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Predictable Stereoselective and Chemoselective Hydroxylations and Epoxidations with P450 3A4

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Supporting Information

ABSTRACT: Enantioselective hydroxylation of one specific methylene in the presence of many similar groups is debatably the most challenging chemical transformation. Although chemists have recently made progress toward the hydroxylation of inactivated C−H bonds, enzymes such as P450s (CYPs) remain unsurpassed in specificity and scope. The substrate promiscuity of many P450s is desirable for synthetic applications; however, the inability to predict the products of these enzymatic reactions is impeding advancement. We demonstrate here the utility of a chemical auxiliary to control the selectivity of CYP3A4 reactions. When linked to substrates, inexpensive, achiral theobromine directs the reaction to produce hydroxylation or epoxidation at the fourth carbon from the auxiliary with *pro-R* facial selectivity. This strategy provides a versatile yet controllable system for regio-, chemo-, and stereoselective oxidations at inactivated C−H bonds and demonstrates the utility of chemical auxiliaries to mediate the activity of highly promiscuous enzymes.

INTRODUCTION

Natural systems are well recognized for streamlining the synthesis of large and complex molecules by iterative C−C bond formation reactions followed by tailoring oxidations at selective positions. Although dramatic progress has recently been made, such a strategy is not yet generally available to chemists, the main difficulty being the poor selectivity achievable for oxidations at inactivated C−H bonds. In particular, the stereoselective hydroxylation of one methylene group in the presence of similar methylenes is probably the most challenging synthetic transformation at the present time. Recent successes by chemists in this area include the regio- and stereoselective hydroxylation of pleuromutilin[1] and the total synthesis of deoxyerythronolide B by late-stage C−H oxidations.[2] The successful methodology uses a bulky, electrophilic iron catalyst and hydrogen peroxide as the oxidant but typically suffers from overoxidation and limited functional group tolerance. The main difficulty arises in the fact that selective hydroxylation of tertiary carbons is favored over reactions at methylene groups. Recent advances in the hydroxylation of inactivated C−H bonds have thus focused on reactions at tertiary sp^3 carbons.[3−7] The electron-rich nature of tertiary C−H bonds and their relative rarity in comparison to methylene and methyl carbons in natural products have made them excellent targets for metal-catalyzed hydroxylation. To our knowledge, however, no strategy using chemical catalysts has been successful at achieving predictable hydroxylation at one inactivated methylene C−H bond among others of similar electronic properties. Enzymes remain unsurpassed at this task. Not only are enzymes typically regio-, chemo-, and stereoselective, but they often perform well in mild aqueous conditions with innocuous oxidants, providing an environment-friendly alternative to organometallic reagents.

Monooxygenases such as cytochrome P450 enzymes (P450s or CYPs) arguably hold the greatest unexploited synthetic potential of any family of enzymes.[8] P450s are ubiquitous enzymes with major roles in mammalian drug metabolism, steroid biosynthesis, and bacterial biosynthesis of secondary metabolites, which are an exceptional source of pharmaceutical agents. Chemists have a special appreciation for P450s because of their impressive ability to catalyze selective hydroxylations at inactivated C−H bonds. To date, however, this potential has remained highly untapped. Commercial applications using P450 enzymes are currently rare and limited to using whole cells.[9−13] Synthetic applications of P450s have been hindered mainly by the need for expensive cofactors, low turnover, and poor stability. These limitations have recently been partly overcome by rational and random mutagenesis,[14−16] yet the use of free enzymes remains nonideal for industrial processes. Research applications of P450s are more accessible considering the smaller scale. Unlike industrial settings, research environments require versatile and promiscuous, yet controllable, catalysts. Mutagenesis has mostly been used to modify the substrate specificity of P450s,[14−16] but rarely to increase their substrate promiscuity.[17] Although numerous P450s are highly promiscuous, product predictions are speculative at best. This problem is of special concern for drug-metabolizing P450s and has beleaguered the pharmaceutical industry for half a century.[18]

The availability of a versatile catalyst for regio-, chemo-, and stereoselective hydroxylations of inactivated methylene groups would create a paradigm shift in synthetic chemistry. Such an advance would however require that the catalyst show reasonable

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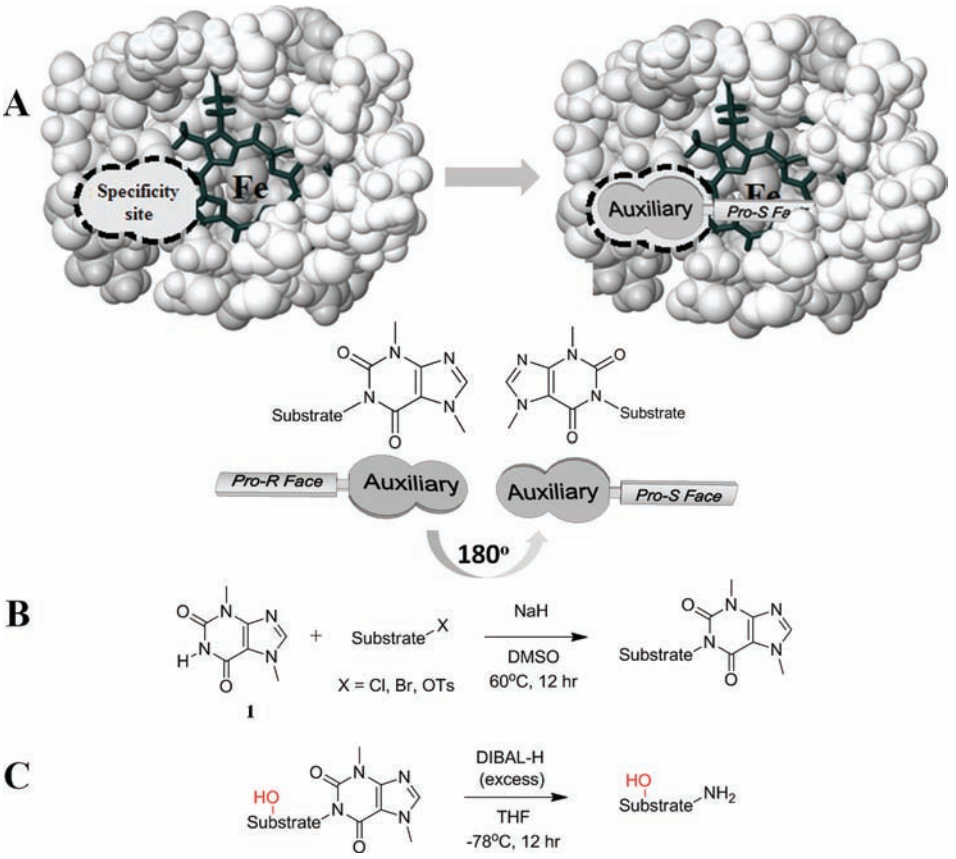

Figure 1. Auxiliary design, attachment to the substrate, and cleavage from the product. (A) The auxiliary was designed to fit a binding pocket (the specificity site) of CYP3A4 near the heme group and display the substrate to orient its *pro-R* face on the proximal face of the reactive iron species. Like a ruler, the distance between the auxiliary and the iron is expected to control the position of hydroxylation relative to the auxiliary. (B) A desired auxiliary should be easy to link to the substrate, yet easily cleaved from the product. The successful auxiliary described here is linked to the substrate via substitution under basic conditions. (C) The hydrophobicity and the chromophoric properties of this auxiliary facilitate recovery of the product from the enzymatic aqueous solution. Next the oxidized product is easily cleaved from the auxiliary.

substrate promiscuity, yet provide highly predictable selectivity. Herein, we report a successful strategy to achieve C−H bond hydroxylations and double bond epoxidations with predictable regio-, chemo-, and stereoselectivity. To this end, we combined the oxidative power of CYP3A4 with the positioning and orienting effects of an inexpensive, easily functionalized, achiral, cell-permeable auxiliary.

RESULTS AND DISCUSSION

Despite decades of sustained investigations (mainly from the pharmaceutical industry), the rules that govern binding selectivity by CYP3A4 and other substrate-promiscuous P450s remain nebulous. Auxiliaries have been very successful in chemical synthesis, and we envisaged that they may provide a new strategy to control the selectivity of P450 enzymes such as CYP3A4 (Figure 1A). A few research groups have reported the use of substrate engineering in biocatalysis, but mostly to increase recognition of non-natural substrates, and not for rational control of selectivity.[19−22] To our knowledge, the only example with P450 enzymes used a carbolide to "anchor" macrolides into P450 PikC.[23] The method did not however control the site of oxidation and generated multiple products. The auxiliary presented here is not only expected to anchor the substrate into the enzyme but to also allow predictable control of the reaction chemo-, regio-, and stereoselectivity. The optimal chemical auxiliary should fit a binding pocket of CYP3A4 near the heme group and display one selected C−H bond of the substrate toward the reactive iron species (Figure 1A). Like a ruler, the distance between the auxiliary and the iron should control the position of hydroxylation. Facial selectivity is also expected to arise from the orientation of the auxiliary relative to the heme prosthetic group.

As inspiration for potential auxiliaries, we considered the structures of several natural substrates of CYP3A4. A number of unsuccessful auxiliaries were tested (e.g., coumarins and fluorescein) until lisofylline attracted our attention. Lisofylline, a molecule with multiple therapeutic activities,[24−30] is metabolized in part by human CYP3A4 via hydroxylation at the fourth carbon from the theobromine group.[31] We therefore envisioned that theobromine (**1**; Figure 1B) may be a useful auxiliary if oxidation is desired at the fourth atom of the substrate from the point of attachment to the auxiliary.

Theobromine is achiral and inexpensive, contains a chromophore, and can penetrate cells. Moreover, it has only one nucleophilic group (a nitrogen atom) and is therefore readily linked to various substrates (Figure 1B). Importantly, this auxiliary is also easily cleaved off after enzymatic reactions, with the generation of an amine or other functional groups depending on

Table 1. Results of CYP3A4 Enzymatic Transformations of Substrate−Auxiliary Molecules

Substrate[a]	Major Product[b]	Regio-selectivity[c] %	Absolute stereochemistry[d] *R* : *S*	Isolated Yield %
Tb **2**	No products detected[e]	N.A.[f]	N.A.	N.A.
Tb **3**	No products detected	N.A.	N.A.	N.A.
Tb **4**	OH Tb **14**	>95%	70:30	60%
Tb **5**	OH Tb **15**	80%	75:25	65%
Tb **6**	OH Tb **16**	>95%	N.A.	80%
Tb **7**	OH Tb **17**	>95%	75:25	80%
Tb **8**	OH Tb **18**	85%	70:30	65%
O Tb **9**	O HO Tb **19**	>95%	50:50	quant.
Tb **10**	OH Tb **20**	65%	70:30	45%
Tb **11**	OH Tb **21**	>95%	60:40	40%
Tb **12**	O Tb **22**	>95%	50:50	40%
Tb **13**	O Tb **23**	>95%	> 99:1	50%

[a] Tb is used to abbreviate the theobromine auxiliary. [b] Determined by comparison to authentic standards. See the Supporting Information for reaction conditions. All reactions were performed at least in duplicate. [c] Percentage of oxidation at the fourth carbon from Tb compared to all other oxidation products. [d] Refers to the stereochemistry at the fourth carbon from Tb (or at the second carbon from Tb for **19**). [e] To a limit of detection of 0.1%. [f] Not applicable.

the conditions used (Figure 1C). To explore the potential of theobromine as a chemical auxiliary for the control of CYP3A4 oxidations, theobromine was functionalized with various small substrates before reaction with CYP3A4. Alkanes and alkenes were selected because of their notorious inertness and of their multiple undifferentiated C−H bonds. Moreover, the expected alcohol products represent versatile chiral synthons with a potentially high commercial value. Under basic conditions (Figure 1B) a series of alkyl halides were reacted with theobromine to generate a range of substrate−auxiliary complexes (compounds **2**−**13**, Table 1) in very good yields.

Compounds **2**−**13** were next allowed to react with CYP3A4 and cofactors in buffer. Product formation and substrate conversion were monitored by HPLC and LC−MS (Figure 2A). For all products detected, the mass increase corresponded to hydroxylation

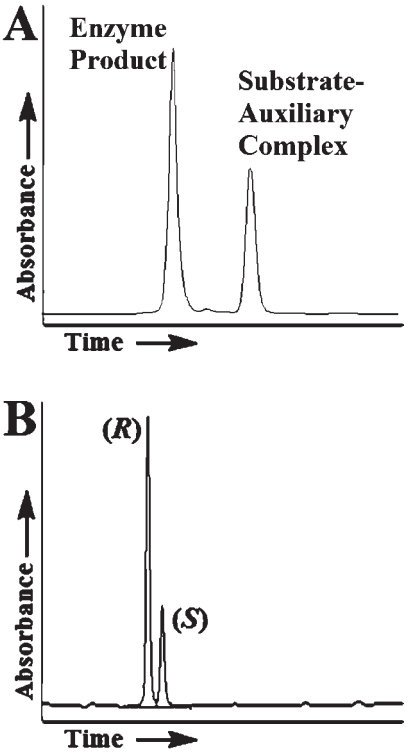

Figure 2. Analysis of the CYP3A4 enzymatic transformations. (A) HPLC trace of an enzymatic reaction mixture showing the oxidized products and the substrate−auxiliary complex. (B) Chiral HPLC trace showing the clean separation of the two enantiomers of the major enzymatic product. See the Supporting Information for the complete list of traces.

or epoxidation. *No product resulting from overoxidation (carbonyl product) could be detected* (<0.1%). Furthermore, the presence of characteristic theobromine fragments verified that all oxidations occurred on the substrate portion of the molecule, and not on the theobromine auxiliary. All products were isolated and purified by achiral chromatography. HPLC analysis on a chiral column (Figure 2B) was used to quantify the enantiopurity of each enzymatic product. The chemical structure of the products and absolute configuration at the oxidation site were determined by comparison with synthetic authentic standards.

Careful analysis of the MS fragmentation patterns provided information about the site of oxidation and was used to plan the syntheses of authentic enantioenriched standards. Authentic alcohol standards were synthesized using a divergent synthetic scheme (Scheme 1A). The racemic synthetic alcohols thus obtained were separated on a chiral HPLC column, and their absolute configuration was determined by a well-established technique[32] involving esterification with enantiopure acids of opposite configuration ((*R*)-MPA and (*S*)-MPA; MPA = α-methoxyphenylacetic acid) to yield a pair of diastereoisomers for each alcohol, followed by comparison of the related diastereoisomers by NMR. Authentic epoxide standards were synthesized by introducing chirality to terminal epoxides using hydrolytic kinetic resolution[33] followed by coupling of the enantioenriched epoxides **34** and **35** to compound **1** (Scheme 1B). The fully characterized authentic enantioenriched standards were assigned to CYP3A4-hydroxylated products on the basis of their retention times and after coinjection on a chiral HPLC column.

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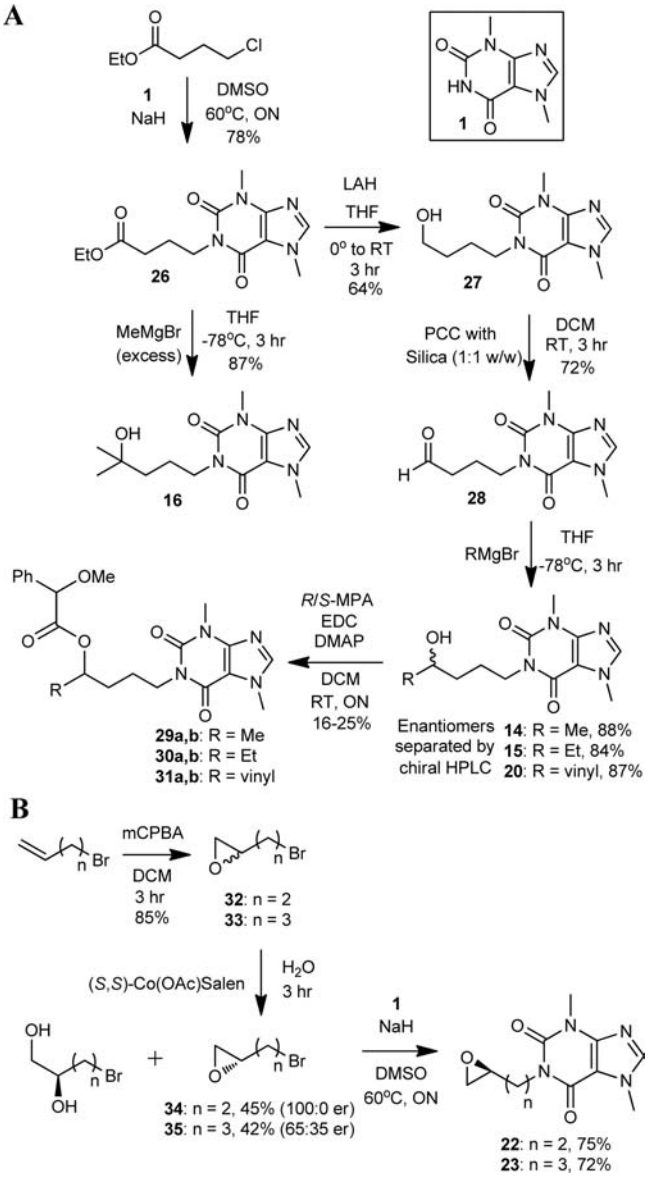
Scheme 1. Synthetic Schemes for the Preparation of Selected Chiral Alcohol (A) and Epoxide (B) Authentic Standards[a]



[a] Racemic alcohols were separated by chiral HPLC. Their absolute stereochemistries were determined by comparing ^{1}H NMR spectra after functionalization with (*R*)- or (*S*)-α-methoxyphenylacetic acid. Compound names with "**a**" were prepared from (*R*)-(−)-α-methoxyphenylacetic acid, and those labeled with "**b**" were prepared from (*S*)-(+)-α-methoxyphenylacetic acid. For the definition of Co(OAc)Salen, see ref 33.

Table 1 lists all products obtained from CYP3A4 transformations for each of compounds **2**−**13**. Our research group has reported that the reaction rate of human CYP3A4 is improved by up to 30% when the natural cofactors, NADPH and cytochrome P450 reductase (CPR), are replaced with the inexpensive cumene hydroperoxide (CHP) or sodium percarbonate (SPC).[34] To take advantage of this, enzymatic transformations were carried out using CHP as a cofactor surrogate. A series of separate experiments using NADPH/CPR revealed that the choice of cofactor used did not affect the stereoselectivity of the reaction, nor did it influence the structure and ratio of products (see the Supporting Information for comparative HPLC traces). After optimization of the conditions, substrate conversion rates were typically around 70% on the basis of HPLC (Figure 2A and Supporting Information), and isolated yields ranged from 40% to quantitative (Table 1).

As predicted, oxidation is detected at the fourth carbon position relative to the theobromine auxiliary for all compounds listed in Table 1, except for compounds **2** and **3**, which were designed not to have a fourth methylene carbon. Moreover, oxidation proceeds with a distinct preference for the *pro-R* face of the substrate and stops at the alcohol or the epoxide. Over-oxidation products are not detected (on the basis of LC−MS analysis of crude mixtures). We expected compound **2** to be too short (no fourth carbon) to achieve the necessary proximity to the heme iron active species, and as expected it is not transformed by CYP3A4 (<0.1%). Compound **3** was designed to have a methyl group at the fourth carbon from the auxiliary. There are very few reports of methyl oxidations by this enzyme;[35] however, the absence of detectable products for the CYP3A4 oxidation of **3** (<0.1%), together with epoxide formation of compound **12** (also four-carbon chain but terminal sp^2 CH_2 instead of CH_3), suggests that electronic factors contribute to the lack of reactivity at the methyl group of **3**. The reduced reactivity of methyl compared to methylene groups is expected from the bond dissociation energy and also well understood for radical processes such as those catalyzed by iron-based small molecules.[36,37] Overall, the fourth-carbon rule consistently allows prediction of which methylene group is hydroxylated by CYP3A4.

The scope of this system was further investigated with compounds containing branching, unsaturations, or a heteroatom at or near the position of oxidation. The tertiary carbon of compound **6** is oxidized by CYP3A4 to a single product (>95%), compound **16**, indicating the potential utility of this system in the production of enantiopure tertiary alcohols. Racemic compounds **7** and **8** are also hydroxylated at the fourth carbon from the auxiliary (products **17** and **18**), in spite of the branching point at either the third or second carbon, respectively. It was interesting to see that the enzyme accepted both enantiomers of compounds **7** and **8** as substrates, although one is transformed with a slight preference over the other (5:2 and 4:3 for **7** and **8**, respectively, absolute configuration not determined). The branching point of compound **9** forms a heterocyclic ring. As anticipated, CYP3A4 proceeds to hydroxylate **9** at the fourth carbon from the auxiliary, with very high selectivity for one fourth carbon (counting clockwise, α to the ether) over the other (counting counterclockwise, β to the ether). This preference was expected on the basis of electronics. Hydroxylation at that position yields a hemiacetal which spontaneously opens to the corresponding γ-hydroxy aldehyde (**19**). The chirality of this product arises from the hydroxyl group, the configuration of which is already determined at the branching point before enzymatic transformation. Here again CYP3A4 accepts both enantiomers of racemic **9**, and as expected for a quantitative transformation, a racemic mixture is obtained. At low conversion rate, however, analysis of the mixture shows a slight preference for one enantiomer (configuration not determined; see the Supporting Information). Although CYP3A4 may not be suitable for resolutions, its tolerance for different enantiomers of the same substrate further demonstrates its substrate promiscuity, which has the advantage here of allowing the generation of different diastereoisomers for comparison. Also in line with the

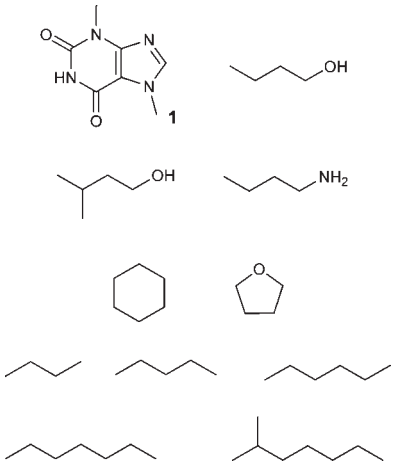


Figure 3. Results of CYP3A4 enzymatic transformations of substrates lacking the auxiliary. None of these compounds were transformed under the same conditions described for Figure 2. From Chefson et al.[39] for the last seven compounds.

fourth-carbon rule and the *pro-R* facial selectivity is the formation of compounds **20** and **21** from **10** and **11**, respectively. Considering that epoxidation of double bonds is thermodynamically favored over sp^3 C−H bond hydroxylation, these examples demonstrate the functional group tolerance of the system. Indeed no epoxidation product was detected (<0.1%) for the transformation of compound **11** by CYP3A4, and LC−MS fragmentation suggests that the terminal epoxide is a minor product (<35%) of oxidation of **10** by CYP3A4 (this explains the low isolated yield, 45%, for product **20**). The functional group tolerance of this enzyme was long established from the selective hydroxylation of approximately half of all clinical drugs, many of which have numerous orthogonal functional groups.[38]

Enantioselective epoxidation of terminal olefins is especially challenging.[33] The use of our auxiliary to control the selectivity of terminal epoxidations was investigated. Two compounds were designed to include a double bond either between C-3 and C-4 (compound **12**) or between C-4 and C-5 (compound **13**). Both underwent CYP3A4-catalyzed epoxidation at the terminal double bond. Lower conversion rates account for the suboptimal isolated yields (40−50%). Further optimization of the reaction conditions may be needed for epoxidations. Unreacted starting material could however be isolated and recycled. While compound **13** generated the enantiopure (>99%) product **23** with the expected *R*-configuration at the chiral carbon, compound **12** was oxidized to the racemic epoxide **22**. This is consistent with the stereoselectivity rules proposed above: stereocontrol is believed to take place at the fourth carbon from the auxiliary, which is not a prochiral carbon in compound **12**.

To demonstrate the synthetic utility of our strategy, scaled up experiments were performed. We were pleased to find that the transformation of 100 mg of **4** affords **14** in 63% isolated yield after purification by flash chromatography (see the Supporting Information for details). Moreover, the regioselectivity and enantioselectivity are similar at both scales.

The necessity of the auxiliary was verified by reacting CYP3A4 with substrates lacking the theobromine group. A number of commercially available molecules resembling the substrate part of compounds **2**−**13** were tested for transformation by CYP3A4 (Figure 3). Also included in Figure 3 are data from a previous

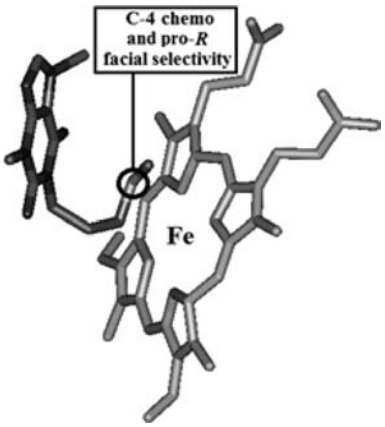


Figure 4. Results of docking studies. Proposed substrate−auxiliary orientation relative to the heme group in the enzyme pocket, as suggested by docking studies (see the text for details).

study.[39] None of the molecules were transformed by the enzyme to any detectable level (<0.1%) without the theobromine auxiliary.

Finally, to determine if the above-described enzymatic selectivity was specific to CYP3A4, enzymatic reactions were performed with human CYP2D6. Although compound **12** was transformed to the corresponding epoxide, **5** and **13** were hydroxylated to mixtures of compounds, and other substrate−auxiliary systems were not oxidized to any detectable level by CYP2D6. Reported crystal structures[40−43] and drug metabolism data for these two enzymes also support a different selectivity.

In an attempt to better understand the reasons for the observed regio- and stereoselectivites, we performed in silico docking studies using the entire scope of substrate−auxiliary complexes, **2**−**13**, and a crystal structure of CYP3A4 available in the protein database.[41−43] Although P450 enzymes are known to be difficult to handle with most in silico applications,[44] this docking survey led to a structure that is consistent with our results. Figure 4 shows the auxiliary bound in proximity to the CYP3A4 heme group with the oxidized C−H bond nearest to the heme iron distal side.

■ CONCLUSION

In summary, we have established a proof-of-concept for the utility of chemical auxiliaries to control the selectivity of enzymatic reactions. We have demonstrated that inexpensive achiral theobromine (**1**) is a powerful chemical auxiliary for CYP3A4 transformations, allowing prediction of the site of oxidation and the facial selectivity. In all cases, oxidation at the fourth carbon from the auxiliary and *Pro-R* facial selectivity are favored. The isolated yields were on the order of ∼70% with purified enzyme at synthetically useful scales, yet we believe that this strategy should also find use with whole-cell transformations. Indeed, theobromine and derivatives similar to compounds **2**−**13** are known to cross cell membranes.[45] This strategy provides easy access to small, enantioenriched or enantiopure, alcohols and epoxides that are otherwise difficult to access, often requiring either expensive enantioselective separation or low-yielding hydrolytic kinetic resolution. Although limited to substrates not considerably larger than the auxiliary, this method has the added advantages of not yielding overoxidation products and of being functional group tolerant (intrinsic to this enzyme).

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Chemical methods for selective oxidations at methylene groups[1,2] preferentially lead to ketone formation and show poor functional group tolerance. Conversely, no carbonyl-containing products were detected here (<0.1%), and this enzyme has a well-established extended functional group tolerance.[38] This was confirmed here with the selective methylene hydroxylation in the presence of a nearby double bond and of the different functionalities present on the auxiliary itself. P450 enzymes, including CYP3A4, are known to catalyze *N*- and *O*-demethylation, yet none of the *N*-methyl groups of the theobromine auxiliary were affected during our transformations. We believe that new auxiliaries can be designed to afford different regio- and stereoselectivities for CYP3A4 transformations. This approach should also apply to other P450s, and to enzymes from other families, to produce a series of biocatalyst/auxiliary systems, each with complementary selectivities. This strategy should therefore find broad application in synthesis.

■ ASSOCIATED CONTENT

Supporting Information. Full experimental methods, compound characterization, selected NMR spectra and HPLC traces, and complete ref 9. This material is available free of charge via the Internet at http://pubs.acs.org.

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