) as well as on the ligand (e.g., ionization pattern) can influence the binding pose and MDS dramatically. Our MDS also did not consider the Fe-bound catalytic oxygen which also can influence the prediction power of our approach.

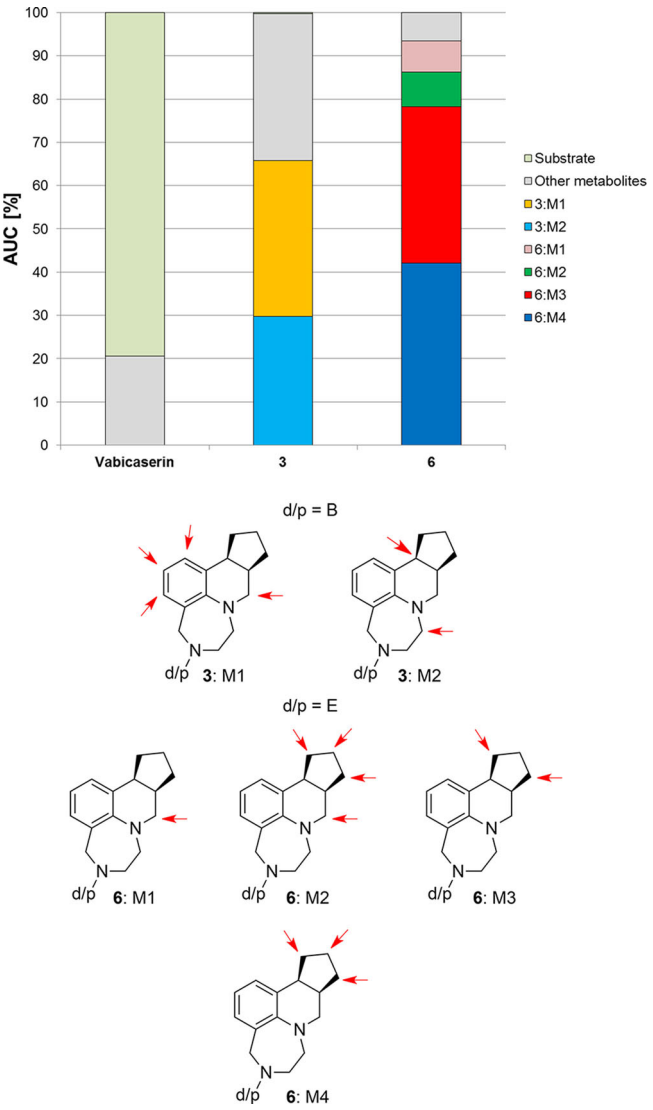

Figure 15. $P450_{BM3}$ mutant library screening results: Bar chart depicts the reaction composition of the selected $P450_{BM3}$ mutant with protected Vabicaserin analogues **3** and **6** determined by LC-MS/MS. The product distribution is shown by the %AUC contribution the M+16 metabolites to the overall reaction composition. Red arrows on the structures mark the possible site of oxidation in the M+16 products (M1 and M2 for reaction with **3** and M1-4 for reaction with **6**). The region of where the oxidation had occurred was determined by the fragmentation pattern in the MS/MS spectrum (in cases where an unambiguous structural assignment based on MS/MS data was not feasible potential sites of oxidation are marked with multiple red arrows). Reaction conditions: Substrate (0.25 mM), "selected $P450_{BM3}$ mutant" (10 mg mL^{-1}), Potassium phosphate buffer (pH 8, 100 mM), MeCN (1.25 %), $NADP^+$ (1 mM), Glucose (25 mM), Glucose Dehydrogenase (0.5 mg mL^{-1}), at r.t.

Combining substrate engineering with protein engineering

In order to demonstrate how d/p groups A–E can facilitate the use of bio-LSO in general synthetic chemistry laboratories, the protected analogues of Vabicaserin **3** and **6** were screened for oxidation with a panel of 23 commercially available $P450_{BM3}$ mutants.[47,48]

LC-MS/MS was used to quickly identify the region where the oxidation had occurred and a $P450_{BM3}$ mutant displaying good conversion and giving access to a diverse range of metabolites was selected from the screening panel (see Figure 15, the full screening results are included in the supporting information). The composition of the reaction of the free amine and the selected mutant was mostly composed of unconverted starting material (owing to likely enzyme inhibition, c.f. Figure 1) and no M+16 products were observed. In contrast, the corresponding reactions of the protected Vabicaserin analogues **3** and **6** saw the reaction composition being dominated by monohydroxylated products (M+16, metabolites M1 and M2 for **3** and M1–4 for **6**, c.f. Figure 15).

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The combination of Vabicaserin and d/p group B allowed access to an aromatic hydroxylated product, whereas the use of d/p group E was gave aliphatic oxidation. The reactions with the selected mutant were scaled up and the products characterized by NMR (see Figure 16 and 17). The use of the

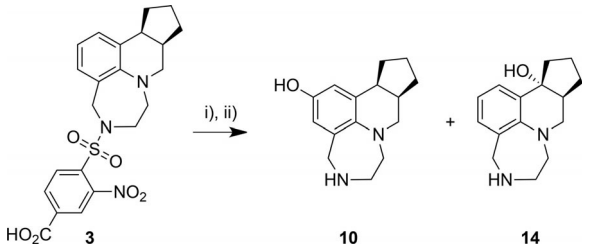

Figure 16. Scale up reactions with the selected $P450_{BM3}$ mutant and Vabicaserin protected with d/p group 2. Reaction conditions: Substrate (0.25 mM), selected $P450_{BM3}$ mutant (10 mg mL^{-1}), Potassium phosphate buffer (pH 8, 100 mM), MeCN (1.25%), $NADP^+$(1 mM), Glucose (25 mM), Glucose Dehydrogenase (0.5 mg mL^{-1}), at r.t. (ii) *N*-acetyl cysteine, Cs_2CO_3, DMSO, r.t., yielding 7% and 11.6% of compounds **10** and **14** respectively.

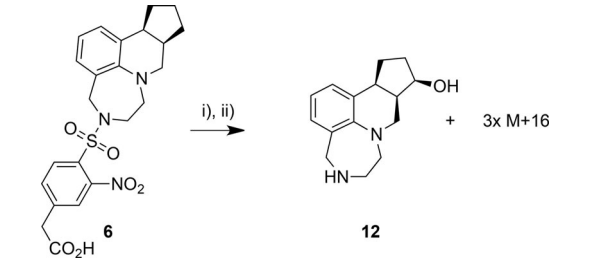

Figure 17. Scale up reactions with the selected $P450_{BM3}$ mutant and Vabicaserin protected with group 5. Reaction conditions: (i) Substrate (0.25 mM), selected P450 mutant (10 mg mL^{-1}), Potassium phosphate buffer (pH 8, 100 mM), MeCN (1.25%), $NADP^+$(1 mM), Glucose (25 mM), Glucose Dehydrogenase (0.5 mg mL^{-1}), at r.t. (ii) *N*-acetyl cysteine, Cs_2CO_3, DMSO, r.t., to give compound **12** in 8% isolated yield.

two different d/p groups in combination with the single selected $P450_{BM3}$ mutant, allowed access to another hydroxylated product **14** in addition to the previously obtained phenol **10** and alcohol **12**, demonstrating the ability of the 2-Ns d/p groups to increase product diversity. In addition, the combination of this d/p group methodology with other P450 panels containing the necessary d/p recognition motif, broadens the accessibility of this bio-LSO technology removing the need for specialist laboratories/ training. Based on the above-mentioned analysis the d/p groups reported here are expected to work best with $P450_{BM3}$ mutants containing a suitably positioned d/p recognition motif.

Conclusions

The combination of substrate engineering with carboxylated 2-Ns amine d/p groups and protein engineering with point mutations has been used successfully to increase conversion of the model compound Vabicaserin to desirable monohydroxylated (M+16) analogues and to alter the regioselectivity producing either corresponding phenols or aliphatic alcohols. The use of commercially available screening panels of $P450_{BM3}$ mutants in combination with this d/p group strategy offers medicinal chemists a readily available toolkit of protecting groups and $P450_{BM3}$ mutants which can be screened to find the best combination for the compound of interest. In addition, the application of high throughput LC-MS/MS to the analysis the oxidation reactions can quickly identify if any products of interest have been generated and thus guide the mutant-protecting group selection for preparative reactions. Scale up and the ease of d/p group cleavage with for example, *N*-acetyl cysteine generated oxidation products in milligram quantities. No attempts were made at this stage to optimize the biocatalytic oxidation for increased turnovers as the activity we realized was sufficient to generate enough material for structural characterization and biological testing. Furthermore, rather than evolving improved catalysts that would not be accessible to the average chemist, we aimed to rely on commercially available enzymes and kept synthesis protocols amenable to standard organic chemistry laboratories. Moreover, the carboxy-Ns d/p groups have been shown to enhance the substrate solubility in aqueous media and thus could be beneficial in combination with other biocatalytic reactions. Future work could be directed towards optimization of d/p group interaction with the recognition motif in the enzyme via further protein engineering thereby tailoring the combination to yield the oxidation products of interest, for example, in $P450_{BM3}$ Y51 and R47 could be shifted to neighbored positions and/or hydrophobic residues could be targeted for mutagenesis to incorporate more polar residues which could interact with the d/p nitro group. Furthermore, modifying the size of hydrophobic active site residues via mutagenesis could broaden both the substrate scope and diversity of metabolites generated.

The combined docking/MDS approach allowed the rationalization of the d/p group related regioselectivity and oxidation pattern, but also showed limitations identifying the SOM with the shortest distance to the heme in some cases. Using additional $P450_{BM3}$ crystal structures as well as longer MD simulation times might overcome these limitations.

Experimental Section

Materials

All commercially available reagents were used without further purification. Deionized water was used for preparation of all aqueous solutions and for work-up of reactions. The enzymes $P450_{BM3}$ and mutant F87V were obtained as crude lyophilized lysates from Almac Sciences Limited. The commercially available $P450_{BM3}$ screening kit used in this work was obtained from Codexis. The sulfonyl chlorides used in preparation of the sulfonylamides were obtained from Enamine Ltd.

Preparative P450 oxidation of 3 with $P450_{BM3\text{-}F87V}$

Oxidation: A solution of **3** (25 mg, 0.052 mmol) in a 1:1 mixture of acetonitrile/100 mM potassium phosphate buffer at pH 8 (2.73 mL), was added to 210 mL of 120 mM potassium phosphate buffer at pH 8 containing the NADPH recycling system: 1.2 mM $NADP+$,

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30 mM Glucose, 0.6 mg mL^{-1} Glucose dehydrogenase. This mixture was then added to a baffled 1 L conical flask containing a solution of $P450_{BM3\text{-}F87V}$ mutant (200 mg in 40 mL of 100 mM potassium phosphate buffer at pH 8), and the reaction mixture shaken at 22 °C, 300 rpm for 2 hours. The reaction mixture was diluted with acetonitrile (300 mL) and stirred for 1 hour at r.t. before the pH was adjusted to pH 2 via addition of HCl (1 M) before the organic solvent was removed in vacuo and the aqueous mixture extracted with EtOAc (3×200 mL), the combined organics were then dried (Na_2SO_4), filtered and concentrated to give a dark yellow residue (14 mg) which was used directly in the next step without further purification.

Deprotection: To a suspension of cesium carbonate (117 mg, 0.36 mmol) and *N*-acetylcysteine (19.45 mg, 0.117 mmol) in DMSO (0.25 mL) was added a solution of the oxidation product isolated in the previous step (14 mg) in DMSO (0.25 mL) and the yellow mixture stirred vigorously at r.t. for 12 hours before the reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3× 10 mL). The combined organics were then washed with water (30 mL) and sat. $NaHCO_3$ (30 mL) before being concentrated to a dark orange oil (6 mg) which was purified via preparative SFC (column: Waters Viridis 2-EP column; mobile phase gradient: 95% CO_2 and 5% modifier (1 min), then 50% modifier (3 min), followed by a wash step. As modifier MeOH with 0.2 vol% of concentrated, aqueous ammonia solution was used), to give the phenol **10** as a colorless oil (1.5 mg, 20.8% yield over two steps).

Preparative P450 oxidation of 6 with $P450_{BM3\text{-}F87V}$

Oxidation: A solution of **6** (53 mg, 0.112 mmol) in a 1:1 mixture of acetonitrile/100 mM potassium phosphate buffer at pH 8 (6.2 mL), was added to 413 mL of 120 mM potassium phosphate buffer at pH 8 containing the NADPH recycling system: 1.2 mM $NADP^+$, 30 mM Glucose, 0.6 mg mL^{-1} Glucose dehydrogenase. This mixture was then added to a baffled 1 L conical flask containing a solution of $P450_{BM3\text{-}F87V}$ mutant (400 mg in 85.7 mL of 100 mM potassium phosphate buffer at pH 8), and the reaction mixture shaken at 22 °C, 300 rpm for 6 hours. The reaction mixture was diluted with acetonitrile (500 mL) and stirred for 1 hour at r.t. before the pH was adjusted to pH 3 via addition of HCl (1 M) before the organic solvent was removed in vacuo and the aqueous mixture extracted with EtOAc (3×400 mL), the combined organics were then dried (Na_2SO_4), filtered and concentrated to give a dark yellow residue (67 mg) which was used directly in the next step without further purification.

Deprotection

To a suspension of cesium carbonate (493 mg, 1.512 mmol) and *N*-acetylcysteine (112 mg, 0.687 mmol) in DMSO (0.2 mL) was added a solution of the oxidation product isolated in the previous step (67 mg) in DMSO (0.5 mL) and the yellow mixture stirred vigorously at r.t. for 2 hours before the reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3×10 mL). The combined organics were then washed with water (30 mL) and sat. $NaHCO_3$ (30 mL) before being concentrated to an orange oil (30 mg) which was purified via preparative SFC (column: Waters Viridis 2-EP column, elution gradient: 95% CO_2 and 5% modifier (5 min) then. 50% modifier (3 min), followed by a wash step. As modifier MeOH with 0.2 vol% of concentrated, aqueous ammonia solution was used) to give phenol **10** and alcohols **11**, **12** and **13** as colorless oils (4.0 mg, 11.9% yield; 2.3 mg, 6.9%; 3.4 mg, 10.1% and 2.6 mg, 7.7% yield, respectively over two steps).

P450 Structure Preparation and Ligand Preparation

Crystal structures of substrate bound P450BM3 were downloaded from the Protein Data Bank (http://www.rcsb.org: code 1FGA and 4KPA). Structures of the corresponding F87V mutations were generated by converting Phe87 into Val using the Mutate Residue tool in the Schrödinger Suite (Ref: Schrödinger Maestro Suite 2016-2, Schrödinger, LLC; New York, NY: 2016.). The substrate-enzyme structures were processed with Schrödingers Protein Preparation Wizard. Bond orders were assigned and adjusted, hydrogen atoms were added and water molecules beyond 5.0 Å from the ligand were deleted as well as water molecules with less than 3 hydrogen bonds to non-waters. The protonation and tautomeric states of the amino acids and all hydrogen bonds were adjusted at pH of 7.0. Finally, the enzyme-substrate complex was minimized with convergence of heavy atoms to an RMSD of 0.3 Å using an OPLS3.

The protected substrates were prepared by using the LigPrep Wizard and the OPLS3 force field in the Schrödinger suite. For each substrate all ionization states were enumerated at a pH of 7.4.

P450 Docking Studies

The docking grid file was generated based on the prepared X-ray structures using the Schrödinger Suite. The bound fatty acid in the crystal structure was used to define the centroid of the substrate binding site. All prepared substrates were docked using Glide, the SP scoring function and default values. The best 5 docked poses for each ligand were selected based on the Glide Score.

Molecular Dynamics Simulations

Molecular Dynamics Simulations were performed for all selected docking poses using the Desmond package in combination with the OPLS3 force field in the Schrödinger suite. Each docked substrate-enzyme result was prepared for MDS by using Schrödingers System Builder tool. The system was solvated using the SPC solvent model and 13 Na^+ ions in an orthorhombic simulation box. For each protected substrate a 10 ns simulation was performed at 300 K and 1.013 bar. The system was relaxed using default values before simulation.

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Conflict of interest

The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication. G.B., F.O. and U.E.W.L. are employees of AbbVie. C.V. and I.P. were employees of AbbVie at the time when they contributed to the study.

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Keywords: docking/protecting group · enzyme catalysis · late-stage oxidation · molecular dynamics · $P450_{BM3}$-mediated oxidation

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[45] C.f. Figure S2 B in the Supporting Information.
[46] The enzymatic oxidation of d/p protected secondary amines of similar size to Vabicaserin worked successfully using P450BM3-F87V. Unpublished results.
[47] The exact nature of the mutations made to these commercially available P450BM3 variants is not disclosed by the vendor and therefore the presence of suitable binding motifs for the d/p groups in the mutants is uncertain as the original carboxylate binding motif (R47, Y51) may have been altered.
[48] The commercially available P450BM3 kit used in this case was obtained from codexis and contained engineered mutants of P450BM3 in which the identity of the mutations made to each mutant is not disclosed. http://www.codexis.com.

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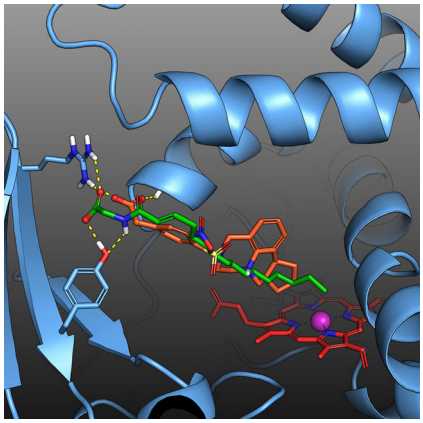
FULL PAPER

Novel solubilizing docking-protecting groups enable the regioselective $P450_{BM3}$-mutant mediated late-stage oxidation of lead compounds by binding the substrate (orange) in a biomimetic fashion (e.g., palmitic acid, green). Enzymatic oxidation, mild deprotection and purification yields hydroxylated derivatives that are difficult to obtain by classical chemical synthesis.



Biocatalysis

*C. Vickers, G. Backfisch, F. Oellien, I. Piel, U. E. W. Lange**

■■ – ■■

Enzymatic Late-Stage Oxidation of Lead Compounds with Solubilizing Biomimetic Docking/Protecting groups

Combining biocatalytic oxidation with protective-group chemistry enables one to modulate the site of oxidation. Lead compounds protected with different solubilizing biomimetic docking/protecting groups yield different hydroxylated derivatives with the same $P450_{BM3}$-mutant. Additional hydroxylation products are obtained when the $P450_{BM3}$-mutant is also varied. Derivatives of this type are difficult to obtain otherwise. Additionally, molecular-dynamic simulations help to rationalize the observed oxidation patterns. For more information, see the Full Paper by U. E. W. Lange et al. on page ■ ■ ff.

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