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Chemical Communications, 2019; 55(34):5029-5032

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Published at: http://dx.doi.org/10.1039/c9cc02060h

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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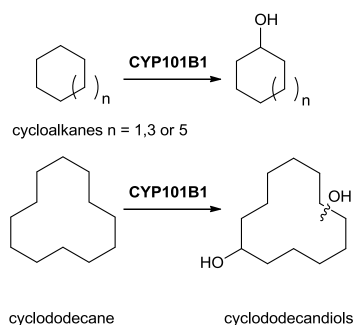
The cytochrome P450 monooxygenase CYP101B1 from *Novosphingobium aromaticivorans* selectively hydroxylated methylene C-H bonds in cycloalkyl rings. Cycloketones and cycloalkyl esters containing C6, C8, C10 and C12 rings were oxidised with high selectively on the opposite side of the ring to the carbonyl substituent. Cyclodecanone was oxidised to oxabicycloundecanol derivatives in equilibrium with the hydroxycyclodecanones.

The selective catalytic functionalisation of carbon-hydrogen bonds in organic molecules would improve synthetic efficiency but is a significant chemical challenge. The regio- and stereo-selective insertion of an oxygen atom into the chemically inert methylene carbon-hydrogen bonds of saturated cyclic hydrocarbons is of interest due to the difficulties associated with these reactions.[1-4] Monooxygenase enzymes, such as the cytochrome P450 superfamily of heme proteins, have the potential to be utilised as biocatalysts for the oxidation of organic molecules.[5-9] However, the monooxygenase activity of many CYP enzymes is often limited by low substrate affinity, poor product selectivity for non-natural substrates or the lack of suitable electron transfer proteins.[10-12] As a result, extensive modification of the enzymes is required before they are able to catalyse the desired reaction. For example, CYP102A1, from *Bacillus megaterium,* which is highly active for fatty acid substrates, has been modified via protein engineering to facilitate the oxidation of the C–H bonds in many substrates.[5, 11, 13-17]

The bacterium *Novosphingobium aromaticivorans* DSM12444 contains the CYP101B1 encoding gene. This enzyme has been shown to bind norisoprenoids, bicyclic terpenoid and tricyclic hydrocarbon acetate esters with high affinity.[12, 18-20] An electron transfer system, comprising a ferredoxin reductase, ArR, and a ferredoxin, Arx has been isolated from the same bacterium and used to support the oxidation activity of CYP101B1 for these substrates with high activity and total turnover numbers (TTN).[12, 18-21] Therefore this system has all the desired attributes for the design of efficient biocatalytic reactions.

We set out to assess if CYP101B1 was capable of acting as an biocatalyst for the selective hydroxylation of cycloalkanes and their derivatives. We initially tested selected cycloalkane substrates ranging in size from C6 through to C12. Cyclohexane was a poor substrate for the enzyme (Table 1, Figure S1 – S3) but cyclooctane and cyclodecane bound to the enzyme with significantly higher affinity (Figure S1 and S2). Both substrates were oxidised by CYP101B1 to the equivalent cycloalkanol (Scheme 1, Figure S3 and S4) with the product formation activity (PFR) and total turnover number (TTN 3400) of cyclooctane being greater than that of the larger cyclodecane (Table 1).



Scheme 1. Oxidation of selected cycloalkanes by CYP101B1.

Cyclododecane bound to CYP101B1 with decreased affinity and was oxidised at lower activity (Table 1). The product of the enzyme catalysed reaction was not cyclododecanol (Figure S3). GC-MS analysis highlighted the formation of double oxidation metabolites as the major products (Figure S4). Cyclododecanol was found to induce a higher spin state shift in CYP101B1 than any of the cyclic alkanes tested above (Table 1, Figure S1 and S2). It was also more efficiently oxidised than cyclododecane (Table 1). Its oxidation resulted in a mixture of cycldodecanediols which coeluted with the major products from cyclododecane oxidation. These included 1,7- and 1,6-cyclododecanediol along with another minor product

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Electronic Supplementary Information (ESI) available: [experimental methods, substrate binding data, gas chromatography, mass spectrometry and NMR data]. See DOI: 10.1039/x0xx00000x

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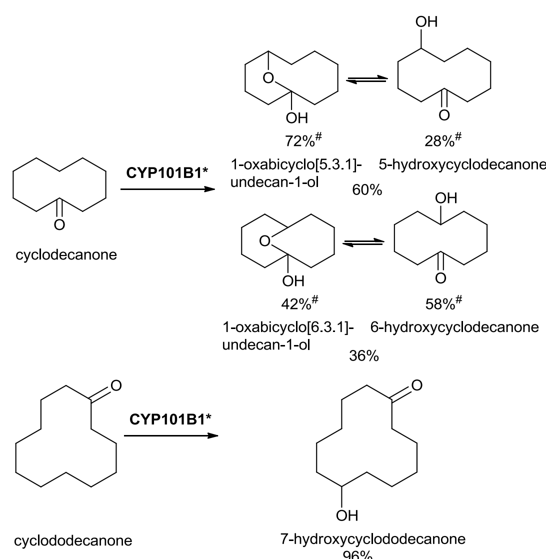
Table 1 Substrate binding and *in vitro* turnover data for CYP101B1 with different substrates. The spin state shift induced by substrate binding is given by the percentage of high spin heme (%HS) and the binding affinity by the dissociation constant (K_d) in micromolar. The NADH oxidation rate is the frequency of NADH oxidation. The coupling efficiency (coupling), is defined as the percentage of NADH utilised for the formation of products. The turnovers were measured using a ArR:Arx:CYP101B concentration ratio of 1:10:1 (0.5 µM CYP enzyme, 50 mM Tris, pH 7.4). Rates (NADH; product formation rate, PFR) are reported as mean ± S.D. (n ≥ 3) and given in nmol.nmol-CYP^{-1}.min^{-1}. TTN is the total turnover number which is the number of turnovers catalysed per enzyme. – no product detected or not measured due to low affinity (Kd) or levels of product formation (TTN).

Substrate	%HS heme	K_d (µM)	NADH	PFR	Coupling (%)	TTN
cyclohexane	15	-	61 ± 9	2 ± 1	3	-
cyclooctane	35	31 ± 4	391 ± 11	209 ± 17	49	3400 ± 150
cyclodecane	40	0.88 ± 0.04	397 ± 15	42 ± 34	11	420 ± 75
cyclododecane	30	0.16 ± 0.03	174 ± 5	15 ± 3	9	-
cyclooctanol	10	-	158 ± 2	-	-	-
cyclododecanol	90	1.5 ± 0.3	198 ± 7	41 ± 1	21	1360 ± 90
cyclooctanone	10	-	93.4 ± 9	-	-	-
cyclodecanone	50	46 ± 2	323 ± 38	149 ± 54	45	1730 ± 50
cyclododecanone	90	0.16 ± 0.03	391 ± 11	283 ± 38	72	5750 ± 280
cyclohexyl acetate	25	540 ± 40	415 ± 56	56 ± 6	14	910 ± 385
cyclohexyl butyrate	60	110 ± 4	603 ± 32	310 ± 62	51	9580 ± 530
cyclohexyl isobutyrate	60	61 ± 5	383 ± 26	220 ± 62	57	3230 ± 60
methyl cyclohexylacetate	95	31 ± 1.5	736 ± 26	261 ± 20	34	9460 ± 540
ethyl cyclohexylacetate	85	40 ± 1	667 ± 29	274 ± 15	42	7250 ± 1720
cyclooctyl acetate	90	1.6 ± 0.2	722 ± 16	223 ± 20	31	8180 ± 1100
cyclooctyl isobutyrate	90	1.3 ± 0.2	732 ± 11	626 ± 30	75	4660 ± 1700
cyclododecyl acetate	95	0.05 ± 0.01	394 ± 8	94 ± 7	23	2990 ± 773

(Figure S5). Cyclooctanol was also tested but was a poor substrate both in terms of binding and productive activity (Table 1).

As we have previously highlighted that substrates with a ketone carbonyl group have enhanced affinity for CYP101B1, as well as higher activity and more selective hydroxylations, we next tested cyclic ketones.[20-22] Cyclooctanone like cyclooctanol did not interact well with CYP101B1 but cyclodecanone and cyclododecanone bound to the enzyme, inducing significant shifts to the high spin form of the enzyme (≥ 50%, Table 1, Figure S1 and S2). Both cyclodecanone and cyclododecanone were efficiently oxidised by CYP101B1. Cyclododecanone was oxidised with high selectivity generating a single product in >95% yield which was identified as 7-hydroxycyclododecanone (Scheme 2, Figure S3, S4 and S5). The minor metabolite (~4%) was unable to be generated in a large enough quantity for characterisation by NMR. However, the mass spectrum was consistent with a monooxygenase product with a mass, m/z 198.35 (Figure S4). Cyclodecanone was oxidised to two major metabolites which were identified as arising from hydroxylation at the 5- and 6-positions (60% and 36%, respectively) with a third minor product making up the remainder (Figure S3, S4 and S5).



Scheme 2. Oxidation of cyclodecanone and cyclododecanone by CYP101B1. * ≤4% minor products. # Distribution from NMR (Figure S5).

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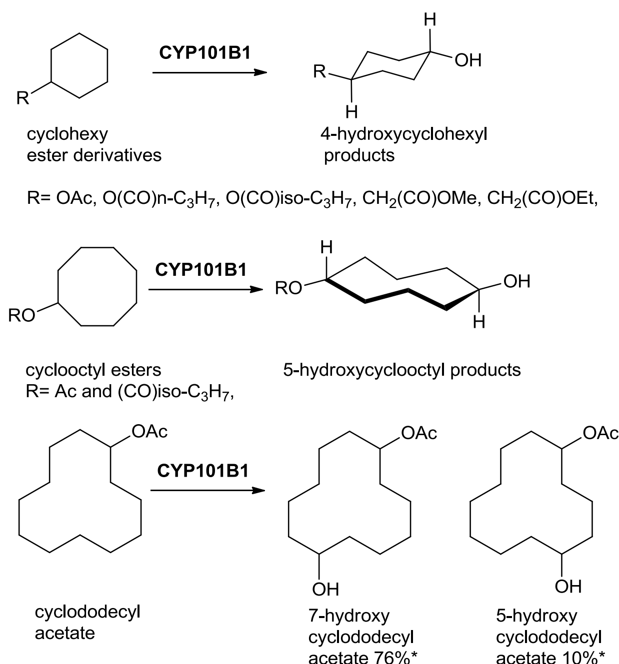
To complicate matters the two major compounds were present as mixtures of the hydroxycyclodecanone and the oxabicycloundecanol isomer which presumably arises from intramolecular nucleophilic attack of the alcohol on the electrophilic carbonyl group to form a cyclic hemiacetal (Scheme 2, Figure S5).[23, 24] We measured the TTN and, in line with the coupling efficiency and PFR, that of cyclododecanone was greater than cyclodecanone (5750 versus 1730) and any of the alkanes or alcohols previously investigated (Table 1).

The selective oxidation of cyclododecanone by CYP101B1 is an encouraging result for the application of this enzyme for the oxidation of large ring cyclic alkyl derivatives. We next examined the effect of adding an ester protecting group to improve the activity of cycloalkanol oxidation. Ester protecting groups, such as acetate and isobutyrate, could act as chemical auxiliaries and directing groups by mimicking the butenone moiety of the norisoprenoids. This would enable improved oxidation and these functionalities were added to cyclooctanol and cyclododecanol using standard methods (ESI and Figure S6).[21, 22, 25-29]

Cyclododecyl acetate bound to CYP101B1 inducing a complete shift in the spin state to the high spin form and bound to the enzyme with nanomolar affinity (Table 1, Figure S1 and S2). It was oxidised to one of the diastereomers of 7-hydroxycyclododecyl acetate (with 74%, regioselectivity, Scheme 3, Figure S3, S4 and S5). The coupling constants of J_{H1} and J_{H7} (J_{H1} = 5.5, 8.1 Hz, J_{H7} = 5.0, 8.0 Hz) inferred the major diastereomer was the *trans* with both substituents in equatorial positions. When isolated this was also found to contain a small amount of the *cis* isomer (2%). One other minor product was characterised as a diastereomer of 5-hydroxycyclododecyl acetate (10%; *trans* H1; J_{H1} = 6.4, 11.9 Hz and H5; J_{H5} = 6.4, 12.4 Hz) as both the C1 and C5 protons coupled strongly to C3 in the HMBC NMR spectrum (Scheme 3, Figure S5).

We next assessed the turnover of the acetate and isobutyrate esters of cyclooctanol. Both displayed enhanced binding and turnover parameters with CYP101B1 compared to cyclooctanol (Table 1, Figure S1 and S2). These esters were also efficiently and selectively oxidised by CYP101B1 with the coupling efficiency and product formation rate of the isobutyrate ester being the greater (Table 1). Both were predominantly hydroxylated to a single major product (Scheme 3 and Figure S3). In each instance, this could be assigned from the ^{1}H NMR coupling constants as the *trans*-diastereomer of the 5-hydroxy metabolite by NMR (Figure S4 and S5). Two minor products were observed in the turnover of the isobutyrate ester one of which was assigned as the other diastereomer of the 5-hydroxy product (~10%) based on the ^{13}C NMR signals (Fig. S5). The oxidation of cyclooctyl acetate by CYP101B1 was more selective and generated *trans*-5-hydroxycyclooctyl acetate in >95% yield with the other diastereomer making up the remainder (Figure S5). Despite the higher product formation rate of the isobutyrate ester, the TTN of the acetate ester was almost two-fold greater (8180 versus 4660) and both were higher than that achieved with cyclododecyl acetate (2990, Table 1).

Cyclohexyl acetate was oxidised by CYP101B1 with moderate activity but high selectivity to *trans*-4-hydroxycyclohexyl acetate (Table 1, Figures S3, S4 and S5). However the dissociation constant was high and this substrate only induced a 25% shift of CYP101B1 to the high spin form upon binding (Table 1, Figure S1 and S2). We hypothesised that the binding of this ester was suboptimal, compared to the cyclooctyl and cyclododecyl equivalents due to the smaller size of the substrate. Therefore we decided to assess the binding of a range of other cyclohexyl derivatives with larger substituents to see if the turnover activity could be improved.



Scheme 3. The Oxidation of cycloalkyl ester derivatives by CYP101B1. * 2% of the product was the *cis* isomer of 7-hydroxyy while the remaining 14% was another hydroxyl species (possibly the other diastereomer of 5-hydroxy).

Cyclohexyl-butyrate and -isobutyrate both displayed enhanced binding parameters to CYP101B1 compared to cyclohexyl acetate (Table 1, Figure S1 and S2). In addition methyl and ethyl cyclohexylacetate, which are ester derivatives of cyclohexanecarboxylic acid, also bound to CYP101B1 (Table 1, Figure S1 and S2) whereas the parent acid did not (data not shown). All of the esters were oxidised with higher product formation activities and coupling efficiencies than cyclohexanol and cyclohexane carboxylic acid, which were not substrates for the enzyme, and cyclohexyl acetate (Table 1, Figure S3). They all resulted in the formation of a single major metabolite in significantly greater quantities (Figure S3). In all instances, this was the 4-hydroxy metabolite (Figure S4 and S5). The CYP101B1 catalysed oxidations of cyclohexylbutyrate and methyl cyclohexylacetate were the most selective (>98%) and displayed the highest TTNs (>9000, Table1). The metabolites were also identified as the *trans*-diastereomer (Figure S5).

The selectivity achieved above is impressive given the lack of other distinguishing features on these cyclic molecules. In general, the addition of double bonds or other functional groups to larger ring structures has been showns to improve the selectivity of P450 catalysed oxidations. For example, the

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activity and selectivity of the CYP102A1 catalysed oxidation of cembrenoid derivatives has been optimised with multiple rounds of mutagenesis and substrate engineering resulting in the formation multiple products at allylic and non-activated C-H bonds but more saturated analogues displayed lower selectivity.[30, 31] The high product formation rates and TTNs for the optimal substrates (PFR > 200 min^{-1} and TTN > 5000 in a 1 hr reaction) combined with the levels of selectivity are favourable compared to other systems such as the monooxygenase system PikC from *Streptomyces venezuelae*.[32] In pioneering work this monooxygenase system was shown to be capable of oxidising cyclic substrates including cycloalkyls, macrolides and macrolactones subsituted with desosamine and related dimethylamine containing synthetic anchoring groups (TTN approx. 500).[27, 28, 32-34] Again the selectivity of these oxidations was reduced for cycloalkyl substrates compared to more functionalised macrolides and macrolactones. For example, 7 products were obtained with the protected cyclododecanol.[27]

In summary, we have identified that the CYP101B1 cytochrome P450 monooxygenase when combined with its physiological electron transfer partner Arx and ArR can efficiently oxidise cyclic hydrocarbon derivatives. The C-H bond abstraction and therefore selectivity of oxidation for a given methylene group was high and could be made almost totally selective by modifying the ester protecting group or functional group on the ring system. Hydroxylation occurred at C-H bonds on the opposite side of the ring to the ketone or ester moiety. The CYP101B1 system is therefore an ideal ideal candidate for study to generate biocatalysts for the selective functionalisation of more complex substrates such as the macrolactone and cembrenoid species described above.

Conflicts of interest

"There are no conflicts to declare".

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