# Probing the substrate specificity of the catalytically self-sufficient cytochrome P450 RhF from a *Rhodococcus sp.*

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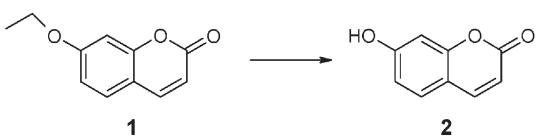
**Analysis of the substrate specificity of the self-sufficient cytochrome P450 RhF revealed that the enzyme tends to catalyse the dealkylation of substituted alkyl–aryl ethers with shorter alkyl moieties more readily than equivalent compounds with longer alkyl groups.**

Cytochrome P450 enzymes belong to the monooxygenase superfamily and are widely distributed in Nature.[1] The mechanism of P450 catalysis involves the sequential delivery of two electrons from redox partner(s), enabling the binding and then reductive activation of molecular dioxygen. In the bacterial system, the required electron equivalents are generally supplied by two auxiliary proteins comprising an iron–sulfur protein (ferredoxin) and a cognate NAD(P)H-ferredoxin reductase.

One well characterised exception to this general rule is the self-sufficient cytochrome P450 BM3 from *Bacillus megaterium*, which comprises a P450 domain fused to a diflavin (FAD, FMN) reductase.[2] Several other cytochrome P450 enzymes with a similar arrangement to BM3 have recently been identified, mainly as a result of genome sequence analysis.[3] An arrangement of this type in which the cytochrome P450 is fused to a reductase partner can support very high catalytic activity. For example, the catalytic activity for cytochrome P450 BM3 is the highest determined for a P450 monooxygenase (17 000 $min^{-1}$ for arachidonate).[4]

Members of the genus *Rhodococcus* are well known for their ability to degrade a wide range of structurally diverse xenobiotic compounds and are therefore likely to be a rich source of potentially useful biocatalysts.[5] We used a PCR-based approach to screen a library of *Rhodococci* for novel cytochrome P450 genes. During the course of this work we cloned a cytochrome P450 from a *Rhodococcus sp*. with a unique structural organization, which we named P450 RhF.[6] Unlike P450 BM3, the reductase domain of P450 RhF comprises a flavin-containing FMN site and a [2Fe2S] ferredoxin-like component. The gene encoding P450 RhF was engineered for heterologous expression in *E. coli* and the recombinant protein purified and characterised.[7,8] However, because the cloning procedure constituted a generic screen for P450-like sequences, the natural function of P450 RhF is unknown. Our initial experiments showed that cytochrome P450 RhF catalyses the *O*-dealkylation of 7-ethoxycoumarin (**1**) to 7-hydroxycoumarin (**2**) (Scheme 1).

In order to characterise the substrate specificity of this enzyme, we report here the *in vitro* biotransformation of several structurally related alkyl aryl ethers. Preliminary screening of several dialkyl ethers gave no detectable activity. α-Tetralone (**11**) was included in the screen as a possible substrate for direct hydroxylation. The structures of the substrates initially screened in this work are shown in Fig. 1.




**Scheme 1**

P450 RhF was purified to homogeneity according to the published procedure.[7] The purified enzyme was able to catalyse the oxidation of **1** to **2** *in vitro* in the presence of the cofactor NADPH. Initial screening of the substrates **3–17** was carried out using a microtiter plate-reader (SpectraMax GeminiXS, Molecular Devices) in a 200 µl volume by monitoring the consumption of NADPH using fluorescence detection ($\lambda_{ex}$ = 340 nm; $\lambda_{em}$ = 440 nm; cut off 435 nm; temperature 30 °C). Reactions that resulted in usage of NADPH with a rate that was at least twice the background oxidation rate were considered potential substrates for P450 RhF. Further analysis by LC-MS was carried out to verify

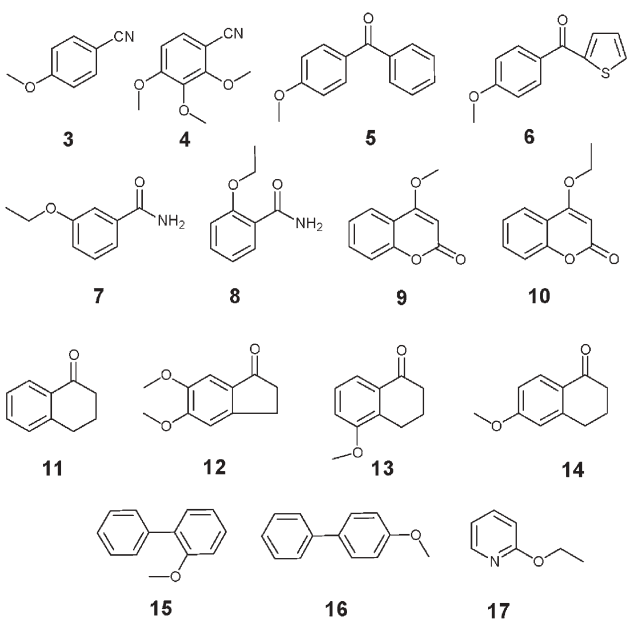



**Fig. 1** The structures of substrates studied.

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**Table 1** Michaelis–Menten parameters determined for each substrate

| Substrate | $k_{cat}$[a]/min$^{-1}$ | *Km*/mM | ($k_{cat}/K_m$)/mM$^{-1}$min$^{-1}$ |
|---|---|---|---|
| **3** | 4.3 ± 0.3 | 1.2 ± 0.2 | 3.6 |
| **4** | 6.2 ± 0.5 | 2.2 ± 0.4 | 2.8 |
| **6** | 4.8 ± 0.1 | ND | — |
| **10** | 2.7 ± 0.2 | 0.8 ± 0.2 | 3.4 |
| **13** | 94 ± 6 | 4.1 ± 0.5 | 23 |
| **14** | 13 ± 2 | 11 ± 2 | 1.2 |

[a] $k_{cat}$ Values are based upon monitoring NADPH turnover. ND: not determined. Michaelis–Menten parameters were determined for substrates shown to be active by LC-MS.

the generation of a reaction product. This analysis is required because not all the NAD(P)H consumed in a P450 system is necessarily coupled to substrate oxidation.[1]

No NADPH turnover was detected using compounds **7**, **8**, **9**, **15**, **16** and **17** and hence no further work was carried out with these substances. By contrast, all the other compounds shown in Fig. 1 displayed significant degrees of NADPH utilization. NADPH consumption could be due to P450 RhF-catalysed substrate oxidation with various degrees of coupling. Alternatively, rather than conversion of substrate to product, binding of a compound to the P450 may cause perturbation in the redox potential of the enzyme, resulting in NADPH usage *via* an oxidative species (hydrogen peroxide, superoxide or even water) while the enzyme returns to the resting state. Compounds **11** and **12** appear to be examples that fall into this latter category because no product formation could be detected. However, substrates that bind close to the heme moiety increase the probability of C–H bond oxidation by the ferryl intermediate, thus suppressing the oxidase-uncoupling pathway. LC-MS analyses confirm product generation for compounds **3**, **4**, **5**, **6**, **10**, **13** and **14** (Table 1). In each case, the mass data indicates that P450 RhF catalyzes the dealkylation of the corresponding alkyl aryl ether. Michaelis–Menten parameters determined for each substrate are given in Table 1. Interestingly, we found a large difference in catalytic efficiency ($k_{cat}/K_m$) for **13** and **14** despite the close structural similarity between these compounds.

The calculations are based on the rate of NADPH consumption, rather than product formation, and therefore represent upper estimates, as no account is taken of possible uncoupled peroxide or oxidase activity. We have, however, determined the coupling efficiency for **13** and **14** to be approximately 3 and 8%, respectively. A low level of coupling such as this is often associated with unnatural substrates.

One advantage of using a fluorescent compound as substrate is the fact that product formation can be measured directly. We decided to investigate the dealkylation of coumarin derivatives to establish the relative rate of reaction versus length of the alkyl moiety. Coumarin compounds bearing a methoxy, ethoxy, propoxy or isopropoxy group at C-7 were tested (Fig. 2). The P450 RhF catalysed formation of 7-hydroxycoumarin was monitored directly by fluorescence spectrophotometry in a 96-well plate format ($\lambda_{ex}$ = 397 nm; $\lambda_{em}$ = 466 nm; cut off 420 nm; temperature 30 °C). Under these conditions, no interference from the fluorescent substrates was detected. A series of eight solutions (0–2 mM final concentration) of each coumarin derivative were prepared by successive dilution from a stock solution (10 mM) in dimethyl sulfoxide into 50 mM potassium phosphate buffer, pH 7.8. The total reaction volume was 200 μl. Typically 5 μl of P450 RhF (3–5 μM final concentration) was added to 185 μl of substrate solution and the solution was allowed to equilibrate for 4 min. Reaction was initiated by the addition of 10 μl of NADPH (10 mM stock). Time-based measurements were recorded every 15 s for 10 min. An extinction coefficient of $\varepsilon$ = 465,000 mM$^{-1}$ cm$^{-1}$, determined using 7-hydroxycoumarin standards, was used to calculate the kinetic parameters. The relative rate of product formation was as follows: methoxy = ethoxy > propoxy > isopropoxy. If the rate of product formation for 7-methoxycoumarin (or 7-ethoxycoumarin) is arbitrarily set to 1.0 the relative rate for the propoxy and isopropoxy derivatives were 0.35 and 0.07, respectively. The kinetic parameters for the dealkylation of 7-methoxycoumarin were $k_{cat}$ = 1.6 min$^{-1}$, $K_m$ = 620 μM.

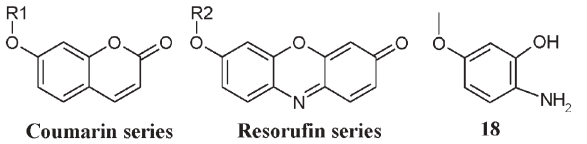


**Fig. 2** R1, methyl, ethyl, *n*-propyl or isopropyl moiety; R2, methyl, ethyl, *n*-propyl or benzyl moiety.

We also investigated whether a series of substituted ethers of resorufin could also act as substrates for P450 RhF. These included the methyl, ethyl, *n*-propyl and benzyl ethers of resorufin (Fig. 2). The dealkylated product in each case was resorufin. Again, product formation was readily detectable by fluorescence spectrophotometry. The protocol was similar to that described for the coumarin series except the excitation and emission wavelengths were adjusted slightly ($\lambda_{ex}$ = 530 nm; $\lambda_{em}$ = 590 nm; cut off 550 nm; temperature 30 °C). An extinction coefficient of $\varepsilon$ = 726 000 mM$^{-1}$ cm$^{-1}$, determined using resorufin standards, was used to calculate the kinetic parameters. The relative rate of product formation was as follows: methoxy > ethoxy > *n*-propoxy > benzyl (*i.e.* 1.0, 0.70, 0.35 and 0.12, respectively). The steady-state parameters for the formation of resorufin from resorufin methyl ether were determined ($k_{cat}$ = 0.35 min$^{-1}$, $K_m$ = 43 μM).

Interestingly, the methoxy derivative of both the coumarin and resorufin series of compounds gave the highest rate of dealkylation with progressively lower rates for compounds bearing longer alkylated substituents, although we cannot rule out the affect of steric hindrance with the isopropoxy and benzyl derivatives. Our observations contrast with what might be expected from the rate of dealkylation of the hydroxylated intermediate in terms of chemical reactivity. The relatively small $K_m$ value for resorufin methyl ether of 43 μM (*cf.* 220 μM for 7-ethoxycoumarin) indicates this compound binds quite well in the active site. Unfortunately, due to the fluorescent properties of the compound it was not possible to assess whether binding of resorufin methyl ether induces a spin state shift.

A feature of the resorufin molecule that may be important for binding is the aromatic ring bearing an alkyl ether moiety with a *meta* oxygen and *para* nitrogen atom. After screening a number of commercially available compounds with these features we found 2-hydroxy-4-methoxyaniline (**18**) gave a significant NADPH turnover in the presence of P450 RhF.

The kinetic parameters were determined as $k_{cat}$ = 29 min$^{-1}$, $K_m$ = 62 μM. Product formation was verified by LC analysis using

authentic compound as standard (on a C18-RP column). Interestingly if the hydroxyl moiety is absent or replaced with another R group (methyl, carboxylic acid, halogen), no NADPH turnover is detectable.

In conclusion, our substrate screening for P450 RhF revealed that the enzyme mediates the dealkylation of substituted aromatic alkyl ethers. The aromatic ring appears to be important for substrate recognition because we were unable to detect enzyme activity using several dialkyl ethers (*e.g.* allyl ethyl ether). Our results indicate that compounds bearing a shorter alkyl moiety tend to undergo dealkylation more readily than those with an extended alkyl chain.

P450 RhF is the first example of a self-sufficient cytochrome P450 with a flavin and FeS containing reductase. Currently, the natural function of P450 RhF and its homologues from phylogenetically unrelated bacteria is unknown. Crystallization of the heme domain of P450 RhF is currently underway. The results of these studies, combined with knowledge of the likely substrate specificity, should help elucidate the natural role of P450 RhF.

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## Notes and references

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